Studying Mitochondrial Role in Cell Growth Regulation in *Drosophila melanogaster*

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

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Certificate of Examination

This is to certify that the dissertation titled "**Studying Mitochondrial Role in Cell Growth Regulation in *Drosophila melanogaster***" submitted by **Mr. Nilmani Singh (Reg. No. MS08036)** for the partial fulfillment 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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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.

Mr. Nilmani Singh

(Candidate)

Dated:

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)

Acknowledgement

Having spent my last year working in supervision of Dr. Sudip Mandal, I would like to express with utmost sincerity, a heartfelt gratitude towards him. He has been an excellent teacher and an inspiring figure all along the way to me. His critical insights and evaluations, patient guidance, enthusiasm and dedication for work, extremely useful anecdotes and lessons about life will always be an asset which will help me in shaping my life.

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Abstract:

Cell growth is the process of increase in mass and size of a cell, which is coordinated through various cell autonomous and non-autonomous factors. The role of many such factors like nutrients, growth factors, signaling cascades, transcription factors have been quite well studied. The process of cell growth is quite energy demanding and requires extensive metabolic activity. Yet, there are no reports about how mitochondrial function is linked with cell growth regulation. The focus of this project is to elucidate the mechanistic basis of cell growth regulation by mitochondrial function. Mitochondrial role in many cellular events like cell cycle regulation, differentiation, and apoptosis is well established and it has been shown to be affecting cell signaling pathways by modulating its outputs e.g. ATP, Reactive Oxygen Species (ROS) etc. We have launched a genome wide loos-of-function screen for nuclear encoded mitochondrial proteins to identify genes which can modify overgrowth phenotype associated with Cyclin D-CDK4 overexpression in adult eyes of *Drosophila*. We have identified three classes of overgrowth modifier; enhancers, suppressors and no change. Thus, we establish that attenuating mitochondrial function can modulate cell growth in positive as well as negative manner. We are doing further cellular level analysis of candidate genes using larval fat body. We have identified a gene mitochondrial acyl carrier protein (mtACP1), a subunit of complex I which when knocked down leads to decrease in cell size. Further analysis shows that mtACP1 knock down cells have high levels of ROS as well as JNK pathway is getting activated in these cells. We have found that in mtACP1 knock down cells, JNK pathway is mediating retardation in cell size. This suggests that attenuating mitochondrial function could affect cell size regulation mediated through JNK pathway.

CHAPTER 1

Introduction, Objective and Materials and Methods

1. Introduction:

Cell growth is a fundamental biological process which corresponds with increase in mass and volume of the cell by accumulation of biomolecules. This process plays a crucial role during development as proper cell size is a pre-requisite for cell division as well as differentiation [1]. Similarly, cell growth influences size of a cell, organ and organism. The size of organisms observed in the biological world is a result of varied distribution of number and size of the cells present in that individual. Although different sizes of organisms are quite obvious in nature, even within an individual different cells attain different sizes. A wide variation in cell size within and between organisms implores to dig deeper into the biology of cell growth regulation. During past few decades, our understanding of cell growth regulation in yeast [2], *Drosophila* [3], *C. elegans* [4] and different model organisms as well as mammalian cell lines have identified several cell intrinsic as well as extrinsic growth regulatory factors. The signaling pathways by which these factors regulate growth are conserved throughout the evolution [5]. Although biomolecular synthesis is an extensive energy demanding process, role of mitochondria the energy hub of cell remains to be explored thoroughly in this process. During past few decades mitochondria has emerged as signaling port of the cell regulating various cellular events e.g. cell cycle regulation [6], differentiation [7], aging and apoptosis [8].

The aim of this project is to elucidate the mechanistic basis of cell growth regulation by mitochondrial activity. Taking advantage of varied genetic tools in *Drosophila* [9] model organism, we employ RNAi based gene knock-out strategy to identify genes that can modify overgrowth phenotype induced by cell autonomous growth regulators CycD-CDK4 overexpression in *Drosophila* adult eye [10]. The screen in adult eye is followed by further molecular genetic analysis in larval fat body. Considering degree of conservation across the animal kingdom [11], understanding cell growth regulation by mitochondrial function in *Drosophila* can be explored to human beings. This further can help us to understand the mitochondrial role in diseases like Cancer and diabetes as well as in the stem cell biology.

1.1 Regulation of cell growth by cell extrinsic factors:

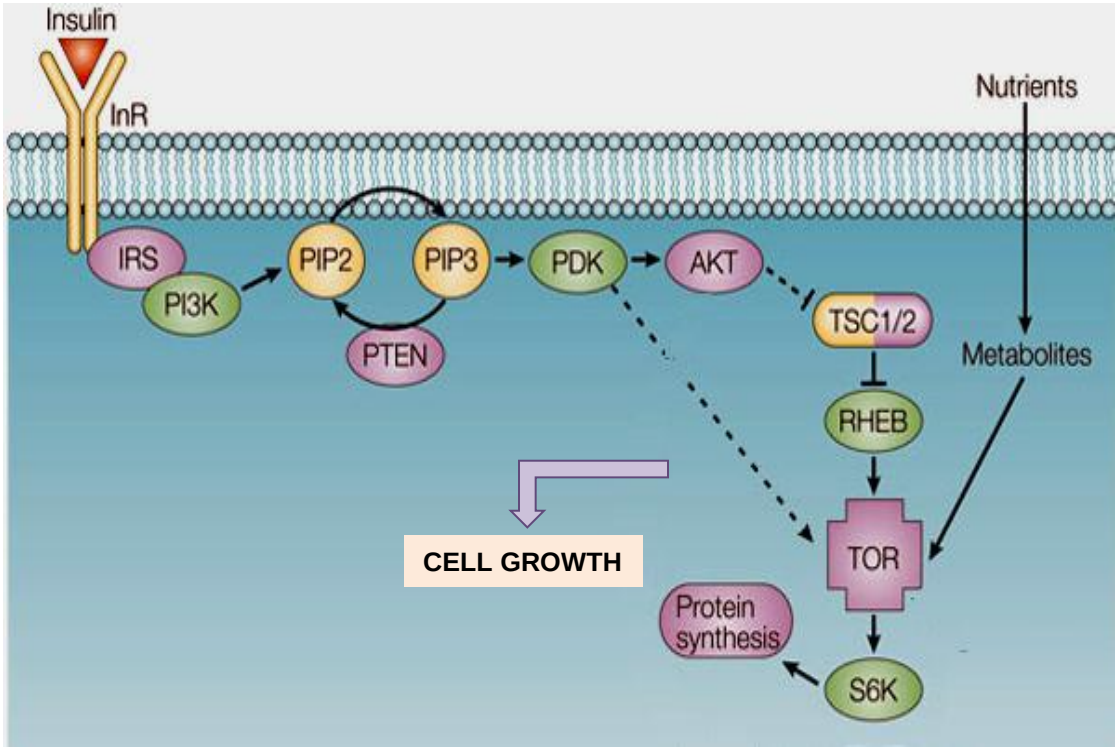
Insulin / Insulin-like growth factor signaling is a major cell growth regulatory pathway which controls transcription of growth regulatory factors and ribosome biogenesis [12].

Insulin binds to a membranous insulin receptor (InR) causing activation of PI3K via adaptor protein Insulin receptor substrate (IRS) (**Fig 1)**. Subsequently, activated PI3K leads to conversion of membrane bound phosphatidylinositol (3,4)-bis-phosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-tris-phosphate (PIP3) PIP3 which with the help of PDK-1 activates protein kinase B, also known as AKT. Phosphatase and tensin homolog (PTEN) converts PIP3 to PIP2 and thus antagonizes PI3K/Akt activity.

Akt regulates activity of mTOR complex which mainly integrates signaling cues from nutrients, growth factors, energy and stress [13]. Major downstream targets of TOR include S6K and 4E-BP, which regulate translation and ribosome biogenesis thus regulating cell growth. Activated AKT protein phosphorylates TSC2 and inhibits TSC1-TSC2 complex formation subsequently blocking its inhibitory role towards Rheb. Active TSC complex dephosphorylates and thus deactivates Rheb protein which is essential for activation of mTOR complex. These events lead to Rheb-GTP mediated activation of TOR complex through PI3K/AKT pathway. Signaling through mTOR complex regulates various processes like protein synthesis, transcription, ribosome biogenesis which affects cell growth in multiple ways. This is a very well conserved signaling pathway during evolution and has been investigated to play active role in oncogenesis.

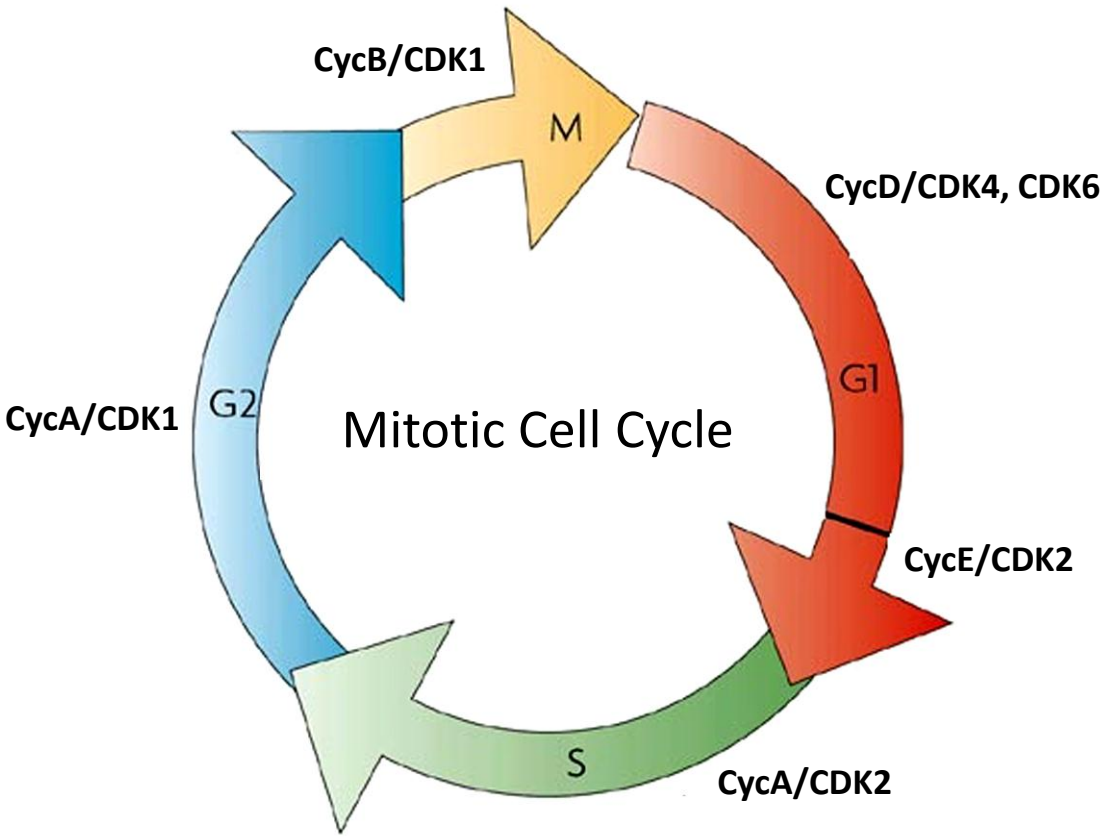
1.2 Regulation of cell growth coupled with cell cycle:

Development of single cell zygote to a multicellular adult requires cell growth, cell division and differentiation being orchestrated in a strictly controlled manner. The turn of events which lead to division of a mitotic cell are categorized by four different phases called gap1 (G1), Synthesis (S), Gap2 (G2) and Mitotic (M) (**Fig 2**). During S phase DNA is replicated while M phase relates to chromosome segregation and cytokinesis. Two Gap phases G1 and G2 provide time delay between S and M phase while cells prepare themselves for upcoming tasks. The transition between these phases is coordinated by regulatory proteins called Cyclins and Cyclin dependent kinases (Cdk). In response to mitogenic signals in G1 phase, Cyclin D gets activated [14] and it binds with CDK4/6 eliciting their protein kinase activity. Cyclin D-CDK4/6 complex phosphorylates Retinoblastoma (Rb) protein which leads to dissociation of E2F transcription factor from Rb protein. E2F family of transcription factors regulates different genes crucial for G1-S transition and DNA synthesis [15]. One of the targets of E2F is Cyclin E, which associates with Cdk2. Cyclin E-Cdk2 complex creates a positive feedback loop again



Modified from Nature Review Genetics (2005) ***6****, 9-23*

Figure 1:
Schematic representation of the signaling pathway which regulates cell growth in response to cell extrinsic factors (Insulin/Growth factors and metabolites) .



*Modified from Nature Reviews Neuroscience (2007), **8**, 438-450*

Figure 2:
Schematic representation of different phases of cell cycle progression during mitotic division of an eukaryotic cell. The different Cyclins and the associated Cyclin Dependent Kinases (CDKs) responsible for phase transition regulations are mentioned.

phosphorylating Rb protein leading to E2F accumulation in cytoplasm and subsequently transition from G1 to S phase [16].

Cyclin D/CDK4 complex in *Drosophila* has been found to be capable of inducing cell cycle progression as well as cell growth [10]. Overexpression of Cyclin D/CDK4 complex in post mitotic cells in *Drosophila* larval tissues leads to overgrowth phenotype. On the other hand, overexpression of cell cycle regulator CycE fails to show such phenotype in spite of the increase in number of cells. It has been shown that ectopic expression of mammalian Cyclin D/CDK4 complex in *Drosophila* stimulates cell growth without interfering with cell cycle [17]. Further work towards elucidating the mechanism of Cyclin D/CDK4 mediated cell growth suggests an Rb independent mechanism [10], but specific mechanism is yet to be worked out.

1.3 Mitochondrial role in cell growth regulation:

Mitochondria are classically viewed as static organelles that function as the ATP generation center and metabolic hub of the cell. In the last few decades of study, this classical image has critically changed to recognize it as a dynamic organelle involved in many cellular processes e.g. apoptosis, proliferation, differentiation and aging etc. Mitochondrial function can produce various signals in form of different metabolites, ATP, ROS and Ca^{2+} etc. along with membrane potential and dynamicity in fission / fusion which ultimately lead to differential response of cell owing to mitochondrial outputs. Collectively these signals are known as mitochondrial retrograde signals. Intensifying focus of researchers towards unraveling the role of mitochondria has established it as an important signaling platform. Mitochondrial function in diseases like cancer, neuromyopathy and cardiomyopathy has been well established [18].

Mitochondrial role in regulation of cell growth is yet not explored. It has been reported that CyclinD-CDK4 mediated cell growth can be suppressed by mutation in *mitochondrial ribosomal protein large subunit 12* (mRpL12) [19]. Homozygous mutant cells for mRpL12 exhibit reduced mitochondrial activity and phenocopy growth defects shown by *CDK4* null mutants. But yet the mechanistic basis of such a process has not been reported. Although, Cyclin D-CDK4 complex has been shown to regulate mitobiogenesis and mitochondrial activity in *Drosophila* [20] but retrograde signaling pathway(s) involved in cell growth regulation needs to be worked out.

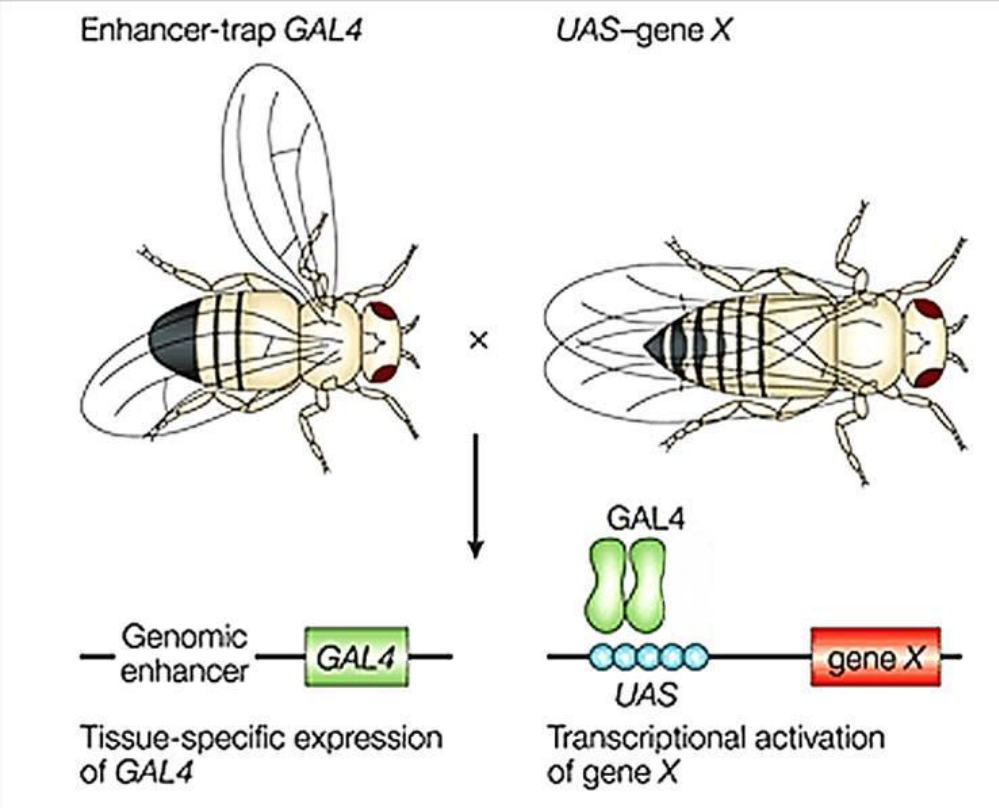
1.4 *Drosophila* as a Model System:

The Golden bug, *Drosophila melanogaster* was first described and later extensively characterized by TH Morgan and his students in the start of 20th century [21]. Being a holometabolous insect, *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. Genome of *Drosophila* which was completely sequenced in the year 2000 [22] has provided unique molecular genetic insights. Availability of multiple tools for genetic manipulations (e.g. UAS-Gal4 system to spatiotemporally control gene expression, generation of somatic clones, and site directed mutagenesis for creating transgenes) and ease of generating heritable genetic changes in *Drosophila* genome makes it the most tractable organism to do genetic studies [23]. Researchers over the decades have generated a very rich collection of mutants which are available for procurement from various stock centers. Studying *Drosophila* has been made easier by availability of phenotypically distinguishable molecular markers for different chromosomes.

Even though *Drosophila* and Human are separated through millions years of evolution, around 75 percent of human disease genes listed in OMIM (Online Mendelian Inheritance in Man) have related sequences in *Drosophila melanogaster* [24]. 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 find a counterpart in *Drosophila*.

1.4.a UAS – GAL4 System:

UAS-GAL4 system was first described by Brand and Perrimon [25] which was 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 responder line. When the driver and responder lines are crossed, the GAL4 and UAS elements come into one genome in F1 generation (**Fig 3**). Thus, expression of



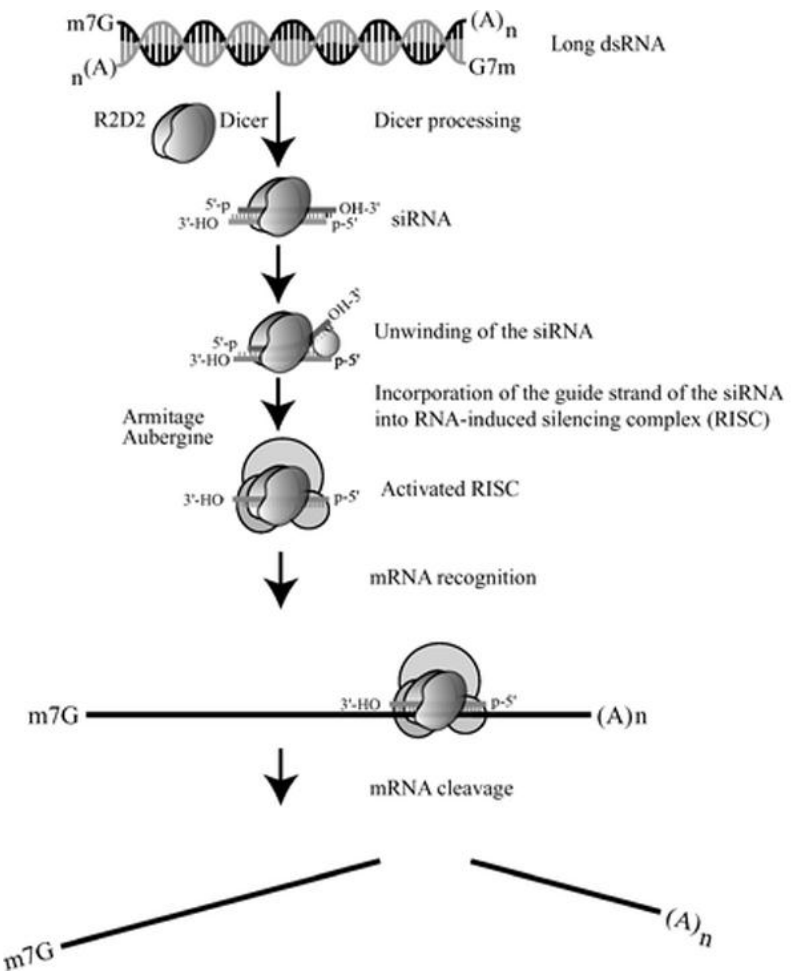
Modified from Nature Reviews Genetics (2002) **3**, 176-188

Figure 3:
Schematic representation demonstrating the mechanism by which ectopic gene expression in a tissue specific manner can be achieved using the UAS-Gal4 system in *Drosophila melanogaster*.

GAL4 protein switches on transcription of target genes downstream of UAS element. Employing this approach, expression pattern of a target gene can be spatiotemporally controlled using proper leverage viz. enhancer of 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 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* [26].

1.4.b RNAi based genetic Screen in *Drosophila*:

RNA interference was first described in *C. elegans* by Andrew Fire and Craig Mellow [27] 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 RNAi based gene knock-down strategy was employed by Kennerdell et al. in 1998 [28] 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, 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 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.



Modified from Annual Review of Medicine (2005) **56**, 401-423

Figure 4:
Schematic representation demonstrating the mechanism by which RNAi mediated gene silencing is achieved in *Drosophilamelanogaster*.

As mentioned earlier, a bipartite approach using genomic enhancer with GAL4 and UAS-RNAi line could be highly successful for functional analysis of genes and pathways. Considering the promising aspects of this technique, attempts have been made to generate libraries of transgenic UAS-RNAi lines. Due to such comprehensive efforts to generate UAS-RNAi lines covering almost whole genome of fly, large collections of UAS-RNAi transgenes are available from different *Drosophila* stock centers which include National Institute of Genetics (NIG-FLY), Japan, Transgenic RNAi Project (TRiP), Harvard Medical School, USA and Vienna *Drosophila* RNAi Center(VDRC), Austria.

1.4.c Adult eye and larval fat body as a model for cell growth screen:

In a developing organism, cell growth and cell cycle progression are intricately linked [1]. Sooner the mitotic cells attain a threshold growth, they undergo cell division. Therefore modulation of cell growth causes changes in cell cycle progression, leading to change in cell number compared with normal conditions. On contrary when cells exit cell cycle, it attains a particular volume before differentiation. Hence the tissue of interest were chosen wherein the growth regulation can be analysed in post mitotic cells e.g., Adult *Drosophila* eye and larval fat body.

Developing eye of *Drosophila* has been used as a model system to study various cellular processes like cell cycle [29], cell interactions during development [30], apoptosis [31]. The adult *Drosophila* compound eye consists of around 800 unit eyes or ommatidia where each ommatidium is a hexagonal structure and the compound eye resembles honeycomb like structure. Each ommatidia further consists of eight photoreceptor neurons, four lens secreting cone cells, and two primary pigment cells. This complex yet precisely ordered structure of compound eye emerges from 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 indentation called morphogenetic furrow [32]. 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. It has also been shown that the adult eye is

not essential for fly survival; mutations wherein eye fails to develop are viable [33]. Hence analysis of cell growth in post mitotic cells of adult *Drosophila* eye was done for the primary screen in this study.

Larval fat body is mesoderm derived tissue which is set out during embryonic development of *Drosophila*. This is a single layer, translucent tissue consisting of around 2200 cells [34]. Number of cells is 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 also secretes important molecules which have a systemic effect on development of *Drosophila* [35]. When a larva is undergoing metamorphosis in pupal stage, dissociation of fat body provides nutrition and energy for different processes taking place inside the pupae.

1.5 Objectives and Design of the Project:

This project aims to unravel the mechanistic basis of mitochondrial retrograde response in cell growth regulation. Employing RNAi based gene knock down strategy, we are carrying out a genome wide screen for nuclear encoded mitochondrial proteins based on genes listed in Mitodrome [36]. A figurative description of the screening strategy has been shown in **Fig 5**. As shown in fig, an eye specific Gal4 driver GMR Gal4 which is expressed in late 3rd instar drives the expression of Cyclin D/CDK4 under control of UAS element leading to overgrowth eye phenotype. Here GMR is Glass Multimeric Repeats which expresses in post mitotic cells of developing *Drosophila* eye [37]. In the above mentioned genome, another construct having UAS-RNAi is also brought in. Subsequently this will lead to silencing of the gene targeted by specific dsRNA expression. Knock down of genes can have three possible effects on overgrowth adult eye phenotype associated with Cyc D/CDK4 overexpression.

a). Genes having no role in cell growth: No change in overgrowth in adult eye

b). Genes involved in positive regulation of cell growth: Suppression of overgrowth in adult eye

c). Genes involved in negative regulation of cell growth: Enhancement of overgrowth in adult eye

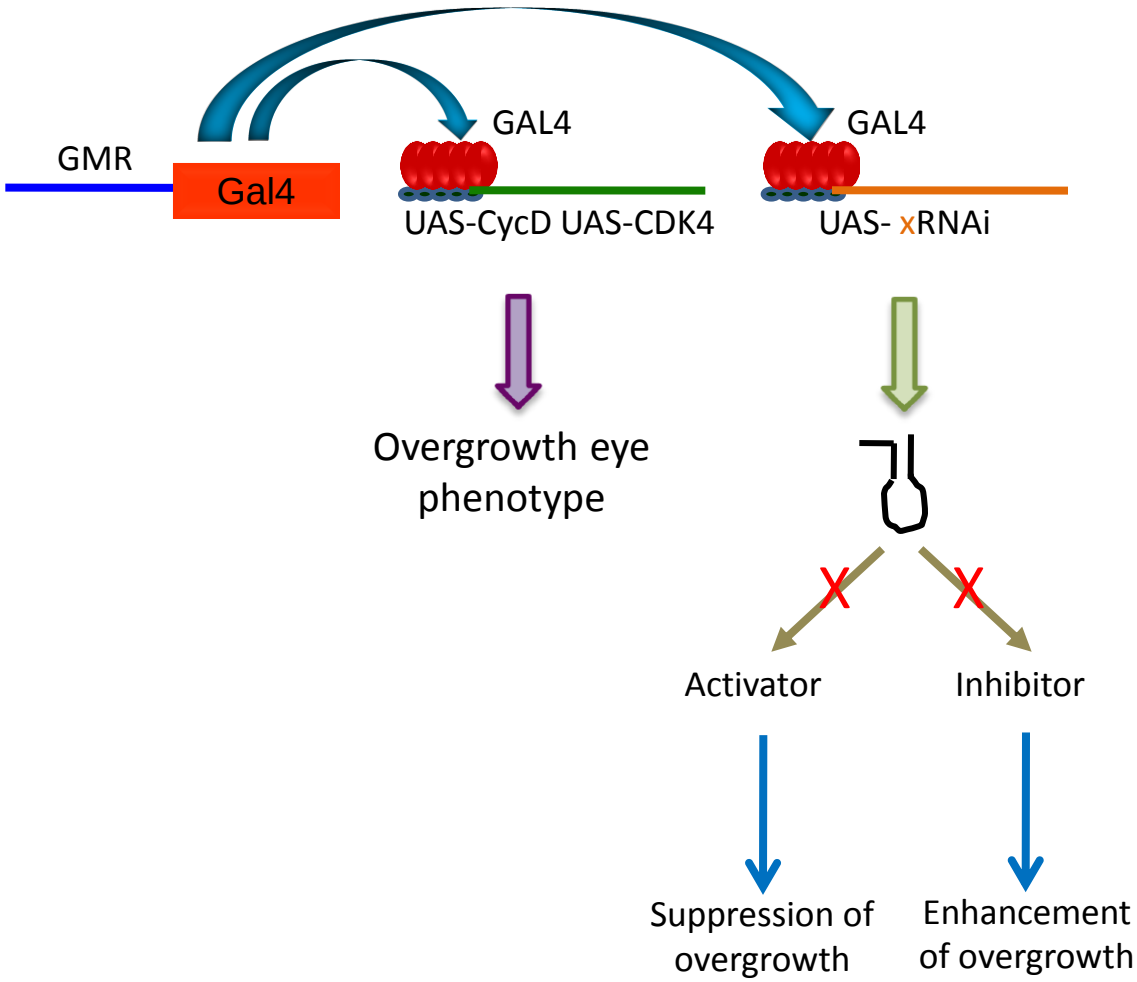


Figure 5:
Schematic representation of the strategy for genome wide loss of function RNAi screen to identify modifiers of cell growth in flies.

Once, candidate genes have been identified from the screen further molecular genetic characterization which is being carried out in larval fat body will shed light on its response pathway(s).The advantages of this strategy include ease, rapidity and reliability in screening and large collection of genes can be screened in lesser amount of time.

2. Materials and Methods:

2.1 Fly Stocks and Genotypes:

Many *Drosophila* lines have been procured 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) ***yw; r4 Gal4:*** This transgenic line was created by Jea H. Park (2004) with the Gal4 insertion on chromosome 3. Previous reports [38] and analysis in our laboratory show that this driver is predominantly expressed in larval fat bodies from early first instar and continues till late third instar.

b) w; GMR Gal4/Cyo; UAS CycD, UAS CDK4: This recombinant line was created 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. The crossing scheme to generate this recombinant line is detailed in **Box 1**.

c). w, UAS Bsk^{DN}: This transgenic line was generated by Adachi-Yamada (1999). This has an insertion on first chromosome which expresses a dominant negative form of *Drosophila* JNK Basket under UAS control.

c). w; puc-LacZ/TM3, Ser: This transgenic line was generated by Ring and Martinez Arias (1993). This has an insertion on third chromosome which expresses β-galactosidase under enhancer region of puckered gene.

d). UAS RNAi lines: Various UAS-RNAi used in this study has been obtained from Bloomington Stock Centre, Indiana, USA. These lines were generated by Transgenic

BOX 1: Scheme of the genetic crosses to generate a line with *GMR Gal4* and *UASCycD, UASCDK4*

yw; GMR Gal4/CyO; +/+ **X** *w/w; Sco/Cyo; TM3Sb/TM6B,Tb*

w; Sco/CyO; TM3Sb/TM6B, Tb **X** *w/w; +/+; UASCycD, UASCDK4*

w; GMR Gal4/Sco; +/TM6B,Tb **X** *w/w; +/CyO; UASCycD, UASCDK4/TM3Sb*

w/w; GMR Gal4/Cyo; UASCycD, UASCDK4/TM6B,Tb **X** *w; GMR Gal4/Cyo; UASCycD, UASCDK4/TM6B,Tb*

w; GMR Gal4/Cyo; UASCycD, UASCDK4/UASCycD, UASCDK4

BOX2 : Scheme of the cross involved to screen a representative RNAi line

w/w; GMR Gal4/CyO; UASCycD, UASCDK4 **X** *yv; +/+; UASmtACP1RNAi*

w; GMR Gal4/ +; UASCycD, UASCDK4 /UASmtACP1RNAi

(non Curly males)

RNAi Project (TRiP) of Harvard University, USA. The stock number of transgenic lines used in this project is mentioned in **Table 1**.

2.2 Genetic Crosses and Recombination:

2.2.a Crosses for Genetic Screen:

As discussed earlier, overexpression of CycD/CDK4 leads to overgrowth in post-mitotic cells in *Drosophila*. We have generated a recombinant line combining GMR-Gal4/Cyo (2^{nd} chromosome) with UAS CycD UAS CDK4 (3^{rd} Chromosome Homozygous), which leads to overgrowth in adult eyes. We cross this transgenic line with UAS RNAi (3^{rd} Chromosome Homozygous) lines as per scheme shown in **Box 2**. The F1 progeny which are non-curly carries genes of our interest which is GMR Gal4, UAS CycD, UAS CDK4 and UAS RNAi. The non-curly males are selected for adult eye SEM imaging.

2.2.b Crosses for cell size analysis in larval fat body:

For our studies we crossed r4 Gal4 line with UAS-RNAi lines to analyse larval fat body cell size as per the crossing scheme shown in **Box 3**.

2.2.c Genration of w; +/ +; r4-Gal4, puc-LacZ / UAS mtACP1 RNAi line: In order to generate this transgenic line we have followed the crossing scheme described in **Box 4**.

2.2.d Generation of UAS Bsk^{DN}; +/+; UASmtACP1RNAi /r4Gal4: We have followed the crossing scheme described in **Box 5** to obtain males of this phenotype.

2.3 Analysing Adult Eye Phenotype:

The *Drosophila* adult eye was imaged with Scanning Electron Microscope (Jeol JCM 5000) at 100X, 170X and 1000X. Newly emerged adults from screening crosses were collected every morning and evening. Properly aged adult flies (maintained at 25^{o}C) were selected for further analysis. Adult non-curly males from each collection were selected for imaging in scanning electron microscope.

2.4 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 40 minutes and then three washings with 0.1% PBT. Tissues were incubated in Phalloidin Alexa 594nm (Molecular Probes) using

BOX 3: Scheme of the genetic cross to knock down mtACP1 in larval fat bodies

yw/yw; +/+; r4 Gal4 **X** *w; +/+; UAS mtACP1RNAi*

↓

yw OR yw/w; +/+; r4 Gal4/UAS mtACP1RNAi

BOX 4: Scheme of the genetic crosses to assay PucLacZ expression in mtACP1 knock down fat body

yw; +/+; r4Gal4 **X** *w/w ; +/+; PucLacZ/TM6B,Tb*

↓

y,w; +/+; r4Gal4/ PucLacZ **X** *w; Sco/CyO; TM3Sb/TM6B, Tb*

↓

w/w; UASnlsGFP/ UASnlsGFP; MKRSTM6B, Tb **X** *w; +/CyO;* **X***/TM6B, Tb*

X ➔ *r4 Gal4; pucLacZ; r4 Gal4,pucLacZ*

↓

w; UASnlsGFP/ +; r4Gal4,pucLacZ/ TM6B, Tb **X** *w; +/+; TM3, Sb/TM6B, Tb*

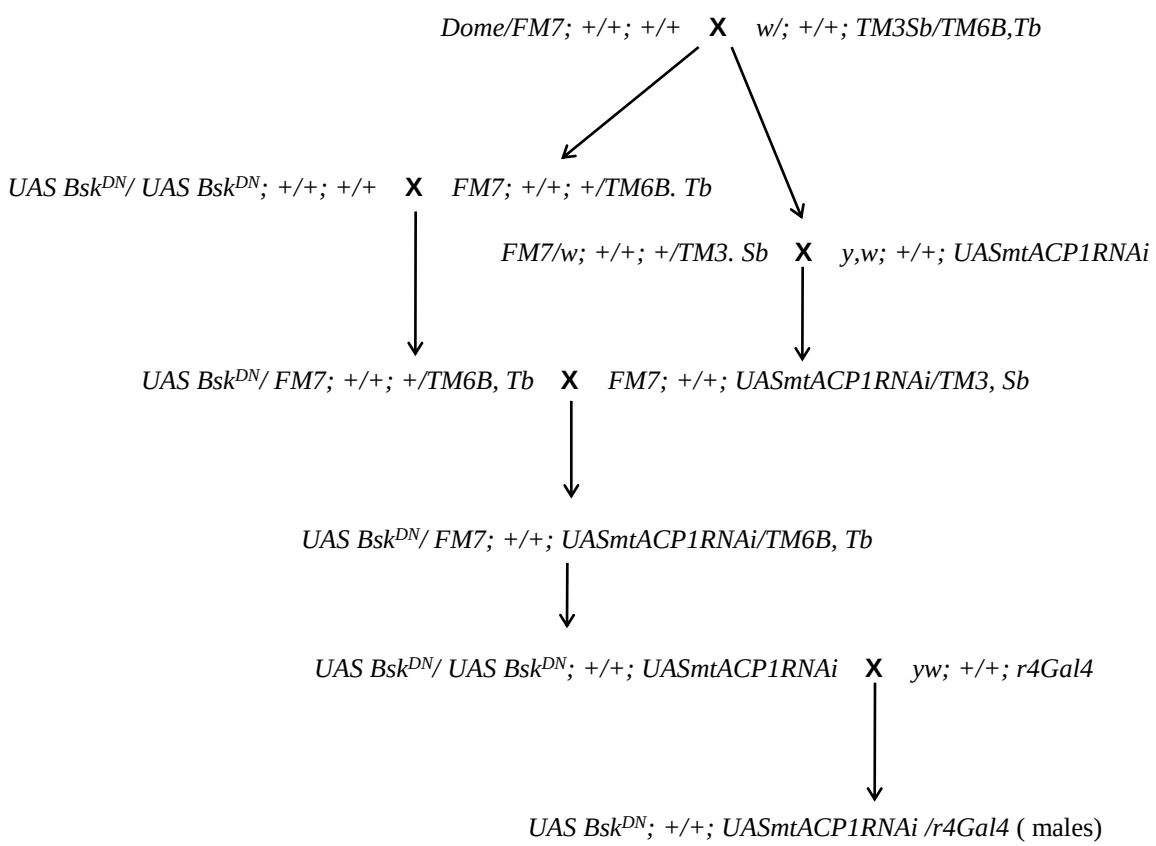
↓

yv; +/+; UASmtACP1RNAi **X** *w; +/ +; r4Gal4,pucLacZ/ TM6B, Tb*

↓

w; +/ +; r4Gal4,pucLacZ/ UASmtACP1RNAi

BOX 5: Scheme of the genetic crosses to co-express UAS Bsk^{DN} and UASmtACP1RNAi in larval fat body



a dilution of 1:100 in 1X PBS. Tissues were washed with 0.1% PBT and mounted in DAPI supplemented Vecta-Shield (Vector labs). Imaging was done using fluorescence microscope (Carl Zeiss) at 20X. Cell size of fat body cells were measured in AxioVision software and statistical analysis was done in Microsoft Excel.

2.5 Measurement of developmental delay:

The lines r4Gal4 and UAS mtACP1 RNAi were crossed and synchronous egg laying was collected and transferred to 29^{o}C along with wild type control w^{1118} in triplicates. The time of pupation was calculated by recording number of new pupae formed in a unit time.

2.6 ROS staining in larval fat body:

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 8% formaldehyde for 10 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.

2.7 Immunohistochemistry

Late third instar larvae crawling on food surface, were dissected in 1X PBS, fixed in 8% paraformaldehyde. Followed by brief washings with 1X PBS, tissues were incubated in 0.3% Triton in 1X PBS for half an hour. After a few washings with 1XPBS, blocking was done with 3% milk powder in 1X PBS. Primary antibody mouse Anti-β-galactosidase antibody (Promega) 1:100 was prepared in blocking solution and fat bodies were incubated overnight at 4^{o}C. Secondary antibody antimouse IgG Alexa 594nm was used in 1:400 dilution. Tissues were mounted in Vectashield with DAPI (Vector Labs) and observed in confocal microscope LSM780 (Zeiss). Images were analysed in ImageJ (NIH) and Adobe Photoshop.

CHAPTER 2

Results and Discussion

3. Results:

3.1 Knocking-down nuclear genes encoding mitochondrial proteins modulate overgrowth eye phenotype

For this study we performed a loss of function genetic screen involving adult eyes of *Drosophila* to isolate nuclear genes encoding mitochondrial proteins, which when mutated, alter cell overgrowth induced by over-expression of CycD/CDK4 complex. We are knocking down nuclear genes encoding mitochondrial proteins using RNAi in a genetic background where CycD/CDK4 are overexpressed in the same cell. As earlier reported [10], over-expression of CycD/CDK4 in post mitotic cells of developing *Drosophila* eyes leads to overgrowth of the adult eye. Closer examination of this overgrowth phenotype revealed that the adult eyes demonstrating overgrowth bulge out laterally compared to the wild type control eyes (**Fig 6 A-C**). Importantly, the increase in size is more evident in individual ommatidial cluster of these eyes (**Fig 6 B, E)**. In our genetic screen, using the screening strategy outlined in **Box 2**, we have identified three categories of overgrowth modifiers; Enhancers, Suppressors and No Change. We have thus far screened 58 UAS - RNAi lines corresponding to 49 nuclear encoded mitochondrial genes (**Table 1**). Among these 49 genes, 24 genes when knocked down do not modify the overgrowth phenotype associated with CycD/CDK4 overexpression in the adult eye. This class of genes suggests that not at all times attenuated mitochondrial will cause modulation in overgrowth.

In spite of the idea that restricted mitochondrial function will lead to decrease in metabolism and hence leading to decrease in cell growth, surprisingly we have identified a class of genes knocking down of which lead to further enhancement of the overgrowth eye phenotype. As shown in **Fig 6 C, F** one of the genes ND23, that codes for one subunit of the Complex I of the mitochondrial electron transport chain, when knocked-down exhibit significant increase in overgrowth phenotype associated with CyclinD/CDK4 over-expression. In consistence we also observed a remarkable increase in the size of individual ommatidial clusters. This enhancement in the ommatidial cluster size lead to overlapping of neighboring ommatidial clusters. This not only disturbs the typical hexagonal array of ommatidial clusters but also renders a rough appearance of the adult eyes. This phenotype could be due to extra secretion of lens material in the eyes.

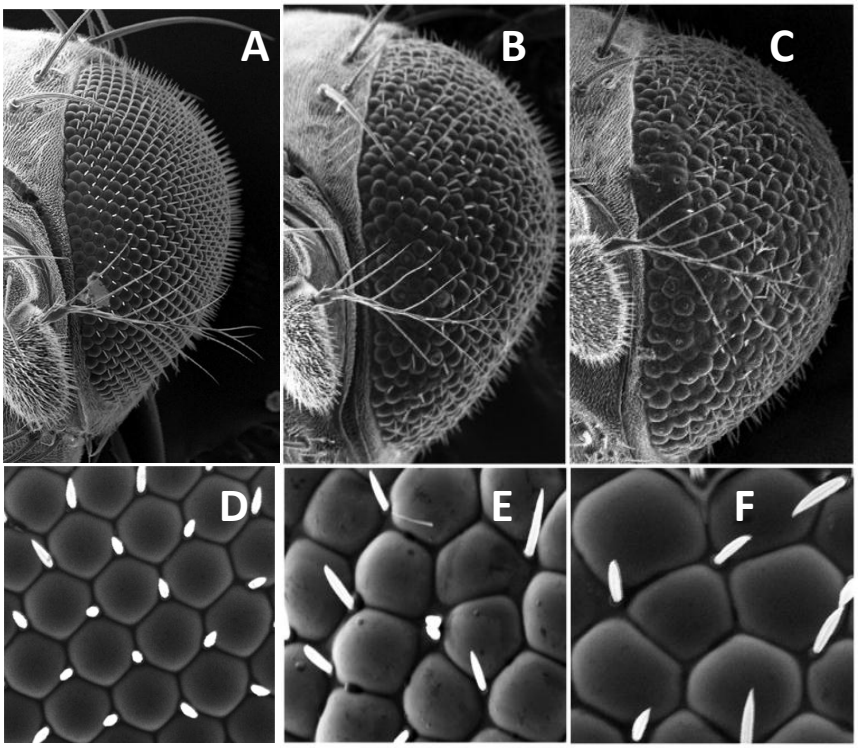


Figure 6:
Knocking down ND23, a component of Complex I leads to enhancement of the overgrowth phenotype associated with CycD CDK4 over expression in adult *Drosophila* eyes. Scanning Electron micrograph of wild type adult eyes (lateral view) appear normal **(A**). In contrast the adult eyes of GMR Gal4; UAS CycD, UAS CDK4 flies (**B**) exhibit overgrowth phenotype. Further enhancement is observed upon knocking down of ND23 in this genetic background (**C)**. (**D to E**): High magnification images of portion of adult eyes as shown in A to C respectively demonstrating the increase in size of ommatidial clusters.

Table 1: screening results			
Class of Modifier	**Bloomington Stock No.**	**Gene disrupted**	**Gene Function**
Suppressor	29528	Mtacp1	NADH:Ubiquinone Oxidoreductase Acyl Carrier Protein
	34915	Wal	Subunit Of Electron Transfer Flavoprotein
	26005	Tom40	Translocase Of Outer Membrane 40
	32496	Sod2	Superoxide Dismutase
	32983	Sod2	Superoxide Dismutase
	34074	CG8728	Metalloendopeptidase Activity
	36083	Arc42	Acyl-Coa Dehydrogenase
	36874	Yip2	Acetyl-Coa C-Acyltransferase Activity
No Change	34729	Hsp60	Heat Shock Protein 60
	28743	Dare	NADPH-Adrenodoxin Reductase
	27083	Debcl	Apoptosis Regulator Bcl-2
	34916	CG14508	Subunit Of Complex IV Of ETC
	35464	CG1746	Subunit Of Complex V Of ETC
	28573	CG1970	Subunit Of Complex I Of ETC
	26744	TFAM	Positive Regulation Of Transcription
	32873	CG4389	Subunit Of Acyl-Coa Dehydrogenase
	35219	Dnk	Deoxynucleoside Kinase Activity
	30511	CG5548	Subunit Of Complex I Of ETC
	32876	CG5599	Lipoamide Acyltransferase
	29572	Porin	Voltage-Gated Anion Channel
	27700	Ppl	Glycine Cleavage H-Protein
	28576	CG8680	Subunit Of Complex I Of ETC
	35142	Pdk	Pyruvate Dehydrogenase Kinase
	28635	Pdk	Pyruvate Dehydrogenase Kinase
	36695	CG3214	Subunit Of Complex I Of ETC
	30500	CG6661	Delta-1-Pyrroline-5-Carboxylate Dehydrogenase
	36608	CG7433	4-Aminobutyrate aminotransferase
	34546	Thiolase	Acetyl-Coa C-Acyltransferase activity
	34343	CG6512	Afg3-Like Protein 2
	36072	Adk3	GTP:AMP Phosphotransferase
	34586	CG8798	Lon Protease
	34028	Acon	Aconitate Hydratase Activity
	38332	CG2118	Methylcrotonyl-Coa Carboxylase Subunit
Enhancer	34973	CG8993	Disulfide Oxidoreductase
	30487	ND23	Subunit Of Complex I Of ETC
	32436	CG12262	Acyl-Coa Dehydrogenase
	32437	CG12262	Acyl-Coa Dehydrogenase
	33878	Cype	Subunit Of Complex IV Of ETC
	27548	Cova	Subunit Of Complex IV Of ETC
	33911	ND75	Subunit Of Complex I Of ETC
	27739	ND75	Subunit Of Complex I Of ETC
	28059	Blw	Subunit Of Complex V Of ETC
	32998	ND42	Subunit Of Complex I Of ETC

	Bloomington Stock No	Gene disrupted	Gene Function
Enhancer	34526	ND42	Subunit Of Complex I Of ETC
	28723	ATP syn-γ	Subunit Of Complex V Of ETC
	29592	Pdsw	Subunit Of Complex I Of ETC
	36701	CG9140	Subunit Of Complex I Of ETC
	36915	CG10924	Phosphoenolpyruvate Carboxykinase
	35294	Adk2	Adenylate Kinase-2
	34066	CPTI	Carnitine O-Palmitoyltransferase
	33740	ATP Syn D	Subunit Of Complex V Of ETC
	28894	ND42	Subunit Of Complex I Of ETC
	34585	CG6459	Calcium-Mediated Signaling
	28062	ATP Syn B	Subunit Of Complex V Of ETC
	33910	ND75	Subunit Of Complex I Of ETC
	29534	CG 9140	Subunit Of Complex I Of ETC
	38251	Tko	Subunit Of Ribosome

This category of modifiers, which is termed as enhancers, includes different genes that are associated with different processes while most of them are components of electron transport chain complex I, complex IV and complex V. Since the loss of function of these genes lead to further increase in size of the ommatidial clusters associated with CyclinD/CDK4 over-expression it clearly demonstrates that in wild type condition these genes might function as negative regulators of cell growth.

Finally we isolated the third category of genes that when mutated suppress the overgrowth eye phenotype observed in flies where CycD/CDK4 is over-expressed in developing adult eyes. In this category we have isolated around seven genes. Of these seven genes the gene that demonstrates maximum suppression of the over-growth phenotype is mtACP1 *(mitochondrial acyl carrier protein 1*) (**Fig 7)**. The gene mtACP1 codes for NADH:Ubiquinone Oxidoreductase Acyl Carrier Protein which required for assembly and organization of complex I of electron transport chain [39]. We found that knocking out these genes in post mitotic cells of the developing eye brings about a significant suppression in the overall size of the adult eye. Even at the level of ommatidial clusters this suppression is obvious and the eyes in general do not exhibit the roughness as observed for the overgrown eyes due to CyclinD/CDK4 over-expression. Isolation of this group of suppressors in turn clearly demonstrates that mitochondrial function can positively regulate cell growth. Together, the isolation of suppressors and enhancers, establish that mitochondrial function can modulate cell growth either positively or negatively in a context dependent manner.

3.2 Larval fat body cells knocked down for mtACP1 are smaller in size

To assess the role of the genes isolated in the primary screen in controlling growth at the cellular level we knocked down these genes 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. Employing the cross scheme mentioned **Box 3** mtACP1 gene was knocked down in the larval fat body cells using r4-Gal4 as the driver line. Analyses of cell size upon knocking down mtACP1 reveal that the individual cells are smaller when compared to the wild type cells. Upon analysis of cell sizes of around seventy fat body

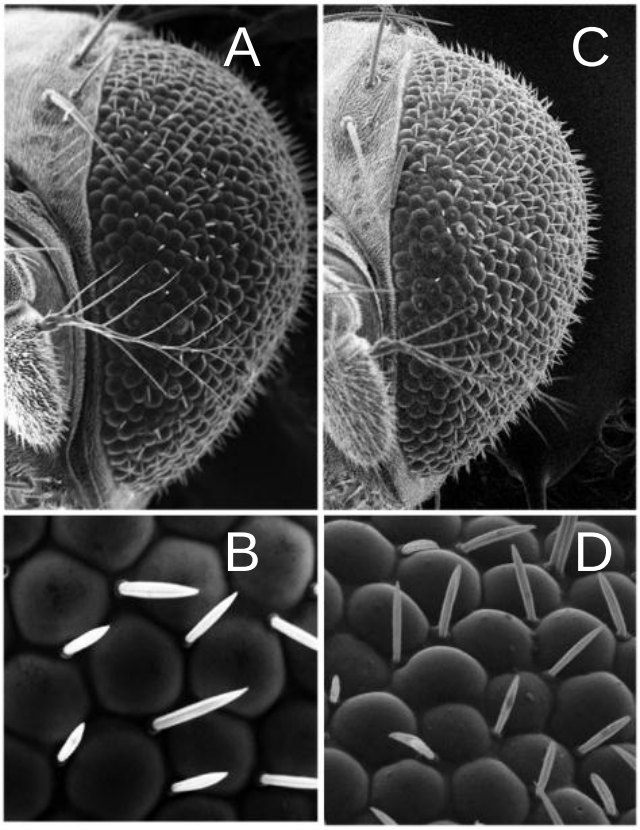


Figure 7:
Adult eye overgrowth phenotype of CycD CDK4 overexpression is suppressed by by knocking down mtACP1 in CycD CDK4 background. Scanning Electron micrograph of adult eyes of GMR Gal4; UAS CycD, UAS CDK4 flies (lateral view) exhibit overgrowth phenotype. .wild type adult eyes (lateral view) appear normal **(A**). In contrast a significant suppression of the overgrowth phenotype is observed upon knocking down of mtACP1 in this genetic background (**C)**. (**B & D**): High magnification images of portion of adult eyes as shown in A & C respectively demonstrating the decrease in size of ommatidial clusters.

cells obtained from seven different larvae we found that knocking down mtACP1 causes decrease in cell size by 40% in comparison to controls (**Fig. 8**). Further statistical analysis shows that this decrease in cell size is significant. Interestingly we did not find any change in the size of the nuclei of mtACP1 knock-down cells when compared to wild type fat body cells. This result suggests that although the mtACP1 knock down cells are smaller in size, the process of DNA replication is not affected in these cells. For understanding the mechanistic basis of cell growth regulation by mitochondrial function, we selected mtACP1 for subsequent analysis.

3.3 Levels of cellular Reactive Oxygen Species (ROS) are high in mtACP1 knock down cells

In an attempt to understand how attenuation of mitochondrial function in mtACP1 knock-down cells cause retardation in cell growth to start with, we were interested to find how does the mitochondria behave in these cells. Previous studies have demonstrated that attenuating mitochondrial activity can lead to generation of different signals such as decrease in cellular ATP level or an increase in cellular ROS level. mtACP1 being involved in organization and activation of Complex I of electron transport chain (ETC) [39], we checked the level of cellular ROS in these cells. ROS moieties generated in cells upon mitochondrial dysfunction can either be superoxide or peroxide molecules. For superoxide detection, we used Dihydroethidium (DHE) a dye that can freely permeate through the cell membrane and gets converted into bright red colour fluorescent molecule 2-dihydroxyethidium upon reacting with superoxide molecules. Staining fat body cells knocked down for matACP1 with DHE, we found that these cells have elevated levels of cellular superoxide when compared to wild type controls (**Fig 9 A, B**). Similarly, peroxide levels were assayed with 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) dye which generates a green fluorescent compound as it reacts with peroxide molecules. In a manner similar to that observed for DHE we found that cells knocked down for mtACP1 activity have higher levels of cellular peroxide when compared to wild type fat body cells (**Fig 9 C, D**). Put together our results establish that knocking down mtACP1 in cells lead to an overall increase in the cellular levels of ROS that includes both superoxides and peroxides.

3.4 JNK pathway is activated in mtACP1 knock down larval fat body cells:

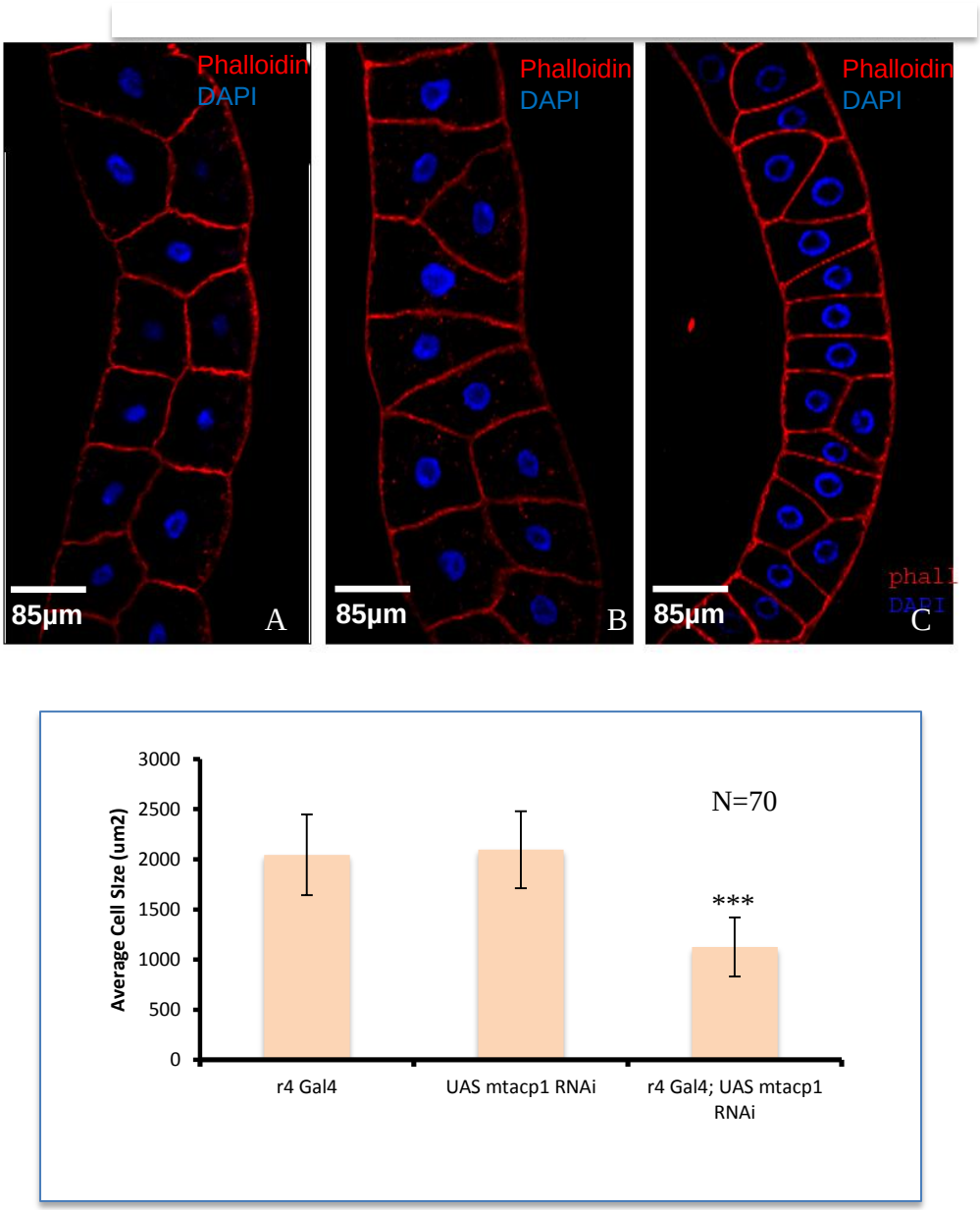


Figure 8:
Cell size analysis of larval fat body in mtACP1 knock down shows that cells of mtACP1 knock down organisms **C)** r4Gal4>UAS mtACP1 RNAi are smaller than wild type controls **A)** r4Gal4 and **B)** UAS mtACP1 RNAi. Quantitative analysis of decrease in cell size shows that the decease is statistically significant with $p<0.001$
(40x, zoom 1)

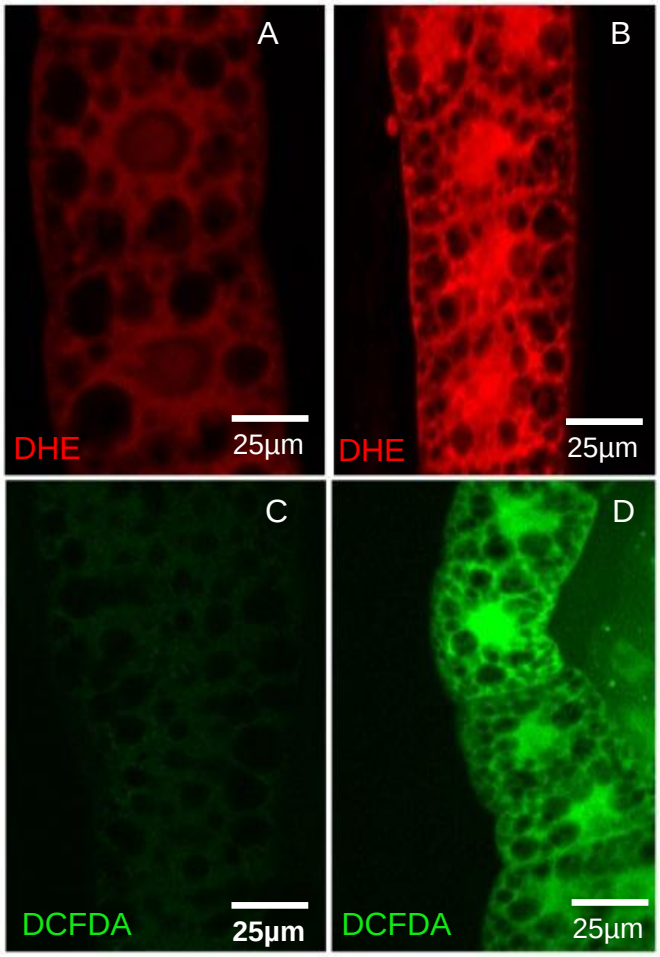


Figure 9:
Fat body cells knocked down for mtACP1 have elevated levels of ROS. The tissues were either stained with Dihydroxy Ethydium (DHE) to detect the levels of superoxide radicals or with DCFDA to detect the levels of peroxide radicals. Compared to control fat body cells (**A and C**) a robust increase in DHE staining (red, **B**) and DCFDA staining (green, **D**) is observed in fat body cells knocked down for mtACP1 activity. (40x zoom 1)

One of the known downstream signaling cascades, which gets activated by high ROS level is the JNK pathway [40]. As you can see in **Fig. 10**, the JNK pathway involves a series of kinases that eventually lead to activation of JNK by phosphorylation. One of the downstream transcriptional targets of JNK is *puckered* that itself codes for a phosphates that negatively regulates JNK. In an attempt to decipher whether JNK pathway is anyway involved in regulating cell growth in mtACP1 knocked down cells we set out to check puckered expression in these cells. To perform this experiment I generated the recombinant stock of *r4-gal4* and *puc-Lacz*, following the genetic scheme as mentioned in **Box 4**. We have done anti β-Gal immunostaining to detect expression of *puc-LacZ* in mtACP1 knock down fat body cells. As shown in the **Fig. 10**, while no puckered expression is observed in wild type late third instar larval fat body cells, expression of puckered is observed in mtACP1 knock down fat body cells of similar age. Our temporal expression studies revealed that the onset of puckered expression takes place even during early-mid third instar larval transition, thereby suggesting the activation of JNK pathway as an early event during the process of cell size reduction.

3.5 Inactivation of JNK pathway leads to rescue of phenotype associated with mtACP1 knock down

The results mentioned in the previous section provide a correlative evidence of the involvement of the JNK pathway in reduction of cell size due to knocking down of mtACP1. To have a functional correlate we performed a genetic experiment to determine whether inactivation of the JNK pathway can prevent reduction of cell size in mtACP1 knock-down cells. For this purpose we knocked down mtACP in fat body cells of larvae that are otherwise expressing a dominant negative form of *basket* (*bsk*), the JNK in flies. It is expected that expression of a dominant negative form would lead to inactivation of Bsk protein, which would then result in inhibition of JNK pathway. As shown in **Fig. 11**, we found that while inhibiting JNK pathway in wild type background do not affect cell growth, knocking down mtACP1 leads to decrease in size of larval fat body cells as demonstrated earlier. Interestingly we found that knocking down mtACP1 in cells expressing dominant negative form of Basket leads to restoration of cell size in mtACP1 knock down fat body cells. In an attempt to have a quantitative estimate of the extent of restoration we analysed the cell size of 66 cells of each genotype. As revealed in **Fig 11** we found that the increase in cell size is statistically significant. This result in conjunction

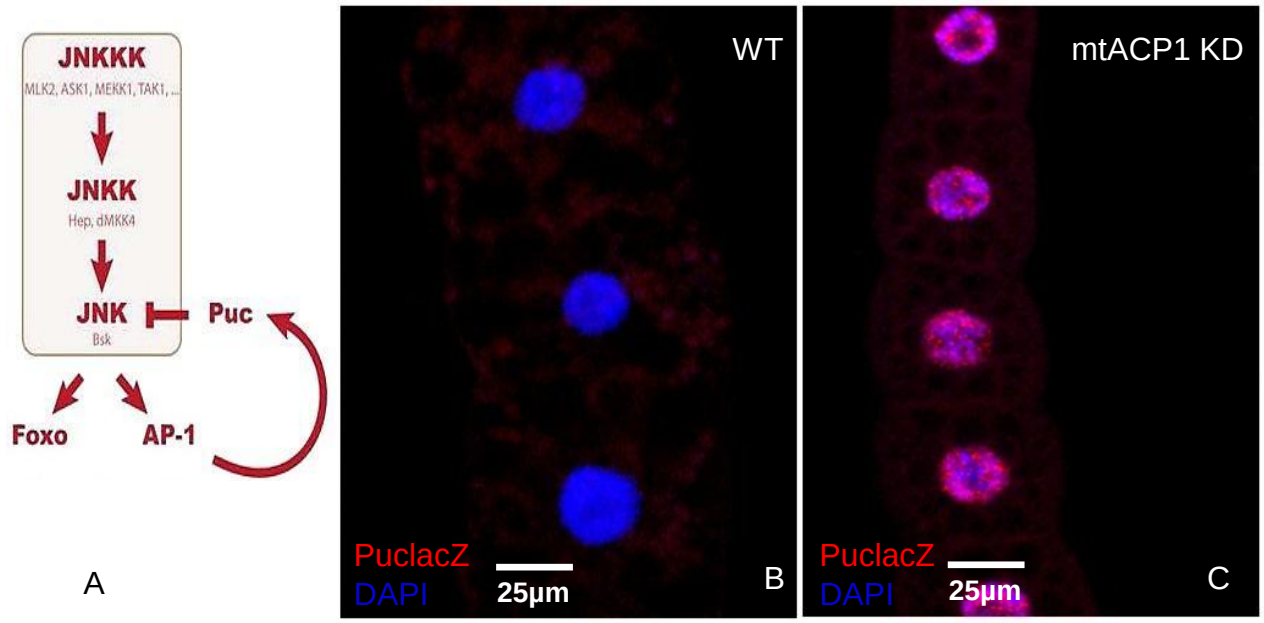


Figure 10:
Schematic representation of JUN Kinase (JNK) signaling Pathway (**A**). Fat body cells from *puckered-lacZ* (puc-lacZ) larvae do not show any lacZ expression in larval fat body cells (B). In contrast knocking down mtACP1 activates JNK pathway leading to expression of puc-lacZ in fat body cells (**C**). (40x, zoom 1)

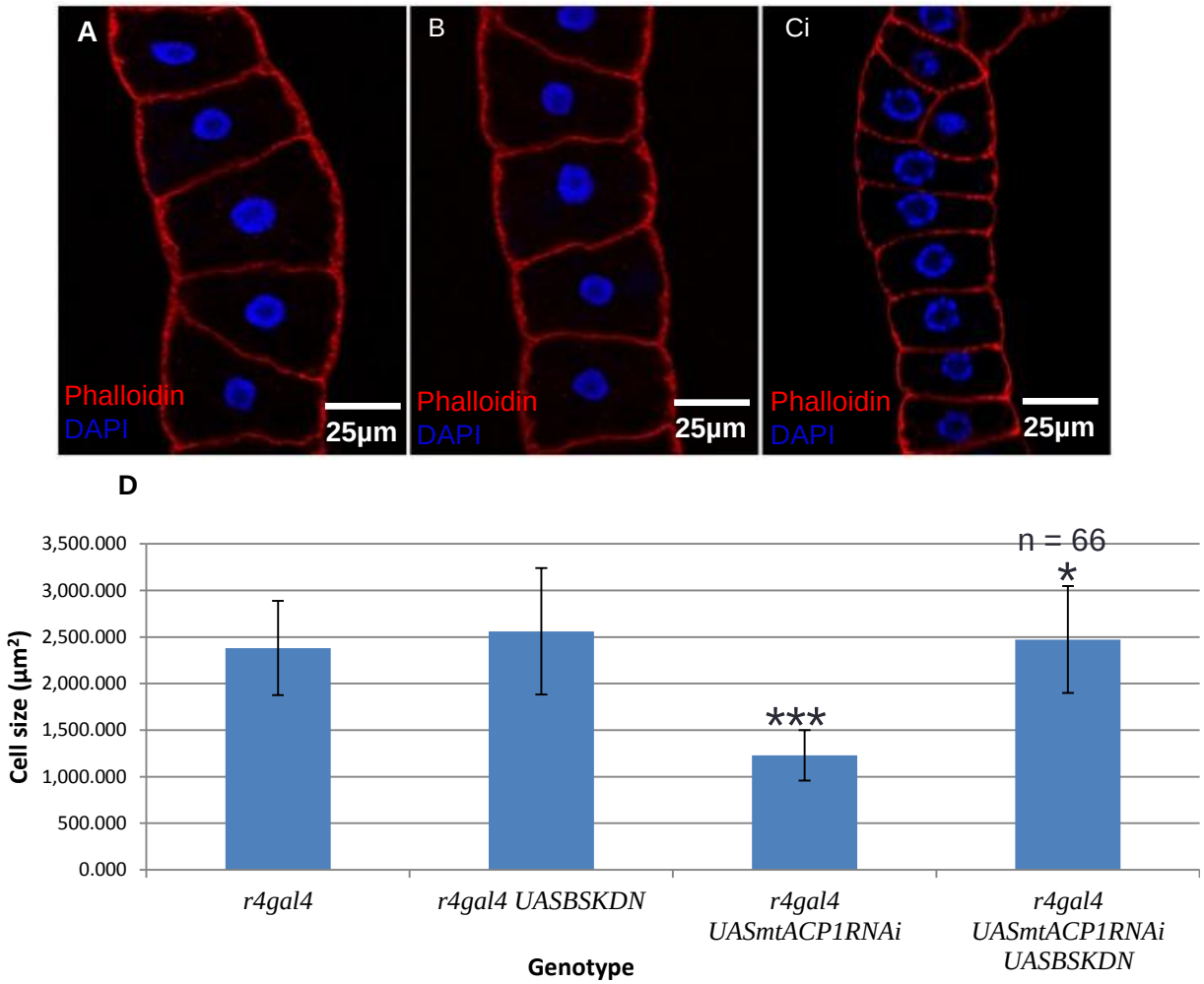


Figure 11:
Inactivation of the JNK pathway by expressing a dominant negative mutation of *Bsk* rescues the decreased cell size phenotype associated with *mtACP1* knock down fat body cells. The size of fat body cells from *r4-Gal4 > UAS Bsk*^{DN} larvae (**A**) are comparable to that of wild type fat body cells. While knocking down mtACP1 leads to smaller cell size (C), expression of Bsk^{DN} in r4Gal4 > UAS Bsk^{DN} ,UAS mtACP1RNAi larval fat body cells rescues this decreased cell size (**B**). Quantitative analysis of cell size shows that the rescue is significant. (* $p<0.1$, *** $p<0.0001$)

with the ectopic expression of puckered in mtACP1 knock-down cells establish that the reduction in cell size of thes cells is mediated by the JNK signaling pathway.

4. Discussion:

Investigation into the molecular genetic events regulating cell growth has emerged as an important task for biologists during past few decades. Although the role of many key factors that include nutrients, growth factors, and members of the TSC-TOR signaling pathway has been extensively worked out, very limited information is available about how the growth homeostasis is maintained by mitochondrial activity in a cell autonomous manner. Here, we report that mitochondria, a dynamic signaling port, when attenuated for its function can cause reduction as well as enhancement in cell growth. Being a metabolic hub, mitochondrial function is in general, linked positively to the cell growth which is an energy requiring process. Surprisingly, in our genetic screen we have also enlisted 14 genes which upon knock down has the potential to further enhance the overgrowth phenotype associated with CycD/CDK4 over-expression. Therefore, we establish that mitochondria can act as both, an inducer as well as an inhibitor of the growth regulatory pathway.

For analysis of the growth modifiers at cellular level, larval fat body was chosen for being post mitotic cells and its enormous growth during larval life. These cells are arranged in a monolayer which is helpful to dissect, stain the F-actin with phalloidin to visualize the cell membrane and eventually measure the cell size for quantitative analysis. Knocking down mtACP1, mitochondrial acyl carrier protein 1 in larval fat body cells shows decrease in cell size suggesting that mtACP1 has an inductive role on cell growth. mtACP1 exists in two forms, soluble in mitochondrial matrix and insoluble membranous form [39]. In matrix, mtACP1 regulates mitochondrial fatty acid synthesis FASII, independent of cytoplasmic fatty acid synthesis [41] and is required for biosynthesis of the lipoic acid. It has been shown in mammalian cells that knocking down mtACP1 causes inactivation of pyruvate dehydrogenase and Glycine cleavage system which require the lipoic acid moiety for its activity. In its membranous form, it is required for activity and assembly of complex I of the electron transport chain. In conjunction with these previous studies our results suggest that knocking down mtACP1 would significantly affect mitochondrial activity both at the levels of the electron transport chain

and biochemical activities happening in the mitochondrial matrix. Similar kind of effect is expected to happen if transport of matrix and membrane proteins is blocked across the mitochondrial membrane. In tune with this argument we observed that knocking down Tom40, a mitochondrial outer membrane transporter protein, also lead to reduction in cell size in manner similar to mtACP1 knock down.

Role of mitochondria in modulating different cellular processes like cell cycle, cell differentiation, aging and apoptosis has been attributed to the retrograde signaling from mitochondria to nucleus. The retrograde signaling pathway can get triggered by diverse signaling molecules including ROS [6], ATP [29], and Ca^{2+} [42] generated by the mitochondria in a context dependent manner. These signaling molecules in turn are sensed by different sensor proteins present in the cytoplasm that in turn activates other proteins of specific signaling pathways to evoke a cellular response. So far we have found that in cells knocked down for mtACP1 there is a significant increase in the level of cellular ROS. Interestingly mtACP1 knocked down cells have higher levels of both superoxide and peroxide molecules. Therefore to have a functional correlate of this observation it would be interesting to determine whether scavenging the higher levels of ROS can lead to restoration of normal cell size in mtACP

1 knock-down cells. For this purpose we have already generated fly stock where we can over express superoxide dismutase or catalase in the cells knocked to for matACP1 to scavenge superoxides and peroxides respectively. Analysis of ROS levels in these cells along with the size of these cells would establish the connection between higher levels of ROS and reduced cell size in mtACP1 knock-down cells.

Reactive Oxygen Species have been reported to activate the Stress Activated Kinases like JNK and p38 [43]. In this study we have shown that knocking down mtACP1 leads to activation of the JNK reporter Puc-LacZ expression, suggesting activation of JNK in mtACP1 knocked down cells. Further, upon suppressing JNK activity by expressing a dominant negative form of Basket, *Drosophila* homologue of JNK I obtained a significant rescue of the reduction in cell size associated with mtACP1 knock down. It will be therefore very interesting to identify how the JNK pathway is activated in mtACP1 knock down cells. There are several upstream kinases of JNK that are known to get activated by diverse stress signals. These molecules include Tak1, Sliper and the Apoptosis Signaling Kinase 1 (Ask1) a JNKKK which might sense the ROS levels and relay the signal

transmission [44]. We are therefore poised to identify the involvement of these molecules in the activation of JNK in mtACP1 knock-down cells. Moreover to establish that the activation of JNK pathway is indeed due to higher levels of ROS we need to determine whether scavenging of ROS in mtACP1 knock down cells can lead to loss of puckered-lacZ expression in these cells. Currently we are involved in generating the appropriate fly line in which this can be studied.

More importantly for complete understanding of the pathway it is essential to identify the downstream targets of the JNK pathway that lead to retardation of cell growth. One of the transcriptional factors that gets activated by the JNK pathway is Foxo [45]. Studies in mammalian cell lines [46] have demonstrated that one of the downstream targets of Foxo is *Thor*, the *Drosophila* homolog of 4EBP. Interestingly, 4EBP which inhibits protein synthesis is downstream target of the canonical well conserved Tsc-Tor growth signaling pathway. Activation of Tor in this pathway leads to inactivation of 4EBP and activation of S6Kinase, together leading to increased protein synthesis essential for cell growth. We hypothesize that in mtACP1 knock down cells activation of JNK pathway might lead to activation of 4EBP in a Foxo dependent manner. 4EBP being a negative regulator of protein synthesis [47], when activated by JNK might lead to reduced cell growth. We are currently involved in generating appropriate fly stocks for molecular genetic dissection of this pathway.

5. Conclusion and Future Directions:

In the current study, we report that mitochondrial function is activator as well as inhibitor of cell growth. Particularly, suppression of cell growth by mitochondrial function, here in mtACP1 knock down cells leads to activation of JNK pathway. It is assumed that activation of the JNK pathway is mediated by accumulation of superoxide and peroxide ions. Although it has been demonstrated that knocking down mtACP1 leads to increased levels of ROS, it needs to be established that the growth retardation demonstrated by these cells is due to high levels of ROS. For that purpose we need to find out whether scavenging the extra ROS from these cells can prevent the reduction in size of these cells. This can be achieved by ectopic expression of genes such as *superoxide dismutase* or *catalase* in mtACP1 knocked down cells and analyse the cell size of these cells. Parallel

experiments are to be done to find that indeed the levels of ROS get reduced upon activation of *superoxide dismutase* and *catalase* genes in mtACP1 knock down cells. In a similar manner the connection between higher levels of ROS and activation of the JNK pathway needs to be established. In that direction it is essential to identify the sensor molecules of the JNK signaling pathway which respond to elevated levels of ROS. Importantly the downstream targets of the JNK pathway need to be identified to unravel the mechanistic basis of growth regulation by activated JNK pathway. In this direction we need to perform epigenetic analysis to identify the candidates downstream of the JNK pathway that get triggered in cells with attenuated mitochondrial function due to mtACP1 knock down.

Given the high degree of conservation between *Drosophila* and the vertebrates, both at the level of transcription factors and signaling pathways associated with regulation of cell growth, understanding this mechanism in flies would help us understand better the role of mitochondrial function in modulating cell growth in higher vertebrates. Since many of the key players linked to the TSC-Tor signaling pathway are either tumor suppressor genes or have oncogenic versions, understanding how mitochondrial function modulate the activity of the growth regulatory pathway, will have long term implications in designing strategies to limit the growth of transformed cells. Likewise the outcome of this study holds the potential to regulate the growth of self-renewing embryonic stem cells by modulating mitochondrial function and thereby help maintaining pluripotency.

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