Analysis of mitochondrial metabolism control on organism development

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M.S. Thesis

Under the guidance of

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Certificate

This is to certify that this dissertation entitled ‘Analysis of mitochondrial metabolism control on organism development’ towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Pooja Naik at IISER Pune under the supervision of Dr. Richa Rikhy, Assistant Professor, Biology Division, IISER Pune during the academic year 2012-2013.

Dr. Richa Rikhy

Assistant Professor

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Declaration

I hereby declare that the matter embodied in the thesis entitled ‘Analysis of mitochondrial metabolism control on organism development’ are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Richa Rikhy, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

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Abstract

Mitochondria are energy-generating organelles of the cells – they produce most of the ATP required for various cellular processes. They also have dynamic morphology, and can under fusion and fission, generating structures which range from small and circular to long and reticular. The mitochondrial fission protein Drp1 has been known to affect the fate of a cell during development via the different signalling pathways, such as EGF and Notch. Mitochondrial morphology proteins are likely to produce such effects by interplay with energy metabolism during development. I have used the *Drosophila* oogenesis as a model system to ascertain a link between mitochondrial morphology, metabolism and developmental signalling pathways. *Drosophila* mutants of the electron transport chain, CoVa in Complex IV and Pdsw in Complex I of the electron transport chain show that there is a change in mitochondrial organization and membrane potential in ovarian follicle cells. Notch mediated signalling is delayed in both follicle cells and bristle development in the adult fly. In addition there is an increase in polarized protein distribution on the plasma membrane in these metabolism mutant cells. Future studies on interactions between morphology and metabolism proteins will enable a study of interaction between morphology and metabolism mediated by the mitochondria.

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Introduction

Mitochondria are one of the most important organelles of the eukaryotic cell. These are thought to be endosymbionts, meaning they were once bacteria which were engulfed by other single-cellular organisms (Andersson et al., 2003; Gray et al., 2001; Poole and Penny, 2007). These organelles are thus double-membranous.

Mitochondria are often called as the powerhouses of the cell. This is due to their energy-producing capabilities. These organelles generate most of the adenosine triphosphate (ATP) required by the cell for all its energetic needs. Metabolic energy is required for growth and development and several cellular processes.

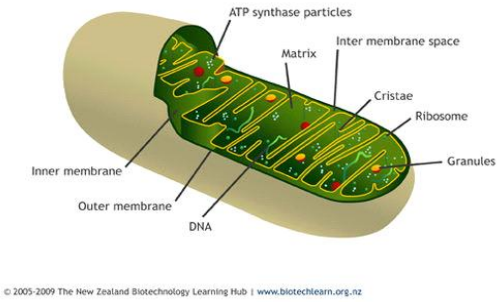

Figure 1: A schematic representation of a mitochondrion. Reproduced from www.bioltechlearn.org.nz

Without the mitochondria, energy is just produced by the process of glycolysis, which occurs in the cytoplasm. This leads to a net output of 2 ATP molecules per molecule of glucose. However in the presence of mitochondria, aerobic respiration can take place, leading to an output of 36 (or 38 in plants) ATP molecules per molecule of glucose (Cox and Nelson, 2004).

The number of mitochondria in each cell depends on the organism, and the tissue type (Voet and Voet, 2010). There are four different compartments in a mitochondrion – the outer membrane, the inter membrane space, the inner membrane, and the matrix (Ventura et al., 2004). The outer membrane is generally smooth, while the inner one is highly convoluted and has folds known as cristae which considerably increase the surface area of the membrane.

Mitochondria are semi-autonomous organelles: they are only partially dependent on the cell for growth and replication. They have their own DNA and ribosomes, and can

synthesize their own proteins. Their genome is quite similar to bacterial genomes (Gray, 1998), which is congruent with the fact that these are endosymbionts.

Apart from generating energy for the cell, mitochondria are also involved in cell signalling, growth and differentiation. They are involved in the process of apoptosis, aging and can regulate the cell cycle as well (McBride et al., 2006).

Mitochondria can be fused or fragmented:

Mitochondria are not static and unchanging – they are dynamic organelles, and change their morphology in living cells by fission and fusion of their membranes (Bereiter-Hahn and Voth, 1994). Several of the proteins involved in this process are dynamins, which are similar to large GTPases (Okamoto and Shaw, 2005). Recent studies indicate that mitochondrial shape and its regulation are important for the physiology of the cell (Cereghetti and Scorrano, 2006) and new proteins needed for this regulation are still being discovered.

For fusion of the mitochondrial outer membrane, there are two important proteins called Mitofusins 1 and 2 (in mammalian systems). Both of these are dynamin-related GTPases (Santel and Fuller, 2000). Inner membrane fusion is carried out by yet another dynamin-related GTPase known as Optic atrophy-1, or Opa-1. (Cipolat et al., 2004)

Mitochondrial fission is regulated by cytosolic dynamin-related protein-1 (Drp1) (Smirnova et al., 2001) and an outer membrane protein called FIS1 (James et al., 2003) in mammalian cells. Drp1 must be activated and recruited onto the mitochondria to cause fission and a few reports suggest that FIS1 is the receptor for Drp1 on the outer membrane (Yoon et al., 2003).

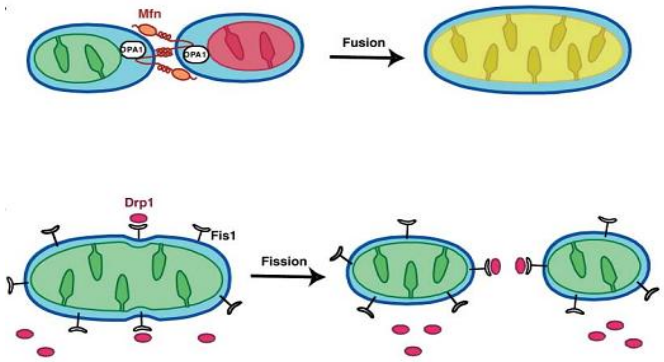

Figure 2: Mitochondrial dynamics – fusion and fission and the proteins involved. Reproduced from Chen and Chan 2005 (Chen and Chan, 2005)

Why are dynamic mitochondrial networks evolutionarily conserved? Why does fusion and fission take place at all? One reason could be that mitochondrial connectivity and fusion leads to genetic complementation, i.e. if two mitochondrial genomes have different defects, then once fusion occurs, each genome can code for whatever component is missing or defective in the other. Another hypothesis (Skulachev, 2001) proposes that if the mitochondrial network is connected, then even hypoxic regions of the cell might be able to generate ATP, since the mitochondrial membrane potential could be transmitted across the connected network. An important role of fission is during mitosis and meiosis, when mitochondria need to be distributed to daughter cells (Taguchi et al., 2007).

Mitochondrial metabolism and the electron transport chain:

The mitochondrion gets its energy-producing capabilities through the electron transport chain.

The electron transport chain is present in the inner membrane of the mitochondria. This comprises of four different complexes (known as Complex I, II, III and IV) which pass electrons to each other, ultimately converting water to oxygen. A proton gradient is generated across the inner membrane as the complexes pump hydrogen ions from the matrix into the inter-membrane space. This transfer of electrons is coupled to a fifth complex known as the ATP synthase which uses the proton gradient to create ATP from ADP. This coupling between the electron transport chain and the synthesis of ATP is called Oxidative Phosphorylation.

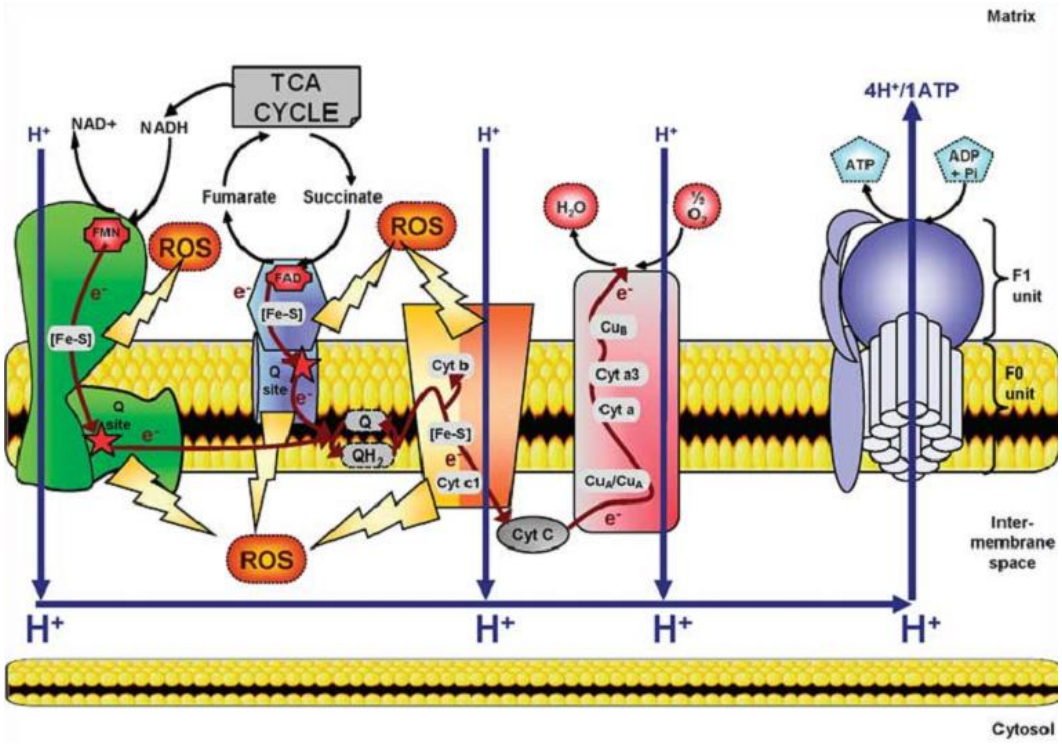

Figure 3: A representation of the electron transport chain in the inner membrane of mitochondria. There are four complexes and the ATP synthase. The direction of movement of electrons and the hydrogen ions are indicated as well. Reproduced from Lemarie and Grimm 2011 (Lemarie and Grimm, 2011)

Mitochondrial morphology and possible regulation of developmental signalling cascades:

Recent work has shown that there is a possible link between morphology of the mitochondria and various cellular signalling pathways. It has been observed that the Hippo pathway plays a role in mitochondrial regulation in *Drosophila* as well as human cells (Nagaraj et al., 2012).

In case of any loss-of-function mutations which are upstream in the Hippo pathway, Yorkie does not get phosphorylated. In this situation Yorkie acts as a transcriptional cofactor and leads to an overgrowth phenotype (Dong et al., 2007; Oh and Irvine, 2008). As this pathway has an extreme effect on growth, and is involved in the progression of cancer, it was a logical leap to study a possible link between this and metabolism, which is why the involvement of mitochondria was hypothesized.

Nagaraj et al. (2012) observed that when Yorkie activity increases, fusion of mitochondria is also increased, and this is due to direct transcriptional activation of *opa1* and *marf,* both of which are mitochondrial fusion proteins. It appears that the Hippo pathway can activate the cell cycle, repress apoptosis, and leads to mitochondrial and metabolic changes, which might all come together to cause tumour growth. Another interesting observation made was that when Yorkie modified mitochondrial morphology, antioxidant enzymes and complex I subunits are upregulated along with a significant reduction in the intercellular ROS levels.

Mitra et al. (2012) showed that Drp1, which is a mitochondrial fission protein, is also linked to certain signalling pathways. (Mitra et al., 2012) This study was done in the epithelial follicle cells of egg chambers of *Drosophila melanogaster*, and the relationship between mitochondrial morphology and determination of cell fate was observed. Mitotic clones of drp1KG in the posterior follicle cells did not show any expression of Hindsight, (which is a homeodomain gene, downstream of Notch) unlike the surrounding background cells. These clones also fail to differentiate. After a series of experiments, it was seen that mitochondrial fission activity is upstream of the Notch signalling pathway which leads to the differentiation of the posterior follicle cells. It was also observed that EGF signalling is upstream of mitochondrial morphology changes.

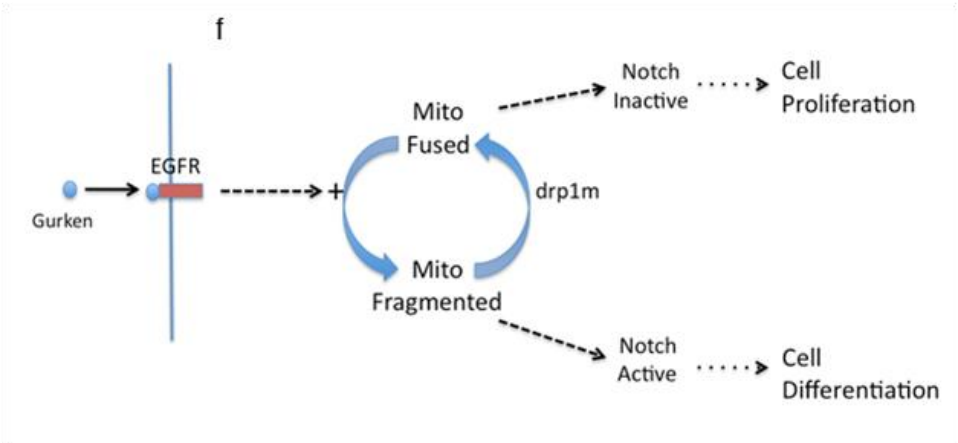

Figure 4: The role of Drp1 mutation on mitochondrial morphology, and how it affects the Notch signalling pathway, and consequently cell fate. Reproduced from Mitra et al 2012.

Thus, it can be clearly seen from literature that there is a connection between the morphology of mitochondria, and various signalling pathways.

Mitochondrial metabolism and possible regulation of developmental signalling cascades:

There have been a few instances where proteins which are involved in the production of energy in the mitochondria have been linked to signalling pathways as well.

It has been observed that mutations in certain metabolic proteins of the electron transport chain can cause the cell cycle to arrest in the G1-S stage (Owusu-Ansah et al., 2008a). Here, two different proteins were studied, and while they both block the cell cycle at the same stage, it is by two different mechanisms. Pdsw is a subunit of Complex I, and when this is knocked down, the ROS production of the cell goes up nearly fivefold. The ATP levels, on the other hand, do not show any significant change. The increased ROS activates the JNK pathway, and once this happens, the levels of Dacapo rise. Dacapo is an inhibitor of the Cyclin E-CDK2 complex. Since Cyclin E is required to cross the G1-S check point, the cell cycle gets arrested at this stage.

CoVa is a subunit of Complex IV, and encodes for cytochrome c oxidase Va. A loss-of-function mutation in this causes the ATP production of the cell to reduce by 60%. The ROS levels in this case do not rise; instead, there is actually a slight drop which is observed. CoVa mutations also cause a cell cycle block, but in this case, it is due to the lowered concentration of ATP. This leads to an increase in the amount of AMP, which activates AMPK and then p53. This causes a loss of Cyclin E, again blocking the G1-S transition.

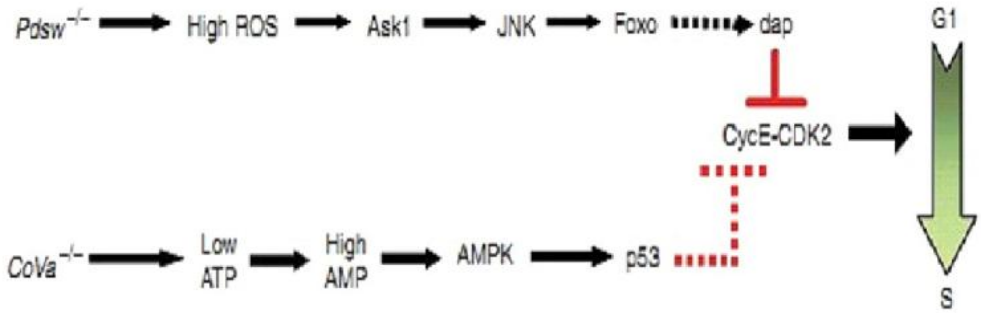

Figure 5: A model depicting two different pathways by which mitochondrial metabolism proteins arrest the cell cycle. Reproduced from Owusu-Ansah et al 2008 (Owusu-Ansah et al., 2008a)

These *in vivo* experiments performed in *Drosophila* lead us to the conclusion that the mitochondrion coordinates signalling pathways for halting the progression of the cell cycle, which are activated by at least two different metabolic signals - ROS and ATP, for halting the progression of the cell cycle. It can be hypothesized that when the mitochondrion is not performing at optimal levels, it might simultaneously activate both pathways because of the increased ROS and AMP levels, and arrest the cell in the G1 stage. This way, the cell can ensure that the ATP-demanding process of mitosis is delayed, and repairs can be carried out on any oxidatively damaged molecules.

This sort of regulation could serve several *in vivo* functions. For example, under hypoxia conditions, cells might be able to survive energy-starved conditions by halting the cell cycle temporarily, and once normal oxygen levels are obtained, can continue proliferating. Also, the functioning of the mitochondria might be used to control when the cell proliferates and when it differentiates. There might be even more signalling mechanisms by which the mitochondrion ensures energy control, by balancing the progression of the cell cycle with the specification of cell fate.

It has been observed that impairments in the mitochondrial electron transport chain, such as in proteins like Pdsw (a component of complex I), CoVa (a component of Complex IV) and mRpL4 (a mitochondrial ribosomal protein) in cells which are Ras-activated can cause over-growth in surrounding tissue (Ohsawa et al., 2012). This was studied in the larval eye discs of *Drosophila*.

The mechanism for this was found out to be the activation of the JNK pathway, via induction of oxidative stress. The JNK pathway was seen to interact with Ras, and leads to the inactivation of the Hippo pathway, which induces certain targets of Yorkie, causing overgrowth of the surrounding tissue.

This study shows that mitochondrial dysfunction is involved with several different signalling pathways and might be linked to certain kinds of cancer. Mutations in mitochondrial electron transport chain are known to arrest the cell cycle, (Owusu-Ansah et al., 2008a) and it was observed that E-cadherin and discs large (Dlg) were also down-regulated in the cells with activated Ras in the background of mitochondrial metabolism defects. This suggests that there might be some kind of disruption in the epithelial integrity, and could also add to the inactivation of the

Hippo pathway. These results also support the Warburg hypothesis about how defects in mitochondria can play a role in causing cancer.

Role of Reactive Oxygen Species in developmental regulation of signalling cascades:

Reactive Oxygen Species, or ROS refer to several different molecules such as the superoxide radical O_2^-, the hydroxyl radical HO, nitric oxide NO and the carbonate radical CO_3^-, most of which are produced by the mitochondria during the process of oxidative phosphorylation (Murphy, 2009). These molecules are thought to play a crucial role in signalling pathways and cell functioning, but also have a negative impact on the state of the cell by triggering degradation processes such as mitochondrial dysfunction, apoptosis and necrosis, aging and disease.

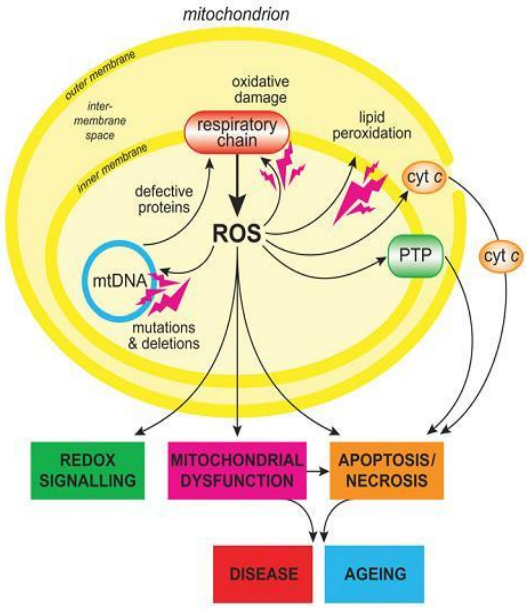

Figure 6: An overview of ROS production in mitochondria. ROS production can lead to oxidative damage to mitochondrial proteins, membranes and DNA causing a lot of problems in ATP production and metabolism. PTP: permeability transition pore; cyt c: cytochrome c. Adapted from Murphy 2009 (Murphy, 2009)

Complex I and Complex III are known as producers of ROS, (Boveris et al., 1976; Lambert and Brand, 2004), while Complexes II and IV either do not produce ROS

themselves, (Barros, 2003; Yankovskaya et al., 2003) or, in the case of Complex IV, might even act as a mitochondrial antioxidant (Skulachev, 1998).

Knowing this, it can be expected that Pdsw, being a component of Complex I, will increased the ROS levels when it is knocked down, which CoVa, being in Complex IV, might either have no effect on ROS quantities when mutated, or possibly reduce the levels of ROS in the cell. This is consistent with what is observed in mutant clones in the larval eye discs of Drosophila (Owusu-Ansah et al., 2008a).

To protect itself from the harmful effects of ROS, such as damage to proteins, lipid membranes and nucleic acids (Orrenius et al., 2007), the cell has developed some defence mechanisms. Superoxide dismutase (SOD) is a protein which reduces the superoxide radical to hydrogen peroxide, and while this is also a species of ROS, it is less reactive. Enzymes such as catalase and glutathione peroxidase further reduce hydrogen peroxide to water.

Motivation for the project

Based on known information about mitochondrial morphology, metabolism and signalling, several interesting questions arise. Is there any link between the shape of mitochondria and the energy output? Do metabolic proteins, for instance Pdsw and CoVa have a role in development? What is the state of the mitochondria and the cell if metabolic proteins are knocked down, and if these have a bearing on morphology, can any resulting phenotypes be rescued by morphology proteins?

Goals of the project

Some effects of CoVa and Pdsw have been studied and analysed in terms of mitochondrial activity and morphology, developmental signalling pathways, cell cycle and some polarity markers.

Materials and Methods

1) *Fly stocks:*

- *w; FRT82B CoVa/TM6, Tb*
- *y[d2] w[1118] P{ry[+t7.2]=ey-FLP.N}2 P{ry[+t7.2]=GMR-lacZ.C(38.1)}TPN1; P{w[+mC]=lacW}Pdsw[k10101] P{ry[+t7.2]=neoFRT}40A / CyO y[+]*
- *hsflp; tub Gal4 UAS CD8 GFP/CyO; Gal80 FRT 82B/TM6*
- *yw hsflp; FRT40A tubGal80/CyOActGFP; tubGal4UASmCD8GFP/TM6*

2) *Creating fly lines:*

Mutant clones were created using the Mosaic Analysis using a Repressible Cell Marker, or the MARCM technique. It combines the GAL4/UAS, GAL80, and FLP/FRT systems to label the mutant cells (Duffy, 2002). This is a very useful strategy, since it positively labels the cells of interest. It targets a UAS-FLP responder with a GAL4 driver specific to the tissue of interest. Mitotic recombination then takes place in the target tissue.

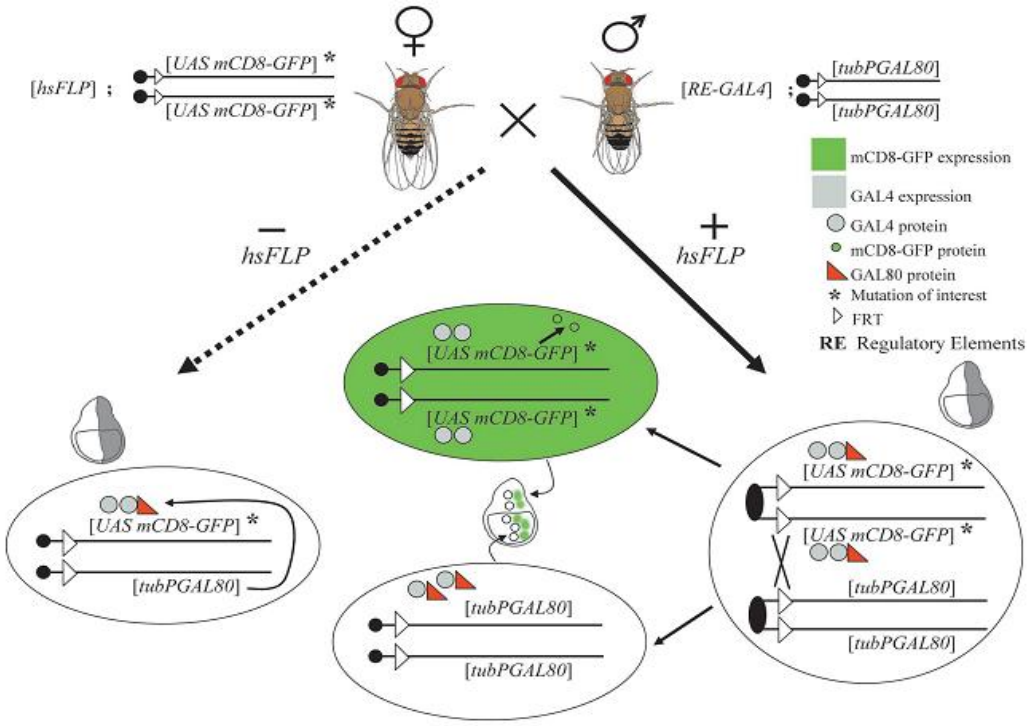

Figure 7: A schematic of the MARCM strategy. Reproduced from Duffy, 2002 (Duffy, 2002).

For the MARCM strategy, they fly six transgenes: two for homologous FRT sites, and one each for the FLP recombinase, a UAS marker, a Gal4 driver and the *tubP-GAL80* transgene. The GAL4-induced UAS-marker expression is prevented by the GAL80 in heterozygous (background) cells, and hence these will be GFP-negative. It is only in homozygous mutant cells that the GAL80 transgene is absent, and the GFP is expressed.

3) Heat Shock:

To obtain follicle cell clones in CoVa and Pdsw mutant flies, the F1 generation was given a heat shock of 37.5°C for 1 hour. Females were kept in a food vial with yeast and some males for up to 10 days, so that they produced several eggs.

4) Ovary Dissection:

Drosophila oogenesis was used as a model system. This is due to the fact that the epithelial follicle cells of the egg chamber are big enough to visualize the mitochondrial morphology clearly. Also, since there are several egg chambers at different stages of development (Roth, 2001), these can be observed together, and comparison studies can be done quite easily in a stage-specific manner without having any experimental errors which might arise if multiple experiments were required.

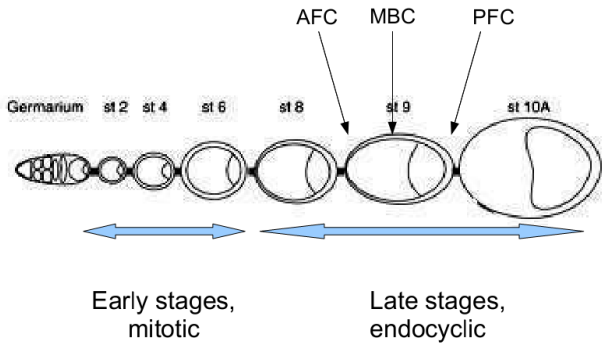

Figure 8: Different stages of oogenesis. In later stage egg chambers, the position of the follicle cells is indicated as well: anterior follicle cells (AFC), main body cells (MBC) and posterior follicle cells (PFC).

The protocol for dissecting ovaries is as follows:

- An adult female fly grown on yeast for up to 10 days was lifted by the wing using forceps and was placed in some Schneider's medium.
- The head and thorax was held firmly by the forceps in the left hand, and with another pair of forceps in the right hand, the 2nd segment from the abdomen was pulled.
- Two almond-shaped structures (the ovaries) were released.
- The fat and extra tissue around the ovaries was removed.
- The fibrous sheath was removed by holding the ovary with forceps, and slicing it longitudinally using a pin.
- The ovaries were stored in Schneider's medium.

5) Staining:

Several different antibodies were used for immunostaining. For looking at mitochondrial activity via ATP levels, AMPK antibody was used. To check Notch signalling, Hindsight (Hnt 1G9-c, anti-mouse, 1:100 dilution, DSHB), Cut (Cut 2B10-c, anti-mouse, 1:100 dilution, DSHB), Notch Intracellular Domain or NICD (Notch intra C17.9C6-c, anti-mouse, 1:100 dilution, DSHB) and Notch extracellular Domain or NECD (Notch extracellular domain F461.3B-c, anti-mouse, 1:100 dilution, DSHB) were used. To check for the EGF pathway, ERK antibody was used. Mitochondria was labelled using Streptavidin (Streptavidin 568, 1:1000 dilution, Invitrogen), and the DNA was labelled with Hoechst. Polarity markers such as Cadherin (Dcad2, anti-rat, 1:10 dilution, DSHB) and Dlg (4F3 Dlg, anti-mouse, 1:10 dilution, DSHB) antibodies were used.

- After obtaining the ovaries, the Schneider's medium was removed. The ovaries were fixed in 4% HCHO in PBS for 15 minutes.
- The ovaries were washed thrice in PBS containing 0.3% Triton-X (PBST) for 5 minutes each, and then blocked in 2% BSA in PBST on the shaker for 30 minutes.
- The block was removed, and the primary antibody was added at a specified dilution in 2% BSA in PBTA. This solution was left overnight at 4 °C on a shaker.

- The antibody was removed, and the ovaries were washed thrice in 0.3% Triton in PBST for 5 minutes each.
- Secondary antibody was added at a dilution of 1:1000 in 0.3% Triton solution in PBS, and left for 1 hour on the shaker.
- The secondary antibody was removed, the ovaries washed, and Hoechst (again at a dilution of 1:1000) was added and left for 10 minutes on the shaker. The ovaries were washed again.

6) Mounting:

A drop of antifade (Slowfade) was placed on a microscope slide, and the ovaries were spread using forceps and needle so that the single ovary developmental stages were visible. A cover slip was placed on top, and the sides were sealed with nail polish.

7) Imaging:

Imaging was done using a Zeiss laser scanning confocal microscope. The tracks used were 405, 488, 568 and 633 nm, for Hoechst (which binds to DNA in the nuclei), GFP (which was already present and labelled all the mutants clones), and the other two channels for specific antibodies.

Other Assays:

ROS detection: Live Imaging DHE assay for detection of ROS, modified from Owusu-Ansah's Nature Methods protocol (Owusu-Ansah et al., 2008b):

- Ovaries were dissected in Schneider's medium
- 0.3 M stock solution of DMSO in Schneider's medium was prepared
- Working solution: the stock solution was diluted 20,000 times in the same medium.
- The ovary tissue was incubated in the working solution of the dye for 5 minutes in a dark chamber at room temperature on an orbital shaker.
- The tissue was washed thrice for 5 minutes in Schneider's medium in a dark chamber at room temperature on an orbital shaker

- Poly-l-lysine was added on a coverslip bottom chamber and left for 5 minutes, before washing off with PBS.
- The tissue was mounted on the coverslip bottom chamber.
- The ovarioles were separated, and some amount of Schneider's was left in the chamber to prevent the tissue from drying up.
- Confocal imaging was performed

Membrane Potential: An assay for the detection of the mitochondrial membrane potential, using MitoTracker Red CMXRos (M 7512 – Invitrogen) was performed. This is a cell-permeant probe which can be retained in the mitochondria even after fixation. This probe labels active mitochondria, based on the membrane potential across the inner membrane. It contains a mildly thiol-reactive chloro-methyl moiety for labelling mitochondria (Invitrogen).

The protocol is as follows:

- Preparation of stock solution: the dye was warmed to room temperature, and then he lyophilized product was dissolved in anhydrous DMSO to a final concentration of 1 mM.
- Working solution: The stock solution was diluted 5,000 times to prepare a working solution of 200 nM.
- This staining solution was prewarmed to 37°C.
- Drosophila ovaries were dissected and incubated in the staining solution for 30 minutes.
- The tissue was washed 3 times in Schneider's medium for 5 minutes each.
- The tissue was fixed in a solution of 3.7% HCHO in PBS at 37°C for 15 minutes
- The tissue was rinsed once in Schneider's medium
- The sample was then permeabilized in a solution of 0.3% Triton X-100 in PBST, along with the addition of Hoechst at a dilution of 1:1000 to stain the DNA. Only one wash of 10 minutes' duration was performed.
- The sample was mounted on a slide

EdU staining: A protocol to observe the S phase of the cell cycle in ovaries was standardized. Imaging kit used for this was the Click-iT EdU Alexa-Fluor 594 Kit (C 10339) by Invitrogen.

EdU stands for 5-ethynyl-2'-deoxyuridine, and is a nucleoside analogue of thymidine (Invitrogen). This gets incorporated into the DNA during active DNA synthesis. He detection is based on a click chemistry reaction – it is a copper-catalysed covalent reaction between an azide (present in the Alexa Fluor dye), and an alkyne (in EdU).

The components of the kit are as follows:

(A) EdU
(B) Alexa Fluor azide
(C) DMSO
(D) Click-iT EdU reaction buffer
(E) CuSO4
(F) Click-iT EdU buffer additive
(G) Hoechst 33342

The standardized protocol is as follows:

Preparation of stock solutions:

- 10 mM stock solution of EdU was prepared.
- A working solution of reaction buffer was prepared by adding 4 ml of the buffer to 36 ml of deionized water.
- A 10X stock solution of buffer additive was prepared.
- 70 µl of DMSO was added to the Alexa Fluor dye and mixed well.

Labelling with EdU:

- A 2X working solution of EdU was prepared, to make up a final concentration of 10 µM.
- The ovaries were dissected, and then incubated in the 10 µM solution for 1 hour.

Fixation and permeabilization:

- After incubation, the solution was removed. 1 ml of 3.7% HCHO in PBS was added, and the tissue was incubated in this for 15 minutes.
- The fixative was removed.
- The tissue was washed twice in 1 ml of 3% BSA in PBS.
- The wash solution was removed. 1 ml of 0.5% Triton X-100 in PBS was added and left undisturbed for 20 minutes

EdU detection:

- A 1X solution of Click-iT EdU buffer additive was prepared by diluting the 10X solution in deionized water.
- A reaction cocktail was prepared by adding the following solutions in the listed order to make up a solution of approximately 500 µl
  (1) 1X Click-It reaction buffer (430 µl)
  (2) CuSO4 (20 µl)
  (3) Alexa Fluor azide (1.2 µl)
  (4) Reaction buffer additive (50 µl)
- The permeabilization buffer was removed from the tissues, and two washes with 1 ml of 3% BSA in PBS were performed. The wash solution was removed.
- The Click-iT reaction cocktail was added to the tissue within 15 minutes of its preparation. The tissue was thus incubated for 30 minutes at room temperature in a dark chamber.
- The cocktail was removed, and the tissue was washed once with 1ml of 3% BSA in PBS.

DNA staining:

- The tissue was washed once with 1 ml of PBS
- Hoechst was diluted 1:2000 in PBS to get a 1X solution (concentration being 5 µg/ml)
- 1 ml of 1X Hoechst was added to the tissue, and it was incubated for 30 minutes at room temperature in a dark chamber
- Hoechst was removed. Tissue was washed twice with 1 ml of PBS.

The tissue was then mounted on a slide, and imaged using a confocal microscope.

Calcium levels: Another assay to check for calcium levels was standardized during the course of this project. This assay used a fluorescent dye called Fluo 4. The protocol is as follows: (Invitrogen)

- 1 µM solution of Fluo 4 was prepared by diluting in Schneider's medium.
- Ovaries were dissected out of the fly, and the tissue was incubated in the Fluo 4 solution for 30 minutes in a dark chamber on an orbital shaker.
- The tissue was washed thrice in Schneider's medium for 5 minutes each.
- The tissue was then mounted on a coverslip bottom chamber in Schneider's medium. Some of the medium was removed so as to ensure that the tissue did not float. However, if too much medium is removed, the tissue dries up, so an optimum is required.
- Imaging was performed immediately since this is a live assay.
- Calcium levels in wild type follicle cells seen in different developmental stages.

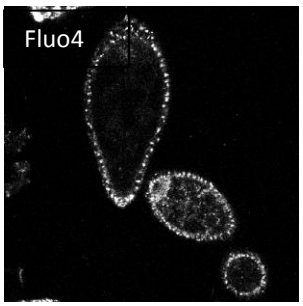

Figure 9: Fluo 4 staining for Calcium in wild type ovaries. The distribution of calcium does not look completely mitochondrial; it seems to be present in the cytoplasm also.

ATP assay: An assay to measure the amounts of ATP produced was also standardized. This uses an ATP detection kit. (Invitrogen)

This is based on a bioluminescence assay using the enzyme luciferase, which catalyses the conversion of luciferin to oxyluciferin, which utilizes ATP. This way it is possible to get a quantified value of how much ATP is used.

The kit consists of the following:

(A) D-luciferin

(B) Luciferase, firefly recombinant
(C) Dithiothreitol (DTT)
(D) 5 mM ATP
(E) 20X reaction buffer

First, a standard curve is required, which shows the amounts of luminescence for different quantities of ATP. The procedure is as follows:

Reagent Preparation:

- 1 ml of 1X reaction buffer was prepared by diluting the 20X solution in deionized water
- This was added to 1 vial of D-luciferin. The vial was protected from light.
- A 100 mM DTT stock solution was prepared, and then 10 aliquots of 160 µl were made and stored at less than - 20°C.
- Low concentration ATP standard solution was made by diluting the 5 mM ATP solution in deionized water. Typical concentrations range from 1 nM to 1 µM.

To prepare the standard reaction solution of 10 mL, the following components were added in the indicated amounts:

- 8.9 ml of deionized water
- 0.5 ml of 20X reaction buffer
- 0.1 ml of 0.1 M DTT
- 0.5 ml of 10 mM D-luciferin
- 2.5 µl of firefly luciferase (5 mg/ml stock solution)

The tube was inverted to mix the contents, as the enzyme would get denatured on vortexing.

For generating a standard curve,

- Appropriate volume of the standard reaction solution made in the last step was added in the luminometer, and the background luminescence was measured.
- Desired amounts of diluted ATP standard solution were added, and luminescence readings were noted. These volumes must always be less than 10% of the total assay volume.

- The background luminescence was subtracted.
- A standard curve was generated for a series of ATP concentrations.

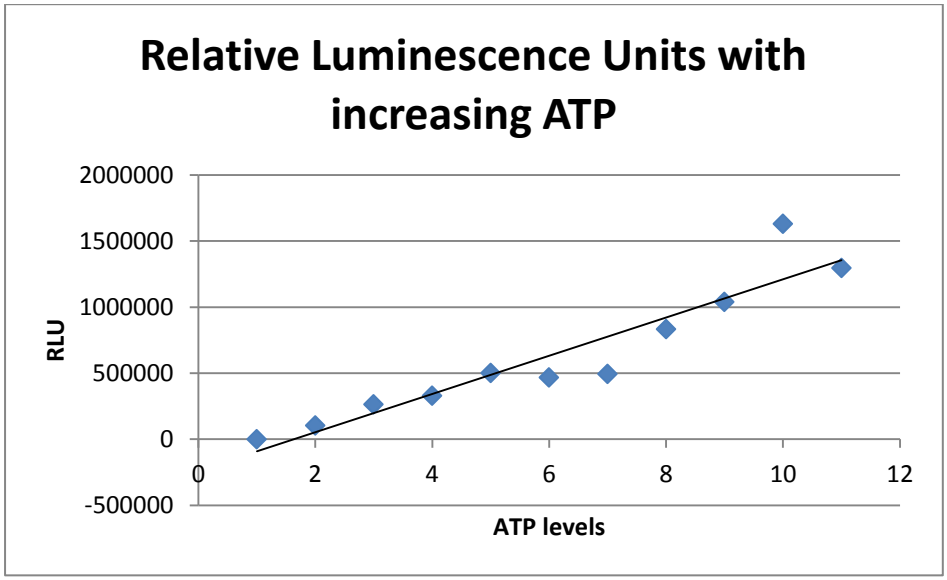

Figure 10: Standard curve generated for relative luminescence units as a function of increasing ATP levels.

For sample analysis, the ATP containing samples must be substituted for the ATP standard solution, keeping the volumes same as before. Now the ATP amounts in the sample can be calculated from the standard curve.

The sample analysis was not performed, because it was tough to estimate how much of ovary tissue should be crushed to obtain ATP values in the range of the standard curve. Protein estimation was considered, but this strategy would not be useful for CoVa and Pdsw MARCM clones (as we require the entire tissue to have the defects). Hence this assay could be performed on RNAi lines for CoVa and Pdsw, driven by tissue-specific drivers.

Image Analysis:

A stage-specific analysis was carried out for some of the observations. The MARCM clones in the developing egg chambers were classified as early/transition and late stages. For graphical analysis of the later stages, these were further categorized based on the position in the egg chamber – as anterior, main body, or posterior follicle cell clones.

For analysing intensities of a specific antibody in the clone with respect to the heterozygous background, Image J software was used. An area was chosen over the clone region, making certain the boundaries of cells were taken in the area, and the mean intensity of that area was calculated. This intensity was a mean of all the intensities per pixel in the chosen area. This way the intensity got normalized over the entire cell, no matter where the antibody staining is localized within the cell.

This procedure was repeated over a few cells in the background, in the same stage and position (anterior, main body or posterior) as the clone, to get the background mean intensity. Then a ratio was taken of the mean intensity in the clone region to the mean intensity in the corresponding background. This was done to normalize the intensities over their backgrounds, and not have any disparities between images taken under different conditions of laser power and noise.

Since ratios have been taken, all the values obtained are greater than, equal to or less than 1. This provides a very clear indication of whether the intensity had increased or decreased in the clones as compared to their heterozygous background. Graphs were plotted in MS PowerPoint.

Results

Electron transport chain mutants were created and studied in follicle cells during oogenesis. Several different immunostaining assays and live imaging assays were performed on these to study mitochondrial activity and morphology, cell cycle and DNA size, Notch and EGF signalling as well as plasma membrane polarity.

Analysis of mitochondrial electron transport chain mutants on mitochondrial activity and morphology:

The electron transport chain present in the inner membrane of the mitochondria is where production of energy takes place. This is where the four complexes transport electrons to each other, generating a proton gradient across the membrane which is then used to drive the ATP synthase and produce ATP. In this process oxygen is also reduced to water. If this reduction does not occur properly, then ROS are produced. Since CoVa is a part of Complex IV and Pdsw is a part of Complex I of the

electron transport chain, knocking these proteins down might cause several problems: increased production of ROS, change in the ATP output, and disrupted mitochondrial membrane potential. It has been seen that Pdsw mutants in developing eye discs have a fivefold increase in ROS levels, while the CoVa mutants show no change (Owusu-Ansah et al., 2008c).

1) ROS levels: ETC mutants might cause the oxygen to be only partially reduced, leading to elevated levels of ROS. This is especially possible for Pdsw knockdown since it is present in Complex I, and this complex is known to increase ROS (Boveris et al., 1976). Hence, measuring ROS levels in follicle cell clones mutant for CoVa and Pdsw might give a good indication of how the mitochondria are functioning in the absence of these proteins. This is being performed using dihydroethidium (DHE), which reacts with any superoxide ions present to form a fluorescent product called 2-hydroxyethidium. An analysis of uptake of DHE in CoVa and Pdsw mutant follicle cells (marked with GFP) shows no difference as compared to the background control cells (negative for GFP). A careful characterization of levels of DHE uptake was carried out in early and late mutant cells and no appreciable accumulation of ROS was obtained in cells mutant for CoVa [Figures 11 and 12] and Pdsw [Figures 13 and 14].

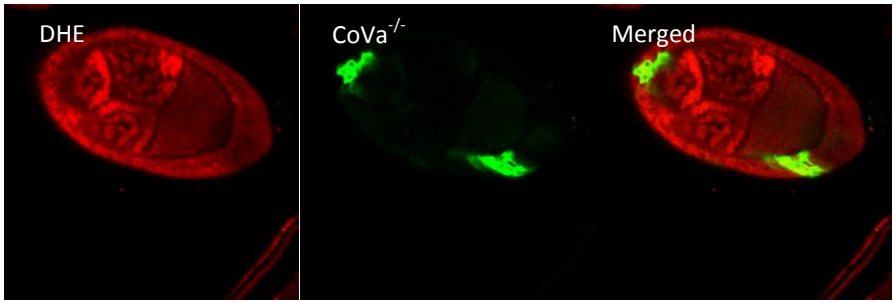

Figure 11: ROS levels depicted by the levels of DHE staining in early stage CoVa clones. There is no significant change in the staining in clones with respect to the background.

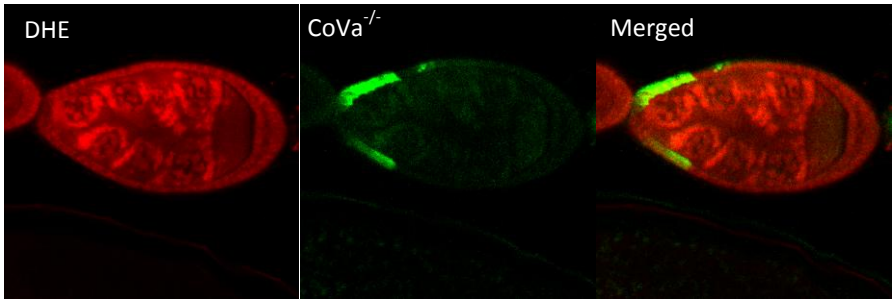

Figure 12: ROS levels depicted by the levels of DHE staining in late stage CoVa clones. There is no significant change in the staining in clones with respect to the background.

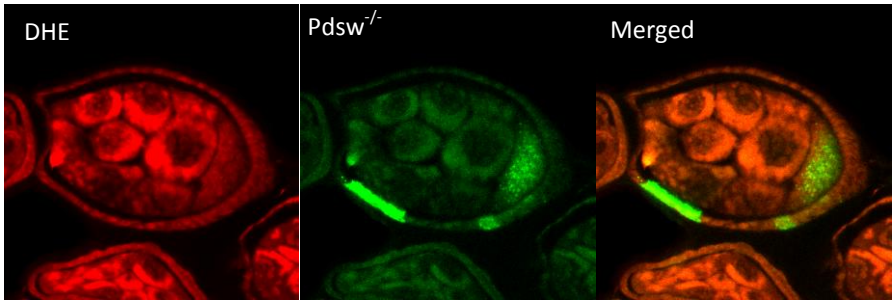

Figure 13: ROS levels depicted by the levels of DHE staining in early/transition stage Pdsw clones. There is no significant change in the staining in clones with respect to the background.

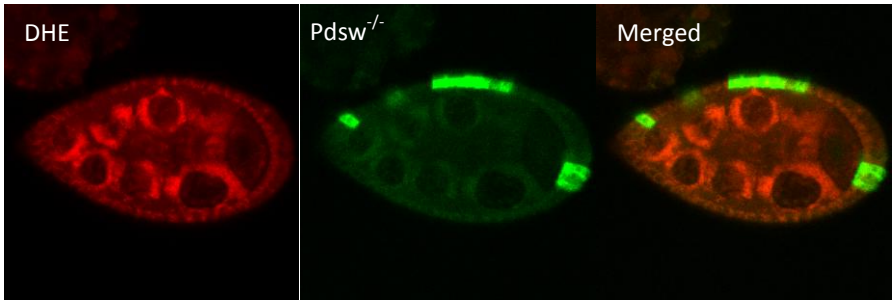

Figure 14: ROS levels depicted by the levels of DHE staining in late stage Pdsw clones. There is no significant change in the staining in clones with respect to the background.

2) ATP levels: Electron transport chain mutants could also cause changes in the net production of ATP by the mitochondria. This is a very direct indication of the quality of mitochondrial function. An indirect read-out of ATP was obtained via immunostaining with AMPK. AMPK senses and responds to the AMP to

ATP ratio, and when ATP levels go down; AMPK levels are expected to increase. In mitochondrial metabolism mutants, it is expected to see elevated AMPK staining. However there was no change in levels of AMPK in early or late follicle cells mutant for CoVa suggesting that there is no significant loss of ATP to activate AMPK [Figures 15, 16 and 17].

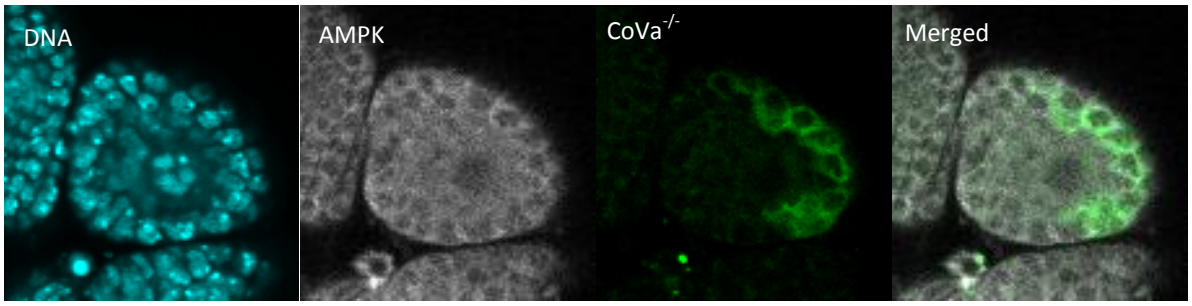

Figure 15: AMPK staining in early stage CoVa clones. There is no significant change in the staining in clones with respect to the background.

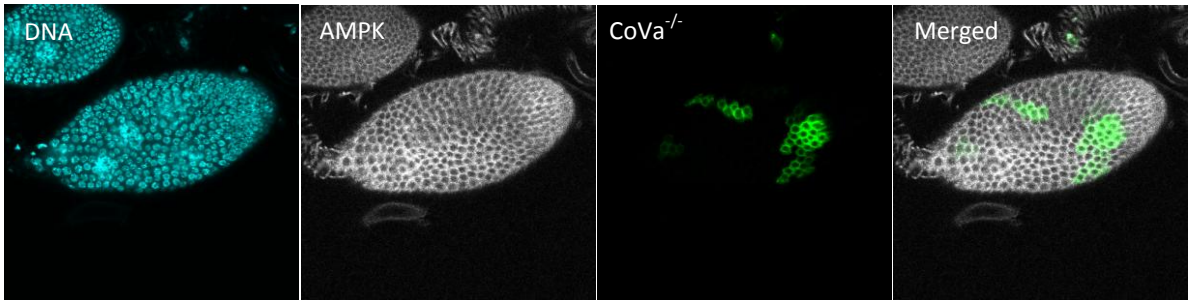

Figure 16: AMPK staining in late stage CoVa clones. There is no significant change in the staining in clones with respect to the background.

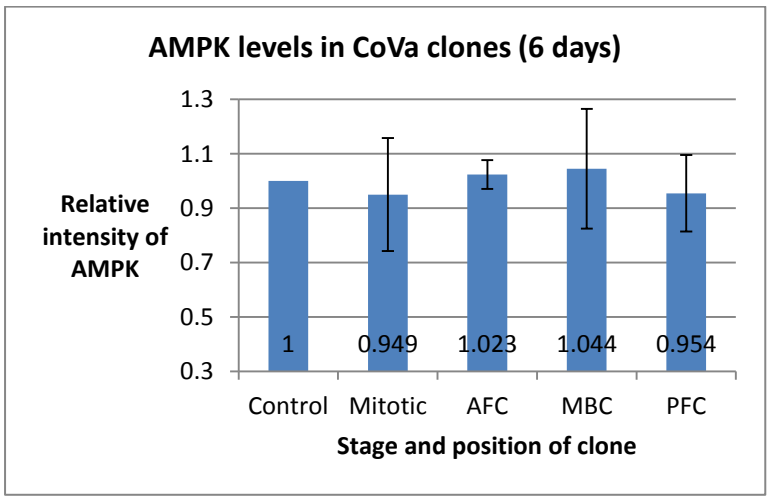

Figure 17: Relative levels of AMPK in CoVa clones: the levels remain close to 1 in all stages

3) Mitochondrial membrane potential: If the electron transport chain does not function properly, then the complexes may not pump enough hydrogen ions into the intermembrane space and a normal proton gradient across the membrane may not be obtained. Therefore checking the membrane potential is also a good indicator of mitochondrial functioning and its possible disruption due to mutations in the ETC proteins. This is checked by CMXRos MitoTracker Red, which is a cell-permeant probe and can be retained in the mitochondria even after fixation. This probe labels active mitochondria, based on the membrane potential across the inner membrane. It contains a mildly thiol-reactive chloro-methyl moiety for labelling mitochondria (Invitrogen). The protocol given by Invitrogen was followed. A decrease in dye intensity and therefore membrane potential was observed in follicle cells mutant for CoVa (GFP positive) at early mitotic and late follicle cell stages compared to control background cells (negative for GFP) [Figures 18 and 19].

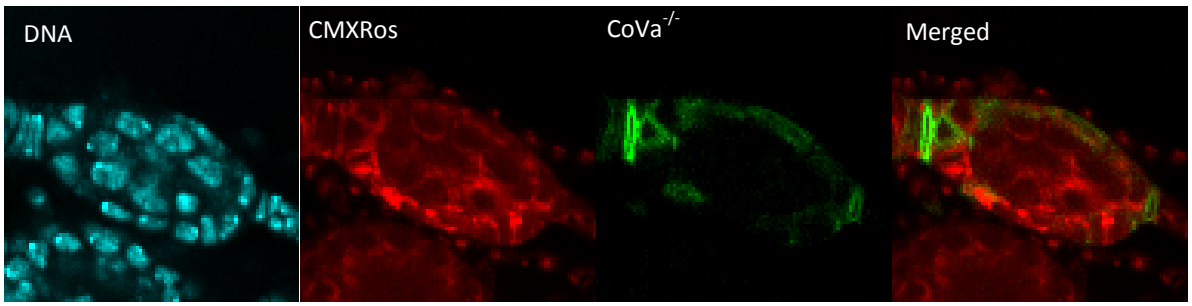

Figure 18: Mitochondrial membrane potential depicted by a CMXRos probe in early stage CoVa clones. The probe labels only active mitochondria. It is observed that the mitochondria in the clones are not active, in contrast to those in the background which are labelled with the probe.

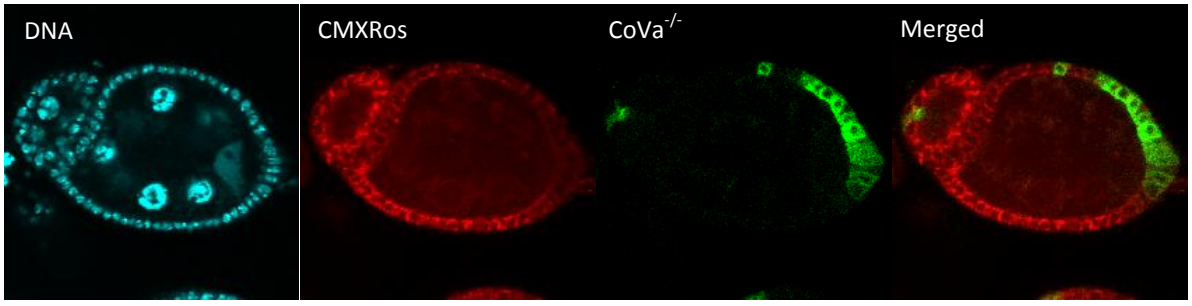

Figure 19: Mitochondrial membrane potential depicted by a CMXRos probe in late stage CoVa clones. The probe labels only active mitochondria. Mitochondria in the clones are not active, but the background cells are.

Analysis of mitochondrial electron transport chain mutants on mitochondrial morphology:

It might be possible that there is a link between how much energy is being produced by the mitochondria and its morphology. It has been observed that in mammalian cells, mitochondria undergo fusion which is dependent on the membrane potential (Ishihara et al., 2003). The membrane potential is dependent on the functioning of the ETC components. To check if the mitochondrial shape is different in the ETC mutant clones, immunostaining was performed with Streptavidin. This binds very strongly to biotin, which is present in the mitochondria. A change in the morphology and distribution of mitochondria was observed in follicle cells mutant for CoVa (GFP positive) at late follicle cell stages as compared to control background cells (negative for GFP) [Figures 20, 21]. It is seen that the distribution of mitochondria in the mutant clones look circular and more evenly distributed when compared to the background. Also, there was an increase in dye intensity in follicle cells mutant for CoVa at late follicle cell stages as compared to control background cells [Figures 22, 23].

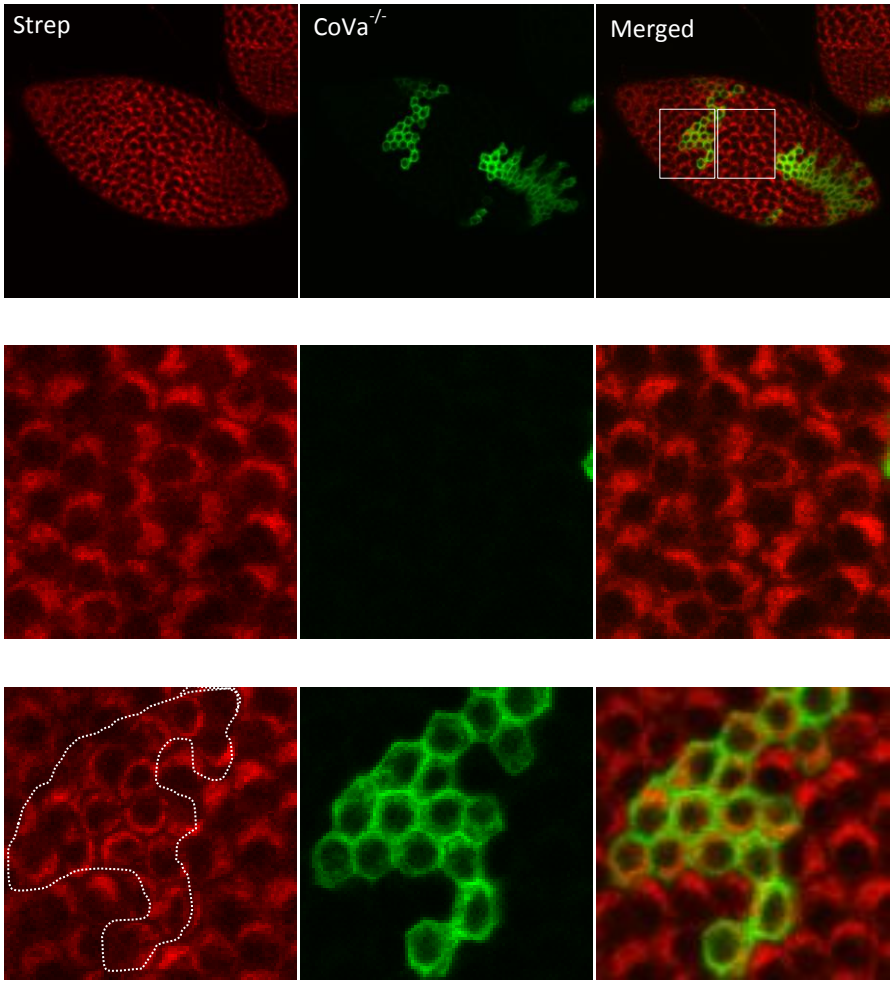

Figure 20: Mitochondrial morphology depicted by Streptavidin staining in later stage CoVa clones. The morphology is circular and more distributed in the clones, as compared to polarized and crescent-shaped in the background.

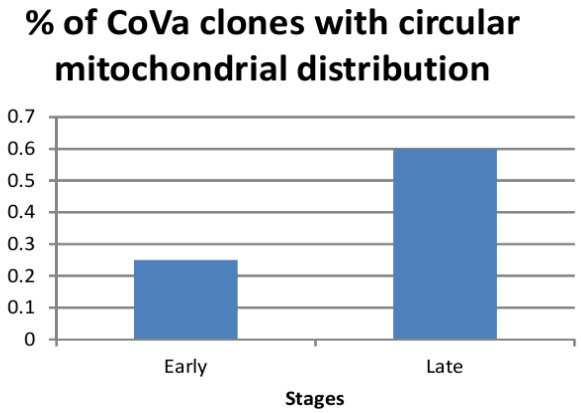

Figure 21: The percentage of CoVa clones which have a circular distribution, in a stage-specific manner. In the later stages, around 60% of clones have this distribution.

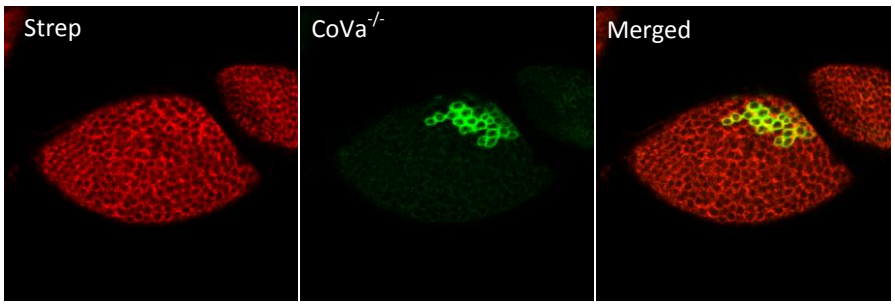

Figure 22: Intensity of mitochondrial staining in later stage egg chambers. The intensity seems more in the CoVa clones as compared to the background.

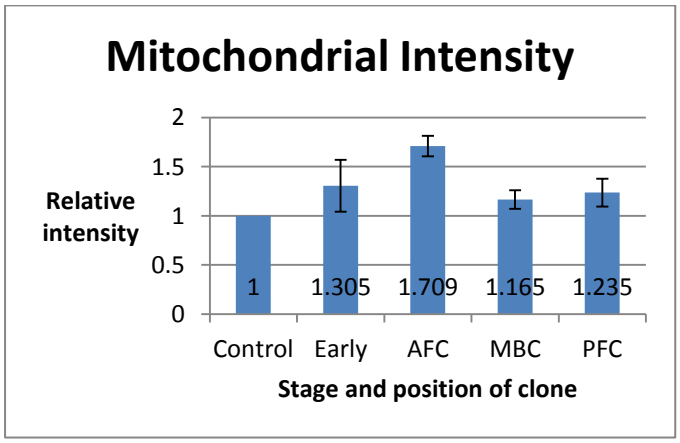

Figure 23: Relative intensity of mitochondrial staining in CoVa clones as compared to the background. It increases in later stages, and seems more in the AFCs.

Analysis of cell cycle and DNA size of follicle cells in electron transport chain mutant animals:

CoVa and Pdsw have been observed to block the cell cycle in the G1-S transition phase (Owusu-Ansah et al., 2008a) in the larval eye discs, and it would be interesting to check if this holds for the cell cycle as well. It could be the case that any defects in signalling might be a result of stalled or slowed cell cycle. DNA size is a good indication of the health of the cell – if the nucleus is very small in the clones with respect to the background, it could indicate cell death. DNA of follicle cells in ETC mutant clones has been observed by staining with Hoechst, and the size has been measured using ImageJ software. An assay to study the cells in the S phase of the cell cycle using EdU staining has also been standardized.

1) DNA size of follicle cells in clones at different developmental stages. A decrease in the size of the nuclei was observed in follicle cells mutant for CoVa (GFP-positive) at late follicle cell stages as compared to control background cells (Negative for GFP) [Figures 24, 25, 26].

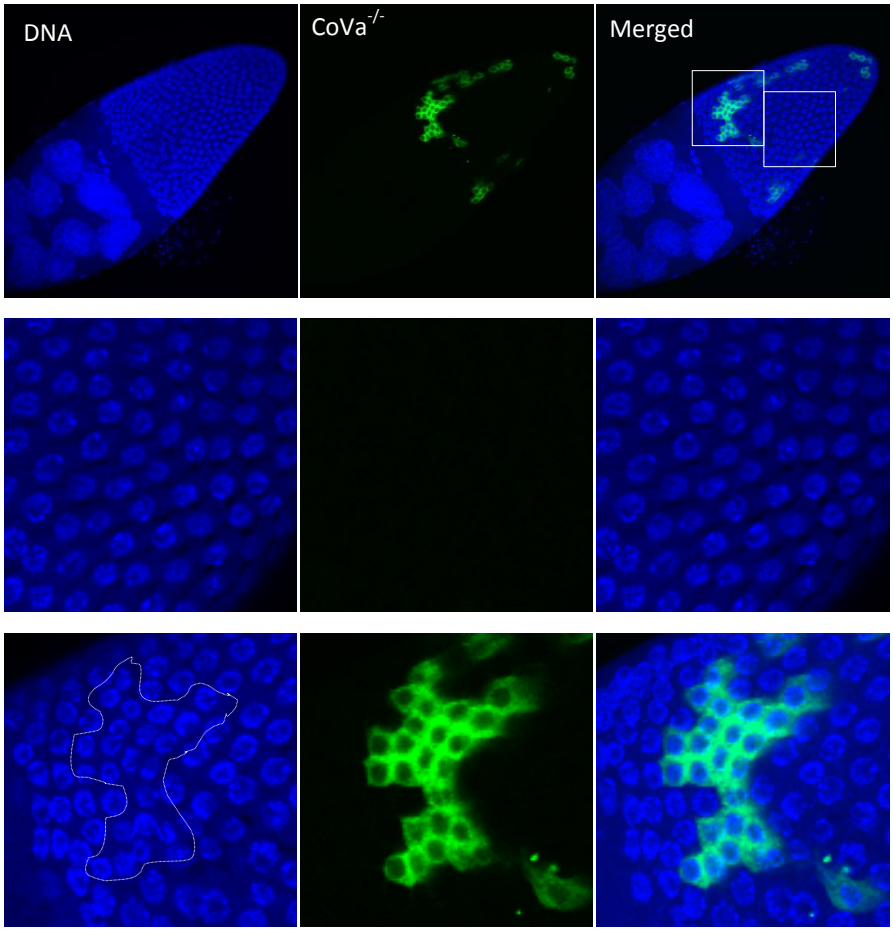

Figure 24: Hoechst staining depicting the DNA in late stage CoVa clones, zoomed-in background, zoomed-in clones. The nuclear size of the clones is lesser than in the background cells.

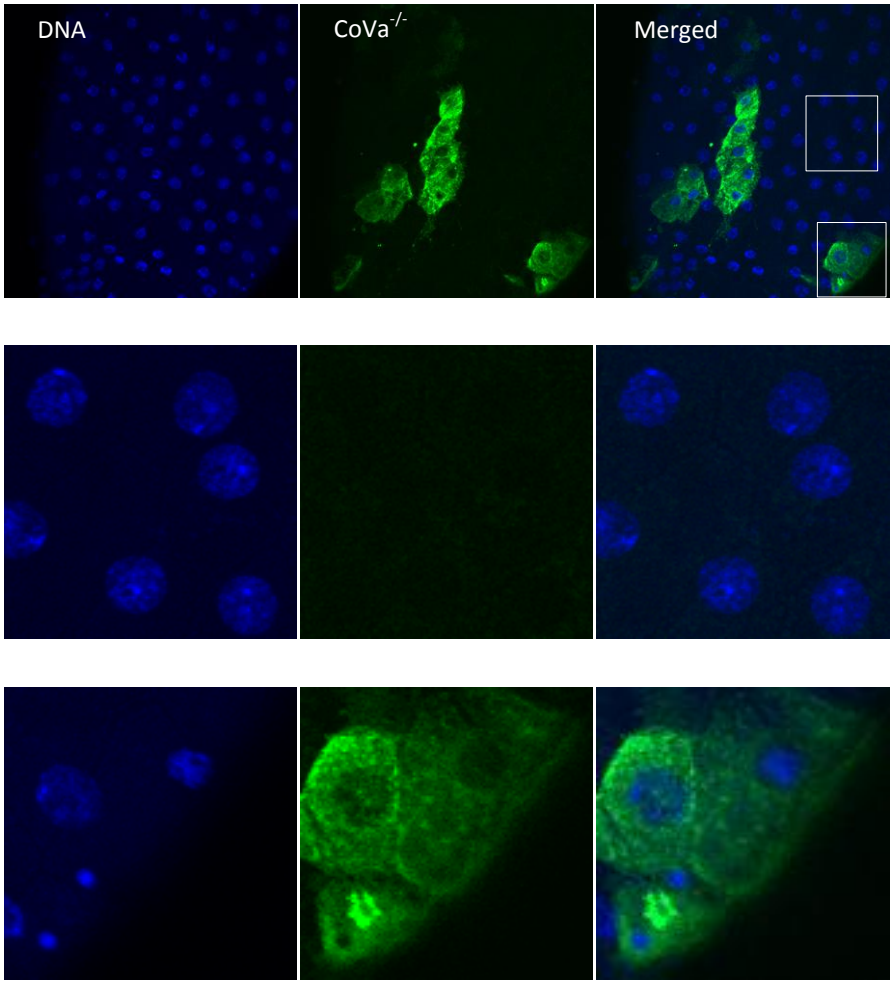

Figure 25: Hoechst staining depicting the DNA in very late stage CoVa clones, zoomed-in background, zoomed-in clones. The nuclear size of the clones seems to be lesser than that in the background cells.

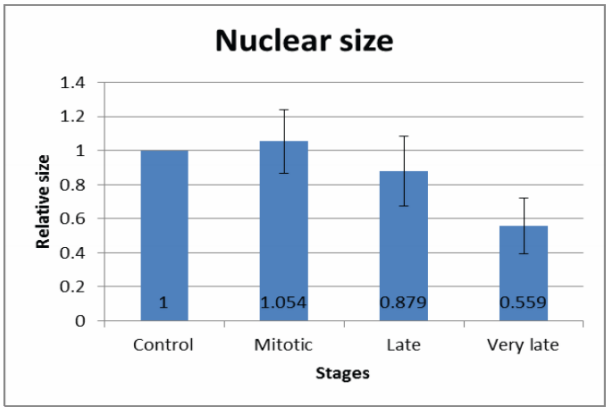

Figure 26: Nuclear size in CoVa clones is decreasing in a stage-specific manner.

2) EdU staining to depict cells in the S phase of the cell cycle in wild type ovaries

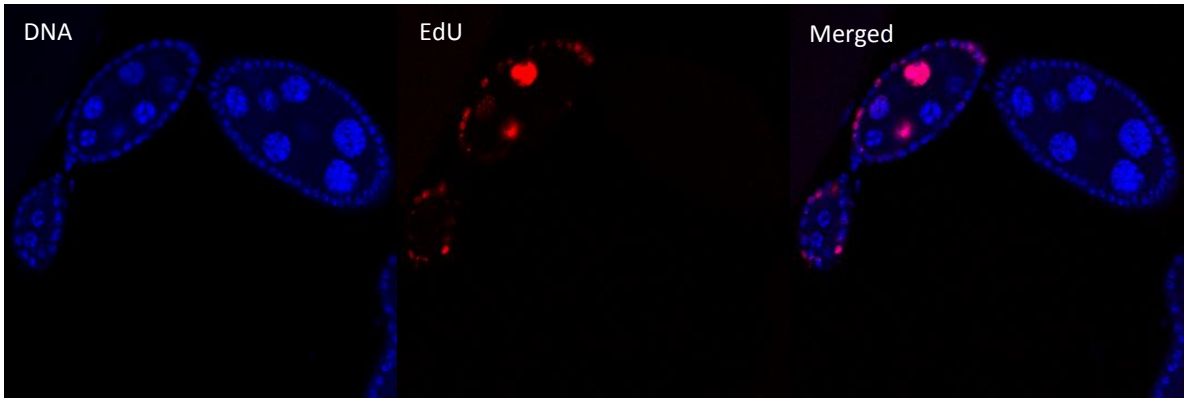

Figure 27: EdU staining depicting the cells which are in the S phase of the cell cycle in wild type ovaries.

Analysis of Notch and EGF signalling in electron transport chain mutant follicle cells and adults:

In oogenesis, there are two waves of Notch activity – one wave at the very beginning, at which stage Cut is expressed, and then another wave around stage 6, where Hindsight is expressed (Sun and Deng, 2007) and Cut is downregulated (Sun and Deng, 2005). This is a transition stage, where the cells switch from the mitotic cycle to the endocycle under the action of Notch (López-schier and Johnston, 2001). We know that morphology proteins such as Drp1 are involved in both Notch and EGF signalling (Mitra et al, 2012). Also, metabolism proteins have been implicated in

signalling pathways such as the JNK and Hippo pathways (Ohsawa et al., 2012). If there is a link between mitochondrial morphology and metabolism, it might be possible that CoVa and Pdsw may also be involved in the regulation of Notch and EGF signalling.

For possible effects on Notch signalling, bristle phenotype of adult flies was studied. Immunostaining was also performed to check for different Notch markers such as Hindsight, Cut, NICD and NECD in the follicle cells during oogenesis. EGF signalling was checked by immunostaining with ERK antibody.

1) Notch signalling: The bristle phenotype observed in adult flies mutant for both CoVa and Pdsw showed a shortened or missing bristle at the microkitae position [Figure 28 and 36] Hindsight staining is not seen at the transition stage in follicle cells mutant for CoVa (GFP-positive), while it is expressed in the background cells (negative for GFP) [Figure 29] but recovers at the later stages [Figure 30]. Cut staining shows retention in follicle cells mutant for Pdsw (GFP-positive) at the transition stage while it is seen to go down in background cells (negative for GFP) [Figure 37]. There is no staining for Cut in late stage follicle cells for both mutant and background cells [Figure 38]. NICD and NECD staining show no significant change in the mutant clones as compared to the background cells [Figures 32, 33, 34, 35, 39 and 40]. There is no significant change in ERK staining in the mutant clones and background control cells in follicle cells mutant for CoVa at either early or late stages [Figures 41,42].

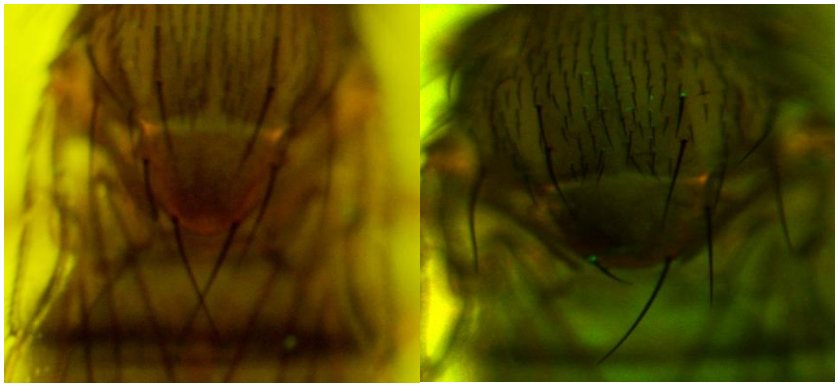

Figure 28: Bristle phenotype in adult flies. In order: Wild type fly, Drp1 mutant with shortened bristle, CoVa mutant fly with shortened bristle, missing bristle, and bright field image of CoVa mutant fly with a missing bristle.

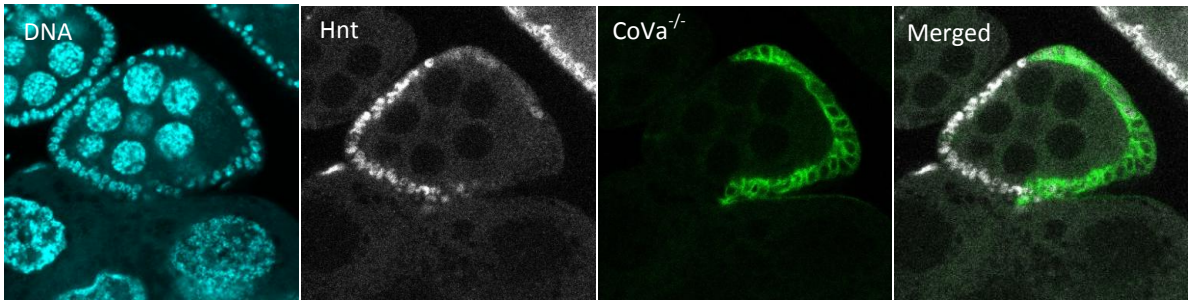

Figure 29: Hindsight staining as a marker for Notch in transition stage CoVa clones. Hindsight staining is present in the background cells, but not in the mutant clones at this stage, indicating that Notch signalling has not occurred at this stage in the clones.

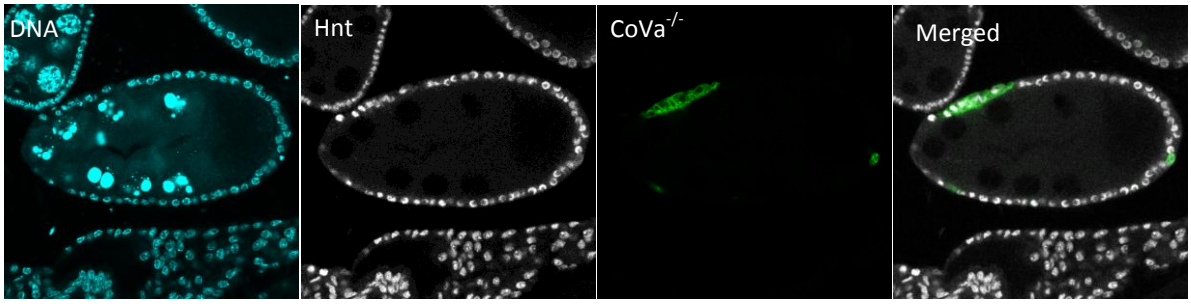

Figure 30: Hindsight staining as a marker for Notch in late stage CoVa clones. Hindsight staining is present in mutant clones as well as in the background cells, indicating that Notch activity has been rescued.

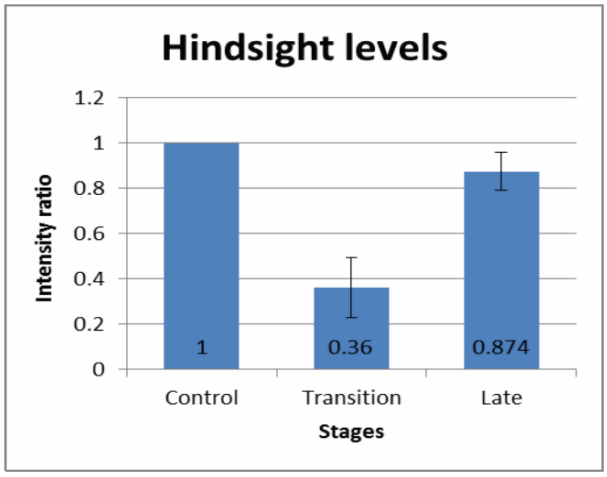

Figure 31: Hindsight levels in different stages of oogenesis in CoVa clones. At the transition stage, very less Hindsight is seen in the clones as compared to the background. This expression recovers in later stages.

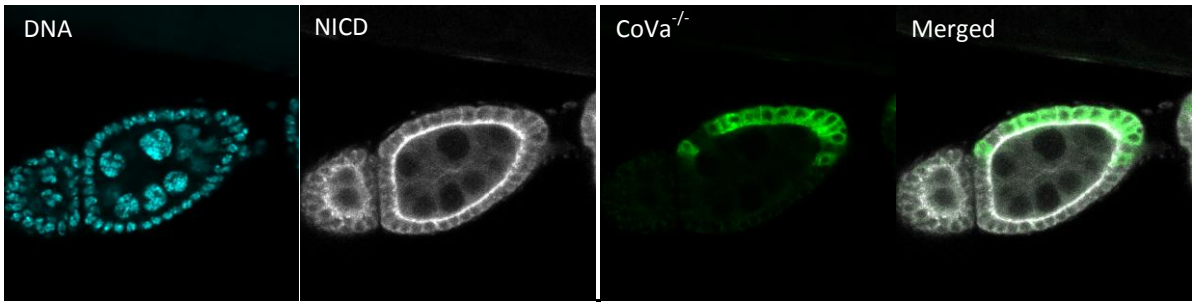

Figure 32: NICD staining in transition stage CoVa clones as an indicator of Notch activity. NICD levels do not look significantly different in the clones and the background.

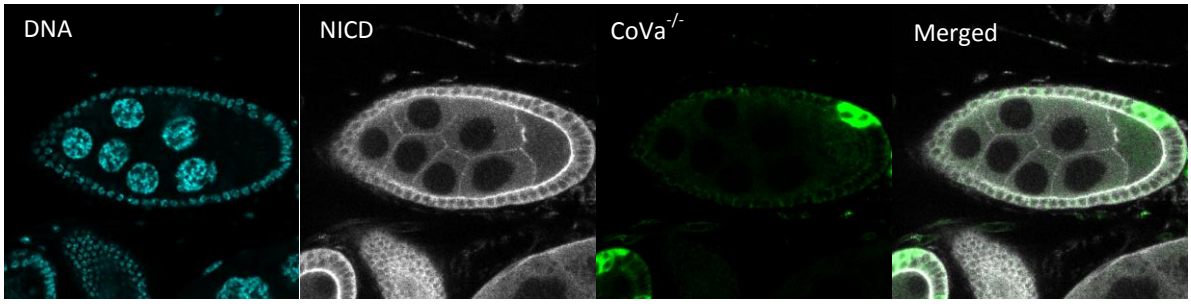

Figure 33: NICD staining in late stage CoVa clones as an indicator of Notch activity. NICD levels do not look significantly different in the clones and the background.

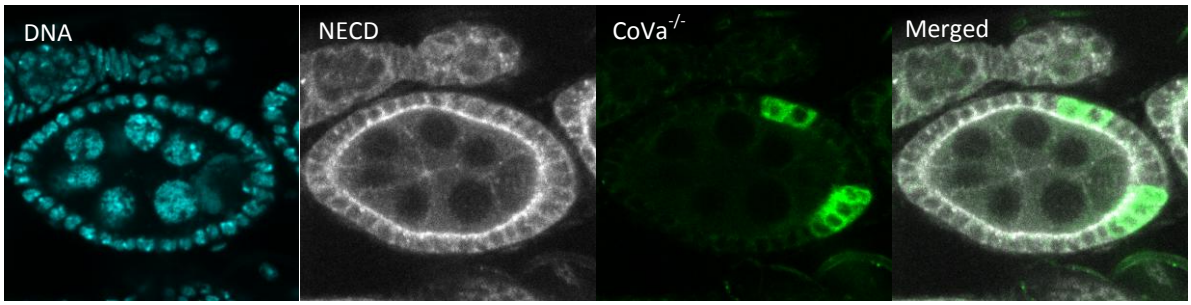

Figure 34: NECD staining in transition stage CoVa clones as an indicator of Notch activity. NECD levels do not look significantly different in the clones and the background

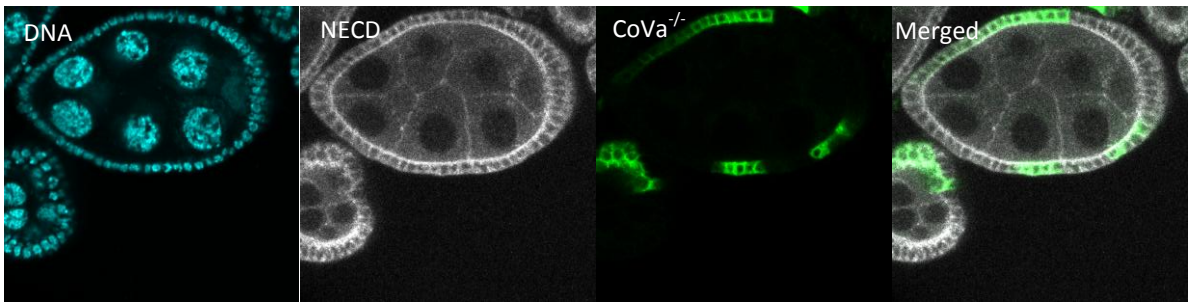

Figure 35: NECD staining in late stage CoVa clones as an indicator of Notch activity. NECD levels do not look significantly different in the clones and the background.

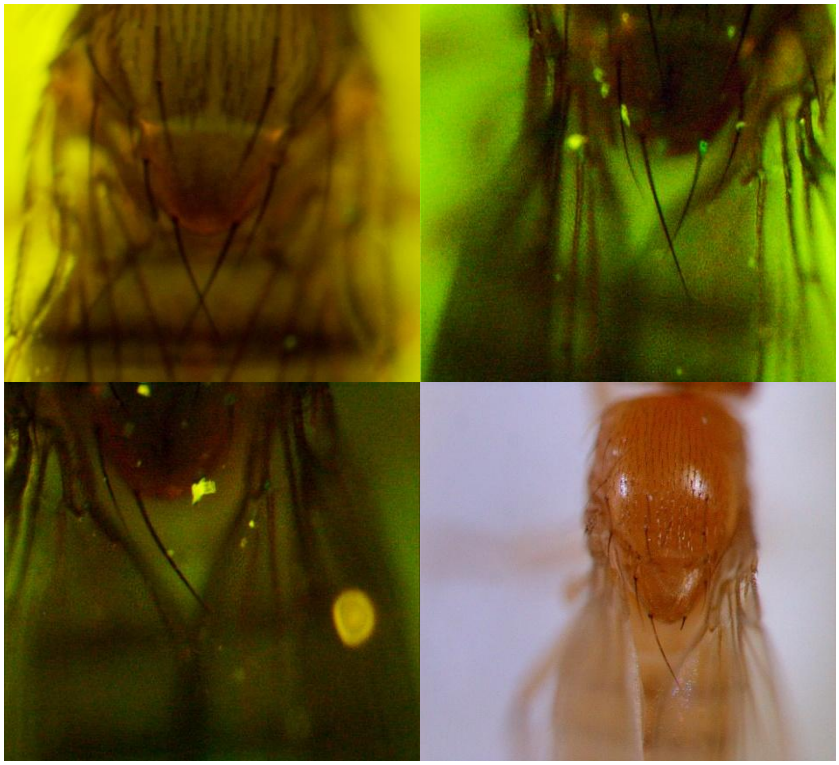

Figure 36: Bristle phenotype in adult flies. Wild type fly, Pdsw mutant fly with shortened bristle, missing bristle, and bright field image of a Pdsw mutant fly with a shortened bristle.

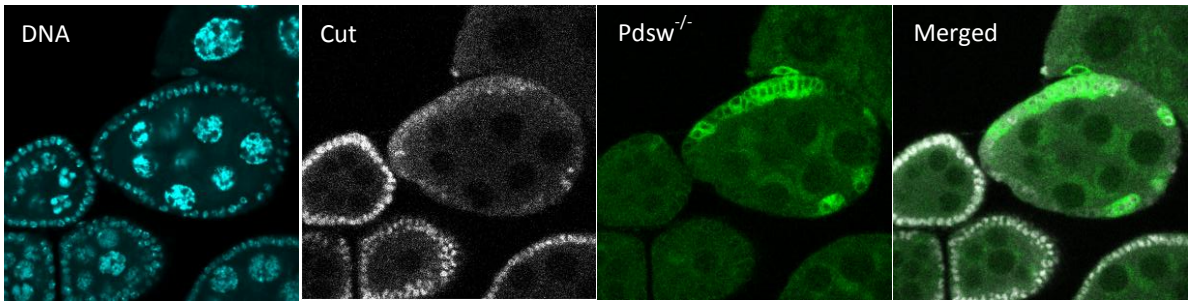

Figure 37: Cut staining as a marker for Notch in transition stage Pdsw clones. Cut should be downregulated at this stage in all cells, but it can be observed that it is retained in the mutant clones, while being downregulated in the background. This indicates that Notch functioning has been affected.

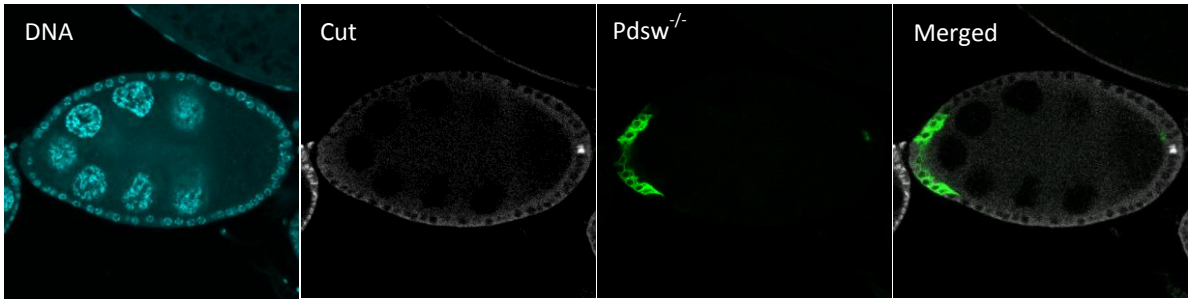

Figure 38: Cut staining as a marker for Notch in later stage Pdsw clones. Cut is not seen in the mutant clones or in the background cells, indicating that Notch signalling has been rescued at this stage.

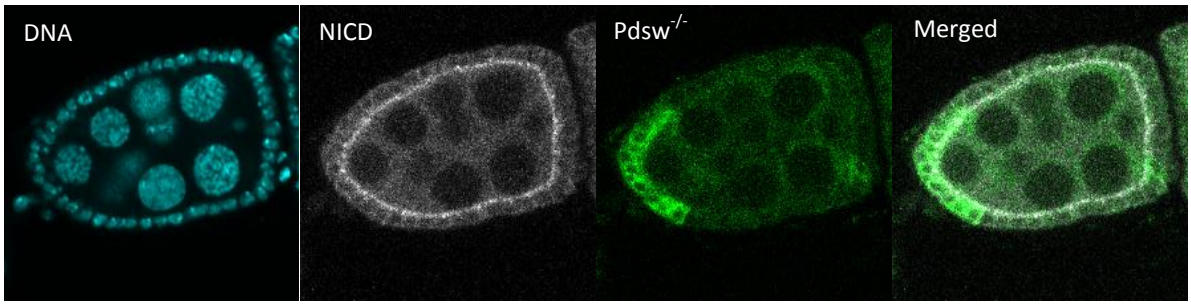

Figure 39: NICD staining as a marker for Notch activity in transition stage Pdsw clones. NICD staining does not look significantly different in the mutant clones from the background.

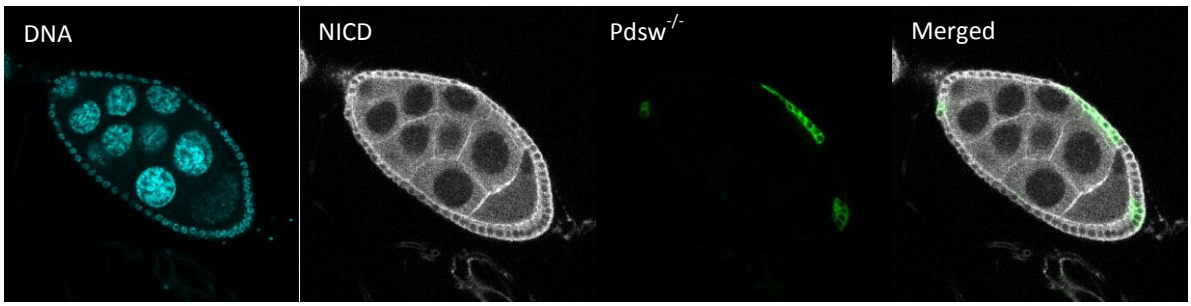

Figure 40: NICD staining as a marker for Notch activity in later stage Pdsw clones. NICD staining does not look significantly different in the mutant clones from the background.

2) EGF signalling in electron transport chain mutants studied by ERK

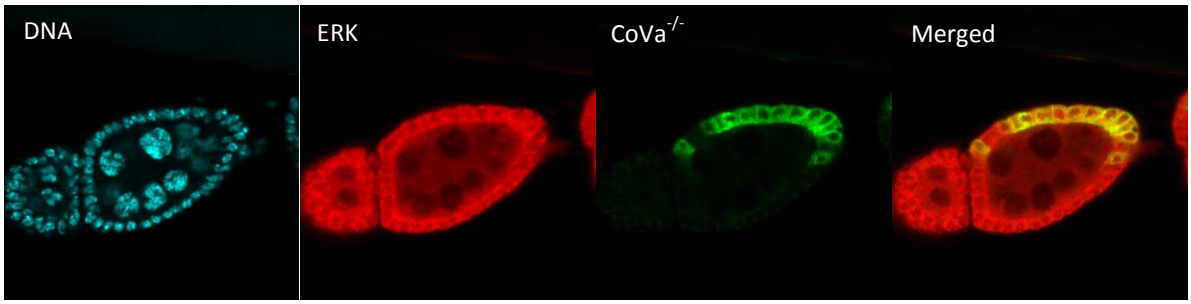

Figure 41: ERK staining as a marker for EGF activity in transition stage CoVa clones. ERK staining does not look significantly different in the mutant clones from the background.

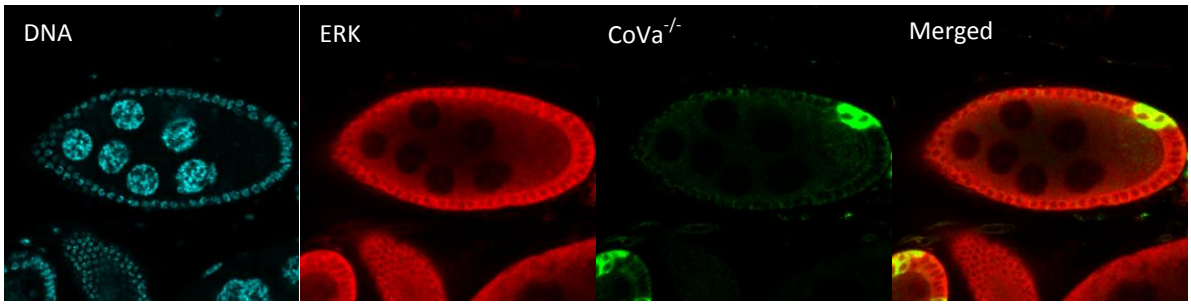

Figure 42: ERK staining as a marker for EGF activity in transition stage CoVa clones. ERK staining does not look significantly different in the mutant clones from the background.

Analysis of plasma membrane polarity in follicle cells mutant for the electron transport chain

CoVa and Pdsw mutations have been observed to decrease the functioning of follicle stem cell niche (Wang et al., 2012), and this phenotype is rescued when Cadherin in overexpressed, indicating that Cadherin is required in the maintenance of the follicle stem cell niche. Also, Cadherin is known to help in positioning of the oocyte during development (Godt and Tepass, 1998). Studying Cadherin in follicle cells of ETC mutant clones can indicate if plasma membrane polarity is linked with metabolism. Other polarity markers such as Dlg were also studied. Immunostaining was performed for both Cadherin and Dlg proteins in ETC mutant clones. A slight increase in Cadherin levels is noticed in follicle cells mutant for both CoVa and Pdsw (GFP-positive) [Figures 43, 46] as compared to the background cells (negative for

GFP) at early stages in development. This increase is more pronounced in mutant follicle cells at later stages in oogenesis [Figures 44, 47]. There is no significant change in Dlg staining in follicle cells mutant for CoVa (GFP-positive) when compared to the control background cells (negative for GFP) at early or late stages [Figures 48, 49].

1) Cadherin

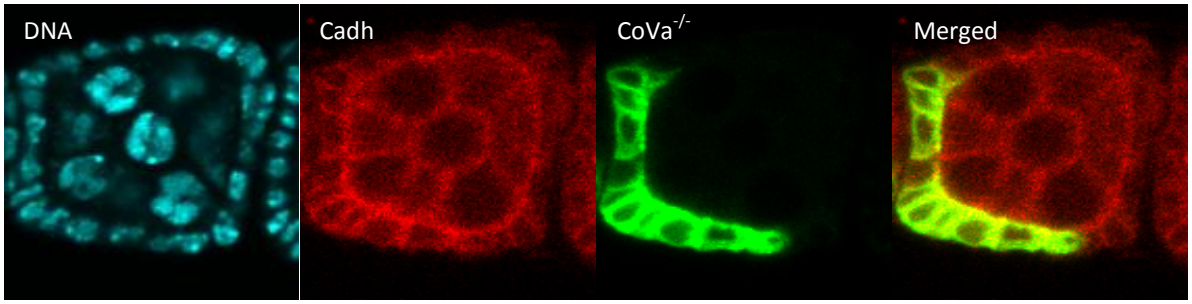

Figure 43: Cadherin staining in transition stage CoVa clones. The staining looks slightly higher in the clones as compared to the background.

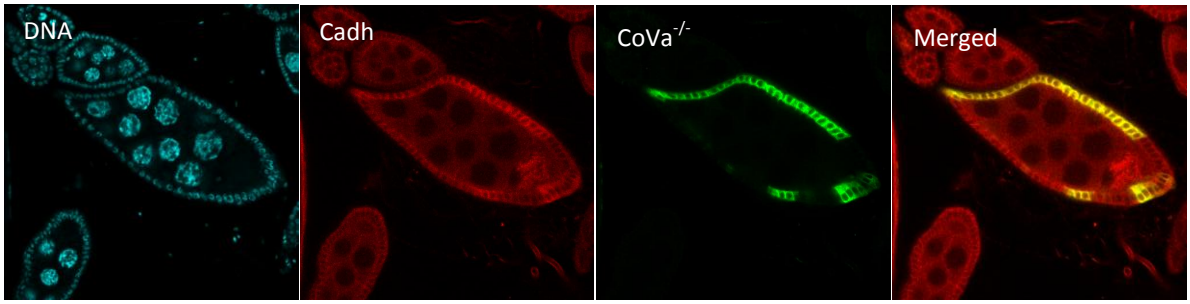

Figure 44: Cadherin staining in late stage CoVa clones. The staining looks higher in the clones as compared to the background.

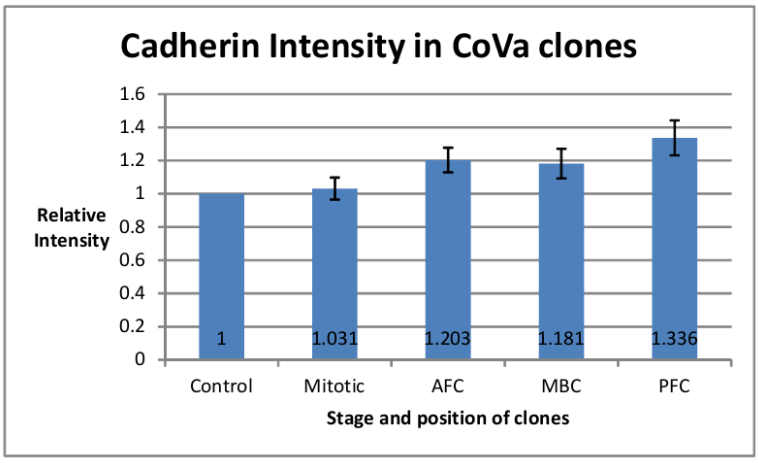

Figure 45: Intensity of cadherin staining in clones as compared to the background. It can be seen that while there is not a very significant increase in cadherin at early stages, it is quite prominent in the later ones.

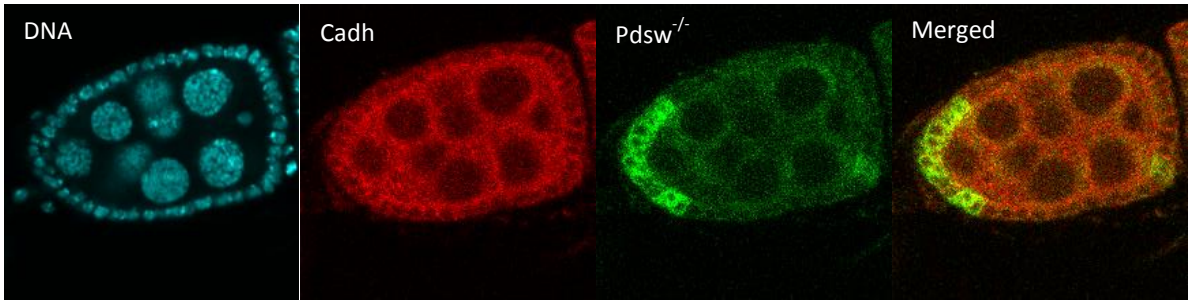

Figure 46: Cadherin staining in transition stage. The staining looks quite similar in the clones as compared to the background.

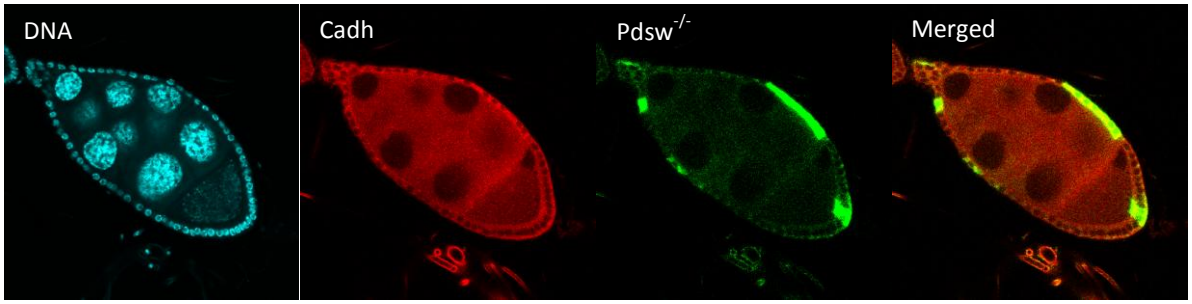

Figure 47: Cadherin staining in late stage Pdsw clones. The staining looks higher in the clones as compared to the background.

2) Dlg

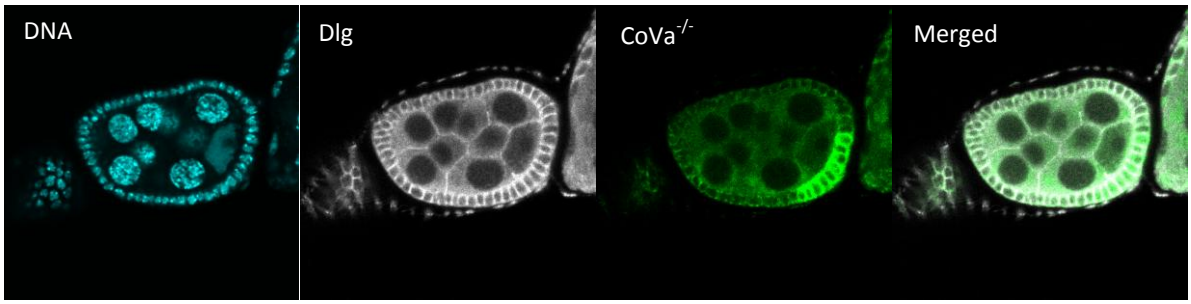

Figure 48: Dlg staining in transition stage CoVa clones. The staining looks similar in the clones and the background.

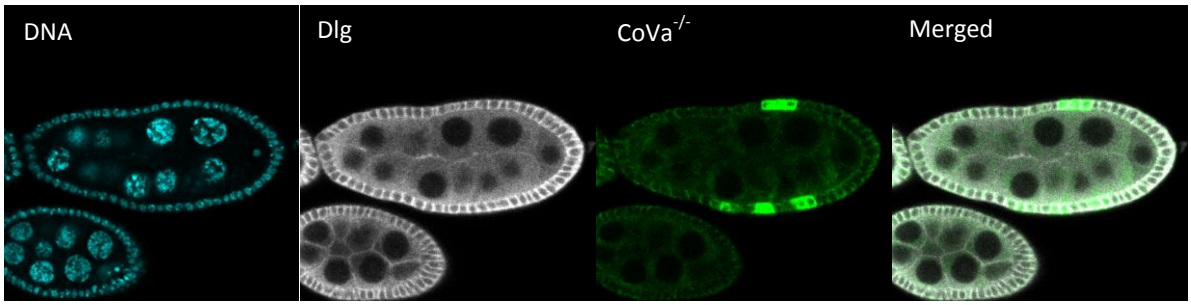

Figure 49: Dlg staining in late stage CoVa clones. The staining looks similar in the clones and the background.

Discussions:

Potential regulation of mitochondrial activity by a functional electron transport chain:

1) **ROS levels:** One of the most obvious ways to see if mitochondrial output changes is to check the levels of ROS produced after knocking down certain proteins which are involved in the electron transport chain. In the above experiments, we see that the DHE assay does not show any observable increase in staining in either CoVa or Pdsw, both at early and later stages. This is contrary to what might be generally expected, since any malfunctioning in the electron transport chain might be reflected in increased levels of oxidative stress. However, DHE is a probe to measure the levels of superoxide ions, which is just

one specific reactive oxygen species. It might be possible that knocking down either or both of these proteins might actually cause oxidative stress by increasing the levels of some other kind species of ROS. Studies done in the larval eye disc (Owusu-Ansah et al., 2008a) indicate that while CoVa clones do not show significant changes in DHE staining as compared to the background, the Pdsw clones show fivefold increase in this staining, indicating that knocking down of Pdsw causes a very noticeable increase in superoxide levels. So while the CoVa results we obtained are congruent with this paper, Pdsw results are different. This might be because the ovaries and eye discs are different tissues, and there might possibly be different energy requirements or signalling pathways triggered.

2) **ATP levels:** Since it was tough to estimate the amount of tissue required to be crushed to generate measurable ranges of ATP, a more indirect method was performed. This involved staining with AMPK. If the levels of ATP drop, then AMP values rise, which is sensed by AMPK (Carling et al., 2011). This is what was expected in CoVa mutant clones from literature (Owusu-Ansah et al., 2008a) However, the AMP levels remain more or less the same in these clones as in the background. This was initially seen in egg chambers of flies dissected 6 days after the heat shock, and we guessed that if we let flies survive till 10 days after the heat shock, the cells might be stressed enough to elevate AMPK levels, However, even after ten days there was no significant change in the AMPK levels. This might be a problem in the specific antibody staining itself, and needs to be verified by staining with other components of the AMPK pathway.
3) **Mitochondrial membrane potential**: The complexes of the electron transport chain pump out protons from the matrix to the intermembrane space, generating a proton gradient across the inner mitochondrial membrane. Normally functional mitochondria will be negatively charged. The dyes used to measure membrane potential are positively charged and cell-permeant. These dyes, including MitoTracker Red CMXRos, will stain only those mitochondria which have a sufficiently negative membrane potential, thereby indicating only those which are functioning properly. As in seen in our results, there is no CMXRos staining in the mutant CoVa clones, striking a clear contrast against the intensely stained mitochondria in the background cells. This result is congruent with what is expected, because cells which are mutant for CoVa will not have proper

functioning of the electron transport chain, and hence may not be able to generate a sufficient proton gradient. These mitochondria will thus not have sufficiently negative charge to get stained with CMXRos.

Potential regulation of mitochondrial morphology by a functional electron transport chain:

The mitochondrial morphology in CoVa and Pdsw clones looks different from the background based on Streptavidin staining. The mitochondrial distribution in the background cells look polarised and crescent-shaped, while the ones seen in the clones look more circular and evenly distributed around the boundary of the cell. This indicates that there is some relation between metabolism proteins and the shape and distribution of mitochondria. Additionally, the intensity of the Streptavidin staining is significantly higher in the clones as compared to the background. Interestingly, this is what is observed in mutants for Marf. It might be interesting to see what happens to the mitochondrial staining in tissues which have a mutation in Marf or Drp1 in the background of CoVa or Pdsw mutations, and see if the phenotype is rescued or aggravated.

Potential regulation of follicle cell signalling by the electron transport chain activity:

Two developmental signalling pathways were checked: Notch and EGF.

1) Notch signalling: Drosophila adults which are mutant for CoVa and Pdsw show a phenotype of shortened or missing bristles. The phenotype of missing microkitae is similar to what is seen in flies which are mutant for Notch. (Purcell and Artavanis-Tsakonas, 1999). Additionally, even Drp1 mutants show missing bristles, and we know that Drp1 mutants inactivate the Notch pathway (Mitra et al., 2012). This indicates at a gross level that the Notch signalling pathway is being affected in both CoVa and Pdsw mutants.

   This was further studied in follicle cell development during oogenesis, using immunostaining techniques, and using transcription factors like Hindsight and Cut which are downstream of Notch. In oogenesis, there are two waves of Notch

activity – one wave at the very beginning, at which stage Cut is expressed, and then another wave around stage 6, where Hindsight is expressed (Sun and Deng, 2007) and Cut is downregulated (Sun and Deng, 2005). This is a transition stage, where the cells switch from the mitotic cycle to the endocycle under the action of Notch (López-schier and Johnston, 2001). It was observed that at the transition stage, when the background cells start expressing Hindsight, mutant clones for CoVa and Pdsw fail to show Hindsight staining. This indicates that the Notch functioning in these mutants is being affected. However, when a later stage of oogenesis is observed, Hindsight in the clones is being expressed, similar to the background. This means that the expression of Notch is merely delayed, and the function recovers in some time.

Similarly, Cut is expressed normally in the early stages of development, but at the transition stage where it is downregulated in the background cells, Cut is still retained in CoVa and Pdsw clones. At later stages, however, Cut is not seen in either the clones or the background. This corroborates with the Hindsight results, and shows that again, there is simply a delay in the functioning of Notch and not a total inactivation of the pathway.

In Drp1 clones, there is a complete inactivation of Notch (Mitra et al., 2012). So we can see that mitochondrial morphology has a role in developmental pathways, and so does metabolism, but the effects are not the same, so while it looks like there might be some interconnection between morphology and metabolism, it does not look like a direct one-to-one correlation.

Immunostaining has also been carried out for Notch Intracellular and Extracellular domains. NICD is released and enters the nucleus after the Notch receptor binds to its ligand and is cleaved via Presinilin-mediated proteolysis (Struhl and Greenwald, 1999).This converts the factor Suppressor of Hairless from a transcriptional repressor to an activator (Struhl and Adachi, 1998). Based on the Hindsight and Cut results, it is expected that the levels of NICD and NECD will be more at the plasma membrane at the transition stage in the clones as compared to the heterozygous background, as this would indicate that Notch is inactive at this stage. However, preliminary results do not indicate any noticeable rise in either NICD or NECD. This is in contrast to what is observed in Drp1 mutant clones, where there are higher levels of NICD and NECD retained on the plasma membrane (Mitra et al., 2012). This might again indicate that Notch functioning is

not completely downregulated in metabolism mutants, as is the case in morphology mutant clones.

2) ERK staining was performed to see if the EGF signalling pathway is also affected by mitochondrial metabolism. There was no significant rise or fall in the levels of ERK on CoVa clones, which seems to indicate that there is no effect of this on EGF pathway. This is in contrast to what is known about Drp1 mutants, which are seen to be downstream of EFG signalling (Mitra et al., 2012). Also, EGF receptor signalling has been observed to specify posterior follicle cells (Van Buskirk and Schüpbach, 1999). However, ERK staining did not show a significant rise even in the posterior follicle cell clones. More staining has to be carried out (such as by MAPK and Ras) to check and corroborate this. Also, analysis of oocyte positioning might give better insights on a possible connection between EGF signalling and electron transport chain mutants.

Potential regulation of cell cycle by electron transport chain activity:

The size of the nucleus is seen to be decreasing in the CoVa and Pdsw clones in a stage-specific manner. At the later stages, the nuclei in clones are noticeably smaller than the background, and this can be explained by the fact that these nuclei have switched from mitosis to the endocycle, and being mutant, they are endocycling slower than those in the background cells. At even later stages, some of the nuclei look exceptionally small. This is indicative of cell death. This can be understood as these cells do not have proper metabolism, and probably do not have enough energy to survive till very later stages.

There are other markers for the cell cycle such as staining for EdU (protocol has been standardized) which might indicate if the mutant cells are going through the cell cycle normally, or are stuck at the G1-S checkpoint (Owusu-Ansah et al., 2008a). Also, one can stain for pH3, which is phosphorylated specifically during mitosis and meiosis at a particular space and time (Thomson et al., 1999) and Cyclin E which might be good indications of the cell cycle progression.

Potential regulation of plasma membrane polarity in follicle cells mutant by electron transport chain activity:

Staining was also done using cadherin, which is an adhesion protein and helps in oocyte positioning during oogenesis (Godt and Tepass, 1998). It was seen that cadherin was upregulated in both CoVa and Pdsw clones. This is a rather surprising result, and there is no satisfactory explanation so far. It is known that CoVa and Pdsw mutations decrease the functioning of follicle stem cell niche (Wang et al., 2012). Normal functioning is obtained in these mutants when cadherin is upregulated, indicating that Cadherin is required in the maintenance of the follicle stem cell niche. Based on this, one hypothesis could be that the follicle cells mutant for CoVa and Pdsw try to retain normalcy of function by upregulating Cadherin, and compensating for the loss in CoVa and Pdsw. Another possible cause for the increase in Cadherin in the clones could be that the fly lines used to generate the mutant clones has a GFP tag which binds to the membrane, and this might possibly increasing the affinity of membrane markers to the membrane. However, staining for discs large (Dlg), which is another membrane marker, rules out this possibility. Dlg levels remain similar in the clones and the background and are not being affected by the lack of metabolism proteins.

References:

Andersson, S.G.E., Karlberg, O., Canbäck, B., and Kurland, C.G. (2003). On the origin of mitochondria: a genomics perspective. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences *358*, 165–77; discussion 177–9.

Barros, M. (2003). H2O2 generation in Saccharomyces cerevisiae respiratory pet mutants: effect of cytochrome c. Free Radical Biology and Medicine *35*, 179–188.

Boveris, B.A., Cadenas, E., and Stoppani, A.M. (1976). Role of Ubiquinone in the Mitochondrial Generation of Hydrogen Peroxide. 435–444.

Van Buskirk, C., and Schüpbach, T. (1999). Versatility in signalling: multiple responses to EGF receptor activation during Drosophila oogenesis. Trends in Cell Biology *9*, 1–4.

Carling, D., Mayer, F. V, Sanders, M.J., and Gamblin, S.J. (2011). AMP-activated protein kinase: nature's energy sensor. Nature Chemical Biology *7*, 512–518.

Cereghetti, G.M., and Scorrano, L. (2006). The many shapes of mitochondrial death. *1*, 4717–4724.

Chen, H., and Chan, D.C. (2005). Emerging functions of mammalian mitochondrial fusion and fission. Human Molecular Genetics *14 Spec No*, R283–9.

Cipolat, S., Brito, O.M. De, Zilio, B.D., and Scorrano, L. (2004). OPA1 requires mitofusin 1 to promote mitochondrial fusion. *2004*, 1–6.

Cox, M., and Nelson, D. (2004). Lehninger, Principles of Biochemistry.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Sarah, A., Gayyed, M.F., Anders, R.A., Maitra, A., and Pan, D. (2007). Elucidation of a Universal Size-Control Mechanism in Drosophila and Mammals. Cell *130*, 1120–1133.

Duffy, J.B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis (New York, N.Y.□ : 2000)*34*, 1–15.

Godt, D., and Tepass, U. (1998). Drosophila oocyte localization is mediated by differential cadherin-based adhesion. Nature *395*, 387–391.

Gray, M.W. (1998). Rickettsia , typhus and the mitochondrial connection. Nature *396*.

Gray, M.W., Burger, G., and Lang, B. (2001). Minireview The origin and early evolution of mitochondria. 1–5.

Invitrogen MitoTracker ® Mitochondrion-Selective Probes. 1–6.

Invitrogen (2005). ATP Determination Kit (A22066) Quick Facts. 1–3.

Invitrogen (2009). Fluo-4 Direct ™ Calcium Assay Kits.

Invitrogen (2011). Click-iT® EdU Imaging Kits. 2–5.

Ishihara, N., Jofuku, A., Eura, Y., and Mihara, K. (2003). Regulation of mitochondrial morphology by membrane potential, and DRP1-dependent division and FZO1-dependent fusion reaction in mammalian cells. Biochemical and Biophysical Research Communications *301*, 891–898.

James, D.I., Parone, P. a, Mattenberger, Y., and Martinou, J.-C. (2003). hFis1, a novel component of the mammalian mitochondrial fission machinery. The Journal of Biological Chemistry *278*, 36373–36379.

Lambert, A.J., and Brand, M.D. (2004). Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). The Journal of Biological Chemistry *279*, 39414–39420.

Lemarie, a, and Grimm, S. (2011). Mitochondrial respiratory chain complexes: apoptosis sensors mutated in cancer? Oncogene *30*, 3985–4003.

López-schier, H., and Johnston, D.S. (2001). Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. 1393–1405.

McBride, H.M., Neuspiel, M., and Wasiak, S. (2006). Mitochondria: more than just a powerhouse. Current Biology : CB*16*, R551–60.

Mitra, K., Rikhy, R., Lilly, M., and Lippincott-schwartz, J. (2012). DRP1-dependent mitochondrial fission initiates follicle cell differentiation during. *197*.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. The Biochemical Journal *417*, 1–13.

Nagaraj, R., Gururaja-Rao, S., Jones, K.T., Slattery, M., Negre, N., Braas, D., Christofk, H., White, K.P., Mann, R., and Banerjee, U. (2012). Control of mitochondrial structure and function by the Yorkie/YAP oncogenic pathway. Genes & Development *26*, 2027–2037.

Oh, H., and Irvine, K.D. (2008). In vivo regulation of Yorkie phosphorylation and localization. Development (Cambridge, England) *135*, 1081–1088.

Ohsawa, S., Sato, Y., Enomoto, M., Nakamura, M., Betsumiya, A., and Igaki, T. (2012). Mitochondrial defect drives non-autonomous tumour progression through Hippo signalling in Drosophila. Nature *490*, 547–551.

Okamoto, K., and Shaw, J.M. (2005). Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annual Review of Genetics *39*, 503–536.

Orrenius, S., Gogvadze, V., and Zhivotovsky, B. (2007). Mitochondrial Oxidative Stress□ : Implications for Cell Death. Annual Review of Pharmacology and Toxicology *47*, 143–183.

Owusu-Ansah, E., Yavari, A., Mandal, S., and Banerjee, U. (2008a). Distinct mitochondrial retrograde signals control the G1-S cell cycle checkpoint.

Owusu-Ansah, E., Yavari, A., and Banerjee, U. (2008b). A protocol for _in vivo_ detection of reactive oxygen species.

Owusu-Ansah, E., Yavari, A., Mandal, S., and Banerjee, U. (2008c). Distinct mitochondrial retrograde signals control the G1-S cell cycle checkpoint. Nature Genetics *40*, 356–361.

Poole, A.M., and Penny, D. (2007). Evaluating hypotheses for the origin of eukaryotes. BioEssays□ : News and Reviews in Molecular, Cellular and Developmental Biology *29*, 74–84.

Purcell, K., and Artavanis-Tsakonas, S. (1999). The Developmental Role of warthog, the Notch Modifier Encoding Drab6. The Journal of Cell Biology *146* , 731–740.

Roth, S. (2001). Drosophila oogenesis: coordinating germ line and soma. Current Biology□ : CB*11*, R779–81.

Santel, A., and Fuller, M.T. (2000). Control of mitochondrial morphology by a human mitofusin.

Skulachev, V.P. (1998). Cytochrome c in the apoptotic and antioxidant cascades. *423*, 275–280.

Skulachev, V.P. (2001). Mitochondrial filaments and clusters as intracellular power-transmitting cables. *26*, 23–29.

Smirnova, E., Griparic, L., Shurland, D., and Bliek, A.M. Van Der (2001). Dynamin-related Protein Drp1 Is Required for Mitochondrial Division in Mammalian Cells. *12*, 2245–2256.

Struhl, G., and Adachi, a (1998). Nuclear access and action of notch in vivo. Cell *93*, 649–660.

Struhl, G., and Greenwald, I. (1999). Presenilin is required for activity and nuclear access of Notch in Drosophila. Nature *398*, 522–525.

Sun, J., and Deng, W. (2007). Article Hindsight Mediates the Role of Notch in Suppressing Hedgehog Signaling and Cell Proliferation. 431–442.

Sun, J., and Deng, W.-M. (2005). Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in Drosophila follicle cells. Development *132* , 4299–4308.

Taguchi, N., Ishihara, N., Jofuku, A., Oka, T., and Mihara, K. (2007). Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. The Journal of Biological Chemistry *282*, 11521–11529.

Thomson, S., Mahadevan, L.C., and Clayton, a L. (1999). MAP kinase-mediated signalling to nucleosomes and immediate-early gene induction. Seminars in Cell & Developmental Biology *10*, 205–214.

Ventura, A., Maccarana, M., Raker, V.A., and Pelicci, P.G. (2004). A Cryptic Targeting Signal Induces Isoform-specific Localization of p46Shc to Mitochondria. Journal of Biological Chemistry *279* , 2299–2306.

Voet, D., and Voet, J.G. (2010). Biochemistry, 4th edition.

Wang, Z. a, Huang, J., and Kalderon, D. (2012). Drosophila follicle stem cells are regulated by proliferation and niche adhesion as well as mitochondria and ROS. Nature Communications *3*, 769.

Yankovskaya, V., Horsefield, R., Törnroth, S., Luna-Chavez, C., Miyoshi, H., Léger, C., Byrne, B., Cecchini, G., and Iwata, S. (2003). Architecture of Succinate Dehydrogenase and Reactive Oxygen Species Generation. Science *299* , 700–704.

Yoon, Y., Krueger, E.W., Oswald, B.J., and Mcniven, M.A. (2003). The Mitochondrial Protein hFis1 Regulates Mitochondrial Fission in Mammalian Cells through an Interaction with the Dynamin-Like Protein DLP1. *23*, 5409–5420.