Analysis of phospholipid regulation of epithelial-like architecture in the *Drosophila* blastoderm embryo

A Thesis

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BS-MS Dual Degree Programme

by

Gayatri Mundhe

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

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Supervisor: Dr. Richa Rikhy, Dept. of Biology, IISER, Pune
Thesis Adviser: Dr. Aurnab Ghose, Dept. of Biology, IISER, Pune

Certificate

This is to certify that this dissertation entitled “Analysis of phospholipid regulation of epithelial-like architecture in the *Drosophila* blastoderm embryo”towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Gayatri Mundhe at Indian Institute of Science Education and Research, Pune under the supervision of Dr. Richa Rikhy, Associate Professor, Department of Biology, IISER, Pune during the academic year 2017-2018.

Dr. Richa Rikhy

Gayatri Mundhe

Committee:

Dr. Richa Rikhy

Dr. Aurnab Ghose

Declaration

I hereby declare that the matter embodied in the report entitled “Analysis of phospholipid regulation of epithelial-like architecture in the *Drosophila* blastoderm embryo” 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.

Dr. Richa Rikhy

Gayatri Mundhe

Abstract

Distinct apico-basal domain identity is achieved in epithelial cells via the presence of apical microvilli and basolateral surface with adherence junctions. Syncytial cells in *Drosophila* embryogenesis display similar onset and maintenance of asymmetric identities, which makes it an excellent and tractable system to study the role of plasma membrane (PM)-actin interactions. Previous studies in the lab have shown an onset of epithelial-like organization at cycle 11. My studies show that the apical cap expands followed by stabilization and adhesion to form the lateral furrow in each syncytial cycle. Phospholipids and their interaction with actin are known to regulate polarity and PM remodeling processes in other organisms. We found that genetically increasing the dosage of phosphatidylinositol(3,4,5)-trisphosphate (PIP_3) binding tGPH (tubulin promoter-GFP-PH domain of GRP1) tag resulted in defective polygonal epithelial-like architecture and short lateral furrows. Down regulation of PI3-Kinase, which presumably lowers PIP_3 levels or supposedly increasing PIP_3 via its antagonist PTEN phosphatase resulted in global defects in cortical actin and desynchronized division cycles. An imbalance in either of these two phospholipids resulted in the loss of cap stabilization and short lateral furrows. Actin remodeling protein Arpc1 is enriched at the actin cap edges during expansion and formin Dia is enriched during cap adhesion and furrow extension. Cap expansion is disrupted in *arp3* RNAi whereas expanded caps are visible in *dia* RNAi. Recruitment of adhesion and actin remodeling proteins such as DE-Cadherin, Rac1 and Dia but not Arp2/3 are lowered in PI3K and PTEN mutant embryos. These studies together show that phospholipid balance is crucial for the recruitment of Dia during cap stabilization and formation of furrow during the syncytial division cycle.

List of Abbreviations

Arp2/3 – Actin related proteins 2 and 3

Dia – Diaphanous

E-Cadh – DE-cadherin

GEF – Guanine nucleotide exchange factor

GFP – Green fluorescent protein

tGPH – Green fluorescent protein under constitutively active tubulin promoter with PH domain

PH-PLCδ- Marker binding to PIP_2

Myo-II – Myosin-II

PI3K – Phosphatydilinositol 3-kinase

PIP_2 –Inositol 4,5-biphosphate

PIP_3 –Inositol 3,4,5-triphosphate

PTEN – Phosphatase and tensin homolog

RNAi – RNA interference

NPFs-Nucleation Promoting Factors

WASP-Wiskott-Aldrich Syndrome protein

N.C-Nuclear cycle

LatA- Latrunculin A

PM- Plasma membrane

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Acknowledgements

“For us, there is only the trying.
The rest is not our business.”

-T.S.Eliot, “East Coker”.

No thesis work is a cake walk! I realized this and the essence of the above phrase during my year-long MS thesis project. All through this journey was I was blessed and nurtured by many. I wish to begin by expressing my gratitude towards them.

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Index

1. Introduction

The introduction section is divided into three sections.

The first part is focused on the structure and morphology of epithelial cells and their functions. Asymmetric distribution of organelles and proteins in epithelial cells will be introduced. Interaction of membrane with the cytoskeleton and how regulation of morphology is orchestrated by them. In particular, the pathways regulating phospholipids and the underlying actin cytoskeleton.

The second part is focused on morphogenesis during *Drosophila* syncytial development and the role of PIP_3 and actin regulators in coordinating membrane expansion and extension. Further, examples of PIPs interacting with regulators like Arp and Dia will be reviewed in epithelial cells.

The third part introduces *Drosophila* syncytial embryo as a model system to study epithelial tissue morphogenesis and interplay between membrane and cytoskeleton. Finally, effects of phospholipid balance in maintaining cell shape and ensuring proper morphogenesis during development are summarized. Objectives of this study are then listed.

1.1. Role of membrane-cytoskeleton interactions during transport, migration, cytokinesis, and development

Cellular plasma membranes (PM) guard the interior against the fluctuating outside, in order to maintain homeostasis. Antagonistic to this viewpoint, PM is an active participant and not just a mere defender. It interacts with the outside world and undergoes complex morphological reorganization along with regulation of cytoskeletal elements. Underlying cortical actin interacts with the PM to balance forces for shape maintenance. Tight regulation of these components is necessary for mitosis, motility, cell shape maintenance, and endocytosis (Fig1).

Sheet-like lamella and lamellipodia, finger-like filopodia and microvilli are actin superstructures evolved for specific cellular functions. The leading edge of migrating cells is marked by the presence of lamellipodia where a dense network of actin polymerizes at the plus-ends (Chhabra and Higgs, 2007). Cell migration and

lamellipodial protrusions were inhibited on the addition of polymerization inhibitor, Latrunculin A (Yarmola et al., 2000). Dorsal wound closure and neural growth cones involve assembly and persistence of filopodia. (Faix et al., 2009) Providing additional

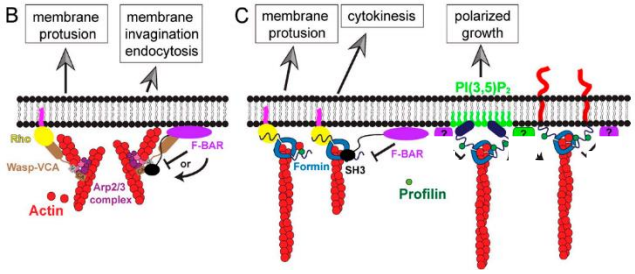


Figure1: Regulation of the actin cytoskeleton by phospholipid $PIP_2$and PIP_3 during the processes of cell migration, vesicular transport, polarized growth, and cytokinesis. Adapted from Bezanilla et al., 2015.

surface area and absorption are primary functions of microvilli. Endocytosis pathways utilize this excess membrane for inward movement and vesicle formation.

Precise positioning of the contractile ring directs coordinated movements of Myo-II with actin. Failure in such organization leads to defects in spindle positioning and overall cell division. Structural maintenance and positioning along with furrow progression are mediated via signaling by phospholipid PIP_2 (Logan and Mandato, 2006). The absence of PIP_3 from the furrow but its accumulation at the poles was detected whereas PTEN localized on the furrow in *Dictyostelium* (Janetopoulos and Devreotes, 2006). Another feature of membrane-cytoskeleton interactions is the maintenance of cell polarity and domain organization. Most widely studied epithelial cells are focused in this study.

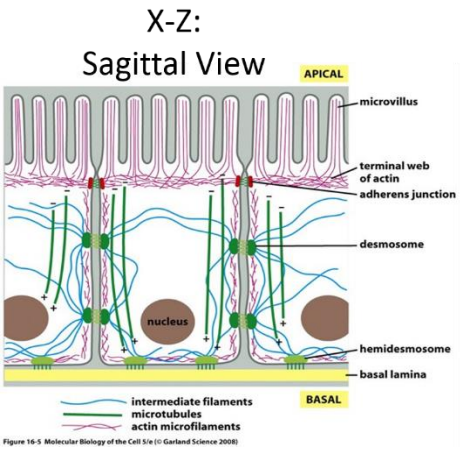
1.2. Epithelial cells exhibit apico-basal polarity and polygonal architecture

Metazoan cells began incorporating added features as their evolution progressed. This evolving complexity demanded an equally efficient level of organization and functioning. In this context, epithelial cells evolved as barriers of cell interior, thereby protecting the insides of the mouth, alimentary canal, and gut. Excretion from the skin surface, absorption of water and essential nutrients and secretory functions as glands represent a diverse fauna of its functions. Diffusion and other transport across cells with the perception of stimuli are also orchestrated by epithelial cells (Alberts et al., 2002).

The polarized organization of the PM is a unique feature of epithelial cells (Fig2). Establishment of this cell anisotropy can be attributed to an asymmetry in lipid and

protein composition, their downstream interactions and membrane-cytoskeleton interactions. Characteristics include (I) presence of apical microvilli to maintain surface area, (II) basolateral domain with adherence and tight junctions, (III) strong adhesion between neighboring cells, (IV) tight polygonal cell packing and (V) domain-dependent localization of polarity proteins, phospholipids, actin-regulators, proteins regulating endocytosis (Bergstralh and Johnston, 2012). In *Drosophila* syncytium, nuclear divisions are devoid of cytokinesis (Mazumdar and Mazumdar, 2002). Though incomplete PM boundaries exist, these cells depict apico-basally polarized domains, which can be referred as 'pseudo-epithelial cells' (Mavrakis et al., 2009).

Overall cell morphology is to be maintained such that maximum surface area is utilized with the usage of minimum surface energy. This is ensured via optimal cell packing into tight, polygonal arrays of epithelial monolayer. In proliferating epithelium, each cell division is associated with the establishment of contacts between the cell edges. Newer sides of the polygon are stabilized with each division, forming increased contacts between cells. Hexagonal dominance with a gaussian distribution of other polygons is observed in the tail epidermis of *Xenopus*, epidermis in *Hydra* and wing discs in *Drosophila*. Conservation of this topology is observed across diverse phyla (Gibson et al., 2006). Polarized morphology in these cells is differentially regulated by



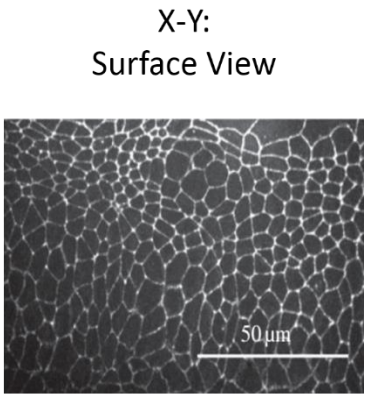


Figure2: Two primary characteristics of epithelial cells include apico-basally polarized PM and tight polygonal architecture. Adapted from Alberts et al., 2002 and Kursawe, 2016.

phospholipids PIP_2 and PIP_3 and the cytoskeleton (Shewan et al., 2011). Dynamics of these two players and their interactions during syncytial development remains a subject of study.

1.3. Structure, function, and dynamics during development of

a) Phospholipids

Cellular lipid bilayers are majorly composed of phospholipids. Amongst these, phosphatidylinositol bisphosphate (PIP_2) and phosphatidylinositol triphosphate (PIP_3) are of interest for this study. Phosphatidylinositol is comprised of a glycerol backbone, tagged with two fatty-acid non-polar tail groups, and a phosphate group substituted with a polar inositol head. The homeostatic balance of PIP_2 and PIP_3 is maintained by 3'-OH phosphorylation activity of PI3-Kinase and its antagonist PTEN phosphatase. PI3-Kinases are under the regulation of GPCRs and tyrosine kinase receptors whereas PTEN is has a tyrosine phosphatase domain. A multitude of proteins, containing pleckstrin homology (PH) domain are recruited to the PM via binding to PIPs. These include Ser/Thr kinases, Akt, Rac, Arf family, Cdc42, RhoGEFs, cytoskeletal proteins like dynamin and PLC to name a few.

During development of *Xenopus* embryos, an overexpression of PI3-Kinase led to an additional head development and dorsal axes. Conversely, lowering in embryos led to axial developmental defects and small tail (Peng et al., 2004). Gastrulation in *Xenopus* is also under PI3-Kinase control (Nie and Chang, 2007). Cell growth and size regulation are under PI3-Kinase and PTEN control during wing disc development in *Drosophila*. Akt control of cell size leads to over proliferation in PTEN mutants and PI3-K overexpression (Gao et al., 2000).

b) Actin

Polymerization into linear filaments (F-actin) is achieved by monomers of G-actin (43kDa protein) via a process of nucleation of new filaments. Actin tread-milling occurs when the barbed and pointed ends of actin filaments polymerize or hydrolyze monomers using ATP. Dissociation constants and critical concentrations do play a vital role in this process. Actin is organized into either branched networks or parallel bundles. WASP and Arp2/3 complex are primary regulators of branched actin whereas Diaphanous (formins), RhoGEFs associate with bundled actin (Chhabra and Higgs, 2007).

Sea urchin embryo development was first studied to observe a very dynamic actin cytoskeleton. Various actin regulators like Rac1 and Cdc42 are studied for their role during epithelial development in *C.elegans* embryo. During development in

Drosophila, the process of dorsal closure, germband retraction, and ventral furrow formation highlight the role of dynamic actin cytoskeleton (Jacinto and Baum, 2003). In vertebrate development, lung patterning is synchronized by the actin cytoskeleton (as reviewed in Pickering, 2013).

1.4. The interaction between membrane and underlying actin govern tissue morphogenesis

During morphogenesis, global rearrangements of the underlying cytoskeleton take place which in turn, regulate remodeling of the PM. In *Drosophila*, lumen formation is under control of actin-associated proteins Cdc42 and formins, during heart development. Activated expression of these proteins led to mislocalization of non-muscle myosin II encoding Zipper and ectopic lumen formation (Vogler et al., 2014). Abrogation of Arp2/3 complex during ring canal formation of *Drosophila* oocyte led to collapsed rings whereas depletion of Diaphanous expanded these ring canals which depicted defective structures (Kindred et al., 2016). Cell polarity is maintained via cytoskeleton and its regulatory proteins during development of embryonic denticles in *Drosophila*. Signaling cues like *Wingless* and *hedgehog* also trigger assembly and accumulation of actin and cytoskeletal rearrangements (Price et al., 2005).

Syncytial development during *Drosophila* embryogenesis involves cycles of ingression and retraction of cleavage furrows (Mazumdar and Mazumdar, 2002). RhoGEF and the formin Diaphanous play a vital role in precise assembly and invagination of these furrows. The absence of RhoGEF demonstrates failure in Diaphanous recruitment, which polymerizes actin and hence, concurrent loss of furrow formation (Großhans et al., 2005). Role of RhoGEF is also further studied in the same system for assessing its function in positioning the furrows in accordance with the central division spindle and guiding ingression encompassing as opposed to bisecting the spindle axis. Ectopic activation of RhoGEF led to the formation of ectopic furrows which were perpendicular to the dividing spindle (Crest et al., 2012).

Nucleation-promoting-factors (NPFs) are increasingly associated with formins where the primary limiting step is polymerization. Spire-Capu and Adenomatous Polyposis Coli 2 (APC2)-Dia are studied in *Drosophila* for their role oogenesis and

syncytium (Jaiswal et al., 2013; Quinlan, 2013). Formation and extension of cleavage furrows is regulated by APC2 binding to Diaphanous which is independent of RhoGEF2 and Rho1 activity. Mutants for APC2 display defective furrow which worsen on the reduction of Diaphanous (Webb et al., 2009) (Fig5). Rocket launcher mechanism was proposed to explain this in vertebrate APC2-Dia interactions. APC2 acts as a nucleation seed which binds and recruits actin monomers, further binding to Diaphanous which acts as an elongation catalyst driving polymerization (Breitsprecher et al., 2012). Formation of the APC2-Dia nucleation complex and its consequent detachment propels effective furrow formation.

Along with furrow formation, regulation of apical microvilli, which act as membrane reservoirs requires cytoskeletal assistance. During syncytial development in *Drosophila*, actin regulator Enabled is under control of proto-oncogenic Abelson Kinase. Expression of Abelson led to increased actin microvilli without furrow assembly. These embryos demonstrated mislocalization of Enabled. Guiding Enabled and polymerization of actin are primary functions of Abelson as mutants demonstrated ectopic accumulation of Enabled and loss of actin microvilli. Arp2/3 and Diaphanous mislocalization added to further defects (Grevengoed et al., 2003) (Fig5). Studies on dorsal closure in *Drosophila* revealed that Enable driven-Diaphanous is necessary to maintain robustness (Nowotarski et al., 2014). Ventral furrow formation is shown to be driven by Abelson and RhoGEF activity for stable cell constriction in *Drosophila* (Fox and Peifer, 2007).

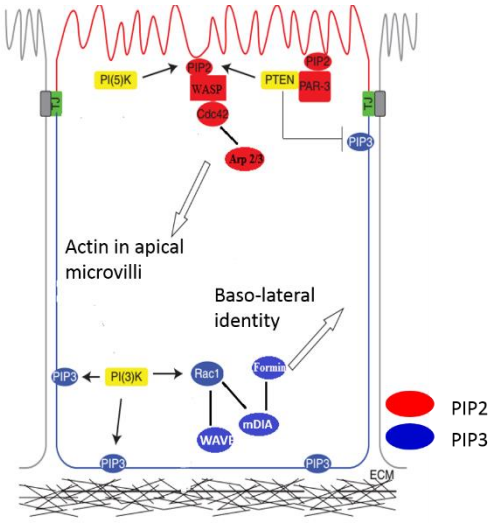


Figure3: Interaction of phospholipid PIP_2 with WASP and PIP_3 with Rac1 and thereby underlying actin cytoskeleton regulates polarized domain identity in epithelial cells. Redrawn with adaptation from Shewan et al., 2011.

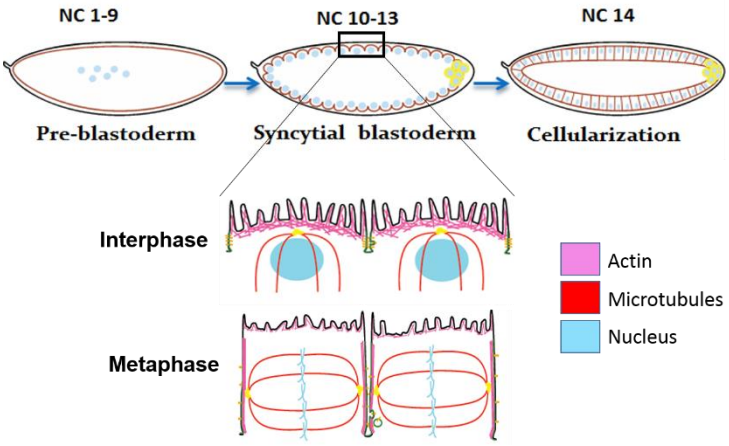


Figure4: Drosophila syncytium as a model system for studying apical microvilli and cleavage furrow dynamics. Distinct interphase and metaphase morphologies exist during syncytial divisions.

1.5. *Drosophila* syncytium as a model system for studying the epithelial-like structure and furrow dynamics

Syncytial blastoderm in *Drosophila* develops without formation of complete cell boundaries. Consecutive cycles of nuclear division without cytokinesis is a highlight of this process. Heavy maternal deposition fast-forwards these division cycles as about 6000 nuclei line the embryo surface in a span of about 2-3 hours. Post-fertilization 1-3 cycles of division occur in the embryo interior. The axial expansion marks the cycles 4-6. Within the next three cycles, cytoskeleton remodeling pushes and migrates these nuclei to the cell cortex. The process of cortical migration arranges cells into a monolayer which synchronously divides around the embryo.

At the end of cycle 9, PM accumulates around each nucleus such that inter-cell exchange is still possible. To ensure proper spindle orientation and compartmentalization during mitosis, pseudo-cleavage furrow extends into the embryo interior. Subsequent cycles require a higher threshold of furrow length for mitosis to occur. Cycle 14 marks the highest ingression of the furrow, followed by cytokinesis to form complete cells (Fig4). This is trailed by gastrulation post which normal, asynchronous division is resumed. These rearrangements play a crucial role in establishment of proper morphogen gradients and in turn, direct formation of body axes (Mavrakis et al., 2009; Mazumdar and Mazumdar, 2002; Sokac and Wieschaus, 2008).

Although the absence of complete boundaries arises during syncytium, sharing is restrictive between adjacent cells. Distinct cellular architecture resembles epithelial-like

morphology. Apical domain with microvilli, a short lateral domain with adhesion and septate junctions and a basal domain persist in these pseudo-cells. This makes syncytium an excellent system for studying dynamics of the apical cap and furrow ingression. Unlike epithelial cell development, time-scale for division is restricted to a few hours during syncytium. Cycles of furrow ingression and retraction are observed from interphase to metaphase of each division cycle from 10-14 (Mazumdar and Mazumdar, 2002). Ease of studying 6000 synchronously dividing nuclei make it an effective model organism. In-vivo dynamics can be studied using live imaging via expression of transgenes. Tissue-specific and temporally-restricted phenotypes can be easily visualized in transparent, dechorionated embryos (Mavrakis et al., 2008).

1.6. Phospholipids PIP_3 and PIP_2 in regulation of shape changes and actin dynamics.

Befitting the role of a second messenger, PIP_3 instructively signals remodeling of the actin cytoskeleton. Predominantly, studies focusing on the role of PIP_3 in coupling signaling with the actin cytoskeleton. Inhibiting or mutating PIP_3 led to interference in signaling polymerization of actin in neutrophils, fibroblasts and also *Dictyostelium*. Cell polarity and motility were triggered on the addition of in-vitro PIP_3 which was attributed to increased actin polymerization (as reviewed in Insall and Weiner, 2001). The mechanism to achieve this is attributed to players like Rac, WASP, RhoGEF, and Cdc42.

In epithelial cells, formation and maintenance of the basolateral identity are attributed to regulation via PIP_3. The shift from apical to basal identity was observed in cells induced with in-vitro PIP_3 at the apical domain. On inhibition of PI3-Kinase, lowering of PIP_3 levels led to the disruption of lateral surfaces (Gassama-Diagne et al., 2006). *Drosophila* follicular epithelium employs PIP_2 to maintain apicobasal polarity.

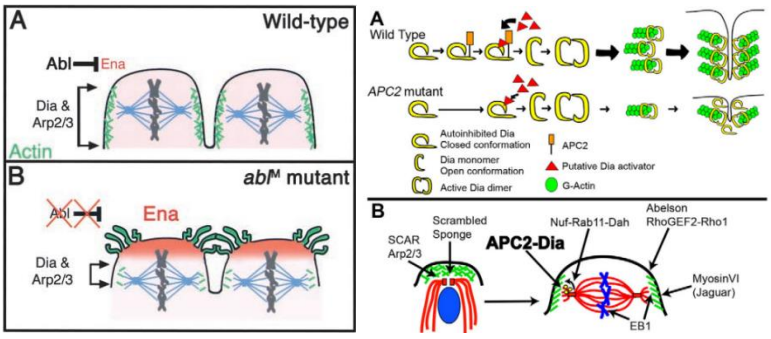


Figure5: Model proposed to explain mechanism of apical microvilli regulation (left) and furrow formation (right). Abl and Ena feeback and APC2-Dia association is primary in these processes. Adapted from Gravengoed et al., 2003 and Webb et al., 2009.

Abrogation of 4,5-OH kinase *Skittles* led to disorganization of the cytoskeleton, disassembly of adherence junctions along with apical constriction. This effect was ascribed to the apical recruitment of polarity protein PAR-3/Bazooka. Any alteration in PIP_2 from apex resulted in cell shape changes(Claret et al., 2014). In *Drosophila* pupal notum and during wing disc development, the difference in relative levels of PIP_3 is generated in clones which alters membrane tension via actin. Tissue invasion is a result of added cell-cell intercalation which is also promoted by mutations in PTEN phosphatase (Levayer et al., 2015).

Association of PIP_2 and PTEN during cell polarity has been studied in numerous systems. In *Drosophila* oocytes and embryos, regulation of the cytoskeleton is at mercy of link between PAR/aPKC polarity complex and apical localization of PTEN (von Stein, 2005). In cell culture, addition of PIP_2 ectopically at the basal surface led to recruitment and localization of apical proteins and interrupts normal domain organization and lumen formation (Martin-Belmonte et al., 2007).

As the cellular levels of PIP_3 are only about 2-5% of the cellular PIP_2 levels, a small amount of PIP_3 can act as an instructive signal for downstream activation. The same isn't true of PIP_2 which floods the cell boundaries. Alternative to the instructive role, PIP_2 functions to adjust the membrane tension. The spread of PIP_2 distribution could restrict actin polymerization and thereby, ensure filament growth in the right direction. Presence of similar mechanisms in the regulation of apical microvilli and furrow extension during *Drosophila* embryogenesis remain yet to be studied. To follow up on previous literature, there was a need to characterize the PM and actin during different phases of cap and furrow dynamics. Observing the actin and PM *in-vivo* in all four dimensions and at increased resolution is crucial. This guides us to the following questions: What is the distribution of phospholipids, PIP_2 and PIP_3 along with other actin-related proteins like Arp2/3, Diaphanous, Rac and WASP during syncytial division? How do these players regulate shape transition from interphase to metaphase? What are the effects of phospholipid depletion on overall embryo morphology and actin cytoskeleton? What interactions of the actin and PM regulate this shape transition?

1.7. Aims of the study

1.7.1. Study of F-actin and PM architecture in-*vivo* during syncytial division cycles in *Drosophila* by using confocal microscopy

1.7.2. Effect of phospholipid mutants on actin and PM morphology in syncytial cells

1.7.3. Observing actin in WT embryos using super-resolution microscopy

1.7.4. Role of actin-regulatory proteins in phospholipid mutant embryos during syncytial division.

2. Materials and Methods

2.1. Buffers and Solutions

1. **1X PBS:** Phosphate Buffered Saline

2. **4% PFA:** Para Formaldehyde

For 40 mL: 10mL 16% PFA + 4mL 10X PBS + 26 mL Distilled water

3. **0.3% PBST:** Phosphate Buffered Saline with Triton

For 200 mL: 20mL 10X PBS + 600µL Triton X-100 + 179.4 mL Distilled water

4. **2% PBTA:** Phosphate Buffered Saline with Azide

For 10mL: 0.2g BSA powder + 200µL Azide (10mg/mL stock)

2.2. *Drosophila* stocks and crosses

Stock Name	Source	ID
Stocks for live imaging		
tGPH	BDSC	BL-8164
nanos Gal4	BDSC	BL-4937
*maternal-alpha-G*al4	BDSC	BL-7062
UASp-LifeAct-RFP	BDSC	BL-58713
UASp-Arpc1-GFP	BDSC	BL-26692
UASp-Dia-EGFP	BDSC	BL-56751
Ub-Synd-GFP/CyO; MKRS/TM6Tb	T. Takeda	-
RNAi line		
PI3K 68D: y^1 sc* v^1; P{y[+t7.7] v[+t1.8]=TRiP.GL00159}attP2	BDSC	BL-35265
PTEN: y^1 v^1; P{TRiP.HMS00044}attP2	BDSC	BL-33643
Arp3: y^1 sc^* v^1; P{TRiP.HMS00711}attP2	BDSC	BL-32921
Dia: y^1 sc^* v^1; P{TRiP.HMS00308}attP2	BDSC	BL-33424
Mutant stock		
ShotgunΔJM depletion: w^{1118}; P{UASp-shg.ΔJM}3	BDSC	BL-58444
CRISPR Lines		
PI3Kinase gRNA: y1 v1; K10dual v+/K10 dual v+; +/+	Raghu	-
dPTEN gRNA: y1v1, +/+, P5dual-CG5671-dpten v+/P5dual-CG5671-dpten v+	Raghu	-
vasa cas9	BDSC	BL-56552
nanos cas9	BDSC	BL-54591

Table 1: List of *Drosophila* stocks used with source and ID. (BDSC: Bloomington *Drosophila* Stock Centre)

6% yeast medium containing agar, cornmeal and sugar is used and flies are reared in bottles. All the RNAi crosses were maintained at 28°C and lines were grown at 25°C.

2.3. Embryo lethality estimation

Embryos were collected after 6 hours or overnight collection and put on a sieve. After washing with distilled water, they were arranged onto an agar plate into a 10x10 matrix. After incubation at required temperature, the number of unhatched embryos were counted after 24 and 48 hours.

2.4. Immunohistochemistry

After emerging of crosses, F1 flies were selected and put into cages with 3% agar and sugar medium with yeast paste. Embryos were collected, after 2 hours at 28°C and 2 hours 30 min at 25°C, into a sieve, washed and dechorionated with bleach for 1 min. The embryos were transferred into vials and fixed with heptane-4%PFA for 25 min at RT on shaker. Embryos were hand devitellinized after storing in heptane for over an hour. Blocking for 1 hour at RT was done with 2% BSA. Primary antibody was diluted in 1XPBTA and kept overnight at 4°C on shaker. Fluorescently tagged secondary antibodies were added (Alexa Fluor 488, 568) in 1:1000 in PBST with fluorescently tagged Phalloidin (1:100) for 1 hour at RT on shaker. Adapted from Chowdhary et al., 2017.

	Primary Antibodies	Organism	Dilution	Lab/ Company
1.	Arp2/3	Rabbit	1:500	William Theurkauf
2.	Dia	Rabbit	1:500	Wasserman Lab
3.	Rac1	Mouse	1:5	DSHB
4.	DE-Cadherin	Rat	1:5	DSHB
5.	Anti-GFP	Mouse	1:1000	Invitrogen
6.	Anti-PIP_2	Mouse	1:500	DSHB
7.	Anti-PIP_3	Mouse	1:500	DSHB
8.	Lamin DmO	Mouse	1:75	AbCam
	Secondary Antibodies	Organism	Dilution	Lab/Company
1.	Alexa488	-	1:1000	Invitrogen
2.	Alexa568	-	1:1000	Invitrogen
3.	Alexa647	-	1:1000	Invitrogen
	Dyes	Organism	Dilution	Lab/Company
1.	Phalloidin488/568/647	-	1:100	Invitrogen
2.	Hoechst	-	1:1000	Sigma

Table 2: List of Antibodies used with details of organism from which it was derived, the dilution used and the source from which it was procured. (DSHB: Developmental Studies Hybridoma Bank)

2.5. Microscopy and Image Analysis

2.5.1. Live-embryo time-lapse imaging

Embryos were collected for 1-1.5 hour and dechorionated with bleach for 1 min. After washing, they were placed on a LabTek chamber with PBS (Mavrakis et al., 2008). Embryos were imaged in 4D with 20-25 z-stacks (each stack about 1µm thickness) every 30s with averaging 2, scan speed 9 and 40X objective on Zeiss Confocal microscope.

2.5.2. Imaging of fixed samples

Confocal imaging was done on fixed embryos at room temperature with an inverted LSM-710 or 780 inverted microscope (Carl Zeiss, Inc) at microscopy facility, IISER, Pune. Excitation was achieved with lasers 488 nm and 633 nm and emission with PMT filters. An oil immersion objective of 40X/1.3 N.A (for LSM710) or 1.4 N.A (for LSM780) with 3X zoom, pinhole 1A.U, averaging 2 and scan speed of 9 was used for imaging z-stacks (step size of 1µm) with Zen software.

2.5.3.Super resolution imaging on STED

Fixed embryos were imaged at 25°C with Leica TCS SP8 STED 3X microscope with excitation at 488 nm and emission with HyD detectors. A 100X oil immersion objective was used with pinhole 1AU, averaging 2, speed 100, format 512X512 and zoom 1.6 for confocal images and averaging 5, format 1500x1500 and zoom 3.5 with 775 nm depletion laser for super-resolutio images on LAS X software.

2.6. Image processing

Images of apical caps are presented as Maximum Intensity Projection (MIP) using ImageJ (https://imagej.nih.gov/ij/). Immunostainings are represented in a single optical plane for surface (X-Y) and sagittal (X-Z) views. The images were de-convolved with Huygens Professional version 17.04 (Scientific Volume Imaging, The Netherlands, __http://svi.nl__) using CMLE algorithm with SNR 20 for confocal and 7 for STED and 40 iterations.

2.7. Image Analysis and Quantification

All the measurements were done on time-lapse movies of syncytial nuclear division cycle 12. Area under cap expansion was measured from first visible section by manually tracing circular ROIs along the edges of apical caps from interphase to metaphase using ImageJ. To quantify extension of cleavage furrows, freehand lines were drawn along the length of the furrow from interphase to metaphase using Zen. Cap area and furrow length was plotted against time for 5 caps and 5 furrows per embryo for a minimum of 3 embryos/genotype using GraphPad™. Linear regression analysis were done on curves where time was fixed as the independent variable and predictions about the trend in dependent variables. In order to compare two curves plotted against the same variable, non-parametric t-test with Welch's correction (assumption of unequal variances) was utilized for analysis using GraphPad™.

3. Results

3.1. Phospholipid PIP_3 and underlying actin choreograph apical cap expansion accompanied by furrow ingression during syncytial cycles

During pseudo-epithelial sheet formation in *Drosophila*, continuous cell shape change accompanied by proliferation and dynamic rearrangements of PM and cortical actin are observed (Fig4). Both localization and function of actin remodeling proteins rapidly change across one cycle of nuclear division from interphase to metaphase. We studied syncytial *Drosophila* embryos to visualize dynamics of $PI(3,4,5)P_3$ (PIP_3) and cortical actin and their role during phases of cap expansion, stabilization and furrow extension, retraction.

3.1.1 Actin cap expansion and shift from circular to polygonal architecture is accompanied with furrow ingression in the syncytial embryo

To study the role of PIP_3 in developing embryos, we chose fluorescently tagged transgenic fly line which ubiquitously expressed PH domain of PIP_3 binding protein GRP1, tagged to GFP under tubulin promoter (Britton et al., 2002). It labels the lateral plasma membrane in complete epithelial cells and depicts a characteristic tight polygonal architecture (Britton et al., 2002). Time-lapse live imaging of tGPH in wild-type background fly demonstrated differential shape changes occurring in all four dimensions. The stage chosen was nuclear cycle 12 of division from early interphase to late metaphase. Observing developing syncytial embryos, expressing constitutive GFP signal for tGPH in both X-Y (surface view) and X-Z (sagittal view) revealed dramatic alterations at the apical surface in early prophase and interphase followed shortly by basolateral rearrangements during metaphase. The interphase marks the first appearance of the apical cap with tGPH marking the rim of the cap (Fig 6a). Devoid of any intercap signal, this stage represents circular cap expansion. As the edges of the caps begin to touch, the cleavage furrow journeys towards the basal membrane. Continual ingression of the furrow thereby stabilizes the apical caps and drives the cells towards metaphase. These incomplete cells establish contacts which, on expansion organize into a sheet of polygonal cells. Completion of one cell cycle is marked by

subsequent retraction of these pseudo-cleavage furrows and cells proceed for another division. Four distinct phases can be identifiedduring morphogenesis: cap expansion, cap stabilization, furrow ingression and furrow retraction (Fig 6b). To analyze this, we fitted a linear equation with time as the independent variable in order to predict the trend in the dependent variable, furrow length. Linear regression analysis of furrow length demonstrated a linear increase from interphase to metaphase and biphasic trend in area of the cap (both linearly increasing and constant). Such dynamic, synchronous and coordinated rearrangements have been previously observed in *Drosophila* syncytial blastoderm (Webb et al., 2009). Fluorescent tag expression did not lead to any adverse effects on the successful development of the organism, as about 90-95% embryos hatched at ambient temperature (n=300 embryos).

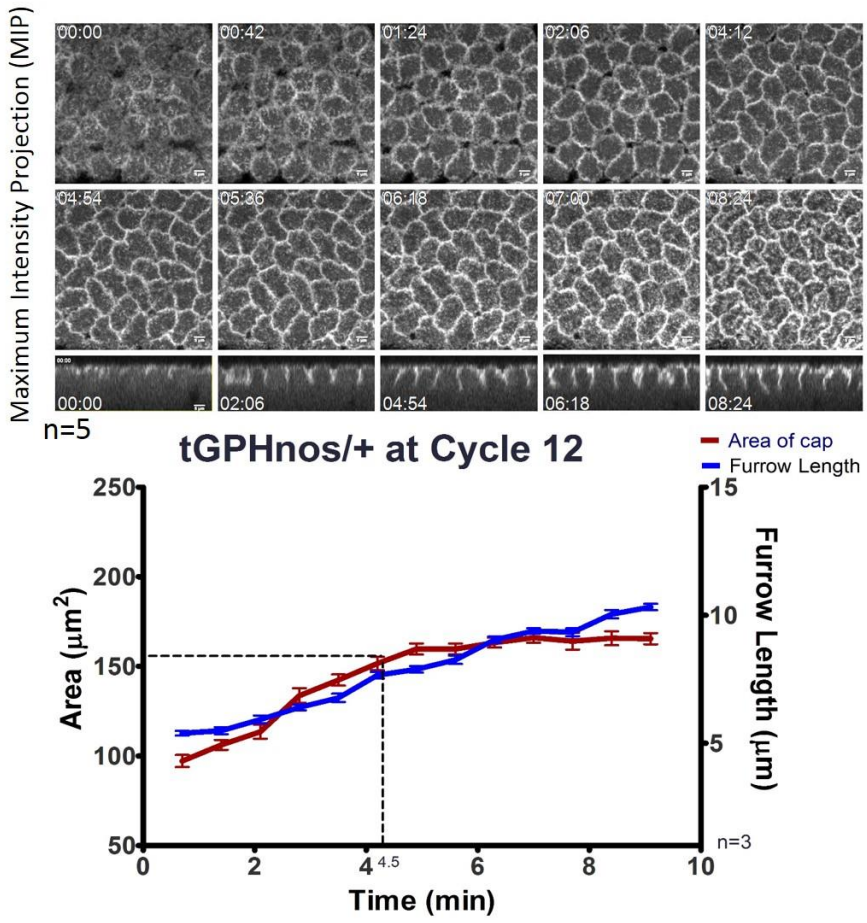


Figure6: a: Nuclear cycle 12 of syncytial embryo labelled with tGPH to visualize PIP3 on the PM. Time-lapse X-Y sections depict phases of cap expansion, stabilization and X-Z sections represent furrow extension and retraction phases (indicated by arrow heads). b: Plot of area of cap expansion along time on left axis and length of furrow ingression on right axis. Here, analyzed (15,3) (Number of caps/furrows measured, number of embryos assessed)

3.1.2. Differential localization of PIP_2 and PIP_3 is seen during early nuclear division cycles in *Drosophila* embryo

In complete epithelial cells, polarized morphology into apical, lateral and basal surfaces is maintained. A multitude of proteins and cytoskeleton function to achieve this asymmetric distribution. In addition to this, phospholipids $PI4,5P_2$ (PIP_2) and PIP_3 distribute themselves into two non-overlapping zones: apically enriched PIP_2 and basolateral dispersion of PIP_3 (Leslie et al., 2008). During cellularization in *Drosophila*, apical identity was maintained by PIP_2 whereas basal-lateral surface is governed by PIP_3 (Claret et al., 2014). Like tGPH, the PH domain of phospholipase $C\delta$ ($PLC\delta$)specifically binds to the PIP_2 on the PM. Precise localization of PHPLC on the membrane with low cytoplasmic and nuclear signal, on interaction with PIP_2 was visualized in transfected NIH-3T3 cells (Varnai and Balla, 1998). We adapted constitutive expression of PHPLC-CFP and tGPH-GFP in order to mark PIP_2 and PIP_3 respectively. To study establishment of differential domain-dependent localization ofthese two phospholipids, first onset of apical caps at cycle 10 is crucial. These actin caps initial expand without lateral ingression, when the nuclear:cytoplasmic ratio is low. An increase in this ratio, with subsequent cycles, is accompanied by linear increase in furrow length. Furrow ingression increases with each cycle terminating at cellularization,where PM extends fully to form complete cells. When flies expressing dual fluorescent tags were imaged live in 4D, earlier cycles 10 and 11 were observed in addition to cycle 12. Due to the presence of very short or no furrows during metaphase of nuclear cycle 10 and 11, only interphase embryos are studied from apical to basal surface. PIP_2 and PIP_3 are differentially distributed across cycles (Fig7a). The initial appearance of caps is marked by the positioning of PHPLC-CFP in the intercap region, collectively with PIP_3 accumulated around the cap rim. As the division cycle progresses, caps expand and make contacts. Both PIP_2 and PIP_3 co localize when the apical caps touch each other and the intercap area reduces. Intercap intensities are maximum at the topmost surface, readily decreasing towards the basal surface. Here, cycle 10 depicts the maximum intercap area and hence, is considered for normalization. No significant differences are observed between cycle 11 and 12 in normalized intensity of PIP_2. In contrast, there is a rise in mean normalized intensity of PIP_3 at cycle12 (Fig7b). In following cycles, the increase in number of dividing caps is coupled to a reduction in individual cap diameter. Conversion of intercap area into area occupied by apical caps

is synchronized with an accumulation of PIP_3 for cap stabilization and a switch to furrow ingression.

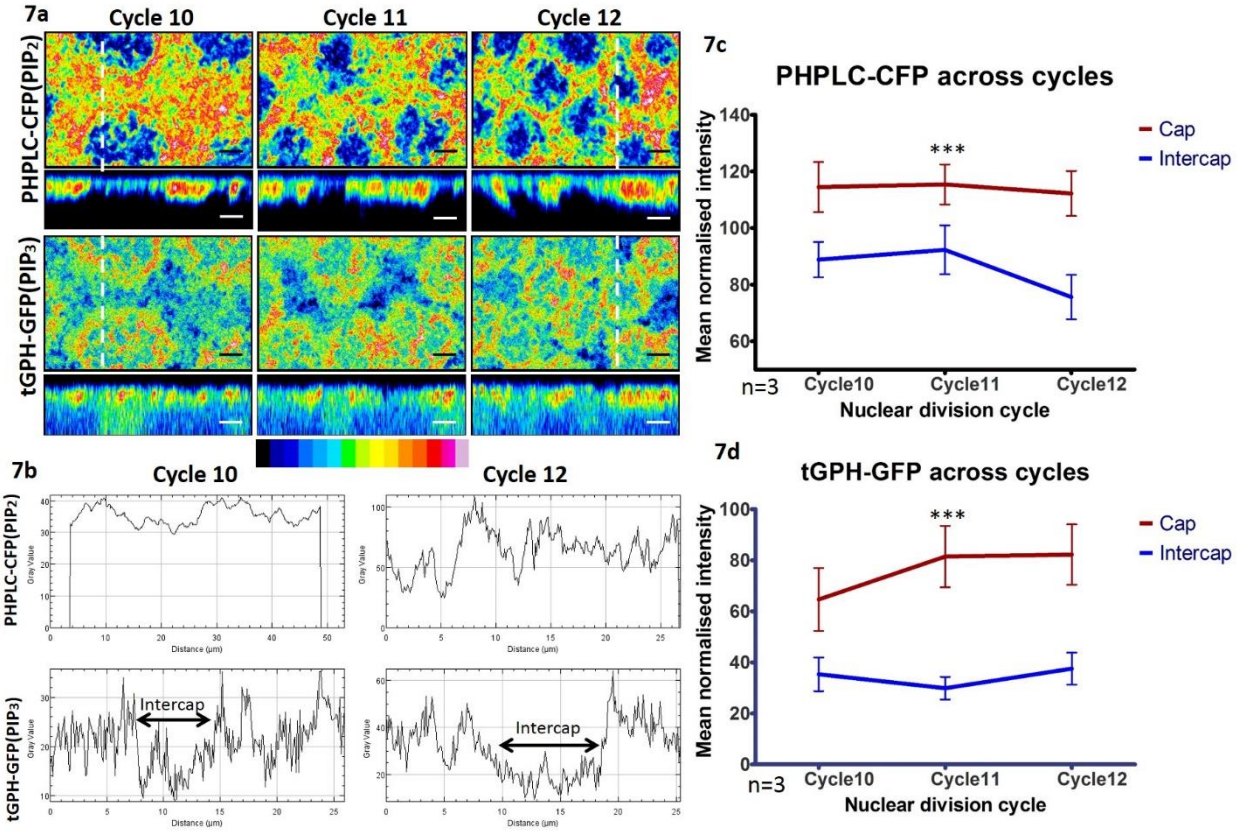


Figure7: a: Localization of PHPLC-CFP marking PIP2 and tGPH-GFP marking PIP3. Intercap PIP2 and around cap PIP3 is seen at cycle 10. Different columns represent different cycles of nuclear division. b: Line scan plots of PHPLC and tGPH at cycle 10 and 12 where absence of intercap PIP3 signal is depicted. Lines across images in a represent position of the plot. c: Mean intensity of cap and intercap plotted across cycles for PHPLC and tGPH (d) Cap increase of PIP3 at cycle 12 significantly different from cycle 10. Here, analyzed (15,3) (Number of caps/furrows measured, number of embryos assessed). p value<0.05, unpaired t-test with Welch's correction.

3.1.3. Loss of apical microvilli from interphase to metaphase is observed with STED microscopy

Syncytial cell shape changes shows an increase in cap area (Fig6a). It is hypothesized that the membrane for pseudo-cleavage furrow extension is supplied by apical microvilli-like projections. Prevalence of such membrane reservoirs has been studied during *Drosophila* cellularization (Figard and Sokac, 2014). Regulation of the PM was determined to be under control of dynamic assembly and disassembly of F-actin, which

deform the microvilli, fuelling the ingression of cleavage furrow (Figard & Sokac, 2016). In order to investigate the apical cap dynamics, we visualized F-actin in fixed *Drosophila* syncytial embryos via staining with Phalloidin in interphase and metaphase of cycle 12. Apical projections are seen in interphase followed by tight membrane devoid of microvilli in metaphase (Fig8). Due to typical pseudo-epithelial characteristics, syncytial cells show apical actin caps which are absent at the baso-lateral surface. Dispersed intensity peaks corresponding to apical microvilli are observed only at apical surface in interphase whereas sharp peaks corresponding to tight membrane persist in metaphase. Presence of cortical actin as apical microvilli clearly provides for the membrane extension phase and thereby is completely lost during metaphase even during syncytial division cycles.

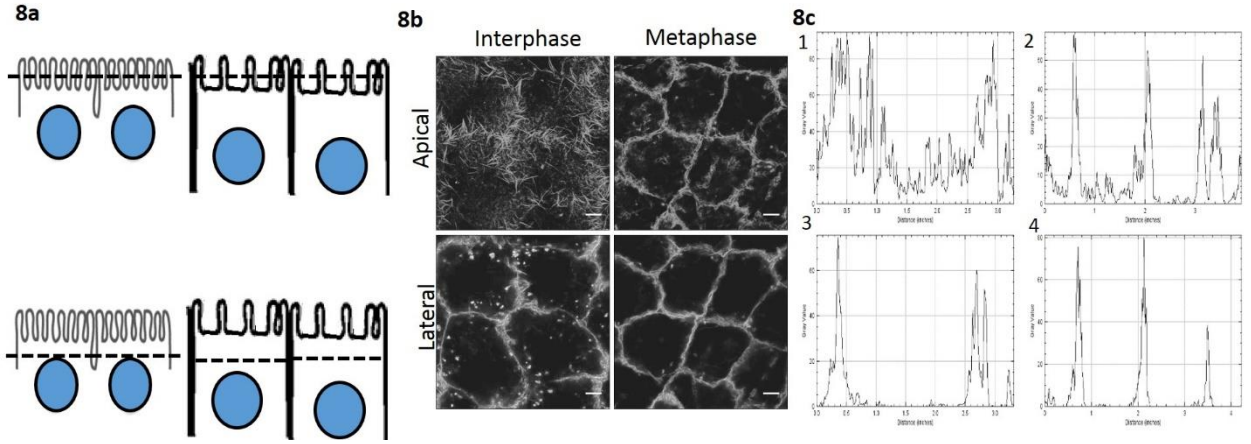


Figure8: a: Schematic of apical and lateral views in interphase and metaphase marked with the section observed b: Syncytial embryos stained with Phalloidin to visualize F-actin via STED at both interphase and metaphase. Dotted lines indicate regions for linescan plots. c: Line scan analysis across single cells depicting a spread in interphase (1,3) due to apical caps but sharp peaks are seen in metaphase (2,4). Here, analyzed (12,3) (Number of caps/furrows measured, number of embryos assessed)

3.1.4. Tight polygonal architecture with furrow formation is observed with Structured Illumination Microscopy (SIM)

Imaging multiple z-stacks is a major drawback of STED as the signal intensity bleaches with successive depletions in the same ROI. To overcome this and achieve high resolution in X-Z, we fluorescently labeled F-actin with Phalloidin and imaged fixed syncytial embryos in both interphase and metaphase in all 3 dimensions using structure illumination microscopy (SIM). Corroborating with STED results, apical membrane is

seen in microvilli-like projections which are lost at metaphase. This loss of membrane reservoir is reflected as an increase in furrow length (Fig9a). Lateral surface at metaphase is organized into a tight sheet of polygons confirms the shift in architecture from spherical caps to tightly packed geometric sheet (Fig9b). Plotting intensity profile across the apical membrane in interphase depicts a spread in distribution (Fig9c). During metaphase, membrane reservoir loss leads to shrinking of the distribution into a peak. Coordinated pseudo-cleavage furrow ingression with apical reservoir loss is observed with SIM. Added temporal resolution can be obtained via live super-resolution imaging during cap expansion and furrow ingression phases of nuclear division cycle.

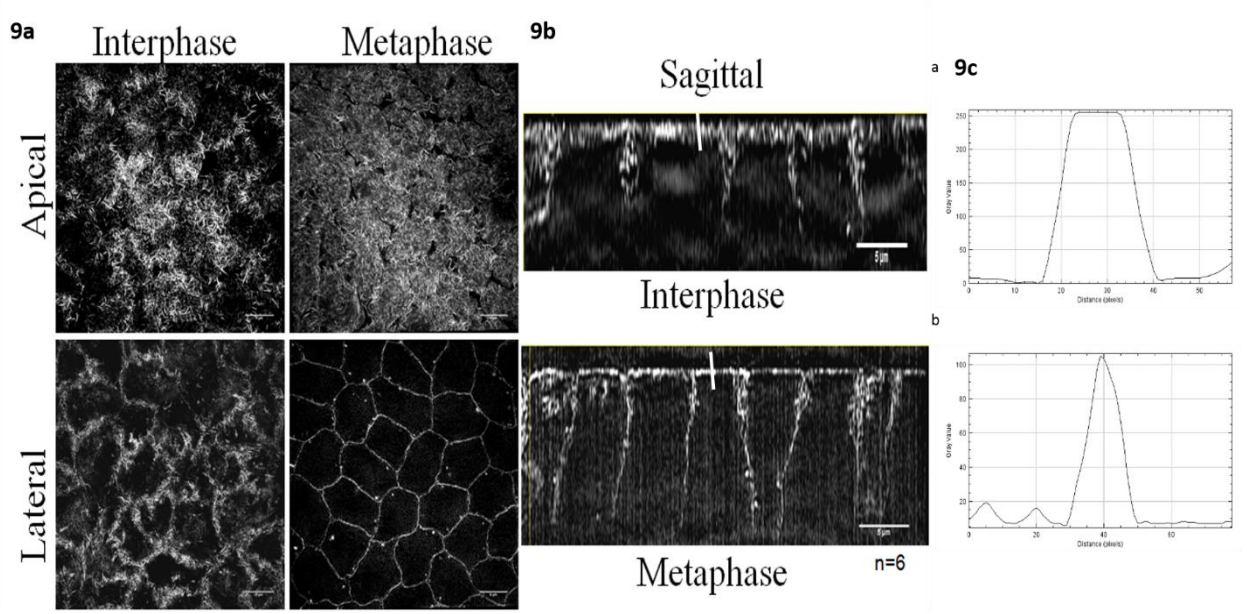


Figure9: a: Fixed syncytial embryos stained for Phalloidin imaged with SIM. X-Y surface views depict apical caps in interphase and polygons in metaphase. b: X-Z sections represent the loss of apical membrane and ingression of furrows in metaphase. c: Interphase (1) and metaphase (2) line scan plots across the apical membrane (white line). Here, analyzed (15,3) (Number of caps/furrows measured, number of embryos assessed)

3.2 Change in Phospholipid PIP_3 causes an alteration in morphogenesis in the syncytial embryo

Specification of membrane domains like apical vs basolateral is regulated by the asymmetric distribution of phosphoinositides. PTEN phosphatase associates with a variety of polarity proteins to maintain apical PI(4,5)P2 localization whereas PI3Kinase stabilizes the basolateral domain with PI(3,4,5)P3 (Shewan et al., 2011). Dynamics of

the underlying actin cytoskeleton are governed by the membrane phospholipids either by interacting with actin-binding proteins, scaffolding proteins in addition to Rho family of GTPases. As a result, PIP_3 localization and function are studied in the context of cell migration, growth and cell survival (Gao et al., 2000; Leevers et al., 1996; Zhou, Bokoch and Traynor-Kaplan, 1998). In *Drosophila*, epithelial cells in follicular epithelium, wing discs and syncytial embryos were disrupted for either of two phospholipids, PIP_2 or PIP_3. Subsequent effects of polarity, actin cytoskeleton, and myosin contractility were hence observed (Claret et al., 2014; Levayer et al., 2015; von Stein, 2005). To ensure proper tissue morphogenesis and shape change, it was hypothesized that an optimal balance of PIP_2/PIP_3 is vital (Reversi et al., 2014). We studied the effects of modulating PIP_3 in developing syncytial embryos.

3.2.1. Increase in dosage of PIP_3 label, tGPH results in defects in polygonal architecture and shorter furrows

In developing embryos, a single chromosomal copy of tGPH with WT led to no apparent effect on lethality or membrane localization of the tag (Fig6). We studied the effect of the increase of copy number i.e genetic dosage of tGPH at cycle 12 from interphase to metaphase. Live embryos were imaged to obtain 4D resolution. In transgenic flies homozygous for tGPH, no drastic alterations occurred during expansion and stabilization of the apical cap. The cap area reaches about 150μm^2. Also, the caps form cell-cell contacts thereby stabilizing the prior cap expansion. Tight polygonal architecture fails to arise even in metaphase like WT. Pseudo-cleavage furrows begin ingressing in interphase but remain short and incomplete throughout metaphase. In these embryos, early onset of metaphase and short nuclear division cycles leading to premature termination at about 7mins and continuation of next round of division (Fig10a). Biphasic cap expansion and stabilization aren't significantly affected but untimely abortion of linear furrow ingression maintains the length to as short as about 5µm (Fig10b). About 28-30% of these embryos fail to hatch within 48hours post egg-laying suggesting effects on normal growth and development (n=300 embryos). These data suggest that the PIP_3 may be getting sequestered when the genomic copies of

tGPH are increased and therefore may be unavailable to perform its function at the

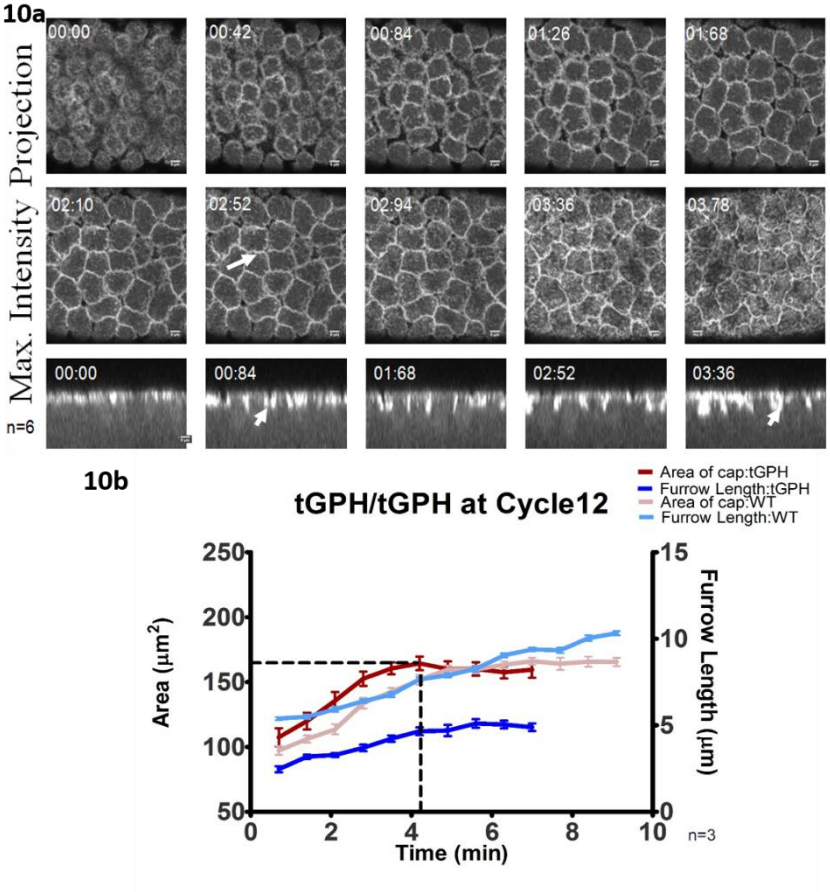


Figure10: a: Time-lapse movie of syncytial embryo with two copies of tGPH tag. Arrow heads indicating cap expansion, extension in X-Y and short furrows in metaphase in X-Z. b: Plot of cap area and furrow length with time from interphase to metaphase. Linearly ingressing short furrows and short division cycle was analyzed for (15,3) (Number of caps/furrows measured, number of embryos assessed)

plasma membrane, thus causing defects in furrow extension and lethality of embryos.

3.2.2. Actin architecture is disrupted on increasing genetic copies of tGPH

In *Drosophila*, oocytes, epithelia, wing disc and syncytial embryos showed disrupted actin cytoskeleton on abrogation of PIP_2 or PIP_3 during shape changes (Claret et al., 2014; Levayer et al., 2015; Reversi et al., 2014; von Stein, 2005). We stained fixed syncytial embryos with Phalloidin for visualizing F-actin cytoskeleton. On imaging in 3D, WT embryos formed polygonal arrays during metaphase accompanied by the ingression of pseudo-cleavage furrows. Increasing dosage of tGPH led to deformation of actin architecture into intercap regions. Consequently, actin dynamics leading to furrow assembly and ingression are affected. Short actin furrows are maintained even in metaphase at cycle12. Patchy accumulation of actin is observed at the point of furrow initiation and intercap regions are visible in sagittal section (Fig11). As about 70%

embryos develop defects in actin architecture, a link between PIP_3 and cortical actin in syncytial embryos can be established. This is consistent with the observation that tGPH alters the functionality of PIP_3 just by binding via PH domain. Sequestering, blocking or functional inactivation by tGPH binding can thereby lead to downstream effects on furrow dynamics.

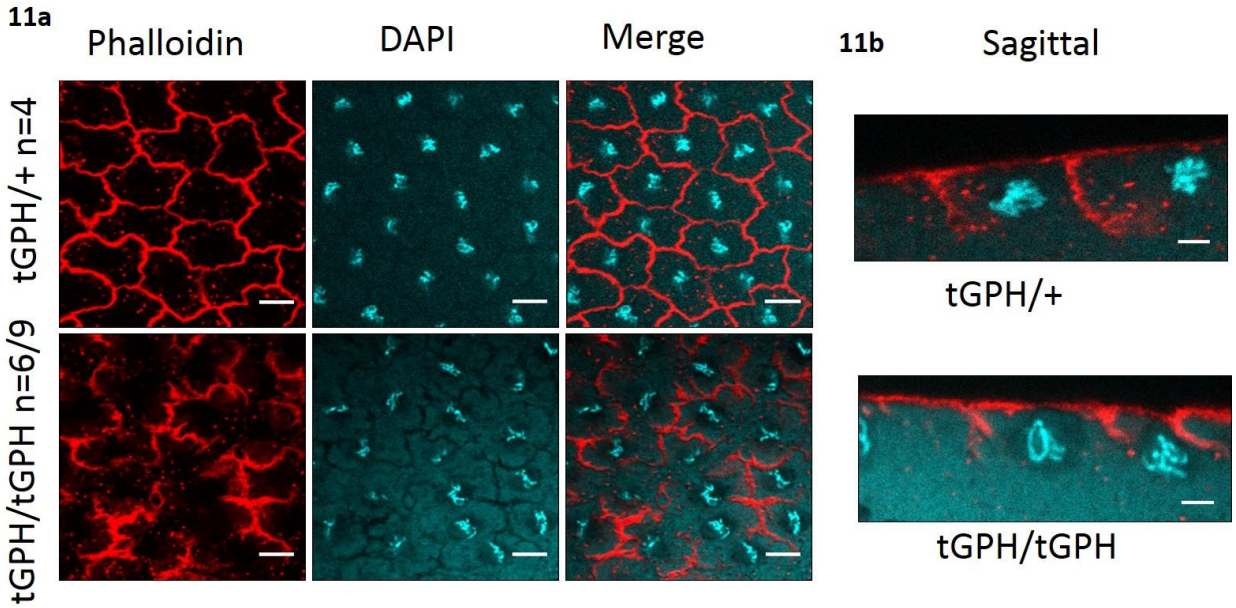


Figure11: a: Fixed syncytial embryos stained for Phalloidin and DAPI to visualize actin (red) and DNA (cyan). Defective actin seen in tGPH/tGPH marked with arrow head in X-Y and intercap actin accumulation in X-Z (b).

3.2.3. PI3Kinase and PTEN phosphatase are crucial for synchronous pseudo-epithelial sheet formation.

Signaling pathways activated by PI3Kinase and PTEN play a role in polarity formation and spindle orientation in different cell types including epithelial cells (Noatynska et al., 2012). In *Drosophila,* actin-based mechanisms via pseudo-cleavage furrows prevent spindle crosstalk along with orchestrating the synchronous mitotic waves from the poles towards the center (Foe, Odell & Edgar 1993; Schejter & Wieschaus, 1993, Koke et al., 2014). Class I PI3-Kinases were discovered to be deployed at the PM in response to interaction with specific receptors or adaptor molecules. They are known to regulate the conversion of PI(4,5)P2, by phosphate addition, to PI(3,4,5)P3 (Auger et al., 1989).

Antagonistic to PI3Kinase, PTEN phosphatase dephosphorylates local PI(3,4,5)P3 to PI(4,5)P2. In epithelial cells in *Drosophila*, this 3-OH phosphatase is known to strongly associate with polarity protein Bazooka, which in combination correlated with $PI(4,5)P_2$ presence. They hypothesized Bazooka driven employment of PTEN to police PIP_3 localization by conversion to PIP_2 (von Stein, 2005). We studied syncytial embryos defective for PI3kinase and PTEN fluorescently labeled for actin and DNA. De-synchronous nuclear division cycles are seen in both PI3K-KO and PTEN-KD embryos. Uniform apical caps are observed in interphase whereas tight polygons arise in metaphase. As opposed to this, lower or higher levels of PIP_3 shifts the equilibrium to slow, para synchronous divisions (Fig12b). The regularity of the underlying actin cytoskeleton is thereby affected leading to the presence of caps, tight polygons and intermediate stages can be visualized in the same embryo (Fig 12a). The viability of these embryos is severely compromised as about 93-97% (n=300 embryos) of PI3K-KD and about 95-100% (n=300 embryos) of PTEN-KO embryos fail to hatch after 48 hours post egg-laying .

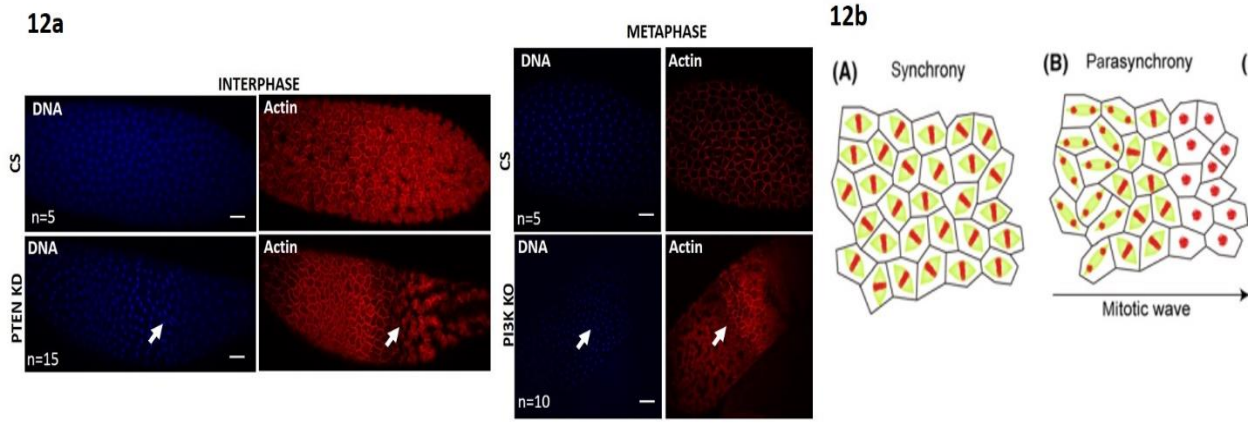


Figure12: a: Disruption of PI3kinase and PTEN phosphatase in syncytial embryos stained with Phalloidin (red) and DAPI (blue) to visualize actin and DNA. Arrow heads indicating desynchronous divisions and globally defective actin cytoskeleton in PI3K-KO and PTEN-KD. b: Categories of synchronous mitotic timings, maximum at synchrony and intermediate with mitotic waves in parasynchrony. Adapted from Ogura and Sasakura, 2017

3.3 PI3K and PTEN mutants show decrease in pseudo-cleavage furrows in syncytial *Drosophila* embryos.

Specification and maintenance of membrane domain identity is vital in polarized cells like epithelial and syncytial systems. Asymmetric phosphoinositide distribution is also found to be essential for maintaining polarity complexes. Such a polarity determining role of PI3kinase and its counter PTEN phosphatase has been studied in *Drosophila*epithelial cells and neuroblasts. Differences in the lipid composition due to shifting PM balance of PIP_2 and PIP_3 contributed to mislocalized domain formation and disturbed polarity (Pinal et al., 2006; Skwarek and Boulianne, 2009; von Stein, 2005; Wu et al., 2007). During cellularization in *Drosophila* embryogenesis, an increase in PIP_2 levels or decrease in PIP_3 levels induces early actomyosin contractions and thereby trapped nuclei in these rings. The ratio of PIP_2:PIP_3 was hypothesized to regulate PM expansion and downstream actomyosin contraction for proper cell shape and size (Reversi et al., 2014). In order to study effects of regulating PIP_2:PIP_3 balance, we studied syncytial embryos down-regulated for PI3Kinase and PTEN via RNAi or

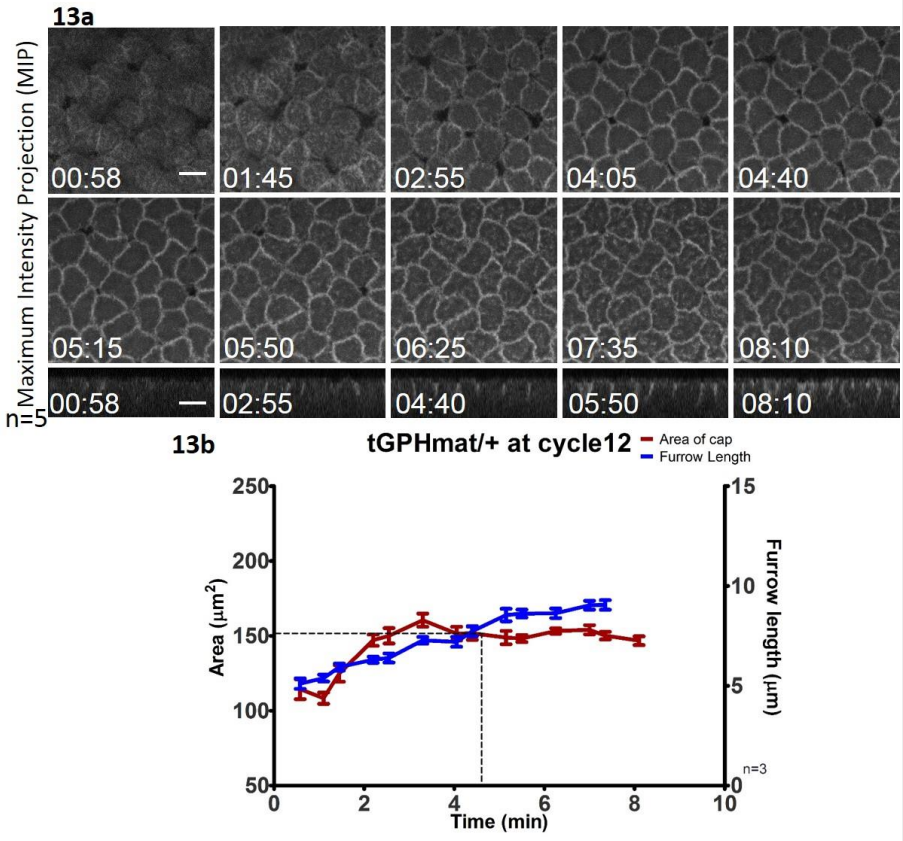


Figure13: a: Nuclear cycle 12 of syncytial embryo labelled with tGPH driven by maternal gal4 at 29 degree. Time-lapse X-Y sections represent one interphase to metaphase journey and X-Z sections represent furrow extension and retraction phases. *b: Plot of area of cap expansion along time on left axis and length of furrow ingression on right axis. Here, analyzed (15,3) (Number of caps/furrows measured, number of embryos assessed)*

knocked out via CRISPR strategy.

3.3.1. Knockdown of PI3Kinase leads to defective apical cap expansion accompanied with short furrows

In *Drosophila*, cell growth and survival are regulated by three distinct PI3-Kinases, PI3K_92E, PI3K_59F and PI3K_68D (Leevers et al., 1996). To study the effect of lowered PIP_3 in the syncytium, we used an RNA interference-based approach. A maternal gal4 system was used to drive the *pi3kinase* RNAi (PI3K_68D) in embryos expressing tGPH to visualize the membrane dynamics. On imaging live embryos in 4D, we observed irregularly formed caps linearly expanding till mitosis. Step-wise increase in cap area is seen through interphase in these embryos, to reach a maximum cap area around end-metaphase (Fig14a). Forgoing cap stabilization phase drastically affects furrow ingression with a maximum at 6µm (Fig14b) as compared to control embryos (Fig13a). Defective mitotic cycles due to the absence of ingressed furrows led to

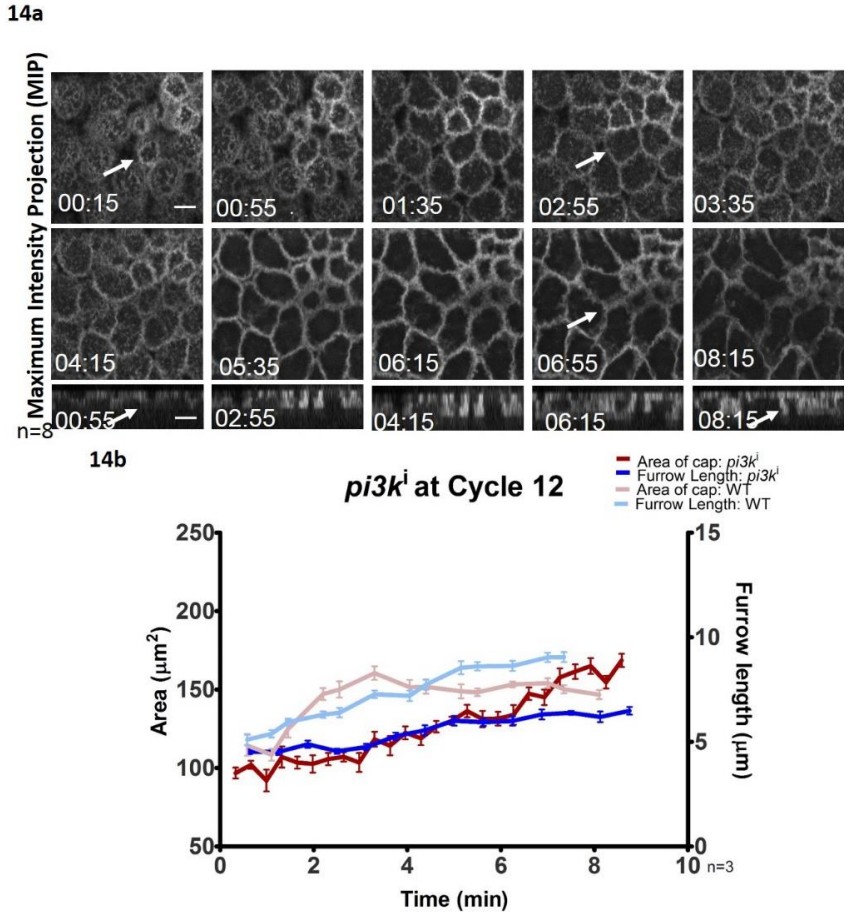


Figure14: a: Time-lapse movie for syncytial nuclear cycle 12 in embryos defective for PI3Kinase marked with tGPH. Defective PM dynamics is governed by drastic nuclear fall-outs and short furrows. Arrows indicate cap expansion and failure of subsequent cap stabilization leading to short furrows in metaphase. b: Plot of cap area and furrow length from interphase to metaphase, Short division cycles are seen here accompanied by failure of cap stabilization and incomplete furrows. Here, analyzed (15,3). (Number of caps/furrows measured, number of embryos assessed)

nuclear fallouts and collapse of the adjacent membrane. With progressive cycles, these disorganized syncytial cells cause massive fallouts leading to a global membrane and cytoskeletal catastrophe. PI3Kinase regulates the cap stabilization and ensuring optimal furrow length.

3.3.2. Decreased furrow formation and loss of cap stabilization is observed on disrupting PTEN phosphatase

Functions of domain maintenance are carried out via Annexin-II recruitment and in turn Cdc42 activation. This triggers an association with proteins of the apical-domain promoting proteins like Par family proteins. Cell motility and lumen formation are driven by these cascades (Martin-Belmonte et al., 2007). Live embryos expressing maternal gal4 driven tGPH were knocked down for PTEN to study the effect of high PIP_3 at cycle 12 of syncytium. At interphase, apical cap expands and progressively achieve cell contacts. In PTEN-KD, cap stabilization is disrupted and caps expand till delayed

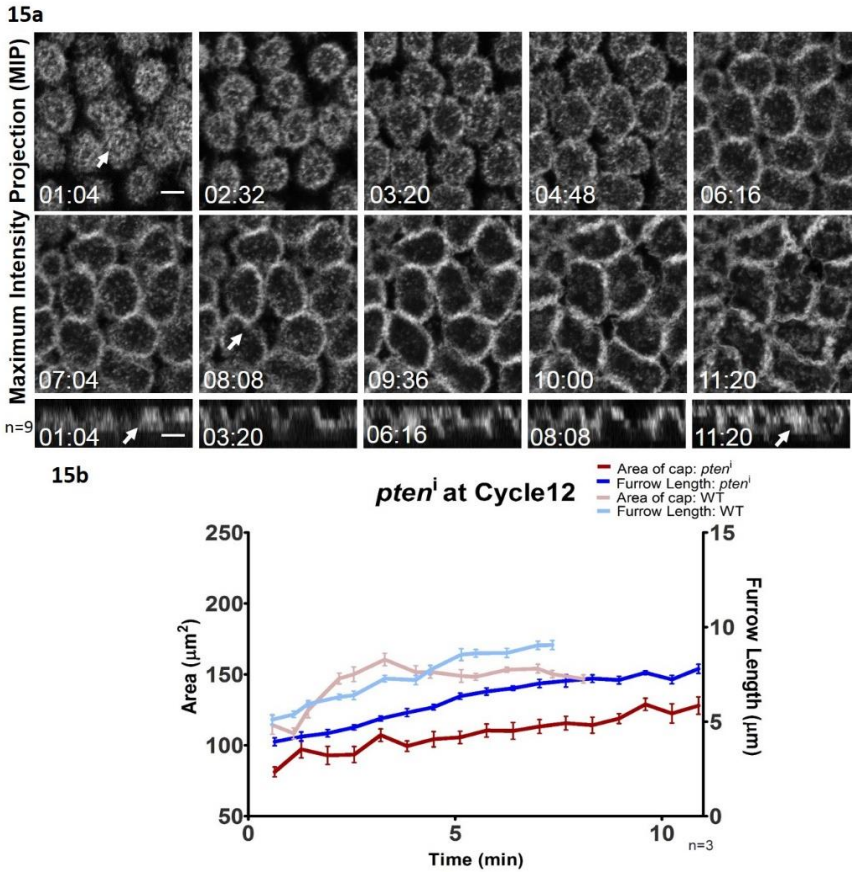


Figure15: a: Time-lapse movie for syncytial nuclear cycle 12 in embryos defective for pten^i marked with tGPH. Defective PM dynamics is governed by drastic nuclear fall-outs and short furrows. Arrows indicate cap expansion and failure of subsequent cap stabilization leading to short furrows in metaphase. b: Plot of cap area and furrow length from interphase to metaphase, Failure of cap stabilization and incomplete furrows are observed. Here, analyzed (15,3). (Number of caps/furrows measured, number of embryos assessed)

mitosis is achieved as compared to control embryos (Fig15). Furrow lengths are decreased for both PTEN-KD and PI3K-KD as compared to control embryos (Fig13a).
In summary, an imbalance of phospholipid led to de-synchronization of nuclear division cycles in addition to failure of cap stabilization and presence of short lateral furrows. Global defects in actin architecture were also observed, and hence analyzing actin remodeling proteins will be key to understanding the defects in cap expansion and furrow extension arising due to changes in membrane composition.

3.4. Actin remodeling proteins like Arp2/3 complex governs apical cap dynamics whereas Diaphanous is essential for extension of pseudo-cleavage furrows in early *Drosophila* embryogenesis

Two diverse kinds of actin polymerization mechanisms have been consistently demonstrated by Arp2/3 and Diaphanous. Initiation and polymerization of actin at 70° angle is achieved by two protein Arp2 and Arp3 (Amann and Pollard, 2001; Mullins et al., 1998). As opposed to this, Diaphanous bundles actin into parallel or anti-parallel unbranched filaments (Zigmond, 2004). During syncytium in *Drosophila*, Arp2/3 complex acts as a trigger for the expansion of apical caps. In Arpc1 mutants, small caps form which fails to expand and consequently incomplete furrow formation occurs (Stevenson et al., 2002). Dia defective embryos demonstrated heavy actin defects and were found to be indispensable at the furrow tip for metaphase ingression (Afshar et al., 2000). To study the role of these actin regulators, we imaged syncytial embryos over-expressing GFP domain in 4D. Knockdown of these regulators via RNAi demonstrated their function during nuclear cycle 12 from interphase to metaphase.

3.4.1. Arp2/3 is localized in earlier stages of cap expansion as compared to Diaphanous in syncytial division cycle

Arpc1-GFP, a subunit of the Arp2/3 complex is localized on the cap at the start of interphase. As the cycle progresses, it accumulates around the rim of the caps during expansion and disappearing completely during metaphase (Fig16a). A peak of Arp2/3 is marked by late cap expansion stage till the caps completely stabilize to form cell

contacts (Fig16c). Arpc1 distribution decreases during metaphase when the furrows are elongated. Intercap regions depict Diaphanous localization during cap expansion and this peaks around metaphase (Fig16b). No apical signal is detected from Diaphanous, like that seen in Arp2/3. We measured membrane to cytosolic ratio of both the lateral section for Arpc1 and furrow tip for Diaphanous. Six different time points in cycle 12 were analyzed: prophase (~1.5min), prometaphase (~3min), cap stabilization (4-5min), furrow extension (6-7min), maximum furrow ingression (~8min) and spindle formation

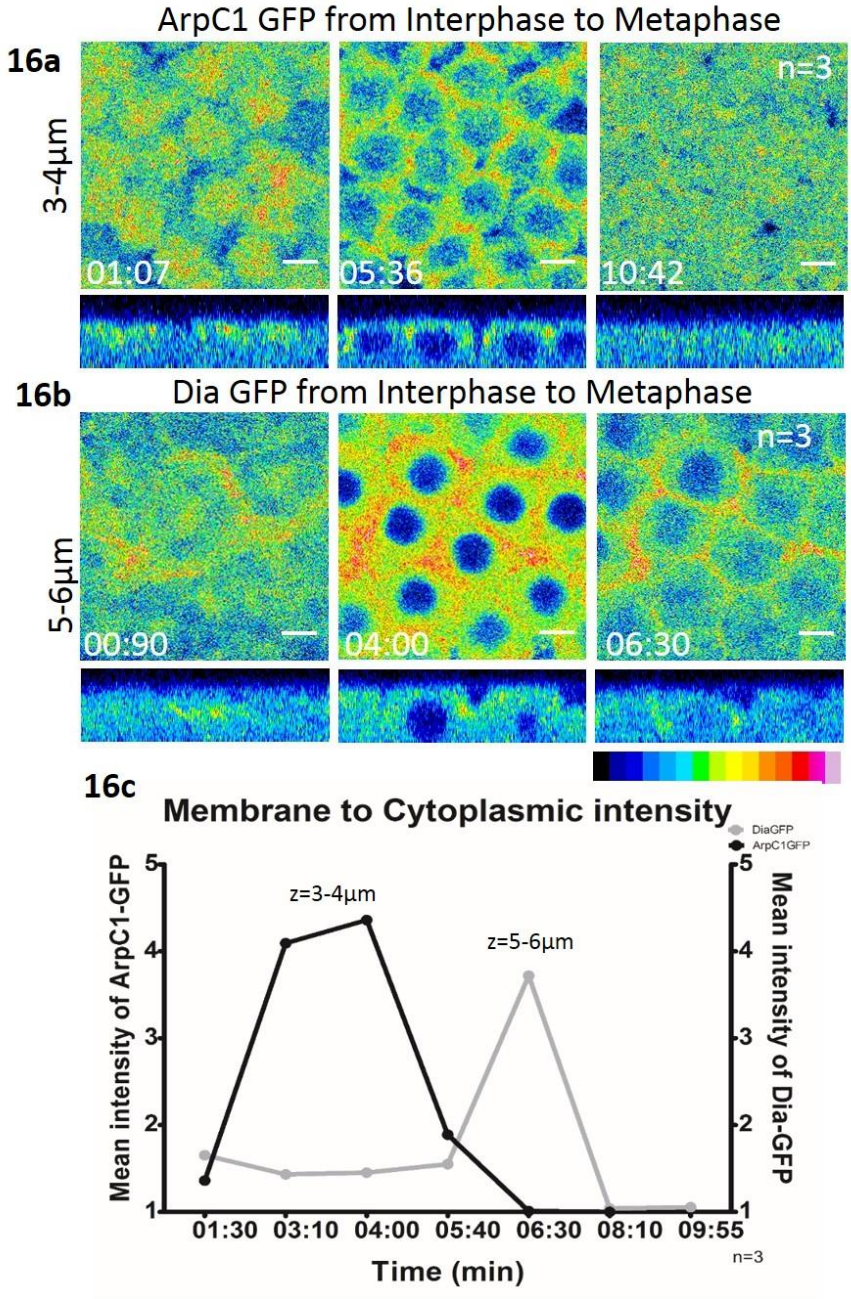


Figure16: a: Time lapse movie of Arpc1-GFP at z section 3-4µm from apical surface. X-Y sections indicate interphase cap intensity followed by cap rim intensity at mid-interphase. No metaphase membrane signal is observed. b: Time lapse movie of Dia-GFP where X-Y sections indicate intercap Dia signal in interphase followed by a membrane signal in metaphase. c: Plot represents peak of intensities of Arpc1 and Diaphanous at different z sections, across cycle 12. Cap expansion and stabilization Arpc1 peak and furrow extension Dia peak is followed by mitosis. Here, analyzed (15,3). (Number of caps/furrows measured, number of embryos assessed)

(~10min). Phase-shifted to Arp2/3 peak, furrow extension phase is governed by Diaphanous peak (Fig16c).

3.4.2. Disruption of Arp2/3 leads to small caps with the absence of metaphase furrows

The Arp2/3 enriched edges drive actin polymerization and thereby, caps touch and form cell-cell contacts. To study the extent of cap expansion under Arp2/3 control, tGPH expression was imaged in knockdown embryos for ArpC1. As interphase begins, small caps form which fails to stabilize although cell contacts are established. Pseudo-cleavage furrows extension is completely lost in these embryos (Fig17a). Cap area remains staggered at around 70-80µm unlike stabilized WT caps augmented by very short 5µm furrows (Fig17b). High embryonic lethality and loss of Arp2/3 immunostaining is accompanied by defective actin and nuclear cycle dynamics on down-regulating Arpc1 (previously done in lab by Prachi and Vishnu). These observations together support the previous reports that establish the role of Arpc1 in expansion and stabilization of apical actin caps (Stevenson et al., 2002).

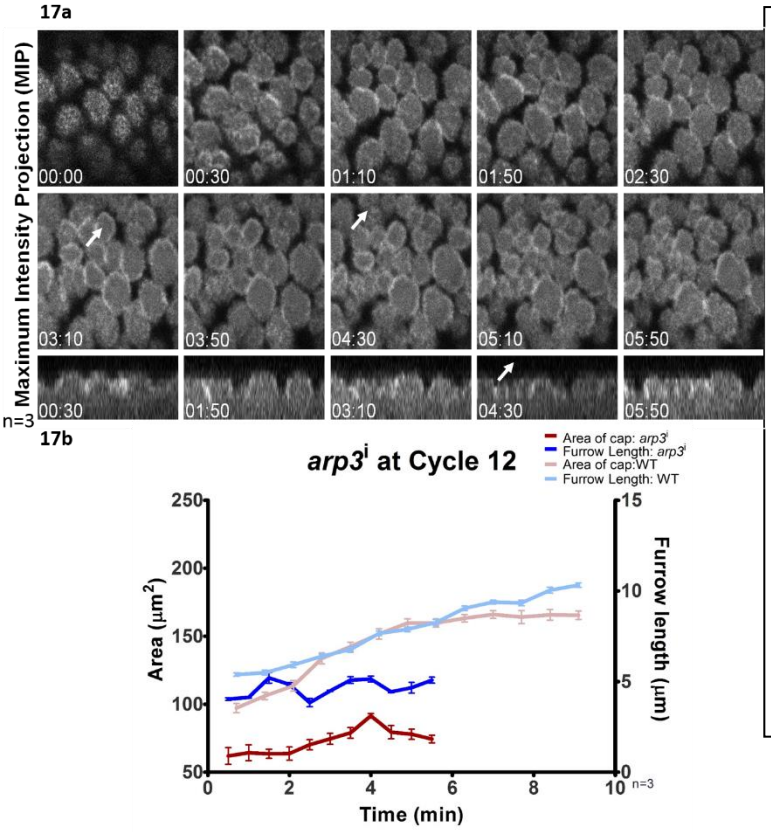


Figure17: a: Time-lapse imaging of arp3 RNAi embryos in which membrane is visualized with tGPH. From interphase to metaphase, small caps are seen which fail to expand and stabilize. Arrows indicate cap initiation phase followed by cell contact formation but progressively lost by metaphase. X-Z sections depict very short furrows ingressing at start of interphase but persist without extension till mitosis. b: The graph represents faulty cap expansion as cap area ceases to increase from interphase to metaphase. Furrow extension is staggered without any linear increase till mitosis. Here, analyzed (15,3) (Number of caps/furrows measured, number of embryos assessed).

3.4.3. Knockdown of Diaphanous is accompanied by loss of shift from cap expansion to furrow ingression

In syncytial *Drosophila* embryos, Adenomatous Polyposis Coli2 (APC2) joins Diaphanous for initiation and elongation of the pseudo-cleavage furrow (Webb et al., 2009). Gal4 driven expression of Diaphanous RNAi at 29 degrees would enable us to study its functional and temporal significance. High embryonic lethality and loss of Dia immunostaining is accompanied by defective actin and nuclear cycle dynamics on down-regulating Dia (previously done in lab by Prachi and Vishnu). Live syncytial embryos, expressing tGPH to mark the membrane, we imaged from interphase to metaphase at nuclear cycle 12. In these embryos, accelerated cap expansion phase is followed by formation of stable cell contacts. These caps further expand throughout interphase but no furrow ingression occurs. Loss of Diaphanous fails to constrain expansion thereby leads to a linear increase in cap area. Switch to furrow extension phase is absent in these embryos (Fig18a). The furrow remains short at about 5µm with over-expanded caps of about 230µm (Fig18b). The complete absence of Diaphanous at the furrow tip in metaphase is observed. Altogether, this data corroborates with earlier reports implicating the role of Diaphanous in cap stabilization and furrow extension (Afshar et al., 2000).

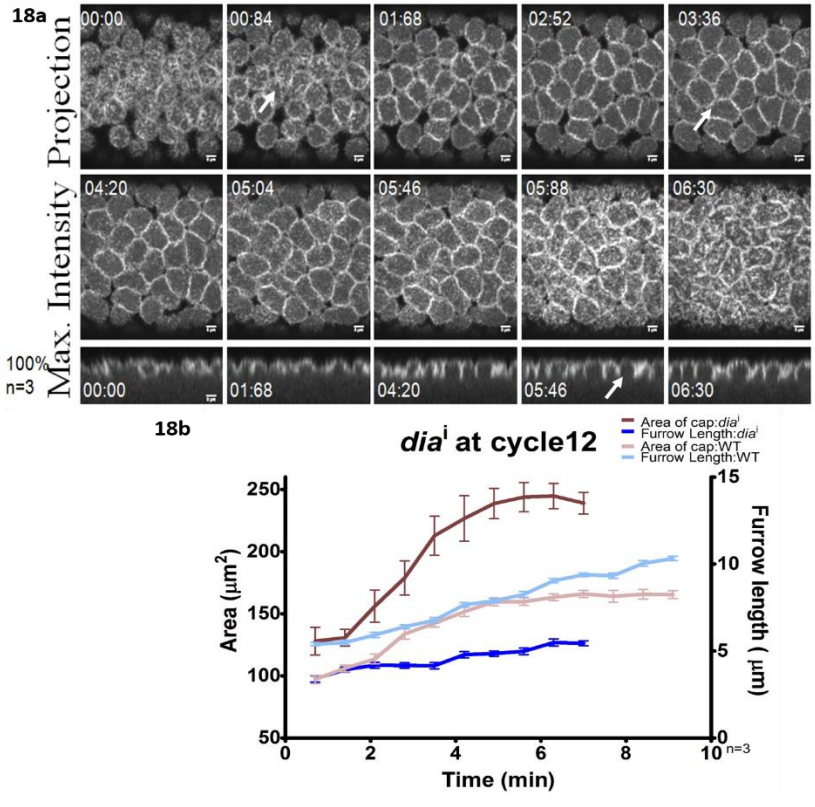


Figure18: a: Time-lapse imaging of DiaRNAi embryos expressing membrane tag, tGPH. Arrows indicate rapid cap expansion in X-Y but incomplete furrow extension in X-Z. Right: b: Plot of cap expansion and furrow length over time representing linear cap expansion and constant furrow ingression. Here, analyzed (15,3) (Number of caps/furrows measured, number of embryos assessed).

3.4.4. Arp2/3 localization remains unaffected in phospholipid deficient syncytial embryos

On altering the ratio of PIP_2:PIP_3, we observed failure in cap stabilization coupled with short pseudo-cleavage furrows. Defective PM architecture has instant repercussions on the underlying actin cytoskeleton. Actin regulators like Arp2/3 are known to regulate actin polymerization guiding cap expansion during syncytial interphase (Stevenson et al., 2002). In addition, WASP family of proteins binds and switches "ON" Arp2/3 dependent polymerization (Papayannopoulos et al., 2005). PI(4,5)P2 binding to N-WASP domain affects this switch (Papayannopoulos et al., 2005; Ridley, 2001; Saarikangas et al., 2010) To study effect of PIP_2:PIP_3 imbalance on actin architecture and Arp2/3 localization and function, we stained syncytial embryos with Phalloidin (F-actin) and Arp2/3 complex. Underlying actin mirrors disruptive cap stabilization and short metaphase furrow with ingression defects or apical microvilli. Early interphase

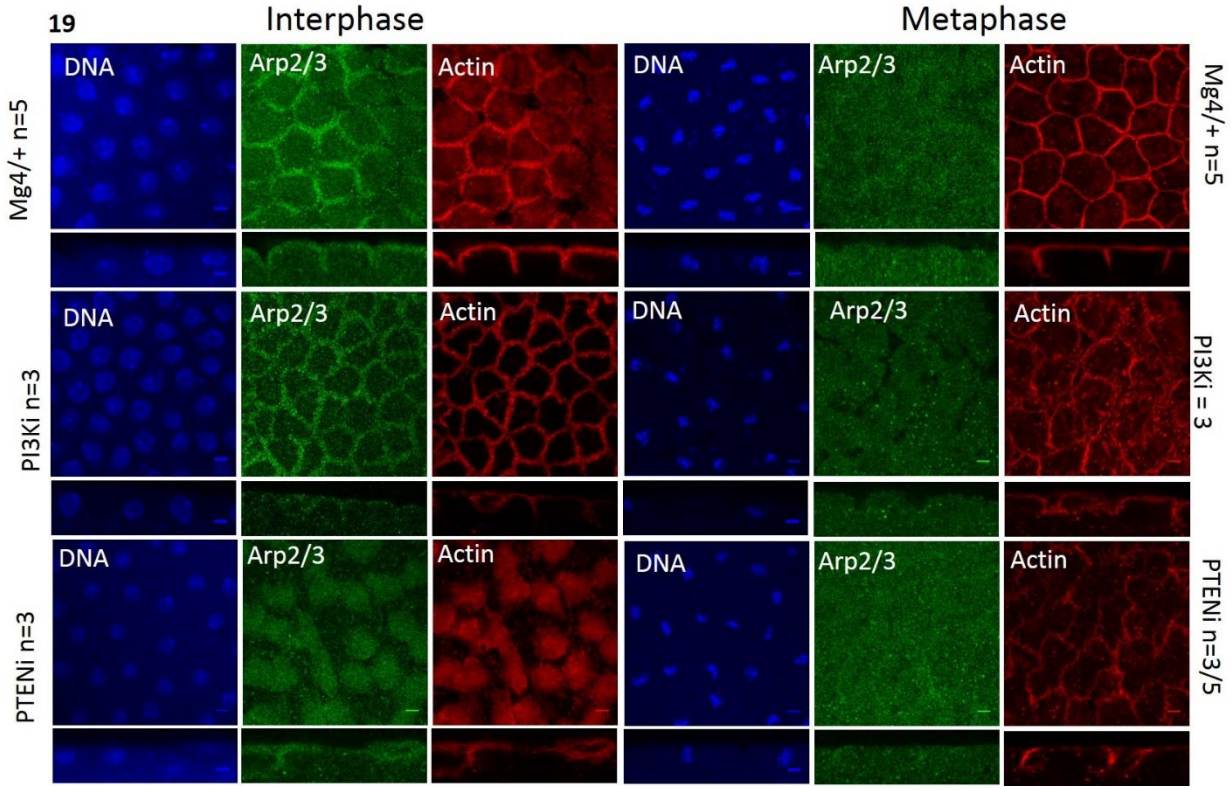


Figure19:Antibody staining of syncytial embryos for Phalloidin to stain F-actin and Arp2/3 at both interphase and metaphase. Remnant Arp2/3 is resident on membrane in PI3K-KO. Defective PTEN-KO embryos exhibit disorganized caps with Arp2/3 localization. Arp2/3 localization around cap rim is maintained in PI3K-KO embryos. All images at same settings.

depicts no significant differences in Arp2/3 localization around cap edges (Fig19). This suggests, recruitment of Arp2/3 temporally precedes the role of PIP_2:PIP_3 balance during syncytial cycles. Also, an effect on the PM is observed in embryos defective for Arp3 complex (discussed in 3.4.2). Further analysis of PIP_2:PIP_3 balance in these embryos would reveal the feedback mechanism at play.

3.4.5. PIP_2:PIP_3 imbalance causes mislocalization of Diaphanous on pseudo-cleavage furrow and its lowering or absence in interphase

Consistently short furrows in metaphase implicated role of phospholipid balance in the extension of pseudo-cleavage furrows. Diaphanous is known to localize at the tip of metaphase furrows during syncytium (Afshar et al., 2000). Rac, a member of Rho family of GTPases, is known to stimulate various formins to polymerize actin bundling. PIP_3 binding at PH domain triggers conversion to RacGTP and thereby, activate Diaphanous (Saarikangas et al., 2010; Shewan et al., 2011). Also, Rac stimulates PI(4,5)P2 to regulate various actin-associated proteins and assist in actin polymerization (Watanabe et al., 1997). We studied the final effector, Diaphanous in embryos defective for PI balance. Lowering or absence of Diaphanous in interphase is observed which is accompanied by a similar effect on the furrow. During metaphase, Diaphanous localizes on the membrane into tight polygons and at tips of extending pseudo-cleavage furrows. On balance perturbation, we observe lowered diaphanous on the polygonal membrane and its complete disappearance from the furrow tip (Fig20). This suggests a temporal delay in Diaphanous recruitment on the PM during metaphase. A balance is an essential factor for proper localization of Diaphanous at the tip of pseudo-cleavage furrows. Further studies on this balance in syncytial embryos downregulated for Diaphanous would provide further insights.

On altering the ratio of PIP_2:PIP_3, we observed failure in cap stabilization and short pseudo-cleavage furrows. As shown in the above sections defective PM architecture could occur due to defects in the underlying actin cytoskeleton. WASP family of proteins binds and switches “ON” Arp2/3 dependent polymerization (Papayannopoulos et al., 2005). PI(4,5)P2 binding to N-WASP domain affects this switch (Papayannopoulos et al., 2005; Ridley, 2001; Saarikangas et al., 2010). To study effect of PIP_2:PIP_3 imbalance on actin architecture and Arp2/3 localization and function, we stained

syncytial embryos with Phalloidin (F-actin) and Arp2/3 complex. Underlying actin mirrors disruptive cap stabilization and short metaphase furrow with ingression defects or apical microvilli. Interphase shows recruitment of Arp2/3 localization around cap edges. This suggests, recruitment of Arp2/3 temporally precedes the role of PIP_2:PIP_3 balance during syncytial cycles. Also, an effect on the PM is observed in embryos defective for Arp3 complex (discussed in 3.4.2). Further analysis of PIP_2:PIP_3 balance in these embryos would reveal the feedback mechanism at play.

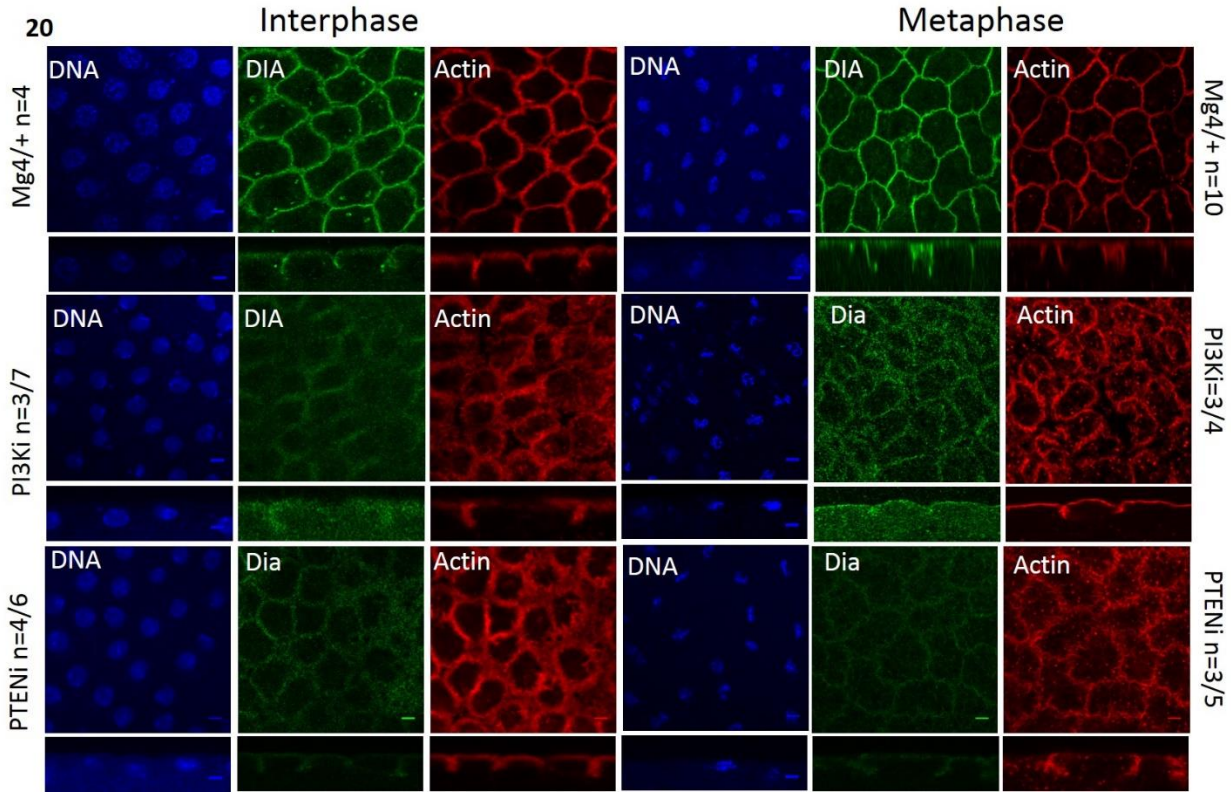


Figure20: Fixed syncytial embryos imaged for Antibody staining for Diaphanous and F-actin. Mislocalization of Diaphanous from the metaphase furrow tip. Embryos defective for PTEN show absence of Diaphanous in interphase as small caps arise in prophase. Disrupted actin architecture is globally observed. All images at same settings.

4. Discussion

4.1. Model of apical cap expansion and pseudo-cleavage furrow dynamics in the early syncytial embryo

Syncytial division cycles occurring in the early *Drosophila* embryo comprise of a series of nuclear divisions devoid of cytokinesis. The outcome of this process establishes polarized pseudo-epithelial cells in regular geometric arrays. Consequent cycles depict a stepwise increase in the nuclear: cytoplasmic ratio, as dividing nuclei flood in a constant syncytial cortex. Maintenance of cell size, shape and ensuring maximal surface area in minimal surface energy requires dynamic rearrangements of the PM and the underlying cytoskeleton. Membrane domain identities and proper nuclear division have to be guaranteed at every division. My work shows that at interphase, apical microvilli dominate in small cortical caps, which successively expand. Further, as cell contacts are established, these caps stabilize without further expansion. As a consequence, membrane from the microvilli is supplied for the ingressing pseudo-cleavage furrow in metaphase. A linear increase in furrow length is accompanied by tight polygonal architecture. At maximum furrow ingression, mitosis is triggered and the nuclei divide. Subsequent cycles witness higher threshold of furrow length to promote mitosis.

Many studies in *Drosophila* have implicated this syncytial architecture which mimics epithelial cell characteristics (Mazumdar and Mazumdar, 2002). Apical PM flattening in metaphase provides for syncytial and cellularization furrows (Figard and Sokac, 2014; Mavrakis et al., 2009; Turner and Mahowald, 1976). Five phases can be traced from interphase to metaphase in one division cycles: (I) Initial actin polymerization, (II) Cap expansion, (III) Cap stabilization, (IV) Furrow extension and polygon formation and (V) furrow retraction post mitosis. Dynamics of cap expansion and furrow extension involve the underlying actin cytoskeleton, various actin-related proteins, Myo-II activity, endocytosis, adhesion, BAR domain and polarity proteins (Afshar et al., 2000; Cao et al., 2008; Claret et al., 2014; Fabrowski et al., 2013; Holly et

al., 2015; Lee et al.; Sherlekar and Rikhy, 2012; von Stein, 2005; Stevenson et al., 2002a).

4.1.1. Remodeling of apical caps into cleavage furrows is regulated by PIP_3 and cortical F-actin.

The five phases marked above have distinct morphology and distribution of actin. Actin accumulates under the caps in interphase to form microvilli-like structures.Further polymerization expands these caps synchronously along the embryo surface. A linear increase in cap area is noted in this phase. As the neighboring caps begin to touch, a variety of adhesion and junction proteins flood to stabilize them. Flattening of the linear cap area is observed till mitosis. The apical microvilli now flatten to supply additional membrane for extending pseudo-cleavage furrows. This apical loss of PM is likely to fuel into the linearly increasing metaphase furrows. This can however be proven by photoactivation of the apical membrane in order to progressively follow of the signal from apical to lateral directions. Proper spindle positioning is ensured when maximum furrows ingress to trigger mitosis. Progressive cycles mark the recruitment of more membrane PIP_3 with increasing N/C ratio (Fig7). Underlying cortical F-actin traces similar phases via polymerization in apical microvilli, branching in expanding caps, and finally depolymerizing into bundled furrows.

Shape change from spherical to polygonal with loss of apical microvilli can be hypothesized to be regulated by plasma membrane tension. Forces for membrane deformation could arise due to membrane-cytoskeleton adhesion energy or membrane tension within X-Y plane (Diz-Muñoz et al., 2013; Sheetz and Dai, 1996). Myosin increases during the phase of cap expansion and furrow length elongation and falls off when the furrow has reached its maximum length thus suggesting that there is an increase in tension during the cap expansion and furrow formation phases (Royou et al., 2004).

4.1.2. Cap stabilization and pseudo-cleavage furrow extension are guided by PM phospholipid balance.

Sequestration of PIP_3 on the membrane due to over-expression of tGPH tag disturbed normal progression of syncytial division. Linear expansion in cap area fails to stabilize at metaphase. Short furrows ingress linearly without reaching the maximum value in metaphase. Binding/blocking or sequestration via tGPH can affect the PM balance of PIP_2:PIP_3, thereby disrupting the underlying actin cytoskeleton. On lowering or augmenting membrane PIP_3, global de-synchronization of mitotic division is seen. Loss of extended furrows allows spindle cross-talk leading to fused nuclei and nuclear fall-outs. These results further emphasize the importance of pseudo-cleavage furrows as described earlier (Foe et al., 2000; Schejter and Wieschaus, 1993).

Perturbing balance of membrane PIP_2:PIP_3 was achieved in syncytium via knockdown of PI3Kinase and its antagonist, PTEN phosphatase. Defective cap stabilization and incomplete furrow extension persist throughout metaphase in short (PI3K-KD) and long (PTEN-KD) mitotic division cycles. Thus an imbalance disrupts homeostatic and synchronous nuclear division waves, actin architecture and optimal furrow extension. Preceding recruitment of Arp2/3 remains unaffected but subsequent cap stabilization and furrow extension proteins, Diaphanous are lowered or absent in metaphase.

4.1.3. Switch from actin branching Arp2/3 during cap expansion to bundled furrow extension by Diaphanous occurs during syncytial division.

During syncytial division cycles, initial apical caps are localized with branched actin regulator Arp2/3. Expanding actin caps peak with most Arp2/3 accumulating at the cap rim. This guides actin polymerization till caps form contacts and are stabilized. A drop in Arp2/3 at this stage marks the peak of Diaphanous localization. Diaphanous localizes to intercap regions and stabilizes these caps and allows for membrane supply into the extending furrow. Localization at the furrow tip during metaphase is observed in syncytium. The absence of cap expansion on Arp3 decrease is visualized as small caps without any extending furrows. On the contrary, Diaphanous knockdown shows expanded caps persist till metaphase. These results are consistent with defects observed in cap expansion defects and furrow extension defects observed in Arpc1 and Dia mutant embryos observed earlier (Afshar et al., 2000; Stevenson et al., 2002).

Edges of interphase caps begin accumulating Arp2/3, thereby polymerizing actin for further cap expansion. On further reduction in intercap area, maintaining PIP_2:PIP_3 balance can be inferred to flatten apical microvilli into the extending furrow. Downstream activation of Rac1, in turn, activates formins. Stabilization of cap expansion is achieved presumably via BAR domain protein Syndapin and Diaphanous. Adhesion protein DE-Cadherin potentially maintains these cell contacts into tight polygons. Recruitment of Cdc42 and other RhoGTPases accompany Myo-II and Diaphanous at the pseudo-cleavage furrow. Synchronous mitotic division is now terminated with proper anaphase and telophase (Fig21). Absence of Dia in phospholipid mutant embryos suggests that the recruitment of Dia is regulated via phospholipid polarity whereas Arp2/3 remains unaffected suggesting its recruitment upstream of regulation by phospholipid balance. This study speculates an emerging role of phospholipid balance in localization and recruitment of downstream effectors. However, future investigation is required to elucidate mechanisms underlying these effects.

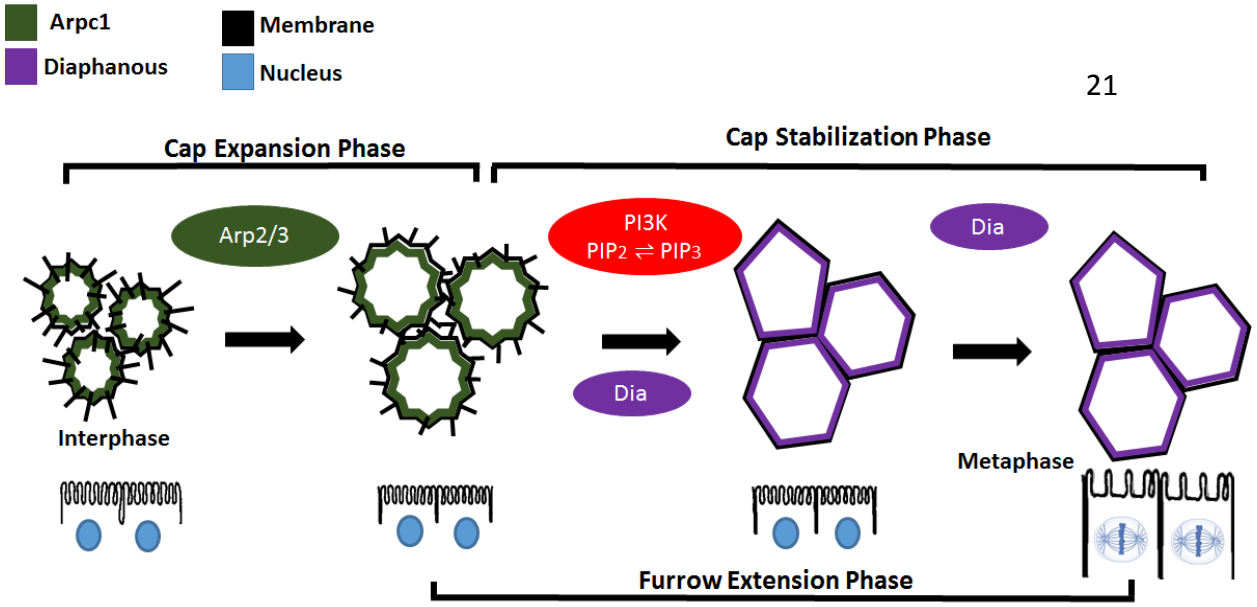


Figure21: Model of regulation of phospholipid and actin-binding proteins in orchestrating apical membrane remodeling and furrow extension during Drosophila syncytium. Different phases of cap expansion, stabilization and furrow ingression are regulated by branched or bundled actin regulators in addition to PM phospholipid balance.

4.2. Ongoing experiments and future perspectives

Phospholipid balance of the PM is essential for recruitment of downstream effectors. It will be interesting to rescue the levels of these proteins in imbalanced syncytial embryos. Expressing GFP tagged to both immediate effectors like Rac1 and WASP and terminal effectors like Arp2/3 and Dia using the UAS-gal4 system would lead to tissue specific expression. On crossing with phospholipid imbalanced flies, a rescue in cellular levels of these proteins along with absence of furrow defects would be observed if the balance is essential for recruitment and localization of proteins building the furrow. Ectopic addition of PIP_3 to PI3-Kinase knockdown embryos also should restore morphological and cytoskeletal defects. To gain deeper insights into furrow dynamics, regulating proteins like Myo-II, RhoGEF and Cdc42 need to be characterized by disruption of phospholipid balance. Further understanding of this imbalance in affecting cell polarity and membrane domain maintenance is necessary.

De-synchronization due to phospholipid imbalance needs to be further explored by studying Cdk1 and Chk1 during syncytium. Regulation of endocytosis pathways in syncytium will provide for the role of Rab5, Rab8, RalA, and SecA in membrane remodeling.In addition, addressing large-scale tissue-level forces, adhesion energy and membrane tension would help in understanding the functional utility of such early onset of membrane polarization in pseudo-epithelial cells.

5. References

1. Afshar, K., Stuart, B., and Wasserman, S. a (2000). Functional analysis of the Drosophila diaphanous FH protein in early embryonic development. Development *127*, 1887–1897.
2. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell, 4th edition.
3. Amann, K.J., and Pollard, T.D. (2001). The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. Nat. Cell Biol. *3*, 306–310.
4. Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., and Cantley, L.C. (1989). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell *57*, 167–175.
5. Bergstralh, D.T., and Johnston, D.S. (2012). Epithelial cell polarity : what cancer. 129–140.
6. Breitsprecher, D., Jaiswal, R., Bombardier, J.P., Gould, C.J., Gelles, J., and Goode, B.L. (2012). Rocket launcher mechanism of collaborative actin assembly defined by single-molecule imaging. Science (80-.).
7. Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., and Edgar, B.A. (2002). Drosophila ' s Insulin / PI3-Kinase Pathway Coordinates Cellular Metabolism with Nutritional Conditions. *2*, 239–249.
8. Brückner, B.R., Pietuch, A., Nehls, S., Rother, J., and Janshoff, A. (2015). Ezrin is a Major Regulator of Membrane Tension in Epithelial Cells. Nat. Publ. Gr.
9. Cao, J., Albertson, R., Riggs, B., and Sullivan, W. (2008). Nuf, a Rab11 effector, maintains cytokinetic furrow integrity by promoting local actin polymerization. J. Cell Biol. *182*, 31–313.
10. Cao, J., Crest, J., Fasulo, B., and Sullivan, W. (2010). Cortical actin dynamics facilitate early-stage centrosome separation. Curr. Biol. *20*, 770–776.
11. Castrillon, D.H., and Wasserman, S.A. (1994). Diaphanous is required for cytokinesis in Drosophila and shares domains of similarity with the products of the limb deformity gene. Development *120*, 3367–3377.
12. Chhabra, E.S., and Higgs, H.N. (2007). The many faces of actin: matching assembly factors with cellular structures. Nat. Cell Biol. *9*, 1110–1121.
13. Claret, S., Jouette, J., Benoit, B., Legent, K., and Guichet, A. (2014). PI(4,5)P2 produced by the PI4P5K SKTL controls apical size by tethering PAR-3 in drosophila epithelial cells. Curr. Biol. *24*, 1071–1079.
14. Crest, J., Concha-Moore, K., and Sullivan, W. (2012). RhoGEF and Positioning of Rappaport-like Furrows in the Early Drosophila Embryo. Curr. Biol. *22*, 2037–2041.
15. Diz-Muñoz, A., Fletcher, D.A., and Weiner, O.D. (2013). Use the force: membrane tension as an organizer of cell shape and motility. Trends Cell Biol. *23*, 47–53.
16. Fabrowski, P., Necakov, A.S., Mumbauer, S., Loeser, E., Reversi, A., Streichan, S., Briggs, J.A.G., and De Renzis, S. (2013). Tubular endocytosis drives remodelling of the apical surface during epithelial morphogenesis in Drosophila. Nat. Commun. *4*, 1–12.
17. Faix, J., Breitsprecher, D., Stradal, T.E.B., and Rottner, K. (2009). Filopodia: Complex models for simple rods. Int. J. Biochem. Cell Biol. *41*, 1656–1664.
18. Figard, L., and Sokac, A.M. (2014). Unfolding the plasma membrane to fuel cell shape change A membrane reservoir at the cell surface. 39–46.
19. Figard & Sokac, 2016 (2016). Membrane supply and demand regulates F-actin in a cell surface reservoir. Dev. Cell *165*, 267–278.
20. Foe, V., Christine, F., and Odell, G. (2000). Microtubules and mitotic cycle phase modulate spatiotemporal distributions of F-actin and myosin II in Drosophila syncytial blastoderm embryos. Development *127*, 1767–1787.
21. Fox, D.T., and Peifer, M. (2007). Abelson kinase (Abl) and RhoGEF2 regulate actin organization during cell constriction in Drosophila. Development *134*, 57–578.
22. Gao, X., Neufeld, T.P., and Pan, D. (2000). Drosophila PTEN Regulates Cell Growth and Proliferation through PI3K-Dependent and -Independent Pathways. Dev. Biol. *221*, 404–418.
23. Gassama-Diagne, A., Yu, W., ter Beest, M., Martin-Belmonte, F., Kierbel, A., Engel, J., and Mostov, K. (2006). Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. Nat. Cell Biol. *8*, 963–970.
24. Gibson, M.C., Patel, A.B., Nagpal, R., and Perrimon, N. (2006). The emergence of geometric order in proliferating metazoan epithelia. *442*, 2–5.
25. Glover, D.M. (1991). Mitosis in the Drosophila embryo-in and out of control. Trends Genet. *7*, 125–132.
26. Goehring, N.W., and Grill, S.W. (2013). Cell polarity: mechanochemical patterning. Trends Cell Biol. *23*, 72–80.
27. Grevengoed, E.E., Fox, D.T., Gates, J., and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. J. Cell Biol. *1213*, 21–9525.
28. Großhans, J., Wenzl, C., Herz, H.-M., Bartoszewski, S., Schnorrer, F., Vogt, N., Schwarz, H., and Müller, H.-A. (2005). RhoGEF2 and the formin Dia control the formation of the furrow canal by directed actin assembly during Drosophila cellularization. Development.
29. Guo, S., Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J.D., Sheetz, M.P., Meyer, T., and

Carolina, N. (2000). Raucher D , Stauffer T , Chen W , Shen K , Guo S , York JD et al .. Phosphatidylinositol 4 , 5- bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhe ... as a Second Messenger that Regulates Cytoskeleton – Plasma Me.
30. Holly, R.M., Mavor, L.M., Zuo, Z., Blankenship, J.T., Albertson, R., Riggs, B., Sullivan, W., Albertson, R., Cao, J., Hsieh, T. -s., et al. (2015). A rapid, membrane-dependent pathway directs furrow formation through RalA in the early Drosophila embryo. Development *142*, 2316–2328.
31. Houk, A.R., Jilkine, A., Mejean, C.O., Boltyanskiy, R., Dufresne, E.R., Angenent, S.B., Altschuler, S.J., Wu, L.F., and Weiner, O.D. (2012). Membrane Tension Maintains Cell Polarity by Confining Signals to the Leading Edge during Neutrophil Migration. Cell *148*, 175–188.
32. Insall, R.H., and Weiner, O.D. (2001). PIP3, PIP2, and Cell Movement - Similar Messages, Different Meanings? Dev. Cell *1*, 743–747.
33. Jacinto, A., and Baum, B. (2003). Actin in development. Mech. Dev. *120*, 1337–1349.
34. Jaiswal, R., Stepanik, V., Rankova, A., Molinar, O., Goode, B.L., and Mccartney, B.M. (2013). Drosophila Homologues of Adenomatous Polyposis Coli (APC) and the Formin Diaphanous Collaborate by a Conserved Mechanism to Stimulate Actin Filament Assembly * □ S.
35. Janetopoulos, C., and Devreotes, P. (2006). Phosphoinositide signaling plays a key role in cytokinesis. J. Cell Biol. *174*, 485–490.
36. Kindred, A.M. (2016). The Role of Diaphanous in Ring Canal Development in Drosophila melanogaster.
37. Koke, C., Kanesaki, T., Grosshans, J., Schwarz, U.S., and Dunlop, C.M. (2014). A computational model of nuclear self-organisation in syncytial embryos. J. Theor. Biol. *359*, 92–100.
38. Lee, D.M., and Harris, T.J.C. (2014). Coordinating the cytoskeleton and endocytosis for regulated plasma membrane growth in the early Drosophila embryo. Bioarchitecture *4*, 68–74.
39. Lee, D.M., Rodrigues, F.F., Yu, C.G., Swan, M., and Harris, T.J.C. PH Domain-Arf G Protein Interactions Localize the Arf-GEF Steppke for Cleavage Furrow Regulation in Drosophila.
40. Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., and Waterfield, M.D. (1996). The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J. *15*, 6584–6594.
41. Leslie, N.R., Batty, I.H., Maccario, H., Davidson, L., and Downes, C.P. (2008). Understanding PTEN regulation: PIP2, polarity and protein stability. Oncogene *27*, 5464–5476.
42. Levayer, R., Hauert, B., and Moreno, E. (2015). Cell mixing induced by myc is required for competitive tissue invasion and destruction. Nature *524*, 476–480.
43. Logan, M.R., and Mandato, C.A. (2006). Regulation of the actin cytoskeleton by PIP2 in cytokinesis. Biol. Cell *98*, 377–388.
44. Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke±, V., and Mostov, K. (2007). PTEN-mediated segregation of phosphoinositides at the apical membrane controls epithelial morphogenesis through Cdc42. Cell *128*, 383–397.
45. Mavrakis, M., Rikhy, R., and Lilly, M. (2008). Current Protocols in Cell Biology Fluorescence Imaging Techniques for Studying Drosophila Embryo Development.
46. Mavrakis, M., Rikhy, R., and Lippincott-Schwartz, J. (2009). Plasma Membrane Polarity and Compartmentalization are Established Before Cellularization in the Fly Embryo. Dev. Cell *16*, 93–104.
47. Mazumdar, A., and Mazumdar, M. (2002). How one becomes many : blastoderm cellularization in Drosophila melanogaster. 1012–1022.
48. Mullins, R.D., Heuser, J.A., and Pollard, T.D. (1998). The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc. Natl. Acad. Sci. U. S. A. *95*, 6181–6186.
49. Nie, S., and Chang, C. (2007). PI3K and Erk MAPK mediate ErbB signaling in Xenopus gastrulation. Mech. Dev. *124*, 657–667.
50. Noatynska, A., Monica Gotta, and Patrick, M. Mitotic spindle (DIS)orientation and DISease: Cause or consequence? J. Cell Biol. *199*, 1025–1035.
51. Nowotarski, S.H., Mckeon, N., Moser, R.J., and Peifer, M. (2014). The actin regulators Enabled and Diaphanous direct distinct protrusive behaviors in different tissues during Drosophila development. Mol. Biol. Cell *25*.
52. Papayannopoulos, V., Co, C., Prehoda, K.E., Snapper, S., Taunton, J., and Lim, W.A. (2005). A Polybasic Motif Allows N-WASP to Act as a Sensor of PIP 2 Density 2002). The two best studied inputs are the small GTPase Cdc42 and PIP 2 , which act in a combinatorial manner: although both are independent activators, they are far. Mol. Cell *17*, 181–191.
53. Peng, Y., Jiang, B.-H., Yang, P.-H., Cao, Z., Shi, X., Lin, M.C.M., He, M.-L., and Kung, H.-F. (2004). Phosphatidylinositol 3-kinase signaling is involved in neurogenesis during Xenopus embryonic development. J. Biol. Chem. *279*, 28509–28514.
54. Pickering, K.A. (2013). Regulation of actin dynamics by phosphoinositides during epithelial closure A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences.
55. Pinal, N., Goberdhan, D.C.I., Collinson, L., Fujita, Y., Cox, I.M., Wilson, C., and Pichaud, F. (2006). Article

Regulated and Polarized PtdIns(3,4,5)P3 Accumulation Is Essential for Apical Membrane Morphogenesis in Photoreceptor Epithelial Cells. Curr. Biol. *16*, 140–149.
56. Price, M., Roberts, D., McCartney, B., Jezuit, E., and Peifer, M. (2005). Cytoskeletal dynamics and cell signaling during planar polarity establishment in the Drosophila embryonic denticle. J. Cell Sci. *119*, 403–415.
57. Quinlan, M.E. (2013). Direct interaction between two actin nucleators is required in Drosophila oogenesis. Development *140*, 4417–4425.
58. Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J.D., Sheetz, M.P., and Meyer, T. (2000). Phosphatidylinositol 4,5-Bisphosphate Functions as a Second Messenger that Regulates Cytoskeleton–Plasma Membrane Adhesion. Cell *100*, 221–228.
59. Reversi, A., Loeser, E., Subramanian, D., Schultz, C., and De Renzis, S. (2014). Plasma membrane phosphoinositide balance regulates cell shape during Drosophila embryo morphogenesis. J. Cell Biol. *205*, 395–408.
60. Ridley, A.J. (2001). Rho GTPases and cell migration. J Cell Sci *114*, 2713–2722.
61. Royou, A., Field, C., Sisson, J.C., Sullivan, W., and Karess, R. (2004). Reassessing the Role and Dynamics of Nonmuscle Myosin II during Furrow Formation in Early Drosophila Embryos □ D□ V. Mol. Biol. Cell *15*, 838–850.
62. Saarikangas, J., Zhao, H., and Lappalainen, P. (2010). Regulation of the Actin Cytoskeleton-Plasma Membrane Interplay by Phosphoinositides. Physiol. Rev. *90*, 259–289.
63. Schejter, E.D., and Wieschaus, E. (1993). bottleneck acts as a regulator of the microfilament network governing cellularization of the Drosophila embryo. Cell *75*, 373–385.
64. Sheetz, M.P., and Dai, J. (1996). Modulation of membrane dynamics and cell motility by membrane tension. Trends Cell Biol. *6*, 85–89.
65. Sherlekar, A., and Rikhy, R. (2012). Drosophila embryo syncytial blastoderm cellular architecture and morphogen gradient dynamics : Is there a correlation ? *7*, 73–82.
66. Shewan, A., Eastburn, D.J., and Mostov, K. (2011). Phosphoinositides in cell architecture. Cold Spring Harb. Perspect. Biol. *3*, 1–17.
67. Skwarek, L., and Boulianne, G. (2009). Great Expectations for PIP: Phosphoinositides as Regulators of Signaling During Development and Disease. Cell Press *12*.
68. Sokac, A.M., and Wieschaus, E. (2008). Zygotically controlled F-actin establishes cortical compartments to stabilize furrows during Drosophila cellularization.
69. von Stein, W. (2005). Direct association of Bazooka/PAR-3 with the lipid phosphatase PTEN reveals a link between the PAR/aPKC complex and phosphoinositide signaling. Development *132*, 1675–1686.
70. Stevenson, V., Hudson, A., Cooley, L., and Theurkauf, W.E. (2002). Arp2/3-dependent psuedocleavage furrow assembly in syncytial Drosophila embryos. Curr. Biol. *12*, 705–711.
71. Turner, F.R., and Mahowald, A.P. (1976). Scanning electron microscopy of Drosophila embryogenesis: 1. The structure of the egg envelopes and the formation of the cellular blastoderm. Dev. Biol. *50*, 95–108.
72. Vogler, G., Liu, J., Iafe, T.W., Migh, E., Mihály, J., and Bodmer, R. (2014). Cdc42 and formin activity control non-muscle myosin dynamics during Drosophila heart morphogenesis. J. Cell Biol *206*, 909–922.
73. Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., and Narumiya, S. (1997). p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin microinjection of Rho causes rapid formation Kazuwa Nakao 2 , Brigitte M.Jockusch 4 and inactivation of Rho by botulinum C3 ADP-ribosyltransfer- endothelin-or GTPγS-induced tyrosine phosphorylation. EMBO J. Cell Motil. *16*, 3044–3056.
74. Webb, R.L., Zhou, M.-N., and McCartney, B.M. (2009). A novel role for an APC2-Diaphanous complex in regulating actin organization in Drosophila. Development *136*, 1283–1293.
75. Wu, H., Feng, W., Chen, J., Chan, L.-N., Huang, S., and Zhang, M. (2007). PDZ domains of Par-3 as potential phosphoinositide signaling integrators. Mol. Cell *28*, 886–898.
76. Yarmola, E.G., Somasundaram, T., Boring, T.A., Spector, I., and Bubb, M.R. (2000). Actin-latrunculin A structure and function:differential modulation of actin-binding protein function by latrunculin A. J. Biol. Chem. *275*, 28120–28127.
77. Zhou, Bokoch and Traynor-Kaplan, 1998 (1998). Disruption of Dictyostelium PI3K genes reduces [32P]phosphatidylinositol 3,4 bisphosphate and [32P]phosphatidylinositol trisphosphate levels, alters Factin distribution and impairs pinocytosis. J. Cell Sci. *111*, 283–284.
78. Zigmond, S.H. (2004). Formin-induced nucleation of actin filaments. Curr. Opin. Cell Biol. *16*, 99–105.