Molecular Characterization of Inception of Polarity in the Plasma Membrane During Early *Drosophila* Embryogenesis

Final year thesis

by

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Certificate

This is to certify that this dissertation entitled ‘Molecular Characterization of inception of polarity in the plasma membrane during early *Drosophila* embryogenesis’ towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by **Ramya Balaji** at IISER Pune under the supervision of Dr. Richa Rikhy, Assistant Professor, Biology Division, IISER Pune during the academic year 2012-2013.

Dr. Richa Rikhy

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Declaration

I hereby declare that the matter embodied in the thesis entitled '**Molecular Characterization of inception of polarity in the plasma membrane during early *Drosophila* embryogenesis** 'are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Richa Rikhy, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

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Abstract

Epithelial cells have a characteristic Plasma (PM) organization in all organisms, with different polarity protein complexes marking apical, lateral and basal membrane. The syncytial embryo of *Drosophila* is a stage prior to the de novo formation of epithelial cells. Despite the absence of complete PM boundaries around each nucleus, the syncytium has been shown to exhibit polarized distribution of some molecules found in epithelial cells. The syncytium is a good model to study the temporal onset of polarized protein distribution and the mechanisms which govern this. Rapid remodeling of the PM during the syncytial division cycles makes it especially suited for observing the onset of polarized distribution of PM *de novo*. I have used time lapse confocal laser scanning microscopy of fluorescently tagged transgenes present in epithelial cells for their temporal distribution on the PM in the syncytial division cycle. This work shows that at least three PM domains, one apical and two lateral, are formed during last four syncytial nuclear division cycles before the embryo forms complete epithelial cells. The plasma membrane is completely marked on the apical and lateral domain by phospholipid binding proteins. Transmembrane proteins capable of interlinking across adjacent PM like DE-Cadherin and Toll mark the entire lateral membrane, whereas PM associating proteins like septins, Bazooka and PatJ are localized to the lower regions of the lateral membrane. Finally, preliminary results using Anillin mutants and laser ablation of centrosomes shows that they are important for lateral membrane organization in the syncytium.

List of figures

List of graphs

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Introduction

Cells are organized into different types of tissues in multicellular organisms enabling different kinds of functions for these tissues. Plasma membrane (PM) of these cells have different kinds of organization which enable functions of cells, like secretion and absorption by epithelial cells lining the gut, migration of single cells, neuronal signal transmission and collective migration of cells during development of an organism. In each of these processes, the PM of cells has asymmetries in its constitution. These asymmetries could be in the form of lipid constitution, protein constitution andlocalization or the manner of cytoskeletal attachment to the PM. The result of these asymmetries is different shapes and structures of the PM which give rise to functions of the cell or group of cells.

During embryogenesis of plants and animals, a single fertilized egg divides to give rise to thousands of cells, which eventually organize to form germ layers that give rise to a multicellular organism. PM asymmetries are very important for the cell division process and for patterning of the embryo. In the mouse embryo, at the 8-cell stage, a redistribution of the PM and cytoskeletal elements occurs, which allows different kinds of cell divisions, symmetric, asymmetric or oblique- the end result of which is the formation of two layers of cells. The outer layer, trophoectoderm, has polarized PM whereas the inner cell mass is unpolarized(Rossant, Chazaud, and Yamanaka 2003; Zernicka-Goetz 2005). In *Caenorhabditis elegans* embryo,asymmetry in the PM in the single-celled egg post fertilization has been well studied. The cortex of the embryo undergoes actomyosin contractions that enable the partitioning of proteins belonging to the PAR family(Munro et al. 2004). This asymmetry guides the first cell division that gives rise to daughter cells destined for different developmental fates that further divide to give rise to cells of different sizes and functions eventually. In *Drosophila melanogaster*, a series of nuclear divisions happen post-fertilization following with the first layer of cells form which are epithelial in nature and have their characteristic PM domain architecture. In development, epithelial cells form the first layer of organized tissue.

Polarity in the plasma membrane of epithelial cells and neuron formation

What is required to make a polarized membrane? The components one can think of as most important for molecular asymmetries to arise on the membrane are- a cue that initiates polarity, molecules that become polarized in the organization responding to the cue, and molecules that stabilize or maintain the established polarity on the plasma membrane. Given the highly complex environment inside even each single cell, deciphering how polarity arises in the plasma membrane of cells remains a challenging question. Below is a review of efforts been in the field to address questions of what molecules and mechanisms constitute the above components of plasma membrane polarity.

Cell polarity has been well-studied in the epithelial cells and neuroblasts of the *Drosophila melanogaster*. Neuroblasts, the stem cells of the nervous system, undergo asymmetric cell division to give rise to daughter cells, one of which maintains its stemness. This asymmetric division is remarkably aided by the presence of polarized localization of a number of proteins on the apico-basal axis(Wodarz 2005; Betschinger and Knoblich 2004). Cells of wing imaginal discs, follicle cells of ovaries, cellularised blastoderm of the embryo are some epithelial tissues used for studying polarity *in vivo* in *Drosophila.* 3D cultures of epithelial cells have been developed on basement membrane cultures which also retain polarity in their PMto perform *in vitro* functional studies of epithelial cells of different mammalian tissues and in cancer(Debnath and Brugge 2005; Eritja et al. 2010).

PM domains in epithelial cells

From various model systems there is some consensus about protein complexes in the plasma membrane of epithelia, which serve a function of forming creating zones of adhesion to form a intact sheet of epithelial cells. However, there are subtle differences in the various adhesion zones and junctions that form in vertebrates and invertebrates. Vertebrate cells have adherens junctions (AJ) and tight junctions(TJ) on their lateral membrane. Invertebrate epithelia have spot adherens junctions and septate junctions (SJ) instead. The function of TJs and SJs is similar in both organisms and prevents passage of molecules in between cells. AJs serve to link adjacent cells together and they bind a network of actomyosin in the interior of the cell. This actomyosin is regulated by the AJs during cell morphogenesis

events(Lecuit and Munro 2011; Levayer and Lecuit 2012). AJs typically have E-Cadherin, a transmembrane protein with an extracellular domain to cross-link with Cadherin of adjacent cells and its intracellular binding partners alpha- and beta-catenin (Harris and Tepass 2010).

The apico-basal polarity in the PM of epithelia is characterized by various proteins that mark different regions of the membrane. Some of the molecular players establishing apical membrane identity in a typical epithelial cell are various PDZ containing proteins like Par-6, Atypical Protein Kinase C (aPKC) and Par-3/Bazooka (Baz). aPKC and Baz show mutual dependence on each other for apical localization (Wodarz et al., 2003). Later on the apical identity is maintained by the by Crumbs (Crb) and Stardust(Sdt). The former protein is a transmembrane protein recruited by the Par complex which in turn recruits Sdt. Feedback and antagonistic interactions of Crb with basolateral proteins like Lethal giant larvae (Lgl), Scribble and Discs large (Dlg) help in polarizing it to the apical membrane in follicle cells (Fletcher et al., 2012). These proteins are highly conserved and are key players implicated with the polarization of most epithelial cells.

What comes first -PM polarization or junctions?

In *Drosophila,* a lot of aspects of epithelial cell polarity and junction formation has been studied in follicular epithelial cells of the ovaries and the blastoderm embryo. The follicle cells are specialized epithelial cells that line the egg chamber and help in positioning of the oocyte amongst other functions. The blastoderm epithelium first forms after 13 rounds of nuclear division in the embryo in a stage aptly called cellularisation (alternatively Nuclear Cycle (NC) 14).It is thought that epithelial AJs are important for delimiting the apical and the baso-lateral domains (Assémat et al., 2008). However, there are conflicting viewpoints and evidences to the importance of AJs for polarization of the epithelium (Bivic, 2005). What is important to remember is that the mechanisms of polarizations have been seen to differ slightly from one epithelial system to another. In each of these systems, the formation of epithelium is different as regards how the cues for polarity are generated, time of polarization and mechanism of polarization (Franz and Riechmann, 2010). The steps of polarization in the follicular epithelium have been identified to involve Baz, Arm and Dlg defining domains in the membrane. Baz and its interacting partners aPKC-Par-6 are inhibited

from the lateral membrane by Dlg. These interactions help decide the location of AJs which in turn prevent Dlg spread into the apical domains. The authors of this study propose that the specification of the apical membrane is the second polarization step after AJ formation reflected by the accumulation of Par-6 and aPKC at the apical membrane domain, followed by a third step of Crb-Sdt localization to the apical membrane (Franz and Riechmann, 2010).

In contrast to follicular epithelial polarization stands the cellular blastoderm polarization. Cellularization is when the first layer of epithelial cells that form *de novo* in the embryo. This layer of cells has characteristic apico-lateral membrane polarity and architecture of a typical epithelial cell. There is apical accumulation of the apical cue Baz, formation of AJs labeled by *Drosophila* E-Cadherin (DE-Cadh) apico-laterally and lateral membrane labelled by Dlg. Absence of AJ components does not impede with apical localization of Baz but AJ localization to right place on the PM requires Baz. However, AJs are required for the maintenance of epithelial architecture and maintenance of basolateral cues (Harris and Peifer, 2004). The apical localization of Baz has been found to be through Dynein mediated apical transport (Harris and Peifer, 2005). It is seen that Baz has a more lateral localization even as cellularisation is taking place and gets relocated apically at the end of cellularisation. This translocation is aided by Par-1 mediated dispersion in addition to MT mediated transport mechanisms that have not been fully understood (McKinley and Harris, 2012).

Unknown cue to polarity initiation

In both these systems, the cue for initiating polarity in the membrane has still not been found. Polarity cues or initiation have been studied better in the *Caenorhabditis elegans* embryo, where asymmetric localization of the Par protein family is seen during the first nuclear division. The cue for polarity establishment is brought by the sperm which regulates the actomyosin contractile properties in a manner such that Par-2 localizes to the posterior end (St Johnston and Ahringer, 2010). The cue is unknown but is known to be brought along with the centrosomes from the sperm. Ablation of centrosomes affects establishment of polarity but not the maintenance (Auxin et al., 2004).

Syncytium of fly as a model to study PM polarity

The syncytium of *Drosophila* embryo is the stage between fertilization and cellularisation of the developing embryo. The prospect of finding a cue to polarity initiation and polarity in the plasma membrane in this stage cannot be overlooked. In the syncytial embryo of *Drosophila*, nine nuclear divisions occur in the interior of the embryo, following which nuclei migrate to the periphery of the embryo. Centrosomes are associated with these nuclei and arrive to the cortex with the nuclei. In the next four nuclear division cycles (NC10-13), the plasma membrane undergoes activity and remodeling accompanying the various phases of division, such that one can associate distinct organization with each of the division phases namely, interphase, prophase, metaphase, anaphase and telophase. The PM over each nucleus looks like a cap with villi-like protrusions in interphase (Fig.1.1a). By metaphase, the PM has ingressed into the embryo, to form furrows which separate each of the spindles (Fig.1.1b). These furrows regress upward by telophase and resume their cap like structure for the next interphase. The PM is lined by F-actin filaments throughout their length and the apical villi have branched actin too(Warn, Magrath, and Webb 1984; Stevenson et al. 2002).

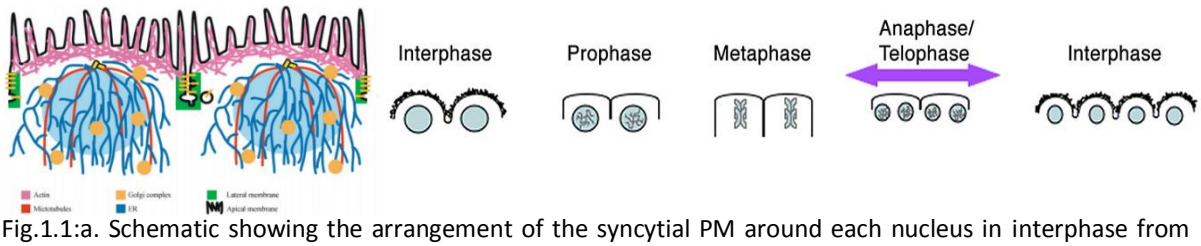


Fig.1.1:a. Schematic showing the arrangement of the syncytial PM around each nucleus in interphase from NC 10 onward(Sherlekar and Rikhy 2011), b. Remodeling of the PM in the syncytial embryo during each NC(Sokac and Wieschaus 2008)

Organelles like ER and Golgi are also seen to associate with each nucleus as they come to the cortex. One would think that these organelles would be shared between nuclei, but diffusion studies show that they are compartmentalized in their organization. Further, diffusion studies on the PM also show that the PM is not freely shared between nuclei either(Mavrakis, 2009; Mavrakis and Rikhy, 2010).This phenomenon is remarkable in that there is no complete cell in the system despite which compartmentalized ‘cell-like’ characteristics are seen. All these evidences

point out to formation of some barriers in the PM which could be resulting in the compartmentalization. Barriers in the PM can be thought of to form with the formation of junctions between adjacent PM. If there are primitive junctions like AJs or their components thereof, it points towards the presence of polarity molecules that might be directing their formation or vice versa. Taken together with the idea of identifying the polarity cue that might be arising with the centrosomes that migrate to the periphery, the syncytial system is good stage to study how polarity in the PM arises.

Motivation for the project

The biggest open question motivating this work is- what is the cue to the inception of PM organization in the syncytium? But prior to addressing this question one needs to develop an understanding of the organization of the PM in the syncytium, since such a study has not been done meticulously till date. Some of the questions the work embarked upon are- How does the PM of the syncytial embryo organize? Which members of various polarity complexes are present in the syncytium? Do adhesions junctions form in the syncytial PM and do they form prior to a polarized membrane formation? The debate of whether AJs or components of AJs are present on the membrane before polarization of apical complexes has been followed here.

Aims

To understand the organization of the syncytial embryo with focus on its PM

To find out which members of the epithelial polarity complexes are present in the syncytial PM

To study the role of centrosomes and actin remodeling on PM organization and ingression and its effect on polarity of the proteins studied.

Materials and Methods

Fly stocks and genetics

All fly stocks and crosses were grown and maintained at 25^{O}C under standard conditions. All fly lines, unless otherwise mentioned, are available from Bloomington stock centre, Indiana, USA.

Fly lines used:

CS - wildtype control for fixed embryos

Transgenic fly lines used for imaging:

Ubi-DE-Cadherin GFP- Ubiquitin promoter driven DE-Cad GFP line

tGPH- GFP tagged PH domain construct that labels PIP3

Nanos gal4- the gal4-UAS strategy was used to express some of the transgensic lines. This particular gal4 driver is maternally expressed in the embryo.

UAS PH-PLC delta Cerulean FP(CeFP) – tagged PH domain of Phospho Lipase C that labels PIP3 ,UASp-Toll Venus (Manos Mavrakis)

UAS Baz-GFP

UAS Sep2 GFP (Andrew Wilde)

Polo GFP, Dlg GFP- protein trap lines (Alan Spradling)

AnillinPQ51, AnillinRS23 (Christian Field) - for Anillin mutant embryos, AniPQ51 flies were crossed with AniRS23 flies and the non-balancer progeny were selected to lay embryos(Field et al. 2005).

For laser ablation experiments, Polo GFP flies were crossed to DE-Cad flies and the appropriate progeny flies were selected using balancers and the embryoslaid by the females were used for imaging.

Immunofluorescence

Three hour embryo collections from flies in cages were dechorionated and washed. Two different fixation protocols were used.The fixative for staining Anillin, Peanut, Diaphanous, PatJ was 4% paraformaldehyde in 1XPBS for 15 minutes, followed by removal of vitelline membrane by hand using a tungsten needle. For Dlg, embryos were boil fixed in 1X wash buffer (7%NaCl, 0.5% Tween-20 in water) and popped in methanol. Embryos were blocked in 2% BSA in 1X PBS for one hour at room temperature (RT) and incubated overnight (O/N) in primary antibodies at 4^{O}C. Following this, they were washed thrice for 15 minutes in 1XPBS-T and treated with secondary antibodies for 1.5 hours at RT. Hoechst staining was included in one of the subsequent washing steps. Embryos were mounted on microscopy slides with Gold AntiFade reagent (Invitrogen).

Antibodies

Anti-Dia, Rabbit polyclonal, 1:1000 (Wasserman lab), Anti-PatJ, Rabbit polyclonal, 1:1000 (Bellen lab), Anti Pnut, -Dlg, Mouse monoclonal, 1:5, (DSHB), Anti-Baz, Rabbit, 1:1000 (Anuradha Ratnaparkhi)

Imaging

For immunofluorescence and live imaging, the samples were imaged on Zeiss LSM 710 and 780 inverted microscopes (IISER Pune microscopy facility). Laser ablation experiments were carried out on Zeiss LSM 780 inverted microscope.Generally, the imaging of embryos was carried out with a 40X, oil objective and NA of 1.3 (LSM 710) and of 1.4 (LSM 780), pinhole of 90.07um, at averaging of 2 for live imaging. For imaging of Dlg, a highly sensitive (40% more sensitive than the PMTs) detector called GaAsP detector was used because the protein trap line had weak expression.

Live imaging of Drosophila embryos

Flies were kept in a cage at 25^{O}C. 1.5 – 2 hour embryo collections were made on an agar plate. Embryos were washed in water and dechorionated in bleach. They were placed horizontally in coverslip chambers (Mikrotek) and covered with 2 ml of 1XPBS for imaging.

Centrosome ablation by multiphoton laser

800nm laser beam (Mai Tai) was used at 5% laser power to micro-ablate centrosomes labelled with GFP tagged centrosomal proteins. The tracking of centrosomes and live imaging post-bleaching was carried out using 488 nm (Argon) laser. A range of laser powers between 0.2% and 6% were tested for ablation. For laser power up to 4%, only bleaching was achieved, as signal recovery in the region where the beam was shot was seen. At 5% laser power, there was no recovery of centrosomal signal and hence this power was chosen for the ablation experiments.Standardization of the laser power to ensure no other microstructures are destroyed is still in progress.

Image analysis

Open source software available online were used for image analysis and processing, namely, ImageJ, Zen 2011 Blue edition and GIMP Image Editor. Graphs and statistical analysis were done on MS Excel 2013.

Description of measurements for studying organization of the syncytial embryo

Each of the live imaging experiments labelled with different transgenic proteins which were fluorescently tagged had 11-15 Z- sections taken at intervals of 30-45 seconds to cover NC 10 to 13 and sometimes NC 14 which is cellularisation. tGPH movies were used to for this section of measurements. Interphase (I), late prophase (LP) and metaphase (M) were identified by visual examination of the Z-sections for characteristic membrane shapes and nuclear architecture.

1. **Area of nucleus in the NC 10-13-** tGPH labels the nuclear membrane also apart from the PM.The nuclei were assumed to be circular at each Z- section and the area was measured at the section with the largest radius for the nuclei. Values were calculated by averaging radii of three nuclei.
2. **Membrane depths in NC 10-13-** The depth of the membrane was calculated as the thickness of each Z- section (1.08um) times the number of Z-sections. The number of Z- sections was decided from the clearest top-most section (typically one can make out apical villi) to the section up to which lateral membrane is visible in each of the phases mentioned above (Fig. 2.1).

3. **Compartment depths in NC 10-13-** Compartments are defined as the set of Z-sections from the clearest top-most section to the one up to which the nuclei are clearly visible for each of the phases mentioned above (Fig. 2.1)
4. **Frequency distribution of polygonal packing in NC 10-14-** Polygonal packing is visible in from metaphase of NC12-13 and in cellularization (NC14). They have been visually marked for the number of edges (and hence which polygonal shape) in each compartment using the Z-section with most compact metaphase packing (Fig.2.2).

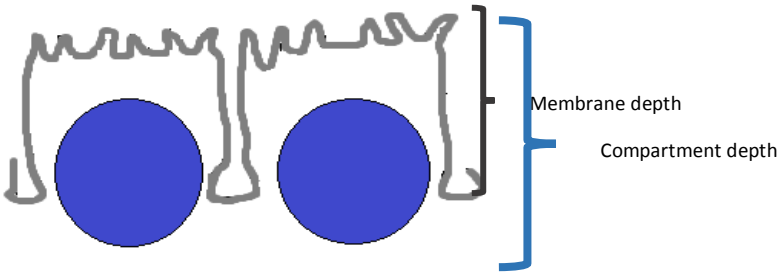


Fig.2.1:Schematic to show membrane and compartment depth measured to understand the organization of the syncytial embryo

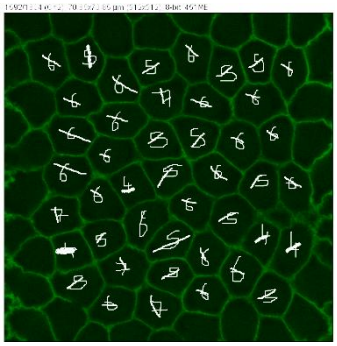


Fig.2.2: Polygonal packing and edge counting- representative image

Description of the intensity analysis

At least three movies for each fluorescently tagged transgene were made. This analysis has data from a single movie for each label. It is representative of the kind of trends in the membrane intensities to supplement visual data about the label's dynamics through each NC.

1. **Total intensity and intensity density**

Intensity measurements in each movie comprised identifying interphase and metaphaseof each NC and marking three kinds of ROIs. In interphase, one kind of ROI was called ‘apical cap’ which was the top-most Z-section at that time point discernible as different from the background noise (labelled as ‘I-apical cap’ in graphs). The second kind was called the interphase lateral membrane which comprises the lateral membrane in the sections below the apical cap till whichever section it is completely visible (labelled as ‘ I –lateral membrane’ in graphs). This is taken at the same time point as the apical cap ROI. In NC 10, this section below has very membrane signal or none. So, only the section immediately below the apical one is taken as I-lateral membrane in NC 10. The third kind of ROI was called the metaphase lateral membrane (labelled as ‘M-lateral membrane in graphs). This comprised of the lateral membrane of Z-sections in the time point where the deepest membrane ingressions are seen. The intensity was calculated using the Time series Analyzer plugin in ImageJ. They are in arbitrary units and are not scaled to be compared across graphs for each of the membrane labels. The ‘polygon selection’ tool was used to draw apical cap ROIs and the ‘segmented line’ tool (thickness of 5) was used for lateral membrane ROIs (Fig.2.3). Three regions were drawn for each kind of ROI in the movies. The total intensity and intensity density were calculated as averages of the values obtained from three regions values from three chosen regions. For the lateral membrane calculations, intensity values from each Z- section were summed before being averaged for the three regions.

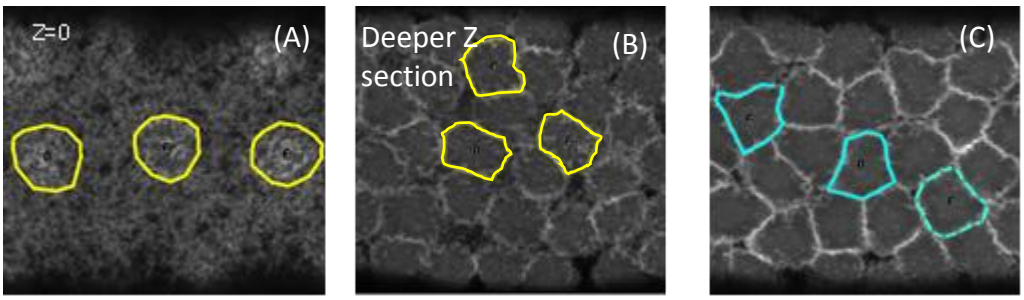


Fig.2.3: Representative Z-sections chosen as ROIs- (A)Apical caps, (B)Interphase lateral membrane and (C)Metaphase lateral membrane

2. Intensity profile across metaphase furrows of NC 13

The XZ orthogonal projection created by Zen 2011 software was used to identify the metaphase furrows of NC 13. Here the length of the furrows was measured by using the ‘line tool’ in ImageJ and an intensity profile across the length of the drawn line was measured.

3. Lateral membrane intensity density - intensity density calculation at new and old contact edges formed

‘Line tool’ was used to measure the intensity density in the lateral membrane labelled with DE-Cad that forms in prophase between two nuclei. The new membrane that forms between two daughter nuclei is loose and the contact edge can be termed weak, as compared to the rest of the contact edges around each of the nuclei. At a suitable Z-section where the lateral membrane is visible intensity density was measured across lines of varying thickness so as to span the edge (Fig.2.4). The average intensity density has been reported for NC 11-13 in two categories – strong contact edges and weak contact edges. This exercise can be carried out for all the imaged membrane markers, but only data for DE-Cad is shown here.

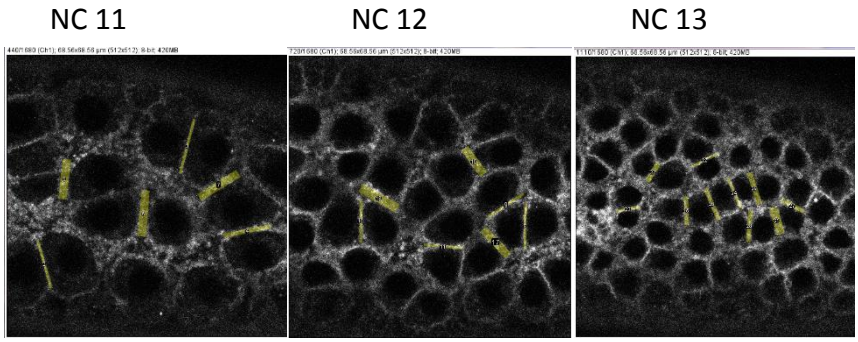


Fig.2.4: Representative Z- sections chosen to measure intensity density in the newly formed versus old lateral membrane. The thick yellow lines represent new membrane, the thin lines represent the old lateral membrane

Results

I. Organization of the syncytial embryo studied using a generic PM label - tGPH

To understand the onset of polarity and organization of the PM, a description of the syncytium as its regard its general nuclear organization and plasma membrane packing and depth is in order. The PM of the embryo labelled by tGPH, a PIP3 marker, before NC 10 shows ruffling on the surface that seems to have no particular pattern (Fig.3.1.A.a). As the nuclei reach the periphery of the embryo in NC 10, there is a sudden dramatic appearance of circular caps with villi-like protrusions becoming more prominent in intensity (Fig.3.1.B.b, see arrow).With each successive division from NC 10 to 13, the size of the nuclei under each cap becomes smaller as measured from the area of the nucleus at the section in which its diameter is largest. Thus with increasing nuclear density in the syncytium the nuclear size is decreasing (Graph 3.1).The depth of the PM at interphase is about 3 um and it ingresses up to 4 um in NC 10. The PM ingression in the earlier NCs (10 & 11) is shallower compared to the later ones (12 & 13). In fact, the PM in metaphase of NC 13 ingresses up to 12 um in the embryo (Graph3.2). The compartment depth around each nucleus however remains the same in Interphase across each NC and increases in length in late prophase and metaphase (Graph 3.3).

It is observed that as the nuclear density of the syncytium increases, the membrane caps around each nucleus are more apposed to each other. In other words the packing of the membrane improves with NCs. The lateral membrane ingression in metaphase acquires a polygonal shape only from NC 12 onward and the shapes are very prominent in metaphase of NC 13 (Fig.3.1m, outlined in yellow is a hexagon). On examining the deepest metaphase Z section for polygonal shape of each compartment, more than 50-60% are hexagons and 30% are pentagons. The remaining have compartments have four or seven sided contacts (Graph3.4). .

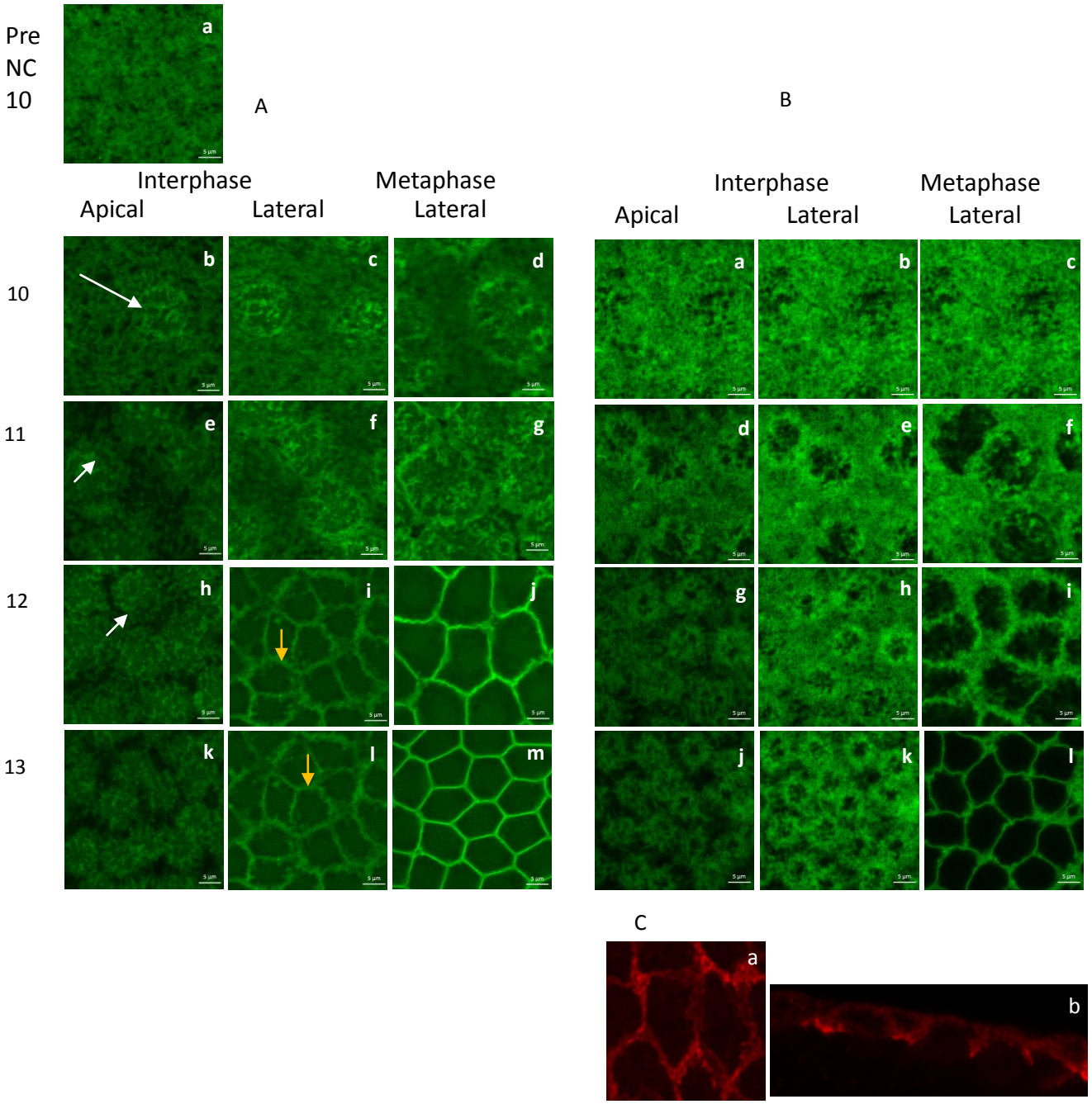
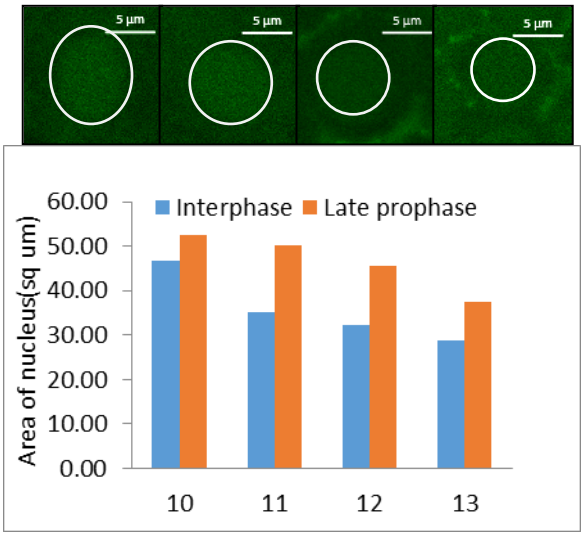
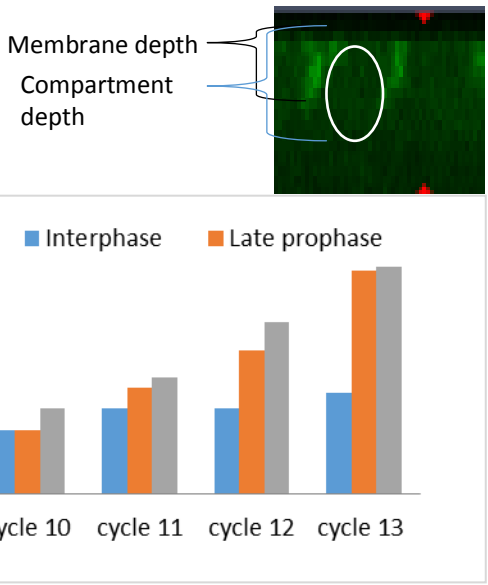


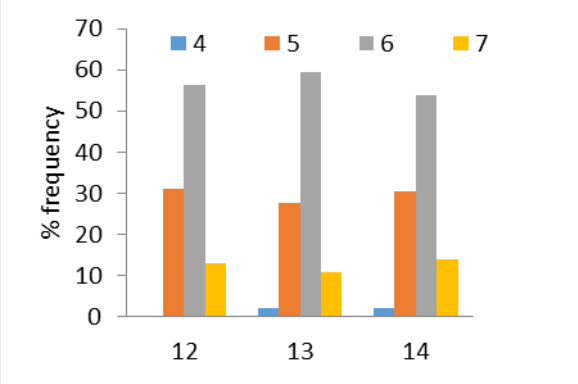
Fig.3.1: A.PM labelled with tGPH: a. before NC 10, villi protrusions, b-m show z-sections of apical and lateral membrane through interphase and metaphase in different NCs. B. PM labelled with PH-PLC delta, a-l showing z-sections of apical and lateral membrane. C. Immunostaining of WT embryo- a. surface view showing Anillin on lateral membrane, b. sagittal view showing Anillin labelling the apical and the whole length of the lateral membrane. (Explanation of arrows described in Results section I and II)



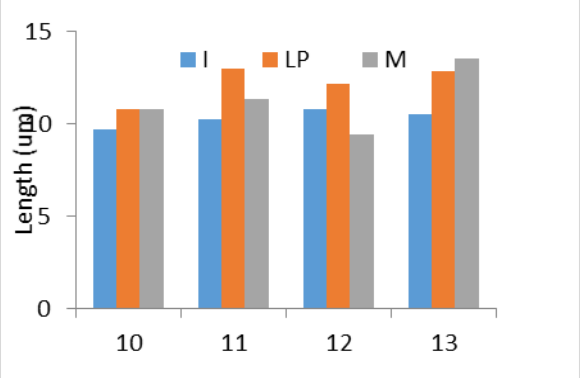
Graph3.1:Area of nucleus in the syncytial embryo from NC 10-13



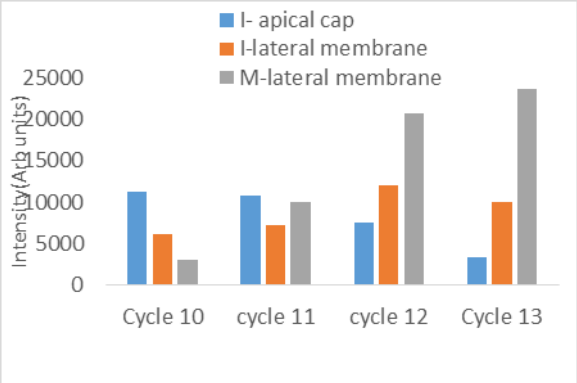
Graph3.2: Membrane depth in the syncytial embryo from NC 10-13



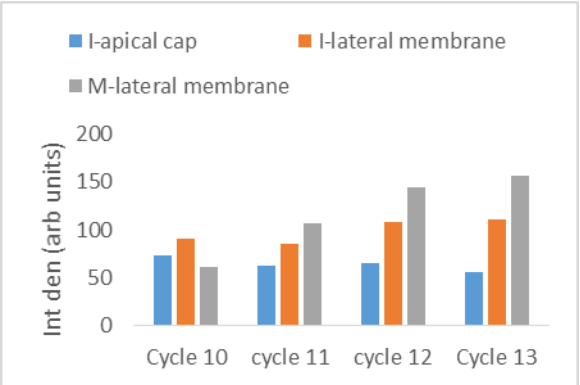
Graph3.4:Frequency distribution of polygon shapes in from NC 12-14



Graph3.3:Compartment depth in the syncytial embryo from NC 10-13



Graph3.5: Total intensity in the PM labelled with tGPH in NC 10-13



Graph3.6: Intensity density in PM labelled with tPGH from NC 10-13

I. tGPH and PH-PLC serve as complete PM markers

These markers label the apical villi and the lateral membrane completely (Fig.3.1.A.b, e,h,k) show the apical villi labelled with tGPH, arrows point the villi labelling in the apical caps in interphase). The lateral membrane in interphase becomes more prominent in NC 12 and 13(Fig.3.1i, l, yellow arrows). The total intensity in the apical caps decreases with each NCwhereas that of the lateral membrane increases both in interphase and metaphase (Graph3.5). The intensity density, for the apical cap is constant throughout the NCs, but that of the lateral membrane increases in both the phases (Graph3.6).

PH-PLC delta, a PIP3 marker is highly overexpressed causing high amount of membrane ruffling and sub apical signal but similar PM remodeling like tGPH is seen (Fig.3.1.B). F-actin remodeling protein Anillin is also seen on the whole of the PM (Fig.3.1.C). Since tGPH, PHPLC and Anillin all mark the apical villi and lateral membrane, this set of markers are termed ‘Class I-Complete’ markers.

II. Plasma membrane associated proteins and transmembrane proteins belonging to various polarity and junction complexes are found in the syncytium from NC 10-13

Immunostaining and live imaging of embryos with various proteins was done to check their presence and distribution on the PM.

DE-Cad and Toll are transmembrane proteins which localize to the lateral membrane

On live imaging DE-Cad, the transmembrane component of AJs in epithelial cells, it can be seen to label the villi on apical caps very distinctly in NC 10 (Fig.3.2a, yellow arrow points to villi). The signal from the apical most sections for DE-Cad drops and moves more into Z- sections of the interphase that show the lateral membrane (Fig2.3a,e,i,l, follow yellow arrows). This is corroborated from total intensity quantification from apical sections which drops with each NC and the concomitant rise in lateral membrane total intensity both in interphase and metaphase (Graph3.7). During metaphase of each NC, DE-Cad very clearly labels the lateral membrane completely. The intensity density in the lateral membrane increases with each NC (Graph 3.8).

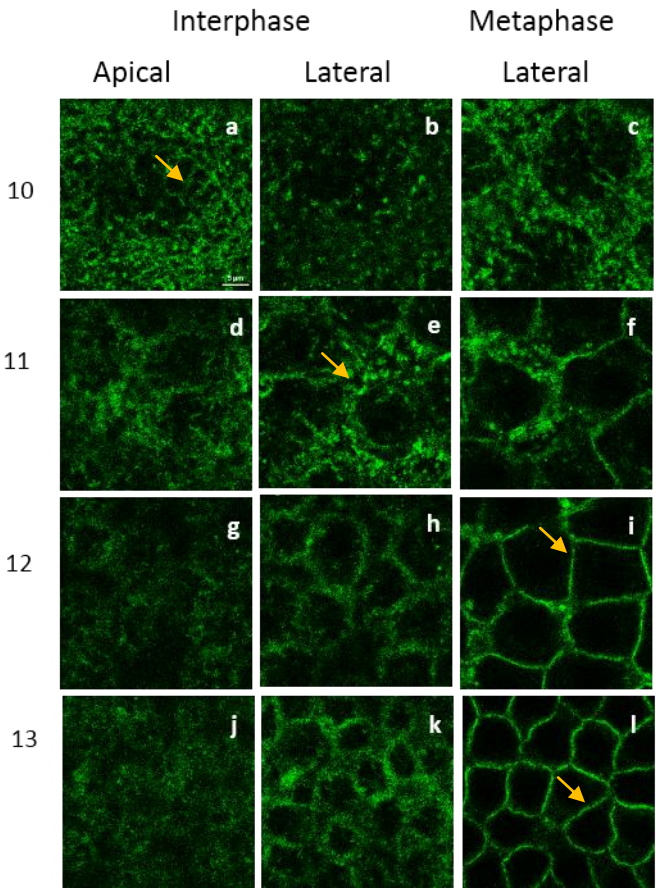
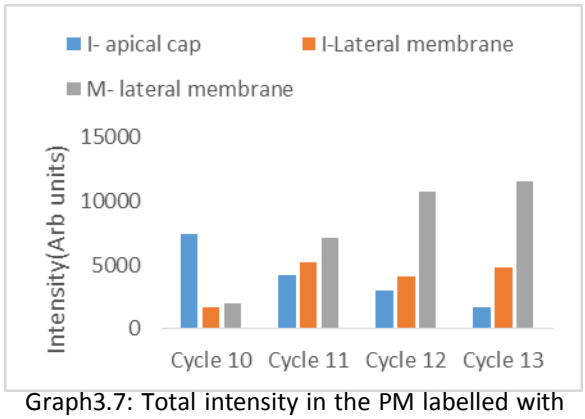
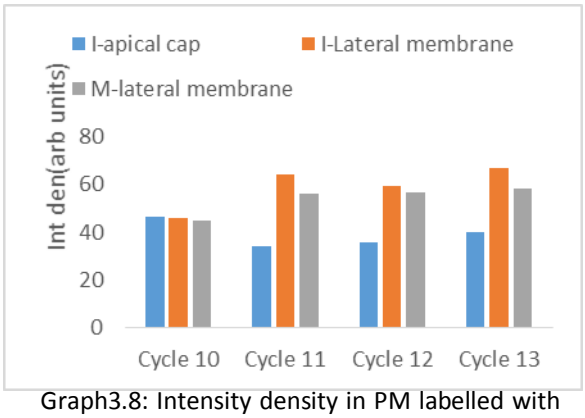


Fig.3.2: PM labelled with DE-Cad: a-l show z-sections of apical and lateral membrane through interphase and metaphase of different NCs. (Explanation of arrows described in Results section II)



Graph3.7: Total intensity in the PM labelled with DE-Cad in NC 10-13



Graph3.8: Intensity density in PM labelled with DE-Cad from NC 10-13

Toll is a transmembrane receptor which is important for the activation of dorso-ventral patterning in the embryo. When it is expressed in mammalian cell cultures, it can cause cells to adhere through interactions presumably from its extracellular domain(Yuki et al. 2013). This function is similar to that of junctional protein DE-Cad. Toll distribution in the PM is follows a similar trend as can be visually discerned (Fig3.3). A large cluster of vesicular structures labelled by Toll are seen in the deeper sections of interphase and metaphase in NC 10 and 11 which clear out in the NC 12 and 13(Fig3.3c, e, yellow arrows point to punctae).The intensity analysis can be predicted to be the same. However, the Venus fluorescent protein tag is highly prone to bleaching and hence the bleaching corrections had to be introduced. The intensity values obtained for each measurement was corrected by dividing them with the corresponding values for the whole embryo. The total intensity in the apical cap shows the same trend as DE-Cad.

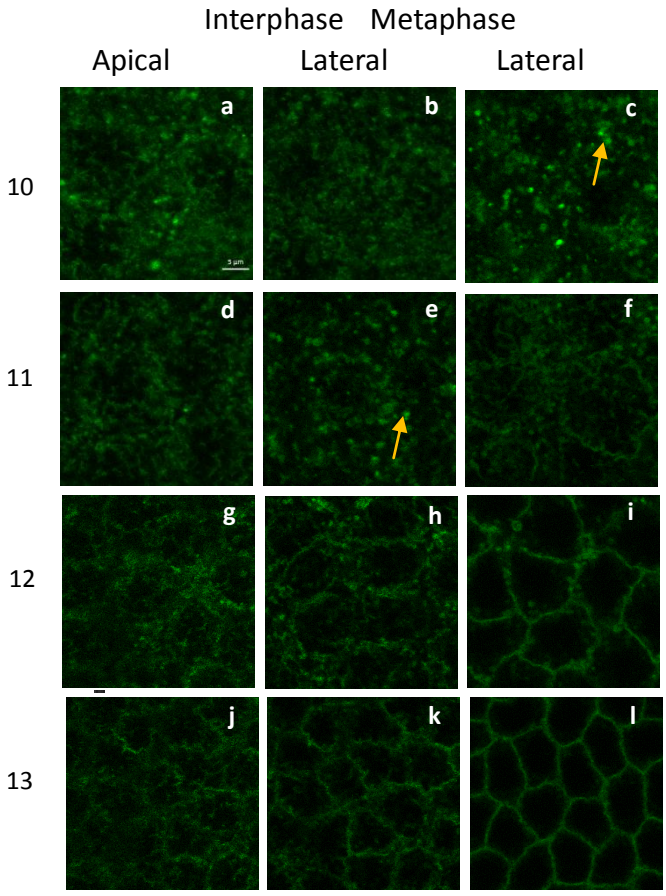
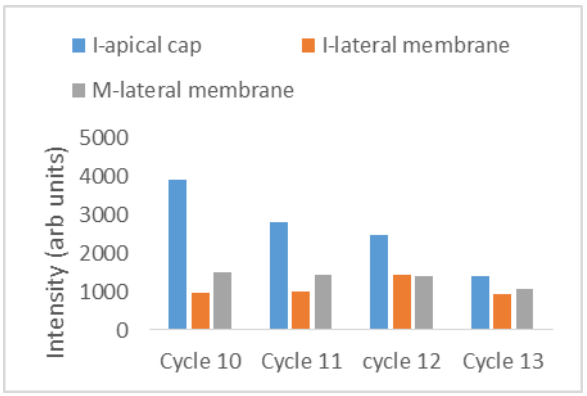
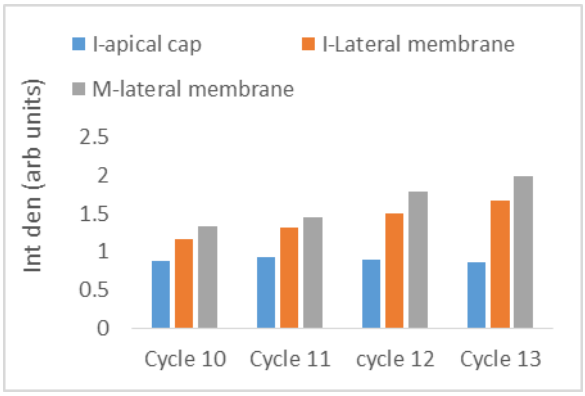


Fig.3.3: PM labelled with Toll: a-l show z- sections of apical and lateral membrane through interphase and metaphase of different NCs. (Explanation of arrows described in Results section II)



Graph3.9: Total intensity in the PM labelled with Toll in NC 10-13



Graph3.10: Intensity density in PM labelled with Toll from NC 10-13

But, the increase in the lateral membrane intensity does not come out to be as much or higher in magnitude (Graph3.9). The expected trend was not obtained because the effect of the division with large overall intensity values of the embryo is nullifying the steep rise in the numerical value of the lateral membrane intensity. The intensity density is a better read out for Toll as it shows a clear increase for the lateral membrane and a decrease for the apical membrane with each NC(Graph3.10).

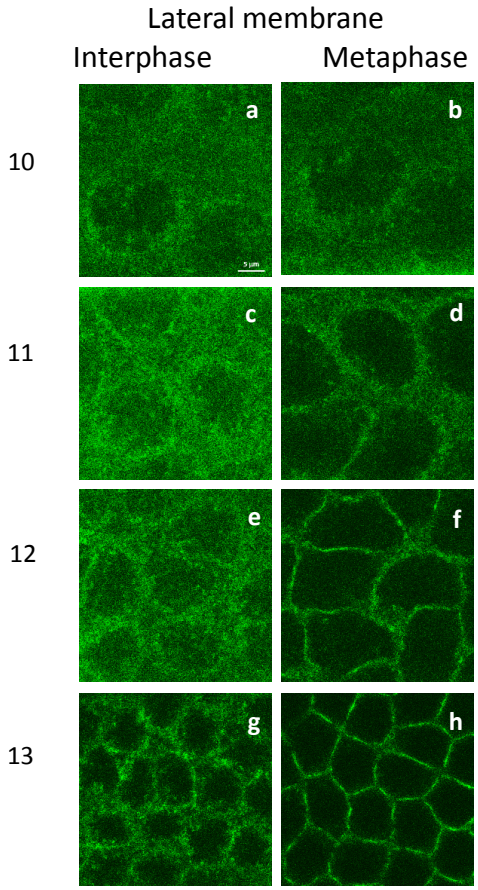
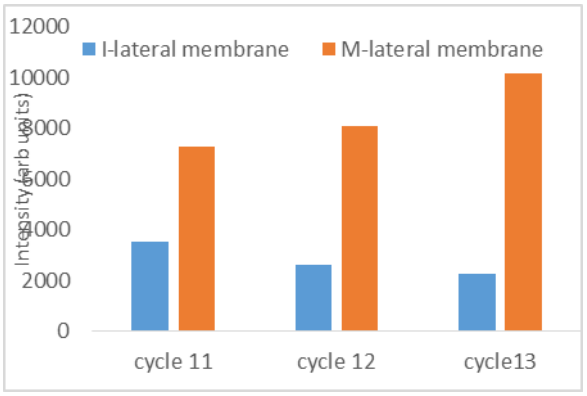
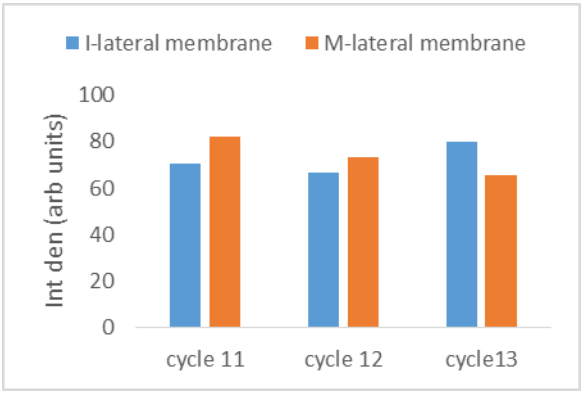


Fig.3.4: Dlg distribution in the syncytial embryo on the lateral membrane z-sections through interphase and metaphase of different NCs.



Graph3.11: Total intensity in the PM labelled with Dlg in NC 10-13



Graph3.12: Intensity density in PM labelled with Dlg from NC 10-13

Dlg labels the lateral membrane

Dlg is a lateral membrane marker in epithelial cells and is implicated with tumor suppressor functions(Mccaffrey and Macara 2011). On live imaging the embryo of protein trap line of Dlg, the apical sections had a high amount of autofluorescence and hence could not be discerned very clearly. Thus only observations about the lateral membrane are reported here. The lateral membrane is difficult to identify in the NC 10 because of background signal and there seems to be a more cytoplasmic localization of Dlg in this cycle (Fig.3.4a, b). The membrane organization is clearer NC 12 onwards. The total intensity of the lateral membrane decreases in the lateral membrane of interphase and increases in each metaphase. The intensity density does not change too much with the NCs (Graph3.12). The intensity profile for Dlg has not been shown because the XZ reconstruction of the XY sections shows too many pixels with high gray values apically. This is because of autofluorescence and the intensity profile would be corrupted by it.

Since this set of PM markers are seen on the lateral membrane asdescribed above, they are henceforth termed as ‘Class II: lateral’ markers.

Sep2, Baz and PatJ label only the lower region of the lateral membrane

Sep2 is one of the five septins (Sep1, 2, 4, 5 and Peanut) found in *Drosophila*. This family of proteins forms heteroligomers with each other and is important for contractile ring assembly in dividing cells(Weirich, Erzberger, and Barral 2008). The syncytium shows presence of Sep2 and Peanut. The distribution of Sep2 was analyzed. Sep2 does not label the villi but still marks the apical most section clearly. It demarcates each cap at its rim distinctly (Fig.3.5a, yellow circle showing rim). This rim moves into the lateral section in interphase 12 and 13(Fig.3.5h, k, yellow circles). The lateral membrane in metaphase also shows Sep2 (Fig.3.5i, l). Total apical intensity for Sep2 drops with each NC and the lateral membrane intensity rises (Graph3.13). However, the rise in the lateral membrane intensity is not in proportion or greater than the magnitude of intensity value drop in the apical cap. This observation is different from the previous two classes of proteins, both of which show is steep rise in lateral membrane intensities.

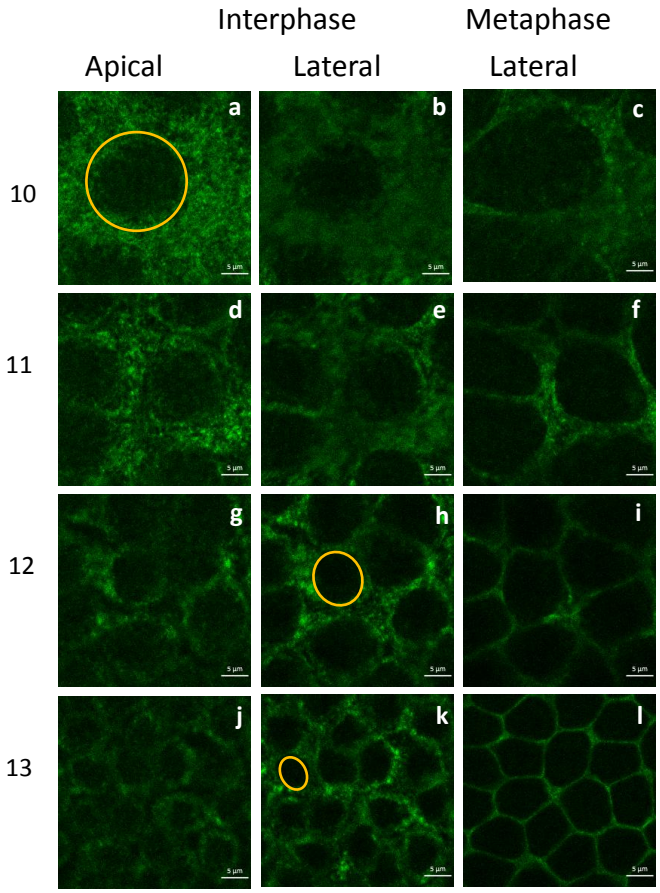
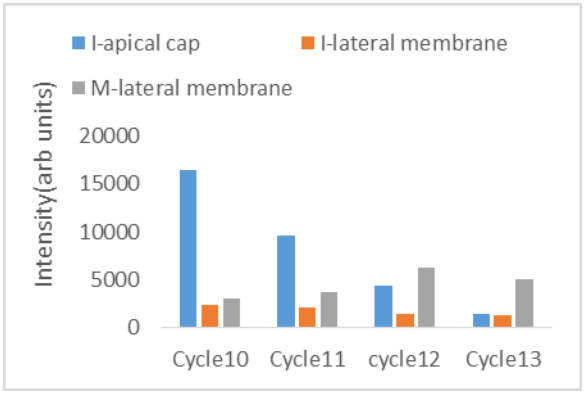
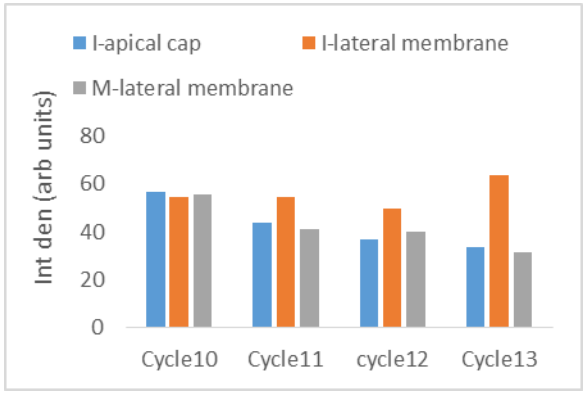


Fig.3.5: Sep2 distribution in the syncytial embryo on the apical and lateral membrane z-sections through interphase and metaphase of different NCs. (Explanation of marked regions described in Results section II)

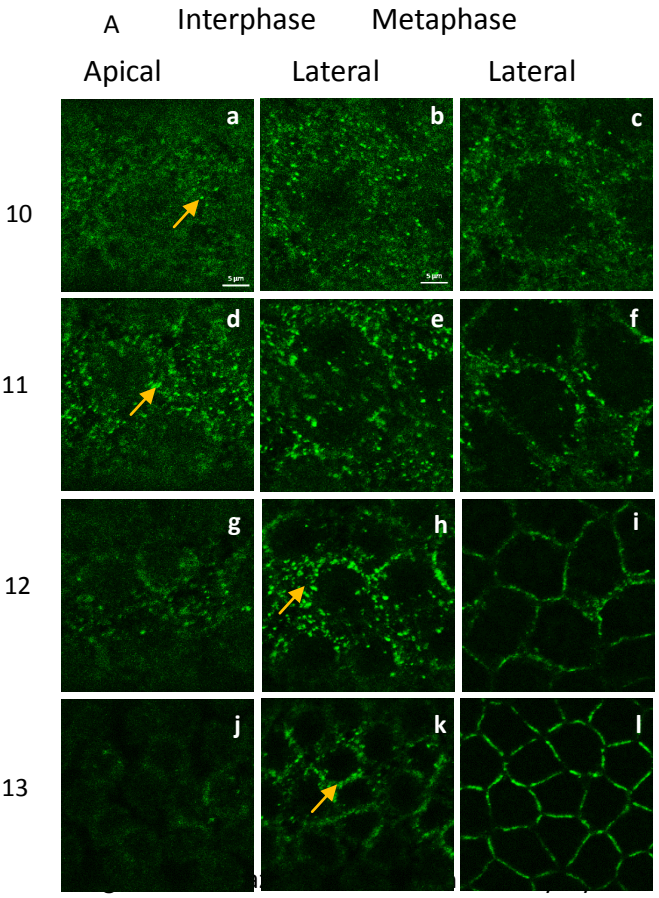


Graph3.13: Total intensity in the PM labelled with Sep2 in NC 10-13

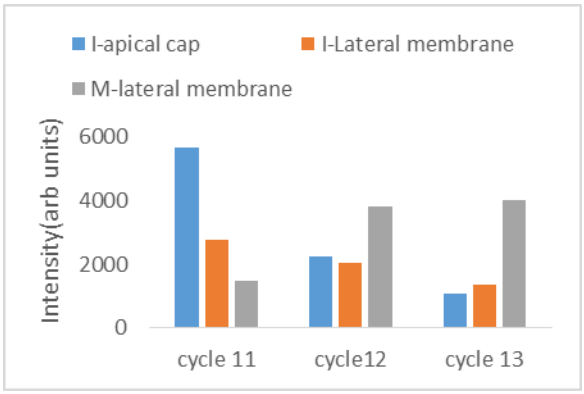


Graph3.14: Intensity density in PM labelled with Sep2 from NC 10-13

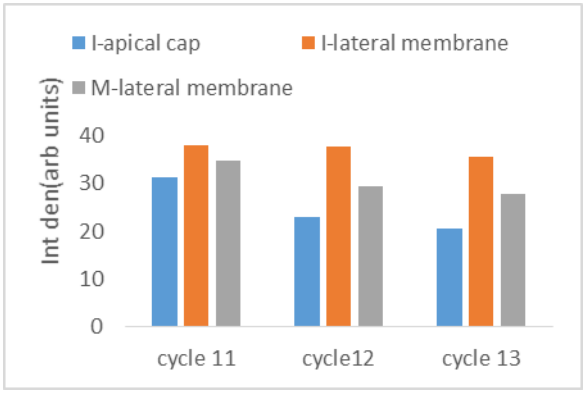
Baz/Par-3 is a protein of the apico-lateral complex seen in typical epithelial cells. Numerous tiny punctae of Baz are seen in the apical most section which cannot be made out to be labelling villi or any region of the PM in particular (Fig.3.6.A.a, d arrows). However, these punctae seem to partition into the lateral membrane sections in interphase 11 and 12 and are seen distinctly on the lateral membrane in interphase 13. The total intensities in the apical caps and the lateral membrane show a trend similar to that of Sep2 (Graph3.15) and the intensity density in the lateral membrane is almost constant through all the NCs (Graph3. 16).



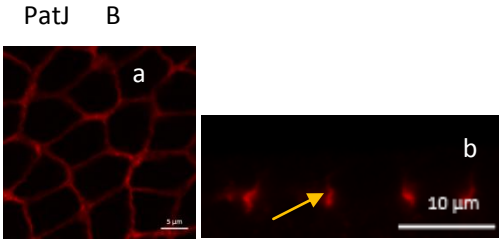
embryo on the apical and lateral membrane z-sections through interphase and metaphase of different NCs, B. a. PatJ immunostaining of WT embryo showing PatJ on the lateral membrane, b. on the furrow tips. (Explanation of arrows described in Results section II)



Graph3.15: Total intensity in the PM labelled with Baz in NC 11-13



Graph3.16: Intensity density in PM labelled with Baz from NC 11-13



PatJ, the member of the Crb-Sdt-PatJ apical complex in epithelial cells, on immunostaining in the syncytium shows a very clear labelling of tips of the metaphase furrows (Fig.3.6.B.b, arrow)Immunostaining of Baz GFP with PatJ antibody shows its localization to be lower than that of Baz (Fig.3.12b).

These three proteins can be termed to belong to a third category of protein markers for the PM. ‘Class III: Furrow tip’.

III. Relative distribution of tGPH, DE-Cad and Baz in the background of PH-PLC delta

Dual imaging of PHPLC with TGPH shows both markers labelling apical and lateral PM completely (Fig.3.7). Dual imaging of PHPLC and DE-Cad shows absence of the latter from the apical sections in NC 13, while PHPLC labels the apical surface. The lateral membrane in the deepest section imaged in metaphase 13 has both PHPLC and DE-Cad signal (Fig.3.8 top and bottom panels show apical section and a deeper lateral section respectively).Double imaging of Baz and PHPLC shows absence of Baz signal in the apical sections of metaphase in NC 13. However, the deepest section in metaphase imaged for NC 13 shows both the markers (Fig.3.9). These images again confirm that both DE-Cad and Baz have a lateral localization.

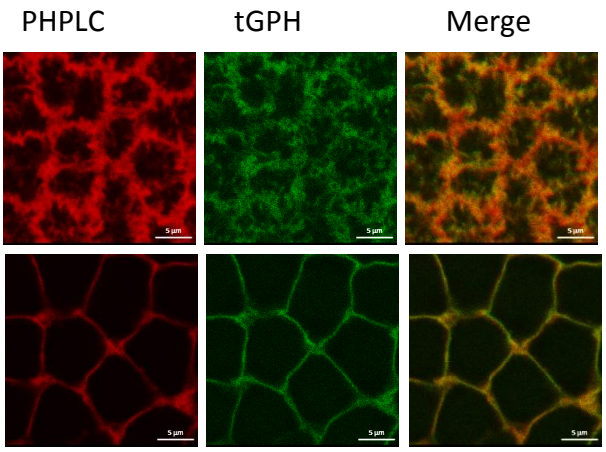


Fig.3.7: Top panel shows an apical section of the embryo, the bottom panel shows the lateral membrane in the deepest Z-section imaged of an embryo carrying both tGPH & PHPLC membrane labels

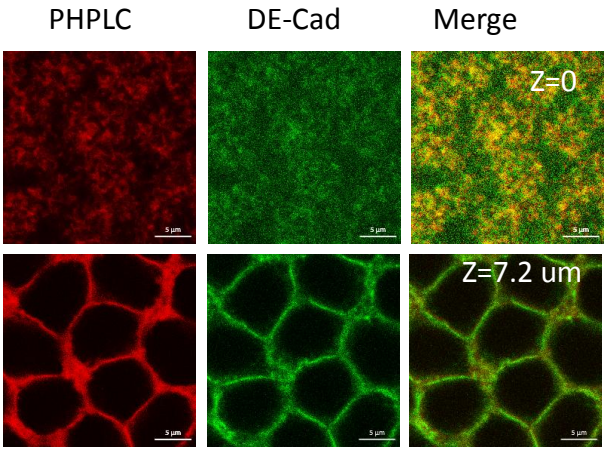


Fig.3.8: Top panel shows an apical section of the embryo, the bottom panel shows the lateral membrane in the deepest Z-section imaged of an embryo carrying PHPLC and DE-Cad membrane markers

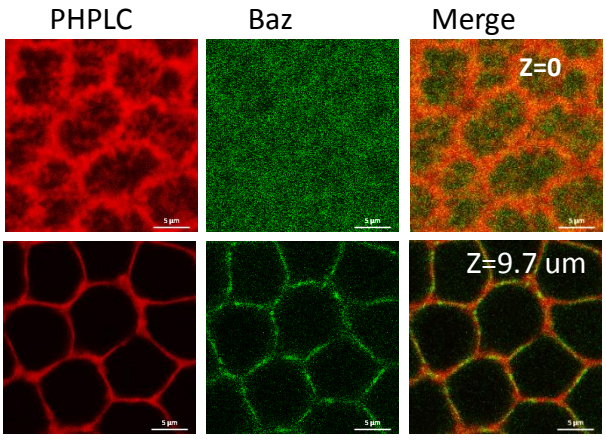


Fig.3.9: Top panel shows an apical section of the embryo, the bottom panel shows the lateral membrane in the deepest Z-section imaged of an embryo carrying PHPLC and Baz

IV. The intensity profile of metaphase furrows of NC 13 show at least two distinct regions of localization of Class II and III proteins

On plotting an intensity profile of orthogonal (xz) sections for the various classes of PM markers, different kinds of profiles are seen for each of the classes. For tGPH, the higher gray values are concentrated in a 0-4 um depth from the surface (Fig.3.10a, Graph3.17a).The figures have been shown as a thermal intensity with red being the high gray values and blue showing low gray values of intensity. The intensity profile of DE Cad in the metaphase of NC 13 shows higher gray values in the lateral membrane at a depth of 4-9 um (Fig.3.10b, Graph3.17b).On inspecting the XZ intensity profile or the Z-sections visually, Toll labels the whole length of the lateral membrane. The intensity profile of Toll in metaphase of NC13 shows high gray values from a depth of 2 to 8um (Fig.3.10c, Graph3.17c). However,for Sep2 the high intensity pixels are confined to depth of 10-12 um of the metaphase lateral membrane (Fig.3.10d, Graph13.17d).Similarly, the intensity profile of Baz shows high gray values in a selective region of the lateral membraneat a depth of 8-10 um in the furrow (Fig.3.10e, Graph3.17e). In both these cases, the depth at which high gray values are seen is only a sub-region of the whole length of the furrow, unlike a marker like TGPH or DE-Cad. Both of the latter labels have a broader intensity profile along the length of the furrow.

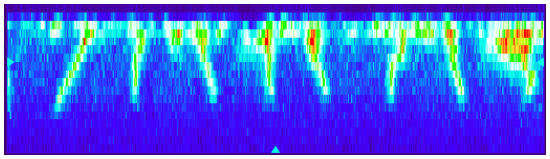
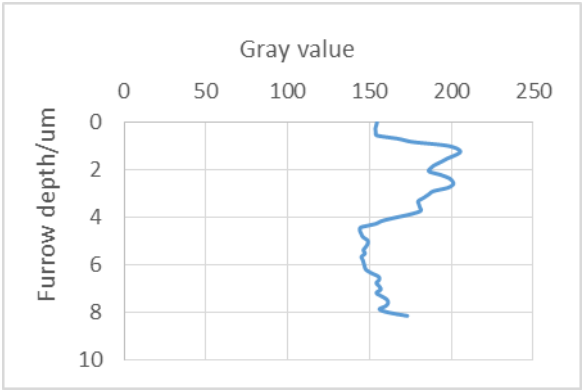


Fig3.10a: Intensity map of tGPH- XZ orthogonal section showing metaphase furrows of NC 13



Graph3.17a:Intensity profile in metaphase furrows of NC 13 labeled withtGPH

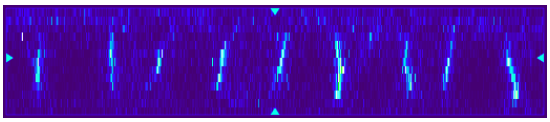
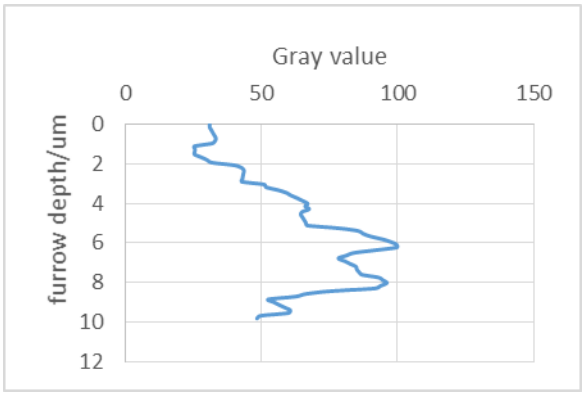


Fig3.10b: Intensity map of DE-Cad- XZ orthogonal section showing metaphase furrows of NC 13



Graph3.17b: Intensity profile in metaphase furrows of NC 13 labelled with DE-Cad

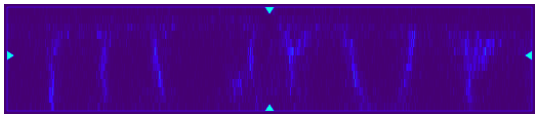
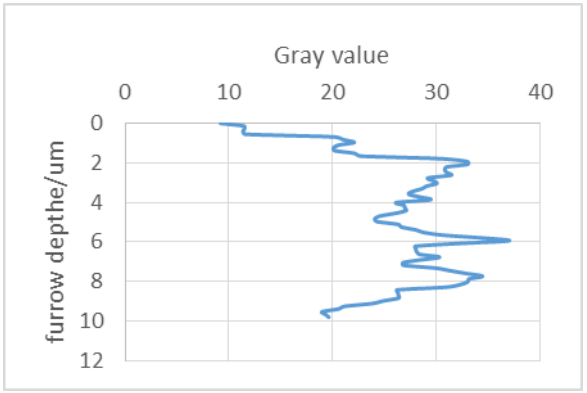


Fig3.10c: Intensity map of Toll- XZ orthogonal section showing metaphase furrows of NC 13



Graph3.17c:Intensity profile in metaphase furrows of NC 13 labelled with Toll

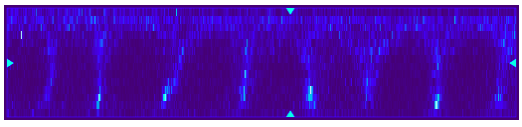
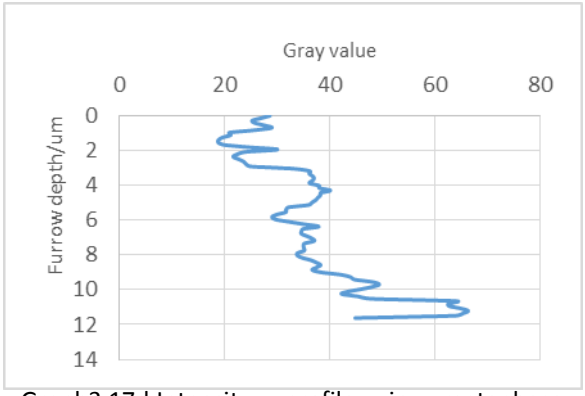


Fig3.10d: Intensity map of Sep2- XZ orthogonal section showing metaphase furrows of NC 13



Graph3.17d:Intensity profile in metaphase furrows of NC 13 labelled with Sep2

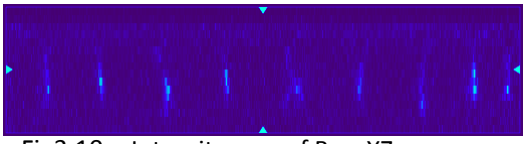
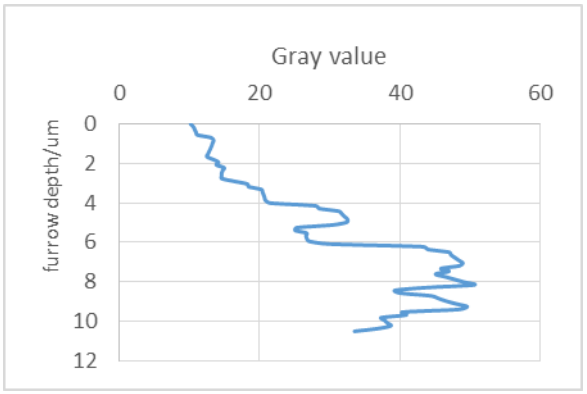


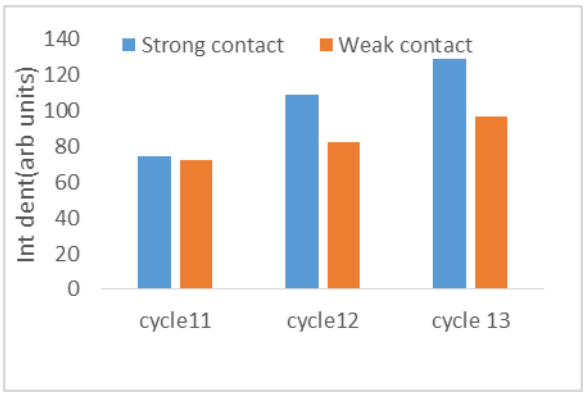
Fig3.10e: Intensity map of Baz- XZ orthogonal section showing metaphase furrows of NC 13



Graph3.14e:Intensity profile in metaphase furrows of NC 13 labelled with Baz

IV. Lateral membrane edges from any previous cycle have more DE-Cad than a newly formed one

Two types of contact edges can be seen in early prophase of each NC from 11-13- termed strong and weak contact edges (Fig.3.11, yellow arrow shows a strong contact and whitearrow shows a weak contact edge). Contact edges are difficult to make out for NC 10. Strong contact edges have higher intensity density of DE-Cad in them and they are the lateral edges that are remnant from the previous NC. Whereas, the weak contact edges are seen between two newly formed daughter nuclei. Consequently, the intensity density at strong contact edges is higher than that at weak contact edges (Graph3.13).



Graph3.18: Intensity density at contact edges in early prophase

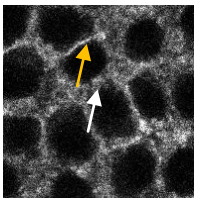


Fig.3.11: Lateral membrane of DE-Cad: white arrow shows a new membrane forming, yellow arrow marks an old lateral membrane

V. Edges and vertices are labeled with different proteins in metaphase of NC 13

The polygonal architecture of the PM of the deepest section in metaphase 13 also shows another remarkable partitioning of proteins. Membrane markers tGPH, PHPLC, DE-Cad, Toll, Sep2, PatJ mark the entirety of any polygon. In contrast, Dlg and Bazooka more distinctly mark only the edges of the polygons than the vertices (Fig.3.12, arrow points to missing Baz from a vertex). Immunostaining of PatJ in Baz GFP embryos shows PatJ aggregation more in the vertices of the polygons and Baz more in the edges of polygons (Fig.3.13a)

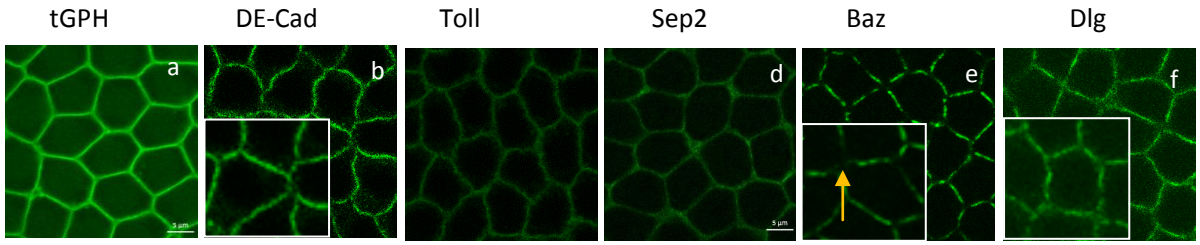


Fig.3.12: Lateral membrane of labelled by various protein markers a-f- insets in b, e, f show labelling at a vertex present in b, absent in e and f. Arrow in e points to a vertex with no Baz

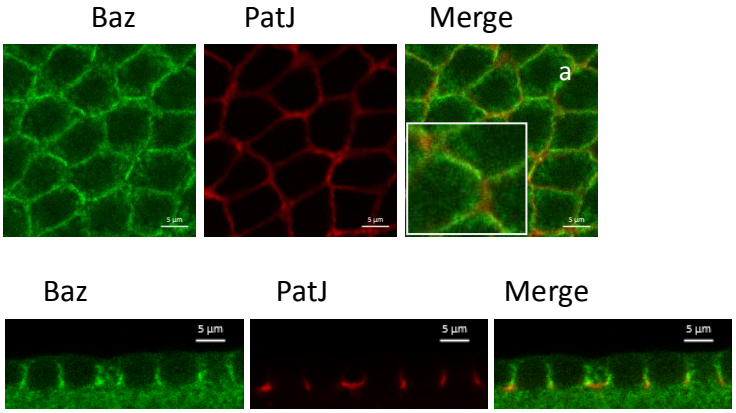


Fig.3.13 a. Surface view of nos gal4>UASBaz-GFP embryo immunostained with PatJ. Merge inset shows a vertex with no Baz staining. b. Sagittal section shows PatJ labelling below Baz.

VI. Effect on membrane architecture and polarity in Anillin mutants

Embryonic lethality of mutants

The anillin PQ/RS mutants have 98% embryonic lethality in 24 hours.

Pnut is lowered in the metaphase furrow tips of Anillin mutants

At cellularisation the architecture of the contractile ring created by the broadening furrow canals is lost. Pnut is not seen uniformly along the circumference of each contractile ring. Instead there is a more apical distribution of Pnut (Fig.3.14.a, b, yellow arrows). The nuclear membrane gets labelled with Anillin allowing visualization of nuclei. The overall shape of a cellularising compartment is loose, with the nuclei not aligned in a single row (Fig.3.14b, (ii) white arrow). In the syncytium too, Pnut does not localize at the furrow tips during furrow ingression. There is Pnut in the whole length of the furrow, but the extent of membrane association is lowered. There is more cytosolic Pnut.staining (Fig.3.14a, (i), (ii) follow white arrows).

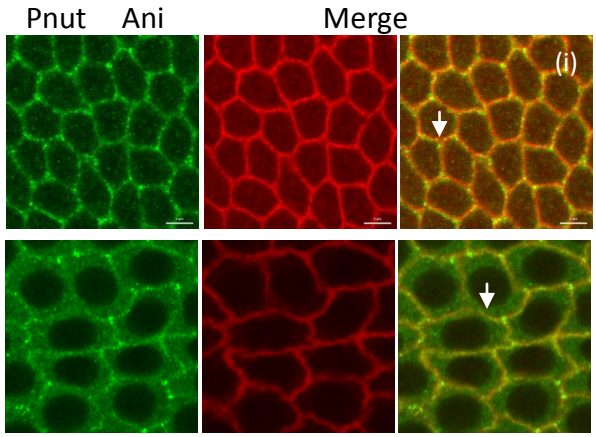
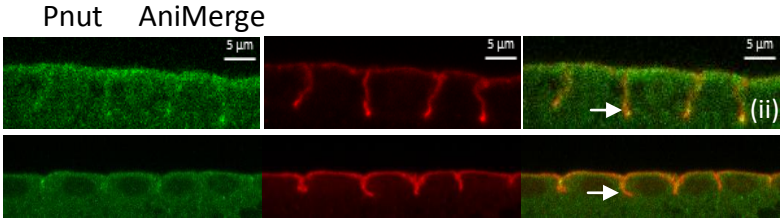


Fig.3.14a: (i) Surface section -Top panel shows WT syncytial embryo immunostaining with Pnut and Anillin,, (ii) Sagittal section- top panel showing WT embryo with Pnut in the furrow tips (arrow), bottom panel showing absence in the furrow tip (arrow)



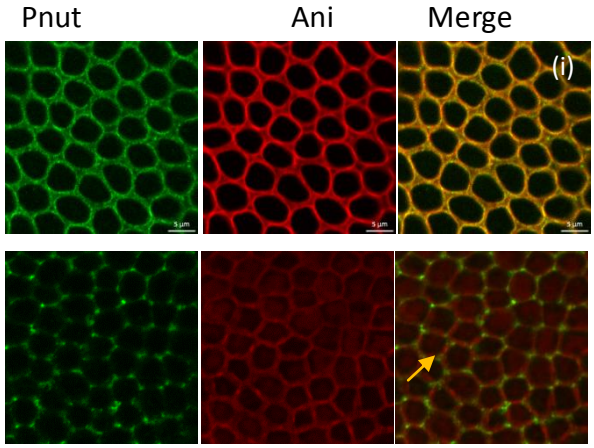
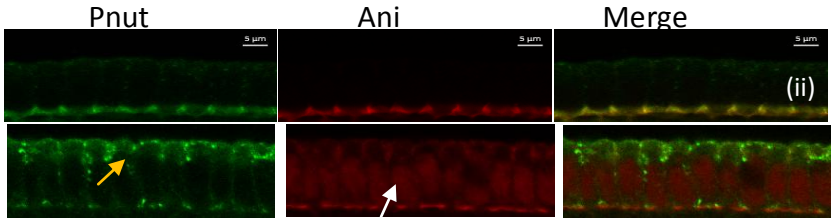


Fig.3.14b: (i) Surface view- top panel shows WT staining at late cellularisation, bottom panel shows staining in Ani mutants- yellow arrow shows Pnut mislocalisation, (ii) sagittal view- Yellow arrow in the bottom panel shows mislocalised apical Pnut and white arrow shows misaligned nucleus



Dlg is lowered in the lateral membrane

Dlg, staining in the syncytial embryos is also lowered on the lateral membrane and mislocalised more apically (Fig.3.14c, follow white arrows). There is more cytosolic signal for this protein in the mutant embryos. At cellularisation too, the Dlg signal on the lateral membrane is very fuzzy and looks cytoplasmic (Fig.3.14c, follow yellow arrows).

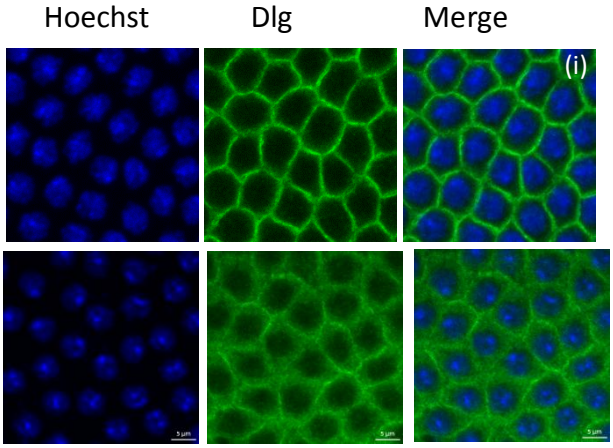
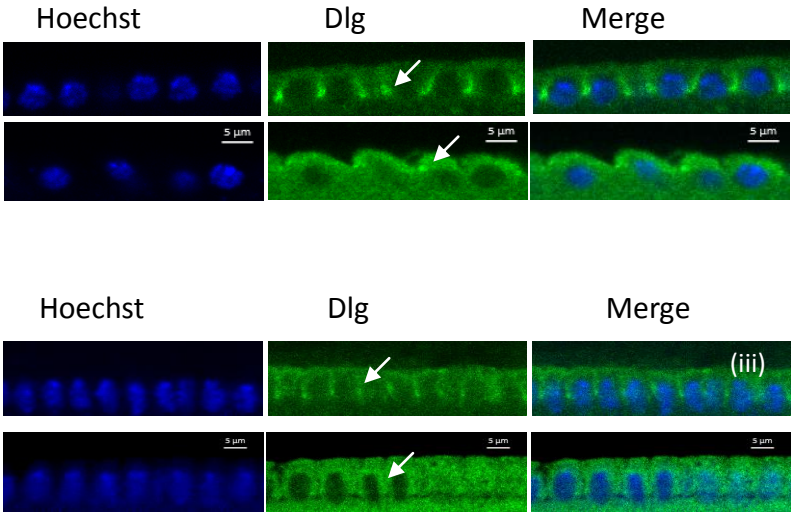


Fig.3.14c: (i) Surface view- top panel shows WT staining at late cellularisation, bottom panel shows staining in Ani mutants with lowered Dlg from the membrane (ii) sagittal view- white arrows in the bottom panel shows mislocalised apical Dlg, (iii) sagittal view of a cellularising embryo- white arrow in bottom panel showing mislocalisation of Dlg



Failure of metaphase furrow ingressions is common- with missing Dia and F-actin

There are frequent breaks in the membrane polygonal shape due to improper, non-uniform membrane ingressions. Diaphanous and F-actin staining is not visible at such sites and Pnut staining is diffuse (Fig.3.14d, arrows show breaks in Dia and absence of Pnut on the lateral membrane).

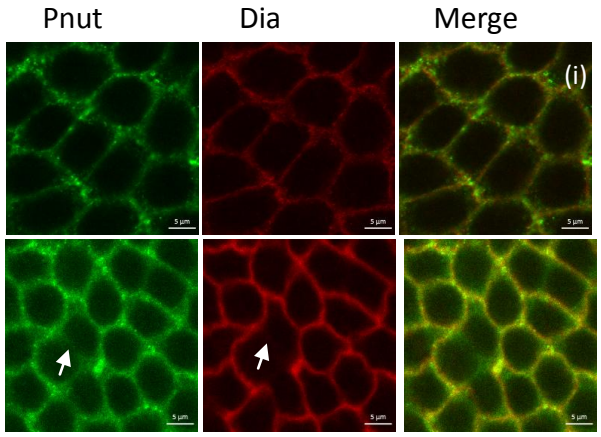
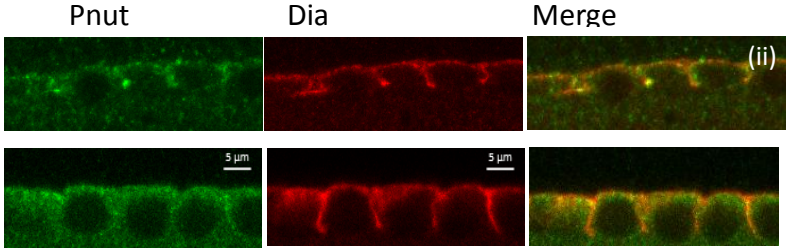


Fig.3.11d: (i) Surface view- top panel shows WT staining in the syncytium, bottom panel shows staining in Ani mutants- white arrow points to breaks in the lateral membrane



VI. Laser ablation of centrosomes causes disruption in the lateral membrane architecture

Centrosomes were visualized using Polo GFP and the membrane was labelled with DE-Cad GFP. Centrosomes were laser ablated and the dynamics of the PM followed post-ablation by live imaging of DE-Cad (Fig.3.14a, shows ablation in red circle). On the side in which centrosome was ablated, the PM failed to ingress as a furrow and the shape of the polygon in a deeper metaphase section was disrupted (Fig.3.14b, red region). The edges formed improperly on the side of centrosomal ablation whereas the edges were were properly formed on the side where the second centrosome was unaffected (Fig3.14b, yellow region).

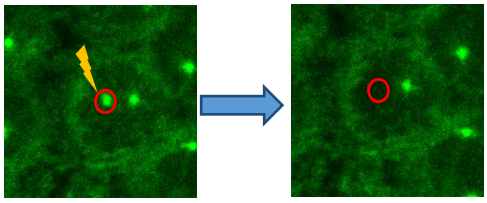


Fig.3.15a: Region marked by circle contains a centrosome and was ablated

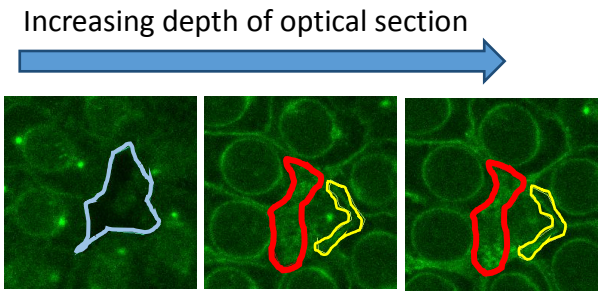


Fig.3.15b: Region marked in blue is the portion of apical PM that has fallen in loosely into deeper sections, where it is followed in red outline. The yellow region marks lateral membrane which shows proper labelling with DE-Cad

Discussion

Hexagonal organization in the syncytial NC 12 and 13 suggests epithelial architecture formation in the syncytium

The shape of the syncytial lateral membrane around each nucleus defines its packing. Hexagonal packing is known to be a minimal interfacial energy packing(Lecuit 2008).Proper membrane packing is essential for epithelial tissue. Here we see hexagonal packing arising from NC 12 and the percentage of hexagons in metaphase are the highest. Hexagonal packing involves adhesion and junction remodeling, which is believed to be modulated by E-Cadherin dynamics in epithelial cells (Lecuit and Lenne 2007; Cavey, Rauzi, and Lecuit 2008). DE-Cadherin containing exocyst vesicles are directed by planar cell polarity proteins for localization to the membrane during shrinking and growth of edges in wing epithelia. (Classen et al. 2005) The presence of planar cell polarity proteins has not been studied in the syncytium, but the existence of hexagonal packing suggests that adhesion junctions are being formed which shape it and these might also be getting remodeled with each NC as new PM is laid between daughter nuclei. The presence of higher amounts of DE-Cad on lateral membrane existent from a previous NC compared to that forming newly between nuclei also indicates that the lateral membrane may have formed strong junctions across PM of adjacent nuclei. There is also the possibility of packing of PM between nuclei being governed by the interplay between the nuclear array in the syncytium and F-actin in the caps. (Kanesaki et al. 2011)

At least two lateral membrane domains are forming in the syncytial embryo by NC 13

The PM of the syncytium is very dynamic in its organization. From studying the distribution of various proteins known to bind with the PM and an analysis of their intensity profiles, three classes of protein distribution are evident. Class I labels the complete PM. The transgenic lines which labels PIP2 and PIP3 namely, tGPH and PH-PLC delta belong to this category. Class II proteins primarily are transmembrane proteins which differentially mark the lateral membrane more clearly with each NC. Class III proteins are chiefly membrane associating proteins which mark only a part of the lateral membrane in metaphase, typically the lower regions of the lateral

membrane. These distributions become very clear only by NC 12 and 13 when the depth of the lateral membrane has increased sufficiently to discern this clearly from the apical membrane.

The intensity analysis of the distribution of proteins gives an idea of how the lateral membrane is organized. For each of the proteins considered, the total intensity in the apical caps decreases with increasing NC. The apical cap size decreases with each cycle. Thus, the intensity per unit area in the apical caps is almost constant in each NC. There is more lateral membrane in interphase of each NC with an accompanying increase in the protein distribution to it. This means that a lateral membrane forms in interphase of each cycle which tends to accumulate the PM markers studied.

The total lateral membrane intensity in metaphase increases with each NC for Class I and II PM markers. The double imaging with a Class I marker of proteins like PH-PLC delta with DE-Cad shows that the apical membrane indeed clears off these proteins in the later NCs and distributes on the lateral membrane. This means that the apical cap protein pool is moving into the lateral membrane in interphase and later in metaphase. Of note is the observation that the increase in the intensity in lateral membrane is over and above the magnitude of decrease in total apical intensity for these Class I and II markers. This suggests some new addition of the proteins to the lateral membrane in addition to redistribution from the apical caps. To test this idea, it would be required to tease apart the two ways of protein additions to the lateral membrane. One way could be to use photoactivable transgenic lines, which can be activated apically and the distribution and movement of the proteins from the apical sections can be tracked into the lateral membrane by live imaging. Colocalisation of a photoactivable fluorescent tag and a normal fluorescent protein tagged line of the same protein in the lateral membrane would help in confirming if indeed new protein recruitment is occurring along with redistribution from the apical caps. For Class III markers Sep2 and Baz, there is again an increase in the lateral membrane intensity during metaphase with each NC, but the increase is almost only about as much in magnitude as the drop in the apical cap intensity. This suggests only a relocalization into the lateral membrane from the apical cap for these molecules. Correspondingly, the intensity density in the lateral membrane is almost the same through each NC or decreases as the proteins localize only to certain

sections of the lateral membrane even as it ingresses deeper. This section of localization turns out to be the lower region of the membrane. It is difficult to eliminate the possibility of new addition of the proteins to the lateral membrane altogether, since there could be a highly dynamic exchange of the total protein pool associated with the membrane and that present in the cytoplasm which is balancing out to a net effect of constancy in the density of the signal observed. Photoactivation experiments would help in understanding the dynamics of these proteins better too.

Intensity profiles of each of these proteins in the metaphase furrows of NC 13 too show that the lateral membrane is not marked uniformly by these proteins. In particular for Class III proteins, the higher intensity pixels are shifted to the deeper sections of the furrow at a depth ranging from 8-11um from the surface of the embryos.

In all at least three domains form on the syncytial PM by NC 13

These observations taken together show that, the PM polarizes in its protein distribution on the apical and lateral membrane and that at least three different kinds of localizations are seen in the PM- one apical domain and two lateral membrane domains at the minimum. A characterization of the apical membrane apart from the villi labelling with PIP2, PIP3 and actin has still not been possible. The presence of apical domain proteins like aPKC, Cdc42 should be tested. Fig.4.1 summarizes the model of the organization of the PM with various polarity proteins in the syncytium at metaphase.

Transmembrane proteins like DE-Cad is possibly the first to organize in the lateral membrane

The question of which is the first protein or first set of polarized proteins present on the PM is still not effectively answered. The results suggest that transmembrane proteins like DE-Cad could be the first to organize in the PM in a polar manner, since they are present on the membrane from NC 10. Baz localization to the lateral membrane only becomes prominent from NC 12. Besides, apical localization of Baz is not seen during the NC which might be important for its polarizing functions. It has been shown that Baz is indeed relocalized apically from the tips of furrow canals only during cellularisation with the help of Par-1 and Dynein mediated directed

transport(Harris and Peifer 2005). The presence of Par-1 in the syncytium or directional transport of Baz has not been studied in the syncytium yet and is worth looking into. It would be interesting to check what happens to the localization of Baz in the *shotgun* (DE-Cad) mutants in the syncytium and that of DE-Cad in Baz mutants. If the localization of DE-Cad goes unaffected in Baz mutants, it would mean that junction formation is above apical polarity establishment in contrast to what has been previously reported to happen during cellularisation. (Harris and Peifer 2004)

A caveat to this analysis is that except for Dlg, all the other proteins imaged have been over-expressed genetically in the embryo. However, some reports on using the DE-Cadherin GFP show that the overexpression is not significantly higher than the endogenous levels of DE-Cad in the syncytium. (Abreu-Blanco, Verboon, and Parkhurst 2011) It has been difficult so far to achieve an immunostaining for DE-Cad in the syncytium to compare the endogenous versus overexpressed protein localization in this line.

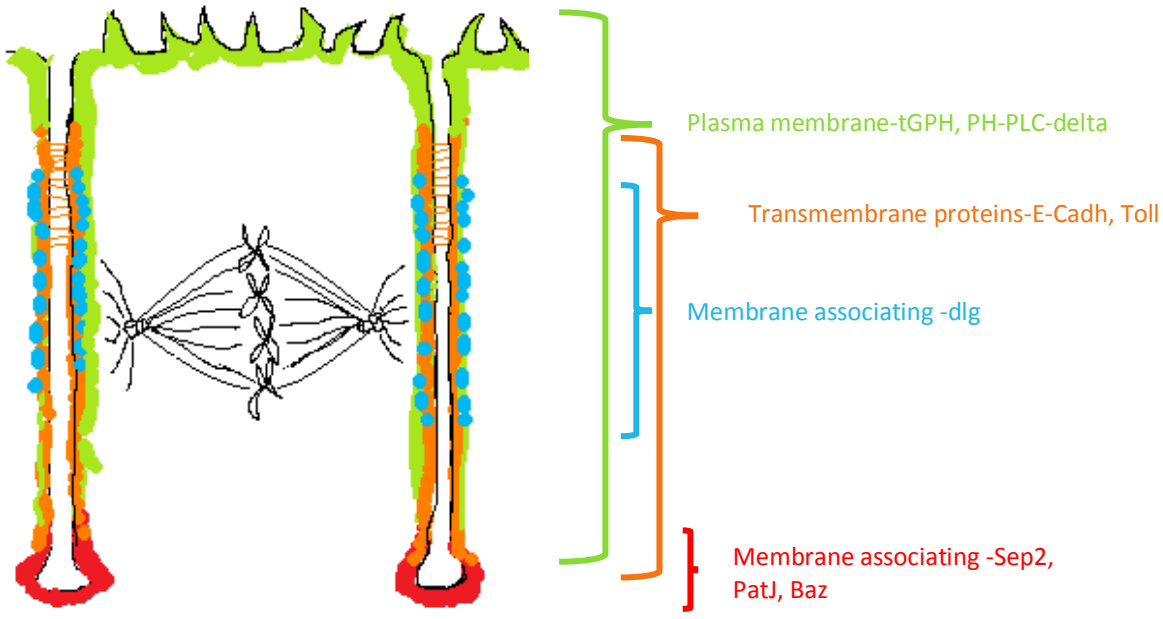


Fig.4.1: Schematic model of the PM organization in the metaphase of NC 12 or 13 in the syncytial embryo

Partitioning barriers between edges and vertices exist in the polygonal lateral membrane architecture of the NC 12 and 13

The partitioning of Baz and Dlg out of vertices in the metaphase lateral membrane of NC 12 and 13 that is observed indicates the presence of a barrier or inhibitory cue to their localization to the vertices. Exactly what is different about the vertices compared to the edges except for more number of adjacent cap-to-cap contacts at vertices than just two adjacent cap contacts along an edge is still unclear. FRAP analysis at edges and vertices with the various fluorescently tagged PM markers could be one way to determine the presence or absence of diffusion barriers in the lateral membrane.

Disruption of a complete PM marker and actin remodeling protein Anillin mislocalises the Septin- Pnut

The immunostainings in cellularising embryos with Anillin mutant for Pnut binding show mislocalised Pnut as previously reported.(Field et al. 2005) The loss in membrane architecture at cellularisation could be replicated here and clearly points out that correct localization of Pnut is required for this process. In addition, the absence of Pnut in the metaphase furrow tips of the syncytial embryo has been found which implicates roles for Anillin in Pnut recruitment in this stage too. Dlg localization is disrupted from the membrane of Anillin mutant embryos. Whether this phenotype is a result of Pnut mislocation or another effect of the point mutations of Anillin used here has still to be deciphered. If Pnut mutants replicated Dlg mislocalisation then the former possibility can be confirmed and the role of Pnut in generation of proper PM protein domains stronger. Pnut which is a septin, heteroligomerises with other septins in the syncytium, to create polymeric structures, whose roles as membrane scaffolds in the syncytium is as yet unexplored. These observations coupled with other Pnut defects observed (data not shown) in membrane-bending mutants open up this intriguing possibility. Broken lateral membrane in these mutants, with missing Actin and its remodeling protein Diaphanous indicates that Actin remodeling is affected in the ingressing PM. The possibility that the PM itself is not ingressing cannot be completely eliminated from the stainings done, and a marker labelling PM would be needed to check its presence or absence at these sites.

Centrosomes are important for lateral membrane organization

Preliminary experiments of laser micro-ablation of centrosomes suggest very important role for them in maintenance of PM shape and dynamics. Further experiments of ablation would reveal which components of the PM they directly affect the localization of-the transmembrane proteins or PM associated proteins or the PM linked actin cytoskeleton. The question of where the initial cue for PM organization and polarization from- centrosomes or the nuclei they migrate is unanswered. Centrosomes have been found to be able to migrate to the periphery and organize tubulin, actin and the PM when DNA synthesis is inhibited (Sullivan 1996; Raff and Glover 1988). Study shows that centrosome replication can be decoupled from that of the nuclei and that these nuclei-independent centrosomes can independently migrate to the cortex of the embryo and influence the PM (McCleland and O'Farrell 2009). It would be interesting to do similar decoupling experiments to find which of these carries the potential cue for the subsequent PM dynamics of the syncytium. This is especially interesting in the light of a recent paper showing importance of centrosomes in the polarity of C.elegans embryo immediately after fertilization.(Auxin et al. 2004)

Conclusion and future directions

This work is the first time a characterization of the organization PM of the syncytium has been done. It highlights that at leastthree PM domains form very distinctly in the syncytial embryo. In other words, the PM is polarized in its organization. The existence of PM domains in a system that does not have complete cells is surprising and may explain the compartmentalization of the organelles in the syncytium as was previously observed (Mavrakis and Rikhy 2010). It would be interesting to study how on disrupting PM organization the organelle compartmentalization gets affected. The syncytium of Drosophila has been well studied for various developmental morphogen gradients (Jaeger and Reinitz 2012). The dynamic studies of some of these indicates the absence of a free diffusion model(Daniels et al. 2012; Kanodia et al. 2009; Lander, Nie, and Wan 2002). Can the PM architecture of the syncytium affect gradient dynamics and distribution in the embryo is a whole area unexplored. Some preliminary work indicated such influence of the PM on the morphogen gradient shapes(Mavrakis and Rikhy 2010). Lastly, how close are these domains in their

similarity to the various protein domain organization and architecture of the PM of a typical epithelial cell is as yet unanswered.

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