Role of Drosophila Rap/Fzr (Cdh1) in retinal axon targeting and its interactions with Loco, Liprin-a and Ras

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Title: Role of Drosophila Rap/Fzr (Cdh1) in retinal axon targeting and its interactions with Loco, Liprin-α and Ras

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Thesis submitted as part of the requirements for the Masters degree

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CITY COLLEGE OF NEW YORK
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A. ABSTRACT

The development of the wild type *Drosophila* compound eye involves stereotypical targeting of photoreceptor axons to the specific layers of the optic ganglion, medulla and lamina, in the third instar larvae. To test the hypothesis that ubiquitin ligases play an important role during retinal axon targeting we have examined the patterns of axon targeting in the developing eye of the *retina aberrant in pattern (rap/fzr)* mutants. Rap/Fzr is a homolog of mammalian Cdh1, an activator of anaphase promoting complex (APC), an E3 ubiquitin ligase. Previous work has shown that Rap/Fzr is required in cell cycle regulation, glia differentiation and pattern formation during eye development. Results from our experiments show that Rap/Fzr is required for proper retinal axon targeting in the developing eye. Our studies using *ro-tau-lacZ* show that the R2-R5 axons fail to stop in the lamina and mis-target to the medulla levels. Mosaic analyses experiments using FLP-FRT and GAL4-UAS techniques show that Rap/Fzr functions in a cell autonomous manner. To test for possible role of other signaling molecules and interactions with Rap/Fzr, we have examined *rap* phenotypes in double mutant combinations with Loco, Liprin-α, and *RasV12* mutants. Our studies suggest that Rap/Fzr is required for proper axon targeting during *Drosophila* visual system development. These results are consistent with other mammalian studies reporting a role of Cdh1 in axon growth and targeting and provides further insights into neuronal functions of the ubiquitin ligase APC_{Cdh1}.
B. INTRODUCTION

Proper functioning of the nervous system requires the formation of precise neuronal connections. Understanding how these connections are formed is a central problem in neurobiology. The work presented in this thesis was conducted to test the hypothesis that Rap/Fzr, a Drosophila homolog of the mammalian Cdh1 is important for correct axon targeting. Rap/Fzr/Cdh1 is a regulatory subunit of the Ubiquitin ligase, the anaphase promoting complex (APC). The fruit fly Drosophila is a preferred experimental model system for study in many areas of biology. Drosophila offers an array of genetic tools that allow for a precise and accurate manipulation of genes in a tissue and stage-specific manner. The well characterized visual system of Drosophila comprised of the highly ordered compound eye and the optic ganglion is an excellent system to study the cellular and molecular mechanisms underlying the formation of precise neuronal connections.

B1. Structure and organization of Drosophila compound eye

The compound eye is made of about 750 individual units called ommatidia. Ommatidium has eight photoreceptor cells (R cells, R1-R8), four cone cells and two primary pigment cells as well as shared structures such as secondary and tertiary pigment cells and sensory bristles (Ou, Pi et al. 2003). In each ommatidium there is a stereotypic arrangement of the photoreceptor cells (R cells), there are six outer R cells (R1-R6) and two inner R cells (R7 and R8). R1-R6 express opsin Rh1 necessary for the detection of blue light and R7 cells express UV-sensitive opsins Rh5 or Rh4. R8 cells express either blue-sensitive Rh5 or green-sensitive Rh6 opsin (Ting and Lee 2007). The ommatidia can be classified into subclasses based on the type of rhodopsin, they include, the pale subtypes that make about 30% and express the UV-sensitive Rh3 in R7 and the blue-sensitive Rh5 in R8, and the yellow subtype that has UV sensitiveRh4 in R7 and green-Rh6 in R8. R7 and R8 can also detect polarized light (Morante and Desplan 2004).


The adult eye develops from a monolayer of epithelial tissue, the eye-antennal imaginal disk which is derived from a group of about 20 cells in the embryo. During the third larval instar stage the eye imaginal disk begins to differentiate into an adult compound eye. R cell differentiation starts as a wave at the posterior end of the eye disk and moves anteriorly. This wave is marked by a groove in the disk called morphogenetic furrow (MF). Cells posterior to the furrow differentiate into photoreceptor neurons while cells anterior to the furrow are unpatterned and undifferentiated. Two mitotic domains are seen in the developing eye disk. The first mitotic domain is diffuse and is seen anterior to the MF. The second mitotic domain is seen as a compact band immediately posterior to the MF. Cells in the MF are arrested in the G1 stage of the cell cycle (Ou, Pi et al. 2003). The secretion of Hedgehog (Buschhorn and Peters 2006) signal in the posterior part of the eye disc facilitates MF progression. Induction of Hh causes further induction of Hh in more anterior cells and the MF moves in a wave. It has been shown that Cubitus interruptus (Ci) a downstream effector of Hh can act as a transcriptional activator or repressor depending on its length (Ou, Pi et al. 2003). Due to the action of Hh, Ci155 accumulates at MF. In addition, Decapentaplegic (Dpp) allows the initiation of MF movement, and is initially expressed in the posterior part of the disc and later is limited just to the MF. The expression of
Wingless (Wg) in the posterior part of the disc, suppresses Dpp which inhibits Wg expression. Wg at the posterior part is suppressed by Unpaired, a ligand for the JAK/STAT signaling pathway. In addition, it has been shown that Hh induces Atonal (Ato), [a HLH transcription factor], expression in the MF and initiates neuronal development and neural induction of R8. Ato expression is suppressed by Hairy (H) which is turn is activated by Dpp (Greenwood and Struhl 1999).

As the MF moves anteriorly, R cell differentiation begins posterior to it in a precise sequence. The R8 cell differentiates first and it recruits sequentially R2, R5 followed by R3, R4 forming a pre-cluster of five photoreceptors. Following a second mitotic division R1 and R6 differentiate and are recruited into the pre-cluster. The R7 cell is the last R cell to differentiate forming a mature cluster of eight photoreceptors. Cone and pigment cell differentiation is completed during the pupal stage. Thus the R8 cell plays a key role in recruiting other R cells. R8 secretes the ligand Spitz, which activates EGF-receptor pathway needed for recruitment of other R cells to the cluster, and Argos, which sequesters Spitz (Siles, Yuva-Aydemir et al. 2010). When R cells start to differentiate, the R cell axons of each cell forms and moves through the optic stalk toward the brain lobes. Each R cell has a specific target optic ganglion it stops at. Thus, R1-R6 terminate at the lamina but R7 and R8 terminate in the medulla (Siles, Yuva-Aydemir et al. 2010). At the late third instar larvae stage retinal axon extend and target induction begins followed by the retinotopic map formation. R7 and R8 are the first to do layer-specific targeting in the medulla at an early pupa stage followed by R1-R6 into the lamina (Ting and Lee 2007).

B3. Photoreceptor axon targeting in the central nervous system

In the wild type Drosophila the axons move from the eye disc via the optic stalk and terminate in the lamina or in the medulla. Axons of the R1-R6 cells will terminate in the lamina, while R7 and R8 axons will continue to move just beneath the R7 into the deeper layer, the medulla, where they terminate in two distinct layers. R8 will terminate in the M3 while R7 will terminate in M6 layer (see Figure 1) (Tayler and Garrity 2003). As the larva enters the pupal stage, additional nerve fibers enter the medulla. The R cell axons follow stereotypical axon projections into the optic lobe. R1-R6, neurons target to the lamina where they synapse with lamina neurons, L1-L5 while R7, R8 and L1-L5 form connections in single columns within the medulla thus, each column has the lamina neurons responsible for the motion processing and color vision, R1-R6, looking at a single point in space. It is currently unknown what restricts synaptic connections to a single column (Millard, Flanagan et al. 2007). The decision making is often regulated by a variety of growth factors and stop signals. For example a gene eddy will prevent proper eye development because the axons will not innervate the brain but will stop at the beginning of the optic stalk and will show clumping of the glia cells (Martin, Poeck et al. 1995). The function of eddy is not well understood, but it was proposed that it encodes an extracellular molecule expressed in the brain or optic stalk that promotes outgrowth of the R cells (Martin, Poeck et al. 1995).
Fig 1. This image shows photoreceptor axon projections from the eye disc to the optic lobe. R1-R6 project to the lamina while R7 and R8 project to the medulla (Tayler and Garrity 2003).

B4. Retinotopic axon projections and topographic representation of space in the *Drosophila* visual system

Interestingly, in *Drosophila* the visual information from a single point in space converges onto a lamina cartridge and a medulla columns, thus each synaptic unit needs projection from 6 different ommatidia to visualize one point in space (Clandinin and Zipursky 2002). Thus, the fly has a topographic representation of the visual field. Different R cells will project to different optic ganglion layers with R1-R6 projecting to the lamina cartridge, while R7 and R8 project to the medulla cartridge. The lamina neurons project in the following order: L1 to M1, L2 to M2, L3 to M3, L4 to M4 and L5 to M5. A cartridge is a synaptic unit within the lamina from which lamina monopolar neurons project to the medulla, also called a column (see Figure 2) (Clandinin and Zipursky 2002). The precise mechanisms of axon targeting have been widely studied but the exact numbers of factors involved are not known. The fly optic ganglion is subdivided into the lamina, medulla, and the lobula complex. The lamina is organized into columns and has about 6000 cells and R1-6 synapse on the lamina forming functional unit. The unit is made of 5 monopolar cells (L1-L5), one or two amacrine cells, three medulla neurons (C2, C3 and T1) and three glia cells. The medulla receives inputs from the inner photoreceptors and is responsible for the color vision. Medulla has about 40,000 cells divided by serpentine layer and axons of tangential neurons dividing it into a distal (outer medulla) and a proximal (inner medulla) (Morante and Desplan 2004).
Fig 2. Information from a single point in space converges onto a lamina cartridge and a medulla column (Clandinin and Zipursky 2002).

**B5. Factors affecting eye disc axon targeting to the optic lobe in the third instar larvae**

Important factors affecting the axon targeting are determined by the growth cones which change with development as they innervate appropriate target. At the beginning, R1-R6 will initially extend the growth cones into the lamina as a single fascicle. While in the pupal stage of development, growth cones tend to extend out of the cluster and innervate specific columns of lamina (Clandinin and Zipursky 2002). Studies have shown that R cell interaction with each other also plays a role in axon targeting to the lamina. Another receptor molecule needed for proper R8 axon pathfinding expressed in growth cones is Gogo, Golden goal. It is involved in axon-axon interaction and is needed for the proper maintenance of the distance between the R cells, helping R8 to reach and recognize its target in the columns of the medulla (Tomasi, Hakeda-Suzuki et al. 2008). *Drosophila* insulin receptor (DlnR) has also been shown to be involved in R cell axon targeting. DlnR is used as a guidance receptor for Dock/Nck that is the adaptor protein (Song, Wu et al. 2003).

**B6. Roles of retinal basal glia on the target selection**

Glia cells play several significant roles in the nervous system. They provide nutritional support for the neurons and, play role in axon guidance and signal transmission. Retinal basal glia (RBG) cells provide positional information for the R cell axons to enter the optic stalk and the brain (see Figure 3) (Tayler and Garrity 2003). The retinal basal glia are born in the optic
stalk then migrate into the basal surface in the eye disc where they contact R cell axons. R cell axons provide cues which signal the differentiation and development of the target cells in the optic ganglion (Clandinin and Zipursky 2002). Without RGB the axons will not project to the proper layers in the optic lobe. Interestingly, the glia cells do not need axons for the proper targeting to the eye disc but the axons need glia to extend to the optic stalk (Chotard and Salecker 2004). Thus, disruption of glia distribution causes R1-R6 axon mistargeting as shown in the nonstop mutants (Martin, Poeck et al. 1995).

The molecules that guide the process of axon targeting are largely unknown but a few candidates have been proposed and they include Hedgehog and Dpp. Axons will stop or redistribute if glia cells are absent or disorganized. Mutants lacking Hh or Dpp will not project axons to the brain due to the stop signal and other axons will not migrate because the glial cells are disorganized or missing. Clumping of glia at the top of the optic stalk may lead to the disorganization of the axon projections as was shown in the gish mutant. gish encoded casein kinase I-γ, loss of gish leadsto the premature entry of the RBG to the eye disc before they have a chance to fully differentiate. The axons will turn away from the optic stalk (Tayler and Garrity 2003).

**B7. Visual system mutations and other factors affecting axon targeting**

Many *Drosophila* mutants have been identified with defects in the targeting of the photoreceptor axons to the optic ganglion. Some of the phenotypes include mistargeting, hypo or hyperinnervation, and clumping of axons (Martin, Poeck et al. 1995; Lee, Herman et al. 2001; Kaminker, Canon et al. 2002; Lee, Clandinin et al. 2003). The above studies used behavioral screens to identify several genes involved in R cell target choice. Some of the most recently discovered genes required for visual system wiring are flamingo (Lee, Clandinin et al. 2003),

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**Fig 3.** Glia cell locations in the optic lobe (Chotard and Salecker 2007).

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brakeless (Senti, Keleman et al. 2000), non-stop, headcase, and sequoia (Berger, Senti et al. 2008). These genes are involved in axon growth, lamina and medulla targeting, defects in R cells, growth cone morphology, undershoots, R cell bypass of its target, and many other morphological defects that are listed in Berger’s paper. Similarly, R cell specification is still not fully understood. For example, Flamingo is responsible for cell-cell interactions between the growth cones and its levels will determine the outgrowth path (Chen and Clandinin 2008).

Another gene identified, Sequoia is involved in the layer-specific targeting of R7 and R8 axons. It is a nuclear protein first expressed in R8 and then in R7. Loss of Sequoia leads to R7 cells mistargeting to R8 layer (Berger, Senti et al. 2008).

Many mutants have specific axon projection morphology defects. For example, a flamingo mutant will have disorganized axons forming bundles and the medulla will be disturbed. Flamingo is a Cadherin-regulated cell surface protein. It allows R1-R6 cells to select the proper target in the lamina. flamingo also is responsible for the regulation of the dendritic patterning. Contrary to many other mutants, the synapses of the flamingo mutants are normal but made to the inappropriate targets. The study further investigated individual R cells to explain axon abnormalities and has shown that N-catherin and Lar contribute to the axon extensions. Lar and Flamingo were identified using behavioral assays for defects in the R cells but further work is required to investigate other potential targets and interactions of the flamingo (Lee, Clandinin et al. 2003).

Another aspect of the axon targeting is shown by the role of certain receptors such as Baboon. It will prevent R7 axons terminals to go to the right target. Baboon is the Activin receptor that prevents R7 cells overlap with other columns of R7 cells. It was suggested that dActivin is a molecule that will restrict R7 growth cone movement. Activin action will lead to axon repulsion from the neighboring columns of the R7 cells. The importance of this molecule is that it provides an example of a molecule that restricts axons to the same column but if expressed in the improper layer in the optic lobe and will lead to improper axon targeting. The molecular signaling pathway is not well understood. Thus, a loss of Activin or mutations in Babo will lead to improper column infestation of the axons (Ting and Lee 2007). Also, expression of a gene in the wrong cell may force a specific cone to differentiate into another cone. For example, Runt a transcription factor from the Runt domain famil, is expressed in two inner R cells (R7 and R8). Upon misexpression of Runt in the R2 and R5 the axons will be misguided from their normal path (Kaminker, Canon et al. 2002). Axons in Drosophila have highly specific targets have the ability to retreat or bypass their target. For example, a Lar mutant will go to its target but will retreat to the wrong position, while the brakeless will not care about the stop signals but will go through the tissue to the last layer in the brain, as the name suggests without brakes, terminating in the medulla. Brakeless is responsible for the proper targeting of the R1-R6 and is present in the nuclei of all R-cell types (Tayler and Garrity 2003).

B8. The ubiquitin-proteosome pathway

Protein degradation is an important element of normal cellular maintenance. It plays a role in the elimination of damaged, mislocalized or foreign proteins. One of the well studied pathways involved in protein degradation is the ubiquitin-proteosome pathway. Ubiquitin is a
small, 76 amino acid protein which is attached to the target proteins. The ubiquitin has an attachment sites on the C-terminus where ubiquitin is added to ε-amino group of a lysine residue. The enzymes involved in the attachment are E1, E2, and E3 (Murata, Yashiroda et al. 2009). E1 is the ubiquitin-activating enzyme found in the nucleus and the cytoplasm. E2 is an ubiquitin-conjugating enzyme while E3 is ubiquitin-protein ligase responsible for the recognition of the protein substrates. E1 will transfer activated ubiquitin to E2 forming a thioester bond and then ubiquitin if transferred from E2 via E3 to the target site (McNaught, Olanow et al. 2001). RING-E3 binds to E2 and a substrate to help E2 with the transfer of ubiquitin to a substrate while HECT-E3 uses thioster bond with the ubiquitin before it is transferred to the substrate (see Figure 4) (Murata, Yashiroda et al. 2009). The E3 Ubiquitin ligase contains RING finger domain in which Cysteine and Histidine residues are ligands to two Zinc ions. RING E3 allows for the direct transfer of ubiquitin from E2-Ubiquitin to substrate (Deshaies and Joazeiro 2009). In general the monoubiquitination of proteins has regulatory effects while polyubiquitionation of 4 or more residues targets proteins for degradation by 26S proteosome (Deshaies and Joazeiro 2009). Monoubiquitination of different lysine residues can have diverse effects on the proteins due to the posttranslational effects on the protein function, structure, assembly and localization (Deshaies and Joazeiro 2009).

![Fig 4. An E3 ubiquitin-proteosome pathway (Ou, Pi et al. 2003).](image)

The 26S proteosome is responsible for degradation of cytoplasmic, nuclear and membrane proteins where ubiquitin is recycled but not degraded. Eukaryotic proteosome are large protein complexes of about 2000 kDa consisting of 20S particles, core and a lid. The core particle is made of 7-membered rings and two types of subunits, alpha and beta, where only beta subunits are catalytically active. The lid plays a role in regulation of the activity and is involved in the energy dependent steps (Adams 2004). The 20S particle is made of about 14 different subunits and relies of 5 different cofactors for its assembly. The base has six ATPase subunits (Rpt1-6) that form hexameric ring with Rpn1 and Rpn2 and relies on chaperones for proper folding, p27, P28, S5b and Rpn14 in mammals (Besche, Peth et al. 2009) but the exact assembly sequence is not well known. Also, ubiquitin-proteosome system was shown to affect axon pruning needed
for a refinement of the neuronal connections in animals; thus, protein degradation plays a crucial
role in axon pruning and Wallerian degeneration which can be inhibited by UPS (Korhonen
and Lindholm 2004). APC is a E3 ubiquitin ligase responsible for conjugation of Lys-48-linked
polyubiquitin chains to the substrate proteins and subsequent targeting them to the 26S
proteosome (Passmore 2004).

B9. Anaphase Promoting Complex/Cyclosome

APC/C (APC) was first described in *Xenopus laevis* in 1995 as a multi-subunit protein
with ubiquitin ligase activity needed for cell-cycle transitions (see Figure 5). It was named after
its main role, being necessary during the anaphase in yeast and mammalian cells (King, Peters et
al. 1995). The complete picture of the functions of APC is currently unknown but multiple
pathways were shown to be involved. The discovery that APC is expressed in the brain tissue
allowed the exploration of additional roles for the APC. It was shown that Cdh1, a regulatory
subunit, has a high expression levels in the postmitotic neurons especially in the cerebellum,
cerebral cortex and hippocampus while Cdc20, another APC activator, is practically absent in
these regions (Gieffers, Peters et al. 1999). Cdh1 binds substrates through its interaction with the
D box (destruction box), which is made of nine amino acids, RxxLxxIxN, found on its substrates
such as cyclin B. Mutations in the D-box prevent protein degradation (Bashir, Dorrello et al.
2004). Cdh1 also recruits substrates that have KEN box (KENxxxN), A-box, and CRY box
(Kaplow et al., 2007(Kim and Bonni 2007). APC has a V shape with APC11 and APC 2 on the
outside and Cdh1 on the inside thus it was suggested that the APC E3 ligase activity is on the
inside while ubiquitination on the outside of the complex (Kim and Bonni 2007). APC-Cdh1 is
regulated by phosphorylation and dephosphorylation of Cdh1. Phosphorylation inhibits APC-
Cdh1 binding as well as its interactions with other inhibiting proteins such as Emi1 and Mad2b
(Kim and Bonni 2007). Cdc20 and Cdh1 contain WD-40 repeats in the C-terminus forming a
seven-bladed propeller structure needed for the protein-protein interactions that recruit substrates
for ubiquitination. Cdc20 plays a role in the activation of APC in mitosis but to remain active in
late mitosis APC must rely on Cdh1 (Gieffers, Peters et al. 1999).
Fig 5. Composition of Anaphase Promoting Complex/Cyclosome (Peters 2006).

B10. *rap, (retina aberrant in pattern), a Drosophila homolog of mammalian Cdh1*

`rap (retina aberrant in pattern)` encodes the Fizzy-related protein (Fzr) which is an activator of Anaphase Promoting Complex, APC, an ubiquitin ligase complex. Rap/Fzr is a homolog of a mammalian Cdh1. Previous studies have shown that Rap/Fzr plays significant roles in eye development (Karpilow, Pimentel et al. 1996). Additionally, recent data have shown that *rap* regulates the glia differentiation in the developing CNS through interactions with Loco. Partial loss-of function mutations cause an increase in number of surface glia in the third instar larvae, while a gain-of-function mutations lead to an increase in neurons (Kaplow, Korayem et al. 2008). Overexpression of Rap/Fzr has shown progressive neurodegeneration, temperature-sensitive paralysis and life span reduction (Mino, et al., unpublished data). In addition, unpublished data suggest possible roles of *rap/fzr* in synaptic plasticity and bouton morphology and structure (A. Wise, unpublished data). A genetic modifier screen has identified 39 other genes which interact with Rap/Fzr and are involved in ubiquitin mediated proteolysis, signal transducers, transcription regulation (Kaplow, Mannava et al. 2007).

B11. Neuronal roles of mammalian Cdh1

In the mammalian nervous system Cdh1 expression is localized to the brain neurons in the cortex, hippocampus, and cerebellum while Cdc20 is very limited in the brain (Kim and Bonni 2007). It was shown that APC

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\text{Cdh1}^\text{Cdh1}
\]

may play a role in the axonal growth and control of patterning. Depletion of Cdh1 by RNAi in neurons of the rat cerebellar cortex caused axonal elongation, but not the dendritic growth. The phenotype can be rescued by RNAi-resistant Cdh1 (Konishi, et al., 2004). It was also suggested that expressing APC11 or Emi1 that normally inhibits APC, also increased axonal length (Konishi, Stegmuller et al. 2004). Surprisingly, with Cdh1 knock down neurons can grow in the presence of inhibitory myelin thus suggests potential therapeutic regenerative possibilities of neurons (Konishi, Stegmuller et al. 2004).

B12. The role APC/Cdh1 in regulation of axonal length in mammals

Several laboratories began to seek ubiquitinated substrates that play a role in the axonal outgrowth. One is SnoN, a transcriptional co-repressor, expressed in post-mitotic neurons. RNAi knockdown of SnoN decreased the length of axons, suggesting that SnoN promotes axonal elongation. Interestingly, SnoN-RNAi inhibits axonal growth in RNAi-Cdh1 cultures (Stegmuller, Konishi et al. 2006). SnoN physically interacts with Cdh1, which causes SnoN ubiquitination and proteosomal degradation in primary cerebellar granule neurons. Thus, RNAi-SnoN prevents axonal outgrowth (Stegmuller, Konishi et al. 2006). SnoN interacts with p300, a transcriptional co-activator, to promote axon growth; thus, SnoN plays two roles, and it can activate or repress transcription in a cell-specific or gene-specific manner (Ikeuchi, Stegmuller et al. 2009). The roles of transcriptional mechanisms used in axonal elongation and stabilization are not fully understood. Ubiquitination starts to emerge as a powerful “cell-intrinsic regulator” in the determination of the axonal lengths. For example, it was shown that ubiquitinated proteins
play a crucial role in the regulation of axonal growth (Stegmuller, Konishi et al. 2006). Identification of substrates needed for APC-Cdh1 is necessary to determine what controls the axonal development. Additional proteins involved in inhibition of axonal growth are Sema3F, Jagged-2, Unc5A, Notch1 and Nogo receptor. The mechanism involved was suggested to involve Cdh1 knockdown that is able to stabilize Id2 and inhibit E protein-mediated Nogo receptor transcription, since Id2 is a substrate for APC-Chd1 (Kim and Bonni 2007). Another Cdh1-APC substrate that plays a role in the axon growth is Id2, inhibitor of DNA binding 2, that is recognized by the D-box and is ubiquitinated and degraded; thus, it promotes axon growth since mutated D-box promotes it; similarly, Id2 with mutated D-box overcomes the inhibitory functions of myelin and axons grow (Yang, Kim et al. 2010).

Axon guidance and growth utilizes cues via the cell surface receptors which use the cytoskeleton to attract or repulse the axon growth cone (Dent and Gertler 2003). Cdh1 and Cdc20 are modified post-translationally by ubiquitination, phosphorylation, and interaction with APC inhibitor (Yang, Kim et al. 2010). Cdh1 regulates axon morphogenesis while Cdc20 is involved in dendrite morphogenesis, and Cdh1-APC operates mainly in the nucleus (Yang, Kim et al. 2010). Cdh1 may be phosphorylated at 11 sites in yeasts and 9 residues in mammals; phosphorylation on Ser40, 151, and 163, Thr121 promotes the nuclear export of mammalian Cdh1 (Huynh, Stegmuller et al. 2009). Phosphorylation of Cdh1 at Cdk sites disrupts the association of Cdh1 with the APC core protein Cdc27 and promotes cytoplasmic accumulation of Cdh1 in granule neurons (Huynh, Stegmuller et al. 2009). Cdh1 is able to autoubiquitinate itself; thus, phosphorylation of Cdh1 at Cdk sites may prevent autoubiquitination of Cdh1 (Huynh, Stegmuller et al. 2009).

B13. Liprin-α

When we look at the molecular level of the axon targeting there are many unknowns. In the literature we can find specific molecules that act as the stop and go signals. We find an array of proteins that seem not to be connected with each other, but at the end influence the phenotype in dramatic ways. These proteins are often multifunctional and their concentration and locations affect the axon targeting as well. Thus, expression of the protein in the wrong place can cause axon misrouting or can cause all axons to go to only one target resulting in numerous projections. One interesting molecule is Liprin-α, which is a scaffolding protein required for proper axon targeting. Liprin-α is localized at the synapse, and is a substrate for APC/Cdh1. Mutations in the Liprin-α will affect axon target selection in R1-R6 photoreceptors (Choe, Prakash et al. 2006). Liprin-α is a protein with N-terminal coiled-coil domain and C-terminal LAR-binding liprin homology domain, which is made of three sterile alpha motif (SAM) domains (Hofmeyer, Maurel-Zaffran et al. 2006). Liprin-α plays a role in the NMJ, anterograde transport and accumulation of synaptic vesicles presynaptically (Prakash, McLendon et al. 2009). Liprin-α is also involved in the regulation of synaptic function and is targeted by APC\textsuperscript{Cdh1} acting as a substrate at the neuromuscular junction (van Roessel, Elliott et al. 2004). Liprin-α mutants show defective axon guidance and are required presynaptically to mediate adhesive events between pre- and postsynaptic cells (Choe, Prakash et al. 2006). Hofmeyer showed that Liprin-α overexpression can partially restore R7 targeting and its removal may worsen the targeting when LAR is absent (2006).
**B14. Loco and its known roles**

The regulators of G-protein signaling (RGS) are an important family of proteins critical in the regulation of signaling mediated by G-coupled receptors [GPCR] (Yu, Wang et al. 2005). GPCR s are found at the cell surface and are linked to the G protein to initiate a signaling cascade. G proteins are made of G\textsubscript{α} subunit and G\textsubscript{βγ} heterodimer (Granderath and Klambt 2004). G\textsubscript{α} is inactive when it is bound to the GDP and is a part of the G\textsubscript{βγ} complex. Once activated, it replaces GDP with GTP. Then, G\textsubscript{α} subunit separates from the complex and is able to activate downstream targets. G\textsubscript{α} has an intrinsic GTPase activity to hydrolyze GTP (Granderath and Klambt 2004). RGS proteins activate the GTPase activity of G\textsubscript{α} subunits and allows for a faster transfer of GTP-bound active to GDP-bound inactive form.

In *Drosophila*, nine genes, such as *axin (a scaffold protein)*, *gprk 2(G-protein-coupled receptor kinase2)* and *loco* (McGurk, Pathirana et al. 2008), are known to encode RGS proteins in *Drosophila. locomotion defects (loco)* encodes a member of an RGS protein family and is involved in the G-protein signaling. It was identified using P-element enhancer trap insertions and the loco gene encodes four possible transcripts by alternative splicing, LocoP1-P4 encodes different Loco isoforms of the following amino acid length: 829, 1175, 872, and 1541 (Granderath and Klambt 2004). Loco has RGS domain and Go Loco motif that plays a role as a Guanine nucleotide dissociation inhibitor (GEF). In addition, *loco* is expressed primarily in the neuroblasts and in the lateral and midline glia cells in the central nervous system. Loco is required for the correct development of the blood brain barrier and the ensheathment of the neuronal bodies and axons. It was shown to physically interact with the G\textsubscript{αi} using yeast-two hybrid screen while having the highest homology to mammalian RGS12 and RGS14 (Granderath, Stollewerk et al. 1999; Ponting 1999). Loco P1 is expressed in the central nervous system, primarily in the lateral glia cells throughout the development and in adult (Granderath and Klambt 2004).

Very little is known about *loco* expressions in non-glia cells but it was shown to be involved in the dorso-ventral patterning and circadian regulation of gene expression (Claridge-Chang, Wijnen et al. 2001; Pathirana, Zhao et al. 2001). Different alleles of *loco* reduce the lifespan and impair locomotion, thus giving *loco* its name (Granderath, Stollewerk et al. 1999). Loco was also found to play a role in male reproductive system differentiation (McGurk, Pathirana et al. 2008). The primary roles of Loco are the formation, extension and migration of glia cells and the asymmetric division of neuroblasts (Yu, Wang et al. 2005). It was shown that the *loco* gene is required for glial differentiation and triggers glial migration or differentiation in the brain but the exact mechanisms are not known (Siles, Yuva-Aydemir et al. 2010).

**B15. Ras and the APC complex**

A possible interaction between of Ras and APC/Cdh1 was suggested through studies on Ets2. Ets2 is a transcription factor that is activated by Ras-Raf-MAPK signaling and induces cyclin D1. Increased expression of Ets2 has been implicated in several cancers. APC-Cdh1 regulates the process since it targets Ets2 for degradation and thus limits the expression of cyclin D1 (Li and Zhang 2009). This suggested a possible interaction of Ras and APC-Cdh1 and its role
as a tumor suppressor. Interestingly, the Ras interaction with APC-Cdh1 is not well characterized. It was also suggested that the APC-Cdh1 may have an inherent tumor suppressor activity, due to its roles in cell cycle regulation and thus may play a role in the genomic stability and generation of cancer. It was also shown in various cancers that the several mutated APC components were tumorigenic (Turnell and Mymryk 2006) but the exact role of APC in the process is unknown. Thus, the suggestion that overexpression of APC substrates may lead to genomic instability and tumorigenesis (Turnell and Mymryk 2006) needs to be further investigated. Turnell et al. (2006) cites several cases of cancers caused by the APC/C substrate problems. RasV12 mutation was shown to cooperate to cause tumor growth and invasion when combined with loss-of-function scribbled which is a tumor suppressor gene with the involvement of JAK/STAT signaling pathway. It was shown that RasV12 causes cell overgrowth in the eye imaginal disc of Drosophila and is an oncogenic form of Drosophila Ras85D (Wu, Pastor-Pareja et al. 2010). The Valine mutation makes the Ras constitutively active.

C. RESULTS

C1. rap mutants exhibit axonal targeting abnormalities.

The partial loss-of-function mutations in the Drosophila rap gene cause a rough external phenotype when compared to the wild type Canton. It was previously shown that these mutations cause additional aberrant mitotic cell cycles in the developing eye (Pimentel and Venkatesh 2005). Examination of the protein expression patterns of Rap/Fzr showed that it is expressed in the nucleus of the post mitotic photoreceptor neurons; thus, it lead us to investigate whether Rap/Fzr has a neuronal function (Pimentel and Venkatesh, 2005). The partial loss-of-function alleles in the rap mutants cause a range of severity in the external rough eye phenotype as compared to the laboratory wild type, Canton S flies. Thus, to test whether Rap/Fzr in the developing R cell neurons plays role in axon targeting, we used Drosophila eye as an experimental model to examine axon projection patterns in developing eyes from rap loss-of-function mutants. The R cells have stereotypical axonal projections, R1-R6 innervate the lamina while R7 and R8 innervate the medulla in a stereotypical manner. To address the question of axonal targeting we utilized the development of Drosophila eye which develops from the eye imaginal disc that is attached through the optic stalk to the optic lobes in the third instar larvae.

We used six different rap alleles previously generated by various methods of mutagenesis such as X-rays (rapx-2 and rapx-3), EMS (rapE2, rapE4, and rapE6), and P-element (w;rap3) by Tadmiri Venkatesh laboratory. These alleles were analyzed using immunohistochemistry and visualized using confocal microscopic imaging for the abnormalities in the axonal projection patterns. The photoreceptor axons were stained with Mab 24B10, which was previously identified to stain photoreceptor cells and their axons in the eye imaginal disc (Zipursky, Venkatesh et al. 1985). The wild type third instar eye discs of CS and w^{1118} (white mutation, no pigment expression, previously shown to have normal photoreceptor axon projection patterns) flies show stereotypical axon projections from the eye imaginal disc, through the optic stalk, with well organized lamina and medulla axons and a well defined lamina plexus (Fig 6. A, B). Rap mutant alleles showed aberrant retinal axon projections. The different rap mutants examined show allele specific variation in phenotype, with rapx-3, rapE4, and rapE2, (Fig. 6. D, E, F) showing photoreceptor axon overgrowth. All mutants show gaps in the lamina plexus, axonal
clumping, disorganized lamina and medulla layers (Fig. 6. C-H). The rap alleles show specific variations in the abnormal phenotype suggesting that Rap operates in a gene dosage dependent manner.
Fig 6. The wild type (CS and w^{118}) Drosophila eye shows proper innervations in the lamina and medulla. Each is a stack image composed of 20-30 focal planes, each advancing 1.5 µm along the z-axis. The axon projections do not show clumping or disorganization. They follow stereotypical patterns of targeting. rap mutants show allele specific abnormalities, lamina gaps (shown with arrows), disrupted medulla and axonal overgrowth. Shown axon projection patterns, stained with 24B10. Arrows point to the gaps in lamina plexus, arrowheads show sample overgrowth areas of axons.

Since, we saw abnormal axonal projection patterns in the photoreceptor axons of rap alleles; we performed a detailed analysis of the six rap alleles by measuring their axonal lengths. The measurements included: a) the lengths of R1-R6 from the base of the optic stalk to the lamina plexus, b) R7-R8 from the base of the lamina plexus to the end of R7-R8 growth cones, c) the width of the lamina plexus, and d) the width of the optic stalk at the base of the eye disc. The lengths were quantified using projected images obtained from z-stack images generated using LSM Image Browser.

A statistical analysis of the results shows significant changes in the lengths of the lamina plexus and R cell axon length. The average lengths of the measured axonal projections, their significance and standard error are plotted in the Figure 7. The percent increase of the statistically significant alleles was the following: rap^{E2} showed significant differences in the length of the R1-R6 cells (33.18%, P value=0.0032, St. Dev.=5.259, n=8) and the lamina plexus (40.84%, P value=0.0054, St. Dev.=21.47, n=8). rap^{E4} allele showed significant differences in the R7-R8 cells (49.63%, P value=0.0038, St. Dev.=24.76, n=7) and the lamina plexus (19.61%, P value=0.0010, St. Dev.=24.76). rap^{E6} allele showed significant differences in R1-R6 cells (24.65%, P value=<0.0001, St. Dev.=4.934, n=7). rap^{X-3} showed significant differences in R1-R6 cells (5.58%, P value=<0.0001, St. Dev.=9.869, n=11). w,rap^{3} showed significant differences in R7-R8 cells (37.87%, P value=0.0385, St. Dev.=55.78, n=15) (see Table A1-3 in Appendix). Additional parameters are needed to assess the severity of axonal projection patterns. Those include quantification of the number of axons, in mutants that the number cannot be easily distinguished; measuring number of clumps per square centimeter may be a good way to assess for the more severe phenotypes since the clumps make it difficult to accurately measure the number of axons present.

Our results are consistent with function of Rap/Fzr as an inhibitor of axon growth and elongation. Similar results were shown in the mammalian homolog Cdh1, where knock down of Cdh1 using siRNA in the axons in the cerebellar cortex showed growth and elongation (Konishi, Stegmuller et al. 2004). The alleles, rap^{x-2}, rap^{x-3}, and w, rap^{3} (Fig 6. C, D, and H) showed additional gaps in the lamina plexus, axon bundling in the medulla and lamina as well as the increase in the overall length of the projections (Fig 6. F-H). The results indicate that Rap/Fzr plays a role in the determination of axonal growth and inhibits axonal growth; thus, partial loss of function clones show an increased length of the photoreceptor axons (Figure 7, Table 1 in the Appendix). The data is supported by the mammalian studies using Cdh1 mammalian homolog of rap, that show that axonal growth in the central nervous system is affected by the loss-of-function Cdh1 (Huynh, Stegmuller et al. 2009).
Figure 7. Histogram representing rap allele R cell photoreceptor axon measurements. The percent increase of the statistically significant alleles was the following: rap$^{E2}$ showed significant differences in the length of the R1-R6 cells (33.18%, P value=0.0032, St. Dev.=5.259, n=8) and the lamina plexus (40.84%, P value=0.0054, St. Dev.=21.47, n=8). rap$^{E4}$ allele showed significant differences in the R7-R8 cells (49.63%, P value=0.0038, St. Dev.=24.76, n=7) and the lamina plexus (19.61%, P value=0.0010, St. Dev.=24.76). rap$^{E6}$ allele showed significant differences in R1-R6 cells (24.65%, P value=<0.0001, St. Dev.=4.934, n=7). rap$^{x-3}$ showed significant differences in R1-R6 cells (5.58%, P value=<0.0001, St. Dev.=9.869, n=11). w;rap$^{3}$ showed significant differences in R7-R8 cells (37.87%, P value=0.0385, St. Dev.=55.78, n=15).

C2. Photoreceptor cell axons R2-R5 show mistargeting in rap mutants.

The 24B10 antibody used to visualize rap mutants and labels all R cells. Thus, it does not distinguish among the inner (R1-R6) and outer (R7 and R8) photoreceptor axons. In order to determine which photoreceptor axon group causes the abnormal phenotype, a ro-tau-LacZ reporter was utilized. The reporter ro-tau-LacZ permits the selective labeling of the R cell axons R2-R5 (Fan, Soller et al. 2005). The ro-tau-LacZ marker was crossed into the rap mutant.
background and the axon projections examined. The wild type, CS, and the ro-tau-LacZ (parental strain) show well defined lamina axons stained with anti-beta-galactosidase antibody (Fig 8. A-B). rap alleles show R2-R6 photoreceptor axons crossing the lamina plexus and terminating in the medulla. Also, in many instances R cells tend to clump at the lamina plexus, causing gaps (Fig 8. D-I). It was also noticed that ro-tau-LacZ reported was leaky. It was previously utilized using horseradish peroxidase staining, which is not as specific as antibody staining; thus, some of the expression was also seen in the R7-R8 cells, but the expression was at a lower level in the parental strain than in the double mutant cells. The data suggests that in rap partial loss-of-function alleles of rap cause developmental axonal changes, possibly changing the photoreceptor outgrowth or affecting proper R cell axon targeting.

Fig 8. R2-R5 axons were visualized selectively with a ro-tau-lacZ reporter to assess targeting to the lamina in third instar optic lobe. A) ro-tau-lacZ parental strain; B) ro-tau-lacZ/Y male parental stock which shows some leakiness to the R7-R8 cells. The double mutant crosses rap and ro-tau-LazZ: C) LocoP452/+ ; ro-tau-lacZ/++; D) rapE2/Y; ro-tau-lacZ; E) rapE4/Y; ro-tau-lacZ; F) rapE6/Y; ro-tau-lacZ; G) rapx2/Y; ro-tau-lacZ; H) rapx3/Y; ro-tau-lacZ; I) w, rap3/Y; ro-tau-lacZ. Arrows point the R2-R5 cells bypassing the lamina plexus.
C3. Rap/Fzr functions cell autonomously in retinal axon targeting

Mosaic analysis is a useful tool in studying lethal mutations, allowing certain defects to be expressed only in particular places, tissues or cells; thus, permitting for an in vivo comparison of the wild type and mutant phenotypes. Mutant clone phenotypes are a common way to assess for cell-autonomous functions. Mosaic analysis methods are widely used to study mutations in a tissue-specific or time-specific manner to see additional developmental influences of the mutants to the wild type background. Eye-antenna disc was one of the first tissues used for the generation of clones in a living organism (Xu and Rubin 1993). As previously shown by other groups, many other mosaics also showed defects in the axonal projections and when compared to the wild type phenotypes it was seen that the axons projected to the wrong target taking unusual routes, misguided by the wrong signals. For examples, in the Flamingo mutant, mosaics showed that mutations in Flamingo would lead to the mistargeting of the axons in the medulla (Takeichi 2007). The axons were seeking the wrong target but often times the synapses formed were normal. In this case, homozygous flamingo mutant was lethal and could not be studied by conventional ways.

Similarly, rap expression is necessary for the survival of Drosophila since rap null alleles are lethal; thus, rap is an essential gene for the development of the fruit fly. The assessment of whether the rap mutation is cell autonomous or non-cell autonomous, in an organism that cannot survive the complete loss-of-function, the generation of clones is necessary. To assess whether Rap/Fzr function in axon targeting is cell autonomous, genetic mosaic analysis was performed by generating mitotic clones in the developing visual system. Mosaic studies were used to test cell-autonomous functions of the rap gene regulating axon projections to their targets. The advantages of using mosaic studies is that patches of tissue of the animal will express normal patterns while other parts will express the mutant phenotype; in other words, we will obtain homozygous null rap-/rap- mutant clones in the overall homozygous wild type, rap+/rap+ twin spot, background.

A system widely used to generate mosaic clones is FLP-FRT system in Drosophila which allows for an efficient mitotic clone generation using the heat shock promoter driving Flippase expression combined with FRT. This site-specific recombination system allows for a deletion of a particular gene of interest. FLP is a site-specific recombinase which recognizes FRT (FLP recombination target) sites (Theodosiou and Xu 1998). Heat shock and eye Flp promoters were used to express the Flippase enzyme. GFP fusion reporter was used to localize and visualize the clones. FLP will cause recombination by excising the DNA fragment between the FRT sites and remove the rap allele.

Our preliminary data show that the regions of the rap-/rap-clones identified by the absence of GFP staining show aberrant axon projections. The eye disc morphology of rap-/rap-clones is shown in Fig 9. Clumping of axons and various gaps in the lamina plexus were observed in the clone patches but the general morphology of the surrounding cells is normal. The phenotype appears cell autonomous since the surrounding genotypically heterozygous or wild type tissue is phenotypically normal. The clones were generated using two different reporter lines with Hsp FLP on different chromosomes to test the signal strength (Fig 10. A-D, I-L). The
clones are enclosed with a white line (Fig 10, E-I, M-P). The visualization of clones in the third instar larva poses a challenge, only the clones in the optic lobe surface can be seen; thus, the images do not show clones on the interior of the lobe. This explains why axons in other brain regions besides the enclosed clone areas shown on the Fig 10. have abnormal axonal patterns, forming clumps, gaps and disorganized lamina plexus. These data suggest that Rap/Fzr function is cell autonomous. Additional experiments need to be performed for more definitive conclusions such as MARCM, mosaic analysis with a repressible marker, that allows visualization of a single cell clone in the heterozygous wild type background (Lee and Luo 2001).

**Clones:**

![Image of clone visualization](image)

**Fig 9. Loss of function** clones of rap-/rap- using FRT site specific recombination. Genotype: w67c23 P{lacW}rapG0418 P{neoFRT}19A/FRT19,UbiGFP,eyFLP; P{ey-FLP.D}5/+. The arrows point to the areas where rap-/rap- clones were generated in the eye disc, showing abnormal photoreceptors. RED=24B10, GREEN=GFP.
Figure 10. The loss-of-function rap clones. A-D and I-L. Show parental strains used to generate the clones. The image shows axonal clumping, mistargeting of photoreceptor axons, disorganized lamina plexus and the overall morphology of the clones is affected. Images D, H, L, and P are merges of GFP, 24B10, and anti-beta-galactosidase staining. The genotype are: A-D: FRT19,UbiGFP,eyFLP1/Y;+/+; +/+; and I-L: FRT19,UbiGFP/Y; +/+; hs.FLP/+; E-H. and M-P. Show the loss-of-function clones outlined in white. Genotypes: E-H: w67c23 P{lacW}rapG0418 P{neoFRT}19A/FRT19,UbiGFP,hs.FLP1; P{ey-FLP.D}5/+; +/+; and M-P: w67c23
C4. Gain-of Function experiments show that Rap/Fzr is required for proper axon targeting.

The data suggested that loss-of-function caused an increase in the R cell length, we wanted to examine whether the gain-of-function will cause the opposite, axonal shortening. To test the effects of over expression of Rap/Fzr, gain-of-function \textit{rap/fzr} clones were generated and screened for photoreceptor axon projection using two systems: UAS/GAL4 and FLP/FRT. \textbf{Thus, we asked whether the gain-of-function in Rap/Fzr modulates proper axon targeting and length.}

The gain-of-function experiments are a useful way to study the effects of the overexpression of a given protein. This experiment utilizes UAS/GAL4 system (Duffy 2002) combined with FLP/FRT system (Golic, Rong et al. 1997). The gain-of-function study ensures that the protein in question is causing the alteration of the phenotype. UAS/GAL4 system allows direct assessment of the effect of expression of a gene of interest with the ability to report and visualize its activity. GAL4 is a transcriptional activator from yeasts, and is expressed in tissue specific manner while UAS is a transgene, an upstream activator sequence (St Johnston 2002). The \textit{rap} gene was cloned downstream of a UAS sequence and the gene expressed in the presence of GAL4 protein that binds to the UAS and activates transcription. Both GAL4 and UAS-\textit{rap/fzr} are carried in different strains, allowing the parental viability. It also allows for the GAL4 to be expressed in a spatial pattern; thus, depending on the tissue promoter used the gene under UAS can be overexpressed in a variety of tissues if crossed to the GAL4 line (McGuire, Roman et al. 2004).

The experiment utilizes the UAS-\textit{fzrII.I}, \textit{UAS-GFP} as a visible reporter gene, and combines the UAS/GAL4 system with heat shock FLP and Actin>CD2>FRT-GAL4 UAS-GFP cassette, (where > are FRT sequences). Under a heat shock, Flippase will be activated causing site-specific recombination of the FRT sequences and excision of the FRT>CD2 flanking sequence. The excision will allow the expression of GAL4 from the Actin promoter and binding of the GAL4 protein to the UAS sequences and activating the expression of \textit{rap/fzr} and GFP reporter gene. All cells labeled with GFP will also overexpress Rap/Fzr protein allowing for the visualization of the affected cells.

The heat shock was administered 24 (Fig 11. A-D, Fig 12. A-D) or 48 hours (Fig 11. E-P, Fig 12. F-T) after egg laying at 37°C water bath. The gain of function clones show axon clumping in early induced clones (Fig 11). It was previously shown that gain-of-function of \textit{rap} causes an increase in the neuroblasts and decrease in the glia numbers in the optic lobe of \textit{Drosophila} (Kaplow, Korayem et al. 2008). Thus, the gain-of-function of \textit{rap/fzr} in the eye imaginal disc causes axonal clumping, disorganization of lamina plexus with many gaps. \textbf{These data suggest that Rap/Fzr is necessary for proper axonal targeting and proper development of the \textit{Drosophila} visual system that begins in the third instar larvae.} Interestingly, the lamina plexus shows clusters of R cells fluctuating from the proper orientation in a step-wise
fashion. It suggests that the overexpression of Rap causes an increased number of neurons that cannot fit onto the plane of the lamina plexus and cause the disorganization of that region. Although, the photoreceptor axons were not measured the length of the R cells is significantly decreased. Further measurements are necessary to assess the significance of the decrease.

Fig 11. Gain-of-function rap clones expressing GFP and FZR. The gain of function clones show axon clumping (shown with arrow), axonal bundles and shortening of axons in early induced clones. A-D heat shocked after 24 hours, E-P heat shocked after 48 hours. D is a merge of A-C; H is a merge of E-G; L is a merge of I-K; and P is a merge of L-O. Genotype: hsp.FLP/+; UAS-fzr II.1/++; Act>Gal4 UAS-GFP/+. Staining: 24B10=RED, GFP=GREEN; DAPI=WHITE.
Fig 12. Additional data showing gain-of-function rap clones expressing GFP and FZR. The gain of function clones show axon clumping (shown with arrow), axonal bundles and shortening of axons in early induced clones. A-D heat shocked 24 hours after egg laying, F-T heat shocked 24 hours after egg laying at 37°C water bath for 15 minutes. E is a merge of A-D; J is a merge of F-I; O is a merge of K-N; and O is a merge of P-S. Genotype: hsp.FLP/+; UAS-fzr II.I/+; Act>Gal4 UAS-GFP/+. Staining: 24B10=RED, GFP=GREEN; REPO=BLUE; DAPI=WHITE.

C5. Loco dominantly interacts with rap to regulate axon targeting

Previous studies have shown that Rap, an ubiquitin ligase, targets Loco for ubiquitination and degradation (Kaplow, Korayem et al. 2008), thus we decided to investigate possible interactions of Rap and Loco in axonal targeting asking whether Loco dominantly interact with rap to regulate axon targeting. Loco was previously shown to affect the number of neurons and glia (Kaplow, Korayem et al. 2008); thus, it may play a role in axonal targeting.

To test for dominant interactions between Rap and Loco we constructed rap loss-of-function mutants with a single copy of the loco loss-of-function mutation. The double mutants were screened for the axonal projection patterns. The locoP452 allele is a homozygous lethal with rare third instar escapers that are characterized by locomotion problems and pupal lethality (Yu,
Wang et al. 2005). Third instar larvae escapers were stained for photoreceptor axons (Fig 13. B) and showed a diffuse lamina plexus with generally wild type axonal projections to the medulla. The cross to ro-tau-LacZ reported did not show mistrargeting or R2-R6 into the medulla thus Loco may act in a different pathway in the normal rap background (Fig. 8C).

To study a possible interaction of rap/fzr with the loco$^{P452}$ allele, a double mutant crosses were performed and third instar larvae were dissected and stained for the R cells in the eye imaginal disc. Surprisingly, loco which was previously shown to dominantly suppress rough eye phenotype in the adult Drosophila eye in genetic modifier screen (Kaplow, Mannava et al. 2007), enhanced the axon bundling and mistargeting of the R axons in the lamina, medulla and lamina plexus in the third instar larvae. The lamina plexus shows an increased number of gaps and axon bundling than the single mutant rap alleles (Fig 13).
Fig 13. Phenotypes of the rap mutants crossed to Loco^P452. The rap phenotype is enhanced by the single copy of Loco^P452. Axons show an increased bundling, more lamina plexus gaps and overall abnormal morphology of axonal projections. Shown axon projection patterns, stained with 24B10 in green. Arrows point the gaps in lamina plexus.
Double mutant *rap/loco* phenotype showed abnormal axon morphologies; thus, we decided to investigate whether *loco* itself has axonal length abnormalities. We measured retinal axon lengths, as described above, in *Loco*\(^{P452}\) allele and the significance of the results varied across the *rap* alleles and has a wide range of P values. Interestingly, the *Loco*\(^{P452}/Tb\) exhibited significant increase in the lengths of the R cells suggesting a possible misregulation. The standard deviation and standard error are plotted in the Graph 2 and summarized in Table 2-3 in the Appendix. Also, the P values and their significance are listed in Table 3. The axonal length in the *Loco*\(^{P452}/Tb\) stock showed a significant increase in the axonal projections and the average lengths of the axonal projections, their significance and standard error are summarized in Table 4 in the Appendix and Figure 14, (33.15%, P value=<0.0001, St. Dev.=5.855, n=8; 37.86%, P value=0.0385, St. Dev.=34.26, n=8; 40.83%, P value=0.0026, St. Dev.=53.24, n=8 for R1-R6 cells, R7-8 cells, lamina plexus lengths, respectively). These results suggest that *rap/fzr* plays a role in the axonal development, targeting and length while *loco* helps to coordinate the axonal length. It also suggests that Loco and Rap may be involved in another pathway that allows for a cross-talk since double mutant Rap/Loco do not show the same phenotype but an enhancement of the *rap* phenotype.

![Graph 2](image_url)

**Figure 14.** Histogram representing *Loco*\(^{P452}/Tb\) allele R cell photoreceptor measurements. The measurements shows the following 33.15%, P value=<0.0001, St. Dev.=5.855, n=8; 37.86%, P
value=0.0385, St. Dev.=34.26, n=8; 40.83%, P value=0.0026, St. Dev.=53.24, n=8 for R1-R6 cells, R7-8 cells, lamina plexus lengths, respectively.

C6. Rap/Fzr and Liprin-α interact in retinal axon targeting

Liprin-α has been recently shown to be involved in photoreceptor axonal targeting (Choe, Prakash et al. 2006; Hofmeyer, Maurel-Zaffran et al. 2006); thus, it remains unknown whether Rap/Fzr and Liprin-α interact in retinal axon targeting. Liprin-α was shown to play a significant role in the neuromuscular junction where it acts as a scaffolding molecule that binds RNA and lipid membranes via SAM motifs (Kaufmann, DeProto et al. 2002). To investigate whether Rap/Fzr interacts with Liprin-α in axon targeting in the third instar larvae, double mutants were generated. Previous work has shown that Liprin-α is a substrate for APC/Cdh1 and that it affects the axon target selection in R1-R6 photoreceptors (Choe, Prakash et al. 2006). Liprin-α mutants showed defective axon guidance and were shown to be required presynaptically to mediate adhesive events between pre- and postsynaptic cells (Choe, Prakash et al. 2006). Hofmeyer (2006) showed that Liprin-α overexpression can partially restore R7 targeting and its removal may worsen the targeting when LAR is absent.

To study a possible interaction of rap/fzr with the Liprin-α alleles, a double mutant crosses were performed and third instar larvae were dissected and stained for the R cells in the eye imaginal disc. The data showed axonal clumping, disorganized lamina and medulla with gaps in the lamina plexus. The preliminary data suggests that Liprin-α enhances the rap partial-loss-of function phenotype. Two Liprin-α stocks were mutated with a P-insertion in the open reading frame of Liprin-α located on 2L (27A1) for 8561 20478 stock and 22459. While stock 8563 was generated by an imprecise excision of the P{lacW} element, removing the Liprin-α coding region. Some of the Liprin-α 5’ UTR and some P-element sequences are still present (Kaufmann, DeProto et al. 2002).

The preliminary data shows an enhancement of the rap phenotype in the photoreceptor axon targeting. Axons appear clumped, with disorganized lamina plexus and gaps. The double mutant eye imaginal discs seem to add up their phenotypes and enhance the overall appearance (Fig 15 and Fig 16). Our results show that the partial loss-of-function rap phenotype is enhanced by a single copy of the mutant Liprin-α. This is consistent with a role for Rap/Fzr in the degradation of Liprin-α as previously suggested (Choe, Prakash et al. 2006). Thus, it is possible that Rap and Liprin-α may be involved in a cross-talk regulating axon targeting.
Figure 15. Liprin-α and rap double mutant crosses show axon projection patterns. Stained with 24B10 = GREEN. The rap phenotype is enhanced by a single copy of Liprin-α by causing additional number of gaps in the lamina plexus, increased axonal clumping, disorganized lamina plexus and medulla. Genotypes: A) +/Y; P{y[+t7.7] w[+mC]=wHy} Liprin-alpha[DG23609]/++; +/+ ; B) rap^{E2}/Y; P{y[+t7.7] w[+mC]=wHy} Liprin-alpha[DG23609]/++; +/+ ; C) rap^{E4}/Y; P{y[+t7.7] w[+mC]=wHy} Liprin-alpha[DG23609]/++; +/+ ; D) rap^{E6}/Y; P{y[+t7.7] w[+mC]=wHy} Liprin-alpha[DG23609]/++; +/+ ; E) rap^{x-2}/Y; P{y[+t7.7] w[+mC]=wHy} Liprin-alpha[DG23609]/++; +/+ ; F) rap^{x-3}/Y; P{y[+t7.7] w[+mC]=wHy} Liprin-alpha[DG23609]/++; +/+ ; G) w, rap^{x-3}/Y; P{y[+t7.7] w[+mC]=wHy} Liprin-alpha[DG23609]/++; +/+ ; H) +/Y; P{w[+mC] y[+mDint2]=EPgy2} Liprin-alpha[EY21217]/++; +/+ ; I) rap^{E2}/Y; P{w[+mC] y[+mDint2]=EPgy2} Liprin-alpha[EY21217]/++; +/+ ; J) rap^{E4}/Y; P{w[+mC] y[+mDint2]=EPgy2} Liprin-alpha[EY21217]/++; +/+ ; K) rap^{E6}/Y; P{w[+mC] y[+mDint2]=EPgy2} Liprin-alpha[EY21217]/++; +/+ ; L) rap^{x-3}/Y; P{w[+mC] y[+mDint2]=EPgy2} Liprin-alpha[EY21217]/++; +/+ ; M) w, rap^{x-3}/Y; P{w[+mC] y[+mDint2]=EPgy2} Liprin-alpha[EY21217]/++; +/+
Figure 16. Liprin-α and rap double mutant crosses show axon projection patterns. Stained with 24B10=GREEN. This image shows Liprin-α/Rap double mutant side-by-side single rap mutants. Genotypes: A) rapE2; B) rapE4; C) rapE6; D) rapx-3; E) rapE2/Y; Df(2L)Liprin-alpha[R60], Liprin-alpha[R60]/+; +/+ ; F) rapE4/Y; Df(2L)Liprin-alpha[R60], Liprin-alpha[R60]/+; +/+ ; G) rapE6/Y; Df(2L)Liprin-alpha[R60], Liprin-alpha[R60]/+; +/+ ; H) rapx-3/Y; Df(2L)Liprin-alpha[R60], Liprin-alpha[R60]/+; +/+  

C7. Rap/Fzr shows dominant interactions with Ras

Loco and Ras were shown to interact in mammals. Loco acts as scaffolding protein for Ras. Thus, it is unknown whether Rap and Ras interact. Ras protein plays critical roles in the regulation of cell growth and differentiation and a myriad of cancers have mutated Ras genes. Ras-binding domain (RBD) of Raf is homologous to Loco; thus, there may be a “cross-talk” between the Ras and Rap/Loco pathways (Ponting 1999). Thus, to investigate possible interaction of rap with Ras in the development of Drosophila adult eye, rap/RasV12 double
mutants were generated, crossing 22 lines of Ras\textsuperscript{V12} to all \textit{rap} alleles. We asked whether Rap/Fzr show dominant interactions with Ras. \textit{rap} loss-of-function allele mutants were crossed to Ras\textsuperscript{V12}, a constitutively active oncogene. Ras is a well studied oncogene that can be activated by single amino acid substitution (Tabin, Bradley et al. 1982). RasV12 is a glycine to valine mutation at residue 12 in the P-loop. This prevents GTPase domain to be inactivated by GAP and makes it constitutively active (Wu, Pastor-Pareja et al. 2010).

Our results show an enhancement of the rough eye phenotype that is allele specific and gene dosage sensitive. Stronger \textit{rap} loss-of-function alleles caused stronger effect with Ras\textsuperscript{V12} on the phenotype. Many lines of RasV12 crossed to \textit{w,rap}\textsuperscript{3}, caused an increased female lethality. Rap alleles show an allele specific phenotype suggesting a dosage dependent phenotype. This means that the more severe the \textit{rap} allele is the more effect it may have on the Ras/Rap interaction. In addition, females are homozygous for the \textit{rap} allele while males are hemizygous what may play an effect since the \textit{rap} females were used for the cross and may have an increased expression of the Rap protein and possible enhancement of the phenotype in the \textit{rap/Ras} double mutants.

Dissecting the pupal lethal larvae showed abnormal eye morphology which most likely caused the lethality. Stronger alleles such as \textit{rap}\textsuperscript{x-2}, \textit{rap}\textsuperscript{x-3}, and \textit{w, rap}\textsuperscript{3} caused additional abnormalities such as formation of holes in the adult eye, formation of black tumorigenic tissue deposits of the epithelial tissue, and often caused a decrease in the overall eye size. The holes in the eyes were located vertically in the anterior-posterior direction. Interestingly, the wild type, \textit{CS}, flies showed a similar to the double mutant phenotype thus it suggests that partial loss-of-function of \textit{rap} may provide a protection from the tumor formation when the heterozygous \textit{rap} mutations are less severe and cause less severe rough eye. The stocks evaluated carried the same P insertion element with RasV12 and all of the stocks evaluated showed the above described trend with slight variations on the severity of the phenotype observed. It was surprising to see that the hemizygous \textit{rap} double mutants were less severely affected than the heterozygous \textit{rap} females. It suggests that although together with Rap, RasV12, functions are partially suppressed by the weak alleles of the loss-of-function \textit{rap}. The data and the number of flies observed and analyzed are summarized in Tables 6-12. The adult eye phenotypes are shown below (Fig 17-23, 26-34). Fig 26. shows heat shock induced expression of P\{sevhs-Ras\textsuperscript{N17}\} for comparison, that shown a slight reduction is the eye size but with no tumorigenic phenotype as shown in RasV12. Similarly, Fig 24. shows only slight enhancement, using Ras85D mutation, in the rough eye phenotype in the stronger \textit{rap} alleles: \textit{rap}\textsuperscript{x-2}, \textit{rap}\textsuperscript{x-3}, and \textit{w, rap}\textsuperscript{3}.

\textbf{Ras data}
### Table 5.

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**Scoring**:
- Cs
  - rapE2
  - rapE4
  - rapx-2
  - rapx-3
  - w,rap3

**Control stocks**:

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**Cattering stocks**:

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Table 6. Compares the adult eye phenotype of the F1 from double mutant crosses of rap and RasV12
Table 7. Compares the adult eye phenotype of the F1 from double mutant crosses of rap and RasV12
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Table 8. Compares the adult eye phenotype of the F1 from double mutant crosses of rap and RasV12
Table 9. Compares the adult eye phenotype of the F1 from double mutant crosses of rap and RasV12

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**Table 10.** Compares the adult eye phenotype of the F1 from double mutant crosses of *rap* and RasV12
Table 11. Compares the adult eye phenotype of the F1 from double mutant crosses of rap and RasV12.
Table 12. Compares the adult eye phenotype of the F1 from double mutant crosses of rap and RasV12.
Figure 17. Shows the adult eye phenotypes of the F1 from the double mutant *rap* and RasV12 crosses
A) (520) +/+; Df(2R)E3363/CyO, P{sevRas1.V12}FK1; +/+; A') CS male, representative of the wild type; B) (520) +/Y; Df(2R)E3363/CyO, P{sevRas1.V12}FK1; +/+; C) Cs; D) Cs/+; CyO, P{sevRas1.V12}FK1/+; +/+; E) Cs/Y; CyO, P{sevRas1.V12}FK1/+; +/+; F) rapE2; G) rapE2/+; CyO, P{sevRas1.V12}FK1/+; +/+; H) rapE2/Y; CyO, P{sevRas1.V12}FK1/+; +/+; I) rapE4; J) rapE4/+; CyO, P{sevRas1.V12}FK1/+; +/+; K) rapE4/Y; CyO, P{sevRas1.V12}FK1/+; +/+; L) rapE6; M) rapE6/+; CyO, P{sevRas1.V12}FK1/+; +/+; N) rapE6/Y; CyO, P{sevRas1.V12}FK1/+; +/+; O) rapx-2; P) rapx-2/+; CyO, P{sevRas1.V12}FK1/+; +/+; Q) rapx-2/Y; CyO, P{sevRas1.V12}FK1/+; +/+; R) rapx-3; S) rapx-3/+; CyO, P{sevRas1.V12}FK1/+; +/+; T) rapx-3/Y; CyO, P{sevRas1.V12}FK1/+; +/+; U) w,rap3; W) w,rap3/+; CyO, P{sevRas1.V12}FK1/+; +/+; W) w,rap3/Y; CyO, P{sevRas1.V12}FK1/+; +/+
Figure 18. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses
A) (754)+/+; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; A’ ) CS male, representative of the wild type; B) (754)+/Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; C) Cs; D) Cs/+; CyO, P{sevRas1.V12}FK1/+; +/+; E) Cs/Y; CyO, P{sevRas1.V12}FK1/+; +/+; F) rapE2; G) rapE2/+; CyO, P{sevRas1.V12}FK1/+; +/+; H) rapE2/Y; CyO, P{sevRas1.V12}FK1/+; +/+; I) rapE4; J) rapE4/+; CyO, P{sevRas1.V12}FK1/+; +/+; K) rapE4/Y; CyO, P{sevRas1.V12}FK1/+; +/+; L) rapE6; M) rapE6/+; CyO, P{sevRas1.V12}FK1/+; +/+; N) rapE6/Y; CyO, P{sevRas1.V12}FK1/+; +/+; O) rapx-2; P) rapx-2/+; CyO, P{sevRas1.V12}FK1/+; +/+; Q) rapx-2/Y; CyO, P{sevRas1.V12}FK1/+; +/+; R) rapx-3; S) rapx-3/+; CyO, P{sevRas1.V12}FK1/+; +/+; T) rapx-3/Y; CyO,
Figure 19. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses
A) (2526) w[1118]; wdl[1]/CyO, P{ry+[t7.2]=sevRas1.V12}FK1 (5689), sev[14]; Ras85D[e1B]/TM3, Sb[1]

P{sevRas1.V12}FK1/++; U) w, rap3; W) w, rap3/+; CyO, P{sevRas1.V12}FK1/++; W) w, rap3/Y; CyO,
P{sevRas1.V12}FK1/++; +/+
Figure 20. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses
A) (5581) +/+; +/+; Df(3L)XS2182/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; A’) CS male, representative of the wild type; B)
(5581) +/-; +/+; Df(3L)XS2182/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D) +/-; +/+; TM3,
P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; E) Cs/Y; +/-; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; F) RapE2; G) rapE2/+; +/-;

(5582), Df(3L)XS543/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

No figure, little change
Figure 21. Shows the adult eye phenotypes of the F1 from the double mutant *rap* and RasV12 crosses
A) (5583) +/+; */+; Df(3L)XS572/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; A’) CS male, representative of the wild type; B) (5583) */Y; */+; Df(3L)XS572/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1], P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D) */+Cs; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; E) Cs/Y; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; F) RapE2; G) rapE2/+; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; H) rapE2/Y; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; I) rapE4; J) rapE4/+; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; K) rapE4/Y; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; L) rapE6; M) rapE6/+; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; N) rapE6/Y; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; O) rapx-2; P) rapx-2/+; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; Q) rapx-2/Y; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; R) rapx-3; S) rapx-3/+; */+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; T)
Figure 22. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses
A) (5584) +/-; +/+; Df(3L)XS705/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; A') CS male, representative of the wild type; B) (5584) +/-Y; +/-; Df(3L)XS705/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]; P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D) +/-Cs; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; E) Cs/Y; +/-; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; F) RapE2; G) rapE2/+; +/-; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; H) rapE2/Y; +/-; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; I) rapE4; J) rapE4/+; +/-; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; K) rapE4/Y; +/-; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; L) rapE6; M) rapE6/+; +/-; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; N) rapE6/Y; +/-; TM3,
Figure 23. Shows the adult eye phenotypes of the F1 from the double mutant *rap* and RasV12 crosses

A) (5683) +/+; +/+; ksr[S-627]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; A’) CS male, representative of the wild type; B) (5683) +/Y; +/+; ksr[S-627]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D) +/Cs; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; E) Cs/Y; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; F) RapE2; G) rapE2/+; +/+; (5683), ksr[S-627]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]
Figure 24. Shows the adult eye phenotypes of the F1 from the double mutant *rap* and Ras85D crosses.
A) (5689) +/+; +/+; sev[14]; Ras85D[e1B]/TM3, Sb[1]; A’) CS male, representative of the wild type; B) (5689) +/Y; +/+; sev[14]; Ras85D[e1B]/TM3, Sb[1]; C) Cs; D) w1118/Cs; sev[14]; Ras85D[e1B]/+; +/+; E) Cs/Y; sev[14]; Ras85D[e1B]/+; +/+; F) rapE2; G) rapE2/+; sev[14]; Ras85D[e1B]/+; +/+; H) rapE2/Y; sev[14]; Ras85D[e1B]/+; +/+; I) rapE4; J) rapE4/+; sev[14]; Ras85D[e1B]/+; +/+; K) rapE4/Y; sev[14]; Ras85D[e1B]/+; +/+; L) rapE6; M) rapE6/+; sev[14]; Ras85D[e1B]/+; +/+; N) rapE6/Y; sev[14]; Ras85D[e1B]/+; +/+; O) rapx-2; P) rapx-2/+; sev[14]; Ras85D[e1B]/+; +/+; Q) rapx-2/Y; sev[14]; Ras85D[e1B]/+; +/+; R) rapx-3; S) rapx-3/+; sev[14]; Ras85D[e1B]/+; +/+; T) rapx-3/Y; sev[14]; Ras85D[e1B]/+; +/+; U) w,rap3; V) w,rap3/+; sev[14]; Ras85D[e1B]/+; +/+; W) w,rap3/Y; sev[14]; Ras85D[e1B]/+; +/+;

(5777) HS P[w+[mW.hs]=sevhs-Ras1[N17]]; JA1
Figure 25. Shows the adult eye phenotypes of the F1 from the double mutant *rap* and hsRas crosses

A) +/+; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1; A') CS male, representative of the wild type; B) +/Y; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1; C) rapE2; D) rapE2/+; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; E) rapE2/Y; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; F) rapE4; G) rapE4/+; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; H) rapE4/Y; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; I) rapx-2; J) rapx-2/+; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; K) rapx-2/Y; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; L) rapx-3; M) rapx-3/+; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; N) rapx-3/Y; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; O) w, rap3; P) w, rap3/+; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+; Q) w, rap3/Y; +/+; P{w[+mW.hs]=sevhs-Ras1[N17]}JA1/+
Figure 26. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses

A) (6561) +/+; +/+; SR3-2[S-432]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]; A’) CS male, representative of the wild type; B) (6561) +/Y; +/+; SR3-2[S-432]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]; P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D) +/+Cs; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; E) Cs/Y; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; F) RapE2; G) rapE2/+; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; H) rapE2/Y; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; I) rapE4; J) rapE4/+; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; K) rapE4/Y; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; L) rapE6; M) rapE6/+; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; N) rapE6/Y; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; O) rapx-2; P) rapx-2/+; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; Q) rapx-2/Y; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; R) rapx-3; S) rapx-3/+; +/+; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/+; T)
Figure 27. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses
A)(6562) +/++; +/++; Su(Tpl)[S-192]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; A’) CS male, representative of the wild type; B) (6562) +/+; +/++; Su(Tpl)[S-192]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]; P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; C) Cs; D) +/Cs; +/++; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; E) Cs/Y; +/++; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; F) RapE2; G) rapE2/++; +/++; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; H) rapE2/Y; +/++; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; I) rapE4; J) rapE4/++; +/++; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; K) rapE4/Y; +/++; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; L) rapE6; M) rapE6/++; +/++; TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]/++; N) rapE6/Y; +/++; TM3,
Figure 28. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses

A) (6563) +/+; +/+ Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; A') CS male, representative of the wild type; B) (6563) +/+; +/+ Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D) +/Cs; +/+; TM3,

(6564), SR3-8[S-131]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]
Figure 29. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses

(6565), 14-3-3epsilon[S-696]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]
Figure 30. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses
Figure 31. Shows the adult eye phenotypes of the F1 from the double mutant *rap* and RasV12 crosses
A) (6573) +/++; +/++; ER3-2[XE-2551]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; A') CS male, representative of the wild type; B) (6573) +/Y; +/++; ER3-2[XE-2551]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D) +/Cs; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; E) Cs/Y; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; F) RapE2; G) rapE2/++; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; H) rapE2/Y; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; I) rapE4; J) rapE4/++; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; K) rapE4/Y; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; L) rapE6; M) rapE6/++; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; N) rapE6/Y; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; O) rapx-2; P) rapx-2/++; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; Q) rapx-2/Y; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; R) rapx-3; S) rapx-3/++; +/++; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/++; T)
Figure 32. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses

A) (6574) +/+; +/+; ER3-3[XE-571]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; A’) CS male, representative of the wild type; B) (6574) +/+; +/+; ER3-3[XE-571]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; C) Cs; D) +/Cs; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; E) Cs/Y; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; F) RapE2; G) rapE2/+; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; H) rapE2/Y; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; I) rapE4; J) rapE4/+; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]/+; K) rapE4/Y; +/+; TM3, P{ry[+t7.2]=sevRas1.V12}FK2,
Figure 33. Shows the adult eye phenotypes of the F1 from the double mutant *rap* and RasV12 crosses
A) (6575) +/+; +/+; ER3-4[XE-2178]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; A') CS male, representative of the wild type; B) (6575) +/Y; +/+; ER3-4[XE-2178]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]; C) Cs; D)

(6576), ER3-5[XE-2335]/TM3, P{ry[t7.2]=sevRas1.V12}FK2, Sb[1]

Figure 34. Shows the adult eye phenotypes of the F1 from the double mutant rap and RasV12 crosses
This study examined the mitotic disregulation level in the rap third instar eye discs.

It was previously shown that rap causes additional cell-cycle divisions in the brain lobes (Kaplow, Korayem et al. 2008). Also, the rap mutants show an abnormal phospho-histone3 (Ser10) staining with cell division not restricted to the MF and differentiated regions of the eye disc. These results serve as a control for the future examination of the third instar eye imaginal disc in the double rap and RasV12 mutants to see if the RasV12 causes additional cell-cycle deregulation or suppresses the phenotype (Fig 35.).

![Figure 35](image)

**Figure 35.** Shows rap allele phospho-histone 3 (Ser10) staining with deregulated mitotic cell division. Genotypes: A-D) Cs/Y; E-H) rap^{X-2}/Y; I-L) rap^{X-3}/Y; M-P) w,rap^{3}/Y

**D. CONCLUSION**

The work presented in this thesis was conducted to test the hypothesis that Rap/Fzr, a Drosophila homolog of the mammalian Cdh1 is important for correct axon targeting. The results show that rap plays a role in axon length determination. rap alleles showed abnormal axonal projections with clumping or photoreceptor axons, causing abnormal lamina, lamina plexus, and medulla morphology. rap alleles showed allele specific variations in the abnormal phenotype suggesting that Rap operates in a gene dosage dependent manner. Measurements of the
photoreceptor axons confirmed overgrowth as previously shown in mammalian data (Konishi, Stegmuller et al. 2004). The results indicate that Rap/Fzr plays a role in the determination of axonal growth and inhibits axonal growth; thus, partial loss of function shows an increased length of the photoreceptor axons. The data utilizing ro-tau-lacZ reporter suggests that in rap partial loss-of-function alleles cause developmental axonal changes, possibly changing the photoreceptor outgrowth or affecting proper R cell axon targeting since some R2-R5 crossed the lamina plexus and terminated in the medulla, a normal target only for R7 and R8.

Loss-of-function mosaic studies utilizing FLP/FRT system suggest that Rap/Fzr functions cell autonomously in retinal axon targeting. Our preliminary data show that the regions of the rap-/rap-clones show aberrant axon projections. Clumping of axons and various gaps in the lamina plexus were observed in the clone patches but the general morphology of the surrounding cells is normal. These data suggest that Rap/Fzr function is cell autonomous but additional experiments need to be performed for more definitive conclusions. One possible way would be to use MARCM, mosaic analysis with a repressible marker, that allows visualization of a single cell clone in the heterozygous wild type background (Lee and Luo 2001).

In addition gain-of-function experiments utilizing GAL4/UAS and FLP/FRT systems showed that Rap/Fzr modulates proper axon targeting and length. Gain-of-function of rap/fzr in the eye imaginal disc causes axonal clumping, disorganization of lamina plexus with many gaps. Lamina plexus shows clusters of R cells fluctuating from the proper orientation in a step-wise fashion. It suggests that the overexpression of Rap causes an increased number of neurons that cannot fit onto the plane of the lamina plexus and cause the disorganization of that region. Although, the photoreceptor axons were not measured the length of the R cells is significantly decreased. Further measurements are necessary to assess the significance and severity of the decrease. These data suggest that Rap/Fzr is necessary for proper axonal targeting and proper development of the Drosophila visual system.

To test for additional Rap interactions we generated double mutants with Loco, Liprin-α, and Ras mutations. Previous studies have shown that Rap, an ubiquitin ligase, targets Loco for ubiquitination and degradation (Kaplow, Korayem et al. 2008), thus we decided to investigate possible interactions of Rap and Loco in axonal targeting asking whether Loco dominantly interact with rap to regulate axon targeting. Loco was previously shown to affect the number of neurons and glia (Kaplow, Korayem et al. 2008); thus, it may play a role in axonal targeting. Surprisingly, loco which was previously shown to dominantly suppress rough eye phenotype in the adult Drosophila eye in genetic modifier screen (Kaplow, Mannava et al. 2007), enhanced the axon bundling and mistargeting of the R axons in the lamina, medulla and lamina plexus in the third instar larvae. The lamina plexus shows an increased number of gaps and axon bundling than the single mutant rap alleles. Statistical analysis of Loco showed significant increase in the lengths of the R cells suggesting a possible misregulation. These results suggest that rap/fzr plays a role in the axonal development, targeting and length while loco helps to coordinate the axonal length. It also suggests that Loco and Rap may be involved in another pathway that allows for a cross-talk since double mutant Rap/Loco do not show the same phenotype but an enhancement of the rap phenotype.
In addition, Liprin-α has been recently shown to be involved in photoreceptor axonal targeting (Choe, Prakash et al. 2006; Hofmeyer, Maurel-Zaffran et al. 2006); thus, it remains unknown whether Rap/Fzr and Liprin-α interact in retinal axon targeting. To investigate whether Rap/Fzr interacts with Liprin-α in axon targeting in the third instar larvae, double mutants were generated. The data from rap/fzr/Liprin-α double mutant crosses showed axonal clumping, disorganized lamina and medulla with gaps in the lamina plexus. The preliminary data suggests that Liprin-α enhances the rap partial-loss-of function phenotype. Our results show that the partial loss-of-function rap phenotype is enhanced by a single copy of the mutant Liprin-α. This is consistent with a role for Rap/Fzr in the degradation of Liprin-α as previously suggested (Choe, Prakash et al. 2006). Thus, it is possible that Rap and Liprin-α may be involved in a cross-talk regulating axon targeting.

Also, Loco and Ras were shown to interact in mammals. Loco acts as scaffolding protein for Ras. Thus, it is unknown whether Rap and Ras interact. Ras protein plays critical roles in the regulation of cell growth and differentiation and a myriad of cancers have mutated Ras genes. Ras-binding domain (RBD) of Raf is homologous to Loco; thus, there may be a “cross-talk” between the Ras and Rap/Loco pathways (Ponting 1999). Thus, to investigate possible interaction of rap with Ras in the development of Drosophila adult eye, rap/RasV12 double mutants were generated, crossing 22 lines of RasV12 to all rap alleles. We asked whether Rap/Fzr show dominant interactions with Ras. rap loss-of-function allele mutants were crossed to RasV12, a constitutively active oncogene. Ras is a well studied oncogene that can be activated by single amino acid substitution (Tabin, Bradley et al. 1982). RasV12 is a glycine to valine mutation at residue 12 in the P-loop. This prevents GTPase domain to be inactivated by GAP and makes it constitutively active (Wu, Pastor-Pareja et al. 2010).

Our results show an enhancement of the rough eye phenotype that is allele specific and gene dosage sensitive. Stronger rap loss-of-function alleles caused stronger effect with RasV12 on the phenotype with some female pupa lethality. Rap alleles show an allele specific phenotype suggesting a dosage dependent phenotype. This means that the more severe the rap allele is the more effect it may have on the Ras/Rap interaction. The data suggest that although together with Rap, RasV12, functions are partially suppressed by the weak alleles of the loss-of-function rap.

Additional data is needed on the protein expression levels in rap and double mutants to check for the allele specific phenotypes. Also, identification of the mutations is rap is necessary to see whether the regulatory regions of rap are affected or whether it is a structural change in the Rap protein that causes the phenotype. In addition, future studies should investigate the axonal lengths of the double mutant crosses with rap and possible look for additional Rap targets. Axonal elongation may play a role in treating axonal regeneration thus additional experiments that would test the rap effect in the injury may shed a light on the axonal regeneration properties. The mammalian data has shown that Cdh1 mutants were able to extend axons in the presence of myelin, an inhibitor of axonal regeneration and lengthening.

E. MATERIALS AND METHODS

Fly stocks and culture

The stocks were maintained at 25°C on standard corn meal-agar medium.
**Rap stocks used:** *rap* mutants alleles were generated by (Pimentel and Venkatesh 2005) using EMS and X-ray mutagenesis: *rap*<sup>x-3</sup>, *rap*<sup>x-2</sup>, w,<sup>rap</sup>, *rap*<sup>E2</sup>, *rap*<sup>E4</sup>, *rap*<sup>E6</sup>

For R2-R6 axonal pattern we used ro-tau lacZ (gift from Iris Salecker).

Overexpression studies were performed with the following stocks: Repo-Gal4/Tb, (733) UAS-fzr II.1.

For mosaic analysis we used FRT19, Ubi-GFP, eyFLP1 (gift from Jessica Treisman), (111943) w<sup>67c23</sup>P{lacW}<sup>rapG0418</sup>P{neoFRT}19A/FM7c; P{ey-FLP.DN}5 (Kyoto Institute of Technology), hsp.FLP; Act>CD2>Gal4 UAS-GFP/TM6B.

**Stocks used for balancing:**

w; If/CyO; MKRS/Tb; w; Tm3,Sb/Tm6,Tb; yw; lwr<sup>3-4(G)</sup>/CyO-Act-GPF (gift from Shuba Govind)

**Bloomington Drosophila Stock Center Stock list**

**Ras stocks used:**

(520), Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1

(754), Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1

(2526), w[1118]; wdl[1]/CyO, P{ry[+t7.2]=sevRas1.V12}FK1 (5689), sev[14]; Ras85D[e1B]/TM3, Sb[1] (5777), P{w+[mW.hs]=sevhs-Ras1[N17]}JA1

(5581), Df(3L)XS2182/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

(5582), Df(3L)XS543/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

(5583), Df(3L)XS572/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

(5584), Df(3L)XS705/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

(5683), ksr[S-627]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

(5689), sev[14]; Ras85D[e1B]/TM3, Sb[1]

(6561), SR3-2[S-432]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

(6562), Su(Tpl)[S-192]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

(6564), SR3-8[S-131]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
(6565), 14-3-epsilon[S-696]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
(6573), ER3-2[XE-2551]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
(6574), ER3-3[XE-571]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
(6575), ER3-4[XE-2178]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
(6576), ER3-5[XE-2335]/TM3, P{ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
(8340), P{w+[mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]
(16011), y[1] w[1118]; PBac{w+[mC]=5HPw+}Ras85D[A014]/TM3, Sb[1] Ser[1]

**Liprin stocks used:**

(8561), w[*]; Df(2L)Liprin-alpha[R60], Liprin-alpha[R60]/CyO, P{w+[mC]=ActGFP}JMR1
(8563), Liprin-alpha[F3ex15]/In(2LR)Gla, wg[Gla-1] Bc[1]
(20478), y[1] w[67c23]; P{y+[t7.7] w+[mC]=wHy}Liprin-alpha[DG23609]/SM6a
(22459), y[1] w[67c23]; P{w+[mC] y+[mDint2]=EPgy2}Liprin-alpha[EY21217]/CyO

**Loco-stocks used:**

Loco^{6452}/Tb; (10009), P{ry[+]=lacZ-un1}loco[rC56]; (14421), y[1]; ry[506] P{y+[mDint2] w[BR.E.BR]=SUPor-P}loco[KG02176]/TM3, Sb[1] Ser[1]; (19915), y[1] w[67c23]; P{w+[mC] y+[mDint2]=EPgy2}loco[EY04589]; (25055), w[1118]; Df(3R)BSC527/TM6C, Sb[1]; (26883), w[*]; P{w+[mC]=EP}loco[GE24954]

<table>
<thead>
<tr>
<th>Rap allele</th>
<th>Phenotype</th>
<th>Classification</th>
<th>Mutagen</th>
</tr>
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<tr>
<td>rap^{x-3}</td>
<td>Rough eye in males</td>
<td>Partial loss of function</td>
<td>x-rays</td>
</tr>
<tr>
<td>rap^{x-2}</td>
<td>Rough eye in males</td>
<td>Partial loss of function</td>
<td>x-rays</td>
</tr>
<tr>
<td>w;rap^{3}</td>
<td>Rough eye in males</td>
<td>Partial loss of function</td>
<td>p-element</td>
</tr>
<tr>
<td>rap^{E2}</td>
<td>Rough eye in males</td>
<td>Partial loss of function</td>
<td>EMS</td>
</tr>
<tr>
<td>rap^{E4}</td>
<td>Rough eye in males</td>
<td>Partial loss of function</td>
<td>EMS</td>
</tr>
<tr>
<td>rap$^{E6}$</td>
<td>Rough eye in males</td>
<td>Partial loss of function</td>
<td>EMS</td>
</tr>
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</tbody>
</table>

The detailed schematics of the all the crosses used are listed in the Appendix at the end of the paper.

**Immunohistochemistry**

Third instar larval eye discs and brains were dissected in PBS and stained with the primary antibodies and counterstained with a secondary antibody. 4% paraformaldehyde solution was used to fix the tissues with pH of 7.4. Primary antibodies were incubated for 2 hours at room temperature or overnight at 4°C. Primary antibodies used: Mab24B10 (Zipursky, Venkatesh, and Benzer, 1985) were used to visualize photoreceptor axons (1:5) supernatant obtained from Developmental Studies Hybridoma Bank; Mab24B10 specifically stains photoreceptor cells in the retina and their axonal projections to the optic ganglion (Zipursky, Venkatesh et al. 1985); Anti-phospho-histone3 (Ser10); Phosphorylation on Ser 10 of histone H3 is a mitotic marker; anti-rabbit Mitosis Marker from Millipore (1:100); ELAV-9F8A9-S anti-mouse (1:5) from Developmental Studies Hybridoma Bank; anti-β-galactosidase polyclonal rabbit (1:500) from Bioscience, rabbit anti-GFP (1:500) polyclonal rabbit (1:500) from Chemicon; rabbit anti-Repo polyclonal (1:500). The tissues were washed in 1XPBS three times for 20 minutes and incubated in the secondary antibody for 90 minutes at room temperature. Secondary antibodies used: Fluorescein (FITC)-conjugated AffinityPure goat Anti-Mouse IgG (H+L) (1:100), Rhodamine (TRITC)-conjugated affinity pure goat anti-mouse (1:100), Rhodamine (TRITC)-conjugated affinity pure goat anti-rabbit (1:100), Cy5-conjugated affinity pure F(ab’)2 goat anti-rabbit (1:100), goat-anti rabbit FITC IgG (H+L) (1:100) Jackson-ImmunoResearch Laboratories. The tissues were washed three times with 1XPBT and mounted using Vectashield Mounting Medium, Vector Laboratories. The fluorescent samples were visualized using confocal microscope LSM 510 (Zeiss). LSM Image Browser was used to capture, filter and visualize z-stacks of the preparations. All images were visualized at 40x magnification 0.7 to 1.5 zoom and z-stack projections planes were compiled using LSM Image Browser. Images were edited with Adobe Photoshop CS3 and CS5.

**Mosaic analysis**

A mosaic analysis was performed by heat shocking first instar larvae for 15 or 30 min at 37°C water bath 24 or 48 hours after egg lying for gain of function experiments. Ras (5777) crosses to rap flies was performed by heat shocking second instar larvae 48-72 hours after egg lying at 37°C for 20 minutes. Then the immunohistochemistry experiments were performed as described above.

Adult eye images were generated in the following way: flies were collected 3-7 days after eclosion and placed into eppendorf tubes and stored in 70% ethanol at 4°C. The flies were placed on a glass slide and the adult head was visualized with Nikon Digita DS F1 digital camera using Nikon SM1000 dissecting microscope. The images were captured using NIC-Elements F 3.0. Images were edited with Adobe Photoshop CS3 and CS5.
Statistical analysis

Analyses were performed with Prism 5 software (GraphPad Software). All statistical tests, significance was set to p<0.05. The two-tailed unpaired Student’s t-test and column statistics were used to analyze photoreceptor axon lengths. Sample size (n) represents individual animals.
F. REFERENCES:


## G, APPENDIX

<table>
<thead>
<tr>
<th>rap alleles</th>
<th>Cs</th>
<th>rap(^{E_2})</th>
<th>rap(^{E_4})</th>
<th>rap(^{E_6})</th>
<th>rap(^{x-2})</th>
<th>rap(^{x-3})</th>
<th>w.rap(^3)</th>
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<td><strong>Optic stalk length</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Average (µm)</td>
<td>19.537</td>
<td>26.02</td>
<td>16.298</td>
<td>18.082</td>
<td>15.906</td>
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<td>75.08-133.106</td>
<td>60.238-79.68</td>
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<td><strong>Length of the lamina plexus</strong></td>
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<td>Average (µm)</td>
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<td>170.023</td>
<td>204.561</td>
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<td>Range (µm)</td>
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<td>143.4-282.98</td>
<td>162.11-185.3</td>
<td>137.37-278.96</td>
<td>133.65-296.06</td>
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**Table 1.** rap allele R cell photoreceptor measurements

### Statistics:

**Column statistics using Prism; optic stalk length:**

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<tr>
<th>Optic stalk</th>
<th>Cs</th>
<th>rap(^{E_2})</th>
<th>rap(^{E_4})</th>
<th>rap(^{E_6})</th>
<th>rap(^{x-2})</th>
<th>rap(^{x-3})</th>
<th>w.rap(^3)</th>
<th>lo xo(^{P452}/Tb)</th>
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<tr>
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<tr>
<td><strong>Std. Error</strong></td>
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<td>1.642</td>
<td>2.975</td>
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<tr>
<td><strong>1-6 R cells</strong></td>
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<tr>
<td></td>
<td>Std. Deviation</td>
<td>Std. Error</td>
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<td>7-8 R cells</td>
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<td>53.24</td>
<td>18.82</td>
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Table 2. Shows the standard deviations and standard error for the measurements of the *rap* and *loco* axonal projections

T test results for optic stalk using Prism

<table>
<thead>
<tr>
<th>Cs vs.</th>
<th>rap&lt;sup&gt;E2&lt;/sup&gt;</th>
<th>rap&lt;sup&gt;E4&lt;/sup&gt;</th>
<th>rap&lt;sup&gt;E6&lt;/sup&gt;</th>
<th>rap&lt;sup&gt;x-2&lt;/sup&gt;</th>
<th>rap&lt;sup&gt;x-3&lt;/sup&gt;</th>
<th>w,rap&lt;sup&gt;1&lt;/sup&gt;</th>
<th>loco&lt;sup&gt;P452/Tb&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Optic stalk</td>
<td></td>
<td></td>
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<tr>
<td>P value</td>
<td>0.1983</td>
<td>0.4468</td>
<td>0.3870</td>
<td>0.3335</td>
<td>0.3454</td>
<td>0.9357</td>
<td>0.2441</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<td>No</td>
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<tr>
<td>1-6 R cells</td>
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<td>P value</td>
<td>0.0032</td>
<td>0.4661</td>
<td>&lt; 0.0001</td>
<td>0.2318</td>
<td>&lt; 0.0001</td>
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<td>Significant</td>
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<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
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<td>7-8 R cells</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Lamina</td>
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Table 3. Shows the t-test results with P values and significance of the measurements for the *rap* and *loco* axonal projections

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Cs</th>
<th>Loco&lt;sup&gt;Ps2&lt;/sup&gt;/Tb</th>
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</thead>
<tbody>
<tr>
<td><strong>Optic stalk length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average (µm)</td>
<td>19.537</td>
<td>26.02</td>
</tr>
<tr>
<td>Range (µm)</td>
<td>12.58-43.42</td>
<td>18.38-34.91</td>
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<tr>
<td><strong>1-6 receptor length</strong></td>
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<tr>
<td>Average (µm)</td>
<td>81.351</td>
<td>108.32025</td>
</tr>
<tr>
<td>Range (µm)</td>
<td>55.674-112.758</td>
<td>84.194-183.274</td>
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<tr>
<td><strong>7-8 receptor lengths</strong></td>
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<tr>
<td>Average (µm)</td>
<td>80.051</td>
<td>110.365</td>
</tr>
<tr>
<td>Range (µm)</td>
<td>39.552-111.932</td>
<td>73.078-168.786</td>
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<tr>
<td><strong>Length of the lamina plexus</strong></td>
<td></td>
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<tr>
<td>Average (µm)</td>
<td>184.923</td>
<td>260.44</td>
</tr>
<tr>
<td>Range (µm)</td>
<td>119.55-237.25</td>
<td>212.47-256.63</td>
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</tbody>
</table>

Table 4. Loco<sup>Ps2</sup>/Tb allele R cell photoreceptor measurements

**Genetic Crosses made:**

1. *rap* alleles and ro-tau lacZ

   rap<sup>E2</sup>/rap<sup>E2</sup>; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ  
   rap<sup>E4</sup>/rap<sup>E4</sup>; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ  
   rap<sup>E6</sup>/rap<sup>E6</sup>; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ  
   rap<sup>x-2</sup>/rap<sup>x-2</sup>; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ  
   rap<sup>x-3</sup>/rap<sup>x-3</sup>; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ  
   w,rap<sup>3</sup>/w,rap<sup>3</sup>; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ  
   Cs/Cs; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ  
   w<sup>1118</sup>/w<sup>1118</sup>; +/-; +/- x w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ
+/+; +/+; Loco\textsuperscript{P452}/Tbx w/Y; Bl/CyO; ro-tau lacZ/ro-tau lacZ

2. Gain of function \textit{rap}

+/+; UAS-fzrII.I/UAS-fzrII.I; +/- x hs.FLP/Y; +/+; Act>CD2>GAL4 UAS-GFP/TM6b
+/+; UAS-fzrII.I/UAS-fzrII.I; +/- x +/-; +/+; Repo-GAL4/Tb

3. \textit{rap} alleles and Loco\textsuperscript{P452}

rap\textsuperscript{E2}/rap\textsuperscript{E2}; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
rap\textsuperscript{E4}/rap\textsuperscript{E4}; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
rap\textsuperscript{E6}/rap\textsuperscript{E6}; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
rap\textsuperscript{x2}/rap\textsuperscript{x2}; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
rap\textsuperscript{x3}/rap\textsuperscript{x3}; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
w,rap\textsuperscript{3}/w,rap\textsuperscript{3}; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
Cs/Cs; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
w\textsuperscript{1118}/w\textsuperscript{1118}; +/-; +/- x +/-; +/+; Loco\textsuperscript{P452}/Tb
+/-; +/-; Loco\textsuperscript{P452}/Tb x +/-; +/-; Loco\textsuperscript{P452}/Tb

4. Loss of function \textit{rap} alleles

(111943) w\textsuperscript{[67c23]} P\{w\textsuperscript{[+mC]}=lacW\}rap\textsuperscript{[G0418]} P\{neoFRT\}19A/FM7c; P\{ey-FLP.D\}5/ P\{ey-FLP.D\}5; +/- x FRT19, Ubi-GFP, eyFLP1/Y; +/-; +/+ 

(111943) w\textsuperscript{[67c23]} P\{w\textsuperscript{[+mC]}=lacW\}rap\textsuperscript{[G0418]} P\{neoFRT\}19A/FM7c; P\{ey-FLP.D\}5/ P\{ey-FLP.D\}5; +/- x FRT19, UbiGFP/Y; +/-; hs.FLP/TM6B

5. \textit{rap} alleles crosses to \textit{Liprin-alpha} alleles

a) Crosses or \textit{rap} alleles to 8561

rap\textsuperscript{E2}/rap\textsuperscript{E2}; +/-; +/- x w\textsuperscript{*}/Y; Df(2L)Liprin-alpha[R60], Liprin-alpha[R60]/CyO,
P\{w\textsuperscript{+[mC]}=ActGFP\}JMR1; +/-

rap\textsuperscript{E4}/rap\textsuperscript{E4}; +/-; +/- x w\textsuperscript{*}/Y; Df(2L)Liprin-alpha[R60], Liprin-alpha[R60]/CyO,
P\{w\textsuperscript{+[mC]}=ActGFP\}JMR1; +/-
c) Balancing 20478 stock for third instar marker

yw/Y; lwr^{3-4(b2)}/CyO-Act-GFP; ++ x (20478) y[1] w[67c23]; P{y[t7.7] w[mC]=wHy} Liprin-alpha[DG23609]/SM6a; ++

d) Cross with balanced third instar marker on 20478 stock

rap^{E2}/rap^{E2}; ++; ++ x yw/Y; P{y[t7.7] w[mC]=wHy} Liprin-alpha[DG23609]/CyO-Act-GFP; ++
rapE4/rapE4; +/-; +/- x yw/Y; P {y[t7.7] w[+mC]=wHy} Liprin-alpha [DG23609]/CyO-Act-GFP; +/-

rapE6/rapE6; +/-; +/- x yw/Y; P {y[t7.7] w[+mC]=wHy} Liprin-alpha [DG23609]/CyO-Act-GFP; +/-

rap x-2/rap x-2; +/-; +/- x yw/Y; P {y[t7.7] w[+mC]=wHy} Liprin-alpha [DG23609]/CyO-Act-GFP; +/-

rap x-3/rap x-3; +/-; +/- x yw/Y; P {y[t7.7] w[+mC]=wHy} Liprin-alpha [DG23609]/CyO-Act-GFP; +/-

w.rap3/w.rap3; +/-; +/- x yw/Y; P {y[t7.7] w[+mC]=wHy} Liprin-alpha [DG23609]/CyO-Act-GFP; +/-

Cs/Cs; +/-; +/- x yw/Y; P {y[t7.7] w[+mC]=wHy} Liprin-alpha [DG23609]/CyO-Act-GFP; +/-

w1118/w1118; +/-; +/- x yw/Y; P {y[t7.7] w[+mC]=wHy} Liprin-alpha [DG23609]/CyO-Act-GFP; +/-

e) Balancing 22459 stock for third instar marker

yw/Y; lwr3-4(2)/CyO-Act-GFP; +/- x (22459) y[1] w[67c23]/y[1] w[67c23]; P {w[+mC] y[+mDint2]=EPgy2} Liprin-alpha [EY21217]/CyO; +/-

f) Cross with balanced third instar marker on 22459 stock

rapE2/rapE2; +/-; +/- x yw/Y; P {w[+mC] y[+mDint2]=EPgy2} Liprin-alpha [EY21217]/CyO-Act-GFP; +/-

rapE4/rapE4; +/-; +/- x yw/Y; P {w[+mC] y[+mDint2]=EPgy2} Liprin-alpha [EY21217]/CyO-Act-GFP; +/-

rapE6/rapE6; +/-; +/- x yw/Y; P {w[+mC] y[+mDint2]=EPgy2} Liprin-alpha [EY21217]/CyO-Act-GFP; +/-

rap x-2/rap x-2; +/-; +/- x yw/Y; P {w[+mC] y[+mDint2]=EPgy2} Liprin-alpha [EY21217]/CyO-Act-GFP; +/-

rap x-3/rap x-3; +/-; +/- x yw/Y; P {w[+mC] y[+mDint2]=EPgy2} Liprin-alpha [EY21217]/CyO-Act-GFP; +/-

w.rap3/w.rap3; +/-; +/- x yw/Y; P {w[+mC] y[+mDint2]=EPgy2} Liprin-alpha [EY21217]/CyO-Act-GFP; +/-
Cs/Cs; +/+; +/- x yw/Y; P{w[+mC] y[+mDint2]=EPgy2}Liprin-alpha[EY21217]/CyO-Act-GFP; +/- 

w^{1118}/w^{1118}; +/-; +/- x yw/Y; P{w[+mC] y[+mDint2]=EPgy2}Liprin-alpha[EY21217]/CyO-Act-GFP; +/-

6. **rap** alleles crosses to **Ras** alleles for adult eye phenotype

a) Crosses or **rap** alleles to 520

rap^{E2}/rap^{E2}; +/-; +/- x +/-Y; Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{E4}/rap^{E4}; +/-; +/- x +/-Y; Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{E6}/rap^{E6}; +/-; +/- x +/-Y; Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{x2}/rap^{x2}; +/-; +/- x +/-Y; Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{x3}/rap^{x3}; +/-; +/- x +/-Y; Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

w,rap^{3}/w,rap^{3}; +/-; +/- x +/-Y; Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

Cs/Cs; +/-; +/- x +/-Y; Df(2R)E3363/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

b) Crosses or **rap** alleles to 754

rap^{E2}/rap^{E2}; +/-; +/- x +/-Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{E4}/rap^{E4}; +/-; +/- x +/-Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{E6}/rap^{E6}; +/-; +/- x +/-Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{x2}/rap^{x2}; +/-; +/- x +/-Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{x3}/rap^{x3}; +/-; +/- x +/-Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

w,rap^{3}/w,rap^{3}; +/-; +/- x +/-Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

Cs/Cs; +/-; +/- x +/-Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

c) Crosses or **rap** alleles to 2526

rap^{E2}/rap^{E2}; +/-; +/- x w[1118]/Y; wdl[1]/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{E4}/rap^{E4}; +/-; +/- x w[1118]/Y; wdl[1]/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/- 

rap^{E6}/rap^{E6}; +/-; +/- x w[1118]/Y; wdl[1]/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/-
rap\textsuperscript{x-2}/rap\textsuperscript{x-2}; +/+; +/+ x w[1118]/Y; wdl[1]/CyO, P\{ry[+t7.2]=sevRas1.V12\}FK1; +/+ 
rap\textsuperscript{x-3}/rap\textsuperscript{x-3}; +/+; +/+ x w[1118]/Y; wdl[1]/CyO, P\{ry[+t7.2]=sevRas1.V12\}FK1; +/+ 
w,rap\textsuperscript{3}/w,rap\textsuperscript{3}; +/+; +/+ x w[1118]/Y; wdl[1]/CyO, P\{ry[+t7.2]=sevRas1.V12\}FK1; +/+ 
Cs/Cs; +/+; +/+ x w[1118]/Y; wdl[1]/CyO, P\{ry[+t7.2]=sevRas1.V12\}FK1; +/+ 

d) Crosses or rap alleles to 5581 
rap\textsuperscript{E2}/rap\textsuperscript{E2}; +/+; +/+ x +/Y; +/+; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{E4}/rap\textsuperscript{E4}; +/+; +/+ x +/Y; +/+; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{E6}/rap\textsuperscript{E6}; +/+; +/+ x +/Y; +/+; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{x-2}/rap\textsuperscript{x-2}; +/+; +/+ x +/Y; +/+; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{x-3}/rap\textsuperscript{x-3}; +/+; +/+ x +/Y; +/+; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
w,rap\textsuperscript{3}/w,rap\textsuperscript{3}; +/+; +/+ x +/Y; +/+; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
Cs/Cs; +/+; +/+ x +/Y; +/+; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 

e) Crosses or rap alleles to 5582 
rap\textsuperscript{E2}/rap\textsuperscript{E2}; +/+; +/+ x +/Y; +/+; Df(3L)XS543/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{E4}/rap\textsuperscript{E4}; +/+; +/+ x +/Y; +/+; Df(3L)XS543/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{E6}/rap\textsuperscript{E6}; +/+; +/+ x +/Y; +/+; Df(3L)XS543/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{x-2}/rap\textsuperscript{x-2}; +/+; +/+ x +/Y; +/+; Df(3L)XS543/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{x-3}/rap\textsuperscript{x-3}; +/+; +/+ x +/Y; +/+; Df(3L)XS543/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
w,rap\textsuperscript{3}/w,rap\textsuperscript{3}; +/+; +/+ x +/Y; +/+; Df(3L)XS543/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
Cs/Cs; +/+; +/+ x +/Y; +/+; Df(3L)XS543/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 

f) Crosses or rap alleles to 5583 
rap\textsuperscript{E2}/rap\textsuperscript{E2}; +/+; +/+ x +/Y; +/+; Df(3L)XS572/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{E4}/rap\textsuperscript{E4}; +/+; +/+ x +/Y; +/+; Df(3L)XS572/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1] 
rap\textsuperscript{E6}/rap\textsuperscript{E6}; +/+; +/+ x +/Y; +/+; Df(3L)XS572/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]
rap x-2/rap x-2; +/+; +/- x +/Y; +/-; Df(3L)XS572/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
rap x-3/rap x-3; +/-; +/- x +/Y; +/-; Df(3L)XS572/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
w, rap3/rap3; +/-; +/- x +/Y; +/-; Df(3L)XS572/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
Cs/Cs; +/-; +/- x +/Y; +/-; Df(3L)XS572/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]

g) Crosses or rap alleles to 5584

rap E2/rap E2; +/-; +/- x +/-; +/-; Df(3L)XS705/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
rap E4/rap E4; +/-; +/- x +/-; +/-; Df(3L)XS705/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
rap E6/rap E6; +/-; +/- x +/-; +/-; Df(3L)XS705/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]

w, rap3/rap3; +/-; +/- x +/-; +/-; Df(3L)XS705/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]

Cs/Cs; +/-; +/- x +/-; +/-; Df(3L)XS705/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]

i) Crosses or rap alleles to 5683

rap E2/rap E2; +/-; +/- x +/-; +/-; ksr[S-627]/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
rap E4/rap E4; +/-; +/- x +/-; +/-; ksr[S-627]/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]
rap E6/rap E6; +/-; +/- x +/-; +/-; ksr[S-627]/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]

w, rap3/rap3; +/-; +/- x +/-; +/-; ksr[S-627]/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]

Cs/Cs; +/-; +/- x +/-; +/-; ksr[S-627]/TM3, P {ry+[t7.2]=sevRas1.V12}FK2, Sb[1]

j) Crosses or rap alleles to 5689

rap E2/rap E2; +/-; +/- x +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]
rap E4/rap E4; +/-; +/- x +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]
rap E6/rap E6; +/-; +/- x +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]
rap^{x-2}\text{/rap}^{x-2}; +/+; +/- x +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]
rap^{x-3}\text{/rap}^{x-3}; +/-; +/- x +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]
w.rap^{3}\text{/w.rap}^{3}; +/-; +/- x +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]
Cs/Cs; +/-; +/- x +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]

k) Crosses or rap alleles to 5777

rap^{E2}\text{/rap}^{E2}; +/-; +/- x +/-; +/-; P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1 / P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1

rap^{E4}\text{/rap}^{E4}; +/-; +/- x +/-; +/-; P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1 / P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1

rap^{E6}\text{/rap}^{E6}; +/-; +/- x +/-; +/-; P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1 / P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1

rap^{x-2}\text{/rap}^{x-2}; +/-; +/- x +/-; +/-; P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1 / P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1

rap^{x-3}\text{/rap}^{x-3}; +/-; +/- x +/-; +/-; P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1 / P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1

w.rap^{3}\text{/w.rap}^{3}; +/-; +/- x +/-; +/-; P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1 / P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1

Cs/Cs; +/-; +/- x +/-; +/-; P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1 / P\{w[+mW hs]=sevhs-Ras1[N17]\}JA1

l) Crosses or rap alleles to 6561

rap^{E2}\text{/rap}^{E2}; +/-; +/- x +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap^{E4}\text{/rap}^{E4}; +/-; +/- x +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap^{E6}\text{/rap}^{E6}; +/-; +/- x +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap^{x-2}\text{/rap}^{x-2}; +/-; +/- x +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap^{x-3}\text{/rap}^{x-3}; +/-; +/- x +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

w.rap^{3}\text{/w.rap}^{3}; +/-; +/- x +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

Cs/Cs; +/-; +/- x +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]
m) Crosses or rap alleles to 6562

rap\textsuperscript{E2}/rap\textsuperscript{E2}; +/+; +++ +/+; +++; Su(Tpl)[S-192]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{E4}/rap\textsuperscript{E4}; +++; +++ x +/+; +++; Su(Tpl)[S-192]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{E6}/rap\textsuperscript{E6}; +++; +++ x +/+; +++; Su(Tpl)[S-192]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{x2}/rap\textsuperscript{x2}; +++; +++ x +/+; +++; Su(Tpl)[S-192]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{x3}/rap\textsuperscript{x3}; +++; +++ x +/+; +++; Su(Tpl)[S-192]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

w,rap\textsuperscript{3}/w,rap\textsuperscript{3}; +++; +++ x +/+; +++; Su(Tpl)[S-192]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

Cs/Cs; +++; +++ x +/+; +++; Su(Tpl)[S-192]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

n) Crosses or rap alleles to 6563

rap\textsuperscript{E2}/rap\textsuperscript{E2}; +++; +++ x +/+; +++; Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{E4}/rap\textsuperscript{E4}; +++; +++ x +/+; +++; Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{E6}/rap\textsuperscript{E6}; +++; +++ x +/+; +++; Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{x2}/rap\textsuperscript{x2}; +++; +++ x +/+; +++; Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{x3}/rap\textsuperscript{x3}; +++; +++ x +/+; +++; Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

w,rap\textsuperscript{3}/w,rap\textsuperscript{3}; +++; +++ x +/+; +++; Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

Cs/Cs; +++; +++ x +/+; +++; Df(3L)XS-5R, AP-2[XS-5R] SR3-7[XS-5R]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

o) Crosses or rap alleles to 6564

rap\textsuperscript{E2}/rap\textsuperscript{E2}; +++; +++ x +/+; +++; SR3-8[S-131]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{E4}/rap\textsuperscript{E4}; +++; +++ x +/+; +++; SR3-8[S-131]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

rap\textsuperscript{E6}/rap\textsuperscript{E6}; +++; +++ x +/+; +++; SR3-8[S-131]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]
p) Crosses or rap alleles to 6565

rap\(^{x-2}\)/rap\(^{x-2}\); +/+; +/+ x +/Y; +/+; SR3-8[S-131]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
rap\(^{x-3}\)/rap\(^{x-3}\); +/+; +/+ x +/Y; +/+; SR3-8[S-131]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
w.rap\(^{3}\)/w.rap\(^{3}\); +/+; +/+ x +/Y; +/+; SR3-8[S-131]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
w,rap\(^{3}\)/w,rap\(^{3}\); +/+; +/+ x +/Y; +/+; SR3-8[S-131]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
w,rap\(^{3}\)/w,rap\(^{3}\); +/+; +/+ x +/Y; +/+; SR3-8[S-131]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]

q) Crosses or rap alleles to 6573

rap\(^{x-2}\)/rap\(^{x-2}\); +/+; +/+ x +/Y; +/+; 14-3-3epsilon[S-696]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
rap\(^{x-3}\)/rap\(^{x-3}\); +/+; +/+ x +/Y; +/+; 14-3-3epsilon[S-696]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
w.rap\(^{3}\)/w.rap\(^{3}\); +/+; +/+ x +/Y; +/+; 14-3-3epsilon[S-696]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
w,rap\(^{3}\)/w,rap\(^{3}\); +/+; +/+ x +/Y; +/+; 14-3-3epsilon[S-696]/TM3, P\{ry\(+t7.2\)=sevRas1.V12\}FK2, Sb[1]
r) Crosses or \textit{rap} alleles to 6574

\begin{itemize}
  \item \textit{rap}^E2/\textit{rap}^E2; +/+; +/+ x +/Y; +/+; \text{ER3-3[XE-571]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^E4/\textit{rap}^E4; +/+; +/+ x +/Y; +/+; \text{ER3-3[XE-571]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^E6/\textit{rap}^E6; +/+; +/+ x +/Y; +/+; \text{ER3-3[XE-571]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^x-2/\textit{rap}^x-2; +/+; +/+ x +/Y; +/+; \text{ER3-3[XE-571]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^x-3/\textit{rap}^x-3; +/+; +/+ x +/Y; +/+; \text{ER3-3[XE-571]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{w,rap}^3/\textit{w,rap}^3; +/+; +/+ x +/Y; +/+; \text{ER3-3[XE-571]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{Cs/Cs}; +/+; +/+ x +/Y; +/+; \text{ER3-3[XE-571]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
\end{itemize}

s) Crosses or \textit{rap} alleles to 6575

\begin{itemize}
  \item \textit{rap}^E2/\textit{rap}^E2; +/+; +/+ x +/Y; +/+; \text{ER3-4[XE-2178]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^E4/\textit{rap}^E4; +/+; +/+ x +/Y; +/+; \text{ER3-4[XE-2178]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^E6/\textit{rap}^E6; +/+; +/+ x +/Y; +/+; \text{ER3-4[XE-2178]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^x-2/\textit{rap}^x-2; +/+; +/+ x +/Y; +/+; \text{ER3-4[XE-2178]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^x-3/\textit{rap}^x-3; +/+; +/+ x +/Y; +/+; \text{ER3-4[XE-2178]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{w,rap}^3/\textit{w,rap}^3; +/+; +/+ x +/Y; +/+; \text{ER3-4[XE-2178]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{Cs/Cs}; +/+; +/+ x +/Y; +/+; \text{ER3-4[XE-2178]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
\end{itemize}

t) Crosses or \textit{rap} alleles to 6576

\begin{itemize}
  \item \textit{rap}^E2/\textit{rap}^E2; +/+; +/+ x +/Y; +/+; \text{ER3-5[XE-2335]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^E4/\textit{rap}^E4; +/+; +/+ x +/Y; +/+; \text{ER3-5[XE-2335]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^E6/\textit{rap}^E6; +/+; +/+ x +/Y; +/+; \text{ER3-5[XE-2335]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^x-2/\textit{rap}^x-2; +/+; +/+ x +/Y; +/+; \text{ER3-5[XE-2335]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{rap}^x-3/\textit{rap}^x-3; +/+; +/+ x +/Y; +/+; \text{ER3-5[XE-2335]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
  \item \textit{w,rap}^3/\textit{w,rap}^3; +/+; +/+ x +/Y; +/+; \text{ER3-5[XE-2335]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]}
\end{itemize}
Cs/Cs; +/+; +/+ x +/Y; +/+; ER3-5[XE-2335]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

u) Crosses or rap alleles to 8340

rap^{E2}/rap^{E2}; +/+; +/+ x P{w[+mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]/Y; +/+; +/+  
rap^{E4}/rap^{E4}; +/+; +/+ x P{w[+mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]/Y; +/+; +/+  
rap^{E6}/rap^{E6}; +/+; +/+ x P{w[+mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]/Y; +/+; +/+  
rap^{x-2}/rap^{x-2}; +/+; +/+ x P{w[+mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]/Y; +/+; +/+  
rap^{x-3}/rap^{x-3}; +/+; +/+ x P{w[+mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]/Y; +/+; +/+  
w.rap^{3}/w.rap^{3}; +/+; +/+ x P{w[+mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]/Y; +/+; +/+  
Cs/Cs; +/+; +/+ x P{w[+mC]=vgMQ-Ras85D.V12}1, y[1] w[1118]/Y; +/+; +/+  

v) Crosses or rap alleles to 16011

rap^{E2}/rap^{E2}; +/+; +/+ x y[1] w[1118]/Y; +/+; PBac{w[+mC]=5HPw[+]Ras85D[A014]}/TM3, Sb[1] Ser[1]  
rap^{E4}/rap^{E4}; +/+; +/+ x y[1] w[1118]/Y; +/+; PBac{w[+mC]=5HPw[+]Ras85D[A014]}/TM3, Sb[1] Ser[1]  
rap^{E6}/rap^{E6}; +/+; +/+ x y[1] w[1118]/Y; +/+; PBac{w[+mC]=5HPw[+]Ras85D[A014]}/TM3, Sb[1] Ser[1]  
rap^{x-2}/rap^{x-2}; +/+; +/+ x y[1] w[1118]/Y; +/+; PBac{w[+mC]=5HPw[+]Ras85D[A014]}/TM3, Sb[1] Ser[1]  
rap^{x-3}/rap^{x-3}; +/+; +/+ x y[1] w[1118]/Y; +/+; PBac{w[+mC]=5HPw[+]Ras85D[A014]}/TM3, Sb[1] Ser[1]  
w.rap^{3}/w.rap^{3}; +/+; +/+ x y[1] w[1118]/Y; +/+; PBac{w[+mC]=5HPw[+]Ras85D[A014]}/TM3, Sb[1] Ser[1]  
Cs/Cs; +/+; +/+ x y[1] w[1118]/Y; +/+; PBac{w[+mC]=5HPw[+]Ras85D[A014]}/TM3, Sb[1] Ser[1]  

w) Crosses or rap alleles to 16839

rap\textsuperscript{E4}/rap\textsuperscript{E4}; +/-; +/- x y[1] w[67c23]/Y; +/-; P{w[+mC] y[+mDint2]=EPgy2}\text{Ras85D[EY07538]/TM3, Sb[1] Ser[1]}

rap\textsuperscript{E6}/rap\textsuperscript{E6}; +/-; +/- x y[1] w[67c23]/Y; +/-; P{w[+mC] y[+mDint2]=EPgy2}\text{Ras85D[EY07538]/TM3, Sb[1] Ser[1]}

rap\textsuperscript{x-2}/rap\textsuperscript{x-2}; +/-; +/- x y[1] w[67c23]/Y; +/-; P{w[+mC] y[+mDint2]=EPgy2}\text{Ras85D[EY07538]/TM3, Sb[1] Ser[1]}

rap\textsuperscript{x-3}/rap\textsuperscript{x-3}; +/-; +/- x y[1] w[67c23]/Y; +/-; P{w[+mC] y[+mDint2]=EPgy2}\text{Ras85D[EY07538]/TM3, Sb[1] Ser[1]}

w,rap\textsuperscript{3}/w,rap\textsuperscript{3}; +/-; +/- x y[1] w[67c23]/Y; +/-; P{w[+mC] y[+mDint2]=EPgy2}\text{Ras85D[EY07538]/TM3, Sb[1] Ser[1]}

Cs/Cs; +/-; +/- x y[1] w[67c23]/Y; +/-; P{w[+mC] y[+mDint2]=EPgy2}\text{Ras85D[EY07538]/TM3, Sb[1] Ser[1]}

7.\textit{rap} alleles crosses to \textit{Loco} alleles for adult eye phenotype

\textbf{a) Crosses or \textit{Ras} alleles to \textit{Loco}\textsuperscript{P452}}

+/-; +/-; \textit{Loco}\textsuperscript{P452}/Tb x (754) +/-; Y; Df(2R)vg-C/CyO, P\{ry[+t7.2]=sevRas1.V12\}FK1; +/-

+/-; +/-; \textit{Loco}\textsuperscript{P452}/Tb x (2526) w[1118]/Y; wdl[1]/CyO, P\{ry[+t7.2]=sevRas1.V12\}FK1; +/-

+/-; +/-; \textit{Loco}\textsuperscript{P452}/Tb x (5581) +/-; +/-; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

+/-; +/-; \textit{Loco}\textsuperscript{P452}/Tb x (5584) +/-; +/-; Df(3L)XS705/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

+/-; +/-; \textit{Loco}\textsuperscript{P452}/Tb x (5683) +/-; +/-; ksr[S-627]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

+/-; +/-; \textit{Loco}\textsuperscript{P452}/Tb x (5689) +/-; +/-; sev[14]; Ras85D[e1B]/TM3, Sb[1]

+/-; +/-; \textit{Loco}\textsuperscript{P452}/Tb x (6561) +/-; +/-; SR3-2[S-432]/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]

\textbf{b) Crosses or \textit{Ras} alleles to 10009}

+/-; +/-; P\{ry[+\#]=lacZ-un1\} loco[rC56]/P\{ry[+\#]=lacZ-un1\} loco[rC56] x (5581) +/-; Y; +/-; Df(3L)XS2182/TM3, P\{ry[+t7.2]=sevRas1.V12\}FK2, Sb[1]
c) Crosses or Ras alleles to 19915


c) Crosses or Ras alleles to 26883

w[*]/w[*]; +/++; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (754) +/Y; Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1; +/+ 

w[*]/w[*]; +/++; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (5581) +/Y; +/++; Df(3L)XS2182/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

w[*]/w[*]; +/++; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (5584) +/Y; +/++; Df(3L)XS705/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

w[*]/w[*]; +/++; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (5683) +/Y; +/++; ker[S-627]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

w[*]/w[*]; +/++; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (6561) +/Y; +/++; SR3-2[S-432]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]
w[*]/w[*]; +/+; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (6562) +/Y; +/+; Su(Tpl)[S-192]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]


w[*]/w[*]; +/+; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (6573) +/Y; +/+; ER3-2[XE-2551]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

w[*]/w[*]; +/+; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (6574) +/Y; +/+; ER3-3[XE-571]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]

w[*]/w[*]; +/+; P{w[+mC]=EP}loco[GE24954]/P{w[+mC]=EP}loco[GE24954] x (6575) +/Y; +/+; ER3-4[XE-2178]/TM3, P{ry[+t7.2]=sevRas1.V12}FK2, Sb[1]