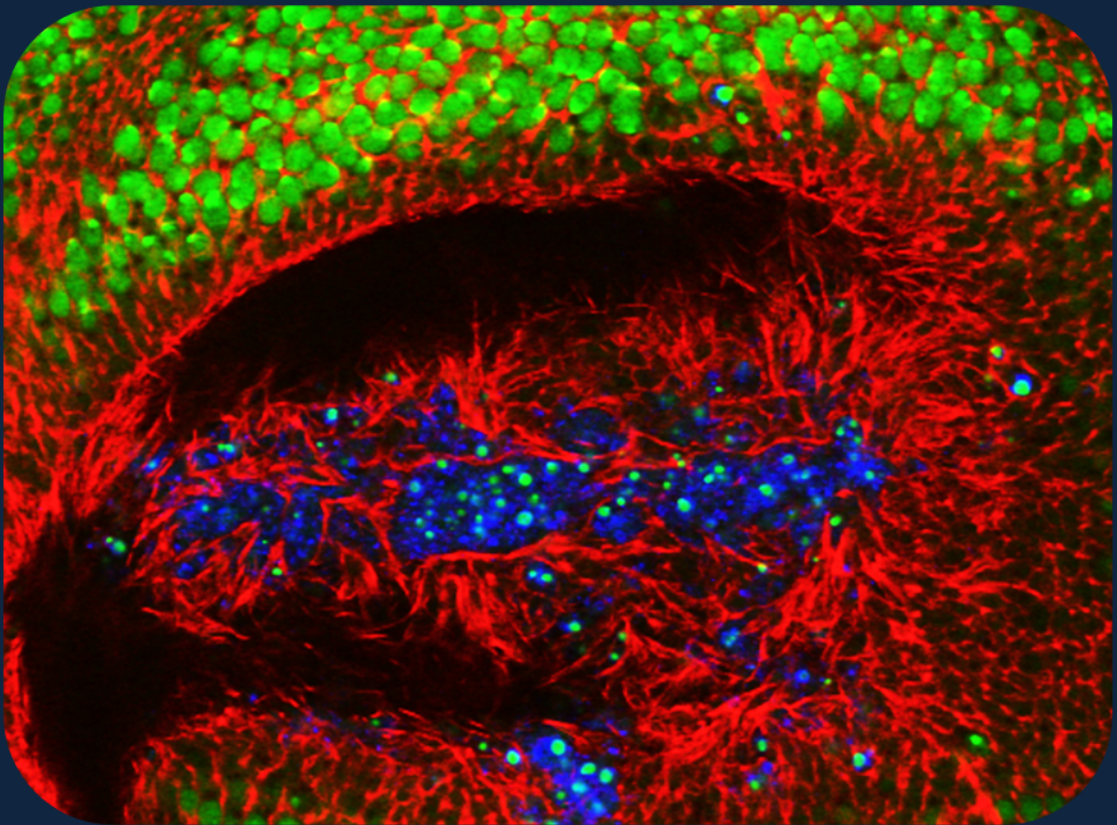


The role of Actin-Capping Protein and Src signalling in tissue growth and apoptosis during *Drosophila* wing development

Barbara Zofia Jezowska



Dissertation presented to obtain the Ph.D degree in
Developmental Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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Esta dissertação é o resultado do meu próprio trabalho desenvolvido entre Outubro de 2007 e Outubro de 2011 no laboratório da Doutora Florence Janody, no Instituto Gulbenkian de Ciência, em Oeiras, Portugal, no âmbito do Programa Doutoral do Instituto Gulbenkian de Ciência PGD2007. Todas as colaborações estão indicadas em cada capítulo, nas secções de ‘Material and Methods’ e ‘Acknowledgments’. Os resultados do Capítulo 3 representam o artigo publicado:

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SUMMARY

The actin cytoskeleton controls numerous cellular processes, including cell morphology and polarity, endocytosis, intracellular trafficking, contractility and cell division. Actin filament growth, stability and disassembly are controlled by a plethora of actin-binding proteins. Among them Capping Protein is a highly conserved $\alpha\beta$ heterodimer, which binds the barbed ends of actin filaments, inhibiting addition or loss of actin monomers. Loss of Capping Protein results in the accumulation of the excessive actin filaments. Interestingly, cells mutant for *Capping Protein* display a tissue-specific behaviour in the *Drosophila* wing imaginal disc. In the most distal domain, loss of *Capping Protein* triggers actin filaments accumulation around the entire cell cortex, cell extrusion and apoptosis. While in the proximal domain, mutant cells maintain a polarized epithelial architecture and accumulate F-actin at the apical cell surface. These observations argue that Capping Protein regulates diverse populations of actin filaments in the cell that have tissue-specific functions.

During my PhD, I aimed to understand the role of Capping Protein in maintaining tissue integrity in the epithelium of the most distal wing disc domain. My results argue that Capping Protein does not affect diverse populations of F-actin along the proximo-distal axis of the wing disc epithelium, but restricts the formation of the apical network of actin filaments in the whole wing disc epithelium (Chapter 1). Interestingly, in the whole tissue, loss of *Capping Protein* reduces Hippo pathway activity and leads to ectopic expression of several Yorkie target genes that promote cell survival and proliferation, including the Adherens Junctions component *DE-cadherin* (Fernández et al. 2011) (Annex 1). Surprisingly, in the most distal wing disc domain, DE-Cadherin provides an active signal, which prevents Wingless signalling and promotes JNK-mediated apoptosis of cells lacking *Capping Protein*. But, when such cells are kept alive, the activity of the JNK pathway and Yorkie oncogene trigger massive proliferation.

However, Yki-dependent increase of DE-Cadherin levels is unlikely to be sufficient to trigger apoptosis of cells knocked down for Capping Protein in the distal wing disc domain since mutant clones for Hippo pathway components also accumulate DE-Cadherin but do not extrude from the wing disc epithelium. In addition to preventing transcription of *DE-cadherin*, Capping Protein maintains DE-cadherin localization specifically in the most distal wing disc domain. The dual effect of Capping Protein loss on DE-Cadherin is likely sufficient to trigger the elimination of mutant cells, preventing them from proliferating (Chapter 2) (Jezowska et al., 2011).

With the goal of understanding how Capping Protein maintains the localization of DE-cadherin at Adherens Junction, I observed that increased levels of the Src oncogene in the distal wing disc epithelium fully recapitulate the outcomes of cells lacking *Capping Protein*. These observations suggest that in this domain, Capping Protein function may restrict Src signalling activity. Indeed, I found that Capping Protein prevents Src signalling activity downstream of the *btk family kinase at 29A* and inhibits Src phosphorylation. My results suggest that the balance between activated Src and Capping Protein is critical to control Src signalling activity. I propose that misregulation of actin cytoskeletal genes, such as Capping Protein, contributes to tumorigenesis through Src signalling, whose expression and activity becomes progressively elevated in a broad spectrum of cancers (Chapter 3).

My data argue that transcriptional upregulation of *DE-cadherin* contributes to elimination of *capping protein* mutant cells in a process of JNK mediated apoptosis. Putting this finding into the perspective, that both Src and Yki overexpression, give rise to DE-cadherin upregulation these data suggest that DE-cadherin transcription could serve as an early mechanism of tumor suppression. E-cadherin could promote the elimination of deleterious cells, possibly through inhibition of Wingless signalling. Malignant cells may compete successfully by losing the overall E-cadherin expression and upregulating mesenchymal cadherins such as N-cadherin to reinforce their fitness.

SUMÁRIO

O citosqueleto de actina controla inúmeros processos celulares, nomeadamente morfologia celular e polaridade, endocitose, tráfego intracelular, contractilidade e divisão celular. O crescimento, a estabilidade e a desagregação dos filamentos de actina são controlados por uma plethora de proteínas que se ligam à actina. Entre elas, a Capping Protein é um heterodímero $\alpha\beta$ altamente conservado, que se liga aos ‘barbed’ terminais dos filamentos de actina, inibindo a adição ou perda de monómeros de actina. A perda de Capping Protein resulta na acumulação excessiva de filamentos de actina. É interessante notar que células mutantes para Capping Protein exibem um comportamento específico do tecido em que estão inseridas no disco imaginal da asa de *Drosophila*. No domínio mais distal, a perda de Capping Protein desencadeia acumulação de filamentos de actina em todo o córtex celular, bem como extrusão celular e apoptose. No domínio proximal, as células mutantes mantêm uma arquitetura epitelial polarizada e acumulam filamentos de actina na superfície apical das células. Estas observações sugerem que a Capping Protein regula diversas populações de filamentos de actina na célula, que têm funções específicas em cada tecido.

Durante o meu doutoramento, procurei entender o papel da Capping Protein na manutenção da integridade dos tecidos no epitélio do domínio mais distal do disco da asa. Os meus resultados demonstram que a Capping Protein não afecta diversas populações de filamentos de actina ao longo do eixo proximal-distal do epitélio do disco da asa, mas restringe a formação de uma rede apical de filamentos de actina em todo o epitélio do disco da asa (Chapter 1). Interessantemente, em todo o tecido, a perda de Capping Protein reduz a actividade da via de sinalização de Hippo e leva à expressão ectópica de vários genes-alvo de Yorkie (Yki), que promovem a sobrevivência celular e proliferação, incluindo o componente das Junções Aderentes *DE-cadherin* (Fernández et al., 2001) (Annex 1). Surpreendentemente, no domínio mais distal do disco da asa, DE-

cadherin proporciona um sinal activo, que impede a sinalização por Wingless e promove a apoptose mediada por JNK das células sem Capping Protein. Porém, quando essas células são mantidas vivas, a actividade da via de sinalização de JNK e Yki, um oncogene, provoca proliferação massiva. No entanto, o aumento dependente de Yki dos níveis de DE-cadherin é improvável que seja suficiente para provocar apoptose das células mutantes para Capping Protein no domínio distal do disco da asa, uma vez que clones mutantes para componentes da via de sinalização de Hippo também acumulam DE-cadherin, mas não extrudem do epitélio do disco da asa. Além de prevenir a transcrição de *DE-cadherin*, Capping Protein mantém a localização da DE-cadherin especificamente no domínio mais distal do disco da asa. O duplo efeito da perda de Capping Protein na DE-cadherin é provável que seja suficiente para promover a eliminação das células mutantes, evitando que proliferem (Chapter 2) (Jezowska e tal., 2011).

Com o objectivo de compreender como Capping Protein mantém a localização da DE-cadherin nas Junções Aderentes, verifiquei que níveis elevados do oncogene Src em 64B no epitélio distal do disco da asa reproduz completamente os mesmos resultados de células sem Capping Protein. Estas observações sugerem que, neste domínio, a função de Capping Protein poderá limitar a actividade de sinalização de Src. De facto, descobri que Capping Protein previne a actividade de sinalização de Src a jusante da cinase da família Btk em 29A e inibe a fosforilação de Src. Os meus resultados sugerem que o equilíbrio entre Src activado e Capping Protein é crucial para o controlo da actividade de sinalização de Src. Assim, proponho que a desregulação dos genes do citosqueleto de actina, como a Capping Protein, contribui para a tumorigenese através da sinalização de Src, cuja expressão e actividade torna-se progressivamente elevada num largo espectro de cancros (Chapter 3).

Os meus resultados que a sobreactivação transcriptional de *DE-cadherin* contribui para a eliminação das células mutantes para Capping Protein num processo de apoptose mediado por JNK. Colocando esta descoberta em perspectiva, que a sobreexpressão de ambos Src e Yki originam a sobreactivação de *DE-cadherin*, estes resultados sugerem

que a transcrição de *DE-cadherin* pode servir como um mecanismo inicial de supressão tumoral. DE-cadherin pode promover a eliminação de células deletérias, possivelmente através da inibição da sinalização de Wingless. Células malignas podem competir com sucesso, perdendo expressão de DE-cadherin global e sobreactivando Cadherins mesenquimais, tais como a N-Cadherin para reforçar a sua aptidão.

LIST OF ABBREVIATIONS

ABPs	Actin Binding Proteins
ADF	Actin depolymerizing factor
AJ	Adherens Junctions
AP	Anterio-posterior
Arm	Armadillo (β Catenin)
Arp 2/3	Actin-related protein complex 2/3
ARPC	Arp complex
bp	base pair
Bsk	Basket
CAP	Adenylate Cyclase Associated Protein
Cora	Coracle
CP	Capping Protein
Cpa	Capping Protein α
Cpb	Capping Protein β
Csh	Csk kinase homolog
Csk	C-terminal Src kinase
CycE	CyclinE
DAAM	Dishevelled-associated activator of morphogenesis
Dia	Diaphanous
DIAP1	<i>Drosophila</i> inhibitor of apoptosis 1
Dlg	Disc-large
DRFs	Diaphanous related formins
Ds	Dachsous
DV	Dorso-ventral
E-cad	E- cadherin

ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
Ex	Expanded
FA	Focal Adhesion
F-actin	filamentous actin
FAK	Focal Adhesion Kinase
FH	Formin-homology
FJ	Four-jointed
Frz	Frizzled
Frz2	Frizzled 2
Hep	Hemipterous
Hpo	Hippo
JNK	Jun N-terminal Kinase
JNKK	Junk kinase kinase
JNKKK	Jun Kinase kinase kinase
Jra	Jun-related antigen
Kay	Kayak
Lats1/2	Large tumor suppressor 1 and 2
Lgl	Lethal giant larvae
MAD	Mother against Dpp
MAP	Mitogen activated pathway
MAST1/2	mammalian STE-20 kinase 1 and 2
Mats	Mob as tumor suppressor
mDia	murine Diaphanous
Med	Medea
Mer	Merlin

Mob	Msp-one-binder
Neu IV	Neurexin IV
NPFs	nucleation promoting factors
N-WASP	neuronal Wiskott–Aldrich syndrome protein
Omb	Optomoto-blind
PCR	Polimerase chain reaction
PKC	Protein kinase C
pMAD	phosphorylated MAD
RNAi	RNA interference
RT	Room temperature
Salm	Spalt
Sav	Salvador
Scrib	Scribble
Sd	Scalloped
SJ	Septate junctions
Src	Rous sarcoma virous
TGF	Transforming growth factor
Tkv	Thickveins
TSG	Tumor supresor gene
Vg	Vestigial
WASP	Wiskott–Aldrich syndrome protein
WAVE	WASP-family veroprolin homolog
Wts	Warts
Yki	Yorkie
Yrt	Yurt
α PKC	Atypical kinase C

TABLE OF CONTENTS

Declaração	I
Acknowledgments/Agradecimentos	III
Summary.....	V
Sumário	VII
List of Abbreviations.....	XI
Chapter 1 – General Introduction	1
1. Epithelial tissue organization	3
1.1. Apico-basal cell polarity.....	3
1.2. Organization of Adherens Junctions components	6
1.3. Organization of cell -matrix adhesion structures	7
1.4. The Cross-talk between Adherens Junctions and cell-matrix adhesion structures.....	9
2. Actin filaments organization and dynamics	12
2.1. Nucleation of actin filaments	13
2.2. Depolymerization of actin filaments and sequestration of actin monomers	15
2.3. Termination of actin filaments	15
2.4. Capping Protein and its role in organization of actin filaments	16
3. Role of actin filaments in organization and remodeling of epithelial junctions ...	19
3.1. Actin filaments in Adherens Junctions stability.....	19
3.2. Role of actin filaments in maturation of cell–matrix adhesion structures....	20

4.	The wing imaginal disc as a model system	21
4.1.	Structure of the wing imaginal disc	21
4.2.	Patterning of the wing imaginal disc	22
4.3.	Wing imaginal disc as a model to study tissue growth and homeostasis	25
5.	Tumorigenesis as an example of the loss of epithelial tissue homeostasis	30
5.1.	The hallmarks of epithelial derived cancers	30
5.2.	<i>Drosophila</i> as a model to study tumorigenesis, advances and limitations ...	32
5.3.	Tumour suppressors genes and oncogenes in <i>Drosophila</i> epithelial tissues	33
5.4.	The components of Hippo pathway as examples of tumour suppressor genes	34
5.5.	Role of JNK signalling in epithelial-derived tumours	35
5.6.	Contribution of loss of cell polarity and adhesion to tumorigenesis	36
5.7.	Src as an example of oncogene	37
6.	References	41
Chapter 2 – Capping Protein restricts apical actin filaments evenly in a whole wing imaginal disc		49
1.	Summary	51
2.	Introduction	52
3.	Material and Methods	54
3.1.	Generation of UAS- <i>cpa</i> ^{WT} and UAS- <i>cpa</i> ^{ΔABD} transgenic flies	54
3.2.	Design and amplification of <i>cpa</i> ^{WT} and <i>cpa</i> ^{ΔABD}	54
3.3.	Fly husbandry	55
3.4.	Mutants and <i>Drosophila</i> strains used	55

3.5. Antibody staining.....	56
3.6. Imaging	56
4. Results	56
4.1. Capping Protein maintains components of Adherens Junctions in the distal wing disc epithelium.....	56
4.2. Capping Protein prevents apical actin filaments accumulation and does not seem to affect the F/G actin ratio	61
4.3. The apical localization of Capping Protein depends on its ability to bind to actin filament.....	64
4.4. Capping Protein prevents the accumulation of three distinct pools of apical actin filaments	67
4.5. Capping Protein may indirectly contribute to Focal adhesions organization	70
5. Discussion and conclusions	72
6. References	76
Chapter 3- A dual function of <i>Drosophila</i> Capping Protein on DE-cadherin maintains Epithelial integrity and prevents JNK-mediated apoptosis	79
1. Author contribution.....	81
2. A dual function of <i>Drosophila</i> Capping Protein on DE-cadherin maintains epithelial integrity and prevents JNK-mediated apoptosis	83
3. Supplementary Data.....	101
Chapter 4 – Capping Protein mediates signalling through the Src oncogenes in restricted <i>Drosophila</i> Epithelial Tissues	111
1. Summary.....	113
2. Introduction.....	114

3. Material and methods	117
3.1. Fly husbandry.....	117
3.2. Mutants and <i>Drosophila</i> strains used.....	117
3.3. Antibody staining.....	118
3.4. Western Blotting.....	118
3.5. Imaging	119
4. Results	119
4.1. Capping Protein loss or Src overexpression affects identical target genes, disrupts polarity and induces apoptosis.....	119
4.2. Capping Protein and Src64B control the balance between tissue growth and apoptosis.	124
4.3. Loss of Capping Protein cooperates with Src64B in the distal wing disc epithelium.....	129
4.4. DE-Cadherin promotes apoptosis and prevents growth of Src64B overexpressing tissue	130
4.5. Capping Protein acts downstream of Src64B	133
4.6. Capping Protein is epistatic to Btk29A	135
4.7. Capping Protein acts in a feedback loop mechanism to attenuate Src activity 136	
4.8. The balance between activated Src and CP is critical for tissue homeostasis 139	
5. Discussion and Conclusions	144
6. Acknowledgments and author contribution	148
7. References	149

Chapter 5 – General Discussion.....	155
1. Capping Protein regulates multiple signaling pathways	157
1.1. Role of Capping Protein in Hippo signalling pathway	157
1.2. Capping Protein regulates Wg, JNK and Src signalling	157
1.3. Interplay between Hippo, Src, JNK and Wg signalling.....	160
1.4. Molecular and cellular roles of Capping Protein in Hippo and Src signalling 161	
2. Signal transduction pathways regulate Actin dynamics.....	163
2.1. Hippo pathway regulates the actin cytoskeleton.....	163
2.2. Src signalling regulates the actin cytoskeleton.	164
3. The role of Actin dynamics in the regulation of signal transduction pathways..	165
3.1. Regulation of the Hippo pathway by Actin dynamics	165
3.2. Regulation of Src activity by Actin dynamics.....	167
4. References.....	171
Appendix.....	177
1. Actin-Capping Protein and the Hippo pathway regulate F-actin and tissue growth in <i>Drosophila</i>	179

CHAPTER 1 – GENERAL INTRODUCTION

1. EPITHELIAL TISSUE ORGANIZATION

1.1. APICO-BASAL CELL POLARITY

A common feature of all eukaryotes is that their cells are organized into multicellular tissues and organs. Epithelia are the most common type of tissues in metazoan with ~60% of mammalian cell types being epithelial or of epithelial origin (Schock and Perrimon, 2002). Cells forming epithelia are polarized meaning that they exhibit compartmentalization of the plasma membrane into distinct domains. The apical domain faces the external environment or lumen. The lateral domains make contact with neighboring cells through Adherens Junctions (AJs), located at the interface between the apical and lateral membrane (Fig.1.1.A). While the basal domain is attached to the Extracellular matrix (ECM) (Fig.1.1). The lateral and basal domains are commonly referred as basolateral domain.

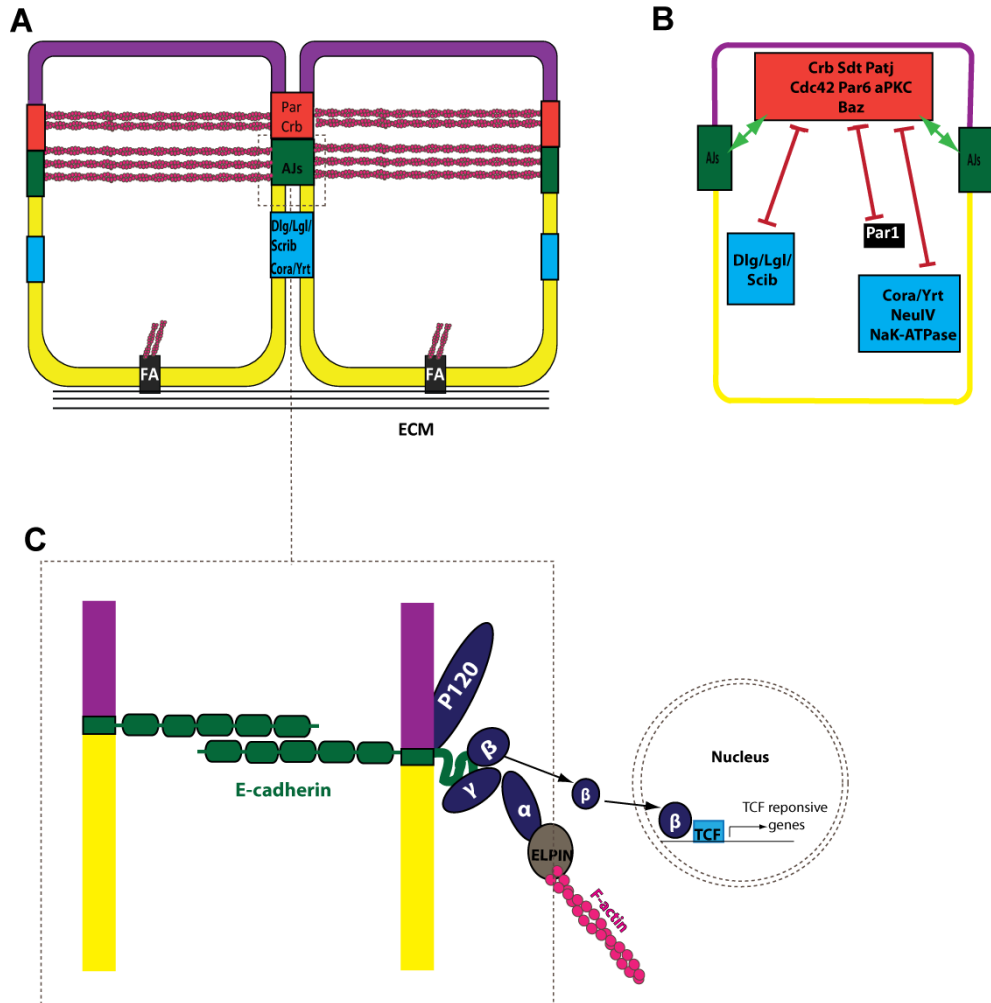


Figure 1.1 Diagrams representing the major complexes that maintain apical-basal polarity and adhesion in *Drosophila* epithelial tissue.

(A) Schematic representation of the apical, basal and lateral domains in epithelial cells. The major complexes are depicted with these color associations: subapical complexes (orange) AJs (green), SJs (blue) and Fas (grey). The apical actin belt associated with AJs and FAs is represented as pink helices. (B) A cartoon representing the global regulatory network of apical and basolateral polarity complexes in *Drosophila* epithelial tissue (adopted from Laprise and Tepass 2011). Apical polarity proteins (Crb, Patj, Cdc42, Par6, α PKC, and Baz) and AJs show mutually supportive interactions (represented in green), while apical factors and the basolateral polarity proteins (Lgl, Dlg, Scrib, Par1, Yrt, Cora, NrXIV, Na⁺/K⁺ ATPase) interact antagonistically (represented in red). The apical and basolateral membrane are represented in magenta and yellow, respectively. (C) Cartoon representing the major components of AJs and their organization (adapted from (Pinho et al., 2011)). The E-cadherin from adjacent cells is involved in homophilic interactions *via* extracellular domains. The intracellular domain of E-cadherin form a complex with Catenins (β and γ Catenins) (represented in blue) that in turn interact with α -Catenin allowing for the linkage with

F-actin (pink helixes) via association with ABPs such as ELPIN (represented in grey). When released from AJs, β -Catenin (Arm in *Drosophila*) can enter the nucleus and together with TCF trigger activation of TCF responsive genes.

The polarity of the membrane is maintained due to the functions of polarity markers that are membrane and cortical molecules, highly conserved between species (reviewed in Laprise and Tepass, 2011). Polarity markers are organized into several functional complexes associated with the apical or basolateral domains. In *Drosophila* epithelial tissues the apical domain contains two complexes, referred as subapical complexes due to their localization, apical to AJs (Fig.1.1.A, B). First, the Par complex consists of atypical protein kinase C (α PKC), its regulatory subunit Par6, the small GTPase Cdc42 and the scaffolding protein Bazooka (Baz) (a homolog of human Par3 protein). The second polarity complex organizes around the transmembrane protein Crumbs (Crb), which binds to Stardust (Sdt), Lin7, the FERM proteins Moesin, Yurt (Yrt) and β_{Heavy} -Spectrin (reviewed in Laprise and Tepass, 2011). Par complex has a role in establishing polarity in most of cell types, while the Crb complex is specific for epithelial tissues. Similarly to the subapical complexes, the basolateral domain, localized basally to AJs, contains two polarity complexes. First, Disc-large (Dlg), Lethal giant larvae (Lgl) and Scribble (Scrib) form the Septate Junctions (SJs), which serve as paracellular barrier. In mammals, however, this function is performed by Tight Junctions (TJs) that localize apically to AJs (reviewed in Matter and Balda, 2003).. Second, the Yrt/Cora complex includes Yurt (YrT), Coracle (Cora), the Na^+/K^+ -ATPase and Neurexin IV (Neu IV). In addition, the kinase Par1, which supports apico-basal polarity, also localized to the basolateral membrane, (reviewed in Laprise and Tepass, 2011).

Members of the subapical polarity modules and AJs components show mutually supportive interactions that are essential for polarization and polarity maintenance. For example in *Drosophila* embryo, when the polarity is established, Crb recruits AJs components that in turn recruit α PKC and Par complexes (reviewed in Laprise and Tepass, 2011). In contrast, the apical and basolateral complexes show mutually

suppressive interactions (Fig.1.1.B),(reviewed in Laprise and Tepass, 2011). However, the molecular bases of the regulatory negative fed back loop between the apical and basolateral polarity complexes are not completely understood. One of the mechanisms involved includes the phosphorylation of Lgl by α PKC that restricts Lgl localization to basolateral membrane. In turn, Lgl, together with Dlg and Scrib, prevents the expansion of the apical markers to the lateral membrane and ensures proper localization of AJs (reviewed in Laprise and Tepass, 2011). While the phosphorylation of Baz and Par complex by Par1 prevents their lateral expansion. Similarly to the Lgl complex, the Yrk/Cora complex has antagonistic regulatory interactions with the apical determinant Crb (Fig.1.1.B). In summary, loss of the apical determinants leads to the expansion of the basolateral membrane. Conversely, disruption of the basolateral polarity components triggers the expansion of the apical membrane.

In addition Integrin based cell-ECM junctions ensure adhesion between cells and components of the ECM (Fig.1.1.A).

1.2. ORGANIZATION OF ADHERENS JUNCTIONS COMPONENTS

AJs are highly organized multimolecular complexes located in between the apical and basolateral domains (Fig.1.1.A), (Schock and Perrimon, 2002). AJs organization majorly depends on the homophilic binding between Cadherins of adjacent cells (Tepass et al., 1996). E-cadherin (E-cad) is generally considered as a prototype of all Cadherins. It is a membrane-spanning glycoprotein whose extracellular domain forms homophilic interactions between cells. The cytoplasmic domain interacts directly with β -Catenin (β -Cat) and plakoglobin (γ -catenin), which in turn interact with α -Catenin (α -Cat) to provide a linkage to the underlying actin cytoskeleton via interactions with Actin Binding Proteins (ABPs) such as ELPIN (Pinho et al., 2011), (Fig.1.1). The interaction of the transmembrane domain of E-cad with p120-catenin (p120) prevents E-cad degradation (Pinho et al., 2011). E-cad is involved in the establishment of apico-basal polarity by

promoting the localization of the subapical complexes, including α PKC and Par, and the basolateral markers, such as Neu IV (Jeanes et al., 2008). In addition to their structural function, E-cad and β -Cat also possess signalling capabilities. β -Cat transduces the Wnt/Wingless signalling pathway by entering into the nucleus and stimulating transcription of TCF/ β -Cat responsive genes (reviewed in Jeanes et al., 2008), (Fig.1.1.C). By tethering β -Cat E-cad can suppress activation of Wnt/Wingless signalling (reviewed in Jeanes et al., 2008). E-cad can also functionally interact with other signalling pathways. For example E-cad clustering downregulates signalling initiated downstream of epidermal growth factor receptor (EGF-R) (Jeanes et al., 2008). EGF-R is a transmembrane glycoprotein that belongs to the family of erbB of tyrosine kinase receptors. Binding of EGFR to its ligands (including EGF and transforming growth factor (TGF) α) leads to autophosphorylation of receptor tyrosine kinase and subsequent activation of signalling pathways that are involved in regulating cellular proliferation, differentiation, and survival (reviewed in Herbst, 2004). In *Drosophila* epithelial tissues, AJs are organized in homologous manner to mammalian AJs. The orthologs of E-cad and β -Cat, DE-cadherin (DE-cad) and Armadillo (Arm) respectively, also have a dual function in mediating cell-cell adhesion and Wg signalling (reviewed in Matter and Balda, 2003; Bilder, 2004).

1.3. ORGANIZATION OF CELL -MATRIX ADHESION STRUCTURES

Several different structures that provide adhesion to extracellular matrix (ECM) can be form in cells in culture (reviewed in Worth and Parsons, 2010). Depending on the protein composition and the lifespan, these structures can range from small, short-lived focal contacts to more stable and bigger Focal adhesions (FAs) or projections known as invadopodia (reviewed in Worth and Parsons, 2010), (Fig1.2.). These structures can be referred as cell to extracellular matrix (C-ECM) adhesions. Regardless of these differences, the function of C-ECM adhesions depends on the presence of Integrins, that

form an heterodimers through transmembrane interaction of an α and β subunits. The extracellular domain of Integrin recognizes and binds to ECM components, whereas the intracellular domain interacts with the actin cytoskeleton through adaptor proteins such as Talin, Tensin and α -actinin. Due to these interactions, Integrins provide a linkage between the underlying actin filaments and the ECM. Integrins also interact with various signalling molecules, including kinases and phosphatases such as Focal Adhesion Kinase (FAK) (reviewed in Worth and Parsons, 2010), (Fig.1.2.).

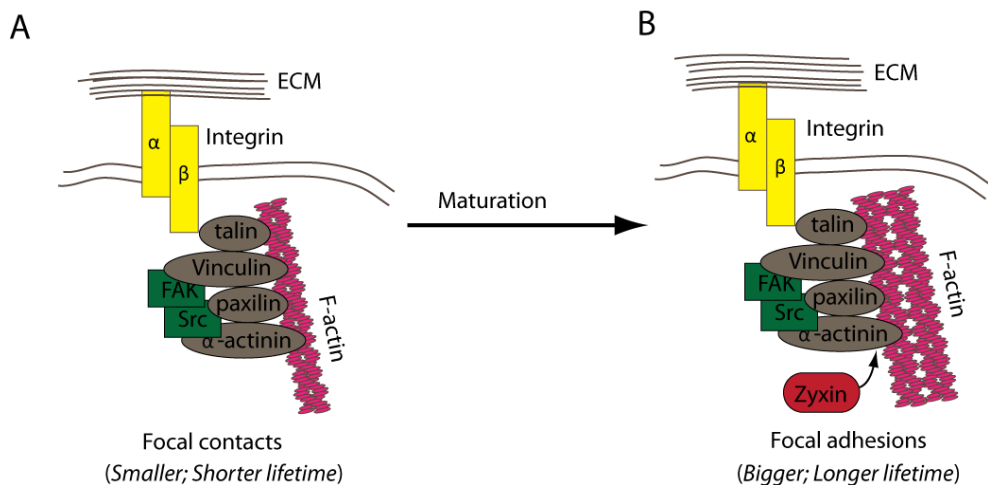


Figure 1.2 Cartoons representing the major components of cell-matrix adhesion structures and the process of their maturation.

Cell-matrix adhesion structures are composed of Integrins (yellow rectangles) that link actin filaments (pink helix) to the components of the Extracellular matrix (ECM). Actin filaments interact with the cytoplasmic domain of β -Integrin through the adaptor proteins (grey squares) that form the cytoplasmic plaque. Many components of the cytoplasmic plaque have signalling properties (green rectangles). (A) Focal contacts are small and have a short life-span. They can mature into Focal adhesions (B) that are larger and more stable. The process of cell-matrix adhesion maturation occurs by the addition of the new proteins into the cytoplasmic plaque (red rounded rectangle) and can be initiated by the application of mechanical forces (adapted from Worth and Parsons, 2010).

Cytoplasmic components of C-ECM adhesions are collectively called cytoplasmic plaques (reviewed in Worth and Parsons, 2010). The composition of cytoplasmic plaques can change depending on the degree of their maturation. For example, focal contacts can mature into Focal adhesions that are bigger, have longer life-span and a different

protein composition (Fig.1.2.). In *Drosophila* epithelial tissues, the composition of the cytoplasmic plaques of C-ECM adhesions can change during development (Delon and Brown 2009) thus, for simplification, I will refer to C-ECM adhesions as FAs in this manuscript.

1.4. THE CROSS-TALK BETWEEN ADHERENS JUNCTIONS AND CELL-MATRIX ADHESION STRUCTURES

AJs and FAs have many common features. First, cadherins and Integrins are both transmembrane receptors that share many signalling molecules and are linked to common scaffolding and cytoskeletal elements. Second, they have the ability to influence crucial downstream functions such as cell growth, survival and transcriptional activity. Due to these common features, both types of adhesion interact with each other at many levels that can be classified into four distinct groups (reviewed in Weber et al., 2011), (Fig.1.3.).

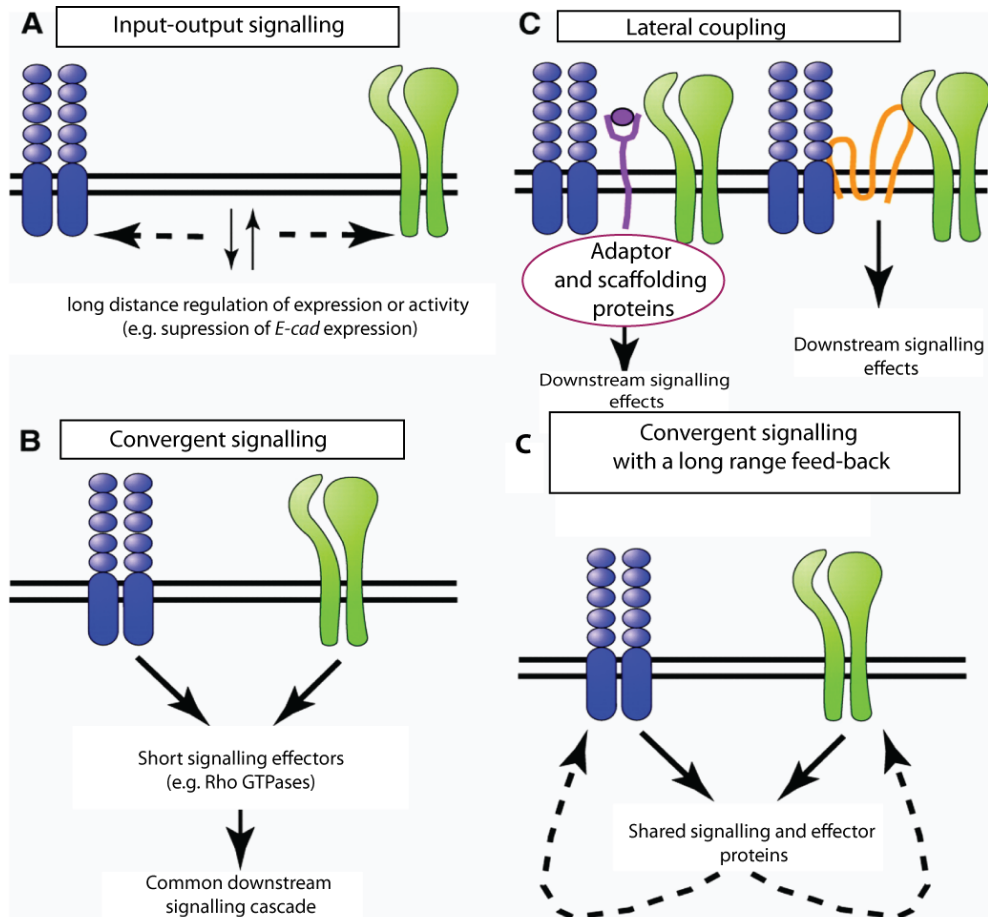


Figure 1.3 The Schematic representation of the types of interactions between Adherens Junctions and Focal adhesions.

(A) to (C) Adherens Junctions are represented in blue and FAs in green. (A) Input-output signalling occurs when the signalling from one adhesion receptor modulates the expression or activity of another adhesion type. (B) Convergent signalling occurs when Cadherins and Integrins signal to common downstream effector molecules. These shared molecules include kinases, adaptor proteins and cytoskeleton. (C) Lateral coupling of adhesion occurs when Integrins and E-cadherins form macromolecular complexes often through interactions with other transmembrane proteins such as growth factor receptors. Lateral coupling allows for signalling in the absence of extracellular matrix or cell-cell adhesion. (D) All three modes shown in A-C work in conjunction with one another to interact in convergent signalling with long-range feedback (adapted from Weber et al., 2011).

First, a “long-range- input-output” signalling in which signalling initiated downstream of adhesion junctions influences the organization of adhesion junctions elsewhere in the cell (Fig.1.3.A.). For example, adhesion of Integrins to distinct ECM proteins can alter expression of genes encoding for AJs components or regulators. During the formation of the mouse embryonic salivary glands, adhesion of Integrins reduce *E-cad* expression, thus altering AJs organization and cell morphology (Onodera et al., 2010). Another example is the activation of Src signalling within FAs that leads to either strengthening or loss of E-cad-mediated adhesion, depending on the levels of Src activation (Martinez-Rico et al., 2010; Avizienyte et al., 2002). A moderate level of Src activation within FAs enables acto-myosin contractility that supports E-cad adhesion in epithelial cells (Martinez-Rico et al., 2010). In contrast, signalling resulting from activation of a constitutively active form of Src (vSrc), propagated upon Integrins clustering, leads to E-cad misslocalization and loss of adhesion in colon cancer cells (Avizienyte et al., 2002).

A second way by which AJs and FAs interact with each other is by sharing common components, such as the scaffolding proteins Vinculin and small GTPases such as Rac that allows for “convergent signalling” (Fig.1.3.B). For example, activation of Rac GTPase occurs in the redundant manner downstream of Integrins or E-cad clustering, which in turn triggers synthesis of cyclin D and higher rate of proliferation in epithelial cells (Fournier et al., 2008). Moreover, scaffolding structures such as the actin cytoskeleton, which allows for the short-range interactions, are also shared between AJs and FAs. For example, FAs clustering induces actin cytoskeleton contractility that can lead to loss of E-cad mediated adhesion (reviewed in Weber et al., 2011).

AJs and FAs can also interact through “lateral coupling”, which involves short-range interactions within the plane of cellular membranes. The difference between “lateral coupling” and “convergent signalling” is that the first does not necessarily involved clustering of the adhesion junctions. The lateral associations between AJs and FAs are achieved through adaptor proteins. For example, epithelial-like growth factor receptor 1 (EGF1R) enables physical coupling of E-cad and Integrin in the plane of membrane.

Activation of EGF1R signalling leads to loss of Integrin and E-cad mediated adhesion (Fig.1.3.C), (reviewed in Weber et al., 2011).

Finally each of these interactions may converge on common signalling pathways giving rise to complex feed-back loops that modulate both initiating signals (Fig.1.3.D). For instance, Src signalling can be activated downstream of both AJs and FAs in mammalian cells and as a result, can alter adhesion mediated by E-cad and Integrins (reviewed in Weber et al., 2011).

2. ACTIN FILAMENTS ORGANIZATION AND DYNAMICS

Actin exists in cell in two main forms: monomeric globular actin (G-actin) and polymeric filamentous actin (F-actin). Formation of F-actin occurs by the polymerization of G-actin that is initiated by slow formation of a nucleation centre, consisting of three G-actin monomers. Once the nucleation centre is formed, new G-actin monomers can be added at higher rate (reviewed in dos Remedios et al., 2003). ATP bound G-actin favourably incorporates into growing filaments at the barbed (fast growing) end. As actin monomers are shifted along the filaments towards the opposite end of F-actin, ATP undergoes slow hydrolysis into ADP that ultimately leads to the release of ADP-G-actin from the F-actin point end (Fig.1.4.A, B). This process is called F-actin treadmilling. When the rate of F-actin ATP -G-actin incorporation and ADP-G-actin hydrolysis is equal the length of F-actin is constant and reflects the steady state of F-actin treadmilling. In *vivo* F-actin polymerization and dynamics of F-actin treadmilling is controlled by a large variety of Actin- binding proteins (ABPs) that can be divided into distinct groups based on their functions on either G or F-actin (reviewed in dos Remedios et al., 2003). Furthermore some ABPs function to organize F-actin into higher ordered-networks or link actin filaments to cellular organelles or apparatus (Michelot and Drubin, 2011).

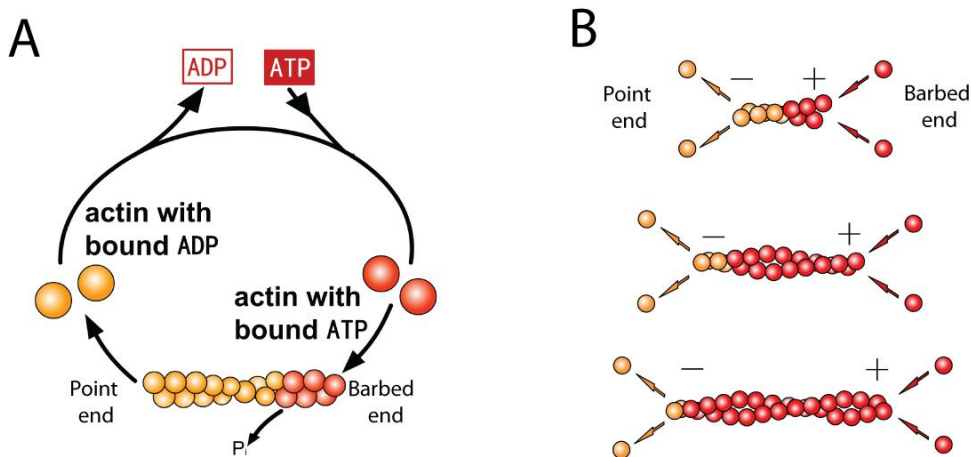


Figure 1. 4 Diagram representing the mechanism of the actin filament treadmilling.

(A) At the physiological concentration of Actin monomers, ATP-G-actin preferentially binds to the barbed end of F-actin (depicted in red), while G-actin-ADP dissociates from the point end of F-actin (depicted in yellow). (B) The actin monomers can flow through the filament by attaching preferentially to the barbed end of the filament and dissociating from the pointed end. The oldest subunits of the filaments are in the highest proximity to point end (adapted from a webpage of Sichuan University <http://jpkc.scu.edu.cn/>).

2.1. NUCLEATION OF ACTIN FILAMENTS

The nucleation of F-actin is an energetically unfavourable event, catalysed by F-actin nucleators *in vivo*. The best known examples are Actin-related protein (Arp) complex 2/3 and Formins (reviewed in Mattila and Lappalainen, 2008). The Arp2/3 complex is a 220kDa module, composed of seven stably-associated polypeptides: Arp2, Arp3 and five additional subunits (ARPC), highly conserved in all eukaryotic organisms. Arp2/3 can generate stable nucleation centers along the side of actin filament, producing new branches of F-actin with $\sim 70^\circ$ of Y-branching angle. The newly formed filament is capped at its pointed end and the barbed end is free for elongation (reviewed in Campellone and Welch, 2010). Activation of the Arp2/3 complex is triggered by nucleation promoting factors (NPFs), which allow the binding of Arp2/3 along the side of actin filaments. The best described NPFs are the Wiskott–Aldrich syndrome protein (WASP) family (including WASP and neuronal WASP (N-WASP) and a group of WASP-family veroprolin homologs (WAVE -1 to WAVE-3), (reviewed in Campellone and Welch, 2010).

Both WASP and WAVE enable the coupling of F-actin branched networks into the membrane. (reviewed in Campellone and Welch, 2010). Arp2/3, together with NPFs, has been described to contribute to cell junctions assembly, endocytosis, membrane ruffling, lamellipodia dynamics and filopodia formation (reviewed in Campellone and Welch, 2010), (Fig.1.5.).

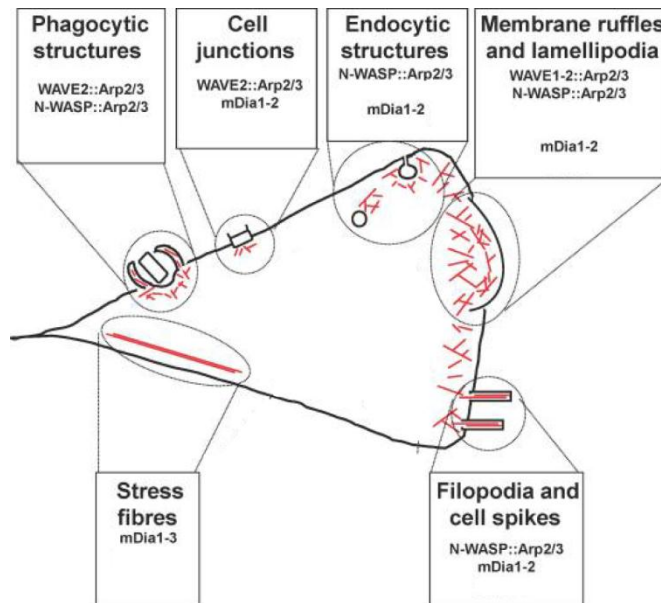


Figure 1.5 Schematic representation of the localization and function of the Arp2/3 complex and Formins in mammalian cells.

Actin filaments (red lines) are nucleated and organized into networks by the Arp2/3 complex and its nucleation promoting factors (WASP, N-WASP and WAVE1-2) or are generated into unbranched structures by Formins. Functional roles for Arp2/3 and Formins are depicted: during phagocytosis, cell junction assembly, membrane ruffling and lamellipodia dynamics, filopodia and stress fibers formation. Abbreviations used: Arp2/3, actin-related protein 2/3; mDia, murine Diaphanous; N WASP, neuronal-WASP, WASP, Wiskott-aldrich syndrome protein, WAVE, WASP verprolin homologous (adapted from Campellone and Welch, 2010).

Unlike the Arp2/3 complex, Formins promote formation of unbranched F-actin network (reviewed in Mattila and Lappalainen, 2008). Formins bind to the barbed end of F-actin, preventing addition of ABPs that terminate F-actin elongation (reviewed in Mattila and Lappalainen, 2008). Formins are present in all eukaryotic organisms. Their defining

feature is the presence of conserved Formin-homology (FH) domains, FH1 and FH2 (reviewed in Mattila and Lappalainen, 2008). The best characterized mammalian Formins are Diaphanous related Formins (DRFs), named based on their similarity to Diaphanous, the first Formin identified in *Drosophila*. DRFs are commonly named murine Diaphanous (mDia, mDia1 to mDia3) and they precipitate in formation of various cellular structures (Fig.1.5.). The best studied examples are stress fibres that underlie FAs, membrane ruffles involved in cell migration, and contractile ring formed during cytokinesis (reviewed in Campellone and Welch, 2010). Moreover DRSF contribute to organization of cell junctions and coordinate F-actin assembly during endocytosis (reviewed in Campellone and Welch, 2010), (Fig.1.5).

2.2. DEPOLYMERIZATION OF ACTIN FILAMENTS AND SEQUESTRATION OF ACTIN MONOMERS

In addition to F-actin nucleators, the activities of actin depolymerizing factors also regulate the extent and spatial pattern of F-actin organization. The best described examples are Actin depolymerizing factor (ADF)/Cofilin family proteins that sever F-actin and increase the rate of G-actin dissociation from a point end that ultimately increase F-actin turn-over (Mazur et al., 2010). G-actin binding proteins such as Adenylate Cyclase Associated Proteins (CAPs) ensure the availability of G-actin by sequestering them and preventing from incorporation into F-actin. On a contrary, Profilin, that binds to G-actin, increase the rate of F-actin elongation by facilitating the exchange rate of G-actin ADP to G-actin ATP (Witke, 2004).

2.3. TERMINATION OF ACTIN FILAMENTS

Termination of actin filaments occurs mainly through the binding of ABPs to the barbed end of F-actin. The best characterized molecules that have the ability to bind to barbed end of F-actin are Gelsolin superfamily and Capping Proteins (CPs). Gelsolin has also F-actin severing properties (Gremm and Wegner, 2000). Whereas, CP forms a highly

conserved heterodimer consisting of Capping Protein α (Cpa) and Capping Protein β (Cpb). CP binds to the barbed end of F-actin, thereby preventing addition and loss of G-actin. CP inhibition leaves the barbed end of F-actin uncapped allowing for incorporation of new G-actin and binding of actin nucleators allowing for the formation of the long actin filaments (reviewed in Cooper and Sept, 2008).

2.4. CAPPING PROTEIN AND ITS ROLE IN ORGANIZATION OF ACTIN FILAMENTS

The structure of the CP heterodimer is highly conserved, found in every eukaryotic organisms and expressed in almost all metazoan cell types (reviewed in Cooper and Sept, 2008). As established in a structure function analysis in yeast and chicken cells, the C-terminal regions of both Cpa and Cpb are important for binding to F-actin (259-286 amino acid residues in Cpa and 264-276 amino acid residues in Cpb) (Hug et al., 1992; Kim et al., 2004), (Fig.1.6.). Although the Cpa subunit binds to F-actin with high affinity (less than 1nM) in the ratio of 1:1 (reviewed in Cooper and Sept, 2008), deletion of the C-terminal region of either Cpa or Cpb leads to loss of efficient F-actin binding (Kim et al., 2004). In the reconstituted actin based motility assay, CP acts by preventing polymerization of short branched F-actin networks mediated by the Arp2/3 complex. This promotes the formation of short branched F-actin networks and maintains the pool of G-actin, by preventing their addition to the barbed ends of F-actin (reviewed in Cooper and Sept, 2008). CP, is also involved in the early stages of endocytosis in yeast cells in which it enables scission of the endocytic vesicles from the plasma membrane (Kim et al., 2004). In addition, since Cpa and Cpb contain highly hydrophobic domains, predicted by *in silico* studies to interact with distinct lipids, CP has been proposed to link F-actin to cellular membrane in yeast and chicken cells (reviewed in Cooper and Sept, 2008)(Smith et al., 2006), (Fig.1.6.). The activity of CP and its ability to bind to F-actin are regulated by many molecules (Tab.1.1). For example, *in vitro* and in cultured cells, Protein kinase C (PKC) has an ability to phosphorylate Cpa and inhibits its activity (Canton et al., 2006). Moreover, CARMIL family proteins inhibit CP through direct

interactions (reviewed in Cooper and Sept, 2008). Furthermore, other molecules that bind to the barbed end of F-actin can compete with CP and indirectly inhibit its function (reviewed in Mattila and Lappalainen, 2008).

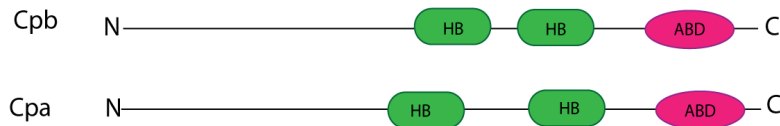


Figure 1.6 Cartoon representing the actin binding domains and predicted hydrophobic domains in Cpa and Cpb.

Representation of the aminoacid sequence of Cpb and Cpa. Both Cpa and Cpb had two hydrophobic domains (HB, green) that had been predicted to interact with the cellular membrane lipids and the actin binding domains (ABD, pink). The hydrophobic domains predicted *in silico*; located in (134-151; 215-232 aa residues) Cpb or (113-130;225-242 aa residues) Cpa. The localization of the ABDs established in the structure function analysis in yeast and chicken cells; located in the (264-287 aa residues) for Cpb and (259-286aa residues) for Cpa.

Representation of the aminoacid sequence of Cpb and Cpa. Both Cpa and Cpb had two hydrophobic domains (HB, green) that had been predicted to interact with the cellular membrane lipids and the actin binding domains (ABD, pink). The hydrophobic domains predicted in silico; located in (134-151; 215-232 aa residues) Cpb or (113-130;225-242 aa residues) Cpa. The localization of the ABDs established in the structure function analysis in yeast and chicken cells; located in the (264-287 aa residues) for Cpb and (259-286aa residues) for Cpa.

In cells, CP seems to contribute to the formation of distinct F-actin networks promoted by the Arp2/3 complex or Formins respectively (Cooper and Sept, 2008). Branched F-actin networks triggered by the Arp2/3 complex and CP, have been proposed to contribute to lamellipodia and membrane ruffles formation (Fig.1.7.A,B). In addition, Arp2/3 activity triggers the formation of F-actin networks involved in endocytosis, cell migration and junctional stability (reviewed in Campellone and Welch, 2010), (Fig.1.5), suggesting that CP is also involved in these processes. CP activity also prevents the formation of F-actin networks promoted by Formins. It does so by

competing with Formins at filament barbed ends (Mattila and Lappalainen, 2008). When CP activity is inhibited, it enables binding of Formins and F-actin elongation (Fig.1.7.C). This mechanism has been proposed to contribute to filopodia formation (reviewed in Cooper and Sept, 2008). Since Formins also promote the formation of other actin-based structures, such as stress fibres, CP might also be involved in this process (reviewed Campellone and Welch, 2010), (Fig.1.5) .

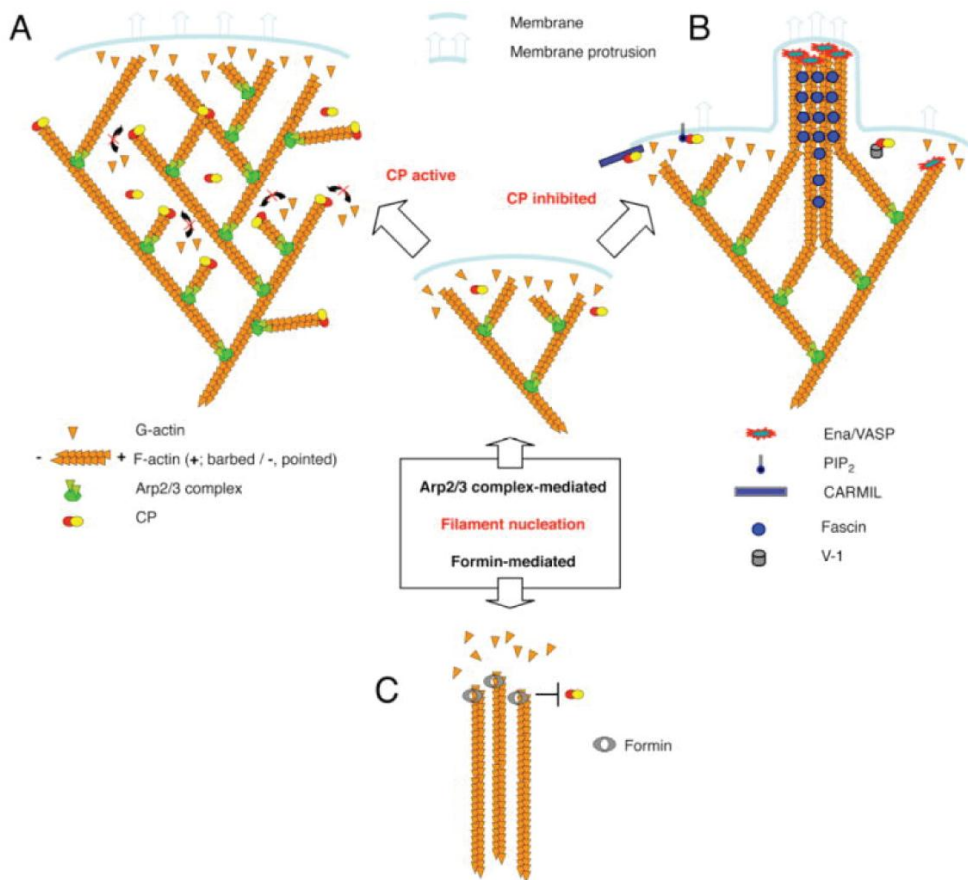


Figure 1.7 Schematic representations of the contribution of Capping Protein into the formation of distinct actin filaments-based structures.

(A) and (B) represent the interactions of CP with F-actin when the latter is linked to membrane. (A) The presence of active CP together with Arp2/3 promotes the formation of the branched F-actin network, which in turn contributes to the formation of lamellipodia. (B) CP inactivation by inhibitory molecules such as CARMIL triggers the elongation of F-actin to produce thin protrusions that can form filopodia. (C) CP might contribute to the formation of the F-actin network not associated with cellular membrane by outcompeting Formins. When CP activity is

inhibited Formins can bind to the barbed ends of F-actin and promote their elongation (Cooper and Sept, 2008).

Table 1 Capping Protein interactors

MAPK→CapZIP	Modulate the actin binding ability of CP	In spleen cells and muscles	Eyers et al., 2005
S 100B	Interact with CP	In vitro	Ivanenkov et al., 1995 Schafer et al., 1996
Twinfilin	CP promote Twf localization in cells	In yeast	Falck et al., 2004
PIP2/PIP3	Uncapping Activity	In vitro	Schafer et al., 1996
Arp2/3 Myosin I CP	CARMIL Cell Migration Uncapping Activity → Cell Migration	In vitro/Colocalize in Migrating cells In vitro/in Mammals cells	Jung et al., 2001 Yang et al., 2005
Cin85/CMS CD2AP	CD2 internalization; clustering, signaling Inhibit capping activity of CP	T Cells In vitro	Hutchings et al., 2003 Bruck et al., 2006
Phosphatidic Acid (PA)	Pollen tube tip growth	In Arabidopsis	Huang et al., 2006
Protein Kinase PKC CKIP-1	Inhibit capping activity of CP Cell morphology	In Vitro In osteosarcoma cells	Canton et al., 2005 Canton et al., 2006
Myotrophin (V-1)	Inhibit capping activity of CP Co-localize	In vitro In cells	Taoka et al., 2003 Bhattacharya et al., 2006
Coactosin	Inhibit capping activity of CP	In vitro/Dictyostelium	Rohrig et al., 1995
Dynein-----Arp1	Vesicle trafficking (?)	In vitro/Droso	Schafer et al., 1994

3. ROLE OF ACTIN FILAMENTS IN ORGANIZATION AND REMODELING OF EPITHELIAL JUNCTIONS

3.1. ACTIN FILAMENTS IN ADHERENS JUNCTIONS STABILITY

The crucial role of F-actin in promoting the assembly, stabilization, and remodelling of AJs has been extensively studied in cell culture models. First, F-actin promotes membrane extensions, allowing the membrane of two cells to be apposed to each other and form adhesions. Second, F-actin forms adhesion belts that evolve into adhesion zippers, allowing for stable E-cad homophilic interactions. Once cell-cell contacts are established, F-actin polymerization and acto-myosin tension are necessary to stabilize AJs. (reviewed in Cavey and Lecuit, 2009). Recently, the work from the laboratory of

Tomas Lecuit had described two distinct F-actin pools that contribute to AJs stability in the early *Drosophila* embryo (Cavey et al., 2008).. They show that a pool of stable actin filaments, localizes in small patches adjacent to spots AJs (sJAs), contributes to the maintenance of homophilic DE-cad dimmers (Fig.1.8.A), whereas, a pool of F-actin, which forms the circumferential F-actin cable, is necessary to immobilize sJAs within AJs and restricts their proper localization (Cavey et al., 2008), (Fig.1.8.B).

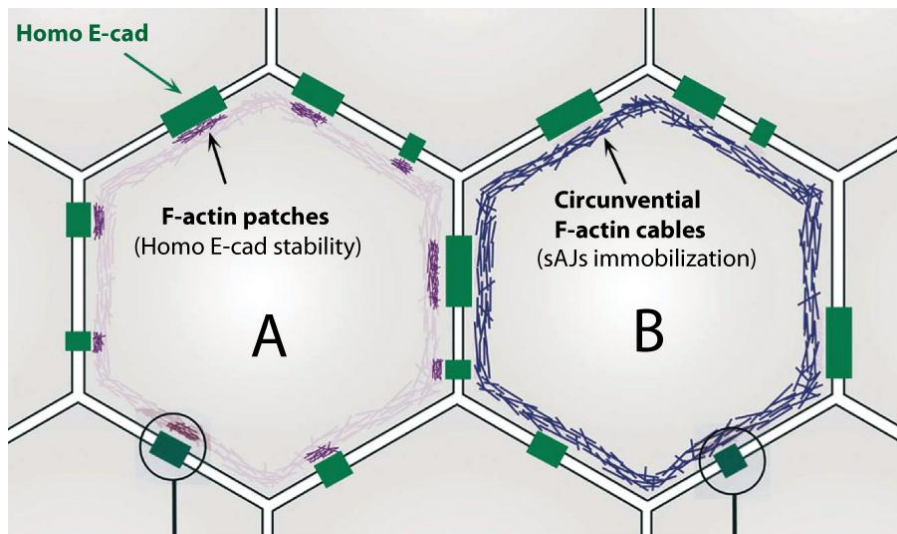


Figure 1.8 Diagram representing two different F-actin pools that contribute to Adherens Junctions stability.

Schematic representation of the cellular apical sites. Homo E-cadherin (green) represents the E-cadherin homophilic complexes from 2 adjacent cells. Cell A presents the organization of small F-actin spots that contribute to the stability of E-cadherin homophilic interactions. Cell B represents the circumferential F-actin cables that immobilize AJs spots (sAJs) within AJs (Cavey et al., 2008).

3.2. ROLE OF ACTIN FILAMENTS IN MATURATION OF CELL–MATRIX ADHESION STRUCTURES.

F-actin that underlies FAs is organized into bundled radial fibers that form stress fibres (SFs) (Oakes et al., 2012). SFs terminate in the cytoplasmic plaque of FAs and interact with adaptor proteins including Talin, α -actinin and Tensin, which in turn link stress fibres to the cytoplasmic domain of Integrins (Delon and Brown, 2007; reviewed in

Worth and Parsons, 2010). Application of mechanical forces triggers Myosin II and mDia1 mediated contractility of actin filaments, which contributes to FAs maturation (Riveline et al., 2001; Geiger and Yamada, 2011). Moreover, SFs have been proposed to serve as platform on which components of the cytoplasmic plaque assemble to contribute to FAs maturation (Oakes et al., 2012).

4. THE WING IMAGINAL DISC AS A MODEL SYSTEM

4.1. STRUCTURE OF THE WING IMAGINAL DISC

The wing imaginal disc had emerged as a powerful model to study mechanisms of epithelial cell polarity, tissue integrity and homeostasis (reviewed in Hariharan and Bilder, 2006). Wing imaginal disc forms during embryogenesis from clusters of 20 to 50 cells that proliferate during larval development to reach a final size of 20,000 to 50,000 cells (reviewed in Herranz and Milán, 2008). They organized into a columnar monolayer epithelium, covered by a thin layer of squamous epithelium called the peripordial membrane (reviewed in Hariharan and Bilder, 2006).

During larval development, wing imaginal discs are subdivided into spatially distinct compartments of different genetic identities, established by the expression of selector genes. The identity of each compartment is maintained as cells keeps dividing through processes that prevent cells of distinct compartments to intermingle. (reviewed in Herranz and Milán, 2008). At the Anterior-Posterior (A-P) and Dorsal-Ventral (D-V) boundaries, two signalling pathways through the long-range morphogen Decapentaplegic (Dpp) and Wingless (Wg) respectively, act as “organizer”, to establish the A-P and D-V compartments (Fig.1.9.A). Consequently, the wing disc has a concentrically organized proximal-distal (PD) axis, subdivided into three distinct compartments: the primordium of the wing blade is in the centre, surrounded by the wing hinge primordium, with the notum and pleura at the periphery (Fig.1.9.B.). For simplicity, I will refer in this manuscript to the proximal wing disc domain for notum and the hinge and to the distal wing disc domain for the blade (Fig.1.9.B).

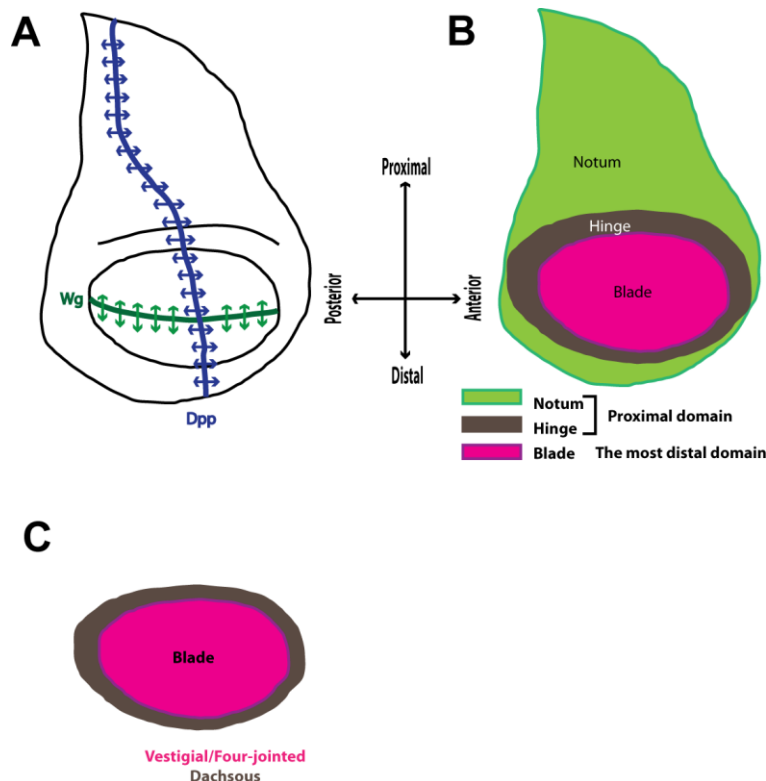


Figure 1.9 Schematic representation of the structure of the 3rd instar wing imaginal disc of *Drosophila melanogaster*.

(A) The schematic representation of the wing imaginal disc with the indicated domains of expression of *Dpp* (blue) and *Wg* (green). The expression of *Dpp* partitions the disc into the posterior and the anterior domain while *Wg* partitions the wing disc into the dorsal and the ventral domain. (B) Schematic representation of the distinct compartments of the 3rd instar wing imaginal disc along the dorso-ventral border. Notum (light green) and hinge (grey) are referred as proximal wing disc domain and blade (magenta) is referred as the most distal wing disc domain. (C) The blade is characterized by the expression of the *Vestigial* transcription factor, which promotes the expression of *four-jointed* and represses *dachshous*. The gradient between *Four-jointed* and *Dachshous* promotes Yki-dependent growth of the wing disc blade.

4.2. PATTERNING OF THE WING IMAGINAL DISC

The anterior-posterior (A-P) boundary is first established in response to the expression of *Dpp* (reviewed in Schwank and Basler 2010), (Fig.1.9.A). *Dpp* is an homolog of transforming growth factor- β (TGF β), involved in cell proliferation, growth,

differentiation and survival in mammalian cells (reviewed in Taylor et al., 2011). Binding of Dpp to its receptor Thickveins (Tkv) promotes phosphorylation and activation of the transcriptional factor Mothers against Dpp (MAD). Phosphorylated MAD (pMAD), in conjugation with Medea (Med), activates target genes, in part by inhibiting the expression of the transcriptional repressor Brinker (Brk) (Fig.1.10.A.). The activities of Brk and pMAD allow for the restricted expression of *spalt (salm)* *optomoto-blind (omb)* and *vestigial (vg)*, that promote differentiation and patterning of the wing imaginal disc (reviewed in Herranz and Milán, 2008). Because Dpp acts as a morphogen, cells localized closer to the source of Dpp expressed different sets of target genes than cells located further away from the Dpp source (reviewed in Affolter and Basler, 2007), (Fig.1.10.A). Later when the wing disc consists of about few hundred cells, the D-V boundary is established by Wg expression (reviewed in Wartlick et al., 2011), (Fig.1.9.A). Wg is the founding member of Wnt family, comprises a large family of secreted glycoproteins that control a variety of developmental and adult processes in all metazoan organisms (reviewed in Kikuchi et al., 2011). Secreted Wg diffuses on each side of the D-V boundary, providing a signal that acts as a morphogen to contribute to the specification of the wing imaginal disc (Baeg et al., 2004; Zecca and Struhl 2010). Binding of Wg to its two receptors Frizzled (Frz1) and Frizzled 2 (Frz2) prevents the degradation of the cytoplasmic pool of Arm. This allows for Arm nuclear translocation where it acts as a co-activator of T-cell factor (TCF) family transcriptional factors to induce the expression of genes involved in wing specification, including *scalloped (sd)* and *vg* (reviewed in Städeli et al., 2006) (Fig.1.10.B).

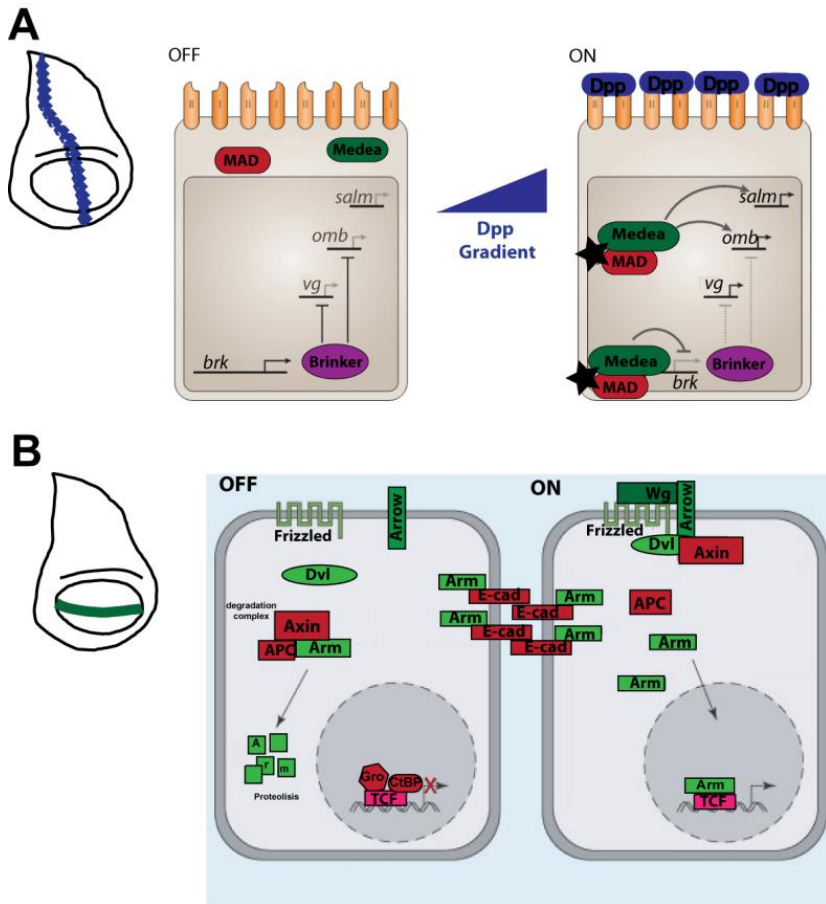


Figure 1.10 Cartoon representing the major cellular events triggered by Decapentaplegic or Wingless signalling.

Schematic representation of the interactions of (A) Dpp or (B) Wg with their receptors and the cellular outcomes of receptors clustering. (A) In the absence of Dpp (cell on the left (OFF)) the level of the Brinker (Brk) transcriptional repressor is high. Brk represses most Dpp target genes including *omb* and *salm*. Dpp binding to its receptor promotes phosphorylation and the activation of the transcriptional factor Mother against Dpp (MAD). Phosphorylated MAD (pMAD) in turn phosphorylates Medea and this complex is translocated into the nucleus. Medea-pMAD represses Brk. Both pMAD and the repression of Brk result in the activation of distinct Dpp target genes, depending on the pMAD interactors and the gradient of Dpp concentration (adapted from Affolter and Basler, 2007). (B) Simplified overview of the Wg signalling pathway. In the absence of Wg ('OFF' stat, left cell) Armadillo (Arm) protein levels are downregulated by the complex 'degradation complex' which contains the Adenomatous polyposis coli (APC) protein and Axin. In addition, the corepressor (Gro) TLE and the Carboxyl-terminal protein (CtBP) are bound to the T-cell factor (TCF) that acts as a transcription factor. The cell on the right represents the 'ON' state of the pathway. The Wg ligand binds Frizzled and its co-receptor (Arrow). In these conditions Axin is bound to Arrow, thereby disrupting the 'degradation complex'. Arm accumulates in the

cytoplasm, it enters the nucleus and displaces Gro from TCF. As a part of Adherens Junctions, Arm/ β -Cat binds to DE-cadherin. The negative and positive regulators of Wg signaling are represented in red and green respectively (adapted from (Lin, 2004; Stadeli et al., 2006)).

4.3. WING IMAGINAL DISC AS A MODEL TO STUDY TISSUE GROWTH AND HOMEOSTASIS

Tissue growth results from the coordinated action of three processes: cell growth (increase in cell mass), proliferation (cell division) and cell survival. Each of these processes is controlled in a cell autonomous manner by highly conserved signalling pathways, whose activities are modulated by inputs provided from the external environment or systemic release of hormones (non-autonomous input) (reviewed Neto-Silva et al., 2009). However, growth of the wing imaginal disc is highly autonomous since immature wing discs, when transplanted into the abdomen of the adult host, proliferate at a similar rate as in larvae and stop growing when reach a correct final size (Bryant and Simpson, 1984). Cell autonomous growth of the wing disc is thought to be controlled by Dpp and Wg signalling that promote cell proliferation and survival, linking in this way wing patterning with tissue growth. Recently Wg and Dpp have been shown to interact with the highly conserved Hippo (Hpo) signalling pathway. The Hpo pathway is a major signalling cascade that controls tissue growth by restricting the nuclear localization of the Yorkie (Yki) co-transcriptional activator (reviewed in Zhao et al., 2011). It consists of three interlinked parts: the Hpo core kinase components, the downstream transcriptional machinery and the upstream regulatory components. The core kinase module consists of Salvador (Sav), Hpo, Warts (Wts) and Mob as tumor suppressor (Mats), whose activity leads to Yki phosphorylation, preventing its nuclear translocation (Fig.1.11. A).

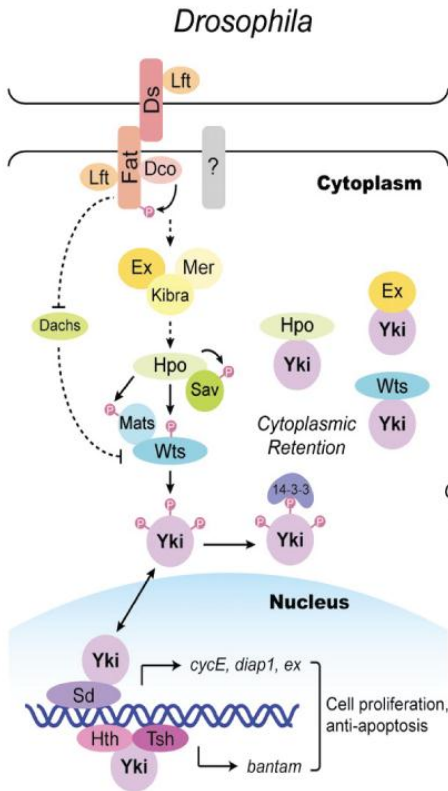
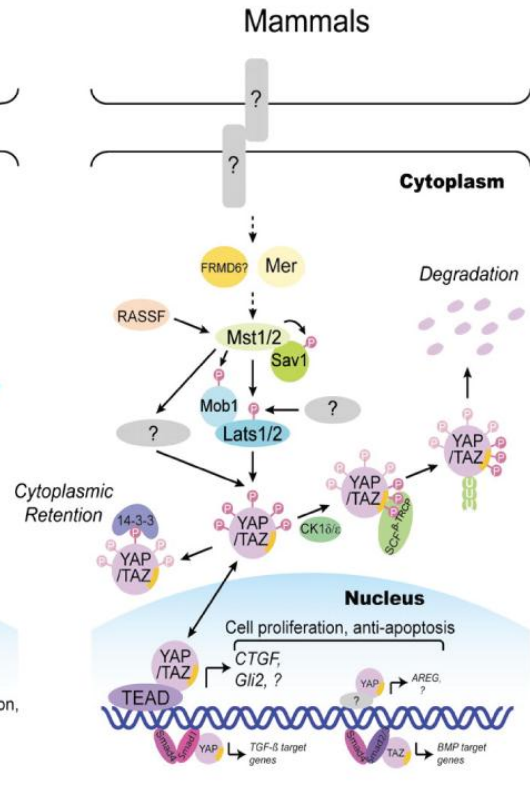
A**B**

Figure 1.11 Schematic representation of the model of the interactions of the Hippo pathway components in *Drosophila* and mammals.

Cells (outlined in grey, nuclei in green) are shown with adherens junctions (AJ) and basolateral junctions (BLJ). (A,B) Hippo pathway components in (A) *Drosophila* and (B) vertebrate are shown in various colors, with pointed and blunt arrowheads indicating activating and inhibitory interactions, respectively. Continuous lines indicate direct interactions, whereas dashed lines indicate unknown mechanisms. See text for further details. Abbreviations: Ajub, Ajuba; App, Approximated; Crb, Crumbs; Dco, Discs overgrown; Dlg, Discs large; Ds, Dachous; Ex, Expanded; Fj, Four-jointed; Hth, Homothorax; Jub, *Drosophila* Ajuba; Lats, Large tumor suppressor; Lft, Lowfat; Lgl, Lethal giant larvae; Mer, Merlin; Mats, Mob as a tumor suppressor; Mob1A/B, Mps1 binder; Mst, Mammalian sterile 20 like; Rassf, Ras-associated factor; Sav, Salvador; Scrib, Scribble; Sd, Scalloped; Taz, transcriptional co-activator with PDZ-binding motif; TEAD, TEA domain protein; Tsh, Teashirt; Yap, Yes associated protein; Yki, Yorkie (Halder and Johnson, 2011).

Wts and Hpo, together with an upstream regulatory component Expanded (Ex) also regulate Yki localization through a phosphorylation-independent mechanism, by direct binding to Yki, sequestering it in the cytosol and preventing its nuclear activity (Badouel et al., 2009; Oh and Irvine, 2011). When the Hpo pathway is inactive, Yki translocates to the nucleus where it acts as co-transcriptional factor and induces the expression of genes involved in cell proliferation and survival, including *CyclinE* (*CycE*), *dMyc*, *Drosophila inhibitor of apoptosis 1*(*DIAP1*), the *bantam* microRNA, and mitogens including *wg* and the ligands of the epidermal growth factor receptor (EGFR) *vein*, *Keren* and *spitz* (reviewed in Pan, 2010; Zhao et al., 2011). Many upstream regulators of the core Hpo kinase cassette have been identified, including Ex and Merlin (Mer) (Hamaratoglu et al., 2006), the polarity genes Crb, Lgl and aPKC (Robinson et al., 2010) and the atypical Cadherins Fat and Dachshous (Ds), whose activity is partially controlled by the Four-Jointed (FJ), kinase located within the Golgi complex (Fig.1.11.) (reviewed in Chan et al., 2011). In the wing imaginal disc the expression of *ff* and *ds* are spatially separated into the blade and hinge respectively (Fig.1.9.C). This stimulates Yki nuclear localization, and consequently growth and expansion of the wing disc (Zecca and Struhl, 2010; Willecke et al., 2008). It is still not clear how the gradients of Fj and Ds are established and maintained but it has been recently suggested that the activity of the Wg target gene *Vg*, expressed as a gradient in the blade, represses *ds* and activates *ff* expression. In turn,, activated Yki stimulates the expression of *vg* in the outer cells of the hinge in a feed-forward mechanism that further contribute to the blade expansion (Zecca and Struhl 2010). In addition, pMAD induced downstream of Dpp signalling, acts as a Yki co-transcriptional partner to induce the expression of the *bantam* microRNA and promotes wing disc growth (Oh and Irvine, 2011).

The same signalling pathways appear to be involved to stimulate proliferation in response to tissue damage or removal. This phenomenon enables the regrowth and replacement of body parts after injury or amputation. An important insight into regeneration was provided by the observation that when cells initiate apoptosis, they

produce mitogenic signals, thereby stimulating the proliferation of neighbouring cells. This process, termed compensatory cell proliferation, was first characterized in the developing imaginal discs of *Drosophila*, but similar phenomena occur in other systems. Compensatory cell proliferation has been observed in *Drosophila* imaginal discs upon induction of cell death by X-irradiation, by expression of pro-apoptotic genes, or by mutation of the anti-apoptotic gene *thread* (*Diap1*) (Perez-Garijo et al., 2004; Ryoo et al., 2004; Huh et al., 2004). Compensatory cell proliferation is associated with the induction of *Wg* and *Dpp* in apoptotic cells, which are believed to induce proliferation of neighbouring cells, allowing the regrowth of the lost tissue and the maintenance of proper tissue size. Secretion of *Wg* is mediated by activation of the Jun-kinase (JNK) signalling pathway in dying cells (Ryoo et al., 2004). In addition, JNK signalling triggers non-autonomous activation of *Yki* in neighbouring cells, which stimulates their proliferation (Sun and Irvine, 2011). JNK is a highly conserved Mitogen activated pathway (MAP), whose activity is triggered by various cellular stresses, including irradiation, reactive oxygen species, infection, aging, disruption of cell polarity, cytoskeletal changes, and induction of apoptosis (reviewed in Igaki, 2009). In *Drosophila*, activation of JNK signalling is triggered in response to the activation of various membrane receptors bind to their ligand, including: *Eiger* (a homolog of Tumor Necrosis Factor (TNF)) and its receptor *Wengen*; PDGF- and VEGF-related factor 1 (PVF) (a homolog of human platelet-derived growth factor (PDGF)) and the PVF receptor (PVR) and finally *Wg* and *Frz*. This induces activation of a JNK kinase kinase (JNKKK), such as TGF- β activated kinase 1 (*dTAK1*), Protein kinase at 92B (*DASK1*), Slipper (*Slpr*) and *Mekk1* through adaptor proteins. In turn, JNKKK activates the JNK kinase (JNKK) Hemipterous (*Hep*), which in turn activates *Bsk* (Fig.1.12).

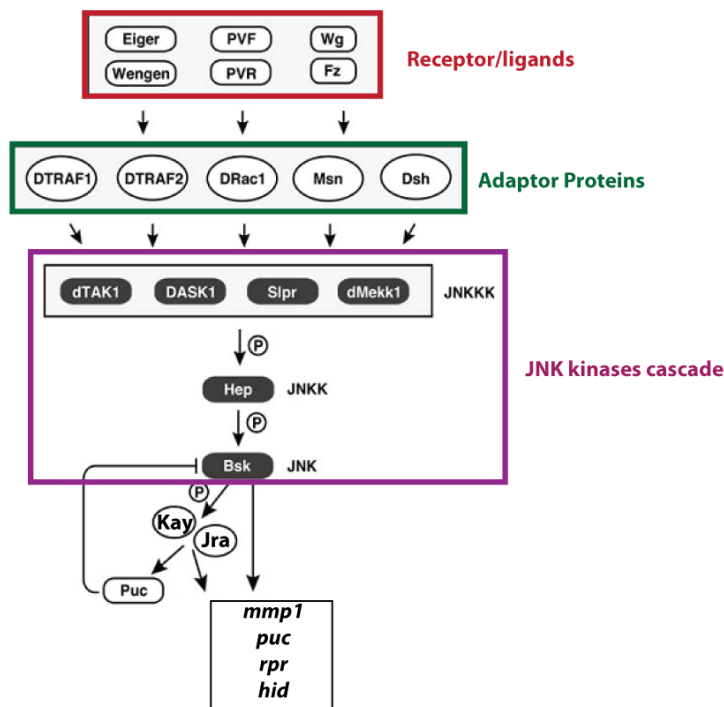


Figure 1.12 A Schematic representation of the major components of the JNK pathway in *Drosophila*.

Drosophila JNK signalling pathway can be activated by cell surface ligand/receptor system such as (Eiger/Wegen PVF/PVR; PDGF/PDGFR and Wg/Fr (represent in red). Adaptor proteins such as TRFAs, Dsh, small GTPase (such as Rac) and Msn (represent in green). Adaptor proteins mediate the signal to activate the core JNK signalling module consisting of JNKKK (dTAK1, DASK1, Slpr and dMekk1), JNKK (Hep), and JNK (Bsk). Activation of the JNK kinase cascade leads to phosphorylation of transcription factors Jra and Kay to induce transcription of *msn*, *mmp1*, *rpr*, *hid*, *puc*. Abbreviations used Bsk-Basket; Dsh- Dishavelled; Wg-Wingless; Fr- Frizzled; PVF-PVF/PVR- platelate- derived growth factor/receptor; PDGF/PDGFR- Platelate growth factor/PDGF receptor; JNK- Jun kinase; JNKK- JNK kinase; JNKKK- JNK kinase kinase; Hep-Hemipterous; dTAK1- TGF- β activated kinase 1; DASK1- Protein kinase at 92B (DASK1), Slpr-Slipper ; Msn- Misshapen; rpr-reaper; mmp1- Matrix metaloprotease 1; Jra-Jun-related antigen; Kay- Kayak; Puc-Puckered (Adapted from (Igaki, 2009).

Activation of JNK signalling induces the transcriptional expression of many target genes, including the pro-apoptic genes (*reaper* (*rpr*) and *hid*); but also *matrixmetaloprotease 1* (*mmp1*), which digests components of the ECMs, *misshapen* (*msn*) that acts as an adaptor protein of JNK signalling and the negative regulator of Bsk *puckered* (*puc*)

(reviewed Igaki, 2009). Activation of JNK signaling has distinct outcomes depending on the tissue type and developmental context (reviewed in Igaki, 2009). It is crucial for cell migration during dorsal closure and wound healing (Martin and Parkhurst, 2004). JNK has also important pro-apoptotic functions (reviewed in Igaki, 2009; Kanda and Miura, 2004). Activated JNK triggers the expression of *reaper*, which in turn leads to degradation of the caspase inhibitor *DIAP1* (Kuranaga et al., 2002). As a result, activated caspases mediate apoptosis. JNK pathway has been also proposed to induce apoptosis independently of caspases (reviewed in Igaki, 2009). By eliminating developmentally aberrant cells from a tissue, JNK signalling has been proposed to maintain tissue homeostasis, as well as protect organisms against tumor development (Brumby and Richardson, 2003; Igaki et al., 2006; Uhlirova et al., 2005) (Uhlirova and Bohmann, 2006).

5. TUMORIGENESIS AS AN EXAMPLE OF THE LOSS OF EPITHELIAL TISSUE HOMEOSTASIS

5.1. THE HALLMARKS OF EPITHELIAL DERIVED CANCERS

In mammals, cancer progression of tissues from epithelial origins involves the stepwise acquisition of a number of neomorphic traits by tumour cells (reviewed in Hanahan and Weinberg, 2000). First, transformed cells sustain the ability to proliferate independent of external growth control mechanisms (self-sufficiency in growth signals), (Fig.1.13.).

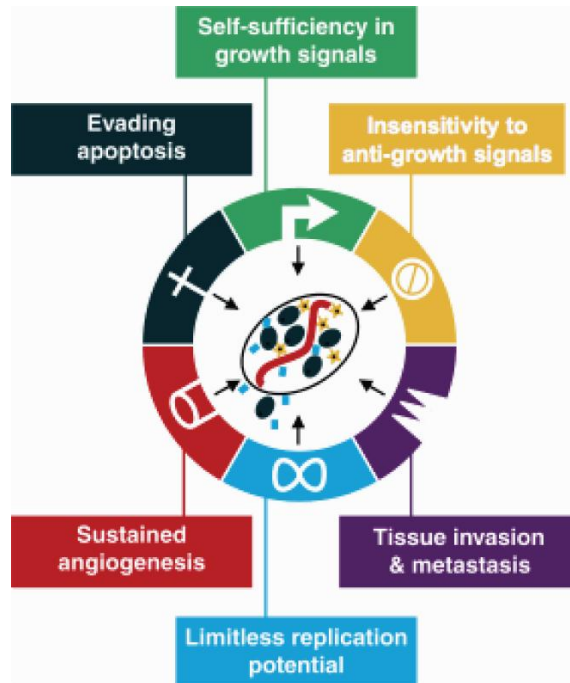


Figure 1.13 Schemat representing major hallmarks of cancer cells.

Tumour cells need to acquire distinct features to survive, multiply and invade distinct tissues. These features can be referred as hallmarks of cancer and include: self-sufficiency in growth signals; insensitivity to anti-growth signals; ability to evade apoptosis; sustained angiogenesis; limitless replication potential; tissue invasion and metastasis (adapted from Hanahan and Weinberg, 2011).

This can be achieved by distinct means. Cells can start to express both a morphogen and its receptor or can signal to neighbouring cells to promote morphogen synthesis or simply increase the synthesis of the morphogen receptors. Cancer cells can also alter the activity of signalling pathways activated upon clustering of morphogen receptors. Second, cancer cells evade growth suppressor mechanisms that ensure robustness of the tissue (insensitivity to anti- growth signals), (Fig.1.13). For instance by mutations of *retinoblastoma* (*rb*) which links signals from distinct pathways, to regulate cells cycle progression or termination. Third, cancer cells have to evade apoptosis since apoptotic cell death has been proposed to serve as a mechanism that protects the organism from the mutated and potentially dangerous cells (Fig.1.13.) Cancer cells can avoid apoptosis

by distinct means; the most common strategy is the loss of *P53*, which induces apoptosis upon cellular stresses. Fourth, cancer cells need to enable the replicative immortality (limitless replication potential), (Fig.1.13.). Normally, most healthy cells undergo only a limited number of cell divisions before going into a non- proliferative viable state and finally senescence. However, cancer cells keep dividing without these restrictions. One of the most common means by which cancer cells keep proliferating is by expressing telomerase, which elongates telomere ends. The fifth hallmark of cancer cells is their ability to induce angiogenesis (sustained angiogenesis), (Fig.1.13.). The multiplying cancer cells reach blood vessels in order to get nutrients, oxygen and remove wastes and carbon dioxide. This brings a necessity for a formation of new blood vessels, able to feed highly proliferating cancer cells. Finally, cancer cells get the ability to invade and metastases (tissue invasion and metastasis) into distant tissues to form secondary tumours (Fig.1.13), (reviewed in Hanahan and Weinberg 2011).

Missregulation of most genes or pathways involved in cancer progression result in only one or two of the hallmarks of cancer and in most cases net tissue-overgrowth is not observed, because there is also induction of compensatory apoptosis. Loss or gain of function mutations in genes that give rise to at least one of the hallmark of cancer are named tumour suppressor genes (TSGs) and oncogenes respectively (reviewed in Hanahan and Weinberg 2011). Therefore, most mammalian cancers are likely to originate from cooperative interactions between both TSGs and oncogenes (Brumby and Richardson, 2005).

5.2. *DROSOPHILA* AS A MODEL TO STUDY TUMORIGENESIS, ADVANCES AND LIMITATIONS

Over the past decade, *Drosophila*, in particular the larval brain, imaginal discs and the follicular epithelium surrounding the egg chamber, has emerged as an important model to study tumour progression (reviewed in Miles et al., 2011; Hariharan and Bilder, 2006). Many distinct features of larval imaginal discs make it useful as a model to study

tumorigenesis. First, growth of imaginal disc is mainly autonomous (Bryant and Simpson, 1984). Second, signalling pathways involved in human cancers are conserved in *Drosophila* (reviewed in Bilder, 2004). Indeed, our understanding of Hpo, Wnt, Dpp and JNK signalling, which all are missregulated in cancer has been mainly extended from studies in flies (reviewed in Miles et al., 2011). Finally, because the *Drosophila* genome is smaller and much less redundant than in humans, it allows for large scale screening for new TSGs and oncogenes (reviewed in Miles et al., 2011). Larval tumours can occur either spontaneously or may be induced in mutagenesis screens. They can develop in the brain, imaginal discs or both tissues (reviewed in Bilder, 2004). Larval epithelial tumours are classified as hyperplastic, when the tissue overgrowth but cells are still arranged into a monolayer. In contrast, neoplastic tumours are characterised by the loss of cell morphology, cells appear rounder and tissue architecture is affected. The induction of neoplastic tumours usually results in the death of the animal after prolonged larvae stages. Moreover when transplanted into the abdomen of an adult fly tumour cells continue to proliferate, cross the basal lamina and invade distant tissues (reviewed in Bilder, 2004; Hariharan and Bilder, 2006). However, as any model systems, *Drosophila* also has several limitations. The process of angiogenesis cannot be modelled in *Drosophila* since its lymphatic and hematopoietic systems are completely different than in mammals. In addition, *Drosophila* does not contain Telomerase.

5.3. TUMOUR SUPPRESSORS GENES AND ONCOGENES IN *DROSOPHILA* EPITHELIAL TISSUES

Genetic screens conducted in *Drosophila* helped to identified many TSGs and oncogenes that appeared to contribute to tumorigenesis in mammals such as components of the Hpo pathway, *hCDC4* (named *archipelago* in *Drosophila*) or *scrib* (reviewed in Bilder, 2004). Mutations for hyperplastic TSGs, such as upstream components of the Hpo pathway, result in excessive cell proliferation and evasion of apoptosis,(reviewed in Hariharan and Bilder, 2006). In contrast, loss of neoplastic TSGs does not increase the

rate of cell proliferation. In some cases, mutant cells even proliferate at slower rate than wildtype cells. Due to this property, when cells homozygote mutant for neoplastic TSGs are induced in small patches of cells (clones) in an heterozygote background, they are outcompeted by wildtype cells around and undergo JNK-mediated apoptosis (reviewed in Hariharan and Bilder, 2006). Moreover, in contrast to hyperplastic TSGs, mutant cells for neoplastic TSGs fail to terminate proliferation and do not differentiate. However, when the whole tissue is mutant for a neoplastic TSG, it form highly proliferative tumours, characterized by the loss of epithelial tissue architecture and an invasive behaviour (reviewed in Bilder, 2004; Hariharan and Bilder, 2006). Neoplastic TSGs consist of two major groups, polarity markers of the *scrib*, *dlg* and *lgl* group and genes that encode for components of the endocytic machinery (including *avalanche* (*avl*), *rab-protein 5* (*rab5*)). The best-described oncogenes in flies are the activated forms of *Ras* (*Ras*^{V12}), *Notch* (*Notch*^{Act}) and *Src*. They cooperate with mutations in TSGs in oncogenic transformation. For example, overexpression of *Ras*^{V12} triggers hyperplastic overgrowth but when combined with a mutation in *scrib*, *dlg* or *lgl* promotes massive tissue overgrowth, loss of tissue architecture and invasive behaviour (reviewed in Miles et al., 2011).

5.4. THE COMPONENTS OF HIPPO PATHWAY AS EXAMPLES OF TUMOUR SUPPRESSOR GENES

Most of the components of Hpo pathway were discovered in the genetic screen in *Drosophila* as hyperplastic TSGs. The first components to be identified were *wts*, *sav*, *hpo* and *mats*. Mutations in any of these genes lead to extensive tissue overgrowth due to increase proliferation and loss of apoptosis. Later Yki was identified to act downstream of the Hpo pathway. Yki is an oncogene and its overexpression result in the tissue overgrowth (reviewed in Chan et al., 2011). The oncogenic potential of Yki depends on its ability to promote transcription of distinct genes involved in cell survival, growth and proliferation. Yki-dependent *dIAP1* expression is essential for the survival of

Yki overexpressing tissues. Moreover by promoting transcription of *dMyc* that acts as an inducer of ribosome biogenesis, Yki promotes cell growth. Furthermore, the expression of *cyclinE* and of the *bantam* microRNA increases the proliferation rate. *bantam* has also a role in blocking apoptosis. Yki also stimulates transcription of the EGFR ligands *vein*, *Karen* and *Spitz* (Zhao et al., 2011). The Hpo pathway is conserved from fly to human (Fig.1.11.A, B). Human components of the Hpo pathway, including YAP (ortholog of Yki), Large tumor suppressor 1 and 2 (Lats1/2, orthologs of Wts), mammalian STE-20 kinase 1 and 2 (Mst1/2; orthologs of Hpo) and Msp-one-binder (Mob1, ortholog of Mats) are able to functionally rescue the corresponding mutations in *Drosophila*. Deregulation of the Hpo pathway components is often associated with cancer in humans. For example *NF2*, the human ortholog of *Merlin* is a known TSG implicated in various signalling pathways including Rac1 GTPase and Src that together with the control of Yki nuclear localization serves as a tumour suppressor mechanism in mammals. Mutations of *NF2* are found in patients with neurofibromatosis, associated with higher risks of developing tumours in the nervous system. The loss of activatory post-transcriptional modifications of Mst1 and Mst2, together with decrease of YAP phosphorylation, is observed in ~30% of human hepatocellular carcinoma (reviewed in Chan et al., 2011). Finally nuclear YAP accumulates in samples from distinct types of cancers, including colonic adenocarcinoma, lung adenocarcinoma, and ovarian cysadenocarcinoma, when compared to the healthy tissues (reviewed in Chan et al., 2011).

5.5. ROLE OF JNK SIGNALLING IN EPITHELIAL-DERIVED TUMOURS

Because of its pro-apoptotic function, JNK activation is thought to have a protective anti-tumor function, which would eliminate aberrant cells, rather than allowing them to proliferate and form tumours (Brumby and Richardson, 2003; Uhlirova and Bohmann, 2006; Igaki et al., 2006). However, when apoptosis is blocked using the Caspase inhibitor P35, cells remain alive “undead” and JNK signalling can promote cell proliferation and tissue overgrowth (Ryoo et al., 2004; reviewed in Pérez-Garijo et al., 2009). JNK signaling

has been linked to proliferative and metastatic features of tumours associated with disruptions of apical-basal polarity in epithelial cells, induced by *lgl*, *dlg* and *scrib* mutations (Igaki et al., 2006; Uhlirova and Bohmann, 2006). Loss of *lgl* triggers JNK signalling, which in turn, promotes Yki nuclear localization, resulting in neoplastic tissue overgrowth (Sun and Irvine, 2011). In addition, JNK signalling contributes to the invasive behaviour of tumour cells induced by *lgl* loss by promoting *mmp1* transcription (Uhlirova and Bohmann, 2006).

5.6. CONTRIBUTION OF LOSS OF CELL POLARITY AND ADHESION TO TUMORIGENESIS

The loss of epithelial polarity and tissue architecture is the primary diagnostic of malignant cancers in tissues such as breast, prostate, and colon (reviewed in Bilder, 2004). Many of the known TSGs belong to the group of adhesions and polarity markers, including *lgl*, *dlg* and *scrib* (reviewed in Bilder, 2004). Moreover, α PKC that directly phosphorylates and restricts Lgl localization to the subapical domain, when overactivated in *lgl* mutant clones promotes tumorigenesis (reviewed in Lee and Vasioukhin, 2008). Moreover, overactivation or promotion of α PKC cortical localization induces larval tumour formation indicating that α PKC acts as an oncogene (Grifoni et al., 2007). Lgl, α PKC and Crb are all upstream regulators of the Hpo pathway and negatively regulate Yki nuclear localization (reviewed Genevet and Tapon, 2011). Moreover, loss of apico-basal polarity also induces activation of JNK pathway that can contribute to tumorigenesis (reviewed in Igaki, 2009). Human orthologs of Scrib, Dlg, Lgl, α PKC have been found misregulated in several human cancer types (reviewed in Martin-Belmonte and Perez-Moreno, 2012)

E-cadherin also plays a pivotal role in epithelial cell polarity, cell signalling and tumor suppression. Disruption of E-cad, through mutations, transcriptional silencing and posttranscriptional modifications, is associated with distinct types of human cancers (reviewed Jeanes, Gottardi, and Yap 2008). Although, loss of E-cad alone is not sufficient

to induce tumorigenesis, it contributes to tumour formation and cancer progression by distinct means. First, loss of E-cad triggers β -Cat release into the cytoplasm and consequently ectopic activation of Wnt signalling pathway, which has been associated with early events of tumorigenesis such as in colorectal cancers (reviewed in Jeanes et al., 2008). Second, loss of E-cad leads to increase EGF-R signalling (reviewed Jeanes et al., 2008). Increased EGF-R signalling has been also associated with poor prognosis and decreased patients survival and resistance to chemotherapy and radiation treatment in tumour cells (reviewed in Herbst, 2004). However, it is still not clear if E-cad directly inhibits EGF-R activation upon E-cad clustering or down-regulates EGF-R signalling activity (reviewed in Jeanes et al., 2008). Independent of the nature of their interactions E-cad clustering can inhibit cell responsiveness to EGF stimulation, revealed by a decrease in cell proliferation and Ras signalling (Qian et al., 2004; Perrais et al., 2007).

Therefore, the ability of polarity markers to interact with signalling pathways may be a general mechanism by which loss of polarity can contribute to tumorigenesis (reviewed in (Martin-Belmonte and Perez-Moreno 2012; Genevet and Tapon 2011).

5.7. SRC AS AN EXAMPLE OF ONCOGENE

Src is a membrane-linked non-receptor kinase, required for regulation of adhesion and cytoskeleton reorganization, cell cycle progression and migration (reviewed in Miles, Dyson, and Walker 2011). Mammals contain nine Src family kinases (SFKs) whose activities are negatively regulated by two kinases: The C-terminal Src kinase (Csk) and Csk homology kinase (CHK) (Chong, Mulhern, and Cheng 2005). *Drosophila* has only two genes encoding for Src: *Src42A* and *Src64B* and one homolog of Csk, *Drosophila* Csk (dCsk) (Stewart et al. 2003; Read et al., 2004). While mutations that activate Src are found rarely if at all in human cancers, elevated levels of SFK have been observed in the broad repertoire of human cancers including those of colon, liver, lung, breast and pancreas origins (reviewed in Ishizawar and Parsons 2004; Summy and Gallick 2006; Guarino 2010). Moreover, SFK activities progressively increase in the later stages of

tumorigenesis and are associated with metastatic behaviour (reviewed in Yeatman 2004). Although the precise mechanism by which high levels of Src activation is triggered during oncogenic transformation is not completely understood, distinct mechanisms have been proposed (reviewed in Irby and Yeatman, 2000). Src protein kinase activity may be increased by direct or indirect interaction with receptor tyrosine kinases, such as epidermal growth EGF-R platelet derived growth factor receptor (PDGF-R) fibroblast growth factor receptor (FG-R) colony stimulating factor-1 receptor (CSF-1R) HER2/neu), and hepatocyte growth factor receptor (c-Met) and also upon E-cad and Integrins clustering (reviewed in Finn, 2008; Irby and Yeatman, 2000). In turn, activated Src may synergistically interact with these pathways (reviewed in Finn, 2008). This leads to diverse outcomes, including tumour growth, invasion and metastasis (Fig.1.14.A) (reviewed in Finn, 2008).

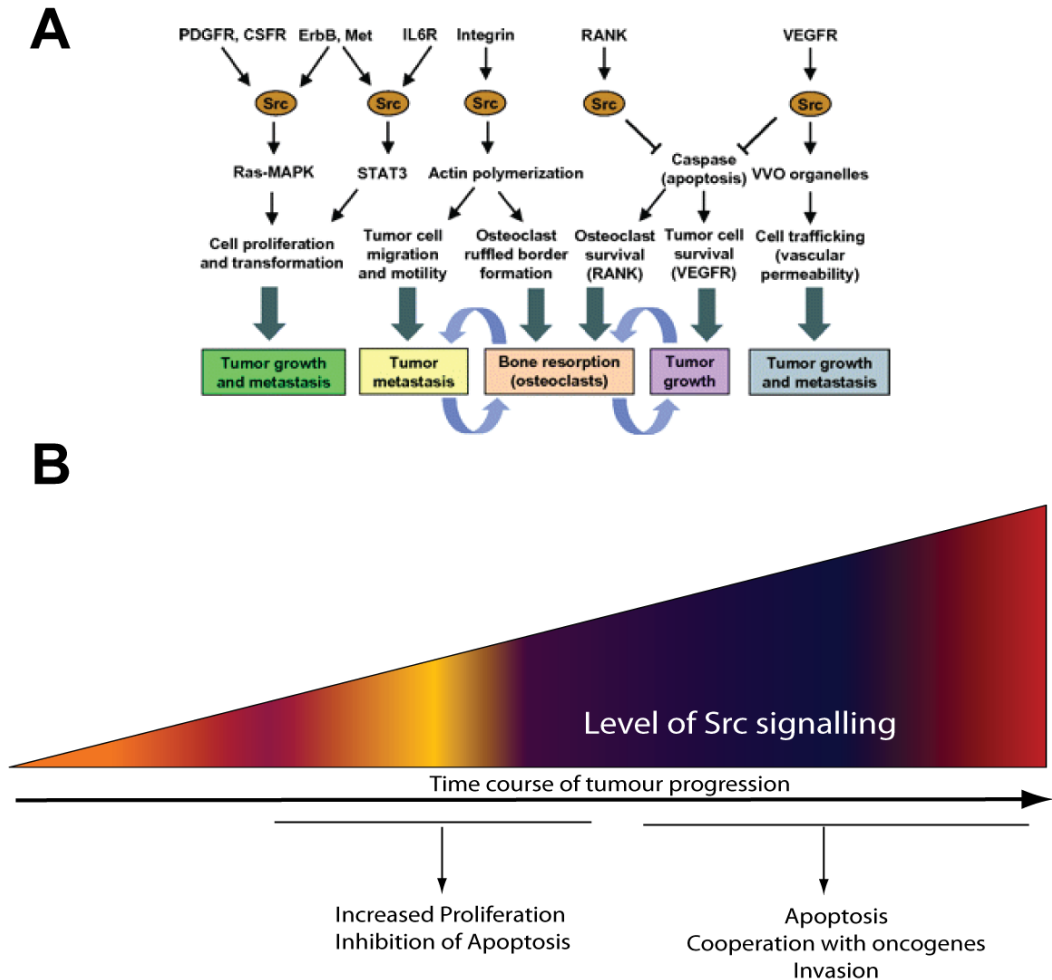


Figure 1. 14 Schematic representation of Src activation, functions and contribution to tumour formation and progression.

(A) Src activation may occur upon clustering of distinct receptors including: EGF-R, PDGF-R, FGF-R, Integrins. Upon activation Src can function synergistically with these signalling pathways to promote different cellular outcomes, such as proliferation, survival or angiogenesis. These processes may contribute to tumour formation and survival. Increased levels of Src signalling trigger distinct cellular responses (Finn, 2008). (B) Model proposed by Vidal explaining the contribution of Src signalling into distinct stages of tumourigenesis. A slightly elevated level of Src signalling induces higher proliferation and provides anti-apoptotic signals. Further increase of Src signalling induce apoptosis and can cooperate with oncogenic mutations such as *Ras*^{V12} to induce metastasis and invasion. Lower levels of Src signalling are associated with early stages of tumorigenesis while higher levels correlate with later stages of cancer and metastasis (adapted from Vidal et al., 2007).

Although the role of Src in tumorigenesis has been extensively studied, the exact mechanisms by which Src contributes to tumourigenesis and tumour progression is still not clear. For instance, overactivation of Src results in distinct cellular outcomes ranging from cell proliferation, differentiation and apoptosis depending on the cell lines used (reviewed in Brown and Cooper, 1996). Moreover, mutations of Src in cancers are rarely identified while the elevated levels of Src protein and its activities is often observed in distinct types of cancer including those of colon, lung, breast and pancreas (reviewed in Irby and Yeatman, 2000) . However, studies from Cagan's laboratory have extended our understanding on how Src signalling contributes to distinct stages of tumour progression. In this studies Vidal and co-authors used *Drosophila* imaginal discs to assayed the effect of increased Src signalling activity, either by knocking down dCsk or by overexpressing Src (Vidal et al., 2006; Vidal et al. 2007). They have demonstrated that mild overexpression of Src64B or hypomorphic mutations of *dCsk* triggers cell proliferation and inhibition of apoptosis (Vidal et al., 2006; Vidal et al. 2007). In contrast, strong Src42A overexpression or null mutation for *dCsk*^{Q156Stop} triggers apoptosis. Interestingly, when combined with Ras^{V12}, only high Src signalling activity cooperate with Ras^{V12} overexpression giving rise to neoplastic overgrowth associated with loss of the ECM component Laminin A and invasion into the larval brain (Vidal et al. 2007). The authors purpose a model in which slight increase of Src signalling induces proliferation and provides anti-apoptotic signals that contribute to the early stages of tumorigenesis. At later stages, higher Src signalling activity, combined with oncogenic mutations, contributes to metastasis and invasion (Vidal et al. 2007), (Fig.1.14.B).

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CHAPTER 2 – CAPPING PROTEIN RESTRICTS APICAL ACTIN FILAMENTS EVENLY IN A WHOLE WING IMAGINAL DISC

1. SUMMARY

Capping Protein, which regulates actin polymerization, has a tissue-specific function in the wing disc epithelium. While in the proximal domain, cells lacking Capping Protein accumulate F-actin apically, sustain polarity and survive, in the distal domain, cells show ectopic F-actin around the entire cell cortex, mislocalize Adherens Junction components, extrude and die. In this chapter, I investigated which population of actin filaments regulated by Capping Protein, triggers loss of polarity and basal extrusion of distal wing disc cells. I show that reducing Capping Protein α levels using RNAi does not induce cortical F-actin accumulation in distal wing disc cells but triggers the accumulation of three distinct populations of actin filaments localized apically and one F-actin population apposed to the basal cell surface. Apical F-actin accumulation is still associated to mislocalization of the Adherens Junctions components Armadillo and DE-cadherin. Moreover, the apical accumulation of an HA-tagged form of Capping Protein α is dependent of its actin-binding domain. Therefore, my results argue that Capping Protein regulates identical F-actin networks in the distal and proximal wing disc domains. However, Adherens Junctions of distal wing disc cells are more sensitive to F-actin accumulation. Basal F-actin accumulation or a reduction in the pool of G-actin are unlikely to affect Adherens junction stability since loss of Capping Protein does not disrupt Integrins localization, nor significantly reduces the G-actin levels. Instead, my results are in agreement with a model in which in the distal, as in the proximal wing disc domains, Capping Protein regulates a population of apical actin filaments, which stabilizes Adherens Junction. Following loss of Capping Protein, apical F-actin accumulation affects Adherens Junction integrity only in distal wing disc cells, triggering loss of cell polarity and F-actin accumulation around the entire cell surface.

2. INTRODUCTION

Capping Protein (CP) is composed of the two highly conserved α (Cpa) and β (Cpb) subunits. CP binds to the barbed (fast growing) end of actin filaments (F-actin) and prevents addition and loss of actin monomers (G-actin). In vivo studies in *Drosophila* epithelial tissue showed that mutations in CP lead to accumulation of ectopic F-actin (Janody and Treisman 2006). Interestingly CP does not seem to have the same functions in all the regions of the developing *Drosophila* wing disc epithelium since loss of either cpa or cpb subunits in the wing imaginal discs give rise to distinct phenotypic outcomes. Along the antero-posterior (A-P) axis the wing imaginal disc can be subdivided into three fate-defined regions: the notum, in the most proximal part, then the hinge and finally the blade in the most distal part (Fig.1.9.). For simplicity, the hinge and the notum will be referred as the proximal wing disc domain, whereas the blade will be called the distal wing disc domain. In CP mutant clones induced in the distal domain, the major components of AJs, Armadillo (Arm) and DE-cadherin (DE-cad), mislocalize to the basolateral position, mutant cells suffer basal extrusion and die by apoptosis. Interestingly, the requirement of CP to maintain cells within the epithelium is restricted to the distal wing disc domain since CP mutant cells that develop in the proximal wing disc domain sustain polarity and survive (Janody and Treisman, 2006). These behaviors are associated to distinct sites of F actin accumulation. In the distal domain, F-actin accumulates throughout the entire cell cortex, while in the proximal domain, ectopic F actin is observed mainly at apical cell surfaces (Janody and Treisman, 2006). These data suggest that CP regulates different F-actin populations. In this scenario, the F-actin population regulated by CP in the distal wing domain would be critical to maintain Adherens Junctions (AJs) and prevents in this way cell extrusion and apoptosis. In agreement with this possibility, both AJs stability and turn-over are controlled by distinct apical F-actin pools. In *Drosophila*, two distinct F-actin pools have been shown to regulate the stability or mobility of homophilic DE-cad clusters at AJs. Stable, small F-actin patches may stabilize homophilic DE-cad interactions. While a dynamic,

contractile F-actin network tethers homophilic DE-cad within AJs and limits their lateral mobility. These two F-actin pools are distinct in term of dynamics and function and are probably intermingled at AJs (Cavey et al., 2008). Moreover, a F-actin population has also been described to regulate DE-cad endocytosis, enabling AJs turn-over (Georgiou et al., 2008). Thus, CP might specifically be required to regulate one of these F-actin pools in the distal but not in the proximal wing disc domains.

Alternatively CP may affect identical apical F-actin populations in the whole wing imaginal disc. However, because distal cells are more sensitive to apical F-actin disruption, cells lose epithelial polarity and as a result, accumulate F-actin around the entire cell cortex. In agreement with this possibility, the cells in the most distal wing disc domain are under higher tension than in the proximal domain (Major and Irvine, 2005; Landsberg et al., 2009; Classen et al., 2005; Farhadifar et al., 2007; Canela-Xandri et al., 2011). Interestingly distinct adhesion complexes can interact to influence the functions and organization of each other (reviewed in Weber et al., 2011). In epithelial tissues, in addition to AJs, which provide the linkage between adjacent cells, another type of junctions- Focal adhesions (FAs) allow for cellular attachment to Extracellular matrix (ECM). The organization and functions of FAs depend on the presence of Integrins that binds to ECM components. The intracellular domain of Integrins interacts with the cytoplasmic components of FAs named cytoplasmic plaque. Similarly, like AJs, FAs are also linked to F-actin, organized into stress fibers. Moreover AJs and FAs share signalling and scaffolding molecules (reviewed Weber et al., 2011). Functions of AJs and FAs influence each other stability and signalling by diverse means. For example, F-actin contractility initiated upon Integrins clustering can inhibit E-cad mediated adhesion (Avizienyte et al., 2004) or suppress E-cad transcription (Onodera et al., 2010). Moreover signalling initiated upon clustering of Integrins and E-cad, such as Src signalling, can influence adhesion mediated by these molecules (reviewed in Weber et al., 2011). This rise the possibility that following CP loss, misslocalization of AJs components and epithelial cell polarity loss results from a F-actin defect at FAs. Either CP may only affect

F-actin linked to FAs in the distal but not the proximal wing disc domains or CP affects F-actin linked to FAs in the whole wing disc epithelium but only cells located in the distal domain are sensitive to change at FAs.

In this chapter, I aimed to analyze if CP restricts different F-actin populations along the Proximo-Distal axis of the wing imaginal disc. To do so, I investigated the effects of reducing CP levels using RNA interference (RNAi) on apical and basal F-actin populations, and on the stability of AJs and FAs in the distal wing domain.

3. MATERIAL AND METHODS

3.1. GENERATION OF UAS-*CPA*^{WT} AND UAS-*CPA*^{ΔABD} TRANSGENIC FLIES

Both UAS-*cpa*^{WT} and UAS-*cpa*^{ΔABD} fly strains were obtained through the site-specific ϕ C31 integrase method (Bischof et al., 2007). The pUASattB vector (GenBank EF36240) was used.

3.2. DESIGN AND AMPLIFICATION OF *CPA*^{WT} AND *CPA*^{ΔABD}

Both constructs were fused to HA tag to monitor their cellular localization. UAS-*cpa*^{WT} was obtained by exertion of the fragment from previously reported *cpa* construct HA-miro that completely rescue *cpa* loss phenotype (Janody and Treisman, 2006). XhoI and HindIII restriction enzymes (Promega) were used to obtain a fragment containing *cpa*^{WT} and 5 UAS sites (UAS-*cpa*^{WT}). UAS-*cpa*^{WT} was inserted into pUASattB vector. The presence of the UAS-*cpa*^{WT} insertion was confirmed by PCR reactions (primers 5'pUASattB and 3'pUASattB in Table.2.1 were used) and sequencing. UAS-*cpa*^{ΔABD} was obtained by PCR reaction using HA-miro as a template (primers 5'-HA and 3'*cpa*^{ΔABD} in Tab.2.1 were used). The PCR reaction products were analyzed by electrophoresis through agarose gel and purified using the NucleoSpinExtract II (Macherey-Nagel). *cpa*^{ΔABD} was inserted into pGEM®-T Easy Vector. The colonies were tested in X-Gal- and IPTG-containing agar. *cpa*^{ΔABD} was extracted with NotI and BglII (Promega) followed by the ligation into pUASattB. The presence of *cpa*^{ΔABD} was confirmed by PCR reaction

(primers 5'pUASattB and 3'pUASattBin Table.2.1 were used) and sequencing. Both UAS-*cpa*^{WT} and UAS-*cpa*^{ΔABD} were integrated into *Drosophila* genome at the 3R-89E region by BestGene (<http://www.thebestgene.com/>).

Table2.1- Primers used to generate UAS-*cpa*^{WT} and UAS-*cpa*^{ΔABD} constructs.

Name of the primer	Sequence
5'-HA	ATAGATCTATGGGATACCCGTACGATG
3'- <i>cpa</i> ^{ΔABD}	ATGCGGCCGCTTAGTCGATCTTGGTCCTGGT
5'- pUASattB	GACTAGGCTAGAATAACTTCGTA
3'-pUASattB	TATGTCCAATTATGTCACACCACA

3.3. FLY HUSBANDRY

Flies were raised at 22°C (unless otherwise indicated) under standard conditions (Roberts, 1998). Crosses were cultured in small vials and 3rd instar larvae were collected for dissections of the wing imaginal discs.

3.4. MUTANTS AND *DROSOPHILA* STRAINS USED

Fly stocks used were *cpa*^{69E}, FRT40A; UAS-HA-*cpa*^{WT} and UAS-*cpa*^{ΔABD}; UAS-*cpa*-IR^{C10} (Fernández et al., 2011); *sd*-Gal4 (Klein and Arias 1998); *hh*-Gal4, a gift from T. Tabata.

To generate *cpa*^{69E} clones marked by the absence of GFP in the wing disc *y, w*; FRT42D, *cpa*^{69E}/CyO, P(*y*⁺) males were crossed to *y, w, hsflp*; FRT42D, *ubi*-GFP females. To generate UAS-*cpa*^{C10} mutant clones *y, w*;; UAS-*cpa*-IR^{C10} FRT40A /CyO, P(*y*⁺) males were crossed to *y, w, hsFLP122, tubGal4, UAS-GFP*; *tub*-Gal80 FRT40A females. The offspring were heat-shocked for 1 h at 37 °C 24h (for *cpa*^{69E} clones) or both 24h and 48 h (for UAS-*cpa*-IR^{C10} clones) after a 24 hour egg collection, corresponding to the first (24h) and second (48H after egg collection) larval instar.

3.5. ANTIBODY STAINING

3rd instar wing imaginal discs were dissected, fixed and stained using the procedure described in (Lee and Treisman, 2001). Between 10 and 15 discs were analyzed for each genetic combination. Antibodies used were mouse anti-Arm (N2 7A1, 1:10; DSHB), rat anti-DE-Cad (CAD2; 1:50, DSHB), mouse anti-Dlg (4F3, 1:50; DSHB), mouse anti-Cor (C615.16, 1:200, DSHB), mouse anti-G-actin (A3853, clone AC-40, 1:200, Sigma Aldrich) and rabbit anti-activated Caspase 3 (1:500; BD Bioscience). Rhodamine-conjugated Phalloidin (Sigma) was used at a concentration of 0.3 μ M and Alexa Fluor® 594 DNase I (Sigma) was used at the concentration of 0.8 μ M. Secondary antibodies were from Jackson ImmunoResearch, used at 1:200 (donkey anti-rabbit TRITC #711-025-152; donkey anti-rabbit Cy™5 #711-175-152; donkey anti-mouse Cy™5 #715-175-150; donkey anti-mouse TRITC #715-025-151; donkey anti-rat TRITC #712-025-153).

3.6. IMAGING

Fluorescence images were obtained on Leica SP5 TCS NT or LSM 510 Zeiss confocal microscopes. The NIH Image J software was used to perform measurements. To quantify the intensity of Alexa Fluor® 594 DNase I signals, the posterior and anterior compartment of *hh > cpa-IR^{C10}* wing discs were outlined separately for each disc and the intensity levels were calculated as the sum of the grey values of all the pixels in the selection divided by the number of pixels for each compartment.

4. RESULTS

4.1. CAPPING PROTEIN MAINTAINS COMPONENTS OF ADHERENS JUNCTIONS IN THE DISTAL WING DISC EPITHELIUM

To investigate whether CP regulates different F-actin populations in the distal and proximal wing disc domains, I looked for experimental conditions in which reducing CP levels triggers ectopic actin filaments formation without affecting cell polarity of distal wing disc cells, in order to analyze where F-actin accumulate. To do so, I expressed *cpa*

double stranded RNA (UAS-*cpa-IR*) using the *hedgehog*-Gal4 (*hh*-Gal4) driver, together with UAS-*mCD8:GFP* to mark the targeted tissue (*hh>-cpa-IR;GFP*). Since *hh*-Gal4 drives the expression of the desired UAS construct in the posterior compartment of the wing disc, the anterior compartment can be used as an internal control. Then, I analyzed the polarity status of Cpa-depleted cells by staining wing disc with diverse polarity markers. DE-cad and Arm were used to mark AJs, while Coracle (Cora) and Disc-large (Dlg) were used to mark Septate Junctions (SJs).

Standard confocal sections of the apical cell surfaces showed that Cpa-depleted cells (marked with GFP) displayed decreased levels of DE-cad (Fig.2.1. B-B') and Arm (Fig.2.1. F-F'), compared to the anterior wildtype compartment. Optical cross sections through the wing disc epithelium confirmed that DE-cad (Fig.2.1. D-D') and Arm (Fig.2.1. H-H') were reduced apically in Cpa-depleted cells. To confirm that this effect was due to Cpa depletion, I stained discs that only expressed GFP under the *hh*-Gal4 control.

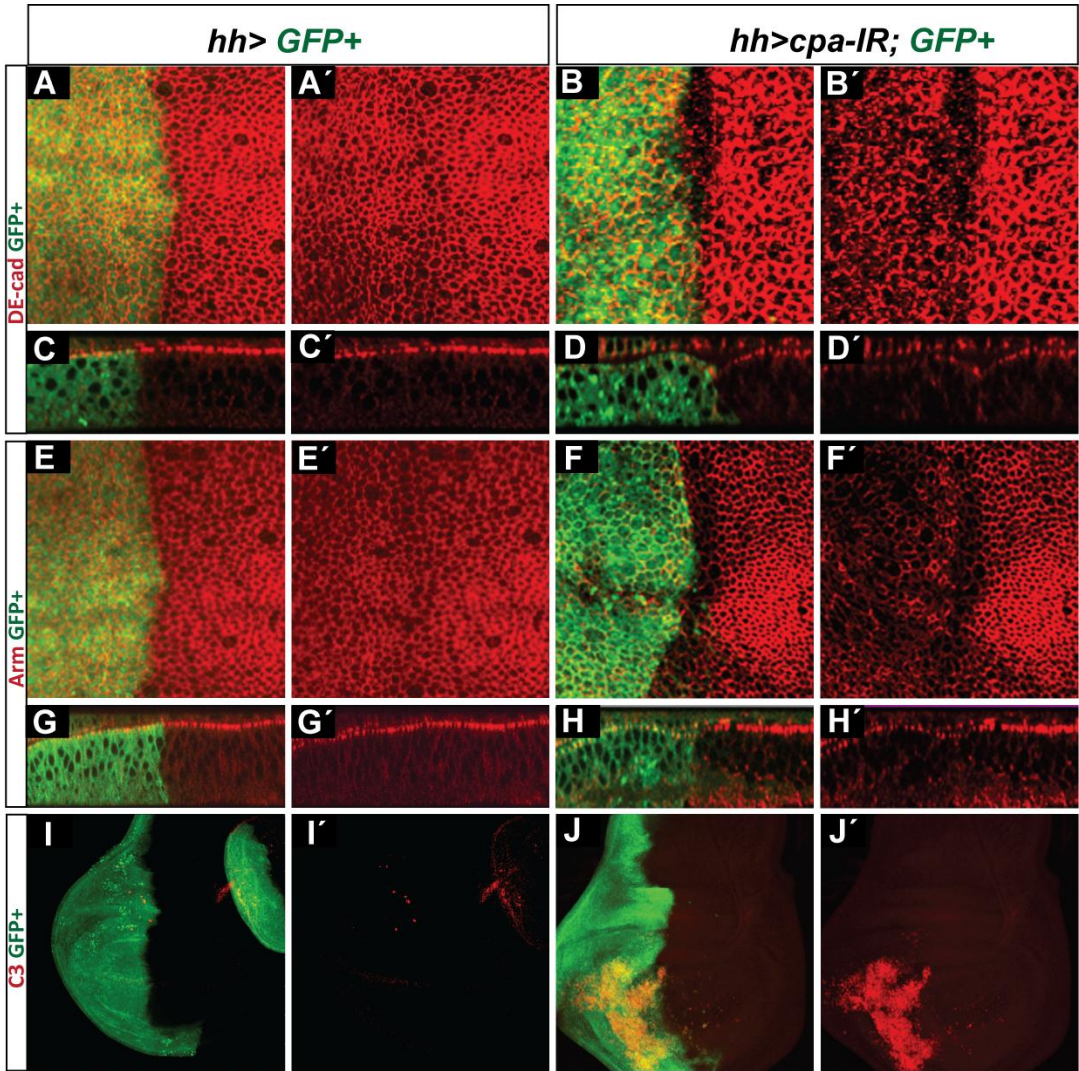


Figure 2.1 Depletion of Capping Protein triggers the misslocalization of Adherens Junctions components at the the apical cell surface and induces apoptosis in the distal wing disc epithelium.

All panels show third instar wing imaginal discs with posterior side to the left. (A- A', B-B', E-E'; F-F', I-I', J-J') standard confocal sections of (A- A', B-B', E-E'; F- F') the apical or (I-I', J-J') basal cells surfaces with a dorsal side up. (C-C', D-D', G-G', H- H') optical cross sections through the distal wing discs epithelium. All the panels shows *hh*-Gal4 driving (A-A', C-C', E-E', G-G', I-I') UAS-*mCD8:GFP* or (B-B', D-D', F-F', H-H', J-J') UAS-*mCD8-GFP* with UAS-*cpb-IR*^{C10} (green in A to H). Discs are stained with (A-D, A'-D') anti-DE-cad (red) or (E-E', H'- H') anti-Arm or (I-I', J-J') anti-activated C 3 (red).

These discs showed uniform honey comb-like pattern of DE-cad (Fig.2.1. A-A') and Arm (Fig.2.1. E-E') at the apical cell surface, uniformly distributed between the anterior and posterior compartment. Moreover, optical cross sections through the epithelial tissues did not reveal differences in the levels of DE-cad and Arm between the anterior and posterior GFP-expressing compartments. Instead DE-cad and Arm was misslocalized to baso-lateral positions (Fig.2.1. C-C' or G-G' respectively). Interestingly, in the domain depleted of Cpa (Fig.2.1. B-B'), there was the appearance of cells accumulating brighter GFP, resembling apoptotic cells. To investigate if indeed the reduction of DE-Cad and Arm levels at AJs is associated to apoptosis, I tested the presence of activated Caspase 3 (C3). As expected, *hh>GFP* control discs did not display activated C3-positive cells, (Fig.2.1. I-I'). In contrast, when Cpa was depleted in the posterior wing compartment, many cells localized basally in the distal domain and expressed activated C3 (Fig.2.1. J-J'). Therefore, in tissue knocked down for Cpa, reduced levels of Arm and DE-cad at AJs are associated to basal cell extrusion and apoptosis. However, many Cpa-depleted cells that show decreased levels of Arm and DE-cad at AJs are still maintained within the epithelium (arrows in Fig.2.1. D-D' and H-H'). This suggests that in these cells, the levels of Arm and DE-cad at AJs are sufficient to maintain cell polarity and prevent cell extrusion and apoptosis.

I then investigated whether knocking down CP affects the localization of SJs. Cora has been reported as a core structural SJs components in *Drosophila*, whereas Dlg establishes the basolateral domain (Oshima and Fehon, 2011). Standard confocal sections of the apical surface of *hh>GFP* control tissues, showed that Cora (Fig.2.2. A-A') and Dlg (Fig.2.2. E-E') were expressed homogenously in the anterior and posterior GFP-expressing compartments. Optical cross sections through the wing disc epithelium revealed that Cora (Fig.2.2. C-C') and Dlg (Fig.2.2. G-G') were mainly localized apically and homogenously distributed along the anterior-posterior axis. Depletion of Cpa in *hh>GFP* tissues did not affect Cora (Fig.2.2. B-B' and D-D') or Dlg (Fig.2.2. F-F' and H-H') localization and protein levels. These results are in agreement with the behavior of *cpa*

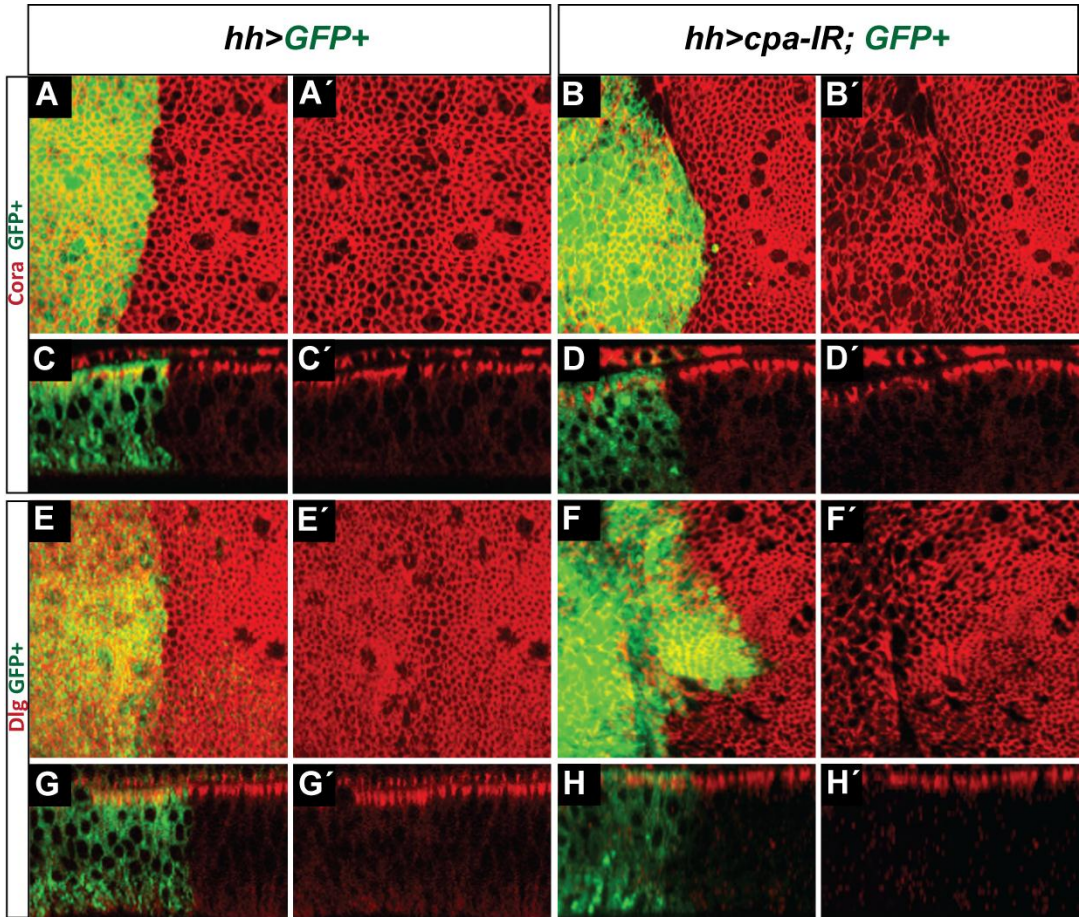


Figure 2.2 Septate Junctions components are not affected upon Capping Protein depletion in the distal wing disc epithelium.

All panels show third instar wing imaginal disc with posterior side to the left. (A-A', B-B', E-E' and F-F') standard confocal sections of the apical cells surfaces with dorsal side up. (C-C', D-D', G-G' and H-H') optical cross sections through the distal wing epithelium with apical side up. (A-A', C-C', E-E' and G-G') *hh*-Gal4 driving UAS-*mCD8:GFP* (green in A, C, E and G). (B-B', D-D', F-F' and H-H') *hh*-Gal4 driving UAS-*mCD8:GFP* (green in B, D, F and H) with UAS-*cpa-IR*^{C10}. Discs are stained with (A-A' to D-D') anti- Cor (red) or (E-E' to H-H') anti- Dlg (red).

mutant cells, which misslocalize Arm and DE-cad at lateral positions without affecting Dlg localization (Janody and Treisman, 2006). Taken together, these data demonstrate that like mutations for null alleles of *cpa* or *cpb*, reducing Cpa levels using RNAi disrupts the apical localization of AJs components and triggers apoptotic cell death. However, while *cpa* mutant cells extrude from the distal wing disc tissue and cannot be recovered

72h after their induction, CP depleted tissues, in which CP function is not completely abolished, can maintain a polarized columnar shape and residual Arm and DE-cad at AJs. Only some cells extrude and undergo apoptosis.

4.2. CAPPING PROTEIN PREVENTS APICAL ACTIN FILAMENTS ACCUMULATION AND DOES NOT SEEM TO AFFECT THE F/G ACTIN RATIO

Although knocking down Cpa affects the localization of the polarity determinants Arm and DE-cad, many depleted cells were still maintained within the epithelium (Fig.2.1). If, in the distal domain, accumulation of F-actin throughout the cortex of *cpa* mutant cells is a consequence of cell polarity loss, I expect that cells depleted of Cpa, that still maintained a polarized architecture, will not accumulate F-actin around the entire cell cortex but will display excess apical F-actin. I therefore investigated the subcellular localization of F-actin in Cpa knocked down wing disc by Phalloidin staining, which marks specifically polymerized actin. Similar to *cpa* mutant cells localized in the proximal wing domain (Janody and Treisman 2006), proximal wing disc tissue expressing UAS-*cpa-IR* and UAS-*mCD8-GFP* under *hh*-Gal4 control showed strong apical F-actin accumulation (Fig.2.3. A-A'). In distal wing disc tissues, cells that are maintained within the epithelium also exhibited strong apical ectopic F-actin (Fig.2.3. B-B'). This suggests that one main function of CP is to prevent apical F-actin accumulation in the whole wing disc epithelium. However, because loss of *cpa* disrupts the apical-basal polarity of distal wing disc cells, excess F-actin in basolateral regions of *cpa* mutant cells is likely a consequence of polarity loss.

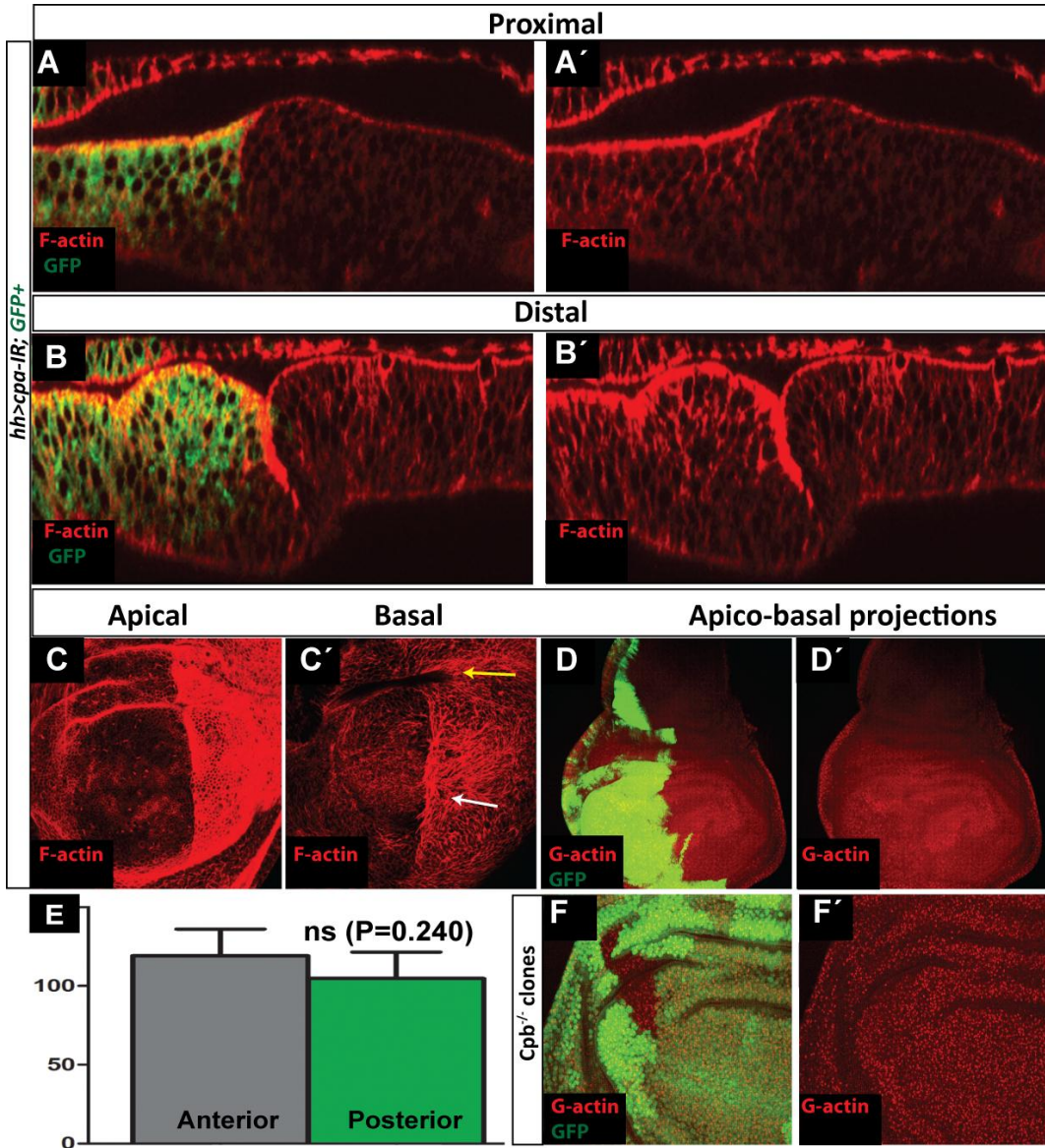


Figure 2.3 Capping Protein depletion leads to apical actin accumulation in a whole wing imaginal disc and does not seem to affect pool of G-actin.

All panels beside (E) show third instar wing imaginal discs with a posterior side to the left. (A-A' to D-D') *hh*-Gal4 driving UAS-CD8:GFP with UAS-*cpa-IR*^{C10}. (F-F') *hs*-FRT induced FRT40A,*cpb*^{M143} clones marked by absence of GFP. (A-A' and B-B') Optical cross sections through (A-A') proximal or (B-B') distal domains of wing imaginal discs. (C-C' and D-D') standard confocal sections of (C) apical or (C') basal cells surfaces. The white or yellow arrows indicate proximal or distal wing disc domains respectively. (D-D' and F-F') The projections of standard confocal sections through the whole wing discs. Discs are stained with (A-A' to C-C') TRITC- Phalloidin to mark F-actin (red) or

(D-D') anti-G-actin (red) or (F-F') TRITC-DNAse1 to mark G-actin (red). Mean intensity of TRITC-DNAse1 fluorescence signal in the posterior was 118 and the anterior compartments was 137 of wing imaginal discs expressing *hh>UAS-CD8:GFP* with *UAS-cpa-IR^{C10}* (n=9).

Interestingly, in addition to prevent apical F-actin accumulation, knocking down Cpa also increased F-actin populations of cortical (Fig.2.3. A-A' and B-B') and basal (Fig.2.3. C') F-actin. Excess basal F-actin appeared to form arrays that resemble actin stress fibers present in both proximal (yellow arrow in Fig.2.3. C') and distal wing disc domains (white arrow in Fig.2.3.C'). However, cortical and basal F-actin accumulation of Cpa-deleted tissues did not appear as drastic as the concentration of F-actin observed at the apical cell membrane. These observations suggest that while CP prevents F-actin accumulation at multiple locations within the cell, its main function might be to inhibit ectopic F-actin formation at the apical cell membrane.

The molecular role of CP is to restrict actin filaments elongation by preventing the addition of new actin monomers (G-actin) to the filament (reviewed in Cooper and Sept, 2008). Therefore, upon CP loss, more G-actin molecules are incorporated in filaments. Thus, it is expected that following CP loss, while the pool of F-actin increases, the pool of G-actin decreases. Monomeric actin has been demonstrated to regulate signaling events. For instance, G-actin has been shown to prevent nuclear translocation of the co-transcription factor MAL, which, together with Serum Response Factor (SRF), induces transcription of many genes responsible for actin dynamics and also for cell survival and proliferation (Vartiainen et al. 2007). Therefore, I considered the possibility that the CP knocked down phenotypes result from an increase in the F-actin pool and/or from a decrease in the G-actin pool. Cell extrusion and death of *cpa* mutant cells is unlikely to result from an increase of MAL nuclear transport as clones double mutant for *cpa* and *blistered (bs)*, which encodes for the *Drosophila* SRF were still extruded from the wing epithelium (Janody and Treisman, 2006). However the G-actin pool could be critical to regulate additional signaling events that might contribute to the outcome of cell losing CP. To explore this possibility, I compared the levels of G-actin between wildtype and

Cpa-depleted discs, using DNase1 conjugated with Alexa Fluor® 594, which specifically labels G-actin (Mannherz et al. 1980). Posterior wing disc cells expressing *cpa-IR* and *mCD8-GFP* seemed to show a weak decrease in DNase1 staining compared to the wildtype anterior compartment (Fig.2.3.D-D'). To quantify the levels of G-actin in Cpa-depleted tissues, I measured the mean intensity of DNase1 fluorescent signal in the Cpa-depleted posterior compartment and in the wildtype anterior compartment. Although posterior cells knocked down for Cpa showed a weak decreased of DNase1 intensity signals compared to anterior wildtype cells, this difference was not statistically significant (n=9, with a p value of 0.240) (Fig.2.3. E). This suggests that the pool of G-actin might not be significantly affected upon CP depletion. To further analyzed this issue, I investigated the level of G-actin using an antibody that has been reported to specifically recognize G-actin in *Drosophila* tissue (Grusche et al., 2009). In *cpb*^{M143} mutant clones induced in wing imaginal disc and marked by the absence of GFP, I could not observe differences in DNase1 intensity signal between mutant clones and the surrounding tissue (Fig.2.3. F-F'). These results suggest that monomeric actin might not be significantly affected upon CP depletion. However, since actin is the most highly expressed protein in cells (Pollard et al., 2000), upon CP depletion, a decrease in the G-actin pool might be undetectable by immunofluorescence.

4.3. THE APICAL LOCALIZATION OF CAPPING PROTEIN DEPENDS ON ITS ABILITY TO BIND TO ACTIN FILAMENT

So far I showed that loss of CP affects preferentially apical actin pools and affects the apical localization of AJs components Arm and DE-cad. Since the major molecular role of CP is to restrict actin filaments elongation (Kim et al., 2004), it is highly possible that loss of AJs components is a direct consequence of accumulation of apical actin populations. Alternatively, CP may regulate Arm and DE-cad localization independently of its role on F-actin since CP is also thought to stabilize the barbed end of the Arp1 microfilament in the Dynactin complex, which is required for vesicular transport along microtubules

(Schafer et al., 1994). To distinguish between these possibilities, I aimed to analyze the role of the Actin-Binding Domain (ABD) of Cpa in maintaining Arm and DE-cad localization. To do so, I generated transgenic flies carrying an HA-tagged form of Cpa full length (UAS-*HA-cpa*^{WT}) and an HA-tagged form of Cpa lacking the ABD (UAS-*HA-cpa*^{ΔABD}), under the control of the UAS regulatory sequences. To ensure the same expression level of both constructs, I used the attB/attP system that enables direct integration into the *Drosophila* genome, permitting therefore to express both constructs at the same levels. I first analyzed the localization of both constructs by targeting their expression with the *scalloped*-Gal4 (*sd*-Gal4) driver, which directs expression in the whole distal wing tissue (Fig.2.4.). Cross sections through the distal wing disc epithelium showed that HA-Cpa^{WT} was targeted to the apical cell membrane and co-localized with DE-cad (Fig.2.4. B-B'). Moreover, in agreement with a role of CP in restricting cortical and basal F-actin accumulation (Fig.2.3.), HA-Cpa^{WT} could also be detected along the apico-basal axis (yellow arrow in Fig.2.4.B'). Interestingly, standard confocal section of the apical cell surface showed that while DE-cad localized around the cell edge in an honey-comb pattern, *HA-Cpa*^{WT} was not restricted around the cell edge but spread on the whole apical surface (Fig.2.4. A-A').

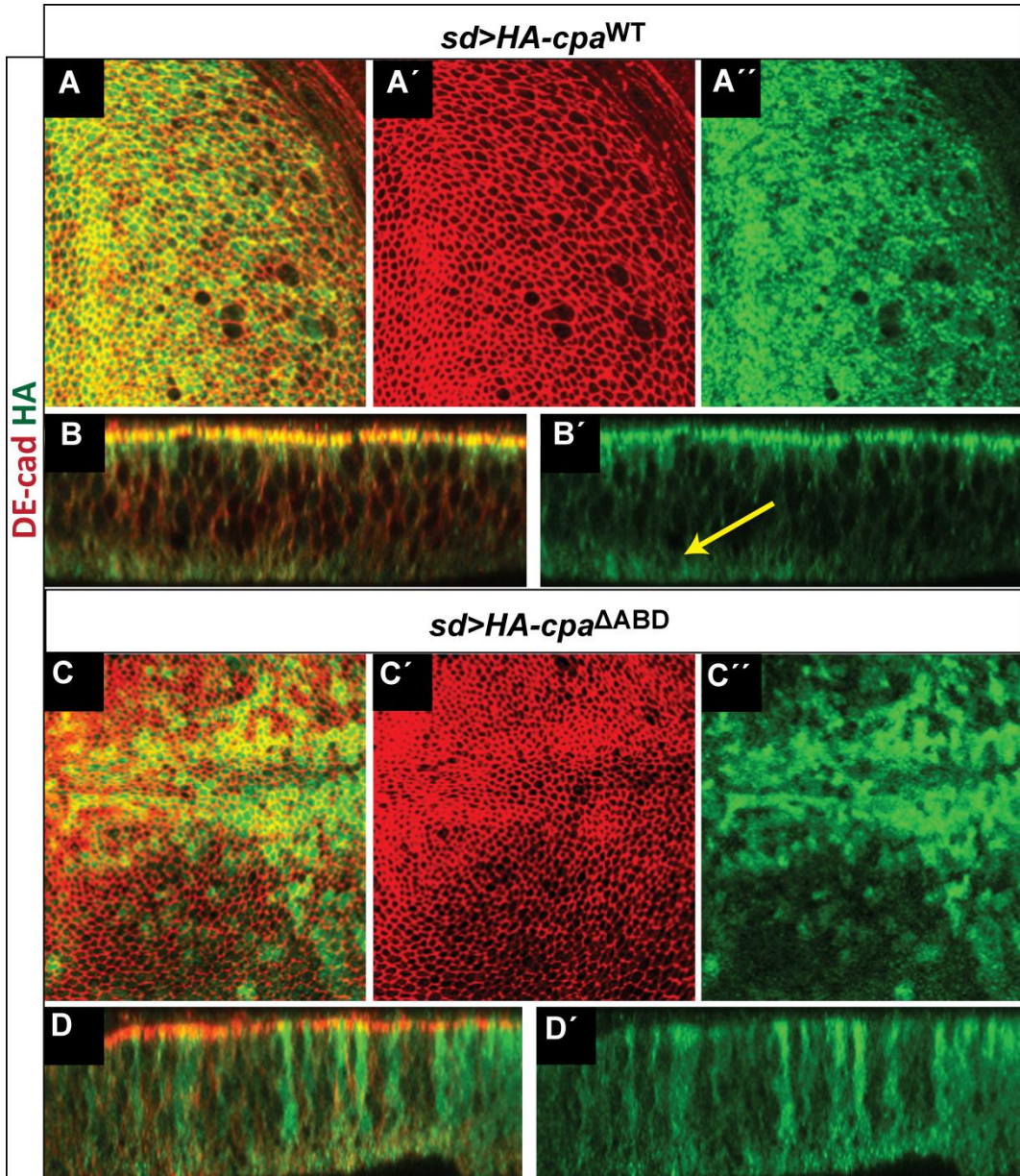


Figure 2.4 The apical localization of Capping Protein α depends on the presence of the Actin Binding Domain.

All panels show third instar wing imaginal discs. (A-A'' and C-C'') standard confocal sections of apical cells surfaces. (B-B' and D-D') optical cross sections through the wing disc epithelium with apical side up, yellow arrow in (B') indicates basal fraction of UAS-HA-*cpa*^{WT}. (A-A'' and B-B') *sd*-Gal4 driving UAS-HA-*cpa*^{WT}. (C-C' and D-D'') *sd*-Gal4 driving UAS-HA-*cpa*^{ΔABD}. Discs are stained with anti-dE-Cad (red) and anti-HA (green).

In contrast, *HA-Cpa*^{ΔABD} expressed under *sd-Gal4* control did not concentrate at the apical cell membrane and weakly co-localized with DE-cad but spread along the entire apico-basal axis of the epithelium (Fig.2.4. C-C'' and D-D''). These results argue that Cpa has a main function on F-actin at apical sites and indicates that apical localization of Cpa depends on its ABD.

Because of time constraints, I did not analyze the ability of *HA-cpa*^{ΔABD} to rescue the defects in Arm and DE-Cad localization in tissues mutant for *cpa*. However I do not expect that this form of Cpa will restore Arm and DE-Cad localization, since the loss of the ABD impedes its apical localization. Taken together, these results argue that apical localization of CP depends on its ability to bind to F-actin. Moreover, it suggests that CP role in maintaining AJs could be due to its function in restriction of apical F-actin population.

4.4. CAPPING PROTEIN PREVENTS THE ACCUMULATION OF THREE DISTINCT POOLS OF APICAL ACTIN FILAMENTS

The defects of CP loss on Arm and DE-cad localization may result from alteration of the small F-actin patches adjacent to AJs or circumferential F-actin pool that immobilizes DE-cad within AJs (Cavey et al., 2008). To investigate which F-actin pool was affected as a result of CP loss, I compared at higher magnification the apical F-actin network in cells depleted of Cpa and positively marked with GFP in the posterior wing disc compartment to cells of the wildtype anterior compartment (Fig.2.5).

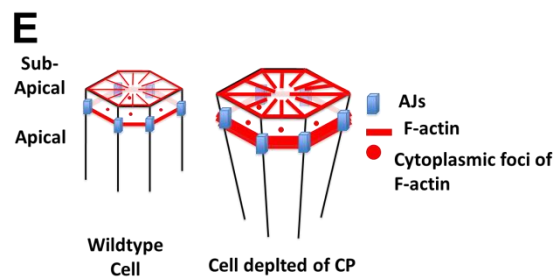
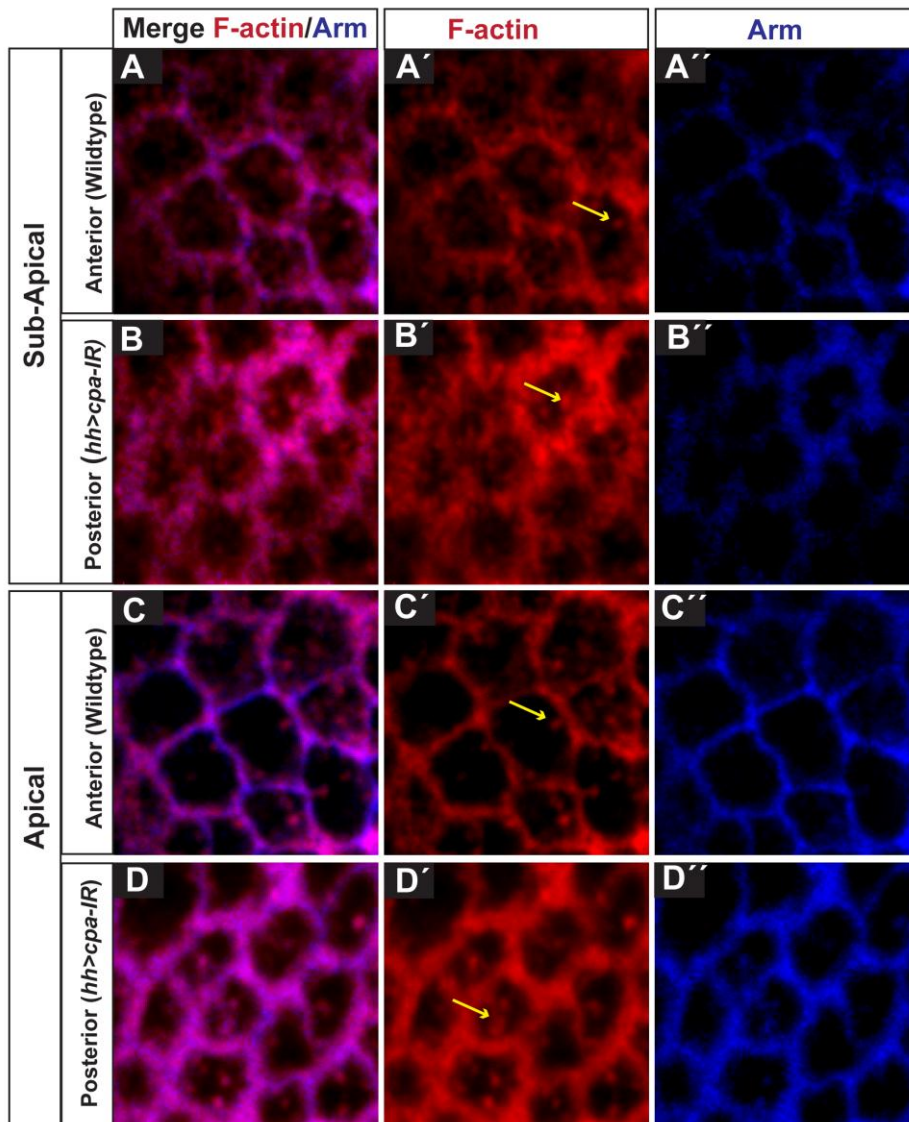


Figure 2.5 Capping Protein depletion affects three distinct apical F-actin populations.

All panels show standard confocal sections of (A-A'' to B-B'') sub-apical or (C-C'' to D-D'') apical domains of wing imaginal discs. (A-A'', B-B'', C-C', D-D'') *hh-Gal4> UAS-mCD8:GFP* with *UAS-cpa-IR^{C10}*. (A-A'' and C-C'') anterior (WT) or (B-B'' and D-D'') posterior (*cpa-IR^{C10}* expressing tissue) sides of wing imaginal discs. The discs are stained with (A to D and A'-D') TRITC-Phalloidin (red) or (A to D and A'' to D'') Arm (blue). (E) Schematic representation of epithelial cells of wing imaginal disc (The baso-lateral view). Wildtype cell (on the left) or cell depleted of CP (on the right). The blue squares represents AJs, red lines represents F-actin.

In the control compartment, high magnification of the apical F-actin network revealed three pools of F-actin, formed at distinct focal planes. In the apical domain, F-actin cables organized parallel to the cellular membrane (referred as the circumferential F-actin cable) strongly co-localized with the AJs component Arm (Fig.2.5. C-C'' and schematic in E.), More apically, a meshwork of F-actin fibers running along the entire apical cell surface (referred as the sub-apical F-actin meshwork) could be observed (Fig.2.5. A-A' and schematic in 2.6.), In addition, F-actin puncta could be detected that localize within the cytoplasm at the apical and sub-apical confocal planes (yellow arrows in A' and C'). However, I was unable to distinguish the stable, small F-actin patches concentrated at AJs from the circumferential F-actin pool described by Thomas Lecuit's Laboratory (Georgiou et al., 2008). In the posterior compartment knocked down for Cpa, all three pools could be observed: the circumferential F-actin belt appeared much broader (compare D-D' to C-C' in Fig.2.5. and schematic in E.), F-actin puncta showed more intense Phalloidin staining (yellow arrow in Fig.2.5. B' and D' and schematic in E.), and the sub-apical F-actin meshwork was expanded (compare B-B' to A-A' in Fig.2.5.). These results argue that loss of CP affects all three pools of apical F-actin, including the circumferential F-actin cable, which co-localizes with AJs components. These observations are in agreement with the sub-cellular localization of HA-Cpa^{WT}, which not only co-localized with DE-cad, but also marks the whole apical cell surface (Fig.2.4. A-A').

4.5. CAPPING PROTEIN MAY INDIRECTLY CONTRIBUTE TO FOCAL ADHESIONS ORGANIZATION

Integrins can influence AJs stability either by promoting cell contractility at cell–cell contacts, disrupting in this way cadherin-mediated adhesions, or by inducing signalling cascades, that directly modulate AJs stability (reviewed in Weber et al., 2011). Because depletion of CP induced accumulation of an F-actin arrays localized basally (Fig.2.3.C') and that HA-*cpa*^{WT} also localized on the basal surface of the wing disc epithelium (arrow in Fig.2.4. B'), I investigate the possibility that the defects in Arm and DE-cad localization of CP-depleted tissue result from a dysfunction of FAs. If this is the case, I expect that FAs would also be affected upon CP depletion. The core components of FAs are Integrins, forming an α/β heterodimer of two large transmembrane proteins. In *Drosophila*, Integrins were named PS, from Position Specific. The genome contain one β subunit (β_{PS}) and two α subunits (α_{PS1} and α_{PS2}). While β_{PS} is expressed in the whole wing imaginal disc, α_{PS1} is expressed only in the dorsal compartment, while α_{PS2} only in the ventral compartment (Walsh and Brown, 1998). Mutations in any of the PS subunits are lethal, but when introduced in patches of cells in wing discs trigger blisters in the adult wings. Moreover, mutations in any cytoplasmic component of FAs also result in blister formation (Walsh and Brown, 1998). To investigate whether loss of CP affect FAs, I examined first if loss of CP induces blistering in the adult wings. Because mutant clones for either *cpa* or *cpb* extrude from the distal wing disc and are eliminated from the tissue 72h after clone induction (Janody and Treisman, 2006), I could not analyze their phenotype at adult stage. I therefore induced clones expressing *cpa-IR* at early larval stages and analyzed the presence of wing blistering in the adult. Blisters at a frequency of 1 out of 9 wings with a ratio of 2 blisters per wing were recovered in the adult wings (Fig.2.6.A). Moreover, like tissues expressing a chimeric form of β_{PS} subunit, that act as dominant negative form of PS Integrins (Domínguez-Giménez et al., 2007), Knocking down Cpa in the whole distal wing disc, by driving UAS-*cpa-IR* under *scalloped*-Gal4 (*sd*-Gal4) or *nubbin*-Gal4 (*nub*-Gal4) control, induced the formation of ectopic folds in

the epithelium (white arrows in Fig.2.6. B and B'). This suggests that loss of CP affects FAs function.

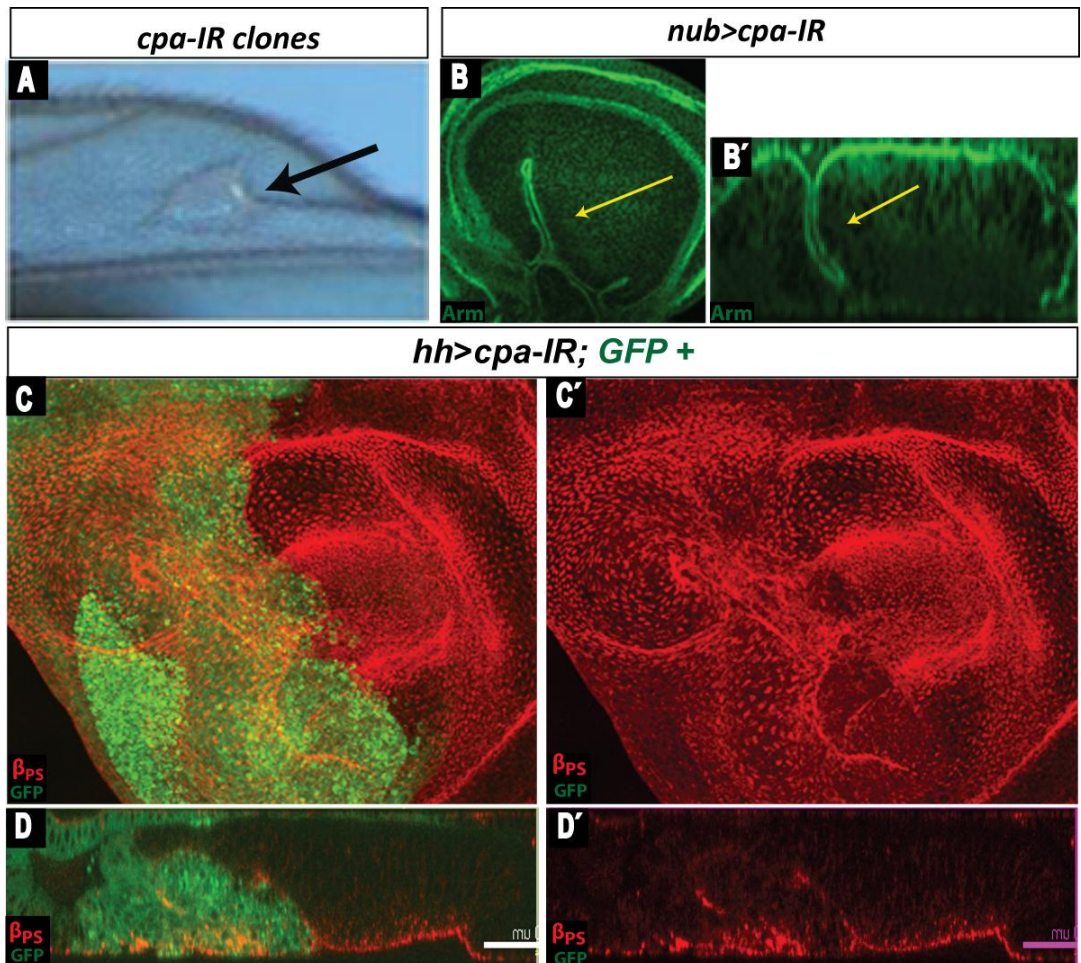


Figure 2.6 Capping Protein depletion recapitulates phenotypes of Integrins loss but do not affect β_{ps} Integrin localization in the wing imaginal disc.

(A) Adult wing containing *hs-FRT* induced *FRT40A*, *UAS-cpa-IR*^{C10} clones. (B-B', C-C' and D') third instar wing imaginal discs. (B- B') *nub>UAS-cpa-IR*^{C10} (C-C' and D-D') *hh>UAS-cpa-IR*^{C10} and *UAS-mCD8:GFP* (green in C and D). (B) Standard confocal section of of baso-lateral cell surface. (B'and D-D') cross section through the wing disc epithelium, yellow arrow in B and B' indicate ectopic fold in a distal domain. (C-C') Standard confocal sections of the basal cell surface of the distal wing disc domain. Discs are stained with (B-B') anti- Arm (green) and (C-C' and D- D') anti- β_{ps} Integrin (red).

I then investigated whether the integrity of FAs was affected following CP loss. To do so, I analyzed the expression of β_{ps} Integrin in posterior wing disc cells Cpa-

depleted versus cells of the anterior wildtype compartment by driving *UAS-cpa-IR* and *UAS-mCD8:GFP* with *hh-Gal4*. Standard confocal sections showed that in the wildtype anterior compartment, β_{PS} Integrin localized at the basal membrane, forming dot-like foci (Fig.2.6. C-C' and D-D'). In the Cpa-depleted, GFP positive posterior compartment, β_{PS} still localized in dot-like foci (Fig.2.6. C-C' and D-D'). Optical cross sections through the wing disc epithelium showed that although Cpa-depleted tissues formed folds, PS Integrin was still localized on the basal surface with no obvious differences in its levels (Fig.2.6. D-D'). These results suggest that although CP might stabilized FAs, it does not do so by promoting Integrins localization.

5. DISCUSSION AND CONCLUSIONS

In this chapter I have investigated the possibility that the differential behavior of cells that lack CP in the distal versus proximal wing disc epithelia results from different F-actin populations being affected. Divers observations argue that CP regulates identical F-actin networks in the distal and proximal wing disc domains. First, cells in which CP levels are reduced using RNAi, mislocalizes the AJs components Arm and DE-Cad only in the distal wing disc domain but accumulate apical F-actin regardless of the region where CP is knocked down (Fig.2.3.). Second, an HA- tagged form of Cpa is enriched apically, in both the distal and proximal wing disc domains (Fig.2.5.). Therefore, while CP affects identical F-actin populations in the whole wing disc, only cells located in the distal domain mislocalize Arm and DE-Cad, extrude and die. This argues that in wing disc's cells located in the distal domain, AJs are more sensitive to F-actin disruption.

However, which F-actin network regulated by CP stabilizes AJs in the distal wing disc epithelium? Different populations of actin filaments are affected upon loss of CP: at apical site, loss of CP triggers the formation of a wide circumferential F-actin cable, a dense sub-apical F-actin meshwork and ectopic F-actin foci. Moreover, I also observed a dense F-actin network formed at the basal cell membrane in the distal and proximal wing disc domains (Fig.4.3.). Because in cells in culture the functions and organization of

FAs can affect AJs stability (reviewed in Klusza and Deng, 2011), in the distal wing domain, F-actin disruption at basal sites could possibly affect FAs signalling in this domain and triggers loss of AJs integrity. However, the localization of Integrins, the main component of FAs, did not appear affected upon CP depletion (Fig.2.6.). I do not neither favor a model in which loss of AJs stability results from a reduction of the pool of G-actin since in the distal wing disc epithelium, I did not observe a major reduction in the levels of G-actin in cells knocked down for CP (Fig.2.3.). Moreover, removing blistered the SRF transcription factor, which affects cellular adhesion in mammals (Ragu et al., 2010; Holtz and Misra, 2011) do not rescue extrusion of *cpa* mutant cells (Janody and Treisman, 2006). In distal wing disc cells, CP may have additional functions, such as stabilizing the barbed end of Arp1 microfilaments of the Dynein/Dynactin complex that is required for vesicle transport along microtubules (Schafer et al., 1994). However, double mutant cells for *cpa* and kinesin heavy chain (*khc* K13314) that counteracts Dynein/Dynactin-based transport, are still extruded from the tissue (Janody and Treisman, 2006).

Instead, I favor a model in which distal wing disc cells are more sensitive to apical F-actin accumulation following CP loss. Diverse apical F-actin populations appear to be controlled by CP. In agreement with a role of CP in regulating the organization of a sub-apical F-actin meshwork (Fig.2.4.), an HA-tagged form of Cpa localizes in a pattern that covers the whole apical cell surface (Fig.2.5.). Disruption of this F-actin meshwork could affect the localization of the sub-apical complexes, which maintain AJs (reviewed in Laprise and Tepass, 2011). Consistent with this possibility, the sub-apical complexes have been shown to be linked to prominent F-actin cables (reviewed in Matter and Balda, 2003). Moreover, CP depletion results in the mislocalization of α PKC, a member of the sub-apical complex (Cláudia Mendes, Master Thesis). Alternatively, disruption of F-actin patches by CP loss, localized in the cell cytoplasm (Fig.2.4.), may affect endocytosis and recycling of AJs components since in yeast, CP regulates the early endocytic phases (Kim et al., 2004). Finally, CP also affects a circumferential F-actin cable, apposed to AJs. (Fig.2.4.). CP could be involved in organizing the small F-actin patches, proposed to

stabilizes homophilic DE-cad (homo DE-cad) interactions and/or the contractile F-actin network that immobilizes homo DE-cad within the plane of AJs (Cavey et al., 2008). In agreement with this possibility, in mammalian model, depletion of the ABP Elpin, that links AJs components to apical F-actin, results in excessive accumulation of apical F-actin and misslocalization of AJs components (Abe and Takeichi, 2008). Moreover, the Arp2/3 complex, which cooperates with CP to nucleate branched F-actin (Cooper and Sept, 2008), is involved in the organization of apical F-actin that recycles DE-cad, contributing in this way to AJs stability (Georgiou et al., 2008). Thus, it is possible that CP together with Arp2/3 control the apical F-actin network involved in DE-cad recycling. The role of CP might also be to link F-actin to cellular membranes (Hutchings et al., 2003; Schafer et al., 1995; Schafer et al., 1998), permitting in this way the formation of a contractile F-actin network that maintain AJs components at apical sites. However, in CP-depleted cells, the F-actin cable was still apposed to the cell membrane. Moreover, deletion of the Cpa Actin Binding Domain (ABD) results in the loss of the apical localization of Cpa (Fig.2.5.), indicating that Cpa localization is not dependent of the apical cell membrane but dependent of its binding with F-actin. Instead, a tight regulation of the balance between polymerization and depolymerization of an F-actin population linked to AJs might be critical to maintain the proper organization of AJs components. Taken together, my results argue that in the distal wing disc domain CP maintains AJs organization, through regulation of apical F-actin.

However, why, following CP loss, AJs stability would be more sensitive to apical F-actin accumulation in distal wing disc cells? These cells are from a different identity compared to proximal wing disc cells, and may express molecules that upon disruption of apical F-actin destabilize AJs. Consistent with this possibility, expressing the wing specification gene *vestigial* (*vg*), whose endogenous expression is restricted to the distal wing disc domain (reviewed in de Celis, 1999), triggers extrusion of *cpa* mutant cells in the proximal domain (Janody and Treisman, 2006). Therefore, *Vg* induces the expression of molecules that modulate the outcome of cells lacking CP. Although I cannot

completely exclude that the presence of Vg affects CP activity or the activity of molecules that regulate the organization of apical F-actin, my observations suggests that Vg modulates the expression of AJs components, or components that control AJs remodeling. Alternatively, differences in external mechanical stimuli between the distal and the proximal wing disc domains may affect the outcome of cells lacking CP. In agreement with this possibility, cells in the distal domain are submitted to the higher mechanical stress than in the proximal domain (Major and Irvine, 2005; Landsberg et al., 2009; Farhadifar et al., 2007; Classen et al., 2005; Canela-Xandri et al., 2011). Therefore, the apical F-actin network formed upon CP loss may destabilize AJs only when AJs are mechanically more challenged. If so, Vg might induce the expression of molecules that increase intracellular forces, counteracting in this way higher mechanical stresses in the distal wing domain. If so, cell extrusion may result from increase cell contraction of the acto-myosin network due to the presence of Vg and the loss of CP. Indeed, contraction of a dense actin and myosin ring is required for apoptotic cell extrusion (Slattum et al., 2009)

All together, my observations are in agreement with a model in which CP does not have a tissue-specific effect on F-actin but regulates three distinct apical and sub-apical F-actin networks in the whole wing imaginal disc that maintain AJs components of cells located in the distal wing disc domain. Following CP loss these F-actin pools might not be properly organized resulting in the destabilization of AJs. This, associated with other factors, may ultimately lead to loss of polarity and cell extrusion.

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CHAPTER 3- A DUAL FUNCTION OF DROSOPHILA CAPPING
PROTEIN ON DE-CADHERIN MAINTAINS EPITHELIAL INTEGRITY
AND PREVENTS JNK-MEDIATED APOPTOSIS

The following chapter includes data that were published in *Developmental Biology*. Briefly we have reported the phenotypic outcomes of Capping Protein (CP) depletion in the most distal wing disc domain. We found that knocking down CP has a dual effect on DE-cadherin: first it misslocalizes DE-cad from Adherens Junctions, second it prevents DE-cadherin transcription. DE-cadherin acts as an active signal that lead to JNK-mediated apoptosis in the most distal wing disc domain. Suppression of apoptosis leads to hyperplastic overgrowth, mediated by Yorkie oncogene. We proposed that the transcriptional up-regulation could serve as an early event of tumor suppression the.

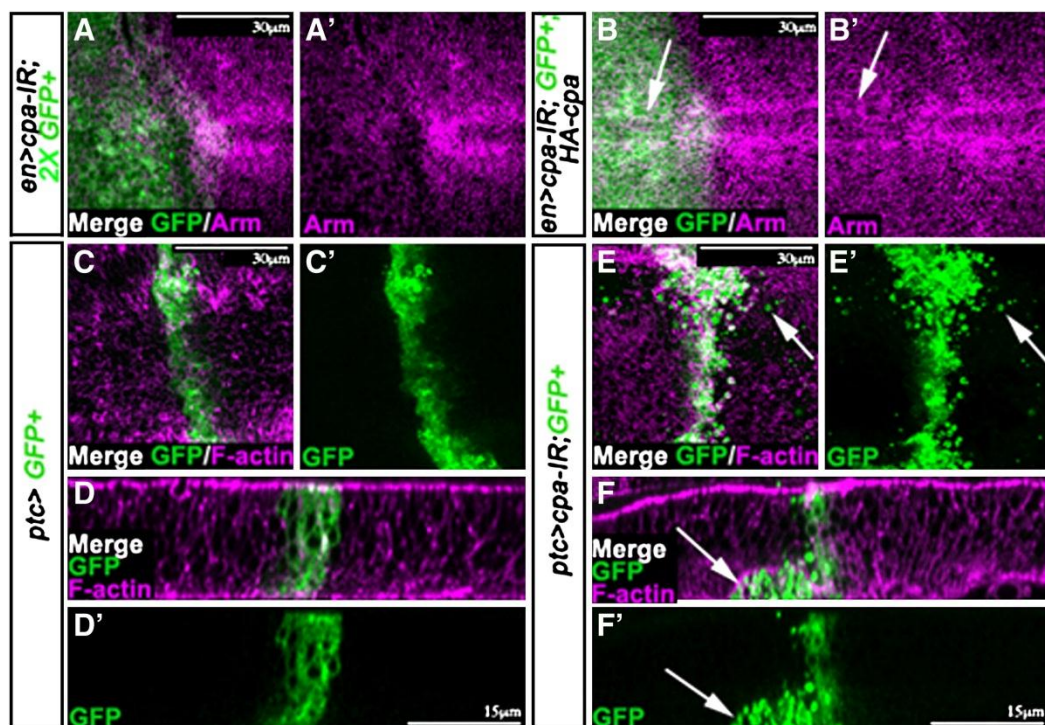
1. AUTHOR CONTRIBUTION

Most of the experiments were planned by me and my supervisor Florence Janody. The following experiments were performed by the co-authors:

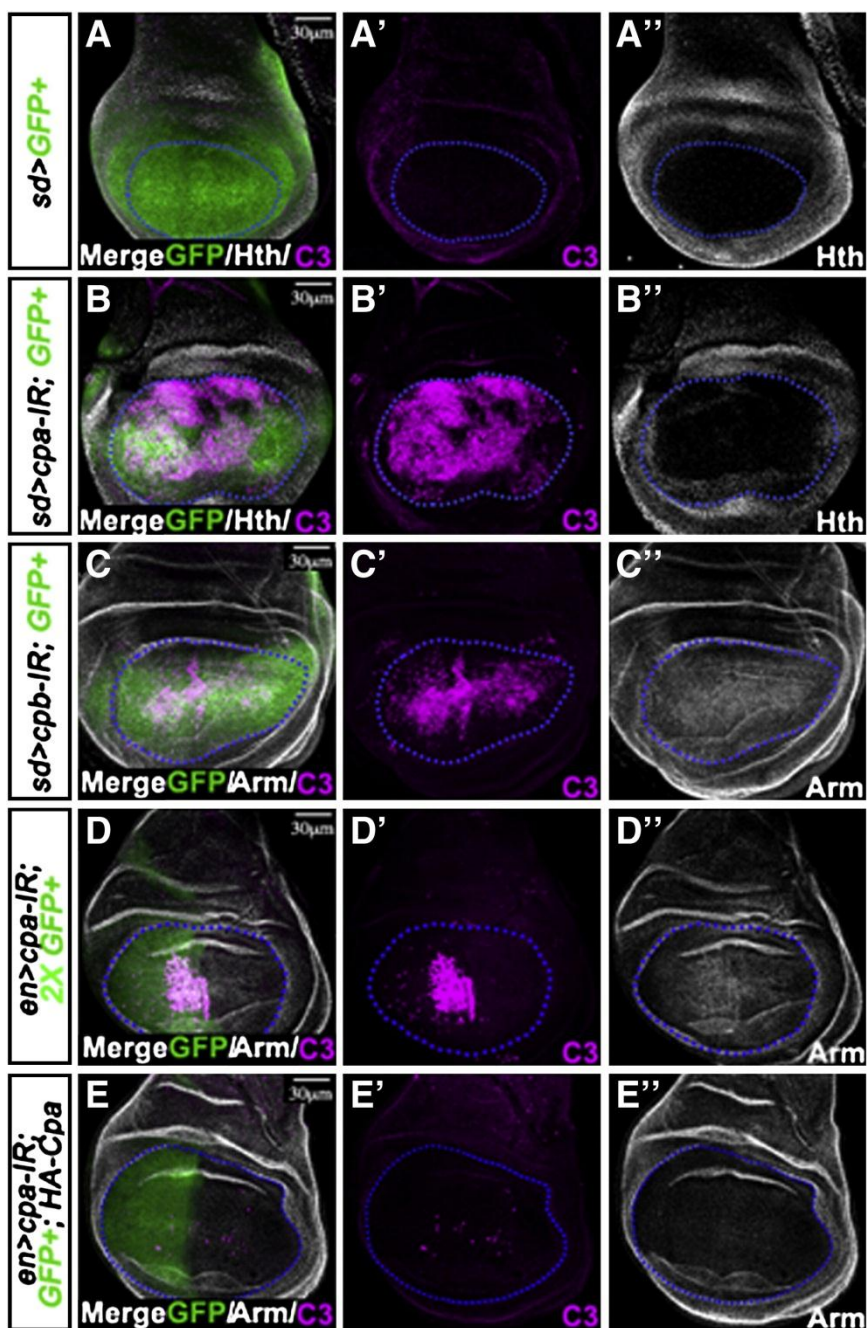
The immunostainings (anti-N-Cad, Phall, anti-MMP1) in a *sd>UAS-cpa-IR^{C10}*; *p35*, anti-DIAP1 in *hh>UAS-cpa-IR^{C10}*, Phall staining in *ptc> UAS-cpa-IR^{C10}* was done by Beatriz García Fernández. Western blot analysis with anti- DE-cad and anti-Armadillo was performed by Ana Rita Amândio. The immunostaining with anti-DE-cadherin in *hh>cpa-IR^{C10}* was done by Cláudia Mendes. The detection of *shg-lacZ* in *cpa^{107E}* mutant clones was done by Catarina Alexandra Pereira.

**2. A DUAL FUNCTION OF *DROSOPHILA* CAPPING PROTEIN ON DE-CADHERIN
MAINTAINS EPITHELIAL INTEGRITY AND PREVENTS JNK-MEDIATED APOPTOSIS**

3. SUPPLEMENTARY DATA

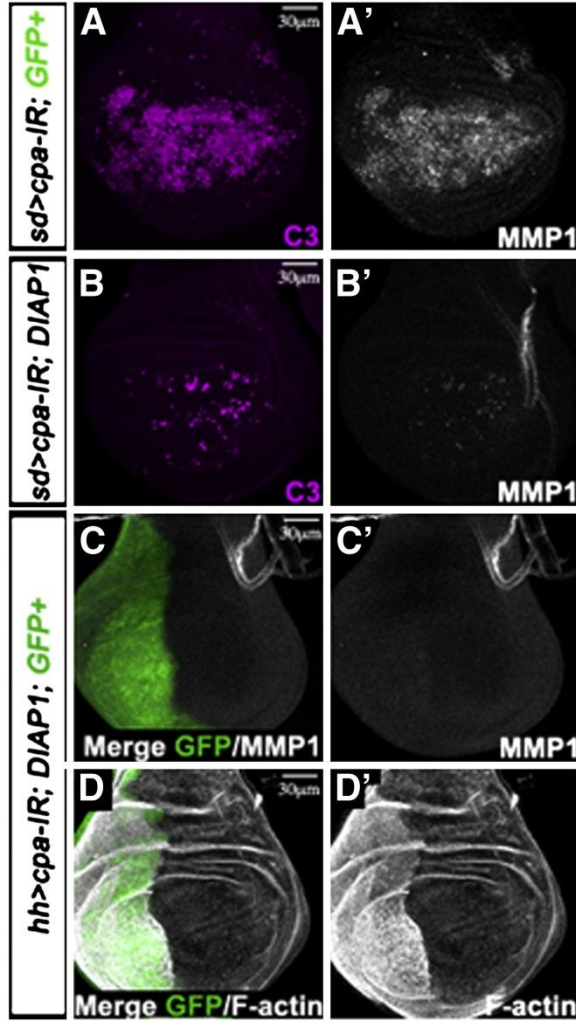


Supplementary Fig. S1. Loss of CP disrupts the localization of Arm at AJs and causes cell extrusion and invasion. All panels show third instar wing imaginal discs with posterior side to the left. (A–A', B–B', C–C' and E–E') standard confocal sections of the (A–A' and B–B') apical or (C–C' and E–E') basal cell surface. (D–D' and F–F') optical cross sections through the distal disc epithelium with apical side up. (A–A') en-Gal4 driving UAS-cpa-IRC10 and two copies of UAS-mCD8-GFP (green in A). (B–B') en-Gal4 driving UAS-cpa-IRC10, one copy of UAS-mCD8-GFP (green in B) and UAS-HA-cpa. Discs are stained with anti-Arm (magenta). The white arrows in B and B' indicate the recovery of Arm staining in Cpa-depleted tissues expressing HA-Cpa. (C–C' and D–D') ptc-Gal4 driving UAS-mCD8-GFP (green). (E–E' and F–F') ptc-Gal4 driving UAS-mCD8-GFP (green) and UAS-cpa-IRC10. Discs are stained with Phalloidin to mark F-actin (magenta in C, D, E and F). The white arrows in E–E' and F–F' indicate Cpa-depleted cells moving away from the ptc expression stripe basally. Note that cells expressing cpa-IR and GFP extrude from the normal epithelium and move away from the ptc expression stripe basally. Scale bars represent 15 or 30 mm as indicated.

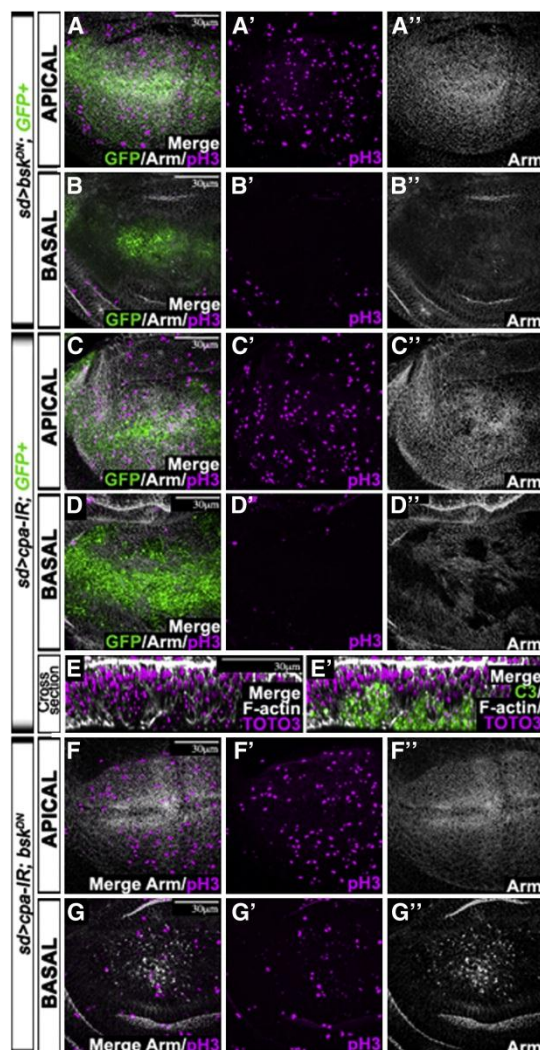


Supplementary Fig. S2. Loss of CP induces massive cell death in the distal wing disc epithelium. All panels show standard confocal sections of third instar wing imaginal discs with posterior side to the left and dorsal side up. (A–A'') *sd*-Gal4 driving UAS-mCD8-GFP (green in A). (B–B'') *sd*-Gal4 driving UAS-mCD8-GFP (green in B) and UAS-*cpa*-IR7009. (C–C'') *sd*-Gal4 driving UAS-mCD8-GFP (green in C) and UAS *cpb*-IR45668. (D–D'') *en*-Gal4 driving UAS-*cpa*-IRC10 and two copies of UAS-

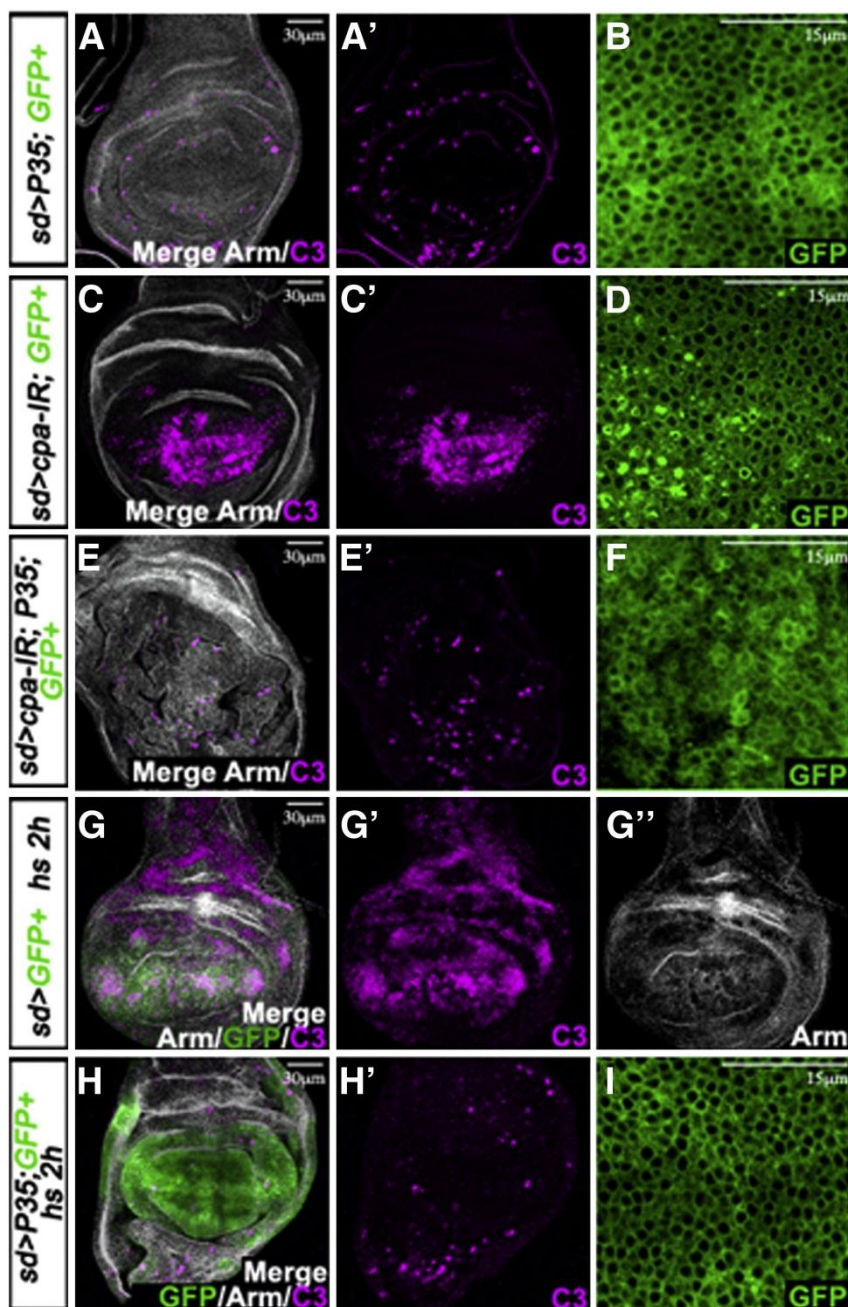
mCD8-GFP (green in D). (E–E'') en-Gal4 driving UAS-cpa-IRC10, one copy of UAS-mCD8-GFP (green in E) and UAS-HA-cpa. Discs are stained with anti-C3 (magenta in A, A', B, B', C, C', D, D', E and E') and anti Hth (white in A, A'', B and B'') or anti-Arm (white in C, C'', D, D'', E and E''). The dashed blue lines outline the distal wing domain. Scale bars represent 30 mm.



Supplementary Fig. S3. DIAP1 overexpression in Cpa-depleted tissues suppresses apoptosis and ectopic MMP1 expression but not F-actin accumulation. All panels show standard confocal sections of third instar wing imaginal discs with posterior side to the left and dorsal side up. (A–A' and B–B') *sd*-Gal4 driving UAS *cpa*-IRC10 and (A–A') UAS-mCD8-GFP or (B–B') UAS-DIAP1. Discs are stained with anti-C3 (magenta in A and B) and anti-MMP1 (white in A' and B'). (C–C' and D–D') *hh*-Gal4 driving UAS-mCD8-GFP (green in C and D), UAS *cpa*-IRC10 and UAS-DIAP1. Discs are stained with (C–C') anti-MMP1 or (D–D') Phalloidin to mark F-actin. Scale bars represent 30 μm.

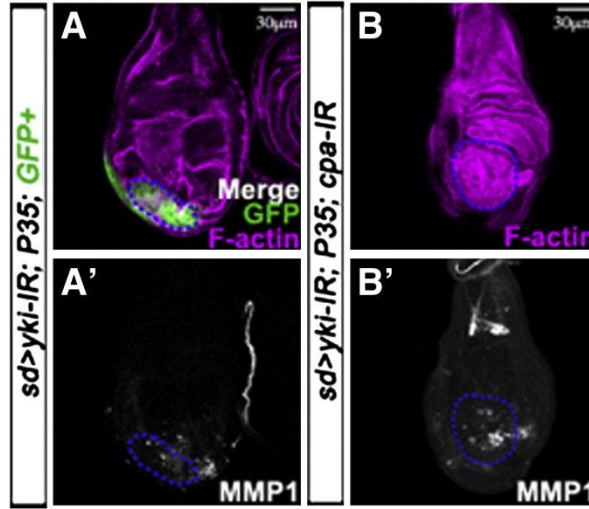


Supplementary Fig. S4. Cpa-depleted cells expressing bskDN proliferate on the basal surface of the distal wing disc epithelium. All panels show third instar wing imaginal discs. (A–A'' to D–D'' and F–F'' to G–G'') standard confocal sections of the (A–A'', C–C'' and F–F'') apical or (B–B'', D–D'' and G–G'') basal surfaces of the epithelium with posterior side to the left and dorsal side up. (E–E') optical cross sections through the distal disc epithelium with apical side up. sd-Gal4 driving (A–A'' and B–B'') UAS-bskDN and UAS-mCD8-GFP (green in A and B) or (C–C'' to E–E'') UAS-cpa-IRC10 and UAS-mCD8-GFP (green in C and D) or (F–F'' and G–G'') UAS-cpa-IRC10 and UAS-bskDN. (A–A'' to D–D'' and F–F'' to G–G'') discs are stained with anti-Arm (white in A, A'', B, B'', C, C'', D, D'', F, F'', G and G'') and anti-pH3 (magenta in A, A', B, B', C, C', D, D', F, F', G and G'). (E–E') disc is stained with anti-C3 (green in E'), Phalloidin to mark F-actin (white) and TOTO3 to mark the cell nuclei (magenta). Scale bars represent 30 mm.



Supplementary Fig. S5. Expressing P35 suppresses apoptosis of Cpa-depleted tissues but does not trigger massive overgrowth of stress-induced apoptotic tissues. All panels show standard confocal sections of third instar wing imaginal discs with posterior side to the left and dorsal side up. *sd*-Gal4 driving (A–A', B, H–H' and I) *UAS*-P35 and *UAS*-mCD8-GFP (green in B, H and I) or (C–C' and D) *UAS*-cpa-IRC10 and *UAS*-mCD8-GFP (green in D) or (E–E' and F) *UAS*-cpa-IRC10, *UAS*-P35 and *UAS*-mCD8-GFP (green in F) or (G–G'') *UAS*-mCD8-GFP (green). (G–G'' to I) discs were heat

shocked for 2 h at 37 °C at first instar larvae. Discs are stained with anti-C3 (magenta) and anti-Arm (White). Scale bars represent 15 and 30 mm as indicated.



Supplementary Fig. S6. Knocking down Yki in “undead” Cpa-depleted cells does not fully suppress ectopic MMP1 expression. All panels show standard confocal sections of third instar wing imaginal discs with dorsal side up. (A–A′) *sd*-Gal4 driving UAS-mCD8-GFP (green in A), UAS-yki-IR4005R-2 and UAS-P35. (B–B′) *sd*-Gal4 driving UAS-yki-IR4005R-2, UAS-P35 and UAS-*cpa*-IRC10. Discs are stained with Phalloidin to mark F-actin (magenta in A and B) and anti-MMP1 (white in A′ and B′). The blue dashed lines outline the distal wing disc domain. Scale bars represent 30 mm.

CHAPTER 4 – CAPPING PROTEIN MEDIATES SIGNALLING
THROUGH THE SRC ONCOGENES IN RESTRICTED *DROSOPHILA*
EPITHELIAL TISSUES

1. SUMMARY

Previously we have demonstrated that knocking down Capping Protein in the distal wing disc epithelium disrupts DE-cadherin and Armadillo localization at Adherens Junctions and upregulates *DE-cadherin* transcription. In turn, DE-cadherin provides an active signal, which promotes JNK-mediated apoptosis (Jezowska et al., 2011). Here I show that like cells lacking Capping Protein, overexpression of the *Src64B* oncogene upregulates *DE-cadherin* transcription, accumulates Matrix Metalloproteases and reduces *Drosophila* Inhibitor of Apoptosis 1. Interestingly, DE-cadherin also promotes apoptosis and prevents growth of *Src64B* overexpressing tissues. Furthermore, decreasing Capping Protein or increasing *Src64B* levels in the distal wing disc epithelium triggers a switch in behavior from tissue overgrowth to apoptosis. Moreover, both Capping Protein and *Src64B* synergize to trigger apoptosis. These observations suggest that Capping Protein and Src act together in the same pathway to control epithelial tissue homeostasis. Interestingly, overexpressing *Capping Protein* suppresses the growth defect of *Src64B* overexpression. Because removing one copy of the *Btk family kinase at 29A*, which mediates Src activity, does not suppress apoptosis of Capping Protein-depleted tissues, this suggests that Capping Protein acts downstream of Btk29A to mediate Src signaling activity. Surprisingly, knocking down *Capping Protein* also prevents Src phosphorylation. However, reducing *Src42A* levels does not suppress death of Capping Protein-depleted cells. Moreover, overexpression of *Drosophila* C-terminal Src kinase or ankyrin-repeat, SH3-domain, and Proline-rich-region containing Protein, two negative regulators of Src, enhances the phenotype of Capping Protein-depleted cells. All together, these observations argue for a dual function of Capping Protein on Src signaling activity: Capping Protein prevents Src signaling activity downstream of Btk29A and inhibits Src phosphorylation, which would otherwise enhance Src signaling activity. Finally, my results suggest that the balance between activated Src and Capping Protein is critical to control Src signaling activity. I propose that missregulation of actin cytoskeletal genes,

such as *Capping Protein* contributes to tumorigenesis through Src signaling. Moreover, one way by which Src expression and activity becomes progressively elevated in a broad spectrum of cancers might be through disruption of the actin cytoskeleton.

2. INTRODUCTION

Rous Sarcoma Virus (v-Src) was identified almost 100 year ago by Peyton Rous as an “agent” that could induce solid tumours in birds. More than 50 years later, this “agent” has been described as a viral oncogene responsible for cellular transformation, and named v-SRC (Rous Sarcoma Virus). Shortly after, Varmus demonstrated that v-Src has its cellular counterpart named c-Src (for cellular Src), which encodes a membrane bound tyrosine kinase, acting as proto-oncogene. Activation of Src kinase leads to distinct cellular processes such as proliferation, apoptosis and migration (reviewed in Aleshin and Finn, 2010). c-Src belongs to a family of tyrosine kinases (SFKs), which includes nine members, that share structural and functional homology and have redundant functions in many biological processes (reviewed in Aleshin and Finn 2010; Thomas and Brugge, 1997) . This renders the study of Src biology in mammalian models complicated. *Drosophila* contains only two *Src* genes: *Src42A* and *Src64B* (Takahashi et al., 1996; Simon et al., 1985), which makes flies a useful model to study Src functions and its contribution to tumorigenesis. Both *Drosophila* Src are regulated by one negative regulator, *Drosophila* C-terminal Src kinase (dCsk), whose activity is positively regulated by Ankyrin-repeat, SH3-domain, and Proline-rich-region containing Protein (dASSPP) (Langton et al. 2007). dCsk inhibits Src42A and Src64B activity by phosphorylation of the conserved Tyrosin residues Tyr511 for Src42A and Tyr547 for Src64B (Read et al., 2004; Pedraza et al., 2004). These inhibitory phosphorylations promote a “closed” conformation of Src, inhibiting the interactions of their kinase domain with their substrates (Engen et al., 2008). While in mammalian, SFKs activate five different kinases of the Tec family kinases (reviewed in Bradshaw, 2010), only one Tec kinase, Btk family kinase at 29A (Btk29A), is involved in Src signal transduction in *Drosophila*. *Drosophila*

Btk29 A is directly phosphorylated by Src64B (Hamada et al., 2005) and seems essential to trigger JNK activation and F-actin remodeling downstream of Src (Langton et al., 2007; Lu et al., 2004; Roulier et al., 1998). Moreover, in the *Drosophila* wing imaginal discs, reduction of one copy of *Btk29A* almost completely rescues apoptosis associated with loss of *dCsk* (Langton et al. 2007), suggesting that Btk29A acts downstream of both Src42A and Src64B. Src42A and Src64B may act in a redundant manner since removing one copy of *btk29A* restores very efficiently the viability of *dCsk* mutant animals, while the loss of one genomic copy of *Src42A* or of *Src64B* does not (Read et al., 2004).

Interestingly activation of Src in mammalian has also been demonstrated to depend on F-actin integrity and on the activity of actin regulators, including Rho family proteins and Formins (Sandilands et al., 2004; Timpson et al., 2001; Fincham et al., 1996). F-actin is involved in Src translocation to the membrane, required for Src activation (Sandilands et al., 2004). Moreover, F-actin integrity is essential for Src activation that occurs upon mechanical stimulation (Wang et al., 2005). In *Drosophila*, mechanical stimulation also activates Src42A signaling during germ band retraction (Desprat et al., 2008).

In turn, Src activation leads to reorganization of F-actin. For instance, in colon cancer cells, Src signalling triggers the re-organization of cortical F-actin and the formation of F-actin enriched protrusions (Avizienyte et al., 2007). Moreover, in malignant glial cells, Src has been shown to contribute to actin turn-over at the leading edge (Angers-Loustau et al., 2004). In *Drosophila*, Src induced F-actin rearrangement to promote ring canals growth (Dodson et al., 1998) and germ-line cysts packing (O'Reilly et al., 2006) in the egg chamber but also to induce morphogenesis of the tracheal system (Matusek et al., 2006) and ommatidium development (Takahashi et al., 1996). Therefore, Src may have a constitutive role in actin assembly and turnover. However, the exact mechanism by which Src signalling influences actin cytoskeleton organization is not fully understood. Src is unlikely to control actin cytoskeleton remodelling through regulation of a single Src substrate since many actin regulators have been shown to be activated downstream

of Src, including the regulation of Rho family members (Rho1, Rac1 and Cdc42A) (Tanji et al., 2010; Timpson et al. 2001) and the direct phosphorylation of the ABP Cortactin (Wu et al., 1991), which activates Arp2/3 and N-WASP mediated F-actin nucleation (Aspenström et al., 200). Moreover, Src has been linked to G-actin phosphorylation in colon cancer cells and Src can directly phosphorylate G-actin *in vitro* (Avizienyte et al., 2007). Therefore, Src activity and F-actin regulation may control each other through feed-back loop mechanisms. Although increased Src level or/and activity is correlated to oncogenic transformation (reviewed in Aleshin and Finn, 2010) its role in tumorigenesis is not fully understood. Studies of Src biology in cell culture models often result in contradictory data. For example, the phenotypes resulting for v-Src expression or c-Src overactivation can range from apoptosis to increase in cell proliferation or transformation (Biscardi et al., 1999; Brown and Cooper, 1996). Studies from Cagan's laboratory in flies have extended our understanding of the variability of phenotypes mediated by elevated levels of Src signalling (Vidal et al., 2006; Vidal et al., 2007). In this studies Vidal and co-authors used *Drosophila* imaginal discs to assay the effect of increased Src signalling activity, either by knocking down *dCsk* or by overexpressing *Src64B* and *Src42A* (Vidal et al., 2006; Vidal et al. 2007). They have demonstrated that mild overexpression of *Src64B* or hypomorphic mutations of *dCsk* triggers cell proliferation and inhibition of apoptosis (Vidal et al., 2006; Vidal et al. 2007). In contrast, strong *Src42A* overexpression or null mutation for *dCsk*^{Q156Stop} triggers apoptosis. Interestingly, when combined with *Ras*^{V12} expression, only high Src signalling activity gives rise to neoplastic overgrowth associated with loss of the ECM component Laminin A and invasion into the larval brain (Vidal et al. 2007). The authors proposed a model in which slight increase of Src signalling induces proliferation and provides anti-apoptotic signals that contribute to early stages of tumourigenesis. At later stages, higher Src signalling activity, combined with oncogenic mutations contributes to metastasis and invasion (Vidal et al. 2007), (Fig.1.14.B).

I have previously shown in (Chapter 3) that CP has a dual function in regulating DE-Cad: it stabilizes DE-Cad at cell-cell junctions, thereby preventing loss of epithelial integrity and inhibits upregulation of the *DE-cad* gene. DE-Cad would otherwise provide an active signal, which affects Wg signalling and promote JNK-mediated apoptosis. However, when cells lacking *CP* are kept alive, JNK is converted into a potent inducer of proliferation (Jezowska et al., 2011). Interestingly, Src42A antagonizes DE-Cad-mediated cell adhesion and stimulates the transcription of *DE-cad* (Shindo et al., 2008). Moreover, in the distal wing disc epithelium, dCsk, the major inhibitor of Src, maintains AJs stability, prevents JNK-mediated apoptosis, whereas halving the genetic dose of *DE-cad* suppresses the apoptotic phenotype of dCsk-depleted cells (Vidal et al., 2006). Since CP and mammalian c-Src both regulate F-actin (Cooper and Sept, 2008; Frame et al., 2002), which in turn, impacts on the kinase activity of c-Src (Desprat et al., 2008; Giannone and Sheetz, 2006; Kim et al., 2010) the main role of CP in the distal wing disc might be to regulate Src activity through the control of F-actin. In this chapter I aimed to investigate the role of Capping Protein in Src signalling activity in *Drosophila* epithelial tissues.

3. MATERIAL AND METHODS

3.1. FLY HUSBANDRY

Flies were raised at 22°C (unless otherwise indicated) under standard conditions (Roberts, 1998). Crosses were cultured in small vials and 3rd instar larvae were collected for dissection of 3rd instar wing imaginal discs.

3.2. MUTANTS AND *DROSOPHILA* STRAINS USED

To generate *cpa*^{69E} clones marked by the absence of GFP in the wing disc *y, w; FRT42D, cpa*^{69E}/CyO P(*y*⁺) males were crossed to *y, w, FRT42D, ubi-GFP* females. The offspring were heat-shocked for 1 h at 37 °C both 24h (for *cpa*^{69E} clones) and at 24h and 48 h (for UAS-*cpa-IR*^{C10} clones) after a 24 h egg collection, corresponding to the first (24h) and second (48h) larval instar. Fly stocks used were *cpa*^{69E}; UAS-HA-*cpa*^{1D} (Janody and

Treisman 2006) and UAS-*cpb*⁷ (Janody and Treisman 2006); UAS-*cpa-IR*^{C10} (Fernández et al. 2011); UAS-*cpb-IR*⁴⁵⁶⁶⁸ (Vienna *Drosophila* Research Center, VDRC); *shg*^{k03401} (Uemura et al. 1996); UAS-*Src64B*^{UY1332} (Nicolaï, Lasbleiz, and Dura 2003) UAS-*Src42A*^{YF.hs.sev} and UAS-*Src64B*^{Y434F}, gift from T. Xu (M Tatenos, Nishida, and Adachi-Yamada 2000); UAS-*dASSP* (Langton et al. 2007); UAS-*dCsk*; UAS-*Src42A-IR*⁷⁸⁷³ (Vienna *Drosophila* Research Center); *sd*-Gal4 (Klein and Arias 1998); *hh*-Gal4, a gift from T. Tabata; *nub*-Gal4, a gift from G.Morata (Calleja et al. 1996).

3.3. ANTIBODY STAINING

The immunohistochemistry staining were performed on the wing imaginal disc of third instar larvae using the procedure described in (Lee and Treisman, 2001). Between 10 and 15 discs were analysed for every genetic combination. Antibodies used were mouse anti-Arm (N2 7A1, 1:10; Developmental Studies Hybridoma Bank), rabbit anti-activated Caspase 3 (1:500; BD Bioscience), rabbit anti-*Src64B* (1:50, gift from M.A.Simon (Dodson et al., 1998), rabbit anti-p*Src42A* (1:200 gift from S. Hayashi (Takahashi et al., 2005) mouse anti-MMP1 (cocktail 1:1 of 5H7B11 and 3B8D12; 1:50, DSHB), mouse anti-DIAP1 (1:200 from B. Hay) mouse anti-β-galactosidase (1:200; Promega). Secondary antibodies were from Jackson ImmunoResearch, used at 1:200 (donkey anti-rabbit TRITC #711-025-152; donkey anti-rabbit CyTM5 #711-175-152; donkey anti-mouse CyTM5 #715-175-150; donkey anti-mouse TRITC #715-025-151).

3.4. WESTERN BLOTTING

For each genotype, either expressing UAS-*mCD8:GFP* or UAS-*cpa-IR*^{C10} or UAS-*Src42A*^{YF.hs.sev} or UAS-*Src64B*^{Y434F} under the control of *sd*-Gal4, proteins were extracted from three 3rd instar larvae wing imaginal discs. Wing discs were homogenized in 5 μl of lysis solution (2%SDS in MiliQ) in the presence of protease inhibitors (Roche #04693159001) and phosphatase inhibitors (2mM Na₃VO₄ and 1mM NaF). Samples were frozen in liquid Nitrogen, boiled for 5 min in 5 μl sample buffer 2×, spun at 13,000 g for 1 min, loaded on a 10% SDS-PAGE gel and transferred to a PVDF membrane

(Amersham Hybond™-P, GE Healthcare). Proteins were visualized by immunoblotting using rabbit anti-H3 (1:1000; Cell Signalling) and mouse rabbit anti-pSrc (1:1000; Bioss International). To quantify the relative amounts of pSrc protein in each genetic background, Western blots were scanned and analyzed using the ImageJ software (NIH; <http://rsb.info.nih.gov/ij/>). Following a background correction step, the intensity levels of pSrc signal was calculated as the sum of the grey values of all the pixels in the selection. Values were then normalized with those of the anti-H3 signals.

3.5. IMAGING

Fluorescence images were obtained on a LSM 510 Zeiss confocal microscope using either a 20× dry or 40× oil objectives. The ImageJ software was used to perform measurements. To quantify the intensity of C3 signal, the most distal wing domain of *sd > UAS-cpb-IR⁴⁵⁶⁶⁸* or *sd > UAS-dASPP; UAS-cpb-IR⁴⁵⁶⁶⁸* wing discs were outlined separately for each disc and the intensity levels were calculated as the sum of the grey values of all the pixels in the selection divided by the number of pixels in the outlined area. To measure the size of the most distal domain the area of the wing primordium and the first ring of the hinge primordium was outlined and then the number of the pixels of the outlined area was calculated using ImageJ software in 2 different phenotypes *sd-Gal4>UAS-Src64B^{UY1332}* and *sd-Gal4;UAS-Cpa>UAS-cpb;UAS-Src64B^{UY1332}*

4. RESULTS

4.1. CAPPING PROTEIN LOSS OR SRC OVEREXPRESSION AFFECTS IDENTICAL TARGET GENES, DISRUPTS POLARITY AND INDUCES APOPTOSIS

We have previously demonstrated that CP-depletion induces distinct outcomes in the most distal wing disc domain. First, the misslocalization of Armadillo (Arm) and DE-cadherin to baso-lateral position and the induction of *DE-cad* transcription (Fig.1. and Fig.6.; Chapter 3). Second, activation of JNK-mediated apoptosis, associated to activation of the JNK target genes: such as *Matrix Metalloproteases (MMP1)* (Fig.4.; Chapter 3) and

the two reporters of JNK signalling, the LacZ enhancer trap insertions in the *misshapen* (*msn-LacZ*) and *puckered* (*puc-lacZ*) genes (Fig.2.; Chapter 3). Finally, loss of CP induces reduction of *Drosophila* Inhibitor of Apoptosis 1 (DIAP1) at a protein level (Fig.2., Chapter 3). Interestingly, the elimination of dCsk-depleted cells from *Drosophila* imaginal discs also requires JNK signalling (Vidal et al., 2006). Moreover, expression of a constitutive active form of *Src42A* triggers loss of Arm and DE-cad from AJs and transcriptional upregulation of *DE-cad* in the developing *Drosophila* tracheal system (Shindo et al., 2008). Furthermore, DIAP1 protein levels has been reported to be reduced upon dCsk-depletion in the wing disc tissue (Vidal et al., 2006). Therefore, cells overexpressing *Src* appear to recapitulate the behavior of CP-depleted cells.

To confirm that overexpressing *Src* in the distal wing disc epithelium affects the same targets than those affected by the loss of CP, I overexpressed *Src64B* by crossing flies carrying UAS insertions into the *Src64B* locus (*Src64B*^{UY1332}) with flies expressing UAS-*mCD8:GFP* under *hedgehog* (*hh*)-Gal4 control. *hh*-Gal4 targets gene expression in the posterior wing disc compartment, allowing to use the anterior compartment as an internal wildtype control. To test the level of *DE-cad* transcription; we used an enhancer trap insertion into the *shg* gene (*shg-LacZ*). As presented in (Fig.6, Chapter 3), in control *mCD8:GFP*-expressing tissues, *DE-cad* showed high levels of expression in cells flanking the D/V boundary and in posterior cells apposed to D/V boundary. Interestingly *shg-LacZ* was upregulated in the whole posterior domain overexpressing *Src64B* (Fig.4.1 A-A').

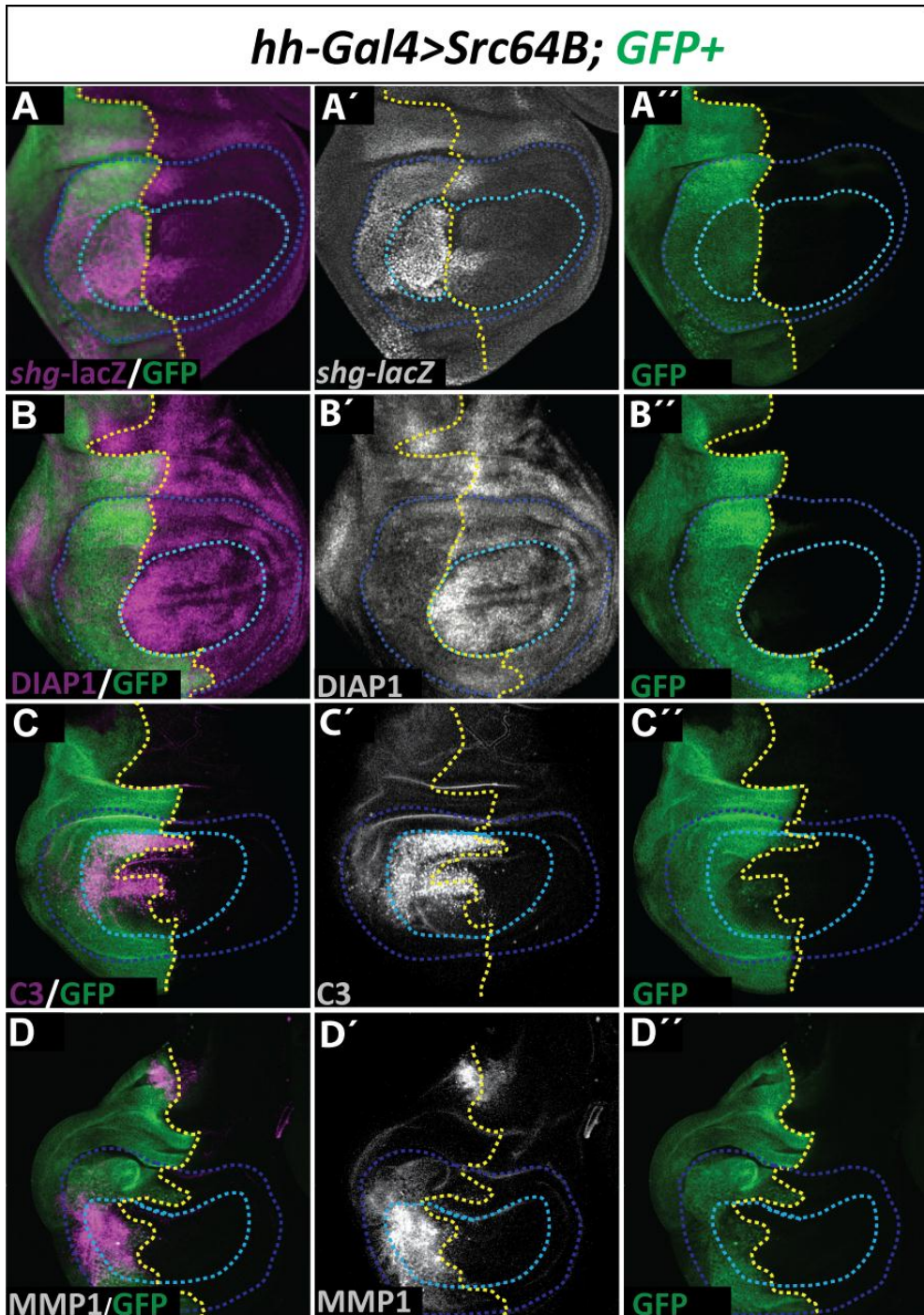


Figure 4.1 *Src64B* overexpression induces *MMP1* and *DE-cadherin* expression; reduces *DIAP1* levels and triggers apoptosis in the most distal wing disc epithelium.

All panels show standard confocal sections of third instar wing imaginal discs with posterior sides to the left and dorsal up. *hh*-Gal4 driving UAS-*mCD8:GFP* (green in A, A'', B, B'', C, C'', D and D'') and *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) and (A-A'') carrying a *LacZ* insertion into the *shg* locus (*shg*^{k03401}). Discs are stained with (A-A'') anti-β-galactosidase to reveal *shg*-LacZ (magenta in A or white in A') or (B-B'') anti-DIAP1 (magenta in B or white in B') or (C-C'') anti-activated C3 (magenta in C or white in C') or (D-D'') anti-MMP1 (magenta in D or white in D'). Dashed lines outline the distal wing disc domains (light blue) or the first ring of the hinge domain (dark blue).

This indicates that *Src64B* overexpression induces *DE-cad* transcription. Moreover, DIAP1 protein level was decreased in the posterior compartment of *hh>Src64B* overexpressing tissues compared to the wildtype control compartment (Fig.4.1 B-B''). Reduction of DIAP1 was associated with apoptosis, monitored with an anti-activated Caspase 3 (C3) antibody, in the posterior compartment of the distal wing disc domain (Fig.4.1.C-C''). Finally, MMP1, accumulated in the posterior compartment of *hh>Src64B*-overexpressing wing discs (Fig.4.1.D-D'), indicating that JNK signaling is activated upon *Src64B* overexpression. Moreover, *Src64B* overexpression reduced Arm (Fig.4.2.A-A'') and DE-Cad (Fig.4.2.B-B'') levels at AJs. Optical cross-sections showed that both appeared displaced from AJs. (Fig.4.2.C-C'' and D-D''), arguing that *Src64B* overexpression affects epithelial cell polarity and consequently triggers cell extrusion and apoptosis.

All together, these observations demonstrate that increase *Src* or decrease *CP* levels affects identical target genes in the distal wing disc tissue. This suggests that *CP* and *Src* act together to control epithelial tissue homeostasis.

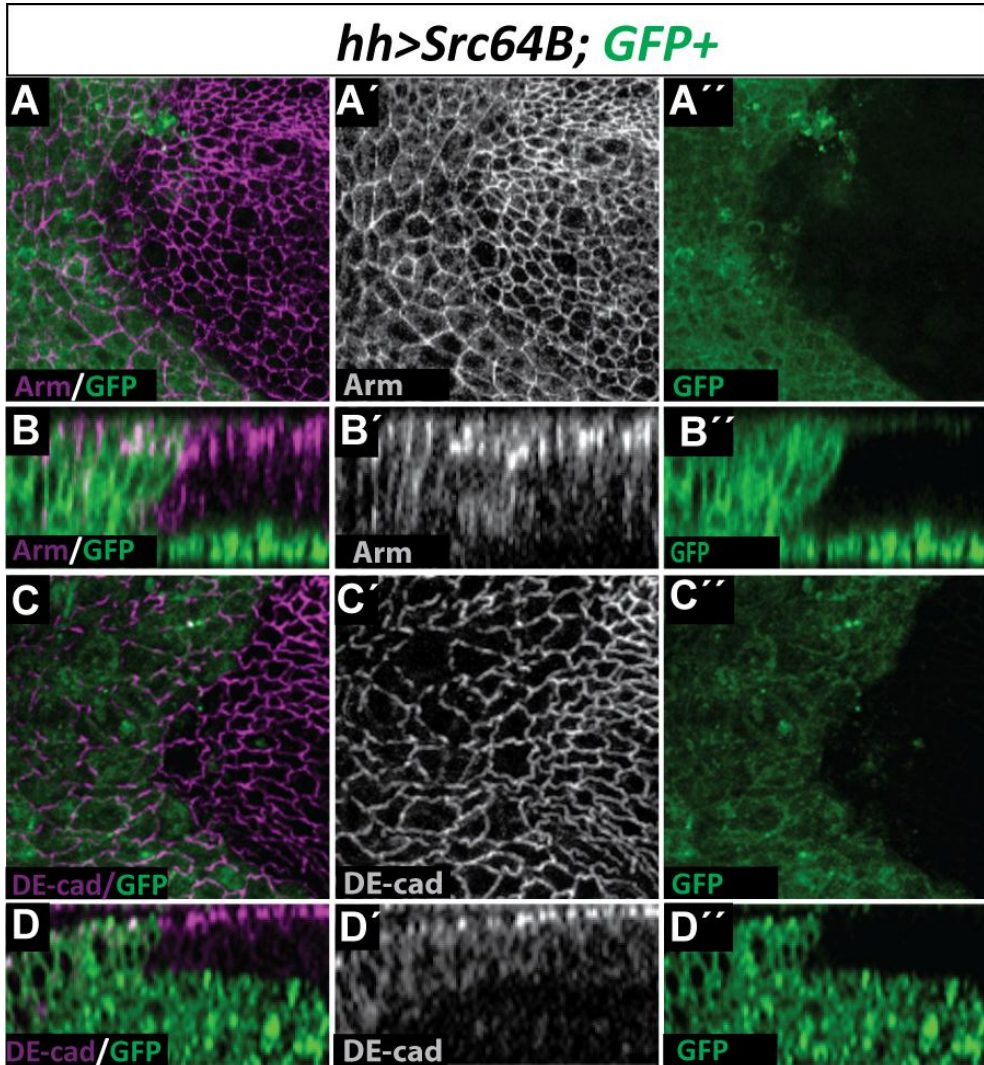


Figure 4.2 Src64B overexpression triggers baso-lateral misslocalization of the components of Adherens Junctions.

All panels show third instar wing imaginal discs with posterior sides to the left. (A-A'' and C-C'') standard confocal sections of the apical cell surface with dorsal up. optical cross sections through the distal disc epithelium with apical side up. (A-A'' to D-D'') *hh*-Gal4 driving *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) and UAS-*mCD8:GFP* (green in A, A'', B, B'', C, C'', D and D''). Discs are stained with (A-A'' and B-B'') anti-Arm (magenta in A and B or white in A' and B') or (C-C'' and D-D'') anti-DE-cad (magenta in C and D or white in C' and D').

4.2. CAPPING PROTEIN AND SRC64B CONTROL THE BALANCE BETWEEN TISSUE GROWTH AND APOPTOSIS.

Reducing levels of dCsk have "opposite" outcomes in epithelia *in situ* (Vidal et al. 2007). Based on these observations, Vidal et al. proposed that anti-apoptotic signals and proliferation is triggered specifically at low levels of Src signalling, while high levels direct apoptotic cell death (Vidal et al. 2007). In agreement with this hypothesis, driving overexpression of *Src64B* (*Src64B*^{UY1332}) with *nubbin*-Gal4 (*nub*-Gal4) did not induced cell death but triggered overgrowth of the distal wing disc tissue, (compare A'' to B'' in Fig.4.3.). In contrast, when driven with *scalloped*-Gal4 (*sd*-Gal4), *Src64B* induced massive apoptosis (Fig.4.3 E') of the distal wing disc tissue, associated to a defect in growth (compare E'' to D'' in Fig.4.3), when compared to *sd*>GFP-expressing control tissues (Fig.4.3.D-D'').

If CP and Src act together in the distal wing disc epithelium, I expect that modulating CP levels would also induce "opposite" outcomes, from tissue overgrowth to massive apoptosis. To test this hypothesis, I analyzed the outcomes of tissues knocked down for *cpa* by driving the expression of dsRNA that target *cpa* to degradation (*cpa*-IR) under *sd*-Gal4 or *nub*-Gal4 control. Knocking down *cpa* using *nub*-Gal4 resulted in mild overgrowth of the distal wing disc tissue (Fig.4.3. C-C'') when compared to *nub*>GFP-expressing control tissues (Fig.4.3. A-A'') and the appearance of few Caspase 3-positive cells (Fig.4.3. C'). In contrast, driving *cpa*-IR with *sd*-Gal4 did not trigger tissue overgrowth but resulted in massive apoptosis (Fig.4.3. F-F').

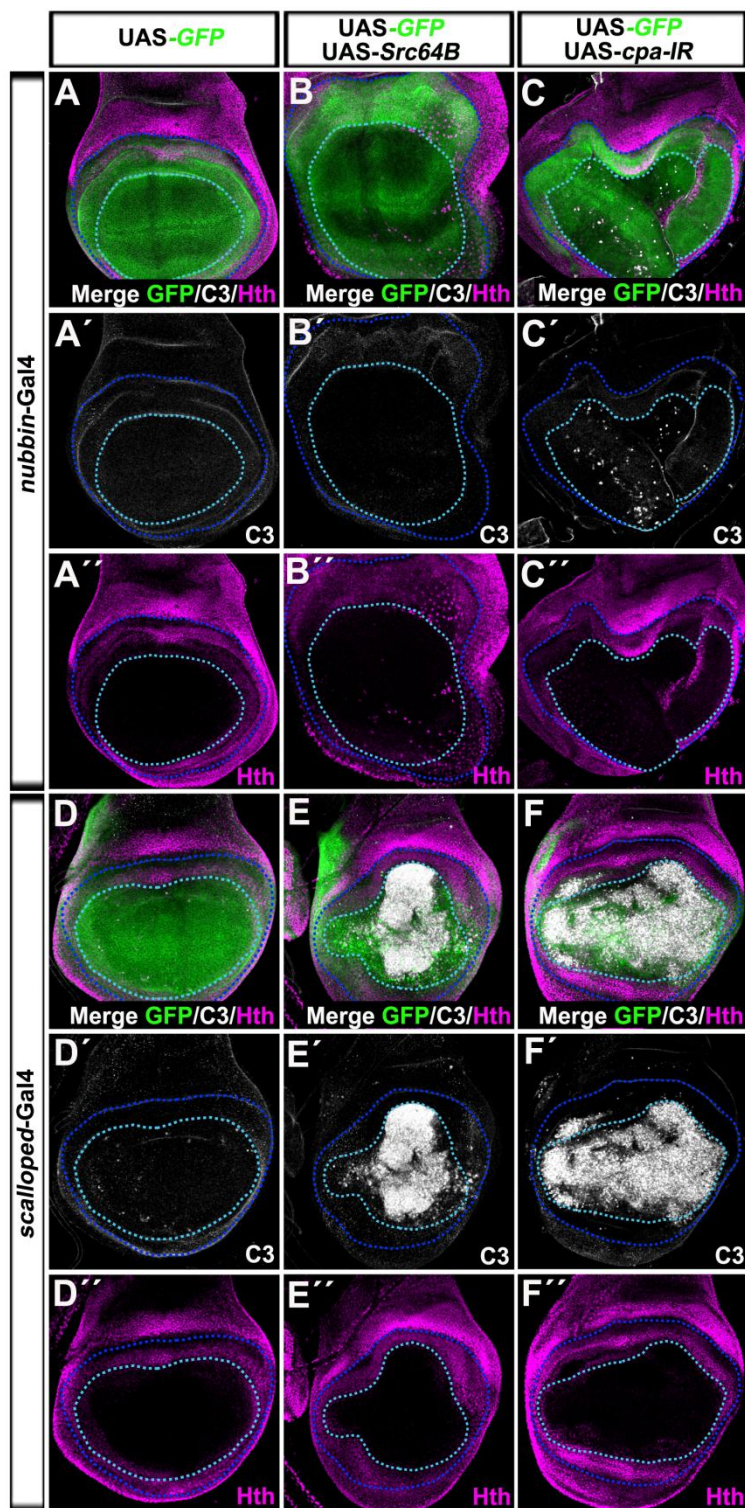


Figure 4.3 Cpa-depletion or Src64B overexpression induces the same phenotypic outcomes in the wing imaginal disc.

All panels show standard confocal sections of third instar wing imaginal discs with posterior sides to the left and dorsal up. (A-A'', B-B'' and C-C'') *nub*-Gal4 driving UAS-*mCD8:GFP* (green in A, B and C) and (B-B'') *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) or (C-C'') UAS-*cpa-IR*^{C10}. (D-D'', E-E'' and F-F'') *sd*-Gal4 driving (D-D'') UAS-*mCD8:GFP* (green in D, E and F) and (E-E'') *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) or (F-F'') UAS-*cpa-IR*^{C10}. Discs are stained with anti-Hth (Magenta in A, A'', B, B'', C, C'', D, D'', E, E'', F and F'') and anti-activated C3 (white in A, A', B, B', C, C', D, D', E, E', F and F'). Dashed lines outline the distal wing disc domains (light blue) or the first ring of the hinge domain (dark blue).

I conclude that loss of CP and Src overexpression not only affects identical targets but also triggers identical "opposite" outcomes in the distal wing domain. Thus, CP might be involved in restriction of Src signaling in the most distal wing domain. Moreover, these observations suggest that in *nub>cpa-IR*-expressing tissues, Cpa levels is slightly decreased and induces a weak activation of Src signalling activity. In contrast, *sd*-Gal4 likely triggers a potent downregulation of Cpa expression, which may induce higher levels of Src signaling.

To determine if the outcomes of tissues overexpressing *Src64B* or expressing *cpa-IR* result from differential expression levels of Cpa or Src64B. I modulated the activity of the *sd*-Gal4 drivers using temperature since at 18°C, Gal4 activity is low but increases at higher temperatures (Phelps and Brand, 1998). The outcomes of *nub>Src64B* and *nub>cpa-IR*-expressing tissues, maintained at 22°C or 28°C and of *sd>Src64B* and *sd>cpa-IR* wing discs, maintained at 18°C or 25°C, were classified based on the strength of overgrowth (weak, medium, strong or no overgrowth) and of the intensity of activated C3 signals (no apoptosis; weak apoptosis; medium apoptosis or strong apoptosis). Maintaining *sd>Src64B* tissues at 18°C did not induce apoptosis (Fig.4.4.D) but triggered tissue overgrowth in 55% of the discs, that range from weak (30%) to medium (25%) (n=20). However, when incubated at 25°C, none of the discs showed overgrowth but all exhibited strong apoptosis (n=5). These results are in agreement with Vidal et al., 2006, arguing that low levels of *Src64B* overexpression

triggers overgrowth, while high levels induces massive apoptosis. At 18°C, knocking down Cpa with *sd*-Gal4 let to a weak overgrowth phenotype for 25% of the discs (Fig.4.4.B), associated to medium levels of apoptosis (Fig.4.4.D) (n=16). When the temperature of incubation was raised to 25°C, none of the discs exhibited overgrowth (Fig. 4.4.B) but all showed strong levels of apoptosis (Fig.4.4.D) (n=12). These observations suggest that a slight decrease of Cpa levels triggers tissue overgrowth, while a higher decrease induces cell death.

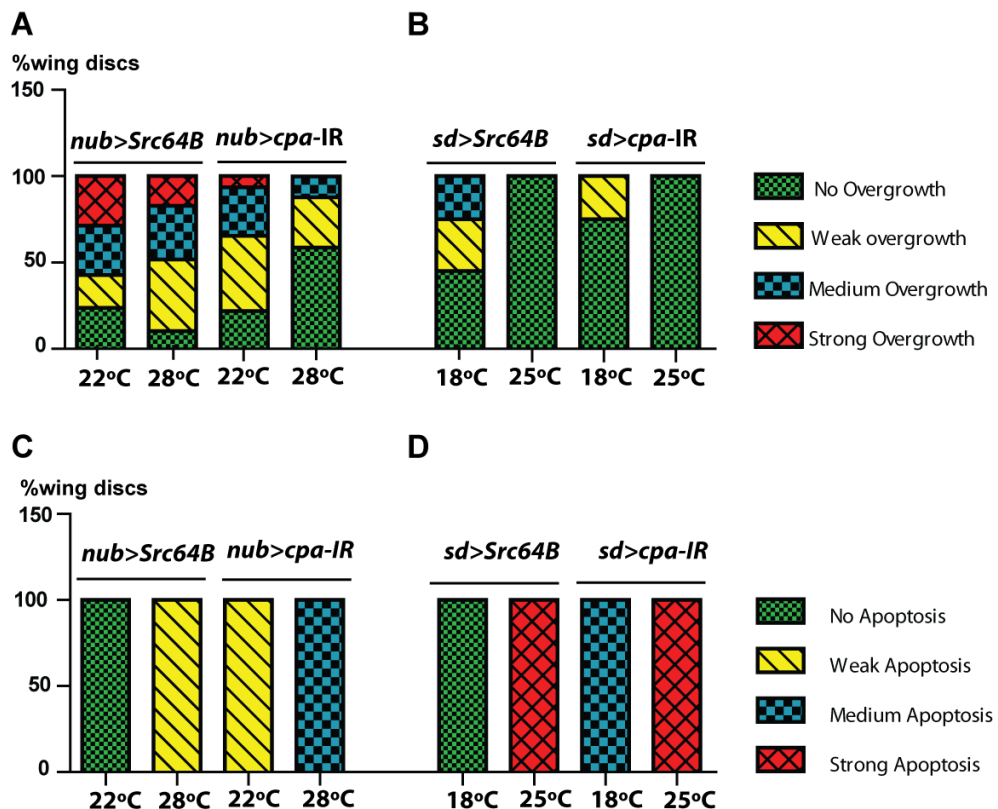


Figure 4.4 Decreasing Cpa or increasing Src64B levels triggers a switch from tissue overgrowth to apoptosis in the most distal wing disc epithelium.

Quantification of the strength of (A and B) tissue overgrowth or (C and D) apoptosis of wing disc epithelia in which (A and C) *nub*-Gal4 drives *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) or UAS-*cpa-IR*^{C10} at 22°C or 28°C or (B and D) *sd*-Gal4 drives *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) or UAS-*cpa*-

IR^{C10} at 18°C or 25°C. Discs were classified into 4 different groups to evaluate (A-B) tissue overgrowth (no overgrowth or weak or medium and strong overgrowth) or (C-D) apoptosis (no apoptosis or weak or medium or strong apoptosis). The vertical axis represents the percentage of discs of each phenotype: *nub>Src64^{UY1332}* discs at 22°C displayed: 23.8% no overgrowth, 19% weak, 28.6% medium, 28.6% strong overgrowth and 100% no apoptosis (n=21); at 28°C displayed 10.4% no overgrowth, 41.4 weak, 31% medium, 17.2% strong overgrowth (n=29); *sd>Src64^{UY1332}* discs at 18°C displayed 45% no overgrowth, 30% weak; 25% medium, 0 strong overgrowth and 100% weak apoptosis (n=20); at 25°C 100% displayed no overgrowth (n=5); *nub>cpa-IR^{C10}* discs at 22°C displayed 21.9% no overgrowth; 43.7% weak; 28.1% medium, 6.3% strong overgrowth and 100% displayed weak apoptosis (n=32); at 28°C displayed: 58.6% no overgrowth; 29.3% weak, 12% medium, 0 strong overgrowth and 100% displayed medium apoptosis (n=58); *sd>cpa-IR^{C10}* discs at 18°C displayed: 75% no overgrowth; 25% weak; 0 medium; 0 strong overgrowth and 100% displayed medium apoptosis (n=16); at 28°C 100% displayed no overgrowth and 100% displayed strong apoptosis (n=12).

I then used the *nub-Gal4* driver to confirm these data. When *nub>Src64B* tissues were incubated at 22°C, almost 80% of the discs exhibited overgrowth, ranging from strong (28.6%), medium (28.6%) to weak (19%), while 23.8% did not overgrowth (Fig.4.3 A); (n=21). None of these discs displayed apoptosis (Fig.4.4.C) (n=21). Increasing the temperature to 28°C decreased the severity of *nub>Src64B*-dependent overgrowth with 17.2% of disc showing strong overgrowth, compared to 28.8% at 22°C (Fig.4.4.A); (n=29). Moreover, raising the temperature induced weak apoptosis (Fig.4.4.C); (n=29). Similar observations were made by knocking down Cpa with *nub-Gal4*. At 22°C, almost 80% of discs showed overgrowth ranging from weak (43.7%), medium (28.1%) to strong (12%). 21.8% did not exhibit overgrowth (Fig.4.4.A); (n=32). In these discs, overgrowth was associated with low levels of apoptosis (Fig.4.4.C); (n=32). Growing larvae at 28°C decreased significantly the number of overgrowing discs with 58.6% that did not exhibit any overgrowth (58.6%) and none that showed strong overgrowth (Fig.4.4.A), while apoptosis increased (Fig.3.3.C) (n=58). All together, these data demonstrate that depending of their levels, Src64B overexpression or Cpa depletion triggers similar "opposite" outcomes in the distal wing domain. While a reduction of Cpa or an increase of Src levels induces tissue overgrowth, a stronger Cpa reduction or Src64B increase induces apoptosis and reduce overgrowth.

4.3. LOSS OF CAPPING PROTEIN COOPERATES WITH SRC64B IN THE DISTAL WING DISC EPITHELIUM

Because knocking down *cpa* or overexpressing *Src64B* affect identical target genes (Fig.4.1. and 4.2.) and give rise to similar outcomes in the distal wing disc epithelium (Fig.4.3. and Fig.4.4.), both are likely involved in the same pathway to control tissue homeostasis. One possibility is that CP restricts Src signaling activity. If so, I expect that decreasing Cpa levels in *Src64B*-dependent overgrowing tissues will enhance Src signaling activity and trigger apoptosis. As previously shown (Fig.4.3. A and C), overexpressing *Src64B*, together with *UAS-mCD8:GF*, under *nub*-Gal4 control, induced tissue overgrowth without major signs of apoptosis (Fig.4.5. A-A'). When Cpa was knocked down, using *nub*-Gal4, it resulted in slight tissue overgrowth and the induction of few apoptotic cells (Fig.4.5.B-B').

However, when both *cpa*-IR and *Src64B* were driven with *nub*-Gal4, this led to massive apoptosis (Fig.4.5. C-C'). These observations suggest that CP restricts Src signaling activity. CP may act upstream of Src by restricting its activation. Alternatively, CP may restrict Src signaling activity downstream of Src.

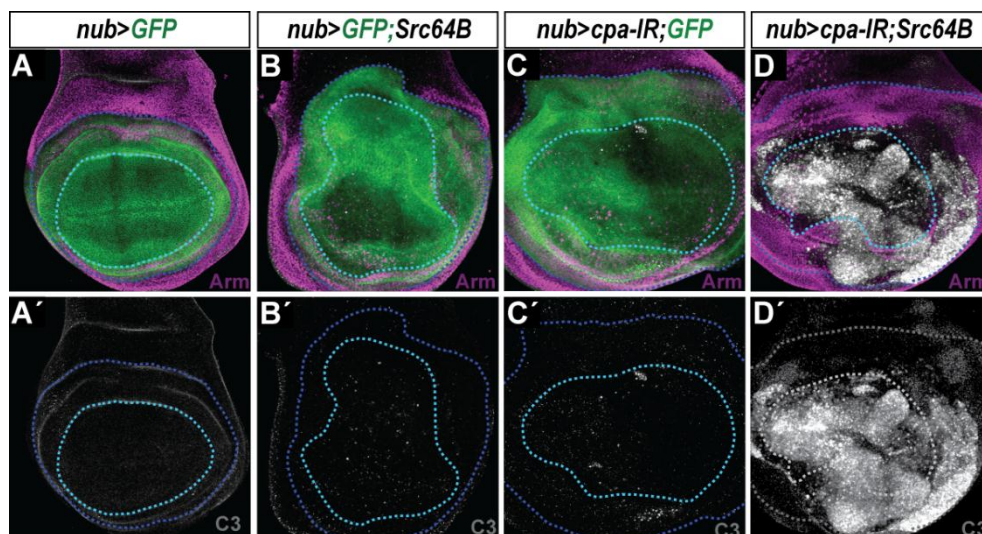


Figure 4.5 Loss of Cpa and *Src64B* overexpression synergize to trigger apoptosis in the wing imaginal disc.

All panels show standard confocal sections of third instar wing imaginal discs with dorsal sides up. *nub-Gal4* driving (A, A') UAS-*mCD8:GFP* (green A) or (B, B') UAS-*mCD8:GFP* (green in B) and *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) or (C, C') UAS-*mCD8:GFP* (green in C) and UAS-*cpa-IR*^{C10} or (D, D') UAS-*cpa-IR*^{C10} and *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}). Discs are stained with anti-Arm (magenta in A, B, C and D) and anti-activated C3 (white). Dashed lines outline the distal wing disc domains (light blue) or the first ring of the hinge domain (dark blue).

4.4. DE-CADHERIN PROMOTES APOPTOSIS AND PREVENTS GROWTH OF SRC64B OVEREXPRESSING TISSUE

Previously we have demonstrated that apoptosis of *cpa*-depleted cells in a distal wing disc can be partially suppressed when one genomic copy of *shg* is removed (Jezowska et al. 2011). Because loss of CP or increase of Src activity has similar cellular (Fig.4.2.) and tissue (Fig.4.3. Fig.4.4.) outcomes; affects identical targets (Fig.4.2.) and when combined induce apoptosis (Fig.4.5), decreasing DE-cad levels may also prevent apoptosis of Src overexpressing cells. In agreement with this possibility, Vidal et al., proposed that DE-cad is part of a signalling network that directs the removal of *dCsk*-depleted cells induced at the anterior-posterior boundary of wing imaginal disc (Vidal, Larson, and Cagan 2006). To investigate the role of DE-cad in apoptosis of Src overexpressing cells, I compared the outcome of wing imaginal discs overexpressing *Src64B* together with UAS-*mCD8:GFP* that were either wildtype for *shg* or heterozygote mutant for the *shg*^{k03401} allele. Indeed, removing one copy of *shg* partially suppressed apoptosis of *Src64B*-overexpressing tissues (Fig.4.6 compare E-E'' with C-C').

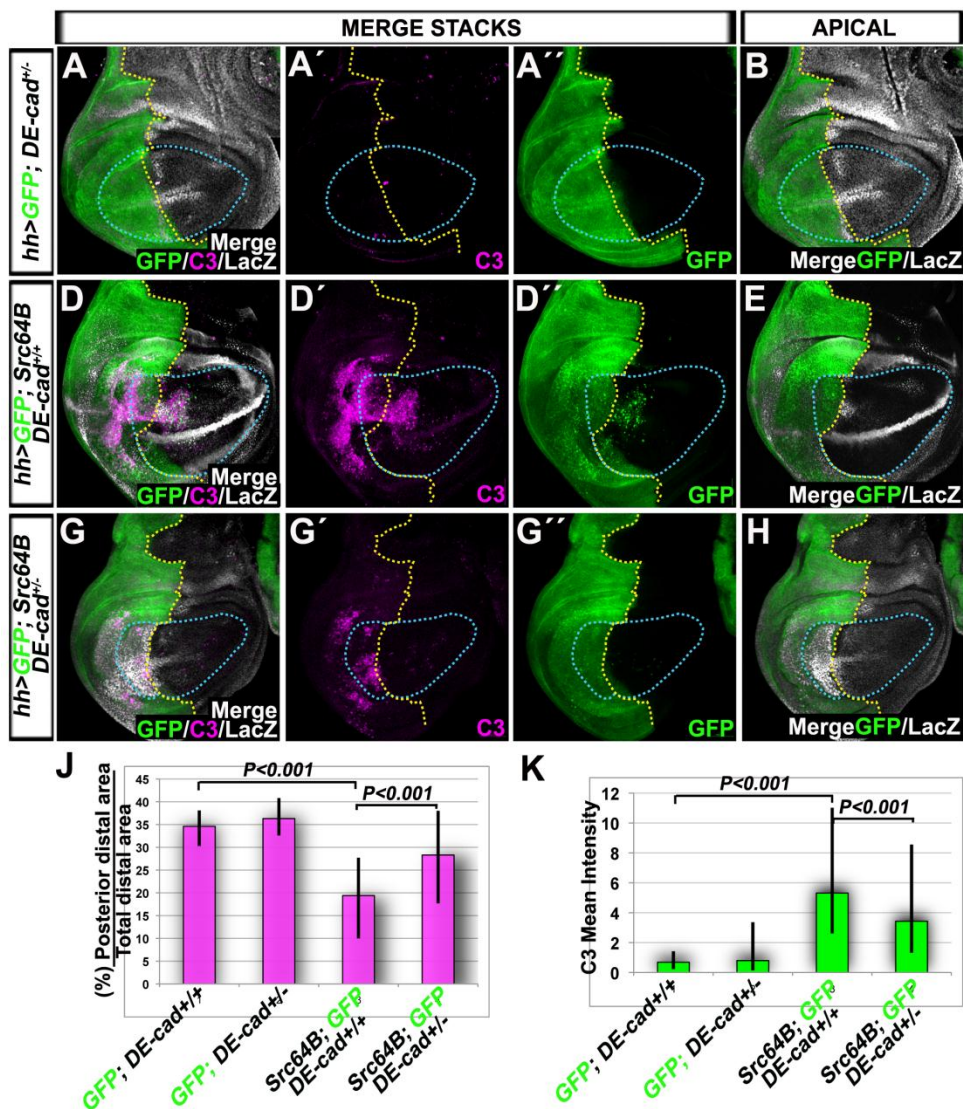


Figure 4.6 Removing one copy of *shg* in tissues overexpressing *Src64B* partially suppresses cell death and restores tissue growth.

(A-A'' to H) standard confocal sections of third instar wing imaginal discs. (A-A'', C-C'' and E-E'') apico-basal projections. (B, E and H) apical cell surfaces (A-A'' and B) discs heterozygote for *shg*^{k03401}, expressing UAS-*mCD8-GFP* (green in A, A'' and B) under *hh*-Gal4 control. (C-C'' and D) wildtype discs carrying the sibling Cyo^{wg-LacZ} balancer chromosome, expressing UAS-*mCD8-GFP* (green in B, B'' and C) and overexpressing *Src64B* (*Src64B*^{UY1332}) under *hh*-Gal4 control. (E-E'' and F) discs heterozygote for *shg*^{k03401}, expressing UAS-*mCD8-GFP* (green in E, E'' and F) and overexpressing *Src64B* (*Src64B*^{UY1332}) under *hh*-Gal4 control. Discs are stained with anti-C3 (magenta in A, A', C, C', F and F') and anti- β -galactosidase to reveal *shg*-LacZ (white in A, B, E and F) or *wg*-LacZ (white in C and D). (G) Ratio in percentage of the posterior distal wing discs area

over the total distal wing disc in each indicated genetic background. The mean for *hh>mCD8-GFP, shg^{+/+}* is 34.6, for *hh>mCD8-GFP, shg^{k03401/+}* is 36.3, for *hh>mCD8-GFP, Src64B^{UY1332}, shg^{+/+}* is 19.4, for *hh>mCD8-GFP, Src64B^{UY1332}, shg^{k03401/+}* is 28.3. Error bars indicate s.e.m.. $P<0.001$ for comparison of *hh>mCD8-GFP, shg^{+/+}* and *hh>mCD8-GFP, Src64B^{UY1332}, shg^{+/+}* and for *hh>mCD8-GFP, Src64B^{UY1332}, shg^{+/+}* and *hh>mCD8-GFP, Src64B^{UY1332}, shg^{k03401/+}*. (H) Mean intensity of C3 signal in each indicated genetic background. The mean for *hh>mCD8-GFP, shg^{+/+}* is 0.682, for *hh>mCD8-GFP, shg^{k03401/+}* is 0.792, for *hh>mCD8-GFP, Src64B^{UY1332}, shg^{+/+}* is 5.315, for *hh>mCD8-GFP, Src64B^{UY1332}, shg^{k03401/+}* is 3.432. Error bars indicate s.e.m.. $P<0.001$ for comparison of *hh>mCD8-GFP, shg^{+/+}* and *hh>mCD8-GFP, Src64B^{UY1332}, shg^{+/+}* and for *hh>mCD8-GFP, Src64B^{UY1332}, shg^{+/+}* and *hh>mCD8-GFP, Src64B^{UY1332}, shg^{k03401/+}*.

I quantified this effect by measuring the intensity signals of C3 staining in each genetic background. Caspase 3 activation was reduced by 34% ($P<0.001$; $n=14$) when one copy of *shg* was removed in *hh>Src64B* wing imaginal discs (Fig.4.6. H). Control wing discs either wildtype for *DE-cad* or heterozygote mutant for the *shg^{k03401}* allele that expressed UAS-*mCD8:GFP* under *hh-Gal4* control, did not displayed significant apoptosis (Fig.4.6.A-A'' and H.).

Interestingly, increasing *Src64B* levels in the posterior compartment strongly reduced the size of the posterior distal wing domain (Fig.4.6.C'' and D), when compared to *hh>GFP* control discs (Fig.4.6.A'' and B). Removing one copy of *DE-cad* in *hh>Src64B* wing imaginal discs restored growth of the posterior distal domain (Fig.4.6.E'' and F). I quantified this effect by calculating the ratio of the posterior distal domain over the total area of a distal domain (area outlined with dashed blue line in Fig.4.6. A-A'' to F) in each genetic background (Fig.4.6.G). Control wing imaginal discs in which expression of UAS-*mCD8:GFP* was driven with *hh-Gal4* showed that the posterior distal domain represented 34.6% of the total area of the distal domain ($n=8$). Overexpressing *Src64B* under *hh-Gal4* control strongly decreased the size of the posterior distal domain to 19.4% ($P<0.001$; $n=19$). While removing one copy of *DE-cad* in *hh>Src64B* wing disc restored growth of the posterior distal domain to 28.3% ($P<0.001$; $n=14$). This effect was specific for *Src64B* overexpression since removing one copy of *DE-cad* did not significantly affect growth of the posterior distal domain of control wing discs expressing UAS-*mCD8:GFP* under *hh-Gal 4* control (Fig.3.5.G) ($n=17$).

Taken together, I conclude that in the distal wing disc epithelium, DE-cad promotes apoptosis and prevents growth of Src64B overexpressing tissues.

4.5. CAPPING PROTEIN ACTS DOWNSTREAM OF SRC64B

Interestingly, CP and Src both regulate F-actin organization in mammalian cell models and in *Drosophila* (Dodson et al., 1998; Somogyi and Rørth, 2004). Src kinase directly phosphorylates Cortactin (Nada et al., 1994), which in turn, promotes activation of N-Wasp (Kowalski et al., 2005) and the Arp2/3 complex (Weaver et al., 2001). Moreover, Src signalling activates Formins, which also triggers F-actin polymerization (Timpson et al., 2001; Tanji et al., 2010). CP activity has been shown to compete with Formins for binding to F-actin barbed end (Harris et al., 2004) and proposed to contribute to formation of F-actin networks mediated by Formins and the Arp2/3 complex (Cooper and Sept, 2008). Therefore, CP may prevent Src signaling by counteracting the Src-dependent effect of Formins or Arp2/3 on F-actin. Alternatively, Src may regulate F-actin by inhibiting the activity or localization of CP.

If CP prevents F-actin polymerization, downstream or in parallel to Src, then, forcing the capping of free F-actin barbed ends should prevent F-actin polymerization and suppress the Src-overexpression phenotypes. To test this possibility, I analyzed the effect of overexpressing both, *cpa* and *cpb* in tissues overexpressing *Src64B* in the whole distal wing disc using the *sd*-Gal4 driver. Overexpressing *cpa* and *cpb* under *sd*-Gal4 control did not have major effects on morphology of the wing imaginal disc but seemed to affect growth and induce a weak apoptotic response (Fig. 4.7. compare B-B' with A).

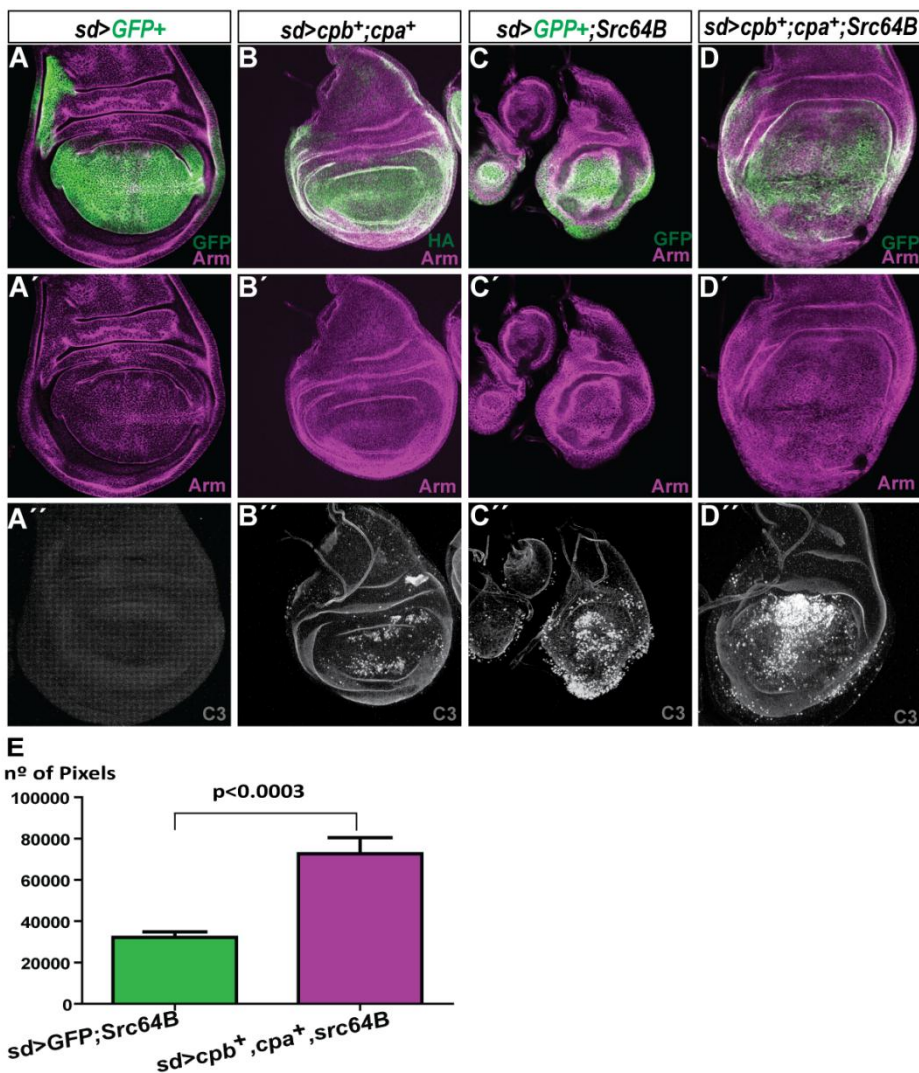


Figure 4.7 Overexpressing *cpa* and *cpb* suppresses the growth defect of *Src64B*-overexpressing tissues.

(A-A'' to D-D'') standard confocal sections of third instar wing imaginal discs. *sd*-Gal4 driving (A-A'') *UAS-mCD8:GFP* (green in A) or (B-B'') *UAS-cpb⁷* and *UAS-HA-cpa^{1D}* or (C-C'') *UAS-mCD8:GFP* (green in C) and *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B^{UY1332}*) or (D-D'') *UAS-cpb⁷*, *UAS-HA-cpa^{1D}* and *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B^{UY1332}*). Discs are stained with anti-Arm (magenta in A, A', B, B', C, C', D and D') and anti-activated C3 (white in A'', B'', C'' and D'') and (B-B'' and D-D'') anti-HA (green in B and D). (E) Quantification of the mean intensity of C3 staining in wing imaginal discs in which *sd*-Gal4 drives *UAS-mCD8:GFP* and *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B^{UY1332}*) (green column) or *UAS-cpb⁷*, *UAS-HA-cpa^{1D}* and *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B^{UY1332}*) (magenta

column). The mean for *sd>GFP; Src64B* is $72670 \pm \text{SEM} (7798)$ and for *sd>cpb⁺; HA-cpa⁺; Src64B* is $32200 \pm \text{SEM} (2688)$; $P < 0.0003$ ($N=9$).

As previously reported (Fig. 4.3) overexpressing *Src64B* in the *sd*-Gal4 domain strongly reduced tissue growth and triggered massive apoptosis (Fig. 4.7. C-C''). When *cpa* and *cpb* were overexpressed it restored the growth defects of *sd>Src64B* overexpressing tissues (Fig. 4.7. D-D''). However, these discs still contained a large number of C3-positive cells. To quantify tissue growth of *sd>Src64B* and *sd>cpa⁺;cpb⁺;Src64B* expressing tissues, I measured the area of the distal wing domain (Fig. 4.7. E). When *cpa* and *cpb* were overexpressed, it restored the size of the distal domain of *sd>Src64B* overexpressing tissues to 46 % ($P < 0.0003$; $n=11$).

I conclude that CP is involved in mediating Src signaling activity in the distal domain of the *Drosophila* wing imaginal disc. CP may act upstream of Src by preventing its activation. Alternatively, CP may restrict Src signaling activity downstream of Src.

4.6. CAPPING PROTEIN IS EPISTATIC TO BTK29A

Src64B has been shown to regulate F-actin through the Tec family kinase Btk29A (Dodson et al., 1998). The control of F-actin might be critical to mediate Src signalling activity since in the *Drosophila* wing imaginal disc, reduction of one copy of *Btk29A* almost completely rescue apoptosis associated to the loss of *dCsk* (dCsk) (Langton et al. 2007). If CP prevents Src activation, I expect that decreasing Btk29A levels will also suppress apoptosis of CP-depleted cells. In contrast, if CP regulates the F-actin network downstream of Src, decreasing Btk29A levels will have no effect on the outcome of cells knocked down for CP. To distinguish between these hypothesis, I first confirmed that Btk29A triggers death of Src64B overexpressing cells. As expected, removing one copy of *btk29A*, strongly prevented apoptosis of *sd>Src64B*-overexpressing wing discs (Fig.4.8. compare A' to B').

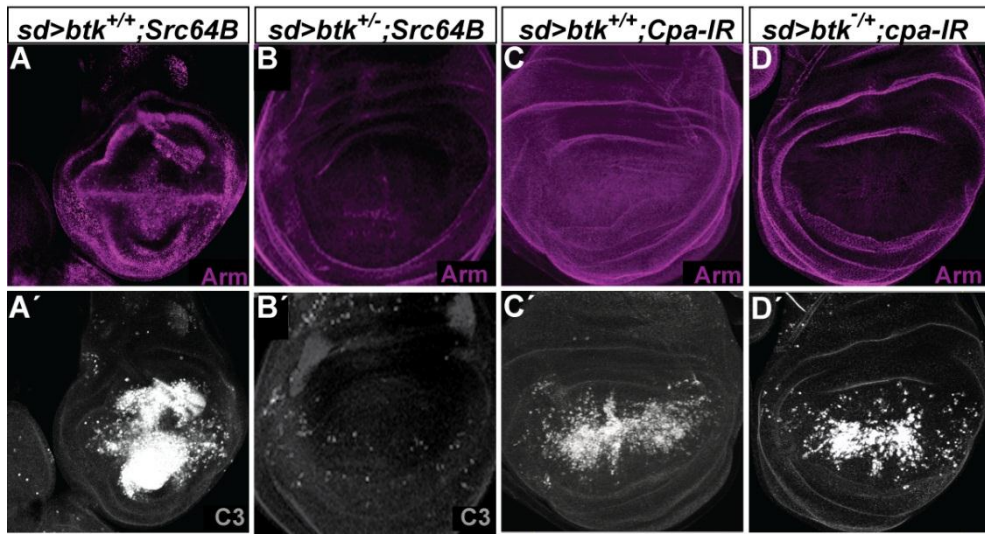


Figure 4.8 Removing one copy of *btk29A* suppresses cell death mediated by *Src64B* overexpression but not triggered by CP depletion.

All panels show standard confocal sections of third instar wing imaginal disc. *sd*-Gal4 driving (A-A' and B-B') *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}) that are either (A-A') wildtype for *btk29A* or (B-B') heterozygote mutant for the *btk20*^{K00206} allele. (C-C' and D-D') *sd*-Gal4 driving UAS-*cpa-IR*^{C10} that are either (C-C') wildtype for *btk29A* or (D-D') heterozygote mutant for the *btk20*^{K00206} allele. Discs are stained with (A, B, C and D) anti-Arm (magenta) and (A', B', C' and D) anti-activated C3 (white).

In contrast, *sd>cpa-IR*-expressing wing discs that were heterozygote mutant for the *btk29A*^{K00206} allele displayed as many C3-positive cells as *sd>cpa-IR*-expressing tissue that were wildtype for *btk29A* (Fig. 4.8. compare C' to D'). These results argue that CP does not prevent Src activation and support a role of CP on Src signaling activity downstream or in parallel to Btk29A kinase.

4.7. CAPPING PROTEIN ACTS IN A FEEDBACK LOOP MECHANISM TO ATTENUATE SRC ACTIVITY

So far, my results demonstrate that CP acts antagonistically to Src signaling (Fig.4.1. to Fig.4.4.) and suggests that CP act downstream of Src (Fig.4.7. and Fig.4.8.). To rule out the possibility that CP inhibits Src activation, I directly assayed the phosphorylation status of *Drosophila* Src in tissues depleted of Cpa using an phospho-specific antibody raised against the Tyr418 residue of Src (pSrcY₄₁₈). This antibody has been successfully

used on *Drosophila* tissues and seems to recognize preferentially the active phosphorylated form of Src42A (Shindo et al. 2008). First, I analyzed the phosphorylation status of Src by Western Blot on wing disc extracts in which *sd*-Gal4 drove either UAS-*mCD:8GFP* or UAS-*cpa-IR*, or a constitutive active form of Src42A (UAS-*Src42A*^{YF.hs.sev}) or Src64B (UAS-*Src64B*^{Y434F}) used as positive controls (Fig. 4.9.C).

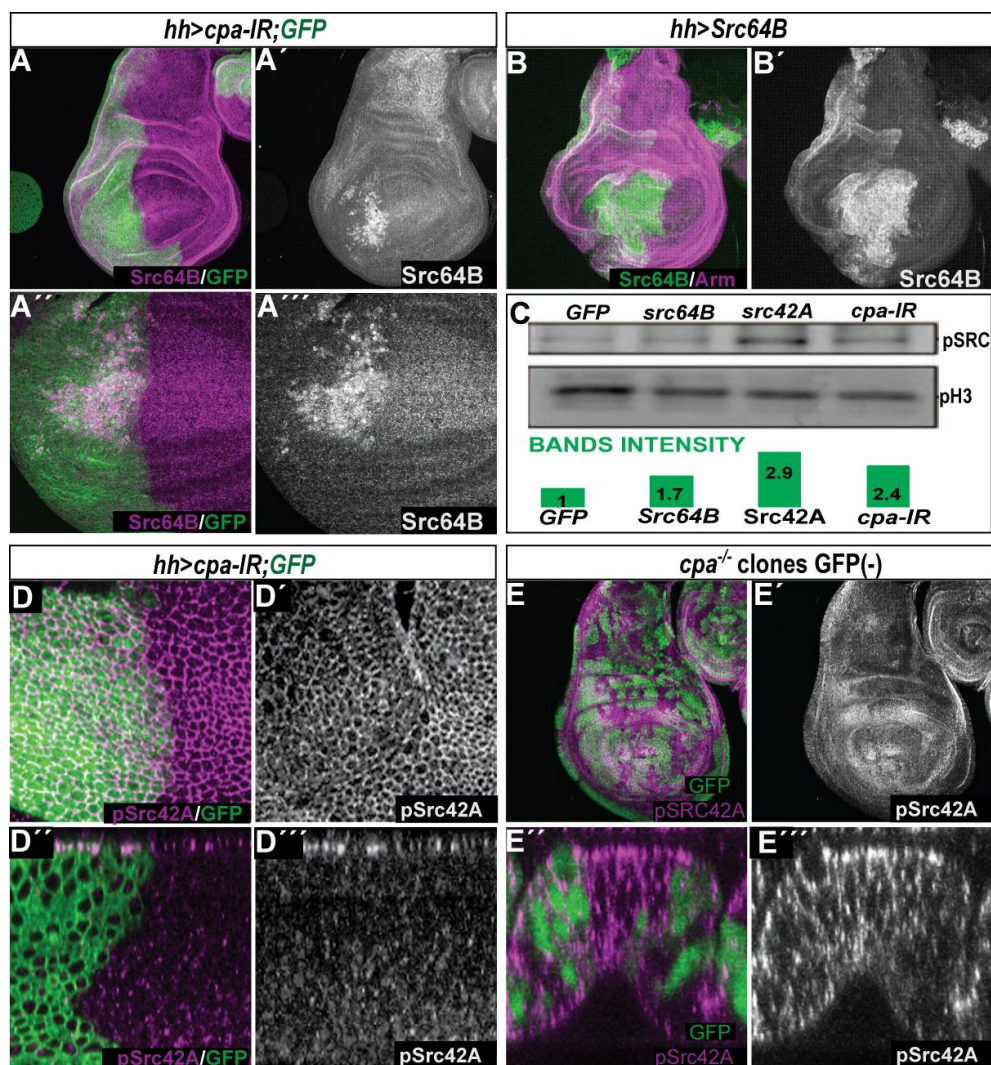


Figure 4.9 Tissues depleted of CP accumulate Src.

(A-A', B-B', D-D' and E-E'') third instar wing imaginal discs with posterior sides to the left. (A-A'', B-B'', D-D' and E-E') standard confocal sections of the (A-A'' and B-B') basal or (D-D' and E-E') apical cell surface with dorsal up. (D'-D'' and E'-E'') optical cross sections through the distal disc epithelium with apical side up. (A-A'' and D-D'') *hh*-Gal4 driving UAS-*cpa-IR*^{C10} and UAS-

mCD8:GFP (green in A, A'', D and D''). (B-B') *hh*-Gal4 driving *Src64B* overexpression through UAS sites inserted into the *Src64B* locus (*Src64B*^{UY1332}). (E-E'') *T155*-Gal4; UAS-*flp*-induced *cpa*^{69E} mutant clones marked by absence of GFP (green in E and E''). Discs are stained with (A-A'' and B-B') anti-*Src64B* (magenta in A and A'' or white in A', A''' and B' or green in B) and (B-B') anti-Arm (magenta in B) or (D-D'' and E-E'') anti-p*Src42A* (Magenta in D, D'', E, E'' or white in D', D''', E', E'''). (C) western blot on protein extracts from *sd*-Gal4 driving (lane 1) UAS-*mCD8:GFP* or (lane 2) UAS-*Src64B*^{Y434F} or (lane 3) UAS-*Src42A*^{YF.hs.sev} or (lane 4) UAS-*cpa-IR*^{C10}; blotted with anti-p*Src* (upper panel) and anti-H3 (lower panel). The values of the anti-p*Src* staining after normalization are 1 *sd*>UAS-*CD8:GFP*; 1.7 *sd*>*Src64B*^{Y434F}; 2.9 *sd*> *Src42A*^{YF.hs.sev}; 2.4 *sd*>*cpa-IR*^{C10}.

As expected, extracts expressing *Src42A*^{YF.hs.sev} contained higher levels of phosphorylated *Src* compared to control tissues expressing UAS- *mCD8:GFP*. To quantify the relative amount of p*Src* signal, I measured the levels of p*Src* intensity after normalization with the corresponding H3 signals, used as a loading control (lower panel in Fig. 4.9.C). *Src42A*^{YF.hs.sev} showed a 2.4 fold increase in p-*Src*. Overexpressing *Src64B*^{Y434F} or depleting *Cpa* did not appear to significantly alter the intensity signal of p*Src*. However, after normalization, quantification showed that knocking down *cpa* or overexpressing *Src64B*^{Y434F} increased the levels of p*Src* to 2.4 and 1.7 fold respectively (lower panel in Fig.4.9.C). Three independent experiments confirmed that *Cpa*-depletion increased the level of p*Src* from 1 to 2.4 folds. These results argue that CP prevents accumulation of the active phosphorylated form of *Src*.

To confirm that CP prevents *Src* phosphorylation, I stained wing imaginal discs with a phospho-specific antibody against *Src42A* (p*Src42*) (Takahashi et al., 2005). Discs expressing UAS-*cpa-IR* together with UAS-*mCD8:GFP* in the posterior compartment using the *hh*-Gal4 driver showed that p*Src42A* is localized mostly apically, forming a honey-comb pattern (Fig. 4.9. D-D'). Cross sections through the wing disc epithelium revealed that *Cpa*-depleted cells accumulated higher levels of p*Src*, when compared to the wildtype anterior compartment (Fig. 3.8.D'-D''). To confirm this result, I analyzed the intensity staining of p*Src42A* in cells mutant for *cpa*. p*Src42A* also accumulated in clones mutant for the *cpa*^{69E} allele (Fig. 4.9. E- E''). All together, these results argue that CP prevents activation of the *Src* kinases.

Increase Src activity of tissue knocked down for Cpa can either result from phosphorylation of the pool of Src present in the cell or can be a consequence of increase Src levels. To distinguish between these possibilities, I analyzed the levels of Src64 using an antibody that recognizes both the active (phosphorylated at Tyr434) and inactive (unphosphorylated) forms of Src64B (O'Reilly et al., 2006). As expected, Src64B accumulated in the posterior compartment of wing imaginal discs overexpressing *Src64* and *UAS-mCD8:GFP* under *hh-Gal4* control (Fig. 4.9.B-B'). Surprisingly, knocking down Cpa in the posterior compartment of the wing imaginal disc also induced Src64B accumulation (Fig.4.9.A-A'). However, increased Src64B levels in Cpa-depleted cells appeared to localize basally, indicating that only extruded cells that undergo apoptosis accumulated Src64B. This observation suggests that Src64B accumulation is not a primary consequence of Cpa loss but rather a consequence of cell extrusion and/or activation of the apoptotic machinery.

Taken together, these data demonstrate that upon loss of CP, Src phosphorylation is induced. Increased pSrc levels might therefore trigger ectopic activation of Src targets and participate in this way in the enhancement of Src signaling activity.

4.8. THE BALANCE BETWEEN ACTIVATED SRC AND CP IS CRITICAL FOR TISSUE HOMEOSTASIS

So far, my results argue that CP has a dual function on Src activity. It prevents Src signaling downstream of the kinase Btk29A (Fig.4.8.) but also inhibits Src activity through phosphorylation. If in CP-depleted cells, increased activity of the Src42A kinase enhances Src signalling activity, I expect that decreasing Src42A levels will partially suppress the outcome of CP-depleted cells. To test this possibility, I analyzed whether removing one copy of *Src42A* rescue apoptosis of Cpb-depleted tissue. However, loss of one copy of *Src42A* in *sd>cpb-IR* expressing wing imaginal disc did not significantly affect the amount of C3-positive cells (Fig. 4. 10. compare D-D' with C-C'). In control, removing one copy of

Src42A did not trigger apoptosis or affect the morphology of wing imaginal discs in which *UAS-mCD8:GFP* was expressed under *sd*-Gal4 control (Fig. 4. 10. compare B-B' with A-A').

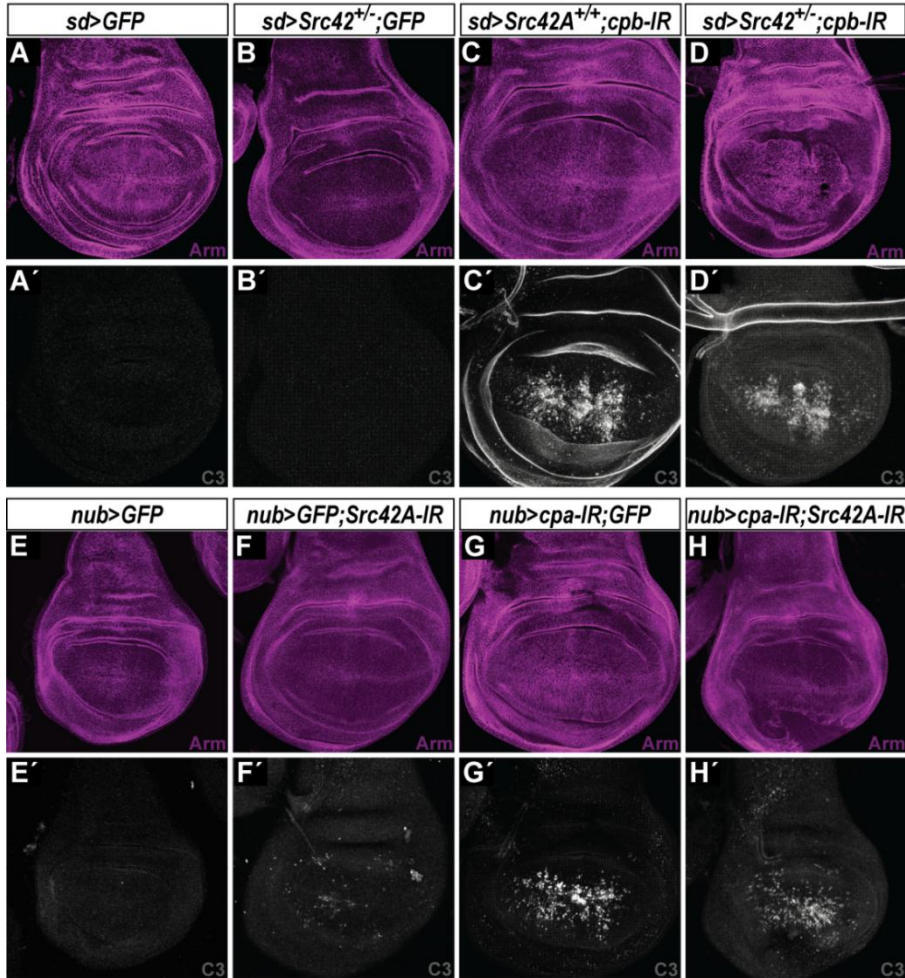


Figure 4.10 Reducing *Src42A* levels does not suppress death of *Cpa*-depleted cells.

All panels show standard confocal sections of third instar wing imaginal discs with dorsal up. (A-A' and B-B') *sd*-Gal4 driving *UAS-mCD8:GFP* in a tissue (A-A') wildtype for *Src42A* or (B-B') heterozygote mutant for the *Src42A*^{E1} allele. (C-C', D-D') *sd*-Gal4 driving *UAS-cpa-IR*^{C10} in a tissue (C-C') wildtype for *Src42A* or (D-D') heterozygote mutant for the *Src42A*^{E1} allele. (E-E' to H-H') *nub*-Gal4 driving (E-E') *UAS-mCD8:GFP* or (F-F') *UAS-mCD8:GFP* and *UAS-*Src42A-IR** or (G-G') *UAS-mCD8:GFP* and *UAS-cpa-IR*^{C10} or (H-H') *UAS-cpa-IR*^{C10} and *UAS-*Src42A-IR**. Discs are stained with (A, B, C, D, E, F, G and H) anti-Arm and (A', B', C', D', E', F', G' and H') anti-activated C3 (white).

To confirm that reduction of Src42A level is not sufficient to partially rescue death of CP-depleted cells, I used RNAi to knock down *Src42A*. Expressing UAS-*Src42A-IR* under *nub-Gal4* control had no visible effect on the morphology of the wing imaginal disc and triggered the appearance of a few apoptotic cells (Fig.4.10 compare F-F' with E-E'). When *Src42A-IR* was expressed together with *cpa-IR* it did not visually affect the number of C3 positive cells of *cpa-IR*-expressing tissues (Fig. 3.9. compare H-H' with G-G'). These results argue that upon loss of CP, increased Src42A activity does not have a main contribution in triggering apoptosis of CP-depleted cells.

Because removing one genomic copy of *Src42A* or *Src64B* does not restore the viability of *dCsk* mutant animals, while the loss of one copy of *btk29A* does (Read et al., 2004), in CP-depleted tissues in which Src42A levels was reduced, Src64B might compensate for the reduction in Src42A activity. To investigate whether decreasing the kinase activities of both Src42A and Src64B partially prevents apoptosis of CP-depleted tissues, I overexpressed *dCsk*, in *sd>cpa-IR* wing discs. Surprisingly, discs overexpressing UAS-*dCsk* under *sd-Gal4* control that should contain decrease activity levels of both Src, resembled tissues overexpressing Src64B (Fig.4.3.), characterized by a reduction of the size of the tissue (Fig.4.11. compare B with A), associated to massive apoptosis in the distal domain (Fig.4.11. compare B' with A'). Depleting Cpa with the *sd-Gal4* driver also triggered massive cell death (Fig. 3.10.C-C') but did not affect tissue size (Fig.4.4.). Overexpressing *dCsk* in *sd>cpa-IR* tissues seemed to prevent tissues growth and appeared to reduce the levels of apoptotic cells (Fig. 4.10. D-D'). Because the outcome of these tissues resembles the one of tissues overexpressing *dCsk* (Fig. 4.11. compare D-D' with B-B'), blocking the activity of Src42A and Src64B may completely prevent the excess Src signaling activity of CP-depleted cells. Alternatively, I cannot exclude that *dCsk* overexpression affects additional processes, independently of its control on Src activity.

Because *dCsk* overexpression is very deleterious when expressed in the wing imaginal disc, I overexpressed *dASPP*, a positive regulator of *dCsk* (Langton et al. 2007).

In contrast to tissues overexpressing *dCsk*, overexpression of UAS-*dASPP* under the control of the *sd*-Gal4 driver did not induce any visible reduction in tissue growth when compared to *sd>GFP* control discs (Fig.4.11. compare E to A), nor induced apoptosis (Fig.4.11 E').

Expression of UAS-*cpb-IR* using the *sd*-Gal4 driver triggered a medium apoptotic response (Fig.4.11. F'). Surprisingly, overexpressing *dASPP* enhanced cell death of UAS-*cpb-IR* tissues (Fig. 4.11. compare G' with F'). Quantification of the intensity signals of C3 staining showed a 1.27 fold increase of apoptotic cells in *sd>dASPP; cpb-IR* compared to UAS-*cpb-IR*⁴⁵⁶⁶⁸ ($P < 0.007$; $n = 11$). These data suggest that decreasing Src activity enhances death of Cpb depleted cells. Because increasing Src activity also synergizes with loss of CP to trigger apoptosis (Fig.4.5.), the balance between activated Src and CP might be critical to control Src signalling activity. Alternatively, I cannot rule out that the synergic effect observed between overexpressed *dASPP* and loss of Cpb is independent of the control on Src activity.

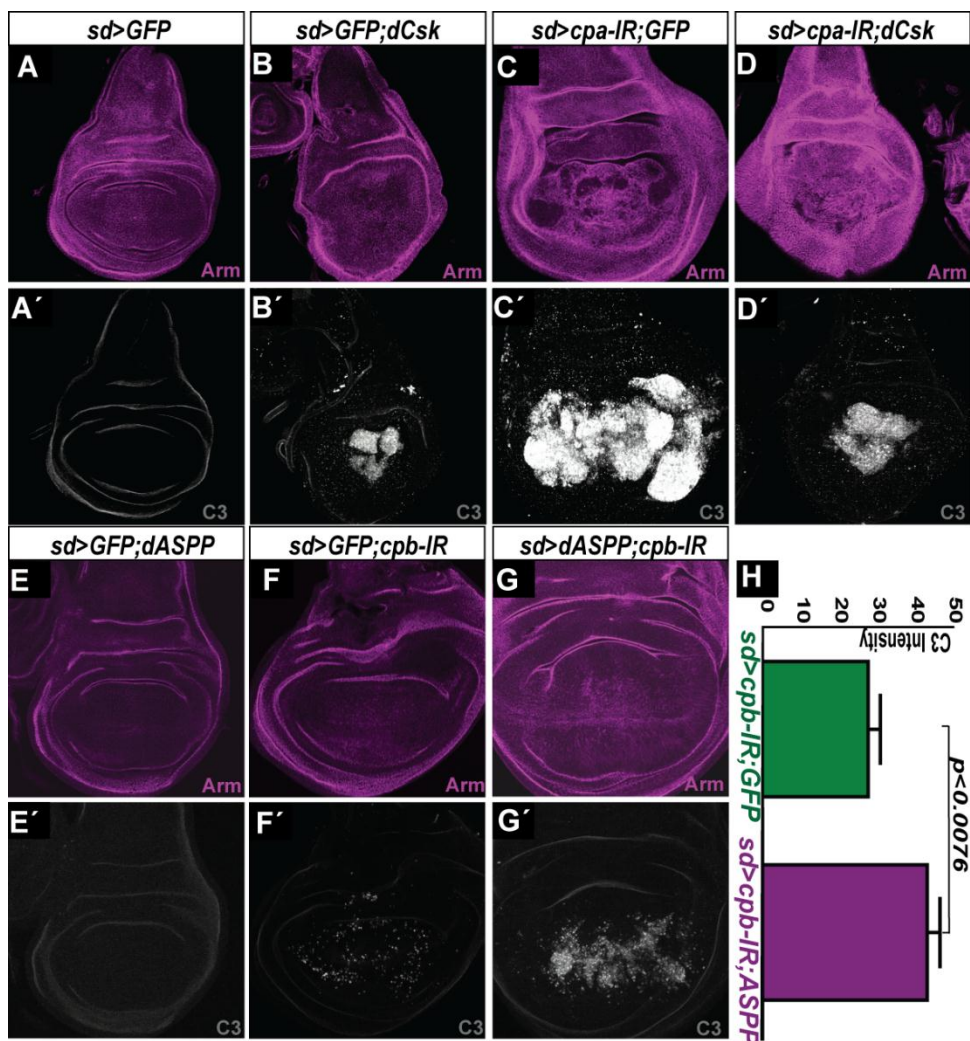


Fig.4.11. *dCsk* or *dASPP* overexpression enhances the phenotype of *Cpa*-depleted cells in the wing imaginal disc.

(A-A' to G-G') standard confocal sections of third instar wing imaginal discs in which *sd*-Gal4 drives (A-A') UAS-*mCD8:GFP* or (B-B') UAS-*mCD8:GFP* and UAS-*dCsk* or (C-C') UAS-*mCD8:GFP* and UAS-*cpa-IR*^{C10} or (D-D') UAS-*cpa-IR*^{C10} and UAS-*sCsk* or (E-E') UAS-*mCD8:GFP* and UAS-*dASPP* or (F-F') UAS-*mCD8:GFP* and UAS-*cpb-IR*⁴⁵⁶⁶⁸ or (G-G') UAS-*dASPP* and UAS-*cpb-IR*⁴⁵⁶⁶⁸. Discs are stained with (A, B, C, D, E, F and G) anti-Arm (magenta) or (A', B', C', D', E', F' and G') anti-activated C3 (white). (H) Quantification of the mean intensity of C3 staining in wing imaginal discs in which *sd*-Gal4 drives UAS-*mCD8:GFP* and UAS-*cpb-IR*⁴⁵⁶⁶⁸ (green column) or UAS-*dASPP* and UAS-*cpb-IR*⁴⁵⁶⁶⁸ (magenta column). The mean for *sd>cpa-IR; GFP* is $43.12 \pm (\text{SEM}) 3.41$ ($n=11$) and for *sd>dASPP;cpb-IR* is $27.67 \pm \text{SEM} (3.23)$ ($n=9$); $P < 0.0076$ for comparison of both genotype ($n=12$).

5. DISCUSSION AND CONCLUSIONS

In this chapter I have shown that in the distal wing disc epithelium, both CP and Src act in the same pathway that controls the balance between tissue growth and apoptosis. First, *Cpa* reduction or *Src64B* overexpression affects identical target genes (Fig.4.1. and 4.2.). Second, decreasing *Cpa* levels or overexpressing *Src64B* shows similar outcomes: while tissues expressing *cpa-IR* or overexpressing *Src64B* with the *nub*-Gal4 driver overgrowth, when expressed using the *sd*-Gal4 driver, induces massive apoptosis (Fig.4.3. and Fig.4.4.). Third, in conditions where *Src64B* overexpression or *Cpa* depletion triggers tissue overgrowth, when combined, induces apoptosis (Fig.4.5.). Fourth, overexpressing *cpa* with *cpb* partially restores the size of tissues overexpressing *Src64B* (Fig.4.7.). Fifth, like loss of *dCsk*, loss of *cpa* cooperates oncogenically with *Ras*^{V12} overexpression (Vidal et al., 2007; Jezowska et al., 2011 respectively). Sixth, like loss of *cpa*, overexpression of *Src64B* cooperates with mitogenic signals sent by P35-expressing "undead" cells (B. Garcia Fernández, personal communication). The model proposed by Vidal et al., argues that a slight induction of Src signalling contributes to tissue overgrowth, while higher Src signalling activity induces apoptosis (Vidal et al., 2007). According to this model, CP loss may trigger an increase in Src signalling activity. Reducing CP levels in Src overexpressing discs likely enhances the excess Src signalling activity, switching the tissue response from overgrowth into apoptosis. Taken together, my observations argue for a role of CP in preventing Src signalling activity.

Capping Protein acts downstream of Src

Diverse observations support a role for CP downstream of Src. First, overexpressing *cpa* and *cpb* restores the growth defect of *Src64B* overexpressing tissues (Fig.4.7). Second, removing one copy of the Src target *Btk29A* does not suppress apoptosis of *Cpa*-depleted tissues (Fig. 4.8.), indicating that CP acts downstream or in parallel to *Btk29A*. The CP heterodimer binds to the barbed ends of actin filaments, preventing F-actin polymerization (Janody and Treisman, 2006; Cooper and Sept, 2008; Delalle et

al., 2005). Interestingly, like loss of CP, overexpressing *Src64B* or knocking down *dCsk* triggers apical F-actin accumulation in the distal wing disc epithelium (B. Garcia-Fernández, personal communication). Therefore, the control of an F-actin network by Src and CP may regulate Src signalling activity. Src may regulate F-actin by phosphorylating ABPs that promote F-actin polymerization since one direct target of Src is Cortactin, which stimulates actin polymerization through direct interaction with the Arp2/3 complex (Wu et al. 1991). However, removing one copy of *Btk29A* in *Src64B* overexpressing tissues not only suppresses apoptosis but also F-actin accumulation (F.Janody, personal communication), indicating that Src controls F-actin through Btk29A. In agreement with this hypothesis, during ring canals morphogenesis of the *Drosophila* egg chamber and during membrane invagination of the *Drosophila* blastoderm embryos, Btk29A acts downstream of *Src64B* to directly or indirectly regulate ABPs that form microfilament rings (Roulier et al., 1998; Thomas and Wieschaus, 2004). One way by which the Btk29A kinase may regulate F-actin is by inactivating CP. Indeed, Cpa and Cpb possess many putative phosphorylation sites (data not shown). CP phosphorylation by Btk29A may prevent CP from binding to the barbed ends of F-actin, leaving the barbed end free for the addition of new actin monomers. However, Btk29A may also inhibit CP activity by phosphorylating and inactivating a positive regulator of CP. Many distinct molecules had been reported to regulate CP activity such as CARMIL proteins, myotrophin or PIP2 (reviewed in Cooper and Sept 2008), (Tab.1.1.; Chapter 1). If Btk29A controls F-actin only by inhibiting CP activity, removing one copy of *btk29A* in tissue with reduced levels of Cpa would be expected to stimulate the activity of Cpa molecules still present in the cell and consequently partially suppresses apoptosis. However, tissues knocked down for *cpa* that are either wild-type or heterozygote mutant for *btk29A* appear to show similar apoptotic levels (Fig. 4.8.). Alternatively, Btk29A may activate ABPs, which outcompetes CP at the barbed end of F-actin and/or stimulates F-actin polymerization at filament-barbed ends (Fig.4.12.A).

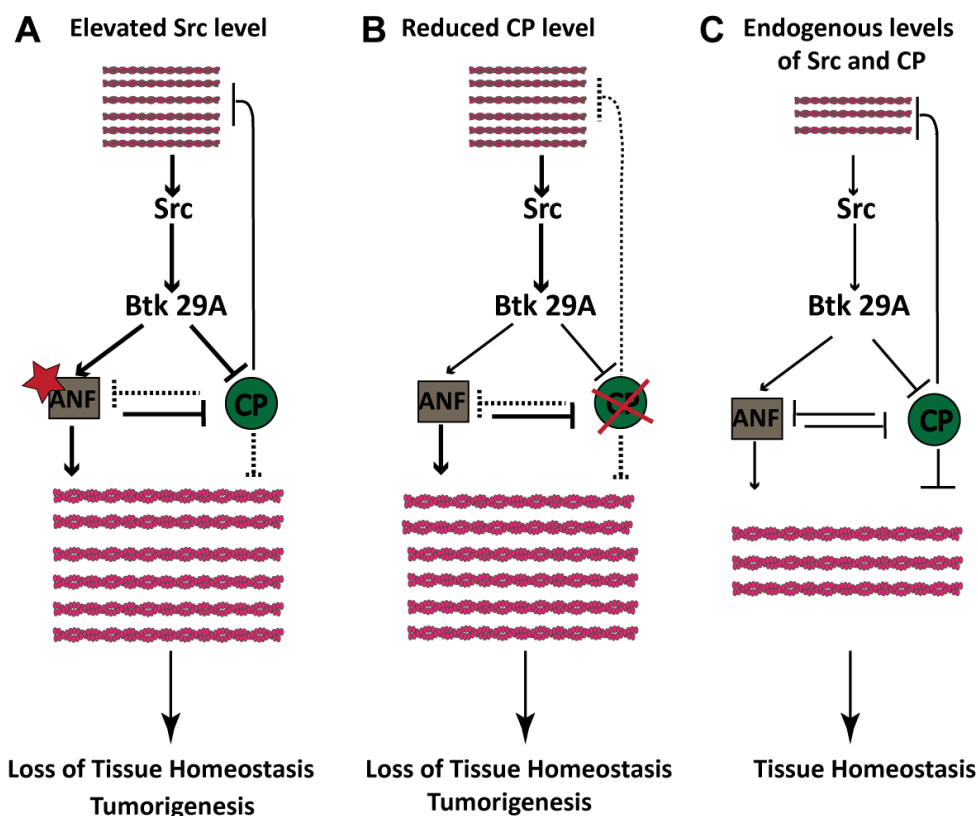


Figure 4.12 Model of the dual function of Capping Protein on Src signaling activity.

(A) In tissues overexpressing Src, activated Src triggers F-actin accumulation through Btk29A, which activates an actin-nucleating factor (ANF) and possibly inhibits the capping activity of CP at filament barbed ends. Decrease CP activity may also affect an F-actin network, which prevents Src phosphorylation and activation. The F-actin network formed downstream of Src and CP, induces loss of tissue homeostasis and tumorigenesis when cells are prevented to die. (B) Loss of CP uncap F-actin barbed ends, allowing the incorporation of new actin monomers to filaments by the ANF and promoting the formation of an F-actin network, which induces loss of tissue homeostasis and triggers Src phosphorylation and activation. (C) In wildtype tissues that contain endogenous levels of Src and CP, CP counteracts the effect of Src on F-actin, promoting in this way tissue homeostasis.

In agreement with this possibility, Src signalling activates Rho family proteins (Tanji et al. 2010; Timpson et al. 2001) and actin nucleating factors (ANFs), such as Formins, WASP and Arp2/3 (Aspenström et al., 2006; Weaver et al. 2001; Kovacs et al., 2011). All together my data argue that Src and CP regulate an F-actin network that mediates the different outcomes of Src signalling, including tissue growth, apoptosis and oncogenic

transformation. In conditions of increased Src activity, phosphorylation of Btk29A activates an ANF and/or prevents CP from capping the barbed ends of F-actin. Consequently a specialized F-actin network is formed that leads to loss of tissue homeostasis and tumorigenesis if apoptosis is blocked (Fig.4.12.B). The same F-actin network is affected when CP is lost. In this condition, an ANF adds new actin monomers to the barbed ends of this specialized F-actin network, free of the capping activity of CP. (Fig.4.12.C).

Capping Protein affects Src activity

Although many observations support a role of CP downstream of Src signalling, knocking down *cpa* also triggers accumulation of the phosphorylated form of Src kinase in the wing disc tissue (Fig.4.9.). Therefore, the F-actin network regulated by CP may also acts upstream of Src to prevent its phosphorylation. This idea is supported by the findings that F-actin depolymerization prevents Src phosphorylation (Fincham et al. 1996; Wang et al. 2005; Kim et al., 2010). Moreover, both Rho family proteins and Formins that induce F-actin polymerization, positively regulate Src activity (Tanji et al. 2010; Timpson et al. 2001). However, in Cpa depleted tissues, increase Src activity is unlikely to play a major role in enhancing Src signalling activity since reducing one copy of *btk29A*; *Src42A* or depleting *Src42A* by RNAi do not suppress apoptosis of Cpa depleted tissues (Fig.4.10.). However, because *Src42A* and *Src64B* seem to act in a redundant manner (Read et al., 2004), I cannot exclude that in condition of *Src42A* reduction, *Src64B* activity is enhanced and contribute to promote the different outcomes of Cpa-depleted tissues. Therefore, to determine whether activated Src contributes to the different outcomes of tissues knocked down for CP, it would be necessary to reduce the levels of both *Src42A* and *Src64B*.

Several results are surprising. First, overexpression of *dASPP*, a positive regulator of *dCsk*, slightly enhances apoptosis of Cpa-depleted tissue (Fig.4.11.). Second, *dCsk* overexpression seems to partially rescue apoptosis triggered by Cpa loss but reduces the size of the distal wing imaginal disc (Fig.4.11). Strikingly, strong activation or reduction of

Src signalling, both triggers apoptosis suggesting that proper levels of Src signalling activity maintain tissue homeostasis (Fig.4.3 and Fig.4.10 and data not shown). dCsk or dASPP overexpression blocks Src activity. Therefore, in CP-depleted tissues, increase Src signalling activity downstream of activated Src requires Src to be active. Complete loss of Src activity might prevent the activity of an actin nucleating factor required to add new actin monomers to actin filaments barbed ends uncapped due to the loss of CP. However, I cannot exclude that *dCsk* overexpression, in addition to inhibit Src activity, affects other signalling pathways since it also phosphorylates Warts *in vitro* (Stewart et al., 2003). Moreover, the *dASPP* overexpression construct has only been used *in vitro* to demonstrate that dASPP phosphorylates and physically interacts with dCsk (Langton et al. 2007) but has not been validated *in vivo*. Therefore, dASPP may affect signalling pathways other than Src signalling.

In conclusion, my data argue for a dual function of CP on Src signalling activity: CP prevents Src signalling downstream of Btk29A and inhibits activated Src through phosphorylation, which may otherwise enhance Src signalling activity. Although, Src signalling activity is unlikely to contribute to apoptosis mediated by CP loss since, Src42A reduction does not rescue apoptosis of CP depleted tissue. The role of CP on Src signalling is likely through regulation of a specialized F-actin network, which controls both Src signalling and phosphorylation. Finally, my results suggest that the balance between activated Src and CP is critical to control Src signalling activity and to maintain in this way tissue homeostasis and prevent tumorigenesis.

6. ACKNOWLEDGMENTS AND AUTHOR CONTRIBUTION

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CHAPTER 5 – GENERAL DISCUSSION

1. CAPPING PROTEIN REGULATES MULTIPLE SIGNALING PATHWAYS

1.1. ROLE OF CAPPING PROTEIN IN HIPPO SIGNALLING PATHWAY

Findings from our laboratory and the group of Georg Halder argue that Capping Protein (CP) functions to inhibit Yki nuclear translocation and activation of several Yki target genes that promote cell survival and proliferation in distinct *Drosophila* tissues and in S2 cells (Fernández et al., 2011; Sansores-Garcia et al., 2011). However, it is not clear at which level of the Hpo pathway CP intersects. Our data argue that CP positively regulates the activity of the core Hpo kinase cassette since wing discs depleted of CP exhibit lower level of phosphorylated Yki (Fernández et al., 2011). However it could be possible that in addition, CP promotes Yki cytoplasmic retention independent of the Yki phosphorylation. In this scenario CP may promote the interaction of Yki with Wts and/or Ex and/or Hpo, which sequester Yki into the cytoplasm and inhibits its nuclear translocation. Alternatively, CP may promote the interaction of Yki with other molecules that are able to sequester Yki in the cytoplasm independent of Ex/Wts/Hpo.

1.2. CAPPING PROTEIN REGULATES WG, JNK AND SRC SIGNALLING

CP depletion triggers activation of Yki in the whole wing disc (Fernández et al., 2011). This triggers inappropriate tissue growth in the proximal wing disc epithelium. However, cells in the distal wing disc domain do not overgrow and undergo apoptosis in a JNK-dependent manner (Fig.3. in Chapter 3). The activation of JNK signalling could arise from both loss of cell polarity (Fig.1. and 11 in Chapter 3) and increase DE-cad protein level (Fig.6. and 11 in Chapter 3). Increased DE-cad may trigger reduction of Wg signalling, by the sequestration of the transcriptional co-activator Arm in the cytoplasm. Reduction of Wg signalling may in turn induce JNK-dependent apoptosis in the most distal wing disc domain (Adachi-Yamada et al., 1999), (Fig. 7. in Chapter 3). Interestingly, in the distal wing disc domain, overexpressing *Src64B* also affects cell polarity (Fig.4.2. in Chapter 4), up-regulates *DE-cad* transcription, triggers JNK signalling activity and

apoptosis (Fig.4.1. in Chapter 4). Moreover, decreasing *DE-cad* levels partially suppresses apoptosis and restores the tissue growth defects mediated by *Src64B* overexpression (Fig.4.6. in Chapter 4). Therefore, in the distal wing disc epithelium the outcomes of cells lacking CP, including loss of cell polarity, increased *E-cad* transcription, activation of JNK pathway and apoptosis recapitulates the phenotypes of tissues overexpressing Src. CP and Src may antagonize each other to regulate DE-cad transcription and AJs localization, which in turn, controls Wg signalling and JNK-mediated apoptosis. In agreement with this model, I found that overexpressing *cpa* and *cpb* restores the growth defects of tissues overexpressing *Src64B*, while knocking down *cpa* triggers increase Src phosphorylation. Since decreasing Btk29A levels suppresses apoptosis of *Src64B* overexpressing tissues but not of tissues depleted of Cpa, my observations indicate that CP has a dual effect on Src signalling: it prevents Src signalling downstream of Btk29A and inhibits Src phosphorylation and activity (Fig.4.7 to 4.9 and 4.12 in Chapter 4), (Fig.5.1.).

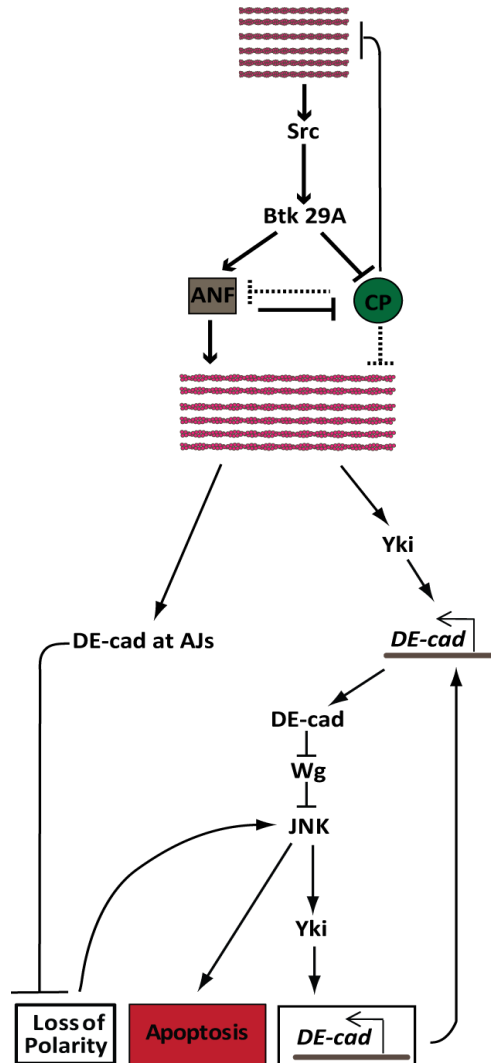


Figure 5.1 Model through CP function and Src signalling activity antagonize each other in the distal domain of wing imaginal disc.

CP and Src signalling activity may antagonize each other in the processes of the formation of F-actin networks involved in DE-cad recycling and Hpo pathway inhibition. Increased level of Src signalling activity or loss of CP may trigger accumulation of ectopic F-actin within these networks resulting in misslocalization of DE-cad from AJs and Yki activation. Upon DE-cad misslocalization cell polarity is disrupted activating JNK pathway. Activation of Yki triggers *DE-cad* transcription that inhibits Wg signalling activity potentiating the activity of JNK pathway and apoptosis. In turn activated JNK pathway may stimulate Yki nuclear localization and *DE-cad* transcription that may act as a fed back loop to further inhibits Wg, activate JNK and apoptosis.

1.3. INTERPLAY BETWEEN HIPPO, SRC, JNK AND WG SIGNALLING

CP represses Yki nuclear localization both in *Drosophila* and mammalian cells (Fernández et al., 2011; Sansores-Garcia et al., 2011). In addition, in the distal wing disc domain, CP loss results in the reduction of Wg target genes expression and JNK pathway activation (Fig.3. and 7 in Chapter 3.) It is important to consider that all these signalling pathways clearly interact *in vivo*. Starting from JNK signalling that is activated upon Src signalling in mammalian (Yoshizumi et al., 2000) and in *Drosophila* (Tateno et al., 2000). Moreover, Src signalling and Hpo pathway might interact at many distinct levels. dCsk that catalyzes the inhibitory phosphorylation of Src (Read et al., 2004), also phosphorylates Warts *in vitro* and *dCsk* and *wts* synergistically interact in a genetic assays in *Drosophila* (Stewart et al. 2003). These data suggest that dCsk, could at the same time prevent Yki nuclear localization in *Drosophila* tissue. In addition, several data indicate that Src signalling can regulate Hpo pathway activity. First, the rough eye phenotype, triggered by the overexpression of the form of *Src64B* insensitive to *dCsk* (*Src64B*^{Δ540}) can be suppress by removing one copy of *wts* (Vidal et al., 2007). Second, *Src64B* overexpression triggers transcription of *exp*, *wg* (data not shown) and *DE-cad* (Chapter 4 Fig.4.1.) all reported as Yki target genes (reviewed in Heallen et al., 2011). These data argue that Src signalling could positively regulate Yki activity. Finally, JNK signalling can induce Yki activation in a process of compensatory proliferation in *Drosophila* wing imaginal disc (Sun and Irvine, 2011). Interestingly nuclear Yki trigger transcription of *DE-cadherin* which has been shown to sequester β-catenin in cytoplasm and prevent it from participating in Wg signalling (Sanson et al., 1996; Widmann and Dahmann, 2009). Activated JNK might potentiate *DE-cad* transcription and reduction of Wg signalling in the most distal wing disc domain.

1.4. MOLECULAR AND CELLULAR ROLES OF CAPPING PROTEIN IN HIPPO AND SRC SIGNALLING

CP binds to the F-actin barbed end, preventing the addition or loss of actin monomers (G-actin), inhibiting in this way actin filaments (F-actin) elongation (reviewed in Cooper and Sept, 2008). Additionally, CP is thought to stabilize the barbed end of Arp1 microfilaments of the Dynactin complex that is required for vesicle transport along microtubules (Schafer et al., 1994). Finally, CP has been proposed to link F-actin to cellular membrane (Hutchings et al., 2003; Schafer et al., 1995; Schafer et al., 1998). Although I cannot rule out that in cells lacking CP, the defects of Hpo and Src signalling pathways are independent of F-actin regulation, I favor a model in which CP regulates both signalling pathways through its role on F-actin. In agreement with this model, F-actin polymerization, mediated by expression of a constitutive active form of Diaphanous (*Dia*^{CA}), which promotes F-actin polymerization or by knocking down Capulet (Capt), which sequesters G-actin, also affects Hpo signalling activity (Sansores-Garcia et al., 2011; Fernández et al., 2011). Conversely, F-actin depolymerization induced by drug treatments inhibits nuclear localization of YAP associated with the reduction of expression of YAP reporter genes (Sansores-Garcia et al., 2011). Therefore, F-actin regulation is critical to control Hpo signalling activity. However, several observations indicate that F-actin polymerization *per se* does not trigger Yki activation. First, F-actin accumulation upon loss of *twinstar* (*tsr*) does not induce upregulation of Yki target genes (Fernández et al., 2011). Second, overexpression of a mutant form of Actin (Actin^{V159N}), which stabilizes F-actin, does not induce YAP nuclear translocation in embryonic mammalian stem cells (Dupont et al., 2011). This argues that a specific F-actin population regulated by CP, Dia and Capt controls Hpo pathway activity.

Activation of Src through phosphorylation also depends on F-actin integrity (Fincham et al., 1996; Wang et al., 2005). Src co-localizes with F-actin in both colon cancer and mouse macrophage-like (RAW264.7) cells (Kim et al., 2010; Avizienyte et al., 2007).

F-actin depolymerization using drugs or actin depletion, decreases the association of Src with F-actin, resulting in inhibition of Src phosphorylation and loss of its kinase activity (Kim et al., 2010). Moreover, the integrity of F-actin is involved in Src translocation into the cell periphery, necessary for Src phosphorylation and its kinase activity (Fincham et al., 1996; Sandilands et al., 2004), (Fig.5.2.).

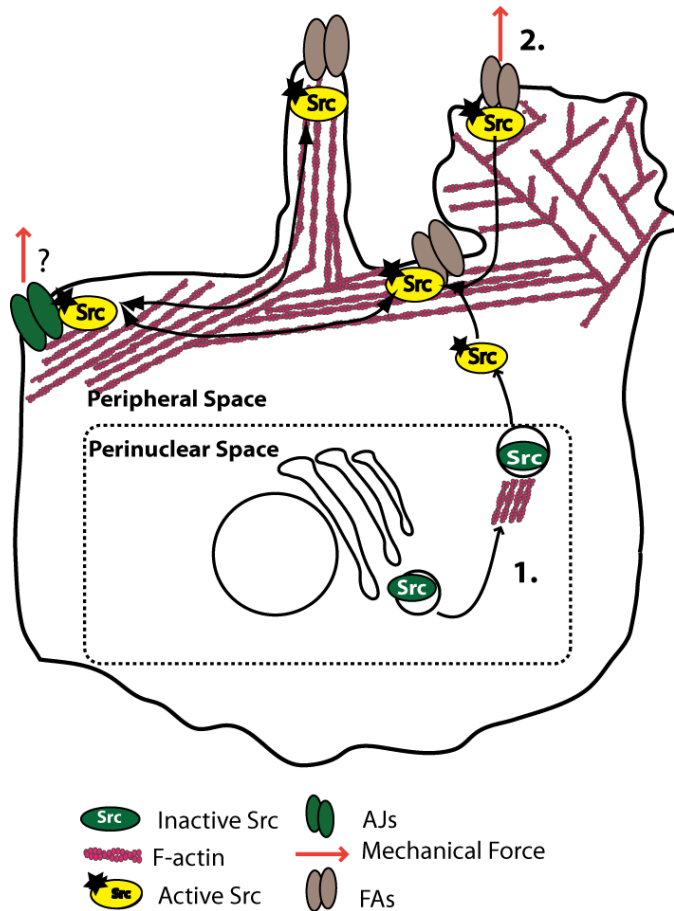


Figure 5.2 Modes of Src activation in mammalian cultured cells.

(1) Upon serum stimulation inactive Src is translocated from perinuclear space to cell periphery allowing for Src activation. Active Src associates within FAs. Src translocation occurs in endocytic vesicles linked to F-actin “clouds” which motility depends on RhoB, possibly mDia and Scar1 (proposed by Sandilands E.). (2) Application of the pulling force leads to the local activation of Src within FAs. Active Src initiates the wave of Src activation transduced through F-actin. The transduction of the wave of Src activation is possibly regulated by Rac1, Cortactin and Arp2/3 (proposed by Wang Y).

In addition mechanical stretching of the F-actin cytoskeleton contributes to Src activation (Tamada et al., 2004; reviewed in Giannone and Sheetz, 2006) (Fig.5.2.). Finally, F-actin depolymerization results in loss of the spatial transduction of Src activation, shown to occur upon the mechanical stimulation applied to HeLa cells transfected with Src reporter (Wang et al., 2005). In turn, Src signalling regulates F-actin organization in both physiological and pathological conditions (Avizienyte et al., 2007; Timpson et al., 2001; Sandilands et al., 2004).

2. SIGNAL TRANSDUCTION PATHWAYS REGULATE ACTIN DYNAMICS

2.1. HIPPO PATHWAY REGULATES THE ACTIN CYTOSKELETON

Interestingly, members of the core kinase cassette of the Hpo pathway are involved in inhibiting F-actin accumulation, both in *Drosophila* (Fernández et al., 2011) and in mammalian model system (Cole et al., 2008; Yang et al., 2004; Nola et al., 2011). In *Drosophila* mutations for *ex*, *hpo*, *sav*, *wts* and *mats* result in apical F-actin accumulation (Fernández et al., 2011). Importantly, F-actin accumulation is independent of Yki activity, suggesting a direct involvement of the core kinase cassette in F-actin organization. The exact mechanism by which Hpo pathway activity prevents F-actin accumulation is not known. The kinase activity of the Hpo pathway may regulate some actin-regulatory proteins through phosphorylation. Indeed, in mammalian cells, Lats1, the Wts homolog, inhibits LIMK1 kinase, which in turn promotes F-actin polymerization (Yang et al., 2004). Moreover, Ajuba, recently identified as a negative regulator of Hpo pathway activity is involved in Rac activation (Nola et al., 2011). Therefore, Hpo pathway activity may regulate F-actin by multiple ways. In turn, regulation of F-actin downstream of Hpo pathway activity is required to regulate the right amount of Yki going to the nucleus.

2.2. SRC SIGNALLING REGULATES THE ACTIN CYTOSKELETON.

The role of Src signalling in regulating the actin cytoskeleton has already been described in many biological processes, both in mammals and *Drosophila* (Saltel et al., 2004; Phillips et al., 2004; Newsome et al., 2004; Dodson et al., 1998). In mammalian model system, Src signalling can affect many actin-related targets and consequently the dynamics of F-actin (Ammer and Weed, 2008; Avizienyte et al., 2007; Timpson et al., 2001; Sandilands et al., 2004; Tanji et al., 2010). For example, one of the direct target of Src kinase activity is the Actin binding protein Cortactin (Cartwright et al., 1989). Phosphorylation of Cortactin triggers activation Arp2/3 and WASP that nucleate F-actin and trigger the formation of branched F-actin network (Ammer and Weed, 2008). In addition Src signalling regulates the activity of the family of Rho GTPase, which in turn, affects F-actin organization (Timpson et al., 2001; Sandilands et al., 2004). One mechanism by which Rho GTPase acts on F-actin includes activation of the mDia, a member of the Formin family that promotes F-actin nucleation and the formation of unbranched F-actin networks (Tanji et al., 2010). Finally Src kinase can *in vitro* phosphorylates β -actin that has been proposed to contribute to the rapid re-arrangements of F-actin organization in colon cancer cells (Avizienyte et al., 2007). In *Drosophila*, Src signalling mediates F-actin reorganization that contributes to ring canals growth in the egg chamber during oogenesis (Kelso et al., 2002; Thomas and Wieschaus, 2004; Lu et al., 2004). In addition during tracheal system development, DAAM that belongs to the Formin family regulates apical F-actin organization, but unlike in mammals, has been proposed to act upstream or in parallel to Src signalling (Matusek et al., 2006). In the distal wing disc epithelium, Src overactivity also results in apical F-actin accumulation (B. Garcia Fernández personal communication) through the activity of the Btk29A kinase (F. Janody, personal communication). Because I have shown that CP, which regulates F-actin polymerization controls Src signalling activity downstream of Btk29A (Chapter 4), My results argue that CP and Src control a apical F-actin network that regulates Src signalling activity.

3. THE ROLE OF ACTIN DYNAMICS IN THE REGULATION OF SIGNAL TRANSDUCTION PATHWAYS

3.1. REGULATION OF THE HIPPO PATHWAY BY ACTIN DYNAMICS

Induction of F-actin polymerization may affect trafficking of upstream regulators of the Hpo pathway. Actin cytoskeleton is essential for vesicle trafficking (reviewed in Merrifield, 2004) and CP contributes to the early events of endocytosis in yeast cells (Kim et al., 2006). Moreover, in *Drosophila* loss of CP is associated with membrane accumulation of crumbs (P. Gaspar, Master Thesis). However, F-actin depolymerization also affects vesicle trafficking (Durrbach et al. 2000) but does not trigger nuclear translocation of Yki. Thus, the role of the F-actin in regulating vesicle trafficking of the Hpo pathway components is unlikely.

A reduction of the pool of Actin monomers (G-actin) due to increase incorporation into actin filaments might regulate Hpo signalling activity, G actin may compete with Yki for binding to nuclear pore complexes (NPC), preventing Yki to be transported to the nucleus or may bind and tether Yki into the cytoplasm. However, overexpression of a mutant form of actin (actin R62D), which is unable to polymerize (Posern et al., 2002), does not affect YAP nuclear localization in cultured fibroblasts (Dupont et al., 2011), arguing that G-actin does not play a role in the Hpo signalling activity

F-actin could also act as a linker for the upstream components of Hpo pathway. Both Merlin and Expanded belong to FERM family proteins that act as a linkage between F-actin and the membrane (McClatchey and Giovannini, 2005). Increase in F-actin accumulation could tether Merlin and Expanded, preventing in this way sequestration of Yki in the cytoplasm, whereas F-actin depolymerization could trigger Merlin and Expanded release. However, both Merlin and Expanded localize normally in cells that loss CP function (Fernández et al., 2011; Sansores-Garcia et al., 2011), arguing that F-actin is unlikely to inhibit Yki nuclear localization through this mechanism.

Interestingly, upstream regulators of the Hpo pathway, such as Expanded, Merlin, Fat, Kibra, Lgl, α PKC and crumbs localize apically (reviewed in Genevet and Tapon, 2011). Moreover, apically localized pool of Hpo has been reported in the eye imaginal disc and a significant portion of Mats is apically localized (reviewed in Genevet and Tapon, 2011). Furthermore, forcing the apical membrane tethering of the MST1 (a mammalian homolog of Hpo), by adding a myristylation signal, leads to its activation (Praskova et al., 2004). Finally, many members of the pathways contain PPxY and WW domains and directly interact between each other (reviewed in Genevet and Tapon, 2011). These data suggest that the apical membrane is the active site, which regulates Yki activity. Because loss of *CP* or *capt* induces apical F-actin accumulation (Baum et al., 2000; Janody and Treisman, 2006) and activation of Yki (Fernández et al., 2011; Sansores-Garcia et al., 2011), this support the idea that in *Drosophila* epithelia, an apical F-actin population acts as a scaffold to tether Hpo pathway components apically regulating in this way Yki activity.

Alternatively, F-actin polymerization could induce Yki nuclear localization through mechanical stimulation. In agreement with this hypothesis, fibroblast cultured on ridge substrates, that mimics conditions of force application, exhibit robust F-actin based stressed fibers that underlie focal adhesions, and nuclear localization of YAP. Conversely, on softer substrates, YAP is cytoplasmic and stress fibers are smaller (Dupont et al. 2011). In a tissue context, mechanical forces applied by neighboring cells are sensed and propagated through AJs (reviewd in Gomez et al., 2011). Since E-cad-mediated adhesion suppresses Hpo pathway activity (Kim et al., 2011), mechanical forces propagated through AJs may be senses by the actin cytoskeleton, which responds by controlling the right levels of active Yki in the cell. Accumulation of an apical F-actin network, due to *CP* or *capt* loss or *Dia*^{CA} expression, may increase intracellular forces and trigger Yki nuclear translocation.

3.2. REGULATION OF SRC ACTIVITY BY ACTIN DYNAMICS

In contrast to mammalian cells, in which FAs are considered as the primary sites of Src activation (reviewed in Cabodi et al., 2010) in *Drosophila*, activated Src (pSrc) is enriched mostly within AJs (Vidal et al., 2006; Shindo et al., 2008; Takahashi et al., 2005), (Fig.3.8 in Chapter 3). In addition *Drosophila*, Src42A co-immunoprecipitates with Arm and DE-cad, in embryonic extracts (Takahashi et al., 2005). These data suggest that in *Drosophila* epithelial tissues, the activation of Src through phosphorylation occurs within AJs. Following loss of CP, a defect in the apical F-actin network may directly affect the phosphorylation status of Src since F-actin integrity is essential for Src activation in mammals.

Different mechanisms can explain how F-actin accumulation, as a result of CP loss, enhances the activation of Src through phosphorylation. The F-actin network regulated by CP may be required to control Src trafficking. Src is translocated to the cellular membrane in vesicles, whose transit depends on F-actin based structures called F-actin “clouds”, regulated by RhoB and Arp2/3 (Sandilands et al., 2004). Loss of CP may stimulate the formation of F-actin “clouds”, resulting in higher rate of Src delivery to the cellular membrane and consequently higher levels of activated Src (Fig.5.3.).

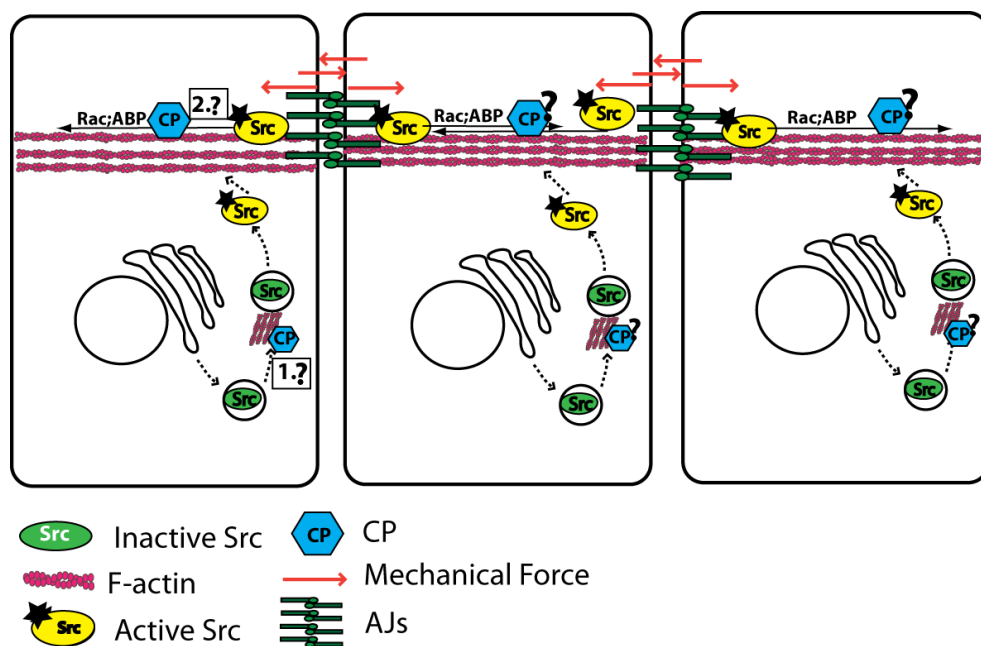


Fig.5.3. Model of Src activation in *Drosophila* epithelial tissue and the potential role of CP in the process of Src activation.

(1.) CP may be involved in the formation of the F-actin clouds and translocation of Src to the cellular membrane. (2.) Application of pulling and pushing forces by the adjacent cells may trigger local Src activation that is transduced through apical F-actin. CP loss could result in increase of the mechanical forces that trigger local Src activation or together with Arp2/3 and Cortactin contribute to the transduction of Src activation.

Consistent with this possibility, CP depletion leads to enrichment of F-actin foci in the cytoplasm (Fig.2.5. in Chapter 2) that could resemble F-actin “clouds”. Moreover, reduction of one copy of *Rho1*, which in mammals is involved in Src membrane translocation (Fincham et al., 1996), suppresses apoptosis of dCsk-depleted (Vidal et al., 2006) or *Src64B* overexpressing wing disc tissues (F- Janody, personal communication). However, trafficking of F-actin “clouds” has been proposed by Emma Sandilands to be regulated in the same principles as Vaccinia virus internalization (Sandilands and Frame, 2008). In contrast, CP activity is predicted to increase the movement of virus containing vesicles (Weisswange et al., 2009; Akin and Mullins, 2008). Thus, if CP would be involved in the formation of F-actin “clouds” its loss would result in the reduction of the velocity of the Src-containing endosomes.

Alternatively the F-actin network, formed upon *CP* loss, may be enriched in Myosins generating forces that contribute to Src activation (reviewed in Giannone and Sheetz, 2006). In agreement with this possibility, Src activity seems also to be regulated by tension in *Drosophila* since mechanical stimulation of the embryo, triggers Src42A-dependent nuclear translocation of Arm (Desprat et al., 2008). In addition, F-actin accumulation may also increase the spatial transduction of Src activation (Fig.5.3.).

C. Regulation of Src signalling activity by F-actin

In cancer cells, Src signalling has a well known role in regulating the organization of the cortical F-actin that contributes to cell invasion and metastasis (Saltel et al., 2004; Avizienyte et al., 2007). My results argue that regulation of the actin cytoskeleton by CP and Src activities mediates all the effects of Src, including proliferation and apoptosis (Chapter 3 and 4). However, what is the role of this F-actin network in controlling the balance between proliferation and apoptosis downstream of Src is not known. Interestingly, in the distal wing disc epithelium, either loss of *CP* or *Src64B* overexpression triggers DE-cad loss from AJs (Fig.6. in Chapter 3; Fig.4.2. in Chapter 4). Src42A regulates AJs turnover in *Drosophila* tracheal epithelial cells, (Shindo et al., 2008) and DE-cad turnover is regulated by endocytosis through the Rho GTPase Cdc42 and Arp2/3-dependent F-actin remodeling in the pupae wing disc (Georgiou et al., 2008). Thus, the F-actin network regulated by Src and CP may control endocytosis-mediated DE-cad recycling (Fig.5.4.). Consistent with this possibility, Src controls endocytosis through regulation of the Rho GTPases (Sandilands et al., 2004). In addition, in yeast, CP together with Arp2/3 contribute to the early stages of endocytosis (Kim et al., 2006). Moreover, in *Drosophila* wing disc, CP co-localizes with Rab5, a marker of early endocytic vesicles (P. Gaspar, Master Thesis). The defects in DE-cad recycling can give rise to loss of DE-cad from AJs and its baso-lateral mislocalization (Georgiou et al., 2008). The F-actin network downstream of Src and CP may also inhibit Hpo signalling activity, triggering an increase in DE-cad levels through elevated Yki, activity. Thus, I propose that

the F-actin network formed upon high levels of Src activity or reduced levels of CP, triggers DE-cad mislocalization from AJs, and an increase in DE-cad protein levels, which both synergize to induce activation of the JNK pathway and apoptosis in the distal domain of the wing disc (Fig.5.1 Chapter 5). Upon weak increase or decrease of Src or CP activities respectively, the F-actin network formed might be sufficient to affect Hpo pathway activity and trigger increase in DEcad levels but un-sufficient to disrupt AJs turnover and the loss of AJs components. Consequently, the tissue kept an epithelial architecture and overgrowth due to disruption of Hpo pathway activity.

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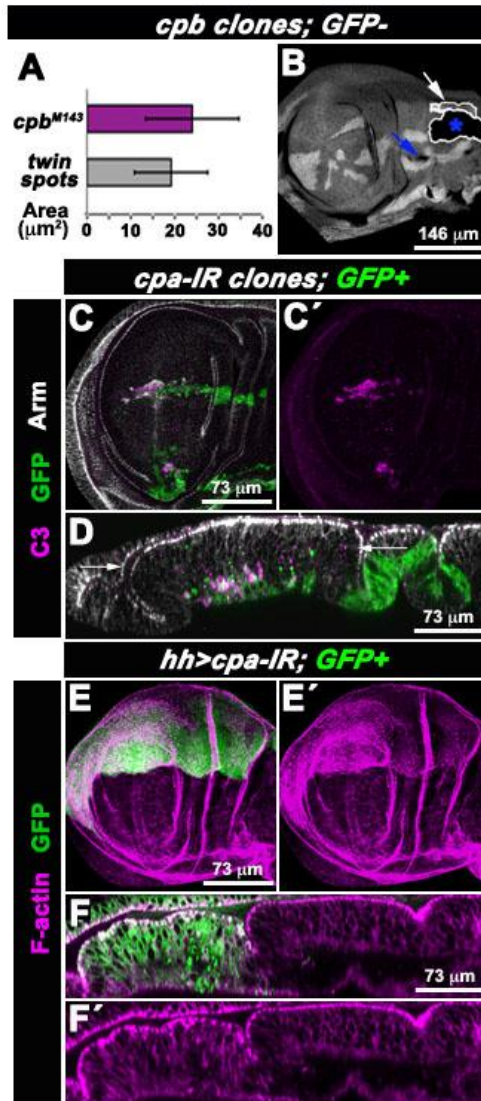
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APPENDIX

1. ACTIN-CAPPING PROTEIN AND THE HIPPO PATHWAY REGULATE F-ACTIN AND TISSUE GROWTH IN *DROSOPHILA*

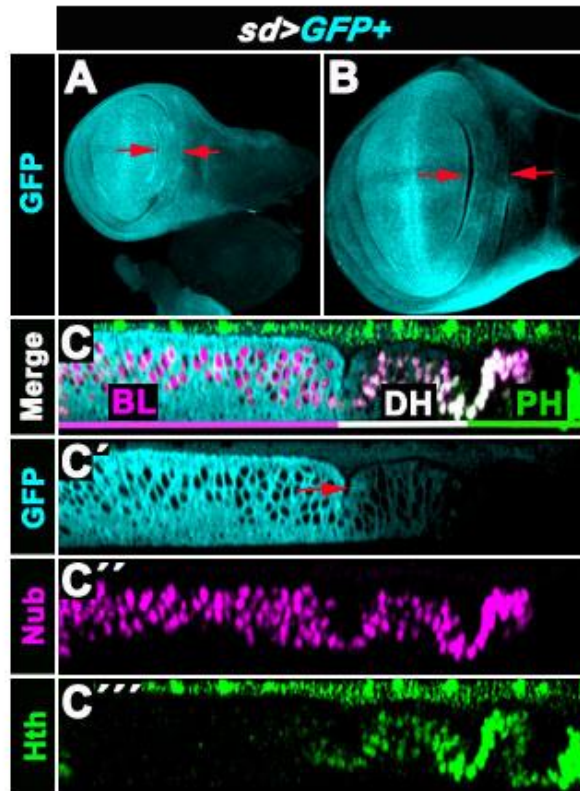
2. SUPPLEMENTARY DATA



Supplemental Figure S1

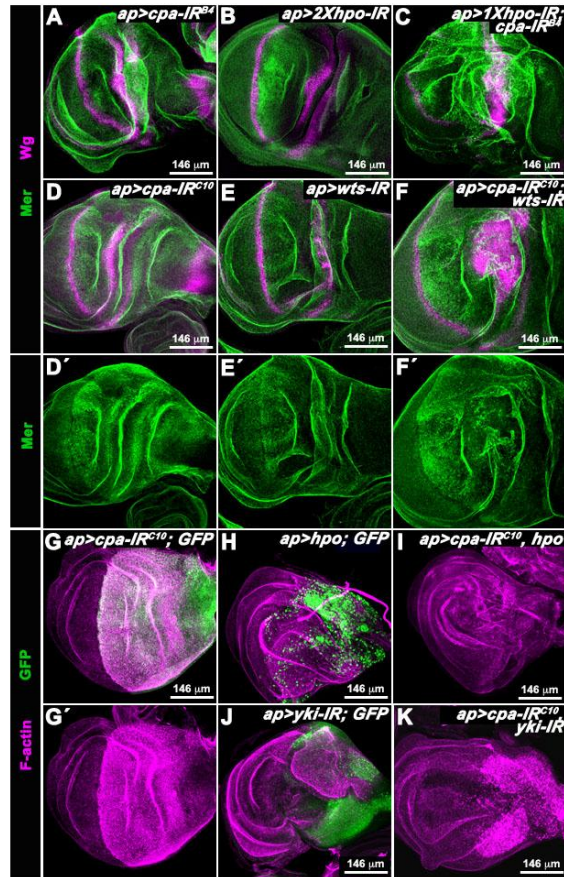
cpa-IR recapitulate the CP mutant allele phenotype. (A,B) *cpbM143* mutant clones are larger on average than wild-type twin spots. (A) Surface area of *cpbM143* mutant clones with no GFP and their wild-type twin spots with two copies of GFP in the proximal wing disc epithelia. The mean for *cpbM143* is 24, for wild-type twin spot is 19.18. Error bars indicate s.e.m. $P < 0.328$. (B) *cpbM143* mutant clones marked by the absence of GFP (white). The blue asterisk indicates a large *cpb* mutant clone outlined in white with no GFP. The white arrow indicates the sibling twin spot with two copies of GFP outlined in white. The blue arrow indicates a small *cpb* mutant clone with no GFP. Note that within the proximal wing disc epithelium, small and large *cpb* mutant clones are recovered, whereas *cpb* mutant clones do not grow within the distal wing disc epithelium. (C-F') Third instar imaginal discs. Standard confocal sections (C,C',E,E') and optical

cross-sections (D,F,F') through disc epithelia. (C-D) Clones expressing UAS-cpa-IRC10 and UAS-GFP (green in C and D) stained with anti-Arm (white in C and D) and anti-Caspase 3 (magenta). Note that clones extrude and die in the wing blade epithelium but are maintained in the remainder of the wing disc. The white arrows in D define the wing blade region. (E-F') hh-Gal4 driving UAS-cpa-IRC10 and UAS-GFP (green in E and F) stained with TRITC-Phalloidin to reveal F-actin (magenta).



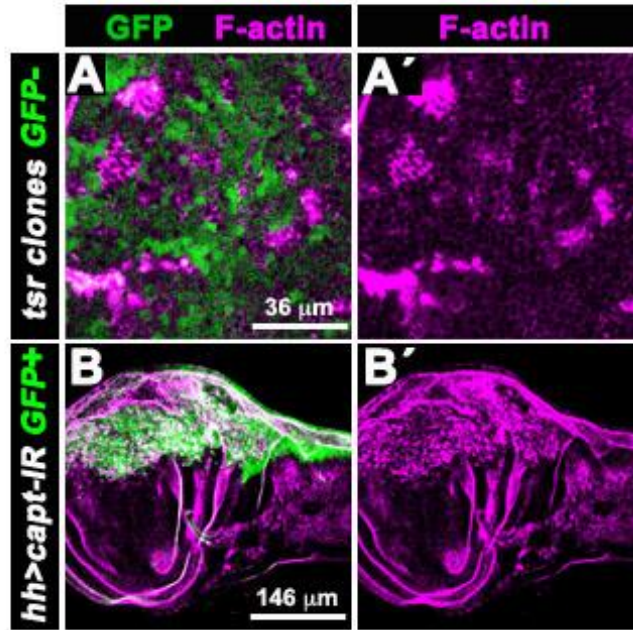
Supplemental Figure S2

UAS-GFP under *sd*-Gal4 control is expressed in the distal hinge domain at mid-third instar larvae, but faded down by the end of third instar larvae. (A-C''') Third instar imaginal discs in which *sd*-Gal4 drives UAS-GFP (cyan in A-C') shown as standard confocal sections (A,B) or optical cross-sections (C-C''') through disc epithelia. A shows mid-third instar larvae; B-C''' show end of third instar larvae. Discs are stained with anti-Nub (magenta in C,C'') and anti-Hth (green in C,C'''). The proximal domain, stained only with anti-Nub (magenta bar in C) represents the blade (BL). The domain stained with both anti-Nub and anti-Hth (white bar in C) represents the distal hinge (DH) and the domain stained only with anti-Hth (green bar in C) represents the proximal hinge (PH).



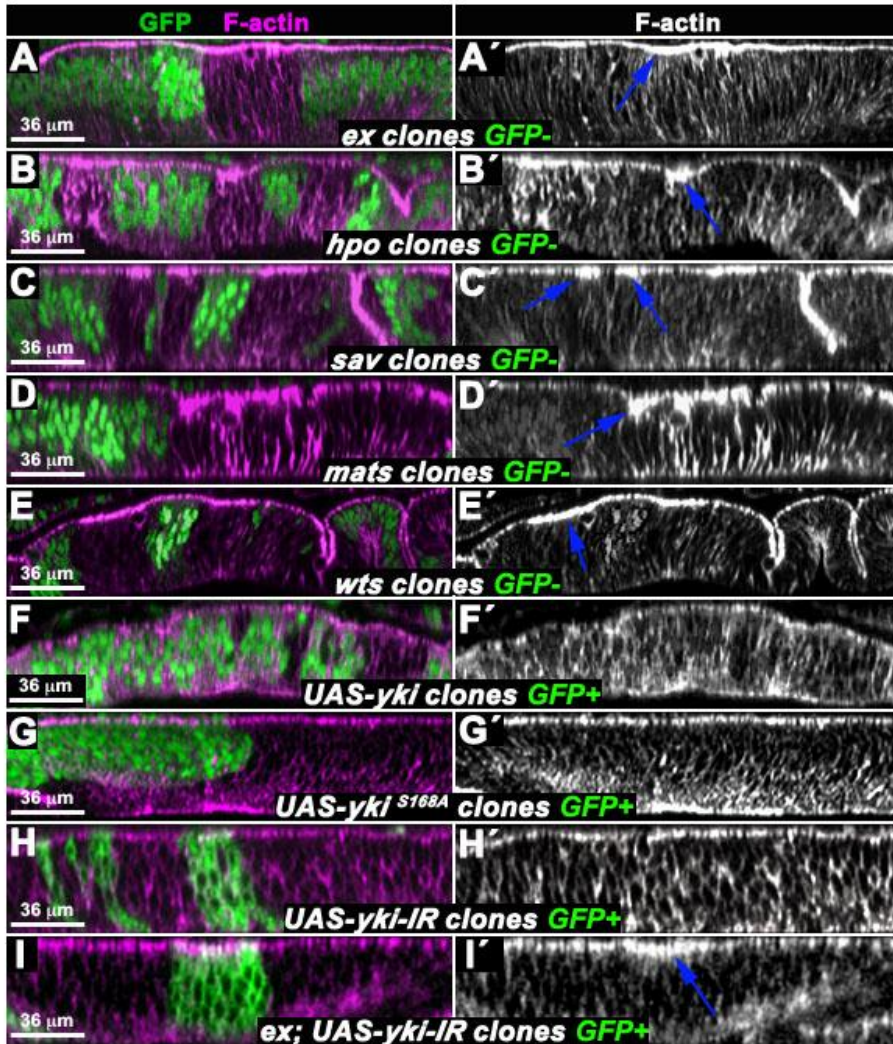
Supplemental Figure S3

CP and the Hpo pathway have additive effects on proliferation and survival genes. (A-K) All panels show third instar imaginal discs. *ap*-Gal4 driving UAS-*cpa*-IRB4 (A); two copies of UAS-*hpo*-IR (B); UAS-*cpa*-IRB4 and one copy of UAS-*hpo*-IR (C); UAS-*cpa*-IRC10 (D,D'); UAS-*wts*-IR12072R-1 (E,E'); UAS-*cpa*-IRC10 and UAS-*wts*-IR12072R-1 (F,F'); UAS-*cpa*-IRC10 and UAS-GFP (G,G') or UAS-*hpo*M11.1 and UAS-GFP (H); UAS-*hpo*M11.1 and UAS-*cpa*-IRC10 (I); UAS-*yki*-IR4005R-2 and UAS-GFP (J); or UAS-*yki*-IR4005R-2 and UAS-*cpa*-IRC10 (K). Discs are stained with (in A to F) anti-Mer (green) and anti-Wg (magenta) or (in G to K) TRITC-Phalloidin to reveal F-actin (magenta).



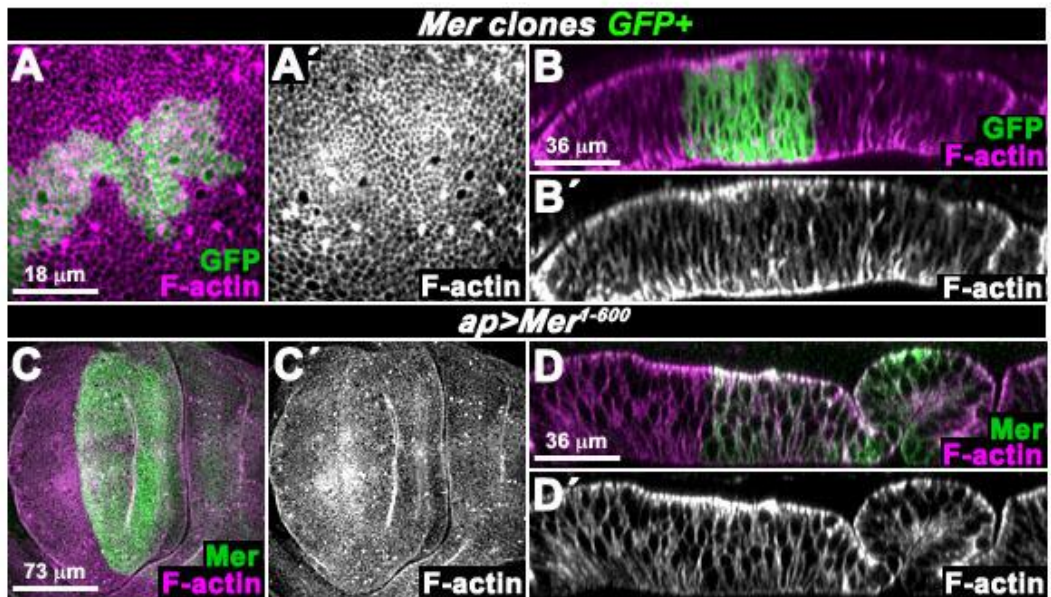
Supplemental Figure S5

Reducing Capt levels or Tsr triggers F-actin accumulation. All panels show standard confocal sections of third instar wing imaginal discs stained with TRITC-Phalloidin to reveal F-actin. (A,A') Mutant clones for *tsr110M* marked by the absence of GFP (green in A). (B,B') *hh*-Gal4 driving expression of UAS-capt-IR5061R-2 and UAS-GFP (green in B).



Supplemental Figure S6

Loss of *ex*, *hpo*, *wtls* or *mats* but not *yki* overexpression induces apical F-actin accumulation. All panels show optical cross-sections through third instar wing imaginal disc stained with TRITC-Phalloidin to reveal F-actin (magenta in A-I and white in A'-I'). (A-E') Clones marked by the absence of GFP (green in A,B,C,D,E), mutant for *exe1* (A,A'), *hpo42-47* (B,B'), *sav3* (C,C'), *matse235* (D,D'), or (E,E') *wtslatsX1*. (F-I') Clones positively labelled with GFP (green in F,G,H,I), overexpressing *UAS-yki* (F,F'), *UAS-yki^{S168A}* (G,G') or *UAS-yki-IR4005R-2* (H,H') and mutant for *exe1*(I,I').



Supplemental Figure S7

Mer has no major effect on F-actin. All panels show third instar wing imaginal discs. A, A', C and C' show standard confocal sections; B, B', D and D' show optical cross-sections through the epithelia. (A-B') Mer4 mutant clones positively labelled with GFP (green in A,B). (C-D') ap-Gal4 driving UAS-Mer1-600. Discs are stained with TRITC-Phalloidin to reveal F-actin (magenta in A,B,C,D and white in A',B',C',D') and anti-Mer (green in C and D).

Table S1. Wing disc growth classification for various genetic contexts where levels of Cpa and/or components of the Hpo pathway were altered

Genotype*	No growth [§]	Wild type [§]	Mild [§]	Moderate [§]	Strong [§]	n [‡]
<i>aP>cpa-IR^{C10}</i>	–	6.7%	30%	50.3%	13%	38
<i>aP>2Xhpo-IR</i>	–	–	14.2%	85.7%	–	7
<i>aP>hpo-IR; cpa-IR^{B4}</i>	–	–	–	14.2%	85.7%	14
<i>aP>wts-IR</i>	–	10%	40%	30%	20%	10
<i>aP>cpa-IR^{C10}; wts-IR</i>	–	–	5%	25%	70%	20
<i>aP>hpo; GFP</i>	100%	–	–	–	–	7
<i>aP>cpa-IR^{C10}; hpo</i>	100%	–	–	–	–	5
<i>aP>yki-IR; GFP</i>	100%	–	–	–	–	15
<i>aP>cpa-IR^{C10}; yki-IR</i>	100%	–	–	–	–	9

*UAS-*cpa-IR^{C10}* UAS-GFP (row 1) or UAS-*hpo-IR* (row 2) or UAS-*cpa-IR^{B4}* and UAS-*hpo-IR* (row 3) or UAS-*wts-IR^{12072R-1}* (row 4) or UAS-*cpa-IR^{C10}* and UAS-*wts-IR^{12072R-1}* (row 5) or UAS-*hpo^{M11.1}* and UAS-GFP (row 6) or UAS-*cpa-IR^{C10}* and UAS-*hpo^{M11.1}* (row 7) or UAS-*yki-IR^{4005R-2}* and UAS-GFP (row 8) or UAS-*cpa-IR^{C10}* and UAS-*yki-IR^{4005R-2}* (row 9) driven under *apterous*-Gal4 driver.

[§]Percent of wing discs that fail to grow (no growth), that do not display any obvious phenotype (wild-type), that displayed weak hinge overgrowth without the appearance of folding (mild), that showed clear dorsal overgrowth, associated with extra folds in the hinge region (moderate) or that displayed extensive overgrowth with many folds formed within the tissue (strong).

[‡]Total number of wing discs analysed.