Functional Analysis Of Mitochondrial Targeted Protein Ppr (Pentatricopeptide Repeat) In Oogenesis In *Drosophila melanogaster*

A Thesis

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Certificate

This is to certify that this dissertation entitled **"Functional analysis of mitochondrial targeted protein Ppr (pentatricopeptide repeat) in oogenesis in *Drosophila melanogaster*"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Adikrishna K K** at IISER Pune under the supervision of **Dr.Richa Rikhy, Associate professor, Department of biology, IISER Pune** during the academic year 2018-2019.

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Declaration

I hereby declare that the matter embodied in the report entitled **"Functional analysis of mitochondrial targeted protein Ppr (pentatricopeptide repeat) in oogenesis in *Drosophila melanogaster*"** are the results of the work 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.

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Contents

Abstract

Mitochondria are semi-autonomous double membrane organelle with an extrachromosomal circular DNA of approximately 19.5 kb in size and known to encodes 37 genes. Mitochondrial genome encodes 11 mRNAs which gets translates to 13 polypeptide that all are subunits of oxidative phosphorylation. Ppr is a trans acting nuclear encoded protein which regulate post transcriptional modification of mitochondrial genome. During stem cell differentiation mitochondrial morphology and metabolism shift towards an increased OXPHOS activity and mitochondrial DNA content is also known to increase. Here we focused on the role of Ppr on mitochondrial DNA translation and the following effect on differentiation, using *Drosophila* oogenesis as a model for stem cell differentiation. We observed that Ppr is expressed in a differential manner in different stages of oogenesis and is required in an increased level in certain stages associated with Notch and EGFR signalling pathways. Depletion of *ppr* is resulting in clustered mitochondrial morphology and a decreased mitochondrial membrane potential, surprisingly with no evidence of energy stress. Additionally depletion in *ppr* in nurse cells shows a defective oocyte nuclear migration. We observed an increased level of cytoplasmic dpERK, an EGFR read out suggesting the direct or indirect role of Ppr in EGFR signalling. Ppr mutant cells show a delay in Cut depletion with no change in NICD levels. These data together indicate a role for Ppr in maintaining mitochondrial activity and EGFR signaling in follicle cells.

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1.Introduction

1.1. Mitochondrial functions and dynamics

Mitochondria are maternally inherited double membrane organelle of eukaryotic cells which are famously known for cellular energy metabolism where it produces ATP through oxidative phosphorylation. Apart from this, mitochondria are also responsible for cell signalling by reactive oxidation species(ROS), regulation of calcium homeostasis and apoptosis. Outer membrane of mitochondria separates organelle from cytosol and the inner membrane, which define inner matrix of mitochondria carry five enzyme complexes of oxidative phosphorylation (Chen and Chan, 2004; Rambold and Pearce, 2018).

Mitochondria are highly dynamic and undergo continuous cycles of fission and fusion in response to cellular environments and requirements. Two GTPase proteins Mitofusin and Optic atrophy 1 (Opa1) control outer membrane and inner membrane fusion respectively, while Dynamin related protein 1(Drp1) govern mitochondrial fission(Bliek, Shen, and Kawajiri, 2013). Fused mitochondrial morphology results in an increase in oxidative phosphorylation and ATP synthesis and calcium ion uptake which are reversed in fragmented mitochondria in *Drosophila* oocyte(Sieber, Thomsen, and Spradling, 2016).

1.2. Mitochondrial dynamics and metabolism role in stem cell differentiation

Mitochondria are shown to have a direct involvement in stem cell fate determination which is associated with its property of oxidative phosphorylation. In mouse and human embryonic stem cells (ESC), mitochondria are observed to be peri nuclearly localised, immature and with poorly developed cristae(Prowse AB et al.,2012; Lonergan T et al.,2016) where as in differentiated cells such as fibroblasts, mitochondria are rich in cristae and are elongated in morphology(Varum S et al.,2011). In *vitro* differentiation of hESC have shown elongation of mitochondria and maturation of cristae and reprogramming of somatic cells into induced pluripotent stem cells shows an opposite phenotype (Prigione A et al.,2010; Folmes CD et

al.,2011). Pluripotent stem cell differentiation is also associated with a metabolic shift from glycolysis in PSC to OXPHOS-based metabolism in differentiated cells. Mitochondrial activities such as ATP production and oxygen consumption are shown to increase during differentiation and a lowered lactate production shows reduced glycolysis. Reprogramming of somatic cells into iPSC (in mouse) shows reverse effects such as an increase in glycolytic enzymes and downregulation of mitochondrial respiratory complexes I and II (Panopoulos AD et al.,2012; Folmes CD et al.,2011). Inhibition of mitochondrial respiratory complex III by antimycin A has shown to promote pluripotency marker Nanog and have a reduction of mRNAs required for differentiation (Pereira SL et al.,2013). Over expressing a mitochondrial biogenesis promoter, peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1a (PGC-1a) in mouse iPSCs enhances their differentiation into adipocytes (Huang PI et al.,2011). This suggests that stimulating mitochondrial biogenesis results in stem cell differentiation or lineage commitment. Also, there have been evidences for inhibiting metabolic shift from glycolysis to OXPHOS resulting in aberrated differentiation in both humans and mice (Wanet,Anais et al.,2015).

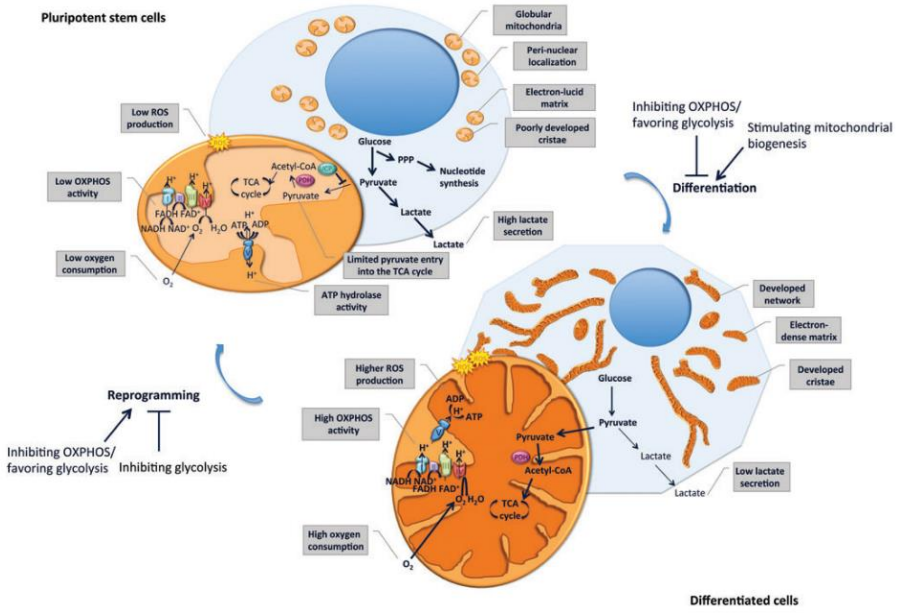


Figure 1.1: Schematic representation of mitochondrial metabolic shift during differentiation and reprogramming (Wanet, Anais et al.,2015)

In mouse HSCs the mitochondrial mass increases during differentiation, while mitochondrial respiration defects, caused by mitochondrial DNA depletion result in aberrated differentiation (Mantel C et al.,2010). This points to the possibility that mitochondrial genome amplification and transcription is likely to take place in a regulated manner during differentiation events and several nuclear genome transcribed proteins will be responsible for executing this function.

1.3. Mitochondria are semi-autonomous organelles with their own DNA

Mitochondria are characterised by an extrachromosomal circular DNA. The mitochondrial DNA (mtDNA) of *Drosophila melanogaster* is of ~19.5-kb in size and encodes 37 genes. The gene content is similar as in mammals which encodes 11 mRNAs translated to 13 polypeptides that all are oxidative phosphorylation (OXPHOS) complex subunits, 2 rRNAs and 22 tRNAs (M.Jaiswal et al.,2015)

Regardless of the presence of its own genome, mitochondria are not self-supporting entities. The transcription, translation and replication of mitochondrial genome are known to be dependent on several trans-acting nuclear encoded proteins. One such family of protein is Ppr (pentatricopeptide unit).

1.4. Ppr (Pentatricopeptide) protein is a mitochondrial regulatory protein

To protect and stabilize the RNAs, RNA binding proteins are required. Ppr(penta-tri-co-peptide) family proteins are well-known example of the RNA binding protein in eukaryotes including plants and animals but is not present in the prokaryotes (Manna.S et al.,2015). The proteins of this family were discovered in *Arabidopsis thaliana* and have Ppr motif and controls RNA metabolism such as splicing, editing, stability and translation of mitochondrial RNA.The mitochondrial gene expression and the turn-over of resultant RNA is controlled by the post transcriptional editing such as splicing and polyadenylation (Baggio.F et al.,2014).

The evidences on the Ppr family proteins suggests the role in the neurodegenerative diseases in the human (Baggio.F et al.,2014). Ppr proteins are found in all eukaryotes and two different homologs of Ppr, DmLRPPRC1 or bicoid

stability factor and DmLRPPRC2(Liu.J.M et al.,2016 and Baggio.F et al.,2014) are present in *Drosophila melanogaster*. Ppr proteins are shown to localise on mitochondria in *Drosophila* larval muscle(figure 1.2) and involved in the regulation of ATP synthesis activity as mutation of *ppr* causes reduction in the ATP production and reduction in the mitochondrially encoded transcripts for ETC complexes except complex I (figure 1.3.A,B)(M.Jaiswal et al.,2015) .

The mitochondrial DNA content, when normalised to nuclear DNA was showing approximately 4 fold increase which indicates a compensatory mechanism via increased mitochondrial biogenesis in response to loss of mtRNA (figure 1.3.C) (M.Jaiswal et al.,2015).

A

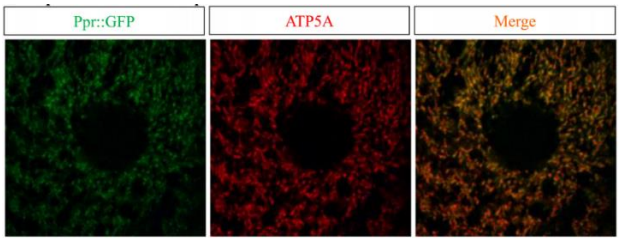
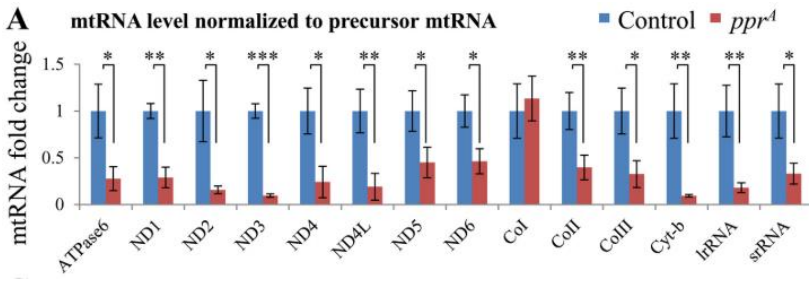


Figure 1.2: Colocalization of Ppr with mitochondrial complex V (M.Jaiswal et al.,2015)



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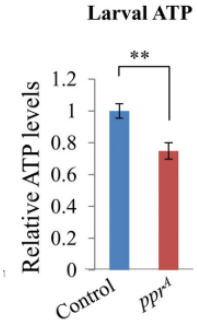

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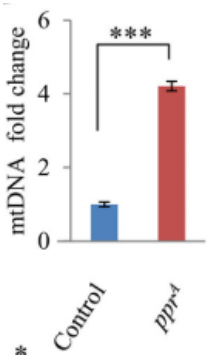


Figure 1.3: Mitochondrial genome transcription in *ppr* mutants**(A)** mtRNA levels normalised to the precursor RNA is decreased upon *ppr* mutation**(B)** Relative ATP level in *ppr* mutant is reduced**(C)**Relative mtDNA content, normalized to nuclear DNA show around 4 fold increase upon *ppr* mutation (M.Jaiswal et al.,2015)

1.5. *Drosophila* oogenesis as a model system

Drosophila oogenesis is used as a model system to study the role of mitochondria in stem cell differentiation. *Drosophila* have a pair of ovary, each consisting of approximately 18 ovariole. Each ovariole is a thread of developing egg chambers starting from germarium. Germarium consists of somatic and germline stem cells which differentiate into egg chambers. Each egg chamber consists of 16 germline cells (15 nurse cells and an oocyte) which form from a single germline stem cell divide asymmetrically. As a result of incomplete cytokinesis, these cells are interconnected through ring canals, which allow the transfer of RNAs and protein to the oocyte (Nystul and Spradling,2007). Somatic stem cells give rise to follicle cells which forms an epithelial layer around germline cells (Nystul and Spradling,2007; castanieto A. et al.,2014). Patterning and differentiation of egg chambers happens in response to the EGFR(epidermal growth factor receptor) and Notch signalling pathway. Based on morphological development, oogenesis is divided into 14 stages (Roth.S et al.,2009). Notch pathway is activated at stage 6 and regulates follicle cell differentiation. EGFR signalling is activated around stage 8 in the posterior follicle cells and is responsible for determination of oocyte polarity.

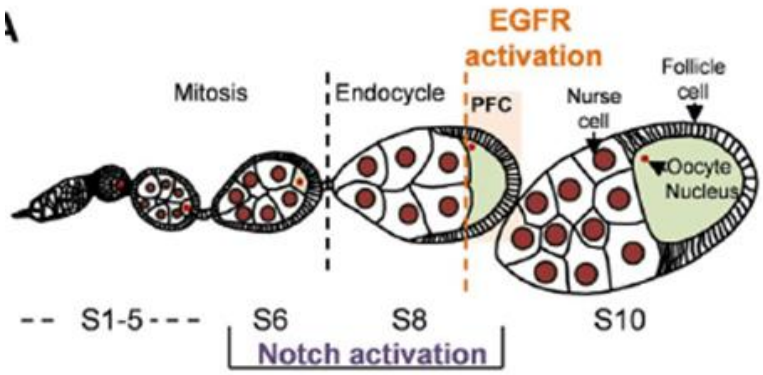


Figure 1.4: Schematic representation of *Drosophila* oogenesis (D.Tomer et.al.,2018)

The model system can be efficiently used to study the signalling pathways and cross talks between somatic and germline cells during development owing to numerous method of genetic manipulations. It has been reported that mitochondrial fission mediated by the protein Drp1 is essential for follicle cell differentiation during oogenesis in the *Drosophila melanogaster* ovary (Mitra et al., 2012).

1.6. Objectives

In the literature discussed above, we know that the volume of mitochondria and mitochondrial numbers increase during the oogenesis in the follicle cells, nurse cells and oocyte. Subunits of mitochondrial respiratory complex II is encoded entirely by nuclear genome where complex I,III,IV,V are encoded by both nuclear and mitochondrial DNA (J.E.Walker,1995). Since an inevitable fraction of the subunits of respiratory complexes are encoded by mitochondrial genome, this addresses the level of transcription of mitochondrial genome during differentiation. Considering these facts, the proposed hypothesis was that transcription and translation of mitochondrial genes are regulated during stem cell differentiation. The established role of mitochondrial regulatory protein Ppr in regulation of mitochondrial genome translation raises the question whether *ppr* have an effect in *Drosophila* oogenesis.

The objectives of my study are as follows:

- Assess the distribution of Ppr during *Drosophila* oogenesis in follicle and germ cell lineages
- Assess the mitochondrial morphology and activity of follicle cell and germ cell mutant for mitochondrial transcriptional regulatory protein Ppr
- Assess the impact of depletion of Ppr on EGFR and Notch signalling

2. Materials and methods

2.1. Fly stocks

Drosophila fly stocks and crosses were maintained in standard corn meal agar at 25^0C. *Nanos-Gal4>UAS-mito-GFP* and *Nanos-Gal4* were used to express *ppr* RNAi (Bloomington stock number: 65950). Stocks were obtained from Bloomington stock centre. The stocks *ppr::GFP,* mutant ppr^A and *hsflp; ubi-GFP FRT19A* were obtained from Manish Jaiswal (TIFR, Hyderabad).

2.2. Generation of follicle cell clones

Homozygous *ppr* mutant clones were generated in the background of heterozygous tissue using flippase(FLP)- FRT mediated site specific recombination. ppr^A *-FRT19A* flies were crossed with *hsflp;ubiGFP-FRT19A*. 2-3 days old adult F1 females of genotype ppr^A *FRT 19A/ubiGFP FRT 19A;hs FLP* were heat shocked at 37^0C for 1 hour to generate *ppr*-mutant follicle cell clones which are negatively marked by GFP. The heat pulsed animals were maintained at 25^0C for 5-8 days and dissection was performed on 8 day old flies.

2.3. Immunostaining and microscopy

Ovaries were dissected in Schneider's medium at room temperature and were immediately fixed in 4% PFA (paraformaldehyde) prepared in 1X PBS (phosphate buffer saline) for 20 minutes, at room temperature. Fixed ovaries were permeabilized in 0.3% triton-X-100 in PBS (0.3% PBST) by 3 washes, 10 minutes each. Tissues were blocked in PBST containing 2% BSA for 1 h and treated with primary antibody for overnight at 4^0C. Ovaries were washed by 0.3% PBST for 3 times, 5 minutes each. Treated with conjugated secondary antibodies in 0.3% PBST for 1 h at room temperature. Followed by nuclear staining with Hoechst 33258 for 10 minutes. The tissue was washed in 0.3% PBST for 10 minutes and mounted in SlowFade Antifade (Invitrogen) mountant. 5-10 days old flies were dissected and 8-10 animals were used for each staining. The list of antibodies used are as follows.

Primary Antibody	Dilution	Company
anti-GFP	1:1000	Invitrogen
anti-ATPβ	1:100	Abcam
anti-Hsp60	1:50	Cell signalling
anti-pAMPK	1:100	Abcam
anti-Cut	1:100	DSHB
anti-NICD	1:20	DSHB
anti-dpERK	1:200	Cst 4370
Secondary antibody		
Alexa Flour 488	1:1000	Invitrogen
Alexa Flour 568	1:1000	Invitrogen
Alexa Flour 633	1:1000	Invitrogen
Dyes		
Hoechst 33258	1:1000	Invitrogen

Table 2.1: List of antibodies and dyes used

Ovaries were stained for mitochondria with fluorescently coupled Streptavidin (Molecular Probes, Thermo Fischer Scientific, USA) for 1 hour in 0.3% PBST at RT. They were washed with 0.3% PBST for 2 times, 10 mins each. Followed by Hoechst 33258 staining for 10 minutes, and final wash with 0.3% PBST for 10 minutes. Ovaries were mounted in SlowFade Antifade (Thermo Fischer Scientific, USA). 5-10 days old flies were dissected and 5-10 animals were dissected for each experiment. Fluorescent probes used were: streptavidin 568, streptavidin 633 (1:1000), phalloidin 647 (1:1000). Imaging was done using the Zeiss 710 confocal microscope and 40x/1.4 oil objective, and Leica TCS SP8 confocal microscope with a 40x/1.4 oil objective. Image analysis was done using ImageJ.

2.4. MitoTracker-Red (CMXRos) staining

Ovaries were dissected in Schneider's medium. Incubated in 1 μM CMXRos red for 45 minutes and washed twice in fresh medium. The ovaries were fixed with 4% PFA in 1X PBS followed by 3 times washes of 0.3% PBST. The nuclear staining

with Hoechst 33258 was performed as mentioned in immunostaining protocol. The ovaries were mounted in Slowfade antifade.

2.5. Glycolysis inhibition

Ovaries were dissected in Schneider's medium and incubated with 2DG for 30 minutes. 2DG was reconstituted in DMSO and then added to Schneider's medium to final concentration of 100µM. Control treatment was done with the equivalent amount of DMSO. Followed by 2 time washes of 1XPBS, 5 minutes each. Tissue was fixed with 4% PFA for 20 minutes, followed by 2 time washes with 0.3% PBST. The rest of the procedure as same as the immunostaining for pAMPK staining.

2.6. ROS detection

Ovaries were dissected and washed in the Schneider's medium followed by the (30uM) DHE treatment for 10 minutes to detect the ROS. The ovaries were washed in the Schneider's medium for 5 minutes. To image the ROS, the ovaries were separated and mounted in the Schneider's medium on the dish containing poly-L lysine coating. To prepare poly-L lysine coating, the 0.3% W/V solution of poly-L lysine (T8787,sigma) was dilute in 1X PBS and allowed to air dry at room temperature overnight. The images were taken at 40X magnification.

2.7. Quantification of oocyte nucleus position

Oocyte nucleus migrates to dorsal anterior position in response to EGFR signalling at stage 7. Nucleus which are on antero-posterior axis of oocyte are considered to be mis localized.

2.8. Quantification of Ppr-GFP

The distribution of Ppr::GFP is quantified using ImageJ. The mean intensity of Ppr in the desired ROI is measured and the ratio to the mitochondrial intensity (stained with streptavidin) is taken.(N=number of cells, number of egg chamber). The stages of egg chambers were quantified based on morphology and plane of maximum area for entire study (Dongyu Jia et al.,2016).

2.9. Quantification of mitochondrial signal in *ppr*^{A} follicle cell clones

Mean intensity of mitochondria in clones are measured and their ratio to the mean intensity of mitochondria in non-clonal cells are taken.(n=data point from single clone from the single egg chamber).

2.10. Quantification of embryonic lethality

Cages were set using 50-60 flies of 2-3 days old in agar media plates without cornflour providing yeast paste for nutrition at 25^{0}C. Plates were changed every day. After 3 days of acclimatization period, embryos were collected and plated in media plates. Percentage of lethality were counted at 24 h and 48 h. Each experiment repeated 3 times.

2.11. Statistical analysis of the data

The data obtained from the ImageJ was stored in the excel sheet and analysed by Graphpad prism software. To conclude the data the unpaired-t test was used.

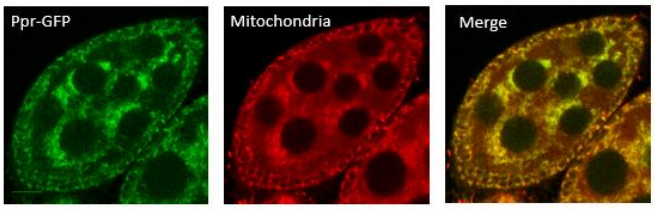
3. Results

3.1. Ppr::GFP localises on mitochondria in nurse cells and follicle cells in *Drosophila* oogenesis

The *Drosophila* Ppr protein has been shown to have important function in transcriptional regulation of mitochondrial DNA. *Drosophila* oogenesis occurs in ovarioles which contain two classes of stem cells. The germ line stem cells (GSC) giving rise to the oocyte and nurse cells and the follicle cell stem cell(FSC) giving rise to the follicle cells. The follicle cells encompass the 15 nurse cells and an oocyte forming a complete egg chamber that proceeds to further stages from 1-14. It is likely that specific stages of egg chambers show differentiation of these lineages and may require transcriptional regulation by Ppr.

As a first aim, we tried to observe the distribution of Ppr using GFP labelled transgene *ppr::GFP* in the germ cells and follicle cells. As shown in the figure(3.1.A,B), the Ppr::GFP shows colocalization with mitochondrial marker streptavidin in follicle cells and germ cells across the stages of oogenesis. Thus Ppr-GFP is localized to mitochondria in follicle cells and germ cells in *Drosophila* oogenesis.

A



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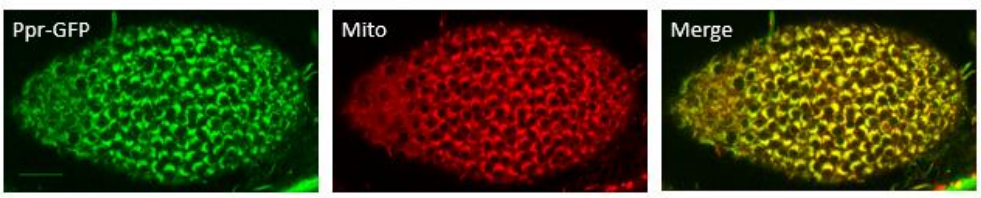
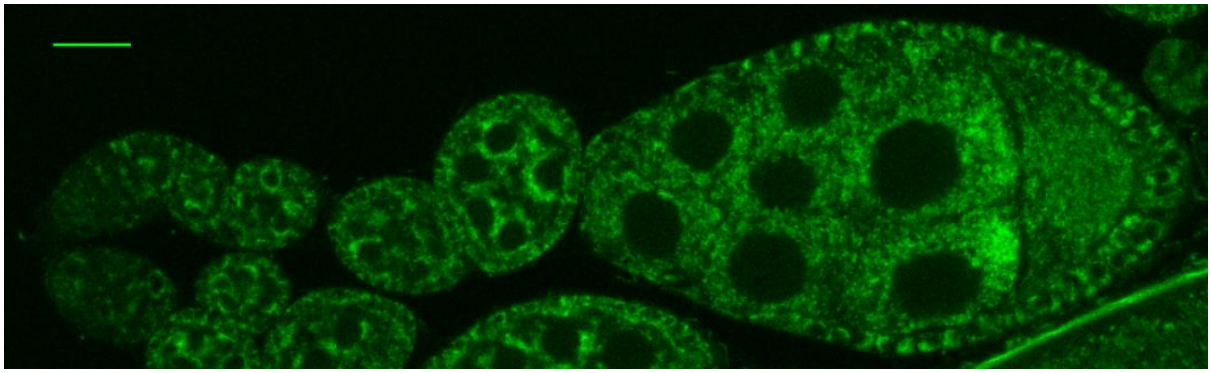


Figure 3.1: Distribution of Ppr::GFP on mitochondria **(A)** In nurse cells **(B)** In follicle cells(n=60)

3.2. Ppr::GFP shows differential expression in stages of *Drosophila* oogenesis

As the results in the 3.1 suggest a localization of Ppr::GFP in the mitochondria, we tried to check the expression of Ppr::GFP across all the stages of oogenesis in the follicle cells and the germ cells. The expression of Ppr::GFP observed to be different along different stages of oogenesis in both follicle cells and germline cells as shown in the figure (3.2). Differential expression of Ppr::GFP was observed across stages of germ cell and follicle cell development and in the next sections, we systematically quantified the levels of increase of the Ppr::GFP expression.

A



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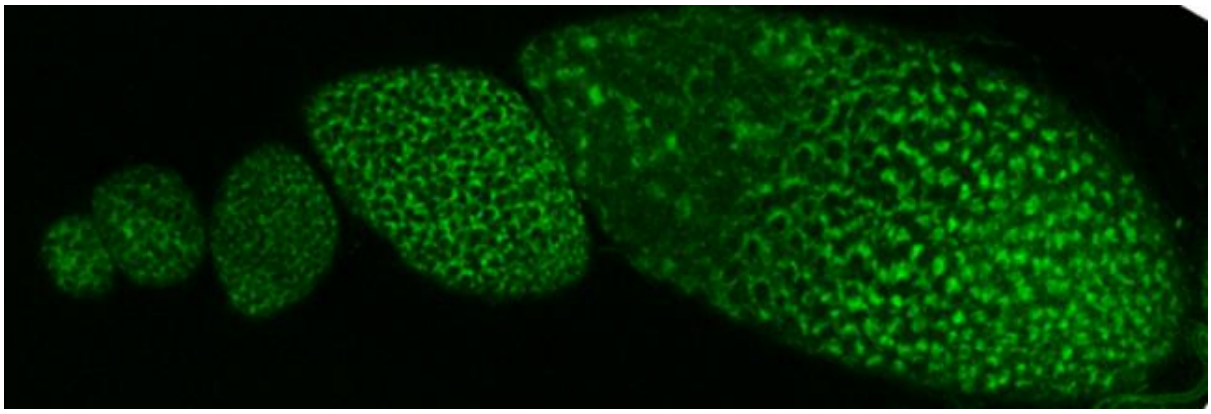


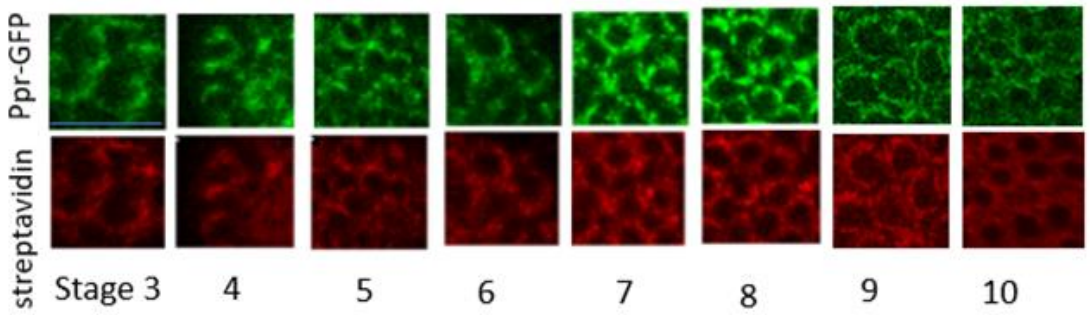
Figure 3.2: Differential expression of Ppr::GFP along stages of oogenesis in **(A)**Nurse cells **(B)**Follicle cells. Scale bar=10µm

3.3. Expression of Ppr::GFP in follicle cells increases in the beginning of endocycle (stage 7)

In continuation of qualitative analysis of Ppr::GFP in 3.2, we tried to quantify the level of Ppr::GFP in all the stages of oogenesis in the follicle stem cell lineage

and germ stem cell lineage. Ppr::GFP was quantified as a ratio to fluorescently labelled Streptavidin which targets to mitochondria. Expression of Ppr::GFP shows a varying intensity in follicle cells across the stages of oogenesis. Ppr::GFP intensity shows a decrease till stage 6 of oogenesis, which is followed by a an increase in intensity when the cells enter endocycle which is at stage 7 as shown in figure (3.3.A). Intensity of Ppr::GFP keep increasing till stage 8 and then decreases to the initial levels by stage 10 which gives an insight to the requirement of Ppr protein in follicle cells during the initial stages of endocycle(figure 3.3.B).

A



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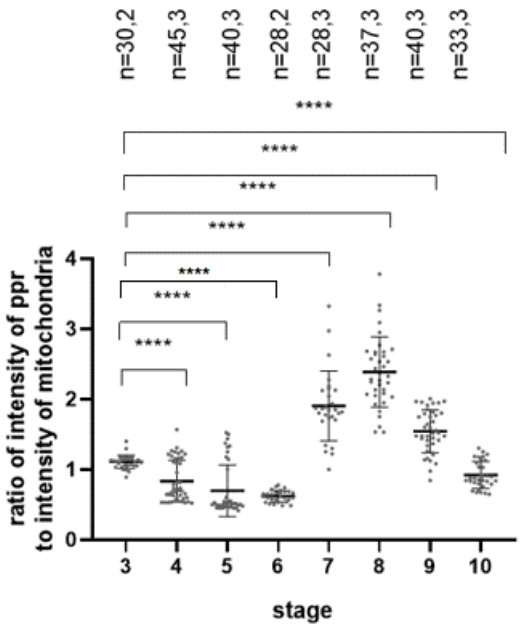
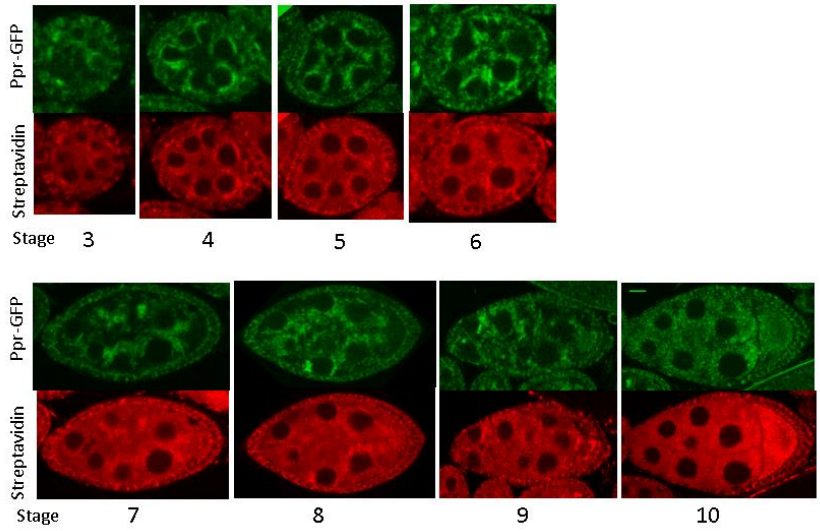


Figure 3.3: Expression of Ppr::GFP in follicle cells across different stages of *Drosophila* oogenesis.**(A)**Images for stage wise expression of Ppr::GFP in follicle cells. Scale bar=15µm**(B)** Quantification of the ratio of intensity of Ppr::GFP to the mitochondria. Analysis was done using unpaired t-test (n=No.of follicle cells, No.of egg chamber)

3.4. Expression of Ppr::GFP in the nurse cells increases prior to endocycle (stage6)

Expression of Ppr::GFP in nurse cells vary across stages during *Drosophila* oogenesis. Intensity of Ppr::GFP shows a nearly constant value in the initial stages, and shows an increase prior to entering the endocycle, at stage 6 after which it decreases to the initial levels as shown in the figure (3.4.A-B).

A



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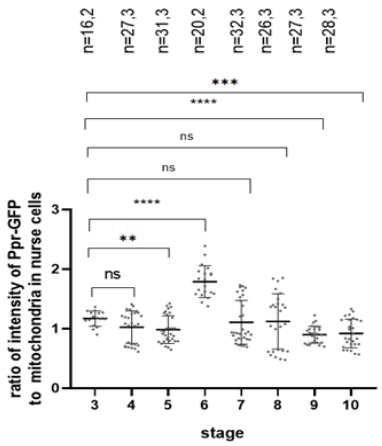


Figure 3.4: Expression of Ppr::GFP in nurse cells across the stages of *Drosophila* oogenesis
(A)images for expression of Ppr::GFP in nurse cells at different stages. Scale bar=15 µm)
(B) Quantification of the ratio of intensity of Ppr::GFP to the mitochondria. Analysis was done using unpaired t-test(n=No.of nurse cells, No.of egg chamber)

3.5. Ppr-GFP expression in the oocyte increase in the later stage of endocycle (stage 8)

As oocyte is also derived from germ cells, we tried to quantify Ppr distribution in oocyte. In addition, oocyte being metabolically inactive, inherit RNAs and protein from nurse cells as it is connected via ring canals. Quantification of ratio of intensity of Ppr::GFP to the mitochondria in oocyte shows an increased value in later stages of endocycle, which comes back to the initial values after stage 8 as shown in the figure(3.5).

A

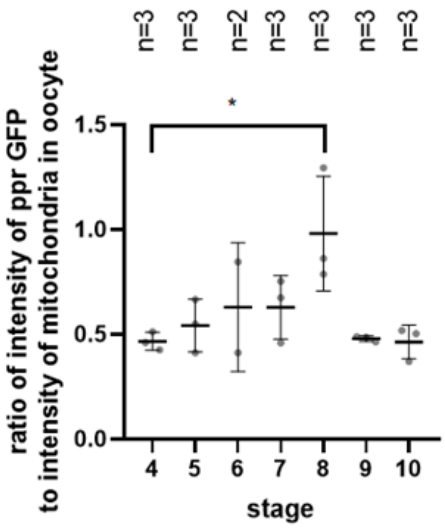


Fig 3.5: Expression of Ppr::GFP in oocyte vary across stages of *Drosophila* oogenesis**(A)** quantification of the ratio of intensity of Ppr::GFP to the mitochondria in oocyte(n=No.of oocyte)

3.6. RNAi mediated depletion of *ppr* in the germ line (nurse cells) and follicle cells results in clustered mitochondrial morphology

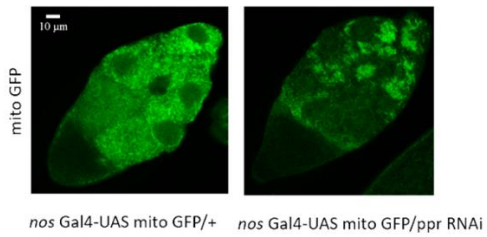
Depletion of Ppr has been shown to result in increase in clustered mitochondria in the muscle (Manish Jaiswal, personal communication). We next tested the effect on oogenesis of depletion of *ppr* . The depletion of *ppr* in germline caused a clustered mitochondrial morphology in nurse cells throughout the stages(figure 3.6 A)

We also monitored the phenotype of mitochondrial clustering in follicle cells mutant for *ppr* by using the ppr^{A} allele. For this we created clones using FRT-FLP technique for mitotic recombination. Like the *ppr* RNAi expression in nurse cells, the mitochondria appeared clustered in the the ppr^{A} follicle cell clones also (figure.3.6 B).

Along with the clustered mitochondrial phenotype, the streptavidin intensity was found to be increased in ppr^{A} mutant follicle cell clones (figure.3.6.C). This could be an indication of increased biosynthesis of mitochondria. The quantification shows consistent increase in the intensity of streptavidin in *ppr* mutants compared to background cells in all stages and a maximum value of ratio of the same at stage 7 (figure.3.6.D).

Hsp60 is a mitochondrial protein which acts as a molecular chaperon under stress conditions. Hsp60 is a mitochondrial resident protein and it marks mitochondria. ppr^{A} clones are characterised by an increased Hsp60 compared to non-mutant background(figure.3.6 E). The ratio of intensity of Hsp60 of ppr^{A} mutant to the background consistently shows a value greater than 1.

A



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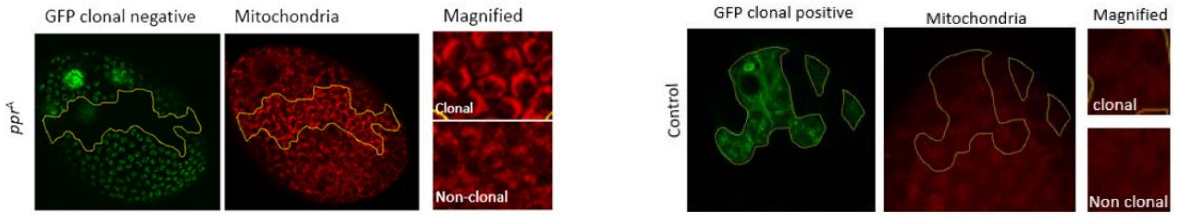

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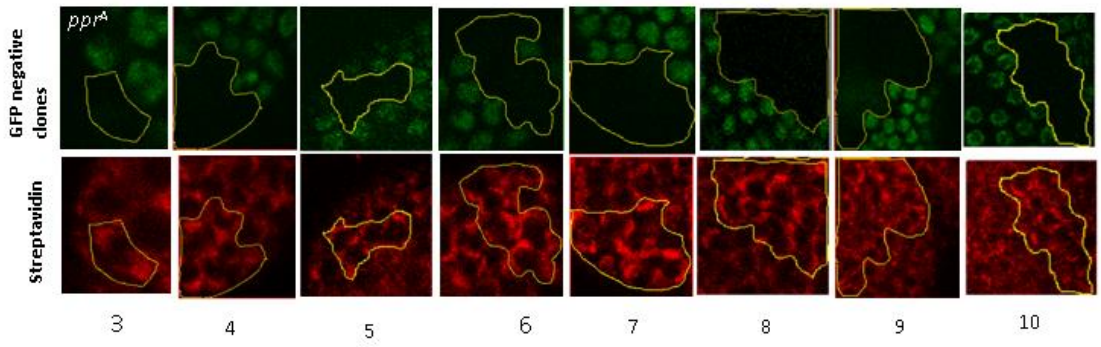

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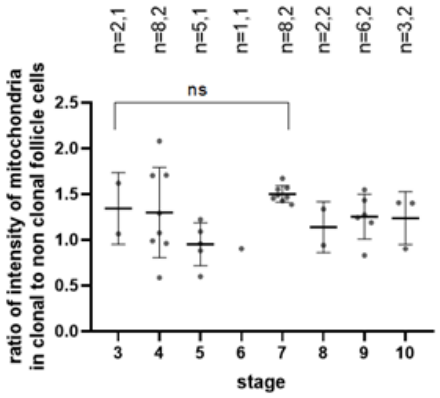

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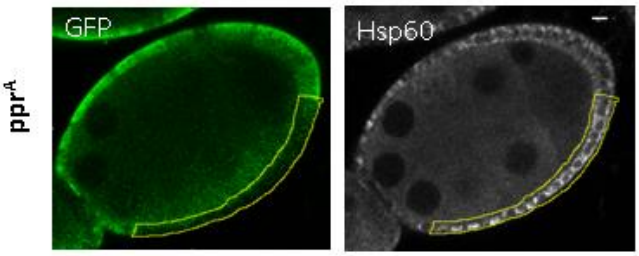


F

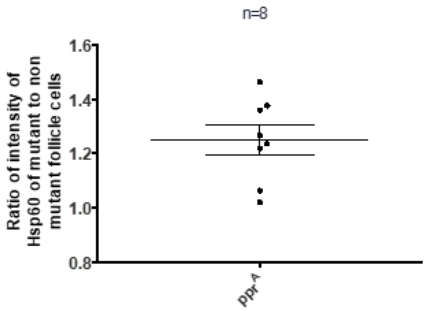


Figure 3.6: Depletion of *ppr* caused clustered mitochondrial morphology **(A)** RNAi mediated depletion of *ppr* causes clustered mitochondrial morphology in nurse cells **(B)** Mitochondrial morphology in *ppr* A mutant follicle cell clone and control **(C)** Increased mitochondrial intensity in *ppr*^{A} mutant follicle cell

across stages of oogenesis **(D)** quantification of ratio of mitochondrial intensity in clonal to non-clonal follicle cell in *ppr* ^{A} (n=No.of clones, No.of egg chambers)**(E)**Increased intensity of Hsp60 in *ppr*^{A} clonal cells**(F)** Quantification of ratio of intensity of Hsp60 in clonal to non-clonal follicle cell(n= number of egg chambers). Scale=10µm

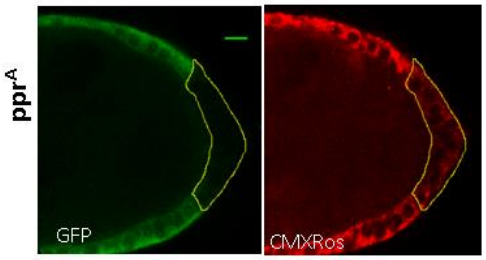
3.7. Mitochondrial membrane potential is lowered in *ppr*^{A} mutant follicle cells

The previous results of clustered mitochondrial morphology let us to know the status of mitochondrial membrane potential (MMP) as it translates to ATP production in the cell (Zorova.L.D,2018). The mitochondrial potential was analysed using the CMXRos dye in the *ppr*^{A} mutant. It accumulates in mitochondria proportional to the MMP (Pendergrass et al,2004).

CMXRos was used to measure the membrane potential by taking a ratio of fluorescence at GFP negative *ppr*^{A} clones to the background. The CMXRos staining appeared to be decreased in *ppr*^{A} clones when compared to the background and the ratio of intensity is consistently less than 1 as shown in the figure(3.8 A,B). considering the fact that no change in phenotype in clones and background would have given a ratio of 1, this indicates a lowered membrane potential in *ppr* mutants.

The lowered membrane potential can be correlated with lowered ATP production. The ATP-synthesis activity of mitochondria is proportionate with the production of ROS in the cell. Thus we checked the reactive oxidative species (ROS) levels in *ppr* mutants using Dihydroethidium (DHE), a fluorescent dye which give fluorescence upon reacting with super- oxides in live cells. DHE was showing a lowered fluorescence in *ppr*^{A} clones compared to the background as shown in figure (3.8.C). Quantification of ratio of intensities of DHE in clones to the background is giving a value less than 1 which confirms the lower ROS levels in *ppr* mutants as shown in figure (3.8.D).

A



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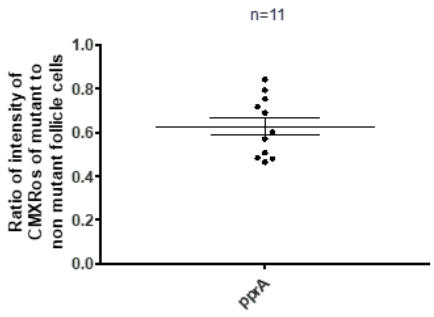


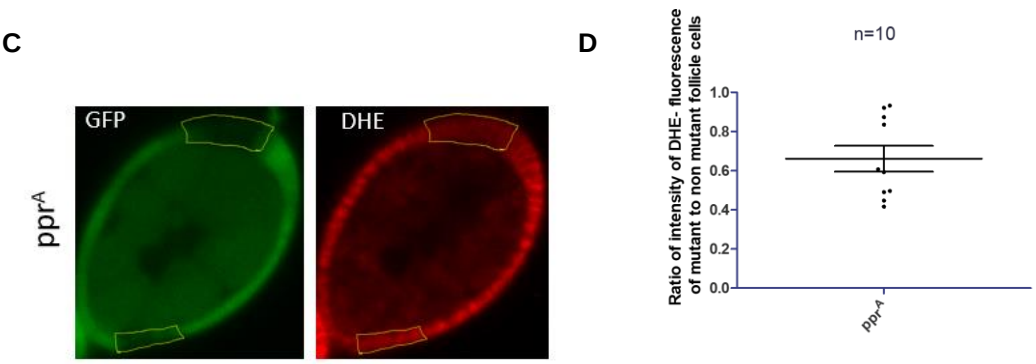


Figure 3.7: (A) Decreased level of CMXRos in *ppr*^{A} clonal cell compared to heterogeneous background **(B)** Quantification of ratio of intensity of CMXRos in mutant to non-mutant follicle cell **(C)** Decreased DHE fluorescence in *ppr*^{A} mutants **(D)** Quantification of ratio of DHE fluorescence in *ppr*^{A} mutants to non-mutant (n=No. of egg chambers). Scale=10µm

3.8. Mutation of *ppr* does not show increase in energy stress markers

The low level of ATP induces the minimization of ATP consumption. Metabolically active cells undergo continuous conversion of ATP into ADP and AMP. The cell starving for energy has lower ATP level and experiences an energy stress. In response to energy stress, AMP-activated protein kinase(AMPK)-a sensor of low ATP levels in the cells gets activated. The phosphorylation of AMPK (pAMPK) is required for the activation of AMPK(Hardie D G,2006).

Though the CMX-Ros levels were decreased, no change in pAMPK was observed in *ppr*^{A} mutant cells compared to heterogeneous background as shown in the figure(3.8.A). The mechanism behind unaltered pAMPK level is yet to be tested.

Effects of inhibition of glycolysis using 2-Deoxy Glucose (2DG), a glucose analogue which cannot serve as a substrate to glycolysis was tested. No change in AMPK levels were observed upon 2DG treatment in *ppr* mutants compared to non-mutant background.

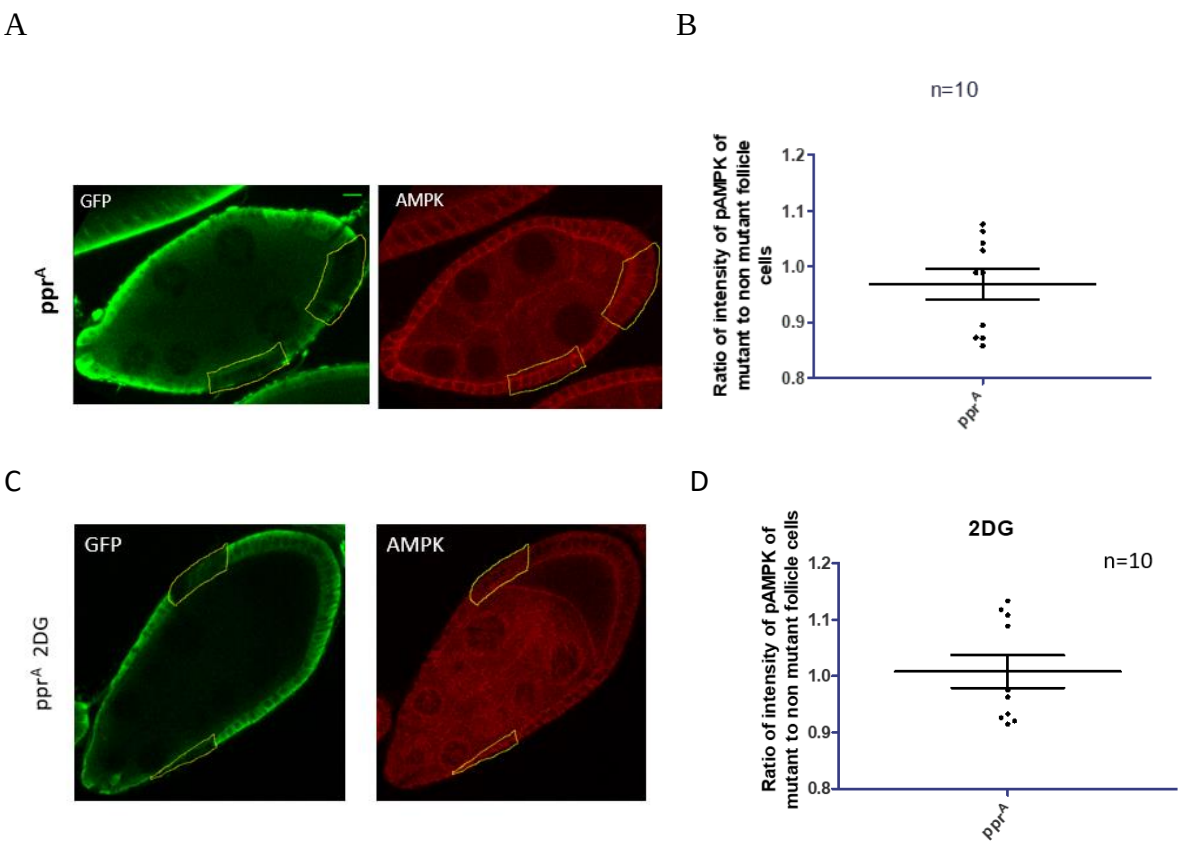


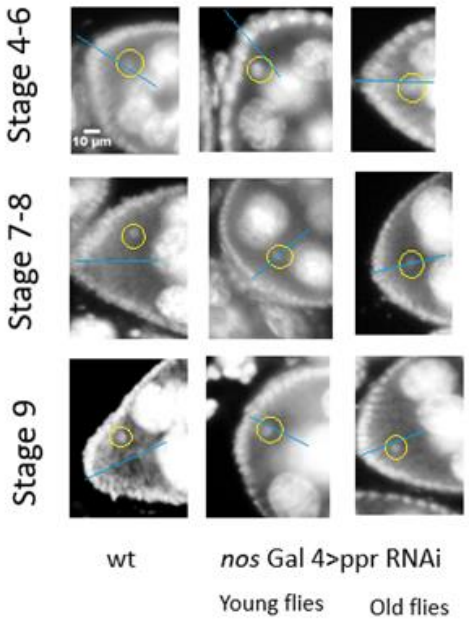
Figure 3.8: (A) No change of level of pAMPK in *ppr*^{A} mutant clones compared to non-clonal background **(B)** Quantification of ratio of intensity of pAMPK in mutant to non-mutant follicle cell **(C)** No change in level of pAMPK in 2DG treated *ppr*^{A} mutant clones compared to non-mutant follicle cell **(D)** Quantification of ratio of intensity of pAMPK in mutant to non-mutant follicle cell. Scale=10µm

3.9. RNAi mediated depletion of *ppr* in nurse cells results in loss of oocyte nucleus migration to the dorso-anterior position

The observed increase in Ppr intensity in *Drosophila* ovary during different stages is closely associated with transition to the endocycle and onset of EGFR (Epidermal growth factor receptor)signalling. Hence we checked if there is a defect in EGFR signalling driven oocyte migration to the dorso-anterior position at stage 7 of oogenesis. We assessed the oocyte migration pattern in the *ppr* RNAi egg chambers in both young (5 -7days) flies and old flies (12-15 days). The oocyte is present at a central position until stage 6 in wild type ovarioles. When EGFR signalling is

activated at stage 7, the cytoskeleton reorients and results in oocyte migration to the dorso-anterior position. Depletion of Ppr protein by using RNAi in the germ line lineage results in loss of oocyte migration in both young and old flies as shown in the figure (3.9.A). The oocyte position was in the centre at the posterior on Ppr depletion thus showing an EGFR signalling loss of function phenotype. The defect in migration of oocyte nucleus appeared to get worse on aging as shown in figure (3.9.B).

A



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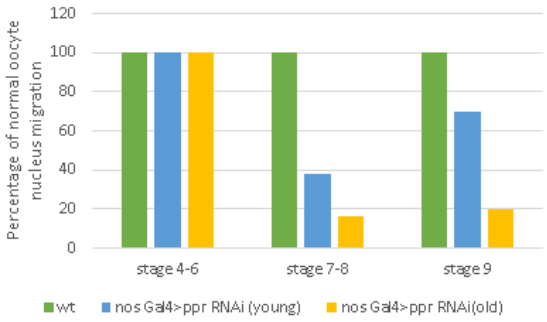

Figure 3.9:RNAi mediated depletion of *ppr* in nurse cells caused loss of oocyte nucleus migration **(A)**oocyte nucleus position in *ppr* depleted egg chambers **(B)** quantification of oocyte nucleus migration(n=20).

3.10. *ppr*^{A} mutant posterior follicle cells show elevated levels of dpERK

From the previous data of oocyte nucleus migration defect, we extended our study to know the status of EGFR signalling in the *ppr* mutant as EGFR signalling pathway is required for oocyte patterning in *Drosophila* oogenesis. In early oogenesis, an interaction between Gurken and EGFR determines a posterior follicle cell fate and thereby anterio-posterior polarity of an egg chamber. By stage 8 of oogenesis, a signal back from posterior follicle cells to germ cells drives

reorganisation of cytoskeleton and migration of oocyte nucleus to dorso-anterior position(Nilson & Schüpbach,1999).

We have seen an increase in levels of *ppr* in oocyte during stage 8 of oogenesis which coincide with EGFR signalling. Hence we planned to assess the impact of *ppr* in EGFR signalling.

EGFR signalling acts via downstream cues like Ras and ERK. Here we checked the levels of dpERK in follicle cells in ppr^{H} clones. An increase in dpERK is observed in ppr^{H} mutant posterior follicle cells as compared to the background heterogeneous cells as shown in figure(3.10.A). This was seen as an increase in ratio of dpERK staining above 1 as shown in figure(3.10.B). Also it is worth noting that the dpERK was present all over the cytoplasm of the cell which is a deviation from the normal where dpERK localises in nucleus. This could be leading to a loss of EGFR signaling and nucleus migration to the dorsal anterior position. This effect was not observed in anterior follicle cells.

A B

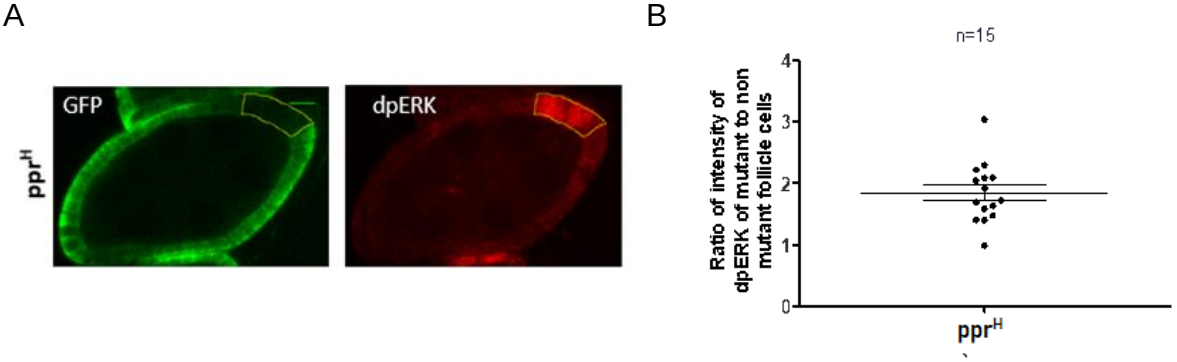

Figure 3.10: (A) Increased level of dpERK in ppr^{H} mutant clones compared to non-mutant background **(B)**Quantification of ratio of intensity of dpERK in mutant to non-mutant follicle cells (n=No.of egg chambers). Scale=10µm

3.11. The *ppr* mutant clones show a relative increase in Cut level

During mitotic division of early stages (1-6), follicle cells express a homeobox transcription factor Cut. Once the Notch signaling is activated it induces hindsight(Hnt) expression which in turn inhibit Cut expression, and the Cut expression starts decreasing from stage 6 (Sun and Deng, 2007).

In *ppr*^{A} mutant follicle cells, Cut shows a higher intensity compared to heterogeneous background as shown in the figure(3.11.A). This is shown as an increase in the ratio of Cut immunostaining in *ppr* mutant follicle cells as compared to the background to more than 1(figure 3.11.B). The depletion of *ppr* in follicle cells using RNAi also shows a longer persistence of Cut compared to the control showing a delayed inhibition of Cut as shown in the figure(3.11.C). And the quantification of the Cut intensity along the stages of oogenesis in *ppr* depleted follicle cells and control is shown in figure (3.11.D)

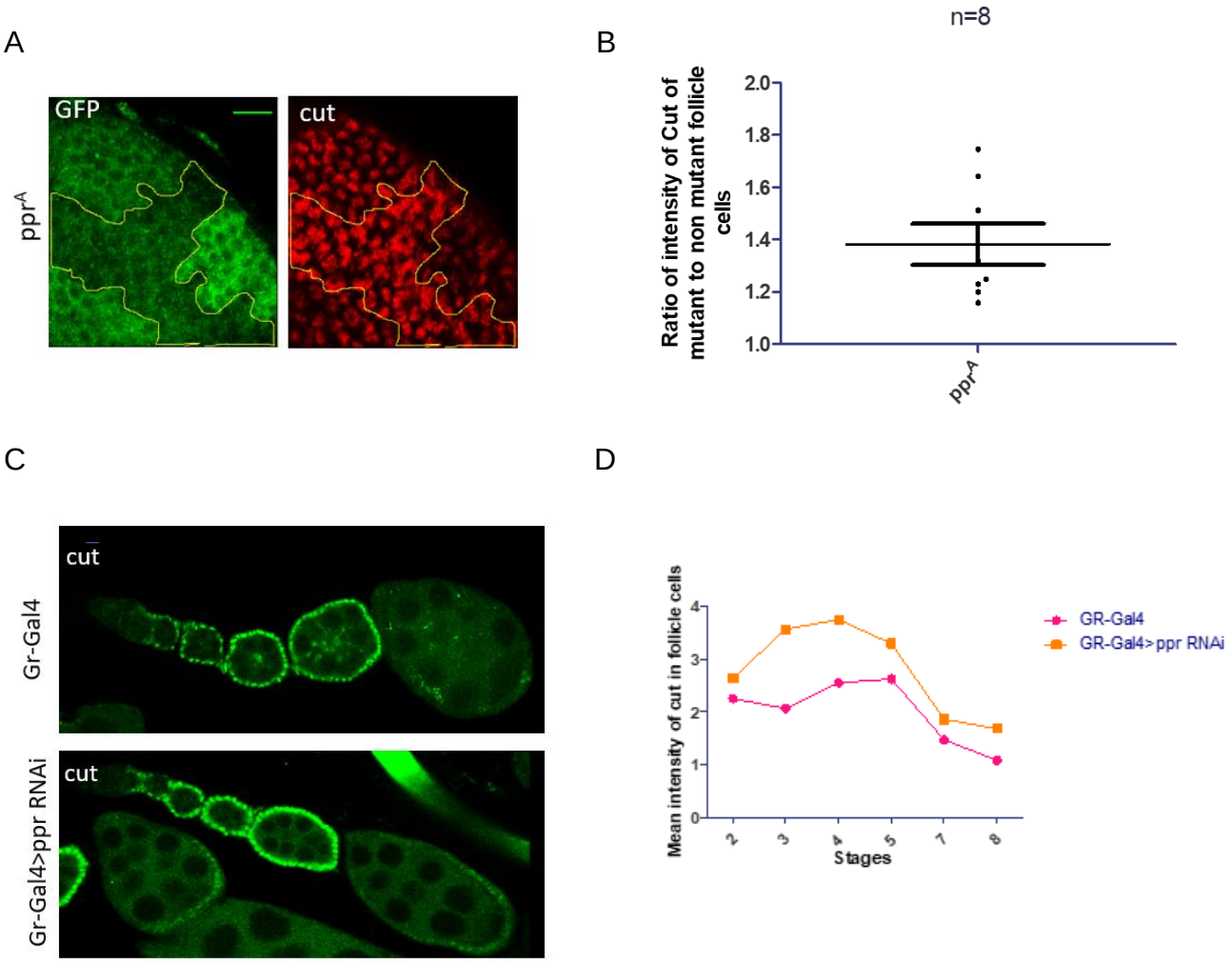

Figure 3.11: Delayed degradation of Cut **(A)**In *ppr*^{A} mutant clones compared to heterogeneous background **(B)**Quantification of ratio of intensity of Cut of mutant to non-mutant follicle cell (n=No.of egg chamber) **(C)** In RNAi mediated depletion of *ppr* in follicle cells **(D)**Quantification of stagewise intensity of Cut in RNAi mediated depletion of *ppr* and wild type(n=5,n=No.of ovarioles). Scale=10µm

3.12. The *ppr* mutant clones show no change in the Notch signaling in the endocycling stages.

Notch is a conserved signalling pathway which help in stem cell maintenance and differentiation. At the end of stage 6 of oogenesis, the epithelial follicle cells are known to enter endocycle where they undergo continuous cycles of DNA replication without cytokinesis(Xu and T. Gridley,2012). This switch from immature to differentiated follicle cell is happening with the help of Notch signalling which gets activated after stage 6(W.M.Deng et al.,2001). Notch signalling gets activated when ligand binds to the Notch receptor. The ligand binding to Notch receptor give rise to proteolytic cleavage of intracellular cytoplasmic domain of Notch receptor known as NICD (Notch intracellular domain) which get transported to nucleus and regulate the transcription of DNA (Struhl and Adachi, 1998) .

To assess the change in Notch signalling pathway, we monitored the plasma membrane accumulation of NICD using a specific antibody in ppr^{A} clones. NICD fluorescence intensity did not show any significant difference in ppr^{A} mutants compared to the background control follicle cells as shown in the figure (3.12.A). The quantification of ratio of intensity of NICD in *ppr* mutant cells to the background gives a value of 1(figure 3.12.B) same as what a control clone would have shown. Taken together there was a delay in clearance of Cut and no defect in NICD processing in *ppr* mutant follicle cells.

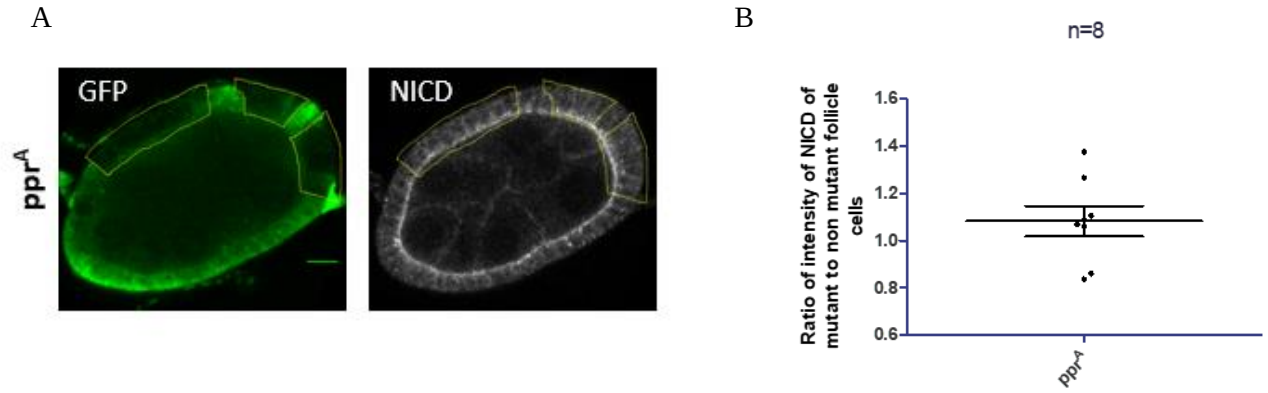

Figure 3.12: (A) No change in level of NICD accumulation on membrane in ppr^{A} compared to heterogeneous background **(B)**Quantification of ratio of intensity NICD of mutant to non-mutant follicle cell. Scale=10µm.

4. Discussion

The study so far agrees with the localization of Ppr onto mitochondria and it's requirement for maintaining normal morphology and function of mitochondria. Ppr most likely plays a role in oogenesis by interacting with the EGFR signaling pathway. We discuss the implications of the observations in the following sections below.

4.1. Implication of differential expression pattern of Ppr during oogenesis

The increased expression of Ppr in specific stages of *Drosophila* oogenesis which are associated with events such as endocycle and activation of EGFR signaling pathway proposes that there could be an increased requirement of proteins which are encoded by mitochondrial genome since the major known function of Ppr is transcriptional and post-transcriptional regulation of the RNA. Particularly the increased expression of *ppr* in oocyte at stage 8 of oogenesis concur with the EGFR signal activation at the same stage.

4.2. Depletion of *ppr* and change in mitochondrial morphology and activity

The depletion of *ppr* using RNAi in nurse cells shows a clustered mitochondrial morphology. The follicle cell mutants for *ppr* also shows a clustered mitochondrial morphology. Also, they show an increased mitochondrial intensity which could be because of the increased mitochondrial biosynthesis in response to the depletion in *ppr.* Also,this appears to have a maximum value at stage 7 where the intensity of Ppr started peaking in follicle cells. The consistent increased value of mitochondrial protein Hsp60 agrees with the results and points to the possibility of increased mitochondrial biosynthesis.

Regardless of the increased mitochondrial intensity, follicle cell mutants of *ppr* show a decreased CMX-Ros level, which is a read out of mitochondrial membrane potential. The decreased membrane potential suggests a lowered level of ATP in *ppr* mutants that requires further confirmation using ATP assay. Lowered ROS levels in *ppr* mutants observed agrees with the reduced mitochondrial potential detected by

CMXRos. Since a fraction of respiratory complexes are encoded by mitochondrial genome, the transcriptional defect which could be followed by depletion in *ppr* is likely to be the reason for impaired oxidative phosphorylation, ATP synthesis, and ROS production observed.

Decreased levels of ATP can cause cellular stress and the stress marker AMPK was expected to be increased. But AMPK levels were not showing any significant difference in *ppr* mutants compared to non-mutant. This indicates that these cells could compensate this depletion of ATP with other means possibly by changing the uptake of Glucose of these cells. Future experiments which test the uptake of Glucose in these cells will be useful to understand the same. 2-Deoxy Glucose treatment was not resulting in any change in pAMPK levels which indicates that glucose doesn't serve as a major substrate for ATP production. This points to the possibility of fat oxidation being the major mechanism of ATP production during oogenesis.

4.3. Effect of depletion of *ppr* in Notch and EGFR signalling

The oogenesis in the fly is patterned by the various signalling pathways in different stages of egg chambers. The stages of oogenesis is categorized in to a) mitotic stage (1-6) and b) an endocycling stage (7-9). The mitotic stage is recognized by the Hindsight signaling read out Cut and an endocycling stage is recognized by Notch signaling read out Hindsight. The EGFR signaling defines polarity and nuclear migration of the oocyte and axis patterning during oogenesis. Therefor an analysis of different signaling pathways along stages of oogenesis may show anomalies in response to *ppr* mutation.

The EGFR signaling is lost in follicle cells containing fused mitochondria due to the depletion of mitochondrial fission protein drp1 with an increased mitochondrial membrane potential (D.Tomer et al.,2017).). In *drp1* mutant cells dpERK accumulated in the cytoplasm. Mitochondrial membrane potential is lowered in cells containing the ERK RNAi. The data of mitochondrial membrane potential (CMXRos) loss in *ppr* mutant follicle cells suggests a possibility of alteration in the EGFR signaling during the oocyte nucleus migration in *ppr* mutant. The increased level of

the cytoplasmic dpERK, an EGFR read out, in the *ppr* mutant suggest an important role of Ppr in the oocyte nucleus migration via indirectly or directly regulating the mitochondrial membrane potential via its morphology modification or modulating the mitochondrial biosynthesis.

The stage dependent requirement of Ppr from the data can be correlated to the endocycling stages where Notch is activated as the Ppr::GFP is higher in the stage 7 & 8. The activation of Notch requires a proteolytic cleavage of the Notch to yield a cytoplasmic NICD that goes to the nucleus during stage 7-9. The unchanged pattern of an NICD in the *ppr* mutant follicle cells suggests that Notch signaling is unlikely to be affected. The Notch signaling is known to activate Hindsight expression. In contrast to the lack of change in the Notch signaling, the Cut persists beyond the stage 6 in the *ppr* mutant follicle cells. The increased level of Cut in the mutant cells can be correlated with delayed degradation of Cut or a delayed inhibition of Hh by defective Notch signaling.

There has been evidences that Notch and EGFR signaling works antagonistically in *Drosophila* eye cell fate specification (Rohrbaugh et al., 2002). The observed increase in activated dpERK, a read out of EGFR pathway, in *ppr* mutant follicle cell indicates an elevated EGFR signaling. However the oocyte migration that is dependent upon EGFR signaling does not take place, possibly because of accumulation of dpERK in the cytoplasm.

So far, our studies add to the evidence that *ppr* plays an inevitable role during stem cell differentiation. We have shown that the function of *ppr* is highly correlated with mitochondrial activity during differentiation using *Drosophila* oogenesis as a model system. Also we have shown there are aberrations in EGFR signaling when mitochondrial genome translation is decreased which leads to the loss of oocyte nucleus migration (figure 4.1.A). The probable mechanism of action of *ppr* from our study is through maintaining appropriate mitochondrial mass and activity which will help the normal EGFR signaling (figure 4.1.B).

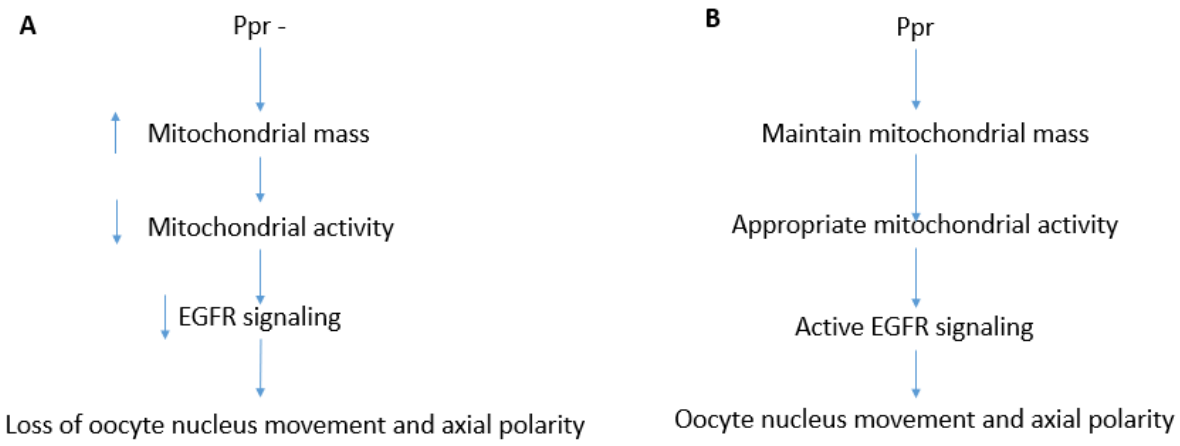

Figure 4.1: Probable mechanism of of action of Ppr **(A)** Resulting phenotypes of *ppr* depletion observed so far **(B)** Possible mechanism of action of *ppr* based on the results.

Mutation of drp1, a mitochondrial fission protein has been shown to cause loss of embryo patterning through an abrogation in EGFR signaling. Mutation in *ppr* shows a comparable mitochondrial phenotype and increase in ERK accumulation in cytoplasm which results in loss of oocyte patterning. But the mitochondrial membrane potential and electron transport chain activity are decreased in contrast to the observations in *drp1* mutants (figure 4.2.A,B). This suggest that the regulation of embryo patterning by Drp1 is acting through functional Ppr as a downstream element.

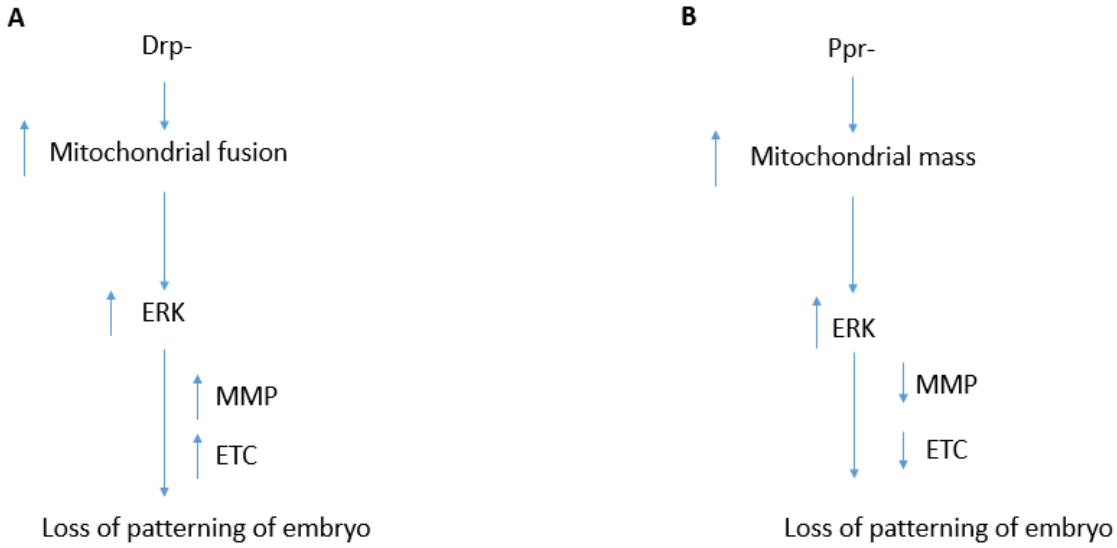

Figure 4.2: Correlation of effect of depletion of Drp1 and Ppr in oogenesis **(A)** Schematic representation of correlation of effects caused by depletion of Drp1(D.Tomer et al.,2017) **(B)** Schematic representation of correlation of effects caused by depletion of Ppr

Mutation of drp1, a mitochondrial fission protein has been shown to cause loss of embryo patterning through an abrogation in EGFR signaling. Mutation in *ppr* shows a comparable mitochondrial phenotype and increase in ERK accumulation in cytoplasm which results in loss of oocyte patterning. But the mitochondrial membrane potential and electron transport chain activity are decreased in contrast to the observations in *drp1* mutants (figure 4.2.A,B). This suggest that the regulation of embryo patterning by Drp1 is acting through functional Ppr as a downstream element.

An interesting question which rises next is the effect of mitochondrial morphology regulating proteins in *ppr* mutants. Disrupting the mitochondrial morphology in *ppr* mutants using depletion of mitochondrial fusion proteins Opa1 and Marf can tell us whether the defects observed are because of the change in mitochondrial functions associated with morphology change.

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