Molecular and Genetic Characterization of *rugose* (*rg*), a Gene Involved in Signal Transduction and Pattern Formation in the *Drosophila* Compound Eye

by

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ABSTRACT

Molecular and Genetic Characterization of *rugose* (*rg*), a Gene Involved in Signal Transduction and Pattern Formation in the *Drosophila* Compound Eye.

By

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Advisor: Professor Tadmiri R. Venkatesh

Cellular pattern formation and cell fate determination are essential for the proper development of multicellular organisms. Previous studies have shown that the cytological location of the *rg* gene is on the 4E-4F region of the X chromosome (Salz, 1992; Shamloula, 1996, dissertation). *rg* mutations result in a rough eye phenotype (Salz, 1992; Refranz and Benzer, 1989; Lindsley and Zimm, 1992; Shamloula, 1996, dissertation) and some alleles exhibit various wing vein phenotypes (Refranz and Benzer, 1989; Shamloula, 1996, dissertation). My present studies have focused on the role of cell-cell interactions in cell fate determination and pattern formation. Genetic characterization of *rg* shows that *rg* interacts with other genes involved in the *EGFR* and *Delta Notch* pathway suggesting that *rg* may function in a related pathway. Molecular studies show that *rg* encodes a PKA anchoring protein (AKAP)

implicating the PKA signaling pathway in cell fate determination in the *Drosophila* compound eye. mRNA expression data shows that *rg* is expressed in the third larval instar eye antennal disc and various stages of the embryonic development. This is consistent with the *rg* mutant phenotype results that show that Rg is required for the differentiation of accessory cells in the late third larval instar and pupal stages in the *Drosophila* compound eye. Rg may act to localize the PKA activity in presumptive cone cells possibly augmenting signaling by other biochemical pathways like those mediated by *EGFR* and *Delta Notch* pathway. In *rg* mutants, signaling by other pathways may not be sufficient for cone cell differentiation due to a reduced signaling by PKA. My results highlight the role of biochemical pathways in cell-cell signaling during development and how different pathways can be utilized for differentiation within the context of an individual cell.

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It is never possible to express thanks because words are definable and feelings are not. This acknowledgement seems so inadequate because I can not express my gratitude fully to all the people who have been part of my studies directly or indirectly. The following is a sorry attempt to do so. My mentor, Dr. Venkatesh, and my advisors for believing in me and encouraging me when I lost my confidence; my friends and fellow sojourners, Angel Pimentel and Hoda Shamloula, and all those good friends for crying with me when I was at my lowest. I am grateful to all my family, especially my parents, Mkawasi and Mwamburi Mbogho, who started it all, for their unconditional love and support, and for teaching me values that have stood me in good stead, and also for providing opportunities that have made me who I am today. This has been a difficult road but I hope I can say it was worth it. In all my endeavors it was, and still is my mother's and father's unshakable faith that has made me prevail. In the end, this is their work. They deserve it more than any one else in the world. Lastly, but not least, I would like to acknowledge Jomo Kenyatta University of Agriculture and Technology for supporting my studies. Thank you.

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CHAPTER I

GENERAL INTRODUCTION

In multicellular organisms cellular pattern formation is essential for the proper development of the individual. Patterning events are initiated in the developing oocyte before fertilization. In *Drosophila* the establishment of the anteroposterior and dorsoventral axes of the oocyte and the surrounding somatic (follicle) cells marks the foundation upon which later embryonic and adult cell fate decisions are determined. The establishment of polarity during oogenesis results in the unequal distribution of signals and maternal morphogens in the form of mRNA that set up embryonic polarity. While *Drosophila* embryonic development seems unique in that there is no cellularization of the early blastoderm and morphogens can easily diffuse to form gradients, the basic principle of unequal distribution of molecules in the unfertilized egg and embryo is maintained in other systems (*C. elegans* and vertebrates) where unequal daughter cells or rearrangement of cytoplasmic components upon fertilization establish polarity in the developing embryo.

Molecular and genetic studies in *Drosophila* have shown that *EGFR* (Epidermal growth factor receptor) gene product and its ligand Gurken mediate interactions of the follicle cells and the developing oocyte in the establishment of their anteroposterior and dorsoventral polarity by the activation of the downstream signaling by the receptor/ligand binding (Gonzalez-Reyes and St. Johnson, 1998;

Gonzalez-Reyes et al., 1995; Roth et al., 1995; Neuman-Silderberg and Schupbach, 1996). EGFR signaling is also required for other cell fate decisions during development including establishment and survival of the embryonic ventral ectoderm cells and central nervous system (Clifford and Schupbach, 1992; Price et al., 1989; Raz and Shilo, 1992, 1993), and the wing vein and eye development (Freeman, 1994; Tio et al., 1994, Kolodkin et el., 1994; Sturtevant, et al., 1993). EGFR is a receptor tyrosine kinase (RTK). RTK signaling is therefore repetitively used by different tissues at different times in *Drosophila* development. The characterization of structural and functional homologs of EGFR in other evolutionarily disparate organisms underscores the developmental importance of this conserved pathway. In *C. elegans*, activation of the EGFR homolog, Let-23 by the binding of its ligand Lin-2 in the anchor cell is required for the vulval cell fate determination during gonadogenesis, hermaphrodite fertility, larval survival and spicule formation (Hill and Sternberg, 1992; Aroian and Sternberg, 1991; Aroian et al., 1994; Katz et al. 1996). Mammalian systems have also been shown to require RTK function for epidermal cell proliferation in much the same way as described for *C. elegans* and *Drosophila* (reviewed in Schlessinger and Ullrich, 1992; Fantl et al.,1993).

Neurogenesis in *Drosophila* requires the function of a group of genes called the *achaete scute* complex. These genes include *achaete*, *scute*, *lethal of scute* and *asense*, and they all code for basic helix-loop-helix (HLH) transcription factors. The *achaete scute* complex promotes neural cell fate determination and differentiation and controls the expression of neuron specific genes like *asense*. The number of cells that

become sensory organ precursor cells may be limited by the lateral inhibition of *Delta Notch* signaling. Transcription control by the HLH proteins is made more efficient by dimerization and DNA binding is optimized by heterodimerization (rather than homodimerization) with another HLH protein Daughterless (Da). Extramicrochitae (Emc), a HLH protein that lacks the basic domain binds to Achaete and Daughterless and thereby inhibit their binding to the DNA site, providing a negative control for the neurogenesis process.

A mammalian process that is analogous to neurogenesis in *Drosophila* is myogenesis. Myogenesis is initiated by the determination of some cells, from a group of cells called somites (mesodermal cells found lateral to the neural plate) during embryogenesis, to form myoblasts that migrate to skeletal tissues where they differentiate. The genes that control myogenesis in mice code for bHLH proteins (Myo-D, Myogenin, Myf5 and Mrf4) and also bind to DNA as dimers. The binding to DNA by myogenic transcription factors is also optimized by heterodimerization with another bHLH protein, E2A. In a process analogous to the negative regulation of neurogenesis in *Drosophila*, myogenesis is inhibited by Id HLH protein that lacks the basic domain, by binding to the myogenic transcription factors and preventing their DNA binding and activation of transcription. Proneural genes of the *achaete scute* complex are very similar in both structure and function to the genes controlling myogenesis in mammals. Achaete/Scute homologs have been identified in other organisms like *C. elegans* where these genes are involved in vulval differentiation. Neurogenic pathways involving Delta (ligand) and Notch (receptor)

proteins have been shown to have structure and functional homologies in diverse organisms such as *Drosophila*, *C. elegans*, *Xenopus* and mice. The function of neurogenic genes may be to mediate the selection of a single cell from a group of equipotential cells by lateral inhibition thereby making it responsive to the differentiating signals.

Timing of developmental signals may be as crucial as location of a cell in relation to its neighbors. Sequential or consecutive exposure of a cell or group of cells to differentiating signals progressively narrows down the choices of that cell(s), and each cell has to interpret the signals as a function of time and space. It is becoming clearer that most of the developmentally important genes are highly conserved either at the molecular, biochemical or functional levels in diverse systems. While I can not mention all the genes that have been identified that are required for proper pattern formation and cell fate determination in all organisms, let alone *Drosophila*, the examples I mention underscore the complexity of conserved developmental systems and their interactions, sometimes maintaining functional similarities and other times acquiring diverse functions. It is amazing that a cell bombarded with a different signals more often than not makes the right choice, and equally intriguing is the varied responses to the same signaling in that reiterative use of a single signaling pathway results in different cell fates. Most developmental signals are effected through cell-cell signaling making it a major mechanism in cell fate determination. To understand the role of cell-cell interaction in cell fate determination it is crucial to elucidate how genetic and molecular mechanisms function at the cellular level.

The *Drosophila* compound eye is an ideal model to study cell-cell interactions and signal transduction because of its amenability to both genetic and molecular manipulations. Furthermore the compound eye is genetically a non-essential structure and eye mutations do not necessarily affect the viability of the fly. The structure of the *Drosophila* compound is composed of photoreceptor (neural) cells and accessory cells (cone cells, pigment cells and cells of the mechanosensory complex) which are stereotypically organized. In the *Drosophila* compound eye various studies have shown that cell lineage plays no role in cell fate determination of the photoreceptor, cone and pigment cells, implicating cell-cell interactions. My studies will focus on the genetic and molecular understanding of cone cell development.

Structural Organization of the Compound Eye

The *Drosophila* compound eye is composed of about 800 unit eyes called ommatidia comprising eight photoreceptor cells (R1-R8), four cone cells, six pigment cells and four cells of the mechanosensory complex. The ommatidia are arranged in hexagonal arrays to form an appearance of a smooth eye [Fig. 1 A and B, Wolff and Ready, 1993, 1278]. The central face of the R cell becomes multiply folded into an elaborate microvillar structure, the rhabdomere, that contains the photopigments (rhodopsin). The photoreceptors can be classified by the position of their rhabdomere in the ommatidial cluster, synaptic connectivity, spectral sensitivity and size. The rhabdomere of R1-R6 are larger and occupy the outer positions surrounding R7 and R8, and their membranes extend the length of the retina. The rhabdomeres of R7 and R8 are smaller and centrally placed in the same plane. R7 rhabdomere is apical to the

basal R8; their rhabdomeres do not extend the length of the retina. R1-R6 axons terminate in the first optic ganglion, the lamina and R7 and R8 axons terminate in the second optic ganglion, the medulla. The four cone cells are placed on top of the photoreceptor cells and they secrete the crystalline lens. They extend their membranes down the length of the retina, insulating the photoreceptor cells and forming cone cell feet basally which contribute to the structural integrity of the fenestrated basement membrane [Fig. 2, Wolff and Ready, 1993, 1280]. The cone cells can be identified according to their positions within the ommatidial cluster. The anterior cone cell lies above R1-R3, the posterior cone cell lies above R4-R6, the polar cone cell lies above R3 and R4 and the equatorial cone cell lies above R1, R6 and R7. The six pigment cells can be divided into primary, secondary and tertiary pigment cells. The two primary pigment cells enclose the four cone cells and their membranes do not extend to the floor of the retina. Together with the cone cells, the two primary pigment cells secrete the acellular crystalline lens and the pseudocone. The outer lattice is formed by the secondary and tertiary pigment cells and insulates and separates each ommatidia. The membranes of the secondary and tertiary pigment cells extend the length of the retina, insulating and separating each ommatidial cluster. The cells of the bristle complex have a mechanosensory function and are found at alternate apices of the ommatidia.

Development of the *Drosophila* compound eye

The *Drosophila* compound eye develops from the eye antennal imaginal disc which is a group of about 20 cells set aside during embryogenesis. Differentiation of

the compound eye starts during the third larval instar. The differentiation events are initiated by the formation of the morphogetic furrow, a dorso-ventral indentation, that sweeps across the imaginal disc in a wave like manner from the posterior to the anterior. Anterior to the furrow the cells remain undifferentiated while posterior to the furrow cells begin to differentiate and assemble into clusters. Several studies have established the sequential stereotypic inductive events that culminate in the formation of the ommatidial clusters (Tomlinson and Ready, 1987 a and b; Lawrence and Green, 1979; Ready et al., 1976). The first cell to differentiate is R8 followed by the pairwise recruitment of R2/R5, R3/R4, R1/R6 and finally R7 is added into the cluster to complete the differentiation of the full complement of the eight photoreceptors. Two mitotic waves are associated with the developmental events in the *Drosophila* compound eye, namely, the first mitotic wave which is more diffuse and occurs ahead of the morphogenetic furrow and the more restricted second mitotic wave which occurs behind the morphogenetic furrow. The precluster cells are derived from the first mitotic wave while the second mitotic wave results in the recruitment of R1, R6 and R7 (Wolff and Ready, 1991; 1993). The cell recruitment and differentiation is also associated with the apical migration of the nuclei. The migration of the R7 nuclei is followed by the pairwise recruitment of the anterior and posterior cone cells at the two cone cell stage and then the equatorial and polar cone cells to complete the four cone cell stage (Tomlinson, 1985; Wolf and Ready, 1993). Subsequent stages of retinal differentiation are accomplished during the pupal stages. During the early pupal stages (20 hours pupation) the two primary pigment cells are added to the

cluster, first flanking the anterior and posterior cone cells and later encompassing the four cone cells at 40 hours post pupation. The secondary and tertiary pigment cells are then added to the cluster and finally the cells of the mechanosensory complex. At 60 hours post pupation all the ommatidial cells have differentiated (Fig. 3). The fine tuning of pattern formation involves elimination of cells that make inappropriate decisions and also the extraneous cells by programmed cell death, rearrangements of cell contacts, and the reorientation of the ommatidia to form the dorso-ventral mirror image symmetry (Wolff and Ready, 1991).

The Role of Signal Transduction in *Drosophila* Compound eye Development:

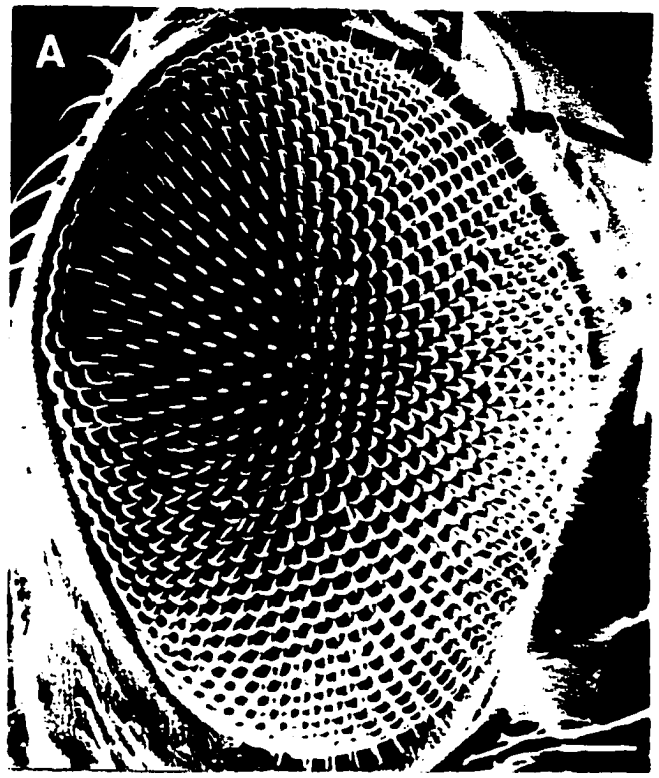
Molecular and classical genetics techniques have contributed enormously to the progress that has been made in recent times on the understanding of developmental processes. Mutations that affect eye development and pattern formation have allowed for the identification of genes encoding components of biochemical pathways involved in these complex events. Furthermore, genetic interaction studies have simplified the search for genes involved in the same or related biochemical pathways. These approaches have been used to identify and clone genes involved in the development of the *Drosophila* compound eye. Signaling by receptor tyrosine kinases has been implicated in cell-cell interaction and cell fate determination. The *Drosophila* Epidermal Growth Factor Receptor (DER), a tyrosine kinase, has been shown to be involved in various cell fate decisions through out development.

In order to try and understand cell fate determination and pattern formation I have chosen to study a gene called *rugose (rg)*. The *Drosophila* compound eye

develops in a specific manner and the retinal cells are arranged in a stereotypic pattern. Lesions in genes involved in pattern formation can be identified by isolating mutations that disrupt the normal patterning of the eye. Mutations in *rugose* cause a rough eye phenotype. The cellular basis for this phenotype is the improper differentiation of the cone cells and the subsequent disruption of the fenestrated basement membrane (Shamloula, 1996 dissertation). Previous studies have suggested that cone cell differentiation may require the same signaling events as those found in photoreceptor differentiation. Recent studies suggest that cone cell development may utilize the same signal transduction pathways as photoreceptor cells. Using dominant negative expression of DER (DN-DER) Freeman (1996) has shown its requirement for cone cell recruitment; furthermore, he also showed that over expression of *spitz*, a gene that encodes EGFR ligand, results in over recruitment of cone cells. Three other genes have been shown to specifically affect non neural accessory cells in the *Drosophila* compound eye. *spa* (Fu and Noll, 1997), *cno* (Miyamoto et al., 1995; Matsuo et al., 1997) and *cut* (Blochlinger et al., 1993) are specific to the development of cone and pigment cells and do not seem to be required in the photoreceptor cells. *cno* has been shown to interact with downstream components of EGFR signaling pathway, *Ras* and *raf* (Matsuo, et al, 1997). Genetic interaction studies are a straight forward way to screen for possible genes that interact with my gene of interest *(rg)*. In my studies I have endeavored to answer the following questions: What is the molecular nature of the *rg* gene? What is the pattern of *rg* mRNA expression? What other genes interact with *rg*?

What is a possible role of *rg* in cell fate determination in the *Drosophila* compound eye?

Fig. 1: External and internal morphology of the *Drosophila* compound eye. Scanning electron micrographs showing (A) wild type (*Canton-S*) compound eye with a smooth surface and regular ommatidial facets [Magnification = 220X]. Tangential sections through the wild type eye (B) shows a reiterated arrangement of the photoreceptor cells and the hexagonal pigment cell lattice. [Bar = 35] (Shamloula, 1996).



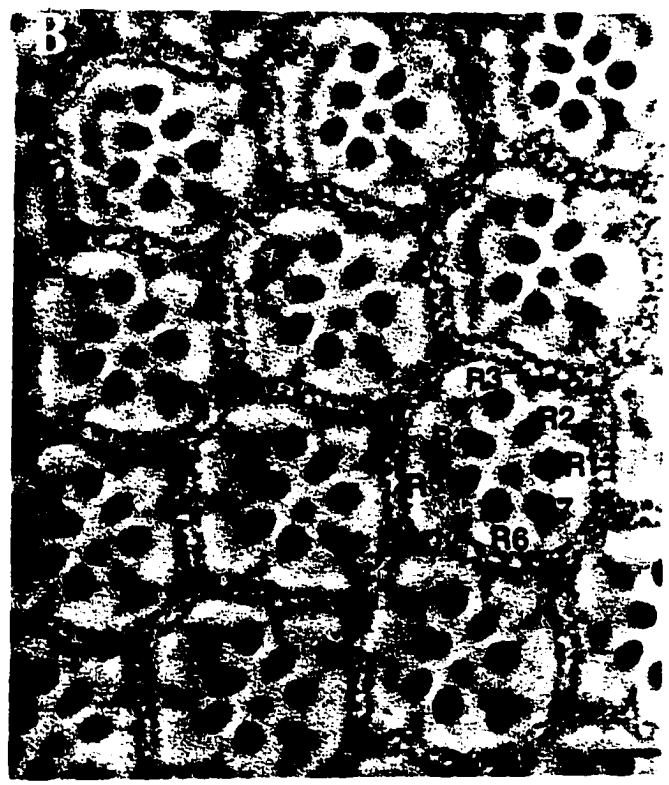


Fig. 2: A schematic of the structural arrangement of the retinal cells in the *Drosophila* compound eye. The eight photoreceptors (R1-R8) are arranged in a hexagonal shape occupying stereotypic positions within the cluster. On the central side the photoreceptors have multiply folded membranes called rhabdomeres that contain the photosensitive pigment. The rhabdomeres for R1-R6 are placed on the outside, are larger and their axons project to the to the first optic ganglion (lamina). The rhabdomeres for R7 and R8 occupy the same plane centrally with R7 apical to R8. R7and R8 project their axons to the second optic ganglion (medulla). The four cone cells are placed on top of the R cells and secrete the lens; and project their membranes basally to form cone cell feet. The two primary pigment cells encompass the cone cells and do not project to the fenestrated basement membrane. The secondary and tertiary pigment cells are shared between ommatidia, insulating each cluster and also contributing to the fenestrated basement membrane. The cells of the bristle complex occupy alternate apices (Wolff and Ready, 1993 with permission).

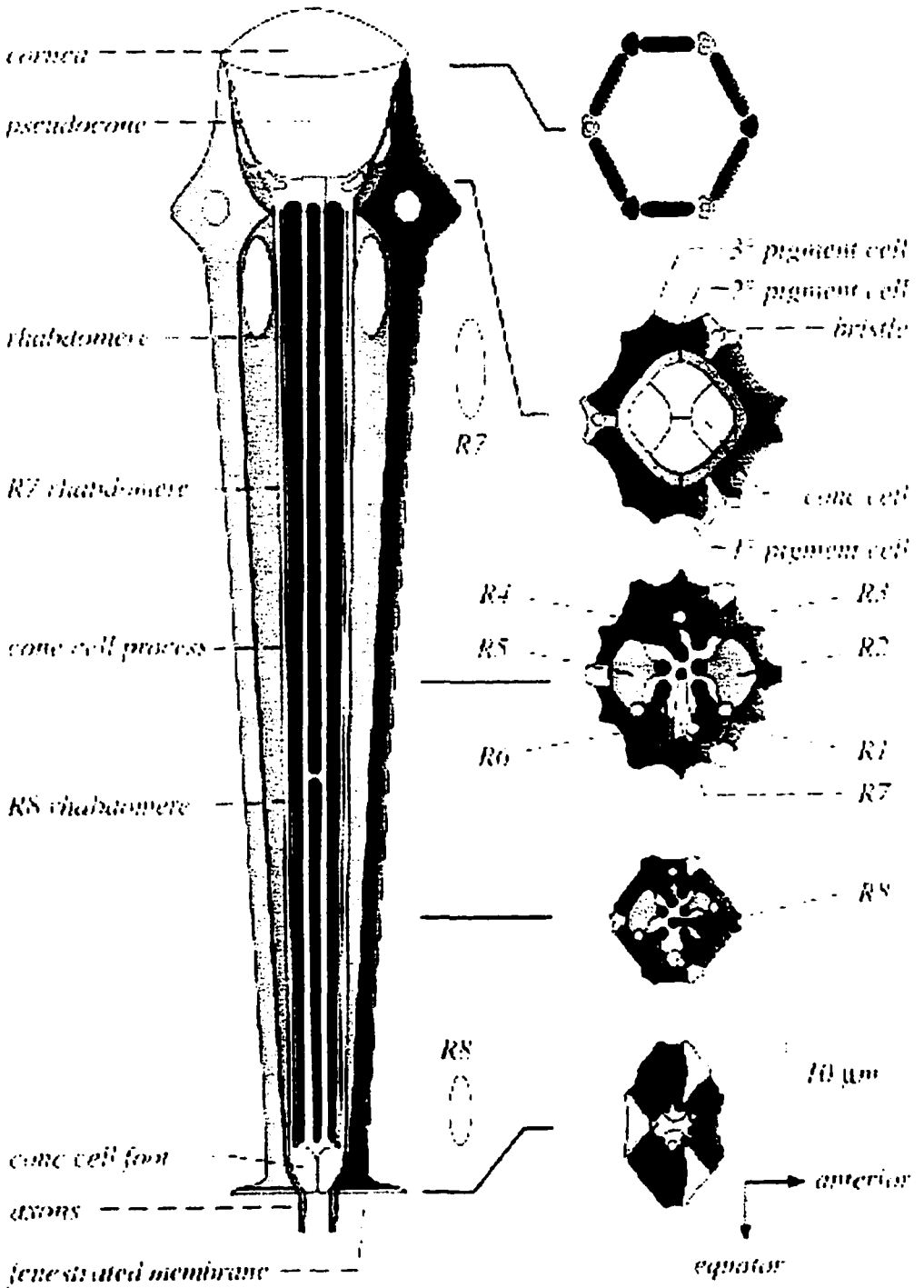
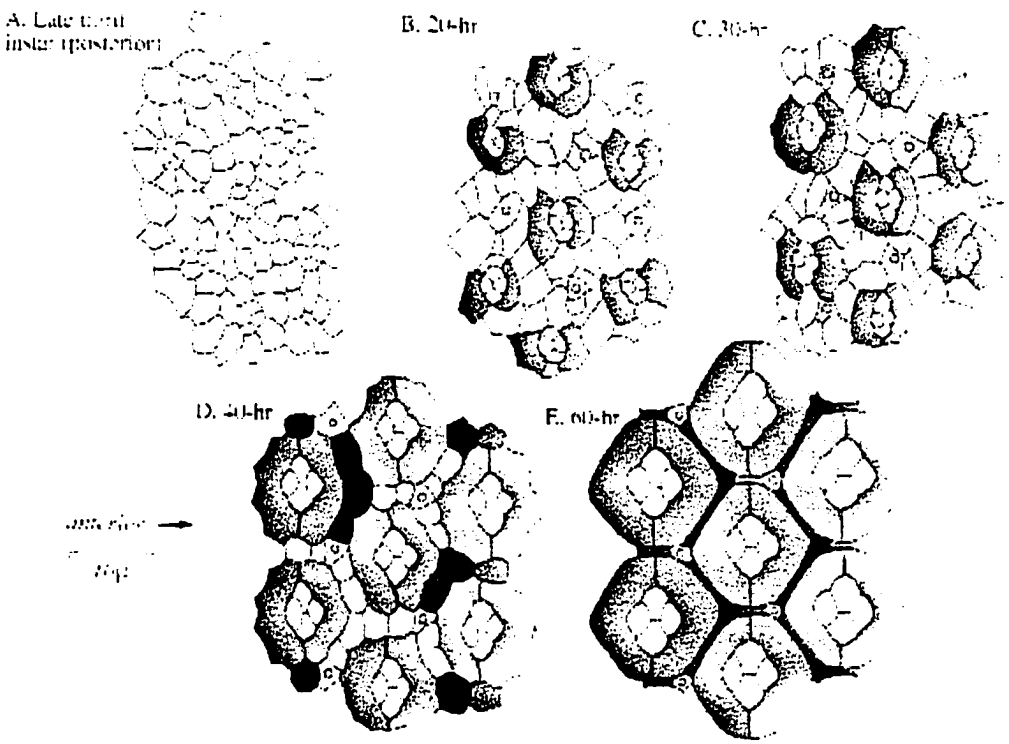


Fig. 3: The development of the non-neural cells of the *Drosophila* compound eye. The anterior and posterior cone cells are added after R7 differentiation during the late third larval instar (A). At 20 hours post pupation the primary pigment cells are added to the cluster (B). At 40 hours post pupation the secondary, then the tertiary pigment cells are added to the cluster (C and D) and by 60 hours after pupa formation the eye differentiation is complete (with permission Wolff and Ready, 1993).



CHAPTER II

ISOLATION AND PHENOTYPIC CHARACTERIZATION OF *rugose* MUTANTS

INTRODUCTION

Differentiation of the accessory cells begins after the last photoreceptor cell, R7 has differentiated. The first accessory cells to differentiate are the cone cells followed by the primary then secondary pigment cells. rg^{1} is a spontaneous hypomorphic allele that has a rough eye phenotype and was the only available allele at the beginning of these studies; other *rg* phenotypes include reduced viability in the stronger alleles, that have since been lost, and various wing vein defects (Refranz and Benzer, 1989; Salz, 1992; Lindsley and Zimm, 1992; Shamloula, 1996, dissertation). Electron microscopy studies show that hexagonal arrangement of ommatidia in *rg* mutants is disorganized [Fig 1 A and B, Shamloula, 1996 dissertation] and sometimes whole ommatidia are missing [Fig. 1 C, Shamloula, 1996], a disorganization of the pigment lattice [Fig. 1 C and 1 D, Shamloula, 1996] where there is an over recruitment of secondary and tertiary pigment cells [Fig. 3 C, D and E, Shamloula, 1996] and improper cell shape [Fig. 3 F, Shamloula, 1996]. *rg* mutations also show a disrupted fenestrated basement membrane [Fig. 2 B and 2 D] (Shamloula, 1996, dissertation). Cell death and cell differentiation markers during the third instar *rg* eye disc show a normal development pattern comparable to the wild type (Refranz and

Benzer, 1989; Shamloula, 1996, dissertation) suggesting that only the later stages of the *Drosophila* compound eye are affected by the *rg* gene product. Photoreceptors develop earlier than cone and pigment cells and using enhancer trap lines Shamloula (1996) has shown that their development is normal in *rg* mutants suggesting that Rg is not required for photoreceptor differentiation. Cobalt staining highlights cellular membranes and late third larval instar and pupal eye discs show that cone cells are reduced in number in *rg* mutants in a dose dependent manner (Fig. 3; Shamloula, 1996) implicating Rg requirement in their development.

In this chapter I will present data on the isolation of more *rg* alleles and phenotypic characterization of *rugose* (*rg*) mutations. This work was done in collaboration with Shamloula (1996). These results show that *rg* is required for cone cell differentiation and subsequent structural integrity of the fenestrated basement membrane.

MATERIALS AND METHODS

Isolation of Gamma ray *rg* alleles:

Three day old wild type w^{1118} *(Canton S)* males were gamma irradiated (3000 rad) to induce random mutations and mated to virgin *Df(1)JC70/FM7* females. Non balancer females were screened for rough eyes. Out of 16,000 flies screened forty eight rough eyed females were isolated and individually mated to *FM7 Y* males and maintained as a stock. Potential *rg* white eyed males from the stock were also mated to virgin attached X females and also maintained as a stock. The rough eyed males

were tested for complementation by mating with rg^1/rg^1 females. Homozygous stocks were established by mating each individual rough eyed male to wild type virgin (*Canton-S*) females. F1 virgin females were collected and back crossed to the parental males. The new alleles were also tested for any dominant effect by crossing to wild type (*Canton-S*) virgins and screening for rough eyed females. The *rg* mutants were analyzed for levels of gene function by placing over a deficiency that uncovers the *rg* gene.

P element induced *rg* alleles:

P element alleles were generated by a hybrid dysgenic cross remobilizing a P element inserted in the *deadhead(dhd)* gene found adjacent to *rg* (Salz, et al., 1994). The P element in dhd^{p4} was remobilized by crossing $w;dhd^{p4}/FM7$ males to *C(1)Dx,y,ywf; Δ2-3SbTM3 -; - -* virgin females providing the source of transposase (Robertson et al., 1988). The F1 progeny was mated interse and F2 males screened for rough eyes. Any rough eyed males were mated to rg^1/rg^1 virgin females to test for complementation. Rough-eyed males that failed to complement rg^1 were individually crossed to attached X females and maintained as a stock. Two of the P element induced alleles (rg^{p2} and rg^{p5}) were used for reversion analysis in a hybrid dysgenic cross in order to determine if the P element induced mutation was insertional. If so, such alleles could be used to clone the *rg* gene by using the P element as a molecular tag.

Deficiency Mapping:

The genetic locus of *rg* was mapped using deficiency stocks that uncover the

4E-4F region of the X chromosome. *rg* males were mated to three day old females of the following genotypes: *Df(1)A113/FM7*, *Df(1)RC40/FM7*, *Df(1)JC70/FM7*, *Df(1)ovoG6/FM7*, *Df(1)svbEh/FM7*, *Df(1)DEB4D/FM7*, *Df(1)GA56/FM7* and the F1 females carrying *rg* mutation over the deficiency (non balancer) were scored for eye phenotype.

Temperature shift experiments:

Temperature sensitive alleles are useful in providing a sensitized system where the temporal requirement of a gene product can be determined. Using the temperature sensitive allele rg^{ts} flies were grown in 17°C . Different stages of fly development were shifted to the non permissive temperature 29°C starting from egg, first, second and third larval instar and pupal stages. The adult flies were scored for severity of the roughness of the eye. A reciprocal downshift experiment was carried out.

Electron microscopy:

Flies were dehydrated in increasing ethanol concentration then critical point dried, mounted and coated with 25 mm gold coat (Karpilow et al., 1989). Transmission microscopy was as described in Longley and Ready (1995). Tissues were dissected in fixative 1% formaldehyde and 0.88% in sodium cacodylate buffer (pH 7.4) then fixed for four hours at room temperature. The tissues were then incubated overnight in 1% tannic acid in fixative, washed in buffer for 30 minutes, incubated in 2% osmium tetroxide (in buffer) for two hours and washed thrice for ten minutes in distilled water. After washing, the tissues were incubated overnight in 2%

uranyl acetate and dehydrated in 50, 70, 80, 90 and 100% ethanol series for 6 minutes each. The tissues were then incubated in ethanol/propylene (1:1) mixture for ten minutes followed by 100% propylene oxide for fifteen minutes, propylene oxide/EMbed 812 mixture (1:1) four hours, EMbed 812 (100%) overnight and embedded in 100% EMbed 812 and DMP-30 for 36 hours at 60°C then sectioned by ultramicrotome.

Light Microscopy:

The cobalt sulfide staining was used to visualize the retinal apical surface of pupal eye discs (Melamed and Trujillo-Cenoz, 1975; Wolf and Ready, 1991a). Dissection of 50 hour pupal eyes (pupae were staged so that white pupae were considered time zero and grown at 23-25°C) was done in 2% glutaraldehyde in PBS solution for 10 minutes, briefly washed in distilled water and transferred into 5% cobalt nitrate for 15 minutes. The eye discs were rinsed for 5 seconds in distilled water then transferred to a drop of 2% ammonium sulfide, washed in water for 5 minutes and mounted in 70% glycerol.

RESULTS

Isolation of new *rg* alleles:

rg^{1} is a spontaneous hypomorphic allele that has a rough eye phenotype and shows reduced viability (Lindsley and Zimm, 1992) and was obtained from the Indiana stock center. New alleles were generated by gamma ray irradiation and by the mobilization of a P element in a dysgenic cross. Of the 16,000 flies screened in the

gamma ray induced alleles eleven new alleles were generated (Table 1). The severity of the eye phenotype was dependent on the severity of the mutation. The strength of the alleles was tested by placing the mutant chromosome over a deficiency. The alleles that behaved as null alleles when tested against a deficiency in the *rg* locus showed no change in the rough eye phenotype; *rg rg* females, *rg Y* males and *rg* deficiency females all had the same eye phenotype and were classified as genetic nulls. The alleles that had *rg* gene residual function showed an increase in the rough eye phenotype when placed over a deficiency; *rg rg* females had a less severe phenotype as compared to the *rg* deficiency females and these were classified as genetic hypomorphs (Table 1). The P element dysgenic cross yielded nine new *rg* alleles out of 30,000 F2 flies screened. Two of these alleles (rg^{p2} and rg^{p5}) were tested for insertional mutation. Results showed that both alleles were insertional mutations because remobilization of the P element caused partial or complete reversion of the roughness of the eye phenotype.

Phenocritical period of *rg* function in eye development:

rg^{ts} is a temperature sensitive allele of *rg* showing a severely rough phenotype at 29°C and mildly rough phenotype at 17°C. Shifting rg^{ts} eggs, first, second and third instar larvae from 17°C to 29°C caused the eye to be very rough while shifts during pupal stages resulted in mildly rough eyes. Reciprocal experiments of shifting eggs, first and second larval instar from 29°C to 17°C resulted in a mildly rough eye while shifts in the third larval instar and pupal stages resulted in a severely rough eye. These results suggest that *rg* gene function is required during the third larval instar

and pupal stages for proper eye formation in *Drosophila*.

DISCUSSION

The expected frequency of obtaining γ alleles at 4000 roentgens with sperm irradiation is 1:5000 (Lindsley and Zimm, 1992). In our screen the frequency for obtaining new *rg* alleles was about 1:1450 which was higher than expected. One reason for this type of recovery of *rg* mutant could be due to a hypermutable site. P element mutagenesis has a lower expected frequency of recovery as compared to irradiation mutagenesis because P element preferentially inserts in regulatory regions of most genes. Our recovery was 1:900 which was much higher than the expected 1:50,000 (Lindsley and Zimm, 1992). One reason as suggested above is a hypermutable region, possibly a large regulatory region. Two of these *rg* alleles rg^{p2} and rg^{p3} are insertional mutations because revertant analysis showed a corresponding reversion to incomplete or complete smooth eye. All the new alleles were classified according to the severity of the rough eye phenotype. A total of seventeen *rg* alleles were established, five of these, rg^{p3}, rg^{p5}, $rg^{γ1}$, $rg^{γ3}$ and $rg^{γ6}$ were classified as genetic nulls suggesting a complete loss of function. The null alleles had severely rough eyes and and had 1-2 cone cells, some of the ommatidia having no cone cells at all. The rest of the *rg* alleles were genetic hypomorphs and showed varying eye roughness in a dose dependent manner suggesting that they had residual *rg* gene function. Hypomorphs also showed varying number of cone cells. The weak alleles showed fewer abnormal ommatidia with 2-3 cone cells per ommatidia. No hypermorphs were

recovered because none of the alleles had more than the normal four cone cells neither were any neomorphs identified. This is not to say that *rg* function is not required for other developmental events and may possibly have phenotypes not recovered in our screens because our initial screens were limited to the rough eye phenotype. Also any neomorphs may have been lost in our screen for the same reason. An EMS mutagenesis was not done in these studies but had previously been done and is currently continuing in our lab. New alleles may include hypermorphs and neomorphs because EMS generally causes point mutations by a GC to AT transition and is less toxic to the animals, unlike γ-ray and X-ray irradiation which may cause single and multi hits including rearrangements, small and multilocus deletions (Lindsley and Zimm, 1992).

The anatomy of *rg* mutants show a disrupted fenestrated basement membrane. This seems to be an indirect effect due to the reduced cone cell number and disorganized pigment lattice. As a result some of the retinal cells lose their basal support and some of the cells fall into the brain. A similar phenotype has been observed in the *strawberry notch* (*sno*) mutants (Coyle-Thompson and Benarjee, 1993). These observation are consistent with the earlier results that showed that the R cell differentiation is not affected in *rg* mutants and therefore any reduction in R cell number observed in the tangential sections could be due to the cells falling into the optic ganglia. The question still remains as to where the presumptive cone cells go because there is no abnormal cell proliferation or cell death. One idea that the presumptive cone cells become secondary and tertiary pigment cells though not

conclusive is consistent with the observation that the undifferentiated cells that occupy the cone cell space in the third larval instar and early pupal stages though determined may fail to develop into a cone cell in the *rg* mutants (Shamloula, 1996). Mutations in *rg* also show wing defects (Shamloula, 1996). This is an interesting phenotype because some conserved developmental pathways like the *Ras* and *Delta Notch* signaling have been shown to be involved in different cell fate decisions all through out development including eye and wing development.

In this chapter I have presented data on the isolation and characterization of new *rg* alleles. Obtaining new alleles was crucial to this project because different alleles provide a comprehensive understanding of the gene functions because they may represent different lesions within the gene which will exhibit different phenotypes. The eye phenotype shows that *rg* function is required for the proper differentiation of the cone cells and pigment lattice (Shamloula, 1996). Using the temperature sensitive allele rg^{1} I have shown that *rg* function is required during the late third larval instar and pupal stages, a time when accessory cells differentiate. *rg* may also be required at other times during development and a molecular analysis of *rg* mRNA and protein would identify the other tissues where the *rg* gene product is required. While we may have a genetic null this may not be a true null because we do not observe a complete loss of cone cells in all the ommatidia. On the other hand, if we do have a true null our results may reflect redundancy.

Table 1: New *rugose* alleles

New *rugose* alleles	*rugose* eye Phenotype	Mutagen
rg^{p1}	hypomorph	P element
rg^{p2}	hypomorph	P element
rg^{p3}	genetic null	P element
rg^{p5}	genetic null	P element
rg^{p6}	hypomorph	P element
rg^{p9}	hypomorph	P element
rg^{v1}	genetic null	Gamma ray
rg^{v2}	hypomorph	Gamma ray
rg^{v3}	genetic null	Gamma ray
rg^{v4}	hypomorph	Gamma ray
rg^{v5}	hypomorph	Gamma ray
rg^{v6}	genetic null	Gamma ray
rg^{v7}	hypomorph	Gamma ray
rg^{v8}	hypomorph	Gamma ray
rg^{v9}	hypomorph	Gamma ray
rg^{v10}	hypomorph	Gamma ray
rg^{v11}	hypomorph	Gamma ray

Fig. 1: Phenotype of the wildtype and *rg* mutant compound eyes. Scanning electron micrographs showing of (A) wildtype (*Canton-S*) compound eye with a smooth surface and regular ommatidial facets and (B) rg^{vo} mutant eye showing a rough eye phenotype. Magnification = 220X. Tangential sections through the wild type eye (C) shows a reiterated arrangment of the photoreceptor cells and the hexagonal pigment cell lattice. In the rg^{vo} mutant (D) eye the section shows aberrant pigment cell lattice (white star) and disorganized photoreceptors (arrows) [Bar = 35] (Shamloula, 1996).

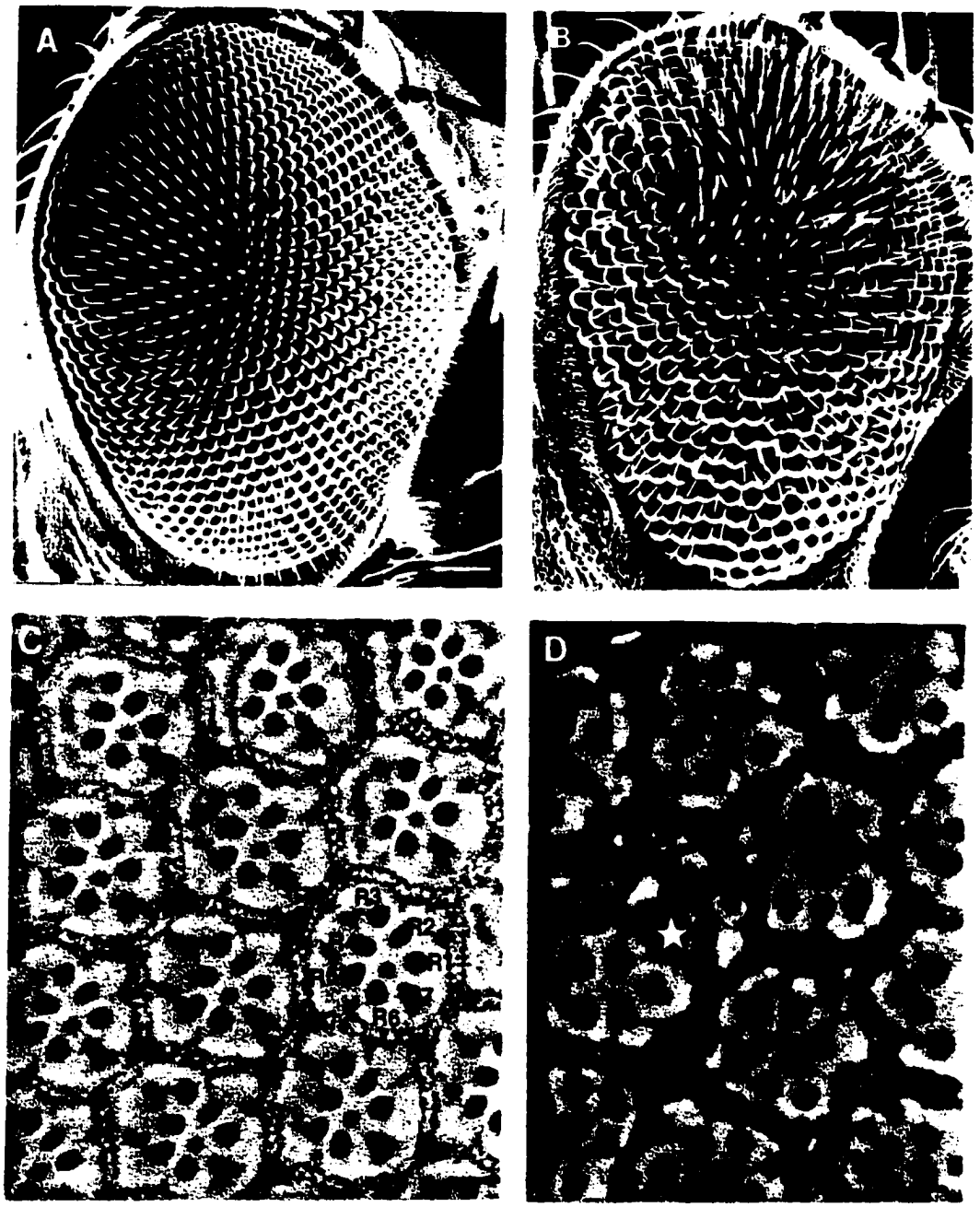


Fig. 2: Transverse sections of wildtype (A) and (C), and *rg* mutant (B) and (D) compound eyes. Note the disrupted fenestrated membrane in the rg^{rn} mutant retina. Panels C and D show higher magnification views of the basement membrane regions, revealing collapsed photoreceptor cells in the lamina region of the optic ganglion. R=retina, L=lamina, M=medulla, fm=fenestrated basement membrane [A and B bar=50μ; C and D bar=30μ] (Shamloula, 1996)

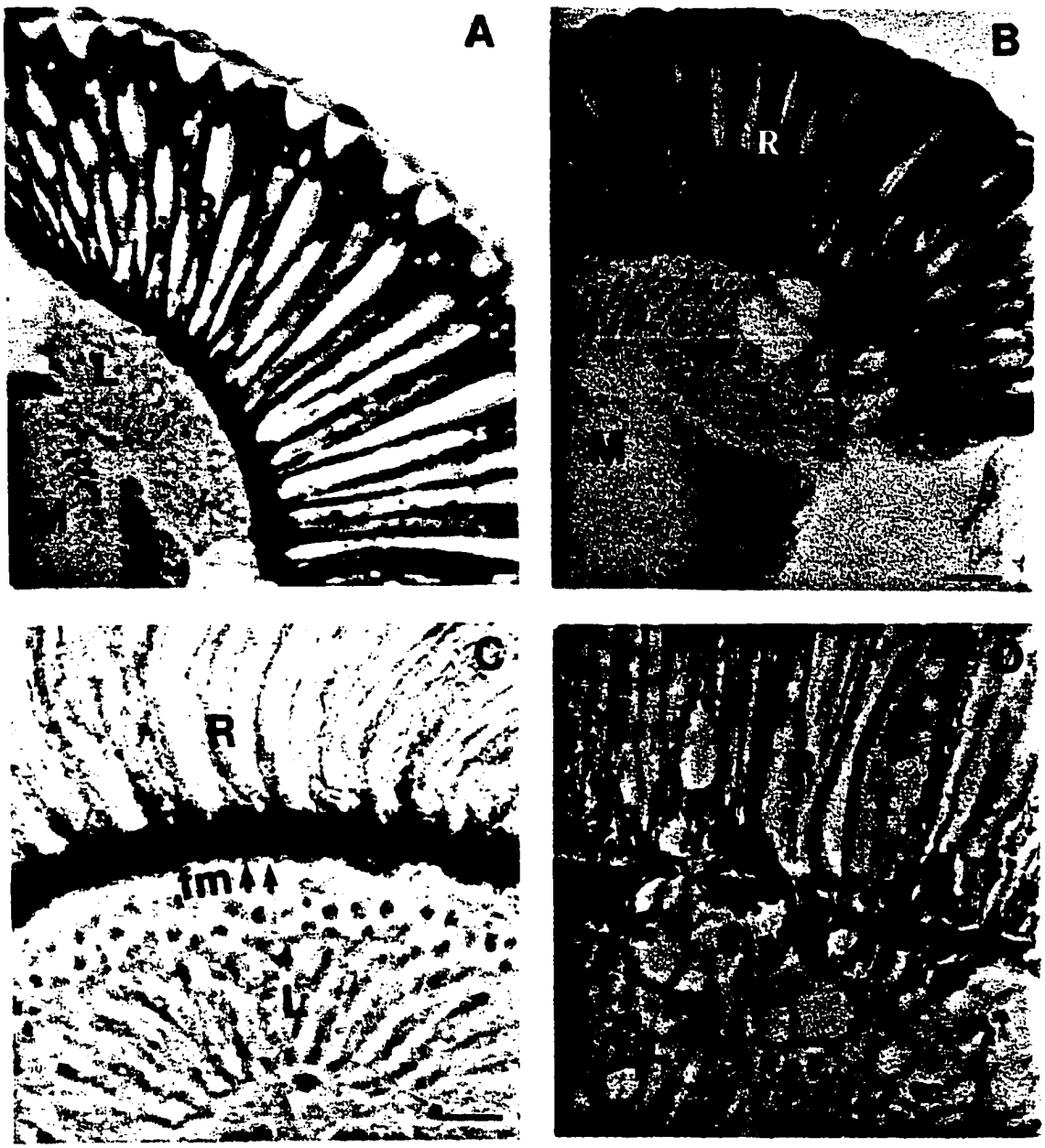
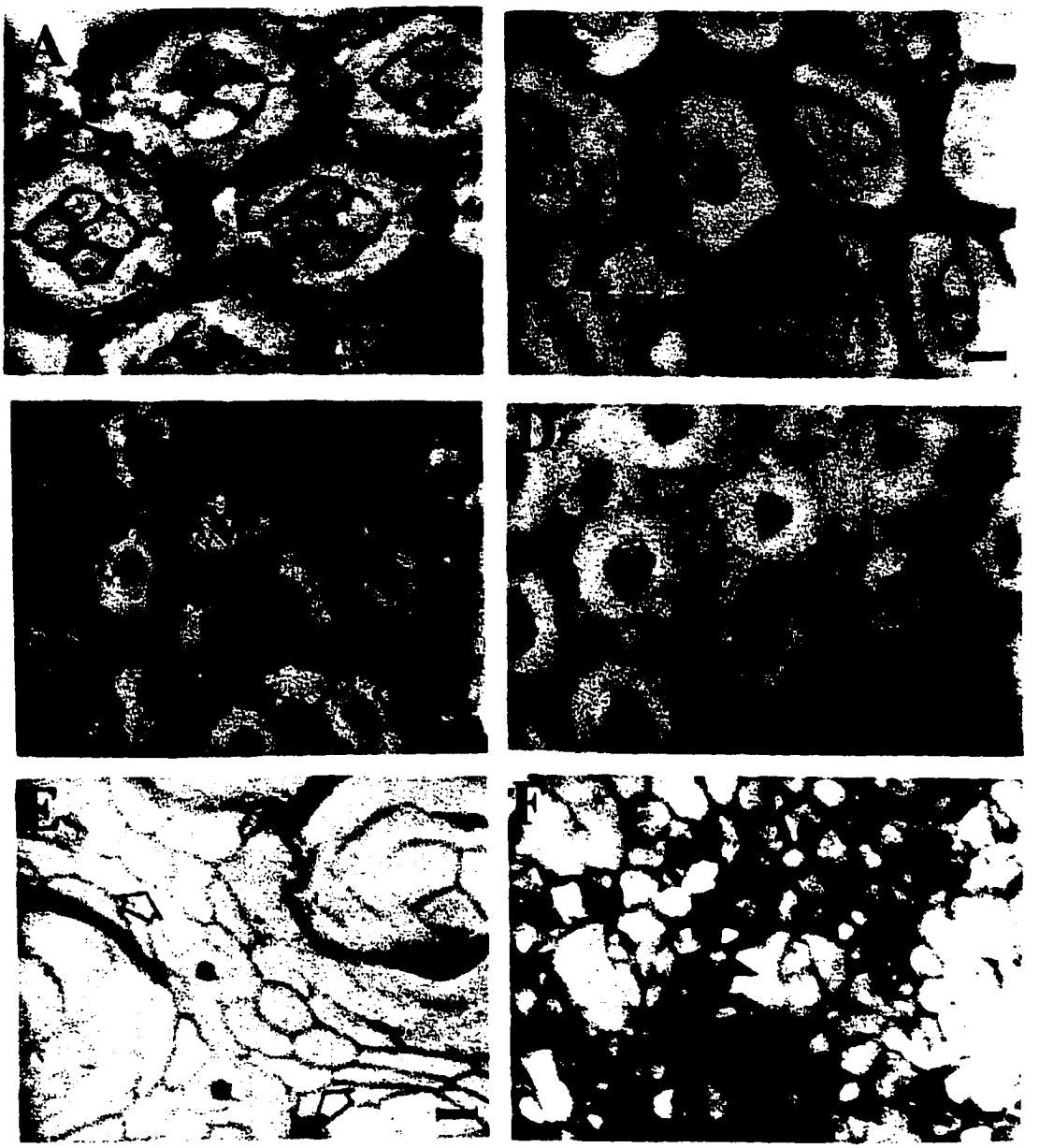


Fig. 3: *rg* mutants show aberrant number of cone and pigment cells. Cobalt sulfide stained preparations of 50 hour pupal stage eyes from *rg* mutant alleles show abundance of secondary pigment cells resulting in the aberrant arrangement of secondary and tertiary pigment cells and a disorganized pigment cell lattice. (A) Wild type shows four cone cells enclosed by two primary pigment cells and each ommatidia is surrounded by hexagonal lattice of secondary and tertiary pigment cells. Cell types were identified according to their positions within the cluster. In the *rg* alleles (B) rg^{r10} (C) rg^{p5} the pigment lattice is disorganized due to excess secondary and tertiary pigment cells (open arrow shows 1-2 cone cell per ommatidia and closed arrow shows increased number of secondary and tertiary pigment cells). Arrows in (D) show a full complement of two primary pigment cells in rg^{p3}. In (E) a severe allele rg^{rn} there is an accumulation of secondary and tertiary pigment cells (open arrows). (F) In the null allele rg^{r3} very few cone cells are seen surrounded by large number of putative pigment cells. [Bar=35 for A-D; 25 in F] (Shamloula, 1996)



CHAPTER III

STUDIES ON THE GENETIC INTERACTIONS OF *rg*

INTRODUCTION

While mutations are useful in identifying and isolating genes of interest, genetic interaction experiments can provide information on how various gene products may function along a biochemical pathway. If two proteins interact directly or indirectly mutations in both proteins may produce a much more severe phenotype than either one alone. The second gene is an enhancer of the first gene mutant phenotype. Second site mutations can also suppress a mutant phenotype if the second mutation compensates for the defect caused by mutations in the first gene. Dominant modifiers can therefore be identified by screening for enhancers and suppressors of a mutant phenotype in an F1 screen for double mutants.

In this chapter I screened for dominant modifiers of *rg* rough eye phenotype. I started with a broad screen using second and third chromosome deficiency kits from the Indiana Stock center. Any modifiers were identified and their cytological locus and break points determined. I then searched the flybase for specific genes that have

been identified within the break points. This was important to ascertain whether the modifications in eye phenotype were due to any known genes within the break points because the deficiencies include more than one gene. For known genes several alleles were obtained to test directly for interactions. If possible both loss-of-function and gain-of-function alleles were used. In this way several genes were identified that interact with *rg*.

My results show that *rg* genetically interacts with components of the *EGFR* pathway and the *Delta Notch* pathway.

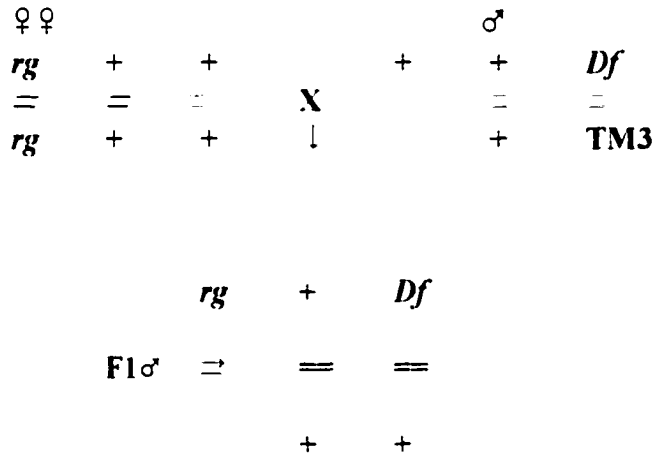
MATERIALS AND METHODS

Identification of Deficiency Strains in the Second and Third Chromosomes that Interact with *rg*:

I screened for dominant interactions of *rg* in an F1 screen. Fly stocks carrying autosomal deficiencies (obtained from Indiana University Fly Stock Center) were screened for dominant interactions with *rg* in an F1 screen. Homozygous *rg* virgin females were crossed to autosomal deficiencies over a balancer. Non balancer F1 males were screened for modified *rg* eye phenotype. Any known genes within the chromosomal region interacting with *rg* were identified using the fly database

(flybase). In order to confirm the results obtained from the broad screening with deficiencies multiple alleles of the identified autosomal genes were tested with various *rg* alleles. This genetic screen provided a simple, sensitized and quick method to identify *rg* modifiers by reducing the function of the interacting gene by half in a *rg* mutant background.

Diagram Showing the F1 Screen for the Modification of *rg* Phenotype



Double mutants (males) *rg*/Y; *Df*/+ scored for enhancement or suppression.

RESULTS

Deficiencies Interacting with *rg*:

I screened 51 second chromosome and 68 third chromosome deficiency stocks obtained from Indiana Stock Center and tested with both severe and hypomorphic alleles of *rg*. About 64% of the second chromosome and 73% of the third chromosome were covered under this scheme (http://flybase.bio.indiana.edu). 21 deficiency stocks showed dominant interactions with *rg* (Table 1). Genes previously mapped within the deficiency breakpoints (Flybase and Lindsley and Zimm, 1992), were identified and different alleles obtained and tested for interactions directly with various *rg* alleles (Table 2). For some of the interacting deficiencies no known genes have yet been characterized. Some of the deficiencies uncovered known genes. Some of these genes identified were found to belong to the *EGFR Ras* signaling pathway and hence further characterization of *rg* in this pathway was pursued (Table 1, 2 and 3). *Delta* which belongs to the neurogenic group of genes was also identified in this screen as a strong enhancer of the *rg* phenotype (Table 2 and 3).

Ras is an enhancer of *rg*:

A dominant negative form of *Ras1*, Ras^{N17} has an asparagine substitution at

position 17 within the nucleotide binding domain (Segal and Shilo; 1986, Feig and Cooper 1988). Under the sevenless promoter Ras^{V17} flies have a rough eye phenotype due to a reduction in the number of photoreceptor and cone cells (Freeman, 1996). *rg* rough eye phenotype is severely enhanced by Ras^{V17} (Fig. 1F and 2F). A constitutively active form of *Ras1*, Ras^{V12} results in over recruitment of all retinal cells causing a rough eye phenotype. Eyes of Ras^{V12} -; *rg rg* did not show any conclusive results because most of the alleles tested did not modify *rg* eye phenotype except rg^{r3} which showed mild suppression suggesting that either overexpression of *Ras1* does not modify *rg* eye phenotype or the genetic test used was not sensitive enough to detect such interactions. A hypomorphic *Ras1* loss-of-function mutation also has a rough eye phenotype due to a reduced number of retinal cells including cone cells. Mutations in *Ras1 (sevd2;E(e.1)* -), enhance the *rg* mutant phenotype (Fig. 1E and Fig. 2E). Taken together these results strongly suggest that *rg* may interact with *Ras* directly or indirectly.

***rolled* is an enhancer of *rg*:**

Loss-of-function mutations in *rolled* (rl^{EMS64} *CyO*) are recessive and show no external eye phenotype (Fig. 3B; Yamamoto, et al., 1996). rl^{EMS64} is a nonsense point mutation at codon 353 predicted to result in a truncation of 24 amino acids in the C terminal (Biggs et al., 1994). Homozygous flies have a reduced number of

photoreceptors and cone cells. Reduction in 50% of the *rl* gene product enhances *rg* eye phenotype (Fig. 3F). Gain-of-function *rl* mutation, rl^{Su23} *CyO* causes a rough eye phenotype due to over recruitment of all retinal cells (Fig. 3C; Sawamoto, et al., 1996). rl^{Su23} is a nucleotide substitution resulting in an amino acid substitution from aspartate to asparagine at position 334, in the kinase subdomain XI and has been shown to have the same molecular lesion as rl^{Sem} (Brunner et al., 1994; Lim et al., 1999), also a gain-of-function mutation. In a rl^{Su23} background *rg* rough eye phenotype is suppressed (Fig. 3E). *rl* is a MAPKinase and has been shown to be downstream of Ras and Raf but upstream of Sina and Yan transcription factors in the DER pathway; also dominant gain-of-function *rl* allele can suppress *sev*, *DER*, *Ras* and *raf* loss-of-function mutations (Brunner et al., 1994). Transgenic flies carrying a constitutively active $sevRas1^{V12}$ construct have rough eye phenotype due to multiple R7 cells and a complete loss-of-function in *rl* mutations suppress this phenotype. Multiple R7 phenotype caused by the constitutive activation of *raf* is also suppressed by loss-of-function *rl* mutation. These results show that *rl* is downstream of both *Ras* and *raf*. The findings that loss-of-function *rl* enhances *rg* phenotype and dominant gain-of-function *rl* suppresses *rg* mutant phenotype are consistent with the idea that *rg* may be involved in the *DER* signaling pathway (Fig. 3E and F).

argos* is a suppressor of *rg

Argos is a secreted protein (Freeman et al., 1992) that has been shown to regulate DER in a negative way and to interact competitively with Spitz (DER ligand), Rhomboid and Star (Kretzschmer et al., 1992; Okano et al., 1992; Freeman et al., 1994; Brunner et al., 1994; Sawamoto et al., 1994; Okabe et al., 1996). Other downstream components of the *DER* signaling pathway (*Dsor1*, *Ras*, *Raf* and *rolled*) have also been shown to interact with Aos implicating Aos as an important negative regulator of the DER signaling pathway in all the retinal cells of the *Drosophila* compound eye (Sawamoto et al., 1994; Golembo, et al., 1996; Sawamoto et al., 1996). Loss-of-function mutations in *aos* result in over recruitment of all retinal cells and over expression of *aos* results in a reduction in the number of photoreceptors, cone and pigment cells indicating that *aos* is a negative regulator of cell fate determination in the *Drosophila* compound eye. I therefore tested to see if *aos* interacts with *rg*. Loss-of-function mutations in *aos*, aos^{25^-} aos^{25^-}, suppress *rg* mutant phenotype (Fig. 4E and Fig. 5E) and restore the cone cell number (Fig. 6). aos^{25^-} is a mutation caused by the imprecise excision of P{lacW} insertion in aos^{P2} causing a deletion of the first exon including the translation initiation site (Okabe et al., 1996; Sawamoto et al., 1996). Wemmer and Klambt (1994) have identified closely linked genes, *bulge* and *soba* that modify *aos* phenotype. bul^{6d^-} is a loss-of-function mutation that enhances *aos* eye phenotype (Wemmer and Klambt, 1994).

bul^{6d7}/*TM6B* have slightly rough eye phenotype (Fig. 4Cand 5C; Wemmer and Klambt,1994)). Double mutant flies of *bul*^{6d7}/+; *rg*/+ genotype show that mutations in enhancers of *aos* enhance *rg* phenotype consistent with *aos* being a suppressor of *rg* phenotype. These results further support the idea that *rg* functions as a positive effector in the *DER* pathway.

***Star, spitz* and *rhomboid* interact with *rg*:**

Star (*S*), *spitz* (*spi*) and *rhomboid* (*rho*) belong to a group of genes known as the "spitz group". Mutations in these genes results in defects in the ventral ectoderm of *Drosophila* embryos. Furthermore these genes have also been shown to be involved in the development of the wing and the *Drosophila* compound eye. In the compound eye development the *spitz* group is required specifically in R8, R2 and R5 (Kolodkin et al., 1994). *S* encodes a putative membrane protein, and genetic studies have shown that *S* interacts with *Ras* and *aos* (Heberlein, et al. 1993; Sawamoto et al., 1996). While the function of Spi is well characterized as a ligand for DER, the roles of Star and Rhomboid are not clear though they are thought to positively modulate the Spi/DER interactions. Mutations in *S*, *S*^{54}/*CyO* show a slightly rough eye phenotype (Fig. 7C; Kolodkin et al., 1994) but removing one copy of *S* causes severe enhancement of the loss-of-function *rg* mutant eye phenotype (Fig. 7D, Table 1 and 2). Over expression of *S* under the heat shock promoter (*S*^{hs8}/*CyO*) completely

suppresses the *rg* mutant phenotype (Fig. 7E). The interactions of *S* with *rg* are quite dramatic and could imply a more direct modulation at the biochemical level. Deficiencies that uncover *spi* and *rho* also showed interactions with *rg* (Table 1 and Table 2). However results from further characterization of various *spi* and *rho* loss-of-function alleles with *rg* showed mild eye phenotype modification suggesting that this genetic screen may not have been sensitive enough or that *rg* may function in a separate but related pathway.

***rg* interacts with *sparkling (spa)*:**

A small number of genes that are specifically involved in the development of the accessory cells of the *Drosophila* compound eye have been identified. Among these, *sparkling* is required for the proper development of the cone cells and the primary pigment cells (Fu and Noll, 1997). Mutations in *spa*, spa^{pol1} spa^{pol1}, have a reduced number of cone and primary pigment cells (Fu and Noll, 1997) while *rg* mutations have a reduced cone cell number, normal primary pigment cell number and abnormal pigment lattice (Shamloula, 1996). A reduction in *spa* function by half enhances the *rg* rough eye phenotype (Table 2).

***Delta* interacts with *rg*:**

Delta belongs to a group of neurogenic genes that are required for the determination of the correct number of cells that take up a neural cell fate during

embryogenesis by inhibition of neural cell fate. Notch is a receptor in this signaling pathway and Delta is a transmembrane receptor. In the *Drosophila* compound eye, the Delta/Notch pathway is required for the establishment of the proper number of photoreceptor cells, cone cells, primary pigment cells and bristle cells (Cagan and Ready, 1989a; Parks et al., 1995). A deficiency that uncovers *Delta* {*Df(3R)DlBX12 ss e/+/ro TM6B*}, showed genetic interactions with *rg* mutations. I therefore tested different *Delta* alleles with various *rg* alleles. My results showed that mutations in *Delta* loss-of-function mutation (example Df^{oB3} *CyO* shown) severely enhanced *rg* mutant eye phenotype (Fig.8, Table 1 and Table 2). The double mutant phenotype was dependent on the *rg* gene dosage.

rg interacts with a deficiency that uncovers PKA-RII gene:

PKA signaling has been shown to be required in oogenesis and embryogenesis in *Drosophila* and may therefore play a crucial role in the early patterning events that determine egg and embryo polarity. In the *Drosophila* compound eye PKA has been shown to interact with *hedgehog* (*hh*), *decantaplegic* (*dpp*) and *wingless* (*wg*) for the initiation of the patterning events (Pan and Rubin, 1995; Heberlein and Moses, 1995; Burke and Basler, 1996; Huang and Kunes, 1996; Dominguez and Hafen, 1997; Chanut and Haberlein, 1997; Pignoni and Zipursky, 1997). The role of PKA in cone cell specification has not been studied. My results show that mutations in the

deficiency that uncovers the regulatory subunit of PKA-RII, *(DF(1)2R^{k1}/CyO*, enhance the *rg* rough eye phenotype (Table 2) suggesting a role of PKA signaling in cone cell differentiation.

DISCUSSION

Transmembrane Spitz is ubiquitously expressed in the eye imaginal disc and its activity is restricted by *Star* and *rhomboid* processing whose expression is initially restricted to R8, R2 and R5 and may induce and initiate the differentiation of subsequent photoreceptor cells (Freeman, 1996; Schweitzer and Shilo review, 1997), and later cone and pigment cells. It has been proposed that diffusion of secreted form of Spitz is controlled by Argos thereby ensuring spatio/temporal inductive cycles by DER activation (Freeman, 1996). I show here that a 50% reduction in the Argos function suppresses *rg* mutant phenotype and restores the cone cell number. *Star* is a severe enhancer of the *rg* phenotype. If *rg* is involved in the *DER* pathway, *argos* acts to negate the function of *rg*. These results are consistent with the finding that Argos over expression enhances *rhomboid* loss-of-function wing phenotype and *Star* eye phenotype (Sawamoto et al., 1994; Sawamoto et al., 1996). These observations

on genetic interactions are not conclusive as to whether *spitz* and *rg* act closely. One explanation for this could be that while *rg* may be involved in the *DER* pathway, other genes that modulate this involvement may be required such that the molecular interactions within the signaling cascade are not direct and hence may be masked in the genetic interaction screening carried out here. Alternatively *rg* may belong to a separate pathway that in concert with other signaling pathways like the *DER* pathway regulate cell development events that involve cone cell differentiation in the *Drosophila* compound eye.

The MAPKinase *rolled* plays a key role at all developmental stages involving *DER* and acts downstream of *Ras* (Brunner, et al., 1994). One of the downstream functions of *rl* gene product is to promote photoreceptor differentiation by modulating the activities of Yan and Pointed transcription factors; Pointed seems to be a positive regulator of differentiation and Yan is down regulated upon phosphorylation in differentiating cells, failure of which results in cell death (Rebay and Rubin, 1995; Brunner, et al. 1994; Lai and Rubin., 1992). It has also been proposed by Karim, et al., (1996) that Yan and Pointed act antagonistically to control *phyllopoid* gene which is required for the development of R1, R6 and R7 and *sina* gene which is required for R7 differentiation. R7 is the last photoreceptor to differentiate and may be involved in the next inductive cycle in the compound eye that

recruits the cone cells. It is therefore very tempting to speculate that *rg* may be involved in a cone cell promoting pathway either by direct receipt of activating or release from repressive state by some signal(s) from R7. Our observation that *rg* mutants also show an increased number of pigment cells (Shamloula, 1996) suggests to us that *rg* may be involved in inductive cycle that recruits the cells of the pigment lattice but not primary pigment cells. However a separate but related *rg* pathway independent or partially related to the Ras/MAPKinase pathway can not be ruled out. The Delta/Notch pathway is also a conserved pathway and has been shown to be repeatedly used during development in the specification of the correct number of cells that become neural cells in *Drosophila*. It has been shown to be required during oogenesis, embryogenesis and later stages for the establishment of adult structures in the third larval instar and pupal stages. Mutations in *Delta* enhance the *rg* mutant eye phenotype. These results are consistent with the notion that Rg functions with other genes in the *Drosophila* compound eye during cone cell specification. Further genetic and biochemical characterization of Rg interactions with Delta/Notch signaling will further elucidate how different signals are integrated during cell differentiation to elicit appropriate cellular responses.

Fig. 1: *rg* interacts with *Ras1* mutations. (A) is a scanning electron micrograph showing a smooth wild type eye (Canton-S) and (B) shows a *Ras1* eye. *sev*^{E}*;E(e.1b)* - allele, with almost a wild type appearance. In (C) a dominant negative mutant allele of *Ras*, *w*^{1118}*/w+,sevhs-Ras*^{V17}*/ CyO*, showing a rough eye phenotype. (D) is a micrograph of *rg*^{Y6} mutant eye showing a rough eye phenotype. (E) and (F) are double mutant eyes that show that *Ras* is a strong enhancer of *rg*. (E) *rg*^{Y6} *Y; sevd2;E(e.1b)* - (F) *rg*^{Y6} *Y; /w+,sevhs-Ras*^{V17} -. Magnification=220X (Shamloula, 1996).

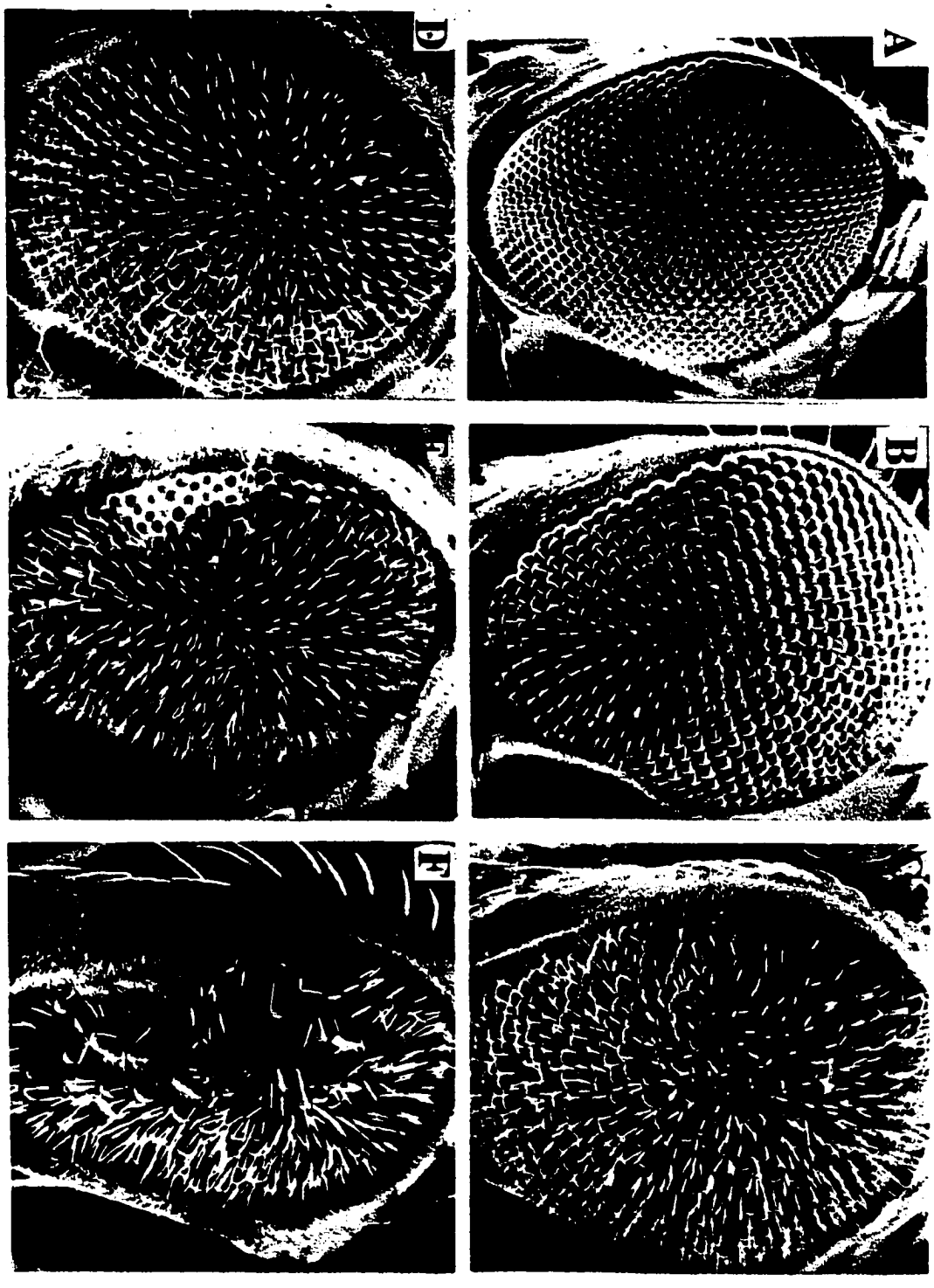


Fig. 2: Tangential sections through compound eyes from wild type, *rg* mutant, *Ras* mutant and *rg*; *Ras* double mutants. (A) *Canton-S*, (B) *Ras* allele sev^{E}; *E(e.1b)* - showing missing R7 cell (curved arrow) but some ommatidia have normal photoreceptor number and arrangement (arrow), (C) w^{1118}; *[w-sevhs-*Ras^{V1} *CyO* showing a disorganized pigment lattice (arrows), (D) rg^{m} *Y*, (E) rg^{m} *Y; [w-,sevhs-*Ras^{V1}*]/+*. A single copy of the *Ras* mutation results in strong enhancement of the *rg* phenotype leading to severely disorganized retina, missing photoreceptors cells, and breakdown of the hexagonal pigment cell lattice. [Bar=35μ] (Shamloula, 1996).

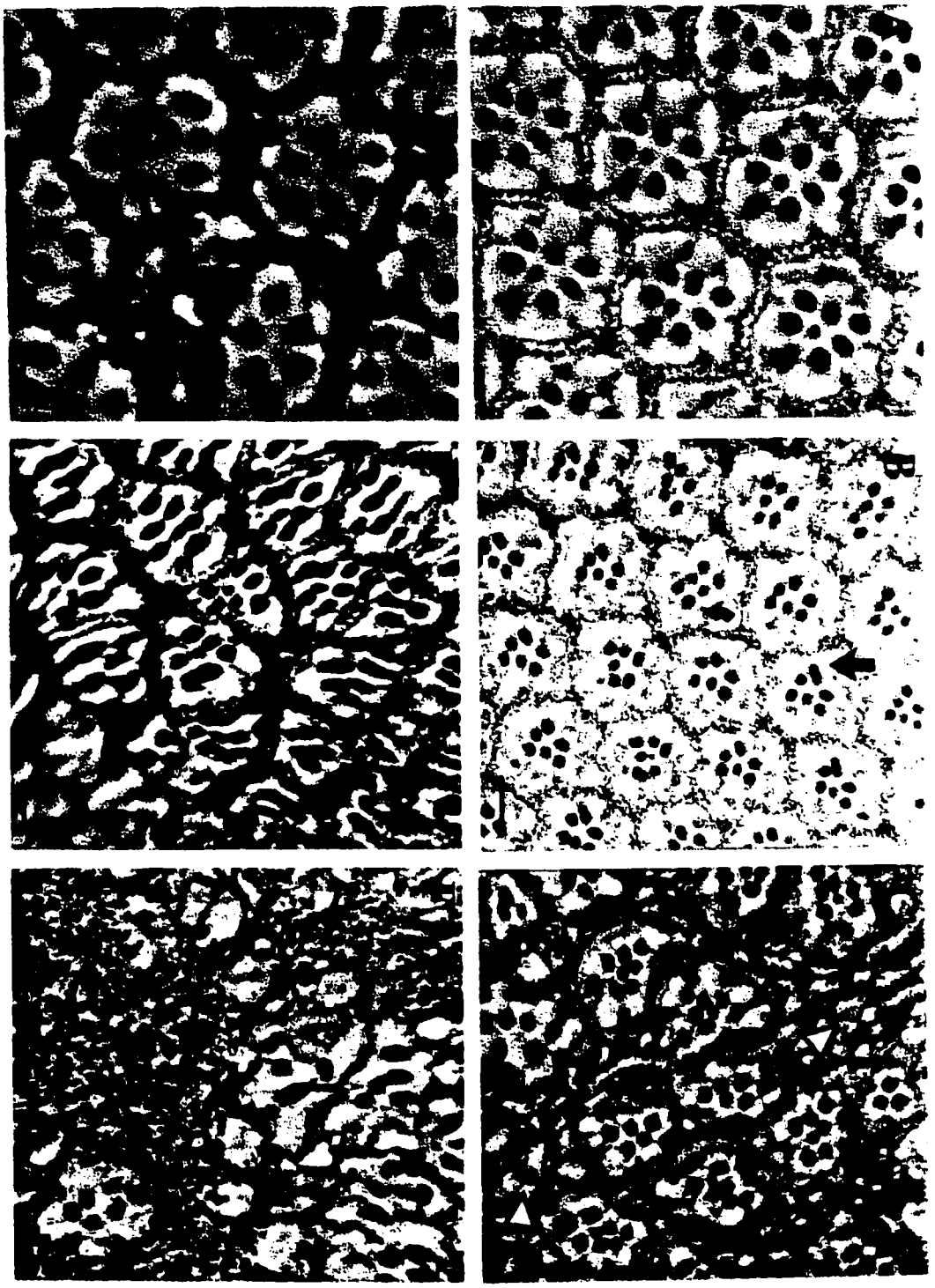


Fig. 3: *rg* interacts with *rolled* (MAP kinase). (A) Scanning electron micrograph of a wild type compound eye (*Canton-S*). (B) Compound eye from a rl^{EMS64} *CyO*, appears wild type (loss-of-function mutation). (C) The *rolled* gain-of-function allele (rl^{Su23} *CyO*) has a mild rough eye phenotype. (D) The compound eye from rg^{Y3} - shows a rough eye phenotype. (E) A single copy of the *rolled* gain-of-function mutation suppresses *rg* rough eye phentoype (rg^{Y3} *Y*; rl^{Su23} -). (F) A single copy of *rolled* loss-of-function mutation acts as an enhancer of *rg* eye phenotype (rg^{Y3} -; rl^{EMS64} -). [Magnification=250X]

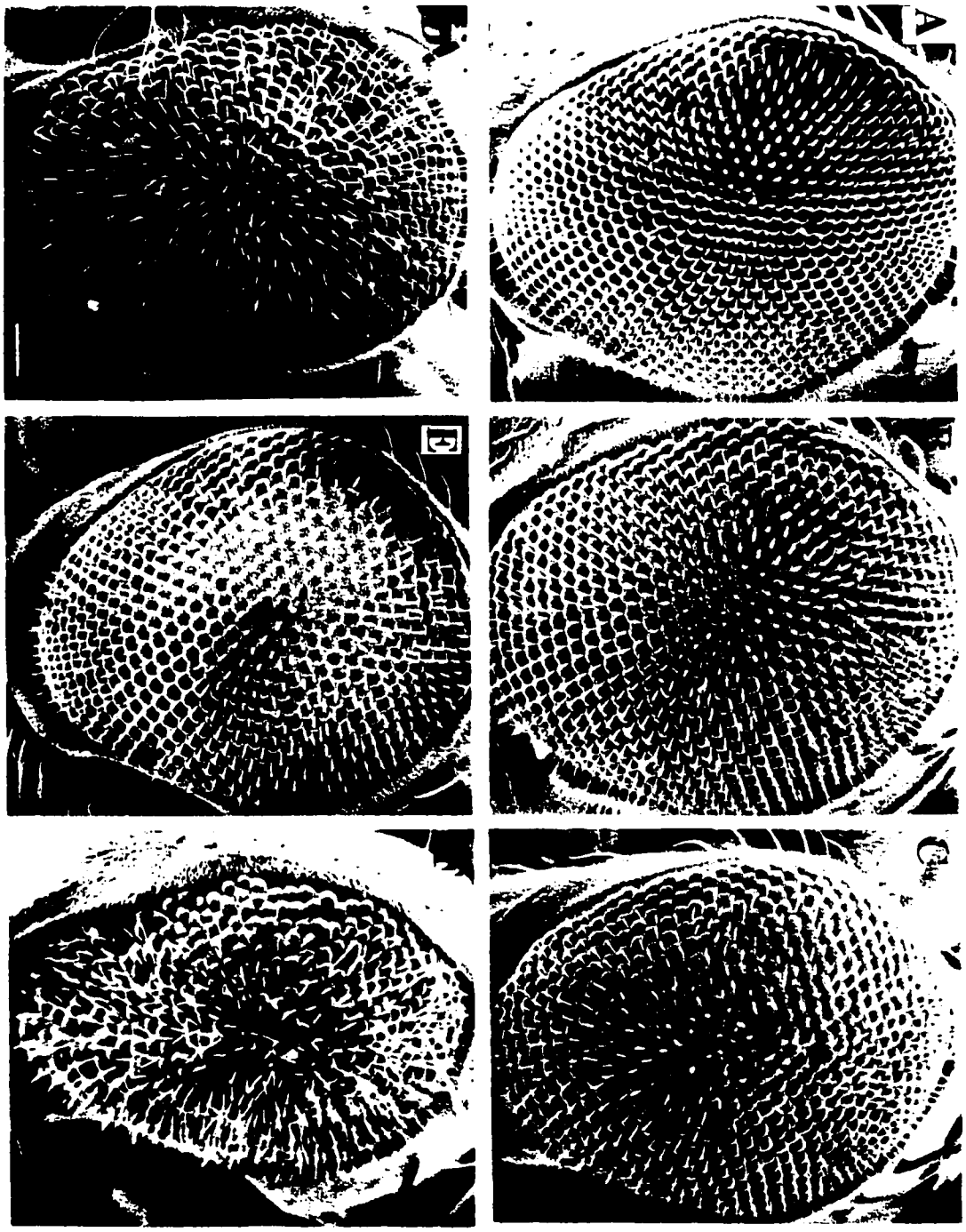


Fig. 4: *argos* mutation acts as a strong suppressor of *rg* eye phenotype. Scanning electron micrographs of the compound eye of (A) wild type, *Canton-S*, (B) rg^{Y10}/Y showing a moderately rough eye, (C) bud^{6d7}, a suppressor of *argos* showing a mildly rough eye phenotype, (D) $argos^{svn1}$ *TM3*. (E) a single copy of *argos* acts as a strong suppressor of the *rg* rough eye phenotype (rg^{Y10} *Y*; $argos^{svn1}$ -). (F) rg^{Y10} *Y*; bul^{6d7} - double mutant showing that a single copy of bul^{6d7} enhances *rg* rough eye phenotype. [Magnification=220X].

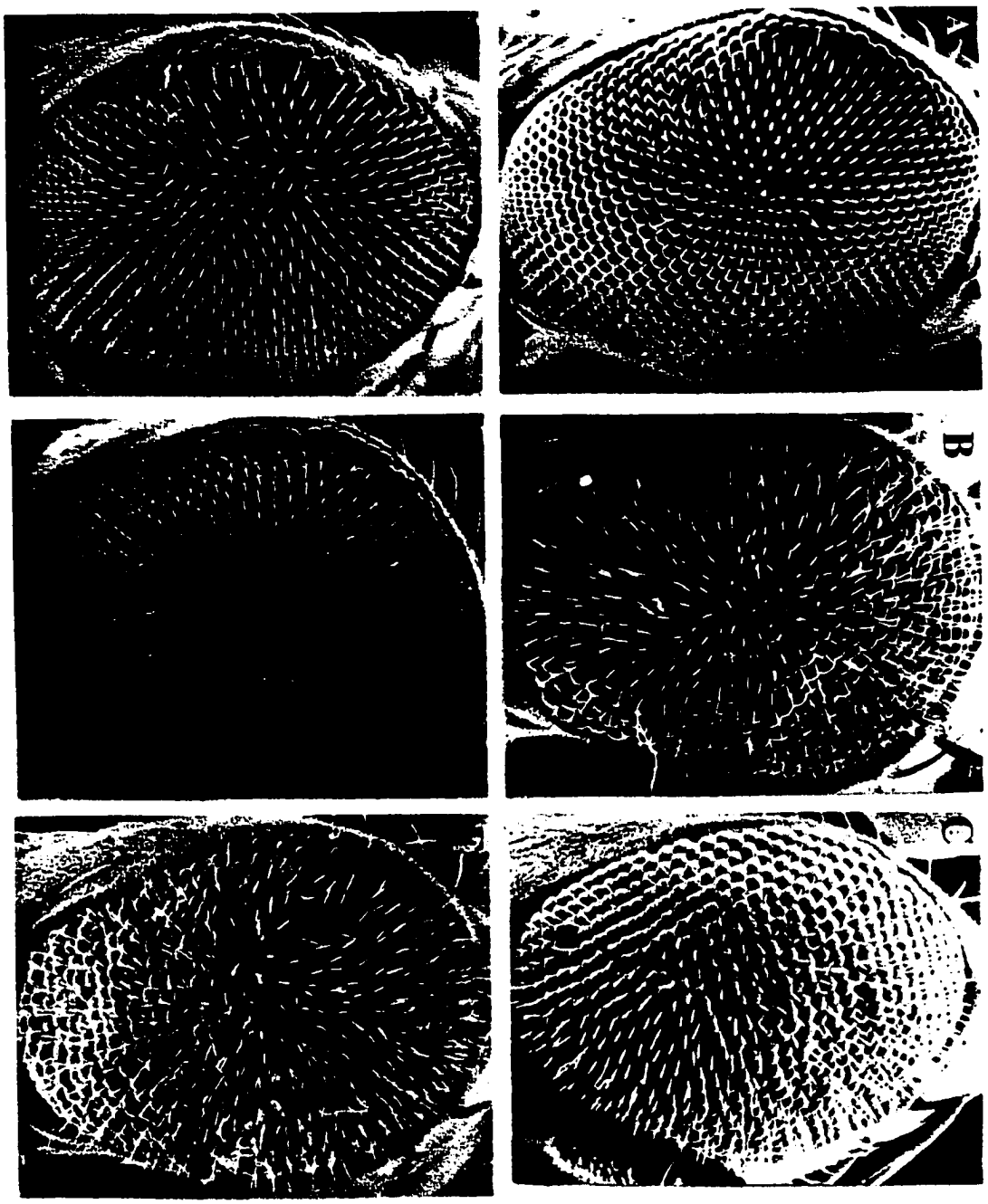


Fig. 5: Anatomy of the *argos* and *rg* double mutants. Light micrographs of the compound eyes tangential sections. (A) Wild type (*Canton-S*) normal hexagonal pigment lattice and the reiterated arrangement of the photoreceptor cells in each ommatidium. (B) rg^{Y10} *Y* eye showing disrupted pigment cell lattice (arrow head) and disorganized photoreceptor cell arrangement. In some of the ommatidia, some of the photoreceptor cells are missing due to the collapse of the basement membrane. (C) $bul^{b\alpha\Gamma}$ *TM3* ommatidia appear to be normal with slight disorganization of the pigment lattice. (D) $argos^{sn^{1}}$ *TM3* have normal ommatidial arrangement. (E) rg^{Y10} *Y*; $argos^{sn^{1}}$ - double mutant showing that a single copy of *argos* completely suppresses the *rg* phenotype restoring normal pigment cell lattice and arrangement of the photoreceptor cells. (F) rg^{Y10} *Y*; $bul^{b\alpha\Gamma}$ - showing that a single copy of $bul^{b\alpha\Gamma}$ enhances the rough eye phenotype of *rg*. There is a greatly disorganized retina, excess pigmentation, distorted pigment cell lattice (arrow). [Bar=30μ]

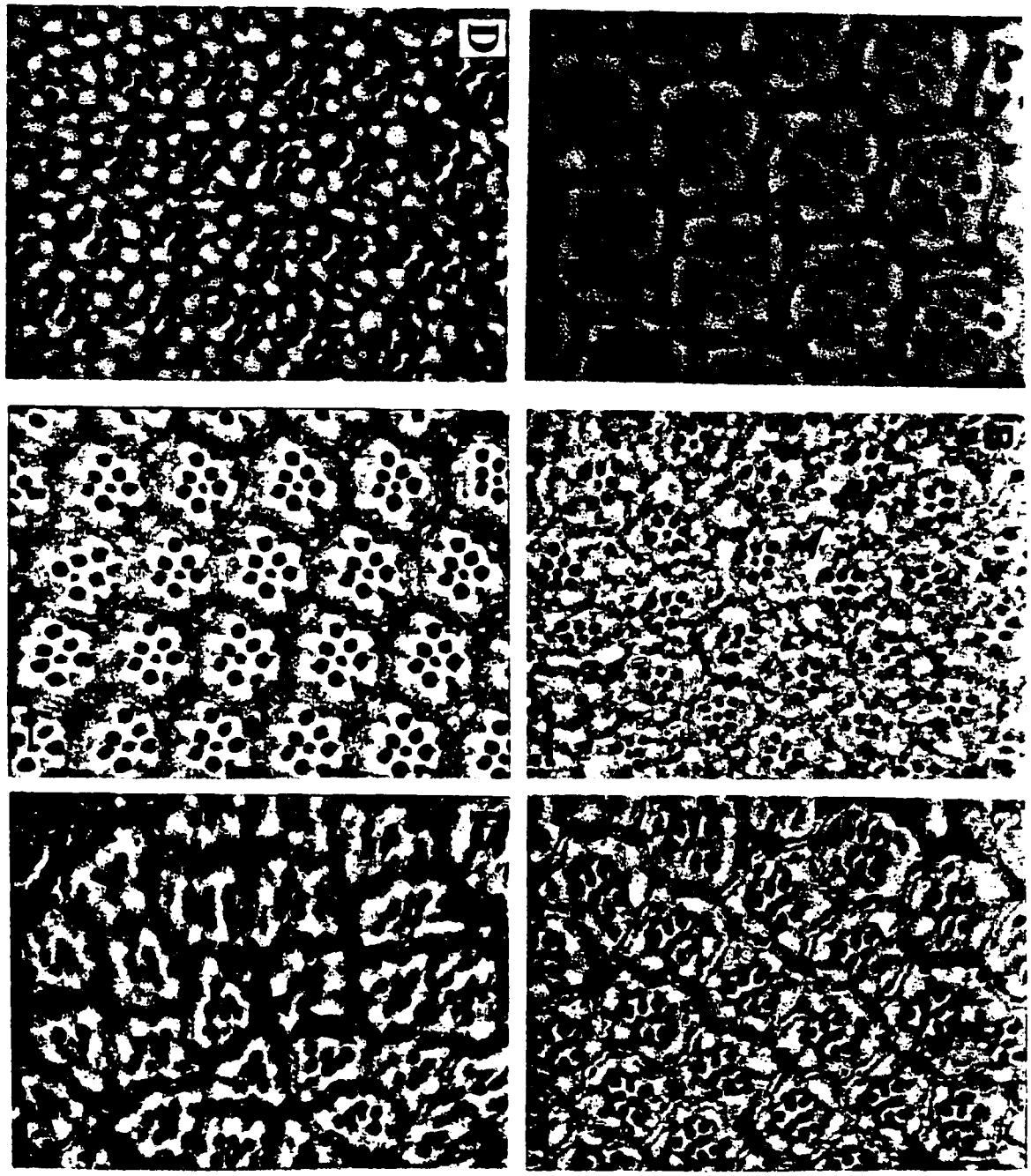


Fig. 6: The *argos* interactions with *rg* were confirmed with Cut antibody staining (Staining procedure as explained in chapter IV material and methods). (A) *Canton-S* Cut antibody staining showing four cone cell clusters. (B) $argos^{25^-}$ *TM3* also shows normal four cone cell clusters. (C) rg^{rn} *Y;* $argos^{25^-}$ – double mutant shows that the *rg* cone cell number is restored to normal.

A

B

C

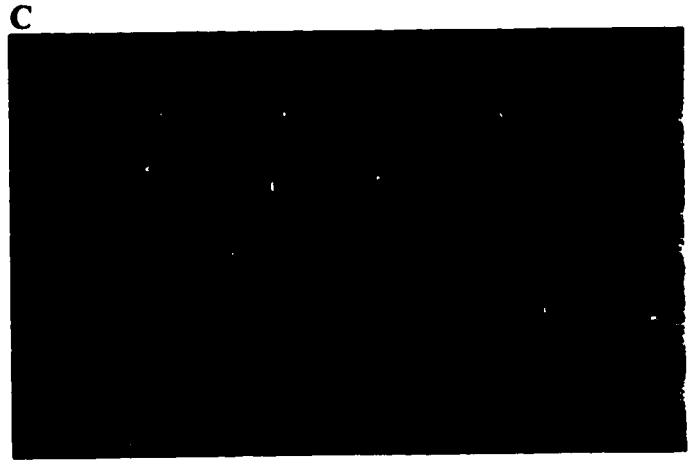


Fig. 7: *Star* mutations act as strong enhancers of the *rg* eye phenotype. (A) Wild type (*Canton-S*), (B) rg^{Y6} *Y*, (C) S^{54} *CyO*, a mildly rough eye phenotype. (D) rg^{Y6} *Y*; S^{54} + double mutant shows severe enhancement of the *rg* rough eye, (E) rg^{Y6} *Y*; S^{Hsd}/+ fly. A heat shock promoter induced expression of Star protein suppresses the *rg* eye phenotype. [Magnification=250X]

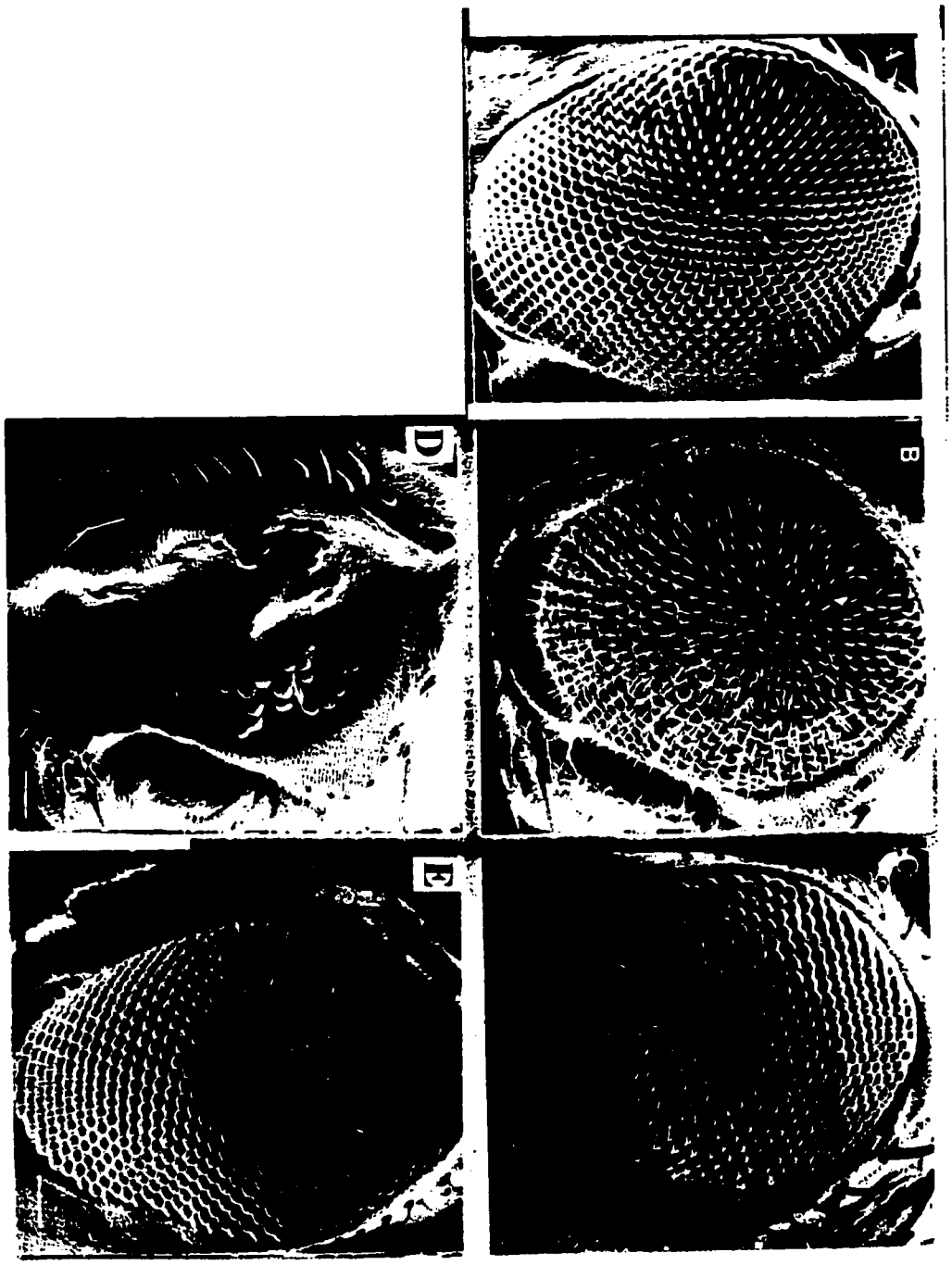


Fig. 8: *Delta* interacts with *rg*. Scanning electron micrographs of (A) *Canton-S* flies showing a smooth eye. (B) rg^{vo} *Y* showing a rough eye phenotype. (C) is a *Delta* loss-of-function mutant, Dl^{nB3} */TM6c* mildly rough eye phenotype. Reducing the *Delta* function by 50% enhances the rough eye *rg* phenotype (D). [Magnification=160X]

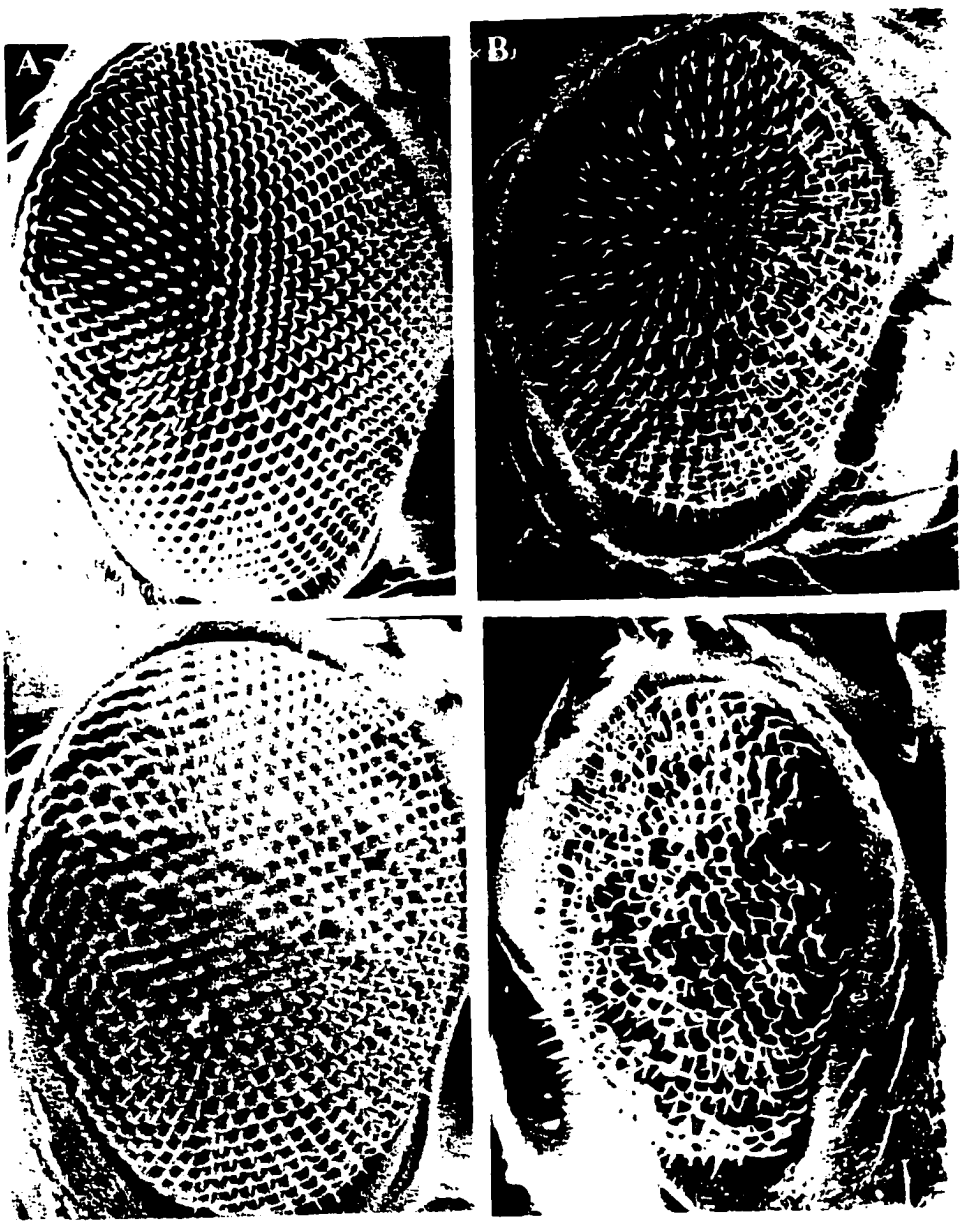


Table 1 : Deficiencies Interacting with *rugose*

Stock #	Genotype	Breakpoints	Phenotype	Gene Identified
3079	*Df(2L)Prl/CyO*	032F01-03; 033F01-02	Enhancer	
3189	*Df(2L)TW50,cn CyO,Dp(2;2)M (2)m[+]*	036E4-F01; 038A06-07, 036F+?	Suppressor	
1473	*Df(2R)Px4,Dp (2L)Px4,In(2LR) Px4,dpb CyO*	060B;060D 01-02,021D 01;022A03, 022A;060B; 021C08;060 D;042A;058A	Enhancer	duplication of *Star*
745	*Df(2L)S2 In(2L+ 2R)CY,CyE(S)*	021C06-D01; 022A06-B01	Enhancer	*Star*
3012	*Df(3R)DlBX12, ss e[4]ro/TM6B*	091F01-02;0 92D03-06	Enhancer	*Delta*
3650	*Df(3L)M21,ri p[p]/Dp(3;3)T33 [L]fl9[R]*	062F;063D,0 62A;064C	Enhancer	62-daughter of *sevenless(dos)*
3647	*Df(3L)HR370/D p(3;3)T33[L]fl9 [R]*	063A01;063D 10,062A;064C	Suppressor	
1893	*Df(3R)by62,T(2; 3)by[62],red e/T M1*	085D11-14;08 5F06041h;085 F06	Enhancer	*RasD85* (GTP binding protein)
2998	*Df(3L)81K19/T M6B*	073A03;74F	Suppressor	*argos*

Table 2 continued

Stock #	Genotype	Breakpoints	Phenotype	Gene identified
3687	*Df(3L)GN50/TM 8, 1(3)4[DTS]th st Sb e;w[1118]*	063E01-02;06 4B17	Suppressor	
3068	*Df(3L)Pc-MK T M3*	078A03;079 E01-02	Suppressor	
2425	*Df(3R)e-N19 TM2*	093B;94	Suppressor	
3649	*Df(3L)HR119 T M6B*	063C06;063E	Suppressor	
1547	*Df(2R)PC4/CyO*	055A;055F	Enhancer	
3368	*Df(2R)cn9 Cy Roi*	042E;44C	Suppressor	
3344	*Df(2R)prdl,7, b Adh[n2] pr cn* sca/CyO	033B02-03;03 4A01-02	Enhancer	
3648	*Df(3L)HR232 T M6B*	063C01;063 D03	Suppressor	
430	*Df(3R)3450 TM 6B;w[1118]*	098E03;099 A06-08	Suppressor	
3084	*Df(2L)ast2/SM1*	021D01-02;02 2B02-03	Enhancer	
3649	*Df(3L)HR119/T M6B*	063E01-02;06 4B17	Suppressor	
2547	*Df(3L)vin2/TM3*	067F02-03068D06	Enhancer	

Table 2: Genes Interacting with *rugose*

Interacting Genes	Alleles tested	*rugose* alleles	Interaction
argos	*argos*$^{25^-}$	*rg*$^{\gamma 3}$. *rg*$^{\gamma 6}$. *rg*γ^-. *rg*$^{\gamma 10}$	Suppressor
	argos^{stv1} *TM3*		
	argos^{stv2} *TM6B*		
	hs agos=4		Enhancer
argos modifiers	*soba*^{sal} *TM3*	*rg*$^{\gamma 1}$. *r*$^{g\gamma 6}$. *rg*$^{\gamma 10}$	Enhancer
	1040^{bulD} *TM3*		
	bul$^{6d^-}$ *TM3*		
Ras1	*sev*^{d2}.*E(e.1.B)th.st.ca TM3*	*rg*$^{\gamma 1}$. *rg*$^{\gamma 6}$. *rg*$^{\gamma 10}$	Enhancer
	sev$_{d2}$:*E(e.1.B)* isogenic		
	Ras^{v12} *CyO*		suppressor
Ras$^{N17^-}$ (dominant negative form)	*[Ras*$^{N17^-}$*]A CyO*	*rg*$^{\gamma 1}$. *rg*$^{\gamma 5}$ *rg*$^{\gamma 6}$. *rg*$^{\gamma 10}$	Enhancer
	[Ras$^{N17^-}$*]C CyO*		
	[Ras$^{N17^-}$*]E CyO*		
Star	*S*^{1} *CyO*	*rg*^{1}. *rg*^{1}. *rg*^{2}. *rg*γ^{5}. *rg*$^{\gamma 4}$. *rg*$^{\gamma 5}$. *rg*$^{\gamma 6}$. *rg*$^{\gamma^-}$. *rg*$^{\gamma 8}$. *rg*$^{\gamma 9}$. *rg*$^{\gamma 10}$. *rg*$^{\gamma 11}$.	Enhancer
	S^{2} *CyO lm*		
	S^{11N23} *CyO*		
	w$_{1}$ *S*$_{1}$ *ho/CyO*		
	cn bw/SM6a IKE 4.28.1		

Table 2 cont'd

Interacting genes	Alleles tested	*rugose* alleles	Interaction
Star alleles	*cn bw SM6a IV KE 2.4.2*	rg^{1}. rg^{1}. rg^{2}. $rg\gamma^{3}$. $rg^{\gamma 4}$. $rg^{\gamma 5}$. $rg^{\gamma 6}$.	Enhancer
	cn bw SM6a VII KE 4.1.1	$rg^{\gamma 7}$. $rg^{\gamma 8}$. $rg^{\gamma 9}$. $rg^{\gamma 10}$. $rg^{\gamma 11}$.	
	VII 30 E1 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 8.E1cn bw SM6a IX KE 4.0.1		
	E3R12X cn bw SM5		
rolled (MAPKinase)	*rl/1/* (loss of function)	$rg^{\gamma 1}$. $rg^{\gamma 2}$. $rg^{\gamma 5}$. $rg^{\gamma 6}$. $rg^{\gamma 8}$. $rg^{\gamma 10}$	Enhancer
	rl^{Su23} *CyO* (loss-of function)		Enhancer
	rl^{ems64} *SM1* (gain-of function)		Suppressor
EGFR	*Ellipse CyO*	$rg\gamma^{3}$. $rg\gamma^{4}$. $rg\gamma^{6}$. $rg^{\gamma 10}$. $rg^{\gamma 11}$	Suppressor
	pn cn top^{1} *bw CyO*		Enhancer
	co stw pwn CyO		Enhancer
	2C82 CyO		
rhomboid	*Df(3L)HR370*	$rg^{\gamma 6}$. $rg^{\gamma 10}$	Suppressor
spitz	*Df(TW50)cn*	$rg^{\gamma 6}$. $rg^{\gamma 10}$	Suppressor
RII (PKA regulatory subunit)	*Df(2R)X1/CyO*	$rg^{\gamma 6}$. $rg^{\gamma 10}$	enhancer

Table 2 cont'd

Interacting Genes	Alleles tested	*rugose* alleles	Interaction
sparkling	spa^{pol}	rg^{x3}, rg^{x6}	Enhancer
	$w;spa^{pol}$		
Delta	Dl^{RF} *TM6c*	rg^{x3}, rg^{x6}	Enhancer
	$Dl(3R)^{M2}$ *TM6c*		
	Dl^{9B39} *es ca TM6c*		
	$ssDl^{6B3?c}$ *TM6* c		

Table 3: A summary of genes that genetically interact with *rg*.

Identified genes	**Genetic Interactions with *rg***	**Cellular/functional requirement in the eye**	**Biochemical pathway**
argos	suppressor	R cells, cone cells as a negative regulator	EGFR
argos suppressors	enhancers		
Star	enhancer	R8, R2 and R5; modulates receptor ligand interactions	EGFR
Ras	enhancer	all retinal cells	EGFR, Sevenless
rolled (MAPKinase)	enhancer	all retinal cells	EGFR, Sevenless
sparkling	enhancer	cone cells, primary pigment cells	?
EGFR	enhancer	all retinal cells	EGFR
Delta	enhancer	all retinal cells	Delta/Notch neurogenic pathway
RII-PKA	enhancer	cone cells?	PKA signaling

Table 4: Flies used in this study

Genetic Locus	Genotype	Source	References
argos	*argos*^{sty1} *TM3*	Dr. Okano (University of Osaka)	Sawamoto et al., 1996
	argos$^{25^-}$		
	argos^{sty2} *TM6B*		
	hs-argos=4 (transgenic)		
argos modifiers	*soba*^{sal} *TM3*	Dr. Klambt (University of Kohn	Wemmer and Klambt, 1995
	bul$^{6d^-}$ *TM6*		
	1040$^{bul\ d}$ *TM6*		
ras1 (loss-of-function	*sev*^{d2} *;E(e.1.B)th st ca T21A*	Dr. Simon (Stanford University.)	Simon, 1991
Ras$^{N1^-}$ (dominant negative)	*sev*^{d2} *;E(e.1.B) T21A* isogenic		Matsuo et al., 1997
	w^{1118} *;P[w−,sev hs-Ras*$^{N1^-}$ *] A Cyo*		
	w^{1118} *;P[w−,sev hs-Ras*$^{N1^-}$ *] C CyO*		
	w^{1118} *;P[w−,sev, hs-Ras*$^{N1^-}$*] E CyO*		
ras^{v12} (constitutively active)	*CRI, Cyo,dp,pr,cn P[ry, sev-ras1*^{v12} *]/Sco*		Dickson et al., 1992

Table 4 cont'd

Genetic locus	Genotype	Source	Reference
	CR2, CyO, P[sev-ras^{v12}] Sco		
	TM3, P[ry,sev-ras^{v12}] e,ftz, ry	Dr. Benarjee (UCLA)	
Star	*S^{2} CyO*	Dr. Benarjee (UCLA)	Kolodkin, 1994
	S^{54} CyO		
	S^{11N23},cn, bw, sp CyO		
	$w_{1}S^{hs8}$ CyO		
	cn bw SM6a IKE 4.28.1	Dr. Zipursky (UCLA)	
	cn bw SM6a IV KE 2.4.2		
	cn bw SM6a VIII KE 4.1.1		
	VII 30 E1 cn bw SM6a		
	E3X12X cn bw SM6a		
	cn bw SM6a VII KE3.22		
	cn bw SM6a II KE 1.0.4		
	cn bw SM6a VIII KE 3.6. C		
	II.8.E1 cn bw SM6a IX KE 4.0.1		
	E3R12X cn bw SM5		

Genetic locus	Genotype	Source	Reference
	E3R34Xcn bw SM5		
Star	*E3RIIX cn bw SM5*		
rolled	*lt rl[1]*	Bloomington stock center	Bigg et al., 1994
	rl[1]		
	rl^{ems64} *SM1*	Dr.Stevens (Albert Einstein School of Medicine)	Sawamoto et al., 1996
	rl^{su23} *CyO*		
deadhead	$w;dhd^{pN}$	Dr. Salz (Case Western Reserve University)	Salz, 1992
DER	$pn\ cn\ top^{1}\ bw\ CyO$	Dr. Schupbach Princeton University)	Clifford and Schupbach, 1994
	co stw pwn CyO)		
	2C82 CyO		
	IP02 CyO		
	$b\ pr\ tor^{RL3}\ cn\ CyO$		
	Y9, cn bw SM1		
	Elp/CyO	Dr. Baker (Albert Eintein College of Medicine)	Baker and Rubin, 1992
RII (PKA regulatory subunit	$Df(2R)X^{1}/CyO$	Dr. Kalderon (Columbia University, NY)	Kalderon and Rubin, 1988
sparkling	$w;\ spa^{pol}/spa^{pol}$	Dr. Noll (University of Zurich)	Fu and Noll, 1997

Table 4 cont'd

Genetic locus	Genotype	Source	Reference
	spa^{pol}	Dr. Muskavitch (Indiana University)	Parks and Muskavitch, 1993
Delta	*Dl*^{RF} *TM6c*		
	Dl(3R)^{M2} *TM6c*		
	Dl^{9B39} *es ca TM6a*		
	ssDl$^{6B3^{-}e}$ *TM6c*		
Suppressor of Hairless	*Su(H)/1)In(2L)Cy,In(2R)Cy, Cy[1]pr[1]*	Bloomington stock center	Sweisguth and Posakony, 1992
source of transposase	*w w;2-3SbTM3 Drop*	Dr. Salz	Salz, 1992
rg duplication	*ywf,ywsnf210;RDUP(w-) -*		Salz, 1992
Deficiency stocks	*Df(1)A113 C(1)DX,ywf;Dp (1;2)w[=64b] -*	Bloomington stock center	Salz, 1992
	Df(1)JC70 FM7c,sn[-]		
rugose	*rg*^{1} *rg*^{1}	Bloomington stock center	Salz, 1992

CHAPTER IV

MOLECULAR CHARACTERIZATION OF THE *rugose* GENE

INTRODUCTION

Phenotypic and genetic analysis as discussed in the previous chapters showed that *rg* gene function is required for the proper differentiation of the *Drosophila* compound eye and may function in a signaling pathway involving *Ras* signaling. To understand the nature of Rg function a molecular characterization of *rg* gene was undertaken. This work was facilitated by the fact that the genetic region 4 E-4 F has been well characterized and the *rg* gene locus had been placed in this region by Salz (1992). In order to understand the molecular nature of *rg* gene and its expression patterns, I undertook to clone the DNA from the *rg* locus and determine the mRNA expression patterns. In this chapter I will present evidence of cloning of the *rg* gene and show that *rg* encodes a *Drosophila* protein kinase A anchoring protein (AKAP), DAKAP550. mRNA expression data shows that *rg* is expressed dynamically during embryogenesis and also in the third larval instar eye disc consistent with the DAKAP550 protein expression data (Han et al., 1997). My data on PKA regulatory subunit (RII) distribution shows that it is expressed in the *Drosophila* eye imaginal disc during the third larval instar and colocalizes with monoclonal antibody Mab22C10, a neuron marker. In the *rg* mutants the distribution of RII is diffuse suggesting that PKA requires AKAP for proper

localization in the *Drosophila* compound eye.

MATERIALS AND METHODS

In order to clone *rg*, various strategies were utilized. First, because some of the mutants generated were by the remobilization of a single P element that contains a P lac Z and an ampicillin resistance gene an attempt to clone the gene by plasmid rescue was made. Initially this was a promising method and a few positive colonies were isolated, and it was hoped that they could be used to isolate the flanking sequences to initiate a genomic walk. However, further analysis of the DNA flanking the P element produced inconsistent results that made continuing the genomic walk impossible.

Cytological Mapping of *rg*:

Strategies to clone and characterize *rg* gene are as outlined in the flow chart (Appendix B).

Deficiency Mapping:

Virgin females from seven different strains that carry deficiencies in the following cytological regions were crossed with rg^{ro} males: *Df(1)RC40/FM7*, (4B1-4F1); *Df(1)A113 C(1)DX, y w f*, (3D6-4F5); *Df(1)JC70/FM7c*, (4C15-5A); *Df(1)ovoG6/FM7*, (4C5-4F2); *Df(1)svbEH/Blmsc*, (4C-4F1,2); *Df(1)y w DEB4D/FM7*, (4E1,2-4F11,12);

Df(1)GA56 FM7, (4C5-4D); *Df(1)ovo44 FM7*, obtained from the Indiana stock center (Banga et al., 1986; Oliver et al., 1988). The F1 non balancer females heterozygous for *rg* were scored for rough eye phenotype.

Duplication Mapping:

yw/f,XX,ywsmf210; RDUP(w+) - flies carry the duplication of the 4C11,12-4F12,12 regions on the second chromosome. To determine if this duplication contains the *rg* locus, rg^{m} males flies carrying the duplication were scored for eye phenotype.

Southern Blot Analysis:

Drosophila genomic DNA was isolated as per Rubin Laboratory Protocols (1990). Restriction enzyme digestion was as recommended by the manufacturer (New England Biolabs). Digested DNA was resolved on a 0.8% agarose gel in 0.5X TBE at 100 volts for 90 minutes. DNA from six overlapping P1 phage clones was isolated (P1 clones were obtained from Dr. Spradling Carniege Institute, Baltimore), using Quiagen columns. Isolation of fragments from agarose gel was done using Geneclean kit. The radioactive labeling using $^{\alpha\text{-}32}$P dCTP random-hexamer DNA labeling kit from Boehringer Mannhein was as per the manufacturer (specific activity of the probe was 4.0×10^{7}cpm/ug). Southern blot and hybridization was done according to Maniatis et al., (1987) using high stringency washes. cDNA probes were labeled using the same protocol.

Genomic Walking:

As part of the Berkeley *Drosophila* genome project, the entire *Drosophila* genome has been subcloned into P1 phage vectors, facilitating cloning of genes. P1 clones contain 80-100 Kb genomic DNA. Six P1 clones (see table 1) containing genomic DNA from the 4E-4F region were obtained from The Berkeley *Drosophila* genome project (courtesy of Dr. Spradling, Carniege Institute, Baltimore). A 2.5 Kb DNA fragment from the *deadhead* region in Bluescript KS+/- (obtained from Dr. H. Salz) was used to initiate a genomic walk by probing a Southern blot containing EcoR1 digested DNA from the P1 clones.

Subcloning of *rg*CD10:

Putative *rg* cDNA (*rg*CD10) was subcloned into a Bluescript pBSKII(+/-) plasmid vector (Stratagene) and transformed into XL1-Blue MRF' *E. coli* cells as described by the manufacturer (Stratagene).

Sequencing of *rg*CD10 and analysis:

The sense and antisense 1.7 Kb *rg*CD10 strands were sequenced by automated DNA sequencing (The Yale University Boyer Center Sequencing Facility) and analyzed using the Blast program. The two strands were 100% percent consistent with one another. Sequence homology was determined by searching the DNA database.

Synthesis of sense and antisense cDNA probes:

Digoxigenin-UTP labeling of RNA probes was as per Dr. Leslie Pick (Mt. Sinai Hospital, NY) modification of Tautz and Pfeifle (1989) procedure. The sense and antisense

probes were synthesized from 1.7 Kb *rg*CD10 which was subcloned into a Bluescript vector, pBSKII+/-, using a Boehringer Mannheim non-radioactive labeling Kit. After 2 hours synthesis at 37°C, hydrolysis was at 65°C in 2X carbonate buffer (120mM Na_2CO_3, 80mM $NaHCO_3$, pH 10.2) and the reaction was stopped in stop buffer (0.2M NaAc, pH 6.0 with acetic acid). The probe was precipitated in 5 μl of 20 mg/ml tRNA (phenol/chloroform extracted), 300 methanol and 10 μl of 4M LiCl at -20°C for 15 minutes and pelleted for 20 minutes at 4°C, washed in 70% ethanol and resuspended in 150 μl hybridization mix (50% formamide, 5X SSC, 100 μg/ml autoclaved salmon sperm DNA, 50 μg/ml Heparin and 0.1% Tween 20). 1 μl of labeled probe in 50 μl hybridization mix was used per reaction.

Whole mount *in situ* hybridization

Embryo Collection:

4-24 hour mutant and wild type embryos were collected and dechorionated in 50% sodium hypochlorite (3-5 minutes). The embryos were fixed for 30 minutes with rocking, in fixation buffer (100 μl 1.3X PBS containing 67 mM EGTA, 100 μl 37% formaldehyde, 400 μl heptane). The lower aqueous phase was removed and methanol added, shaken vigorously (10 seconds) and left to settle. Further washing was done twice in methanol and 3 times in ethanol. Embryos were stored in ethanol at -20°C.

Dissection of Eye Discs:

Eye discs with optic lobes attached were dissected in Ringer's Solution and washed

twice for 5 minutes in PBS. Carcasses were fixed for antibody preabsorption. Eye discs were fixed in 4% formaldehyde, 0.6% Triton X-100 in PBS, for 15 minutes, washed 3 times for 5 minutes each in PBT (0.1% Tween-20 in PBS) and digested with Proteinase K (100 μg/ml in PBT). Digestion was stopped by 1X glycine in PBT for 10 minutes, washed twice for 5 minutes each in PBT and post-fixed for 15 minutes in 4% formaldehyde, 0.1-0.2% glutaraldehyde in PBS, then washed 5 times for 5 minutes in PBT.

Hybridization:

Single stranded mRNA in-situ hybridization was carried out according to Tautz and Pfeifle (1989) and as modified by Dr. Leslie Pick (Mount Sinai Hospital, NY) at 55°C overnight for ovaries and embryos, and 48°C for eye discs for 16-40 hours.

Immunohistochemistry:

Third larval instar eye antennal discs were dissected in 1X PBS and fixed in 4% paraformaldehyde for 15 minutes, washed in 0.6 % Triton-X in PBS and incubated in 1:100 dilution monoclonal antibody MAb22C10 and 1:50,000 dilution polyclonal antibody anti-RII (dilutions in PBT) for 3 hours at room temperature or overnight at 4°C. The eye antennal discs were then washed and incubated in goat anti-mouse Rhodamine (for MAb22C10) and goat anti- Rabbit FITC (for anti-RII) for 2 hours at room temperature, washed and mounted in N-propylene gallate in glycerol (0.08 grams N-propylene gallate in 15 ml PBS and 5 ml glycerol)

Cut antibody staining was carried out as above. White prepupae were collected and considered time zero of the pupation period. The pupae were staged for 50 hours (23-25°C) postpupal stage and the eye discs were dissected in 1X PBS. A 1:100 dilution Cut antibody was used and 1:200 FITC conjugated anti-mouse secondary antibody.

PCR analysis of wild type and *rg* mutant genomic DNA:

Standard methods were used to amplify a genomic product from the wild type and the *rg* mutants from the *rg* gene locus. Primers were synthesized from the PKA anchoring protein, DAKAP550 cDNA (RGS1-CTTGTCCTCACCTCAATGAA and RGS4-AACTGCTGTTGCTGACCG). Amplified products were gel purified using 0.8% agarose gel. To determine any RFLPs the DNA PCR products were EcoR1 digested and resolved on an agarose gel. Because the primers were synthesized from within the *rg*CD10 region any EcoR1 sites would indicate the presence of an intron and RFLPs would indicate DNA lesions within the amplified region amongst the different *rg* mutant alleles. Standard Southern blot and labeling procedures were used to test if any *rg*CD10, a putative *rg* cDNA hybridized with the PCR products.

RESULTS

Cytological location of *rg*:

By using deficiencies the *rg* gene was mapped to the 4F1,2 region (Fig. 1). In order to confirm the deficiency mapping results, rg^{ro}/rg^{ro} females were crossed with the flies carrying a duplication containing 4E-4F region, and F1 rg^{ro} *Y* males carrying the duplication were scored for eye phenotype. The duplication which spans the 4C11,12-4F11,12 complemented the *rg* rough eye phenotype confirming that *rg* is contained within the 4E-4F region (Shamloula, 1996). These results were consistent with those obtained by Salz (1992), placing *rg* in the duplication 4C11,12-4F11,12 during a screen to find mutations in *Df(1)JC70* that are either female lethal, sterile, sonless or daughterless.

Chromososmal walking using P1 clones:

To initiate a chromosomal walk in the *rg* region, a 2.5 Kb genomic fragment from the *dhd* region was used. The 2.5 Kb *dhd* fragment hybridized to a 5.8 Kb fragment on a genomic Southern blot containing DNA from wild type and all γ and P element induced *rg* mutant alleles, confirming that this was indeed a genomic fragment from outside the *rg* region because no Restriction Fragment Length Polymorphisms (RFLPs) were detected (Shamloula, 1996, dissertation). The 2.5 Kb *dhd* fragment was used to initiate a walk in the P1 clones. Two of these P1 clones, #82-63 and #5-14 hybridized with the *dhd* fragment (Fig. 2). The

hybridization of the *dhd* fragment was much darker with P1 #5-14 than with P1 #82-64. P1 clones contain up to 100 Kb of genomic DNA (Smoller et al., 1991; Sternberg, 1990) and so these two clones were further analyzed to test which one contained the *rg* gene DNA locus. Only one of these clones, P1 #82-63, detected RFLPs. These results make sense because *dhd* must be contained in P1 #5-14 while *rg* is contained in P1 #82-63. Further genomic walk obtained a single 3.5 Kb EcoR 1 fragment which was designated *rg*D1, that detected RFLPs ranging from 3.5-12 Kb (Fig. 3 and Fig. 4).

Isolation of *rg* cDNAs (done in collaboration with Shamloula, 1996):

Of the 500,000 recombinant phage screened, ten candidate cDNA clones were obtained and designated *rg*CD1-*rg*CD10, with EcoR1 inserts ranging in size from 0.5-3.2 Kb (Shamloula, 1996 dissertation). To confirm that the cDNAs were derived form the *rg* gene, the inserts were isolated and tested on a genomic Southern blot containing *rg* and wild type DNA to detect any RFLPs. The cDNAs were also mapped back to the P1 clone # 82-63 from which *rg*D1 was obtained. In order to determine if the various cDNAs were related, they were hybridized to each other. Screening 590,000 of the head cDNA library did not yield any positive clones. *rg*CD1, *rg*CD3 and *rg*CD6 were spuriously isolated because further analysis showed that they are not derived from the 4E-4F region; they did not detect RFLPs on a Southern blot and failed to hybridize to *rg*D1 and as such they were excluded from further characterization. Characterization of *rg*CD10 indicated that this cDNA could have been

derived from the *rg* gene region because it detected RFLPs ranging in size from 3.5 Kb to 12 Kb, on EcoR1 Southern blots containing DNA from *rg* mutants and wild type flies (Fig. 5 and 6). These results were confirmed by an EcoR1/Pst1 double digested *rg* mutant and wild type genomic DNA Southern blot which also showed RFLPs as detected by *rg*CD10 (Fig. 7). Hybridization of *rg*CD10 to the other cDNAs namely *rg*CD2, *rg*CD4, *rg*CD5, *rg*CD7, *rg*CD8 and *rg*CD9 showed crosshybridization of *rg*CD10 with *rg*CD4 (weak), *rg*CD7 (strong) and *rg*CD2 (weak). These results suggested that *rg*CD2, *rg*CD4, *rg*CD7 and *rg*CD10 are derived from the same genomic region and could be part of the same message. *rg*CD5, *rg*CD8 *and rg*CD9 did not hybridize with *rg*CD10 suggesting that they may be derived from another genomic loci.

Southern Blot analysis of putative *rg* cDNAs:

In order to understand the relationship between the seven cDNAs isolated using *rg*D1 as a probe I carried out further characterization of the cDNAs by Southern blotting. One question was whether the seven cDNAs were derived from the same genomic region and if so, were they contigous? Secondly, I tested whether any of the cDNAs detected RFLPs in the *rg* mutant Southern blot analysis. The isolated cDNAs (*rg*CD2, *rg*CD4, *rg*CD5, *rg*CD7, *rg*CD8, *rg*CD9 and *rg*CD10) were used in Southern blot analysis. *rg*CD10 detected RFLPs in an EcoRI blot (Fig. 5 and 6). *Canton-S*, *dhd* (which was a parental stock for P alleles) and *RDUP−* (containing a duplication of *rg* gene locus) showed two bands, 4 Kb and 7 Kb while

all the mutant alleles showed a one 12 Kb band. While these results provided evidence that suggested that *rg*CD10 was derived from the *rg* region the fact that all the mutant alleles showed a similar pattern in the RFLP analysis, the 12 Kb band, was disturbing because this suggested that all the *rg* mutant alleles had the same or similar mutation at the molecular level. In order to further verify this observation *rg*CD10 was used as a probe for an EcoRI/Pst1 double digest Southern blot (Fig. 7). *Canton-S*, *dhd*, *Oregon R* and *RDUP*− − showed four common bands of molecular weight 2, 1.2, 1.0 and 0.75 Kb. *Canton-S* also showed an extra 1.6 Kb band. All the gamma rays (γ) generated alleles showed a common 2.6 Kb band. Nine of these alleles (rg^{1}, $rg^{\gamma 1}$, $rg^{\gamma 2}$, $rg^{\gamma 3}$, $rg^{\gamma 5}$, $rg^{\gamma 7}$, $rg^{\gamma 8}$, $rg^{\gamma 10}$ and $rg^{\gamma 11}$) also lacked the 1.2 Kb band. $rg^{\gamma 4}$ showed an extra band (2.0 Kb) and $rg^{\gamma 6}$ and $rg^{\gamma 9}$ showed similar RFLPs, with three extra bands (3.0 Kb, 2 Kb and 1.8 Kb). All the P alleles revealed similar RFLPs, a 3 Kb band, 0.8 and 0.5 Kb band as compared to the wild type (2, 1.6, 1.2, 1.0 and 0.75 Kb bands) but different from the γ alleles; in addition $rg^{p5.1}$ showed a 1.7 Kb band. These results strongly suggested that *rg*CD10 was derived from the *rg* genomic locus. The other cDNAs were also used as probes on a Southern blot containing *rg* and wild type genomic DNA. As expected *rg*CD7 detected hybridization patterns identical to *rg*CD10. *rg*CD2 detected RFLPs on the EcoRI/Pst1 genomic blot that were distinct from *rg*CD10 (Fig. 8). In the *Canton-S*, *Oregon R* and *RDUP*−, *rg*CD2 hybridized with a 3.7 and a 2.6 Kb band; *Canton-S* had an extra 2.8 Kb band and *RDUP*+ had a 6.0, a 4.5 and a 4.0 Kb band. rg^{1} and

five γ alleles ($rg^{\gamma 1}$, $rg^{\gamma 2}$, $rg^{\gamma 5}$, $rg^{\gamma 7}$, $rg^{\gamma 8}$) and *JC70/+* (a deficiency that uncovers *rg* region) showed banding pattern similar to *dhd*. In $rg^{\gamma 3}$ and $rg^{\gamma 6}$ alleles, *rg*CD2 detected hybridization patterns identical to *RDUP+*. $rg^{\gamma 4}$ had the two bands common to all alleles and the controls and a 4.5 Kb band. $rg^{\gamma 9}$ showed a 5.5 Kb unique band and $rg^{\gamma 10}$ a 5.0 Kb unique band. These results could mean that there was incomplete digestion of genomic DNA because all the unique bands were high molecular weight or could reflect real DNA changes in the *rg* mutants. The most interesting observation was that the *rg*CD10 and *rg*CD2 recognized a common 2.6 Kb band (Fig. 7 and 8). *rg*CD4 did not show any convincing polymorphisms but strongly hybridized with a 3.7 Kb band, a band that also hybridized with *rg*CD2 but not *rg*CD10 (Fig. 9). *rg*CD5 strongly hybridized to a 4.2 Kb band and *rg*CD8 a 2.4 Kb band; both detected no RFLPs. *rg*CD9 did not recognize any fragments on a genomic Southern blot suggesting that it could have been picked up spuriously and does not really reflect a product of mRNA synthesis.

Mapping of *rg*CDNAs:

*rg*CD10 was further mapped on to *rg*D1 showing that *rg*CD10 was derived from *rg*D1 genomic area but extended beyond the 3.5 Kb *rg*D1 fragment because some of the *rg*D1 EcoR1 bands did not hybridize with *rg*CD10. Probing *rg*D1 with *rg*CD10 showed that *rg*D1 fragment contained some fragments that did not hybridize with *rg*CD10 indicating that *rg*D1 may contain intron sequences. *rg*CD10 was mapped back onto P1 clone. *rg*CD10 hybridized

strongly to two EcoR1 bands, a 3.5 Kb fragment corresponding to the *rg*D1 band, and a 2.5 Kb band indicating that this lower molecular weight band is also derived from the *rg* region. There was also a 2.0 Kb band that showed weak hybridization (Fig. 10).

I tested to determine whether the *rg* cDNAs were overlapping. In order to do this I attempted to establish the relationship between *rg*CD10 and the other cDNAs by mapping them back on to each other and to the P1 clone #82-63. *rg*CD7 hybridized strongly with *rg*CD10 and recognized the same P1 EcoRI bands. *rg*CD2 and *rg*CD4 hybridized to a single 1.2 Kb band on the P1 EcoRI blot. *rg*CD5 and *rg*CD9 did not recognize any P1 bands. *rg*CD4 also cross hybridized with *rg*CD7 but not as strongly as *rg*CD7 and *rg*CD10 hybridization. *rg*CD8 showed hybridization with several bands that were hard to characterize. *rg*D1 strongly hybridizes to *rg*CD10 and on an EcoRI genomic Southern blot *rg*CD10 identified RFLPs similar to but not identical with *rg*D1.

Sequence Analysis of *rg*CD10:

1.7 Kb *rg*CD10 was subcloned into a 3.2 Kb Bluescript pBSKII(+/-) plasmid vector as described by the manufacturer (Stratagene) and transformed into XL1 Blue MRF'. The *rg*CD10 sequence would help predict the type of protein encoded by *rg* and its biochemical function. The predicted amino acid sequence of *rg*CD10 shows alanine rich regions (Fig 13). The amino acid sequence was predicted from the Block server database (Henikoff, S. and Henikoff, J.G. 1991). The predicted amino acid sequence data of *rg*CD10 product showed

99-100% identity to a recently cloned *Drosophila* Protein Kinase A anchoring protein, DAKAP550 (Fig. 12 and 13; Han et al., 1997) that maps to 4F1,2 of *Drosophila* first chromosome and high homology to a *C. elegans* Protein Kinase A anchoring protein (Fig. 11 and 13). The predicted size of DAKAP550 protein is 2300 amino acids suggesting that *rg*CD10 codes for part of the DAKAP550 protein.

PCR analysis of wild type and *rg* mutant genomic DNA from *rg* gene locus:

Primers from the cDNA sequences of the DAKAP550 that are 2 Kb apart were synthesized and used to amplify a product that is about 9.5 Kb from the wild type and *rg* mutant genomic DNA. The expected product from the cDNA sequence data was about 2.0 Kb. This suggested that the 9.5 Kb genomic sequence of the PCR product contained an intron of about 7 Kb. The 9.5 Kb genomic PCR product was analysed by restriction digestion with EcoR1 and size fractionation on an agarose gel (Fig. 14 A). The DNA PCR product from the *rg* mutants showed two bands, 5 Kb and 3.5 Kb in size while the wild type PCR product DNA showed three bands, 3.5 Kb, 3 Kb and 2.5 Kb. These results showed that the *rg* mutants lost an EcoR1 site (Fig. 14 B). To determine if the amplified genomic product encoded *rg*CD10, a Southern blot of the EcoR1 restriction digest was probed with labeled *rg*CD10. The results showed that *rg*CD10 hybridizes with the 5 Kb band in the *rg* mutants and two bands, the 3.0 Kb and the 2.5 Kb in the wild type (Fig. 14 C).

***rg*CD10 Embryonic Expression:**

Protein and mRNA expression gives an indication of the requirement of a gene during development. *rg*CD10 mRNA expression is ubiquitously expressed throughout the embryonic stages. The earliest expression is observed before stage 5 at which time it shows a general cytoplasmic expression that becomes enriched anteriorly, ventrally and posteriorly up to the region of the pole cell invagination (Fig. 15 A). In the *rg* mutants the *rg*CD10 mRNA expression is reduced and comparable to sense strand staining (Fig. 15 B and C). The late stage 5 embryos also show enriched mRNA expression at the region of cephalic furrow formation (Fig. 15 D) At stage 6 the expression continues to show intensified expression of *rg*CD10 anteriorly including the region of the cephalic furrow formation, ventrally and posteriorly as the pole cells invaginate (Fig. 15 E). In the late stage 6 to stage 7 *rg*CD10 expression continues with the same pattern of expression found in stage 6 but also becomes maximized at the region of anterior midgut rudiment; this expression persists up to stage 8 (Fig. 15F). In the later stages of embryonic development (stages 13-17), *rg*CD10 expression is restricted to the central nervous system (Fig. 15 G). There is also some low expression anteriorly at the antenomaxillary complex. In the later stages of embryonic development in the *rg* mutants the dramatic expression of *rg* CNS expression is missing (15H). In all the embryonic stages little or *rg* mRNA expression was detected in the *rg* mutants suggesting strongly that the lesions in the *rg* mutation as detected by cDNA *rg*CD10 reduced or abolished mRNA expression.

Expression of *rg*CD10 in the Eye Disc:

Development of adult structures begins during the third larval instar. The compound eye begins to differentiate during late third larval instar. *rg*CD10 mRNA is expressed ubiquitously during the late third larval instar in the eye antennal disc (Fig. 16 A). This expression is reduced in the *rg* mutants; the expression is comparative to *rg*CD10 sense expression (Fig. 16 B and C). The *rg*CD10 expression also seems to be enriched at the dorsal and ventral parts of the eye disc and in some it is also enriched in the morphogenetic furrow.

RII and Cut antibody expression:

Protein Kinase A anchor proteins bind to the RII, PKA regulatory subunit, and tethers the whole enzyme complex to a specific subcellular site. To test if RII (and indirectly PKA) distribution was altered in the *rg* mutants, I localized RII-PKA subunit expression using antibody staining in *rg* mutants and wild type flies. In the wild type the RII staining colocalizes with MAb22C10 which labels differentiating photoreceptors (Fig. 17 B). In the *rg* mutant, the distribution of RII is altered suggesting that *rg* gene product is required for the normal distribution of PKA activity (Fig. 17 A).

In the *Drosophila* compound eye Cut is expressed in the cone cells (Fig.17C and D), Blochlinger, et al., 1993). Recent studies have shown that *cut* gene expression is regulated by PKA activity during oogenesis in *Drosophila* (Jackson and Blocklinger, 1997). To test if Cut expression or cellular distribution is regulated by *rg* during cone cell development in

the *Drosophila* compound eye I used Cut antibody staining. In the *rg* mutants the Cut expression is present but is lacks normal distribution as compared to the wild type pupal eye disc (Fig. 17), an observation that is consistent with the idea that Cut requires PKA activity for its biochemical functions.

DISCUSSION

Molecular analysis is an important step in understanding the structure and functional roles of a gene in a biochemical pathway. Genetic and phenotypic analysis of *rg* showed its requirement in the *Drosophila* eye development and also suggested its requirement during other stages in development. The 2.5 Kb fragment from the *dhd* region initiated a genomic walk which yielded a 3.5 Kb genomic fragment that detected RFLPs with various *rg* alleles on a Southern blot. This 3.5 Kb fragment was designated *rg*D1 and was used for further molecular analysis to screen cDNA libraries. Ten clones were isolated from an eye disc library but three of these (*rg*CD1, *rg*CD3 and *rg*CD6) were discarded after analysis showed they did not hybridize to *rg*D1 and did not detect any RFLPs on Southern blots. Screening the head cDNA library did not yield any positive clones suggesting that *rg* may be downregulated in the adult tissues. *rg*CD10 showed RFLPs on an EcoR1 and an EcoR1/Pst1 Southern blots and also *rg*CD10 mapped back to *rg*D1. *rg*CD10 was subcloned, sequenced

and used to characterize the other cDNAs. DNA sequence data showed that *rg*CD10 was identical to a PKA anchoring gene that encodes the DAKAP550 protein isolated by Han et al. (1997). The predicted size of DAKAP550 CDNA is 7.1 Kb and *rg*CD10 is 1.7 Kb. The other cDNAs were therefore further analyzed to see if they could be overlapping clones. *rg*CD10 hybridized strongly to *rg*CD7 and also recognized identical bands on the Southern blots and the P1#82-63 clone hybridization, suggesting that they could be one and the same. *rg*CD7 also showed weak hybridization with *rg*CD4 but *rg*CD4 hybridized to different bands on the Southern blot. *rg*CD2 also hybridized weakly with *rg*CD7 and also showed a distinct banding pattern on the Southern blot. However, *rg*CD2 had a 2.6 Kb band common to *rg*CD7 and *rg*CD10 suggesting that *rg*CD2 may be from the same region and may be an overlapping clone. *rg*CD4 also showed a distinct banding pattern but had a common band with *rg*CD2 again suggesting that the two cDNAs overlap and they are from the same region. *rg*CD5 and *rg*CD8 did not detect any RFLPs neither did they cross hybridize with any of the other cDNAs. This suggests that *rg*CD5 and *rg*CD8 are not from the *rg* region but these results do not preclude the possibilty that they could be derived from another part of a large and complex *rg* gene. The data from DAKAP550 analysis indicates that *rg* genomic organization is very complex and could encopmass as much as 20 Kb (Han et al., 1997).

Sequence analysis of *rg*CD10 shows that *rg* is a Protein Kinase A (PKA) anchoring protein and could in fact be the same as DAKAP550 gene. Primers obtained from

DAKAP550 sequences amplified a 9.5 Kb in both the *rg* mutants and the wild type genomic DNA. The primers used were 2 Kb apart and therefore the amplified product was much bigger than expected. These results suggested that the amplified product contained a large intron (about 7 Kb). The amplified DNA product was the same size in all *rg* alleles tested (rg^{r3}, rg^{r6} and rg^{r9}) and the wild type. To test if the amplified product contained any DNA changes due to the mutations in *rg* a restriction analysis was carried out. The 9.5 Kb PCR product showed two bands in the *rg* mutants (5 Kb and 3 Kb) and three bands in the wild type (3.5 Kb, 3.0 Kb and 2.5 Kb). The *rg* mutants have lost an EcoR1 site. This was further evidence that the amplified PCR product was from the *rg* locus and that the mutation modified an EcoR1 site. This also provided evidence that *rg* encoded DAKAP550 because primers from the DAKAP550 cDNA were used for this analysis. Further support for the suggestion that *rg* encodes the anchor protein DAKAP550 was provided by the Southern blot analysis. The EcoR1 PCR product was probed with *rg*CD10. In the *rg* mutants the 5 Kb band hybridized with *rg*CD10 but not the 3.0 Kb band. This 3.0 Kb band could be part of the 7 Kb intron. In the wild type *rg*CD10 hybridized with two bands, the 3.0 Kb and 2.5 Kb, providing further evidence that the *rg* mutants have lost an EcoR1 site. These results support the genomic Southern blot analysis which showed similar DNA changes in the mutants and provides evidence that *rg* in fact encodes the PKA anchor protein, DAKAP550.

Expression of *rg*CD10 mRNA shows that it is found in embryonic and larval stages.

In the embryo the expression is dynamic but enriched ventrally and anteriorly at all times. The expression is reduced or missing in *rg* mutants comparable to sense strand staining. This pattern of expression suggests a role of *rg* in the developing nervous system and posteriorly may function in the pole cell invagination. In the later stages of embryonic development *rg*CD10 expression becomes restricted to the central nervous system. The expression of *rg*CD10 is similar to that observed in the DAKAP550 antibody staining where at stage 5 of embryonic development the expression is ubiquitous and in the later stages it becomes enriched in the invaginating ventral furrow and the flanking mesodermal layer (Han et al., 1997). At stage 16 the DAKAP550 expression is elevated in the central nervous system and it is especially intense in the ventral nerve cord similar to *rg*CD10 expression. DAKAP550 is also expressed in the gut endothelium, tracheal system and salivary glands an observation not found in *rg*CD10 staining. This discrepancy could reflect a real difference between mRNA and protein expression or the poorer resolution of in-situ hybridization as compared to antibody staining which is much more sensitive. In the third larval instar eye disc *rg*CD10 is expressed ubiquitously throughout the eye antennal disc. In contrast, DAKAP550 is enriched behind the morphogenetic furrow and is found within the differentiated photoreceptors (Han et al., 1997). Again this expression of *rg*CD10 may suggest that post transcriptional modifications may reorganize the protein distribution and ultimately determine the spatiotemporal requirement of *rg* gene product. This differential protein expression may

be crucial to the functioning of *rg* (DAKAP550) in tethering PKA activity where it is required.

PKA is the main effector of cAMP activity which has been shown to mediate several signaling activities in *Drosophila*. Loss of function mutations in the main catalytic subunit (DC0) of PKA results in female sterility, abnormal oogenesis, embryonic and larval lethality, maternal effect phenotypes (Lane and Kalderon, 1993). In the eye, mitotic recombination experiments showed that PKA is not required for general functions suggesting that it mediates specific functions in cell-cell interactions (Lane and Kalderon, 1993). Another catalytic subunit, DC2 is only expressed in some cells during development and shows a different mutant phenotype from DC0. DC2 is expressed ventrally during the early embryonic stages and in later stages the expression is resolved into seven stripes and an enriched expression at the cephalic region (Melendez et al., 1995). Using β-galactosidase reporter gene DC2 was shown to be expressed in the developing leg and wing discs; in the optic lobe, the expression was found to be in a crescent shape in the lamina (Melendez et al.,1995). The pattern of expression of DC2 in the embryo and optic lobe is reminiscent of the *rg*CD10 expression. Mutations in DC2 do not seem to have deleterious effects, neither can the DC2 function compliment DC0 mutations(Melendez et al., 1995). An interesting DC2 phenotype shows a rough eye phenotype and it will be interesting to know if the cellular requirement of DC2 is the eye is the same as *rg* gene product (Melendez, et al., 1995). It may be that the

expressions of *rg* and DC2 are just coincident but it is also possible that Rg may be required for anchoring of DC2.

I have provided molecular evidence of the cloning of the *rg* gene and that *rg* infact encodes DAKAP550, a PKA anchoring protein. The deficiency mapping data places both *rg* and the gene encoding DAKAP550 in the 4F1-4F3 region of the X chromosome. The cloned genomic DNA and the cDNAs from the *rg* region map to a P1 clone #82-63 which is derived from the 4E-4F region and detect RFLPs on Southern blots of genomic DNA from *rg* mutant alleles. The mRNA expression data using *rg*CD10 shows distinct patterns in the embryo and the eye antennal disc which appear reduced or missing in the *rg* mutant alleles; the wild type expression is similar to the DAKAP550 protein expression. The DNA sequence analysis of *rg*CD10 shows a 99-100% identity to DAKAP550 and primers derived from the DAKAP550 cDNA sequence amplify a genomic product in the wild type and *rg* mutants that hybridized with *rg*CD10. In the *rg* mutants this DNA product shows a modified EcoR1 site which remains unchanged in the wild type.

PKA exists as a tetramer in its inactive form with two regulatory subunits (R) and two catalytic subunits (C). It has been proposed that this enzyme complex is tethered at appropriate cytoplasmic sites. The binding of cAMP to the R subunit activates and releases the C subunit which goes on to phosphorylate target substrate molecules (see review Taylor et al., 1990; Francis and Corbin 1994). I determined the distribution of PKA-RII regulatory

subunit by antibody staining of wild type and *rg* mutant third larval instar eye discs. The wild type eye disc distribution of RII as determined by the antibody staining shows that it is expressed in the developing photoreceptors and axons (where it colocalizes with MAb22C10) and around photoreceptor clusters. In the *rg* mutants the RII distribution is diffuse and some of the consistency with which it colocalizes with MAb22C10 is lost. These results suggest that *rg* may interact with RII in anchoring PKA enzyme complex. In the absence or reduced function of *rg* gene product the RII-PKA complex may be distributed randomly within the cell. This may result in the enzyme not being activated if it is not tethered in areas of high cAMP concentrations or the enzyme may be activated but the substrate may be unavailable. In either scenario, the signaling is lost and the cell is unable to make appropriate responses.

The *cut* gene has been shown to genetically interact with PKA and *Notch* during oogenesis in *Drosophila* (Jackson and Blochlinger, 1997). My results also show that the distribution of the Cut protein is more diffuse when compared to the wild type localization in the *rg* mutants. These results agree with the idea that *rg* encodes a PKA anchor protein and that mutations in *rg* affect Cut expression or localization. In the Rg reduced function, the RII and the PKA enzyme complex distribution and activity may become randomized. If the nuclear localization of the Cut protein is partly dependent on the PKA signaling then some of the Cut protein may become mislocalized. The finding that Cut protein is abnormally distributed suggests that other mechanisms are required for its nuclear localization and/or

expression and PKA by itself is not sufficient in regulating the Cut function. This notion is supported by the genetic interaction data that has implicated various other genes that may function in concert with *rg* (and the PKA pathway) in regulating cone cell differentiation.

Taken together these results provide evidence for the cloning of the *rg* gene. The mRNA expression data shows that *rg* gene product may be required during all the stages of embryogenesis and eye development. The RII antibody staining provides further evidence that *rg* is an anchoring protein and mutations in *rg* affect the distribution and hence biochemical activities of PKA-RII.

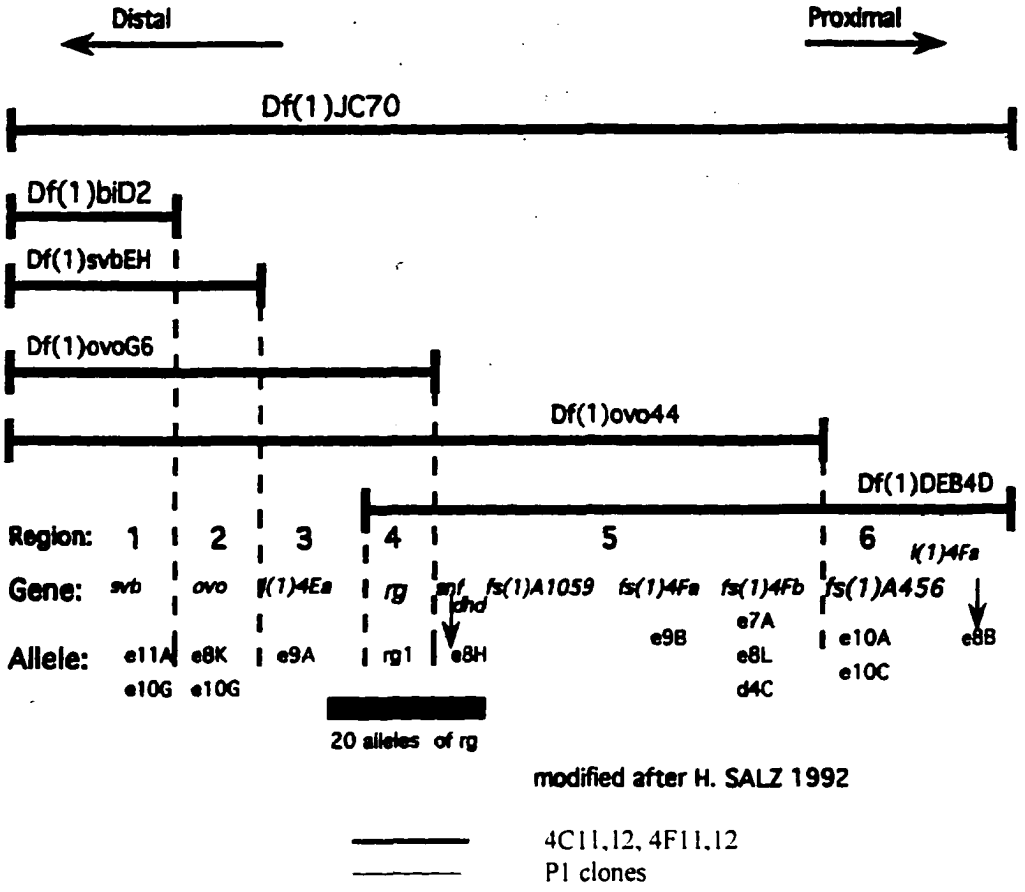


Fig 1: Overlapping deficiencies from the 4E-4F genomic region uncover *rg*.

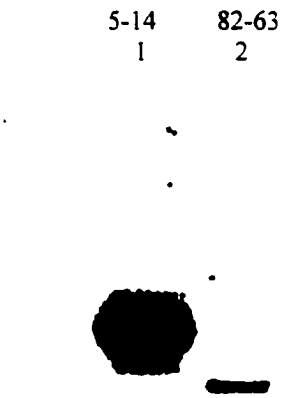


Fig. 2: P1 clones probed with *deadhead* (*dhd*) 2.5 Kb genomic fragment. Two P1 clones from the 4E-4F region hybridized with the 2.5 Kb *dhd* genomic fragment that was obtained from Dr. Salz. *dhd* is a gene that is near *rg*. On a Southern blot the *dhd* fragment does not detect RFLPs. Lane 1. P1#5-14 and Lane 2. P1#82-63.

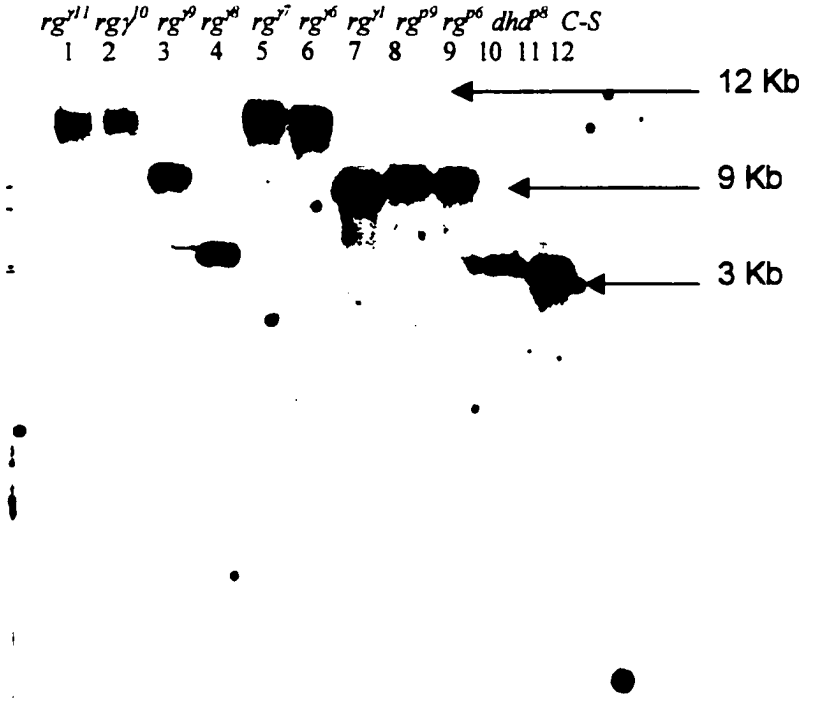


Fig. 3: EcoR1 genomic blot probed with *rg*D1. A genomic fragment, *rg*D1 detected RFLPs on a genomic Southern blot containing DNA from wild type and *rg* mutant flies. Lanes 1. $rg^{\gamma 11}$, 2. $rg^{\gamma 10}$, 3. $rg^{\gamma 9}$, 4. $rg^{\gamma 8}$, 5. $rg^{\gamma 7}$, 6. $rg^{\gamma 6}$, 7. rg^{1}, 7. rg^{p9}, 8. rg^{p6}, 9. rg^{p6}, 10, dhd^{p8}, 11. *Canton S*

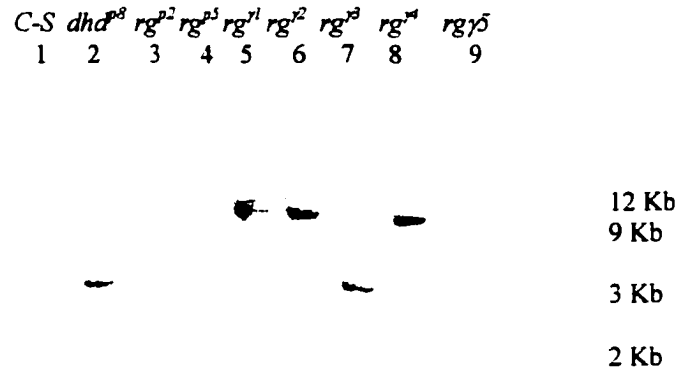


Fig. 4: EcoR1 blot probed with *rg*D1 detects RFLPs on a genomic southern blot containing *rg* mutant and wild type genomic DNA.

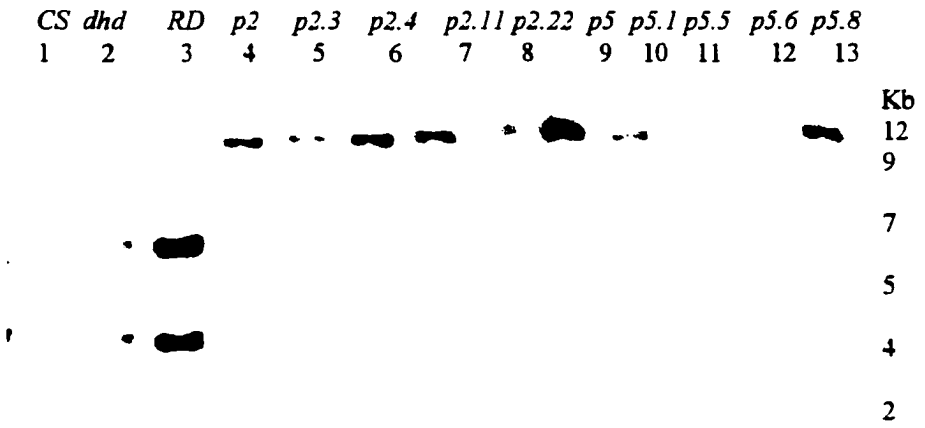


Fig . 7: EcoR1 blot probed with *rg*CD10. An EcoR1 genomic blot containing wild type and *rg* DNA was probed with *rg*CD10 putative *rg* cDNA which detected RFLPs. Lanes 1. *Canton S*, 2. dhd^{P8}, 3. *RDUP1+*, 4. rg^{p2}, 5. $rg^{p2.3}$, 6. $rg^{p2.4}$, 7. $rg^{p2.11}$, 8. $rg^{p2.22}$, 9. rg^{p5}, 10. $rg^{p5.1}$, 11. $rg^{p5.5}$, 12. $rg^{p5.6}$, 13. $rg^{p5.8}$

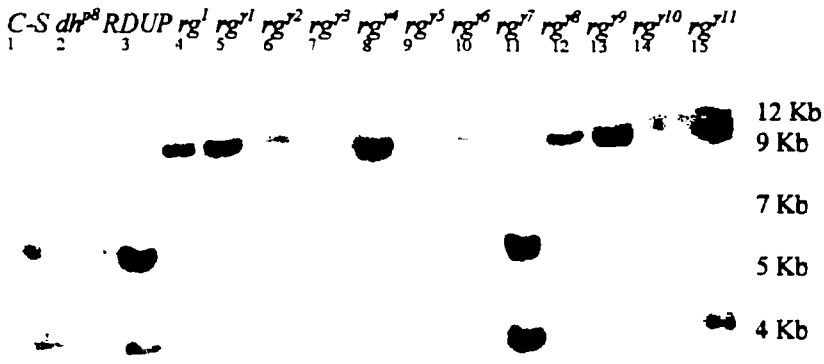


Fig 6: EcoR1 genomic blot probed with *rg*CD10. *rg*CD10 detects RFLPs on a genomic Southern blot containing wild type and *rg* mutant genomic DNA digested with EcoR1.

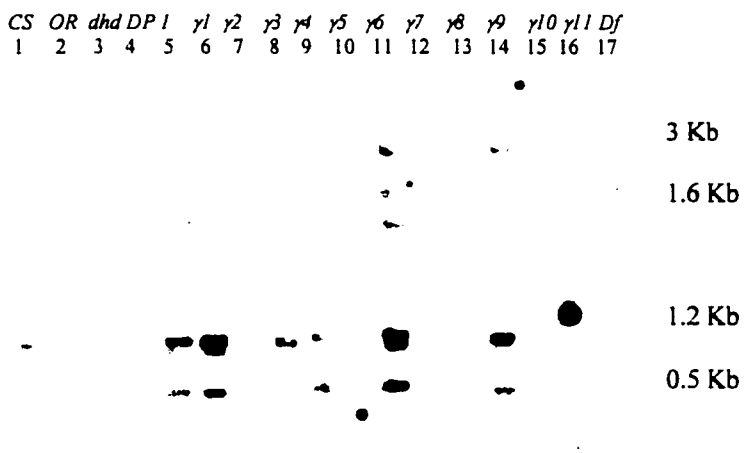


Fig. 7: EcoR1 /Pst1 blot probed with *rg*Cd10. An EcoR1 and Pst1 blot was probed with a putative *rg* cDNA, *rg*CD10 which detected RFLPs in the different *rg* mutants. Lanes 1. *Canton S*, 2. *Oregon R*, 3. dhd^{P8}, 4. *RDUP+/+*, 5. rg^{1}, 6. $rg^{\gamma 1}$, 7. $rg^{\gamma 2}$, 8. $rg^{\gamma 3}$, 9. $rg^{\gamma 4}$, 10. $rg^{\gamma 5}$, 11. $rg^{\gamma 6}$, 12. $rg^{\gamma 7}$, 13. $rg^{\gamma 8}$, 14. $rg^{\gamma 9}$, 15. $rg^{\gamma 10}$, 16. $rg^{\gamma 11}$, 17. *Df(1)JC70/+*

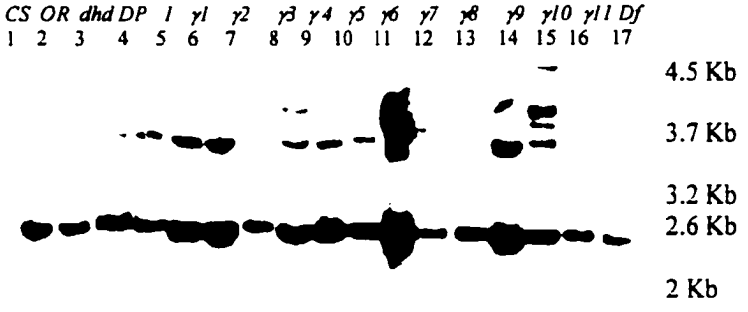


Fig. 8: An EcoR1/Pst1 blot probed with *rg*CD2. The putative *rg*CD2 detects RFLPs on an EcoR1 and Pst1 double digest blot containing wild type and *rg* mutant genomic DNA. Lanes 1. *Canton S*, 2. *Oregon R*, 3. dhd^{P4}, 4. *RDUP/+*, 5. rg^{1}, 6. $rg^{\gamma 1}$ 7. $rg^{\gamma 2}$ 8. $rg^{\gamma 3}$, 9. $rg^{\gamma 4}$ 10. $rg^{\gamma 5}$, 11. $rg^{\gamma 6}$, 12. $rg^{\gamma 7}$, 13. $rg^{\gamma 8}$, 14. $rg^{\gamma 9}$, 15 $_{rg}{}^{\gamma 10}$, 16 $rg^{\gamma 11}$, 17. *Df*

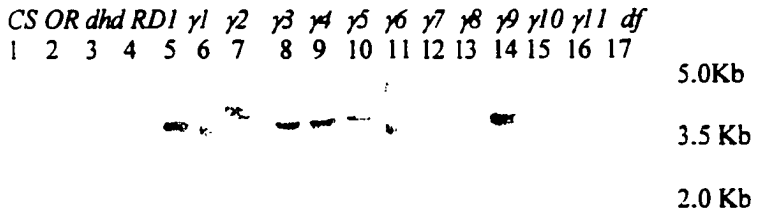


Fig. 9: EcoR1/Pst1 blot probed with *rg*CD4. A genomic blot containing wild type and *rg* mutant genomic DNA was double digested with EcoR1 and Pst1. *rg*CD4 did not detect RFLPs in most of the *rg* mutants except $rg^{\gamma 6}$. Lanes 1. *Canton S*, 2. *Oregon R*, 3. dhd^{P8}, 4. *RDUP/+*, 5. rg^{1}, 6. $rg^{\gamma 1}$, 7. $rg^{\gamma 2}$, 8. $rg^{\gamma 3}$, 9. $rg^{\gamma 4}$, 10. $rg^{\gamma 5}$, 11. $rg^{\gamma 6}$, 12. $rg^{\gamma 7}$, 13. $rg^{\gamma 8}$, 14. $rg^{\gamma 9}$, 15. $rg^{\gamma 10}$, 16. $rg^{\gamma 11}$, 17. *Df(1)JC70/+*

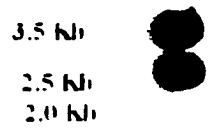


Fig. 10: The putative *rg* cDNAs *rg*CD7 and *rg*CD10 detect three fragments on an EcoR1 blot of P1#82-63. The 3.5 Kb band is *rg*D1 and the 2.5 Kb and 2 Kb bands are unique to the cDNAs.

Fig. 11: (A) *rg*CD10 predicted amino acid sequence shows high homology with *C. elegans* protein, F10F2.1. A search in the data base shows that *rg*CD10 encodes a protein that has regions of high homology to F10F2.1 (Han et. al., 1997, this study)

Fig. 12: *rg*CD10 shows 100% identity to DAKAP550 gene. The nucleotide sequence of DAKAP550 genomic shows that it encompasses a genomic locus of more than 20 Kb with complex introns. DAKAP550 cDNA nucleotide sequence is about 7.1 Kb (Han et. al., 1997)

Fig. 13: DAKAP550 amino acid sequence shows that the predicted protein product is about 2,300 amino acids and is highly acidic (Han et. al., 1997).

Fig. 11: Rg amino acid sequence has high homology with *C. elegans* F10F2.1

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

gi|2393880       (AF003622) A-kinase anchor protein DAKAP5...   842  1.6e-199   3
gnl|PID|e348766 (Z35598) F10F2.1 [Caenorhabditis elegans]      245  6.3e-50    4


gi|2393880 (AF003622) A-kinase anchor protein DAKAP550 [Drosophila
           melanogaster]
           Length = 2359

 Score = 604 (280.5 bits), Expect = 1.6e-199, Sum P(3) = 1.6e-199
 Identities = 111/111 (100%), Positives = 111/111 (100%)

Query:     1 MIAIVYYIYNRWTKSEIKCLVNGQLASSTEMAWFVSTNDPFDKCYIGATPELDEERVFCGQ 60
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Query:    61 MSAIYLFSEALTTQQICAMHRLGPGYKSQFRFDNECYLNLPDNHKRVSHFQ 111
             MSAIYLFSEALTTQQICAMHRLGPGYKSQFRFDNECYLNLPDNHKRVSHFQ
Sbjct:   176 MSAIYLFSEALTTQQICAMHRLGPGYKSQFRFDNECYLNLPDNHKRVSHFQ 226

 Score = 153 (71.0 bits), Expect = 1.6e-199, Sum P(3) = 1.6e-199
 Identities = 31/31 (100%), Positives = 31/31 (100%)

Query:   164 EQEARAIDWSDEKLDLNAAFVKIRAVLTARN 194
             EQEARAIDWSDEKLDLNAAFVKIRAVLTARN
Sbjct:   279 EQEARAIDWSDEKLDLNAAFVKIRAVLTARN 309

 Score = 842 (391.0 bits), Expect = 1.6e-199, Sum P(3) = 1.6e-199
 Identities = 166/204 (81%), Positives = 167/204 (81%)

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Query:   301 XXXADTEEVNQLKAVLYDGKLSNAIVFMYNPVATDGQLCLQSSPKGNVSYFVHTPHALML 360
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Sbjct:   415 VGSADTEEVDQLKAVLYDGKLSNAIVFMYNPVATDGQLCLQSSPKGNVSYFVHTPHALML 474

Query:   361 QDVKAVVTHSIHCTLNSIGGIQVLFPLFSQLDMAHEGLGDIKRDPTLCSKLLGFICELVE 420
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Sbjct:   475 QDVKAVVTHSIHCTLNSIGGIQVLFPLFSQLDMAHEGLGDIKRDPTLCSKLLGFICELVE 534

Query:   421 TSQTVQQHMIQNRGFLVISFMLQR 444
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Sbjct:   535 TSQTVQQHMIQNRGFLVISFMLQR 558

 Score = 242 (112.4 bits), Expect = 1.4e-24, P = 1.4e-24
 Identities = 49/58 (84%), Positives = 51/58 (87%)

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Fig. 13: DAKAP550 amino acid sequence

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1981 qqqqqqqqqq qqqqhyyqqq qqqqvvhnhs hhhmtaayyq qqqhqqvaag qqqhsptpla
2041 thstsssass tatsqpasss slsslasqsq qqshrqlhkq qqqqqqqqqq qqphyhphqp
2101 hyglinghqq hpqlngkhya engstagyhp hshphphggy mrngdtstlq qngmpdyqav
2161 glmnghgsgg tgnnnnssim nnmrnlrngg gansssppg shqviqgvat agavnanaav
2221 gvgmggavgv gmggvaggld ggvvyktsai nnnyryngrn asagtggrqi qdsdyeiivv
2281 dennpsvlad ndshssgpps iksvdsdvgs lnmnstgktk cprwshpsei liddhkpshs
2341 ndeswtdvnl nedaavqaa

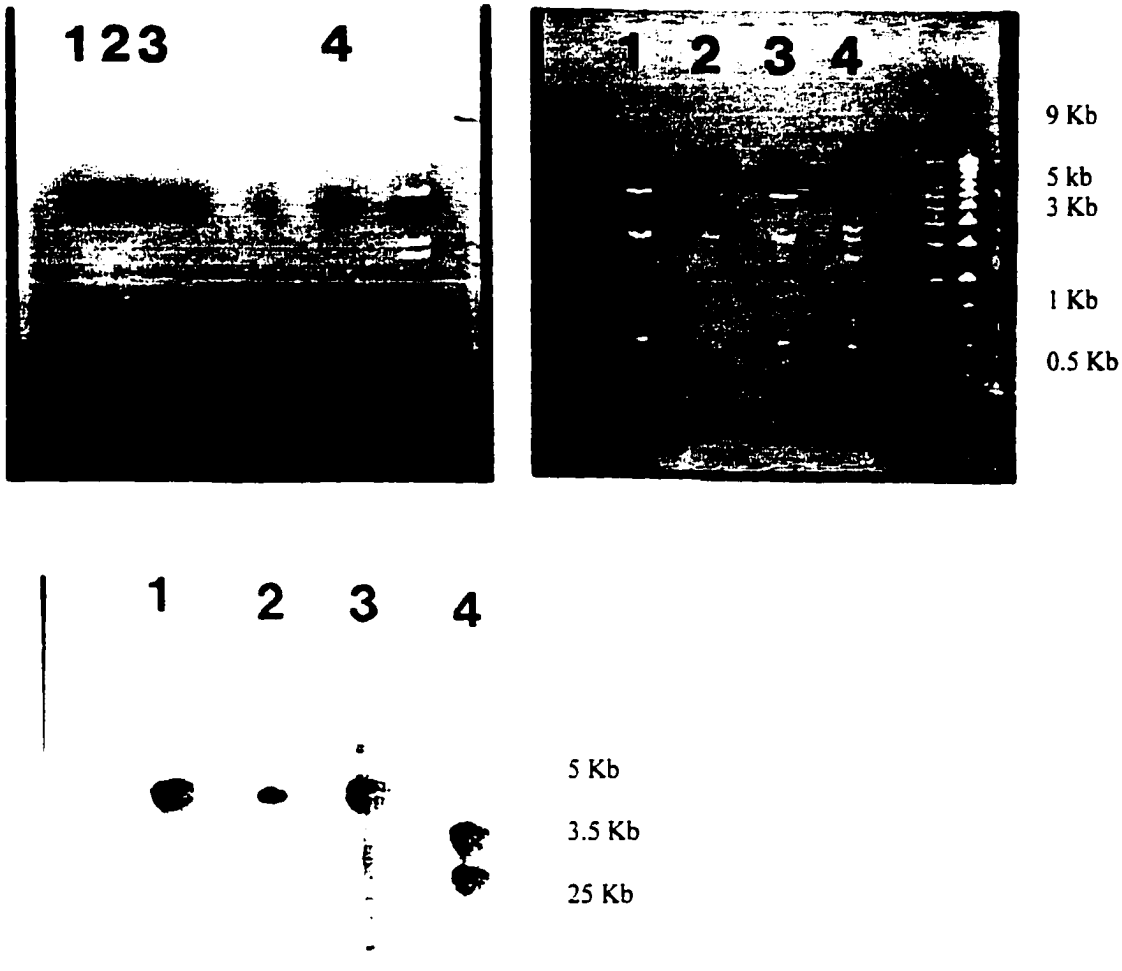
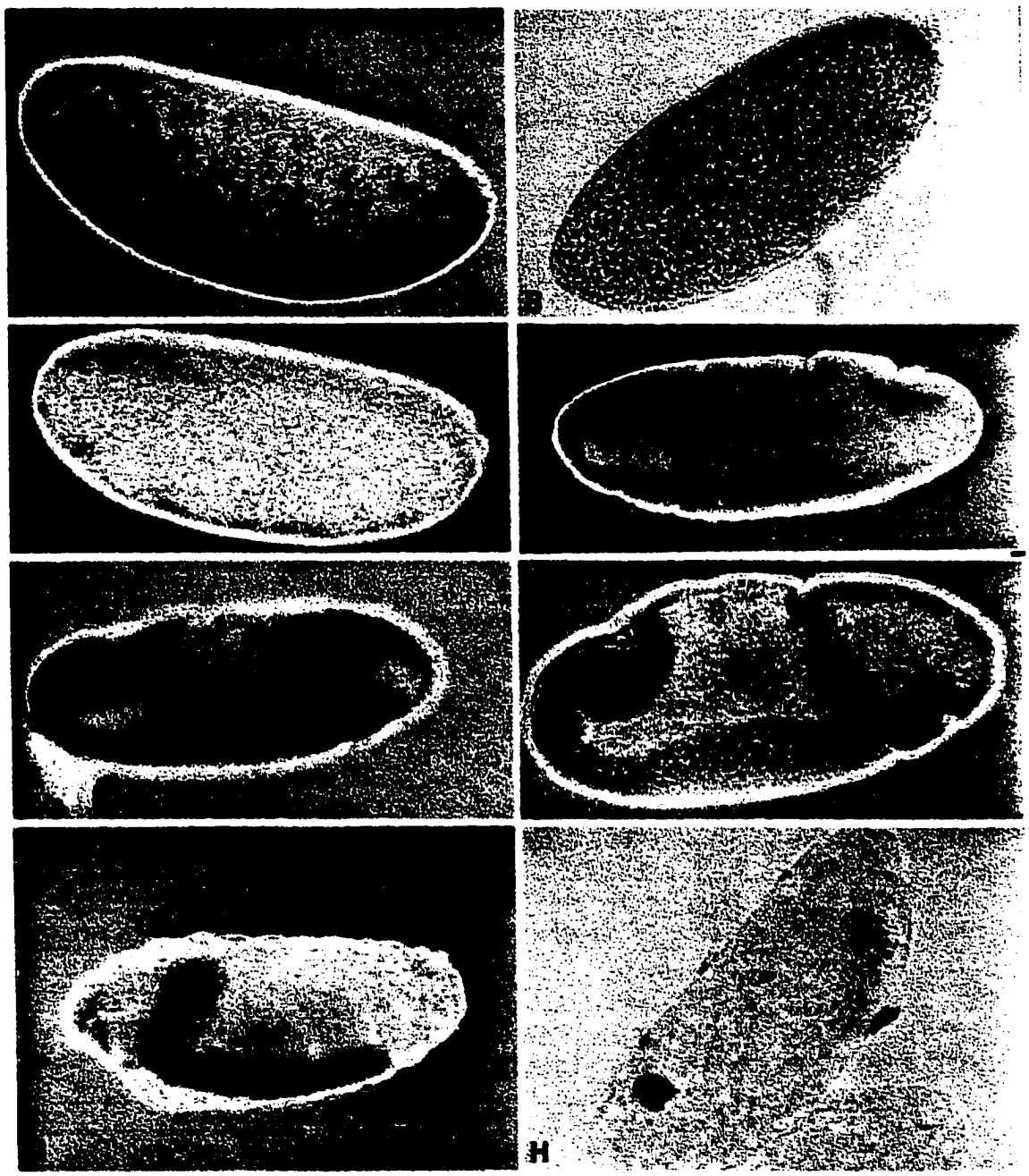


Fig. 14: PCR analysis of the genomic DNA from the wild type and *rg* mutants. A, B, and C agarose gel showing PCR products from *rg* mutants (lanes 1-3) and wild type (lane 4). (A) shows a 9.5 Kb amplified PCR product from *rg* mutants and *Canton S.* (B) is an EcoR1 digest of the 9.5 PCR product showing that *rg* mutants have lost an EcoR1 site . (C) The digested DNA in B was probed with *rg*CD10. The larger 5 Kb *rg* band and two of the EcoR1 wild type bands hybridize.

Fig. 15: Embryonic mRNA expression of *rg* as detected by in situ hybridization with *rg*CD10 probe. (A) In the earliest stages of embryo development there is ubiquitous expression of *rg* which becomes predominantly ventralized during stage 5. (B) In the mutant rg^{r10} embryos of the equivalent stage (stage 5) this expression is highly reduced. (C) *Canton S* sense strand staining was used as a negative control. (D) During the late stage 5 the *rg* expression is enriched in the anterior regions and in the cephalic furrow formation. (E) There is enriched expression anteriorly, posteriorly including the pole cell invagination and the cephalic furrow formation. (F) In the late embryonic stage 6 and 7 the same pattern of expression continues anteriorly, posteriorly, cephalic furrow formation and ventrally. The expression broadens at this stage. (G) At later stages of embryonic development (13-17) the *rg* expression becomes restricted to the central nervous system and the anterior part of the embryo also shows low levels of expression. (H) In the null allele (rg^{r6}) at the later stages of embryo (stages 13-17) development show that the *rg* expression is abolished (compare to G)



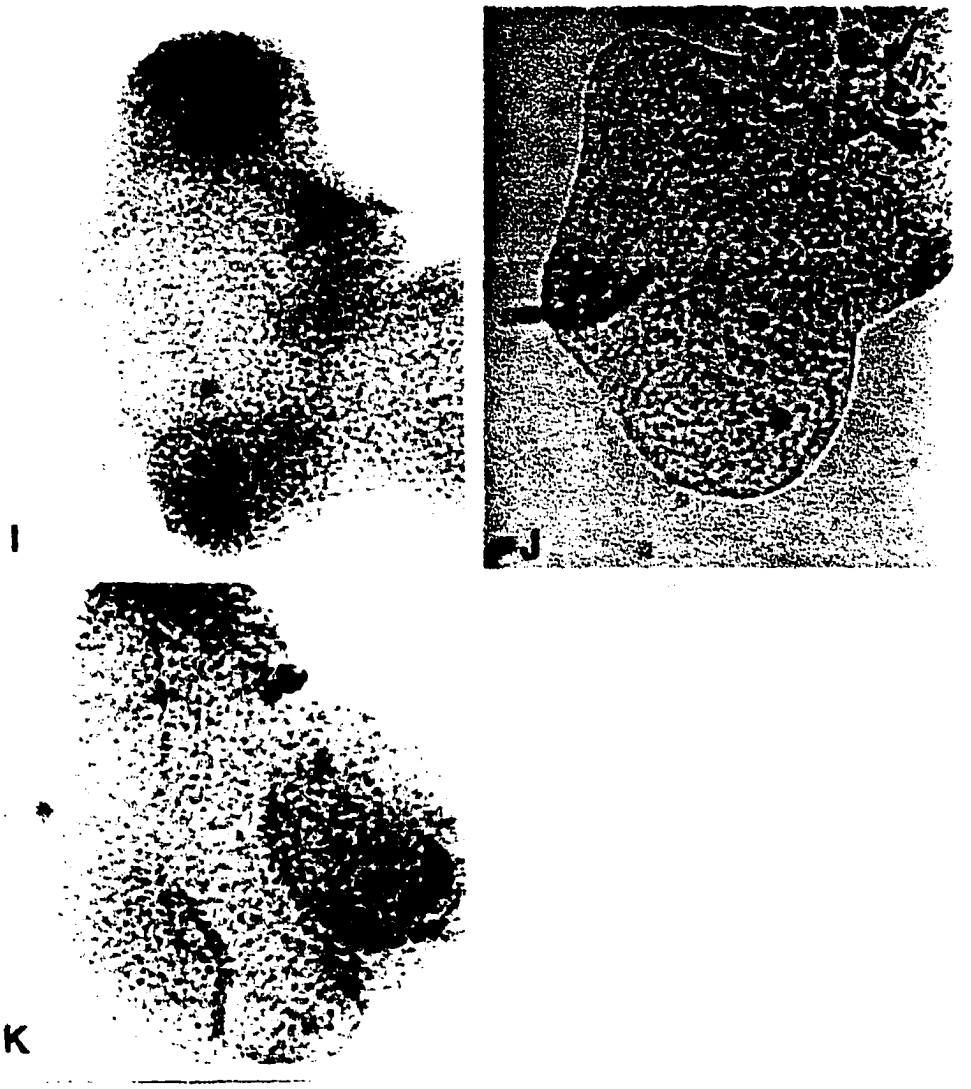
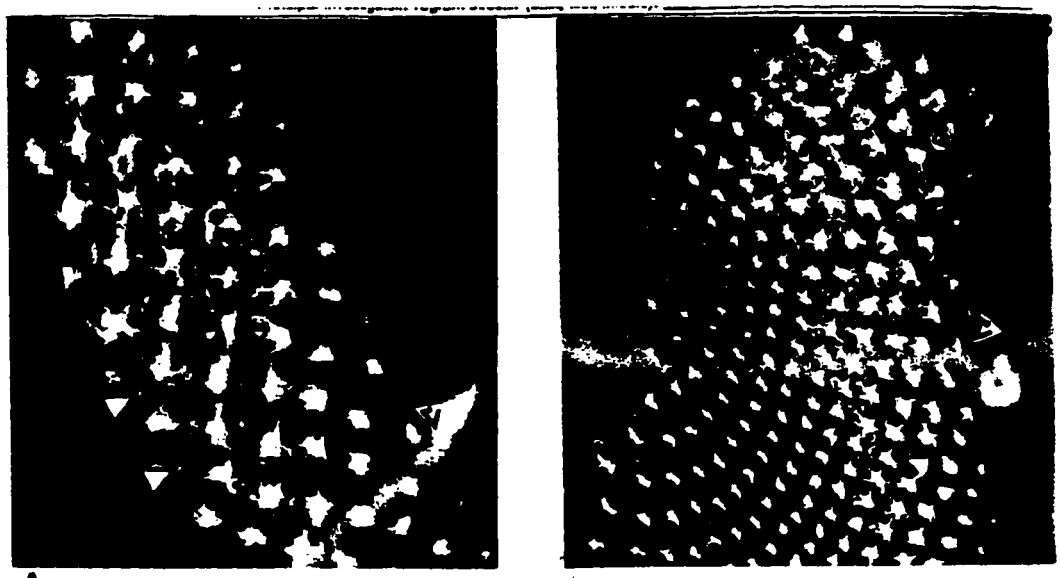


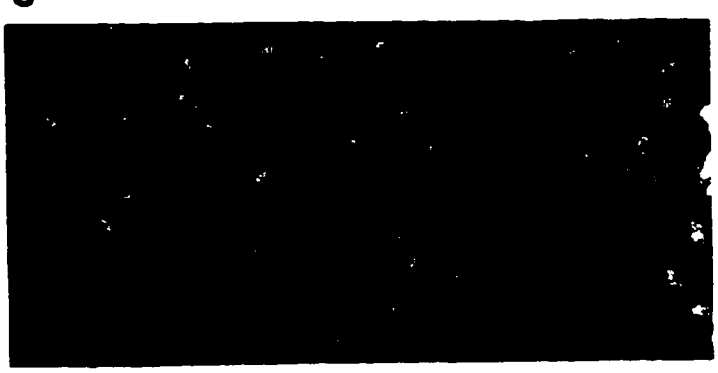
Fig. 16: *rg*CD10 mRNA expression in the developing late third larval eye disc: In the wild type developing eye imaginal disc the *rg* mRNA (*rg*CD10) is expressed throughout the disc I and K and some eye discs show an enriched expression in the morphogenetic furrow (K). In the rg mutant, $rg^{\chi 6}$ no expression is seen (J).

Fig. 17: The RII-PKA antibody staining in the third larval instar eye disc and Cut antibody staining in the 50 hour pupal eye disc. The monoclonal antibody MAb22C10 labels photoreceptors in the developing third larval eye imaginal disc (green) and RII-PKA colocalizes (yellow) but also around the ommatidial clusters (red) in the *Canton S* (B). In the *rg* mutant ($rg^{\gamma 3}$) the RII-PKA localization is abnormal (arrows) and in some places the MAb22C10 (green) does not colocalize (A). Cut antibody specifically labels the cone cells. In the 50 hour pupal eye the wild type shows four cone cell clusters with an apparent nuclear localization (C). This four cone cell cluster arrangement and nuclear localization is highly disorganized in the $rg^{\gamma 3}$ mutant 50 hour pupal eye disc (D).



A

C



D

Table 1: P1 clones from the 4E-4F genomic region used to clone the *rg* gene.

P1 clone	#	Genomic locus
DS02520	27-24	4D3-4E1
DS004457	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 #82-63 hybridized with 2.5 Kb *dhd* genomic fragment and also detected RFLPs on a Southern blot containing *rg* mutant and wild type genomic DNA. It was used for further molecular analysis. *P1 #05-14 also hybridized with the 2.5 Kb *dhd* genomic fragment but further probing on a Southern blot it did not detect RFLPs in the *rg* mutants.

CHAPTER V

SUMMARY AND CONCLUSIONS

A number of the physiological effects of hormones and neurotransmitters are mediated by phosphorylation and dephosphorylation by kinases and phosphatases in a highly regulated manner. Extracellular signals activate specific receptors which in turn activate GTP binding proteins (G proteins). The G proteins activate and increase the synthesis of Cyclic AMP (cAMP), an enzyme utilized as a second messenger in response to the hormonal stimuli (Scott, 1991; Francis and Corbin, 1994). cAMP (3'5'-cyclic adenosine monophosphate) activation results in the activation of protein kinase A (PKA). PKA is a tetrameric holozyme containing two regulatory (R) subunits and two catalytic (C) subunits (Beebe and Corbin, 1986). Upon activation of PKA by the binding of four cAMP molecules to the R subunits the C subunits are released and activated, which then go on to phosphorylate a variety of effector molecules (Taylor et al., 1990). The R subunits are also associated with protein kinase A anchoring proteins (AKAPs) that target the enzyme to specific subcellular sites. The anchoring of PKA to discrete subcellular sites provides further regulation of the activities of this multifunctional enzyme. Current evidence shows that the anchoring of R subunits of PKA type II (RII) to AKAPs targets PKA in close proximity to relevant substrates conveying specificity to cAMP/PKA mediated signaling (Scott, 1991; Rubin, 1994). The first AKAP to be identified was Microtubule associated Protein 2 (MAP 2)

anchoring PKA acitvity to microtubule networks in neurons (Lohmann et al., 1984; Shwartz and Rubin, 1985). Numerous AKAPs have now been identified with distinct subcellular localization (Scott and McCartney, 1994; Faux and Scott, 1996). Most AKAPs act as molecular anchors by tethering the enzymes associated with them to subcellular structures near the site where PKA activity is required. Several anchoring proteins have been identified that anchor single kinases or phosphatases to their specific sites. The AKAPs therefore have at least two binding domains, one for the R subunit and the other for the subcellular target site. Various AKAPs are cell and tissue specific (Scott and McCartney, 1994; Faux and Scott, 1996; Eide et al., 1998; Chen et al., 1998; Li et al., 1996). However some of these AKAPs may act as scaffolds, assembling multiprotein complexes. One such example is the mammalian AKAP79 which has been shown to associate with three signaling enzymes namely protein kinase C (PKC), calcineurin (CaN) which is a calcium and calmodulin-dependent protein phosphatase 2B and PKA (Klauck et al., 1996). AKAP220 has also been shown to be a multivalent anchoring protein binding to PKA and type 1 phosphatase (PP1); immunocytochemical analysis also shows overlapping staining patterns for PKA and PP1 in the rat hippocampal neurons, further suggesting that PKA and PP1 activity may be coordinately regulated by AKAPs (Schillace and Scott, 1999). Targeting of kinases and phosphatases by a common anchoring protein suggest a model of reversible phosphorylation and dephosphorylation of substrates. Initially AKAPs were thought to bind only RII subunits exclusively but emerging data shows that PKA can also bind and be localized by RIα subunits (Skalhegg et al.,

1994; Levy et al., 1996; Imaizumi-Scherrer et al., 1996). RI and RII have distinct but overlapping determinants for dimerization and docking and the dimerization domains show strong similarities and subtle differences (Banky et al., 1998). AKAPs are therefore required to physically and functionally organize protein complexes by associating with RI and/or RII PKA subunits, and thereby integrate upstream and downstream effector molecules of the cAMP mediated signaling pathway. However, AKAPs do more than just anchor PKA complexes. Felicello et al. (1997) have shown that AKAP75 anchors PKA activity and enhances the translocation of the signal to the nucleus in HEK cells. A truncated form of AKAP75 (AKAP45) that lacks the anchoring domain but has RII binding domain showed considerable less nuclear catalytic subunit activity with decreased magnitude and sensitivity. These results also showed that binding of AKAP to PKA is necessary but not sufficient for signaling and that anchoring of PKA to a subcellular site by AKAP increases sensitivity and potency to low levels of cAMP (Felicello et al., 1997). cAMP mediates water channels translocation from intracellular vesicles to apical membranes in response to antidiuretic hormone. Blocking o5f PKA binding to AKAP inhibits the activities of antidiuretic hormone in primary cultured rat inner medullary collecting duct cells underscoring the importance of PKA tethering to subcellular sites (Klassman et al., 1999). It has also been shown by Ali et al. (1998) that AKAP is required in the ROMK kidney channels believed to be K+ secretory channels. Activation of Xenopus oocytes coexpressing ROMK channels and AKAP79 significantly increased K+ current but not when ROMK was expressed alone. This and other current data on

AKAPs suggest that tethering of PKA by AKAPs is required for PKA activity and in addition AKAPs may be required for further biochemical activities like increased sensitivity and potency to activating molecules.

In these studies a gene called *rg* was identified in *Drosophila*. *rg* is required for the proper differentiation of *Drosophila* compound eye. Mutations in *rg* result in a rough eye phenotype due to a reduced number of cone cells in a dose dependent manner. The development of photoreceptors does not require *rg* gene product. In addition the fenestrated basement membrane is disrupted, a secondary effect due to the disorganized pigment lattice and reduction in cone cell number. Mutations in *rg* have also been shown to be required for proper wing venation (Shamloula, 1996, dissertation). Genetic interaction studies showed that *rg* gene product interacts with the *Delta Notch* and *Ras* pathway. Phenotypic analysis of *rg* eye phenotype showed that *rg* gene product is required for cone cell differentiation (Shamloula, 1996). Two other genes that specifically affect cone and pigment cell differentiation are *sparkling* and *canoe* (Fu and Noll, 1997; Matsuo et al., 1997). My results show that *rg* genetically interacts with *sparkling*. Cut is a transcription factor that is specifically expressed in the cone cells in the *Drosophila* compound eye (Blochlinger et al., 1993). Immunochemistry studies show that the expression of *cut* is disorganized in the *rg* mutants suggesting that proper localization of Cut may require the function of Rg and PKA signaling.

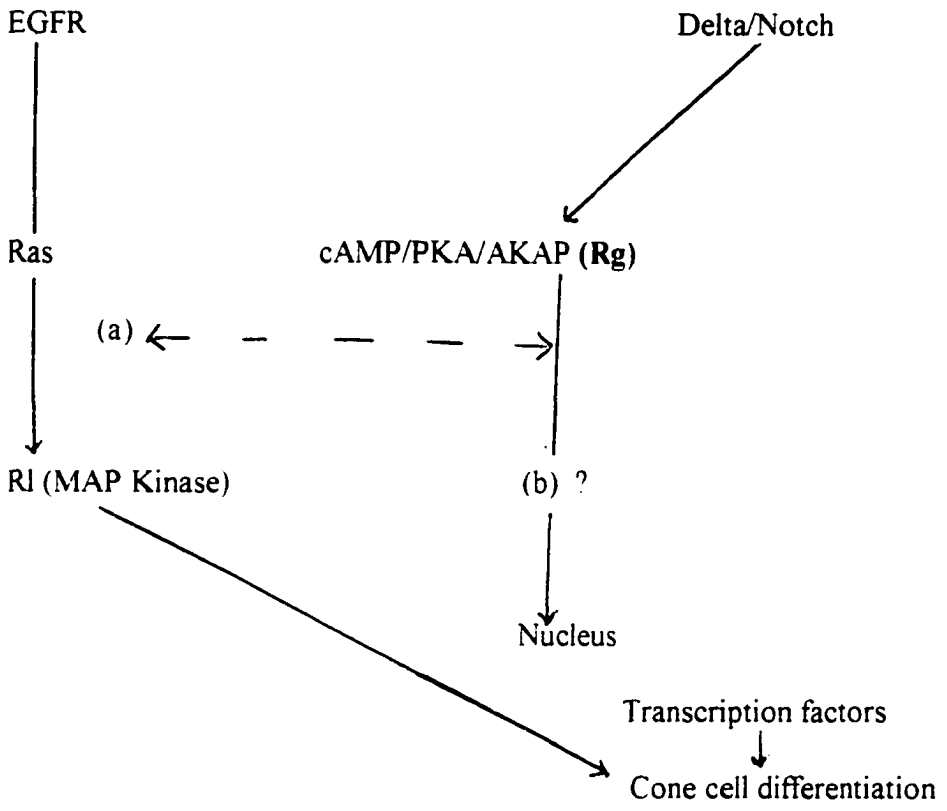
Molecular analysis has shown that *rg* encodes a *Drosophila* PKA anchor protein, DAKAP550. Both mRNA and protein expression data suggests that Rg is

required during oogenesis, embryogenesis and morphogenesis (Han et al., 1997; this study). The observations reported here are consistent with the widespread functions of cAMP/PKA mediated signaling in various systems. In *Drosophila*, PKA activity is required for various developmental events. Mutations in the main catalytic subunit of PKA, DC0, results in female sterility, abnormal oogenesis and embryonic lethality (Lane and Kalderon, 1993). These results are consistent with the present observations of *rg* mRNA distribution (this study) and protein (Han et al., 1997) during oogenesis, embryogenesis and the differentiation of adult structures. PKA signaling interacts with other signaling pathways involving *Ras/rolled* (MAP kinase), *hedgehog* (*hh*), *decapentaplegic* (*dpp*), *wingless* (*wg*), *Delta Notch*, *cut*, *canoe* and *scaborous* (Li et al., 1995; Kalderon, 1995; 1997; Jiang and Struhl, 1995; Jackson and Blochlinger, 1997; Miyamoto et al., 1995), in pattern formation during different developmental stages in *Drosophila*. The anchoring of PKA to specific subcellular sites is crucial to its function. PKA anchoring proteins have distinct domains that bind PKA and subcellular sites and this binding greatly influences the activity of the enzyme due to the proximity of activating cAMP concentrations and target substrate protein.

Cell fate determination involves complex molecular and genetic events. Spatial-temporal molecular cues are orchestrated in a highly sequential and stereotypic manner. Cells involved in the earlier differentiation decisions influence the later decisions according to where a specific cell is located. The biochemical pathways used in different organisms for pattern formation have been highly conserved. Rg is

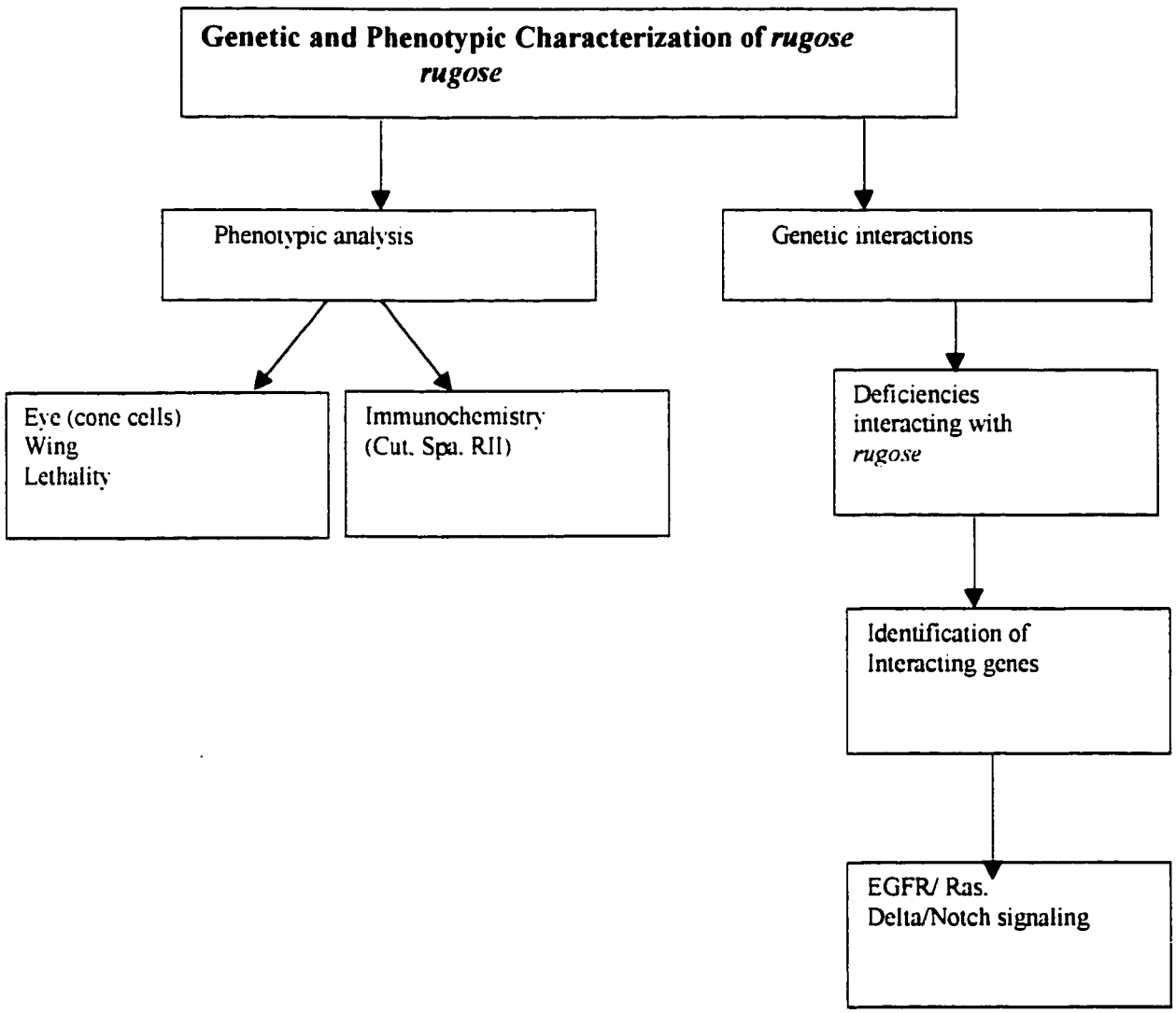
the first AKAP to be identified in *Drosophila*. Futhermore, this is the first study to show the importance of PKA signaling in cone cell differentiation in the *Drosophila* compound eye. How might PKA influence cone cell differentiation in the *Drosophila* compound eye? One model is that PKA signaling augments signaling by the *EGFR* and possibly *Delta Notch* pathway by elevating kinase activity to a threshold that is required for cone cell differentiation. In this model AKAP is required to anchor PKA activity at sites near the substrate molecules which could be other cytoplasmic proteins or some nuclear factors. Reduced AKAP as in mutations in *rg* the PKA is mislocalized and may be far away from its substrates and/or activating levels of cAMP, resulting in reduced signaling levels. And therefore in *rg* mutants the reduced signaling through cAMP/PKA may result in below threshold level of signaling that is required specifically for cone cell differentiation. Further biochemical studies to determine Rg distribution and its direct binding to PKA in the cone cell would shed light on the mechanisms of cone cell differentiation in the *Drosophila* compound eye.

Model of Rg function in cone cell differentiation.

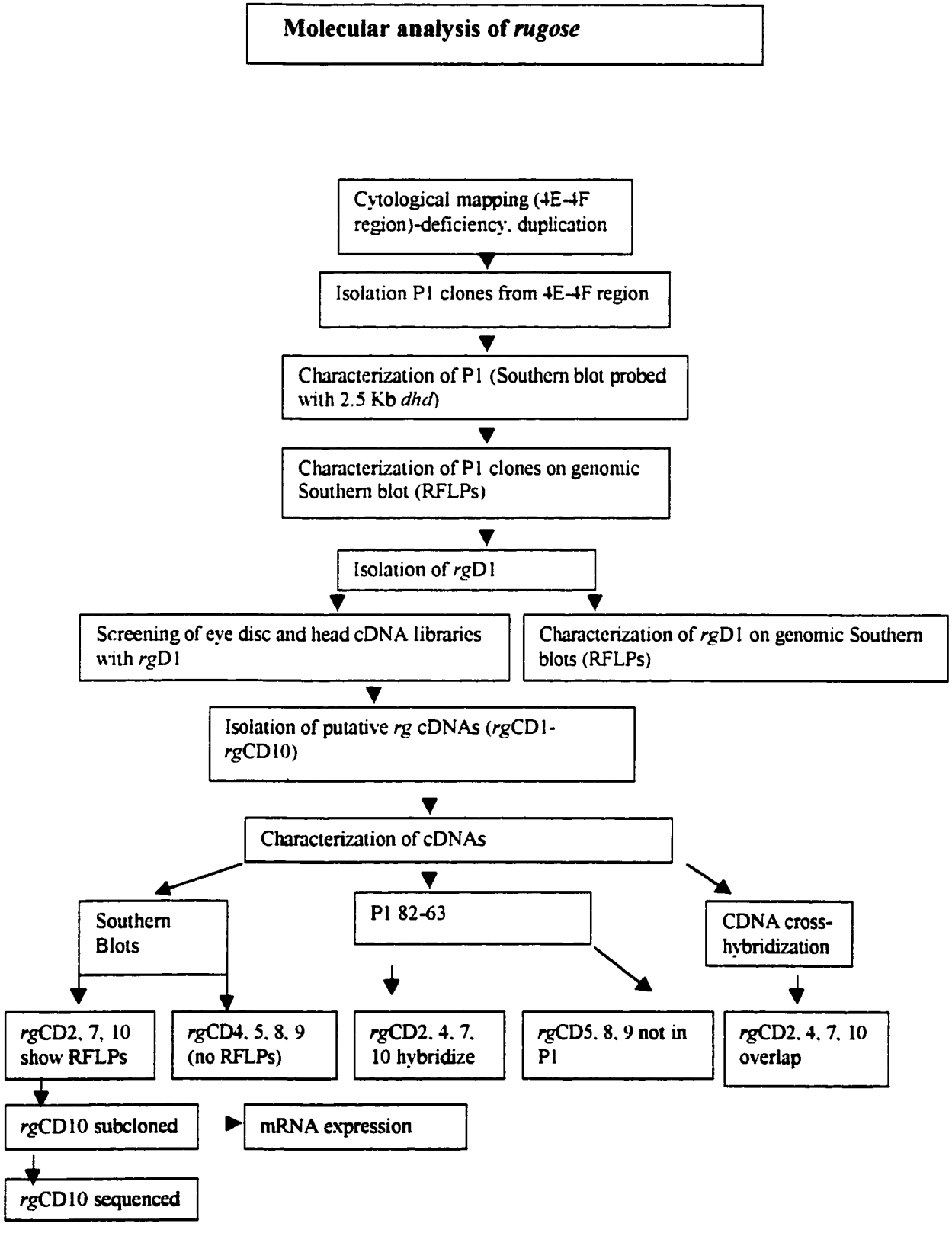


Rg may anchor PKA signal that activates cytoplasmic target substrates that may directly interact with the MAPkinase pathway (a) or augment its signal through a separate pathway (b). The threshold of signals reaches the nucleus where the threshold is enough to activate transcription factors that are cone cell specific.

Appendix A



Appendix B



REFERENCES

ALI, S. CHEN, X., LU, M., XU, J. Z., LEREA, K. M., HERBERT, S. C. and WANG, H. H. (1998). The A kinase anchoring protein is required for mediating the effect of protein kinase A on ROMK1 channels. Proc. Natl. Acad. Sci. USA 95: 10274-10278

AROIAN, R. V., LESA, G. M. and STERNBERG, P. W. (1994). Mutations in the *Caenorhabditis elegans let-23* EGFR-like gene define elements important for cell-type specificity and function. EMBO J. 13: 360-366.

AROIAN, R. V. and STERNBERG, P. W. (1991). Multiple functions of *let-23*, a *C. elegans* receptor tyrosine kinase gene required for vulval induction. Genetics 128: 251-267.

BAKER, N. E. and RUBIN, G. M. (1992). *Ellipse* mutations in the *Drosophila* homologue of *EGFR* receptor affect pattern formation, cell division, and cell death in eye imaginal discs. Dev. Biol. 150:381-396

BANGA, S. S., BLOOMQUIST, B.T., BRODBERG, R. K., PYE, Q. N., LARRIVEE, D.C., MASON, J. M., BOYED, J. B., AND PAK, W. L. (1986). Cytogenetic characterization of the 4BC region on the X-chromosome of *Drosophila melanogaster*: Localization of *mei-9*, *norp A* and *omb* genes. Chromosoma 93: 341-346.

BANKY, P., HUANG, L. J., and TAYLOR, S. S. (1998). Dimerization/docking domain of the type I-alpha regulatory subunit of cAMP-dependent prorein kinase. Requirements for dimerization and docking are distinct but overlapping. Journal Biol. Chem. 273:35048-35055

BEEBE, S. J. AND CORBIN, J. D. (1984). Rat adiposse tissue cAMP-dependent protein kinase: a unique form of type II. Mol. Cell Endocrinol. 36:67-78

BIGGS, W. H., ZAVITZ, K. H., DICKSON, B., van der STRATEN, A., BRUNNER, D., HAFEN, E., ZIPURSKY, S. L. (1994). The *Drosophila rolled* locus encodes a MAP kinase required in the *sevenless* signal transduction pathway. EMBO J. 13:1628-1635.

BLOCHLINGER, K., JAN, L. Y. and JAN, Y. N. (1993). Postembryonic patterns of expression of *cut*, a locus regulating sensory organ identity in *Drosophila*. Development 117: 441-450.

BRUNNER, D., OELLERS, N., SZAD, J., BIGGSIII, W. H., ZIPURSKY, S. L. and HAFEN, E. (1994). A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell 76: 875-888.

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

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

CAGAN , R. L. and READY, D. F. (1989b). The emergence of order in the *Drosophila* pupal retina. Developmental Biology 136: 346-362.

CLIFFORD, R. and SCHUPBACH, T. (1992). The *torpedo* (DER) receptor tyrosine kinase is required multiple times during *Drosophila* embryogenesis. Development 115: 853-872.

CHEN, N., GALLAHER, N., GOODMAN, N. H. and SMOLIK, S. M. (1998). Protein kinase A directly regulates the activity and proteolysis of *cubitus interruptus*. Proc. Natl. Acad. Sci. 95:2349-2354

CHANUT, F. and HEBERLEIN, U. (1997). Retinal morphogenesis in *Drosophila*: hints from an eye-specific *decapentaplegic* allele. Dev. Genet. 20: 1197-207.

COYLE-THOMPSON, C. A. and BANARJEE, U. (1993). The *strawberry notch* gene functions with *Notch* in common developmental pathways. Development 119: 377-395.

DOMINGUEZ, M. and HAFEN, E. (1997). Hedgehog directly controls initiation and propagation of retinal differentiation in the *Drosophila* eye. Genes and Dev. 1997: 3254-3264.

DICKSON, B., SPRENGER, F., MORRISON, D., HAFEN, E. (1992). Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature 360:600-603

EIDE, T., COGHLAN, V., ORSTAVIK, S., HOLSVE, C., SOLBERG, R., SKALHEGG, B. S., LAMB, N. J., LANGEEBERG, L., FERNANDEZ, A., SCOTT, J. D. JAHNSEN, T., and TASKEN, K. (1998). Molecular cloning, chromosomal localization, and cell cycle-dependent subcellular distribution of A-kinase anchoring protein, AKAP95. Exp. Cell Res. 238:305-316

FELICIELLO, A. LI, Y. AVVEDIMENTO, E. V. GOTTESMAN, M. E. and RUBIN, C. S. (1997). A-kinase anchor protein 75 increases the rate and magnitude of cAMP signaling to the nucleus. Curr. Biol. 7: 1011-1014.

FANTL, W. J., JOHNSON, D. E. and WILLIAMS, L. T. (1993). Signaling by receptor tyrosine kinases. Ann. Rev. of Biochem. 62: 453-481.

FAUX, M.C. and SCOTT, J. D. (1996). Molecular glue: Kinase anchoring and Scaffold

proteins. Cell 85: 9-12.

FEIG, L. A. and COOPER, G. M. (1988). Inhibition of NIH 3T3 cell proliferation by a mutant *Ras* protein with preferential affinity for GDP. Mol. Cell Biol. 8:3235-3243

FORTINI, M. E. and ARTAVANIS-TSAKONAS, S. (1994). The Suppressor of Hairless protein participates in *Notch* receptor signaling. Cell 79: 273-282.

FORTINI, M. E., SIMON, M.A. and RUBIN, G. M. (1992). Signaling by the Sevenless protein tyrosine kinase is mimicked by *Ras1* activation. Nature 355: 559-561.

FRANCIS, S. H. and CORBIN, J. D. (1994). Structure and function of cyclic nucleotide-dependent protein kinases. Ann. Rev. Physiol. 56: 237-272.

FREEMAN, M., KIMMEL, R. E., and RUBIN, G. M. (1992). Identifying targets of the *rough* homeobox gene of *Drosophila*: evidence that *rhomboid* functions in the eye development. Development 116: 335-346.

FREEMAN, M. (1994). The *spitz* gene is required for photoreceptor determination in *Drosophila* eye where it interacts with EGF receptor. Mechanisms of Dev. 48: 25-33.

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

FU, W., DUAN, H., FREI, E. and NOLL, M. (1998). *shaven* and *sparkling* are mutations in separate enhancers of the *Drosophila Pax2* homolog. Development 125: 2943-2950.

FU, W. and NOLL, M. (1997). The Pax2 homolog *sparkling* is required for the development of cone cells in the *Drosophila* eye. Genes and Dev. 11: 2066-2078.

GOLEMBO, M., SCHWEITZER, R., FREEMAN, M. and SHILO, B. Z. (1996). *argos* transcription is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop. Development 122: 223-230.

GONZALEZ-REYES, A. and ST. JOHNSTON, D. (1998). Patterning of the follicle cell epithilium along the antero-posterior axis during *Drosophila* oogenesis. Development 125: 2837-2846.

GONZALEZ-REYES, A. ELLIOT, H. and ST. JOHNSTON, D. (1995). Polarization of both major body axes in *Drosophila* by *gurken-torpedo* signaling. Nature 375: 654-658.

GRAY, P. C., JOHNSON, B. D., WESTENBROEK, R. E., HAYS, L. G., YATES, JR 3RD, SHEUER, T., CATTERALL, W. A. and MURPHY, B. J. (1998). Primary structure and

function of an A kinase anchoring protein associated with calcium channels. Neuron 20: 1017-1026

GRAY, P. C., SCOTT, J. D. and CATTERALL, W. A. (1998). Regulation of ion channels by cyclic AMP-dependent Protein Kinase and A-kinase anchoring proteins. Current Opinions in Neurobiology 8: 330-334.

HAN, J. D., BAKER, N. E. and RUBIN, C. S. (1997). Molecular characterization of a novel anchor protein from *Drosophila melanogaster*. Journal of Biological Chemistry 272: 26611-26619.

HANAHAN, D. Techniques for transformation of *E. coli* in DNA cloning, Vol. I, A practical approach, Chapter 6, Ed Glover, D. M., Oxford, IRL press (1985).

HEBERLEIN, U., HARIHARAN, I. K. and RUBIN, G. M. (1993). *Star* is required for neuronal differentiation in the *Drosophila* retina and displays dosage-sensitive interactions with *Ras1*. Developmental Biology 159: 001-0012.

HEBERLEIN, U. and MOSES, K. (1995). Mechanisms of *Drosophila* retinal morphogenesis: The virtues of being progressive. Cell 81: 987-990.

HEBERLEIN, U. and RUBIN, G. M. (1991). *Star* is required in a subset of photoreceptor cells in the developing *Drosophila* retina and displays dosage sensitive interactions with *rough*. Developmental Biology 144: 353-361.

HILL, R. J. and STERNBERG, P. W. (1992). The *lin-3* gene encodes an inductive signal for the vulval development in *C. elegans*. Nature (London) 358: 470-476.

HENIKOFF, S. and HENIKOFF, J. G. (1991). Automated assembly of protein blocks for database searching. Nucleic Acids Research 19: 6565-6572.

HUANG, Z. and KUNES, S. (1996). Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. Cell 86: 411-422.

HAUSKEN, Z. E. and SCOTT, J. D. (1996). Properties of A-kinase anchoring proteins. Biochem. Soc. Trans. 24: 986-991.

JACKSON, S. M. and BLOCHLINGER, K. (1997). *cut* interacts with *Notch* and Protein Kinase A to regulate egg chamber formation and to maintain germline cyst integrity during *Drosophila* oogenesis. Development 124: 3663-3672

JIANG, J. and STRUHL, G. (1995). Protein kinase A and *hedgehog* signaling in *Drosophila* limb development. Cell 1995: 80: 563-572.

JOHNSON, B. D., SCHEUER, T. and CATTERALL, W. A. (1994). Voltage-dependent potentiationof L-type Ca2+ channels in the skeletal muscle cells requires anchored cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. 91: 11492-11496.

IMAIZUMI-SCHERRER, T., FAUST, D. M., BENICHOU, J. C., HELLIO, R. and WEISS, M. C. (1996). Accumulation in fetal muscle and localization to the neuromuscular junction of cAMP-dependent protein kinase A regulatory and catalytic subunits RI alpha and C alpha. J. Cell Biol. 134:1241-1254

KALDERON, D. (1997). Hedgehog signaling: Ci complex cuts and clasps. Current Biology 7: 759-762.

KALDERON, D. (1995). Morphogetic signaling. Responses to *hedgehog*. Curr. Biol. 5: 580-582.

KALDERON, D. and RUBIN, G. M. (1988). Isolation and characterization of *Drosophila* cAMP-dependent protein kinase genes. Genes Dev. 2;1539-1556

KARIM, F. D., CHANG, H. C., THERRIEN, M., WASSARMAN. D. A., LAVERTY, T. AND RUBIN, G. M. (1996). A screen for genes that function downstream of *Ras1* during *Drosophila* eye development. Genetics 143: 315-329.

KARPILOW, J., KOLODKIN, A., BORK,T. and VENKATESH, T. (1989). Neuronal development in the *Drosophila* compound eye: *rap* gene function is required in photoreceptor cell R8 for ommatidial pattern formation. Genes and Development 3: 1834-1844.

KATZ, W. S., LESA, G. M., YANNOUKAKOS, D., CLANDININ, T. R., SCHLESSINGER, J. and STERNBERG, P. W. (1996). A point mutation in the extracellular domain activates *let-23*, the *Caenorhabditis elegans* Epidermal Growth Factor Receptor homolog. Molecular and Cell Biology 16: 529-537.

KLAUCK, T. M., FAUX, M. C., LABUDDA, K., LANGEBERG, L. K., JAKEN, S. and SCOTT, J. D. (1996). Coordination of the three signaling enzymes by AKAP79, a mammalian scaffold protein. Science 271: 1589-1592.

KOLODKIN, A. L., PICKUP,A. T., LIN, D. M., GOODMAN, C. S. and BENARJEE, U. (1994). Characterization of Star and its interactions with *sevenless* and EGF receptor during photoreceptor cell development in *Drososphila*. Development 120: 1731-1745.

KRETZESCHMAR, D., BRUNNER, A., WIERSDORFF, V., PFLUGFELDER, G. O., HEISENBERG, M., SCHNEUWLY, S. (1992). *giant lens*, a gene involved in cell determination and axon guidance in the visual system of *Drosophila melanogaster*. EMBO

J. 11:2531-2539

LAI, Z. C. AND RUBIN, G. M. (1992). Negative control of photoreceptor development in *Drosophila* by the product of the *yan* gene, an ETS domain protein. Cell 70: 609-620.

LANE, M. E. and KALDERON, D. (1995). Localization and functions of Protein Kinase A during *Drosophila* oogenesis. Mechanisms of Development 49: 191-200.

LANE, M.E. and KALDERON, D. (1994). RNA localization along the anteroposterior axia of the *Drosophila* oocyte required PKA-mediated signal transduction to direct normal microtubule organization. Genes and Development 8: 2986-2995.

LANE, M. E. and KALDERON, D. (1993). Genetic investigation of cAMP-dependent Protein Kinase function in *Drosophila* development. Genes and Development 7: 1229-1243.

LAWRENCE, P. A. AND GREEN, S. M. (1979). Cell lineage in the developing retina of *Drosophila.* Developmental Biology 71: 142-152.

LEVY, F. O., RASMUSSEN, A. M., TASKEN, K., SKALHEGG, B. S., HUITFELDT, H. S., FUNDERUD, S., SMELAND, E. B. and HANSSON, V. (1996). Cyclic AMP-dependent protein kinase (cAK) in human B cells: co-localization of type I cAK (RI alpha 2 C2) with the antigen receptor during anti-immunoglobin-induced B cell activation. Eur. J. Immunol. 26:1290-1296.

LI, Y., NDUBUKA, C. and RUBIN, C. S. (1996). A kinase anchor protein 75 targets regulatory (RII) subunits of cAMP-dependent protein kinase II to the cortical actin cytoskeleton in the non neuronal cells. J. Biol. Chem. 271:16862-16869

LI, W, OHLENMEYER, J., LANE, M. E. and KALDERON, D. (1995). Functions of protein kinase A in *hedgehog* signal transduction and *Drosophila* disc development. Cell 80: 553-562.

LIM, Y. M., NISHI, Y., TSUDA,L., INUOE, Y.H., NISHIDA,Y. (1999). Genetic analysis of *rolled* which encodes a *Drosophila* mitogen-activated protein kinase. Genetics 153:763-771

LINDSLEY, D. L. AND ZIMM, G. G. (1992). "The genome of *Drosophila melanogaster*". Academic Press

LOHMAN, S. M., DeCAMILLI, P., EINIG, I. and WALTER, U. (1984). High affinity binding of the regulatory subunit (RII) of cAMP-dependent protein kinase to microtubule-associated and other cellular proteins. Proc. Natl. Acad. Sci. USA 81:6723-6727

LONGLEY, R. L. and READY, D. F. (1995). Integrins and the development of three dimensional structure in the *Drosophila* compound eye. Developmental Biology 171: 415-433.

MANIATIS,T., FRISTCH, E. F. and SAMBROOK, J. (1982). Molecular Cloning. Cold Spring Harbour, New York, USA.

MANIATIS, T., FRISTCH, E. F. and SAMBROOK, J. (1987). Molecular Cloning. Cold Spring Harbour, New York, USA.

MATSUO, T., TAKAHASHI, K., KONDO, S., KAIBUCHI, K., and YAMAMOTO, D. (1997). Regulation of cone cell formation by Canoe and Ras in the developing *Drosophila* eye. Development 124: 2671-2680.

MELAMED, J. and TRUJILLO-CENOZ, O. (1975). The fine structure of the eye imaginal discs in *Muscoid* flies. Journal of Ultrastructure Research 51: 79-93.

MELENDEZ, A., LI, W and KALDERON, D. (1995). Activity and function of a second *Drosophila* Protein Kinase A catalytic subunit gene. Genetics 141: 1507-1520.

MEYEROWITZ, E. M., and KANKEL, D. R. (1978). A genetic analysis of visual system development in *Drosophila melanogaster*. Developmental Biology 62 : 112-142.

MIYAMOTO, H., NIHONMATSU, I., KONDO, S., UEDA, R., TOGASHI, S., HIRATA, K., IKEGAMI, Y. and YAMAMOTO, D. (1995). *canoe* encodes a novel protein containing a GLGF/DHR motif and functions with *Notch* and *scaborous* in common developmental pathways in *Drosophila*. Genes and Development 9: 612-625.

MUSKAVITCH, M. A. T. (1994). *Delta-Notch* signalling and *Drosophila* cell fate choice. Developmental Biology 166: 415-430.

NEUMAN-SILBERBERG, F. and SCHUPBACH, T. (1996). The *Drosophila* TGF-α like protein Gurken: expression and cellular localization during *Drosophila* oogenesis. Mechanisms of Development 59: 105-113.

OKABE, M., SAWAMOTO, K., OKANO, H. (1996). The function of the *Drosophila argos* gene product in the development of embryonic chordotonal organs. Developmental Biology 175:37-49

OKANO, H., HAYASHI,S., TANIMURA, T., SAWAMOTO, K., YOSHIKAWA, S., WANATABE, J., IWASAKI, M., HIROSE, S., MIKOSHIBA, K., MONTELL, C. (1992). Regulation of *Drosophila* neural development by a putative secreted protein. Differentiation 52:1-11.

OLIVER, R. B., PERRIMON, N. and MAHWOWALD, A. P. (1988). Genetic evidence that the *sansfille* locus is involved in *Drosophila* sex determination. Genetics 120: 159-171.

PAN, D. and RUBIN, G. M. (1995). cAMP-dependent protein kinase and hedgehog act antagonistically in regulating decapentaplegic transcription in Drosophila imaginal discs. Cell 1995 80: 543-552.

PARKS, a. L. and MUSKAVITCH, M. A. (1993). *Delta* function is required for bristle organ determination and morphogenesis in *Drosophila*. Dev. Biol. 157:484-496

PARKS, A. L., RUDOFF-TURNER, F. AND MUSKAVITCH, M. A. T. (1995) Relationships between complex *Delta* expression and the specification of retinal cell fates during *Drosophila* eye development. Mechanisms of Development 50: 201-216.

PAWSON, T. and SCOTT, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science 278: 2075-2080.

PIGNONI, F. and ZIPURSKY, S. L. (1997). Induction of *Drosophila* eye development by *decapentaplegic*. Development 124: 271-278.

PRICE, J. V., CLIFFORD, R. J. and SCHUPBACH, T. (1989). The maternal ventralizing locus *torpedo* is allelic to *faint little ball*, an embryonic lethal, and encodes the *Drosophila* EGF receptor homolog. Cell 56: 1085-1092.

REBAY, I. and RUBIN, G. M. (1995). Yan functions as an inhibitor of differentiation and is negatively regulated by activation of Ras1/MAPK pathway. Cell 81: 857-866.

RAZ, E. and SHILO, B. Z. (1992). Dissection of the *faint little ball* (*flb*) phenotype: determination of the development of the *Drosophila* central nervous system by early interactions in the ectoderm. Development 114: 113-123.

RAZ, E. AND SHILO, B. Z. (1993). Establishment of ventral cell fates in the *Drosophila* embryonic ectoderm required DER, the EGF receptor homolog. Genes and Development 7: 1937-1948.

READY, D. F., HANSON, T. E. and BENZER, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. Developmental Biology 53: 217-240.

REFRANZ, P. J. and BENZER, S. (1989). Monoclonal antibody probes discriminate early and late mutant defects in development of the *Drosophila* retina. Developmental Biology 136: 411-429.

ROBERTSON, H. M., PRESTON, C. R., PHILLIS, D. M., JOHNSON-SCHLITZ, bENZ,

W. K. and ENGELS, W. R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. Genetics 118: 461-470.

ROSENMUND, C., CARR, D. W., BERGENSON S.E. NILAVER, G. SCOTT, J. D. and WESTBROOK G. L. (1994). Achoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. Nature 368: 853-856.

ROTH, S., NEUMAN-SILBERBERG, F. S., BARCELO, G. and SCHUPBACH, T. (1995). *cornichon* and the EGF receptor signaling process are necessary for both anterior-posterior and the dorso-ventral pattern formation in *Drosophila*. Cell 81: 967-978.

RUBIN, C. S. (1994). A kinase anchor proteins and the intercellular targeting of signals carried by cyclic AMP. Biochim. Biophys. Acta 1224:467-479

SALZ, H. K. (1992). The genetic analysis of *snf*: A *Drosophila* sex determination gene required for activation of *sex-lethal* in both the germline and the soma. Genetics 130: 547-554.

SALZ, H. K., FLICKINGER, T. W. MITTENDROF, E., PELLICENA-PALLE, A., PETSCHEK, J. P. AND ALBRIGHT, E. B. (1994). The *Drosophila* maternal effect locus *deadhead* encodes a thioredoxin homolog required for female meiosis and early embryonic development. Genetics 136: 1075-1086.

SAWAMOTO, K., OKANO, H., KOBOYAKAWA, Y., HAYASHI, S., MIKOSHIBA, K. and TANIMURA, T. (1994). The functionof *argos* in regulating cell fate decisions during *Drosophila* eye and wing vein development. Developmental Biology 164: 267-276.

SAWAMOTO, K., OKABEI, M., TANIMURA, T., MIKOSHIBA, K., NISHIDA, Y. and OKANO, H. (1996). The *Drosophila* secreted protein Argos regulates signal transduction in the Ras/MAPK pathway. Developmental Biology 178: 13-22.

SCHLESSINGER, J. and ULLRICH, A. (1992). Growth factor signalling by receptor tyrosine kinases. Neuron 9: 383-391.

SCHWARTZ, D. A. and RUBIN, C. S. (1985). Identification and differential expression of two forms of regulatory subunits (RII) of cAMP-dependent protein kinase II in Friend erythroleukemic cells. Differentiation and 8-bromo-cAMP elicit a large and selective increase in the rate of biosynthesis of only one type of RII. J. Biol. Chem. 260:6296-6303

SCHWEITZER, R. and SHILO, B. Z. (1997). A thousand and one role for the Drosophila EGF receptor. Trends in Genetics: 13: 191-196

SCHWEITZER, R., HOWES, R. SMITH, R., SHILO, B. Z. and FREEMAN, M. (1995).

Inhibition of *Drosophila EGF* receptor activation by the secreted protein Argos. Nature 376: 699-702.

SCHILLACE, R. V. and SCOTT, J. D. (1999). Association of the type 1 protein phosphatase PP1 with the A-kinase anchoring protein AKAP220. Curr. Biol. 9:321-324.

SCOTT, J. D. (1991). Cyclic nucleotide-dependent protein kinases. Pharmacol. Ther. 50:123-145

SCOTT, J. D. and McCARTNEY, S. (1994). Localization of A-kinase through anchoring proteins. Mol. Endocrinol. 8:5-11.

SEGAL, D. and SHILO, B. Z. (1986). Tissue localization of *Drosophila melanogaster ras* transcripts during development. Mol. Cell Biol. 6:2241-2248

SIMON, M. A., BOWTELL, D. D. L., DODSON, G. S., LAVERTY, T. R. and RUBIN, G. M. (1991). Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the Sevenless protein tyrosine kinase. Cell 67: 701-716.

SKALHEGG, B. S., TASKEN, K., HANSSON, V., HUITFELDT. H. S., JAHNSEN, T. and LEA, T. (1994). Localization of cAMP-dependent protein kinase type I with the TCR-CD3 complex. Science 263:84-87

SHAMLOULA, H. K. (1996) A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

SMOLLER, D. A., PETROV, D., HARTL, D. L. (1991). Characterization of bacteriophage P1 library containing inserts of *Drosophila* DNA of 75-100 Kilobase pairs. Chromosoma: 100:487-494

STURTEVANT, M. A., ROARK, M. and BIER, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. Genes and Development 7: 961-973.

SWEISGUTH, F. and POSAKONY, J. W. (1992). *Suppressor of Hairless*, the *Drosophila* homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. Cell: 69:1199-1212

TAUTZ, D. and PFEIFLE, C. (1989). A non radioactive in situ hybridization method for the localization of specific RNAs in the *Drosophila* embryos reveals a translational control of the segmentation gene ***hunchback***. Chromosoma 98: 81-85.

TAYLOR, S. S., BUECHLER, J. A. and YONEMOTO, W. (1990). cAMP-dependent protein kinase: framework for diverse family of regulatory enzymes. Annu. Rev. Biochem. 59:971-1005

TIO, M., MA, C. and MOSES, K. (1994). *spitz*, a *Drosophila* homolog of transforming growth factor-α, is required in the founding photoreceptor cells of the compound eye facets. Mechanisms of Development 48: 13-23.

TOMLINSON, A. (1985). The cellular dynamics of pattern formation in the eye of *Drosophila*. Journal of Embryology and Experimental morphology 89: 313-331.

TOMLINSON, A., BOWTELL, D. D. L., HAFEN, E. and RUBIN, G. M. (1987). Localization of Sevenless protein, a putative receptor for positional information, in the eye imaginal disc of *Drosophila*. Cell 49: 143-158.

TOMLINSON, A. and READY, D. F. (1987a). Neuronal differentiation in the *Drosophila* ommatidium. Developmental Biology 120: 366-376.

TOMLINSON, A. and READY, D. F. (1987b). Cell fate in the *Drosophila* ommatidium. Developmental Biology 123: 264-375.

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

WEMMER, T. AND KLAMBT, C. (1995). A genetic analysis of the Drosophila closely linked interacting genes *bulge*, *argos*, and *soba*. Genetics 140: 629-641.

WOLFF, T. AND READY, D. F. (1993). Pattern formation in the *Drosophila* retina (chapter 22). Cold Spring Harbour.

WOLFF, T and READY, D. F. (1991a). Cell death in normal and rough eye mutants in *Drosophila*. Development 113: 825-839.

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