Analysis of mitochondrial morphology dynamics in the maintenance of epithelial polarity in Drosophila oogenesis

A thesis

submitted in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

By

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20163447

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH

PUNE

Dedicated to my parents and teachers…

CERTIFICATE

It is certified that Mr. Bhavin Uttekar's thesis, ***"Analysis of mitochondrial morphology dynamics in the maintenance of epithelial polarity in Drosophila oogenesis"*** which he submitted, represents the original work he conducted while a student at IISER in Pune under my direction and supervision from August 2016 to March 2023. Any portion of the work provided here has never been a part of a thesis that was previously submitted for the granting of a degree or certificate from another university or institution. I further attest that, to the best of my knowledge, the claims he made above regarding his thesis are true.

Date: 15th March 2023

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DECLARATION

I, Bhavin Uttekar, affirm that this writing expresses my views in my own words and that I have properly attributed and referenced any outside ideas I have included. In addition, I affirm that I have followed all rules governing academic honesty and integrity and that I have not created or manipulated any idea, data, fact, or source in my submission. I am aware that breaking any of the aforementioned rules will result in disciplinary action from the Institute and may also result in penalties from the sources who were improperly cited or from whose proper permission was not obtained when necessary. The work that I have reported in my thesis is original work that I completed under Dr. Richa Rikhy's direction.

Date: 15/03/2023

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TABLE OF CONTENT

List of Figures		vii
List of abbreviations		x
Abstract		xiii
Synopsis		xiv
Chapter 1:	**Introduction**	
1.1	Mitochondria dynamics and function	1
1.1.1	Mitochondrial morphology and dynamics	2
1.1.2	Regulation of mitochondrial morphology	3
1.1.3	Mitochondrial morphology and associated changes in mitochondrial activities	6
1.2	Mitochondria and cell signaling pathways	8
1.3	Mitochondria and epithelial cell differentiation	10
1.3.1	Differential distribution and metabolism of mitochondria in stem cells and differentiated cells	10
1.3.2	Epithelial cell differentiation and polarity program	11
1.3.3	Mitochondrial morphology and distribution in epithelial cell differentiation	13
1.4	Mitochondrial morphology in normal development and in diseased condition	14
1.4.1	Mitochondrial morphology during the development of an organism	14
1.4.2	Mitochondrial morphology in diseases	15
1.5	Aims and objectives of the thesis	17
Chapter 2:	**Materials and methods**	
2.1	Material and reagents	18
2.1.1	Fly stocks	18
2.1.2	Primary and secondary antibodies	18

2.1.3	Dyes for mitochondrial activities	19
2.2	Experimental procedures and methods	19
2.2.1	Selection of *Drosophila* oogenesis as a model system	19
2.2.2	Fly genetics and MARCM technique to generate mitotic clones in *Drosophila* ovaries	20
2.2.4	Immunostaining of *Drosophila* ovaries	21
2.2.5	Live imaging using mitochondrial dyes	22
2.2.6	Microscopy and image acquisition techniques	22
2.2.7	Quantification of the Data and statistics	23
	a) Identification of stages of development in ovarioles:	23
	b) Quantification of the formation of multilayer:	23
	c) Quantification of mitochondrial morphology:	23
	d) Quantification of of aPKC, Dlg, PatJ and Hnt:	23
	e) Quantification of apical constriction	24
	f) Quantification of the dpERK:	24
	g) n values and Statistical analysis:	24
Chapter 3:	**Study of mitochondrial morphology in different mitochondrial morphology mutants and analysis of follicle epithelial cell organization during *Drosophila* oogenesis**	
3.1	Introduction	25
3.2	Materials and methods	25
3.2.1	*Drosophila* genetics:	25
3.2.2	Follicle clone generation using MARCM method:	26
3.2.3	Immunostaining of *Drosophila* ovaries:	26
3.2.4	Microscopy and image acquisition techniques:	26
3.3	Results	26
3.3.1	Mitochondrial morphology mutants change the shape of mitochondria in *Drosophila* follicle cells	26

3.3.2	Analysis of follicle cell shape and organization in different mitochondrial morphology mutants in endocyclic stages of *Drosophila* oogenesis	28
3.3.3	Analysis of follicle cell shape and organization in different mitochondrial morphology mutants in mitotic stages of *Drosophila* oogenesis	30
3.4	Discussion	32
Chapter 4:	**Study of follicle epithelial cell polarity in different mitochondrial morphology mutants during *Drosophila* oogenesis**	
4.1	Introduction	34
4.2	Materials and methods	35
4.2.1	*Drosophila* genetics:	35
4.2.2	Follicle clone generation using MARCM method:	35
4.2.3	Immunostaining of *Drosophila* ovaries:	35
4.2.4	Microscopy and image acquisition techniques:	35
4.3	Results	35
4.3.1	Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells of different mitochondrial morphology mutants in endocycling stages of *Drosophila* oogenesis	35
4.3.2	Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells of different mitochondrial morphology mutants in mitotic stages of *Drosophila* oogenesis	37
4.3.3	Analysis of lateral polarity protein disc large (Dlg) in follicle cells of different mitochondrial morphology mutants in endocycling stages of *Drosophila* oogenesis	39
4.3.4	Analysis of lateral polarity protein disc large (Dlg) in follicle cells of different mitochondrial morphology mutants in mitotic stages of *Drosophila* oogenesis	40
4.4	Discussion	41

Chapter 5:	**Study of mitochondrial activities and effect on epithelial cell polarity in different antioxidant enzyme mutants during *Drosophila* oogenesis**	
5.1	Introduction	43
5.2	Materials and methods	46
5.2.1	*Drosophila* genetics:	46
5.2.2	Follicle clone generation using MARCM method:	46
5.2.3	Immunostaining of *Drosophila* ovaries:	46
5.2.4	Microscopy and image acquisition techniques:	46
5.3	Results	46
5.3.1	Analysis of mitochondrial ROS in follicle cells depleted for antioxidant enzymes	46
5.3.2	Analysis of mitochondrial morphology in follicle cells depleted for antioxidant enzymes	48
5.3.3	Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells depleted for the antioxidant enzyme in endocycling stages of *Drosophila* oogenesis	53
5.3.4	Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells depleted for the antioxidant enzyme in mitotic stages of *Drosophila* oogenesis	55
5.4	Discussion	57
Chapter 6:	**Study of signaling pathways responsible for follicle cell polarity and differentiation, including EGFR and Notch signaling pathways during *Drosophila* oogenesis**	
6.1	Introduction	59
6.2	Materials and methods	63
6.2.1	*Drosophila* genetics:	63
6.2.2	Follicle clone generation using MARCM method:	63
6.2.3	Immunostaining of *Drosophila* ovaries:	63

6.2.4	Microscopy and image acquisition techniques:	63
6.3	Results	64
6.3.1	Analysis of EGFR signaling in follicle cells depleted for mitochondrial morphology proteins and antioxidant enzymes in endocycling stages of *Drosophila* oogenesis	64
6.3.2	Analysis of EGFR signaling in follicle cells depleted for mitochondrial morphology proteins and antioxidant enzymes in mitotic stages of *Drosophila* oogenesis	66
6.3.3	Analysis of EGFR signaling in follicle cells depleted for Erk, a component of EGFR signaling pathway, in endocycling and mitotic stages of *Drosophila* oogenesis	68
6.3.4	Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells depleted for Erk, a component of EGFR signaling pathway, in mitotic and endocycling stages of *Drosophila* oogenesis	71
6.3.5	Analysis of oocyte positioning in follicle cells depleted for mitochondrial morphology proteins, antioxidant enzymes, and for erk (EGFR) mutant in endocycling stages of *Drosophila* oogenesis	73
6.3.6	Analysis of Notch signaling in multilayered follicle cells depleted for mitochondrial morphology proteins and antioxidant enzymes	75
6.4	Discussion	77
7	**Thesis summary and future directions**	
7.1	Analysis of follicle cell architecture on mitochondrial fusion by depletion of mitochondrial fission protein Drp1	79
7.2	Analysis of apical polarity protein aPKC in follicle cells on depletion of mitochondrial fission protein Drp1	80
7.3	Analysis of signaling pathways associated with the regulation of follicle cell polarity	80

7.4	Future directions	82
8	**Appendix**	
A1	Fission mutant Drp1 show either absent or mislocalized and accumulated sub-apical market PatJ	83
A1.1	Sub-apical polarity marker PatJ mislocalizes or remains absent in the follicle cell clones depleted for Drp1 proteins at endocycling stages.	83
A1.2	Sub-apical polarity marker PatJ mislocalizes or remains absent in the follicle cell clones depleted for Drp1 proteins at mitotic stages.	84
A2	Follicle cell organization in other fission mutants where mitochondria are fused	85
9	**References**	87

LIST OF FIGURES

Synopsis:

Figure 1: Interaction between mitochondrial morphology, ROS and EGFR and Notch signaling in *Drosophila* FC differentiation

Chapter 1: Introduction

Figure 1.1: Structure and functions of the mitochondria

Figure 1.2: Proteins regulating the mitochondrial morphology

Figure 1.3: Organization of electron transport chain complexes in cristae

Figure 1.4: Mitochondrial morphology, distribution, and activities in stem cells and in differentiated cells

Figure 1.5: Epithelial cell and polarity protein complexes

Chapter 2: Materials and methods

Figure 2.1: *Drosophila* oogenesis as a model system to study epithelial cell polarity

Figure 2.2: Mosaic analysis with the cell repressible marker (MARCM) technique

Figure 2.3: Mechanism to detect mitochondrial ROS

Chapter 3: Mitochondrial morphology and epithelial cell organization analysis in mitochondrial morphology protein deficient follicle cells

Figure 3.1: Mitochondrial morphology in fission and fusion mutants

Figure 3.2: Arrangement of endocycling stage follicle cells of different mitochondrial morphology mutants

Figure 3.3: Arrangement of mitotic stage follicle cells of different mitochondrial morphology mutants

Chapter 4: Study of follicle epithelial cell polarity in different mitochondrial morphology mutants during *Drosophila* oogenesis

Figure 4.1: Drp1 mutant follicle cell clones show loss of aPKC in endocycling stages and are partially recovered in $drp1^{KG};opa1^{i}$

Figure 4.2: Drp1 mutant follicle cell clones show loss of aPKC in mitotic stages and are partially recovered in *drp1*^{KG}*;opa1*^{i}

Figure 4.3 Distribution of basolateral polarity protein (Dlg) in follicle cells of control and *drp1*^{KG} at the endocycling stage

Figure 4.4 Distribution of basolateral polarity protein (Dlg) in follicle cells of control and *drp1*^{KG} at the mitotic stage

Chapter 5: Study of mitochondrial activities and their effect on follicle epithelial cell polarity during *Drosophila* oogenesis

Figure 5.1: Mitochondrial ROS and antioxidant enzymes

Figure 5.2: Mitochondrial ROS in mitochondrial morphology mutants of Drp1, Opa1, and in antioxidant enzyme mutants

Figure 5.3: Mitochondrial morphology analysis in FCs depleted of Drp1 and ROS scavengers

Figure 5.4: Mitochondrial morphology analysis in FCs depleted of Drp1 and FCs co-depleted for Drp1 and ROS scavengers

Figure 5.5: Mitochondrial morphology analysis in sagittal view of FCs depleted of Drp1 and FCs co-depleted for Drp1 and ROS scavengers

Figure 5.6: Multilayering in PFC clones of *drp1*^{KG} is reduced in *drp1*^{KG};*sod2*^{i}, and *drp1*^{KG};*catalase*^{i}

Figure 5.7: Drp1 mutant follicle cell clones show loss of aPKC in endocycling stages and are partially recovered in *drp1*^{KG}*;sod2*^{i} and *drp1*^{KG};*catalase*^{i}

Figure 5.8: Drp1 mutant follicle cell clones show loss of aPKC in mitotic stages and are partially recovered in *drp1*^{KG}*;sod2*^{i} and *drp1*^{KG};*catalase*^{i}

Chapter 6: Study of interaction of change in reactive oxygen species with signaling pathways responsible for follicle epithelial cell polarity and differentiation

Figure 6.1: Mechanism of EGFR signaling

Figure 6.2: Mechanism of Notch signaling and Trans/Cis activation

Figure 6.3: Increase in dpERK in Drp1 depleted clones is rescued on additional depletion of Opa1 and ROS scavengers at the endocycling stage

Figure 6.4: Increase in dpERK in Drp1 depleted clones is rescued on additional depletion of Opa1 and ROS scavengers at mitotic stage

Figure 6.5: ERK depletion shows a decrease in accumulation of dpERK in *drp1*^{KG}*;erk*^{i} in endocycling and mitotic stage follicle cell clones

Figure 6.6: The extent of multilayering is reduced in *drp1*^{KG}*;erk*^{i} compared to *drp1*^{KG}

Figure 6.7: aPKC depletion in Drp1 depleted FC clones recovers on additional depletion of ERK

Figure 6.8: Oocyte migration defects in *drp1*^{KG} mutant egg chambers do not get suppressed on additional depletion of Opa1, and ROS scavengers

Figure 6.9: Notch differentiation defects in Drp1 depleted PFC clones are suppressed on additional depletion of Opa1, ROS scavengers, and ERK

Appendix

Figure A1: Drp1 mutant follicle cell clones show the absence or mislocalization of PatJ in mitotic stages that are not rescued in *drp1*^{KG}*;opa1*^{i}

Figure A2: Drp1 mutant follicle cell clones show the mislocalization and accumulation of PatJ in mitotic stages that are not rescued in *drp1*^{KG}*;opa1*^{i}

Figure A3: Mitochondrial morphology and follicle cell arrangement in fission mutant *Drp1*^{SG}

LIST OF ABBREVIATIONS

AarF domain-containing kinase 1	ADCK1
Adherence junctions	AJ
Apoptosis signal-regulated kinase 1	ASK1
Atypical PKC isotype-specific interacting protein	ASIP
Atypical protein kinase C	aPKC
Autosomal dominant optic atrophy	ADOA
Bazooka/Partitioning defective 3	Baz/Par3
Big MAP kinase 1	BMK1
cAMP response element binding protein	CREB
Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)	CCCP
CBF1, Su(H), LAG1	CSL
c-Jun N-terminal Kinase	JNK
Crumbs	Crb
cyclic Adenosine monophoshate	cAMP
Cysteine	Cys
Delta, Serrate, and Lag-2	DSL
Disc large	Dlg
Doubly phosphorylated extracellular signal related kinase	dpERK
Downstream Receptor kinase	DRK
Drosophila optic atrophy 1	dOpa1
Dynamin related protein 1	Drp1
Electron transport chain	ETC
Embryonic stem cells	ESCs
Epidermal Growth Factor	EGF
Epidermal Growth Factor Receptor	EGFR
Extra cellular matrix	ECM
Extra cellular signal related kinases	ERK
Follicle cells	FCs
Follicle stem cells	FSCs

Fuzzy Onion	fzo
Germ stem cells	GSCs
Green Fluorescene protein	GFP
Guanosine triphosphatases	GTPase
Heat shock protein 60	Hsp60
Hindsight	Hnt
Indirect Flight Muscle	IFM
Inner mitochondrial membrane	IMM
Intestinal stem cells	ISCs
Intracellular pH	pHI
Lethal giant larvae	Lgl
Long-Optic atrophy protein 1	L-OPA1
Mitochondrial associated regulator factor	Marf
mitochondrial DNA	mtDNA
Mitochondrial dynamin like protein 1	MGM1
mitochondrial processing peptidase	MMP
Mitochondrial reactive oxygen species	mtROS
Mitofusin-1	MFN1
Mitofusin-2	MFN2
Mitogen-activated protein kinase kinase	MAPKK
Mosaic analysis with the cell repressible marker	MARCM
Mouse embryonic fibroblast	MEF
NF-E2 related factor 2	Nrf2
Non-small-cell lung cancer	NSCLC
Notch Intracellular-Domain	NICD
Optic atrophy 1	Opa1
Outer mitochondrial membrane	OMM
Oxidative phosphorylation	OXPHOS
Partitioning defective	Par
PALS1-associated TJ protein	PatJ
Permeability transition pores	PTP

Phosphatase and tensin homolog depleted on chromosome 10	PTEN
phosphatidylinositol 3,4,5 triphosphate	PIP3
phosphatidylinositol 4,5 bisphosphate	PIP2
phosphoinositide 3-kinase	PI3K
Planar cell polarity	PCP
Platelate derived growth factor	PDGF
Pleckstrin homology domain	PH Domain
Pluripotent Stem Cells	PSCs
Posterior follicle cells	PFCs
Pre-follicle cells	pFCs
presenilins-associated rhomboid-like	PSARL
Protein tyrosine phosphatases	PTPs
Reactive oxygen species	ROS
Respiratory chain supercomplexes	RSC
Scribble	Scrib
Short-Optic atrophy protein 1	S-OPA1
Stardust	Sdt
Super oxide dismutase 1	SOD1
Super oxide dismutase 2	SOD2
Tight Junction	TJ
Transforming growth factor	TGF
triple-negative breast cancer cells	TNBC
Unfolded protein response	UPR

ABSTRACT

A number of stem cells in *Drosophila* and mammalian systems are partially polarised epithelial cells that have a lateral and basal domain but no apical domain. As a result of their differentiation, an apical domain develops, resulting in a completely polarised epithelial cell. Signaling and metabolic processes combine to control stem cell differentiation. The suppression of the EGFR signaling pathway is coincident with the apical domain formation in *Drosophila* follicle stem cells and intestinal stem cell differentiation. Previous research has demonstrated that the lack of the mitochondrial fission protein Drp1 results in abnormal EGFR and Notch signaling as well as the loss of follicle cell differentiation. We have attempted an analysis of the role of mitochondrial dynamics in regulating mitochondrial activity and epithelial polarity in follicle cell differentiation. We have found that loss of Drp1 leads to the presence of follicle cells in multiple layers. This is not seen in the depletion of mitochondrial fusion protein Opa1. Multilayering is coincident with a cytoplasmic accumulation of EGFR signaling component ERK and loss of apical polarity protein aPKC. Interestingly, this loss of apical polarity is regulated by levels of reactive oxygen species (ROS). An increase in ROS by depletion of SOD2 and Catalase leads to mitochondrial fragmentation and suppression of apical polarity defect in Drp1-depleted follicle cells similar to Opa1. We find that EGFR signaling pathway activation with ERK inhibits apical polarity in Drp1 depleted follicle cells. Further, mitochondrial fragmentation and an increase in ROS lead to apical polarity and Notch-mediated differentiation in Drp1 depleted follicle cells. Our studies implicate an important interaction between signaling, mitochondrial activity in the form of ROS, and apical polarity formation during differentiation in *Drosophila* follicle cells.

SYNOPSIS

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1. Introduction:

ATP is famously produced by mitochondria, a double-membrane cell organelle (Jouaville et al. 1999; Osellame, Blacker, and Duchen 2012; McQuibban et al. 2006). From the ancestors of -proteobacteria, eukaryotic cells acquired mitochondria as an endosymbiont (Urbauer, Rath, and Haller 2020; Martin, Garg, and Zimorski 2015; Zachar and Boza 2020; T. Ishihara, Kohno, and Ishihara 2015; Nunnari and Suomalainen 2012; Gray, Burger, and Lang 1999; Frohman 2010). The discovery that mitochondria serve as a place for oxidative phosphorylation helps the cell by supplying energy for a variety of cellular functions (Chaban, Boekema, and Dudkina 2014). In addition to producing energy, mitochondria also perform a number of other tasks, including the generation of ROS, calcium buffering, metabolic control, the synthesis of phospholipids and heme, cell cycle control, apoptosis, and other signals (Cipolat et al. 2004; Urbauer, Rath, and Haller 2020; Brand et al. 2013; Benard et al. 2010; N. Ishihara et al. 2009; Romanello et al. 2010; Mayr et al. 2011; Seo, Yoon, and Do 2018). One of the crucial roles that mitochondria play in cell stress is also known (Kasahara and Scorrano 2014). Being present in the cell, mitochondria communicate with the nucleus and cause the cell to undergo unfolded protein response (UPR) (Favaro et al. 2019). Thus, mitochondria perform diverse functions in the cell and many of them are yet to be understood in detail. The shapes of mitochondria alter dynamically and are advantageous to the cells. Specific proteins are needed to assist in the transformation of mitochondria from one type to another. Several of these proteins work to produce either fused or fragmented mitochondria depending on the needs of the cell (Scott and Youle 2010; Y. J. Liu et al. 2020). A Dyanamin relation protein-1 (Drp1) protein causes the fission, which results in the fragmentation of

mitochondria, in a very ordered manner (N. Ishihara et al. 2009; Basu et al. 2017; Frank et al. 2001; Zhu et al. 2004). There are two sets of proteins required for the fusion of the mitochondria. The outer mitochondrial membrane is fused by an OPA1, a dynamin-related GTPase, in the cell (van der Bliek 1999; N. Ishihara et al. 2006; Kowaltowski et al. 2019). Mitofusin is required to fuse the outer mitochondrial membrane (OMM) (Kasahara et al. 2013; Rana et al. 2017). Mitochondrial metabolism is regulated by the action of these fusion and fission proteins in the cell. The quality control of mitochondrial cristae is taken care of by these fusion proteins. The change in the equilibrium of mitochondrial dynamics leads to a change in mitochondrial activities. Impairment of fusion events promotes ROS production in the cell (Millet et al. 2016; Quintana-Cabrera et al. 2018, [a] 2021). The ROS regulates apoptosis (X. Liu et al. 1996; Eleftheriadis et al. 2016), cell signaling, etc (Tait and Green 2012; Zhang et al. 2016; Rodríguez-Colman et al. 2017). The mitochondrial amount and shape play a vital role in the control of cell differentiation, cell fate specification, and ongoing organismal development (Madan et al. 2021). The process of stem cell differentiation occurs extremely early in an organism's development. We've gathered research on mitochondria's potential significance in stem cell development. To distribute them into freshly produced or differentiated daughter cells, the proliferative stem cells need fragmented and smaller mitochondria (H. Chen and Chan 2017). Differentiation of C2C12 cells and HC11 (Mouse mammary epithelial cell line) requires efficient oxidative phosphorylation (OXPHOS) (Remels et al. 2010; Elswood et al. 2021). An increase in mitochondrial biogenesis, mitochondrial potential, ATP, and mitochondrial ROS are also linked to the differentiation of human embryonic stem cells (hESCs) (Cho et al. 2006). Since disrupting respiratory chain complexes impairs even cardiomyocyte differentiation, oxidative phosphorylation is also a need for stem cell differentiation (Chung et al. 2007). In order for mice cells to differentiate into heart tissue from embryonic stem cells (ESCs), Mfn1/2 and Opa1 fusion proteins should function to fuse the mitochondria (Kasahara et al. 2013). In *Drosophila*, the fusion of the mitochondria and an increase in the mitochondrial pyruvate metabolism are necessary for intestinal stem cells to differentiate into enterocytes (Schell et al. 2017; Deng et al. 2018).

This literature helps us understand how the shape of mitochondria can regulate cell differentiation. An example of such differentiation is the formation of an epithelial cell from its precursor cell. An epithelial cell's polarity complexes and cell-cell junctions are both well-structured when it first forms during embryogenesis (Thowfeequ, Stower, and Srinivas 2022; Louvet-Vallée et al. 2001).

The epithelial cells in multicellular species, like *Drosophila*, polarise from their progenitor epithelial stem cells or from already-existing epithelial cells (Castanieto, Johnston, and Nystul 2014; J. Chen and St Johnston 2022). There are three domains a) apical, b) lateral, and c) basal in each polarised cell (J. Chen et al. 2018). An apical domain possesses a complex containing atypical protein kinase C(aPKC)- Bazooka(PAR3)- PAR6 proteins and a subapical complex containing Crumbs(Crb)-PatJ-Stardust(Sdt) proteins, a lateral domain complex containing Scribble(Scrb)-Disclarge (Dlg)- Lethal giant larvae (Lgl) proteins, and a basal domain possess integrin proteins and these proteins are conserved across species (Tepass et al. 2001; Assémat et al. 2008; Royer and Lu 2011). The apical and basal domains of polarized cells are separated by the presence of Adherens junctions (AJs) composed of DE-Cadherin in *Drosophila* (Müller 2000; Royer and Lu 2011). The order of the polarity establishment is distinct when the epithelial cells polarize. The polarity establishment helps in embryogenesis, organ formation, and collective cell migration (Leptin 2005; Montell 2008; Heisenberg and Bellaïche 2013). Thus, a formation of an appropriate polarity in the epithelial cells makes them capable of functioning and migrating together which is necessary for tissue formation. The role of mitochondria, while epithelial cells are differentiating and getting their polarity, is not well understood. Very few pieces of evidence are there stating the role of mitochondria in epithelial cell differentiation and in the formation of polarized epithelial cells. Elongated mitochondria and an oxidative phosphorylation process are required for hepatocyte development where polarity is formed gradually during the differentiation process (Fu et al. 2013). During the development of a mouse tooth, the differentiation of epithelial cells and a shift in metabolism are necessary for the production of enamel (Imhof et al. 2020). Additionally, to convert crypt-based columnar epithelial cells (CBCs) into paneth cells (PCs) with low mitochondrial activity, variations in mitochondrial activity are required (Ludikhuize et al. 2020). *Drosophila* intestinal epithelial differentiation from the intestinal stem cells (ISCs) is supported by a fused mitochondrial morphology (Deng et al. 2018). We are motivated to learn more about this subject by the paucity of literature on the examination of mitochondrial shape in controlling epithelial cell differentiation. We employ *Drosophila* oogenesis to study the formation of epithelial cells and the relevance of mitochondrial shape and mitochondrial activities in light of the aforementioned literature.

Each of the two ovaries in the *Drosophila* has between 16 and 20 ovarioles. Each ovariole includes 16 germ cells in its developing egg chambers, which are encircled by a layer of follicular

epithelial cells. Follicle stem cells and germ stem cells are located in the germarium at the beginning of each ovariole (Fadiga and Nystul 2019). Based on their capacity for selective gene amplification and proliferation, the egg chambers are divided into three stages: stage 1-6 mitotic, stage 7-10a endocycling, and stage 10b-13 gene amplification (Jia, Huang, and Deng 2015). Stages 1-6 are mitotic egg chambers because follicle cells continue to divide at this point. The follicle cells stop dividing but continue to duplicate DNA numerous times in stages 7 to 10a, which are endocycling egg chambers. The egg chambers selectively amplify the genomic region in stages 10b–13. At the conclusion of oogenesis, the stage 14 egg chamber develops through the aforementioned three stages. These stages display finely tuned signaling pathways, including EGFR signaling in follicle stem cell differentiation, dorsoventral axis patterning, and the development of respiratory appendages in the stage 14 egg chamber (Castanieto, Johnston, and Nystul 2014; Van Buskirk and Schüpbach 1999; Papadia et al. 2005; Wasserman and Freeman 1998), Notch signaling in the transition from the mitotic to endocycling stage (Sun and Deng 2007), an endocycling to gene amplification stage transition involving ecdysone signaling (Sun et al. 2008). During *Drosophila* oogenesis, a number of biological events can be observed, including follicle stem cells differentiating into follicle epithelial cells, changes in the shape of follicle epithelial cells, and follicle epithelial cells undergoing apoptosis. (Margolis and Spradling 1995; Wu, Tanwar, and Raftery 2008; Castanieto, Johnston, and Nystul 2014). In addition to controlling signaling, follicle cells can take on various morphologies, including triangular follicle stem cells, cuboidal mitotic follicle cells, columnar endocycling posterior follicle cells, and squamous follicle cells in later stages of oogenesis. (Wu, Tanwar, and Raftery 2008). As a result, Drosophila oogenesis is a useful model system for investigating a variety of epithelial cell events in relation to follicle cell differentiation, polarity establishment, signaling modulation, and follicle cell shape transitions, among other things.

In the present study, we are trying to understand how mitochondrial shape regulates the follicle epithelial cell polarity during the *Drosophila* oogenesis process. Further, we have investigated how these changes in mitochondrial shape affect the mitochondrial activities and the signaling pathways in follicle epithelial cells during *Drosophila* oogenesis.

2. Results:

2.1 Mitochondrial fusion in Drp1 (*drp1*^{KG}) mutant causes the formation of multiple layers of follicle epithelial cells at the endocycling stage during *Drosophila* oogenesis

We try to analyze mitochondrial morphology in various mitochondrial morphology mutants including fission protein mutant *drp1*^{KG}, fusion protein mutant *opa1*^{i} , and a double mutant *drp1*^{KG}*;opa1*^{i} . We use the MARCM technique to specifically label the follicle cells having a homozygous mutation of fission protein Drp1 or the follicle cells having a homozygous mutation of fission protein Drp1 with UAS-driven expression of the desired gene. Streptavidin is used to designate the mitochondrial morphology for analysis because it is known to bind with the biotinylated proteins present in the mitochondrial matrix (Hollinshead, Sanderson, and Vaux 1997; Chowdhary et al. 2017; Yoon et al. 2019). The loss of Drp1 function in follicle cells shows clustered mitochondria and is also reported to have similarly clustered mitochondrial morphology in follicle cells and in neuroblasts (Mitra et al. 2012; Tomer et al. 2018; Dubal et al. 2022). Another mutant, where the fusion protein Opa1 is suppressed using *UAS-opa1*^{RNAi}, shows fragmented mitochondria. The same mutant was also used to show similarly fragmented mitochondria in the neuroblasts (Dubal et al. 2022). The combination of *drp1*^{KG}*;opa1*^{i} shows a clustering of mitochondria that can not be optically resolved. Thus, altering an equilibrium of proteins that regulate mitochondrial morphology shows altered mitochondrial shapes in follicle cells during *Drosophila* oogenesis.

These mutants are analyzed to see the shape of the follicle cells in mitotic and endocycling stages. The follicle cells of stages 1-6, known as the mitotic stage, keep on dividing until they enter the endocycling stage (Sun and Deng 2007). These cells do not show any defects in their monolayer arrangement when mitochondrial are either fused or fragmented in *drp1*^{KG} and *opa1*^{i} mutants, respectively. The double mutant *drp1*^{KG}*;opa1*^{i} follicle cells are also not altered from their monolayer arrangement. Categorization of the formation of multiple layers suggests that the fusion in *drp1*^{KG} forms 3 layers of cell in most of the egg chambers which gets reduced in a double mutant of *drp1*^{KG}*;opa1*^{i}. Thus, we show that the unopposed fusion of mitochondria in the follicle cells forms multiple layers at the endocycling stage during *Drosophila* oogenesis.

2.2 Mitochondrial fusion in Drp1 (*drp1*^{KG}) mutant causes loss of an apical polarity protein aPKC from follicle epithelial cells in both mitotic and endocycling stages

The fission mutant *drp1*^{KG} exhibits clustered mitochondria and a formation of multiple layers at the endocycling stage. Taking this into account, we further show that the formation of multiple layers in *drp1*^{KG} mutant is because of the loss of an apical protein aPKC. In addition, we also observe that the early-stage egg chambers from the mitotic stage also show the loss of aPKC. This loss of an apical polarity protein gets rescued using the double mutant of *drp1*^{KG}*;opa1*^{i} in mitotic and endocycling stage follicle cells. These observations help us to understand that the reduction of multiple layers could be because the polarity is rescued in the double mutant *drp1*^{KG}*;opa1*^{i}. Thus, an excessive clustering of the mitochondria in *drp1*^{KG} could be a reason for the loss of polarity. Hence, we have further tried to find out the mechanism of how a mitochondrial shape can regulate the polarity proteins.

2.3 Increasing ROS in Drp1 (*drp1*^{KG}) mutant causes reduction of multilayers at endocycling stages and recovery of aPKC at both mitotic and endocycling stages

Now, we have seen that by blocking a fusion protein Opa1 in *drp1*^{KG}*;opa1*^{i} recovers the aPKC of Drp1 mutant follicle cells. The impaired function of Opa1 leads to the formation of mitochondrial ROS in the cell is known (Yarosh et al. 2008; Tang et al. 2009; Jang and Javadov 2020; Quintana-Cabrera et al. 2021b). We also observe the increase in Mitochondrial ROS in *opa1*^{i} follicle cell clones. The reactive oxygen species are also known to cause the fragmentation of the mitochondria (Muliyil and Narasimha 2014). We, therefore, explore a mutant to cause a similar increase in ROS by depleting antioxidant enzymes such as mitochondrial superoxide dismutase 2 (SOD2) and catalase using *sod2*^{i} and *cat*^{i}. The fluorescence intensity of mitoSOX is found to be higher in *sod2*^{i} and *cat*^{i} follicle cell clones as compared to neighboring cells. Furthermore, these antioxidant mutants also show a fragmented mitochondrial morphology in *sod2*^{i} and *cat*^{i}. The double mutants drp1KG;sod2i, and drp1KG;catalasei also show less compactness of mitochondrial clusters. These observations allow us to further check if the multiple layers are reduced in these double mutants. We find that these mutants show a reduction in multiple layers as compared to the drp1KG mutant alone. As these double mutants show rescue of multilayering of the follicle cells similar to *drp1*^{KG}*;opa1*^{i}, the aPKC analysis is performed further. This analysis shows a recovery

of aPKC back to the membrane of these double mutants drp1KG;sod2i, and drp1KG;catalasei similar to *drp1*^{KG}*;opa1*^{i} as compared to *drp1*^{KG} in mitotic and endocycling stage.

2.4 Elevated EGFR signaling in Drp1 (*drp1*^{KG}) mutant is responsible for the formation of multiple layers and loss of aPKC

Increased dpERK, an indicator of EGFR signaling, is present in the germarium's follicle stem cells, whereas aPKC, a protein that helps cells maintain apical polarity, is absent. In ovarioles that contain follicle cell clones devoid of Drp1, it has been demonstrated that EGFR signaling is increased. (Tomer et al. 2018). In addition, we showed that during the endocycling and mitotic stages, the apical polarity protein aPKC was lost from these drp1KG mutant cells. However, this loss was partially restored in double mutants of drp1KG;opa1i, drp1KG;sod2i, and drp1KG;cati. We wished to determine from these findings whether the enhanced dpERK level (EGFR signaling) of the drp1KG follicle cells was diminished in these double mutants. We observed that in the follicle cells of these double mutants, the amount of dpERK was decreased during the endocycling and mitotic stages. Therefore, the loss of aPKC from the multilayered epithelium of drp1KG follicle cells could be caused by the enhanced EGFR signaling in endocycling follicle cells.

To prove that the loss of aPKC is because of the enhanced EGFR signaling, we suppressed the ERK, a downstream component of the EGFR signaling pathway, using erk1i in the background of Drp1 mutant follicle cells. Upon suppression of the EGFR signaling pathway, the formation of a multilayer was reduced in a double mutant of *drp1*^{KG}*;erk*^{i} as compared to *drp1*^{KG}. Furthermore, the same double mutant showed a recovery of an apical polarity protein aPKC. The observations of the reduction of multilayers and recovery of aPKC helped us to understand that the EGFR is downstream of the formation of multilayers and loss of aPKC in Drp1 mutant cells.

2.5 Abberant EGFR signaling in Drp1 (*drp1*^{KG}) mutant is responsible for the oocyte migration defects that do not get rescued in double mutants

The migration of the oocyte into the dorso-anterior location as well as the patterning of the posterior follicle cells are caused by the activation of EGFR signaling in follicle cells. Ovarioles containing posterior follicle cells deficient of Drp1 show a defective oocyte migration to the dorso-anterior location and the elevated level of the downstream component of EGFR signaling, doubly phosphorylated ERK (dpERK), in the cytoplasm (González-Reyes and St Johnston 1998; Mitra et

al. 2012; Tomer et al. 2018). Reduced EGFR signaling causes mitochondrial fragmentation and mitochondrial activity to decline (Tomer et al. 2018; Mitra et al. 2012). We measure the frequency of defective oocyte migration further in double mutants where we see a rescue in multilayering and loss of aPKC phenotype of $drp1^{KG}$. Surprisingly, migration of oocyte to the dorso-anterior position remains similarly defective in $drp1^{KG}$ and the combinations of $drp1^{KG};opa1^{i}$, $drp1^{KG};sod2^{i,}$ and $drp1^{KG};cat^{i}$. These results demonstrate that although dpERK levels in the cytoplasm of drp1KG;opa1i, drp1KG;sod2i, and drp1KG;cati are lowered, this is not enough to drive the oocyte to migrate to the dorso-anterior position.

2.6 Defective Notch signaling in Drp1 ($drp1^{KG}$) mutant is rescued in double mutants of $drp1^{KG};opa1^{i}$, $drp1^{KG};sod2^{i}$, $drp1^{KG};cat^{i}$, and $drp1^{KG};erk1^{i}$

We see multiple layers of the follicle cells in the Drp1 mutant at endocycling stage. This stage is marked by the active Notch signaling during oogenesis. The interaction of Delta ligand from germ cells with the Notch receptor of follicle cells is required to initiate the signaling (López-Schier and St Johnston 2001). Hindsight (Hnt) is expressed in response to active Notch signaling in posterior follicle cells surrounding the egg (Ruohola et al. 1991; Kim-Yip and Nystul 2018). The posterior follicle cells of $drp1^{KG}$ with defective mitotic to endocycling transition show a loss of Hnt, whereas this defect is partially reversed by a decreasing mitochondrial activity, blocking fusion, and depleting ERK level (Mitra et al. 2012; Tomer et al. 2018). We see various defects of Drp1 such as the formation of multilayers, loss of aPKC, and increased dpERK level that goes away partially with the double mutants $drp1^{KG};opa1^{i}$, $drp1^{KG};sod2^{i}$, $drp1^{KG};cat^{i}$, and $drp1^{KG};erk1^{i}$. We hypothesize further to check if these double mutants rescue the defective Notch activation of $drp1^{KG}$ mutant follicle cells. As per the expectation, these double mutants exhibit a partial rescue in Hnt expression as compared to fission mutant $drp1^{KG}$. These data indicate that the defective Notch signaling is partially rescued by blocking additional mitochondrial fusion, elevation in ROS, or decreasing excessive dpERK in Drp1 mutant follicle cells.

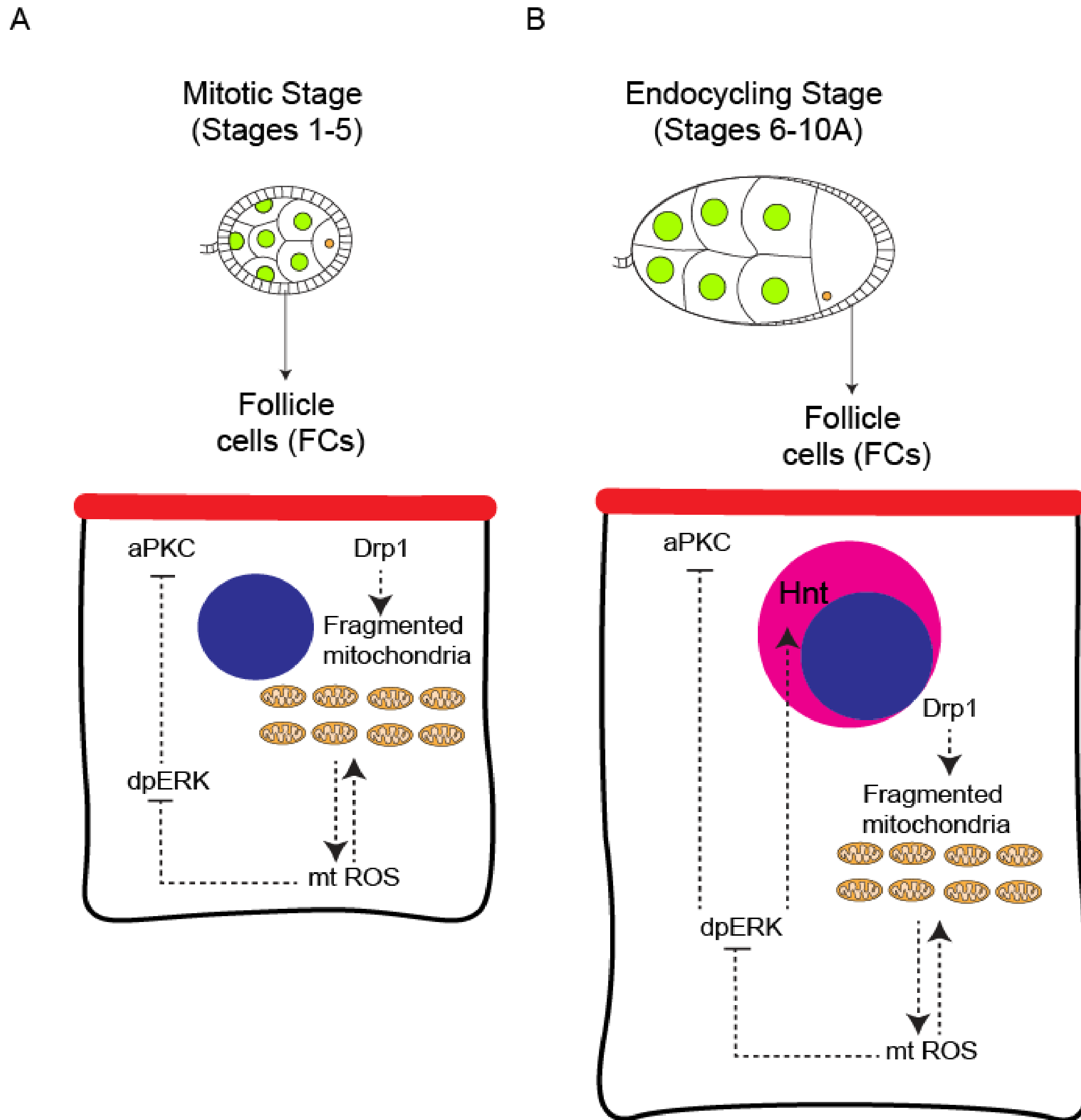

Figure 1: Interaction between mitochondrial morphology, ROS and EGFR and Notch signaling in *Drosophila* FC differentiation

(A-B) The schematic shows the interaction between mitochondrial fission regulated by Drp1, ROS, and polarity protein aPKC (Red) in mitotic and endocycling FCs of *Drosophila* oogenesis. Drp1 is required for the mitochondrial fission thereby controlling the levels of ROS and appropriate activation of dpERK in turn affecting the distribution of aPKC on the apical membrane in FCs in mitotic stages **(A)**. In PFCs in endocycling stages, Drp1 is required for the mitochondrial fission thereby controlling the levels of ROS for appropriate activation of dpERK thereby regulating the accumulation of aPKC at the apical membrane and the activation of Notch signaling **(B)**.

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CHAPTER 1: Introduction

1.1 Mitochondria dynamics and function

Mitochondria, famously known for ATP production, are double membrane organelle of the cell (Figure 1.1) (Jouaville et al. 1999; Osellame, Blacker, and Duchen 2012; McQuibban et al. 2006; Giacomello et al. 2020). Mitochondria was incorporated as an endosymbiont in the eukaryotic cells from α-proteobacteria ancestors (Urbauer, Rath, and Haller 2020; Martin, Garg, and Zimorski 2015; Zachar and Boza 2020; T. Ishihara, Kohno, and Ishihara 2015; Jodi Nunnari and Suomalainen 2012; Gray, Burger, and Lang 1999; Frohman 2010). The observation of mitochondria as a site for oxidative phosphorylation serves the purpose of providing energy to the cell for different cellular processes (Chaban, Boekema, and Dudkina 2014). Other than energy production, mitochondria possess several functions such as reactive oxygen species (ROS) production, calcium buffering, metabolic regulation and synthesis of crucial components of the cells such as phospholipids and heme, cell cycle regulation, apoptosis, and various signaling, etc. (Cipolat et al. 2004; N. Ishihara et al. 2009; Benard et al. 2010; Romanello et al. 2010; Mayr et al. 2011; Brand et al. 2013; Seo, Yoon, and Do 2018; Urbauer, Rath, and Haller 2020). The role of mitochondria in cell stress is also one of the important functions observed (Kasahara and Scorrano 2014). Unfolded protein response (UPR) is a mechanism where the cell responds to the accumulation of unfolded proteins in the cell. Mitochondria, being present in the cell, communicates to the nucleus and triggers UPR in the cell (Favaro et al. 2019). In addition to nuclear communication, the mitochondrial quality control protein Hsp60, also known as a mitochondrial molecular chaperone, is involved in the unfolded protein response and maintenance of the stemness of an intestinal stem cells in the mouse that indirectly states the function of mitochondrial chaperones in cell fate specification (Berger et al. 2016). Thus, mitochondria perform diverse functions in the cell and many of them are yet to be understood in detail.

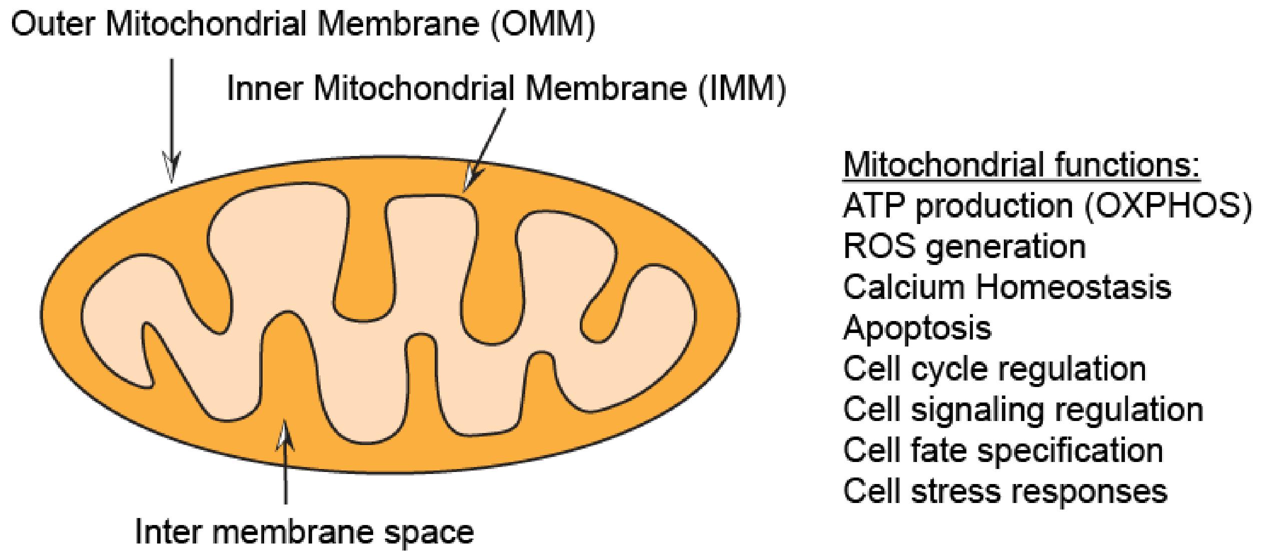

Figure 1.1: Structure and functions of the mitochondria

Mitochondria are double membrane organelles with intermembrane space present between the outer membrane and the inner membrane. The inner membrane is folded to form cristae. Mitochondria perform various functions as listed in the diagram.

1.1.1 Mitochondrial morphology and dynamics

The functions and distribution of the mitochondria vary depending on the shape, ranging from fused to fragmented forms, of the mitochondria (H. Chen et al. 2003; J. Nunnari et al. 1997). The cell maintains an equilibrium between a pool of fused and fragmented mitochondria as the mitochondrial shape is known to regulate the metabolism of the cell and thereby regulate the fate of the cells. The reports on mitochondrial shape suggest that the stem cell mitochondria remain fragmented and depend on glycolytic metabolism, whereas the differentiated cells show fused mitochondria with elevated oxidative phosphorylation (OXPHOS). Many examples of differentiated cells also exhibit different proportions of fused and fragmented mitochondria. The *Drosophila* embryo has fragmented mitochondria, whereas *Drosophila* muscle cells exhibit fused mitochondria to meet the energy need of the muscles. The changes or shifts in the equilibrium of the mitochondrial morphology perturb several cellular functions, signaling, and cell fate specification. Mitochondrial functions are regulated by the dynamic transitions of their shapes from fused to fragmented and fragmented to fused forms. To regulate the output of the mitochondrial energy during several cellular events, mitochondria undergo a cycle of fusion and

fission (Varuzhanyan et al. 2019; H. Chen and Chan 2017). The role of fusion and fission is not only to change the mitochondrial shape but it helps post-mitotic cells to keep a quality check for the damaged mitochondria, which are then removed by autophagy (Favaro et al. 2019). The quality of mitochondria and their matrix is also maintained by mitophagy, a process that removes damaged or dysfunctional mitochondria in a fission-dependent manner (Abeliovich et al. 2013). Thus, the changes in mitochondrial morphology provide assistance in the quantity and quality determination of mitochondria in the cell. Additionally, the change in mitochondrial morphology help in conferring the cell its metabolic status.

1.1.2 Regulation of mitochondrial morphology

As discussed in the previous section, mitochondria undergo dynamic changes in their shapes, and these changes are helpful for the cells. Transforming mitochondria from one type of shape to another type of shape requires help from specific proteins. Several such proteins are functioning to derive either fused or fragmented mitochondria as per the requirement of the cell (Scott and Youle 2010; Y. J. Liu et al. 2020).

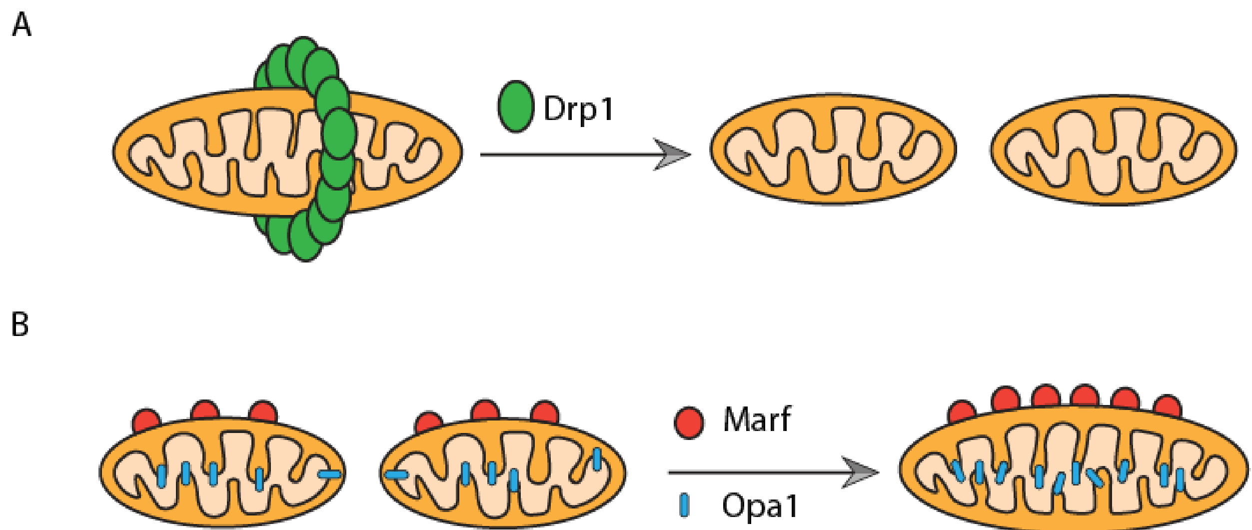

Figure 1.2: Proteins regulating the mitochondrial morphology

Drp1 wraps around the large mitochondrion (Singular) in oligomeric association and breaks it into smaller and fragmented mitochondria **(A)**. Marf assists fusion of the outer mitochondrial membrane (OMM) and Opa1 assists in the fusion of the inner mitochondrial membrane (IMM) **(B)**.

The fission, leading to the fragmentation of mitochondria, is caused by a Dynamin related protein-1(Drp1) protein in a very organized manner. The Drp1 protein, across all the metazoans, possesses GTP binding and GTPase domain (Zhu et al. 2004). The Drp1 remains cytoplasmic and is placed onto the mitochondria in an oligomeric fashion and pinches the longer mitochondria into the shorter form (N. Ishihara et al. 2009; Basu et al. 2017; S. Frank et al. 2001; Zhu et al. 2004) (Figure 1.2). The recruitment of Drp1 from the cytosol to mitochondria requires an adaptor protein Fis1, Mitochondrial dynamics protein of 49 kDa (Mid49), mitochondrial fusion factor (Mff), and Mitochondrial dynamics protein of 51 kDa (Mid51) to potentiate a process of fragmentation (James et al. 2003; Y. Yoon et al. 2003; Losón et al. 2013; Palmer et al. 2011). To recruit the Drp1 protein in mitochondria, the phosphorylation process at a particular serine (ser) residue is more important (Z. Zhang et al. 2016). Based on the position of serine phosphorylation, Drp1 exhibits active or inactive forms. Phosphorylation on Ser-616 of Drp1 is needed to translocate the Drp1 from the cytosol to the mitochondrial membrane (L. Xie et al. 2020). Ser-656 and Ser-637 phosphorylation of Drp1 by cAMP-dependent protein kinase A (PKA) inhibit fragmentation by reducing the GTPase activity of the Drp1 (Jahani-Asl and Slack 2007; L. Xie et al. 2020). The phosphorylation of Drp1 is also affected by kinases such as phosphoglycerate mutase family member 5 long (PGAM5L) form (Xu et al. 2015), GSK3-β (Loh et al. 2015), AMPK, Cyclin-dependent kinase (Q. Xie et al. 2015), Calcium-Calmodulin dependent kinase (Q. Xie et al. 2015). Fusion and fission by Drp1 are reported to have a role in the survival of *C.elegans* and *D.melanogaster,* respectively (Chaudhari and Kipreos 2017; Rana et al. 2017). A function of Drp1, to get fragmented mitochondria, is required in all phases of the cell division process (Horbay and Bilyy 2016). As discussed earlier in this section, Drp1 in mammalian cells, Drp1 homolog Dnm1 in yeast, plays a critical role in mitophagy to remove dysfunctional mitochondria (M. Frank et al. 2012; Mao and Klionsky 2013). Drp1-mediated fission helps in the formation of smaller mitochondria so that they can be transported easily as compared to larger mitochondria (Campello et al. 2006; Amiri and Hollenbeck 2008). Drp1, being a pro-fission protein, is associated with the downstream activation of the Caspases during the developmental cell death in *Drosophila* (Goyal et al. 2007). Drp1 suppression blocks the apoptosis in the *Arabidopsis thaliana*, proving that Drp1 mediated fission is required to initiate the apoptosis (Abdelwahid et al. 2007). Drp1-mediated fission causes loss of mitochondrial membrane potential, cytochrome c release, and subsequent apoptosis in COS7 cells (S. Frank et al. 2001). Thus, mitochondrial fragmentation by Drp1

precedes and regulates the process of apoptosis. This literature tells us why cells require a function of Drp1 to produce fragmented mitochondria.

The fusion of the mitochondria requires the assistance of Mfn1 and Mfn2 in mammalian cells to fuse the outer mitochondrial membrane and Opa1 (also denoted as OPA1) to fuse the inner mitochondrial membrane (Figure 1.2). An OPA1, a dynamin-related GTPase, maintains the mitochondrial network in the cell (van der Bliek 1999; N. Ishihara et al. 2006; Kowaltowski et al. 2019). A deficiency of Opa1 causes Optic Atrophy in humans (Katti et al. 2021; H. Chen et al. 2010; Agier et al. 2012). The proteolytic cleavage of OPA1 is helpful in regulating the function of Opa1. The yeast Mgm1, a homolog of human OPA1, is processed by a rhomboid protease to regulate the mitochondrial morphology (N. Ishihara et al. 2006). An OPA1 exists in long (L-OPA1) that remains bound to the inner mitochondrial membrane and the soluble short (S-OPA1) isoforms depending on the mitochondrial potential dependent cleavage by a metalloprotease (overlapping with m-AAA protease) OMA (Gilkerson, De La Torre, and St Vallier 2021). Low mitochondrial potential and permeability transition pores (PTP) induced mitochondrial swelling triggers OMA1 to cleave L-OPA1 to S-OPA1 and that hampers the fusion-promoting event mediated by L-OPA1 (Gilkerson, De La Torre, and St Vallier 2021; Jang and Javadov 2020; Griparic, Kanazawa, and van der Bliek 2007). Although the presence of any isoform of OPA1 can help maintain the cristae structure and mitochondrial energy output (Del Dotto et al. 2017). S-OPA1 does not help in fusion activity like that of L-OPA1 but it protects mouse embryonic fibroblast (MEF) cells against necrosis (H. Lee et al. 2020). Loss of OPA1 from the HeLa, NIH-OVCAR-3, and RGC-5 cells causes a lowering of the mitochondrial potential and subsequent fragmentation leading to apoptosis (Olichon et al. 2003; Ju et al. 2009). Mitofusin is required to fuse the outer mitochondrial membrane (OMM) (Rana et al. 2017). The MFN1 and MFN2 are reported to form dimers in homomeric and heteromeric patterns to carry out the fusion of the IMM (Kasahara and Scorrano 2014). Mutation in humans causes Charcot-Marie-Tooth type 2 A disease (Katti et al. 2021; H. Chen et al. 2010). The ubiquitination of Mfn by Parkin, an E3 ubiquitin ligase, helps the release of the Mfn from the outer mitochondrial membrane and helps in mitophagy in mouse and S2R+ cells (Ziviani, Tao, and Whitworth 2010; McLelland et al. 2018). As opposed to Drp1, the MFN2- a Marf homolog protein is known to positively regulate calcium uptake in the mitochondria (Kowaltowski et al. 2019). Mfn is essential for the interaction between the ER and mitochondria in addition to taking part in the fusion of the outer membrane (de Brito and Scorrano

2008). Thus, the regulation of mitochondrial morphology, assisted by a set of fusion and fission proteins, helps in quality and quality assurance inside the cell.

1.1.3 Mitochondrial morphology and associated changes in mitochondrial activities

Mitochondrial ETC complexes help in extracting energy from the respective substrates, and as a result, they generate a mitochondrial potential which is shown in (Figure 1.3). Complex I and II donate their electrons to ubiquinone (Q) and are reduced to ubiquinol (QH2). The reduced ubiquinol transfers electrons to complex III which circulates electrons to cytochrome C further. The reduced cytochrome then donates electrons to complex IV carries out the reduction of molecular oxygen in the mitochondrial matrix and forms metabolic water (Schon and Przedborski 2011; Nolfi-Donegan, Braganza, and Shiva 2020; Vercellino and Sazanov 2022). The passing of electrons from complex II does not transfer protons in the inter-membrane space of the mitochondria, whereas other complexes involve themselves in proton movement across the membrane, which leads to the formation of mitochondrial membrane potential. Mitochondrial architecture is more important for ATP production in the cell as it regulates the assembly and localization of these electron transport chain proteins. An electron transport chain is comprised of five complexes, complex I, II, III, IV, and V, and they all are placed appropriately on the inner membrane of mitochondria to extract energy from the respective substrates (Figure 1.3) (Nolfi-Donegan, Braganza, and Shiva 2020; Vercellino and Sazanov 2022). And hence the structure of the inner mitochondrial membrane (IMM) is crucial for mitochondrial metabolism. The state of mitochondrial morphology is helpful in maintaining the efficiency of mitochondrial activity such as mitochondrial membrane potential, ATP formation, ROS generation, etc. As we know that the mitochondrial morphology is mainly regulated by Drp1, Opa1, and Marf, we need to understand how these proteins can affect mitochondrial activities.

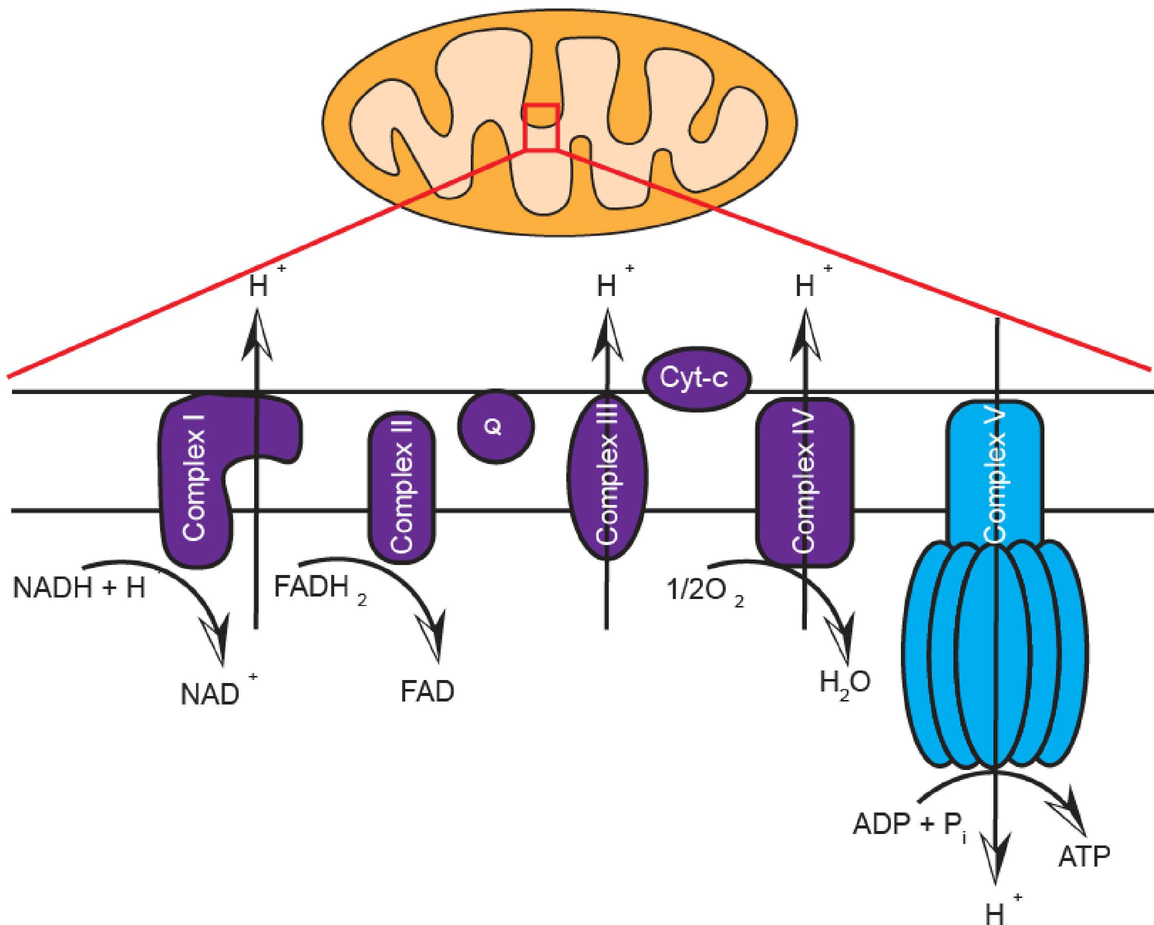

Figure 1.3: Organization of electron transport chain complexes in cristae and ATP formation
The folded structure of the inner mitochondrial membrane, known as the cristae, contains protein complexes I to V for the electron transport chain. The diagram shows a path of electron transport from various complexes to generate ATP through oxidative phosphorylation (OXPHOS). The reducing equivalents from NADH and FADH2 are extracted and passed on to complex I and complex II, respectively. The rest of the path remains the same for electron transport. Complex IV does not transport electrons but transports protons back inside the matrix for the synthesis of ATP.

The study in *Drosophila* follicle cells mutant for Drp1 protein shows increased mitochondrial membrane potential (Tomer et al. 2018). The fusion of mitochondria in Drp1 mutant *Drosophila* embryos reduces ROS (Chowdhary et al. 2020). Contrary to that, blocking the function of Drp1 is also reported to decrease ATP output and elevate mitochondrial ROS (L. Xie et al. 2020). The fusion protein Opa1 maintains a cristae structure of the IMM and also stabilizes the ATP synthase complex to reduce ROS accumulation (Quintana-Cabrera et al. 2018, 2021). The

overexpression of Opa1 also promotes increased mitochondrial ATP synthesis in cells with galactose supplemented (Quintana-Cabrera et al. 2021). The haploinsufficiency in optic atrophy patients is reported to have increased ROS due to reduced mitochondrial respiration (Millet et al. 2016). Blocking the function of Mfn1/2 causes a reduction in OXPHOS during spermatogenesis in mice (Varuzhanyan et al. 2019). Recently, it has been shown that the cristae morphology and the ATP content in the cell also regulate the proteins, such as YME1L and OMA1, necessary to regulate the proteolytic processing of the mitochondrial morphology proteins (MacVicar and Langer 2016). Thus, mitochondria morphology proteins are the key elements in deciding the shape of mitochondria and thereby the activities associated with the shape.

As discussed earlier, calcium entry into the mitochondrial matrix is required by the dehydrogenases of the Krebs (TCA) cycle, which further increases mitochondrial metabolism (Szabadkai et al. 2006). The fusion proteins Opa1 and Marf1 also induce more mitochondrial calcium entry and could promote mitochondrial metabolism is a question to be studied. Not only that, but the availability of metabolic substrates also determines mitochondrial morphology and their activities. For example, the hyperglycemic condition increases mitochondrial fragmentation and subsequent production of mitochondrial ROS (Yu, Robotham, and Yoon 2006). Tumor cells rely on Glycolytic metabolism but Glutamine metabolism also helps tumor cells grow (Yoo et al. 2020). Glutamate intake also promotes the TCA cycle by forming TCA cycle intermediates (Yoo et al. 2020; Yang, Venneti, and Nagrath 2017). Studies have shown that glutamate deprivation increases ROS, dissipates mitochondrial potential, and reduces ATP production in breast cancer cell lines (Gwangwa, Joubert, and Visagie 2019). Inhibition of Complex II exerts more invasive properties on the cells due to succinate accumulation and associated changes (Vercellino and Sazanov 2022). Such metabolic adaptations and their effect on mitochondrial shape and function affect cellular events and make them more interesting to explore further.

1.2 Mitochondria and cell signaling pathways

The recent role of mitochondria in the regulation of signaling pathways has attracted more interest in mitochondrial biology. Many such signaling pathways associated with development are regulated by mitochondrial shape and related changes in mitochondrial activities. In addition to the mitochondrial regulation of cell signaling pathways, there are several reports telling regulation of mitochondrial shape via cell signaling pathways.

Mitochondria play an important role in viral infection as mitochondrial antiviral signaling protein (MAVS) tagged on the outer mitochondrial membrane help in the binding of pattern recognition receptor proteins (Kawai et al. 2005; R. B. Seth et al. 2005). Mitochondrial membrane potential, affected by prostaglandin (PGE2), signals to regulate nuclear gene activation in macrophages (Sanin et al. 2018). Mitochondria oppose or participate in cell apoptosis with the help of antiapoptotic or proapoptotic proteins (C. Wang and Youle 2009). Bcl2 (B-cell lymphoma 2) protein is one of the antiapoptotic proteins that reside in an inner mitochondrial membrane of the mitochondria (Hockenbery et al. 1990). The release of mitochondrial residential protein cytochrome c helps in activating caspases and the formation of apoptosome and promote the apoptotic signaling cascade (X. Liu et al. 1996; Eleftheriadis et al. 2016). In addition to its role in apoptosis, cytochrome c level acts as an important marker of hepatic injuries and kidney injuries (Miller et al. 2008; Zager, Johnson, and Hanson 2004; Oberst, Bender, and Green 2008). Mitochondrial ROS can activate the RIPK3 and can initiate necrosis of the cell (Tait and Green 2012). Mitochondrial ROS also stabilizes hypoxia-inducing factor (HIF), which further regulates gene expression required for metabolism under the low amount of oxygen (N. S. Chandel et al. 1998; Navdeep S. Chandel et al. 2000). The metabolic products from the TCA cycle can influence the gene expression profile through epigenetic modifications (Lisowski et al. 2018). During crypt-based columnar cells (CBCs) differentiation into Paneth cells, Drp1-dependent fission of the mitochondria is promoted by suppression of FOXO/Notch signaling (Ludikhuize et al. 2020). The CBCs show more ROS and subsequent activation of MAPK, whereas panteth cell differentiation shows less mitochondrial ROS with lowered mitochondrial activity (Rodríguez-Colman et al. 2017). In the *Drosophila* follicle cells, the mutation in mitochondrial ribosomal protein 4 (*mRPL4*) and a complex I subunit (*pdsw)* gene increases ROS production and activates JNK signaling (Z. A. Wang, Huang, and Kalderon 2012). Elevated mitochondrial membrane potential in fused mitochondria of Drp1 mutant follicle cells (FCs) increases the EGFR signaling pathway and suppresses the Notch signaling in endocycling FCs (Mitra et al. 2012; Tomer et al. 2018). Apart from that, EGFR signaling increases the β-oxidation of fatty acids and induces mitochondrial biogenesis and mitochondrial potential (C. Zhang et al. 2022). The above observations from different studies indicate that the mitochondria remain involved in the regulation of various signaling pathways through their metabolic activities and morphology.

1.3 Mitochondria and epithelial cell differentiation

1.3.1 Differentiatial distribution and metabolism of mitochondria in stem cells and differentiated cells

The diagram (Figure 1.3) shows a path of electron transport from various complexes to generate ATP through oxidative phosphorylation (OXPHOS). The reducing equivalents from NADH and FADH2 are extracted and passed on to complex I and complex II, respectively. The rest of the path remains the same for electron transport. Complex IV does not transport electrons but transports protons back inside the matrix for the synthesis of ATP.

Mitochondrial quantity and morphology play an essential function in the regulation of cell differentiation, cell fate specification, and further development of an organism (Madan et al. 2021). Stem cell differentiation is a very early process in the development of an organism. In this section, we have compiled literature that suggests the role of mitochondria during stem cell differentiation (Figure 1.4). The proliferative stem cells require fragmented and smaller mitochondria to distribute them into newly formed or differentiated daughter cells (H. Chen and Chan 2017). One such differentiation was observed in the lactogenic differentiation of the HC11 (mouse mammary epithelial cell line), where higher OXPHOS and glycolysis are observed (Elswood et al. 2021). It is evidenced that the C2C12 cells while undergoing myogenic differentiation, show increased gene expression for mitochondrial biogenesis and OXPHOS, too (Remels et al. 2010). The proliferative stem cells require fragmented and smaller mitochondria to distribute them into newly formed daughter cells (H. Chen and Chan 2017). Cardiomyocyte differentiation from embryonic stem cells (ESCs) in mice requires a function of Mfn1/2 and Opa1 fusion proteins to fuse the mitochondria (Kasahara et al. 2013). The human embryonic stem cell (hESC) differentiation is also accompanied by an increase in mitochondrial biogenesis, mitochondrial potential, ATP, and mitochondrial ROS (Cho et al. 2006). Even cardiomyocyte differentiation was impaired upon disrupting respiratory chain complexes and thus oxidative phosphorylation is also a necessary event for stem cell differentiation (Chung et al. 2007). Intestinal stem cell differentiation to enterocytes requires fusion of the mitochondria and an increase in the mitochondrial pyruvate metabolism in *Drosophila* (Schell et al. 2017; Deng et al. 2018). Paneth cell differentiation from the crypt-based columnar cells, as discussed earlier, requires low mitochondrial activity and is dependent on glycolysis (Rodríguez-Colman et al. 2017; Ludikhuize et al. 2020). There is one more phenomenon where the cell first differentiates and then again dedifferentiates. During the dedifferentiation of

the cell, the cell attains fragmented mitochondrial morphology (Noguchi and Kasahara 2018). There are arguments over why stem cells keep lowering OXPHOS to reduce ROS-mediated damage (Lisowski et al. 2018). Thus, mitochondrial morphology is a very important aspect to be studied in the field of stem cell differentiation.

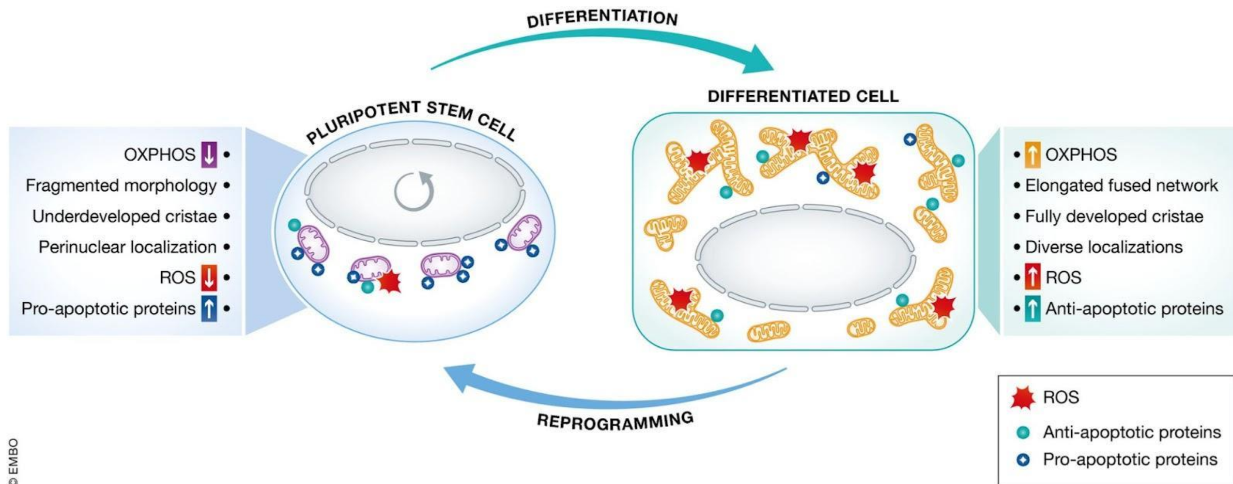

Figure 1.4: Mitochondrial morphology, distribution, and activities in stem cells and in differentiated cells

The stem cell and differentiated cells show variation in mitochondrial distribution, mitochondrial morphology, mitochondrial OXPHOS capacity, and ROS content. Image adapted from (Lisowski et al. 2018)

1.3.2 Epithelial cell differentiation and polarity program

The literature discussed in 1.3.1 states the significance of mitochondrial morphology and its importance in cell differentiation. One such differentiation process happens when an epithelial cell forms from its precursor cell. An epithelial cell, that first comes into existence during embryogenesis, possesses well-structured cell-cell junctions and polarity complexes (Thowfeequ, Stower, and Srinivas 2022; Louvet-Vallée et al. 2001). The formation of the epithelial cell starts from embryogenesis and passes through different stages of development and is important in building a highly organized multicellular organism (Hebbar and Knust 2021; Montell 2008; J. Chen et al. 2018). In multicellular organisms such as *Drosophila*, the epithelial cells polarize from their precursor epithelial stem cells or from previously existing epithelial cells (Castanieto, Johnston, and Nystul 2014; J. Chen and St Johnston 2022). Each polarized cell contains three

domains a) apical, b) lateral, and c) basal (J. Chen et al. 2018). An apical domain possesses a complex containing atypical protein kinase C(aPKC)- Bazooka(PAR3)- PAR6 proteins and a subapical complex containing Crumbs(Crb)-PatJ-Stardust(Sdt) proteins, a lateral domain complex containing Scribble(Scrb)-Disclarge (Dlg)- Lethal giant larvae (Lgl) proteins, and a basal domain possess integrin proteins and these proteins are conserved across species (Figure 1.5) (Tepass et al. 2001; Assémat et al. 2008; Royer and Lu 2011). The apical and basal domains of epithelial cells are separated by the presence of Adherens junctions (AJs) composed of DE-Cadherin which is encoded by a *shotgun* gene in *Drosophila* (H. A. Müller 2000; Royer and Lu 2011). The order of the polarity establishment is unique during the polarization of the epithelial cell.

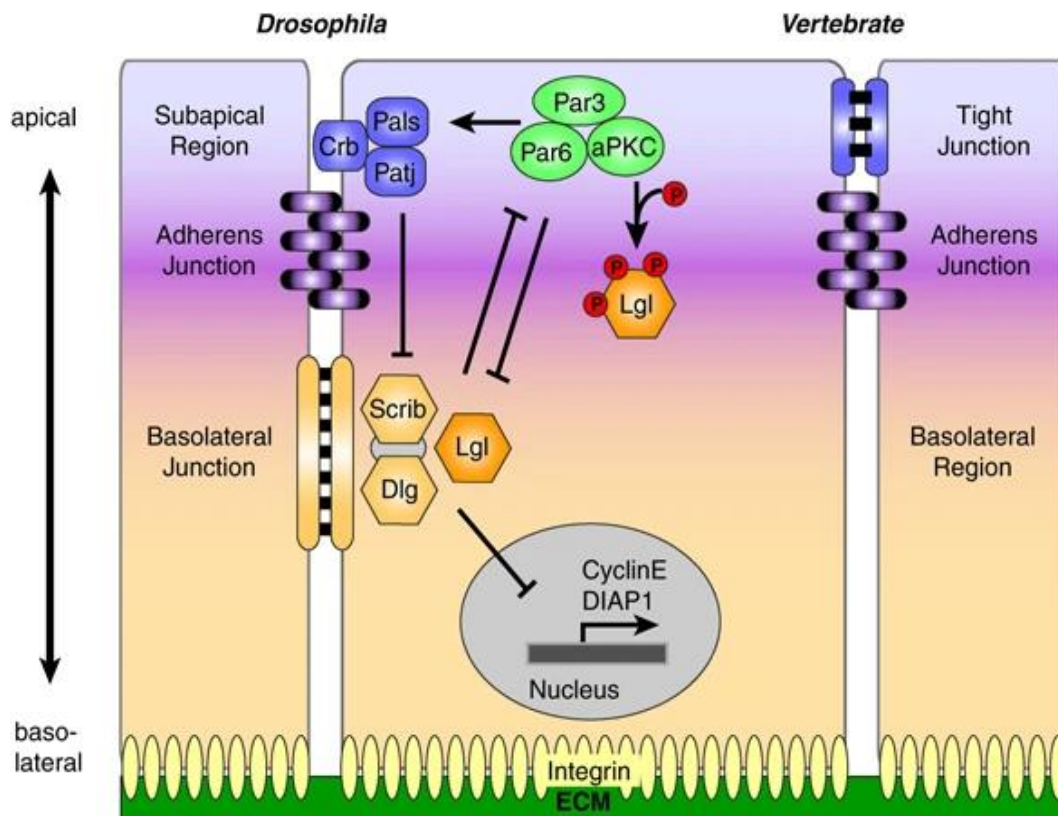

Figure 1.5: Epithelial cell and polarity protein complexes

The differentiated epithelial cell with the apical, lateral, and basal domains. The domains show a presence of apical protein complexes, lateral protein complexes, and basal protein complexes. Image adapted from (Humbert et al. 2008)

The establishment of the polarity renders the cell in a specific shape to perform cellular functions. One of the major functions of the epithelial cell polarity is to align and place the various signaling receptors in the correct place of the stimulus (Li et al. 2017). The association of the cytoskeleton with the polarity of the cell is required to generate tensions and help maintain the cells in their shape and spatial arrangement (Heisenberg and Bellaïche 2013). The interconnections developed between cells also help in collective cell migration and movements such as *Drosophila* gastrulation (Montell 2008; Leptin 2005). Thus, a formation of an appropriate polarity in the epithelial cells makes them capable of functioning and migrating together which is necessary for tissue formation. From the previous discussion, the polarity of the cell help in maintaining the shape, structure, functions, and motility of the cell during different stages of development (Montell 2008). There are several instances where polarity is seen to be lost during development or during disease progression. Many reports have noticed that the loss of polarity complexes leads to impaired tissue organization with the development of tumor-like growth (Li et al. 2017; H.-A. J. Müller 2018).

1.3.3 Mitochondrial morphology and distribution in epithelial cell differentiation

As the work in the thesis is focused on epithelial cells, we have tried to understand the role of mitochondrial morphology and activity taking various examples of epithelial cell differentiation into consideration. Hepatocyte differentiation where polarity is established in a gradual manner during the differentiation process requires elongated mitochondria and oxidative phosphorylation (Fu et al. 2013). The enamel formation requires the differentiation of epithelial cells and a change in metabolism during tooth development in mice (Imhof et al. 2020). *Drosophila* intestinal epithelial differentiation from the intestinal stem cells (ISCs) is supported by a fused mitochondrial morphology (Deng et al. 2018). In addition to that, the differences in mitochondrial activity are necessary to differentiate crypt-based columnar epithelial cells (CBCs) into paneth cells (PCs) with low mitochondrial activity (Ludikhuize et al. 2020). In this part, we have tried to understand how specifically epithelial cell differentiation is regulated by mitochondrial morphology directly or indirectly. We are motivated to enhance our understanding of this subject by the paucity of literature on the examination of mitochondrial shape in controlling epithelial cell differentiation.

1.4 Mitochondrial morphology in normal development and in diseased condition

1.4.1 Mitochondrial morphology during the development of an organism

Mitochondrial quantity and morphology play an essential function in the regulation of cell differentiation, cell fate specification, and further development of an organism (Madan et al. 2021). The fission of the mitochondria is involved in the differentiation of the intestinal stem cells into paneth and goblet cells in organoid and mouse models (Ludikhuize et al. 2020). Mitochondrial fission protein drp1 deficient mice are embryonic lethal and a specific known down of Drp1 in neurons causes early death of mice (N. Ishihara et al. 2009). The mice lacking in fission protein, Drp1, show compromised oocyte health and organellar defects (Udagawa et al. 2014). Drp1 deficient follicle cells in *Drosophila* exhibit differentiation defects and do not proceed to endocycling stages during oogenesis (Mitra et al. 2012). The fusion defects due to Mfn2 knockout in mouse causes impaired spermatogenesis (T. Wang et al. 2022). The fuzzy onion (Fzo), the first protein discovered in *Drosophila* for mitochondrial fusion and a homolog to Mfn, is required for the development of spermatids in *Drosophila* (Hales and Fuller 1997; Deng et al. 2008). Blocking fusion of mitochondria by Mfn1 and Mfn2 is associated with spermatogenesis defects due to failure in metabolic regulation (Varuzhanyan et al. 2019). In the testes of male *Drosophila*, mitochondrial fusion, and lipid metabolism helps maintain the germ stem cells (GSCs) in the testes (Sênos Demarco et al. 2019). Similarly, the female GSCs are also regulated by the OXPHOS metabolism dependent on the fusion of the mitochondria (Garcez et al. 2020). And thus mitochondrial morphology and associated metabolism play a critical role in the reproductive health of an animal.

Early muscle development in *Drosophila* also requires Mfn-mediated fusion of mitochondria (Katti et al. 2021). Mouse also showed muscle defects, reduced respiratory capacity, and increased mitochondrial mutation upon blocking a fusion of mitochondria (H. Chen et al. 2010). Overexpression of Drp1 in mouse skeletal muscle causes similar defects as Mfn2 knock-out mice showed for mitochondria including (Touvier et al. 2015). The fragmentation of mitochondria leads to insulin resistance in mouse skeletal muscles (Jheng et al. 2012; R. Liu et al. 2014). Thus, a fusion of mitochondria is very essential for muscle development in mice. The fusion of mitochondria helps in synapse formation during larval development in *Drosophila* (Sandoval et al. 2014). The midgut development in *Drosophila* shows a requirement for the fusion of the mitochondria during intestinal stem cell differentiation (Deng et al. 2018). Resultantly,

mitochondrial morphology probably plays a significant role in the development of tissues and organs.

1.4.2 Mitochondrial morphology in diseases

Mitochondrial quality and quantity are two important factors observed to be altered in several human disorders. In many pathological situations, the equilibrium of fusion and fission proteins gets perturbed and affects mitochondrial distribution and morphology in cells. Defective fission of the mitochondria leads to the early, after 37 days, death of a girl with several organ levels and physiological abnormalities (Waterham et al. 2007). Impaired mitochondrial respiratory functions are associated with neurodegenerative diseases directly or indirectly (Schon and Przedborski 2011). Many of the neurons related disorders have shown mitochondrial OXPHOS dysfunctions (DiMauro and Schon 2003). Leigh syndrome (affecting neurons), and Kearns-Sayre syndrome (affecting extraocular muscles and retina) where mitochondria show impaired Oxphos and cause serious consequences leading to fatality (Schon and Przedborski 2011; Schubert Baldo and Vilarinho 2020). A disease that causes blindness in patients, Leber's Hereditary Optic Neuropathy (LHON), has mitochondrial complex I mutation involved which further leads to increased ROS and apoptosis of retinal ganglionic cells (Sadun, La Morgia, and Carelli 2011; Finsterer and Zarrouk-Mahjoub 2016; Fiedorczuk and Sazanov 2018). A mutation in cytochrome b is also involved in causing akinetic rigid syndrome and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) (De Coo et al. 1999; El-Hattab et al. 2015; Vercellino and Sazanov 2022). Reduction of the cytochrome c oxidase (COX) in a patient causes sporadic encephalomyopathies causing spinocerebellar ataxia (Silvestri et al. 2000). Mitochondrial enzymes DNA polymerase and helicase Twinkle are important for mitochondrial DNA replication. Mutation of these enzymes causes infantile-onset spinocerebellar ataxia with mitochondrial DNA level reduction in patients (Hakonen et al. 2008). Friedreich's ataxia (FRDA) is also reported to have a mutation in proteins required for ETC component ubiquinone biosynthesis (Gerards et al. 2010). There are several other reports which suggest a similar role of mitochondria in the progression of neurodegenerative disorders in humans.

Nowadays, mitochondria are reported to have importance because many cancers show variation in the mitochondrial shapes, a mutation in mitochondrial genome and impaired mitochondrial functions (Zong, Rabinowitz, and White 2016; Maiuri and Kroemer 2015;

Chatterjee, Dasgupta, and Sidransky 2011; Schöpf et al. 2016). The mitochondria were first correlated with cancers in 1920 to understand the Warburg effect (Roberts and Thomas 2013). In many of the cancer cells, mitochondrial respiration is observed to be suppressed leading to the transition of metabolism from OXPHOS to glycolysis to form lactic acid (Roberts and Thomas 2013). The metabolite, succinate, is known to be an oncogenic metabolite as it can further confer epigenetic changes in the cell and is responsible for many cancer types in humans (Tannahill et al. 2013; Mills et al. 2016). Complex II-derived ROS is associated with insulin resistance in mice (Fazakerley et al. 2018). Mitochondrial accumulation in certain tumors, such as Birt-Hogg-Dube (BHD) syndrome, increases OXPHOS and helps tumor cells grow further (Hasumi et al. 2012). The presence of larger mitochondria helps in proliferation and protection from the apoptosis of the lung cancer cell line. Enlargement of mitochondria helps mitochondria to preserve the functions and ATP generation capacity in these cells (Chiche et al. 2010). Mitochondrial ROS is not quenched by mitochondrial antioxidant systems causing more accumulation of ROS in the cell (Sabharwal and Schumacker 2014). Increased oxidative stress in cancer cells induces DNA damage leading to further enhancement of tumorigenicity (Roberts and Thomas 2013; Yang Ai et al. 2013). In non-smoker lung cancer patients, the mitochondrial DNA mutation is found to be associated with complex-I which further exacerbates the oncogenic phenotypes in the patients such as increased proliferation, invasiveness, and ROS production (Dasgupta et al. 2012; Ishikawa et al. 2008). Thus, mitochondrial activities regulate the progression of the tumors in cancer patients and the progression of the disease. The above literature helps us to understand the role of mitochondria in several human disorders indicating the importance of mitochondrial functions in the pathogenesis of these diseases including neurodegenerative and oncogenic diseases. The importance and requirement of the mitochondria are fair enough to provide energy during the cell differentiation process in many cases (Urbauer, Rath, and Haller 2020). Mitochondria help cells to differentiate by potentiating the epigenetic changes associated with the metabolic transitions from glycolysis to OXPHOS (Tsogtbaatar et al. 2020). The literature discussed here suggests that the mitochondria, other than providing energy to the cell, participate in various cellular processes such as cell survival, differentiation, apoptosis, etc.

1.5 Aims and objectives of the thesis

It is evident from the description above that mitochondria are crucial for many cellular activities, from energy production to apoptosis. Here, we also talk about the shape of mitochondria in stem cells and various differentiated cells. We have also discussed the importance of mitochondria in the differentiation of epithelial cells and polarity formation. We are motivated to examine the role of mitochondria in this context since there is a lack of data on how mitochondrial morphology and related metabolism change epithelial cell shape, polarity, and differentiation. Using *Drosophila* as a model organism, this study aims to comprehend how mitochondrial morphology affects epithelial cell development in the following objectives.

1) Analyze the effects of mitochondrial morphology on epithelial cell arrangement in mitochondrial morphology mutants

2) Analysis of epithelial polarity defects in mitochondrial morphology mutants in follicle cells

3) Functional analysis of mitochondrial morphology and activity in the mutants for mitochondrial morphology and epithelial polarity

4) Epistatic analysis of signaling pathways associated with the polarity in the mitochondrial morphology mutant

2.1 Material and reagents

2.1.1 Fly stocks

Drosophila fly strains are grown and maintained at 25 C on a standard cornmeal agar medium. The flies stocks obtained from the Bloomington Stock Center are FRT40A-drp1KG (BL13510), UAS-opa1 RNAi (y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00349}attP2, Bloomington stock number # BL32358) UAS-erk RNAi (y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00173}attP2, Bloomington stock number # 34855), UAS-sod2 RNAi (y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01989}attP2, Bloomington stock number # BL25969), UAS-hSOD1 A4V (w[1118]; P{w[+mC]=UAS-hSOD1.A4V}9.1/TM6B, Tb[1], Bloomington stock number # BL33607), UAS-catalase RNAi (y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00990}attP2 ,Bloomington stock number # BL34020). For the experiments, hsflp; Gal80-FRT40A/CyO; tub-Gal4, UAS-CD8::GFP/TM6 is procured from Nicole Grieder and FRT40A/SM6a is obtained from Mary Lilly. The standard genetic crosses are performed to make combinations for the experiments and they are drp1KG-FRT40A/ Cyo; 32358/TM6 drp1KG-FRT40A/ Cyo; 34855/TM6,drp1KG-FRT40A/ Cyo; 25969/TM6, drp1KG-FRT40A/ Cyo; 34020/TM6, drp1KG-FRT40A/ Cyo; 33607/TM6, FRT40A/ Cyo; 34855/TM6, FRT40A/ Cyo; 25969/TM6, FRT40A/ Cyo; 34020/TM6, FRT40A/ Cyo; 33607/TM6.

2.1.2 Primary and secondary antibodies

The details of the primary antibodies used are 1: 500 Chi-GFP (A10262, ThermoFisher), 1:500 Rabbit aPKC, 1:500 Mouse aPKC, 1:10 Mouse Dlg (4F3, DSHB), 1:10 Mouse-Hindsight (1G9, DSHB), 1:10 Mouse-Cut (2B10, DSHB), 1:200 Rabbit-dpERK (4370, CST), 1:200 Rabbit-Caspase, 1:200 Mouse-ATPb (ab14730, Abcam), 1:200 Rabbit-pH3 (PA5-17869, ThermoFisher).

The secondary antibodies are used with 1:1000 dilution and the details are Anti-chicken 488 (A11039, ThermoFisher), Anti-Mouse 568 (A11004, ThermoFisher), Anti-Mouse 633 (A21050, ThermoFisher), Anti-Mouse 647 (A21235, ThermoFisher), Anti-Rabbit 568 (A11011, ThermoFisher), Anti-Rabbit 633 (A21070, ThermoFisher), Anti-Rabbit 647 (A21245, ThermoFisher).

2.1.3 Dyes for mitochondrial activities

MitoSOX dye with a final concentration of 5uM (M36008, ThermoFisher)

2.2 Experimental procedures and methods

2.2.1 Selection of *Drosophila* oogenesis as a model system

The *Drosophila* possesses two ovaries, and each contains 16-20 ovarioles. Each ovariole has developing egg chambers with 16 germ cells surrounded by a sheet of follicle epithelial cells, as shown (Figure 2.1). Each ovariole, at its beginning, hosts a germarium where follicle stem cells (FCs) and germ stem cells (GSCs) are positioned (Fadiga and Nystul 2019). The egg chambers are characterized into stage 1-6 mitotic, stage 7-10a endocycling, and stage 10b-13 gene amplification stages based on their proliferation and selective gene amplification capacity (Jia, Huang, and Deng 2015). Stage 1-6 is mitotic egg chambers as follicle cells keep on dividing into these stages. Stage 7-10a is endocycling egg chambers as the follicle cells stop division but replicate DNA multiple times in these stages. In later stages 10b-13 stages, the egg chambers selectively amplify the genomic region. The development of the stage 14 egg chamber passes through the above three stages at the end of oogenesis.

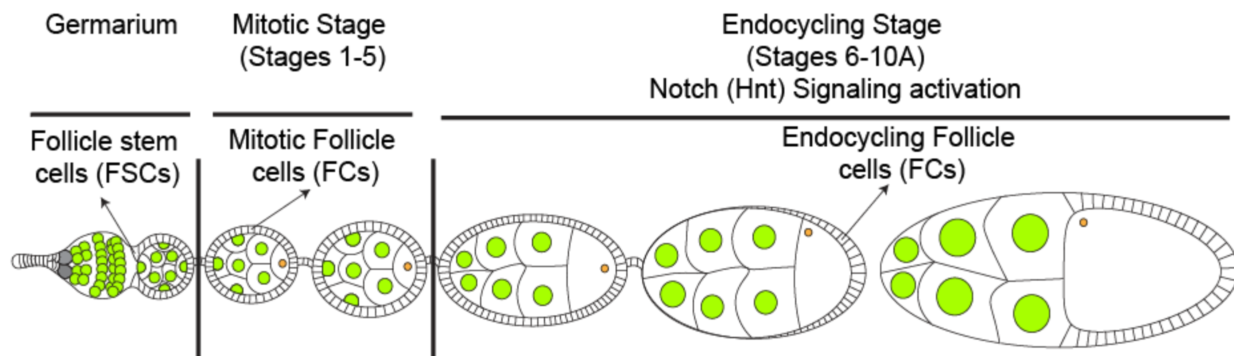

Figure 2.1: *Drosophila* oogenesis as a model system to study epithelial cell polarity

The diagram shows an individual ovariole from the *Drosophila* ovary. The germarium, on the left, has triangular follicle stem cells lacking an apical domain. Each ovariole comprises developing egg chambers from stages 1 to 14. Each egg chambers have follicle epithelial cells surrounding 16 germ cells. Stages 1-5 are mitotic egg chambers, whereas stages 6-10A are endocycling egg chambers.

These stages exhibit fine-tuned signaling pathways such as Epidermal Growth Factor Receptor (EGFR) signaling in follicle stem cell differentiation, dorsoventral axis patterning, and a formation of respiratory appendages seen in stage14 egg chamber (Castanieto, Johnston, and Nystul 2014; Van Buskirk and Schüpbach 1999; Papadia et al. 2005; Wasserman and Freeman 1998), Notch signaling in mitotic to endocycling stage transition (Sun and Deng 2007), an ecdysone signaling in endocycling to gene amplification stage transition (Sun et al. 2008). Various cellular processes such as follicle stem cell differentiation to follicle epithelial cells, change in morphology of follicle epithelial cells, and apoptosis of follicle epithelial cells are seen during the *Drosophila* oogenesis (Margolis and Spradling 1995; Wu, Tanwar, and Raftery 2008; Castanieto, Johnston, and Nystul 2014). Apart from signaling regulation, the follicle cells also appear in different shapes such as triangular follicle stem cells, cuboidal mitotic follicle cells, columnar endocycling posterior follicle cells, and squamous follicle cells in the later development of oogenesis (Wu, Tanwar, and Raftery 2008). Thus, *Drosophila* oogenesis is one of the good model systems to study diverse epithelial cell events in context with follicle cell differentiation, polarity establishment, signaling regulation, follicle cell shape transitions, etc.

2.2.2 Fly genetics and MARCM technique to generate mitotic clones in *Drosophila* ovaries

The flies having genotype drp1KG-FRT40A/ SM6a are crossed to hsflp; Gal80-FRT40A/CyO; tub-Gal4,UAS-CD8::GFP/TM6. F1 having a genotype hsflp/+; drp1KG-FRT40A/ Gal80-FRT40A; tub-Gal4, UAS-CD8::GFP/+ are heat shocked at 37.5 °C for 60 minutes in a water bath to induce follicle cell clones in the ovaries followed by a transfer of flies to the vials having fresh media with yeast sprinkled. The entire mechanism of mitotic recombination-based clone generation is shown in the diagram (Figure 2.2). The flies are reared and transferred to fresh media every 3 days until they aged 10 days (i.e 10 days post heat shock). To perform RNAi-mediated knockdown needed for epistatic analysis flies carrying FRT40A/ Cyo; 34855/TM6 and drp1KG-FRT40A/ Cyo; 34855/TM6, FRT40A/ Cyo; 34020/TM6 and drp1KG-FRT40A/ Cyo; 34020/TM6, FRT40A/ Cyo; 33607/TM6 and drp1KG-FRT40A/ Cyo; 33607/TM6 are crossed with hsflp; Gal80-FRT40A/CyO; tub-Gal4, UAS-CD8::GFP/TM6 to get desired genotype.

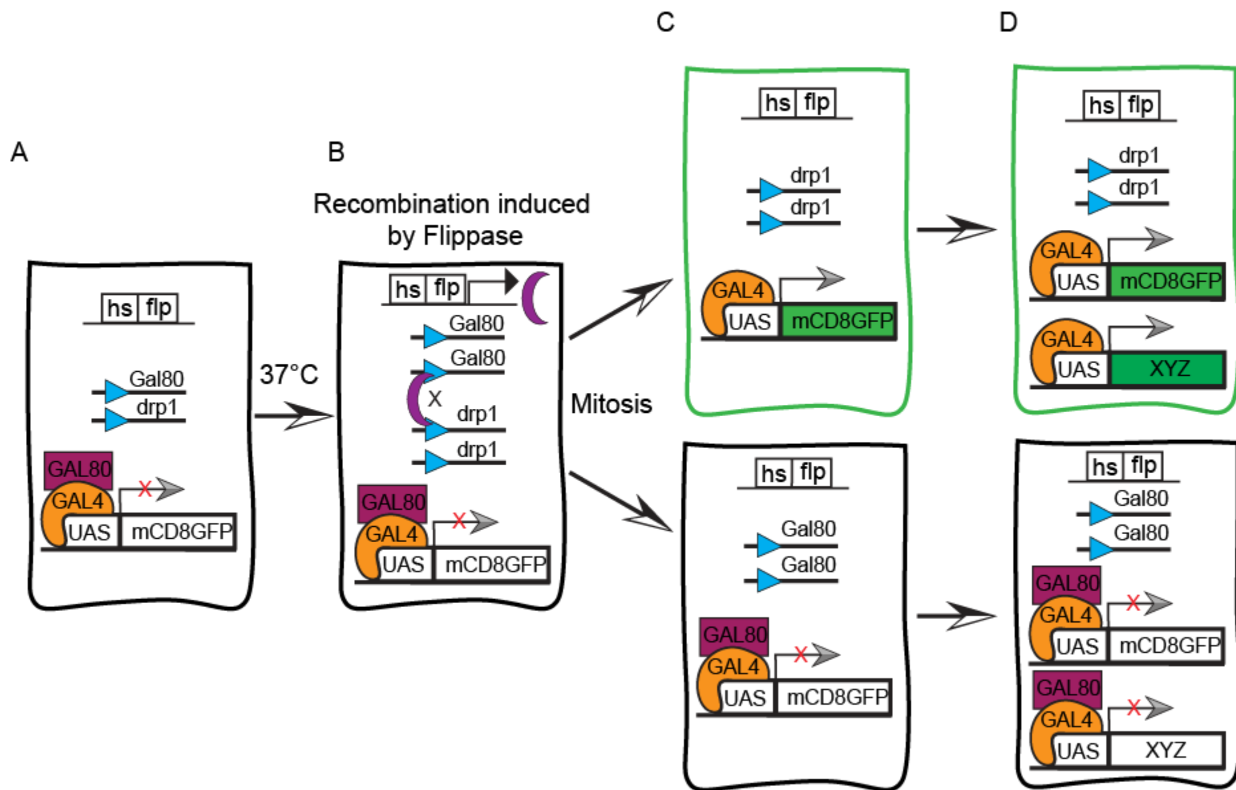

Figure 2.2: Mosaic analysis with the cell repressible marker (MARCM) technique

The somatic cells with no heat shock were given **(A)**. The recombination is introduced using heat shock at 37 °C for 1 hour. The heat-shock promoter drives the expression of the flippase enzyme (purple) in somatic cells at 37 °C **(B)**. The formation of the daughter cells, after mitosis, is marked by the presence and absence of the green fluorescence proteins (GFP). The cell showing GFP receives no Gal80 but desired mutation in homozygous condition, whereas the cell with no GFP receives Gal80 **(C)**. The expression of UAS constructs using the Gal4 expression in GFP marked cells for epigenetic analysis **(D)**.

2.2.4 Immunostaining of *Drosophila* ovaries

The fly ovaries are dissected out 10 days post heat shock in a serum-free Schneider's insect medium and ovarioles were separated using fine needles. The dissected ovaries are washed in a fresh Schneider's insect medium to remove the debris released during dissection. The ovaries are fixed using freshly prepared 4% PFA for 20-25 minutes followed by three washes of 0.3% TritonX-100 (T8787, Sigma) dissolved in 1x PBS (further called 0.3% PBST) for 10 minutes each to remove excessive PFA. The ovaries are then transferred to a blocking solution containing 2% BSA (MB083, HIMEDIA) dissolved in 0.3% PBST for 1 hour. After blocking, the ovaries are

incubated with primary antibodies for 16-18 hours followed by three 10-minute washes with 0.3% PBST. The secondary antibodies, dissolved in 0.3% PBST, were added for 2 hours to let the secondary antibodies bind with the primary antibodies. The surplus secondary antibodies are washed using three washes of 0.3% PBST for 5 minutes each. The second wash of 0.3% PBST contain a Hoechst solution, with 1:1000 dilution, to mark the nucleus. The ovarioles are separated and are mounted on a glass slide using Slowfade (S36937, ThermoFisher).

2.2.5 Live imaging using mitochondrial dyes

The MitoSOX (M36008, ThermoFisher) dye gets oxidized by mitochondrial superoxides and gives fluorescence (Figure 2.3) (Robinson et al. 2006). The fly ovaries are dissected and are washed in Schneider's medium as described earlier. The ovaries are immersed in Schneider's medium containing MitoSOX dye with the final concentration of 5uM for 15 minutes followed by a 5-minute wash with Schneider's medium (Parker, Moran, and Mitra 2017). The ovaries are immediately transferred to a glass slide and mounted using Schneider's medium for the imaging. The images are acquired within 30 minutes post-mounting.

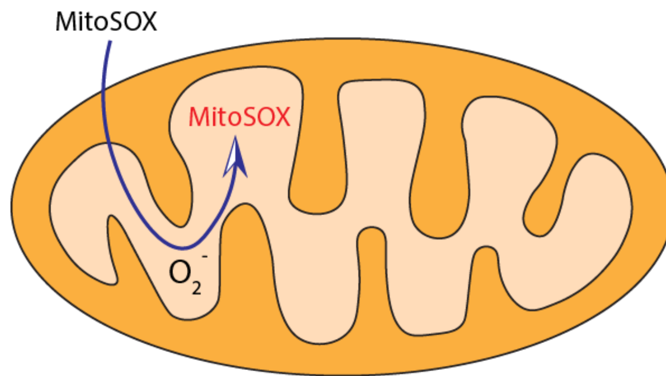

Figure 2.3: Mechanism to detect mitochondrial ROS

MitoSOX specifically enters mitochondria where it gets oxidized by mitochondrial superoxide to give fluorescence.

2.2.6 Microscopy and image acquisition techniques

Images are acquired on Zeiss LSM 710 or Zeiss LSM 780 using a 40X oil objective with 1.4 numerical aperture (NA). The fluorescence intensity captured is between 0-255 grey values on an 8-bit scale.

2.2.7 Quantification of the Data and statistics

a) Identification of stages of development in ovarioles:

The stage identification is performed by measuring the surface area of the middle stack of the egg chambers (Jia et al. 2016). Images are quantified with the help of Fiji and ImageJ (Schneider, Rasband, and Eliceiri 2012; Schindelin et al. 2012).

b) Quantification of the formation of multilayer:

For PFCs in both controls and mutants, the number of FC layers is estimated. To quantify the multilayering phenotype in the GFP-positive clones, the rows of cells per clone are counted.

c) Quantification of mitochondrial morphology:

To evaluate the mitochondrial morphology, the Streptavidin or ATP-β staining of the mitochondria in the GFP-positive follicular cell clones and GFP-negative neighboring cells are compared visually. Streptavidin or ATP-β staining in the follicle cells that are more punctate distributed is used to characterize the fragmented mitochondria. The Compactness of the mitochondrial staining and a punctate appearance from the ATP-β staining are used to distinguish between mitochondria that are more and less clustered. Furthermore, the follicle cells' mitochondrial morphology, which lack discernible variations, are classified as intermediate. From the observations, the percentage of egg chambers with the respective mitochondrial morphology are estimated.

d) Quantification of aPKC, Dlg, PatJ and Hnt:

For the analysis of cells containing polarity protein aPKC, we estimate the numbers of FCs per clone deficient of aPKC at the apical membrane in the GFP-positive clones. We express them as a percentage of the total cells in the clone. To quantitate the rescue of the aPKC on the membrane, the percentage of PFCs showing the presence of aPKC are counted in the layer of cells adjacent to the oocyte. For analysis of Notch signaling mediated differentiation, we calculate the percentage of egg chamber where at least one cell shows Hnt recovery.

We also have done intensity measurement for the aPKC, Dlg, and PatJ proteins. We have not included in the thesis as they show non-significant differences across genotypes.

e) Quantification of apical constriction:

We measure apical surface reduction by using Dlg as a marker. The distance between two adjacent arms showing Dlg strips in the fluorescence image is calculated in the follicle cells from clone and from non-clonal part. The ration of the two is taken to represent the apical constriction of the follicle cells.

f) Quantification of the dpERK:

We defined a region of interest (ROI) around the clone labeled with mCD8-GFP and surrounding follicle cells for the measurement of dpERK. Additionally, background ROI was used to subtract the noise from the signal. The dpERK in the cells was quantitated using the thresholding method in ImageJ/Fiji. The ratio of a clone's thresholded dpERK intensity value to that of its neighboring cell was calculated.

g) n values and Statistical analysis:

Each data point (n) represents quantification from a distinct clone in different egg chambers. A minimum number of 8 egg chambers are used for the statistics. The data for each genotype is shown with its respective mean and SEM. The statistical test applied for two groups is Student’s t-test and for more than two groups is a One-way analysis of variance with Tukey’s multiple comparison test.

Chapter 3: Study of mitochondrial morphology in different mitochondrial morphology mutants and analysis of follicle epithelial cell organization during *Drosophila* oogenesis

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3.1 Introduction

Mitochondria, as discussed in Chapter 1, are dynamic organelles and undergo fusion and fission cycles to regulate several mitochondrial functions. Mitochondrial fusion and fission help in the regulation of mitochondrial distribution, mitochondrial transport, mitochondrial metabolism, and mitochondrial quality control. In addition to the above functions, mitochondrial dynamics also participate in several cellular processes such as stem cell maintenance, cell proliferation, cell differentiation, cell death, and epigenetic modifications in the cell. Mitochondria behave differently when they are either fragmented or fused. The Drp1 protein is responsible for the fragmentation of the mitochondria, whereas Opa1 and Marf1 carry out the fusion of the inner mitochondrial membrane and outer mitochondrial membrane, respectively (Chapter 1, Figure 1.2). Recent evidence has shown that mitochondrial morphology and associated changes in mitochondrial metabolism and activities affect the state of the cell.

The variation in mitochondrial shapes is engaged in cell shape regulation, as evidenced by several reports. The *Drosophila* intestinal stem cells (ISCs) that are triangular in shape and without an apical domain differentiate into mature enterocyte with an apical domain and requires mitochondrial fusion (Deng et al. 2018). The follicle cells in *Drosophila* depleted for the fission protein Drp1 are reported to alter the shape of follicle epithelial cells (Mitra et al. 2012; Tomer et al. 2018). The egg chambers showed impaired morphological features when germ cells were depleted for the mitochondrial morphology regulatory proteins such as Drp1, Marf1, and Opa1 (Garcez et al. 2020). This limited evidence shows a regulation of cell shape through mitochondrial morphology directly or indirectly. We attempted to assess the role of mitochondrial morphology in regulating follicle cell shape and a monolayer organization of the follicle cells around the developing egg chamber during the *Drosophila* oogenesis.

3.2 Materials and methods

3.2.1 *Drosophila* genetics:

The fly stocks used are listed in section 2.1.2 of Chapter 2.

3.2.2 Follicle clone generation using MARCM method:

The mitotic recombination-based clone generation was performed using the MARCM method as described in section Chapter 2.

3.2.3 Immunostaining of *Drosophila* ovaries:

The description of the procedure for the immunostaining is in section 2.2.4, the list of antibodies is in section 2.1.2, and the dyes are in section 2.1.3 of Chapter 2.

3.2.4 Microscopy and image acquisition techniques:

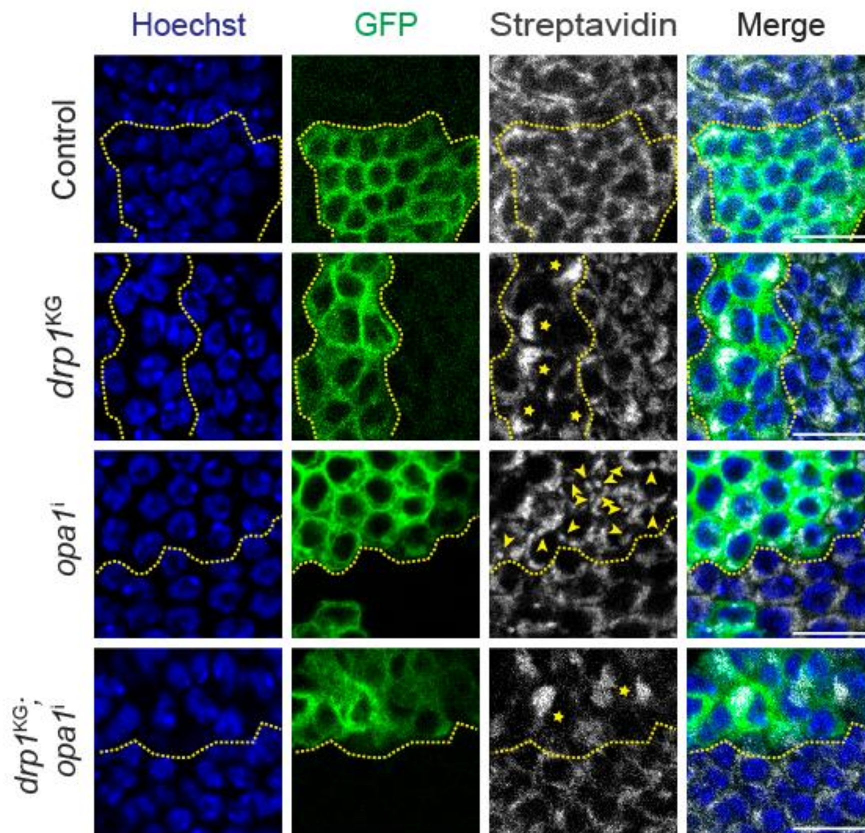
The details for image acquisition are discussed in section 2.2.6 of Chapter 2.

3.3 Results

3.3.1 Mitochondrial morphology mutants change the shape of mitochondria in *Drosophila* follicle cells

In this section, we try to analyze mitochondrial morphology in various mitochondrial morphology mutants. We use the MARCM (Chapter 2, Figure 2.2) technique to specifically label the follicle cells having a homozygous mutation of fission protein Drp1. For the rescue and control experiments of (Upstream activating sequence) UAS-driven constructs of different mutants, we exploit GAL4 to drive UAS-driven constructs of different mitochondrial morphology-related mutants in these mitosis-induced recombination-based follicle cell clones (Chapter 2, Figure 2.2). The Drp1 mutant clones of epithelial cells have transposon-mediated null mutant $drp1^{KG03815}$ ($drp1^{KG}$). To analyze the mitochondrial morphology, we use streptavidin to mark the mitochondrial morphology as streptavidin is known to bind with the biotinylated proteins found in the mitochondrial matrix. And streptavidin has been previously used to show that it binds specifically to mitochondria and can be used to show mitochondrial morphology in the *Drosophila* embryo (Hollinshead, Sanderson, and Vaux 1997; Chowdhary et al. 2017; W. Yoon et al. 2019). The nucleus of the follicle cells is stained with Hoechst. These results validated the mitochondrial mutants that we are using in this study. In Figure 3.1, the loss of Drp1 function in follicle cells shows clustered mitochondria and is also reported to have similarly clustered mitochondrial morphology in follicle cells and in neuroblasts (Mitra et al. 2012; Tomer et al. 2018; Dubal et al.

2022). Another mutant, where the function of Opa1 is blocked using *UAS-opa1*^{RNAi}, shows fragmented mitochondria (Figure 3.1) and is also used to show similarly fragmented mitochondria in the neuroblasts (Dubal et al. 2022). The combination of *drp1*^{KG}*;opa1*^{i} still shows a clustering of mitochondria (Figure 3.1) that can not be optically resolved. Thus, altering an equilibrium of proteins that regulate mitochondrial morphology in follicle cells during *Drosophila* oogenesis.



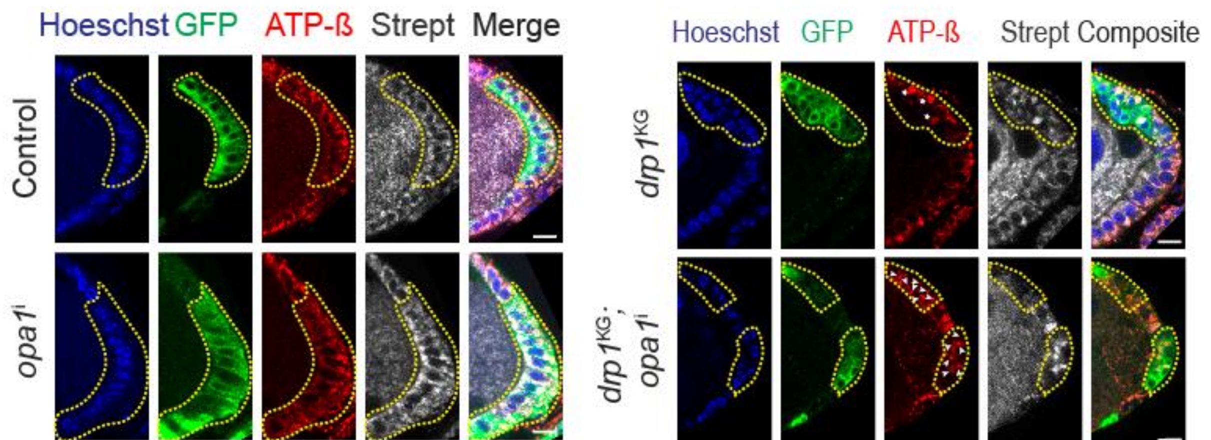


Figure 3.1: Mitochondrial morphology in fission and fusion mutants

(A-B) Representative images show mitochondria stained with Streptavidin (greyscale) in FCs in the endocycling stages in the genotypes control FRT40A (100% intermediate, n=21), *drp1*^{KG} (92% clustered, n=26), *opa1*^{i} (92% fragmented, n=24) and *drp1*^{KG}*;opa1*^{i} (100% clustered with decrease in compaction as compared to *drp1*^{KG} alone, n=7) **(A)**. Representative images showing mitochondria stained with ATP-β (red) and Streptavidin (greyscale) in FCs in the sagittal view of endocycling follicle cells in the genotypes control FRT40A, *drp1*^{KG}, *opa1*^{i}, and *drp1*^{KG}*;opa1*^{i} **(B).** mCD8-GFP (green) marks the clones in PFCs. The nucleus (blue) is stained with Hoechst. Yellow asterisks mark clustered mitochondria and yellow arrowheads mark fragmented mitochondria. Scale bar=10µm.

3.3.2 Analysis of follicle cell shape and organization in different mitochondrial morphology mutants in endocyclic stages of *Drosophila* oogenesis

We have discussed that *Drosophila* oogenesis is a sound model system for studying epithelial cell arrangement and shape transitions (see chapter 2, section 2.2.1) (Wu, Tanwar, and Raftery 2008). The cuboidal follicle epithelial cells from mitotic stage 1-6 transit to columnar epithelial cells, specifically in the posterior part of egg chambers of endocyclic stage 7-10a (González-Reyes and St Johnston 1998; Jia, Huang, and Deng 2015). The follicle cells in the endocycling stage do not divide but keep on multiplying their DNA. We try to assess if these columnar cells, known as posterior follicle cells (PFCs), show altered cell shape when mitochondrial morphology is impaired in these cells. The loss of Drp1 in these cells causes the formation of multiple layers in the endocycling stages. We try to characterize the egg chambers showing multiple layers based on the number of cell layers present in that particular egg chamber. We observe that the number of cell layers per clone in the *drp1*^{KG} depleted posterior follicle cells varies from a monolayer of cells to a maximum of 6 layers of cells per clone (Figure 3.2). Most egg chambers show an average of three cell layers per clone in *drp1*^{KG} mutant follicle cells. We count the percentage of egg chambers with 3 or more multiple cell layers and that is 66% (n=50) for the egg chambers having Drp1 mutant clones (Figure 3.2C). We try to suppress additional fusion using *UAS-opa1*^{RNAi} in follicle cells depleted for Drp1 protein. To do that, we use a combination of *drp1*^{KG}*;opa1*^{i} which show a 42% percentage of egg chambers with three or more cell layers which is a partial rescue of the multilayer phenotype we see in *drp1*^{KG} mutant follicle cells.

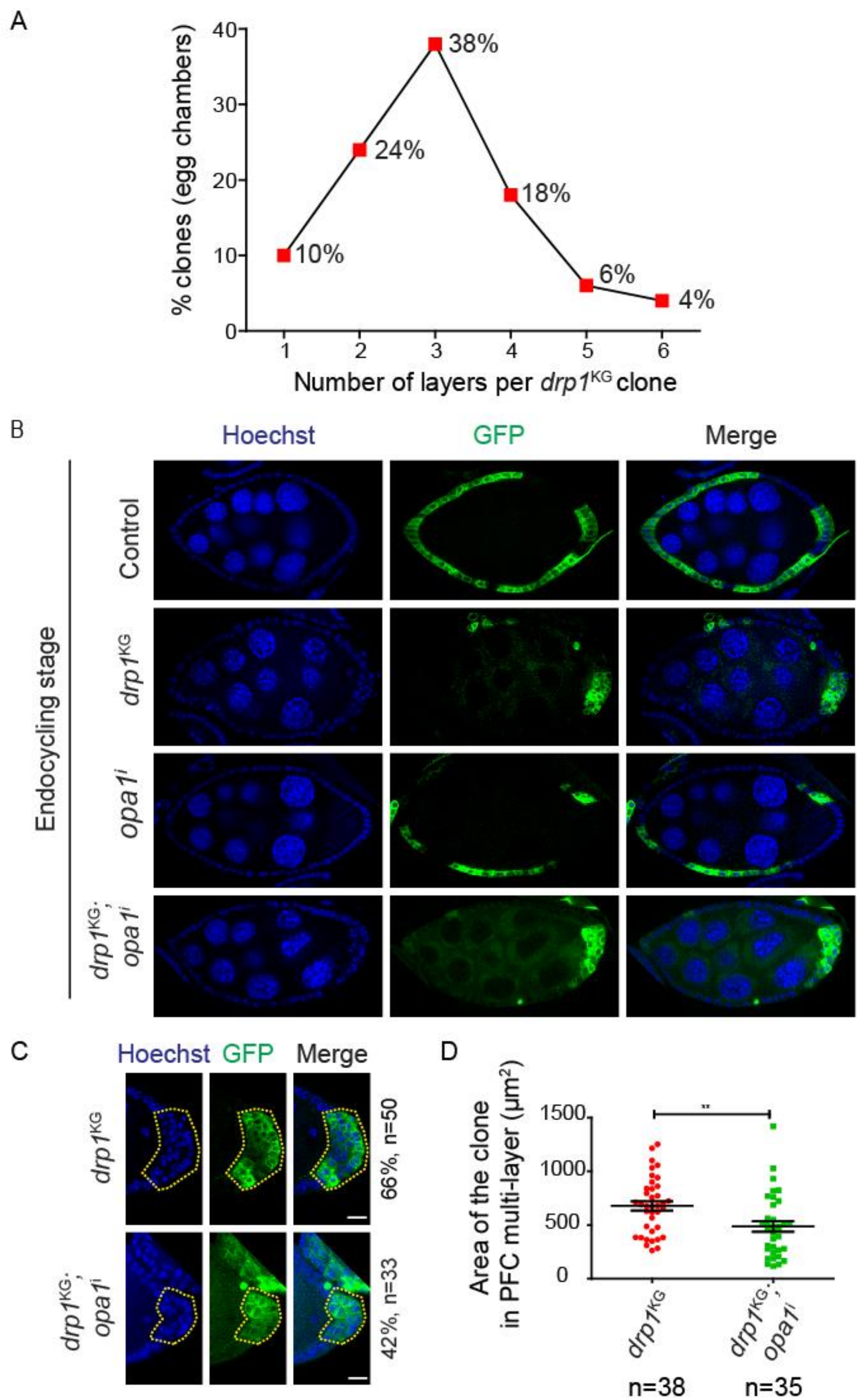


Figure 3.2: Arrangement of endocycling stage follicle cells of different mitochondrial morphology mutants

(A-D) Graph categorizing the % of the *drp1*^{KG} mutant egg chambers with follicle cells arranged in one layer (10%,n=50, N=6), two layers (24%,n=50, N=6), three layers (38%,n=50, N=6), four layers (18%,n=50, N=6), five layers (6%,n=50, N=6), six layers (4%,n=50, N=6) **(A)**. Representative images of FC arrangement in control FRT40A, *drp1*^{KG}, *opa1*^{i}, and *drp1*^{KG};*opa1*^{i} PFC clones at endocycling stage **(B)**. Representative images showing multiple layers of PFC clones in *drp1*^{KG} (66% clones have 3 layers or more, n=50) and *drp1*^{KG}*;opa1*^{i} (42% clones have 3 layers or more, n=33) (also see Fig. S1B) **(C)**. The graph shows a comparison of the PFC clone area between *drp1*^{KG} (n=38) and *drp1*^{KG};*opa1*^{i} (n=35) **(D)**. The plot shows Mean+SEM. Each data point (n) represents a clone in a separate egg chamber. The statistical test performed for clone area measurement is Student's t-test. ns=non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. Scale bar=10µm.

Additionally, we attempt to measure the area of the follicle cell clones from these egg chambers. We also find that the area of the clone is higher when Drp1 is depleted from the follicle cells but gets reduced while using *drp1*^{KG}*;opa1*^{i} (Figure 3.2D). Blocking Opa1 protein alone using *UAS-opa1*^{RNAi} causes fragmentation of the mitochondria but does not alter the cell shape in endocycling follicle cells. These data tell us that the clustered or fused mitochondria are responsible for the impaired arrangement of the posterior follicle cells in the endocycling stage egg chambers of the *drp1*^{KG} mutant. The fragmented mitochondrial morphology is necessary to maintain the cellular architecture of the endocycling follicle cells.

3.3.3 Analysis of follicle cell shape and organization in different mitochondrial morphology mutants in mitotic stages of *Drosophila* oogenesis

From the previous results of 3.3.2, we can observe that the epithelial organization defects are visible in endocycling stages during oogenesis when the function of fission protein Drp1 is inhibited. We then analyze if these defects arise early in the mitotic stage itself. The cuboidal follicle cells of the mitotic stage envelope around the germ cell to form a mitotic egg chamber from stage 1 to stage 6. These cells are arranged in a monolayer and keep dividing to give more follicle epithelial cells until they enter the endocycling stage (Sun and Deng 2007). These cells do not show any changes in their monolayer arrangement when mitochondrial were either fused or fragmented in Drp1 and Opa1 mutants, respectively (Figure 3.3). The double mutant *drp1*^{KG}*;opa1*^{i} follicle cell clones are not affected too.

These observations show that the fused mitochondria are responsible for the impaired arrangement of follicle cells in the endocycling stage but not in the mitotic stage egg chambers. The fragmented mitochondrial morphology also does not change the organization of follicle epithelia in mitotic stage egg chambers.

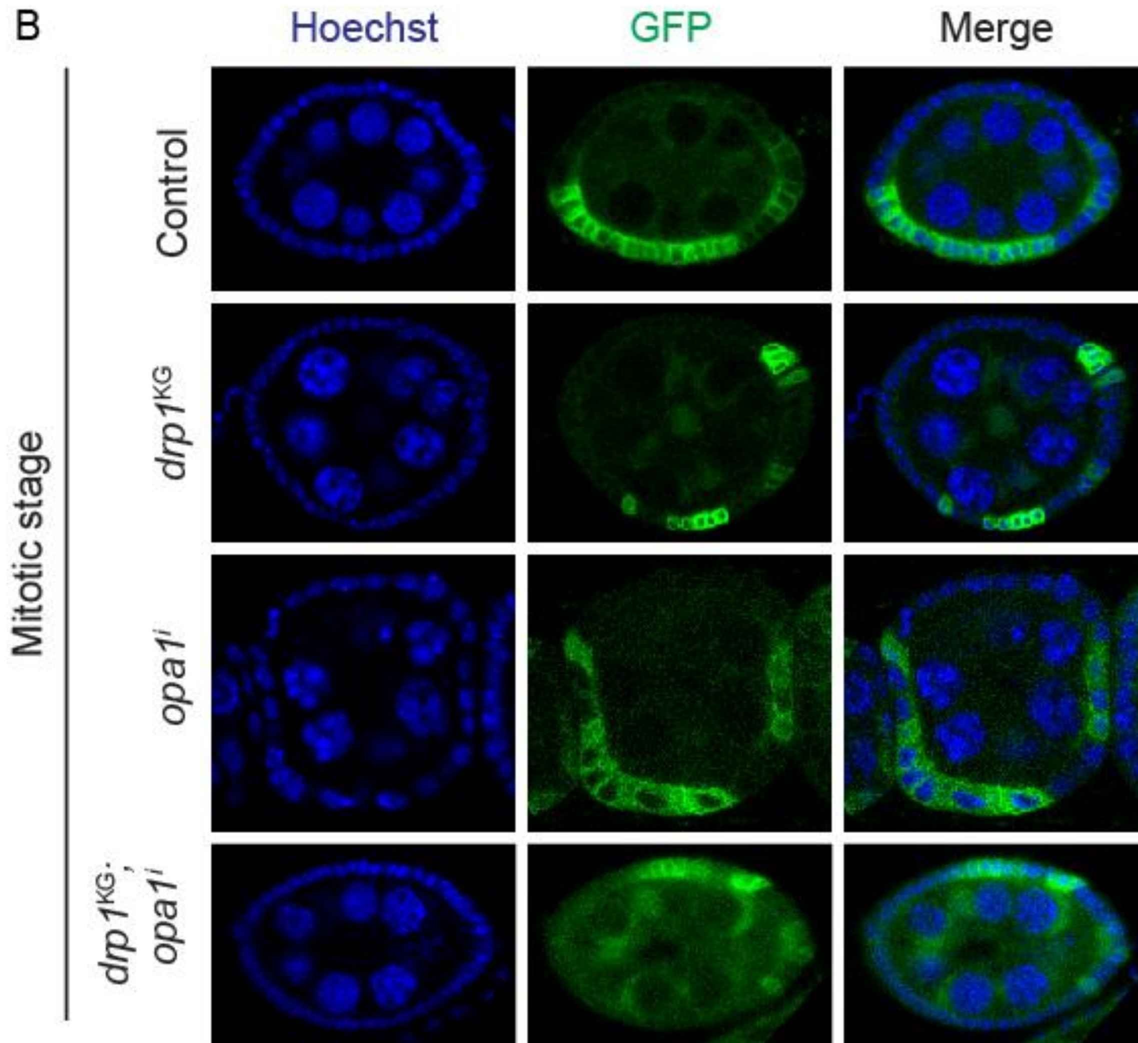


Figure 3.3: Arrangement of mitotic stage follicle cells of different mitochondrial morphology mutants
Representative images of FC arrangement in control FRT40A, *drp1*^{KG}, *opa1*^{i}, and *drp1*^{KG}*;opa1*^{i} follicle cell clones at the mitotic stage. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst.

3.4 Discussion

In this chapter, we have discussed how mitochondrial morphology is affected by blocking the function of various proteins required for maintaining the mitochondrial shape. In the field of epithelial cell biology, very little evidence is available to understand the role of mitochondria in maintaining epithelial integrity and architecture. The mitochondria quantity and quality are also necessary to maintain developing egg chambers during *Drosophila* oogenesis (Tourmente et al. 1990). But it is not clear how mitochondria can affect epithelial development during oogenesis. It is also unclear how the mitochondria are distributed in follicular epithelia and what activities they do as the development proceeds further in oogenesis. There is a report where an increase in intracellular pH (pHI) is reported to have a regulatory role in pre-follicle cell (pFCs) differentiation from follicle stem cells (Ulmschneider et al. 2016). These observations lead to the curious question of how mitochondrial morphology and its activities are regulated throughout this epithelial development during oogenesis.

The mitochondrial morphology, as we know from 1.1.2, is regulated by several proteins. The Drp1 regulates the fission of the mitochondria. The fusion proteins Opa1 and Marf1 regulate the fusion of the mitochondria. Any change that impairs the equilibrium of these proteins alters mitochondrial morphology. Blocking the function of fission protein Drp1, in *drp1*^{KG} mutant follicle cells, causes clustered mitochondria, whereas blocking fusion protein Opa1 causes fragmentation of mitochondria as per observed in 3.3.1 (Figure 3.1). The reason behind these clustered mitochondria is a lack of fission by Drp1 in these cells. In Drp1-depleted follicle cells, the fusion of the mitochondria is thought to be unopposed as there is no Drp1 present from the beginning of the formation of follicle cells due to the mitotic recombination event in the MARCM technique. The same cells have fusion proteins Opa1 and Marf1 that further add up to the fusion of the mitochondria. This unopposed fusion, due to the absence of Drp1 and the agonistic role of Opa1 and Marf1 in fusion events, help mitochondria to be more clustered in these cells. To suppress mitochondria from undergoing additional fusion in *drp1*^{KG} mutant, we have used *drp1*^{KG}*;opa1*^{i} combination where the function of Opa1 protein is suppressed using *UAS-opa1*^{RNAi}. We do not see effectively resolved mitochondria in *drp1*^{KG}*;opa1*^{i} combination as blocking the fusion protein Opa1 can only inhibit some extent of a fusion event in *drp1*^{KG,} whereas the function of Marf1 is still there. In addition, the lack of fission protein Drp1 does not help in the fragmentation of the mitochondria.

Although the mitochondrial morphology is not that drastically resolved in *drp1*^{KG}*;opa1*^{i}, these endocycling follicle cells show a reduction in the multilayer defect of the *drp1*^{KG} mutant (Figure 3.2B, C). The data also suggests that the fission of mitochondria does not alter the follicle cell organization, as seen in *UAS-opa1*^{RNAi}. Thus, it is more important to understand the role of fused mitochondria in the epithelial organization during *Drosophila* oogenesis. Surprisingly, the early-stage mitotic follicle cells do not undergo the formation of multiple layers of the cells in any of the mitochondrial morphology mutants, which shows the importance of mitochondrial morphology in regulating the stages of oogenesis. In the following chapter, we will try to understand the reasons for the formation of multiple layers.

Chapter 4: Study of follicle epithelial cell polarity in different mitochondrial morphology mutants during *Drosophila* oogenesis

4.1 Introduction

Epithelial cell differentiation is a process where an epithelial stem cell or pre-existing epithelial cell divides to form a daughter epithelial cell. The differentiation of the epithelial cell involves an establishment of polarity. As discussed earlier, an epithelial cell gains three domains apical, lateral, and basal. All of these three domains exhibit a set of proteins that forms domain-specific complexes (Chapter 1, Figure 1.6). An apical domain possesses a complex having an atypical protein kinase C(aPKC)- Bazooka(PAR3)- PAR6 proteins and a subapical complex having Crumbs(Crb)-PALS1 associated tight junction protein(PatJ)-Stardust(Sdt) proteins. A lateral domain complex is composed of Scribble(Scrb)-Disclarge (Dlg)- Lethal giant larvae (Lgl) proteins whereas a basal domain possesses integrin proteins and these proteins are conserved across species (Tepass et al. 2001; Assémat et al. 2008). *Drosophila* oogenesis, as discussed earlier, is a good model system to study the epithelial cell polarity (Chapter 2, Figure 2.1). *Drosophila* embryogenesis, *Drosophila* imaginal disc epithelial cells, *Drosophila* midgut epithelial cells, and *Drosophila* follicle epithelial cells are explored in the field to understand the mechanism of the polarity establishment (Goode et al. 1996; Baum and Perrimon 2001; Bergstralh, Lovegrove, and St Johnston 2013; Khoury and Bilder 2020; Bilder, Li, and Perrimon 2000; H. A. Müller 2000).

Much evidence suggests that the establishment of polarity and differentiation in these cells are regulated by mitochondrial morphology and associated mitochondrial activities. The hepatocyte polarization requires fused mitochondria and efficient OXPHOS (Fu et al. 2013). The intestinal stem cell that does not possess an apical domain undergoes differentiation and forms enterocytes with an apical domain due to increased mitochondrial fusion (Deng et al. 2018). In addition, the follicle cells of *Drosophila* also show altered cells shape and epithelial organization when Drp1 was depleted in them (Tomer et al. 2018). This literature helps us to understand the involvement of mitochondrial morphology in the polarization of epithelial cells. But it is poorly understood how mitochondria regulate the developing epithelial cells in the context of their polarity.

The *Drosophila* oogenesis process is a good model to understand the progression of the establishment of the polarity. The follicle epithelial cells during oogenesis pass through multiple stages of development which helps understand the cell shape transitions along with remodeling in epithelial polarity. In this section of results, we have attempted to understand the role of mitochondria morphology in the regulation of the apical-basal polarity of the follicle epithelial cells during various stages of oogenesis.

4.2 Materials and methods

4.2.1 *Drosophila* genetics:

The fly stocks used are listed in section 2.1.2 of Chapter 2.

4.2.2 Follicle clone generation using MARCM method:

The mitotic recombination-based clone generation was performed using the MARCM method as described in section Chapter 2.

4.2.3 Immunostaining of *Drosophila* ovaries:

The description of the procedure for the immunostaining is in section 2.2.4, the list of antibodies is in section 2.1.2 and the dyes are in section 2.1.3 of Chapter 2.

4.2.4 Microscopy and image acquisition techniques:

The details for image acquisition are discussed in section 2.2.6 of Chapter 2.

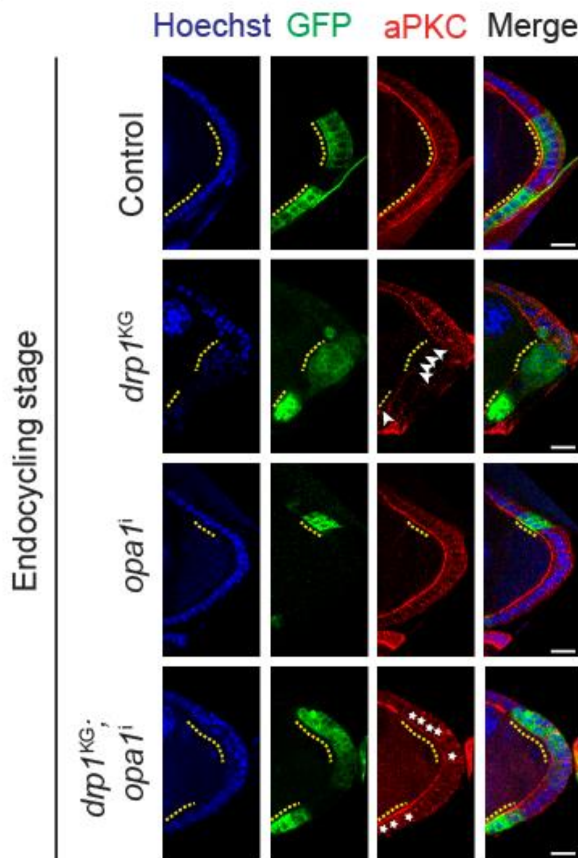
4.3 Results

4.3.1 Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells of different mitochondrial morphology mutants in endocycling stages of *Drosophila* oogenesis

We have seen the multilayer formation and an altered epithelial organization in $drp1^{KG03815}$ ($drp1^{KG}$) mutant follicle cell clones in endocycling stage egg chambers (Chapter 3, Figure 3.2B, C). The next step is to identify if these cells lose the polarity proteins and become so altered. To assess the reason behind the formation of the multiple layers, we analyze the distribution of

apicobasal polarity proteins. As discussed in the introduction, a mature follicle cell attains all the polarity complexes on their respective domains. In addition, the multilayer has been observed in follicle cells mutant for polarity proteins. The interesting observation about multilayering of Drp1 mutant follicle cells is that they are seen mostly at endocycling stages as discussed in (Chapter 3, Figure 3.2B, C). We check an apical polarity protein aPKC on the apical membrane of the Drp1 mutant follicle cells of multilayered epithelia of the endocycling stage. We find that the cells of this multilayer, those who are in contact with the oocyte, have lost the membrane aPKC as shown by arrowheads (Figure 4.1). Furthermore, we also observe that the multilayer formation is partially reduced when additional fusion events are blocked using *drp1*^{KG}*;opa1*^{i} (Chapter 3, Figure 3.2C). Based on these rescues, we also expect that the apical polarity protein aPKC comes back on the membrane of the double mutant *drp1*^{KG}*;opa1*^{i} follicle cell clones that are absent in *drp1*^{KG}. The percentage of cells, those in contact with the oocyte, losing aPKC is lowered in this double mutant of *drp1*^{KG}*;opa1*^{i} to as compared to *drp1*^{KG} follicle cell clones (Figure 4.1).

A



B

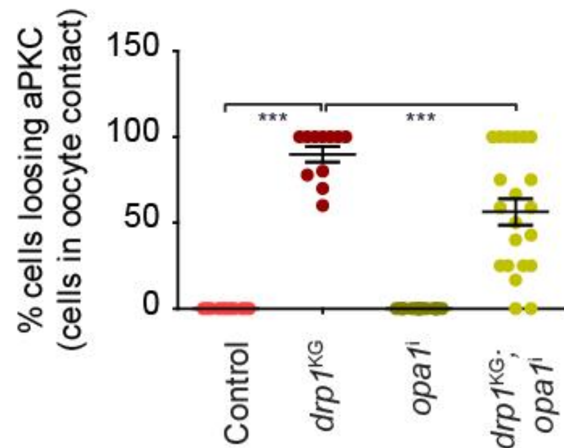


Figure 4.1: Drp1 mutant follicle cell clones show loss of aPKC in endocycling stages and are partially recovered in *drp1*^{KG}*;opa1*^{i}

(A-B) Representative images showing aPKC (red) in PFC clones from *drp1*^{KG} and *drp1*^{KG}*;opa1*^{i} at the endocycling stage **(A)**. The graph shows the percentage of cells per PFC clone adjacent to the oocyte losing aPKC in *drp1*^{KG} and *drp1*^{KG}*;opa1*^{i} at the endocycling stage **(B).** mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. White arrowheads show loss of aPKC in FCs. White asterisks mark FCs that show the presence of aPKC in double mutants. The graphs show data in Mean+SEM. Each data point (n) in the endocycling stages represents the percentage of cells losing aPKC in a single clone of a separate egg chamber. The n values for the genotypes control FRT40A, *drp1*^{KG}, *opa1*^{i}, and *drp1*^{KG}*;opa1*^{i} is given as (n, N; n is the number of clones, N is the number of independent replicates) being (n=11,11,22,21 and N=3,5,3,4 respectively) for endocycling stages. The statistical test performed is one-way ANOVA with Tukey's multiple comparisons. ns=non significant, * P<0.05, ** P<0.01, *** P<0.001, Scale bar=10µm.

4.3.2 Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells of different mitochondrial morphology mutants in mitotic stages of *Drosophila* oogenesis

We do not see the multilayers of cells and altered epithelial organization in *drp1*^{KG03815} (*drp1*^{KG}) mutant follicle cell clones in mitotic stage egg chambers (Figure 3.3). But to check if the impairment of apical polarity protein aPKC starts in early stages, we assess the polarity protein aPKC in these *drp1*^{KG} mutant follicle cells in mitotic stages. Although the follicle cells are arranged in monolayer in *drp1*^{KG} mitotic stage, they show a loss of the membrane aPKC as shown by arrowheads (Figure 4.2). Furthermore, we also observe that the loss of aPKC phenotype is partially rescued when additional fusion events are blocked using *drp1*^{KG}*;opa1*^{i} (Figure 4.2). The fragmentation of mitochondria at this stage does not affect the distribution of aPKC in follicle cells depleted for Opa1 protein using *UAS-opa1*^{RNAi}. This evidence suggests that the early loss of aPKC in Drp1 depleted follicle cells is not enough to cause the formation of multiple layers of the cells but sustaining the aPKC loss further in endocycling stages causes the formation of the multilayers.

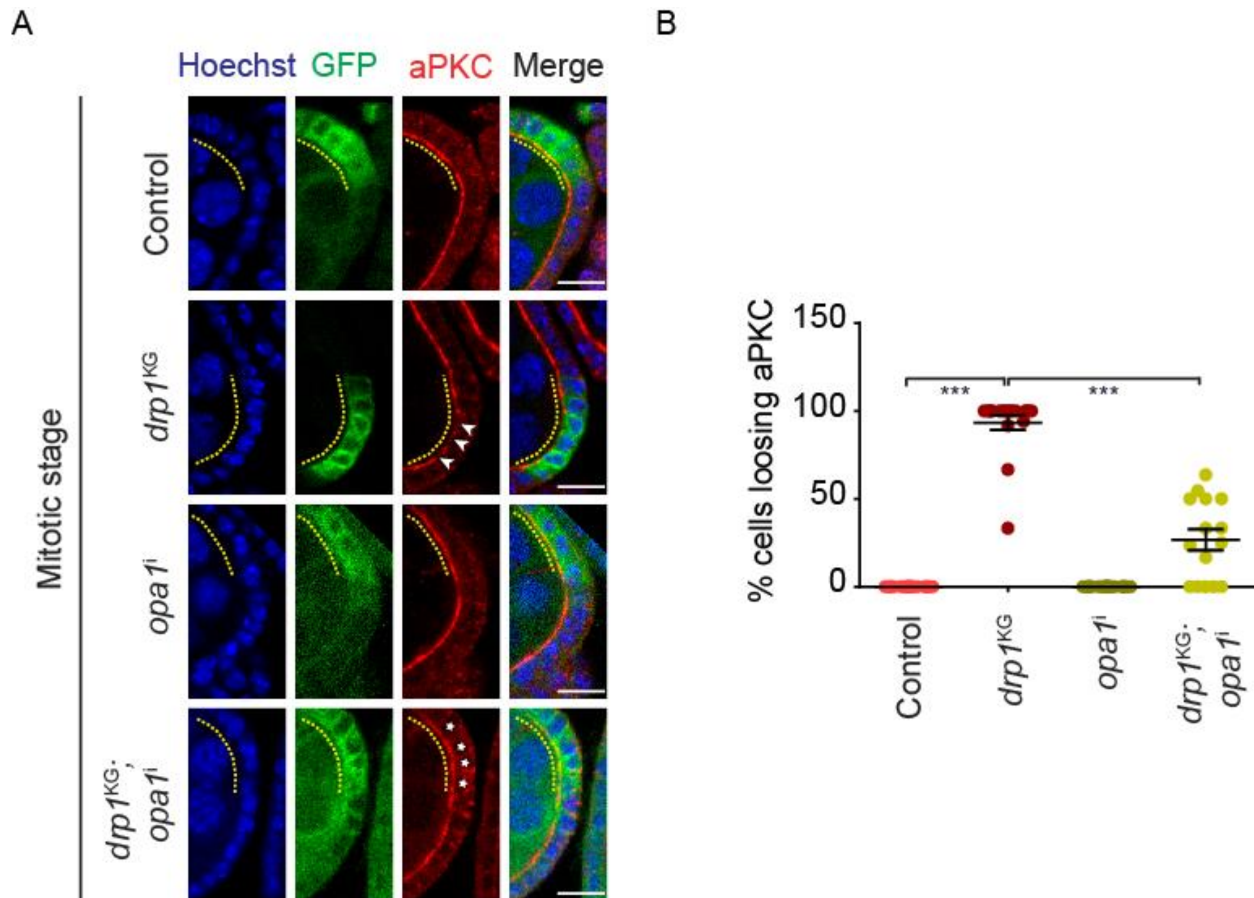


Figure 4.2: Drp1 mutant follicle cell clones show loss of aPKC in mitotic stages and are partially recovered in *drp1*^KG^*;opa1*^i^

(A-B) Representative images showing aPKC (red) in *drp1*^{KG} and *drp1*^{KG}*;opa1*^{i} in mitotic stage FCs **(A)**. The graph shows a significant rescue in % of cells losing aPKC in *drp1*^{KG}*;opa1*^{i} as compared to *drp1*^{KG} mutant follicle clones in mitotic stages **(B)**. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. White arrowheads show defective in FCs. White asterisks mark FCs that show the presence of aPKC in double mutants. The graphs show data in Mean+SEM. Each data point (n) in the endocycling and mitotic stages represents the percentage of cells losing aPKC in a single clone of a separate egg chamber. The n values for the genotypes control FRT40A, *drp1*^{KG}, *opa1*^{i}, and *drp1*^{KG}*;opa1*^{i} is given as (n, N; n is the number of clones, N is the number of independent replicates) being (n=17,17,21,15 and N=3,5,3,4 respectively) for mitotic stages. The statistical test performed is one-way ANOVA with Tukey's multiple comparisons. ns=non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Scale bar=10µm.

4.3.3 Analysis of lateral polarity protein disc large (Dlg) in follicle cells of different mitochondrial morphology mutants in endocycling stages of *Drosophila* oogenesis

The apical protein aPKC, as discussed earlier, is not only an apical polarity protein but it functions as a kinase that phosphorylates and restricts basal protein Dlg on the basal domain (Golub et al. 2017; Schmidt and Peifer 2020). From the results discussed in 4.3.1, it is expected that the Dlg can be mislocalized from the basal domain to the apical domain as the aPKC is no longer there to restrict the Dlg. So to check that, we immunostained the *drp1*^{KG} follicle cells for the Dlg protein where we see a loss of aPKC. The observations of the endocycling follicle cells depleted for the Drp1 proteins do not show mislocalized basal protein Dlg as shown by the arrow (Figure 4.3). Thus the partial loss of aPKC is likely to be responsible for the Drp1 depleted follicle cells being present in multiple layers in endocycling stages.

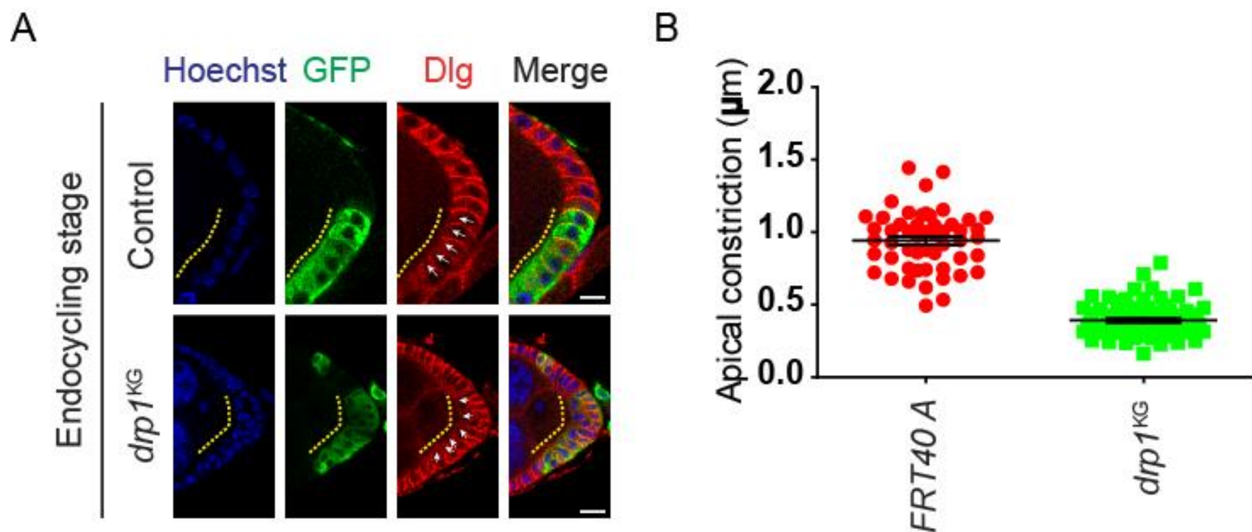


Figure 4.3: Distribution of basolateral polarity protein (Dlg) in follicle cells of control and *drp1*^{KG} at the endocycling stage

Representative images showing the distribution of Dlg (Red) in control FRT40A (100% Dlg present on the lateral domain, n=10) and *drp1*^{KG} (100% Dlg present on the lateral domain, n=10) follicle cell clones in endocycling stages **(A)**. The graph shows a significant apical constriction in *drp1*^{KG} (n=80) as compared to control FRT40A (n=52) follicle clones in endocycling stages **(B).** The plot shows Mean+SEM. Each data point (n) represents an apical length of an individual follicle cell of a clone normalized to the apical length of an adjacent follicle cell in a separate egg chamber. The statistical test performed for clone area measurement is Student's t-test. ns=non significant, * P<0.05, ** P<0.01, *** P<0.001. mCD8-GFP (green)

expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. White arrows mark the apical domain of the FCs, note that Dlg is present in the lateral domain in PFCs in the endocycling stage adjacent to the oocyte and FCs in the mitotic stage. Scale bar=10µm.

4.3.4 Analysis of lateral polarity protein disc large (Dlg) in follicle cells of different mitochondrial morphology mutants in mitotic stages of *Drosophila* oogenesis

Similar to the result 4.3.2, we see a loss of aPKC from the apical domain of the follicle cells depleted for Drp1. We expect that the Dlg should be mislocalized to the apical domain as an apical domain is now devoid of aPKC regulating the localization of Dlg on the basal domain. We observe that the Dlg is normally localized to the lateral domains in mitotic follicle cells depleted of Drp1 as shown with arrows (Figure 4.4). The results in 4.3.3 and 4.3.4 are parallel with the fact the loss of aPKC in *drp1*^{KG} mutant follicle cells does not cause a change in the localization of basal polarity protein Dlg. The examination of cell shape reveals that there is a constriction of the apical surface of Drp1-depleted mitotic follicle cells and the percentage of egg chambers with such constriction is 91% (n=43) even when the Dlg is not mislocalized.

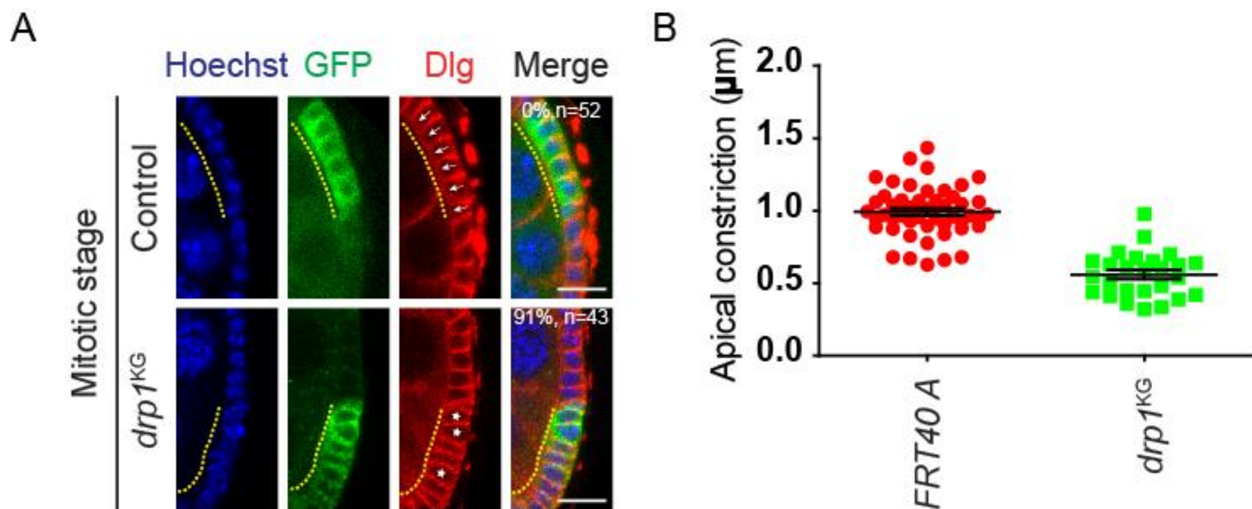


Figure 4.4: Distribution of basolateral polarity protein (Dlg) in follicle cells of control and *drp1*^{KG} at mitotic stage

Representative images showing the distribution of Dlg (Red) in control FRT40A (100% Dlg present on the lateral domain, n=11) and *drp1*^{KG} (100% Dlg present on the lateral domain, n=10) follicle cell clones in

mitotic stages. The clones have apically constricted or narrow FCs in *drp1*^{KG} (91%, n=43) as compared to control FRT40A (0%, n=52) **(A).** The graph shows a significant apical constriction in *drp1*^{KG} (n=26) as compared to control FRT40A (n=51) follicle clones in mitotic stages **(B).** The plot shows Mean+SEM. Each data point (n) represents an apical length of an individual follicle cell of a clone normalized to the apical length of an adjacent follicle cell in a separate egg chamber. The statistical test performed for clone area measurement is Student's t-test. ns=non significant, * P<0.05, ** P<0.01, *** P<0.001. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. White arrows mark the apical domain of the FCs, note that Dlg is present in the lateral domain in PFCs in the endocycling stage adjacent to the oocyte and FCs in the mitotic stage. Scale bar=10µm.

4.4 Discussion

In this chapter, we have tried to understand why multilayers are formed in Drp1 depleted follicle cells in the endocycling stage of *Drosophila* oogenesis. Depletion of polarity proteins aPKC, Baz, Crumbs, Scribble, Dlg, and Lgl leads to the distribution of follicle cells in multiple layers (Ventura et al. 2020; Khoury and Bilder 2020; Luo et al. 2016; Sun and Deng 2005; Romani et al. 2009; Benton and St Johnston 2003; Bilder, Li, and Perrimon 2000; Fletcher et al. 2012; Dent et al. 2019; Q.-Q. Wang et al. 2021; Woolworth, Nallamothu, and Hsu 2009; Baum and Perrimon 2001; Bergstralh, Lovegrove, and St Johnston 2013; Moreira et al. 2019). The similar multilayering of follicle cells in endocycling egg chambers was observed in aPKC mutants follicle cells in late-stage egg chambers but not in the early-stage egg chambers (Ventura et al. 2020; Bergstralh, Lovegrove, and St Johnston 2013). The apical protein aPKC is one of the proteins in the apical polarity complex aPKC-PAR3-PAR6 complex. Being a kinase, the apical protein aPKC phosphorylates several other polarity proteins including Dlg, Bazooka, Crumbs, and Lgl regulates epithelial cell polarization and thus serves as one of the important apical polarity protein (Golub et al. 2017; Morais-de-Sá, Mirouse, and St Johnston 2010; Sotillos et al. 2004; Betschinger, Mechtler, and Knoblich 2003). The loss of aPKC from Drp1 depleted follicle cells in both stages might behave like aPKC mutant cells. The role of aPKC is also known to regulate the mitotic spindle orientations which are necessary for the correct and symmetric division of the cells (Guilgur et al. 2012). Thus, a loss of aPKC from the *drp1*^{KG} mutant follicle cells could be one of the reasons behind the formation of the multilayer in the endocycling stages. But the loss of aPKC in mitotic follicle cells of *drp1*^{KG} mutant does not cause the multilayer formation is surprising. The

mitotic follicle cells, during division, show a loss of aPKC but still remains in monolayer in the wild type. And hence a loss of aPKC in both stages give a differential phenotype in terms of multilayer formation. The literature also suggests that a basolateral protein Dlg but not the apical protein aPKC plays a role in the orientation of the mitotic spindle (Bergstralh, Lovegrove, and St Johnston 2013). aPKC depletion results in the formation of gaps and multilayered epithelia on optogenetic inactivation. This occurs due to the activation of Myosin II finally leading to multilayering (Osswald et al. 2022). Besides the loss of aPKC, the basolateral marker Dlg in these cells is not mislocalized. These results help us to understand the reason behind multilayer formation and open a question that how mitochondrial morphology in the follicle cells of two different stages regulate their polarity. Additionally, we have not checked the EMT markers for the same. This can be done using Snail immunostaining as the Snail protein is reported to be one of the EMT markers and suppresses *Drosophila* E-Cadherin (DE-Cadh). Surprisingly, Snail overexpression is reported to have caused no multilayer formation in the same article (Tseng et al. 2016). Yet, it would be nice to see if snail increases in these Drp1 mutant multilayered cells.

The observations from the literature and our results tell us that the maintenance of the epithelial sheet around developing egg chambers requires a strictly regulated polarity complex. The change or loss of these polarity loses the integral regulation of other polarity complexes and gives such multilayer formations in the epithelia as we observe in *Drosophila* follicle cells.

Chapter 5: Study of mitochondrial activities and effect on epithelial cell polarity in different antioxidant enzyme mutants during *Drosophila* oogenesis

5.1 Introduction

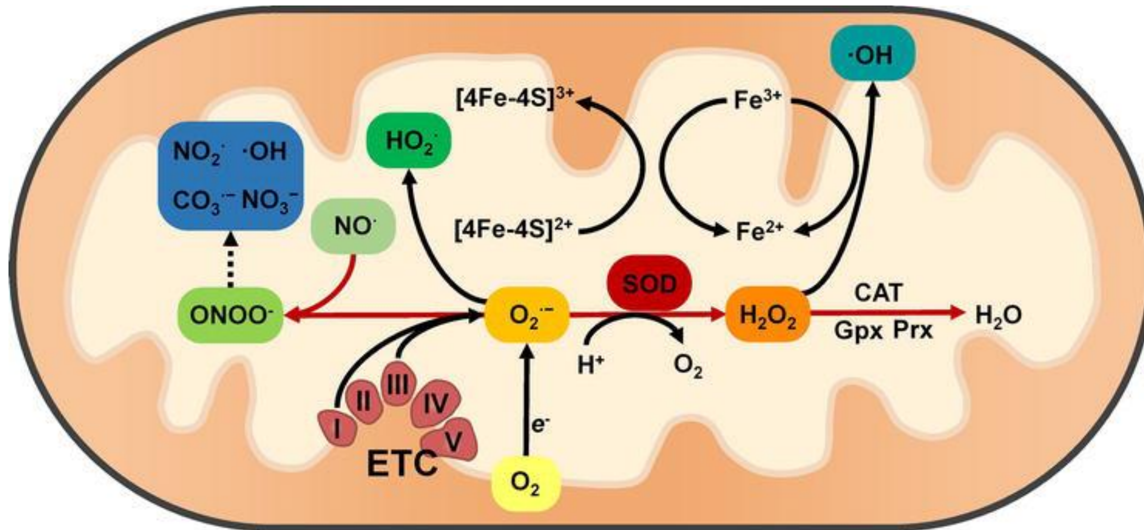


Figure 5.1: Mitochondrial ROS and antioxidant enzymes

Mitochondria generate ROS through complexes I and III of the ETC during oxidative phosphorylation. The Superoxide Dismutase converts superoxide to hydrogen peroxide. The catalase, and glutathione peroxidase convert hydrogen peroxide into water. Image adapted from (https://www.intechopen.com/chapters/69153)

The mitochondria, while performing oxidative phosphorylation, can generate reactive oxygen species (ROS) as a byproduct of the electron transport chain (ETC) or as a result of impaired mitochondrial functions (Thannickal and Fanburg 2000; Semenzato et al. 2008; Murphy 2012; Cid-Castro and Morán 2021). Thus mitochondria, being a major source of ROS generation, are more prone to be affected by ROS (Miyazono et al. 2002). These ROS are superoxide, hydroxyl, and hydrogen peroxide (Chance, Sies, and Boveris 1979; Murphy 2009; Ray, Huang, and Tsuji 2012; Holmström and Finkel 2014; Helmut Sies et al. 2022). ROS are known to oxidize biomolecules of the cell, including the lipids, proteins, and nucleotides, and causes damage to them (Perry et al. 2000; Nishikawa 2008; Ray, Huang, and Tsuji 2012; Holmström and Finkel 2014; Lisowski et al. 2018). ROS, a key secondary signaling molecule, regulates several cellular processes starting from cell proliferation to apoptosis (Holmström and Finkel 2014; Li et al. 2017;

Scialò et al. 2020; Graham et al. 2022). The role of ROS is thought to be involved in the fragmentation of the mitochondria too (Muliyil and Narasimha 2014). ROS are often associated with cytotoxic effects and impairment of cell polarity (Li et al. 2017; Hebbar and Knust 2021). The loss of polarity affects the cell architecture followed by cell shape and makes them more invasive in nature. Excessive ROS leads to tumorigenesis in many cancers and is responsible for their metastatic properties (Nishikawa 2008; F. Chen et al. 2021). Thus, the scavenging of ROS is also necessary for the maintenance of an optimal oxidizing environment inside the cell. To do that, a cell has a defense system that comprises several antioxidant enzymes including superoxide dismutase, catalase, peroxidase, glutathione peroxidase, etc (Das and Roychoudhury 2014). These enzymes have a unique role in quenching the ROS generated in the cell and the mechanism for it is shown in Figure 5.1. Mutating these enzymes involves an increase in ROS and causes detrimental or beneficial effects on the cells in a context-dependent manner. These differential effects of ROS for the cell remain unelucidated in the redox regulation of cellular events.

Several signaling pathways that regulate the polarity of the epithelial cell have components that can be activated or deactivated by the ROS. The protein tyrosine phosphatases (PTPs) can be regulated through the oxidation of Cysteine (Cys) residues present in their structure by ROS (Holmström and Finkel 2014). Recent research articles suggest a possible mechanism that participates in the organism's development, including *C.elegance*, *D.melanogaster*, Mouse, etc. Evolutionarily conserved MAPK signaling-associated components, including Extracellular signal-related kinases (ERK1/2), c-Jun N-terminal Kinase (JNK), p38, the big map kinase 1 (BMK1/ERK5) are regulated by oxidative stress (Ray, Huang, and Tsuji 2012). An epidermal growth factor (EGF), Platelet-Derived Growth Factor (PDGF), or insulin-stimulated production of H2O2 deactivates a phosphatase called Phosphatase and Tensin Homolog (PTEN) by oxidizing Cys residue resulting in the elevation of phosphatidylinositol triphosphate (PIP3) and regulating PI3K-Akt signaling in the cells (Kwon et al. 2004). Changing oxidizable Cys residue to the serene (Ser) in the nuclear respiratory factor2 (Nrf2) protein reduces the Nrf's ability to induce antioxidant gene induction, which proves that the Nrf2 oxidation regulates the quenching of the ROS in the cell (Bloom, Dhakshinamoorthy, and Jaiswal 2002). The cAMP response element binding protein (CREB) signaling regulates the hypoxia-related response of the cell in a redox-dependent regulation through its oxidizable Cys residues (Goren et al. 2001). Cytoskeletal remodeling via F

actin polymerization and calmodulin kinase II (CaMKII) are regulated through their Met residue (Erickson et al. 2008; Hung et al. 2013).

An excessive amount of ROS is detrimental to the cell, and hence the amount of ROS in the cell has to be taken care of by antioxidant systems, including antioxidant enzymes (H. Sies 1991; Ray, Huang, and Tsuji 2012). The quenching of ROS is accomplished by antioxidant enzymes such as Sod1, Sod2 (Mn-Sod), Catalase, Glutathione peroxidase, and Peroxidase. Although the role of ROS is correlated with polarity loss in several instances, the regulation of polarity by ROS is yet to be clarified in detail and opens a broad field of ROS biology to be studied (Li et al. 2017). The quenching of ROS is associated with polarity reprogramming in nonpolarized T4-2 cells (Li et al. 2017). Thus, ROS is an important molecule to be studied in context with the signaling pathways.

However, an optimal amount of ROS is required to promote several developmental events. Few observations have been made about ROS in the field to support ROS as a developmentally essential component for the organism (Gauron et al. 2016; Hung et al. 2013). Drp1-mediated fission reduces ROS and regulates myosin negatively during the cellularization of *Drosophila* embryogenesis (Chowdhary et al. 2020), whereas Mitochondrial ROS (mtROS) is helpful in the dorsal closure of the pupal development in *Drosophila* by regulating the adherens junctions and myosin activation (Hunter et al. 2018). Hydrogen peroxide regulates axonal growth in zebrafish development, modulating hedgehog signaling (Gauron et al. 2016). ROS can help Zebrafish regenerate their fins and heart and tail in Xenopus, helping in controlling compensation to catastrophic events (Gauron et al. 2013; Love et al. 2013). The regeneration process in planaria is preceded by the induction of ROS followed by the activation of the EGFR pathway (Jaenen et al. 2021). Mitochondrial ETC component complex I produce H2O2 that increases a lifespan via the stress adaptation pathway in *Drosophila (Scialò et al. 2020; Graham et al. 2022)*. Contrary to that, reducing ROS also increases the lifespan in *Drosophila* compromised for mitochondrial morphology regulator AarF domain-containing kinase 1 (dADCK1) where fused mitochondria are seen (W. Yoon et al. 2019). The contribution of ROS as a secondary messenger is well-known in the field as discussed earlier. Accumulation of ROS can damage or oxidize mitochondrial proteins, which can further translate into impaired mitochondrial functions. The functions of the ROS are not well characterized concerning the formation of epithelia and the establishment of the polarity complexes during the regeneration and normal development. And hence, we are trying to

understand how ROS can change mitochondrial morphology and affect the polarity of the epithelial cells.

5.2 Materials and methods

5.2.1 *Drosophila* genetics:

The fly stocks used are listed in section 2.1.2 of Chapter 2.

5.2.2 Follicle clone generation using MARCM method:

The mitotic recombination-based clone generation was performed using the MARCM method as described in section Chapter 2.

5.2.3 Immunostaining of *Drosophila* ovaries:

The procedure described for the immunostaining is in section 2.2.4, the list of antibodies is in section 2.1.2, and the dyes are in section 2.1.3 of Chapter 2.

5.2.4 Microscopy and image acquisition techniques:

The details for image acquisition are discussed in section 2.2.6 of Chapter 2.

5.3 Results

5.3.1 Analysis of mitochondrial ROS in follicle cells depleted for antioxidant enzymes

Opa1 depletion rescues the aPKC loss of Drp1 deficient follicle cells, as discussed in 4.3.1 (Figure 4.1) and 4.3.2 (Figure 4.2). Loss of Opa1 function increases mitochondrial ROS, and overexpression of Opa1 in synergy with the ATP synthase complex oligomerization reduces mitochondrial ROS (Tang et al. 2009; Jang and Javadov 2020; Quintana-Cabrera et al. 2021). Mitochondrial ROS (mtROS) was shown to be elevated in the Opa1-mutant eye cells in *Drosophila (Yarosh et al. 2008)*. We stained ovaries containing living mutant or control follicle cell clones with a fluorescent dye mitoSOX to detect mitochondrial superoxides in these cells. Depletion of Opa1 leads to an increase in the mitoSOX fluorescence in the follicle cell clones compared to their neighboring follicle cells (Figure 5.2). The ROS generated is quenched by antioxidant enzymes such as Sod1, Sod2, Catalase, and Glutathione peroxidase. We, therefore, use

a mutant of antioxidant enzymes to mimic a similar increase in ROS by depleting mitochondrial superoxide dismutase 2 (SOD2) (Kirby et al. 2002; Mukherjee et al. 2011; Celotto et al. 2012) and catalase using shRNA expression. The fluorescence intensity of mitoSOX is found to be higher in *sod2*^{i} and *cat*^{i} follicle cell clones as compared to neighboring cells. The intensity of ROS is measured on a rainbow scale (Figure 5.2).

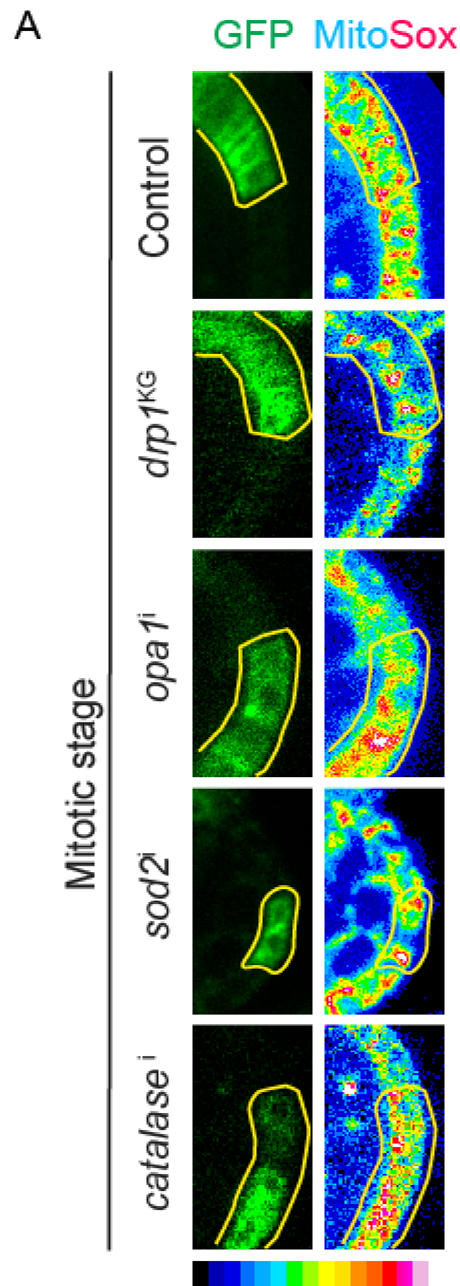


Figure 5.2: Mitochondrial ROS in mitochondrial morphology mutants of Drp1, Opa1, and in antioxidant enzyme mutants

Representative images showing the fluorescence intensity in a rainbow scale (red pixels are higher intensity and blue pixels are lower intensity) of MitoSOX in control FRT40A, *drp1*^{KG}, *opa1*^{i}, *sod2*^{i}, and *catalase*^{i}. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with solid or dashed yellow outlines.

5.3.2 Analysis of mitochondrial morphology in follicle cells depleted for antioxidant enzymes

Blocking antioxidant enzymes in the cell not only increases ROS but induces mitochondrial fragmentation. Sod2 depletion has also affected mitochondrial dynamics leading to fragmentation during *Drosophila* embryogenesis (Muliyil and Narasimha 2014). And therefore, we assess mitochondrial morphology in *sod2*^{i} and *cat*^{i} follicle cell clones by immunostaining for an antibody against ATP-β. Mitochondria are more punctate in appearance in the clone regions depleted of antioxidant enzymes sod2 and catalase as compared to their neighboring cells in the surface view of the follicle cells (Figure 5.3) and sagittal view of the follicle cells (Figure 5.5A).

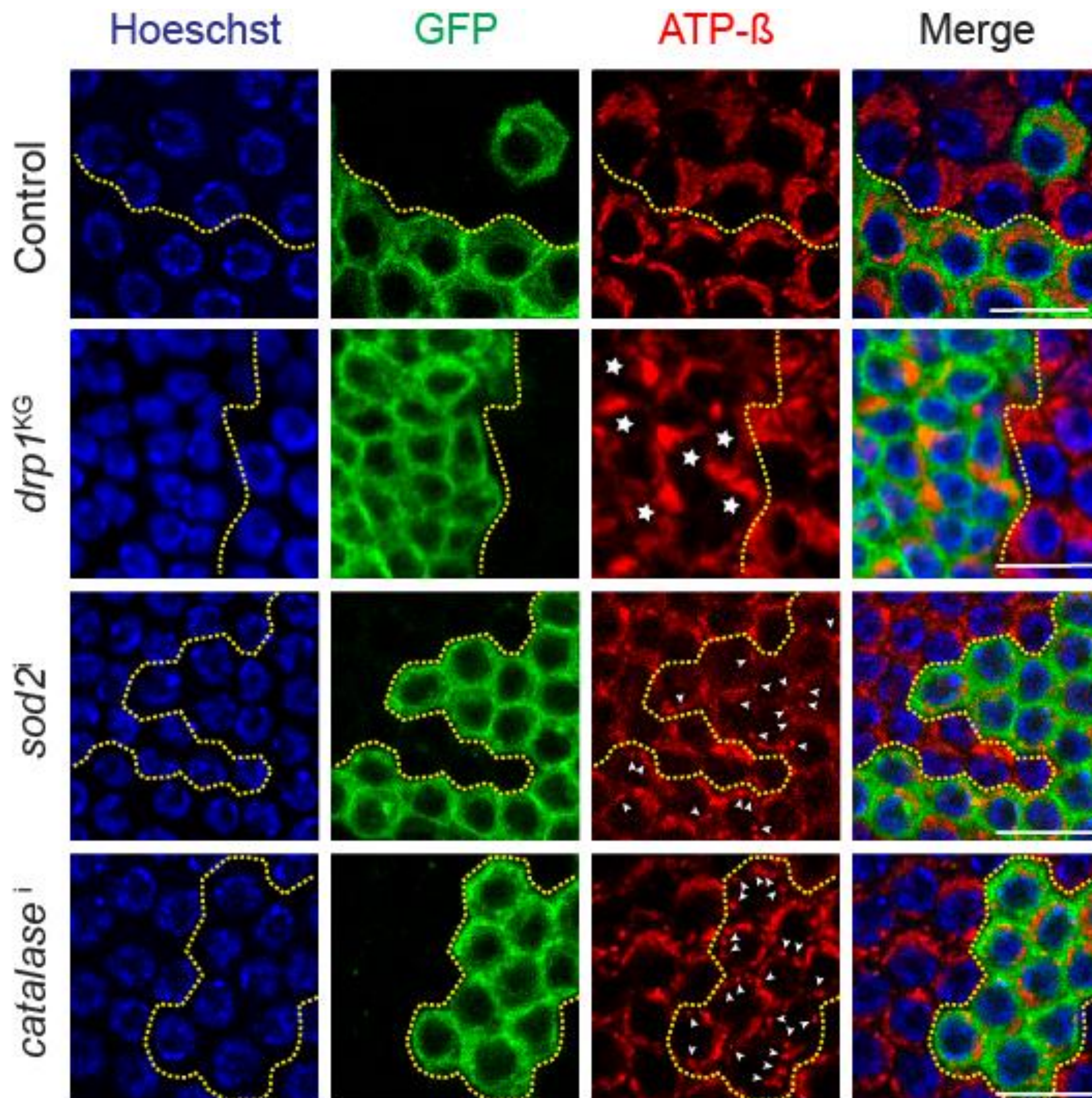


Figure 5.3: Mitochondrial morphology analysis in FCs depleted of Drp1 and ROS scavengers

Mitochondria stained with ATPβ (red) are shown in representative images from control FRT40A (100% intermediate, n=21), *drp1*^{KG} (92% more clustered, n=26), *sod2*^{i} (82% fragmented, n=28), and *catalase*^{i} (79% fragmented, n=14). mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. Asterisks mark clustered mitochondria and Arrowheads mark the punctate mitochondria in surface view. The nucleus (blue) is stained with Hoechst. Scale bar=10μm.

Further, the analysis of mitochondrial morphology in *drp1*^{KG};*sod2*^{i,} and in *drp1*^{KG};*cat*^{i} showed a relaxed mitochondrial network as compared to the compact mitochondrial network in Drp1 depleted follicle cells (Figure 5.4).

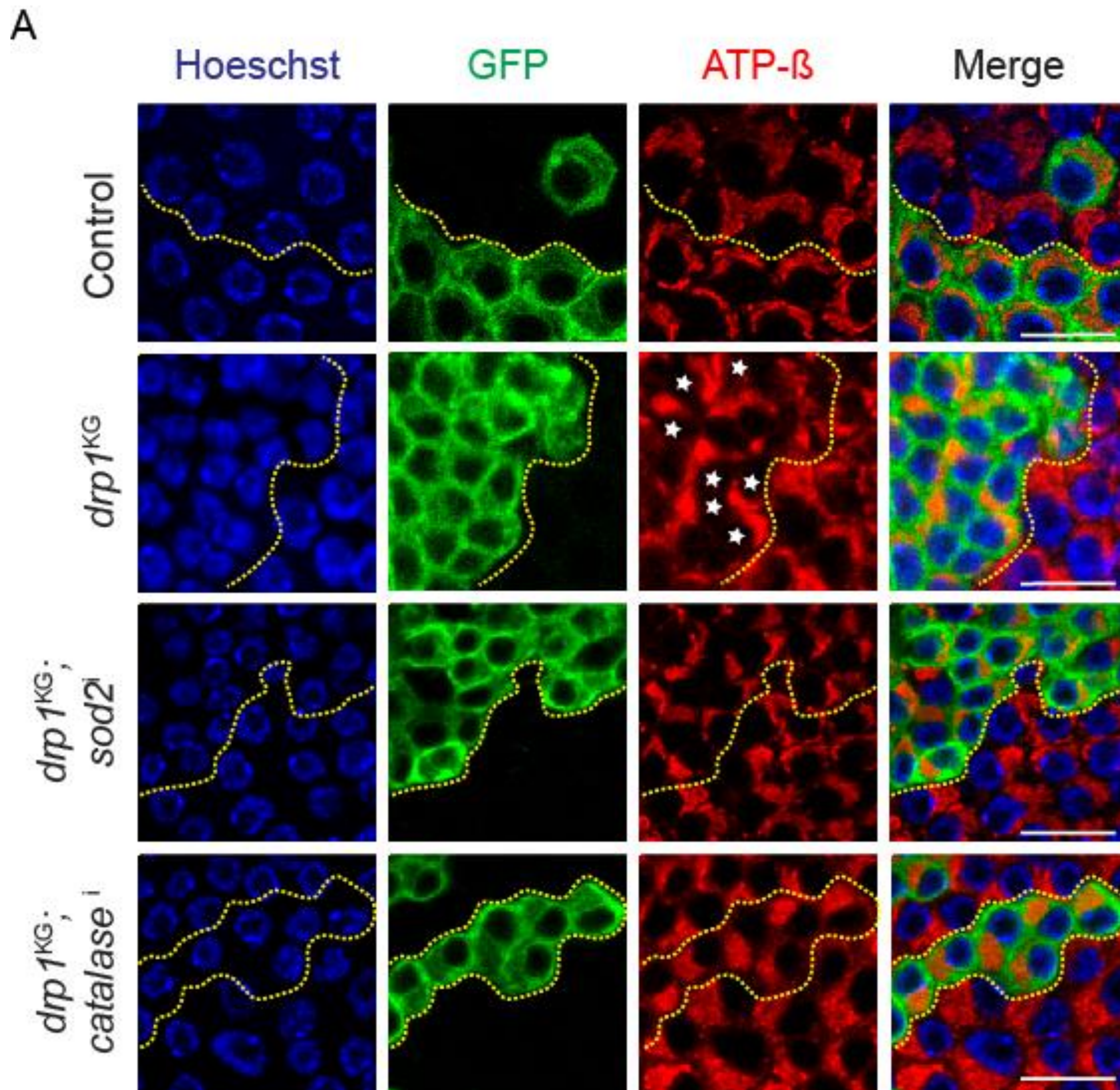


Figure 5.4: Mitochondrial morphology analysis in FCs depleted of Drp1 and FCs co-depleted for Drp1 and ROS scavengers

Representative images showing mitochondria stained with ATP-β (red) in FCs in the endocycling stage in control FRT40A (100% intermediate, n=21), *drp1*^{KG} (92% more clustered, n=26), *drp1*^{KG};*sod2*^{i} (92% less clustered, n=13) and *drp1*^{KG};*catalase*^{i} (79% less clustered, n=14). mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. Scale bar=10µm.

We have analyzed that the mitochondria remain clustered on one side of the follicle cells when Drp1 depleted in the follicle cells whereas *drp1*^{KG};*sod2*^{i} and in *drp1*^{KG};*cat*^{i} show scattered and more relaxed clusters in the follicle cells in sagittal view (Figure 5.5B).

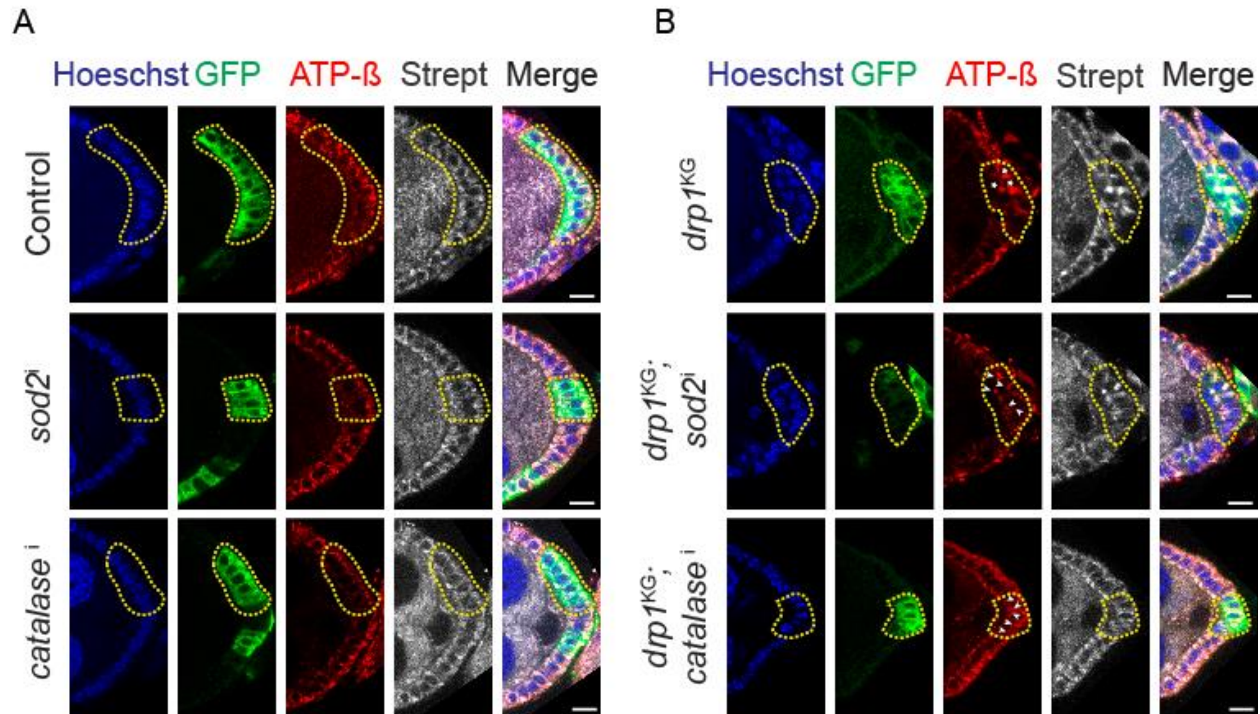


Figure 5.5: Mitochondrial morphology analysis in sagittal view of FCs depleted of Drp1 and FCs co-depleted for Drp1 and ROS scavengers

(A-B) Representative images showing mitochondria stained with ATP-β (red) in FCs in the sagittal view of endocycling follicle cells in control FRT40A (100% intermediate, n=11), *sod2*^{i} (72% fragmented, n=11), and *catalase*^{i} (71% fragmented, n=7) **(A)**. Mitochondrial morphology in *drp1*^{KG} (86% clustered, n=14), *drp1*^{KG};*sod2*^{i} (88% clustered with a decrease in compaction as compared to *drp1*^{KG} alone, n=8), and *drp1*^{KG};*catalase*^{i} (71% clustered with decrease in compaction as compared to *drp1*^{KG} alone, n=7) **(B)**. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. Arrowheads mark the relaxed mitochondria. The nucleus (blue) is stained with Hoechst. Scale bar=10μm.

Additionally, the loss of antioxidant enzymes in follicle cells mimics the increase in ROS that we see in *opa1*^{i} follicle cells. We observe that the combinations of *drp1*^{KG};*sod2*^{i} and in *drp1*^{KG};*cat*^{i} follicle cells rescue the multilayer defect and increased the clone area of *drp1*^{KG} too

(Figure 5.6). Thus, these results suggest that ROS can regulate mitochondrial morphology, which indirectly participates in the organization of epithelia during oogenesis.

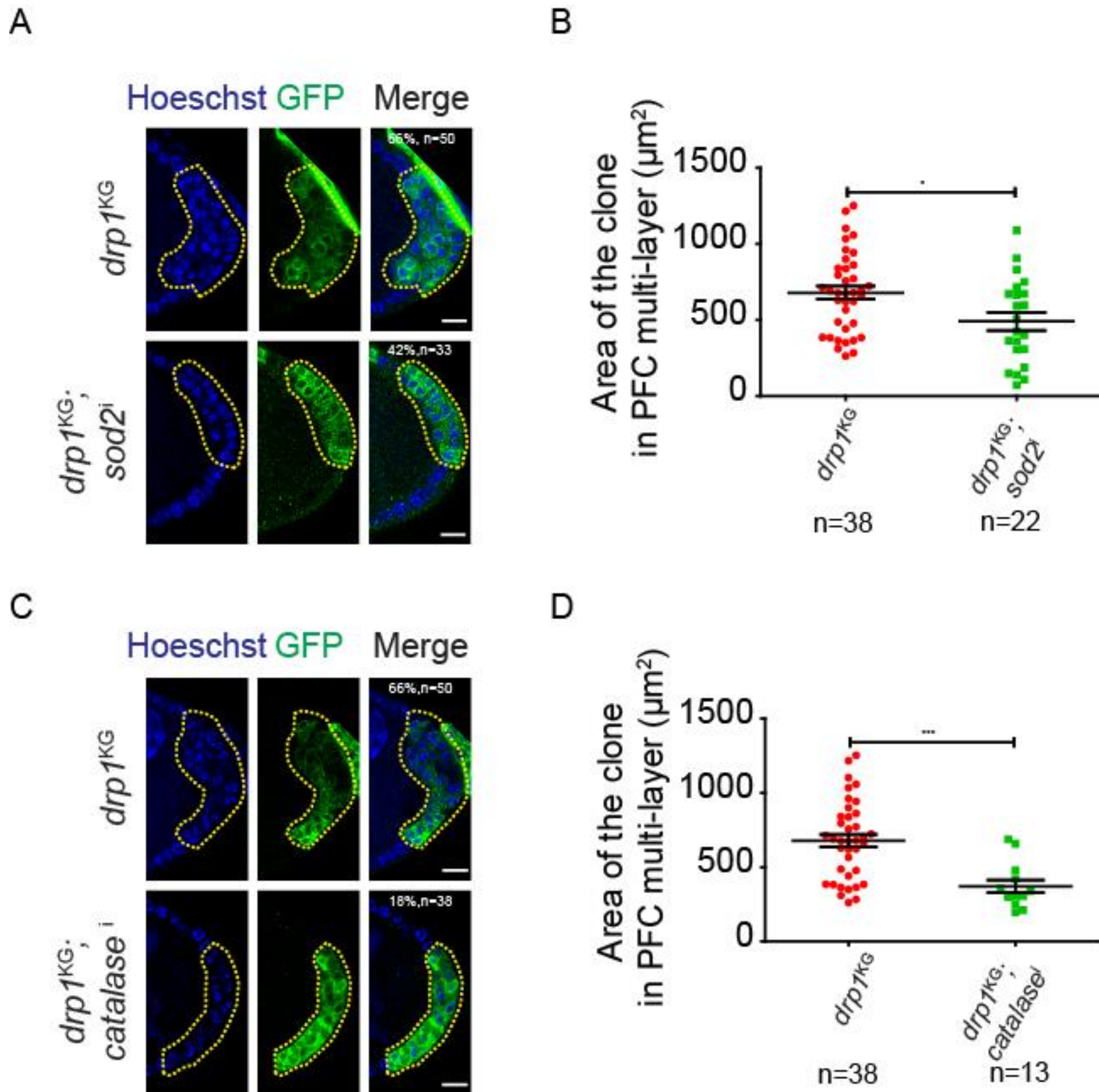


Figure 5.6: Multilayering in PFC clones of *drp1*^{KG} is reduced in *drp1*^{KG};*sod2*^{i}, and *drp1*^{KG};*catalase*^{i}
(A-D) Representative images of PFC clones in *drp1*^{KG} showing 3 or more cell layers per clone at a frequency of 66%,n=50, and the frequency of *drp1*^{KG};*sod2*^{i} is 42%,n=33 **(A)** and *drp1*^{KG};*catalase*^{i} is 18%,n=33 **(C)**. The graph shows a comparison of the PFC clone area of *drp1*^{KG} (n=38), *drp1*^{KG};*sod2*^{i} (n=22), and *drp1*^{KG};*catalase*^{i} (n=13) **(B, D)**. mCD8-GFP (green) expressing FC clones of the indicated

genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. The statistical test performed for clone area measurement is Student's t-test. ns=non significant, * P<0.05, ** P<0.01, *** P<0.001. Scale bar=10µm.

5.3.3 Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells depleted for the antioxidant enzyme in endocycling stages of *Drosophila* oogenesis

An increased amount of mitochondrial ROS and more fragmented mitochondria in follicle cells depleted for ROS-scavenging enzyme mutants are similar to those depleted for Opa1. In addition, the rescue of multilayer defects in double mutants of *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*cat*^{i} is similar to the rescue we see in *drp1*^{KG}*;opa1*^{i}. Therefore, We further analyze if depletion of ROS scavengers in Drp1 deficient follicle cells rescues the loss of aPKC seen in Drp1 deficient follicle cells in endocycling stages. As per the expectation, the level of aPKC in endocycling stage follicle clones of *drp1*^{KG};*sod2*^{i,} and *drp1*^{KG};*cat*^{i} increase on the apical membrane as compared to *drp1*^{KG} alone (Figure 5.7). This increase in aPKC was similar to that found in *drp1*^{KG}*;opa1*^{i} clones at endocycling stages (Figure 4.1). This partial rescue for the number of multilayers and loss of aPKC observed in *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*cat*^{i} suggests an important role of ROS in regulating the mitochondrial morphology and regulation of the apical polarity protein aPKC in follicle epithelial cells.

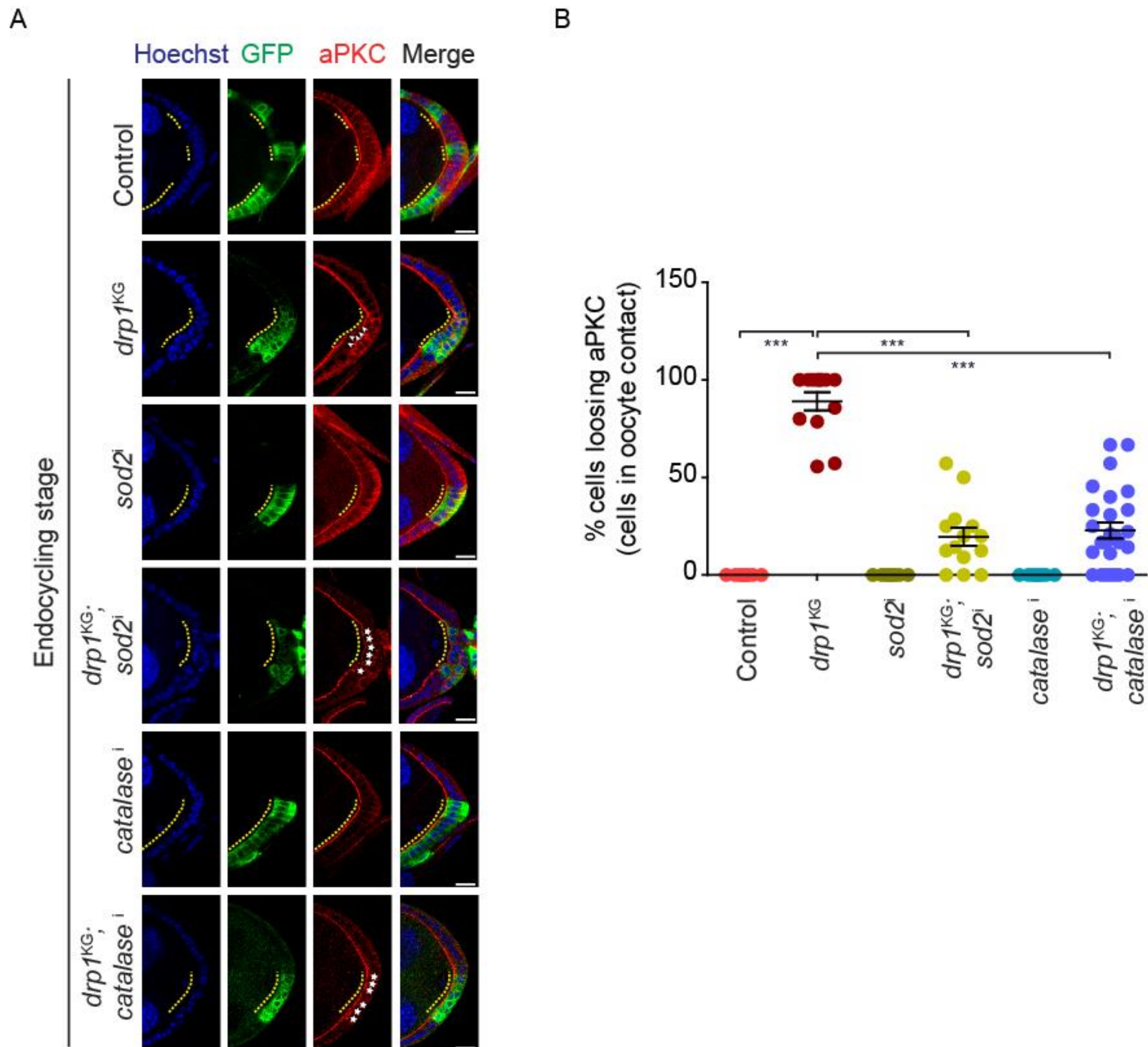


Figure 5.7: Drp1 mutant follicle cell clones show loss of aPKC in endocycling stages and are partially recovered in $drp1^{KG}$;$sod2^{i}$ and $drp1^{KG}$;$catalase^{i}$

(A-B) Representative images showing aPKC (red) staining in control FRT40A, $drp1^{KG}$, $sod2^{i}$, $catalase^{i}$,$drp1^{KG}$;$sod2^{i}$ and $drp1^{KG}$;$catalase^{i}$ PFC clones **(A)**. The graph shows quantification of the percentage of cells losing aPKC in each PFC clone adjacent to the oocyte in control FRT40A, $drp1^{KG}$, $sod2^{i}$, $catalase^{i}$,$drp1^{KG}$;$sod2^{i}$ and $drp1^{KG}$;$catalase^{i}$ **(B)**. Representative images showing aPKC (red) staining in control FRT40A, $drp1^{KG}$, $sod2^{i}$, $catalase^{i}$,$drp1^{KG}$;$sod2^{i}$ and $drp1^{KG}$;$catalase^{i}$ FC clones in mitotic stages **(C)**. The graph shows the quantification of the percentage of cells losing aPKC in each FC clone in mitotic stages in control FRT40A, $drp1^{KG}$, $sod2^{i}$, $catalase^{i}$,$drp1^{KG}$;$sod2^{i}$ and $drp1^{KG}$;$catalase^{i}$ **(D)** mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The

nucleus (blue) is stained with Hoechst. White arrowheads mark FCs deficient in aPKC. White asterisks mark FCs that show the presence of aPKC in double mutants. The graphs show data in Mean±SEM. Each data point (n) in the endocycling and mitotic stages represents the percentage of cells losing aPKC in a single clone of a separate egg chamber. The n values of the genotypes control FRT40A, *drp1*^{KG}, *sod2*^{i}, *drp1*^{KG};*sod2*^{i}, *catalase*^{i,} and *drp1*^{KG};*catalase*^{i} given as (n, N; n is the number of clones, N is the number of independent replicates) (n=12,13,9,14,13,26 and N=3,5,3,3,3,3 respectively) for endocycling stages. The statistical test performed is One way ANOVA with Tukey's multiple comparisons. ns=non significant, * P<0.05, ** P<0.01, *** P<0.001, Scale bar=10μm.

5.3.4 Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells depleted for the antioxidant enzyme in mitotic stages of *Drosophila* oogenesis

We observe a rescue in terms of recovery of aPKC back on the apical membrane in double mutants *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*cat*^{i} as compared to Drp1 depleted follicle cells in the endocycling stage as discussed in Figure 5.7. We further expect a similar rescue in the mitotic stage too and hence we immunostain the follicle cells with aPKC antibody. In mitotic stages. As per the expectation, the level of aPKC in mitotic and endocycling stage follicle clones of *drp1*^{KG};*sod2*^{i}, and *drp1*^{KG};*cat*^{i} increase on the apical membrane as compared to *drp1*^{KG} alone (Figure 5.8). This increase in aPKC was similar to that found in *drp1*^{KG}*;opa1*^{i} clones (Figure 4.2). This partial rescue for the number of multilayers and loss of aPKC observed in *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*cat*^{i} suggests an important role of ROS in regulating the mitochondrial morphology and regulation of the apical polarity protein aPKC in follicle epithelial cells.

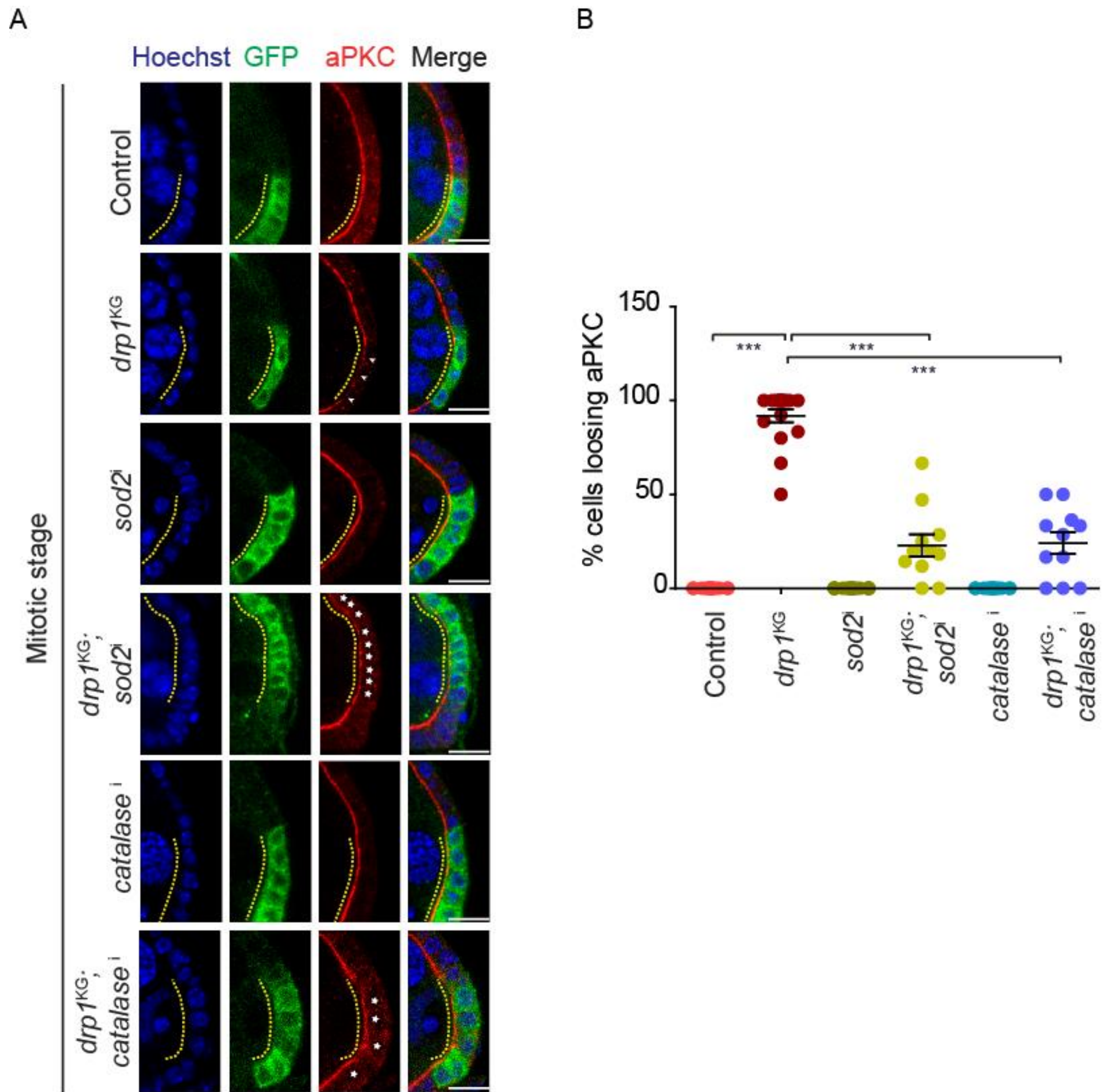


Figure 5.8: Drp1 mutant follicle cell clones show loss of aPKC in mitotic stages and are partially recovered in *drp1*^{KG}*;sod2*^{i} and *drp1*^{KG};*catalase*^{i}

(A-B) Representative images showing aPKC (red) staining in control FRT40A, *drp1*^{KG}, *sod2*^{i}, *catalase*^{i},*drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*catalase*^{i} PFC clones **(A)**. The graph shows quantification of the percentage of cells losing aPKC in each PFC clone adjacent to the oocyte in control FRT40A, *drp1*^{KG}, *sod2*^{i}, *catalase*^{i},*drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*catalase*^{i} **(B)**. Representative images showing aPKC (red) staining in control FRT40A, *drp1*^{KG}, *sod2*^{i}, *catalase*^{i},*drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*catalase*^{i} FC clones in mitotic stages **(C)**. The graph shows the quantification of the percentage of cells losing aPKC in each FC clone in mitotic

stages in control FRT40A, *drp1*^{KG}, *sod2*^{i}, *catalase*^{i} ,*drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*catalase*^{i} **(D)** mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. White arrowheads mark FCs deficient in aPKC. White asterisks mark FCs that show the presence of aPKC in double mutants. The graphs show data in Mean±SEM. Each data point (n) in the endocycling and mitotic stages represents the percentage of cells losing aPKC in a single clone of a separate egg chamber. The n values of the genotypes control FRT40A, *drp1*^{KG}, *sod2*^{i}, *drp1*^{KG};*sod2*^{i}, *catalase*^{i,} and *drp1*^{KG};*catalase*^{i} given as (n, N; n is the number of clones, N is the number of independent replicates) being (n=17,17,26,11,15,11 and N=3,5,3,3,3,3 respectively) for mitotic stages. The statistical test performed is One way ANOVA with Tukey's multiple comparisons. ns=non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Scale bar=10µm.

5.4 Discussion

We describe the formation and the functions of ROS in the introduction of this chapter. Later on, we try to understand the importance of ROS in the regulation of mitochondrial shape. The fragmentation of the mitochondria because of ROS or oxidative stress is evidenced in several papers (Muliyil and Narasimha 2014; Tsushima et al. 2018). Any impairment to the mitochondrial functions or disturbance to the defense system of the cell elevates ROS (Willems et al. 2015). Many times, the ROS in the cell is detrimental to its survival but the physiological level of ROS does not cause apoptosis (Zhou, Shao, and Spitz 2014). In our study, we see a fragmentation of the mitochondria when antioxidant enzymes Sod2 or Catalase are suppressed in the follicle cells. Moreover, these mutants increase the ROS (Figure 5.2), detected with the help of mitoSOX dye, which could be the reason for fragmented mitochondria as reported in previous literature in the field (Muliyil and Narasimha 2014). Literature suggests that the fragmentation of the mitochondria through ROS involves the fusion proteins as a target. The oxidation of the Opa1 protein leads to the fragmentation of the mitochondria when ROS is increased (Semenzato et al. 2008). Additionally, the fusion proteins Opa1 and Marf need to be on the mitochondrial membrane for their activity. The increase in ROS releases Opa1 from the mitochondrial membrane and causes the fragmentation of the mitochondria in HT22 cells (Sanderson, Raghunayakula, and Kumar 2015). Thus, the fragmentation of mitochondria in cells, where the functions of antioxidant enzymes are blocked, could be because of the ROS affecting fusion proteins and their localization (Figure 5.3).

The ROS in the cell is associated with the alteration of polarity components. The observations in CaCO2 and ECV304 cells indicate that the ROS can impair the tight junction (TJ) assemblies of the cells (N. Wang et al. 2012; A. Seth et al. 2008). Additionally, ROS control PKC activation in human peritoneal mesothelial cells (HPMC) (H. B. Lee et al. 2004). The further results of reduction of multilayering in double mutants *drp1*^{KG};*sod2*^{i} and *drp1*^{KG}; *cat*^{i} (Figure 5.6A, C) are very much similar to the *drp1*^{KG}*;opa1*^{i} (Figure 3.2C). The loss of aPKC in the Drp1 mutant is also rescued using double mutants of *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*cat*^{i} (Figure 5.7-5.8) which is again similar to what we observe in *drp1*^{KG}*;opa1*^{i} (Figure 4.1-4.2). The fragmented mitochondrial morphology in mutants *sod2*^{i} and *cat*^{i} might involve the suppression of the Opa1 protein which needs to be tested further. The Drp1 is also activated by the oxidative stress in the cell and causes mitochondrial fragmentation but the double mutants used here are deficient for the Drp1 and can not be a cause of fragmentation here (Cid-Castro and Morán 2021). The oxidation of the Opa1 protein remains a question to be addressed further in these mutants. The activation of apoptosis in the cell can result from excessive ROS, but an appropriate increase in ROS can also have various impacts on the cell. In the case of elevated ROS in the Drp1 mutant, we see a rescue of multilayered cells, and loss of aPKC so far, and further investigation of signaling pathways is to be done in the next chapter.

Chapter 6: Study of signaling pathways responsible for follicle cell polarity and differentiation, including EGFR and Notch signaling pathways during *Drosophila* oogenesis

6.1 Introduction

Drosophila oogenesis is a process where there are a lot of cellular events that happen in the context of follicle epithelial cell development, as discussed in section 2.2.1 of chapter 2. These cellular events include follicle stem cell differentiation, self-renewal of follicle stem cells, polarity establishment of the follicle epithelial cells, change in cell morphology, apoptosis of the follicle epithelial cells (Wasserman and Freeman 1998; Dobens and Raftery 2000; Castanieto, Johnston, and Nystul 2014). During these developmental events, follicle stem cells and follicle epithelial cells show unique signaling pathways regulating these cellular events. EGFR signaling is one of such pathways that is required for the FSCs maintenance and differentiation of the FSCs to pre-follicle cells. EGFR signaling also regulates the morphology of FSCs by negatively controlling the formation of an apical domain via suppressing an apical domain protein aPKC (Castanieto, Johnston, and Nystul 2014). The same EGFR signaling pathway, through EGF receptor and Gurken ligand interaction, regulates oocyte positioning during the later stages of development (Neuman-Silberberg and Schüpbach 1996; Nilson and Schüpbach 1998; Wasserman and Freeman 1998). Later during development, EGFR signaling helps form respiratory appendages (Wasserman and Freeman 1998). To operate the EGFR signaling in flies, the EGF receptor requires four different types of ligands, including Gurken, Spitz, Vein, and Keren (Lusk, Lam, and Tolwinski 2017). These ligands are broadly categorized into 1) Transforming growth factor (TGF) -α ligands that are Gurken, Spitz, and Keren, and 2) a single neuregulin-like ligand that is Vein (Rutledge et al. 1992; Schnepp et al. 1996; Steinhauer et al. 2013).

The EGFR signaling starts with receptor dimerization and cross phosphorylation followed by activation of Downstream Receptor kinase (DRK). The son of sevenless (SOS), along with DRK, activates Ras which further activates Raf. Raf further activates mitogen-activated protein kinase kinase/extra cellular-related kinase (MAPKK/MEK) through phosphorylation which further activates mitogen-activated protein kinase/extra cellular-related kinase (MAPK/ERK) by phosphorylation. In *Drosophila*, rolled is an example of MAPK/ERK, and its double phosphorylated form (dpERK) can be used as a readout of activation of EGFR signaling pathway

as shown in (Figure 6.1) (Lusk, Lam, and Tolwinski 2017). The dpERK enters the nucleus and initiates a gene expression of related genes (Figure 6.1).

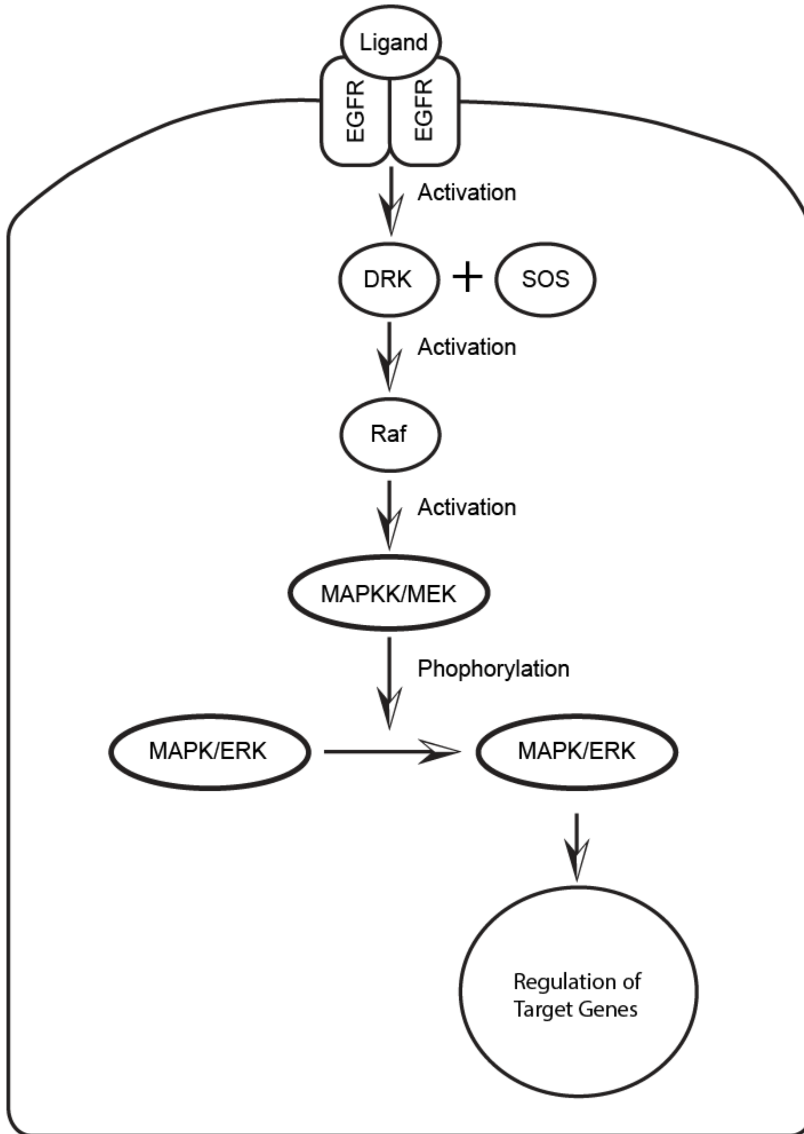


Figure 6.1: Mechanism of EGFR signaling

The Delta-ligand of the signal-sending cell interacts with the Notch receptor on the signal-receiving cell. The Notch Intracellular-Domain (NICD) is released into the cytoplasm when the Notch receptor is proteolytically cleaved. Target genes are controlled by the NICD once it enters the nucleus.

The EGFR signaling pathway discussed above plays an essential role during various cellular events, including stem cell maintenance, cell proliferation, cell differentiation, cell death, etc (C. Zhang et al. 2022). Along with that, these pathways also regulate mitochondrial

morphology and interact with mitochondria (Tomer et al. 2018). The phosphorylation of EGFR on Tyr-845 enables binding to cytochrome C oxidase subunit II in cell line study (Demory et al. 2009). EGFR is also observed to be higher in invasive non-small-cell lung cancer (NSCLC) cells where EGFR moves to mitochondria and induces fission and increases ATP to make cells more migratory (Che et al. 2015; T.-H. Wang et al. 2017). Even, the translocation of EGFR to mitochondria is also reported to escape apoptosis in cancer cell lines (Cao et al. 2011). EGFR regulates mitochondrial potential and mitochondrial morphology in *Drosophila* ovarian follicle cells depleted for pro-fission Drp1 protein (Tomer et al. 2018). Recently, EGFR has been shown to promote *Drosophila* intestinal stem cell maintenance by increasing β-oxidation, which further increases mitochondrial potential (C. Zhang et al. 2022). In the last two studies, the mitochondrial potential and EGFR are interlinked and are positively regulated by each other. It will be interesting to reveal if this mechanism is being operated in every tissue.

Besides EGFR signaling, the Notch signaling in metazoans plays an important role in cell proliferation, cell differentiation, and stem cell maintenance. It is a highly conserved signaling pathway in the animal kingdom (Artavanis-Tsakonas, Rand, and Lake 1999; Baonza and Garcia-Bellido 2000; Guo and Ohlstein 2015). During oogenesis in *Drosophila*, the transition of follicle cells from mitotic to the endocycling stage is marked with the appearance of Hindsight (Hnt) protein- a readout of Notch signaling (X. Wang, Adam, and Montell 2007). Notch signaling is also required in later stage9 for the border cell migration (X. Wang, Adam, and Montell 2007). The same signaling pathway helps in cuboidal to stretched squamous epithelium transition in later stages 8-10a of *Drosophila* oogenesis (Jia et al. 2022). The self-renewal process of intestinal stem cells in *Drosophila* is regulated by the Notch signaling (Ohlstein and Spradling 2007).

The Notch signaling gets activated when Receptor-Ligand interactions happen. Notch receptors on one cell interact with Delta, Serrate, and Lag-2 (DSL) family ligands of neighboring cells (Baonza and Garcia-Bellido 2000; D'Souza, Meloty-Kapella, and Weinmaster 2010; Lieber, Kidd, and Struhl 2011). Upon interaction of the Notch receptor and its ligand, the Notch receptor is cleaved to release Notch Intracellular Domain (NICD) which further goes into the nucleus and binds to DNA binding proteins CBF1, Su(H), LAG1 (CSL) to activate the transcription of downstream genes (D'Souza, Meloty-Kapella, and Weinmaster 2010; Kopan 2012; L. Chen et al. 2018). The signal-receiving cell with Notch receptor present on the surface and signal-sending cell with ligands present on the cell participate in the signaling pathway. The Fate of the signal-

receiving and signal-sending pathway is decided by this receptor-ligand interaction. The mechanism is shown in schematics (Figure 6.2). The Cis and Trans activation concept of Notch signaling makes Notch signaling inactive in one cell and active in another cell as shown in (Figure 6.2).

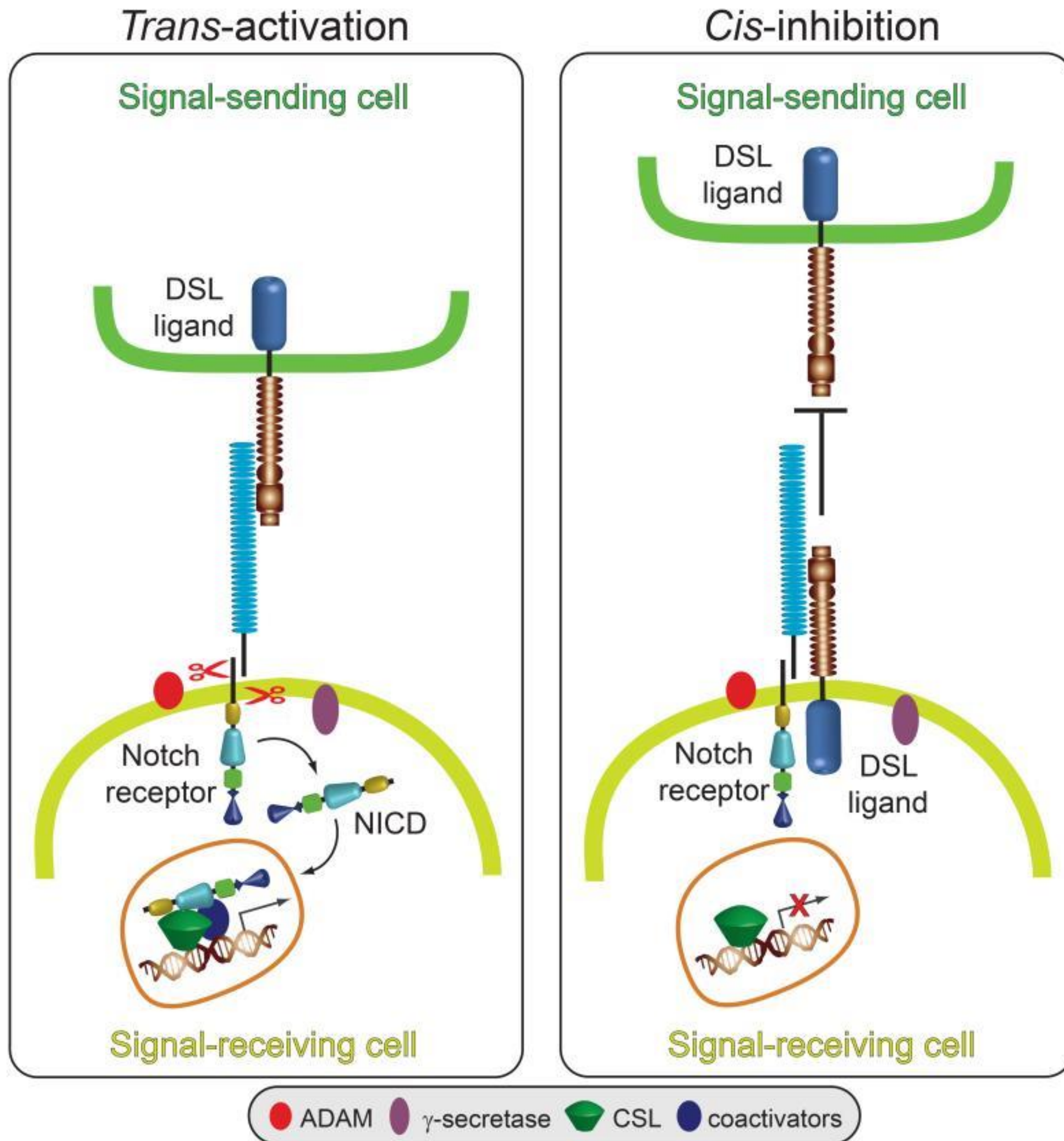


Figure 6.2: Mechanism of Notch signaling and Trans/Cis activation

In Trans activation, the Notch receptor on the signal-receiving cell interacts with the Delta-ligand of the signal-sending cell. The Notch receptor is cleaved proteolytically to release the Notch Intracellular-Domain (NICD) in the cytoplasm. The NICD enters the nucleus and regulates the target genes. In Cis activation, the Ligand and receptors of the same cells interact suppressing the Notch activation. Image adapted from (D'Souza, Meloty-Kapella, and Weinmaster 2010)

Notch signaling also interacts with the mitochondrial morphology proteins and regulates mitochondrial morphology. There is a positive feedback mechanism between Notch signaling and Drp1 mediated fission of the mitochondria in triple-negative breast cancer cells (TNBC) and confers cell survival and proliferation (L. Chen et al. 2018). Ablation of the fusion proteins Marf1 and Opa1 in embryonic stem cells (ESCs) impairs the differentiation into cardiomyocytes by increasing Notch1 signaling (Kasahara et al. 2013). Similar results were observed in Drp1 depleted *Drosophila* follicle cells where defective Notch signaling was observed (Mitra et al. 2012; Tomer et al. 2018). Contrary to the above literature, a study in type-II neuroblast in *Drosophila* shows decreased Notch signaling upon blocking Opa1 function (Dubal et al. 2022). Thus, the role of mitochondrial morphology and Notch signaling in the differentiation process remains poorly understood. Here, we have attempted to check the mechanism of the Notch in the Drp1 depleted *Drosophila* follicle cells and the double mutant combinations where we could see a rescue in the formation of multiple layers, and loss of aPKC as discussed in chapters 3,4, and 5.

6.2 Materials and methods

6.2.1 *Drosophila* genetics:

The fly stocks used are listed in section 2.1.2 of Chapter 2.

6.2.2 Follicle clone generation using MARCM method:

The mitotic recombination-based clone generation was performed using the MARCM method described in Chapter 2.

6.2.3 Immunostaining of *Drosophila* ovaries:

The procedure described for the immunostaining is in section 2.2.4, the list of antibodies is in section 2.1.2, and the dyes are in section 2.1.3 of Chapter 2.

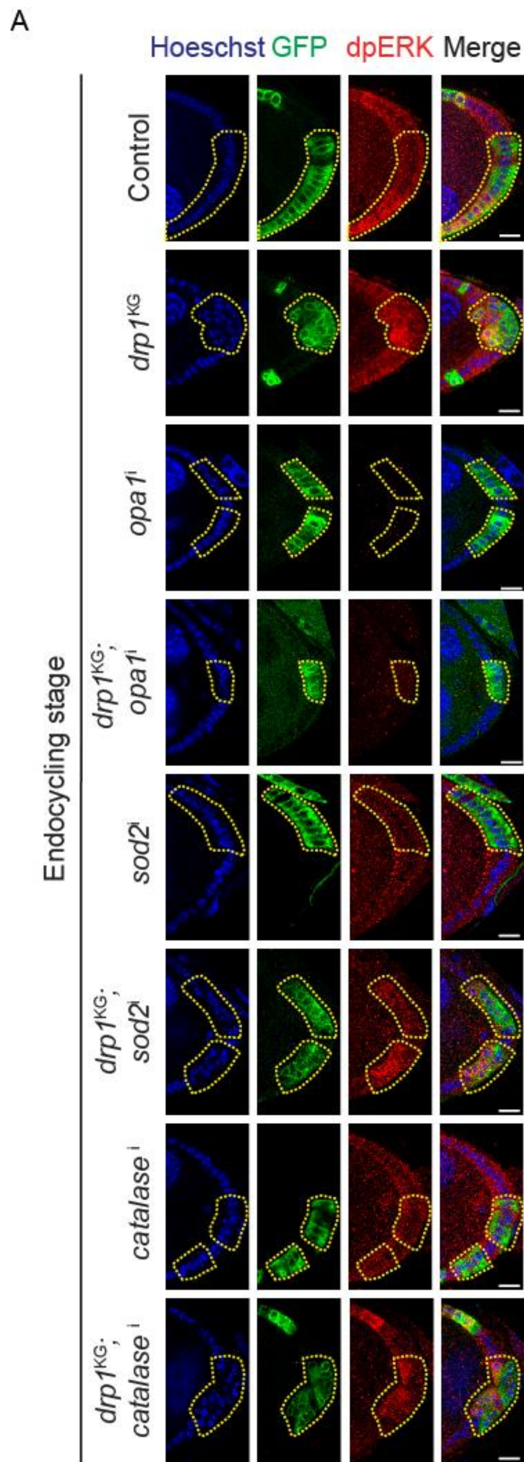
6.2.4 Microscopy and image acquisition techniques:

The details for image acquisition are discussed in section 2.2.6 of Chapter 2.

6.3 Results

6.3.1 Analysis of EGFR signaling in follicle cells depleted for mitochondrial morphology proteins and antioxidant enzymes in endocycling stages of *Drosophila* oogenesis

The follicle stem cells in the germarium show increased dpERK as an EGFR signaling read out and absence of apical polarity protein aPKC. EGFR signaling has been previously shown to be elevated in ovarioles which contain follicle cell clones depleted of Drp1 (Tomer et al. 2018). In addition to that, we show that an apical polarity protein aPKC is lost from these $drp1^{KG}$ mutant cells in endocycling stages, which is partially rescued in double mutants of $drp1^{KG};opa1^{i}$, $drp1^{KG};sod2^{i}$, and $drp1^{KG};cat^{i}$ as discussed in 4.3.1 (Figure 4.1) and 5.3.3 (Figure 5.7). From these observations, we want to assess if an increased dpERK level (EGFR signaling) of $drp1^{KG}$ follicle cells gets reduced in these double mutants. We find that the follicle cells of these double mutants show a reduction in dpERK level in the endocycling stages (Figure 6.3). Thus, the increased EGFR signaling in endocycling follicle cells could be responsible for the loss of aPKC from the multilayered epithelium of $drp1^{KG}$ follicle cells.



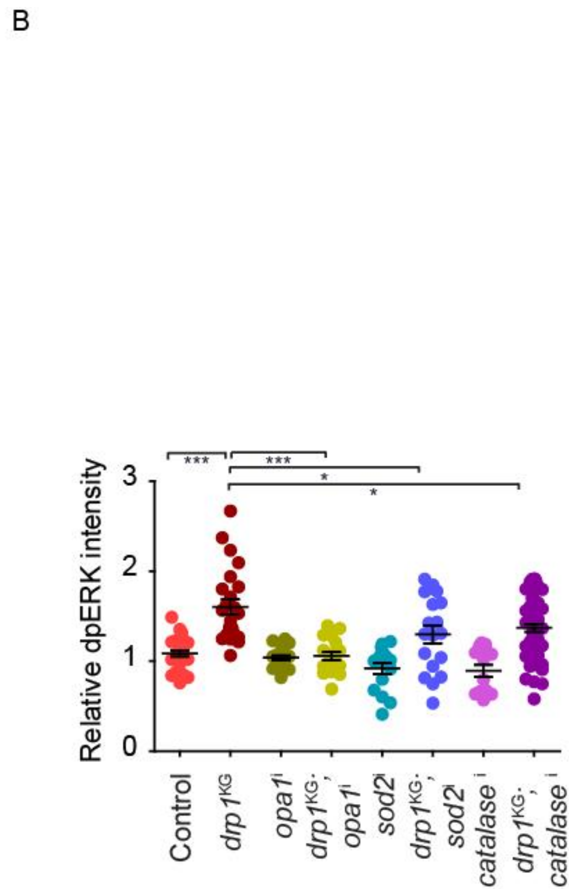


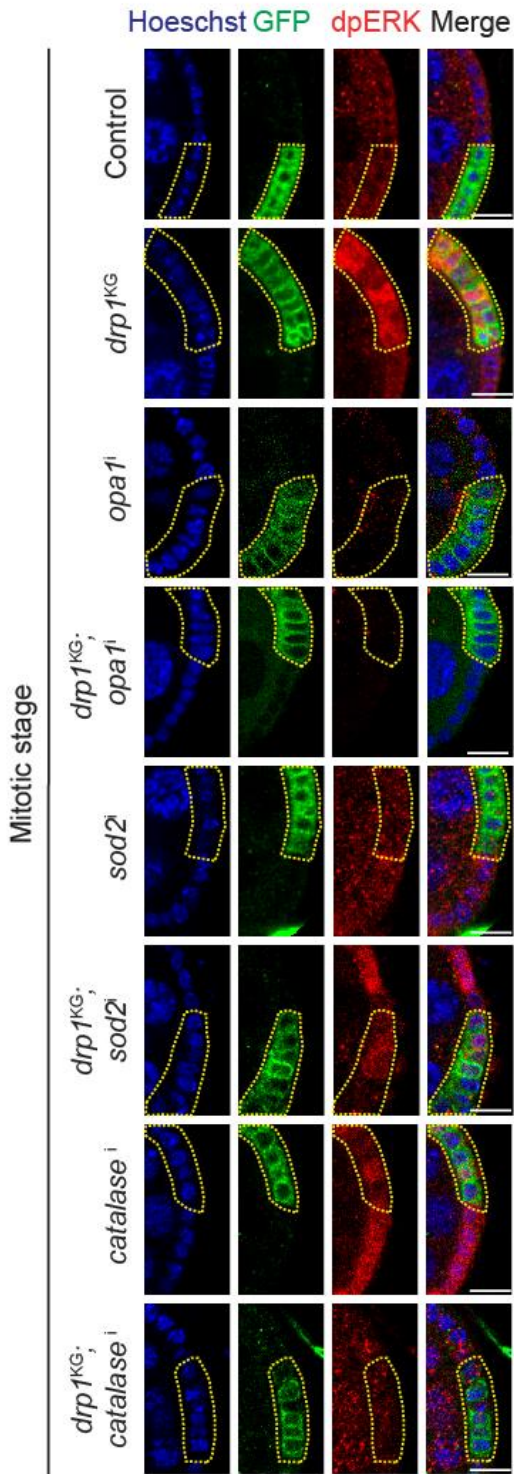
Figure 6.3: Increase in dpERK in Drp1 depleted clones is rescued on additional depletion of Opa1 and ROS scavengers at the endocycling stage

(A-B) Representative images showing dpERK (red) in control FRT40A, *drp1*^{KG}, *opa1*^{i}, *sod2*^{i}, *catalase*^{i}, *drp1*^{KG};*opa1*^{i}, *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*catalase*^{i} PFC clones in endocycling stages **(A)**. The graph shows

the quantification of relative dpERK fluorescence compared to neighboring control cells in control FRT40A, *drp1*^{KG}, *opa1*^{i}, *sod2*^{i}, *catalase*^{i}, *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG};*sod2*^{i}, and *drp1*^{KG};*catalase*^{i} PFC clones **(B)**. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. The data of each genotype is presented with its respective mean and SEM. The graphs show data in Mean+SEM. Each data point (n) in the endocycling and mitotic stages represents the relative fluorescence intensity of dpERK from a single clone from a separate egg chamber. The n values for genotypes control FRT40A, *drp1*^{KG}, *opa1*^{i}, *drp1*^{KG}*;opa1*^{i}, *sod2*^{i}, *drp1*^{KG};*sod2*^{i}, *catalase*^{i}, and *drp1*^{KG};*catalase*^{i} are given as (n, N; n is the number of clones, N is the number of independent replicates) (n=25,24,20,18,16,18,14,55 and N=4,4,3,4,3,3,3,6 respectively) for endocycling stages. ns=non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Scale bar=10μm.

6.3.2 Analysis of EGFR signaling in follicle cells depleted for mitochondrial morphology proteins and antioxidant enzymes in mitotic stages of *Drosophila* oogenesis

The elevated dpERK (EGFR signaling) in multilayered *drp1*^{KG} follicle cells is responsible for the loss of aPKC (Figure 6.3). The *drp1*^{KG} follicle cells in the mitotic stage, despite being arranged in a monolayer, show the loss of apical polarity protein aPKC. This loss of aPKC is partially rescued in double mutants of *drp1*^{KG}*;opa1*^{i} , *drp1*^{KG}*;sod2*^{i} and *drp1*^{KG}*;cat*^{i} as discussed in 4.3.2 (Figure 4.2) and 5.3.4 (Figure 5.8). From these observations, we hypothesize that an increased EGFR signaling is responsible for the loss of aPKC in mitotic stage follicle cells of *drp1*^{KG} too. We also see aPKC getting rescued in double mutants, so we expect the level of dpERK to be reduced in these double mutants. As per expectation, we find that the follicle cells of these double mutants show a reduction in dpERK level in mitotic stages (Figure 6.4). Thus, it is possible that the increased EGFR signaling in mitotic follicle cells could be responsible for the loss of aPKC from monolayered epithelium *drp1*^{KG} follicle cells.



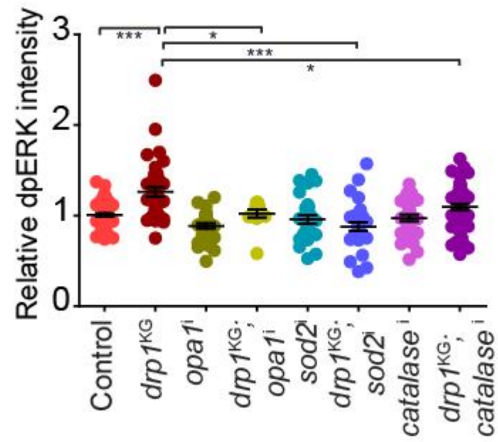


Figure 6.4: Increase in dpERK in Drp1 depleted clones is rescued on additional depletion of Opa1 and ROS scavengers at mitotic stage

(A-B) Representative images showing dpERK (red) in control FRT40A, *drp1*^{KG}, *opa1*^{i}, *sod2*^{i}, *catalase*^{i}, *drp1*^{KG};*opa1*^{i}, *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*catalase*^{i} FC clones in mitotic stages **(C)**. The graph shows the

quantification of relative dpERK fluorescence compared to neighboring control cells in control FRT40A, *drp1*^{KG}, *opa1*^{i}, *sod2*^{i}, *catalase*^{i}, *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG};*sod2*^{i} and *drp1*^{KG};*catalase*^{i} FC clones in mitotic stages **(D).** mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. The data of each genotype is presented with its respective mean and SEM. The graphs show data in Mean+SEM. Each data point (n) in the endocycling and mitotic stages represents the relative fluorescence intensity of dpERK from a single clone from a separate egg chamber. The n values for genotypes control FRT40A, *drp1*^{KG}, *opa1*^{i}, *drp1*^{KG}*;opa1*^{i}, *sod2*^{i}, *drp1*^{KG};*sod2*^{i}, *catalase*^{i}, and *drp1*^{KG};*catalase*^{i} are given as (n, N; n is the number of clones, N is the number of independent replicates) being (n=52,40,31,12,27,30,30,47 and N=5,4,2,4,3,3,2,5 respectively) for mitotic stages. ns=non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Scale bar=10μm.

6.3.3 Analysis of EGFR signaling in follicle cells depleted for Erk, a component of EGFR signaling pathway, in endocycling and mitotic stages of *Drosophila* oogenesis

An increased EGFR signaling in follicle cells depleted for fission protein Drp1 could be one of the mechanisms behind the loss of an apical polarity protein aPKC in the mitotic and endocycling stages (Figure 6.3,6.4). To prove that the aPKC is lost when dpERK is higher in the Drp1 mutant, we deplete ERK protein using *erk*^{i} in Drp1 mutant follicle cells. As per expectation, follicle cells of *erk*^{i} alone and a double mutant *drp1*^{KG}*;erk*^{i} show a reduction in dpERK level in mitotic and endocycling stages (Figure 6.5).

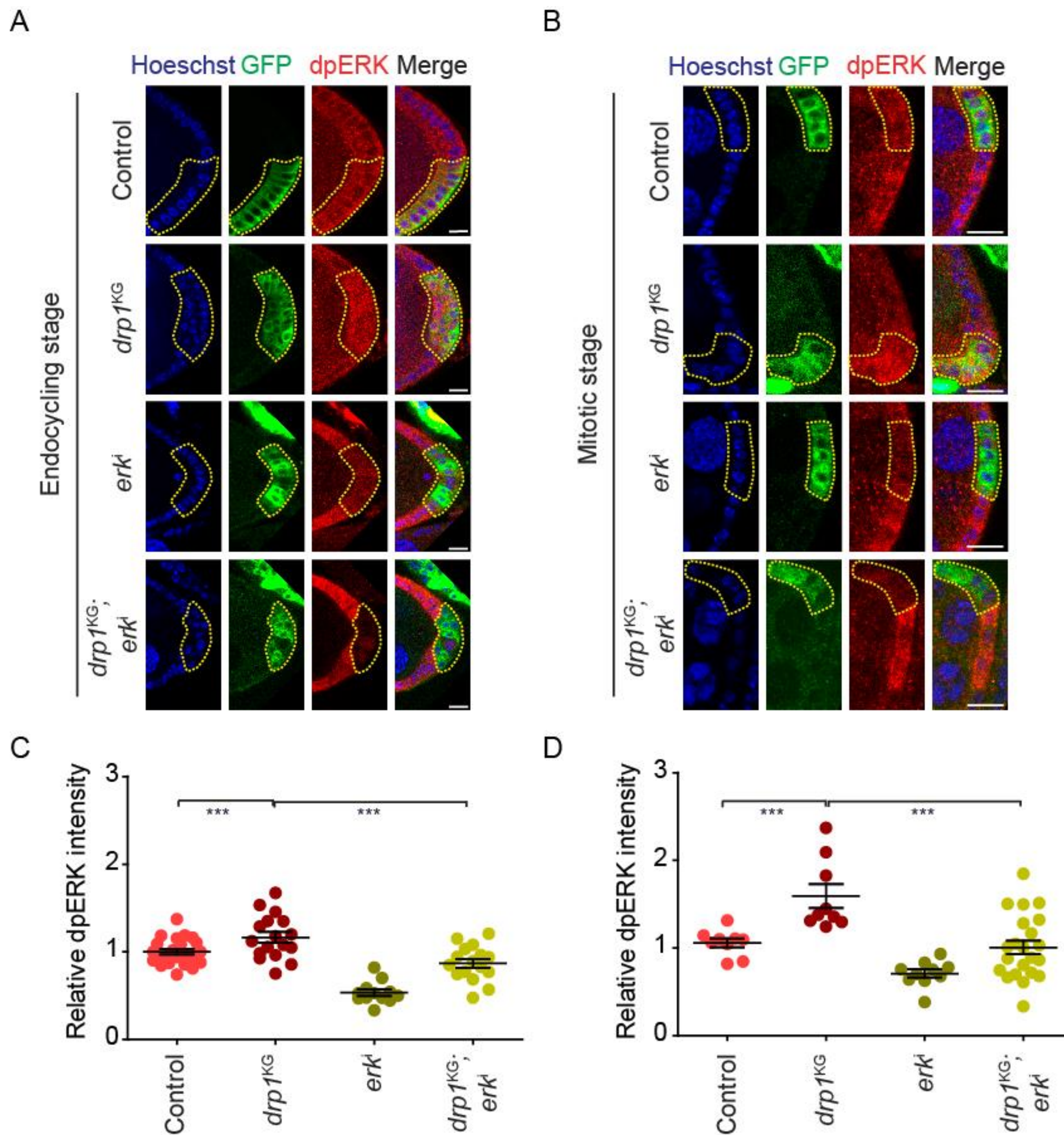


Figure 6.5: ERK depletion shows a decrease in accumulation of dpERK in ***drp1*****^{KG}*****;erk*****^{i} in enodocycling and mitotic stages**

(A-D) Representative images showing dpERK (red) in control FRT40A, *drp1*^{KG}, *erk*^{i}, and *drp1*^{KG}*;erk*^{i} PFC clones in endocycling stages **(A)** and FCs in mitotic stages **(B)**. The graph shows the quantification of relative dpERK fluorescence compared to neighboring control cells in control FRT40A, *drp1*^{KG}, *erk*^{i}, and *drp1*^{KG}*;erk*^{i} PFCs in endocycling stages **(C)** and FCs in mitotic stages **(D)**. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. The data of each genotype is presented with its respective mean and SEM. The graphs show data in Mean+SEM. Each data point (n) in the endocycling and mitotic stages represents the relative

fluorescence intensity of dpERK from a single clone from a separate egg chamber. The n values for the genotypes control FRT40A, *drp1*^{KG}, *erk*^{i}, and *drp1*^{KG}*;erk*^{i} is given as (n, N; n is the number of clones, N is the number of independent replicates) (n=9,9,10,22 and N=3,3,3,3 respectively) for endocycling stages and (n=24,18,12,16 and N=5,4,3,3 respectively) for mitotic stages. The statistical test performed for clone area measurement is Student's t-test. ns=non significant, * P<0.05, ** P<0.01, *** P<0.001. Scale bar=10μm.

Additionally, blocking EGFR also rescues the multilayer defects observed in *drp1*^{KG} follicle cells (Figure 6.6A). There is a significant reduction in the area of the clone in *drp1*^{KG}*;erk*^{i} as compared to *drp1*^{KG} (Figure 6.6B). These results help us to validate the suppression of EGFR signaling in the single and double mutants and their further usage to understand the relation of the EGFR signaling pathway in the regulation of polarity protein aPKC.

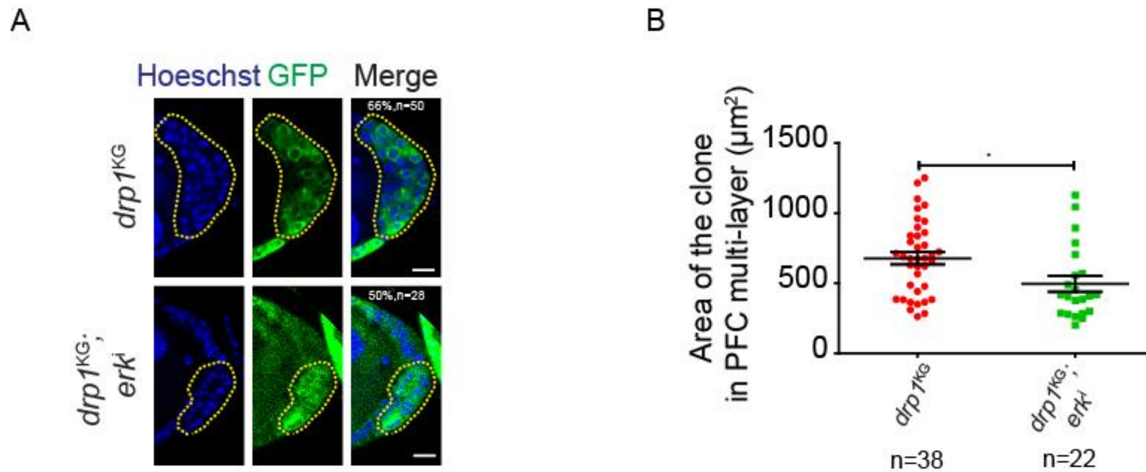


Figure 6.6: The extent of multilayering is reduced in *drp1*^{KG}*;erk*^{i} compared to *drp1*^{KG}

(A-B) Representative images of PFC clones in *drp1*^{KG} showing 3 or more cell layers per clone is at a frequency of (66%,n=50) and that of *drp1*^{KG}*;erk*^{i} is at a frequency of (50%,n=28) **(A)**. The graph shows a comparison of the PFC clone area between *drp1*^{KG} (n=38) and *drp1*^{KG}*;erk*^{i} (n=22) **(B)**. The statistical test performed for clone area measurement is Student's t-test. ns=non significant, * P<0.05, ** P<0.01, *** P<0.001. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. Scale bar=10μm.

6.3.4 Analysis of apical polarity protein atypical protein kinase-C (aPKC) in follicle cells depleted for Erk, a component of EGFR signaling pathway, in mitotic and endocycling stages of *Drosophila* oogenesis

The double mutants $drp1^{KG};opa1^{i}$, $drp1^{KG};sod2^{i}$ and $drp1^{KG};cat^{i}$ show a reduction of dpERK level in the follicle cells of endocycling and mitotic stages as compared to $drp1^{KG}$ clones (Figure 6.3-6.4). The suppression of an ERK protein, a component of the EGFR signaling pathway, using the erk^{i} in Drp1 mutant follicle cells also reproduces a similar reduction in EGFR (Figure 6.5). We hypothesize that the loss of aPKC in Drp1 mutant follicle cells should also get rescued using a combination of $drp1^{KG};erk^{i}$ like other double mutants $drp1^{KG};opa1^{i}$, $drp1^{KG};sod2^{i}$ and $drp1^{KG};cat^{i}$. As per the expectation, the double mutant $drp1^{KG};erk^{i}$ shows decreased % of the cells losing aPKC from the membrane in endocycling and mitotic follicle cells in comparison to $drp1^{KG}$ (Figure 6.7). These observations help us to understand the role of EGFR in regulating the aPKC in follicle cells depleted for Drp1.

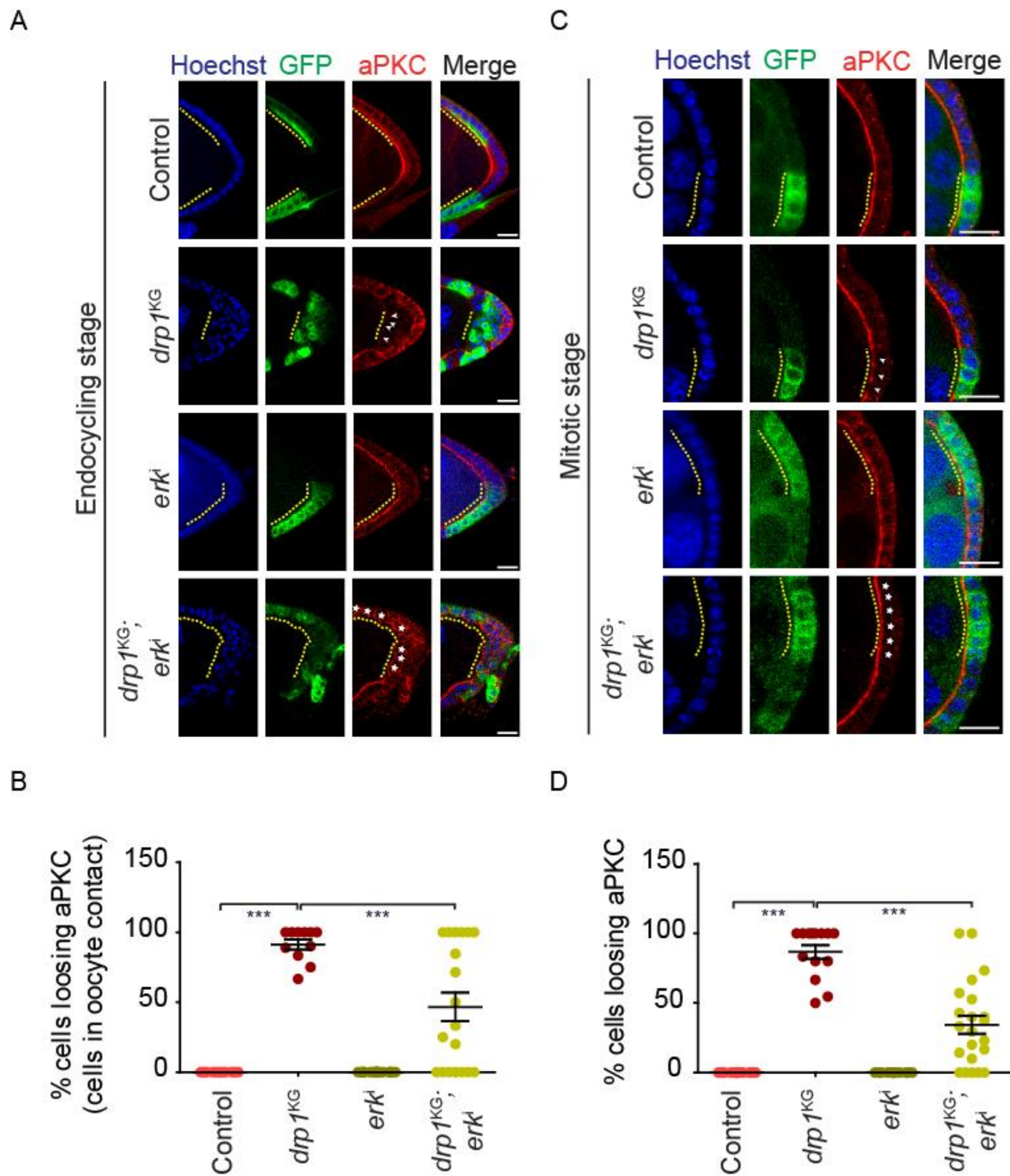


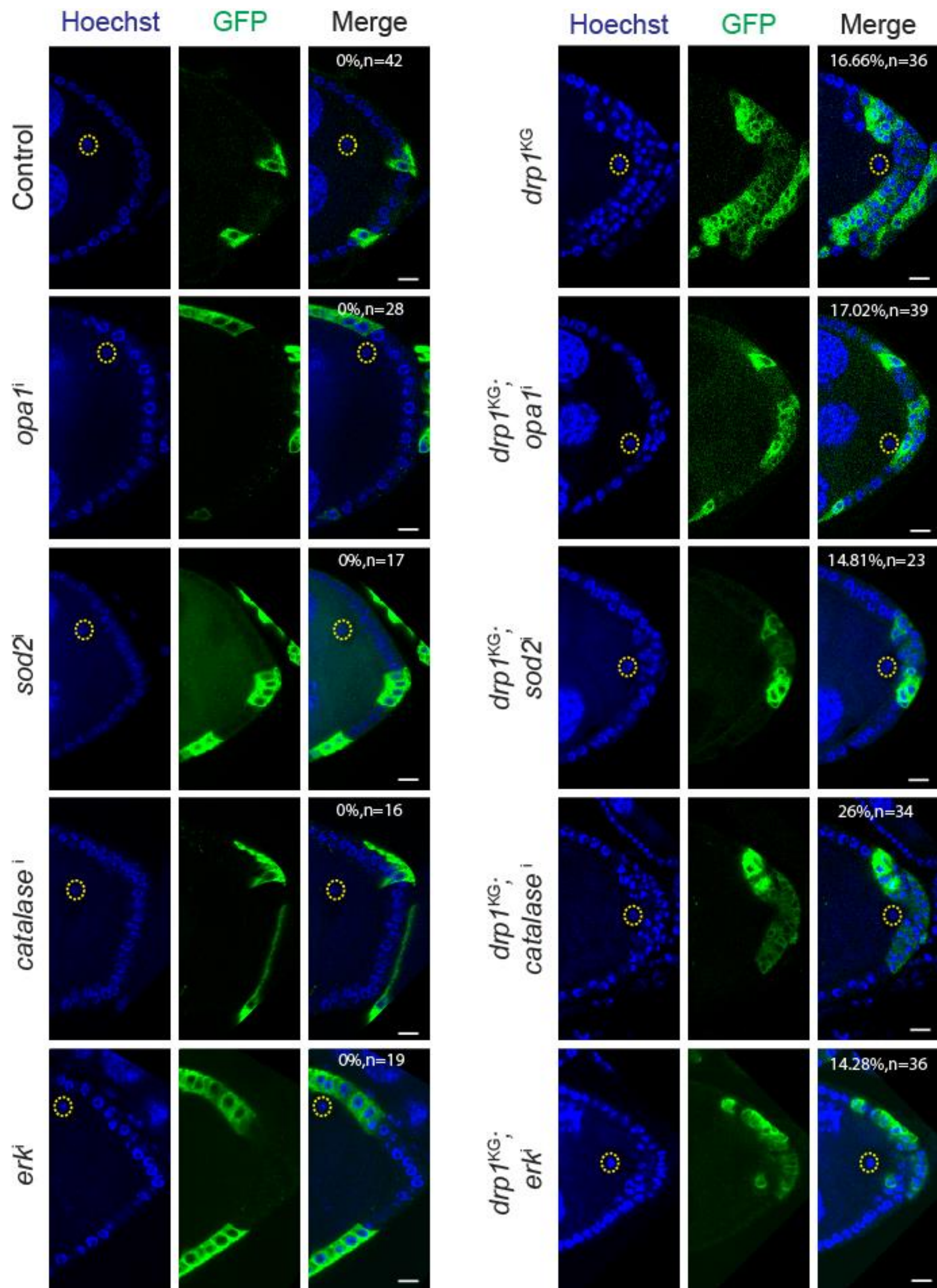
Figure 6.7: aPKC depletion in Drp1 depleted FC clones recovers on additional depletion of ERK

(A-D) Representative images showing aPKC (red) in control FRT40A, *drp1*^{KG}, *erk*^{i,} and *drp1*^{KG}*;erk*^{i} PFC clones at endocycling stages **(A)**. Representative images showing aPKC (red) in control FRT40A, *drp1*^{KG}, *erk*^{i,} and *drp1*^{KG}*;erk*^{i} FC clones at mitotic stages **(B).** The graph shows the quantification of the percentage of cells losing aPKC in each PFC clone adjacent to the oocyte in control FRT40A, *drp1*^{KG}, *erk*^{i,} and *drp1*^{KG}*;erk*^{i} at endocycling stages **(C)**. The graph shows the quantification of the percentage of cells losing

aPKC in each FC clone in control FRT40A, *drp1*^{KG}, *erk*^{i}, and *drp1*^{KG}*;erk*^{i} at mitotic stages **(D).** mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. White arrowheads mark FCs deficient in aPKC. White asterisks mark FCs that show the presence of aPKC in double mutants. The graphs show data in Mean+SEM. Each data point (n) in the endocycling and mitotic stages represents the percentage of cells losing aPKC in a single clone of a separate egg chamber. The n values for the genotypes control FRT40A, *drp1*^{KG}, *erk*^{i,} and *drp1*^{KG}*;erk*^{i} is given as (n, N; n is the number of clones, N is the number of independent replicates) (n=9,11,10,19 and N=3,5,3,4 respectively) for endocycling stages and (n=23,14,12,22 and N=5,5,3,3 respectively) for mitotic stages. ns=non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Scale bar=10µm.

6.3.5 Analysis of oocyte positioning in follicle cells depleted for mitochondrial morphology proteins, antioxidant enzymes, and for erk (EGFR) mutant in endocycling stages of *Drosophila* oogenesis

Activation of EGFR signaling in follicle cells leads to the migration of the oocyte in the dorso-anterior position and patterning of the posterior follicle cells. Ovarioles containing posterior follicle cells deficient of Drp1 show a decrease in oocyte migration to the dorso-anterior location and the accumulation of the downstream effector of doubly phosphorylated ERK (dpERK) in the cytoplasm (González-Reyes and St Johnston 1998; Mitra et al. 2012; Tomer et al. 2018). Depletion of EGFR signaling leads to a decrease in mitochondrial activity and fragmentation (Tomer et al. 2018; Mitra et al. 2012). We analyzed the frequency of oocyte migration and accumulation of dpERK as a readout of EGFR signaling in *drp1*^{KG} mutant depleted of Opa1 and ROS scavengers. Migration of oocyte to the dorso-anterior position was similarly aberrant in *drp1*^{KG} and the combinations of *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG}*;sod2*^{i,} and *drp1*^{KG}*;cat*^{i} (Figure 6.8).



Hoechst
GFP
Merge
Hoechst
GFP
Merge
Control
0%,n=42
opa1i
0%,n=28
sod2i
0%,n=17
catalase i
0%,n=16
erki
0%,n=19
drp1KG
16.66%,n=36
drp1KG;
opa1i
17.02%,n=39
drp1KG;
sod2i
14.81%,n=23
drp1KG;
catalase i
26%,n=34
drp1KG;
erki
14.28%,n=36

Figure 6.8: Oocyte migration defects in *drp1*^{KG} mutant egg chambers do not get suppressed on additional depletion of Opa1, and ROS scavengers

Representative images showing the dorso-anterior migration of the oocyte in stage egg chambers in control FRT40A (0% oocyte at the posterior in stage 9 or 10 egg chambers,n=42, N=5), *drp1*^{KG} (17%,n=36, N=5), *drp1*^{KG}*;opa1*^{i} (17%,n=39, N=5), *drp1*^{KG};*sod2*^{i} (15%,n=23, N=3), *drp1*^{KG};*catalase*^{i} (26%,n=34, N=4) and *opa1*^{i} (0%,n=28, N=5), *sod2*^{i} (0%,n=17, N=4), *catalase*^{i} (0%,n=16, N=4). mCD8-GFP (green) expresses in FC clones of the represented genotype. Nucleus (blue) is stained with Hoechst and the nucleus is marked with yellow circular outlines. Scale bar=10µm.

These data showed that even though the levels of dpERK were reduced in the cytoplasm of *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG}*;sod2*^{i,} and *drp1*^{KG}*;cat*^{i} but it was not sufficient to cause migration of the oocyte to the dorso-anterior position.

6.3.6 Analysis of Notch signaling in multilayered follicle cells depleted for mitochondrial morphology proteins and antioxidant enzymes

We know that the follicle cells of the Drp1 mutant cause multiple-layer formation in an endocycling stage, as discussed in 3.3.2 (Figure 3.2). The activation of Notch signaling is required in this endocycling stage during oogenesis. This signaling, at this stage, is initiated by the interaction of Delta ligands from germ cells with the Notch receptor of follicle cells (López-Schier and St Johnston 2001). As a result, Hindsight (Hnt) is expressed in response to active Notch signaling in posterior follicle cells around the oocyte during this stage transition phase of follicle cells (Ruohola et al. 1991; Kim-Yip and Nystul 2018). The posterior follicle cells of *drp1*^{KG} with defective mitotic to endocycling transition showed a loss of Hnt, whereas this defect is partially reversed by a decreasing mitochondrial activity, blocking fusion, and depleting ERK level (Mitra et al. 2012; Tomer et al. 2018). We see various defects of Drp1 such as the formation of multilayers, loss of aPKC, and increased dpERK level that goes away partially with the double mutants *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG};*sod2*^{i}, *drp1*^{KG};*cat*^{i}, and *drp1*^{KG}*;erk1*^{i}. We hypothesize further to check if these double mutants lead to a rescue in Notch activation defect of *drp1*^{KG} mutant follicle cells. Parallel to the hypothesis, we find that these double mutants show partial rescue in Hnt as compared to *drp1*^{KG} (Figure 6.9). These data show that the loss of Notch signaling mediated differentiation is rescued partially by blocking additional mitochondrial fusion, increase in ROS, or reducing elevated dpERK in follicle cells depleted for Drp1.

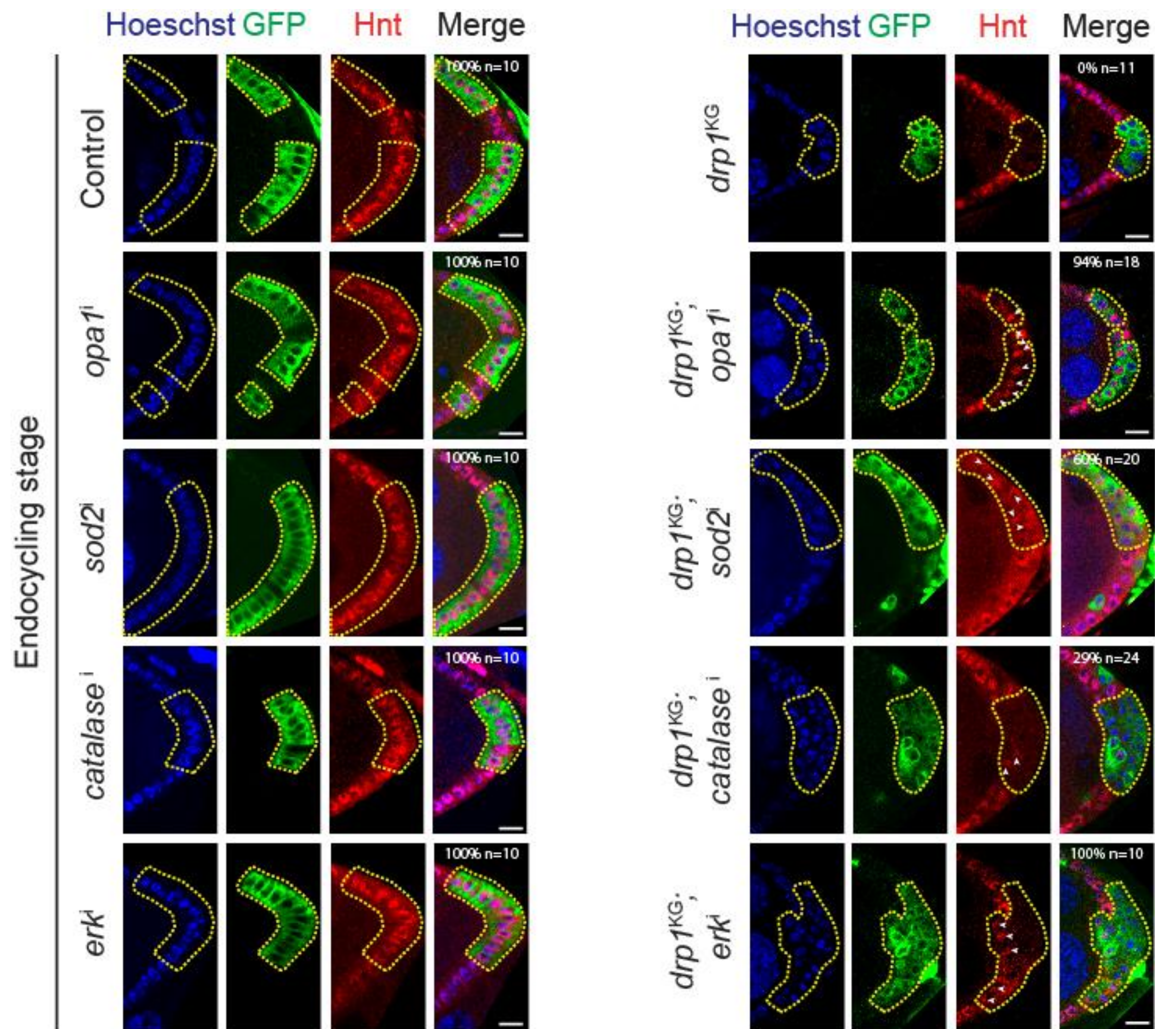


Figure 6.9: Notch differentiation defects in Drp1 depleted PFC clones are suppressed on additional depletion of Opa1, ROS scavengers, and ERK

Representative images showing Hnt (red) in PFCs in the genotypes control FRT40A (100% show Hnt, n=10), *opa1*^{i} (100%,n=10), *sod2*^{i} (100%,n=10), *catalase*^{i} (100%,n=10) and *erk*^{i} (100%,n=10) on the left side and *drp1*^{KG} PFCs is (0%,n=11), *drp1*^{KG}*;opa1*^{i} (94%,n=18), *drp1*^{KG};*sod2*^{i} (60%,n=20), *drp1*^{KG};*catalase*^{i} (29%,n=24), *drp1*^{KG}*;erk1*^{i} (100%,n=10) on the right side. mCD8-GFP (green) expressing FC clones of the indicated genotype are marked with dashed yellow outlines. The nucleus (blue) is stained with Hoechst. White arrowheads mark PFCs showing the presence of Hnt. The numbers in each image represent the percentage of PFC clones showing the presence of Hnt. Scale bar=10µm.

6.4 Discussion

In this section, we have tried to understand the role of the EGFR signaling pathway in causing a loss of an apical polarity protein and a Notch signaling pathway that is indispensable for the mitotic to endocycling stage transition. We have assessed a double phosphorylated (dpERK) level as a readout of EGFR signaling, as mentioned in earlier literature (Castanieto, Johnston, and Nystul 2014; Tomer et al. 2018). During oogenesis, it has been observed that the FSCs are triangular in shape and do not have an apical domain and aPKC. They show higher dpERK, a readout of EGFR signaling, which is responsible for the lack of aPKC and lack of an apical domain in these cells (Castanieto, Johnston, and Nystul 2014). The Intestinal stem cells in *Drosophila* are also triangular in shape with increased dpERK levels and do not have an apical domain (Deng et al. 2018). The fusion of mitochondria in follicle cells depleted for Drp1 causes multiple-layer formation, a loss of an apical protein aPKC, and increased EGFR signaling (Tomer et al. 2018). The follicle cells of Drp1 mutant exhibit an elevated level of dpERK in endocycling (Figure 6.3 and 6.5A, C) and mitotic stages (Figure 6.4 and 6.5B, D), a readout of an active EGFR signaling, which might be causing the follicle cells to retain a follicle stem cell-like feature lacking aPKC on their membrane.

Increased EGFR signaling pathway is associated with many cancers, including colon cancer and lung adenocarcinoma in mice (Johnson et al. 2001; Calcagno et al. 2008; Jiang et al. 2011). Additionally, all the double mutants *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG};*sod2*^{i}, *drp1*^{KG};*cat*^{i}, where dpERK level is reduced, show reduction of multiple layers and recovery of aPKC as compared to *drp1* mutant follicle cells. Thus, this literature and our observations suggest that EGFR signaling might provide an important link for the loss of aPKC in Drp1 mutant follicle cells. The reduction in multiple layers (Figure 6.6) and recovery of aPKC (Figure 6.7) in *drp1*^{KG}*;erk1*^{i} strongly supports the role of EGFR in the regulation of aPKC protein. We also expect the dorso-anterior positioning defect of the oocyte should be rescued in the double mutants but we don't observe the same for these double mutants (Figure 6.8). The partial rescue in the multiple layer formation and partial recovery of the polarity protein aPKC is not enough to reverse all EGFR-related defects in these double mutants. Surprisingly, overexpression of the constitutive form of EGFR does not cause the formation of multiple layers in the clones of follicle cells in our study. This indicates that either the level of EGFR is not enough to trigger the formation of multiple layer formation in this mutant or the synergy between fusion of mitochondria and elevated level of the EGFR signaling

altogether causes the formation of multiple cell layers. Although our analysis shows that the aPKC defects are partially reversed when EGFR signaling is suppressed by additional fragmentation or increasing ROS, I speculate that the oxidation may change the status of proteins related to EGFR signaling in the cell. Yet, I need to prove if these proteins are oxidized and cause hyperactivation, hypoactivation, or change in the quantitative and qualitative redistribution of aPKC or its target proteins, and other processes including transcription and translation.

Notch signaling is reported to induce the differentiation of *Drosophila* mitotic cells to endocycling follicle cells (Mitra et al. 2012; Tomer et al. 2018), *Drosophila* intestinal stem cells to epithelial cell lineages (Patel, Dutta, and Edgar 2015), *Drosophila* hematopoiesis (Blanco-Obregon et al. 2020). These reports suggest the important role of Notch in the differentiation process. Additionally, the double mutants of *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG};*sod2*^{i}, *drp1*^{KG};*cat*^{i}, *drp1*^{KG}*;erk1*^{i} show the reduction of multiple layers of Drp1 mutant (Figure 3.2C, Figure 5.6A,C and Figure 6.6A). These observations are of importance as these multilayers are formed at the endocycling stages where Notch signaling gets activated. We observe a reduction of the Notch defect in these double mutants where multiple layers are also reduced. There is a possibility that appropriate cell morphology is required to initiate signaling through a ligand-receptor-based interaction. Although the Hnt recovery in *drp1*^{KG};*cat*^{i} is not predominant as we see in *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG};*sod2*^{i} ,*drp1*^{KG}*;erk1*^{i} (Figure 6.9). These observations suggest that the Hindsight recovery is dependent more on mitochondrial morphology and mitochondrial ROS rather than cytoplasmic ROS. Thus, ROS plays an important role in the transition of the egg chambers from the mitotic to the endocycling stage.

7 Thesis summary and future directions

Mitochondria are double membrane organelles important for ATP production as shown in Figure 1.1 (Jouaville et al. 1999; Osellame, Blacker, and Duchen 2012; McQuibban et al. 2006; Giacomello et al. 2020). Apart from energy production, mitochondria perform diverse functions including reactive oxygen species (ROS) production, calcium homeostasis, synthesis of important components such as phospholipids and heme, cell cycle regulation, apoptosis, unfolded protein response (UPR), etc. (Cipolat et al. 2004; N. Ishihara et al. 2009; Benard et al. 2010; Romanello et al. 2010; Mayr et al. 2011; Brand et al. 2013; Kasahara and Scorrano 2014; Berger et al. 2016; Seo, Yoon, and Do 2018; Favaro et al. 2019; Urbauer, Rath, and Haller 2020). The mitochondria undergo a diverse range of shape modifications with the help of mitochondrial morphology proteins such as Drp1, Opa1, and Marf (Figure 1.2) (Scott and Youle 2010; Y. J. Liu et al. 2020). The shape of the mitochondria in stem cells and differentiated cells holds significance as their abundance and shape in these cells regulate the fate specification and associated changes as shown in Figure 1.4 (Lisowski et al. 2018). Mitochondrial fusion and OXPHOS are required for the differentiation of hepatocytes (Fu et al. 2013). Even a differentiation of Intestinal stem cells to enterocytes requires a fusion of the mitochondria and mitochondrial pyruvate metabolism. They have not studied the role of these metabolic changes and polarity formation in enterocytes (Schell et al. 2017; Deng et al. 2018). Thus, the role of mitochondria morphology in epithelial cell differentiation and associated signaling pathways are not clearly understood (Castanieto, Johnston, and Nystul 2014; Schell et al. 2017; Deng et al. 2018).

The above descriptions of mitochondria help us predict the diverse roles of mitochondria during the development of an organism at the cellular level. The role of mitochondria in the epithelial cell differentiation process and polarity establishment is less explored. We have tried to understand the mechanisms by which mitochondrial fusion leads to an abrogation of follicle cell differentiation and their organization during development using *Drosophila* as a model organism.

7.1 Analysis of follicle cell architecture on mitochondrial fusion by depletion of mitochondrial fission protein Drp1

We have shown that the posterior follicle cells (PFCs), show disorganized cell architecture when mitochondria are force fused using Drp1 mutant (*drp1*^{KG}). A similar observation was already

reported (Mitra et al. 2012; Tomer et al. 2018). We have shown that the loss of Drp1 induces the formation of multiple layers in the endocycling stages but not in the mitotic stages. 66% of the egg chambers show 3 or more than 3 cell layers per clone (Figure 3.2C). Suppressing fusion of the mitochondria or increasing ROS in follicle cells depleted for Drp1 reduces the extent of multiple-layer formation. These observations suggest that the clustered or fused mitochondria lead to the impaired architecture of the posterior follicle cells in the endocycling stage. The analysis of mitotic follicle cells does not show the formation of multiple layers (Figure 3.3). These observations help us understand the requirement of fragmented mitochondria during the process of oogenesis and follicle cell development.

7.2 Analysis of apical polarity protein aPKC in follicle cells on depletion of mitochondrial fission protein Drp1

We have shown that the fusion of mitochondria leads to the formation of multilayers of the follicle cells in *drp1*^{KG} mutant egg chambers at the endocycling stage but not at the mitotic stage. We have shown that the apical polarity protein aPKC is lost from these multilayered follicle cells at the endocycling stage of the same *drp1*^{KG} mutant. Apart from that, follicle cells from mitotic stages also lose the aPKC. Furthermore, blocking additional mitochondrial fusion or increasing ROS in follicle cells depleted for *drp1* helps reduce multilayer formation and rescue loss of aPKC (Figure 3.2C, 4.1, 4.2, 5.6-5.8). Reactive oxygen species in the cell may change the activities of the proteins by oxidizing their amino acid residues. Apart from that, ROS can also regulate the activities of kinases and phosphatases (Ray, Huang, and Tsuji 2012; Corcoran and Cotter 2013). In our case, we see a recovery of aPKC, one of the kinases, on the membrane of the follicle cells in double mutants of *drp1*^{KG}*;opa1*^{i}, *drp1*^{KG};*sod2*^{i}, and *drp1*^{KG};*catalase*^{i}. The oxidation and stabilization of aPKC could also be possible when ROS in the cell increases. We need to further investigate how an increase in ROS can actually affect aPKC or the signaling pathways that regulates aPKC such as EGFR (Castanieto, Johnston, and Nystul 2014).

7.3 Analysis of signaling pathways associated with the regulation of follicle cell polarity

The fusion of the mitochondria is reported to have increased dpERK, a read-out of EGFR signaling, in the multilayered follicle cells depleted for Drp1 (Tomer et al. 2018). We show that the dpERK increases in follicle cells of both stages. Suppressing EGFR signaling reduces the

extent of the formation of multiple layers and also helps rescue the loss of aPKC in follicle cells depleted for Drp1. The above observation is supported by the fact that EGFR regulates aPKC negatively in follicle stem cells (Castanieto, Johnston, and Nystul 2014) This data puts EGFR signaling upstream of the loss of aPKC. The over-expression of aPKC in Drp1 mutant follicle cells will help us understand if the formation of a multilayer is due to the loss of aPKC from these cells at endocycling stages. Contrary to that, loss of aPKC in mitotic stages does not cause the formation of multiple layers. Apart from that, the mutants for lateral polarity proteins (Dlg-Lgl-Scribble) also cause elevated dpERK, defective Notch signaling, and show multiple layers (Li et al., 2008; Li et al., 2009; Tian and Deng, 2008). Similarly, the loss of aPKC causes the formation of multiple layers. But the EGFR and Notch signaling are yet to be understood in these multilayered cells (Kim et al., 2009; Wodarz et al., 2000). These observations also suggest that the stage of follicle cells and loss of aPKC have differential defects.

The transition from the mitotic to the endocycling stage is supported by the activation of Notch signaling. The previous literature shows that the follicle cells depleted for Drp1 are defective in Notch signal activation (Mitra et al. 2012; Tomer et al. 2018). We show that reducing EGFR signaling in these cells by suppressing additional fusion or increasing ROS help rescue the Notch activation defects partially. Thus, aberrant EGFR signaling in Drp1-depleted follicle cells causes a lack of Notch activation. But it is not clear whether the upregulation of Notch in this cell can rescue the defects. It is a little tricky to do that because Notch overexpression can promote the proliferation of stalk cells connecting two egg chambers leading to the formation of multiple layers. We have also observed elevated EGFR which is proven to be an antagonist for Notch signaling (Hasson et al., 2005). Apart from this, the loss of aPKC and subsequent loss of Notch signaling is also reported in *the Drosophila* gut (Goulas et al., 2012). These findings correlated antagonistic roles of EGFR and Notch signaling and their link to the polarity protein aPKC.

In our study, the elevated EGFR and defective Notch signaling pathways are rescued partially by either depleting fusion protein Opa1 or increasing ROS in follicle cells depleted for Drp1. These rescues might have happened due to ROS-mediated inactivation of fusion proteins or activation of other machinery that can suppress the mitochondrial fusion events (Kashatus et al., 2015). It is also possible that the increased ROS may be suppressing the downstream target of EGFR signaling and hence we see a rescue in multilayering and loss of aPKC. A decrease in EGFR

signaling could also be one of the possible mechanisms to rescue the Notch defects (Hasson et al., 2005).

7.4 Future directions

We aimed to address the role of mitochondria and associated changes in the regulation of epithelial cell differentiation and polarity formation. The role of mitochondrial morphology in other examples of the epithelial cell remains yet to be discovered. The Drp1-mediated fission of the mitochondria could be important in the regulation of signaling pathways like EGFR and Notch. We do not know if other pathways of stem cell differentiation are still getting affected when mitochondrial morphology is altered. The increase in ROS in Drp1 depleted follicle cells helps rescue multilayer formations, loss of aPKC, impaired EGFR, and Notch signaling pathways. We need to still understand the mechanism by which ROS affects all these phenotypes of Drp1 mutants.

The suppression of polarity protein aPKC also increases myosin activation and helps in the constriction of the follicle cells (Osswald et al. 2022). Additionally, it has been observed that the loss of aPKC in Drp1 depleted follicle cells shows constricted apical surface. And hence, we expect myosin to be hyperactivated in these follicle cells. We still have to figure out if the fusion of mitochondria can also activate myosin in these cells and become an upstream event for the apical constriction and formation of smaller cells. Suppression of myosin activation in these Drp1 depleted follicle cells may answer if the myosin can regulate the cell size downstream of Drp1 mediated fission of mitochondria. We also have to understand how mtROS can also participate in myosin inactivation.

The reports suggest that endocytic vesicles get enriched near the apical surface of the cell at the site of apical constriction during *Drosophila* and *Xenopus* development (Lu and Bilder 2005; J.-Y. Lee and Harland 2010). We need to correlate the function of Rab-5 and Rab11-mediated apical constriction in these Drp1-depleted follicle cells. This experiment will help us understand the fact that if aPKC is actually absent because of altered Rab5-mediated endocytosis or Rab11-mediated recycling of aPKC.

8 Appendix

A1 Fission mutant Drp1 show either absent or mislocalized and accumulated sub-apical market PatJ

The *Drosophila* follicle cells are a good model system to study the polarity establishment (Wu, Tanwar, and Raftery 2008). The polarity proteins present on the follicle cells during development are under special control of signaling pathways (Castanieto, Johnston, and Nystul 2014). Apart from that, the polarity complexes get restricted to their respective domain via phosphorylation by other protein kinases such as aPKC. The presence of aPKC, being kinase, helps localize the other polarity markers such as Dlg, Bazooka, Crumbs, and Lgl via phosphorylating them (Golub et al. 2017; Morais-de-Sá, Mirouse, and St Johnston 2010; Sotillos et al. 2004; Betschinger, Mechtler, and Knoblich 2003). Thus, aPKC loss may affect the localization of these polarity proteins too. We already know that the aPKC is lost in follicle cells depleted for Drp1 at mitotic and endocycling stages and mitotic stages. Hence, we analyze sub-apically located and one of the Crumbs complex proteins that are PatJ in these follicle cells.

A1.1 Sub-apical polarity marker PatJ mislocalizes or remains absent in the follicle cell clones depleted for Drp1 proteins at endocycling stages.

The follicle cells depleted for Drp1 protein show the formation of multiple cell layers and loss of aPKC (Chapters 3-5). It is interesting to know the status of sub-apical protein PatJ if it shows any defects in these multilayers of cells. Analysis of PatJ in Drp1 depleted follicle cells at endocyclic stages shows mislocalization of PatJ. Additionally, the PatJ that is mislocalized gets accumulated on the membrane of follicle cells (Figure A1). The further analysis of double mutant *drp1*^{KG};*opa1*^{i} shows that this mislocalization or accumulation does not get reversed upon additional suppression of the mitochondrial fusion in *drp1*^{KG}.

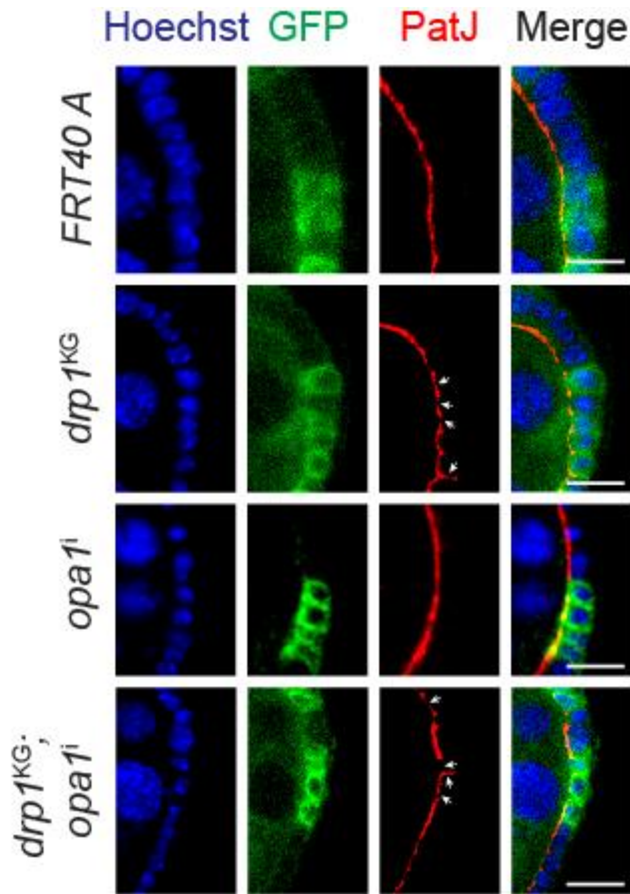


Figure A1: Drp1 mutant follicle cell clones show the absence or mislocalization of PatJ in mitotic stages that are not rescued in *drp1*^{KG}*;opa1*^{i}

Representative images showing PatJ (red) in PFC clones from *drp1*^{KG} and *drp1*^{KG}*;opa1*^{i} at the endocycling stage. mCD8-GFP (green) marks FC clones. The nucleus (blue) is stained with Hoechst. White arrows show defects of PatJ in FCs. Scale bar=10μm.

A1.2 Sub-apical polarity marker PatJ mislocalizes or remains absent in the follicle cell clones depleted for Drp1 proteins at mitotic stages.

Although the follicle cells depleted for Drp1 protein show mono-layer of the cells, they show loss of aPKC from the apical surface (Chapters 3-5). The analysis of sub-apical protein PatJ in these cells shows mislocalization and accumulation on the follicle cell membrane. Additionally, the PatJ is absent on the membrane of a few follicle cells (Figure A2). The further analysis of double mutant *drp1*^{KG}*;opa1*^{i} shows that the PatJ defects do not get reversed upon additional suppression of the mitochondrial fusion in *drp1*^{KG}.

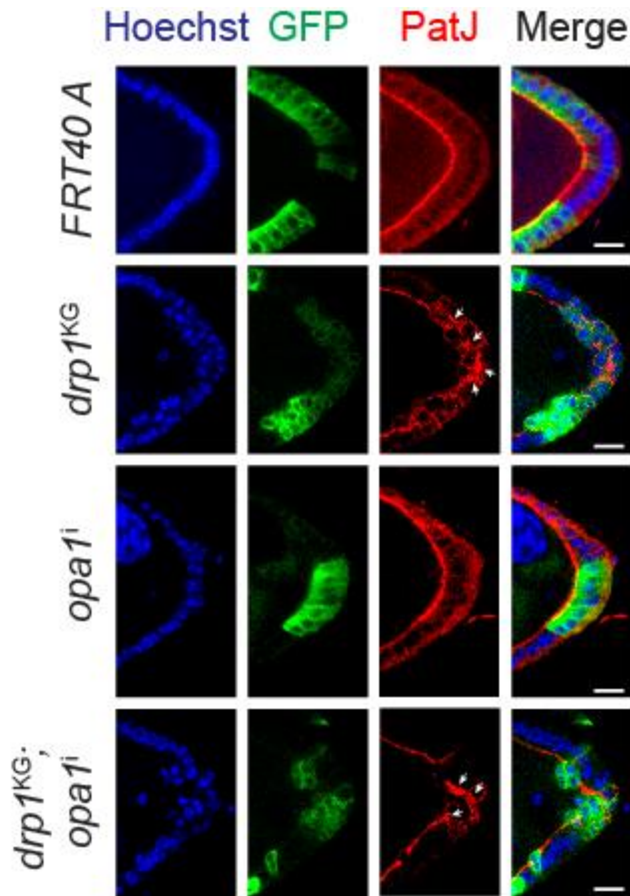


Figure A2: Drp1 mutant follicle cell clones show the mislocalization and accumulation of PatJ in mitotic stages that are not rescued in *drp1*^{KG}*;opa1*^{i}

Representative images showing PatJ (red) in PFC clones from *drp1*^{KG} and *drp1*^{KG}*;opa1*^{i} at the endocycling stage. mCD8-GFP (green) marks FC clones. The nucleus (blue) is stained with Hoechst. White arrows show defects of PatJ in FCs. Scale bar=10μm.

A2 Follicle cell organization in other fission mutants where mitochondria are fused

We have seen that the fusion caused by the depletion of Drp1 induces fused mitochondria and the same has been shown by previous literature too (Mitra et al. 2012; Tomer et al. 2018). We wanted to know if the fusion of mitochondria in other mutants also causes similar defects. We used p*UASp-Drp1*^{SG} (*drp1*^{SG}) to drive its expression in GFP-positive cells using MARCM as described in Chapter 2. The same mutant was used to cause mitochondrial fusion in the past too (Chowdhary et al. 2020; Dubal et al. 2022). The GFP-positive egg chambers of *drp1*^{SG} show fused mitochondria but do not show multiple-layer formation as we observe in *drp1*^{KG} mutant egg chambers (Figure

A3). We also have to remember that these GFP-positive cells are heterozygous mutants for Drp1 proteins as compared to GFP-positive cells in the homozygous mutant *Drp1*^{KG}. It suggests that the complete lack of Drp1 gives this phenotype.

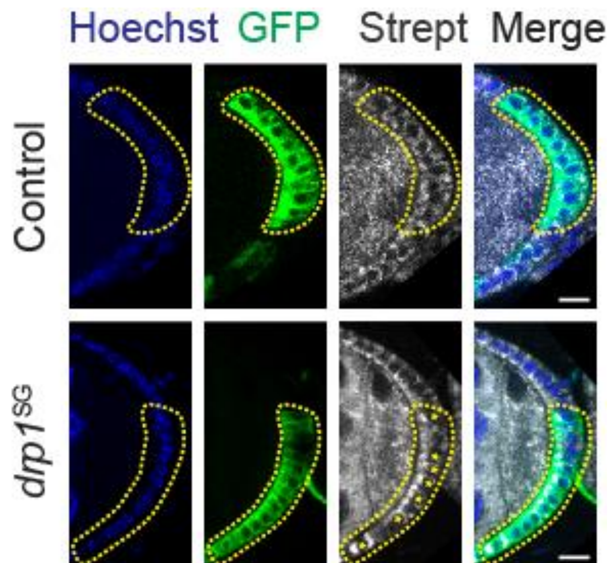


Figure A3: Mitochondrial morphology and follicle cell arrangement in fission mutant *Drp1*^{SG}
Representative images show mitochondria stained with Streptavidin (greyscale) in FCs in the endocycling stages in the genotypes control FRT40A, *drp1*^{SG}. mCD8-GFP (green) marks the clones in PFCs. The nucleus (blue) is stained with Hoechst. Yellow asterisks mark clustered mitochondria. Scale bar=10µm.

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