

The role of Actin regulators in polarity and membrane organization of epithelial like cells in early *Drosophila* embryo

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By

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BS-MS Dual Degree Program



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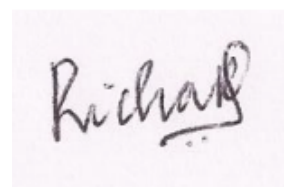
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Certificate

This is to certify that this dissertation entitled “**The role of actin regulators in polarity and membrane organization of epithelial like cells in early *Drosophila* embryogenesis**” towards the partial fulfilment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Vishnu MS** at IISER Pune under the supervision of “**Dr. Richa Rikhy**, Assistant Professor, IISER Pune Biology Department” during the academic year 2014-2015.

A handwritten signature in black ink, appearing to read 'Richa Rikhy', is centered within a light pink rectangular box.

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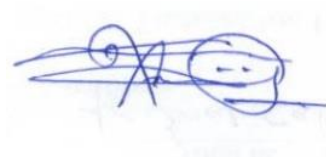
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Declaration

I hereby declare that the matter embodied in the report entitled “**The role of actin regulators in polarity and membrane organization of epithelial like cells in early *Drosophila* embryogenesis**” are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER), Pune, under the supervision of **Dr. Richa Rikhy** and the same has not been submitted elsewhere for any other degree.



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Abstract

The syncytial *Drosophila* embryo has an epithelial like plasma membrane (PM) with an apical like and lateral like membrane domains even in the absence of complete cells. Previous studies in lab showed polarised distribution of plasma membrane proteins like Bazooka, Peanut and Patj and the hexagon dominant membrane packing of epithelial like cells in *Drosophila* early embryogenesis. We are studying the role of the actin cytoskeleton in maintaining plasma membrane polarity and polygonal packing in *Drosophila* early syncytial and cellularisation stages. RNAi mediated knockdown, OvoD1 germline clone strategy and pharmacological inhibitors are used to generate embryo mutants for branched and bundled actin regulators such as Arp2/3, WASP and Rhogef2 and Diaphanous respectively. Our studies show that Bazooka and Peanut are delocalised in the XY plane of the plasma membrane of RNAi mediated knockdown of bundled actin mutant embryos. Patj distribution remains largely unaffected and reveals morphogenetic defects in branched and bundled actin mutant embryos. Polygon analysis showed a shift towards greater numbers of pentagons in branched and bundled actin mutant embryos. Short metaphase furrow canals and loose membrane are seen during syncytial stages for embryos mutant for RhoGEF2 RNAi. We are able to show actin cytoskeleton possibly plays an important role in the localisation of Bazooka during early syncytial stages and maintains polygonal organisation of epithelial cells in early *Drosophila* embryogenesis.

List of Figures.

Fig.No.	Legend	Page No.
1.1	Asymmetric distribution of junctional component in epithelial cell.	
1.2	Bundled and branched actin network.	
1.3	Conserved hexagonal dominant packing in plant and animal kingdom.	
1.4	Previous studies in the lab.	
3.1	Latrunculin A treatment of embryo.	
3.2	Knock down of RhoGEF2 in RhoGEF2 RNAi.	
3.3	Giant nuclei phenotype of RhoGEF2.	
3.4	Knock down Dia in Dia RNAi.	
3.5	Knock down of Arp in Arp66b RNAi.	
3.6	Knockout of Dia and Arp in dia5 and arpC1 mutants.	
3.7	WT localisation pattern on Peanut during syncytium and cellularisation.	
3.8	WT localisation pattern on Bazooka during syncytium and cellularisation.	
3.9	WT localisation pattern on Patj during syncytium and cellularisation.	
3.10	Polarized distribution of Bazooka and Peanut defective in RhoGEF2 RNAi.	
3.11	Polarized distribution of Bazooka and Peanut in Dia RNAi.	
3.12	Polarized distribution of Bazooka and Peanut in Arp RNAi and WASp RNAi.	
3.13	Polarized distribution of Patj in cellularisation for mutants of Arp, WASp and RhoGEF2.	
3.14	Polygon packing in syncytial and cellularisation embryos for WT and embryos mutant for actin regulators.	

3.15	Polygon distribution of WT embryo during 12th and 13th syncytial stages.	
3.16	Polygon distribution of RhoGEF2 RNAi embryo during 12th and 13th syncytial stages.	
3.17	Polygon distribution of Dia RNAi embryo during 12th and 13th syncytial stages.	
3.18	Polygon distribution of Arp66b RNAi embryo during 12th and 13th syncytial stages.	
3.19	Polygon distribution of WASp RNAi embryo during 12th and 13th syncytial stages.	
3.20	Loose membrane phenotype of RhoGEF2 RNAi.	
3.21	Line scan analysis for WT and RhoGEF2 RNAi.	
3.22	Metaphase furrow canal length of embryos mutant for actin regulators.	
3.23	Graph showing metaphase furrow canal length of embryos mutant for actin regulators	
4.1	Model for bundled actin regulation of polarised distribution of Bazooka, Patj and Peanut and the membrane architecture of epithelial cell.	

Table No.	Legend	Page No.
2.1	List of fly lines used in the project	
2.2	List of antibodies and dyes used in the project.	
3.1	Percentage lethality of RNAi mutants.	
4.1	Summary of the analysis of polarised distribution of polarity proteins Bazooka, Peanut and Patj in embryos mutant for actin regulators.	
4.2	Summary of the polygon analysis in embryos mutant for actin regulators.	

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1. Introduction

1.1 Epithelial cells are the morphologically distinct cell type in metazoan.

Epithelial cells are one of the first formed cell types in metazoan ontogeny and they serve the fundamental function of segregating and protecting internal compartment from the external environment. They also perform functions like tissue morphogenesis, organogenesis, vectorial transport of nutrient or ions etc. Epithelial cells are characterized by asymmetric distribution of organelles and proteins and a polarised plasma membrane with discrete domains which are morphologically and functionally different from each other. They consist of an apical domain containing microvilli, a lateral domain containing junctions and a basal domain contacting the substratum (Mostov, Su et al. 2003). Several molecular players are responsible for the formation and maintenance of these different domains in an epithelial cell.

1.2 The epithelial cell plasma membrane comprises 3 asymmetrically distributed junctional components

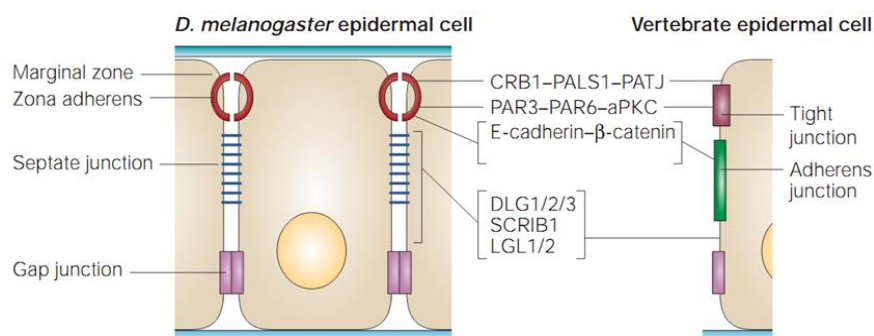


Figure 1.1. Showing the asymmetrically distributed junctional components in invertebrate and vertebrate epithelial cell (Macara, 2004).

Depending upon the differential localisation of proteins, epithelial cells are characterised to have different domains in the apico-basal axis. The apical domain mainly contains two complexes namely PAR-complex and the crumbs complex. PAR-3 (Bazooka), PAR-6 and aPKC constitute the PAR complex (Knust and Bossinger 2002) and Crumbs, PALS1 (Stardust), Patj makes the Crumbs complex (Bilder, Schober et al. 2003). In mammalian system, these two complexes bind to the tight junction that separates apical and baso-lateral domain. In *Drosophila*, these proteins localise to the subapical complex (also known as marginal zone), which is the border between apical and baso-lateral complexes. Adherens junction is present

below the tight junction (below sub apical complex in *Drosophila*), where E-cadherins and Nectins are present which helps the cells to adhere each other. α -catenin and β -catenin are known to regulate the E-Cadherins and Afadin plays a role in regulating Nectins (Shin, Fogg et al. 2006) (Fig. 1.1).

The baso-lateral domain of the epithelial cells mainly contain two sets of protein complexes; Scribble complex which contains Disc-large (Dlg), Lethal giant larve (Lgl) and Scribble (Scrb) (Bilder, Li et al. 2000) and Coracle complex that contains Coracle, Neurexin IV and Na,K-ATPase (Laprise, Lau et al. 2009). In *Drosophila*, Scribble complex protein localises to the septate junction (similar to tight junction in mammals). On the lateral domain, Gap junctions are present which mediates intercellular communications. Basal domain contain integrins that communicate with the extra cellular matrix.

1.3 The epithelial cell polarisation is conserved across metazoan kingdom.

The regulation of epithelial polarity formation is highly conserved across metazoan kingdom. Cnidarians are considered to be the simplest metazoan which has an epithelium by definition of having proper domain in apico-basal axis (Tyler 2003). Even though sponges are reported to have similar molecular marker binding apical domain, it lacks junctional domain and baso-lateral domain (Fahey and Degnan 2010). The higher order invertebrates and vertebrates follows conserved mechanism of epithelial cell polarisation during their development. For example, in *C.elegans* embryogenesis polarity of the embryo is evident from the very first event of fertilisation, where the signal from sperm centrosome polarises the zygote in to A/P axis (St Johnston and Ahringer 2010). A/P axis of the embryo is also mediated by PAR proteins similar to the apico-basal polarity of epithelial cells. The anterior region of the embryo is enriched with PAR-3, PAR-6 and aPKC and posterior region is enriched with PAR-1 and PAR-2. During 4 cell stage of embryo development, the embryo starts polarising radially and the apical PAR complex proteins start relocating to the contact free surfaces of the cells and PAR-2 and PAR-1 localised in a complimentary fashion to the cell contact sites. The mutual exclusion among these complexes maintain its asymmetric localisation. PAC1 (RhoGAP) is responsible for the reallocation of PAR protein upon contact dependant cues in *C.elegans* (Nance and Priess 2002). In *Xenopus*, blastomeres adopt epithelial character and tight

junction (TJs) assembly during the first cleavage itself (Fesenko, Kurth et al. 2000). Here also, it is the same PAR proteins aPKC and PAR6b responsible for apical cortex formation and aPKC antagonises the baso-lateral regulators PAR1 and Lgl2 from localising to the apical domain (Ossipova, Tabler et al. 2007). In a vertebrate system like mouse embryo, radial polarisation starts at 8 cell stage which involves the asymmetric localisation of PAR proteins (Louvret, Aghion et al. 1996). PAR-3, PAR6b and aPKC becomes restricted to apical contact free surfaces and PAR1/EMK1 is localised to the baso-lateral contacted surfaces (Vinot, Le et al. 2005).

1.4 Actin cytoskeletal dynamics in a cell.

Actin cytoskeleton integrity is important for the maintenance of cell shape and structure. Continuous remodelling of actin network in response to external stimuli is important for cell motility and formation of functionally specialised structures such as filopodia, lamellopodia, stereocilia, microvilli etc. Thus actin cytoskeleton plays an important role in cell signal transduction pathway. For example, actin dynamics is important for the absorptive function of intestinal epithelium, mechanosensing in the ear, phagocytosis by a macrophage during an immune response etc. The regulation of actin network is through a set of actin binding proteins which are known as actin regulators. There are two types of actin organisation in an actin network such as parallel unbranched bundles of filaments and highly branched, interlaced filament networks. Bundled actin network is known to be regulated through actin nucleators such as Diaphanous, Formin etc. Rho1 GTPase activated by RhoGEF2 is known to regulate the activation Diaphanous. Branching of actin filaments are regulated through the actin nucleator Arp2/3 which is inturn regulated through the protein WASp (Fig.1.2). These bundled and branched

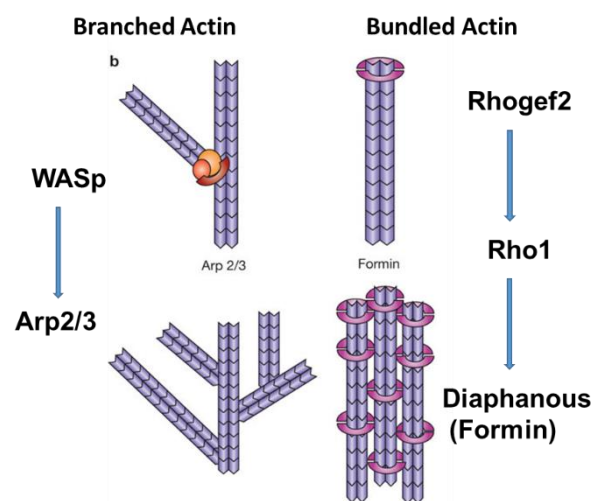


Figure 1.2. Showing the branched and bundled actin network. Branched actin network is regulated through WASp and Arp2/3 and bundled actin is regulated through RhoGEF2, Rho1 and Dia. (adapted from Chhabra and Higgs 2007)

actin network together controls the dynamics of a cell (Revenu, Athman et al. 2004, Chhabra and Higgs 2007).

1.5 Actin cytoskeletal network plays a crucial role in epithelial cell polarity.

In *C.elegans*, PAR-3, PAR-6 and aPKC is distributed uniformly across the cortex of the embryo till 30 minutes after the fertilisation. The embryo contains a dynamic and contractile actomyosin network that appears to be destabilized near the point of sperm entry. The asymmetric actomyosin network creates an imbalance and initiates a flow of cortical nonmuscle myosin and F-actin towards the opposite future anterior pole of the embryo. PAR-3, PAR-6 and aPKC which are known be associated with F-actin network also moves towards the anterior pole in this cortical flow of actin and thus they create their asymmetric distribution (Munro, Nance et al. 2004). In *Drosophila* cellularisation stage, it has been shown that Bazooka which is analogous to PAR-3 localised to the apical domain with the help of actin cytoskeletal cues. This actin cytoskeletal cues mediates the basal to apical Bazooka transport as well as supports the binding of Bazooka to the apical scaffold (Harris and Peifer 2005). In *Drosophila* oogenesis, small GTPase Cdc42 plays a crucial role in maintaining oocyte polarity. Cdc42 itself is asymmetrically localised to the anterolateral cortex of the oocyte and it regulates the stability of oocyte actin network through which it mediates the apical localisation PAR-6/aPKC/Bazooka complex (Leibfried, Muller et al. 2013).

1.6 Epithelial cells are packed in a polygonal array with a predominant distribution of hexagons

Epithelial cell packing is another unique feature which is conserved across the metazoan kingdom. Topology, the connectivity among cells in a tissue forms a regular pattern of polygonal arrangements among metazoans. In fact, this topological structure of epithelial cell packing is also conserved across plant kingdom and shows similarities

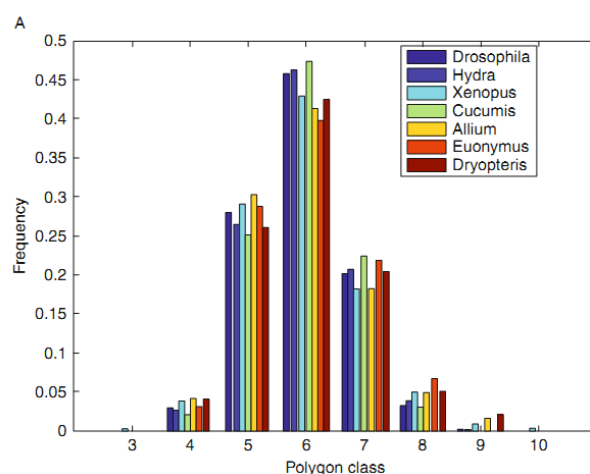


Figure 1.3. Showing the hexagonal dominant packing of epithelial cell in plant and animal kingdom (adapted from Gibson and Gibson 2009).

with other cellular structures like compressed soap bubble, honeycombs, insect retinal cells etc. The observation that apical section of proliferative epidermal cells have constant distribution of polygon type was first made by Lewis in cucumber. This special geometric order of epithelial cells has a dominant fraction of hexagons followed by pentagons, then heptagons and lesser frequencies of other polygons (Gibson and Gibson 2009) (Fig. 1.2). The reason that many biological and non-biological system adapt to such predictable geometric array is to minimize surface energy or maximize space filling (Hayashi and Carthew 2004). Gibson et. al. proved mathematically and experimentally that, “a few cycles of topology changes by the mechanism of asymmetric cell division is sufficient enough to create an equilibrium topology in which ~50% of cells become hexagonal followed by variable frequencies of pentagons and heptagons, regardless of the initial conditions”. The division mechanism of epithelial cells are governed by the mitotic spindle orientation and cleavage plane on the cell cortex (Strauss, Adams et al. 2006). In plants, the preprophase band, a ring of microtubules and F-actin defines the future site of cleavage plane on the cell cortex (Jurgens 2005). In *Drosophila*, adherens junction components such as E-cadherin, Bazooka and Canoe are known to regulate the spindle orientation mechanism (Le Borgne, Bellaiche et al. 2002, Speicher, Fischer et al. 2008). Another aspects which affects the geometric order of the epithelial cells is the cortical tension which is mediated through cell adhesion and apical constriction. In metazoans, Myosin II and actin cytoskeleton plays a crucial role in regulating cell adhesion and apical constriction (Fox and Peifer 2007). E-cadherins accumulates at the cell-cell contact surfaces and helps in the formations and stability of adherens Junctions (AJs) in epithelial cells. RhoGEF2, activator of Rho1 GTPase plays an important role in the regulation of assembly and disassembly of AJs during apical constrictions (Laplanche and Nilson 2006).

1.7 The syncytial *Drosophila* blastoderm embryo forms epithelial like cells with distinct domains and polygonal organization

The syncytial *Drosophila* embryo has an epithelial like plasma membrane (PM) with polarized distribution of protein in apical like and lateral like membrane domains even in the absence of complete cells (Fig 1.4A) (Mavrakakis, Rikhy et al. 2009). The *Drosophila* syncytium and cellularisation is a good model system to study onset of polarity formation and epithelial topology de novo. In *Drosophila* embryogenesis, nuclei remain centrally until the 9th division after which they migrate to the periphery of the embryo. In nuclear cycle 11-14, the PM partially encases each nucleus. Previous studies in lab gave us some exciting incites on the polarized distribution of plasma membrane proteins like Bazooka, Peanut and Patj and the hexagon dominant membrane packing of epithelial like cells in *Drosophila* early embryogenesis. Peanut belongs to the septin family of proteins which has a function of cytokinesis through its interaction with actin filament (Adam, Pringle et al. 2000). Bazooka protein in *Drosophila* is a homolog of PAR-3 proteins in *C.elegans*. One of the main role of Bazooka in early *Drosophila* development is the maintenance of

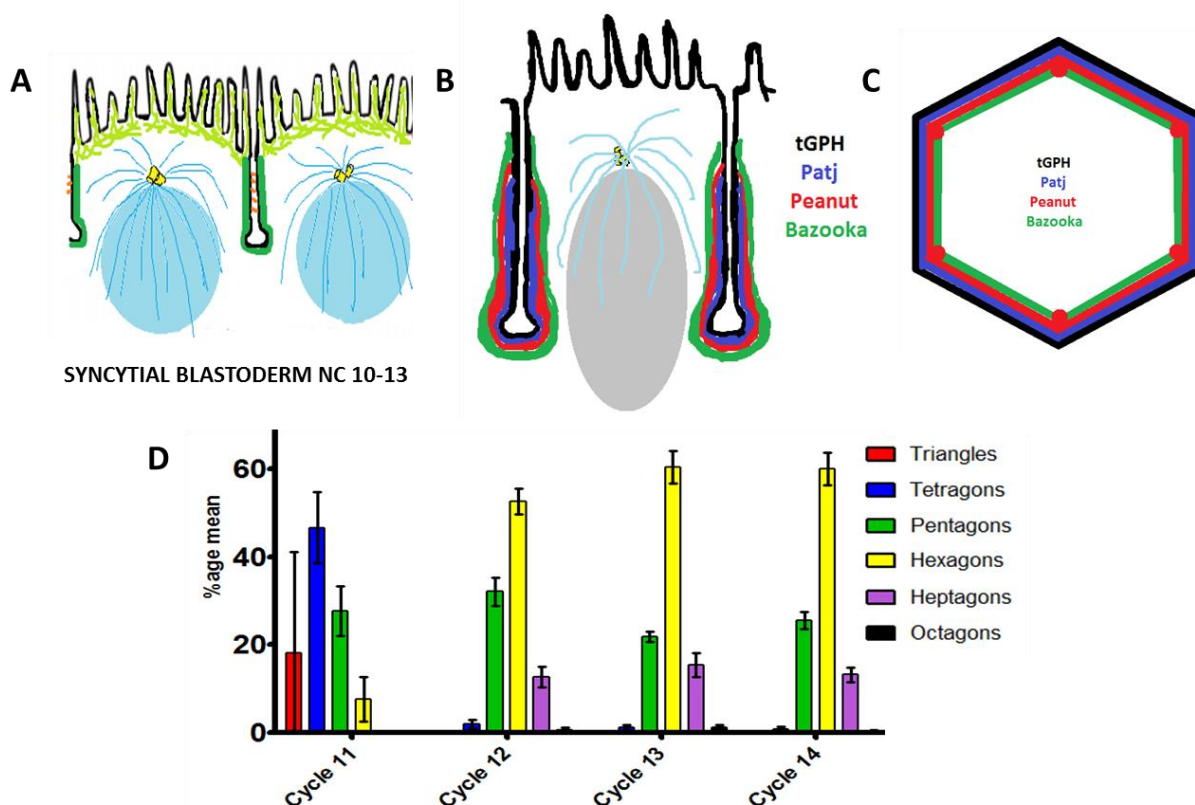


Figure 1.4. Previous studies done in the lab. A) Epithelial like plasma membrane of syncytial *Drosophila* embryo. B) Proteins present in different domains along the lateral membrane of an epithelial like cell. C) Differential localization of proteins X-Y axis in an epithelial like cell. D) Graph that shows hexagon dominant packing of epithelial cells arises as early as 12th syncytial stage.

apico-basal polarity of epithelial cells associating with PAR-6 and aPKC (Harris and Peifer 2005). Patj (Pals1 associated tight junction protein) plays a crucial role in the formation of epithelial cell polarity. Patj along with Crumbs and Stardust regulates the formation and stability of adherens junction in *Drosophila* epithelial cells (Roh, Fan et al. 2003). We showed that, the epithelial like cells of *Drosophila* syncytial embryo are polarized in both X-Y and X-Z axis. Bazooka is localized in the furrow canal and along the edges of the PM. Sep2/Peanut is found in contractile ring and at vertex (Fig. 1.3B & C). We also found that, the hexagon dominated packing of epithelial like cells already arises in cycle 12 (Fig. 1.3D). The syncytial blastoderm has synchronous division cycles and this shows a polygonal distribution similar to fully formed epithelia despite the absence of complete cells questioning the dogma of its formation due to asynchronous divisions.

1.8 The role of actin cytoskeleton in maintaining epithelial like cells plasma membrane polarity and polygonal packing in *Drosophila* early embryogenesis.

The very obvious questions next to ask is how do these polarity proteins manage to get organized in a polarized fashion in the epithelial like plasma membrane? And what helps maintain this polarity? And what helps the plasma membrane pack in hexagonal dominant fashion in the embryo? If we carefully observe the epithelial like cells during early *Drosophila* development, one of the striking factor is the thick meshwork of actin beneath the membrane which is actively remodelling along with the membrane during each cell division stages. This actin meshwork is comprised of bundled and branched actin. RhoGEF2 and Diaphanous are known regulators of bundled actin and WASp and Arp regulates the filamentous actin. It has been shown that Bazooka is localised to sub apical membrane with the help actin cytoskeletal cues during cellularisation of *Drosophila* embryogenesis (Harris and Peifer 2005). But the role actin in polarised distribution of Bazooka during early syncytial stages are not known. Patj proteins are associated with apical crumbs complex, which are known to interact with apical actin scaffold for its polarised distribution. So actin may indirectly affect the localisation of Patj to sub apical plasma membrane. Peanut is known to regulated actin polymerisation and make curved actin bundles at the furrow tip during membrane invagination (Mavrakakis, Azou-Gros et al. 2014) but an inverse relation of actin regulating Peanut polarisation is yet to be identified. When it comes to polygonal organisation of epithelial cells, correct cell division, maintenance of

surface tension, cell adhesion and apical constriction are the factors that plays a crucial role. The actin cytoskeleton is reported to play a quintessential role in all these factors. So role of actin cytoskeleton in polygonal organisation will be the most interesting to look at. The main questions we are trying to ask through this project are; whether these actin regulators play any role in polarized distribution of above said polarity proteins in early syncytial and cellularising embryo? How the membrane architecture is going to change if we disrupt this actin regulators during these stages? Does the plasma membrane still be able to maintain a hexagon dominant packing in the absence of these actin regulators?

1.9 Aims of the project

1.9.1 Generation of embryos mutant for regulators of actin cytoskeleton remodelling in the *Drosophila* embryo using RNAi knockdown, OvoD1 strategy and Pharmacological inhibitors.

1.9.2 Analysis of polarized distribution of Bazooka, Peanut and Patj in embryos mutant for actin regulatory proteins.

1.9.3 Analysis of polygonal packing in the syncytial plasma membrane of embryos mutant for actin regulatory proteins.

2. Materials and methods

2.1 *Drosophila* Stocks and crosses

GAL4 lines	RNAi lines	Germline mutants	Other Stocks
nanos-GAL4	Dia RNAi (y1 sc* v1; P{TRiP.HMS00308}attP2)	Dia ⁵ FRT40A	Ovo ^D FRT40A
tGPH; nanos- GAL4	WASp RNAi (y1 sc* v1; P{TRiP.HMS01534}attP40)	Arpc1 FRT40A	CS(+/+)
	RhoGEF2 RNAi (y1 sc* v1; P{TRiP.HMS01118}attP2)		
	Arp66B RNAi (y1 sc* v1; P{TRiP.HMS00711}attP2)		

Table 2.1. The list of fly lines used in the project

3% yeast medium containing cornmeal, sugar and agar is used for rearing flies. All the RNAi crosses were maintained at 28°C and germline mutant clones were grown and maintained at 25°C. The list of fly lines used in the project are given in the table 2.1.

2.1.1 Targeted gene expression using UAS-GAL4 system.

The UAS-GAL4 system was used as tool to targeted knock down of proteins. GAL4 is a regulatory gene sequence which encodes for a protein of 881 amino acids which binds to specific sequence called Upstream Activating Sequences (UAS) and acts as enhancer for the activation. It results in the transcription of downstream gene sequences or RNAi. The maternally expressed gene *nanos* was used as a promoter for GAL4 to express the targeted genes or RNAi during oogenesis. The F1 generation female flies containing a combination of the targeted gene and RNAi were crossed to males and embryos were collected for further analysis. For live imaging of plasma membrane dynamics transgenic RNAi lines were expressed in the background of GFP tagged PH domain of GRP and nanos Gal4 [tGPH; nanos GAL4]. tGPH is, a PIP3 (phosphatidyl-inositide phosphate 3) marker that labels the entire plasma membrane.

2.1.2 Creating germline clone embryos using dominant sterile Ovo^{D1} mutation strategy.

Homozygous germline clonal embryos were generated using FLP-FRT method containing a dominant female sterile mutation *Ovo*^{D1}. Female flies heterozygous for *Ovo*^{D1} do not lay eggs. FLP recombinase was incorporated in the X-chromosome of the flies and was controlled by the heat shock protein promoter. FLP-FRT site specific recombination system was used to generate embryos homozygous for the mutant chromosome by heat pulsing during oogenesis. The F1 generation heterozygous with FRT-‘mutant’/FRT-*Ovo*^{D1} were heat pulsed in a water bath at 37.5°C for 1 hour during larval, pupal and adult stage. *dia*⁵ and *arp*^{C1} alleles with FRT40A3 were used to generate germline clonal embryos using the *Ovo*^{D1} FRT 40A3 stock.

2.2 Immunostaining

Embryos were collected for 3 hours at 28°C for RNAi or at 25°C for germline clones crosses, dechorionated in 100% bleach for 1 minute and then fixed with 4% formaldehyde in PBS and equal volume of heptane for 20 minutes. Then the embryos were either methanol devitelinized (equal volume of methanol and heptane) or hand devitelinized (in 1X PBS) depending on the antibody used for staining. Washes were given with 1X PBST (0.3% Triton-X100) for 5 minutes and 2% BSA in PBST was used for 1 hour blocking. After overnight primary antibody incubation at 4°C, fluorescently coupled secondary antibodies were added in PBST for 1

hour as per the standard protocol. After PBST washes and staining with Hoescht, embryos were mounted on slides with Slow Fade Gold Antifade reagent from Invitrogen. Antibodies used in the project are given in the table 2.2.

Primary Antibodies	Dilution	Lab/Company
Bazooka	1:1000	Andreas
Peanut	1:5	DHSB
Patj	1:1000	Hugo Bellen
Arp2/3	1:1000	Stevens
Dia	1:1000	Wasserman
Rhogef2	1:5000	Grosshans
Amphiphysin	1:1000	Gabrielle Boulianne
Syndapin	1:150	Vimlesh Kumar
Secondary Antibody	Dilution	Lab/company
Alexa488	1:100	Invitrogen
Alexa568	1:1000	Invitrogen
Alexa633	1:1000	Invitrogen
Dyes	Dilution	Lab/company
Phalloidin 488/568/633	1:100	Invitrogen
Hoechst	1:1000	Sigma

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

2.3 Microscopy

2.3.1 Imaging of fixed samples.

All confocal imaging of fixed embryos were done at room temperature (23°C) using an LSM-710 or 780 inverted microscope (Carl Zeiss, Inc. and IISER Pune microscopy facility) with excitation at 488 nm, 543 nm and 633 nm and emission collection with PMT filters. A Plan Apochromat 40x/ 1.3 NA (for LSM 710) and 1.4 NA (for LSM 780) oil objective, with pinhole of 90.03, averaging of 2, acquisition speed of 8, zoom of 3 was used for imaging with the help of Zen software.

2.3.2 Live imaging

Embryos were collected on yeast coated agar plate for 1 – 1.5 hours at 28°C and dechorionated in 100% bleach for 1 minute. After washing with distilled water, they were placed dorso- or ventro-laterally in coverslip chambers (LabTek). After covering the laid embryos with 2 ml of 1X PBS, imaging was done with above mentioned microscopes with averaging of 2, scan speed of 10 and zoom of 3. Z-stacks were taken from the apical surface of the embryos touching the coverslip to approximately 20 -25 slices inside the embryo (length of each Z-stack was 1.08µm).

2.4 Image analysis

Open source software such as ImageJ and Gimp Image editor were used for image compilation and analysis. Graphs and statistical analysis were done using GraphPad Prism. Two-tailed unpaired student's T-test was performed to check the significance.

2.4.1 Frequency distribution of polygons in membrane packing.

In order to estimate the frequency distribution of polygons in membrane packing, time lapse images of early embryo development of transgenic tGPH fly lines were taken. Then they were visually marked for number of vertices in 12th and 13th cell division stages using the z-section with most compact metaphase packing. The analysis was done for atleast 3 time lapse images and average of the percentage polygon distribution from all the embryo were used for plotting the graph. Standard Error of Mean (SEM) was used in the error bar for all the graphs. Two-tailed unpaired student's T-test was performed to check the significance of frequency

distribution. Significant values $p < 0.05$, $p < 0.01$, $p < 0.001$ have been denoted by in the graph by *, **, *** respectively.

2.4.2 Line scan analysis for measuring membrane tightness.

Time lapse images of transgenic tGPH fly lines were taken. Segmented line tool of ImageJ was used to draw straight lines crossing the edges (excluding vertices) of the membrane. The gray value profile (proxy for intensity) for the line segments were plotted in the graph. Tight membranes are represented by sharp peaks and loose membranes are represented by broad peaks.

2.4.3 Measuring the length of furrow canal membrane.

Time lapse images of transgenic tGPH fly lines were taken. Using the orthogonal section view in the ImageJ, XZ axis view and YZ axis view of the time lapse movie was obtained. Then marked the furrow canal region using line tool at 12th and 13th division cycle using the z-section with most compact metaphase packing. The analysis was done for atleast 3 time lapse images. The same statistical analysis mentioned above is followed here also.

2.5 Pharmacological treatment of embryos to study actin disruption

Embryos were collected for 3-4 hours at 25°C and treated with 100% bleach for 1 minute for dechoriation. After washing, embryos were incubated in 1:1 mixture of limonene (Sigma) and heptane for 1 minute. Then the embryos were incubated with specific pharmacological drug in serum free Schneider medium for 15 minutes. Afterwards embryos were fixed with 4% formaldehyde for 20 minutes. Three PBST (0.3% TritonX-100) washes were given and then devitinilised using equal volume of methanol and heptane. Further, above said protocol for immunostaining was followed.

2.6 Embryo lethality estimation

Embryo were collected in sieve and washed and arranged on an agar plate in a matrix fashion. Then incubated at required temperature. Number of unhatched embryos were counted after 24 hours and 48 hours.

3. Results

3.1 Latrunculin A treatment in *Drosophila* embryogenesis causes general actin delocalization and morphogenetic defects in the plasma membrane architecture.

In order to study the effect of actin network on plasma membrane organization in the syncytial *Drosophila* embryo, we chose to disrupt actin using Latrunculin A (Lat A) which affects the actin polymerisation in a dominant negative fashion by binding to the site of actin nucleation. For drug delivery in to the embryo, we standardized an

embryo permeabilization protocol using a combination of limonene and heptane in 1:1 ratio. We used a concentration range of Lat A from 200 nM to 20 μ M. We have got 71.4 % embryos that showed morphogenetic defects and actin delocalization by 20 μ M Lat A treatment. Membrane showed severe architectural defects such incomplete or missing furrow canal and general actin delocalization. The nuclear organization in the embryo was also affected in Lat A treated embryo (Fig. 3.1). In order to study the role of actin more carefully we moved to studying defects caused by reduction of specific proteins which give rise to branched and bundled actin described in the next section.

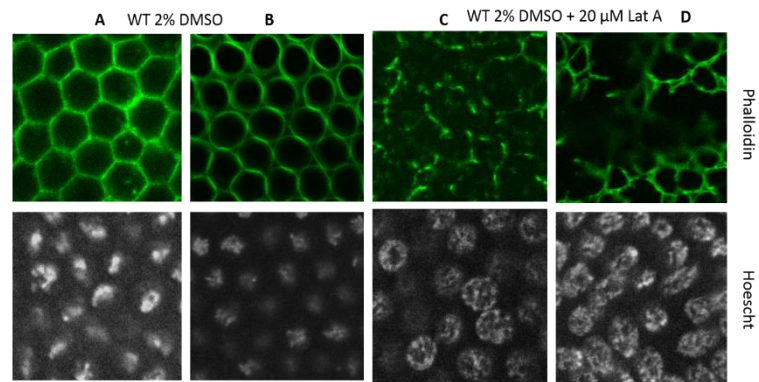


Fig. 3.1. Latrunculin A treatment of embryo. A&B) WT control treated with 2% DMSO. Polygonal arrangement and contractile rings are shown respectively in A & B. C & D) The treatment with 20 μ M of Latrunculin A disrupts the membrane architecture and contractile ring. Nuclear organisation also seems to be affected.

3.2 Generation of *Drosophila* embryos mutant for branched and bundled actin dynamics using RNAi and hypomorphic alleles.

To study the role of the actin network in maintaining plasma membrane organization and epithelial cell polarity in the syncytial embryo, we targeted RhoGEF2 and Diaphanous which are bundled actin regulators, WASp and Arp which are branched actin regulators. We used RNAi mediated knockdown and Ovo^{D1} germline clone strategy to create knockout for these actin regulators.

3.2.1 RNAi mediated knockdown caused differential lethality of embryos and resulted in decrease of corresponding protein during *Drosophila* embryogenesis.

Using nanos-Gal4/UAS- RNAi system, knockdown mutants are generated for actin regulators Rhogef2, Arp66b, WASP and Diaphanous. The percentage of lethal embryos were quantified after two days in an agar plate (Table 3.1). Rhogef2 showed the maximum lethality of 84.25%, WASp showed 18.2% lethality, Dia showed 25% lethality and Arp66b showed the least of 6.2%. Immunostaining with antibodies of respective actin regulators (except WASp) were done for confirming a loss of the protein due to RNAi mediated knockdown. In wild type embryos, RhoGEF2 protein is localized on the apical membrane as well as in furrow canal on both the stages. The RhoGEF2 RNAi showed a 100% loss of RhoGEF2 protein during both syncytium and cellularisation stages and did not show any localisation of RhoGEF2 protein on the membrane (Fig.

RNAi Lines	% Lethality (n)
Rhogef2	84.25 (335)
Diaphanous	25 (200)
Wasp	18.2(400)
Arp	6.2(320)
WT	2(300)

Table 3.1. Percentage lethality for RNAi mutants of actin regulators.

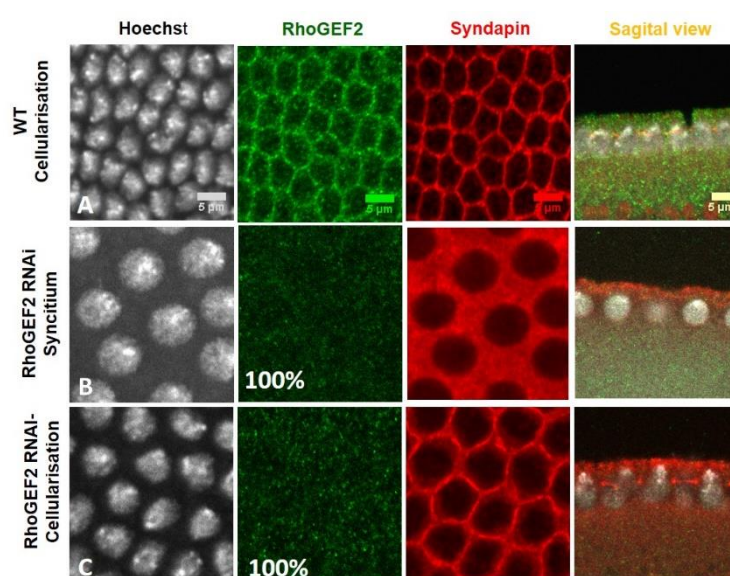


Figure 3.2. Knockdown of RhoGEF2 in RhoGEF2 RNAi.
A) WT staining of RhoGEF2 protein during cellularisation.
B&C) Showing the knock down RhoGEF2 in RhoGEF2 RNAi during both syncytium and cellularisation stage.

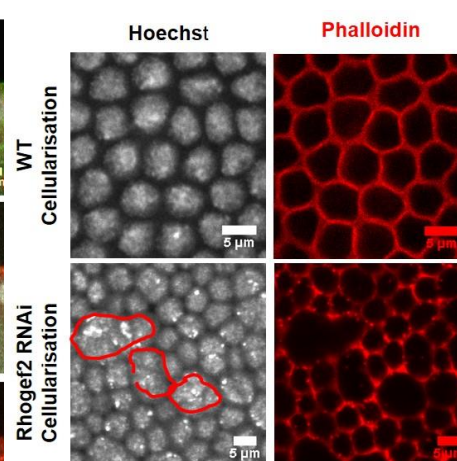


Figure 3.3. Giant nuclei phenotype seen in RhoGEF2 RNAi (marked by red in Hoechst staining). Phalloidin staining shows the missing lateral membrane associated with large nuclei.

3.2). Syndapin, which is a membrane binding BAR-domain protein was used as a positive control and it was not affected in cellularisation stage. One of the striking phenotype of RhoGEF2 RNAi was the multiple nuclei or giant nuclei. All these giant nuclei phenotype was associated with incomplete or missing lateral membrane (Fig. 3.3).

In WT syncytial stages, Dia is localized to both apical membrane and furrow canal and in the cellularisation stage, Dia is not found on the apical membrane but enriched on the furrow canal. In embryos containing Dia RNAi showed a weak knockdown of Dia protein. In both syncytium and cellularisation, only 40% showed reduction of Dia protein on the membrane (Fig. 3.4).

During syncytial stages, Arp is localized to the apical membrane during interphase and then it is localised to the furrow canal towards the

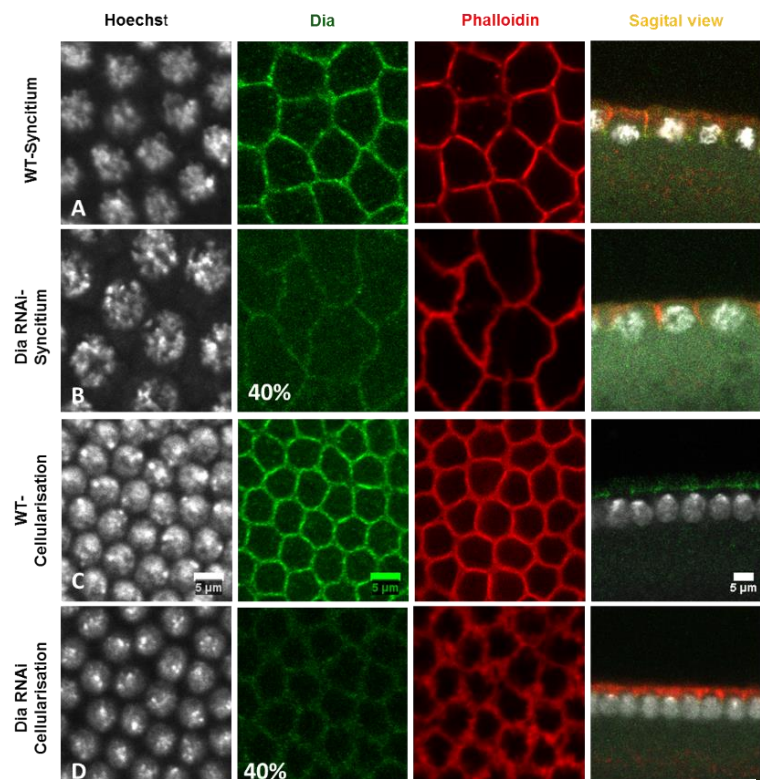


Figure 3.4. Knockdown of Dia in Dia RNAi. A & C) WT staining of Dia protein during syncytium and cellularisation. B&D) Showing the knockdown of Dia protein in Dia RNAi during both syncytium and cellularisation stage.

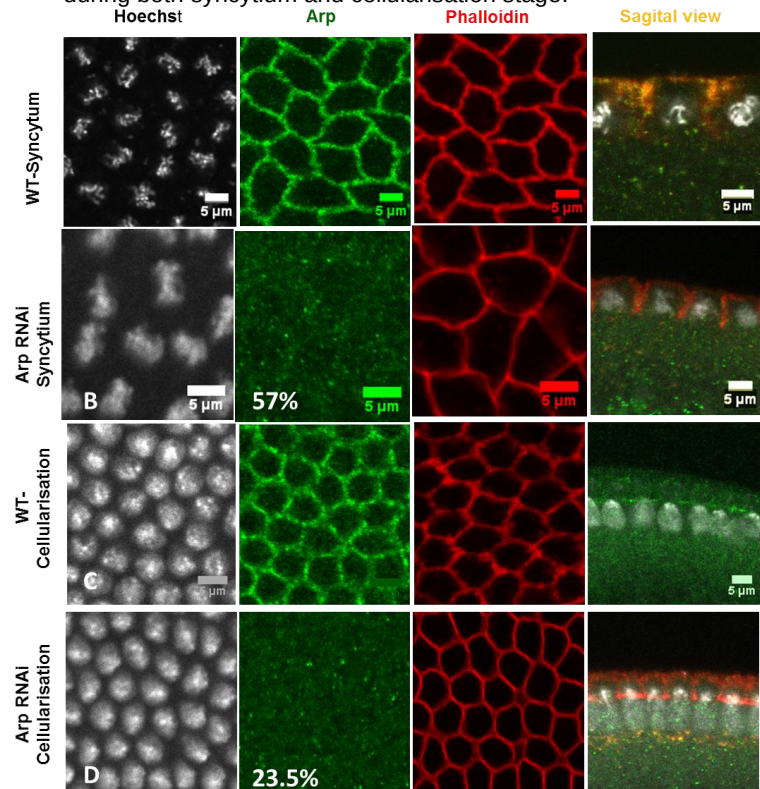


Figure 3.5. Knockdown of Arp in Arp66b RNAi. A&C) WT staining of Arp protein during syncytium and cellularisation. B&D) Showing the knockdown of Arp in Arp RNAi during both the stages.

metaphase. In WT, during cellularisation Arp is not localised to apical membrane but enriched at the furrow canal. Arp66b RNAi showed knockdown of Arp in 57% of embryos in syncytium and 23.5% of embryos in cellularisation and they showed a complete loss of Arp protein on the membrane as well as furrow canal in both syncytial and cellularisation stages (Fig. 3.5).

3.2.2 Hypomorphic embryo mutant *dia*⁵ and *arpC1* results in decrease in Dia and Arp with severe defects in membrane architecture.

*Dia*⁵ and *arpC1* germ line clones have been used previously to study the effect of loss of Diaphanous and Arp proteins on actin remodelling in the syncytial division cycles in the *Drosophila* embryo. *Dia*⁵ has been shown to be important for metaphase furrow formation and *arpC1* has been shown to be important for actin cap expansion during the syncytial division cycle. We have used these germline mutant clones to further see an effect on plasma membrane organisation and polarity in syncytial *Drosophila* embryo. Both mutants were severe such that embryo arrest development at the early embryonic stages. Very short or absent furrow canals, clustering of nuclei or large nuclei, defective membrane architecture were the phenotypes common to both the germline clones.

Both Dia and Arp protein levels were lowered in these mutant embryos (Fig. 3.6). Studying epithelial cell polarity aspect was not feasible in these mutants due to

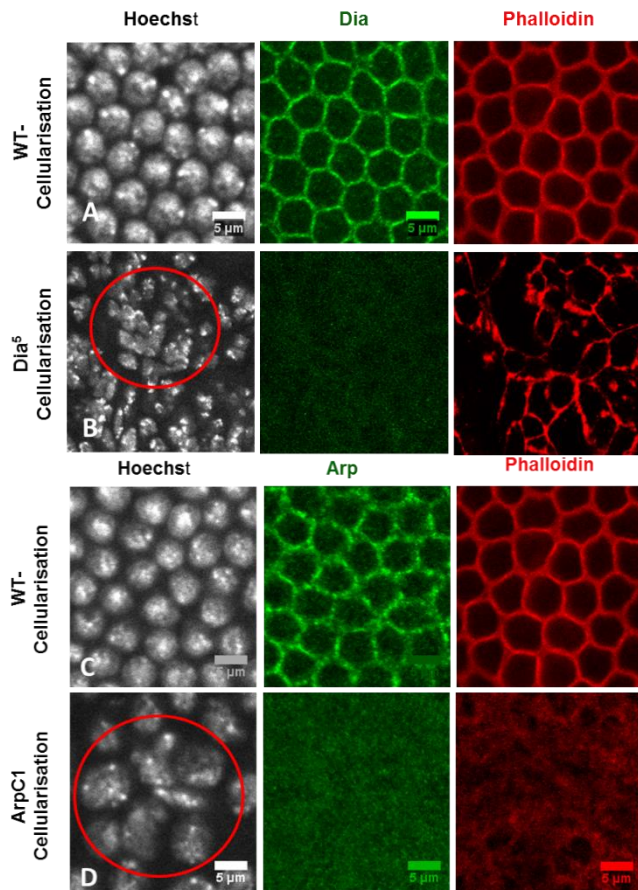


Figure 3.6. Knockout of Dia and Arp in *dia*⁵ and *arpC1* mutants. A) WT staining of Dia protein during cellularisation. B) Showing the knockout of Dia in *dia*⁵ during cellularisation stage. C) WT staining of Arp protein during cellularisation. D) Showing the knockout of Arp in *arpC1* during cellularisation stage. Red circles show the clustering of nuclei. Phalloidin staining indicates disruption of membrane architecture due to the mutant.

multiple severe morphological defects. We have decided to use the RNAi lines for further studies.

3.3 Analysis of polarized distribution of Bazooka and Peanut in the X-Y plane of the plasma membrane in actin regulatory mutant embryos.

Previous studies in the lab described the polarised distribution of Bazooka and Peanut in syncytial as well as cellularisation stages in WT embryo. Peanut belongs to the septin family of protein which localize basally with respect to the junctional protein, in the furrow canal of ingressing plasma membrane during syncytium (Fig 3.7A). During cellularisation, it localises differentially along the XZ axis of the epithelial cell. Peanut shows a punctate staining on the apical membrane below the junctional complexes (Fig. 3.7B) and as we down in Z-axis, Peanut shows enrichment of localization on the vertices where multiple cell membrane meet (Fig. 3.7C) and

finally it forms a circular ring like structure on the furrow canal region of the cellularising epithelia (Fig. 3.7D). Bazooka protein in *Drosophila* is a homolog of PAR-3 proteins in *C.elegans*. It is localized asymmetrically in the baso- lateral domain of the plasma membrane in the syncytium (Fig. 3.8A) and gets transported sub-apically in cellularisation. During the sub-apical localization, Bazooka is specially enriched on the edges rather than vertices (Fig. 3.8B). Patj (Pals1 associated tight

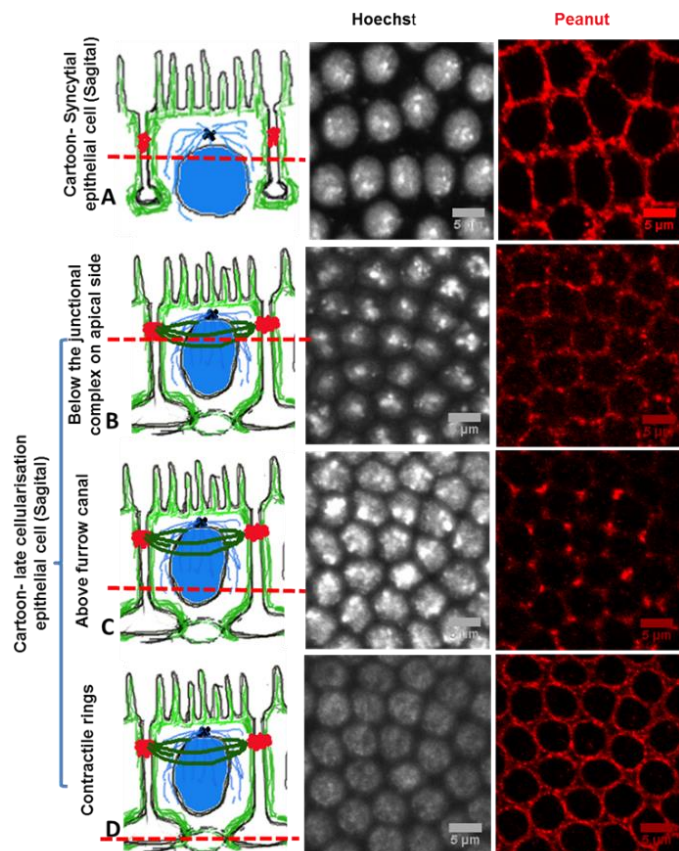


Figure 3.7. WT localisation pattern of Peanut during syncytium and cellularisation. A) Peanut is localised to the lateral like membrane in syncytium. In cellularisation, B) Shows faint and punctate apical staining below the junctional complexes, B) Vortex enriched localisation just above the furrow canal and C) Circular ring like localisation around the contractile rings. In the cartoon: Red line- plane of imaging, green – actin filaments, red- junctional complex, blue- nuclei, Dark green- microtubule

junction protein) plays a crucial role in the formation of epithelial cell polarity. In WT embryos, Patj is found to be associated with the plasma membrane tightly at the adherent junctions in the syncytium and cellularisation (Fig. 3.9).

We have immunostained these polarity proteins in embryos mutant for actin regulatory proteins to study the role of actin in their polarised distribution in plasma membrane.

3.3.1 Bazooka and Peanut are delocalised in the XY plane of the plasma membrane of RNAi mediated knockdown of bundled actin mutant embryos.

Polarized distribution of Bazooka on epithelial cell membrane is the most affected in embryos mutant for bundled actin regulators. In RhoGEF2 RNAi, all the embryos in the syncytial stage and 85% of embryos in cellularisation stage showed a defect in the localization of Bazooka on the membrane (Fig. 3.10 B,D&E). The sharp plasma membrane localization of Bazooka was absent and the protein was diffused into the cytoplasm. 15% of embryos in cellularisation showed a Bazooka staining which was not diffused

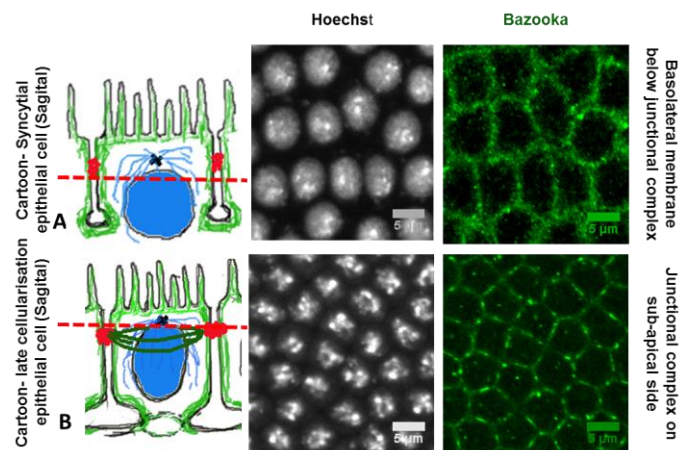


Figure 3.8. WT localisation pattern of Bazooka during syncytium and cellularisation. A) Distribution of Bazooka during syncytium. B) Localization of bazooka on the edges of the membrane in cellularisation. In the cartoon: Red line- plane of imaging, green – actin filaments, red- junctional complex, blue- nuclei.

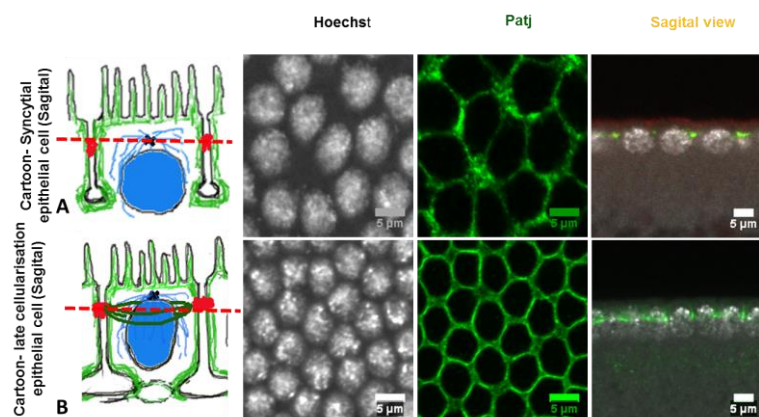


Figure 3.9. WT localisation pattern on Patj during syncytium and cellularisation. A&B) Patj localizes specifically to the tip of the furrow canal in both cellularisation and syncytium. Cartoon: Red line- plane of imaging, green – actin filaments, red- junctional complex, blue- nuclei.

in to the cytoplasm but still they do not show the edge enriched Bazooka localization pattern compared to WT (Fig. 3.10F). Dia RNAi also showed similar phenotype for Bazooka localization on the membrane. Bazooka delocalization from the membrane was in 83% of syncytial embryos (Fig. 3.11B). During cellularisation, 33% of embryo showed a diffused Bazooka staining phenotype (Fig. 3.11D).

Localisation of Peanut on the membrane also became diffuse in embryos mutant for bundled actin regulators. All the syncytial embryos in RhoGEF2 RNAi showed the diffused Peanut distribution phenotype (Fig. 3.10B). In cellularisation, 38% of embryos showed a diffused distribution of Peanut on the plasma membrane (Fig. 3.10E). Dia RNAi did not show diffused Peanut

localization in both the stages (n=20, Fig. 3.11B&D). But in 33% of embryos in cellularisation, Peanut was found uniformly across the membrane in contrast to its vertex enriched localisation in WT cellularisation embryos (Fig. 3.11D).

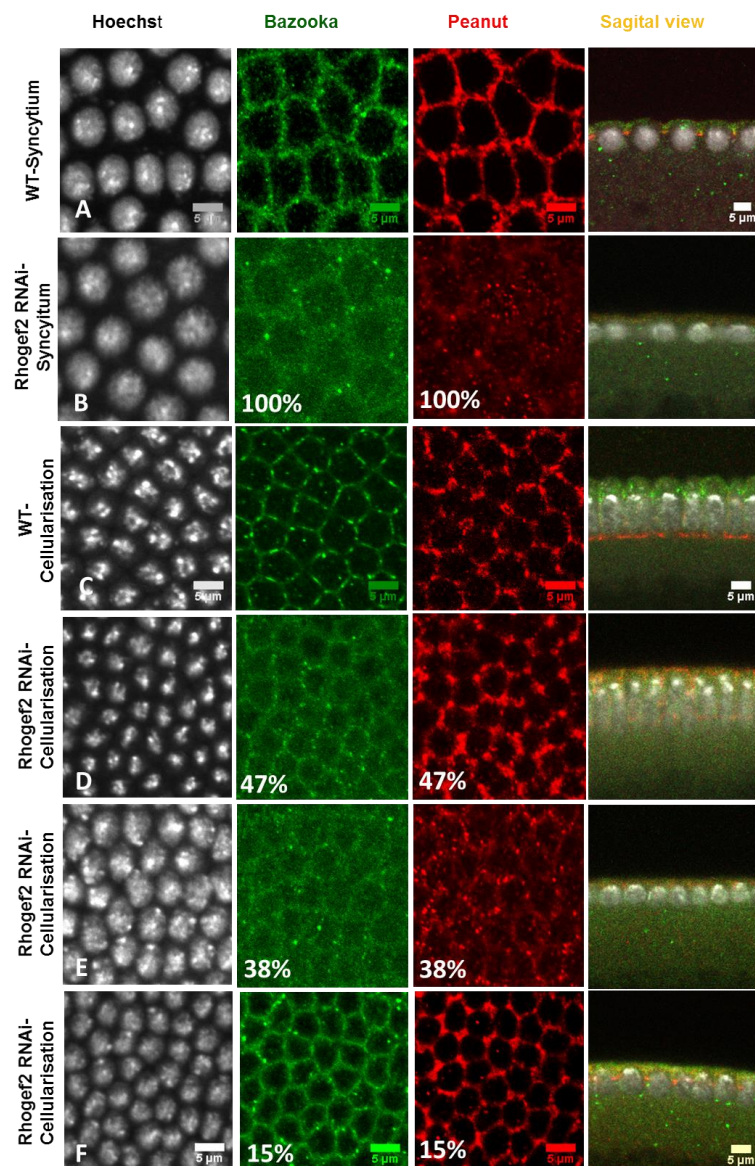


Figure 3.10. Polarized distribution of Bazooka and Peanut defective in RhoGEF2 RNAi. B) All syncytial stages showed defective localization of both Bazooka and Peanut. D) In cellularisation, 47% of embryos showed diffused distribution of Bazooka but almost unaffected localization of Peanut on to the vertices of the membrane. E) 38% of embryo showed defective localization of both Bazooka and Peanut. F) Only 15% of the embryo showed localization of both Bazooka and Peanut similar to the WT.

3.3.2 Bazooka and Peanut distribution is variable in the XY plane of the plasma membrane of branched actin mutant embryos.

WASp RNAi did not showed any considerable change the localisation of Bazooka and Peanut compared to the WT in both the stages (n=11, Fig. 3.12B&G). In Arp66b RNAi, embryos in the syncytium stage did not show any defect (n=10, Fig. 3.12C) and 23% of embryos in cellularisation showed a diffused phenotype of Bazooka and Peanut (Fig. 3.12B). 77% embryos of Arp66b RNAi in cellularisation showed Bazooka and Peanut localisation comparable to WT.

3.4 Patj distribution

remains largely unaffected and reveals morphogenetic defects in branched and bundled actin mutant embryos.

Patj protein localisation on the membrane was not affected in any of these embryos mutant for actin regulators (n>10, Fig. 3.13). If the lateral membrane is present, Patj will be recruited to furrow canal. RhoGEF2 mutant showed a loose membrane phenotype during the syncytial stages so Patj staining also showed loose pattern but it was not diffused into cytoplasm. The sagittal view shows that, Patj was still able bind to the plasma membrane even though the membrane was loose in RhoGEF2 RNAi.

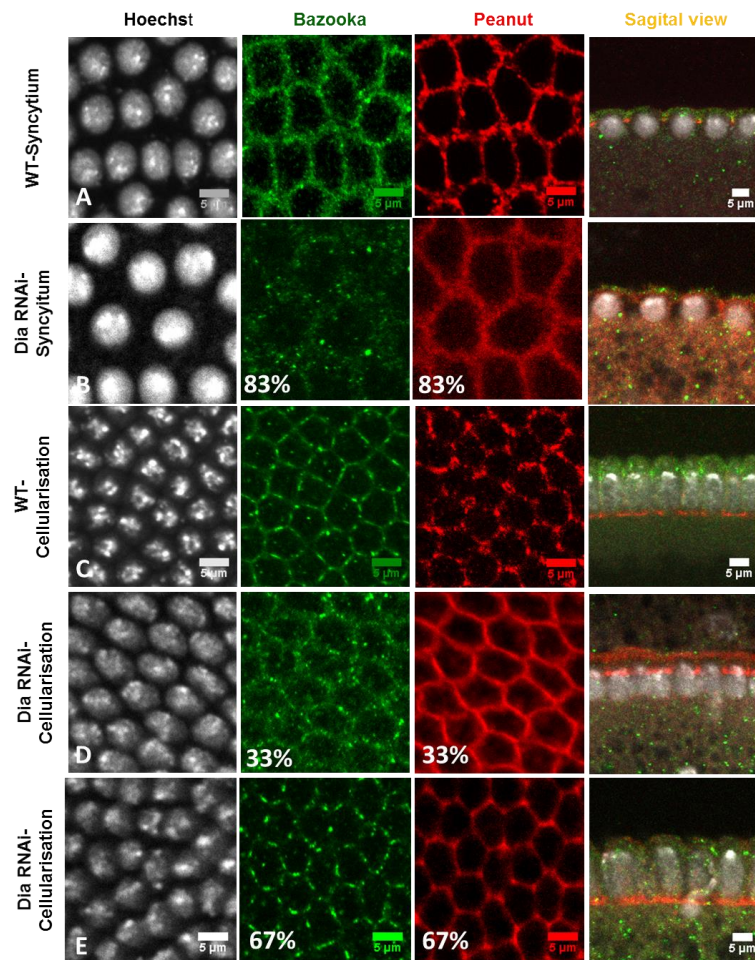


Figure 3.11. Polarized distribution of Bazooka and Peanut in Dia RNAi. B) 83% of embryos in syncytial stage showed defective localization of Bazooka but Peanut is normal. D) In cellularisation, 33% of embryos showed diffused distribution of Bazooka but peanut is found throughout the membrane in contrast to WT. E) 67% of embryos in cellularisation resembled the WT.

3.5 Live imaging of plasma membrane architecture during embryogenesis in bundled and branched actin mutant embryos.

Polygon packing was analysed using tGPH marker in all the embryos. tGPH, GFP tagged PH domain of GRP, a PIP3 (phosphatidyl-inositide phosphate 3) marker labels the entire plasma membrane and demonstrates the polygonal architecture of epithelial cells (Britton, Lockwood et al. 2002). In WT embryo, pentagons were dominant over tetragons and heptagons in 12th syncytial stage. The difference between pentagons and hexagons were not significant at this stage. In 13th syncytial stage, hexagons dominate over all other polygons. After hexagons, pentagons were dominating over tetragons and heptagons (Fig 3.14A, 3.15). Appropriate polygonal packing may be a result of correct divisions and plasma membrane tension and

characterizing these in the embryos mutant for actin regulatory proteins will help us analyse their role in these properties. We have also tried analysing the role of actin regulatory mutants in other features associated with membrane architecture such as

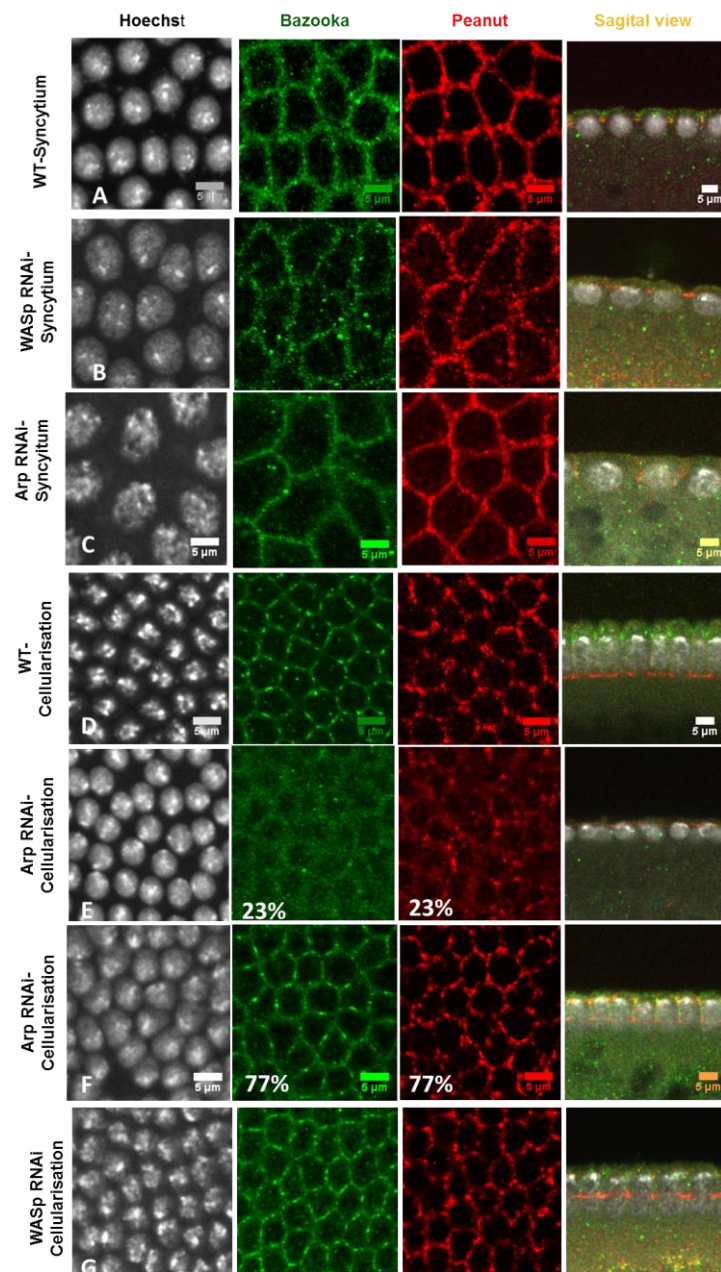


Figure 3.12. Polarized distribution of Bazooka and Peanut in Arp RNAi and WASp RNAi. B&C) In both WASp and Arp RNAi, Embryos in syncytial stage did not showed any defective localization of both Bazooka and Peanut. E) For Arp RNAi, during cellularisation 23% of embryos showed diffused distribution of both Bazooka and Peanut (F) and 77% of embryos in cellularisation resembled the WT. G) WASp RNAi did not show any defect in Bazooka and Peanut localisation during cellularisation.

membrane tightness and metaphase furrow length from these time lapsed tGPH movies.

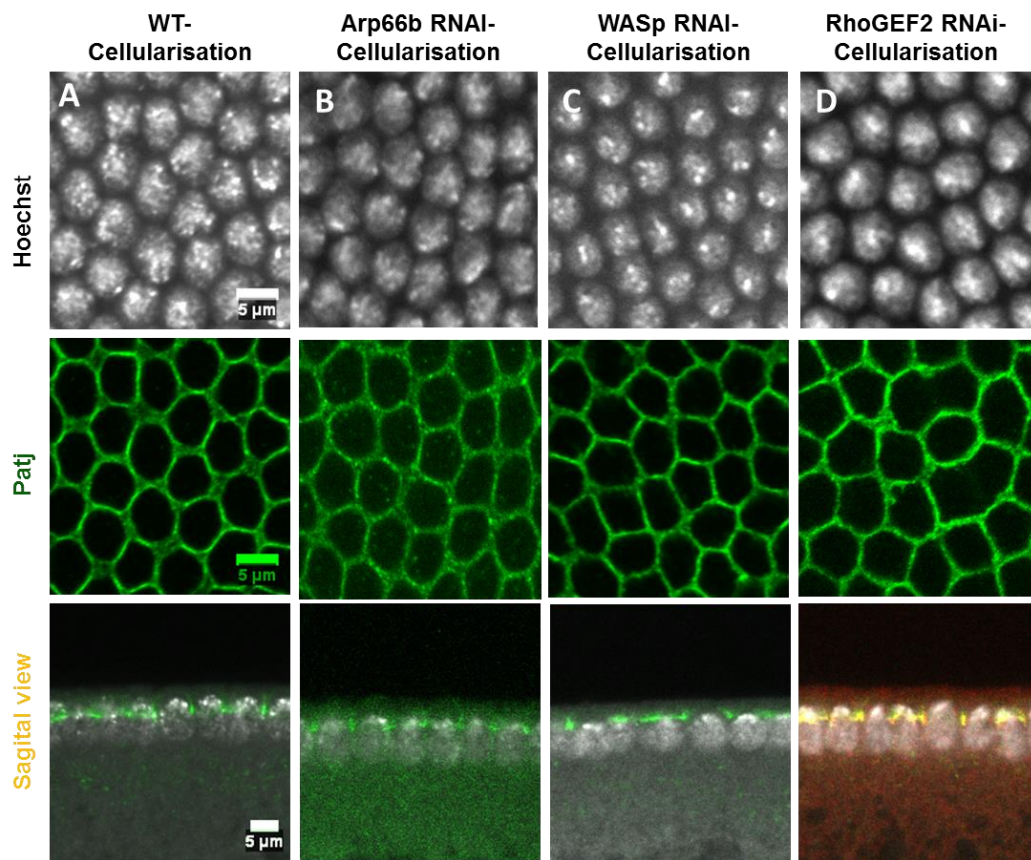


Figure 3.13. Polarized distribution of Patj in cellularisation for mutants of Arp, WASp and RhoGEF2. Localisation of Patj on to the membrane did not have any defect in any of these mutants compared to WT.

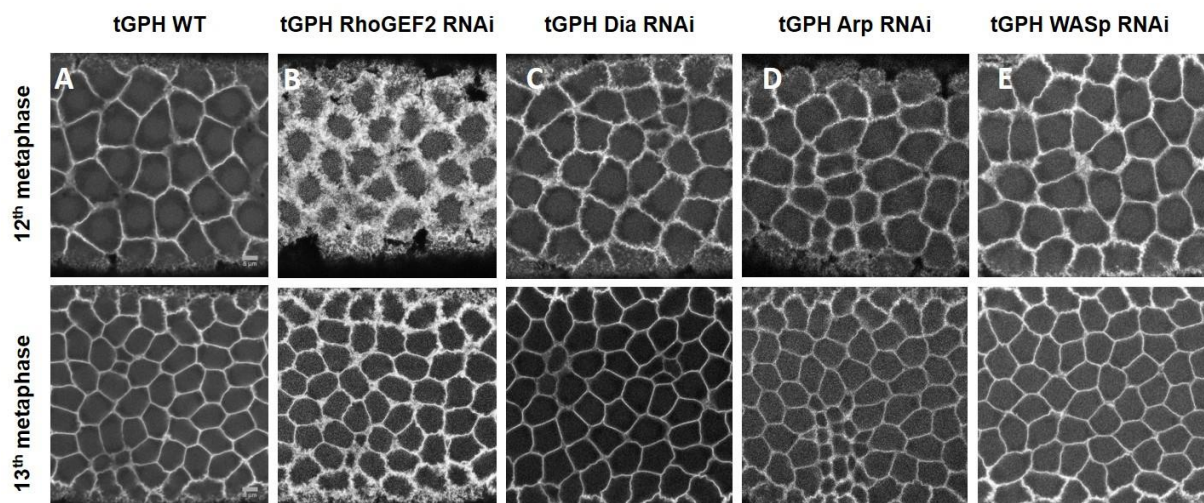


Figure 3.14. Polygon packing in syncytial and cellularisation embryos for WT and embryos mutant for actin regulators. Number of pentagons are increasing in embryos mutant for actin regulators compared to the WT.

3.5.1 Polygon analysis shows a shift towards greater numbers of pentagons in branched and bundled actin mutant embryos.

RhoGEF2 RNAi showed dominance of pentagons and tetragons over hexagons in 12th syncytial stages. There was no significant difference between pentagons and tetragons at this stage. During 13th syncytial stages, pentagons showed a clear dominance over both tetragons and hexagons (Fig. 3.14B, 3.16). In Dia RNAi, during 12th syncytial stages pentagons exhibited a clear dominance over tetragons and hexagons. During 13th syncytial stages, the difference between pentagons and hexagons were not significant but these two sets dominated over tetragons and heptagons (Fig. 3.14C, 3.17).

Number of pentagons were significantly larger than the number of tetragons and hexagons at 12th syncytial stages of Arp66b RNAi. At 13th syncytial stages, the number of pentagons and hexagons were equally dominating and hexagons were significantly more in number than tetragons and heptagons (Fig. 3.14D, 3.18). Data was not significant enough to comment on the dominance among tetragons, pentagons and hexagons at 12th syncytial stages for WASp RNAi. At

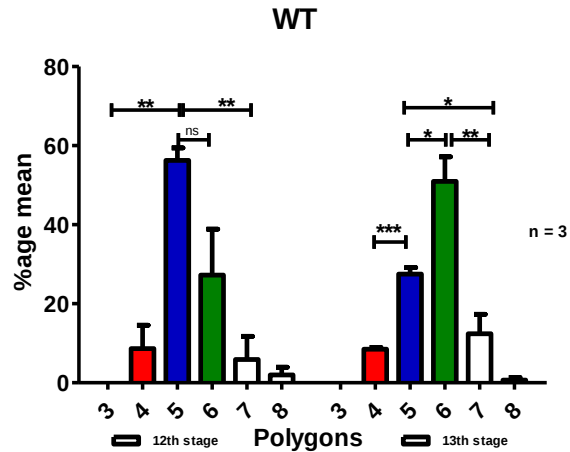


Figure 3.15. Polygon distribution of WT embryo during 12th and 13th syncytial stages.

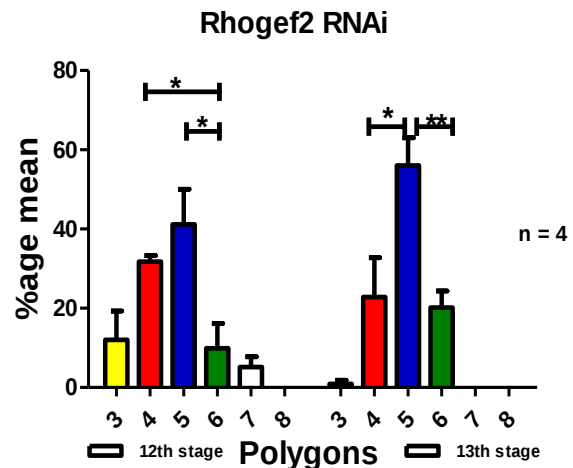


Figure 3.16. Polygon distribution of RhoGEF2 RNAi embryo during 12th and 13th syncytial stages.

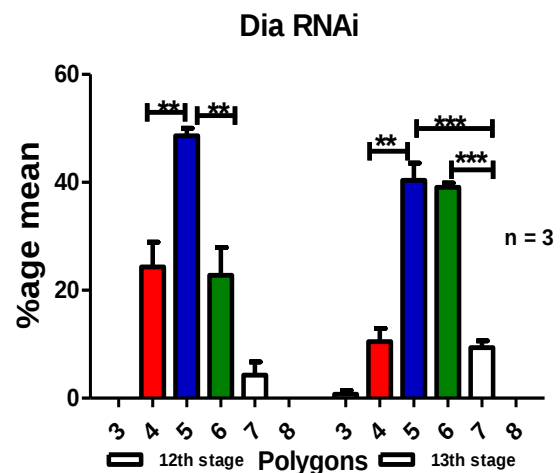


Figure 3.17. Polygon distribution of Dia RNAi embryo during 12th and 13th syncytial stages.

13th syncytial stages, pentagons and hexagons were equally dominant over tetragons and heptagons (Fig. 3.14E, 3.19).

3.5.2 RhoGEF2 RNAi showed loose membrane at 12th metaphase and immediate recovery at 13th metaphase.

One of the striking phenotype of RhoGEF2 RNAi was its loose and wavy membrane. At 12th metaphase, the membrane was thick and diffused and as it progressed towards telophase, membrane became wavy in nature. This phenotype was recovered at the 13th metaphase and the membrane became compact in the mutant compared to the WT (Fig. 3.20). In WT embryo, the line scan analysis of the plasma membrane showed sharp peaks at the edges of the plasma membrane for 12th and 13th metaphase. But line scan analysis for RhoGEF2 RNAi showed broad peaks at 12 metaphase and sharp peaks at 13th metaphase (Figure 3.21).

3.5.3 Metaphase furrow length was affected in few embryos containing RNAi mediated knockdown for actin regulators.

In WT embryo, average 12th metaphase furrow length is 6.3 μ m and 13th metaphase furrow length is 9.3 μ m. RNAi mediated knockdown showed two categories of phenotype on live imaging with tGPH. Severe RNAi mutant embryos for RhoGEF2 (2 out of 8) and Arp (2 out of 5) showed an arrest of development before cellularisation stages. Very short or absent metaphase furrows were seen in them. Mild mutants

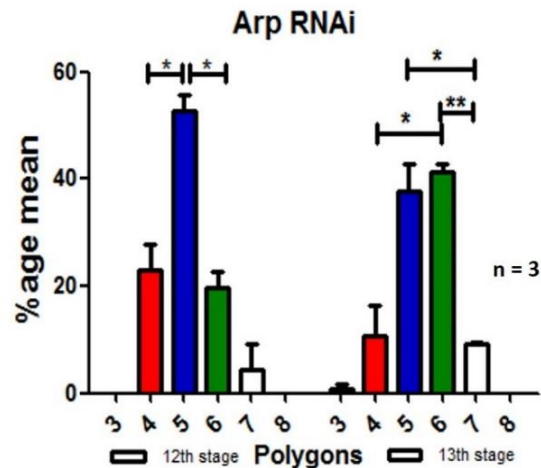


Figure 3.18. Polygon distribution of Arp66b RNAi embryo during 12th and 13th syncytial stages.

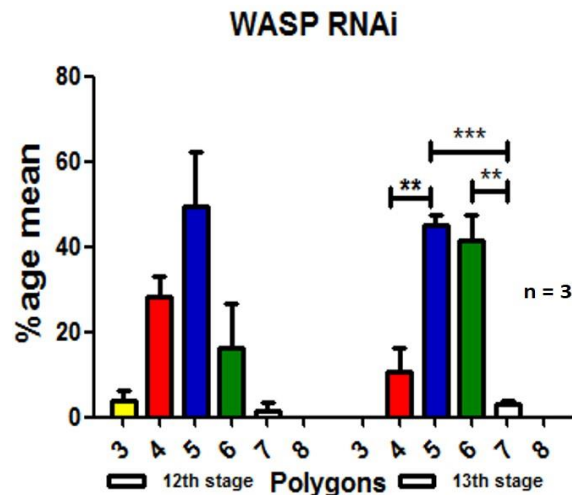


Figure 3.19. Polygon distribution of WASP RNAi embryo during 12th and 13th syncytial stages.

for RhoGEF2 (6 out of 8) and Arp (3 out of 5) did not show a significant change in metaphase furrow length compared to the WT embryo in 12th metaphase and 13th metaphase stages (Figure 3.22, 3.33).

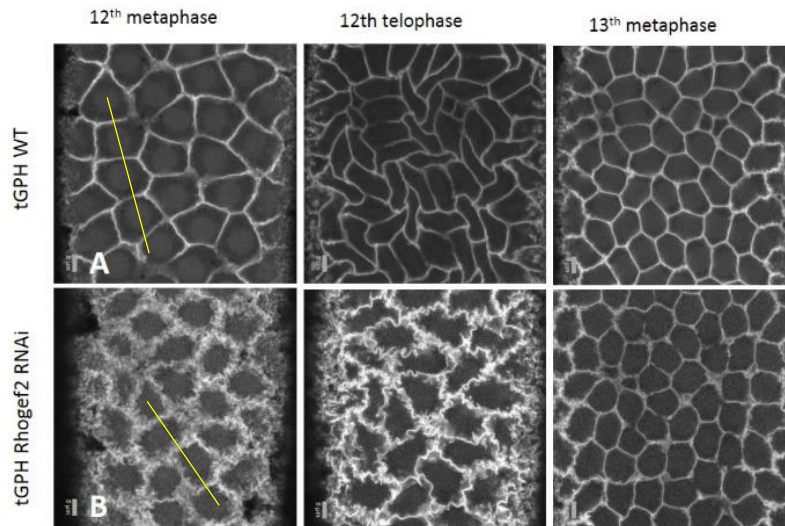


Figure 3.20. Loose membrane phenotype of RhoGEF2 RNAi.

A) tGPH WT embryo shows tight membrane from 12th metaphase to 13th metaphase. B) tGPH RhoGEF2 RNAi showing thick loose membrane at 12th metaphase and wavy membrane at 12th telophase and finally showing tight membrane at 13th metaphase. Line scan analysis is done through the yellow lines.

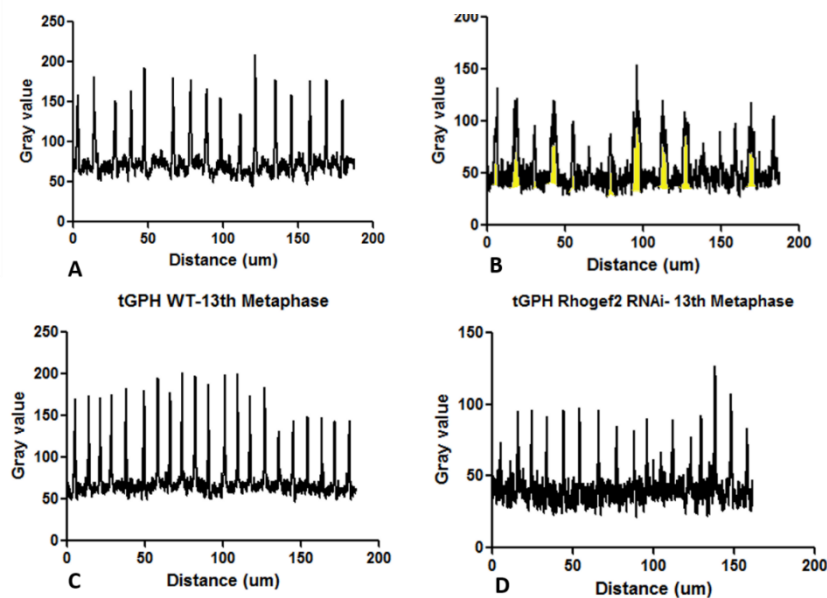


Figure 3.21. Line scan analysis for WT and RhoGEF2 RNAi showing the loose membrane phenotype of RhoGEF2 RNAi. A & C) WT shows sharp peaks along the edges at 12th and 13th metaphase. B) RhoGEF2 RNAi shows a broad peak (yellow colour) at 12th metaphase. D) RhoGEF2 RNAi shows sharp peaks along the edges during 13th metaphase.

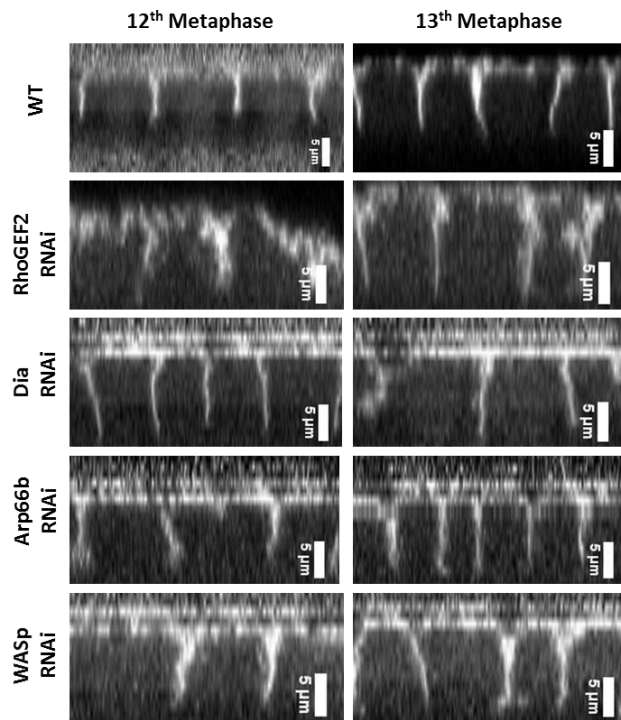


Figure 3.22. Metaphase furrow canal length of embryos mutant for actin regulators during 12th and 13th syncytial stages. Metaphase furrow canal length did not change significantly in embryos mutant for actin regulators.

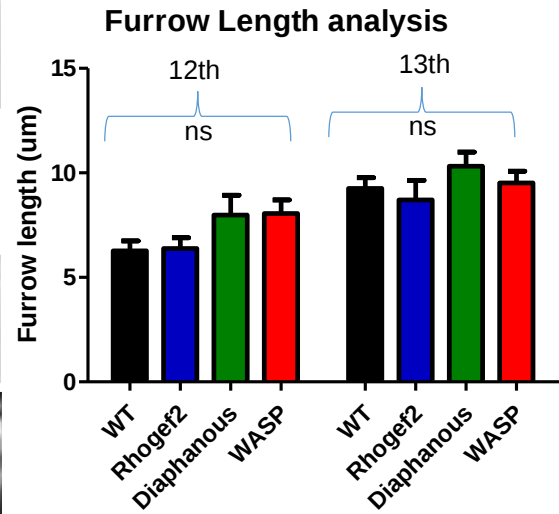


Figure 3.23. Graphical representation of metaphase furrow canal length of embryos mutant for actin regulators during 12th and 13th syncytial stages.

Discussion

4.1 Actin cytoskeleton regulation of plasma membrane polarity

For studying the role of actin cytoskeleton, we have adopted pharmacological drug treatment, RNAi strategy and OvoD¹ germline clone strategy against actin regulators RhoGEF2 and Diaphanous which regulates bundled actin, Arp66b and WASp which regulates branched actin. Latrunculin A treatment against actin polymerisation in early *Drosophila* embryogenesis caused general actin delocalisation and morphogenetic defect in the plasma membrane such as incomplete or missing furrow canal. The nuclear organisation in embryo was also affected.

RNAi Mutants	% Lethality	Stage	Rhogef2 (%affected)	Dia (%affected)	Arp (%affected)	Bazooka (%affected)	Pnut (%affected)	Patj (%affected)	Phalloidin (%affected)
Rhogef2	84.5	Syncytium	100			100	100	0	0
		Cellularisation	100			85	38	0	0
Dia	25	Syncytium		40		83	0	0	0
		Cellularisation		40		33	0	0	0
Arp	18.2	Syncytium			57	0	0	0	0
		Cellularisation			23.5	23	23	0	0
WASP	6.2	Syncytium				0	0	0	0
		Cellularisation				0	0	0	0

Table 4.1. Summary of the analysis of polarised distribution of polarity proteins Bazooka, Peanut and Patj in embryos mutant for actin regulators.

RNAi mediated knockdown of actin regulators caused differential lethality of embryos and resulted in decrease in corresponding protein during both syncytium and cellularisation stages. The lowering of protein in cellularisation will be lesser than syncytium because of maternal to zygotic transition of proteins. High lethality of an RNAi mutant indicates that the respective protein is knocked down and development of embryos is not supported in the absence of the protein. If the RNAi mutant shows less lethality, it can be either because of the redundancy of protein in development or recovery of the phenotype by zygotic protein or the inefficiency of the RNAi to cause significant depletion in the level of protein. RhoGEF2 RNAi is expected to cause maximum defects in the embryo compared to others since it has given maximum lethality and showed 100% reduction in the level of RhoGEF2 protein in both syncytium and cellularisation. Embryos of RhoGEF2 RNAi showed phenotypes like multiple nuclei, large nuclei, incomplete or missing lateral membrane and loose membrane. Dia RNAi and WASp RNAi showed partial knockdown and only 40% of

Dia RNAi embryo showed a loss of protein. Even though the percentage lethality was 8.3 in Arp66b, 57% of embryos showed a reduction in protein during syncytium which indicates that recovery of loss of protein might have happened due to maternal to zygotic transition and thus decreased the lethality (Table 4.1).

When we studied the polarised distribution of Bazooka and Peanut in the XY plane of plasma membrane in actin regulatory mutants, we found that Bazooka and Peanut are delocalised from the plasma membrane in embryos mutant for RhoGEF2 and Diaphanous. Bazooka was diffused into the cytoplasm during syncytium in 100% of embryos mutant for RhoGEF2 and 83% of embryos mutant for Diaphanous. This phenotype was still persistent in cellularisation in 83% of embryos of RhoGEF2 RNAi and 33% of embryo of Dia RNAi. Peanut showed a diffused staining pattern only in RhoGEF2 RNAi but not in Dia RNAi (Table 4.1). During cellularisation stage of *Drosophila* embryogenesis, it is known that Bazooka is recruited to the membrane with the help actin cytoskeletal cues and Bazooka will be delocalised in to lateral membrane in the absence of an actin scaffold (Harris and Peifer 2005). We show that, during syncytial stages also Bazooka is localised to the sub-apical membrane with the help of actin scaffold and bundled actin network possibly plays a crucial role in supporting Bazooka to bind onto the sub-apical membrane during syncytial stages. Bazooka delocalisation can also be because of the defective membrane architecture. RhoGEF2 RNAi causes membrane to become loose and defective in organisation and this may result in the change of lipid composition of the membrane where Bazooka is unable to find its binding partner. In fact delocalisation of Bazooka from the membrane itself going to cause change in phosphoinositide composition of the apical membrane. In apical Bazooka/PAR-6/aPKC complex, Bazooka is known to recruit to PTEN which is a phosphatase that converts PIP3 into PIP2 (Stein, Ramrath et al. 2005). As a result, Cdc42 and aPKC are down regulated in the apical membrane under normal circumstances since Cdc42 is recruited to the membrane by PIP3. In RhoGEF2 RNAi, we predict that the amount of PIP3 level will increase in the apical membrane and as a result Cdc42 will be recruited to the apical membrane. Cdc42 is known to regulate branched actin reorganisation through WASp and Arp2/3. Our result also predicts that, RhoGEF2 RNAi mutant will lead to an increase of branched actin network on the apical cortex of epithelial cell. This explains the fact that Phalloidin staining of actin is always present in all the embryo mutant for actin

regulators and also it is possible that actin network is regulated through multiple pathways.

Peanut regulation of F-actin to form curved actin bundles are reported in the literature but the regulation of Peanut localisation with the help of actin is not known. We speculate that, Peanut delocalisation from the membrane of RhoGEF2 RNAi embryos was mainly because of the defective loose membrane phenotype. We could find 47% of embryos which are defective for bazooka localisation but showed Peanut localisation similar to WT. Even in *Dia* RNAi, 33% of embryos in cellularisation showed absence of Bazooka on the membrane but Peanut staining was uniformly across the membrane (Table 4.1). This shows that, Peanut localisation is indirectly affected by the mutants for bundled actin regulators. Peanut localisation on the lateral membrane is essential for formation of furrow canal. In RhoGEF2 RNAi mutant, Peanut delocalisation might be a reason for the incomplete furrow canals (Fig. 4.1).

In the mutants for branched actin regulators, the localisation of Bazooka and Peanut was not severely affected compared to bundled actin mutants. We cannot conclude anything about the importance of branched actin network for Bazooka and Peanut localisation since these branched actin mutants showed only partial knockdown and less lethality. Only with the help of more efficient RNAi mutant or through pharmacological drug treatment study against branched actin regulators could tell us any useful information regarding the role bundled actin regulators in plasma membrane polarity.

Patj distribution remained largely unaffected in all these embryos mutant for actin regulators. Patj was localised to sub-apical membrane irrespective of these mutants for actin regulators and the membrane defective phenotypes caused by them. This clearly shows that polarised localisation of Patj is independent of bundled actin regulators.

Hypomorphic mutants *dia*⁵ and *arpc1* showed a reduction of Diaphanous and Arp protein in early syncytial stages but it showed severe mutant phenotypes such as very short or absent metaphase furrow canal, clustering nuclei and defective membrane architecture. Studying epithelial cell polarity aspect was not feasible in these mutants due to multiple severe morphological defects.

4.2 Actin cytoskeleton regulation of plasma membrane tension and polygonal architecture.

WT (n=3)		Rhogef2 (n=4)		Dia (n=3)		Arp (n=3)		WASP (n=3)	
12 th	13 th	12 th	13 th	12 th	13 th	12 th	13 th	12 th	13 th
• 5 Sided is dominant over 4 & 7 • 5 sided is found more than 6 but not significant	• 6 sided is dominant over all. • 5 sided is dominant over 4, 7	• 4 & 5 sided polygons are dominant over 6	• 5 sided polygons is dominant over all.	• 5 Sided polygons is dominant over all	• 5 & 6 sided polygon is dominant over others	• 5 sided is dominant over all.	• 5 & 6 sided polygons are dominant over others	• Data not significant .	• 5 and 6 sided polygon is dominant over other

Table 4.2. Summary of the polygon analysis in embryos mutant for actin regulators.

Polygon analysis showed that, there is a shift towards greater number of pentagons in branched and bundled actin mutants. In WT, pentagons and hexagons are equally dominant in 12th syncytial stages and hexagons show a significant dominance over all other polygons in 13th syncytial stages. In RhoGEF2 RNAi, tetragons and pentagons became equally dominant in 12th syncytial stages and pentagon showed a clear dominance over all other polygons in 13th syncytial stages. For other mutant for actin regulators, the number of pentagons increased and became equally dominant with hexagons in both syncytial stages (Table 4.2). Hexagonal topology is reported to have minimum surface energy and maximum packing (Gibson and Gibson 2009). Mechanism of nuclear division, maintenance of membrane surface tension, adhesion of epithelial cells and apical constriction are reported to play an important role hexagon dominant packing of epithelial cells. It has been shown that, the Rho kinases Rok, myosin II and an integral F-actin network are required for anaphase cell elongation by polar relaxation of cortex (Hickson, Echard et al. 2006). These mutants for actin regulators showed clustering of nuclei and giant nuclei phenotype because of irregular cell division which in turn affects epithelial cell topology. E-Cadherin along with its dynamic interactions with cortical actin and actin organisation helps in the cell adhesion through adherens junctions. The adhesion between the cells positively regulate the tissue surface tension in epithelial cells.

These mutants for actin regulators showed striking phenotype like thick and loose

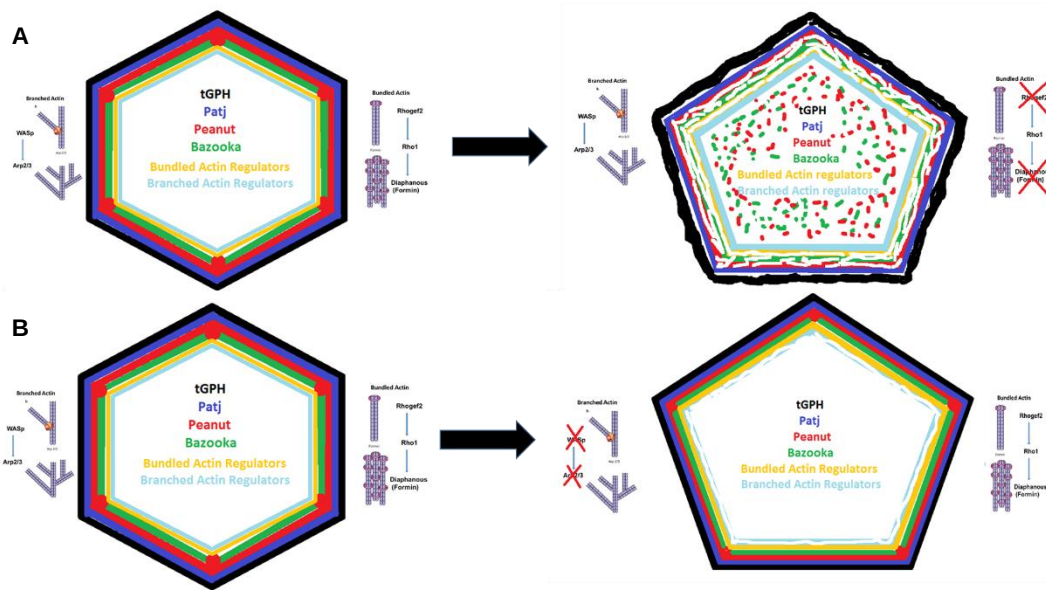


Figure 4.1. Model for bundled and branched actin regulation of polarised distribution of Bazooka, Patj and Peanut and the membrane architecture of *Drosophila* epithelial like cell. A) Mutant for bundled actin regulators affects the polarised distribution of Bazooka and Peanut. They are also responsible for the pentagon dominant epithelial like cell packing. B) Mutant for branched actin regulators does not affect the polarised distribution of Bazooka, Peanut and Patj but it is responsible for the increase the number of pentagons in epithelial like cell packing.

membrane. We speculate that it is because of the defective adhesion between epithelial cells which decreases the surface tension. We predict that, Cadherin localisation should be affected in RhoGEF2 RNAi that reduced the adhesion and surface tension of epithelial cells which further affected its epithelial cell topology. Nuclear fall outs are another phenotype which is seen in these embryos mutant for actin regulators which may affect the topology of epithelial cells. Studies show that Rok is required for apical constriction in flies and it is activated in most cases by RhoGEF2 and Rho. The Rok helps in tethering acto-myosin to AJs which in turn undergo complex disassembly and reassembly process through which it regulates the apical constriction (Hayashi and Carthew 2004). The mutants for RhoGEF2 and Arp66b showed severe phenotype such as very short or absent metaphase furrow canal and developmental arrest. Here we provide evidence that correct nuclear division, maintenance of cell adhesion and surface tension and apical constriction are indeed essential factors that determines the topology of the epithelial cells. These essential factors are deregulated in embryos mutant for actin regulators which caused in them a change in topology of epithelial cell packing. This shows that actin

regulators play a very important role in hexagon dominant epithelial cell packing, maintaining the surface tension and furrow canal invagination.

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