Functional analysis of mitochondrial metabolism in *Drosophila* oogenesis and embryogenesis

Exploring the role of mitochondrial metabolism proteins in development of ovary and embryo of *Drosophila melanogaster*

A thesis report submitted towards the partial fulfillment of

BS-MS Dual degree program

By

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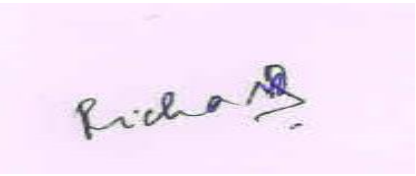
Certificate

This is to certify that this dissertation entitled **"Functional analysis of mitochondrial metabolism in *Drosophila* oogenesis and embryogenesis"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by Abhijeet Petkar at Indian Institute of Science Education and Research,Pune under the supervision of Dr.Richa Rikhy, Assistant Professor, Biology Department, during the academic year 2015-2016.



Signature of the Supervisor **Date: 28th March, 2016**

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Declaration

I hereby declare that the matter embodied in the report entitled **"Functional analysis of mitochondrial metabolism in *Drosophila* oogenesis and embryogenesis"** are the results of the investigations carried out by me at the Department of biology, Indian Institute of Science Education and Research, Pune under the supervision of Dr. Richa Rikhy and the same has not been submitted elsewhere for any other degree.

Signature of the Student **Date: 28th March, 2016**

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Abstract

Mitochondria synthesizes ATP which is the major source of cellular energy during oxidative phosphorylation in electron transport chain present in inner membrane of mitochondria. Previous studies showed the importance of mitochondrial dynamics and its function during oogenesis in *Drosophila melanogaster.* In this thesis I have attempted to examine the role of mitochondrial metabolism during embryogenesis and oogenesis. Inhibition of genes involved in mitochondrial metabolism was done using RNAi mediated knockdown with Nanos Gal4 expressed during oogenesis and embryogenesis and c306 Gal4 in follicle cells. RNAi mediated knockdown of subunits of components of complex 1, 3 and 5 of mitochondrial electron transport chain caused lethality in embryos. RNAi mutants of key proteins in these complexes resulted in increased pAMPK caused by a decrease in ATP levels. This caused disruption in actomyosin ring shape during cellularisation and mitochondrial transport across the apicobasal axis in the embryo. Future studies on mitochondrial shape and function change in these mutants along with analysis of the developmental pathways will yield an analysis of steps of *Drosophila* embryogenesis that will depend on mitochondrial metabolism.

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1. Introduction

1.1 Mitochondrion is a subcellular organelle in a eukaryotic cell which functions in ATP synthesis

Mitochondrion is a membrane enclosed structure found in most eukaryotic cells. It is a double membrane organelle. It is known as the power house of the cell because of its abilities to generate ATPs (Adenosine tri-phosphates) or cellular energy. There are four different compartments in a mitochondrion -1.Outer Membrane 2. Inner membrane space 3. Inner membrane 4. Matrix. Mitochondria have their own DNA and ribosomes and can synthesize their own proteins. Their genome is quite similar to bacterial genomes (Lynn Margulis et al., 1970). In human, 16.6 kb mitochondrial genome encodes 37 proteins which includes 13 proteins involved in respiratory chain, 22 tRNAs and 2 rRNAs which are important for translation of mitochondrial DNA genes. (Phillipa J. Carling et al., 2011). In *Drosophila melanogaster*,8.3kb fragment of mitochondrial DNA encodes seven proteins, 12 tRNAs and 3' ends of CO III genes and 16S rRNA ((Garesse, 1988). Mitochondria are also involved in apoptosis, cell signaling, growth and differentiation (Mitra et al., 2012). Mitochondria are dynamic organelles .They undergo change in morphology in a cell by fusion and fission of their outer and inner membranes.

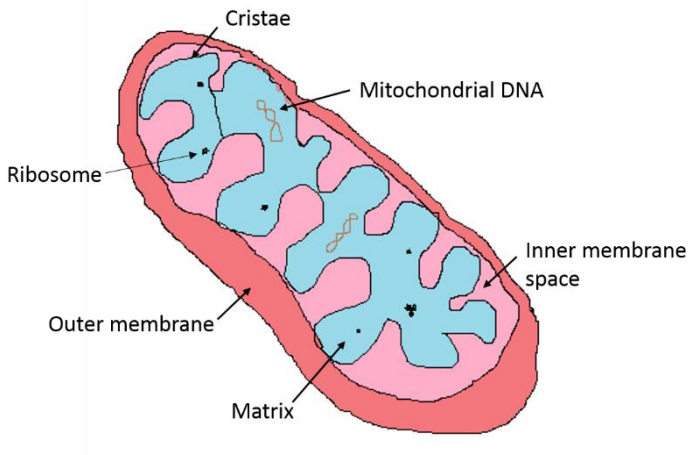


Fig 1.1: A schematic representation of the mitochondrion. It consists of four compartments, 1. Outer membrane, 2. Inner membrane space, 3. Cristae (folding of inner membrane) and 4. Matrix. It also contains its own DNA.

1.2 Electron transport chain is the main driver for the production of energy in mitochondria.

The Electron Transport Chain which produces ATP (chemical energy needed for cell) is present in inner membrane of mitochondria. ETC consists of five different complexes (Fig 2):

1. Complex I : NADH dehydrogenase
2. Complex II : Succinate dehydrogenase
3. Complex III : Cytochrome bc1
4. Complex IV : Cytochrome c oxidase
5. Complex V : F1F0-ATP synthase

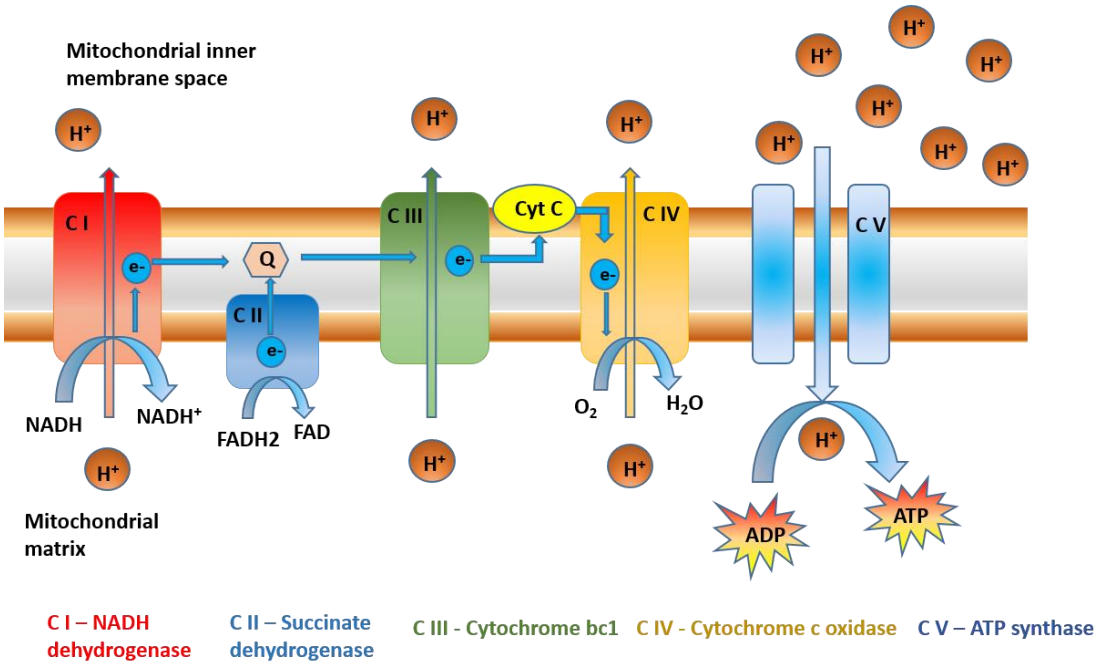


Fig 1.2: A schematic representation of the electron transport chain in the inner membrane of mitochondria. The electrons from donors like NADH and FADH2 are transferred within these complexes and proton gradient is generated at inner membrane space which is used by ATP synthase to produce ATP.

The electrons are transferred within these complexes with the help of membrane embedded ubiquinone and soluble cytochrome c both which are mobile carrier (Leonid A. Sazanov et al., 2015). These electrons are transferred from donor molecules such as NADH and succinate which are by-products of Krebs's cycle to these complexes. During the transfer of electrons these complexes produce proton gradient across the

inner membrane of mitochondria. ATP synthase uses this proton gradient to produce ATP (*Karp, Gerald et al., 2008).*

1.3 Electron transport chain complexes are made up of several subunits which help in electron transport

NADH dehydrogenase (complex I) is the largest complex of ETC which catalyze the first step of oxidative phosphorylation in mitochondria. It oxidizes NADH and transfers two electrons via Flavin mononucleotide and iron-sulfur centers to ubiquinone and produces proton gradient across the inner membrane of mitochondria. The mammalian complex I enzyme consists of ~45 polypeptides (subunits). It is in L shaped structure with one arm fixed in the inner membrane and another one perpendicular and directed inside the mitochondrial matrix (*J. Mark Skehel et al., 2006).*

Succinate dehydrogenase, complex II of electron transport chain performs dual role in mitochondria. It oxidizes succinate to fumarate in Kreb's cycle and in oxidative phosphorylation it transfers electrons from succinate to ubiquinone. It is made up of four nuclear encoded subunits: SDHA, SDHB, SDHC, SDHD (Katarina Kluckova et al., 2012).

Cytochrome bc1 is the complex III of respiratory chain of mitochondria. It transfers electrons from ubihydroquinone to cytochrome c and produce proton gradient across the inner mitochondrial membrane. The mammalian cytochrome bc1 complex is made up of 11 subunits, with 3 common subunits (cytochrome b, cytochrome c1 and Rieske [2Fe-2S] protein), 2 core and 6 low molecular weight proteins (So Iwata et al.,1998).

Cytochrome c oxidase is the complex IV of electron transport chain in mitochondria. It is the terminal component of the ETC chain which receives four electrons from four cytochrome c molecule and reduces molecular oxygen present in the mitochondrial matrix to two molecules of water. During this process, it translocates four H^+ ions into the inner-membrane space and generate proton gradient. This proton gradient is then used by ATP synthase to produce ATP. It consists of 13 structural subunits which require accessory factors to assemble them into fully functional enzyme. It has been observed that mutations in these assembly factors which eventually lead to COX

deficiency is one of the most frequent causes of mitochondrial respiratory chain defects in human (Leticia Martínez-Morentin et al., 2015).

F1F0 ATP synthase is the important enzyme which synthesize ATP from ADP and inorganic phosphate,Pi using proton gradient across the inner membrane of mitochondria. It is comprised of two sub complexes, F1 and Fo. F1 subcomplex is large and present inside the matrix. It is made up of 3 copies of each alpha and beta subunits and one copy of each gamma, delta and epsilon subunits. The Fo subcomplex is proton pore embedded in the inner membrane, consists of one a, two b and 10 c subunits. The main characteristic of this complex is the rotary movement of assembly of different subunits which transport protons across it and produce ATP (adenosine tri-phosphate) (Robert K. Nakamoto et al., 2008).

1.4 Defects in the mitochondrial metabolism and respiratory chain complex proteins lead to several clinical conditions

It has been found that deficiency in mitochondrial metabolism proteins and the defects in respiratory chain complexes lead to different clinical conditions. Defects in oxidative phosphorylation system of mitochondria produce high level of superoxide (ROS) which eventually affects the other cell functions and leads to cellular aging. It has been found that congenital deficiency of Complex II (SDH) subunits (SDHA, SDHB and SDHAF1) of electron transport chain of mitochondria lead to different childhood disease like Leigh syndrome, cardiomyopathy and infantile leukodystrophies (Attje S. Hoekstra, Jean-Pierre Bayley et al.,2013). It has been observed that mutations in mitofusins (Mfn2) lead to Charcot-MarieTooth subtype 2A (CMT2A), a group of disorders which affect the function of peripheral nerves which eventually lead to muscle weakness and sensory loss in distal limbs (David C. Chan et al., 2006). It has been well studied that autosomal dominant optic atrophy (DOA) is caused by mutations in gene OPA1 which mitochondrial dynamin related protein. The condition DOA features loss of retinal ganglion cells which lead to loss of sharp vision in patients (David C. Chan et al., 2006).

1.5 Mitochondrial function in embryonic stem cells (ESCs).

Embryonic stem cells exhibit two features, one is their self-renewal and other is capacity to generate different types of cells through differentiation(Wanet et al., 2015). It has been studied that proper mitochondrial function is required for differentiation and proliferation of embryonic stem cells(Mandal et al., 2015). During self-renewing state, ESCs contain fewer mitochondria with poor cristae developed and limited oxidative capacity. After differentiation of these ESCs, mitochondria increase in number and develop more cristae and attain tubular structure. In order to find out the importance of mitochondrial function in ESCs, mouse and human ESCs were treated with CCCP (carbonyl cyanide m-chloro phenyl hydrazine), a drug which disrupts the oxidative phosphorylation. These experiments found that disrupting mitochondrial function resulted in reduction of proliferation rate of ESCs without compromising their ability of pluripotency (Mandal et al., 2015). During undifferentiated state of embryonic stem cells, mitochondrial oxidative phosphorylation capacity is restricted with low level of ATP production. These undifferentiated stem cells are mostly dependent on glycolytic pathways. Upon differentiation of these stem cells, they require lot of energy to perform the specific function. So, upon differentiation of these embryonic stem cells, cells become mostly oxidative phosphorylation dependent with reduction in glycolytic pathways (Hu et al., 2016). It has been studied that oxidative phosphorylation during differentiation of embryonic stem cells is required for regulation of transcriptional activation of essential genes necessary for early embryonic differentiation (Hu et al., 2016).

1.6 Mitochondrial ATP synthase complex is required specifically for germ cell differentiation in *Drosophila melanogaster* ovary

The differentiation of stem cells during animal development is a key process which involves changes in cellular properties and give rise to new set of cells with different identity and function. In *Drosophila melanogaster,* germ cells reside at anterior tip of ovary near somatic niche. After division of germline cell, one daughter cell reside close to somatic niche maintaining its stem cell identity and other called cyst undergoes differentiation and 4 rounds of cell division to form 15 nurse cells and an oocyte. It has been studied that mitochondrial ATP synthase is crucial for this differentiation to occur independent of its role in oxidative phosphorylation (Fig.1.3). During the differentiation,

it promotes the maturation of mitochondrial cristae through dimerization and specific upregulation of itself (Teixeira et al., 2015)

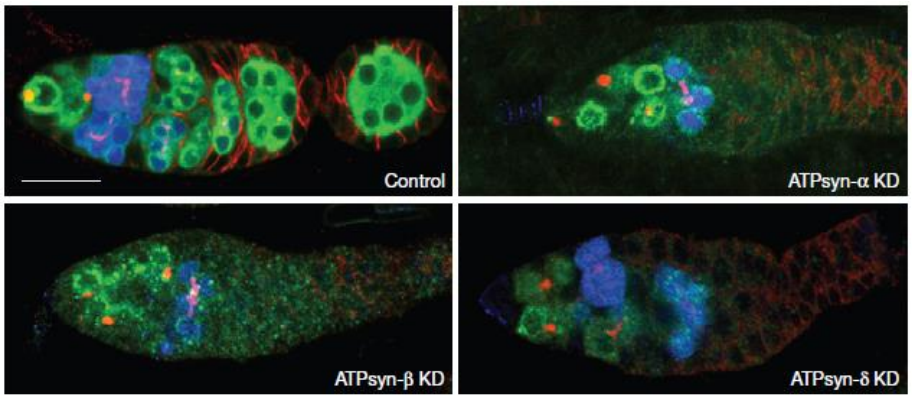


Fig 1.3: ATP synthase plays an essential role in differentiation of germ cells. Here it shows control and ATP synthase knockdown germaria which express the reporter of differentiation, bag of marble-GFP (bamP-GFP) immunostained with anti-vasa (green) which labels germ cells. Anti-GFP labeled in blue detects bamP-GFP and anti-1B1 (red) labels spectrosomes, fusosomes and somatic cells(Teixeira et al., 2015).

Previous studies have shown the important role of complexes of electron transport chain during germ cell differentiation of *Drosophila*(Teixeira et al., 2015). It has also been studied that defects in mitochondrial function lead to several neurological disorders(Chan, 2006). It has been studied that mitochondria are required for differentiation and proliferation embryonic stem cells(Mandal et al., 2015). Here we are trying to ask what is role of proteins involved in mitochondrial metabolism during oogenesis and embryogenesis in *Drosophila melanogaster*. In particular we have studied the impact of electron transport chain mutants on actin contractile ring morphology in cellularization. Cellularisation is process which give rise to epithelial cells of embryo during early development. These cells the undergo differentiation to form different structures in fly. It is very much important to understand the role of oxidative phosphorylation during these process which will decode the complete process of differentiation of cells from early undifferentiated cells. Here we are using oogenesis and embryogenesis of *Drosophila melanogaster* as model system to study the role of mitochondrial metabolism proteins in the differentiation of the cells.

1.7 *Drosophila* oogenesis is a model system to study role of mitochondrial metabolism during follicle cell differentiation

Drosophila has 2 ovaries. Each ovary has approximately 16 ovarioles. The anterior portion of each ovariole is called germarium. The germarium has a germ cell (stem cell) that undergoes asymmetric cell division to produce one daughter cell and one cyst. The cyst undergoes 4 mitotic divisions to produce 16 cells of which one becomes oocyte and remaining 15 become nurse cells (Fig 1.4). Nurse cells provide nutrition and proteins to developing oocyte. The ovarian cells give rise to follicle cells. These surround the oocyte. These follicle cells progress through different developmental stages. During stage 1-5 (S 1-5) most of these follicle cells undergo mitotic divisions. Some cells exit the mitotic cycle under the influence of Notch activation to form stalk cells separating consecutive egg chambers. During stage 6-8 (S 6-8) all follicle cells exit the mitotic cycle and endocycle. Follicle cell epithelium gets patterned into posterior follicle cells (PFC), Main body cells (MBC), and anterior follicle cells (AFC). During stage 9 majority of follicle cells migrate over the surface of egg chamber. Stage 10 follicle cells secret vitelline membrane around the oocyte. (Li He, Xiaobo Wang et al., 2011). It has been shown that changing mitochondrial morphology affects follicle cell differentiation in association with EGFR pathway (Mitra et al., 2012). Since, there is an interconnection between mitochondrial morphology and energetics, it is important to study role of electron transport chain during these stages.

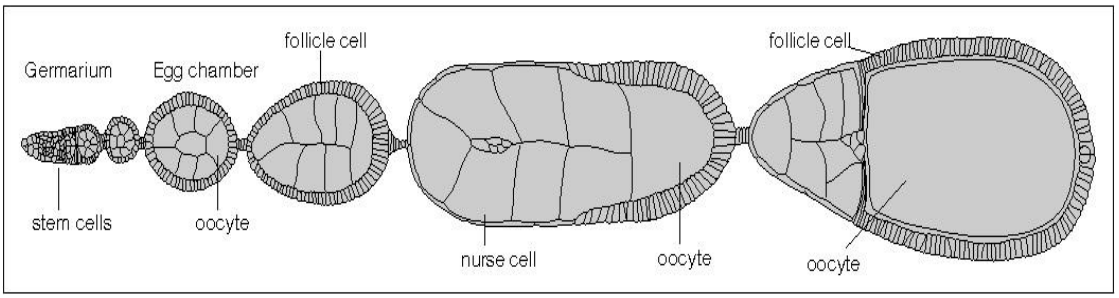


Fig 1.4: A schematic representation of *Drosophila* oogenesis (Adapted from: **http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_02/DBNDros.html)**

1.8 *Drosophila* embryogenesis as a model system to study the role mitochondrial metabolism in cellular processes

During *Drosophila* embryogenesis nuclei start dividing in a common cytoplasm (Fig 1.5). After 9 divisions, nuclei migrate towards the periphery of the egg and are partially enclosed with plasma membrane forming pseudo epithelial cells. This stage is called syncytial blastoderm. Some of the nuclei migrate towards the posterior end of the embryo to form pole cells or germ cells. After 13 mitotic divisions (around 3 hours after fertilization) plasma membranes ingress into the cytoplasm to form complete cells. This stage is referred to as cellularization which is followed by gastrulation. It has been shown that mitochondrial localization and morphology is important for embryonic survival. Hence, it is also important to understand underlying mechanisms.

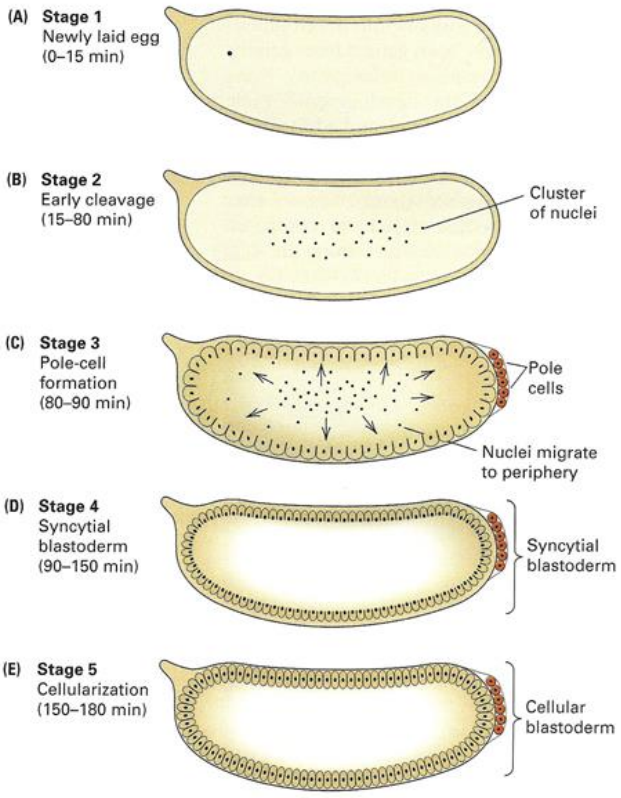


Fig 1.5: A schematic representation of *Drosophila* embryogenesis (Adapted from: **http://www.discoveryandinnovation.com/BIOL202/notes/lecture21.html)**

1.9 Mitochondria as an important factor for the proper development of human oocyte and embryo

Mitochondria are maternally inherited organelles which develop from very few mitochondrial population and are amplified during oogenesis. After having enough number of mitochondria in fully grown oocyte, they don't increase in number during early development. Mitochondria are distributed in the embryo which is thought to play roles in long term viability of blastomere and also patterning and axes defining in embryos (Rémi Dumollard et al., 2007). Proper balance of ATP supply and demand is very much essential for proper fertilization oocyte and development of embryo. It has been studied that the imbalance in these ATP supply/demand could be a factor which lead to several developmental defects such as 1. Chromosomal segregation disorders, 2. Maturation and fertilization failure, 3. Arrested cell division and 4. Abnormal cytokinesis and fragmentation. It has been proposed that high levels of fragmentation leads to destructive elimination of blastomeres during early development. It has been studied in mouse that small increase in mitochondrial superoxides (ROS) lead to follicular hyperstimulation and are related to number and spacing of repeated cycles. (Jonathan Van Blerkom et al., 2010). Embryonic development involves different process like patterning of tissues and organs, cell fate decisions and also morphogenesis. These processes involve several signalling pathways. It has been studied that calcium is universal messenger involved during gastrulation and play important role in these signalling pathways (Webb and Miller et al., 2003).

To study role of mitochondrial energetics components in these developmental stages of *Drosophila melanogaster* we are using knockdown strategies using RNAi driven using Gal4s specific to these stages. We are analysing mitochondrial localization, morphology and cell shape in the knockdown embryos and ovaries.

1.10 Aims of the project

1.10.1. To do a lethality screen using RNAi against mitochondrial metabolism genes in *Drosophila* oogenesis and embryogenesis.

1.10.2. To study the effect of RNAi mediated knockdown of mitochondrial metabolism genes on contractile ring formation, mitochondrial distribution and morphology.

2. Materials and Methods

2.1 *Drosophila melanogaster* stocks and crosses :

Gal4 lines used:

1. Nanos Gal4 – expresses at germ cells and at embryos
2. C306 Gal4- express at interfollicle cells and border follicle cells

Food used for rearing flies

3% yeast (15gm) medium containing cornmeal (75gm), malt (30gm), sugar (80gm) and agar (10gm).

For the below mentioned RNAi crosses, some of them were maintained at 29^0 Celsius and some of them were maintained at 25^0 Celsius. The detailed information of crosses is provided in the lethality table in the results section. The list of fly lines used in the project is given in the table 2.1.

Sr. No.	Stock no.	RNAi against genes	Genotype
1	51855	NADH dehydrogenase 24kDa subunit	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03429}attP40
2	52922	NADH dehydrogenase 39 kDa subunit	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03662}attP40
3	52939	NADH dehydrogenase 51 kDa subunit	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ21591}attP40
4	50577	NADH dehydrogenase 19 kDa subunit	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLC01699}attP2
5	51807	Succinyl coenzyme A synthetase α subunit	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03366}attP40
6	51357	Cytochrome b-c1 complex subunit 8	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03242}attP2
7	42948	COX15/CtaA family(Heme A synthase)	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02641}attP40
8	28059	ATPsynthase alpha domain	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02896}attP2
9	28056	ATPsynthase beta subunit 1	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02892}attP2
10	27712	ATPsynthase beta subunit 2	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02792}attP2
11	51714	ATP Synthase Coupling factor 6-subunit F6	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03238}attP2/TM3, Sb[1] Ser[1]
12	28062	ATP synthase beta subunit 3	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02899}attP2
13	50543	ATP synthase gamma subunit	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLC01662}attP2
14	36871	Superoxide dismutase 2 (Mn)	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL01015}attP40
15	38906	NADPH oxidase	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00677}attP40/CyO
16	26744	Mitochondrial transcription factor	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02307}attP2
17	42842	Glutathione S transferase	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02534}attP40
18	34609	Calmodulin	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01318}attP2
19	33634	Glycogen metabolism	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00032}attP2
20	40849	Mitochondrial calcium regulation	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02016}attP40

Table 2.1: The list of fly lines used in this project.

2.1.1 UAS-Gal4 system is a genetic method to study targeted gene expression in *Drosophila*

Gal4 is a yeast transcription activator which has no effect on other organisms like *Drosophila*, human cells. It binds to UAS (upstream activating sequence) and activates transcription of target genes or RNAi. Nanos which express at germ cells (maternally) and at embryos is used as promoter for Gal4 to express targeted RNAi sequences during embryogenesis. C306 (follicle cell gal4) which expresses at border follicle cells and inter follicle cells is used as promoter for Gal4 to express targeted RNAi sequences during oogenesis. The F1 generation female flies and males flies containing both targeted RNAi sequence and specific gal4 were collected in a cage. After 3-3.5 hrs of feeding the flies with food containing agar (2.5 %) and sugar (3 %) and a small drop of yeast paste over the plate, the embryos of F1 flies were collected and used for the further experiments and analysis.

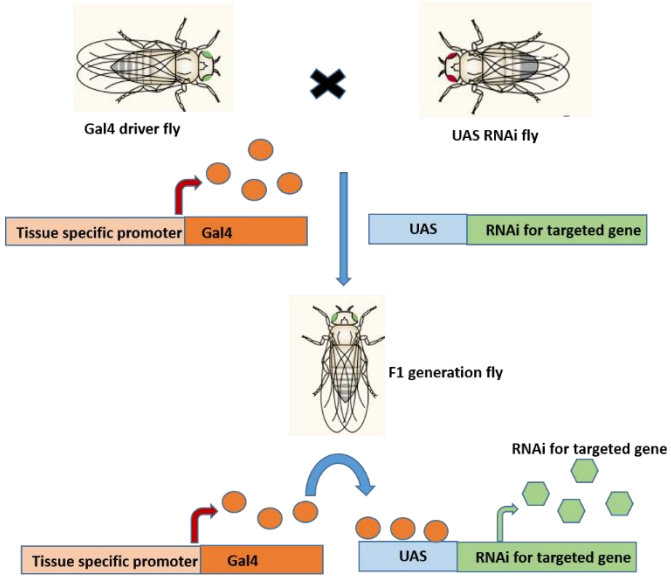


Fig 2.1: The UAS-Gal4 system for tissue specific expression of particular protein in *Drosophila melanogaster.*

2.2 Immunostaining of the embryos :

Embryos were collected after 3-3.5 hours at 25°C for targeted RNAi and 29°C for other RNAi, dechorionated them in 100% bleach for 1 minute and then fixed with 4% formaldehyde in PBS and equal volume of heptane for 20 minutes. Then the embryos were either methanol devitelinized (with equal volume of methanol and heptane) or hand devitelinized (in 1X PBST (0.3% Triton-X100)) depending on the antibody used for staining. Then the embryos were washed thrice with 1X PBST (0.3% Triton-X100) for 5 minutes each and then 2% BSA in 1X PBST (0.3% Triton-X100) was used for 1 hour blocking. Primary antibody was added and kept for overnight incubation at 4°C. After the incubation primary antibody was removed, embryos were washed thrice with 1X PBST (0.3% Triton-X100) and fluorescently labelled secondary antibodies were added in 1X PBST (0.3% Triton-X100) as per their dilution and kept it for 45 minutes on rotor. Embryos were then washed thrice with 1X PBST (0.3% Triton-X100) and Hoescht was added in second wash. After the last wash embryos were mounted on slides with slow fade Gold antifade reagent from Invitrogen. Antibodies used in this project are listed in the table 2.2 given below.

Primary antibodies	**Dilution**	**Company/Lab**
pAMPK-Rb	1:200	Cell signalling
Secondary antibodies		
Alexa 488	1:1000	Invitrogen
Dyes		
Phalloidin 488/568	1:400	Life technologies
Streptavidin 488/568	1:1000	Invitrogen
Hoechst	1:1000	Invitrogen
CMXROS	1:10000	Invitrogen

Table 2.2: The list of antibodies and dyes used in this project.

2.3 Immunostaining of the ovaries :

After the dissection of almond shaped ovaries from 10 days old adult female flies, the extra fats and tissue surrounding the ovaries were removed using forceps and small needle and then they were stored in Schneider's medium. The ovaries were fixed with 4% formaldehyde for 15 minutes followed by washing them thrice with 1X PBST (0.3% Triton-X100) for 5 minutes each. Then 2% BSA in 1X PBST (0.3% Triton-X100) was used for 30 minutes of blocking. Primary antibody was added and kept for overnight incubation at 4°C. After the incubation primary antibody was removed, ovaries were washed thrice with 1X PBST (0.3% Triton-X100) and fluorescently labelled secondary antibodies were added in 1X PBST (0.3% Triton-X100) as per their dilution and kept it for 45 minutes on rotor. Ovaries were then washed thrice with 1X PBST (0.3% Triton-X100) and Hoescht was added in second wash. After the last wash ovaries were mounted on slides and separated using needles so that individual developmental stages can be seen and then put coverslip with slow fade Gold antifade reagent from Invitrogen on it.

2.4 Microscopy

2.4.1 Imaging of fixed samples

The confocal images of fixed sample of embryos were taken using an LSM-710 or 780 inverted microscope (Carl Zeiss, Inc. and IISER Pune microscopy facility) with excitation at 358 nm, 488 nm and 568 nm and emission collection with PMT filters at room temperature (23°C). A Plan Apochromat 40x/ 1.3 NA (for LSM 710) and 1.4 NA (for LSM 780) oil objective was used and pinhole of 90.03, averaging of 2, acquisition speed of 11 and zoom of 3 was set for imaging with the help of Zen software.

2.4.2 Live Imaging of embryos

Embryos were collected after 1–1.5 hours after feeding the flies in a cage on yeast coated agar plate in a sieve at 25°C. Then they were dechorionated in 100% bleach for 1 minute. After washing with distilled water for some time, they were arranged dorso-or ventro-laterally on coverslip chambers (LabTek). Embryos were then covered with 2 ml of 1X PBS and the live imaging was done with above mentioned

microscopes with averaging of 2, scan speed of 11 and zoom of 3. Z-stacks were taken from the apical surface of the embryos touching the coverslip to approximately 30-32 slices inside the embryo (length of each Z-stack was 1.08µm).

2.5 Image analysis

Open source software such as ImageJ and Zenlite were used for image compilation and analysis. Graph Pad Prism software was used for plotting the graph and doing statistical analysis. Two-tailed unpaired student's T-test was used to check the significance of the results.

2.5.1 Calculating the circularity of actin contractile ring in the embryos

In order to find the circularity of actin contractile ring in the embryos, z stacks where actin contractile ring can been seen were superimposed with maximum intensity (Z projection) and a single image was obtained. Using polygonal tool in imageJ, the boundary of each actin contractile ring was measured manually. Using ImageJ the dimensions of ring like area, perimeter were measured. And the circularity was calculated using the formula.

$$\text{circularity, } C = \frac{4\pi \text{ area}}{\text{perimeter}^2}$$

The analysis was done for at least 10 cells per embryo and the graph of circularity for different embryos in experiments was plotted in Graph Pad. Two-tailed unpaired student's T-test was used to check the significance of the results. Significant values such as $p<0.05$, $p<0.01$, $p<0.0001$ were put up in the graph using the labels such as *, **, *** respectively.

2.5.2 Measuring the apical-basal mitochondrial intensity in the embryo

For measuring the apical-basal mitochondrial intensity in the embryos, sagittal section of embryo was chosen. The circular ROIs were drawn at apical region (above the nucleus) and basal region (below the nucleus) of the cells in sagittal section of embryo to measure the mitochondrial intensity. Apical and basal mitochondrial intensity in each cell was measured using Image J software with formula (Maximum intensity-minimum intensity). The mitochondrial intensity was measured for 5 cells per embryo.

The ratio of apical to basal mitochondrial intensity was calculated.The graph of apical to basal mitochondrial intensity ratio was plotted using GraphPad. Two-tailed unpaired student's T-test was used to check the significance of the results. Significant values such as $p<0.05$, $p<0.01$, $p<0.0001$ were put up in the graph using the labels such as *, **, *** respectively.

2.6 Drug treatment of embryos to study mitochondrial distribution

Embryos were collected for 3 hrs at 25^0 Celsius and then washed with distilled water. They were dechorionated with 100% bleach for 1 minute and then washed again. After that embryos were added in a 1:1 mixture of limonine and heptane and drug (Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) with dilution (1:1000) was added to it and incubated for 15 minutes. After the incubation, the mixture was removed and 4% formaldehyde and heptane (equal volume) was added and kept for incubation for 20 minutes. After the fixation,embryos were either methanol devitelinized (with equal volume of methanol and heptane) or hand devitelinized (in 1X PBST (0.3% Triton-X100)) depending on the antibody used for staining. Then the embryos were washed thrice with 1X PBST (0.3% Triton-X100) for 5 minutes each and then 2% BSA in 1X PBST (0.3% Triton-X100) was used for 1 hour blocking. Primary antibody was added and kept for overnight incubation at 4°C. After the incubation primary antibody was removed, embryos were washed thrice with 1X PBST (0.3% Triton-X100) and fluorescently labelled secondary antibodies were added in 1X PBST (0.3% Triton-X100) as per their dilution and kept it for 45 minutes on rotor. Embryos were then washed thrice with 1X PBST (0.3% Triton-X100) and Hoescht was added in second wash. After the last wash embryos were mounted on slides with slow fade Gold antifade reagent from Invitrogen.

2.7 Calculating the embryonic lethality

Embryo were collected in sieve for 3-3.5 hours and washed with distilled water. Then they were arranged on an agar plate in a matrix fashion (10X10). Then they were kept for incubation for 2 days at 25^0 Celsius or 29^0 Celsius depending upon the experiment. The lethal embryos which were not hatched were counted after 24 hours and 48 hours.

3. Results and Discussions:

3.1 RNAi mediated knockdown of subunits of complex I, III, IV and V of ETC of mitochondria using Nanos Gal4 caused embryonic lethality in *Drosophila melanogaster*.

In order to study the role of different mitochondrial metabolism proteins in *Drosophila* embryogenesis, the target genes were chosen from a preliminary RNAi screen done collectively by the lab to screen for phenotypes in wings and nervous system of *Drosophila*. These genes encode proteins for ETC: Complex 1, Complex 2, Complex 3, Complex 4, Complex 5 (Table 3.1.1) and for proteins involved in Glucose uptake, calcium homeostasis and in other mitochondrial metabolism processes (Table 3.2.1). Here, the screening was done at 25^0 C and 29^0 C in order to knockdown the genes which encode the different subunits of ETC complexes in *Drosophila* embryo using RNAi technique. The RNAi for specific genes was expressed using NanosGal4 during *Drosophila* oogenesis and embryogenesis. The experiment was carried out at two different temperature in order to modulate the expression of the Gal4 and therefore the RNAi levels. The embryos from the mutants were collected and arranged in matrix fashion on agar plates. The percentage of lethal embryos (those which didn't hatch) after 24 hrs and 48 hrs was obtained. It has been found that inhibition of the expression of these genes caused lethality in the embryos (Table 3.1.1 and 3.2.1). RNAi mediated knockdown of subunits of complex I caused lethality in the embryos. Complex I subunits help it in transporting electrons to ubiquinone. Disruption in this process lead to leakage of electrons which produce reactive oxygen species (ROS) which is detrimental to different cellular processes in the cell. These subunits are part of core assembly of complex I. Depletion of these subunits could have disassembled the structure of complex I which in turn could have disrupted the process of oxidative phosphorylation. Succinyl Co A synthetase, an enzyme which catalyse reversible reaction of succinyl co A to succinate. Complex II gets electrons from succinate. Inhibition of this subunit caused low lethality in the embryos. It is possible that RNAi titer in order to knockdown it was low or the subunit is long lived and hence difficult to be eliminated. Complex III is important in generating proton gradient and it transfers the electrons to cytochrome c which then delivers them to complex IV. Depletion of subunit 8 of complex III caused high embryonic lethality as compared to control. This subunit is one of the core assembly proteins of complex III. Its depletion could have

disrupted the structure of complex III and in turn could have hampered the process of oxidative phosphorylation. Complex IV is important in oxidative phosphorylation. It reduces a molecule of oxygen and produce two molecule of water. During this process, it also generates proton gradient across the inner membrane. Complex IV subunit mutant flies didn't lay embryos at 29 ^{0}C which indicates that this particular subunit is important in early development of egg (Table 3.1.1). Complex V ATP synthase is made up of F0 and F1 sub-complexes. Rotary action of these sub-complexes utilise the proton gradient across the membrane to produce ATP for different cellular processes. These sub-complexes are made up of several subunits. Here it has been found that RNAi mediated knockdown of different subunits of ATP synthase caused lethality in the embryos when the experiment was carried out at 29 ^{0}C (Table 3.1.1). These subunits (ATP synthase alpha domain, beta subunit 1, 2 and 3) form the catalytic core of F1 sub-complex of ATP synthase which produces ATP by phosphorylating ADP. ATP synthase gamma subunit is the central stalk that joins F0 sub-complex to F1 sub-complex. And ATP synthase coupling factor 6 F6 subunit form the peripheral stalk that attaches F0 and F1 sub-complexes together. It is possible that depletion of these subunits could have disrupted the core assembly and function of ATP synthase and in turn hampered ATP production. RNAi lines against succinyl CoA synthetase alpha subunit (51807), blw-ATP synthase alpha domain (28059), ATP synthase beta subunit (28056), ATP synthase beta subunit (27712) and NADH dehydrogenase 24kDa subunit (51855) have showed low lethality because the RNAi titer may be low. It is also possible that these subunits are long lived and hence difficult to be eliminated. During development of embryo different cellular metabolism processes are dependent on oxidative phosphorylation. Depletion of these subunits might have affected these process. Therefore, the results obtained for embryonic lethality after depletion of different subunits of ETC complex I, III, IV and V suggest that proper oxidative phosphorylation is necessary for early embryo development.

Sr.No.	RNAi used against	Lethality (24 Hrs)	Lethality (48 Hrs)	n
	Wild type	4.90%	3.80%	261
Complex I(NADH dehydrogenase) subunits				
AT 25 ^{0}C				
1	NADH dehydrogenase24kDa subunit	4.34%	4.34%	115
2	NADH dehydrogenase 39 kDa subunit	9.66%	8.00%	300
AT 29 ^{0}C				
3	NADH dehydrogenase51 kDa subunit	49.50%	40.18%	107
4	NADH dehydrogenase 24kDa subunit	88.88%	77.77%	9
5	NADH dehydrogenase 19 kDa subunit	No embryos laid		
6	NADH dehydrogenase 39 kDa subunit	No embryos laid		
Complex II(Succinate dehydrogenase) subunit				
AT 25 ^{0}C				
1	Succinyl coenzyme A synthetase α subunit	5.00%	5.00%	200
Complex III (cytochrome bc1) subunit				
AT 25 ^{0}C				
1	Cytochrome bc1 complex subunit 8	59.25%	55.45%	54
Complex IV(Cytochrome c oxidase)- subunits				
AT 25 ^{0}C				
1	COX15/CtaA family(Heme A synthase)	11.11%	9.63%	135
AT 29 ^{0}C				
2	COX15/CtaA family ((Heme A synthase)	No embryos laid		
Complex V(ATP synthase) - subunits				
AT 25 ^{0}C				
1	ATPsynthase alpha domain	3.00%	1.33%	300
2	ATPsynthase beta subunit 1	4.00%	2.00%	300
3	ATPsynthase beta subunit 2	6.00%	4.33%	300
4	ATP Synthase Coupling factor 6-subunit F6	17.11%	15.58%	263
AT 29 ^{0}C				
5	ATP Synthase Coupling factor 6-subunit F6	13.46%	11.53%	52
6	ATP synthase beta subunit 3	13.66%	13.66%	300
7	ATP synthase gamma subunit	No embryos laid		

Table 3.1.1. **Percentage lethality of embryos obtained from RNAi mediated knockdown of electron transport chain complexes.** Screening was done at 25 ^{0}C and 29 ^{0}C in order to check lethality of embryos of mutants of different subunits of complex I, II, III, IV and V of ETC of mitochondria.

3.2 RNAi mediated knockdown of different mitochondrial associated proteins involved in glycogen metabolism, mitochondrial calcium regulation, calcium signal transduction pathway using Nanos gal4 caused embryonic lethality in *Drosophila* embryos

Apart from the role of synthesising energy for the cell, mitochondria play important role in processes like apoptosis, cell signaling, growth and differentiation(Mandal et al., 2015). Several metabolic processes like Kreb's cycle, oxidation of fatty acids and amino acids etc occur inside the mitochondria. In this screen, the genes which encode mitochondria metabolic proteins were inhibited using RNAi technique. The RNAi for specific genes was expressed using NanosGal4 in *Drosophila* embryo. Here, the screening was done at 29^0 C in order to modulate RNAi levels. The embryos from the mutants were collected and arranged in matrix fashion on agar plates. The percentage of lethal embryos (those which didn't hatch) after 24 hrs and 48 hrs was obtained. These genes were 1. Superoxide dismutase which controls the ROS level in the cell(Sedensky and G. Morgan, 2006), 2. NADPH oxidase which is an enzyme present on plasma membrane of neutrophils facing the extracellular space which generates oxygen radical from inside the cell which kills the bacteria and viruses (Vlahos et al., 2011) 3. Gene which encode protein important in glycogen metabolism. In humans, glycogen, a polysaccharide of glucose is stored in the cells of liver and muscles (Kreitzman SN et al., 1992), 4. Gene which is important in calcium regulation which regulates calcium homeostasis in the cell, 5. Gene encoding mitochondrial transcription factor, which help in replication of mitochondrial genome (fly base) 6. Calmodulin which is a calcium ion binding messenger protein in eukaryotic cells. It is part of calcium signal transduction pathway in the cell. 7. Glutathione S transferase, which is involved in glutathione metabolic process in the cell (fly base). It has been found that inhibition of NADPH oxidase in mice induces influenza A virus induced lung inflammation (Vlahos et al., 2011). Glycogen is a source of energy in cells. Inhibition of proteins involved in its metabolism will affect the energy level in cells that will eventually hamper several other processes in the cell. Here I depleted these genes using RNAi and found that their depletion caused lethality in the embryos (Table 3.2.1). These genes are necessary for proper cellular function. It is possible that depletion of these genes could have affected the cellular processes involved in early embryonic development and caused lethality.

Sr. No.	RNAi used against	Lethality (24 Hrs)	Lethality (48 Hrs)	n
1	Wild type	4.90%	3.80%	261
2	Superoxide dismutase 2 (Mn)	12.58%	10.88%	294
3	NADPH oxidase	15.33%	12.66%	300
4	Mitochondrial transcription factor	19.04%	19.04%	42
5	Glutathione S transferase	27.60%	24.39%	246
6	Calmodulin	49.13%	44.78%	230
7	Glycogen metabolism	52.50%	52.50%	217
8	Mitochondrial calcium regulation	65.50%	60.34%	232

Table 3.2.1. **Percentage lethality of embryos of obtained from RNAi mediated knockdown of different mitochondrial associated proteins.** Screening was done at 29 ^{0}C in order to check the lethality of embryos of mutants of different mitochondrial associated proteins.

3.3 RNAi mediated knockdown of subunits of different complexes of ETC of mitochondria using C306 Gal4 didn't produce F1 generation flies in *Drosophila melanogaster*.

In *Drosophila melanogaster*, oogenesis is an excellent model system to study the role of mitochondrial metabolism during follicle cell differentiation. It has been shown that changing mitochondrial morphology affects follicle cell differentiation in association with EGFR pathway (Mitra et al., 2012). Since, there is an interconnection between mitochondrial morphology and energetics, it is important to study role of ETC during these stages. As mentioned earlier, it has been observed that mitochondrial ATP synthase complex which functions in producing ATP is necessary for germ cell differentiation in *Drosophila melanogaster* ovary (Teixeira et al., 2015). Hence, the experiment of studying the role these mitochondrial ETC complex proteins was carried out. Here, the screening was carried out at 25^0 C and 29^0 C in order to modulate RNAi levels. The genes which encode subunits of different complexes of ETC of mitochondria were knockdown using RNAi technique. Here, C306Gal4 which expresses at border follicle cells and inter follicle cells was used as promoter for Gal4 to express targeted RNAi. It has been found that, depletion of these genes during oogenesis didn't produce progeny (F1 generation flies) when the experiment was carried out at 29^0 C. And when the same experiment was carried out 25^0 C, F1 generation flies were obtained from some of the crosses (Table 3.3.1)

As explained earlier in UAS-Gal4 system, the F1 generation flies will have both gal4 and RNAi expressed. Here, it could be possible that this particular gal4, is expressed at some early developmental stages of embryos which eventually bind to UAS sequence of RNAi for targeted genes. Inhibiting the function of these genes at early stages of embryo development could have hampered its development and which resulted in no progeny formation in all cases. It means that the RNAi lines are functional. The experiment which will show the expression pattern of this C306 gal4 during oogenesis and embryogenesis need to be done in order to prove the hypothesis. For some of the crosses set up at 25^0 C, it could be possible that due to low RNAi expression for particular genes, their embryos have overcome the developmental barrier and produced F1 generation flies. The ovary dissection of the parent flies which didn't produce progeny was not done. It could have helped us to understand whether there is any problem in ovary development or not.

Sr. No.	ETC Complex	RNAi used against	Result
At 29 ^{0}C			
1	Complex I	NADH dehydrogenase 39 kDa subunit	No progeny
2		NADH dehydrogenase 24kDa subunit	No progeny
3	Complex IV	COX15/CtaA family(Heme A synthase)	No progeny
4	Complex V	ATP Synthase Coupling factor 6-subunit F6	No progeny
5		ATP synthase beta subunit 2	No progeny
6		ATP synthase alpha domain	No progeny
7		ATP synthase beta subunit 1	No progeny
At 25 ^{0}C			
8	Complex I	NADH dehydrogenase 39 kDa subunit	No progeny
9	Complex IV	COX15/CtaA family(Heme A synthase)	No progeny
10	Complex V	ATP synthase beta subunit 2	No progeny
11		ATP Synthase Coupling factor 6-subunit F6	Normal
12		ATP synthase alpha domain	Normal
13		ATP synthase beta subunit 1	Normal

Table 3.3.1The results of RNAi mediated knockdown of subunits of different complexes of electron transport chain of mitochondria using C306 Gal4. Screening was done at 29 ^{0}C and 25 ^{0}C.

3.4 RNAi mediated knockdown of subunits of complex I, II and V caused increased pAMPK implying a possible fall in ATP levels during late cellularisation.

In eukaryotic cells, sufficient levels of energy need to be maintained for proper functioning of cells. During fall in these energy levels due to some reason or stress, cells need to have some mechanism to balance the energy. AMP-activated protein kinase (AMPK) fulfils this role of energy homeostasis in the cell. AMPK detects the ratio of AMP/ATP in the cell. During energy (ATP) deprived state of cell, AMPK gets activated and promotes the activation of catabolic pathways and inhibit the anabolic pathways in the cell (David Carling et al., 2011). Here I have depleted the proteins involved in oxidative phosphorylation. Depletion of these proteins could affect the process of oxidative phosphorylation and which in turn affect ATP production. So, one of the readout to check whether ATP production is affected or not is to check the status of energy sensor (AMPK) in the cell. pAMPK is the activated form of AMPK. Here I have stained the wild type and mutant embryos with phosphorylated AMPK (pAMPK) antibody which labelled pAMPK in the cell (Fig 3.1). I measured the pAMPK intensity using Image J software and found that knockdown of targeted subunits of complex I, II and V of ETC caused significant increase in pAMPK intensity during late cellularisation of embryo (Fig 3.2). Increase in pAMPK in these mutant embryos implies that depletion of these different subunits of ETC complex have lowered the sufficient energy level required to perform several cellular processes during late cellularisation stages of embryogenesis. Hence, these subunits are important for ATP production in the cell.

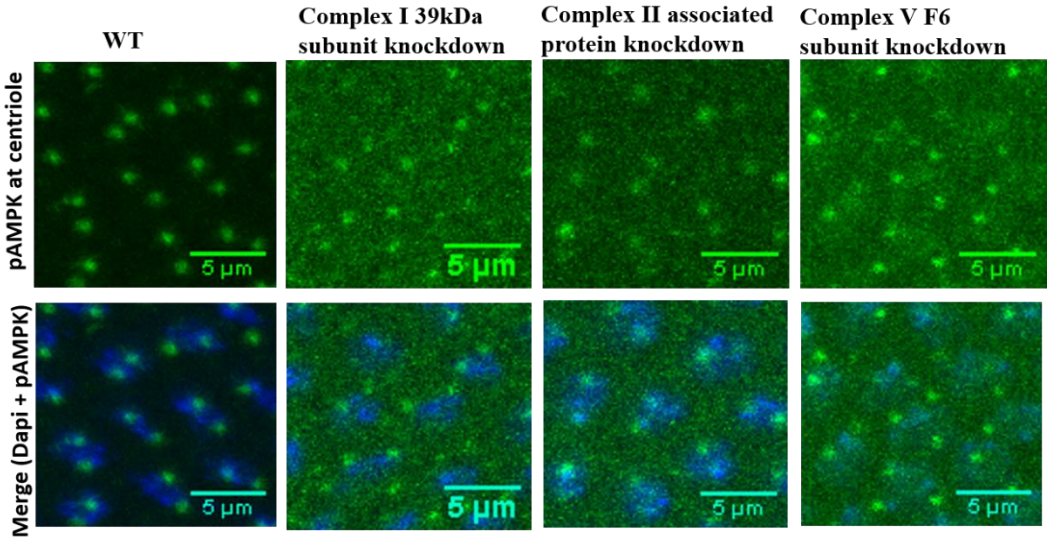


Fig 3.1. pAMPK intensity levels during late cellularisation of *Drosophila* embryogenesis. Top panel shows the pAMPK staining (mainly present at centrioles) in the late cellularised embryos of wt and mutants of complex I, II and V of ETC of mitochondria. The bottom panel shows composite image of pAMPK and dapi (which labels nucleus). Green- pAMPK, Blue-Nucleus

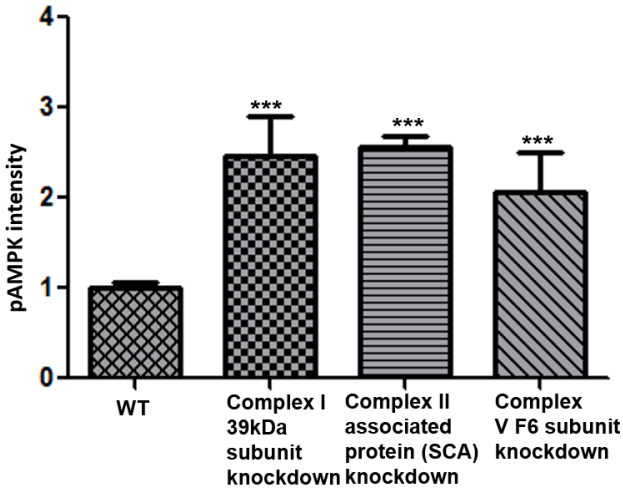


Fig 3.2. Bar representation of average pAMPK intensity (normalised to wild type) during late cellularisation of *Drosophila* embryogenesis. Here, n= (20, 2) for WT, n= (20,2) for complex I 39kDa subunit knockdown, n=(20,2) for complex II associated(SCA) subunit knockdown and n=(50,5) for complex V F6 subunit knockdown. The error bars represent standard deviation (SD). Two-tailed unpaired student's T-test was used to check the significance of the results. *** On error bars represent Significant p values $p<0.0001$

3.5 Analysis of circularity of actin contractile ring in different ETC complex's subunits mutants during *Drosophila melanogaster* embryogenesis

Cellularization of *Drosophila* embryo, which starts after the 13th nuclear cycle of syncytial blastoderm is characterised by the invagination of plasma membrane around the peripheral nuclei of embryo to form complete columnar epithelial cells (Tritarelli et al., 2004). It has been found that both actin and myosin II play a role during this process. They provide constricting force for membrane invagination. Myosin II is localised within the cytoskeletal caps associated with somatic nuclei present at the embryonic surface during syncytial blastoderm which form myosin rings present during cellularisation after the final syncytial division(Young et al., 1991). It has been observed that proper localisation of myosin and it's timing during cellularisation produce force required for complete cellularisation and other cell shape changes during embryogenesis(Young et al., 1991). During early cellularisation, actin form hexagonal network around the nucleus and as the cellularisation progress,these proteins accumulate at leading edge of invaginating membrane. They reorganize and form rings which will constrict the membrane basally and form complete epithelial

cells(Tritarelli et al., 2004). It has been observed that myosin II play an essential role at end of the cellularisation when it is recruited to the furrow front from basal cytoplasmic reservoir by its apical movement using microtubules ((Tritarelli et al., 2004).

Plasma membrane invagination is a dynamic process in *Drosophila* embryogenesis. F-Actin, myosin II and several other proteins assemble at the tip of invaginating plasma membrane and form contractile ring. Myosin is an ATPase which require energy for its motor activity (Rayment et al., 1996).Since this process is energy dependent, so it is essential to check whether depletion of energy producing proteins affect the formation of contractile ring or not. So in order to look at process of formation of contractile ring during plasma membrane invagination in the developing embryo, actin was stained with phalloidin which binds to it. Now one of the read out to check the success of formation of contractile ring is the circularity of the ring. It is calculated with a formula which takes into account it's all dimensions, that is radius, diameter, perimeter, area etc. The circularity of contractile ring was calculated as:

$$\text{circularity, } C = \frac{4\pi \text{ area}}{\text{perimeter}^2}$$

Circularity C= 1 means complete circular and C<1 means less circular

In order to analyse the physiological impact of ETC complex downregulation on crucial steps of *Drosophila* embryogenesis, the following targeted RNAi lines were chosen on the basis of their embryonic lethality (Table 3.1.1) and reduction in circularity of actin contractile ring formed during cellularisation of embryo.

1. **52922** RNAi-against NADH dehydrogenase 39kDa subunit (complex I) – embryonic lethality (8.0 % at 25 ^{0}C and no embryos laid at 29 ^{0}C), 2. **51807** RNAi- against Succinyl coenzyme A synthetaseα subunit (complex II) - embryonic lethality (5.0 % at 25 ^{0}C), 3. **51357** RNAi- cytochrome bc1 complex 8 subunit (complex III) - embryonic lethality- 55.45 % at 25 ^{0}C and 4. **51714** RNAi –against ATP synthase coupling factor F6- subunit F6- embryonic lethality- 15.58 % at 25 ^{0}C and 11.53 % 29 ^{0}C.

The different data of targeted RNAi lines in this project for which comparable stages images of cellularisation during *Drosophila* embryogenesis available were only those put up in the results and graphs. I need to repeat the experiments in order to get those comparable stage results.

3.5.1 Inhibition of 39kDa subunit of complex I of ETC of mitochondria results in reduction in circularity of actin contractile ring during early cellularisation stage of embryogenesis.

Apart from actin and myosin, contractile ring consists of several other core proteins. Among them are septins, which polymerise into higher order structure and bind to cell membrane, anillin, which crosslink actin filaments at cytokinetic ring and non-muscle myosin-II etc (Mavrakis et al., 2014). These proteins stabilize at the tip of furrow and form the the ring which will constrict the invaginating membrane. Here circularity of actin contractile ring was measured by drawing its shape manually and its value was calculated using formula given earlier (Fig 3.3). Here the circularity of actin contractile ring was significantly reduced (p value < 0.0001) in the early cellularised embryos of mutant of 39kDa subunit of complex I of ETC as compared to wild type (Fig 3.4). The circularity of actin contractile ring is also reduced in the early cellularised embryos of mutant of F6 subunit of complex V of ETC of mitochondria, but the reduction is less significant (p value < 0.05) as compared to wild type (Fig 3.4). As explained earlier, formation of contractile ring involve several proteins which require ATP. Here, complex I 39 kDa subunit and complex V F6 subunit are important in energy production. It is possible that depletion of these subunits could have led to lowering of sufficient energy level which in turn could have disturbed the formation of contractile ring during early cellularisation. The circularity of contractile ring in complex II associated protein knockdown embryos is similar to that of wild type. Since complex II provide additional electrons to ubiquinone, so it is possible that the effect on lowering of ATP due to inhibition of the complex II associated protein was rescued by sufficient energy production by other complexes in ETC. Proper balance of ATP in the cell could have rescued the phenotype of reduction in the circularity of contractile ring in complex II associated protein knockdown embryo.

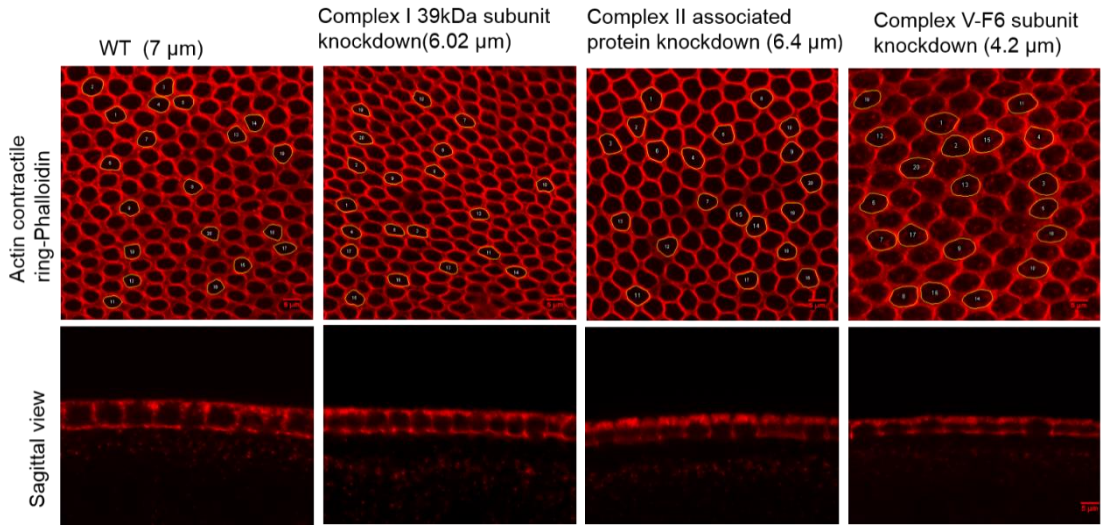


Fig 3.3 Actin contractile ring during early cellularisation stage of embryos. Here it shows actin contractile ring in early cellularised embryos of WT and subunits of ETC complex I, II and V knockdown of (Top panel).Below panel shows sagittal view of the same embryo. The furrow length of each embryo is put up in bracket at the top of panel. Red- Phalloidin.

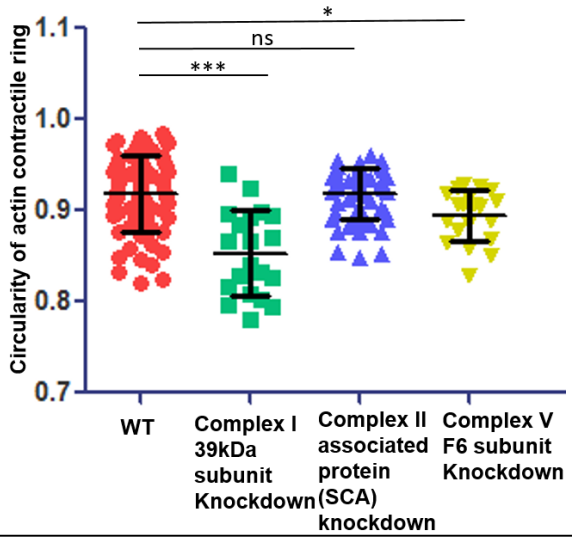


Fig 3.4. **Scatter plot representation of the circularity of actin contractile ring in early cellularisation stage embryos of wild type and different subunits of ETC complex I, II and V knockdown.** Here n= (70,4) for WT, n= (20,1) for complex I 39kDa subunit knockdown, n=(20,1) for complex II associated protein (SCA) knockdown and n=(20,1) for complex V F6 subunit knockdown. The error bars represent standard deviation (SD). Two-tailed unpaired student's T-test was used to check the significance of the results. *** = represent Significant p values p<0.0001, ns=not significant, *=p<0.05

3.5.2 RNAi mediated knockdown of 39kDa subunit of complex I and complex II associated protein (SCA) caused significant reduction in circularity of actin contractile ring during mid cellularisation stage of embryogenesis.

During mid cellularisation of *Drosophila* embryo, plasma membrane invagination reach half of nucleus. Actin, myosin and several other proteins organise at tip of invaginating furrow and form contractile ring(Tritarelli et al., 2004). Circularity of actin contractile ring was significantly reduced (p value < 0.0001) in the mid cellularised embryos of mutant of 39kDa subunit of complex I of ETC as well as in mutant embryos of complex II associated protein (SCA) compared to wild type(Fig 3.5 and 3.6). Here it is possible that proper formation of contractile ring would require a certain threshold of energy. Since these subunits play important role in energy production in the cell, it could be possible that due to their depletion cell couldn't produce that threshold of energy. Hence these subunits of complex I and complex II are important for formation of contractile ring during mid cellularisation of embryogenesis.

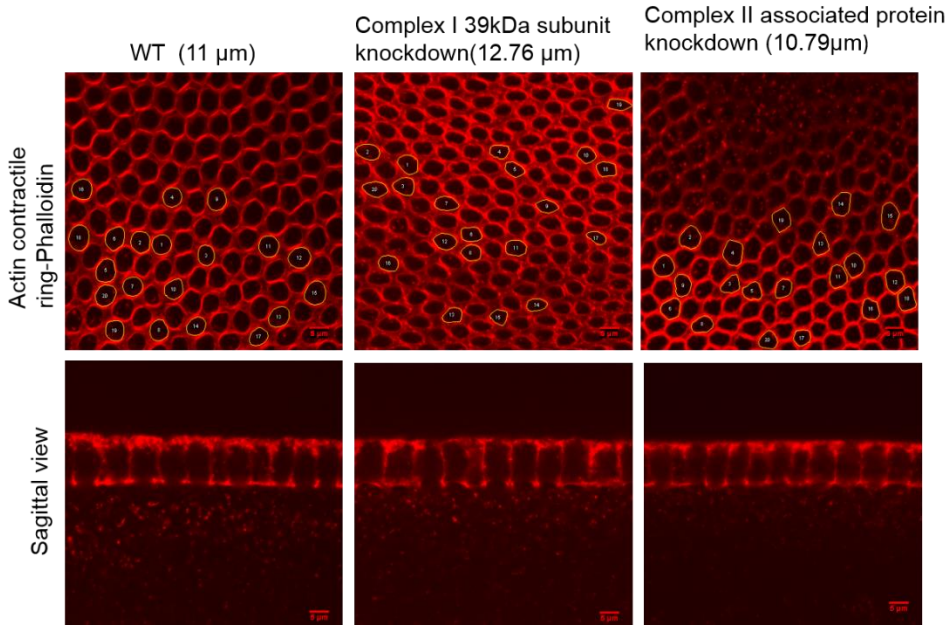


Fig 3.5 Actin contractile ring during mid cellularisation stage of embryos. Here it shows actin contractile ring in mid cellularised embryos of WT and different subunits of ETC complex I and II knockdown (Top panel). Below panel shows sagittal view of the same embryo. The furrow length of each embryo is put up in bracket at the top of panel. Red- Phalloidin.

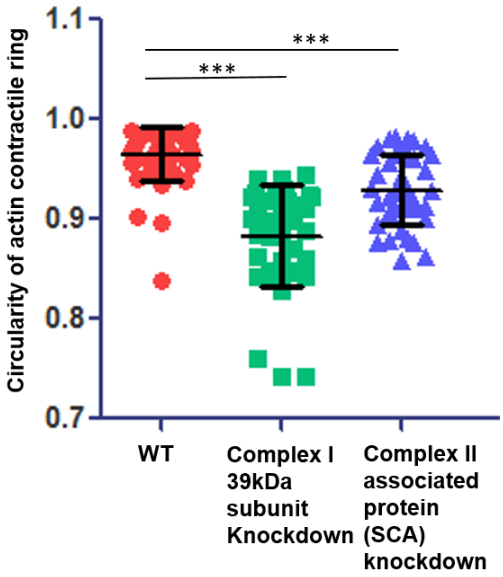


Fig 3.6. **Scatter plot representation of the circularity of actin contractile ring in mid cellularisation stage embryos of wild type and different subunits of ETC complex I and II knockdown.** Here n= (50, 4) for WT, n= (40,2) for complex I 39kDa subunit knockdown and n=(40,3) for complex II associated protein (SCA) knockdown. The error bars represent standard deviation (SD). Two-tailed unpaired student's T-test was used to check the significance of the results. *** On error bars represent significant p values $p<0.0001$

3.5.3 RNAi mediated knockdown of subunits of complex I, III and V caused significant reduction in circularity of actin contractile ring during late cellularisation stage of embryogenesis.

End of cellularisation marks the complete closure of invaginating plasma membrane around the nucleus and produce columnar epithelial cells. During this process myosin and actin provide constricting force for invaginating plasma membrane(Tritarelli et al., 2004). Here,circularity of actin contractile ring is significantly reduced (p value < 0.0001) in the late cellularised embryos of mutants of subunits of complex I,III and V of ETC of mitochondria compared to wild type (Fig 3.7 and 3.8).

The inhibition of complex I 39kDa subunit during embryogenesis caused reduction in the circularity of contractile actomyosin ring during early, mid and late cellularisation of embryo. It is possible that RNAi mediated knockdown of this subunit caused reduction in the sufficient energy level required dynamic contractile ring formation. The

inhibition of succinyl coA synthetase alpha subunit caused significant reduction in circularity of actomyosin ring during mid cellularisation and that reduction is recovered during late cellularisation of embryo. It is possible that during late cellularisation that energy reduction barrier due to its knockdown is overcome by some other means of energy source in the embryo. The knockdown of subunits of complex III and complex V also resulted in reduction in circularity of contractile ring during late cellularisation. Subunits of complex III and V are involved in energy production. It is possible that RNAi mediated knockdown of this subunit caused reduction in the sufficient energy level required dynamic contractile ring formation.

It has been observed that septins are necessary for producing curved and tightly packed actin filaments. It has been studied in *Drosophila* embryogenesis that in septin mutants, the circularity of contractile ring was reduced (Manos Mavrakis et al., 2013). Septins are GTPase and require GTP for their polymerization (Christine S et al., 2008). It is possible that GTP levels may get affected by a possible reduction of ATP in ETC mutants. Here the proteins directly involved in energy production are inhibited. So it is possible that due lack of threshold of energy during cellularisation of embryo, these processes disturbed in their function which in turn affected formation of contractile ring and shape of the ring (Fig 3.3, 3.5 and 3.7).

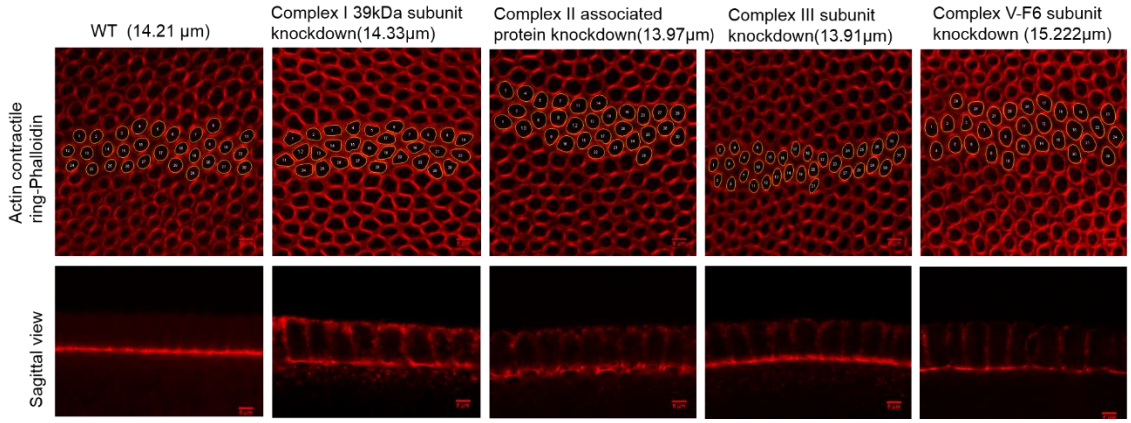


Fig 3.7 Actin contractile ring during late cellularisation stage of embryos. Here it shows actin contractile ring in late cellularised embryos of WT and different subunits of ETC complex I, II, III and V knockdown (Top panel). Below panel shows sagittal view of the same embryo. The furrow length of each embryo is put up in bracket at the top of panel. Red- Phalloidin.

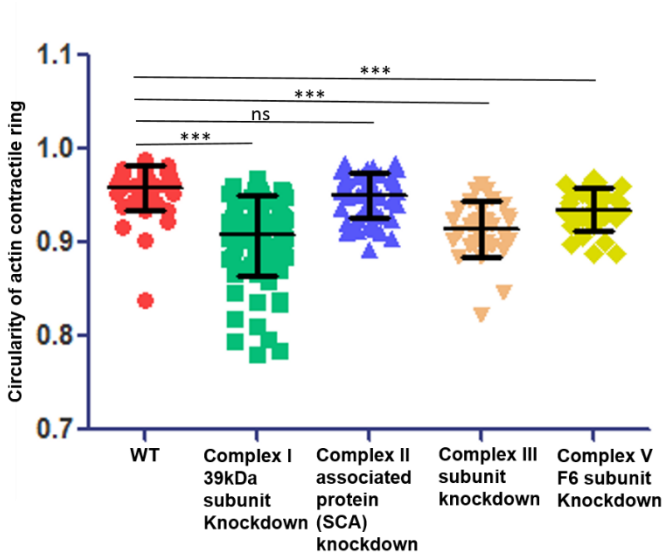
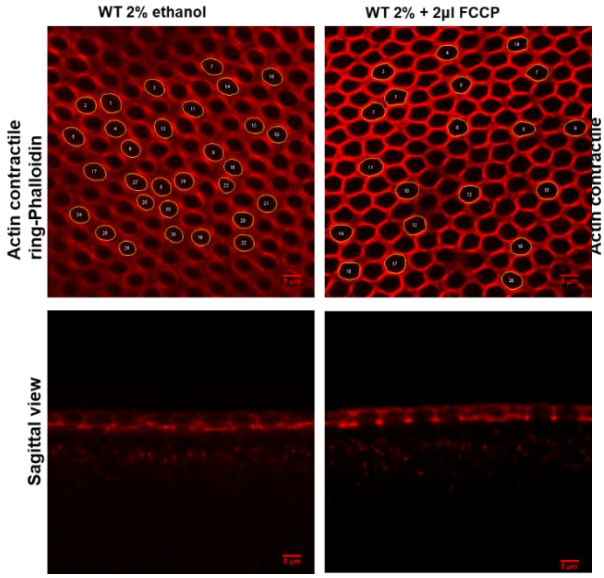


Fig 3.8. Scatter plot representation of the circularity of actin contractile ring in late cellularisation stage embryos of wild type and different subunits of ETC complex I, II, III and V knockdown. Here n= (50, 2) for WT, n= (110, 5) for complex I 39kDa subunit knockdown and n= (50,2) for complex II associated protein (SCA) knockdown, n=(30,1) for complex III subunit knockdown and n= (30,1) for complex V F6 subunit knockdown. The error bars represent standard deviation (SD). Two-tailed unpaired student's T-test was used to check the significance of the results. *** On error bars represent Significant p values p<0.0001, ns= not significant.

3.6 Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) treatment in *Drosophila* embryogenesis caused reduction in circularity of actin contractile ring.

Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP), is an uncoupling agent which disrupts the transmembrane ion concentration gradient by allowing ions to pass through lipid membrane. It also disrupts the process of energy production in the cell. In order to study the role of transmembrane potential and also of FCCP on membrane organisation, I decided to disrupt the membrane potential of cell using FCCP and study plasma membrane organisation during cellularisation stages of *Drosophila* embryogenesis. It has been found that disruption of membrane potential and ATP synthesis caused significant reduction in circularity of actin contractile ring during transition stages of celllularisation (between early and mid cellularisation) (Fig 3.11).There was no significant reduction in the circularity of actin ring during late cellularistion stages of embryogenesis (Fig 3.12). Disruption of membrane potential and energy in the cell caused reduction in circularity of actin contractile ring during transition stages of cellularisation of embryogenesis. The contractile ring formation

require sufficient amount of energy in the cell. It is possible here that due to disruption of energy level in cells this process get affected. Experiment to find out energy levels using pAMPK activity need to be done.



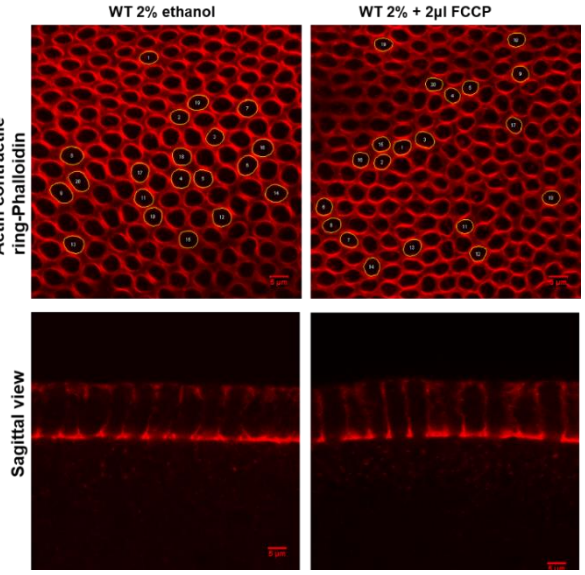
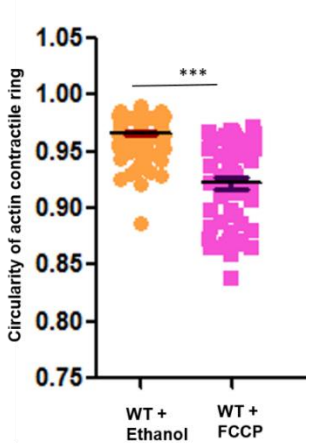


Fig 3.9. Actin contractile ring during transition (between early to mid) cellularisation stage of FCCP treated embryos. Here it shows actin contractile ring in transition stage of cellularised embryos of WT treated with 2% ethanol and WT2% treated with FCCP.

Fig 3.10. Actin contractile ring during late cellularisation stage of FCCP treated embryos. Here it shows actin contractile ring in late cellularised embryos of WT treated with 2% ethanol and WT2% treated with FCCP.



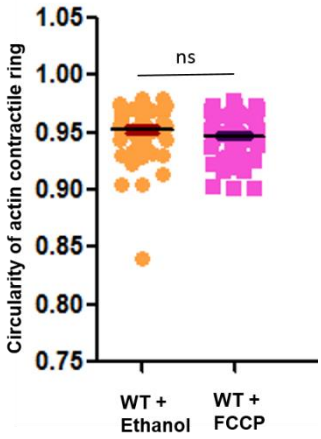


Fig 3.11. Scatter plot representation of the circularity of actin contractile ring during transition stage (between early to mid) cellularisation. Here n= (120, 5) for WT 2% ethanol and n= (60, 3) for WT 2% ethanol treated with FCCP. Error bars= standard error on mean. ***p value <0.0001. Significance test= two-tailed unpaired student's T-test.

Fig 3.12. Scatter plot representation of the circularity of actin contractile ring during late cellularisation of *Drosophila* embryogenesis. Here n= (60, 3) for WT 2% ethanol and n = (60,3) for WT 2% ethanol treated with FCCP. Error bars= standard error on mean. ***p value <0.0001. Significance test= two-tailed unpaired student's T-test.

3.7 Mitochondria are less punctate in complex I,II, III and V subunits knockdown embryos and more diffused in complex V F6 subunit knockdown embryos at contractile ring

The inhibition of subunits of different complexes of ETC of mitochondria resulted in the change of the shape of the contractile ring possibly due to destabilisation of the F-actin. Mitochondria are known to move on microtubules and actin network (Boldogh and Pon, 2006). Actin also plays a role in keeping the mitochondria at site of high ATP requirement (Laura L Lackner et al., 2013). It is important to find out whether the shape of the contractile ring is affected due to lack of mitochondrial transport to the contractile ring in mutants which have a defective electron transport chain. Here, we have analysed the actin and mitochondrial intensity at contractile actin ring during late cellularisation of embryo. We plotted their intensity profile as well (Fig 3.13). It has been found that the mitochondria are punctate and distributed along the actin ring in wild type. RNAi mediated knockdown of subunits of different ETC complexes (I, III and V) showed less punctate mitochondria. Mitochondria were peripherally associated in at contractile ring. Mitochondria in complex V F6 subunit knockdown embryos were uniformly diffused in the cell. Mitochondria which is a source of energy in the cell is distributed differently from cell to cell type, developmental stages (Laura L Lackner et al., 2013). It is possible that reduction in the energy levels in the cell due inhibition of energy producing proteins affected transport of mitochondria to the actin ring. It might be possible that microtubules and actin network didn't form in a proper way due to lack of threshold of energy.

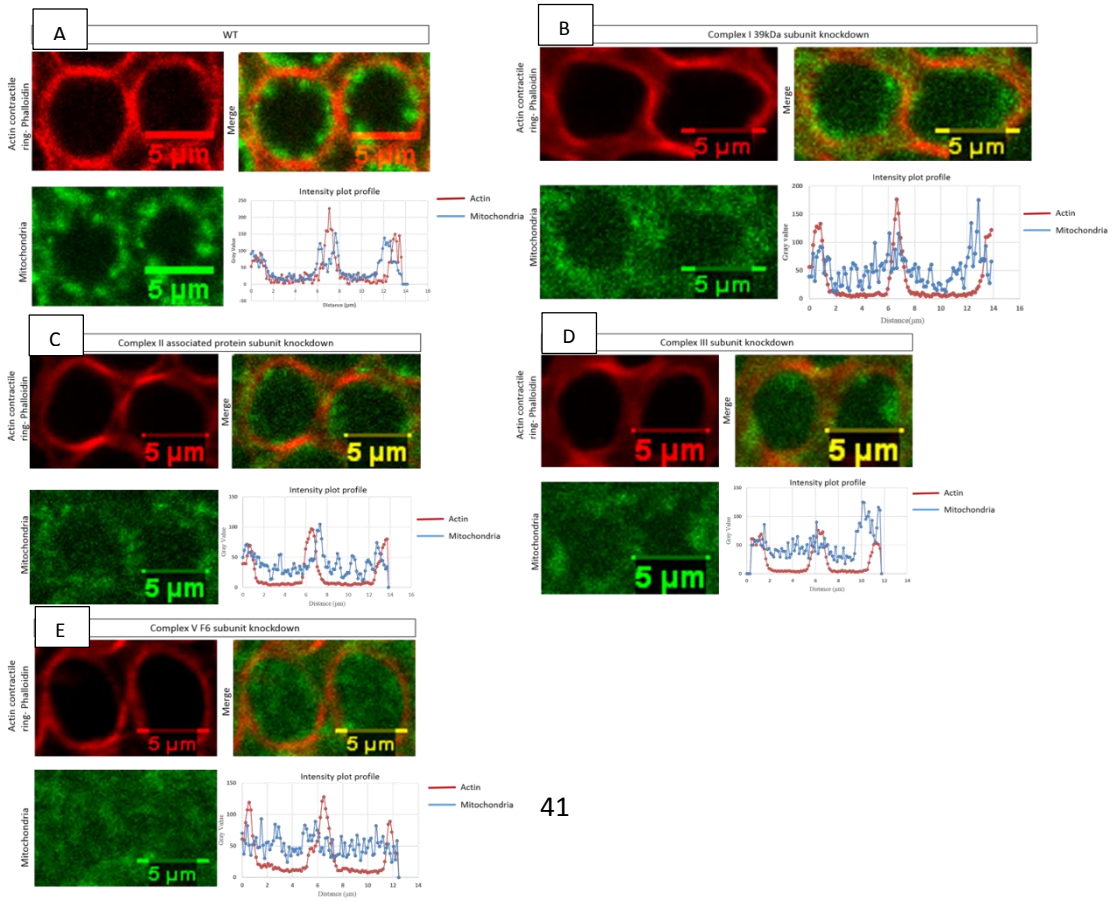


Fig 3.13. Mitochondrial distribution at contractile ring in WT and different subunits of ETC complex I, II, III and V knockdown *Drosophila* embryos. Here mitochondrial and actin intensity profile is plotted across a single line drawn at centre across two contractile actin rings shown in each panel. A = WT (Sayali Chowdhary,RR lab) B= complex I 39kDa subunit knockdown, C= complex II associated subunit (SCA) knockdown, D= complex III subunit knockdown, E= complex V F6 subunit knockdown. Red – phalloidin, Green- Mitochondria (labelled using streptavidin). In intensity plot profiles, Red- actin intensity, Blue- mitochondrial intensity.

3.8 Ratio of Apical to Basal mitochondrial intensity is affected in ETC complex II, III and V subunits knockdowns.

Since mitochondrial intensity changed somewhat at the contractile ring, I further assessed if mitochondrial trafficking along the apicobasal axis during cellularization was affected. During cellularisation stage of *Drosophila* embryogenesis, mitochondria migrate from basal region to apical region (Sayali Chowdhary and Richa Rikhy, personal communication). The mitochondria migration require motor proteins which eventually require energy (ATP)(Lackner, 2013). So in order to check whether knockdown of targeted genes affect this migration of mitochondria. We measured the apical and basal mitochondrial intensity in both wild type and in mutants. Here RNAi mediated knockdown of these selected subunits of complex II, III and V in embryos caused migration defects in mitochondria form basal to apical region of a cell. Inhibition of complex I 39kDa subunit didn't cause migration defect during late cellularisation (Fig 3.14) Analysis of more stages of embryos in these mutants needs to be done in order to quantify the results. Mitochondrial distribution in the cell is important for different cellular and organelle functions and cell survival. Movement of mitochondria requires different motor proteins like kinesin, dynein and myosin which help in transporting mitochondria to different parts of the cell(Lackner, 2013). These motor proteins require energy to move along the cytoskeleton tracks in the cell. It is possible that energy required for their assembly and movement is reduced due to knockdown of targeted subunits of complexes of ETC. Complex III and V subunit mutant showed much difference in ratio of mitochondrial intensity from apical to basal region. The F6 subunit is a part of ATP synthase F0 sub-complex, is a part of peripheral stalk which joins F1 and F0 sub-complex together. Inhibition of this subunit will result into disruption these sub-complexes which will hamper ATP production in the cell. Similarly, other subunits of different complexes are part of the machine which generate energy for different cellular processes in the cell.

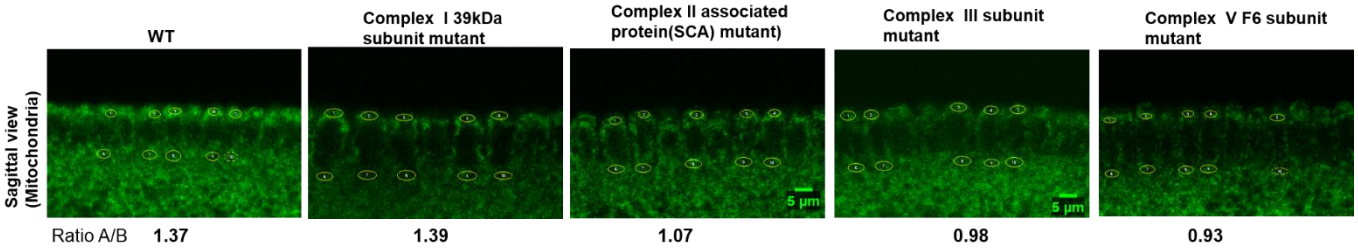


Fig 3.14. Ratio of apical to basal mitochondrial intensity in late cellularisation of *Drosophila* embryos. The sagittal view of embryo showing apical-basal mitochondrial distribution. Mitochondrial intensity is measured using Image J software. Apical (A) region above the nuclei and Basal (B) denotes region below the nuclei. ROI are drawn to measure Apical and Basal mitochondrial intensity. Values at below represent the average value of ratio of apical to basal mitochondrial intensity. Green- Mitochondria (labelled using streptavidin)

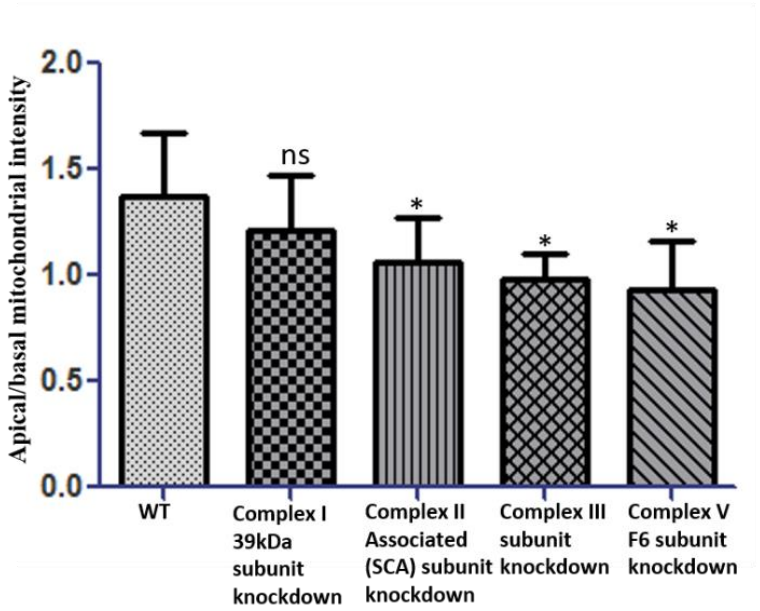


Fig 3.15. Bar representation of average apical to basal mitochondrial intensity during late cellularisation of *Drosophila* embryos. Here n= (5,1) for WT,n= (15,3) complex I 39kDa subunit knockdown ,n=(10,2) for complex II associated protein knockdown, n=(5,1) for complex III subunit knockdown and n=(5,1) for complex V F6 subunit knockdown. The error bars represent standard deviation (SD). Two-tailed unpaired student's T-test was used to check the significance of the results. * On error bars represent Significant p values $p<0.05$, ns = not significant.

3.9 Live imaging of mitochondrial movement during cellularisation stages of *Drosophila* embryogenesis.

In a cell, several processes and signalling pathways happen continuously. Mitochondria, which is an essential organelle in inside the cell provides ATP and calcium signal for these process to happen in a systematic manner. In order to deliver ATP and calcium signal to different parts of cell, it migrates along the cytoskeletal tracks. In neurons, mitochondria are required at synapse in order to balance the energy level. It has been observed that mitochondria co-localize with actin cables and move along it *in vivo* and its movement is dependent on actin dependent motor activity in budding yeast. It has been observed that mitochondrial dynamics and its distribution was stopped due to disruption of actin network by pharmacological drug treatment or duet to deletion of proteins which bind to actin in budding yeast. (Rebecca L. Frederick et al., 2007). RNAi mediated knockdown of targeted subunits of complexes of ETC caused destabilization of actin filaments which caused polygonal shape of actin contractile ring (Fig 3.3, 3.5 and 3.7). Therefore it is important to find out whether mitochondrial distribution is affected during cellularisation stage of *Drosophila* embryogenesis in mutants. Here live imaging of embryos containing transgenic mitogfp which labels mitochondria was carried out. The transgenic line which was used to knockdown target gene consist of single nanos Gal4 which drives expression of both mitogfp and RNAi for target gene

3.9.1 Mitochondrial distribution pattern during cellularisation stages of *Drosophila* embryogenesis is maintained in complex I 39kDa subunit knockdown mutant embryos.

RNAi mediated knockdown of complex I 39kDa subunit caused reduction in circularity of actomyosin contractile ring (Fig 3.3, 3.5 and 3.7) during early, mid and late cellularistion stages of embryogenesis. So it is important to understand whether destabilization of actin filaments at contractile ring cause defects in mitochondrial migration or distribution in different cellularisation stages of embryogenesis. In wild type *Drosophila* embryo, mitochondria migrate from basal region to apical region as embryo progress in development different cellularisation stages of embryogenesis (Fig 3.16 and 3.18). Here we analysed the mitochondrial intensity from apical region to basal region of nuclei during early, mid and late cellularisation stages of embryogenesis. The live imaging of mitochondria in embryos of complex I 39kDa subunit mutant showed similar pattern of mitochondrial distribution when it undergoes development from early to late cellularisation stages (Fig 3.17 and 3.19).

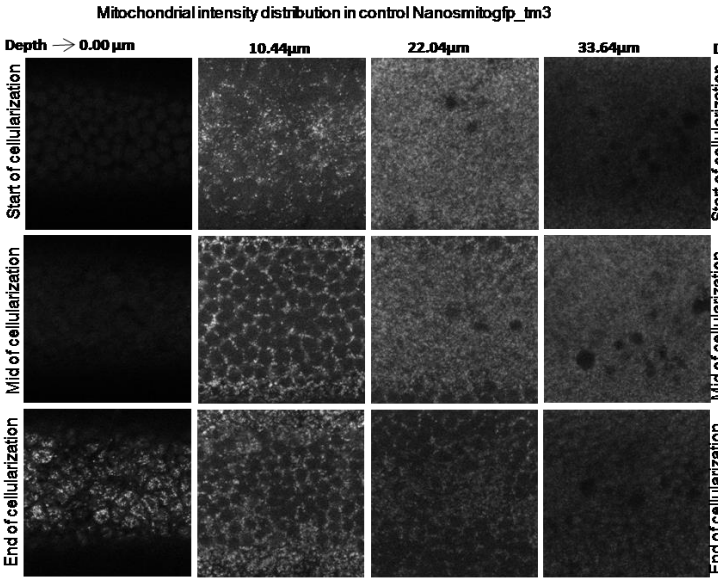


Fig 3.16. Mitochondrial distribution pattern during cellularisation stages of embryogenesis in control embryos (Nanosmitogfp_tm3). Top panel shows start of cellularisation, middle panel shows mid of cellularisation and bottom panel shows end of cellularisation. Values on the top of figure represents depth of embryo (in µm) from apical region to basal region.

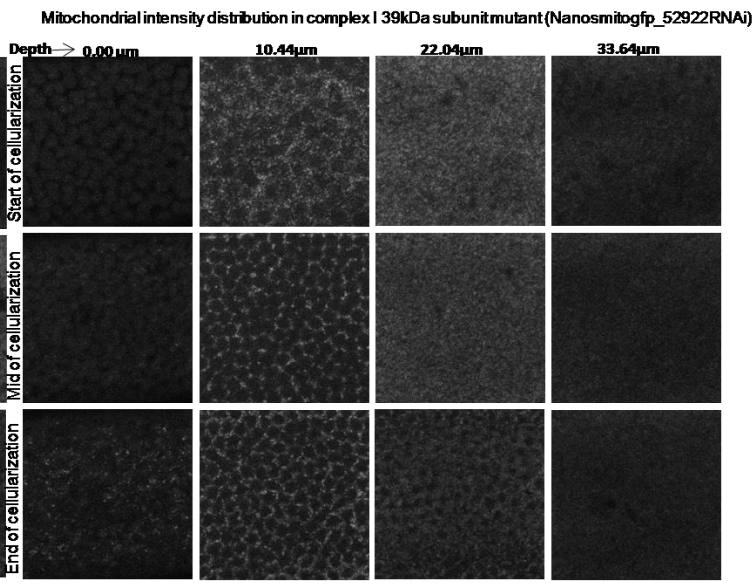
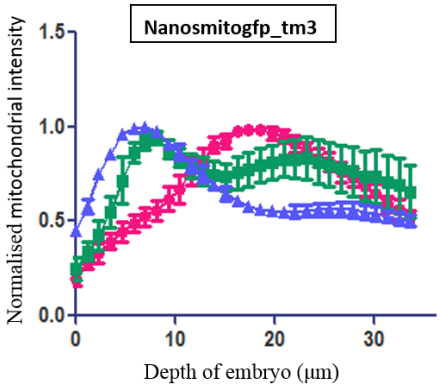


Fig 3.17. Mitochondrial distribution pattern during cellularisation stages of embryogenesis in complex I39kDa subunit mutant embryos (Nanosmitogfp_52922RNAi). Top panel shows start of cellularisation, middle panel shows mid of cellularisation and bottom panel shows end of cellularisation. Values on the top of figure represents depth of embryo (in µm) from apical region to basal region.



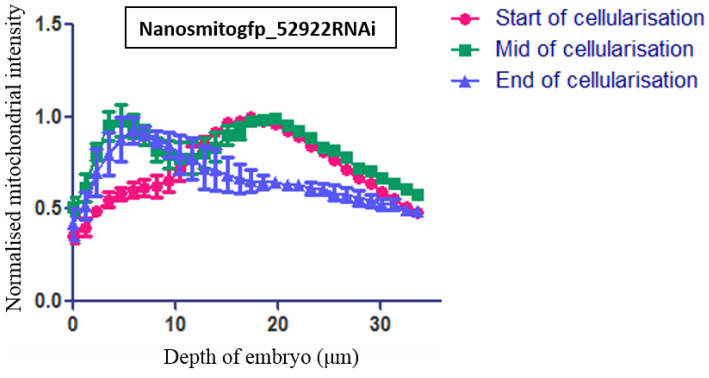


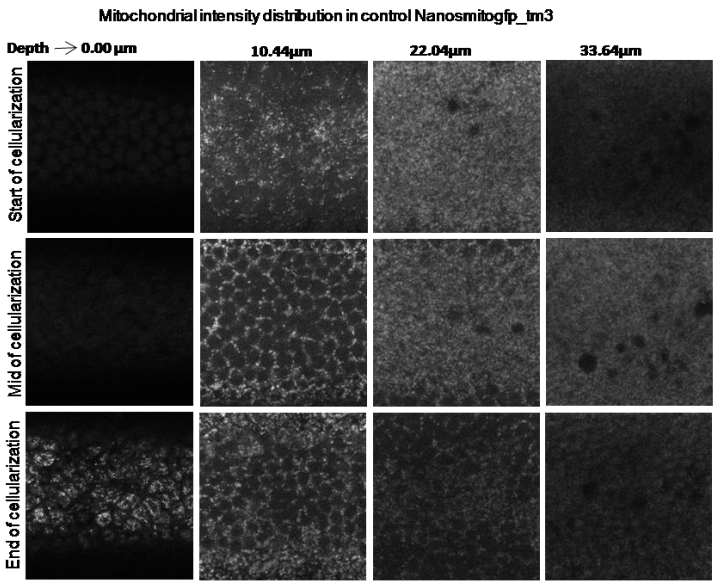
Fig 3.18. The graphical representation of mitochondrial distribution pattern during cellularisation stages of embryogenesis in control embryos (Nanosmitogfp_tm3). Here n=3 embryos of control. Depth is measured from top surface of embryo. The graph is plotted in GraphPad prism version 5 scientific software.

Fig 3.19. The graphical representation of mitochondrial distribution pattern during cellularisation stages of embryogenesis in Complex I 39kDa subunit knockdown embryos (Nanosmitogfp_52922RNAi). Here n=3 for complex I 39kDa subunit knockdown embryos. Depth is measured from top surface of embryo. The graph is plotted in GraphPad prism version 5 scientific software.

3.9.2 Mitochondrial distribution pattern during cellularisation stages of *Drosophila* embryogenesis is affected during early cellularisation in complex V subunit knockdown mutant embryos.

As mentioned above for complex I 39kDa subunit mutant embryos, inhibition of complex V- F6 subunit using RNAi caused significant reduction in circularity of actomyosin contractile ring (Figs 3.7) during late cellularistion stage of embryogenesis. And since this F6 subunit mutant embryos also showed defects in ratio of apical to basal mitochondrial intensity during late cellularisation, I wanted to study whether destabilization of actin filaments at contractile ring cause defects in mitochondrial migration or distribution in different cellularisation stages of embryogenesis. Here mitochondrial distribution pattern was seen to be similar as in control during cellularisation stages of embryogenesis. Here it shows increase in mitochondrial intensity at apical region during early cellularisation of complex V mutant which is recovered during mid cellularisation (Fig 3.23). It is possible that motor activity of motor proteins is affected in this muntant.

The mitochondrial distribution pattern during cellularisation of *Drosophila* embryos in both the mutants is seen to be similar to in control. The previous results of ratio of apical to basal mitochondrial intensity which is found to be affected in Complex V F6 subunit mutant embryos (Fig 3.15). It tells us that there is defect in migration of mitochondria in the cell during cellularisation of embryo. Here it is possible that the proteins are not completely knockdown in order to produce defects in mitochondrial migration because single Gal4 is divided equally for driving the expression of both mitogfp and RNAi for complex I 39kDa subunit and complex V F6 subunit. Since the experiments were carried out at low temperature (25 ^{0}C), it could be possible that RNAi tier was low in order to knockdown the subunits of complex I and V. Hence, we need to completely inhibit the subunits and then analyse the results for migration of mitochondria during cellularisation of embryo.



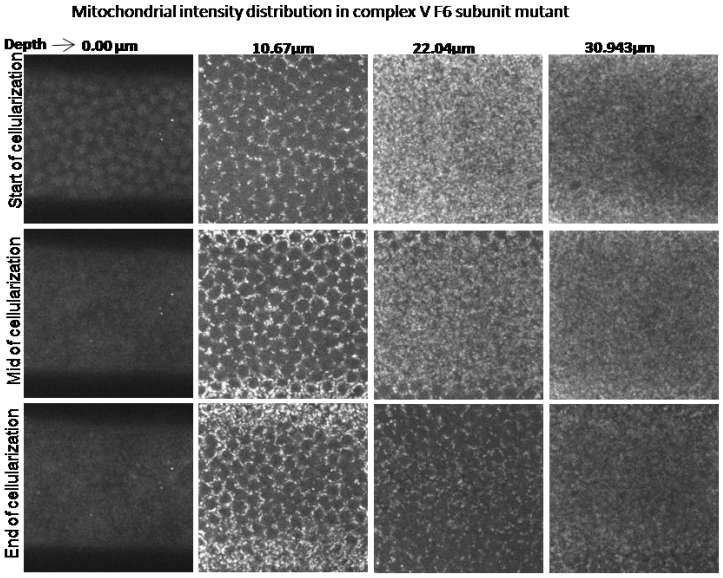


Fig 3.20. Mitochondrial distribution pattern during cellularisation stages of embryogenesis in control embryos (Nanosmitogfp_tm3). Top panel shows start of cellularisation, middle panel shows mid of cellularisation and bottom panel shows end of cellularisation. Values on the top of figure represents depth of embryo (in µm) from apical region to basal region. Here intensity is measured for n=3 embryos of control.

Fig 3.21. Mitochondrial distribution pattern during cellularisation stages of embryogenesis in complex V F6 subunit knockdown embryos (Nanosmitogfp_51714RNAi). Top panel shows start of cellularisation, middle panel shows mid of cellularisation and bottom panel shows end of cellularisation. Values on the top of figure represents depth of embryo (in µm) from apical region to basal region.

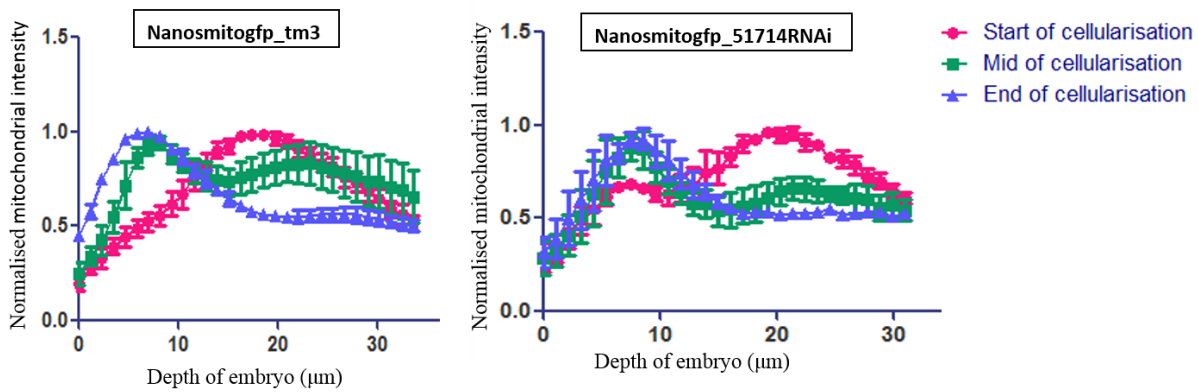


Fig3.22The graphical representation of mitochondrial distribution pattern during cellularisation stages of embryogenesis in control embryos (Nanosmitogfp_tm3). Here n=3 embryos of control. Depth is measured from top surface of embryo. The graph is plotted in GraphPad prism version 5 scientific software.

Fig3.23The graphical representation of mitochondrial distribution pattern during cellularisation stages of embryogenesis in complex V F6 subunit knockdown embryos (Nanosmitogfp_51714RNAi). Here n=3 for complex V F6 subunit knockdown embryos. Depth is measured from top surface of embryo. The graph is plotted in GraphPad prism version 5 scientific software.

3.9.3 Live imaging of embryos for complex I 39kDa subunit and complex V F6 subunit knockdown didn't show significant difference in apical to basal mitochondrial intensity during cellularisation of *Drosophila* embryogenesis

In order to correlate the results obtained for apical to basal mitochondrial intensity in fixed embryos, I took the orthogonal section of embryos from live images for wild type and also for mutants of complex I 39kDa subunit and complex V F6 subunit. Here I measured the apical and basal mitochondrial intensity using Image J software. Here, inhibition of 39kDa subunit of complex I and F6 subunit of complex V didn't show significant difference in apical to basal mitochondrial intensity during different stages of cellularisation (start, mid and end). As explained earlier, it is possible that the proteins are not completely knockdown in order to produce defects in mitochondrial migration because single Gal4 is divided equally for driving the expression of both mitogfp and RNAi for complex I 39kDa subunit and complex V F6 subunit. And also the experiments were carried out at low temperature (25 ^{0}C), it could be possible that RNAi tier was low in order to knockdown the subunits of complex I and V. Hence, we need to completely inhibit the subunits and then analyse the results for migration of mitochondria during cellularisation of embryo.

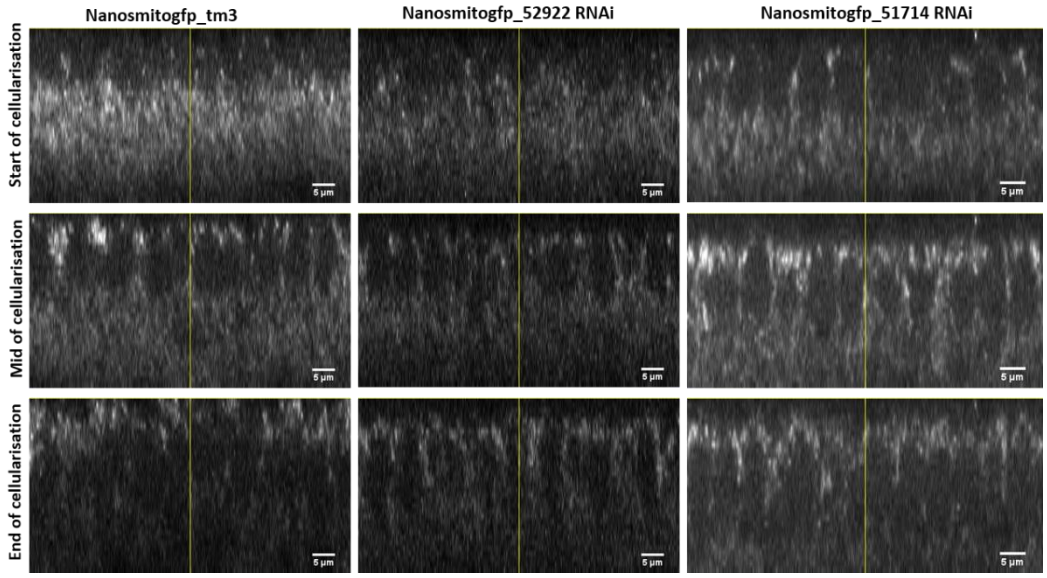


Fig 3.24. Orthogonal section of live embryos of WT and mutants of subunits of complex I and V showing apical to basal mitochondrial intensity in cellularisation of *Drosophila* embryos. The sagittal view of embryo showing apical-basal mitochondrial distribution. Mitochondrial intensity is measured using Image J software. Apical (A) region above the nuclei and Basal (B) denotes region below the nuclei. ROI are drawn to measure Apical and Basal mitochondrial intensity. First panel- start of cellularisation, second panel- mid of cellularisation and third panel- end of cellularisation. Mitochondria here is labelled using mitogfp (white)

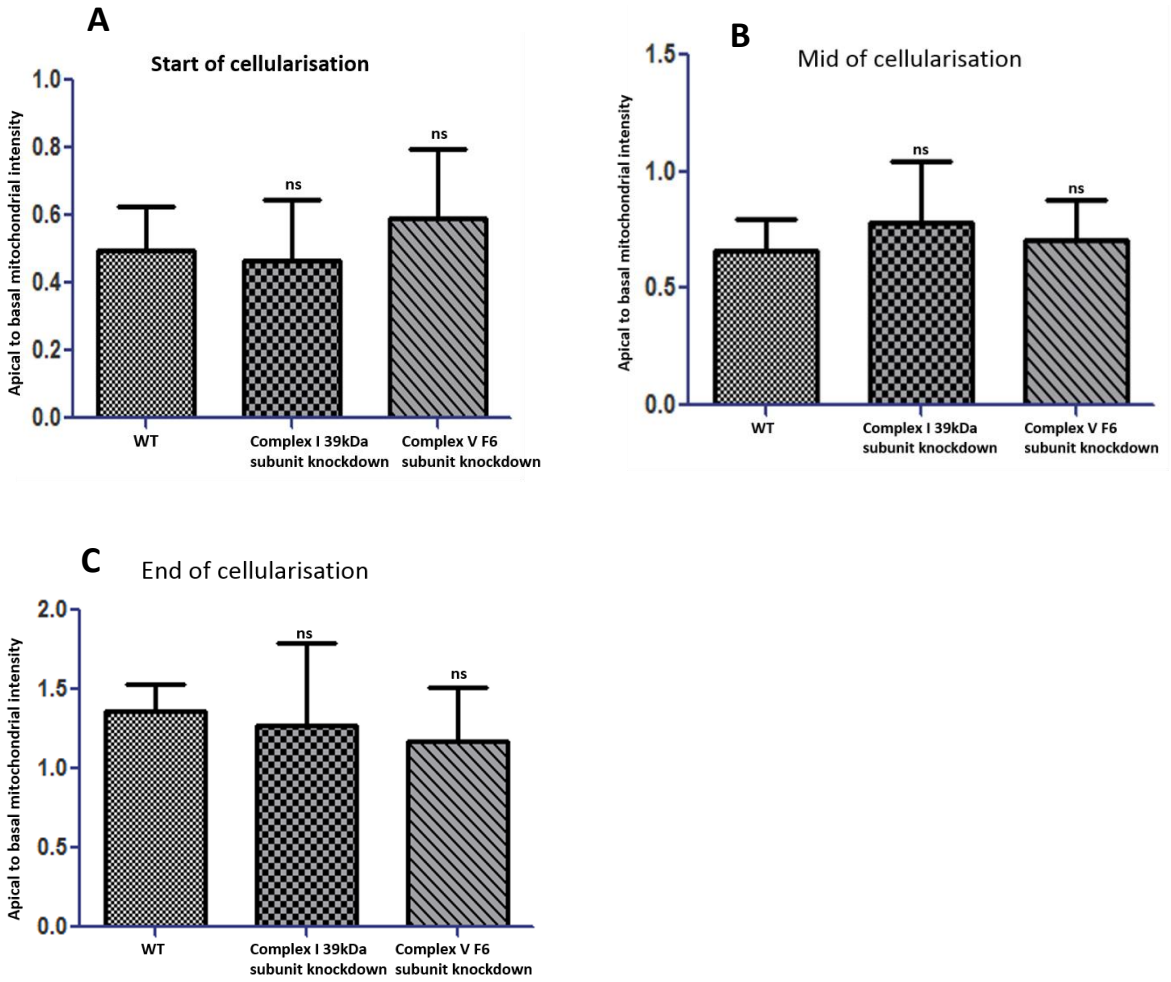


Fig 3.25. Bar representation of average apical to basal mitochondrial intensity during cellularisation of *Drosophila* embryos (live image analysis). Here n= (15, 3) for WT (Nanosmitogfp_tm3), n= (15,3) complex I 39kDa subunit knockdown (Nanosmitogfp_52922RNAi) and n=(15,3) for complex V F6 subunit knockdown (Nanosmitogfp_51714RNAi). **A**= start of cellularisation, **B**= mid of cellularisation and **C**= end of cellularisation. The error bars represent standard deviation (SD). Two-tailed unpaired student's T-test was used to check the significance of the results. ns = not significant.

4. Conclusions

In this project, I wanted to study role of set of mitochondrial metabolism proteins during *Drosophila* oogenesis and embryogenesis which consisted of most of them involved in oxidative phosphorylation and some of them involved in ROS regulation, calcium regulation and glycogen metabolism etc. Depletion of these proteins during oogenesis and embryogenesis produced lethality. It implies that the functions performed by these proteins are essential for early embryonic development of *Drosophila melanogaster.* It was observed that depletion of targeted subunits of ETC complex I, II and V led to fall in sufficient cellular energy level. Since the processes like contractile ring formation during cellularisation and mitochondrial transport are energy dependent. It is important to check whether these processes are affected due to depletions of subunits of ETC complexes. I observed that the circularity of contractile ring was significantly reduced in the embryos of ETC complex I, III and V subunits knockdown. It is also observed that mitochondria became less punctate in complex I, II, III and V subunits knockdown and with more diffused distribution in complex V F6 subunit knockdown as compared to wild type. It has been found that RNAi mediated knockdown of ETC complex subunits led to defect in mitochondrial transport during cellularisation of *Drosophila* embryogenesis.

5. Future perspectives

Depletion of subunits of ETC complexes have shown to affect mitochondrial transport from basal region to apical region during cellularisation and also shape, in future it would be worth checking whether mitochondrial transport to the apical region in cellularisation may be dependent on mitochondrial electron transport chain and morphology. Rate of transport of mitochondria from basal to apical axis with live imaging in mutants of ETC and morphology can be checked which will help to understand role of ETC and mitochondrial morphology in mitochondrial transportation in cell. And future studies to check the effect of signalling pathways on correct localisation and function of mitochondria which may be important during gastrulation and embryo patterning will provide an analysis of steps of *Drosophila* embryogenesis that will depend on mitochondrial metabolism.

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