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Contribution of Ref(2)p to regulation of *Drosophila* notum epithelial cell apico-basal polarity and phenotype

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Abstract

Cell polarity impacts on the maintenance of cell shape, cell-cell junction integrity, and protrusions formation and dynamics. Further, polarity regulates cell movement, proliferation and differentiation. Conversely when cells lose their polarity they may be susceptible to dysfunction that may underlie degenerative disorders and tumour progression. Cell polarity and polarity protein complexes are highly conserved between different organisms from unicellular to multicellular, and from invertebrates to vertebrates. The focus of this study is the apico-basal polarity that is established normally in epithelial cells.

The protein p62 has been revealed to have a role in epithelial cells phenotypic alteration. It is considered as a multifunctional scaffold protein and acts as a signalling hub for different pathways, and through interactions with the polarity protein aPKC we hypothesise that it may regulate apico-basal polarity. Ref(2)p is the *Drosophila* homologue of p62 and using *Drosophila melanogaster* as a model system we investigated the effects of *Ref(2)p* mutation or overexpression on epithelial cell apico-basal polarity, cell shape and protrusion dynamics. Our data suggests that Ref(2)p is required to maintain normal cell size, cell-cell junction stability, and protrusion dynamics and formation. Both the PB1 and UBA domains of Ref(2)p were recognized to be essential for localizing aPKC apically. As a multifunctional protein, Ref(2)p showed a further role in cell division, chromosome segregation and tumour repression. *Ref(2)p* mutants, as well as *aPKC* mutants, showed a decrease in cell division rate and phenocopied a blebbing phenotype detected in *SCAR* mutant dividing cells. Mechanistically, these phenotypes are likely at least due to Ref(2)p's interaction with aPKC and on broader scale due to changes in Ref(2)p mediated autophagy on polarity proteins. Since levels of autophagic activity, mediated by Ref(2)p, have potential effects on polarity proteins levels, which affect apico-basal polarity, cell size and actin cytoskeleton organization.

List of publications

- Shohayeb, B., Georgiou, M. & Layfield, R. (2015), Ref(2)p, p62 homologue, has a further role in regulating apico-basal polarity: presentation abstract accepted in LINK'15 conference (Student-led Interdisciplinary Research Conference), held at the University of Nottingham on 20th of July, 2015.

- Shohayeb, B., Georgiou, M. & Layfeld, R. (2015), Contribution of Ref(2)p to regulation of *Drosophila* notum epithelial cell apico-basal polarity and phenotype: poster abstract accepted for publication in 78th Harden Conference: Protein Kinases in Health and Disease organized by UK Biomedical Society in Winchester on 16th of September, 2015.

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1. Introduction

1.1. Cell Polarity Overview

Cell polarity is an essential characteristic in all cell types from single-cell organisms, such as bacteria, to multi-cellular organisms, such as *C. elegans*, *Drosophila* and mammalian cells [1]. The significance of cell polarity is apparent in controlling cell behaviour, outlining the asymmetry of cell shape, controlling the distribution of different proteins, and maintaining cell morphology. Cell polarity has an importance in cell migration, cell division, and cell differentiation [2, 3]. Investigations in mammalian cell culture revealed, that cell-cell contact and cell-extracellular matrix has a significant impact on maintaining cell polarity [4]. There are different types of polarity, however using *Drosophila* as a model the main focus of this study is on the apico-basal polarity that is basically established in epithelial cells. In epithelial tissue the cells are organized in a sheet like architecture, where neighbouring cells are attached to each other by junctions and each epithelial cell has an apical domain facing the luminal space or the external environment and basal domain facing extracellular matrix or the basement membrane [5].

Different model system studies suggest that there are evolutionary conserved polarity protein cassette complexes in different organisms. These complexes in *Drosophila* are the Crumbs (Cbs) complex including Cbs/Pals/Dlt at the apical domain, the Par complex Bazooka (Baz)/Par6/aPKC in the subapical domain, and the Scribble (Scrib) complex Scrib/ Disc Large (DLG)/Lethal Giant Larvae (Lgl) at the basal domain, and near to Septate Junction (SJ). The SJ starts to form a halfway through *Drosophila* embryogenesis however, mammalian cells do not have SJs [6]. The Par complex is central in cell polarity and junctional maintenance [7]. The difference in cell shape from one cell type to another and from one organism to another is an indication of different cell polarity mechanisms, however still the basic core mechanisms of polarity is conserved in all eukaryotic cells [1].

Generally, the Cbs and Par complexes localize to and establish the apical domain of the cell, while the Scrib complex localizes to and establishes the basolateral domain through a process known as 'mutual exclusion' [8]. Basically, aPKC prevents the apical localization of Lgl by its phosphorylation, since Lgl competes with Par3, Baz homologue in *Drosophila,* to form an independent complex with Par6/aPKC. Therefore,

this is an indication of the two complexes with mutual dependency in maintaining apico-basal polarity (Figure 1-E) [7, 9]. Studies of overexpressing Lgl elucidate the excess formation of Lgl/Par6/aPKC complex at the expense of Par3/Par6/aPKC, which apparently leads to suppression of epithelial junction formation, and thus an increase in apical domain area. In the context of cell polarity regulation, Par6/aPKC interacts with either Lgl or Par3 and this is regulated by aPKC phosphorylation activity [7].

Baz is essential in cell polarity maintenance. Baz interacts with Par6/aPKC and functions in promoting apical domain identity and the formation of cell-cell junctions. Baz localizes and is restricted at the Adherens Junctions (AJ) as being antagonized on the apical domain by Cbs, which is a transmembrane protein. Cbs competes with Baz for the PDZ domain region of Par6. Besides, aPKC antagonizes Baz by phosphorylation and once Baz becomes phosphorylated it cannot bind to aPKC. On the lateral domain Baz is inhibited by Par1 phosphorylation as well through Scrib regulation to keep it recruited at the junctions [10] (Figure 1-E). A study using *Drosophila* epithelial cells indicated that the Baz/Par6/aPKC complex is required for cell polarity initiation and functions in defining the apical domain. The apical signalling activation of Crb/Sdt/Dlt complex by Baz/Par6/aPKC complex counteracts the influence of Scrib/Lgl/DLG complex at the apical domain as Cbs complex inhibits Scrib complex effect apically [6]. Although aPKC showed no influence on Baz localization in early developmental stages and at the initiation of the polarity in the first instance in cellularization, still *aPKC* and *Par6* mutants showed failure in forming belt junctions at gastrulation and later developmental stages by Baz at AJ [5].

Genetic analysis in *Drosophila* revealed the functional and physical relationships between more than 30 polarity proteins involved in the maintenance and establishment of apico-basal polarity [11]. Par6 in the Par complex is essential for cell polarity as it co-localizes with activated Cdc42 GTPase, a key regulator in apico-basal polarity [12]. *Drosophila* Par6 localises above the AJ, however in mammalian cells exists at the tight junction (Figure 2-A-B) [5]. Par6 mediates transferring the cell polarity signal from Cdc42 to aPKC [13]. The GTPase Binding Domain (GBD) of Par6 inhibits aPKC enzymatic activity, until Cdc42 becomes in its GTP form (Figure 1-C), which in turn enhances the enzymatic activities of aPKC by repressing the inhibitory effect of Par6 on aPKC [14]. The local activity of Cdc42 is either inhibited by GTPase Activating Poteins (GAPs) such as Rich, or activated by Guanine Nucleotide Exchange Factors (GEFs) such as ECT2 [15]. Primarily, cell polarity is generated and regulated by extracellular stimuli, for instance, cell-cell contact receptors such as Cadherins, physical stress in integrins, and also chemotactic signals. These stimuli are regulated

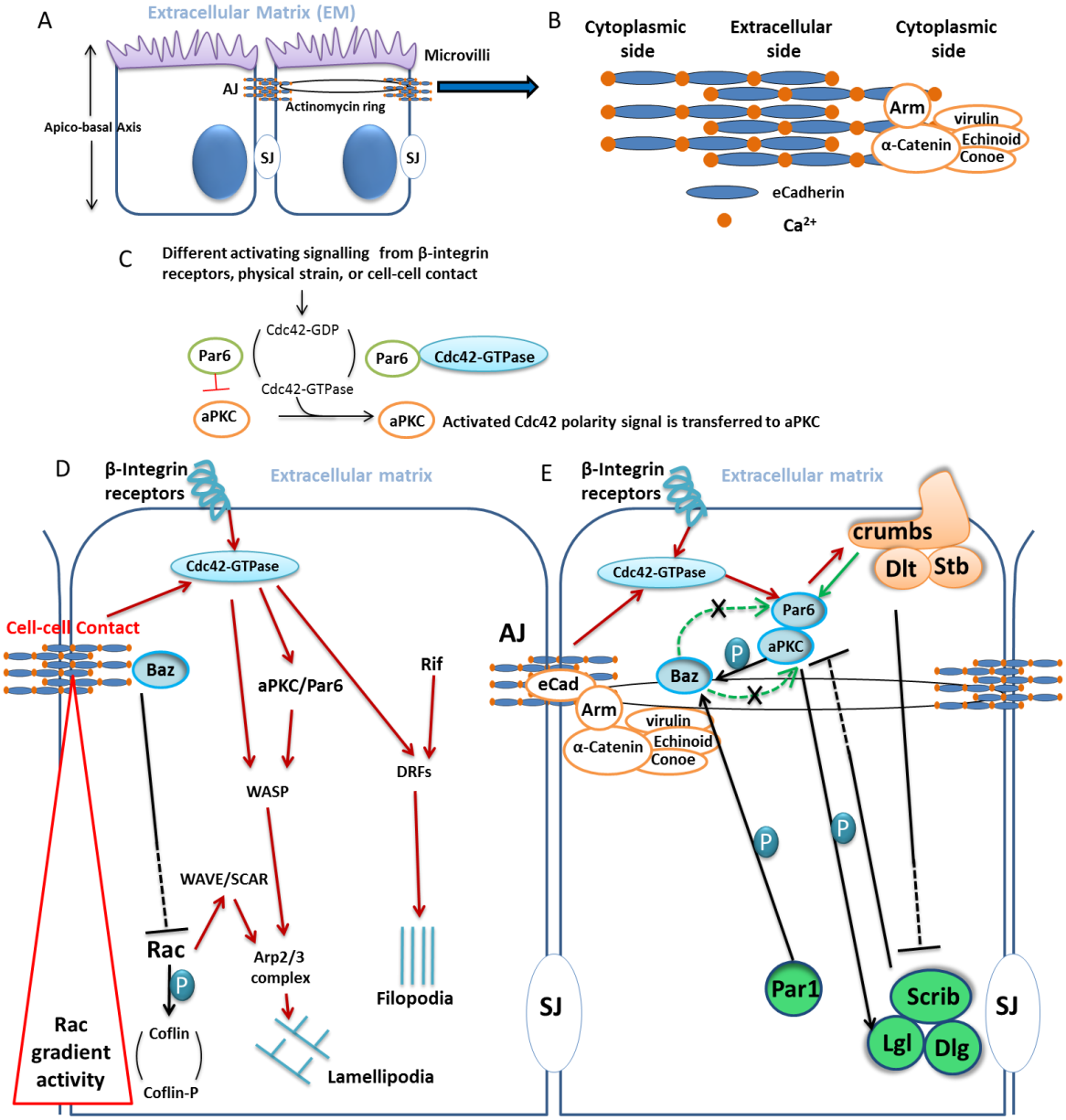


Figure 1: Schematic cell polarity diagram of epithelial cells

(A) shows the apico-basal axis of epithelial cells, indicating AJ and SJ position. (B) At AJ E-Cadherin exists and has a negatively charged motif that binds to Ca^{2+} and forms a rod-like template. These rod-like templates bind to each other homophilically. In the extracellular side E-Cadherin rods are opposing and interacting with each other. From the cytoplasmic side E-Cadherin interacts with Armadillo (Arm) with which α-Catenin interacts and forms the Cadherin-Catenin complex, to which F-actin binding proteins as virulin, echinoid, and Conoe binds. (C) shows how the polarity signalling transferred from Cdc42 after activation to aPKC by repressing the inhibitory effect of GBD domain of Par6 on aPKC. (D) Cdc42 Rho GTPase activity is required for protrusion formation. Cdc42 activates WASP directly or mediated by aPKC/Par6 then WASP activates Arp2/3 complex that contribute to criss-cross bundles formation of lamellipodia. Also, Rac activates Arp2/3 complex mediated by WAVE/SCAR. Rac further inhibits cofilin activity by phosphorylation as cofilin depolarizes F-actin. Baz localizes close to the junctions and according to unpublished work in Georgiou lab it inhibits Rac activity, thereby it generates Rac activity gradient along the apico-basal axis (personal communication). Cdc42 and Rif activate Diaphanous-Related Formins (DRFs) which then initiates filopodia formation in parallel actin bundles. (E) Once Cdc42 is activated from

extracellular signalling or cell-cell contact signals, it activates Par complex (Baz/Par6/aPKC) and Par complex activates Cbs complex. There is mutual antagonism between polarity complexes as the Cbs complex supresses the Scrib complex from localizing in the apical domain and the Scrib complex represses the Par complex from localizing at the basal domain. Baz is recruited to the junctions by phosphorylation from aPKC and Par1, as well as competitive binding of Cbs to PDZ domain with Par6 which prevents Baz form binding to Par6. Red arrows indicate activation signalling, Black arrows indicate phosphorylation, Green arrows indicate an interaction occurs and dashed lines indicate inhibitory action.

and mediated by Cdc42 and consequently lead to the recruitment and downstream activation of Cdc42 [16] (Figure 1-E), afterwards Cdc42 activates different polarity pathways and certainly the Par complex, with which Cdc42 interacts and transfers polarity signalling to aPKC for establishing apico-basal polarity, and then the mutual cross talk antagonism between polarity complexes is embarked.

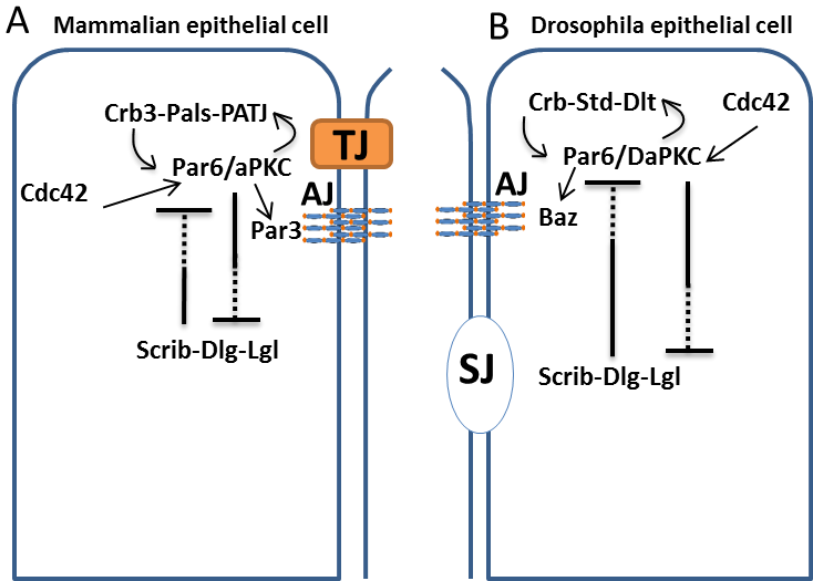


Figure 2: Differences between mammalian and *Drosophila* epithelia.

(A) shows the polarity complexes and proteins localization relatively from the tight junction and AJ in mammalian epithelial cells, whereas (B) shows the polarity complexes and proteins localization relatively from AJ and SJ in *Drosophila* epithelial cells.

1.2. Cell junctions mediate apico-basal cell polarity

The protein complexes processed by epithelial cells and required for the maintenance of apico-basal polarity are involved in cell junction formation as well. Cell junctions are initiated by cell-cell contact signals and activation of Rho GTPases. From the mammalian cells side, junctions include tight junctions, AJs, and desmosomes for preserving epithelial cells structure and polarity [1, 17]. Instead in *Drosophila* there are the subapical region, AJs and SJs [6]. AJs are localized apically on the lateral membrane. They connect adjacent epithelial cells together and define the apico-basal axis of the cells (Figure 1-A) [18]. AJs were described as the primary hallmark of epithelial cell polarity [5]. E-Cadherin, a member of the large Cadherin superfamily, is a transmembrane protein and a main component in the AJ. In mammalian cells there are at least 80 members of the Cadherin superfamily shown to be expressed in a

single cell, on the contrary there are only 6 members in *Drosophila*. The extracellular portion of E-Cadherin has negatively charged Ca^{2+} binding motifs [19], when Ca^{2+} is bound, forms a rod-like extracellular domain [17] and allows homophilic interactions between E-Cadherin molecules, either in cis or in trans [20]. The E-Cadherin cytoplasmic region binds to Arm (the β-Catenin orthologue in *Drosophila*). α-Catenin binds to Arm to form the Cadherin-Catenin complex that interacts with actin filaments and actin binding proteins, such as vinculin and nectin-afadin, which is an echinoid-canoe homologue in *Drosophila* [20]. The Cadherin-Catenin complex is the core of AJs and it is known to interact with a number of polarity proteins to maintain the integrity of cell junctions and polarity along the apico-basal cell axis (Figure 1-B) [21]. Moreover, the E-Cadherin cytoplasmic domain transduces signals established upon cell-cell contact to the nucleus and consequently it further regulates gene expression. Cdc24, Rho, and Rac are members of the Rho family, which is a part of the Ras superfamily of GTPases. These Rho GTPases play a fundamental role in the assembly of AJs in *Drosophila* and they have been shown to be regulated by E-Cadherin mediated initial cell-cell contact. Additionally, Rho small GTPases are considered to contribute to the stability of junctions by regulating actin filament assembly [22]. Cdc24, RhoA, and Rac alongside the E-Cadherin junctional complex and polarity protein complexes are orchestrating and significantly essential in maintaining cell polarity, stabilizing cell-cell junctions and regulating the actin cytoskeleton [23]. RhoA has an importance in maintaining cell-cell adhesion of E-Cadherin complexes through DRFs and non-muscle myosin II. The Myosin II molecule has been elucidated to have two separate isoforms and they regulate junctional integrity in different mechanistic ways. Myosin IIA enhances the homophilic adhesion of E-Cadherin; however myosin IIB contributes to junctional integrity by supporting the apical actin ring and actomyosin dynamics. This is apparently involved in maintaining the homeostasis and integrity of the junctions [23]. Upon Rac activation actin-based protrusions start to form, to establish a contact mediated by E-Cadherin between epithelial cells, and the other way round occurs as when the initial contact between neighbouring cells is being established, this enhances the formation of lamellipodia and/or filopodia [23]. Cdc42-Par6-aPKC has a significant role in AJ organization and stability as inhibition of any of *Cdc42-Par6-aPKC* affects the continuity of the junctions and results in ectopic junctional formation in epithelial cells of *Drosophila* notum. This Cdc42-Par6-aPKC regulatory role of the AJ is independent from their role in targeting Baz, Lgl, and Rac [24].

1.3. Cell polarity and actin cytoskeleton based protrusions

Extracellular signalling and cell-cell adhesion activate a number of Rho GTPase family molecules [25]. A general conservation of Rho GTPase family members exist in all eukaryotes. Besides regulating cell polarity Rho GTPases have a significant role in regulating cell movements and protrusion formation, through their influence on actin cytoskeleton filament polymerization, membrane trafficking and cell adhesion [26]. In different models as in *Drosophila* dorsal closure and *C. elegans* epidermal cell development, and cultured mouse keratinocytes or generally in mammalian cells; it is indicated that folipodia and/or lamellipodia protrude from cells and run into adjacent cells. This apparently shows the intimate implication of cell-cell contact and the Rho GTPase family, especially Rac and Cdc42, in protrusion formation and in polarizing actin filaments. 22 Rho GTPases are found in mammals, however only RhoA, Cdc42 and Rac1 are well studied. Extracellular laminin is correctly assembled in the presence of Rac, which then helps to control apico-basal axis orientation. Therefore, particularly Cdc42 and Rac are well studied and coordinate the perseverance of cell polarity intimately [22, 27]. Almost all members of the Rho GTPase family are found in the form of GTP as an active form or GDP as an inactive form. GEFs are responsible for the removal of GDP and allow its replacement with GTP; therefore Rho proteins become active after this replacement and activate downstream signalling effectors. In contrast, GAPs down regulate Rho proteins by GTP cleavage from Rho proteins and in turn Rho proteins become inactive [26]. Mammalian epithelial cells are characterized by dynamic actin filament based protrusions that are classified into microvilli, filopodia/ microspike and Lamellipodia. Interestingly even in invertebrate eukaryotes, such as *Drosophila,* it has been indicated that epithelial cells are not that simple, but they possess apparent different dynamic actin based protrusions along the apico-basal axis including microvilli in the apical domain, lateral sheet like lamellipodia protrusions in the intermediate level of the cells and eventually filopodia at the basal domain of the cells [26, 28]. Activated GTP form of Rac activates PAK, which is a serine/threonine kinase, and then later PAK activates Cofilin by phosphorylation, as Cofilin in its unphosphorylated from depolymerizes actin filaments (F-actin) in lamellipodia and filododia (Figure 1-D) [26].

One side of this study focuses on Ref(2)p, p62 homologue in mammalian cells, role in dynamic protrusions formation generated by actin polymerization including lamellipodia and filopodia in *Drosophila* notum. In lamellipodia the actin filaments are organized like a network or in criss-cross arrangements that end up with broad sheet like protrusions. Lamellipodia are suggested to be regulated by actin filament

nucleation factors such as the Arp2/3 complex. Arp2/3 complex is activated by Rac and/or Cdc42 through the WAVE/SCAR complex and WASP, nucleation promoting factors, respectively. Arp2/3 localizes at the front of the lamellipodia, polymerizes actin filaments and leads to the formation of new polymerization of actin branching from already formed actin filaments [26, 29]. In contrast to the network organization of actin filaments in lamellipodia, actin filaments are organized in long parallel tight bundles in filopodia, as finger-like shaped protrusions. Filopodia have a significant role in sensing other cells or soluble signals in the extracellular environment. Additionally, Cdc42 alongside Rif is the major regulator of filopodia extension by activating DRFs, however Cdc42 null fibroblastoid cells still maintain formation of cell filopodial protrusions, so there are other mediators that substitute the loss of Cdc42 as Rif. Recent studies elucidated that DRFs are the major regulator of filopodia formation, since DRFs interacts with the barbed positively charged terminals of actin filaments and polymerize them into parallel bundles. Although, it has been suggested that filopodia are formed beyond lamellipodial boundaries as a convergent elongation, where there is an absence of the Arp2/3 complex in the periphery, lamellipodia are not essential for filopodia extension (Figure 1-D) [26, 29, 30]. Besides the pattern difference in actin filaments organization in lamellipodia and filopodia, filopodia elongates and extend continuously, whereas lamellipodia after short period of extension they become capped, so they are relatively shorter in comparison to filopodia. This is the reason why filopodia requires Ena/VASP family proteins that counteract the capping and terminating events in the cytoplasm and those family proteins concentrate in filopodial ends [30].

Mechanistically, it has been suggested that the formation of dynamic protrusions require the intimate regulatory corporation between Rho GTPases and apical polarity proteins. The apical polarity proteins interact with Rac and Cdc42 to enhance the formation of two classes of overall three classes of dynamic protrusions along the baso-lateral domain, which include lamellipodia and filopodia; but not microvilli which is basically on the apical surface [5, 23]. This Rac activity gradient along the apico-basal axis is antagonistically regulated by Cdc42-Par6-aPKC and Baz, where Cdc42-Par6-aPKC is responsible for actin filaments formation and protrusion dynamics by activating Rac with highest activity at the basal domain. In contrast Baz inhibits Rac activity by inhibiting Sif, which is a GEF and able to activate Rac GTPase. Sif is TIAM1 homologue in *Drosophila*. This inhibitory action of Baz is suggested to increase as moving up to the apical domain as Baz is recruited to the junctions [28]. It has been revealed that in different polarized cell systems ,Baz/Par3 binds and regulates the activity of Sif/TIAM [31]. Consequently, in epithelial cells of *Drosophila* notum

formation of filopodia along the entire apico-basal axis was apparently seen in Sif/TIAM1 overexpression or Baz/Par3 null mutant cells, and this accompanied with a decrease in the sheet-like protrusions [28]. Additionally, it has been suggested that Baz/Par3 may sequester Sif/TIAM1 to prevent its association with Rac. Recent unpublished work in the Georgiou lab has shown Rac activity exists in a gradient pattern as being highly active at the basal domain therefore induces the formation of finger like protrusions filopodia at the basal domain of epithelial cells; however the level of Rac activity becomes lower while moving up to the apical domain, where preferentially sheet like protrusions are formed (personal communication). This role of Baz in regulating protrusions is apart from Baz role in Par complex in outlining and determining the apical domain of epithelial cells [28, 32]. Thus, any change in polarity determinants that can act as modifiers and influence Baz/Par3 subapical localization, will affect Rac activity gradient and influence filopodia or/and lamellipodia formation (Figure 1-D). Interestingly, Ref(2)p is implicated in protrusion formation in *Drosophila* haemocytes and mouse macrophages. This is significantly important for the spreading of haemocytes in epidermal wounds in *Drosophila* [33], however at the molecular mechanistic level it is not known how is that regulated. This study will investigate mechanistically how Ref(2)p significant in protrusions formation, which will be discussed later.

1.4. Ref(2)p *Drosophila* Homologue of p62

Early investigations established on *Ref(2)p* (refractory to sigma p) revealed that *Ref(2)p* is a polymorphic locus and was named after sigma rahbdovirus for its effect on the virus multiplication. The *Ref(2)p* locus is located on the left arm of chromosome 2 in the *Drosophila* genome [34]. As a result of sigma viral multiplication in dipterans nervous system, an irreversible paralysis occurs upon exposure to high concentration of carbon dioxide [35]. Further investigations were performed in this area and showed that *Ref(2)p* exists in a number of allelic forms occurring naturally in *Drosophila*. Those alleles are classified by comparing their influence on sigma virus multiplication in different strains into permissive Ref(2)p^{o} and restrictive Ref(2)p^{p}. Only the restrictive from of Ref(2)p has the ability to control sigma virus infection [34]. It has been shown that Ref(2)p interacts with sigma virus capsid P protein and Ref(2)p shares a common epitope with N viral protein, however at the molecular level it is not known how Ref(2)p controls sigma virus multiplication [36]. It has been shown experimentally that p62, the Ref(2)p homologue in mammalian cells, interacts with capsid viral protein, to drive it into autophagosome [37]. Recently, Ref(2)p has been detected in rod-like aggregates in the fly egg chamber, which are suggested to be

aggregates of viruses or bacteria and Ref(2)p might control sigma virus multiplication through targeting it to autophagy [38].

Nonsense mutations in the *Ref(2)p* gene following diepoxybutane exposure results in the occurrence of several mutations according to the loss in the exons. The nonsense mutation in the permissive allele *Ref(2)p^{o}* includes *Ref(2)p^{od2}*, and *Ref(2)p^{od3}*. The *Ref(2)p^{od3}* mutation leads to a premature stop and a loss of part of exon 2 and all of exon 3, which consequently lacks the ubiquitin-associated (UBA) domain at the C-terminal of the protein. Ref(2)p^{od2} has a deletion in the first 80 amino acids and lacks the Phox and Bem1p domain (PB1) domain localized in exon 1, at the N-terminal of Ref(2)p. They are all considered non-functional in terms of controlling sigma virus multiplication [35, 39]. Although, *Ref(2)p^{o}* flies are homozygous are viable, males are sterile [35]. At the molecular level there is no significant clear explanation of the sterility condition in males, yet electron microscopy imaging showed degeneration and myelin figures around spermatids in homozygous permissive alleles including *Ref(2)p^{od3}* and *Ref(2)p^{od2}*. Also, a significant variation in mitochondrial sizes was observed that appeared degenerated [35]. The study here will focus on *Ref(2)p^{od2}* and *Ref(2)p^{od3}* mutants in another research aspect, namely that of cell polarity.

According to a search done on Flybase, Ref(2)p indicated a general similarity in overall structural domains of human p62/sequestosome-1, as p62 is considered as the mammalian orthologue of Ref(2)p in *Drosophila* [40]. Human p62 (Figure 3-A') has 440 amino acids. This multi-functional scaffold protein has a number of structural domains and functional motifs and is considered as a signalling hub in a variety of cellular pathways. In the N-terminus of p62 it has the PB1 domain, that allows p62 to oligomerise with itself, and mediates interactions with different protein kinases including MEKK3, MEK5, ERK, and atypical protein kinase C including PKCζ, and PKCλ/ι [41, 42]. Then, there is the ZZ zinc finger domain to which receptor-interacting serine-threonine kinase 1 (RIP1) binds. Those are followed by the TRAF6-binding domain, LC3-interacting region (LIR) motif, and Keap1 interacting region (KIR) motif to which Keap1 binds, as well as UBA domain in the C-terminus. LC3-interacting region (LIR) is the motif to which LC3 binds on the phagophore to form the autophagosome in autophagy process, which is a protective mechanism processed by the cell to degrade undesired waste proteins and broken organelles. LC3 is the Atg8a homologue in *Drosophila* [42, 43]. In autophagy p62/Ref(2)p acts as a cargo for protein aggregates and binds to ubiquitylated proteins through the UBA domain and still mediate the initiation of autophagy by binding to the LC3/Atg8 on the inner member of the phagophore that later extend around protein aggregates and form an

autophagosome [41, 44]. Thereafter, the autophagosome fuses with a lysosome to from the autolysosome and degrade its contents. Additionally, p62 has been revealed to have a role in NF-kappa β signalling activation, and consequently p62 has a critical role in directing cell decision either to cell survival or cell apoptosis [42].

In *Drosophila*, Ref(2)p (Figure 3-A'') has 599 amino acids and a PB1 domain in its N-terminus followed by ZZ zinc finger and then a UBA domain in its C-terminus [45]. Also, it has been found that Ref(2)p interacts with the *Drosophila* TRAF6 homologue, dTRAF2, that plays a role in the toll-signalling pathway, which is similar to NF-kappa β signalling pathway in mammalian cells [40]. After analysing the conserved motif to which TRAF6 can bind across spices, Pro-x-Glu-x-x was assigned as a conserved motif in the TRAF6 binding domain (TB) (Figure 3-B) [46]. By using the COBLIT online alignment tool it has been revealed that this motif is conserved in *Drosophila* as well between amino acids 283-288. Bioinformatics analysis performed previously showed that Ref(2)p has a putative LIR motif between amino acids 451-458 and also a putative KIR motif between amino acids 484-496, however further investigation of these putative motifs should be performed experimentally [38]. The similarity between the human p62 and Ref(2)p in amino acid sequence is 23.9% with 17.9% identity [40], however as explained previously there is a similarity in structural and functional domains that are highly conserved between both proteins including the PB1 domain, ZZ zinc finger, TB domain, LIR and KIR motifs, and lastly the UBA domain (Figure 3-A'-A'').

Interestingly, Ref(2)p in *Drosophila* interacts physically with aPKC as does p62 with aPKC in mammalian cells in activating NF-kappa β and we hypothesize that this interaction might influence apico-basal polarity. This shows the high conservation of p62-aPKC signalling [40]. *Drosophila* has single orthologues, which means it has only one isoform of p62 and aPKC that makes it an ideal and simple model for studying p62/aPKC signalling pathways [38, 40]. This study will investigate the role of Ref(2)p in regulating the apico-basal polarity of epitheial cells using *Drosophila melanogester* as a model system.

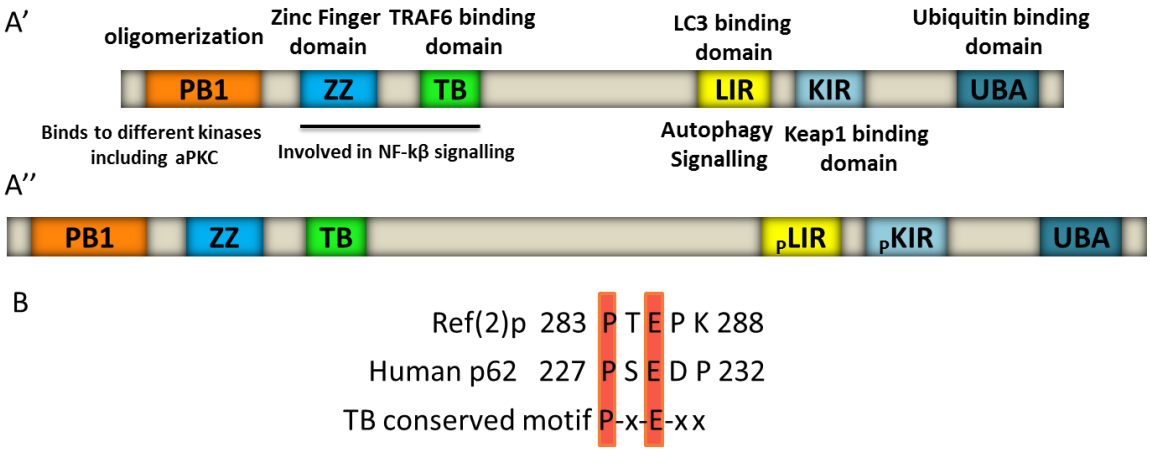


Figure 3: Ref(2)p homology with p62

Schematic diagram shows the high conserved homology between human p62 (A') and Ref(2)p (A'') domains and motifs. TRAF6 binding motif conserved in *Drosophila* Ref(2)p, which was analysed using COBLIT online alignment tool (B).

2. Aims and Objectives

- Investigate Ref(2)p's role in maintaining *Drosophila* epithelial cell phenotypes in regard to apico-basal polarity by examining different Ref(2)p mutants and expression levels.

- Investigate the role of Ref(2)p mediated autophagy in *Drosophila* epithelial cell apico-basal polarity.

- Provide a mechanistic explanation of Ref(2)p phenotypes observed in *Drosophila* epithelial cells.

3. Methods

3.1. Genetic Manipulation

Making the most use of *Drosophila* genetics we were able to study cell polarity in fly notum in relation to *Ref(2)p* mutants *od2* and *od3* [35, 39], a UAS-RNAi construct against *Ref(2)p* (VDRC,108193), and a UAS-mCherry-*Ref(2)p* construct (I. Nezis) [45] (see appendix 1, Figure 22). Homozygous *Ref(2)p* mutant flies were generated to study the impact of these mutations on cell shape, junction stability and polarity proteins. Gal4 drivers, used to drive expression of UAS constructs included Pannier-Gal4 (Pnr-Gal4) and Neuralized-Gal4 (Neu-Gal4). These drive expression in specific cells within the fly notum: Pnr-Gal4 drives expression in a central stripe on the back of the fly [47]; Neu-Gal4 drives expression specifically within the progenitor cell of the external sensory organ, the pI cell, and its progeny [48]. To analyse protrusion dynamics and cell morphology, individual cells could be imaged using Neu-Gal4 to drive expression of the UAS-Moesin (moe)-GFP construct [28]. Neu-positive cells are not yet committed to a progenitor cell fate, and therefore are representative of all epithelial cells in the notum [49]. Moe-GFP consists of the actin binding domain of Moesin fused to GFP, thereby targeting GFP to the actin cytoskeleton [50].

Pnr-Gal4 was used to drive the expression of UAS-RNAi constructs of *Ref(2)p* and *Atg1* (Bloomington), as well as overexpressed mCherry *Ref(2)p* and *Atg8a* (Bloomington) under the control of UAS promotor. Additionally, homozygous *Ref(2)P* mutant flies including *od2*, that lacks PB1 domain, and *od3*, that lacks UBA domain, were used. The w^{1118} stock (Bloomington) was used as a wild type control. Ubx-FLP [24], with ubi-GFP [51], was used to generate negatively marked clones for *aPKC* mutants. In contrast mosaic analysis with a repressible marker (MARCM) system with pnr-Gal4-meo-GFP could be used to generate positively marked clones of mutant cells with RNAi constructs of *Ref(2)p* in a background of wild tye cells, however MARCM and Pnr-Gal4 were used to generate clones for mCherry flag-tagged *Ref(2)p*, those flies were dissected and immunostained. Pnr-Gal4-UAS-moe-GFP and Ubi-His-RFP (Georgiou lab) were used in homozygous files of *Ref(2)p* mutants including *od2* and *od3*, and *aPKC* null flies besides wild type flies to analyse cell division rate and other phenotypes might be observed. Ubi-GFP-E-Cadherin files [24] were used to study the localization of Ref(2)p along the apico-basal axis by crossing them to UAS-mCherry flag-tagged *Ref(2)p* flies. To magnify the signals in western blotting, Act5C-Gal4 flies were crossed with UAS-RNAi construct flies of *Ref(2)p* and UAS-mCherry flag-tagged overexpressing *Ref(2)p* flies, as Act5C-Gal4 drives a global expression of genetic constructs under the UAS promotor [52] (See the full stocks genotypes in Table 3 and genetic constructs details in Table 4 used in this study in Appendix 3).

3.1.1. Gal4/UAS system

Gal4/UAS system (Figure 4) was first described in the yeast *Saccharomyces cerevisiae* and has a role in regulating gene expression, as it binds to the Upstream Activity Sequence (UAS). This system has been shown to regulate transgenes efficiently in *Drosophila*, since Gal4 acts as a transcriptional activator protein that identifies and binds to UAS followed by the transgene of interest. Consequently, in the presence of Gal4, the UAS transgene is expressed, such as a GFP reporter [52]. Gal4 expression can be under the control of different drivers, such as Pnr [47], Neu [48] and Act5C [52].

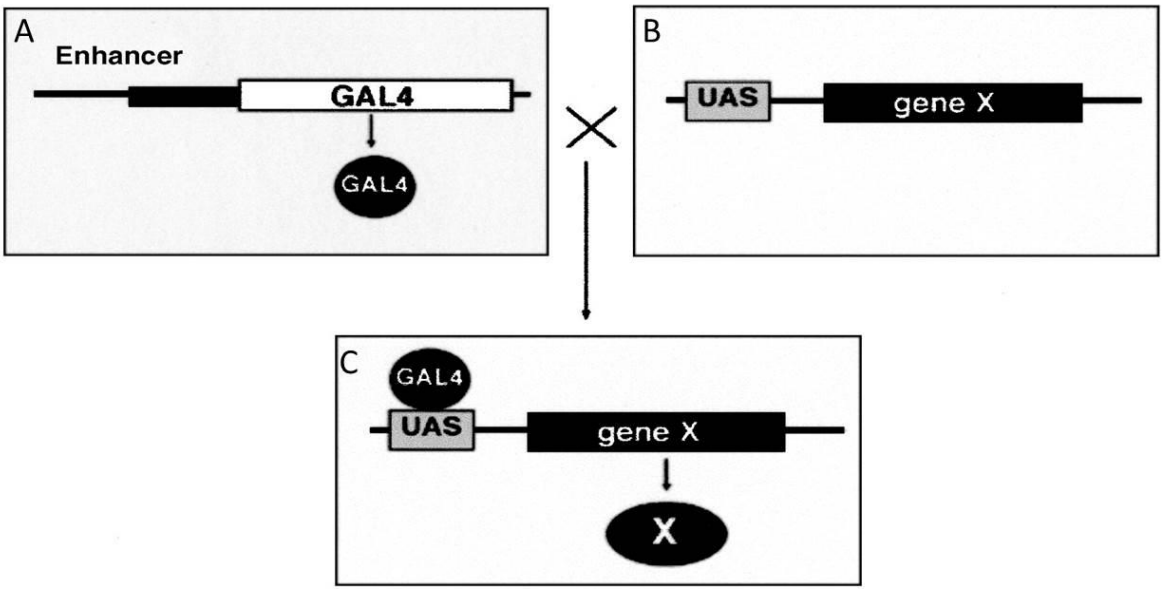


Figure 4: Gal4/UAS system

A genetically modified female fly (A) carrying the Gal4 driver under the control of an enhancer or a promotor crossed to (B) a male fly that carries the transgene of interest downstream of a UAS sequence. (C) The progeny has the Gal4 protein that activates the expression of the transgene under the control of UAS promotor [53].

3.1.2. Flp/FRT system

For manipulating *Drosophila* genetic background the Flippase (Flp)/ Flippase Recognition Target (FRT) system could be used to generate mitotic clones and it is site-specific recombination sequence. Flp has been identified in yeast *Saccharomyces cerevisiae*. Post-mitotic recombination takes place by the induction of Flp at a site-specific identical FRT region on homologous chromosomes. After the formation of recombinant chromosome arms they are segregated into two daughter cells. Eventually, one daughter cell represents a wild type cell with homozygous wild type alleles and the other daughter cell represents a mutant cell with two homozygous mutant alleles. These mutant cells could be positively or negatively marked (see below Figure 5). In our experiments the Flp enzyme was expressed either under the control of the

Ultrabithorax promotor (Ubx-Flp, limits expression of Flp to the fly notum) or a heat shock promoter (hs-Flp). The FRT sites used in this study are FRT13G, FRT40A and FRT19A [51]. The numbers indicated with FRT represent the location of these FRT sites on the chromosome arm. Flp/FRT is the most suitable and safer technique for enhancing specific sites of recombination in chromosome arms in comparison to other techniques, such as exposing files to ionizing radiation [54].

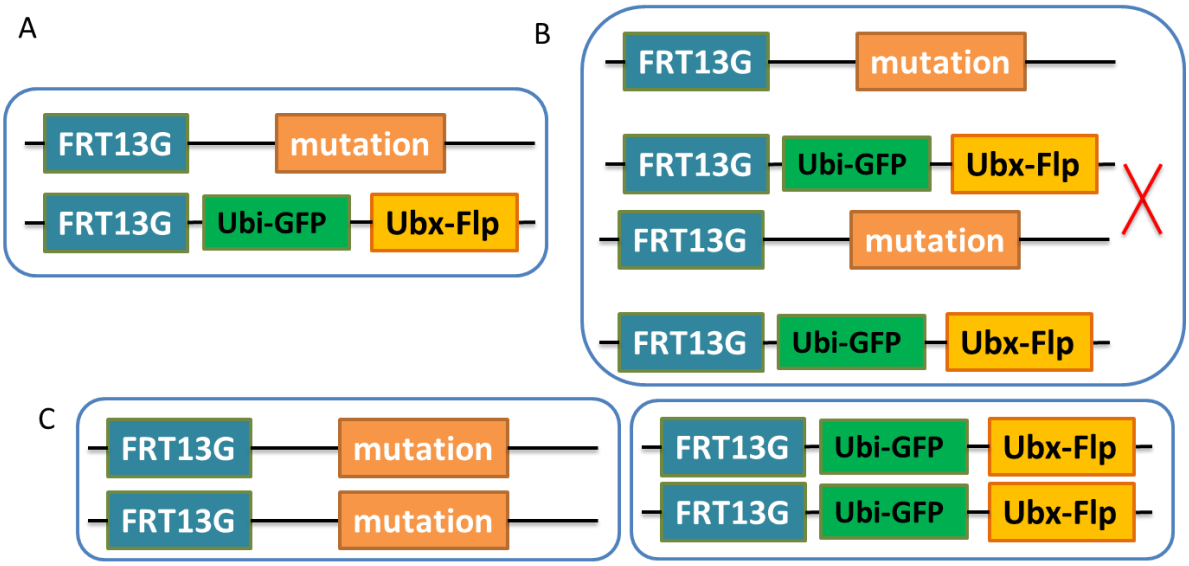


Figure 5: Flp/FRT system

Heterozygous parenteral cell with mutant allele and FRT site on one arm, whereas on the other arm Ubx-Flp, an identical FRT site and Ubi-nuclear-GFP, which expresses GFP in the nucleus ubiquitously (A). After DNA replication in S phase, post-mitotic recombination induced by Flp between the FRT sites of two chromosome arms leads to site-specific exchange of chromosomes segments (B). Two non-identical homozygous daughter cells result, as one is wild type homozygous whilst the other one is mutant homozygous. Homozygous mutant cells have lost the Ubi-nls-GFP construct and therefore the mutant clones are said to be negatively marked (all non-homozygous-mutant cells are nuclear-GFP-labelled) (C).

3.1.3. MARCM system

MARCM is a more sophisticated system in which Flp/FRT, Gal4/UAS and Gal80 are combined. Gal80 is expressed throughout the fly, under the control of the tubulin promotor. Gal80 prevents Gal4-mediated expression of UAS constructs [55]. After the induction or expression of Flp, post-mitotic recombination occurs. Non-identical daughter cells are generated as one becomes homozygous mutant and the other cell with homozygous tub-Gal80 with identical FRT region. In homozygous mutant cells, when the GFP reporter and the mutant transgene not on the same chromosome, however both under the control of UAS promotor; they will be expressed concomitantly and generate positively marked clones. This because homozygous mutant cells will have

lost Gal80 suppressor effect and allow Gal4 to activate the expression of genetic constructs downstream UAS sequence [55].

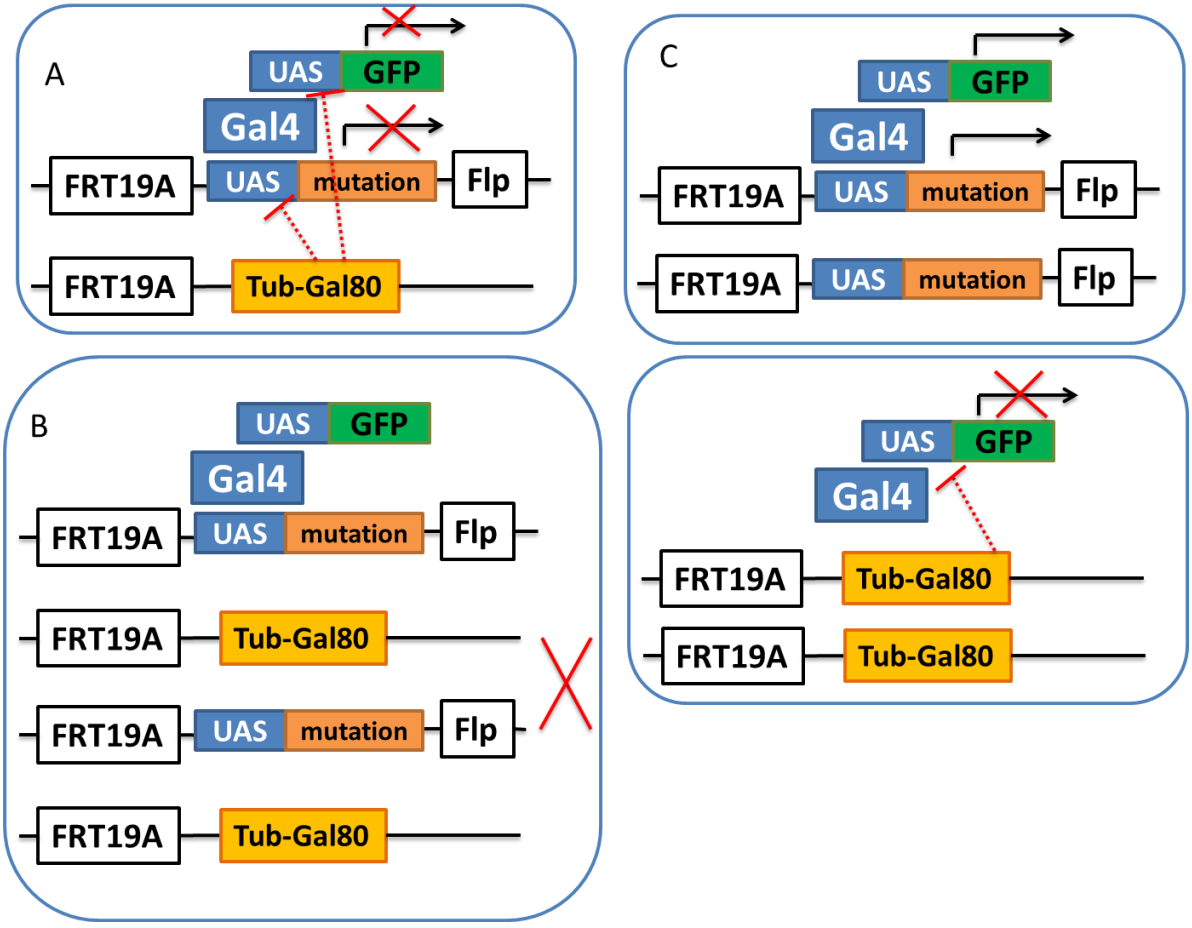


Figure 6: MARCM system

(A) shows a heterozygous parenteral cell with one FRT chromosome arm together with a transgene under UAS control and Flp. The other chromosome arm, possessing an identical FRT site also has Tubulin-Gal80, which represses the activating effect of Gal4 on UAS in both the transgene and UAS-reporter GFP. (B) DNA amplification in S phase and then recombination induced by Flp in G2 phase. (C) Homozygous mutant daughter cells express the transgene and the GFP reporter by Gal4 activation due to the absence of Gal80; the other cell is homozygous tub-Gal80, which represses UAS-GFP expression. Therefore clones of mutant cells will be specifically labelled with GFP (positively marked clones).

3.1.4. Balancer Chromosomes

Balancer chromosomes are commonly used in *Drosophila melanogaster* to maintain stocks of a heterozygous mutation or a transgene. Balancer chromosomes are also used for mutagenesis screening. Additionally they suppress homologous chromosomes cross-over in meiosis in germline cells. They always carry a dominant genetic marker and are considered as homozygous lethal. The dominant genetic markers are easily identifiable, for example bristle length or eye shape and colour could be affected, and these markers are used to confirm the presence of the balancer chromosome, so that the mutation can be tracked through generations [56] (See Table 2 in Appendix 2).

3.2. Fly Pushing

Flies were raised in plastic vials plugged with cotton stoppers. Bloomington fly food recipe was selected as a standard food for flies (7340 ml Tap water, 565 ml golden syrup, 127 g yeast, 73 g soya flour, 535 g cornmeal, 42 agar and 35.4 ml propionic acid). Flies were stored in incubators adjusted at 25^{o}C and 18^{o}C, since flies take the half time period at 18^{o}C rather than 25^{o}C for development from laid eggs to emerge flies passing through the larvae stage. Normally files take at 25^{o}C 9-10 days from eggs to flies. Flies were flipped to new vials every 10 days when kept at 18^{o}C or 2-3 days when kept at 25^{o}C. Flies were anaesthetized using carbon dioxide porous polyethylene gas diffusers and screened under Leica M60 microscopes and males were identified from female by the ventrally located clasper at the posterior end of the male abdomen. For setting genetic crosses virgins were collected every 8 or 16 hours at 25^{o}C or 18^{o}C respectively as female flies cannot mate with males in the first 8 hours of adulthood. In crosses the males to females ratio was 1:2 or 1:3. Balancer chromosomes were used to repress meiotic recombination that occurs normally in females. Additionally, it enables for genetic tracking along the crossing schemes [57].

3.3. Dissections and immunostaining

Pupae were dissected at the age of 17-24 hours after puparium formation (APF) in cold PBS at room temperature. UAS-mCherry *Ref(2)p* pupae were an exception as they were dissected at the age of 16hrs, 23hrs and 32hrs APF. Pupae were fixed from the posterior end facing up with two pins crossing each other on a small rubber dish. The outer case of the pupae were removed and the heads were cut and a longitudinal cut performed in the pupae abdomen and a Pasteur pipette was used to flush the gut content (Figure 7). The central stipe of the notum were fixed in 4% paraformaldehyde for 25 minutes, then 3 washes with PBST (PBS containing 0.1% Triton X-100) with 2 minutes interval. Then the primary antibodies were prepared in PBST 0.1% and left with notum tissues overnight at 4^{o}C, then washed 3 times with PBTS 0.1%. Secondary antibodies were prepared in PBST 0.1% and left on notum tissues for 1 hour at room temperature. After that tissues were washed 3 washes with PBST 0.1% with 2 minutes interval, then they were left in PBS containing 50% Glycerol overnight at 4^{o}C. Tissues were mounted on a microscopic slide and 22x22 mm coverslips with 0.13-0.17 mm thickness were used for generating the interface. Primary antibodies used in staining were Rat anti E-Cadherin antibody (DSHB,1:100), Rabbit anti aPKC (Santa Cruz, 1:1000), mouse anti Discs Large (DSHB, 1:100), mouse anti Arm (DSHB, 1:100), mouse anti Fasciclin (Fas) III (DSHB, 1:400) and guinea pig anti Baz (1:300) from A. Wodarz. Secondary anitbodies were purchased from Molecular Probes including Alexa Fluor 488 anti-rat, Alexa Fluor 488 anti-mouse, Alexa Fluor 546 anti-rat, Alexa Fluor

546 anti-mouse, Alexa Fluor 647 anti-rabbit and Alexa Flour 546 anti-guinea pig with dilution used 1:300 for of all secondary antibodies.

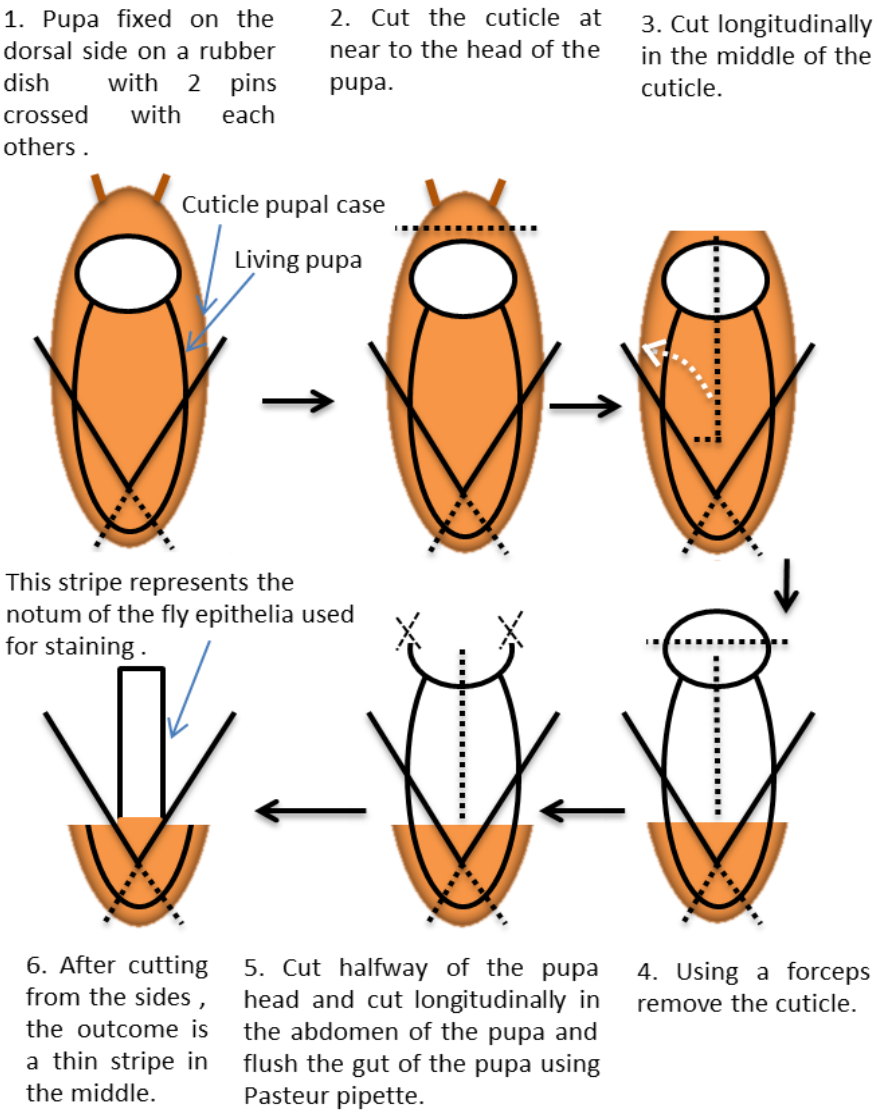


Figure 7: Schematic diagram shows the procedures of notum dissection

Diagram shows steps of dissecting a fly notum, which starts with removing the cuticle and then cutting in the pupa head and abdomen to reach a small thin stripe of the fly notum epithelia that is fixed afterwards and stained.

3.4. Mounting and Confocal imaging

Pupae were collected at 0 hours APF once they pupate and kept at 25°C. Between the ages of 12 to 14 hours they are placed ventral side down onto a microscopic slide using double-sided sticky tape. Then the notum was exposed by removing part of the cuticle. On both sides of the microscopic slide small 22x22 mm coverslips with 0.13-0.17 mm thickness were used on posterior and anterior ends to generate a stage on which a long 24x50 mm coverslip (0.16-0.19 mm think) covered with thin layer of Oil 10S will fit and create the interface with the notum (Figure 8). Imaging was performed using a Zeiss LSM510 Confocal Fluorescence Microscope with 40x/1.3 oil Ph3 objective. In live and

fixed cells imaging z-stacks were taken with 1 µm planes from the cuticle to basal lamina at a resolution of 1024x1024. Fiji (ImageJ) and Volocity software were used for image analysis. For measuring the fluorescence intensity of different polarity proteins staining, an average of a minimum of 20 and a maximum of 50 staining intensities for the same protein were measured at different junctions between cells, in a minimum of four pupal notum tissues, and quantified by setting a line around each junction and the mean intensify tool in Fiji was used to measure the mean fluorescence intensity of different polarity proteins immunestaining. The length of the cell cycle and cell invasion overtime were measured in Zeiss LSM510 software as the time intervals were adjusted at 90 secs for imaging a single 6 µm z-stack with 1 µm for each plane then the same 6 µm z-stack were imaged about 10 times for 90 secs each in cell division or cell invasion, therefore the time interval at the same plane over time is 90 secs.

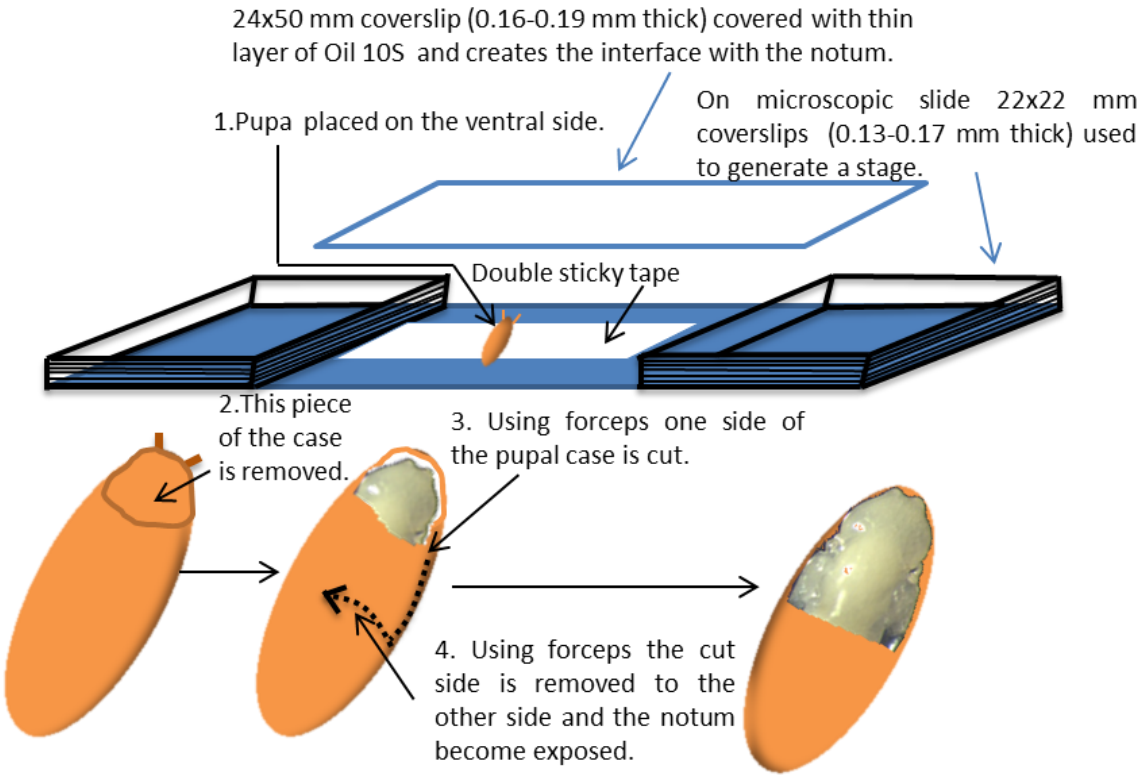


Figure 8: Schematic diagram showing steps of pupae mounting

The figure shows the steps of mounting of removing the case on the head first and then exposing the notum using a forceps.

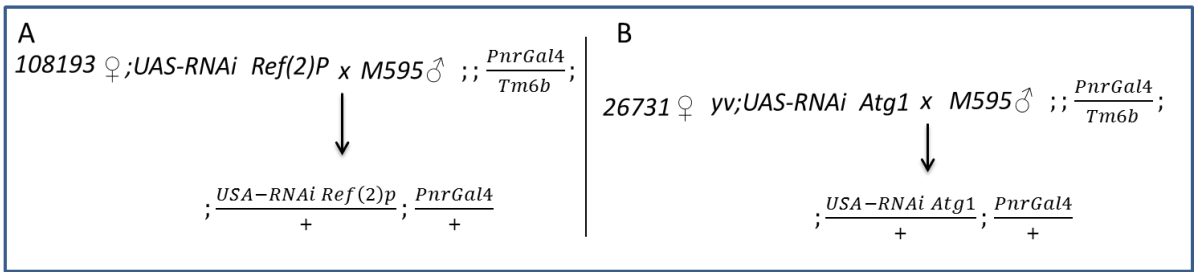
3.5. Genetic Crossing Schemes

The genetic schemes illustrated below show the multi steps performed to reach the genotypes of interest. Some genotypes were simplified in the crossing schemes (The full detailed genotype could be found in Table 3 in Appendix 3).

3.5.1. Pnr-Gal4 Crosses with different transgenes of UAS-constructs

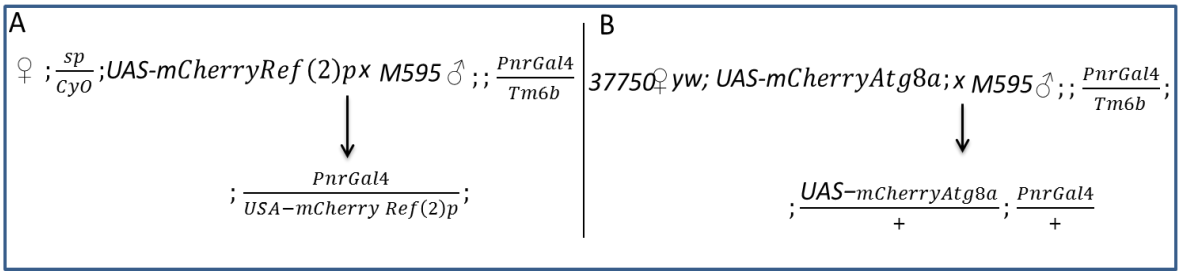
Ref(2)p RNAi construct, mCherry tagged overexpressed *Ref(2)p* transgenes, *Atg1* RNAi construct and mCherry overexpressed *Atg8a* are under the control of UAS promotor therefore they are crossed with Pnr-Gal4 driver flies to drive the expression of these transgenes in the central region of flies notum. Wild type w^{1118} was used as a control.

i. UAS-RNAi constructs of *Ref(2)p* and *Atg1*



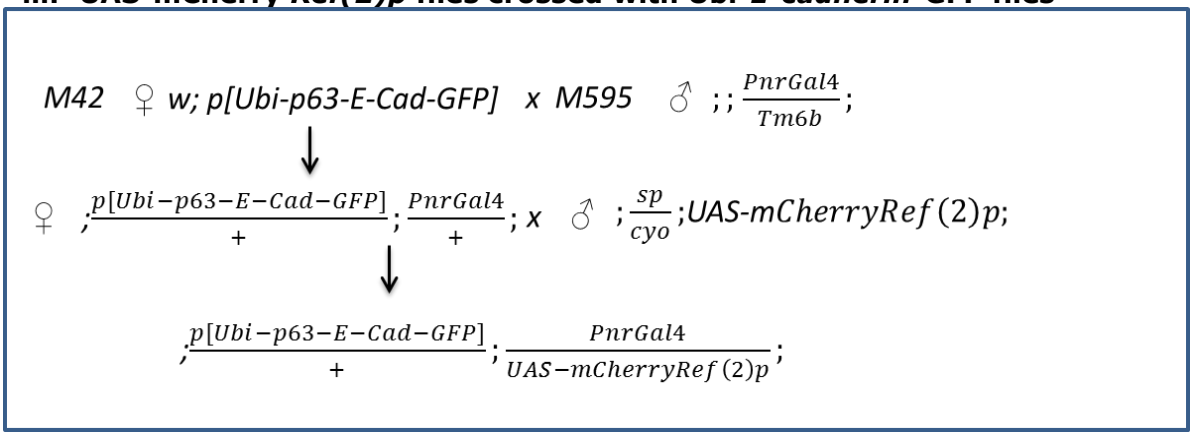
Pnr-Gal4 diver flies crossed with UAS-*Ref(2)p* RNAi construct (A), and UAS-*Atg1* RNAi construct (B), to drive the expression of these constructs in the central region in the back of the flies.

ii. UAS-mCherry *Ref(2)P* and UAS-mCherry *Atg8a* overexpression



Pnr-Gal4 diver flies crossed with UAS-mCherry overexpressed *Ref(2)p* construct (A), and UAS-mCherry overexpressed *Atg8a* construct (B), to drive the expression of these constructs in the central region in the back of the flies.

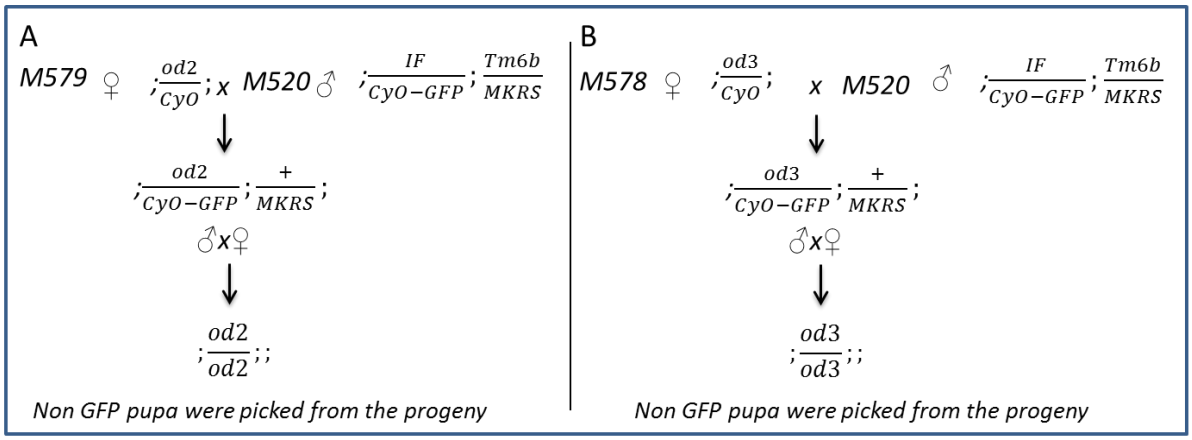
iii. UAS-mCherry *Ref(2)p* flies crossed with Ubi-*E-cadherin*-GFP flies



This Crossing scheme is to generate flies expressing both UAS-mCherry *Ref(2)p* and Ubi-*E-Cadherin*-GFP for live imaging, so that the localization of mCherry Ref(2)p could be checked in comparison to E-Cadherin-GFP.

3.5.2. Homozygous *Ref(2)p* mutants

The CyO-GFP balancer for the second chromosome was used to generate heterozygous *Ref(2)p* mutant flies and then negatively marked (GFP-negative) pupae were picked as homozygous *Ref(2)p* mutants for dissection and imaging.

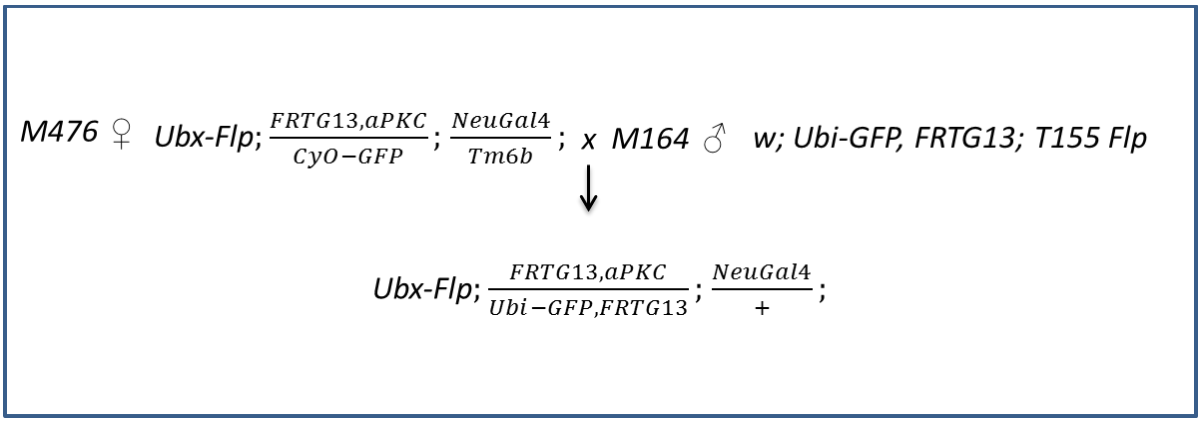


Crossing scheme to obtain homozygous *Ref(2)p* mutants including *od2* mutation that lacks PB1 domain (A) and *od3* mutation that lacks UBA domain (B).

3.5.3. Negatively marked clones

Flp/FRT and Ubi-nls-GFP used to make negatively marked clones in *aPKC* null flies in a background of wild type cells expressing ubiquitously GFP in nucleus.

i. *aPKC* negatively marked clones

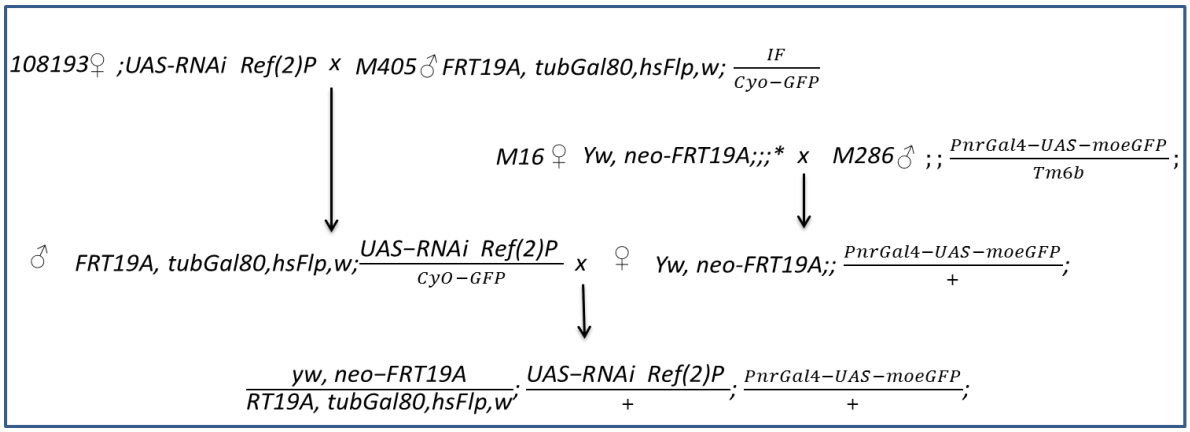


Using Flp/FRT system in this cross scheme is to generate negatively marked clones of homozygous *aPKC* null cells in background of nuclear GFP wild type labelled cells.

3.5.4. MARCM

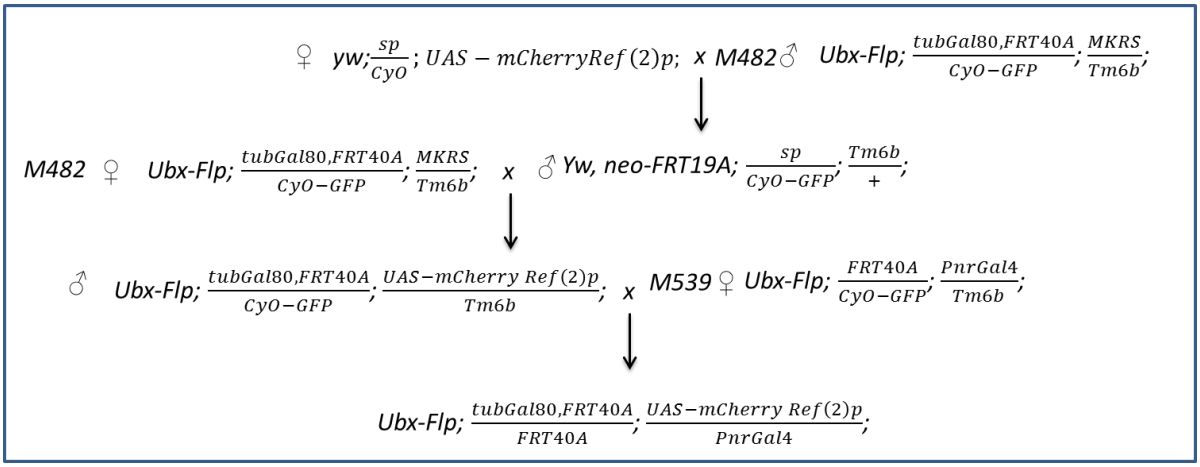
MARCM is used here for generating positively marked clones in a background of wild type cells in UAS-*Ref(2)P* RNAi construct and UAS-mCherry *Ref(2)P* overexpression.

i. UAS-*Ref(2)p* RNAi positively marked clones



This cross scheme is for generating positively marked clones of UAS-*Ref(2)p* RNAi. Using Gal80, under the control of Tubulin promotor, represses the activating effect of Gal4 on UAS promotor in wild type cell of homozygous Gal80 as consequence of post mitotic recombination mediated by Flp/FRT system. In contrast positively marked clones were generated of UAS-*Ref(2)p* RNAi construct labelled in moe-GFP as mutants cells lack tub-Gal80.

ii. UAS-mCherry flag-tagged *Ref(2)p* positively marked clones

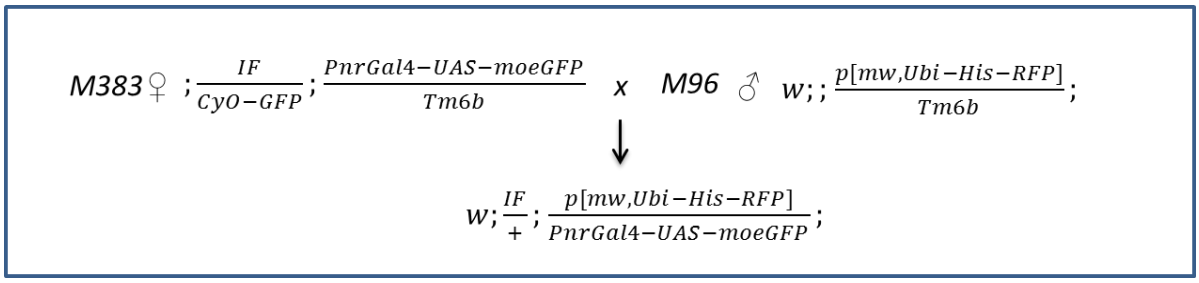


This cross scheme is for generating positively marked clones of UAS-mCherry overexpressed *Ref(2)p*. Using Gal80, under the control of Tubulin promotor, represses the activating effect of Gal4 on UAS promotor in wild type cell of homozygous Gal80 as consequence of post mitotic recombination mediated by Flp/FRT system. In contrast positively marked clones were generated of UAS-mCherry overexpressed *Ref(2)p* labelling *Ref(2)p* in mCherry as mutants cells lack tub-Gal80.

3.5.5. Cell division experiment

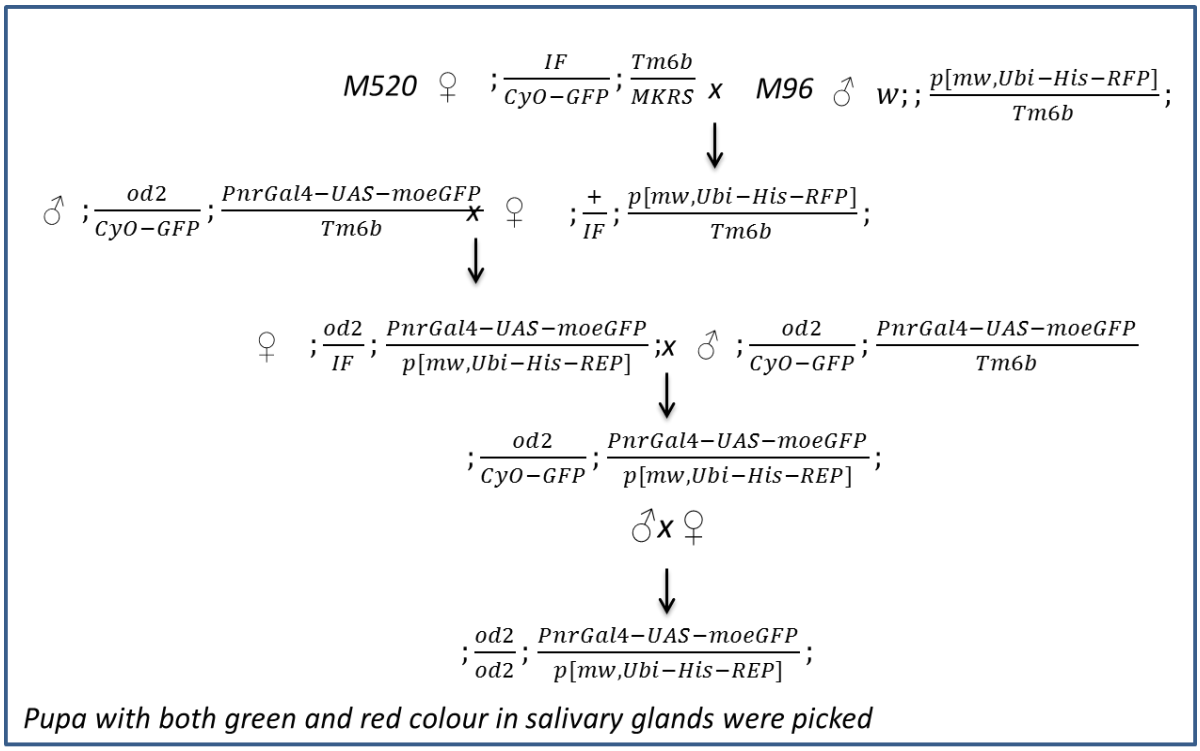
Cell division experiments were performed in fly notum to examine the rate of cell division in *Ref(2)p* mutants of *od2* and *od3*, as well as *aPKC* null flies in comparison to wild type to check if the increase in cell size is due to cell division failure, any change in cell morphology during division or defects in actin cytoskeleton.

i. Wild type



This crossing scheme is to generate a wild type flies have both Pnr-Gal4-UAS-moe-GFP and Ubi-His-RFP in the notum expressed. Crossing Pnr-Gal4-UAS-moe-GFP which drives GFP expression in mosein of actin cytoskeleton and Ubi-His-RFP flies which has RFP expressed in the histones. Pupae with both green and red colour in the salivary glands were picked for imaging.

ii. *Ref(2)p* Mutants

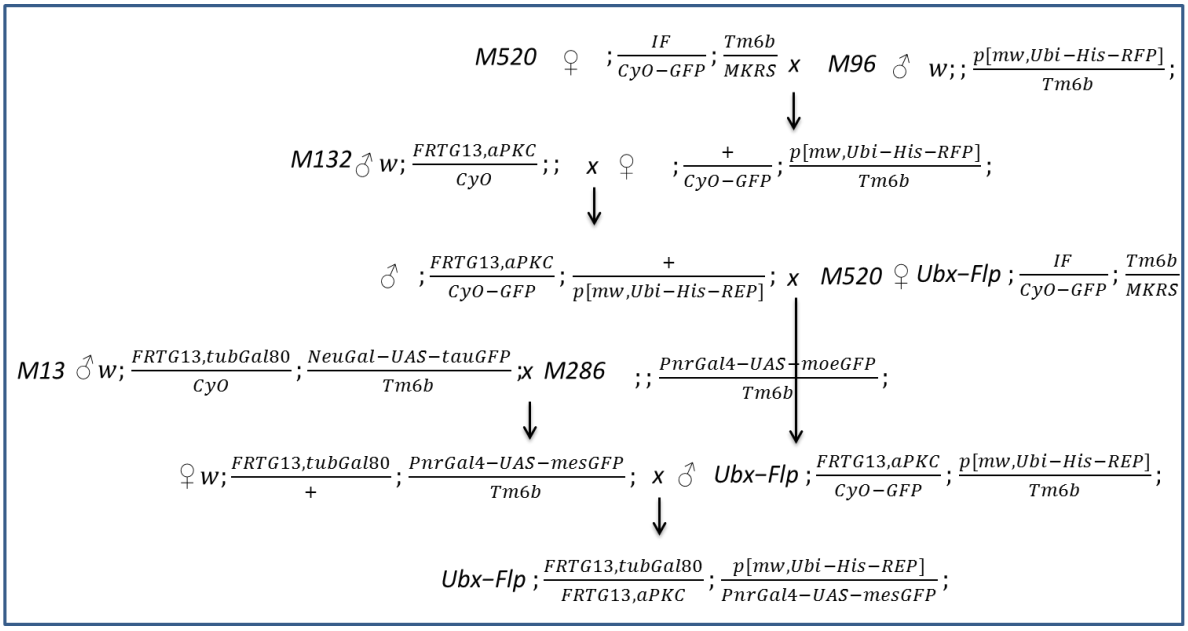


Pupa with both green and red colour in salivary glands were picked

This crossing scheme is to end up with homozygous *od2 Ref(2)p* mutant flies with Pnr-Gal4-UAS-moe-GFP coupled with Ubi-His-RFP. Pupae with both green and red colour in

the salivary glands were picked for imaging. The same crossing scheme was done for *od3 Ref(2)p* mutation as well.

iii. *aPKC* null flies

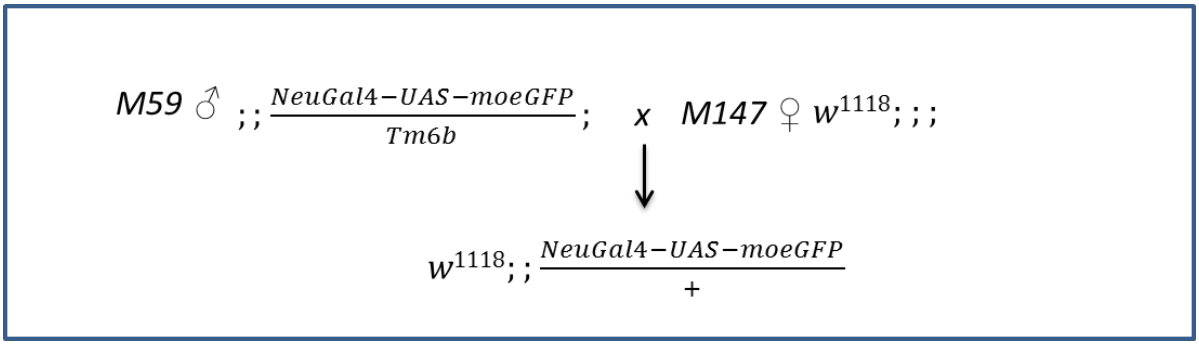


To look at cell division in *aPKC* null flies MARCM system was used as *aPKC* null flies are homozygous lethal. A crossing scheme used to generate clones of homozygous *aPKC* null cells in flies notum by tub-Gal80 and Flp/FRT system. This coupled with using Pnr-Gal4-UAS-moe-GFP and Ubi-His-RFP. The pupae picked should be females and the clones of *aPKC* should be detected as only 50% of the females will have Ubx-Flp.

3.5.6. Cell morphology and Protrusion dynamics experiment

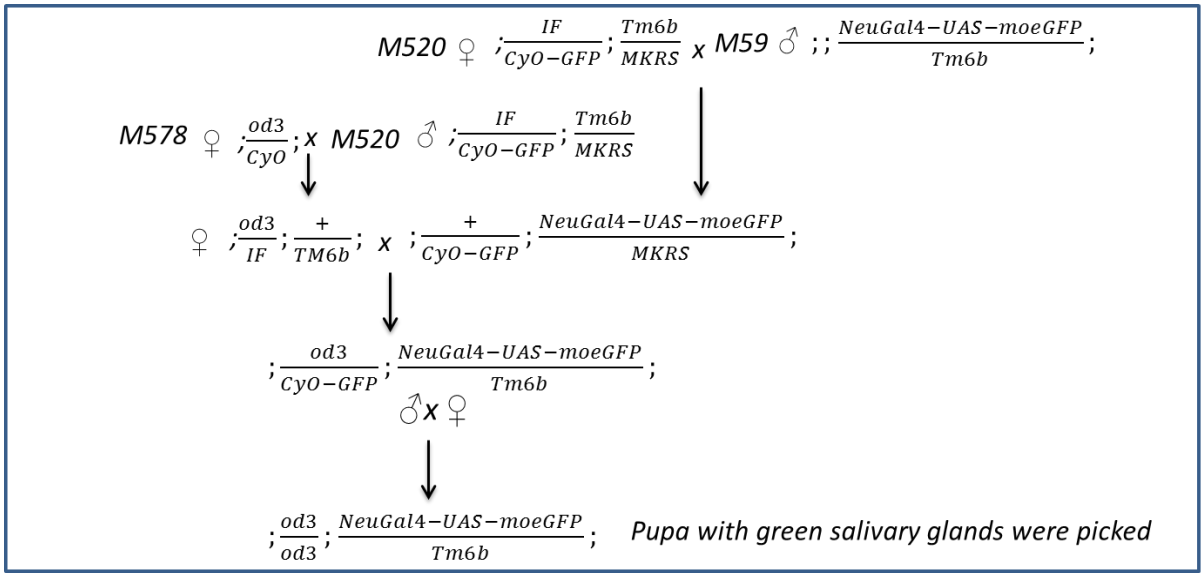
Neu-Gal4 drives the expression in the sensory organ pI cells and at the time of imaging these cells will not have been decided on their fate, therefore they are representative for all epithelial cells in the notum. By labelling individual cells with moe-GFP by using Neu-Gal4-UAS-moe-GFP construct, cell morphology and protrusions could be easily studied [48].

i. Wild type



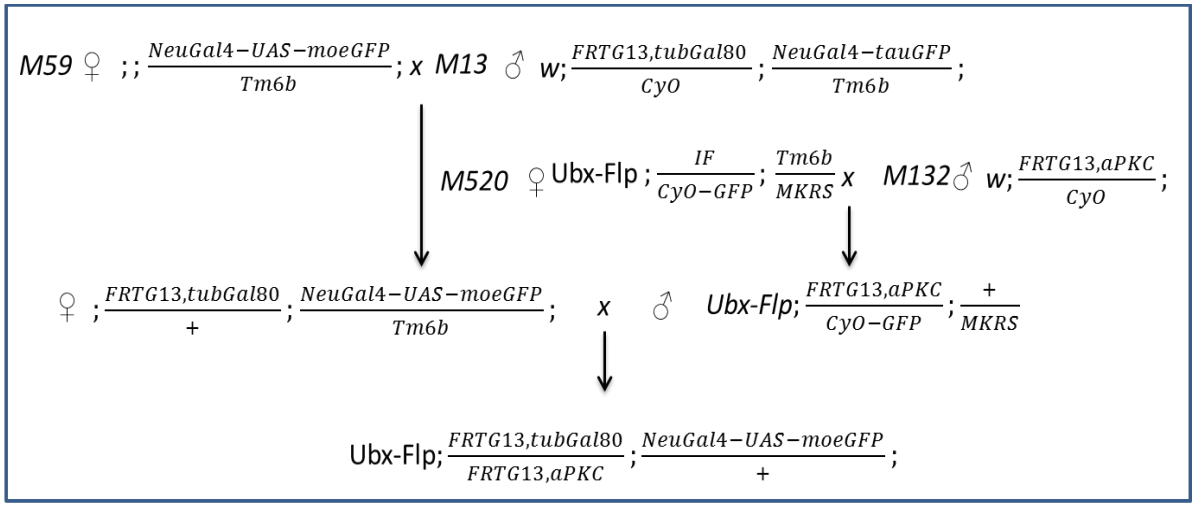
Wild type flies with white eye gene on X-chromosome crossed with Neu-Gal4-UAS-moe-GFP flies to allow for imaging and looking at cell protrusions and cell morphology of individual pI cells, which are representative for the rest of notum epithelial cells.

ii. *Ref(2)p* mutants



Neu-Gal4-UAS-moe-GFP flies crossed with *od2 Ref(2)p* mutation balanced over CyO-GFP to enable of picking homozygous mutant negatively labelled GFP pupae. Neu-Gal4-UAS-moe-GFP enables for looking at protrusions behaviour and cell morphology in *Ref(2)p* homozygous mutant cells. The same crossing scheme performed in *od3 Ref(2)P* mutation.

iii. *aPKC* null flies



As homozygous null *aPKC* is lethal, so MARCM system and Neu-Gal4-UAS-moe-GFP were used to drive the expression in individual cells with homozygous null *aPKC* in flies notum to look at cell protrusions and cell morphology.

iv. UAS-mCherry *Ref(2)P* and UAS-mCherry *Atg8a* overexpressing files

A

$$♀ ; \frac{sp}{CyO} ; UAS\text{-}mCherryRef(2)p; \; x \; M59 \; ♀ ;; \frac{NeuGal4-UAS-moeGFP}{Tm6b};$$

$$\downarrow$$

$$;; \frac{NeuGal4-UAS-moeGFP}{UAS-mCherryRef(2)p}$$

B

$$37750♀ ; UAS\text{-}mCherryAtg8a;; \quad x \; M59 \; ♀ ;; \frac{NeuGal4-UAS-moeGFP}{Tm6b};$$

$$\downarrow$$

$$;; \frac{NeuGal4-UAS-moeGFP}{UAS-mCherryAtg8a}$$

UAS-mCherry *Ref(2)p* (A) and UAS-mCherry *Atg8a* overexpressing flies (B) crossed with Neu-Gal4-UAS-meo-GFP flies to study the morphology change in Autophagy overactive flies and autophagy influence on protrusion formation.

3.5.7. Cell invasion experiment

Ref(2)p effects on apico-basal polarity indicated that it might have tumour repressor activity to identify that wild type, homozygous *Ref(2)p* mutants and both UAS-constructs of *Ref(2)p* and *Atg1 RNAi* were combined Pnr-Gal4 drivers expressing UAS-meoGFP through crosses with Pnr-Gal4-UAS-meo-GFP flies.

i. Wild type

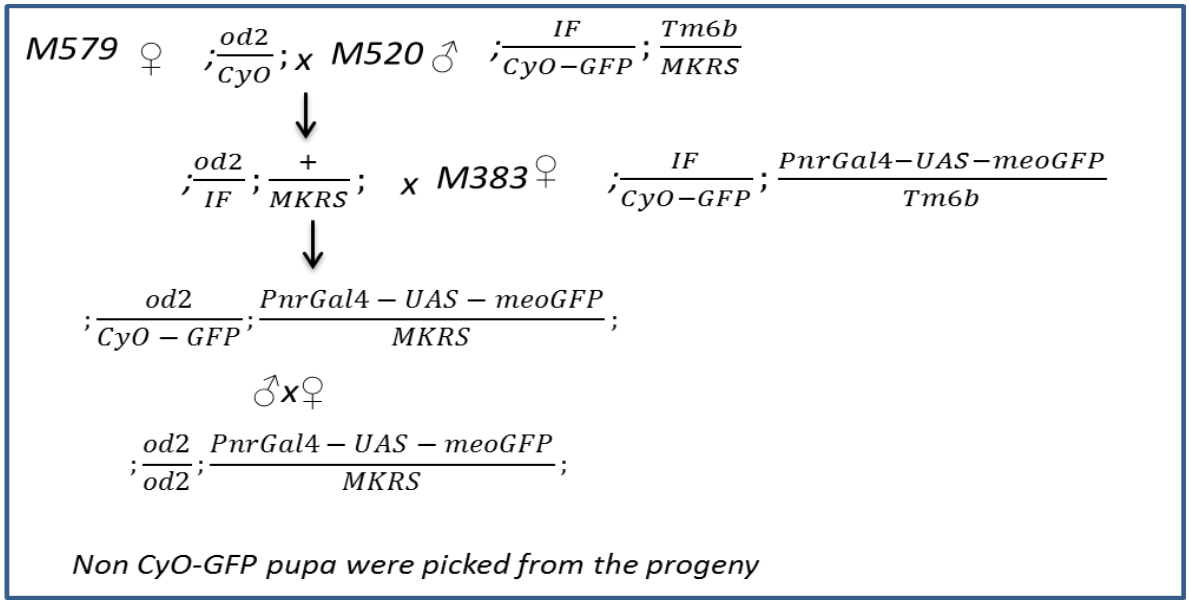
$$M147 ♂ \; w^{1118};;; \quad x \quad M286♀ \quad ;; \frac{PnrGal4-UAS-moeGFP}{+};$$

$$\downarrow$$

$$w^{1118};; \frac{PnrGal4-UAS-moeGFP}{+};$$

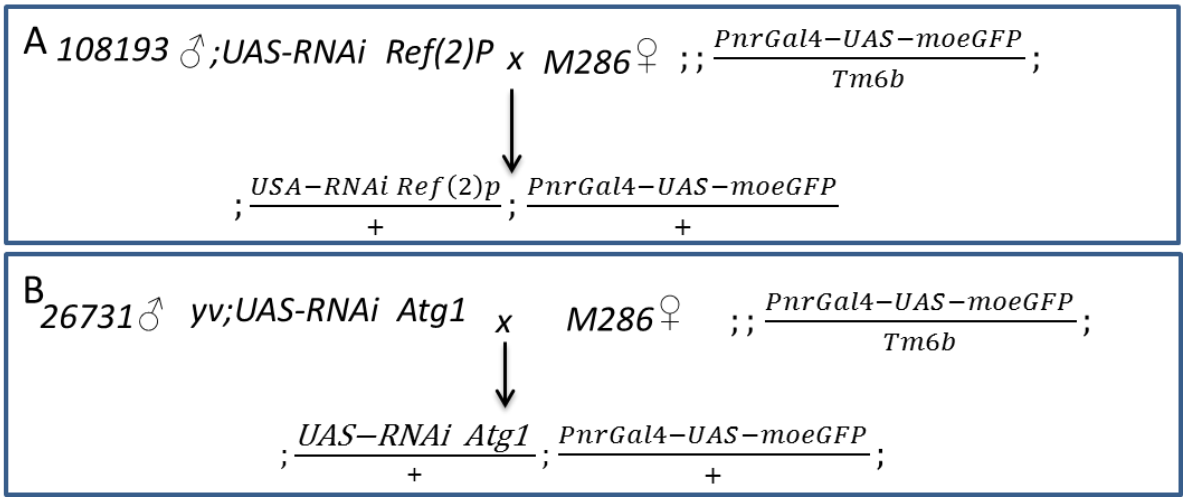
Wild type cell invasion was studied by crossing wild type having white eye gene on X chromosome with Pnr-Gal4-UAS-moe-GFP flies. Then, the invading cells could be detected labelled in moe-GFP.

ii. *Ref(2)p* mutants



Ref(2)p homozygous mutant *od2* combined with Pnr-Gal4-UAS-moe-GFP pupae were generated through a multistep crossing scheme, so the invading cells could be detected labelled in moe-GFP. CyO-GFP was used to generate and track homozygous *Ref(2)P* mutant as at the end of the crossing scheme non GFP pupae were picked. The same crossing scheme was done for *od3 Ref(2)p* mutation.

iii. UAS-*Ref(2)p* RNAi and UAS-*Atg1* RNAi constructs



Straight forward cross done for UAS-*Ref(2)p* RNAi flies and UAS-*Atg1* RNAi flies (both stocks have RNAi constructs on the second chromosome) with Pnr-Gal4-UAS-moe-GFP expressing flies on the third chromosome, so that the invading cells could be detected labelled in moe-GFP.

3.6. Immunoblotting

Proteins were resolved on a gradient (5-20%) polyacrylamide resolving gel (30% (w/v) acrylamide solution, 1.5M Tris-HCl pH 8.8, 10% (w/v) SDS, 10% (w/v) APS, TEMED) which were cast on casting plate. Once poured, the resolving gel was overlaid with isopropanol to form an even horizontal line and get rid of any bubbles. Filter paper was used to remove the isopropanol and then stacking gel was poured on top, along with a comb. This was then allowed to set. Around 20-25 Pupae notum was dissected as previously described (Section 3.3) and spun in 30µl lysis buffer (50% mM Tris, 1% Triton, 150mM NaCl, 1 mM EDTA and 1 Protease inhibitor cocktail tablet) or two flies crossed with Act5C-Gal4 squashed properly in 40µl lysis buffer and then spun and the supernatant taken. Protein amounts in samples were normalized using Bradford assay. Then the gel plate was placed in the gel tank filled with electrode buffer (25mM Tris, 186mM Glycine, and 0.1% (w/v) SDS) electrophoresis was carried out at 40 mA (500 V max). After removal of the stacking gel, the resolving gel was submerged in transfer buffer (25mM Tris, 192mM glycine, 20% methanol) to transfer proteins to a nitrocellulose membrane and a blotting sandwich is made for that. Proteins were transferred overnight at 40 mA.

The blot was then blocked for 2 hour at RT in 5% marvel in PBS to block non-specific sites on the membrane. The blot was then incubated overnight at 4^{o}C in the appropriate primary antibody, diluted in blocking buffer overnight. Primary antibodies used were rabbit anti Ref(2)p (Ab123009, 1:100) and mouse anti GAPDH as a loading control (Ab9484, 1:1000). The blot was washed 3 times on a rocker with TBS-Tween (0.05% tween) for 2 mins per wash, and incubated for another hour secondary antibody diluted in blocking buffer. Secondary antibody used from Dako including HPR anti Rabbit and HRP anti mouse all used at the dilution of 1:2000. Then, blots were washed a further 3 times with TBS only and developed using an ECL chemiluminescence detection kit. The blot was incubated in ECL reagent for 1min and wrapped in cling-film. This was then placed in an x-ray cassette and overlaid with film for the required exposure time and developed in a dark room.

4. Results

4.1. Ref(2)p has a role in regulating apico-basal polarity

Epithelial cells preserve their architecture through maintaining the apico-basal polarity. As long as epithelial cells are polarized they have distinct domains along the apico-basal axis classified into the apical domain, the junctional region and the baso-lateral domain [58]. To examine the hypothesized notion of Ref(2)p implications in apico-basal polarity maintenance via interaction with aPKC in epithelial cells of dorsal thorax of *Drosophila* m*elanogaster*, two genetic mutants of *Ref(2)p* were studied including *od2* and o*d3* mutations (Figure 9-B-C) and *Ref(2)p* RNAi (Figure 9-D). Both mutants and

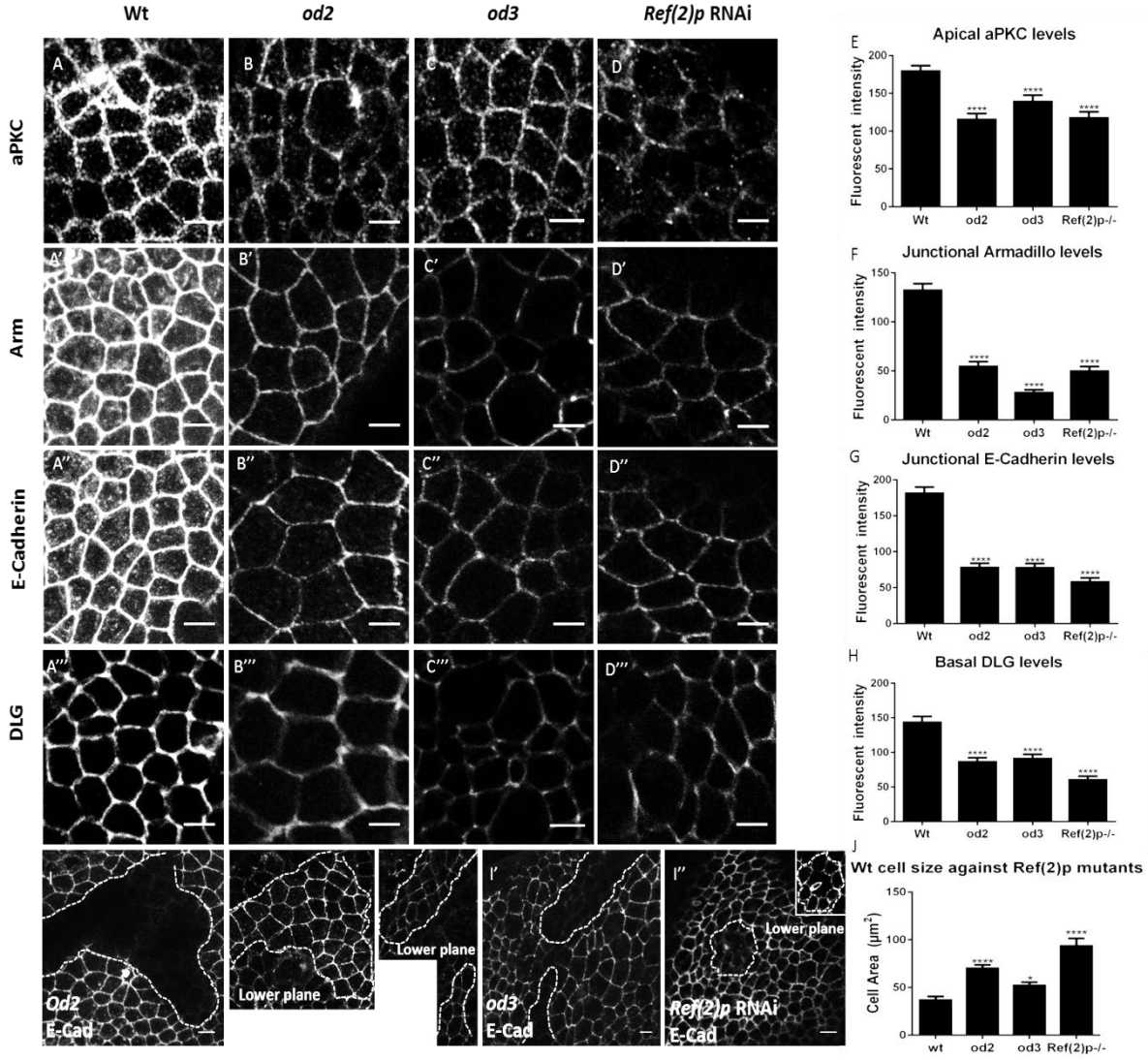


Figure 9: Ref(2)p epithelial cell phenotypes

Od2 homozygous (B), *od3* homozygous (C) mutations and UAS-Ref(2)p-RNAi driven on the back of the fly with Pnr-Gal4 (D) showed junctional breaks and increased cell size (J) in comparison to wild type (A). A broad decrease in polarity proteins levels were observed in all mutants, including aPKC (E), Arm (F), E-Cadherin (G), and DLG (J). Further these mutants showed folding of epithelial tissue in fly notum (I). Scale bars represent 5 microns (P value *<0.05 and ****<0.001).

RNAi showed an increase in cell size (Figure 9-J) and junctional discontinuities in comparison to wild type (Figure 9-A). Further, a disturbance in polarity protein localisation was observed as aPKC, E-Cadherin, Arm and DLG antibodies showed a reduced level of protein at the cell cortex (Figure 9-E-H). Another phenotype observed was epithelial tissue folding as seen in (Figure 9-I). Interestingly, these epithelial phenotypes closely match phenotypes seen in *aPKC* null clones generated in the *Drosophila* notum where tissue folding and junctional discontinuities were observed [24] and as shown in (Figure 10-A-A'). A decrease in junctional E-Cadherin levels were observed (Figure 10-C) and an increase in cell size as well (Figure 10-B). Another experiment done by generating positively marked clones of *Ref(2)p* RNAi clones in wild type tissue using MARCM system and Pnr-Gal4-UAS-moe-GFP construct confirmed the phenotypes of increasing cell size and decrease in aPKC levels in *Ref(2)p* RNAi mutants (Figure 10-D). This indicates that Ref(2)p has a role in regulating apico-basal polarity of epithelial cells and these phenotypes might be due to an interaction occurs between Ref(2)p and aPKC via PB1 domain and localizing aPKC apically. The observed disturbance in other polarity proteins is likely due to the decreased levels/mis-localization of aPKC from the apical domain.

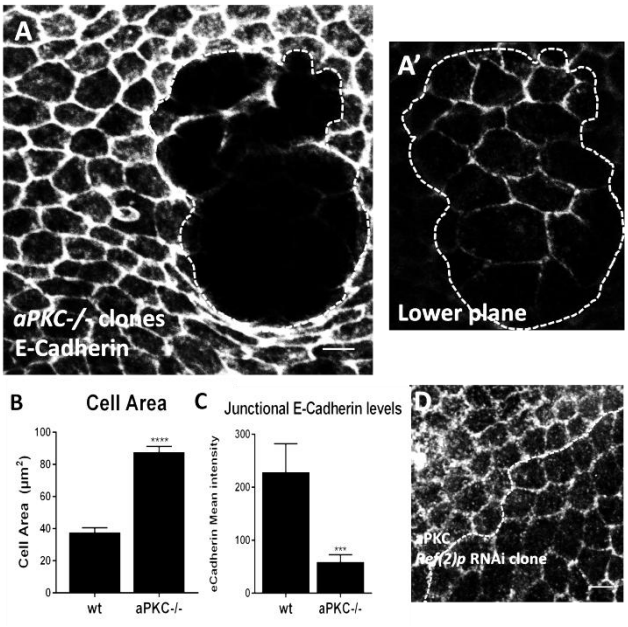


Figure 10: aPKC's role in maintaining cell shape and Ref(2)p RNAi clones

The Flp/FRT system was used to generate negatively marked clones of homozygous *aPKC* null cells in *Drosophila* notum using a Ubi-nls-GFP construct, which expresses nuclear GFP in wild type cells (A). Tissue folding and cell size increase was observed in *aPKC* null clones (A'-B) in the lower plane. E-Cadherin levels decreased in *aPKC* mutant cells (C). Positively marked clones of *Ref(2)p* RNAi were generated using MARCM system with Pnr-Gal4 driving the expression of a UAS-moe-GFP construct. The dotted line shows the upper part of wild type cells and lower part of *Ref(2)p* RNAi mutant cells. Increase in cell size and decrease in aPKC levels were detected (D) scale bars represent 5 microns (P value *** and ****< 0.001).

Studying cell morphology was performed using Neu-Gal4, which drives expression of moe-GFP in well-spaced cells on the back of the fly. Cell morphology differences from wild type cells were observed in *Ref(2)p* mutant flies in terms of cell volume, protrusion

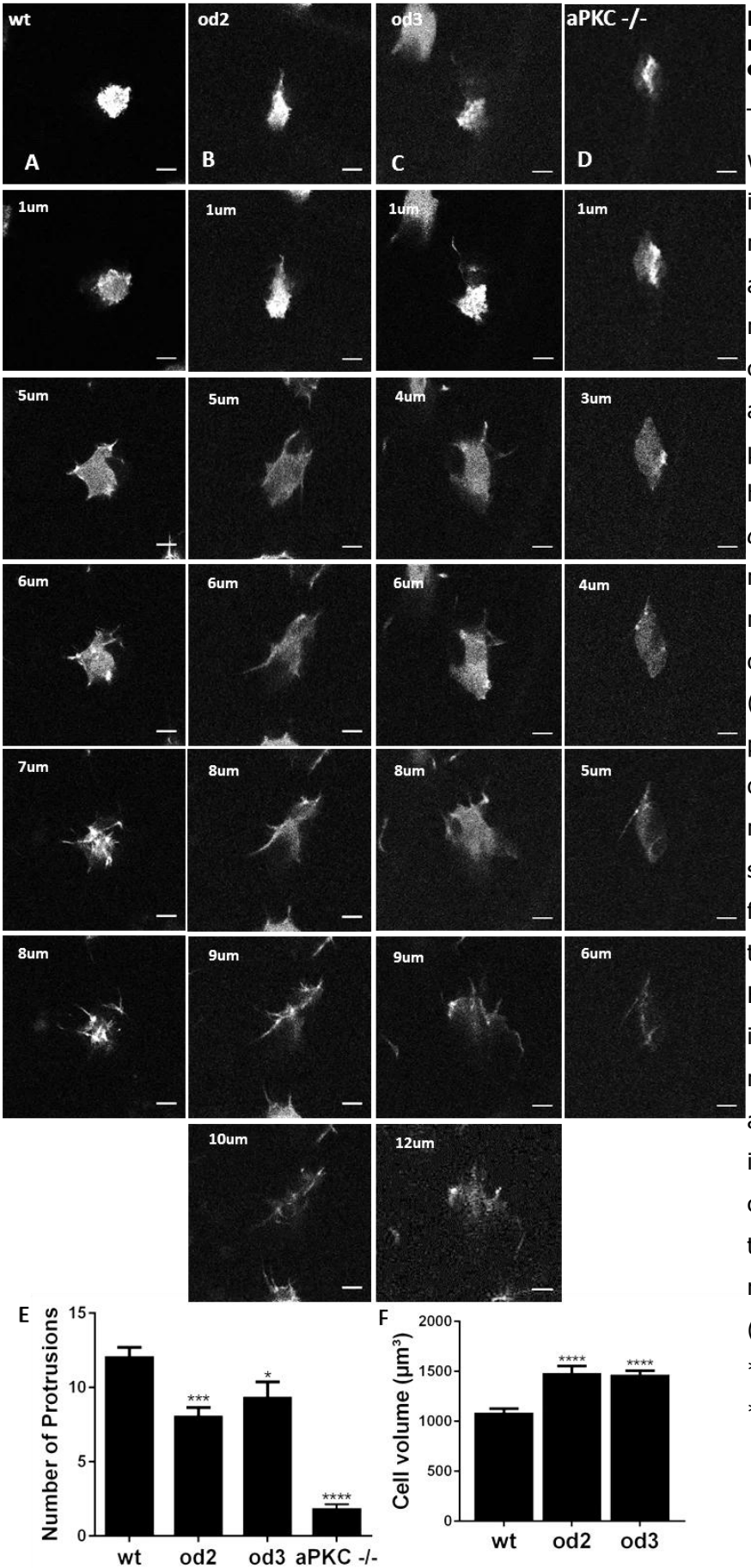


Figure 11: Ref(2)p mutants impact on cell morphology

The moe-GFP reporter was used to study individual cell morphology in *od2* and *od3* homozygous mutants and wild type cells. Wild type cells appear in (A) with 8 µm cell length; however *od2* (B) and *od3* (C) mutants are more longitudinal. The number of protrusions decreased in mutants (E), however protrusions almost disappeared in *aPKC* null mutant cells, sometimes few filopodia appeared in the basal domain (D). Further, there is an increase in *Ref(2)p* mutants cell volume and cell area at the intermediate level in comparison to wild type cells. Scale bars represent 5 microns (P value *<0.05 and *** and ****<0.001).

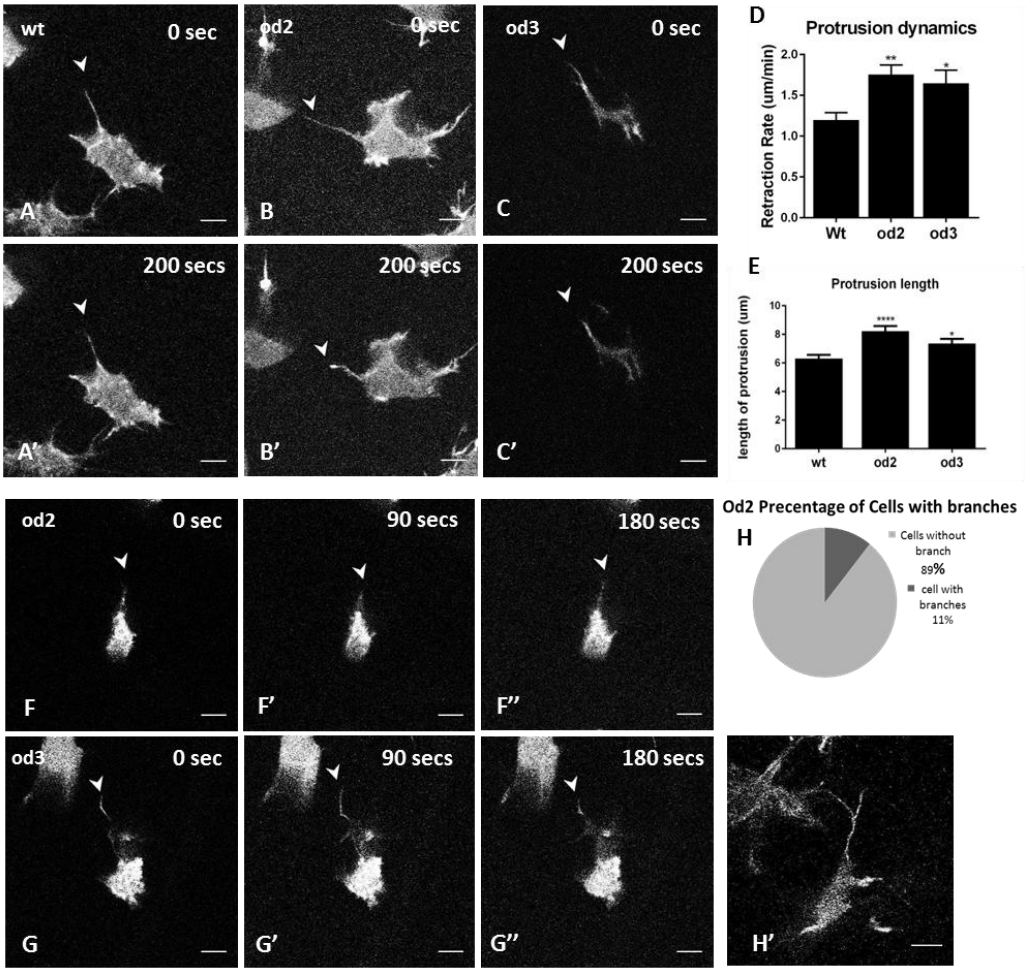


Figure 12: Ref(2)p influences on protrusion dynamics and morphology

Using the moe-GFP reporter in *od2* and *od3* homozygous mutants, a change was observed protrusion dynamics in in *od2* (B) and *od3* (C) *Ref(2)p* mutants, as shown in comparison to wild type (A) and this is represented in the histogram in (D) as there is an increase in protrusion retraction rate detected in *Ref(2)p* mutants over time. A lower number of protrusions found in *Ref(2)p* mutants, however they were longer in length. Other phenotypes were detected including the dynamic apical protrusions over time in both mutations *od2* (F) and *od3* (G) and protrusion branching in *od2* mutation (H, H'). About 11% of *od2* mutant cells were detected with protrusion branching, however in wild type no protrusion branching was observed. Scale bars represent 5 microns (P value *<0.05, **<0.01 and ***<0.001).

length, count, dynamics and branching (Figure 11 and Figure 12). An increase in cell volume (Figure 11-F) besides an increase in cell area at the intermediate levels was found in both mutations of *Ref(2)p* domains including *od2* and *od3* (Figure 11-B-C), and shrinkage in the apical area of the cells similar to the phenotype seen in *Cdc42*, *Par6* or *aPKC* null cells (Figure 11-D) in comparison to wild type cells (Figure 11-A) [28]. *aPKC* null cells had almost no protrusions, sometimes they displayed a very small number at the basal domain, which means that the actin cytoskeleton is disrupted [28]. In contrast both *Ref(2)p* mutants showed a decrease in protrusion count (Figure 11-E) and an increase in protrusions length (Figure 12-E). More phenotypes were found in *Ref(2)p*

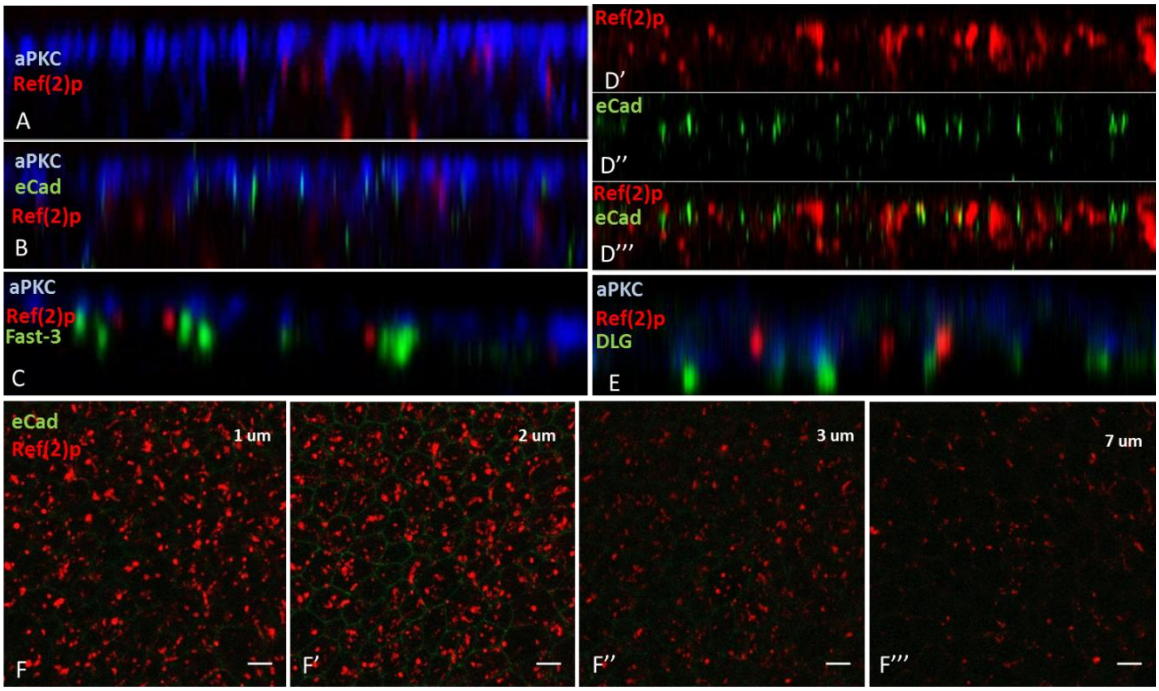


Figure 13: Ref(2)p localizes apically along the apico-basal axis

Using flies of mCherry flag-tagged *Ref(2)p* under the control of UAS promotor crossed with Pnr-Gal4 flies to drive the expression in the fly notum and staining for aPKC (A) in the apical domain, E-Cadherin (B) at junctions and Fas III (C) and DLG (E) at the basal domain. Also, live imaging of E-Cadherin-GFP and mCherry Ref(2)p pupae showed apical localization of mCherry overexpressed Ref(2)p protein(D'-D''). Volocity software was used to generate YX view. Z stack from 1µm to 7 µm down showed the decrease of Ref(2)p moving down to basal of the cell (F-F'''). Scale bars represent 5 µm.

mutants including significant increases in retraction rate (Figure 12-A-D), however extension rate showed no significant difference from wild type. 50 protrusions were examined for the retraction and the extension rate in both *Ref(2)p* mutations and wild type. The mean retraction rates of wild type was 1.19 µm/min, with 1.75 µm/min and 1.65 µm/min for *od2* and *od3* respectively. The mean extension rates were 1.11 µm/min, 1.58 µm/min and 1.46 µm/min for wild type, *od2* and *od3* respectively. Additionally, protrusion branching was seen in *od2* mutants (Figure 12-H). Taken together, the phenotypes of the increase in the cell, junctional instability and epithelial tissue folding could rationalized by the mis-localization of aPKC from the apical domain as shown in the immunofluorescence experiment previously and suggest a partial disruption in actin cytoskeleton in *Ref(2)p* mutants. Further evidence supporting this is the localization of mCherry flag-tagged overexpressed Ref(2)p in the apical domain of epithelial cells in *Drosophila* Notum (Figure 13).

4.2. Polarity proteins are regulated by Autophagy

Ref(2)p was suggested to be a multifunctional protein as is its mammalian homologue p62 and involved in many cellular processes including autophagy [45], therefore the

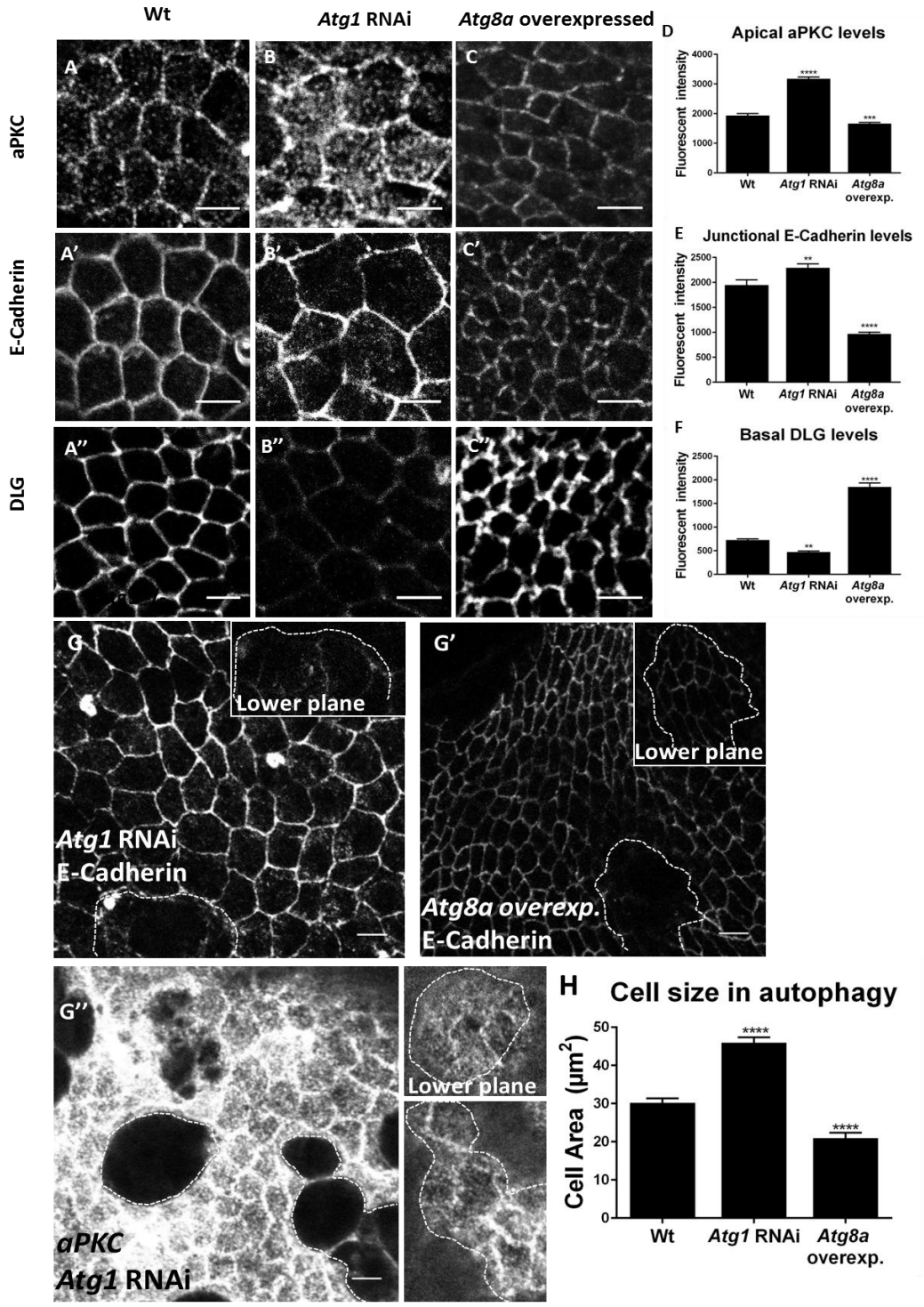


Figure 14: Autophagy phenotypes on epithelial cells

The Pnr-Gal4 driver was used to express UAS-*Atg1* RNAi (B), which is considered as an autophagy deficient model, or UAS-mCherry-*Atg8a* (C), which is considered as an autophagy overactive model. Compared to wild type (A) there are junctional defects in both *Atg1* RNAi and *Atg8a* overexpressed, increase in aPKC and E-Cadherin levels and decrease and DLG levels in *Atg1* RNAi, however the opposite occurs in *Atg8a* overexpressed (D-F). Tissue folding was found in both *Atg1* RNAi (G and G'') and *Atg8a* overexpressed (G'). An increase in cell size was found in *Atg1* RNAi cells, however a decrease in size was found in *Atg8a* overexpressing cells (H). Scale bars represent 5 microns (P values ** <0.01 and *** and **** < 0.001).

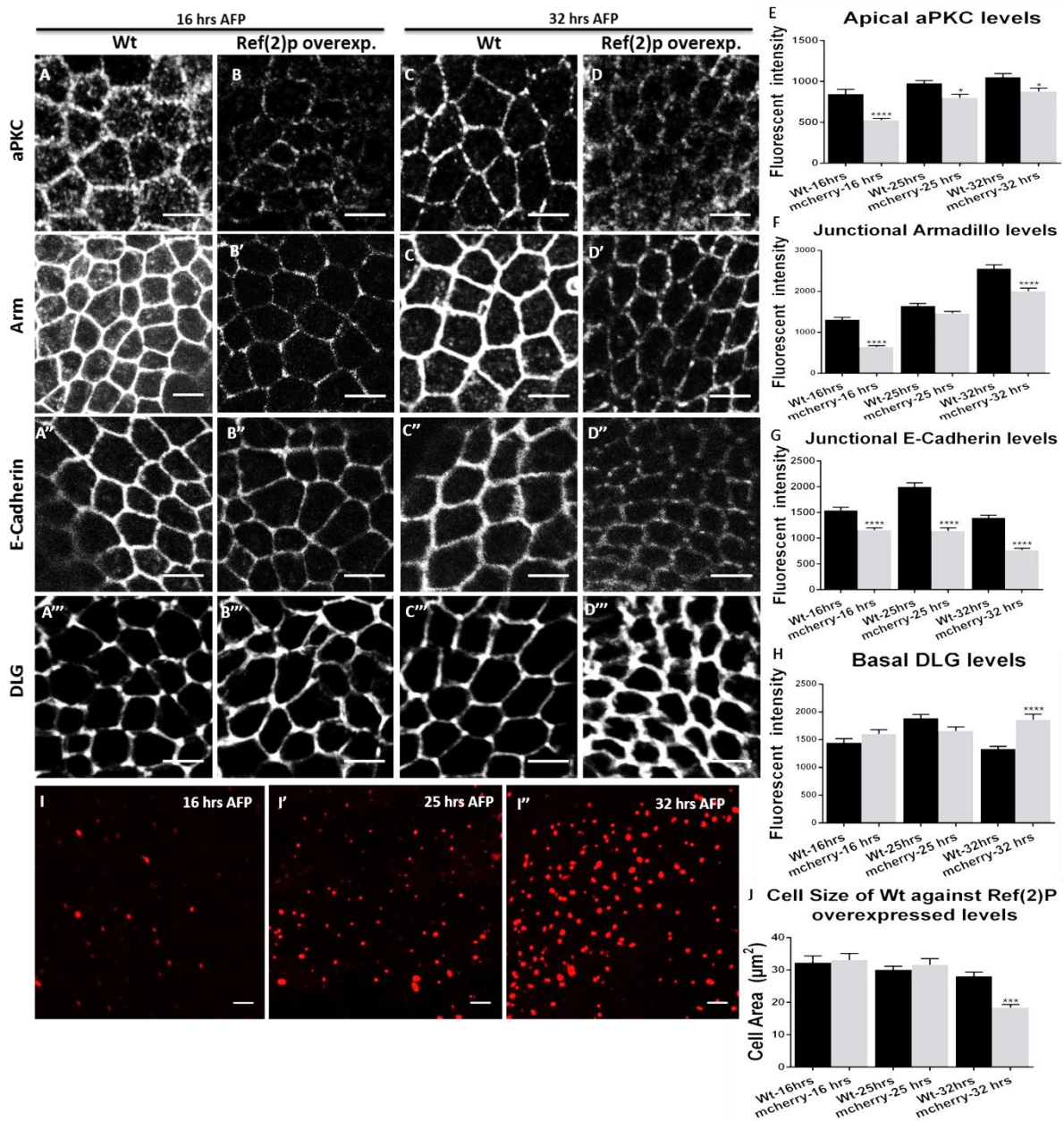


Figure 15: Ref(2)p effects on polarity proteins and junctions upon aging

This study showed the effects of overexpressing *Ref(2)p* upon aging in comparison to wild type as the left panel shows wild type cells (A) and *Ref(2)p* overexpressing cells (B) at 16 hrs APF. The right panel shows wild type cells (C) and *Ref(2)p* overexpressing cells (D) at 32 hrs APF. *Ref(2)p* overexpression showed a decrease in aPKC, E-Cadherin, Arm, however an increase in DLG was detected (E-H) respectively. In terms of cell size no significant difference occurred at the age of 16 hrs and 25 hrs APF, however a significant decrease observed at the age of 32 hrs APF in *Ref(2)p* overexpression (J). mCherry flag-tagged *Ref(2)p* overexpressed under the control of UAS promotor forms Ref(2)p aggregates which increase in number and size over time (I). Scale bars represent 5 μms (P value *** and **** < 0.001).

question was whether Ref(2)p's role in autophagy has an effect on regulating polarity proteins, especially as *od3* mutation, which lacks the UBA domain and considered as an essential domain in autophagy, showed a decrease in aPKC levels and its mis-

localization. Flies with reduced Atg1 (through RNAi) or increased Atg8a (UAS-*Atg8a* overexpression) were used as an autophagy deficient model and autophagy overactive model respectively. Atg1 is very important in the early stages of Autophagy initiation and Atg8a is important for autophagosomes formation [44]. Strikingly, in comparison to wild type (Figure 14-A) an unexpected increase was observed in aPKC levels in cortical and cytoplasmic compartments of *Atg1* RNAi (Figure 14-B) cells accompanied with an increase in E-Cadherin and decrease in DLG. In contrast, *Atg8a* overexpressing cells (Figure 14-C) showed the opposite phenotypes (Figure 14-D-F). This is an indication that aPKC is regulated by autophagy degradation ubiquitously. In terms of cell size an increase was found in *Atg1* RNAi and a decrease was found in *Atg8a* overexpressing cells (Figure 14-H). Interestingly, when cell size decreases and DLG levels increase, as in *Atg8a* overexpression (Figure 14-C''), SJs integrity is affected. Abnormal junctional breaks and discontinuities were observed in both autophagy deficient and autophagy overactive cells. Further tissue folding (Figure 14-G) was found in both *Atg1* RNAi and *Atg8a* overexpressing epithelial sheets. This could be interpreted as a disturbance in polarity along the apico-basal axis of the epithelial cells, including aPKC in the apical domain, which has been proven to have role in junctional stability [24].

More investigations were performed in *Ref(2)p* overexpressing epithelial cells as another autophagy overactive model to examine polarity protein levels relative to autophagic activity over time. Therefore, epithelial cells of fly notum overexpressing mCherry Ref(2)p protein were dissected and stained for different polarity proteins including aPKC, E-Cadherin, Arm and DLG at different pupal ages including 16 hrs, 23 hrs and 32 hrs APF. Overexpressed flag-tagged mCherry *Ref(2)p* showed aggregates of Ref(2)p that increased over time in number and size (Figure 15-I). Interestingly, *Ref(2)p* overexpression phenocopied *Atg8a* overexpression (a decrease in aPKC, E-Cadherin and Arm levels, as well as an increase in DLG levels) (Figure 15-E-H). Junctional abnormalities and junctional discontinuities were found (Figure 15-D'-D'') together with tissue folding (Figure 16-C). For additional confirmation, clones were generated for *Ref(2)p* overexpressing cells and these pupae were dissected at the age of 25 hrs APF. An apparent decrease in aPKC levels and E-Cadherin levels were detected in these clones of *Ref(2)p* overexpressing cells (Figure 16-A), besides breaks in the junctions appearing in E-Cadherin staining. Additionally, wild type cells showed breaks in the junctions shared with mutant cells, where a wild type cell is facing a mutant cell (Figure 16-B). A slight decrease in cell size could be observed, however it is not significant and the significant decrease was detected at older pupal ages.

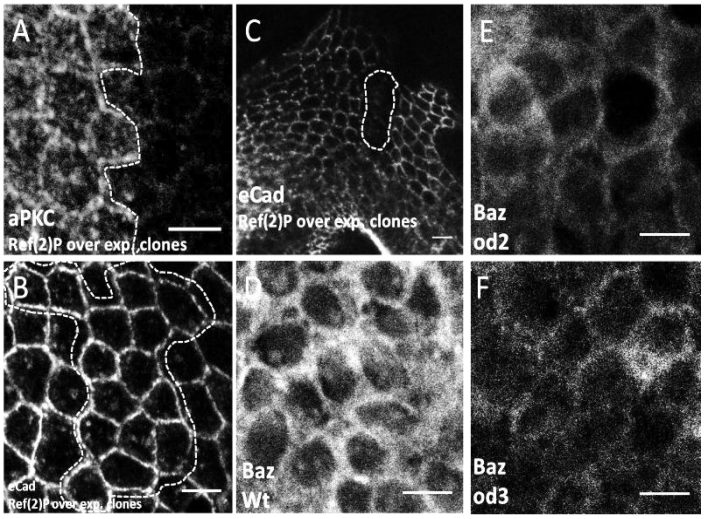


Figure 16: *Ref(2)p* overexpressing clones and Baz staining

Ref(2)p overexpressing clones were generated using MARCM system and showed an apparent decrease in aPKC and E-Cadherin levels (A-B) and Junctional breaks in E-Cadherin staining (B). These tissues were imaged at the age of 25 hrs APF, therefore the cell size was still not significant. Tissue folding occurred in *Ref(2)p* overexpressing cells (C). Baz levels decreased in *od2* (E) and *od3* (F) mutants in comparison to wild type (D) scale bars represent 5 microns.

Then the morphology of individual cells in *Ref(2)p* (Figure 17-B) and *Atg8a* (Figure 17-C) overexpressing flies were studied using the moe-GFP reporter together with Neu-Gal4. This study showed almost all lamellepodia were abolished at the intermediate level and when *Ref(2)p* overexpressing cells were imaged at the age of 12 hrs APF an increased number of filopodia (Figure 17-D) were identified, however at older ages at around 16 hrs APF the filopodia starts to diminish. The overall protrusion numbers decreased in comparison to wild type cells (Figure 17-A). Both *Ref(2)p* and *Atg8a* overexpressing cells were found to be longer than wild type and interestingly apical protrusions were observed in *Ref(2)p* overexpressing cells. A decrease in the apical area was apparent in *Atg8a overexpressing cells*, however this was not the case in *Ref(2)p* overexpressing cells as the significant decrease in cell size was observed at later stages, at the age of 32 hrs APF.

4.3. Ref(2)p role as a tumour repressor and in cell division

There is a relationship between cell polarity and cell invasion found in tumour metastasis [59]. Ref(2)p has a role in regulating cell polarity and interestingly, occasional invasive cells were traced over time underneath the epithelial sheet lamina in *Ref(2)p* mutants, *Ref(2)p* RNAi and *Atg1* RNAi. In this case the mutant cells are no longer polarized. In contrast, in wild type the cells are not migratory, as they maintain their polarity (Figure 18).

Interestingly, cell division could be investigated in *Ref(2)p* mutant flies and *aPKC* null flies through generating *od2 Ref(2)p* and *od3 Ref(2)p* homozygous mutant flies, and *aPKC* null flies combined with Pnr-Gal4-UAS-moe-GFP and Ubi-His-RFP. The MARCM system was used to generate *aPKC* mutant clones, as homozygous mutants of null *aPKC* are not viable. Cell division was found to take longer, about 9 mins in both *Ref(2)p*

mutants and *aPKC* null cells, whereas in wild type it took around 6 mins (Figure 20-A-D). Strikingly, mitotic blebbing was observed in *Ref(2)p* mutants and *aPKC* null cells (Figure 20-B-D) respectively, which similar to that observed in *SCAR* mutants [60]. Additionally, defective chromosome segregation during mitosis was observed in 25% *od3 Ref(2)p* mutant cells and 12.5% in *aPKC* null cells analysed (Figure 21-B-C).

5. Discussion

5.1. Ref(2)p has a role in maintaining cell junctions, cell shape and cell morphology

Apico-basal polarity has a significant role in the maintenance of epithelial cell shape, cell junction integrity and cell morphology. Polarity is essential in controlling cell behaviour, cell symmetry and asymmetry directing several cellular events, such as cell division or cell migration [2, 3]. Once the columnar epithelial cell is polarized it has distinct sub-cellular regions including the apical, junctional and baso-lateral domains [58]. The Par complex including Baz/Par6/aPKC is considered as a central polarity complex in maintaining the apico-basal axis of epithelial cells and apical domain identification. Also, aPKC as part of Cdc42-Par6-aPKC has a regulatory role on AJ stability, and actin cytoskeleton organization [24]. p62, the Ref(2)p homologue in mammalian cells, has a PB1 domain at its C-terminus which allows it to oligomarize with itself or interact with different kinases including aPKC in NF-kappa β activation [41]. Alongside the high homology reported between Ref(2)p and p62, a physical interaction between Ref(2)p and aPKC via PB1 was also revealed in toll-signalling pathway in *Drosophila* that is similar to NF-kappa β signalling pathway in mammalian cells [40].

Drosophila is in ideal model system to study p62/aPKC signalling as it has only single isoforms of Ref(2)p and aPKC. Using immunofluorescence in *Ref(2)p* mutants, *od2* and *od3*, and *Ref(2)p* RNAi showed an increase in cell size accompanied with junctional breaks and a global decrease in several polarity proteins including aPKC in the apical domain, E-Cadherin and Arm at the AJ and DLG at the SJ, within the basolateral domain. Epithelial tissue folding is another phenotype detected in different *Ref(2)p* mutants. Further investigation of aPKC and E-Cadherin levels and cell size in relation to Ref(2)p were performed by generating positively marked clones of *Ref(2)p* RNAi in a background of wild type cells using MARCM system, Pnr-Gal4 and UAS-moe-GFP. These clones confirmed a decrease in aPKC and E-Cadherin besides increase in cell size. These phenotypes match along with the phenotypes seen in *aPKC* mutant clones of increasing

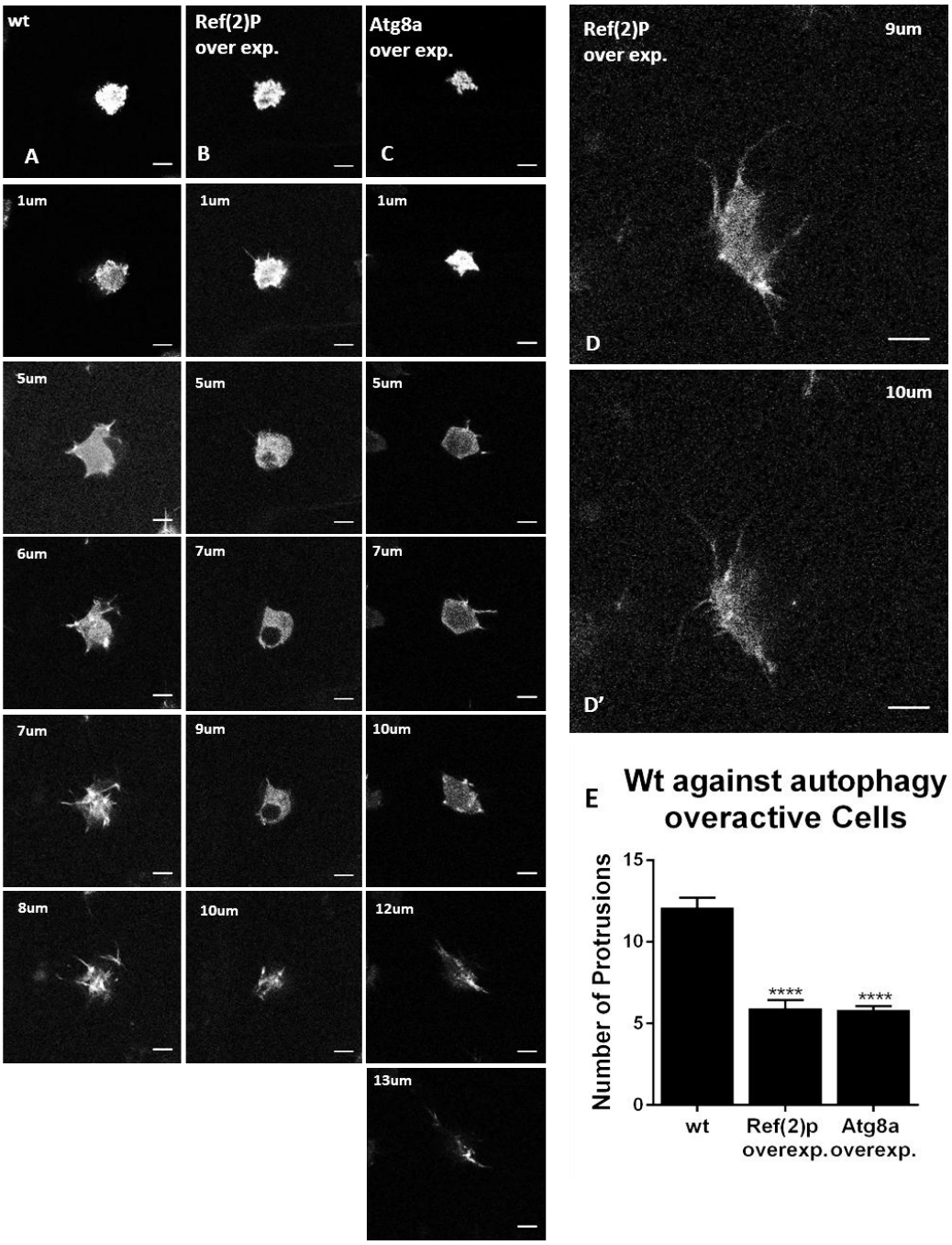


Figure 17: *Ref(2)p* and *Atg8a* overexpressing cells as an autophagy model for studying cell morphology

The Neu-Gal4 driver was used to study the individual cell morphology in *Ref(2)p* (B) and *Atg8a* (C) overexpressing cells. Both *Ref(2)p* and *Atg8a* overexpressing cells showed an increase in cell length and fewer protrusions when compared to wild type. (E). Also both mutants showed almost loss of the intermediate protrusions at the age of (16 hrs APF) *Atg8a* overexpressing cells showed a decrease in apical area. In *Ref(2)p* at early stages (12 hrs APF) of pupal development (D-D') many filopodia appeared which disappeared at later stages (16 hrs APF) (B). Apical protrusions were observed in *Ref(2)p* overexpressing cells (B). Scale bars represent 5 microns (P value ****< 0.001).

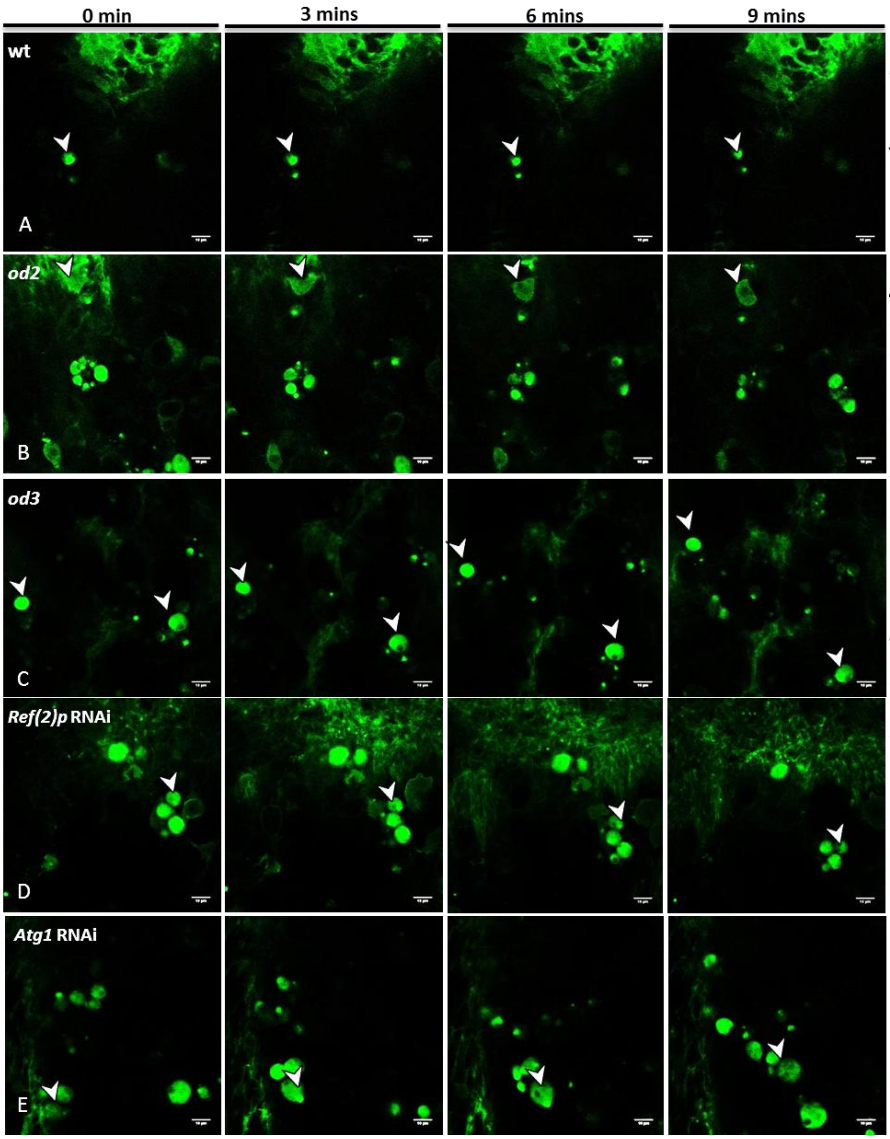


Figure 18: Ref(2)p and cell invasion

To study the involvement of Ref(2)p in cell invasion *Ref(2)p* mutants (B-D) and *Atg1* RNAi (E), as well as wild type (A), were crossed to Pnr-Gal4-UAS-moe-GFP flies to drive GFP expression in cytoskeleton and visualize epithelial cells in fly notum in GFP. *Ref(2)p* mutants showed migratory invasive cells under the epithelial lamina as seen in *Atg1* RNAi, however wild type cells were polarized. Scale bars represent 5 microns.

in the cell size, appearance of ectopic junctions with discontinuities and epithelial tissue folding. Therefore Ref(2)p with its two domains PB1 domain and UBA domain are important for maintaining epithelial cell shape and junctional integrity. The phenotypes observed in *od2 Ref(2)p* mutants and *Ref(2)p* RNAi are suggested to be due to the failure of interaction between aPKC and od2 Ref(2)p mutant protein via PB1 domain, so mis-localization of aPKC from the apical domain and consequently the disturbance in other polarity proteins levels (Figure 19). Notably, these phenotypes were found in *od3* mutation as well. This suggests that the UBA domain in Ref(2)p has importance ,as well as the PB1, domain in maintaining polarity. An increase in cell size was reported in osteoclasts in Paget's disease where the p62 UBA domain is mutated [61]. Interestingly, it has been revealed recently that p62 has a role in epithelial phenotypic alteration and controlling junctional protein levels in mammalian epithelial cells such as MDCK cells [62].

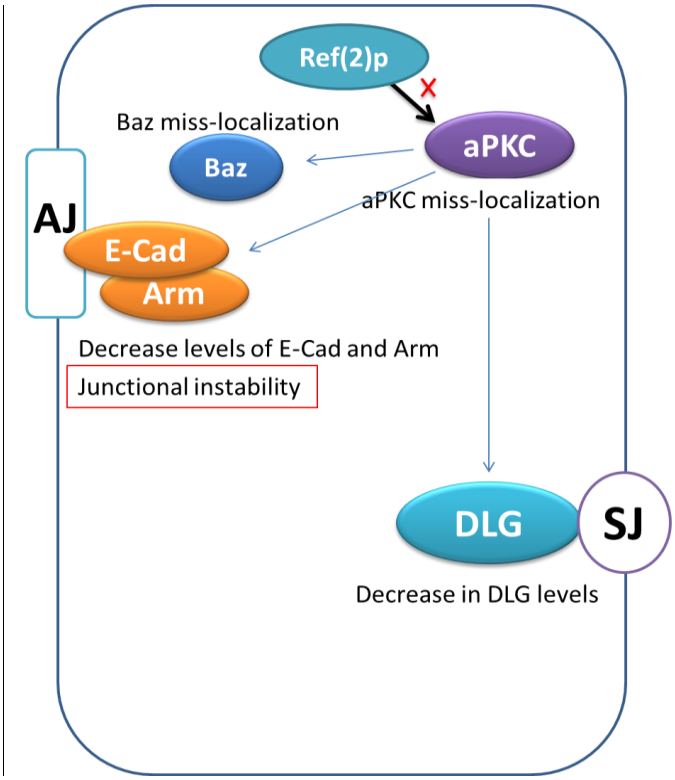


Figure 19: Proposed model of polarity proteins disturbance in *Ref(2)p* mutants and RNAi

The diagram shows aPKC miss-localization effects, due to the failure of the interaction with Ref(2)p in both *od2* and *od3* mutations and RNAi, on other polarity proteins including Baz, E-Cadherin, Arm and DLG.

Turning to cell morphology, different phenotypic differences in cell morphology were detected in *Ref(2)p* mutants. *Ref(2)p* mutants effects on cell shape phenocopied those of *aPKC* mutant cells, such as a decrease in apical area, an increase in cell length, and a decrease in protrusion number. *aPKC* mutant cells lose almost all protrusions, due to a disruption of the actin cytoskeleton; aPKC as a part of Cdc42-Par6-aPKC is involved in maintaining AJ stability and actin cytoskeleton organization. A mutation in any of the proteins in this complex manifests actin cytoskeleton defects [28]. A significant variance in protrusions dynamics were observed in *Ref(2)p* mutants including *od2* and *od3* with a significant increase in retraction rate, however no significant effect was found in protrusions extension rate. Dynamic apical protrusions were observed in both *Ref(2)p* mutants and considered abnormal in comparison to wild type. These phenotypes are suggested to be due to the mis-localization of aPKC as a failure of interaction with Ref(2)p mutants. In regard to the apical protrusions observed in both *Ref(2)p* mutants, aPKC role in recruiting Baz at the junction by phosphorylation might be implicated in that. This interpretation for the apical protrusions phenotype is because *aPKC* null epithelial cells showed decrease in Baz levels at the junctions and junctional instability [5]. As mentioned before from unpublished work in Georgiou lab Baz is required to generate Rac gradient along the apico-basal axis with highest Rac activity at the basal domain and less Rac activity moving up to the apical domain of the cells (personal communication). Rac is responsible for crowded filopodial protrusion formation at the basal domain of the cells and lamellepodia at the intermediate level. Fewer protrusions

are found closer to the apical domain [28]. To have a more accurate answer for that; staining for Baz in both *Ref(2)p* mutants were performed and the results showed mis-localization of Baz at the junctions (Figure 16-D-F).

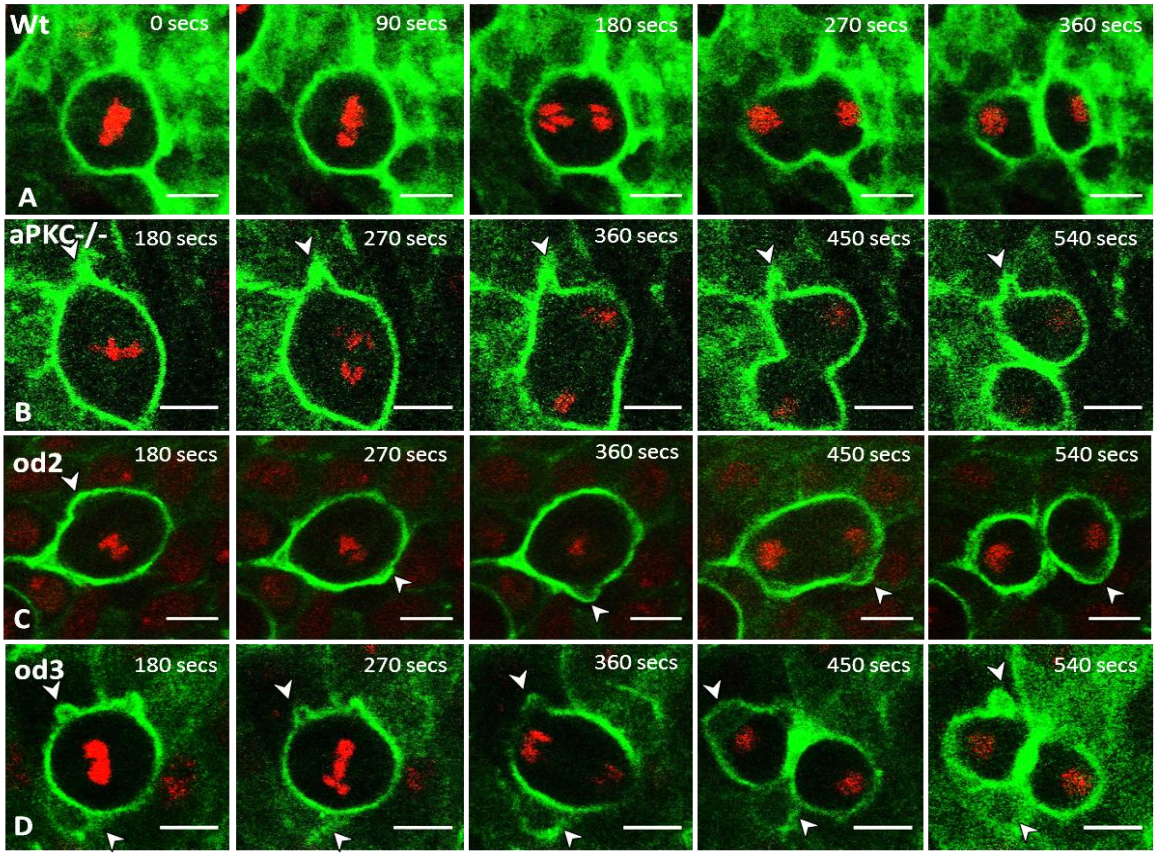


Figure 20: Ref(2)p impacts on cell division

Through a number of crosses *Ref(2)p* homozygous mutants and null *aPKC* mutant flies were combined with Pnr-Gal4-UAS-meo-GFP and Ubi-His-RFP. And this generated a model for studying cell division. *od2* mutation (B), *od3* mutation (C) and *aPKC* null epithelial cells showed decrease in cell division rate as they took longer time for dividing in 540 sec in comparison to wild type dividing in 360 secs (A) Also, all mutants showed blebbing of the cells during division (arrowheads). Scale bars represent 5 microns

Interestingly, Ref(2)p, an important cargo in selective autophagy, besides Atg1, a main autophagy mediator, have been shown to have a role in protrusion formation in *Drosophila* haemocytes, which is important for haemocyte spreading in epithelial wounds. Given the pervious information, Ref(2)p was suggested to mediate autophagic degradation of ubiquitylated substrates, that has role in protrusion formation [33]. It is known that Rho GTPases are involved in protrusion regulation and actin cytoskeleton organization [28] and therefore an interrelation between Rho components pathway and autophagy pathway has been suggested [33]. The molecular mechanism of Ref(2)p involvement in protrusion formation is still unclear, however the work done in this study gives more insight into the context of the mechanistic explanation. As aPKC has been shown before to be essential in protrusions formation [24] and this study elucidated the

importance of the interaction occurs between Ref(2)p and aPKC in maintaining aPKC localization apically. The mis-localization of aPKC in *Ref(2)p* mutants and *Ref(2)p* RNAi is a possible explanation of protrusions defects and decreased numbers observed in *Ref(2)p* mutants. This is applied in *od2* mutation and *Ref(2)p* RNAi whose lack PB1 domain, via which we hypothesized Ref(2)p would interact with aPKC. Surprisingly, *od3* mutation, that lacks the UBA domain and is an important domain in autophagy signalling, showed aPKC mis-localization as well. These findings suggest that both PB1 and UBA domains of Ref(2)p have a role in localizing aPKC apically. Ref(2)p mutants showed similar phenotypes to those observed in any of *Cdc42-Par6-aPKC* mutants including junctional breaks, cell morphology changes and epithelial tissue folding [24, 28]. Because of the phenotypic similarity this underlies a potential role of Ref(2)p in regulating actin cytoskeleton as Cdc42-Par6-aPKC has a role in regulating actin cytoskeleton and actin based protrusions formation [28]. The next step was to think

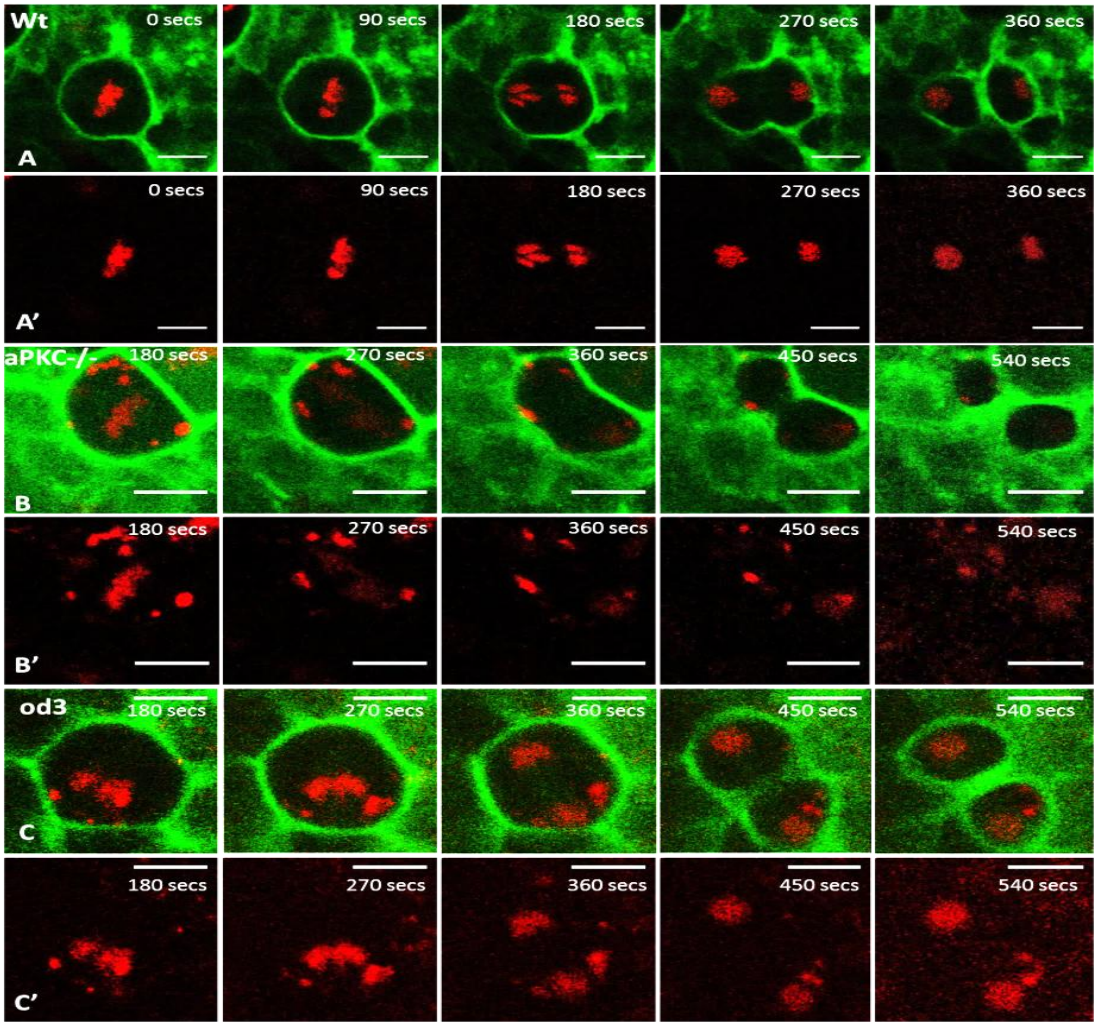


Figure 21: Ref(2)p and chromosome segregation

Defects in chromosome segregation appeared in *aPKC* null cells and *od3* homozygous mutants as shown in (B-C) respectively compared to normal wild type chromosome segregation in (A). Scale bars represent 5 microns.

about whether these phenotypes observed in *Ref(2)p* mutants are due to only mis-localization of aPKC as a failure of interaction with Ref(2)p or there might be a broader influence of Ref(2)p mediated autophagy.

5.2. Autophagy is implicated in regulating polarity proteins and consequently protrusion formation

Autophagy is a catabolic cellular process to degrade unwanted macromolecules as ubiquitylated proteins and non-functional organelles [63]. Ref(2)p is an important mediator in selective autophagy as it acts as a cargo for delivering ubiquitylated proteins and organelles to autophagosome [64]. Ref(2)p further localizes and has a role in brain protein aggregate formation upon flies aging and in autophagy deficient flies [45] and in epithelial cells of flies notum as well (Figure 15-I). Previous research work has shown that aPKC degradation is mediated by ubiquitylation in yeast [65] and *Drosophila* [33]. Surprisingly, the results showed that aPKC levels/localization (together with other polarity proteins) are very much affected by the level of autophagic activity. An increase in aPKC and E-Cadherin levels and a decrease in DLG levels were seen in *Atg1* RNAi cells, whereas there were a decrease in aPKC and E-Cadherin levels and an increase in DLG levels in *Atg8a* overexpressing cells. Finding in this study suggests that DLG levels oppose the levels of apical and junction proteins including aPKC, E-Cadherin and Arm as observed in these experiments. It is unclear why this is not the case in *Ref(2)p* mutants as an overall decrease in aPKC, E-Cadherin, Arm and DLG levels were observed. *Ref(2)p* overexpressing cells phenocopied *Atg8a* overexpressing cells in terms of polarity proteins levels, junctional breaks, cell size, and apical area. For further confirmation *Ref(2)p* overexpressing clones were generated using the MARCM system and at the age of 25 hrs APF showed a decrease in aPKC levels and junctional breaks, however a significant decrease in cell size only became apparent at the age of 32 hrs APF. Taken together, this suggests that autophagy has a role in the ubiquitin mediated degradation of aPKC and consequently has a role in regulating other polarity proteins downstream aPKC. The homeostasis of polarity proteins in the normal levels is essential to maintain apico-basal polarity and junctional stability as disturbance of apico-basal polarity were subsequent to increase or decrease of aPKC, E-Cadherin and DLG without keeping the orchestration between them. Interestingly, in *Ref(2)p* overexpressing cells aPKC decreased levels was accompanied with a decrease in Arm levels, as Arm is dependent on aPKC activity. It has been reported that increasing the activity of aPKC will increase the levels of Arm as Arm is activated and phosphorylated by aPKC [66].

Because of the decreased levels of aPKC observed in autophagy overactive cells, as expected, looking at cell morphology of *Atg8a* and *Ref(2)p* overexpressing cells, the findings were close to *aPKC* mutant cells morphology phenotypes of losing protrusions

and decreasing in apical area of the cell [24]. Both *Atg8a* and *Ref(2)p* overexpressing cells showed a decrease in protrusion number. Sometimes almost no protrusions detected in *Ref(2)p* overexpressing cells. Interestingly, at earlier stages of 12 hrs APF of pupae development the cells lose their lamellepodia at the intermediate level, however the basal domain showed increase in filopodia number, which disappeared at later developmental stages at the age of 16 hrs APF. Apical protrusions were identified in *Ref(2)p* overexpressing cells, which relied on the disturbance upon decrease in aPKC levels and consequently mis-localization of Baz from being recruited at the junctions and then the Rac activity gradient lost and apical protrusions starts to appear [5, 28].

In terms of cell size, an increase in the cells size was found in *Atg1* RNAi representing an autophagy deficient model, however decease in cell size was seen in *Atg8a* and *Ref(2)p* overexpressed representing an autophagy overactive model. Supportive to these findings in *Drosophila* larvae stage fat body cells showed a decrease in cell size in *Atg1* overexpression and a growth advantage in *Atg1* mutant cells [67]. Additionally, reduced size or shrinkage of midgut cells in larvae requires autophagy [68]. *aPKC* mutant cells showed an increase in cell size, however autophagy signalling has the upper hand in regulating cell size, as autophagy deficient cells showed higher increase in cell size rather than the increase in cell size seen in *aPKC* null cells, although aPKC levels were high. Additionally, autophagy overactive cells showed a decrease in cell size accompanied with decrease in aPKC level. Therefore, aPKC has a role in regulating cell size; however autophagic activity has the upper hand in controlling the cell size. One of the pathways that regulates both autophagy and cell growth relatively to cell size is mTor signalling pathway [69]. According to the results presented above, interpreting the increase in cell size in *Ref(2)p* mutants could be as follows; in *od2* mutation the increase in the cell size is merely due to the mis-localization of aPKC in the apical domain, while the increase in cell size in *od3* mutation and *Ref(2)p* RNAi could be suggested due to autophagy deficiency, as well as aPKC mis-localization.

5.3. Ref(2)p localizes apically and requires both PB1 and UBA domains to localize aPKC apically

The results revealed that there is mis-localization in aPKC form the apical domain in *od2*, which is interpreted by the lack of the interaction between Ref(2)p and aPKC via PB1 domain as the od2 mutant protein lacks the PB1 domain. This interaction is important in NF-kappa β signalling pathway [40], however this study proved a further role of this interaction for localizing aPKC apically. The increase in cell and junctional discontinuities in the *od2* mutant is an issue of localizing aPKC apically and autophagy is not involved in that. Surprisingly, the *od3* mutant also showed a decrease in aPKC apical levels and yet *od3* mutant cells are considered as autophagy deficient for lacking

UBA domain. On the contrary, in our autophagy deficient model (*Atg1* RNAi) we observed an increase in aPKC levels in both the cortical and cytoplasmic compartments of the cells, which is not the case in *od3* mutation although it is considered as autophagy deficient. This provides evidence that the Ref(2)p UBA domain, as well as the PB1 domain, has a role in localizing aPKC apically. Further evidence that support these findings is Ref(2)p localization along the apico-basal axis of the epithelial cells. Staining for a number of polarity determinants along the apico-basal axis and live imaging pupae of expressing GFP labelled E-Cadherin with mCherry Ref(2)p under the control of UAS control, revealed that Ref(2)p localizes almost apically and its levels decreases moving down to basal domain.

5.4. Ref(2)p implication in tumour repression and cell division

There is an intimate connection between the loss of cell polarity and cell invasion as a phenotype of tumour progression and metastasis [59]. It has been revealed that activating *aPKC* inappropriately leads to the appearance of tumorigenic phenotypes, similar to tumour suppressor mutant phenotypes including *DLG* and *Lgl* [66]. Several studies were done on p62 to investigate its role in cancer; some studies showed that lower level of p62 leads to cancer progression, however others showed that p62 overexpression leads to cancer, which means that p62 levels must be regulated appropriately [42]. As Ref(2)p does affect cell polarity and *Ref(2)p* mutants have shown polarity defects, it is expected to be a tumour repressor. To investigate cell invasion in *Ref(2)p* mutants and *Atg1* RNAi*, they* were crossed with Pnr-Gal4-UAS-moe-GFP. Unlike wild type, in *Ref(2)p* mutants and *Atg1 RNAi* the invasive unpolarised cells are broken up from the epithelial sheet below epithelial lamina and start to migrate over time. This is an indication of losing the polarized nature of cells in the epithelial sheet, while wild type cells maintain their polarity and they stay in the epithelial sheet.

Looking at cell division in *Ref(2)p* mutants and *aPKC* null cells revealed a decrease in cell division rate as the cell division takes longer than normal, which might be a possibility of actin cytoskeleton failure in cytokinesis. Also, some other determinants that affect the cell division could be considered as microtubules and chromosome alignment. Further, mitotic blebbing was found in both *od2* and *od3 Ref(2)p* mutants, as well as in *aPKC* mutants. Interestingly, this phenotype of mitotic blebbing is very similar to that seen in *SCAR* mutants during cell division as SCAR has an important role in regulating mitotic cell division and it is further implicated in cell adhesion and cell migration by activating Arp2/3 complex [60]. Cdc42-Par6-aPKC were shown to regulate Arp2/3 for maintaining junctional and actin cytoskeleton stability, thereby aPKC might act upstream of SCAR indirectly affecting other effectors of SCAR, such as Arp2/3. Additionally, the blebbing phenotypes seen in both *Ref(2)p* mutants confirms the

importance of both Ref(2)p domains in localizing aPKC apically in the apico-basal axis of the cells. This suggests that Ref(2)p acts upstream SCAR and regulates SCAR effectors indirectly via aPKC in either of two ways. First, Ref(2)p localizes aPKC apically then aPKC with Cdc42 and Par6 regulate Arp2/3 which is one of the SCAR effectors. The other way is that aPKC when localized apically; it recruits Baz to the junctions and, from recent unpublished work in Georgiou lab, Baz keeps the Rac gradient along the apico-basal axis of the cells (personal commination). Rac is responsible for activating SCAR [26]. So, the suggested signalling is either Ref(2)p/aPKC/Arp2/3 or Ref(2)p/aPKC/Baz/Rac/SCAR/Arp2/3 (Figure 1-D), which need further investigations.

In order to enable daughter cells in mitosis to maintain the continuance of life, replication and segregation of the genetic material are essential [70]. Centrosomes, microtubules based mitotic spindles, and kinetochores at centromeres of opposite chromatids are essential in chromosome segregation [71, 72] and any imbalance occurs in any of these structures contribute to chromosome mis-segregation and aneuploidy [73]. Chromosome segregation defects underlie chromosomal instability, which observed in various types of cancer cells [73]. Interestingly, a defect in chromosome segregation is observed during mitotic cell division in both *od3* mutants and *aPKC* null epithelial cells, which might be multinucleated as well and this is a further evidence of a possible disruption of cytokinesis or microtubules based spindle defects and kinetochore mis-attachment in *Ref(2)p* mutants and *aPKC* null cells during cell division. Comparing to *SCAR* mutants phenotypes, when SCAR loses its Abi subunit the cells become multinucleated which indicates a defect in cytokinesis [74]. This gives an indication that Ref(2)p dependently or mediated by aPKC may affect one of the chromosome segregation determinants including centrosome, kinetochores attachment and mitotic microtubule spindles, which require further investigations. A collective comparison of phenotypes observed in *aPKC*, *Ref(2)p* and autophagy mutants is illustrated in (Table 1).

Table 1: Phenotypes comparison of *aPKC*, *Ref(2)p* and autophagy mutants

Phenotypes	Autophagy		Ref(2)p				aPKC
	Atg1 RNAi	*Atg8a* overexpr.	*Od2*	*Od3*	*Ref(2)p* RNAi	*Ref(2)p* overexpr.	*aPKC -/-*
Cell size	high increase	decrease	increase	increase	increase	decrease	increase
Junction breaks	yes	yes	yes	yes	yes	yes	yes
No. of Protrusion	_____	severe decrease	decrease	decrease	_____	severe decrease	lost
Apical Protrusions	_____	no	yes	yes	_____	yes	no
aPKC levels	increase	decrease	decrease	decrease	decrease	decrease	lost
Arm Levels	_____	_____	decrease	decrease	decrease	increase	_____
eCad levels	increase	decrease	decrease	decrease	decrease	decrease	decrease
DLG levels	decrease	increase	decrease	decrease	decrease	increase	_____
Blebing during cell division	_____	_____	yes	yes	_____	_____	yes
Cell division rate	_____	_____	decease	decease	_____	_____	decease
Chromosome Segregation	_____	_____	No	yes	_____	_____	yes
Cell invasion	yes	_____	yes	yes	yes	_____	_____

6. Conclusion

Ref(2)p, the fly homologue of p62, is a multifunctional protein [38], since it has been shown to be involved in the toll signalling pathway [40], localized and implicated in brain protein aggregate formation in autophagy deficient and old flies [45], and protrusions formation [33]. The work done in this study provides another potential of Ref(2)p multifunctional nature in cellular processes. Ref(2)p localizes at the apical domain of epithelial cells and has a role in regulating apico-basal polarity. It requires both the PB1 and UBA domains in localizing aPKC apically. Consequently, Ref(2)p has a role in maintaining junctional stability, cell size, and protrusions formation and dynamics. These phenotypes are similar to those observed in *Cdc42-Par6-aPKC* mutants and they were shown to have a role in regulating the actin cytoskeleton [24]. This study provides greater insight into the molecular mechanism of Ref(2)p in protrusions formation, which is regulated by a number of polarity proteins, Rho GTPases and cytoskeletal regulators. Strikingly, Ref(2)p mediated autophagy has a potential role in regulating aPKC levels and other polarity determinants, so again the same phenotypes that were observed in *Ref(2)p* mutants were also detected in autophagy deficient and autophagy overactive cells. Polarity protein homeostasis should be very tightly regulated as an increase or decrease in concentration, or mis-localisation of any particular polarity protein will affect the distribution of other polarity proteins and as a result will affect cell junction stability, protrusion formation and cell size. As a cell polarity regulator Ref(2)p additionally has been revealed as a tumour suppressor, since invading cells were detected in *Ref(2)p* mutants. Further *Ref(2)p* mutants, as well as *aPKC* mutants, showed defects in chromosome segregation during cell division. Cell division itself took longer, therefore the rate of cell division decreased and a blebbing of cells was observed during cell division as well. This phenotype of blebbing is similar to the phenotype observed in *SCAR* mutants [60], which suggests that Ref(2)p mediated by aPKC acts upstream of SCAR indirectly on one of its effectors, Arp2/3.

7. References

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Appendix 1: Validating *Ref(2)p* mutant flies.

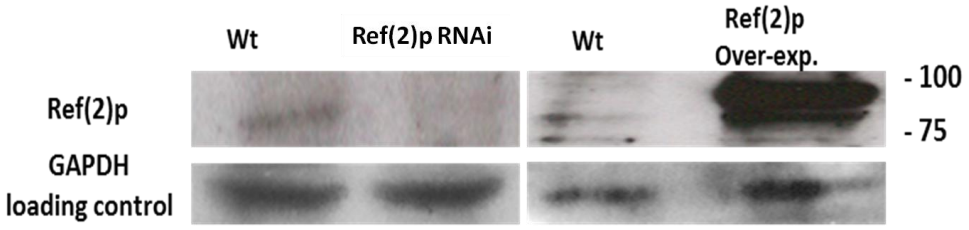

Figure 22: Immunoblotting for *Ref(2)* RNAi and *Ref(2)p* overexpressed

Western blotting was done for *Ref(2)p* RNAi flies left panel (A) and *Ref(2)p* overexpressing flies right panel (B).

Appendix 2: Balancer Chromosomes

Table 2: [75] Balancer Chromosomes and Genetic Markers

Name	Chromosomes	Type	Phenotype
CyO (Curly of Oster)	Second	Balancer	Curly wing
TM3, Ser	Third	Balancer	Stubble (short bristles) or serrate wings
Tm6b	Third	Balancer	Tubby (short, fat body), Humoral (extra macrochaetes)
IF (Irregular Facets)	Second	Marker	Small rough eyes with irrigular shape
MKRS	Second	Marker	Stubble
Dr	Third	Balancer	Nearly eyeless with very small and rough eyes

Appendix 3: Full Genotypes of Stocks

Table 3: Full Genotypes of Stocks

Stock Number	Genotype	Origin
M579	;od2/CyO	Georgiou Lab
M578	;od3/CyO	Georgiou Lab
M520	Ubx-flp; IF/CyO-GFP; MKRS/TM6B	Georgiou Lab
M595	;;PnrGal4/Tm6b	Georgiou Lab
M59	;;P[mw,Gal4][neu], P[mw,UAS-Moe-GFP] / TM6b;;*	Georgiou Lab
	Yw;sp/cyo;UAS-mCherry-Ref(2)P/TM3, Ser	Ioannis Nezis
	y^1,w^{1118}; P{UASp-mCherry-Atg8a}2; Dr^1/TM3; Ser^1	Bloomington 37750
	y^1v^1; P{TRiP.JF02273}attP2	Bloomington 26731
M482	ubxflp; tubgal80, FRT40A/CyO-GFP; MKRS/TM6b	Georgiou Lab
M539	Ubx-flp; FRT40A/CyO-GFP; PnrGAL4/TM6b	Georgiou Lab
M580	;P{KK105338}VIE-260B	VDRC (108193)
M405	FRT19A, tub-Gal80, hsFLP,w; IF/CyO-GFP	Georgiou Lab
M413	ubi-GFP, FRT19A; IF/CyO-GFP; MKRS/TM6B	Georgiou Lab
M286	w;; Pnr-Gal4, UAS-moe-GFP/TM6b	Georgiou Lab
M383	w, Ubx-FLP; IF/CyO-GFP; Pnr-Gal4, uas-moe-GFP/TM6b	Georgiou Lab
M42	w;P[Ubi-p63E-cad-GFP][5];;;*	DGRC Kyoto
M96	w;;P[mw,Ubi-His-RFP][14.3] / TM6b;;*	M. Georgiou
M147	$w^{[1118]}$;;;;*	Bloomington
M231	y[1] w[*]; P{Act5C-GAL4-w}E1/CyO	Bloomington 25374
M16	Yw, neo-FRT19A;;;*	Bloomington 1744
M13	w;P[mw,FRT][G13],P[mw,Tub-Gal80] / CyO;P[mw,Gal4][neu], P[mw,UAS-tau-GFP] / TM6b;;*	Y. Bellaiche
M132	w;P[mw,FRT][G13],aPKC / CyO;;;*	Y. Bellaiche
M476	Ubx-FLP; FRTG13, aPKC/CyO-GFP; NeuG4/TM6b	Georgiou Lab
M164	w;Ubi-GFP, FRT G13;T155 Flp;;*	B. Baum

Appendix 4: Genetic constructs

Table 4: Genetic constructs

Genetic construct	Function
Pnr-Gal4	Gal4 activates the expression of constructs downstream UAS and is driven by Pnr in the central region of *Drosophila* notum [47].
Tub-Gal80	Gal80 is expressed ubiquitously under the control of Tubulin promotor and represses Gal4 activity [55].
FRT	FRT exists at several sites of chromosome arms and those are the sites where the recombination is induced by Flp [54].
Neo-FRT19A	Neomycin-resistant flies, which grow selectively on G418 food and considered to have FRT19A [54].
Ubx-Flp	Flp, under the control of Ultrabithorax, is induced specifically in cells of the dorsal thorax of the flies [24].
Hs-Flp	Flp is induced by Heat shock [51].
Ubi-nls-GFP	It drives the expression of GFP in the nucleus ubiquitously [51].
UAS-moe-GFP	GFP, under the control of UAS promotor, is driven in actin-binding domain of moesin, so GFP localizes in actin cytoskeleton [28].
Act5C-Gal4	It drives global expression of Gal4 in all cells of the fly [52].
UAS-transgene	Transgene downstream UAS sequence is activated by Gal4 [52]. RNAi contracts and mCherry tagged constructs are under the control of UAS promotor.
Ubi-His-RFP	Ubi-promotor drives the expression of RFP ubiquitously into the full coding region of histone A2.
Neu-Gal4	It drives the expression of GFP into the pI cells and their progeny in *Drosophila* notum [48].
Od2	*Ref(2)p* mutation that lacks the first 80 amino acids due to deletion mutation, with no PB1 domain is the translated protein [35].
Od3	*Ref(2)p* mutation that lacks part of the distal part of second exon and the whole third exon sequence of full Ref(2)p sequence, with no UBA domain is the translated protein [35].
CyO-GFP	It drives the expression of GFP is in abdomen under Curly of Oster promotor control accompanied with curly wings [76].