CHARACTERIZATION OF GENES INVOLVED IN PHOTORECEPTOR DIFFERENTIATION

by

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To my family

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ABSTRACT

CHARACTERIZATION OF GENES INVOLVED IN PHOTORECEPTOR DIFFERENTIATION

The one receptor - one neuron rule is a common phenomenon observed in sensory organs. In the Drosophila eye, each photoreceptor expresses one of six different rhodopsin genes. The expression patterns of inner photoreceptors divide ommatidia into three distinct subtypes: pale, yellow and DRA. In the pale subtype R7 and R8 cells express rh3 and rh5 subsequently while in the yellow subtype rh4 and rh6 are expressed in the inner PRs. Photoreceptor specification in *Drosophila* is a step-wise process, which requires activation of specific genes. Although certain transcription factors like *spalt*, *prospero*, *senseless*, *ortodenticle* and *spineles* have been identified so far, the whole picture and the pathways are still unknown. Aiming to identify novel genes playing a role in PR specification, an enhancer trap screen was previously performed. In this screen, the transposable element piggyBac was used as a vector with the aim of targeting novel genes. According to molecular functions of the genes identified and their expression patterns, 13 genes were selected for further characterization. In the course of this study the detailed expression, loss of function and gain of function analysis of those candidate genes were performed. Possible functions in eye development of five of the genes (*cropped, headcase, hephaestus*, *stubble* and *taranis*) were defined. Three of them (*cropped, headcase* and *hephaestus*) function possible early in eye development and eye growth. While the role of the *Stubble* gene is suggested as Rh6 de-repression in a subset of pale R8 cells. *Taranis* is believed to have a function in maintenance of rhabdomere size in photoreceptor cells.

The characterization of the expression patterns of a number of enhancer-trap lines, their molecular as well as their initial functional characterization is the basis for future functional work of some of these genes in rhodopsin regulation and retinal patterning in general.

ÖZET

FOTORESEPTÖR FARKLILAŞMASINDA ROL ALAN GENLERİN KARAKTERİZASYONU

Bir reseptör-bir sinir hücresi kuralı bütün duyu organlarında görülen yaygın bir fenomendir. Drosophila gözündeki herbir fotoreseptör altı rhodopsin geninden birini ekpres etmektedir. İç fotoreseptörlerin rhodopsin ekspresyonlarına göre retina üç ana tip omatidyaya ayrılmıştır; soluk (‘pale’), sarı (‘yellow’) ve DRA. Soluk tip omatidyada R7 veR8 hücreleri sırasıyla rh3 ve rh5 moleküllerini ekpres ederken, sarı tip omatidyaların iç fotoreseptörlerinde rh4 ve rh6 ekpresyonu gözlemlenmektedir. Drosophila göz gelişiminde, fotoreseptörlerin özelleşmesi adım adım ilerleyen bir süreçtir ve bu süreç belirli bazı genlerin aktivasyonunu gerektirir. Şu ana kadar, bu süreçte görev alan p*rospero, senseless, ortodenticle* ve *spineles* gibi bazı transkripsiyon faktörleri bulunmuştur. Fakat bütün mekanizmanın nasıl işlediği bu genlerle açıklanamamaktadır. Daha önce, fotoreseptör özelleşmesi sürecinde etkili olan yeni genleri bulmak amaçlı bir bir ‘yükseltici yakalama’ (enhancer-trap) taraması yapılmıştır. Bu taramada vektör olarak piggybac transpozıbıl elemanı kullanılmıştır. Bulunan genlerin moleküler fonksiyonlarına ve ekspresyon verilerine dayanarak 13 gen üzerlerinde detaylı analiz amaçlı seçilmiştir. Bu çalışma kapsamında seçilen genlere detaylı ekpresyon analizi, işlev kaybettirme ve işlev kazandırma çalışmaları yapılmıştır. Göz gelişiminde muhtemel rolü olan 5 gen (*cropped, headcase, hephaestus, stubble* ve *taranis*) bulunmuştur. Bunların üçü (*cropped, headcase* and *hephaestus*) erken göz gelişimi ve göz büyümesinde rol almaktadır. *Stubble* geninin fonksiyonu Rh6 baskılanmasını ortadan kaldırması olarak önerilmiştir. Taranis geninin fonksiyonu ise rhabdomere boyutlarını düzenlemek olarak önerilmiştir.

Bu çalışmada incelenen genler ve onların detaylı analizleri, ileride yapılacak kapsamlı çalışmalar için bir temel oluşturacaktır.

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LIST OF ABBREVIATIONS

bp	Base pairs
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CNS	Central nervous system
Del	Deletion
DNA	Deoxyribonucleic acid
DRA	Dorsal rim area
ds RNA	Double stranded RNA
EGFR	Epidermal growth factor receptor
FRT	Flip recombinase targets
GFP	Green fluorescent protein
lGMR	Long glass multiple reporter
N	Notch
NF-Y	Nuclear factor Y
NF-YC	C-subunit of nuclear factor Y
PBS	Phosphate buffered saline
pBGay	Piggybac element
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Power of hydrogen
PR	Photoreceptor
Pros	Prospero
PTB	Polypyrimidine tract binding protein
Rh5	Rhodopsin5
Rh6	Rhodopsin6
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Real time PCR
UAS	Upstream activating sequence
UV	Ultraviolet

1. INTRODUCTION

According to the famous philosopher, Immanuel Kant, our knowledge of the outside world depends on our modes of perception. In humans there are five senses: vision, smell, taste, touch, and hearing. All together they function in formation of the outside perception. Among them, vision is probably the most developed sense. Consequently, the eye, organ of vision has been the most studied sensory organ.

1.1. Human Eye Versus *Drosophila* Eye

Although the compound eye of *Drosophila melanogaster*, which consists of ~ 800 stereotypical unit eyes (ommatidia), and the simple eye of humans seem to be distantly related by physical appearance, they share significant similarities. The human retina is composed of two types of photoreceptors (PRs); cones and rods. Cone cells are involved in color discrimination while rod cells play a role in black and white vision under dim light conditions (Wernet and Desplan, 2004). The *Drosophila* ommatidia also contains two types of photoreceptors; inner and outer PRs (named because of their positions in each ommatidia). The inner PRs are like cone cells in human and are responsible for color perception and the outer PRs detect motion in dim light similar to rods. Furthermore, color discriminating cells in the human and the *Drosophila* retina are distributed stochastically. Humans have a trichromatic visual system which includes S-, M- and L- cone cells whose opsins absorb short (<500 nm, blue), medium (~ 530 nm, green) and long (~560 nm, red) wavelengths respectively, while the *Drosophila* retina contains UV-, blue- and green-sensitive PRs. The spatial arrangement of the different types of cones in the photoreceptor mosaic in the human retina is shown in Figure 1.1A. Because of these resemblances, the *Drosophila* retina is a powerful system to study eye development.

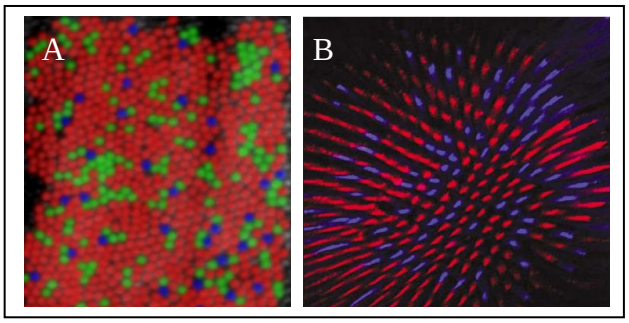

Figure 1.1. Spatial distribution of photoreceptors in the human and the Drosophila retina. (A) Pseudocolour image of the trichromatic cone mosaic from a living human retina. Blue, green and red colors represent the S, M and L cones, respectively (Roorda and Williams, 1999). (B) Confocal image of a wild type whole mount Drosophila retina stained with anti-Rh5 in blueand anti−Rh6 in red (Mikeladze-Dvali *et al*., 2005).

1.2. Photoreceptors in *Drosophila*

The *Drosophila* visual system is composed of two compound eyes and three ocelli. In the compound eye of *Drosophila*, each ommatium is a single eye unit that has eight photoreceptor cells (R1-R8), four lens-secreting cone cells and two primary pigment cells. Additionally, several secondary and tertiary pigment cells with interommatidial bristles border neighbouring ommatidia (Frankfort *et al*., 2004; Mikeladze-Dvali *et al*., 2005). Eight PRs can be divided into two subsets; inner and outer according to their morphology, functions and axonal projections.

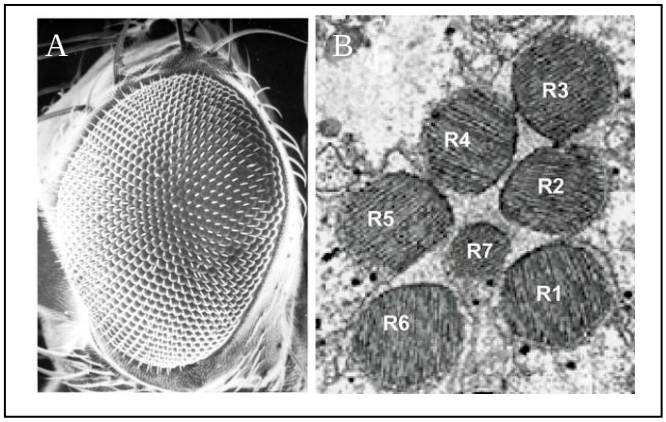

Figure 1.2. The Drosophila compound eye. (A) Scanning electron micrograph of an adult Drosophila compound eye, composed of ~800 ommatidia. (Developmental Biology, 8th edition) (B) Cros-section through an adult ommatidium. The light gathering structures

(rhabdomeres) of seven photoreceptors are visible (R8 is under R7). The rhabdomere diameter is larger for outer PRs (R1-R6) while R7 has a smaller rhabdomere diameter (Mikeladze-Dvali *et al*., 2005).

The outer PRs (R1-R6) are responsible for motion detection and image formation, have large rhabdomeres spanning the entire thickness of the retina (Figure 1.2B). And their axons extend to the lamina.

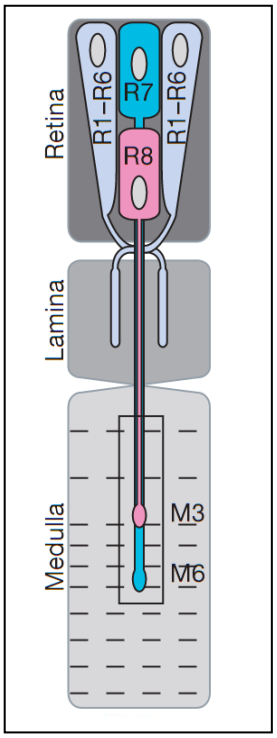

Figure 1.3. Axon projections of outer and inner photoreceptors. Schematic representation of inner photoreceptor axonal projection. R7 cell axon project to M6 layer of medulla and R8 cell axon project to M3 layer of medullain Drosophila visual system (Morey *et al*., 2008).

The inner PRs - R7 and R8 - which are responsible for color discrimination have smaller rhabdomeres than outer PRs (Figure 1.2B) and they project their axons to the medulla part of the optic lobe. However, their target layers differ within the medulla. The axon of an R7 cell makes connections with secondary neurons in the M6 layer of the medulla while the M3 layer of medulla is the target region for R8 neurons (Figure 1.3) (Mikeladze-Dvali *et al*., 2005; Morey *et al*., 2008).

Some of the factors playing a role in this differential synaptic targeting have been reported (Lee *et al*., 2001; Nern *et al*., 2005; Clandinin *et al*., 2001; Morey *et al*., 2008). For example, a subunit of NF-Y (nuclear factor Y), which is a transcription factor, NF-YC is responsible for the repression of the R8 target program in R7 cells. In the absence of *NF-YC*, R7 axons project to M3 layer (R8 axons target region). This mistargeting is shown to be due to derepression of *senseless* (*sens*) in R7 cells. Additionally, *sens* directly binds to a R8-specific cell surface protein, Capricious, which is believed to regulate R8 target specificity. Consequently, it was suggested that *sens* plays a role in R8 axon targeting (Morey *et al*., 2008).

Three ocelli are located on the vertex of the *Drosophila* head. Ocellar photoreceptors express the Rhodopsin 2 molecule, which confers ultraviolet sensitivity and their axons project to the corresponding ocellar ganglion. Each giant ocellar interneuron from each ganglion projects into the brain via the ocellar nerve. The ocelli are involved in simple visual guidance and modulation of sensitivity of the compound eyes during phototaxis (Hu *et al*., 1979).

1.3. Ommatidial Subtypes

The opsin protein, which is a G-protein-coupled receptor linked to a retinal chromophore, is the photosensitive molecule for most animal species. Depending on their absorption spectra, opsins are classified as UV, blue, green and red opsins.

The “one receptor cell-one receptor molecule” rule is a common phenomenon in most sensory systems to avoid sensory overlap. Human cone cells, for example, choose one out of three opsins; S, M and L whose opsins absorb short (<500 nm, blue), medium (~530 nm, green) and long (~560nm, red) wavelengths, respectively. This is also true for the *Drosophila* visual system. Each photoreceptor cell expresses only one of the six rhodopsins (rh1-rh6), which differ in their wavelength specificity. The outer PRs in each ommatidium always express rh1. The inner PR cell R7 expresses either UV-sensitive rh3 or rh4 and the R8 cell expresses either blue-sensitive rh5 or green-sensitive rh6. According to the rhodopsin expression profile of the inner PRs mainly three different types of ommatidia can be distinguished (Figure 1.4).

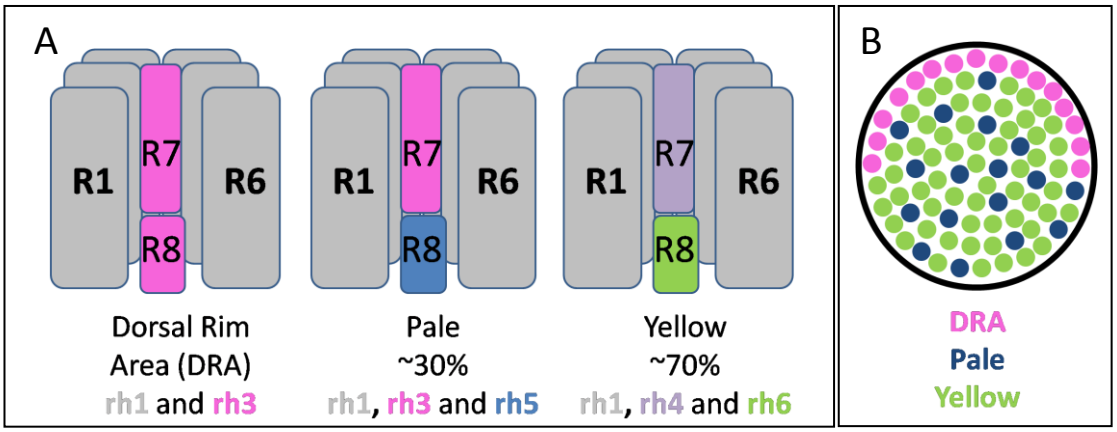

Figure 1.4. The Drosophila retinal mosaic. (A) Inner PRs opsin expression specify ommatidial subtype. (B) Schematic representation of the distribution of ommatidial subtypes throughout Drosophila retina.

The dorsal two rows of ommatidia constitute the dorsal rim area (DRA). These ommatidia are responsible for polarization vision and express UV-sensitive rh3 in both R7 and R8 (Figure 1.4.) (Wernet *et al.*, 2003). The inner PRs of the DRA not only differ in their rhodopsin expression pattern but also they have an enlarged rhabdomere diameter and the rhabdomeres of R7 and R8 are perpendicular to each other. It is believed that they function in detection of polarized light, which helps the fly to navigate itself depending on the sun light (Labhart *et al*., 1999).

Unlike the DRA subtype, the other two ommatidial subtypes "yellow" and "pale" are distributed stochastically throughout the *Drosophila* retina. The inner PRs of both subtypes show coupling in their rhodopsin expression pattern. In the pale (p) subtype of ommatidia, which constitutes 30% of the retina, R7 and R8 cells express rh3 and rh5, respectively. And the remaining 70% of the *Drosophila* eye is composed of the yellow (y) subtype, in which rh4 expression in R7 cells is coupled with rh6 expression in R8.

However, there are some exceptions to those couplings and the one receptor cell-one receptor molecule rule. In 1996, Chou *et al*. described atypical odd-coupled rh3/rh6 ommatidia in wild type fruitflies. This type of ommatidia is found to be outside of the DRA with a low (6%) ratio. One other exception is rh3 and rh4 co-expression in R7 cells in a region near the dorsal edge of the eye, outside the DRA. This newly identified class of ommatidia is called "dorsal y" subtype (Mazzoni *et al*., 2008).

As indicated earlier, different ommatidial subtypes are classified according to the rhodopsin expression pattern of their inner PRs. But how does a PR cell choose to become an inner PR and express rh3 rather than rh4, rh5 or rh6? Which genes/molecules play a role in this choice? Even though some genes playing crucial roles in photoreceptor differentiation have been identified so far, the pathways linking those genes are not well understood.

1.4. Photoreceptor Differentiation

Early photoreceptor formation in *Drosophila* has been shown to occur in a two-step process (Mollereau *et al.*, 2001). The first step, which takes place during larval development, involves neuronal commitment of PRs and targeting of their axons to the optic lobe. In the second step, terminal differentiation of PRs occurs, during pupal development. The terminal differentiation steps include rhabdomere morphogenesis and rhodopsin expression.

1.4.1. Photoreceptor Specification

The retinal determination genes *eyeless* (*ey*), *eye gone* (*eyg*), *sine oculis* (*so*), *eyes absent* (*eya*) and *dachshund* (*dac*) are required for the specification of the eye primordium. It is known that mutations affecting any of these genes cause reduction or absence of the eye. The gain of function studies carried out with these genes, proved that expression of them, alone or in combinations, is sufficient to form ectopic eyes (Jang *et al*., 2003; Morante *et al*., 2007). The two *Drosophila* homologs of *Pax6* genes, *eyeless* and *twin of eyeless* (*toy*), orchestrate expression of other retinal determination genes. The *Pax6* gene and its homologs are referred to as master regulators of eye development because in most species they are capable of inducing ectopic eyes (Morante *et al.*, 2007).

After the determination of the eye imginal disc during embryogenesis –which will form the adult eye-, differentiation of cells starts from the morphogenetic furrow and progresses like a wave through undifferentiated cells. *Decapentaplegic* (*dpp*) and *Hedgehog* (*hh*) signals positively affect this process, while wingless acts as a negative regulator (Morante *et al*., 2007).

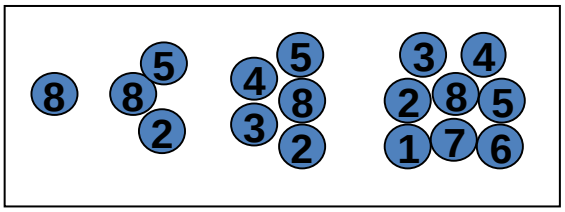

Figure 1.5. Specification of photoreceptors in the larval eye imaginal disc. Schematic representation of PRs get specified; first R8, then R2 and R5, later R3 and R4, and then R1 and R6, and finally R7.

From a cluster of equipotential epithelial cells, one cell chooses to become an R8 cell. Then, other PR cells are recruited by the R8 cell with the help of epidermal growth factor (EGFR) and Notch (N). Later, R2-R5 cells, R3-R4 cells and R1-R7 cells are recruited sequentially (Figure 1.5) (Morante *et al*., 2007).

In 2001, Frankfort *et al*. showed that in *senseless* mutants, R8 differentiation is disrupted and the presumptive R8 cell chooses R2/R5 fate. Additionally, misexpression of *senseless* causes expansion of repression of *rough* in non-R8 cells. And these results suggested that repression of *rough* by *senseless* in R8 cells is sufficient and required for generation of an R8 fate. However, the task of R8 cell differentiation from an equivalence group requires complicated mechanisms rather than a single gene effect. A recent article written by Pepple *et al*. suggests that R8 selection is a two step process; initial selection is done according to lateral inhibiton while the second selection depends on derepression of *senseless* by the transcription factor *rough* with the help of *atonal* (Pepple *et al*., 2008).

1.4.2. Late Photoreceptor Differentiation

Photoreceptor differentiation takes place during pupal development in a stepwise process (Figure 1.6). The first step begins with the choice of becoming an inner versus an outer PR. . Inner PR differentiation starts with generation of color PR fate which is established through the action of *sal*. The differential expression of *pros* and *sens* in R7 and R8 cells respectively causes discrimination of R7 and R8 cells from each other. After that, ommatidial subtype specification process begins. DRA subtype is specified by the expression of *hth* while pale and yellow subtypes require *otd* and *ss*, respectively.

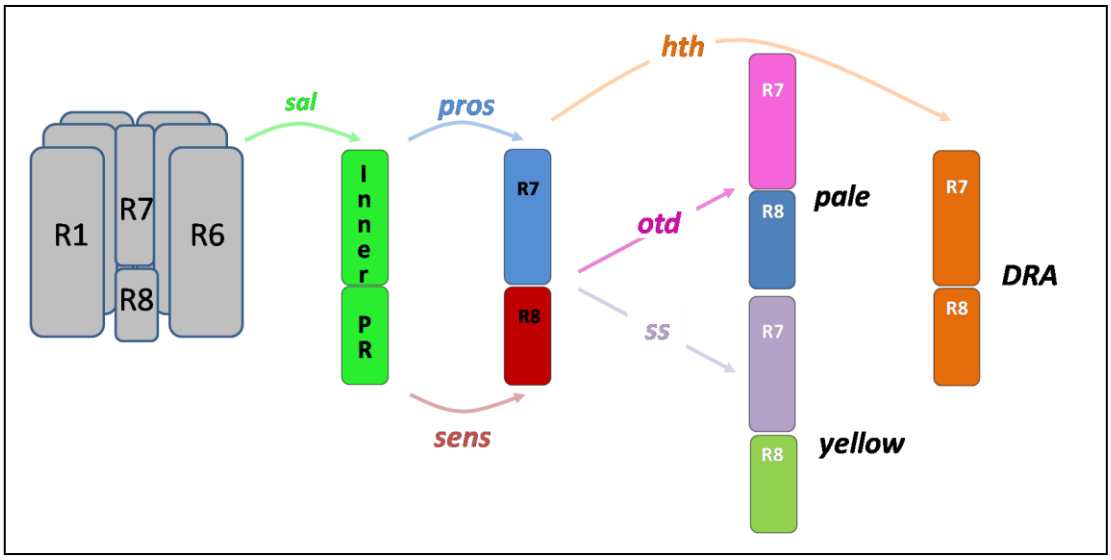

Figure 1.6. Differentiation of inner photoreceptors. Photoreceptor differentiation is a stepwise process.

The *spalt* (*sal*) gene complex encodes two zinc finger proteins; *sal* major (*salm*) and *sal* related (*salr*). In most tissues, these two transcription factors have almost identical expression patterns. Both of them are expressed in R3, R4 and cone cells during larval development. But in *salm* mutant larva, no significant change in the eye imaginal disc was observed. In the pupal stagethe *spalt* genes are expressed exclusively in R7 and R8 cells. Loss of function studies of *spalt* resulted in absence of inner PRs with small rhabdomere diameter and generation of two new PRs with large rhabdomeres (Figure 1.7B left). Additionally, the new cells express rh1 rather than inner PR specific rhodopsins (Figure 1.7B center) (Mollereau *et al.*, 2001). Yet their axons still project to the medulla (Figure 1.7B right) which proves the hypothesis that PR differentiation is a two step process. During larval stages, axonal projections are established as the first step. Then terminal differentiation and rhodopsin expression of photoreceptors take place in the pupa. Since these two processes are independent of each other, in *spalt* mutant flies, axonal projection patterns of photoreceptors are not disrupted while rhodopsin expression patterns of inner photoreceptors are altered. Thus, in *spalt* mutants, inner PRs are transformed into outer PRs and *spalt* is required for the specification of inner PRs (Mollereau *et al*., 2001).

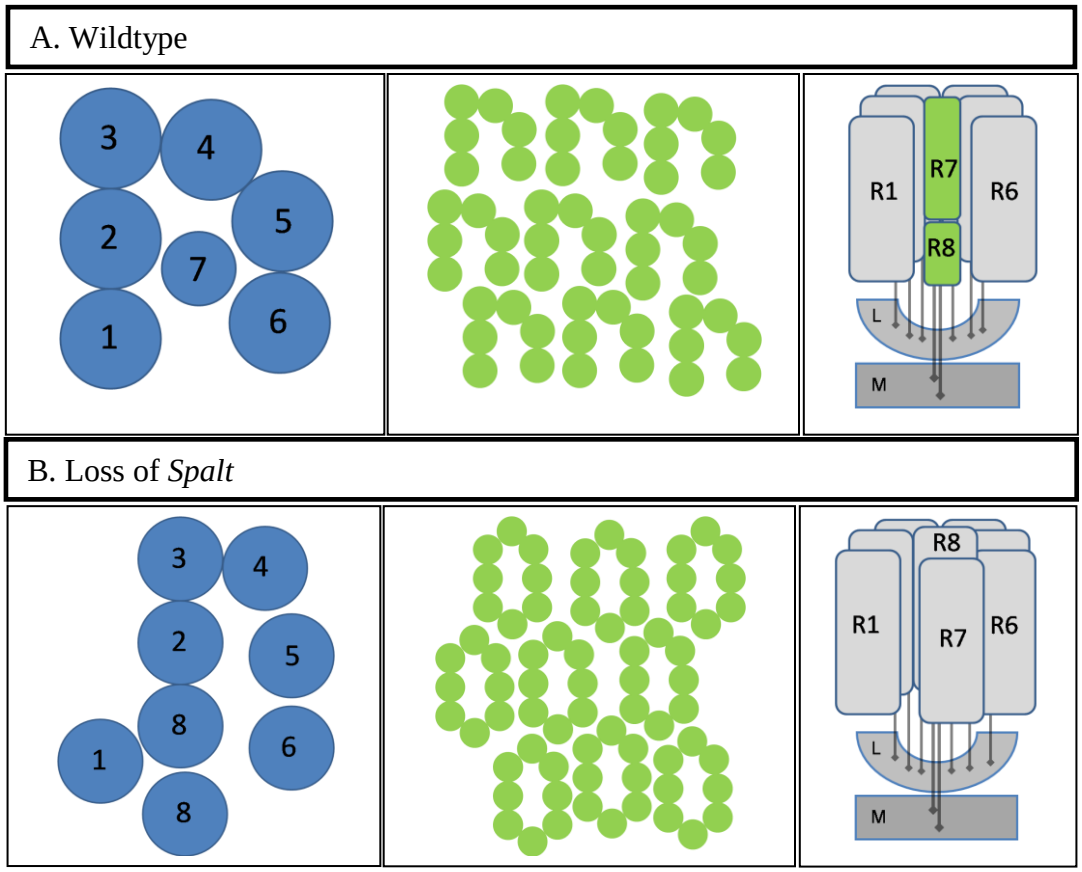

Figure 1.7. Specification of inner PRs: the role of *spalt.*

After acquiring a generic inner PR fate, R7 and R8 differentiation takes place. The transcription factor *prospero* (*pros*) binds specifically the promoters of R8 specific rhodopsins and represses them. *pros* is exclusively expressed in R7 and cone cells in the *Drosophila* eye. In *pros* null mutant eyes rh5 and rh6 expression is derepressed in R7 cells. On the other hand misexpression studies of *pros* resulted in repression of R8 specific rhodopsins in both inner PRs (Figure 1.8). Thus, *pros* directly represses R8 specific rhodopsin expression in R7 cells and is both required and sufficient for R7 specification. Consequently, R7 and R8 distinction is achieved with *pros* expression in R7 cells (Cook *et al*., 2003).

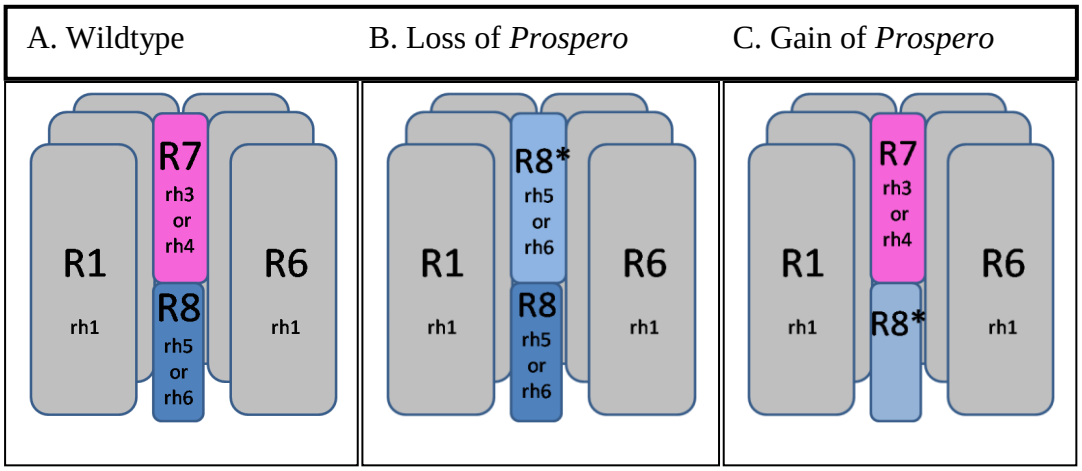

Figure 1.8. Specification of R7 cells: role of *prospero.* (A) In wildtype flies, rh3 or rh4 is expressed in R7 cells. (B) In the absence of *prospero*, R7 cells loose their R7 cell characteristics and express one of R8 specific opsins, rh5 or rh6. (C) Misexpression of *pros* results in repression of R8 specific opsins in R8 cells. These results suggest that *pros* is both required and sufficient for repression of a default inner PR fate, R8, in R7 cells (Cook *et al*., 2003).

The function of the *senseless* (*sens*) gene in R8 cell specification is known to be essential. In *sens* mutants, the presumptive R8 cell chooses the R2/R5 fate. However, senseless has also a late function. In late loss of *sens* mutants R8 specific opsin expressing PRs were significantly reduced and cells were expressing rh3 instead of rh5/rh6 (Xie *et al*., 2008). Additionally, the positions of nuclei in R8 cells were at the center of the retina instead of at the base of the retina. The rhabdomere position of R8 cell was also altered in *sens* mutants. While normally the R8 cell lies under the R7 cell in late loss of *sens* mutants, R8 cells rhabdomeres extended into the R7 layer (Figure 1.9). All of those results suggest that *sens* is necessary for R8 cell differentiation. In order to show that *sens* is also sufficient for R8 cell differentiation, the authors performed misexpression studies. When R7 cells ectopically express *sens*, they start to express rh6 instead of R7 specific rhodopsins. And the nuclear positions of many *sens*-misexpressing R7 cells are altered. Additionally, *pros* expression was significantly reduced in R7 cells. Thus, *sens* is both required and sufficient for R8 cell differentiation (Xie *et al.*, 2008).

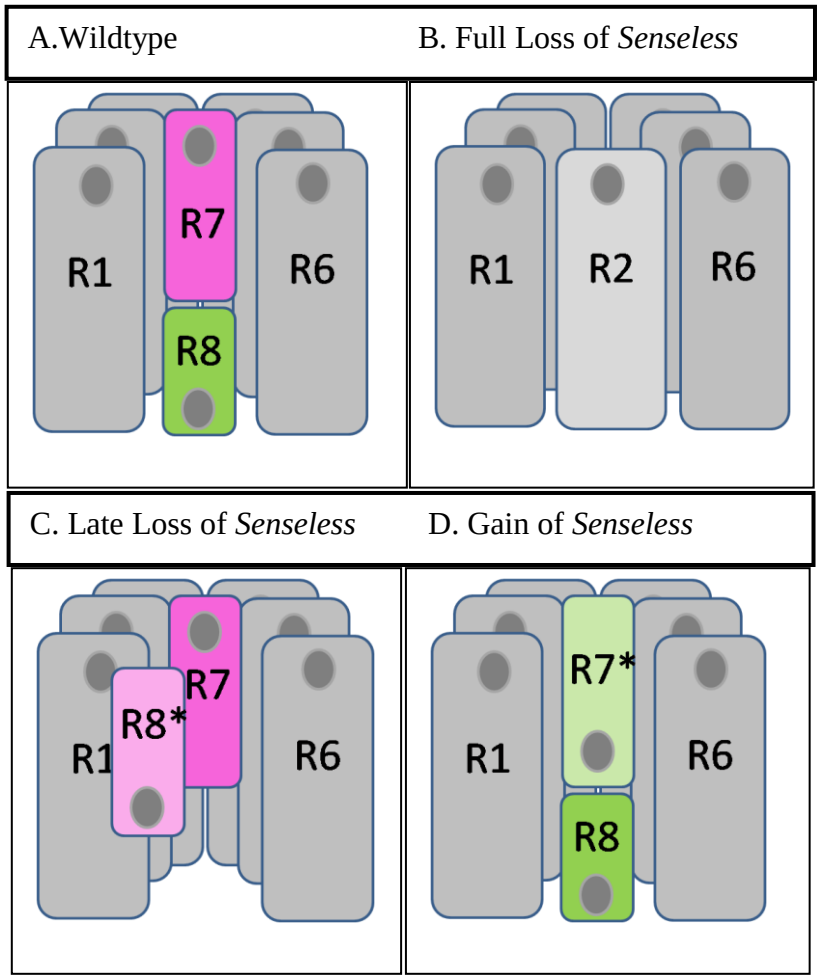

Figure 1.9. Specification of R8 cells: the role of *senseless*. (A) Schematic representation of PRs and their nuclear position in wildtype flies. (B) Full loss of *sens* phenotype. (C) Late loss of function phenotype. R8* represents *sens*-negative R8s (D) Misexpression of *sens* results in R7 cells (R7*) with R8 characteristics, like distal nuclei and rh5/rh6 expression.

After inner PR specification and R7-R8 discrimination, ommatidial subtype specification with differential rhodopsin expression starts. The DRA subtype consists of rh3 expressing both R7 and R8 cells. This specific expression pattern requires a homeodomain transcription factor, *homothorax* (*hth*). It is expressed in R7 and R8 cells of the DRA specifically. Loss of function studies of *hth* revealed transformation of the inner DRA PRs into color sensitive inner PRs by losing their typical enlarged rhabdomeres and rh6 expression in R8 cells. The rh3 expression in R7 cells is not altered in *hth* mutants. Thus, in the absence of *hth* atypical odd-coupled ommatidia are formed instead of y or p subtypes. When *hth* is misexpressed in the eye, rh3 expression expands to all inner PRs

throughout the retina. *Homothorax* is both necessary and sufficient for DRA subtypespecification (Wernet *et al*., 2003).

The two main subtypes in the retina, p and y are stochastically distributed, preserving a particular ratio (30% pale, 70% y). The mechanisms and the genes that are responsible for this distribution are partly known. *Orthodenticle* (*Otd*) is one of the first characterized genes involved in p/y specification. *Otd* is a K_{50} homeoprotein which can bind K_{50} sites and is expressed in all PRs. Loss of *otd* resulted in the loss of rh3 and rh5 expression throughout the retina and the expansion of rh6 expression to outer PRs (Figure 1.10). Additionally, rh1 expression was expanded to a subpopulation of inner PRs. This proves that *otd* is required for pale subtype formation and plays a dual role during this process. It is essential for the expression of pale subtype rhodopsins (rh3 and rh5) and it represses the expression of yR8 specific rhodopsin, rh6, in the pale subtype inner PRs. However, the misexpression studies do not reveal any alterations of rhodopsin expression patterns. Consequently, *otd* is not sufficient to generate p subtype ommatidia on its own, and requires other factors to create ectopic p subtype ommatidia (Tahayato *et al*., 2003).

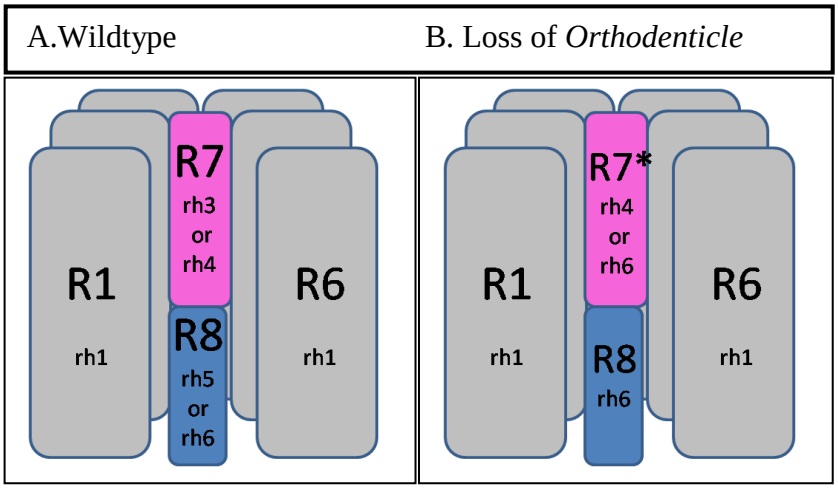

Figure 1.10. Pale subtype specification: role of *otd*. (A) Opsin expression in the wildtype flies (B) Loss of *otd* results in a change of opsin expression inR7 cells. Either one of the two yellow subtype specific opsins, rh4 or rh6, are expressed in R7 cells. Furthermore, in *otd* mutants, all R8 cells become a yellow subtype R8 cell and express only rh6.

The key regulatory gene of p/y specification, *spineless* (*ss*) was defined recently (Wernet *et al.*, 2006). PR-specific *spineless* gene expression is first oberved at mid-

pupation, which is one day before rhodopsins are expressed. 60-80% of R7 cells express ss, which corresponds to the y subtype. Like most of the genes playing a role in terminal differentiation of PRs, ss encodes a transcription factor. Loss of function studies of *spineless* shows a striking phenotype. Rh4 expresssion is diminished while rh3 expression is expanded into all R7 cells. Additionally, there is an increased number of rh6 expressing R8 cells but rh5 expression is not lost like rh3 expression (Figure 1.11B). Remaining yR8 cells in the dorsal third of the eye are coupled with rh3 expressing R7 cells and they form odd-coupled subtype as in the case of absent *hth*. Ectopic expression of ss in developing PRs results in rh4 expression in all PRs including outer PRs and R8s (Figure 1.11C).

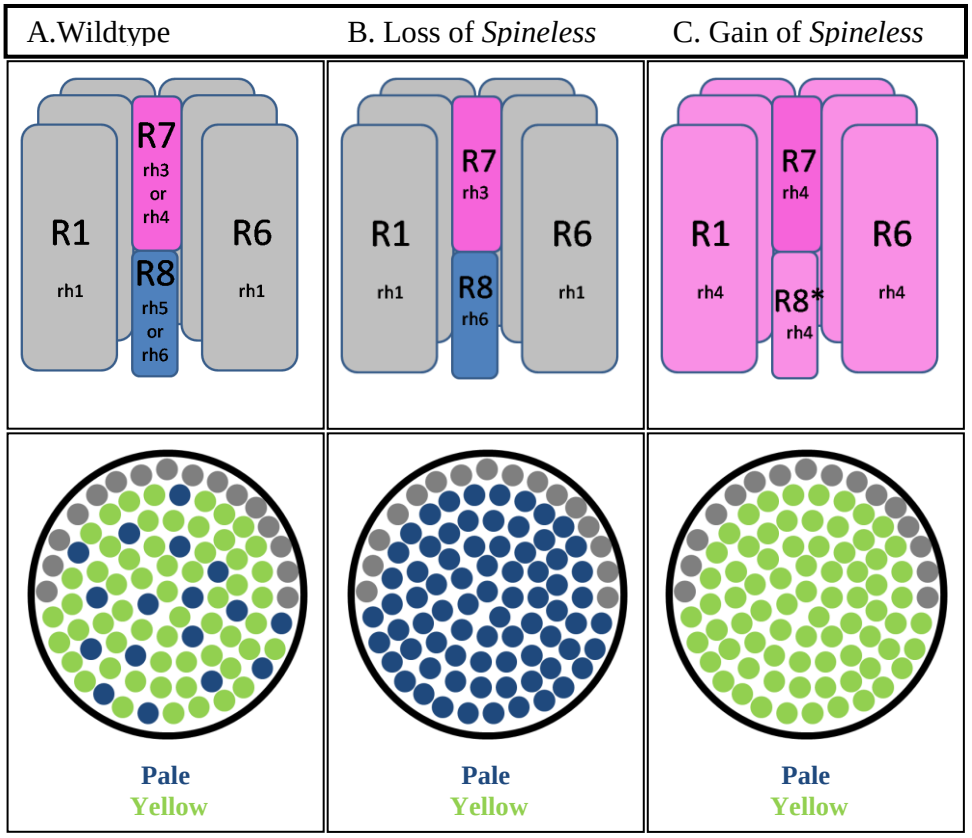

Figure 1.11. Pale and yellow subtype specification: the role of *spineless*. (A) Opsin expression in the wildtype flies (top). The distribution of DRA (gray), pale (30%, blue) and yellow (70%, green) R7 cells throughout the retina (bottom). (B) Loss of *spineless* phenotype. (C) Misexpression of *spineless* phenotype .

The downstream activators/repressors of p/y R8 are two growth regulators *warts* (*wts*) and *melted* (*melt*). They form a bistable loop, in which each of them has the ability to repress the other one. They are expressed in a complementary manner in the pR8 and yR8.

The *wts* mutant flies show rh5 expression throughout the retina without any rh6 expression. In contrast, rh6 expression expands to all R8 cells in *melt* mutant flies (Figure 1.12B and 1.12C). Furthermore, the over-expression of both genes reveals similar phenotypes. When *wts* is misexpressed, rh5 is replaced by rh6 in all R8 cells and when *melt* is misexpressed, rh6 expression is lost (Figures 1.12D and 1.12E).

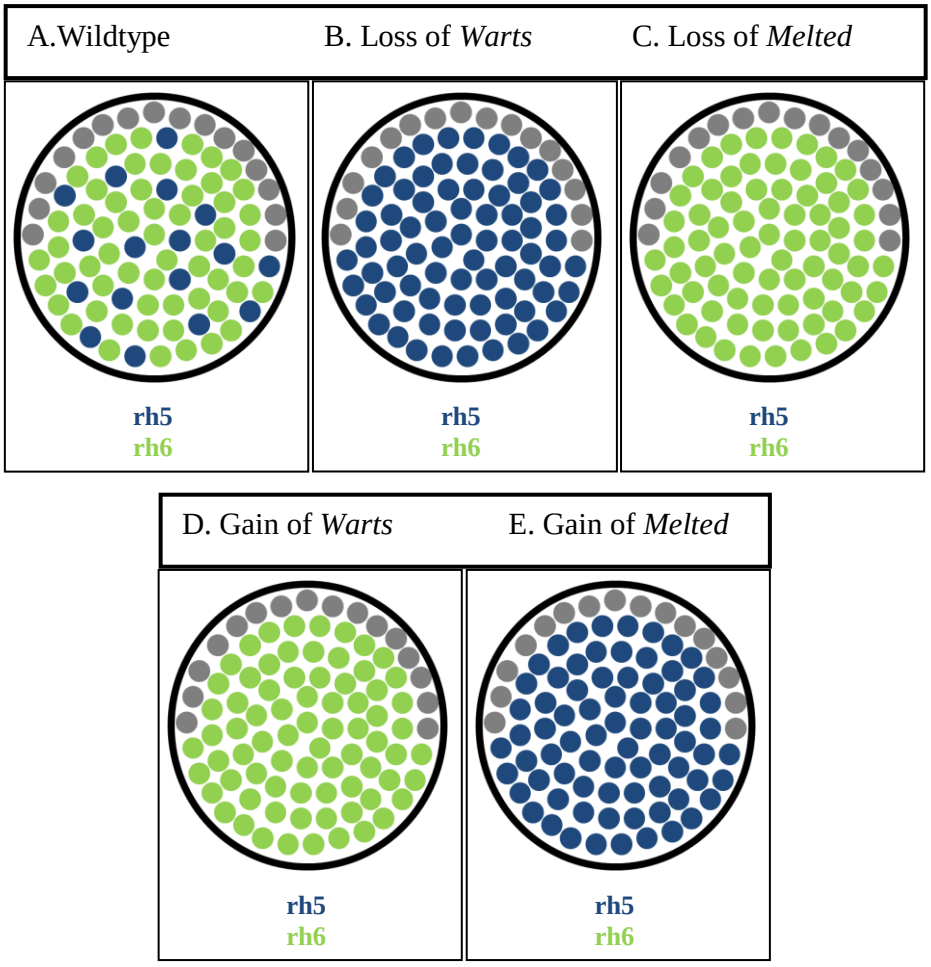

Figure 1.12. Pale and yellow subtype specification: the role of *warts* and *melted*. (A) Schematic representation of opsin expression pattern of R8 cells in wildtype flies: DRA (gray), rh5 (blue) and rh6 (green). (B) Loss of *warts* phenotype. (C) Loss of *melted* phenotype. (D) *Warts* misexpression phenotype. (E) M*elted* misexpression phenotype.

To determine whether *wts* and *melt* repress each other, expression pattern of *melt* was analyzed in *wts* misexpressed flies. As expected, *melt* expression was lost. In conclusion, a model was suggested by Mikeladze-Dvali *et al*., in which *melt* and *wts* repress each other in order to maintain a single pR8 or yR8 fate with the help of *hippo* (*hpo*) and *salvador*

(*sav*). When there is an instruction from a pR7, *melted* expression dominates and it represses *wts*. If there is no instruction, the default state, *wts* expression is dominated and it represses *melt* and prevents the cell from acquiring the yR8 fate (Mikeladze-Dvali *et al*., 2005).

1.5. The Current Model for Ommatidial Subtype Specification

Taken together all those results, the model for retinal patterning seems to involve a two-step mechanism (Figure 1.13). The first step involves the selection of yR7 by *ss*, the "choice" step. The second one is the "instruction" step in which committed yR7 cells send signals to the underlying R8 cells to become pR8.

The choice of becoming a pale versus a yellow ommatidium is first made in the R7 cell depending on *ss* expression. R7 cell expressing *ss* become a yellow R7 cell, and the remaifning R7 cells choose to express rh3 and become a pale R7 cell. Then, upon an instruction coming from neighboring R7 cell, R8 cell become a pale R8 cell and express rh5. It is proposed that in yellow subtype there is no instruction since rh6 expression of R8 cell is the default R8 cell state (Wernet *et al*., 2006).

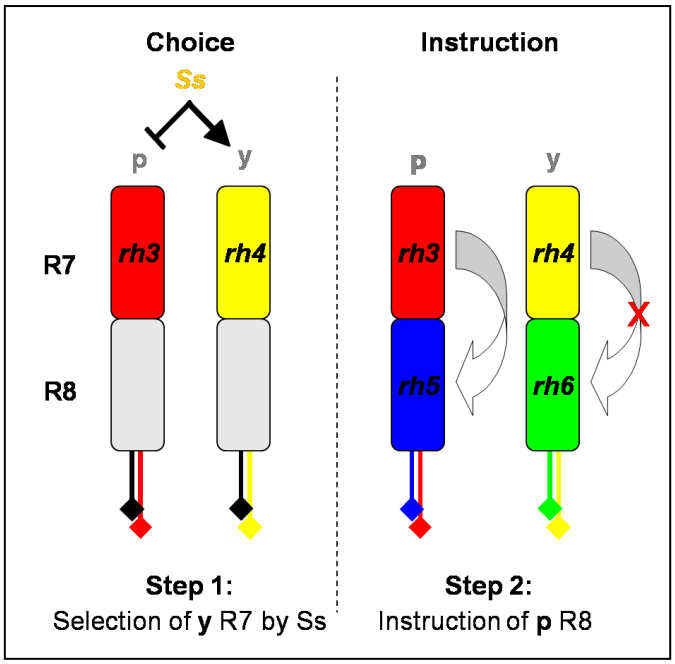

Figure 1.14. The current model for ommatidial subtype specification (Wernet *et al*., 2006).

1.6. The GAL4 Enhancer Trap Screen

In 1993, Brand and Perrimon established a novel method to identify unknown enhancers. The main idea of this method involves the *S. cerevisiae* GAL4/UAS system. After the introduction of the P-element vector containing the GAL4 gene with a minimal promoter into flies, the vector is mobilized by a P-element transposase. When the vector inserts itself is nearby a regulatory region the regulatory region of the gene activates the minimal promoter in the vector, and GAL4 protein synthesis takes place. In the presence of a reporter construct (like UAS-GFP), GAL4 binds to UAS and causes the activation of the reporter gene. Enhancers showing expression in the tissue of interest which can then be selected for further analysis. The general mechanism of enhancer trapping is schematized in Figure 1.14.

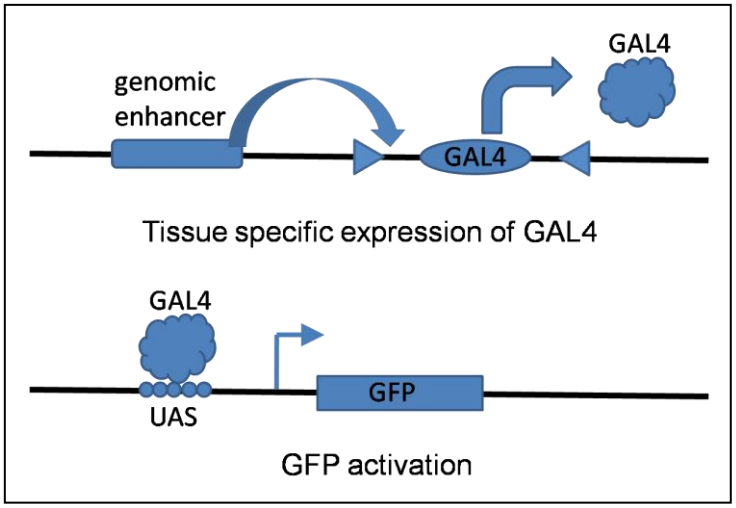

Figure 1.14. General mechanism of enhancer trap screening. A transgenic P-element (region between the two triangles) containing a GAL4 sequence and a minimal promoter comes under the influence of a tissue specific genomic enhancer. When the genomic enhancer is active, GAL4 protein is synthesized. Then GAL4 binds to UAS which causes expression of reporter gene, GFP.

Since the establishment of this method many enhancer-trap screens and other P-element based screening methods have been performed. However, in none of these cases a saturation of the genome was possible. Only 4,000 of 14,000 genes have been associated with P-element insertions due to hot-spots and cold-spots of insertion (Ryder and Russell, 2003). The piggyBac element has a different insertional specificity than P-element and was

reported not to have hot or cold spots of insertion. A piggyBac based enhancer-trap screen was previously performed in order to identify novel genes that might be involved in photoreceptor differentiation (A. Çelik and C. Desplan, unpublished data). In the framework of this study a subset of enhancer-trap lines was analyzed in detail for their expression patterns and their potential role in rhodopsin regulation, which is the last step in PR differentiation.

2. PURPOSE

Photoreceptor specification in Drosophila is a step-wise process which requires activation of specific genes. Although certain transcription factors like *spalt*, *prospero*, *senseless*, *ortodenticle* and *spineless* have been identified so far, the whole picture and the pathways are still not known. In the framework of this thesis, I aimed to identify novel genes involved in photoreceptor differentiation in *Drosophila* using a previously established GAL4 enhancer-trap screen. The strategy of this study maybe summarized as follows:

1. Selection of candidate genes
   - Molecular localization of GAL4 enhancer trap screen lines with inverse PCR protocol.
   - Expression pattern analysis by co-staining with cell specific markers like neuronal cell marker ElaV, R7 cell marker Prospero and glial cell marker Repo.
2. Functional analysis
   - Loss of function analysis with RNA interference method using ey-GAL4, lGMR-GAL4 as drivers to downregulate the targeted gene activity in the eye tissue specifically, and in deficient lines
   - Gain of function analysis using lGMR-GAL4 as a driver to overexpress the targeted gene in all photoreceptors.

3. MATERIALS AND METHODS

3.1. Biological Material

Flies were raised at 25°C with a 12:12 day:night cycle in temperature and humidity-controlled incubators unless otherwise noted. The humidity of incubators was 60%. Commercially available fly food (Applied Scientific, USA) was used (113g fly food: 700ml water) and prepared freshly every week. Fly strains used in the experiments are listed and described in Table 3.1.

Table 3.1. Fly strains used in the course of the study

Name of Line	Chromosome	Description
GAL4 drivers		
lGMR-GAL4	II	(5x) long glass multiple reporter 38 bp binding site driving *S. cerevisiae*
ey-GAL4	II	Eyeless promoter driving *S. cerevisiae* GAL4
UAS constructs		
UAS-*CG7985*	III	UAS containing P-element, P{EPgy2}, in the upstream region of *CG7985*
UAS-Dicer2	II	UAS fused to Dicer2 cDNA
UAS-*fas*	II	UAS containing P-element, P{EPgy2}, in the upstream region of *faint*
UAS-*hdc*	III	UAS containing P-element, P{EPgy2}, in the upstream region of *headcase*
UAS-*heph*	III	UAS containing P-element, P{EPgy2}, in the upstream region of *hephaestus*
UASnGFP	I	UAS fused to nuclear GFP
UAS-*pyd*	III	UAS containing P-element, P{EP}, in *polychaetoid*
UAS-*rdo*	II	UAS containing P-element, P{EPgy2}, in the upstream region of *reduced*
UAS-RNAi-*caps*	II	UAS fused to *capricious* dsRNA
UAS-RNAi-*CG11360*	III	UAS fused to *CG11360* dsRNA
UAS-RNAi-*CG33259*	III	UAS fused to *CG33259* dsRNA
UAS-RNAi-*crp*	II, III	UAS fused to *cropped* dsRNA
UAS-RNAi-*fas*	II, III	UAS fused to *faint sausage* dsRNA
UAS-RNAi-*hdc*	II	UAS fused to *headcase* dsRNA
UAS-RNAi-*heph*	III	UAS fused to *hephaestus* dsRNA
UAS-RNAi-*pyd*	III	UAS fused to *polychaetoid* dsRNA
UAS-RNAi-*rdo*	II	UAS fused to *reduced ocelli* dsRNA
UAS-RNAi-*Sb*	III	UAS fused to *Stubble* dsRNA
UAS-RNAi-*tara*	II	UAS fused to *taranis* dsRNA
UAS-*tara*	III	UAS containing P-element, P{EPgy2}, in the upstream region of *taranis*
Chromosomal Deficiency Lines		
Def-*caps*	III	Df(3L)ED4502 chromosomal deficiency spanning *capricious*
Def-*CG7985*	III	Df(3R)ED6361 chromosomal deficiency spanning *CG7985*

Name of Line	Chromosome	Description
Def-*hdc*	III	Df(3R)ED6332 chromosomal deficiency spanning *headcase*
Def-*pyd*	III	Df(3R)ED5330 chromosomal deficiency spanning *polychaetoid*

3.2. Chemicals and Supplies

All chemicals used in this study were from Sigma, Roche, Fisher Scientific, or Molecular Probes unless stated otherwise.

3.2.1. Enzymes

Restriction enzymes and buffers were used from New England Biolabs, Go Taq polymerase. Buffers were used from Promega, T4 ligase and T4 ligase buffer were used from New England Biolabs.

3.2.2. Chemical Supplies

Ethidium Bromide Solution	:	Sigma Life Sciences, U.S.A.
Goat Serum	:	Millipore
Lithium Chloride	:	Merck, Germany
Marker X	:	Roche Applied Science
Paraformaldehyde	:	Sigma-Aldrich, U.S.A.
Phenol:Chloroform: Isoamyl alcohol	:	Sigma-Aldrich, U.S.A.
SeaKem LE Agarose	:	Cambrex Bio Science, U.S.A.
Tween 20	:	Sigma-Aldrich, U.S.A.
Triton-X	:	Roche

3.2.3. Buffers and Solutions

Solutions were autoclaved for 20 min. at 121 bar, glassware was autoclaved and oven baked for at least 2 hours at 65°C. All buffers and solutions were prepared following protocols by Sambrook and Russel (1989) if not stated otherwise.

Table 3.2. Contents of buffers and solutions utilized in the study

Buffer/Solution	Content
PBS	137mM NaCl 2.7 mM KCl 10mM Na_2HPO_4 1.8mM KH_2PO_4
PBX3	PBS 0.3% Triton X-100
PBX5	PBS 0.5% Triton X-100
10x Loading Buffer	50% Glycerol 0,0005% Bromophenol Blue
1x TAE Buffer	40 mM Tris.HCl 1 mM Ethylenediaminetetraacetic acid (EDTA) 0.1% Acetic acid
Buffer A	100mM Tris (pH 7,5) 100mM EDTA 100mM NaCl 0.5% SDS
BNT Solution	PBS % 0.1 BSA % 0.1 Tween20 250 mM NaCl

3.2.4. Oligonucleotide Primers

Primers were diluted with double distilled sterile water to obtain a final concentration of 100pmol/ul. Diluted primers kept at -20^{0}C.

Table 3.3. Primers used in the course of the study

Primer	Primer Sequence (5'-3')	T (^{0}C)
AC19	CTTGACCTTGCCACAGAGGACTATTAGAGG	72
AC20	CAGTGACACTTACCGCATTGACAAGCACGC	73
AC37	GGTCAACTTCAAAGTCCACCAG	62
AC38	CGGCGACTGAGATGTCCTAAATG	65

3.2.5. Antibodies

Antibodies used during experiments are listed in Table 3.4.

Table 3.4. Antibodies used in the course of the study

Name	Antigen	Species	Dilution	Supplier
Primary Antibodies				
ElaV	ElaV	rat	1:50	Hybridoma Bank
GFP	GFP	rabbit	1:500	Hybridoma Bank
Pros	prospero	mouse	1:10	Hybridoma Bank
Repo	repo	mouse	1:20	Hybridoma Bank
Rh5	Rhodopsin5	mouse	1:50	S.Britt
Rh6	Rhodopsin6	rabbit	1:100	C.Desplan
Secondary Antibodies				
Alexa 488	rabbit	goat	1:1000	Invitrogen
Alexa 555	mouse	goat	1:1000	Invitrogen
Alexa 647	rabbit	donkey	1:1000	Invitrogen
Alexa 647	rat	donkey	1:1000	Invitrogen
Cy3	mouse	donkey	1:1000	Jackson Laboratory
Cy3	rat	donkey	1:1000	Jackson Laboratory

3.2.6. Embedding Media

Vectashield (Vector) Embedding Medium, which is a good bleaching retardant was used for fluorescent substrates and dyes in the course of this study.

3.2.7. Disposable Labware

Test tubes, 15 ml	:	Greiner Bio-One, Belgium
Test tubes, 50 ml	:	Greiner Bio-One, Belgium
Microscope Cover Glass	:	Fisher Scientific, UK
Microscope Slides	:	Fisher Scientific, UK
Filter Tips	:	Greiner Bio-One, Belgium
PCR Tubes	:	Bio-Rad, U.S.A

3.2.8. Equipment

Autoclave	:	Astell Scientific Ltd., UK
Centrifuges	:	Eppendorf, Germany (Centrifuge 5424, 5417R)
Cold Room	:	Birikim Elektrik Soğutma, Turkey

Electronic Balances	:	Sartorius, Germany (TE412)
Electrophoresis Equipment	:	Bio-Rad Labs, USA (ReadySub-Cell GT Cells)
Environmental Test Chamber	:	Sanyo
Fluorescence Stereomicroscope	:	Leica Microsystems, USA(MZ16FA)
Freezers	:	Arçelik, Turkey Thermo Electron Corp., USA (Thermo Forma 723)
Gel Documentation System	:	Bio-Rad Labs, USA (Gel Doc XR)
Heating Block	:	Fisher Scientific, France (Dry-bath incubator)
Heating magnetic stirrer	:	IKA, China (RCT Basic)
Incubator	:	Weiss Gallenkamp, UK (Incubator Plus Series)
Inverted Microscope	:	Zeiss, USA (Axio Observer, Z1)
Laboratory Bottles	:	Isolab, Germany
Micropipettes	:	Eppendorf, Germany (Research)
Microwave oven	:	Vestel, Turkey
Nutating Mixer	:	Labnet
pH meter	:	WTW, Germany (Ph330i)
Refrigerators	:	Arçelik, Turkey
Rotamax	:	Heidolph
Stereo Microscope	:	Olympus, USA (SZ61)
Thermal Cycler	:	Bio-Rad Labs, USA (c1000 Thermal Cycler)
Vortex Mixer	:	Scientific Industries, USA (Vortex Genie2)
Water Bath	:	Grant Instruments, UK (JB Aqua 12)

3.3. Molecular Biological Techniques

3.3.1. Isolation of Genomic DNA

15 flies were homogenized with a tissue homogenizer in a 2ml Eppendorf reaction tube containing 200µl Buffer A. After incubation at 65°C for 30 minutes, 114µl 6M LiCl and 286µl 5M KoAc were added in each tube and tubes were incubated on ice for 10 minutes. Then tubes were centrifuged at 14000rpm for 15 minutes and DNA in the supernatant was extracted using 600µl phenol: chloroform (1:1). The DNA was precipitated by 0,7 volume of isopropanol and centrifugation at 14000 rpm for 15 minutes at room temperature. The pellet was washed with 70% EtOH and again centrifuged for 10 minutes. After 10 minutes air drying, pellet was solved in 75µl distilled water.

3.3.2. Restriction Digestion of DNA

5µl of genomic DNA solution were digested with 10 units of restriction enzyme TaqI (NEB) in a total volume of 30µl at 65°C for 3 hours.

3.3.3. Ligation

10µl of the restriction mix were ligated in a total volume of 400µl, using 2µl T4 DNA ligase (NEB) at 16°C overnight in the presence of 40µl T4 DNA ligase buffer (10x). Then 1100µl % EtOH and 40µl 5M NaOAc were added in each tube and tubes were incubated at -20°C for one hour. The DNA was precipicated by centrifugation at 14000 rpm for 15 minutes at 4°C. The pellet was washed with 70% EtOH and again centrifuged for 10 minutes at 14000 rpm, 4°C. Finally, the pellet was airdried and resusspended in 65µl distilled water.

3.3.4. Inverse PCR

The main idea of inverse PCR is the identification of genomic sequences flanking a transposable element (Figure 3.1). First genomic DNA is digested with the restriction enzyme TaqI. There is a TaqI site in the pBGay 5’ end. When there is another TaqI site in

the nearby gene a digestion product containing both piggyBac and the nearby gene is formed. After ligation with T4 DNA ligase, all digestion products are circularized including our digestion product of interest. The primers designed for inverse PCR bind to piggyBac DNA but amplify outside of the vector because of their position. Therefore, using the piggyBac specific primers the nearby gene region can be amplified and identified by sequencing.In our case primers pointing outward of the pBGay vector were used in PCR reactions on previously digested and circularized DNA. Primer sequences are given in primer Table 3.3.

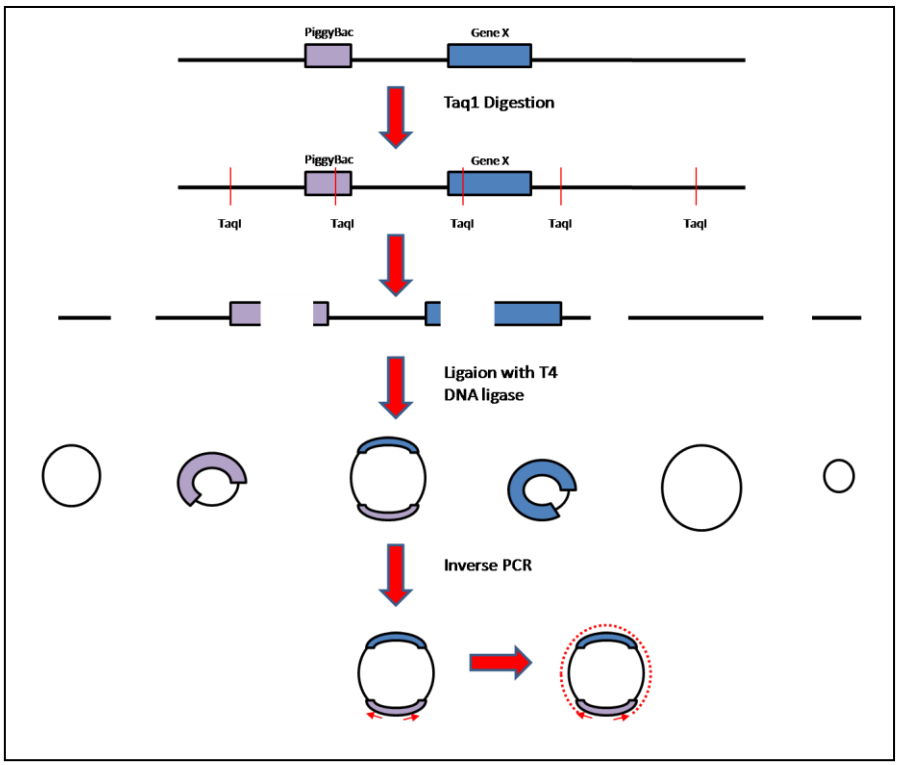

Figure 3.1. Schematic representation of inverse PCR protocol. The inverse PCR protocol of a line in which PiggyBac element (purple box) inserted itself nearby a gene (blue box) is schematized.

The inverse PCR protocol involves two PCR reactions. In the first PCR, 15µl circularized DNA is used as template. Reactions were performed in a total volume of 50µl with 0,5µl dNTP (10mM), 1µl Taq polymerase, 5µl Roche Taq polymerase buffer and 2µl of each primer (AC19 and AC20, 10 pmol/µl). The reaction conditions were: 5’ 95°C, (30’’ 95°C, 1’ 62°C, 2’ 72°C) X 40, 7’ 72°C. For the second PCR, 2µl of first PCR product was used as template without any purification. The reaction conditions were the same as

the first PCR except the primers (AC37 and AC38) and the reaction conditions: 5’ 95ºC, (30’’ 95ºC, 1’ 65ºC, 2’ 72ºC) X 35, 7’ 72ºC. PCR products of both reactions were run on a %1 agarose gel side by side.

3.3.5. Agarose Gel Electrophoresis

A 1% agarose gel was prepared with 1x TBE buffer and 30 ng/ml ethidium bromide solution. Samples were prepared by addition of 1µl loading dye to 50µl of PCR product and loaded on an agarose gel. The gel was run at 120V for 40 minutes and visualized under a transilluminator (Bio-Rad, California, USA).

3.3.6. Gel Extraction of DNA

Agarose gel fragments containing the right bands were excised with a clean scalpel. To obtain DNA fragments of the desired molecular weight QIAquick Gel Extraction Kit (Qiagen) was used according to the manufacturer’s suggestions. DNA was eluted in 30µl of Elution Buffer. The samples are stored at -20°C.

3.3.7. Sequencing Analysis

Purified DNA samples were directly subjected to sequencing. Samples were sequenced at Iontek (Istanbul, Turkey) or Macrogen Inc. (Korea). In order to identify the insertion site of pBGay in each line, the sequencing results were compared against the whole *Drosophila* genome by using the FlyBase service of Indiana University.

3.4. Histological Techniques

3.4.1. Immunohistochemistry

3.4.1.1. Antibody Stainings On Eye Imaginal Discs. Wandering third instar larvae were dissected in PBS (1x). Imaginal disc-brain complexes were fixed in 4% PFA (paraformaldehyde in PBS) for 20 minutes at room temperature. After three washes in PBX3, the samples were blocked for one hour in BNT. The imaginal discs were incubated

in primary antibodies overnight at 4°C. After removal of primary antibody, imaginal disc-brain complexes were washed in PBX3 for three times. Secondary antibodies were added and discs were incubated at room temperature for 2 hours. After three washes in PBX3, eye imaginal discs were seperated from thebrains and mounted with Vectashield (Vector Laboratories). Primary and secondary antibodies and their working dilutions are listed in Table 3.4.

3.4.1.2. Whole Mount Adult Eye Antibody Staining Technique. Adult flies anesthetized on CO_2 pads and their heads and bodies were separated. By using two sharp forceps, proboscis/labrum section was removed and the eye tissue was cleared from remaining brain tissue and cuticle. The dissected eyes were collected on ice. Eyes were fixed with 4% PFA for 15 minutes at room temperature. Lamina parts of fixed eyes were removed. After three washes with PBX5, the samples were blocked with 10% goat serum (in PBS) for 20 minutes at room temperature. Then primary antibodies were added and samples incubated at 4°C for two overnights. After another three washes with PBX5, the tissues were again fixed with 4% PFA for 5 minutes at room temperature. Samples were washed with PBX5 for three times and secondary antibodies were added. After incubation for 4 hours at room temperature and three washes with PBX5, tissues were mounted with Vectashield (Vector Laboratories) and analyzed under a fluorescent microscope (Zeiss) or a confocal microscope (Leica). Primary and secondary antibodies and their working dilutions are listed in Table 3.4.

3.5. Experiments For Functional Knock-down of Selected Gene Products

3.5.1. Knockdown by RNAi

The Vienna Drosophila Research Center has generated UAS-RNAi lines for every gene in the Drosophila genome. These lines are publicly available. RNAi lines for 11 candidate genes were ordered and crossed lGMR-GAL4, ey-GAL4 drivers to knock-down gene function in the eye specifically. It was previously reported that RNA interference method is more efficient in neurons when there is Dicer endonuclease in the cells (Barry Dickson, personal communication). UAS-Dicer constructs were added to the driver line before crossing them with the UAS-RNAi lines. F1 flies carrying all constructs were

selected and whole-mount eyes were dissected (Figure 3.2). After dissection, whole mount adult eye antibody staining technique was performed to analyze any changes in the Rh5/Rh6 ratio. Additionally, Only UAS-RNAi construct carrying transgenic lines were dissected and stained as a control.

3.5.2. RNAi lines

RNAi lines used in the course of the study were ordered from Viena Drosophila RNAi Center and are listed in Table 3.1.

3.5.3. Drivers

In order to knock down the genes tissue specifically, eyeless gene regulatory region (ey-GAL4) and lGMR (lGMR-GAL4) were used as drivers.

3.5.4. Crosses

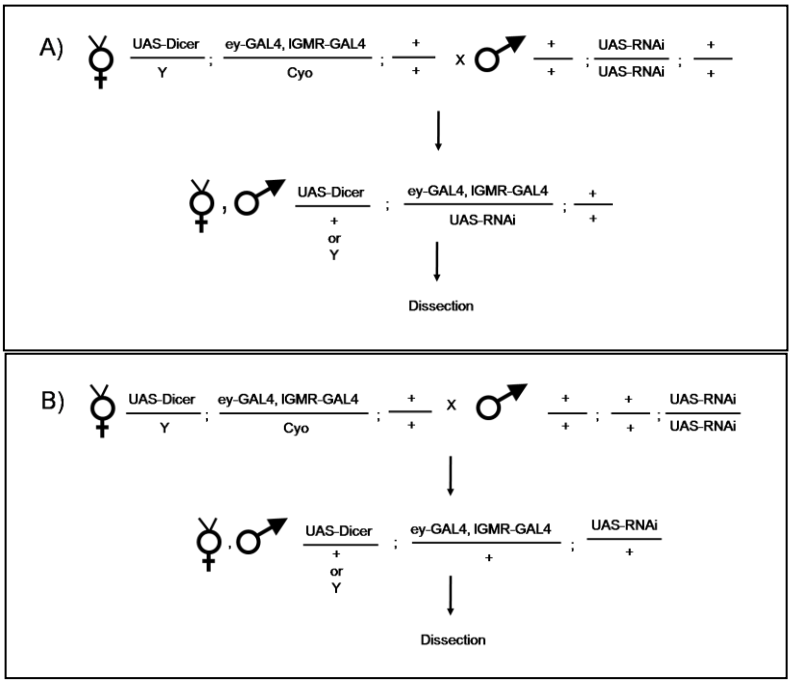

Figure 3.2. Crossing scheme for downregulation of genes with RNA interference.

Figure 3.2A is the crossing scheme of UAS-RNAi construct on the second chromosome. In the F1 generation, flies which do not carry CyO were selected for dissection. Figure 3.2B is the crossing scheme of UAS-RNAi construct on the third chromosome.

3.5.5. Analysis

After dissection of flies, whole mount adult eye antibody staining technique was performed to check rh5/rh6 proportion changes upon inducing genes with RNA interference. Mounted samples were analyzed under a confocal microscope (Leica or Zeis). Stack sizes were changing between 25µm – 35µm depending on the size of the sample Images were merged with Adobe Photoshop CS2 program.

3.6. Experiments For Misexpression of Selected Gene Products

For tissue specific gain of function studies, yeast GAL4/UAS system was used. In this system, depending on nearby sequence of GAL4, tissue specificity can be achieved. The activated GAL4 in the tissue of interest can bind to UAS sequences, which leads to activation of the gene coupled to UAS even if that gene is normally inactive in that tissue and/or for that time.

3.6.1. UAS Constructs and Lines

Flies carrying UAS construct in the upstream region of the selected genes were ordered from Bloomington Drosophila Stock Center (Table 3.1).

3.6.2. Drivers

With the aim of misexpressing selected gene products in all photoreceptor cells, lGMR-GAL4 was used as a driver. Additionally, to avoid auto-fluorescence in red eyes an antisense construct against white was crossed in (UAS-pWIZ).

3.6.3. Crosses

Crossing scheme for misexpression of genes in all photoreceptors are shown in Figure 3.3. In order to select flies carrying both driver and UAS construct, flies were selected against CyO in the F1 generation.

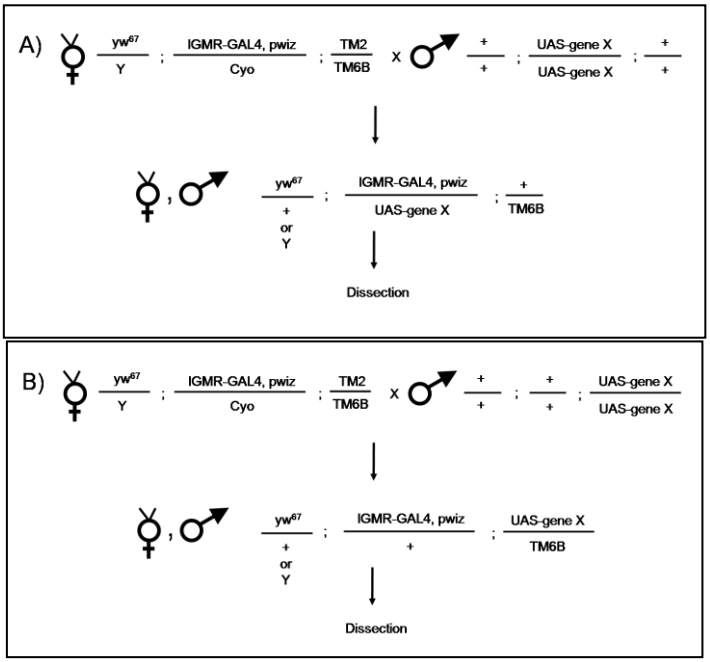

Figure 3.3. Crossing scheme for misexpression of genes in all photoreceptors.

Figure 3.3A is the crossing scheme of UAS-gene X construct on the second chromosome. In the F1 generation flies, which do not carry CyO (curled wing marker) were selected for dissection. Figure 3.3B is the crossing scheme of UAS- gene X construct on the third chromosome.

3.6.4. Analysis

After dissection of flies, whole mount adult eye antibody staining technique was performed to check rh5/rh6 proportion changes upon misexpression of genes. Mounted samples were analyzed under a confocal microscope (Leica or Zeis). Stack sizes were changing between 25µm – 35µm depending on the size of the sample. Images were merged with Adobe Photoshop CS2 program.

4. RESULTS

4.1. The GAL4 Enhancer Trap Screen

In order to characterize novel genes involved in opsin gene regulation and late retinal patterning, a piggyBac enhancer trap screen was previously performed for genes expressed in photoreceptor subsets in pupae (Çelik and Desplan, unpublished results). 258 lines showing expression in the eye were retained. In the framework of this study a subset of these lines were analyzed genetically and molecularly. In particular, 34 lines were molecularly mapped to the *Drosophila* genome by inverse PCR and sequencing. 60% of insertions (18) were found to be in intronic regions. The remaining 40% were mapped to upstream regions. Some gene regions were hit more than one time (*faint sausage*, *CG1907* and *capricious*), and the insertion sites of 3 pairs of lines were exactly the same (Table 4.1).

Table 4.1. Enhancer trap lines retained from the pBGay/UAS-GFP screen. 34 lines were molecularly mapped based on their expression pattern in photoreceptors or the optic lobes.* lines selected for further characterization.

Line #	Gene Name	Chr.	Molecular Function
707*	cropped	2L	transcription factor
711*	polychaetoid	3R	guanylate kinase
724*	headcase	3R	transcription factor
730	CG5017	3R	Unknown
735	Ecdysone-induced protein	3L	heme binding activity
748*	G33259	3L	Unknown
757*	hephaestus	3R	nucleic acid binding and translation repressor activity
783*	unzipped	2R	unknown, involved in axonogenesis
792*	Stubble	3R	serine-type endopeptidase activity
799	CG4835	3L	unknown, suggested to have role in chitin binding
736* & 806*	faint sausage	2R	*Drosophila*-specifc IgSF protein and cell surface protein
827	frizzled	3L	transmembrane receptor activity
832	Odorant receptor 45b	2R	olfactory receptor activity
837	CG12054	3R	unknown, suggested to have zinc ion binding
878	Jumeau	3R	unknown, predicted to have transcription factor activity
884	Leucokinin receptor	3L	leucokinin receptor
887*	CG7985	3R	unknown, suggested to have hydrolase activity
929	CG17838	3R	Unknown,suggested to have mRNA binding activity
946 & 962	CG1907	3R	unknown, predicted to have transmembrane transporter activity
963	fringe	3L	UDP-glycosyltransferase activity
972	Furin 1	3R	serine-type endopeptidase activity
992	CG10750	2L	unknown
1002* & 1012*	capricious	3L	cell adhesşon molecule functioning in R8 PRs axon guidance
1018*	CG11360	4	unknown, predicted to have RNA binding

Line #	Gene Name	Chr.	Molecular Function
1019*	reduced ocelli	2L	unknown, involved in ocellus devolopment
1029	eiger	2R	protein binding
1046	pipsqueak	2R	DNA binding
1048 *& 1078*	taranis	3R	nuclear protein of trithorax group
1049	CG34172	2L	unknown
1055	C-terminal Src kinase	3R	protein tyrosine kinase activity

The functional categories of targeted genes are listed in Table 4.2. In the largest fraction, 30% of the genes are categorized as genes with unknown gene product. The molecular functions of some of these genes are predicted from their sequences, while some of them were shown to be involved in certain biological processes, like axonogenesis (Zhao *et al*., 1998). 4 genes in this category show no homology to previously characterized genes and their molecular functions are unknown. Cell surface proteins and receptors are represented with the second highest percentage, 20%. While 16,7% of the targeted genes are nuclear proteins and transcription factors. When grouped together, enzymes and kinases constitute 13,3% of the trapped genes. Finally, 6,7% of the targeted genes are localized to the cytoplasm.

Table 4.2. Screen results: targeted genes grouped into functional categories. Total number of genes listed is lower than the number of enhancer traps retained, as some genes were targeted multiple times independently. Nearly half of the targeted genes (43,3 %) were either with unknown gene product or with no homologies.

Gene Product	# of genes	percentage	Enhancer Traps
Kinase	1	3	AC1055
Cytoplasmic	2	7	AC735, AC1029
Enzyme	3	10	AC792, AC963, AC972
No homologies	4	13	AC730, AC748, AC992, AC1049
Nuclear Proteins /Transcription Factors	5	17	AC707, AC724, AC757, AC1046, AC1078
Cell Surface Proteins / Receptors	6	20	AC711, AC806, AC827, AC832, AC884, AC1002
Unknown Gene Product	9	30	AC783, AC799, AC837, AC878, AC887, AC929, AC962, AC1018, AC1019

Depending on their expression pattern in the larval eye imaginal discs and the molecular function of the nearby gene, 16 enhancer-trap lines were selected for further characterization (Table 4.1). AC736, AC1012 and AC1018 lines died during this study so further characterization of those lines could not be performed.

In parallel with the localization studies enhancer-trap lines were analyzed for their expression pattern by crossing the enhancer-trap line to UAS-nuclear GFP carrying transgenic flies. The nuclear localization signal in front of the GFP ensures that the GFP protein will be localized to the nucleus and thus will allow the co-localization with other markers. The markers used in this study are usually transcription factors and thus localized to the nucleus. Imaginal disc-brain complexes were dissected and stained with GFP antibodies and the neuronal marker ElaV to visualize all photoreceptors.

Our strategy in functional studies was to analyze Rh5 and Rh6 distribution in R8 cells. The current model for Rhodopsin expression states that a decision is taken by the expression of *spineless* in the R7 cells and this information is then communicated to the R8 cells. Thus, any change in this instruction step should be detectable by checking R8 cell opsin expression.

4.2. Enhancer Trap Lines with Inner PRs Expression Pattern

4.2.1. A GAL4 Enhancer Trap Insertion in *Hephaestus*

One of the insertions of the piggyBac element pBGay (line AC757) was chosen for further characterization because of its particularly interesting expression pattern and molecular function of the nearby gene, *hephaestus* (*heph*). The *Hephaestus* gene encodes the mammalian homologue of polypyrimidine tract binding protein (PTB).

In vertebrates, PTB binds to intronic polypyrimidine tracts preceding many 3′ pre-mRNA splice sites (Garcia-Blanco *et al*., 1989). Additionally, there is experimental evidence that PTB's have many other functions, including the control of alternative exon selection (Cote *et al*., 2001), translational control or internal ribosome entry site (IRES) use (Kim *et al*., 2000), mRNA stability (Tillmar *et al*., 2002) and mRNA localization (Cote *et al*., 1999). PTB may also act as a transcriptional activator (Rustighi *et al*., 2002).

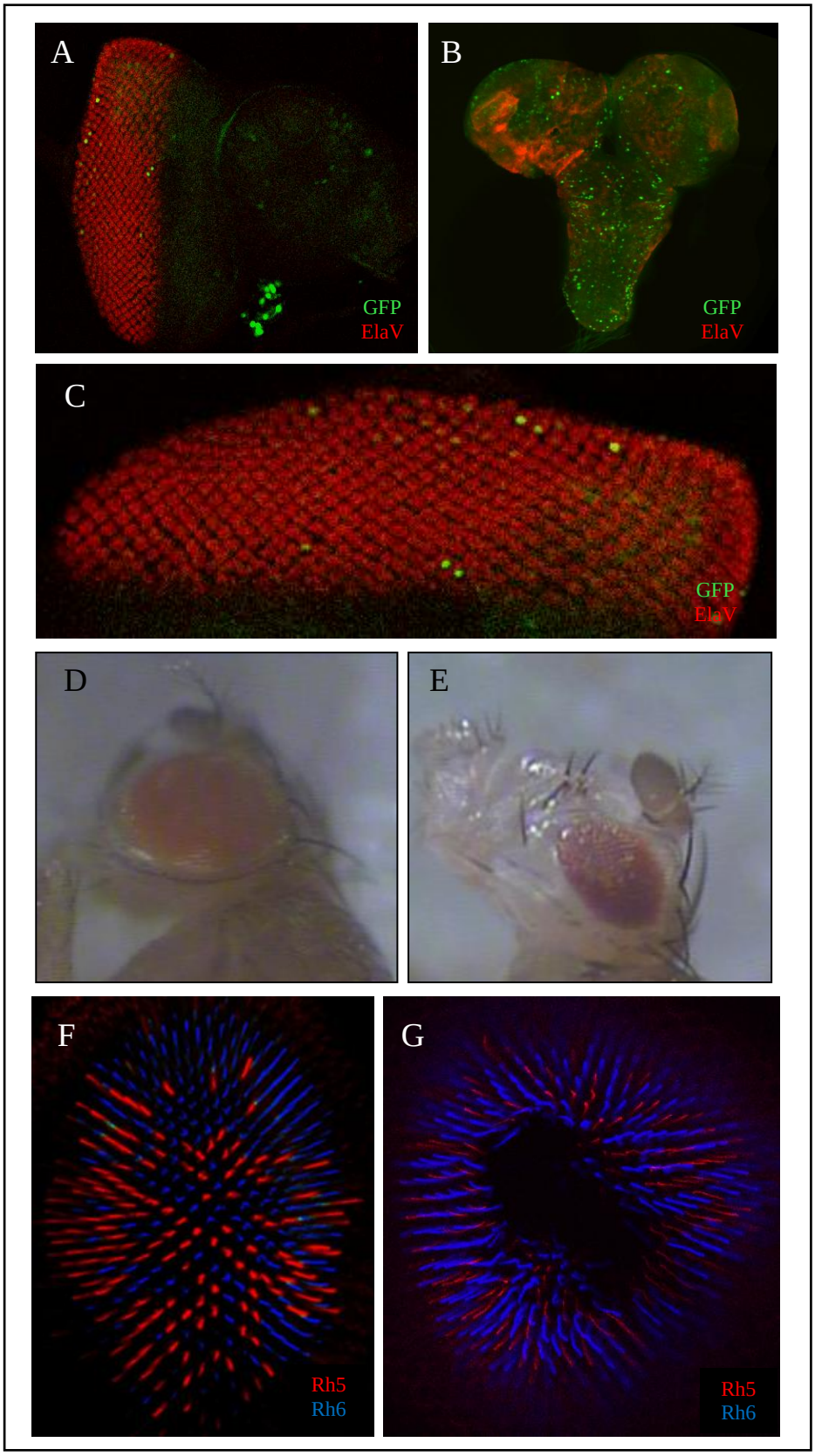

Figure 4.1. AC757 is expressed in R2 or R8 photoreceptors and downregulation of *hephaestus* causes a small eye phenotype.

The *Hephaestus* gene was initially identified in a genetic screen as a player of spermatogenesis (Castrillon *et al*., 1993). Later it was shown that *hephaestus* affects the wing margin and wing vein pattern formation by attenuating Notch activity and it was suggested that *heph* acts as a repressor of Notch activation (Dansereau *et al*., 2002).

4.2.1.1. *Hephaestus* is Expressed in R8 Cells. *Heph* expression was analyzed in third instar larval imaginal discs and larval brains. Larval brains were analyzed as well as any expression in the optic lobe would be particularly interesting for defining the color vision circuit. In the eye imaginal disc *heph* expression was observed in a small subset of ommatidia in R2 and R8 PRs, mostly in R8 cells (Figures 4.1A and 4.1C). In the brain *heph* expression was observed in motor neurons in the ventral nerve cord (Figure 4.1B).

4.2.1.2. Downregulation of *Hephaestus* Expression Leads to Reduction in Eye Size. To investigate a possible role of *heph* in PR differentiation and rhodopsin regulation *heph* was down-regulated using eyGAL4, lGMR-GAL4, UAS-Dicer2 as a driver and UAS-*heph* RNAi lines generated in the Vienna Drosophila Resource Center. Upon down-regulation of *heph* a significant reduction in eye sizes was observed. The eyes in 2 analyzed flies (Figure 4.1E) were approximately half the size of wild-type eyes (Figure 4.1D). The observed phenotype indicates that *heph* might function in early eye development and may be important for eye growth.

4.2.1.3. Misexpression of *Hephaestus* Has No Effect on Ommatidial Subtype Choice. Using lGMR-GAL4 as a driver and UAS-*heph*, *heph* was ectopically expressed in all photoreceptors. Then R8 specific rhodopsin gene expression in adult eyes of flies carrying both constructs was analyzed. The distribution of Rh5 and Rh6 expressing cells were similar to wild type retinas (Figure 4.1F) and no obvious change in the ratio of Rh5%/Rh6 was detected (Figure 4.1G).

4.2.2. A GAL4 Enhancer Trap Insertion in *Unzipped*

Inverse PCR analysis followed by sequencing revealed that the AC783 line molecularly maps into the first intron of the gene *unzipped*. The *unzipped* gene codes for a putative integral membrane protein and is believed to be involved in axon recognition or

axon adhesion. It was shown that *unzipped* mutants affect axonal fasciculation in the CNS and was suggested that un*zipped* is an essential component required for the establishment of the normal pattern of axons during late neural development (Zhao *et al*., 1998).

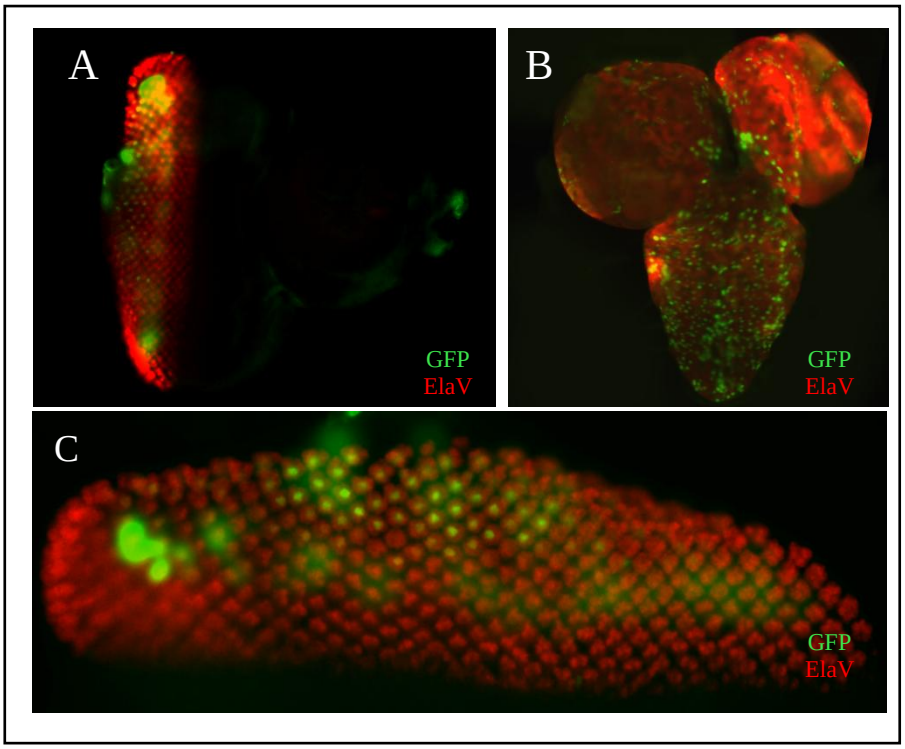

Figure 4.2. AC783 is expressed in R8 photoreceptors.

4.2.2.1. *Unzipped* is Specifically Expressed in R8 Photoreceptors. The expression pattern of *unzipped* is very interesting. In third instar larval eye imaginal discs expression of *unzipped* starts around the 7th row of ommatidia and is restricted to one cell per ommatidium (Figures 4.2A and 4.2C). From its position within the ommatidum we presume that the labeled cells are R8 cells. This expression pattern is very interesting as no other gene has been observed that shows this kind of late R8 specific expression in the eye disc. In the larval brain, *unzipped* expression was observed in optic lobes and some motor neurons in the ventral nerve cord (Figure 4.2B).

4.2.3. A GAL4 Enhancer Trap Insertion in *Capricious*

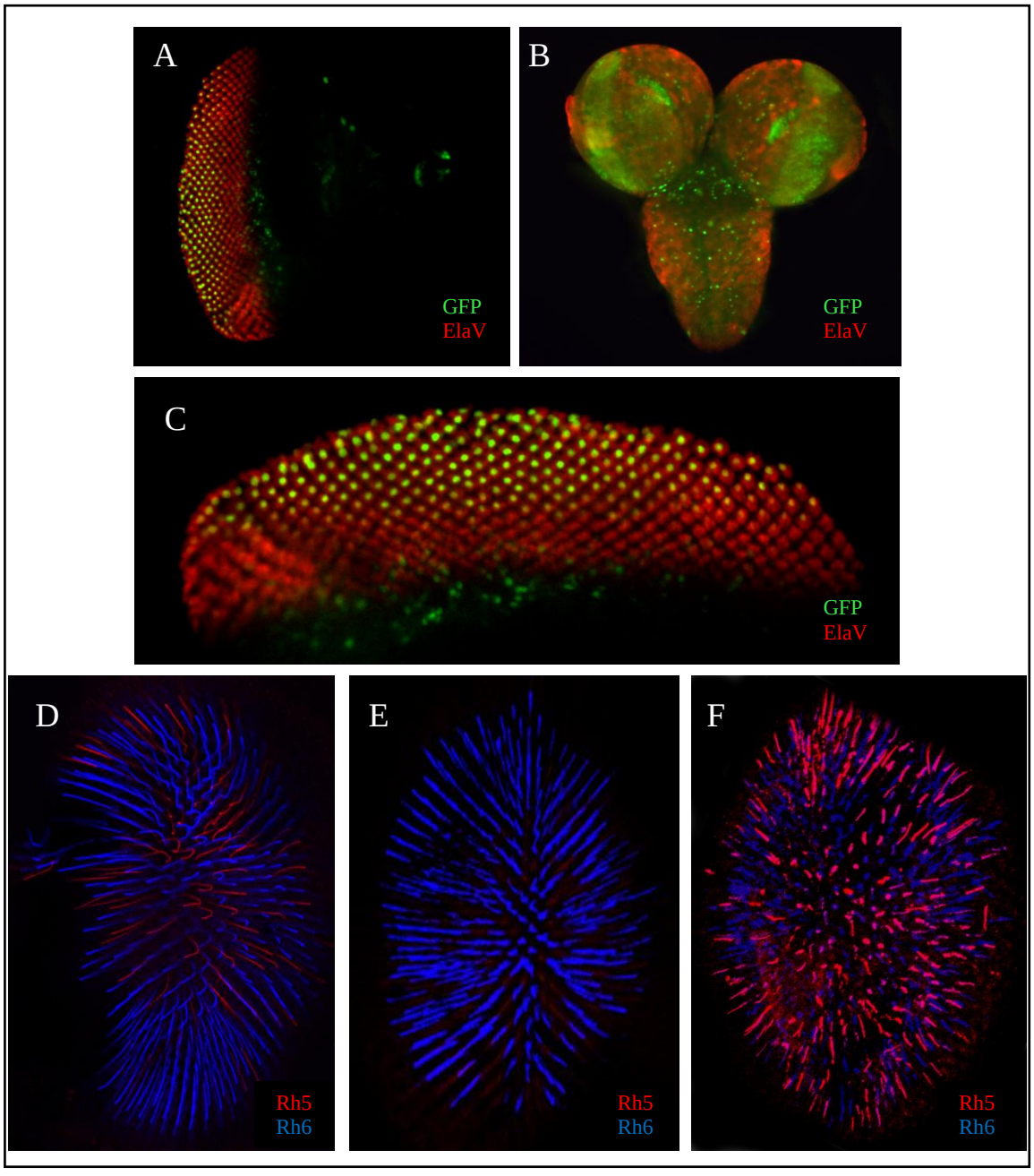

Figure 4.3. AC1002 is expressed in R8 photoreceptors.

In our screen, two lines -AC1002 and AC1012- were mapped to the same region, 3,5kb upstream of the *capricious* gene. Unfortunately the AC1012 line was not very healthy and the stock died after DNA extraction was performed. Consequently, the expression pattern could not be determined for that line. The *capricious* gene (*caps*), a transmembrane protein with leucine-rich repeats (LRRs), is shown to be an essential

component of R8 axon projection. In the absence of *caps*, local targeting errors by R8 axons were observed. On the other hand, misexpression of *caps* in R7 cells cause projection of R7 cell axons to the R8 target layer, M3 (Shinza-Kameda *et al*., 2006).

During development, a gene can play different roles in the same cells at different times. For example, the *senseless* gene functions in both specification of R8 cells in third instar larval stages and in terminal differentiation of R8 cells and rhodopsin expression during pupal stages (Frankfort *et al*., 2001; Xie *et al*., 2007). In the light of this, *caps* was chosen for functional analysis in order to understand if it might have a later role in retinal differentiation.

4.2.3.1 *Capricious* is Expressed in R8 Cells. In previous studies, *caps* was shown to be expressed specifically in R8 cells in the eye imaginal disc and throughout pupal stages (Shinza-Kameda *et al*., 2006). Gene expression of the enhancer-trap line AC1012 in our study correlates with previous results. In the third instar larval eye imaginal disc, *caps* expression was detected in one photoreceptor cell per ommatidia and its position indicated that it is an R8 cell (Figures 4.3A and 4.3C). In the brain, *caps* expression was observed in optic lobes and some motor neurons in the ventral nerve cord (Figure 4.3B).

4.2.3.2. Loss of *Capricious*. For loss of function studies, *capricious* gene activity was down-regulated using the RNA interference method. Genes were down-regulated specifically in the eye using eyGAL4, lGMR-GAL4, UAS-Dicer2 as a driver. Then, expression of Rhodopsin5 and Rhodopsin6 in R8 cells of whole mount adult eyes was analyzed. When compared to wild type eyes, no changes in the Rh5/Rh6 expression pattern were observed (Figure 4.3D). But in the driver line Rh5/Rh6 co-expression was observed in 107 cells (~39%) out of total 272 cells counted. And Rh6 expression was observed in 262 cells (~96%). While Rh5 expression (~40%) was close to the wild type ratio (~30%) (Figure 4.3E). Consequently, there was an expansion of Rh6 expression. And it was suggested that excess amount of Dicer2 in those flies interact with the Rh5/Rh6 gene expression pathway which requires RNA interference for gene regulation. But the details of the pathway or how this interaction takes place are not known. Thus, when *caps* loss of function phenotype is compared to driver line phenotype, it seems the effect of excess

Dicer was removed by down-regulation of *caps*. These results indicate that *caps* might function in Rh5/Rh6 expression pattern.

Furthermore, *caps* deletion line which consists of a large deletion (Df(3L)ED4502) on 3rd chromosome spanning *caps* gene, was ordered from Bloomington Drosophila Stock Center. Due to deleted numerous genes, the line was homozygous lethal. Consequently, R8 opsin expression analysis was performed on heterozygote *caps* mutants. The Rh5/Rh6 ratio of heterozygote *caps* mutants revealed no changes compared to wild type (Figure 4.3F).

In conclusion, as deduced from its RNAi phenotype *caps* seems to have a role in Rh5/Rh6 expression regulation in R8 cells according to its RNA interference phenotype. Yet, as seen from the analysis of the deficiency line of one copy is not enough to characterize *caps* function. Full knock-down of *caps* is necessary to make any conclusive conclusions.

4.2.4. An Enhancer-trap Insertion in *CG7985*

The insertion of the enhancer-trap line AC887 was mapped to the first intron of the *CG7985* gene. Based on its sequence *CG7985* has been predicted to have O-Glycosyl hydrolase activity. Glycosyl hydrolases are responsible for hydrolization of the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a noncarbohydrate moiety (Henrissat *et al*., 1995). Depending on their substrate type, O- or S-glycosides, glycoside hydrolases are classified into two groups: O-glycosyl hydrolases and S-glycosyl hydrolases. It was previously reported that *O*-linked glycosylation functions in secretion to form components of the extracellular matrix, adhering one cell to another by interactions between the large sugar complexes of proteoglycans. Since we are particularly interested in how communication between R7 and R8 cells can be established and maintained the suggested function for *CG7985* appeared very interesting and thus we tried to pursue it functionally.

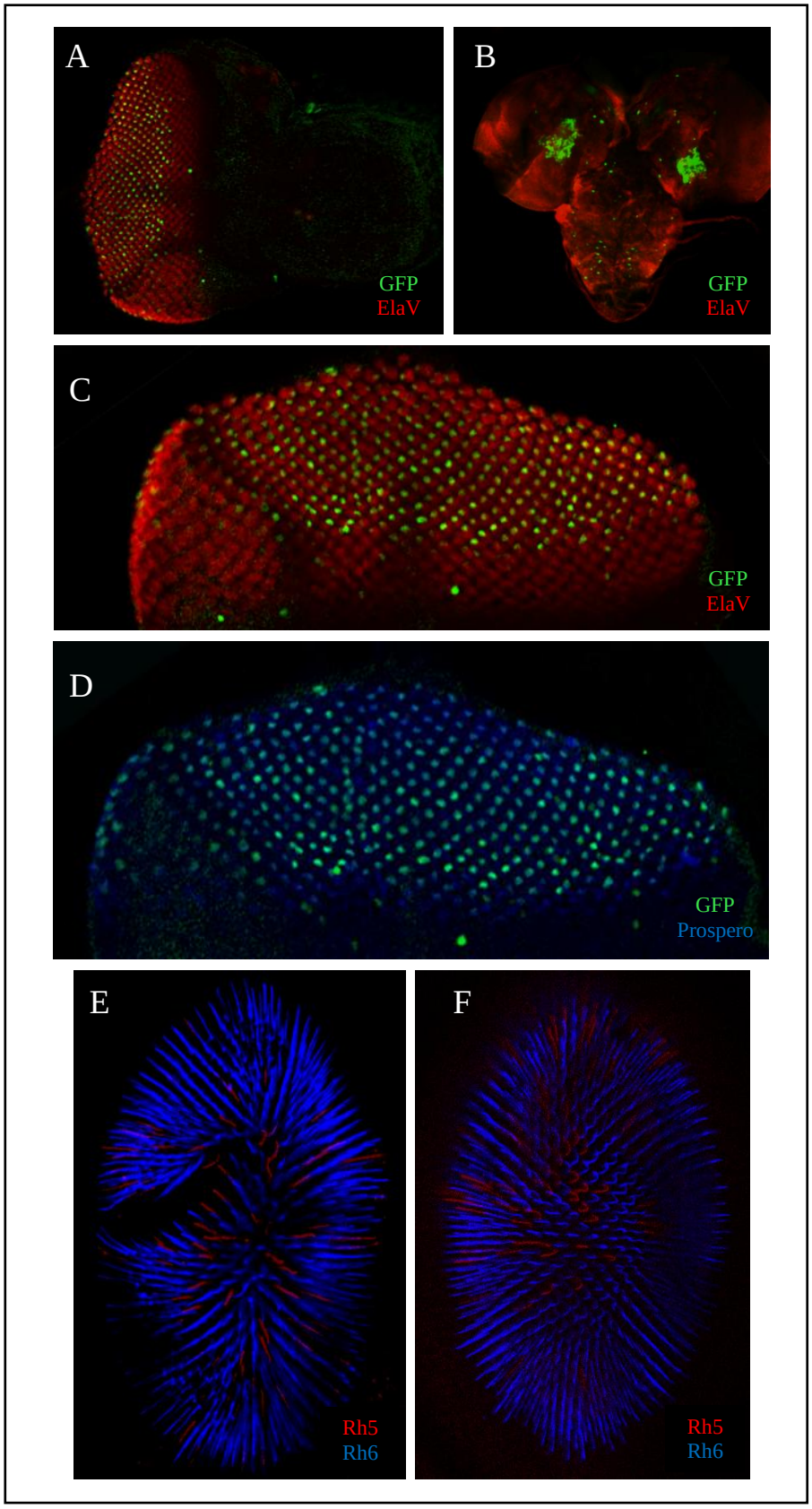

Figure 4.4. AC887 is expressed in R7 photoreceptors.

4.2.4.1. *CG7985* is Expressed in R7 Cells. *CG7985* expression in third instar larval imaginal discs was found to be specific to one cell per ommatidium (Figures 4.4A and 4.4C). By co-staining with the R7 cell marker prospero, it could be shown that there is a 100% overlap of *CG7985* expressing cells and prospero-positive cells (Figure 4.4D). During the recruitment of photoreceptor cells, R7 cells are the last ones to be specified. Consequently, prospero expression starts at around the 8^{th} row of ommatidia while the onset of *CG7985* expression is around two rows later. This indicates that *CG7985* might have a role in R7 specification, but could also have a role in differentiation of R7 cells. In the brain *CG7985* expression was observed in some neurons in the ventral nerve cord and the antennal lobe, which is important for olfactory processing (Figure 4.4B).

4.2.4.2. Analysis of *CG7985* Deficient Flies. Aiming to identify whether *CG7985* has a function in photoreceptor differentiation and rhodopsin gene regulation, *CG7985* deficient flies were analyzed. The deficiency (Df(3R)Exel6178) has a large deletion on chromosome arm 3R where *CG7985* is located and is homozygous lethal. Consequently only hetrozygote *CG7985* mutant flies could be analyzed. Whole mount adult eyes were stained for Rh5 and Rh6 to check for changes in the rhodopsin ratio, however no changes in the distribution of yellow and pale ommatidia were observed (Figure 4.4E). Analysis should be repeated in homozygous mutants.

4.2.4.3. Gain of Function Analysis of *CG7985*. *CG7985* was ectopically expressed in all photoreceptors, using lGMR-GAL4 as a driver and UAS-*CG7985* line and stained for Rh5 and Rh6. Gain of function analysis revealed no changes in rhodopsin ratio when compared to wild type eyes (Figure 4.4F), which indicates that *CG7985* does not have a role in R8 rhodopsin regulation. Any changes in R7 rhodopsin expression that have no impact on changes in the R8 rhodopsin expression as well as any effects on axonal projection patterns will be investigated later.

4.3. Enhancer Trap Lines With Outer PRs Expression Pattern

Several enhancer trap lines were found to have specific expression patterns in outer PRs. Since so far it cannot be excluded conclusively that outer PRs don't have any function in inner PR specification analyzing those is still important. Besides their function in adult

flies, two outer PRs, R3 and R4, play a role in establishment of planar cell polarity during larval stages. Their correct specification is required for establishment of proper ommatidial chirality (Domingos *et al*., 2005).

4.3.1. A GAL4 Enhancer Trap Insertion in *Cropped*

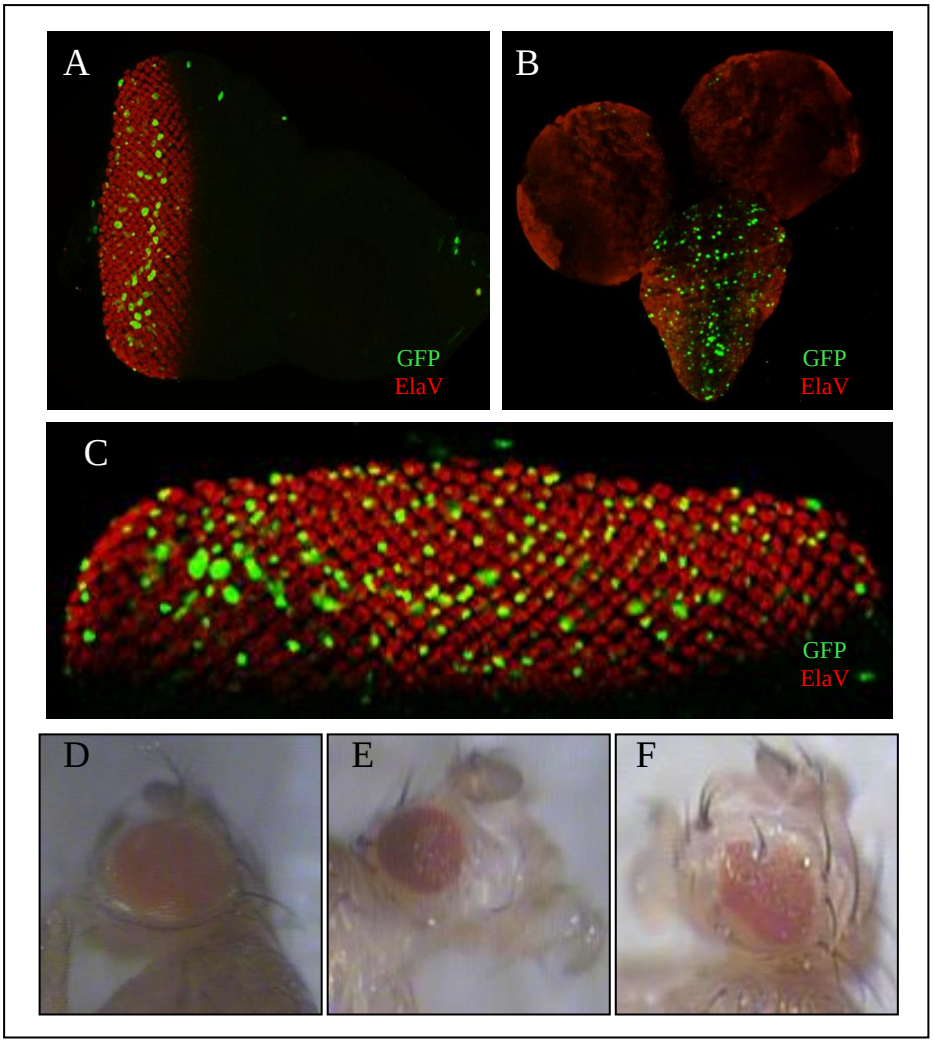

Figure 4.5. AC707 is expressed in R3 and R4 photoreceptors, and downregulation of *cropped* causes a small eye phenotype.

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The enhancer-trap line AC707 was chosen for further characterization because of the molecular function of the nearby gene, *cropped* (*crp*). The *cropped* gene encodes a basic Helix-Loop-Helix (bHLH) transcription factor. The bHLH motif is characterized by two α-helices connected by a loop. The bHLH transcription factors play important roles during the development of various metazoans including flies, nematodes, and vertebrates.

BMAL1-Clock, which is a core transcription complex in the molecular circadian clock, and c-Myc, which have been linked to cancer due to their effects on cell growth and metabolism, are two examples of bHLH family members in humans.

There are 56 bHLH genes in *Drosophila melanogaster* genome. For some of them involvement in eye development were previously shown. For instance, the *atonal* gene is a bHLH family member and is essential for R8 cell recruitment during larval stages (Jarman *et al*., 1994). Another example of bHLH family transcription factor is *spineless*. *Spineless* is transiently expressed in pupal stages and it has an essential role in ommatidial subset specification in eye development (Wernet *et al*., 2006). The *Cropped* gene has known interactions with *sisyphus*, the *Drosophila* myosin XV homolog, which plays a role in dorsal closure (Liu *et al*., 2008). With the aim of identifying whether *cropped* gene is involved in eye development, functional analyses were performed.

4.3.1.1. *Cropped* Shows Expression in R3 and R4 Cells. *Crp* expression was observed in third instar larvae in both eye-imaginal discs and the larval brain (Figures 4.5A and 4.5B). In almost every ommatidium, *crp* expression was observed mostly in both R3 and R4 cells while in a small subset of ommatidia it was observed only in R3 or R4 cells (Figure 4.5C). Expression in cells overlying the photoreceptors was also apparent. These cells are most probably glial cells. Analysis of *crp* expression in the brain revealed expression in motor neurons in the ventral nerve cord and few dorsal neurons (Figure 4.5B).

4.3.1.2. Loss of Function Analysis of *Cropped*. A possible role of *crp* on PR differentiation and rhodopsin regulation was investigated by down-regulating the expression of *crp* using eyGAL4, lGMR-GAL4, UAS-Dicer2 as a driver in two UAS-*crp*-RNAi lines (26885 and 26886). As a result, flies with significantly reduced eye sizes were observed (Figures 4.5E and 4.5F). In all (9) flies analyzed the size of the eyes was reduced by half as compared to wild type (Figure 4.5D). The observed phenotype indicates that *crp* might function in the early eye development and/or eye growth.

4.3.2. A GAL4 Enhancer Trap Insertion in *Polychaetoid*

The piggyBac element in the enhancer-trap lineAC711 was localized close to the *polychaetoid* (*pyd*) gene. *Polychaetoid* (PYD) encodes a membrane-associated guanylate kinase (MAGUK) protein that localizes to adherens junctions. MAGUK proteins act as protein scaffolds by coordinating protein interactions through three types of domains and the PDZ domain is one of them (Gonzalez-Mariscal *et al*., 2000). PYD is a PDZ domain protein whose sequence is closely related to mammalian Zona Occludens proteins, ZO-1, ZO-2 and ZO-3 (Takahisa *et al*., 1996). MAGUK family members function was interpreted as to localize, recruit, and organize other proteins at the plasma membrane; in order to allow extracellular signals to initiate a response from intracellular signal transduction pathways, and/or to act as molecular scaffolds or anchoring proteins that are crucial in controlling cytoarchitecture and maintaining cell junction structure (Dimitratos *et al*., 1999).

Pyd expression was previously shown in two classes of support cells within the pupal eye, interommatidial precursor cells and cone cells, at 25 h after puparium formation (Seppa *et al*., 2008). Decreasing *pyd* activity leads to defects in the patterning of those support cells, indicating the involvement of *pyd* in supportive cell pupal development in the eye. But *pyd* expression in photoreceptors and whether or not it has a function in those cells have not been reported yet.

4.3.2.1. Expression Pattern of *Polychaetoid.* *Pyd* expression analysis in third instar larvae in both eye-imaginal discs and larval brain revealed expression in both structures (Figures 4.6A and 4.6B). As shown in Figure 4.6C, *pyd* expression was observed in almost every ommatidium starting right after the morphogenetic furrow mostly in R3 and R4 cells. In later rows additional expression in R6 cells was also apparent in a small subset of ommatidia. Additionally, expression of *pyd* in a small subset of glial cells was shown by co-staining with the glial marker Repo (Figure 4.6D). In the larval brain localized expression *of pyd* in the optic lobes could be observed (Figure 4.6C).

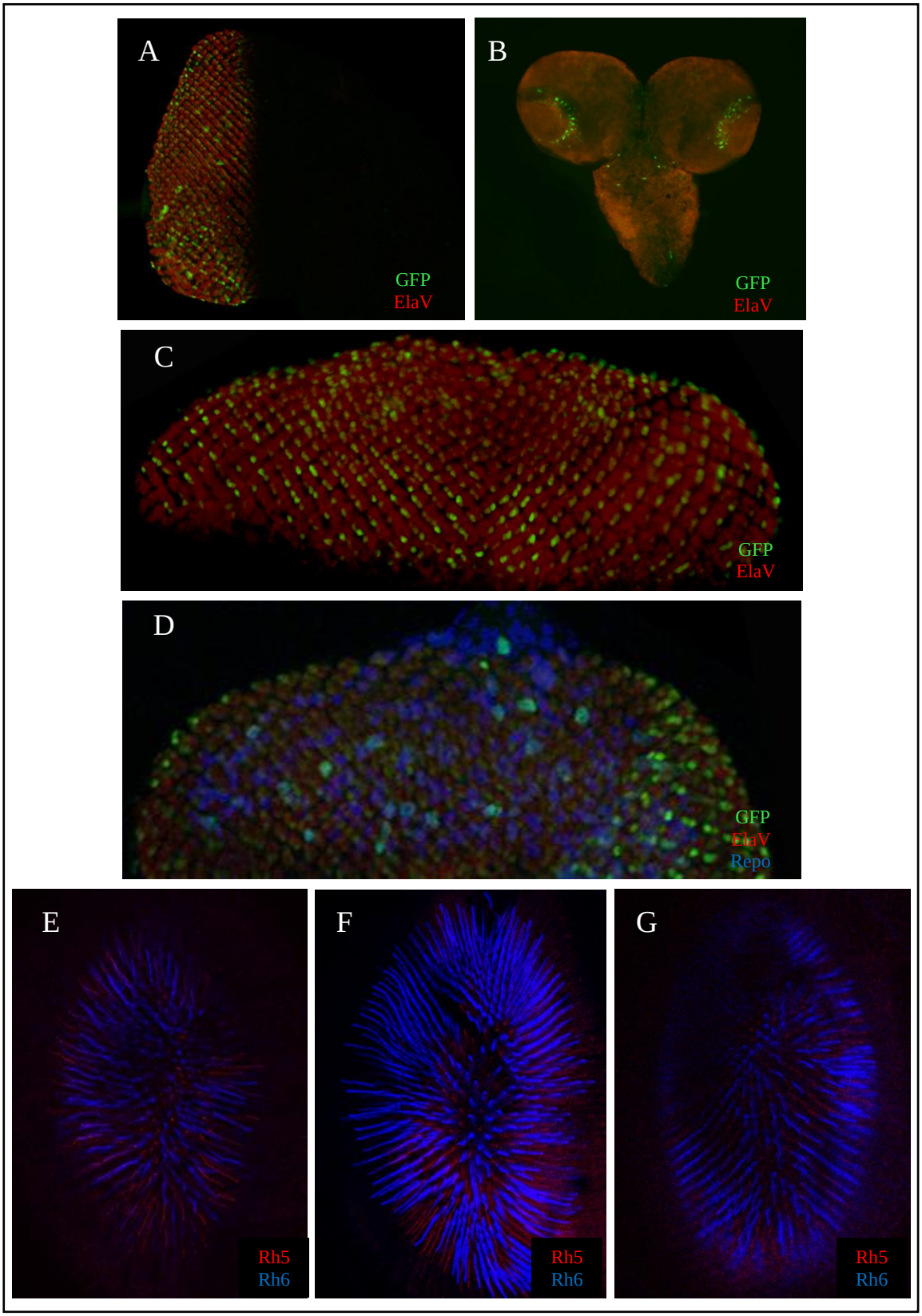


Figure 4.6. AC711 is expressed in a subset of outer photoreceptors and glia.

4.3.2.2. Loss of Function Analysis of *Polychaetoid.* With the aim of investigating a possible role of *pyd* in PR differentiation and rhodopsin regulation, expression of *pyd* was down-by RNAi. Additionally, a *pyd* deletion line with a chromosomal deficiency (Df(3R)ED5330) on the 3rd chromosome was analyzed. The deficiency line was homozygous lethal and thus heterozygote flies were dissected directly and whole mounted eyes were subjected to immunohistochemical staining with antibodies against Rh5 and Rh6. No phenotype was observed after either down-regulation or one copy loss of *pyd* expression (Figures 4.6E and 4.F)

4.3.2.3. Misexpression Analysis of *Polychaetoid.* Misexpression of *pyd* using lGMR-GAL4 as a driver and UAS-*CG7985*and subsequent analysis of Rh5/Rh6 expression revealed a wild type Rh5/Rh6 expression pattern (Figure 4.6G) indicating that *pyd* does not have a function in rhodopsin regulation.

4.3.3. A GAL4 Enhancer Trap Insertion in *Headcase*

The piggyBac enhancer-trap element in line AC724 inserted ~2kb upstream of the *headcase* (*hdc)* gene. The *hdc* was firstly identified as a head specific gene whose homozygous mutants revealed missing or disrupted head capsule phenotype (Weaver and White, 1995). *hdc* was characterized as a negative regulator of terminal differentiation. Later, it was reported that *hdc* encodes a nuclear transcription factor which functions in tracheal development where it acts in an inhibitory signaling mechanism (Steneberg *et al*., 1998). The human homolog of the *hdc* protein (HECA) plays an important role in human carcinogenesis and is believed to act as a tumor suppressor in head and neck cancer (Dowejko *et al*., 2009).

4.3.3.1. Expression Pattern of *Headcase*. *Hdc* expression in third instar larvae was observed in both eye-imaginal discs and larval brain (Figures 4.7A and 4.7B). In the eye imaginal disc *hdc* is expressed in two outer PRs, R3 and R4, in most of the ommatidia (Figure 4.7C). Additionally, *headcase* expression was also observed in glial cells as confirmed with co-staining with Repo (Figure 4.7D). *Hdc* expression was also observed in optic lobes and in some motor neurons in the ventral nerve cord (Figure 4.7B).

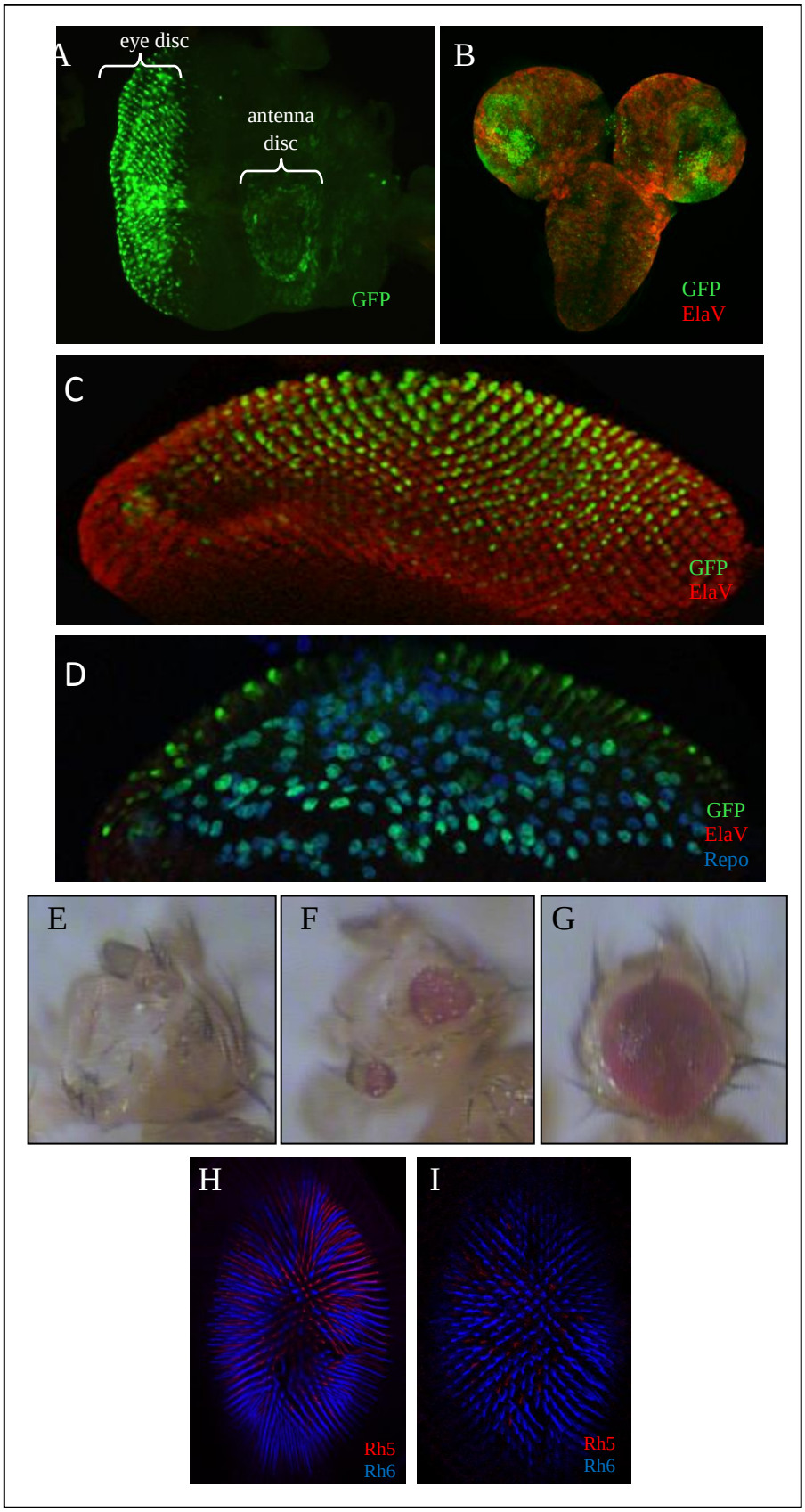


Figure 4.7. AC724 is expressed in R3 and R4 photoreceptors and glia, and downregulation of *headcase* cause loss of eye or largening in the eye size.

4.3.3.2. *Headcase* Loss of Function Analysis. To understand whether *hdc* has a possible role in PR differentiation and rhodopsin regulation, *hdc* expression was down-regulated using RNAi and eyGAL4, lGMR-GAL4, UAS-Dicer2 as a driver. Upon down-regulation various eye phenotypes were observed. *Hdc* mutant flies had either significantly large and round eyes (Figure 4.7E) (1), or no eyes (Figure 4.7F) (1), or significantly small and disrupted eyes (Figure 4.7G) (2). Additionally, we analyzed the eyes of heterozygous viable *hdc* deficient lines (Df(3R)ED6332). The morphology of the *hdc* heterozygote mutant eyes was not disrupted and analysis of the Rh5/Rh6 expression pattern of deletion lines revealed also a wild type phenotype (Figure 4.7H). This can be due to function of remaining one copy in *hdc* heterozygote mutants. In conclusion, according to RNAi down-regulation phenotype it was suggested that that *hdc* functions in early eye development.

4.3.3.3. *Headcase* Gain of Function Analysis. In addition to loss of function studies, *hdc* was misexpressed in all photoreceptors by using lGMR-GAL4 as a driver and UAS-*hdc*. Ectopic *hdc* expression did not reveal any changes in Rh5/Rh6 ratio in the retina of the resulting flies (Figure 4.7I).

4.3.4. A GAL4 Enhancer Trap Insertion in *CG33259*

After inverse PCR and sequencing, the insertion site of the piggyBac element in the enhancer-trap line AC748 was found to be an intergenic region. The nearest gene with the right orientation was the gene *CG33259*. In the literature there are no reports on *CG33259*. And as indicated in Table 4.2, no homologies of *CG33259* to previously characterized genes are known.

4.3.4.1. Expression Pattern of *CG33259*. Reporter gene expression analysis of the enhancer-trap line AC748 in third instar larvae, revealed expression in both eye-imaginal discs and the larval brain (Figures 4.8A and 4.8B). GFP expression was observed in every ommatidium starting at the morphogenetic furrow in two outer PRs, R3 and R4 cells in the eye imaginal disc (Figure 4.8C). Additionally, *CG33259* expression was observed in some motor neurons in the ventral nerve cord and in the optic lobes in the brain (Figure 4.8B).

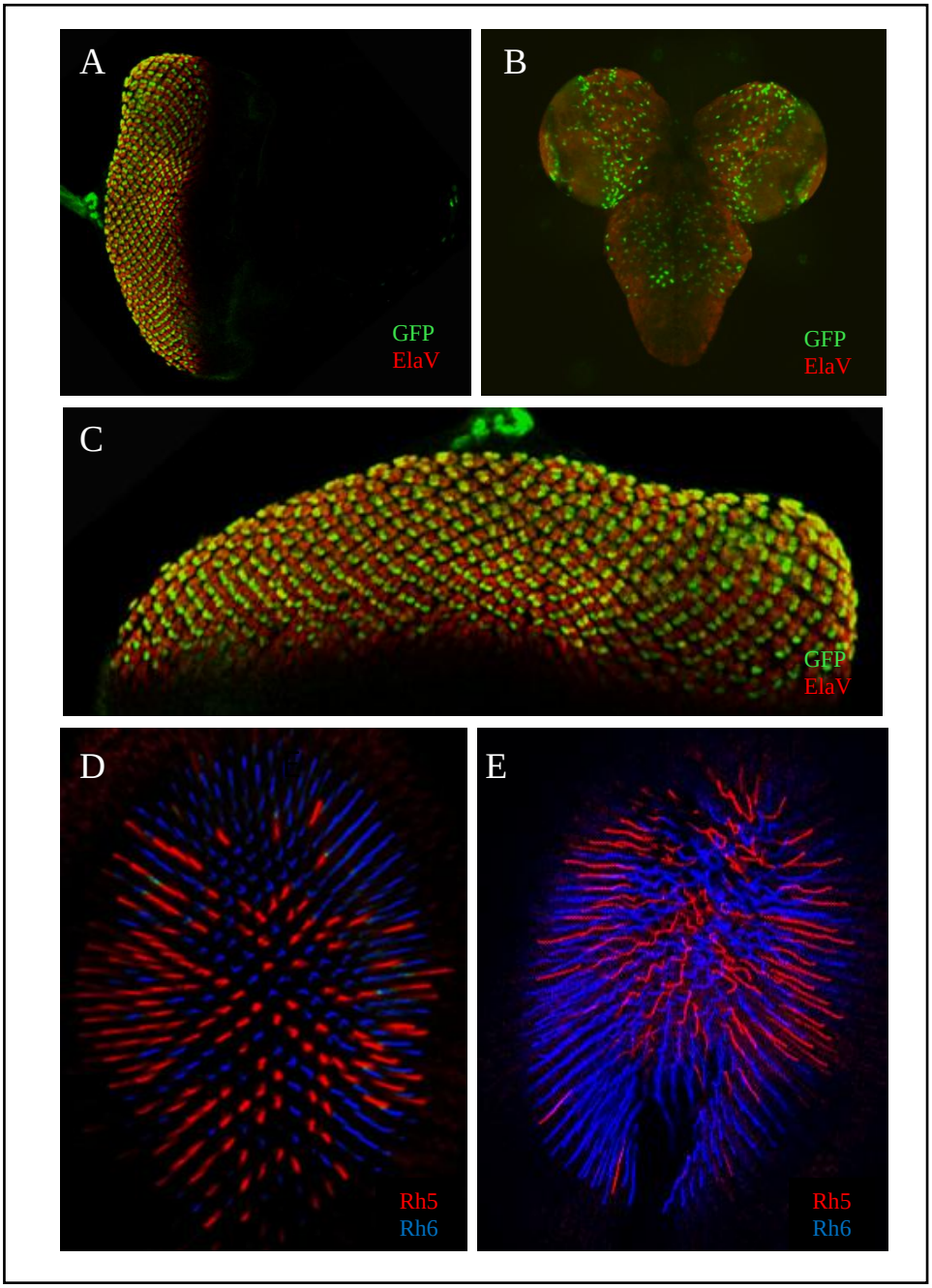


Figure 4.8. AC748 is expressed in R3 and R4 photoreceptors.

4.3.4.2. Loss of Function Analysis of *CG33259.* Expression of *CG33259* was down-regulated using eyGAL4, lGMR-GAL4, UAS-Dicer2 as a driver and UAS-*CG33259* RNAi lines. The resulting flies had eye phenotypes (Figure 4.8E) like wild type (Figure 4.8D) which indicate down-regulation of *CG33259* should have interfered with the action of excess amount of Dicer. So that, the effect of excess Dicer (Rh5/Rh6 co-expression and Rh6 expression expansion) was removed by down-regulation of *CG33259*. To conclude, it

was suggested that *CG33259* has a role in Rh5/Rh6 expression regulation but further experiments, like full knock-down, should be performed in order to understand the exact function of *CG33259*.

4.3.5. A GAL4 Enhancer Trap Insertion Upstream of *Stubble*

The piggyBac element insertion in line AC792 is located between two genes, *Akt1* and *Stubble* (*Sb*) which are oppositely oriented in the genome. The pBGay insertion site is upstream of *Sb* and downstream of Akt1, so it was supposed that the reporter gene expression is related to *Sb*. The *Sb* gene encodes a type II trans-membrane serine protease (TTSP). TTSPs are a class of proteins which are linked to human developmental abnormalities and pathology (Hooper *et al*., 2001). Apart from having serine protease motifs, their numerous and varied non-proteolytic domains are likely to mediate interactions with proteolytic substrates and inhibitors as well as other proteins and ligands, resulting in non-proteolytic activities like transducing signals as in the case of *Sb*. The *Sb* gene is required for epithelial morphogenesis of imaginal discs, including the formation of bristles, legs, and wings (Appel *et al*., 1993).

4.3.5.1. Expression Pattern of AC792. Analysis of the expression pattern of the AC792 line revealed a low number of expressing cells in third instar larval eye-imaginal discs and larval brain (Figures 4.9A and 4.9B). The few cells showing expression could not be identified unequivocally, but we think that it might be PRs R1 and or R6 judging from their position within the ommatidia (Figure 4.9C).

4.3.5.2. Loss of Function of *Stubble*. The role of *Sb* in PR differentiation and rhodopsin regulation expression was analyzed by down-regulating *Sb* expression using eyGAL4, lGMR-GAL4, UAS-Dicer2 as a driver and a UAS-*Sb* RNAi line. Upon down-regulation, Rh5/Rh6 co-expression was observed in a small subset of ommatidia (~4%), in 14 cells (Figure 4.9D). This phenotype was also observed in eyGAL4, lGMR-GAL4, UAS-Dicer2 driver line (~39%) (Figure 4.3E). And it was suggested that excess amount of Dicer2 in those flies interact with the Rh5/Rh6 gene expression regulation.

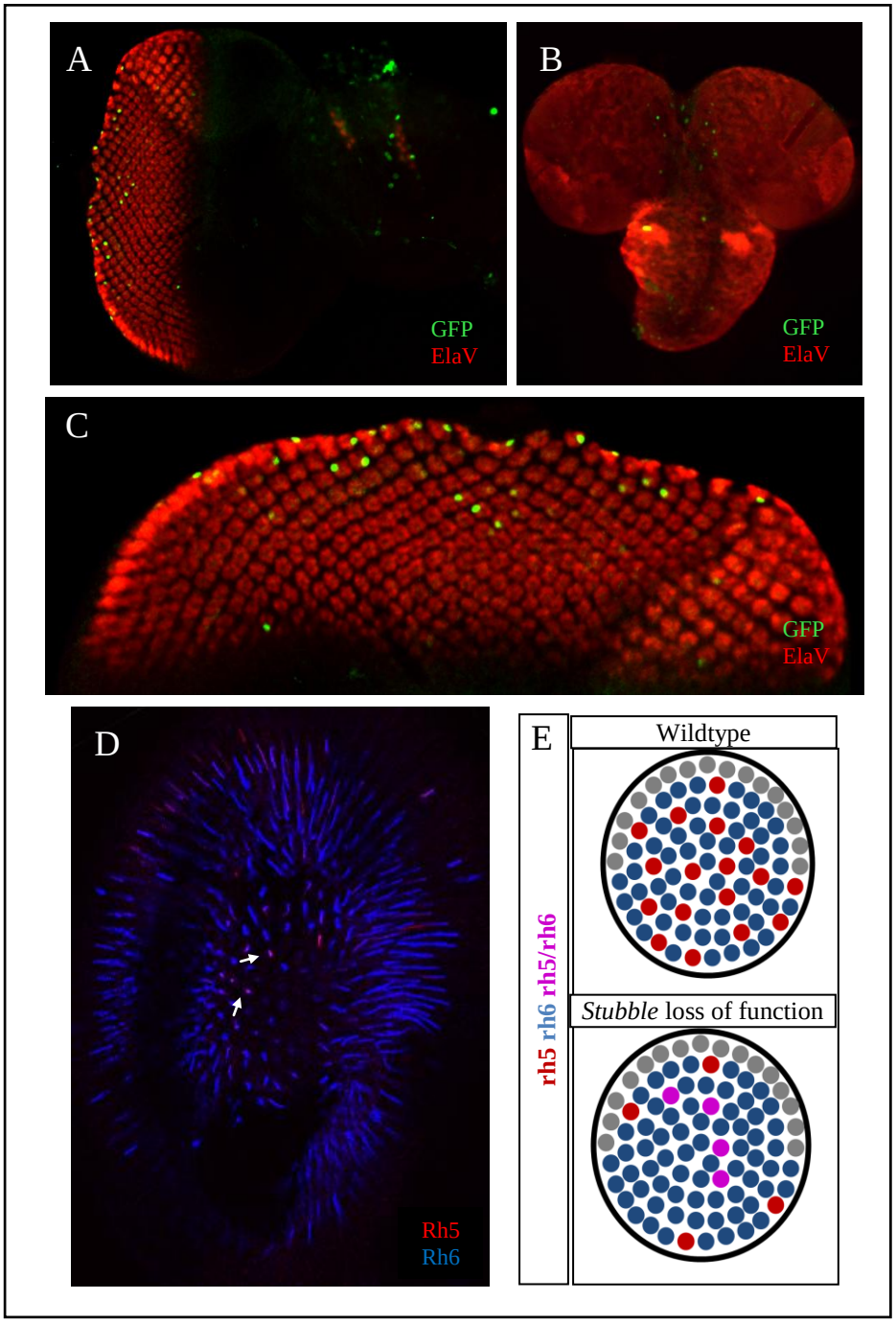


Figure 4.9. AC792 is expressed in R2 photoreceptors and downregulation of S*tubble* cause Rh5/Rh6 co-expression. Rh5/Rh6 co-expression is pointed out with arrows.

Furthermore, out of total counted 336 cells, only 55 of them were expressing Rh5 (~16%). The down-regulation of S*b*, resulted in an overall reduction in R8 cells expressing Rh5. This reduction was not observed in either of the controls, UAS-*Sb* RNAi line and eyGAL4, lGMR-GAL4, UAS-Dicer2 line. On the other hand, there were 295 cells with Rh6 expression (~87%). The proportion of Rh6 expressing R8 cells was induced. Thus,

down-regulation of *Sb* resulted in opsin expression change in almost half of pale R8 cells (~15%) and expansion of Rh6 expression. All these results indicate that *Sb* function in Rh6 de-repression regulation in a subset of pale R8 cells. The expression of genes cannot be down-regulated with one hundred percent efficiency via RNA interference method because of penetrance issues. For the role of *Sb* in Rh6 de-repression regulation, total knock down of S*b* should give us more information about whether the function of the gene is limited to a subset of pale R8 cells or not.

4.3.6. A GAL4 Enhancer Trap Insertion in *Faint Sausage*

Two of the piggyBac element insertions (lines AC736 and AC806) were in *faint sausage* (*fas*). The *fas* gene encodes a GPI (Glycosylphosphatidylinositol) anchored adhesion molecules of the immunoglobulin superfamily (IgSF). The IgSF proteins are either soluble or they are attached to the membrane by a trans-membrane helix or a GPI anchor. And they are known for their roles in cell-cell recognition and communication during embryonal development (Vogel *et al*., 2003). FAS protein was reported to be involved in nerve cell migration and establishment of axonal pathways in the developing nervous system of *Drosophila* (Levken *et al*., 1998).

4.3.6.1. Expression Pattern of *Faint Sausage*. Analysis of *fas* expression was done via co-staining of third instar larvae eye-imaginal discs and larval brain with GFP and the neuronal marker ElaV. Expression of *fas* was observed in both structures (Figures 4.10A and 4.10B). In the eye imaginal disc *fas* expression was observed in every ommatidium. In the early ommatidia right after the morphogenetic furrow *fas* is expressed in two outer PRs, R3 and R4 cells, while after row 5the expression pattern changes, and two additional outer PRs - R1 and R6 cells - also express the *fas* gene (Figure 4.10C). Additionally, *fas* expression was also observed in supportive glial cells in eye imaginal disc as demonstrated by co-staining with the glial marker Repo (Figure 4.10D). In the brain *fas* expression was observed in mainly neuroblasts as well as in the optic lobe (Figure 4.10C).

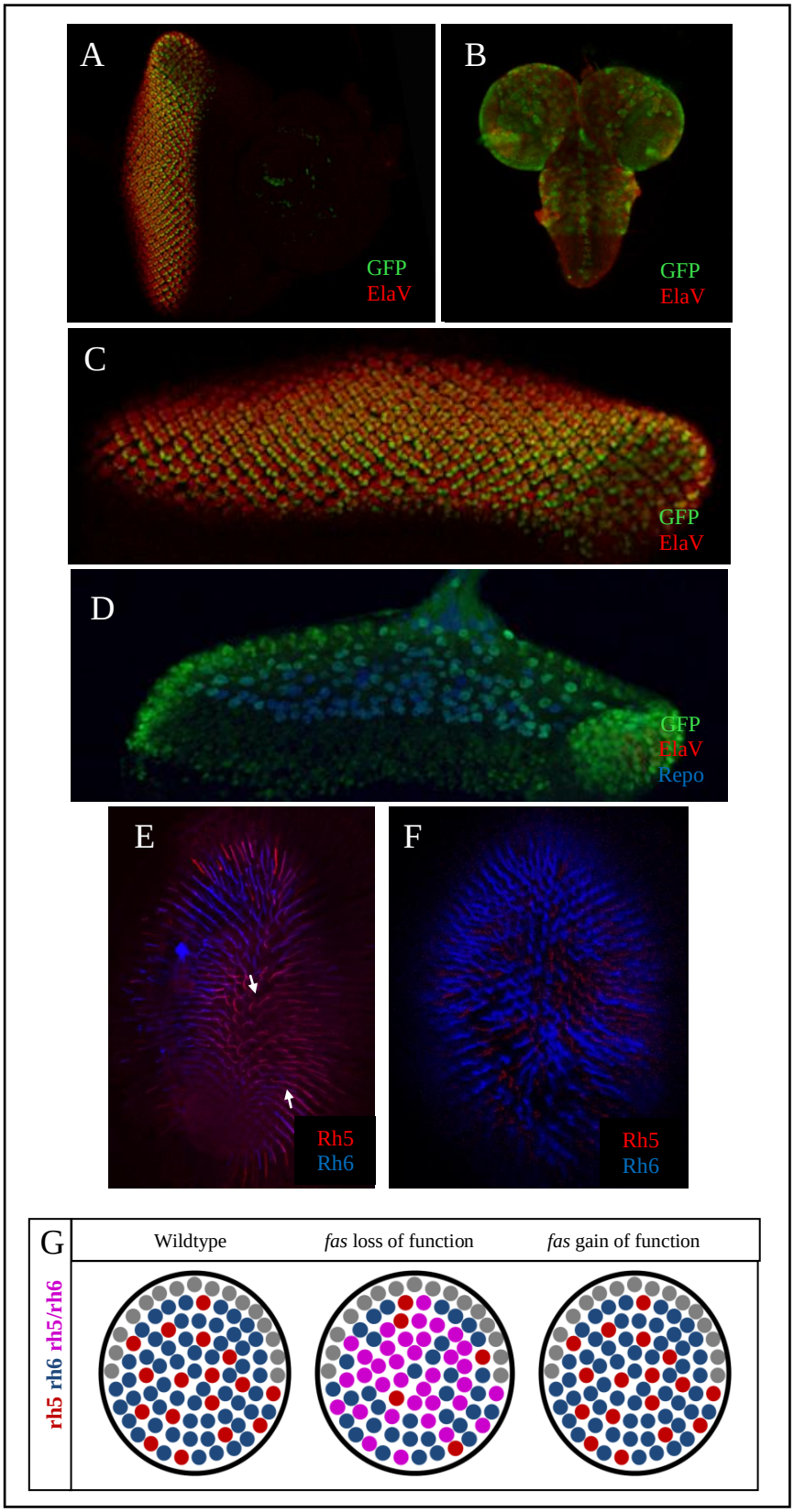


Figure 4.10. AC806 is expressed in a subset of outer photoreceptors and downregulation of *faint sausage* cause Rh5/Rh6 co-expression. Rh5/Rh6 co-expression is pointed out with arrows.

4.3.6.2. Downregulation of *Faint Sausage* by RNA Interference. Expression of *fas* was downregulated using the RNA interference method. In the retina of the resulting flies, Rh5/Rh6 co-expression was observed in 80 cells (out of total 143 cells) with a high ratio of 55%. Additionally, there were significant changes in the proportion of Rh5 expressing R8 cells (97 cells, ~67%) and Rh6 expressing R8 cells (126 cells, ~88%) (Figure 4.10E). Co-expression of Rh5/Rh6 (~39%) upon expansion of Rh6 expression (~96%) was also observed in the driver line (Figure 4.3E). It was suggested that excess Dicer activity caused that phenotype. But an induction in Rh5 expressing R8 cells was not observed in any of control lines, the driver line and UAS-*fas* RNAi line. Consequently, these results indicate that *fas* might have a function in Rh5/Rh6 expression regulation in 8 cells. But full knock-down will give us more information about the role of *fas* in Rh5 expression regulation.

4.3.6.3. Misexpression Analysis of *Faint Sausage.* In order to understand whether *fas* is also sufficient to repress Rh6, UAS-*fas* line was ordered and lGMR-GAL4 was used to drive *fas* expression in all photoreceptors. After staining of whole mount retinas with antibodies against Rh5 and Rh6 no rhodopsin phenotype was observed (Figure 4.10F). These results indicate that *fas* is required but not sufficient for Rh6 repression in a subset of pale R8 cells.

4.3.7. A GAL4 Enhancer Trap Insertion in *CG11360*

Although the expression pattern of line AC1018 could not be determined, the nearby gene, *CG11360* was chosen for further characterization because of its predicted molecular function. The product of the gene has not been identified yet. But based on sequence similarities, it is predicted that the molecular function of *CG11360* is RNA binding (FlyBase). In the literature, there is one report showing involvement of *CG11360* in alternative splicing of two genes; *Dscam*, an axon guiding molecule, and *para*, a voltage-gated action potential sodium channel (Park *et al*., 2004).

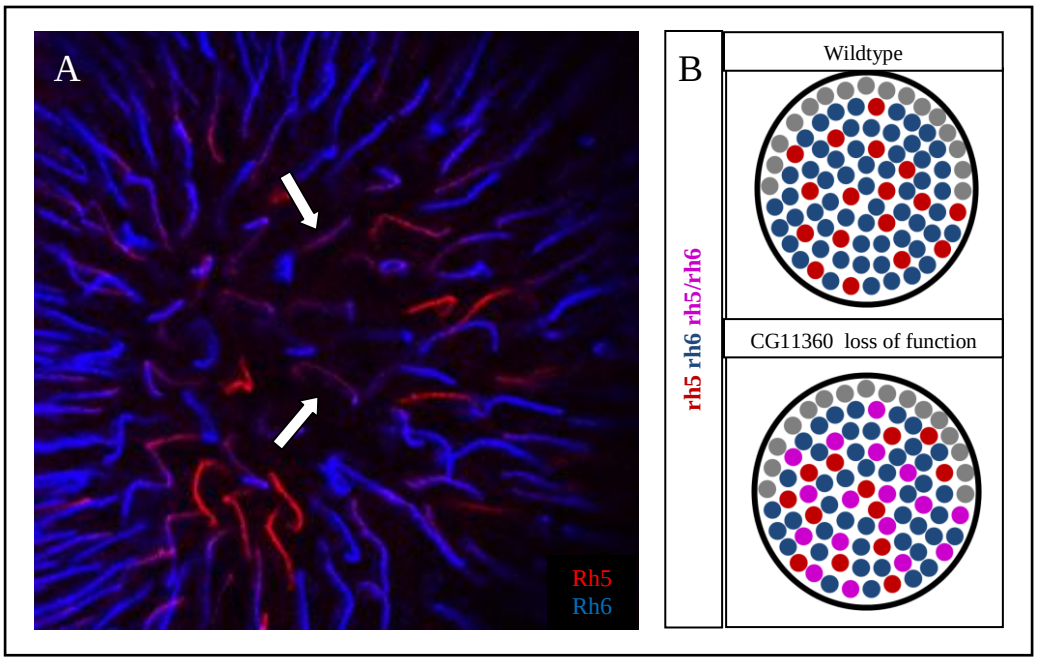


Figure 4.11. Downregulation of *CG11360* cause Rh5/Rh6 co-expression. Rh5/Rh6 co-expression is pointed out with arrows.

4.3.7.1. Loss of Function Analysis of *CG11360*. Loss of function analysis of *CG11360* was performed in order to investigate a possible role of *CG11360* in PR differentiation and rhodopsin regulation. *CG11360* expression was down-regulated by RNA interference followed by antibody staining against Rh5 and Rh6. Analysis of whole mount adult eyes showed Rh5/Rh6 co-expression in 25% of ommatidia (86). There was an induction in Rh6 expressing R8 cells (273, ~81%) while Rh5 expressing R8 cells proportion was close to wild type ratio (144, ~43%) (Figure 4.11A). These phenotypes were also observed in the driver line. So, it was concluded that full knock-down of *CG11360* is required in order to further investigate a possible role of *CG11360* in PR differentiation.

4.3.8. A GAL4 Enhancer Trap Insertion in *Reduced Ocelli*

One of the insertions of the piggyBac element (line AC1019) was in the first intron of the *reduced ocelli* (*rdo*) gene. The *rdo* gene is predicted to encode a leucine rich repeat (LRR) protein. Leucine rich repeats are 20-29 base pair motifs predicted to be involved in a great diversity of biological functions that require protein-protein interaction (Kobe and

Kajava, 2001). Mutations of the *rdo* gene show that it is involved in ocellus development (Caldwell *et al*., 2007).

4.3.8.1. Expression Pattern of *Reduced Ocelli.* Expression analysis revealed that *rdo* is expressed in ocelli, eye-imaginal discs and larval brain (Figures 4.12A and 4.12B). In eye imaginal discs, *rdo* expression was observed in every ommatidium in two outer PR cells, R3 and R4 (Figure 4.12C). Expression of rdo in the brain was observed in the optic lobes (Figure 4.12B).

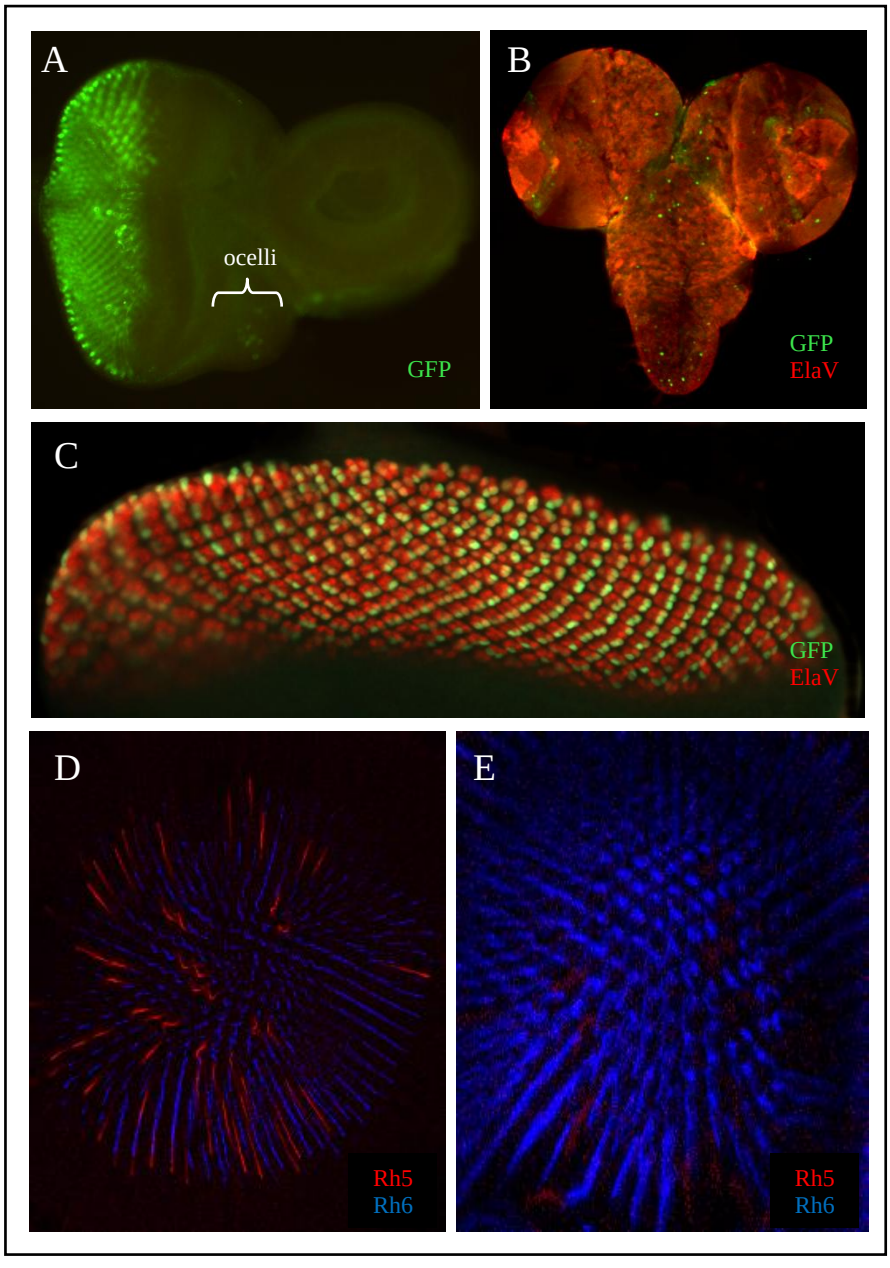


Figure 4.12. AC1019 is expressed in R3 and R4 photoreceptors, and ocelli.

4.3.8.2. Downregulation of *Reduced Ocelli* by RNAi. Aiming to investigate a possible role of *rdo* in PR differentiation and rhodopsin regulation, *rdo* was down-regulated using UAS-*rdo* RNAi lines followed by staining of whole mount adult retinas against Rh5 and Rh6. Down-regulation of *rdo* resulted in a wild-type Rh5/Rh6 expression pattern (Figure 4.12D). In a comparison to the driver line phenotype, loss of function phenotype of *reduced ocelli* indicates *rdo* function in Rh5/Rh6 expression regulation. R8 specific opsin expression analysis of driver line revealed Rh5/Rh6 co-expression (~39%) and an expansion of Rh6 expression (~96%) and this phenotype is believed to be resulted from excess amount of Dicer. Consequently, the wild type phenotype observed as a result of *rdo* down-regulation indicates that the effect of excess Dicer removed by down-regulation of *rdo*. Loss of function analysis on homozygous mutants should give us more information about the role of *rdo* in Rh5/Rh6 expression regulation.

4.3.8.3. Misexpression of *Reduced Ocelli.* *Rdo* misexpression in all photoreceptors using lGMR-GAL4 as a driver and an UAS-*rdo* line followed by antibody staining against Rh5 and Rh6 revealed no changes in rhodopsin expression pattern of R8 cells (Figure 4.12E).

4.3.9. A GAL4 Enhancer Trap Insertion in *Taranis*

The first intron of *taranis* (*tara*) seems to be a hot spot for piggyBac element insertion. Two of our piggyBac lines, AC1048 and AC1078, localized to the same region in the *tara* gene. *Tara* is a trithorax-group gene which encodes two closely related 96-kD protein isoforms (TARA-α/-β) (Calgaro *et al*., 2002). Trithorax-group proteins are chromatin regulatory proteins which generally act to maintain gene expression. *Tara* is homologous to a family of mammalian proteins, including human p34SEI-1/TRIP-Br1 (Calgaro *et al*., 2002), which regulate the transcriptional activation of CDKs involved in cell cycle regulation (Hsu *et al*., 2001).

4.3.9.1. Expression Pattern of *Taranis.* Analysis of *tara* expression in third instar larvae in both lines AC1048 and AC1078 revealed different expression patterns in eye-imaginal discs (Figures 4.13A and 4.14A) while they had a similar expression pattern in larval brains (Figures 4.13B and 4.14B). Three types of GFP expression in AC1048 line was

observed in most of the ommatidia throughout eye imaginal disc: expression in only R3 cells, expression in only in R4 cells and expression in both R3 and R4 cells (Figure 4.13C).

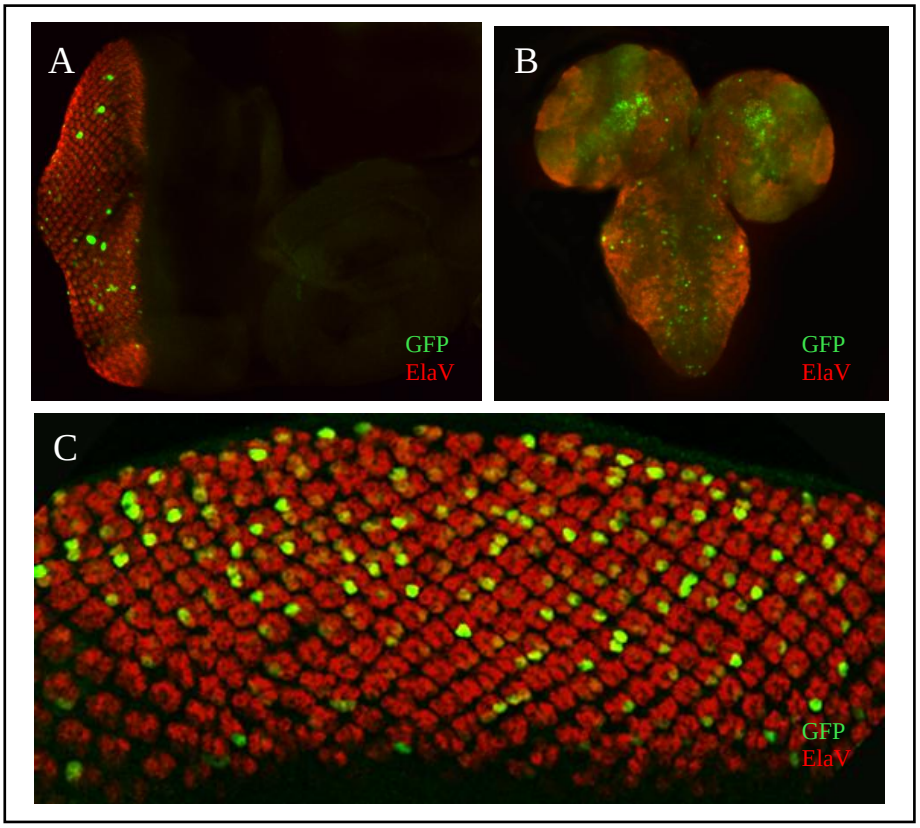


Figure 4.13. AC1048 is expressed in R3 and R4 photoreceptors

On the other hand, GFP expression in AC1078 line revealed expression in both R3 and R4 cells in almost every ommatidium (Figure 4.14C). Furthermore, *tara* expression in supportive glial cells was also observed in line AC1078 as deduced by co-staining with the glial cell marker Repo (Figure 4.14D). In the brain *tara* expression was observed in the optic lobe, antennal lobe and some motor neurons in the ventral nerve cord.

4.3.9.2. Downregulation of *Taranis.* The possible role of *taranis* in PR differentiation and rhodopsin regulation was investigated by down-regulating expression of *taranis* using eyGAL4, lGMR-GAL4, UAS-Dicer2 as a driver and UAS-*tara*-RNAi. The resulting flies had same phenotype with the driver line. Rh5/Rh6 co-expression was observed in 56 cells out of 195 total cells counted (~28%), there was an induction in Rh6 expressing R8 cells (185 cells, ~94%) while the proportion of Rh5 expressing R8 cells was close to wild type ratio (65 cells,~33%)(Figure 4.14E).

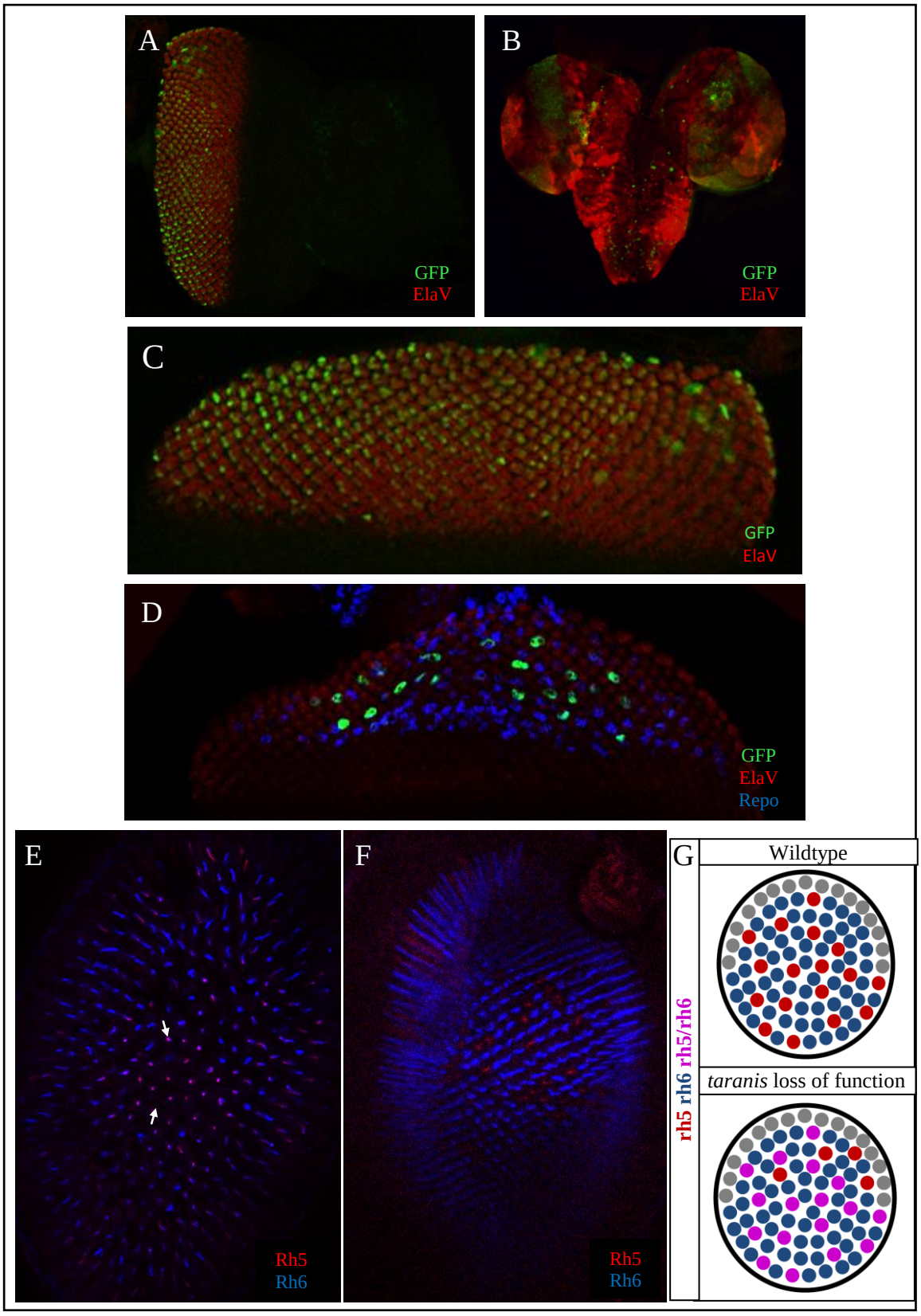


Figure 4.14. AC1078 is expressed in R3 and R4 PRs, and glial cells in eye imaginal disc, and downregulation of *taranis* cause Rh5/Rh6 co-expression and a reduction in rhabdomere size in R8 cells.

Upon down-regulation of *tara*, the rhabdomere size of R8 cells throughout the retina were significantly reduced (Figure 4.14E). The rhabdomeres are composed of thousands of tightly-packed microvilliare which are surface area to house the tens of millions of rhodopsin molecules. This phenotype indicates that *tara* might have a function in rhabdomere structure formation in R8 cells.

4.3.9.3. Misexpression of *Taranis.* In order to further characterize *tara* and its role in rhodopsin regulation and ommatidial subtype specification, UAS-*tara* line was used together with the lGMR-GAL4 driver to mis-express *tara* in all photoreceptors. *Tara* gain of function resulted in flies with a wild type Rh5/Rh6 ratio (Figure 4.14F). In conclusion, it was suggested that *tara* might have a role in rhabdomere structure formation in R8 cells.

5. DISCUSSION

Previous screens have shown that enhancer trapping is a powerful system to identify novel genes on the basis of their expression pattern (Bellen *et al*., 1989; Torok *et al*., 1993). In the framework of this thesis we have analyzed 34 enhancer-trap lines. Their expression in the eye disc and brain during pupal stages was shown in a preliminary screen (Çelik and Desplan, unpublished results). The aim of the enhancer-trap screen was to identify novel photoreceptor specific genes using a less known transposable element, piggyBac. PiggyBac has a different insertional specificificity compared to the P-element, and was suggested to insert preferentially to introns and not to have hot or cold spots of insertion (Thibault *et al*., 2004).

The 34 lines that were analyzed in this re-screen were molecularly mapped to 30 genes (Table 4.1). Looking at the insertion sites in 60% of the lines the piggyBac element was found to be inserted in upstream regions, while in 40% of the lines it was found to be inserted into introns. Although small in number, these results are in line with previous observations by Thibault *et al*., 2004. The function of 13 (43%) out of 30 genes were not defined previously. Furthermore, all the identified genes with known molecular functions fall into all major protein classes, (kinases, cytoplasmic proteins, enzymes, nuclear proteins/transcription factors and cell surface proteins/receptors (Celniker and Rubin, 2003). In conclusion, our enhancer trap strategy using the piggyBac element for screening to identify novel genes could be considered successful as previously not identified genes could be targeted and expression in photoreceptors could be shown.

In the course of this study, 13 genes were selected for further characterization, which involved detailed expression, loss of function and gain of function analysis. Expression patterns resulting from the enhancer-traps were analyzed by co-staining with the neuronal marker ElaV to reveal any expression in photoreceptor cells. Additionally, the glial cell marker Repo and the R7 cell marker Prospero were used to define specific cell types where necessary.

Detailed expression analysis revealed that out of the 13 candidate genes analyzed, 4 of them were expressed in the inner PRs, while other 9 were expressed in the outer PRs. The ommatidial subtypes are classified according to the opsin expression of the inner PRs and it is believed that there is a communication between the neighboring R7 and R8 cells. Since there is an instruction from the R7 cell and regulating the R8 cell opsin expression, any change in this instruction should be detectable by analyzing R8 cell opsin expression. Our strategy for functional studies was to analyze R8 opsin expression, this would reveal also changes happening in the R7 cells.

On the other hand it is not known whether the outer PRs have a role in ommatidial subtype specification. Consequently, the functional analysis of genes, which are expressed in the outer PRs was also carried out to investigate their possible role in late retinal patterning.

In this study, functional analyses of 12 genes were performed. For most of the genes, loss of function analysis was performed using an RNA interference method. The UAS-RNAi carrying transgenic flies were ordered from the Vienna Drosophila Research Center, where UAS-RNAi lines for every gene in the *Drosophila* genome were prepared. Usually more than one line for each gene is available due to the transgenes, which can insert into different regions of the genome. Several of these lines should be tested and should exhibit the same phenotype in order to make sure that a "real" phenotype is observed. In the course of this study we were able to analyze only one of these RNAi lines (except for two lines in one case) due to restricted availability of a suitable acquisition system (confocal microscope). Additionally, in all cases the extent of knock-down by RNAi were not assessed, thus one cannot be sure in cases where no phenotype was observed that gene knock-down has really happened. This could and should be assessed by RT-PCR methods for example and could be done for each of the analyzed genes and lines.

To knock-down genes with RNA interference, eyGAL4, lGMR-GAL4, UAS-Dicer2 was used as a driver. As a result of control experiments, an unexpected phenotype was observed in the driver line. There was co-expression of Rh5 and Rh6 in R8 cells (which is normally not observed in wild type) in 39% of ommatidia and an induction in Rh6 expressing R8 cells constituting 96% of ommatidia (Figure 4.3E). We suggested that the

excess amount of Dicer2 in those flies interact with an unknown Rh5/Rh6 gene expression pathway which requires RNA interference for gene regulation. Maybe, as a result of increased Dicer2 levels, specific genes playing a role in Rh5/Rh6 regulation are repressed, which cause co-expression of R8 opsins and expansion of Rh6 expression. Consequently, genes giving wild type eye phenotype as a result of down-regulation with RNA interference method (*caps*, *CG33259*, *pyd* and *rdo*) were interpreted as having a role in Rh5/Rh6 regulation. In other words, down-regulation of those genes seems to somehow eliminate the effect of excess Dicer2 thus they should be functioning in Rh5/Rh6 regulation.

For some genes chromosomal deficiency lines spanning the target gene regions were available but due to large deletions on the chromosome, they were homozygous lethal. Deletion lines for the genes *caps*, *hdc*, *CG7985* and *pyd* were analyzed in heterozygous animals. In all four lines a wild type expression of Rh5 and Rh6 were observed. It was concluded that the deletion of one copy was not enough to characterize the function of those genes. Homozygous mutants will be more informative. In order to be able to analyze these mutations in a homozygous state these deficiencies should be recombined onto FRT chromosomes. The recombined alleles could then be used to generate somatic mutations, e.g. homozygous mutant eyes (when crossed to eyeless-flp lines) in a heterozygous background.

For seven genes mis-expression analyses could be performed as UAS-lines were available in various stock centers. These genes were: *fas*, *CG7985*, *heph*, *hdc*, *pyd*, *rdo*, *tara*. After misexpressing the target genes in all photoreceptors, R8 opsin expression pattern analysis revealed no changes in any of the gain of function studies. Although lGMR-GAL4 is a commonly used driver for misexpression studies in the eye, expression levels of targeted genes should be assessed by for example RT-PCR methods or *in situ* hybridization, in order to make sure that misexpression has really occurred and our gain of function results are trustable.

Table 5.1. RNAi knock-down phenotype and expression pattern of the candidate genes.

Gene Name	Expression Pattern	RNAi Knock-Down Phenotype
Genes with inner PRs expression		
hephaestus	R2 and R8	Reduced eye size
unzipped	R8	---
capricious	R8	Wild type
CG7985	R7	---
Genes with inner PRs expression		
cropped	R3 and R4	Reduced eye size
polychaetoid	R3, R4 and R6	Wild type
headcase	R3 and R4	Reduced eye size
CG33259	R3 and R4	Wild type
Stubble	R1 and R6	An overall reduction in Rh5 expressing R8 cells
faint sausage	R1, R3, R4 and R6	Rh5/Rh6 co-expression
CG11360	---	Rh5/Rh6 co-expression
reduced ocelli	R3 and R4	Wild type
taranis	R3 and R4	Reduced rhabdomere size of R8 cells throughout the retina

In the early eye development e*yeless*, *eye gone*, *sine oculis*, *eyes absent* and *dachshund* are required for the specification of the eye primordium. It is known that mutations affecting any of these genes cause reduction or absence of the eye (Morante *et al.*, 2007). In this study, as a result of *hdc*, *heph* and *crp* RNAi knock-down, reduction in the eye size was observed. It was suggested that they have a possible role in the early eye development and eye growth. In order to identify their exact roles in the early eye development, the possible interactions of *hdc*, *heph* and *crp* with e*yeless*, *eye gone*, *sine oculis*, *eyes absent* and *dachshund* will be assessed in the future studies.

It was previously reported that *Sb* encodes a type II trans-membrane serine protease, which is required for development of legs and wings (Appel *et al*., 1993). It is a common phenomenon that the same factors play similar roles during development of different tissues. Although *Sb* was not highly expressed in larval eye imaginal discs, tissue specific down-regulation of *Sb* reveals interesting results. Rh5/Rh6 co-expression was observed in a small subset of ommatidia (~4%), which was also observed in the driver line. And there was an overall reduction in number of Rh5 expressing R8 cells (~16%). While, the proportion of Rh6 expressing R8 cells was induced (~87%)(Figure 4.9D). Thus, down-regulation of *Sb* resulted in opsin expression change in almost half of pale R8 cells (~15%) and expansion of Rh6 expression. All these results indicate that *Sb* function in Rh6 de-

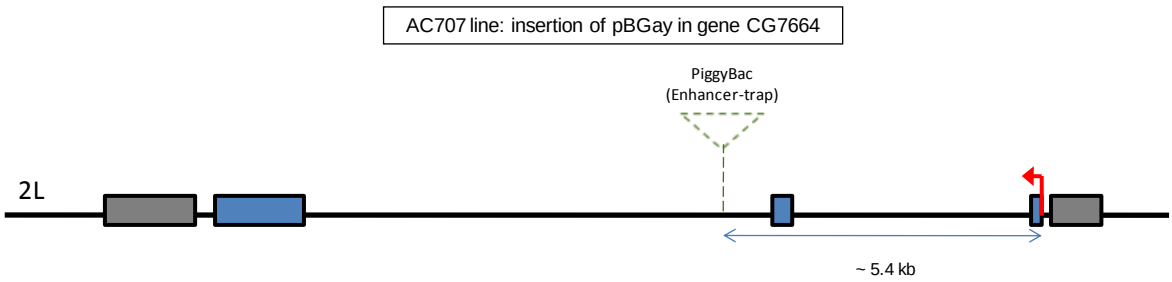
repression regulation in a subset of pale R8 cells. The expression of genes cannot be down-regulated with one hundred percent efficiency via RNA interference method because of penetrance issues. For the role of *Sb* in Rh6 de-repression regulation, total knock down of S*b* should give us more information about whether the function of the gene is limited to a subset of pale R8 cells or not.

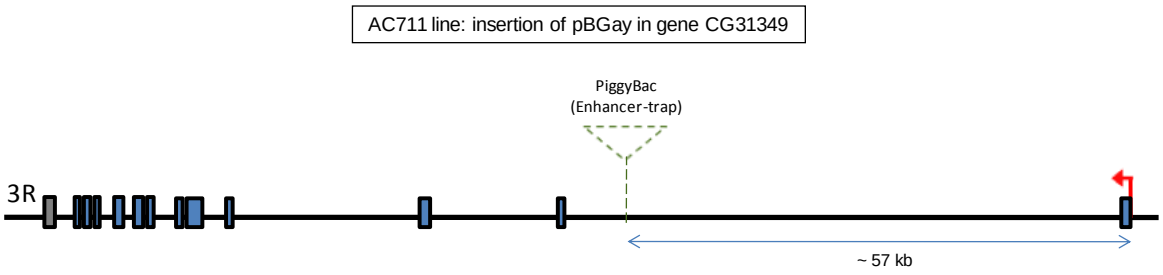
Tara encodes a trithorax-group protein which function in chromatin regulation for maintaining gene expression (Calgaro *et al*., 2002). Because of its molecular function, *taranis* was selected as a candidate gene since most of the identified genes involved in PR differentiation are transcription factors. *Tara* loss of function analysis resulted in a similar Rh5/Rh6 expression phenotype with the driver line. Additionally, the rhabdomere size of R8 cells throughout the retina was significantly reduced in the absence of *tara* (Figure 4.14E).. The rhabdomere morphogenesis is an unknown mechanism for the most part. Only a few genes' involvement in this process are reported; *Pph13*, *Spacemaker* and *Prominin* (Zelhof *et al*., 2003; Zelhof *et al*., 2006). The *tara* loss of function phenotype indicates that *taranis* might function in rhabdomere morphogenesis. The possible interactions of *tara* with identified genes in rhabdomere morphogenesis mechanism will be assessed in the future studies.

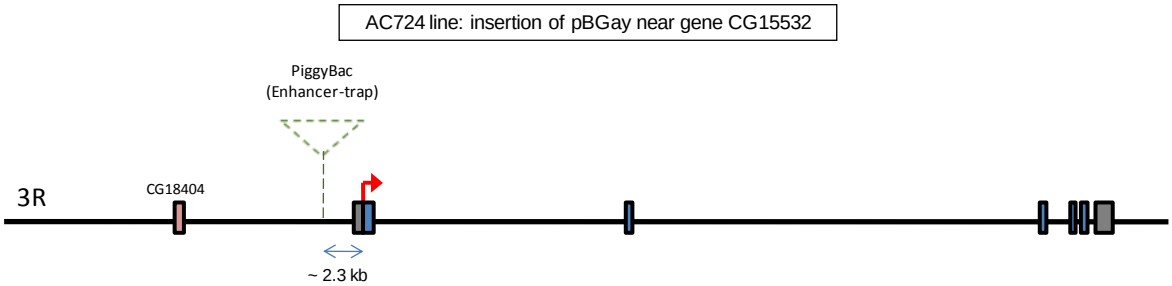
This study provides the preliminary results for further analysis of the candidate genes found in the enhancer trap screen. The expression pattern of these genes and the analysis of changes in photoreceptor subtype ratios in adult flies when these genes are misexpressed and supressed, gives an idea on whether these genes have a role in subtype specification or not. Those genes which are thought to be involved in this process, should be analyzed further and correlated with the pathways involved in the regulation of these subtypes. A detailed expression pattern analysis, total deletion of the target gene, and epistasis analysis would give a more detailed perspective on the functional role of these genes.

APPENDIX: MOLECULARLY LOCALIZED GAL4 ENHANCER TRAPS

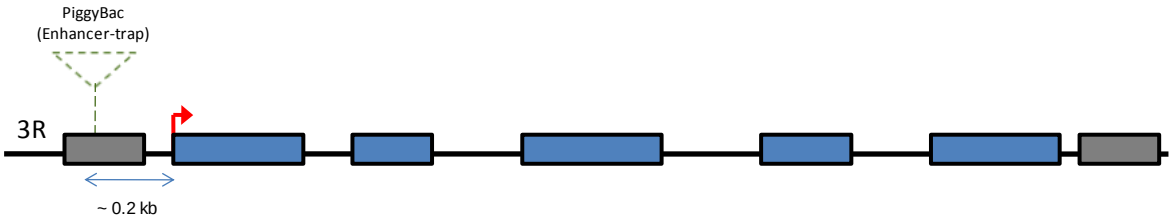
Gene regions of molecularly localized 34 GAL4 enhancer-trap lines. Grey boxes stand for 5'UTR's and 3'UTR's, exons are represented by blue boxes, and red arrows show both the location of the transcription start site and the orientation of transcription.



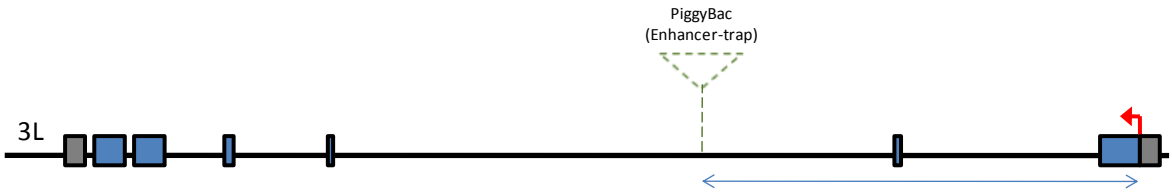




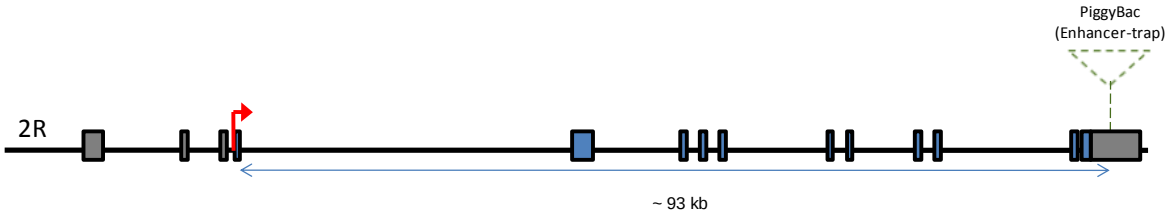
AC730 line: insertion of pBGay in gene CG5017



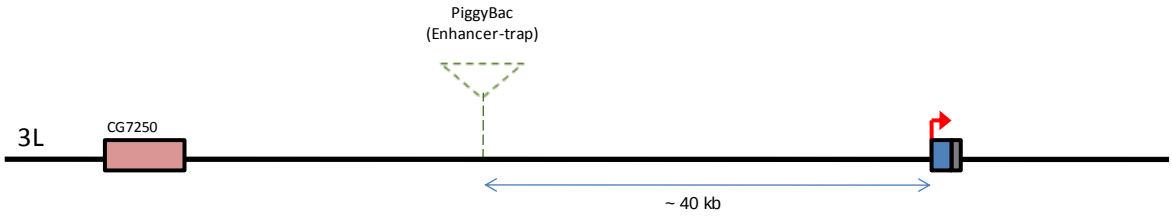
AC735 line: insertion of pBGay in gene CG8127



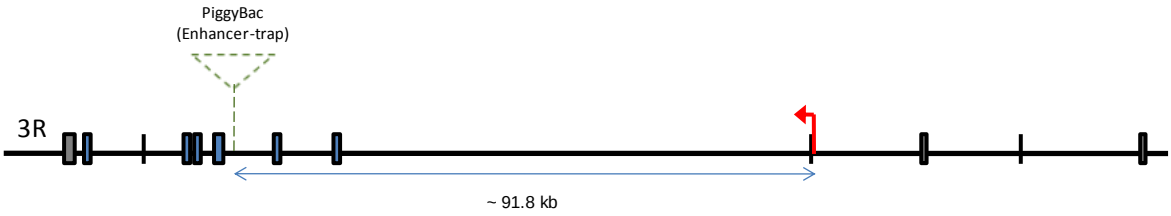
AC736 line: insertion of pBGay in gene CG17716



AC748 line: insertion of pBGay near gene CG33259



AC757 line: insertion of pBGay in gene CG31000



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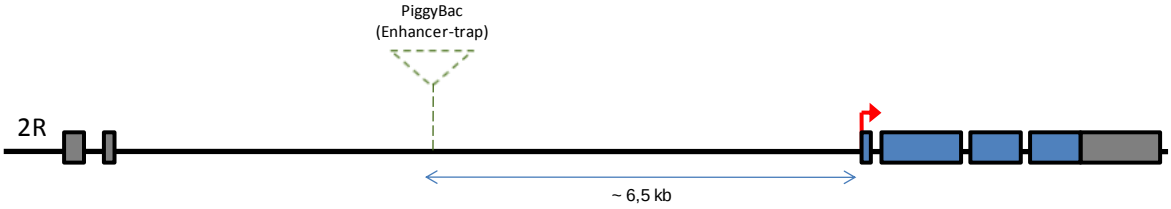
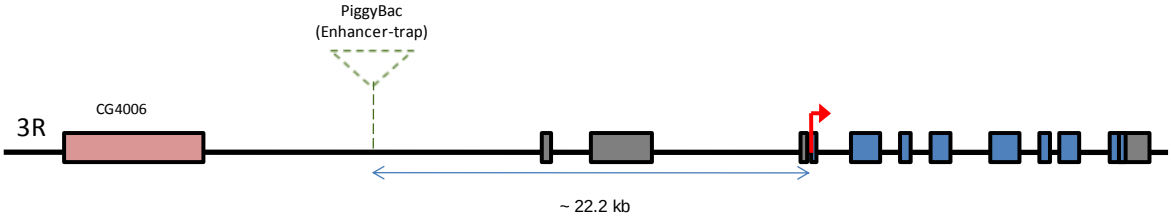
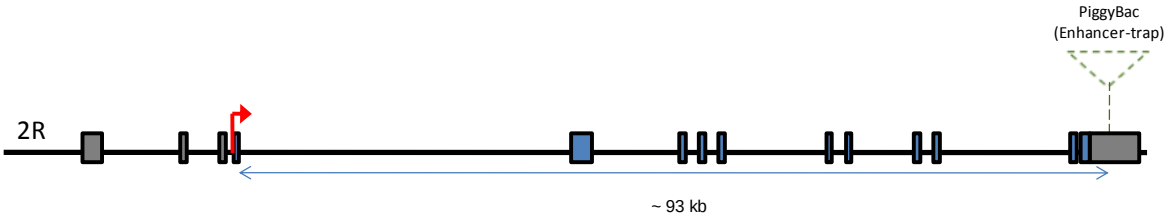


AC792 line: insertion of pBGay in near gene CG4316



AC799 line: insertion of pBGay near gene CG4835

AC806 line: insertion of pBGay in gene CG17716



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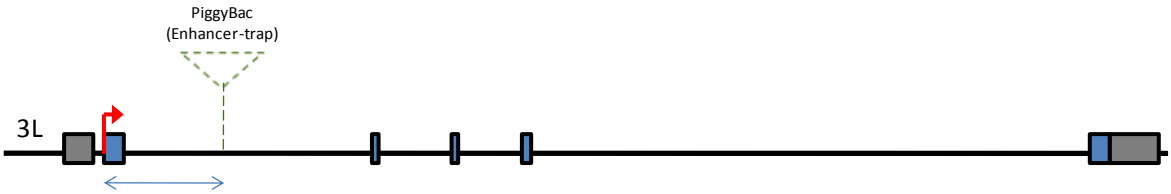
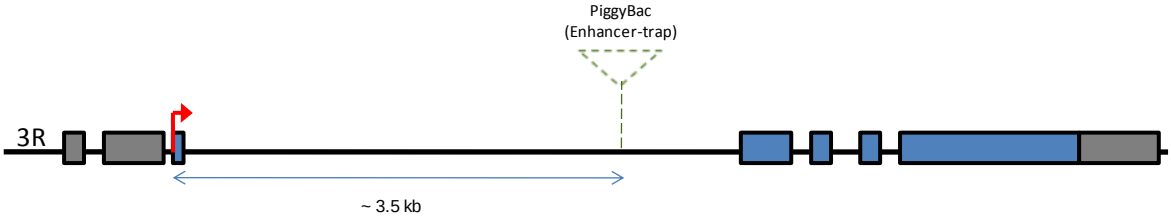


AC832 line: insertion of pBGay near gene CG12931

AC837 line: insertion of pBGay in gene CG12054



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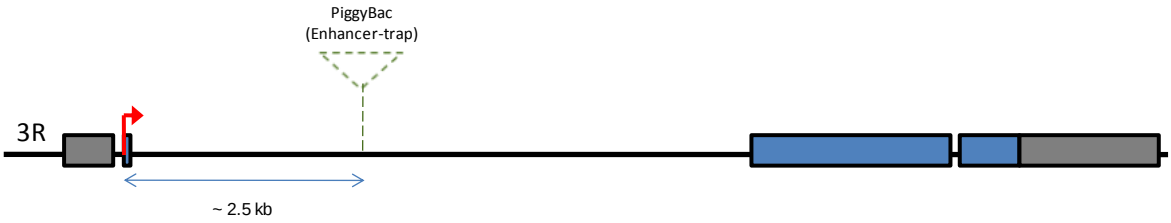

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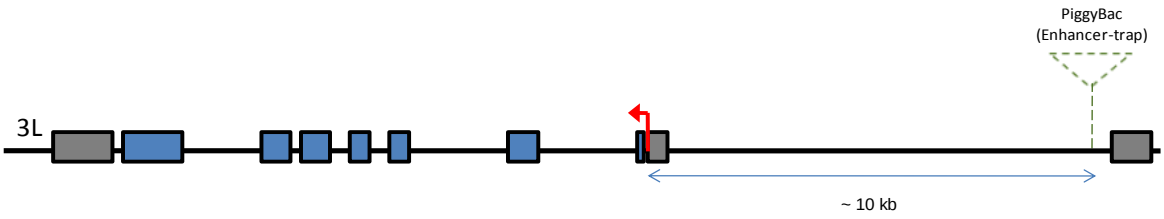

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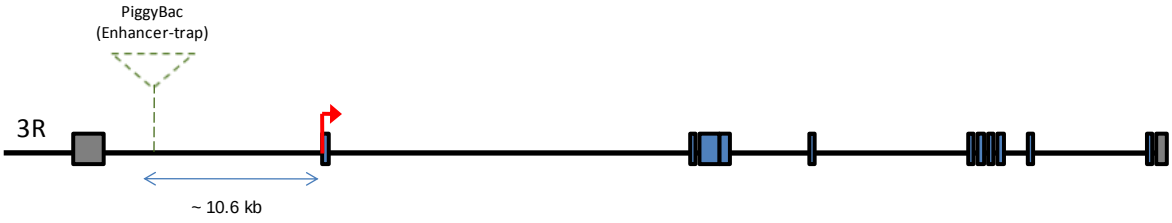
AC929 line: insertion of pBGay in gene CG17838



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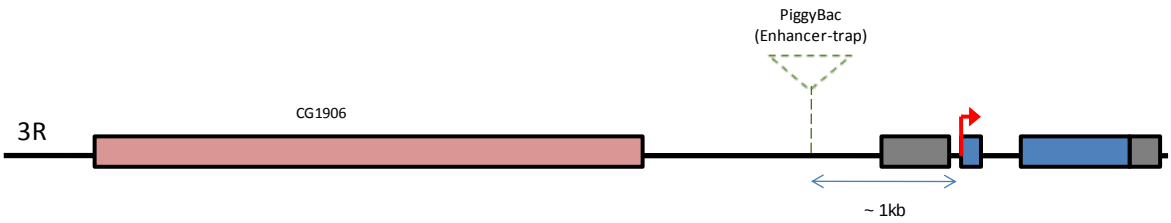

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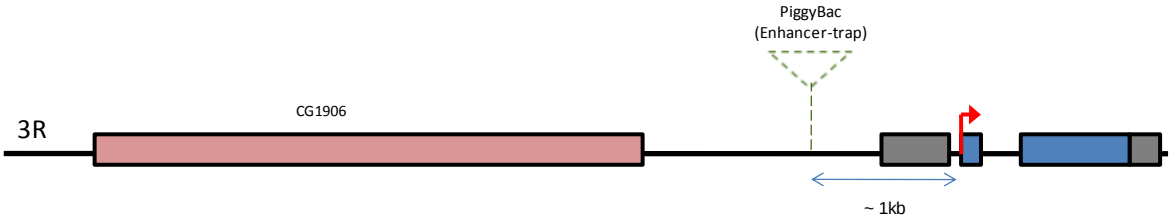

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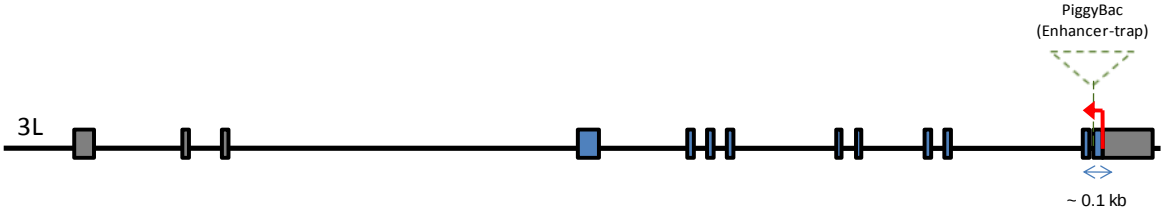

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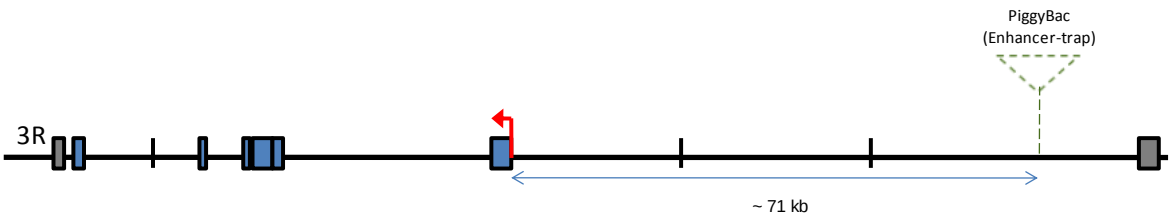

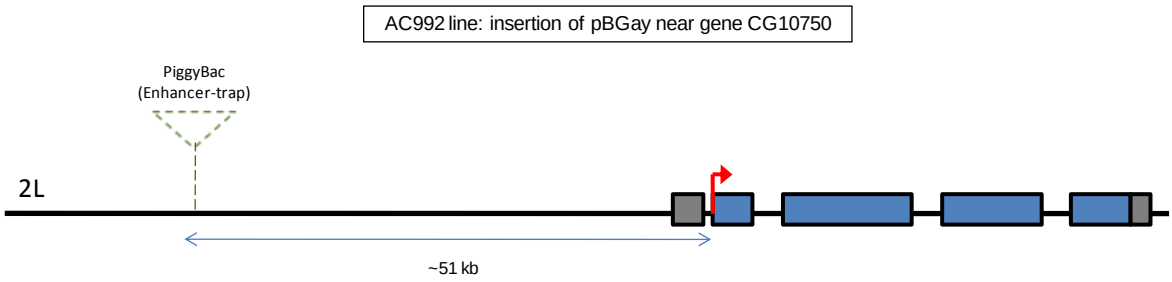
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PiggyBac
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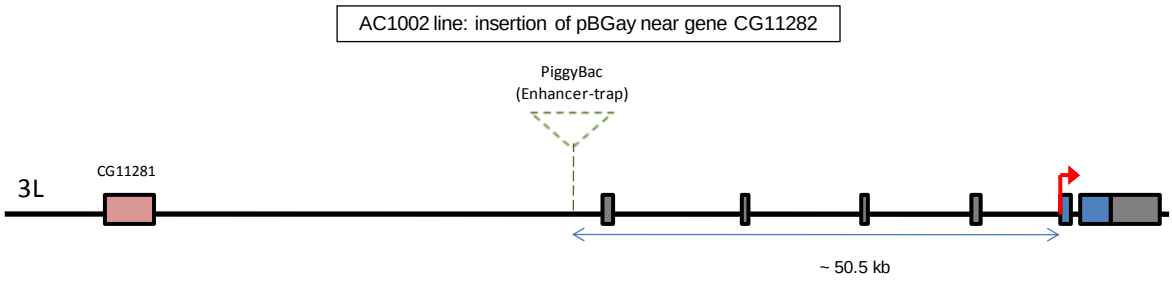
2L
~51 kb


AC1002 line: insertion of pBGay near gene CG11282
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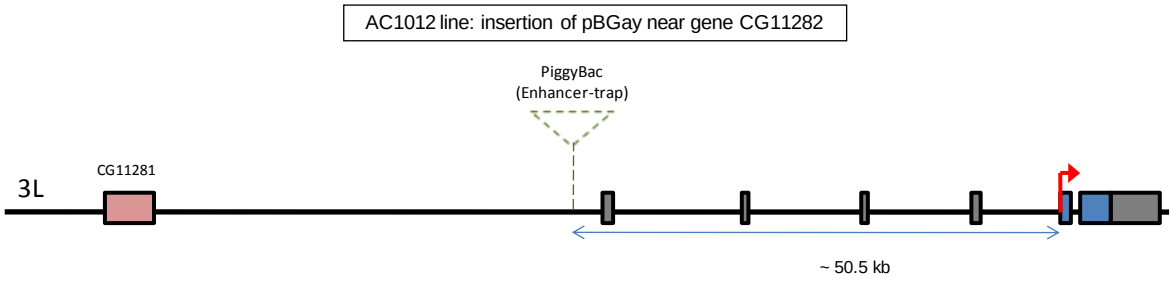
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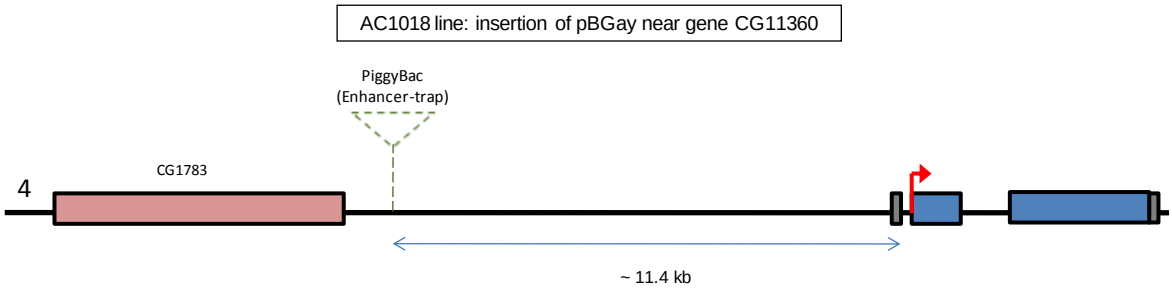
~ 50.5 kb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(Enhancer-trap)
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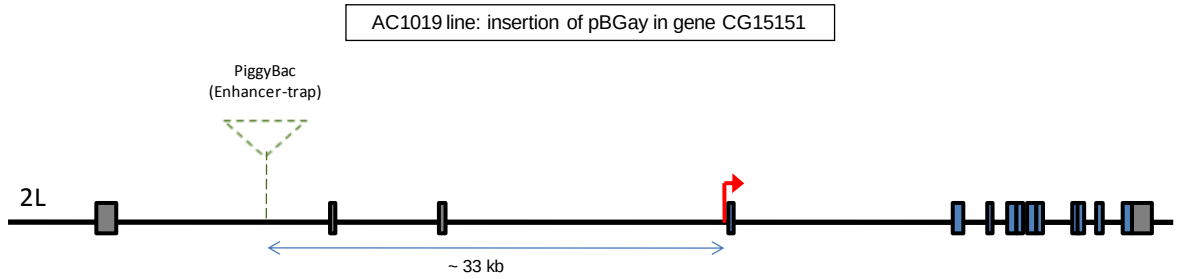
~ 11.4 kb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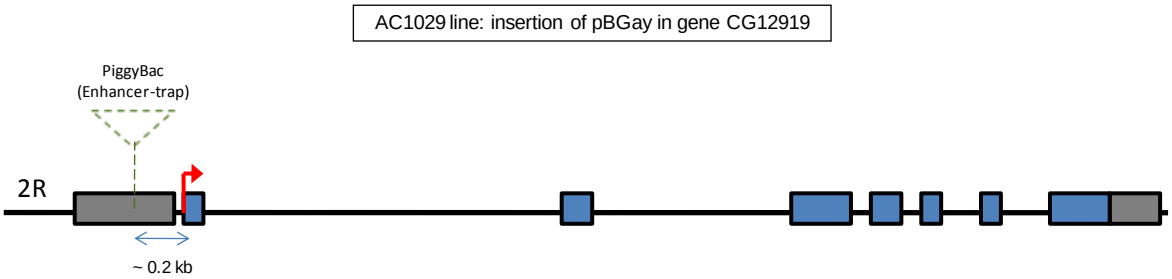
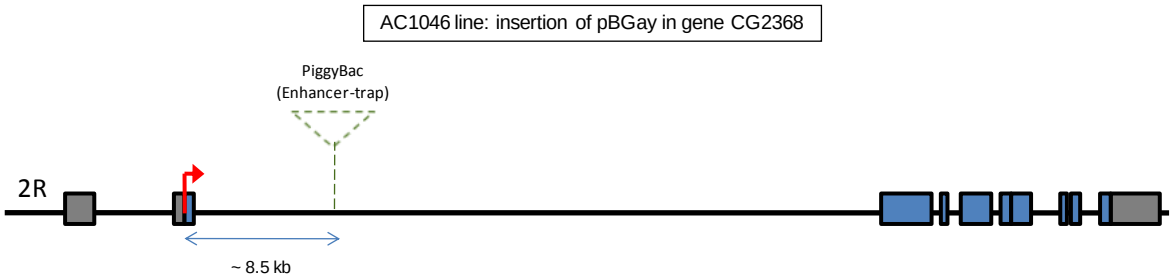
2L
~ 33 kb


AC1029 line: insertion of pBGay in gene CG12919
PiggyBac
(Enhancer-trap)
2R
~ 0.2 kb


AC1046 line: insertion of pBGay in gene CG2368
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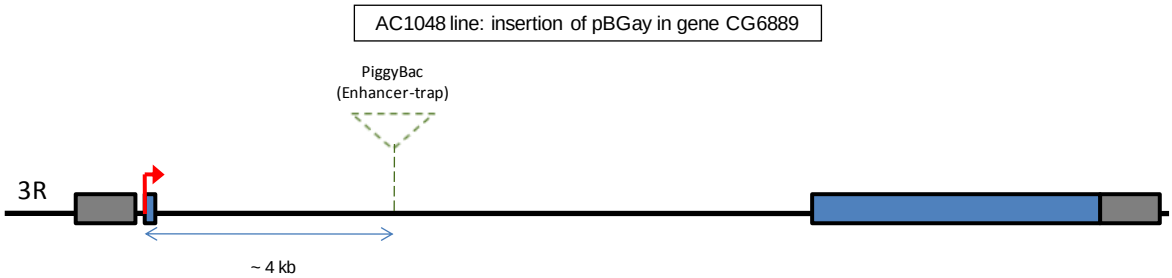
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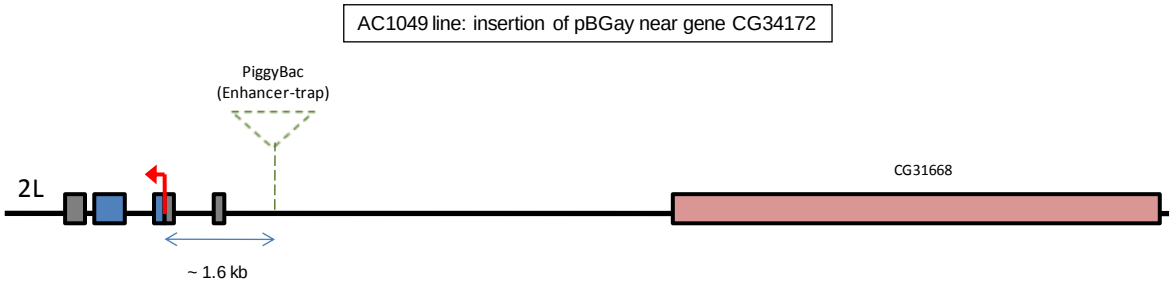
(Enhancer-trap)
3R
~ 4 kb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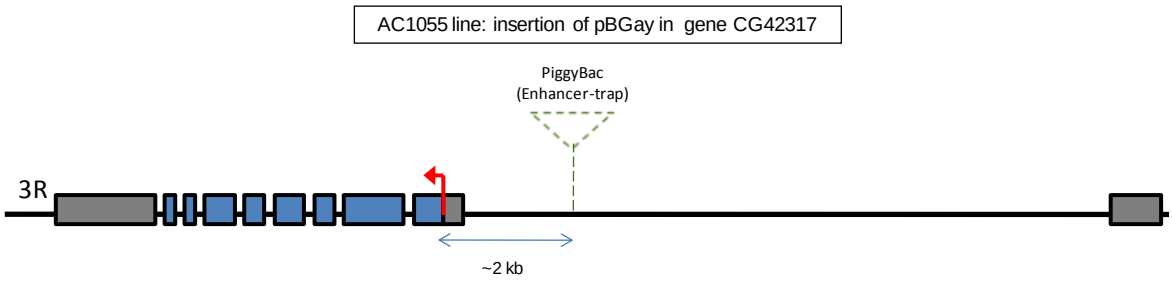
(Enhancer-trap)
2L
CG31668
~ 1.6 kb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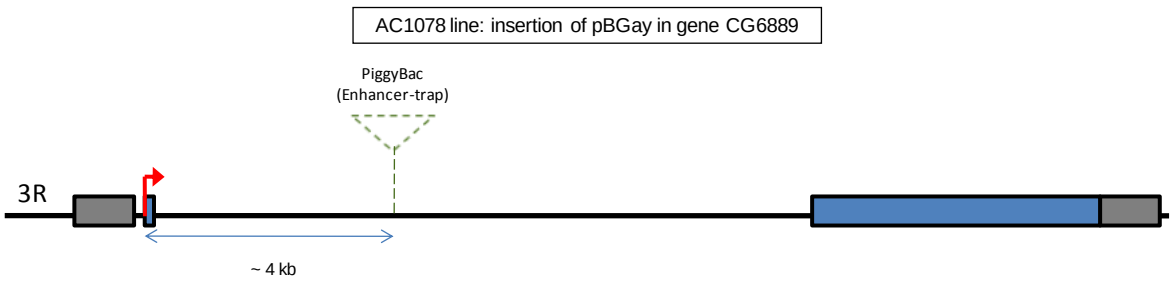
PiggyBac
(Enhancer-trap)
3R
~2 kb


AC1078 line: insertion of pBGay in gene CG6889
PiggyBac
(Enhancer-trap)
3R
~ 4 kb

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