Analysis of regulation of epithelial-like architecture formation by mitochondrial dynamics in *Drosophila* embryogenesis

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

Bhavesh Anil Ohal

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

April, 2023

Supervisor: Dr. Richa Rikhy, IISER Pune

Expert: Dr. Nagaraj Balasubramanian, IISER Pune

Certificate

This is to certify that this dissertation entitled ‘Analysis of regulation of epithelial-like architecture formation by mitochondrial dynamics in *Drosophila* embryogenesis’ towards the partial fulfilment of the BS-MS dual degree programe at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Bhavesh Anil Ohal at Indian Institute of Science Education and Research under the supervision of Dr. Richa Rikhy, Professor, Department of Biology, during the academic year 2022-2023.

Dr. Richa Rikhy

Committee:

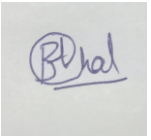
Dr. Richa Rikhy

Dr. Nagaraj Balasubramanian

This thesis is dedicated to all those who suffer from mental health issues but still fight back!

Declaration

I hereby declare that the matter embodied in the report entitled ‘Analysis of regulation of epithelial-like architecture formation by mitochondrial dynamics in *Drosophila* embryogenesis’ are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Richa Rikhy and the same has not been submitted elsewhere for any other degree



Bhavesh Anil Ohal

Date: 31/03/2023

Chapter 1 : Contents

List of Figures

Chapter 1: Introduction

Chapter 3: Results and Discussion

Abstract:

Mitochondrial morphology and dynamics play a crucial role in epithelial cell formation and maintenance, however the mechanisms by which they affect these processes remains to be completely investigated. Here we have attempted to study the role of mitochondrial fission in the onset of polarity during epithelial cell formation in *Drosophila* embryogenesis. Our previous studies show that the depletion of the mitochondrial fission protein Drp1 leads to fused mitochondria which accumulate basally of the cells during cellularization. We further elucidated the changes in epithelial polarity proteins in embryos depleted of Drp1. We find that though the apical cell area remains the same as controls and there is depletion of apical and basal adherens' junction protein DE-cadherin in Drp1 depleted embryos. Reactive oxygen species have been previously found to be decreased in Drp1 depleted embryos. We find that restoration of ROS in the mitochondrial SOD2 RNAi expressing embryos leads to a reversal of the defect in DE-cadherin distribution in Drp1 depleted embryos. Subsequently, we also observed reduction in levels of Bazooka protein which helps recruit DE-Cadherin apically. The basal activation of actomyosin ring constriction is decreased in Drp1 depleted embryos and myosin II is enriched in the cytoplasm. The polarity protein PatJ which is present at the apical membrane and at the ring is seen to be increased in the cytoplasm in Drp1 depleted embryos. Dlg, a lateral membrane protein appears to be increased basally in Drp1 depleted embryos. We further tested the role of trafficking pathways in mediating these polarity protein defects by staining for amphiphysin, Rab5, Rab11 and Rab7 in Drp1 depleted embryos. We found that amphiphysin, a marker for endocytosis is accumulated at the ring. Amphiphysin labelled endocytic tubes at the furrow are increased indicating that there is a delay in endocytosis. Further there is a depletion and mislocalization of endocytic pathway components Rab5, Rab11 and Rab7. Together these data indicate that epithelial polarity proteins are likely to affected at the plasma membrane during their formation in cellularization in Drp1 depleted embryos due to a defect in trafficking. It is likely that decrease in mitochondrial ROS may play a role in regulating these defects.

- **Acknowledgements:**

I would sincerely like to thank my supervisor for the project Dr. Richa Rikhy for giving me the opportunity to work in the lab and explore developmental biology. I would also like to thank Dr. Girish Ratnaparkhi for instilling enormous interest in the field of developmental biology during his teaching. I would also express my vote of thank to my expert Dr. Nagaraj Balasubramanian for the critical comments in the project. I would also like to thank the microscopy imaging facility at IISER Pune for the extensive amounts of imaging performed throughout the course and the facility managers Vijay and Santosh were kind enough to solve any discrepancy occurring during imaging. Fly media facility members comprising of Yogesh and Snehal were helpful in providing the transgenic flies and the fly media for the experiments. The incredible team of our lab members including Harsh, Somya, Bhavin, Shashwat, Aparna, Sanjana, Rahul, Debasmita, Anirban, Shweta, Chaitanya and Atharva were very supportive and friendly throughout the course. They helped contribute to my understanding for the subject in all possible ways and the fruitful discussions with them helped escalate my interest in the subject. Dr. Girish Ratnaparkhi's lab members including Kundan, Vidyadheesh, Namrata, Amruta, Mrunal and Lovleen were helpful with fly work and the technical assistance in the lab space. I am grateful to Samyuktha and Ninad for the timely help with the schematics. Finally, I would thank my family and friends for the persistent support throughout.

Chapter 2: Introduction

1. *Mitochondria morphology and dynamics:*

Mitochondria as cellular organelles help in cell survival, growth, metabolism and regulate signalling cascades involved in pluripotency and differentiation(Kasahara and Scorrano, 2014; Giacomello *et al.*, 2020). The smooth outer membrane is where signals involving development converge and allow for changes in mitochondrial morphology and dynamics, whilst the inner membrane harbors ATP-generating machinery. These developmental signalling pathways affect mitochondrial morphology and dynamics and in turn, the mitochondrial morphology regulates the production of ATP and ROS spatio-temporally in the cell. The outcome of the signaling pathways therefore occurs due an interaction between mitochondrial dynamics and activity. Fused mitochondria increase ATP, decreases ROS levels and compensates for material in deficient mitochondria while fission helps clear defective mitochondria, increases ROS in the cell and have escalated dynamics being able to access various subcellular locations. The morphology depends on cellular requirements with fusion depending on linking of the outer membrane Mitofusin proteins MFN1 and 2 and the OPA1 inner mitochondrial membrane protein while fission being regulated by Dynamin-related protein Drp1. It is normally kept inactive but if activated by phosphatases, binds the outer mitochondrial membrane with subsequent oligomerization and GTP-dependent hydrolyses partitioning the membrane(Giacomello *et al.*, 2020).

The supporting roles of mitochondrial morphology have been studied in many developmental contexts in *Drosophila* embryogenesis such as syncytial blastoderm cycles, cellularization and dorsal closure(Chowdhary *et al.*, 2017, 2020). Here we will study the role of mitochondrial dynamics in regulating the onset of epithelial polarity in *Drosophila* embryogenesis.

2. Drosophila cellularization as a model system to study the role of mitochondrial morphology regulation in epithelial cell formation:

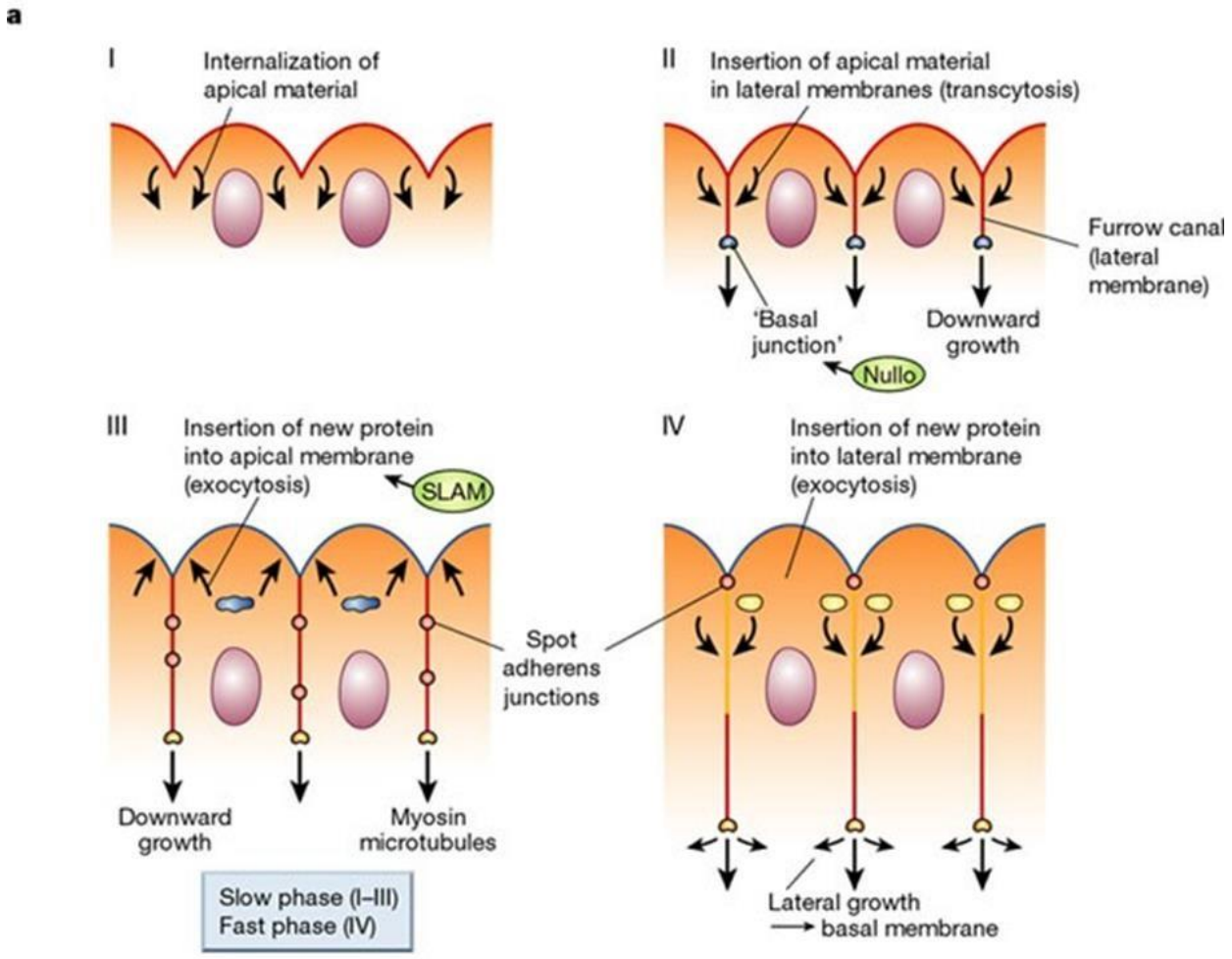
Drosophila embryogenesis begins with 9 successive nuclear divisions in the embryo interior with cycles 10-13 occurring in the periphery and in the interphase of cycle 14, cellularization occurs with ingression of membranes and occurrence of around 6000 epithelial cells(Sokac and Wieschaus, 2008). Mitochondria enriches around nuclei even before it reaches the cortex and till cycle 13, it enriches basally tothe nuclei. They are equally distributed to the daughters, restricted around their own nuclei and have microtubule-based transport. Inhibiting oxidative phosphorylation inhibits metaphase furrow formation. Mitochondria travel apically during cellularization via microtubule-dependent transport. The mitochondrial fission protein Drp1, if mutated, shows basal accumulation of mitochondria. These embryos show a decrease in contractile ring constriction with larger contractile rings and defective membrane extension which was partly rescued via increasing the ROS levels in the cells (Chowdhary *et al.*, 2017, 2020). Mitochondrial activity and morphology dynamics have been preliminarily shown to regulate cell shape changes in the early stages of *Drosophila* embryogenesis. However, whether their role is needed for epithelial polarity formation in embryogenesis is not known.

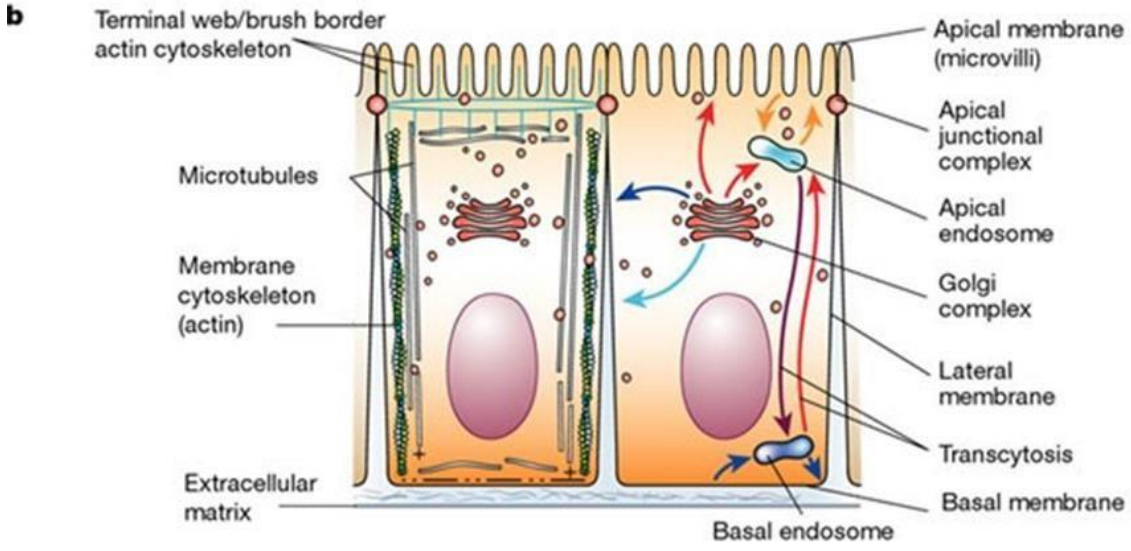
3. Mitochondria in epithelial cell formation and polarity maintenance:

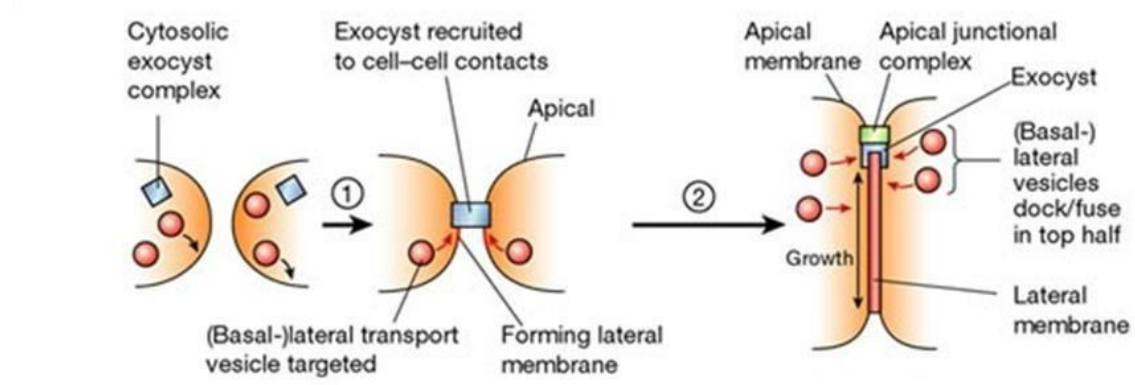
3.1. Introduction to cell polarity:

Cell polarity refers to the differential organization of cellular components inside the cell which affects its shape and structure to ultimately induce different functions to different regions of the cell. Epithelial cells demonstrate apico-basal polarity wherein lipids and proteins move to the uniquely defined positions on the plasma membrane and function in those particular domains along with a polarized cytoskeleton. The molecules inducing polarity are well-conserved across many species though their downstream effectors might vary(Nelson, 2003; Buckley and St Johnston, 2022).

Apico-basal polarity sets up the unique cell membrane domains like the apical domain facing the external environment, the basal membrane facing the extracellular matrix and the lateral membrane is which contacts the neighbouring cell. The structures of the polarity proteins are such that they possess different domains which can help physically interact or indirectly regulate the activity of other polarity proteins(St Johnston and Sanson, 2011).


a
I
Internalization of apical material
II
Insertion of apical material in lateral membranes (transcytosis)
Furrow canal (lateral membrane)
'Basal junction'
Downward growth
Nullo
III
Insertion of new protein into apical membrane (exocytosis)
SLAM
Spot adherens junctions
Downward growth
Myosin microtubules
Slow phase (I–III)
Fast phase (IV)
IV
Insertion of new protein into lateral membrane (exocytosis)
Lateral growth
basal membrane


b
Terminal web/brush border actin cytoskeleton
Apical membrane (microvilli)
Apical junctional complex
Microtubules
Apical endosome
Membrane cytoskeleton (actin)
Golgi complex
Lateral membrane
Transcytosis
Extracellular matrix
Basal membrane
Basal endosome


c
Cytosolic exocyst complex
Exocyst recruited to cell–cell contacts
Apical
①
②
Apical membrane
Apical junctional complex
Exocyst
(Basal-) lateral vesicles dock/fuse in top half
Growth
Lateral membrane
(Basal-)lateral transport vesicle targeted
Forming lateral membrane

Schematic 1: Mechanisms regulating polarity formation in Drosophila embryogenesis: Polarity induction in Drosophila embryogenesis' cellularization stage. a(I-III): Slow phase has heavy insertion of apical markers on lateral side and recycling of membrane and proteins back to the reservoir, Fig a(IV): Late cellularization has polarity markers inserted laterally. b: Polarized epithelial cell showing cytoskeletal structures on left and vesicular transport on right directly via Golgi and indirectly via endocytic travel of endosomes. c: Exocyst complex help create lateral domain. Exocyst is cytosolic and vesicles fuse basally but when Cadherin forms cell-cell contacts, Exocyst is recruited sub-apically and vesicles are now targeted laterally [6]. Adapted from (Nelson 2003).

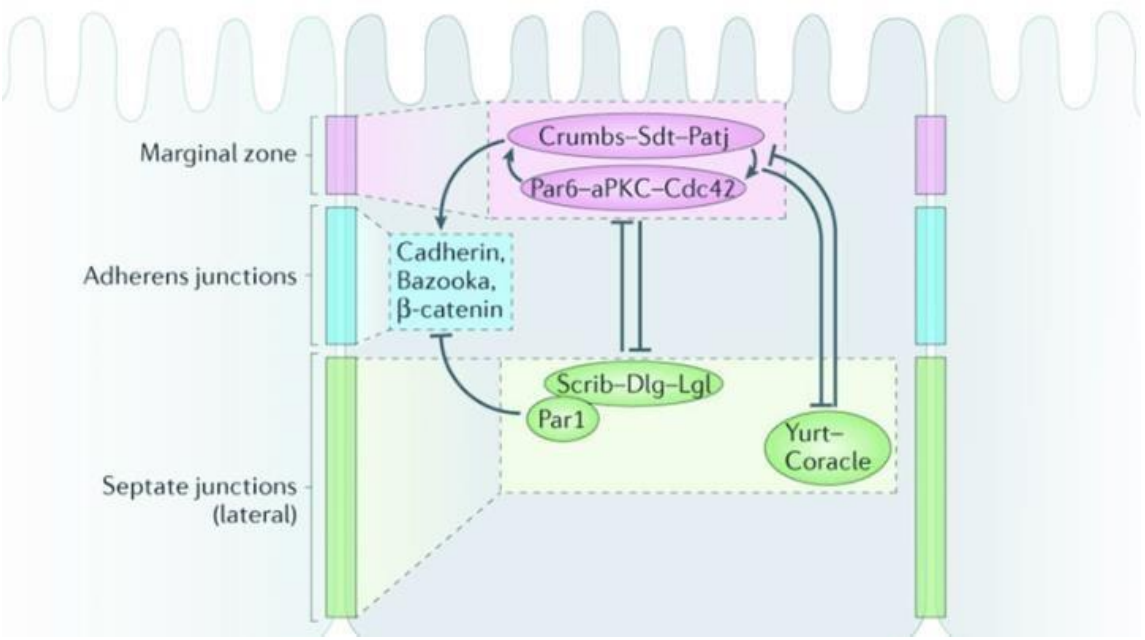
3.1.1. The Apical domain's polarity markers and regulators :

The apical domain possesses transporters and ion channels alongside different modifications like microvilli which can also be seen on the apical side of *Drosophila* embryonic cells. They act as a reservoir of actin which unfolds during cellularization. The polarity modules of Partitioning defective(PAR) includes Bazooka/Par3, Par6 and Atypical Protein Kinase C(aPKC) and the Crumbs module includes Crumbs, Stardust and PatJ proteins and these 2 modules help ensure apical membrane identity. aPKC first binds to the Par6 complex which acts as its adaptor and regulates its kinase functioning while other proteins interact with aPKC directly as well as via Par6. On the other hand, Crumbs module is a collective complex where Crumbs is transmembrane and its intracellular domain binds Stardust and Stardust binds PatJ. Bazooka seems to be the initial signal for apical polarity as it directly binds Par6-aPKC via its PDZ-binding motif and also attaches to Stardust and ultimately, recruits Par6 and Crumbs module apically.

Once Bazooka reaches the apical destination via microtubules, aPKC phosphorylates Bazooka and Cdc42 replaces it and the whole complex of Cdc42-Par6-aPKC becomes active now (Riga, Castiglioni and Boxem, 2020). aPKC via its polybasic domain binds the lipids expressed anteriorly like Phosphatidylinositol 2 and 4 phosphate. A plethora of sorting signals exist for the proteins targeted to apical domain like those of attaching N- and O-linked glycans, GPI-linkages or unique tail signal sequences. Many of the apical proteins end up in glycolipid rafts with their movement depending highly on actin. These apical proteins go via different endocytic compartments marked by Rab4, Rab8 and Rab11 endosomal markers before reaching their destination (Fölsch, Mattila and Weisz,2009).

The separation of the apical and lateral domains occurs by Adherens Junction formation characterized by the Cadherin-Catenin complexes and positioned and stabilized by Par3. aPKC inhibits the Par3 complex so that it does not go apically and the lateral marker Par1 does not allow it to come on the lateral side ultimately

restricting the localisation of Par3 at the interface of apical and lateral domains. Adherens' Junctions provide connectivity between adjacent cells, help induce and maintain polarity in a variety of organisms, transmits the environmental bio-mechanical signals to the cell and maintain overall cellular morphology. The Cadherin molecules from the Trans-Golgi network are transported to these junctions via Rab8-Rab11 and RalA GTPases (Buckley and St Johnston, 2022).



Schematic 2: Polarity modules establishing apico-basal polarity in Drosophila: Distinct polarity modules mark the different regions in plasma membrane for polarisation to occur. Apical domain is marked by Crumbs and aPKC module while the Adherens Junction has Cadherin-Catenin and Bazooka complexes and at the basolateral domain are Scribble module, Par1 and Yurt-Cora complex. Mutual antagonism exists amongst the members of different domains to ensure the appropriate domain size. Adapted from (Burki 2017).

3.1.2. The basolateral domain polarity markers' and regulators:

The lateral domain aids adhering of the cell to the neighbouring cells and mediates communication amongst them and thus, important adhesive markers reside on the lateral membrane. The basolateral domain marks the Scribble complex consisting of DLG (Discs Large), LGL (Lethal Giant Larvae) and Scribble wherein DLG binding to lateral membrane recruits Scribble to create the septate junctions not allowing cell-to-cell diffusion of molecules. DLG in turn via its GUK domain also binds LGL which is a prime target of aPKC for phosphorylation so that LGL does not enter apically. This domain is also maintained by the Par1/MARK complex which phosphorylates

and recruits' proteins laterally. They also prevent LGL phosphorylation by aPKC at the lateral membrane (Riga, Castiglioni and Boxem, 2020). The basolateral proteins have dileucine, tyrosine or both peptide groups in their tails which is recognized by clathrin adaptor protein AP1B and puts the cargo in vesicles which ultimately fuse with the exocyst localized at the basal junctions. The process is highly aided by Rab10 GTPase as mutations in it help transport the cargo apically (Fölsch, Mattila and Weisz, 2009). The lateral membrane proteins have domains like those of PDZ which can help in the sequential recruitment of other proteins containing PDZ.

Though cells might be structurally and functionally diverse, the mechanism to induce polarity remains the same with localized assembly of molecular cues which recruit cytoskeleton locally for directed and polarized vesicle trafficking (Nelson, 2003).

3.2. Maintenance of cell polarity:

Cellularization helps form the first complete epithelial cell membrane in *Drosophila* embryogenesis, and also shows the onset of zygotic genome activation. Membrane extension is accompanied with the formation of epithelial polarity with differential insertion of proteins and lipids on the invaginating membrane (Lecuit, 2004). Polarity establishment in cellularization occurs via polarized insertion of new membrane and proteins as membrane ingresses. Firstly, basal junction separates furrow canal from the somatic buds and as membrane starts to invaginate, new membrane inserts apically while the apical membrane goes on the lateral side and in the fast phase, membrane insertion shifts from apical to the lateral side (Lecuit and Wieschaus, 2000). The secretory pathway allows membrane insertion and setting of polarity markers on lateral side and the process is aided by microtubules.

Polarity is induced and maintained by diverse polarity protein regulators, phosphoinositide's of the cell membrane, the dynamics of the cytoskeleton and the GTPases. Once established, these domains are maintained via vesicular targeting as well as by junctional proteins not allowing the components to move out of their unique domains thus, elucidating the importance of maintenance of these domains. The Trans-Golgi network is the primary sorting place for synthesized proteins for migration to different membrane regions via different pathways .Correct vesicular targeting of the polarized membrane seem to be dependent on the exocyst complex localisation during cellularization, the pairing of V and T-Snare as apical region has Syntaxin-3 T-Snare while the basal side has Syntaxin-4 T-Snare as well as on the differences in lipid composition as PI(4,5)P2 is seen on apical side while PI(3,4,5)P3

localizes on basal side. Rab GTPases affect the stability of exocyst and Snare complexes(Wj and C, 2001; Spiliotis and Nelson, 2003).

3.2.1. Mutual interactions amongst the polarity markers to maintain the domain sizes:

The sizes and positions of the respective domains are determined by antagonistic interactions of the domain markers as apical markers inhibit lateral markers and vice-versa (Schematic 2) (St Johnston and Sanson, 2011). LGL and Par2 are lateral membrane markers and bind via the hydrophobic effect as well as by their basic groups but aPKC on the apical side phosphorylates these lateral markers to diminish these charge effects so that these markers do not bind apical membrane. aPKC also phosphorylates Par1/MARK of lateral membrane and Bazooka to not accumulate it apically. LGL, in turn, physically binds Par6-aPKC to inhibit membrane binding and kinase functioning in the lateral membrane. Par1/MARK of lateral membrane inhibits Bazooka accumulation laterally via phosphorylation and also prevents the activity of aPKC downstream. Recently, Yurt proteins are also characterized which via oligomerization, negatively affect aPKC and Crumbs localisation laterally and also restrict their activity to some extent apically and aPKC reciprocally acts to inhibit Yurt by disallowing its oligomerization(Riga, Castiglioni andBoxem, 2020; Buckley and St Johnston, 2022).

3.2.2. Role of the endocytic components to maintain polarity:

Though most studies hint towards polarized exocytosis in inducing polarity, the role of endocytic regulators is also getting unraveled. Genetic screens help identify vital endocytic polarity regulators such as Avalanche which helps in early endosome creation but its mutations mis localized apical proteins at the basal side and also resulted in faulty adherens' junction formation. In non-epithelial cells like those of the *C.elegans* embryo, Par6 polarity proteins localize anteriorly while Par2 goes posteriorly. Par6 and Par2 constantly endocytose using dynamin, enrich in the early endosomes and again re-localizes apically and basally respectively. The endosomes formed can also mature in late endosomes and if late endosomes do not form, Par2 levels drop down at the posterior side. Similarly, Oskar mRNA localizes posteriorly in *Drosophila* oocytes and is maintained by constant endocytosis and recycling posteriorly and mutation in early endosomes causes rapid accumulation of Oskar mRNA in the cytosol(Shivas *et al.*, 2010). The above examples illustrate the role of endocytic regulators in maintaining polarity and thus, it stresses the importance of

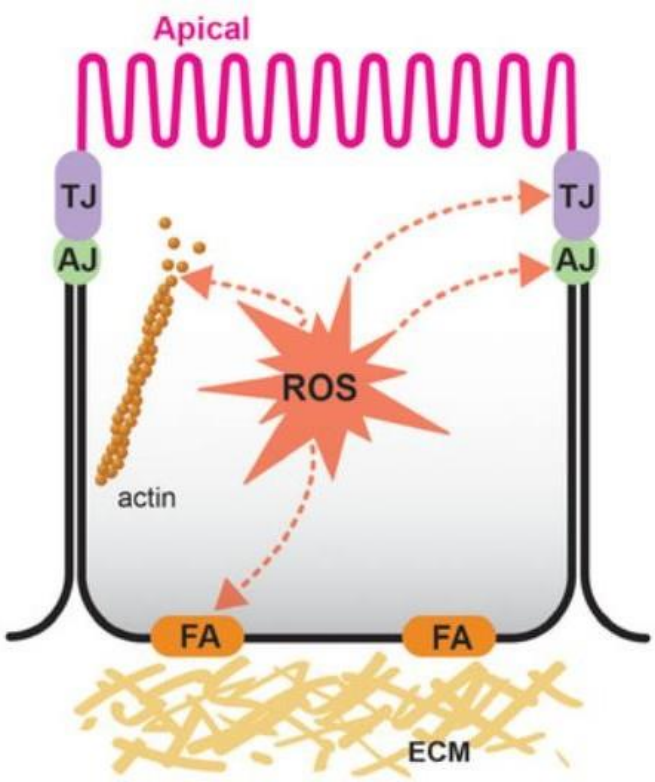
studying the role of early, late and recycling endosomes in induction of polarity in the *Drp1*^{SG} embryos.

3.3. Importance of cell polarity:

The polarity established helps in a range of functions from selective absorption and secretion to maintaining the overall cellular architecture and integrity. The dynamic nature of polarity molecules does aid in developmental processes like apical constriction and wound healing owing to immediate changes in cell shape and function which can allow or inhibit cellular movement. Apical-basal polarity mediates morphogenesis and determines relative sizes of apical, basal and lateral domains with the respective proteins marking them. Lateral domain connects adjacent cells; increasing sizes of apical and basal domains can increase surface area of cell, make it cuboidal to squamous and thin the epithelium but if lateral domain increases, the surface area decreases, cells turn cuboidal to columnar and epithelium thickens. Polarity markers also affect actomyosin contractility as certain apical markers like Crumbs and aPKC help organize actomyosin network and contractility respectively (Spiliotis and Nelson, 2003). Mutations in the proteins regulating polarity do cause a variety of diseases like that of microvillus inclusion disease where syntaxin-3 is mutated and Rab11-vesicle delivery is severely affected as well as defects in neural tube formation occur if lateral domain proteins like Scribble are mutated (Golachowska, Hoekstra and van IJzendoorn, 2010). Cancers seem to affect the polarity markers of Par3 and Par6 which maintain the adherens junctions to acquire migratory abilities (McCaffrey *et al.*, 2012). This explains the importance and maintenance of the polarity markers in their respective domains.

3.4. Mitochondria's role in inducing and maintaining polarity:

The mitochondrial dynamics in cellularization (Chowdhary *et al.*, 2017, 2020) is studied and it is highly possible of mitochondria involving in establishing polarity, helping regulate and maintain the subcellular locations of polarity proteins and regulating their functions via intermediates like ATP, ROS and calcium(Giacomello *et al.*, 2020) . The polarity markers can also affect the dynamics of mitochondria as well. The mitochondria-polarity crosstalk has surfaced recently.



<u>Schematic 3: Polarity modules as targets of ROS:</u> It is now known that Reactive Oxygen Species (ROS) can act on tight and adherens' junction components as well as Focal Adhesion and actin and thus, play an important role in establishingand maintaining polarity modules. Adapted from (Hebbar et, al. 2021).

Reactive Oxygen Species stem from the products of molecular diatomic oxygen to produce free radicals and reactive molecules. Mitochondrial electron transport chain is amongst the key producers of ROS and their abundance is tightly regulated in the cell by antioxidants such as superoxide dismutase (SOD). ROS can affect proteins involved in polarity like that of oxidation of Src tyrosine kinase important in DE-Cadherin recycling and can also cause independent activation of small GTPases like Rho, Rac and Cdc42 which maintain the polarity markers. Due to ROS oxidation of Src kinase in retinal cells, phosphorylation of p120 catenin occurs which generally remains a part of adherens' junction but its oxidation causes N-Cadherin to be globally removed and thus, cells lose their contacts. In various morphogenetic processes like wound healing, dorsal closure and delamination in thorax, it is well-established that mitochondrial ROS helps recruit actin and myosin at the respective sites by affecting contractility regulators like ROCK(Hebbar and Knust, 2021). It will be interesting to note that Drp1 mutant embryos also have lessened mitochondrial ROS and if this ROS can turn out to be the defining factor in myosin recruitment at the cellularization tip. In differentiating *Drosophila* midgut cells, if mitochondrial ROS escalates, it affects the localisation of adherens' junction components such that cells detach from one another and differentiation ceases. In embryonic wound healing, mitochondrial ROS allows E-cadherin trafficking and myosin accumulation around wounds via action on Src42 proteins vital in junctional protein dynamics(Hunter *et al.*,

2018). Epithelial-to-Mesenchymal transition displays changing mitochondrial morphology from fused to spherical allowing less ATP and more ROS generation ultimately leading to the detachment of polarity markers to acquire migratory ability. This explains the importance of ROS in maintaining the polarity markers in healthy cells and thus, it will be critical to know if the reduced levels of ROS in *Drp1*^{SG} mutants affect the onset of polarity. Inducing fused mitochondria in *Drosophila* follicle cells affect the localisation and activity of important polarity proteins like aPKC. Escalating oxidative stress via mitochondrial fission aids in EMT and promotes glycolytic switch in hepatocytes and MCF7 cells. Lung cancer studies indicate that altered mitochondrial function downregulates epithelial markers like E-Cadherin and upregulates mesenchymal marks (Madan *et al.*, 2021).

Polarity markers themselves can also induce changes in ROS levels of the cells. The Crumbs protein which helps define the identity of the apical domain controls Rac1 GTPase activity so as to reduce the cellular ROS. It can also affect the trafficking pathways which helps maintain polarity wherein Crumbs mutants form abnormal *Drosophila* eyes and Rab11-dependent transport of eye components severely reduces due to ROS levels altered (Hebbar and Knust, 2021). Fat Cadherin has one of its subunits cleaved which migrate towards complex 1 of mitochondria to reduce the ROS levels but if the binding is altered in any way, ROS levels tend to increase and ultimately hamper the planar cell polarity in the eye (Sing *et al.*, 2014). Thus, we are interested in evaluating the role of mitochondrial morphology regulation in establishing and maintaining polarity in cellularization.

□

- **Aims of the project**:

1. Studying plasma membrane and actin remodelling in fission mutants of mitochondria

2. Studying epithelial architecture formation in fission mutants of mitochondria

3. Understanding the mechanisms that lead to defects in epithelial polarity formation in mitochondrial fission mutants

- # Chapter 3: Material and methods

1. Drosophila stocks and genetics:

-Cornmeal agar medium was fed to the flies which were kept at 28 degrees Celsius
- The UAS-Gal4 method is used to create mutations in flies as females with the Gal4 construct if crossed with males of UAS construct fused with the gene of interest will express the gene in progeny. Mitochondrial fission involves Dynamin-related protein and thus, to study mitochondrial fission mutants, UASp-*Drp1*^{SG} mutants were made which has the conserved Ser-193 in the GTPase domain mutated to glycine(Chowdhary *et al.*, 2020). The recombinant line *nanos*-Gal4: UAS-mito-GFP was used as the driver with mito-GFP marking mitochondria while *nanos*-Gal4 producing Gal4 protein. We expressed *Drp1*^{SG} using the recombinant fly stock *nanos*-Gal4: UAS-mito-GFP line and then collected the eggs of the F1 progeny because it is this embryo which has the defect due to maternal dumping. For control embryos, the recombinant line was crossed to the flies having *w*^{1118} genotype conferring white eyes. For the *Drp1*^{SG} phenotype epistasis experiments via increased levels of ReactiveOxygen Species, superoxide dismutase 2 mutants were used. For control, we crossed lines of *nanos*-Gal4 with UAS-Sod2RNAi at 28 degrees Celsius and collected the F1 progeny.. For the 'Drp1^{SG}; Sod2i' line, we similarly crossed *nanos*-Gal4 with the recombinant line UAS-*Drp1*^{SG} ; UAS-Sod2RNAi. Thus, it simultaneously allows the Gal4 to drive *Drp1*^{SG} and Sod2RNAi phenotype in the F1 eggs. For DE-CadherinGFP live imaging, the line 'EndoCadGFP/ Sm6A; nanosGal4/ Tm6Tb' was crossed at 25 degrees Celsius with W1118 for control and *Drp1*^{SG} for the mutant and non-balancer flies are selected. For BazookaGFP live imaging, the line 'BazGFP/ CyO; nanosGal4/ Tm6Tb' was crossed at 28 degrees Celsius with W1118 for control and *Drp1*^{SG} for the mutant and non-balancer flies are selected.

2. Immunostaining:

The 2.5-3-hour old embryos were collected from the egg chamber and are subjected to the standardized embryo staining protocol via hand devitellination. Collected eggs are first treated with bleach for a minute to remove the outer chorionic layer and later with heptane and PFA to remove the waxy layer with heptane trapping PFA and allowing the transport across vitelline to fix the egg tissues and later, the vitelline is removed by needle in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4). 3 PBST (Triton X-100,0.3 %)

washes for 5 minutes allow the removal of membranes and blocking was done for 1 hour with 2 % BSA. Primary antibody is added in embryos in the PBTA solution and kept for 12- 16 hours and again washed with PBST 3 times for 5 minutes. Secondary antibodies are added and kept in for an hour in dark conditions and again washed with 3 times with PBST with second wash being of the nuclear stain Hoechst. Mounting was aided by Slow fade Gold and slides were stored at 4 degrees Celsius.

For antibody staining of amphiphysin, myosin II, DLG and PatJ, methanol devitellination was used. The collected eggs are first treated with bleach for a minute to remove the outer chorionic layer and later with heptane and methanol. The scintillation vial is shaken rigorously for a minute to remove the waxy and the vitelline layer and the eggs which have these coverings removed go basally in heptane which are then collected. These embryos can be stored at cold temperatures and used later or can be given subsequent washes of PBS or PBST. The primary and secondary antibodies are added and subsequently mounted and slides can be stored at 4-degree Celsius.

Live cell imaging is done by collecting the eggs in the egg chamber and putting in bleach for 1 minute followed by washes with distilled water and complete drying by soft tissue. Dried embryos are mounted in LabTek Chamber and it is then filled with PBS for subsequent imaging.

Concentrations of the antibodies are: Phalloidin Alexa 647 (1:500), Streptavidin Alexa 488 (1:500), Discs Large Homolog (1:500) raised in mouse, Amphiphysin (1:300) raised in rabbit, Ras analog in brain Rab5 (1:500) raised in rabbit, DE- Cadherin (1:10) raised in rat, Ras analog in brain 7 (1:500) raised in rabbit, PatJ(1:100) raised in rabbit, Ras analog in brain 11 (1:500) raised in rabbit, myosin II(Spaghetti Squash 1:500) raised in mouse , and Hoechst dye for DNA (1:1000)

3. Imaging:

The confocal laser scanning microscopes of Zeiss LSM 710 or 780 were used having the oil immersion objective of Plan Apochromat 63X/ 1.4 Numerical Aperture. X-Y and sagittal images were acquired at constant frame size of 71*71 microns with scan speed of 9. Z-stacks were obtained at 1 micron thickness with scan speed of 9 and optical zoom of 1.9X. Z-stacks are taken from apical to basal side while sagittal view is taken when nuclei are properly aligned. Imaging was done on 8-bit scale in the dynamic range of 0-255. It was ensured that the image pixels are not saturated during imaging. Live cell imaging had similar settings with 1 micron as the interval between slices and 3 minutes as total time for stack to be completed. Image

representation is done by cropping an image of 256*256 pixels from the appropriate slice of the Z-Stack. X-Z view of the image is represented by ‘Orthogonal View’ wherein it is ensured that most of the nuclei remain aligned when selecting for the ortho view.

4. Quantification:

‘Fiji’ was the software used all throughout for image analysis and ‘GraphPad Prism’ was the software used for plotting and data analysis.

1. Cell size was measured using Polygon tool in Fiji by drawing polygon along the cell membrane and measuring its area while furrow lengths are measured using Line tool in Fiji where the length of a line drawn along the furrow was measured in a sagittal image. Similarly, length of Amphiphysin endocytic tubules is also measured by Line tool. Basal Area is also measured using Polygon tool wherein a polygon is drawn along the ring and its area is measured. While measuring the apical and basal area, care was taken that the orthogonal view in Early images range from 5-6 microns, mid stage having the rangefrom 9-13 microns and Late having the range from 18-24 microns. For cell area quantification in Syncytial Cycle 13, only those images across both genotypes are considered where the orthogonal length is above 8.5 microns because at lengths below this, immense variation in cell size is seen across both genotypes. Also, the cell area highly depends on a variety of factors during sample preparation and imaging. Those images are neglected from the quantification where there were mounting issues so that squished embryos do not present any challenges in the quantification. Also, the cell size does not remain uniform across the embryo and it seems that the size reduces at the edges so that the images under quantification donot show this area up. Only the area of hexagonal cells is taken and not of the pentagonal or other polygonal cells which also show up.
2. Apical intensity of mitochondria was done by sum projection of slices from the slice where cell started up to the slice where nucleus is seen and measured using Fiji and was normalised to the nucleus as background and to the number of stacks.
3. For quantification of DE-Cadherin at the apical adherens’ junction, we first took the sum intensities for the slices in Z-stack which showed the adherens’ junction and measured the intensity of DE-Cadherin using the segmented line tool in Fiji around 10 cells. For background, we go deep inside the nucleus and return to the DE-Cadherin channel to see the darkest region and measure it. For all the cells, the DE-Cadherin intensity is divided by the number of slices in the apical adherens’ junction and further divided by the background. The quantification is done only in the mid and

late stages as apical adherens' junction is seen only there and the quantification is done for Control, *Drp1*^{SG} mutant, Sod2 RNAi and *Drp1*^{SG}; Sod2 RNAi genotypes. The same is done for basal DE-Cadherin quantification but while choosing the background, that slice is selected prior to which the first signal for DE-cadherin appears. In live cell imaging, apical belt is quantified wherein at late stage where the belt is seen, sum projection of the slices is taken, intensity is measured along the membrane and divided by number of stacks and background.

4. For BazookaGFP quantification, orthogonal views are opened and sum projection of the slices is taken along the furrow where Bazooka is seen and is divided by the background and the number of stacks.

5. For PatJ quantification in the *Drp1*^{SG} mutant, the cytoplasmic PatJ dots are individually counted across the cells. In FIJI software, 'Auto Threshold' was used with the thresholding method set to Default with the subsequent selection of 'Set Threshold' icon. This was done so that PatJ puncta having intensity higher than the set value by the software will be counted in the quantification. The cells are further classified having 1,2 and greater than 2 cytoplasmic PatJ dots.

6. For DLG intensity quantification at the basal junction, we first chose the plane where the ring actually starts and PatJ channel was used here. We then chose 3 slices above this plane and made sum slices for it. DLG intensity was measured around 10 cells using polygon tool in Fiji. For background, we go deep inside the nucleus and return to the DLG channel to see the darkest region and measure it. For all the cells, the DLG intensity is divided by the number of slices in the basal junction and further divided by the background.

7. For BazookaGFP quantification, orthogonal views are opened and sum projection of the slices is taken along the furrow where Bazooka is seen and is divided by the background and the number of stacks. Plots are done using GraphPad Prism software.
8. For Rab11 quantfication, 3 planes are chosen right below the centriolar sub-apical plane, sum projection is done and Rab11 intensity is measured along the membrane and is divided by the cytoplasmic background.

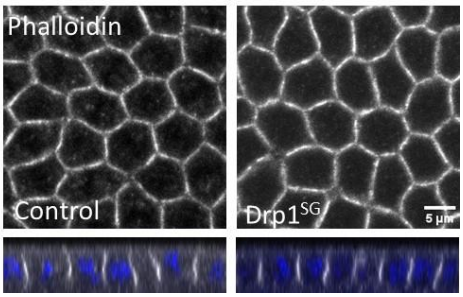
- # Chapter 4: Results and Discussion

The current work involves assessing the role of mitochondrial fission in plasma membrane remodeling and polarity formation in early *Drosophila* embryogenesis. Over expression of the *Drp1*^{SG} mutant was carried out in *Drosophila* embryos to further examine the defects in epithelial polarity formation in cellularization. The results first examine the defects in epithelial polarity markers and then a role of trafficking pathways is regulating this polarity in Drp1 depleted embryos.

1. *Analysis of plasma membrane remodelling in syncytial cycle 13 and cellularization in embryos deficient for mitochondrial fission protein Drp1:*

Cells from syncytial cycle 13 embryos stained with phalloidin

A.



B.

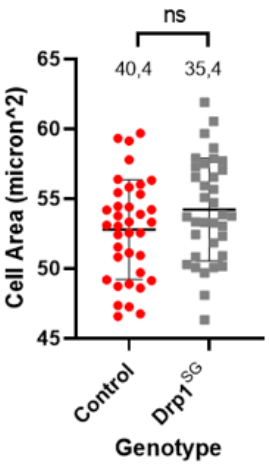


Figure 1: Apical area quantification in metaphase of syncytial cycle 13.

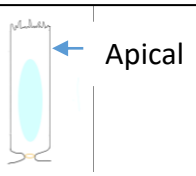
A. *Representative images of from embryos in metaphase of syncytial cycle 13 stained with phalloidin. Shown below are the orthogonal views respectively.*

B. *The cell area is represented as average ± SD. The statistical analysis of the data is carried out via Mann Whitney t-test. n=4 embryos (40 cells) in control and Drp1*^{SG} *mutants had n=4 embryos (35 cells) analyzed.*

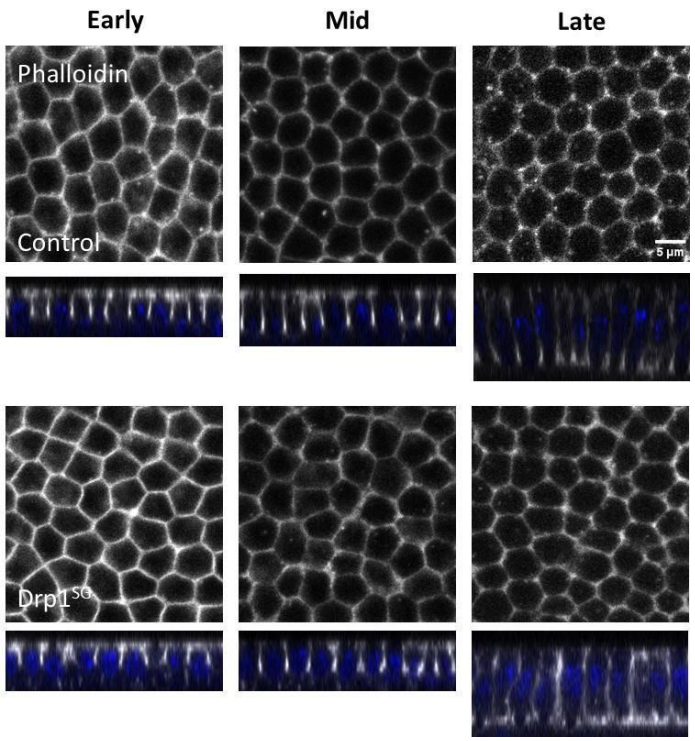
In the syncytial division cycles in the *Drosophila* embryo, there is membrane furrowextension in metaphase in between adjacent nuclei. We expressed *Drp1*^{SG} and tested if there were changes in apical area. *Drp1*^{SG} expressing embryos have similar apical area with respect to control embryos in cycle 13 (Figure 1). Cellularization shows an increase in membrane length to form complete polarised cells which are approximately 40 microns in height (Sokac and Wieschaus, 2008). We estimated the change in apical area in early, mid and late stages of cellularization. We found that the apical area remained similar in embryos across all the cellularisation stages (Figure 2). This suggests that cortical actin tension, the players regulating it and the overall cellular architecture is similar in *Drp1*^{SG} embryos as compared to the control across all cellularization stages

It was shown that mitochondrial mutants having mutations in the complexes involved in the Electron Transport Chain (ETC) have reduced furrow extension in the syncytial cycles (Chowdhary *et al.*, 2017). It is observed that even in embryos with highly fused mitochondria, furrow extends to similar levels as seen in control and thus, verifies that ATP levels do not affect furrow extension in the *Drp1*^{SG} embryos.

Cells across cellularization stained with phalloidin



A.



B.

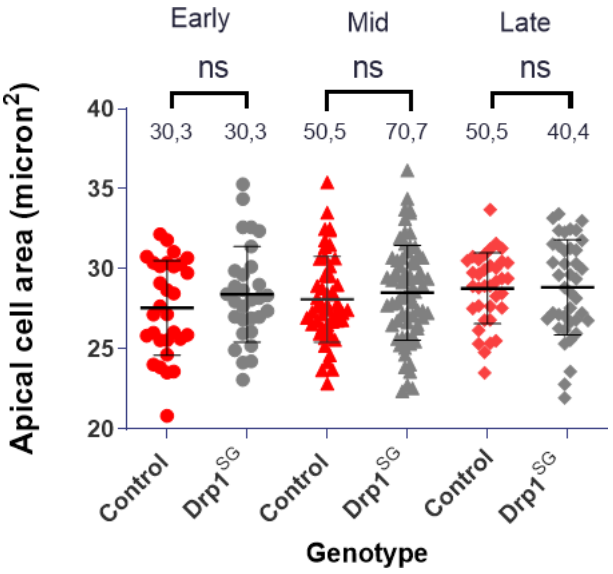


Figure 2: Estimation of apical area during cellularization.

A. *Sample images stained with phalloidin staining performed and similar apical surface area was observed for Drp1^{SG} mutant all across the early, mid and late stages. Shown below are the orthogonal views respectively.*

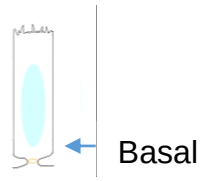
B. *The apical area is represented as average ± SD. The statistical analysis was carried out by Mann-Whitney t-test. n=3 embryos (30 cells) in control early, n=5 embryos (50 cells) in control mid, n=5 embryos (50 cells) in control late. For Drp1^{SG}, n=3 embryos (30 cells) in mutant early, n=7 embryos (70 cells) in mutant mid and n=4 embryos (40 cells) in mutant late*

Cell shape and size is highly controlled by the cytoskeleton and the mechanobiological forces that a cell experience. In *Arabidopsis thaliana* epidermal cells, it was seen that the conical shape of the cell is maintained by optimal ROS levels and decreased levels of ROS directly affected the microtubule organisation and thus, caused alteration in the shape of the epidermal cells (Dang *et al.*, 2018). However, this is in complete disagreement with the phenomena in the *Drp1^{SG}* embryos. It is known that the reduced levels of ROS in *Drp1^{SG}* embryos do not affect the microtubule organisation and the microtubule arrays are very similar to control embryos (Chowdhary *et al.*, 2020). As microtubules are important mediators of cellular architecture and form, it is highly possible that as it is not affected in the *Drp1^{SG}* embryos, we do not see a striking change in the cell shape. It might be probable that reduced levels of ROS do not directly affect the players involved in regulating the overall cellular architecture or that the embryo has other redundant mechanisms that allow it to maintain its size and shape. The actin polymerisation and depolymerization in the cortex remains dynamic. It is well-established that excess levels of ROS in the cells do affect the players involved in regulating the cell shape determinants negatively. The excess ROS help form disulphide bonds on B-Actin to reduce its stability and polymerisation as well as add glutathione on certain amino acids in the actin monomers to prevent their addition. Tyrosine Kinase Src is an important regulator of actin at the cortex and is crucial in mediating polarity and excess levels of ROS can oxidise it to affect the cell size (Hebbar and Knust, 2021). Comparing this to the fact that the levels of ROS reduce in *Drp1^{SG}* mutants, it can be believed that certain proteins vital in maintaining cellular architecture do get prevented from the detrimental effects of ROS and thus, the overall cellular

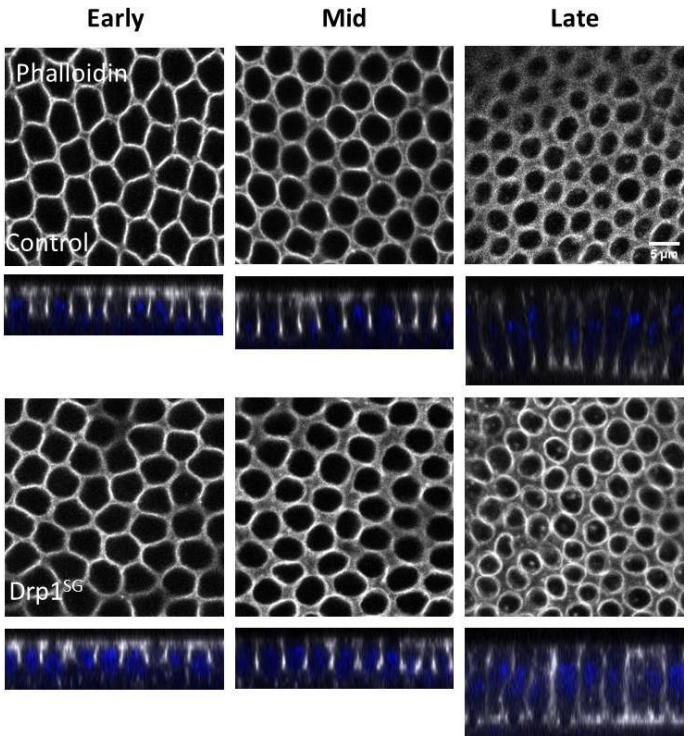
morphology remains unperturbed in the mutant.

Further in my work, I demonstrate that polarity factors do get affected in terms of their localisation and intensity in the *Drp1*^{SG} mutant. As the cell size remains similar, it is highly possible that the altered distribution of the polarity factors do not affect the overall cell size in the non-epithelial cells and it is when the cells acquire complete epithelial characters at the end of cellularisation, the introduced polarity factors' dynamics affect the cell morphology dynamics. It seems that correct cytoskeletal architecture and appropriate cortical tension are sufficient to ensure proper polygonal shape even in the *Drp1*^{SG} mutants. Thus, we can see that in *Drp1*^{SG} embryos having non-epithelial cells of syncytial cycles and cellularisation where polarity is just being set up, polarity factors do not seem to be a major candidate to maintain the cellular morphology.

Basal acto-myosin rings in cellularisation stained with phalloidin



A.



B.

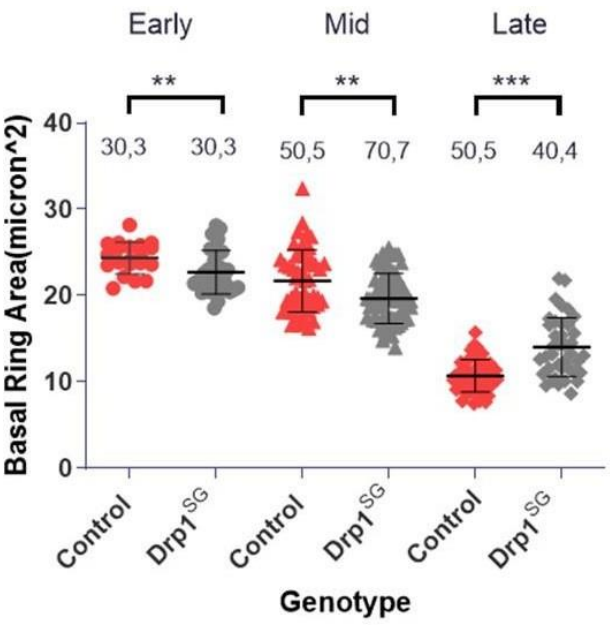


Figure 3: Estimation of basal ring area in cellularization.

A. *Phalloidin staining done and increased basal ring area was observed for* $Drp1^{SG}$ *mutant all across the early, mid and late stages. Shown below are the orthogonal views respectively.*

B. *The basal ring area is represented as average ± SD. The statistical analysis was carried out by Mann-Whitney t-test. n=3 embryos (30 cells) in control early, 5 embryos (50 cells) in control mid, n=4 embryos (40 cells) in control late. For* $Drp1^{SG}$*,n=3 embryos (30 cells) in mutant early, n=7 embryos (70 cells) in mutant mid and n=4 embryos (40 cells) in mutant late.*

We estimated the change in basal ring area in early, mid and late stages of cellularization. We do see that the rings form in the early stage, they slowly start to constrict throughout the mid stage because this demonstrates slow phase of cellularisation and as soon as the late stage initiates, rapid furrow ingression commences and the ring completely constricts via the action of actin and myosin. We found that the basal ring area did not remain similar in embryos across all the cellularization stages (Figure 3) as contrary to the apical cell area. We did see premature ring constriction right from the early stage of the $Drp1^{SG}$ mutant and thering remains hyper-constricted even in the mid stages. Interestingly, as pointed in previous work, we also did observe defects in ring constriction in the late stage. Thus, the epithelial cells are not completely formed as the ring does not pinch off completely during cellularization and thus, we observe larger ring size during at the end of cellularization. This shows that the rate of ring constriction differs as we move from the mid to the late stage in the $Drp1^{SG}$ mutant.

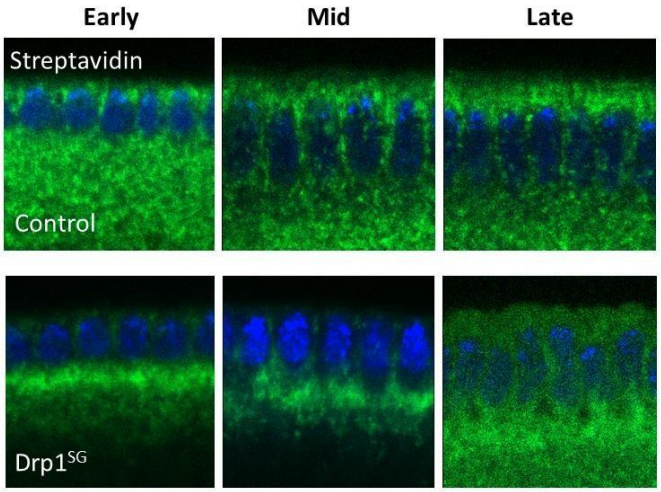
The early stage marks premature constriction and it is highly possible that the furrow canal forms much earlier in the $Drp1^{SG}$ mutant than the control and this

furrow canal might constrict in the mutant at the end of early cellularization. Genes crucial in cellularization like Nullo and Serendipity-alpha help recruit actin at the basal ring and it is possible that these proteins work much early in the mutant when the furrow canal forms. Alongside it, genes like Dunk can also work earlier in mutant which helps the recruitment of myosin II from the apical cortex and the accumulative effect of all these proteins in early recruitment of actin and myosin II might account for the decrease in the basal ring area(He, Martin and Wieschaus, 2016). Bottleneck is a gene which does not allow for the basal ring constriction and thus, regulates constriction and this can precisely be defective in the *Drp1*^{SG} mutant thus, allowing for constriction right from the early stages(Sokac and Wieschaus, 2008). From 10-12 microns of furrow ingression, we see that the rings did constrict in the control but the amount of constriction seem to be increased in the *Drp1*^{SG} mutant as the ring area severely reduces. The forming of the furrow canal also marks the migration of important polarity factors like Discs Lost (dlt) and PatJ in the forming furrow canal and this might account for the early settling of the polarity factors as well as of the genes recruiting acto-myosin in early cellularization.

The late stage wherein rings do not constrict also ensure for shorter furrow membrane in the *Drp1*^{SG} mutant. As actin and myosin II remain key players for constriction, myosin II seems the one which is affected in the *Drp1*^{SG} mutant. ROS in many systems helps recruit actin and myosin II and inhibiting the levels of ROS adversely affect the processes like embryonic wound healing and dorsal closure(Mateus *et al.*, 2011; Madan *et al.*, 2021). It might be possible that reduced ROS levels in the embryo might inhibit the components of the signalling pathways which help recruit myosin. Slam is one of the genes which help recruit myosin II from mid-cellularization and continues to late stage and Slam can be a potential candidate whose functioning remains deactivated in *Drp1*^{SG} embryos in late cellularization(He, Martin and Wieschaus, 2016). Optimum levels of ROS have been involved in activating certain amino acid residues of the active site of proteins and it can be possible that proteins in myosin II recruitment or myosin II itself do require oxidation of certain residues which is just not possible in the *Drp1*^{SG} embryos. The shorter furrows owing to the larger ring size will definitely have impact on the localisation of polarity factors because in completely formed epithelial cells, we see their appropriate localization but as rings do not completely constrict in the mutant, it will be diificult for the polarity factors to ensure their complete localisation.

Mitochondria in cellularization stained with streptavidin

A.



B.

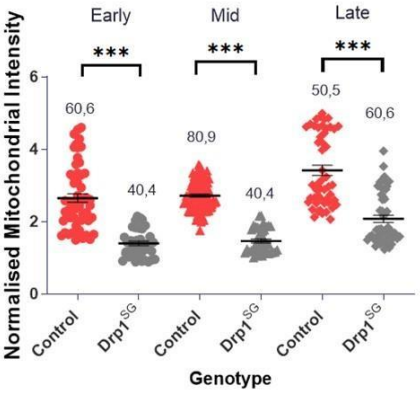


Figure 4: Estimation of mitochondrial intensity in the apical sections during cellularization.

- ***A.*** *Streptavidin is used to stain mitochondria and it was observed that mitochondria do not travel apically in Drp1^{SG} mutant and can be seen across early, mid and late stages. Images show fused mitochondria residing basally in the mutant. The orthogonal views are represented.*
- ***B.*** *The normalised mitochondrial intensity is represented as average ± SD. The statistical analysis was carried out by Mann-Whitney t-test. n=6 embryos (60 cells) in control early, n=9 embryos (80 cells) in control mid, n=5 embryos (50cells) in control late. For Drp1^{SG}, n=4 embryos (40 cells) in mutant early, n=4 embryos (40 cells) in mutant mid and n=6 embryos (60 cells) in mutant late*

Mitochondria have been previously shown to accumulate at the base in *Drp1*^{SG} expressing embryos(Chowdhary *et al.*, 2020). We estimated the apical mitochondrial intensity in our experiments by staining the embryos for mitochondria with fluorescently coupled streptavidin. In control images, we see that little amounts of mitochondria rise up at the apical side at the end of early cellularization and the mitochondria continue migrating at the mid cellularization as seen from the plots of normalised intensity. They fully complete their migratory activity at the end of late cellularization. As expected from previous studies, we found that there is decreased mitochondrial intensity in apical regions in *Drp1*^{SG} expressing embryos and this occurs due to lack of migration of mitochondria to apical regions (Figure 4). We see little to no accumulation of the mitochondria at the apical side in early and mid-stages for the mutant and only a few of them travel apically in late cellularization. . The mitochondrial migration happens via dynein motors on the microtubule network and dynein mutants show similar basally accumulated mitochondria as that of the *Drp1*^{SG} mutants. Dynein helps transport autophagosomes and fuse it to the lysosomes in artery myocytes and ROS has been a key player to improve Dynein-based ATPase activity and the autophagosome movement(Xu *et al.*, 2014). It can be possible that reduced ROS levels in the *Drp1*^{SG} mutants do not allow for the dynein-based activity to initiate. The favorable case still remains that it is more convenient and easier for dynein to carry fragmented mitochondria instead of long, tubular ones and thus, we observe diminished apical mitochondrial intensity. Differing mitochondrial morphologies are also seen in systems like those of the neurons where dendrites have denser and fused mitochondria as compared to the sparse ones at the axons wherein the mitochondria at the post-synapse produce ATP for supporting the dynamic cytoskeletal architecture as well as neurotransmitter regulation(Seager *et al.*, 2020). The functional consequence of the apical migration in *Drosophila* cellularization is yet to be elucidated.

To summarize, we did not observe noticeable defects in the cell surface area in the *Drp1*^{SG} mutants but we do see changes in the basal ring area all across cellularization with less constriction and larger rings at the end of cellularization. As expected, mitochondria are fused and remain basally all throughout cellularization.

Next, we analysed the different polarity markers and saw if defects were observed in the *Drp1*^{SG} embryos.

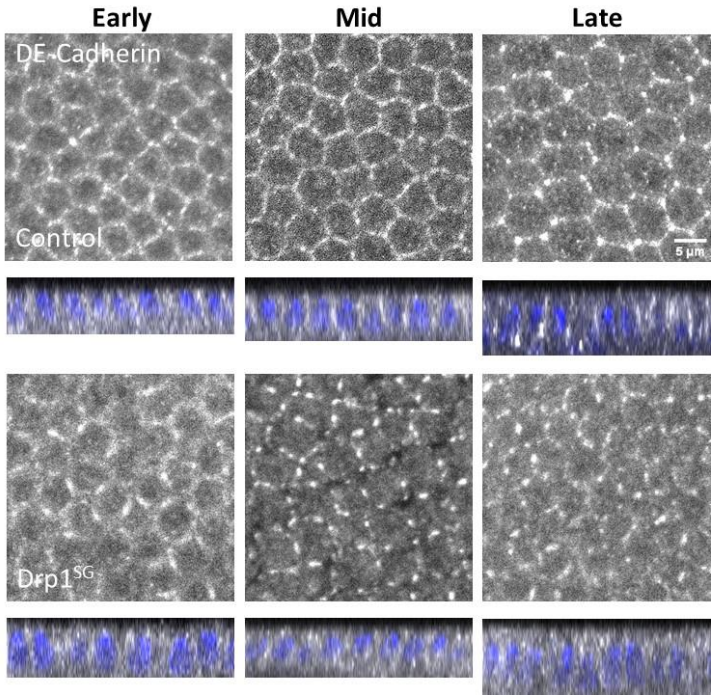
2. Analysis of polarity protein distribution in embryos deficient for mitochondrial fission protein Drp1:

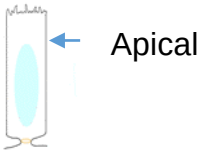
The following section highlights the study of polarity proteins in terms of their localisation and intensity throughout cellularization in control and *Drp1*^{SG} mutant embryos. Broadly, the Adherens' junction protein DE-Cadherin is examined to visualize its positioning in the apical and basal adherens' junction, Bazooka is studied in apical zone which help recruit DE-Cadherin .Discs large Homolog (DLG) is studied to look at the lateral membrane development and Pals-Associated Tight Junction (PatJ) is studied for its basal junction localisation.

2.1.1. Analysis of Adherens' Junction proteins in embryos deficient for mitochondrial fission protein

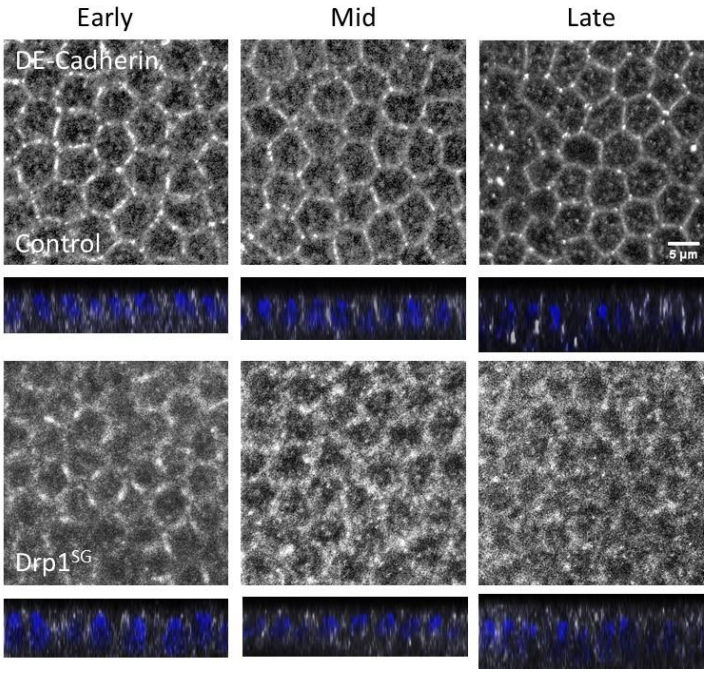
Drosophila epithelial Cadherin (DE-Cadherin) in adherens' junction connects adjacent cells via its homo and heterophilic interactions with the extracellular domain of other DE-Cadherin molecules and also conveys signals to the actin cytoskeleton via its intracellular domain(Halbleib and Nelson, 2006). It is one of the most influential players in establishing and maintaining polarity as many epithelial cells start depositing their polarity factors on the membrane once DE- Cadherin reaches the Adherens' junction(West and Harris, 2016). Though in *Drosophila* embryogenesis, that is not exactly the case as polarity markers get deposited on their respective locations alongside DE-Cadherin. It becomes important to study its localisation to visualize defects in cell-cell contacts(Wang *et al.*, 2004). Increasing its levels on membrane helps form more adhesive contacts and decrease cellular re- arrangements while decreasing its level has the opposite effect. Its levels are regulated by the endocytic and recycling pathways with Par-3/Bazooka going on the apical side of lateral membrane and stabilizing it on the adherens' junctions during late cellularization(St Johnston and Sanson, 2011). DE-Cadherin mutants show defective furrow ingression and altered nuclear anchorage.

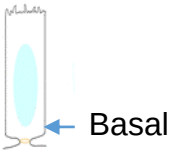
A. *DE-Cadherin at apical side across cellularization*





B. *DE-Cadherin at basal side across cellularization*





<u>Figure 5</u>: Analysis of DE-Cadherin distribution in cellularization.

A. *DE-Cadherin staining shown on the apical side across early, mid and late-stages clearly showing defects in DE-Cadherin accumulation on the apical*

belt for the Drp1^{SG} mutant. The early stage shown for both the genotypes clearly indicate a broken pattern for the mutant. Shown below are the orthogonal views respectively.

B. *Basal junction visualized in mid stages for control and Drp1^{SG} mutant clearly indicate that basal junctions do not form properly in the Drp1^{SG} embryos. Shown below are the orthogonal views respectively. n=5 embryos (50 cells) in control early, n=7 embryos (70 cells) in control mid, n=4 embryos (40 cells)in control late. For Drp1^{SG}, n=5 embryos (50 cells) in mutant early, n=4 embryos (40 cells) in mutant mid and n=4 embryos (40 cells) in mutant late.*

In cellularization, DE- Cadherin first resides on the forming basal junction which help separate the ingressing furrow canal from the lateral side and persists there until the initiation of late cellularization. It then enriches as spots on the apical side of lateral region wherethe apical adherens' junction is just forming and continues its accumulation to form spot adherens' junction and completion of the complete apical belt happens further in gastrulation(Wang *et al.*, 2004).

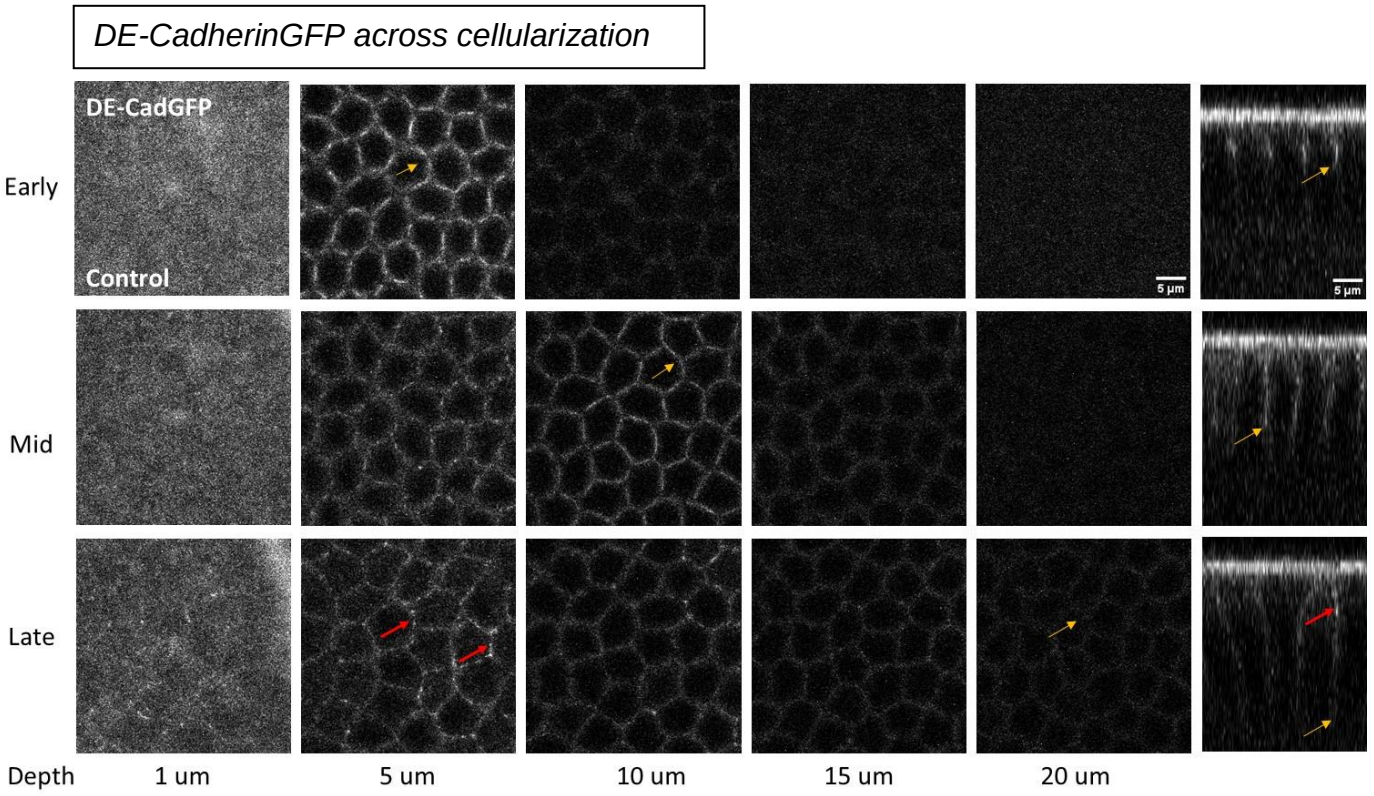
In the early stage of cellularization, the basal junction forms after deposition of DE-Cadherin but in the *Drp1^{SG}* mutant, we can already see that deposition at the basal junction is faulty as it is enriched only on certain edges of the cell while majorly being absent in the other areas of the cell (Figure 5). The basal junction begins to form as soon as membrane starts to invaginate in cellularization and DE-Cadherin starts accumulating here and completion of the junction happens at the end of early cellularization. Similarly, we observed that basal junctions do not form appropriately in mid cellularization and thedefective basolateral transfer of DE-Cadherin persists even in the mid stage. DE- Cadherin's removal from the Trans-Golgi network happens when it binds ankyrin. It then further binds PIPKIy661 lipid motifs allowing it to interact with the adaptor proteins of the endosomes which ultimately fuse at the basolateral membrane(Fölsch, Mattila and Weisz, 2009). The defect in the DE-Cadherin accumulation in the *Drp1^{SG}* mutant can be as a result of a defect in the above-
mentioned steps. ROS are critical in activating phosphatidyl inositol's in breast cancer cells and phosphatidyl inositol's can activate ROS in cases of salt tolerance in plant cells(Liu *et al.*, 2021). Thus, it might not be possible for the reduced levels of ROS in *Drp1^{SG}* mutant to activate these lipid motifs critical for DE-Cadherin's basal

accumulation. If the forming basal junction remains hampered in the *Drp1*^{SG} mutant, it is possible that components of the furrow canal and the forming lateral membrane do not remain associated at their respective positions and might migrate to the positions on the other side of the basal adherens' junction and if this remains true, the polarity proteins meant to be localizing at the lateral membrane might mis localise at the ingressing tip.

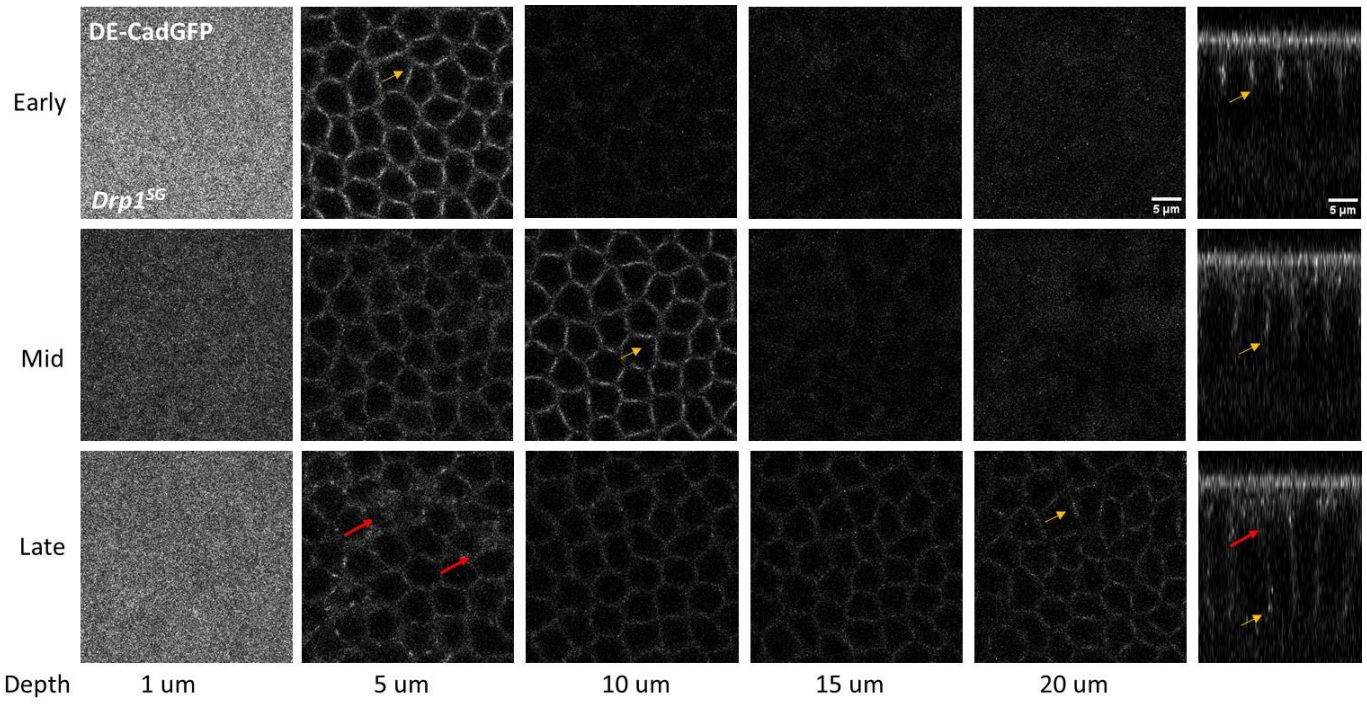
From the mid stage, DE-Cadherin starts to accumulate at the position of the adherens' junction which is just at the apical side of the forming lateral membrane. This apical belt is of paramount importance to segregate the depositing apical polarity determinants from those of the basal ones and also serve as mediators of cell-cell contacts during cellularization. We can see that DE-Cadherin starts to accumulate apically during mid cellularization and increases it gradually in late cellularization for control but the accumulation in the mid stage is just not seen in the *Drp1*^{SG} mutant and the clustering seems to be little enhanced as the embryo approaches late stage (Figure 5). We are not able to see proper DE-Cadherin accumulation on the edges as we see it uniformly in the control. One can also make out the drastic increase in DE-Cadherin's intensity at the apical belt as we transition from the mid to late stage but the progress of accumulation also seems to be a problem as less of it accumulates in the late stage for the *Drp1*^{SG} mutant. We could see some images for the mutant where we could see the apical pattern in the mid and late stage while some images also showed no membrane localisation and it seemed that heavy intracellular accumulation of DE-Cadherin happened in those embryos.

We also visualised DE-Cadherin using the endogenous DE-CadherinGFP tag in the *Drosophila* embryos. In control embryos, the basal junction can be seen in the early, mid and late stages at depths of 5, 10, and 20 microns respectively and is marked by the yellow arrows (Figure 6). The basal junctions are also seen across the cellularization stages in *Drp1*^{SG} embryos as well and are marked by yellow arrows (Figure 6). We also did see DE-Cadherin accumulating at the membrane of the apical side in late stage and is marked by the red arrows (Figure 6) and this is very similar to the observations in fixed imaging. However, we did not see similar apical accumulation in late stage of *Drp1*^{SG} embryos. The membrane bound intensity of DE-Cadherin at the apical side of late stage embryos also reduced as seen from the quantification (Figure 6).

A.


DE-CadherinGFP across cellularization
DE-CadGFP
Control
Early
Mid
Late
5 µm
Depth
1 um
5 um
10 um
15 um
20 um

B.


DE-CadGFP
Drp1SG
Early
Mid
Late
5 µm
Depth
1 um
5 um
10 um
15 um
20 um

C.

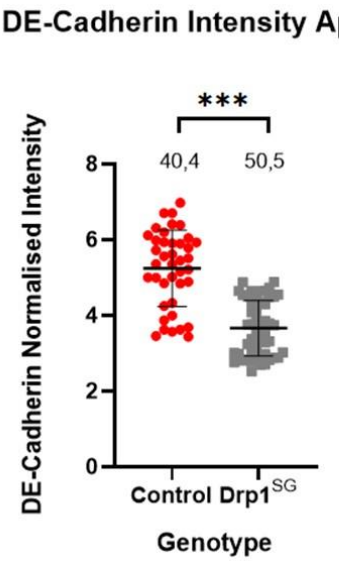

DE-Cadherin Intensity Apically
***
40,4
50,5
DE-Cadherin Normalised Intensity
8
6
4
2
0
Control
Drp1SG
Genotype

Figure 6: Analysis of DE-Cadherin distribution via live cell imaging in cellularization.

A. *DE-Cadherin live cell imaging shown at depths of 1,5, 10, 15 and 20 microns for early, mid and late-stage for control. One can visualize the basal junction marked by yellow arrowheads across cellularization and also see apical DE-Cadherin accumulation at the late stage marked by red arrowhead.*

B. *DE-Cadherin live cell imaging shown at depths of 1,5, 10, 15 and 20 microns for early, mid and late-stage for $Drp1^{SG}$. One can visualize the basal junction marked by yellow arrowheads across cellularization. The apical DE-Cadherin accumulation at the late stage marked by red arrowhead seems to be defective in $Drp1^{SG}$ and appropriate membrane localisation seems to be missing.*

C. *The normalised DE-Cadherin intensity at the apical membrane in late stage for control and Drp1SG are represented as average + SD and one can see increased membrane-associated intensity in control. The statistical analysis was carried out by Mann-Whitney t-test. n=4 embryo movies in control and n=5 embryo movies embryos for Drp1SG.*

The experiment thus, offers support to the evidence that apical localisation of DE-Cadherin in late stage of *$Drp1^{SG}$* embryos is lowered and they do not localise to the membrane appropriately.

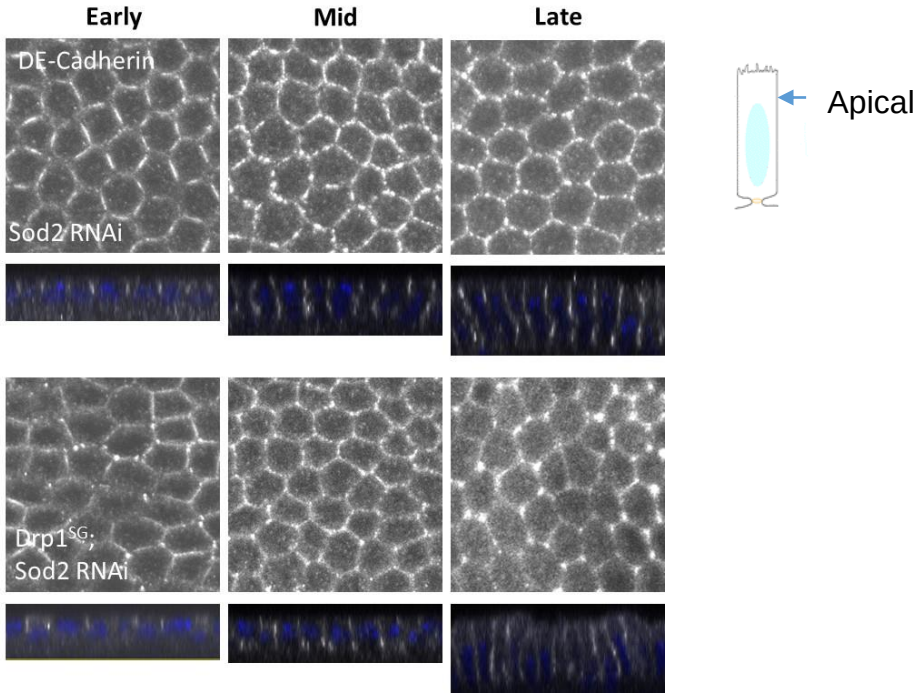
For apical trafficking of DE-Cadherin, the endosomes sequentially accumulate Rab4, Rab8 and Rab11 and classify as recycling endosomes. Phosphatidyl inositol phosphates bind to it to recruit exocyst complexes and finally, the entry of RalA GTPase to the recycling endosomes help ensure the complete transfer to the apical membrane(Polgar and Fogelgren, 2018). As the apical trafficking remains defective in the *$Drp1^{SG}$* mutant, it is highly possible to have defects in any of the pathway mediating its apical transfer. ROS are known to activate GTPases along with GEFs by oxidising certain critical residues in the active site but in the *$Drp1^{SG}$* mutant, as ROS levels reduce, these GTPases might not be activated thus, blocking the apical transfer of DE-Cadherin. In cases of escalated oxidative stress, RalA GTPase is activated by ROS which in turn activates tumour suppressor genes like FOXO ahead(Ferro *et al.*, 2012). Maybe, in the *$Drp1^{SG}$* mutant, reduced ROS levels might not sufficiently activate RalA GTPase thus, altering DE-Cadherin's apical targeting. This result is so critical because it might suggest of defect in complexes

like Par3/ Bazooka which mediate the apical accumulation of DE-Cadherin and if that is the case, its mutual antagonism with the apical and basolateral markers can also be affected which can surely cause defect in other polarity markers' localisation. Another way of thinking suggests that as the mutant cells do not have proper apical belt forming, the intermixing of apical and basolateral determinants can happen which can give rise to polarity proteins' mislocalisation all throughout. If the adherens' junction has a defect in its formation, then the corresponding cell-cell contacts might be weakin the *Drp1*^{SG} embryos suggesting of loose adhesion among cells which can surely affect future developmental processes. This is suggested because cells with lowered DE-Cadherin contacts often remain detached from each other and are less rigid(West and Harris, 2016). To recapitulate, DE-Cadherin analysis reveals that exocytic pathways regulating its dynamics are altered in *Drp1*^{SG} embryos.

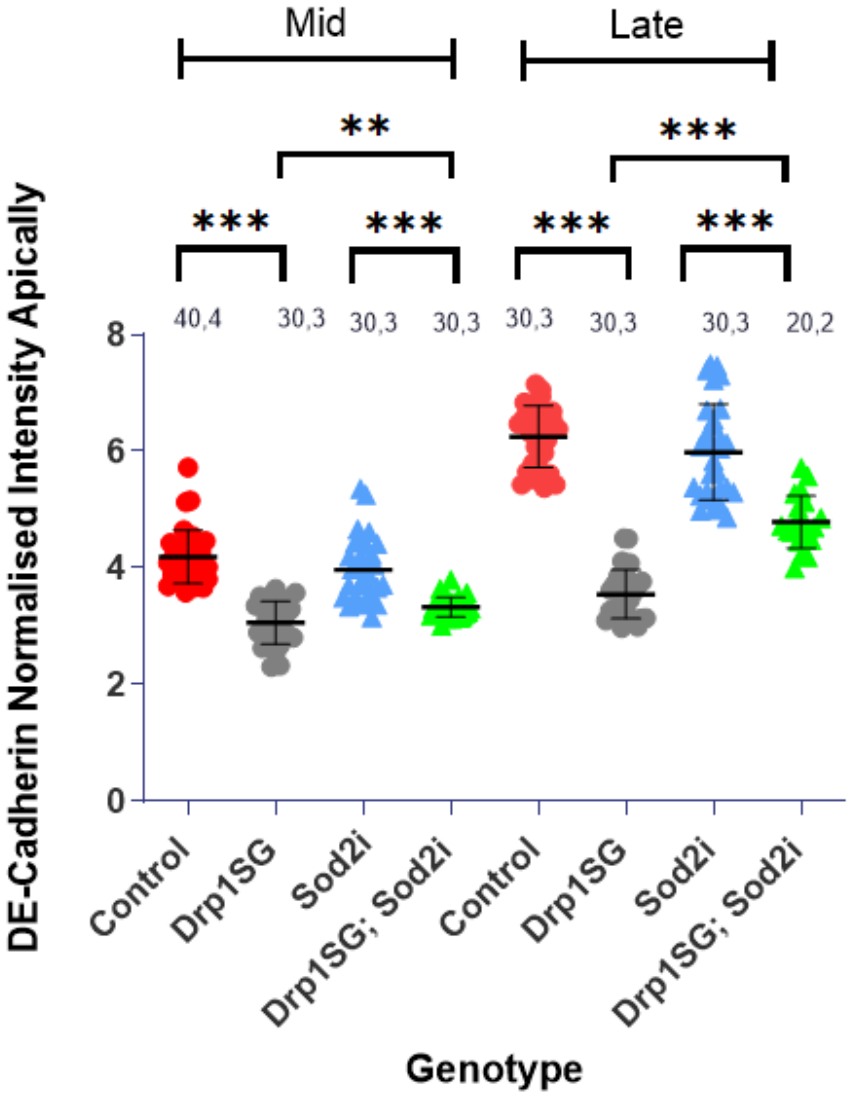
2.1.2. Analysis of Adherens' Junction proteins in embryos deficient for mitochondrial fission protein Drp1 and rescued by increasing the global ROS levels:

As global ROS levels seem to be a major factor in determining the correct localisation and levels of polarity proteins, the global ROS levels can be indeed increased via genetic means to visualise the rescue of the defect seen in the *Drp1*^{SG}mutant(Chowdhary *et al.*, 2020). It was shown previously that fused mitochondria which generally reside basally throughout cellularization undergo fission and travel apically once the ROS levels increase via the expression of Superoxide dismutase 1(Sod1) RNA interference. Sod1 is a protein which uses Zinc and Copper as cofactors to scavenge the cytoplasmic levels of ROS. We used Superoxide dismutase 2 (Sod2) RNA interference method to verify change in the mitochondrial dynamics in the *Drp1*^{SG} mutant. Sod2 enzyme specifically targets ROS generated in the mitochondria via Manganese cofactor. Indeed, we observed change in the mitochondrial dynamics as Sod2 RNA interference if expressed in the *Drp1*^{SG} embryos did cause partial fission of the mitochondria and these mitochondria did travel apically all as cellularization progressed. The following result proved that increasing the levels of mitochondrialROS which get completely reduced in the *Drp1*^{SG} mutant rescues the phenotype of the mutant. We still remain inconclusive as to how the sustained levels of ROS in thedouble mutant cause mitochondria to fission out. We then further looked if the increased apical transfer of mitochondria and its fission alongside the overall increased ROS levels affect the localisation and intensity of DE-Cadherin on the forming Adherens' Junction.

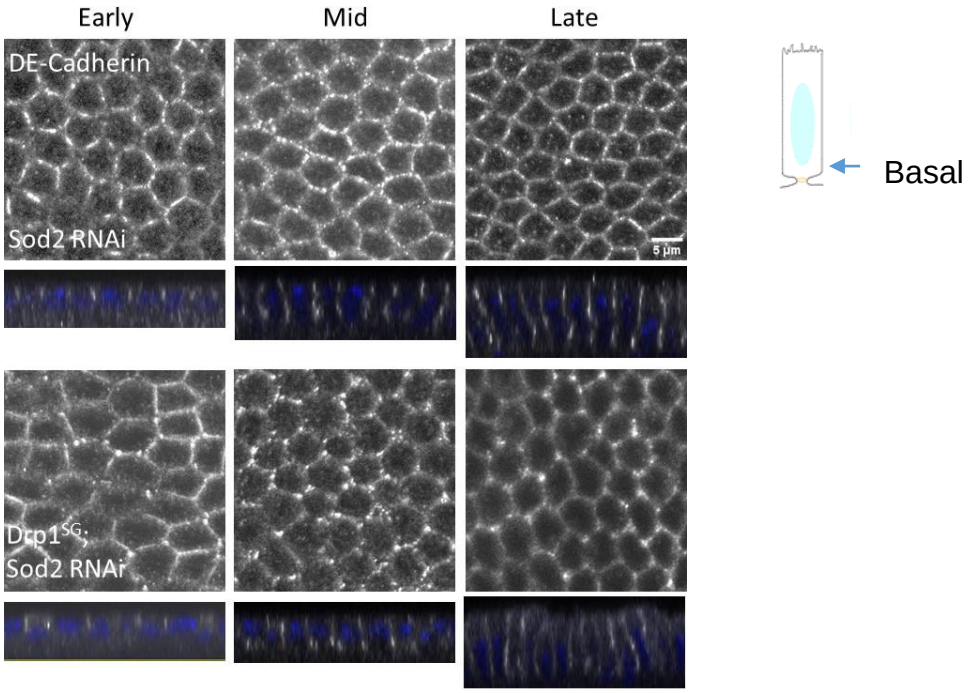
A. *DE-Cadherin at apical side across cellularization*



B.



C. *DE-Cadherin at basal side across cellularization*



D.

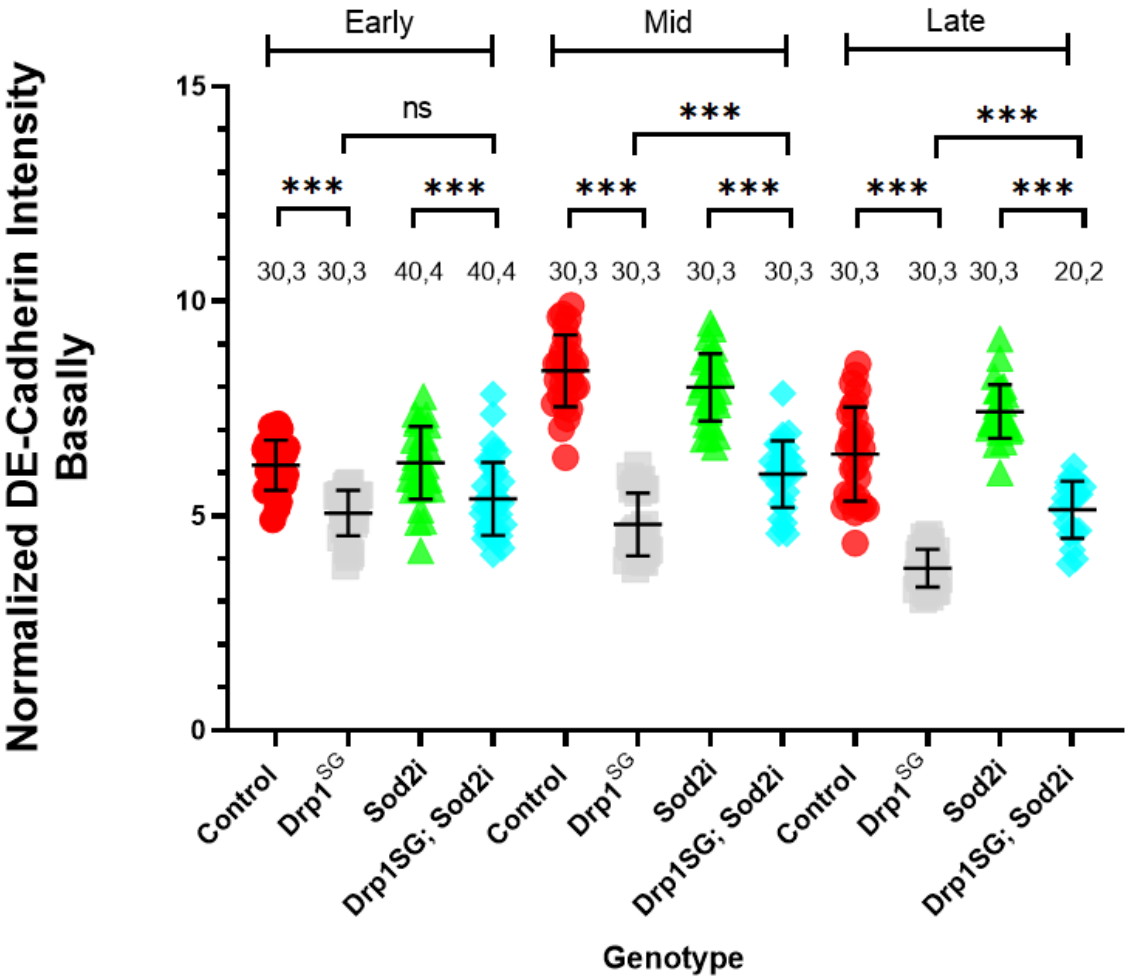


Figure 7: Analysis of DE-Cadherin distribution in cellularization in embryos expressing only Sod2 interference and Sod2 interference in Drp1^{SG} embryos

A. *DE-Cadherin staining shown on the apical side across early, mid and late-stages clearly showing that DE-Cadherin does come on the apical belt in Sod2i embryos and the localisation and intensity seem to be rescued in the Drp1^{SG} embryos expressing Sod2i. The basal junction for early stage is shown for both the genotypes clearly indicating DE-Cadherin accumulation at the edges in boththe So2i and Drp1^{SG}; So2i embryos. Shown below are the orthogonal views respectively.*

B. *The normalised DE-Cadherin intensity for control, Drp1^{SG}, Sod2i and Drp1^{SG}; Sod2i are represented as average ± SD. The statistical analysis was carried out by Mann-Whitney t-test. n=4 embryos (40 cells) for control mid, , n=3 embryos (30cells) for control late, n=3 embryos (30 cells) for Drp1^{SG} mid, n=3 embryos (30 cells) for Drp1^{SG} late, n=3 embryos (30 cells) for Sod2i mid, n=3 embryos (30 cells) for Sod2i late, n=3 embryos (30 cells) for Drp1^{SG}; Sod2i mid and n=2 embryos (20 cells) for Drp1^{SG}; Sod2i late*

C*. DE-Cadherin staining shown on the basal junction across early, mid and late- stages clearly showing that DE-Cadherin does come on the basal junction in Sod2i embryos and the localisation and intensity seem to be rescued in the Drp1^{SG} embryos expressing Sod2i. The basal junction for early stage is shown forboth the genotypes clearly indicating DE-Cadherin accumulation at the edges in both the So2i and Drp1^{SG}; So2i embryos. Shown below are the orthogonal views respectively.*

D*. The normalised DE-Cadherin intensity for control, Drp1^{SG}, Sod2i and Drp1^{SG}; Sod2i are represented as average ± SD. The statistical analysis was carried out by Mann-Whitney t-test. n=3 embryos(30 cells) for control early, n=3 embryos (30 cells) for Drp1^{SG} early, n=4 embryos (40 cells) for Sod2i early, n=4 embryos (40 cells) for Drp1^{SG}; Sod2i , n=3 embryos (30 cells) for control mid, , n=3 embryos (30 cells) for control late, n=3 embryos (30 cells) for Drp1^{SG} mid, n=3 embryos (30 cells) for Drp1^{SG} late, n=3 embryos (30 cells)*

for Sod2i mid, n=3 embryos (30 cells) for Sod2i late, n=3 embryos (30 cells) for Drp1^{SG}; Sod2i mid and n=2 embryos (20 cells) for Drp1^{SG}; Sod2i late

When the global ROS levels were increased by expressing Sod2 RNA interference construct in the control and the *Drp1^{SG}* mutant, we did observe changes in the DE-Cadherin dynamics (Figure 7). We see that in mid stage, Sod2i embryos have similar levels of DE-Cadherin accumulating at the apical side as compared to the control while the *Drp1^{SG}*; Sod2i construct has increase in DE-Cadherin accumulation at the apical belt than its *Drp1^{SG}* counterpart. The levels seem to be enhanced in the double mutant as we see less clustering of DE-Cadherin on the vertices and more localisation on the edges. The same can also be seen in the basal junction at the early stage (Figure 7). In mid stage, 10 out of 13 embryos show the rescue in the double mutant and the remaining 3 embryos have clustering very similar to the mid stage of Drp1^{SG} embryo. In late stage, Sod2i embryos also have similar DE-Cadherin clustering at the apical side and *Drp1^{SG}*; Sod2i embryos also seem to show a partial rescue as DE-Cadherin gets enriched on the edges with an appropriate localisation and do not show clustering as in its *Drp1^{SG}* counterpart. Out of the 5 embryos stained for the double mutant, 2 show the rescue phenotypes in late stage. In both the mid and late stages, where the rescue is not seen, we observed highly fused mitochondria with no apical migration of mitochondria.

The basal junction membrane intensity (Figure 7) does show increased levels from early to mid in control and Sod2i embryos while *Drp1^{SG}* hardly showed any increase at the membrane. '*Drp1^{SG}; Sod2i*' rescue line had greater membrane-bound intensity than its *Drp1^{SG}* counterpart in the mid stage suggesting efficient membrane-bound delivery once ROS levels rose up. The basal junction disassembles as late stage progresses and that can be seen in control, Sod2i and '*Drp1^{SG}; Sod2i'* rescue embryos . This does suggest that fission of the mitochondria, the ROS metabolites and the apical migration collectively can act to maintain polarity markers on the membrane.

Mitochondrial ROS is involved in oxidising proteins like Src42A which help trafficking of DE-Cadherin around the wound and ultimately reduces it. In prohemocyte differentiation of *Drosophila*, it has been established that increasing ROS levels reduce the expression of DE-Cadherin, increases the expression of DE-

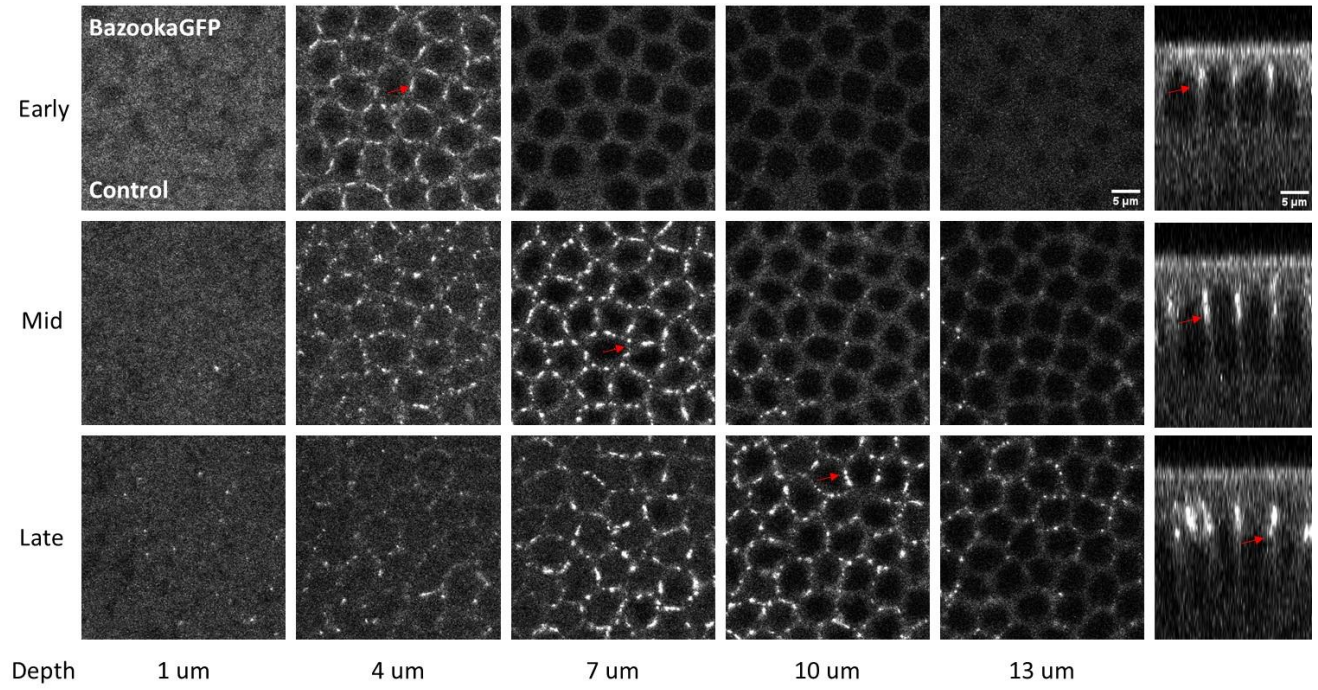
Cadherin repressors and when they also increased ROS via Superoxide Dismutase mutants, they got reduce levels of DE-Cadherin. In retinal cells of *Drosophila*, important proteins like Src Kinase can be oxidised in times of oxidative stress and lead to phosophorylation of the components binding the intracellular domain of N-Cadherin and ultimately leads to its internalization(Hebbar and Knust, 2021). When prostate cancer cells were provided with hydrogen peroxide, it was seen that E-Cadherin levels severely dropped down and cells do acquire invasive properties(Lim *et al.*, 2005). This is highly contradictory to what we see in our system because when we increase the ROS levels, DE-Cadherin starts accumulating more on the apical and basal junction. In all of the above systems, E-Cadherin is already present on the cells and increasing the levels of ROS can decrease the E-Cadherin levels but in *Drosophila* cellularization, we see that increasing the ROS levels in the $Drp1^{SG}$ embryo does positively affect the levels of DE-Cadherin. Here, the ROS levels might directly impact the trafficking pathways of DE-Cadherin and might not directly impact DE- Cadherin itself. It can be highly possible that some proteins involved in trafficking of DE-Cadherin might get activated and now allow for appropriate apical settling of DE-Cadherin. Also as more DE-Cadherin accumulates at the membrane, it is highly possible that the strength of the cell-cell contacts improve in the double mutant as well as the junction becomes more functionally relevant as the polarity factors now remain at their respective positions. Thus, one might expect rescue in the phenotypes of other polarity factors as well if they remain defective in the $Drp1^{SG}$ mutant. If the apical and basal transfer of DE-cadherin improves, that might suggest that transfer of other polarity proteins might also improve if given that increased ROS levels affect the trafficking pathways directly. To recapitulate, we saw that DE- Cadherin levels reduce at the apical and basal membrane in the $Drp1^{SG}$ mutant but the phenotype is rescued once the levels of mitochondrial ROS are increased.

2.1.3. Analysis of apical domain proteins in embryos deficient for mitochondrial fission protein Drp1:

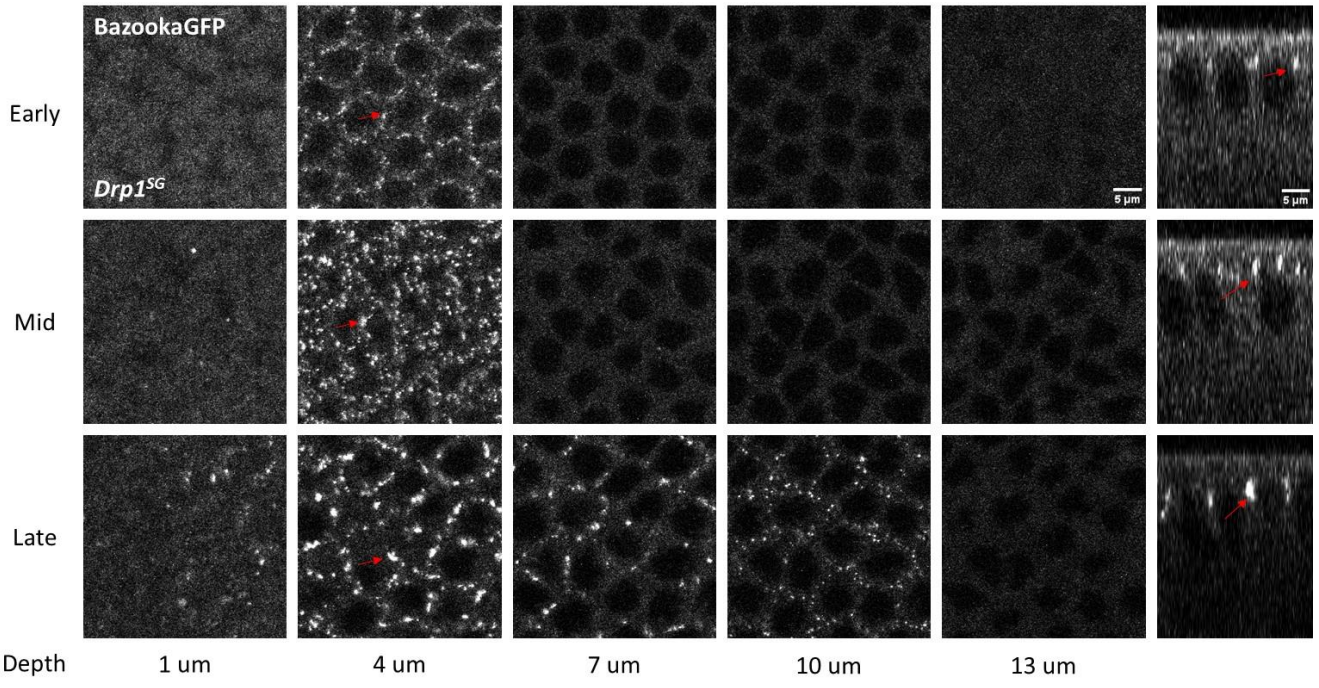
As adherens' junction proteins like DE-Cadherin remain defective in $Drp1^{SG}$ embryos, we wished to visualize polarity factors above in the hierarchy which help recruit DE-Cadherin. Bazooka is one of the apical polarity factors which help establish polarity in non-polarising cells and also recruit apical polarity complexes

like aPKC-Par6 and Crumbs module apically. In embryos where Bazooka is mutated, adherens' junctions do not form apically thus, it serves to recruit the junctional components in the apical zone. Once DE-Cadherin is recruited apically, Bazooka helps assemble it into spots by the end of cellularization and these spots coalesce into a continuous belt at the end of gastrulation(Harris and Peifer, 2004).

A.



B.



C.

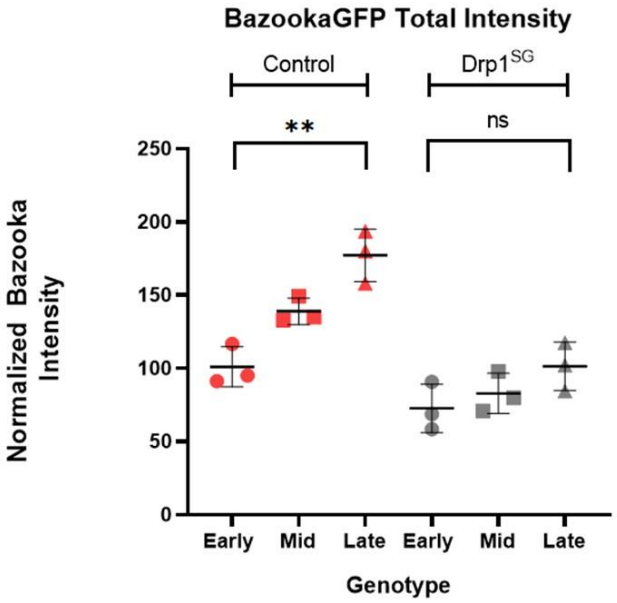


Figure 8: Analysis of Bazooka distribution via live cell imaging in cellularization.

A. *BazookaGFP live cell imaging shown at depths of 1,4, 7, 10 and 13 microns for early, mid and late-stage for control. One can visualize the basal junction marked by red arrowheads in early cellularization and progressively see its migration at the sub-apical side as cellularization progresses.*

B. *BazookaGFP live cell imaging shown at depths of 1,4, 7, 10 and 13 microns for early, mid and late-stage for $Drp1^{SG}$ embryos. One can visualize the basal junction with reduced membrane-associated intensity marked by red arrowheads in early cellularization and we do not see Bazooka migration towards the sub-apical side as cellularization progresses.*

C. *The normalised BazookaGFP intensity across the furrow for control and Drp1SG are represented as average + SD. One can see increasing intensity in control as cellularization progresses but the overall intensity in $Drp1^{SG}$ remains reduced . The statistical analysis was carried out by One-Way Anova. n=3 embryo movies in control and n=3 embryo movies embryos for Drp1SG.*

Live imaging for BazookaGFP endogenous tag reveals that in the early stage, it associates with the basal junction and moves down in the apical zone of the mid-stage once new membrane is added apically in cellularization (Figure 8). More

Bazooka is added as cells progress in the faster late cellularization and Bazooka clusters position at the sub-apical region right where adherens junction forms. The quantification of the total Bazooka intensity (Figure 8) also shows the increasing levels of the protein added apically during cellularization. Strikingly, in the *Drp1^{SG}* embryos, we see that Bazooka levels drop down (Figure 8) across cellularization, it does not associate with the membrane appropriately and in the late stages, we see lesser Bazooka clusters around the membrane. Moreover, the migration of Bazooka towards the sub-apical region is also reduced and might mark towards the non-addition of new membrane in cellularization.

In *C. elegans,* it is noted that microfilaments help stabilize Bazooka orthologue (Severson and Bowerman, 2003)and in *Drp1^{SG}* embryo, it can hint towards a non-stable actin scaffold at the end of cellularization. It seems that the overall translation of the protein is reduced or it can be possible that its trafficking towards the apical zone by the Dynein motors (Benton and St Johnston, 2003)be affected in *Drp1^{SG}* embryos. Bazooka oligomerization into clusters also seem to be affected in the mutant. The overall implication of lesser Bazooka at the apical side suggests of lesser recruitment of DE-Cadherin in the sub-apical region. Bazooka is a critical polarity component and in *Drp1^{SG}* embryos where it is defective, then it can also be inferred that Bazooka-dependent recruitment of aPKC and Crumbs apical modules seem to be defective. In this case, appropriate size of apical zone is not formed an it can be tested if this defective apical zone prevents the entry of basolateral markers there.

2.1.4. Analysis of Baso-Lateral Membrane proteins in embryos deficient for mitochondrial fission protein Drp1:

The lateral membrane protein of DLG1 (Discs Large MAGUK Scaffolding protein 1) is analysed in control and *Drp1^{SG}* mutants just to visualize if its localisation and intensity get affected in the mutant. It is critical in maintaining cell-cell contacts as well and also recruits specific channels and receptors to the plasma membrane. It helps establish epithelial polarity and also act as tumour suppressor gene. In *Drosophila* cellularization, it is known that DLG1 first accumulates above the furrow canal in the early stage and as cellularization progresses, DLG1 keeps on adding uniformly on the lateral membrane. DLG mutants have altered plasma membrane formation and polarity factors like Neurexin supposedly localising to the lateral region go in the apical side in the mutant and opposite to that, overexpression experiments do suggest that Neurexin now has an enlarged accumulation on the furrow(Nelson,

2003; Dudu, Pantazis and González-Gaitán, 2004). Thus, it becomes critical to analyse the levels of DLG and see if it remains defective in the *Drp1^{SG}* mutant.

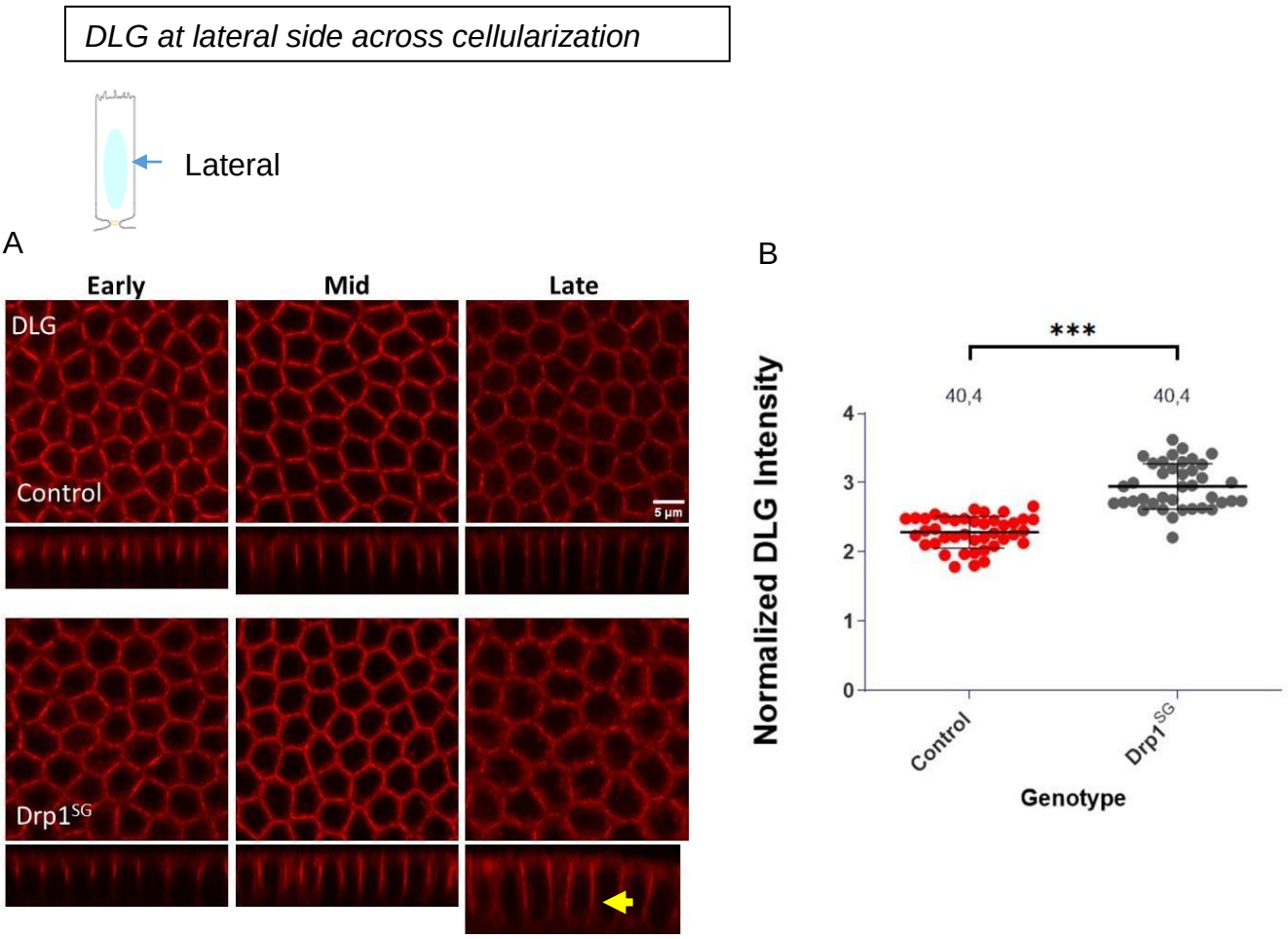


Figure 9: Analysis of DLG distribution as a lateral membrane marker in cellularization.

A. *DLG staining shown on the lateral side across early and mid-stages clearly showing that DLG intensity and localisation is unaffected in Drp1^{SG} mutants but in the late stage, we did observe basal accumulation of DLG1 in the mutant shown by yellow arrowhead in orthogonal view. Shown below are the orthogonal views respectively.*

B. *The normalised DLG intensity at the basal junction for both the genotypes is represented as average ± SD. The statistical analysis was carried out by Mann-Whitney t-test. n=4 embryos (40 cells) in control and n=4 embryos (40 cells) in Drp1^{SG} mutant.*

We can see that DLG deposition starts from the early stage in cellularization and it remains accumulated just above the furrow canal and as the furrow canal

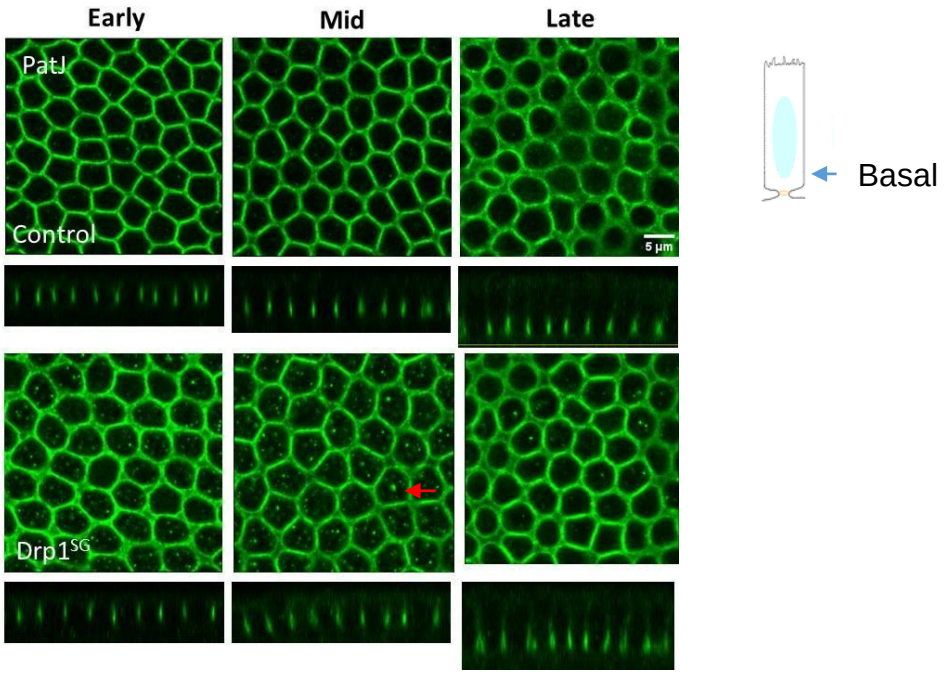
progresses down, DLG progressively adds on the lateral membrane. DLG deposition and intensity remains unaffected in the early and mid-stages and even in images where we see highly fused mitochondria, the deposition just did not get affected. In late stage, we see that DLG deposition happens at the apical side of lateral membrane in control but surprisingly, in the *Drp1*^{SG} mutant, we see that DLG deposition happens at 2 places across the furrow. It enriches on the apical side of the lateral region as well as on the basal side of the lateral region. In images of highly fused mitochondria in the late stage of cellularization, the basal deposition seems very prominent (Figure 9).

The defect in DLG deposition can occur due to persistent basolateral accumulation in the late stage of *Drp1*^{SG} embryos. The membrane protein Strabismus helps localise the lateral membrane proteins and via its 2 PDZ-containing domains, it binds DLG which has 3 PDZ domains and help it recruit from the post-Golgi vesicles to the basolateral membrane. The reduction in ROS can cause changes in the redox-sensitive sites of Strabismus such that it remains persistently activated and continues binding DLG to recruit it basally(Dudu, Pantazis and González-Gaitán, 2004). In *Drosophila* follicular and epithelial cells, it is shown that DLG has a polybasic domain and being positively charged, it binds the negatively charged Phosphatidyl inositol's on the lateral membrane(Lu *et al.*, 2021). ROS has been known to oxidise proteins and inactivate it by inserting carbonyl groups on the positively charged amino acids. Decreased ROS levels in the *Drp1*^{SG} mutant might just not be able to do it onto the basal membrane selectively and DLG thus, actively targets itself on the basal membrane. It also seems that Phosphatidyl inositol levels do not decrease at the basal region of the furrow in the Drp1^{SG} mutant and thus, DLG can continuously accumulate itself on the basal region. DLG also interacts with Scrib protein which escalates DLG deposition at the plasma membrane electrostatically and it might be the case that the reduced levels of ROS just cannot inhibit these interactions. If the electrostatic interactions still persist, then Scrib and Lethal Giant Larvae (LGL) can also be expected to give the same phenotype as DLG helps recruit them to the lateral membrane. This explains that the lateral polarity factors might be defective in terms of their localisation and thus, the global ROS levels ultimately affect the localisation of lateral polarity markers.

As polarity factors seem defective at the basal junction, PatJ (PALS1-Associated Tight Junction) Protein was also tested in the *Drp1*^{SG} mutant and is checked for its intensity and localisation. PatJ protein has various PDZ-domains through which it

interacts with other proteins and acts as a scaffolding protein onto the membrane. PatJ is the polarity marker vital in regulating myosin II activity as it binds myosin II phosphatase, does not allow the enzyme to phosphorylate myosin II regulatory light chain proteins and thus, keeps the myosin II active. In *Drosophila* photoreceptor and follicle cells, it stabilizes the Crumbs-Stardust apical complex and also forms a core part of the Crumbs module. PatJ is crucial for adherens' junction stability as in epithelial cells where one copy of E-Cadherin is affected, if PatJ mutation is introduced, it leads to the complete damage of the adherens' junction with cells losing their shape and polarity factors like DLG accumulating in cytoplasm leading to intense polarity loss(Sen, Nagy-Zsvér-Vadas and Krahn, 2012; Zhou and Hong, 2012).In human intestinal cell lines where PatJ is knocked down, the associated Stardust and Crumbs do not localise on the tight junctions and accumulate intracellularly while some of the other tight junction proteins enter lateral membrane. In mammalian epithelial cells, PatJ RNA interference causes a delay in tight junction formation(Wang, Lyu and Li, 2021). Thus, PatJ can be thought of having a role in the maintenance of Crumbs module and other proteins at the tight junction. Throughout cellularization, it associates with the invaginating furrow, co-localises with myosin II and might regulate its constriction while in the initiation of gastrulation, it is recruited at the apical domain. PatJ null mutants do not affect the apical-basal polarity(Zhou and Hong, 2012).

A. *PatJ at basal side across cellularization*



B.

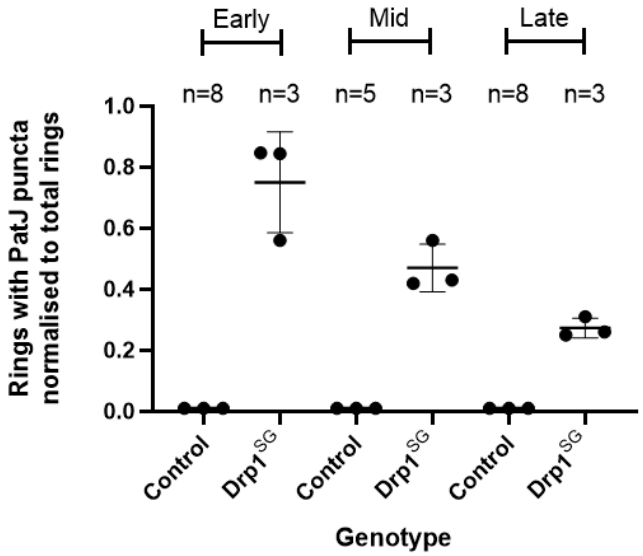


Figure 10: Analysis of PatJ distribution as a basal membrane marker in cellularization.

A. *PatJ staining shown on the basal junction across early, mid and late stages clearly showing that PatJ remains cytoplasmic (red arrowhead) in the* $Drp1^{SG}$ embryos*. Shown below are the orthogonal views respectively.*

B. *The number of basal rings involving accumulation of PatJ are normalised to the total number of rings in the slice and is represented as average ± SD. PatJ accumulation persists highest in the early stage when furrow canal is just forming. In early stage, n=8 and n=3 embryos analysed in control and* $Drp1^{SG}$ *respectively; in mid stage, n=5 and n=3 embryos analysed in control and* $Drp1^{SG}$ *respectively; in late stage, n=8 and n=3 embryos analysed in control and* $Drp1^{SG}$ *respectively*

PatJ deposits itself on the invaginating tip as cellularization initiates and at the end of early cellularization, PatJ deposits itself completely on the contractile ring. We see that PatJ continues to deposit itself on the invaginating tip in the mid as well as late cellularization. Interestingly, in the *Drp1*^{SG} mutant, we observed PatJ deposition on the contractile ring as well as its accumulation in the cytoplasm severly in the early stage (Figure 10). The cytoplasmic accumulation appears in the mid stage as well and the phenotype still persist in late cellularisation for some images.

From the quantification (Figure 10), one can easily see that the basal rings having PatJ dots and rings with 2 or greater than 2 dots are high in the early stage and reduce progressively in the mid and late stages. This leads to an overall decrease in the number of PatJ dots and is also seen when total PatJ dots are normalised with the total rings in an image. This result suggests that the number of PatJ dots as well as the number of rings having the PatJ dots seem to be enhanced in the *Drp1*^{SG} early stage.

This does suggest that PatJ initially accumulates in the early stage in the *Drp1*^{SG} mutant. Initially, it did seem like PatJ could possibly be removed from the basal ring and accumulate in the cytoplasm but the intensity of PatJ at the basal junction was similar to the early and mid-stages in control. PatJ first deposits itself on the basal membrane from the apical microvilli alongside myosin II from the point when cellularization initiates and does not come as vesicles from the Golgi network. Also, no cytoplasmic vesicles of PatJ are observed in control all throughout cellularization in control and this evidence suggest that PatJ does not come via vesicular trafficking but still inthe *Drp1*^{SG} mutant, we can visualize it in the cytoplasm. This leaves us with a possibility that the somehow endocytosis can happen unevenly all throughout the furrow in the mutant and this can cause the removal of PatJ from the furrow. Maybe,this can hint us towards the basal junction not forming properly as suggested from reduced DE-Cadherin at the basal junctions and because of this, PatJ can leak into the cytoplasm. PatJ null mutants do not affect apico-basal polarity in any way but fused mitochondria and reduced levels of ROS did hamper the localisation of PatJ. PatJ and DE-Cadherin at the basal junction seem to be defective and because of this, it can be possible that the initial basal junction does not form properly in the *Drp1*^{SG} mutant thus, ineffectively

separating the furrow canal compartments from the forming lateral membranes. PatJ has also been suggested in supporting adherens' junction stability but if PatJ itself has a faulty localisation, then this can affect the basal adherens' junction in the early and mid-stages. Thus, we conclusively state that basal markers like PatJ do get severely affected in the *Drp1*^{SG} mutant.

PatJ is an apical polarity marker as it is a part of the Crumbs module but still in cellularization, it localizes in the basal cytoplasmic ring which has actin and myosin II accumulation. Myosin II is recruited from the apical cortex in the invaginating tip and so does PatJ. Recent evidences do suggest that in *Drosophila* larvae, PatJ directly binds and recruit myosin II and also inhibits Myosin Phosphatase(Sen, Nagy-Zsvér-Vadas and Krahn, 2012). This phosphatase inactivates myosin II and PatJ inactivates this phosphatase by allosteric regulation and these lines of evidence do suggest that PatJ can be possibly involved in recruiting myosin II during cellularization as PatJ and myosin II, both remain at the invaginating furrow tip. As PatJ remains cytoplasmic throughout cellularization and can also be in principle aid in recruiting myosin II, we had to analyze myosin II localization and intensity in cellularisation.

Myosin II recruitment in the early stage is aided by the Dunk gene and in the mid and late stage, it is recruited with the help of Slam gene. The cortical flow of actin and myosin II end up forming the cytoplasmic acto-myosin contractile rings.

A. *Myosin II at basal side across cellularization*

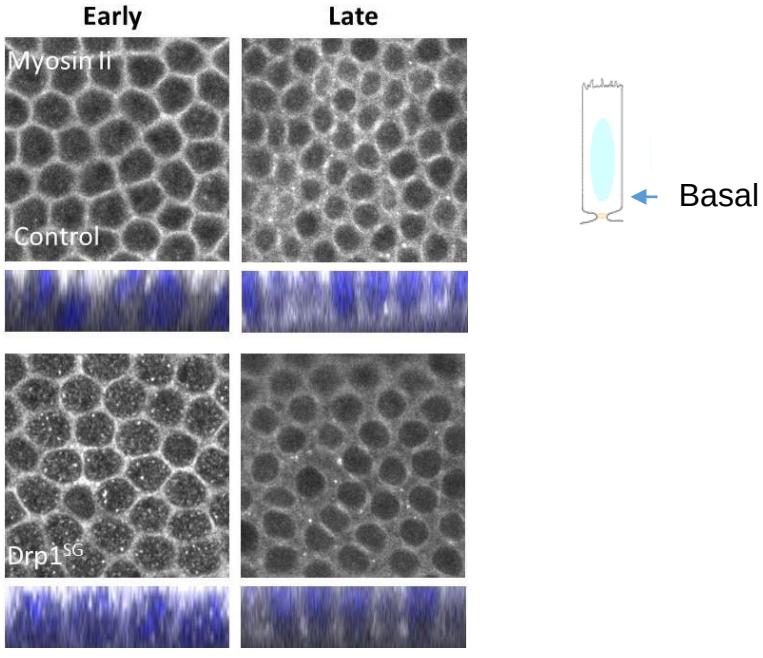


Figure 11: Analysis of myosin II distribution as a basal membrane marker in cellularization.

A. *Myosin II staining shown on the invaginating furrow across early and late stages clearly showing that myosin II remains cytoplasmic in the early stage in the Drp1^{SG}* embryos *and has reduced intensity in late stages. Shown beloware the orthogonal views respectively. n=6 embryos for control early, n=4 embryos for control late, n=6 embryos for Drp1^{SG} early and n=5 embryos for Drp1^{SG} late stage.*

From the myosin II staining, we do see that in control, myosin II remains on the cytoplasmic ring in the early stage with hardly any signal in the cytoplasm whereas, in the *Drp1^{SG}* mutant, in the early stage, we can see myosin II on the contractile ringas well as accumulated in the cytoplasm. Similarly, in the late stage, we do find high myosin II signal at the basal ring in control but we see it completely reduced in the *Drp1^{SG}* mutant (Figure 11). We verify that clustering of myosin II in the early stage and its intensity reduction in the late stage occur severely in those images where mitochondria remains heavily fused.

The myosin II phenotype is highly similar to the PatJ cytoplasmic accumulation in the early stage. Thus, it is highly possible that because of endocytosis or by any other means, PatJ can leak out of the furrow and along with PatJ, myosin II can also be recruited in the cytoplasm. If this is true, then this further supports that basal adherens' junction do remain faulty as myosin II also exits out from the invaginating tip. This can further support the fact that as adherens' junction remains defective, components like those of the polarity factors might move out from their respective sites and enter new sites. As myosin II is recruited through Dunk gene, it can be possible that reduced levels of ROS in the *Drp1^{SG}* mutant can affect the functioning of this protein and thus, we see altered localisation of myosin II and this altered localisation of myosin II can also in some way contribute to the reduced ring size that we observed in the early and mid-stages (Figure 3).

Mitochondrial ROS is known to be involved in recruiting myosin II as seen in the examples of wound healing and dorsal closure and inhibiting mitochondrial ROS in these processes do not allow for myosin II recruitment(Mateus *et al.*, 2011; Hunter *et al.*, 2018). Similarly, we can infer that in *Drp1^{SG}* mutants where the mitochondrial

ROS are depleted, myosin II recruitment in the late stages is affected and we thus, see depleted levels of myosin in the late stage. Similarly, we can infer that reduced ROS levels can affect the activity of Slam gene in the embryo and thus, the effective myosin II recruitment does not happen. The contractility in the ring also depends on Rho-GTP activity which gets activated by Rho-GEF2 and is negatively regulated by GRAF(Sharma and Rikhy, 2021). As ROS are known to functionally activate Rho molecules by oxidising important amino acid residues, it can be possible that reduced levels of ROS cannot allow the Rho-GTP activation and thus, contractility remains defective in the *Drp1*^{SG} embryos. The functional consequence of it remains that the rings do not fully constrict in the late stage and thus, we have shorter furrows at the end of cellularisation. These shorter furrows then can ultimately affect the localisation of the polarity molecules as in principle, the lateral domain reduces. Thus, we conclusively prove that PatJ and myosin II acquire defective localisation in the *Drp1*^{SG} mutant and this an in turn, alter the contractility dynamics.

3. Analysis of membrane recycling pathways in embryos deficient for mitochondrial fission protein Drp1:

Endocytosis, exocytosis and recycling work together to form the lateral furrow during cellularization and also help in the polarised insertion of lipids and membranes. Targeted exocytosis of the polarity factors towards the plasma membrane is the most studied method in inducing polarity and they depend on a variety of factors like the Rho and Rac GTPases, polarised cytoskeleton and biosynthetic sorting signals from the Trans-Golgi network(Fölsch, Mattila and Weisz, 2009). Recently, the role of endocytosis is also unravelling in terms of targeted insertion of membrane and polarity factors. The cargo for endocytosis is pinched from the plasma membrane and can fuse with the early endosomes. The endocytic machinery in flies can help in internalization, recycling and sustaining the levels of polarity factors and endocytic regulators can help remove misfolded or altered proteins and ultimately help separate the apical and basolateral domains(Shivas *et al.*, 2010). Cellularisation in *Drosophila* provides a great model where all the 3 processes collectively interact to establish polarity and thus, the membrane dynamics are studied in *Drp1*^{SG} embryos. As these mutant embryos have reduced final length of furrow ingression, we expect that membrane insertion and recycling pathways are also affected in the mutants which thus, also affect the insertion of their respective polarity factors. The following sections demarcate the role of early, late and recycling endosomes and the membrane recycling pathways in inducing

polarity in the *Drp1*^{SG} embryos.

3.1. Analysis of Early Endosomes' Dynamics in embryos deficient for mitochondrial fission protein Drp1:

For understanding the importance of endocytic pathways in inducing polarity in cellularization, Rab5 was tested in cellularization which helps mark the early endosomes. Ras Analog in Brain 5 (Rab5) is a GTPase involved in endocytosis and membrane transport, helps recruit effectors helping in internalization of vesicles and help fuse the endocytosed vesicle with the early endosomes(Yuan and Song, 2020). In *Drosophila* cellularization, it is known that Rab5 concentrates apically and deposits itself on the plasma membrane and endocytoses microvilli to form sharp hexagonal cellular shape on the apical side(Fabrowski *et al.*, 2013). Rab5 was checked for its localisation and intensity throughout cellularization in *Drp1*^{SG} embryos.

A. *Rab5 early endosomal marker at apical and basal side across cellularization*

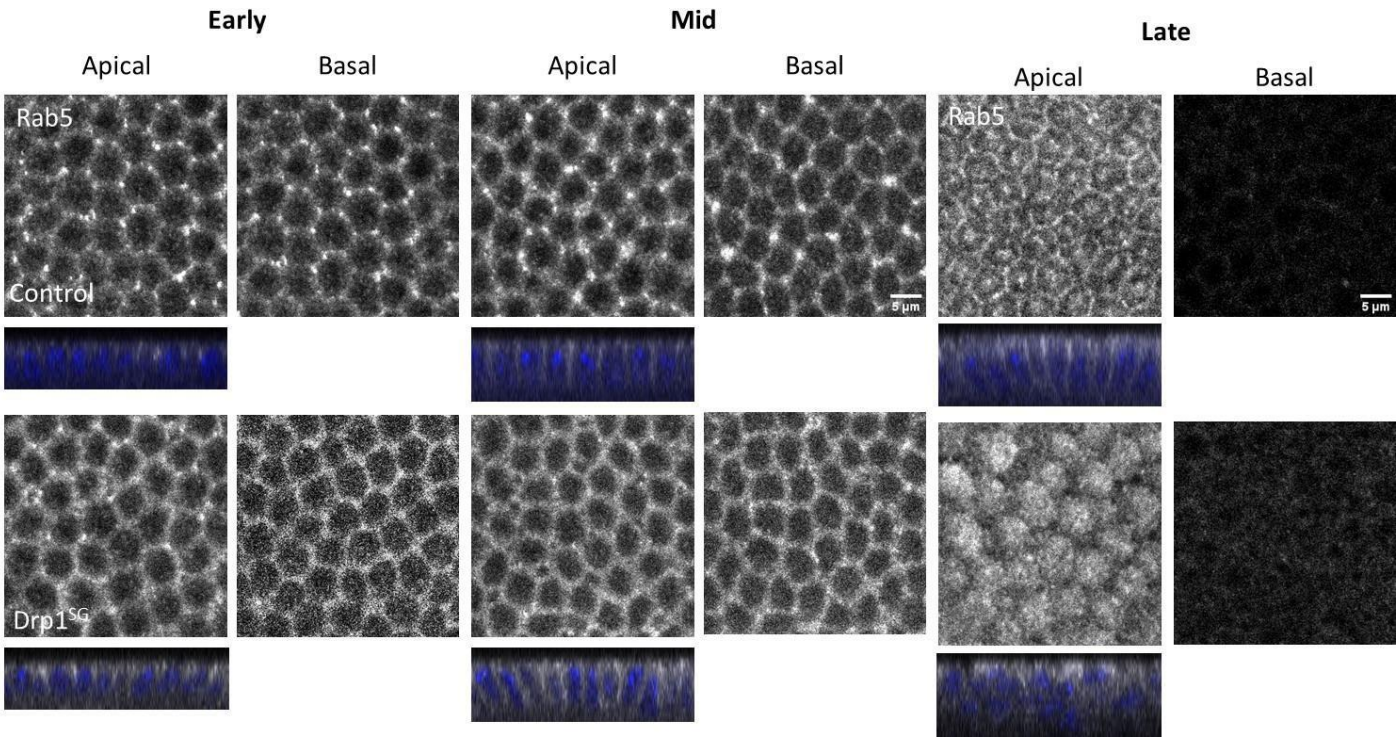


Figure 12: Analysis of Rab5 distribution as early endosome marker in basal junction at cellularization.

***A.** Rab5 staining shown on the basal junction across mid and mid-stages clearly showing that Rab5 remains as clusters in the early and mid-stage in the control* embryos *but has a membrane-associated pattern in the Drp1^{SG} embryos. Shown below are the orthogonal views respectively. n=4 embryos for control early, n=3 embryos for control mid, n=3 embryos for Drp1^{SG} early and n=3 embryos for Drp1^{SG} mid stage. Rab5 staining on the apical area in late stage clearly showing that Rab5 localises on membrane and the endocytosed cargo in control but high intracellular accumulation is observed in the mutant. n=3 in control late and n=3 in Drp1^{SG} late*

In control images of the early stage, it seems that Rab5 increases its intensity basally and appears as clusters around the membrane (Figure 12). In the mid stage, the clustering increases around the membrane and intensifies heavily suggesting that Rab5 has increased localisation and perhaps functioning. In the *Drp1^{SG}* mutant, the clustering is never really observed and Rab5 seems to be uniform across the membrane in the early stage and the membrane-association persists even in the midstage. Rab5 intensity increases apically in the mid stage for both the genotypes. In *Drp1^{SG}* early and mid-embryos as well, we encountered certain embryos having clustering of Rab5 but not to that extent as seen in control.

Basally, the clustering of Rab5 at certain locations in the membrane might signify the shaping of the hexagonal ring by Rab5 as it might help endocytose the membrane at those particular locations to form the contractile ring. The increased clustering of Rab5 in the mid stage might signify increased endocytosis of the membrane and thus, reduction in the size of the contractile ring. In *Drp1^{SG}* embryos, the uniform membrane-associated pattern of Rab5 might hint towards increased endocytosis at the base and thus, we might get reduced ring size as we observed in the early and mid-stages (Figure 3). Rab5 GDP dissociation inhibitor (GDI) binds Rab5-GDP and help localise it to the membrane and Rab5-GEF allows it the transitioning to Rab5-GTP(Yuan and Song, 2020) and it seems that lowered levels of ROS in the *Drp1^{SG}* embryos hint to the stimulated functioning of Rab5-GDI or Rab5-GEF. Upon nutrient stress in *S. cerevisiae,* Rab5 isoforms increased their functioning and prevented ROS levels to rise up and this can indicate that the already diminished levels of ROS can be further reduced by the escalated levels of Rab5 in

the *Drp1*^{SG} mutant (Nakatsukasa *et al.*, 2014). If endocytosis increases at the base and if it is due to the Rab5 action in the mutant, it can surely affect the polarity markers in the basal junction and the invaginating tip. It is highly possible that PatJ and myosin II can be recruited at the cytoplasm due to this increased Rab5 endocytic activity in the mutant. There can be another case where Rab5 accumulates because endocytosis is just not happening in the mutant due to defect in other components of the endocytic machinery and increased Rab5 is attempting for this endocytosis to happen. If that is true, then it affects the membrane recycling activity from the furrows and the excess membrane from the furrow is unremoved.

In late stage, we do observe increased apical localisation in control and Rab5 can be seen onto the membrane as well as on the endocytosed cargo but interestingly, in *Drp1*^{SG} mutant, high intracellular accumulation of Rab5 and no localisation on the membrane is observed (Figure 12). Thus, it is revealed that Rab5 has dual roles and localisation patterns in early and late stages. If endocytosis does not happen apically in the mutant, then the excess microvilli can persist even when gastrulation initiates and it might affect the overall cellular morphology at the apical side. This can hint that in late stage for the mutant, Rab5-GdI and Rab5-GEF activity can be strongly suppressed due to lowered ROS levels and thus, Rab5 cannot enter the membrane in late stages. This hypothesis seems doubtful because membrane-association was high in the basal area of early and mid-stages for the mutant but this association is absent completely in the apical area of late stages. Maybe, the ROS levels affect the functioning of Rab5-GTP activators differently in cell regions or can affect it differently during different cellularization stages. The high intracellular accumulation in the mutant might suggest that high Rab5 levels allow endocytosis to happen but endocytosis is just not happening due to the overall endocytic machinery being affected. The excess membrane in late stage can affect the localisation of apical polarity markers as these markers rightfully had to localise to the sharpened hexagons formed but in the *Drp1*^{SG} embryos where sharpened hexagons are not formed in the late stage, the markers localise to the excess membrane protruding and not on the actual hexagonal boundaries. Staining for apical polarity markers and not observing its hexagonal localisation at the end of late cellularisation can prove this. The crowding of Rab5 compartments intracellularly can spatially obstruct the travel of the apical polarity marker vesicles.

To conclude, Rab5 defects seen in late cellularization stages suggest defects in formation and migration of early endosomes from the apical membrane and defects

in earlier stages suggest of increased endocytosis from the membrane because of increased membrane localisation.

3.2. Analysis of Recycling Endosomes' Dynamics in embryos deficient for mitochondrial fission protein Drp1:

Early endosomes formed are either recycled back to different regions of plasma membrane or degraded. The recycling endosomes are marked by the Rab11 marker which encompass the cargo in early endosomes and puts it in apical or basolateral domains. Apical membrane is endocytosed and the early endosomes formed are recycled back on the forming lateral membrane in cellularization and thus, Rab11 marked recycling endosomes are critical for membrane invagination as they insert these vesicles at the lateral surface. It is known that Rab11 endosomes recruit and maintain Crumbs and aPKC polarity modules in the apical domain and also recruit adherens' junction components like DE-Cadherin(Golachowska, Hoekstra and van IJzendoorn, 2010). In cases like Dynamin mutants where membrane insertion is altered, one can see polarity markers like Neurotactin trapped inside Rab11 vesicles and thus, it explains the role of Rab11 in introducing polarity markers at the furrow. Rab11 interacts with Nuclear fallout Protein (Nuf) at the pericentriolar recycling endosomes and both are vital in delivering the membrane at the growing furrows(Pelissier, Chauvin and Lecuit, 2003). Thus, it was critical to analyse Rab11 localisation during cellularization in the *Drp1^{SG}* mutants.

A. *Rab11 recycling endosomal marker at apical and basal side across cellularization*

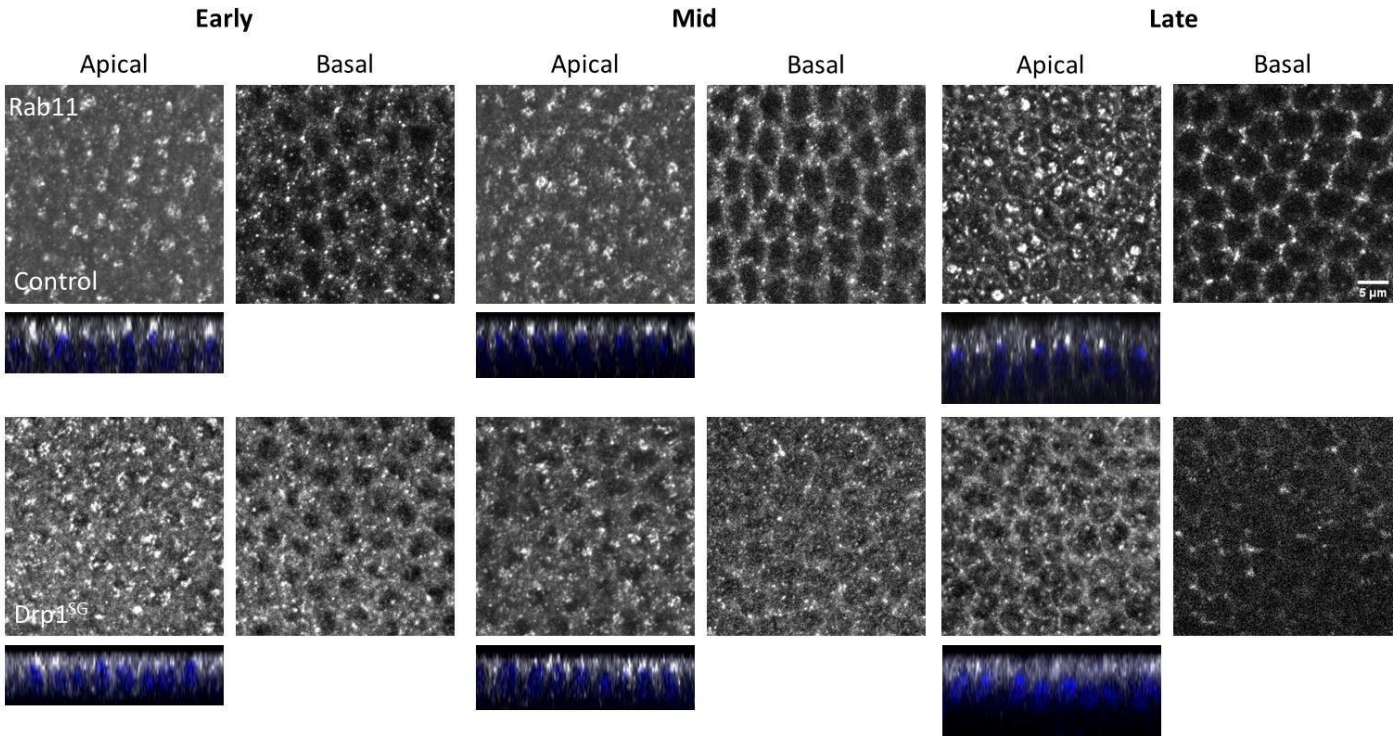


Figure 13: Analysis of Rab11 distribution as recycling endosome marker in basal junction at cellularization.

A. *Rab11 staining shown on the basal junction across early and mid-stages clearly showing that Rab11 accumulates properly at the basal junction in control early and mid-stage but the membrane localisation of Rab11 seems defective in* $Drp1^{SG}$ *early and mid-stage. Shown below are the orthogonal views respectively.n=6 embryos for control early, n=5 embryos for control mid, n=3 embryos for* $Drp1^{SG}$ *early and n=6 embryos for* $Drp1^{SG}$ *mid stage. Rab11 staining on the sub- apical area in late stage clearly showing that Rab11 localises on membrane in control but this accumulation is defective in the* $Drp1^{SG}$ *mutant. n=3 embryos for control late and n=3 embryos for* $Drp1^{SG}$ *late stage.*

We can see that Rab11 localises appropriately on the basal membrane in early and mid-stage but this membrane association seems defective in the $Drp1^{SG}$ mutant (Figure 13). In the early as well as the mid-stage in the mutant, the pattern of Rab11 appears very hazy and is never seen crisply on the membrane. It seems that Rab11 transport to the lateral membrane or the Rab11 binding to the membrane is affected in the mutant. If this is the case, then membrane endocytosed from the apical side will not reach the lateral side and does not contribute significantly to the growth of lateral side as seen in control. The Rab5 endosome which transitions to Rab11 recycling endosomes encompasses membrane along with the polarity factors which is deposited on the basal side and if this deposition is defective in the mutant, then one an in principle infer that polarity markers like DE-Cadherin going to the basal side are affected and not efficiently transported to the basal side and thus, accumulated in the cytoplasm. ROS scavengers are known to disassemble microtubule organisation in *A. thaliana* cells(Dang *et al.*, 2018) and it can be expected that microtubule organisation is also affected in the $Drp1^{SG}$ embryos which affect the Rab11 transport. It is known that microtubule organisation is unaffected in $Drp1^{SG}$ embryos and thus, lowered ROS affecting Rab11 transport on the microtubules seem less likely. Rab11 is prenylated at C-terminus and this, post-translational modification allows Rab11 binding to the membrane. The prenyl-binding domains are targeted in cancers to not allow Rab11-mediated membrane delivery and it seems that reduction of ROS levels can affect the Rab11-prenylation(Lee, Wickner and Song, 2020). It is also possible that Rab11 GEFs are not activated in

the presence of lowered ROS in the mutant, thus ultimately affecting its membrane binding.

Similarly, in late stage, we can see that Rab11 associates with the membrane at the sub-apical side mediating the delivery of apical polarity proteins and adherens' junction components like DE-Cadherin. In *Drp1^{SG}* embryos, we can see that membrane-association is hampered and again, this can be due to the defect in recycling endosomes transport to the membrane or Rab11 association at the membrane which can be affected by prenylation or GEF dynamics. This explains that polarity molecules targeted to the apical side and adherens' junction via Rab11-mediated vesicular delivery is altered in the *Drp1^{SG}* embryos and thus, they do not localise properly at the sub-apical membrane and this, then explains the lowered delivery of DE-Cadherin at the sub-apical membrane. (Figure 6)

In both the genotypes, Rab11 associates properly with the pericentriolar region where nuclear fallout protein is localised and it indicates that lowered ROS levels do not affect Rab11 pericentriolar localisation. Thus, we conclude that Rab11 membrane-association is altered basally in early and mid-stage and sub-apically in the late stage for the *Drp1^{SG}* embryos.

3.2. Analysis of Degradation Endosomes' Dynamics in embryos deficient for mitochondrial fission protein Drp1:

Early endosomes cargo, if has to be degraded, transition as degrading endosomes marked by Rab7 (Ras Analog in Brain 7) marker. The Rab7 GTPase helps mature early endosomes to late endosomes, fusion of late endosomes to lysosomes for degradation and helps microtubule transport of late endosomes. Rab7 helps in mitophagy wherein isolation membrane is put around damaged mitochondria and Rab7 allows for autophagosome membrane growth, the oxidised and unfolded proteins from mitochondria also bud off as vesicles and are marked by Rab7 for degradation. They also can degrade excess amounts of membrane, proteins and lipids which are dysfunctional and helps maintain their optimal levels(Guerra and Bucci, 2016). In cases where mitochondria fuse, it is known that mitochondrial degradation reduces as punctuated mitochondrion is not available. We also observed cytoplasmic localisation of polarity factors like PatJ and myosin II which could possibly not be degraded (Figure 9 and 10). Also, apical membrane is not endocytosed by Rab5 (Figure 11) and thus, degradation might not happen. Thus, we had to check for the degradation dynamics in cellularisation to see for the

defect in the *Drp1^{SG}* embryos.

A. *Rab7 degradation endosomal marker at apical and basal side across cellularization*

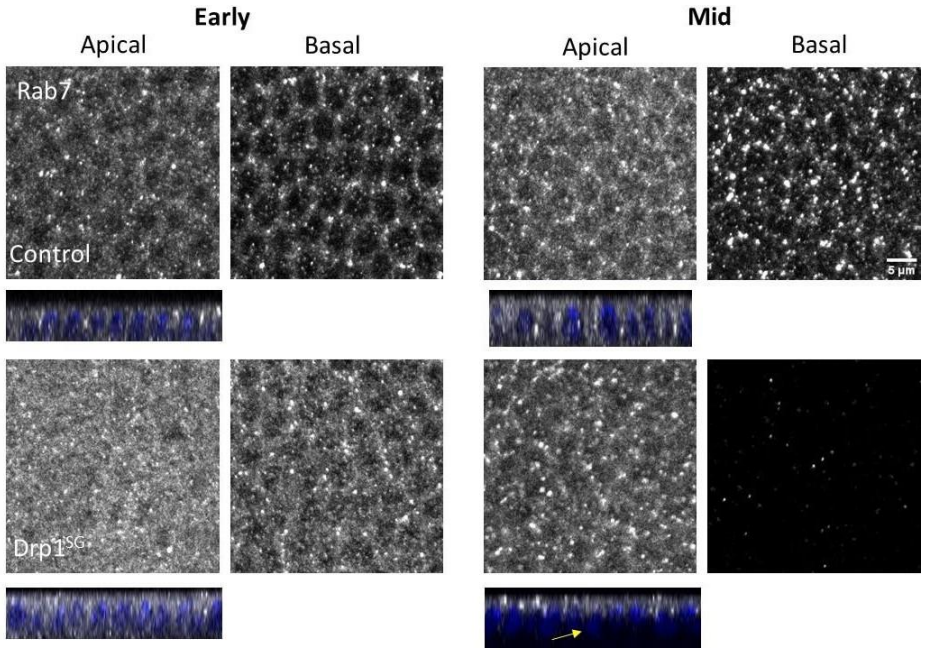


Figure 14: Analysis of Rab7 distribution as degradation endosome marker in apical and basal side at cellularization.

A. *Rab7 staining shown on the apical and basal side across early stage clearly showing that Rab7 accumulates extensively at the apical and basal side in Drp1^{SG} early. In mid, the apical side show similar levels of degrading endosomes in both the genotypes but Rab7 heavily reduces at the basal side in Drp1^{SG} embryos (Yellow arrow marks the reduction of Rab7 at the basal side). Shown below are the orthogonal views respectively. n=3 embryos for control early, n=6 embryos for control mid, n=3 embryos for Drp1^{SG} early and n=6 embryos for Drp1^{SG} mid stage.*

In early cellularization, we can see that Rab7 degrading endosomes appear apically and can be seen on the basal side in control (Figure 14). The Rab7 marker accumulates extensively on the apical and basal side in early stage of *Drp1^{SG}* embryos and all throughout the embryo, we can see increased Rab7 accumulation. In the mid stage, Rab7 again accumulates on the apical side and there is a characteristic clustering right at the basal side in control. In mid-stage of *Drp1^{SG}* embryos, we could see it on the apical side but the signal is lost completely from the

basal side.

This is an interesting result because we did expect Rab7 to have reduced intensity because excess membrane is not endocytosed, mitophagy is reduced and polarity factors persist in cytoplasm for the *Drp1^{SG}* mutant. But we observed the exact opposite where Rab7 has increased intensity at the early stage both in the apical and basal side. In dorsal ruffles which are actin-rich protrusions, Rab7 help form and organise it (Mascia *et al.*, 2016) and it can be possible that Rab7, in apical side of early and mid-stage of both the genotypes maintain the structures of actin-rich microvilli present there. Maybe, it can be case that as degradation is just not happening in the *Drp1^{SG}* embryos, Rab7 is attempting for degradation just by accumulating heavily. The accumulation of Rab7 does not support functioning because it is well known that Reactive Oxygen Species and Reactive Nitrogen Species target the nucleotide-binding NKCD motif in Rab7 for its activation(Ferro *et al.*, 2012) and as *Drp1^{SG}* embryos have it reduced, activation of Rab7 might not happening and it is just not functional.

In mid stage however, we just do not see Rab7 accumulation on the basal side in the *Drp1^{SG}* mutant. This does support PatJ and myosin II result as in the basal region of mid staged embryo, their accumulation can increase because Rab7 overall has reduced intensity on the basal side. Maybe, the Rab7 activators like GEF can affected by the reduced levels of ROS in the mutant and thus, we do not see it activated on the basal side. After Rab5-mediated endocytosis of apical membrane, it is known that apical membrane travel basally and might encounter degradation basally(Fabrowski *etal.*, 2013). Thus, in the mid stage for the control, we observed huge clusters of Rab7 at the base and it is highly possible that Rab7 mediates degradation here. In the mutant, we did not observe this Rab7 basal clustering and we can infer that Rab7- mediated degradation of membrane and proteins does not happen in the *Drp1^{SG}* embryos. It is crucial to determine that Rab7 accumulation in the early stage for the mutant is in its active or inactive state and exactly what is the cargo undergoing degradation. As polarity factors persist in mid-stage of *Drp1^{SG}* embryos, we can infer that this is due to the reduction of Rab7 in the mid stage. If this is really the case, then polarity proteins which do not localise properly on their respective plasma membrane domains might not be degraded and as overall degradation pathway is affected, unfolded and non-localised proteins will still persist in the cell and might affect the overall localisation and functioning of other polarity markers. Thus, we conclude that diminished intensity of the Rab7 at the basal side

in mid-stage marks the defects in degradation pathway in *Drp1*^{SG} mutants.

3.3. Analysis of Basal Membrane endocytosis in embryos deficient for mitochondrial fission protein Drp1:

The majority of membrane in the furrow comes from the unfolding of apical membrane reservoirs in the early and mid-stage of cellularization(Figard *et al.*, 2016). The furrow canal assembles completely in the early stage when the nuclei first become spherical to elongated and the furrow ingresses about 5 microns. During this formation of the furrow canal, some of the furrows can ingress much deeper and this can lead to asynchrony because it is expected that almost all the cells ingress similarly in space and time. To regulate this, the excess membrane ingresses is endocytosed in the form of membrane tubules which is marked by amphiphysin protein and endocytosed membrane again re-localises back to the apical microvilli. It can also engulf excess contractile proteins from the fast ingressing furrows and thus, provides a mechanism for efficient synchrony in ingression. Amphiphysin has the characteristic BAR domain allowing membrane curvature, mediates endocytosed tubule formation and organisation. The pinching off the tubules depend on actin and dynamin as well(Sokac and Wieschaus, 2008, p. 200; Su *et al.*, 2013). Thus, amphiphysin as a basal membrane recycling marker is checked to see for its localisation and functioning in *Drp1*^{SG} embryos to verify if these dynamics are altered in the mutant.

Amphiphysin on basal membrane in early stage and as tubules in early and mid-stages

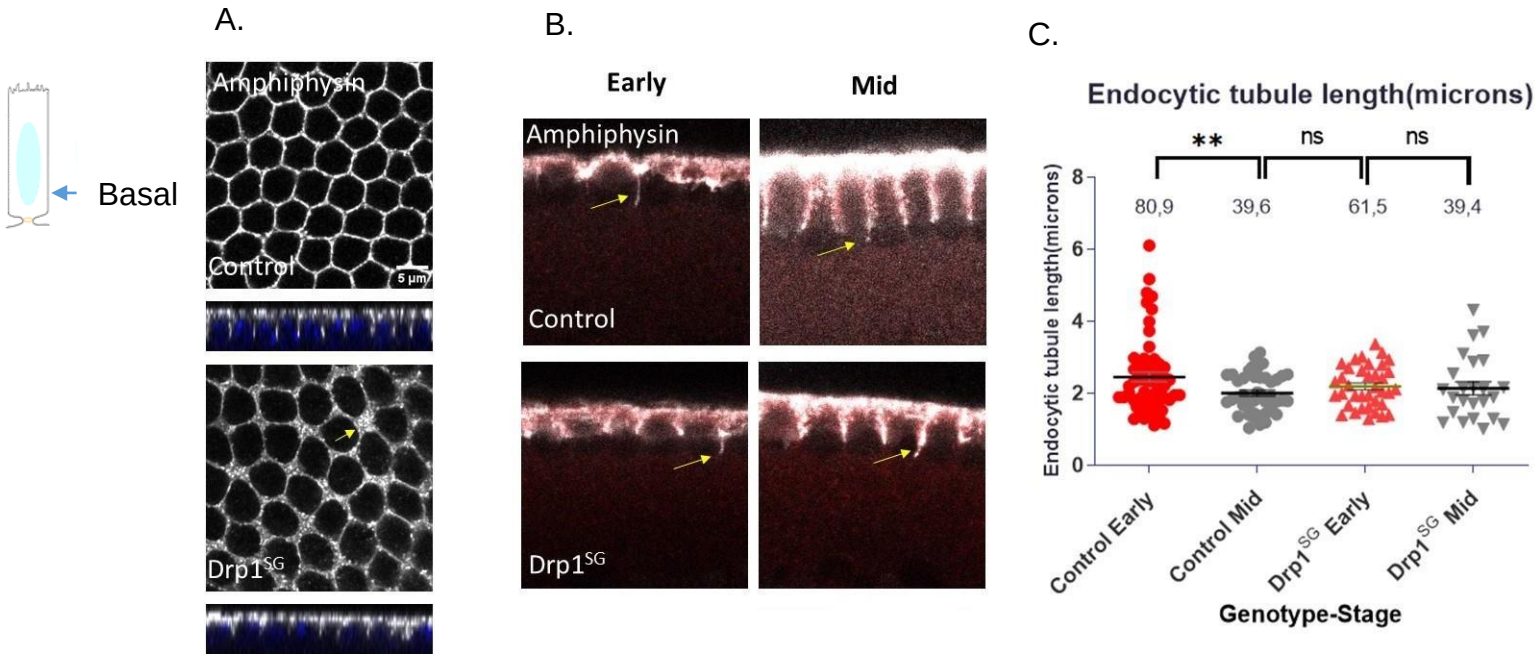


Figure 15: Analysis of amphiphysin as basal ring marker in early and mid-cellularization.

***A**. Amphiphysin staining shown on the basal ring across early stage clearly showing that it associates properly at the basal ring in control early but the membrane localisation of amphiphysin seem defective in Drp1^{SG} early. Arrow denotes amphiphysin clustering in the mutant. n=4 embryos for control early andn=4 embryos for Drp1^{SG} early. Shown below are the orthogonal views respectively.*

***B**. Representative images depicted for early and mid-cellularization endocytic tubules marked with DLG in red as the membrane marker and amphiphysin in yellow suggesting that tubules do not decrease in length in Drp1^{SG} embryos and might attribute to its persistence from early stages. n=9 (80 tubules) and n=6 (39tubules) embryos in control early and mid- respectively while in Drp1^{SG} mutant case, n=5 (61 tubules) and n=4 embryos (39 tubules) in early and mid- respectively.*

***C**. The length of the endocytic tubules is represented as average ± SD. The statistical analysis was carried out by Mann-Whitney t-test.*

We see amphiphysin localising properly at the basal ring in control embryos at early cellularization but the same is not true for *Drp1*^{SG} as we see amphiphysin clustering around the basal rings (Figure 15). Amphiphysin localisation at the apical side and across the furrow seem similar in control and the mutant but once at the basal side where amphiphysin functions, we observe a change in localisation. Based on the cytoplasmic PatJ and myosin II defects as well as DE-Cadherin at the basal junctions, we did infer that basal junction assembly is severely affected in the *Drp1*^{SG} embryos and if this is true, we can see that amphiphysin which generally localises on the ring can exit the basal junction and localise at places around the ring. Another possibility can be that high numbers of furrows can ingress asynchronously in the *Drp1*^{SG} embryos and thus, amphiphysin has to be localised as clusters to remove off the excess membrane. This can be believed because if the basal junction is damaged, it might not prevent the continuous cortical flow of actin and myosin II which can drive this asynchronous ingression during the formation of furrow canal. If the basal junction itself is problematic, then it is expected that other polarity factors residing in the furrow canal as well as the forming lateral membrane can have altered localisations. The N-terminal helix of amphiphysin inserts itself on the membrane and it specifically binds negative regions on the membrane(Aryal *et al.*, 2022). The reduced levels of ROS in the mutant can affect this N-terminal helix structurally or directly affect the charge on the membrane so that amphiphysin cannot bind the membrane properly.

One can easily visualize amphiphysin tubules in early and mid-stage which emanate from the base of the furrow and the lengths were checked to see if there were any defects in the length of the tubules (Figure 15). Surprisingly, the tubule length reduces in *Drp1*^{SG} mutant early stage and does not decrease in the mutant mid stage. In control, the tubule length decreases in mid because by the time cells enter mid stage, furrow canal is properly formed, there is timely coordination of ingression and owing to the growing synchronous ingression, less furrows ingress faster and thus, shorter tubules are seen. No decrease in tubule length in mutants suggests that these tubules persist from early to mid-stages and do not undergo endocytosis suggesting endocytic defects as well as altering

coordination of the ingressing furrows. Also, the tubules appear shorter in mutants and might be due to the effect of low ROS levels on the workings of the endocytic machinery that help assemble the tubules. Amphiphysin null mutants have shorter cleavage furrow tip tubules(Su *et al.*,2013) and the same is observed in *Drp1*^{SG} embryos suggesting reduction in amphiphysin functioning possibly due to hampered localisation at the base .In Nullo mutants, the amphiphysin tubules still persist and furrow canal components like PatJ migrate in these tubules(Sokac and Wieschaus, 2008). It is highly possible that in *Drp1*^{SG} as well where tubules are not cleaved, polarity factors like PatJ might migrate in these tubules and have reduced functioning and localisation at the furrow. The BAR domain of amphiphysin helps recruit dynamin(Su *et al.*, 2013) but dynamin seems defective as tubules are not cleaved off and thus, in our mutant, it seems that BAR domain or dynamin can be affected by the lower levels of ROS. Thus, the shorter endocytic tubules reveal its persistence in the mid-stage from the early stage in *Drp1*^{SG} mutant and can hopefully suggest for the possible role of mitochondria in regulating endocytic machinery. This might drastically affect the kinetics of furrow ingression as endocytic tubules generated help in the timely ingression of furrows in all the cells but in the mutant case, it seems that the tubules just do not cleave off.

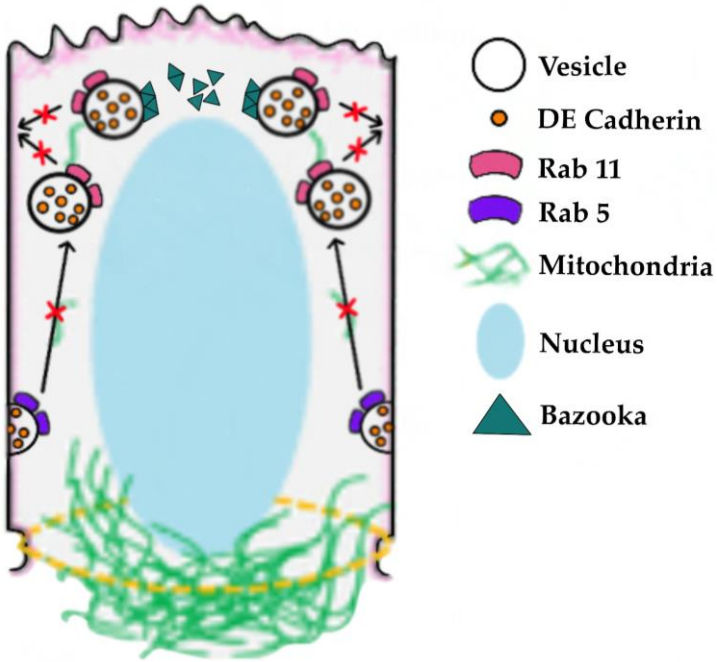
Thus, we conclude that basal components endocytic machinery is affected in the *Drp1*^{SG} embryos.

4. Summary:

	Control	*Drp1^{SG}*
1.Epithelial cell shape analysis		
i. Apical Area	Similar to *Drp1^{SG}*	Similar to control
ii. Basal Area	The rings constrict as cellularization progresses	Reduced in early and mid-stages while relaxed in late
2. Polarity protein analysis		
i. DE-Cadherin		
i) Apical	Accumulates at the apical adherens' junction	Does not accumulate apically at adherens' junction
ii) Basal	Accumulates at basal adherens' junction	Improper accumulation basally
iii) Sod2-mediated rescue	Sod2i show proper accumulation at apical and basal side	Partial accumulation at apical and basal side when global ROS levels rose up
ii. Bazooka	Accumulates apically and migrates to sub-apical side	Less accumulation apically and less migration at sub-apical side seen
iii. DLG	Localises at sub-apical side in late	Localises sub-apically as well as at the basal side of lateral membrane in late
iv. PatJ	Localises at acto-myosin rings across cellularization	Localises at acto-myosin ring as well as in the cytoplasm
v. Myosin II	Localises at acto-myosin rings	Localises at acto-myosin ring

	across cellularization	as well as in the cytoplasm and reduced in late-stage
3.Trafficking markers analysis		
i. Rab5		
Apical	Clustered in early and mid an at membrane and cargo in late	Complete membrane-localised at membrane in early and mid and intracellular accumulation in late
Basal	Clustered in early and mid an at membrane and reduced basally in late	Complete membrane-localised at membrane in early and mid and reduced basally in late
ii. Rab11		
Apical	Onto perinuclear region in early and mid and onto the sub-apical membrane in late	Onto perinuclear region in early and mid and incomplete sub-apical membrane in late
Basal	Basally localised and cytoplasmic near rings in early and mid and onto the membrane in late	Hazily localised in early and reducing intensity at base as cellularization progresses
iii. Rab7		
Apical	Seen apically in early and mid	Seen apically in early and mid like control
Basal	Heavily accumulated at the base in early and mid	Accumulation seen early but completely reduced at mid
iv. Amphiphysin		
	Seen as tubules in early and mid and reduced length at mid	Tubules seen in early and mid and persistent length at mid

The work presented attempts to link mitochondrial morphology and dynamics to developmental biology in terms of polarity induction and maintenance in early *Drosophila* embryogenesis. Drp1-depleted embryos cause mitochondrial fusion which reduce the global ROS levels as well as hamper mitochondrial travel in the process of cellularization. It does not allow the completion of basal acto-myosin ring constriction though the overall apical area is unaffected. Polarity molecules like DE-Cadherin of the adherens' junction do not localise properly on the membrane in the apical and basal junction and hint towards defective adherens' junction formation but the phenotype is partially rescued when global ROS levels escalated in the system. The basal junction markers like PatJ and myosin appear cytoplasmic in the early stages suggesting of defects in basal junction formation while being completely reduced at late cellularization thus, allowing incomplete constriction. The lateral membrane marker of DLG persists at the basal side in late stage suggesting of trafficking defects of lateral proteins. Rab5 as an early endosomal marker remains membrane-bound across the furrow in the mutant and at late stage accumulates at the villi ultimately, not allowing for apical tubular endocytosis. Rab11 as recycling endosomal marker does not remain membrane-attached basally in early and mid-stage hinting towards inefficient membrane and polarity proteins basally and is also not accumulating at sub-apical membrane in late stage suggesting defective adherens' junction components' delivery. Rab7 as degradation marker is completely reduced basally in the mid stage and might hint towards defect in cargo degradation basally. Amphiphysin accumulates heavily at the basal side and the persistence of tubules in the mid stage does suggest of asynchronous furrow ingression and lack of tubule cleavage.



Schematic 4: Pathways hypothesized for mis-localisation of adherens' junction in Drp1^{SG} embryos: Different mechanisms act to recruit DE-Cadherin onto the sub-apical side which might remain defective in Drp1^{SG} embryos. The apical marker of Bazooka might not recruit DE-Cadherin onto the sub-apical side. Rab11 distribution is altered in Drp1^{SG} embryos and might not allow the sub-apical positioning of DE-Cadherin. Rab5 distribution basally is also affected and might hint towards the defective basal to apical translocation of DE-Cadherin.

The work emphasizes onto the faulty positioning of adherens' junction apically as observed in fixed and live cell imaging for DE-Cadherin. Newly formed DE-Cadherin can exit the trans-Golgi network and associate with Rab11 apical endosomes to reach the sub-apical side. Bazooka clusters also help recruit the DE-Cadherin vesicles on the apical side(Harris and Peifer, 2004). DE-Cadherin from the basal side goes apically via the Rab5 and Rab11 pathways(Woichansky *et al.*, 2016). Our data clearly suggests defects in the above pathways (Schematic 4) and might contribute towards the altered positioning and recruitment of DE-Cadherin apically.

It will be of great interest to see the mechanisms by which the ROS levels affect the localisation of polarity molecules, the protein residues or domains critically requiring ROS for activation or deactivation, what exact ROS species' play major roles in the processes as well as the aftermath in future developmental processes like gastrulation. Mitochondrial metabolites like calcium as well as mitochondrial interaction with other organelles in *Drp1^{SG}* embryos can be checked to see its role in maintaining polarity. One can indeed check for the effect of depleting mitochondrial fusion proteins to see if polarity is affected and that will present the complete role of mitochondrial morphology, dynamics and functioning to induce and maintain polarity in cellularization.

- **References:**

Aryal, C.M. et al. (2022) ‘The N-terminal helices of amphiphysin and endophilin have different capabilities of membrane remodeling’, Biochimica Et Biophysica Acta.

Biomembranes, 1864(7), p. 183907. Available at: https://doi.org/10.1016/j.bbamem.2022.183907.

Bell, G.P. et al. (2015) ‘Aurora Kinases Phosphorylate Lgl to Induce Mitotic Spindle Orientation in Drosophila Epithelia’, Current Biology, 25(1), pp. 61–68. Available at: https://doi.org/10.1016/j.cub.2014.10.052.

Buckley, C.E. and St Johnston, D. (2022) ‘Apical-basal polarity and the control of epithelial form and function’, Nature Reviews. Molecular Cell Biology, 23(8), pp. 559– 577. Available at: https://doi.org/10.1038/s41580-022-00465-y.

Chowdhary, S. et al. (2017) ‘Analysis of mitochondrial organization and function in the Drosophila blastoderm embryo’, Scientific Reports, 7(1), p. 5502. Available at: https://doi.org/10.1038/s41598-017-05679-1.

Chowdhary, S. et al. (2020) ‘Mitochondrial morphology and activity regulate furrow ingression and contractile ring dynamics in Drosophila cellularization’, Molecular Biology of the Cell, 31(21), pp. 2331–2347. Available at: https://doi.org/10.1091/mbc.E20-03-0177.

Dang, X. et al. (2018) ‘Reactive oxygen species mediate conical cell shaping in Arabidopsis thaliana petals’, PLOS Genetics. Edited by G.P. Copenhaver, 14(10), p. e1007705. Available at: https://doi.org/10.1371/journal.pgen.1007705.

Dudu, V., Pantazis, P. and González-Gaitán, M. (2004) ‘Membrane traffic during embryonic development: epithelial formation, cell fate decisions and differentiation’,

Current Opinion in Cell Biology, 16(4), pp. 407–414. Available at: https://doi.org/10.1016/j.ceb.2004.06.008.

Fabrowski, P. et al. (2013) ‘Tubular endocytosis drives remodelling of the apical surface during epithelial morphogenesis in Drosophila’, Nature Communications, 4,

p. 2244. Available at: https://doi.org/10.1038/ncomms3244.

Ferro, E. et al. (2012) ‘The Interplay between ROS and Ras GTPases: Physiological and Pathological Implications’, Journal of Signal Transduction, 2012, p. 365769.

Available at: https://doi.org/10.1155/2012/365769.

Figard, L. et al. (2016) ‘Membrane Supply and Demand Regulates F-Actin in a Cell Surface Reservoir’, Developmental cell, 37(3), pp. 267–278. Available at: https://doi.org/10.1016/j.devcel.2016.04.010.

Fölsch, H., Mattila, P.E. and Weisz, O.A. (2009) ‘Taking the scenic route: biosynthetic traffic to the plasma membrane in polarized epithelial cells’, Traffic (Copenhagen, Denmark), 10(8), pp. 972–981. Available at: https://doi.org/10.1111/j.1600-0854.2009.00927.x.

Giacomello, M. et al. (2020) ‘The cell biology of mitochondrial membrane dynamics’, Nature Reviews. Molecular Cell Biology, 21(4), pp. 204–224. Available at: https://doi.org/10.1038/s41580-020-0210-7.

Golachowska, M.R., Hoekstra, D. and van IJzendoorn, S.C.D. (2010) ‘Recycling endosomes in apical plasma membrane domain formation and epithelial cell polarity’, Trends in Cell Biology, 20(10), pp. 618–626. Available at: https://doi.org/10.1016/j.tcb.2010.08.004.

Guerra, F. and Bucci, C. (2016) ‘Multiple Roles of the Small GTPase Rab7’, Cells, 5(3), p. E34. Available at: https://doi.org/10.3390/cells5030034.

Halbleib, J.M. and Nelson, W.J. (2006) ‘Cadherins in development: cell adhesion, sorting, and tissue morphogenesis’, Genes & Development, 20(23), pp. 3199–3214. Available at: https://doi.org/10.1101/gad.1486806.

He, B., Martin, A. and Wieschaus, E. (2016) ‘Flow-dependent myosin recruitment during Drosophila cellularization requires zygotic dunk activity’, Development, 143(13), pp. 2417–2430. Available at: https://doi.org/10.1242/dev.131334.

Hebbar, S. and Knust, E. (2021) ‘Reactive oxygen species (ROS) constitute an additional player in regulating epithelial development’, BioEssays, 43(8), p. 2100096. Available at: https://doi.org/10.1002/bies.202100096.

Hunter, M.V. et al. (2018) ‘Oxidative Stress Orchestrates Cell Polarity to Promote Embryonic Wound Healing’, Developmental Cell, 47(3), pp. 377-387.e4. Available at: https://doi.org/10.1016/j.devcel.2018.10.013.

Kasahara, A. and Scorrano, L. (2014) ‘Mitochondria: from cell death executioners to regulators of cell differentiation’, Trends in Cell Biology, 24(12), pp. 761–770.

Available at: https://doi.org/10.1016/j.tcb.2014.08.005.

Lecuit, T. (2004) ‘Junctions and vesicular trafficking during Drosophila cellularization’, Journal of Cell Science, 117(16), pp. 3427–3433. Available at: https://doi.org/10.1242/jcs.01312.

Lecuit, T. and Wieschaus, E. (2000) 'Polarized Insertion of New Membrane from a Cytoplasmic Reservoir during Cleavage of the Drosophila Embryo', Journal of Cell Biology, 150(4), pp. 849–860. Available at: https://doi.org/10.1083/jcb.150.4.849.

Lee, M., Wickner, W. and Song, H. (2020) 'A Rab prenyl membrane-anchor allows effector recognition to be regulated by guanine nucleotide', Proceedings of the National Academy of Sciences of the United States of America, 117(14), pp. 7739– 7744. Available at: https://doi.org/10.1073/pnas.2000923117.

Lim, S.D. et al. (2005) 'Increased Nox1 and hydrogen peroxide in prostate cancer',

The Prostate, 62(2), pp. 200–207. Available at: https://doi.org/10.1002/pros.20137.

Liu, J. et al. (2021) 'ROS Homeostasis and Plant Salt Tolerance: Plant Nanobiotechnology Updates', Sustainability, 13(6), p. 3552. Available at: https://doi.org/10.3390/su13063552.

Lu, J. et al. (2021) 'Electrostatic plasma membrane targeting contributes to Dlg function in cell polarity and tumorigenesis', Development (Cambridge, England), 148(7), p. dev196956. Available at: https://doi.org/10.1242/dev.196956.

Madan, S. et al. (2021) 'Mitochondria Lead the Way: Mitochondrial Dynamics and Function in Cellular Movements in Development and Disease', Frontiers in Cell and Developmental Biology, 9, p. 781933. Available at: https://doi.org/10.3389/fcell.2021.781933.

Mascia, A. et al. (2016) 'Rab7 Regulates CDH1 Endocytosis, Circular Dorsal Ruffles Genesis, and Thyroglobulin Internalization in a Thyroid Cell Line', Journal of Cellular Physiology, 231(8), pp. 1695–1708. Available at: https://doi.org/10.1002/jcp.25267.

Mateus, A.M. et al. (2011) 'Endocytic and Recycling Endosomes Modulate Cell Shape Changes and Tissue Behaviour during Morphogenesis in Drosophila', PLoS ONE. Edited by A. Bergmann, 6(4), p. e18729. Available at: https://doi.org/10.1371/journal.pone.0018729.

McCaffrey, L.M. et al. (2012) 'Loss of the Par3 Polarity Protein Promotes Breast Tumorigenesis and Metastasis', Cancer Cell, 22(5), pp. 601–614. Available at: https://doi.org/10.1016/j.ccr.2012.10.003.

Murali, A. and Rajalingam, K. (2014) 'Small Rho GTPases in the control of cell shape and mobility', Cellular and molecular life sciences: CMLS, 71(9), pp. 1703–1721.

Available at: https://doi.org/10.1007/s00018-013-1519-6.

Murthy, M. et al. (2010) ‘Sec5, a member of the exocyst complex, mediates Drosophila embryo cellularization’, Development (Cambridge, England), 137(16), pp. 2773–2783. Available at: https://doi.org/10.1242/dev.048330.

Nakatsukasa, K. et al. (2014) ‘The Nutrient Stress-induced Small GTPase Rab5 Contributes to the Activation of Vesicle Trafficking and Vacuolar Activity*’, Journal of

Biological Chemistry, 289(30), pp. 20970–20978. Available at: https://doi.org/10.1074/jbc.M114.548297.

Nelson, W.J. (2003) ‘Adaptation of core mechanisms to generate cell polarity’,

Nature, 422(6933), pp. 766–774. Available at: https://doi.org/10.1038/nature01602.

Parone, P.A. et al. (2008) ‘Preventing mitochondrial fission impairs mitochondrial

function and leads to loss of mitochondrial DNA’, PloS One, 3(9), p. e3257. Available at: https://doi.org/10.1371/journal.pone.0003257.

Pelissier, A., Chauvin, J.-P. and Lecuit, T. (2003) ‘Trafficking through Rab11 Endosomes Is Required for Cellularization during Drosophila Embryogenesis’, Current Biology, 13(21), pp. 1848–1857. Available at: https://doi.org/10.1016/j.cub.2003.10.023.

Polgar, N. and Fogelgren, B. (2018) ‘Regulation of Cell Polarity by Exocyst-Mediated Trafficking’, Cold Spring Harbor Perspectives in Biology, 10(3), p. a031401.

Available at: https://doi.org/10.1101/cshperspect.a031401.

Riga, A., Castiglioni, V.G. and Boxem, M. (2020) ‘New insights into apical-basal polarization in epithelia’, Current Opinion in Cell Biology, 62, pp. 1–8. Available at: https://doi.org/10.1016/j.ceb.2019.07.017.

Seager, R. et al. (2020) ‘Mechanisms and roles of mitochondrial localisation and dynamics in neuronal function’, Neuronal Signaling, 4(2), p. NS20200008. Available at: https://doi.org/10.1042/NS20200008.

Sen, A., Nagy-Zsvér-Vadas, Z. and Krahn, M.P. (2012) ‘Drosophila PATJ supports adherens junction stability by modulating Myosin light chain activity’, The Journal of Cell Biology, 199(4), pp. 685–698. Available at: https://doi.org/10.1083/jcb.201206064.

Sharma, S. and Rikhy, R. (2021) ‘Spatiotemporal recruitment of RhoGTPase protein GRAF inhibits actomyosin ring constriction in Drosophila cellularization’, eLife, 10, p. e63535. Available at: https://doi.org/10.7554/eLife.63535.

Shivas, J.M. et al. (2010) ‘Polarity and endocytosis: reciprocal regulation’, Trends in Cell Biology, 20(8), pp. 445–452. Available at: https://doi.org/10.1016/j.tcb.2010.04.003.

Sing, A. et al. (2014) ‘The atypical cadherin fat directly regulates mitochondrial function and metabolic state’, Cell, 158(6), pp. 1293–1308. Available at: https://doi.org/10.1016/j.cell.2014.07.036.

Sokac, A.M. and Wieschaus, E. (2008) ‘Local actin-dependent endocytosis is

zygotically controlled to initiate Drosophila cellularization’, Developmental Cell, 14(5), pp. 775–786. Available at: https://doi.org/10.1016/j.devcel.2008.02.014.

Spiliotis, E.T. and Nelson, W.J. (2003) ‘Spatial control of exocytosis’, Current Opinion in Cell Biology, 15(4), pp. 430–437. Available at: https://doi.org/10.1016/s0955-0674(03)00074-7.

St Johnston, D. and Sanson, B. (2011) ‘Epithelial polarity and morphogenesis’, Current Opinion in Cell Biology, 23(5), pp. 540–546. Available at: https://doi.org/10.1016/j.ceb.2011.07.005.

Su, J. et al. (2013) ‘The BAR domain of amphiphysin is required for cleavage furrow tip-tubule formation during cellularization in Drosophila embryos’, Molecular Biology of the Cell, 24(9), pp. 1444–1453. Available at: https://doi.org/10.1091/mbc.E12-12- 0878.

Wang, F. et al. (2004) ‘The role of DE-cadherin during cellularization, germ layer

formation and early neurogenesis in the Drosophila embryo’, Developmental Biology, 270(2), pp. 350–363. Available at: https://doi.org/10.1016/j.ydbio.2004.03.002.

Wang, W.-J., Lyu, T.-J. and Li, Z. (2021) ‘Research Progress on PATJ and Underlying Mechanisms Associated with Functional Outcomes After Stroke’, Neuropsychiatric Disease and Treatment, 17, pp. 2811–2818. Available at: https://doi.org/10.2147/NDT.S310764.

West, J.J. and Harris, T.J.C. (2016) ‘Cadherin Trafficking for Tissue Morphogenesis: Control and Consequences’, Traffic (Copenhagen, Denmark), 17(12), pp. 1233– 1243. Available at: https://doi.org/10.1111/tra.12407.

Wj, N. and C, Y. (2001) ‘Protein trafficking in the exocytic pathway of polarized epithelial cells’, Trends in cell biology, 11(12). Available at: https://doi.org/10.1016/s0962-8924(01)02145-6.

Xu, M. et al. (2014) ‘Enhancement of dynein-mediated autophagosome trafficking and autophagy maturation by ROS in mouse coronary arterial myocytes’, Journal of Cellular and Molecular Medicine, 18(11), pp. 2165–2175. Available at: https://doi.org/10.1111/jcmm.12326.

Yuan, W. and Song, C. (2020) ‘The Emerging Role of Rab5 in Membrane Receptor Trafficking and Signaling Pathways’, Biochemistry Research International, 2020, p. 4186308. Available at: https://doi.org/10.1155/2020/4186308.

Zhou, W. and Hong, Y. (2012) ‘Drosophila Patj plays a supporting role in apical- basal polarity but is essential for viability’, Development (Cambridge, England), 139(16), pp. 2891–2896. Available at: https://doi.org/10.1242/dev.08