Characterization of growth enhancing mutant Mitochondrial rho GTPase (Miro) in *Drosophila*

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali
April 2016

Dedicated to my father

Certificate of Examination

This is to certify that the dissertation titled "**Characterization of growth enhancing mutant Mitochondrial rho GTPase (Miro) in *Drosophila***" submitted by Mr. Yashpal Singh (Reg. No. MS11042) for the partial ful-filment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated: April 20, 2016

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Sudip Mandal at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Yashpal Singh

(Candidate)

Dated: April 20, 2016

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr.Sudip Mandal
(Supervisor)

Acknowledgment

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Abstract

The evolutionarily conserved mitochondrial Rho (Miro) is a small GTPase belongs to Ras superfamily member with three unique features. It has two GTPase domains, unlike only one found in other small GTPases, and it also has two EF-hand calcium-binding domains, which allow Ca2+-dependent modulation of its activity and functions. Miro has been shown to act as a key player in mitochondrial transport and dynamics. However, whether Miro function regulates major cell biological processes such as cell growth, cell proliferation, cell death and cell differentiation has not yet worked out. In this study, we report that Miro can enhance the overgrowth phenotype associated with CycD/CDK4 overexpression in the adult eye of *Drosophila*. Moreover, Miro knockdown in fat body cells leads to the increase in fat body cell size, suggesting Miro functions as a negative regulator of cell growth. Furthermore, Miro loss of function cells in larval eye imaginal disc has accelerated proliferation. However, Miro loss of function does not seem to affect other cell biological processes. This study suggests that perturbed mitochondrial dynamics by Miro loss of function can regulate cell growth and consequent proliferation.

CHAPTER 1: Introduction, Objectives, materials and methods

1. Introduction

1.1Mitochondria: A dynamic organelle

The mitochondrion is an endosymbiont-derived organelle [1]. In a classical view, mitochondria are the "powerhouse" of the cell which provides energy, in the form of ATP to be utilized in cellular reactions. Over the years, our grasp on mitochondrial function and dynamics has extended, earlier thought to be rigidly structured, they are now considered as an organelle that constantly, fuse, and divide, and undergo regulated turnover. These dynamic processes enable mitochondrial functioning by regulating mitochondrial recruitment to critical regions within the cell, exchange of content between mitochondria, mitochondrial cross talk with the cytosol and the mitochondrial quality control. Hence, mitochondria can readily adapt to changes as per cellular requirements.

With multiple key roles in eukaryotic various cellular processes, best known as the compartment that has the respiratory electron transport chain, the mitochondrion is also the site of the fatty acid-oxidation and TCA cycle, and it serves to participate in intracellular calcium buffering [2, 3]. However, these organelles have turned out to play vital roles in calcium homeostasis, in the formation of reactive oxygen species and the initiation of apoptosis. It has been reported that mitochondrion plays a significant role in the metabolism of lipids, carbohydrates, and proteins and acts as a center to direct cell growth, proliferation, differentiation and death [4, 5]. Maintenance of mitochondrial dynamic nature and integrity is featured by various diseases linked to mitochondrial dysfunction. These comprise inherited mitochondrial diseases caused by mutations in mtDNA or nuclear DNA, mitochondrial malfunction causes severe defects in cells/tissues with high energy demands such as muscle, brain, kidney and liver [8]. Mitochondrial dysfunction and compromise in its quality control are also associated with aging and common age-related diseases such as neurodegenerative diseases, metabolic diseases, and cancer [6,7,8,9]. Henceforth, it is essential to understand how mitochondria regulate various cell biological processes such as cell growth, proliferation, differentiation, and death.

Maintaining mitochondrial dynamicity

Mitochondrial dynamics have shown to play the important role in various developmental and cellular programs. For example, recent studies have shown that efficient mitochondrial transport towards the synaptic ending for supplying energy is essential for proper neurotransmitter release and recycling since

it meets up with all the energy requirements [15]. This mitochondrial transport is achieved through cytoskeletal tracks which include actin and microtubule network where the former one is the track for short range transport whereas the later involves fast, long range transport [10-12]. Mitochondrial morphology, which also determines the functionality and dynamicity of the organ, is regulated through two complex processes, mitochondrial fission and fusion, where the two mitochondrial membranes act in concert. A wide array of molecules which includes proteins like OPA1 is involved in these processes and absence of these critical molecules has been shown to affect mitochondrial functionality thereby causing various developmental defects.

1.2 The Miro GTPase: At the heart of mitochondrial dynamicity

The Miro GTPases belongs to Ras superfamily, classically known to act as regulators of diverse cellular processes. Initially, Miro1 and Miro2 were identified in mammals as atypical Rho-like small GTPases [16]. They belong to an evolutionarily conserved class of proteins which are unique in their structure, comprising two GTPase domains flanking two EF-hand Ca2+ binding domains and a C-terminal transmembrane domain which localize the protein in the outer mitochondrial membrane [16-18] (Fig.1). In *Drosophila*, dMiro was shown to be interacting with the kinesin motor adaptor Milton, implicating Miro involvement in mitochondrial trafficking [19]. Miro proteins were found to form protein complexes with Milton and kinesin motors [20]. dMiro deletion in *Drosophila* was shown to reduce the number of moving mitochondria in axons. TRAK1 and TRAK2 are the mammalian orthologous of Milton protein, which has been shown to interact with mammalian microtubule motors and Miro1 and Miro2 (Fig.2). The number of moving mitochondria was significantly reduced when Miro1 or TRAKs was depleted in cultured hippocampal neurons [21]. Milton appears to be a highly specific mitochondrial trafficking factor, In *Drosophila*, Milton acts as an essential link between Miro and kinesin motors [20]. There is very strong evidence which shows that the mammalian orthologues of Milton, TRAK proteins are important for mitochondrial trafficking [22]. Initially, TRAK proteins were shown to interact directly with KIF5 motors as similar as we see in the case of Milton, recent studies has also validated that they can also interact with dynein motors via other interacting proteins such P150 [23] (Fig.1B). The same study has also revealed that a conformational change in TRAK2 was responsible for its favored association with dynein, preventing the interaction with kinesin motor. Suggesting the involvement of Miro in regulating the polarized transport of mitochondria by controlling the differential

recruitment of TRAK proteins into the motor complex or by controlling its molecular conformation. In addition to the TRAKs, the mammalian Miro1 has been shown to interact directly to KIF5 [24] (Figure 1B) Other than interacting with motors, TRAK proteins have also been found in complexes with the enzyme OGT [25] (Figure 1B), OGT catalyses O-linked glycosylation of serine and threonine residues with the post-translational modification O-GlcNAc [26]. Recent studies [27] suggest that TRAKs are modified by O-GlcNAc. Recently, Miro has been found to interact with components of the machinery of mitochondrial fusion such as mitofusins (Mfns) [28], dynamin-related GTPases expressed on the outer mitochondrial membrane which is critical for regulating mitochondrial fusion. As shown by mitochondrial trafficking assays in Mfn2-knockout neurons lead to a decrease in mitochondrial motility, which cannot be rescued by Miro2 overexpression suggesting Mfn2 and Miro2 functions in a co-operative manner to regulate mitochondrial trafficking. Further studies showed that overexpression of Miro1 increased mitochondrial length in neurons [29]. To put together, these results suggest that Miro is also likely to be playing an important part in mitochondrial trafficking, fusion, and fission.

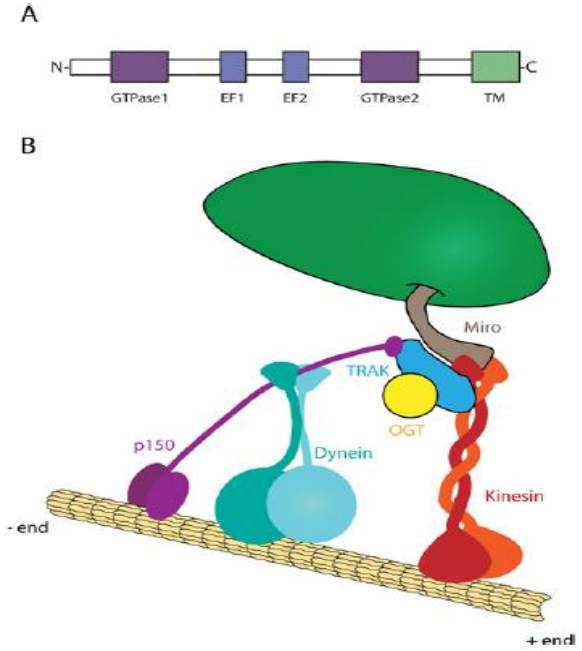


Fig1. (A) Schematic diagram of Miro functional domains. At the N-terminus, two GTPase domains flank the two Ca2+ -binding EF-hand domains, whereas the mitochondrial transmembrane domain (TM) is located at the C-terminus of the protein. (B) Mitochondrial transport along the microtubules is mediated by the interaction between the mitochondrial Miro proteins and the microtubulemotor proteins. Specifically, Miro binds the kinesin motors and the TRAK adaptor molecules directly, whereas dynein-dependent transport is mediated by the interaction between the dynactin subunit p150 and TRAK. The TRAK adaptors also bind OGT. [13]

Calium-dependent mitochondrial arrest by Miro

Miro proteins have shown to play a major role in mitochondrial arrest in a calcium-dependent manner by uncoupling mitochondria from the microtubules through which they are transported. Distinct mechanism has been proposed till now explaining the whole process of Miro mediated mitochondrial arrest. In the first proposed model, increase in Ca2+ level results in dissociation of KIF5, a motor protein involved in mitochondrial transport from the microtubule track which in low Ca2+ levels, KIF5 remains associated with both mitochondria and microtubules thereby facilitating its transport [30]. The second model talks about how the increase in Ca2+ levels can uncouple Miro and mitochondria from the microtubules by inhibiting the interaction of the former with KIF5 [32]. Recently another molecule SNPH(syntaphilin) has also shown to play the role in Ca2+ dependent mitochondrial arrest. According to this model, known as engine switch model, Miro in low Ca2+ concentration remains bound to KIF5 and microtubule-associated syntaphilin whereas when the level of Ca2+ rises, Miro gets dissociated from KIF5 and syntaphilin resulting in mitochondrial stopping [33].

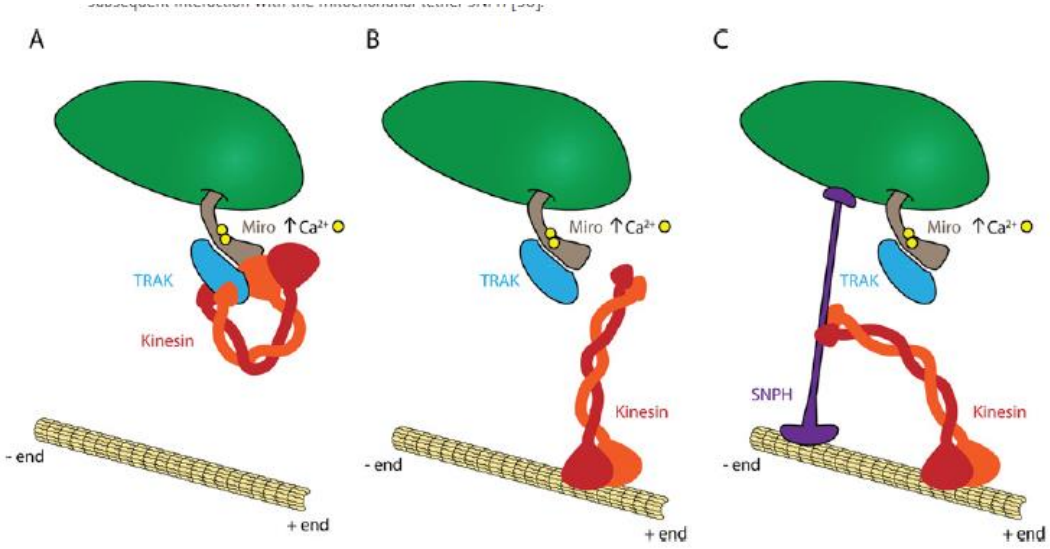


Fig2. Models of Ca2+ -dependent mitochondrial arrest According to the first model (A), Ca2+ binding to Miro EF-hand domains promotes the detachment of the kinesin motors from the microtubules and the interaction of their motor domains with Miro, causing mitochondrial arrest [16]. In the second model (B), Ca2+ binding to Miro causes the release from the kinesin motors, determining the detachment of the mitochondrion from the microtubule TRAKs [9]. A third model (C) shows both the detachment of kinesin from Miro and its subsequent interaction with the mitochondrial tether SNPH [13]

Miro and diseases

Given that Miro functions as a key player in mitochondrial transport and dynamics, this protein could be associated or implicated in diseases with defects in mitochondrial function and movement, predominantly neurodegenerative and neurodevelopmental disorders. Generally, mitochondrial defects have been comprehensively associated with Alzheimer's disease (AD) and Parkinson's disease (PD) [34]. The Parkinson's disease-related proteins Parkin and PINK-1 interact with MIRO and shown to affect mitochondrial motility and Miro degradation [35]. MIRO protein levels were shown to be downregulated in the presenilin 1 E280A mutation associated with familial AD [36]. However, Miro has not been directly related to PD yet. Recently, AD-associated pathology and tauopathy have been linked with perturbation of another MIRO-TRAK interactor, mitofusin 2 [37]. In *Drosophila* axons, Miro loss of function promotes tau phosphorylation and AD pathology [38]. The *Drosophila* orthologue of mitofusin, Marf have been shown to be required for mitochondrial transport along axons, and Marf loss of function resulted in a depletion of mitochondria in neuromuscular junctions [39]. MIRO has been also associated with another neurodegenerative disease, Amyotrophic Lateral Sclerosis (ALS). Miro1 was found to be significantly reduced in the spinal cord tissues of ALS patients [40].

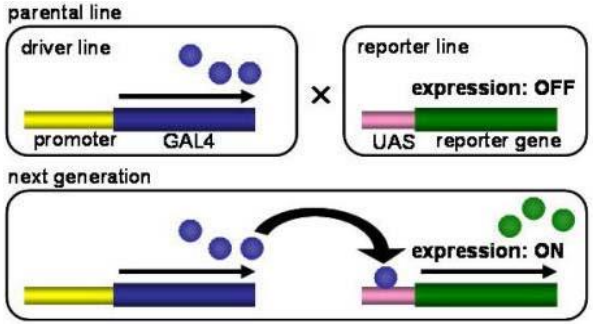
1.3 *Drosophila* as a model system

Drosophila melanogaster, the golden bug was described and extensively characterized by TH Morgan and his students during the onset of the 20th century. The *Drosophila* life cycle involves sequential phases, egg, larvae, pupae, and adult. Having a short generation time, high fecundity, cheap and easy husbandry protocols combined with small genome size makes *Drosophila* a very good model system. The genome of *Drosophila*, which was completely sequenced in the year 2000 has provided unique molecular genetic insights. The availability of multiple tools for genetic manipulations (Gal4-UAS, FLP-FRT, etc.) and ease of generating heritable genetic changes in *Drosophila* genome makes it the most tractable organism to do genetic studies. Over the years, huge numbers of mutants have been generated which are available for use from various stock centers. Studying *Drosophila* has been made easier by an availability of phenotypically distinguishable molecular markers for different chromosomes. Although *Drosophila* and Human are separated through millions of years of evolution, around 75 percent of human disease genes listed in OMIM (Online Mendelian Inheritance in Man) have related

sequences in *Drosophila melanogaster* [41]. This fact alone underlines the relevance of *Drosophila* as a model system for obtaining crucial insights for clinical purposes. Except for this, there are many developmental as well as cell biological processes in Humans which have a counterpart in *Drosophila*.

1.3a Gal4-UAS system

Andrea Brand and Perrimon discovered the Gal4-UAS system which they used to regulate ectopic expression pattern of genes in a spatiotemporally controlled manner. GAL4 protein is a transcriptional activator in the yeast *Saccharomyces cerevisiae* which binds to its target DNA sequence called Upstream Activator Sequence (UAS) and regulates expression of different genes. In *Drosophila*, this is a bipartite arrangement, where one transgenic fly contains GAL4 placed under promoter of a gene, also called driver line; and another transgenic fly carries a target DNA sequence placed under UAS element, a reporter line. When the driver and reporter lines are crossed, the GAL4 and UAS elements come into one genome in F1 generation. Thus, expression of GAL4 protein switches on transcription of target genes downstream of UAS element. Employing this approach, the expression pattern of a target gene can be spatiotemporally controlled using proper leverage viz. enhancer of the appropriate gene in driver line. This also comes with the advantage that lethal genes can be switched on for a small time window in a specific tissue, which allows a better insight into the mechanism of such genes while excluding systemic effects. Now, with the combination of RNAi and UAS-GAL4 system, it is also being used to generate spatiotemporally controlled knock-down of target genes. Another fine-tuning tool in this system is temperature dependent transcriptional activity of Gal4 protein towards UAS element. Gal4 mediated transcription of UAS element is not active at 16^{o}C, whereas it has its maximum activity at 29^{o}C in *Drosophila*.



https://invbrain.neuroinf.jp/static/moth-e/methods.html

Fig3. Schematic representation of Gal4-UAS System: Gal4 transcription activator binds and activates genes downstream to upstream activating sequences (UAS).

1.3b Genetic Screen in *Drosophila* using RNAi technology

The concept of RNA interference was initially described in *C. Elegans* by Andrew Fire and Craig Mellow [42] as they observed that dsRNA could induce gene silencing by degrading mRNA and interfering with normal translation in the cells. With the potency of RNAi to alter gene expression in insects as well, this approach has been routinely employed for conducting large genetic screens in *Drosophila* during last decade. In *Drosophila*, first, the RNAi-based gene knock-down strategy was employed in 1998 [43] establishing that frizzled and frizzled2 act in the wingless pathway. RNAi technique provides an opportunity for in vivo loss of function screen by creating transgenic flies, which when combined with UAS-GAL4 system becomes one of the elegant techniques for mutant phenotype analysis. Using above approach, a function of embryonic lethal genes can be analyzed by regulating gene-specific dsRNA expression in tissue and developmental stage specific manner. The mechanistic basis of RNAi-induced gene silencing is highly conserved among different phyla like animals, insects, plants, etc. As shown in Fig 4, the long double-stranded RNA gets cleaved by the action of an enzyme called dicer into shorter fragments called small interfering RNAs (siRNA, ~20 nucleotides). While one strand of double stranded siRNA is incorporated into a complex called RNA-induced silencing complex (RISC), another strand gets degraded. AGO family protein help in RISC mediates complimentary recruitment of mRNA and subsequently mRNA breakdown is initiated by the complex. The robustness and specificity of RNAi in hampering translation in vitro as well as in vivo has made it a very sought after technique.

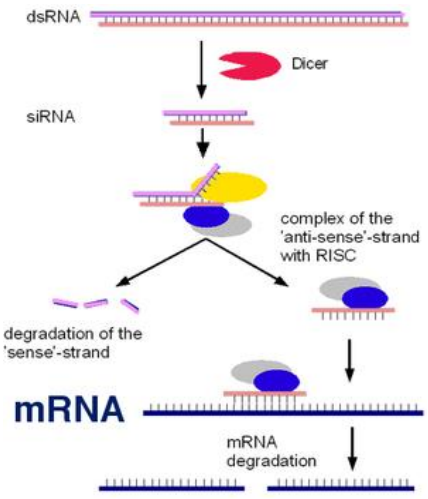


Fig4. Schematic representation showing RNAi technology: Cells can trim double stranded RNA to form small inhibitory RNA (siRNA) by the function of an enzyme dicer. An siRNA can be processed to the single strand anti-sense RNA and used to target mRNAs for destruction. Several proteins (colored ovals) are required for efficient RNA interference. The protein-containing complex was named "RNA-induced silencing complex", RISC.

1.3c FLP-FRT System

FLP-FRT recombination is a site-directed recombination technology, used to manipulate an organism's DNA *in vivo* under controlled conditions. The FLP - FRT system has been extensively used to generate somatic mosaics in *Drosophila*, mouse and zebrafish. Genetic mosaics are very rare in nature. However, this can be achieved by FLP-FRT recombination. It is analogous to Cre-lox recombination but involves the recombination of short flippers recognition, target (FRT) sites by the recombinase (Flp) derived *Saccharomyces cerevisiae*. There exist different variant FRT sites exist, but recombination can usually occur only between two identical FRTs but generally not among non-identical ("heterospecific") FRTs. A general schematic representation for inducing mitotic clones using the site-specific recombinase FLP, its target FRT sites, and cell markers to label mutant cells in *Drosophila* is illustrated in Fig.5. Clones can be marked with cell-autonomous markers, allowing analysis of these cells in both developing and adult tissues.

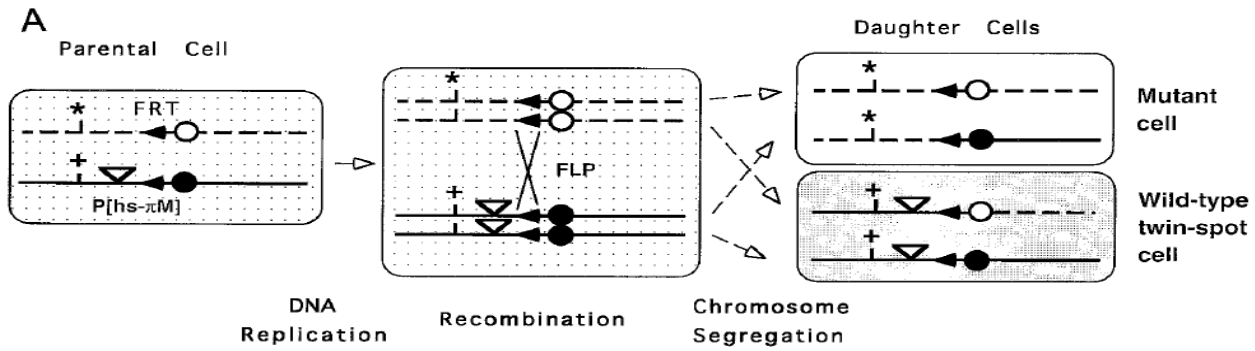


Fig5. FLP-FRT System: Generating and labeling mutant clones using FLP/FRT and cell markers. (A) In a heterozygous parental cell, FLP induces mitotic recombination between FRT sites (solid arrows) on homologous chromosome arms. Segregation of recombinant chromosomes at mitosis produces two daughter cells: a mutant cell bearing two copies of the mutant allele (*) and a wild-type twin-spot cell containing only the wild-type form of the gene (+). The cell marker P[hs-pM] cosegregates with the wild-type gene, labeling the mutant cell by its absence. Subsequent cell divisions result in clones from each of these original daughter cells. [14]

1.3d Model for Cell growth screen: Adult eye and larval fat body

Developing eye of *Drosophila* has been used as a model system to study various cellular processes like cell cycle [45], cell interactions during development [46], apoptosis [47]. The *Drosophila* adult eye serves as a perfect model for analyzing growth regulation as it is a tissue consists of post-mitotic cells. The adult *Drosophila* compound eye consists of around 800 ommatidia where each ommatidium is a hexagonal structure and the compound eye resembles honeycomb-like structure. Each ommatidium consists of eight photoreceptor neurons, four lens secreting cone cells, and two primary pigment cells. It has also been shown that the adult eye is not essential for fly survival; mutations wherein eye fails to develop are viable [48]. Hence, analysis of cell growth in postmitotic cells of adult *Drosophila* eye was done for the primary screen in this study.

The larval fat body is mesoderm-derived tissue which is set out during embryonic development of *Drosophila*. This is a single layer, a translucent tissue consisting of around 2200 cells [49]. A number of cells are decided during embryonic stages and it undergoes extensive cell growth and endoreplication while cell proliferation is absent during larval stages. While its function is analogous to mammalian liver and adipose tissue, it secretes important molecules which have a systemic effect on the development of *Drosophila* [50]. When a larva is undergoing metamorphosis in the pupal stage, dissociation of the fat body provides nutrition and energy for different processes taking place inside the pupae.

1.3e Model for assaying Cell proliferation, apoptosis and differentiation: Third late instar larval eye imaginal disc

A sac of epithelial cells called imaginal disc during larvae. The eye imaginal disc undergoes extensive proliferation during early larval instars and differentiation starts in third instar from posterior to anterior direction. The front edge of differentiation wave can be marked as an depression called morphogenetic furrow [47]. With respect to the furrow, the cells anterior to it keep on dividing mitotically, cells within the furrow are arrested in G1, and cells posterior to furrow undergo synchronous mitotic division before being differentiated. Precision and order in structure of adult eye is dependent on large number of cells, any mutation which affects cell number or size will show pronounced perturbation in ommatidial patterns and hence will be phenotypically easier to detect. Therefore, eye imaginal disc will serve as a perfect model for

assaying the effect of desired mutation in major cellular processes such as proliferation, differentiation and apoptosis.

2. Objectives

This project aims to understand the biological functions of Mitochondrial rho GTPase (Miro). A genome-wide screen for nuclear genes encoding mitochondrial proteins that can regulate cell growth lead to the identification of Miro as a negative regulator of cell growth. It is interesting to know that a mitochondrial protein is negatively regulating cell growth. Therefore, it would be really important to see the role of Miro in controlling cell biological processes as cell growth, proliferation, differentiation, and death. Hence, this projects targets to characterize Miro.

3. Materials and Methods

3.1Fly stocks and genotypes

Many *Drosophila* lines have been obtained from different stock centers and whenever required new lines were generated by crossing or recombining appropriate lines. The flies were reared on food made from agar, maize powder, sugar and yeast at 25^{o}C in standard bottles / vials if not mentioned otherwise. References for different transgenic lines have been mentioned below.

a) ***w; GMR Gal4/Cyo; UAS CycD, UAS CDK4***: This recombinant line was generated in our laboratory. This has GMR Gal4 insertion on second chromosome and third chromosome has homozygous insertion of UAS CycD, UAS CDK4. Adult flies of this transgenic line show an overgrowth in eye.

b) ***UAS Miro RNAi:*** This RNAi line has been obtained from Bloomington Stock Centre, Indiana, USA. These lines were generated by Transgenic RNAi Project (TRiP) of

Harvard University, USA.

c) w; UAS Miro: This fly line was generated by Hugo J. Bellen. It expresses Miro under UAS control on the second chromosome.

d) ***yw; r4 Gal4:*** This transgenic line was created by Jea H. Park (2004) with the Gal4 insertion on chromosome 3. This driver is predominantly expressed in larval fat bodies from early first instar and continues till late third instar.

e) yw, eyeflp; FRT82B/FRT82B: This transgenic line was created by Barry Dickson. Procured from Bloomington stock center.

f) hsflp;FRT82B,Ubi-GFP,M/Tb: This transgenic line was procured from Bloomington stock center.

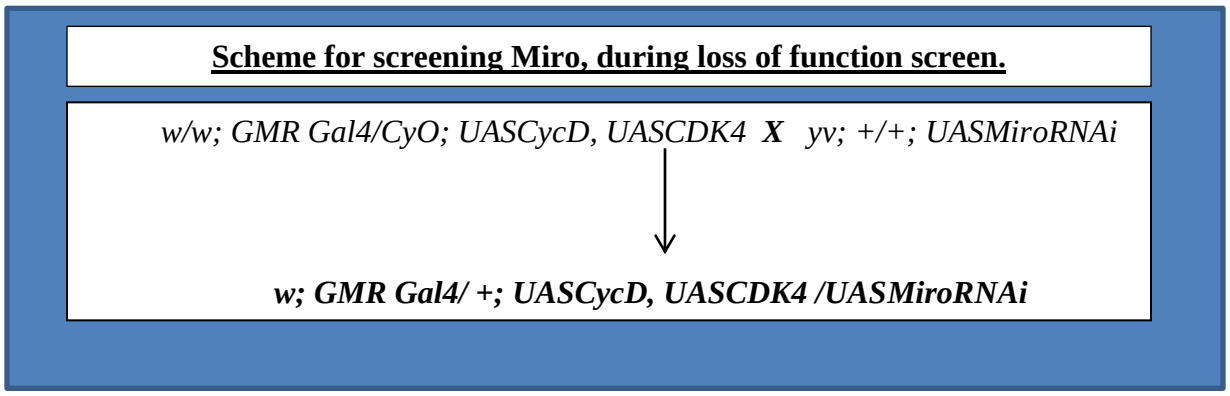
g) yw, eyeflp; FRT82B, Miro^{SD32}/Tb: This transgenic line was created in our lab by bringing eyeflp along with Miro loss of function.

h) yw, eyeflp; FRT82B, Miro^{B6832}/Tb: This transgenic line was created in our lab by bringing eyeflp along with Miro loss of function.

3.2 Genetic crosses and recombination

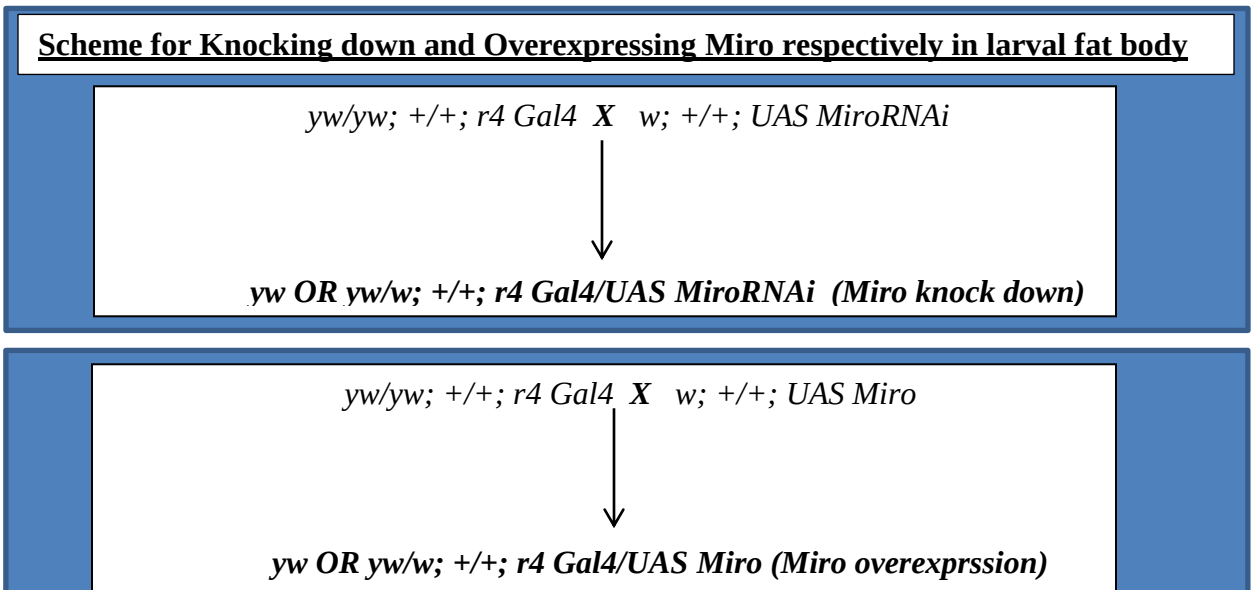
3.2a Identification of Miro from genetic screen

Available recombined line GMR-Gal4/Cyo (2nd chromosome) with UAS CycD UAS CDK4 (3rd Chromosome Homozygous), which leads to overgrowth in adult eyes when crossed with UAS MiroRNAi (3rd Chromosome Homozygous) line. The F1 progeny which are non-curly carries genes of our interest which are GMR Gal4, UAS CycD, UAS CDK4 and UAS MiroRNAi. The non-curly males are selected for adult eye SEM imaging.

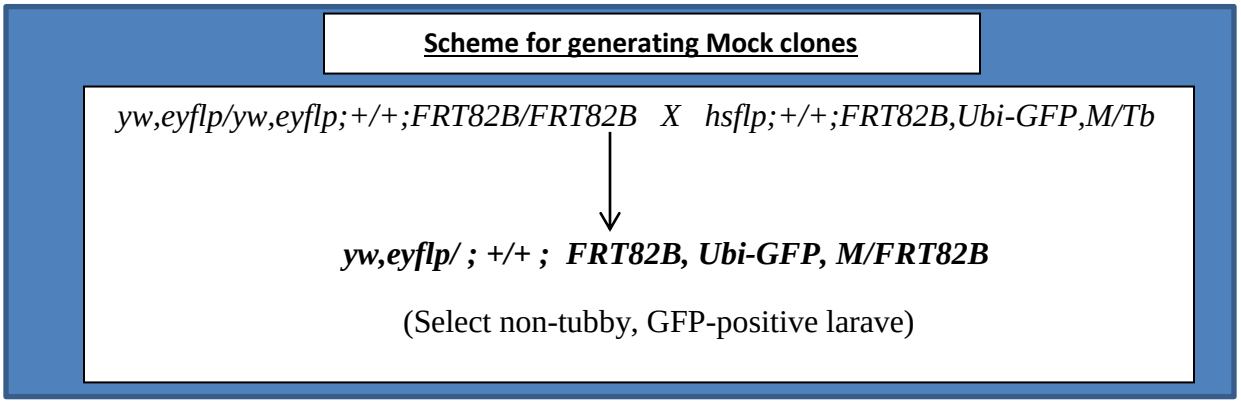


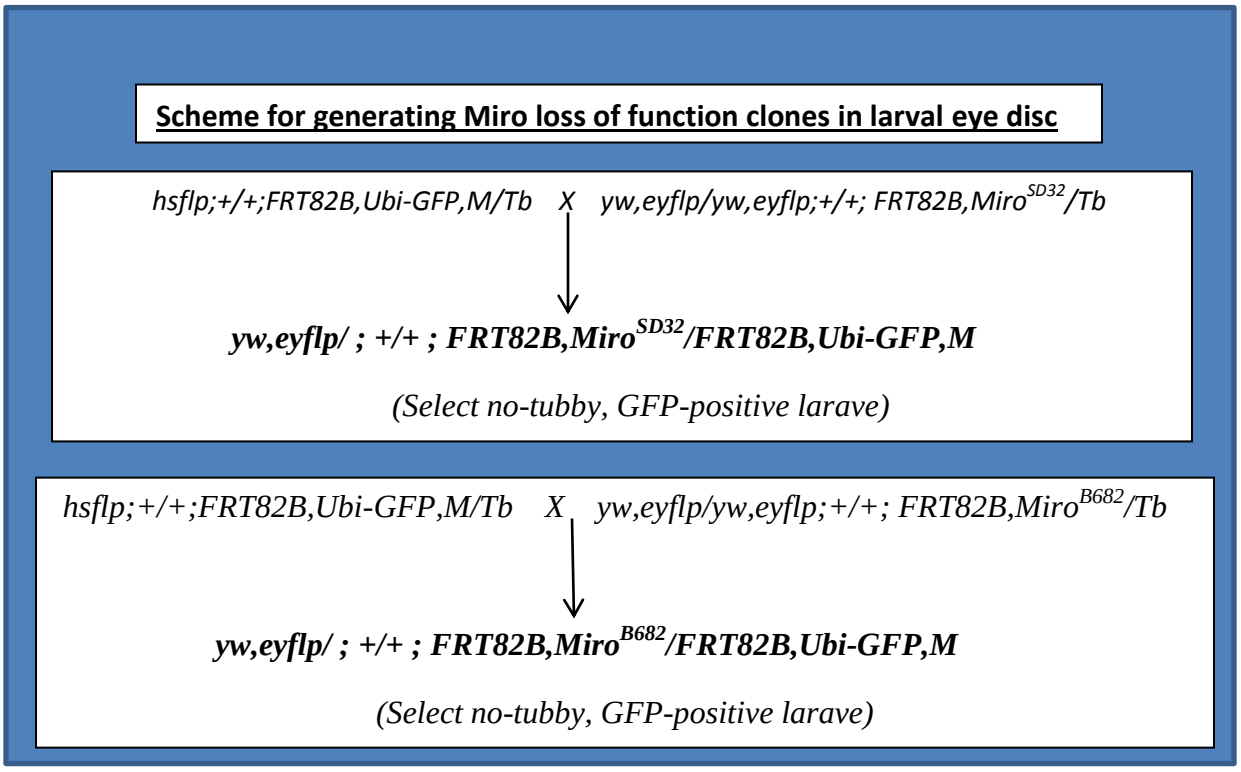
3.2b Crosses for cell size analysis in fat body

To assay cell growth, we crossed r4Gal4 line with UASMiroRNAi and UASMiro respectively.



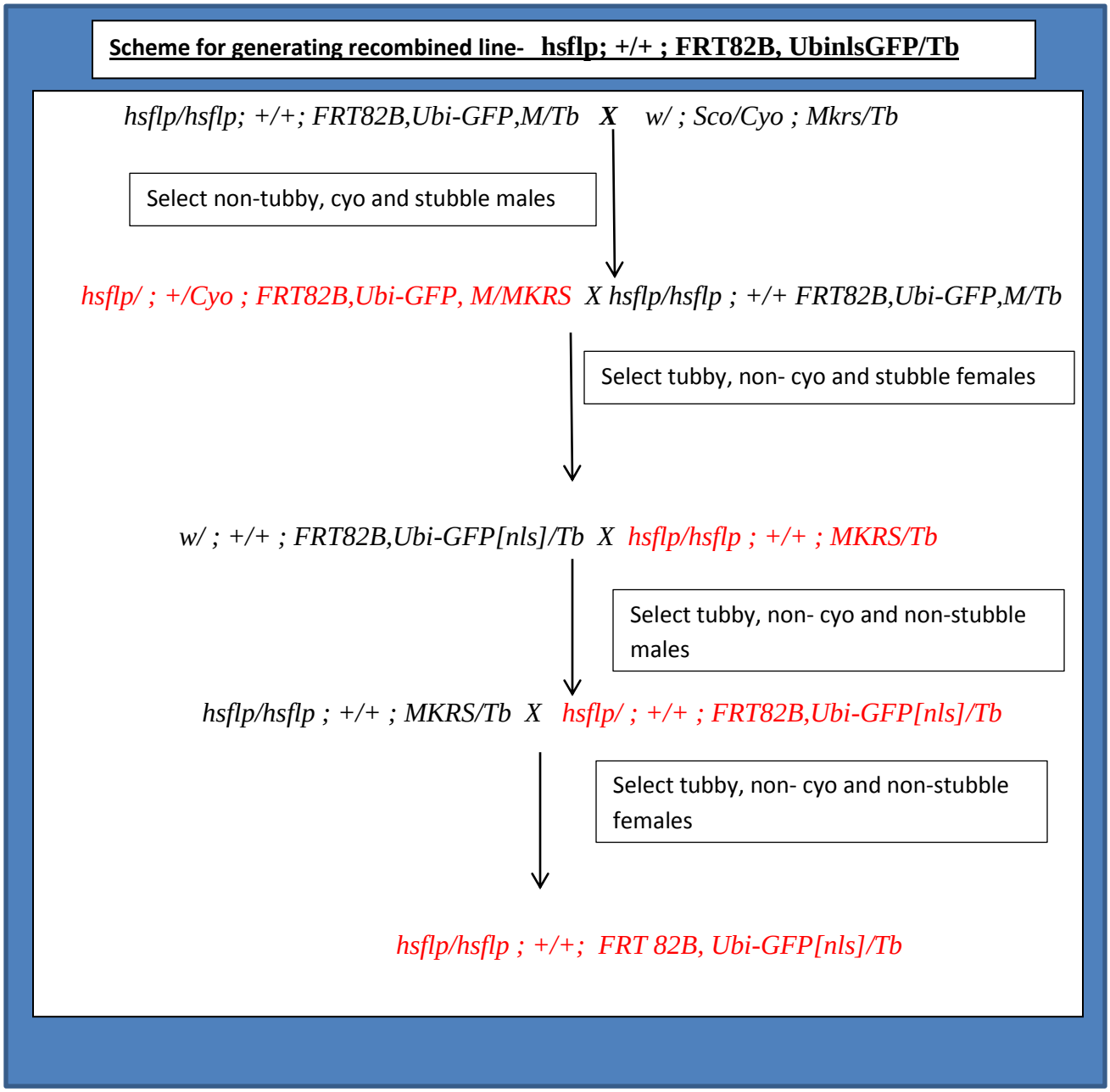
3.2c Crosses for generating Mock and Miro loss of function somatic clones in larval eye disc





3.2d Generation of hsflp; +/+ ; FRT82B, UbinlsGFP/Tb

The aim of creating this line is to remove the ribosomal mutation minute (M) which is present along with the Ubi-GFP sequence. Minute mutation is lethal if homozygous. This recombined transgenic line will be free from minute mutation and hence will be used for future experiments such as twin spot analysis.



3.3: Phalloidin staining and size measurement of fat body cells.

Late third instar larvae were pulled out and fat bodies were dissected in 1X PBS solution followed by 8% paraformaldehyde fixation for 45 minutes and then three washings with 0.3% PBT. Tissues were incubated in Phalloidin Alexa 594nm (Molecular Probes) using a dilution of 1:100 in 1X PBS. Tissues were washed twice with 0.3% PBT and incubated in DAPI for 30 minutes. Tissues were given two wash with 1X PBS and mounted on Vecta-Shield (Vector labs). Imaging was done using fluorescence microscope (Carl Zeiss) at 20X. The cell size of fat body cells was measured in AxioVision software and statistical analysis was done in Microsoft Excel by two-tailed T-test.

3.4: Brdu assay in third late eye imaginal disc.

Third late instar larvae were dissected in 1X PBS and the pull out tissues were incubated in Brdu solution (1:1000 in 1X PBS) for 30 minutes as normal and for 15 minutes as short Brdu exposer at room temperature. 1 quick wash were given to tissues with 1X PBS followed by fixation in 5% paraformaldehyde for 45 minutes and then by using standard immuno staining protocol (as described below) anti-GFP antibody was developed [Primary antibody: anti-GFP mouse (1:100) and secondary antibody: mouse FITC (1:1000)]. Later steps were done in dark. After developing anti-GFP tissues were incubated in 2% HCL in 1X PBS for 25 minutes without shaking followed by a quick wash with 1X PBS and then fixing the tissues in 5% paraformaldehyde for 30 minutes and eventually anti-Brdu antibody was developed using a standard immuno-staining protocol [Primary antibody: anti-Brdu Rat (1:100) and secondary antibody: anti-Rat Cy3 (1:1000)]. After developing anti-Brdu antibody, tissues were washed thrice with 0.1% PBT for 10 minutes each followed by incubation in DAPI for 30 minutes. Finally, the tissues were washed twice with 1X PBS for 5 minutes each and then mounted in Vecta shield.

3.5: TUNEL assay in third late eye imaginal disc.

Third late instar larvae were dissected in IX PBS followed by fixation in 5% Paraformaldehyde for 45 minutes and then 5 washes for 15 minutes each with 0.4% PBT. Tissues were incubated in

100mM sodium citrate in 0.1% PBT for 50 minutes at 70^{o}C followed by cooling the tissues at room temperature for 20 minutes. 3 washes for 10 minutes each were given with 1X PBS. Incubate tissues in Enzyme mix (1:10 dilution with buffer) [Roche, Cell detection kit] @37 °C for 90 minutes. Wash tissues 2 times for 10 minutes each with 1X PBS followed by incubation in DAPI for 30 minutes. Give 2 short wash for 5 minutes each and mount in Vecta shield.

3.6: ROS staining in third late eye imaginal disc.

Early third instar larvae reared at 29^{o}C were dissected in 1X Schneider media followed by incubation in DHE (0.3μM in Schneider media, Molecular Probes) for 5 minute at room temperature in dark. After washings with 1X PBS, brief fixation was done with 5% formaldehyde for 15 minutes. Tissues were mounted in vectashield followed by imaging in Laser Scanning Confocal Microscope (LSM 780, Carl Zeiss). Incubation time for DCFDA (Molecular Probes) was 15 minutes and 1X PBS was used instead of Schneider media in all relevant steps.

3.7: Immunochemistry

Third late instar larvae were dissected in 1X PBS followed by fixation in5% paraformaldehyde for 45 minutes followed by 3 wash for 15 minutes each with 0.3% PBT. Tissues were incubated in 10% BSA in 0.3% PBT for 1 hour and kept on shaker. Tissues were incubated in primary antibody [All primary antibody and dilution used are mentioned below] for 16-18 hours at 4^{o}C. After incubation in primary antibody, tissues were washed in 0.3% PBT for 1 hour (4x15') at room temperature. Prior to secondary antibody tissues were incubated in 10% BSA for 30 minutes. Tissues were incubated in secondary antibody [All secondary antibody and dilution used are mentioned below] for 16-18 hours at 4^{o}C. Tissues were given 3 wash for 15 minutes each with 0.35 PBT followed by incubation in DAPI for 30 minutes. Finally, tissues were washed twice for 5 minutes each with 1X PBS and mounted in Vecta shield.

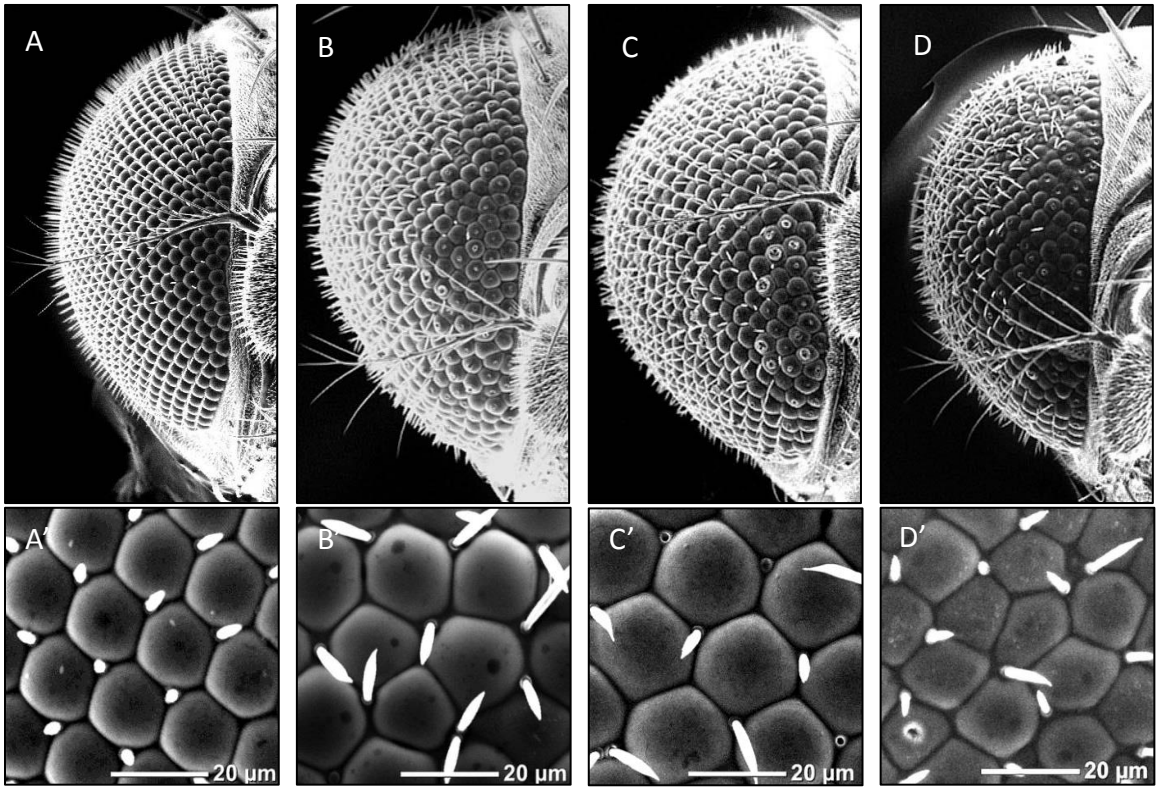
Primary antibody used:		Secondary antibody used:
1. ELAV (1:100)	→	Goat anti-rat Alexa Flour 488 (1:500)
2. Cut (1:5)	→	Mouse Cy3 (1:500)
3. Atonal (1:1000)	→	Mouse Cy3 (1:500)
4. 22C10 (1:50)	→	Mouse Cy3 (1:500)

Chapter 2 – Results and Discussions

1. Results

1.1 Knocking down nuclear encoded mitochondrial gene Miro enhances overgrowth adult eye Phenotype.

A loss of function genome wide RNAi screen to identify nuclear genes encoding mitochondrial proteins that can modulate cell growth lead to the isolation of Mitochondrial rho GTPase(Miro) as an enhancer of overgrowth which when mutated, enhances overgrowth induced by over-expression of CycD/CDK4 complex.



OreR

GMR-Gal4; UAS-CycD, UAS-CDK4

GMR-Gal4; UAS-CycD, UAS-CDK4, UASMiro RNAi

GMR-Gal4; UAS-CycD, UAS-CDK4, UASMiro

Fig6. Miro knock down enhances overgrowth adult eye phenotype caused by CYCD/CDK4 overexpression in post mitotic cells of *Drosophila* eye. Scanning electron microscope images shows lateral view and ommatidia of wild type adult eye (A,A'), Overgrowth adult eye phenotype (B,B'), Knocking down Miro enhances overgrowth (C,C') and Overexpressing Miro suppresses overgrowth phenotype (D,D').

1.2 Larval fat body cells knocked down for Miro are larger in size

To assess the role of Miro isolated in the primary screen in controlling growth at the cellular level we knocked down Miro independently in the larval fat body cells using fat body specific Gal4 drivers. Interestingly larval fat body cells are unique cells in the developing *Drosophila* larvae that do not demonstrate any cell division during the larval period. Rather, these cells just grow in size to be utilized during metamorphosis in the pupal stage. Miro gene was knocked down in the larval fat body cells using r4-Gal4 as the driver line. Analyses of cell size upon knocking down Miro reveal that the individual cells are larger when compared to the wild-type cells. Upon analyzing cell size of around ninety fat body cells obtained from seven different larvae, we found that knocking down Miro causes increase in cell size by 1.5 fold in comparison to controls. Further statistical analysis shows that this increase in cell size is highly significant. On the other hand, when Miro was overexpressed, it leads to decrease in the cell size compare to control which is also very significant as found by statistical analysis (Fig.7). Put together, this suggests that Miro functions as a negative regulator of cell growth.

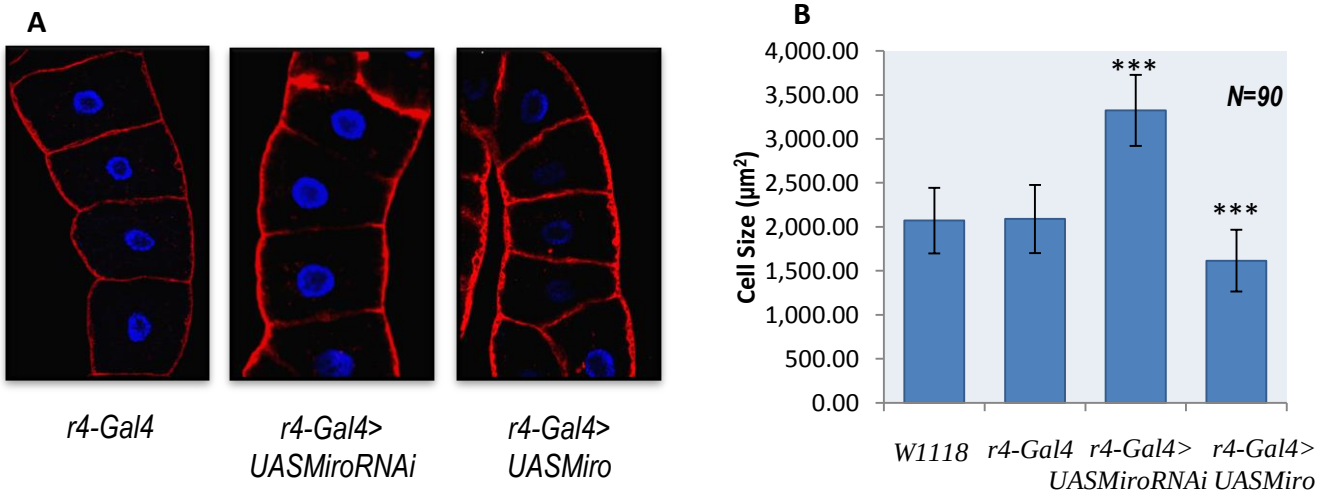


Fig.7. Miro knock down in fat body cells leads to increase in cell size (P_1) and overexpression of miro decreases cell size(P_2). Phalloidin Red and DAPI stained fat body cells (A). Graph showing quantitative analysis (B). $P_1 = 5.81*10^{-48}$ & $P_2=9.04*10^{-21}$

1.3 Miro loss of function leads to increased rate of proliferation in larval eye disc.

The mitochondrial control of proliferation: The Warburg effect underlies the simultaneous increase in glycolytic rate with a reduced mitochondrial respiratory rate [44,45]. It is a kind of mitochondrial dysfunction that is found in tumor metabolic reprogramming pathways. Therefore, in this study where we are generating a mitochondrial dysfunction by creating a complete loss of function of a mitochondrial protein Miro, we wanted to see if this particular mitochondrial dysfunction has any effect on proliferation. Interestingly, it was found that Miro loss of function cells have increased the rate of proliferation when assayed by BrdU incorporation. Fig8. A and A' are Mock third late instar eye disc where both GFP-positive cells and GFP-negative cells are wild types in nature. On the other hand images B and B' are experimental third late eye discs where GFP-positive cells are wild type in nature and GFP-negative cells are homozygous for Miro loss of function. When the control and experimental tissues were exposed to BrdU for 30 minutes, we found an increase in BrdU Incorporation in Miro loss of function clones but not so robust. However, BrdU incorporation for 15 minutes, there is the huge increment in the BrdU signal in Miro loss of function clones as compared to the control wild type clones. This indicates that Miro loss of function causes the cells to enter S phase at a faster rate. Therefore, cell cycle in Miro mutant cells is accelerated.

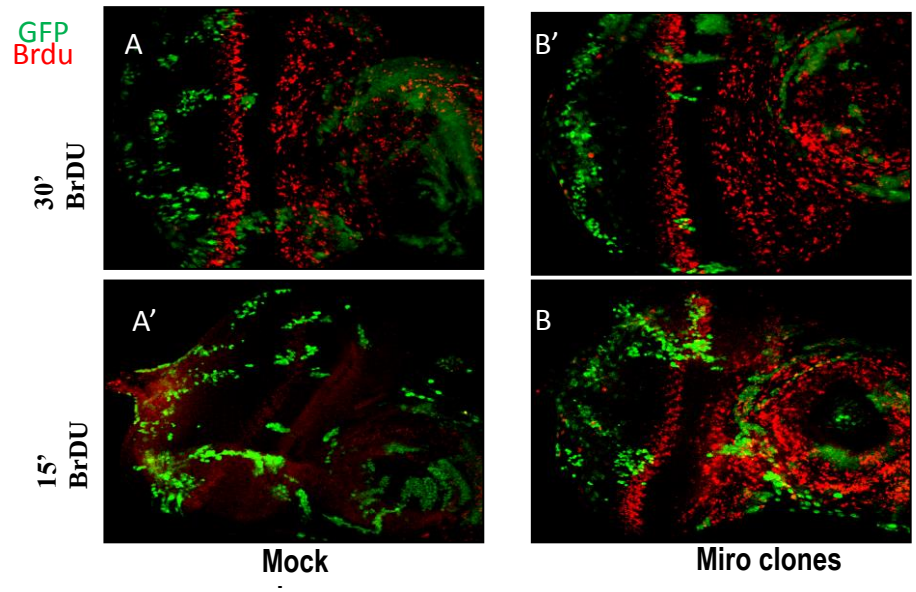


Fig.8. Brdu Incorporation in Mock clones at 15' and 30' in fig. A and Fig. A' respectively. In Miro clones, the Brdu Incorporation at 15' (Fig. B) and at 30' (Fig. B') is higher as compared to mock clones

1.4 Miro loss of function does not lead to aberrant cell death in larval eye disc during early Stages of development.

It is a well-known concept that mitochondria play a key role in apoptotic cell death in mammals, by releasing apoptogenic factors during apoptosis. Therefore, in this study, we wanted to find out whether mitochondrial dysfunction caused by Miro loss of function leads to any aberrant cell death or not. For assaying cell death, we performed TUNEL staining. TUNEL assay marks the dying cells by labelling the fragmented DNA, which is one of the hallmarks of cells undergoing apoptosis. There is no cell death observed in wild type cells in larval eye discs. Therefore, we overexpressed a pro-apoptotic gene hid as a positive control as seen in fig. 9A. The Miro loss of function does not show any ectopic TUNEL staining (9C &9D) suggesting there is no cell death.

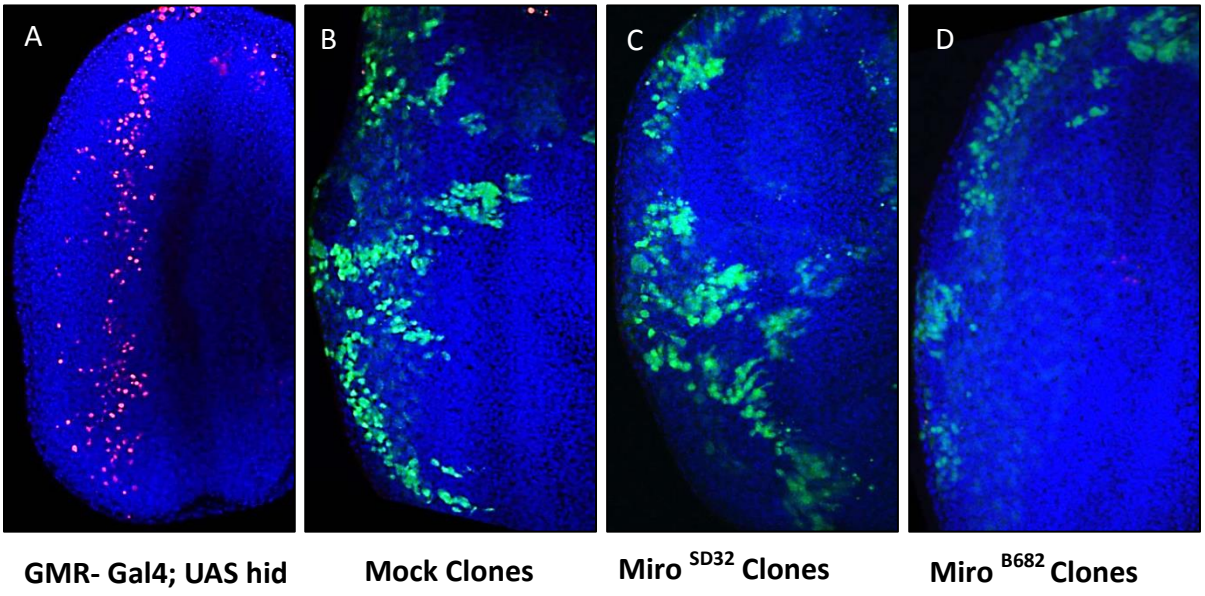


Fig.9. Overexpression of hid in wild type background leads to cell death in third Instar larval eye imaginal disc as seen by TUNEL staining in Red[Positive control] (A). Mock clones (B), do not show TUNEL staining so as the Miro clones (C and D).

1.5 There is no change in ROS level with Miro loss of function.

Previous studies have demonstrated that attenuating mitochondrial activity can lead to the generation of different signals, such as the decrease in cellular ATP level or an increase in cellular ROS level. ROS moieties generated in cells upon mitochondrial dysfunction can either be superoxide or peroxide molecules. Therefore, mitochondrial dysfunction caused by Miro loss of function might or might not lead to the change in ROS levels. Hence, for superoxide detection, we used Dihydroethidium (DHE) a dye that can freely permeate through the cell membrane and gets converted into bright red color fluorescent molecule 2-dihydroxyethidium upon reacting with superoxide molecules. Staining Miro loss of function somatic clones in third late instar eye disc with DHE, we found that these cells have no change in superoxide levels when compared to wild type controls. This shows that Miro mutant cells have no change in ROS levels.

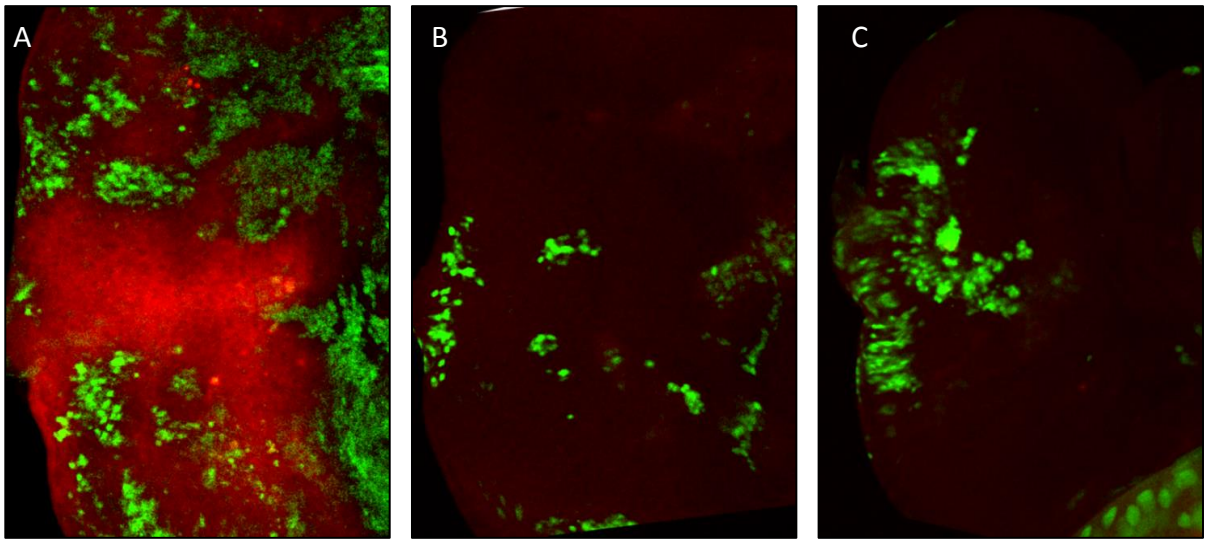


Fig.10 Superoxides stained with DHE (dihydroethedium). DHE level in mtACP1 clones as a positive control (A) whereas there is no change in DHE level in Mock clones (B) as well as Miro clones (C).

1.6 Miro loss of function does not lead to compromised differentiation in larval eye Imaginal disc.

Mitochondrial metabolism and its dynamicity have been fairly reported in regulating differentiation. The metabolic profile distinguishes the undifferentiated state from the differentiated state, with a dynamic mitochondrial morphology and a shift from glycolysis to mitochondrial oxidative phosphorylation (OXPHOS) [46, 47]. Therefore, perturbation in mitochondrial dynamics by Miro loss of function might lead to defects in differentiation. Hence, in this study, we aim to find out if the Miro loss of function hampers cellular differentiation in developing eye imaginal disc of *Drosophila*. By using differentiated cell markers such as ELAV, Cut, Atonal and 22C10 we labeled different differentiated cell types as seen in the fig. By comparing the differentiation in Miro loss of function clones with the Mock clones we found that there is no observable difference in the differentiation pattern of these cells. Hence, suggesting that Miro loss of function does not lead to defects in differentiation of developing eye imaginal disc.

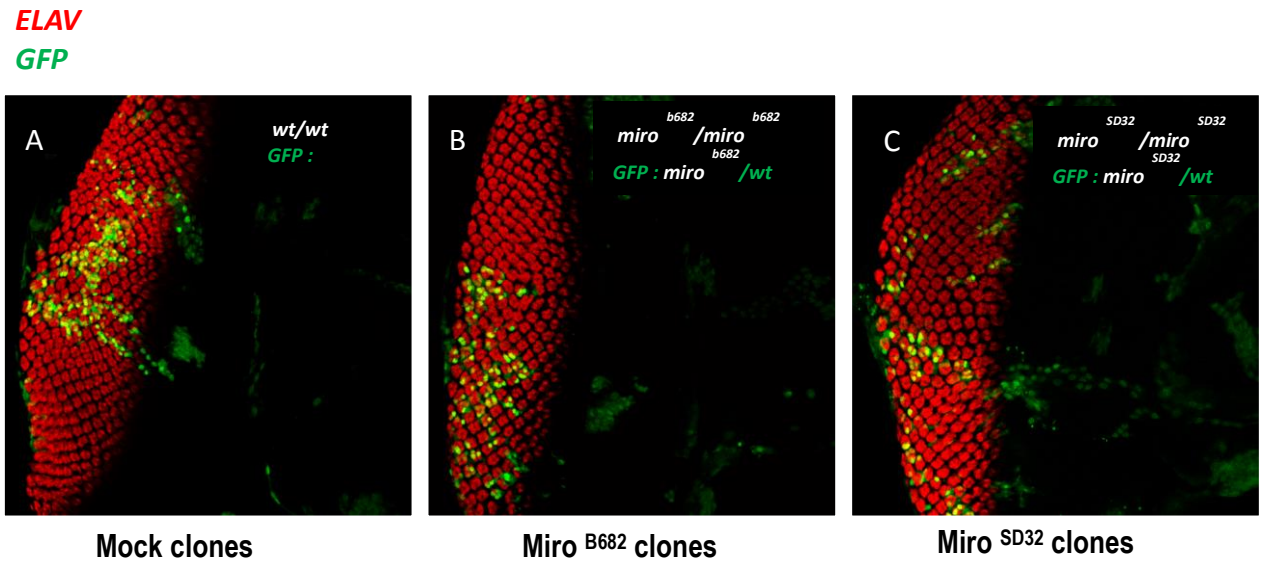


Fig.11 Photoreceptors are marked with ELAV in Mock clones (A) and in Miro clones (B and C). No change in ELAV expression can be observed in Miro clones compared to control Mock clones.

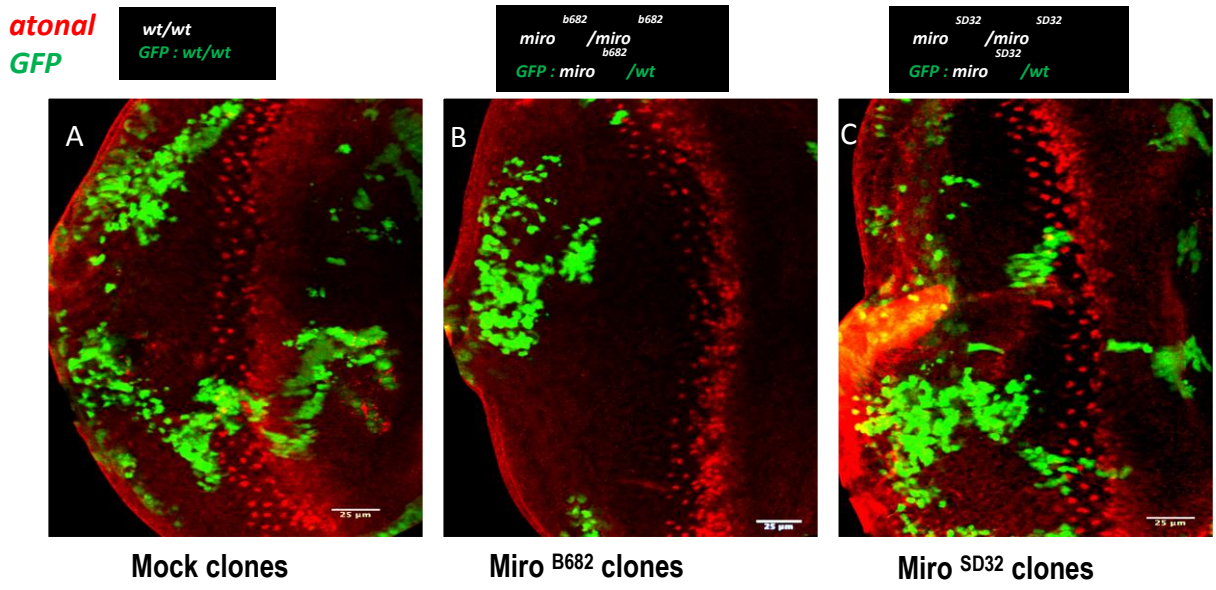


Fig. 12 Proneurons R8 marked with atonal in mock clones (A) and Miro clones (B &C). There is no change in the expression pattern of atonal in miro clones as compared to mock clones**.**

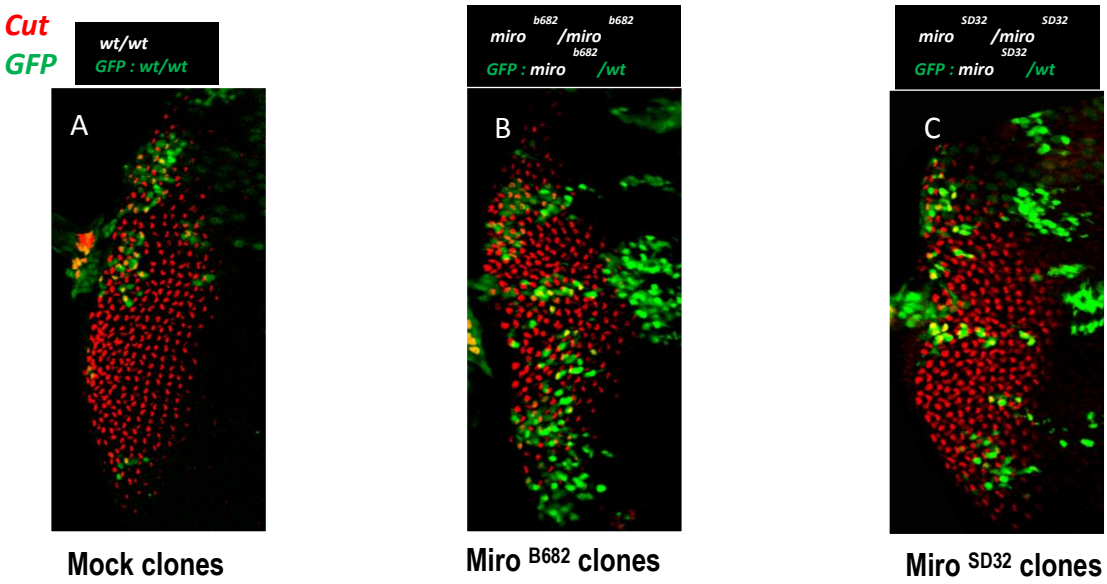


Fig13. Cone cells marked with Cut expression in Mock Clones (A) and in Miro Clones (B & C). Specification of cone cells is not altered in miro loss of function.

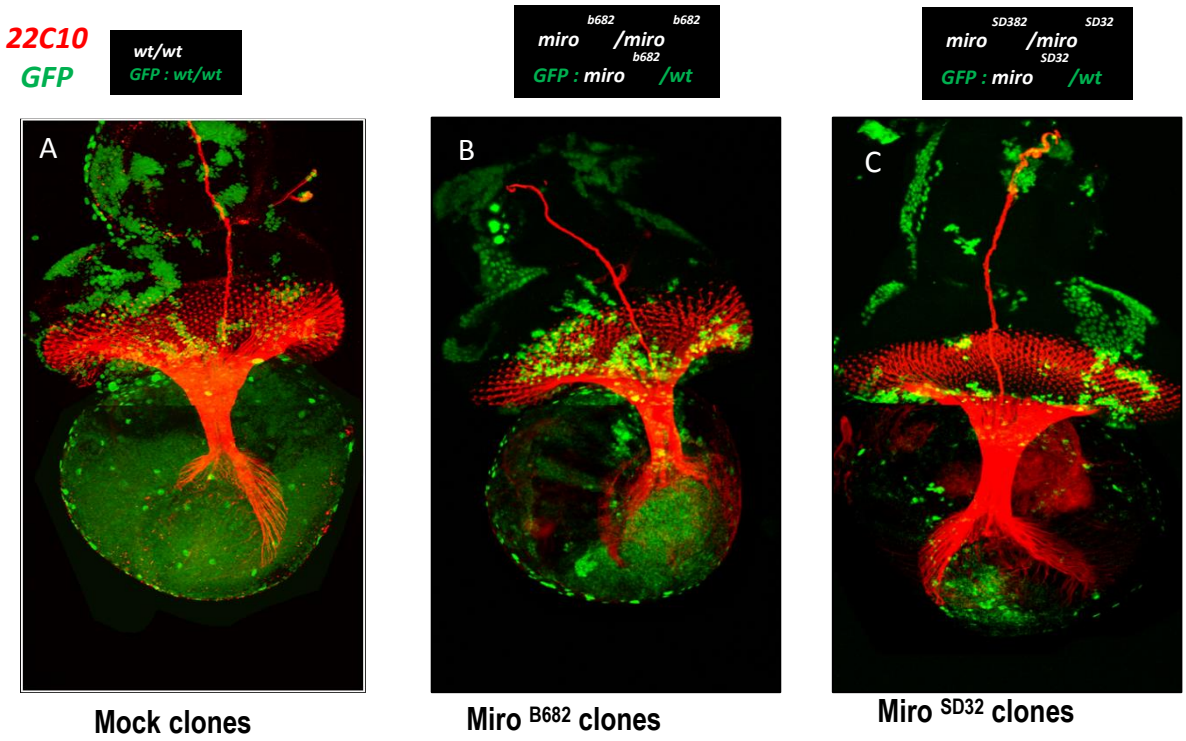


Fig.14 Axonal projection marked with 22C10, from eye imaginal disc to brain in Miro loss of function clones is not altered (B and C) as compared to Mock clones (A).

2. Concluding remarks and Discussions

Most studies on the Miro GTPases have so far been focused on the role of the proteins in neuronal activity and function. They have shown that Miro is a key regulatory component of the complex involved in the transport of mitochondria. However, it is certainly important to remember that the Miro proteins are ubiquitously expressed in mammals and that they are likely to have additional and essential roles in other cell types. Therefore, in this study, using different cell/tissue types in we have characterized Miro. During the characterization of this gene, we have focused on looking the effect of Miro mutation in controlling cell biological processes as cell growth, proliferation, death, and differentiation. Here, we report that the G-S transition is accelerated in Miro mutant cells. Miro mutant cells in eye disc do not show cell death or differentiation defects. Interestingly, we do see that Miro Knock down in CycD/CDK4 overexpression causes an increase in adult eye overgrowth. Whereas, knock down of Miro or Miro overexpression modulates the cell size, dictating its role as a negative cell size regulator in fat body cells. This indicates that, Miro loss regulates cell size depending on cell type and sensitive background such as CycD/CDK4 overexpression.

Since Miro acts as a key player in mitochondrial dynamics it would be really interesting to dissect out how mitochondrial dynamics regulates cell growth. Studies have suggested that Miro loss of function causes aggregation of mitochondria in the cell. Mitochondrial aggregation could be a possible cause for a cell size enhancement in Miro loss and it will be really exciting to understand this process. Also, Miro is seen to regulate cell growth in fat body cells which contains the lipids, it could be possible that Miro might be affecting the lipid synthesis in these fat body cells and the increase in lipid accumulation might be causing the increase in cell size. Therefore, a comprehensive amount of study needs to be done to understand a unique mitochondrial dynamics associated mechanism regulating cell growth.

The role of Miro in mitochondrial dynamics has also provided tempting links with several diseases, particularly neurodegenerative diseases. The links of Miro with neurodegenerative diseases are kind of tentative at the moment. Future studies need to be done to reveal insights into the pathophysiological roles of Miro in various disease models and may shed more light onto those associated with neurodegeneration with defects in mitochondrial dynamics. Recent findings have suggested that Miro can initiate CNS repair upon injury [48] which is highly interesting and could potentially be exploited as a therapeutic strategy in regenerative medicine.

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