

Syndapin Functions in Orchestrated Remodelling of the Membrane and Actin Cytoskeleton

A THESIS

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

**OF THE DEGREE OF
DOCTOR OF PHILOSOPHY**

BY

APARNA SHERLEKAR

20103078



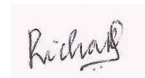
**INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH,
PUNE, 2016**

CERTIFICATE

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The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution. I further certify that the above statements made by her in regard to her thesis are correct to the best of my knowledge.

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(Supervisor)



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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH (IISER), PUNE
 (An Autonomous Institution, Ministry of Human Resource Development, Govt. of India)
 Dr. Homi Bhabha Road, Pashan, Pune – 411 008

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Form for submission of Ph.D. Thesis

Declaration by student

Name of Student: APARNA SHERLEKAR Reg. No.: 20103078

Thesis Supervisor(s): DR. RICHA RIKHY Department: BIOLOGY

Date of joining : 2-08-2010 Date of Pre-Synopsis Seminar : 23-05-2016

Title of Thesis : SYNDAPIN FUNCTIONS IN ORCHESTRATED
 REMODELLING OF THE MEMBRANE AND ACTIN CYTOSKELETON.

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(I/II)

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LIST OF ABBREVIATIONS

Critical concentration	(Cc)
Atomic Force Microscopy	(AFM)
Nucleation promoting factors	(NPFs)
Actin related protein	(Arp)
Actin-related protein complex	(ArpC)
Neuronal Wiskott-Aldrich Syndrome protein	(N-WASp)
Connecting motif	(C)
Acidic motif	(A)
Diaphanous	(Dia)
Formin homology	(FH)
GTPase protein binding domain	(GBD)
Diaphanous inhibitory domain	(DID)
Diaphanous auto-inhibitory domain	(DAD)
Actin-binding domain	(ABD)
Pleckstrin homology	(PH)
Phosphatidylinositol diphosphate	(PI(4,5)P ₂)
Nuclear cycle	(n.c.)
GTPase-activating protein	(GAP)
Guanosine nucleotide exchange factor	(GEF)
Rho-GTPase-activating protein	(RacGAP)
Nuclear fallout	(Nuf)
Recycling endosome	(RE)
Early endosome	(EE)

Bin, Amphiphysin, Rvs	(BAR)
Amphyphysin	(Amph)
WASp homology	(WH)
Neuronal BAR	(N-BAR)
Phox BAR	(PX-BAR)
Cdc42-interacting protein 4	(CIP4)
Fer-CIP4 homology (FCH) BAR	(F-BAR)
Inverted BAR	(I-BAR)
Missing in metastasis	(MIM)
Src Homology 3	(SH3)
Proline Rich	(PR)
Cordon Bleu	(Cobl)
Latrunculin A	(LatA)
Cytochalasin D	(CytoD)
Jasplakinolide	(Jasp)
Synaptic Dynamin binding protein	(Syndapin)
Protein kinase C and casein kinase II interacting protein	(PASIN)
Furrow tip	(FT)
Cleavage furrow tip	(CFT)
Fluorescence Recovery after Photobleaching	(FRAP)
Fluorescence Loss In Photobleaching	(FLIP)
General Receptor for Phosphoinositides-1	(GRP1)
Phospholipase C	(PLC)
Shibire	(Shi)

temperature sensitive

(ts)

Peanut

(Pnut)

ABSTRACT

The syncytial nuclear division cycles during early *Drosophila* embryo development involves orchestrated remodelling of the membrane and cytoskeleton. We have shown that the F-BAR domain protein Syndapin, that is known to have roles in membrane tubulation, endocytosis and cytoskeletal remodelling, is increasingly enriched at the tip of syncytial pseudocleavage furrows with each nuclear cycle. The dynamics of pseudocleavage furrow progression depends on Syndapin function. We showed that Syndapin function was important for recruitment of septin Peanut and organization of the formin Diaphanous at the furrow tip downstream of the furrow initiation protein RhoGEF2. Syndapin-depleted embryos showed disorganized actin on the furrow, also seen in RhoGEF2 mutants. Interestingly, Syndapin overexpression in RhoGEF2-depleted background, restored actin organization and extent of furrow ingression. Additionally, we showed that Syndapin or RhoGEF2 depletion causes reduction in the number of Rab5 puncta; however, Syndapin overexpression in RhoGEF2-depleted background cannot restore the number of Rab5 puncta. Therefore, we conclude that Syndapin can rescue the furrow extension phenotype by actin organization alone and not because of its role in early endocytosis.

Actin turnover is also crucial for dynamic expansion of the apical caps prior to furrow formation. Arp2/3 function is important for expansion of the apical cap. We find that *syndapin* mutants limit cap expansion and show abnormally large actin caps. We are able to rescue this cap expansion phenotype by combining mutants of a component of the Arp2/3 complex, ArpC1 with the *syndapin* mutant. Using TIRF microscopy, we find that remodelling of apical actin structures is defective in these mutants giving rise to the observed phenotypes. Therefore, Syndapin and Arp2/3 complex oppose each other to regulate dynamics of actin cap expansion in the syncytial embryo.

Apical cap expansion defects also correlate with defects in furrow extension and formation of edges of polygonal compartments where the expanding caps meet. Therefore, we also studied effects of Syndapin depletion and also of other actin regulatory proteins on the distribution of polygon classes that are conserved across organized epithelial sheets in various tissues with a maximum of hexagons. The result was a frequency distribution skewed towards pentagons rather than hexagons. Interestingly, however, the recruitment of junctional polarity proteins was not affected in embryos depleted of Syndapin.

Through similar conserved mechanisms, Syndapin is, therefore, crucial to limit apical cap expansion which later also affects furrow ingression and polygonal distribution in syncytial blastoderm embryos of *Drosophila*. These functions are probably conserved in processes like apical emergence and cleavage furrow extension.

SYNOPSIS

Name of the Student: APARNA SHERLEKAR

Registration Number: 20103078

Name of Thesis Advisor: DR. RICHA RIKHY

Date of Registration: 2nd AUG., 2010

Place: Indian Institute of Science Education and Research (IISER), Pune

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Introduction

Various conserved cellular processes like membrane trafficking, cell migration, ciliogenesis, cytokinesis etc. require dramatic membrane deformations that are driven by forces generated from remodelling the actin cytoskeleton. The search for conserved membrane-binding proteins that cooperate with the cytoskeleton in shaping the plasma membrane yielded the Bin–Amphiphysin–Rvs (BAR) domain-containing proteins. The BAR-domain proteins are further classified based on the curvature of the membrane they bind to or help sculpt. The FES-CIP4 homology (FCH) domain is the archetypal feature of all F-BAR proteins. F-BAR domain protein Syndapin has an EFC (extended FCH) domain that interacts with negatively charged phospholipids and the SH3 domain, a protein-protein interaction domain (Quan & Robinson, 2013; Suetsugu, 2016). To mechanistically study the role of Syndapin in orchestrating dynamics of the membrane and actin cytoskeleton in an *in vivo* context, we chose the *Drosophila* syncytial blastoderm embryo as a model in which rapid nuclear division cycles are accompanied by dramatic remodelling of the membrane and actin cytoskeleton.

Unlike other F-BAR domain containing proteins like CIP4 or FCHO1 that generate tubes of low curvature, Syndapins can stabilize tubules of various diameters and can also facilitate membrane constriction *in vitro* giving Syndapin its unique membrane remodelling potential (Bai et al., 2012; Wang et al., 2009). This allows Syndapins to be recruited to and function in the formation and maintenance of membrane structures such as caveolae (Hansen, Howard & Nichols, 2011), clathrin-coated vesicles (Kessels & Qualmann, 2002), synaptic vesicles in

activity-dependent bulk endocytosis (Clayton et al., 2009), ciliated sensory hair cells (Schüler et al., 2013), dendritic spines (Schneider et al., 2014), filopodia (Shimada et al., 2010) and lamellipodia (de Kreuk et al., 2012). *Drosophila* Syndapin was also shown to play a role in cytokinesis and interact in vitro with the scaffold protein Anillin (Takeda et al., 2013) which recruits various actin assembling proteins at the cleavage furrow. In a recent study, we further mechanistically show using the syncytial pseudocleavage furrow of the *Drosophila* embryo as a model that Syndapin is crucial for furrow ingression. Importantly, we found that Syndapin can rescue furrow extension phenotypes by organization of actin in the pseudocleavage furrow in the RhoGEF2-depleted background by regulating actin assembly in the furrows (Sherlekar & Rikhy, 2016).

Syndapins have long been known to interact with N-WASp via its SH3 domain and thereby activate Arp2/3-mediated actin polymerization (Kessels & Qualmann, 2002; Modregger et al., 2000). Syndapin 2 SH3 domain also interacts with another actin nucleator Cordon Bleu (Cobl) (Ahuja et al., 2007; Koch et al., 2011; Schwintzer et al., 2011) and with C-terminus of Rac1. Syndapin 2 negatively regulates Rac1 signalling in cell spreading and wound healing; conversely inhibition of Rac1 promotes membrane tubulation by Syndapin 2 (de Kreuk et al., 2011). Interaction with Cobl mediates formation and regulation of ciliary (Schüler et al., 2013) and microvillar length (Wayt & Bretscher, 2014). In an attempt to reconcile the large body of literature on interactions of BAR proteins with membranes as well as regulatory proteins of the cytoskeleton, Kostan et al. (2014) showed that the F-BAR domain of Syndapin 2 can interact electrostatically with actin filaments and that there exists a competition, albeit weak, between negatively charged liposomes and F-actin for interaction with the F-BAR domain of Syndapin 2 and not other F-BAR proteins like CIP4 or FCHO2 (Kostan et al., 2014).

Along with its role in furrow ingression we also show that Syndapin plays an antagonistic role to the Arp2/3 complex to limit the expansion of the apical actin cap above each nucleus that, in turn, affects furrow ingression. Once these caps expand and come into contact, they form furrows which are organized as a polygonal sheet of compartments with a conserved frequency of polygon sidedness. We also show that Syndapin function is important for maintaining this ratio of sidedness of polygons.

Therefore, we show that Syndapin role is important for regulated dynamics of membrane and actin cytoskeleton – crucial for the development and patterning of the *Drosophila* embryo.

Results and Discussion

1. Syndapin promotes pseudocleavage furrow formation by actin organization in the syncytial *Drosophila* embryo

Since nothing was known about Syndapin localization or function in the *Drosophila* embryo, we first used antibody staining to ascertain its localization. We also used a GFP-tagged Syndapin transgene (Synd-GFP) to study the dynamics of recruitment of Syndapin to the membrane, both by quantifying intensity levels from time-lapse movies and using Fluorescence Recovery After Photobleaching (FRAP) analysis. We found that although Syndapin is present on the entire plasma membrane, it is specifically enriched at the furrow tip compared to the apical membrane.

We then examined the effects of Syndapin depletion using a hypomorphic mutant for Syndapin (*synd*) in the background of tGPH (a GFP-tagged pleckstrin homology domain that labels PIP3 on the membrane) on dynamic remodelling of the plasma membrane during the nuclear division cycles during which the plasma membrane extends into the embryo cortex to form furrows which subsequently regress before the next cycle. We showed that *synd* embryos showed slower furrow dynamics; the furrows during the nuclear cycle were also significantly shorter than wild-type furrows. We were able to restore furrow length by overexpression of Synd-GFP in the *synd* mutant background. Using Kymograph analysis, we also showed that the furrows had slower movement along the XY plane.

Since Syndapin is known to have roles in endocytosis (Kessels & Qualmann, 2002), we checked numbers of Rab5-+ve puncta in wild-type embryos versus those depleted of Syndapin. We found that the numbers of Rab5 puncta were significantly reduced in *synd* mutant embryos compared to wild-type. We also tested whether Syndapin functions in forming cleavage furrow tip (CFT) tubules that are present at the tips of growing furrow membranes and are endocytosed during regression. We found that CFT tubules are shorter in *synd* mutant embryos and thus showed that Syndapin has a role in membrane tubulation.

Next, we tested whether known actin remodelling proteins that also localize to the furrow tips also affect furrow dynamics. It is known that the mutant of scaffold protein Anillin and furrow initiation protein RhoGEF2 affect furrow formation. Consistent with this, we found that *anillin* and *rhogef2* embryos showed slower furrow dynamics than wild-type. We also

found that depletion of the septin Peanut that is recruited by Anillin shows shorter furrow lengths compared to wild-type.

We found that *rhogef2*, *pnut* and *synd* all have defective furrow dynamics and that RhoGEF2 is crucial for initiation of furrow formation; we thus hypothesised that one/both of Syndapin or Peanut proteins may be the key component downstream of RhoGEF2 crucial for actin-driven furrow extension. Therefore, we overexpressed Peanut-mCherry and Synd-GFP (which independently have similar furrow dynamics as control embryos) in the RhoGEF2-depleted background and measured furrow dynamics. Interestingly, we observed a rescue upon over-expressing Syndapin but not Peanut in this background, thereby establishing Syndapin as a key downstream protein that can regulate furrow ingression.

Since our observations showed that Syndapin was crucial to furrow extension, what remained was to test the mechanism by which this could happen. Actin dynamics is known to drive membrane dynamics of the furrow in various contexts. Therefore, we checked if actin organization in the furrow is perturbed in *synd* embryos. With confocal microscopy we found that there were fewer F-actin puncta in *synd* compared to control embryos. We imaged this using Airyscan for better resolution and found that long organized structures seen in wild-type were missing and disorganized ‘blobs’ were present.

Now that we knew that Syndapin and actin regulatory proteins all show defects in furrow morphology and dynamics and Syndapin shows defects in actin organization, we also checked if the actin regulatory proteins Peanut, Diaphanous and RhoGEF2 are affected in *synd* mutants. We found that there is drastic loss of Peanut recruitment to the membrane; Diaphanous that is normally enriched at the furrow tip is spread all along the furrow membrane. However, RhoGEF2 was still localized to the membrane in *synd* embryos. In a converse experiment, we checked if Syndapin recruitment is lost in the actin regulatory protein mutants. Not surprisingly, we found that Syndapin is lost in each of these mutants. We performed a comprehensive analysis of recruitment of each of these proteins in their mutants and found that a complex interplay exists between these proteins and that a linear pathway does not emerge.

Since Syndapin overexpression can rescue furrow extension in RhoGEF2 depleted background, we predicted that this rescue may be a result of the role Syndapin plays in actin organization. Indeed, we found that Syndapin overexpression can rescue the poorly organized

actin seen upon *rhogef2* depletion. Also, Syndapin overexpression could not rescue the number of Rab5-+ve puncta that were lost when RhoGEF2 was depleted. Therefore, we concluded that the rescue of furrow dynamics we observe upon Syndapin overexpression in *rhogef2*^{RNAi} background is due to Syndapin's role in actin organization (Figure 1).

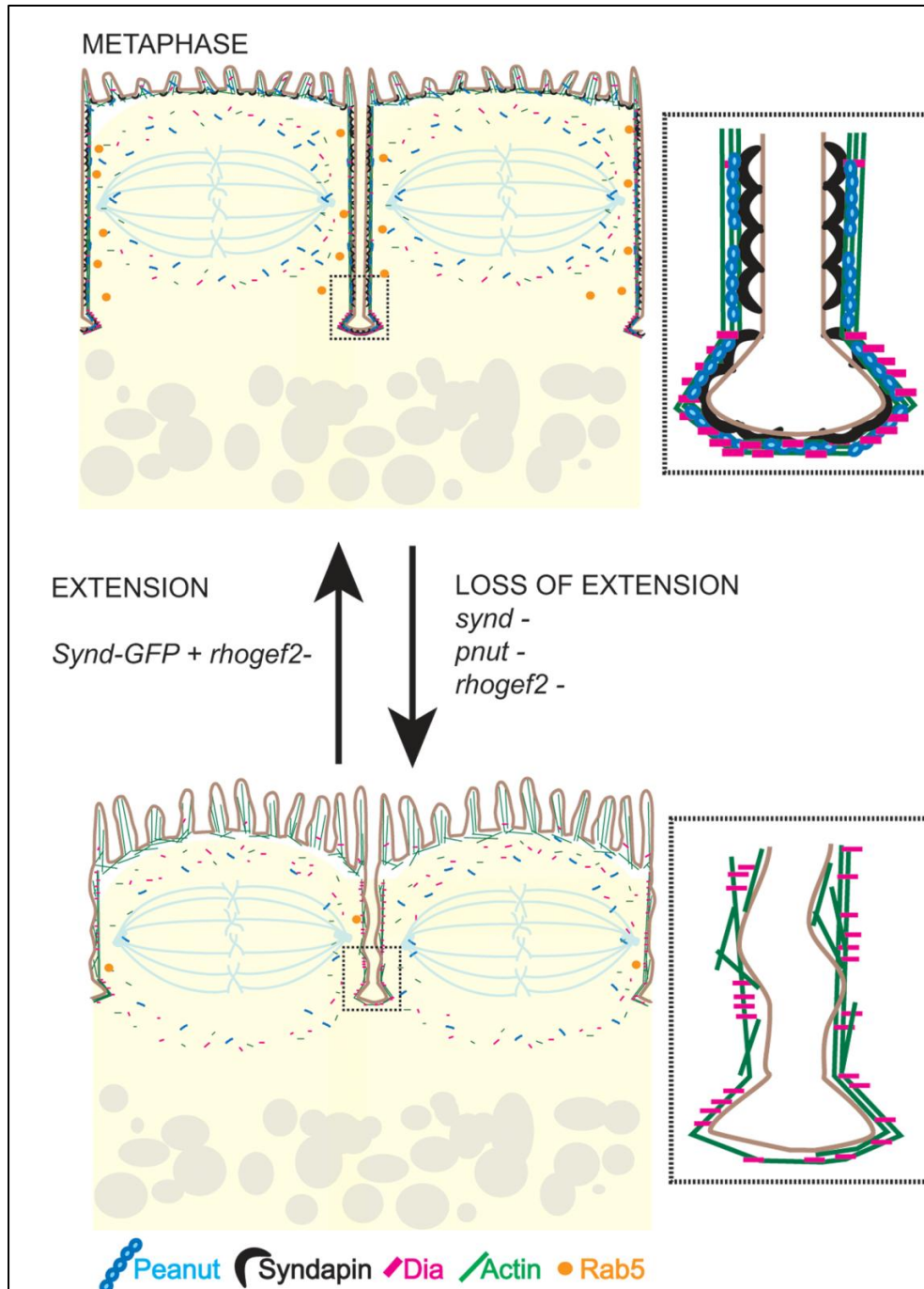


Figure 1. Schematic showing that the furrow defect in *synd*, *rhogef2* and *pnut* mutants is reversed by Synd overexpression in *rhogef2*^{RNAi}. Pseudocleavage furrow extension in metaphase of the syncytial cycle in *synd*, *pnut*, *rhogef2* mutants is decreased due to

recruitment defects in *Synd*, *Pnut*, *Dia* and actin and reversed in *rhogef2* mutants by overexpression of *Synd*.

2. Syndapin controls Arp2/3 complex-dependent actin cap expansion dynamics in *Drosophila* syncytial embryos

Since we showed that Syndapin can affect actin structures in the dynamic process of furrow extension, we also wanted to test Syndapin's role in apical cap expansion. Arp2/3 complex function is known to be important for cap expansion (Stevenson et al., 2002; Cao et al., 2010). Arp2/3 nucleates branched actin and this dendritic network of actin abuts the membrane and is thought, through treadmilling, to generate the forces needed to push the membrane outward. We first tested the dynamic localization of Syndapin and ArpC1 of the Arp2/3 complex and found that they are both enriched at the rim of the expanding cap at a depth of $\sim 3\mu\text{m}$. Thus, we know that both Syndapin and ArpC1 are present at the leading edge of the expanding cap and are thus poised to control actin dynamics in this region.

We then individually depleted Syndapin and Arp2/3 complex to check defects in cap expansion by measuring the change in area of the apical caps as they expanded. Consistent with previously published results with ArpC1 depletion, we found that in *arp3*^{RNAi} embryos, the apical caps fail to expand. Interestingly, in *synd* mutants, the apical caps are abnormally large, significantly bigger than the control caps. Thus, we know that Syndapin function is important to limit apical cap expansion. We, therefore, combined one copy of *arpC1* mutant allele with *synd* mutant and, interestingly, observed a rescue of cap area compared to control.

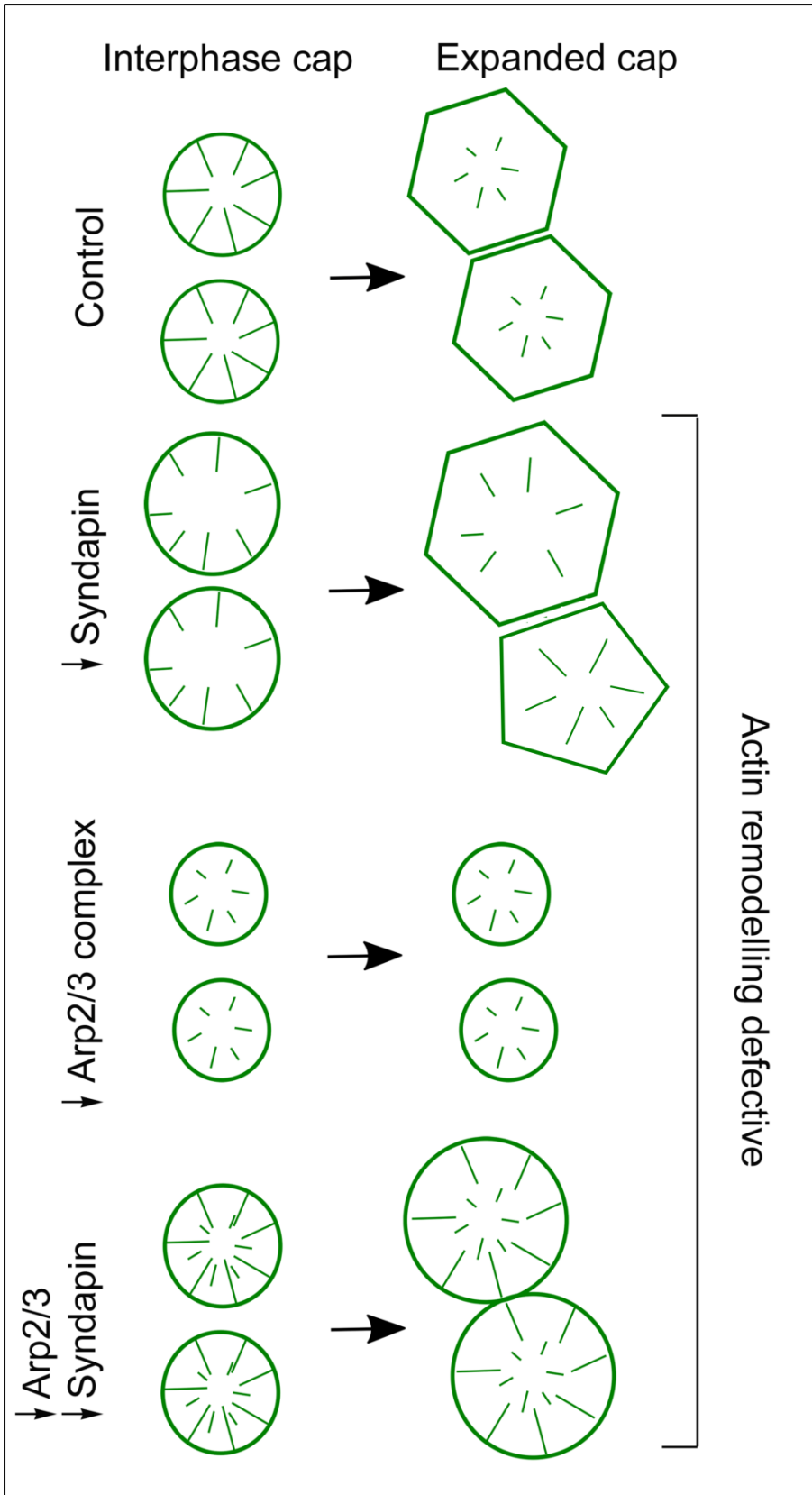
To understand the process mechanistically, we observed cap dynamics using TIRF microscopy and found that cap expansion in control caps involves remodelling of long actin protrusions visible on the surface in interphase caps into shorter protrusions, possibly extending into the depth of the embryo, by the time the caps have expanded. This remodelling of caps is defective in mutants for both ArpC1 and Syndapin. While in ArpC1-depleted embryos, the short protrusions predominate, in *synd* mutants, long protrusions persist throughout the process of cap expansion. In the combination of *synd* with *arpC1*, both long and short protrusions persist and are able to rescue area and rate of cap expansion, although other defects are present (Figure 2).

3. Syndapin regulates the frequency distribution of polygons in the ‘pseudoepithelial’ sheet

Apical cap expansion defects also correlate with defects in furrow extension and formation of edges of polygonal compartments where the expanding caps meet. Acto-myosin dynamics provides the force known as line tension at the cell edges in an epithelial sheet (Farhadifar et al., 2007). The sum of forces at the vertex generates a conserved distribution of frequency of polygon sidedness with a maximum of hexagons. We tested the role of Syndapin in maintenance of this polygon number ratio. We found that in Syndapin mutants, the polygons form what is known as a ‘soft’ network similar to what is observed upon Myosin-II depletion (Farhadifar et al., 2007) in which the polygon number ratio is skewed with larger number of pentagons and fewer hexagons than control, although the number of hexagons is still dominant (Figure 2). When we deplete actin regulatory proteins – Pnut, WASp and RhoGEF2 – we get a further skewed distribution with a maximum of pentagons.

Syndapin depletion, however, does not affect recruitment of polarity proteins that form part of junctional complexes like Caherin, Bazooka and Patj. Therefore, we predict that Syndapin affects a pool of actin that is associated with membrane tension but not with junctional protein assembly.

Figure 2. Model for apical actin cap expansion in syncytial *Drosophila* embryos. Circular apical actin caps expand, contact each other and form polygons as the nuclear cycle progresses from beginning of interphase to the beginning of metaphase in control embryos; correspondingly there is a shortening of apical protrusions in the caps (green lines). When Synd is depleted, the caps undergo runaway expansion and the protrusions do not shorten as the caps expand unlike in the control. When the caps meet to form furrows, increased numbers of pentagons are present compared to control. Caps that are depleted of components of the Arp2/3 complex show the opposite phenotype, the caps fail to expand and compared to the control, the protrusions are very short and remain so. These caps remain circular as the nuclear cycle progresses. When both Synd and ArpC1 are depleted, the caps expand normally but remain rounded perhaps because both long and short protrusions are continually present in both ICs and ECs. In all three mutant scenarios there seems to be lack of remodelling of the actin protrusions that display different phenotypic readouts.



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CHAPTER 1

Introduction

1.1 Membrane-cytoskeletal interactions: Cellular Morphology, Structure and Function

Cytoskeletal-membrane interactions yield morphological structures which facilitate a repertoire of cellular functions like cell motility, sensing the extracellular environment, intracellular/extracellular transport of materials and cytokinesis (Figure 1.1) (Griffiths, 2007). There are at least 15 such structures in metazoan cells and are formed in response to different stimuli that are spatio-temporally regulated in the cell. This is achieved by modulating the dynamics of a thin, interconnected network of actin filaments and non-muscle myosin-II (Myo-II) motors underneath the plasma membrane that together constitute the cell cortex (Clark, Dierkes, & Paluch, 2013). Among mechanical elements in the cell, only actin is closely associated with the membrane at the cortex, microtubules and intermediate filaments are arrayed throughout the cytoplasm. Actin filaments in the cortex provide mechanical support to the plasma membrane allowing it to resist deformation from extracellular forces or internal hydrostatic pressure thereby maintaining cell shape, volume and mechanical properties (Stewart et al., 2011). The actin-rich structures present superimposed on the cell cortex, with important and specific cellular functions, range from sheet-like lamellipodia and lamella to finger-like filopodia and microvilli.

Lamellipodia and Lamella are surface-attached, sheet-like membrane protrusions observed during crawling cell motility and spreading (Abercrombie, Heaysman, & Pegrum, 1971, 1970a, b,c). Lamellipodium is present more distally, extends for a few microns from the leading edge of the motile cell and contains a dense branched network of actin filaments with a majority of growing ends oriented towards the leading edge. It is less adherent, more dynamic, thinner and devoid of organelles compared to the lamellum. The lamellum extends from the lamellipodium to the cell body and has less branched, more stable bundled actin decorated with Myo-II motors (Svitkina & Borisy, 1999; Svitkina, Verkhovsky, McQuade, & Borisy, 1997). The rate of actin polymerization in the lamellipodium is two-fold faster than in the lamellum (Danuser & Waterman-Storer, 2006) and actin polymerization alone drives formation of lamellipodial protrusions (Thomas D. Pollard & Borisy, 2003). Latrunculin A, a metabolite of the *Latrunculia*

sponge that binds to G-actin and prevents it from incorporating into F-actin, concomitantly inhibits lamellipodial protrusion and cell migration (Yarmola, Somasundaram, & Boring, 2000). Other sheet-like structures include ruffles, phagocytic cups, podosomes and invadopodia (Chhabra & Higgs, 2007).

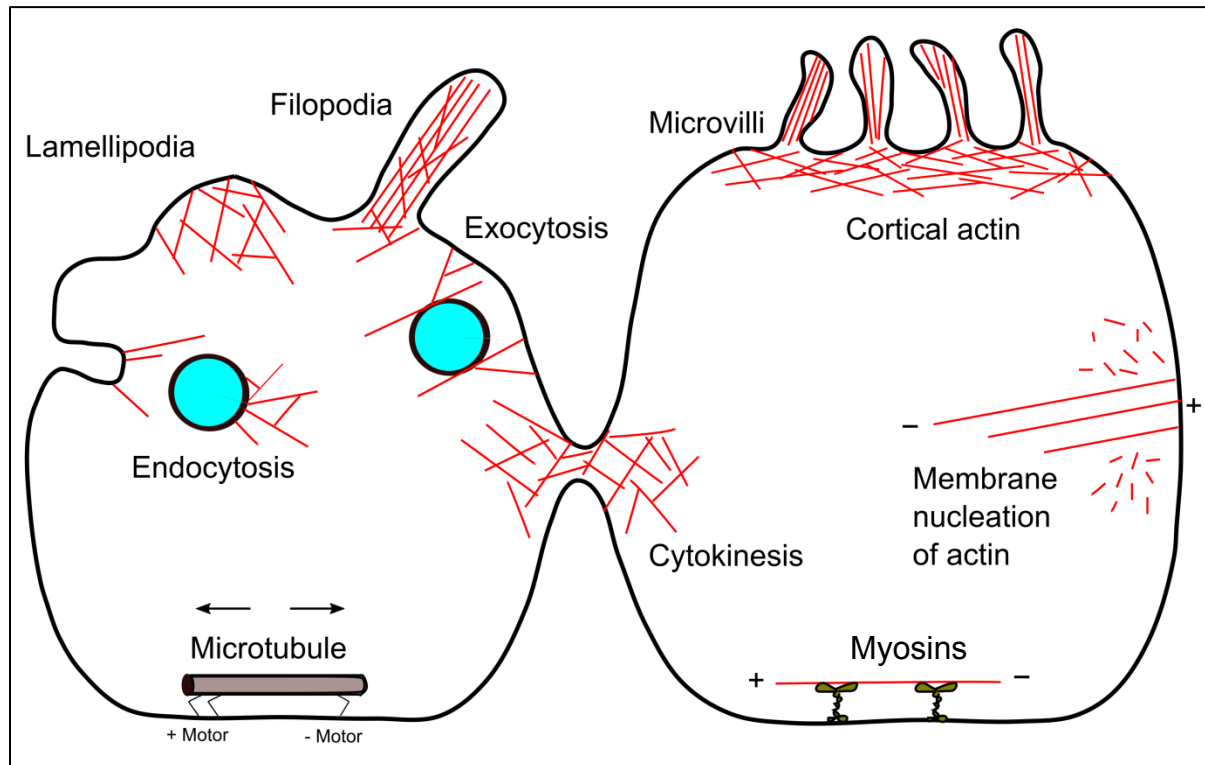


Figure 1.1. Cytoskeletal-membrane interactions facilitate a repertoire of cellular functions. Actin (red) polymerizes with plus-ends abutting the membrane to produce forces that drive essential cellular processes like cytokinesis, cellular motility, vesicle trafficking, protrusive growth of filopodia etc. Myosin motors (like Myo-II) attached to membrane organelles walk along actin; most myosins move towards the plus ends to generate contractile forces. Microtubules also interact with the membrane to transport cargo using molecular motors that walk in a polarized manner on their polymer scaffold (Redrawn with adaptations from Griffiths, 2007).

Filopodia are thin (0.1-0.3 microns in diameter), dynamic (lifetime of the order of tens of seconds), actin-rich (around 10-30 actin bundles each) protrusions (Yang & Svitkina, 2011) that extend from the cell surface of neural growth cones, lamellipodia of migrating cells, epithelial cells undergoing wound closure, etc. (Faix et al., 2009). Filopodial assembly involves filament nucleation, persistent growth of actin filaments and filament bundling.

Microvilli are present on the apical surface of epithelial cells. They may be densely packed and of uniform length like those present on the luminal surface of intestinal and kidney epithelial cells that help increase surface area of absorption (9-16 fold) (Crawley et al., 2014) or, shorter and of more variable length as seen on circulating lymphocytes and dorsal surfaces of many cultured cells, and their formation is likely driven by actin polymerization (Chhabra & Higgs, 2007). Microvilli cover the surface of fertilized mammalian eggs (Reeve & Ziomek, 1981); sea urchin embryos have elongated microvilli that form an 'attachment complex' that help position it concentrically in the perivitelline space (Spiegel et al., 1989) and *Drosophila* embryos too have microvilli on their apical surface during the early syncytial divisions post fertilization (Turner & Mahowald, 1976).

Endocytosis and Exocytosis Dynamic assembly of actin at the cortex near endocytic sites assist the remodelling of the cell surface to allow inward movement of vesicles and is also crucial for vesicle scission (Saarikangas, Zhao, & Lappalainen, 2010; Smythe & Ayscough, 2006). Macropinocytosis is an actin-dependent endocytic pathway and is important for morphological changes in cells by incorporation of membrane components (Florey et al., 2011), for example, retraction of growth cone in repulsive axon guidance (Kolpak et al., 2009). Assembly of a dynamic actomyosin complex around exocytic vesicles post-fusion is required to expand the fusion pore by exerting a force parallel to the plasma membrane, and/or to pull membranes of secretory vesicles toward the plasma membrane by exerting a force tangential to the surface of the vesicle (Porat-Shliom et al., 2013).

Cytokinesis is achieved by a circumferential ring of differentiated cortex, enriched in actin and Myo-II that contracts to divide the cytoplasm (Satterwhite et al., 1992). The positioning of the contractile ring is specified by the mitotic apparatus such that contraction bisects the mitotic spindle, ensuring that division occurs between the separating chromosomes (Rappaport, 1996). Actin dynamics is required in all steps following cleavage furrow positioning; filamentous actin assembly leads to formation of a cytokinetic ring that constricts during cell division (Green, Paluch, & Oegema, 2012). Failure to assemble actin at the cleavage furrow also affects central spindle organization. For example, in *Drosophila* spermatids the result of improper actin organization at the contractile ring is fewer, enlarged and polyploid spermatids with improperly formed spindles (Giansanti, 2004; Giansanti et al., 1998).

Role of plasma membrane in organizing cytoskeleton. Plasma membrane tension feeds back to cellular processes that need close interplay between the membrane and the cytoskeleton like vesicle trafficking, caveola formation, actin polymerization and changes in Myo-II activity (Köster & Mayor, 2016). Actin contractility and plasma membrane tension are regulated by increase or decrease in the rate of these cellular processes in order to maintain membrane tension homeostasis (Diz-Munoz et al., 2013). So far, a comprehensive picture of factors governing membrane tension homeostasis has not emerged.

1.2 Actin dynamics generates enough force to drive shape changes in membranes

The sequence and structure of eukaryotic actins are extremely well conserved, with less than 5% sequence variation among the actin genes found in metazoans and protozoa (Pollard & Cooper, 1986). The actin filament is structurally polar with one end termed as the ‘pointed end’ (or minus end) while the other is called the ‘barbed end’ (or plus end) (Pollard, 2007). This terminology originates from the fact that the S1 fragment of Myo-II binds F-actin in a stereospecific way, and actin decorated with Myo-II S1, used to study F-actin polarity *in vivo*, appears as a string of arrowheads pointing toward the minus end (Holmes et al., 2003; Mooseker & Tilney, 1975). Due to the structural asymmetry and different conformational changes between the plus and minus ends, the rate constant for monomer association (and removal) is always faster for the plus end than for the minus end even if the bound nucleotide (ATP/ADP-Pi/no nucleotide) is uniform throughout the filament (Pollard & Cooper, 1986; Pollard, 2007). However, in the case of uniform bound nucleotide when all monomers in the actin filament are identical, there is no thermodynamic difference between monomer addition to the plus end and monomer addition to the minus end. Therefore, the equilibrium constant for monomer dissociation, called the critical concentration (C_c) will be the same at the two ends of the filament (Howard, 2002). The critical concentration has units of μM and can be expressed as the ratio of the monomer dissociation rate constant (k^{off}) to the monomer association rate constant (k^{on}) (Figure 1.2). This implies that if the plus end has a faster monomer association rate constant than the minus end but the same critical concentration, it must also have a faster monomer dissociation rate constant. For conditions under which all actin monomers are bound to the same nucleotide, as would be the case for the polymerization of ADP-G-actin, the filament then grows at both ends if the G-actin concentration exceeds the critical concentration, shrinks at both ends if the G-actin concentration

is less than the critical concentration, and undergoes random fluctuations in length at both ends when the G-actin concentration equals the critical concentration. On the other hand, if the polymerizable species is ATP-G-actin, which can undergo hydrolysis upon assembly into F-actin, the two ends of the resulting filament will no longer have the same bound nucleotide. ATP-actin has a significantly lower critical concentration than ADP-actin, creating a range of G-actin concentrations between the critical concentrations for ATP- and ADP-actin, in which net assembly happens at the plus end and net disassembly happens at the minus end (Bugyi & Carlier, 2010; Fujiwara et al., 2007; Pollard, 2007). This situation is termed treadmilling (Pollard & Borisy, 2003; Pollard, 2007).

Actin polymerization has long been believed to produce force that drives cell motility (Carlier et al., 2003; Mogilner & Oster, 2003). Initial attempts to measure this force used biomimetic systems that allow measurements of force produced by populations of actin filaments pushing on bacterium, beads or vesicles (Giardini et al., 2003; Marcy et al., 2004; Schwartz et al., 2004; Upadhyaya & Oudenaarden, 2003). Direct measurements of the polymerization force using single-molecule techniques are scarce. A first study used optical traps on bare filaments, giving a force of ~ 1 pN (Footer et al., 2007). By monitoring the buckling of a polymerizing filament trapped between a formin and a Myo-II molecule in the presence of phalloidin, a second study found the force to be ~ 1.3 pN (Kovar & Pollard, 2004). In both cases, the concentration of actin was an order of magnitude lower than *in vivo*, and the measured forces were in fact close to the theoretical maximum under the experimental conditions.

Several biophysical models have attempted to predict the protrusive force that actin network dynamics can generate. The Brownian ratchet model explains how actin filament polymerization converts the free energy of monomer binding to the plus end into a pushing force (Mogilner & Oster, 1996; Mogilner & Oster, 2003; Peskin, Odell & Oster, 1993). In the simplest case, the end of a filament pushes against a fluctuating mechanical load, such as the cell membrane. Whenever Brownian fluctuations of the cell membrane open a space large enough to fit a new monomer onto the end of the filament, a monomer diffuses in and increases the length of the filament, preventing the membrane's average position from returning to its previous value and is thus instead ratcheted forward – hence the name ‘Brownian ratchet’. This model has been tested for bundles of straight actin filaments with an optical trapping experiment and shown to corroborate

experimental results (Footer et al., 2007). It has also been shown that membrane fluctuations can drive branched actin network-based motility (Shaevitz & Fletcher, 2007). The Brownian ratchet model has been extended to incorporate more realistic filament and membrane geometries and mechanics, including the elasticity of filaments at an angle to the membrane and their fluctuations, which could also serve to create space for monomer insertion when the membrane

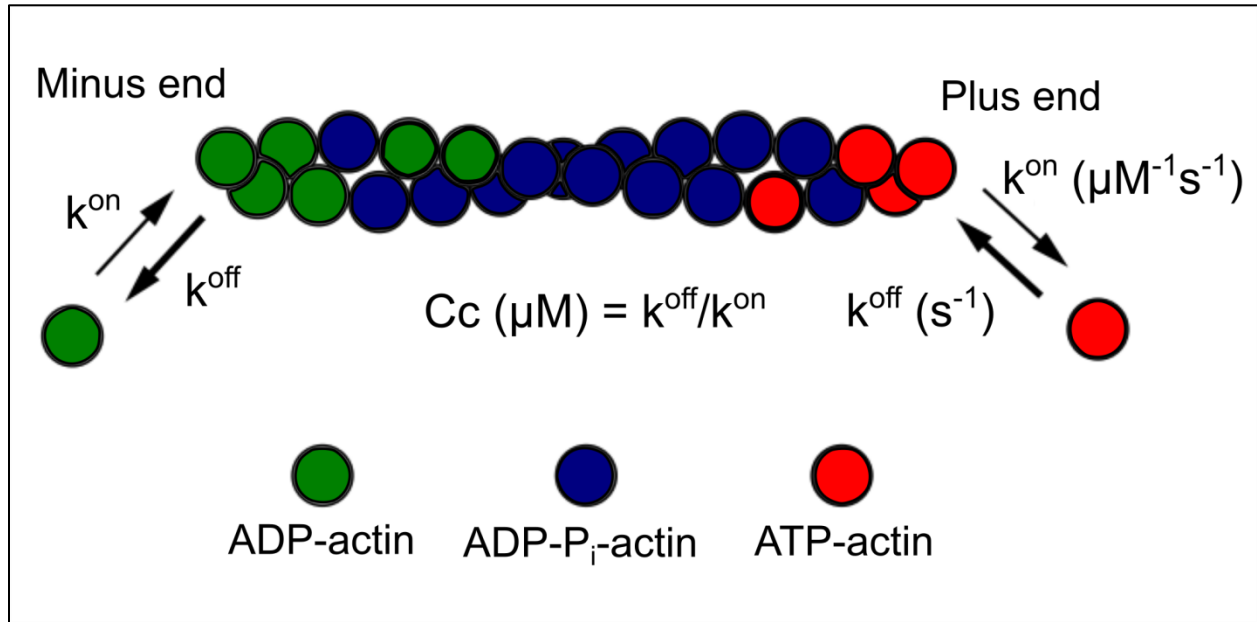


Figure 1.2. Actin treadmilling. Actin monomers associate (at rate k^{on}) and dissociate (at rate k^{off}) from both ends of the polymerizing filament. However, association at plus ends is ten times faster and dissociation occurs twice as fast compared to the minus end at steady state. Steady state ratio of k^{off} to k^{on} termed the critical concentration (C_c) is five times greater at the minus end than at the plus end (Redrawn with adaptations from Lodish, Berk, Zipursky, (2000)).

does not fluctuate (Mogilner & Oster, 1996). The elastic Brownian ratchet model predicts that an angle 48° (close to the observed $\sim 35^\circ$ angle of the filaments to the growth direction) is optimal for force generation (Mogilner & Oster, 1996). The tethered elastic Brownian ratchet model also predicts that the actin network velocity will increase with increased network density, and, at constant nucleation rate, the density of the network increases with the membrane tension while the number of pushing filaments is constant (Mogilner & Oster, 2003).

Polymerization velocity is the flow rate of actin relative to the cell membrane i.e. the difference between the protrusion rate and the retrograde flow rate and depends on the elongation rate of a single average filament at the membrane (Carlsson, 2010). Experiments using atomic force

microscopy (AFM) have probed the force-velocity of actin networks at high forces, upto the point of network stall (Parekh et al., 2005; Prass et al., 2006). The force-velocity in these studies exhibited a regime in which the velocity was force-independent before eventual stall. This observation is consistent with another model of actin network growth called the autocatalytic model (Weichsel & Schwarz, 2010), which, unlike the tethered elastic Brownian ratchet model, assumes that the number of pushing filaments can be increased in response to increasing membrane tension. In the membrane-adjacent zone, new actin filaments are generated in proportion to the amount of actin in the zone. The model predicts that as the membrane tension increases and the growth velocity decreases, filaments spend more time in the branching zone, creating more new filaments and reducing the overall force per filament. At steady state, this process gives rise to a tension-independent growth velocity as observed in the experiments. The autocatalytic model and the tethered Brownian ratchet model can coexist if the nucleation rate and the network architecture depend on the growth velocity, due to which, different force-velocity curves may apply to different network architectures (Weichsel & Schwarz, 2010).

1.3 Accessory Proteins Regulate Actin Polymerization and Assembly

Actin filament turnover is governed by many actin-binding proteins like those that function in monomer capture and delivery and others that promote filament nucleation, elongation, capping, severing or depolymerisation.

Compared to actin filament elongation spontaneous initiation of new filaments, called nucleation, is very slow. *Nucleation proteins* actively catalyze rapid initiation of actin filament polymerization (Campellone & Welch, 2010). The Arp2/3 complex initiates branching on the sides of existing actin filaments by anchoring the minus end of the daughter filament to mother filament at the branch point as the free plus end of the daughter grows away from the complex at a regular 70° branch angle (Amann & Pollard, 2001; Mullins, Heuser, & Pollard, 1998). The Arp2/3 complex consists of seven subunits: two *actin-related proteins* Arp2 and Arp3 maintained in an inactive state by five other subunits ARPC (actin-related protein complex) 1 through 5 of various molecular weights (Machesky et al., 1994). Its natively inhibited state is relieved by cooperative binding to nucleation promoting factors (NPFs), actin filaments and an actin monomer (Figure 1.3). NPFs include Wiskott-Aldrich Syndrome protein (WASp), SCAR/WAVE etc. that have three functional domains – WH2/WASp-homology 2 or V domain, a connecting

(C) motif and an acidic (A) motif. VC binds actin while CA binds the Arp2/3 complex, thus VCA domains bring together the Arp2/3 complex and the first subunit in the daughter filament. Arp2 and Arp3 bind the first and second subunits of the daughter filament as their binding surfaces mimic actin (Beltzner & Pollard, 2004). This interaction requires a conformational change, the details of which are not fully understood although crystallographic and cryo-electron microscopic structures of the Arp2/3 complex and branch junctions have shed some light on this mechanism (Saha & Pollard, 2012).

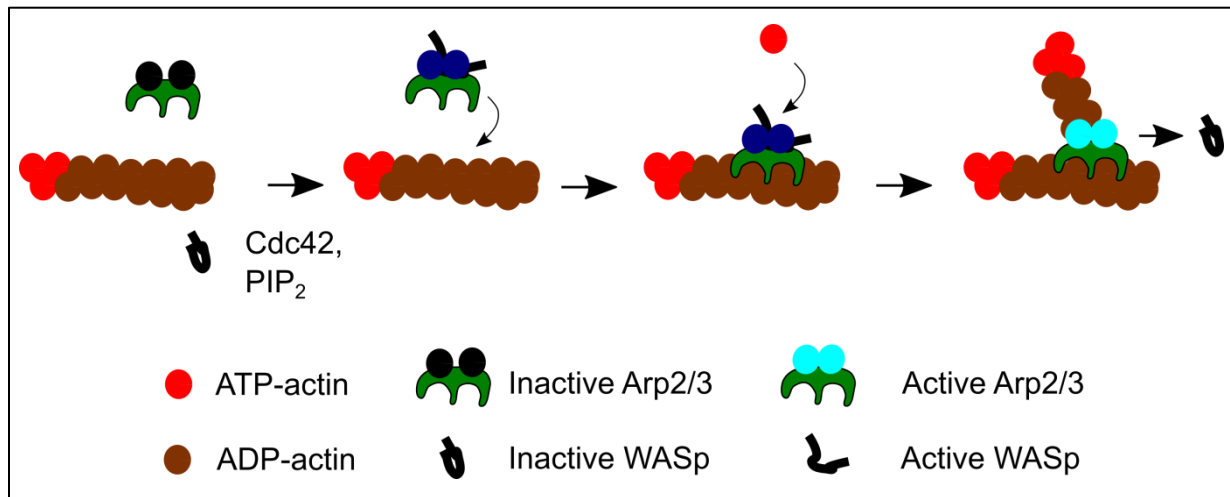


Figure 1.3. Branch nucleation by Arp2/3 complex. NPFs (e.g. WASp; SCAR/WAVE), activated by binding to Cdc42 and PIP_2 membranes bring together the Arp2/3 complex and actin monomers to nucleate actin filaments that form new branches from the side of pre-existing filaments. The Arp2/3 complex remains attached at the minus end of the filament (Redrawn with adaptations from Goley & Welch (2006)).

There exists, an equilibrium, between the different conformational states of the Arp2/3 complex such that only one in a hundred complexes is active at equilibrium, but the nucleation activity of the Arp2/3 complex differs by several orders of magnitude in the absence and presence of NPFs (Machesky & Insall, 1999) perhaps because Arps may have a low affinity for each other in the absence of NPFs (Beltzner & Pollard, 2004). NPFs, mother filaments, and actin monomers cooperate to influence this equilibrium, but individually none of them can activate the complex. Spectroscopy and electron microscopy have documented conformational changes, but the structural, thermodynamic, and kinetic details of the activation process are still incomplete. Nucleotide binding is also an important regulator of Arp2/3 activity – Arp2 and 3 each bind to

ATP with micromolar affinity – any mutations that reduce this affinity cause drastic depletion of Arp2/3 activity (Martin et al., 2005). Ongoing studies are also trying to determine how nucleotide hydrolysis is coupled to branch formation (Dayel, Holleran & Mullins, 2001; Dayel & Mullins, 2004) and disassembly (Martin, Welch & Drubin, 2006).

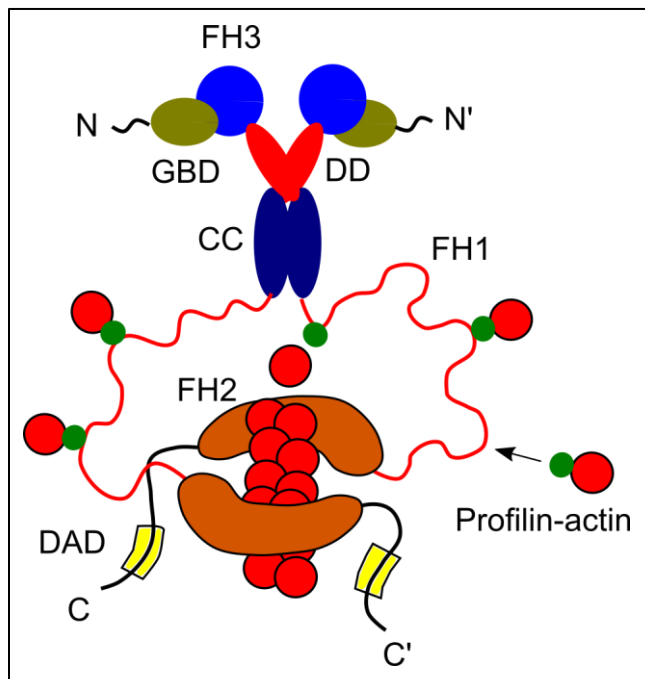


Figure 1.4. Processive actin polymerization by activated formin dimer. The proline-rich FH1 domain recruits profilin–actin complexes in close proximity to the FH2 domain. G-actin molecules are polymerized to F-actin by the dimeric FH2 domain (Redrawn from Kühn & Geyer (2014).

Formins (formin homology proteins) are a class of proteins that also function to stimulate actin polymerization by creating a nucleation site; however, unlike the Arp2/3 complex they generate unbranched filaments (Zigmond, 2004). The presence of two conserved domains, FH1 and FH2 characterize members of the formin family. The FH2 domain which is ~400 amino acids in size is responsible for its actin nucleation activity. Crystallization studies indicate that individual FH2 domains are composed of alpha helices arranged in an overall crescent shape, the inner concave face of which binds actin filaments (Xu et al., 2004). FH2 domains of two formin molecules that constitute a functional dimer form a ring connected by flexible tethers around the actin filament and remain associated with the plus end of the filament as it elongates. This processive motion of formins is unlike the Arp2/3 complex that remains stationary, bound to the filaments' minus end (Campellone & Welch, 2010). An adjacent proline-rich FH1 domain binds the actin monomer-delivery protein profilin. FH1-profilin interactions accelerate filament elongation, most likely because binding of profilin-actin complexes to the FH1 domain increases, several fold over its

diffusion rate, the local concentration of G-actin that can be delivered directly to the barbed end (Kovar et al. 2006; Paul & Pollard, 2008). The best characterized mammalian formins are mDIA1 and 2, orthologues of the *Drosophila* formin Diaphanous (Dia). They have three conserved functional regions. The N-terminus contains regulatory sequences, including a GTPase protein binding domain (GBD) and a partially overlapping Diaphanous inhibitory domain (DID). The DID participates in auto-inhibition by interacting with the C-terminus Diaphanous auto-inhibitory domain (DAD) that prevents the FH2 domain from catalyzing actin polymerization; the C-terminus also contains the FH1–FH2 module. Formin auto-inhibition is released when the formin GTPase-binding domain (GBD) interacts with a GTP bound form of a small GTPase Rho protein (RhoA/Rac/Cdc42) (Higgs, 2005) (Figure 1.4).

Bundling proteins organize actin filaments into bundles of parallel or anti-parallel filaments or into a three-dimensional orthogonal network. Bundling proteins include filamin, α -actinin, spectrin, and fimbrin. Actinin family proteins are large polypeptides that contain an N-terminus actin-binding domain (ABD), 4 spectrin-like repeats and two C-terminus calcium-binding motifs (Oikonomou et al., 2011; Trulsson et al., 2011). Actinins function as homodimers, with the two molecules arranged in an anti-parallel fashion such that the actin binding of one protein and the calcium-binding domain of the other are in close proximity making actinin–actin interactions sensitive to calcium levels. The anti-parallel arrangement of the individual proteins in the actinin homodimer allows the two actin-binding domains to bind different actin filaments and bring them in closer upto a distance equal to the length of the actinin homodimer ~ 40 nm. Interaction of the dimer with actin filaments can create actin bundles with either parallel or anti-parallel filament orientation. Flexibility within spectrin-like domains of Actinins and, between these domains and the actin-binding domain, allows some movement of the actin filaments relative to each other. The spectrin-like repeats can also serve as docking sites for signalling proteins. Like Actinins, Filamins are large proteins with an N-terminus ABD; Filamin also serves as a scaffolding protein that physically links signalling proteins to actin structures. They also contain a C-terminus homodimerization domain that forms functional dimers with a V-shaped structure (Nakamura, Stossel & Hartwig 2011). As a result, actin filaments bound by the homodimer are roughly perpendicular to each other forming a three-dimensional mesh-like structure with gel-like properties (Stossel et al., 2001).

Septins are a group of GTP-binding proteins that self-assemble into heteromeric complexes, which can polymerize into higher-order structures (Saarikangas & Barral, 2011), and bind membranes (Bertin et al., 2010), Anillin (Field et al., 2005; Kinoshita et al., 2002; Oegema et al., 2000) (an actin crosslinker present at the cytokinetic ring in most animal cells) and Myo-II (Joo et al., 2007; Mostowy et al., 2010) in different organisms. Septins were found to be required for generating curved and tightly packed actin filament networks. F-actin bundling and bending activity were shown to be conserved for fly and human septins, it is therefore likely that septins contribute to the assembly, stabilization and contractility of cytokinetic rings (Mavrakakis et al., 2014).

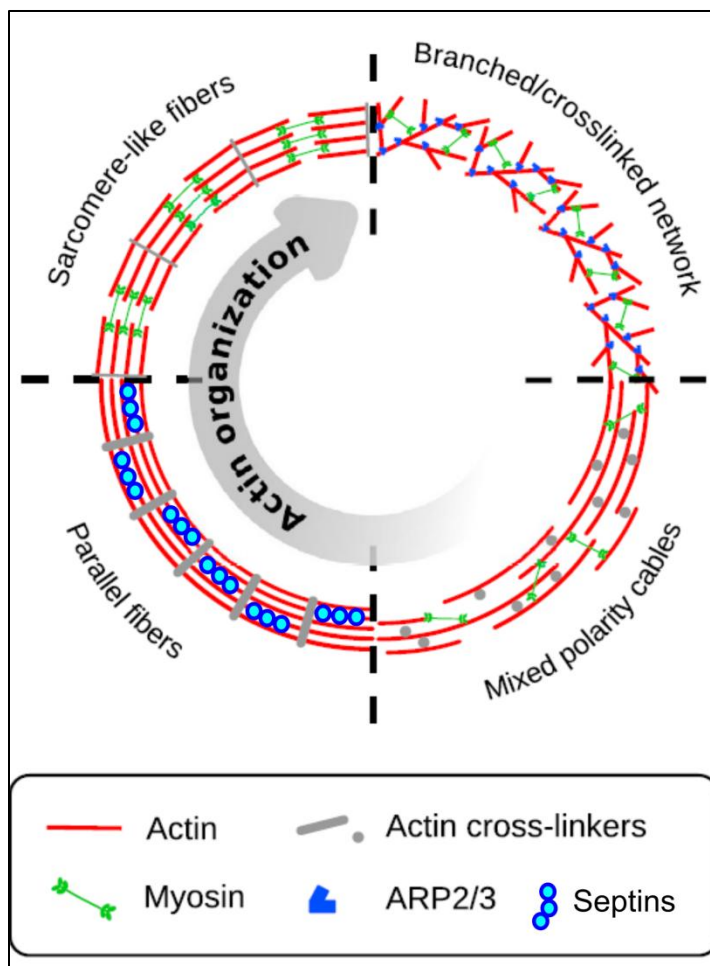


Figure 1.5. Actin Organization. Depending on their function and localization, cells can form distinct actin network structures including contractile, branched actomyosin networks built of cross-linked actin fibers coupled to myosin motors. Anisotropic networks are formed when long actin fibers with mixed polarity are linked together and thus aligned with each other by different cross-linker proteins. Parallel actin fibers are generated when cross-linked actin fibers are aligned in the same direction. They can achieve curved geometries by the bending action of septins. Regular arrays of parallel actin fibers connected by myosin motors are found in sarcomeres, representing the highest level of actin organization. Contractile actin rings are associated with most of these structures (Adapted from Schwyer et al., (2016)).

Capping proteins bind either to the plus or minus ends of actin filaments. Capping at the minus end can shorten filament length by promoting the conversion of ATP-actin or ADP-Pi-actin monomers to ADP-actin. Since actin monomers are continually dissociating from the minus filament ends the net result of plus end capping is also filament shortening (Pollard & Borisy, 2003). Formins and Ena/VASP proteins cap the plus end of actin polymers; however, they recruit profilin-actin to the growing polymer and also prevent other plus-end capping proteins from binding and are hence described as ‘leaky’ caps (Krause et al., 2003).

Severing proteins catalyze internal cleavage of actin filaments and cause filament shortening. Gelsolin and cofilin are the best-studied severing proteins. Cleavage by gelsolin creates two minus ends in place of the original one, since gelsolin remains attached to the plus end of one of the severed filaments effectively capping the polymer and preventing actin reassembly. This results in doubling the rate of F-actin depolymerisation (Sun et al., 1999). Cofilin, on the other hand, preferentially cleaves older actin filaments due to its greater binding affinity to ADP-actin over ATP-actin. At high molar ratios of cofilin to actin, cofilin-mediated disassembly makes free plus ends for monomer addition and stabilizes actin monomers, thus enhancing actin nucleation (Ichetovkin et al., 2002).

Contraction by motor protein Myo-II uses the energy of ATP hydrolysis to create mechanical forces and has the ability to disassemble large actin networks in various cellular contexts (Haviv et al., 2008). The forces generated by Myo-II are utilized for shape changes and migration because Myo-II proteins bind to two adjacent actin filaments and cause these filaments to move relative to one another. Myo-II motors move toward the plus end of filaments and cause filaments that are oriented in an anti-parallel fashion to slide relative to one another; if one of the filaments is fixed in place, this sliding motion causes contraction to occur (Reichl et al., 2008) (Figure 1.5).

1.4 *Drosophila* syncytial blastoderm embryo is an excellent and tractable model to study membrane-cytoskeletal interactions

The *Drosophila* blastoderm embryo develops as a syncytium (Mazumdar & Mazumdar, 2002; Sherlekar & Rikhy, 2016). The first 13 nuclear division cycles in the embryo are synchronous, incomplete and the cytoplasm is not segregated by a plasma membrane boundary. Nuclear cycles

1–9 proceed within the interior of the embryo (Figure 1.6A-B). Cytoskeletal rearrangements occurring during the syncytial cycles 6–9 promote spreading of the nuclei throughout the embryo and migration of the nuclei toward the periphery (Karr & Alberts, 1986). At the end of nuclear cycle 9, nuclei have reached the periphery of the embryo to form the syncytial blastoderm (Figure 1.6C). At this stage, the plasma membrane buds around each nucleus but does not encircle the nucleus leaving a possibility for exchange of molecular components between nuclei. The membrane buds extend into longer membrane extensions/pseudocleavage furrows in metaphase that function to prevent spindle crosstalk between dividing nuclei (Foe, Odell & Edgar 1993; Schejter & Wieschaus, 1993) and may also govern patterning of morphogen gradients crucial for body axes formation (Sherlekar & Rikhy, 2012). By the end of cycle 13 there are 6000 nuclei at the cell periphery. In a prolonged interphase of cycle 14, cellularization takes place by extending the plasma membrane around each nucleus and giving rise to complete epithelial cells (Lecuit, 2004; Figure 1.6D). This is followed by gastrulation after which cells resume dividing, albeit asynchronously.

Studies have shown that the syncytium has a distinct cellular architecture, which implies that there is limited sharing between cytoplasm surrounding adjacent nuclei despite the absence of complete plasma membrane boundaries, and each compartment in this organization (Frescas et al., 2006; Mavrakakis et al., 2009) are termed as ‘pseudocells’. The plasma membrane has distinct domains during nuclear cycles 10–13 in the syncytial blastoderm (Mavrakakis et al., 2009). This can be readily seen during interphase of the syncytial nuclear cycles (n.c.). The apical domain is rich in microvilli which were observed in early studies by scanning electron microscopy (Turner & Mahowald, 1976). They have also been observed by light microscopy in transgenic embryos containing a fluorescently tagged pleckstrin homology (PH) domain from phospholipase C (PLC) or PHPLC δ , which labels phosphatidylinositol diphosphate (PI(4,5)P₂) in the plasma membrane and, using a fluorescently tagged palmitoyl group from Gap43, which gets anchored to the inner leaflet of the plasma membrane. Distinct from this apical domain, is a short lateral domain (~ 2 μ m) that contains junctional proteins and actin remodelling proteins. Thus, the plasma membrane displays epithelial-like characteristics even in the absence of complete epithelial cells (Figure 1.6E) (Mavrakakis et al., 2009).

During the syncytial nuclear cycle, membrane extension of the furrow is accompanied by apical actin cap expansion and probably flattening of apical microvilli (Turner and Mahowald, 1976; Mavrakakis et al., 2009) (Figure 1.6F) which may supply membrane to fuel extension as is observed during cellularization (Figard & Sokac, 2014). Our studies (see Chapters 3-5) and others have shown that the concomitant process of apical cap expansion and membrane elongation during syncytial division cycles is driven by dynamics of the actin cytoskeleton (Afshar, Stuart & Wasserman 2000; Cao et al., 2010; Sherlekar & Rikhy, 2016; Stevenson et al., 2002).

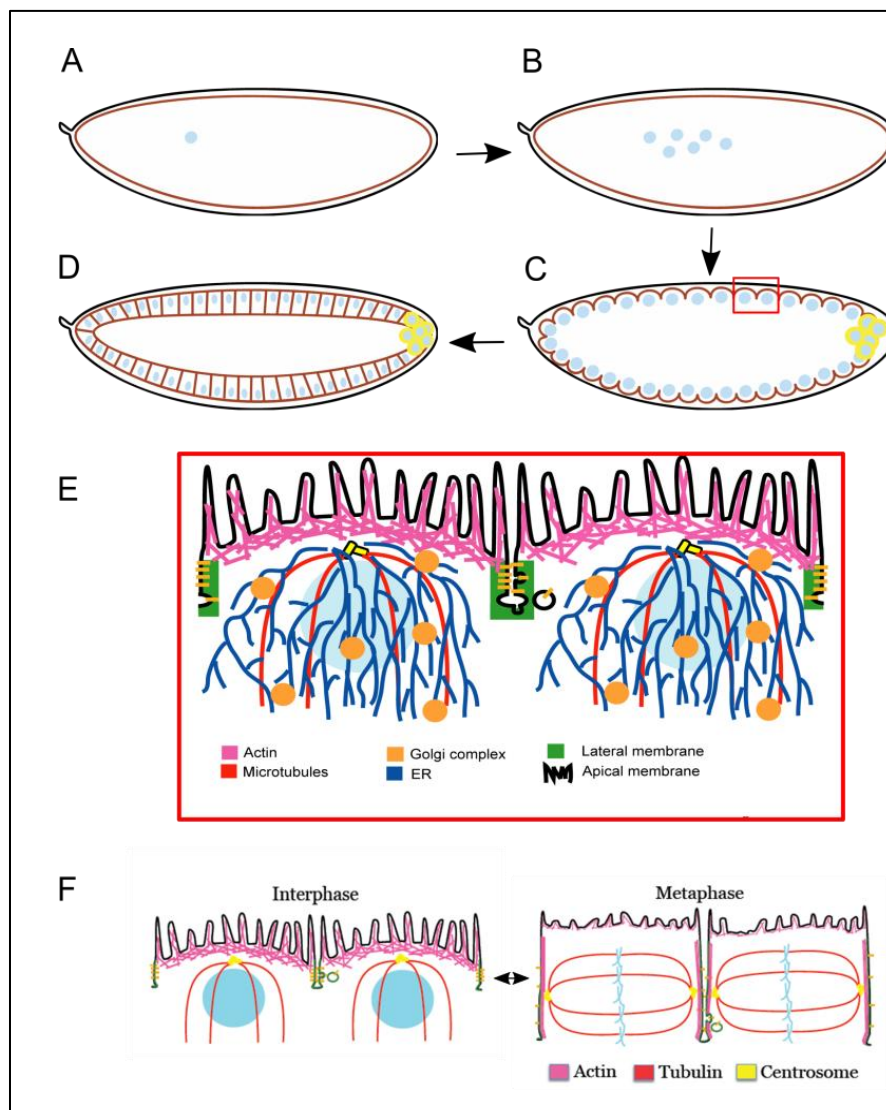


Figure 1.6. Early development of *Drosophila* embryos. The fertilized embryo after egg laying is known as the preblastoderm embryo (A). The nucleus divides (termed nuclear cycles (n.c.)) 9 times within the

embryo. The nuclei then move outwards and line the cortex where they undergo 4 additional nuclear cycles. This stage is termed the syncytial blastoderm embryo (C). Subsequently, the membrane between nuclei extends deep into the cortex in a process known as cellularization (D). The syncytial blastoderm embryo has nuclei surrounded by discrete plasma membrane caps and limited sharing between cytosolic constituents of adjacent compartments or 'pseudocells' (E) zoomed in from the red box in (C). From interphase to metaphase of the nuclear cycle the microvilli in the cap flatten and supply membrane for extension of the pseudocleavage furrow accompanied by dramatic remodelling of the membrane and cytoskeleton (F).

Drosophila embryos are highly amenable to live-cell imaging studies; apart from the relative ease of generating transgenic animals that express fluorescently tagged proteins and the ability to express fluorescently labelled proteins in a specific tissue or at specific developmental times, embryos are transparent upon dechoriation (see Materials and Methods) and can hence easily be imaged using light microscopy. In addition, the multitude of genetic resources available for *Drosophila* provides the unparalleled opportunity to evaluate the *in vivo* behaviour of fluorescently tagged proteins in the embryo in both wild-type and mutant contexts (Mavrakakis et al., 2008).

1.5 Furrow assembly and elongation proteins are functionally conserved

Cytokinesis demands an intricate synchronization of actomyosin ring assembly/contraction (Figure 1.7) and furrow ingression from the cellular cortex by molecular players that are highly conserved across species and with other related cellular processes (Table 1.1). Here, I highlight parallels between cleavage furrow assembly during cytokinesis of complete cells and pseudocleavage furrow formation during the syncytial division cycles in the *Drosophila* embryo (Figure 1.8).

Furrow initiation The onset of furrow assembly involves positioning the division plane between segregating chromosomes during anaphase. Early experiments by Rappaport, (1961) revealed microtubule spindle reorganization into a structure called the central spindle actively govern ed this process. The currently accepted model states that two processes occur to varying extents in different cell types to regulate furrow initiation: first, astral microtubules inhibit furrow formation at the poles – this is termed the 'astral relaxation' model and second, stable astral microtubules contact the equatorial cortex and, reinforced by signals from the central spindle, promote furrow ingression – this is referred to as the 'equatorial and central spindle stimulation'

model (D'Avino, Giansanti & Petronczki 2015). In syncytial *Drosophila* embryos, most crucial molecules associated with the central spindle that initiate furrow formation localize to regions of astral microtubule overlap, also the site for metaphase furrow formation (Crest, Concha-Moore & Sullivan, 2012). Active Rho1 (mammalian RhoA) controls actomyosin assembly and thus provides a key signal for furrow initiation. Rho1 is activated by RhoGEFs, active Rho releases auto-inhibition of formins (like Dia) and thus signals actin polymerization.

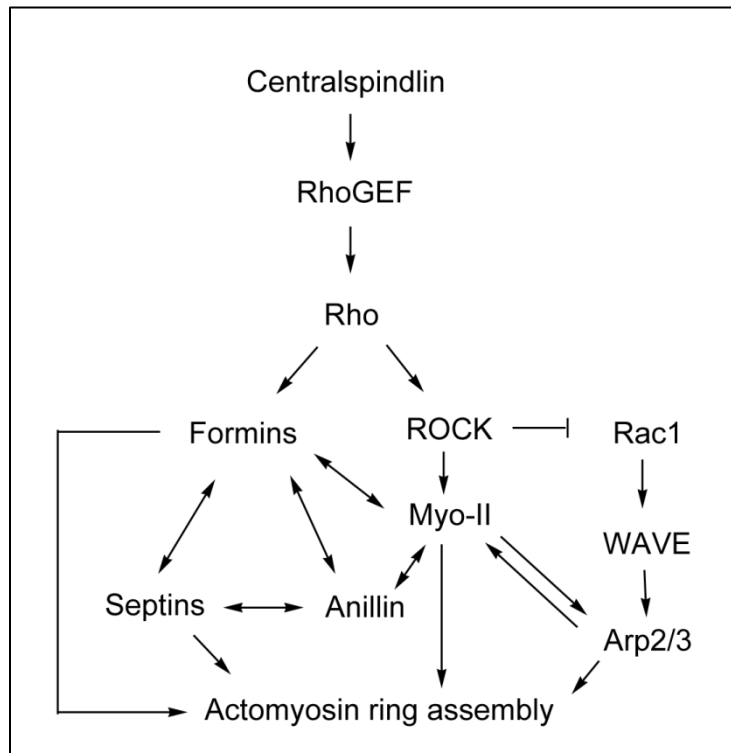


Figure 1.7. Conserved actin regulatory proteins that govern assembly of actomyosin ring. The centralspindlin complex then recruits and activates the RhoGEF, resulting in the generation of an active Rho zone at the division plane. Rho promotes actomyosin ring assembly by activating the actin nucleator formin and the Myo-II activator ROCK. The centralspindlin also accumulates Anillin that recruits Septins that together with Formins, Myo-II and Arp2/3 complex govern actomyosin ring assembly (see text for details and references).

Active Rho also activates Rho kinase that in turn phosphorylates and activates Myo-II (D'Avino et al., 2015). During conventional cytokinesis, central spindle proteins position the furrow through localized activation of RhoA that begins during metaphase, by anaphase a stable stripe of activated RhoA is present through the plane that the cleavage furrows form and finally the signal is turned off in late telophase by a GTPase-activating protein, RhoGAP (Piekny, Werner & Glotzer, 2005; Wagner & Glotzer, 2016; Yüce, Piekny & Glotzer, 2005). The centralspindlin, Ect2 GEF (*Drosophila* RhoGEF Pebble (RhoGEF^{Pbl})) that catalyzes Rho activation, and RhoA have been shown to be engaged in a positive feedback loop that could strengthen and sharpen the zone of RhoA activation (Bement et al., 2015; Zhang & Glotzer, 2015), which may thus contribute to complete furrow ingression during anaphase (Wagner & Glotzer, 2016).

Orthologues of proteins with roles in cytokinesis	Mammalia	<i>Drosophila melanogaster</i>	<i>Caenorhabditis elegans</i>	Function
ACTIN REMODELLING	MgcRacGAP/ RacGAP1	tumbleweed (tum)/ RacGAP50C	CYK-4	GAP (microtubule assembly, recruits Anillin)
	RhoA	Rho1	RHO-1	GTPase (activates formins)
	mDIA1, mDIA2	Diaphanous	CYK-1	Formin (actin assembly)
	Anillin	Scraps/Anillin	ANI-1	Scaffold protein (assembly of contractile ring proteins)
	ECT-2	RhoGEF2 and RhoGEF ^{Pbl}	LET-21	GEF (activates Rho)
	Septin 7	Peanut	Unc-59	Septin (actin remodelling)
	Pacsin/ Syndapin1 and 2	Syndapin	Sdpm-1	Membrane bending (binds Anillin, N-WASP, Rac1 etc.)
VESICLE TRAFFICKING	Dynamin	Shibire/Dynamin	Dynamin	Membrane fission (abscission)
	Arf6	Arf6	ARF-6	ADP-ribosylation (membrane addition by recycling endosomes)
	Rab11	Rab11	RAB-11	Exocytosis of recycling endosomes
	RalA	RalA	RALA	GTPase (tethers the exocyst to the cytokinetic furrow)
	Cytosolin-1	Steppke	GRP-1	Arf-GEF (activates Arfs)
	Pacsin/ Syndapin1 and 2	Syndapin	Sdpm-1	Membrane tubulation, endocytic functions (binds Dynamin, Synaptojanin)

Table 1.1. Proteins with evolutionarily conserved roles in cytokinesis. Table listing mammalian, fruit fly and worm orthologues and their conserved functions in cytokinesis of proteins discussed in the text which are broadly classified based on whether they regulate actin remodelling or membrane trafficking

("UniProt: a hub for protein information," 2015). A comprehensive list of proteins is excluded due to lack of space.

Rho-GTPase-activating protein (RacGAP50C) of the centralspindilin complex positions RhoGEF^{Pbl} at contractile rings. Similarly, another RhoGEF2 functions in pseudocleavage furrows to activate Rho1 for furrow initiation (Buchsbaum, 2007; Schmidt & Nichols, 2004). In syncytial embryos, Rho1 is localized in an apical ring during interphase that extends basally at future sites of pseudocleavage ingression. A subcortical stripe of Rho1 forms during prophase that becomes well-defined by metaphase at the equatorial plate. This localization is lost by anaphase/telophase. Therefore, the position of the cortical stripe of Rho1 is equivalent to that found in cells undergoing conventional cytokinesis; however, the timing is altered such that it coincides with pseudocleavage furrow formation (Crest, Concha-Moore & Sullivan, 2012).

Furrow ingression Studies suggest that in addition to their role in regulating the Rho activation zone, RacGAPs also downregulate Rac GTPases at the cleavage site to reduce cortical stiffness and inhibit cell adhesion, thus allowing robust and rapid furrow ingression (D'Avino et al., 2015). RacGAP50C accumulates Anillin at the furrow (D'Avino et al., 2008; Gregory et al., 2008), and Anillin, in turn, is responsible for both Septin and Myo-II association at the contractile ring (Piekny & Maddox, 2010; Straight et al., 2005). Cleavage furrow ingression is slowed and in *C. elegans* embryos depleted of Rho-kinase and cytokinesis failures increase when embryos are depleted of both Rho-kinase and Anillin/Septins (Maddox et al., 2007). Thus, Anillin and formins together assemble the actomyosin contractile ring and regulate ingression of the cleavage furrow. Furrow ingression involves dynamic growth of actin filaments through profilin-regulated polymerization and the action of anti-capping proteins (like Ena/VASP and Diaphanous-related formins (DRFs)) (Grevengoed et al., 2003).

Extension of the furrow during cellularization in *Drosophila* embryos is driven by an actomyosin contractile ring which, apart from actin and Myo-II also comprises Anillin, Septins, RhoGEF2 and Dia (Lecuit, 2004). Membrane extension has been shown to be fuelled by flattening of apical microvilli as well as Rab11-mediated endocytosis (Figard et al., 2013; Lecuit & Wieschaus, 2000; Pelissier et al., 2003). Although in developing *Drosophila* embryos, contractile rings form only during cellularization, as described earlier, the syncytial pseudocleavage furrows contain most of the proteins present in the contractile ring such as spectrin, cofilin, Arp2/3, Rho1,

RhoGEF2, Dia, Anillin and septins (Afshar et al., 2000; Crest et al., 2012; Grosshans et al., 2005; Miller & Kiehart, 1995; Neufeld & Rubin, 1994; Riggs et al., 2003; Stevenson et al., 2002). Existing studies including ours have implicated nucleators Arp2/3 and Dia, RhoGEF2, Anillin and the septin Peanut in pseudocleavage furrow extension – depletion of any these proteins results in shorter/missing furrows (Afshar et al., 2000; Grosshans et al., 2005; Sherlekar & Rikhy, 2016; Silverman-Gavrila, Hales & Wilde 2008; see Chapters 3-5). The pseudocleavage furrow ingresses the longest during metaphase of the syncytial cycle, and gets progressively longer with each nuclear cycle (Holly et al., 2015; Sherlekar & Rikhy, 2016). Our work has shown that homeostatic control of actin dynamics is required for extension of pseudocleavage furrows, that actin polymerization alone can drive pseudocleavage furrow formation and that the F-BAR protein Syndapin is a key regulator of this process (Sherlekar & Rikhy, 2016; see Chapters 3-4 and Figure 1). We show that recruitment of proteins Dia and Peanut, which function to organize actin in furrows (Grosshans et al., 2005; Mavrikakis et al., 2014), also depends on Syndapin (Sherlekar and Rikhy, 2016; see Chapter 4). Previous studies have shown that overexpression of F-BAR protein Cip4, an activator of Arp2/3 nucleator complex, phenocopies Dia loss-of-function phenotypes (Yan et al., 2013). Cip4 is also known to function synergistically with a related F-BAR protein Nostrin (Zobel et al., 2015), however, their role in furrow formation remains to be investigated.

Concomitant to the force generated by actomyosin ring constriction, successful cytokinesis requires extensive membrane trafficking activities (McKay & Burgess, 2011; Tang, 2012). The dramatic increase in total surface area during furrowing requires the transport of membrane vesicles to be inserted at the cleavage site. Endocytosis mediates both ingression and abscission of the furrow (Prekeris & Gould, 2008). Vesicle traffic to the furrow additionally serves to recycle regulatory proteins and remodelling factors required at different stages of this process (Neto & Gould, 2011) like RhoGEF2 to the syncytial furrow membrane during metaphase (Cao et al., 2008). Mutants for endocytic components like clathrin and dynamin, showed defective cytokinesis both in mammalian cell lines and *in vivo* models (Gerald et al., 2001; Thompson et al., 2002; Wienke et al., 1999) including in *Drosophila* syncytial embryos where furrows fail to form (Rikhy, Mavrikakis & Lippincott-Schwartz 2015). Small GTPases that regulate recycling trafficking – Rab11, Arf6 and Rab35, are required for successful cytokinesis in various cellular contexts (Montagnac, Echard & Chavrier 2008; Schiel & Prekeris, 2013). Rab11 mainly

regulates the recycling of vesicles from recycling endosomes to plasma membrane and was first found to be essential for furrow ingression in *C. elegans* (Skop et al. 2001). In *Drosophila*, Rab11 is crucial for cytokinesis in S2 cells and in spermatocytes (Kouranti et al. 2006; Giansanti et al. 2007) and during cellularization in embryos (Pelissier et al. 2003; Riggs et al. 2003). In *Drosophila* spermatocytes, Rab11 and its effector Nuclear fallout (Nuf) localize to the cleavage furrow. Mutants for Rab11 show defects in both for furrow ingression and actomyosin ring constriction and display a significant incidence of cytokinesis failure (Giansanti et al., 2007). Likewise in mammalian cells, both the Nuf ortholog FIP3/Arfophilin and Rab11 accumulate at the cleavage furrow and depletion of either protein results in cytokinesis failures (Wilson et al., 2005). FIP3 and another Rab11 effector, FIP4 (both orthologous to Nuf) exist in a complex with Arf6 and depend on Arf6 for targeting to the central spindle (Fielding et al., 2005) which, in turn, interacts with the exocyst complex and mediates vesicle fusion with the plasma membrane (Fielding et al., 2005; Prigent et al., 2003). Conserved orthologues to Arf6/FIP3/FIP4 complex are absent in *Drosophila*. Nuf fails to bind Arf6 and, instead, depends on Rab11 for its recruitment (Giansanti et al., 2007; Hickson et al., 2003; Riggs et al., 2003). In turn, Nuf recruits RhoGEF2 to the furrow (Cao et al., 2010) for Rho1 activation. As a result, local Dia-induced actin polymerization and Myo-II activation occurs. However, Arf6 is still required for furrow ingression in *Drosophila* spermatocytes, in which it acts downstream of recycling endosomes (Dyer et al., 2007). We investigated the role of endocytosis in furrow ingression in syncytial *Drosophila* embryos. Interestingly, we found that furrow ingression can be rescued in RhoGEF2-depleted backgrounds solely by rescuing defects in actin organization and remodelling even though endocytic defects are not rescued (Sherlekar and Rikhy, 2016; see Chapter 4). In syncytial and cellularizing *Drosophila* embryos, rather than a role in promoting furrow ingression, endocytosis seems to regulate furrow overgrowth primarily via two mechanisms: an Amphiphysin-based mechanism and an Arf-GEF-induced mechanism (Lee & Harris, 2014). Endocytosis occurs at the tip of the ingressing syncytial furrows and can be visualized in the form of tubules labelled with the N-BAR domain protein Amphiphysin (Sokac & Wieschaus, 2008; see Chapter 3). Their formation depends on actin and Dynamin and defects in endocytosis result in persistent growth of these tubules and slow down rate of furrow ingression (Oegema et al., 2000; Sokac & Wieschaus, 2008; Su et al., 2013), while loss of these tubules could result in longer furrows due to faster growth-rate (Lee & Harris, 2014). The Arf-GEF cytohesin Stepk

promotes endocytosis at pseudocleavage furrows and furrow canals to restrain actomyosin networks at these sites (Lee & Harris, 2013). Without Stepkke-based endocytic inhibition, Lee & Harris (2014) hypothesised that actomyosin networks abnormally expand and inhibit other endocytic events leading to co-expansion of cytoskeleton and plasma membrane at the furrow tips.

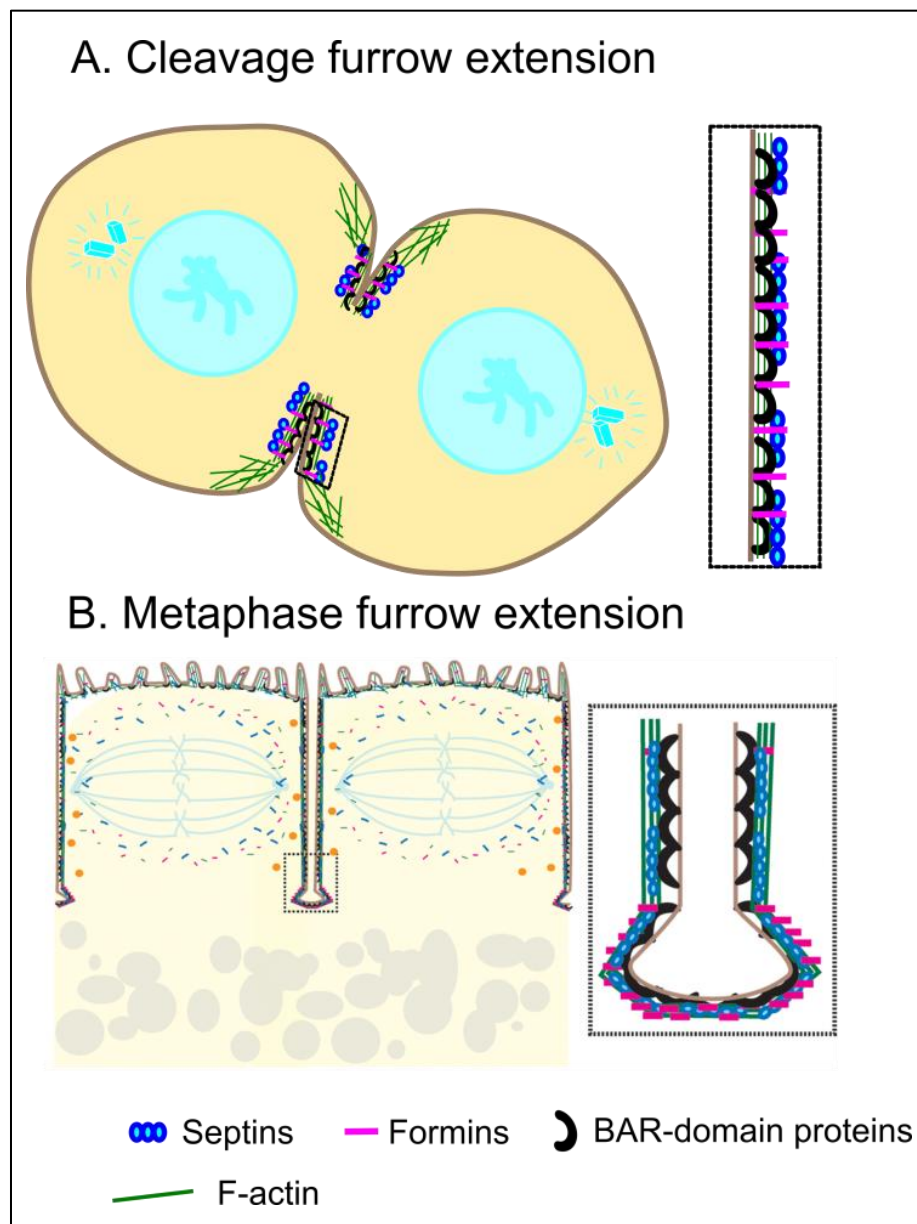


Figure 1.8. Furrow extension is regulated by a conserved machinery of proteins. Cleavage furrow extension during conventional cytokinesis and metaphase furrow extension during nuclear cycles in the syncytial *Drosophila* embryo require a conserved repertoire of proteins including Anillin, Septins (D'Avino

et al., 2009, Caebernard, 2012), actin assembly proteins like Formins (Grosshans et al., 2005) and BAR-domain proteins (Yan et al., 2013, Takeda et al., 2013) that coordinate membrane-cytoskeletal functions (see insets for zoomed-in section of the furrow membrane).

Consistent with a role of secretory vesicle trafficking in furrowing, cytokinesis in *C. elegans* embryos and *Drosophila* spermatocytes was observed to be sensitive to brefeldin A, a fungal toxin that interferes with anterograde transport from ER to the Golgi (Kitazawa et al., 2012; Robinett et al., 2009; Skop et al., 2001). Various studies have demonstrated the need for polarized insertion of new membrane by exocytosis at the apical membrane or cortex to supply membrane for furrow extension both during cellularization (Lecuit & Wieschaus, 2000; Sisson et al., 2000; Skop et al., 2001) and the syncytial nuclear cycles (Holly et al., 2015). RalA is a small-GTPase that functions through the exocyst complex to control directed membrane addition (Moskalenko et al., 2002). Embryos mutant for RalA have short/missing pseudocleavage furrows. F-actin assembly at the furrow is compromised and actin appears punctate along the furrow. The membrane (labelled with a fluorescent tag) also has a punctate distribution (Holly et al., 2015). To test whether poor F-actin organization is a consequence, rather than a cause of loss of membrane integrity, Holly et al. (2015) examined relative distributions of RalA and F-actin in the wild-type furrow and found that F-actin is disassembled prior to furrow retraction, while RalA still remained bound to the membrane. They concluded that RalA-dependent membrane trafficking is central to furrow formation, while F-actin is a mere bystander only serving as a structural support to the membrane (Holly et al., 2015). A study published in the same year by Tran et al. (2015) built on existing data from cell lines (Giner, 2005; Porat-Shliom et al., 2013; Trifaró, Gasman & Gutiérrez 2008) to show in *ex vivo* cultures of salivary glands that F-actin is actively disassembled along the apical plasma membrane at sites of secretory vesicle fusion and reassembled on the vesicle membrane. WASp-mediated activation of Arp2/3 is important for expulsion of secretory cargo and, significantly, for integration of vesicular membranes with the apical plasma membrane (Tran et al., 2015). These studies clearly show that actin dynamics is crucial for several steps of the exocytic pathway. They also strongly suggest that in pseudocleavage furrows also, F-actin assembly by Arp2/3 will be crucial for RalA and the exocyst complex to function for apical membrane addition therefore, actin remodelling is likely to actively govern membrane integrity rather than play a passive role as a membrane scaffold (Figure 1.9). Our study clearly demonstrates that F-actin organization drives pseudocleavage

furrow extension and that Syndapin regulates F-actin dynamics (Sherlekar and Rikhy, 2016; see Chapter 3-4). Syndapin1 function is essential for the assembly of vesicle clusters in presynaptic terminals and regulates actin-based cytoskeletal dynamics, thereby influencing the number of vesicles available for exocytosis (Greengard et al., 1993; Südhof et al., 1989). Since Arp2/3-mediated actin assembly is important for exocyst function (Tran et al., 2015), in *Drosophila* syncytium too Syndapin, in turn, controls Arp2/3 activation by interacting with N-WASp (described in the following section, Kumar et al., 2009), Syndapin function could also regulate apical membrane addition by the exocyst complex.

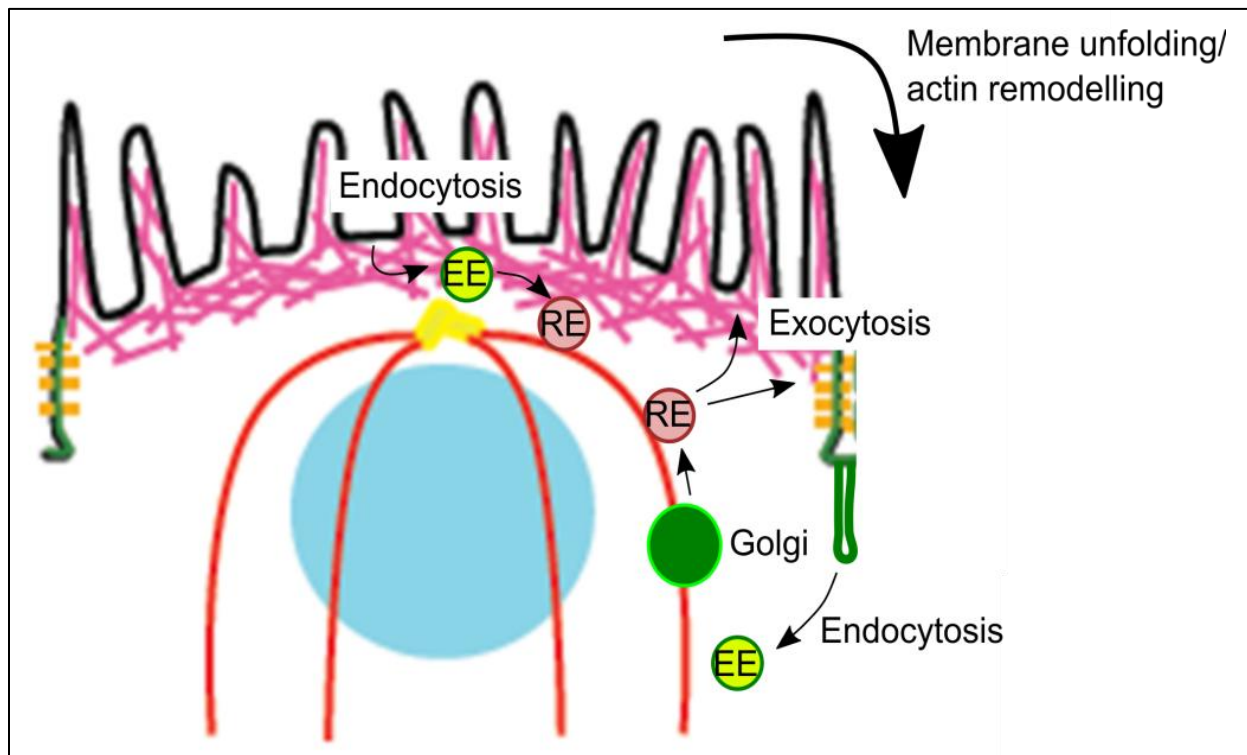


Figure 1.9. Membrane-cytoskeletal remodelling and vesicle recycling regulate the extension of the pseudocleavage furrow. Membrane unfolding and remodelling of the membrane and cytoskeleton drives ingression of the furrow. Vesicle trafficking from Golgi-to-plasma membrane via exocytosis; Golgi-to-recycling endosome (RE)-to-plasma membrane via exocytosis; endocytosis from the apical plasma membrane (EE-early endosome) and transcytosis to lateral plasma membranes via the RE; and basal flow of exocytosed and microvillar membrane may function to restrict overgrowth of the pseudocleavage furrow (Redrawn with adaptations from Lee & Harris, (2013)).

Our work and others suggest (as mentioned previously) that the membrane required to fuel furrow extension in the early *Drosophila* embryo may be provided by flattening of apical

microvilli (Figard & Sokac, 2014; Figard et al., 2013; see Chapter 5). Super-resolution images reveal shortening of cortical actin protrusions along the plane of the apical cap from interphase to metaphase at the same time membrane in the microvillar reservoir is unfolded/remodelled into furrows. Apical surface ‘emergence’ is a cellular behaviour characteristic to epithelial cells governed by actin remodelling underlying processes like epithelial cell extrusion and epithelial folding (Guillot & Lecuit, 2013; Martin & Goldstein, 2014; Sedzinski et al., Wallingford, 2016). In syncytial *Drosophila* embryos, a process akin to apical emergence accompanies pseudocleavage furrow extension termed ‘apical cap expansion’. The apical cap comprises of cortical actin that abuts the membrane forming bulges above each ‘pseudocell’. As the cap expands, neighbouring caps come in contact to form pseudocleavage furrows. Several proteins including nucleators Arp2/3 and Dia have been implicated in apical cap expansion suggesting that actin dynamics is crucial to this process (Stevenson et al., 2002; Cao et al., 2010; see Chapter 5). Our work also confirms these findings; additionally we place Syndapin as a crucial player in this process (see Chapter 5).

1.7 Actin-induced membrane tension governs cell packing and tissue morphology

The actomyosin cytoskeleton and the adhesive properties of cells mechanically control the dynamics of cell packing and morphological properties of cells in a tissue (Farhadifar et al., 2007; Käfer et al., 2007; Landsberg et al., 2009; Major & Irvine, 2006). The cell packing geometry of proliferative epithelia has been studied in *Drosophila* wing disc, Hydra epidermis and tail epidermis of *Xenopus*. The frequency distribution of different classes of polygons (with different sidedness) is highly reproducible between different species. Hexagons are the most frequent, followed by pentagons and heptagons, that are equally prevalent (Figure 1.10, (Gibson et al., 2006). Initial attempts to model frequency distribution of polygons in a proliferating sheet of cells used only topological rules in their description, as a result of which they inaccurately predicted a maximum of 3-4 sided polygonal cells rather than hexagons (Cowan and Morris 1986) or correctly predicted a maximum of six-sided polygons but did not allow for triangles to be present (Gibson et al., 2006). Farhadifar et al. (2007) proposed the first model to describe how cellular mechanics influence cell shape, surface area and frequency distribution of cell neighbour number using *Drosophila* third-instar larval wing disc as a model. The epithelial tissue was modelled as a two-dimensional network in the plane of apical cell junctions in which cells are

represented as two-dimensional polygons. A stable configuration of the two-dimensional network corresponds to a force balance at the vertices, and the only perturbation driving the system out of equilibrium is cell division. They tested different combinations of cell and junctional forces by comparing *in silico* simulations with *in vivo* measurements of cell packing features. To experimentally probe the mechanical properties of cells, they ablated cell edges using a UV laser and analyzed edge relaxation. This experiment allowed the authors to narrow the possible combinations of cortical forces matching *in vivo* analysis. They conclude that cortical elasticity plays an important role in cell mechanics and therefore governs cell packing properties (Farhadifar et al., 2007; Kafer et al., 2007). Their *in silico* simulations generated a phase diagram that predicts interesting features that could possibly govern cell behaviours. For example, decreased actomyosin contractility or increased cell-cell adhesions might lead to a more fluid network where cells may exchange neighbours (as in the case of cell-cell intercalations) or, the frequency distribution of polygons in the network may shift to the lower side such that fewer sided polygons such as pentagons or tetragons dominate rather than hexagons (Farhadifar et al., 2007; Figure 1.11). Networks that contain predominantly hexagons are also predicted to have increasing cell area for increasing sidedness of the polygon, while in soft networks, all cells are predicted to have the same area and perimeter and, as a consequence, the cell area does not depend on neighbour number (Farhadifar et al., 2007). The models explained thus far account for the observed packing geometry globally but do not consider single cells and their neighbour effects. For instance, Gibson et al. (2006) assumes that each cell can gain one vertex from each of the dividing neighbouring cells, independent of its polygon class. But the distribution of mitotic cells depends on their clustering: a cell gains additional sides when its neighbours divide, so the polygon number of a cell when mitosis starts is sensitive to whether its neighbours divide earlier, later or simultaneously.

Sheets of dividing polygonal epithelial cells have also been modelled using Voronoi diagrams with increasing success. A Voronoi tessellation is a partition of space according to certain neighbourhood relations of a given set of points in this space forming convex polygons that do not overlap (Voronoi, 1908). It has been applied to various biological contexts to explain behaviour of various aggregates of cells and swarming animals (Bock et al., 2010). One of the earliest models using Voronoi diagrams predicted that cells would divide in the plane perpendicular to the long axis of the cell, they used simulations to predict polygon distribution

after a full division process of a single layer of cells and found this to match division in a starfish blastula monolayer of cells (Honda, Yamanaka & Dan-Sohkawa, 1984).

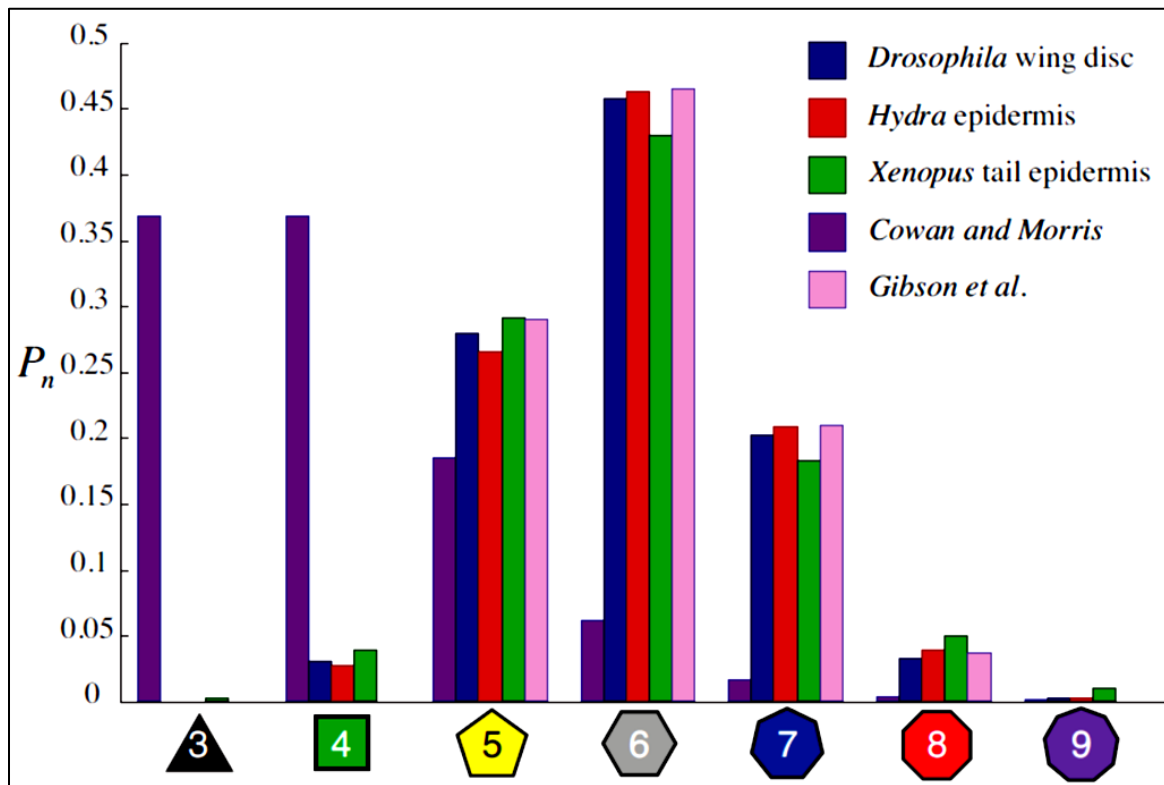


Figure 1.10. Frequency distribution of different classes of polygons in a proliferating sheet of cells in *Drosophila* wing disc, *Hydra* epidermis and *Xenopus* tail epidermis (Gibson et al., 2006). For comparison, are shown the distribution of polygon classes that were found by Cowan and Morris (1986) and Gibson et al. (2006) (Reproduced with adaptations from Farhadifar, 2009).

Brodland and Veldhuis (2002) observed that mitosis can drive autonomous reshaping of epithelia and confirmed that the cleavage plane orientation depends on the long axis of the cell. For a comprehensive mechanical understanding of epithelia, they proposed that it is important to learn how mitosis affects and is affected by the cell shape and epithelial deformation. They classified mitosis into two broad categories and simulated these using Voronoi tessellations: cell-oriented division in which some individual cells divide and frame-oriented division in which the whole sheet of cells divide; both these categories could either be with or without growth. The simulation of cell-oriented division predicts that cells around the recently divided cell will undergo rearrangements and annealing to accommodate the daughter cells within the sheet.

During frame-oriented division, the simulation predicts that the cells' aspect ratio doubles. Just before dividing, the cells in the dividing sheet are stretched so they have their long axes aligned with the direction of stretching causing an alignment of resulting cleavage planes. The cells are constrained along the edges (the cells' edges cannot move individually with respect to its neighbouring edges). Then mitosis can produce significant cell reshaping, thus relieving stress (Brodland & Veldhuis, 2002). In a recent study, Sánchez-Gutiérrez and coworkers (2015) computed an optimal centroidal Voronoi tessellation where the seed of each Voronoi cell is its centroid. They obtained a series of Voronoi diagrams with different frequencies of polygon distributions. This was converted to a continuous function that allowed them to calculate how the percentage of 4-, 5-, 7- or 8-sided cells change with respect to the percentage of hexagons. These

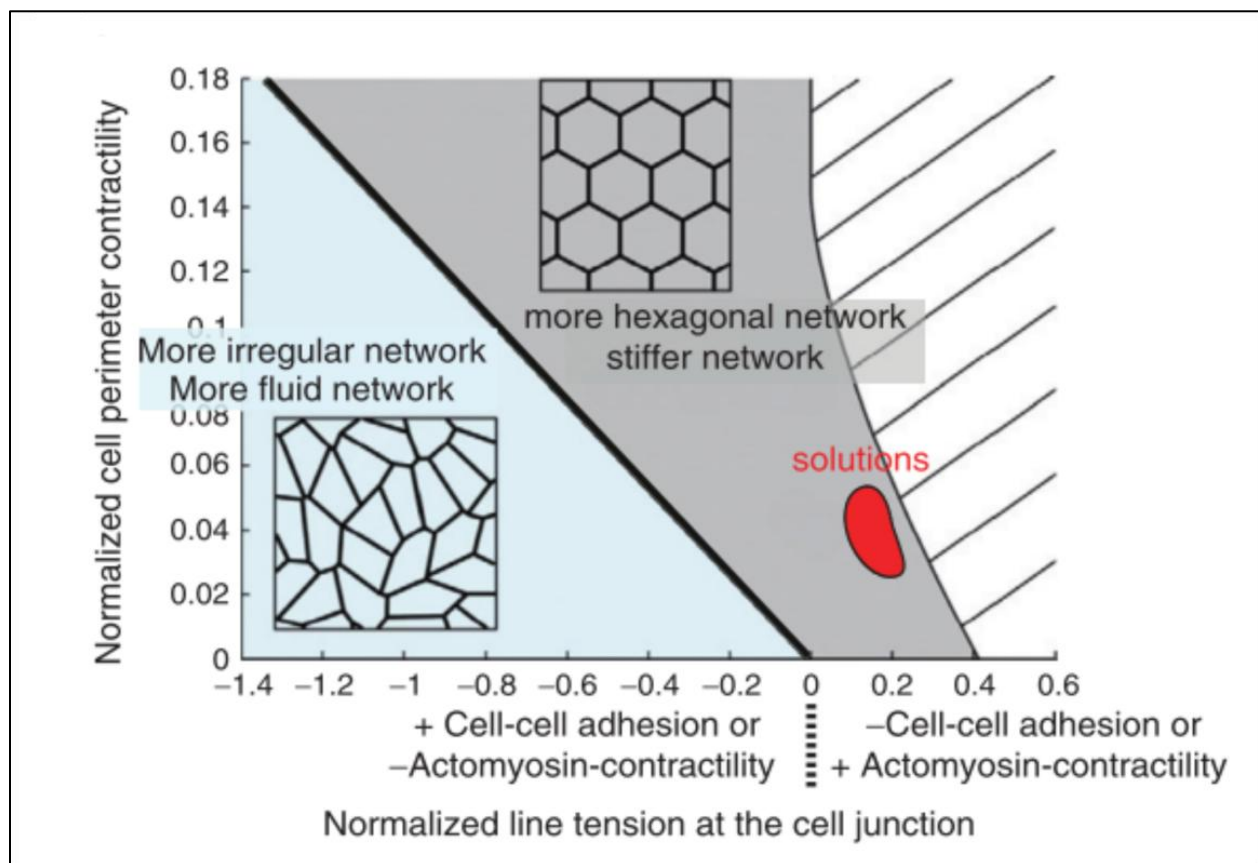


Figure 1.11. Phase diagram of cell packing geometry as a function of cell perimeter contractility and line tension at cell junction (vertex model). *In silico* simulations predict lower line tension will yield more irregular and fluid ('soft') cell networks and more regular and stiffer cell networks will be seen with higher line tension. Solutions (red region) best fit *in vivo* data (Reproduced from Rauzi & Lenne, (2011)).

probabilities were then compared to natural tessellations from real tissues and, importantly, used to identify deviations in diseased states (Sánchez-Gutiérrez et al., 2015).

1.8 The BAR-domain superfamily of proteins in regulation of cytoskeletal dynamics and membrane remodelling

The BAR (Bin, Amphiphysin, Rvs) domain-containing family of proteins are excellent candidates for the concerted remodelling of the cytoskeleton and membrane deformation needed to establish and maintain polarity in cells. The BAR domain was originally identified as an evolutionarily conserved region common between yeast proteins (rescue viability upon starvation proteins, Rvs161 and Rvs167) and metazoan amphiphysins (Amphiphysin and its splice variant, Bin1) (Itoh & De Camilli, 2006). These proteins typically contain a BAR domain and, in addition, actin-binding WH2 (WASP homology 2) domains, RhoGAP/GEF domains, PDZ and SH3 domains that link them to actin-regulatory networks as well as endocytic machinery like Dynamin (De Camilli et al., 1983) and Synaptojanin (McPherson et al., 1996). The Endophilin and Sorting nexin families of proteins were first identified as forming functional homodimers (Ringstad, Nemoto & De Camilli 1997; Worby & Dixon, 2002); now all BAR domain-containing proteins are known to dimerise. All members of this superfamily bind negatively charged phospholipid head groups of the membrane via electrostatic interactions with positively charged residues on the surface of their dimers.

Different members of this group bind/induce different membrane curvatures (Figure 1.12): The classical neuronal or N-BAR domain induces the maximum curvature followed by the phox or PX-BAR domain. Then the Fes-CIP4 homology or F-BAR domain binds to membranes of very large radius of curvature (three times that by N-Bar domain) and finally, as opposed to rest of the BAR domain superfamily, the IRSp53-MIM homology or I-BAR domain containing proteins bind and stabilise negative rather than positive membrane curvatures (Saarikangas et al., 2010). The Pink BAR is the only instance of a BAR protein that promotes zero curvature: its BAR domain shows no intrinsic curvature (Pykäläinen et al., 2011). Many studies on BAR domain-containing proteins have been conducted in cell lines like HeLa cells and in *in vitro* reconstitution assays from mammalian brain tissue. Several proteins of this family have been shown to have roles in actin remodelling and endocytosis in these contexts (Qualmann, Koch & Kessels 2011).

Fourteen BAR domain proteins are highly expressed in the early *Drosophila* embryo (The modENCODE Consortium et al., 2010; Table 1.2). I began by studying the F-BAR domain-containing protein Syndapin for which there exists only a single 65 kDa isoform in flies (Roos & Kelly, 1998; Takeda et al., 2013). Mammalian orthologues of *Drosophila* Syndapin – Syndapin (or Pacsin) 1 and 2 were initially identified as binding partners for Dynamin and neural Wiscott-Aldrich Syndrome protein (N-WASp) via its SH3 domain (Qualmann et al., 1999; Roos & Kelly, 1998). Syndapin 3 is a predominantly muscle-specific isoform of Syndapin (Qualmann et al., 2004). Membrane deformation by Syndapin 1 is subject to release of autoinhibition of the F-BAR domain by binding of its SH3 domain to the proline rich (PR) region of proteins such as Dynamin (Rao et al., 2010).

Molecular mechanisms of membrane bending Crystal structures and molecular dynamics simulations have led to an improved understanding of BAR domain protein structure and function (Mim & Unger, 2012; Britta Qualmann et al., 2011). They led to the prediction that the intrinsic curvature of BAR domain dimers aids membrane remodelling by a ‘scaffolding’ mechanism (Ayton et al., 2009; Blood, Swenson & Voth 2008; Fugier et al., 2011; McMahon & Gallop, 2005) which implies that the membrane adopts a forced shape simply by virtue of their binding to BAR protein dimers based on their intrinsic curvature. In contrast to scaffolding, an alternate mechanism, also backed by experimental and computational evidence, proposes that insertion of amphipathic ‘wedges’ into the bilayer can sense membrane curvature and promote its formation through concerted displacement of lipids in the leaflet proximal to the site of insertion (Bhatia et al., 2009; Gallop et al., 2006). It is clear that both mechanisms are at play, however, the relative contribution of each mechanism remains an area of active investigation.

F-BAR domain-containing proteins link membrane and cytoskeletal function in various processes, including endocytosis, cell shape and polarity, cell motility, and cytokinesis (Roberts-Galbraith and Gould, 2010; Liu et al., 2015). The yeast orthologues of F-BAR protein Cip4 are known to recruit formins and influence their nucleation and elongation activities (Laporte et al., 2011; Yan et al., 2013; Graziano et al., 2014; Willet et al., 2015). In addition, Hof1 (Cip4 in *Saccharomyces cerevisiae*) coiled-coil domain binds Septin (Cdc10) and localizes it to the bud neck (Meitinger et al., 2013; Oh et al., 2013). *Drosophila* Cip4, however, is not essential for formin Dia recruitment to cellularization furrows, and its loss does not result in a defect in

cellularization; however, its overexpression shows *dia* loss-of-function phenotypes (Yan et al., 2013).

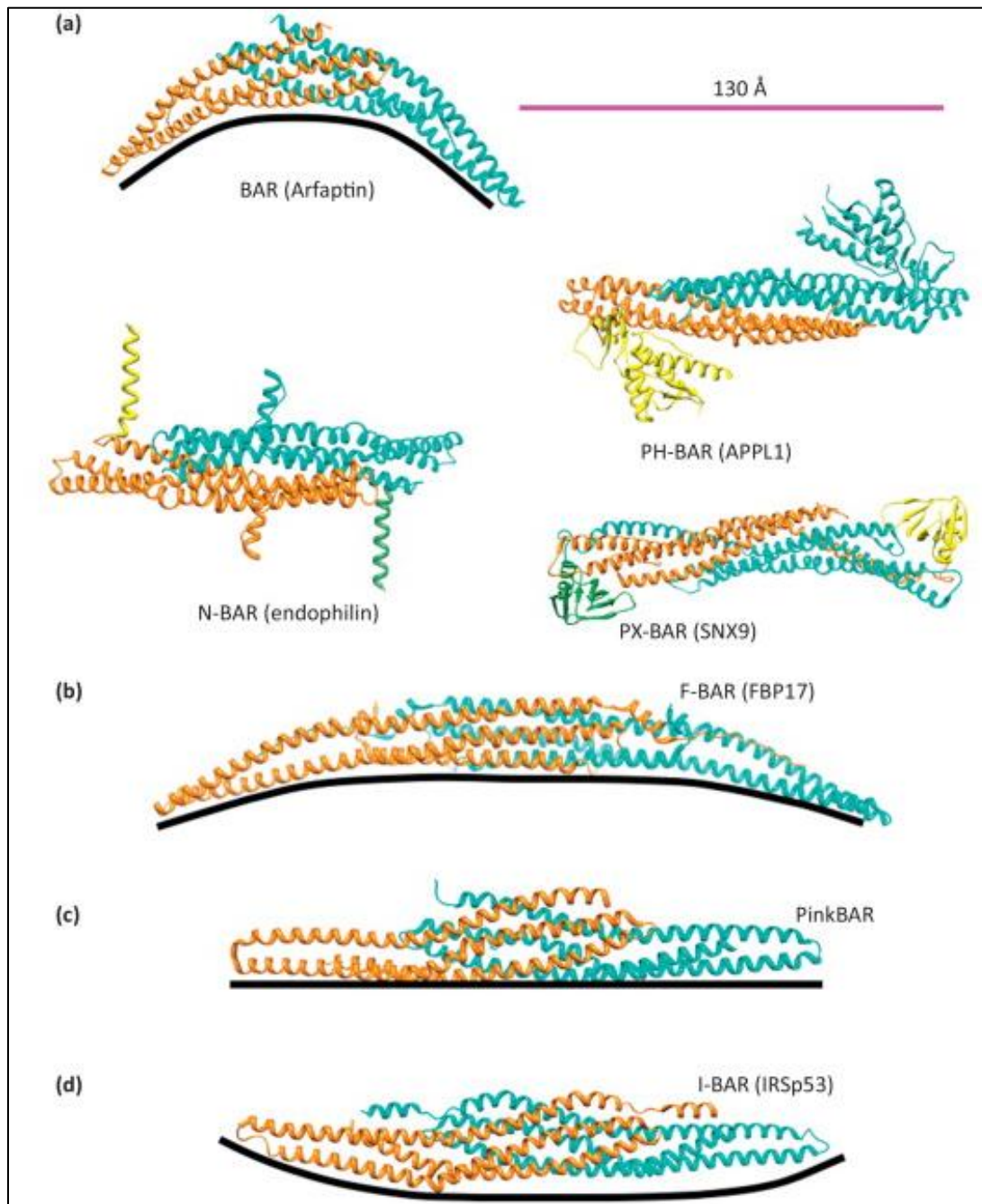


Figure 1.12. Structures of BAR domains from various subfamilies. Structures are depicted as dimers (cyan and orange). The black line underneath each structure outlines the intrinsic curvature of each domain. (a) BAR domain of Arfaptin 1 exhibits the highest positive curvature within the BAR superfamily. Classic BAR domains are further subdivided based on additional membrane binding domains. The N-

BAR domain Endophilin contains an N-terminal helix (yellow and green) and an additional set of amphipathic helices termed 'insert helices' (orange and cyan). APPL1 is a PH-BAR domain that contains an additional pleckstrin homology domain (PH). PX-BAR proteins, for example, Sorting Nexin 9, comprise a phox homology domain (PX) and a BAR domain. (b) F-BAR domain from FBP has a shallow, positive curvature that is characteristic of this subfamily. (c) The PinkBAR is the only BAR domain that shows no intrinsic curvature. (d) I-BAR domain proteins are the only subfamily that can bend membranes to generate structures with negative curvature (scale bar: 130 Å, Reproduced from Mim & Unger, (2012)).

Syndapin has uniquely versatile structural properties Unlike other F-BAR domain-containing proteins like CIP4 or FCHO1 that generate tubes of low curvature, Syndapins can stabilize tubules of various diameters and can also facilitate membrane constriction *in vitro* giving Syndapin its unique membrane remodelling potential (Bai et al., 2012; Wang et al., 2009). This allows Syndapins to be recruited to and function in the formation and maintenance of membrane structures such as caveolae (Hansen, Howard & Nichols 2011), clathrin-coated vesicles (Kessels & Qualmann, 2002), synaptic vesicles in activity-dependent bulk endocytosis (Clayton et al., 2009), ciliated sensory hair cells (Schüler et al., 2013), dendritic spines (Schneider et al., 2014), filopodia (Shimada et al., 2010) and lamellipodia (de Kreuk et al., 2011). *Drosophila* Syndapin was also shown to play a role in cytokinesis and interact *in vitro* with the scaffold protein Anillin (Takeda et al., 2013) which recruits various actin assembling proteins at the cleavage furrow. As mentioned earlier, we further mechanistically showed that Syndapin is crucial for pseudocleavage furrow ingression in *Drosophila* syncytial embryos (Sherlekar & Rikhy, 2016; see Chapters 3-4).

There are two mechanisms by which BAR proteins can achieve membrane bending: rigidity of the protein's secondary structures and by oligomerization to form higher order assemblies. For instance, Syndapin 1 recognizes and stabilizes highly curved tubular membranes in a concentration-dependent manner (Ramesh et al., 2013). BAR domains present positively charged residues that bind negatively charged membranes via electrostatic interactions (Edeling et al., 2009). Syndapins also have two amphipathic alpha-helices or wedge loops; one that binds and inserts into the membrane (Wang et al., 2009) and the other that binds neighbouring dimers through lateral interactions (Frost et al., 2008). However, the wedge loop in Syndapin is unique since the diameter of the tubules does not correlate with the intrinsic structure of the F-BAR

domain. Syndapin dimers also organize in a tip-to-tip manner that form spiral assemblies both in the presence and absence of liposomes (Bai & Zheng, 2013). Lateral and tip-to-tip modes of

No.	Name	Physiological Function
1	F-Bar	
2	Syndapin	Subsynaptic reticulum formation, cytokinesis-cleavage furrow & spindle assembly
3	Nostrin	Adherens junction organization
4	CG8176	Not known
5	Cip4	Synaptic growth regulation at NMJ, adherens junction organization, negative regulation of actin nucleation
6	Nervous wreck	Regulation of actin filament polymerization, Synaptic growth regulation at NMJ
7	Fps85D	Dorsal closure, axon guidance, regulation of adherens junctions
8	N-Bar	
9	Amphiphysin	Muscle T tubule defects, cleavage furrow formation
10	Endophilin A	Synaptic vesicle recycling, lysophosphatidic acid acyltransferase activity
11	Endophilin B	Endocytosis, vitellogenesis
12	RhoGAP92B	Negative regulation of actin filament polymerization, subsynaptic reticulum organization
13	Arfaptin	Synapse growth at NMJ by association with Dynactin, Endocytosis in TGN
14	PICK1	Regulated secretory pathway
15	ICA69	Like Arfaptin, Function Not known
16	PH-Bar	
17	Graf	Like Arfaptin, Function Not known
18	Centaurin beta 1A	Like ARF, Function Not known
19	Asap	Like ArfGAP, Function not known
20	Sorting nexin 16	Synaptic growth regulator, receptor recycling
21	I-Bar	
22	MIM	Border cell migration, germ cell migration, receptor recycling
23	CG32082	Like MIM, Filopodium assembly

Table 1.2. 19 flavours of BAR-proteins in *Drosophila*. The table lists the annotated BAR-domain containing proteins in *Drosophila*, of which 14, highlighted in yellow, are expressed in the syncytial blastoderm embryo (modENCODE Consortium, 2009).

interaction are also seen in other F-BAR domain proteins like CIP4 and FBP17 (Frost et al., 2008; Shimada et al., 2010) but they make more rigid tubes compared to Syndapin 2 (Tanaka-Takiguchi et al., 2013). F-BAR proteins usually generate tubes of low curvature (~100 nm) but tubes generated by Syndapins 1 and 2 have various diameters: low (~100 nm), intermediate (~50 nm) and high curvature (~10 nm) owing to their flexible spiral assemblies. Syndapin 3, however, is like other F-BAR proteins in that it can only make tubes of low curvature and this is owing to its rigid wedge loop (Bai et al., 2012). Flat membranes are not sensed by BAR domains other than Pink BAR, rather they interact with wedge loops: a study therefore proposed that wedge loops are responsible for curvature sensing/bending at low protein concentrations, while the BAR domain takes over the role of controlling membrane curvature at high concentrations (Isas et al., 2014). Experimental evidence however also shows that some BAR proteins without amphipathic helices can sense membrane curvature at low densities (Prévost et al., 2015); thus, the role of helices versus the BAR domain in coupling with membrane curvature and also the precise mechanism by which Syndapins function to bend membranes remains unresolved.

Syndapin role in actin assembly: possible role in regulation of plasma membrane tension

Syndapins have long been known to interact with N-WASp via its SH3 domain and thereby activate Arp2/3-mediated actin polymerization (Kessels & Qualmann, 2002; Modregger et al., 2000). Syndapin 2 SH3 domain also interacts with another actin nucleator Cordon Bleu (Cobl) (Ahuja et al., 2007; Koch et al., 2011; Schwintzer et al., 2011) and with C-terminus of Rac1. Syndapin 2 negatively regulates Rac1 signalling in cell spreading and wound healing; conversely inhibition of Rac1 promotes membrane tubulation by Syndapin 2 (de Kreuk et al., 2011). Interaction with Cobl mediates formation and regulation of ciliary (Schüler et al., 2013) and microvillar length (Wayt & Bretscher, 2014). In an attempt to reconcile the large body of literature on interactions of BAR proteins with membranes as well as regulatory proteins of the cytoskeleton, Kostan et al. (2014) showed that the F-BAR domain of Syndapin 2 can interact electrostatically with actin filaments and that there exists a competition, albeit weak, between negatively charged liposomes and F-actin for interaction with the F-BAR domain of Syndapin 2 and not other F-BAR proteins like CIP4 or FCHO2. Also, at higher molar ratios of Syndapin 2 to

actin, this interaction can cause actin filament stability by decreasing its depolymerization rates without affecting cofilin activity. Cofilin on the other hand increases polymerization rates of actin by making free plus ends for monomer addition even in the presence of Syndapin 2 (J Kostan et al., 2014). Therefore, Syndapin 2 in the presence of monomeric actin and Cofilin will cause rapid polymerization of actin into stable filaments.

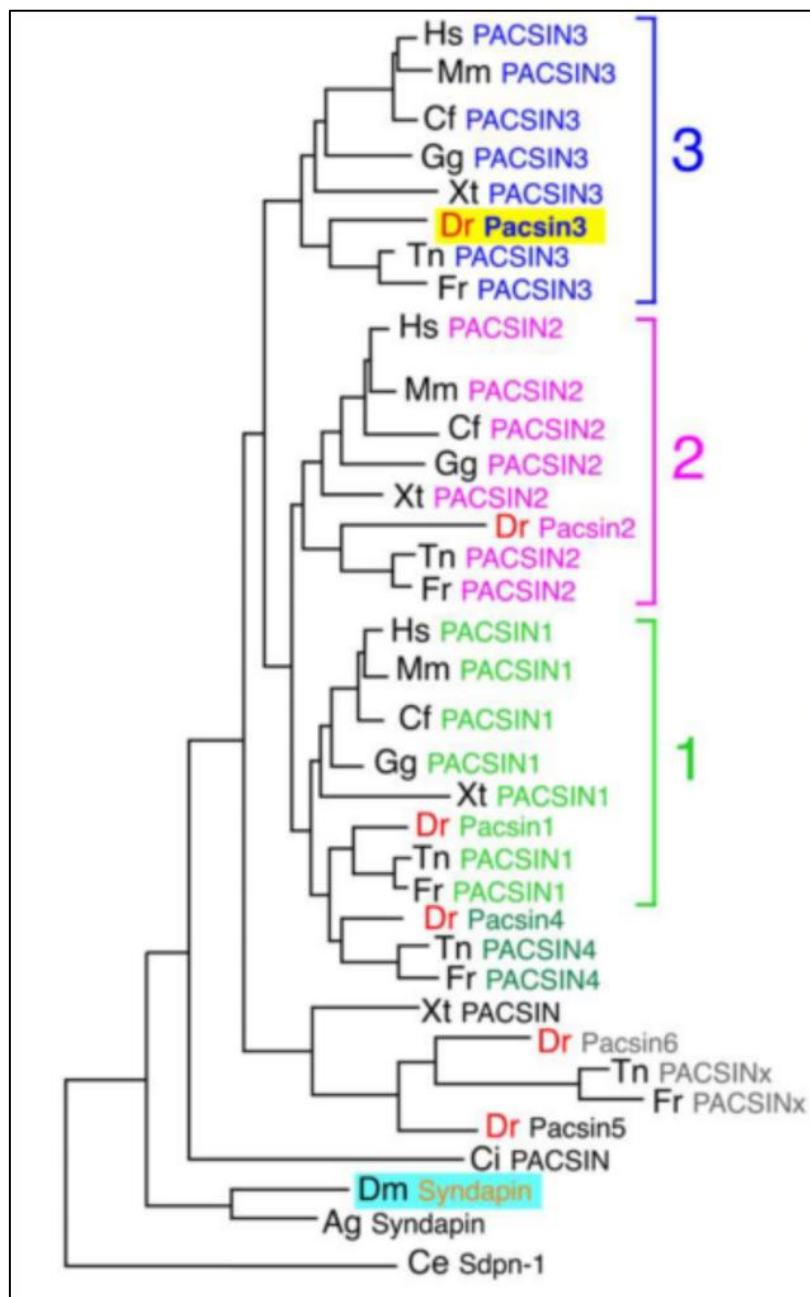


Figure 1.13. Syndapin and its orthologues. PACSIN family dendrogram (TreeFam accession TF313677) includes vertebrate PACSINs 1 (green), 2 (magenta) and 3 (blue) and *Drosophila* Syndapin (Dm). Mm, *Mus musculus*; Cf, *Canis familiaris*; Gg, *Gallus gallus*; Xt, *Xenopus tropicalis*; Dr, *Danio rerio*; Tn, *Tetraodon nigroviridis*; Fr, *Fugu rubripes*; Ci, *Ciona intestinalis*; Ag, *Anopheles gambiae*; Ce, *Caenorhabditis elegans* (Reproduced from Edeling et al., (2009)).

Cortical tension plays a role in the extent of furrow ingression (Wagner & Glotzer, 2016). Assembly/disassembly of BAR proteins on the membrane depends on plasma membrane tension. For instance, Syndapin 2 depletion causes the loss of caveolae from the membrane (Hansen et al., 2011). Caveolin-1 binds to the F-BAR domain of Syndapin 2 and activates it to cause membrane tubulation (Senju et al., 2011) in small localized areas to which a small sub-pool of Syndapin 2 localizes (Koch et al., 2012). Caveolae are known buffers of membrane tension during mechanical stress (Sinha et al., 2011) and so by regulating Caveolae on the membrane, Syndapin 2 can regulate plasma membrane tension. Since Syndapin 2 was also known to associate with proteins that regulate microvillar length (Wayt & Bretscher, 2014), it is possible that other dynamic actin protrusions that like microvilli present on the apical surface of *Drosophila* syncytial embryos may serve as membrane reservoirs for the extension of the pseudocleavage furrow, and Syndapin could mediate this release.

Cleavage furrow growth, like any protrusion from the membrane (Chhabra & Higgs, 2007) requires two concurrent processes to be homeostatically regulated: 1. Membrane tension to supply membrane for extension of the furrow, 2. Tension caused by actin contractility to provide the force for furrow extension which, in turn, is controlled by various actin regulators (Bezanilla, Gladfelter, Kovar, & Lee, 2015; Figard & Sokac, 2014). Actin assembly is promoted by actin nucleators like formins and the Arp2/3 complex. Actin is further processively elongated and bundled by the action of formins (Pruyne et al., 2002; Vizcarra, Bor & Quinlan, 2014) and septins (Mavrakakis et al., 2014) and Cofilin-mediated disassembly makes free plus ends for monomer addition. Our study on *Drosophila* pseudocleavage furrow extension showed that Syndapin can organize the formin Dia and the septin Peanut on pseudocleavage furrow membranes of *Drosophila* blastoderm embryos. Loss of these proteins were shown to cause defects in furrow elongation (Sherlekar & Rikhy, 2016; see Chapter 4). During furrow extension, plus ends of actin filaments are oriented towards and in close proximity to the plasma membrane (few nm apart) so that a single monomer is added every time the filament tip and membrane separate from each other by simple brownian motion (Peskin et al., 1993). Given that Syndapins can bind both the membrane and actin filaments, Syndapin could shuttle between membrane bound and actin bound states and thus modulate membrane tension to drive furrow elongation. We found that Syndapin can rescue furrow extension phenotypes by organization of actin in the pseudocleavage furrow. To mechanistically understand the process by which Syndapin functions

in vivo, we used drugs that affect actin assembly: Latrunculin A (LatA) sequesters free actin monomers and makes them unavailable for filament growth (Coué et al., 1987), Jasplakinolide (Jasp) that blocks actin dynamics by stabilizing actin filaments (Murthy & Wadsworth, 2005) and Cytochalasin D (CytoD) that slows down actin polymerization by allowing monomer addition only at the minus end. Both LatA and Jasp through different mechanisms block actin dynamics. In either case, furrow extension is not rescued in Syndapin-depleted embryos. CytoD treatment, on the other hand, is able to rescue furrow elongation (Sherlekar & Rikhy, 2016; see Chapter 4). It is plausible that slowing down actin polymerization allows sufficient time for actin

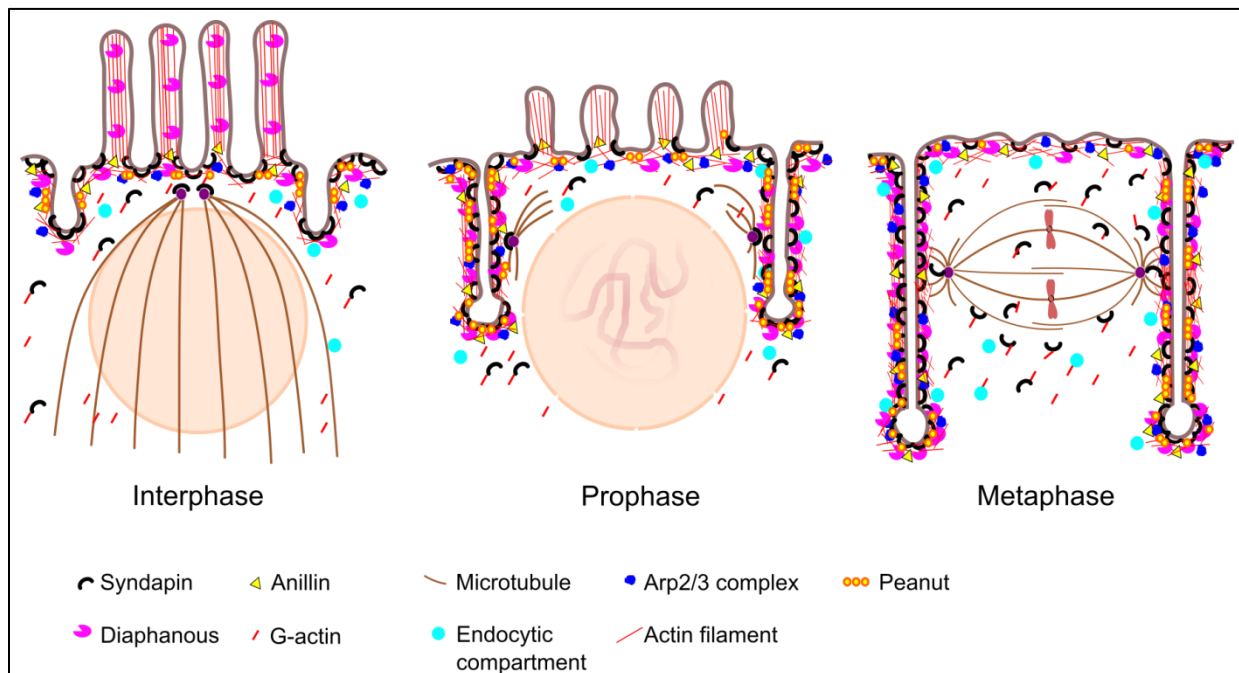


Figure 1.8. Model summarizing Syndapin's role in dynamics of membrane and cytoskeleton. The syncytial *Drosophila* nuclear cycle proceeds from interphase to metaphase through prophase during which pseudocleavage furrow extension occurs. Microvilli-like protrusions on the apical surface most likely flatten as the pseudocleavage furrow extends in prophase and metaphase. Syndapin could recruit to the base of microvilli, and regulate its length. Syndapin recruits to the plasma membrane and associates with actin-remodelling proteins Anillin and Peanut and/or actin to favour its persistent growth into filaments and, therefore, regulate dynamics of the pseudocleavage furrow. Arp2/3 and Diaphanous nucleate actin for growth into networks and bundles to scaffold membrane protrusions.

assembly, disassembly and organization to govern dynamics of furrow growth. It also points to loss of homeostatic control of actin assembly/disassembly when Syndapin is depleted. This, together with the study by Kostan et al. which suggests that Syndapin can slow down

depolymerization rates of actin filaments (J Kostan et al., 2014) clearly demonstrate that Syndapin is important for dynamics of actin filaments to drive furrow extension. Thus, our model suggests that by releasing membrane tension from apical microvilli and organizing actin in the furrows, Syndapin can drive pseudocleavage furrow elongation (Figure 1.14).

The *Drosophila* syncytial embryo was established as a functionally unique model to study cleavage furrow formation nearly two decades ago since the molecular composition of pseudocleavage furrows was indistinguishable from conventional cleavage furrows (Sisson, Rothwell & Sullivan, 1999) such as those of *Drosophila* spermatocytes: a favourite model for cytokinesis studies (Giansanti and Fuller, 2012). Work on these two systems have built on existing knowledge to firmly establish Syndapin as a key molecule crucial for cleavage furrow formation (Sherlekar and Rikhy, 2016; Takeda et al., 2013). We thus propose a model that encompasses all of Syndapin's roles in the context of furrow formation. Firstly, Syndapin can associate/dissociate from the membrane and modulate plasma membrane tension and, with its unique structure, bend membranes into tubes of various diameters. Syndapin can also recruit Anillin, Peanut and Diaphanous to the cleavage furrow, proteins essential for furrow extension. Finally, Syndapin can directly regulate rates of actin depolymerization and decrease actin dynamics which allows control of actin contractility that drives furrow extension. Therefore, our model places Syndapin as a key player in the homeostatic control of membrane and cytoskeletal tension that orchestrate cleavage furrow formation.

CHAPTER 2

Materials and Methods

2.1 *Drosophila* Strains and Genetics

Flies were raised on standard cornmeal agar at 25°C. Embryos from Canton S flies were used in control experiments. Homozygous escaper adults from the transposon tagged mutant *synd*^{1d}/TM6B, Tb¹ [V. Kumar (Kumar et al., 2009)] or adults in with trans-allelic combination of *synd*^{1d}/Df(3R)BSC43 [BL-7413] were used to generate *synd* mutant embryos. These embryos showed a loss of Syndapin immunostaining and high percentage of embryonic lethality by hatch assay (Figure1). The *synd* mutant phenotype was rescued using Synd-GFP [T. Takeda and D. Glover (Takeda et al., 2013)]. *synd*^{RNAi} (BDSC stock) was crossed to nanos-Gal4 and embryos laid by females of the genotype *synd*^{RNAi}/nanos-Gal4 showed a lower embryonic lethality than the mutant (Figure 1).

The following mutant stocks were used, w^{*}; *dia*⁵ FRT40A/CyO [BL-9138], y¹ w^{*}; *rhogef*^{2.1} FRTG13/CyO [BL-9122], *pnut*^{XP} FRT^{G13}/SM6a-TM6B, Tb¹ [M. Mavrakis] and w¹¹¹⁸; P{w^{+mC}=EP}Arp3^{EP3640}/TM6B, Tb¹ [BL-17149]. Females carrying the mutant and heat-shock FLP were crossed to respective FRT *ovo*^D males -*ovo*^D FRT40A/CyO [BL-2121] or *ovo*^D FRTG13/CyO to generate germline clones by heat shocking third instar larvae, pupae and adult flies for 1hr each at 37.5°C. The transheterozygous animals *scra*^{k08255}/ *scra*⁸ [BL-4403, 4286] gave *anillin* (*scraps*) mutant embryos. *shi*^{ts2} [R. Rikhy] mutants were transferred to a restrictive temperature (32°C) for 5min before fixation.

Gap43-Venus (Mavrakis, Rikhy & Lippincott-Schwartz, 2009) with H2A-RFP [BL-25377] was used to show pseudocleavage furrow morphology changes across nuclear cycle 13. Metaphase furrow lengths in nuclear cycles 12 and 13 were measured by a tGPH; nos-Gal4 recombinant line

Stock Name	Source	ID
Mutant stocks		
<i>w</i> [*] ; <i>dia</i> ⁵ FRT40A/CyO	BDSC	BL-9138
<i>y</i> ¹ <i>w</i> [*] ; <i>rhogef2</i> ^{A1} FRTG13/CyO	BDSC	BL-9122
<i>pnut</i> ^{XP} FRT ^{G13} /SM6a-TM6B, Tb ¹	M. Mavrakis	-
<i>w</i> ¹¹¹⁸ ; P{ <i>w</i> ^{+mC} =EP}Arp3 ^{EP3640} /TM6B, Tb ¹	BDSC	BL-17149
<i>ovo</i> ^D FRT40A/CyO	BDSC	BL-2121
<i>scra</i> ^{k08255}	BDSC	BL-4403
<i>scra</i> ⁸	BDSC	BL-4286
Stocks for live imaging		
Gap43-Venus	R. Rikhy	-
H2A-RFP	BDSC	BL-25377
tGPH	BDSC	BL-8163
nos-Gal4	BDSC	BL-4937
UASp-LifeAct-GFP	R. Rikhy	-
UASp-Arpc1-GFP	BDSC	BL-26692
UASp-Dia-EGFP	BDSC	BL-56751
Ub-Synd-GFP/CyO; MKRS/TM6Tb	T. Takeda	-
UASp-mCherry::Pnut	T. Lecuit	-
RNAi lines		
<i>rhogef2</i>	BDSC	BL-34643
<i>dia</i>	BDSC	BL-33424
<i>pnut</i>	VDRC	GD-11791
<i>wasp</i>	BDSC	BL-36647
<i>arp3</i>	BDSC	BL-32921
<i>arpc1</i>	BDSC	BL-31246

Table 2.9. List of *Drosophila* stocks used. With details of the Source and ID number (if procured from a stock centre; BDSC: Bloomington *Drosophila* Stock Center, BL: Bloomington Line, VDRC: Vienna *Drosophila* Resource Center, GD: Genomic disruption (P-element library)).

[BL-8163, 4937]. To visualize actin in apical caps we used UASp-LifeAct-GFP generated in the lab. To assess localization of Arp2/3 and Dia in live embryos we used UASp-Arpc1-GFP [BL-26692], UASp-Arp3-GFP [BL-39723] and UASp-Dia-EGFP [BL-56751].

RNAi lines for *rhogef2* [BL-34643], *dia* [BL-33424], *pnut* (VDRC, Austria), *wasp* [BL-36647], *arp3* [BL-32921] and *arpc1* [BL-31246] were used. Ub-Synd-GFP/+; *synd*^{1d}/Df was used to measure furrow and protein dynamics and Ub-Synd-GFP was combined with *rhogef2*^{RNAi} to test rescue. UASp-mCherry::Pnut [T. Lecuit (Guillot & Lecuit, 2013)] was used to overexpress Pnut in background of *rhogef2*^{RNAi}.

2.2 Immunohistochemistry

Antibody	Organism	Dilution	Source	Catalogue No.
Anillin	Rabbit	1:1,000	C. Field, J. Brill	-
Pnut	Mouse	1:5	DSHB	4C9H4
Dia	Rabbit	1:1,1000	Wasserman lab	-
Dorsal	Rabbit	1:5,000	A. Courey	-
DPatj	Rabbit	1:1,000	H. Bellen	-
Rab11	Rabbit	1:100	Abcam	ab3612
GFP-Tag	Chicken	1:1,1000	Invitrogen	A10262
Amphiphysin	Rabbit	1:1,000	A. Pawson	-
Syndapin	Rat	1:150	V. Kumar	-
Rab5	Rabbit	1:250	Abcam	ab13253
Dlg	Mouse	1:10	DSHB	4F3
Toll	Rabbit	1:1,000	S. Wasserman	-
aPKC	Rabbit	1:1,000	Santa Cruz	C20
Sep2	Guinea Pig	1:250	M. Mavrikakis	-
Sep1	Rat	1:250	M. Mavrikakis	-
Arp3	Rabbit	1:1,000	W. Theurkauf	-
Arm	Mouse	1:10	DSHB	N2 7A1

Table 2.10. List of Antibodies used. With details of Organism from which derived, Dilution used, Source from which procured (DSHB: Developmental Studies Hybridoma Bank) and Catalogue number (if procured from a commercial source).

Embryos were collected for 3hrs at 25⁰C (and at 28⁰C for RNAi experiments) on 3% sucrose agar plates, washed and dechorionated in 100% bleach for 1 min, fixed in 1:1::4% PFA in PBS:Heptane for 15 min, hand/methanol devitellinized, permeabilized with 0.3% Triton-X in PBS (PBST) blocked in 2% BSA (Sigma-Aldrich[®]) in PBST and immunostained with primary antibodies: Syndapin (Rat, 1:150 [V. Kumar]), Amphiphysin (Rabbit, 1:1,1000 [A. Pawson]), Dia (Rabbit, 1:1,000 [S. Wasserman]), Peanut (Mouse, 1:5 [DSHB-4C9H4]), Anillin (Rabbit, 1:1,000 [C. Field and Julie Brill]), Rab5 (Rabbit, 1:200 (Abcam[®])), GFP Tag antibody (Chicken, 1:1,000 [Invitrogen[™]], Sep1 (Rat) and Sep2 (Guinea Pig) (1:250 [M. Mavrakis]) and Arp3 (Rabbit, 1:1,000 [W. Theurkauf]). RhoGEF2 antibody (Rabbit, 1:500 [J. Grosshans]), Dlg (Mouse, 1:10 [DSHB-4F3]) and Arm (Mouse, 1:10 [N2-7A1]) staining was done after heat-fixation followed by 1:1::Heptane:Methanol devitellinization. Fluorescently coupled secondary antibodies (Alexa Fluor[®] 488, 568 and 633, Life technologies[™] (1:1,000)) were used. DNA was labelled with Hoechst 3342 (10 mg/ml) Life technologies[™] (1:1,000) and F-actin was labelled with Alexa Fluor[®] 488/568 Phalloidin (1:100, Life technologies[™]). The embryos were mounted in SlowFade[®] Gold (Life technologies[™]). These samples were imaged using a Plan-Apochromat 40x/1.3 NA oil-immersion objective on the confocal Laser Scanning Microscope (LSM 710, Zeiss[™]). Z-stacks were acquired on sequentially scanned images with a spacing of 1.08 μ m between slices. n values indicate number of syncytial cycle 12 or 13 embryos screened. Embryos defective for a phenotype were calculated as a percentage of n. For quantitative analysis of Rab5-positive vesicles and Amph furrow tubules, images were acquired using a Plan-Apochromat 63x/1.3 NA oil-immersion objective on the Confocal Laser Scanning Microscope (LSM 710, Zeiss[™]) with pinhole set at 1 AU for best signal/noise.

Zeiss[™] LSM 800 with Airyscan (0.15 μ m slice thickness) was used to acquire and process high-resolution images of Hoechst and Phalloidin-stained embryos.

2.3 Drug treatment with actin-binding drugs

Drug	Concentration used	Source	Catalogue No.
LatA	10 μ M	Sigma Aldrich [®]	L5163
Jasp	200 nM	Sigma Aldrich [®]	J4580
CytoD	50 μ M	Sigma Aldrich [®]	C8273

Table 2.11. List of drugs used. With details of Concentration used, the Source and its Catalogue No.

Dechorionated embryos were added to a solution of 1:1::(R)-(+)-Limonene (Sigma):Heptane to which the drug dissolved in DMSO was added to achieve the desired final concentration (200nM Jasp, 50 μ M/ 100 μ M CytoD, 10 μ M LatA or DMSO without drug in case of Control) and incubated with shaking for 5min (Adapted from (Rand et al., 2010; Schulman et al., 2013). The solution was then replaced with fresh Heptane and subsequently fixed, hand-devitenillized and immnostained as mentioned in the protocol above.

2.4 Live-embryo time-lapse imaging

Embryos were collected for 1hr, dechorionated and mounted in a LabTek™ chamber with PBS (Mavrakakis et al., 2008) imaged in 4D (every 30s with 0.48/1.08 μ m thickness) by a Zeiss Plan-Apochromat 40X/1.4 NA oil immersion objective on a Zeiss™ LSM 780 inverted Confocal microscope. Staging of embryos during the cell cycle was done by corroborating presence of nuclear envelope and its breakdown with metaphase furrow length. Since some tGPH is in the cytoplasm these events are easy to distinguish in the live movies to accurately stage the embryos.

2.5 TIRF Microscopy

0-1 hour embryos were collected and dechorionated with 50% sodium hypochlorite. Embryos were lined up on a LabTek™ chamber having their ventral side facing the coverslip in order to maximise the contact area of the embryo to the coverslip. Mounted embryos were immersed in 1ml 1X PBS solution. Embryos were then subjected to time-lapse TIRF imaging on Olympus Biosystems cell TIRF using an Olympus Plan APO 100x oil objective (N.A. 1.49). The angle of incidence was maintained at the critical angle for total internal reflection with small angular

adjustments to sample illuminating angle in each experiment. In order to get optimized signal from the cortex/apical membrane, TIRF illumination plane was set at approximately 100 nm optical section thickness from the interface. For time lapse, images were acquired every 6 seconds. Post acquisition, all time frames were deconvolved using deconvolution algorithm provided by Olympus imaging and processing software package.

2.6 Image Processing

Images were processed with ImageJ (NIH) and compiled with Adobe Photoshop CS5™. Immunostainings are shown from single optical planes for sagittal (XZ) or surface views (XY). A Gaussian smoothing filter with a radius of one pixel was used to reduce noise. Processed Images of F-actin and DNA obtained from Airyscan were imported into Imaris8.1 software (Bitplane) and snapshots of single Z-slice (Slice), 3D surface rendering (Surface) and maximum intensity projection (MIP) were represented.

2.7 Fluorescence Recovery After Photobleaching (FRAP)

For FRAP (Mavrakakis et al., 2008) 3 prebleach scans, 30 bleach iterations (0.03 s each) were taken on living Synd-GFP and tGPH embryos in $\sim 2.5 \mu\text{m}^2$ regions on the apical and FT membrane. Recovery of fluorescence intensity was recorded until a steady-state was reached. The data was background corrected and normalized to the prebleach intensity and transformed on a 0-1 scale. The individual post-bleach intensities were fit with a One-phase exponential association equation ($Y = Y_{\text{max}} \cdot (1 - e^{-k \cdot X}) + c$) using GraphPad™. Nonlinear regression was used obtain the Y_{max} or mobile fraction for Synd-GFP (R^2 for apical 0.83 ± 0.1 S.D. and FT membrane 0.83 ± 0.03 S.D.). The immobile fraction (1-mobile fraction) was calculated and plotted as scatter plots using GraphPad™ for apical and FT membranes.

2.8 Fluorescence Loss in Photobleaching

Continuous photobleaching of the region of interest (ROI) of size $5 \mu\text{m}^2$ was carried out on syncytial embryos expressing LifeAct-GFP with the 488 nm laser at 100% power and 30 iterations. The ROI was bleached after every second. The ROI and neighboring region were monitored for fluorescence depletion. The data was background corrected and normalized to the prebleach intensity, transformed on a 0-1 scale and mean intensities (a.u.) were plotted with standard error using GraphPad™.

2.9 Image analysis and Quantification

Intensity density on membrane regions from confocal images of Syndapin and Amphyphysin was computed using ImageJ. After background subtraction, the ratio of Syndapin:Amphyphysin was plotted as a boxplot using GraphPad™. Non-parametric Mann-Whitney U test was performed to test for significance between means. Intensity of Synd-GFP (in apical, lateral and FT membrane regions) was similarly measured from images in metaphase of cycles 11, 12 and 13 and plotted as a scatter plot using GraphPad™. The mean intensity values were compared for significance using One-Way ANOVA, Tukey's Multiple comparison test. For furrow length, freehand lines along the lateral membrane to FT from orthogonal views at desired time points of time lapse movies were measured and mean $\pm$ S.E.M. of the furrow lengths were plotted using GraphPad™. Lateral furrow broadness was represented as intensity peaks on a line across furrows in orthogonal views using the Plot profile analysis tool of ImageJ.

For numbers of Rab5-positive vesicles, the ImageJ plugin 3D-Objects Counter was used. Only objects in the volume of the pseudocell and below 250 nm diameter were counted. The absolute number was normalized to the number of pseudocells in the ROI and plotted for 3 embryos/genotype using GraphPad™.

To quantify furrow tubule length, images of fixed Amph-stained embryos in prometaphase during cycle 13 were used; freehand lines were drawn along the tubules, their length measured using ImageJ and their number counted. The length and number (per furrow) were plotted for a minimum of 5 embryos/genotype using GraphPad™.

2.10 Quantification of apical actin cap

All measurements were done on time lapse movies of the apical caps in syncytial nuclear cycle 12. The segmented line tool in ImageJ was used to measure intensity of ArpC1-GFP and Synd-GFP along the caps as they expand. Apical cap areas and circularity were measured at a 3 μ m depth from the first visible z stack using the polygon tool of ImageJ.

2.11 Analysis of frequency distribution of Polygon sidedness

The number of edges of each syncytial pseudocell in each nuclear cycle was counted manually and plotted as a histogram with mean $\pm$ S.D. The polygon analysis for wild-type was also done

using the packing analyzer software provided by Benoit Aiguoy and was found comparable to our manual analysis. The packing analyzer detects polygons and overlays on the input image. Manual corrections can be done in case of incorrect detections and the border cells which are not completely in the field of view can be removed. The image is then rendered into a polygon array and colour coded based on the polygon type. An excel sheet is also generated with the area, perimeter, and polygon type of each cell in the image. In case of mutants, since the architecture was disturbed too much to analyze using the software, manual detection and counting of polygons was done for all data sets. The significant differences between control and mutant embryos at n.c. 13 were analyzed using Non-parametric Mann Whitney U test.

2.12 Kymograph Analysis

Lateral membrane movement of metaphase furrows in the XY plane was plotted using multiple Kymograph plugin of ImageJ on single-plane time-series movies of tGPH::GFP labelled embryos. Lines were drawn to generate tracks across 4 metaphase 13 furrows. The displacement was quantified by selecting the initial to final points of furrow movement using the line tool and subsequently, using the macro 'read velocities from tsp' to obtain displacement/velocities. The displacement was plotted using GraphPad™. Unpaired t-test (two-tailed) with Welch's correction was used to test significance between means and F test was used to compare significant difference in variances.

2.13 Cuticle preps

Embryos were collected for 3 hrs at 25⁰C in collection cages on 3% sucrose agar and aged for 20-22 hrs at 25⁰C. They were dechorionated using 100% bleach for 1 min and then successively washed with 100% ethanol and methanol. They were then devitellinised with 1:1 :: Methanol: Heptane and once more washed with methanol. Air-dried embryos were mounted in 85% lactic acid on a pre-warmed slide at 55⁰C and incubated on a slide warmer at the same temperature for 8 hrs to dissolve the guts. The cuticle preps were imaged using dark-field optics (Plan-Neofluar 10x/0.3 Air objective on Zeiss Axio Imager Z1).

CHAPTER 3

Syndapin Function is Essential for Pseudocleavage Furrow Elongation and Membrane Tubulation

3.1 Introduction

The F-BAR protein Syndapin (Synaptic dynamin binding protein) [also known as PACSIN (protein kinase C and casein kinase II interacting protein)], was first identified as a phosphoprotein involved in vesicle formation and transport (Modregger, 2000). It was also shown that Syndapin1 links the recruitment of synaptic vesicles and endocytosis with cytoskeletal rearrangements (Britta Qualmann & Kelly, 2000). Early studies on Syndapin function in *Drosophila* showed that Syndapin interacts with Dynamin and N-WASp via its SH3 domain and that it was highly expressed in the embryo (Kumar et al., 2009a). Over-expression of Syndapin in the larval neuro-muscular junction caused abnormal expansion of the subsynaptic reticulum (Kumar et al., 2009b).

These observations suggested plausible roles for *Drosophila* Syndapin in membrane tubulation, remodelling of the actin cytoskeleton and endocytosis. To further investigate these roles mechanistically, and, in relation to other proteins that influence the cellular homeostasis of these processes, we used the *Drosophila* syncytial embryo. Firstly, as mentioned earlier, Syndapin is highly expressed in the early embryo. Secondly, the syncytial embryo has rapid nuclear cycles with extensive remodelling of the membrane and cytoskeleton that lend us a tractable model to study Syndapin function *in vivo*.

More recently, Syndapin function was implicated in cytokinesis. Syndapin was shown to physically interact with Anillin and together colocalize at the cleavage furrow (Takeda et al., 2013). Anillin is known to recruit Peanut (Pnut) to the furrow membrane during cellularization (Oegema et al., 2000; Field et al., 2005; Liu et al., 2012; Kinoshita et al., 2002). Since syncytial pseudocleavage furrow extension closely mimics that of conventional furrows (Sisson et al., 1999), we also examined the role of proteins involved in cytokinesis like RhoGEF2, Anillin, Dia and Pnut in pseudocleavage furrow formation.

In this chapter, we characterize Syndapin localization and dynamics in wild-type embryos. In addition we describe mutants for Syndapin morphologically and the defects observed in membrane dynamics during the syncytial cycles in mutant embryos compared to wild-type. We assess the role of Syndapin in endocytosis based on levels of the early endosomal marker, Rab5. In addition, we test the ability of Syndapin to generate cleavage furrow tip (CFT) tubules. Significantly, we also demonstrate morphologically that Syndapin overexpression can rescue membrane furrow elongation in *rhogef2*^{RNAi} embryos.

3.2 Results

3.2.1 Syndapin is enriched at *Drosophila* syncytial pseudocleavage furrow tips

To study where Syndapin localizes on the metaphase furrow membrane in the *Drosophila* syncytial embryo, we used an antibody generated against the –SH3 domain of Syndapin (Kumar *et al.*, 2009a). We found that Syndapin localized to the apical and lateral membranes, and was enriched at the furrow tip (FT) where it colocalized with Patj, a marker for the FT (Figure 2.1B) (Mavrikakis *et al.*, 2009). We verified the specificity of the Syndapin antibody by staining embryos mutant for Syndapin (*synd*^{1d}/Df) which shows loss of Syndapin staining from the membrane. *synd*^{1d} is a severe loss of function allele and was generated by mobilizing a P-element (EP(3)409) within the 5' untranslated region (Kumar *et al.*, 2009a). *synd*^{1d} combined with the deficiency allele (null for Syndapin) yields a strong hypomorph for Syndapin. We normalized the intensities of Syndapin in the mutant versus the wild-type to the intensities of Amphyphysin (Amph, an N-Bar domain-containing protein) antibody with which the embryos were co-stained (Figure 2.1C). There is a significant loss of Syndapin antibody intensity in *synd*^{1d}/Df embryos (Figure 2.1D, F). *synd*^{RNAi} embryos driven by nanos-Gal4, homozygous *synd*^{1d} embryos from adult fly escapers and homozygous mutant embryos for Syndapin using *ovo*^D germline mutant strategy also confirm this and show loss of Syndapin staining. *synd*^{1d}/Df embryos showed highest embryonic lethality and hence was used to study defects in furrow morphology (Figure 2.1G). The antibody staining and lethality were rescued using the fluorescently tagged Ub-Syndapin::GFP (Synd-GFP; Synd-GFP expressed with the ubiquitin promotor), indicating the functionality of this GFP-tagged Syndapin (Figure 2.1D-G)

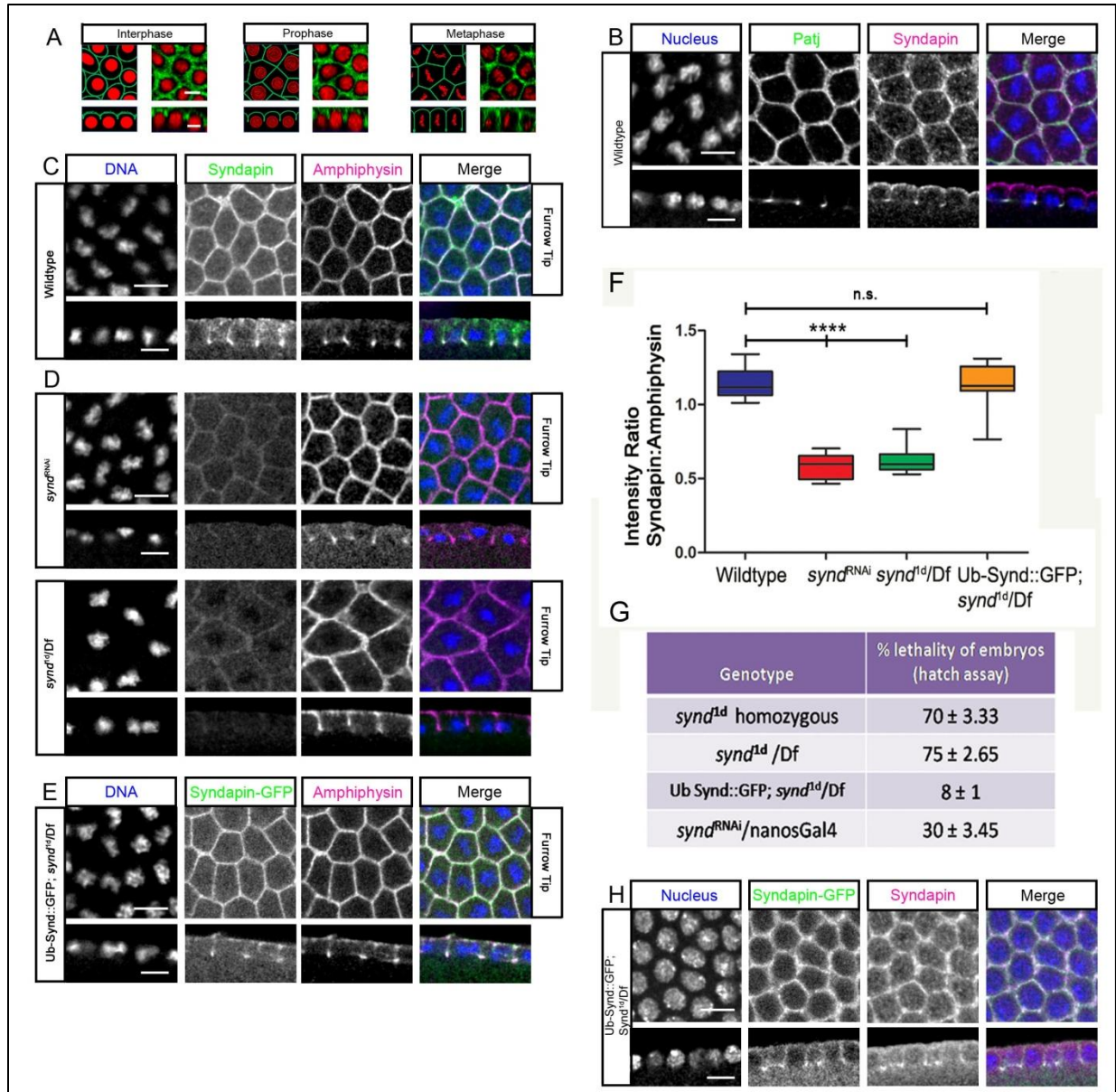
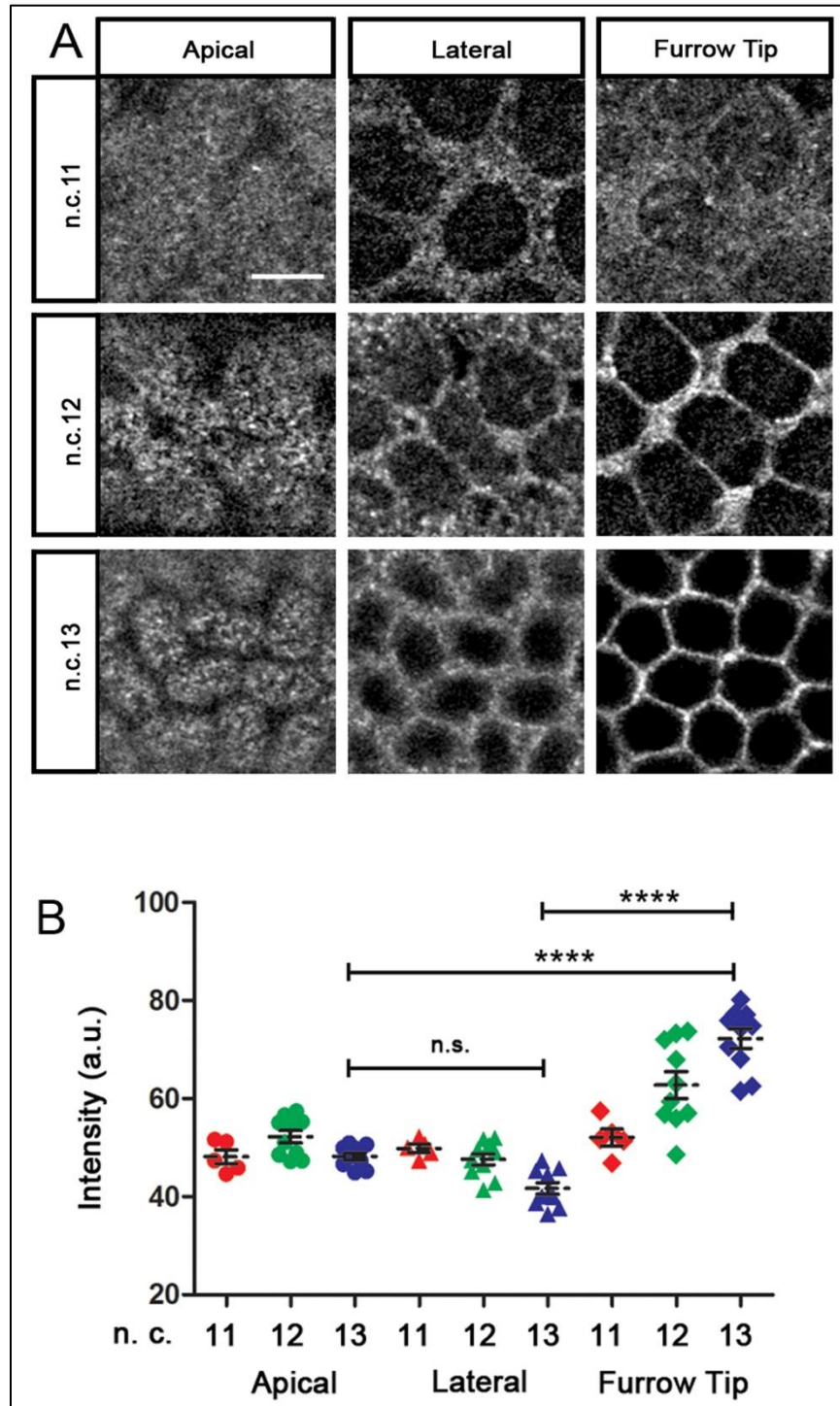
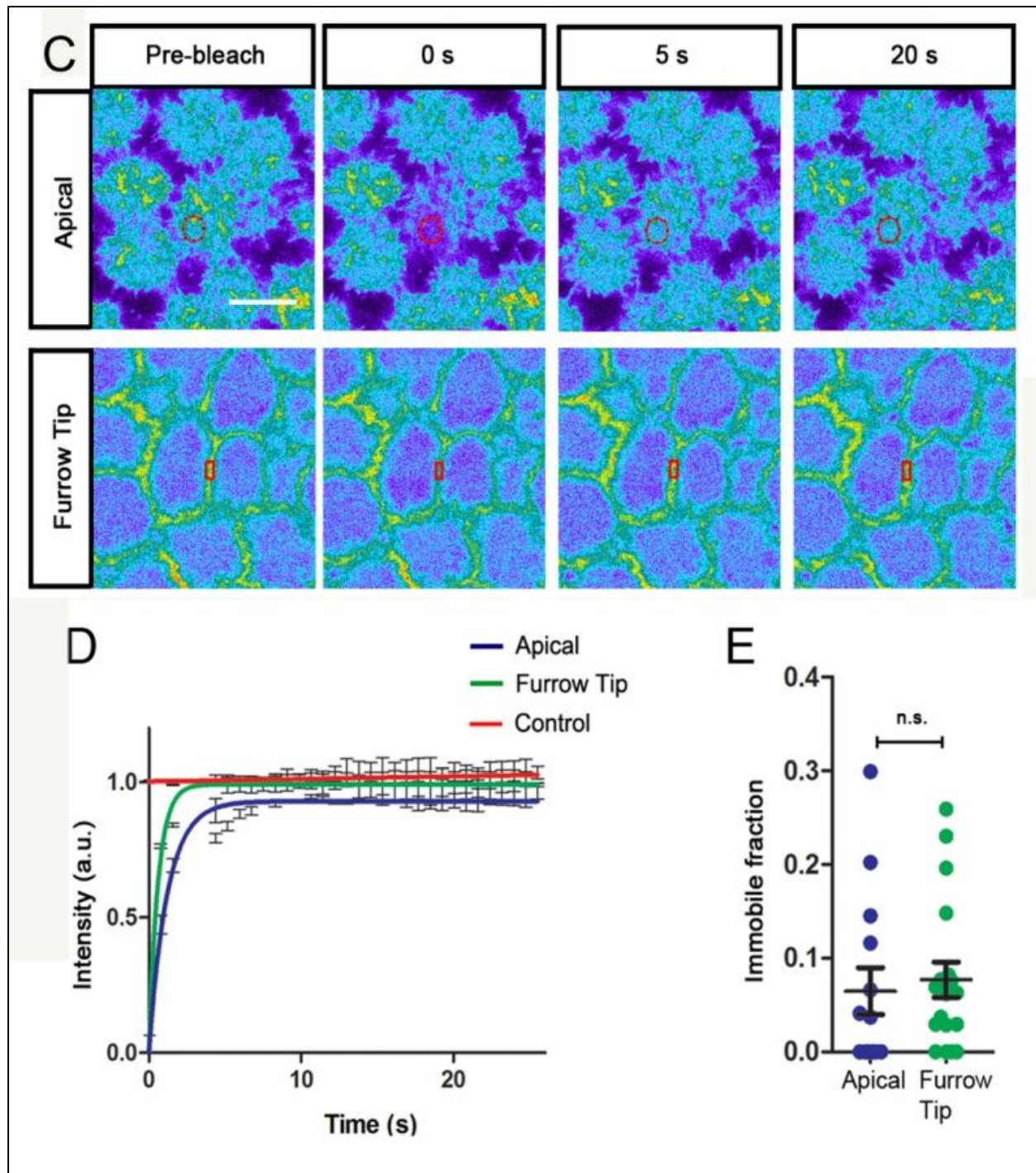


Figure 2.12 Syndapin is enriched at *Drosophila* syncytial pseudocleavage furrow tips.

Pseudocleavage furrow extension visualized in syncytial nuclear cycle 13 with membrane marker Gap43-Venus (green) and Histone-RFP (red) (A). Surface and sagittal views of embryos with Syndapin (magenta) enrichment in Patj positive regions (green) at the FT (DNA, blue) (B). Syndapin antibody/Synd-GFP (green) and Amph (magenta) are localized at metaphase (DNA, blue) furrow tip in wild-type embryos (C). Syndapin immunostaining and embryo viability is reduced in mutants and rescued by Synd-GFP. Syndapin immunostaining ratio to Amph is reduced in *synd*^{RNAi} (60%, n=15) and *synd*^{1d}/Df (85%, n=40) embryos in metaphase as compared to Synd-GFP (25%, n=16) (D-E) and in a box-plot using Syndapin:Amphiphysin fluorescence intensity ratios (F, n=3;15 furrows each, ****, P<0.0001, n.s. not significant by Non-parametric Mann-Whitney U test). Mean ± S.D of 24 hr embryo lethality shows the

highest lethality in *synd*^{1d}/Df which is reversed by Synd-GFP (n=3; 100 embryos/genotype), P<0.0001 (G). Syndapin antibody (magenta) colocalizes with Synd-GFP (green) at the pseudocleavage membrane (H) validating specificity of the syndapin antibody. Scale Bar=10 μ m.





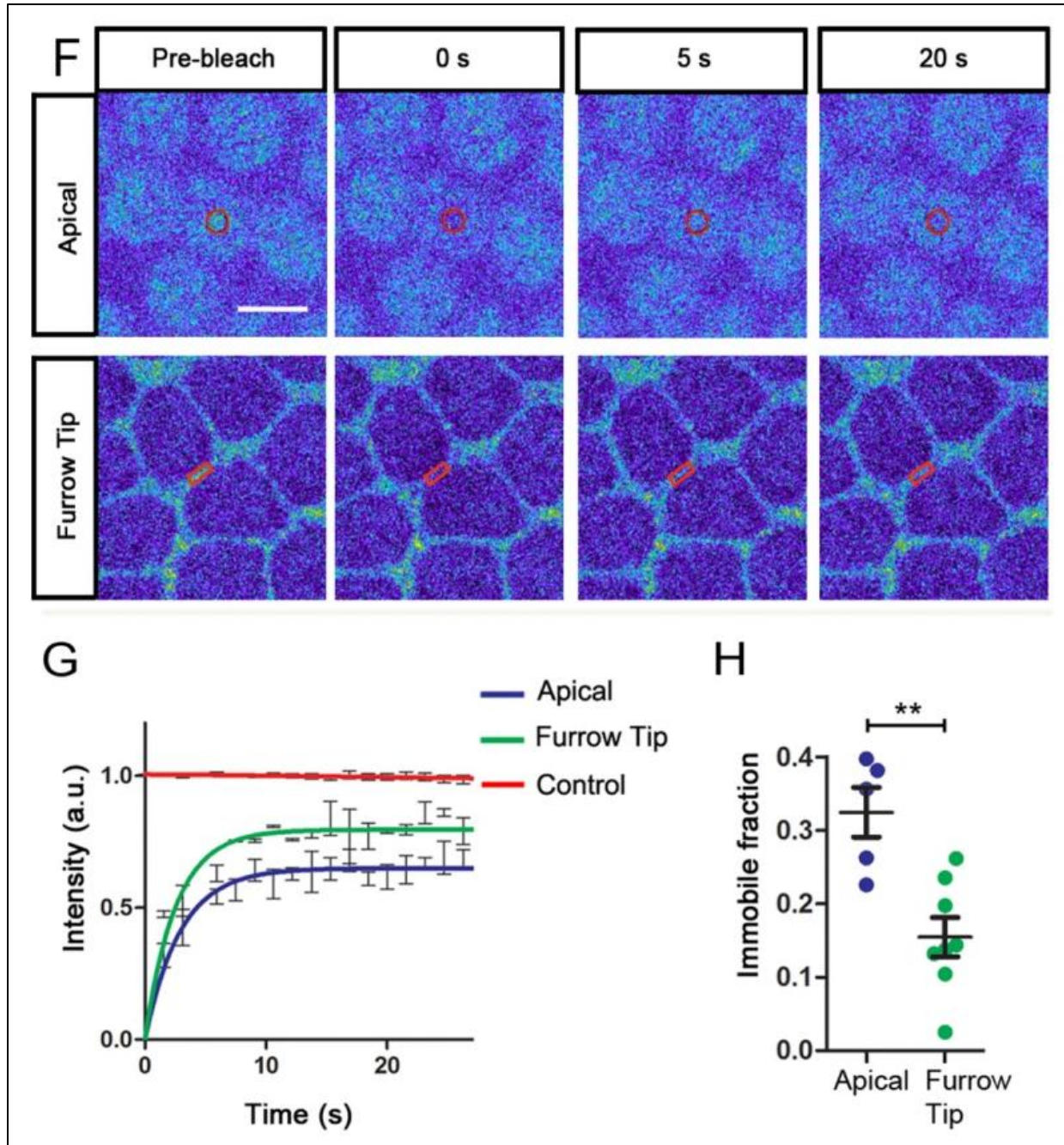


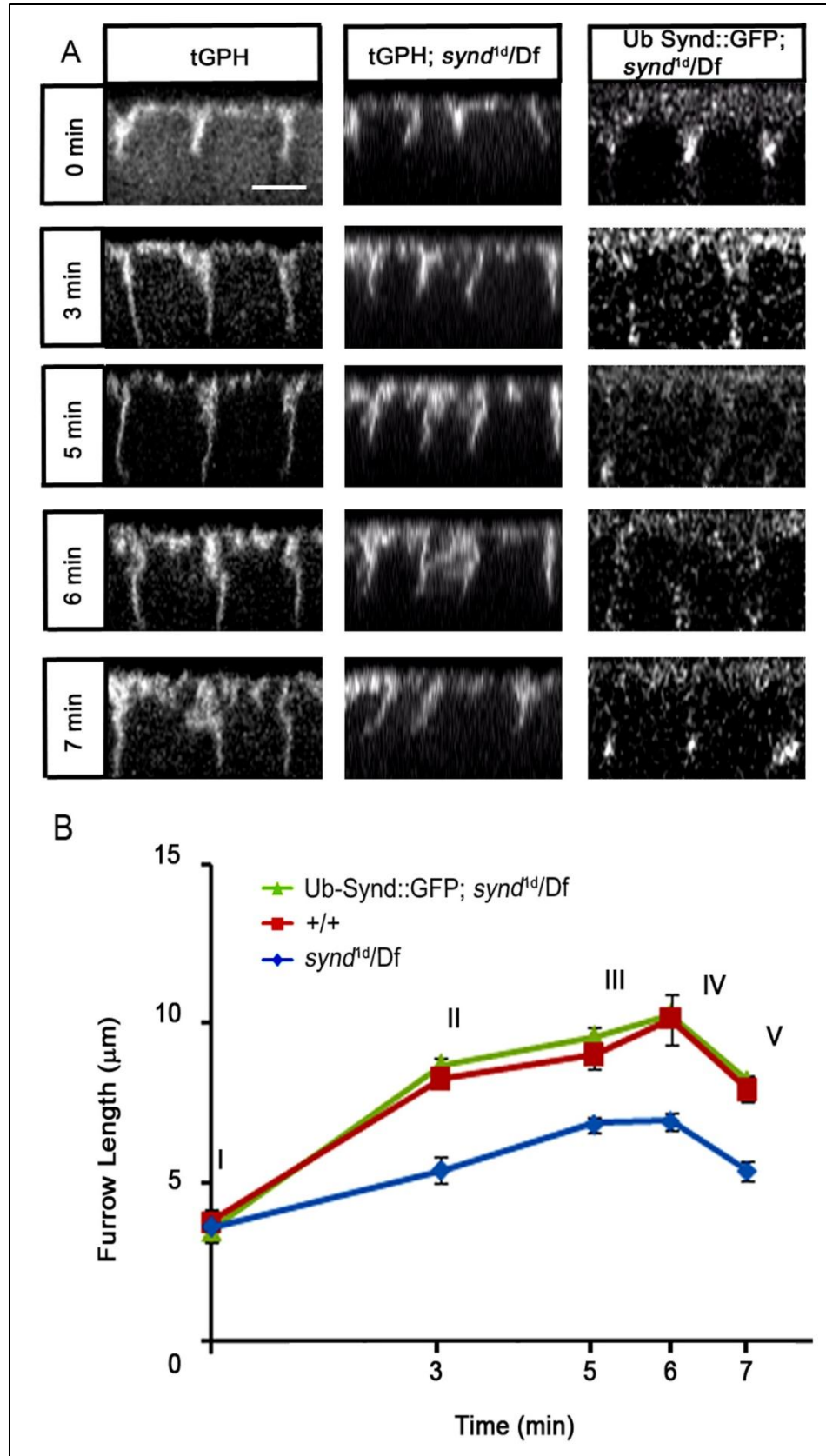
Figure 2.13. Association of Syndapin to the furrow tip increases across syncytial cycles. Synd-GFP fluorescence in Synd-GFP; *synd*^{1d}/Df embryos in metaphase across nuclear cycles (n.c.) 11, 12 and 13 in the apical, lateral and FT membranes (A) is quantified in a scatter-plot (arbitrary units a.u., n=3; 10 membrane ROIs, ****, P<0.0001, n.s. not significant, One-Way ANOVA and Tukey's Multiple comparison test) (B). FRAP with tGPH control at the FT as compared to the apical membrane does not show a significant difference. Photobleaching (black ROI, ~ 2.5 μm^2) of tGPH was performed in apical or FT membranes and images at key time points are shown (C). FRAP recovery occurs to same extent at the

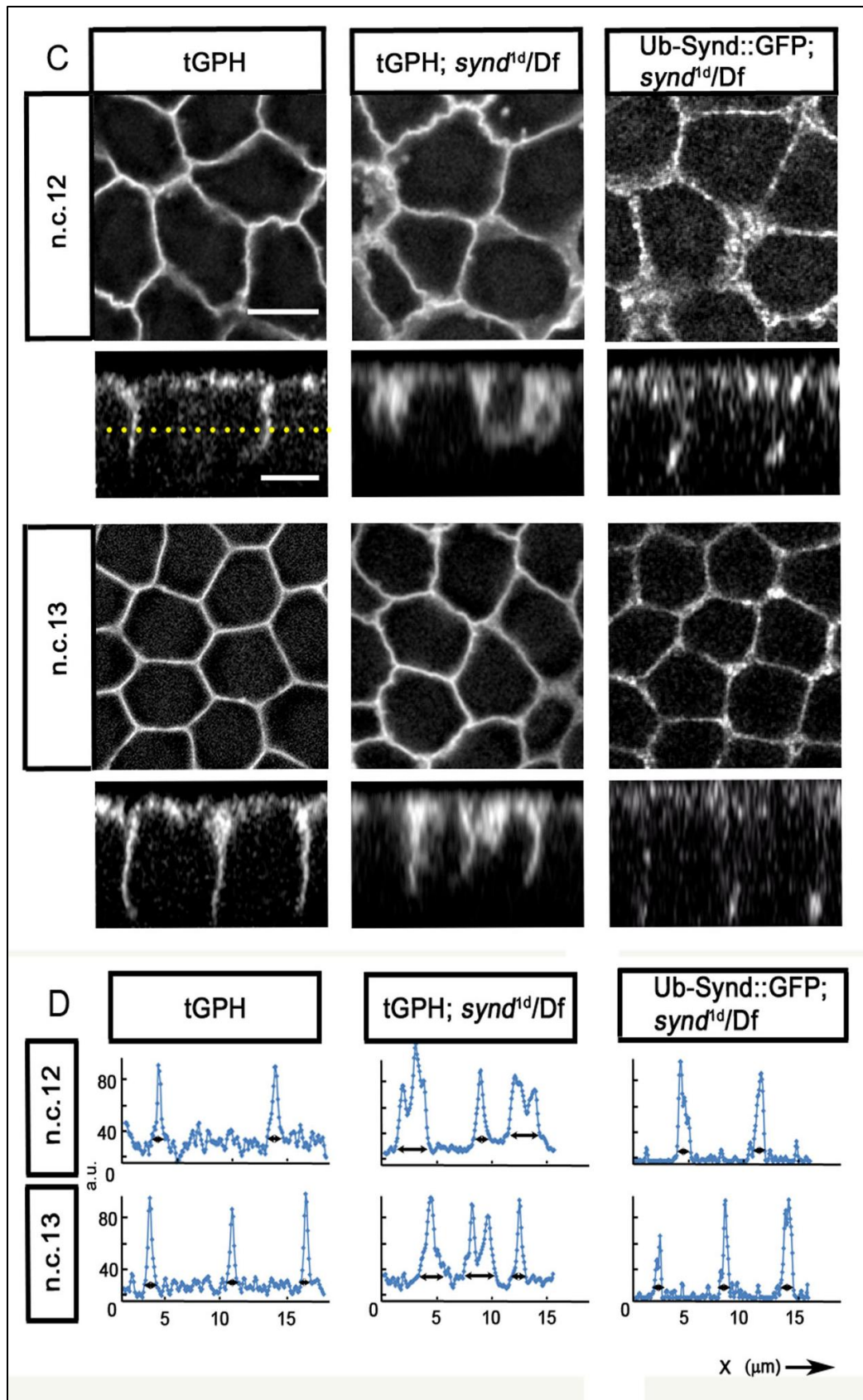
FT and apical membranes (n=5 embryos/region) (D). The immobile fraction from individual FRAP curves in C is shown in a scatterplot (E, n=5, n.s., not significant; Non-parametric Mann-Whitney U test). Synd-GFP shows increased mobile fraction at the FT as compared to the apical membrane. Photobleaching (black ROI, $\sim 2.5 \mu\text{m}^2$) of Synd-GFP was performed in apical or FT membranes and images at key time points are shown (F). FRAP occurs to a greater extent at the FT membrane (n=5 embryos/region) (G). The immobile fraction from individual FRAP curves in (H) is shown in a scatter-plot (K, n=5, *; $P < 0.05$, Non-parametric Mann-Whitney U test). Scale Bar=10 μm .

(Takeda et al., 2013). The Synd-GFP localization overlaps almost completely with Syndapin antibody staining (Figure 2.1H).

The pseudocleavage furrow extends from interphase through prophase to a maximum during metaphase of a nuclear cycle (Figure 2.1A). The dynamics of Syndapin localization to the membrane in pseudocleavage furrows during metaphase of nuclear cycles 11-13 was measured in living embryos expressing Synd-GFP in the *synd^{1d}/Df* background (Figure 2.2A-B). Synd-GFP localization confirms its recruitment to the membrane: we quantified the intensity of Synd-GFP in the apical, lateral and FT membranes and found that Syndapin is increasingly enriched at the FTs as the nuclear cycles progress from 11-13 and the metaphase furrows grow longer with each cycle (Holly et al., 2015) (Figure 2.2A-B).

To assess Syndapin dynamics at the FT compared to the apical membrane, we used Fluorescence Recovery after Photobleaching (FRAP) in these embryos during cycle 13 metaphase in the apical and FT membrane. The FRAP recovery data was fit to a one-phase exponential association equation (for diffusion-uncoupled FRAP); the recovery plateau obtained was higher at the metaphase FTs as compared to apical region with a mobile fraction of $\sim 80\%$ as compared to 60% (Figure 2.2F-H). This difference of mobile fraction was not seen for the GFP-tagged plekstrin homology (PH) domain of general receptor for phosphoinositides-1 (GRP1; tGPH) (Britton, Lockwood, Li, Cohen, & Edgar, 2002) (Figure 2.2C-E). Thus, Syndapin shows increased mobile fraction at the FTs of long metaphase furrows where it is enriched.





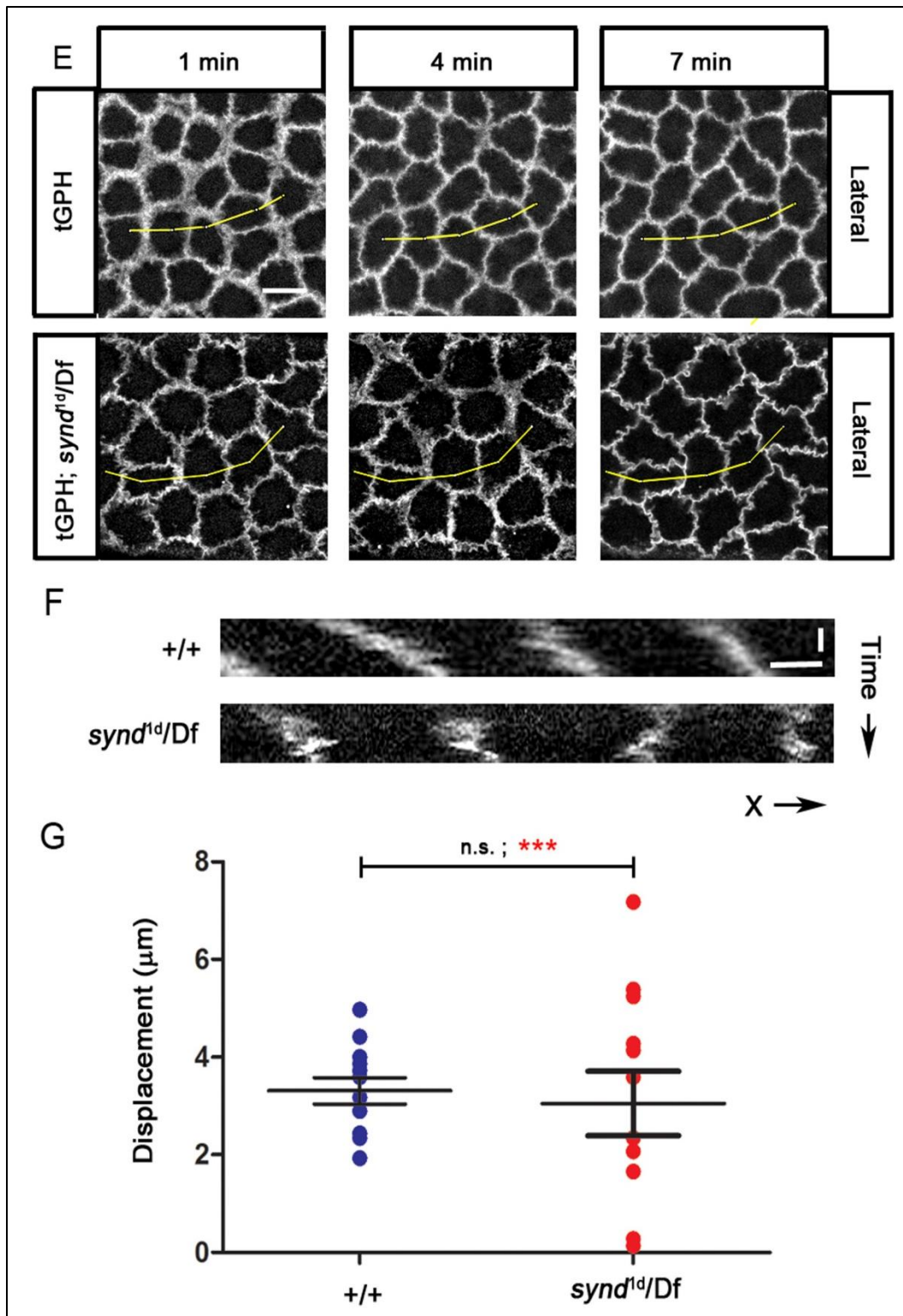


Figure 2.14. *synd* mutant embryos have short and disorganized pseudocleavage furrow membranes. *synd* mutants have shorter metaphase furrow lengths (A-B). Time lapse orthogonal views in tGPH control and *synd*^{1d}/Df embryos are shown at 0, 3, 5, 6 and 7 min (corresponding to I, II, III, IV and

V) in syncytial cycle 13 (A). Graph shows significantly decreased furrow lengths in *synd*^{1d}/Df (B, mean $\pm$ S.E.M., n=3; 5 furrows/embryo). *synd* mutants have loosened metaphase furrow membranes (C-D). Surface and orthogonal views of longest metaphase furrows from tGPH expressing control, *synd*^{1d}/Df and Synd-GFP; *synd*^{1d}/Df embryos in cycle 12 and 13 (C). *synd*^{1d}/Df embryos show increased width (arrows) in line scan intensity profile across lateral furrow membranes as compared to control and Synd-GFP expressing embryos (D). *synd* mutants have decreased XY displacement in the syncytial cycle (E-G). Time lapse surface views are shown at ~1, 4 and 7 min from tGPH containing control and *synd*^{1d}/Df embryos in cycle 13 (yellow line indicates track selected for kymograph generation) (E-F). The average displacement in a scatter plot is not significantly different (n=3; 4 furrows/embryo, P>0.05 n.s. not significant) but variance is increased in *synd*^{1d}/Df (G) (****, P< 0.0001, F test to compare variances Unpaired t-test (Two-tailed) with Welch's correction). Scale Bar=10 μ m. Kymograph Scale Bar= X=2.5 μ m, Y=100 s.

3.2.2 *synd* mutants have shorter and disorganized pseudocleavage furrows

Syndapin enrichment at the FT with corresponding increase in furrow length with each nuclear cycle prompted us to test whether the dynamics of furrow formation may be affected in mutants for Syndapin. We imaged developing embryos containing tGPH as a plasma membrane marker to compare furrow lengths in orthogonal sections. First, we measured pseudocleavage furrow lengths in control tGPH embryos at five time points in cycle 13: (I) end of prophase (prometaphase) when the nuclei are the largest and furrows start to ingress ($3.8 \pm 0.4 \mu$ m), (II) beginning of metaphase (after ~3 min) when the nuclear envelope breaks down (furrow length $8.3 \pm 0.6 \mu$ m), (III) middle of metaphase, (IV) end of metaphase (~6 min) when the furrow is the longest ($10.2 \pm 0.8 \mu$ m) and (V) anaphase when the furrow starts to retract (~7 min) (Figure 2.3A-B). We also measured furrow lengths with other membrane markers and found that furrow dynamics measured in embryos expressing different PH domains (tGPH, PHPLC δ -CFP) and in Synd-GFP; *synd*^{1d}/Df embryos were indistinguishable from each other.

The tGPH; *synd*^{1d}/Df furrows in prometaphase had a similar length as the control ($3.6 \pm 0.5 \mu$ m), however through metaphase, their length remained constant ($6.8 \pm 0.5 \mu$ m,) and was significantly shorter than the control. The anaphase furrow ($5.4 \pm 0.3 \mu$ m) was also much shorter than controls ($7.9 \pm 0.4 \mu$ m) at 7 min. These furrow extension-retraction dynamics were rescued in *synd* mutant embryos containing Synd-GFP (Figure 2.3A-B).

The furrow membrane in *synd* depleted embryos appeared ‘loosened’ or ruffled as compared to a sharp, distinct furrow in tGPH and Synd-GFP; *synd*^{1d}/Df rescue embryos (Figure 2.3D). This was depicted with a line scan across the metaphase lateral furrow membrane that revealed broader peaks in cycles 12 and 13 in mutant embryos as compared to focused peaks in wild-type tGPH and rescue embryos. The furrow plasma membrane moves laterally and synchronously in the XY plane during the syncytial cycles and we reasoned that this displacement will be affected if the furrow membranes are shorter and loosened. Previous studies in *Xenopus* embryos have also shown that loose membranes in planar cell polarity and septin mutants during collective cell migration exhibited increased undulating behavior in kymographs (Kim et al., 2010). A plot of the mean syncytial furrow membrane displacement showed a significantly higher variance in *synd* depleted embryos as compared to control. Also the displacement of adjacent furrows occurred asynchronously in mutant embryos (Figure 2.3E-G). Thus, Syndapin is required for appropriate furrow elongation and its deficiency not only causes defects in furrow extension but also membrane ruffling and correlates with an altered displacement and asynchrony in lateral membrane movement.

3.2.3 *synd* mutants have defects in CFT tubule formation and have fewer Rab5 endosomes compared to wild-type

Syndapin functions in Clathrin/Dynamin-mediated endocytosis and actin remodelling (Edeling et al., 2009; Britta Qualmann & Kelly, 2000). Therefore, we evaluated the contribution of Syndapin’s role in endocytosis for metaphase furrow formation. First, we analyzed the localization of Dynamin/Shibire in *synd*^{1d}/Df embryos. Dynamin levels in immunostained *synd* mutant embryos were unaffected and co-localized with Amph at the furrow tip (Figure 2.4A). Conversely, *shi*^{ts} (a temperature-sensitive mutant allele of Dynamin) embryos showed reduced Syndapin distribution on the membrane (Figure 2.4B).

Endocytosis occurs at the tip of the ingressing syncytial furrows and can be visualized in the form of tubules labelled with Amph (Sokac & Wieschaus, 2008a). Their formation depends upon actin dynamics and recruitment of Dynamin. They persist through early cellularization and are then pinched off by Dynamin-dependent endocytosis. Their dynamics correlates with the rate of furrow ingression during cellularization: slow during the early phase and faster during late

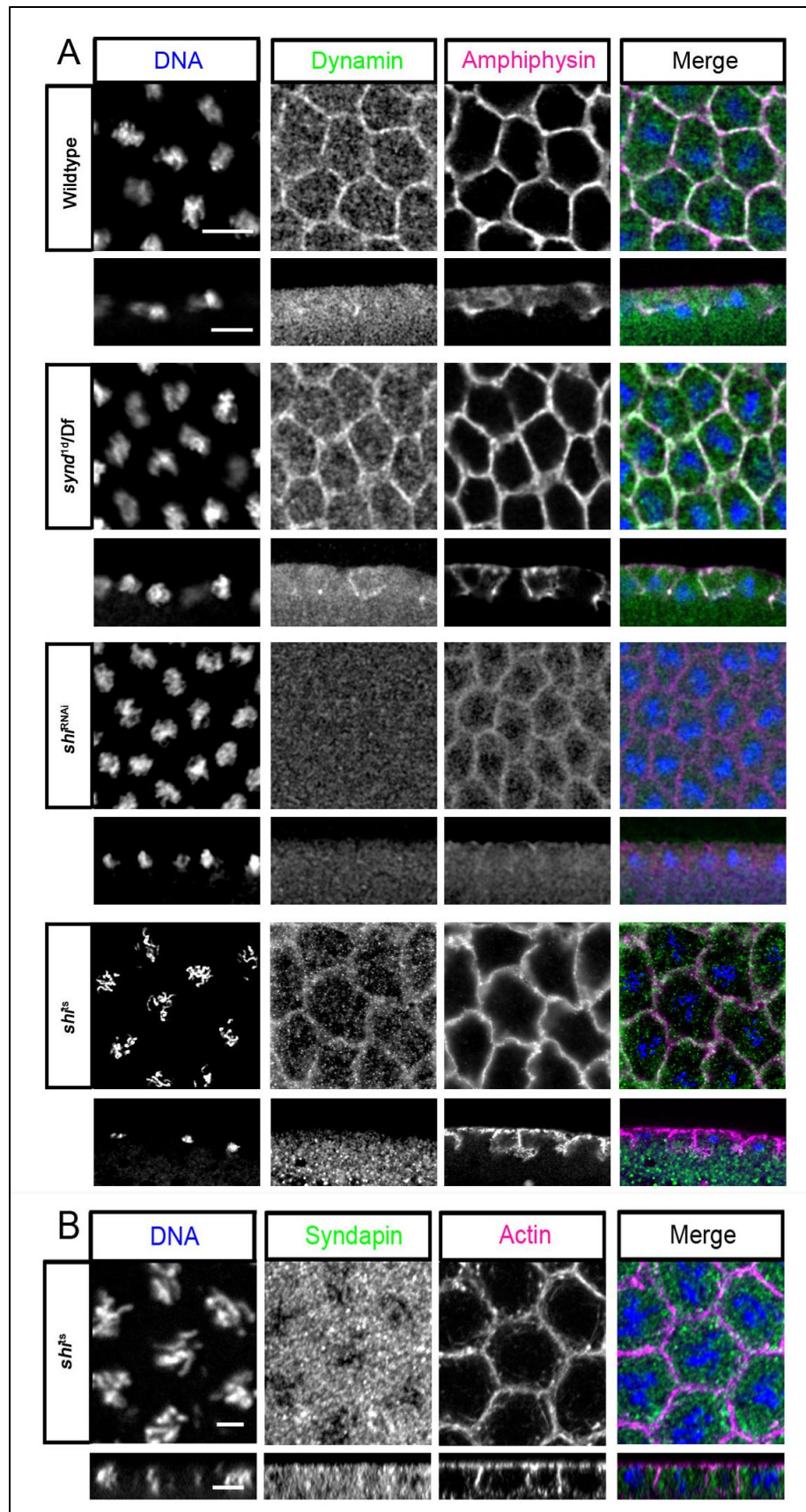
cellularization (Braun, 2005; Oegema et al., 2000; Sokac & Wieschaus, 2008a, 2008b; Su et al., 2013). We quantified the length of tubules in control and mutant embryos during metaphase (of syncytial cycle 13) when the tubules are the longest and most numerous. *synd*^{1d}/Df embryos showed a significant decrease in length of tubules compared to wild-type. Synd-GFP expression in *synd*^{1d}/Df embryos rescued the tubule length defect (Figure 2.4C-D). *shi*^{ts} embryos, at the restrictive temperature, are reported to show an increase in length of tubules during cellularization (Sokac & Wieschaus, 2008a; Su et al., 2013); we also found a significant increase in their length compared to wild-type in syncytial cycles (Figure 2.4C-D).

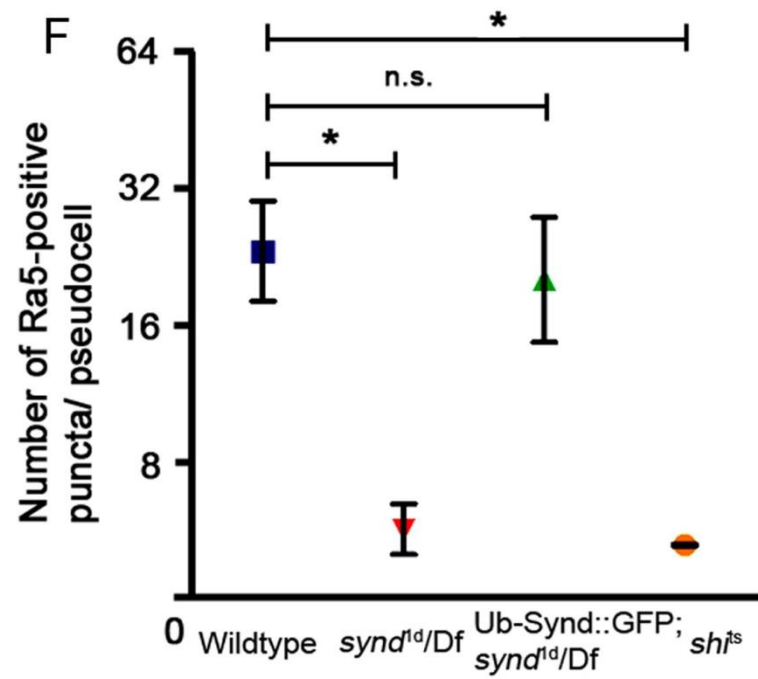
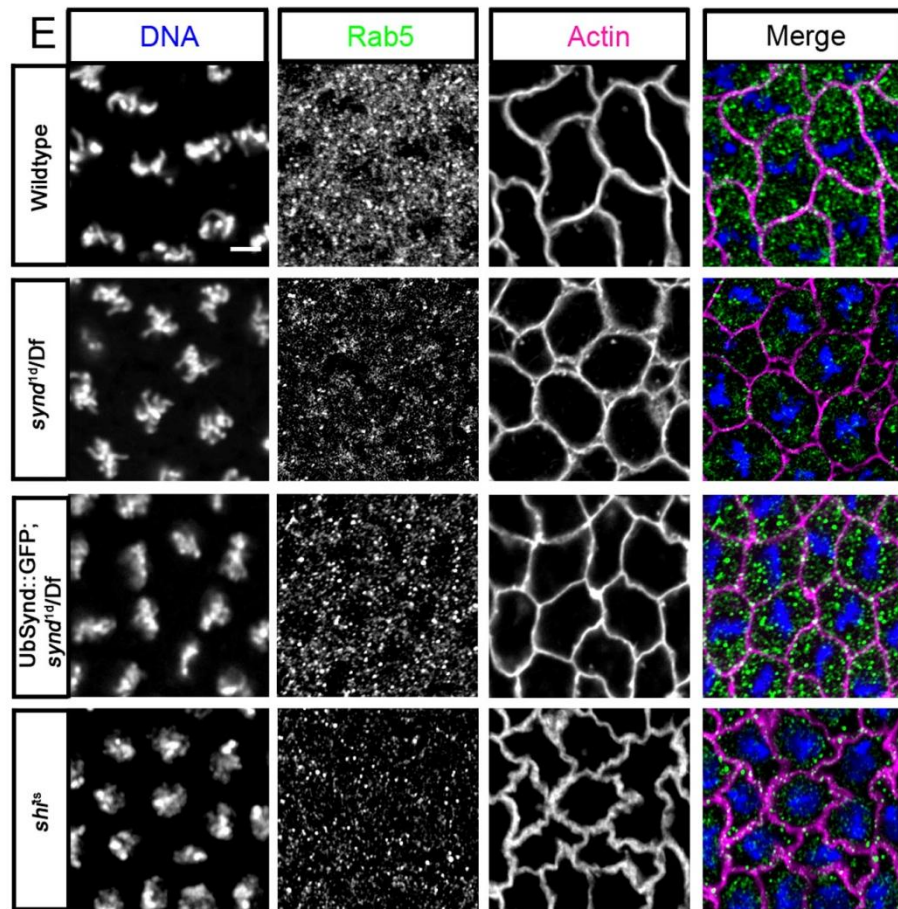
Syndapins have been shown to be crucial for vesicle internalization at a very early step in endocytosis (Braun, 2005). Rab5 is an early endosomal marker, a principle component of early endosomal biogenesis *in vivo* (Zeigerer et al., 2012). Rab5 early endosome vesicles increase when *shi*^{ts} embryos are released from the temperature sensitive block (Rikhy et al., 2015). We found that the number of Rab5 punctae were significantly lowered in *synd* mutant embryos compared to wild-type and phenocopied *shi*^{ts} embryos at the restrictive temperature. Rab5 vesicle numbers were rescued upon overexpression of Synd-GFP in *synd* mutant background (Figure 2.4E-F).

3.2.4 Mutants for *anillin*, *pnut*, *rhogef2* also show defects in furrow morphology

Depletion of either Dia or RhoGEF2 results in short/missing furrows (Afshar et al., 2000; Grosshans et al., 2005). Pseudocleavage furrow dynamics have been described in mutants of *anillin* which fails to recruit Pnut to the membrane. The result was reduction in depth of furrow ingression and premature dissipation of actin in the furrows (Silverman-Gavrila et al., 2008).

Like *anillin*, initiation/extension dynamics in *pnut* and *rhogef2* mutant embryos are affected. We studied this in tGPH-containing embryos depleted for Pnut and RhoGEF2 and found that they phenocopied *synd*^{1d}/Df embryos; the furrow length at 6 min (IV, when the furrow is the longest) was reduced compared to controls (Figure 2.5B). The *pnut*^{XP/+}; *synd*^{1d}/*synd*^{1d} combination had short furrows (II-IV) like *synd*^{1d}/Df embryos. *+anillin*^{PQ}; *synd*^{1d}/*synd*^{1d} also had short furrows; however they did not show the characteristic ingression-retraction phenotype seen with time in the wild-type and also in the other mutant embryos. In other words, combining the mutant allele





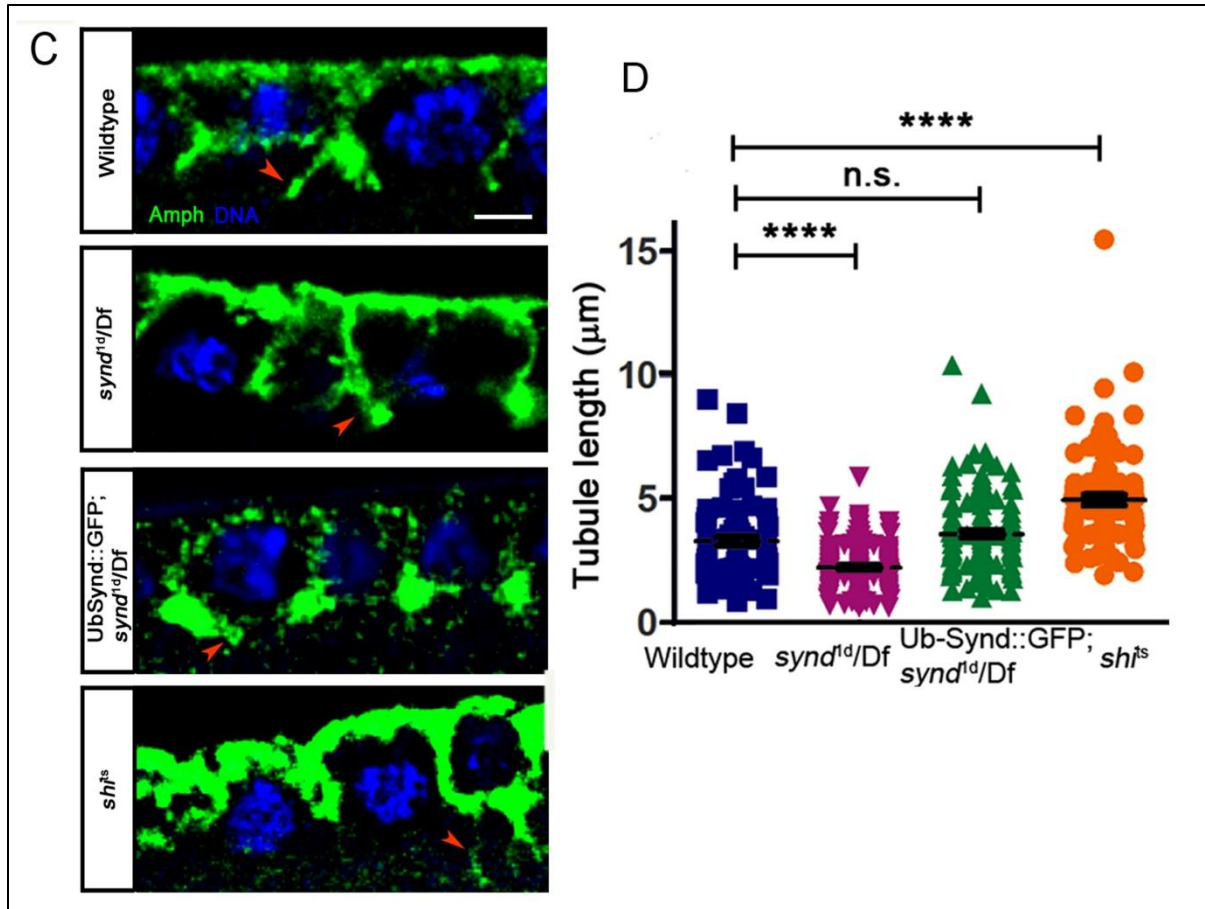
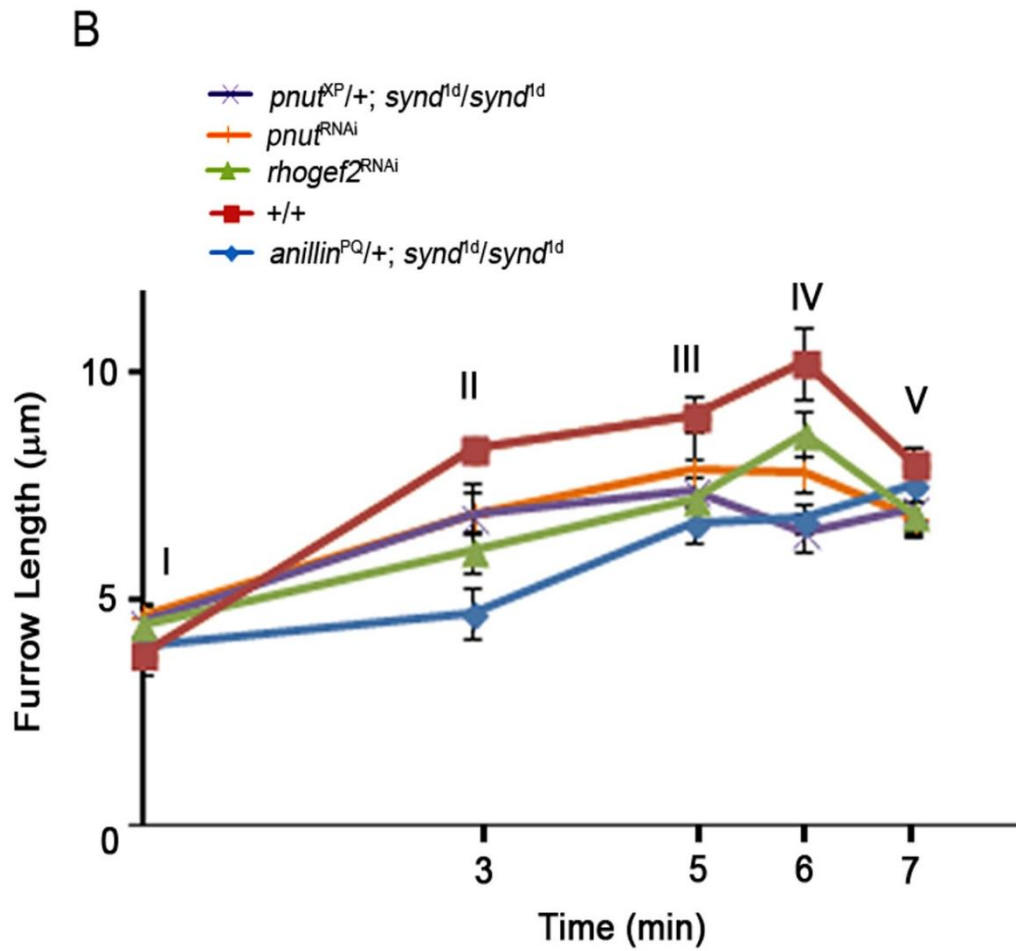
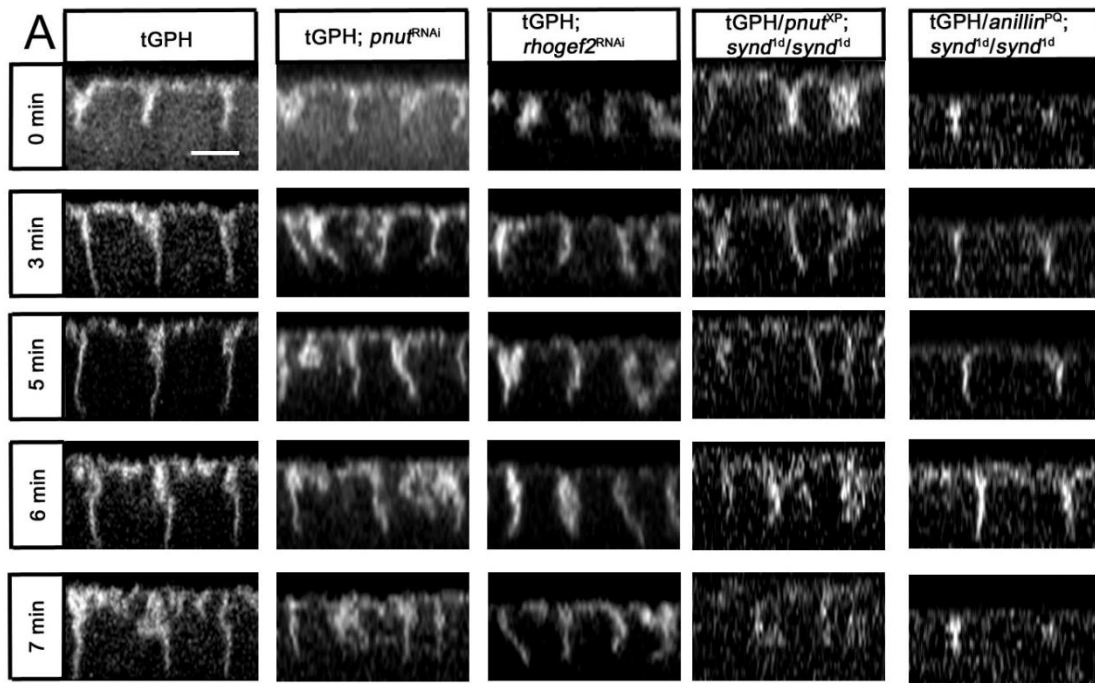
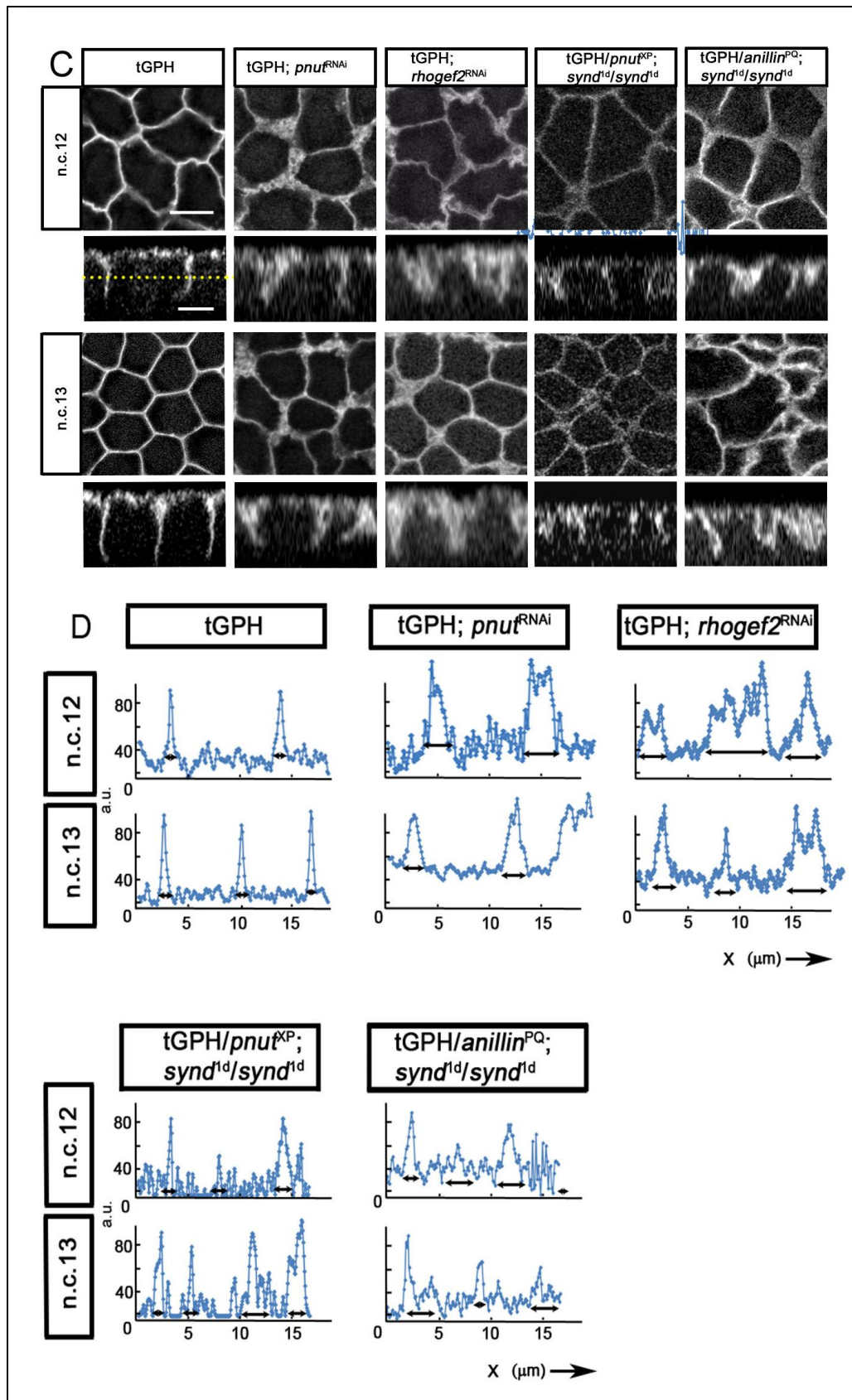


Figure 2.15. *synd* mutants have defects in CFT tubule formation and have fewer Rab5 endosomes compared to wild-type. Dynamin (green) distribution is unaffected in *synd* mutant embryos. Dynamin levels are unaffected in *synd*^{1d}/Df embryos but has a punctate distribution in *shi*^{RNAi} and *shi*^{ts} (100%, n=10) compared to wild-type. Syndapin is reduced from the furrow membrane in *shi*^{ts} embryos (100%, n=12). Orthogonal views of furrow membrane immunostained with Amph (green) and Hoechst (blue) showing tubules at the furrow tips (C). *synd*^{1d}/Df showed significantly shorter tubules while *shi*^{ts} showed significantly longer tubules compared to wild-type (D, n=3, ~20 furrows per embryo, ****, P<0.0001, *, P<0.05, n.s., not significant, Non-parametric Mann-Whitney U test). Red arrow; plasma membrane tubule along furrow membrane. Scale Bar=5 μm. Rab5 vesicle immunostaining and numbers/pseudocell are decreased in *synd*^{1d}/Df and *shi*^{ts}. Rab5 vesicle numbers are rescued in Synd-GFP; *synd*^{1d}/Df embryos (n=3, n.s. not significant or *, P<0.05, Non-parametric Mann-Whitney U test) (E-F). Scale Bar=10 μm.

of Anillin with *synd* mutant worsened the furrow dynamics observed in wild-type, *synd* mutant alone or in combination with *pnut*^{XP}.





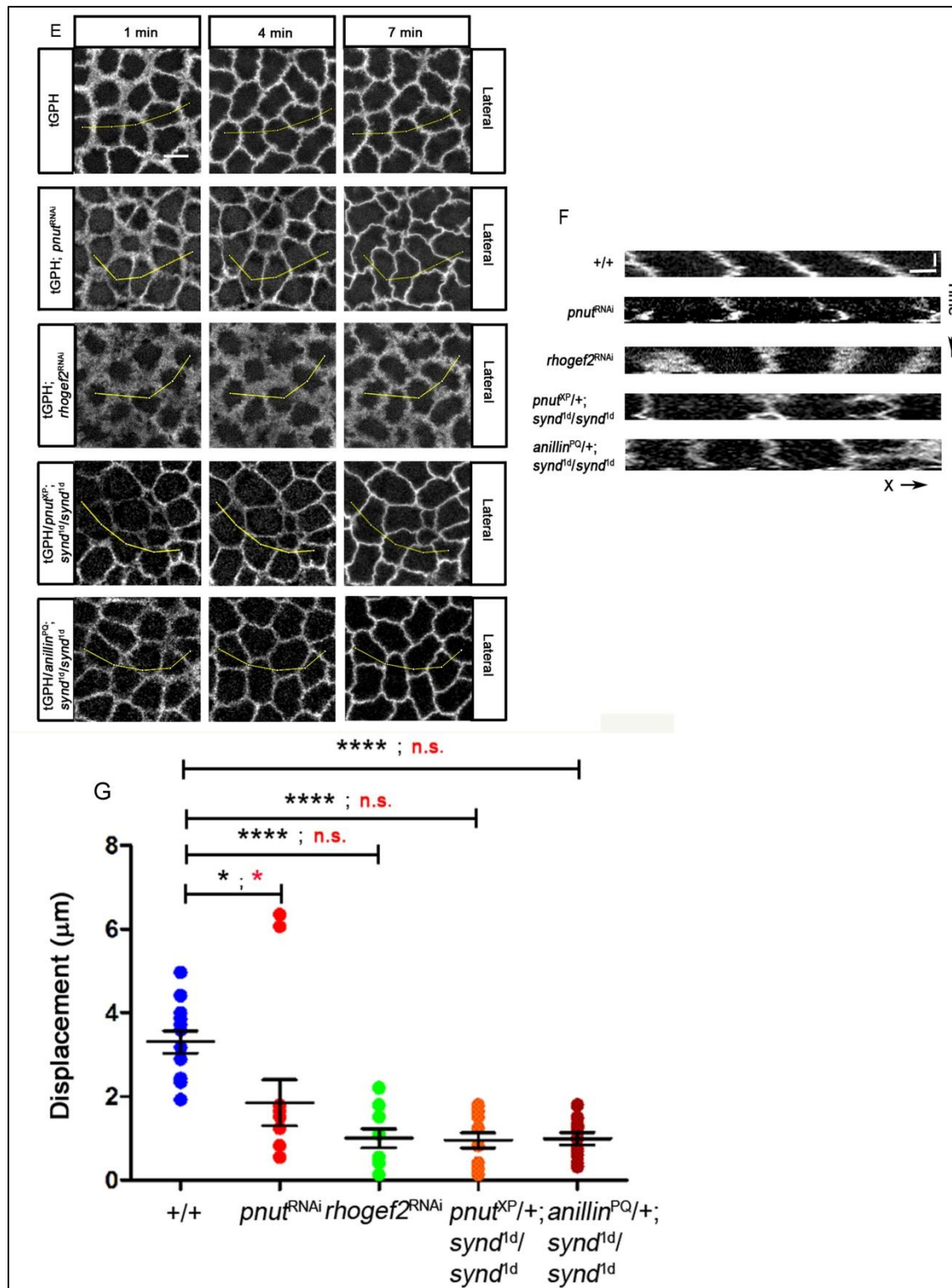
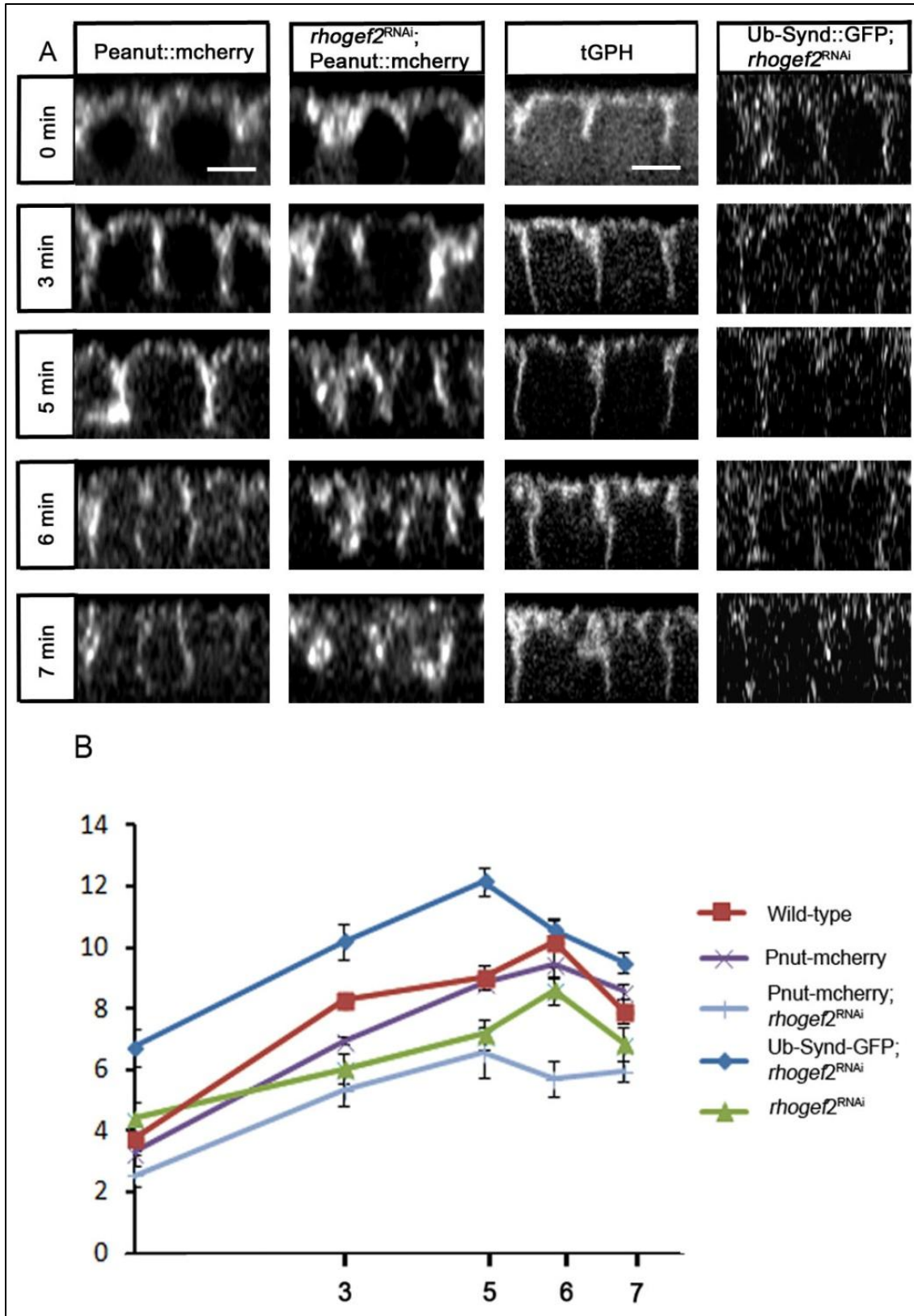


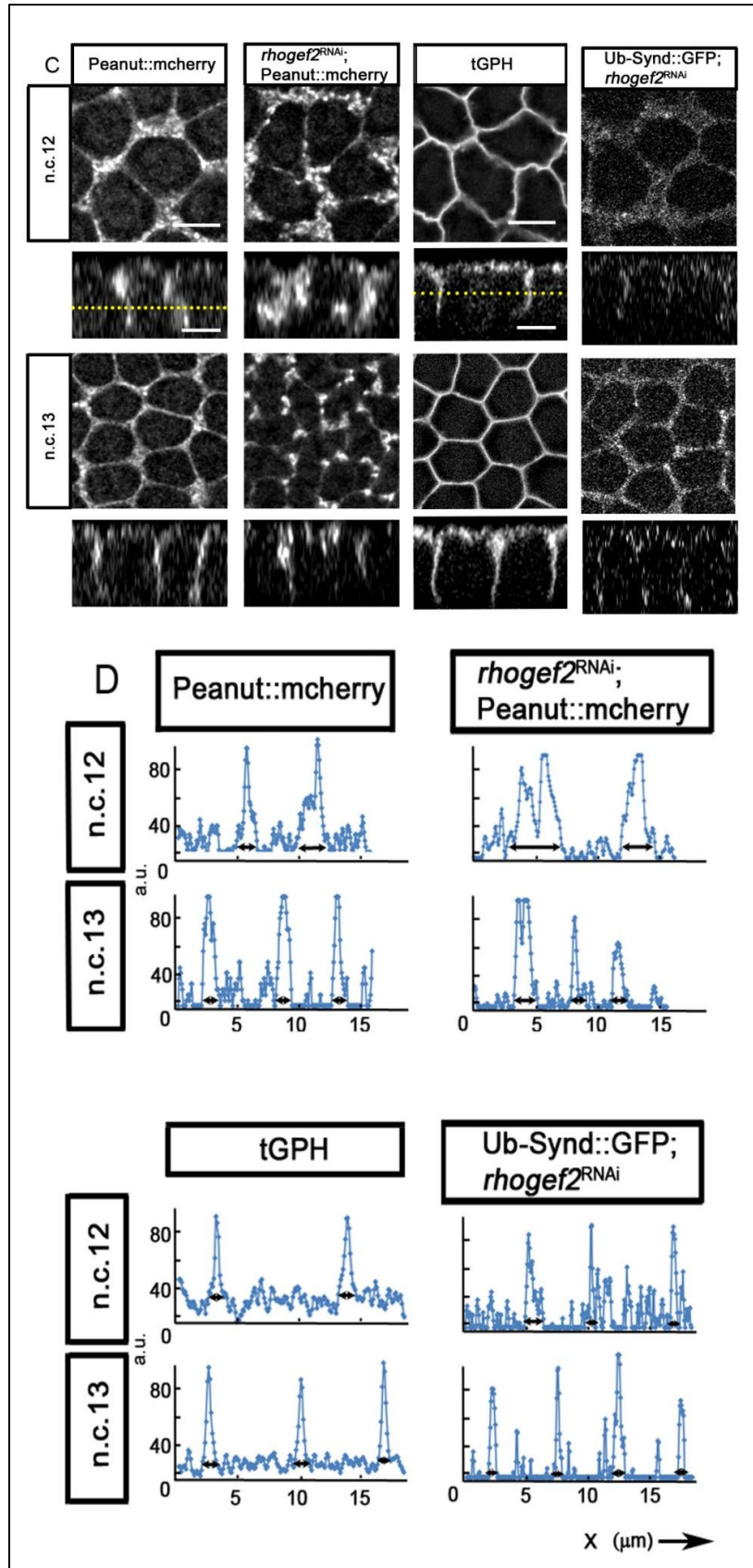
Figure 2.16. *rhogef2*^{RNAi} and *pnut*^{RNAi} phenocopy *synd* mutant furrow extension phenotypes. *pnut* and *rhogef2* mutant embryos have decreased metaphase furrow lengths, loose furrow membranes and decreased XY displacement (A-G). Time lapse orthogonal views (A) at 0, 3, 5, 6 and 7 min

(corresponding to I, II, III, IV and V) of furrow extension in *pnut*^{RNAi}, *rhogef2*^{RNAi}, *pnut*^{XP/+}; *synd*^{1d} and *anillin*^{PQ/RS/+}; *synd*^{1d} mutants expressing tGPH show a decrease in length as compared to wild-type (B) (mean ± S.E.M.) (n=3; 5 furrows/embryo). Surface and orthogonal views of longest metaphase furrows during n.c. 12 and 13 in *pnut*^{RNAi}, *rhogef2*^{RNAi}, *pnut*^{XP/+}; *synd*^{1d} and *anillin*^{PQ/RS/+}; *synd*^{1d} as compared to control embryos (C). Line scan intensity profile across mutant furrow membranes shows a broader area (arrows) (D). Time lapse surface views (E) at ~1, 4 and 7 min of lateral membranes from tGPH containing *pnut*^{RNAi}, *rhogef2*^{RNAi}, tGPH/*pnut*^{XP}; *synd*^{1d} and tGPH/*anillin*^{PQ/RS}; *synd*^{1d} show less displacement (across the yellow line) in the XY plane as compared to control embryos in cycle 13 (F-G) (n=3; 4 furrows/embryo, *, P<0.05; ****, P<0.0001 Unpaired t-test (Two-tailed) with Welch's correction, variance n.s. not significant, or *, P< 0.05, F test to compare variances). Scale Bar=10 µm. Kymograph Scale Bar= X=2.5µm, Y=100s.

Also, as we described in *synd* mutant, *rhogef2*^{RNAi} and *pnut*^{RNAi} mutant embryos had broad furrows and loosening of membrane along the edges revealed by a line scan across orthogonal sections in cycles 12 and 13 (Figure C-D). Multiple kymograph analysis for lateral furrow membrane in the XY plane showed significantly less mean displacement and therefore increased severity in *rhogef2*^{RNAi}, *pnut*^{RNAi}, *pnut*^{XP/+}; *synd*^{1d/synd}^{1d} and *anillin*^{PQ/+}; *synd*^{1d/synd}^{1d} combination mutants as compared to *synd*^{1d/Df} (Figure E-G). Furrow displacement depends on RhoGEF2 and Pnut and *synd* mutant embryos show a significant defect in displacement along with lowering of Pnut in the *pnut*^{XP/+} background and Anillin and Pnut in *anillin*^{PQ/+} background in addition to Syndapin depletion.

In summary, we found that *pnut*, *rhogef2* *pnut*^{XP/+}; *synd*^{1d/synd}^{1d} and mutant embryos phenocopied the furrow extension defects of *synd* and *anillin* mutant embryos. *anillin*^{PQ} in combination with the *synd* mutant showed increased severity in furrow phenotypes suggesting that Anillin is a more upstream component in the pathway of proteins that regulate furrow elongation.





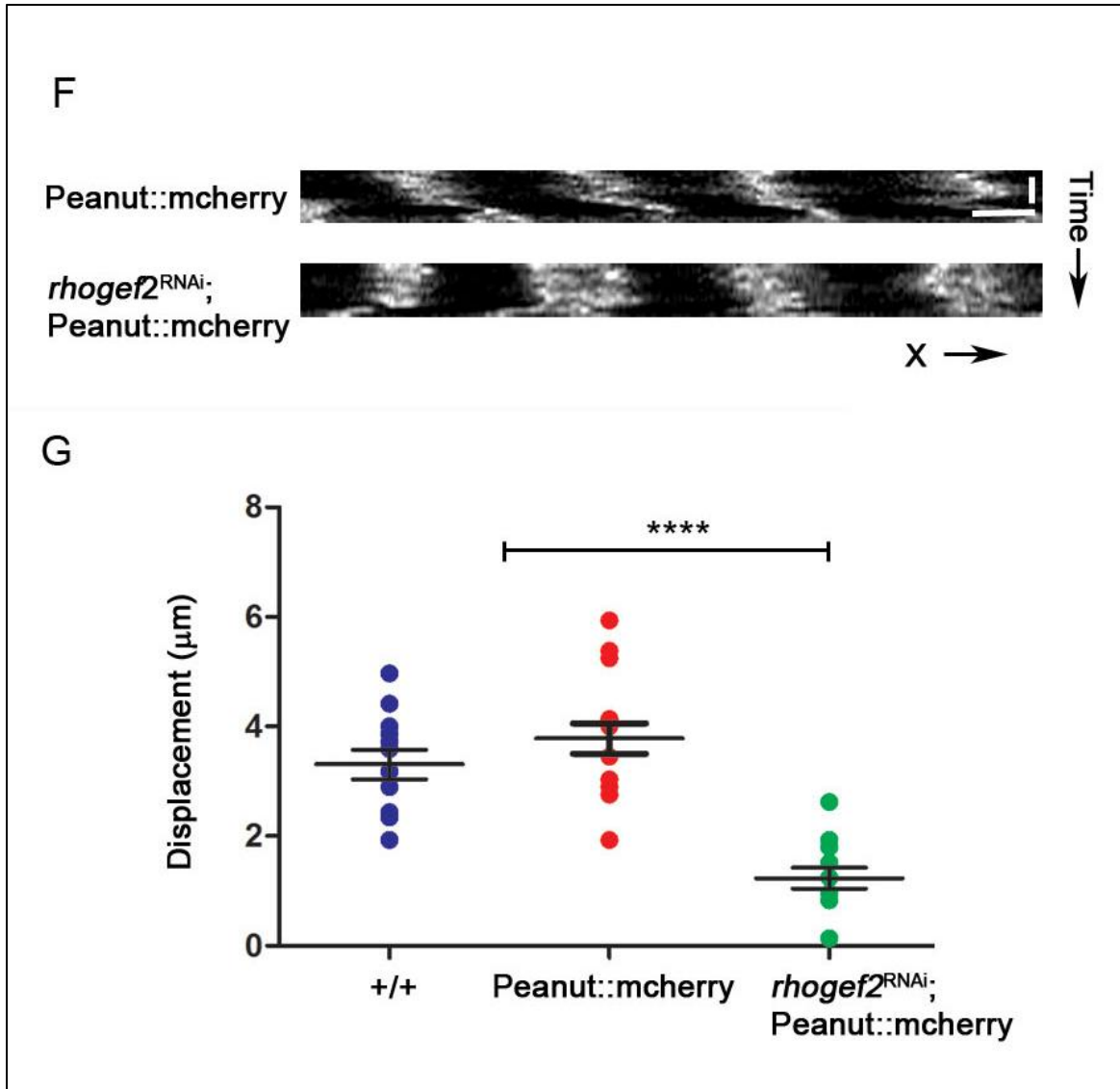


Figure 2.17. *rhogef2*^{RNAi} short, loosened and XY displacement furrow defects are reversed by Syndapin but not Pnut overexpression. Time lapse orthogonal views in Pnut-mcherry and tGPH controls and also Pnut and Syndapin overexpression in *rhogef2*^{RNAi} embryos at 0, 3, 5, 6 and 7 min (corresponding to I, II, III, IV and V) in syncytial cycle 13 (A) shows significantly decreased furrow lengths in *rhogef2*^{RNAi} (mean ± S.E.M., n=3; 5 furrows/embryo). Surface and orthogonal views of longest metaphase furrows from in Pnut-mcherry and tGPH controls and also Pnut and Syndapin overexpression in *rhogef2*^{RNAi} embryos in cycle 12 and 13 (C). Line scan intensity profile across mutant furrow membranes shows a broader area (arrows) which is reversed in the Synd-GFP; *rhogef2*^{RNAi} but not in Pnut-mch; *rhogef2*^{RNAi} (D). Time lapse surface views at ~1, 4 and 7 min from cycle 13 Pnut-mcherry containing control and *rhogef2*^{RNAi} embryos (yellow line indicates track selected for kymograph generation) (E-F) showed significant decrease compared to control (G) (n=3, 4 furrows/embryo, P<0.0001 ****,

Unpaired t-test (Two-tailed) with Welch's correction. n.s., not significant, F test to compare variances). Scale Bar=10 μ m. Kymograph Scale Bar= X=2.5 μ m, Y=100s.

3.2.5 Defects in pseudocleavage furrow extension and morphology in *rhogef2*^{RNAi} are reversed by Syndapin and not Pnut overexpression

RhoGEF2 is known to be upstream of actin-dependent processes like furrow formation and *rhogef2* mutants have disrupted F-actin organization (Grosshans et al., 2005). Syndapins and Pnut have independently been shown to favour persistent growth of actin (Julius Kostan et al., 2014) and actin organization (Mavrakakis et al., 2014) respectively, and we know that *rhogef2*, *pnut* and *synd* all have defective furrow dynamics, we thus hypothesised that one/both proteins may be the key component downstream of RhoGEF2 crucial for actin-driven furrow extension.

To check whether Syndapin or Pnut can promote furrow dynamics in the absence of RhoGEF2, we individually overexpressed Syndapin and Pnut in the *rhoGEF2*^{RNAi} background and quantified furrow extension across the nuclear cycle. A combination of Synd-GFP with *rhoGEF2*^{RNAi} showed a rescue (IV), and the furrows were longer than those observed in either tGPH or Synd-GFP; *synd*^{1d}/Df embryos alone (I-III and V, Figure 2.6A-B,). Overexpression of Pnut in the *rhoGEF2*^{RNAi} background, however, did not abrogate either the furrow length or loosened membrane phenotypes (Figure 2.6A-G). This shows that Syndapin could specifically rescue furrow extension in *rhoGEF2*^{RNAi} expressing embryos.

3.3 Discussion

We approached the question of Syndapin function in the *Drosophila* embryo naïvely, since nothing heretofore was known about its role during embryonic development. We, therefore, first looked at where Syndapin localizes in the embryo. We found that Syndapin recruits to the plasma membrane and is enriched at the tip of the ingressing pseudocleavage furrow. This localization and the fact that Syndapin is known to cause membrane tubulation *in vitro* (Takeda et al., 2013), strongly suggested that Syndapin function may relate to dynamics of furrow formation. This led us to quantify furrow dynamics in live embryos. We found a significant defect in furrow ingression in these embryos. The next obvious question to ask was how Syndapin functions in furrow elongation. From studies on its mammalian orthologues, we know that Syndapins have roles in activity-dependent bulk endocytosis (ADBE), clathrin-mediated

endocytosis (CME), endosomal and vesicle trafficking (Braun, 2005; Koch et al., 2012; Qualmann & Kelly, 2000). However, *Drosophila* Syndapin was shown to be dispensable for synaptic vesicle endocytosis at the larval neuromuscular junction (Kumar et al., 2009a). We tested the levels of Rab5 (early endosome marker) and Rab11 (labels recycling endosomes). Number of Rab5 puncta were distinctly lower in *synd* mutant embryos, Rab11 levels were also lowered (Appendix F). Thus, we conclude that Syndapin does function in endocytosis in the early embryo. We also examined Syndapin's role in membrane tubulation *in vivo* by testing its role in formation of cleavage furrow-tip tubules and found that in embryos mutant for Syndapin, the tubules were much shorter. This confirms (Takeda et al., 2013) Syndapin role in membrane tubulation.

In summary, we know that Syndapin is crucial for furrow elongation. Most significantly, Syndapin overexpression can rescue furrow elongation in the absence of RhoGEF2. We found that Syndapin functions in endocytosis and membrane tubulation in the embryo. Since furrow elongation is an actin-driven process, and Syndapin is known to have roles in actin remodelling, the exact function of Syndapin in actin-driven elongation of the pseudocleavage furrow remained to be investigated. It also begged the question whether Syndapin's role in furrow elongation was a consequence of its function in endocytosis or actin remodelling or both.

In the next chapter, I describe Syndapin's function in actin organization and also its epistatic relationship with other proteins known to be involved in furrow extension.

CHAPTER 4

Syndapin Function Regulates Pseudocleavage Furrow Extension through Actin Organization

4.1 Introduction

Drosophila synd mutants showed decreased pseudocleavage furrow extension. Actin polymerization provides the force that drives furrow elongation. Syndapins are known to interact with actin both by direct binding (Julius Kostan et al., 2014) and, by binding to and regulating the function of actin assembly proteins like N-WASp (thereby activating Arp2/3-mediated actin polymerization (Kessels & Qualmann, 2002)) and Anillin ((Takeda et al., 2013) which in turn recruits actin assembly factors to the cleavage furrow). However, the relationship of Syndapin with other proteins that are known to govern furrow initiation/extension like RhoGEF2, Dia and Pnut was unexplored. We, therefore, performed an epistatic analysis to test the recruitment of Syndapin in mutants of these proteins and conversely checked their localization to the furrow membrane in *synd* mutant embryos.

In order to test how Syndapin can modulate rates of actin polymerization *in vivo* possibly by direct binding, we used drugs that can alter the kinetics of actin polymerization in embryos mutant for *synd* and *rhogef2*. Cytochalasins bind reversibly to actin monomers. Its binding increases the nucleotide triphosphatase activity of actin. Actin-cytochalasin complex binds to the plus-ends of filaments and blocks actin polymerization at that end while still allowing polymerization to occur from the minus-end. Hence, the critical concentration is raised to that of the minus-end. Cytochalasins eliminate the lag phase of actin polymerization during which nucleation occurs by increasing the stability of actin dimers or trimers (Atlas & Lin, 1978; Flanagan & Lin, 1980). Jasplakinolide binds actin trimers reversibly and thus markedly enhances rates of actin nucleation. Unlike cytochalasins, it also lowers the critical concentration of plus-end actin polymerization. Thus, jasplakinolide causes *de novo* nucleation resulting in disordered polymeric actin and insufficient monomeric actin to allow for remodelling of actin structures *in vivo* (Bubb et al., 2000). Latrunculin A binds to monomeric actin and prevents it from polymerizing into filaments, thereby rapidly disrupting the actin cytoskeleton (Coué et al., 1987).

The use of these drugs provided us a handle on controlling rates of actin polymerization and allowed us to make predictions about how Syndapin may regulate actin polymerization *in vivo*.

In this chapter, I discuss how loss of Syndapin impacts actin architecture. I then describe Syndapin's place in relation to other proteins that influence furrow growth. I also dissect the role of Syndapin downstream of RhoGEF2 and describe effects on actin organization and endocytosis upon overexpression of Syndapin in *rhogef2* mutant embryos. Finally, I make deductions about the mechanism of Syndapin function on actin dynamics on the basis of effects of actin drugs on actin organization along pseudocleavage furrows in *Drosophila* embryos mutant for Syndapin and RhoGEF2.

4.2 Results

4.2.1 *synd* shows defects in actin organization in the pseudocleavage furrow

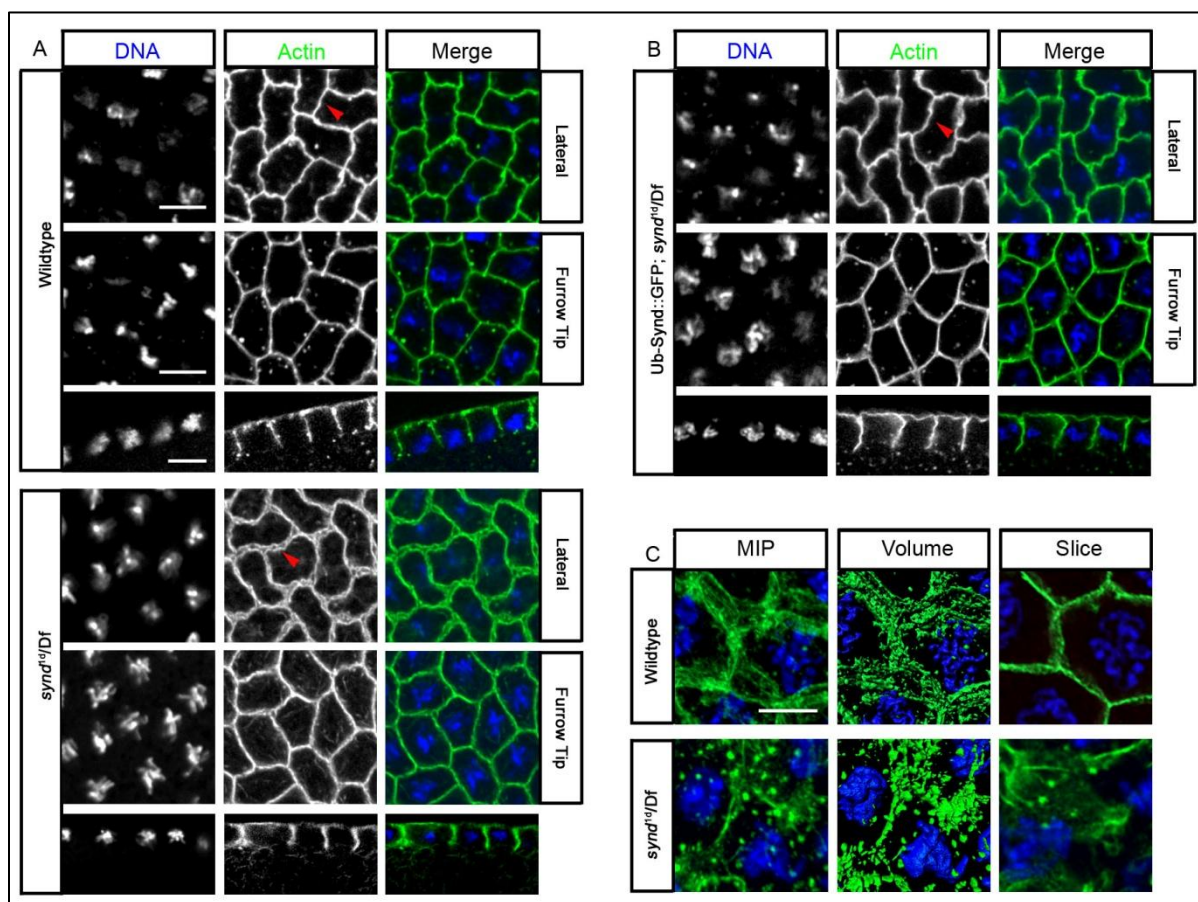


Figure 3.18. *synd* mutants show defects in actin organization. F-actin distribution (green) in *synd*¹⁰/Df reveals loose membrane (red arrowheads) and absence of F-actin puncta below cortex (77%, n=33) as

compared to wild-type (3%, n=50) (A). This phenotype is rescued in Synd-GFP; *synd*^{1d}/Df embryos (25%, n=16) (B). F-actin (green) in wild-type embryos stained with phalloidin is comprised of few punctae and organized linear structures. In *synd* mutants, there are mostly large punctae and disorganized structures along the furrow. Scale Bars=10 µm.

We first used fluorescent Phalloidin to check F-actin levels and distribution. F-actin levels were unaffected in *synd* embryos (Figure 3.1A). However, F-actin staining also revealed ‘looseness’ in the lateral furrow membrane as shown by tGPH and this was rescued with Synd-GFP (Figure 2.3C-D; Figure 3.1A-B, see arrowheads). To gain further insight into morphological defects in F-actin seen in *synd* mutants, we used structured illumination by Airyscan and 3D surface rendering. The maximum intensity projection (MIP) and Surface rendered image (Surface) in wild-type revealed long and short actin along with few punctate structures. In *synd* mutants, however, the long structures were lost; F-actin was disorganized and punctate along the furrow (Figure 3.1C). This clearly demonstrates that Syndapin is essential for proper actin organization in the pseudocleavage furrow.

4.2.2 *synd* mutants show a loss of Pnut and mislocalization of Dia on the pseudocleavage furrow

Defects in furrow extension prompted us to check the effect of *synd* mutant on key players of cleavage furrow initiation/extension. Since the short furrow phenotypes of *synd*, *anillin* and *pnut* phenocopy each other, we first checked localization of Pnut and Anillin in *synd* mutant embryos. Interestingly, we found that in *synd* mutant embryos, Pnut association with the pseudocleavage furrow membrane was lost, a defect which was partially reversed by expression of Synd-GFP transgene (Figure 3.2A). However, Anillin was present on the furrow tip in *synd*^{1d}/Df embryos (Figure 3.2A). Taken together, this suggests that Pnut recruitment to the membrane depends not only on its binding to Anillin (Oegema et al., 2000; Field et al., 2005; Liu et al., 2012; Kinoshita et al., 2002), but also on Syndapin and the Anillin-Syndapin-Pnut interaction is significant for pseudocleavage furrow formation.

Next, we studied the localization of proteins that regulate furrow initiation – RhoGEF2 and its effector Dia (Grosshans et al., 2005); missing and short furrows are caused due to the absence of RhoGEF2 and Dia during syncytial divisions and cellularization (Afshar et al., 2000; Barmchi, Rogers, & Häcker, 2005). In syncytial embryos, RhoGEF2 is cytoplasmic in interphase and

localizes to furrow tips in prophase (Barmchi et al., 2005). Rho1, the downstream RhoGTPase effector of RhoGEF2, is required for Dia recruitment to cellularizing furrows (Grosshans et al., 2005), however, there may be other RhoGEF2-independent mechanisms for Rho1 activation (Yoshida, Bartolini, & Pellman, 2009) that allow some Dia to still be recruited to the membrane in germline clone *rhogef2* mutants (Appendix B) or RhoGTPase-independent mechanisms may be implicated in its recruitment (Ang, Zhao, Lim, & Manser, 2010; Yonetani et al., 2008). RhoGEF2 was unaffected in *synd* mutant embryos and localized to the metaphase furrow (Figure 3.2C) showing that Syndapin presence on the furrow membrane is not important for RhoGEF2 recruitment.

Dia was identified as a gene responsible for cytokinesis (Castrillon & Wasserman, 1994). It initiates furrow formation along with Adenomatous Polyposis Coli2 (APC2) in *Drosophila* syncytial embryos (Webb, Zhou, & McCartney, 2009) and Dia and APC1 collaborate to increase actin nucleation (Jaiswal et al., 2013). In *synd*^{1d}/Df embryos, Dia was present uniformly along the length of the furrow in sagittal views (Figure 3.2B, see arrowhead) unlike wild-type where it was enriched at the furrow tip. Its enrichment at furrow tips was rescued with Synd-GFP (Figure 3.2B). Altogether, we observed that in *synd* mutants, Pnut was completely depleted from the furrow, while F-actin, Anillin and Dia were present on the furrow membrane. These data suggest that Syndapin contributes to stable furrow formation by recruitment of Pnut and enrichment of Dia at the furrow tip.

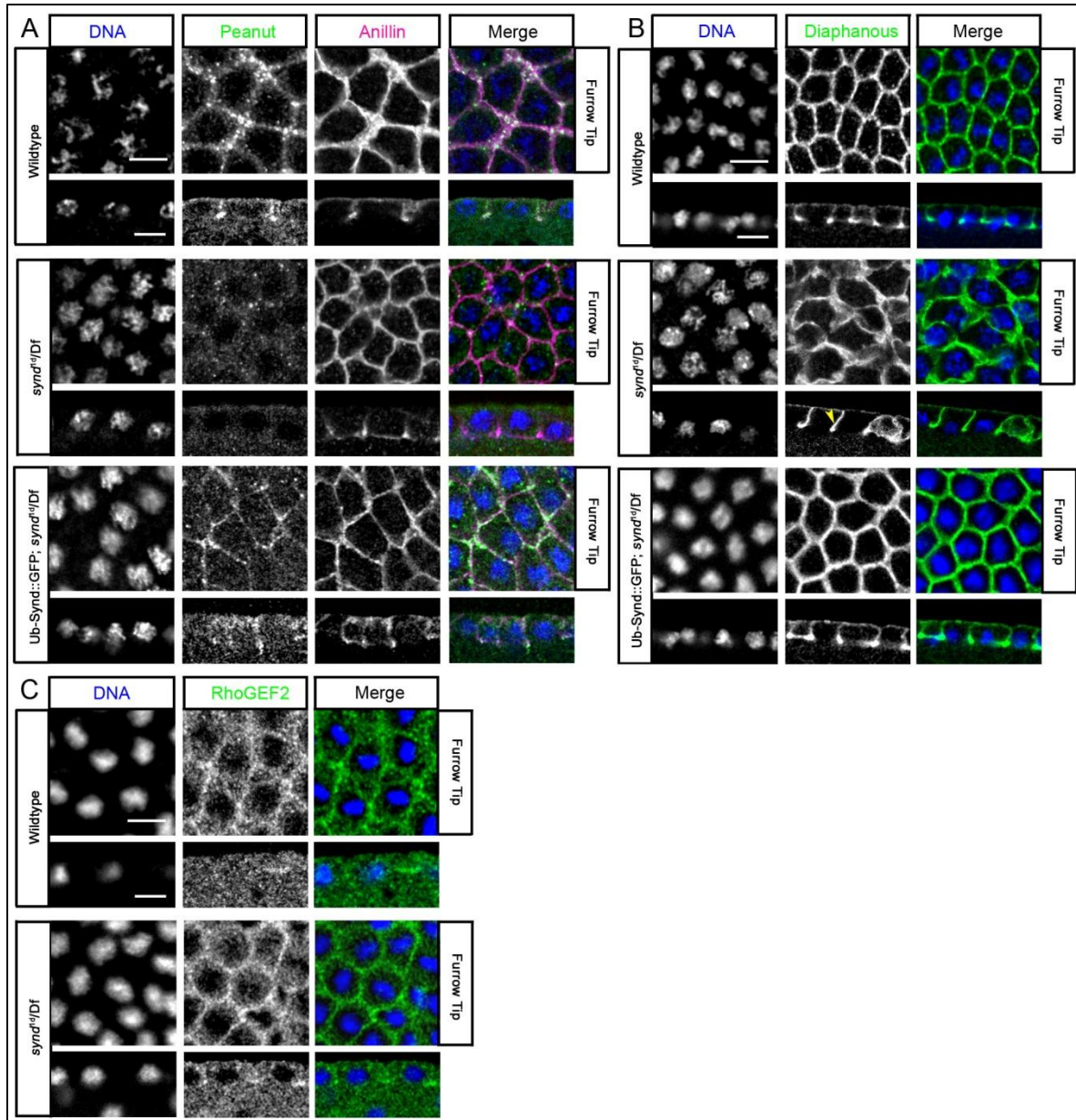


Figure 3.19. *synd* mutants show a loss of Pnut and mislocalization of Dia. *synd*^{1d}/Df embryos show Pnut (green) loss (93%, n=44) as compared to wild-type (2%, n=45) and Synd-GFP; *synd*^{1d}/Df (30%, n=14). Anillin localizes normally to the furrow tip membrane (magenta) (86%, n=44) in metaphase (A). Dia (green) is uniformly distributed along the furrow membrane in *synd*^{1d}/Df (90%, n=50) compared to wild-type (3%, N=50) and Synd-GFP; *synd*^{1d}/Df embryos (25%, n=16) (B). RhoGEF2 (green) antibody distribution is unchanged in *synd*^{1d}/Df (91%, n=11) compared to wild-type embryos (C). Yellow arrowhead; uniform Dia staining along length of furrow (B, D), Scale Bars=10 μm.

4.2.3 RhoGEF2, Dia, Anillin and Pnut are each necessary for the recruitment of Syndapin and organization of actin on the *Drosophila* syncytial pseudocleavage furrow

Conversely, to test whether Syndapin recruitment depends on presence of proteins known to affect furrow formation, we imaged germline clone mutant embryos and/or RNAi lines for *rhogef2*, *dia*, *anillin* and *pnut* for Syndapin antibody localization and found that they are defective for localization of Syndapin (Figure 3.3A). In addition, each of these mutants showed an apical accumulation of F-actin (Figure 3.3A-B, see arrowheads, Appendix B). Most interestingly, these mutants also showed significantly reduced Pnut implying a strong correlation between Syndapin and Pnut localization on the membrane (Figure 3.3A-B, Appendix B). *dia*⁵ mutant embryos also showed a loss of Anillin and Pnut as reported previously (Afshar et al., 2000) and *anillin* mutants in turn showed reduced levels of Dia (Appendix B). In *pnut* mutant embryos, Anillin was present but levels were reduced on furrows (Appendix B). These observations together corroborate earlier reports that implicate Dia in Anillin and Pnut recruitment (Afshar et al., 2000) and also of Anillin in Pnut recruitment (Field & Alberts, 1995; Kinoshita et al., 2002; Oegema et al., 2000).

Since Syndapin was depleted in *pnut* mutants, Pnut was in turn depleted in *synd* (Figure 3.2A; 3.3B) and Dia has been shown to be important for furrow initiation (Grosshans et al., 2005), we doubly depleted Pnut and Dia in *synd* mutants by combining *pnut*^{XP} and *dia*⁵ alleles in one copy each (heterozygous) with *synd*^{1d}/Df to see if the defects were exacerbated. In each case, we obtained very few embryos suggesting an increased severity as compared to *synd*^{1d}/Df, *dia*⁵/+ and *pnut*^{XP}/+. Phalloidin revealed stronger defects such as broader FTs in *pnut*^{XP}/+; *synd*^{1d}/Df and missing furrows in *dia*⁵/+; *synd*^{1d}/Df that have been previously observed in *dia*⁵ germline mutants (Figure 3.2A-B) (Afshar et al., 2000; Grosshans et al., 2005). Thus, the furrow phenotype of *synd*^{1d}/Df was worsened by doubly depleting Dia or Pnut.

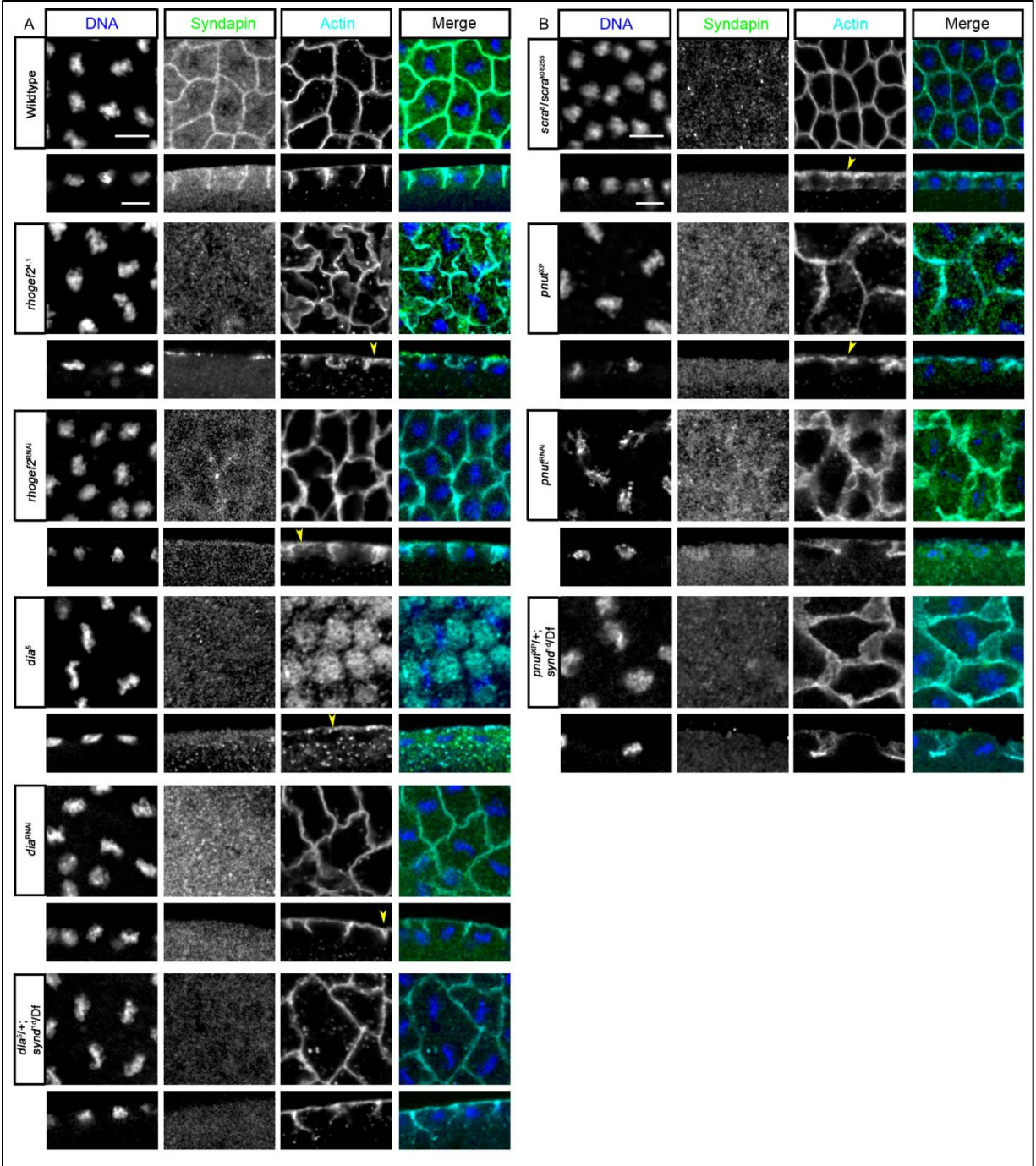


Figure 3.20. *rhogef2*, *dia*, *anillin* and *pnut* mutant embryos show Synd loss from pseudocleavage furrow membranes. Synd (green) immunostaining is lost in mutants for *rhogef2* (*rhogef2^{4.1}* (88%, n=14) and *rhogef^{RNAi}* (87.5%, n=16), *dia* (*dia⁵* (100%, n=15) and *dia^{RNAi}* (50%, n=20) and in *dia^{5/+}; synd^{1d}/Df* (100%, n=10) (A), *anillin* (*scra⁸/scra^{k08255}*) (100%, n=14) and *pnut* (*pnut^{XP}* (99%, n=22) and *pnut^{RNAi}* (44%, n=18) and in *pnut^{XP/+}; synd^{1d}/Df* (91%, n=11) (B) as compared to wild-type in metaphase furrows (DNA, blue). Yellow arrow; apical actin accumulation, Scale Bar=10 μm

4.2.4 Syndapin overexpression can rescue defects in actin organization but not endocytosis in RhoGEF2-depleted embryos

Upon overexpression of Synd-GFP in the *rhogef2*^{RNAi} background and using structured illumination by Airyscan, we found long organized actin with fewer puncta. Even though this organization was not exactly like in the wild-type furrow (there were mostly longer structures and very few short structures), it allowed actin to grow into elongated structures that could restore furrow length (Figure 3.4A). Thus, Syndapin is important for organizing F-actin into more continuous structures at the furrow and can, in the process, rescue furrow phenotypes in *rhogef2* mutants.

Since Syndapin can restore actin organization in the furrow, we also checked if actin assembly proteins like Pnut and Dia that are lost from the membrane in RhoGEF2-depleted embryos can be restored upon Syndapin overexpression. Indeed, we observed a rescue in Dia and Pnut localization to the pseudocleavage furrow membrane compared to *rhogef2*^{RNAi} embryos alone (Figure 3.4B-C). F-actin localization to furrows was uniform and its apical accumulation was lost (Figure 3.4D). However, as predicted, Pnut overexpression in *rhogef2*^{RNAi} could not rescue Syndapin, Dia or F-actin organization on the pseudocleavage furrow (Figure 3.4E).

Once we knew that Syndapin could restore furrow actin growth and assembly, we asked whether RhoGEF2-depleted embryos showed defects in endocytosis and whether Syndapin overexpression could ameliorate these effects. We found that unlike in *synd* mutants, in *rhogef2*^{RNAi} embryos, Dynamin levels are lowered (Figure 3.5A). Also, the number of Rab5 positive puncta are greatly reduced compared to wild-type. When Synd-GFP was overexpressed in this background, there was, in fact, no rescue of these phenotypes (Figure 3.5A-C).

We can therefore conclude that the reversal of furrow phenotypes in *rhogef2* mutant embryos is due to the ability of Syndapin to recruit and organize actin and proteins of the actin remodelling machinery such as Dia and Pnut and not because of its role in endocytosis.

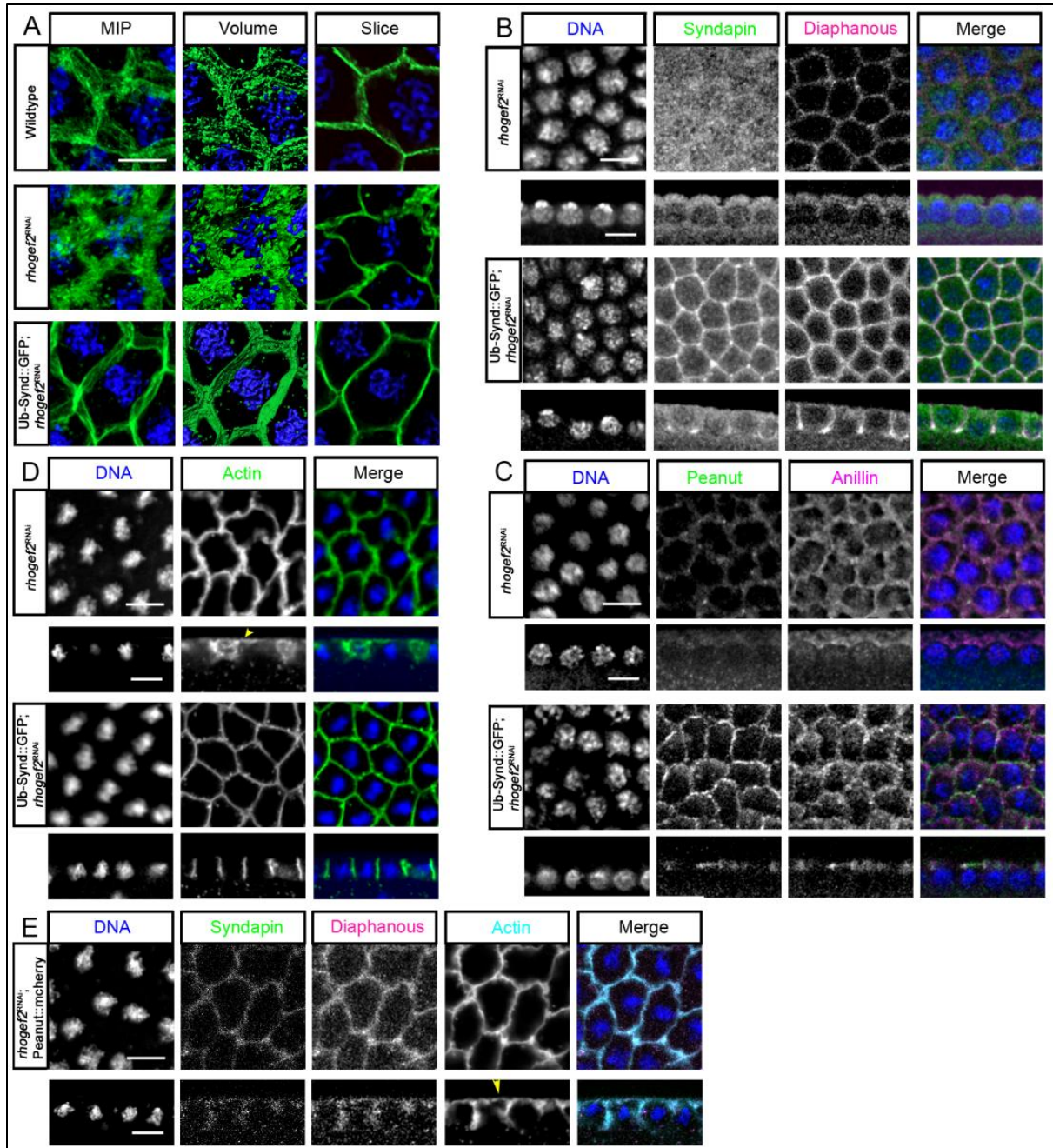


Figure 3.21. Syndapin overexpression can rescue defects in actin organization and recruitment of actin remodelling proteins in *rhogef2^{RNAi}* embryos. F-actin (green) in wild-type embryos stained with phalloidin is comprised of few cytosolic punctae and organized linear structures. In *rhogef2^{RNAi}*, actin is disorganized and linear structures are missing. *rhogef2^{RNAi}* with Synd-GFP rescues this phenotype and yields more linear structures and small cytosolic punctae (A). Dia, Anillin and Pnut recruitment defects in *rhogef2^{RNAi}* are reversed by Synd-GFP. Synd-GFP; *rhogef2^{RNAi}* metaphase (DNA, blue) embryos show a reversion of Dia (magenta) (50%, n=10 as compared to 91%, n=22 in *rhogef2^{RNAi}*) (B), Pnut (green) and Anillin (magenta; 66%, n=12 as compared to 100%, n=14 in *rhogef2^{RNAi}*) (C) apical accumulation of actin

(green) (50%, n=10 as compared to 100%, n=16 in *rhogef2*^{RNAi}) (D). *rhogef2*^{RNAi}; Peanut::mcherry embryos show defects in Syndapin (green) recruitment (100%, n=10) and partial loss of Dia (magenta; 70%, n=10) along with diffuse, cortical F-actin (cyan; 100%, n=10) on furrow membrane (E). Scale Bar=10 μ m.

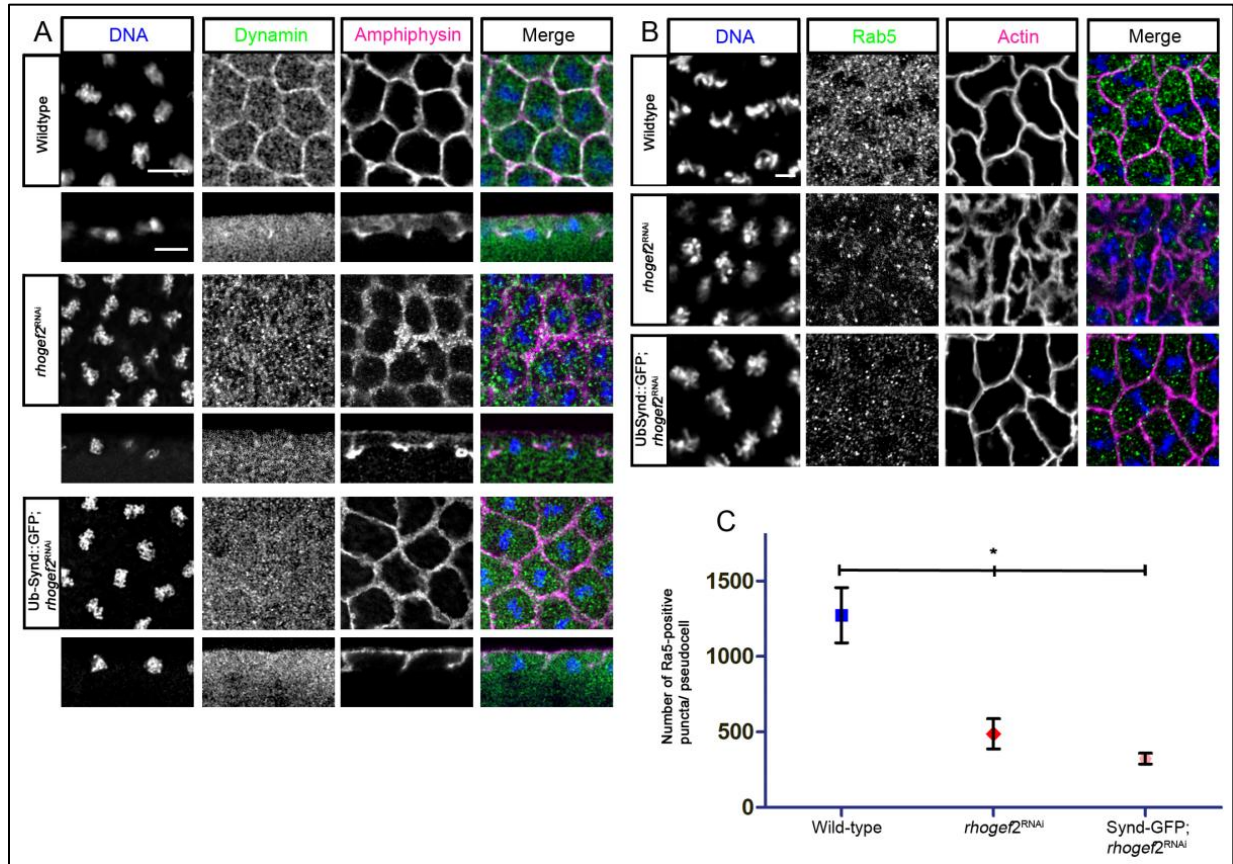


Figure 3.22. Syndapin overexpression cannot rescue endocytic defects observed in *rhogef2*^{RNAi} embryos. Dynamin (green) distribution is punctate in *rhogef2*^{RNAi} and Synd-GFP; *rhogef2*^{RNAi} embryos (100%, n=10) compared to wild-type (A). Rab5 vesicle immunostaining and puncta/ pseudocell are decreased in *rhogef2*^{RNAi}. Rab5 puncta numbers are not rescued in Synd-GFP; *rhogef2*^{RNAi} embryos (n=3, n.s. not significant or *, P<0.05, Non-parametric Mann-Whitney U test) (B-C).

4.2.5 Manipulating rates of actin dynamics in *synd* and *rhogef2* mutants can rescue actin organization and furrow elongation phenotypes

Next, we used drugs that alter rates of actin polymerization in embryos mutant for Syndapin and RhoGEF2. LatA treatment resulted in drastic disruption of actin structures with F-actin either only in caps or very short furrows in both wild-type and mutant embryos (Figure 3.6A). Upon treatment with Jasp, *synd* mutant embryos showed fewer puncta on the membrane, and both *synd*

and *rhogef2* embryos showed more disordered polymerized actin structures and disorganized furrows (Figure 3.6B). Interestingly, CytoD treatment caused continuous structures of actin to form and was able to form elongated furrows in *synd* and *rhogef2* mutant embryos compared to DMSO control (Figure 3.6B). Reports suggest that cytochalasins have greater nucleating and bundling properties when actin networks are relatively labile as in wounded/mutant cells (Goddette and Frieden, 1986) and disruption of actin allows CytoD to reorganize it into thick networks (Ilse Foissner & Wasteneys, 1999) or stable rods (Collings, Wasteneys, & Williamson, 1995) in a concentration-dependent manner (I Foissner & Wasteneys, 2000). However, at twice the above concentration, actin was disorganized and disrupted in wild-type embryos (Figure 3.6C).

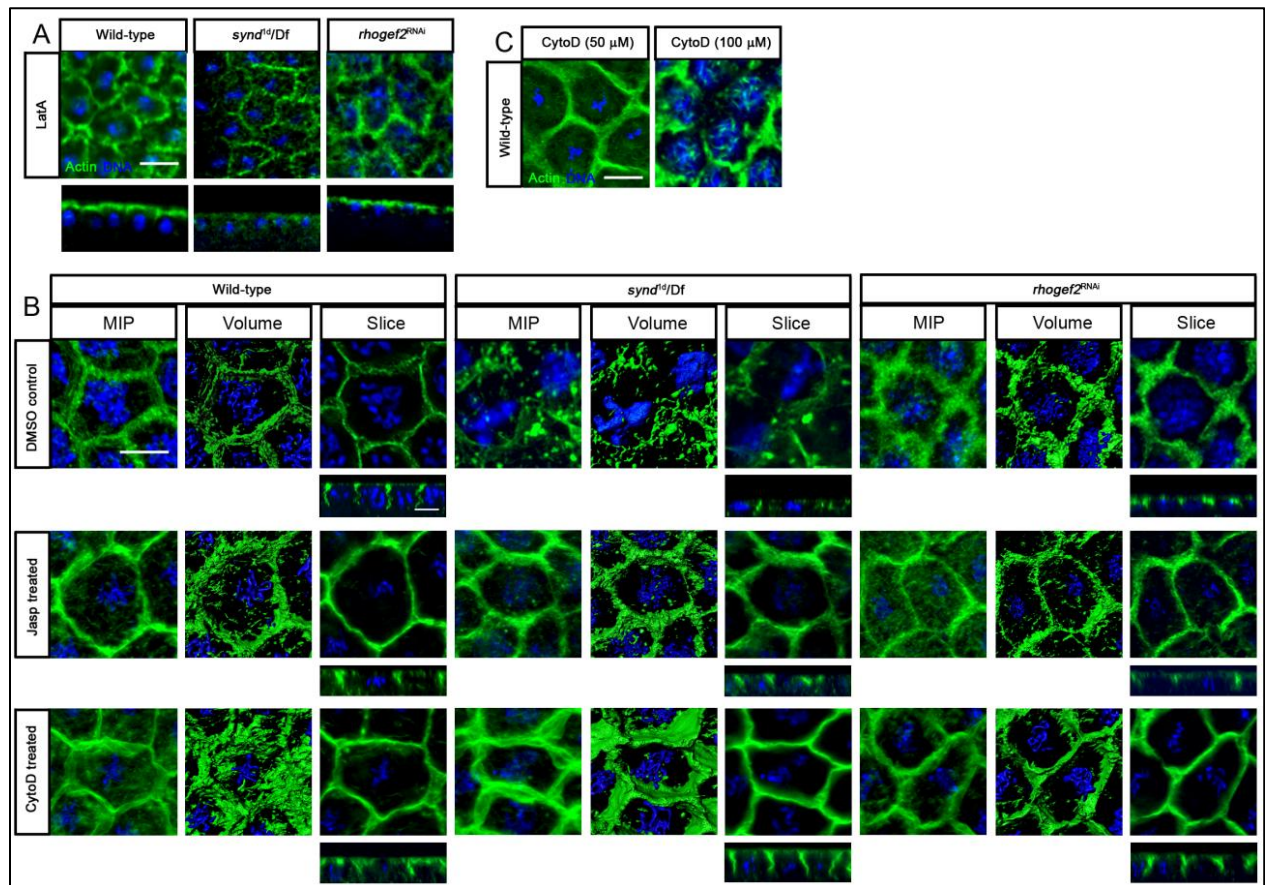


Figure 3.23. Manipulating rates of actin dynamics in *synd* and *rhogef2* mutants can rescue actin organization and furrow elongation phenotypes. Wild-type, *synd*^{1d}/Df and *rhogef2*^{RNAi} embryos treated with 10μM LatA showed short/missing furrows (100%; n=10) (A). Wild-type embryos treated with 100μM CytoD showed furrow breaks and disrupted actin structures while with 50μM CytoD did not (C). Treatment with Jasp increased short actin structures, reduced punctae but showed disorganized furrows in *synd*^{1d}/Df

(100%; n=11) and *rhogef2*^{RNAi} (100%; n=11). CytoD treatment in *synd*^{1d}/Df (60%; n=12) and *rhogef2*^{RNAi} (50%; n=10) embryos restores furrow morphology by converting disrupted actin into organized structures; in wild-type it reorganizes actin into thick, short rods compared to DMSO control (100%; n=10). Scale Bar=10 μ m.

These experiments clearly illustrate that actin growth and assembly is the primary driving force for furrow elongation, and points to a mechanism by which Syndapin is able, either by direct binding to actin or via interaction with proteins that control actin dynamics, to slow down rates of actin assembly/disassembly essential to promote organized growth of furrows.

4.3 Discussion

Our experiments have demonstrated that Syndapin regulates actin polymerization in addition to its roles in endocytosis and membrane tubulation in the *Drosophila* embryo. We have been able to conclusively demonstrate that Syndapin functions to promote furrow formation by organization and elongation of F-actin structures (Figure 3.1). We show that Syndapin is essential for recruitment and distribution of Pnut and Dia on the membrane (Figure 3.2). In turn, Pnut and Dia also affect Syndapin distribution on the membrane (Figure 3.3). RhoGEF2/Dia and Anillin/Pnut have been previously shown to regulate F-actin architecture at cleavage and cellularization furrows (Afshar et al., 2000; Liu et al., 2012; Mavrakakis et al., 2014) and hence loss of Syndapin in *synd* mutants affect actin both directly as well as due to its influence on Pnut and Dia localization. As with other actin-regulated processes, though a linear pathway of association/regulation of these actin remodelling proteins to the furrow membrane is unlikely, our data imply that Syndapin is a key component in the RhoGEF2-Dia-Anillin/Pnut pathway during actin-driven furrow elongation.

Actin stabilization into continuous structures reversed the furrow length defect in *synd* mutant embryos. Jasplakinolide blocks actin turnover at the contractile ring (Murthy & Wadsworth, 2008) and affects cleavage furrow invagination while preserving furrow integrity (Cao, Albertson, Riggs, Field, & Sullivan, 2008b) and hence showed fewer punctae in *synd* and *rhogef2* mutant embryos. Cytochalasin D, on the other hand, allows, albeit slower, actin polymerization at minus ends while blocking turnover at plus ends (Franki, Ding, Gao, & Hays, 1992; Goddette & Frieden, 1986) and therefore, *synd* and *rhogef2* mutant embryos treated with cytochalasin D resulted in more organized actin structures and elongated furrows (Figure 3.6).

This provides mechanistic insight into how Syndapin functions in regulating actin polymerization and may be further investigated through kinetic studies of actin polymerization.

Overexpression of Syndapin and not Pnut in the *rhogef2*^{RNAi} containing embryos partially reversed pseudocleavage furrow recruitment and morphology defects seen in *rhogef2*^{RNAi} and increased the furrow length compared to wild-type. It is probable, as has been suggested earlier (D. M. Lee & Harris, 2014), that Syndapin's role in endocytosis may function to limit furrow

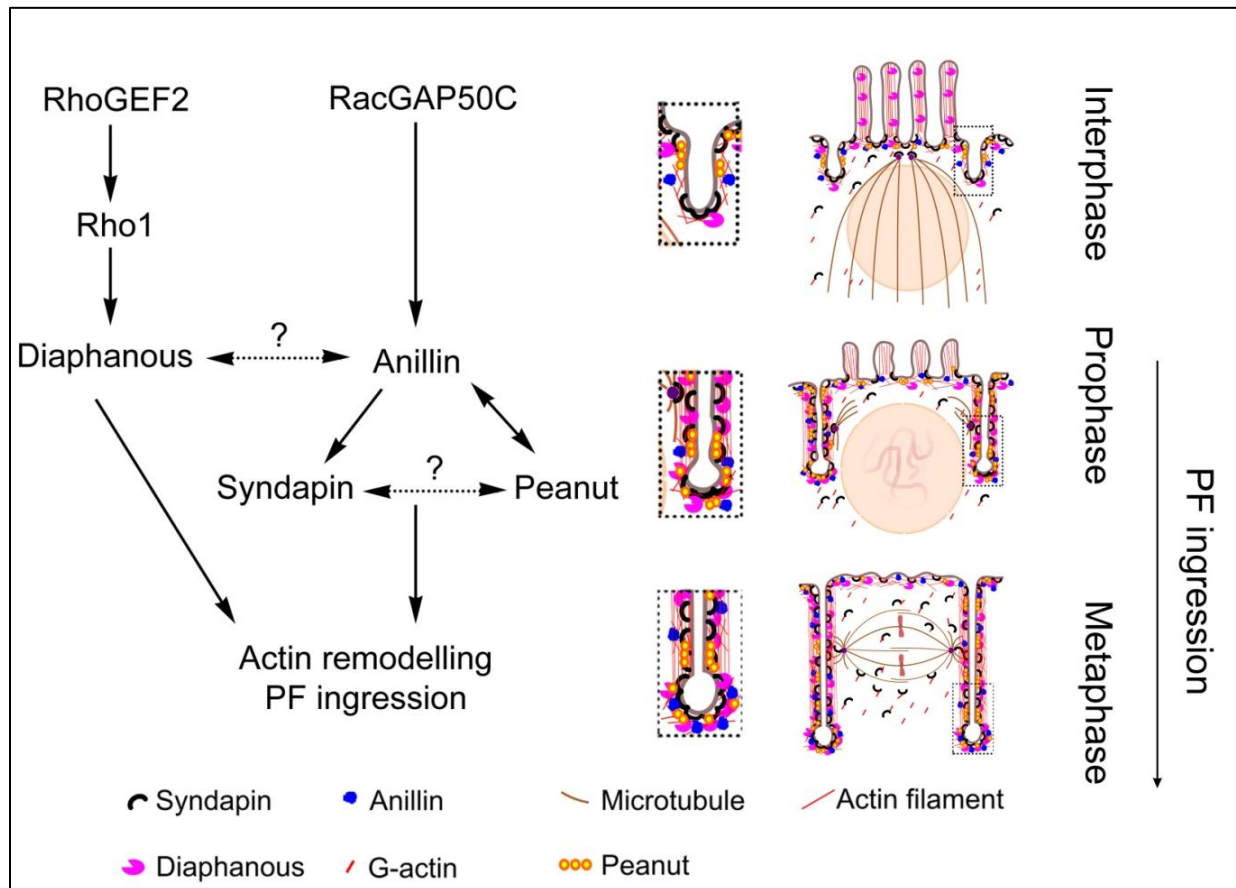


Figure 3.24. Schematic showing that the furrow defect in *synd*, *rhogef2* and *pnut* mutants is reversed by Syndapin overexpression in *rhogef2*^{RNAi}. Pseudocleavage furrow extension in metaphase of the syncytial cycle in *synd*, *pnut*, *rhogef2* mutants is decreased due to recruitment defects in Syndapin, Pnut, Dia and actin and reversed in *rhogef2* mutants by overexpression of Syndapin. Syndapin, Anillin, Pnut and Dia together regulate actin remodelling and, therefore pseudocleavage furrow dynamics.

growth. Another possibility is that Syndapin activity is needed at the pseudocleavage furrow for extension and some as-yet uncharacterized proteins play a role in furrow limitation. It is interesting to compare the functions of F-Bar domain proteins, Syndapin with Cip4 in furrow

elongation and Dia recruitment. Cip4 antagonizes Dia function and its overexpression has *dia* loss of function phenotypes like missing furrows (Yan et al., 2013). It is possible that opposing activities of F-Bar proteins Syndapin and Cip4 with respect to Dia, are in a balance with respect to each other and future experiments can test whether this function plays a role in limiting the growth of pseudocleavage furrows.

Since we have established Syndapin's role in actin dynamics, in the next two chapters, we study two other processes in the syncytial embryo governed by actin remodelling in the context of Syndapin function: apical cap expansion and maintenance of polygonal cell shape in the dividing 'pseudoepithelial' sheet.

CHAPTER 5

Syndapin Controls Arp2/3 Complex-dependent Actin Cap Expansion Dynamics in *Drosophila* Syncytial Embryos

(This work is unpublished and is a manuscript under preparation. In this chapter, I have plotted data from TIRF imaging of embryos acquired and manually quantified by Prachi Richa).

5.1 Introduction

During the syncytial nuclear cycles, pseudocells lack a basement membrane but have a complex organization of the apical membrane into microvilli during interphase that mediates critical functions including transducing signals from the perivitelline space and acting as membrane reservoirs for the extension of pseudocleavage furrows. The apical membrane is lined by a mesh of cortical acto-myosin that forms a cap over each nucleus (Foe et al., 2000). The cap then begins to expand, with actin at the centre and the Arp2/3 complex at the periphery which forms the leading edge of the expanding cap where actin polymerization drives its expansion until nuclear envelope breakdown when the caps meet to form furrows and Myo-II falls off the membrane and is recruited back only during telophase (Royou et al., 2002; Stevenson et al., 2002; Cao et al., 2010). If caps fail to expand, there is also a consequent failure to form furrows. Defects in apical cap expansion were first described in mutants for the protein Scrambled where small actin caps form but fail to expand (Stevenson et al., 2001). Following this study, it was observed that Scrambled is necessary for Arp2/3 localization to the cap margins and that Arp2/3 mediated actin polymerization was critical to drive cap expansion and subsequent furrow formation (Stevenson et al., 2002). Centrosome separation is also defective in mutants that inhibit cap expansion, however it has been demonstrated that cap expansion is upstream of centrosome separation and defects in centrosome separation does not influence cap expansion (Cao et al., 2010).

Another important study found that Myo-II functions to limit Scrambled or Arp2/3 complex-driven cap expansion. Inhibition of Myo-II activity using Rho-kinase inhibitor or RNAi led to abnormally large caps (Sommi et al., 2011). Unlike in *arpC1* or *scrambled* mutants, where actin accumulates in the cap but the cap fails to expand (Stevenson et al., 2001; 2002; Sommi et al., 2011), Myo-II inhibition results in failure of actin accumulation in the cap, the expanded caps are

larger that control caps (Sommi et al., 2011). Thus, Myo-II is necessary to limit cap expansion while Arp2/3 is critical to drive actin cap expansion. However, the mechanistic details of the process by which Myo-II functions are still unknown.

Actin dynamics is crucial for cap expansion; LatA- and Jasp-treated embryos fail to expand caps. Apart from Arp2/3, Dia has also been shown to be important for apical cap expansion (Cao et al., 2010). We have shown that Syndapin role is important for coordinated actin and membrane dynamics. Syndapin is known to physically interact with N-WASp (that recruits Arp2/3) via its SH3 domain and mammalian Syndapin2 interacts with Rac-1 via its F-BAR domain and inhibits Rac-1 activity (which, in turn, recruits Arp2/3 by activating the SCAR/WAVE complex) (de Kreuk et al., 2011; Kumar et al., 2009). Both Syndapin- and Arp2/3-depletion causes defects in furrow formation. The exact nature of the epistatic relationship between Syndapin and Arp2/3 has not been explored. We, therefore, investigated the role of Syndapin in apical cap dynamics (which involves dramatic remodelling of the membrane and cytoskeleton) in relation to Arp2/3 complex function.

5.2 Results

5.2.1 ArpC1 and Syndapin are both present at the boundary of the expanding apical cap

We first looked at where the Arp2/3 complex and Syndapin are localized in the apical region. It has been reported previously that ArpC1 localizes to the rim of the expanding apical cap using fixed images (Stevenson et al., 2002). We used ArpC1-GFP and Synd-GFP to look at their localization dynamics as the embryos proceeded through n.c. 12 at a plane where the apical caps are first visible (0 μm) and at a depth of 3 μm where the boundary of expanding caps are seen. We found that both ArpC1 and Syndapin are present at the rim of the expanding cap (Figure 4.1A). We then quantified levels of each protein as the cap expanded. We found that the levels of Syndapin remained more-or-less constant; at 3 μm , the levels of ArpC1 increased for the first 4 min while the apical levels (0 μm) decreased until the caps came in contact and then remained constant (Figure 4.1B).

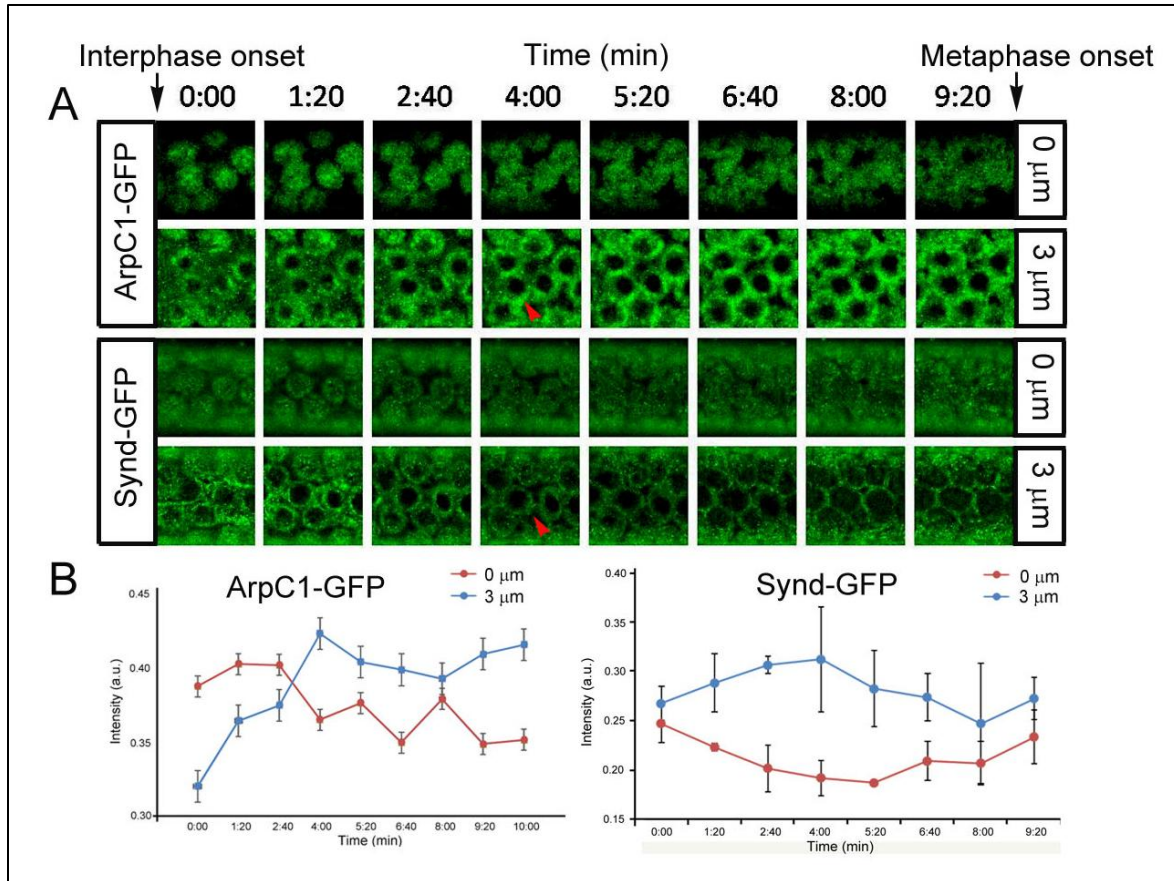


Figure 4.25. ArpC1-GFP and Synd-GFP are localized at the boundary of the expanding apical cap. Time lapse images of expanding apical caps during n.c. 12 showing localization of ArpC1-GFP and Synd-GFP at 0 μ m and 3 μ m especially at boundary of expanding cap (red arrows) (A). At 3 μ m the levels of Syndapin (Normalized Intensity in arbitrary units (a.u.) $\pm$ S.D.) remained more-or-less constant while the levels of ArpC1 increased for the first 4 min until the caps came in contact and then remained constant (B).

5.2.2 Apical cap expansion is affected upon depletion of Syndapin and Arp

LifeAct is a 17 amino-acid peptide used to label actin filaments without interfering with its dynamics *in vivo* (Riedl et al., 2008). We used a LifeAct-GFP line generated in our lab and combined it with the nanos-GAL4 driver to study apical actin cap dynamics from time-lapse movies. We also tested various RNAi/mutant lines in this background to study defects in cap expansion dynamics (Figure 4.2A).

We measured apical cap areas at regular time intervals starting from the onset of interphase of cycle 13 until the onset of prophase of the same cycle (when membranes come into contact with

each other and the cap stops expanding). The cap area was observed to increase as the nuclear cycle progressed in LifeAct-GFP/+ control embryos (Figure 4.2B-C). Consistent with previous studies, depletion of Arp3 of the Arp2/3 complex results in significantly smaller caps that form but do not expand (Figure 4.2C).

Interestingly, when we deplete Syndapin, cap expansion occurs but the caps expand faster and are significantly larger in *synd* than in control embryos. Also, unlike in wild-type, the actin accumulated at the periphery of the cap dissipates as soon as the caps come into contact (Figure 4.2A-B). Thus, we conclude that Syndapin function is important to limit expansion of the apical actin cap.

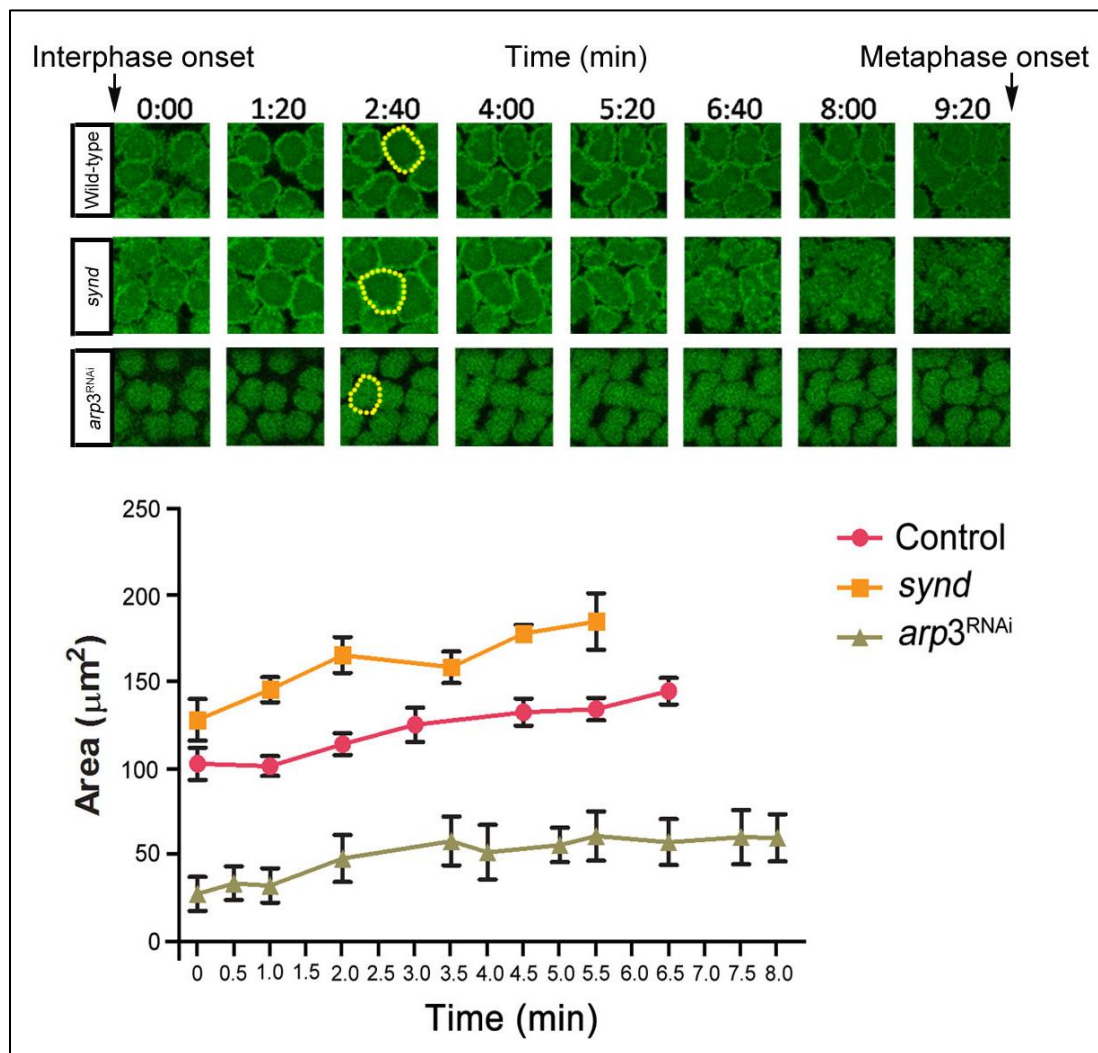


Figure 4.26. Effect of depletion of Syndapin and Arp3 on apical cap expansion. Time lapse images of expanding apical actin caps during n.c. 12 in control, *synd* and *arp3^{RNAi}* embryos in the LifeAct-GFP

background. Red arrow indicates rapid actin dissipation when caps merge in *synd* compared to slower dissipation in control (A). Scale Bar = 10 μm . Plot showing increasing cap area $\pm$ S.D. with time quantified by drawing outlines (yellow dotted lines in (A)) around caps and measuring area for $n \geq 9$ caps/3 embryos, One-Way ANOVA and Tukey's Multiple comparison test (B-C).

5.2.3 Doubly depleting ArpC1 with *synd* mutant can rescue cap area phenotype

Depletion of either ArpC1 (Cao et al., 2010) or Arp3 of the Arp2/3 complex causes apical actin cap expansion to fail. On the other hand, depletion of Syndapin results in formation of abnormally large caps (Figure 4.2A-B). If the model suggested by Sommi et al. (2011) is correct, there should be two opposing forces that regulate apical cap expansion – an outward force that drives expansion and an inward force that limits runaway expansion. In an attempt to restore this balance, we combined one copy of *arpC1* mutant allele with *synd*^{1d}/Df and quantified cap expansion in these embryos (Figure 4.3A-B).

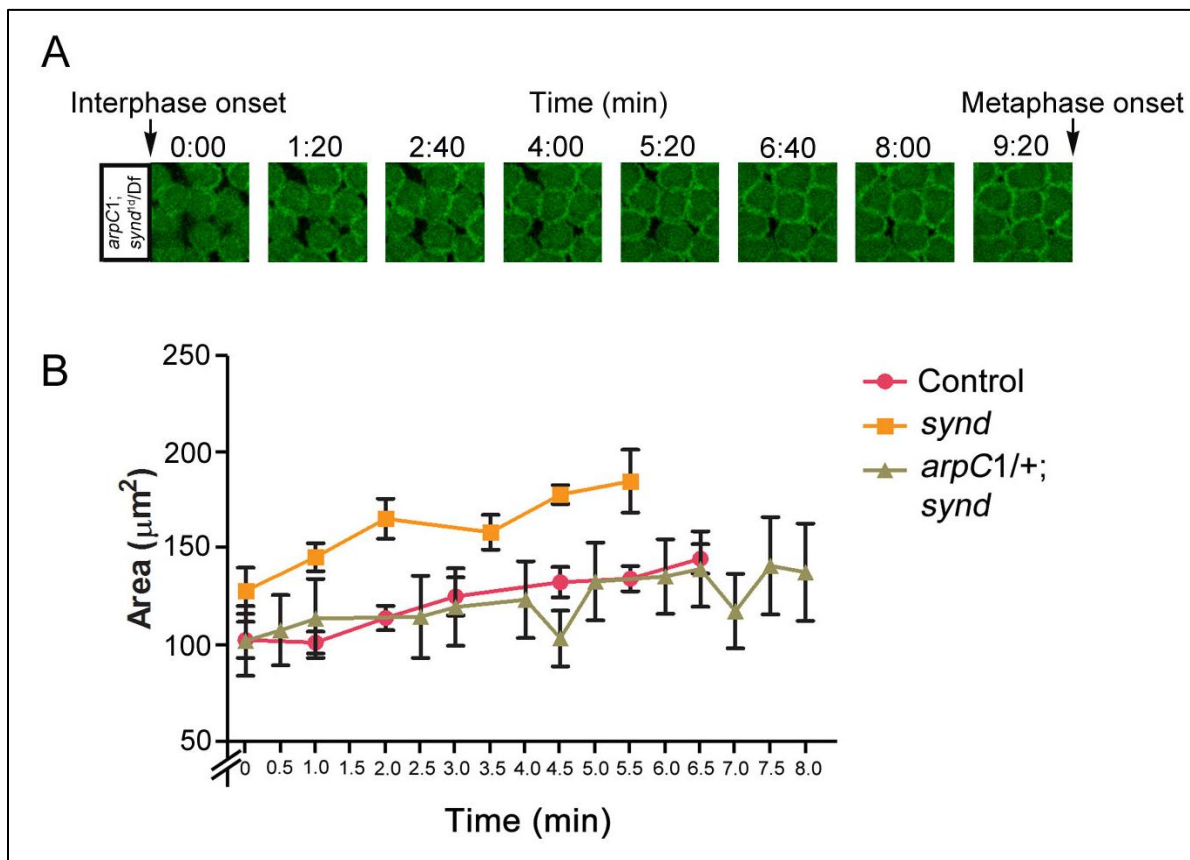


Figure 4.27. A single copy of *arpC1* mutant allele with *synd* can rescue cap expansion phenotype.

Time lapse images of expanding apical actin caps during n.c. 12 in *arpC1^{+/+}; synd* mutant combination (A) shows a rescue in rate of cap expansion similar to control compared to *synd* mutant alone (B). Scale Bar

= 10 μ m. Plot showing increasing cap area $\pm$ S.D. with time quantified by drawing outlines (yellow dotted lines in (A)) around caps and measuring area for $n \geq 9$ caps/3 embryos, One-Way ANOVA and Tukey's Multiple comparison test (B).

Interestingly, we found a rescue of *synd* cap expansion phenotype when one copy of *arpC1* allele was additionally depleted and the caps expanded at the same rate as the control caps. In addition, rate of cap expansion was also slowed compared to *synd*, in fact, the caps took even longer than control caps to expand. Therefore, we can conclude that Arp2/3 complex and Syndapin together regulate the inward and outward forces that govern expansion of apical actin caps although further studies need to be done to ascertain the players downstream of Syndapin that achieve this inward force.

5.2.4 Apical caps are more circular upon depletion of Arp3 or ArpC1 in *synd* background

In wild-type embryos, Arp2/3-driven actin polymerization provides the outward force (f_o) that drives apical cap expansion (Stevenson et al., 2002; Cao et al., 2010) and Myo-II that cycles in response to Cdc2 levels during the nuclear cycle (Royou et al., 2002) provides the limiting inward force (f_i). However, since the caps expand the outward force must exceed the inward force i.e. $f_o > f_i$ (Sommi et al., 2011). Once the caps touch each other, Myo-II levels start to decrease (becomes more cytosolic) (Royou et al., 2002) or f_i reduces; thus, $f_o \gg f_i$ and the membranes come in contact and form polygons after which line tensions come into play. This is reflected in the fact that circularity (defined as $4\pi \left(\frac{\text{area}}{\text{perimeter}^2} \right)$). A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon) in control embryos is initially close to 1 and then it gradually falls (Figure 4.4). In *synd* embryos also, although the area increases much faster than control (Figure 4.2B), the circularity follows the same trend as in the control (Figure 4.4). In *arp3*^{RNAi} embryos apical caps do not expand i.e. $f_o \leq f_i$. Their circularity also drops much more gradually than in control (Figure 4.4). When both ArpC1 and Syndapin are depleted, initially $f_o > f_i$ since the caps expand normally. However, the caps remain rounded even after contact – the circularity falls very slowly similar to Arp3-depleted embryos. Here, absolute values of both f_o and f_i should be lower than control and that once Myo-II dissociates or f_i further decreases, one can speculate that f_o or the force produced by Arp2/3-driven polymerization is too low to rapidly make contacts and form polygons. Since Rho signalling is responsible for activation of both Myo-II and Dia, it is possible

that once Myo-II dissociates, activated Dia levels increase promoting long filamentous actin organization necessary for furrow formation. Since the runaway cap expansion defect of Myo-II (Sommi et al., 2011) and Syndapin depletion (Figure 4.2B) phenocopy each other, it still remains to be tested whether Syndapin depletion has any effect on the recruitment of Myo-II.

To gain further insight into this process, since the molecular players involved are known actin regulators, we used super-resolution microscopy to study how actin is organized in the apical caps.

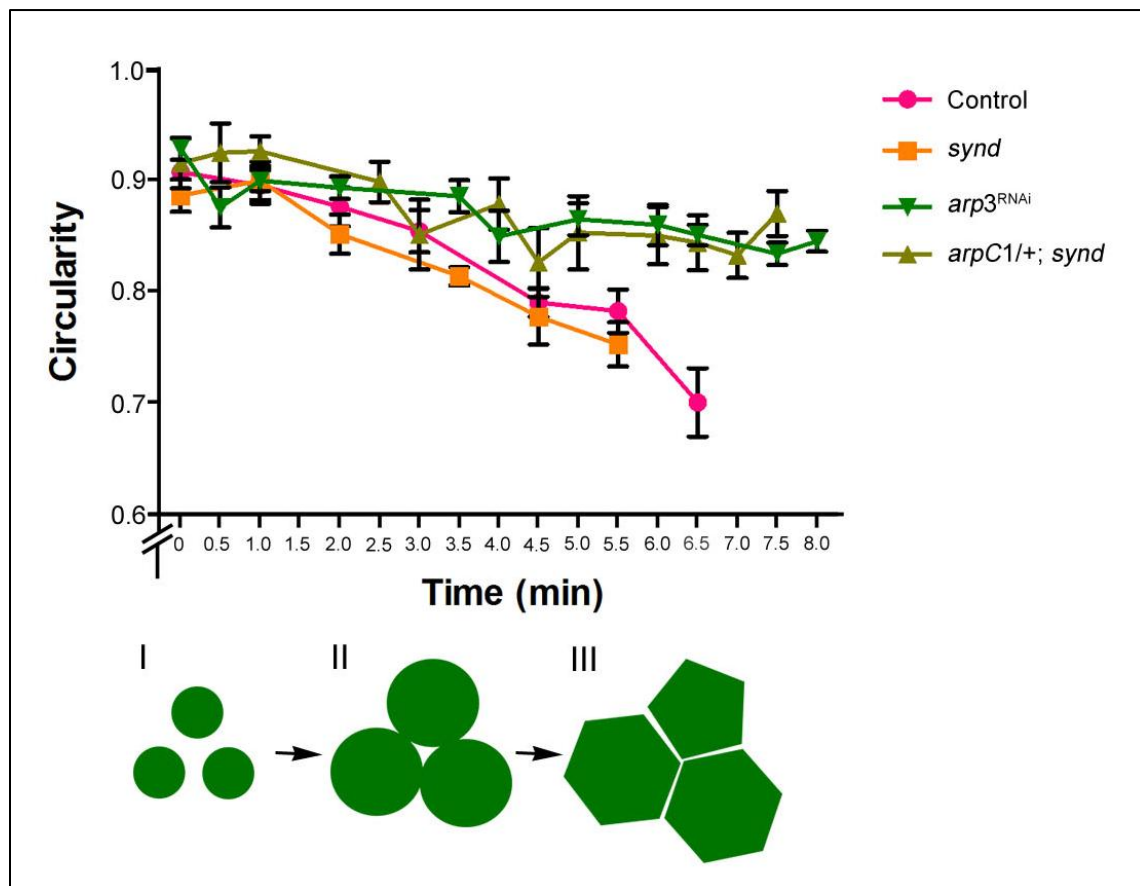


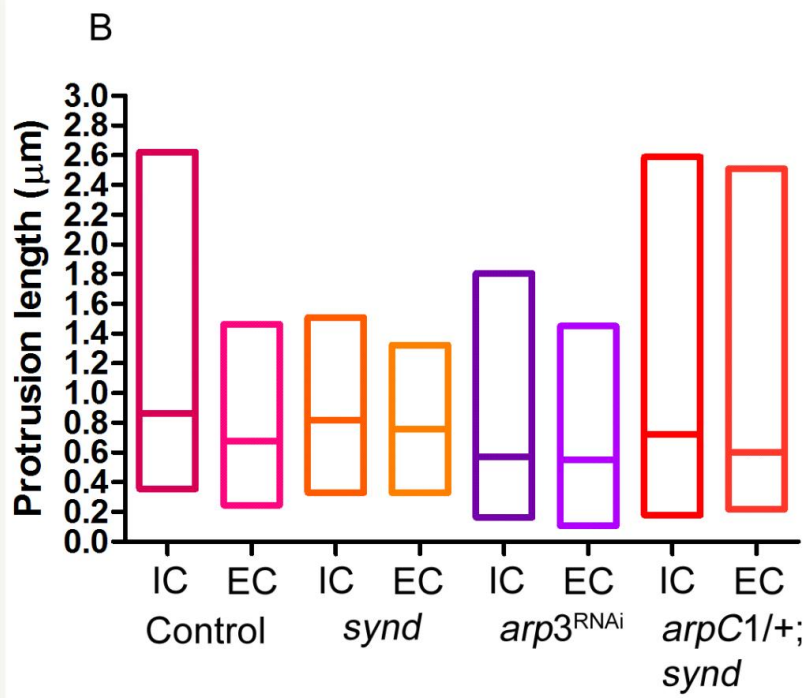
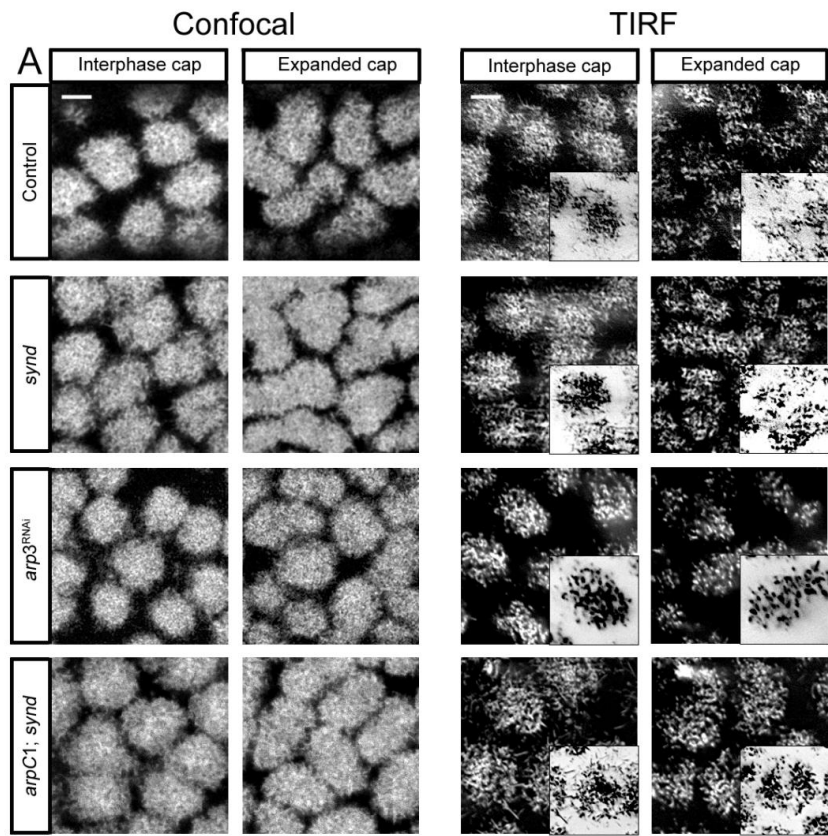
Figure 4.28. Circularity of apical caps during expansion. Plot showing circularity ($\pm$ S.D.) of expanding apical actin caps in control, *synd*, *arp3^{RNAi}* and *arpC1/+; synd* embryos in the LifeAct-GFP background. In control, as the apical caps expand they are initially circular, their circularity decreases (perimeter increases much faster than area) as they come into contact and become polygons (I-III). In *synd* mutants, the caps are larger than control and already in contact but the circularity falls at the same rate as they do in control since their perimeter increase proportionate to the area in the same ratio as the control (II-III). In *arp3^{RNAi}*, the caps take a longer time to expand and come in contact (both perimeter and area increase more slowly than control), thus the caps remain almost circular (I). In the *arpC1/+; synd* combination, the

caps expand but take much longer to form edges of polygons (their perimeter increases much slower than the control) so the circularity of caps again falls much more slowly (I-II).

5.2.5 Cortical actin remodelling is essential for proper actin cap expansion

During interphase of nuclear cycle 14 (early cellularization), the microvilli above each actin cap have been observed to flatten concomitant with the ingression of the cleavage furrows which involves disassembly of the apical actin (Figard et al., 2016; 2013). This process is also likely to occur during the syncytial nuclear cycles. Therefore, we used Total Internal Reflection (TIRF) microscopy to study dynamics of actin organization in apical actin caps (This work was done in collaboration with Prachi Richa). In control embryos it was observed that the optically resolvable LifeAct-labelled actin (~ 100 nm XY resolution) we termed ‘protrusions’ were longer (upto ~ 2.6 μm) in interphase caps (ICs) compared to the expanded caps (ECs) that were shorter (upto ~ 1.5 μm ; Figure 4.5A-D). These protrusions therefore parallel to the plane of the cap possibly abutting the cap circumference (and pushing it outward by the force of treadmilling) in the ICs but are subsequently remodelled to be organized perpendicular to the cap surface after cap expansion in the ECs. This remodelling could also allow for the apical microvilli to flatten and supply membrane and actin bundles for the ingressing furrow.

Interestingly, in *synd* embryos, the mean protrusion lengths in ICs and ECs are not significantly different from each other and from the mean control ICs length (Figure 4.5B-D). However, the difference lies in the distribution of protrusions where it is evident that there are some very short protrusions (~ 0.4 μm) and the very long protrusions (> 1.5 μm) are missing from these mutant caps (Figure 4.5C). This implies that actin protrusions in the caps are not disassembled and remodelled into structures perpendicular to the cap surface (into furrows) upon cap expansion; they remain long and undergo treadmilling along the surface and continue to provide force for cap expansion, thus resulting in runaway expansion of caps. This phenotype may also be explained by the observation that when mammalian Syndapin2 is depleted, Rac1 activity is enhanced. If this were true in *Drosophila* embryos too, up-regulated Rac1 in *synd* mutant embryos could explain a consequent increase in Arp2/3 activity which would result in unregulated cap expansion and, therefore, this interaction needs to be investigated.



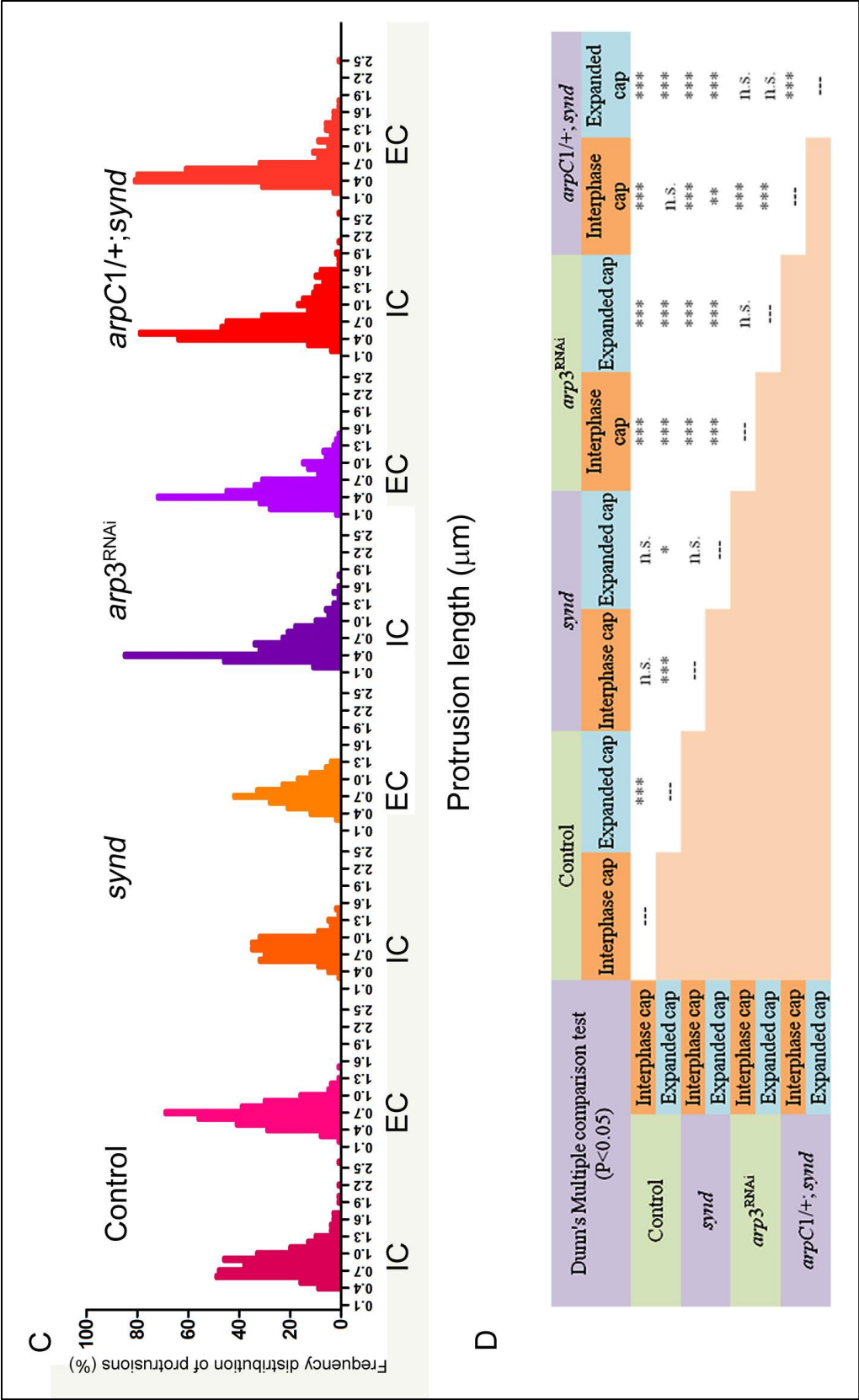


Figure 4.29. Cortical actin remodelling is essential for proper actin cap expansion. Images from time lapse confocal and TIRF (acquired by Prachi Richa) movies of Interphase caps (ICs) and Expanded caps (ECs) at 0 μm during n.c. 12 in control, *synd*, *arp3*^{RNAi} and *arpC1/+; synd* embryos in the LifeAct-GFP background. Scale Bar = 5 μm (A). Box plots and frequency distribution histograms of Protrusion lengths (μm) quantified (by Prachi Richa) in ICs and ECs the above embryos (N= 900 protrusions/9 caps/3 embryos (B-C). Table of comparisons between ICs and ECs in each genotype using Kruskal-Wallis test and Dunn's Multiple comparison test ($P < 0.05$).

In Arp3-depleted embryos, the majority of protrusion in both ICs and ECs are very short ($\sim 0.4 \mu\text{m}$; the mean protrusion length of ICs and ECs are not significantly different), even shorter than those in control ECs. Also unlike the control, some protrusions are extremely short ($0.1 - 0.4 \mu\text{m}$), very close to the resolving power of the microscope (Figure 4.5C). This explains why the caps in *arp3*^{RNAi} embryos do not expand – Arp2/3 function is needed to assemble long actin protrusions that push the cap membrane outward.

Like in the control, in embryos of the mutant combination *arpC1/+; synd*, there is a significant difference in protrusion lengths between ICs and ECs (Figure 4.5B-D). There are a large number of extremely short ($0.1 - 0.4 \mu\text{m}$), short ($0.4 - 0.7 \mu\text{m}$), long ($0.7 - 1.5 \mu\text{m}$) and even some very long ($> 1.5 \mu\text{m}$) protrusions in the both the ICs and ECs. However, while the maximum number of protrusions are $\sim 0.7 \mu\text{m}$ in ICs, they significantly shift to a majority of $\sim 0.4 \mu\text{m}$ in ECs (Figure 4.5C). This explains the rescue of cap expansion phenotype seen in these embryos. Also, since few very long protrusions persist in the ECs, the caps remain circular (Figure 4.4) but some loss-of-function of the Arp2/3 complex prevents runaway expansion. If Syndapin and Myo-II function are somehow linked, this would explain loss of actin protrusion remodelling due to loss of Myo-II mediated disassembly of actin filaments, and this hypothesis merits testing.

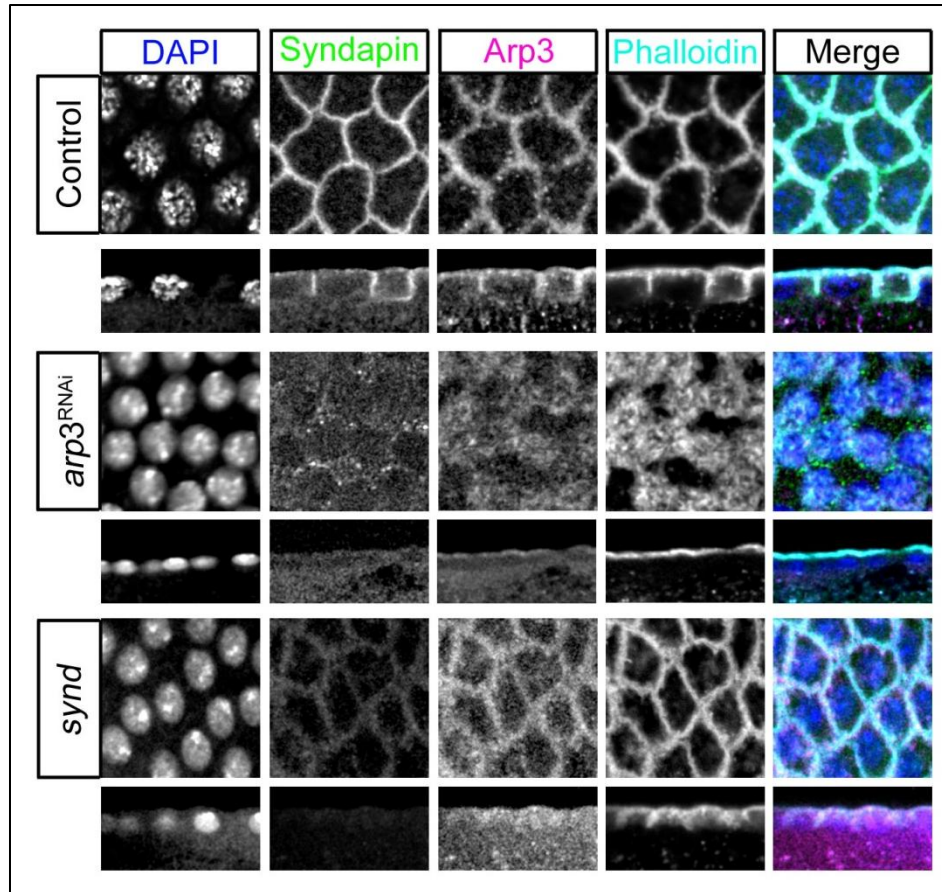


Figure 4.30. Arp3-depleted embryos fail to recruit Syndapin to the membrane. In *arp3*^{RNAi} embryos, Syndapin fails to recruit to the membrane (58%, n = 12) but in *synd* mutant embryos, Arp3 levels are unaffected (83%, n = 12).

5.2.6 Arp3-depleted embryos fail to recruit Syndapin to the membrane

To test the epistatic relationship between Syndapin and Arp2/3, we fixed and immunostained control and mutant embryos using antibodies for Syndapin and Arp3. We found that upon Arp3 depletion, Syndapin fails to be recruited to the membrane. However, interestingly, Arp3 recruitment does not depend on the presence of Syndapin as seen in *synd* embryos in n.c. 12 (Figure 4.6). Thus, we can conclude that the effects of Syndapin depletion including runaway cap expansion are independent of Arp2/3 complex function and depend on some other downstream effectors.

5.3 Discussion

The length of microvilli is regulated by the amount of free monomeric actin available for turnover and longer microvilli have faster actin incorporation and treadmilling rates. Actin crosslinking proteins that control treadmilling can increase the stiffness, stability and length of parallel actin filament bundles in microvilli. Extrinsic factors like membrane tension also negatively regulate microvillar length (Sauvanet et al., 2015). Syndapin 2 has been identified to regulate microvillar length in polarized epithelial cells of placental syncytiotrophoblasts (Wayt and Bretscher, 2014). Since our previous study implicates *Drosophila* Syndapin in actin dynamics during furrow ingression, we studied actin remodelling during apical cap expansion a concurrent process that potentially also involves flattening of the apical microvilli. We found that depletion of Syndapin results in abnormally large apical actin caps similar to the phenotype observed upon depletion of Myo-II (Sommi et al., 2011). This is exactly opposite to what is observed when components of the Arp2/3 complex are depleted (Stevenson et al., 2002; Cao et al., 2010). Therefore, Myo-II/Syndapin and Arp2/3 complex seem to direct forces in opposite directions in order to regulate cap expansion.

An important protein that promotes cell spreading through the activity of the Arp2/3 complex is Rac1. Rac1 and RhoA have been shown to have opposite functions in various cellular contexts since RhoA/Rho1 promotes formin activity and the assembly of linear actin bundles rather than branched actin networks (Chauhan et al., 2011; Parri and Chiarugi, 2010). Syndapin 2 is known to bind and negatively regulate Rac1 function (de Kreuk et al., 2012). If this relationship holds in *Drosophila* also, it is conceivable that Syndapin will inhibit Rac1 and Arp2/3 complex function and thereby promote Rho1 and Myo-II activity. When Syndapin is depleted, Rac1 will no longer be inhibited and will therefore be upregulated. This will promote excessive Arp2/3 function causing uncontrolled/runaway cap expansion.

Actin turnover is also crucial for expansion of the apical caps. This is reflected in our study in which we show that apical actin protrusions are not remodelled upon depletion of either Syndapin or components of Arp2/3 complex or both. Depletion of formins, ArpC1 or using drugs that inhibit actin assembly/turnover like LatA/Jasp negatively regulate cap expansion (Cao et al., 2010). Myo-II which is known to be important for disassembly of dendritic actin networks (Haviv et al., 2007; Wilson et al., 2010), on the other hand, is important to limit cap expansion

and depletion of Myo-II causes formation of abnormally large caps. Although likely, it remains to be determined whether Syndapin functions through the activity of Myo-II to limit cap expansion (Figure 4.7).

In the next chapter, I discuss the role of Syndapin and actin assembly proteins in regulating conserved organization of lateral membrane furrows that form post apical cap expansion into a polygonal array of pseudocells.

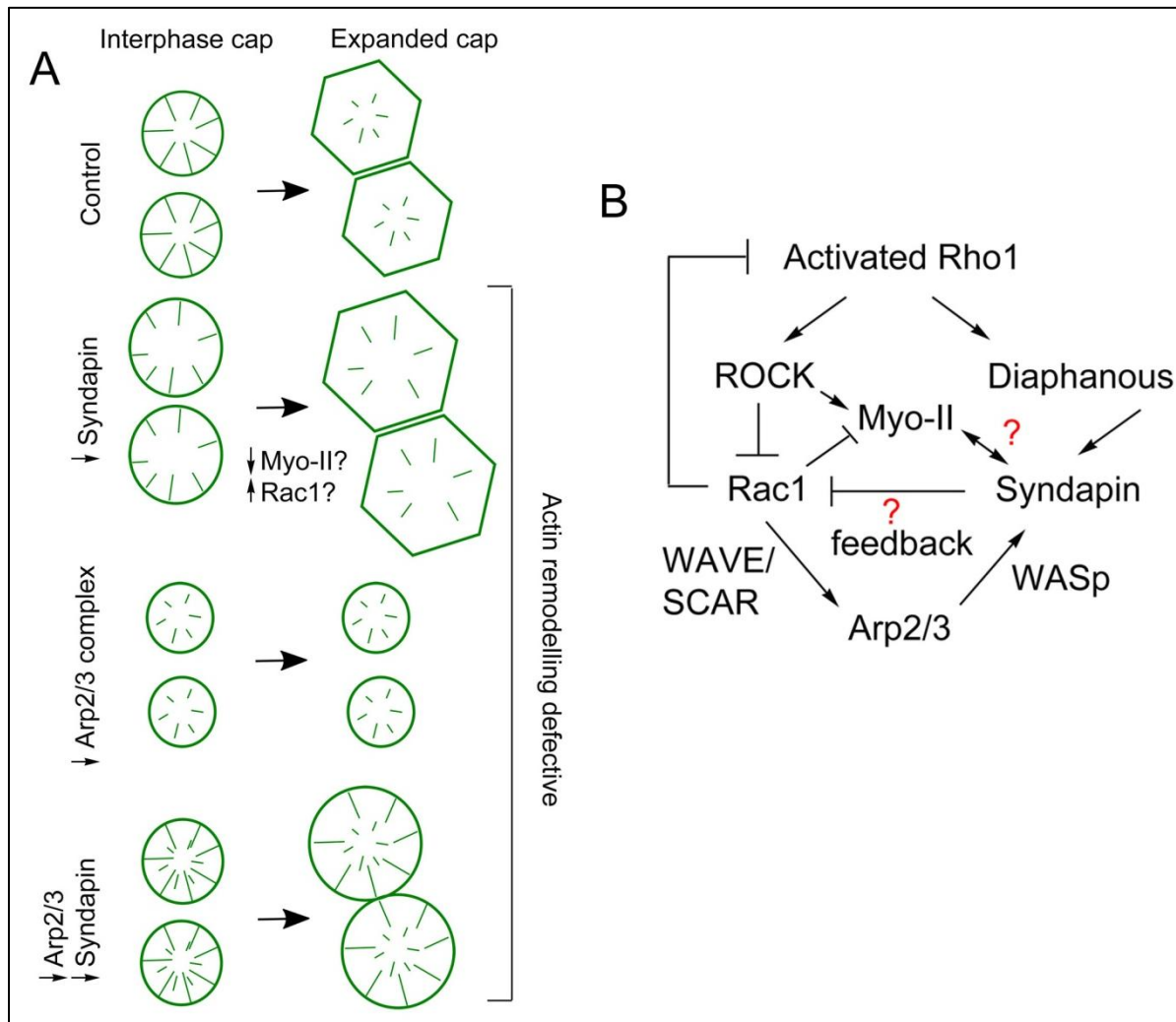


Figure 4.31. Model for apical actin cap expansion in syncytial *Drosophila* embryos. Circular apical actin caps expand, contact each other and form polygons as the nuclear cycle progresses from beginning of interphase to the beginning of metaphase in control embryos; correspondingly there is a shortening of apical protrusions in the caps (green lines). When Syndapin is depleted, perhaps as a consequence of increased Rac1 and/or depleted pMyo-II, the caps undergo runaway expansion and the protrusions do not shorten as the caps expand unlike in the control. Caps that are depleted of components of the Arp2/3

complex show the opposite phenotype, the caps fail to expand and compared to the control, the protrusions are very short and remain so. When both Syndapin and ArpC1 are depleted, the caps expand normally but remain rounded perhaps because both long and short protrusions are continually present in both ICs and ECs. In all three mutant scenarios there seems to be lack of remodelling of the actin protrusions that display different phenotypic readouts (A). Possible role for Syndapin in the pathway that activates/inhibits Arp2/3 complex function. Activated Rho1 is important for Myo-II phosphorylation by Rho-kinase (ROCK) and to release Dia from autoinhibition. ROCK inhibits Rac1 which, in turn, inhibits Myo-II function. Rac1 also inhibits Rho1 activity and is important for activation of the Arp2/3 complex. Syndapin requires Dia and Arp2/3 for localization to the membrane. Syndapin may inhibit Rac1, this and its interaction with Myo-II needs to be further investigated (B).

CHAPTER 6

Syndapin Regulates the Frequency Distribution of Polygons in the ‘Pseudoepithelial’ Sheet

(The work done in this chapter has not been previously published elsewhere, but has served as preliminary work for another manuscript in preparation. However, the results described are interesting in their own right and complete the results described in the previous chapters).

6.1 Introduction

There are several generalizations that apply to polygonal cell layers in packed tissues. *Euler's theorem* states that in a tissue with a large number of cells, the average number of neighbours a cell has is 6. According to *Lewis' law*, the average area (A_n) of an n-sided cell increases linearly with n, i.e. smaller cells have fewer sides. *Aboav-Weaire Law* states, cells with higher number of sides tend to have fewer sided neighbours and *vice versa*. Directly or indirectly these laws imply a relationship between the number of cells, the area they occupy and their distribution of neighbours (Sánchez-Gutiérrez et al., 2015).

The syncytial *Drosophila* embryo grows from 1 to 6000 nuclei in 2 hrs of development. Unlike in an epithelial sheet of cells, the monolayer of nuclei at the cortex of syncytial insect embryos lack membranes separating them into cells, so wall tension does not dominate the dynamics in this system. Syncytial embryos go through a rapid sequence of cell divisions and after each division, the nuclei rapidly self-organise in a few minutes (Foe & Alberts, 1983; Mavrakakis, Rikhy & Lippincott-Schwartz, 2009). The last four syncytial nuclear divisions start at both anterior and posterior poles and propagate to the centre within a minute, in a wave-like manner. During these mitoses, the nuclei are significantly displaced. Regular arrangement of the nuclear array needs to be restored after mitosis in order to conserve uniform polygonal distribution and compartment size; a process that involves conversion of two-way vertices into three-way vertices (Gibson et al., 2006). In particular, a uniform cell size and regular cellular packing have been hypothesised to be important for establishing precise morphogen gradients (Bollenbach et al., 2008; Kavousanakis et al., 2010; Sample & Shvartsman, 2010; Sherlekar & Rikhy, 2012), and

for the establishment of planar cell polarity signalling in later development (Ma et al., 2008). The nuclear rearrangements, and the subsequent maintenance of positioning, are generated within a rapidly changing structural network by constantly remodelling actin and microtubule cytoskeleton (Kanesaki et al., 2011). Kanesaki et al. (2011) found that $56.1 \pm 0.4\%$ of the compartments had six neighbours while $22.1 \pm 0.2\%$ and $19.5 \pm 0.2\%$ had five and seven neighbours, respectively during early cellularization (14th interphase) in the *Drosophila* embryo. In contrast to the report from *Drosophila* wing discs (Classen et al., 2005) in which the proportion of hexagons continually increases from about 40% to 80%, these proportions were found to remain relatively constant during 14th interphase (Kanesaki et al., 2011). Computer simulations by Farhadifar (2009) predicted that if a division cycle occurs without growth, the sidedness shifts towards hexagons or the sheet becomes more regular. Sánchez-Gutiérrez et al. (2015), however, postulated that the polygon distribution does not depend on cell proliferation alone and that the increase in the homogeneity of the cell sizes correlates with a higher number of hexagons, pentagons and heptagons, which is what explains the conserved polygon distribution described in various tissues. In haploid embryos that undergo an extra (14th) nuclear division, Kanesaki et al. (2011) found that order is not increased compared to wild-type, implying that neither cell proliferation nor cell size/cell density can fully govern robust regulation of ordering. However, the syncytial cycles 11-13 were found to have greater variation in order, suggesting that a certain threshold number density is indeed important to achieve ordering (Kanesaki et al., 2011). During the syncytial nuclear cycles, there is breakdown of the actin and microtubule cytoskeleton in anaphase and therefore a complete reordering of compartments takes place between anaphase of one cycle and interphase of the next when the cytoskeleton is rapidly reassembled and order is re-established. Throughout the nuclear cycle, the F-actin caps and microtubules grow and become denser, with the contact area between the caps and microtubules additionally increasing over time. A computational model on ordering and organization in the syncytial embryo predicted that ordering was independent of the nuclear density, although increasing in time (Koke et al., 2014). During the syncytial division cycle, actin caps expand and contact each other. This involves separation of the centrosomes and formation of spindles accompanied by formation of membrane contacts (Stevenson et al., 2002; Cao et al., 2010) which, according to Koke et al., (2014) is a key determinant of correct reordering of the nuclei. Their model is of an attractive tensile network of microtubules, resisted

by actin caps bearing the tensional forces. This antagonistic force mechanism enables the cell to stabilise its shape and resist deformation and the repulsive forces are necessary and sufficient to generate the observed reordering within the embryo. Disordering of the nuclei and the mitotic spindle force was found to be nonlinearly correlated, with the maximum disorder observed in the system determined by the strength of the mitotic spindle. They also correctly predict that nuclei maintain their relative spatial positions across phases of division and rearrangement (Koke et al., 2014; Krzic et al., 2012; Mavrakakis et al., 2009).

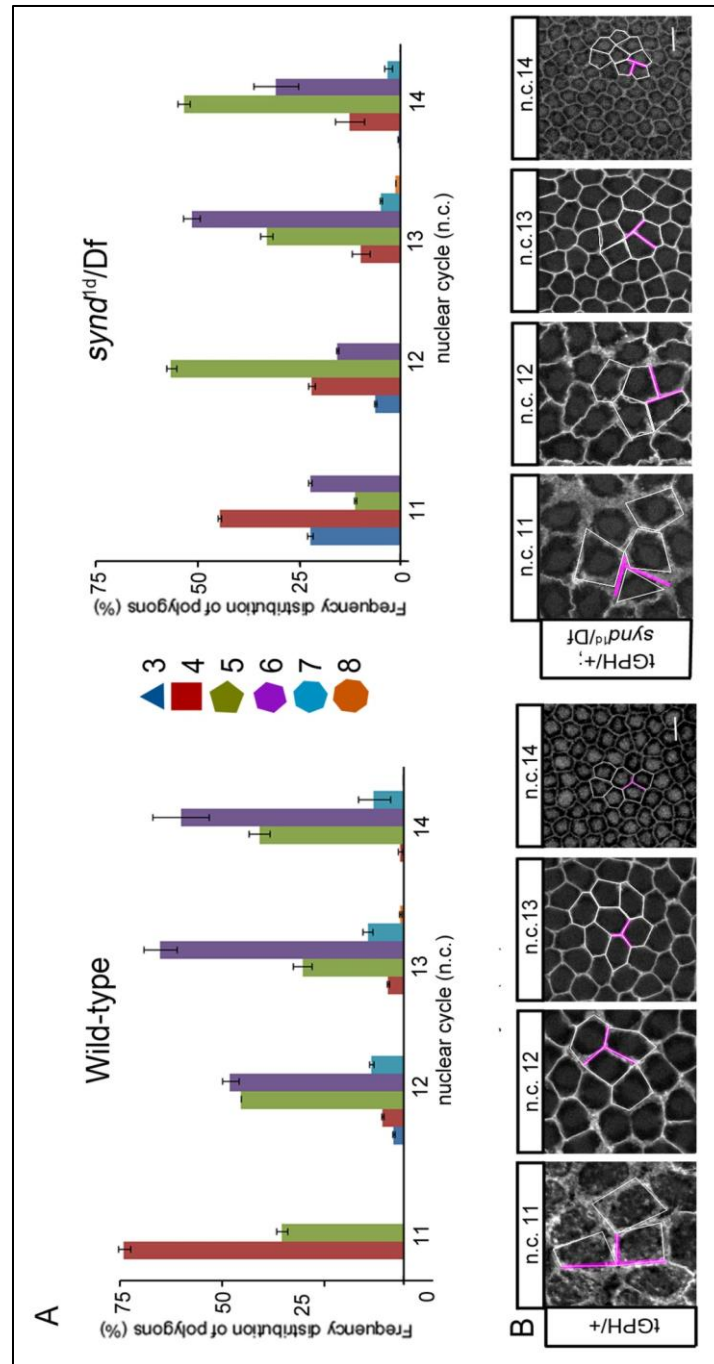
6.2 Results

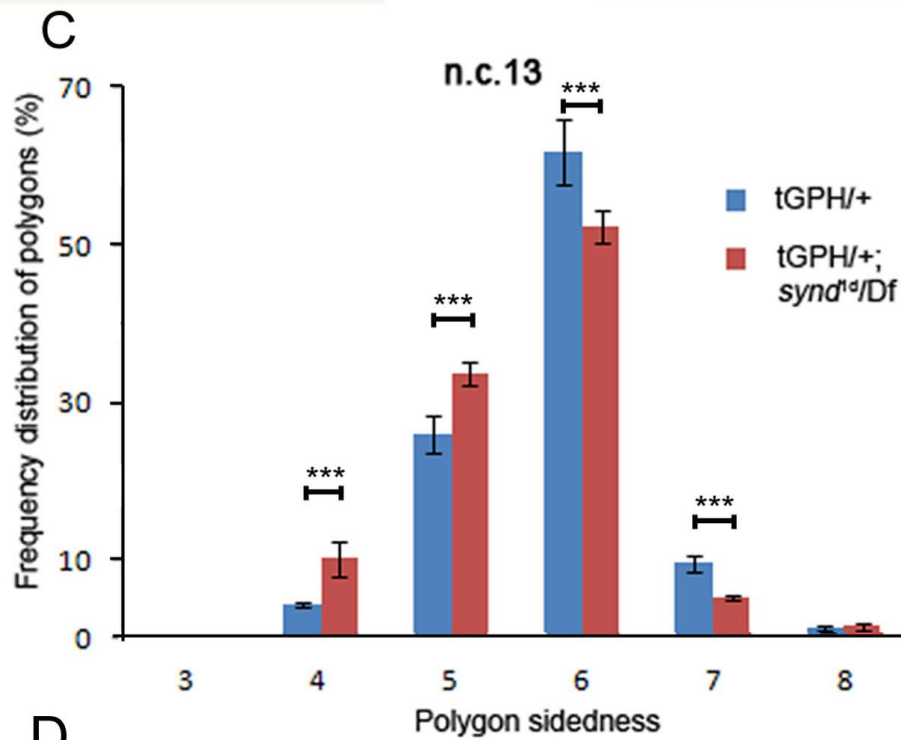
6.2.1 Syndapin is crucial for maintenance of conserved polygon distribution during syncytial nuclear cycles

We characterized the wild-type distribution of polygons during metaphase of nuclear cycles 11-13 and interphase of 14th cycle. This is interesting since it helps us test whether the conserved principles that govern sheets of polygonal epithelia in various tissues apply to syncytial ‘pseudopeithelial cells’. And if they did, we would have a handle to further understand whether perturbing the polygonal distribution can have far-reaching consequences on developmental patterning of the embryo by affecting morphogen gradients as suggested previously. We showed that in wild-type embryos, as nuclei divide and come closer together, the frequency distribution of polygons increasingly shifts towards a regular pattern which is predominantly hexagonal. In n.c. 12, the number of hexagons and pentagons are equal. By n.c. 13, the number of hexagons is more than double the number of pentagons (Figure 5.1 A-D). A reflection of ordering of polygons is the increase in three-way vertices and a decrease in two-way vertices, which in wild-type are extremely rare from cycle 12 (Figure 5.2 B).

Maintenance of tension in the membrane has been shown to involve actin, myo-II and the cadherin-catenin complex (Classen et al., 2005; Farhadifar et al., 2007; Rauzi et al., 2008). We have mechanistically showed that Syndapin function is crucial for actin organization in the syncytial embryo. We, therefore, asked whether depletion of Syndapin would result in formation of a ‘soft’ network and affect the conserved polygon distribution. Indeed, we showed that in *synd* mutants, the distribution is altered in favour of 5-sided rather than 6-sided polygons, similar to what was found previously in other tissues (Farhadifar, 2009). Interestingly, there was also a

much higher preponderance of 3- and 4-sided polygons in *synd* mutants (Figure 5.1A-D). We observed a significantly higher occurrence of two-way vertices in *synd* mutants as quantified in cycle 13 where there are $21.0 \pm 2.4\%$ (n=3) as compared to wild-type which have only $3.0 \pm 0.5\%$ (n=3) (Figure 5.1B). This clearly indicates that the polygon network is less ordered in *synd* mutants compared to wild-type.





D

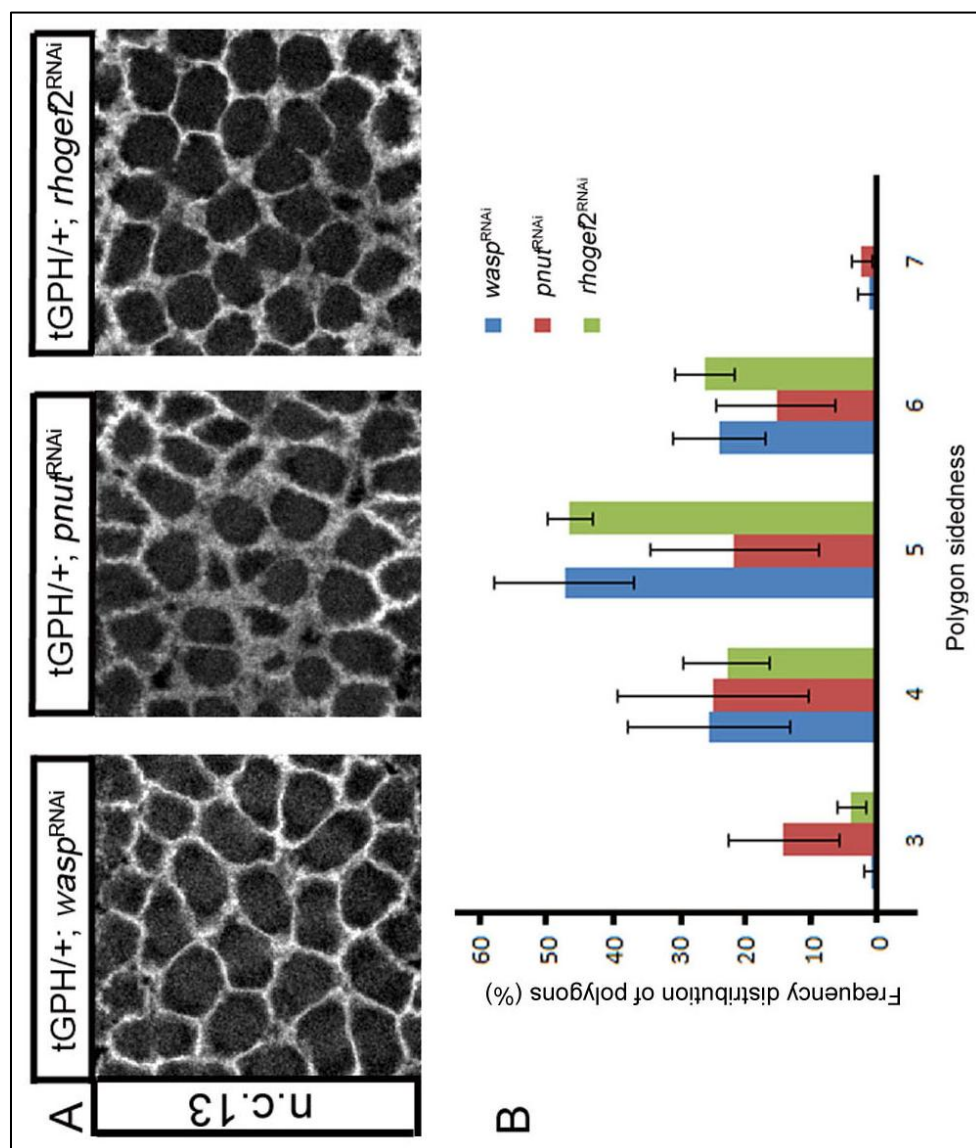
% wild-type	11	12	13	14
3	0	2.70 ± 0.33	0	0
4	69.57 ± 1.45	5.40 ± 0.33	4.04 ± 0.33	1 ± 0.67
5	30.43 ± 1.33	40.54 ± 0.05	25.25 ± 2.33	36 ± 2.64
6	0	43.24 ± 2.02	60.6	55.5 ± 7.02
7	0	8.11 ± 0.58	9.09	7.5 ± 1.15
8	0	0	1.01	0
% <i>synd</i>				
3	22.22 ± 0.67	6.25 ± 0.33	0	0.52 ± 0.33
4	44.44 ± 0.33	21.88 ± 0.88	9.76 ± 2.18	12.5 ± 3.51
5	11.11 ± 0.33	56.25 ± 1.15	32.93 ± 1.52	53.13 ± 1.52
6	22.22 ± 0.33	15.63 ± 0.33	32.93 ± 2.08	30.73 ± 5.48
7	0	0	32.93 ± 0.33	3.13 ± 1.00
8	0	0	32.93 ± 0.33	0

Figure 5.32. Syndapin is crucial for maintenance of conserved polygon distribution during syncytial nuclear cycles. Percentage frequency distribution of polygons in wild-type and *synd* mutant embryos during the syncytial nuclear cycles (n.c.) 11-13 (A) quantified from stills at metaphase from movies in the background of membrane marker tGPH (B). Comparison showing significant difference between percentage frequency distributions of 4-7-sided polygons in wild-type and *synd* mutant embryos during n.c. 13 (n=3, ***; $P < 0.001$, Non-parametric Mann-Whitney U test) (C). Table showing average values with standard deviation across 3 embryos used to plot the frequency distributions of polygons in (A) from wild-type and *synd* mutant embryos (D).

6.2.2 Proteins that affect actin assembly in the pathway with Syndapin also show a skewed polygon distribution

I also tested whether other proteins that we know function with Syndapin in actin assembly/organization affect the frequency distribution of polygons in the syncytium. This would confirm that Syndapin, actin and the actin assembly proteins work in the same pathway to order polygonal epithelia. Polygon distributions from embryos in n.c. 13 depleted of WASp, Pnut and RhoGEF2 were tested (Figure 5.2A-B). As expected, the frequency distribution of polygons was affected compared to wild-type. In *synd* there was a greater preponderance of 5-sided polygons than wild-type, but the number of hexagons was still dominant (though lower than wild-type). However, remarkably, unlike *synd*, depletion of Wasp, Pnut or RhoGEF2 caused the number of pentagons to increase dramatically along with a significant increase in 3-, 4- and 7-sided polygons, because of which the number of hexagons were no longer dominant (Figure 5.2A-B). This indicates that since WASp, Pnut and RhoGEF2 can directly influence actin assembly by regulating actin nucleation and stability, they are key downstream components in maintaining contractile tension while Syndapin is probably upstream in this process. Syndapin depletion also does not affect recruitment of junctional proteins that are responsible for maintenance of polarity in polarized epithelial cells like Armadillo (Arm, *Drosophila* orthologue of β -catenin, of the Cadherin-catenin complex that forms the Adherens junction), Discs large (Dlg, of the Scribble protein complex that forms the Septate junction), PALS1-associated tight junction protein (Patj, of the Crumbs protein complex that forms the Tight junction) or Bazooka (Baz, *Drosophila* orthologue of Par3 also of the Crumbs complex) (Figure 5.2C). So this could mean that Syndapin function is not crucial for actin-related function in junctional protein assembly that directly controls tension and consequent ordering of polygons in the epithelial

sheet. The exact organization and assembly of junctional proteins in during syncytial cycles is also unknown and merits further investigation since they are localized differently until cellularization and several of these proteins like Crumbs and aPKC are missing at this stage of development. However, further mechanisms that affect frequency of polygon distribution upon depletion of Syndapin and proteins that affect actin assembly need to be further investigated by quantifying tension parameters using biophysical techniques like laser ablations and by specifically disrupting junctional proteins in the background of *synd* to study their epistatic relationship with respect to polygonal organization.



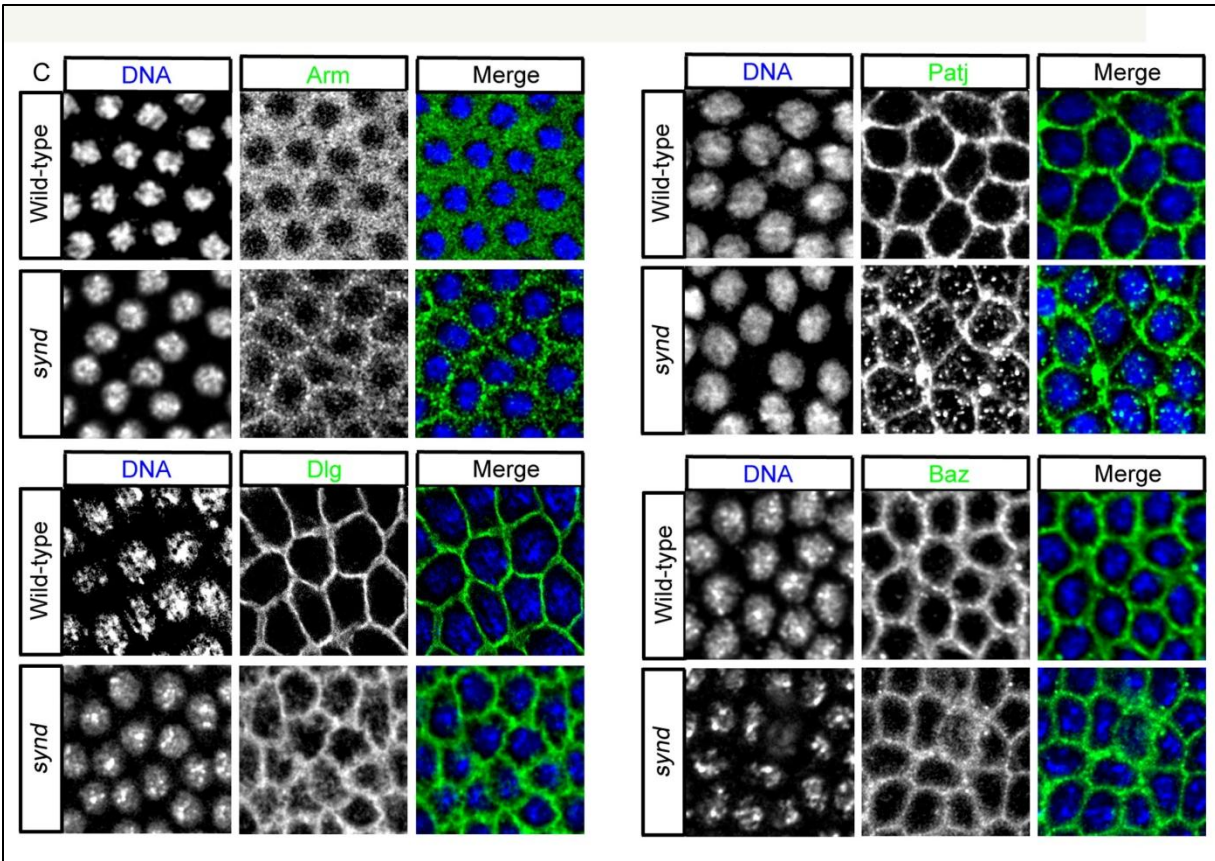


Figure 5.33. Proteins that affect actin assembly in the pathway with Syndapin also show a skewed polygon distribution. Unlike in *synd*, Pnut-, WASp- and RhoGEF2-depletion causes 5-sided polygons to dominate in n.c. 13 (A) which is quantified in the frequency histogram (B) (n=3). Recruitment of polarity proteins Arm, Patj, Dlg and Baz are not affected in *synd* mutant embryos (C).

6.2.3 *synd* mutants show defects in body patterning during final stages of embryonic development

Since regular cellular packing have been hypothesised to be important for establishing precise morphogen gradients (Bollenbach et al., 2008; Kavousanakis et al., 2010; Sample & Shvartsman, 2010; Sherlekar & Rikhy, 2012), we looked for defects in body-axis patterning. To look for patterning defects as a result of early problems in signalling activity, I performed cuticle preps of wild-type and mutant embryos at 22 hrs after egg laying (just prior to hatching) and observed several phenotypes – defective head and tail segments, mild ventralization, merged and missing abdominal denticle bands (Figure 5.3A) quantified in the table (Figure 5.3B). In the syncytial embryo, patterning of the body axes are established by morphogen gradients: Bicoid is important

for determination of the embryo anterior and its gradient decays along the antero-posterior axis, Toll/Dorsal signal to establish the dorso-ventral axis while EGFR/Torso signalling is crucial for patterning the embryo both along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes (Sherlekar and Rikhy, 2012). Thus, defects observed in patterning of the A-P and D-V axes in the cuticle preps of *synd* embryos are a result of early morphogen patterning defects that depend on the membrane-cytoskeletal architecture of the embryo. However, the details of exact nature of these defects including the cellular processes and signalling pathways that govern them merit further investigation.

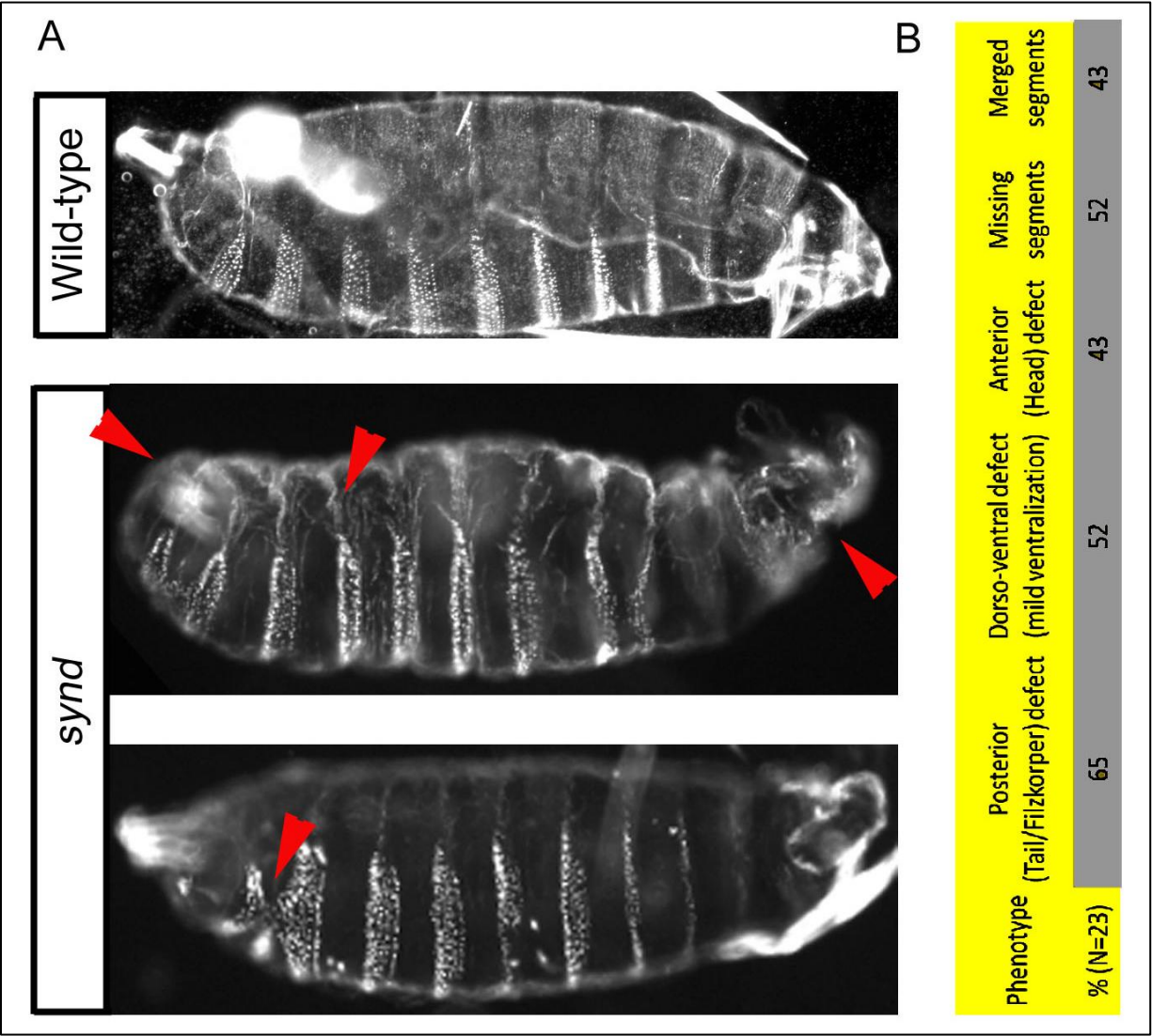


Figure 5.34. Cuticle preps of wild-type and *synd* mutant embryos showing various patterning defects (see arrows) (A). Table showing quantification of observed phenotypes (B).

6.3 Discussion

Our work lends credence to the model by Koke et al., (2014) described above that suggests that in wild-type embryos, when expanding apical actin caps meet during each nuclear cycle, each time, ordering of the polygonal array requires repulsive forces between interacting actin networks which is governed by actin turnover and increasing membrane tension. In Syndapin-depleted embryos, we know that there is loose/floppy membrane in the furrows (Sherlekar & Rikhy, 2016, see Chapter 3). Therefore, the lack of membrane tension as well as poor actin organization results in a ‘soft’ network with a skewed frequency distribution of polygonal classes where the number of hexagons is diminished in favour of pentagons. Other work from the lab has shown that when proteins that form a part of junctional complexes in polarized epithelial cells are depleted like DE-Cadherin or Bazooka, there is a similar shift in the polygonal distribution (unpublished data) as was seen in *synd* mutants. But, surprisingly, all of these polarity proteins including Dlg and Patj were recruited to the membrane in *synd* mutant embryos. This suggests that although in both cases, actin organization is affected, their pathways may not converge and different pools of actin may be involved. Another possibility is that Syndapin functions downstream to these proteins and further investigation is necessary to distinguish between these scenarios.

We found, unexpectedly, that when any of the actin assembly proteins like N-WASp, RhoGEF2 and Pnut are depleted, the polygon distribution defect is much worse than that observed in *synd* mutants. Thus, it is likely that proteins that regulate actin organization directly influence tension caused by the actin cytoskeleton and, as a consequence, the polygon distribution and, probably, function downstream of Syndapin.

An exciting possibility was revealed to us by performing cuticle preps that reflect maintenance/disruption of early events during syncytial development – the establishment of morphogen gradients that govern body axis patterning and also formation of a pattern of denticle bands on the cuticle just prior to hatching. *synd* mutants showed head and tail defects and also defects in denticle band patterning, therefore, suggesting that early polygonal network assembly defects can have far-reaching consequences on the development of the organism. Most of this is still, however, speculative and requires intensive investigation.

7 Conclusions and Future Perspectives

Syndapins are uniquely versatile molecules that can bind and tubulate a variety of membrane curvatures. Syndapins can also interact with a variety of actin regulators and, unlike most other F-BAR proteins (other than PICK1), can directly interact with actin. We, therefore, chose to study the role of Syndapin in orchestrated dynamic remodelling of the membrane and actin cytoskeleton during rapid nuclear division cycles in the *Drosophila* syncytial blastoderm embryo.

Syndapin is crucial for pseudocleavage furrow dynamics

We have found a novel role for Syndapin in regulating the dynamics of syncytial furrows. We showed that Syndapin-depleted embryos fail to recruit septins Pnut and Sep1 to the membrane and also fail to enrich Dia at the furrow tips. We demonstrated that Syndapin functions to organize actin along the furrow membrane into long parallel structures; depletion of Syndapin results in shorter structures with ‘blobs’ of F-actin. Syndapin is able to rescue actin organization in the RhoGEF2-depleted background and restore furrow length. This also suggests a mechanism, other than direct actin interaction, by which Syndapin is able to regulate function of other components downstream of RhoGEF2 such as Rho1 to restore furrow dynamics and this remains to be investigated. Our work has, in addition, shown that Syndapin’s ability to rescue the furrow phenotype is most probably independent of its role in endocytosis. Syndapins are likely to promote furrow extension in other cellular contexts as well like during cytokinesis in metazoans (Takeda et al., 2013).

Syndapin is important to limit expansion of apical actin caps

The activity of the Arp2/3 complex to form dendritic actin networks that push against the plasma membrane is thought to drive apical cap expansion in syncytial *Drosophila* embryos. The process of apical cap expansion is probably accompanied by flattening of apical microvilli and expanded caps meet to form furrows. We showed that Syndapin-depleted embryos have abnormally large caps, similar to those seen in Myo-II-depleted embryos (Sommi et al., 2011). Depleting ArpC1 in the *synd* background rescued cap expansion defects. We also demonstrated that cap expansion is probably a consequence of remodelling of actin protrusions on the surface

of expanding caps, which is defective in both Syndapin- and Arp2/3 complex-depleted embryos. Syndapin directly binds to N-WASp whose function is to recruit Arp2/3 (Mark J Dayel & Mullins, 2004). It is possible that Syndapin antagonizes Arp2/3 complex function through this interaction; however, Syndapin depletion does not affect Arp3 recruitment to the membrane. Mammalian Syndapin2 is known to inhibit Rac1 that is responsible for cell spreading which would, in turn, promote Rho1 and finally Myo-II and Dia activity. These mechanisms merit further investigation in the context of syncytial embryo development.

Homeostasis of many epithelial tissues involves the addition of new cells from basally positioned progenitors; this involves an ill-studied process known as ‘apical emergence’ by which cells expand and integrate into the existing epithelium. This process was shown to be cell autonomous and depends on actin-driven pushing forces regulated by molecules like Dia, RhoA etc. (Sedzinski et al., 2016), these molecules are also involved in expansion of actin caps and furrow formation in the syncytium. It would be interesting to study the role of F-BAR proteins like Syndapin in regulation of the process of apical emergence that is conserved across all epithelial cell types.

Syndapin depletion results in a ‘soft’ network of polygons in the pseudoepithelial sheet

In wild-type embryos, when expanding apical actin caps meet during each nuclear cycle, ordering of the polygonal array requires repulsive forces between interacting actin networks which is governed by actin turnover and increasing line tension between opposing membranes. In Syndapin-depleted embryos, we know that there is loose/floppy membrane in the furrows therefore the lack of membrane tension as well as poor actin organization results in a ‘soft’ network with a skewed frequency distribution of polygonal classes where the number of hexagons is diminished in favour of pentagons.

An interesting result was that when any of the actin assembly proteins like N-WASp, RhoGEF2 and Pnut are depleted, the polygon distribution defect is much worse than that observed in *synd* mutants. It is therefore likely that proteins that regulate actin organization directly influence tension caused by the actin cytoskeleton and, as a consequence, the polygon distribution and could possibly function downstream of Syndapin.

Biophysical measurements using techniques like laser ablations will be the key to understanding the forces (or the lack of) at play at vertices in control versus mutant embryos that govern frequency distribution of polygon sidedness in the dividing pseudoepithelium.

As a consequence of defects in actin organization and membrane morphology in Syndapin mutants, there are also patterning defects in both A-P and D-V axes that we quantified from cuticle preps. Further studies on the influence of membrane and cytoskeletal architecture on morphogen gradient dynamics will help mechanistically elucidate Syndapin's role in developmental patterning.

Syndapin therefore functions in diverse processes that involve dynamic and coordinated remodelling of the membrane and cytoskeleton via remarkably conserved mechanisms.

8 APPENDICES

Appendix A

Loss of function mutants of Syndapin show defects in development

During development in *Drosophila*, polarity is established for the first time with oocyte selection and positioning at the posterior of the egg chamber (González-Reyes & St Johnston, 1994, 1998). The oocyte is localised posteriorly because it preferentially adheres to the posterior stalk cells due to up-regulation of DE-Cadherin in both these cell types (Godt & Tepass, 1998; González-Reyes & St Johnston, 1998). The nucleus randomly moves to either side of the oocyte – the future dorsal side is thus determined by this symmetry breaking event; the oocyte probably lacks dorso-ventral polarity prior to this. Gurken (*grk*) mRNA is targeted to the nucleus at the future dorsal side and Grk mediates EGFR signalling that specifies the posterior follicle cells. The nuclear repositioning is accompanied by a reversal of microtubule polarity – the posterior microtubule organising centre (MTOC) is replaced by one at the lateral and anterior cortex (Steinhauer & Kalderon, 2006). Anterior-posterior polarity in the oocyte is primarily due to transport and localisation of oskar mRNA to the posterior cortex and bicoid mRNA to the anterior cortex (Januschke et al., 2002). Conserved mediators of polarity – Par1 and Lgl – have been implicated in the establishment of polarity in the oocyte (Munro, 2006; Tian & Deng, 2008). Thus, polarity is established well before the *Drosophila* egg is laid.

During stage 6/7 of oogenesis, the EGFR (Epidermal Growth Factor Receptor) ligand Gurken is secreted and activates EGFR in the terminal follicle cells and thus causes induction of the posterior follicle cell (PFC) fate. During stage 8, the nucleus moves to an antero-dorsal position. Gurken signals to the EGFR receptor on the future dorsal follicle cells and delimits the ventral zone within the follicle cell epithelium by repressing Pipe expression in the dorsal region (Peri, Technau & Roth, 2002). When PFCs do not correctly differentiate, the oocyte nucleus does not migrate normally and gurken mRNA localisation is affected. In *synd* mutants, during oogenesis, PFCs which should be differentiated and polarised by stage 7, display a multilayering phenotype (Figure A1 A) and also lack polarity markers like aPKC (70%, n=10). Apart from this, the nucleus of the oocyte fails to migrate from the posterior to an anterior-dorsal location (Figure A1

C). This indicates a defect in EGFR pathway signalling as a consequence of which the microtubules fail to reverse polarity and transport the nucleus and the EGFR ligand Gurken to its new site for further signalling. This is reflected in head and tail defects observed in *synd* mutants shown previously in the embryo cuticle preps (Figure 6.3). We also found that some ring canals observed with phalloidin staining between adjacent nurse cells in the ovary were missing indicating possible cytokinetic defects (Figure A1 G). A study by de Kreuk et al., (2012) showed that Syndapin2 regulates EGFR internalization suggesting a mechanism by which these observable phenotypes may occur.

To assess whether defects in early patterning events in *synd* mutant ovaries can translate into long-term developmental defects in the embryo (Figure A1), we checked dorso-ventral patterning markers. Levels of the membrane receptor protein Toll is also lowered in *synd* mutants and may be more cytosolic (Figure A1 B) in 67% (n=12) of the embryos. Thus, we checked for levels of Dorsal in the ventral nuclei in the syncytial embryo but found no visible lowering of levels; the levels on the other hand may be increased. Strikingly, the Dorsal gradient in cellularization (and cycle 13) extends into nuclei on the dorsal half of the embryo both at the anterior and posterior poles (Figure A1 H). As shown earlier (Chapter 6; Figure 6.3), the embryo cuticle preps show ventralization defects that are likely to be a consequence of this spread of Dorsal from the ventral side of the embryo. Interestingly, the adult cuticle shows defects in melanisation of the ventral cuticle in the abdominal segments (Figure A1 E).

Thus, there are both AP and DV defects which indicate problems in various signalling pathways like EGFR, Toll and Notch. When *synd*^{RNAi} is driven by MS1096 and Sca-GAL4, wing and bristle morphology defects are observed respectively. Flies that eclose at 25°C have missing anterior cross-vein and slightly curled wings (Figure A1 F) as compared to control MS1096/+ with 50% penetrance. At 28°C, 100% of the flies show very defective, curled wings with both long veins and cross veins missing and an uneven wing margin (Figure A1 F).

In flies with *synd*^{RNAi} driven by Sca-GAL4, several scutellar and dorso-central macrochaete are missing in the adult compared to control Sca-GAL4/+ flies (Figure A1 D). In *synd* adult flies the bristles are shorter and the humeral bristle is missing compared to wild-type control (Figure A1 D). These observations also indicate defects in signalling pathways like Notch and EGFR.

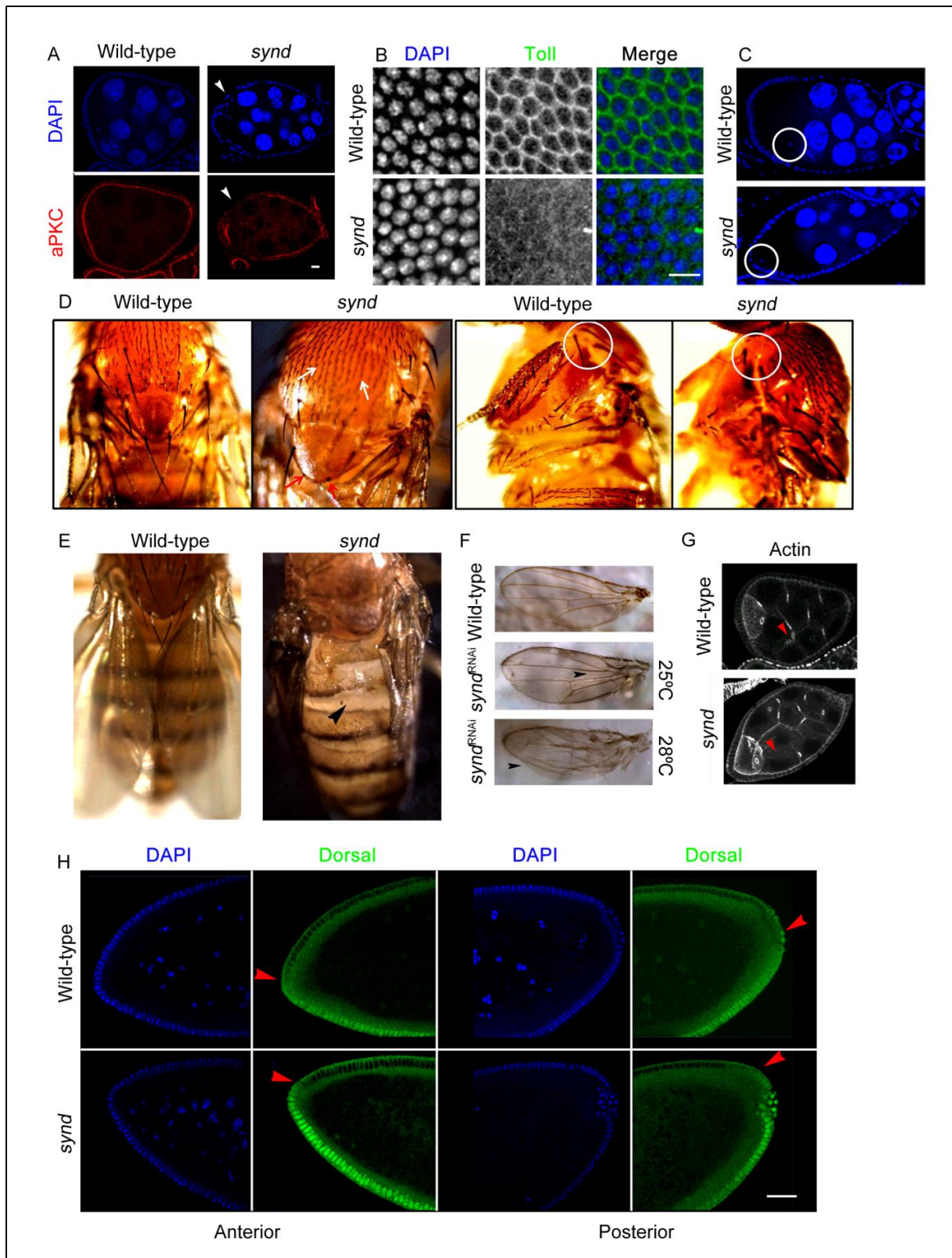


Figure A35. Various developmental phenotypes result from defects in signalling pathways in *synd* mutants at different stages of development. Ovaries from *synd* mutant escaper flies showed

multilayering of PFCs in stage 6/7. The regions where multilayering was observed, there was loss of apical polarity protein aPKC (white arrowheads, 70%, n=10, scale bar = 20 μ m) (A). Toll distribution was more cytosolic in *synd* mutant embryos compared to wild-type (67%, n=44, scale bar = 10 μ m) (B). In stage 7/8 ovaries from *synd* mutant escaper flies, there was a nuclear re-positioning defect (70%, n=15; white circle around the oocyte nuclei). Missing dorso-ventral (white arrows) and scutellar bristles (red arrows) as well as missing/short humeral bristles (white circles) in *synd* mutant escaper adults compared to wild-type (D) which also show decreased pigmentation in ventral abdominal cuticle (black arrowhead) (E). *synd*^{RNAi} adult wings showed missing anterior crossvein at 25°C (50%, n=70) and was curled and organization was completely disrupted at 28°C (100%, n=33) black arrowheads) (F). Some actin ring canals are missing in Synd-depleted ovaries (red arrowheads; 65%, n=15). The Dorsal gradient in the nuclei of *synd* mutant embryos spreads into the nuclei ventral side compared to the wild-type in cycle 14 both in the anterior (87%, n=30) and posterior (75%, n=30) of the embryo (red arrowheads) (H).

Appendix B

Epistasis analysis of actin-remodelling protein mutants and *synd* mutant embryos

Summary of protein distribution in mutant combinations							
Antibody, %phenotype	Syndapin	Synd::GFP	RhoGEF2	Dia	Anillin	Peanut	Actin
Genotype							
<i>synd^{Δ1}/Df</i>	Absent, 85%, n=40	-	Present, 91%, n=11	Uniform along furrow length, 90%, n=50	Present, 86%, n=44	Absent, 93%, n=44	Reveals loose membrane, reduced cytosolic puncta, 77%, n=33
<i>synd^{Δ1}/Df; Ub-Synd::GFP</i>	Rescue, 75%, n=16	100%, n=10	Not done	Rescue, 75%, n=16	Rescue, 70%, n=14	Rescue, 70%, n=14	Rescue, 75%, n=16
Wildtype	Present 97%, n=70	-	Present, 100%, n=20	Present 97%, n=50	Present 98%, n=45	Present, 98%, n=45	Present, 97%, n=50
Furrow initiation mutants							
<i>rhogef2^{Δ1}</i>	Absent, 88%, n=14	-	Absent, 100%, n=12	Reduced, 88%, n=14	Reduced, 100%, n=10	Absent, 100%, n=10	Reveals loose membrane, apical accumulation, 100%, n=14
<i>rhogef2^{RNAi}</i>	Absent, 88%, n=16	-	Absent, 75%, n=16	Reduced, 91%, n=22	Reduced, 100%, n=14	Reduced, 100%, n=14	Reveals loose membrane, apical accumulation, 100%, n=16
Ub-Synd::GFP; <i>rhogef2^{RNAi}</i>	Rescue, 50%, n=10	Present, 100%, n=6	Present, 50%, n=12	Rescue, 50%, n=10	Rescue, 34%, n=12	Rescue, 34%, n=12	Rescue, 50%, n=10
<i>dia⁵</i>	Absent, 100%, n=15	-	Present, 100%, n=12	Absent, 100%, n=15	Absent, 100%, n=15	Absent, 100%, n=13	Apical accumulation/in caps, 100%, n=15
<i>dia^{RNAi}</i>	Absent, 50%, n=20	-	Not done	Absent, 40%, n=20	Absent, 18%, n=20	Absent, 63%, n=16	Reveals loose membrane, apical accumulation, 40%, n=20
<i>dia⁵ +/-; synd^{Δ1}/Df</i>	Absent, 100%, n=10	-	Not done	Reduced, 100%, n=10	Reduced, 80%, n=11	Absent, 100%, n=11	Reveals loose membrane, apical accumulation, 100%, n=10
Furrow extension mutants							
<i>scrd⁸/scrd^{Δ8255}</i>	Absent, 100%, n=14	-	Absent, 80%, n=10	Reduced, 100%, n=11	Reduced, 60%, n=12	Absent, 100%, n=12	Apical accumulation, 75%, n=16
<i>pnut^{xp}</i>	Absent, 95%, n=22	-	Present, 100%, n=12	Reduced, 95%, n=22	Reduced, 100%, n=22	Absent, 100%, n=16	Reveals loose membrane, apical accumulation, 100%, n=11
<i>pnut^{RNAi}</i>	Reduced, 44%, n=18	-	Not done	Reduced, 20%, n=20	Reduced, 55%, n=18	Absent, 55%, n=18	Reveals loose membrane, 20%, n=20
<i>pnut^{xp} +/-; synd^{Δ1}/Df</i>	Absent, 91%, n=11	-	Not done	Reduced, 91%, n=11	Reduced, 57%, n=14	Absent, 57%, n=14	Apical accumulation, 89%, n=18

Table B1. Summary of actin-remodelling protein recruitment defects in mutant embryos. This table summarizes the distribution phenotypes of Synd, Rhogef2, Dia, Anillin, Pnut and F-actin in various mutant combinations shown in Figures 3, 4, 6 and S2. *synd*^{1d}/Df is used for Synd depletion. Rhogef2 is depleted in germline clones with allele *rhogef2*^{4.1} and using RNAi in *rhogef2*^{RNAi}. Dia is depleted in germline clones using *dia*⁵ allele and using RNAi in *dia*^{RNAi}. Pnut is depleted in germline clones with the *pnut*^{XP} allele. Anillin is depleted embryos containing the *scra*⁸/*scra*^{k08255} combination. n represents the numbers of syncytial embryos examined and % phenotype is calculated. *synd*, *pnut*, *rhogef2*, *anillin* and *dia* mutants show a loss of both Synd and Pnut.

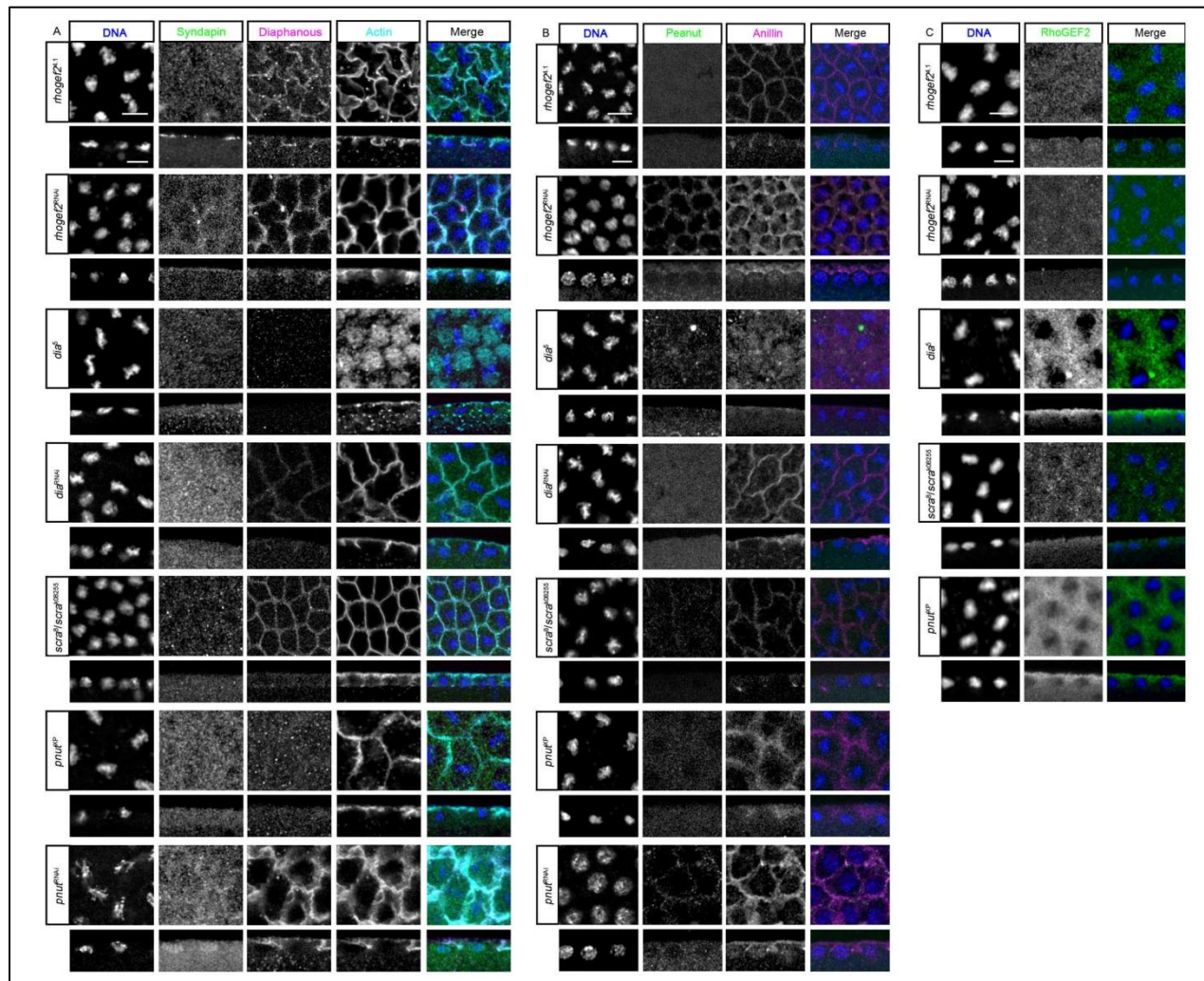


Figure B1. Characterization of *rhogef2*, *dia*, *anillin* and *pnut* mutant embryos. A-C. *rhogef2* mutants show RhoGEF2 loss (*rhogef2*^{4.1}; 100%, n=12 and *rhogef2*^{RNAi}; 75%, n=16) and *rhogef2*^{4.1} mutants show Pnut loss (*rhogef2*^{4.1}; 100%, n=10, *rhogef2*^{RNAi}; 100%, n=14) from the membrane and reduced distribution of Dia (88%, n=14), Anillin (*rhogef2*^{4.1}; 100%, n=10, *rhogef2*^{RNAi}; 100%, n=16) and F-actin accumulated apically (*rhogef2*^{4.1}; 100%, n=14, *rhogef2*^{RNAi}; 100%, n=16). *dia* mutants have decreased Dia (*dia*⁵; 100%,

n=15 and *dia*^{RNAi}; 40%, n=20), Anillin (*dia*⁵; 100%, n=15, *dia*^{RNAi}; 18%, n=20) and Pnut (*dia*⁵; 100%, n=13 and *dia*^{RNAi}; 63%, n=16); F-actin is accumulated apically or in caps (*dia*⁵; 100%, n=15, *dia*^{RNAi}; 40%, n=20). RhoGEF2 presence is unaffected (100%, n=12). *anillin* mutant *scra*⁸/*scra*^{k08255} shows loss of Anillin (60%, n=12), RhoGEF2 (80%, n=10) and Pnut (100%, n=12) and reduced Dia (100%, n=11) and apically accumulated F-actin (75%, n=16). *pnut* mutants show loss of Pnut (*pnut*^{XP}; 100%, n=11 and *pnut*^{RNAi}; 55%, n=18), Dia (*pnut*^{XP}; 90%, n=11; *pnut*^{RNAi}; 20%, n=20), and Anillin (*pnut*^{XP}; 99%, n=22; *pnut*^{RNAi}; 55%, n=18) and apically accumulated F-actin (*pnut*^{XP}; 100%, n=11 and *pnut*^{RNAi}; 20%, n=20); RhoGEF2 is unaffected in this mutant (100%, n=12). Scale Bar=10 μ m.

Appendix C

Summary of phenotypes observed upon Synd-depletion and overexpression of F-BAR and SH3 domains in embryos immunostained with actin and membrane regulatory proteins with possible functional relevance

Protein studied/ Mutant	Synd ^{1d}	Synd ^{F-Bar}	Synd ^{SH3}	Functional relevance
Syndapin	Lowered (77%, N=32)	Both membrane-bound and Cytosolic (75%, N=16)	More cytosolic, increased on membrane (91%, N=11)	F-BAR domain mediates membrane recruitment, SH3 domain is crucial for endocytosis
Peanut	Lowered (42%, N=44)	Lowered on membrane, more apical puncta (67%, N=33)	Lowered Peanut (88, N=14)	Syndapin is important for localization to furrows
Diaphanous	Patchy distribution (67%, N=25)	Increased, Both membrane- bound and Cytosolic (91%, N=22)	Increased on membrane (80%, N=20)	Syndapin is important for distribution along furrows
F-actin	Lowered, especially apical and puncta (65%, N=35)	Not visibly affected?	Not visibly affected?	Syndapin is important for remodelling of actin
Toll	Lowered from membrane (70%, N=44)	Increased, Membrane-bound, Cytosolic and Nuclear in interphase (88%, N=16)	Less cytosolic (60%, N=12)	Syndapin may be important for localisation to furrows
Rab5	Lowered (54%, N=13)	Increased cytosolic (88%, N=14)	Puncta stuck on the membrane (44%, N=18)	Syndapin has a crucial role in early endocytosis
Dlg	Present, patchy in places	Increased cytosolic (95%, N=22)	Increased membrane, also cytosolic (98%, N=45)	Suggests a regulation of endocytic and recycling processes

Table C1. Summary of phenotypes observed upon overexpression of the F-BAR (*synd*^{F-BAR}) and SH3 (*synd*^{SH3}) domains of Syndapin in comparison with full length depletion of Synd (*synd*^{1d}) stained with various actin and membrane remodelling proteins as well as some polarity proteins including the percentage of embryos in which it was observed and the possible functional relevance of the observed phenotypes.

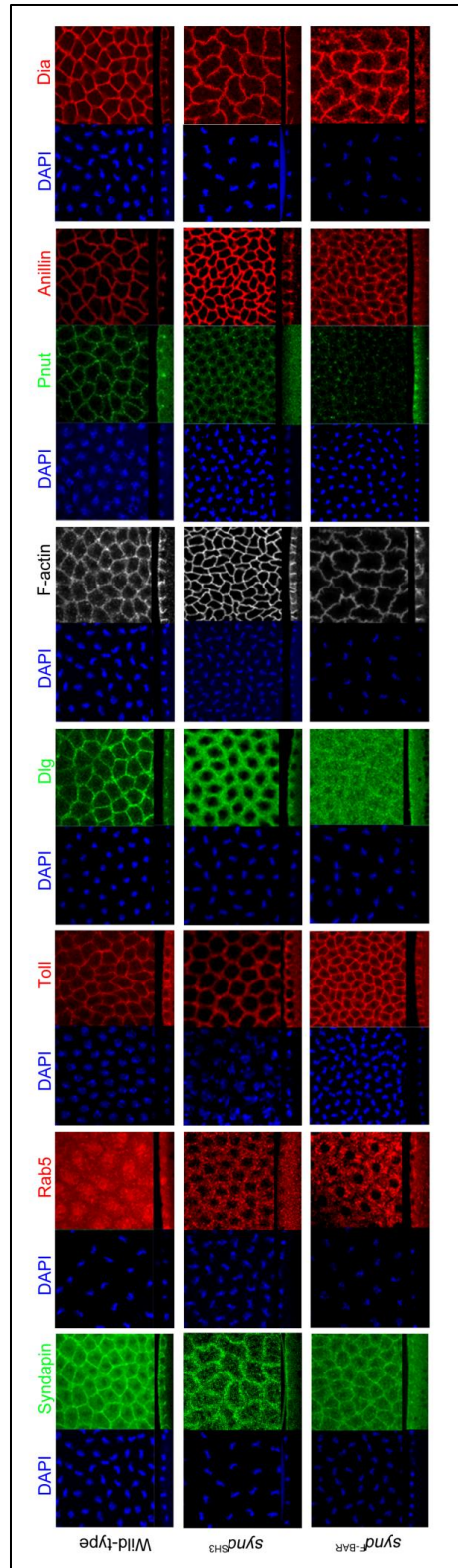


Figure C1. Montage of images of *synd*^{1d}, *synd*^{F-BAR} and *synd*^{SH3} embryos stained with the above mentioned proteins.

Appendix D

synd mutants affect Sep1 but not Sep2 distribution on the membrane

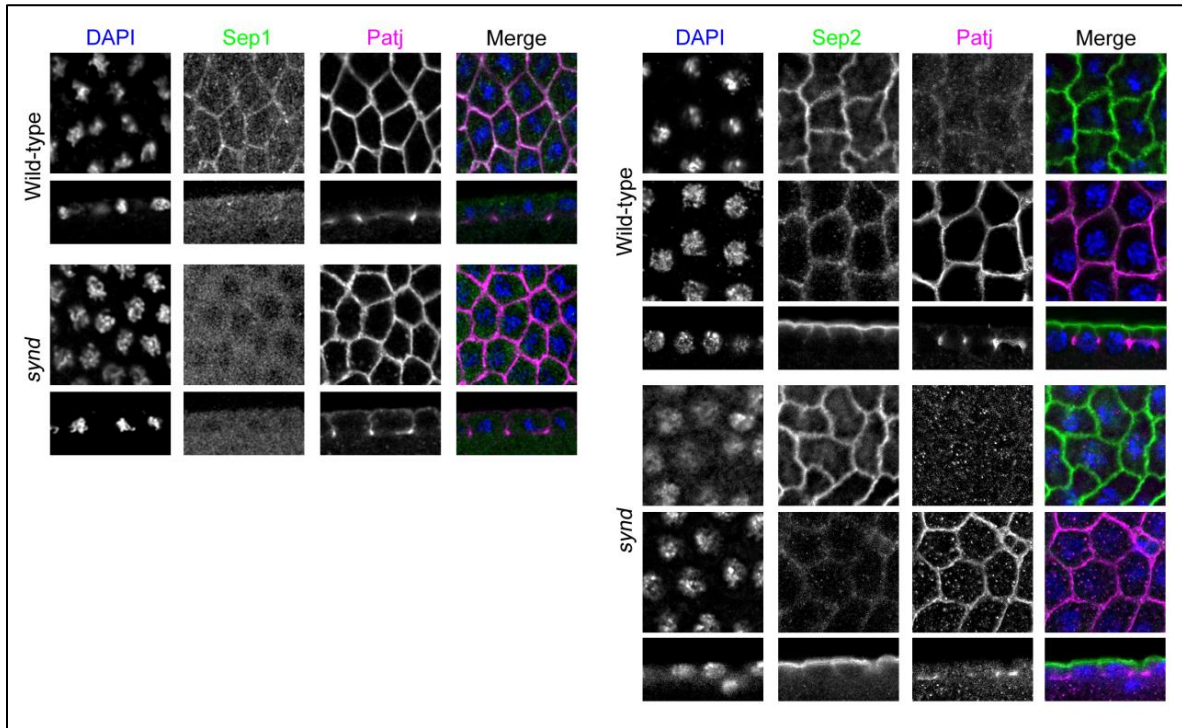


Figure D1. In *synd* mutant embryos, Sep1 levels are affected but Sep2 levels are comparable to wild-type. Syncytial *synd* and wild-type embryos immunostained with Sep1 and Sep2 antibody with Patj staining as control. Scale bar = 10 μ m.

Since septin Pnut levels were depleted in *synd* mutant embryos, we also checked whether other septin proteins are also affected. We found that Sep1 (Septin1) staining was lost, but Sep2 (Septin2) staining was not affected compared to wild-type. It has been reported in a previous study that Pnut and Sep1 exist in the same heterooligomeric complex and need to be in a complex to associate with the membrane, but Sep2 belongs to two different complexes; one with Sep1 and Pnut and the binding to the other complex (perhaps with Sep4 and Sep5) is sufficient to recruit it to the membrane (Adam, Pringle & Peifer, 2000). Interestingly, while Sep1 is mostly localized to the furrow tip like Pnut, Sep2 has predominantly apico-lateral localization.

Appendix E

Actin structures in *synd* apical caps show a slower mobility compared to control

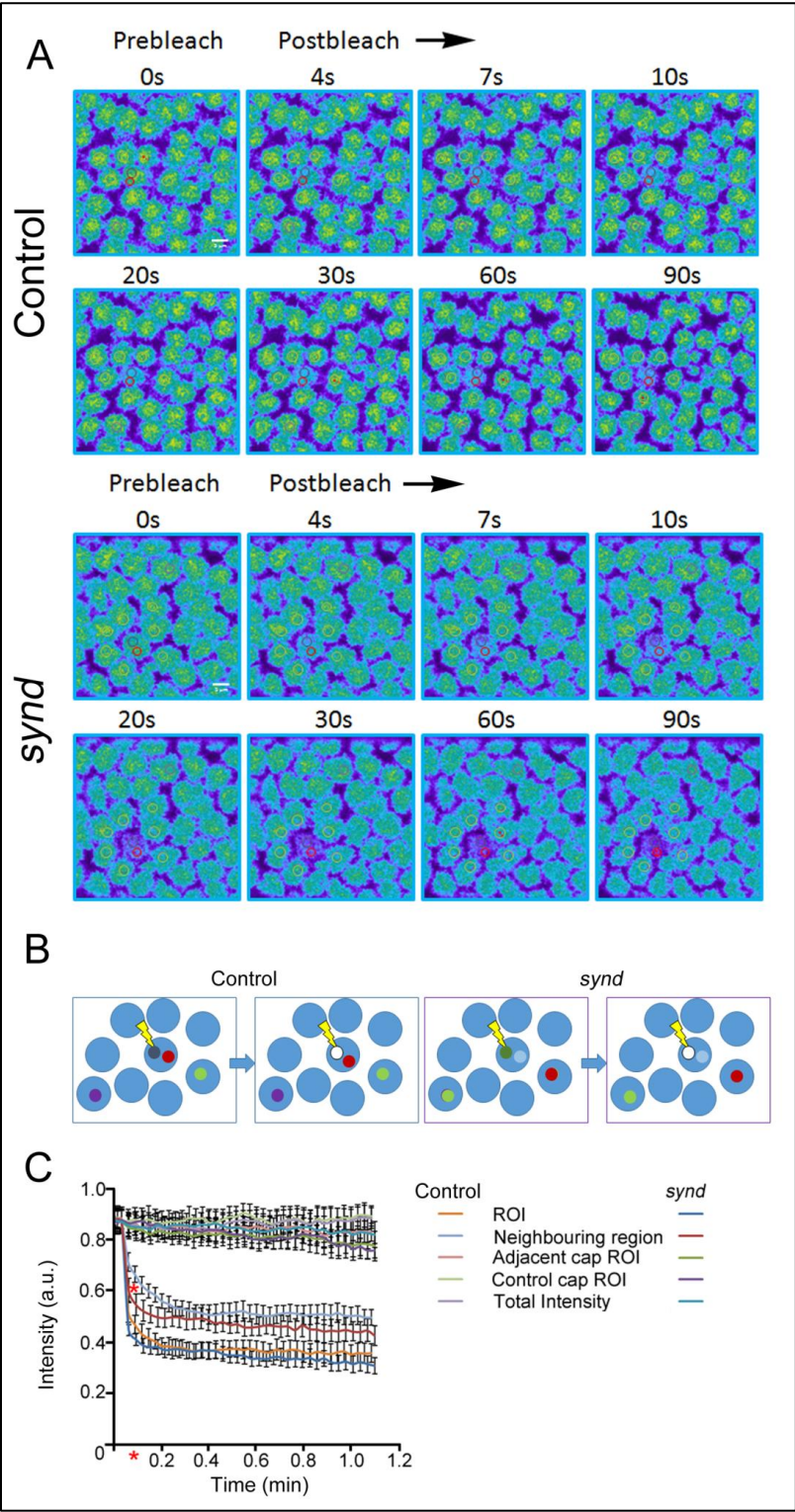


Figure E1. FLIP assay in apical actin caps of control and *synd* embryos. Still time lapse images from the FLIP assay in control and *synd* mutant showing colour coded ROI bleach and control regions. Scale Bar = 20 μm (A). Schematic of bleach experiments in control and *synd* embryo with colour coded ROI bleach and control regions (B). Graph showing normalized fluorescence intensity loss versus time (min) for control and *synd* embryos with colour key for ROI bleach and control regions. The red asterisk indicates the region during first 10s when the intensity loss in the neighbouring is slower in *synd* compared to control (C).

We saw in chapter 5 that apical actin protrusions in *synd* embryo caps did not shorten after cap expansion like they did in wild-type. To demonstrate that there was a defect in exchange of actin in the caps, we performed Fluorescence Loss in Photobleaching (FLIP) assay. We bleached one region (ROI) within the cap $\sim 5 \mu\text{m}^2$ and observed fluorescence intensity loss in this region and a neighbouring region within the same cap (Figure D1 A-B). We found that recovery in the neighbouring region during the first ~ 10 s is comparable to that in the ROI for the control (LifeAct-GFP/+) caps. However, for the *synd* (in LifeAct-GFP background) caps, in the first ~ 10 s, the normalized fluorescence intensity loss is much slower in the neighbouring region compared to the ROI (Figure D1 C). This supports our hypothesis that there is slower mobility of actin in the *synd* apical caps compared to control.

Appendix F

Rab11 levels are lowered in *synd* mutants compared to control

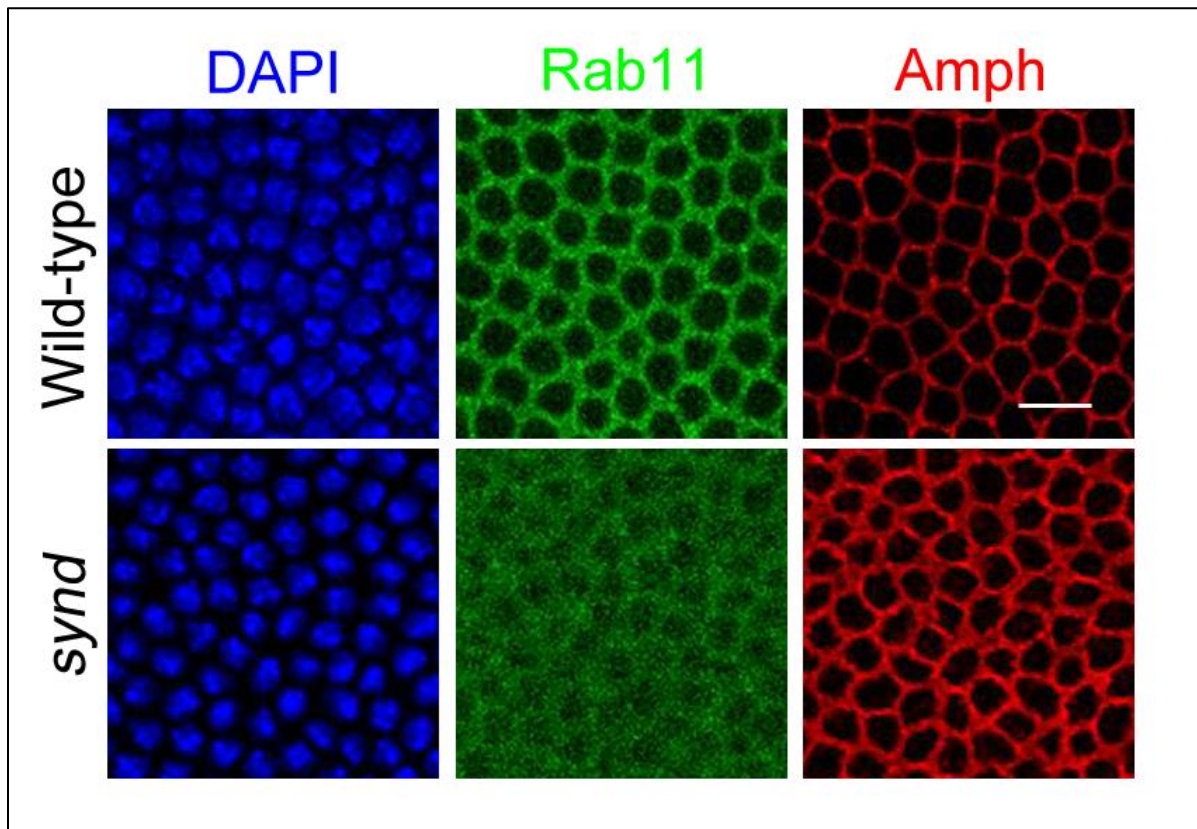


Figure F1. Rab11 levels are lowered in *synd* embryos compared to control. Embryos immunostained with Rab11 show decreased levels in *synd* mutants compared to control (60%, n=15). Amph staining used as control. Scale Bar = 10 μ m.

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