THE ROLE OF UBIQUITIN MEDIATED PROTEOLYSIS IN DROSOPHILA GLIA DEVELOPMENT

by

MARGARITA KAPLOW

A dissertation to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctoral of Philosophy, The City University of New York

2009

©2009

MARGARITA KAPLOW

All Rights Reserved

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

___2/01/09_____ Date

_____Dr.TadmiriVenkatesh
Chair of Examining Committee

____2/01/09_____ Date

_____Dr. Laurel Eckhart
Executive Officer

Dr. Marie Filbin

Dr. Zaven Kaprelian

Dr. Mark Pezzano

Dr. Chris Li
Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

THE ROLE OF UBIQUITIN MEDIATED PROTEOLYSIS IN DROSOPHILA GLIA DEVELOPMENT

By

Margarita Kaplow

Adviser: Professor Tadmiri Venkatesh

Biological processes are dynamic, requiring both simple and complex mechanisms that enable cells to adapt with the ever-changing environment. Ubiquitination is one of many posttranslational modifications that result in a change in cellular activity. Mono-ubiquitination, the addition of a single ubiquitin moeity leads to endocytosis and membrane trafficking, while the addition of a multiple ubiquitin chains primarily results in protein degradation. Rap/Fzr acts as an activator of the E3 ubiquitin ligase, Anaphase Promoting Complex (APC) which has been studied for its role in the timely degradation of cell cycle regulators. My thesis work focuses on novel roles Rap/Fzr during nervous system development and specifically investigates its role during glia development. My results show that Rap/Fzr regulates glia development through its interaction with

Loco, an RGS protein and Nonstop, a ubiquitin specific protease. Bioinformatic analysis revealed that both Loco and Nonstop contain Destruction box (D-box) motifs and KEN box motifs, which are amino acid sequences used by Rap/Fzr for substrate recognition. My thesis work shows that Rap/Fzr targets Loco for ubiquitination, and subsequent degradation and thus, inhibits the formation of glia from dividing neuroblasts. Furthermore, Rap/Fzr together with Nonstop, regulates the migration and the endoreplication of glia cells.

ACKNOWLEDGEMENTS

Foremost, I am grateful to my mentor Dr. Tadmiri Venkatesh who has inspired me to continue pursuing my interest in basic science research. Dr. Venkatesh has supported me in many aspects of scientific growth. Through his guidance, I have improved both my communication and my critical thinking skills.

I would like to thank my husband, Daniel Stasik, who has been patient with me throughout the course of my research. I also thank my parents, David and Emerita Kaplow and my sister, Patrcia Humphries who have given me confidence to pursue a Ph.D.

I would also like to extend my appreciation to the CCNY community, including past and present members of the Venkatesh lab, who have been supportive of my research. Additionally, I am grateful to my committee members for their guidance.

My Ph.D would not have been possible without financial support from the NIH- MBRS RISE program and NYC Louis Stokes Alliance for Minority Participation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1.

Chapter 2.

OVERVIEW

Signal processing from neural networks is accomplished by the diverse repertoire of neuronal and glia subtypes. From purkinje cells of the cerebellum, to the pyramidal cells of the hippocampus, the classification of neurons based on morphology has been well documented. Although the cytology of and glia has been described as early as 1928 by Ramon y Cajal, the functional roles of glia within the nervous system have only begun to be elucidated. To date, there are four type of glia categorized in the mammalian nervous system. Glia within the central nervous system include astrocytes and oligodendrocytes, while Schwann cells lie within the peripheral nervous system. Both oligodendrocytes and Schwann cells ensheathe and myelinate axons terminals, while astrocytes support and maintain the blood brain barrier (Kandel et al., 2000). Similar to vertebrates, the nervous system of *Drosophila melanogaster* consists of different glia subtypes that are also characterized according to function and morphology (Pereanu et al., 2005). For example, subperineurial and perineurial glia located on the exterior surface of the brain are similar to astroctyes, and maintain the blood brain barrier (Bainton et al., 2005; Stork et al., 2008). Furthermore, the genetic tools available in Drosophila, has made it an

excellent model system for identifying molecular pathways involved during glia differentiation and migration.

In Drosophila, glia differentation occurs through a binary switch wherein activation of glia specific genes and simulateneous repression of neurogenic genes leads to glia cell fate of a given neuroblast. The gene *glia cell missing* (*gcm)*, promotes glia cell formation at the expense of neuron differentiation in all Drosophila glia except for the midline glia (Bernardoni et al., 1999; Hosoya et al., 1995). GCM initiates the glia differentiation pathway through the activation of transcription factors, *repo* and *pointed*, which consequently regulates the transcription of *loco,* a gene that encodes for an Regulator of G-protein Signaling (RGS) protein (Granderath et al., 1999; Jones, 2005). Although the functional partners of *loco* such as the gene *moody*, a gene that encodes for G-protein couples receptors (GPCRs) are established, downstream targets of Loco have yet to be identified (Bainton et al., 2005; Schwabe et al., 2005). Furthermore, regulation of glia cell fate specification beyond the level of transcription activation requires additional investigation. Post-translational modifications such as ubiquitination are essential in regulating protein function within multiple developmental programs (Hegde and DiAntonio, 2002; Joazeiro and Weissman, 2000; Pickart, 2004; Stegmuller and Bonni, 2005).

Ubiquitination, a reversible mechanism, ensures timely degradation of cellular proteins and facilitates diverse biological processes that require precise control of constant protein turnover. The sequential addition of ubiquitin to proteins destined for destruction is highly regulated and involves three protein complexes, including E1 activating, E2 conjugating, and E3 ligase enzymes (Pickart, 2004). Recognition of specific substrates targeted for degradation occurs during the final stages of the ubiquitin pathway, and is accomplished by the diversity of E3 ligases (Hershko and Ciechanover, 1998; Pickart, 2001). The Anaphase Promoting Complex, (APC) is an example of an E3 ligase that precisely targets mitotic regulators such as cyclin A, cyclin B, Geminin and Securin for degradation. However, within the last five years, specific subunits of the APC have been implicated for ubiquitinating proteins unrelated to cell cycle function (Konishi et al., 2004; Stegmuller et al., 2006; van Roessel et al., 2004). A regulatory subunit of the APC, Rap/Fzr and its mammalian homologue, Cdh1 regulates nervous system development by targeting the transcription regulator SnoN. In turn, SnoN functions to enhance axonal growth (Stegmuller et al., 2006). The APC is also required for the proper localization of proteins necessary for the asymmetric division of neuroblasts (Slack, 2007; Slack et al., 2006). Our study presents an additional role for the APC during nervous system

development. Our results show that Rap/Fzr regulates two aspects of glia development. Rap/Fzr ubiquitinates the RGS protein Loco preventing the differentiation of glia cells and therefore, promotes neuronal formation of neuroblasts (Kaplow et al., 2008). Furthermore, Rap/Fzr targets the ubiquitin specific protease Nonstop and regulates the endoreplication of glia cells.

CHAPTER 1

A genetic modifier screen identifies multiple genes that interact with Drosophila Rap/Fzr and suggests novel cellular roles

Margarita E. Kaplow[Ψ], Laura J. Mannava[Ψ], Angel C. Pimentel[¥], Hector A. Fermin[Ψ], Vanetta J. Hyatt[Φ], John J. Lee[Ψ] and Tadmiri R. Venkatesh[1Ψ]

[Ψ]Department of Biology, City College and The Graduate Center, City University of New York, 138th Street and Convent Avenue, New York, NY 10031. [¥]Department of Cellular and Molecular Biology, University of Arizona, Tucson, AZ 85721. [Φ] Department of Internal Medicine, Weill Medical College at Cornell University, New York, NY 10021

[1]Author for correspondence: Tadmiri R. Venkatesh

Department of Biology

City College of New York

New York, NY 10031.

Tel: 212-650-8469

Fax: 212-650-8585

Email: venky@sci.ccny.cuny.edu

Running Title: Genetic interactions of Rap/Fzr

J Neurogenet. 2007 Jul-Sep;21(3):105-51.

ABSTRACT

In the developing Drosophila eye, Rap/Fzr plays a critical role in neural patterning by regulating the timely exit of precursor cells. Rap/Fzr (Retina aberrant in pattern/Fizzy related) is an activator of the E3 Ubiquitin ligase, the APC (Anaphase promoting complex-cyclosome) that facilitates the stage specific proteolytic destruction of mitotic regulators, such as cyclins and cyclin dependent kinases. To identify novel functional roles of Rap/Fzr, we conducted an F_1 genetic modifier screen to identify genes which interact with the partial-loss-function mutations in *rap/fzr*. We screened 2741 single P-element, lethal insertion lines and *piggyBac* lines on the second and third chromosome for dominant enhancers and suppressors of the rough eye phenotype of *rap/fzr*. From this screen, we have identified 40 genes which exhibit dosage sensitive interactions with *rap/ fzr* and of these 31 have previously characterized cellular functions. Seven of the modifiers identified in this study are regulators of cell cycle progression with previously known interactions with Rap/Fzr. Among the remaining modifiers, 27 encode proteins involved in other cellular functions not directly related to cell-cycle progression. These fall into at least three groups based on their previously known cellular functions: transcriptional

regulation , regulated proteolysis, and signal transduction. These results suggest that in addition to cell cycle regulation, Rap/Fzr regulates ubiquitin ligase mediated protein degradation in the developing nervous system as well as in other tissues.

Keywords: Anaphase Promoting Complex, cell-cycle, ubiquitination, E3-ligase enzyme, retina patterning, mitotic exit

INTRODUCTION

Ubiquitination of cellular proteins followed by proteosome degradation is an important regulatory process implicated in a multitude of biological processes including cell cycle regulation, axon guidance and synaptogeneis, tumor metastasis (Hegde and DiAntonio, 2002; Kaelin and Maher, 1998; Schaeffer et al., 2004). The ubiquitination pathway involves a series of sequential enzymatic steps whereby ubiquitin, a 76 amino acid polypeptide, is added as a tag to substrates destined for degradation. The sequential addition of ubiquitin involves E1 activating, E2 conjugating, and E3 ligase enzyme; ubiquitinated proteins are subsequently degraded by the 26S proteosome (Pickart, 2004). At least four different classes of E3 ligases, APC, SCF, HECT and Ring/U-box, have been reported (Ou et al., 2003;

Vierstra, 2003). These differ in organization and structure but all function as multi-subunit protein complexes that facilitate the ubiquitination of protein substrates. Within these protein complexes are other polypeptide subunits whose functions are regulatory in nature (Vierstra, 2003). Of the four classes of E3 ligases, the Anaphase Promoting Complex (APC) is a multi-subunit ubiquitin ligase complex that has been characterized primarily for its regulatory role during cell cycle progression. APC facilitates the ubiquitination and timely degradation of key mitotic regulators, such as cyclins, cyclin dependent kinases and securin. Targeting of protein substrates to the APC complex for ubiquitination is dependent on two proteins with a WD (Trp-Asp) repeat domain, Fizzy/Cdc20 and Rap/Fzr (Kashevsky et al., 2002). *Drosophila fizzy (fzy)* was originally discovered in a screen for embryonic lethal genes on the second chromosome (Nusslein-Volhard, 1984) and was later shown to be required for cell cycle progression (Dawson et al., 1993; Sigrist et al., 1995). In a screen to isolate genes similar to *fzy*, Sigrist and Lehner (1997) used a Xenopus *fzy* cDNA as a probe and cloned a homologous gene from Drosophila and named it *fzr* (*fizzy-related*). Subsequently, a previously characterized Drosophila gene, *rap (retina aberrant in pattern*) was shown to encode the Fizzy related protein (Karpilow et al., 1989b; Pimentel and Venkatesh, 2005a).

Rap/Fzr (Retina aberrant in Pattern/Fizzy related) is a regulatory subunit of the APC and is the Drosophila homolog of the mammalian and yeast Cdh1. Rap/Fzr is required during G1 phase for mitotic arrest and mediates the targeting of mitotic regulators such as Cyclin A and Cyclin B to the APC for degradation (Araki et al., 2003; Pimentel and Venkatesh, 2005a; Pimentel and Venkatesh, 2005b; Schaeffer et al., 2004; Sigrist and Lehner, 1997b). The role of Rap/Fzr in mitosis has been demonstrated in a variety of developing tissues, such as the embryo, wing imaginal disc, eye-antennal disc and developing follicle cells (Araki et al., 2003; Schaeffer et al., 2004; Sigrist and Lehner, 1997a). In the developing Drosophila eye, partial loss-of-function mutations in *rap/fzr* result in the unscheduled accumulation of Cyclin B, leading to additional rounds of cell cycles and causing a disorganized pattern of the photoreceptors in the developing eye. The aberrant arrangement of photoreceptors manifests as a rough eye phenotype in the adult compound eye (Pimentel and Venkatesh, 2005b).

The ubiquitin ligase, Anaphase promoting complex (APC) requires either Cdc20/Fzy or Rap/Fzr/Cdh1 for the ubiquitination of protein targets. According to the current model, a set of tandem WD repeats from the WD domain fold into a propeller shape and each propeller blade serves as a platform for binding different cell cycle regulatory proteins (van der Voorn

and Ploegh, 1992). Protein substrates targeted for ubiquitination by the APC complex contain the characteristic N-terminal destruction box (D-box) motif with the consensus amino acid sequence RXXL. While APC/Fzy only binds proteins with D-box sequences, APC/Fzr, uses two additional recognition signals for substrate targeting. The KEN box sequence was first discovered in Fzy/Cdc20, which is targeted by APC/Fzr for degradation but lacks D-box sequences (Listovsky et al., 2004; Littlepage and Ruderman, 2002; Peters, 2002; Zur and Brandeis, 2002). The second recognition signal is the A-box, which is found in Aurora-A kinase, and is comprised of the short sequence QRILGPSNVPQRV with a serine 53 residue that is phosphorylated during mitosis (Littlepage and Ruderman, 2002). Although primarily studied for its role as a regulator of cell cycle, recent studies have implicated the APC in novel cellular functions. In the neuromuscular junction, the APC has been shown to regulate synaptic size and function (van Roessel et al., 2004). The APC has also been shown to be involved in TGFß signaling (Nourry et al., 2004). Similarly, mutations in *cdh1,* the mammalian homolog of *rap/fzr,* have been shown to cause increased axon growth and defasciculation (Konishi et al., 2004), suggesting its involvement in cellular functions unrelated to cell cycle regulation.

In the present study, we undertook a genome-wide modifier screen to

identify genes which interact with Rap/Fzr with the goal of characterizing novel cellular roles for Rap/Fzr. Our data presented here show that, in addition to seven genes encoding cell cycle regulators, *rap/fzr* exhibits dosage sensitive interactions with 33 other genes. These interacting genes encode proteins involved in ubiquitin mediated proteolysis, signal transducers, transcription factors, and several genes of unknown function. Our observations suggest that Rap/Fzr participates in a multitude of cellular functions in addition to cell cycle regulation, consistent with a more widespread cellular role for the ubiquitin-proteosome pathway.

MATERIALS AND METHODS

Drosophila Stocks and Genetics: Fly stocks were reared and crosses were done at 25° C. Isolation and characterization of of *rap/fzr* alleles has been previously described (Jacobs et al., 2002; Karpilow et al., 1989b; Karpilow et al., 1996). Three *rap/fzr* mutants were used for the genetic screen, based on the rough eye phenotype, a strong hypormophic allele, $rap^{x\text{-}3}$ and two weaker hypomorphic alleles, rap^{E6} and rap^{E4} (Jacobs et al., 2002; Karpilow et al., 1996). The stocks used in this study: P-element insertion lethal lines, piggyback insertion lines and EMS induced mutant alleles were all obtained from the Indiana University, Bloomington Stock Center. The entire collection of P-element insertion lethal lines, 2741 lines, at the time we

began the screen, was were used for the initial screening of 2nd and 3rd chromosomes. 279 *piggyBac* stocks available from Bloomington Stock Center were also used in the genetic screen. *piggyBacs* have been recently described as a more efficient method for transposon mutagenesis in Drosophila (Thibault et al., 2004). Two to four day old males carrying autosomal P-lethal or *piggyBac* insertions over balancers were mated to homozygous rap^{E4}/rap^{E4}, rap^{E6}/rap^{E6} and rap^{x-3}/rap^{x-3} virgin females. Non-balancer F1 male progeny, hemizygous for *rap/fzr* and heterozygous for the P-element lethal or *piggyBac* lines were screened for dominant effects on the rough eye phenotype under a Wild dissecting microscope. The rough eye phenotype was classified as an enhancers if the eyes appeared rougher compared to eyes of *rap/fzr* male flies. Suppressors were identified when eyes of F1 males were smoother compared to *rap/fzr* male flies. Initial genetic screen involved at least three investigators who scored the phenotype independently. Initial results were verified in a secondary screen when multiple mutant alleles of modifier were crossed to multiple *rap/fzr* alleles. Each potential suppressor or enhancer was independently scored independently by at least three investigators to minimize the subjective nature of the screen.

Immunohistochemistry: Whole mount preparations of third instar larval eye disks were stained with the appropriate antibodies and counterstained with fluorescent secondary antibodies as described previously (Pimentel and Venkatesh, 2005a; Pimentel and Venkatesh, 2005b) . Anti-phospho-Histone-H3 antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and anti-Cyclin B was purchased from Developmental Studies Hybridoma Bank, University of Iowa. Slides were visualized using a Zeiss Axiocam epi-fluorescence microscope.

Scanning Electron Microscopy: Adult compound eyes were processed for scanning electron microscopy according to the procedure described previously (Karpilow et al., 1989b).

RESULTS

The compound eyes of flies carrying partial loss-of-function (hypomorphic) mutations in the *rap/fzr* gene exhibit a rough external surface as opposed to the smooth eyes of wild type flies. In our modifier screen, we used weak hypomorphic *rap/fzr* alleles,rap^{E4} and rap^{E6}*(Jacobs et al., 2002)* and Korayem *et. al* manuscript in preparation) and looked for either suppression or enhancement of the rough eye phenotype. Our rationale was that the weak rough eye phenotype would provide a sensitized system

for the F_1 modifier screen to identify genes that interact with *rap/fzr***.** We used the autosomal P-element lethal lines and *piggyBack* insertion (Bloomington Drosophila Stock Center) in a simple F_1 dominant modifier screen to look for the effect of a reduction in gene dosage of an unlinked locus on the *rap/fzr* eye phenotype. In the primary screen, we crossed males from the balanced P-element lethal lines to three different *rap/fzr* alleles (*rap*^{E4}*, rap* ^{E6} *and rap*^{X3}). Our screen included 547 P-element lethal lines on the second chromosome and for the third chromosome, we tested 1975 P-element lethal lines and 279 *piggyBac* stocks from the Bloomington Stock Center. Modifiers were classified as either enhancers or suppressors. From an initial screen of 2741 autosomal lines, we isolated 80 putative modifiers (data not shown). In a secondary screen, putative modifiers were crossed to multiple alleles of *rap/fzr*. 40 modifiers of the *rap/fzr* phenotype were isolated from our secondary screen, of which nine were enhancers and 31 were suppressors (Table 1). Of the 40 modifiers, multiple alleles were available for 32 genes based on available data in the flybase (http://flybase.bio.indiana.edu/) and these were tested for interaction. Based on the available data in published literature and flybase we classified the interacting genes into subgroups. Seven modifiers identified in the genetic screen were classified as cell cycle regulators: *Cyclin A*, *Cyclin B, string*,

dally, *encore*, *polo,* and *Rca1*, these have been previously shown to interact with Rap/Fzr. Six genes identified in the screen were involved in proteolytic degradation: *UbcD6*, *Tbp-1, nonstop, Acer, slamdance* and *morula.* Although these genes encode proteins with related cellular function there is no report of their interactions with Rap/Fzr. In addition, 27 genes identified in the genetic modifier screen suggest novel roles for Rap/Fzr. Nine genes detected in the screen were classified for their role in signal transduction. Another six genes detected in the screen were classified for their role during transcription. *heix* and *walrus*, two genes involved in general cellular metabolism were also detected as modifiers of *rap/fzr*. Two genes identified in the screen are involved in nervous system function, *slowpoke* and *14-33ζ*. Although functional roles of *Tropomyosin-1* and *like-AP180* are known genetic interactions between Rap/Fzr has not been previously reported. There is little or very limited information as to the cellular function of six genes identified in the screen and we have classified these as genes of unknown molecular function that interact with *rap/fzr*.

rap/fzr interacts with genes involved in cell cycle regulation

In view of the previously well characterized cell cycle function of Rap/Fzr, our expectation was that the modifier screen would identify genes encoding other cell cycle regulatory molecules. Consistent with our

expectation, several genes that control cell cycle progression were detected in the screen and are briefly summarized below (Table 1).

Cyclin A: We found that the loss of one copy of *Cyclin A* acts as a strong suppressor of the rough eye phenotype of *rap/fzr*. The genetic suppression was further confirmed with scanning electron micrographs showing strong suppression of the *rap/fzr* rough eye phenotype (Figure 1) of the compound fly eye with a single copy of an amorphic allele of *Cyclin A* in combination with the rap^{E4} allele. Cyclin A functions in both S and M phases of the cell cycle, promoting DNA replication and centrosome duplication**.** In addition, it has been shown that Cyclin A levels increase during interphase and Cyclin A is degraded to ensure proper mitotic exit (Lee et al., 2001; Murray, 2004; Sigrist et al., 1995). During oogenesis, *Cyclin A* loss-of-function mutant phenotypes include transition from mitotic cycle to endoreplication cycle (Schaeffer et al., 2004). Conversely, overexpression of *Cyclin A* shows inhibition of endocycling (Schaeffer et al., 2004). In the developing eye disk, Rap/Fzr has not been found to directly control the degradation of Cyclin A. However, studies using follicle cells show an upregulation of *Cyclin A* in *rap/fzr* mutant clones after mitotic to endocycle transition (Schaeffer et al., 2004). Others have also reported that control of *cyclin A* levels is mediated by the Fizzy/Cdc20 protein during late embryogenesis

(Lee et al., 2001). Fizzy/Cdc20 also regulates and targets cyclins to the APC, but is known to be specifically active during metaphase/anaphase transition (Kashevsky et al., 2002).

dally (*division abnormally delayed)***:*** A single mutant copy of *dally* dominantly suppressed the rough eye phenotype of *rap/fzr*. *dally* encodes a proteoglycan of the HSPG (heparan sulfate proteoglycan) family and is thought to acts as a co-receptor for Dpp and Wg signaling (Fujise et al., 2003). In the developing eye and the optic lobe, Dally is required during cell cycle progression, and *dally* mutants disrupt G2-M phase of both lamina precursor cells in the optic lobe and photoreceptor cells in the retina. Molecular analysis has established that the *dally* mutant phenotype is a consequence of Cyclin A accumulation (Nakato et al., 2002). Upregulation of *Cyclin A* in *dally* mutants is similar to the phenotype of *rap/fzr* in follicle cells. Interaction between *rap/fzr* and *dally* suggest the possibility that the two genes play a similar role in the degradation of Cyclin A.

encore: *encore* is a gene implicated in cell cycle progression and was found in our screen as a dominant enhancer of *rap/fzr* phenotype. Mutations in the *encore* gene lead to extra mitotic divisions during oocyte differentiation (Hawkins et al., 1996). Abnormal accumulation of Cyclin A and persistent levels of Cyclin E were also found in *encore* mutants (Ohlmeyer and

Schupbach, 2003). Failure to down regulate Cyclin E maybe a consequence of *encore's* role as a regulator of components of the SCF ubiquitin ligase (Ohlmeyer and Schupbach, 2003). Since Encore functions as a regulator of the SCF ubiquitin ligase, interaction between *rap/fzr* and *encore* supports previous reports linking these two proteolytic pathways in the control of cell cycle (Guardavaccaro et al., 2003).

***Cyclin B*:** *Cyclin B* was detected as a dominant suppressor of the rough eye phenotype of *rap/fzr*. Similar to Cyclin A, Cyclin B levels rise during interphase and fall during mitosis (Murray, 2004). Unlike Cyclin A, which is necessary during S and M phases of the cell cycle, Cyclin B in only crucial during mitosis (Strausfeld et al., 1996). Cyclin B is degraded by Cdc20/Fizzy activation of APC during the metaphase to anaphase transition (Peters, 2002). During the anaphase to G1 transition, Cyclin B is degraded by activation of APC by Rap/Fzr. Previous work from our laboratory showed an accumulation of Cyclin B in the eye-imaginal disks of *rap/fzr* loss-of-function mutants (Pimentel and Venkatesh, 2005b). Similarly studies in follicle cells show an upregulation of Cyclin B in *rap/fzr* mutant clones (Schaeffer et al., 2004). Our results are consistent with targeting of Cyclin B for destruction by the 26S proteosome following ubiquitination by APC.

***polo*:** A single copy of the *polo* mutation acts as a dominant suppressor of the *rap/fzr* rough eye phenotype. Polo is a serine/threonine kinase that plays an important role in cell cycle progression. The phosphorylation of cytoskeletal actin and associated proteins is likely to regulate the structure of the central spindle apparatus. Mutations in the *polo* gene cause failure in cytokinesis during spermatogenesis (Carmena et al., 1998). Furthermore, in the developing nervous system *polo* mutations have been shown to cause arrested cells or cells in a delayed metaphase-state of mitosis (White-Cooper et al., 1996). Interaction between Rap/Fzr and Polo is supported by previous studies suggesting that Polo phosphorylates and activates subunits of the APC (Zachariae and Nasmyth, 1999).

String is a phosphatase necessary during the G2/M transition of the cell cycle. *string* was identified as a dominant suppressor of *rap/fzr* (Figure 1H). In developing oocytes, recent studies have shown that the Notch pathway down regulates *string* at stage 6 for the progression into endocycling (Schaeffer et al., 2004). *rap/fzr* is activated by Notch during endocycling of follicle cells (Schaeffer et al., 2004; Shcherbata et al., 2004). It has been suggested that an interplay between *rap/fzr* activation and *string* down-regulation is required for mitotic-to endocycle transition (Schaeffer et al., 2004).

Rca1 (*Regulator of cyclin A*) has been previously reported as an inhibitor of Rap/Fzr (GROSSKORTENHAUS and SPRENGER 2002). In our genetic modifier screen, *Rca1* suppressed the rough eye phenotype of *rap/fzr* (Figure 1G). *Rca1* is activated during the G2 phase of the cell cycle when it binds and prevents Rap/Fzr from functioning. Furthermore, it has been suggested that Rca1 inhibits Rap/Fzr function by blocking its association with Cdc27, a core component of the APC (Grosskortenhaus and Sprenger, 2002).

Phenotype of genetic modifiers in the eye imaginal disk

To determine if genes implicated in cell-cycle modify *rap/fzr* phenotype in the developing eye disk, we stained double mutants with an antibody against phosphorylated histone-H3, a mitotic marker. During the third instar larval stage, the developing eye shows a stereotypic pattern of mitosis. The morphogenetic furrow (MF) demarcates the differentiated and undifferentiated regions of the eye disk, while cells immediately anterior and posterior to the morphogenetic furrow are actively going through mitosis. Cells in the MF are arrested in G1 phase of the cell cycle (Ou et al., 2003; Pimentel and Venkatesh, 2005a; Pimentel and Venkatesh, 2005b). Our results show that *Cyclin A*, *rca1*, and *string* consistently suppress both the rough eye phenotype of *rap/fzr* mutants and restore mitotic organization of third instar larvae eye disks (Figure 1G-I and Figure 2G-I).

Previous work from our laboratory and work by others have established degradation of Cyclin B as a crucial step for mitotic exit (Pimentel and Venkatesh, 2005; Su et al., 1998). Several studies have shown that Rap/Fzr and Fzy/Cdc20 mediate the cell cycle stage specific ubiquitination and degradation of Cyclin A, Cyclin B and String by the APC (Peters, 2002; Zur and Brandeis, 2002). Our screen identified mutations in *Cyclin A* and *string* as dominant suppressors of the *rap/fzr* rough eye phenotype. To test whether this suppression phenotype in the adult was a result of suppression of the mitotic phenotype in the developing eye disk, we examined the distribution of Cyclin B and Phosphohistone-H3 in the double mutant eye disks. Anti-Cyclin B antibody staining of eye-disks from *rap/fzr* mutant*s* shows an abnormal accumulation of Cyclin B compared to wild type (Figure 2B-C). *rap/fzr* mutants eye disks with a single copy of either *Cyclin A* and *string* mutation show Cyclin B and Phosphohistone-H3 distribution similar to wild type (Figure 2G and Figure 2I) consistent with the role of these genes in cell cycle progression. In contrast, rap^{E4} mutants with a single copy of *Rca1* mutation shows persistent levels of Cyclin B similar to rap^{E4} mutants (Figure 2H). This result suggests a separate cellular role for *Rca1* in addition to cell cycle regulation. Consistent with this idea, the mammalian homolog of *Rca1*, *Emi1* functions

in a biological context other than cell cycle regulation: Emi1 was shown to play a role in axon growth and defasciculation (Konishi et al., 2004). In conclusion, most genes that act as suppressors or enhancers of the rough eye phenotype of *rap/fzr* also modify *rap/fzr* in the developing eye disk as enhancers or suppressors.

Genes involved in proteolytic degradation modify the eye phenotype of *rap/fzr*

In addition to genes involved in cell-cycle regulation, several genes involved in proteolysis also showed interactions with *rap/fzr*. Genes such as *APC2/morula, Tbp-1, slamdance, nonstop, Ubcd6, and Acer* all function in protein degradation pathways and were found as dominant modifiers of *rap/fzr* (Table 1).

APC2: A component of the APC, Morula/APC2 was detected as an enhancer of the rough eye phenotype of *rap/fzr*. Since Morula/APC2 is a subunit of APC, it is not surprising that the phenotype of loss-of function *morula* mutants is similar to that of *rap/fzr* mutants. Initial studies from Drosophila oocytes revealed that Morula/APC2 inhibits mitosis during endocycling of nurse cells: *morula* mutants cause nurse cells to reenter mitosis and chromosomes are arrested in metaphase (Kashevsky et al., 2002). More recent studies reveal a role of Morula/APC2 in nervous system

development: In the Drosophila neuromuscular junction, *morula* mutants have increased number of synaptic boutons (van Roessel et al., 2004).

Tbp-1: Another gene that is involved in the ubiquitin pathway, *Tat-binding protein-1* or *Tbp-1* was also identified as a suppressor of *rap/fzr*. Although Tbp-1 has not been studied extensively, sequence analysis shows that Tbp-1 contains an AAA-ATPase domain which is homologous to a component of the regulatory complex (RC). Two regulatory complexes attach to the 20S proteosome core and act to recognize proteins degraded by the 26S proteosome. The ATPase component of the regulatory complex has the function of unfolding substrates that enter the 20S core (Holzl et al., 2000).

***UbcD6*:** A single copy of the recessive lethal P-element insertion in the gene encoding the ubiquitin conjugating enzyme, *UbcD6* acted as a suppressor of *rap/fzr*. Although very little is known about the cellular roles of UbcD6, sequence analysis has revealed that UbcD6 is the Drosophila homolog of yeast *RAD6,* a DNA repair gene (Koken et al., 1991). Since *UbcD6* encodes a ubiquitin conjugating enzyme (Koken et al., 1991), an interaction between *UbcD6* and *rap/fzr* raises the possibility that UbcD6 is a E2-conjugating enzyme that sequentially delivers ubiquitin to the substrates of the APC.

***nonstop*:** Two genes involved in protein degradation, *nonstop* and

slamdance were both detected as suppressors of the *rap/fzr* eye phenotype. *nonstop* encodes a ubiquitin specific protease (UBP), involved in deubiquitination and can either inhibit or stimulate protein degradation (Poeck et al., 2001b; Wilkinson, 1997). POECK *et al.* (2001) have shown glia migration defects in the developing nervous system of *nonstop* mutants. In *nonstop* loss-of-function mutants, epithelial, marginal and medulla glia fail to migrate from glial precursor areas (GPC) located at dorsal and ventral edges of the R cell projection field. As a result of defective glia migration, R1-R6 axons mistarget to the lamina and terminate into the medulla (Poeck et al., 2001a). Our results suggest that *nonstop* may function in the same ubiquitination pathway as Rap/Fzr. Nonstop might counteract and modulate Rap/Fzr's function by de-ubiquitinating its substrate or by removing the ubiquitin tag from Rap/Fzr itself thereby keeping the APC active for a longer time period.

***slamdance*:** The gene *slamdance* encodes Aminopeptidase N (APN), an ectoenzyme well characterized for its role in various biological functions (Look et al., 1989; Ward et al., 1990; Zhang et al., 2002). APN has been implicated in mediating release of Ca2+ from intracellular stores (Santos et al., 2000). In the nervous system, APN cleaves angiotensin III during the renin-angiotensin pathway (Zini et al., 1996). Although biochemical studies

are necessary to determine the function of APN in *slamdance* mutants, Zhang et al. (2002) show bang-sensitive behavior with *slamdance* mutants. Furthermore, electrophysiological evidence show neuron hyperactivity followed by paralysis with *slamdance* mutants (Zhang et al., 2002).

***Acer*:** The gene *Angiotensin-converting enzyme-related*, (*Acer*), is another gene involved in proteolysis, also suppressed the rough eye mutant phenotype of *rap/fzr*. Acer encodes a peptidyl-dipeptidase and *Acer* mutants exhibit a severe defect in heart morphogenesis (Crackower et al., 2002). Acer mRNA was found to be expressed in the dorsal vessel during embryonic development (Taylor et al., 1996). Acer's mammalian homolog, ACE2, is expressed in the lung and has been shown to inactivate Angiotensin II. More recent evidence further shows that recombinant ACE2 is able to protect mice from severe acute lung injury (Imai et al., 2005; Kuba et al., 2005).

Several unnamed genes involved in degradation pathways also modified *rap/fzr* (Table 1). The gene *CG4080* annotated by the Berkeley Drosophila Genome project (http://www.fruitfly.org/), has no known biological function but was annotated as encoding a protein with a SCF or Ring Finger protein domains. SCF and Ring Finger proteins are characteristic of ubiquitin E3 ligases of a class distinct from the APC (Ou et

al., 2003). Our result that a Rap/Fzr potentially interacts with this gene raises the interesting possibility that Rap/Fzr may regulate SCF like ubiquitin ligases.

rap/fzr interacts with genes involved in signal transduction pathways

Genes involved in multiple signaling pathways were also detected as modifiers of *rap/fzr* (Table 1). Recent evidence suggests that ubiquitination can activate signaling events by processing precursors of a specific pathway or by degrading an inhibitor of a pathway (Chen, 2005). Ubiquitin can also inhibit second messenger systems by degrading the actual signal.

Delta, ebi and pointed: A component of the Notch inductive pathway, *Delta,* was an enhancer of *rap/fzr*. *ebi* and *pointed*, genes that act downstream of the EGFR pathway, were also identified as enhancers of *rap/fzr*. In *Delta, ebi,* and *pointed* animals crossed to rap^{E6} mutants, F1 progeny had a more severe rough eye phenotype compared with *rap/fzr* flies (Figure 1D-F). *ebi* encodes a WD40 domain protein and has been shown to facilitate the degradation of the transcriptional repressor Tramtrak 88 during the differentiation of R7 photoreceptor neurons (Boulton et al., 2000). Ebi limits S-phase entry and prevents cells from proliferating during development (Boulton et al., 2000). Recent work on *ebi* has shown that this gene is also involved in ubiquitination pathways (Tsuda et al., 2002). Ebi is

thought to cause the degradation of the SMRTR repression complex. In turn, SMRTR blocks the inhibitory action of Su (H) on Delta (Tsuda et al., 2002). Ebi's interaction with Rap/Fzr raises the possibility that these genes are working together for the breakdown of Su(H).

Lis-1: Mutations in *Lissencephaly-1* or *Lis-1*, which encodes a microtubule-associated WD 40 protein, acted as a dominant suppressor of the rough eye phenotype of *rap/fzr* mutants. *Lis-1* mutants affect both nervous system development and oocyte differentiation (Lei and Warrior, 2000; Liu et al., 2000). In the developing mammalian brain, the *Lis-1* mutant phenotype includes congenital brain malformation whereby the cerebral surface appears smooth (Morris et al., 1998). During oogenesis, *Lis-1* mutants interrupt cytoblast synchronized divisions. Disruption of rapid cytoblast division results in an abnormal cell number in cysts and these cysts almost never contain an oocyte. In both processes, Lis-1 works in conjunction with cytoskeletal proteins to affect either nurse-cell-to-oocyte transport or neural migration (Liu et al., 1999b; Swan et al., 1999) .

ksr (*kinase suppressor of ras)*: *ksr* was identified as a dominant suppressor of *rap/fzr*. *ksr* is known to function in the MAP kinase signaling pathway and has been shown act as the scaffolding protein for the formation of RAF/MEK complex(Roy et al., 2002). Ksr is known to be degraded by the

ubiquitin ligase, IMP: Impedes Mitogenic Signal propagation (Matheny et al., 2004). Interaction between Ksr and Rap/Fzr suggest the possibility that Rap/Fzr may mediate the ubiquitination of Ksr by other ubiquitin ligases like the APC.

loco: *loco* encodes a protein that belongs to the family of regulators of G-protein signaling (RGS). In our genetic modifier screen, *loco* was able to dominantly suppress the rough eye phenotype of *rap/fzr*. *loco* embryos have no apparent physical defects but fail to hatch. *loco* has been described as a gene that affects late glia differentiation (Granderath et al., 2000). In *loco* mutants, glia fail to properly ensheath longitudinal axon tracts (Schwabe et al., 2005). Furthermore, normal glia-glia cell contacts necessary for the formation of the blood-brain barrier are also disrupted in *loco* mutants (Granderath et al., 1999). Interaction between Loco and Rap suggests that Rap/Fzr may facilitate degradation of proteins involved in glia differentiation.

***Star*:** Star mutations act as strong dominant enhancers of the rough eye phenotype of *rap/fzr*. Like *pointed* and *ebi*, *Star* is also involved in the EGFR signaling pathway (Kolodkin et al., 1994). *Star* belongs to the *spitz*

group of genes which control the development of ventral lateral blastoderm (Bang and Kintner, 2000; Kolodkin et al., 1994) . Genes that belong to the *spitz* group all exhibit a mutant phenotype that affects cuticle structures (Mayer and Nusslein-Volhard, 1988). Together with the intra membrane protease Rhomboid, Star functions in the proteolytic processing of the EGFR ligand Spitz before it can bind and activate the EGFR receptor (Bang and Kintner, 2000). Additionally, Star also plays a role in the trafficking of Spitz from a peripheral nuclear compartment to a location where the protein can then be cleaved (Tsruya et al., 2002). Star and Rap/Fzr may function together to activate Rhromboid-mediated proteolytic processing of Spitz.

spätzle: Two alleles of *spätzle* were analyzed and both were detected as suppressors of *rap/fzr*. *spätzle* was first classified a dorsal group gene, because *spätzle* mutants are dorsalized and lose ventral and lateral patterning during embryonic development (Stein and Nusslein-Volhard, 1992). *spätzle* is a known ligand of the Toll receptor and has been studied as a part of Drosophila immune response during fungal infection. Interestingly, *spätzle* must be cleaved by serine proteases such as Easter before it can bind to the Toll receptor (Chasan et al., 1992; Levashina et al., 1999).

***twins*:** *twins* acts as a dominant enhancer of the *rap/fzr* rough eye phenotype. *twins* encodes a serine/thereonine phosphatase and is involved in several

independent biological processes. For example, *twins* has been shown to be involved in wing development, eye development via the Ras pathway, and peripheral nervous system development (Shiomi et al., 1994; Uemura et al., 1993; Wassarman et al., 1996). During mechano-sensory bristle formation, *twins* mutants exhibit ectopic support cells and inhibit neural cell formation. Twins acts on the Numb protein, which contains a phosphotyrosine binding domain, to promote support cell differentiation (Uemura et al., 1989). Rap/Fzr is inhibited by phosphorylation and activated by dephosphorylation (Listovsky et al., 2000). Genetic interactions between *twins* and *rap/fzr* suggest that Twins may activate Rap/Fzr by dephosphorylation.

Transcriptional regulators act as modifiers of the *rap/fzr* phenotype

Nine genes that were detected as modifiers in our screen encode regulators of transcription (Table 1) suggesting that Rap/Fzr may mediate ubiquitination of transcription factors. Ubiquitination has been shown to regulate transcription factors in multiple ways (Conaway et al., 2002). For example, Pol II transcription factors are known to be degraded by the ubiquitin pathway. Activation of transcription factors following ubiquitination and inactivation of transcription factors without proteosomal degradation has also been reported (Kaiser et al., 2000; Zhang, 2003). The transcription factor, bric a brac1 (*bab1*) was found to be as a suppressor of

rap/fzr. Bab1 is a transcription factor with a conserved BTB/POZ protein domain. BTB/POZ domains are located in the N-terminus of several transcription factors and function to mediate protein-protein interactions (Couderc et al., 2002; Zollman et al., 1994). Current evidence shows that *bab1* plays a role in tarsal differentiation, ovarian development, and sexual dimorphism (Couderc et al., 2002). Bab1 may act as a morphogen since tarsal segments are dependent on graded distribution of Bab1 protein (Couderc et al., 2002; Godt et al., 1993).

cnc *(cap n collar): cnc* was detected as a suppressor of *rap/fzr*. Cap n collar has been classified as a basic leucine zipper and targets Hedgehog and Wingless during anterior and posterior segmentation during embryonic development (Mohler et al., 1995; Mohler et al., 1991). In conjunction with the Hox gene, *Deformed*, *cap n collar* also regulates head patterning. During the larval stages, *cap n collar* mutants exhibit missing mandibles, which are replaced with additional maxillary structures (Mohler et al., 1995).

mod(mdg4) *(modifier of mdg4)***:** *mdg4* was detected as suppressor of *rap/fzr*. The phenotype of *modifier of mdg4* mutants implicates the gene in various processes such as apoptosis, position effect variegation, chromatin insulation, and control of homeotic genes (Dorn et al., 1993; Gerasimova and Corces, 1998; Gerasimova et al., 1995; Harvey et al., 1997). Sequence

analysis from cDNA of *modifier of mdg4* reveals that the gene contains 21 isoforms with a common BTB/POZ domain. It has been suggested that the many splice variants of *modifier of mdg4* contribute to its different functions (Buchner et al., 2000).

tap (*target of Pox-n*)**:** *tap* is a basic helix loop helix (bHLH) transcription factor. *tap* mutants are mild suppressors of *rap/fzr*. *tap* is a proneural gene that encodes proteins similar to other bHLH proteins such as Atonal and Achaete-Scute(Bush et al., 1996; Campuzano and Modolell, 1992; Gautier et al., 1997). However, *tap* differs from *atonal* and *achaete-scute* because it is activated during late neurogenesis immediately before mitotic exit and neural differentation. A downstream target of tap is *Paired box neuro* (*Pox-n*), which is expressed in chemosensory organ lineages. Although *Pox-n* is expressed throughout differentiation of chemosensory organ lineage, *tap* is expressed briefly prior to the onset of differentiation (Gautier et al., 1997; Ledent et al., 1998). *tap* progenitor cells give rise to three types of glia cells located lateral to longitudinal connectives: segmental nerve glia, intersegmental glia, and exit glia (Bush et al., 1996).

Aly: *Aly* was identified as a suppressor of *rap/fzr*. Aly is an RNA binding protein that preferentially binds to mRNA and is a component of the exon-exon junction complex, which is a multiprotein structure that is deposited by

the spliceosome 20-24 nucleotides upstream of splice junctions. Aly has also been implicated in nuclear export of mRNA because it recruits NFX1 to associate with mRNA. NFX1 is a known nuclear export receptor (Gatfield and Izaurralde, 2002; Palacios, 2002).

pointed: As mentioned above, *pointed* acts as an enhancer of *rap/fzr* (Figure 1E and Figure 2E). *pointed* has been extensively studied in numerous processes, R7 photoreceptor differentiation formation, heart development, and ommatidial polarity (Alvarez et al., 2003; Gaengel and Mlodzik, 2003). Although most research on *pointed* focuses on its role during EGFR and Ras signaling, it has also been studied during glial cell differentiation (Klaes et al., 1994; Klambt, 1993). *pointed* encodes two forms of Ets-domain transcription factors, PntP1 and PntP2. PntP1 and PntP2 arise from the use of two alternate promoter regions separated by 50 kb (Klambt, 1993). PntP1 and PntP2 have regions that are homologous to the vertebrate Ets DNA binding domain (Klambt, 1993). *In-situ* hybridization has shown that lateral glial cells express only PntP1 while PntP2 is expressed only by midline glia (Klambt, 1993). Since glia provide nourishment for axons, *pointed,* consequently, affects axon growth, guidance and maintenance. *pointed* mutants were found to form thinner longitudinal connectives and fused

commissures (Klaes et al., 1994; Klambt, 1993). Our preliminary results suggest that Rap/Fzr may target Pointed for degradation.

Branchless: Branchless is a secreted signaling molecule and is a homolog of vertebrate FGF. Branchless binds and activates the Breathless receptor and guides primary branching of tracheal cells (Englund et al., 1999). A single copy of the *branchless* mutation acts a s an enhancer the *rap/fzr* phenotype. Interestingly, Branchless activated signaling pathway induces transcription of the *pointed* gene and our results suggest that Pointed may be a substrate of Rap/Fzr.

Two genes implicated in metabolism interact with *rap/fzr*

heix is a gene involved in coenzyme and lipid metabolism. *heix* acted as a suppressor of *rap/fzr* (Table 1). Although little information is known about *heix*, according to Drsophila genome annotation (flybase) *heix* encodes a UbiA Prenyltransferase. *heix* is necessary for normal wing morphology (Roch et al., 1998), and interacts with the nuclear zinc finger protein, Hindsight (Wilk et al., 2004).

walrus: A single copy of the of the *walrus* mutation suppressed the rough eye phenotype *rap/fzr* (Table 1). From sequence analysis data, it can be inferred that *walrus* encodes an enzyme that functions as an oxidoreductase

(http://www.ensembl.org/Drosophila_melanogaster). Furthermore, *walrus* is expressed within the mitochondrial matrix. *walrus* mutant phenotype include numerous defects during Malpighian morphogenesis, suggesting that *walrus* affects common processes necessary for morphogenesis of epithelial tissue (Liu et al., 1999a).

Genes that regulate nervous system function interact with *rap/fzr*

slowpoke and 14-33ζ : Both *slowpoke* and *14-33ζ* acted as dominant suppressors of *rap/fzr* (Table 1). *slowpoke* encodes a calcium-activated, voltage activated potassium channel that functions to gate current within presynaptic terminals (Yazejian et al., 1997). Since voltage activated potassium channels are integral to proper function of the nervous system, the *slowpoke* mutant phenotype includes a decrease in neurotransmitter release (Warbington et al., 1996). Defects in courtship song have also been observed in two specific alleles of *slowpoke* (Peixoto and Hall, 1998). 14-33ζ is an isoform of the 14-33 protein. 14-33 proteins were first identified as acidic brain proteins (Aitken et al., 1992). It was later discovered that 14-33 proteins act as scaffolding proteins for signaling transduction pathways such as Ras signaling. The mutant phenotype of *14-33ζ* includes several nervous system defects, such as loss of photoreceptors and a failure of olfactory learning tasks (Kockel et al., 1997; Skoulakis and Davis, 1996).

Interestingly, co-immunoprecipitation experiments using adult Drosophila heads show Slowpoke and 14-33ζ interact (Zhou et al., 1999). Further colocalization studies suggest a Slowpoke/ 14-33ζ complex is present at the neuromuscular junction (Zhou et al., 1999).

Tropomyosin 1 was also detected as a suppressor of the rough eye phenotype of *rap/fzr* (Table 1). *Tropomyosin 1* encodes an actin binding protein and has been shown to be alternatively spliced (Basi and Storti, 1986; Johnstone and Lasko, 2001; Tekotte and Davis, 2002). Tropomyosin 1 plays role in many processes including a role during oocyte development for the localization of oskar mRNA (Tekotte and Davis, 2002) and dendrite morphogenesis (Grueber and Jan, 2004).

***like-AP180 (lap*)** : Mutations in *lap* were identified as suppressors of *rap/fzr* (Table 1). *lap* encodes a clathrin binding protein and functions in synaptic vesicle endocytosis (Zhang et al., 1998). When *lap* mutants were examined during larval development, larva appeared sluggish and uncoordinated. Further behavioral and electrophysiological studies revealed motor defects of *lap* mutant larva were due to impaired synaptic vesicle endocytosis (Bao et al., 2005)

Genes with unknown molecular function modify *rap/fzr* phenotype

Several genes identified in the genetic screen were those with unknown molecular function, or these factors had not been studied extensively. For example, disruption of *Ugt86Da* acted as a suppressor of the rough eye phenotype of *rap/fzr* (Table 1). According to its sequence analysis on flybase, *Ugt86Da* encodes a glucouranosyl transferase. Similar to Ugt86Da, little is known about two ribosomal proteins, Ribosomal protein LP0 and Ribosomal protein S26, both of which are also suppressors of *rap/fzr* when disrupted (Table 1). Although many reports have studied the role of ecdysone-induced proteins as hormonal signals necessary for the transitions in a Drosophila's life cycle, the role of *Ecdysone-induced protein 78C* (*Eip 78C*) during larval development has not been well characterized. *Eip 78C* also acted as a suppressor of *rap/fzr* (Table 1). *Eip 78C* mutants show no visible phenotype and are viable and fertile (Russell et al., 1996). Two genes identified as suppressors in the screen, *CG4080*, and *l(2)k08110*, have no or little information known. As mentioned earlier, *CG4080* encodes ubiquitin ligase protein domains, a RING domain. *l(2)k08110* contains no sequence analysis data. The only information known about *l(2)k08110* is that mutant phenotype of the gene affects crystal cells formation (Milchanowski et al., 2004).

Bioinformatic analysis reveals modifiers of Rap/Fzr contain putative Destruction Box, KEN Box, and A-Box Sequences: Experiments using biochemical assays have identified the requirement of a destruction box sequence (D-box) essential for substrate recognition by APC/Fzr and APC/Fzy. In an effort to understand the nature of interaction between candidate genes and *rap/fzr*, a search for D-box sequences using the Destruction Box finder program (Dana Reichman Weizzman Institute) was performed on all isolated modifiers of *rap/fzr*. This program aligns DNA sequences to substrates that have known D-box amino acid sequences, such as *Cyclin B, Cyclin A, geminin*, and *securin*. Twenty one of the forty genes that enhanced or suppressed *rap/fzr* encoded D-box like sequences (Table 2). Most of the genes aligned between 4 to 6 amino acids out of a total 9 amino acid sequence. *CG4080* and *mod(mdg4)* had six amino acids that were identical to Cyclin A's D-box sequences. In contrast, nine genes possessed only four amino acid sequences that matched known D-box sequences. These nine genes include *string*, *Rca1*, *encore, Delta, slamdance, morula, lap, Aly, Lis-1* (Table 2).

E-value- and relevance: For statistical purposes, a given E-value is listed on Table 2 for all D-box alignments. E-values correspond to the expected number of times sequence alignments were made by chance. A

lower E-value indicates a more dependable and true sequence alignment (http://bioinfo.weizmann.ac.il/~danag/d-box/main.html). In Table 2, all D-box alignments had E-values that were less than 1. Cyclin B had the lowest E-value, 1.7x 10^{-5} followed by Cyclin A which had an e-value of 0.003. Slowpoke and Mdg4 also had very low E-values, of 0.021 and 0.0098, respectively. Loco had the highest e-value of 0.93. Although E-values serve as a valuable statistical tool for finding D-box in potential targets of Rap/Fzr, further biochemical analysis is necessary to confirm the functionality of these D-box sequences**.**

KEN Box and A Box

Since APC/Fzr also recognizes KEN box and A-box sequences for target recognition of substrates, we also searched for these two sequences in modifiers of *rap/fzr*. For A-box and KEN box sequence searches, we used SIM alignment tool program, which uses an algorithm for finding the best non-intersecting alignments between two sequences (Huang and Miller, 1991). From this program we found seven modifiers of *rap/fzr* which contained KEN box sequences. These genes include *Cyclin B*, *Cyclin A*, *Rca1*, *string*, *loco*, *nonstop*, and *UbcD6* (Table 3). All seven genes contained 100% sequence identity when aligned with KEN box sequence

(Table 3). When SIM alignment tool was used to determine if modifiers of *rap/fzr* contained A-box sequences, 38 genes aligned with the A-box sequence QRILGPSNVPQRV (Table 4). Most genes showed 30-50% sequence identity when aligned with the A-box sequence. The genes *branchless*, *encore*, and *string* showed the highest percent sequence identity with the A-box. When aligned to nine residues of the A-box sequence, *branchless* and *encore* showed 55.6% sequence identity. *string* showed 50% sequence identity when aligned to ten residues of the A-box sequence. *pointed* and *Rca1* showed the lowest percent sequence identity with the A-box, with 27.3% and 25% sequence identity when aligned to 11 residues, respectively (Table 4) . Taken together, these data show that 39 of the 40 identified *rap/fzr* modifiers (all except *walrus*) contain a D-box, KEN sequence and/or an A-box suggesting that the interacting proteins identified by our screen may be cellular targets of Rap/Fzr.

DISCUSSION

Genetic modifier screens are invaluable in identifying gene interactions and facilitate a genetic dissection of complex biological pathways. In addition, modifier screens also frequently uncover novel interactions suggesting new cellular functions for the gene of interest. Rap/Fzr/Cdh1 is an activator of APC and is characterized primarily for its

role in the timely degradation of mitotic regulators such as cyclins and cdks. However, in recent years, Rap/Fzr/Cdh1 and APC have been shown to function in processes unrelated to cell cycle in postmitotic neurons (Konishi et al., 2004; van Roessel et al., 2004). Our studies on the expression patterns of Rap/Fzr in the developing eye and central nervous system, for instance, showed that Rap/Fzr is expressed in postmitotic neurons (Pimentel and Venkatesh, 2005b) and led us to speculate on possible non-mitotic functional roles of Rap/Fzr. To identify genes that interact with *rap/fzr,* we carried out a F1 modifier screen using the weak hypomorphic alleles of *rap/fzr* as a sensitized system for screening. Our expectation was two fold: 1) our screen would identify known genes which encoded cell cycle regulators required for mitotic progression, and 2) our screen would identify genes with functions not directly related to cell cycle progression. Our results presented here support the idea that Rap/Fzr functions in multiple cellular pathways in addition to cell cycle progression. Although ubiquitin ligase complexes have been best characterized in regard to their role in cell cycle regulation, in recent years, ubiquitination mediated proteolysis has shown to be critical in a multitude of cellular processes (Hegde and DiAntonio, 2002; Muratani and Tansey, 2003; Speese et al., 2003).

Consistent with our initial expectations, our results showed that

rap/fzr interacts with key cell cycle genes such as *string, rca1,Cyclin B* and *Cyclin A*. Loss of one copy of *string, rca1* and *Cyclin A* suppressed the rough eye phenotype of *rap/fzr*. Furthermore, genes like *dally* and *polo,* which are in signaling pathways but also have defective cell cycle phenotypes, acted as suppressors of *rap/fzr*. Since studies have shown that *rap/fzr* activates the APC for degradation of Cyclin A and Cyclin B during mitosis for entry to G1, it is was expected that *string* and *rca1*, genes that regulate the cell cycle during G2/M phase, would act as dominant suppressors of *rap/fzr* (Baonza et al., 2002; Schaeffer et al., 2004; Shcherbata et al., 2004). Rca1 has been shown to act as an inhibitor of Rap/Fzr during G2/M phase of the cell cycle. In turn, Rap/Fzr inhibition guarantees high cyclin levels needed for mitotic entry (Grosskortenhaus and Sprenger, 2002)**.** Additionally, recent evidence suggests that of Rap/Fzr/ Cdh1- mediated degradation of cyclins are no longer restricted to cell-cycle progression. Mammalian studies have shown that Cdh1 targets cyclin B1 in terminally differentiated neurons. Cdh1 knockdown causes accumulation of cyclin B1, and promotes reentry to S-phase, which ultimately leads to cell death (Almeida et al., 2005).

Our results showed that nine genes encoding components of signal transduction pathways act as modifiers of the *rap/fzr* phenotype, suggesting

a link between these signal pathways and regulated proteolysis (Table 1). These results are consistent with other studies which have shown that signal cascades modulate ubiquitin mediated regulated proteolysis. For example, the ubiquitin ligase, Impedes Mitogenic Signal propagation (IMP), suppresses Ras signaling by sequestering and binding to Ksr. Ksr provides a scaffold for the continued phosphorylation and activation of ERK. It has been shown that only a strong, constitutively active form of Ras can overcome the inhibitory effect of IMP. Activated Ras causes autoubiquitination of IMP, thereby preventing IMP from binding to Ksr. These data support the idea that a ubiquitin ligase such as IMP can regulate the sensitivity and threshold of signaling cascades like the Ras pathway (Ory and Morrison, 2004). Similarly, other studies have shown that ubiquitin ligases cooperate to down regulate the EGFR pathway (de Melker et al., 2004; Lipkowitz, 2003). Cbl proteins are classified as E3 ligases and are known to ubiquitinate EGFR upon ligand binding. Ubiquitinated EGFR leads to endocytosis of the receptor and subsequent degradation via lysosome or proteosome (Lipkowitz, 2003). As mentioned earlier, *loco,* a gene involved in G-protein signaling and glia differentiation, acted as a dominant suppressor of *rap/fzr*. Taken together, these studies and results from our genetic screen support the notion that ubiquitination is a

mechanism for the modification of signal transduction pathways. Genetic interactions between *rap/fzr* and genes involved in signaling suggest that Rap/Fzr is able to either terminate signaling cascades or shift the balance between two pathways such as glia differentiation and neural differentiation.

The relationship between ubiquitination and transcriptional regulation is well documented, and ubiquitination has been shown to control the levels of transcription. Transcription factors are recognized by their transcription activation domains for the addition of ubiquitin moiety. The ubiquitin tag then leads to the destruction of transcription factor and the termination of transcription (Conaway et al., 2002; Muratani and Tansey, 2003). A direct role for Pol II as an initiator of ubiquitination has also been proposed and supported by the experimental evidence showing that some of the E3 ligases are components of the Pol II complex (Conaway et al., 2002). Interestingly, the four of the genes identified in our screen, *pointed, modifier of mdg4, ecdysone-induced protein 78C,* and *target of Poxn*, are reported as having RNA polymerase II transcription factor activity (Donze and Kamakaka, 2001). Furthermore, ubiquitin regulation of transcription without protein degradation has also been reported. For example, precursors of known Pol II transcription factors, such as NFκB p52 and p50 subunits, are processed by cleavage through ubiquitination (Karin and Ben-Neriah, 2000). A

suicide model has been proposed for the role of ubiquitin during transcription. This model suggests that active transcription factors promote ubiquitination. In turn, ubiquitination supports a single round of transcription and simultaneously tags the protein for subsequent destruction and termination of signal (Salghetti et al., 2001). Interaction between *rap/fzr* and numerous genes involved in transcription suggests that the APC plays a role in controlling regulated gene expression. Recent evidence has shown that APC binds and promotes transcription of CBP (CREB binding protein)/p300. CBP and p300 are transcriptional coactivators that control cell growth, transformation and development (Goodman and Smolik, 2000; Turnell et al., 2005). Id2 (inhibitor of differentiation 2), a DNA binding protein known to regulate of bHLH transcription factors such as E47 for axon growth, has also been identified as a target for Cdh1-mediated degradation in mammalian studies (Lasorella et al., 2006).

Although modifiers of Rap/Fzr were categorized into primary molecular function as shown in Table 1, most genes have been connected to multiple biological processes. The EtS domain transcription factor Pointed, for example, has been extensively studied for its role as an effector during EGFR signaling and glia differentiation (Klambt, 1993). Other genes like *twins* have been linked to the MAP kinase signal cascade and cell cycle

regulation (Shiomi et al., 1994; Uemura et al., 1993). In recent years, several studies have begun to elucidate the role of ubiquitination in nervous system development and function. Cdh1, the mammalian homolog of Rap/Fzr, has been shown to be necessary for axon fasciculation and axon growth. Through RNA interference and *cdh1* knockout mice, experiments showed that parallel fibers of cerebellum were highly defasciculated. Cdh1 knockdown also increased the total length of granule neuron axons. Furthermore, parallel fibers grew past boundary layers towards the external granule layer (Konishi et al., 2004). The transcriptional corepressor SnoN has been recently identified as a substrate for Cdh1-mediated degradation responsible for promoting axon growth ((Stegmuller et al., 2006). Another gene, *highwire*, has been identified as a negative regulator of synaptic growth. Highwire contains a Ring-H2 Domain, a motif present in a class of ubiquitin ligases. Similar to Cdh1 knockdown, known substrates for Highwire ubiquitination have yet to be determined (Hegde and DiAntonio, 2002; Wan et al., 2000)**.**

For future experiments, it would be interesting to determine the nature of interactions between *rap/fzr* and genes detected in the screen. For example, our recent results have shown direct interactions among Rap/Fzr, Loco, and Nonstop regulate glia specification (Kaplow et al. manuscript in

preparation). Similarly, since *nonstop* is a ubiquitin protease required for glia migration (Poeck et al., 2001a), studies on the interaction between Nonstop and Rap/Fzr may uncover a role for *rap/fzr* in glia migration. The genetic screen described in this study has identified potential substrates for Rap/Fzr. Whether it is through cell-cycle regulation, transcription, axon targeting or changes in synaptic strength, ultimately, ubiquitin can serve as a control mechanism for protein turnover.

Acknowledgments:

We are grateful to Dr. Christine Li, Yuika Goto, and Stephanie Kadison for critical reading of the manuscript. We are thankful to Daniel Fimiarz for assistance with Image J and confocal microscopy. We thank the Bloomington stock center for the supply of Drosophila stocks and the University of Iowa Hybridoma Bank for the antibodies. We are thankful to Dr. Mark Tanouye for *slamdance* stocks. This work was supported by the NIH grants S06GM008168 and SG12RR060 (NIH-RCMI). Margarita Kaplow was supported by the NIH-MBRS-RISE program at City College, and the New York City Alliance for Minority Participation.

Table 1. Genes on the second and third chromosome identified as modifiers of Rap/Fzr

Interacting Locus	Cytological Position	Alleles Tested	Effect
I. Cell Cycle Regulation			
Cyclin A	68E1-	P{PZ}CycA^{03946} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}	Suppressor
		CycA^{C8LR1}/TM3, Sb^{1} P{35UZ}2	Suppressor
		w$^{[1118]}$; PBac{w[+mC]=PB}CycA$^{[c05304]}$/TM6B, Tb[1]	Suppressor
Cyclin B	59B2	y[1] w$^{[67c23]}$; P{w[+mC] y[+mDint2]=EPgy2}CycB$^{[EY08217]}$	Suppressor
		w[*]; CycB[2]/CyO, P{ry[+t7.2]=ftz/lacB}E3	Suppressor
dally	66E1--3	P{PZ}dally^{06464} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}	Suppressor
		w[^{1118}]; PBac{w[+mC]=WH}dally$^{[f01984]}$	Suppressor
encore	64F1--3	y^{1} w^{67c23}; P{SUPor-P}enc^{KG05608} ry	Enhancer
		enc^{R1} st^{1} e^{1} ca^{1}/TM3, Ser^{1}	Enhancer
Rca1	27C1	Rca1^{IX} cn^{1} bw^{1}/SM6a	Suppressor
		P{PZ}Rca1^{03300} cn^{1}/CyO; ry	Suppressor
		Rca1$^{[2]}$ cn$^{[1]}$ bw$^{[1]}$/SM6a	Suppressor
string	99A5	ry^{506} P{PZ}stg^{01235}/TM3, ry^{RK} Sb^{1} Ser^{1}	Suppressor
		ru^{1} h^{1} th^{1} st^{1} cu^{1} sr^{1} e^{s} stg^{4} ca^{1}/TM3, Sb^{1} Ser^{1}	Suppressor
polo	77B2--3	Dp(1;Y)B[S]; ru^{1} st^{1} polo^{1} e^{s} ca^{1}/TM3, Sb	Suppressor
		P{SUPor-P}polo$^{16-1}$/MKRS	Suppressor
		P{PZ}polo^{01673} ry^{506}/TM3, ry^{RK} Sb^{1} Ser	Suppressor
		y^{1}; P{SUPor-P}polo^{KG03033} ry^{506}/TM3, Sb^{1} Ser^{1}	Suppressor
II. Proteolysis			
Acer	29D4	y^{1} w^{67c23}; P{lacW}Acer^{k07704}/CyO	Suppressor

Morula	60A15	y^1 w^{67c23}; P{SUPor-P}$mr^{KG07756}$	Enhancer
		e$(mr)^1$; mr^2/SM6a	Enhancer
		px^1 bw^1 mr^1 sp^1/In(2LR)bw^{V1}, ds^{33k} bw^{V1}	Enhancer
nonstop	75D4	w^{1118}; P{XP}not^{d06314}/TM6B, Tb^1	Suppressor
		P{ry[+t7.2]=PZ}$not^{[02069]}$ $ry^{[506]}$/TM6B, ry[CB] Tb[+]	Suppressor
		$y^{[1]}$ $w^{[67c23]}$; P{y[+t7.7] w[+mC]=wHy}$not^{[DG24306]}$/TM3, Sb[1]	Suppressor
slamdance	97D8	y^1 w^{1118}; PBac{5HPw$^+$}sda^{A427}	Suppressor
		y^1 w^{67c23}; P{EPgy2}$sda^{EY11539}$	Suppressor
Tbp-1	95B1	ry^{506} P{PZ}04210a P{PZ}Tbp-1^{04210b}/TM3, ry^{RK} Sb^1 Ser^1	Suppressor
Ubiquitin Conjugating Enzyme (UbcD6)	82E3--4	y^1 w^{67c23}; P{EPgy2}UbcD$6^{EY04634}$/TM3, Sb^1 Ser^1	Suppressor
III. Signal Transduction			
Delta	92A1--2	Dl^3/In(3R)C, $sprd^1$ e	Enhancer
		Dl^{11}/In(3R)P, Dfd^1 ca	Enhancer
		ss^1 Dl^{6B} e^1/TM6C, Sb	Enhancer
		Dl^{RF}/TM6C, Sb	Enhancer
		ry^{506} P{PZ}Dl^{05151}/TM3, ry^{RK} Sb^1 Ser	Enhancer
ebi	21C2	y^1 w^{67c23}; P{lacW}ebi^{k16213}/CyO	Enhancer
		$w^{[1118]}$; $ebi^{[WKS-24]}$/CyO, P{w[+mC]=GMR-p21.Ex}2	Enhancer
ksr	83A5	ksr^{S-627}/TM3, P{sevRas1.V12}FK2, Sb	Enhancer
		w^{1118}; P{lacW}ksr^{j5E2}/TM3, P{ftz/lacC}SC1, ry^{RK} Sb^1 Ser^1	Suppressor

Lis-1	52F5--7	^{1} w^{67c23}; P{lacW}Lis-1^{k13209}/CyO	Suppressor
		y^{1} w^{67c23}; P{EPgy2}Lis-1^{EY11274}	Suppressor
loco	94B6--8	P{lacZ-un1}locorC56	Suppressor
		y^{1}; ry^{506} P{SUPor-P}loco^{KG02176}/TM3, Sb^{1} Ser^{1}	
pointed	94E2--E13	pnt$^{\Delta 88}$/TM3, Sb^{1} (amorph)	Enhancer
		ru^{1} h^{1} th^{1} st^{1} cu^{1} sr^{1} e^{s} pnt^{2} ca^{1}/TM3, Sb^{1} Ser^{1}	Enhancer
		ry^{506} P{PZ}pnt^{07825}/TM3, ry^{RK} Sb^{1} Ser^{1}	Enhancer
		y^{1}; ry^{506} P{SUPor-P}pnt^{KG04968}/TM3, Sb^{1} Ser	Enhancer
spatzle	97E1-	spz^{2} ca^{1}/TM1	Suppressor
		y^{1} w^{67c23}; ry^{506} P{SUPor-P}spz^{KG05402}	Suppressor
Star	21E4-	y^{1} w^{67c23}; P{lacW}S^{k09530}/CyO	Enhancer
		al$^{[1]}$ S$^{[1]}$ ast$^{[1]}$ dpp$^{[d-ho}$]/SM1	Enhancer
		ex^{1} ds^{1} S^{X} ast^{X}/SM1	Enhancer
twins	85F13--14	P{PZ}tws^{02414} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}	Enhancer
IV.Transcription Regulation			
Aly	84B2	P{PZ}Aly^{02267} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}	Suppressor
branchless	92B2--3	ry^{506} P{PZ}bnl^{00857}/TM3, Sb	Enhancer
		y^{1} w^{67c23}; ry^{506} P{SUPor-P}bnl^{KG00157}/TM3, Sb^{1} Ser	Enhancer
bric a brac 1 (bab1)	61E2--F1	w*; P{GawB}bab1$^{Pgal4-2}$/TM6B, Tb^{1}	Suppressor
		w$^{[*]}$; P{w[+mW.hs]=GawB}bab1$^{[Agal4-5]}$/TM3, Sb$^{[1]}$	Suppressor
cap n collar	94E4--7	ry^{506} P{PZ}cnc^{03921}/TM3, ry^{RK} Sb^{1} Ser^{1}	Suppressor
		y^{1} w^{67c23}; P{wHy}cnc^{DG05305}	Suppressor

mod(mdg4)	93D7--9	y^1 w^{1118}; P{lacW}mod(mdg4)^{L3101}/TM3, Ser	Suppressor
		ry^{506} P{PZ}mod(mdg4)^{03852}/TM3, ry^{RK} Sb^1 Ser^1	Suppressor
target of Poxn (tap)	74A5	tap^{01658} P{PZ}$blot^{01658}$ ry^{506}/TM3, ry^{RK} Sb^1 Ser	Mild
V. Various functions and Unknown Molecular Function			
CG4080	67B--4	$y^{[1]}$ $w^{[67c23]}$; P{y[+mDint2] w[BR.E.BR]=SUPor-P}CG4080$^{[KG08382]}$ $ry^{[506]}$	Suppressor
		$y^{[1]}$ $w^{[67c23]}$; P{w[+mC] y[+mDint2]=EPgy2}CG4080$^{[EY01375]}$/TM3, $Sb^{[1]}$ $Ser^{[1]}$	Suppressor
Ecdysone-induced protein 78C (Eip 78C)	78C2--3	cn^1; In(3L)8h, pur^2 Eip78C^{8h} ry	Suppressor
		$y^{[1]}$; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Eip78C$^{[KG03241]}$ $ry^{[506]}$	Suppressor
like-AP180 (lap)	84C6	y^1; P{SUPor-P}lap^{KG06751} ry^{506}/TM3, Sb^1 Ser	Suppressor
		y^1 w^{67c23}; P{EPgy2}lap^{EY11719} P{EPgy2}^{EY11719}	Suppressor
l(2)k08110	39F1--3	y^1 w^{67c23}; P{lacW}l(2)k08110^{k08110}/CyO	Suppressor
heix		b^1 $heix^1$ pr^1 cn^1 wx^{wxt} bw^1/CyO	Enhancer
Ribosomal protein LP0	79B2	P{PZ}RpLP0^{01544} ry^{506}/TM3, ry^{RK} Sb^1 Ser	Suppressor
RpS26	36F4	P{PZ}RpS26^{04553}/CyO; ry^{506}	Suppressor
		y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}RpS26[KG00230]/SM6a	Suppressor
Tropomyosin1 (Tm1)	88E12--13	y^1 w^{1118}; P{lacW}l(3)L7160a^{L7160a}, P{lacW}L7160b, P{lacW}Tm1^{L7160c}/TM3,	Suppressor
		$y^{[1]}$ $w^{[67c23]}$; P{w[+mC] y[+mDint2]=EPgy2}Tm1$^{[EY12089]}$/TM3,	Suppressor

		$Sb^{[1]}$ $Se^{r[1]}$ $ry^{[506]}$ P{ry[+t7.2]=PZ}$Tm1^{[02299]}$/TM3, $ry^{[RK]}$ $Sb^{[1]}$ $Ser^{[1]}$	Suppressor
slowpoke	96A14--17	y^1 w^{1118}; PBac{5HPw$^+$}A459/TM3, Sb^1 Ser	Suppressor
		w^{1118}; PBac{RB}slo^{e03162}	Suppressor
Ugt86Da	86D4	y^1 w^{67c23}; P{SUPor-P}KG01728 ry^{506}	Suppressor
walrus	48C1--2	y^1 w^{67c23}; P{lacW}wal^{k14026}/CyO	Suppressor
		y^1 w^{67c23}; P{wHy}$wal^{DG30703}$	Suppressor
		cn^1 P{PZ}wal^{02516}/CyO; ry^{506}	Suppressor
	35F6--7	y^1 w^{67c23}; P{lacW}$heix^{k11403}$/CyO	Suppressor
14-33ζ	46E6--8	P{PZ}14-3-3ζ^{07103} cn^1/CyO; ry	Suppressor
		P{lArB}14-3-3ζ^{P1375}/CyO; ry^{506}	Suppressor
		y^1 w^{67c23}; P{EPgy2}$CG2269^{EY03325}$ P{EPgy2}14-3-3$\zeta^{EY03325}$	Suppressor
		P{lArB}14-3-3ζ^{P1188}/CyO; ry^{506}	Suppressor

Modifiers of Rap/Fzr are organized into five groups according to function: cell cycle regulation, proteolysis, signal transduction, transcription regulation and various functions and unknown molecular function. In each functional group, genes are further organized alphabetically. The cytological position of interacting loci are also displayed on the second column. Multiple alleles were available for 32 genes and were tested for interaction. Genotypes of alleles tested are displayed on the third column and was obtained from the Bloomington Stock Center.

Table 2. Destruction Box sequence alignments for genes that interact with Rap/Fzr

Gene Name	D-Box Alignment			E-value
Cyclin B	CG2B_DROME	37	RAALGDLQN	1.7e-05
			|||||||||	
	Unknown	37	RAALGDLQN	
		290	RAVLiDwiN	
Cyclin A	CGA2_DROME	160	RSILGVIQSS	.003
			||||||||||	
	Unknown	160	RSILGVIQSS	
slowpoke	gmn_bommo	17	RRSLKTLQ	.0098
			|| | ||	
	Unknown	927	RRewKMLQ	
mod(mdg4)	gmn_bommo	17	RRSLKTLQ	.080
			|| | ||	
	Unknown	599	RRlqKaLQ	
	CGA_hempu	44	RAALGTITNV	.021
			|| | | ||	
	Unknown	54	RAILnTdTNk	
nonstop	gmnH_xenla	33	RRTLKVIQ	.18
			||| ||	
	Unknown	89	RRTaKVak	
cnc	CGB1_Ranja	22	RNALGDIGN	.24
			| | || |	.89
	Unknown	440	eNqLEDITN	
		466	RLPLdELlN	
		762	nAALGDIcp	
	CGA1_soybn	28	RVVLGELPNL	.84
			| | || |	
	Unknown	466	RlPLdELlND	

CG4080	CGA2_lyces	63	RAVLRDVTNV	.26
			| || | | |	
	Unknown	463	RdVLSDqTSV	
	CG2B_DICDI	50	RGALSDLTN	.35
			| ||| |	
	Unknown	463	SRDVLSDqTs	
slamdance	pim_drome	30	KKPLGNLDN	.30
			| || |	
	Unknown	601	KfAyGNmDR	
spatzle	CGA_hempu	44	RAALGTITNV	.30
			| | || |	
	Unknown	48	kAlLATINNG	
	pim_drome	30	KKPLGNLDN	.35
			| | |	
	Unknown	48	KalLaTiNN	
UbcD6	gmn_bommo	17	RRSLKTLQ	.31
			| | ||	
	Unknown	10	mRdfKrLQ	
	CGA2_DROME	160	RSILGVIQSS	.61
			|| |||	
	Unknown	103	sAILTSIQSL	
pnt	gmnH_xenla	33	RRTLKVIQ	.35
			|| || ||	
	Unknown	19415	RRlLKdIQ	
lap	gmnH_xenla	33	RRTLKVIQ	.37
			|| | |	
	Unknown	179	lkTLpVLQ	

Lis-1	gmn_chick	48	RRTLKMIQ || ||	.42
	Unknown	59	dRTiKMwe	
morula	gmnH_xenla	33	RRTLKVIQ || | |	.43
	Unknown	322	RRrLsfwQ	
Delta	gmnH_xenla	33	RRTLKVIQ | || |	.52
	Unknown	65	RvcLKhyQ	
string	gmn_bommo	17	RRSLKTLQ ||||	.59
	Unknown	38	RRSLelms	
		177	RRcLsMte	
		294	hRdLKsIs	
	CGB1_Brarer	36	RAALGEIGN | | | |	.87
	Unknown	225	RCAavEkEN	
twins	CGA1_CARAU	33	RVVLGVLTEN | | | ||	.65
	Unknown	383	RAkLcSLyEN	
encore	gmn_bommo	17	RRSLKTLQ | | ||	.69
	Unknown	612	RpngKMLQ	
Aly	pim_drome	30	KKPLGNLDN ||||	.73
	Unknown	110	RlivGNLDy	
		79	KfPrGdVNs	
Rca1	gmn_bommo	17	RRSLKTLQ | | ||	.81
	Unknown	197	qRdLerLQ	

loco	CGA2_HUMAN	47	RAALAVLKSG	.93
			|| || |	
	Unknown	356	RAqLArLEDQ	

Of the 40 modifiers of Rap/Fzr, 21 genes encode for amino acids that contain destruction box sequences. Genes are organized from low to high e-values. All alignments were performed using D-box finder server (http://bioinfo2.weizmann.ac.il/~danag/d-box/help_page.html#query). Bottom sequence or Unknown, represents the amino acid sequence that was aligned with previously established proteins that contain RXXL sequence.

Table 3. Seven modifiers of Rap/Fzr contain KEN-Box sequence motiff

Gene Name	KEN box Alignment		% Sequence Identity
Cyclin A	CCNA_DROME	13 KEN	100%
	UserSeq2	1 KEN	
		***	
	CCNA_DROME	123 KEN	100%
	UserSeq2	1 KEN	
		***	
Cyclin B	CCNB_DROME	248 KEN	100%
	UserSeq2	1 KEN	
		***	
loco	Q8IN00_DRO	676 KEN	100%
	UserSeq2	1 KEN	
nonstop	Q95TK9_DRO	72 KEN	100%
	UserSeq2	1 KEN	
		***	
		***	
Rca1	P91666_DRO	214 KEN	100%
	UserSeq2	1 KEN	
		***	
string	MPIP_DROME	231 KEN	100%
	UserSeq2	1 KEN	
		***	
UbcD6	UBCD6_DROM	131 KEN	100%
	UserSeq2	1 KEN	
		***	

Seven modifiers of Rap/Fzr contain KEN sequences. All modifiers have 100% sequence identity to a KEN sequence. Genes are organized alphabetically.

Table 4. A- box Sequence Alignment

Gene Name	A-box Alignment			% Sequence Identity
Aly	Q9V3E7_DRO	147	RSLGTADV	50%
	UserSeq2,	2	RILGPSNV * ** *	
Acer	ACER_DROME	322	RALPPS	66.7%
	UserSeq2,	2	RILGPS * * **	
bab1	BAB1_DROME	452	GPSAEPR	66.7%
	UserSeq2,	5	GPSNVPQ ***	
branchless	P91672_DRO	81	LVPSAVSER	55.6%
	UserSeq2,	4	LGPSNVPQR * ** * *	
cap n collar	CNC_DROME	943	MGSERVP	42.9%
	UserSeq2,	4	LGPSNVP * **	
CG4080	Q9VSX7_DRO	193	RILLIQNAPEKI	41.7%
	UserSeq2,	2	RILGPSNVPQRV *** * *	
Cyclin A	CCNA_DROME	54	GNNNVPR	57.1%
	UserSeq2,	5	GPSNVPQ * ***	
Cyclin B	CCNB_DROME	94	GNGAVPPKV	44.4%

	UserSeq2,	5	GPSNVPQRV	
			* ** *	
dally	DALY_DROME	257	DIPQQV	50%
	UserSeq2,	8	NVPQRV	
			** *	
Delta	DL_DROME	373	EKVLTCSDKP	30%
	UserSeq2,	1	QRILGPSNVP	
			* * *	
ebi	EBI_DROME	183	QASTGGSN	50%
	UserSeq2,	1	QRILGPSN	
Eip 78C	E78C_DROME	475	LHPSHLQQQ	44.4%
	UserSeq2,	4	LGPSNVPQR	
			* ** *	
encore	ENC_DROME	1339	GPPTTPQVV	55.6%
	UserSeq2,	5	GPSNVPQRV	
			** ** *	
heix	Q9V3R8_DRO	210	LFGPISV	42.9%
	UserSeq2,	3	ILGPSNV	
			** *	
ksr	Q24170_DRO	299	QTLLSQSHV	44.4%
	UserSeq2,	1	QRILGPSNV	
			* * * *	
lap	Q9V175_PYR	17	PNRVLER	42.9%
	UserSeq2,	6	PSNVPQR	
			* * *	

Lis-1	LIS1_DROME	400	SVDQTV	50%
	UserSeq2,	8	NVPQRV	
			* * *	
loco	Q8IN00_DRO	33	QQQLGQSSPVRR	41.7%
	UserSeq2,	1	QRILGPSNVPQR	
			* ** * *	
mod(mdg4)	MMD4_DROME	92	GEVNVKQ	57.1%
	UserSeq2,	5	GPSNVPQ	
			* **	
morula	Q95TK2_DRO	16	RILHP	80%
	UserSeq2,	2	RILGP	
			*** *	
nonstop	Q95TK9_DRO	52	QRKCDKAFVP	40%
	UserSeq2,	1	QRILGPSNVP	
			** **	
pointed	PNT1_DROME	544	KLTDPDEVARR	27.3%
	UserSeq2,	2	RILGPSNVPQR	
			* * *	
polo	POLO_DROME	10	TDIPDRL	28.6%
	UserSeq2,	7	SNVPQRV	
			* *	
Rca1	P91666_DRO	386	ERLTPPQRAQNR	25%
	UserSeq2,	1	QRILGPSNVPQR	
			* * *	
RpLP0	RLA0_DROME	29	IVGADNV	57.1%

	UserSeq2,	3	ILGPSNV * * **	
RpS26	RS26_DROME	92	RRIRTP	50%
	UserSeq2,	1	QRILGP ** *	
slamdance	Q9VBA3_DRO	662	ERYLLPS	57.1%
	UserSeq2,	1	QRILGPS * * **	
slowpoke	SLO_DROME	84	LGPNDPKQK	44.4%
	UserSeq2,	4	LGPSNVPQR *** *	
spätzle	SPZ_DROME	212	LQPTDVSSRV	50.0%
	UserSeq2,	4	LGPSNVPQRV * * * **	
Star	STAR_DROME	567	QRKIGRNYFYQRL	38.5%
	UserSeq2,	1	QRILGPSNVPQRV ** * **	
string	MPIP_DROME	74	LLSPEGSPQR	50%
	UserSeq2,	3	ILGPSNVPQR * * ***	
tap	TAP_DROME	56	IPQ	66.7%
	UserSeq2,	9	VPQ **	

Tbp-1	Q9V3V6_DRO	239	KLAGPQLV	37.5%
	UserSeq2,	2	RILGPSNV ** *	
Tm1	TPM1_DROME	89	TSIPQ	40%
	UserSeq2,	7	SNVPQ **	
twins	2ABA_DROME	196	IRDPQNV	57.1%
	UserSeq2,	3	ILGPSNV * * **	
UbcD6	UBCD6_DROM	40	IFGPHDTP	50%
	UserSeq2,	3	ILGPSNVP * ** *	
Ugt86Da	Q9VGT3_DRO	420	QKIINNPEATQRV	38.5%
	UserSeq2,	1	QRILGPSNVPQRV * * ***	
14-33ζ	1433Z_DROM	66	SSIEQK	33.3%
	UserSeq2,	7	SNVPQR * *	

Genes that interact with Rap/Fzr encode for amino acids that have sequence identity to the A-box, QRILGPSNVPQRV. The A-Box is another recognition signal for Rap/Fzr. According to SIM alignment tool, 38 modifiers of Rap/Fzr contained 30-50% sequence identity to the A-box. Genes are arranged alphabetically. Top sequence represents amino acid sequence of modifiers. Bottom sequence, UserSeq2, refers to A-box amino acid consensus sequence.

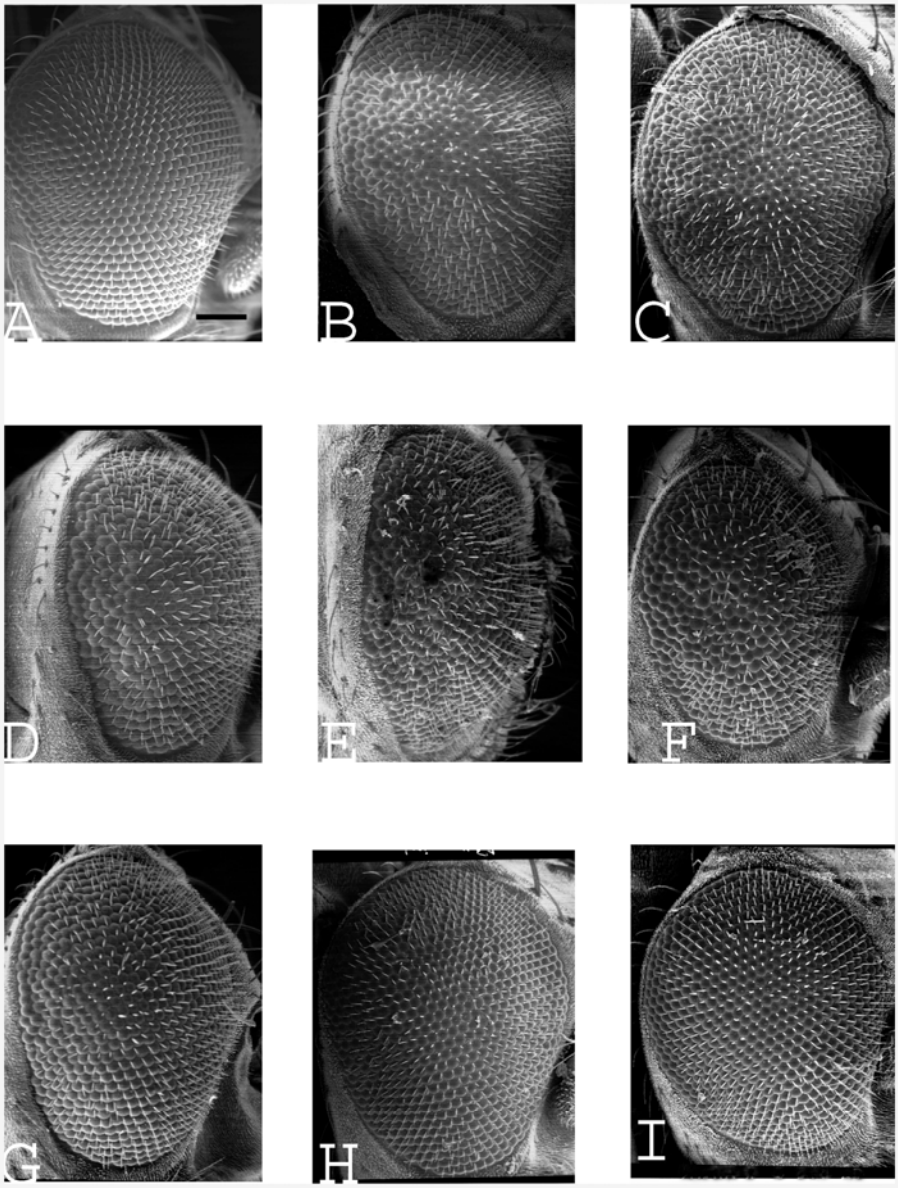
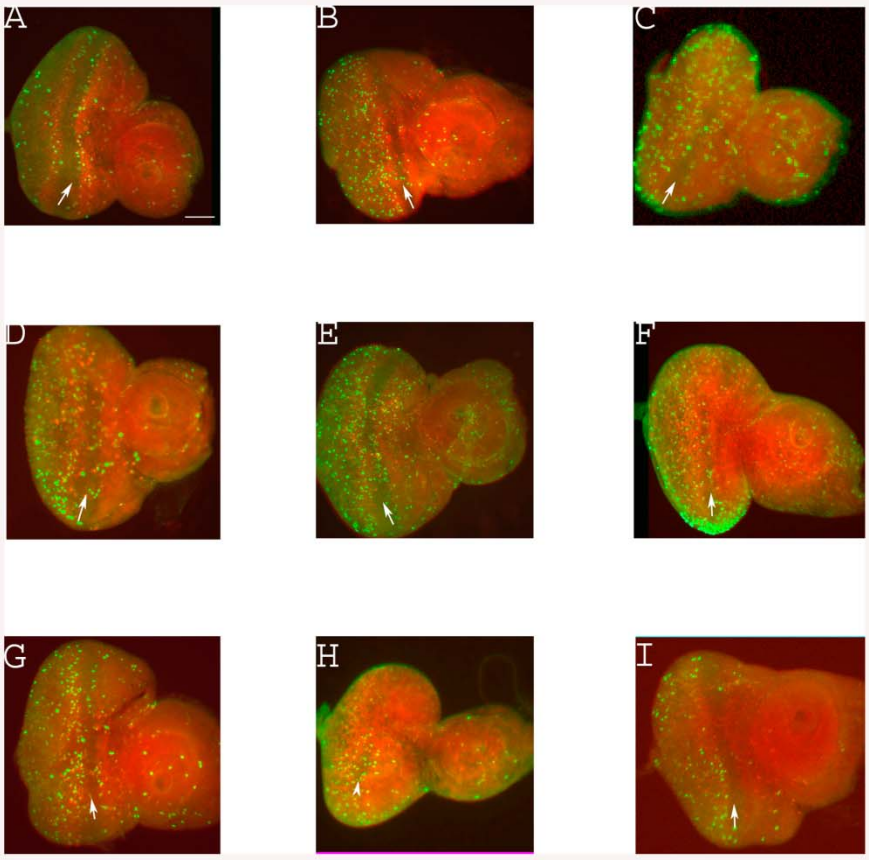


Figure I. Examples of modifiers' phenotypes: Results of scanning electron micrographs from genetic modifier screen. **A**) Smooth appearance of wild type compound eye. **B-C**) *rap* ^{E4}and *rap*^{E6} are two mutant alleles that exhibit a rough eye phenotype due to disorganized arrangement of photoreceptors. **D-F**) Middle panel shows examples of genes categorized as enhancers of Rap/Fzr: *ebi*, *Delta*, and *pointed* respectively. Bottom panel shows examples of suppressors of Rap/Fzr: *Regulator of cyclinA1***(G),** *string***(H)**, and *Cyclin A***(I)**, respectively. Scale bar = 50 μm


A
B
C
D
E
F
G
H
I

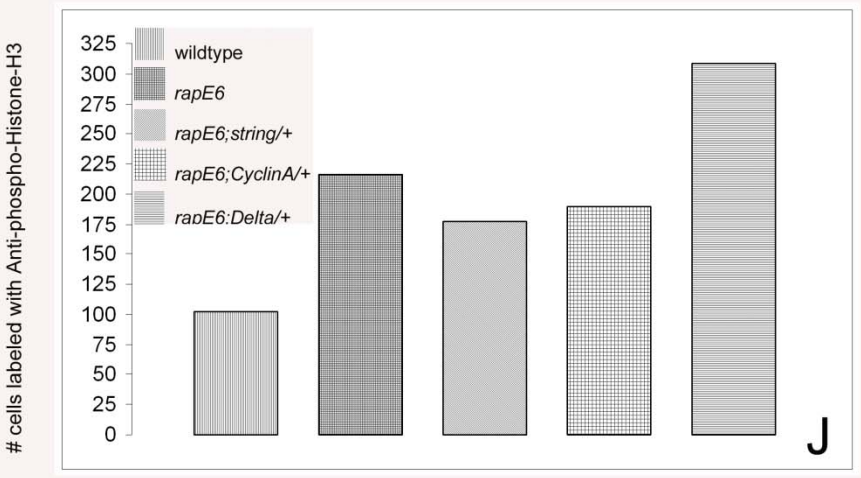

cells labeled with Anti-phospho-Histone-H3
325
300
275
250
225
200
175
150
125
100
75
50
25
0
wildtype
rapE6
rapE6;string/+
rapE6;CyclinA/+
rapE6:Delta/+
J

Figure II. Phenotype of the modifiers in the developing eye. Third instar eye imaginal disks were double stained with anti-cyclin B (red) and mitotic marker anti-phospho Histone-H3 (green) antibodies. **A**) Wild type flies exhibit phospho Histone-H3 and cyclin B staining directly anterior and posterior to the Morphogenetic Furrow (MF). **B-C**) *rap* ^{E4} and *rap*^{E6} are two mutant alleles that exhibit an increased number of cells undergoing mitosis anterior to the MF. **D-F**) Middle panel shows examples of genes categorized as enhancers of Rap/Fzr*: ebi, pointed* and *Delta* respectively. Only *pointed* enhances *rap/fzr* at the level of the eye disk with more phospho-Histone 3 staining anterior and posterior to the MF. **G-I)** Bottom panel are examples of suppressors of *rap/fzr*: *string*, *Regulator of cyclinA1* and *Cyclin A,* respectively. All three genes suppress the phenotype of *rap/fzr* mutants during development since eye disks staining pattern is similar to wild type. Scale bar = 10μm

J) Graphical representation of the number of cells labeled with Anti-phospho-Histone-H3 in third instar eye-imaginal disks. 101 cells were counted for wildtype eye disks. *rap*^{E6} mutant eye disks display 217 cells undergoing mitotis. Examples of suppressors, *Cyclin A* and *string***,** caused an 18% and 10% reduction in cells undergoing mitosis, respectively. *Delta*, acts as dominant enhancer of Rap/Fzr and caused 9% increase in cells labeled with Anti-phospho-Histone-H3.

CHAPTER 2

Regulation of glia number in Drosophila by Rap/Fzr, an activator of the Anaphase Promoting Complex and Loco, an RGS protein

Margarita E. Kaplow, Adam H. Korayem and Tadmiri R. Venkatesh[1]

Department of Biology, City College and The Graduate Center, City University of New York, New York, NY 10031

Running Title: Rap and Loco regulate glia number

Key Words: Differentiation; Drosophila; Glia; Loco; Ubiquitination

[1]Author for correspondence: Tadmiri R. Venkatesh
Department of Biology

City College of New York

New York, NY 10031.

E-mail: venky@sci.ccny.cuny.edu

Genetics. 2008 Apr;178(4):2003-16.

ABSTRACT

Glia mediate a vast array of cellular processes and are critical for nervous system development and function. Despite their immense importance in neurobiology, glia remain understudied and the molecular mechanisms that direct their differentiation are poorly understood. Rap/Fzr is the *Drosophila* homolog of the mammalian Cdh1, a regulatory subunit of the Anaphase promoting complex-cyclosome (APC/C). APC/C is an E3 ubiquitin ligase complex well characterized for its role in cell cycle progression. In this study, we have uncovered a novel cellular role for Rap/Fzr. Loss-of-function of *rap/fzr* leads to a marked increase in the number of glia in the nervous system of third instar larvae. Conversely, ectopic expression of *UAS-rap/fzr* using *repo-GAL4* results in the drastic reduction of glia. Data from clonal analyses using the MARCM (mosaic analysis with a repressible cell marker) technique show that Rap/Fzr regulates the differentiation of surface glia in the developing larval nervous system. Our genetic and biochemical data further show that Rap/Fzr regulates glia differentiation through its interaction with Loco, an RGS (regulators of G-protein signaling) protein and a key effector of glia specification. We propose that in the developing nervous system, Rap/Fzr targets Loco for ubiquitination, thereby regulating glia differentiation.

INTRODUCTION

Glia are major components of the nervous system and are key mediators of several neurodegenerative diseases (Kozuka et al., 2005; Kretzschmar et al., 1997; Qin et al., 2007). In the human brain, glia outnumber neurons by a factor of ten and play a critical role in its response to disease and injury (Edenfeld et al., 2005; Lemke, 2001; Miller, 2005). In addition to their well-known role as homeostatic regulators to provide ionic balance for neurons, glia also play a role in neuroblast proliferation, axon guidance and fasciculation, engulfment of cellular debris, and neurotransmitter uptake (Awasaki and Ito, 2004; Hidalgo et al., 1995; Watts et al., 2004). Despite their importance, however, the molecular mechanisms underlying gliogenesis are poorly understood. The developing nervous system of *Drosophila* offers a superb experimental system for understanding these mechanisms.

In the *Drosophila* embryonic and larval nervous system glia arise from neuroblasts which are pluripotent neural stem cells that serve as precursors for all glia and neurons (Betschinger and Knoblich, 2004; Chia and Yang, 2002; Egger et al., 2007; Jan and Jan, 2001; Slack et al., 2006; Yu et al., 2006). Neuroblasts divide asymmetrically, giving rise to two daughter cells of unequal size. The smaller daughter cell or ganglion mother cell,

GMC, differentiates into a neuron or a glia cell, while the larger cell is capable of self-renewal and has the potential to give rise to additional differentiated cells (Campos-Ortega, 1997; Edenfeld et al., 2002; Skeath and Thor, 2003; Udolph et al., 1993; Urbach et al., 2003). A binary mode of regulation is thought to operate within embryonic neuroblasts, wherein neuroblasts give rise to either neurons or glia. Glia differentiation requires the activation of glia specific effector genes as well as the repression of a neuronal differentiation program (Akiyama-Oda et al., 2000; Badenhorst, 2001; Giesen et al., 1997; Jones, 2005). The transcription factor Gcm (Glial cells missing) has been shown to be a key regulator of glia specification. *Drosophila* embryos lacking Gcm function show a reduction of glia cells while ectopic expression of Gcm causes the formation of extra glia (Bernardoni et al., 1998; Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Gcm activates the transcription of downstream target genes such as *pointed* (*pnt)* and *reversed polarity* (*repo*). Pnt and Repo together in turn promote glia cell fate specification by activating the transcription of the downstream effector *loco* (Granderath et al., 2000; Jones, 2005; Yuasa et al., 2003). Thus, activation of *loco* is a critical step in glia differentiation. Because Gcm also functions to promote lamina neuron formation in the postembryonic larval nervous system (Chotard et al., 2005; Soustelle and

Giangrande, 2007) the key step in glia specification and terminal differentiation is the activation of downstream effectors such as Pnt, Repo and Loco; and the suppression of neurogenic genes such as *atonal*, *asense* and *deadpan*. Thus, the mechanisms that regulate the cellular levels of Pnt, Repo and Loco would be expected to play a critical role in gliogenesis.

loco encodes an RGS (regulator of G protein signaling) domain protein (Granderath et al., 1999). In mammalian systems RGS domain proteins function as GTPase activating proteins (GAPs) and modulate G-protein signaling by promoting GTP hydrolysis (Han et al., 2006). In *Drosophila* however, Yu et al (2005), have shown that through its interaction with Gαi, Loco may function as a guanine nucleotide inhibitor (GDI) and as a GAP.

Disruption of *loco* gene function leads to multiple defects during development in *Drosophila* (Granderath et al., 1999; Pathirana et al., 2001; Yu et al., 2005). In *loco* loss-of-function mutant embryos, failure of glia differentiation leads to improper ensheathing of neuronal axons and consequently the blood brain barrier is disrupted (Granderath et al., 1999; Schwabe et al., 2005). Biochemical studies in the *Drosophila* embryos have shown that Loco regulates these processes by direct physical interaction with G protein subunits (Schwabe et al., 2005).

While the molecular and genetic mechanisms that control asymmetric division of *Drosophila* embryonic neuroblasts have been studied extensively (Betschinger and Knoblich, 2004; Chia and Yang, 2002; Jan and Jan, 1998; Jan and Jan, 2001; Pearson and Doe, 2003), the genetic components that regulate asymmetric division of neuroblasts in the postembryonic nervous system are less well understood. Recently, cell cycle regulators and tumor suppressors, such as, the *lethal giant larvae* (Lee et al, 2006), *brain tumor* (Bello et. al, 2006), *aurora-A Kinase*, *polo*, and *imaginal disks arrested* (Lee et al., 2006a; Slack, 2007; Slack et al., 2006; Wang et al., 2007) have also been found to regulate of neuroblast division during the development of the postembryonic nervous system.

Rap/Fzr (Retina aberrant in pattern/Fizzy related) is a regulatory component of the APC/C and functions to regulate mitotic cell cycle progression (Jacobs et al., 2002; Pimentel and Venkatesh, 2005a). *rap/fzr* mutants have a rough eye phenotype (Karpilow et al., 1989a). Here we show that in the developing larval nervous system loss of Rap/Fzr leads to an increase in glia and, conversely, targeted overexpression of Rap/Fzr leads to a dramatic decrease in glia number. Furthermore, our clonal analysis data derived by the MARCM technique, demonstrates that the Rap/Fzr functions to regulate surface glia lineages. Genetic and biochemical data presented

here suggests that Rap/Fzr regulates gliogenesis through its interaction with Loco, a key effector of gliogenesis. We propose that Rap/Fzr targets Loco for ubiquitination by the APC/C and eventual proteosomal degradation. We show that Rap/Fzr and Loco physically interact in tissue extracts and that Loco is ubiquitinated in vivo. Our results uncover a novel functional role for *rap/fzr* and supports the idea that ubiquitination is a key post-translational regulatory mechanism during glia differentiation.

MATERIALS AND METHODS

Fly stocks and genetic experiments

The following mutant flies were used: *wrap3* and rap^{E6} which have been previously described (Jacobs et al., 2002; Karpilow et al., 1989a), and $loco^{P452}$ which has been previously described (Yu et al., 2005). Enhancer trap lines, *P{lacZ-un1}*$loco^{rC56}$ w^{67c23}, *P{lacW}*rap^{G0326}/*FM7c*, and *repo-GAL4/TM6;Tb* were obtained from the Bloomington Stock Center. *repo-GAL4/TM6;Tb* flies were used to drive expression of *UAS- rap/fzr*, *UAS-loco-GFP* (gift from Dr.Ulrike Gaul). *rap/fzr* mosaic clones were generated using FLP-mediated mitotic recombination (Xu and Rubin, 1993). rap^{G0326}*P{neoFRT}18A/FM7* or *rap/fzr*^{8F3} *P{neoFRT}19A/FM7* (kind gift from Dr. Christian Klambt) recombinants were crossed to *P{neoFRT}18A arm-lacZ*; *hsFLP* (kind gift

from Dr. Ting Xie) and *P{neoFRT}19A arm-lacZ*; *hsFLP* (kind gift from Dr. Nick Baker). .

MARCM clones were generated by the method described by (Lee and Luo, 1999) For generating MARCM clones the following fly stock were used http://flystocks.bio.indiana.edu/Reports/5134.html*P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]; P{UAS-mCD8::GFP.L}LL5/+;repoGAL/+*, *P{neoFRT}19A arm-lacZ/+*; *UAS-rap/fzr/+*, and *rap/fzr*^{8F3} *P{neoFRT}19A/FM7*. For MARCM and FLP/FRT mediated recombination, first and second instar larvae were collected in a separate vials and heat pulsed at 37°C twice at 2 hour intervals (Slack et al., 2006). Eye disc-brain complexes from female third instar larval brains were dissected for all mosaic experiments

Immunonohistochemistry

Whole mount preparations of third instar larval eye discs and brains were stained with the appropriate antibodies and counterstained with fluorescent secondary antibodies as described previously (Pimentel and Venkatesh, 2005a; Pimentel and Venkatesh, 2005b). Anti-Repo mouse (8D12) and anti-Dachsund mouse (Mabdac1-1) supernatants were obtained from Developmental Studies Hybridoma Bank and were used at a dilution of 1:5 for all experiments. Anti phospho-Histone H3 rabbit antibody was

purchased from Upstate Biotechnology (Lake Placid, NY) and was diluted 1:100 for experiments. Anti-Loco C1 antibody was a kind gift from Dr. William Chia and was used at a dilution of 1:250. Anti-Miranda antibody was a gift from Dr. Fumio Matsuzaki and was used at a 1:1000 dilution. Anti-Fzr (human) antibody was purchased from Invitrogen and was used at a dilution of 1:10. Anti-β-galactosidase (rabbit) antibody was purchased from eBioscience and was used at a dilution of 1:50. Polyclonal rabbit anti-GFP antibody (Abcam) was used at 1:250 dilution.

Protein isolation and Western Blot Analysis

Wild type embryonic extracts and *UAS-loco-GFP;repo-GAL4* larval extracts were homogenized using immunoprecipitation buffer (10 mM Tris, pH 7.5, 80 mM glycerophosphate, pH 7.3, 20 mM EGTA, pH 8.0, 15 mM $MgCl_2$, 0.5 mM DTT, 2 mM Na_3VO_4, 10% glycerol, 0.1% NP40). 500 µg of protein lysate was precleared using 50 µL of protein IgG beads (eBioscience). 500 µL of precleared lysate was incubated overnight at 4°C with either 1 µL of Loco C1 antibody (Dr.William Chia), 1 µL of anti-GFP (Abcam) or 1 µL of anti-Rap/Fzr (Pimentel and Venkatesh, 2005a) and 50 µL of protein IgG beads. Beads were washed three times with immunoprecipitation buffer before addition of 50 µl of SDS sample buffer (250 mM Tris, 8.2% SDS,

05% bromophenol blue, 40% glycerol and 200 mM of DTT). Immunoprecipitated protein was extracted by boiling protein IgG beads in SDS sample buffer for 5 minutes.

Protein from larvae were separated by SDS-PAGE using a 12% resolving gel and 5% stacking gel. SDS-PAGE gel was transferred to 0.45 μm nitrocellulose membrane (Pierce) for 1 hour at 100V at 4°C. Blots were blocked in 5% skim milk dissolved in Tris buffered saline containing 0.2% Tween 20 (TBS-T) for two hours. Membranes were incubated overnight in primary antibodies, using a 1:1000 dilution for polyclonal anti-GFP antibody, polyclonal anti-ubiquitin antibody (Abcam), and anti-Loco C1 antibody. Polyclonal anti-Rap/Fzr antibody was used at a 1:150 dilution. After primary antibody incubations, blots were washed three times in TBS-T for a period of five minutes for each wash. Membranes were then incubated for one hour in secondary antibody using a 1:10000 dilution of anti-rabbit IgG-horseradish peroxidase (Jackson Labs) and a 1:1000 anti-rabbit Trueblot antibody (eBioscience). After secondary antibody incubation, membranes were washed for 10 minutes three times in TBS-T. Blocking solution was used for the dilution of both primary and secondary antibodies. For detection of horseradish peroxidase on immunoblots, ECL Western Blotting Substrate kit was used according to the company's protocol

(Pierce). Due to low expression levels of endogenous Loco and limited availability of anti-Loco antibody, we used extracts from transgenic *Drosophila* strains expressing *UAS-loco-GFP*. Expression of Loco-GFP in glia cells was achieved using the *repo-GAL4* as a driver and anti-GFP antibody was used for all larval immunoprecipitation experiments.

Statistics and Quantitative Analysis

All confocal images for third instar larval brains and eye discs were taken using an LSM 510 (Zeiss) at 40X magnification, with an optimum resolution of .252 nm. The following antibodies were used for counting various cell types: glia cells (anti-Repo), neuroblasts (anti-Miranda), mitotic cells (anti-phosphohistone-H3) and neurons (anti-Dachshund). Volocity image analysis software (Improvision) was used for counting cells in a three dimensional structure. For anti-Dachshund staining, confocal image stacks were acquired using the same settings for all samples, with a gain of 815. Analysis of neuron volume was performed using quantitative fluorescence measurements as previously described by (Cuschieri et al., 2006). Image J software (http://rsb.info.nih.gov/ij/) was used for counting surface glia cells and neurons located on the surface of the brain. The top optical sections of confocal stacks were used for analysis of surface glia. Significance and *p* values were derived by Student's *t*-test .

RESULTS

Rap/Fzr regulates glia differentiation

Although gliogenesis and neurogenesis have been primarily studied during embroyonic development, studies by Pereanu et al. (2005) show a rapid increase in glia number during the third instar larval stage. Furthermore 90% of neurons in the adult CNS of *Drosophila* are generated during the third instar larval stage (Arama et al., 2000; Bello et al., 2007; Bello et al., 2006). During the third instar larval stage, glia differentiation occurs in the optic stalk and glia precursor cell areas (GPCs), which are located in the dorsal and ventral margins of the optic ganglion (Dearborn and Kunes, 2004; Poeck et al., 2001a). The subset of glia found in the developing third instar eye imaginal disc, the retina basal glia (RBG) is born in the optic stalk and migrates into the eye imaginal disc (Choi and Benzer, 1994; Rangarajan et al., 1999). Similarly, glial cells of the optic lobe, lamina glia, epithelial glia, and the marginal glia, are initially born in GPC areas and then subsequently migrate into the lamina plexus (Huang and Kunes, 1998; Perez and Steller, 1996). To test whether Rap/Fzr plays a role in glia differentiation during the third instar larval stage, we stained third instar larval brains from *rap/fzr* loss-of-function mutants (*wrap3*) with the glia-specific marker Repo (Figure 1B). We found a significant increase in

glia number in the *rap/fzr* loss-of-function mutant (*wrap3*) compared to wild type (Canton-S) brains ($p<.028$, $n = 17$, Figures 1D). Conversely, to study the effects of ectopic expression of Rap/Fzr, we employed the UAS-GAL4 system (Brand and Perrimon, 1993) and expressed *UAS-rap/fzr* in glia using the *repo-GAL4* driver. *UAS-rap/fzr; repo-GAL4* larval brains showed a significant decrease in glia number ($p<.00000014$; $n=17$, Figures 1C and Figure 1D) compared to wild type (Figure 1A).

To test whether the observed increase in glia number was due to increase in mitosis we stained larval brains with the mitotic marker anti phospho-Histone H3 antibody and estimated the mitotic index. We did not detect significant changes in the mitotic index of third instar larval brains in *wrap3* mutants ($p >.25$, $n=22$, Figure 1E). Similarly, the decreased number of glia cells in the brains of the gain-of-function, *UAS-rap/fzr; repo-GAL4* animals was not due to a change in the mitotic index compared to wild type brains ($p >.12$, $n=21$, Figure 1E). Similar to our results reported here, Pereanu et al., (2005) also reported a change in glia cell number without a significant change in mitotic index and suggested that the additional glia cells arise from the differentiation of secondary neuro-glioblasts located in the surface of the brain.

The binary model of neuron glia differentiation predicts that a given ganglion mother cell (GMC) differentiates into either a neuron or a glial cell (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). To test whether the observed changes in the glia number in Rap/Fzr mutants were accompanied by corresponding changes in the number of neurons, we used anti-Dachshund antibody (neuronal marker) to estimate the number of neurons in third instar larval brains. Due to the inability to resolve individual cells using Dachshund antibody, voxel intensity from confocal microscope images was used to measure changes in neuron volume (See Materials and Methods). *wrap3* loss-of-function mutants showed a significant decrease of Dachshund staining compared to wild type ($p<.017$, $n=17$; Figure 2A, Figure 2B and Figure 2D). Conversely, ectopic overexpression brains (*UAS-rap/fzr; repo-GAL4*) showed an increase in voxel intensity and hence, anti-Dachshund staining compared to wild type flies ($p<.0003$, $n=15$; Figure 2A, Figure 2C, and Figure 2D). These observed changes in the neuronal number are consistent with the binary switch model for neuron and glia differentiation (Hosoya et al., 1995; Jones et al., 1995). The presence of Rap/Fzr causes a switch wherein a neuroblast precursor differentiates into neuron instead of glia cell.

Rap/Fzr regulates in glia differentiation in a cell autonomous manner

The *rap/fzr* loss-of-function (*wrap3*) mutants used above are strong hypomorphs that express residual levels of Rap/Fzr activity (Korayem et. al, manuscript in preparation). To ascertain the phenotypic effects of a complete loss-of-function mutant and to address the issue whether *rap/fzr* controls glia development in a cell autonomous manner, we generated mosaic tissue with null clones of *rap/fzr*. Mitotic recombination was induced with a null allele of *rap/fzr* (Stiles and Klambt, personal communication) using FLP/FRT (Golic et al., 1997; Xu and Rubin, 1993). Because the *Drosophila* brain is a multi-layered structure, single images throughout a confocal stack were analyzed for β-galactosidase expression. The absence of *armadillo-lacZ* (*arm-lacZ*) staining indicated *rap/fzr* mutant clones and Repo positive cells indicated glia cells. These *rap/fzr* mutant patches showed smaller glia cells and an increased number of Repo positive cells (Figure 3A-C and Figure 3A'-3C' white outline). Within the entire brain structure, results were consistent and showed an increased number of glia cells in areas that lacked *arm-lacZ* expression. Most of the *rap/fzr* null clones generated in the brain were concentrated in areas within the optic lobe.

To test whether the significant decrease in glia number in *UAS-rap/fzr;repo-GAL4* animals was due to apoptosis, we stained third instar

larval brains and eye discs with the apoptosis marker anti-Caspase-3 (Baker and Yu, 2001). The reduction in glia number in ectopic overexpression (*UAS-rap/fzr; repo-GAL4*) brains and eye discs was not correlated with increased apoptosis. In fact, both the eye imaginal discs and brains showed a decrease in the level of apoptosis. In contrast, in loss-of-function *rap/fzr* mutants where glia numbers increase, an increase in apoptosis was observed as evidenced by increased anti-Caspase-3 staining (See Supplementary data, Figure S1). This is consistent with our previous report of an increased rate of apoptosis due to *rap/fzr* loss-of-function in *Drosophila* eye discs (Karpilow et al., 1996). Similarly, mammalian studies show that silencing of APC/C^{Cdh1}, the human homolog of *rap/fzr* induces cell-death (Almeida et al., 2005). Taken together, these data suggest that apoptosis is not a contributing factor to the change in glia number observed in *rap/fzr* mutants.

Because ectopic overexpression of *rap/fzr* in glia caused a drastic reduction in glia cell number, we sought to test whether this result was specific or whether the expression of Rap/Fzr in other cell-types could lead to similar results. We expressed *UAS-rap/fzr* in other cell types employing a variety of other GAL4 drivers. For example, expression of *UAS-rap/fzr* with the pan-neural driver, *elav-GAL4* (Yao and White, 1994) had no effect on the number of neurons in the third larval instar brain and eye imaginal disc.

Similarly, Rap/Fzr expression using other GAL4 drivers had no effect (data not shown). These results suggest that changes in glia number upon targeted expression of Rap/Fzr are not due to non specific effects.

MARCM analysis reveals *rap/fzr* specifically regulates surface glia lineages

To test if *rap/fzr* plays a direct role in suppressing glia cell lineages, MARCM (mosaic analysis with repressible marker) clones (Lee and Luo, 1999) were generated to determine the consequence of *rap/fzr* overexpression at the cellular level. MARCM analysis facilitates the tracking of cell lineages and therefore would determine if *rap/fzr* is able to control the cell fate decision of a given neuroblast. Our data from the MARCM based analysis show that overexpression of *rap/fzr* suppresses the formation of surface glia. In the surface glia lineages, when 31 clones were analyzed from eight larval brains only 74% of the clones were Repo negative while 26% of clones were Repo positive (Figure 4A). However, when MARCM clones which overexpressed *rap/fzr* were generated in the optic lobe lineages or interior regions of the larval brain, neuropile lineages frequently detected the presence of glia cells. To test for effect of the loss-of-function of Rap/Fzr at the single level, we examined 16 MARCM clones from surface regions of five larval brains using the *rap/fzr* loss-of-function

allele, *rap/fzr*^{8F3}. Results from these experiments show that loss of *rap/fzr* leads to the formation of surface glia since 81% of clones were Repo positive (Figure 4B). However, when *rap/fzr* loss-of-function MARCM clones were generated within the neuropile or the optic lobe, lineage analysis shows clones which were not Repo positive. Overall, our MARCM based analysis indicates that overexpression of *rap/fzr* suppresses the generation of surface glia while loss of *rap/fzr* function promotes surface glia formation

Rap/Fzr genetically interacts with Loco, a positive effector of glia differentiation

Our genetic modifier studies identified Loco as a dominant suppressor of the *rap/fzr* rough eye phenotype (Kaplow et al., 2007). Loco plays a critical role in embryonic asymmetric cell division of neuroblasts during gliogenesis and also mediates septate junction formation during glia differentiation (Schwabe et al., 2005; Yu et al., 2005). To test whether Loco interacts with Rap/Fzr to regulate glia differentiation in the larval nervous system, we analyzed third instar larval brains in a loss-of-function mutant allele (*loco*^{p452}). A significant decrease in the glia number was seen in loss-of-function mutant *loco*^{p452} compared to wild type ($p<.002$, $n=15$; Figure 5A, Figure 5B, and Figure 5E). This *loco*^{p452} phenotype is similar to the glia phenotype of the *rap/fzr* ectopic overexpression (*UAS-rap/fzr; repo-GAL4*)

mutant. Our genetic interaction studies showed that a single copy of the *loco* mutation acts as a dominant suppressor of the rough eye phenotype. As with *wrap3* mutants, the larval brains of a second *rap/fzr* allele, rap^{E6}/fzr (a weak hypomorphic allele) animals show a significant increase in glia number compared to wild type ($p<.02$, $n=15$; Figure 5C, Figure 5B, and Figure 5E). To examine whether $loco^{p452}$ could suppress the rap^{E6}/fzr phenotype, we generated rap^{E6}/fzr; $loco^{p452}$ /+ animals. A single copy of the $loco^{p452}$ mutation was able to suppress the rap^{E6}/fzr glia phenotype. Larval brains from rap^{E6}/fzr; $loco^{p452}$ /+ show glia numbers similar to wild type (Figure 5B Figure 5D, and Figure 5E). These results suggest that *rap/fzr* and *loco* genetically interact in a dosage sensitive manner to regulate glia number in the larval brain.

Rap/Fzr and Loco Physically interact:

During cell cycle, Rap/Fzr directly interacts with cellular substrates to target them for ubiquitination by the ubiquitin ligase, APC/C. The Rap/Fzr and a substrate interaction requires either a KEN box or a Destruction box motif (D box: RXXLXXXN) within the substrate (reviewed in, (Pfleger and Kirschner, 2000). Bioinformatic analyses of Loco revealed that it contains RGS protein contains both a KEN box and a Destruction box (Kaplow et al., 2007). To test whether Rap/Fzr and Loco physically interact in vivo, we

performed a series of biochemical experiments. Immunoprecipitation from larval brain lysates (*loco-GFP; repo-GAL4*) showed that Rap/Fzr and Loco are found in a complex (Figure 6A). To further validate our results, we also carried out a reciprocal experiment wherein, Rap/Fzr was immunoprecipitated from larval brain extracts of *UAS-loco-GFP; repo-GAL4*. Western blots probed with anti-Loco-GFP antibody confirmed the presence of Loco in a complex with Rap/Fzr (Figure 6B right panel). These results suggest that Rap/Fzr and Loco physically interact in vivo and Loco is a possible substrate for ubiquitination by the APC/C.

To test whether Loco is ubiquitinated in vivo, third instar brain lysate from *UAS-loco-GFP; repo-GAL4* larvae was immunoprecipitated with anti-Loco-GFP protein; and probed with anti-ubiquitin on Western Blots (Figure 6C). Two proteins bands of 100 kDa and 80 kDa, were detected, which most likely correspond to the Loco protein isoforms, and suggests that the Loco protein isoforms are ubiquitinated (Figure 6C). Taken together, these results suggest that Rap/Fzr and Loco physically interact and that Loco is ubiquitinated in vivo. Furthermore, these data argue that changes in *rap/fzr* gene dosage should be reflected in the cellular levels of Loco protein. To test if this was indeed the case, we examined Loco levels in larval brains of wild type, *rap/fzr* loss-of-function and gain-of-function strains using

antibody staining. Immunoflourescence data show higher levels of Loco in *rap/fzr* loss-of-function mutants compared to wild type (See Supplementary data, Figure S2). Conversely, *UAS-rap/fzr; repo-GAL4* larval brains show weak Loco expression compared to wild type larval brains. These results further support the notion that Rap/Fzr directly targets Loco for ubiquitination followed by proteosomal degradation.

Rap/Fzr and Loco are expressed in a subset of glia in the developing larval brain and eye imaginal disc.

Our results presented above predict that Rap/Fzr and Loco are expressed in the same subset of glial cells. Currently, glia from third instar larval brain have been classified into at least three subtypes based on position, morphology and function:

a) surface glia, b) cortex glia, and c) neuropile glia. Surface glia ensheath the brain and play a role in the formation of the blood brain barrier. Cortex glia form around neurons and neuroblasts while neuropile glia, compartmentalize the neuropile, forming axon tracts for neurites (Chotard et al., 2005; Pereanu et al., 2005; Schwabe et al., 2005). Presently, no markers that distinguish the specific glia subtypes in the brain are available and thus we have used the anti-Repo antibody to label glia cells.

To test our prediction that Rap/Fzr and Loco are expressed in glia cells, we monitored expression patterns of Rap/Fzr and Loco in the third instar larval brains and eye imaginal discs. We used two enhancer trap lines with P-element insertions in *rap/fzr*$^{-P2241}$ and *loco*$^{-rC56}$, respectively and examined the tissue expression patterns of both *rap/fzr* and *loco*. Third instar larval brains from *rap/fzr*$^{-P2241}$ and *loco*$^{-rC56}$ were double labeled with anti-β-galactosidase and the glia specific anti-Repo antibody. Results from *rap/fzr*$^{-P2241}$ staining show strong *rap/fzr* expression on the periphery of the brain localized to surface glia (See Supplementary data, Figure S3). Robust *rap/fzr* expression was also observed in cells in the deeper regions of the brain but lack of Repo staining in these cells suggests that these are non-glial cells (See Supplementary data, Figure S3). Unlike *rap/fzr*$^{-P2241}$, which showed expression predominantly localized to surface glia, *loco* enhancer trap line, *loco*$^{-rC56}$ showed expression within most glia cells, including surface, cortex and neuropile glia (See Supplementary data, Figure S4).

Based on our genetic and biochemical data, we expected colocalization between Rap/Fzr and Loco within third instar larval brains. Double labeling with antibodies against *Drosophila* Loco and human anti-Rap/Fzr/Cdh1 followed by confocal microscopy showed colocalization of

Loco (Green) and Rap/Fzr (Red) on the surface of the brain (See Supplementary data, Figure S5). Strong colocalization between Rap/Fzr and Loco is especially prominent along the ventral edge of the brain, near the margin between brain and ventral ganglion. However, Loco and Rap/Fzr distribution within deeper layers of the larvae brain are disparate. Within the cortex, Rap/Fzr expression is present in the optic lobe and in a dispersed cluster of cells on the ventral side of the brain. Loco staining within the cortex is relatively weak compared to Rap/Fzr staining. Only a few cells on the ventral side of the brain are Loco-positive (See Supplementary data, Figure S5). In the optic lobe, Loco expression is almost undetectable.

To test whether both proteins are expressed within glia cells, wild type larval brains were triple-labeled with anti-Loco (blue), anti-Rap/Fzr (red), and anti-Repo (green) antibodies. Our results showed that in the surface regions of the brain, Rap/Fzr and Loco are localized to the cytoplasmic region of the glia whose nuclei are stained with anti-Repo (Figure 7A-E, arrowhead). When localization of Rap/Fzr, Loco and Repo was analyzed in the deeper layers of the brain, distribution of Loco was very diffuse within the cortex. Strong localization of Rap/Fzr was seen within the optic lobe surrounding marginal, lamina, and epithelia glia (Figure 7A'-E').

In our experiments, the data obtained from *lacZ* enhancer trap expression patterns did not completely agree with the protein localization studies carried out using antibodies. For example, expression of the *loco* enhancer trap line was seen in most Repo-positive cells, including glia cells located in the optic lobe while the anti-Loco antibody was restricted to a subset of glia. Similarly, the *rap/fzr* enhancer trap showed a broader expression pattern while the Rap/Fzr protein expression was predominantly within the surface of the brain. The more limited expression seen with the antibodies may be because the antibodies were generated against a particular epitope restricted to a subset of glia and thus may be not reflect the entire expression pattern observed in the enhancer trap. This discrepancy notwithstanding, both enhancer trap line expression and antibody staining confirm that Rap/Fzr and Loco colocalize within surface glia.

Since *rap/fzr* expression is primarily restricted to surface glia, we examined for changes in surface glia number in *rap/fzr* mutants. Markers that specifically label different glia subtypes are not currently available. For this reason, surface glia were identified based on the position in third instar larval brains (See Materials and Methods). Glia located on the superficial regions of larval brains were analyzed in *rap/fzr* mutants. Results show that *rap/fzr* loss-of-function mutants (*wrap3*) show a significant increase in

surface glia number compared to wild type flies (See Supplementary data, p<.002, n = 13, Figure S6A). Conversely, *UAS-rap/fzr; repo-GAL4* larval brains showed a significant decrease in glia number (See Supplementary data, n=14; p<.0002, Figure S6 A) compared to wild type.

We determined the number of neurons in the region of the brain corresponding to surface glia location in *rap/fzr* mutant larvae. Results show that loss-of-function mutants (*wrap3*) show a significant decrease in surface neurons compared to wild type flies. (See Supplementary data, p<.05, n = 17, Figure S6 B). Reciprocally, *UAS-rap/fzr; repo-GAL4* larval brains showed a significant increase in surface neurons (See Supplementary data, n=13; p<.055, n=12, Figure S6 B) compared to wild type flies.

Rap/Fzr and Loco protein are localized within third instar larval neuroblasts

In addition to their expression in surface glia, Rap/Fzr and Loco are also expressed in a subset of cells that are not Repo-positive. Because Loco has been previously shown to play a role in asymmetric division of neuroblasts (Yu et al., 2005), we tested whether these Repo negative cells represented either neuroblasts or GMCs. The nuclear GMC marker, anti-Prospero and the neuroblast marker, anti-Miranda, were used to label neuroblasts and GMCs located in third instar larval brains. Prospero and

Miranda are widely used markers for studying asymmetric division of neuroblasts (Ceron et al., 2006). Although Prospero shows no colocalization with Rap/Fzr and Loco antibodies, Rap/Fzr and Loco are expressed in a subset of Miranda positive neuroblasts (Figures 8 A,C and D), suggesting that Rap/Fzr and Loco also function early, in neuroblasts.

However, *rap/fzr* loss-of-function (*wrap3*) mutants and wild type third instar larval brains showed no significant changes in neuroblast number compared to wild type as monitored by Miranda staining (Figure 9A and 9B). By contrast, ectopic overexpression of Rap/Fzr in glial cells resulted in a significant increase in the number of neuroblasts as evidenced by the increased number of Miranda positive cells (Figure 9C). These data are consistent with a role for Rap/Fzr and Loco during early stages of neuroblast determination as well as later during glia differentiation. To examine whether ectopic neuroblasts formed in *UAS-rap/fzr; repo-GAL4* larvae had the potential to self-renew and give rise to neurons, which would account for the increased staining with the Dachshund neuronal marker in these animals, wild type, *UAS-rap/fzr; repo-GAL4*, and *wrap3* larvae were pulse fed BrdU for 15 hours after 24, 48 and 96 hours larval hatching. *UAS-rap/fzr; repo-GAL4* larvae incorporated a significant amount of BrdU while *wrap3* BrdU incorporation was similar to wild type larvae (Supplementary data, Figure

S7). Taken together, ectopic neuroblasts formed in *UAS-rap/fzr; repo-GAL4* larvae are proliferative and have the potential to give rise to neurons (data not shown). Although total mitotic index was unaltered in *rap/fzr* mutant larvae (Figure 1E), these results were measured after the larvae have developed to third instar larval stage. Pulse feeding of BrdU shows that cells types such as neurons and glia are newly generated and are the cells which continue to divide.

DISCUSSION

The APC/C is a multi-subunit ubiquitination complex that has been well characterized for its role in regulating mitotic exit (Geley et al., 2001; Sudakin et al., 1995). Rap/Fzr/Cdh1 is an activator of APC/C, and plays a key role in the regulation of mitosis by targeting cell cycle regulators, such as cyclins and cyclin dependent kinases for ubiquitination (Pimentel and Venkatesh, 2005a; Schwab et al., 1997; Sigrist and Lehner, 1997b) . Our data uncover a novel role for Rap/Fzr in the regulation of glia differentiation. Loss-of-function *rap/fzr* mutants display an increase in glia number and a corresponding decrease in neuronal number. Conversely, targeted overexpression of Rap/Fzr in glia leads to a severe reduction in glia number with a corresponding increase in neuronal number. Our clonal analysis data derived from MARCM experiments suggest that Rap/Fzr specifically

regulates differentiation of a subset of glia, the surface glia. Several lines of evidence presented here support the idea that Rap/Fzr regulates gliogenesis by targeting the RGS protein, Loco, for ubiquitination. First, our genetic interaction studies show that a single copy of the *loco* mutation is a dominant suppressor of the *rap/fzr* rough eye phenotype, as well as the glia phenotype in the larval brain. Second, our biochemical data show an interaction between Rap/Fzr and Loco in larval brain tissue and ubiquitination of Loco is in larval extracts. We conclude that Loco is targeted for ubiquitination by Rap/Fzr through either its D-box and/or KEN box motifs, two signature ubiquitination targeting motifs by the APC/C (Burton and Solomon, 2001; Hilioti et al., 2001; Pfleger and Kirschner, 2000). Third, results from immunolocalization experiments show that Rap/Fzr and Loco colocalize within surface glia in the postembryonic larval brain.

Loco has been previously shown to be a positive effector of glia development during *Drosophila* embryogenesis (Granderath et al., 2000; Granderath and Klambt, 2004; Granderath et al., 1999). Recently, Loco has also been reported to have a role during the asymmetric cell division of embryonic neuroblasts (Yu et al., 2005). Our results suggest a new role for Loco in glia differentiation during postembryonic development of

Drosophila CNS. We propose (Figure 10) that the cellular level of Loco in the postembryonic GMC is a key positive effector in the binary switch of glia differentiation. Rap/Fzr negatively regulates glia number by targeting Loco for ubiquitination by the ubiquitin ligase (APC/C) and eventual proteosomal degradation. Our model further predicts that alteration in the *rap/fzr* gene dosage would change cellular levels of Loco, which consequently affects glia number.

Although it is unclear if larval neuroblasts divide with a fixed apical and bsal orientation (Slack, 2007), we show that Loco is expressed with Miranda, a protein normally localized within the basal axis and a key mediator of asymmetric division of embryonic neuroblasts (Ikeshima-Kataoka et al., 1997; Shen et al., 1998; Slack et al., 2006). Although Miranda is used as a specific marker for larval neuroblasts, its entire function has not been completely studied in postembryonic development (Slack et al., 2006). We find that Loco and Miranda colocalize with Rap/Fzr (Figure 7), suggesting a possible functional role for these molecules during postembryonic neuroblast division. This hypothesis is supported by our results which show ectopic neuroblasts and strong incorporation of pulse labeled BrdU when *rap/fzr* is overexpressed (See Supplementary data, Figure S7)

Collectively, our data suggest that Rap/Fzr regulates glia differentiation during two phases of development: initially Rap/Fzr controls the proliferation and self-renewal of dividing neuroblasts, and subsequently, Rap/Fzr regulates the differentiation of GMCs. Our model is consistent with evidence from other studies showing proliferation of larval neuroblasts is controlled by other components of the APC/C (Slack et al., 2006), such as, *ida*, a subunit of the APC/C, and Aurora-A kinase, (Lee et al., 2006a; Slack, 2007; Wang et al., 2006) a known target of APC/C mediated ubiquitination during mitotic progression (Littlepage and Ruderman, 2002). Since work by Slack et. al have shown a possible role for *ida* and in turn, the APC, during neuroblast division, it would be interesting to determine if additional components of the APC have roles during later phases of development, in regulating the differentiation of GMCs. When glia number was analyzed in *morula/APC2* mutants flies, results showed a significant increase in *morula/APC2* loss-of-function mutants similar to *rap/fzr* loss-of-function mutants (data now shown). However, the precise roles of additional components of the APC and the mechanism by which the entire complex functions during glia differentiation have yet to be explored. Aurora-A kinase inhibits neuroblast renewal by disrupting cortical localization of aPKC/Numb (Lee et al., 2006a; Wang et al., 2006). Our results show that

Rap/Fzr is able to regulate neuroblast number, by targeting Loco for degradation. Similarly, Rap/Fzr might target proteins involved in cortical polarity, such as Aurora-A kinase for degradation. Rap/Fzr may also regulate centrosome and spindle orientation, which are critical for neuroblast self-renewal leading to the generation of ectopic neuroblasts (Lee et al., 2006a; Wang et al., 2007).

Compartmentalization of Loco within GMCs may promote a glia cell lineage in larval neuroblasts. Loco is colocalized with Miranda and Rap/Fzr in the basal axis, whereas during asymmetric division of embryonic neuroblasts, Loco expressed in the apical axis and functions together with Gαi and Pins (Yu et al., 2005). Some of the same key players maybe involved in both asymmetric division of larval and embryonic neuroblasts. For example, in mutant embryos lacking both Pins and Loco, neuroblasts divide symmetrically (Yu et al., 2005). In contrast, *pins* mutant clones in the developing larvae show a marked decrease in neuroblast number (Lee et al., 2006b). These results suggest that significant differences exist between embryonic and larval neuroblast divisions. With each embryonic neuroblast division, the neuroblast decreases in size, leading to a maximum of 12 divisions (Bossing et al., 1996; Fuse et al., 2003). By contrast, larval neuroblasts are able to self- renew and grow, a few larval neuroblasts are

able to generate thousands of neurons and glia necessary for adult *Drosophila* CNS (Akong et al., 2002; Ceron et al., 2001; Rolls et al., 2003). Our experiments support a model in which components of the ubiquitin ligase complex APC/C mediate a posttranslational regulatory mechanism critical to the differentiation program and thus influence the binary fate decisions of cells to become either neuron or glia.

During the past two years, other studies have also reported novel roles for the APC/C and its components during nervous system development, independent from its function during cell cycle regulation. Mammalian studies have demonstrated a role for Cdh1 in axon growth, through its interaction with the transcriptional corepressor, SnoN (Konishi et al., 2004; Stegmuller et al., 2006). Furthermore, in vitro cell culture studies using neuroblastoma cell lines and silencing of Cdh1 in postmitotic cerebellar granule neurons demonstrate that the DNA binding protein, Id2 (inhibitor of differentiation 2), is a target for Cdh1-mediated ubiquitination (Lasorella et al., 2006). Our results show that Rap/Fzr is involved in glia differentiation and are consistent with other data which demonstrate that Cdh1, the mammalian homolog of Rap/Fzr, targets transcriptional regulators involved in the differentiation program of the developing nervous system. Thus, in addition to its role in the regulation of cell cycle progression, Rap/Fzr

promotes neuron formation and inhibits gliogenesis. Our studies presented here lend further support to the idea that ubiquitination functions as a key regulatory mechanism during nervous system development.

Acknowledgments:

We are grateful to Dr. Chris Li, Dr. Christian Klambt, Dr. Anu Janakiraman and Dr. Stephanie Kadison for critical reading of the manuscript. We gratefully acknowledge Dr. Klambt and Marion Stiles for sharing data and reagents prior to publication. We are thankful to Daniel Fimiarz (CCNY) for assistance with the confocal microscopy. We thank the Bloomington stock center for the supply of *Drosophila* stocks and the University of Iowa Hybridoma Bank for the antibodies. We are also thankful to Dr. Andrea Brand, Dr. Nick Baker, Dr. William Chia, Dr. Ulrike Gaul, Dr. Angela Giangrande, Dr. Jurgen Knoblich, Dr. Ting Xie, Dr. Manzoor Bhat, Dr. Fumio Matsuzaki, and Dr. Veronica Rodrigues for stocks and reagents. This work was supported by the NIH grants S06GM008168 (TRV) and SG12RR060 (NIH-RCMI). Margarita Kaplow was supported by the NIH-MBRS-RISE program at City College, and the New York City Alliance for Minority Participation. The confocal microscope facility at City College was supported by NIH grant 1S10RR020899-01 (TRV)**.**

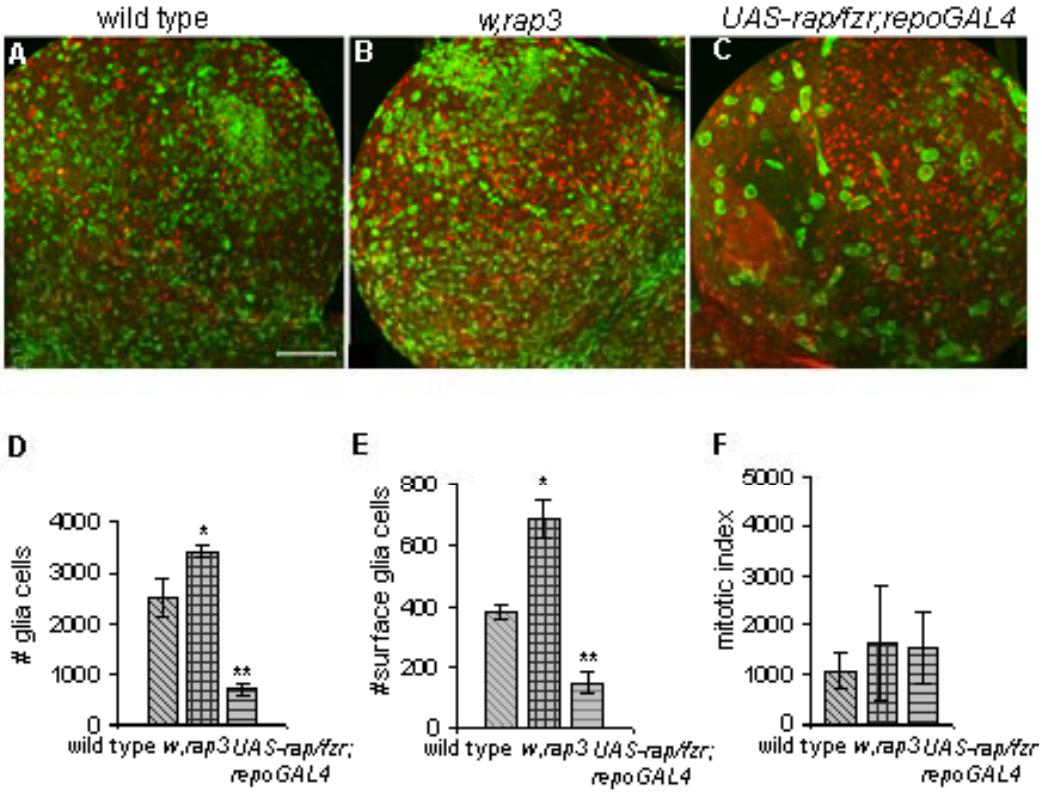
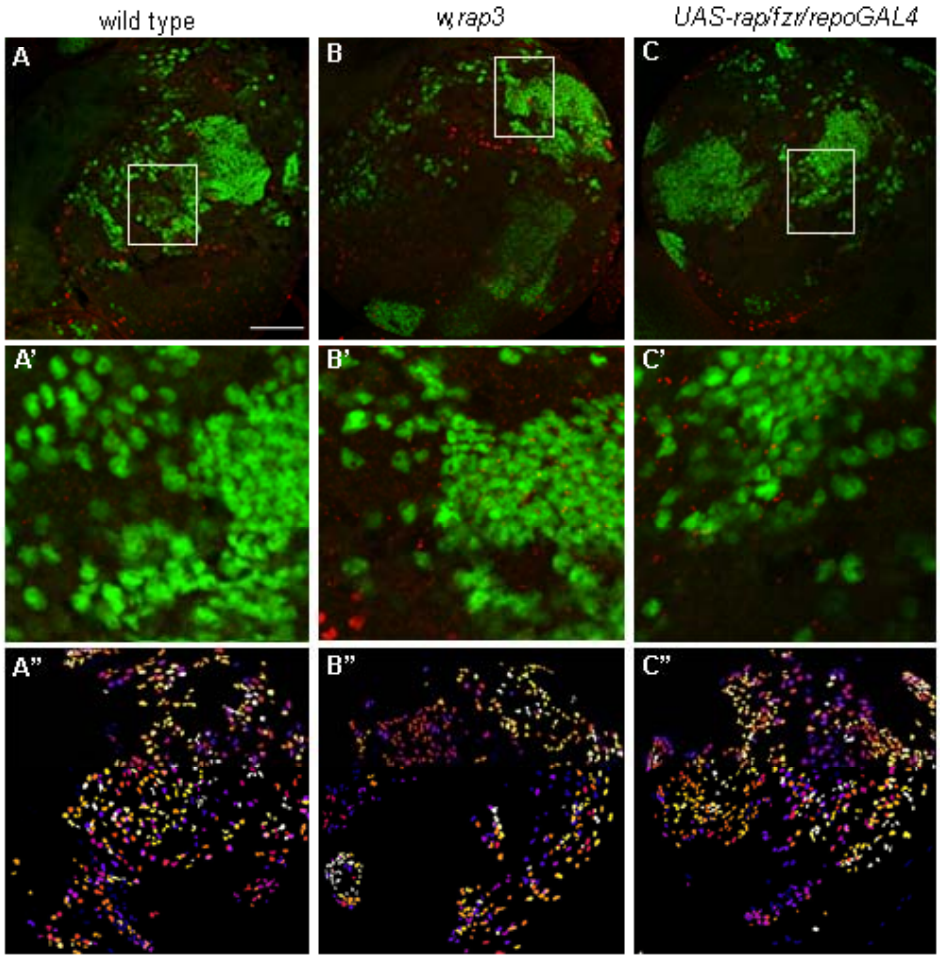


Figure 3. Rap/Fzr regulates glia differentiation in third instar larval brains without altering mitotic index

Larval brains that were stained using the glia specific marker Repo (green) and mitotic marker anti-phospho-Histone H3 (red) (A-C). In third instar larval brain, *rap/fzr* loss-of-function mutant, *wrap3*, (B) shows a significant increase in glia number compared to wild type (A) (glia number for *wrap3* is 3431 cells +/- 375; CS 2390 cells +/- 121, n = 17; p<.028). Conversely, ectopic expression of Rap/Fzr (C) shows a decrease in glia number (glia number for *UAS-rap/fzr; repo-GAL4* is 724 cells +/- 128, n=17; p<.00000014). Although *wrap3* and *UAS-rap/fzr; repo-GAL4* show statistically significant changes in glia number, no significant changes were observed in the mitotic index (*wrap3* 1621 +/- 322; *UAS-rap/fzr;repo-GAL4* 1554 cells +/- 256; CS 1086 +/- 117, n=22, n=21, p >.25 and p >.12). Images are maximum projections taken from LSM 510 confocal microscope. Scale bar, 50 μm. (D) and (E) Graphical representation of glia number and mitotic index in third instar larval brains.

Figure 2. Rap/Fzr promotes neuron formation in third instar larval brain.
(A-C) Third instar larvae brains were stained with the early neuronal differentiation marker, Dachshund (green) and mitotic marker anti-phospho-Histone H3 (red) (A-C). *rap/fzr* loss-of-function mutants, *wrap3* (B) show a significant decrease in neuron density compared to wild type (A) brains (*wrap3* 1.08×10^6 voxels +/- 1.40×10^5; CS 1.62×10^6 voxels +/- 1.41×10^5, n=17 $p<.017$). Ectopic overexpression of Rap/Fzr in *UAS-rap/fzr;repo-GAL4* (C) brains results in an increase in neuronal volume (3.07×10^6 voxels +/- 2.38×10^5, n=15 $p<.0003$). Images are maximum projections taken from LSM 510 confocal microscope. Scale bar, 50 μM.


wild type
w,rap3
UAS-rap/fzr/repoGAL4
A
B
C
A'
B'
C'
A"
B"
C"

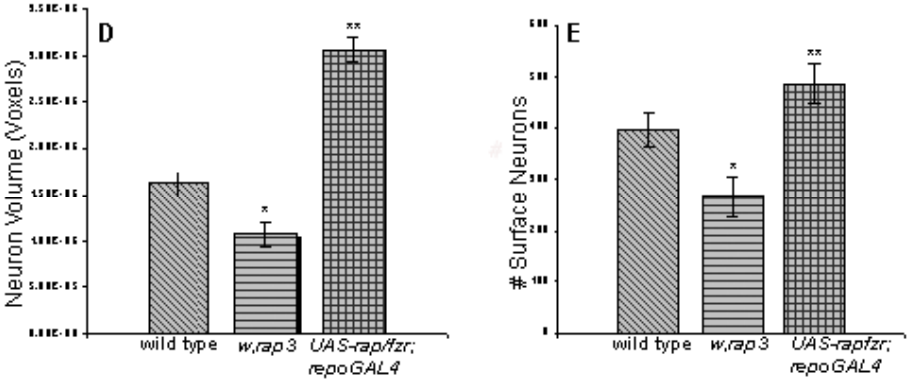

D
Neuron Volume (Voxels)
wild type
w,rap3
UAS-rap/fzr; repoGAL4
E
Surface Neurons
wild type
w,rap3
UAS-rap/fzr; repoGAL4

Figure 2

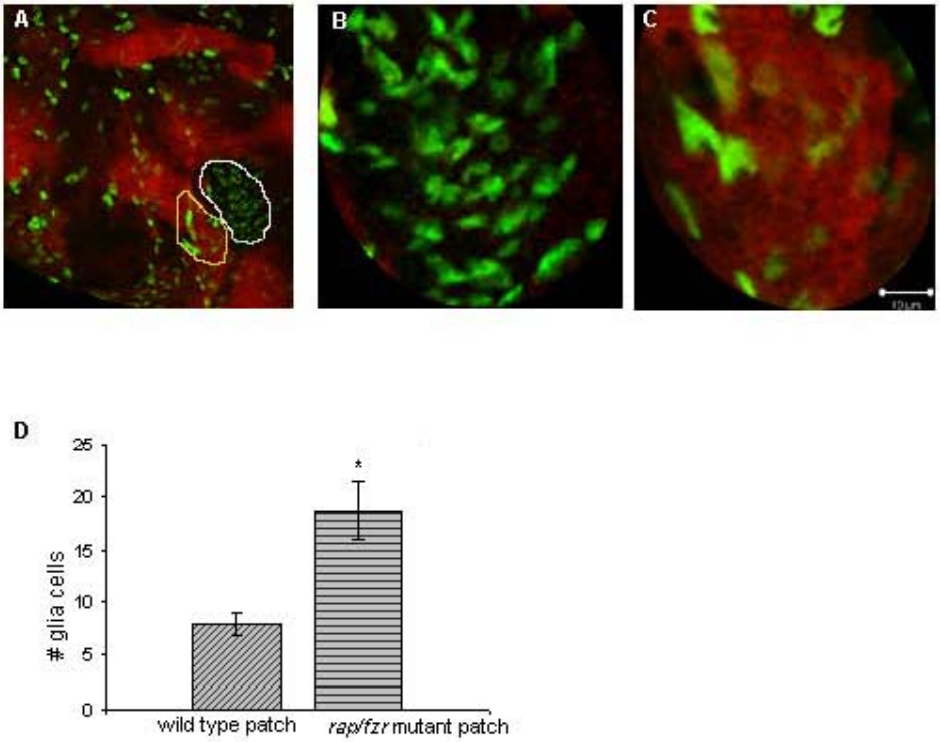


Figure 3. Rap/Fzr functions cell autonomously to regulate glia differentiation.

rap/fzr- null clones were induced by the FLP/FRT technique (see methods for details). Figure shows examples single optical sections from a confocal microscope of mosaic tissue with *rap/fzr*- null clones in the third instar larval brains marked by the lack of anti-β-galactosidase (red) staining. Third instar larval brains were stained with anti-β-galactosidase (red) and anti Repo (green) (A-C and A'-C'. Overlay of images (C, and C') show increased number of Repo positive glia in *rap/fzr*- clones (outlined by a white lines) red anti b-galactosidase staining. Scale bar, 50 μm.

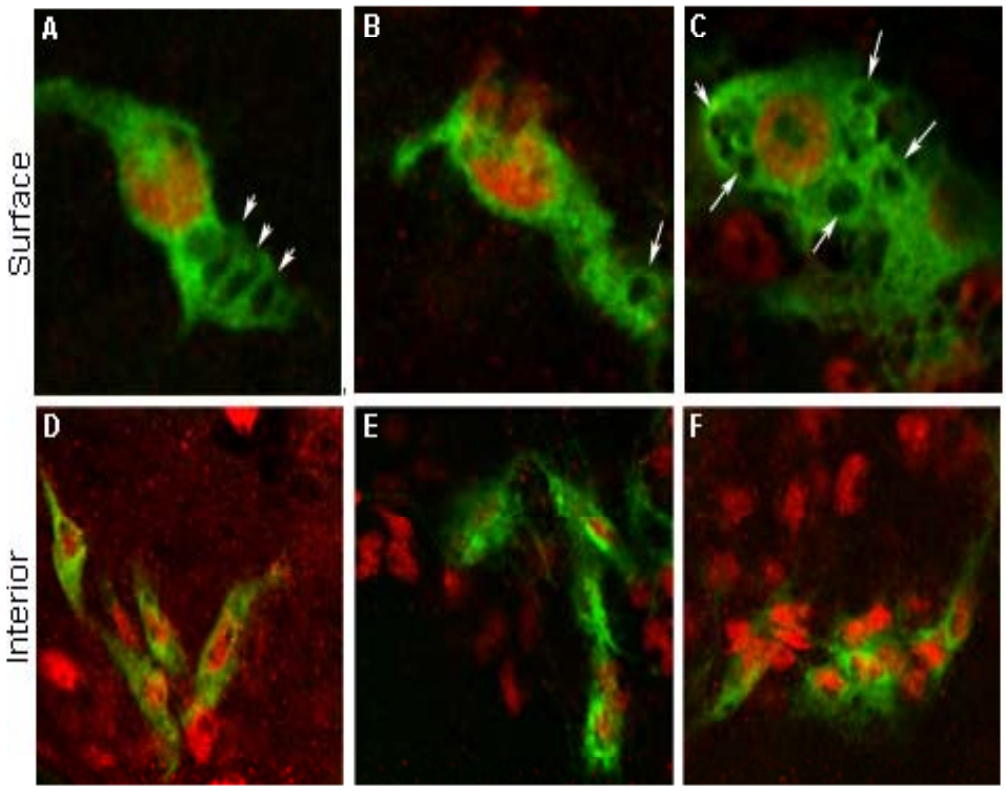


Figure 4. Rap/Fzr regulates development of Surface glia

Single optical sections from a confocal microscope show MARCM clones which are GFP (green) positive. (A) An example of *rap/fzr* overexpression using MARCM based analysis located on the surface of the brain. Several clones do not contain glia cells (red, anti-Repo) arrowhead. (B) *rap/fzr* loss-of-function MARCM clones located within the exterior surface of the brain are Repo-positive cells, arrowheads.
Scale bar, 50 μm.

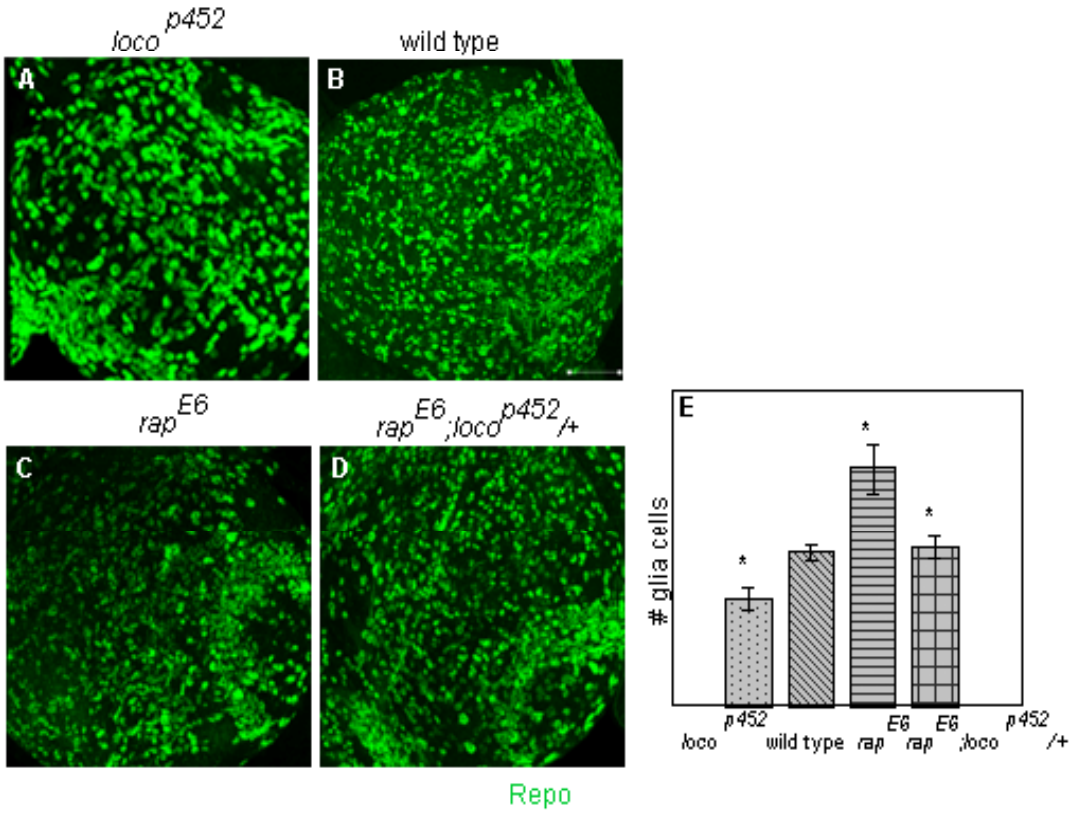


Figure 5. *rap/fzr* and *loco* genetically interact to regulate glia differentiation

Third instar larval brains were dissected and stained with the glia specific marker, Repo. Compared to wild type brains (B), $loco^{P452}$, loss-of-function mutant (A) shows a significant decrease in glia number (1628 +/- 161 Repo positive cells compared to 2390 +/- 126 for wild type, n=15, p<.002). Conversely, the hypormorphic allele rap^{E6} (C) shows a marked increase in glia number compared to wild type brains (3627 +/- 388 Repo positive cells, p<.02, n=15). The rap^{E6} glia phenotype is suppressed with one copy of $loco^{P452}$ (D), (brains analyzed for rap^{E6}; $loco^{P452}$/+, show 2464 +/- 175 Repo positive cells, n=12, p <.03).). Images are maximum projections taken from LSM 510 confocal microscope Scale bar, 50 μm. (E) Graphical representation of glia number in third instar larval brains.

Figure 6. Biochemical evidence shows Loco interacts with Rap/Fzr, and Loco is ubiquitinated in vivo.

A) Protein lysates from *UAS-loco-GFP; repo-GAL4* third instar larval brain-eye complex were used to immunoprecipitate Loco-GFP protein. Western Blot analysis using anti-GFP reveals a band around 100 kDa corresponding to Loco protein in larvae. Negative control shows the absence of Loco protein (Left Panels). Rap/Fzr coimmunoprecipitates with Loco in third instar larval brain-eye complex. A 53 kDa band is detected using Rap/Fzr antibody in larval lysates. Negative Control shows the absence of Rap/Fzr when IgG beads were incubated without GFP antibody (Right panels).

B) Protein extracts from *UAS-loco-GFP; repo-GAL4* third instar larval brain-eye complex were used to immunoprecipitate Rap/Fzr protein. Western Blot analysis using anti-Rap/Fzr reveals a band around 53 kDa corresponding to Rap/Fzr protein in larval brain extracts (left Panels). Negative control shows the absence of Rap protein. Data in the right panels shows that Loco physically interacts with Rap/Fzr in third instar larval brain-eye complex. A 100 kDa band is detected using anti-GFP antibody in larvae, representative of Loco protein. Negative Control shows the absence of Loco protein when IgG beads were incubated without Rap/Fzr antibody.

C) Loco is Ubiquitinated in vivo. Protein lysates from *UAS-loco-GFP; repo-GAL4* third instar larval brains were again used to immunoprecipiate Loco-GFP protein. Western Blot analysis using anti-GFP reveals a band around 100 kDa corresponding to Loco protein in larval extracts (left panel). Negative control shows the absence of Loco protein. Western Blot analysis with GFP-Loco immunoprecipitate reveals the presence of ubiquitin around 80 kDa and 100 kDa (right panel). Small inset (bottom right) represents lighter exposure to visualize bands clearly.

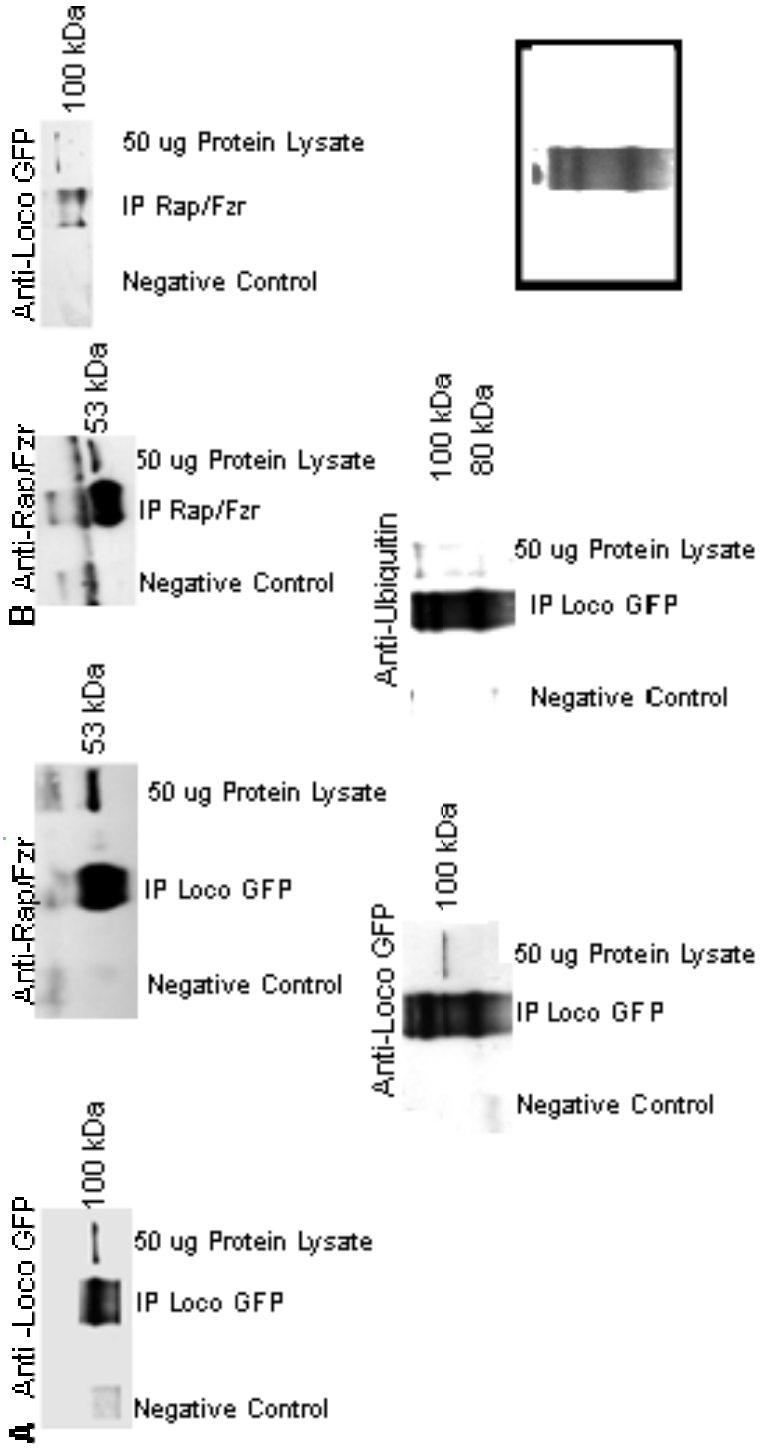

A
Anti -Loco GFP
100 kDa
50 ug Protein Lysate
IP Loco GFP
Negative Control
B
Anti-Rap/Fzr
53 kDa
50 ug Protein Lysate
IP Loco GFP
Negative Control
Anti-Loco GFP
100 kDa
50 ug Protein Lysate
IP Loco GFP
Negative Control
Anti-Rap/Fzr
53 kDa
50 ug Protein Lysate
IP Rap/Fzr
Negative Control
Anti-Ubiquitin
100 kDa
80 kDa
50 ug Protein Lysate
IP Loco GFP
Negative Control
Anti-Loco GFP
100 kDa
50 ug Protein Lysate
IP Rap/Fzr
Negative Control

Figure 6

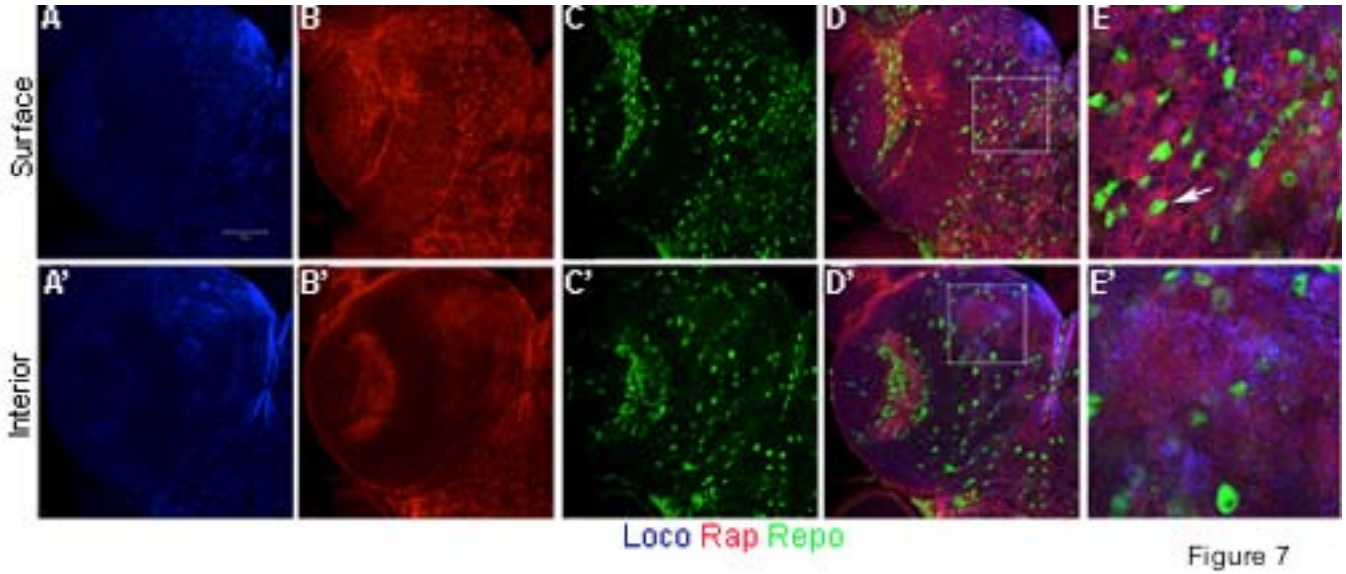


Figure 7. Loco and Rap/Fzr colocalize within a subset of glia cells in third instar larvae brains

Single optical sections from a confocal microscope show Loco (blue), Rap (red) and Repo (green) distribution within the exterior surface of the brain (A-C). Merged image of triple-labeled immunoflurescent brain shows Rap/Fzr (red), Loco (blue) and Repo (Green) localized within a subset of glia cells. Loco and Rap are localized within the cytoplasm of glia indicated by arrowhead (D). Images display the interior of the brain (A'-C'). Loco (Blue) expression is diffuse on the brain's interior and is restricted to the ventral region of the brain. Unlike Loco staining, Rap/Fzr (red) expression is apparent in two regions, the optic lobe and ventral edge of the brain. In the optic lobe, Rap/Fzr colocalizes with Repo staining illustrated by overlay (D') diagram. White boxes in (D) and (D') indicate magnified images displayed in (E and E') diagrams. Scale bar, 50 μm.

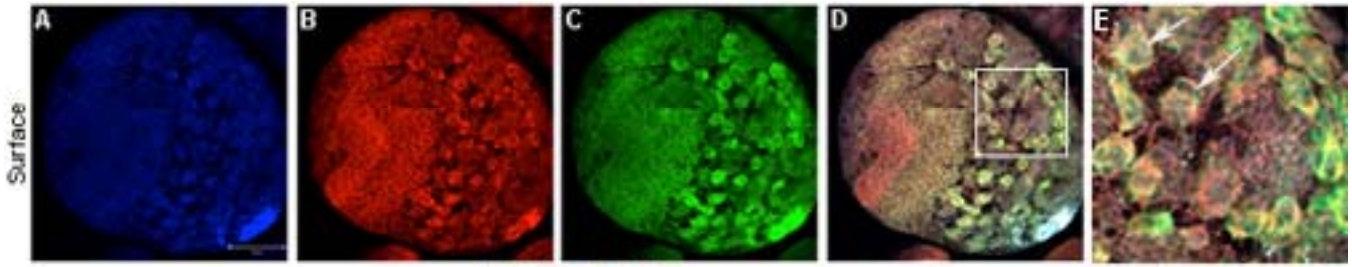


Figure 8. Rap/Fzr and Loco are localized within Neuroblasts located on the brain surface

Single optical sections from a confocal microscope of third instar larval brains showing immunolocalization of, Loco (blue), Rap (red) and Miranda (green) within neuroblasts in the superficial areas (A-C). A merge of the images in (D) shows colocalization of Loco and Rap/Fzr in Miranda-positive cells, confirming Loco and Rap/Fzr expression within neuroblasts (D). White box in (D) indicate magnified image displayed in (E). Scale bar, 50 μm.

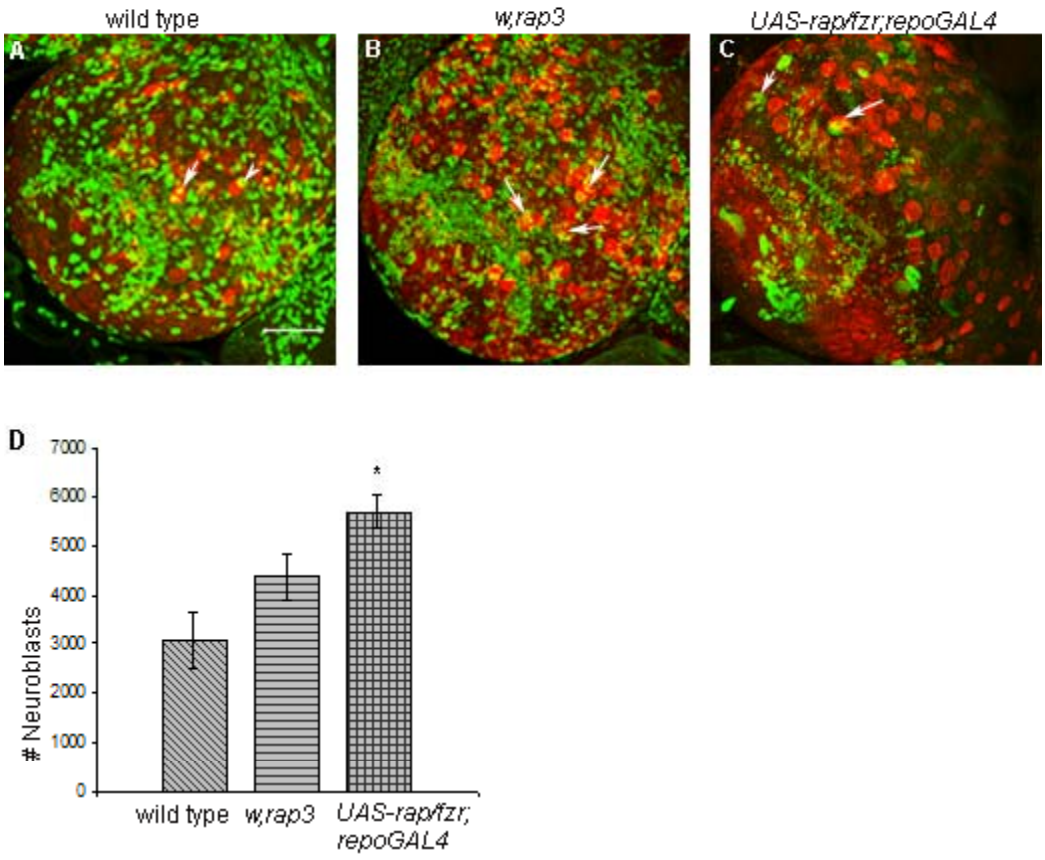


Figure 9. Overexpression of *rap/fzr* generates extra neuroblasts in third instar larvae brains.

Third instar larval brains were labeled with the neuroblast marker, Miranda (red) and Repo (green) antibody. Compared to wild type (A), gain-of-function, *UAS-rap/fzr; repo-GAL4* (C), brains show significant increase in Miranda positive cells (5416 neuroblasts compared to 3067 neuroblasts in normal larval brains, n=15; p <.002). Student *t*-test reveals no significant difference in neuroblast numbers between loss-of -function mutant, *wrap3* (B) and wild type (4356 neuroblast in *wrap3* and 3067 neuroblasts normal larvae, n=15;p<.100) Arrowheads show some Repo positive cells that are also Miranda positive in wild type, *wrap3*, *UAS-rap/fzr;repo-GAL4* larvae. Images are maximum projections taken from LSM 510 confocal microscope. Scale bar, 50 μm Scale bar, 50 μm.

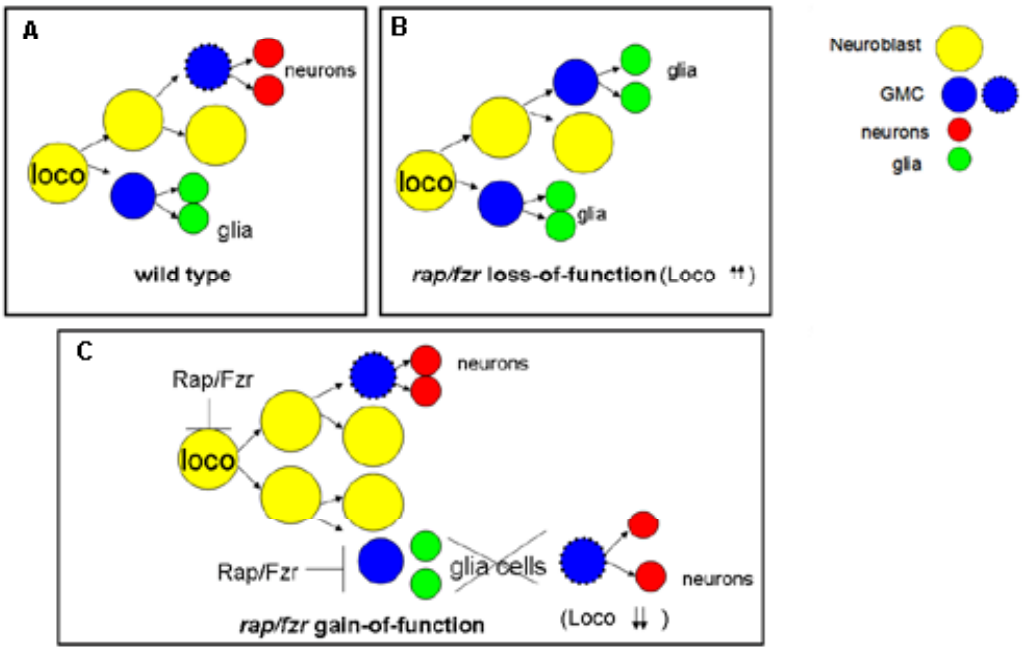
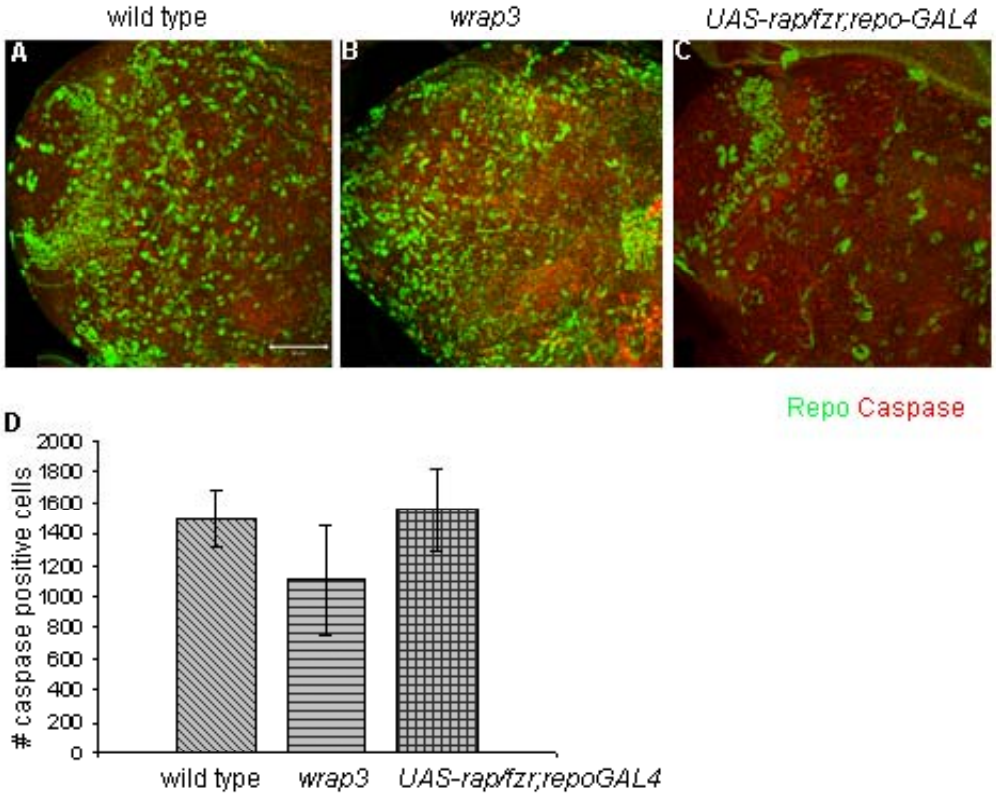


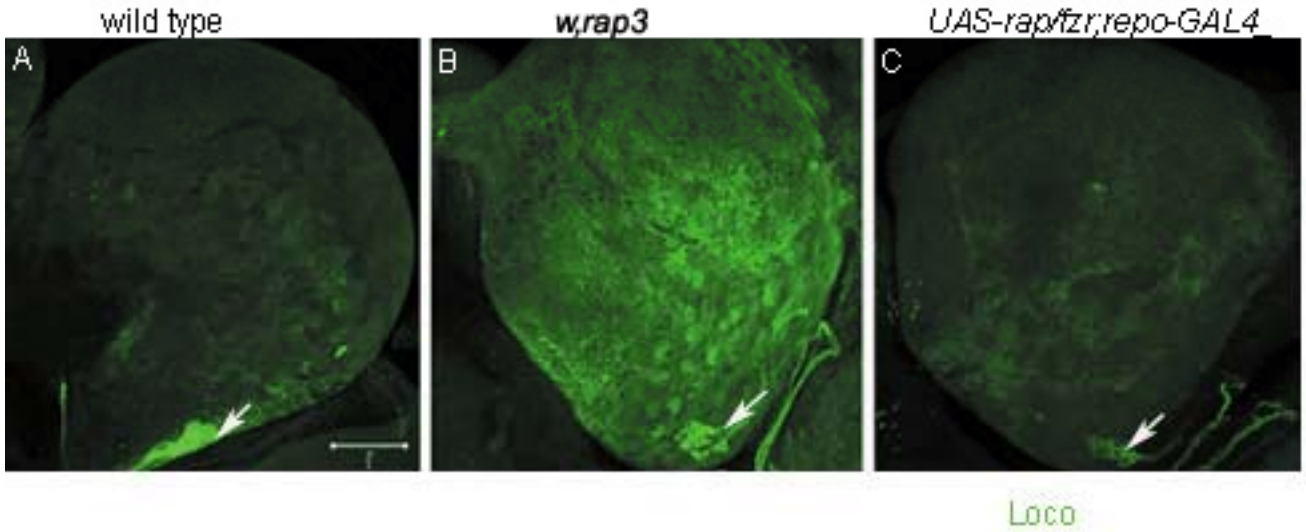
Figure 10. A proposed model for the interaction between Rap/Fzr and Loco.

A. In wild type third instar larvae, neuroblasts located on the exterior surface of the brain divide asymmetrically creating a larger daughter cell a neuroblast, (NB, yellow), and a smaller daughter cell, the ganglion mother cell (GMC, blue). The GMCs will divide once to give rise to either two neurons (red) or two glia (green). Rap/Fzr and Loco are localized within the neuroblasts and function to control neuroblast and glia number. Rap/Fzr targets Loco for ubiquitination by APC/C and regulates its levels. B. *rap/fzr* loss-of-function leads to elevated levels of Loco. Consequently, Loco accumulation within the GMCs leads to an increase in glia number. When Rap/Fzr is overexpressed (C) during third instar larval stage, Loco is targeted for degradation leading to an increase in neuroblast number. Furthermore, destruction of Loco within the GMCs leads to the formation of neurons.



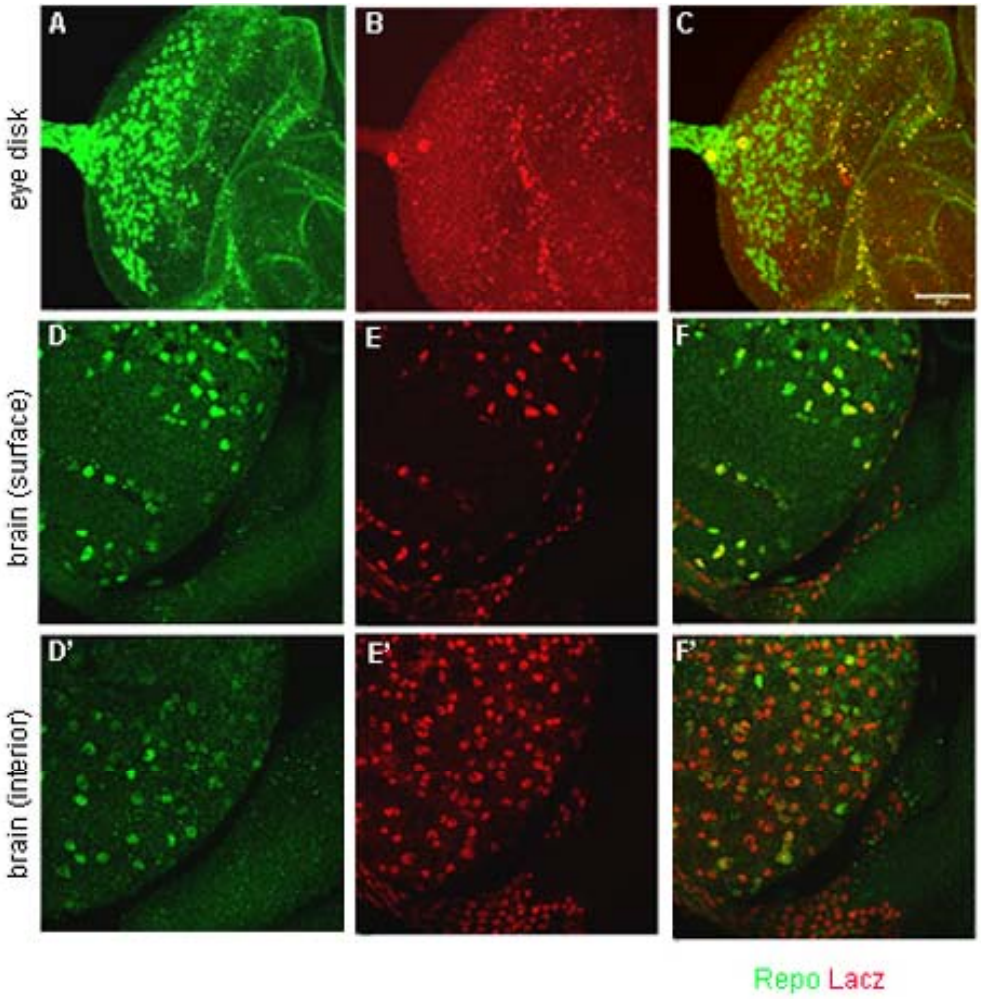
Supplementary Figure 1. Reduction in glia number in *rap/fzr* gain-of-function mutants is not due apoptosis.

The cell death marker, Caspase-3 (red), and the glia specific marker Repo (green) were used to stain third instar larvae brains and eye imaginal discs (A-F). Although *wrap3* loss of function mutants show an increase in cell death in the eye imaginal disc (C) and brain (F), *UAS-rap/fzr;repo-GAL4* (B and E) mutants show similar levels of Caspase-3 staining compared to A) wild type eye disc and brain(D). Scale bar, 50 μm



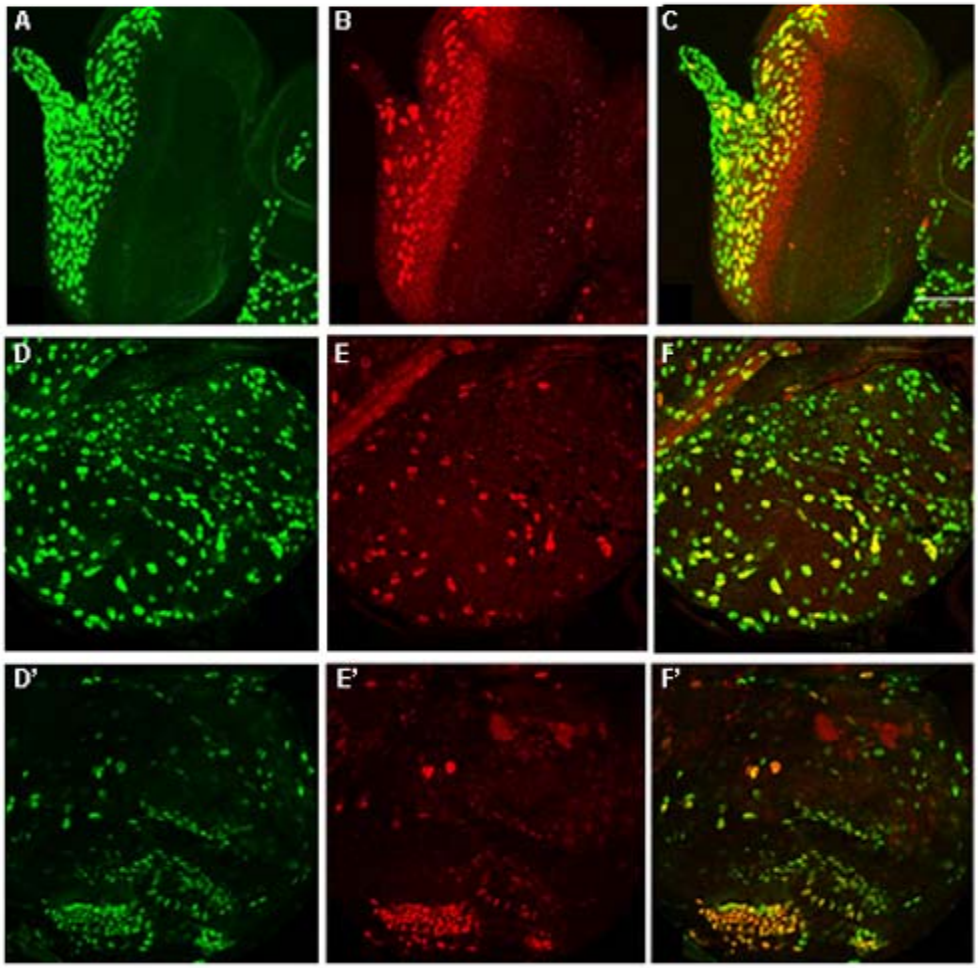
Supplementary Figure 2. Loco expression is elevated in *rap/fzr* loss-of-function mutants.

Loco expression (green) was analyzed in wild type (A), *wrap3* (B) loss-of-function mutants and *UAS-rap/fzr;repo-GAL4* (C), third instar larval brains. Loco protein levels are higher in *wrap3* (B) compared to wild type (A) brains. However, *UAS-rap/fzr;repo-GAL4* larval brains show weaker expression of Loco compared wild type, especially in regions of the where Loco expression is usually distinct (arrowhead). Scale bar, 50 μm.



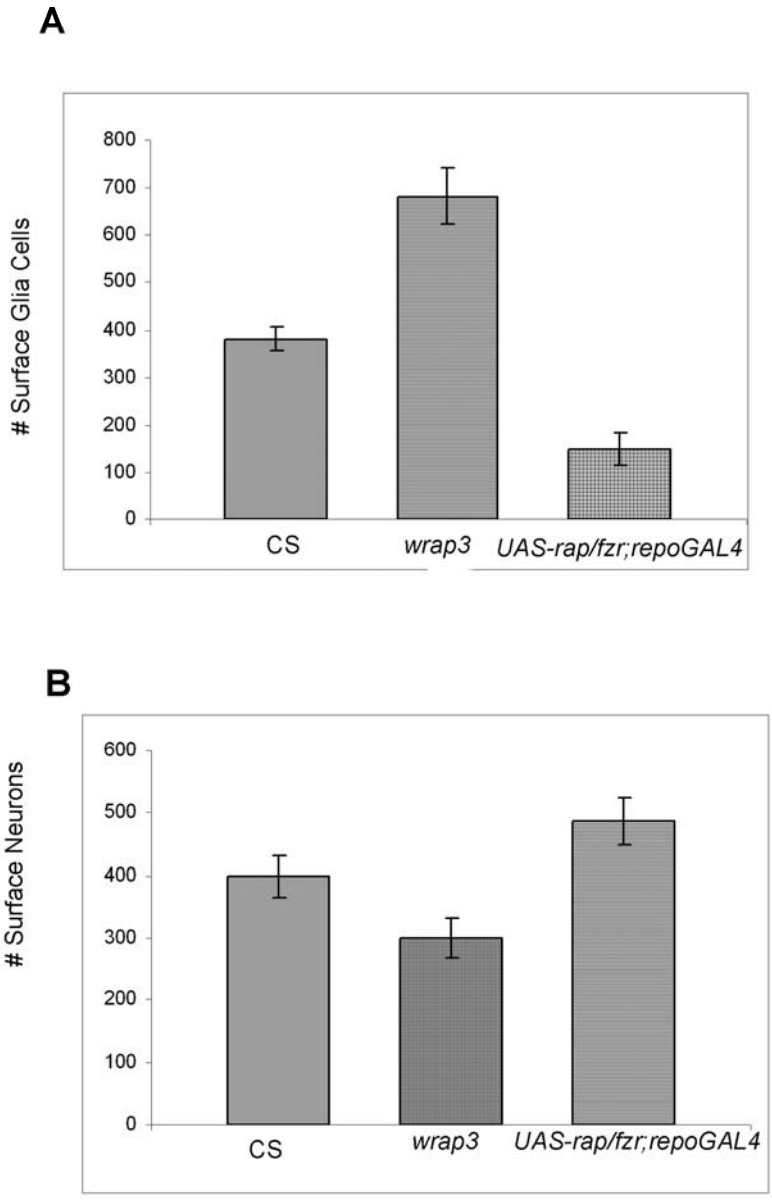
Supplementary Figure 3. *P2241* enhancer trap line reveals expression of *rap/fzr* within glia cells.

Confocal images of third instar eye imaginal discs from the *rap/fzr* enhancer trap line *rap/fzr*^{P2241} double stained with anti β-galactosidase (red) and anti-Repo (green) (A-C). Yellow staining shows localization of *rap/fzr* expression within a subset of retinal basal glia cells. Larval brains from *rap/fzr*^{P2241}were double stained with anti β-galactosidase (red) and anti-repo (green) (D-F). *rap/fzr* expression completely overlaps with Repo staining in confocal images taken from the peripheral layers larval brains. Confocal images of deeper layers within the larvae brain (D'-F') show *rap/fzr* expression is variable within glia cells towards the interior of the brain as shown with only some overlap between β-galactosidase and Repo staining. Scale bar, 50 μm



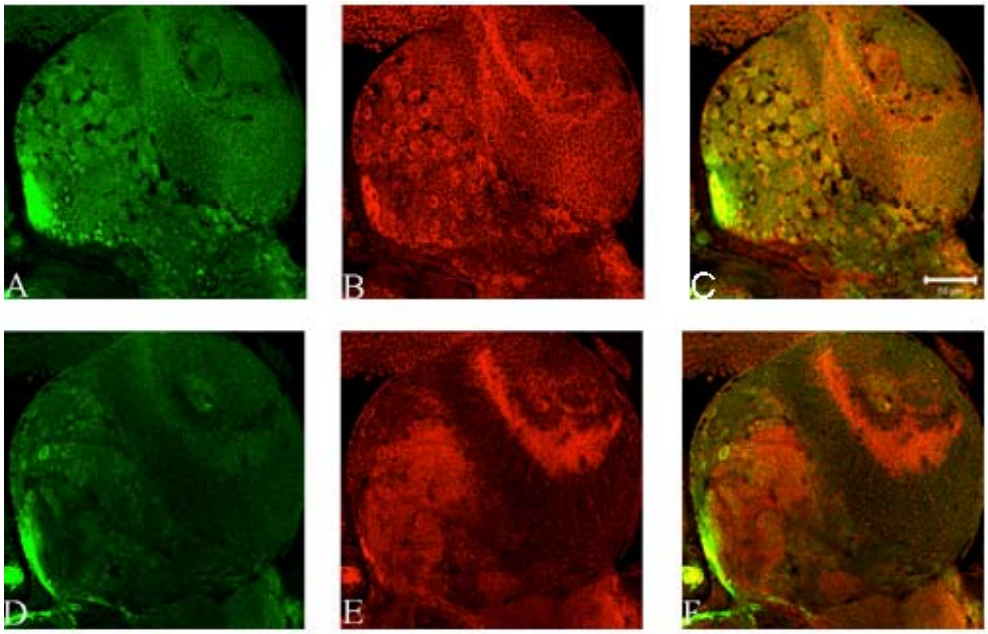
Supplementary Figure 4. *loco* is expressed in glia cells.

Expression pattern of *loco* in retinal basal glia were analyzed using enhancer trap line, loco^{rC56}. Third instar eye imaginal discs were double stained with anti β-galactosidase (red) and anti-Repo (green) (A-C). Yellow staining shows localization of and *loco* expression within retinal basal glia, a subtype of glia cells within the eye-imaginal disc.
loco^{rC56} larval brains were also double stained with anti β-galactosidase (red) and anti-Repo (green)(D-F). *loco* expression overlaps with Repo staining in confocal images taken from the peripheral layers larval brains. (D'-F') Yellow, overlay staining show *loco* expression localized within most glia cells towards the interior of the brain. Scale bar, 50 μm



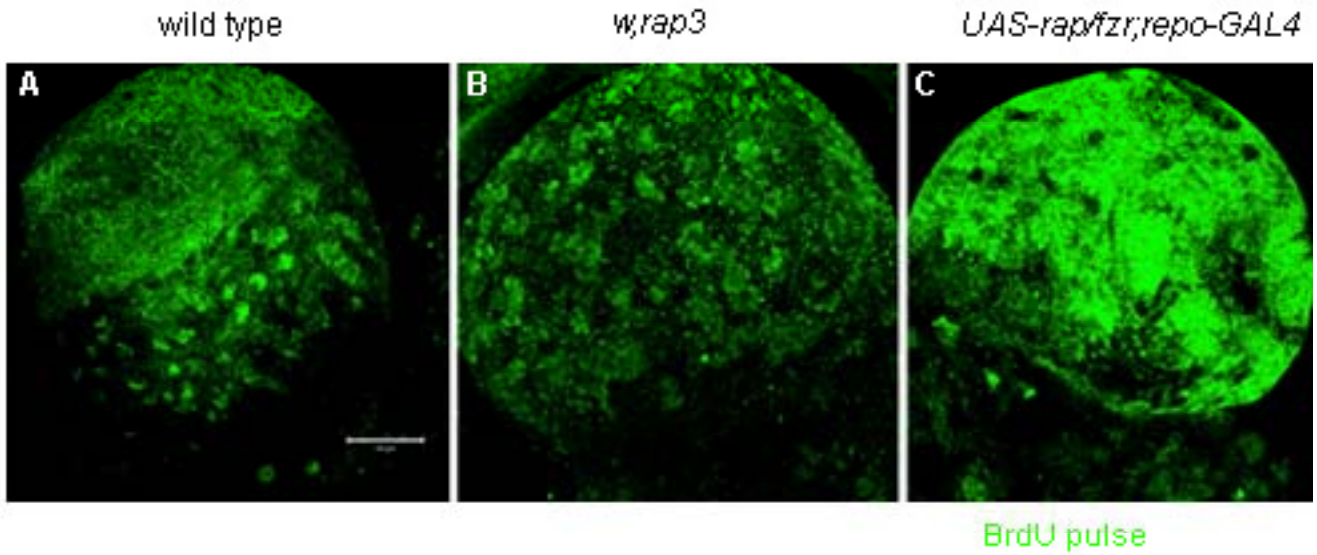
Supplementary Figure 5. Quantification of surface glia in *rap/fzr* mutants.

Image J software was used to analyze the number of surface glia cells (A) and surface neurons (B) in *rap/fzr* loss-of-function and *UAS-rap/fzr; repoGAL4* mutant larvae. Bar graph represents surface glia number and surface neurons in *rap/fzr* mutants.



Supplementary Figure 6. Loco and Rap/Fzr colocalize in the third instar larval brains .

(A-C) Figure shows an image representing the superficial layer from a confocal image stack. Loco (green) and Rap/Fzr (red) colocalize on the surface of the brain. Strong Loco expression is seen on the ventral surface of the brain, close to the boundary between the brain and the ganglion. (D-F) Image shows the middle layer of a confocal stack. Very little colocalization occurs between Loco (green) and Rap (red) on the brain's interior. Although Loco expression is weak on the brain's interior, Rap/Fzr expression is robust in the optic lobe and the ventral side of the brain. Scale bar, 50 μm.



Supplementary Figure 7. Ectopic neuroblasts in *UAS-rap/fzr;repo-GAL4* larvae are proliferative and self-renew.

Wild type, *UAS-rap/fzr; repo-GAL4*, and *wrap3* were collected after larval hatching and fed BrdU for 15 hours. Third instar larvae were then dissected and stained for BrdU incorporation. All images from confocal microscopy were taken with the same gain intensity. Results showed that loss-of-function mutant, *wrap3* (B)had levels of BrdU incorporation similar to wild type (A). Compared to *wrap3* and wild type, *UAS-rap/fzr; repo-GAL4* had immense levels of BrdU incorporation in third instar larval brains. Scale bar, 50 μm

CHAPTER 3

The ubiquitin protease Nonstop interacts with Rap/Fzr during Drosophila development to regulate glia migration and endoreplication

Margarita E. Kaplow, Rosa Mino, Tania Moin and Tadmiri R. Venkatesh*

Department of Biology, City College and The Graduate Center, City University of New York, 138th Street and Convent Avenue, New York, NY 10031.

* Author for correspondence: Tadmiri R. Venkatesh
Department of Biology
City College of New York
New York, NY 10031.
E-mail: venky@sci.ccny.cuny.edu

Running Title: Nonstop and Rap/Fzr regulate glia endocycles

Key Words: ubiquitin specific protease, endocycle, glia, Anaphase Promoting Complex, E3 ligase, and polyploidy

ABSTRACT

The *nonstop (not)* gene, encodes for a ubiquitin specific protease (UBP) which is required for glia migration and axon targeting in the Drosophila nervous system. Rap/Fzr is the Drosophila homolog of the mammalian Cdh1, an activator of the Anaphase Promoting Complex (APC), an E3 ubiquitin ligase. In the following study, we present data that show that Nonstop acts as a dominant suppressor of the *rap/fzr* loss-of-function eye phenotype. Furthermore, we show that *nonstop* and *rap/fzr* genetically interact to control glial cell endocycling. Our data show that Nonstop prevents glia cell endocycling while overexpression of *rap/fzr* promotes endocycling of glia cells. Co-immunoprecipitation studies show that Nonstop physically interacts with Rap/Fzr and is a possible substrate for APC-mediated degradation.

INTRODUCTION

Cell cycle progression requires the timely degradation of mitotic regulators**.** The posttranslational mechanism ubiquitination, precedes degradation, and is essential to the regulation of key mitotic proteins. Ubiquitination of proteins is a tightly controlled process involving a series of enzymes, E1 activating enzyme, E2 conjugating enzyme and E3 ligase enzyme. Each enzyme sequentially adds ubiquitin, a 76 amino acid polypeptide, to each protein complex and predestines a substrate for destruction. The Anaphase promoting complex (APC) is an E3 RING ubiquitin ligase composed of 12 or more protein subunits and plays a critical role towards the final step of targeting proteins to the 26S proteosome. E3 ligases contain specific binding sites for recognizing substrates; therefore, research has primarily focused on the activation of E3 ubiquitin ligases such as the APC (Hershko and Ciechanover, 1998; Pickart, 2001). The role of the APC in targeting proteins involved in cell cycle progression has been well characterized. Two activators of the APC, Cdc20/Fizzy and Cdh1/Rap/Fizzy-Related regulate the APC during two distinct phases of mitosis. Cdc20/Fizzy regulates APC activity during metaphase to anaphase transition while Rap/Fzr modulates APC activity during telophase to G1 transition (Dawson et al., 1993; Dawson et al., 1995; Sigrist et al., 1995;

Sigrist and Lehner, 1997). Until recently, the role of the APC was limited to targeting mitotic proteins such as securin, cyclin A, cyclin B to the 26S proteosome. However, recent evidence shows that the APC has roles in regulating protein levels of differentiated cells. In Drosophila, the APC regulates the size synaptic boutons by targeting liprin-alpha to the 26S proteosome (van Roessel et al., 2004). Furthermore, techniques using siRNA show that silencing Cdh1/Rap/Fzr in the rat cerebellum promotes axonal growth (Konishi et al., 2004; Stegmuller et al., 2008; Stegmuller et al., 2006). Although numerous studies have found novel postmitotic roles for components of the APC and the ubiquitin pathway, regulation of APC activity is not well understood. Recent evidence has shown deubiquitination as a mechanism for controlling the activation of the APC itself. It has been shown that the ubiquitin specific protease, USP44 deubiquitinates cdc20, prevents the dissassembly of Mad-Cdc20 complex, and therefore prevents the initiation of Anaphase (Stegmeier et al., 2007). However, a ubiquitin specific protease (UBP) that modulates Cdh1/Rap/Fzr activity has yet to be discovered. Conversely, a ubiquitin ligase complex such as the APC can modulate the activity of UBPs by targeting UBPs for degradation, ensuring substrates are kept ubiquitinated and therefore degraded.

Previous results from a genetic modifier screen identified Nonstop, a ubiquitin specific protease, as a dominant suppressor of *rap/fzr* during eye development in *Drosophila* (Kaplow et al., 2007). In the following study, we sought to determine the level of interaction between *nonstop* and *rap/fzr*. Our analyses of *nonstop* mutants show a disruption in glia development. *nonstop* loss-of-function mutants exhibit large glia nuclei that are polyploid. Similarly, when *rap/fzr* is ectopically expressed using *repo-GAL4*, these flies also exhibit glia cells which endocycle. These results are consistent with new findings that show Rap/Fzr regulates levels of Geminin during the normal endocycle of follicle cells (Narbonne-Reveau et al., 2008; Zielke et al., 2008). Geminin is a known substrate targeted by Rap/Fzr during mitosis and has been recently identified as a regulatory protein necessary for endocycling (Bermejo et al., 2002). Furthermore, our results show that both Nonstop and Rap/Fzr regulate the migration of retinal basal glia during third instar larval stage. Bioinformatic analyses reveal that Nonstop contains putative Destruction (D-box) and KEN box motifs, amino acid sequences bound by Rap/Fzr for the recognition of substrates. We present biochemical data that show physical interaction between Nonstop and Rap/Fzr. Our results suggest a novel role for Nonstop in the regulation of glia endoreplication and propose a novel substrate targeted by the APC.

RESULTS AND DISCUSSION

In the Drosophila eye, Nonstop acts as a dominant suppressor of Rap/Fzr

From a genetic screen *nonstop* was identified as a dominant suppressor of the rough eye phenotype of *rap/fzr* (Kaplow et al., 2007). Scanning electron micrographs of the ommatidias from rap^{E6} flies, a weak, hypomorphic allele of *rap/fzr*, and $rap^{E6};not^{1}/+$ double mutants, confirmed that *nonstop* suppresses that rough eye phenotype of *rap/fzr* (Figure 1B-C). The ommatidia of $rap^{E6};not^{1}/+$ mutant flies appear smooth similar to the compound eye of wild type flies (Figure 1A-C). Our results further reveal that a single copy of *nonstop* is also able to dominantly suppress the mitotic phenotype of *rap/fzr* in the developing eye-imaginal disc (Figure 1F-G). For detecting cells undergoing mitosis, anti-phospho-Histone-H3 was used for immonolabeling eye discs of wild-type, rap^{E6}, and $rap^{E6};not^{1}/+$ mutant flies. Quantification of phospho-Histone-H3 show 104 cells undergoing mitosis in wild type eye discs compared to 530 cells in rap^{E6} flies (Figure 1G). When eye discs of $rap^{E6};not^{1}/+$ were analyzed, mitotic index was reduced to 249 cells (Figure 1G) showing that a 50% reduction in *nonstop* gene dosage was

able to suppress the abnormal overproliferation phenotype of *rap*^{E6} mutant flies**.**

Although Nonstop has been previously characterized as a ubiquitin specific protease and suppresses the mitotic phenotype of *rap/fzr* mutants, Nonstop function has been limited to glia cell migration (Poeck et al., 2001) and has not been previously implicated in regulating the cell cycle. Because Nonstop has an unknown role in mitosis, we examined the eye imaginal discs of *nonstop* loss-of-function mutants for cells undergoing mitosis. Rap/Fzr promotes mitotic exit, therefore, we expected the converse of Nonstop, and predicted that *nonstop* loss-of-function mutants would display a reduction of cells undergoing mitosis. Compared to wild type eye discs, our results showed *nonstop* loss-of-function mutants displaying similar number of cells undergoing mitosis (Figure 2A-B). Results from Phospho-histone-H3 staining suggest that *nonstop* does not directly regulate mitotic exit in the eye imaginal disc. However, recent studies demonstrate that Nonstop deubiquitinates histone H2B, which ultimately leads to the regulation of mitotic chromatin (Weake et al., 2008). The role of Nonstop in the deubiquitination of histone H2B may, in turn, lead to the suppression of the *rap/fzr* mitotic phenotype.

In contrast to the loss-of-function *rap/fzr* mutant phenotype, where an accumulation of cyclin A and cyclin B causes extra mitotic cycles, precocious expression of *rap/fzr* in follicle cells cause enlarged nuclei and cells undergo ectopic endocycles (Schaeffer et al., 2004; Shcherbata et al., 2004; Sigrist and Lehner, 1997). Similarly, ectopic expression of *rap/fzr* using engrailed-GAL4 leads to the formation of large nuclei, DNA overreplication, and polyploidy in the wing imaginal disc (Sigrist and Lehner, 1997). As a ubiquitin specific protease, Nonstop can presumably counteract Rap/Fzr activity and remove the polyubiquitin tag that is promoted by Rap/Fzr. We tested whether Nonstop and Rap/Fzr function antagonize each other by examining the effect of loss-of function *nonstop* mutants in the wing imaginal discs. If Nonstop acts to inhibit Rap/Fzr function, then it is expected that a loss in Nonstop function would mimic the overexpression phenotype observed in *rap/fzr* mutants. When comparing the wing imaginal discs of *nonstop* mutants and wild type flies, results show no significant changes in the size of DNA nuclei as determined by Hoechst staining (Figure 2B-C). Although polyploidy was not observed in the wing imaginal discs of *nonstop* mutants, the size of the wing disc was considerably smaller in comparison to wild type wing discs (Figure 2D-E). Smaller eye imaginal discs were also observed in *nonstop* mutants (data not

shown) suggesting the possibility that organ development is delayed in *nonstop* loss-of-function mutant flies.

Loss of *nonstop* function leads to glia cell polyploidy

Given that both *nonstop* and *rap/fzr* regulate aspects of glia development (Kaplow et al., 2008; Poeck et al., 2001), we examined this cell type more thoroughly in *nonstop* mutants. When glia cell nuclei were analyzed in *nonstop* mutants, our results show a two-fold increase in the size of glia nuclei compared to wild type (Figure 3A-3F). Although perineurial and subperineurial glia, located within the surface of third instar larval brain have been described for their large morphology (Stork et al., 2008), compared to wild type brains, both *nonstop* and *UAS-rap/fzr;repo-GAL4* mutants exhibit a significant increase in glia nuclei size within the surface of the brain and the optic lobe region (Figure 3G-H). Results from our previous study showed glia numbers significantly decrease and mitotic index was unaffected in *UAS-rap/fzr;repoGAL4* mutants (Kaplow et. al, 2008). When *UAS-rap/fzr;repoGAL4* mutants were reexamined, results from Repo and Hoechst staining reveal giant nuclei within the remaining glia cells (Figure 3G-I). Consistent with previous findings which show that ectopic expression of *rap/fzr* can lead to follicle cell endocycling and DNA polyploidy in wing imaginal discs, our results suggest that ectopic

expression of *rap/fzr* using repo-GAL4, can also lead to glia cell polypoidy. In contrast, loss-of-function *nonstop* mutants display polyploidy exclusively within glia cells.

To further validate that loss of *nonstop* causes polyploidy within glia cells, we used *UAS-RNAi- nonstop* and were able to specifically silence *nonstop* within glia cells. Silencing *nonstop* within glia cells show similar results to loss-of-function *nonstop* mutant, when glia nuclei appear to double in size compared to wild type glia cells (Figure 4A-C). Our results show that both Nonstop and Rap/Fzr function to regulate the glia cell endocycling. Since *nonstop* and *rap/fzr* genetically interact to regulate mitotic exit in the eye imaginal disc, we were curious to learn if Nonstop and Rap/Fzr, together, regulate amount of DNA within glia cells.

Nonstop is a possible substrate for APC-mediated degradation

Since *nonstop* acts as a dominant suppressor of rough eye phenotype of *rap/fzr*, we investigated if *nonstop* and *rap/fzr* genetically interact to modulate the size of glia nuclei. Our results show that a single copy of *nonstop* is able to cause the endoreplication of glia in a loss-of-function, rap^{E6} mutant background (Figure 4D-E). In the developing Drosophila larval nervous system, we show that *rap/fzr* and *nonstop* genetically interact

to regulate mitosis in the eye imaginal disc and the amount of DNA within glia cells. A loss in Nonstop function is able to cause glia cells to endocycle in a *rap/fzr* mutant background, which lead us to further test the level of interaction between these two proteins.

Analysis of Nonstop amino acid sequence revealed that it contains both KEN and D box motifs, known recognition sequences used by Rap/Fzr for APC-dependent ubiquitination (Kaplow et al., 2007). We predicted that Nonstop would physically interact with Rap/Fzr since it contained amino acid sequences detected by Rap/Fzr. Lysates from wild type third instar larval brains and eye discs were used to immunoprecipitate Rap/Fzr protein with a molecular weight of 53 kDa (Figure 4F). Western Blot analysis demonstrates that Nonstop coimmunoprecipitates with Rap/Fzr, and the presence of Nonstop protein, 85 kDa in size, is visible on a Western Blot (Figure 4F). Conversely, when Nonstop was immunoprecipitated using larval extracts, western blot analysis shows Rap/Fzr physically interacts with Nonstop (Figure 4F). Our biochemical experiments show direct binding between Rap/Fzr and Nonstop, and therefore, suggest that Nonstop is a possible target of APC-dependent ubiquitination. Our study suggests both a genetic, and a physical interaction between Rap/Fzr and Nonstop. Because Nonstop has also been described for its role during glia migration, we

determined if Nonstop and Rap/Fzr function together in regulating glia migration.

Nonstop and Rap/Fzr regulate the migration of retinal basal glia

nonstop was originally identified in a screen for genes affecting axon targeting in the larval optic lobe. In wild type larvae, R1–R6 photoreceptor axons terminate within the lamina, while R7 and R8 axons continue within deeper layers of the optic lobe, called the medulla (Poeck, 2001). Further investigation of *nonstop* mutants revealed that axon targeting defects was a direct consequence of the inability of glia cells to migrate to proper locations. Glia cells of the optic lobe migrate from glia precursor cell (GPCs) areas located in the most dorsal and ventral edges of the R-cell projection field. In *nonstop* mutants, glia fail to migrate into the lamina plexus and instead, pool along the ventral margins of the R-cell projection field.

We tested the migration of retinal basal glia (RBGs) in *nonstop* loss-of-function mutants to determine if Nonstop function is restricted to regulating the migration of optic lobe glia. RBGs originate from the optic stalk and migrate through glia-glia interactions into the eye imaginal disc.

RBGs are more favorable than optic lobe glia for studying migration because they can be easily distinguished from the entire glia population. Furthermore, the migration of glia can be easily monitored throughout development, with 20 glia cells migrating into the eye imaginal disc during second instart stage and ~350 glia cells towards the end of third instar larval stage (Siles et.al). Compared to wild type, both *nonstop* null mutants and *nonstop-RNAi* mutants show fewer glia cells that migrate into the eye imaginal disc during late third instar larval stage (Figure 5A-C). Our data have shown that *rap/fzr* and *nonstop* genetically interact to control mitotic events in the eye imaginal disc and endocycling of glia cells during third instar larval stage. To further investigate if Rap/Fzr plays a direct role in the migration of glia similar to Nonstop, we examined the migration of RBGs using FLP/FRT mediated recombination to generate *rap/fzr* null clones. Our results show that in contrast to *nonstop* loss-of-function mutants and *nonstop* null clones, when glia are prevented from entering the eye imaginal disc Figure 5E-E"), *rap/fzr* mutant clones exhibit RBGs the migrate into the morphogenetic furrow (MF) when RBGs normally cease migration before the MF (Figure 5D-D").

In summary, our data reveals that Nonstop functions to regulate the migration of another glia subtype, RBGs, in addition to optic lobe glia. Our

results raise the possibility that Nonstop may also regulate the migratory behavior of other cell types including numerous glia subtypes. Furthermore, we show that Rap/Fzr functions in opposition to Nonstop, during the migration and endoreplication of glia cells. We present a possible model whereby Rap/Fzr and Nonstop compete for the same substrate, which maintain glia in a quiscent state. However, when the balance between Nonstop and Rap/Fzr function is disrupted, for example in *not*^{1} and *UAS-rap/fzr;repoGAL4* mutants, persistent degradation of a particular substrate leads to glia cell endoreplication. Our results show a novel role for posttranslational modifications such as ubiquitin-mediated proteolyis during nervous system development. Identifying precise substrates of Rap/Fzr and Nonstop will be pivotal towards understanding the role of APC-mediated degradation during glia development.

MATERIALS AND METHODS

Fly stocks and genetic experiments

The following mutant flies were used: *rap*^{E6} (Jacobs et al., 2002; Karpilow et al., 1989), and *not*^{1} */TM6;Tb* (Martin et al., 1995; Poeck et al., 2001) have been previously described. *repo-GAL4/TM6;Tb* (Bloomington Stock

Center) was used to drive expression of *UAS-rap/fzr* (Sigrist and Lehner, 1997)and *UAS-nonstopRNAi* (Vienna Drosophila RNAi Center). *nonstop* and *rap/fzr* mosaic clones were generated using FLP-mediated mitotic recombination (Xu and Rubin, 1993). *rap/fzr*^{8F3} *P{neoFRT}19A/FM7* (gift from Dr.Christiam Klambdt) recombinants were crossed to *P{neoFRT}19A arm-lacZ*; *hsFLP*

(gift from Dr. Nick Baker). y*w;not*^{1}*P{FRT80B}/Tb* (gift From Dr. Iris Salecker) flies were crossed *to P{ry[+t7.2]=hsFLP}22, y[1] w[*]; P{w[+mC]=arm-lacZ.V}70C P{ry[+t7.2]=neoFRT}80B* (Bloomington Stock Center).

Histology

Whole mount preparations of third instar larval eye discs and brains were stained with the appropriate antibodies and counterstained with fluorescent secondary antibodies as described previously (Kaplow et al., 2008; Pimentel and Venkatesh, 2005). Anti-Repo mouse (8D12) and was obtained from Developmental Studies Hybridoma Bank and was used at a dilution of 1:5 for all experiments. Anti phospho-Histone H3 rabbit antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and was diluted 1:100 for experiments. Anti-β-galactosidase (rabbit) antibody was purchased from eBioscience and was used at a dilution of 1:50. For labeling DNA,

Hoechst 33342 manufactured by Invitrogen (Eugene, Oregon) was used at a working concentration of .1mg/mL . Hoechst labeling protocol that was used has been previously described by (Page et al., 2005).

Protein isolation and Western Blot Analysis

Wild type, Canton S, larval extracts were homogenized using immunoprecipitation buffer (10 mM Tris, pH 7.5, 80 mM glycerophosphate, pH 7.3, 20 mM EGTA, pH 8.0, 15 mM $MgCl_2$, 0.5 mM DTT, 2 mM Na_3VO_4, 10% glycerol, 0.1% NP40). 500 μg of protein lysate was precleared using 50 μL of protein IgG beads (eBioscience). 500 μL of precleared lysate was incubated overnight at 4°C with either 1 μL of Nonstop 1 antibody (Dr.William Chia),or 1 μL of anti-Rap/Fzr (Pimentel and Venkatesh, 2005) and 50 μL of protein IgG beads. Beads were washed three times with immunoprecipitation buffer before addition of 50 μl of SDS sample buffer (250 mM Tris, 8.2% SDS, 05% bromophenol blue, 40% glycerol and 200 mM of DTT). Immunoprecipitated protein was extracted by boiling protein IgG beads in SDS sample buffer for 5 minutes.

Protein from larvae was separated by SDS-PAGE using a 12% resolving gel and 5% stacking gel. SDS-PAGE gel was transferred to 0.45 μm nitrocellulose membrane (Pierce) for 1 hour at 100V at 4°C. Blots were

blocked in 5% skim milk dissolved in Tris buffered saline containing 0.2% Tween 20 (TBS-T) for two hours. Membranes were incubated overnight in primary antibodies, using a 1:1000 dilution. Polyclonal anti-Rap/Fzr antibody was used at a 1:150 dilution. After primary antibody incubations, blots were washed three times in TBS-T for a period of five minutes for each wash. Membranes were then incubated for one hour in secondary antibody using a 1:10000 dilution of anti-rabbit IgG-horseradish peroxidase (Jackson Labs) and a 1:1000 anti-rabbit Trueblot antibody (eBioscience). After secondary antibody incubation, membranes were washed for 10 minutes three times in TBS-T. Blocking solution was used for the dilution of both primary and secondary antibodies. For detection of horseradish peroxidase on immunoblots, ECL Western Blotting Substrate kit was used according to the company's protocol (Pierce).

Statistics and Quantitative Analysis

All confocal images for third instar larval brains and eye discs were taken using an LSM 510 (Zeiss) For counting cell undergoing mitosis, (anti-phosphohistone-H3). Image J software (http://rsb.info.nih.gov/ij/) was used for counting surface glia cells and neurons located on the surface of the brain. The top optical sections of confocal stacks were used for analysis of surface glia. Significance and *p* values were derived by Student's *t*-test.

Figure 1. *nonstop* dominantly suppresses the eye phenotype of *rap/fzr*

(A-C) Results of scanning electron micrographs from a genetic modifier screen show a smooth appearance of a wild type **(A)** compound eye compared to rap^{E6} **(B),** a hypomorphic mutant allele that exhibits a rough eye phenotype due to disorganized arrangement of photoreceptors. **C**) rap^{E6}; not^{1}/+ double mutant flies show a smooth ommatidia similar to wild-type.
(D-F) Third instar eye imaginal discs were stained with the mitotic marker anti-phospho Histone-H3 (red). **A**) Wild type eye disc shows two discrete mitotic domains marked by anti-phospho-Histone-H3 staining, anterior and posterior to the Morphogenetic Furrow (arrowhead). **B**) Eye discs from rap^{E6} mutants exhibit unregulated mitosis within all regions of the disc. **C)** During development, *nonstop* also suppresses the mitotic phenotype of *rap/fzr* mutants, eye discs of rap^{E6};not^{1}/+ mutants are similar to wild type eye discs.
G) Quantification of cells undergoing mitosis in the eye imaginal disc show 104 +/- 19 cells undergoing mitosis in wild type eye discs, 530 +/- 121 cells in rap^{E6} flies and 249 +/- 19 cells in the eye discs of rap^{E6};not^{1}/+ flies.(n=9, p<.013)

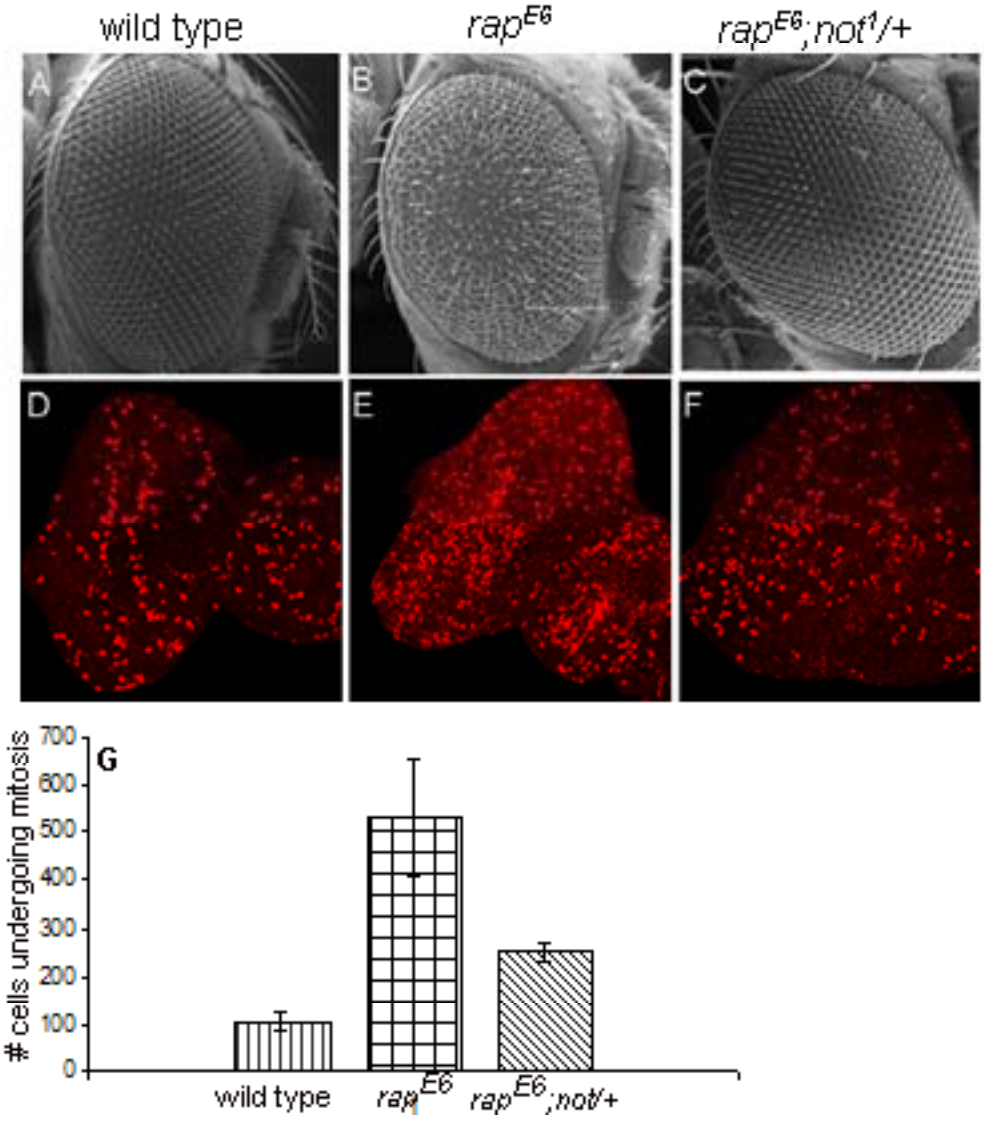

wild type
rap^E6
rap^E6;not^1/+
A
B
C
D
E
F
G
cells undergoing mitosis
700
600
500
400
300
200
100
0
wild type
rap^E6
rap^E6;not^1/+

Figure 1

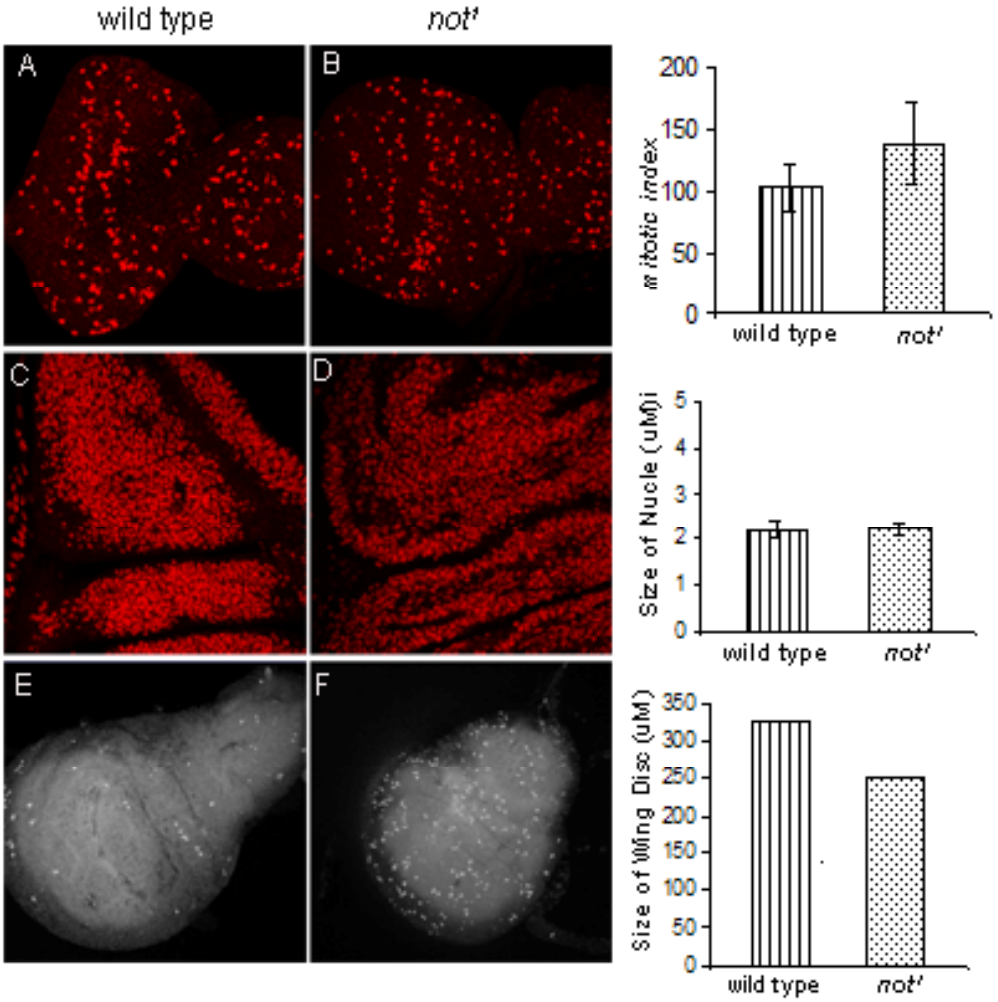


Figure 2. Mutations in *nonstop* affect imaginal disc growth

Anti-phospho-Histone H3 (red) labels cells undergoing mitosis in the eye imaginal disc. Similar to wild type **(A)**, *nonstop* **(B)** amorphic mutants exhibit two mitotic domains (arrowheads). Hoechst staining (green) labels DNA nuclei in wing imaginal discs. DNA nuclei are comparable between wild type **(C)** and *nonstop* null mutant flies **(D)**. *nonstop* **(F)** loss-of-function larvae have smaller wing discs than wild type larvae **(E)**. Images are maximum projections taken from LSM 510 confocal microscope. Scale bar, 50 μm

Figure 3. Glia cell endocycle in loss-of- function *nonstop* mutants and when rap/fzr is ectopically expressed using repo-GAL4 .

(A-A'", B-B'", and C-C'") Single optical sections from a confocal microscope show third instar larval brains stained with the nuclear glia cell marker, Repo (green). Hoechst dye (red) was used to label DNA nuclei. When comparing glia cells in larval brains, *nonstop* null mutants **(B)** display glia cells that are larger than wild type **(A)**. Hoechst staining confirms glia cells in *nonstop*^{1} **(B')** larval brains endocycle, containing large DNA nuclei compared to wild type **(A')**. Similar to the *nonstop* loss-of-function mutants, ectopic overexpression of *rap/fzr* cause glia cells to endocycle **(C-C")**. Images in (**A"')**, (**B"')**, and **(C'')** are overlay images of Repo and Hoechst staining. Images labeled with Hoechst dye are magnified in (**A""-C"')**.
(D) The size of glia nuclei were quantified in *not*^{1}, *UAS-rap/fzr;repoGAL4*, and wild type larval brains. When glia within the surface of the brains were analyzed, wild type glia were 48.18 μM compared to 68.36 μM in *not*^{1}mutants and 172.9 μM in *UAS-rap/fzr;repoGAL4* $p<.044$; $n=22$, $p<.032$;$n=18$.

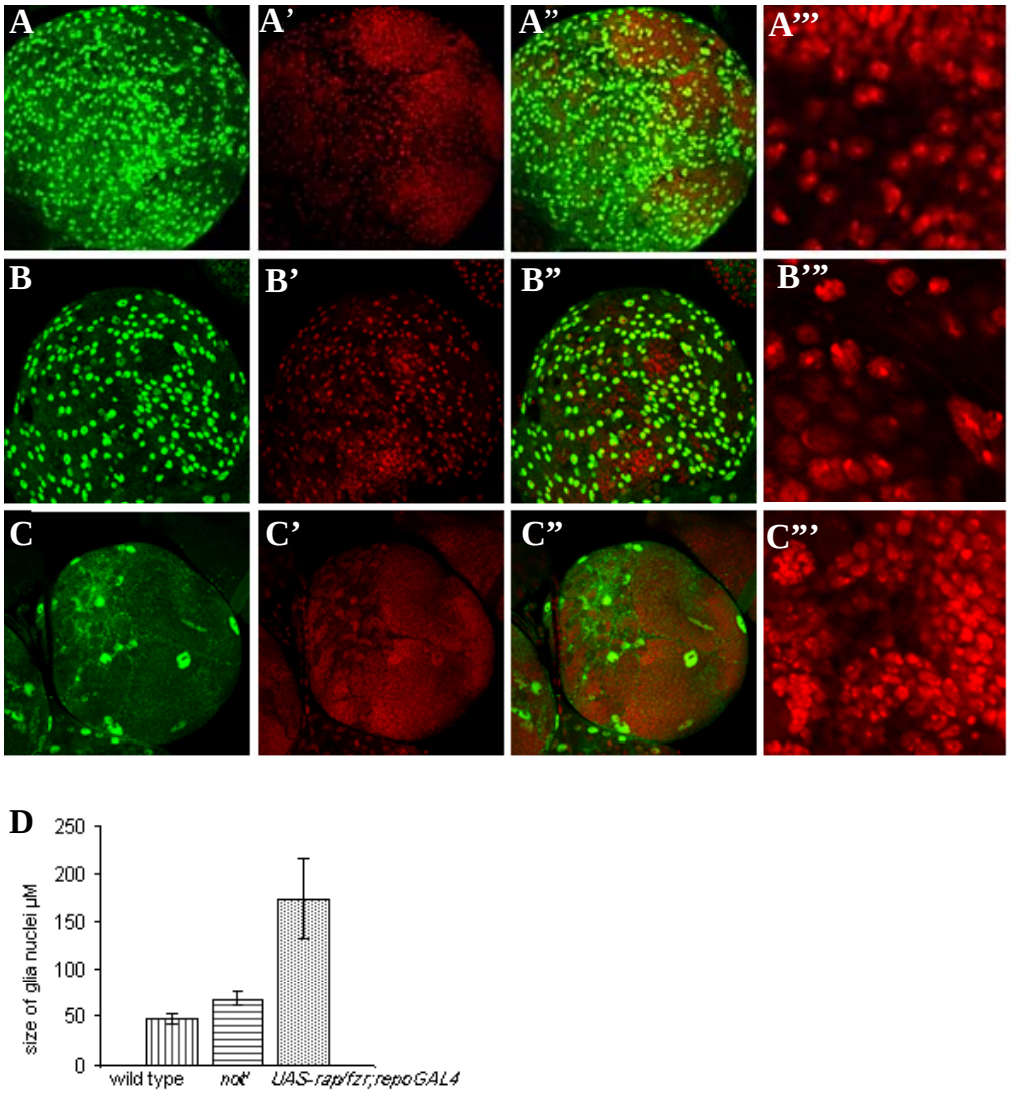

A
A'
A"
A'"
B
B'
B"
B'"
C
C'
C"
C'"
D
size of glia nuclei µM
250
200
150
100
50
0
wild type
not'
UAS-rap/fzr;repoGAL4

Figure 3

Figure 4. Nonstop, together with Rap/Fzr regulate glia cell endocycling.
When *UAS-nonstopRNAi;repoGAL4* larval brains were labeled with Hoechst dye **(A)** and Repo antibody **(B),** glia cells endoreplicate similar to not^{1} loss-of-function mutants. Image displayed in **(C)** is an overlay of (**A)** and **(B)**. Nonstop and Rap/Fzr genetically interact to control glia polyploidy **(D-E)**. When comparing rap^{E6} **(D)** to $rap^{E6};not^{1}/+$ **(E)**, Repo staining shows glia nuclei in rap^{E6} flies are similar in size to wild type glia nuclei. Mutating a single copy of *nonstop* is able to cause some glia cells to endocycle in a *rap/fzr* mutant background **(E)**.
(F) Nonstop binds to Rap/Fzr. When Rap/Fzr protein, identified by a 53 kDa band on a western blot, was immunoprecipated from larval extracts, Nonstop coimmunoprecipitates with Rap/Fzr. The 83 kDa band on the right panel represents Nonstop protein.

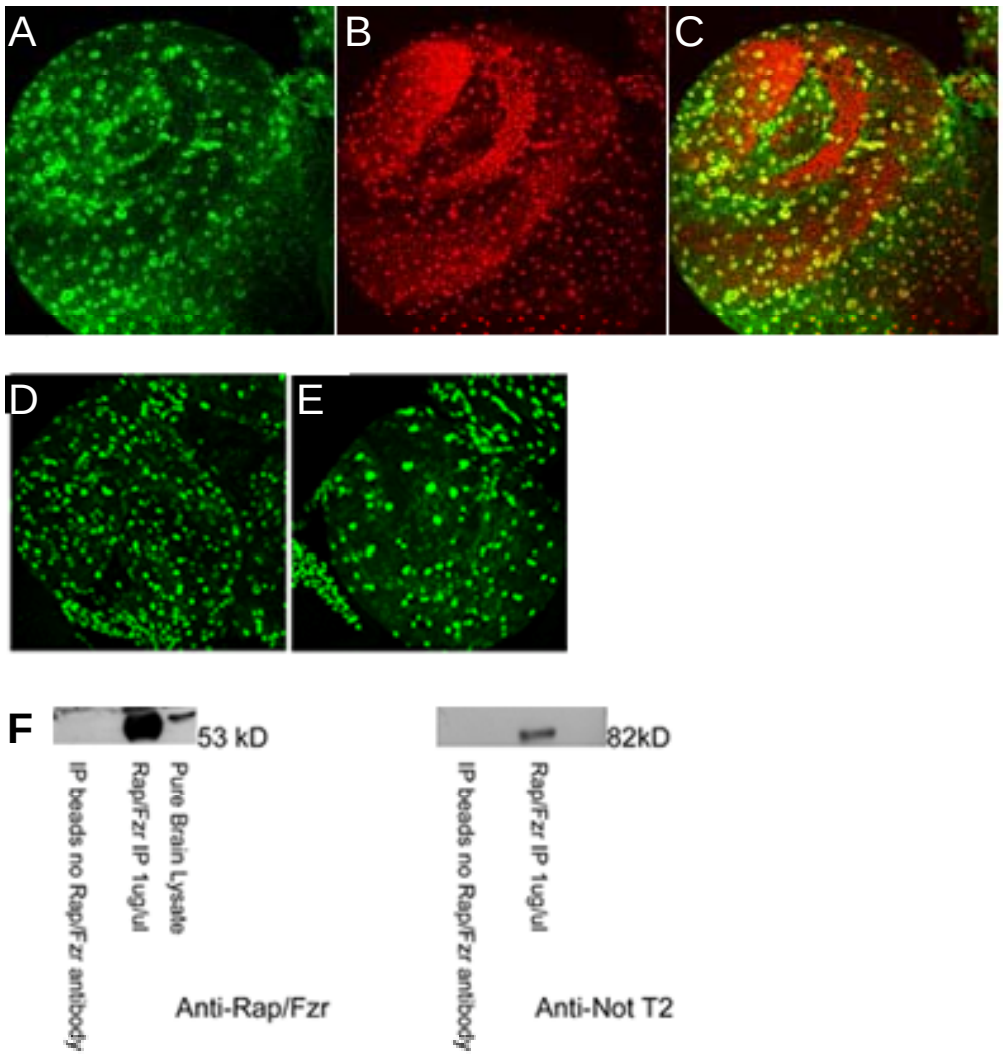

A
B
C
D
E
F
53 kD
82kD
IP beads no Rap/Fzr antibody
Rap/Fzr IP 1ug/ul
Pure Brain Lysate
Anti-Rap/Fzr
IP beads no Rap/Fzr antibody
Rap/Fzr IP 1ug/ul
Anti-Not T2

Figure 4

Figure 5. In the eye imaginal disc, both *not* and *rap/fzr* mutants exhibit glia migration defects

(A-C) Repo antibody label retinal basal glia (RBG) in the eye imaginal disc during third instar larval stage. **(A)** Wild type eye discs exhibit RBG that migrate properly, directly posterior to the morphogenetic furrow (arrowheads). In not^{1} **(B)** mutant larvae, RBG fail to migrate completely in the eye disc. Similar to not^{1} mutants, fewer RBG migrate in the eye imaginal discs of *UAS-nonstopRNAi;repoGAL4* larvae **(C)**.

(D-D"', E-E"') *nonstop* and *rap/fzr* null clones were identified by the absence of β-galactosidase (red) staining and white outline. Repo antibody (green) was used to label glia cells of the eye imaginal disc. Loss of Rap/Fzr function lead RBG to migrate into the morphogenetic furrow. Overlay image in (**D"'**) show glia cell migrating past their normal boundary. Conversely, glia cells in *not* null clones **(E-E"')** are prevented from migrating in the eye imaginal disc.

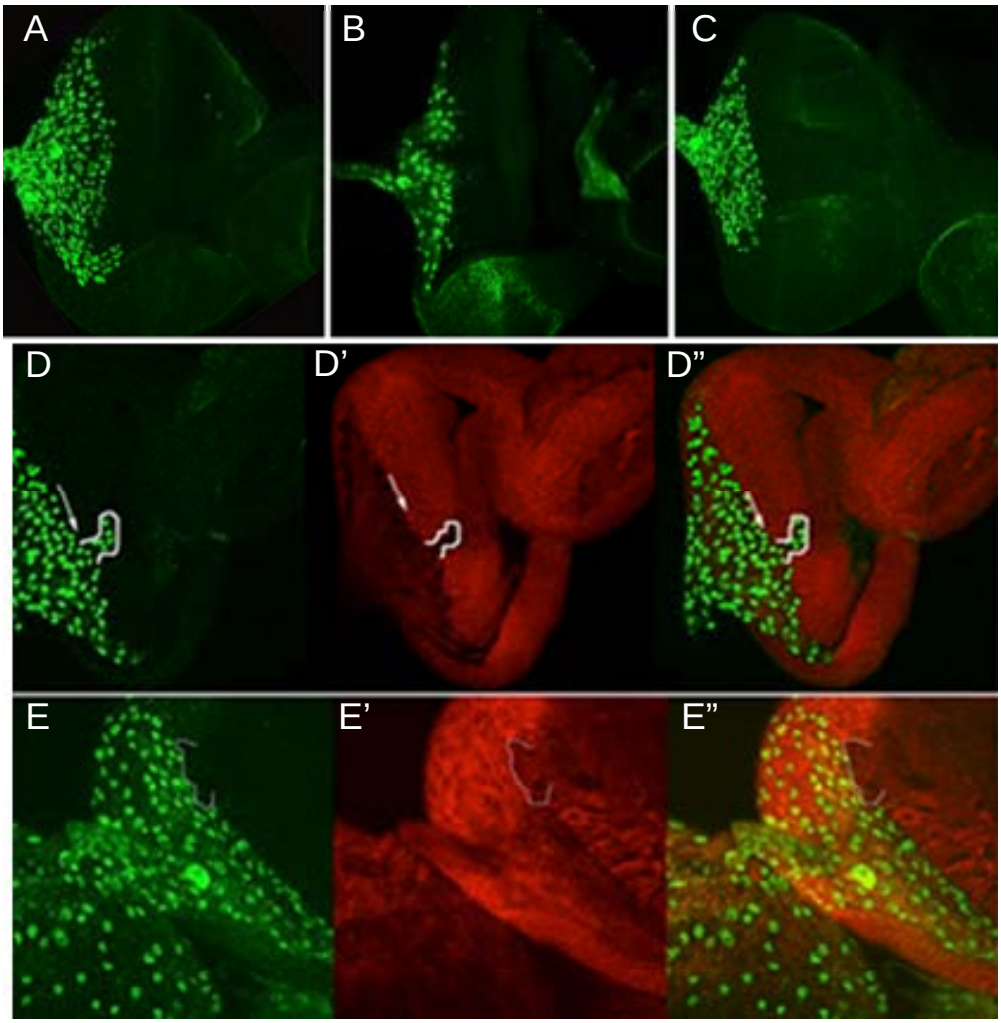
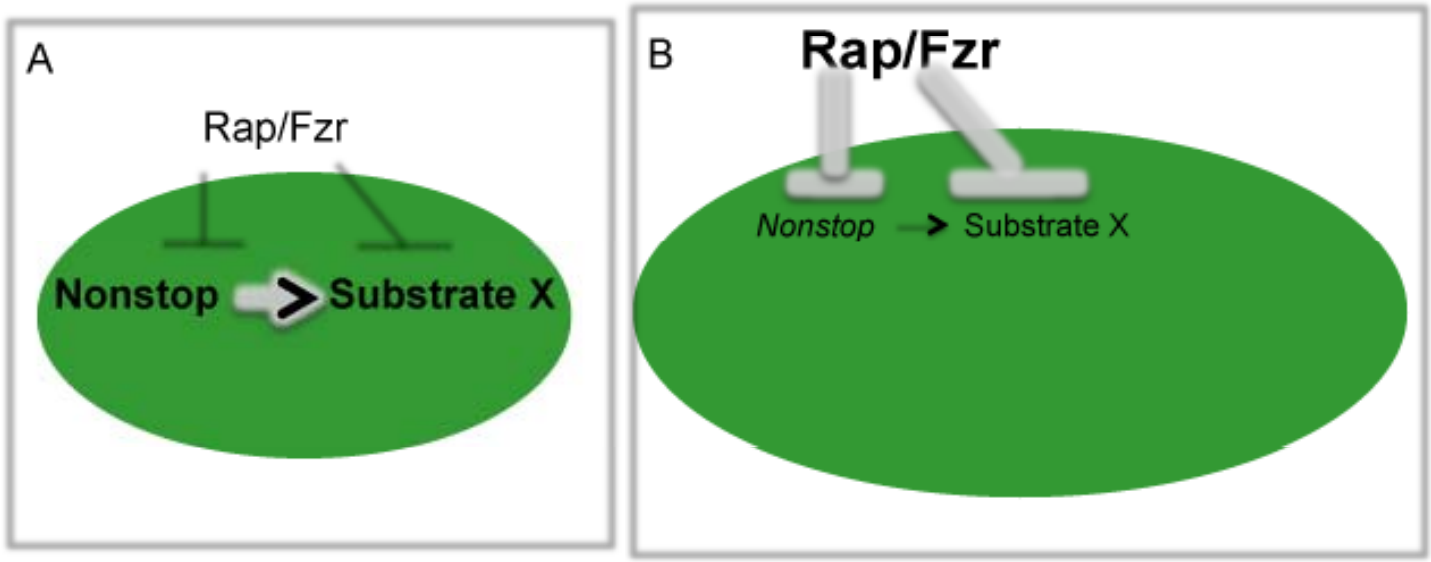


Figure 5

Figure 6. Nonstop and Rap/Fzr Regulate Glia Cell Endoreplication

A model presents a hypothetical mechanism for the regulation of glia cell endocycles. **A)** Under normal conditions, Rap/Fzr targets Nonstop and an unknown substrate/s (Substrate X), possibly Geminin as shown by previous studies for ubiquitination and subsequent degradation. Nonstop prevents the continual degradation of the same substrate/s by the removal of polyubiquitin moieties. Rap/Fzr and Nonstop have opposite functions, promoting degradation and preventing proteolysis, thus resulting in cyclical levels of Substrate X. **B)** When Rap/Fzr is ectopically expressed in glia cells, the balance between Nonstop and Rap/Fzr function is disrupted, which ultimately results in the down-regulation of Substrate X and endoreplication. Similarly, when there is a loss-of Nonstop function, Substrate X is no longer prevented from proteolysis and this leads to glia cell polploidy.


A
Rap/Fzr
Nonstop
Substrate X
B
Rap/Fzr
Nonstop
Substrate X

DISCUSSION

Within the last decade, emerging studies have revealed glia as active components of nervous system development. Glia respond to damage within the nervous system by eliminating debris and thus limits further damage that can be caused by activation of an inflammatory response from the immune system (Kurant et al., 2008; Ziegenfuss et al., 2008). Recent studies have shown glia express NMDA receptors, and therefore raise the possibility that glia are able propagate signals within the nervous system (Muller et al., 1993). However, to determine the complete function of glia within the nervous system requires understanding the genetic mechanisms that regulate their development. The *Drosophila melanogaster* serves as a useful animal model system for studying the genetic pathways involved during glia development. During Drosophila embryonic development, activation of specific gliogenic genes results in the specification of a given neuroblast into glia cells. The fly system has identified several transcription factors such as: Glial Cells Missing, Repo, and Pointed that are involved in glia differentiation (Hosoya et al., 1995; Jones et al., 2004). The gene *loco*, which encodes for an RGS protein has also been implicated in the glia specification pathway(Granderath et al., 2000; Granderath and Klambt, 2004; Granderath et al., 1999). My results have further identified

posttranslational modifications such as ubiquitination as an important mechanism involved in glia development. Rap/Fzr is an integral component of the ubiquitination pathway. Together with the APC and other E3 ubiquitin ligases, Rap/Fzr specifically targets substrates for ubiquitination (Jacobs et al., 2002; Pimentel and Venkatesh, 2005; Sigrist and Lehner, 1997; Zur and Brandeis, 2001). My genetic analyses show that *rap/fzr* interacts with two genes previously characterized for their role during glia development, *loco* and *nonstop*.

Both Loco and Nonstop were first identified as dominant suppressors of the rough eye phenotype displayed in *rap/fzr* loss-of-function mutants. Furthermore, bioinformatic analysis of Nonstop and Loco revealed that both proteins contained putative Destruction (D-box) and KEN box motifs, amino acids sequences recognized by Rap/Fzr for targeting its substrates (Kaplow et al., 2007). The presence of D-box and KEN-box motifs, and coimmunoprecipitaton results suggests that a direct physical interaction exists between Rap/Fzr and the two proteins. However, coimmunoprecipitation data may also suggest that Loco and Nonstop are in complex with Rap/Fzr. Additional experiments such as yeast-two hybrid assay and in vitro binding assays from purified proteins would further confirm direct, physical interaction between Rap/Fzr , Loco and Nonstop.

Western blot experiments also show that Loco is ubiquitinated in vivo which further supports a model in which Rap/Fzr targets Loco to the APC for polyubiquitination. Furthermore, analyses of *rap/fzr* loss-of function mutant larvae show a higher expression of Loco. Conversely, Loco expression is reduced in *UAS-rap/fzr;repoGAL4* larvae, which supports the idea that Rap/Fzr targets Loco for polyubiquitination. Although cell biological experiments suggest a model wherein Rap/Fzr targets Loco to the APC, analysis of Loco protein levels through western blot experiments from *rap/fzr* mutant larvae would have further supported this model. Moreover, quantification of Loco protein is more feasible through Western blot analysis.

Genetic, biochemical, and cell biological data from my work presents a novel role for *rap/fzr*, a regulatory subunit of the APC during Drosophila glia development. My results suggest Rap/Fzr regulates glia differentiation through its interaction with Loco, a positive regulator of gliogenesis. Furthermore, our data propose Rap/Fzr targets Loco for polyubiquitination during asymmetric division of neuroblasts and during the differentiation of GMCs (Kaplow et al., 2008). Our results are consistent with studies which show a role for known substrates of the APC, such as Aurora A kinase during neuroblasts divisions (Lee et al., 2006a; Lee et al., 2006b; Wang et

al., 2006). Analysis of *UAS-rap/fzr;repoGAL4* larvae show a significant increase in neuroblast number. My data also suggest that ectopic neuroblasts are able to self-renew in *UAS-rap/fzr;repoGAL4* larvae because an immense amount of BrdU incorporation is observed within these mutant larvae. Alternatively, BrdU incorporation in *UAS-rap/fzr;repoGAL4* larvae could be in consequence of the endoreplicating glia cells that are found within these mutants larvae. To test proliferation of dividing neuroblast, future experiments could focus on methods such as time-lapse, in vivo imaging, which could definitively determine the renewal of a fluorescently labeled neuroblast. In addition to its role in neuroblast division and glia differentiation, our results show additional roles for Rap/Fzr during the nervous system development.

My studies on the *nonstop* genetic interaction uncover a role for Rap/Fzr during glia migration and endoreplication. Although Nonstop had been previously described for its role during the migration of optic lobe glia, genetic analysis of not^{1} and *UAS-notRNAi;repoGAL4* mutants reveal defective migration of another subytpe of glia, retinal basal glia (RBG)(Poeck et al., 2001). When RBG were observed in not^{1} and *UAS-notRNAi;repoGAL4* mutants, RBG fail to migrate completely into the eye-imaginal disc. Conversely, when *rap/fzr* null clones were generated during

third instar larval stage, loss of Rap/Fzr function resulted in the migration of RBG past their normal boundary. To eliminate the possibility that *not*^{1} and *UAS-notRNAi;repoGAL4* mutants are delayed developmentally and as consequence, glia migrate much later, one could look at RBG past third instar larval stage. Generating large *not*^{1} mutant clones in the eye imaginal disc could also determine if Nonstop definitively regulates RBG. Furthermore, our results also show an increase in the amount of the size of glia nuclei in *not*^{1} and in *UAS-rap/fzr;repoGAL4* . Hoechst labeling revealed glia cells are polyploid in *not*^{1} and in *UAS-rap/fzr;repoGAL* larvae. Overall, my results present novel genetic pathways involved in glia differentiation and migration. My results suggest Rap/Fzr targets Loco for polyubiquitination and regulates the differentiation of surface glia. At the same time, Rap/Fzr is antagonistic of Nonstop function, yet both proteins are involved in regulating glia migration and endoreplication. Determining the precise substrate that is targeted by Rap/Fzr and Nonstop would be an important experiment towards understanding general mechanisms that regulates migratory cells such as glia. For future experiments, one could perform a deubiquitination assay to determine if Rap/Fzr is targeted by Nonstop. Generating *nonstop* homozygous and *rap/fzr* hemizygous mutant larvae would also provide more information on the nature of interaction

between Rap/Fzr and Nonstop. In summary, both genetic and biochemical experiment are needed for future experiments and for determining the feedback mechanism that may be occurring between Nonstop and Rap/Fzr.

BIBLIOGRAPHY

Aitken, A., Collinge, D. B., van Heusden, B. P., Isobe, T., Roseboom, P. H., Rosenfeld, G. and Soll, J. (1992). 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. *Trends Biochem Sci* **17**, 498-501.

Akiyama-Oda, Y., Hotta, Y., Tsukita, S. and Oda, H. (2000). Mechanism of glia-neuron cell-fate switch in the Drosophila thoracic neuroblast 6-4 lineage. *Development* **127**, 3513-22.

Akong, K., McCartney, B. M. and Peifer, M. (2002). Drosophila APC2 and APC1 have overlapping roles in the larval brain despite their distinct intracellular localizations. *Dev Biol* **250**, 71-90.

Almeida, A., Bolanos, J. P. and Moreno, S. (2005). Cdh1/Hct1-APC is essential for the survival of postmitotic neurons. *J Neurosci* **25**, 8115-21.

Alvarez, A. D., Shi, W., Wilson, B. A. and Skeath, J. B. (2003). pannier and pointedP2 act sequentially to regulate Drosophila heart development. *Development* **130**, 3015-26.

Araki, M., Wharton, R. P., Tang, Z., Yu, H. and Asano, M. (2003). Degradation of origin recognition complex large subunit by the anaphase-promoting complex in Drosophila. *Embo J* **22**, 6115-26.

Arama, E., Dickman, D., Kimchie, Z., Shearn, A. and Lev, Z. (2000). Mutations in the beta-propeller domain of the Drosophila brain tumor (brat) protein induce neoplasm in the larval brain. *Oncogene* **19**, 3706-16.

Awasaki, T. and Ito, K. (2004). Engulfing action of glial cells is required for programmed axon pruning during Drosophila metamorphosis. *Curr Biol* **14**, 668-77

Badenhorst, P. (2001). Tramtrack controls glial number and identity in the Drosophila embryonic CNS. *Development* **128**, 4093-101.

Bainton, R. J., Tsai, L. T., Schwabe, T., DeSalvo, M., Gaul, U. and Heberlein, U. (2005). moody encodes two GPCRs that regulate cocaine behaviors and blood-brain barrier permeability in Drosophila. *Cell* **123**, 145-56.

Baker, N. E. and Yu, S. Y. (2001). The EGF receptor defines domains of cell cycle progression and survival to regulate cell number in the developing Drosophila eye. *Cell* **104**, 699-708.

Bang, A. G. and Kintner, C. (2000). Rhomboid and Star facilitate presentation and processing of the Drosophila TGF-alpha homolog Spitz. *Genes Dev* **14**, 177-86.

Bao, H., Daniels, R. W., MacLeod, G. T., Charlton, M. P., Atwood, H. L. and Zhang, B. (2005). AP180 maintains the distribution of synaptic and vesicle proteins in the nerve terminal and indirectly regulates the efficacy of Ca2+-triggered exocytosis. *J Neurophysiol* **94**, 1888-903.

Baonza, A., Murawsky, C. M., Travers, A. A. and Freeman, M. (2002). Pointed and Tramtrack69 establish an EGFR-dependent transcriptional switch to regulate mitosis. *Nat Cell Biol* **4**, 976-80.

Basi, G. S. and Storti, R. V. (1986). Structure and DNA sequence of the tropomyosin I gene from Drosophila melanogaster. *J Biol Chem* **261**, 817-27.

Bello, B., Holbro, N. and Reichert, H. (2007). Polycomb group genes are required for neural stem cell survival in postembryonic neurogenesis of Drosophila. *Development*.

Bello, B., Reichert, H. and Hirth, F. (2006). The brain tumor gene negatively regulates neural progenitor cell proliferation in the larval central brain of Drosophila. *Development* **133**, 2639-48.

Bermejo, R., Vilaboa, N. and Cales, C. (2002). Regulation of CDC6, geminin, and CDT1 in human cells that undergo polyploidization. *Mol Biol Cell* **13**, 3989-4000

Bernardoni, R., Kammerer, M., Vonesch, J. L. and Giangrande, A. (1999). Gliogenesis depends on glide/gcm through asymmetric division of neuroglioblasts. *Dev Biol* **216**, 265-75.

Bernardoni, R., Miller, A. A. and Giangrande, A. (1998). Glial differentiation does not require a neural ground state. *Development* **125**, 3189-200.

Betschinger, J. and Knoblich, J. A. (2004). Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. *Curr Biol* **14**, R674-85.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev Biol* **179**, 41-64.

Boulton, S. J., Brook, A., Staehling-Hampton, K., Heitzler, P. and Dyson, N. (2000). A role for Ebi in neuronal cell cycle control. *Embo J* **19**, 5376-86.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15.

Buchner, K., Roth, P., Schotta, G., Krauss, V., Saumweber, H., Reuter, G. and Dorn, R. (2000). Genetic and molecular complexity of the position effect variegation modifier mod(mdg4) in Drosophila. *Genetics* **155**, 141-57.

Burton, J. L. and Solomon, M. J. (2001). D box and KEN box motifs in budding yeast Hsl1p are required for APC-mediated degradation and direct binding to Cdc20p and Cdh1p. *Genes Dev* **15**, 2381-95.

Bush, A., Hiromi, Y. and Cole, M. (1996). Biparous: a novel bHLH gene expressed in neuronal and glial precursors in Drosophila. *Dev Biol* **180**, 759-72.

Campos-Ortega, J. A. a. H., V. (1997). The embryonic development of Drosophila melanogaster. Berlin: Springer Verlag.

Campuzano, S. and Modolell, J. (1992). Patterning of the Drosophila nervous system: the achaete-scute gene complex. *Trends Genet* **8**, 202-8.

Carmena, M., Riparbelli, M. G., Minestrini, G., Tavares, A. M., Adams, R., Callaini, G. and Glover, D. M. (1998). Drosophila polo kinase is required for cytokinesis. *J Cell Biol* **143**, 659-71.

Ceron, J., Gonzalez, C. and Tejedor, F. J. (2001). Patterns of cell division and expression of asymmetric cell fate determinants in postembryonic neuroblast lineages of Drosophila. *Dev Biol* **230**, 125-38.

Ceron, J., Tejedor, F. J. and Moya, F. (2006). A primary cell culture of Drosophila postembryonic larval neuroblasts to study cell cycle and asymmetric division. *Eur J Cell Biol* **85**, 567-75.

Chasan, R., Jin, Y. and Anderson, K. V. (1992). Activation of the easter zymogen is regulated by five other genes to define dorsal-ventral polarity in the Drosophila embryo. *Development* **115**, 607-16.

Chen, Z. J. (2005). Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol* **7**, 758-65.

Chia, W. and Yang, X. (2002). Asymmetric division of Drosophila neural progenitors. *Curr Opin Genet Dev* **12**, 459-64.

Choi, K. W. and Benzer, S. (1994). Migration of glia along photoreceptor axons in the developing Drosophila eye. *Neuron* **12**, 423-31
.
Chotard, C., Leung, W. and Salecker, I. (2005). glial cells missing and gcm2 cell autonomously regulate both glial and neuronal development in the visual system of Drosophila. *Neuron* **48**, 237-51.

Conaway, R. C., Brower, C. S. and Conaway, J. W. (2002). Emerging roles of ubiquitin in transcription regulation. *Science* **296**, 1254-8.

Couderc, J. L., Godt, D., Zollman, S., Chen, J., Li, M., Tiong, S., Cramton, S. E., Sahut-Barnola, I. and Laski, F. A. (2002). The bric a brac locus consists of two paralogous genes encoding BTB/POZ domain proteins and acts as a homeotic and morphogenetic regulator of imaginal development in Drosophila. *Development* **129**, 2419-33.

Crackower, M. A., Sarao, R., Oudit, G. Y., Yagil, C., Kozieradzki, I., Scanga, S. E., Oliveira-dos-Santos, A. J., da Costa, J., Zhang, L., Pei, Y. et al. (2002). Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* **417**, 822-8.

Cuschieri, L., Miller, R. and Vogel, J. (2006). Gamma-tubulin is required for proper recruitment and assembly of Kar9-Bim1 complexes in budding yeast. *Mol Biol Cell* **17**, 4420-34.

Dawson, I. A., Roth, S., Akam, M. and Artavanis-Tsakonas, S. (1993). Mutations of the fizzy locus cause metaphase arrest in Drosophila melanogaster embryos. *Development* **117**, 359-76.

Dawson, I. A., Roth, S. and Artavanis-Tsakonas, S. (1995). The Drosophila cell cycle gene fizzy is required for normal degradation of cyclins A and B during mitosis and has homology to the CDC20 gene of Saccharomyces cerevisiae. *J Cell Biol* **129**, 725-37.

de Melker, A. A., van der Horst, G. and Borst, J. (2004). Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment. *J Biol Chem* **279**, 55465-73.

Dearborn, R., Jr. and Kunes, S. (2004). An axon scaffold induced by retinal axons directs glia to destinations in the Drosophila optic lobe. *Development* **131**, 2291-303.

Donze, D. and Kamakaka, R. T. (2001). RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in Saccharomyces cerevisiae. *Embo J* **20**, 520-31.

Dorn, R., Krauss, V., Reuter, G. and Saumweber, H. (1993). The enhancer of position-effect variegation of Drosophila, E(var)3-93D, codes for a chromatin protein containing a conserved domain common to several transcriptional regulators. *Proc Natl Acad Sci U S A* **90**, 11376-80.

Edenfeld, G., Pielage, J. and Klambt, C. (2002). Cell lineage specification in the nervous system. *Curr Opin Genet Dev* **12**, 473-7.

Edenfeld, G., Stork, T. and Klambt, C. (2005). Neuron-glia interaction in the insect nervous system. *Curr Opin Neurobiol* **15**, 34-9.

Egger, B., Boone, J. Q., Stevens, N. R., Brand, A. H. and Doe, C. Q. (2007). Regulation of spindle orientation and neural stem cell fate in the Drosophila optic lobe. *Neural Develop* **2**, 1.

Englund, C., Uv, A. E., Cantera, R., Mathies, L. D., Krasnow, M. A. and Samakovlis, C. (1999). adrift, a novel bnl-induced Drosophila gene, required for tracheal pathfinding into the CNS. *Development* **126**, 1505-14.

Fujise, M., Takeo, S., Kamimura, K., Matsuo, T., Aigaki, T., Izumi, S. and Nakato, H. (2003). Dally regulates Dpp morphogen gradient formation in the Drosophila wing. *Development* **130**, 1515-22.

Fuse, N., Hisata, K., Katzen, A. L. and Matsuzaki, F. (2003). Heterotrimeric G proteins regulate daughter cell size asymmetry in Drosophila neuroblast divisions. *Curr Biol* **13**, 947-54.

Gaengel, K. and Mlodzik, M. (2003). Egfr signaling regulates ommatidial rotation and cell motility in the Drosophila eye via MAPK/Pnt signaling and the Ras effector Canoe/AF6. *Development* **130**, 5413-23.

Gatfield, D. and Izaurralde, E. (2002). REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export. *J Cell Biol* **159**, 579-88.

Gautier, P., Ledent, V., Massaer, M., Dambly-Chaudiere, C. and Ghysen, A. (1997). tap, a Drosophila bHLH gene expressed in chemosensory organs. *Gene* **191**, 15-21.

Geley, S., Kramer, E., Gieffers, C., Gannon, J., Peters, J. M. and Hunt, T. (2001). Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. *J Cell Biol* **153**, 137-48.

Gerasimova, T. I. and Corces, V. G. (1998). Polycomb and trithorax group proteins mediate the function of a chromatin insulator. *Cell* **92**, 511-21.

Gerasimova, T. I., Gdula, D. A., Gerasimov, D. V., Simonova, O. and Corces, V. G. (1995). A Drosophila protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. *Cell* **82**, 587-97.

Giesen, K., Hummel, T., Stollewerk, A., Harrison, S., Travers, A. and Klambt, C. (1997). Glial development in the Drosophila CNS requires concomitant activation of glial and repression of neuronal differentiation genes. *Development* **124**, 2307-16.

Godt, D., Couderc, J. L., Cramton, S. E. and Laski, F. A. (1993). Pattern formation in the limbs of Drosophila: bric a brac is expressed in both a gradient and a wave-like pattern and is required for specification and proper segmentation of the tarsus. *Development* **119**, 799-812.

Golic, M. M., Rong, Y. S., Petersen, R. B., Lindquist, S. L. and Golic, K. G. (1997). FLP-mediated DNA mobilization to specific target sites in Drosophila chromosomes. *Nucleic Acids Res* **25**, 3665-71.
Goodman, R. H. and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. *Genes Dev* **14**, 1553-77.

Granderath, S., Bunse, I. and Klambt, C. (2000). gcm and pointed synergistically control glial transcription of the Drosophila gene loco. *Mech Dev* **91**, 197-208.
Granderath, S. and Klambt, C. (2004). Identification and functional analysis of the Drosophila gene loco. *Methods Enzymol* **389**, 350-63
.
Granderath, S., Stollewerk, A., Greig, S., Goodman, C. S., O'Kane, C. J. and Klambt, C. (1999). loco encodes an RGS protein required for Drosophila glial differentiation. *Development* **126**, 1781-91.

Grosskortenhaus, R. and Sprenger, F. (2002). Rca1 inhibits APC-Cdh1(Fzr) and is required to prevent cyclin degradation in G2. *Dev Cell* **2**, 29-40.

Grueber, W. B. and Jan, Y. N. (2004). Dendritic development: lessons from Drosophila and related branches. *Curr Opin Neurobiol* **14**, 74-82.

Guardavaccaro, D., Kudo, Y., Boulaire, J., Barchi, M., Busino, L., Donzelli, M., Margottin-Goguet, F., Jackson, P. K., Yamasaki, L. and Pagano, M. (2003). Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. *Dev Cell* **4**, 799-812.

Han, J., Mark, M. D., Li, X., Xie, M., Waka, S., Rettig, J. and Herlitze, S. (2006). RGS2 determines short-term synaptic plasticity in hippocampal neurons by regulating Gi/o-mediated inhibition of presynaptic Ca2+ channels. *Neuron* **51**, 575-86.

Harvey, A. J., Bidwai, A. P. and Miller, L. K. (1997). Doom, a product of the Drosophila mod(mdg4) gene, induces apoptosis and binds to baculovirus inhibitor-of-apoptosis proteins. *Mol Cell Biol* **17**, 2835-43.

Hawkins, N. C., Thorpe, J. and Schupbach, T. (1996). Encore, a gene required for the regulation of germ line mitosis and oocyte differentiation during Drosophila oogenesis. *Development* **122**, 281-90.

Hegde, A. N. and DiAntonio, A. (2002). Ubiquitin and the synapse. *Nat Rev Neurosci* **3**, 854-61.

Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67**, 425-79.

Hidalgo, A., Urban, J. and Brand, A. H. (1995). Targeted ablation of glia disrupts axon tract formation in the Drosophila CNS. *Development* **121**, 3703-12.

Hilioti, Z., Chung, Y. S., Mochizuki, Y., Hardy, C. F. and Cohen-Fix, O. (2001). The anaphase inhibitor Pds1 binds to the APC/C-associated protein Cdc20 in a destruction box-dependent manner. *Curr Biol* **11**, 1347-52.

Holzl, H., Kapelari, B., Kellermann, J., Seemuller, E., Sumegi, M., Udvardy, A., Medalia, O., Sperling, J., Muller, S. A., Engel, A. et al. (2000). The regulatory complex of Drosophila melanogaster 26S proteasomes. Subunit composition and localization of a deubiquitylating enzyme. *J Cell Biol* **150**, 119-30.

Hosoya, T., Takizawa, K., Nitta, K. and Hotta, Y. (1995). glial cells missing: a binary switch between neuronal and glial determination in Drosophila. *Cell* **82**, 1025-36.

Huang, X. and Miller, W. (1991). A Time-Efficient, Linear-Space Local Similarity Algorithm. *Advances in Applied Mathematics* **12**, 337-357.

Huang, Z. and Kunes, S. (1998). Signals transmitted along retinal axons in Drosophila: Hedgehog signal reception and the cell circuitry of lamina cartridge assembly. *Development* **125**, 3753-64.

Ikeshima-Kataoka, H., Skeath, J. B., Nabeshima, Y., Doe, C. Q. and Matsuzaki, F. (1997). Miranda directs Prospero to a daughter cell during Drosophila asymmetric divisions. *Nature* **390**, 625-9.

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., Yang, P., Sarao, R., Wada, T., Leong-Poi, H. et al. (2005). Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* **436**, 112-6.

Jacobs, H., Richter, D., Venkatesh, T. and Lehner, C. (2002). Completion of mitosis requires neither fzr/rap nor fzr2, a male germline-specific Drosophila Cdh1 homolog. *Curr Biol* **12**, 1435-41.

Jan, Y. N. and Jan, L. Y. (1998). Asymmetric cell division. *Nature* **392**, 775-8
.
Jan, Y. N. and Jan, L. Y. (2001). Asymmetric cell division in the Drosophila nervous system. *Nat Rev Neurosci* **2**, 772-9.

Joazeiro, C. A. and Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**, 549-52.

Johnstone, O. and Lasko, P. (2001). Translational regulation and RNA localization in Drosophila oocytes and embryos. *Annu Rev Genet* **35**, 365-406.

Jones, B. W. (2005). Transcriptional control of glial cell development in Drosophila. *Dev Biol* **278**, 265-73.

Jones, B. W., Fetter, R. D., Tear, G. and Goodman, C. S. (1995). glial cells missing: a genetic switch that controls glial versus neuronal fate. *Cell* **82**, 1013-23.

Kaelin, W. G., Jr. and Maher, E. R. (1998). The VHL tumour-suppressor gene paradigm. *Trends Genet* **14**, 423-6.

Kaiser, P., Flick, K., Wittenberg, C. and Reed, S. I. (2000). Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4. *Cell* **102**, 303-14.

Kandel, E. R., Schwartz, J. H. and Jessell, T. M. (2000). Principles of Neural Science: The McGraw-Hill Companies,Inc.

Kaplow, M. E., Korayem, A. H. and Venkatesh, T. R. (2008). Regulation of Glia Number in Drosophila by Rap/Fzr, an Activator of the Anaphase-Promoting Complex, and Loco, an RGS Protein. *Genetics* **178**, 2003-16.

Kaplow, M. E., Mannava, L. J., Pimentel, A. C., Fermin, H. A., Hyatt, V. J., Lee, J. J. and Venkatesh, T. R. (2007). A genetic modifier screen identifies multiple genes that interact with Drosophila Rap/Fzr and suggests novel cellular roles. *J Neurogenet* **21**, 105-51.

Karin, M. and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18**, 621-63.

Karpilow, J., Kolodkin, A., Bork, T. and Venkatesh, T. (1989a). Neuronal development in the Drosophila compound eye: rap gene function is required in photoreceptor cell R8 for ommatidial pattern formation. *Genes Dev* **3**, 1834-44.

Karpilow, J., Kolodkin, A., Bork, T. and Venkatesh, T. (1989b). Neuronal development in the Drosophila compound eye: rap gene function is required in photoreceptor cell R8 for ommatidial pattern formation. *Genes Dev* **3**, 1834-44.

Karpilow, J. M., Pimentel, A. C., Shamloula, H. K. and Venkatesh, T. R. (1996). Neuronal development in the Drosophila compound eye: photoreceptor cells R1, R6, and R7 fail to differentiate in the retina aberrant in pattern (rap) mutant. *J Neurobiol* **31**, 149-65.

Kashevsky, H., Wallace, J. A., Reed, B. H., Lai, C., Hayashi-Hagihara, A. and Orr-Weaver, T. L. (2002). The anaphase promoting complex/cyclosome is required during development for modified cell cycles. *Proc Natl Acad Sci U S A* **99**, 11217-22.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H. and Klambt, C. (1994). The Ets transcription factors encoded by the Drosophila gene pointed direct glial cell differentiation in the embryonic CNS. *Cell* **78**, 149-60.

Klambt, C. (1993). The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* **117**, 163-76.

Kockel, L., Vorbruggen, G., Jackle, H., Mlodzik, M. and Bohmann, D. (1997). Requirement for Drosophila 14-3-3 zeta in Raf-dependent photoreceptor development. *Genes Dev* **11**, 1140-7.

Koken, M., Reynolds, P., Bootsma, D., Hoeijmakers, J., Prakash, S. and Prakash, L. (1991). Dhr6, a Drosophila homolog of the yeast DNA-repair gene RAD6. *Proc Natl Acad Sci U S A* **88**, 3832-6.

Kolodkin, A. L., Pickup, A. T., Lin, D. M., Goodman, C. S. and Banerjee, U. (1994). Characterization of Star and its interactions with sevenless and EGF receptor during photoreceptor cell development in Drosophila. *Development* **120**, 1731-45.

Konishi, Y., Stegmuller, J., Matsuda, T., Bonni, S. and Bonni, A. (2004). Cdh1-APC controls axonal growth and patterning in the mammalian brain. *Science* **303**, 1026-30.

Kozuka, N., Itofusa, R., Kudo, Y. and Morita, M. (2005). Lipopolysaccharide and proinflammatory cytokines require different astrocyte states to induce nitric oxide production. *J Neurosci Res* **82**, 717-28.

Kretzschmar, D., Hasan, G., Sharma, S., Heisenberg, M. and Benzer, S. (1997). The swiss cheese mutant causes glial hyperwrapping and brain degeneration in Drosophila. *J Neurosci* **17**, 7425-32.

Kuba, K., Imai, Y., Rao, S., Gao, H., Guo, F., Guan, B., Huan, Y., Yang, P., Zhang, Y., Deng, W. et al. (2005). A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med* **11**, 875-9.

Kurant, E., Axelrod, S., Leaman, D. and Gaul, U. (2008). Six-microns-under acts upstream of Draper in the glial phagocytosis of apoptotic neurons. *Cell* **133**, 498-509.

Lasorella, A., Stegmuller, J., Guardavaccaro, D., Liu, G., Carro, M. S., Rothschild, G., de la Torre-Ubieta, L., Pagano, M., Bonni, A. and Iavarone, A. (2006). Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth. *Nature* **442**, 471-4.

Ledent, V., Gaillard, F., Gautier, P., Ghysen, A. and Dambly-Chaudiere, C. (1998). Expression and function of tap in the gustatory and olfactory organs of Drosophila. *Int J Dev Biol* **42**, 163-70.

Lee, C. Y., Andersen, R. O., Cabernard, C., Manning, L., Tran, K. D., Lanskey, M. J., Bashirullah, A. and Doe, C. Q. (2006a). Drosophila Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. *Genes Dev* **20**, 3464-74.

Lee, C. Y., Robinson, K. J. and Doe, C. Q. (2006b). Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. *Nature* **439**, 594-8.

Lee, L. A., Elfring, L. K., Bosco, G. and Orr-Weaver, T. L. (2001). A genetic screen for suppressors and enhancers of the Drosophila PAN GU cell cycle kinase identifies cyclin B as a target. *Genetics* **158**, 1545-56.

Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-61
.
Lei, Y. and Warrior, R. (2000). The Drosophila Lissencephaly1 (DLis1) gene is required for nuclear migration. *Dev Biol* **226**, 57-72.

Lemke, G. (2001). Glial control of neuronal development. *Annu Rev Neurosci* **24**, 87-105.

Levashina, E. A., Langley, E., Green, C., Gubb, D., Ashburner, M., Hoffmann, J. A. and Reichhart, J. M. (1999). Constitutive activation of toll-mediated antifungal defense in serpin-deficient Drosophila. *Science* **285**, 1917-9.

Lipkowitz, S. (2003). The role of the ubiquitination-proteasome pathway in breast cancer: ubiquitin mediated degradation of growth factor receptors in the pathogenesis and treatment of cancer. *Breast Cancer Res* **5**, 8-15.

Listovsky, T., Oren, Y. S., Yudkovsky, Y., Mahbubani, H. M., Weiss, A. M., Lebendiker, M. and Brandeis, M. (2004). Mammalian Cdh1/Fzr mediates its own degradation. *Embo J* **23**, 1619-26.

Listovsky, T., Zor, A., Laronne, A. and Brandeis, M. (2000). Cdk1 is essential for mammalian cyclosome/APC regulation. *Exp Cell Res* **255**, 184-91.

Littlepage, L. E. and Ruderman, J. V. (2002). Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. *Genes Dev* **16**, 2274-85.

Liu, X., Kiss, I. and Lengyel, J. A. (1999a). Identification of genes controlling malpighian tubule and other epithelial morphogenesis in Drosophila melanogaster. *Genetics* **151**, 685-95.

Liu, Z., Steward, R. and Luo, L. (2000). Drosophila Lis1 is required for neuroblast proliferation, dendritic elaboration and axonal transport. *Nat Cell Biol* **2**, 776-83.

Liu, Z., Xie, T. and Steward, R. (1999b). Lis1, the Drosophila homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation. *Development* **126**, 4477-88.

Look, A. T., Ashmun, R. A., Shapiro, L. H. and Peiper, S. C. (1989). Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase N. *J Clin Invest* **83**, 1299-307.

Martin, K. A., Poeck, B., Roth, H., Ebens, A. J., Ballard, L. C. and Zipursky, S. L. (1995). Mutations disrupting neuronal connectivity in the Drosophila visual system. *Neuron* **14**, 229-40.

Matheny, S. A., Chen, C., Kortum, R. L., Razidlo, G. L., Lewis, R. E. and White, M. A. (2004). Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP. *Nature* **427**, 256-60.

Mayer, U. and Nusslein-Volhard, C. (1988). A group of genes required for pattern formation in the ventral ectoderm of the Drosophila embryo. *Genes Dev* **2**, 1496-511.

Milchanowski, A. B., Henkenius, A. L., Narayanan, M., Hartenstein, V. and Banerjee, U. (2004). Identification and characterization of genes involved in embryonic crystal cell formation during Drosophila hematopoiesis. *Genetics* **168**, 325-39.

Miller, G. (2005). Neuroscience. The dark side of glia. *Science* **308**, 778-81.

Mohler, J., Mahaffey, J. W., Deutsch, E. and Vani, K. (1995). Control of Drosophila head segment identity by the bZIP homeotic gene cnc. *Development* **121**, 237-47.

Mohler, J., Vani, K., Leung, S. and Epstein, A. (1991). Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis. *Mech Dev* **34**, 3-9.

Morris, S. M., Albrecht, U., Reiner, O., Eichele, G. and Yu-Lee, L. Y. (1998). The lissencephaly gene product Lis1, a protein involved in neuronal migration, interacts with a nuclear movement protein, NudC. *Curr Biol* **8**, 603-6.

Muller, T., Grosche, J., Ohlemeyer, C. and Kettenmann, H. (1993). NMDA-activated currents in Bergmann glial cells. *Neuroreport* **4**, 671-4.

Muratani, M. and Tansey, W. P. (2003). How the ubiquitin-proteasome system controls transcription. *Nat Rev Mol Cell Biol* **4**, 192-201.

Murray, A. W. (2004). Recycling the cell cycle: cyclins revisited. *Cell* **116**, 221-34.

Nakato, H., Fox, B. and Selleck, S. B. (2002). dally, a Drosophila member of the glypican family of integral membrane proteoglycans, affects cell cycle progression and morphogenesis via a Cyclin A-mediated process. *J Cell Sci* **115**, 123-30.

Narbonne-Reveau, K., Senger, S., Pal, M., Herr, A., Richardson, H. E., Asano, M., Deak, P. and Lilly, M. A. (2008). APC/CFzr/Cdh1 promotes cell cycle progression during the Drosophila endocycle. *Development* **135**, 1451-61.

Nourry, C., Maksumova, L., Pang, M., Liu, X. and Wang, T. (2004). Direct interaction between Smad3, APC10, CDH1 and HEF1 in proteasomal degradation of HEF1. *BMC Cell Biol* **5**, 20.

Nusslein-Volhard, C., Wieschaus,E.,Kluding,H.,. (1984). Mutations affecting pattern of the larval cuticle in Drosophila melanogaster. I: Zygotic loci on the second chromosome. *Wilhelm Roux's archives of developmental biology* **193**, 267-282.

Ohlmeyer, J. T. and Schupbach, T. (2003). Encore facilitates SCF-Ubiquitin-proteasome-dependent proteolysis during Drosophila oogenesis. *Development* **130**, 6339-49.

Ory, S. and Morrison, D. K. (2004). Signal transduction: implications for Ras-dependent ERK signaling. *Curr Biol* **14**, R277-8.

Ou, C. Y., Pi, H. and Chien, C. T. (2003). Control of protein degradation by E3 ubiquitin ligases in Drosophila eye development. *Trends Genet* **19**, 382-9.

Page, A. R., Kovacs, A., Deak, P., Torok, T., Kiss, I., Dario, P., Bastos, C., Batista, P., Gomes, R., Ohkura, H. et al. (2005). Spotted-dick, a zinc-finger protein of Drosophila required for expression of Orc4 and S phase. *Embo J* **24**, 4304-15.

Palacios, I. M. (2002). RNA processing: splicing and the cytoplasmic localisation of mRNA. *Curr Biol* **12**, R50-2.

Pathirana, S., Zhao, D. and Bownes, M. (2001). The Drosophila RGS protein Loco is required for dorsal/ventral axis formation of the egg and embryo, and nurse cell dumping. *Mech Dev* **109**, 137-50.

Pearson, B. J. and Doe, C. Q. (2003). Regulation of neuroblast competence in Drosophila. *Nature* **425**, 624-8.

Peixoto, A. A. and Hall, J. C. (1998). Analysis of temperature-sensitive mutants reveals new genes involved in the courtship song of Drosophila. *Genetics* **148**, 827-38.

Pereanu, W., Shy, D. and Hartenstein, V. (2005). Morphogenesis and proliferation of the larval brain glia in Drosophila. *Dev Biol* **283**, 191-203.

Perez, S. E. and Steller, H. (1996). Migration of glial cells into retinal axon target field in Drosophila melanogaster. *J Neurobiol* **30**, 359-73.

Peters, J. M. (2002). The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol Cell* **9**, 931-43.

Pfleger, C. M. and Kirschner, M. W. (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev* **14**, 655-65.

Pickart, C. M. (2001). Ubiquitin enters the new millennium. *Mol Cell* **8**, 499-504.

Pickart, C. M. (2004). Back to the future with ubiquitin. *Cell* **116**, 181-90.

Pimentel, A. C. and Venkatesh, T. R. (2005). rap gene encodes Fizzy-related protein (Fzr) and regulates cell proliferation and pattern formation in the developing Drosophila eye-antennal disc. *Dev Biol* **285**, 436-46.

Poeck, B., Fischer, S., Gunning, D., Zipursky, S. L. and Salecker, I. (2001). Glial cells mediate target layer selection of retinal axons in the developing visual system of Drosophila. *Neuron* **29**, 99-113.

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., Knapp, D. J. and Crews, F. T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* **55**, 453-62.

Rangarajan, R., Gong, Q. and Gaul, U. (1999). Migration and function of glia in the developing Drosophila eye. *Development* **126**, 3285-92.

Roch, F., Serras, F., Cifuentes, F. J., Corominas, M., Alsina, B., Amoros, M., Lopez-Varea, A., Hernandez, R., Guerra, D., Cavicchi, S. et al. (1998). Screening of larval/pupal P-element induced lethals on the second chromosome in Drosophila melanogaster: clonal analysis and morphology of imaginal discs. *Mol Gen Genet* **257**, 103-12.

Rolls, M. M., Albertson, R., Shih, H. P., Lee, C. Y. and Doe, C. Q. (2003). Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. *J Cell Biol* **163**, 1089-98.

Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. and Therrien, M. (2002). KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev* **16**, 427-38.

Russell, S. R., Heimbeck, G., Goddard, C. M., Carpenter, A. T. and Ashburner, M. (1996). The Drosophila Eip78C gene is not vital but has a role in regulating chromosome puffs. *Genetics* **144**, 159-70.

Salghetti, S. E., Caudy, A. A., Chenoweth, J. G. and Tansey, W. P. (2001). Regulation of transcriptional activation domain function by ubiquitin. *Science* **293**, 1651-3.

Santos, A. N., Langner, J., Herrmann, M. and Riemann, D. (2000). Aminopeptidase N/CD13 is directly linked to signal transduction pathways in monocytes. *Cell Immunol* **201**, 22-32.

Schaeffer, V., Althauser, C., Shcherbata, H. R., Deng, W. M. and Ruohola-Baker, H. (2004). Notch-dependent Fizzy-related/Hec1/Cdh1 expression is required for the mitotic-to-endocycle transition in Drosophila follicle cells. *Curr Biol* **14**, 630-6.

Schwab, M., Lutum, A. S. and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* **90**, 683-93.

Schwabe, T., Bainton, R. J., Fetter, R. D., Heberlein, U. and Gaul, U. (2005). GPCR signaling is required for blood-brain barrier formation in drosophila. *Cell* **123**, 133-44.

Shcherbata, H. R., Althauser, C., Findley, S. D. and Ruohola-Baker, H. (2004). The mitotic-to-endocycle switch in Drosophila follicle cells is executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions. *Development* **131**, 3169-81.

Shen, C. P., Knoblich, J. A., Chan, Y. M., Jiang, M. M., Jan, L. Y. and Jan, Y. N. (1998). Miranda as a multidomain adapter linking apically localized Inscuteable and basally localized Staufen and Prospero during asymmetric cell division in Drosophila. *Genes Dev* **12**, 1837-46.

Shiomi, K., Takeichi, M., Nishida, Y., Nishi, Y. and Uemura, T. (1994). Alternative cell fate choice induced by low-level expression of a regulator of protein phosphatase 2A in the Drosophila peripheral nervous system. *Development* **120**, 1591-9.

Sigrist, S., Jacobs, H., Stratmann, R. and Lehner, C. F. (1995). Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. *Embo J* **14**, 4827-38.

Sigrist, S. J. and Lehner, C. F. (1997). Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* **90**, 671-81.

Skeath, J. B. and Thor, S. (2003). Genetic control of Drosophila nerve cord development. *Curr Opin Neurobiol* **13**, 8-15.

Skoulakis, E. M. and Davis, R. L. (1996). Olfactory learning deficits in mutants for leonardo, a Drosophila gene encoding a 14-3-3 protein. *Neuron* **17**, 931-44.

Slack, C., Overton, P.M., Tuxworth, R.I, Chia, W. (2007). Asymmetric localisation of Miranda and its cargo proteins during neuroblast division requires the anaphase-promoting complex/cyclosome. *Development* **134**, 3781-3787.

Slack, C., Somers, W. G., Sousa-Nunes, R., Chia, W. and Overton, P. M. (2006). A mosaic genetic screen for novel mutations affecting Drosophila neuroblast divisions. *BMC Genet* **7**, 33.

Soustelle, L. and Giangrande, A. (2007). Novel gcm-dependent lineages in the postembryonic nervous system of Drosophila melanogaster. *Dev Dyn* **236**, 2101-8.

Speese, S. D., Trotta, N., Rodesch, C. K., Aravamudan, B. and Broadie, K. (2003). The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy. *Curr Biol* **13**, 899-910.

Stanley, E. R. and Freeman, M. R. (2008). Draper-dependent glial phagocytic activity is mediated by Src and Syk family kinase signalling. *Nature* **453**, 935-9.

Stegmeier, F., Rape, M., Draviam, V. M., Nalepa, G., Sowa, M. E., Ang, X. L., McDonald, E. R., 3rd, Li, M. Z., Hannon, G. J., Sorger, P. K. et al. (2007). Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. *Nature* **446**, 876-81.

Stegmuller, J. and Bonni, A. (2005). Moving past proliferation: new roles for Cdh1-APC in postmitotic neurons. *Trends Neurosci* **28**, 596-601.

Stegmuller, J., Huynh, M. A., Yuan, Z., Konishi, Y. and Bonni, A. (2008). TGFbeta-Smad2 signaling regulates the Cdh1-APC/SnoN pathway of axonal morphogenesis. *J Neurosci* **28**, 1961-9.

Stegmuller, J., Konishi, Y., Huynh, M. A., Yuan, Z., Dibacco, S. and Bonni, A. (2006). Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. *Neuron* **50**, 389-400.

Stein, D. and Nusslein-Volhard, C. (1992). Multiple extracellular activities in Drosophila egg perivitelline fluid are required for establishment of embryonic dorsal-ventral polarity. *Cell* **68**, 429-40.

Stork, T., Engelen, D., Krudewig, A., Silies, M., Bainton, R. J. and Klambt, C. (2008). Organization and function of the blood-brain barrier in Drosophila. *J Neurosci* **28**, 587-97.

Strausfeld, U. P., Howell, M., Descombes, P., Chevalier, S., Rempel, R. E., Adamczewski, J., Maller, J. L., Hunt, T. and Blow, J. J. (1996). Both cyclin A and cyclin E have S-phase promoting (SPF) activity in Xenopus egg extracts. *J Cell Sci* **109 (Pt 6)**, 1555-63.

Su, T. T., Sprenger, F., DiGregorio, P. J., Campbell, S. D. and O'Farrell, P. H. (1998). Exit from mitosis in Drosophila syncytial embryos requires proteolysis and cyclin degradation, and is associated with localized dephosphorylation. *Genes Dev* **12**, 1495-503.

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V. and Hershko, A. (1995). The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Mol Biol Cell* **6**, 185-97.

Swan, A., Nguyen, T. and Suter, B. (1999). Drosophila Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. *Nat Cell Biol* **1**, 444-9.

Taylor, C. A., Coates, D. and Shirras, A. D. (1996). The Acer gene of Drosophila codes for an angiotensin-converting enzyme homologue. *Gene* **181**, 191-7.

Tekotte, H. and Davis, I. (2002). Intracellular mRNA localization: motors move messages. *Trends Genet* **18**, 636-42.

Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H. L. et al. (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. *Nat Genet* **36**, 283-7.

Tsruya, R., Schlesinger, A., Reich, A., Gabay, L., Sapir, A. and Shilo, B. Z. (2002). Intracellular trafficking by Star regulates cleavage of the Drosophila EGF receptor ligand Spitz. *Genes Dev* **16**, 222-34.

Tsuda, L., Nagaraj, R., Zipursky, S. L. and Banerjee, U. (2002). An EGFR/Ebi/Sno pathway promotes delta expression by inactivating Su(H)/SMRTER repression during inductive notch signaling. *Cell* **110**, 625-37.

Turnell, A. S., Stewart, G. S., Grand, R. J., Rookes, S. M., Martin, A., Yamano, H., Elledge, S. J. and Gallimore, P. H. (2005). The APC/C and CBP/p300 cooperate to regulate transcription and cell-cycle progression. *Nature* **438**, 690-5.

Udolph, G., Prokop, A., Bossing, T. and Technau, G. M. (1993). A common precursor for glia and neurons in the embryonic CNS of Drosophila gives rise to segment-specific lineage variants. *Development* **118**, 765-75.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1989). numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. *Cell* **58**, 349-60.

Uemura, T., Shiomi, K., Togashi, S. and Takeichi, M. (1993). Mutation of twins encoding a regulator of protein phosphatase 2A leads to pattern duplication in Drosophila imaginal discs. *Genes Dev* **7**, 429-40.

Urbach, R., Schnabel, R. and Technau, G. M. (2003). The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in Drosophila. *Development* **130**, 3589-606.

van der Voorn, L. and Ploegh, H. L. (1992). The WD-40 repeat. *FEBS Lett* **307**, 131-4.

van Roessel, P., Elliott, D. A., Robinson, I. M., Prokop, A. and Brand, A. H. (2004). Independent regulation of synaptic size and activity by the anaphase-promoting complex. *Cell* **119**, 707-18.

Vierstra, R. D. (2003). The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends Plant Sci* **8**, 135-42.

Vincent, S., Vonesch, J. L. and Giangrande, A. (1996). Glide directs glial fate commitment and cell fate switch between neurones and glia. *Development* **122**, 131-9.

Wan, H. I., DiAntonio, A., Fetter, R. D., Bergstrom, K., Strauss, R. and Goodman, C. S. (2000). Highwire regulates synaptic growth in Drosophila. *Neuron* **26**, 313-29.

Wang, H., Ouyang, Y., Somers, W. G., Chia, W. and Lu, B. (2007). Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. *Nature* **449**, 96-100.

Wang, H., Somers, G. W., Bashirullah, A., Heberlein, U., Yu, F. and Chia, W. (2006). Aurora-A acts as a tumor suppressor and regulates self-renewal of Drosophila neuroblasts. *Genes Dev* **20**, 3453-63.

Warbington, L., Hillman, T., Adams, C. and Stern, M. (1996). Reduced transmitter release conferred by mutations in the slowpoke-encoded Ca2(+)-activated K+ channel gene of Drosophila. *Invert Neurosci* **2**, 51-60.

Ward, P. E., Benter, I. F., Dick, L. and Wilk, S. (1990). Metabolism of vasoactive peptides by plasma and purified renal aminopeptidase M. *Biochem Pharmacol* **40**, 1725-32.

Wassarman, D. A., Solomon, N. M., Chang, H. C., Karim, F. D., Therrien, M. and Rubin, G. M. (1996). Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in Drosophila. *Genes Dev* **10**, 272-8.

Watts, R. J., Schuldiner, O., Perrino, J., Larsen, C. and Luo, L. (2004). Glia engulf degenerating axons during developmental axon pruning. *Curr Biol* **14**, 678-84.

Weake, V. M., Lee, K. K., Guelman, S., Lin, C. H., Seidel, C., Abmayr, S. M. and Workman, J. L. (2008). SAGA-mediated H2B deubiquitination controls the development of neuronal connectivity in the Drosophila visual system. *Embo J* **27**, 394-405.

White-Cooper, H., Carmena, M., Gonzalez, C. and Glover, D. M. (1996). Mutations in new cell cycle genes that fail to complement a multiply mutant third chromosome of Drosophila. *Genetics* **144**, 1097-111.

Wilk, R., Pickup, A. T., Hamilton, J. K., Reed, B. H. and Lipshitz, H. D. (2004). Dose-sensitive autosomal modifiers identify candidate genes for tissue autonomous and tissue nonautonomous regulation by the Drosophila nuclear zinc-finger protein, hindsight. *Genetics* **168**, 281-300.

Wilkinson, K. D. (1997). Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *Faseb J* **11**, 1245-56.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-37.

Yao, K. M. and White, K. (1994). Neural specificity of elav expression: defining a Drosophila promoter for directing expression to the nervous system. *J Neurochem* **63**, 41-51.

Yazejian, B., DiGregorio, D. A., Vergara, J. L., Poage, R. E., Meriney, S. D. and Grinnell, A. D. (1997). Direct measurements of presynaptic calcium and calcium-activated potassium currents regulating neurotransmitter release at cultured Xenopus nerve-muscle synapses. *J Neurosci* **17**, 2990-3001.

Yu, F., Kuo, C. T. and Jan, Y. N. (2006). Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. *Neuron* **51**, 13-20
.
Yu, F., Wang, H., Qian, H., Kaushik, R., Bownes, M., Yang, X. and Chia, W. (2005). Locomotion defects, together with Pins, regulates heterotrimeric G-protein signaling during Drosophila neuroblast asymmetric divisions. *Genes Dev* **19**, 1341-53.

Yuasa, Y., Okabe, M., Yoshikawa, S., Tabuchi, K., Xiong, W. C., Hiromi, Y. and Okano, H. (2003). Drosophila homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors. *Development* **130**, 2419-28.

Zachariae, W. and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev* **13**, 2039-58.

Zhang, B., Koh, Y. H., Beckstead, R. B., Budnik, V., Ganetzky, B. and Bellen, H. J. (1998). Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron* **21**, 1465-75.

Zhang, H., Tan, J., Reynolds, E., Kuebler, D., Faulhaber, S. and Tanouye, M. (2002). The Drosophila slamdance gene: a mutation in an aminopeptidase can cause seizure, paralysis and neuronal failure. *Genetics* **162**, 1283-99.

Zhang, Y. (2003). Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes Dev* **17**, 2733-40.

Zhou, Y., Schopperle, W. M., Murrey, H., Jaramillo, A., Dagan, D., Griffith, L. C. and Levitan, I. B. (1999). A dynamically regulated 14-3-3, Slob, and Slowpoke potassium channel complex in Drosophila presynaptic nerve terminals. *Neuron* **22**, 809-18.

Zielke, N., Querings, S., Rottig, C., Lehner, C. and Sprenger, F. (2008). The anaphase-promoting complex/cyclosome (APC/C) is required for rereplication control in endoreplication cycles. *Genes Dev* **22**, 1690-703.

Zini, S., Fournie-Zaluski, M. C., Chauvel, E., Roques, B. P., Corvol, P. and Llorens-Cortes, C. (1996). Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. *Proc Natl Acad Sci U S A* **93**, 11968-73.

Zollman, S., Godt, D., Prive, G. G., Couderc, J. L. and Laski, F. A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in Drosophila. *Proc Natl Acad Sci U S A* **91**, 10717-21.

Zur, A. and Brandeis, M. (2002). Timing of APC/C substrate degradation is determined by fzy/fzr specificity of destruction boxes. *Embo J* **21**, 4500-10.