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MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABPs | Actin Binding Proteins |
| A-P | Anterior-Posterior |
| AJs | Adherens junctions |
| Arm | Armadillo (β-catenin) |
| aPKC | Atypical protein kinase C |
| Baz | Bazooka |
| CP | Capping protein |
| Cpa | Capping protein α |
| Cpb | Capping protein β |
| Crb | Crumbs |
| Dlg | Discs large |
| Dlt | Discs lost |
| E-Cad | E-Cadherin |
| F-actin | Filamentous actin |
| Lgl | Lethal giant larvae |
| Par | Partitioning defective |
| RNAi | RNA interference |
| SAR | Sub apical region |
| Scrib | Scribble |
| SJs | Septate junctions |
| Std | Stardust |
| TSGs | Tumor supressor genes |
| ZA | Zonula adherens |

# ABSTRACT

Epithelial integrity generally depends on the balance between the stability and remodelling of adherens junctions (AJs). Growing evidence indicates that endocytosis has a central role in maintaining AJs in a state of dynamic equilibrium. However, it is not currently understood how AJs remodelling is achieved without losing epithelial integrity. In this work, I found that Capping Protein (CP), which prevents extension of the barbed ends of actin filaments, interacts genetically with atypical protein kinase C (aPKC), a major effector of the Par-aPKC complex, to maintain epithelial integrity in *Drosophila* wing imaginal disc. This genetic interaction appears to have differential requirements along the wing disc, since decreasing CP and aPKC levels induce extrusion and death of wing blade cells, whereas cells in the distal hinge region seem to overproliferate. Interestingly, loss of *CP* results in a significant reduction of aPKC from the apical membrane, suggesting that CP may restrict apical localization of aPKC, which in turn, is required to control AJs dynamics. In addition, similar to *apkc* mutant tissues, loss of *CP* leads to decreased apical levels of the AJs components, E-Cadherin (E-Cad) and Armadillo (Arm), suggesting that both CP and aPKC promote AJs stability by regulating early endocytic events.

Therefore, by promoting the formation of a highly branched actin network, CP could have a putative role in AJs stability and remodelling by restricting aPKC to the apical membrane and by promoting early endocytic events. Taken together, the presented data highlight the crucial synergy between polarity complexes and actin cytoskeleton in regulating AJs dynamics, which is intimately linked to the maintenance of epithelial integrity.



**Keywords:** Capping Protein, atypical protein kinase C, adherens junctions, endocytosis, *Drosophila* wing disc

# RESUMO

Os tecidos epiteliais possuem duas propriedades contraditórias: têm uma arquitectura robusta, necessária para a sua estabilidade, porém exibem uma extensa plasticidade de modo a permitir eventos celulares tais como divisão e morte celular [1, 2]. Quando ocorre uma perda na regulação deste balanço entre estabilidade e plasticidade, as células epiteliais perdem a adesão entre elas e adquirem a capacidade de sobreproliferar. De facto, a maioria dos tumores malignos surge apartir de epitélios com defeitos no controlo da adesão e proliferação [3, 4].

A adesão entre células epiteliais é em parte devida à acção coesiva das junções aderentes (AJs), cuja principal função é ligar o citoesqueleto de actina entre células vizinhas. As AJs circundam a região apical das células e são principalmente compostas pela proteína transmembranar E-Caderina (E-Cad). Através do seu domínio citoplasmático, E-Cad associa-se a β-catenina (em *Drosophila* codificada por *armadillo*, *arm*) e α-catenina, que por sua vez, promove a ligação ao citoesqueleto de actina [5]. Apesar de terem um papel crucial na manutenção da integridade epitelial, recentes estudos têm revelado que as AJs são constantemente remodeladas através de endocitose [6-8], processo celular pelo qual moléculas extracelulares ou associadas à membrana são internalizadas para o interior das células [9, 10]. No entanto, ainda não é compreendido como é que a remodelação das AJs é estabelecida sem que a integridade do epitélio seja perdida.

Na região apical às AJs existem dois complexos proteicos que interactuam entre si de modo a promover a polaridade dos tecidos epiteliais: o complexo composto por Crumbs (Crb), Stardust and Discs lost, denominado por complexo Crb, e o complexo constituído por Bazooka/Par3, proteína cinase C atípica (aPKC) e Par6, designado por complexo Par-aPKC [11]. O complexo Par-aPKC é activado através da associação entre Par6 e a Rho-*like* GTPase Cdc42. Após esta activação, aPKC fosforila Crb, que por sua vez, promove a estabilização das Ajs [12, 13]. Curiosamente, foi demonstrado que a remoção de *cdc42* ou *apkc* em tecidos epiteliais de *Drosophila*, resulta na acumulação

de proteínas apicais associadas à membrana, tais como Crb, em vesículas endocíticas e à redução dos níveis apicais de E-Cad e Arm. Análises genéticas adicionais sugerem que aPKC actua como efector de Cdc42, regulando a endocitose de proteínas apicais, que por sua vez, tem um efeito estabilizador nas AJs durante processos dinâmicos de reorganização celular [14, 15].

Nos tecidos epiteliais, a região apical das células contém uma banda de filamentos de actina, essencial para o suporte das Ajs [2]. O citoesqueleto de actina desempenha um papel fundamental em numerosos processos celulares, tais como regulação da morfologia celular e manutenção da polaridade celular [16]. Dada a sua dinâmica, é necessária uma regulação eficaz, que é desempenhada por proteínas que se ligam à actina (ABPs) [17, 18]. Uma dessas proteínas é o heterodímero *Capping Protein* (CP), composto pelas subunidades α (Cpa) e β (Cpb), que se ligam à região terminal dos filamentos de actina, prevenindo a perda ou adição de monómeros de actina e, desta forma, a sua polimerização excessiva [19]. A remoção de *CP* no disco imaginal da asa de *Drosophila* leva a diferentes fenótipos celulares que são dependentes da região do disco. Enquanto clones de células mutantes para *CP* mantêm a polaridade e sobrevivem nas regiões do *hinge* e *notum*, células mutantes na *wing blade* sofrem extrusão basal e morrem por apoptose [20]. Semelhante ao fenótipo observado em epitélios mutantes para *apkc*, diminuição dos níveis proteicos de *CP* usando a técnica ARN de interferência (RNAi), resulta na acumulação de Crb em estruturas vesiculares [21]. Isto sugere que, tal como aPKC, CP parece regular a endocitose de proteínas apicais. Curiosamente, foi postulado que CP interactua geneticamente com aPKC no disco imaginal da asa [21]. No entado, a ferramenta genética usada para diminuir a activade de aPKC pode ter efeitos inespecíficos [22] e portanto, a possível interacção genética entre CP e aPKC foi novamente analisada.

Desta forma, o presente trabalho tinha como objectivo compreender o mecanismo pelo qual CP pode cooperar com aPKC na manutenção da integridade epitelial do disco imaginal da asa de *Drosophila*. De modo atingir este objectivo, a técnica RNAi sob o controlo do sistema UAS-Gal4, foi usada para diminuir os níveis proteicos de CP em regiões específicas do tecido (García Fernández, B. and Janody, F.

*et al.*, unpublished data), e um alelo mutante termosensível para aPKC ($apkc^{ts}$) foi usado para modular a actividade cinásica de aPKC consoante a temperatura (Martinho, R. *et al.*, unpublished data).

Quando comparado com o fenótipo anteriormente descrito para células com níveis reduzidos de CP, ao diminuir ambos os níveis de CP e aPKC no disco de asa, uma maior quantidade de células sofreram extrusão basal e morreram por apoptose. Pelo contrário, células mutantes para $apkc^{ts}$ encontraram-se mantidas no epitélio, sem nenhum indício de morte celular. Estes resultados confirmam que CP interactua geneticamente com aPKC na manutenção da integridade epitelial do disco da asa. Esta interacção genética parece ter diferentes requerimentos ao longo do disco da asa. Enquanto que a diminuição dos níveis de CP e aPKC levou à morte das células da *wing blade*, as células na região distal do *hinge* pareceram proliferar. Isto sugere que CP e aPKC mantêm a integridade epitelial e previnem a morte celular na *wing blade*, ao passo que na região do *hinge*, CP e aPKC parecem restringir o crescimento, possivelmente através da regulação da sinalização Hippo [23].

Curiosamente, a diminuição dos níveis de CP resultou numa redução significativa dos níveis apicais de aPKC, sugerindo que, através da sua função como regulador da dinâmica do citoesqueleto de actina, CP restringe a localização de aPKC na membrana apical das células epiteliais. Além disso, semelhante ao fenótipo observado em epitélios mutantes para *apkc*, diminuição dos níveis de CP levou a um decréscimo acentuado dos níveis apicais de E-Cad e Arm. Isto sugere que CP tem um papel putativo na dinâmica das AJs. Por último, o efeito nos níveis apicais de E-Cad após diminuição dos níveis de CP foi semelhante ao observado após sobreexpressão de Src64B, um importante regulador da estabilidade das AJs. Uma vez que a actividade de Src parece ser regulada por CP (García Fernández, B. and Janody, F. *et al.*, unpublished data), este resultado sugere que o efeito de CP nas AJs pode ser dependente da actividade de Src. Baseando nestas observações, são apresentadas no presente trabalho diferentes hipóteses que pretendem explicar o papel de CP na manutenção da estabilidade das AJs, e de como esta função putativa de CP pode estar interligada com a função conhecida de aPKC na regulação da dinâmica das AJs.

É importante referir que as diferentes hipóteses não são mutuamente exclusivas e por isso, de um modo geral, através da sua principal função, prevenir a polimerização dos filamentos de actina, CP parece ter um papel putativo na manutenção da estabilidade das AJs por: (a) restringir a localização de aPKC na membrana apical; (b) regular eventos iniciais de endocitose; e (c) regular a actividade de Src. No conjunto, os dados apresentados neste trabalho apontam para uma crucial sinergia entre complexos de polaridade e o citoesqueleto de actina na regulação da dinâmica das AJs, o qual está intimamente ligado à manutenção da integridade dos tecidos epiteliais.

**Palavras-chave:** *Capping Protein*, proteína cinase C atípica, junções aderentes, endocitose, disco imaginal da asa de *Drosophila*

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# I. INTRODUCTION

## 1. Epithelial tissues

A great variety of epithelia line the walls of cavities, separating two different chemical milieus or, in the case of the epidermis, serve as protection from the external environment [2]. The structure and function of epithelial tissues generally depend on apicobasal polarization, which is achieved and maintained by linking asymmetrically distributed intercellular junctions to the cytoskeleton of individual cells [11]. During aberrant epithelium development, cells often lose adhesion between each other, disrupting the polarity and normal growth of the tissue. In fact, the majority of human tumours arise from epithelial tissues with abnormal cell adhesion and proliferation [3, 4].

### 1.1 Epithelial architecture: polarity and cell-cell adhesion

Simple epithelia generally consisted of a laterally coherent sheet of cells with an apical and a basolateral domain. The apical surface is divided into the free apical domain and a domain where a neighbouring cell is contacted. Similarly, the basolateral domain is further subdivided into a basal domain mediating cell-matrix adhesion and a lateral domain involved in cell-cell adhesion [2, 11] (Fig.).

In *Drosophila* epithelial cells, cell-cell adhesion is mainly accomplished by three junctional complexes: adherens junctions (AJs), septate junctions (SJs), the functional equivalent to the vertebrates' tight junction, and the sub-apical region (SAR) [2].The AJs are a generally circumferential junction located just basal to the apico-basolateral boundary, which major function is to link the actin cytoskeleton of neighbouring epithelial cells. They are composed of the transmembrane protein E-cadherin (E-cad), which is linked on its cytoplasmic side to β-catenin (in *Drosophila* encoded by *armadillo*) and α-catenin. In turn, α-catenin binds to actin filaments, forming a continuous band, the zonula adherens (ZA) [5]. In addition to β-catenin, the Armadillo family also includes several other related molecules, including p120-catenin. Although not directly involved in coupling the cadherins to the cytoskeleton, p120 has emerged

as a critical regulator of cadherin adhesive activity and cytoskeletal organization [24, 25] (Fig.).

The SJs act as permeability barriers between cells and contain a complex of three proteins: Discs large (Dlg), Lethal giant larvae (Lgl) and Scribble (Scrib) (the Scrib complex). These proteins have been proposed to form a biochemical complex that controls and refines the segregation of apical and basolateral membrane domains [26]. SAR is the most apically localized junctional complex and it is defined by the accumulation of two protein complexes: the Crumbs (Crb), Stardust (Std), Discs lost (Dlt) complex (the Crb complex) and the Bazooka/Par3, atypical Protein Kinase C (aPKC), Par6 complex (the Par-aPKC complex) (Fig.1) [2, 14]. Mutations in genes coding for the Lgl, the Crb, and the Par-aPKC complex all disrupt epithelia formation but appear to do so because they are required for the initial generation of epithelial polarity and junctions.

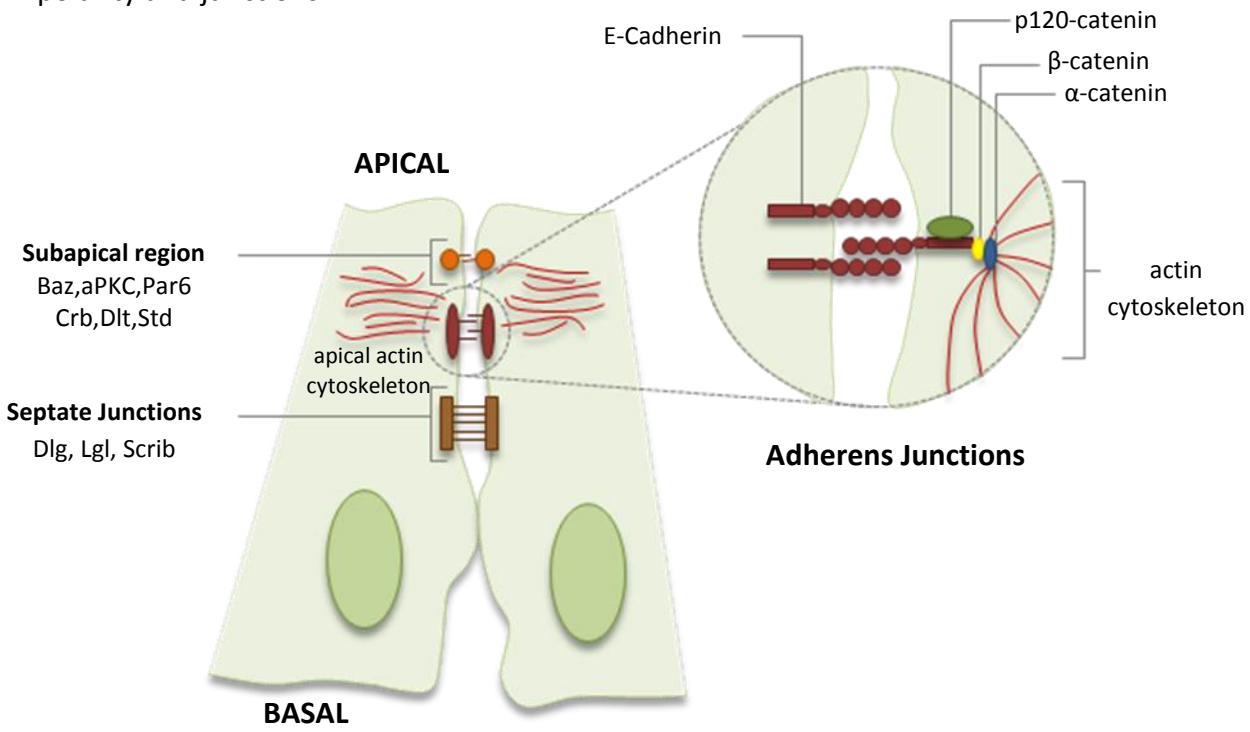



**Fig. 1 - Schematic representation of *Drosophila* epithelial cells.** The major junctional complexes are represented: the apically localized subapical region (SAR), the adherens junctions (AJs) and the septate junctions (SJs). Epithelial polarity complexes are located in stratified regions along the apico-basal axis of cells. The Crb complex along with the Par-aPKC complex is located in the SAR, while the basolateral Scrib complex forms the SJs. Adapted from Shock, F. & Perrimon, N. (2002) [2] and Mosesson, Y. *et al.* (2008)[27].

### 1.2 The Par-aPKC complex

The apico-basal polarity and the early phases of ZA assembly in epithelial cells depends on the Par-aPKC complex, which is composed of two scaffold proteins, Par3 (Bazooka, Baz in *Drosophila*) and Par6, and an atypical protein kinase C (aPKC) [28-30]. In *Drosophila* epithelia, aPKC physically interacts with Par6, and although this interaction tethers aPKC to the apical membrane, it is also though to inhibit aPKC kinase activity. It has been shown that a key event that activates aPKC at apical domain is the binding of the Rho-like GTPase Cdc42 [31] to the CRIB (Cdc42/Rac interactive binding) domain of Par6 [12, 13]. Although Baz, Par6, and aPKC are often assumed to function as a complex, it was recently reported that Baz is excluded from the apical domain by aPKC phosphorylation. This apical exclusion of Baz was shown to be required for the establishment of apical and basolateral membranes [32].

Similar to its interaction with Baz, aPKC-mediated phosphorylation is required for the correct apical localization of Crb and its cytozolic associates, Sdt and Dlt, whose activity is required to further recruit and concentrate sparsely distributed E-Cad into the AJs [22, 33]. Concomitantly, the interaction with the Crb complex upregulates aPKC activity and inhibits the function of basolateral regulators such as the Scrib complex at the apical space [12, 13]. In addition, aPKC phosphorylates Lgl to exclude it from the apical membrane and restricts it to the lateral domain, where it interacts with Dlt and Scrib [34].

### 1.3 The development of epithelial tumours

Epithelial tumour progression involves the stepwise acquisition of a number of neomorphic traits by tumour cells. These traits include disruption of epithelial adhesion and apicobasal polarity, self-sufficiency in growth signalling, resistance to apoptosis, inability to differentiate and acquisition of migratory and invasive abilities [35]. The development of epithelial tumours is thought to involve cooperation between tumour suppressor genes (TSGs) and oncogenes, as well as between tumour and its microenvironment [36].

TSGs are genes that, when mutated, can act cell-autonomously and cause excessive tissue overgrowth. In *Drosophila*, TSGs are generally divided into two categories: hyperplastic whenever they show increased proliferation, although epithelial architecture is maintained, whereas they are considered to be neoplastic if tissue overproliferation occurs concurrently with disruption of epithelial structure [37]. Interestingly, the components of the Par-aPKC complex were identified as neoplastic TSGs [38]. Indeed, the invasive potential of ovarian and breast cancer cells is associated with loss of aPKC from the apical membrane and its recruitment as a positive mediator for proliferation and invasiveness [38-40]. Additionally, aPKC can be regulated by other TSGs. For example, mutations in the TSG von Hippel-Lindau (VHL) results in mislocalization and degradation of aPKC and subsequent loss of epithelial architecture [41]. Thus, failure to restrain aPKC to the apical domain and deregulation of its activity are key factors for cancer progression [38].

One of the oldest and most investigated oncogenes is the non-receptor protein tyrosine kinase Src. In addition to the well-established role of Src in regulating cellular proliferation, there is accumulating evidence that Src also acts to affect adhesion, invasion and motility events, particularly in epithelial cells and in cancer cells during later stages of cancer progression [42]. Src interacts directly with the AJs components, E-Cad and Arm, and its overactivity promotes AJs disassembly, reducing adhesion and promoting motility and invasiveness [42, 43].

### 1.4 *Drosophila* wing imaginal disc as an epithelial model system

The fruit fly, *Drosophila melanogaster*, has been used as a model organism in diverse fields since the turn of the previous century, and today is one of the most widely-used and genetically best-known of all multicellular organisms. *Drosophila* imaginal discs are considered bona fide epithelia and thereby, they are an excellent model to study epithelial integrity. The imaginal discs can be seen in the newly hatched larvae as local invaginating epidermal cell clusters. Whereas most of the larval cells have very limited mitotic capacity, imaginal disc cells proliferate rapidly

and give rise to adult cuticular structures, such as the wings and legs. In this work, I used the wing imaginal disc as an epithelial model due to its well characterized patterning events and ease of dissection in 3rd instar larvae.

The wing imaginal discs are bilayered epithelial tissues consisting of a columnar monolayer epithelium, whose cells are polarized along the apicobasal axis, being covered by a thin layer of squamous epithelium called the peripodial membrane. As pupation begins, the cells at the centre of the disc telescope out to become the most distal region of the wing – the wing blade - , the surrounding cells give rise to the wing hinge and the outmost cells become the proximal structures – the notum and the pleura (dorsal thorax structures) (Fig.2). Early in their development, discs are subdivided into spatially distinct, stable units called compartments, which are developmental fields of cells sharing common ancestry and adhesive properties. Signalling between compartments establishes the anterior-posterior (A-P) and dorsal-ventral (D-V) boundaries [44, 45].

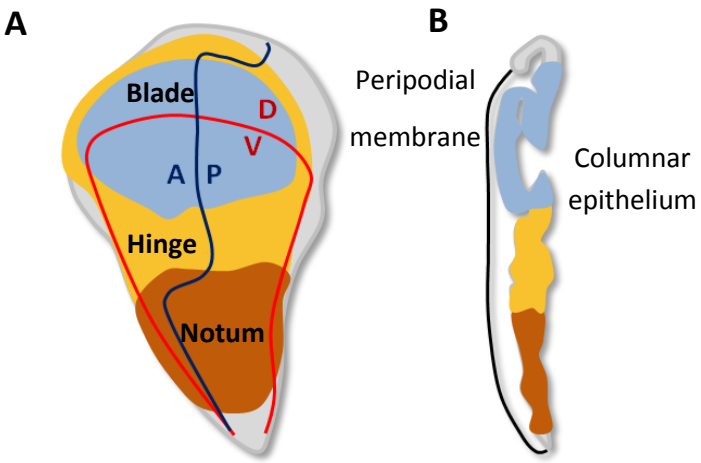



**Fig. 2 – Drosophila wing disc and fate maps.** (A) Fate map of 3rd instar wing imaginal disc showing the anterior-posterior (A-P) and dorsal-ventral (D-V) compartment boundaries and major regions of the disc: the wing blade (blue), which gives rise to the proper wing, the hinge (yellow), that constricts to form a mobile link to the body wall of the fly and the notum (orange), which gives rise to dorsal thorax structures. (B) Cell layers of the wing disc. There are two main cell layers: the peripodial membrane and the columnar epithelium. Adapted from Butler, M.J. *et al.,* (2003) [46].

The A-P boundary is defined during the 1st instar larvae and requires *engrailed* (*en*) expression in the posterior compartment for its maintenance. The Engrailed (En)

transcriptor factor promotes *hedgehog* (*hh*) expression in the posterior compartment and it acts as a short-range morphogen to specify the expression of *decapentaplegic* (*dpp*) in a narrow stripe of cells adjacent to the A-P boundary [30]. Additionally, Dpp is also required for proximal-distal patterning [30]. During the 2$^{nd}$ instar larvae, an antagonist relationship between the epidermal growth factor (EGF) and Wingless (Wg) signalling establishes the D-V boundary and divides the disc into a dorsal region that gives rise to the notum, and a ventral region that forms the wing [31]. The wing is further subdivided into distal and proximal regions by expression of the selector genes *vestigial* (*vg*) and *scalloped* (*sd*) in the wing blade and *homothorax* (*hth*) and *teashirt* (*tea*) in the wing hinge [45].

## 2. Endocytosis

Growing evidence has revealed an important role for vesicle trafficking in maintaining epithelial architecture. Exocytosis, in which recently synthesized proteins are released from the trans-Golgi network to apical or basolateral membrane domains, have received primary emphasis among trafficking routes responsible for epithelial polarity, but accumulating evidence now reveals that endocytosis plays an equally important role in regulating polarity and adhesion of epithelial tissues [8, 47].

### 2.1 Mechanisms of Endocytosis

Endocytosis is a cellular process used by all eukaryotic cells to internalize extracellular or membrane-bound cargoes from the plasma membrane into the cell interior through a series of vesicle compartments. Endocytosis occurs by various mechanisms, which can be divided into those that are clathrin-dependent and those that are clathrin-independent, such as phagocytosis and macropinocytosis [9, 10].

In clathrin-dependent endocytosis, with the help of the Adapter Protein-2 (AP-2), clathrin triskelia form a cage structure around invaginated membrane and the activity of the GTPase dynamin (in *Drosophila* encoded by *shibire, shi*) results in vesicle scission. Newly formed vesicles fuse with other endocytic vesicles to form an early

(sorting) endosomal compartment [9]. These fusion events are regulated by the endocytic syntaxin Avalanche (Avl) and the GTPase Rab5 [48]. The primary function of the early endosome is the sorting of internalized cargo to different intracellular destinations. Cargo destined for degradation remains in the early endosome as it matures into a multivesicular body (MVB). Ultimately, when the endosome reaches its maturity, Rab7 GTPase mediates its fusion with a lysosome, where proteolytic degradation occurs. Alternatively, cargo destined for recycling is sorted into a specialized recycling endosome and then returned via vesicles to the plasma membrane [48, 49]. The GTPase Rab11 mediate slow endocytic recycling through recycling endosomes, whereas Rab4 mediates fast endocytic recycling directly from early endosomes (Fig.3) [48].

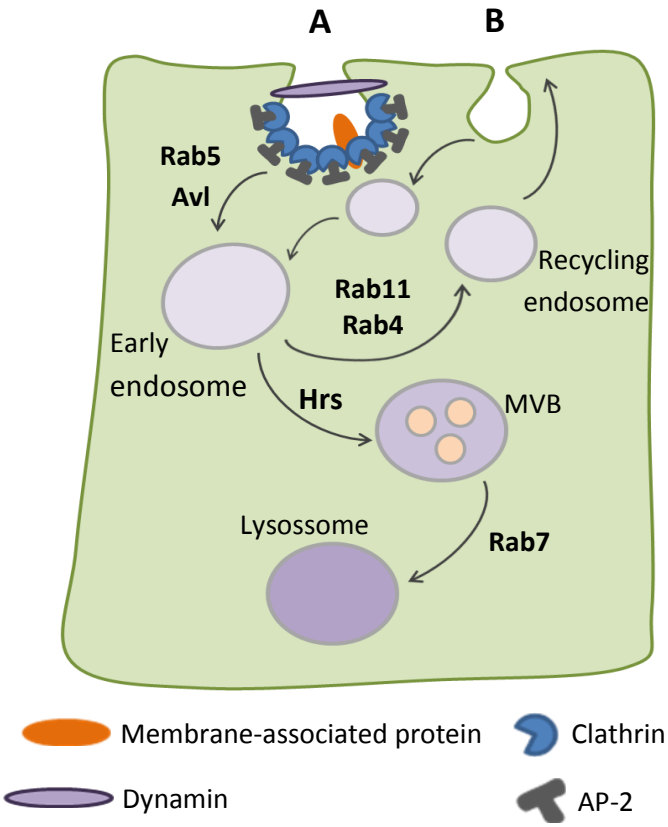



**Fig. 3 - Simplify view of the endocytic pathway.** Clathrin-dependent (A) and clathrin-independent (B) vesicle formation is shown. Internalized membrane-associated proteins either are sent back to the membrane by the recycling pathway or else are degraded in the lysosome. Adapted from Fisher, J.A. *et al.* (2006) [50].

## 2.2 Remodelling of cell-cell adhesion through endocytosis

Although all junctional complexes are functionally significant, recent evidence has shown that AJs play an essential role in regulating the dynamics of epithelial tissues. In fact, the redistribution of E-Cad through regulated endocytosis is crucially important to mediate dynamic adhesion and epithelial integrity during processes of cell division, cell death and cell rearrangement [6-8]. Even though AJs remodelling plays a key role in epithelial integrity, its regulation is still poorly understood.

Studies in mammalian cells and *Drosophila* have pointed to the Rho-like GTPases Cdc42 and Rac as one group of AJs regulators [14, 51]. Knocking-out *cdc42* function causes a disruption of AJs during dynamic cell rearrangements in *Drosophila* neuroectoderm. Interestingly, when cell rearrangement is blocked, neuroectoderm integrity is restored, indicating that epithelia undergoing extensive adhesion remodelling are more susceptible to endocytic defects. Further genetic interaction studies suggested that, the failure to maintain AJs in *cdc42* mutant tissues is a possible secondary consequence of the loss of apical polarity proteins, such as Crb, from the plasma membrane [14]. Interestingly, constitutively active aPKC partially suppressed effects of Cdc42 reduction, strongly implicating aPKC as the key effector in regulating endocytosis of apical proteins, which in turn, has a stabilizing effect on AJs during cell rearrangement [14, 15].

The complete disassembly of AJs in epithelial tissues results in loss of adhesion and the acquisition of a migratory or mesenchymal-like phenotype [52]. For instance, upon Src activation, E-Cad is ubiquitinated and then shuttled to the lysossomal degradation instead of following its normal trafficking route of recycling back to the apical membrane. This results in loss of E-Cad at apical junctional sites, leading to loss of adhesion and disruption of epithelial integrity [53, 54].

## 2.3 Endocytic genes define a novel class of Drosophila tumour suppressor genes

With the introduction of genetic mosaic screening techniques, several neoplastic TSGs have been characterized that are key regulators of cargo entry into

the early endosome and endosomal maturation. Among these new endocytic TSGs are the genes *shibire* (*shi*), *rab5* and *avalanche* (*avl*) [35, 37]. Mosaic clones mutant for *shi*, *rab5* or *avl* are slow-growing and usually eliminated from the disc [35, 55] through a process of cell competition [56]. When the *wild-type* contribution is removed, mutant imaginal discs for *shi*, *rab5* or *avl* accumulate apical proteins, such as Crb and the Notch receptor, at the plasma membrane, disrupt AJs and undergo massive overproliferation, resulting in the development of neoplastic overgrowths [35, 55]. Interestingly, Notch signalling is downregulated in *shi*, *rab5* and *avl* mutant tissues, suggesting that overgrowth in this subset of endocytic TSGs mutants is not a result of increased Notch signalling [57]. In fact, it appears to be due to mislocalization of Crb, since increasing Crb levels in imaginal discs causes neoplastic transformation, whereas interfering with Crb activity through co-expression of a dominant negative form of aPKC ($aPKC^{CAXXDN}$) ameliorates the phenotype [55].

## 3. The actin cytoskeleton

The cytoskeleton comprises a network of protein filaments including microtubules, actin filaments and intermediate filaments, which are held together and linked to subcellular organelles and the plasma membrane by a variety of accessory proteins. A key feature of epithelial cells is the polarized actin cytoskeleton, which plays a central role in numerous cellular processes, such as generation and maintenance of cell shape and polarity, cell division, contractility and motility. A continuous band of actin filaments localized apically and links epithelial cells to each other, forming the ZA. This continuous band of actin filaments is extremely important for the maintenance of the epithelia, since it provides adhesive strength between epithelial cells [2].

### 3.1 Actin dynamics and its regulation by actin binding proteins

Actin is present in cells in two main forms: the monomeric globular actin (G-actin) and the polymeric filamentous actin (F-actin). The filamentous actin is a

polarized structure, in which the fast-growing barbed (+) end is the preferred site for ATP-monomeric-actin addition, whereas depolymerization occurs by loss of ADP-actin subunits from the slow-growing pointes (-) end. This ATP-hydrolysis-driven filament polymerization is a very dynamic process, which requires a tight regulation *in vivo*.

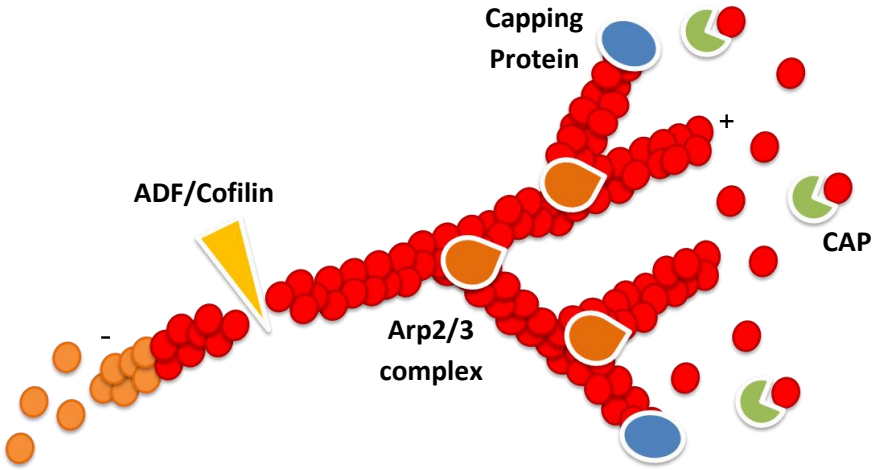



**Fig. 4 – Actin dynamics is controlled by a large array of ABPs.** The actin nucleators, such as Arp2/3 complex, promote de novo actin filament nucleation and branching. ADF/Cofilin factors severe the actin filaments and promote dissociation of the actin monomers from the pointed (-) end. Cyclase-associated proteins (CAP) sequester actin monomers, preventing their incorporation into filaments. Capping proteins (CP) restrict the access to the barbed (+) end, forming a protein cap that prevents further addition of actin monomers. Adapted from Disanza, A. *et al.* (2005) [18].

Actin dynamics and structure are modulated by several actin binding proteins (ABPs), which in turn are under the control of specific signalling pathways (Fig.4) [17, 18]. For instance, the nucleation of actin filaments is an energetically unfavourable event, being promoted by certain ABPs, such as the Arp2/3 complex and formins. Once activated by a member of the evolutionary conserved Wiskott - Aldrich syndrome protein (WASp) family, the Arp2/3 complex nucleates new actin filaments by binding to the side of a pre-existing filament. Importantly, the activity of WASp family is regulated by the Rho-like GTPases Cdc42 and Rac [18, 58]. Among the ABPs that prevent actin polymerization, the actin depolymerizing factor (ADF)/Cofilin family, the Cyclase-associated protein (CAP) and Capping Proteins (CP) are some of the most important. Cofilin severs filaments and enhances dissociation of actin

monomers from the pointed end [19], CAP sequesters actin monomers, preventing their incorporation into filaments [59] and CP restricts accessibility of the barbed end, inhibiting addition or loss of actin monomers [60].

### 3.2 Capping Protein: a highly conserved heterodimer

CP is a highly conserved heterodimer, composed of α (Cpa) and β (Cpb) subunits, which binds the barbed end of actin filaments with high affinity, thereby preventing excessive actin polymerization [61]. Interestingly, *in vitro* studies showed that CP shunts actin monomers away from the barbed ends and onto Arp2/3 complex, promoting more frequent filament nucleation by the Arp2/3 complex [62]. Thus, functional CP, along with the Arp2/3 complex, support the assembly of a dominantly branched network of small actin filaments, generating protruding force and giving rise to the lamellipodia of migrating cells [58]. When capping of actin filaments by CP is inhibited by PIP2, CARMIL or Melanotrophin/V-1, long actin filaments can become bundled, giving rise to filopodia [63]. Moreover, besides having a critical role in the termination of filaments, CP has been suggested to mediate actin filament attachment to the plasma membrane [64, 65].

Although the mechanism and regulation of CP have been studied, most of the available data results from *in vitro* biochemical studies and cell culture. Since *in vitro* analysis do not exactly reproduce the complexities within a tissue, it is crucial to understand the role of CP and its regulation *in vivo*.

### 3.2 CP prevents cell extrusion within restricted regions of Drosophila epithelia

As expected, *in vivo* studies showed that CP prevents excessive actin filaments polymerization, confirming its already known role in actin filament termination [20]. In addition, loss of either *cpa* or *cpb* in *Drosophila* imaginal discs gives rise to identical developmental phenotypes [20, 66], consistent with its role as part of a functional heterodimer [61].

Interestingly, CP has a region-specific requirement in *Drosophila* wing imaginal

disc. While *cpa* or *cpb* mutant clones maintain cell polarity and survive in the hinge and notum regions, mutant clones in the wing blade mislocalize Arm and E-Cad to basolateral domains and undergo cell extrusion and apoptosis [20]. By expressing a HA-tagged form of Cpa, it was observed that Cpa mostly accumulates at the apical regions of the cell [20], suggesting that CP regulates a specific pool of apical actin filaments. However, excessive actin filament accumulation may not be the main cause of extrusion and death of *CP* mutant clones, since mutations in the genes encoding the Cyclase-associated protein Capulet (Capt) and the Cofilin homolog Twinstar (Tsr) also induce strong accumulation of actin filaments, but do not cause cell extrusion [20, 66].

### 3.3 CP prevents accumulation of apical membrane-associated proteins

It has been recently pointed that actin cytoskeleton has a crucial role in invagination and scission of endocytic vesicles [67, 68]. For instance, in epithelial cell lines, the use of Cytochalasin D, an actin-disrupting agent, inhibits endocytosis of membrane-associated proteins and the formation of coated vesicles [69]. Similarly, in budding yeast, when actin polymerization is blocked, the initial movement of endocytic vesicles away from the plasma membrane, which presumably corresponds to vesicle scission and release, is completely blocked [70, 71].

Previous data from F. Janody's lab revealed that RNA interference (RNAi) leading to *CP* depletion in the posterior compartment of *Drosophila* wing disc leads to accumulation in punctuated structures of the membrane-associated proteins, Crb and Notch (Fig.5) [21]. In particular, similar to *shi*, *rab5* and *avl* mutant tissues [57], Notch accumulation is not due to transcriptional upregulation, since Notch target genes, such as *cut* and *m8-lacZ*, are downregulated in *CP* depleted cells [21]. In this sense, Notch accumulation in *CP* depleted cells was suggested to be due to an early endocytic defect, blocking progression to early endosomes where activation of Notch takes place [57]. Accordingly, accumulation of Crb in *CP* depleted cells could also be due to an endocytic defect.

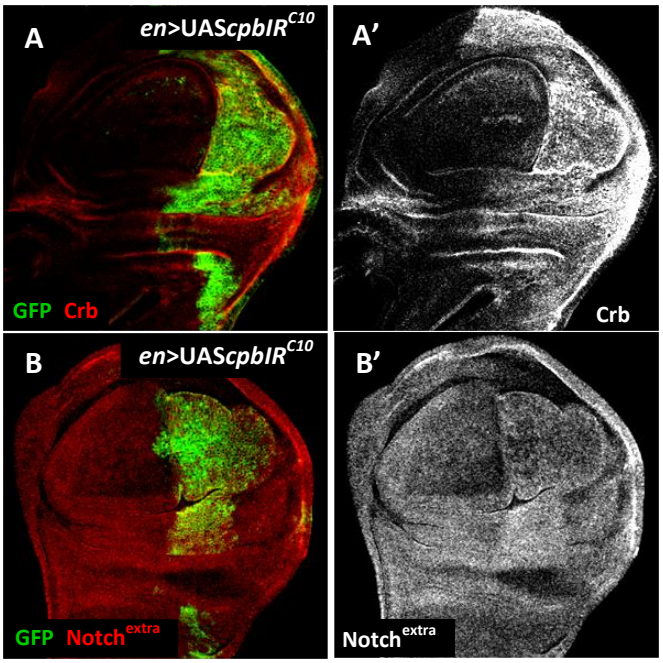



**Fig. 5** - ***CP* depleted cells accumulate the Notch receptor and Crb in the wing imaginal disc.** All panels show apical Z-projections sections of third instar wing discs. (A-B') Wings discs expressing UAS-*cpbIR*$^{C10}$ and UAS-*GFP* (green) under *en*-Gal4 control are stained with either anti-Crb or anti-Notch extracellular domain (red in A and B or grey in A' and B', respectively). Adapted from Gaspar, P. (2008) [21].

### 3.4 CP appears to interact genetically with aPKC to maintain epithelial integrity

As mentioned before, loss of *CP* in *Drosophila* wing discs leads to accumulation of Crb in punctuated structures. In the same study, it was shown that clones overexpressing the intracellular domain of *crb* extrude and die mainly in the wing blade epithelium [21]. This clonal behaviour is reminiscent to the *CP* mutant phenotype [20], suggesting that extrusion and death of *CP* mutant cells could be the result of excessive Crb signalling activity [21]. To investigate this possibility, a 'kinase-dead' form of *aPKC* (*aPKC*$^{CAXXDN}$), that acts as a dominant negative form and is known to reduce the activity of overexpressed *crb* [22, 55], was expressed in cells mutant for *cpa* or *cpb* [21]. Unexpectedly, expression of *aPKC*$^{CAXXDN}$ induced extrusion and death of *CP* mutant cells not only in the prospective wing blade, but also in the hinge and notum epithelia (Fig.6) [21]. Consistent with an enhancement of the CP depletion phenotype, driving both aPKC$^{CAAXDN}$ and *cpb* depletion using *nub*-Gal4, which drives the expression of the UAS-target gene in the wing blade and distal region of the hinge,

results in a severely enhanced adult wing phenotype, when compared to the phenotype of wings depleted of *cpb* alone (Fig.8) [21]. Given that expressing *aPKC*$^{CAXXDN}$ in either clones of cells (Fig.6A-A') or using *nub*-Gal4 (Fig.7B) has no visible effect on wing disc, this suggested that CP and aPKC interact genetically to maintain epithelial integrity of wing disc [72].

However, some problems associated to the use of *aPKC*$^{CAXXDN}$ as a proper kinase dead are raised. Although this construct harbours similar modifications to a *Xenopus* aPKCλ kinase inactive protein that acts as a dominant negative [73], no biochemical analysis were performed to investigate the kinase activity of *Drosophila aPKC*$^{CAXXDN}$. Additionally, the presence of the CAAX sequence enables *aPKC*$^{CAXXDN}$ to be targeted to the cell membrane. Therefore, the possibility that the genetic interaction between *aPKC*$^{CAXXDN}$ and CP results from unspecific effects of *aPKC*$^{CAXXDN}$, which might trap random kinase substrates, cannot be excluded.

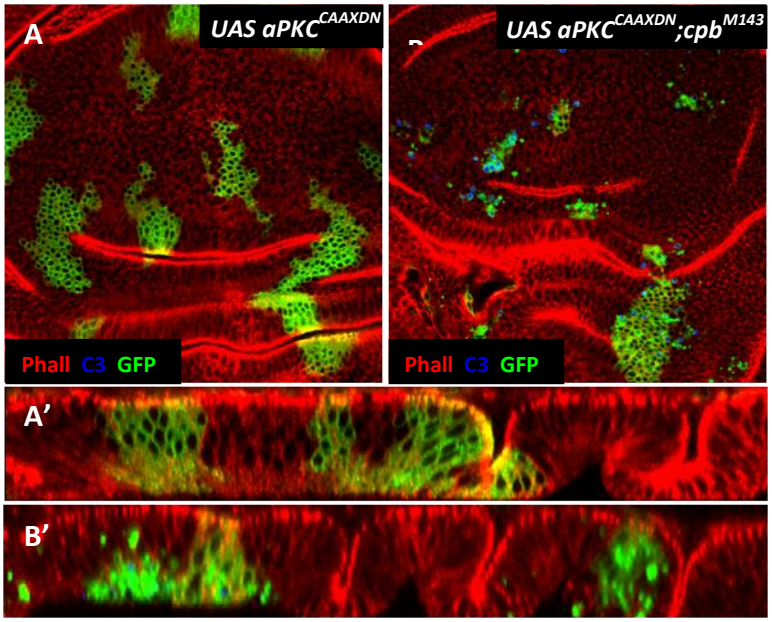



**Fig. 6 - CP interacts genetically with aPKC to prevent loss of epithelial cell polarity and extrusion.** All panels show third instar wing discs with clones positively labelled with GFP (green in A-B'). (A and B) Basal Z-projections. (A' and B') Reconstructed XZ sections through the wing disc epithelium. (A-A') Clones expressing *aPKC*$^{CAXXDN}$, (B-B') *cpb*$^{M143}$ mutant clones expressing *aPKC*$^{CAXXDN}$ are stained with anti-Arm to outline apical cell membrane (red in A-B') and anti-activated Caspase 3, which identifies Caspase-dependent cell death (blue in A-B'). Adapted from Gaspar, P. (2008).

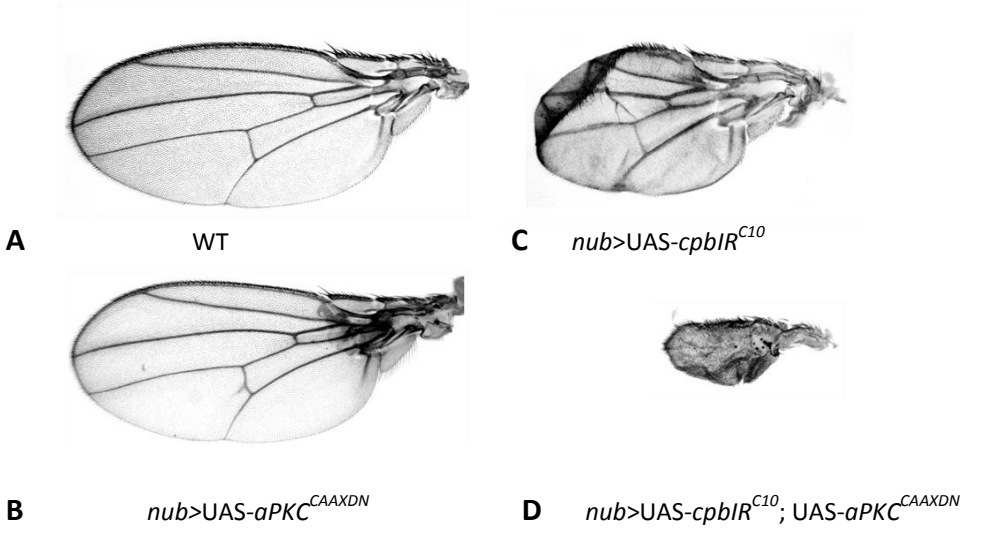



**Fig. 7 - CP genetically interacts with aPKC during wing morphogenesis.** (A) *wild-type* (WT), (B) *nub*>*cpbRI*$^{C10}$, (C) *nub*>UAS-*aPKC*$^{CAAXDN}$ and (D) *nub*>UAS-*cpbIR*$^{C10}$; UAS-*aPKC*$^{CAAXDN}$ adult wings. From Gaspar, P. (2008) [21].

### 3.5 CP cooperates with Src signalling

In the wing blade epithelia, exponential decrease of Cpb levels induces a switch from neoplastic growth to cell death (García Fernández, B. and Janody, F. *et al.*, unpublished data). Interestingly, overexpression of Src64B (*Drosophila* possess two Src kinases, Src64B and Src42A) mimics the effects of decreasing Cpb levels: slight increase induces tissue overgrowth, while strong overexpression promotes cell death (García Fernández, B. and Janody, F. *et al.*, unpublished data) [74, 75]. Moreover, when Cpb depleted tissues also contained a slight increased in Src64B, this results in massive cell death, associated to a strong decrease of the membrane-associated form of Arm. These results suggest that reduced CP levels might cooperate with increased Src64B levels to trigger apoptotic cell death (García Fernández, B. and Janody, F. *et al.*, unpublished data). It was reported that mechanical stretching of cultured cells activates Src through an interaction with actin filaments [76]. Furthermore, actin disruption by Cytochalasin B or knock-down of actin by siRNA strongly inhibit the phosphorylation of Src and its kinase activity [77]. CP might therefore inhibit actin-dependent mechanical activation of Src.

# II. AIMS

The Capping Protein (CP) αβ heterodimer, which regulates actin polymerization, prevents accumulation of apical membrane-associated proteins, such as Crb and Notch [21]. Furthermore, knocking-out *CP* induces mislocalization of E-Cad e Arm to basolateral domains in the wing blade epithelium [20]. Interestingly, the atypical protein kinase C (aPKC), a major effector of the Par-aPKC complex, was shown to control the endocytic uptake of Crb and Notch from the apical membrane and to stabilize AJs during dynamic cell rearrangement [14, 15]. CP might therefore cooperate with aPKC to control the abundance of apical proteins and to regulate AJs stability, which are intimately linked to the maintenance of epithelial integrity. In agreement with this possibility, CP interacts genetically with a dominant negative form of aPKC ($aPKC^{CAXXDN}$). However, this genetic interaction should be further studied, since the $aPKC^{CAXXDN}$ might have unspecific effects [22].

The main goal of this work was to get insight on the mechanism by which CP cooperates with aPKC to maintain epithelial integrity in *Drosophila* wing imaginal disc. To do so, I aimed to: confirm the genetic interaction between CP and aPKC using RNAi lines under the control of the UAS-Gal4 system and a termosensitive mutant allele of aPKC ($apkc^{ts}$) (Guilgur, L. and Martinho, R., unpublished data) (Task 1); analyze whether aPKC localize properly in the wing blade epithelium following *CP* depletion (Task 2); and finally evaluate the putative role CP in AJs stability (Task 3).

# III. MATERIAL AND METHODS

## 1. Fly strains and genetics

### 1.1 Fly husbandry

All flies were raised at 25°C (unless otherwise indicated), according to standard conditions [78]. Fly stocks were maintained by transferring adults to fresh medium every few generations, while crosses were cultured in small vials containing a yeast-glucose-agar medium.

### 1.2 Fly Stocks: mutant allele and transgenic lines available for this study

In order to demonstrate that the genetic interaction between CP and *aPKC*$^{CAXXDN}$ (see Introduction Fig.7 and Fig.8) is due to a reduction of aPKC activity, I used a temperature sensitive allele of aPKC (*apkc*$^{ts}$) bearing a point mutation in a highly conserved residue of the kinase domain, which causes a temperature-dependent reduction of the kinase activity (Guilgur, L.G. and Martinho, R. *et al.*, unpublished data). At permissive temperature (25°C), *apkc*$^{ts}$ maternal mutants show a complete loss of epithelial integrity that leads to embryonic lethality (Guilgur, L.G. and Martinho, R. *et al.*, unpublished data). This phenotype is similar to the one observed in maternal mutants for the *apkc* loss of function allele [79]. However, while zygotic mutants for the *apkc* null allele die as late as second instar larvae [15, 80], *apkc*$^{ts}$ zygotic mutants are viable and morphologically normal at permissive temperature (25°C), but are lethal at restrictive temperature (30°C). At semi-permissive temperature (28°C), the viability is variable and adults show defects in abdominal dorsal closure (Guilgur, L.G. and Martinho, R. *et al.*, unpublished data). In contrast to the *apkc* loss of function allele [15, 79, 80], the use of the *apkc*$^{ts}$ allele (Martinho, R. *et al.*, unpublished data) allowed me to modulate aPKC levels by switching the temperature and thereby gave me the opportunity to determine whether CP genetically interacts with aPKC in maintaining wing epithelial integrity.

The majority of fly stocks used in this work were available at the F.Janody's Lab and some were kindly supplied by R.Martinho's Lab. Oregon R flies were used as the wild-type stock. The Gal4 drivers and UAS-transgenes used in this study are reported in Table 1.

| **Gal4 Drivers** | **Spatial-temporal domain of activity in the wing disc** |
|---|---|
| ***nubbin*-Gal4 (*nub*-Gal4)**<br>(a gift from G.Morata [81]) | From 2nd instar larvae (48h) to end of larval stage in the prospective blade and distal hinge. |
| ***hedgehog*-Gal4 *(hh*-Gal4*)***<br>(a gift from T.Tabata [82]) | From end of embryogenesis to end of larval stage in the posterior compartments. |

| **UAS-*transgene*** | **Usage purpose** | **Transgene location** |
|---|---|---|
| **UAS-*cpaIR$^{\#2}$***<br>(National Institute of Genetics, NIG) | To promote knock-down of *cpa* expression | 3rd |
| **UAS-*cpbIR$^{B4}$***<br>(Vienna Research Centre, VDRC) | To promote knock-down of *cpb* expression | 3rd |
| **UAS-*HA-cpa$^{ID}$***<br>[20] | To overexpress HA tagged form of *cpa* | 3rd |
| **UAS-*apkcIR$^{KK100874}$***<br>(Vienna Research Centre, VDRC) | To promote knock-down of *apkc* expression | 2nd |
| **UAS-*CD8-GFP*** | To mark plasmatic membrane | 3rd |

**Table 1 -** Gal4 drivers and UAS constructs used during the course of this work.

### 1.3 Fly Stocks generated

Stable stocks were generated using standard fly genetic methods and crossed with standard balancer stocks [83] or GFP marked balancer stock (CTG; Cyo, *Twi*-Gal4, UAS-*2xEGFP*) [84]. The fly stocks generated in this work are reported in Table 2.

Both *apkc$^{ts}$* (Martinho, R. *et al.*, unpublished data) and *cpa* [85] mutant alleles are located in the right arm of the 2nd chromosome. Thus, to facilitate the process of generating a transgenic line with reduced levels of aPKC and CP, instead of the *cpa*

mutant allele [85], I used a RNAi line against *cpa*, which is under UAS-Gal4 control (UAS-*cpaIR*$^{\#2}$) and is located in the 3$^{rd}$ chromosome. Although the RNAi technique do not completely abolish the expression of the target gene, I found that overexpressing UAS-*cpaIR*$^{\#2}$ in clones generated by the MARCM system recapitulates the *cpa* loss of function phenotype [20]: accumulation of actin filaments in all regions of the disc and cell extrusion and death specifically in the wing blade (see Results Fig.1E-H' and Fig.2C-C'' and E). Since the UAS-Gal4 system is more active at higher temperatures, *cpa* depletion can be modulated by switching the temperature.

In order to overexpress UAS-*cpaIR*$^{\#2}$ using *nub*-Gal4 driver in an *apkc*$^{ts}$ mutant background, a recombinant line was produced between the *apkc*$^{ts}$ mutant allele (*w*$^{-}$; FRT42D,*apkc*$^{ts}$/CyO) and *w*-; *nub*-Gal4, both located on the second chromosome. To confirm the presence of *nub*-Gal4, recombinant flies (*w*$^{-}$; *nub*-Gal4, FRT42D,*apkc*$^{ts}$/CyO) were crossed either with UAS-*CD8-GFP* or UAS-*cpaIR*$^{\#2}$ and selected either for GFP expression in *nub*-domain or for having *cpa* depleted wings (see Introduction Fig.8C). Since *apkc*$^{ts}$ homozygous females are sterile, recombinant flies were first crossed with *w*$^{-}$; FRT42D,*apkc*$^{ts}$/CyO, and F1 non CyO females were selected for absence of F2 progeny.

| **Fly stocks generated** |
|---|
| *w*$^{-}$; *nub*-Gal4,FRT42D,*apkc*$^{ts}$/CyO |
| *w*$^{-}$; FRT42D,*apkc*$^{ts}$/CyO; UAS-*cpaIR*$^{\#2}$/TM6B |
| *w*$^{-}$; FRT42D,*apkc*$^{ts}$/CTG |
| *w*$^{-}$; FRT42D,*apkc*$^{ts}$/CTG; UAS-*cpaIR*$^{\#2}$/TM6B |
| *w*$^{-}$; FRT42D,*apkc*$^{ts}$/CTG; *hh*-Gal4,UAS-*CD8-GFP*/TM6B |

**Table 2 -** Fly stocks generated during the course of this work.

## 1.4 Genetic tools

### 1.4.1 The UAS-Gal4 system

Expression of UAS constructs was performed at 25$^{o}$C, unless mentioned otherwise, using the Gal4 system [86]. The Gal4 protein is an archetypal eukaryotic transcription factor isolated as an activator of genes responsible for galactose

metabolism in budding yeast [87]. The Gal4 gene has been widely cloned and used to genetically manipulate expression of genes in foreign organisms. For instance, in *Drosophila*, several well characterized enhancer sequences have been 'trapped' by the Gal4 coding gene to yield spatial-temporal specific drivers of gene expression [86]. Promoter sequences containing a consensus binding site for activating Gal factors, termed UAS sequences, have been introduced in a number of eukaryotic expression vectors, allowing for the production of transgenic constructs that are conditionally expressed upon the availability of Gal4. Thus, in *Drosophila*, one transgenic line, the 'driver', expresses Gal4 in a known temporal and spatial pattern and a second transgenic line, the 'responder', contains a UAS-*transgene* in a transcriptionally silent state due to the absence of Gal4. When 'responder' lines are mated to flies expressing the Gal4 driver, the resulting progeny (F1) express the transgene in a pattern that is identical to Gal4 driver expression pattern [88].

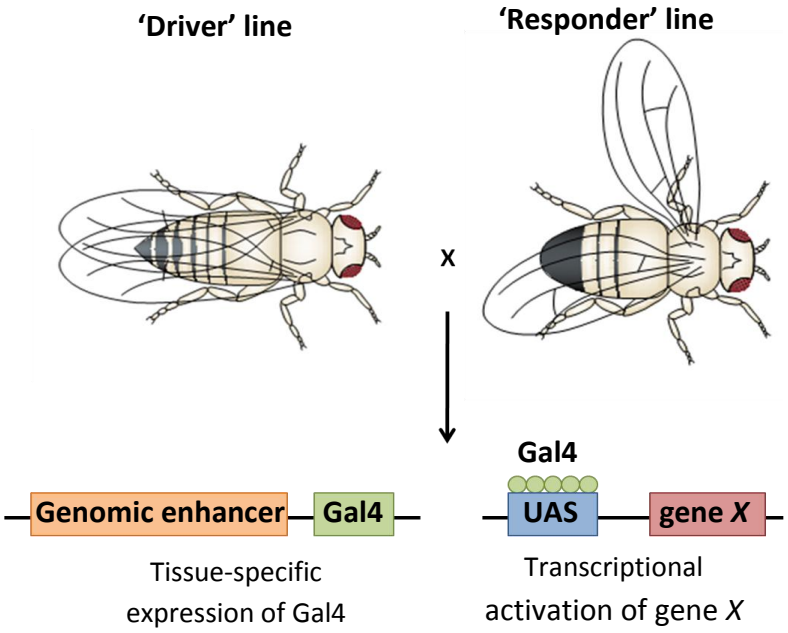



**Fig. 1 - The UAS-Gal4 system in *Drosophila*.** One of the parental stocks carries the Gal4 gene in close proximity to a known enhancer of gene expression. This stock can be crossed to another stock containing a UAS-*transgene* (UAS-gene *X*), and the resulting progeny will express this transgene in the pattern of expression of the Gal4 factor, which is specified by its associated enhancer region. Adapted from St Johnston, D. (2002) [89].

### 1.4.2 Generation of clones by mitotic recombination

The FLP/FRT system was used to generate mitotic clones to study recessive mutations that would cause embryonic lethality if present in the entire organism [89]. The Flipase (FLP) recombinase, a yeast λ integrase, directs site-specific mitotic recombination through recognition of Flipase Recombination Target (FRT) sites on homologous chromosome arms. Several P-element insertion stocks have been generated in *Drosophila,* introducing FRT sites close to the centromere of each chromosome as well as a transgene encoding FLP driven by a heat-shock inducible promoter (hsp70). In this sense, mitotic recombination between FRT sites in homologues chromatids occurs following a 1 hour treatment at 37$^{o}$C of individuals at the stage of interest. As a result, in a heterozygous parental cell, segregation of recombinant chromosomes at mitosis results in the generation of two daughter cells: a homozygous clone for the mutation distal to FRT site and a 'twin spot' clone, which is genetically wild-type [90].

In this study, mosaic analysis with a repressible cell marker (MARCM) was used to generate *apkc$^{ts}$* mutant clones positively marked by GFP, as well as constitutively overexpress UAS-*transgenes* in marked clones. The MARCM technique is based on the combine use of Gal4 together with its repressor Gal80, providing a way of controlling the UAS-Gal4 system. When Gal80 is removed by FRT-mediated mitotic recombination, Gal4 expression is no longer suppressed and can drive the expression of a certain UAS-marker, like UAS-*GFP* or another UAS-*transgene* [90].

The fly stocks containing FLP and FRT sites, used during the course of this work to generate positively marked clones, are indicated in Table 2 and Table 3, and heat-shock was performed at 1$^{st}$ or at 2$^{nd}$ instar larvae in order to analyze whether clones induced at different developmental stages display differences in cell behaviour. Since *apkc$^{ts}$* allele has different outcomes depending on the temperature, after being heat-shocked, larvae were raised at either 25°C or 28°C or 30°C until late 3$^{rd}$ instar larvae.

| FLP/FRT stock | Usage Purpose |
|---|---|
| ***y*$^{-}$,*w*$^{-}$,*hsFLP*$_{122}$, UAS-GFP; FRT42D, *tub-Gal80*; *tub-Gal4*/ TM6B** | To generate clones positively marked by GFP |

**Table 3 -** FLP/FRT system stocks used during the course of this work.

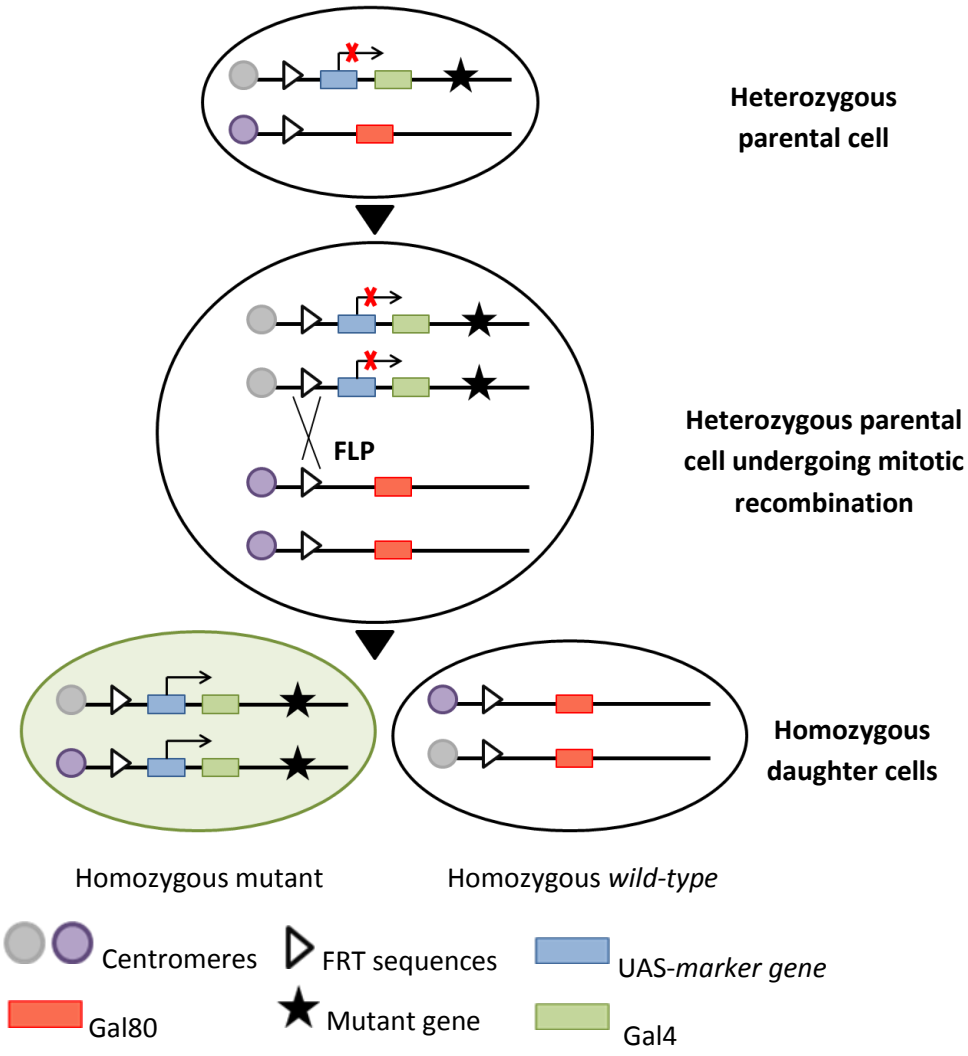



**Fig. 2 - Genetic basis of the mosaic analysis with MARCM system.** After site-specific mitotic recombination, a heterozygous parental cell can give rise to two daughter cells in which the chromosome arms distal to the recombination site become homozygous. Driven by a ubiquitous enhancer, Gal80 efficiently suppresses Gal4-dependent expression of a UAS-*marker gene*. If Gal80, but not Gal4 or UAS-*marker gene*, is inserted on the chromosome arm carrying the *wild-type* gene of interest, the daughter cell homozygous for the mutant gene no longer contains Gal80. Therefore, the *marker gene* can be specifically turned on by Ga4 in homozygous mutant cells. Adapted from Lee, T. & Luo, L. (1999) [91].

## 2. Immunohistochemistry

### 2.1 Antibody staining

Third instar larvae were dissected in 0,1M phosphate buffer (pH 7,2) and fixed in 4% formaldehyde in PEM (0,1M PIPES pH 7,0; 2mM $MgSO_4$; 1mM EGTA) for 30 minutes on ice. Larvae were then washed and permeabilized 3 times, for 20 minutes each, in PBS 0,2% Triton X-100 (PBT) and incubated with the primary antibodies diluted in PBT, supplemented with 10% donkey serum, overnight at $4^oC$. The larvae were then washed 3 times, for 20 minutes each, in PBT and incubated in the dark with secondary antibody diluted in PBT 10% donkey serum, for 2 hours at $4^oC$. Imaginal discs were washed again 3 times for 20 minutes with PBT before mounting between slides and cover-slides in Glycerol 60%. The primary antibodies used were: mouse anti-Arm, (N27A1, 1:10; Developmental Studies Hybridoma Bank (DSHB)); rabbit anti-activated Caspase3 (1:300; Cell Signalling Technology); rabbit anti-aPKC (1:2000; Santa Cruz); rat anti-E-Cad (CAD2, 1:25; DSHB); mouse anti-HA (1:5000; Covance). The secondary antibodies used were Donkey anti-mouse and anti-rabbit conjugated to TRITC or Cy5, (1:200; Jackson Immunoresearch).

### 2.2 Phalloidin staining

A Phalloidin conjugated dye was used to reveal F-actin. Third instar larvae were dissected, fixed and permeabilized as described in the previous section. Before mounting, tissues were incubated with TRITC conjugated Phalloidin, (1:200; Sigma), in PBT for 8-10 minutes and rinsed 3 times 5 minutes, before mounting in Glycerol 60%.

## 3. Image Acquisition and Analysis

Fluorescent images of wing imaginal discs were obtained with a Zeiss LSM 510 Meta confocal microscope using a 20x 0.80NA dry objective lens or a 40x 1.3NA oil objective lens. During confocal image acquisition, the detection parameters were

adjusted to avoid under- or overexposed pixels, and images were acquired though the full thickness of the wing disc either at 1µm for 20x lens or at 0.5 µm for 40x lens. Images of adult wings were obtained either with a Media Cybernetics EvolutionMP CCD camera attached to a Leica DM LB2 microscope using a 10x dry objective lenses for images at 0.48µm scale or with a DFC 420C CCD camera attached to a Leica $MZ7_5$ using 2x dry objective lenses for images at 1.55 µm. Images of adult flies were obtained on a DFC 420C CCD camera attached to a Leica $MZ7_5$ using 1.25x dry objective lenses. The ImageJ (NIH) and Imaris v6.4.2 (Bitplane) softwares were used for image processing and analysis, and for reconstructions of orthogonal XZ sections (in depth) and Z-projections.

For quantification of antibody staining, the average fluorescence intensity measured of three non-overlapping 350$\mu m^2$ area regions in the anterior and posterior wing compartment of maximum Z-projection of the ten most-apical slices. In order to qualitatively evaluate colocalization between fluorophores, binary colocalization masks were obtained with ImageJ, using a cut off point of 60% relative to maximum pixel intensity. Using the same software, quantification of the colocalization was performed using the Mander's Overlap coefficient (plugin available on ImageJ website), which indicates an overlap of the signals and thus represents the true degree of colocalization [92, 93]. All confocal images used for colocalization analysis were first median filtered (radius 2.0) to reduce background noise and to improve the efficiency of the colocalization analysis.

## 4. Classification of cell death observed in mosaic analysis

In order to determine whether reducing aPKC, using the *apkc*$^{ts}$ allele, enhances the phenotype of *cpa* depleted clones, I qualitatively categorized each wing disc based on cell extrusion and anti-activated Caspase 3 staining observed within GFP positive cells : (A) no cell death – wing discs that do not display activated Caspase 3-expressing cells; (B) weak cell death – wing discs showing few activated Caspase 3 positive cells, but the majority of cells marked by GFP are still maintained within the epithelium; (C)

medium cell death – wing discs displaying additional Caspase-dependent cell death and most GFP positive cells are located basally; (D) strong cell death – all basal GFP labelled cells express activated Caspase 3.

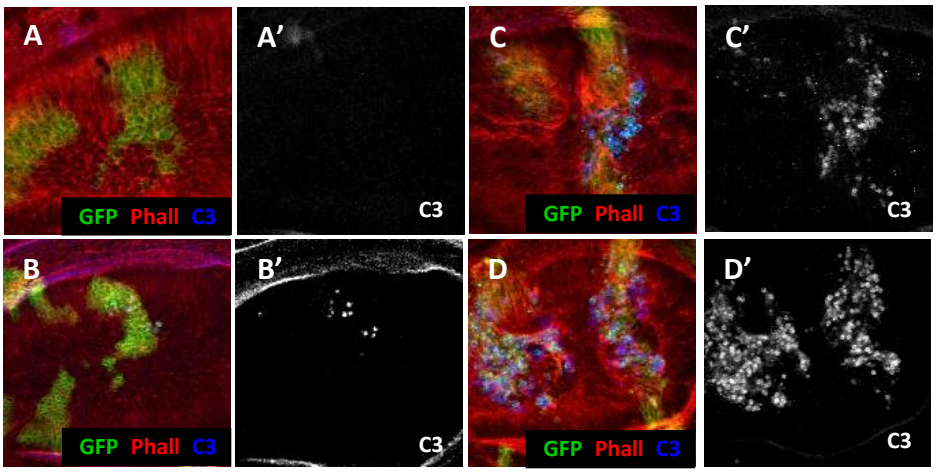



**Fig. 3 – Cell death classification of wing imaginal discs for different experimental conditions where levels of Cpa and/or aPKC were reduced.** All panels show basal Z-projections of third wing discs with clones positively labelled with GFP (green in A-D) and stained with TRITC-Phalloidin to reveal F-actin (red in A-D) and anti-activated Caspase 3, which identifies Caspase-dependent cell death (blue in A-D or grey in A'-D'). Dorsal is downwards.

## 5. Statistical analysis

A two-sample unpaired Student's test two-tail was used to test for differences between *wild-type* wings and *apkc*$^{ts}$ homozygous adult wings. A two-sample paired Student's test one-tail was used to test for differences either in aPKC or E-Cad or Arm staining between *wild-type* cells (anterior wing compartment) and *cpb* depleted cells (posterior wing compartment). All samples followed a normal distribution (Shapiro-Wilk's normality test, $p>0.1$) and variances were equal (Levene's test for equality of variances, $p>0.05$). Statistical tests were computed with the STATISTICA v9 (StatSoft) programme.

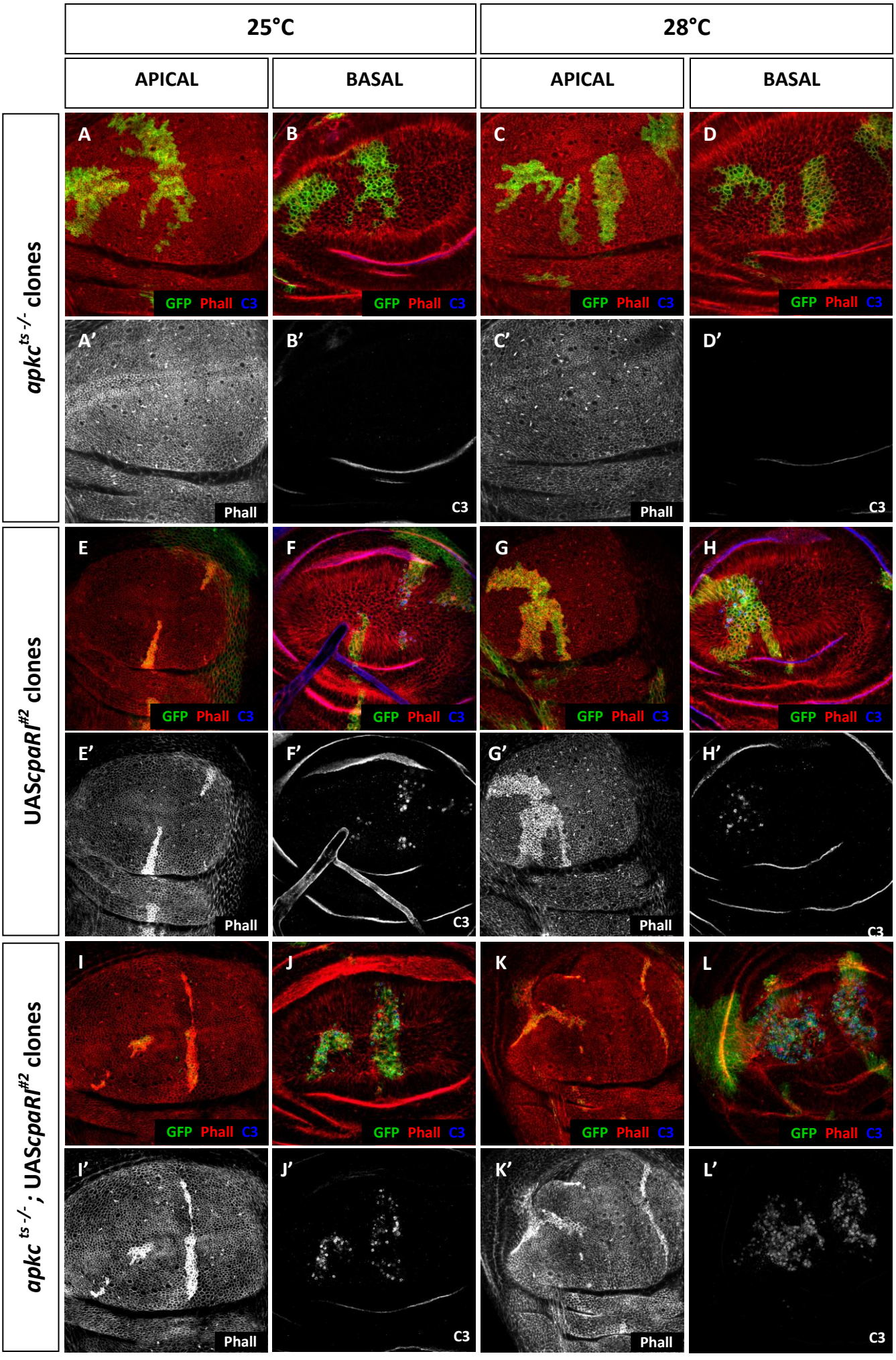
# IV. RESULTS

## 1. Capping Protein cooperates with aPKC

In order to demonstrate that the genetic interaction between CP and *aPKC*$^{CAXXDN}$ is due to a reduction of aPKC activity, I investigated whether decreasing aPKC levels using mutant alleles enhances the *CP* loss of function phenotype. To do so, I used a temperature sensitive allele of aPKC (*apkc*$^{ts}$) bearing a point mutation in a highly conserved residue of the protein kinase domain, which causes a temperature-dependent reduction of aPKC kinase activity (Guilgur, L.G. and Martinho, R. *et al.*, unpublished data). In contrast to *apkc* loss of function alleles [15, 79, 80], the use of the *apkc*$^{ts}$ allele (Martinho, R. *et al.*, unpublished data) allowed me to modulate aPKC levels by switching the temperature and thereby gave me the opportunity to determine whether CP interacts genetically with aPKC in maintaining wing epithelial integrity.

### 1.1 *apkc*$^{ts}$ induces extrusion and death of Cpa-deleted cells in mosaic clones induced in the wing blade

First, I analyzed whether the *cpa* depletion phenotype was enhanced when cells were also homozygote mutant for the *apkc*$^{ts}$ allele. In this sense, I made use of the MARCM system to induce clones homozygous mutant for *apkc*$^{ts}$ that overexpress UAS-*cpaIR*$^{\#2}$. Heat-shock was performed in 1$^{st}$ or at 2$^{nd}$ instar larvae in order to analyze whether clones induced at different developmental stages display differences in cell behaviour. Since *apkc*$^{ts}$ allele has different outcomes depending on the temperature, after being heat-shocked, larvae were raised at 25°C, 28°C or 30°C until late 3$^{rd}$ instar larvae. Discs were stained with anti-activated Caspase 3, which identify Caspase-dependent cell death and with TRITC-Phalloidin, to reveal F-actin. This allowed me to determine whether decreasing aPKC levels modulate cell death and F-actin accumulation of *cpa* depleted cells. It was difficult to obtain either *cpaIR*$^{\#2}$ or


25°C
28°C
APICAL
BASAL
APICAL
BASAL
apkc ts -/- clones
UAScpaRI#2 clones
apkc ts -/- ; UAScpaRI#2 clones
A
B
C
D
A'
B'
C'
D'
E
F
G
H
E'
F'
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I
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GFP Phall C3
Phall
C3

**Fig. 1 - CP and aPKC interact genetically to maintain epithelial integrity in specific regions of the wing disc.** All panels show Z-projections of third instar wing discs with clones positively labelled with GFP (green in A-L) induced at 1st instar and maintained either at permissive (25°C) (A-J') or at semi-permissive temperature (28°C) (C-L'). (A-D') *apkc*$^{ts}$ mutant clones, (E-H') *cpa* depleted clones and (I-L') *apkc*$^{ts}$ mutant clones expressing *cpaRI*$^{\#2}$ are stained with TRITC-phalloidin to reveal F-actin (red in A-L or grey in A', C', E', G', I', K') and anti-activated Caspase 3, which identifies Caspase-dependent cell death (blue in A-L or grey in B', D', F', H', J', L'). Dorsal is downwards.

*apkc*$^{ts\ -/-}$; *cpaIR*$^{\#2}$-expressing clones maintained at 30°C and thereby, I only analyzed clones kept at 25°C and 28°C.

While cells homozygote mutant for *apkc*$^{ts}$ showed no visible consequence on F-actin, as indicated by normal TRITC-Phalloidin staining, when discs were maintained at either 25°C (Fig.1A-A') or 28°C (Fig.1C-C'), *apkc*$^{ts\ -/-}$; *cpaIR*$^{\#2}$-expressing cells, labelled positively with GFP, still displayed a strong TRITC-phalloidin staining, reminiscent to the one observed in cells expressing *cpaIR*$^{\#2}$ (compare Fig.1E-E' with I-I' and G-G' with K-K'). This indicates that unlike *cpa* depletion, *apkc*$^{ts}$ allele has no effect on F-actin. However, I cannot rule out the possibility that aPKC could have subtle roles on F-actin organization, since the Par-aPKC complex is thought to locally regulate cytoskeletal dynamics, through the Rho-like GTPases Cdc42 and Rac [94].

Interestingly, when discs were maintained either at permissive or semi-permissive temperature, *apkc*$^{ts}$ mutant clones expressing *cpaIR*$^{\#2}$ showed a higher amount of activated Caspase 3 positive cells within the wing blade, as compared to clones expressing *cpaIR*$^{\#2}$ (compared Fig.1F-F' with 1J-J' and 1H-H' with 1L-L'). In contrast, *apkc*$^{ts}$ mutant clones did not undergo cell death in any experimental condition tested, as indicated by the absence of activated Caspase 3-expressing cells (Fig. 1B-B' and 1D-D'). Although some variability in anti-activated Caspase 3 staining was detected either in *cpaIR*$^{\#2}$ or *apkc*$^{ts-/-}$; *cpaIR*$^{\#2}$-expressing clones, when I qualitatively categorized each wing disc based on cell extrusion and death observed within GFP positive cells (see Material and Methods), this confirmed that decreasing aPKC activity levels with *apkc*$^{ts}$ enhances death of *cpa* depleted cells (Fig. 2).

Surprisingly, the majority of either *cpaIR*$^{\#2}$ or *apkc*$^{ts\ -/-}$; *cpaIR*$^{\#2}$-expressing clones induced at 2nd instar larvae were maintained in all regions of the wing disc (Fig.S1) and

just a small fraction of clones from either genetic context died in the wing blade. However, similar to clones induced at 1st instar larvae, quantification of this effect indicated that *apkc*$^{ts}$ mutant cells expressing *cpaIR*$^{\#2}$ are more likely to extrude basally and undergo apoptosis than cells depleted of *cpa* alone (Fig.2).

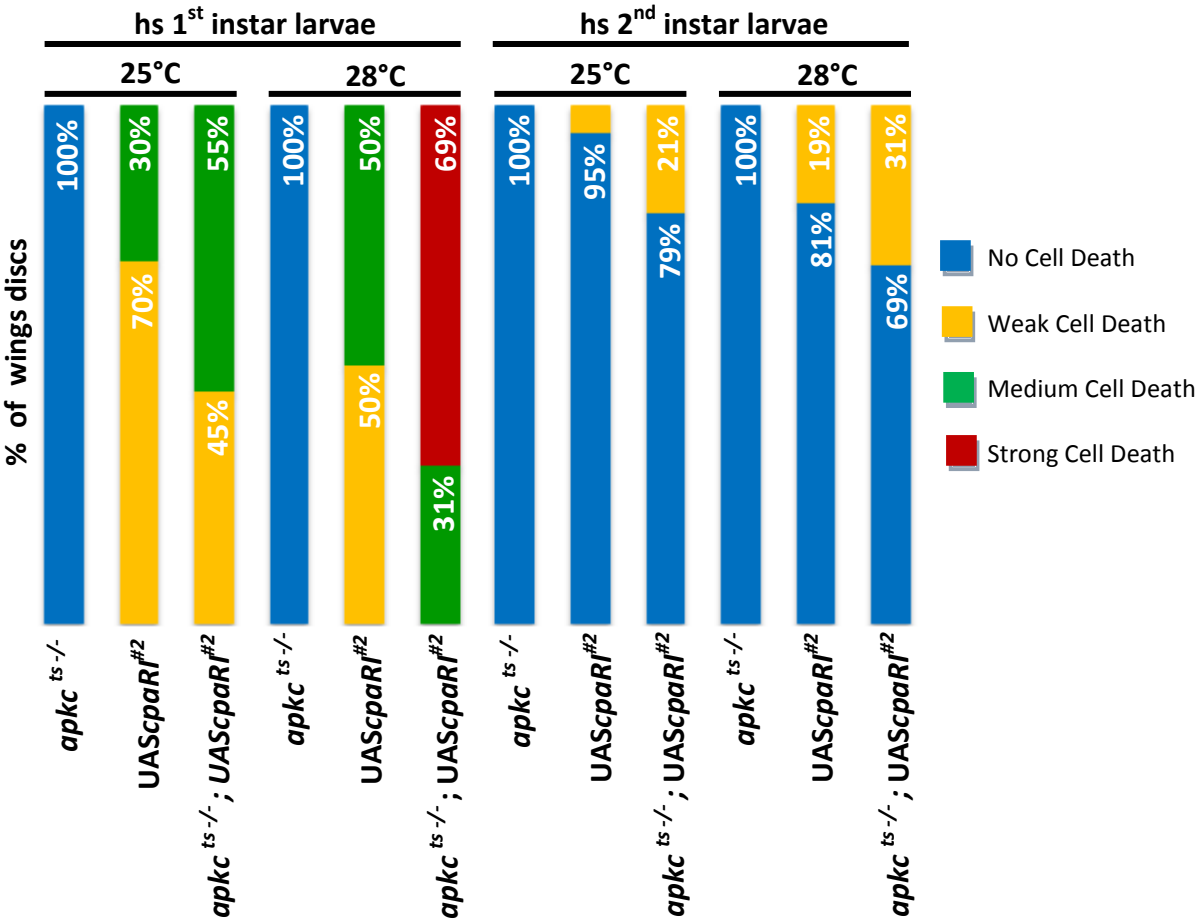



**Fig. 2 – Cell death classification in *apkc*$^{ts}$ mutant cells (*apkc*$^{ts-/-}$), *cpa* depleted cells (UAS*cpaIR*$^{\#2}$) and *apkc*$^{ts}$ mutant cells expressing *cpaIR*$^{\#2}$ (*apkc*$^{ts-/-}$; UAS*cpaIR*$^{\#2}$).** A qualitative criterion was used to categorize the amount of cell death observed in each wing disc (see Material and Methods).The columns represent the data for *apkc*$^{ts}$ mutant cells [hs 1st instar larvae n=10 (25°C) and n=13 (28°C); hs 2nd instar larvae n=16 (25°C) and n=16 (28°C)], *cpa* depleted cells [hs 1st instar larvae n=10 (25°C) and n=14 (28°C); hs 2nd instar larvae n=19 (25°C) and n=16 (28°C)] and *apkc*$^{ts}$ mutant cells expressing *cpaIR*$^{\#2}$ [hs 1st instar larvae n=20 (25°C) and n=13 (28°C); hs 2nd instar larvae n=34 (25°C) and n=13 (28°C)]. hs denotes heat-shock induction.

Unexpectedly, while *aPKC*$^{CAXXDN}$ induced apoptosis of *CP* mutant cells located in the wing hinge and notum [72], decreasing aPKC levels using the *apkc*$^{ts}$ allele did not promoted cell death of *cpaIR*$^{\#2}$-expressing clones in other wing regions than the wing

blade when tissues were grown either at 25°C (data not shown) or at 28°C (Fig.3C-C''). Reconstructed XZ sections through the wing disc showed that in the wing blade epithelium, either *cpaIR*$^{\#2}$- or *apkc*$^{ts-/-}$; *cpaIR*$^{\#}$ -expressing cells were found on the basal surface of the disc rather than within the epithelium (Fig. 3D-E). In contrast, *apkc*$^{ts}$ mutant clones survived equally well in all regions of the disc (Fig.3A-A').

In conclusion, regardless of the different cellular outcomes observed when clones were induced at different developmental stages (heat-shocks performed either at 1$^{st}$ or 2$^{nd}$ instar larvae), both experimental conditions revealed that CP interacts genetically with aPKC to maintain epithelial integrity and to prevent apoptotic cell death (Fig.2). Moreover, these results suggest that the genetic interaction between CP and aPKC have different requirements along the proximal-distal axis of the wing disc, favouring the idea that the wing blade epithelium has a distinct cellular dynamic and organization.

### 1.2 *apkc*$^{ts}$ enhances the Cpa-depletion phenotype in the posterior compartment of the wing disc

Although it was clear that CP and aPKC interact genetically in a mosaic analysis (Fig.1-3), differences in heat-shock timing led to unexpected variance in the final outcomes. To confirm the genetic interaction, I used the UAS-Gal4 system to overexpress UAS-*cpaIR*$^{\#2}$ and UAS-*GFP* using *hedgehog*-Gal4 (*hh*-Gal4) in *apkc*$^{ts}$ homozygous mutant wing discs. *hh*-Gal4 drives the expression of the UAS-target gene in the posterior domain of the wing imaginal disc and thereby, the anterior compartment can be used as *wild-type* control. Driving UAS-*cpaIR*$^{\#2}$ with *hh*-Gal4 causes actin filaments accumulation in the whole posterior compartment of the wing disc and extrusion and cell death in the wing blade (García Fernández, B. and Janody, F. *et al.*, unpublished data). Since I observed that *apkc*$^{ts}$ mutant allele enhanced death of cells expressing UAS-*cpaIR*$^{\#2}$ when discs were maintained at permissive temperature (25°C) (Fig.2), I only analyze the consequence of driving UAS-*cpaIR*$^{\#2}$ under *hh*-Gal4 control in *apkc*$^{ts}$ mutant wing discs at this temperature.

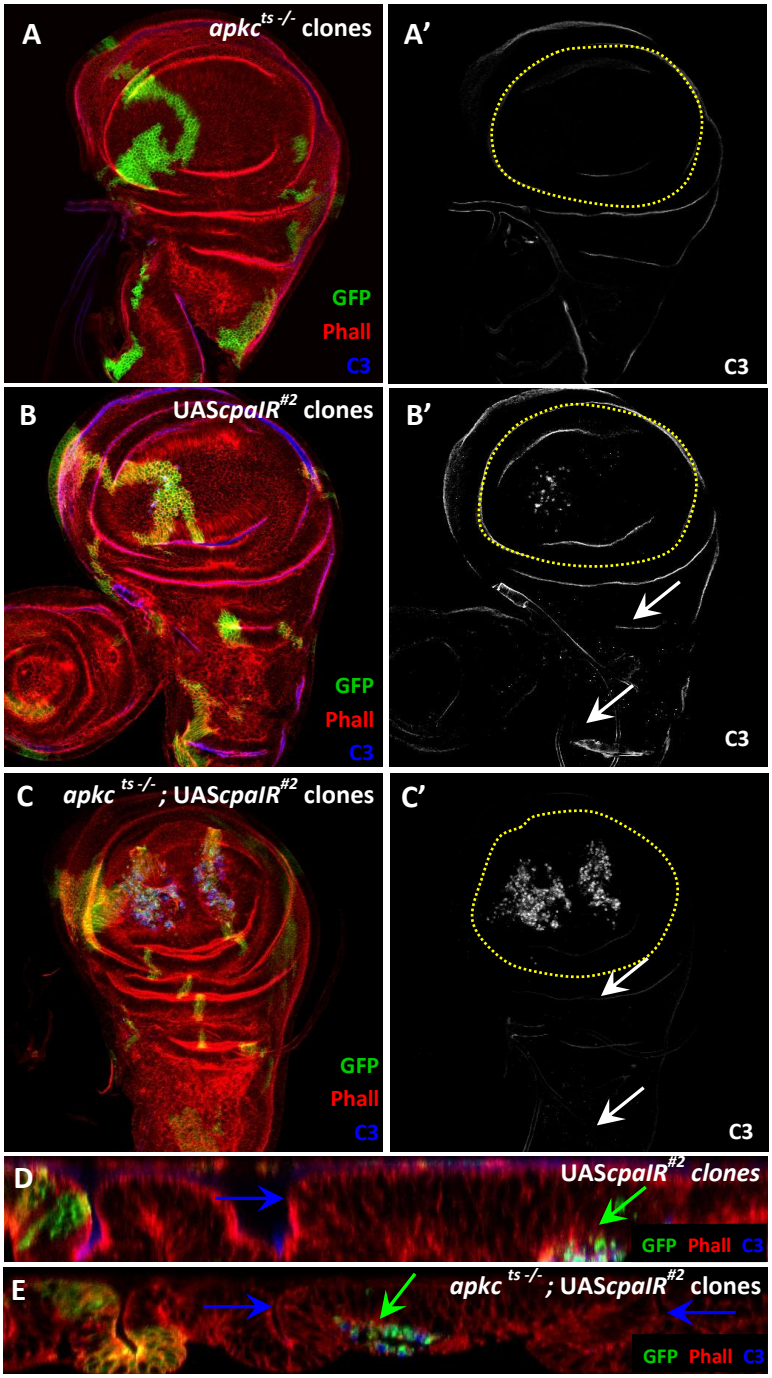



**Fig. 3 - *apkc*$^{ts}$ mutant clones expressing *cpaIR*$^{\#2}$ are extruded basally and undergo apoptosis in the wing blade epithelium.** All panels show third instar wing discs with clones positively labelled with GFP (green in A, B, C, D, E) induced at 1$^{st}$ instar larvae and maintained at 28°C. (A-E) Basal Z-projections. The wing blade is delimited by a punctuated yellow line. (D-E) Reconstructed XZ sections through the wing disc epithelium. (A-A') *apkc*$^{ts}$ mutant clones, (B-B' and D) clones expressing *cpaIR*$^{\#2}$ alone and (C-C' and E) *apkc*$^{ts}$ mutant clones expressing *cpaIR*$^{\#2}$ are stained with TRITC-phalloidin to reveal F-actin (red in A-E) and anti-activated Caspase 3, which identifies Caspase-dependent cell death (blue in A-E or grey in A'-C'). Green arrows in D and E indicate GFP positive cells extruding basally and undergo cell death and blue arrows define the wing blade region. Dorsal is downwards on Z-projections and to the left on reconstructed XZ sections.

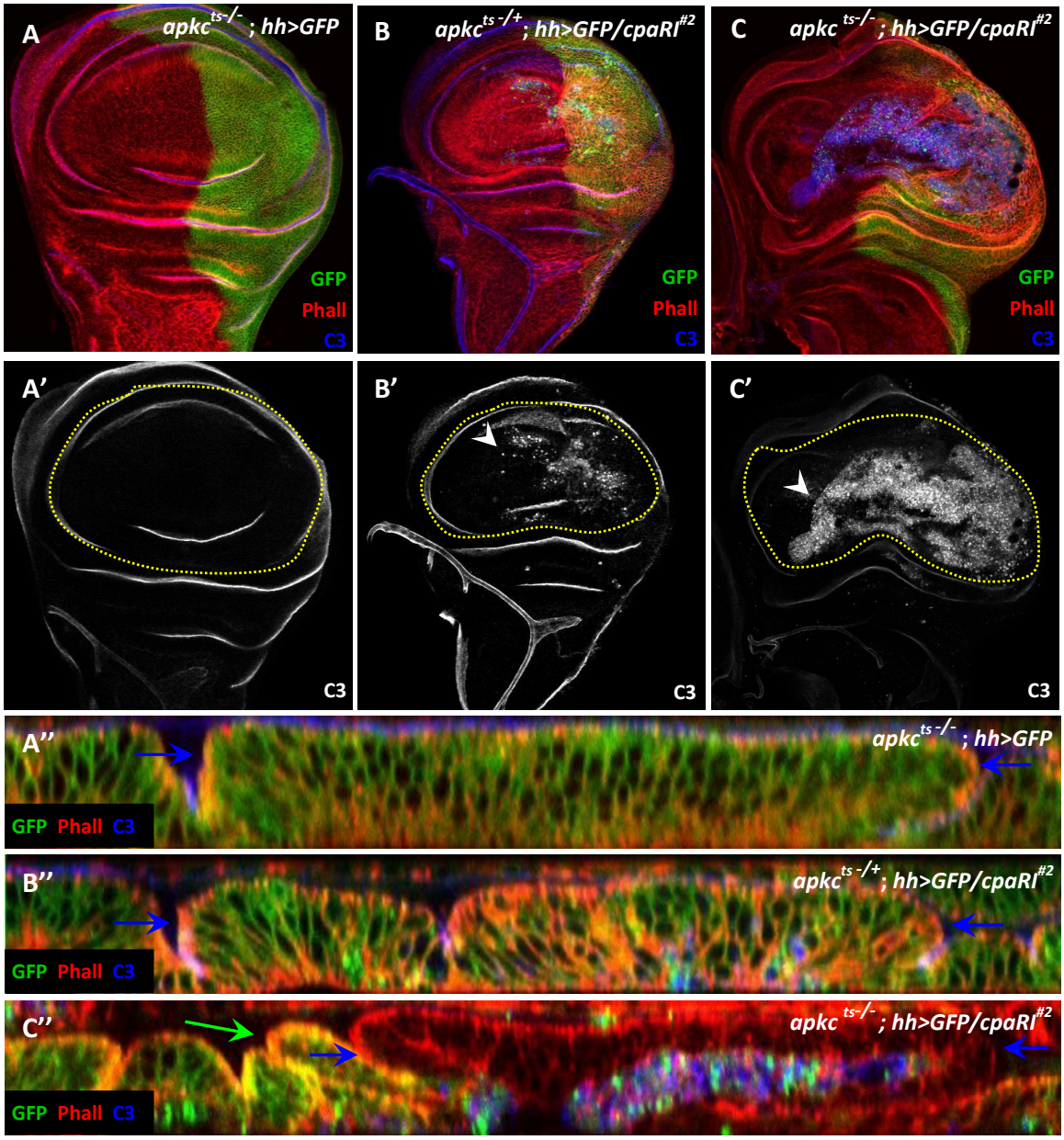



**Fig. 4 – In *apkc*$^{ts}$ mutant wing discs, depletion of *cpa* in *hh*-expressing cells causes massive cell death in the wing blade and might lead to hinge overgrowth.** All panels show third instar wing discs grown at permissive temperature (25°). (A-C') Basal Z-projections. The wing blade is delimited by a punctuated yellow line. (A'', B'', C'') Reconstructed XZ sections through the wing disc epithelium. (A-A'') *apkc*$^{ts}$ homozygous mutant wing discs expressing UAS-*GFP* (green) in the posterior compartment with *hh*-Gal4, (B-B'') *apkc*$^{ts}$ heterozygous mutant wing discs expressing UAS-*GFP* (green) and UAS-*cpaIR*$^{\#2}$ under *hh*-Gal4 control and (C-C'') *apkc*$^{ts}$ homozygous mutant wing discs expressing UAS-*GFP* (green) and UAS-*cpaIR*$^{\#2}$ in *hh*-expression domain stained with TRITC-Phalloidin to reveal F-actin (red in A, A'', B, B'', C, C'') and anti-activated Caspase 3, which identifies Caspase-dependent cell death (blue in A, A'', B, B'', C, C'' or grey in A', B', C'). White arrowheads in B' and C' indicate cells crossing the anterior-posterior compartment boundary and relocalizing to the anterior. Green arrow in C'' indicate the presence of extra foldings in the hinge region. Blue arrows define the wing blade region. Posterior is towards the right and dorsal downards on basal Z-projections. Dorsal is towards left on reconstructed XZ sections.

As expected, *apkc*$^{ts}$ mutant wing discs were morphologically normal and no dying cells were recovered in the whole wing region (Fig. 4A-A''). *hh>GFP/cpaIR*$^{\#2}$-expressing cells in which I removed on copy of *apkc*$^{ts}$ (Fig. 4B-B´') showed the *hh>GFP/cpaIR*$^{\#2}$ phenotype (García Fernández, B. and Janody, F, unpublished data). However, when I drove UAS-*cpaIR*$^{\#2}$ with *hh*-Gal4 in *apkc*$^{ts}$ mutant discs, this led to activation of Caspase 3 in most GFP-positive cells located in the wing blade epithelium, indicating that those cells were dying (Fig. 4C-C''). This confirms that CP cooperates with aPKC to prevent cell death in the wing blade epithelium.

Reconstructed XZ sections through wing disc epithelia showed that *apkc*$^{ts\ -/-}$; *hh>GFP/cpaIR*$^{\#2}$ cells extrude basally and undergo apoptosis in the presumptive blade (Fig.4C''), whereas in the hinge region cells were not only able to survive, but also seemed to overproliferate, as suggested by the presence of extra foldings (green arrow in Fig.4C''). These foldings were not observed in *apkc*$^{ts\ -/-}$ wing discs (Fig.4A'') or in heterozygous mutant discs for *apkc* expressing UAS-*cpaIR*$^{\#2}$ in the *hh*-domain (Fig.4B''), suggesting that in this region, CP and aPKC synergize to restrict growth. In addition, similar to *CP* depleted cells alone (white arrow in Fig.4B' and Fig.8D), *apkc*$^{ts-/-}$ ;*hh>GFP/cpaIR*$^{\#2}$ cells were often found in the anterior compartment of disc (white arrow in Fig.4C'), suggesting that decreasing aPKC levels using the *apkc*$^{ts}$ allele seemed to increase the motility of *cpa* depleted cells.

Consistent with the mosaic analysis, decreasing aPKC levels enhanced cell death of *hh> GFP/cpaIR*$^{\#2}$-expressing cells, arguing for a genetic interaction between CP and aPKC to maintain epithelial integrity and to prevent death of wing blade cells. Moreover, in this experimental condition, I also found that *apkc*$^{ts}$ appears to enhance the ability of *cpa* depleted cells to overproliferate in the hinge region.

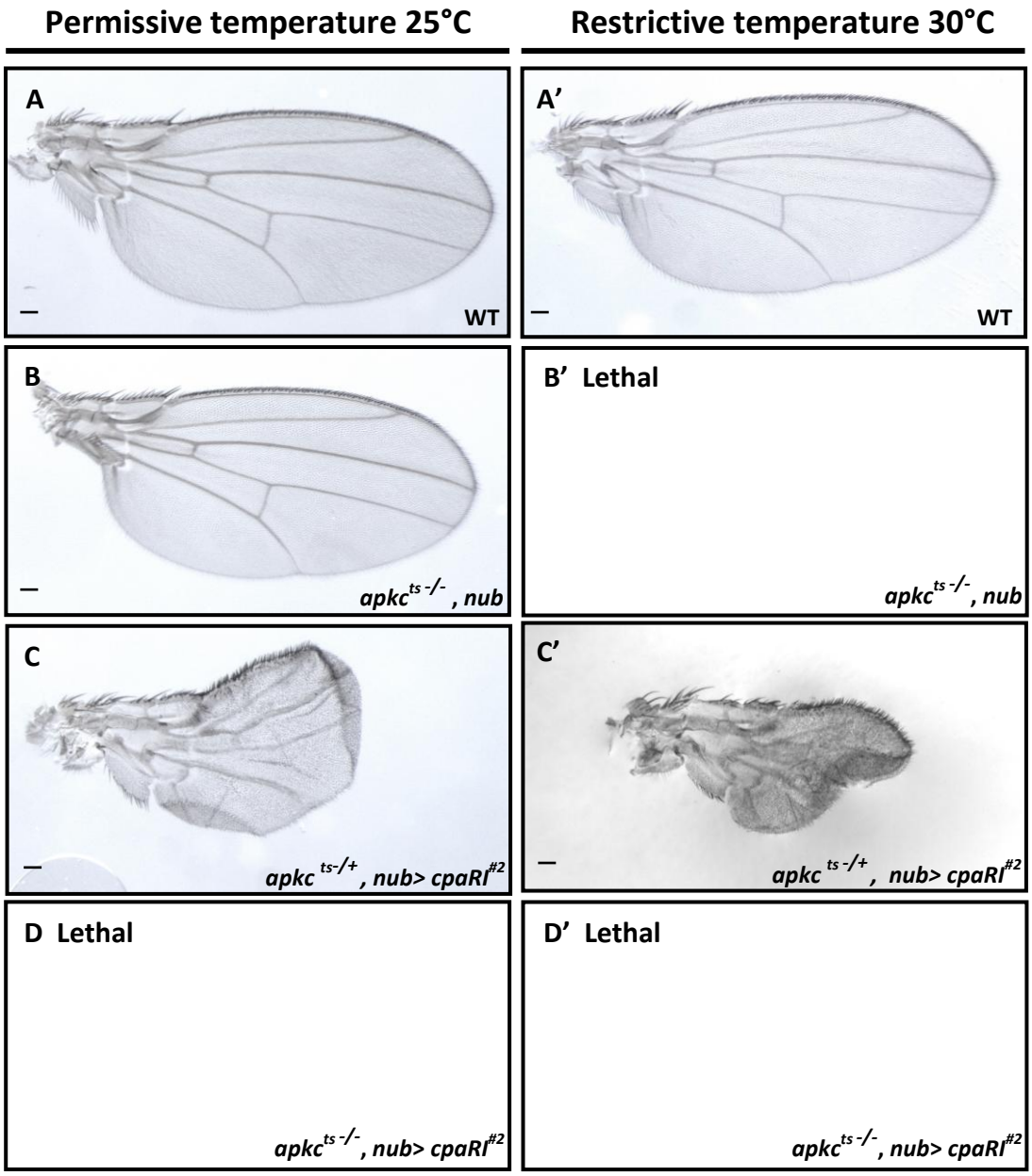
### 1.3 Capping Protein acts synergistically with aPKC during adult wing morphogenesis

It was previously shown that driving expression of the dominant negative form of aPKC (*aPKC*$^{CAXXDN}$), using *nubbin*-Gal4 (*nub*-Gal4), which drives the expression of the

UAS-target gene in the wing blade and the distal region of the hinge, enhances the *cpb* depletion phenotype of adult wings (see Introduction Fig.8). Therefore, I next asked whether decreasing aPKC levels using the *apkc*$^{ts}$ mutant allele also enhance the phenotype of *CP* depleted adult wings. To do so, I expressed UAS-*cpaIR*$^{\#2}$ with the *nub*-Gal4 driver in an *apkc*$^{ts}$ mutant background maintained at either permissive (25°C) or restrictive temperature (30°) and analyzed the adult wing phenotypes.

Adult flies homozygote mutant for *apkc*$^{ts}$ were only viable when maintained at 25°C and mutant wings did not display any patterning defects (compare Fig. 5A with 5B). However, the wing size was significantly reduced when compared to *wild-type* wings (Fig. 5E), suggesting that proliferation or cell survival may be affected in *apkc*$^{ts}$ mutant wings maintained at permissive temperature. Surprisingly, when I drove UAS-*cpaIR*$^{\#2}$ with *nub*-Gal4 in an *apkc*$^{ts}$ mutant background, this led to pupal lethality either at permissive or restrictive temperatures, arguing that reduced aPKC and CP levels synergise to induce pupal lethality. In contrast, removing one copy of *apkc* did not affect the viability of *nub>cpaIR*$^{\#2}$ flies, neither enhanced the *cpa* depletion phenotype of wings maintained at 25°C (compare Fig. 5C with Introduction Fig. 8C).

To further demonstrate that pupal lethality results from decreased aPKC and CP levels, I drove both *cpb* and *apkc* depletion using RNAi lines (UAS-*cpbIR*$^{B4}$ and UAS-*apkcIR*$^{KK100874}$, respectively) with the *nub*-Gal4 driver. Indeed, only one male expressing both constructs was recovered when flies were raised at 25°C (n=119 flies from four independent crosses), arguing that reduced levels of both CP and aPKC in *nub*-expressing cells leads to pupal lethality. Since the lethality of *nub>cpbRI*$^{B4}$, *apkcIR*$^{KK100874}$ flies did not permit to analyze the adult wing phenotype, I next attempted to recover adult flies by maintaining flies at 18°C, thereby reducing Gal4 activity. Indeed, under this experimental condition, flies expressing both *cpbRI*$^{B4}$ and *apkcRI*$^{KK100874}$ with *nub*-Gal4 were viable. At 18°C, *cpb* depleted wings seemed slightly smaller and were downwardly curved when compared to *wild-type* wings (compare Fig.6A with 6B), while expressing *apkcRI*$^{KK100874}$ with *nub*-Gal4 resulted in a severely reduced and thickened wing (Fig. 6C), suggesting that cell death may have occurred during wing development. When I combined both *cpb* and *apkc* depletion using *nub*-



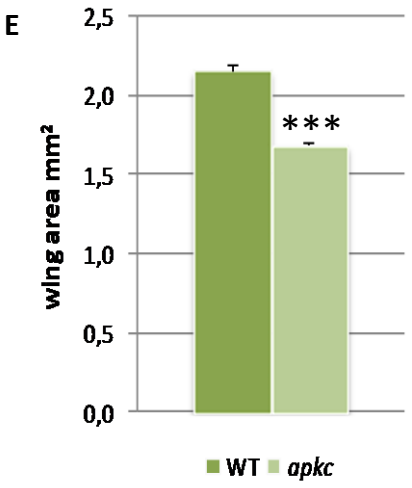




**Fig. 5 - Decreasing *cpa* using *nub*-Gal4 driver in *apkc*$^{M}$ homozygous mutant flies results in pupal lethality.** (A-A') *wild-type* wing (WT), (B-B') *apkc*$^{ts}$ homozygous mutant wing, (C-C') depletion of *cpa* with *nub*-Gal4 in an *apkc*$^{ts}$ mutant background and (D-D') depletion of *cpa* with *nub*-Gal4 in an *apkc*$^{ts}$ mutant background maintained either at permissive (25°C) or restrictive temperature (30°C). (E) *apkc*$^{ts}$ homozygous mutant wings are significantly reduced when compared to WT wings (***p<0.001, n = 30 adult wings in each case). Error bars indicate standard error (SE). Magnification scale bars 100µm.

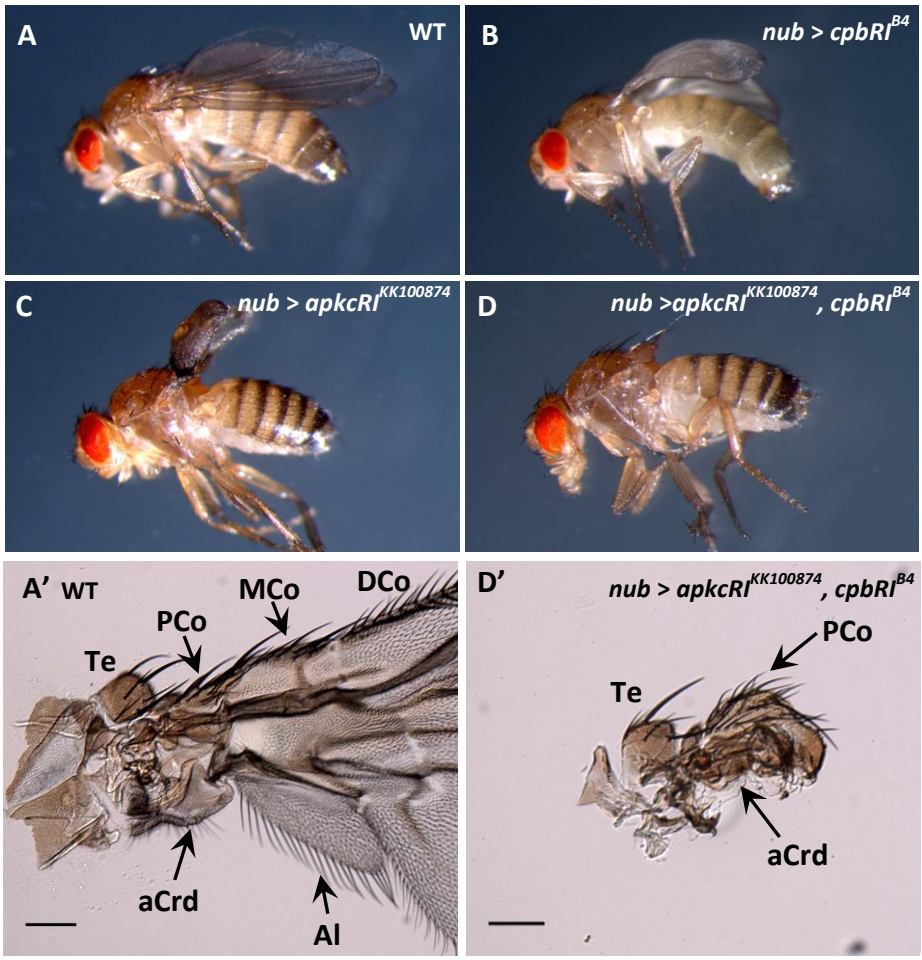



**Fig. 6 – CP acts synergistically with aPKC during wing morphogenesis.** (A) *wild-type* (WT), (B) *nub>cpbRI*$^{B4}$, (C) *nub> apkcRI*$^{KK100874}$ and (D) *nub> nub>apkcRI*$^{KK100874}$; *cpbIR*$^{B4}$ flies raise at 18°C. (A') Close up view of the *wild-type* wing hinge. The more proximal portion of the hinge, which corresponds to the outer ring in the wing imaginal disc, is morphologically demarcated from the rest of the wing and is composed by the proximal costa (PCo) and the axilary cord (aCrd) [95]. The more distal portion of the hinge, which corresponds to the inner ring in the disc, is continuous with the wing blade and contains the allula (Al), the medial costa (MCo) and the distal costa (DCo) [95]. The tegula (Te) is not considered a part of the hinge [96]. (D') Close up view of the *nub>apkcRI*$^{KK100874}$; *cpbIR*$^{B4}$ adult wing. The wing blade and the distal hinge structures, including the medial costa (MCo), the distal costa (DCo) and allula (Al) are missing. The remaining wing tissue seems to have cells with proximal costa (PCo) and axilary cord (aCrd) identity. The tegula (Te) do not seem to be altered. Magnification scale bars 100µm.

Gal4, this led to a complete loss of the proper wing and the distal hinge structures, including the medial costa (MCo), distal costa (DCo) and allula (Al) (compare Fig.6A'with 6D'). Although the remaining tissues seemed to be similar to the proximal costa (PCo) and the axilary cord (aCrd) structures structures, I did not analyze the expression of patterning genes, such as *homothorax* and *wingless*, to confirm the

cellular identity of the remaining wing tissue. The tegula (Te) and the notum (data not shown) are the only structures that do not seem to be altered. Overall, these results indicate that depletion of both *cpb* and *apkc* using *nub*-Gal4 results in a complete loss of the proper wing, suggesting that CP acts synergistically with aPKC to prevent cell death of wing blade cells. However, I did not analyze *nub>apkcRI*$^{KK100874}$; *cpbIR*$^{B4}$ third instar wing discs, and therefore I cannot confirm that cells depleted of both *cpb and apkc* extrude basally and undergo cell death.

## 2. aPKC apical localization is affected by knocking-down CP levels

Since I showed that CP and aPKC interact genetically to maintain epithelial integrity of the wing disc (Fig. 1-6), I next asked whether CP acts in parallel or upstream of aPKC. In this sense, I first determine if CP colocalizes with aPKC at apical sites by expressing an HA-tagged form of Cpa (UAS-HA-*cpa*$^{ID}$) using the *hh*-Gal4 driver and stained the discs with anti-aPKC and anti-HA to reveal HA-Cpa expression. Indeed, I observed that both aPKC and HA-Cpa were mostly localized at the apical surface of wing cells (Fig. 7A-A' and B-B', respectively).

I next qualitatively evaluated the level of colocalization between aPKC and HA-Cpa (see Material and Methods). Colocalization masks above 60% of pixel intensity revealed that HA-Cpa has strong colocalization at apical cell surface with aPKC (white dots in Fig. 7C and D). Reconstructed XZ sections through the wing disc also revealed a colocalization of aPKC with the apical pattern of HA-Cpa (white dots in Fig. 7C' and D'). When I quantified this colocalization using the Mander's Overlap coefficient (see Material and Methods), I found that 73.7% of aPKC and HA-Cpa fluorescent signals overlapped, arguing for an extensive colocalization between CP and aPKC.

Next, I drove the expression of UAS-*cpbRI*$^{B4}$ and UAS-*GFP* using *hh*-Gal4 driver and analyzed whether reducing CP levels could affect the apical localization of aPKC. While aPKC was apically restrict in *hh>GFP* control discs (Fig.8A-A'), *cpb* depleted cells labelled with GFP showed a very diffuse anti-aPKC staining at apical surface (Fig.8B-B'). Reconstructed XZ sections through the wing disc epithelium revealed that aPKC is

largely absent at the apical domain of *cpb* depleted cells, when compared to anterior cells serving as internal control (Fig. 8D-D') or *hh>GFP* control discs (Fig. 8C-C'). Indeed, when I measured the fluorescent intensity of anti-aPKC staining (see Material and Methods), I found that *cpb* depleted cells (posterior domain) displayed a significant reduction of apical aPKC when compared to *wild-type* cells (anterior domain) (Fig. 8E), suggesting that CP restricts the localization of aPKC to the apical cellular domain.

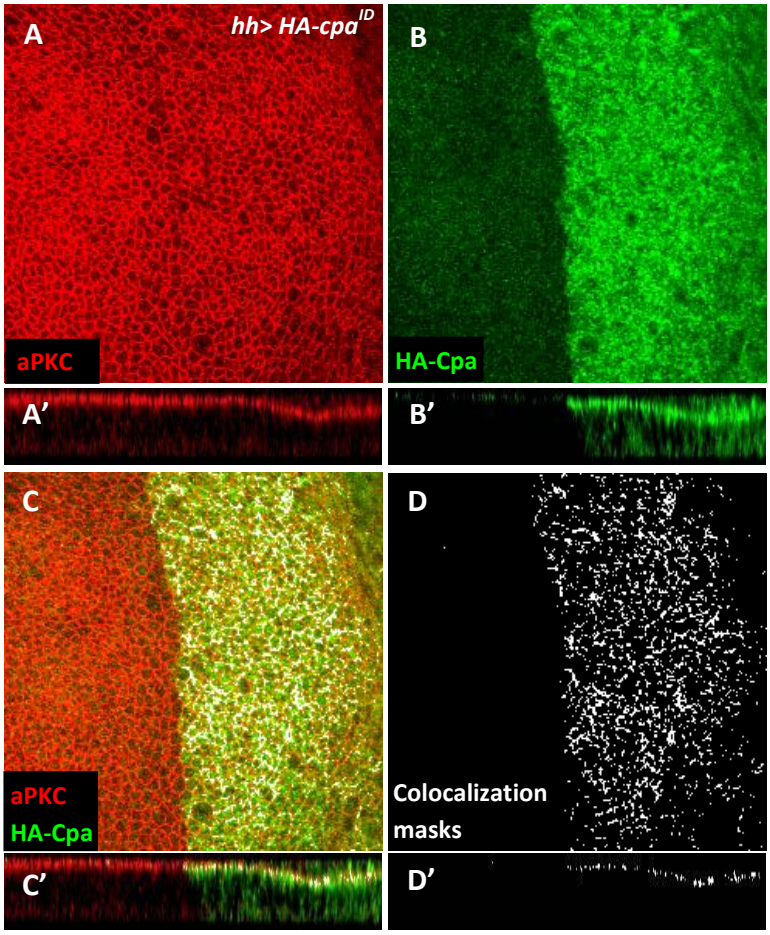
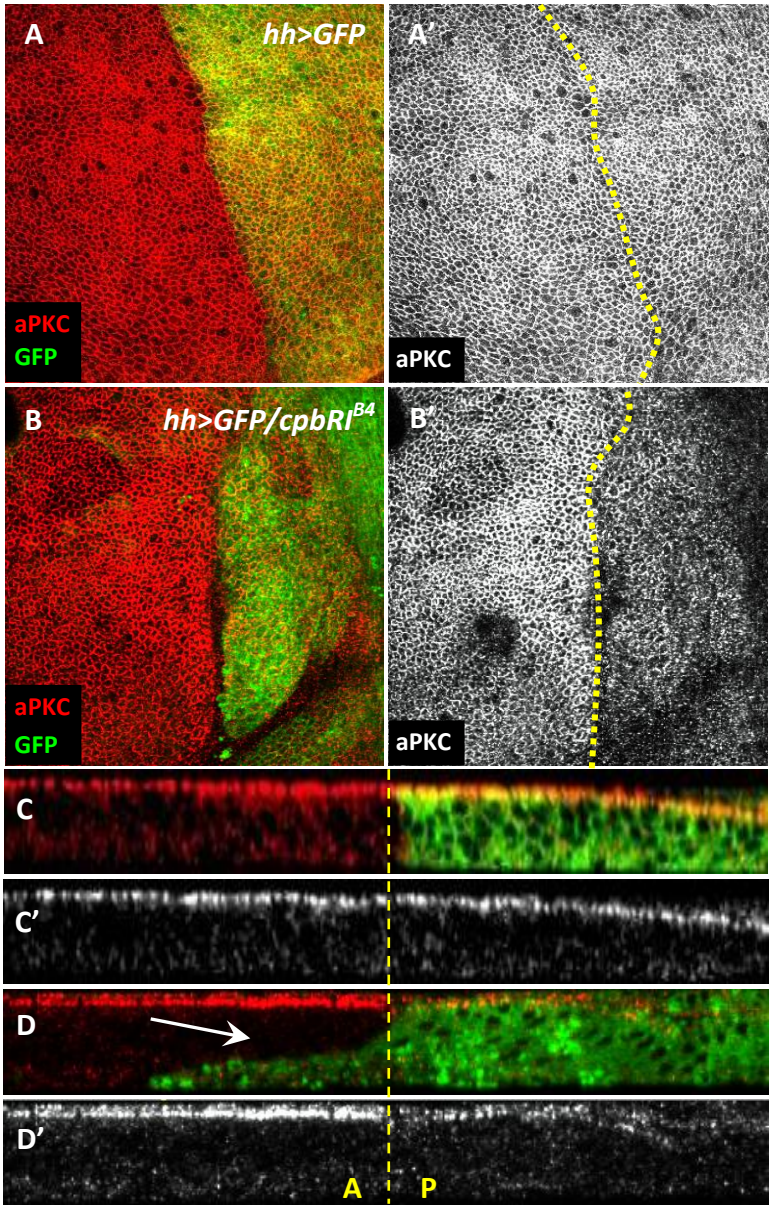



**Fig. 7 - Cpa and aPKC colocalize at apical cell membrane.** (A-C') Third instar wing discs expressing UAS-HA-*cpa*$^{ID}$ in the posterior compartment using *hh*-Gal4 and stained with anti-HA to reveal HA-Cpa expression (green in B-C') and anti-aPKC (red in A-A' and C-C'). (A-D) Apical Z-projections. (A'-D') Reconstructed XZ sections through the wing disc epithelia. (D-D') Colocalization masks above 60% of pixel intensity (white dots) showing overlapping regions of aPKC (red) and HA-Cpa (green). (C-D') HA-Cpa (green) has strong colocalization at the apical cell surface (white dots) with aPKC (red) (Mander's overlap coefficient = 0.737 ± 0.02 (SD), ratio red: green pixels = 1.044 ± 0.06 (SD), n=6 wing discs). Posterior is towards right and dorsal downwards.



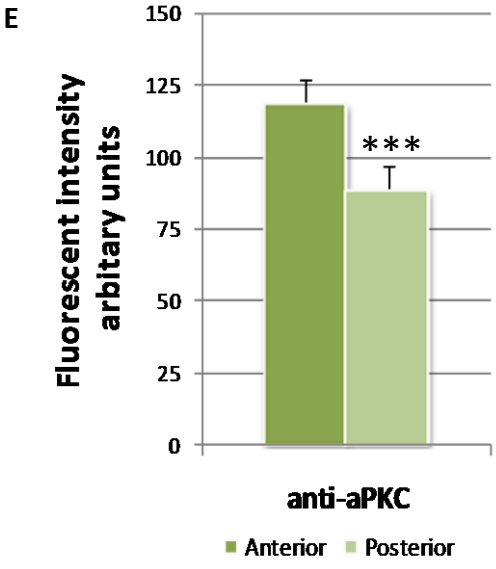




**Fig. 8 – CP restricts the localization of aPKC to the apical sites**. (A-D') Third instar wing imaginal discs expressing UAS-*GFP* alone (green in A and C) or UAS-*GFP*/UAS-*cpbRI*$^{B4}$ (green in B and D) in the posterior compartment with *hh*-Gal4 and stained with anti-aPKC (red in A-D and grey in A'-D'). (A-D) Apical Z-projections. (A'-D') Reconstructed XZ sections through the wing disc epithelia. (E) anti-aPKC fluorescent intensity is significantly lower in posterior cells (*cpb* depleted cells) when compared to anterior cells serving as internal control (***p<0.001, n=12 wing discs). Error bars indicate the standard error (SE). White arrow in D indicates cells crossing the anterior-posterior compartment boundary and relocalizing to the anterior. Posterior is towards right and dorsal downwards. A/P – anterior-posterior boundary.

Furthermore, as previously mentioned (Fig.4), numerous *cpb* depleted cells, expressing GFP, were found beneath the wing epithelium, crossing the anterior-posterior compartment boundary and relocalizing to the anterior (white arrow in Fig.8D), suggesting that CP prevents cell motility.

## 3. Capping Protein maintains AJs components in the apical surface

While loss of Cdc42, Par6 or aPKC activity causes a depletion of the AJs proteins E-Cad and Arm from the plasma membrane [14, 15, 97], the apical transmembrane proteins Crb and Notch accumulate in early endosomal compartments [14]. Interestingly, previous results from F. Janody's Lab revealed that decreasing CP levels by making use of RNAi lines leads to accumulation of Crb and Notch in punctuated structures, suggesting that CP regulates endocytosis of apical transmembrane proteins (see Introduction Fig.5) [72]. Moreover, one of the first consequences of removing CP, using lethal alleles for *cpa* or *cpb*, is the mislocalization of E-cad and Arm along more basolateral domains of the wing blade cells [20]. Therefore, it is conceivable that by promoting aPKC localization, CP regulates the proper localization of apical proteins, such as Notch and Crb, and maintains AJs in a state of dynamic equilibrium through endocytosis of E-Cad and Arm.

To test this possibility, I first determined whether decreasing CP levels by expressing UAS-*cpbRI*$^{B4}$ and UAS-*GFP* using the *hh*-Gal4 driver leads to a reduction of E-Cad level at the apical surface, as previously observed in *apkc* mutant tissues [14, 15, 97]. While E-Cad was apically restricted in *hh>GFP* control discs, as indicated by anti-E-Cad staining (Fig. 9A-A'), *cpb* depleted cells marked by the presence of GFP showed decreased levels of E-cad at the apical domain (Fig. 9B-B'). However, in *cpb* depleted cells E-cad was not mislocalized to basolateral positions (Fig. 9B-B'), as previously described in *cpa* mutant clones induced at early third instar [20]. When I measured the fluorescent intensity of anti-E-cad staining (see Material and Methods), I observed that cells depleted of *cpb* (posterior wing domain) displayed a significantly reduction of E-cad at the apical sites, when compared to anterior cells serving as internal control (Fig.

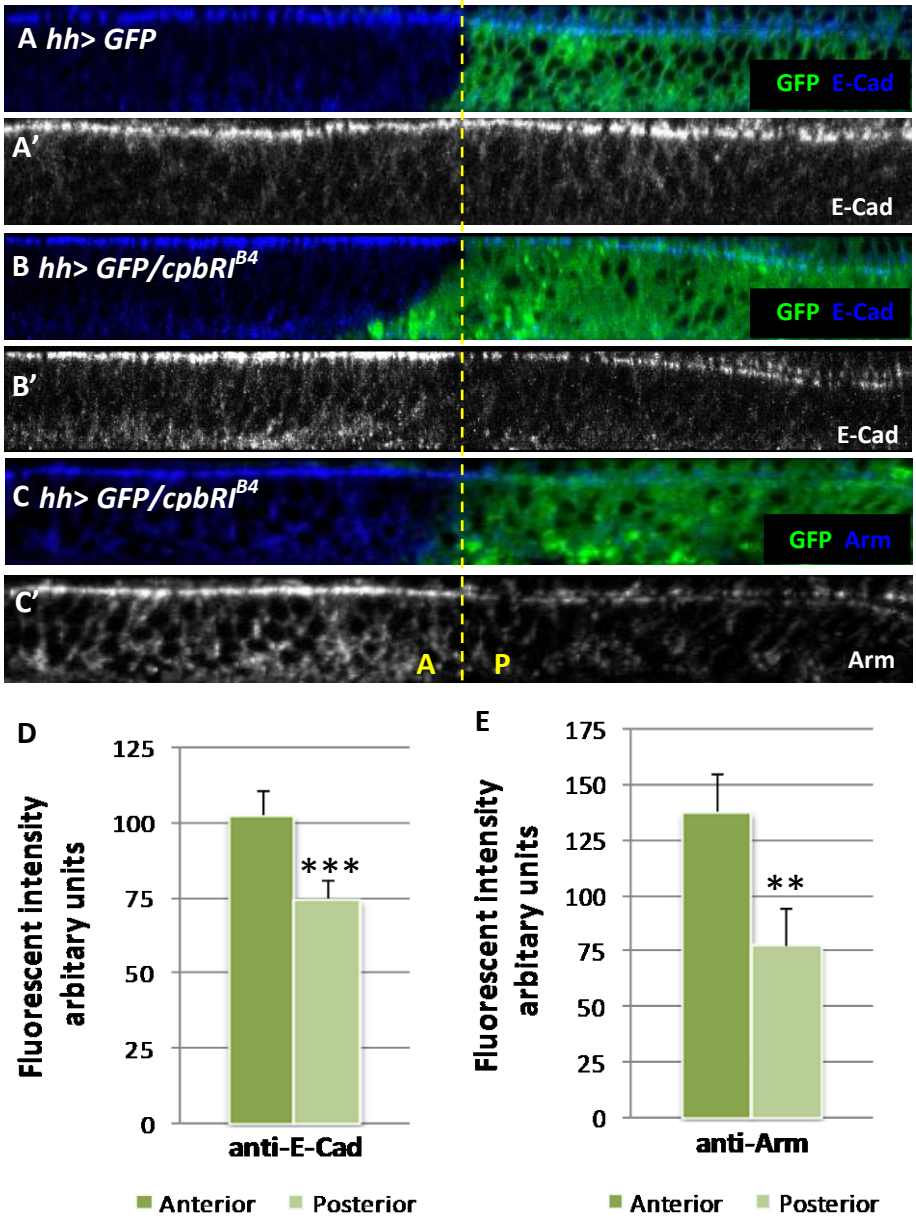



**Fig. 9 - CP restricts the localization of E-Cad and Arm to the apical sites.** (A-C') Third instar wing imaginal discs expressing UAS-*GFP* alone (green in A) or UAS-*GFP*/UAS-*cpbRI*$^{B4}$ (green in B and C) in the posterior compartment with *hh*-Gal4 stained with anti-E-Cad (blue in A, B and grey in A', B') or anti-Arm (blue in C and grey in C'). (A-C') Reconstructed XZ sections through the wing disc epithelia. (D) anti-E-Cad or (E) anti-Arm fluorescent intensity is significantly lower in posterior cells (*cpb* depleted cells) when compared to anterior cells serving as internal control (***p<0.001, n=10 wing discs and **p<0.01, n=5 wing discs).Error bars indicate the standard error (SE). Posterior is towards right. A/P – anterior-posterior boundary.

9D). I next analyzed whether Arm levels were also affected by *CP* depletion. Indeed, depletion of *cpb* in the posterior wing domain resulted in a significantly decrease of Arm at the apical surface (Fig. 9C-C' and E). In addition, like E-Cad (Fig. 9B-B'), Arm was

not mislocalized along the basolateral membrane (Fig. 9C-C'). All together, these results suggests that, similar to *apkc* mutant tissues [14, 15, 97], depletion of *CP* results in a decrease of the junctional proteins E-Cad and Arm at apical membrane, which in turn destabilizes AJs and leads to the disruption of epithelial integrity. Furthermore, in a closer examination of *cpb* depleted cells, I observed gaps in junctional E-Cad (Fig.10A') that were not found in *hh>GFP* control discs (Fig.10A).

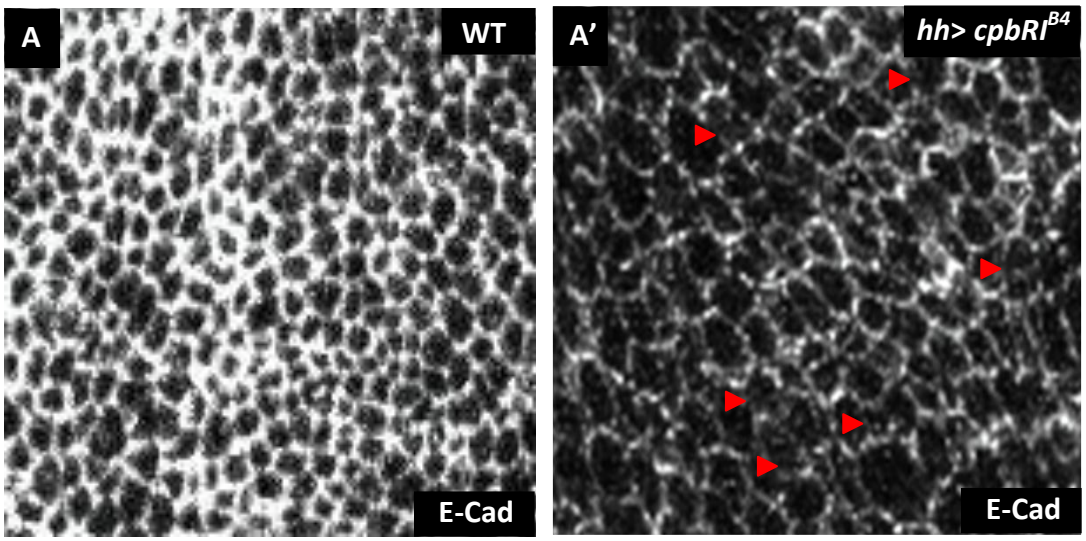



**Fig. 10 – Closer examination of E-Cad defects in *cpb* depleted cells** (A-A') Apical Z-projections of third instar wing imaginal discs stained with anti-E-Cad. (A) *wild-type* (WT) wing disc and (A´) *cpb* depletion in *hh*-expressing cells. Red arrows indicate gaps in E-Cad.

Interestingly, the behaviour of cells with reduced levels of CP recapitulates many aspects of neoplastic transformation resulting from increased Src activity. In fact, it was previously shown that loss of CP cooperates with increased Src64B levels to trigger apoptotic cell death in the wing blade (García-Fernandéz, B. and Janody, F. *et al.*, unpublished data). Since Src interacts directly with AJs components, and when overactivated, disrupts AJs stability [42, 43], I further analyzed whether increased levels of Src64B could justify the reduction of E-Cad at apical domain of *cpb* depleted cells (Fig. 9B-B'). In fact, when I overexpressed UAS-*Src64B*$^{UY1332}$ and UAS-*GFP* using *hh*-Gal4 driver, I found that cells with increased levels of Src64B that were still present within the tissue displayed reduced levels of E-Cad at apical surface, when compared

to anterior cells serving as internal control (Fig. 11B-B') or *hh>GFP* control discs (Fig. 11A-A'). Since the majority of GFP positive cells expressing *Src64B*$^{UY1332}$ were extruded basally (Fig. 11C-C') and undergo cell death (García-Fernandéz, B. and Janody, F. *et al.*, unpublished data), it was difficult to measure the fluorescent intensity of anti-E-cad staining at apical cell surface and therefore, I did not performed statistical analysis. Similar results were obtained for Arm (García-Fernandéz, B. and Janody, F. *e al.*, unpublished data). This suggests that CP might cooperate with Src to maintain the AJs components E-Cad and Arm at apical surface and therein prevent loss of epithelial integrity and cell death in the wing disc.

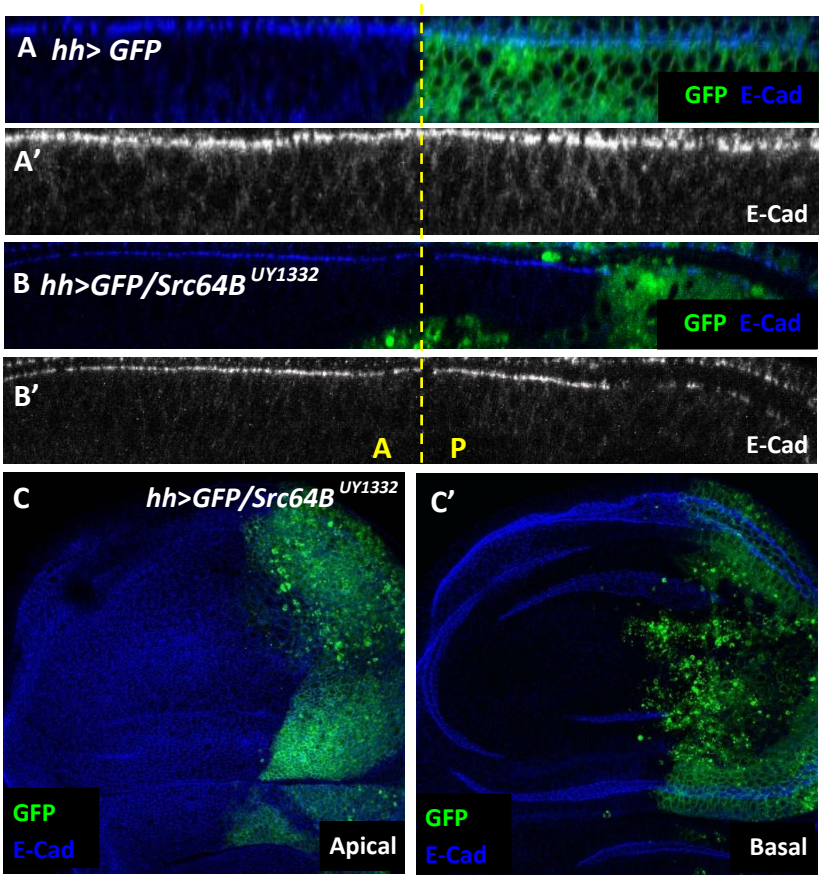



**Fig. 11 - Src64B overexpression mimics the behaviour of *cpb* depleted cells.** (A-C') Third instar wing imaginal discs expressing UAS-*GFP* alone (green in A) or UAS-*GFP*/ UAS-*Src64B*$^{UY1332}$ (green in B and C) in the posterior compartment with *hh*-Gal4 stained with anti-E-Cad (blue in A, B, C, C' and grey in A', B'). (A-B') Reconstructed XZ sections through the wing disc epithelia. (C) Apical or (C') basal Z-projections. Posterior is towards right and dorsal downwards. A/P – anterior-posterior boundary.

# VI. DISCUSSION

## 1. Capping Protein interacts genetically with aPKC

In this report I provide evidence that Capping Protein, an actin binding protein that restricts actin polymerization, and aPKC, a major component of the Par-aPKC complex, interact genetically to maintain epithelial integrity in *Drosophila* wing imaginal disc. The clonal analysis I performed revealed that decreasing aPKC activity, using $apkc^{ts}$ allele, enhanced extrusion and death of *cpa* depleted cells in the wing blade region, while clones located in the hinge and notum were maintained within the epithelium (see Results Fig.1-3). Moreover, knocking-down *cpa* using *hh*-Gal4 driver in an $apkc^{ts}$ mutant background enhanced the phenotype of *cpa* depleted cells, as indicated by massive cell death in the wing blade and formation of extra foldings in the distal hinge region (see Results Fig.4). Taken together, these results argue that CP interacts genetically with aPKC to prevent extrusion and death of wing blade cells, as previously observed in F. Janody's Lab (see Introduction Fig.8) [72], and further suggest that both CP and aPKC may also restrict growth in the distal hinge region.

Surprisingly, I found that depletion of *cpa* with *nub*-Gal4 in an $apkc^{M}$ mutant background led to pupal lethality either at permissive (25°C) or restrictive temperature (30°C) (see Results Fig.5). *nubbin* promotes proliferation and differentiation of the wing blade and distal hinge regions, but is also required during asymmetric division of neuroblasts that give rise to specific motor neurons [98]. Since the Par-aPKC complex [99, 100] and a dynamic actin cytoskeleton [100] are essential for a proper asymmetric division of *Drosophila* neuroblasts, it could be possible that decreased levels of CP and aPKC might result in a deficient neuromuscular system and pupal lethality. This hypothesis is reinforced by the fact that reducing aPKC levels with a different genetic tool (termosensitive mutant allele vs. RNAi lines) also led to pupal lethality of $nub>cpaIR^{\#2}$ flies, suggesting that CP appears to cooperate with aPKC in promoting neuronal differentiation and thereby, in preventing pupal lethality. Such possibility should be further studied.

### 1.1 The interaction between CP and aPKC has differential requirements along the proximal-distal axis

As mentioned above, cells depleted of *cpa* that were also mutant for $apkc^{ts}$ extrude and die only in the wing blade, but not in the hinge or notum (see Results Fig. 1-4). This suggests that wing blade cells have distinct cytoskeletal organization and/or adhesive properties that make them more sensitive to reduced levels of CP and aPKC. Indeed, in *wild-type* discs, cells in the centre of the wing blade have a narrow apical cell surface and express higher levels of F-actin and E-cad, a major component of AJs, when compared to hinge and notum cells [101]. Moreover, it was shown that tissue surface tension, caused by adhesion between cells [1], is uniformly distributed in the wing disc, with maximum compression in the centre of the wing blade [102]. Therefore, in order to allow cell division and cell rearrangement without losing epithelial integrity, the wing blade epithelium might require a highly coordinated balance between stability and remodelling of adhesion contacts [6-8]. As I will discuss further, CP might act upstream or in parallel with aPKC in maintaining AJs stability. Thus, decreasing aPKC, using $apkc^{ts}$ allele, might enhance the AJs defects induced following *cpa* depletion, which in turn can lead to loss of epithelial integrity and massive cell death. Conversely, hinge and notum epithelia may require low remodelling of AJs to allow cell division and thereby, low levels of CP and aPKC might be sufficient to maintain epithelial integrity and prevent cell death.

However, these results contrast with previous data from F. Janody's Lab, where expression of a dominant negative form of aPKC ($aPKC^{CAAXDN}$) [22] in *cpa* or *cpb* mutant clones causes cell extrusion and death in all regions of the wing imaginal disc (see Introduction Fig. 8) [72]. This difference could be a consequence of affecting CP and aPKC levels with different genetic tools. While the *cpa* or *cpb* mutations used in the previous work [72] are null mutations, resulting in a complete knock-out of CP [85], I used RNAi lines that knock-down c*pa* or *cpb* expression, meaning that the reduction of CP levels is partial. Moreover, instead of the $aPKC^{CAAXDN}$ construct to reduce aPKC activity, I used the termosensitive allele $apkc^{ts}$ (Guilgur, L.G. and Martinho, R. *et al.*,

unpublished data). Thus, CP and/or aPKC levels might still be high enough in my experimental conditions to observe a genetic interaction between CP and aPKC in regions outside the wing blade epithelium. Another possibility for these discrepancies may be that death of *CP* mutant cells expressing $aPKC^{CAXXDN}$ in the hinge or notum may not result from decreased aPKC activity, but from unspecific effects of $aPKC^{CAXXDN}$ expression, as this construct may trap random kinase substrates [103].

Knocking-down *cpa* using *hh*-Gal4 driver in an $apkc^{ts}$ mutant background seems to induce the formation of extra foldings in the distal hinge region, suggesting that distal hinge cells acquired the ability to overproliferate (see Results Fig.4). This overproliferation could be due to loss of cell contact inhibition, which ensures that epithelial cells stop proliferating when in contact with each other. The conserved Hippo signalling pathway has been proposed to regulate contact inhibition of growth. A prevailing view is that apical members of the Hippo signalling pathway, Expanded and Merlin, provide 'contact inhibition' cues from adjacent cells to restrain cell proliferation, growth and survival [23]. In this sense, CP and aPKC might provide similar contact inhibition cues that feed into the Hippo signalling pathway to regulate cell proliferation in the distal hinge region. In fact, it has been shown that both CP (García Fernández, B. and Janody, F. *et al.*, unpublished data) and aPKC [104] regulate proliferation and survival by controlling activity of the Hippo signalling pathway.

Overall, my results show that CP interacts genetically with aPKC to prevent death of wing blade cells and to restrict growth in the wing hinge, arguing for differential requirements of CP and aPKC within different regions of the wing disc.

**1.2 The interaction between CP and aPKC is dosage dependent**

Interestingly, I found that most *cpa* depleted clones induced at 2$^{nd}$ instar larvae were maintained within the wing epithelium, even when those cells were also mutant for $apkc^{ts}$ (see Appendix Fig.S1). One possible explanation could be that, in this experimental condition, reduction of CP and aPKC levels only began at 2$^{nd}$ instar larvae, and therefore the amount of CP and aPKC levels in late 3$^{rd}$ instar wing disc could be

sufficient to prevent death of wing blade cells. This suggests that CP and aPKC may have a dosage dependent effect in the wing disc. In agreement with this possibility, when CP and aPKC levels were strongly decreased at semi-restrictive temperature (28°C), a higher number of either *apkc*$^{ts\ -/-}$*; cpaRI*$^{\#2}$- or *cpaRI*$^{\#2}$-expressing clones induced at 2$^{nd}$ instar larvae extrude and die, when compared to permissive temperature (25°C) (see Results Fig.2). Additionally, exponential decrease of CP levels by modulating Gal4 activity using temperature was shown to promote differential cellular outcomes from overgrowth to cell death (García Fernández, B. and Janody, F. *et al.*, unpublished data).

Taken together, the interaction between CP and aPKC seems to have a dosage dependent effect in the wing disc, since modulating their levels leads to differential cellular behaviours (Fig.1).

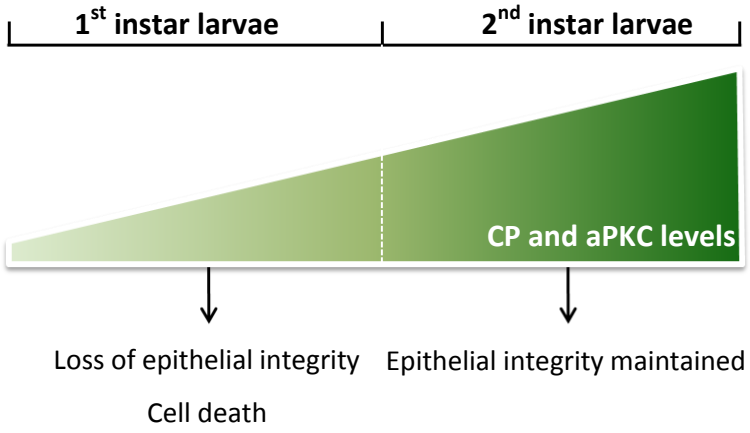



**Fig. 1 – Differential levels of both CP and aPKC lead to differential cellular outcomes.** The graph represents the hypothetical levels of CP and aPKC in late 3$^{rd}$ instar wings discs (when wing discs are dissected and analyzed). When clones are induced at 1$^{st}$ instar larvae, the levels of CP and aPKC in late 3$^{rd}$ instar larvae might be highly reduced, leading to loss of epithelial integrity and cell death within the clones. In contrast, when clones are induced at 2$^{nd}$ instar larvae, the levels of CP and aPKC in late 3$^{rd}$ instar larvae might be sufficient to maintain epithelial integrity.

## 2. CP appears to restrict aPKC localization to the apical membrane

CP is a highly conserved αβ heterodimer that binds to the barbed end of actin filaments and prevents both depolymerization and addition of new actin monomers [61]. Besides having a critical role in the termination of filaments, CP has been

suggested in many systems to provide a stable membrane anchor for actin filaments [64, 65]. Furthermore, an HA-tagged form of Cpa accumulates at the apical sites in all regions of the disc and colocalizes with the AJs components, E-Cad and Arm [20]. Interestingly, I found that the HA-tagged form of Cpa [20] also colocalized with aPKC at apical sites (see Results Fig.7) and depletion of *cpb* resulted in a significant reduction of aPKC levels at apical surface (see Results Fig. 8B-B' and D-D'). It has been shown that failure to restrain aPKC to the apical domain is a key factor for loss of epithelial integrity [38-41]. In this sense, CP could maintain epithelial integrity by promoting the formation of a specific actin network that may act as a scaffold for proper apical localization of aPKC (Fig.2).

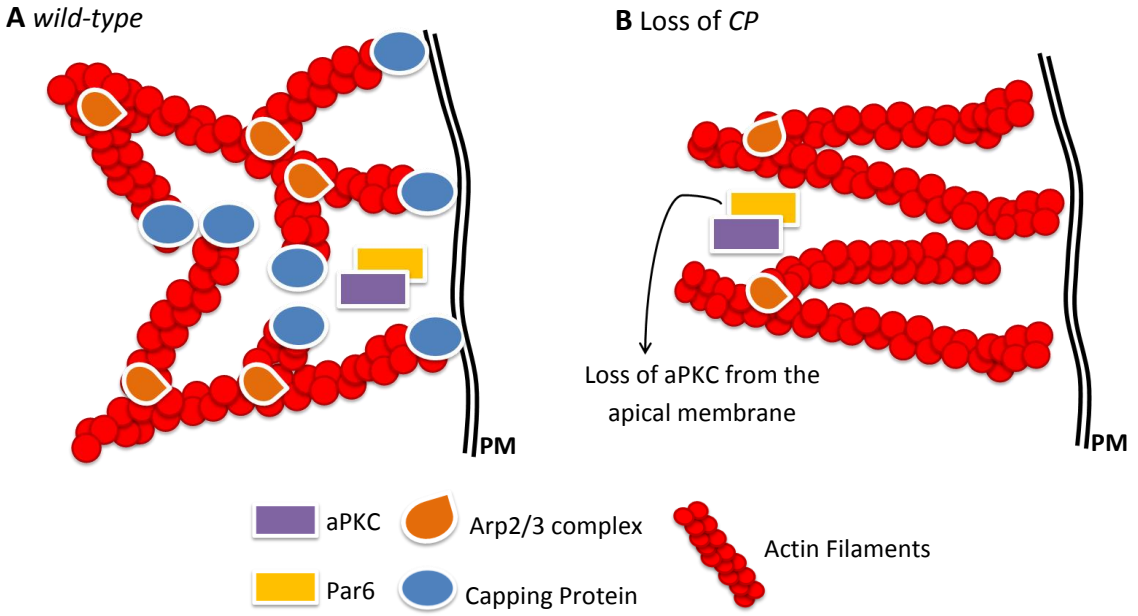



**Fig. 2 – CP restricts aPKC localization to the apical membrane.** (A) A highly branched actin filament network generated by the barbed end capping activity of CP might restrict aPKC localization to the apical membrane. (B) Loss of *CP* might result loss of aPKC from the apical membrane due to apical accumulation of long actin filaments.PM – Plasmatic membrane.

To validate this hypothesis, overexpression of a wild-type form of aPKC incorporating a membrane-tethering CAAX motif (UAS-*aPKC*$^{CAAXWT}$) [22], which was shown to rescue *cdc42* mutant phenotype [14], could be use to attempt to partially rescue the phenotype of *CP* depletion. In addition, it would be interesting to determine the total amount of aPKC protein in *CP* depleted tissues by performing Western Blot

analysis. If aPKC protein levels are reduced in *CP* depleted tissues, this would indicate that loss of CP affects both the localization and stability of aPKC.

Both *in vitro* biochemical studies and cell culture assays have suggested that the major role of CP is to prevent excessive actin polymerization [61]. Therefore, loss of aPKC from the apical domain could be due to excessive actin polymerization rather than loss of CP itself. To further test this, aPKC localization by immunostainning could be analyzed in mutant cells for either *capt* or *tsr*. If, in these genetic contexts, the apical localization of aPKC is not affected, this would suggest that following depletion of *CP*, specific defects in the apical actin network induce loss of aPKC from the apical surface. In addition, it would be interesting to further analyze if the apical localization of other components of the Par-aPKC complex are also affected in *CP* depleted cells.

## 3. CP has a putative role in AJs stability

Epithelial integrity generally depends on the balance between the robustness and plasticity of intercellular junctions [11]. In particular, AJs plays an essential role in regulating the dynamics of epithelial tissues. In this sense, loss of epithelial integrity following reduction of CP and aPKC levels could be due to a disruption of AJs stability. Since it has been well established that Par-aPKC regulates AJs dynamics [14, 15, 105, 106], I will focus on the putative role of CP in the maintenance and remodelling of AJs.

**Hypothesis A: CP regulates AJs stability by restricting aPKC localization to the apical surface**

In addition to a reduction of aPKC apical levels, my results also revealed that depletion of *cpb* leads to a significant decrease of the AJs components E-Cad and Arm at the apical membrane (see Results Fig.9). In contrast, the apical proteins, Crb and Notch, were previously shown to be accumulated in punctuated structures following *CP* depletion in the wing disc (see Introduction Fig.5) [72]. Interestingly, these two outcomes, apical reduction of AJs components (see Results Fig.9) and accumulation of

apical proteins (see Introduction Fig.5) [72], are also observed in *apkc* mutant tissues [14, 15]. It was recently demonstrated that the Par-aPKC complex regulates two distinct endocytic routes in *Drosophila* embryo: regulates the endocytic uptake of apical proteins from the plasma membrane, which is required for AJs stability, and accelerates the processing of apical cargo from the early to the late endosome [6, 14]. Thus, it was proposed that loss of AJs in *apkc* mutant tissues is presumably a secondary consequence of the loss of apical proteins from the plasma membrane [14].

Based on these observations, a possible mechanism by which CP regulates AJs stability is by restricting aPKC localization to the apical membrane. Once in the apical localization, aPKC is able to regulate the endocytic uptake of apical proteins from the plasma membrane and to prevent their accumulation in early endosomes, which in turn contributes to the maintenance of AJs stability (Fig.3).


**Fig. 3 - CP regulates AJs stability by restricting aPKC localization to the apical surface.** According to this hypothesis, CP promotes the formation of a specific actin network that is required for proper apical localization of aPKC, which in turn, regulates the endocytic uptake of apical proteins and controls AJs stability.

**Hypothesis B: Both CP and aPKC regulate AJs stability by promoting early endocytic events**

Although AJs have to be stable to maintain tight cell association, they need to be constantly remodelled to allow cellular processes, such as cell division. Remodelling of AJs required endocytosis of E-Cad and aberrant regulation of this process results in loss of cell adhesion and disruption of epithelial integrity [6-8].

Interestingly, in a closer examination to *CP* depleted cells, I observed gaps in junctional E-Cad (see Results Fig.10). This phenotype resembles the one detected in pupal wings bearing a temperature-sensitive mutation for *Drosophila dynamin* (*shibire*) [107], which mediates the scission of endocytic vesicles from the plasma membrane [108]. It has been shown that actin polymerization generates a compressive or pushing force [70, 109, 110] that is coordinated with vesicle scission and helps separate the budding portion of a endocytic vesicle from its site of formation [111, 112]. In addition, several *in vitro* studies and theoretical models have proposed that short actin filaments are stiffer than long filaments and therefore more effective in generating pushing force [16]. In this sense, by creating short actin filaments, CP might promote the formation of a highly branched actin network, which may be required to provide physical force for proper scission of endocytic vesicles. Thus, CP could have an additional aPKC-independent function in regulating AJs stability by controlling early steps of endocytosis (follow arrow 1 in Fig.4). This hypothesis is reinforced by the fact that, in budding yeast, CP is essential for the initial movement of endocytic vesicles away from the plasma membrane, which presumably corresponds to vesicle scission and release [113]. Additionally, vesicle scission can also occurs in a dynamin-independent manner, where the driven force required to vesicle scission is provided by actin polymerization [114]. To further confirm the role of CP in endocytosis of AJs components, live imaging experiments using a GFP tagged form of E-Cad could be performed [105, 106]. In addition, transmission electron microscopy could also help to uncover the AJs defects induced following *CP* depletion.

The Par-aPKC complex was recently shown to control the distribution and structure of the polarized epithelial actin cytoskeleton [94]. Thus, aPKC might act in parallel with CP to regulate the dynamic of actin cytoskeleton and to maintain AJs in a state of dynamic equilibrium through endocytosis of E-Cad and Arm (follow arrow 2 in Fig.4). However, in my experimental conditions, $apkc^{ts}$ mutant clones (see Results Fig.1) showed no visible effect on F-actin. Alternatively, the Par-aPKC complex has been suggested to recruit dynamin to endocytic sites [105] and thereby, it is possible

that both aPKC and CP could have parallel functions in promoting scission of endocytic vesicles (follow arrow 3 in Fig.4).

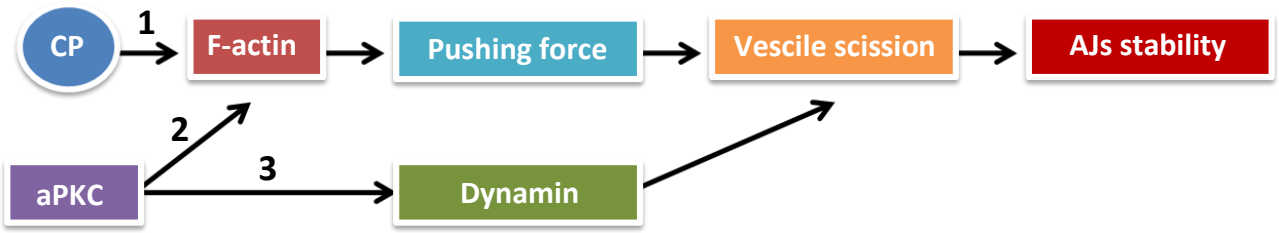



**Fig. 4 - CP acts in parallel with aPKC to regulate early steps of endocytosis.** (A) CP could have an aPKC-independent function in regulating AJs stability by generating an actin filament network that provides the driven force for scission of endocytic vesicles. Loss of CP could result in endocytic defects, leading to AJs disruption. (B) CP could act in parallel with aPKC to regulate the dynamics of actin cytoskeleton, which in turn, promotes early endocytic steps and regulates AJs stability. (C) CP and aPKC could have parallel functions in promoting scission of endocytic vesicles: while CP regulates the formation of a highly branched actin network, which generates pushing force, aPKC recruits dynamin to endocytic sites.

**Hypothesis C: CP regulates AJs stability by inhibiting Src signalling**

During recent years, Src has been identified as one key regulator of AJs stability [42]. Src localizes at AJs and its abnormal activation disrupts AJs by promoting ubiquitination and degradation of E-Cad [53, 54], which results in loss of epithelial adhesion, acquisition of mesenchymal traits and increase motility and invasion [27]. Previous data from F.Janody's Lab suggest that CP might restrict Src activity by inhibiting actin-dependent mechanical activation of Src (García-Fernandéz, B. and Janody, F. *e al.*, unpublished data). Therefore, another mechanism by with CP regulates AJs stability might be through regulation of Src activity (Fig.5). Indeed, similar to *cpb* depletion phenotype (see Results Fig.9B-B'), I found that cells with increased levels of Src64B showed reduced levels of E-Cad at apical surface (see Results Fig.11B-B'). Given that Src promotes degradation of E-Cad, leading to its loss from apical membrane [53, 54], it would be interesting to further determine whether reduction of E-Cad in *CP*

depleted cells is also due to increased degradation of E-Cad. This could provide strong evidence that CP regulates AJs stability by restricting Src activity.

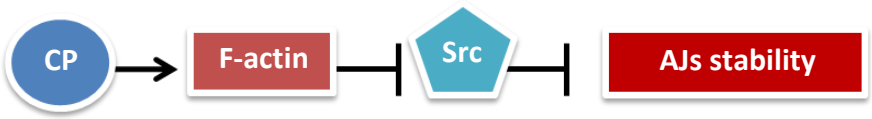



**Fig. 5 - CP regulates AJs stability by inhibiting Src signalling.** CP might regulate Src activity through actin cytoskeletal rearrangements. Loss of CP could lead to abnormal activation of Src, which in turn, would promote degradation of E-Cad, resulting in AJs disassembly.

It is crucial to mention that the presented hypotheses for the putative role of CP in AJs stability are not mutually exclusive. In fact, it was recently shown that aPKC is recruited by Src to promote motility and invasiveness, suggesting that abnormal activation or mislocalization of aPKC may unleash its oncogenic potential [38, 115].

Taken together, through its major function, capping of actin filaments, CP might maintain AJs in a state of dynamic equilibrium by: (A) restricting aPKC localization to the apical surface; (B) generating pushing force required for endocytic vesicle scission; and (C) inhibiting Src signalling (Fig.6). Such a model would be restricted to the wing blade, as *CP* depleted cells are maintained within the hinge and notum epithelia.

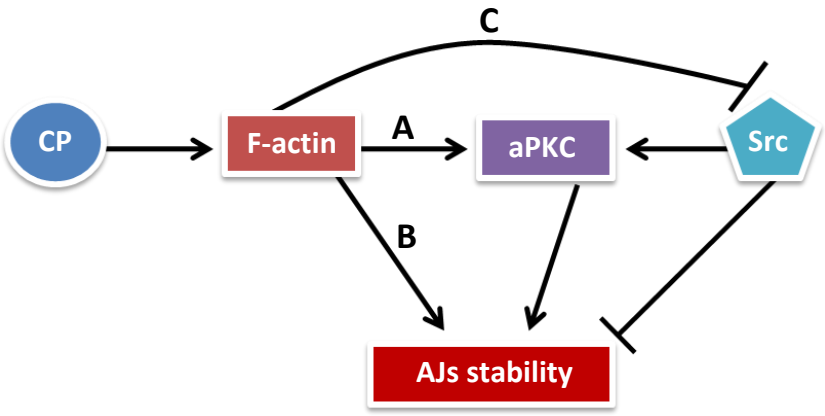



**Fig. 6 – General model for the putative role of CP in AJs stability.** Through its major function, capping of actin filaments, CP could regulate AJs in a state of dynamic equilibrium by: (A) restricting aPKC localization to the apical surface; (B) generating pushing force required for endocytic vesicle scission; and (C) inhibiting Src signalling.

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# Appendix

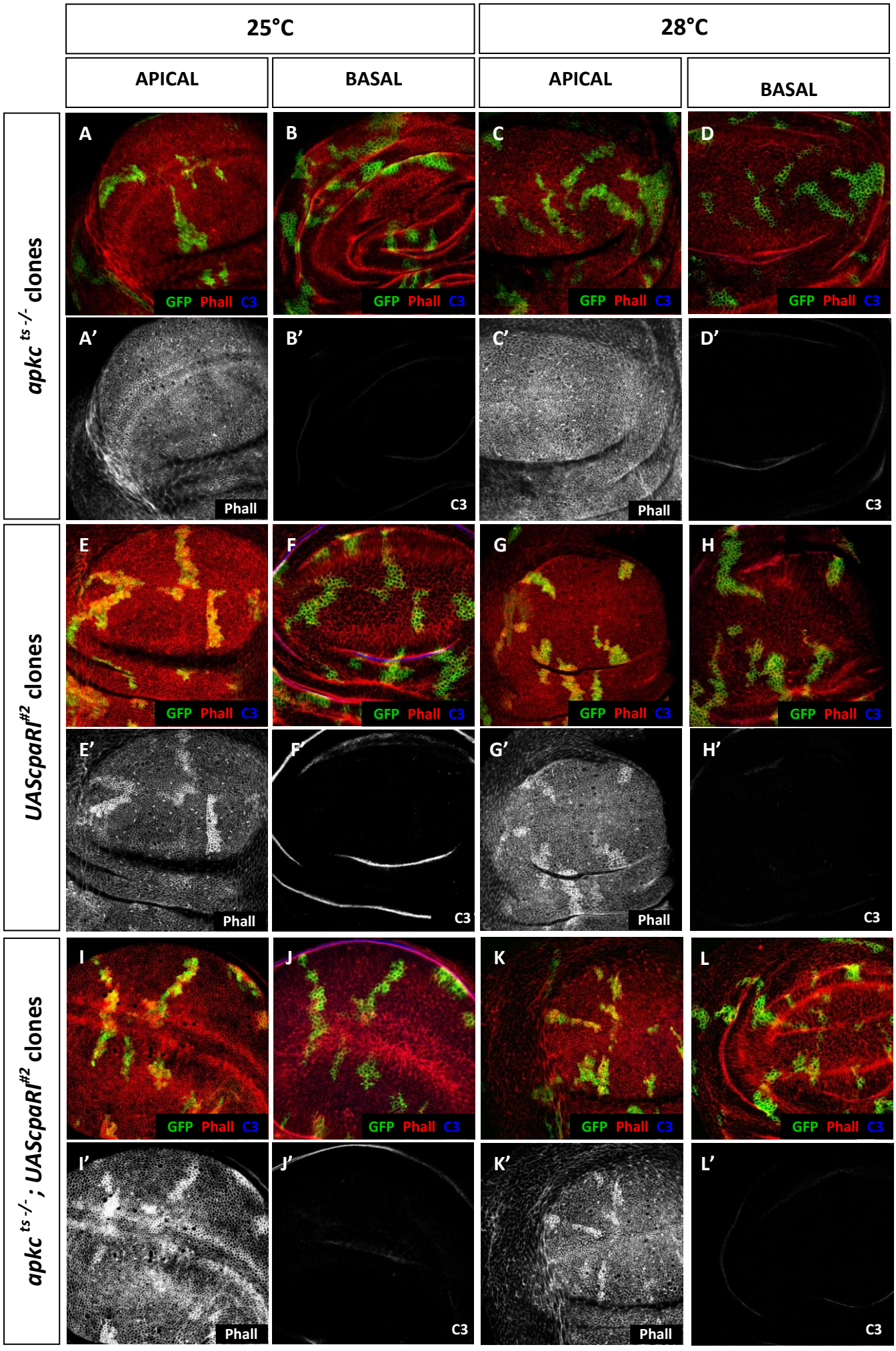
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**Fig. S1 – All clones induced at 2$^{nd}$ instar are maintained within wing disc epithelium.** All panels show Z-projections of third instar wing discs with clones positively labelled with GFP (green in A, B, C, D, E, F, G, H, I, J, K, L) induced at 2$^{nd}$ instar larvae and maintained at either permissive (25°C) (A-J') or semi-permissive temperature (28°C) (C-L'). (A-D') *apkc$^{ts}$* mutant clones, (E-H') *cpa* depleted clones and (I-L') *apkc$^{ts}$* mutant clones expressing *cpaRI$^{\#2}$* are stained with TRITC-Phalloidin to reveal F-actin (red in A-L or grey in A', C', E', G', I', K') and anti-activated Caspase 3, which identify Caspase-dependent cell death (blue in A-L or grey in B', D', F', H', J', L').Dorsal is downwards.