A study of Focal Adhesion Kinase in cancer using *Drosophila melanogaster*

Juan Pablo Macagno

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Beatson Institute for Cancer Research
College of Medical, Veterinary and Life Sciences
University of Glasgow
Glasgow
UK

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Abstract

Cancer is a group of diseases that affects almost every organ of the human body. A normal cell transforms into a cancer cell as a consequence of cumulative failures that alter diverse cellular processes such as cell proliferation, adhesion, migration, and cell death.

Focal Adhesion Kinase (FAK) is a ubiquitous protein that is involved in all these cellular processes. Therefore, it is not surprising that FAK plays important roles in cancer; in fact, it has been linked to tumour progression or regression depending on the cellular and genetic context. We used *Drosophila melanogaster* as a model organism to study FAK's duality in cancer. In this thesis we describe two novel roles of *Drosophila* FAK (FAK56): as a tumour suppressor within receptor tyrosine kinases (RTKs)-driven contexts, and as a tumour promoter by inhibiting cell death in nervous tissues.

We investigated how FAK56 regulates signalling resulting from the overexpression of RTKs RET and EGFR. Our data indicated that FAK is a suppressor of RTKs in fly epithelia. This was also observed in human cancer cell lines, suggesting an evolutionary conserved mechanism. On the other hand, we found FAK56 prevented caspase-dependent cell death and uncovered a novel link between FAK56 and Relish, the *Drosophila* homologue of human NF-κB: *Relish* mutants suppressed *FAK56* loss-induced cell death in the larval central nervous system and eye imaginal discs.

As supported by the results presented in this thesis, FAK may be a good therapeutic target in cancer biology; however, in some contexts it may also behave as a tumour suppressor. Therefore, we conclude it will be necessary to identify the context of FAK activity before designing therapeutic strategies against FAK-expressing tumours.

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Author’s declaration

I hereby declare that all the work reported in this thesis is my own unless otherwise stated. None of the work has been previously submitted for any other degree at any other institution. All sources of information used in the preparation of this thesis are indicated by reference.

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Publications

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Cordero, J.B., Macagno, J.P., Stefanatos, R., Strathdee, K., Cagan, R., Vidal, M. (2010) Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter. *Dev Cell* 18: 999–1011.

Abbreviations

˚C	grades Celsius/Centigrades
Δ	delta/deletion/truncated
aa	amino acid(s)
act	actin
AKT/PKB	Protein Kinase B
ANOVA	Analysis of variance
BCL-2	B-Cell Lymphoma 2
BDSC	Bloomington Drosophila Stock Center
BSA	Bovine serum albumin
Caspase	Cysteine-aspartic protease
CO2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
dcp-1	Death caspase-1
DD	Death domain
DGRC	Drosophila Genetic Resource Center
dH_2O	Distilled Water
DIABLO	Direct IAP-Binding protein with Low PI
DIAP1	Drosophila Inhibitor of Apoptosis Protein 1
dlg	disc large
DMEM	Dulbecco`s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dp53	Drosophila p53
dpp	decapentaplegic
Dredd	Death related ced-3/Nedd2-like protein
DrICE	*Drosophila* interleukin-1beta-converting enzyme
Dronc	Drosophila melanogaster NEDD2-like caspase
DSHB	Developmental Studies Hybridoma Bank
dUTP	Deoxyuracil triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial growth factor
EGFR	Epidermal growth factor receptor
egr	Eiger
ELAV	Embryonic lethal abnormal vision

ERK1/2	Ras-dependent extracellular signal-regulated kinase
ey	eyeless
FADD	Fas-Associated protein with Death Domain
FAK	Focal Adhesion Kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-like interleukin-1 beta-converting enzyme
FLIP	FLICE-inhibitory protein
FLP	Flippase
FRT	Flippase recognition target
G-proteins	Guanine-Nucleotide-Binding Proteins
GFP	Green flourescent protein
GMR	glass multimer reporter
Grb2	Growth Factor Receptor Bound Protein 2
h/hr	hour(s)
HCl	Hydrochloric acid
hid	head involution defective
IMD	immune deficiency
IOPs	Interommatidial precursors
Ird5	immune response deficient 5
JNK	c-Jun N-terminal kinases
kb	Kilobase Pairs
kDa	kilo Dalton
lgl	lethal giant larvae
MAPK	Mitogen activated protein kinase
MARCM	Mosaic analysis with a repressible cell marker
MDM2	Murine double minute 2
MET/ HGFR	Hepatocyte growth factor
mM	miliMolar
MOPS	3-(N-morpholino)propanesulfonic acid
N-Cad	N-Cadherin
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline triton X-100
PCD	Programmed Cell Death

PI3K	Phosphatidylinositol 3- Kinase
ptc	patched
PVDF	Polyvinylidene Fluoride
Ras	Rat sarcoma
RET	Rearranged during transfection
RFP	Red fluorescent protein
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
scrib	scribble
SDS	sodium dodecyl sulfate
SEM	Standard error of the mean
siRNA	Small interfering RNA
Smac	Second Mitochondria-derived Activator of Caspases
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween 20
TNF/TNFR	Tumour necrosis factor/TNF receptor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF2	TNF receptor-associated factor 2
tub	tubulin
TUNEL	Terminal Deoxynucloetidyl Transferase-mediated dUTP Nick End Labelling
UAS	upstream activating sequence
UV	ultraviolet
v	vermilion gene
VDRC	Vienna Drosophila RNAi Center
VEGFR	Vascular endothelial growth factor
w	white gene
W/V	weight per volume
WT	wild type
y	yellow gene

Chapter 1 – Introduction

1.1 Cancer

Cancer is a complex set of diseases that develops as a multistep process and affects almost every organ of the human body. When a cell derails from its normal course and transforms into a hyper proliferative death-resistant cell type it may lead to an overgrowth within the tissue. The environment, composed of different cell types in the vicinity and immune cells, plays a major role in the development or suppression of this abnormal phenomenon (Hanahan and Weinberg, 2011).

A mass of cells growing abnormally in a tissue is called a tumour. These entities form as a result of mutations that activate genes that favour tumour expansion (oncogenes), and/or deactivate genes that suppress cell growth and migration or induce death (tumour suppressor genes). A primary tumour, i.e. the original heterogeneous group of tumour cells in its founding site, can also acquire different properties that make it more aggressive and malignant. The communication with neighbour cells and the immune system has been proved essential for this outcome. Cell migration helps tumour cells to invade surrounding tissues. If this movement leads cells into the blood or lymphatic stream, tumour cells will be able to travel throughout the body and eventually they will find a new niche. This process is called metastasis and derives in the formation of secondary tumours in different locations from its original site. These later stages of tumour progression are typically what cause more cancer-related deaths in humans (Stefanatos and Vidal, 2011, Parisi and Vidal, 2011, Hanahan and Weinberg, 2011).

There are over 200 hundred types of cancer and generally they originate and behave differently based on its location in the body (CRUK, 2012). Therefore, cancer requires intensive studies of its several features in order to better understand its diversity and complexity in all the possible scenarios. Further and more detailed investigation will bring us closer to stop some of the tumour formation steps and thus, reduce cancer-related mortality.

1.2 Discovery of Focal Adhesion Kinase

Focal Adhesion Kinase (FAK) was discovered independently as a tyrosine-phosphorylated substrate of v-Src upon Rous-sarcoma Virus infection of chicken embryo cells (Kanner et al., 1990, Schaller et al., 1992) and downstream of Integrins in mouse fibroblast cells plated onto fibronectin (Guan et al., 1991, Guan and Shalloway, 1992, Hanks et al., 1992).

Focal Adhesion Kinase owes its name to the localisation observed in those cells and for being a functional kinase. Focal adhesions (FA) are sites where cell and extracellular matrix (ECM) interact and FAK was shown to co-localise with known FA proteins such as Tensin, Talin and Integrins themselves. Also, FAK was able to phosphorylate itself and substrates on tyrosine residues (Hanks et al., 1992, Schaller et al., 1992, Guan and Shalloway, 1992).

Overall, these observations indicated FAK was involved in normal processes such as cell-extracellular matrix attachment, but also in abnormal scenarios, such as oncogenic transformation (see section 2.3).

Additionally, FAK was later implicated in signalling pathways downstream of other membrane receptors such as G-protein-coupled receptors (Zachary et al., 1992) and receptor tyrosine kinases (RTKs) (Sieg et al., 2000) (Lu et al., 2001) (Chen and Chen, 2006) (Garces et al., 2006) (Plaza-Menacho et al., 2011), and further scaffolding functions were also uncovered (Lim et al., 2008) (Kurenova et al., 2004) (Schlaepfer et al., 1994) (Kessler and Muller, 2009).

1.3 FAK structure

Nucleotide and protein sequence analysis determined FAK was a 125kDa polypeptide ($p125^{FAK}$), comprised of 1052 amino acid residues and three separate domains: an N-terminal band 4.1, ezrin, radixin, moesin (FERM) domain, followed by a tyrosine kinase domain and a C-terminal focal adhesion targeting (FAT) domain (Figure 1.1).

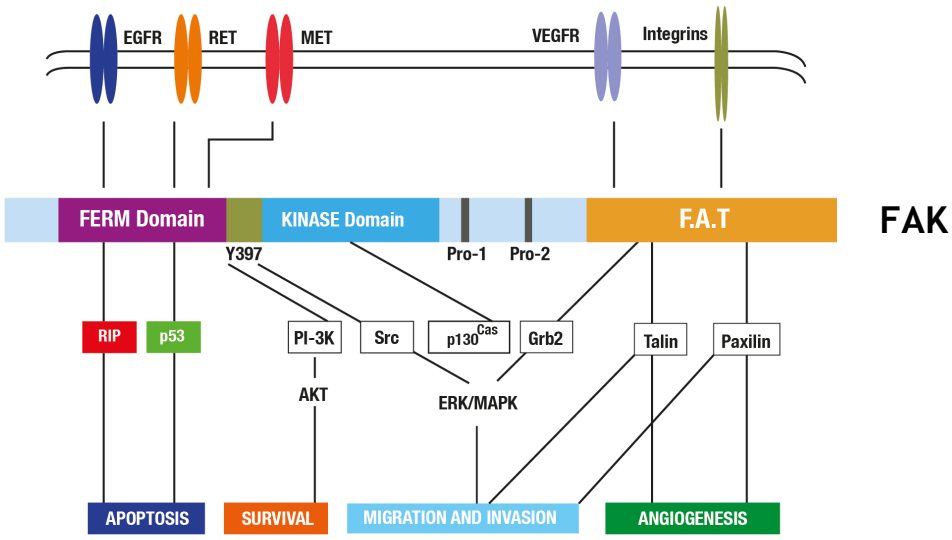

Figure 1.1 Schematic representation of FAK protein and its binding partners
Linear representation of the three domains of FAK: FERM domain (purple), Kinase domain (turquoise) and FAT domain (orange). The autophosphorylation site is also highlighted (Y^{397}). Pro-1 and Pro-2 represent proline-rich sequences in the C-terminal domain. There is another Pro region in the FERM domain not depicted in this representation. FAK is involved in interactions with multiple partners such as RTKs EGFR and RET, p53 and RIP through its FERM domain and focal adhesion proteins like Integrins, Talin and Paxilin through its FAT domain. These interactions involve FAK in several signalling pathways such as PI3K/AKT or Ras/MAPK and in diverse cellular processes such as apoptotic cell death, survival and proliferation, cell migration and invasion, and angiogenesis.

Although the kinase domain is similar to the catalytic domain of all protein kinases, the overall architecture (with the FERM and FAT domains) is unique to FAK meaning that FAK is a distinctive protein tyrosine kinase (PTK). The deduced amino acid sequence does not contain a hydrophobic transmembrane region or acylation site, unlike RTKs or Src-family kinases respectively (Hanks et al., 1992, Schaller et al., 1992).

Thus, FAK is the founding member of a structurally conserved family of cytoplasmic non-receptor protein-tyrosine kinases (PTK). FAK is evolutionary conserved in mammalian species and lower organisms such as frogs, zebrafish and *Drosophila* (Hanks et al., 1992, Schaller et al., 1992, Zhang et al., 1995, Hens and DeSimone, 1995, Henry et al., 2001, Fox et al., 1999, Fujimoto et al., 1999, Palmer et al., 1999). This PTK subfamily so far comprises of two mammalian members: FAK and Pyk2 (Proline-rich tyrosine kinase 2).

Pyk2 has similar domains and shares significant similarity with FAK: 45% overall identity and 60% identity in the kinase domain. (Avraham et al., 1995, Herzog et al., 1996, Sasaki et al., 1995, Lev et al., 1995, Yu et al., 1996). Although they share some interacting partners, Pyk2 binds other proteins as

well, shows a different expression pattern and responds to different stimuli (Menegon et al., 1999) (Xiong and Mei, 2003) (see section 1.4.2).

1.3.1 Domains

1.3.1.1 FERM domain

FERM domains are large modules (≈400 amino acids) normally present in cytoplasmic proteins that link the cell membrane with the cytoskeleton by interacting with proteins or phospholipids. FERM-containing proteins, including FAK, also contain nuclear localisation signals (NLS) and nuclear export signals (NES), thus indicating they are able to bring information to the nucleus in order to coordinate ECM and nuclear events (Frame et al., 2010).

The crystal structure of the FAK FERM domain (Ceccarelli et al., 2006) showed a 'clover leaf' structure consisting of three lobes (F1, F2 and F3) (Figure 1.2). Their intramolecular interactions together with FERM binding proteins control FAK activation and localisation. Several FAK FERM interacting partners have been reported: Integrin–β tails (Schaller et al., 1995), several RTKs such as EGFR, PDGF (Sieb 2000), VEGFR (Garces et al., 2006), RET (Plaza-Menacho et al., 2011) (Sandilands et al., 2012b) and MET (Chen and Chen, 2006), and some novel interactions like receptor interacting protein (RIP) (Kurenova et al., 2004), p53 and MDM2 (Golubovskaya et al., 2005) (Lim et al., 2008)(Figure 1).

1.3.1.2 Kinase domain

FAK kinase domain contains all the structural motifs common to protein kinases such as an ATP-binding site, three residues postulated to interact with the γ-phosphate of ATP, and an aspartate residue predicted to be the catalytic site (Schaller et al., 1992). Two regions containing tyrosine residues are the most important for its activity: the linker segment, connecting FERM with Kinase domain, has the auto-phosphorylation site, Tyr^{397}; and the activation loop contains two tyrosine residues, Tyr^{576} and Tyr^{577}, which are phosphorylated by Src and are required for maximal catalytic activity (Calalb et al., 1995).

FAK is able to auto-inhibit itself. This occurs when the FERM domain folds and binds the catalytic domain; this conformation hides the activation loop and

the autophosphorylation site preventing Src recruitment (Cooper et al., 2003) (Lietha et al., 2007) (Figure 1.2).

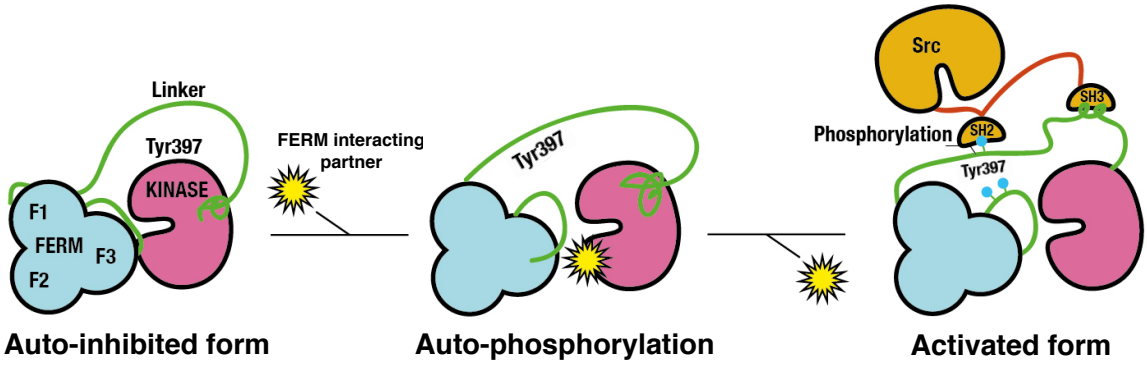

Figure 1.2 Overview of FAK activation
FAK Interaction with an interacting partner such as RTKs and/or phosphatidylinositol 4,5-P_2 (PIP2) lipid leads to conformational changes that unlock the tyrosine-397 (Y^{397}) in the linker region and allows its auto-phosphorylation *in trans*. Phosphorylated Y^{397} recruits SH2 domain of Src. Also, a proline-rich sequence within the FERM domain interacts with SH3 domain of Src. Subsequently, Src phosphorylates tyrsoines-576 and 577 in the activation loop to induce full FAK kinase activity.

Activation of FAK is initiated when a FERM binding partner (Integrins, RTKs or phospholipids) (Frame et al., 2010) or an intracellular pH increase (Choi et al., 2013) unlocks the auto-inhibited conformational state and allowing *in trans* auto-phosphorylation of Tyr^{397}. This creates a binding site for the SH2 (Src Homology 2) domain of Src (Calalb et al., 1995), which further phosphorylates Tyr^{576} and Tyr^{577}. This leads to maximum catalytic activity of FAK and its new conformational state cannot be inhibited anymore as the phosphorylated activation loop now impedes the access to the FERM docking site in the Kinase domain (Frame et al., 2010).

Then, Src-FAK signalling complex subsequently phosphorylate and recruit multiple cellular components including both other focal adhesions-associated proteins (such as paxilin and p130Cas) and signalling proteins (such as phosphoinositide-3 kinase (PI3K), phospholipase Cγ (PLCγ) and growth factor receptors bound protein (Grb2) (Frame et al., 2010). FAK can also be phosphorylated at several tyrosine (397, 407, 576, 577, 861, 925) and serine (722, 732, 843, 910) residues (Hochwald, 2009). Overall, a massive multiprotein complex forms around FAK that integrate and deliver signals from the cortex to the cytoskeleton and nucleus.

1.3.1.3 FAT domain

The C-terminal domain of FAK, also known as FAT domain, contains two proline-rich regions and a focal adhesion targeting sequence (FAT) (Hildebrand et al., 1993). Src homology 3 (SH3) domain containing proteins, such as p130Cas, bind to the proline-rich motifs. Proteins like Paxilin and Talin interact with the FAT domain and bring FAK to the focal adhesions (Parsons, 2003). Also, other proteins interact with this domain, such as Ras-GTPase activating protein Graf, PI3K, and Grb2 (Figure 1.1) (Hochwald, 2009). Therefore, FAK is part of large protein complexes that link ECM to the activation of signaling pathways, such as PI3K/Akt (v-Akt murine thymoma oncogene; also known as Protein Kinase B (PKB)) and Ras/MAPK (Mitogen-Activated Protein Kinase), which ultimately regulate cytoskeleton changes, cell migration, growth and survival. The crystal structure of the FAK FAT domain showed it can form dimmers as well, therefore increasing the number of interacting proteins in the complex (Hayashi et al, 2002) (Prutzman et al., 2004).

Additionally, alternative splicing generates a truncated isoform of FAK, called FAK-related non-kinase (FRNK) (Richardson and Parsons, 1995). This protein, consisting of the C-terminal domain only, acts as a negative regulator of FAK as it competes for binding sites of FAK in the focal adhesions and prevents downstream signaling as it lacks a kinase domain (Schaller et al., 1993).

1.4 Physiological and cancer-related functions of FAK

Due to its two large N- and C-terminal domains, which mediate protein-protein interactions, FAK is a major scaffolding protein. Its kinase activity is induced as a consequence of these interactions but is associated with only a subset of functions (Arold, 2011). Thus, its diverse and vast number of connections makes FAK a pivotal hub where signals are processed and delivered from the cell cortex to the cytoskeleton and nucleus. These signals ultimately affect many cellular processes such as adhesion, migration, survival and proliferation (Parsons, 2003).

All these cellular responses are involved in essential aspects of cancer such as tumour invasion, metastasis, angiogenesis, tumour growth, and cell

death avoidance. Several aspects of the role of FAK within normal cellular/tissue contexts will be described in the next sections together with a summary of how these aspects are altered in oncogenic scenarios.

1.4.1 Gene regulation

The human FAK (or PTK2, protein tyrosine kinase 2) gene has been mapped on chromosome 8. The coding sequence contains 34 exons and codifies a 3.8 Kb mRNA (Corsi et al., 2006), which is expressed ubiquitously. The promoter region spans 600bp and contains binding sites for many transcription factors, among which are p53 and NF-κB (Golubovskaya et al., 2004). Additionally, internal promoters and alternative splicing control the expression of several isoforms identified in different tissues, such as FRNK (a dominant negative isoform) (Richardson and Parsons, 1995) and FAK^{+} (the most abundant form in the brain, containing a 3-amino acid insertion in the C-terminal region) (Menegon et al., 1999).

Soon after the discovery of its contribution to cell transformation (Schaller et al., 1992), FAK was found upregulated in a variety of human tumours, (Weiner et al., 1993). Since then, an enormous amount of information about the participation of FAK in cancer has been accumulated.

Some cancer cell lines, mainly derived from head, neck, lung, breast and colon tumours, showed an increased copy number of the *FAK* gene (Agochiya et al., 1999) but no activating mutations have been reported so far.

Most of the studies showed that FAK was transcriptionally upregulated in a large number of tumour tissues such as thyroid, prostate, breast, colon and ovary (McLean et al., 2005). Also, an increased FAK expression or activity was normally associated with invasive and metastatic tumours (Owens et al., 1995) and poor prognosis (Recher et al, 2004).

Although little is known about the transcriptional control of FAK promoter, the transcriptional factor p53, found loss or mutated in more than 50% of human tumours (Hollstein et al., 1991), can repress FAK expression;

consequently, higher levels of FAK were found in p53-deficient tumours (Golubovskaya et al., 2008a).

1.4.2 Cellular localisation and pattern of expression

In the cell, FAK can be found at the membrane, particularly enriched in focal adhesions, and also in the nucleus (Lim, 2013). Pyk2 also localises to the nucleus but in the cytoplasm is found mainly at perinuclear regions as it lacks a Talin binding site that recruits it to the focal adhesions (Klingbeil et al., 2001).

Their pattern of expression and activating stimuli also differs. While FAK is ubiquitously expressed across the body, Pyk2 expression is more restricted to some tissues, being particularly high in the CNS (Sasaki et al., 1995) (Lev et al., 1995) (Avraham and Avraham, 1997). For example, during CNS development, FAK is predominant but in adult brains Pyk2 expression is higher than FAK and is also found in glia (Menegon et al., 1999). While FAK is activated in response to ECM adhesions and growth factors (Zachary et al., 1992, Sieg et al., 2000) (Schaller et al., 1992), Pyk2 responds to an increase of intracellular Ca^{2+} (hence its other name Calcium-dependent tyrosine kinase (CADTK)) (Lev et al., 1995) (Avraham and Avraham, 1997) .Consequently, there is enough evidence showing that these two related proteins, although very similar in structure, have a differential regulation. Interestingly, overexpression of Pyk2 in some cell lines induces apoptosis while FAK normally prevents it (Frisch et al., 1996b) (Xiong and Parsons, 1997). Also, it was shown that after FAK loss, Pyk2 expression was increased, likely to compensate FAK absent functions (Sieg et al., 1998).

1.4.3 FAK in development

The importance of FAK during development has been demonstrated through mouse genetic studies. A knockout of the FAK gene resulted in embryonic lethality (Ilic et al., 1995). $FAK^{-/-}$ littermates were smaller than wild type at embryonic stage E8.0-8.5 and several reasons explained this lethality: higher levels of apoptosis, a defect in blood vessel morphogenesis and reduced proliferation and cell motility (Ilic et al., 1998, Ilic et al., 2003) (Lim et al., 2008).

1.4.4 Cell motility, invasion and metastasis

Cells need a dynamic regulation of all kind of adhesions with or without the help of matrix metalloproteases (MMPs) in order to move along the basement membrane/ECM or across epithelia. As FAK influences the disassembly of integrin-associated adhesions in migrating cells, it is not surprising FAK is involved in the spread of cancer (Schlaepfer et al., 2004).

The role of FAK in cell motility was apparent in mouse fibroblasts derived from $FAK^{-/-}$ embryos, which exhibited an increased number of focal contact sites, a rounded morphology and decreased rates of cell migration (Ilic et al., 1995). Interestingly, v-Src transformation of $FAK^{-/-}$ fibroblasts as well as re-introduction of FAK rescued the integrin-motility defects (Hsia et al., 2003), whereas ectopic expression of FAK in $FAK^{+/+}$ cells also stimulated cell migration (Cary et al., 1996). Consistently, phosphatases such as PTEN and SHP2 have been shown to cause dephosphorylation of FAK and affect cell motility (Tamura et al., 1998).

As mentioned above, highly invasive and metastatic tumours showed increased expression of FAK (Owens et al., 1995). Normally, more migratory cells have dynamic cell-ECM contacts and loose cell-cell contacts; in fact, FAK phosphorylation (Ilic et al., 1995) and higher focal adhesion turnover was related to deregulation of E-cadherin-dependent adhesion during epithelial-to-mesenchymal transition (EMT) in colon cancer (Avizienyte et al., 2002).

1.4.5 Angiogenesis

The formation of new blood vessels from pre-existing ones is as necessary in embryonic as in cancer development (Ilic et al., 1995) (Lee et al., 2010) (Tavora et al., 2010). The Vascular Endothelial Growth Factor Receptors (VEGFRs), fibroblast growth factor receptor (FGFR) and Integrins play key roles in normal and tumour angiogenesis (Otrock et al., 2007, Taeger et al., 2011) (Hodivala-Dilke et al., 2003). These receptors were shown to activate FAK upon ligand binding in several endothelial cells (Sieg et al., 2000) (Abedi and Zachary, 1997, Shi et al., 2011), with consequent association of adaptor proteins such as

Shc, Grb2, c-Src and stimulation of proliferation and migration (reviewed in (Lechertier and Hodivala-Dilke, 2012)) (Figure 1.1).

Correspondingly, overexpression of FAK in vascular endothelial cells stimulated migration and angiogenesis in mice and humans (Peng et al., 2004, Kornberg et al., 2004), while FAK inhibition impaired FAK-Grb2-MAPK signalling, VEGF expression and reduced tumour vasculature in mouse neuroblastoma, breast and prostate carcinoma (Mitra et al., 2006).

1.4.6 Avoidance of cell death

Apoptosis is an evolutionary conserved form of programmed cell death that eliminates cells that are in excess or become dangerous. FAK was linked to survival signalling when inhibition of its activity led to cell detachment from the basement membrane/ECM and subsequent cell death. This special type of programmed cell death was called anoikis (Frisch et al., 1996a). Resistance to anoikis is essential for tumour cells in order to invade and metastasize (Hanahan and Weinberg, 2000). Supporting this survival function, conditional *fak* deletion in mouse skin led to increased apoptosis and prevented chemically induced papilloma tumour progression (McLean et al., 2004).

FAK promotes survival through different signalling pathways (Figure 1.1). For instance, FAK-overexpressing cells induced expression of inhibitor of apoptosis proteins (IAPs), and the survival Akt and NF-κB pathways upon etoposide treatment, a potent drug that causes DNA breaks (Sonoda et al., 2000).

Additionally, FAK interacts directly with RIP, a serine/threonine kinase with a death domain (DD). Tumour necrosis factor (TNF)-induced death is normally mediated by RIP through the formation of the death inducing signalling complex (DISC), formed by the DD-containing proteins TNFR, TRADD, FADD, RIP and pro-caspase 8 (Stanger et al., 1995). Thus, FAK prevents apoptosis signalling by sequestering the recruitment of RIP to the DISC complex (Kurenova et al., 2004).

Alternatively, FAK can also block p53-dependent apoptosis (Ilic et al., 1998). The FERM domain of FAK interacts with p53 and Mdm2 in the nucleus and promotes ubiquitin-mediated degradation of p53 (Lim et al., 2008), thus precluding its transcriptional activity. With this, it seems clear that there is a feedback regulatory mechanism between these two proteins, given that p53 can also regulate FAK transcription (Golubovskaya et al., 2008a), as discussed in section 1.4.1. The fact that many human tumours present mutations in p53 suggests that FAK might be upregulated and consequently cell death would be inhibited and invasion/metastasis would be favoured.

1.4.7 Non-canonical roles of FAK: tumour suppressor?

A growing body of evidence is showing unexpected roles of FAK. In certain conditions FAK had a negative impact over cell migration and tumour progression. For instance, it has been shown liver metastases had lower FAK levels compared to the primary colorectal carcinoma in humans (Ayaki et al., 2001), while dephosphorylation of FAK increased cell motility, invasion and metastasis in various EGFR-expressing human carcinomas (Lu et al., 2001) (Caceres et al., 2005).

Additionally, ERK activation by the expression of oncogenes H-Ras and K-Ras led to FAK inhibition and hence, loss of focal contacts and promotion of migration and metastasis. This negative regulation of FAK by ERK was supported by an inverse correlation between active ERK and active FAK in glioblastoma samples (Zheng et al., 2009).

These reports suggest FAK could prevent tumour progression, in contrast to its role associated to the progression of the disease. This non-canonical function of FAK has to be further investigated in a greater number of cancer scenarios in order to find out whether this behaviour occurs only in certain tissues, during specific stages of tumour formation or within particular genetic contexts (reviewed in (Zheng and Lu, 2009)).

1.4.8 Development of therapeutics against FAK

As described so far, many studies have shown FAK plays a positive role in the progression, survival and invasion of tumours. Therefore, FAK has been

regarded as a potential target for cancer therapeutics. In fact, some inhibitors designed against the catalytic domain of FAK are in early clinical trials for cancer treatment (Hochwald, 2009).

However, given the high conservation of the catalytic domain of kinase proteins there is a concern about the specificity of these FAK inhibitors. For example, Novartis TAE226 inhibits FAK and Pyk2 activities by preventing Y^{397} autophosphorylation, but also inhibits insulin-like growth factor 1 receptor (IGF1R) (reviewed in (Lechertier and Hodivala-Dilke, 2012)). Even if the inhibitors are specific for FAK, not always they produce the expected results; this is probably due to the scaffolding functions of FAK, which are mostly independent of the kinase domain. For instance, better efforts have to be made in order to block the multiple interactions mediated by the FERM and FAT domains of FAK: RIP and p53 binding sites are good targets as their inhibition would lead to cell death (Cance et al., 2013).

Also, the non-canonical roles of FAK should begin to be considered in the designing of therapeutics strategies for those specific scenarios where FAK acts as a tumour suppressor rather than tumour promoter.

1.5 FAK in *Drosophila*

1.5.1 Discovery and structure

A degenerate PCR approach aiming for conserved residues within tyrosine kinase domains was taken in order to find a *Drosophila* homologue of the vertebrate FAK protein family. This approach detected a single protein in the *Drosophila* genome with the structure characteristics of FAK and Pyk2 (Fujimoto et al., 1999, Palmer et al., 1999, Fox et al., 1999). This protein was named DFAK56D (here after called FAK56), as *in situ* hybridization to polytene chromosomes showed it localised on the second chromosome, band 56D.

FAK56 contains 15 exons encoding a 4.5Kb mRNA and a 1200 amino acid polypeptide (140kDa) . FAK56 exhibits high overall amino acid similarity with human FAK (34%) and Pyk2 (29%), and 63% and 54% of identity within the kinase domains, respectively, including a conserved autophosphorylation site (tyrosine-

Y^{430}). A 24 amino acid kinase insert and a 120aa C-terminal extension are the only features not found in FAK and Pyk2 (Palmer et al., 1999) (Figure 1.3).

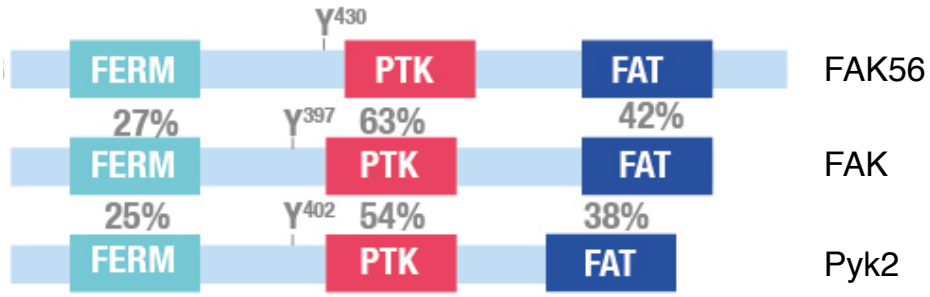

Figure 1.3 FAK56 conserved domains
FERM domains, protein tyrosine kinase (PTK) domains, and FAT domains of *Drosophila* FAK (FAK56) and human FAK and Pyk2 are schematized with their corresponding amino acid identity percentage relative to FAK56.

1.5.2 Characterisation of FAK56

As shown in vertebrates, FAK56 also localised in focal contacts and mediated integrin signals through phosphorylation when FAK56-expressing cells were plated on ECM components such as fibronectin, tiggrin or laminin (Fox et al., 1999, Palmer et al., 1999, Fujimoto et al., 1999).

Although FAK56 is expressed throughout the body at all stages of development, its expression is regulated. High levels were observed during embryonic stage, particularly in the developing Central Nervous System (CNS) and muscle (Fujimoto et al, 1999; Palmer et al, 1999b). A drop in protein levels was detected at early larval stage to then increase towards the end of larval and pupal stage. In adult life, brain and ovaries showed higher levels of FAK56 (Fox et al., 1999).

Despite its ubiquitous expression and important functions described in mammals, FAK56 mutants are viable and fertile, proving it is dispensable for general development. Additionally, integrin-dependent adhesion processes, such as cell attachment or migration, and localisation of associated components are not affected in absence of FAK56 (Grabbe et al., 2004). This suggested the role of FAK56 might become apparent only under stress conditions. In fact, FAK56 mutant flies display a lifespan of about half-life of wild type flies and exhibit sensitivity to mechanical stimuli, suffering seizure and temporal paralysis (Ueda et al., 2008)

On the other hand, overexpression of FAK56 results in lethality when ubiquitously expressed, meaning it is able to inhibit developmental processes. When expressed more selectively it gives phenotypes such as wing blistering or muscle detachment, which are characteristic of loss of integrin function (Palmer et al., 1999) (Grabbe et al., 2004). Thus, FAK family members are not essential in *Drosophila*, but their accessory roles can become important in certain circumstances.

1.5.3 FAK56 in nervous system

Most of the current research about FAK56 in *Drosophila* is carried out in the context of integrin signalling in the field of neurobiology. This is probably due to its enriched expression in CNS, glial cells and muscle (Fujimoto et al., 1999) (Palmer et al., 1999, Ueda et al., 2008), and consistently, FAK56 mutants had abnormal neuromuscular junction growth (NMJ) and defects in optic stalk structure (Murakami et al., 2007, Tsai et al., 2008).

FAK56 expression in neurons at the presynaptic compartment restricts NMJ growth through cAMP activation and interestingly, inhibition of Ras/MAPK pathway (Tsai 2008). This non-canonical relationship between FAK56 and MAPK signalling correlates with other studies in mammals (see section 1.4.7) where an inverse correlation between FAK and ERK signalling has been observed in glioblastoma (Zheng et al., 2009). Further evidence supporting these observations may have implications in cancer biology and future therapeutic strategies against FAK.

Interestingly, FAK56 was involved in nerve conduction in the glial cells surrounding neurons. In a similar way that Pyk2, FAK56 responded to an increase of intracellular calcium levels (Ueda et al., 2008). Therefore, and given FAK56 is the only FAK PTK family member, we cannot discard the possibility that FAK56 behaves as both human FAK and Pyk2 depending on the tissue context.

1.6 *Drosophila* as a model organism for cancer research

This section discusses the history and power of the fruit fly as a model organism, particularly in the modeling of cancer.

1.6.1 History of *Drosophila melanogaster*

There are nine species within the *Drosophila* subgroup, all of which originated in equatorial Africa. *Drosophila melanogaster* became the most cosmopolitan and domestic member of this group since it was able to breed in a wider variety of fruits and habitats than its sister species, which led to a stronger association with humans and therefore a rapid spread around the world (Lachaise, 1988).

The popularity of *Drosophila melanogaster* as a laboratory animal and its success as a human commensal have some common reasons: a short life cycle, a large offspring, the ability to grow on varied and accessible food sources, and the usual presence in human houses, which is also true for another famous research system, the common house mouse (*Mus musculus*) (Keller, 2007).

The first person to use *Drosophila melanogaster* in the laboratory was William Ernest Castle at Harvard University in 1901 (Stephenson and Metcalfe, 2013). A few years later, in 1906, Thomas Hunt Morgan began working with *Drosophila* at Columbia University, New York. Morgan chose *Drosophila melanogaster* because of its short reproduction time and lifespan, ease of culturing, high fecundity and especially, inexpensive maintenance, an important factor at that time when funding was not very accessible (Lewis, 1998). His research produced great advances in genetic research, which ultimately led to the sequencing of the *Drosophila* genome in 2000 (Adams et al., 2000).

1.6.2 Flying into the lab

Since the discovery of the *white* gene and the sex-linked inheritance in 1906 (Morgan, 1910), the fruit fly *Drosophila melanogaster* has been an important model organism that made notorious contributions to many fields of research such as genetics, cell biology and developmental biology, among others (Stephenson and Metcalfe, 2013).

More recently, researchers began to use the fruit fly as a model system to study human afflictions, such as cancer or neurodegenerative diseases (Vidal and Cagan, 2006, Stephenson and Metcalfe, 2013) and also beyond basic research, as

a tool to find new therapeutics compounds for human medicine (Dar et al., 2012).

A model organism is any non-human species that is studied to understand certain biological phenomena (Fields and Johnston, 2005), for instance a human disease such as cancer. The fact all living organisms share a common descendant also means that many fundamental biological functions (developmental, metabolic and signalling pathways) have been conserved during evolution. This makes possible to study different aspects of human biology by using simpler organisms rather than humans themselves, which would be nearly impossible and often unethical.

Normally, a research model system should have the following characteristics: small size, quick generation time, wide accessibility, ease of manipulation, many conserved mechanisms, simple genetics, and low cost. *Drosophila melanogaster* gathers all these traits but, like any other model organism, also has limitations, such as the obvious anatomical and physiological differences with humans.

Nonetheless, for more than 100 years, *Drosophila melanogaster* has proven successful as a research model and offered scientists a good balance between organismal complexity and genetic power. The following sections will describe the main technical and biological aspects that make the fruit fly an attractive model in the laboratory:

1.6.2.1 Generation time and lifetime

The fruit fly is an insect belonging to the orden Diptera and the subclass Pterygota, which characterize for going through four distinctive development stages: embryonic, larval, pupal and adult (summarised in Figure 1.4). This type of insect development is called holometabolism or complete metamorphism (Gateff, 1978). At 25ºC, the whole developmental process takes approximately 11 days and an adult fly lifespan lasts between 4 to 5 weeks, being both timeframes temperature dependent. Female flies have high fecundity, laying roughly 100 eggs per day. Overall, it means quick experimentation with large numbers of offspring: a main difference against mouse models, which take 21

days of gestation, 3 months to reach sexual maturity and give birth to 6 to 10 individuals during lifetime.

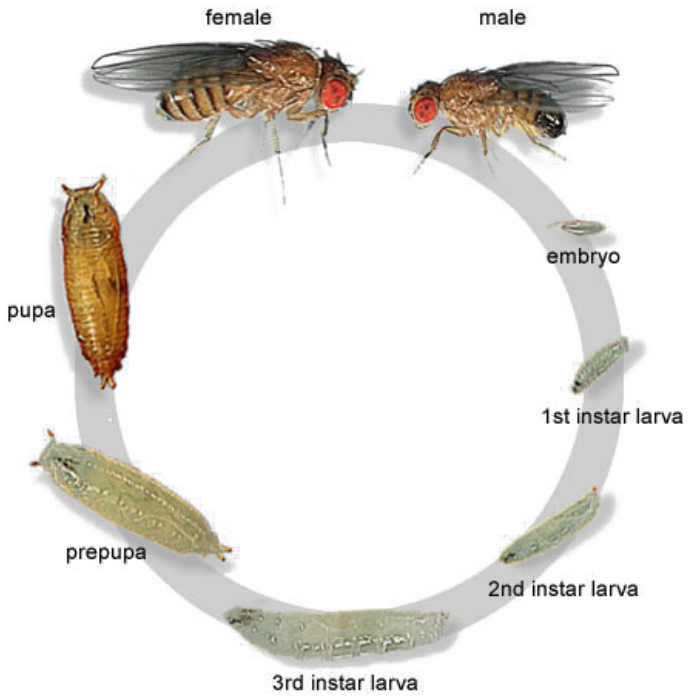

Figure 1.4 Life cycle of *Drosophila melanogaster*
At 25˚C, the entire cycle lasts 11 days. 24h for each of the first three stages, 2 days as third instar larva and 6 days of pupal stage until final eclosion of the adult fly. Source: Fly*Move* (Katrin Weigmann, 2003).

1.6.2.2 Manipulation and costs

Flies grow in a diverse range of food sources, especially spoiled fruits; therefore it is inexpensive to culture them. A simple food recipe consists of yeast, cornmeal, a source of glucose and agar. Also, most of the equipment required for their manipulation is not as costly as it is for human cell cultures or mice. Excluding the costs of common reagents that are necessary in any laboratory, the simplicity of maintenance and manipulation of the flies plus lesser experimental regulations than other more complex systems is what makes them one of the more economic models in research.

1.6.2.3 Complete genome sequence and low genetic redundancy

Drosophila has only 4 chromosomes compared to our 23 pairs and 20 pairs in mice (*Mus musculus*). Since the completion of the fly genome sequence (Adams et al., 2000), large genetic analyses (Reiter et al., 2001, Rubin et al., 2000) have shown that more than 50% of human genes linked with diseases, including cancer, have their homologue in *Drosophila*. Thus, the low

chromosome number simplifies genetics studies and the high genetic conservation plus the low genetic redundancy facilitate functional studies in *D. melanogaster*.

1.6.2.4 Genetic tools

In addition to the abovementioned advantages, the absence of homologous recombination in males, the development of gene targeting technologies through natural transposable elements, known as P-elements (Rubin and Spradling, 1982), the GAL4/UAS system (Brand and Perrimon, 1993) and its variants, the FLP-FRT recombinase system (Golic and Lindquist, 1989, Xu and Rubin, 1993), the creation of balancer chromosomes and RNAi lines, several stocks centers and databases, among many others resources, creates a large arsenal of genetics techniques that makes *D. melanogaster* a powerful genetic tool.

1.6.3 Modelling cancer in flies

Cancer is a multistep process where a normal cell transforms into a more proliferative and invasive, malignant cell type. This transformation requires several genetic alterations that all together overcome several regulatory mechanisms. These errors accumulate over time, which explains why many types of cancer show an age-dependent incidence (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). Given the short lifespan of *D. melanogaster*, it is counterintuitive that fly cells will become malignant during its lifetime (review in (Gonzalez, 2013)). However, recent work has showed testis and intestine tumours occur in adult flies also with an age-increasing frequency (Salomon and Jackson, 2008), demonstrating that ageing also affect tumourigenesis in flies.

Beyond many anatomical and physiological differences that impede the modeling of certain aspects of cancer exactly as it happens in vertebrates, current *Drosophila* cancer models are designed in tissues of adult flies and developing larvae (review in (Gonzalez, 2013)). Fly cells do have the same capabilities as human cells to become malignant when cancer-relevant genes are perturbed. Thus, *Drosophila* can display several hallmarks of cancer such as inhibition of cell death, uncontrolled proliferation, invasion, metabolic

reprogramming and genome instability, which then can be model in several tissue types to study context-dependent tumour initiation (Tipping and Perrimon, 2013).

The high extent of homology and low redundancy of the fly genome makes the flies an excellent system to carry out genetic screens in search of oncogenes and tumour suppressors (Vidal and Cagan, 2006). The best example is the work that identified the first tumour suppressor gene, *lethal (2) giant larvae* (*l(2)gl*) (Bridges, 1944) (see section 2.5.4.2). Another approach to understand cancer is to recreate the scenario of gain and loss of functions found in a particular type of cancer (Gonzalez, 2013). This can be done in homologous or analogous organs, if available, and/or in a complete different tissue in *Drosophila*, thus allowing the study of context-dependent interactions of tumour suppressors and oncogenes (Tipping and Perrimon, 2013). Illustrative examples are the modeling of human colorectal cancer in the fly gut after APC (Adenomatous Polyposis Coli) loss (Cordero et al., 2012) or the mimicking of a thyroid tumour type, Multiple Endocrine Neoplasia, in the fly eye (Read et al., 2005).

1.6.3.1 Keeping a fly eye on cancer

The latter example illustrates how the *Drosophila* eye can be useful for learning about cancer types that do not have an equivalent tissue in the fly. Historically, the fly eye has been an excellent system to identify genetic interactions and dissect signalling pathways (Brumby and Richardson, 2005). And currently, it continues to do so (please see Chapter 3).

The *Drosophila* compound eye is an elegant structure of nature composed of about 750 hexagonal units called ommatidia, which pattern in a honeycomb-shaped array (Figure 1.5). Each ommatidial unit consists of fourteen cells: eight photoreceptor and six supporting cells (four cone cells and two primary pigment cells). The units are optically insulated by the hexagonal lattice, where secondary pigment cells make each side of the hexagon, and three bristle cells and three tertiary pigment cells constitute the corners of the hexagon (Cordero et al., 2004).

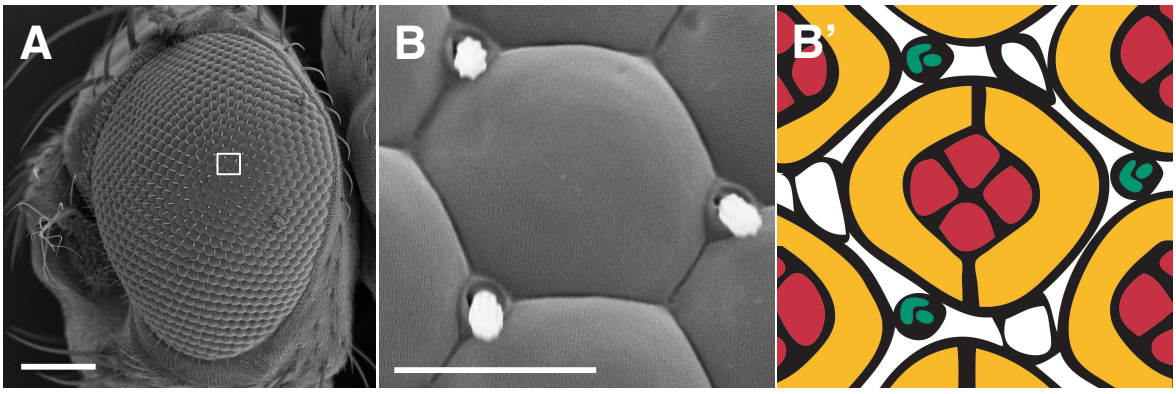

Figure 1.5 ***Drosophila*** **adult eye and ommatidia cellular composition**
(A-B) Scanning electron microscope micrograph of a wild type adult eye. Scale bar: 100μm. (B) External view of an ommatidial unit (white box in A) showing three bristles and the lenses secreted by the cone cells located underneath. Scale bar: 10μm. (B') Ommatidial structure. Four cone cells (red), two primary pigment cells (yellow) and eight photoreceptor cells (not shown) located beneath the cone cells make up an ommatidial unit. Secondary and tertiary pigment cells (white) plus three bristle cell (green) shape the hexagonal architecture.

During the end of larval life, the undifferentiated and proliferating epithelial cells of the eye disc transform into a well-patterned neuroepithelium through a series of ordered differentiation steps. Cell fates in the retina are determined by local signals passed between cells. These signals result in progressive recruitment of undifferentiated cells by their previously differentiated neighbours. Thereby, photoreceptor cell clusters are specified first. They instruct neighbouring cells to differentiate into cone cells, and then primary pigment cells emerge. The final decision is to create the interommatidial lattice: from the sea of undifferentiated interommatidial precursor cells (IPCs) surrounding the ommatidial cores, some cells are recruited as secondary and tertiary pigment cells while remaining cells are removed by developmental programmed cell death between 26 and 30 hours after puparium formation (APF) (Miller and Cagan, 1998). By 42hs APF the final pattern of the ommatidia is fully arranged. If these surplus cells are not eliminated, the adult eye appears roughened because ommatidial units are separated by a varying number of cells. The resulting imperfect alignment of the ommatidia also presumably affects the ability of the fly to see (Brachmann and Cagan, 2003).

This repetitive structure is what makes the adult eye an ideal system for genetic analysis. Any subtle change interfering with cell proliferation, cell death or differentiation during development will be reflected several hundred-fold in the adult eye. Furthermore, alterations of the symmetric hexagonal lattice are visible, easy to score and do not cause lethality, as the fly eye is not essential for survival (Gonzalez, 2013).

Many *D. melanogaster* genes related to cancer or even human cancer genes give rise to a so-called 'rough' phenotype when expressed in the eye. This becomes a sensitive readout in the search for suppressors and enhancers genes or drugs that modulate this phenotype (Read et al., 2005, Vidal et al., 2005). We took a similar approach to study FAK function within an RTK-driven context, please see Chapter 3.

1.6.3.2 Imaginal discs

Unlike the process in vertebrate development, the adult fly is not formed as a result of the continuous development of embryonic tissues. Imaginal precursor cells are established as discrete groups of cells localized to specific regions of the embryo. Two types of precursors determine the larval and adult organs. The larval anlagen differentiate during embryonic development to give origin to the larval tissues, which grow by cell enlargement without division becoming polyploid, e.g. larval salivary glands. The adult organ precursors remain undifferentiated until hatching of the larva from the egg, to then quickly divide and become imaginal discs and cell nests (Lewis I. Held, 2002).

Cell nests include neuroblasts, histoblasts and imaginal rings, which are intimately associated with larval structures and give rise to the abdomen and internal organs of the adult such as the brain, salivary glands and gut. Imaginal discs, on the other hand, are single-layered groups of cells formed from invaginations of the embryonic epithelium, which during metamorphosis transform into the adult external structures such as head, appendages and genitalia (Figure 1.6) (Lewis I. Held, 2002).

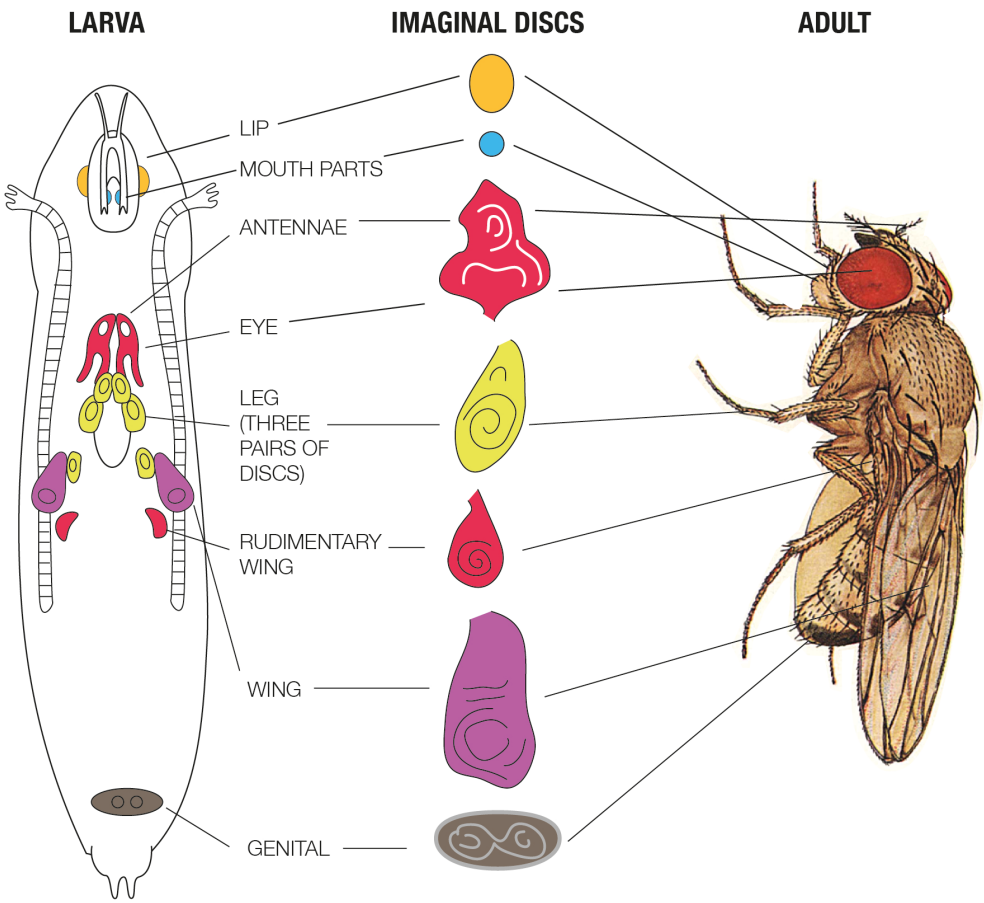

Figure 1.6 Larval imaginal discs and adult structures
Schematic representation of a third instar larva, its imaginal discs and their resultant adult external structures. Note that the eye-antenna disc is actually made of two discs: the upper region is the primordium of the antenna and the lower disc of the fly compound eye. The wing imaginal discs form the wings, the hinges and the notum of the adult fly (for more details see Figure 1.7)

As described before, the eye imaginal disc and adult eye have made numerous contributions to identifying genetic interactions and dissecting signalling pathways. The same applies for the wing imaginal disc and the adult wing, which have helped to find signalling pathways involved in cancer such as Notch signalling and Hippo signalling (reviewed in (Perrimon et al., 2012)).

Like all imaginal discs, the wing disc is a sack-like structure compartmentalised by three axes: anterior/posterior (defined by the expression of *decapentaplegic*), dorso/ventral (define by *vestigial*), and distal/proximal (defined by *distal-less* and *aristaless*) (Figure 1.7). During metamorphosis, the proximal region will become the notum and the distal part will give rise to the adult wing through an invagination at the intersection of the dorsal/ventral axis, defined by Wingless (Lewis I. Held, 2002).

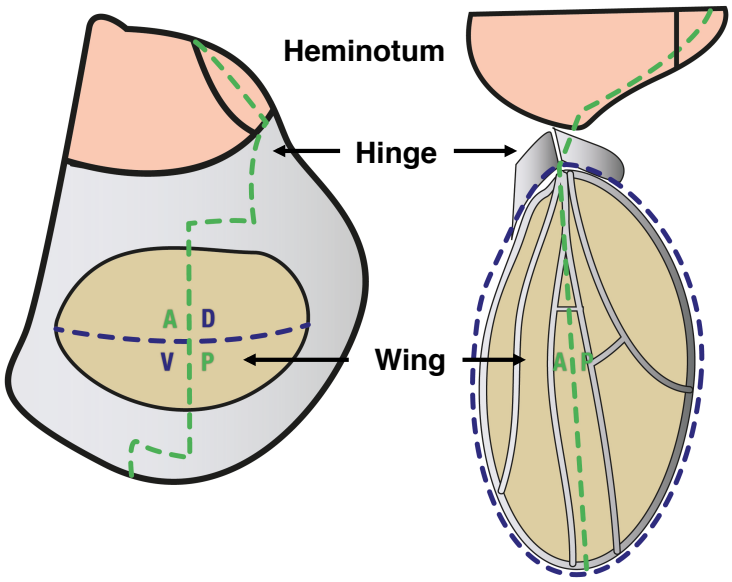

Figure 1.7 Wing imaginal disc and adult wing
Fate map of the wing imaginal disc and dorsal view of the adult wing. The equivalent parts are coloured in pink (Heminotum), silver (Hinge) and light brown (wing pouch and adult wing). A green dashed line defines the anterior/posterior compartment; the dorso/ventral compartment is drawn as a purple dashed line; and proximal/distal axis runs from top to bottom of the figure. During metamorphosis the wing pouch evaginates to form a double-layered epithelium that becomes the adult wing.

Given the amount of information accumulated over decades of study about wing development, several driver lines have been developed to promote expression in particular compartments or boundaries of the wing disc (see section 2.1.2 in Materials and Methods). These tools, such as *decapentaplegic (dpp)-Gal4* or *patched (ptc)-Gal4,* permit gene over-expression or downregulation in a certain population of cells to then investigate how this affects the wild type neighbours (Tipping and Perrimon, 2013) (Vidal et al., 2006, Vidal et al., 2007). In chapter 3, I describe how we used this system to assess RTK/FAK signalling regulation.

Clonal analysis also allows the comparison of two genotypically different cell populations in the same tissue and animal and hence, to study cell autonomous and non-autonomous effects and cell competition mechanisms (Perrimon, 1998) (Morata and Ripoll, 1975). Notably, generation of clones permits the study of otherwise lethal genes, such as the case of the scribble group genes (Gateff, 1978) (Bilder et al., 2000, Woods and Bryant, 1989). Therefore, by adapting this technique to a cancer context, where a population that exhibit malignant behaviour is next to or within a wild type population of cells, it is possible to study aspects of tumour microenvironment. In this regard, several screens made in larval imaginal discs helped to discover genetic

interactions causing tumour formation and/or metastasis (Brumby and Richardson, 2005). It is of particular interest of this thesis the cooperative interaction between the oncogene *Ras* and the tumour suppressor genes of the scribble group, *scribble* (scrib), *lethal (2) giant larvae* (*l(2)gl*) and *disc large* (dlg), which will be deeply described in section 1.6.4.2 and Chapter 5.

1.6.4 *Drosophila* models of cancer used in this study

1.6.4.1 RTK-driven cancer models

Receptor tyrosine kinases (RTKs) are a class of transmembrane proteins with intrinsic inducible tyrosine kinase activity. Their structure consists of three main domains: an extracellular ligand-binding domain, a membrane-spanning domain and a cytoplasmic kinase domain (Lemmon and Schlessinger, 2010). RTKs integrate external stimuli into the cell through signalling cascades that regulate many critical cellular processes such as cell growth and proliferation, differentiation, migration, metabolism and cell death. Given such a vast influence on varied cell fates their activity has to be tightly regulated. When this regulation is lost aberrant signalling occurs. In fact, genetic mutations that increase their activity or protein levels, or release those regulatory constraints have been often found in cancer (reviewed in (Blume-Jensen and Hunter, 2001)).

Consistently with the important processes they influence, RTKs molecular structure, mechanism of activation and many signalling downstream components have been conserved from *C. elegans* to humans (Lemmon and Schlessinger, 2010), including *Drosophila melanogaster*.

As explained before, genetic screens in the fly eye are very useful to study signal transduction pathways. An illustrative example of this is the development of a *Drosophila* model of thyroid cancer (Read et al., 2005, Vidal et al., 2005, Das and Cagan, 2010) achieved by the expression of the receptor tyrosine kinase RET (Rearranged during transformation) in the developing eye.

As its name suggests, the *ret* gene was found to be rearranged in cellular transformation assays (Takahashi et al., 1985). RET associates with glycosyl phosphatidylinositol (GPI)-anchored cell surface receptors (known as GFRα proteins) to bind ligands belonging to the glial-derived neurotrophic factor

(GDNF) family. Activating mutations in RET cause the familial cancer syndrome Multiple Endocrine Neoplasia type 2 (MEN2) and medullary thyroid carcinoma (MTC) (reviewed in (Jhiang, 2000, Leboulleux et al., 2004)). Furthermore, chromosomal translocations involving the ectopic expression of RET are very frequent in Papillary thyroid Carcinoma (PTC), the most common type of thyroid cancer (Grieco et al., 1990, Bongarzone et al., 1994), and pheochromocytomas (Santoro et al., 1990).

In flies, although there are neither GDNF homologues nor GFR co-receptors, *Drosophila* RET was shown to have a functional tyrosine kinase activity when overexpressed (Abrescia et al., 2005). Presumably, its expression may be sufficient to form dimmers and trigger downstream signalling. In mammals, other RTKs such as insulin receptor or epidermal growth factor receptor (EGFR) were reported to be on the cell surface as dimmers/oligomers even in absence of the ligand (Ward et al., 2007, Clayton et al., 2005, Gadella and Jovin, 1995).

The forced expression of the *Drosophila* homologue dRET in the fly eye was used in a genome-wide genetic screen in order to identify signalling downstream components. This screen indicated that CSK/Src and Ras/MAPK pathways, among others, mediate RET-downstream signalling (Read et al., 2005). Also, a drug screen on this model identified a chemical compound (Vidal et al., 2005), which is now under consideration for the treatment of hereditary medullary thyroid cancer (MTC) (Wells et al., 2010, Wells et al., 2012). More recently, this model was used in combination with mammalian systems to improve the efficacy of targeting compounds (Dar et al., 2012). Therefore, this fly model has proven useful and could in the future further help to clarify the RET-downstream signalling steps.

In Chapter 3, I describe how we took advantage of this model to investigate the role of FAK within a cancer-like scenario. Also, we used *Drosophila* EGFR, arguably the most characterised RTK, to expand our findings on other members of the RTK family.

1.6.4.2 Loss of cell polarity model and the tumour microenvironment

Development of cancer, or malignant neoplasia, requires the capacity of cells to divide. Contrarily, post-mitotic cells are unable of neoplastic transformation. Most of the *Drosophila* tissues are post-mitotic but among the few capable of neoplastic growth are the imaginal discs. In vertebrates and flies, there are two types of neoplasms: benign and malignant (Gateff, 1978). Benign neoplasms grow slowly without invasion or metastasis, remain structurally and functionally similar to their tissue of origin and are not lethal. Malignant neoplasms are more aggressive; they grow faster, invade neighbour tissues and often metastasize causing ultimately death to the host. Their cellular composition differs from the original tissue, as structure, function and differentiation are normally lost (Miles et al., 2011).

The group of genes *disc large, lethal giant larvae* and *scribble* are regulators of apical-basal cell polarity (Muller, 2003). Animals fully mutant in those genes lead to loss of epithelial polarity, differentiation defects and tumour-like growth of imaginal discs, which causes ultimately host lethality (Cordero et al., 2010). Hence the name: tumour suppressors. In fact, *lgl* was the first tumour suppressor gene to be discovered (Bridges, 1944).

Interestingly, small patches of cells mutant for these genes are eliminated from the tissue and replaced, when surrounded by wild type cells (Brumby and Richardson, 2003). This phenomenon is a type of cell competition and serves, in this case, as the first defense against potential malignant cells. However, if the mutant cells acquire a mutation that gives them instead an advantage over their neighbours, this cell competition effect can be inverted towards the wild type population. In fact, this is what happens when an activating mutation in the small GTPase Ras (d*Ras85D*^{G12V} *or Ras*^{V12}) occurs in the tumour suppressor mutant cells. Ras is an oncogenic protein able to activate several signalling pathways involved in proliferation and survival; therefore, Ras will cooperate with the tumour suppressor mutation to overcome the deadly signals and grow quickly and in an invasive manner like a malignant neoplasm (Pagliarini and Xu, 2003, Cordero et al., 2010).

In summary, *Drosophila* imaginal discs provide an excellent scenario to study different grades of neoplasia and also, tumour microenvironment. A large and growing body of evidence indicates that the human immune system also plays a role in tumour formation and invasion. In *Drosophila*, it is also the case as hemocytes, the phagocytes of invertebrates, were reported to associate with Ras^{V12} $scb^{-/-}$ tumours (Pastor-Pareja et al., 2008), and Eiger (Egr), the only *Drosophila* homologue of the Tumour Necrosis Factor (TNF) cytokine family, was shown responsible for promoting tumour growth in this scenario (Cordero et al, 2010). In chapter 5, I expand the background of this exciting interaction between the immune system and malignant neoplasia and describe my contribution to this work.

1.7 Aims of this study

Although FAK56 is the sole *Drosophila* FAK family member and is not essential for fly development or viability, there is evidence suggesting it is required in conditions of stress. Also, little is known about FAK56 in cancer-like scenarios in *Drosophila*.

Therefore, considering FAK's multiple interactions and the growing evidence about a confounding behaviour in cancer, we aimed to shed more light on the role of FAK in tumourigenesis using *Drosophila* as a model.

The main objectives of this thesis are:

- To explore the role of FAK56 in RTK-driven scenarios. How does FAK56 behave in this context? (Chapter 3)

- To investigate anti-cell death properties of FAK56 (Chapter 4).

Additionally, I described a novel hemolymph transfusion technique in *Drosophila* larvae entirely developed in our laboratory in order to study the interaction between the immune system and tumours.

Chapter 2 – Materials and Methods

2.1 *Drosophila* husbandry

2.1.1 Fly husbandry

All flies were cultured at 25ºC (unless otherwise stated) on standard media (see Table 2.1) with a controlled 12h-light/12h-dark cycle. Stocks were maintained at 18ºC.

Ingredient	Amount (per litre)
Agar	10 g
Sucrose	15 g
Glucose	33 g
Maize meal	15 g
Wheatgerm	10 g
Treacle	30 g
Soya flour	5 g
Yeast	35 g
Propionic Acid	5 mL
Nipagin (Methylparaben)	10 mL
Tap water	1000 mL

Table 2.1 Standard fly food recipe
Beatson Central Services prepared fly media following this recipe. Nipagin stock concentration: 5% in Ethanol.

2.1.2 Stocks

Strain/Genotype	Description	Source/Reference
Control Strains		
w^{1118}	Strain *white* mutant - flies have white eyes.	Vidal lab stocks
Canton-S	Wild type strain of *Drosophila*	Vidal lab stocks
Driver lines		
w^{1118}, *P[*w^{+mC}*, Glass Multimer Reporter (GMR)-Gal4]*	Posterior to the morphogenetic furrow in differentiating and post-mitotic cells of the developing eye	(Freeman, 1996, Hay et al., 1997)
w^{1118}*; P[*w^{+mC}*, patched-Gal4]*	Within a compartment of cells in imaginal discs.	(Speicher et al., 1994)
w^{1118}*; P[*w^{+mC}*, hemesee-Gal4]*	Drives expression in hemocytes	(Kurucz et al., 2003)
y^1 w^{1118}*; ; P[*w^{+mC}*, act5C-Gal4]/TM6B,* Tb^1	Ubiquitous expression in all cells driven by *actin5C* promoter	Bloomington Drosophila Stock Center (BDSC) – Stock 3954
w^{1118}*; ; P[*w^{+mC}*, decapentaplegic-Gal4]*	Within a compartment of cells in imaginal discs.	(Speicher et al., 1994)

Responder lines (UAS)		
w^{1118}; ; P[w^{+mC}, UAS-dFAK]	UAS-transgene expressing wild type FAK56	(Grabbe et al., 2004, Palmer et al., 1999)
w^{1118}; P[w^{+mC}, UAS-dFAK^{Y430F}]	UAS-transgene expressing FAK56 autophosphorylation mutant - Point mutation that replaces tyrosine 430 for a phenylalanine residue.	(Grabbe et al., 2004, Palmer et al., 1999)
w^{1118}; P[w^{+mC}, UAS-dFAK$^{\Delta 400}$]	UAS-transgene expressing a 400-aa N-terminal deletion of FAK56.	(Grabbe et al., 2004, Palmer et al., 1999)
y^{1} w^{1}; ; P[w^{+mC}, UAS-dRET^{C695R} (dRET^{CA})]	UAS-transgene expressing an activated isoform of dRET - Point mutation that replaces cysteine 695 for an arginine residue	(Read et al., 2005)
w, P[w^{+mC}, UAS-dEGFR]*	UAS-transgene expressing wild type *Drosophila* EGFR	(Freeman, 1996)
w; P[w^{+mC}, UAS-2xEGFP]*	UAS-transgene expressing Green Fluorescent Protein (GFP)	BDSC - Stock 6874
w, P[$w^{+mC,}$ UAS-RFP]*	UAS-transgene expressing Red Fluorescent Protein (GFP)	Vidal lab stocks
w^{1118}; ; P[w^{+mC}, UAS-Ras85D^{V12}]	UAS-transgene expressing an activated isoform of dRas85D - Point mutation that replaces glycine 12 for a valine residue	(Karim and Rubin, 1998)
w^{1118}, P[w^{+mC}, UAS-DICER2]	UAS-transgene expressing the *Drosophila* RNAse III enzyme DICER2.	(Dietzl et al., 2007)
w^{1118}; ; P[w^{+mC}, UAS-debcl]	UAS-transgene expressing Debcl	(Colussi et al., 2000, Quinn et al., 2003)
w^{1118}, P[w^{+mC}, UAS-p35]	UAS-transgene expressing baculovirus caspase inhibitor p35	(Hay et al., 1994)
w; ; P[w^{+mC}, UAS-DIAP1]*	UAS-transgene expressing *Drosophila* Inhibitor of Apoptosis Protein 1.	(Hay et al., 1995)
w; ; P[w^{+mC}, UAS-basket^{K53R} (JNK^{DN})]*	UAS-transgene expressing a dominant negative isoform of c-Jun N-terminal kinase (JNK)	(Weber et al., 2000)
y^{1} w^{1118}; ; P[w^{+mC}, UAS-dp53^{H159N} (dp53^{DN})]	UAS-transgene expressing a dominant negative isoform of dp53 that prevents transcriptional activity	(Brodsky et al., 2000)
y^{1} w^{1118};; P[w^{+mC}, UAS-dp53]	UAS-transgene expressing wild type *Drosophila* p53	(Dichtel-Danjoy et al., 2013)
y^{1} w^{1118};; P[w^{+mC}, UAS-dΔNp53]	UAS-transgene expressing a N-terminally truncated *Drosophila* p53 isoform	(Dichtel-Danjoy et al., 2013)
w^{1118}; P[w^{+mC}, UAS-Eiger] (Strong	UAS-transgene expressing wild type Eiger	(Moreno et al., 2002, Igaki et al.,

transgene)		2002)
RNAi lines (UAS)		
w^{1118}; P[w^{+mC}, UAS-Src42A^{RNAi}]	UAS-RNA interference transgene for Src42A	Vienna Drosophila RNAi Center (VDRC) – Stock v17643
y^1 v^1; P[y^+ v^+, Ras85D^{RNAi}]	UAS-RNA interference transgene for Ras85D	BDSC – Stock 29319
y^1 v^1; P[y^+ v^+, debclD^{RNAi}]	UAS-RNA interference transgene for debcl	BDSC - Stock 27083
Mutants and deficiencies		
y^1 w^{67c23}; FAK^{CG1}	null allele of FAK56 created by P-element excision	(Palmer et al., 1999)
y^1 w^{67c23}; P[SUPor-P]FAK^{KG00304}	hypomorphic allele of FAK56 created by P-element insertion	BDSC – Stock 13080
w^{1118}; P[RS5]FAK$^{5-SZ-3124}$	hypomorphic allele of FAK56 created by P-element insertion	Drosophila Genetic Resource Center – Stock 125903
P[neoFRT]82B, scrib^1/TM6b, Tb^1	loss of function allele of scribble created by EMS mutagenesis	(Bilder et al., 2000)
w^{1118}; ; Rel^{E20}	loss of function allele of Relish created by P-element excision	(Hedengren et al., 1999)
w^{1118}; ; Rel^{E38}	loss of function allele of Relish created by P-element excision	(Hedengren et al., 1999)
Df(3L)H99/TM3, Sb^1	Genetic deficiency that deletes *reaper, hid and grim* genes	(White et al., 1994)
Df(3L)X38/TM6B, Tb^1	Genetic deficiency that deletes *reaper and sickle* genes	(Peterson et al., 2002)
w; egr^1*	loss of function allele of eiger created by P-element excision	(Igaki et al, 2002)
Other lines		
y^1 w^1; ; P[w^{+mC}, GMR-dRET^{C695R} (dRET^{CA})]	Recombinant transgene expressing an activated isoform of dRET (point mutation that replaces cysteine 695 for an arginine residue) under the control of GMR promoter	(Read et al., 2005)
y^1 w^1; ; P[w^{+mC}, GMR-RET]	Recombinant transgene expressing wild type *Drosophila* RET (dRET) under the control of GMR promoter	(Read et al., 2005)
w; ; P[w^{+mC}, GMR-rpr]/TM6B, Tb$^+$*	Recombinant transgene expressing wild type *reaper* under the control of GMR promoter	(White et al., 1996)
w; P[w^{+mC}, GMR-hid]/CyO*	Recombinant transgene expressing wild type *hid* under the control of GMR promoter	(Grether et al., 1995)

w; ; P[w^{+mC}, GMR-grim]*	Recombinant transgene expressing wild type *grim* under the control of GMR promoter	(Chen et al., 1996)
y^{1}, w, eyFLP/+; act5C>y^{+}>GFP/+; P[neoFRT]82B, P[w^{+mC}, tub-Gal80]/TM6B*	Mosaic analysis with a repressible cell marker (MARCM) line	(Lee and Luo, 2001)

Table 2.2 List of *Drosophila* strains used in this study
Please note most of the lines have a *white* mutant background such as w^{1118}, w^{1} or w* when the exact mutation is unkown. This is useful to track the insertion of transgenes carrying a cDNA sequence of the wild-type *white* gene, called *mini-white* (*w*$^{+mC}$), which is sufficient to restore red pigmentation of the eye (Klemenz et al., 1987).

2.1.3 Survival studies

In order to quantify the percentage of survival of a given genotype, survivor and non-eclosed (dead as pupae) flies were scored in three independent experiments. The ratio between survivor flies against total number of animals stands for the percentage of survival.

2.2 *Drosophila* genetics

2.2.1 Gal4/UAS binary system

This system consists of two components: the yeast transcription activator protein Gal4 and the Upstream Activating Sequence (UAS) element, which consists of four related 17bp sites analogous to an eukaryotic enhancer that Gal4 binds to activate gene transcription (Brand and Perrimon, 1993). The *gal4* gene is placed downstream of a tissue or cell type specific promoter, so called the driver line. Thus, Gal4 is only expressed in those tissues where the promoter is active. Importantly, it was shown that Gal4 expression has little or no activity in *D. melanogaster* (Fischer et al., 1988). The UAS element is placed upstream of a reporter gene/transgene construct, the responder line (Duffy, 2002). When the driver and responder lines are combined, the resulting progeny express the reporter gene of interest in a particular pattern determined by the driver line.

Thus, this system allows the overexpression or mis-expression of any particular gene, or RNAi constructs to downregulate the transcription of a gene of interest (Lam and Thummel, 2000, Fortier and Belote, 2000) (see Table 2.2).

2.2.2 Extensions of the Gal4/UAS system: Gal80.

Further developments of the technique have allowed a better spatial and temporal control of gene expression. The Gal80 repressor binds to Gal4 transcriptional activation domain and blocks its activity (Ma and Ptashne, 1987). Thus, expression of Gal80 can be used to prevent Gal4-driven expression (Lee and Luo, 1999). Temperature sensitive variants of Gal80 protein ($Gal80^{TS}$) allow a more precise temporal control over Gal4/UAS expression (Matsumoto et al., 1978, McGuire et al., 2003). Therefore, when a fly line containing the three components of the system is moved from permissive temperatures (18-25ºC) to a non-permissive temperature (29ºC), Gal80 becomes inactive and allows Gal4 to activate the transcription of UAS-linked genes of interest.

2.2.3 Mosaic analysis with a repressible cell marker (MARCM) analysis

In order to study genetic cooperation and tumour microenvironment we generated recombinant clones using the MARCM technique that allows the analysis of cell populations genotipically different from the host tissue (Lee and Luo, 2001). Crosses combining the MARCM line, FRT82B *scrib*^{1} and *UAS-RAS*^{V12} (see Table 2.2) were maintained at 25°C. Wandering giant larvae bearing tumours were collected for hemolymph microinjection experiments (see section 1.3.4).

MARCM allows restricted expression of transgenes in homozygous mutant cells. In our experiment, expression of the FLP recombinase (Flippase) in the developing eye by the *eyeless* promoter produced *scrib*-deficient cell clones expressing GFP and Ras^{V12}, which grew uncontrollably in a tumour-like manner. This clones originated as a consequence of two independent recombination events within a cell. FLP/Flippase recognition target (FRT)-mediated mitotic recombination of FRT82B-bearing arms of the third chromosome produced *scrib* mutant cells and loss of the Gal80 repressor only in these cells; while recombination of the "flip-out" construct *act>y*$^{+}$*>GFP (Ito et al., 1997)* led to Gal4-driven expression of GFP and Ras^{V12}. Additionally, Gal80 expression in the rest of the tissues reduced GFP or Ras^{V12} expression associated to leakiness of the flip-out construct (Lee and Luo, 2001).

2.3 *Drosophila* dissection techniques

2.3.1 Third instar larval tissues

Grabbing the mouth hook and pulling it out the larval body allowed getting whole and intact imaginal discs. Eye and wing imaginal discs were dissected in 1X PBS (Phosphate Buffered Saline), fixed in 4% formaldehyde for 30 minutes at room temperature, and rinsed in PBS-T (PBS, 0.1% Triton X-100).

2.3.2 Pupal retinas

Pupal case was opened at the indicated time points and entire retinas were carefully dissected and separated from the cephalic complex in 1X, then fixed in 4% formaldehyde for 30 minutes at room temperature, and rinsed in PBS-T (PBS, 0.1% Triton X-100).

2.3.3 Adult organs

In order to image adult eyes, heads or male genitalia, flies were collected and froze overnight and then imaged. Wing blades were dissected and mounted on a glass slide in 10% glycerol and then imaged.

2.3.4 Microinjections

Larvae were rinsed in *Drosophila* Schneider 2 (S2) media and anaesthetized with CO_2. 'Donor' larvae were dried and opened with forceps with care not to damage internal tissues. Three larvae were grouped together and their hemolymph loaded immediately into a micro-needle using a Narishige 1M-5A manual microinjector and transfused within 5 min to avoid melanisation and haemocyte adhesion to available surfaces. 'Acceptor' larvae were injected ventrally with approximately 1μl on the posterior third and transferred into a culture vial with excess humidity to recover for 24h. Larvae that died or that displayed excessive melanisation after the transfusion were discarded.

2.4 Staining assays and imaging

2.4.1 Immunofluorescence

After PBS-T washing, tissues were incubated in primary antibody at 4°C overnight, then washed in PBS-T (3 times, 10 minutes each), and incubated again for 2 hours in secondary antibody. Finally, tissues were rinsed in PBS-T and counterstained with DAPI (1µg/ml, SIGMA) for 5 min at RT and then mounted in Vectashield® mounting medium.

2.4.2 Primary and second antibodies

Primary and secondary antibodies working dilutions were prepared in PBS-TB buffer (PBS, 0.1% Triton X-100, 0.3% BSA).

Antibody	Working dilution, animal	Source
Primary antibodies (anti-)		
Armadillo	1:3, mouse	DSHB
Cut	1:50, mouse	DSHB
p(Y418)Src	1:100, rabbit	Cell Signalling
p(Y397)FAK	1:100, rabbit	Invitrogen
p(T202/Y204)MAPK	1:200, rabbit	Cell Signalling
Elav	1:500, rat	DSHB
Prospero	1:30, mouse	DSHB
GFP	1:4000, chicken	DSHB
Dlg	1:50, mouse	DSHB
N-Cadherin	1:20, rat	DSHB
Akt1	1:100, rabbit	Cell Signalling
Secondary antibodies (anti-rabbit, mouse, rat or chicken)		
Alexa 488	1:200	Molecular Probes
Alexa 594	1:100	Molecular Probes
Alexa 633	1:50	Molecular Probes

Table 2.3 Antibodies used in this study
Primary and secondary antibodies used for immunofluorescence assays are listed. DSHB: Developmental Studies Hybridoma Bank.

2.4.3 TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labelling)

Eye discs or retinas were dissected and fixed in 4% formaldehyde in PBS-T for 20 min at RT. Samples were permeabilised in 100mM Sodium Citrate in PBS-T (PBS, 0.1% Triton X-100) at 65° C for 30 min followed by the addition of TUNEL

mix according to the manufactures instructions (In Situ Cell Death Detection Kit, Roche) and incubated at 37ºC for 2 h on a rotating platform.

2.4.4 Confocal microscopy

All immunofluorescence preparations were analysed on a Zeiss 710 upright confocal microscope and images were processed with ImageJ program (NIH).

2.4.5 Light microscopy

Adult eye and male genitalia images were taken with a Leica M205 FA stereomicroscope equipped with Montage software. Eye size measurements were done with ImageJ program (NIH). Wing blades images were taken with an Olympus BX51 FL Microscope.

2.5 Human cancer cells

2.5.1 Cell lines

MDA-MB-231 ATCC® HTB-26™ is a *Homo sapiens* breast adenocarcinoma cell line derived from a metastatic site. The cell line is aneuploid female, lacking chromosomes 8 and 15 and known to express EGF (Davidson et al., 1987) and TGFα receptors (Bates et al., 1990) and the WNT7B oncogene (Huguet et al., 1994).

H1299 *ATCC*® CRL-5803™ is a *Homo sapiens* small-cell lung carcinoma derived from a lymph node metastatic site. These cell lack the expression of the p53 protein and were reported to express high levels of wild type EGFR (Rusch et al., 1993, Amann et al., 2005).

2.5.2 Cell culture, media and supplements

MDA-MB-231 cells were cultured in Dulbecco's modified Eagle medium (DMEM) and H1299 cells were cultured in Roswell Park Memorial Institute medium (RPMI), supplemented with 2mM glutamine and 10% FBS (Fetal Bovine Serum) at 37°C in a humidified 5% CO_2 atmosphere. Subculture was done three 2-3 times a week at subconfluence by removing medium, rinsing the adherent

cells in PBS, incubating cells with trypsin (0.25% (w/v), 1 mM EDTA – 1min, 37ºC) and placing cells into plates containing warm fresh medium.

2.5.3 Transfection of small interference RNA

siGENOME non-targeting (NT) siRNA pool (D-001206-13-05) and Smartpool siRNAs targeting FAK (L-003164-00) were obtained from Dharmacon, 10ul of the 20uM stock was used in each transfection. Non-targeting and FAK siRNAs were transfected into cells using Nucleofector Technology (Nucleofector Solution V, program X-013 (MDA-MD-231) or X-001 (H1299); Lonza) and Nucleofector® II, Amaxa Biosystems. Assays were set at 24 hours (H1299) or 48 hours after transfection to allow maximum efficiency of the siRNAs.

2.5.4 EGF treatment

EGF treatment (30uM, 15 minutes - Millipore) was performed on serum-starved cells, whereas all other experiments were conducted in the presence of serum. Each assay was independently repeated three times.

2.5.5 Human cell imaging and analysis

MDA-MB-231 transfected cells were washed in ice cold PBS, fixed in 4% paraformaldehyde for 10 minutes at room temperature (RT), and permeabilised during 5 minutes in PBS + 0.2% Triton X-100. Then, cells were blocked in 1% BSA/PBS solution for 30 minutes and incubated with primary antibody overnight. Secondary antibody was added to cells for 1 hour at RT and then cells were washed, incubated with FITC-Phalloidin for 10 minutes and finally mounted in Vectashield with DAPI.

Imaging was done with an inverted confocal microscope (FluoView FV1000; Olympus) with FluoView software (Olympus) and processed with Fiji (ImageJ) software (National Institutes of Health). Immunofluorescence intensity values of EGFR were obtained by creating a mask of the cell outline, defining a threshold and measuring fluorescent intensity. Data analysis and Mann-Whitney statistical tests were performed and plotted in GraphPad Prism 6 software.

2.5.6 Freezing and thawing

To freeze: cells were detached (Trypsin 0,25% (w/v), 1 mM EDTA - 1 min, 37ºC), resuspended in medium and centrifuged at 90*g* and 4ºC for 5min. After supernatant was removed, the pellet was quickly resuspended in 1ml of freezing media (FBS) and placed on ice. Cells were stored overnight at -80ºC and transferred to liquid N_2 for indefinite storage.

To thaw: frozen cells were placed in a 37ºC water bath until sides were thawed but centre remained frozen. Then, thawed cells were poured into a 10cm plate with 9ml of pre-warmed media (37ºC). Then, cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere and as soon as cells were attached, medium was changed.

2.6 General Molecular Biology Procedures

2.6.1 Protein extraction

Cells were washed in PBS (Phosphate Buffered Saline), lysed and scraped off the plate in lysis buffer (2% SDS, 100mM Tris-HCl, pH 7.4). Cell protein extracts were incubated at 95ºC for 5 minutes, sonicated and clarified by centrifugation at 10,000*g* for 15 min. Samples were stored at -80ºC until use.

Protein concentration was determined by Bradford method (Bradford, 1976).

2.6.2 Bradford Protein Assay

This colorimetric assay is based on the absorbance change of the dye Coomassie Brilliant Blue G-250. When it binds to proteins, the dye has an absorption spectrum maximum at 595nm. Thus, the absorbance value at 595nm is proportional to the amount of dye bound to protein and therefore, to the protein concentration of the sample.

In order to quantify the protein amount of a sample, a standard curve was prepared from a 2mg/ml BSA solution. The curve consisted of dilutions containing from 0 to 5 ug of BSA. Absorbance values corresponding to each point were plotted and its linear regression trendline with its correspondent equation

calculated. The protein concentration of a given sample was estimated taking into account its absorbance and the trendline equation. All analyses were performed with the Microsoft Office Excel software. Abcam® Optiblot Bradford reagent and Eppendorf® BioPhotometer plus spectrometer were used.

2.6.3 Western Blot

Proteins were resolved by SDS-PAGE and analysed by immunoblotting.

2.6.3.1 Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is a technique that allows separation of molecules based on their mobility within an electric field. SDS-PAGE uses a polyacrylamide-gel matrix to separate denatured proteins moving according to their molecular weight. SDS denatures and binds proteins giving them rod-like shapes and a homogeneous negative charge proportional to the length of the polypeptide; in other words, SDS gives a similar charge/weight ratio. Therefore, following the application of an electric current, the negative-charged proteins migrate towards the positive electrode. Since the shape of the polypeptides as well as the pore-size of the gel matrix is common to all the proteins, their size is the only variable that determines the speed of migration. Thus, smaller polypeptides will move faster through the gel.

Prior to use, protein samples were thawed and a volume corresponding to 30ug was mixed with an equal volume of 2X Laemmli lysis buffer (4% SDS, 20% glycerol, 120mM Tris-HCl pH6.8, 0.02% bromophenol blue). After short vortexing, samples were heated at 95ºC for 5 minutes to allow full denaturation. After short centrifugation, protein samples were loaded into a Invitrogen NuPAGE® Bis-Tris 4-12% gradient gel. 10µL of *BIO-RAD* Precision Plus Protein™ Dual Color Standard was loaded into a well so as to determine the molecular weight of the proteins within the samples. Gels were run in 1X NuPAGE® MOPS SDS running buffer at 130V for approximately 90min, in a Invitrogen XCell *SureLock*™ electrophoresis system.

2.6.3.2 Transfer

Following gel electrophoresis, proteins were transferred from the gel to a nitrocellulose or PVDF membrane. The sponges and paper towels were all soaked carefully in 20% methanol transfer buffer (NuPAGE® Transfer buffer) before use. Methanol maintains dimensional stability of gels minimizing gel swelling and removes SDS from proteins increasing protein binding to the membrane. Proteins were transferred at 100V for 60 minutes in a *BIO-RAD* Mini-PROTEAN® system.

2.6.3.3 Immunoblotting and Imaging

After transfer, the membrane was blocked in 5% BSA/TBST (Tris-buffered saline buffer + 0.1% Tween 20) blocking buffer at room temperature for 60 minutes with constant agitation. After blocking, the membrane was incubated with primary antibody solution diluted in the blocking buffer overnight at 4°C with gentle rotation. The next day, the membrane was washed 3 times in 1X TBST buffer, 15 minutes to get rid of unbound primary antibody and reduce background. After washes, the membrane was incubated with secondary antibodies solution for 1h at room temperate with agitation. Following secondary antibody incubation, the membrane was washed in the 1X TBST buffer 3x10mins to remove unbound secondary antibody. Finally, nitrocellulose membranes were imaged with Odyssey Imager (LI-COR Biosciences) and analysed with Image Studio Lite software.

The following antibodies were used for immunoblotting: anti-ß-actin (1:10000, Abcam), anti-FAK (1:1000, C-20, Santa Cruz Biotechnology), anti-phosphorylated (T202/Y204)-MAPK (pERK1/2) (1:200, Cell signalling), anti-ERK1/2 (1:10000, Promega), anti-EGFR (1:2000, BD Transduction Laboratories). Secondary antibodies were IRDye 680RD anti-mouse (1:10000, LI-COR) and IRDye 800CW anti-rabbit (1:10000, LI-COR).

2.7 Statistics

To statistically analyse eye size measurements and survival we used Graph Pad Prism 6 software and applied Student's parametric t-test or Mann-Whitney non-parametric test to compare two groups of data or One-way ANOVA followed by Bonferroni post-test to compare more than two groups of data. P-

values less than 0.05 were considered statistically significant. The results are shown as the mean ± standard error of the mean (SEM).

Chapter 3 – FAK as a tumour suppressor

3.1 Summary

Receptor Tyrosine Kinase (RTK) and Focal Adhesion Kinase (FAK) direct cellular outcomes through activation of multiple signalling pathways, including mitogen-activated protein (MAP) kinase. FAK is reported to interact with several RTKs, although little is known about how FAK regulates their downstream signalling.

We therefore investigated whether *Drosophila* FAK regulates signalling resulting from the overexpression of the RTKs RET and EGFR. Our results indicate that FAK is a suppressor of RTKs in fly epithelia. In the eye, relative levels of RTK to FAK dictate final cell fate. Mechanistically, FAK suppresses RET via specific downregulation of MAPK signalling; thus acting opposite to its proposed role at the integrin junction. Furthermore, FAK's suppression of RTK/MAPK signalling was also observed in the human breast tumour cell line MDA-MB-231, suggesting an evolutionary conserved mechanism.

FAK is widely considered as a therapeutic target in cancer biology, however it might also have tumour suppressor properties in some epithelial contexts. Therefore, FAK's negative regulation of RTK/MAPK signalling might have potential implications in the designing of therapy strategies for RTK-driven tumours.

3.2 Brief introduction

Research in model organisms can provide important insights on the effects of oncogenic pathways in different *in vivo* environments (Vidal and Cagan, 2006, Tenenbaum, 2003). Particularly, *Drosophila* has made numerous contributions to cancer biology, *e.g.;* by identifying components of several signalling pathways such as the Hippo (Edgar, 2006) and Receptor Tyrosine Kinase (RTK)/Ras/MAPK signalling pathways (Karim et al., 1996, Dickson et al., 1996, Therrien et al., 2000, Rebay et al., 2000, Mariappa et al., 2011).

FAK is a cytoplasmic non-receptor tyrosine kinase that interacts primarily with Integrins at the focal adhesion sub-domains of the plasma membrane (reviewed in (Parsons, 2003)). FAK belongs to a hub where phosphorylation signals are regulated and transferred into the cell, therefore it is implicated in many cellular processes such as adhesion, migration, survival and differentiation (Parsons, 2003, Gelman, 2003) and is normally found over-expressed in migrating and invasive tumour cells (Siesser and Hanks, 2006). The current knowledge suggests that abnormal FAK activation is a key driver of tumour cell motility and survival in conditions that would trigger anoikis (detachment-dependent apoptosis) in normal cells (reviewed in (Zhao and Guan, 2009, Siesser and Hanks, 2006)). Thus, FAK has been regarded as a potential target for cancer therapeutics.

In *Drosophila,* there is a single FAK homolog (*FAK56D*, here after called *FAK56*) (Fujimoto et al., 1999, Palmer et al., 1999, Fox et al., 1999); FAK56 is ubiquitously expressed, with particularly high levels in the developing Central Nervous System (CNS) and muscle (Fox et al., 1999). Consistently, *FAK56* mutants have abnormal neuromuscular junction growth and defects in the optic stalk tubular structure (Murakami et al., 2007, Tsai et al., 2008). Nevertheless, *FAK56* mutants are viable and fertile (Grabbe et al., 2004), proving it is dispensable for general development. This suggests the role of FAK56 may become apparent only under conditions of stress. In fact, *FAK* mutants display sensitivity to mechanical stimuli, suffering seizure and temporal paralysis (Ueda et al., 2008).

To examine the role of FAK56 within a context of oncogenic stress, we initially used a *Drosophila* model of cancer (Read et al., 2005, Vidal et al., 2005, Das and Cagan, 2010) achieved by the expression of the receptor tyrosine kinase RET (Rearranged during transformation). Activating mutations in RET cause the familial cancer syndrome Multiple Endocrine Neoplasia type 2 (MEN2) (reviewed in (Jhiang, 2000, Leboulleux et al., 2004)). Furthermore, chromosomal translocations implicating ectopic expression of RET are frequent in Papillary thyroid Carcinoma (PTC), the most common type of thyroid cancer (Grieco et al., 1990, Bongarzone et al., 1994), pheochromocytomas (Santoro et al., 1990) and breast carcinoma (Boulay et al., 2008).

Several RTKs were described to directly phosphorylate and activate FAK (Sieg et al., 2000, Chen and Chen, 2006). Interestingly, direct interaction and mutual phosphorylation between FAK and RET has also been reported (Sandilands et al., 2012b, Plaza-Menacho et al., 2011). Nevertheless, the functional importance of FAK in RTK signalling *in vivo*, in particular in the context of tumour development, is not yet clear ((Sieg et al., 2000) and reviewed in (Zhao and Guan, 2009)). Therefore, FAK56 is a likely candidate to be activated by *Drosophila* RET (here after called dRET) and mediate its signalling cascade in *Drosophila*.

3.3 Objectives

We aimed to characterise the regulatory role of FAK56 downstream of RTKs and extrapolate it to RTK-driven human cancers.

3.4 Results

3.4.1 RET activates FAK and MAPK

We expressed a constitutively activated form of dRET (hereafter called $dRET^{CA}$) with the *ptc-Gal4* driver, which is active in a stripe of cells immediately anterior of the Anterior/Posterior compartmental boundary of the developing wing imaginal discs (Figure 3.1A and D). GFP expression by itself did not affect this cell population nor produced ectopic activation of the cytoplasmic kinases Src, FAK or MAPK (Figure 3.1A-C). As expected from previous studies (Read et al, 2005), expression of $dRET^{CA}$ (*ptc>dRET^{CA}*) led to phosphorylation of Src, and MAPK on residues that report their activation (Figure 3.1D-E, see methods). Interestingly, we also observed increased phosphorylation of FAK56 (Figure 3.1F).

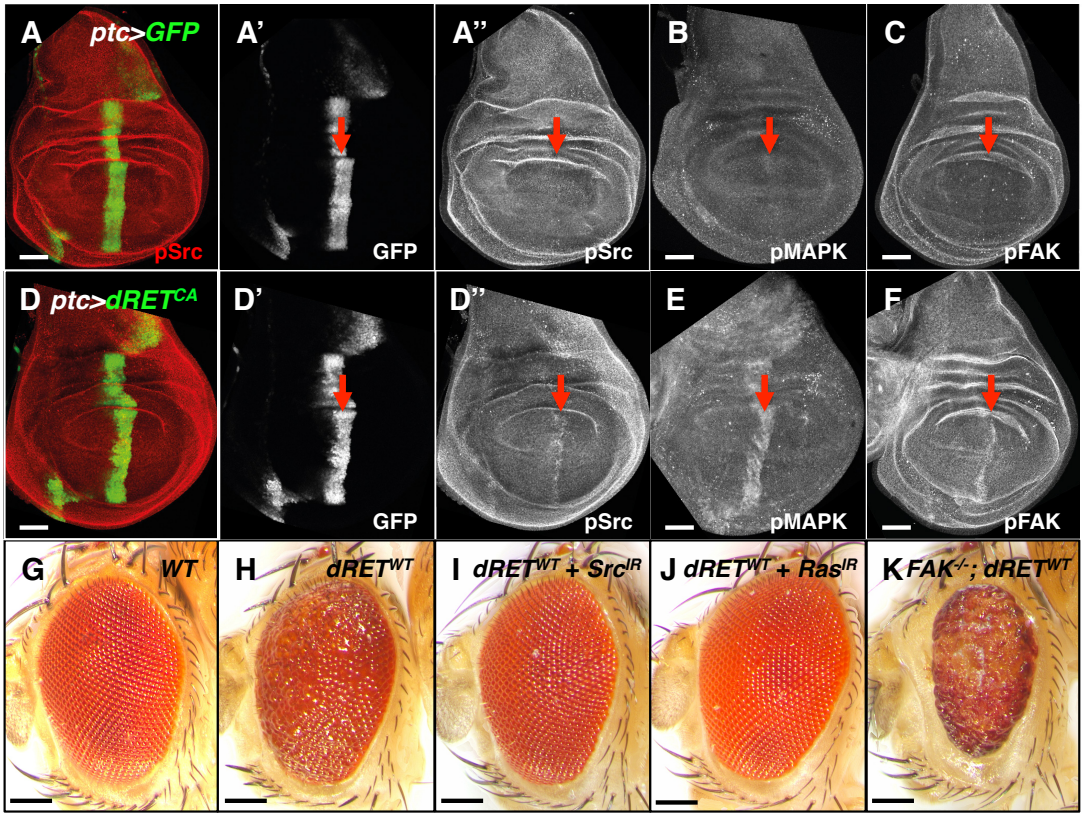

Figure 3.1 A functional relationship between dRET and FAK56
(A-F) Confocal images of wing disc epithelia. Control tissues (A-C) were GFP driven by *ptc-Gal4* (*ptc-Gal4, UAS-GFP*/+). Experimental tissues (D-F) also expressed dRET^{CA} (*ptc-Gal4, UAS-GFP*/+; *UAS-dRET^{CA}*/+). Stainings against pSrc, pMAPK and pFAK proteins (see methods), as a proxy for probing their activation levels, are shown in grayscale panels. Note hyper-phosphorylation of Src, MAPK and FAK after dRET^{CA} expression within the *ptc* domain, indicated by the arrows. Scale bars, 50 µm. (G-K) Micrographs of adult eyes with the indicated relevant genotypes; *GMR-Gal4* was used to drive UAS-linked transgenes. Note that expression of *dRET^{WT}* gave a rough eye phenotype. RNAi knock down of its known effectors Src and Ras suppressed this phenotype, while *FAK56* deletion enhanced it. Genotypes: *w1118 (WT)* // *GMR-dRET^{WT}*/+ *(dRET^{WT})* // GMR-Gal4/+; *GMR-dRET^{WT}*/+; *UAS-Src42A^{RNAi}*/+ *(dRET^{WT} + Src^{IR})* // GMR-Gal4/+; *GMR-dRET^{WT}*/+; *UAS-Ras85D^{RNAi}*/+ *(dRET^{WT} + Ras^{IR})* // *FAK^{CG1}*; *GMR-Ret^{WT}*/+ *(FAK$^{-/-}$*; *dRET^{WT})*. Scale bar, 100 µm. Please see also Figure 3.2.

Next, we tested genetically the importance of Src, Ras/MAPK and FAK downstream of RET. The *Drosophila* compound eye is an elegant structure composed of about 750 hexagonal units called ommatidia, which pattern in a honeycomb-shaped array (Figure 3.1G) (Cordero et al., 2004). This repetitive array makes the eye very sensitive to perturbations in signalling pathways. The ectopic expression of a single wild type copy of the *Drosophila RET* gene (hereafter called *dRET^{WT}*) under the control of the eye-specific GMR promoter (*GMR-dRET^{WT}*) disturbed the normal array of ommatidia, creating a 'rough' eye phenotype (Figure 3.1H; (Read et al., 2005)). In a search of genes involved in RET signalling, previously known members of the Ras/MAPK and Src signalling pathways were identified (*drk* (Grb2), *Sos*, *Ras85D*, *ksr*, *Gap1* (RasGAP), *Src42A*, *Src64B*, *Jra* (c-Jun) and *basket* (JNK)) (Read et al., 2005). As expected, we observed that the dRET-induced phenotype was reversed when Src42A or Ras85D

proteins were knocked down by RNA interference (Figure 3.1I-J), confirming that they play key roles downstream of dRET signalling. Downregulation of these proteins alone in a wild type eye caused no substantial defect (Figure 3.2).

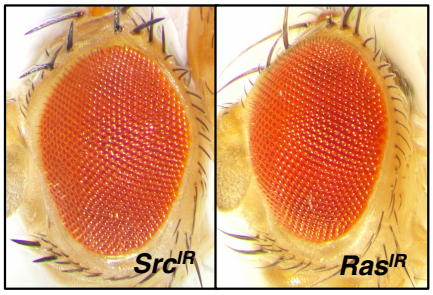

Figure 3.2 Control for expression of Src42A and Ras85D RNA-interference transgenes
Src42^{IR} and *Ras85D*^{IR} independent expression in the eye (*GMR-Gal4/+; UAS-Src42A*^{RNAi}*/+* and *GMR-Gal4/+; UAS-Ras85D*^{RNAi}*/+*) did not affect eye development nor hexagonal organisation of ommatidia in the adult eye. Scale bars, 100 μm.

Contrary to expectations for a potential dRET-signalling effector, loss of FAK56 did not suppress the rough eye phenotype; rather this was exaggerated: animals fully mutant for *FAK56* (please see legend of Figure 3.3 for a clarification of nomenclature) expressing dRET^{WT} in the eye *(FAK*^{CG1}*; GMR-dRET*^{WT}*/+*) completely disrupted the normal ommatidial pattern and led to a smaller eye (Figure 3.1K). To further test whether this effect was caused by loss of FAK56, *i.e.*, that there were no other genetic background mutations influencing the results, we next used two additional *FAK56* mutant allelic combinations. These showed a similar genetic interaction with *GMR-dRET*^{WT} (Figure 3.3).

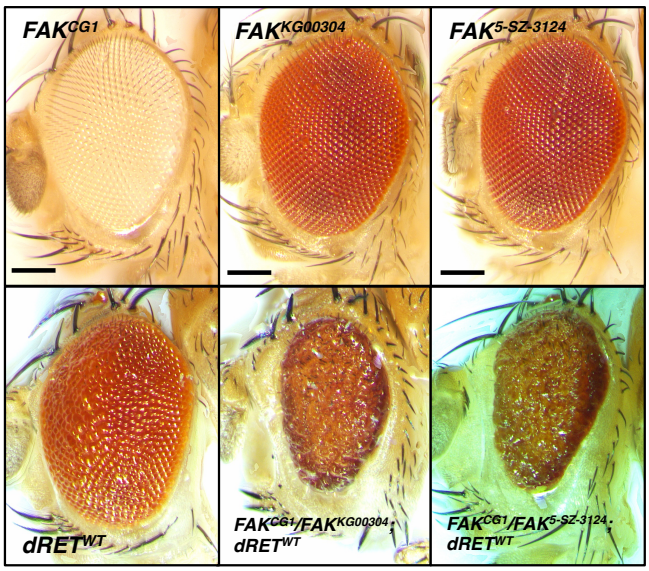

Figure 3.3 Different *FAK56* alleles enhance eye roughness caused by RET expression
Effect of independent *FAK* mutant allelic combinations over RET-driven rough phenotype (GMR-dRET^{WT}/+). Three different *FAK56* mutant lines were combined to produce trans-heterozygous *FAK*56 mutants: *FAK*^{CG1}*/FAK*^{KG00304}*; GMR-dRET*^{WT}*/+* and *FAK*^{CG1}*/FAK*$^{5\text{-}SZ\text{-}3124}$*; GMR-dRET*^{WT}*/+*, which

showed phenotypes similar to *FAK^{CG1}; GMR-dRET^{WT}*/+ (Figure 3.1K). *FAK56^{CG1}* (hereafter referred as *FAK$^{-/-}$* or *FAK^{CG1}*) is a null line consisting of a deletion that removes 1887bp including the initiation codon plus 1260 base pairs of the coding sequence, which correspond to the first 421 amino acids of FAK56; please note this allele was in a white background. The other two *FAK56* lines bear an insertion of different P-elements in the same site of the gene, resulting in hypomorphic lines (Grabbe et al., 2004). Scale bars, 100 μm.

Overall, these data suggest that dRET activates FAK56, which in turn has unanticipated suppressive effects on RET signalling.

3.4.2 FAK suppresses RET in different fly epithelia

To further test whether FAK can inhibit RET signalling we took complementary genetic approaches by combining dRET and FAK56 in different imaginal discs using the *GMR-Gal4, dpp-Gal4* and *ptc-Gal4* drivers. FAK56 overexpression by itself had no apparent phenotype on the adult survival and organs, resembling those of wild type animals (Figure 3.4A, D, G, J).

Drosophila RET expression did affect several tissues of the adult fly and also resulted in developmental toxicity (with a penetrance that depended on the temperature and other factors (Dar et al., 2012) (Figure 3.4B, 2E, 2H, 2J). As mentioned above, when dRET^{WT} was expressed in the eye, it altered the normal pattern of ommatidia (Figure 3.4B, see also 3.1H), which was prevented when FAK56 was simultaneously expressed with dRET (Figure 3.4C). Escaper *ptc>dRET^{CA}* adults showed wing vein defects (Dar et al., 2012), with absence of the anterior cross vein (Figure 3.4E). Remarkably, this phenotype was rescued by the co-expression of FAK56 with dRET^{CA} (Figure 3.4F). Escaper *ptc>dRET^{CA}* adult males also displayed rotation defects in the epandrium (Figure 3.4H). The *patched* gene is expressed in a compartment-specific manner across most imaginal discs in the larva, including the genital disc (Speicher et al., 1994). Therefore, RET expression in this tissue perturbed normal development and rotation of the male genital organ (Gleichauf, 1936, Adam et al., 2003). Importantly, FAK56 also suppressed this dRET-induced phenotype and restored the proper orientation of the male genitalia (Figure 3.4I).

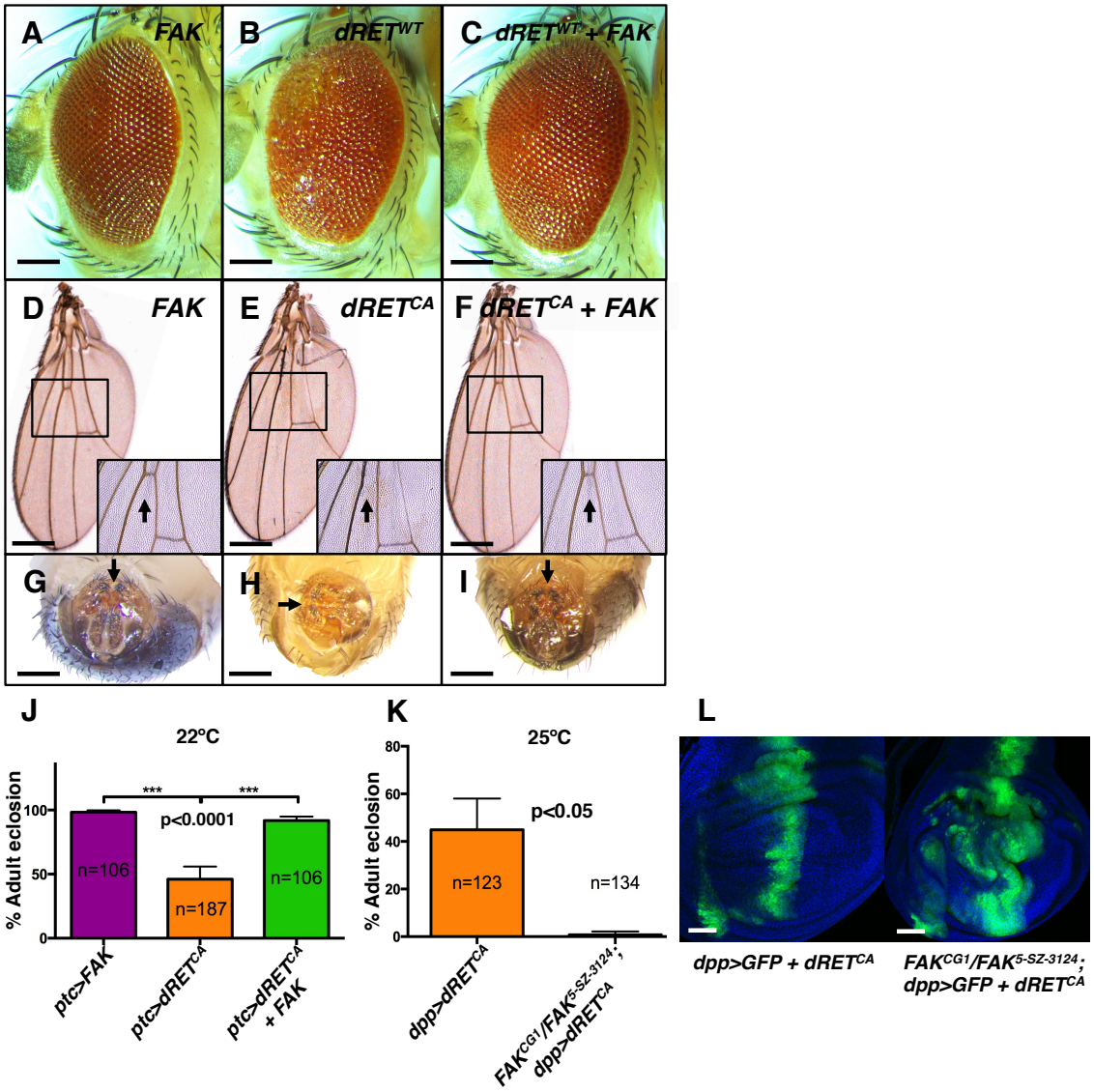

Figure 3.4 FAK56 inhibits dRET-driven effects in different fly tissues
(A-C) Eyes expressing FAK displayed a normal adult eye phenotype, while dRET^{WT} expression (*GMR-dRET^{WT}/+* [*dRET^{WT}*]) perturbed the normal pattern. Co-expression of dRET^{WT} and FAK56 supressed RET-driven mis-patterning defects. Scale bar, 100 µm. (D-F) FAK-expressing wings via *ptc-gal4* showed no detectable defects, while dRET^{CA} expression led to disappearance of anterior cross veins (arrow in inset box). RET-driven wing defects were suppressed by simultaneous expression of FAK56. Scale bar, 500 µm. (G-I) *ptc*-driven dRET^{CA} expression also led to incomplete rotation of the male genitalia (arrows). FAK56 co-expression rescued this phenotype and it did not affect the normal development of the genitalia by itself. Scale bar, 100 µm. (J) Quantification of the penetrance on adult eclosion for the indicated genotypes, note that FAK co-expression rescued significantly the developmental lethality of *ptc>dRET^{CA}* animals. Error bars are standard deviations in this and all plots. P values in one-way ANOVA tests (see methods) are shown. (K) Conversely, *FAK* mutation, which by itself had no effect in viability, enhanced to almost full penetrance the developmental lethality of *dpp>dRET^{CA}* animals. (L) Confocal images from wing discs with the indicated genotypes. Note that FAK mutation enhanced the size and tortuous shape defects associated to ectopic expression of *dRET^{CA}* within the *dpp* stripe. Scale bars, 50 µm.

Correspondingly, 46% of *ptc>dRET^{CA}*-expressing flies made it to adulthood (n=187) in our experimental conditions. Co-expression of dRET^{CA} and FAK56 increased survival up to 92% (n=106) (Figure 3.4J). Conversely, loss of FAK56 enhanced the morphological defects induced by dRET in the imaginal discs

(Figure 3.4L) and reduced the survival rate of *dpp>dRET^{CA}* animals (Figures 3.4K).

Together, these observations demonstrate that FAK inhibits RET-induced phenotypic effects in multiple imaginal disc epithelia.

3.4.3 The N-terminal domain of FAK, but not its kinase activity, is required to suppress RET effects

To gain insight into the mechanism by which FAK suppresses RET signalling, we co-expressed dRET^{CA} and different FAK mutant alleles in the eye. By measuring the eye areas of these phenotypes (Figure 3.5E), we observed that a wild type allele of FAK (Figure 3.5B) significantly restored the eye size when compared to *GMR>dRET^{CA}* eyes (*GMR-Gal4/+; UAS-dRET^{CA}/+*) (Figure 3.5A). Interestingly, and in correlation with other observations made in different systems (Sandilands et al., 2012b, Plaza-Menacho et al., 2011), the N-terminal FERM domain comprised within the first 400 amino-acid residues of the FAK protein seem to be essential for this functional interaction of FAK and RET: expression of a FAK mutant with a deletion of this region (FAK$^{\Delta 400}$) failed to modify the eye size of *GMR>dRET^{CA}* flies (Figure 3.5C). On the other hand, a point mutant allele in the major auto-phosphorylation site of FAK56 (FAK^{Y430F}, equivalent to tyrosine Y-397 in human FAK) did rescue the eye size significantly (Figure 3.5D).

These results suggest that the FERM domain of FAK56 is necessary to inhibit RET signalling, while the major autophosphorylation site — required for full FAK kinase activity (Frame et al., 2010) — is not essential. However, the level of mRNA and protein expression of each transgene should be measured in order to verify they are present in similar amounts and thus, validate the biological significance of these observations.

If that is the case, our results would indicate that in the genetic and functional interaction with RET, FAK could act as a scaffold rather than a kinase protein.

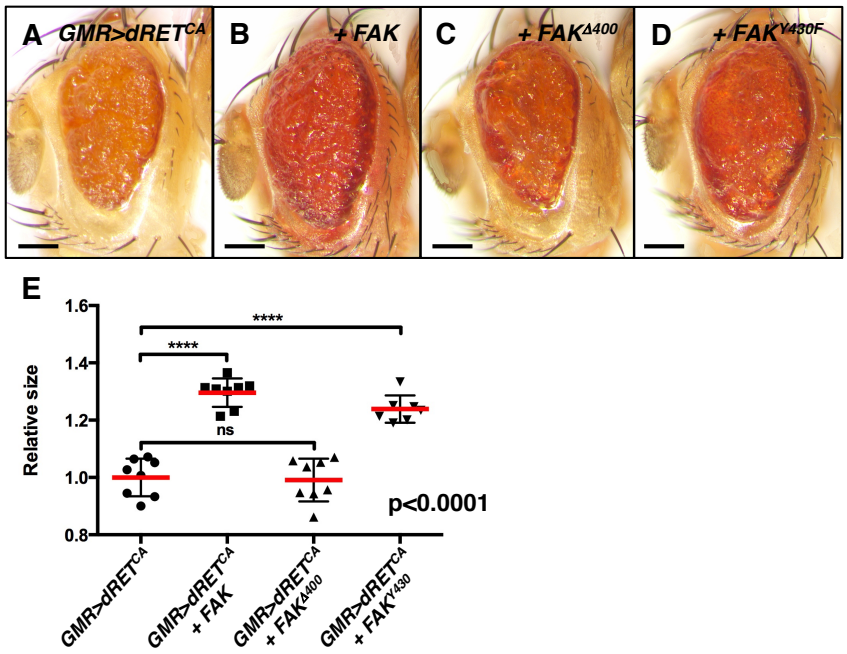

Figure 3.5 FAK56 N-terminal domain scaffolding functions mediate suppression of RET
(A-D) Eye micrographs correspond to the indicated genotypes. Note the difference in size as quantified in E. Scale bar, 100 μm. E, Quantification of the relative eye sizes from the indicated genotypes, shown in panels A-D. Note that while an auto-phosphorylation point mutant (tyrosine (Y)-to-phenylalanine (F) replacement) version of FAK was able to rescue the size of dRET-expressing eye, an amino-terminal deletion mutant was not.

3.4.4 Relative RET/FAK levels regulate signalling output

We next focused on the eye to further characterize the RET/FAK regulatory loop and its signalling output; specifically, we analysed how different experimental conditions that altered RET/FAK relative levels affected eye patterning.

As described above, GMR-driven expression of a wild type allele of dRET (*GMR-dRET^{WT}*) gives a mild rough eye phenotype when compared to a wild type eye (Figure 3.6A-B, also see Figure 3.1G-H). Within this context, eye roughness was gradually enhanced when one or two copies of the wild type *FAK56* gene were lost: loss of one copy of *FAK56* gene (*FAK$^{+/-}$; GMR-dRET^{WT}*) increased the ommatidial disorganization but did not affect eye size significantly (Figure 3.6C). However, the *FAK56* homozygous mutant (*FAK$^{-/-}$; GMR-dRET^{WT}*) displayed no recognizable ommatidial units and significantly reduced eye size (Figure 3.6D and 3.1K). Note that the *FAK56* mutation by itself showed no detectable defects in the adult eye as the ommatidial units displayed a normal pattern (Figure 3.6F). These results suggest that endogenous levels of FAK may suppress RET in a dose-dependent manner; hence, we propose that the relative levels between RET and FAK may determine the phenotypic outcome in this tissue.

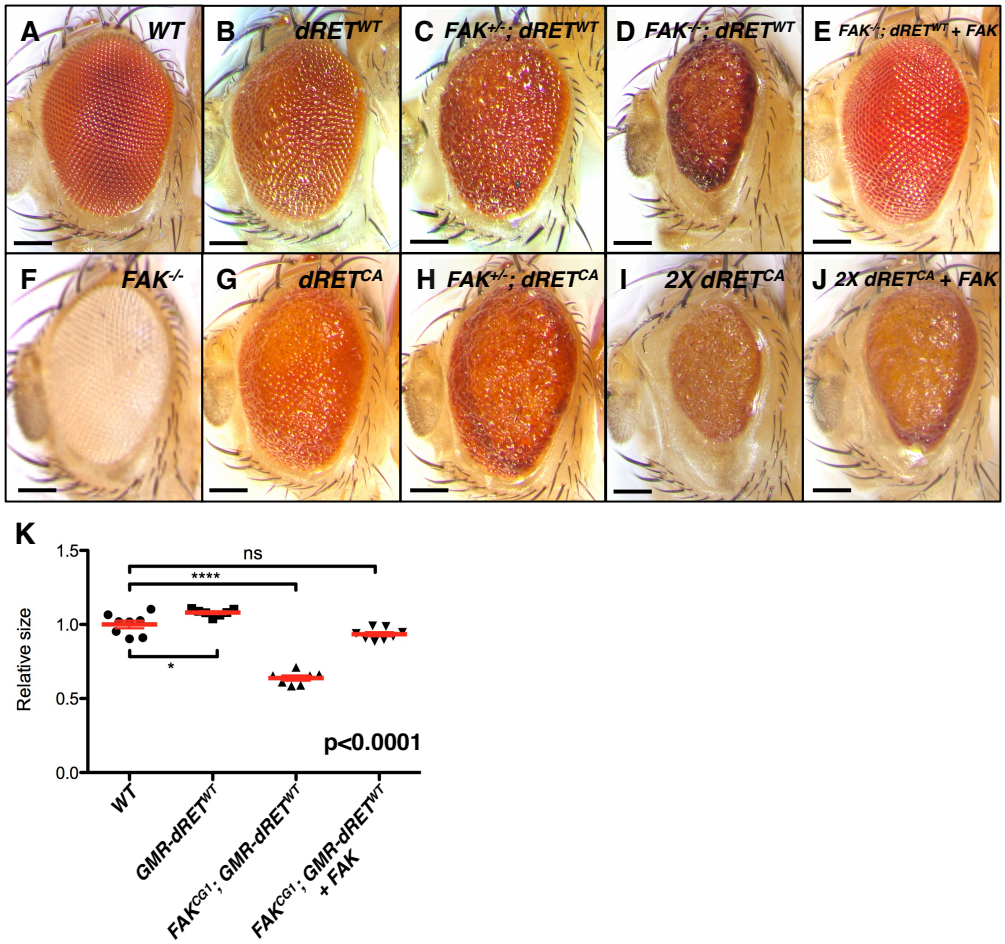

Figure 3.6 RET/FAK ratios regulate RET signalling outcome
Wild Type (A) and *FAK56* (F) animals displayed normal eye patterning. Note that the FAK^{CG1} was in a *white* background (see Figure 3.3). (B) $dRET^{WT}$ expression caused a rough phenotype. (C-D), lowering the genetic dose of *FAK56* gene enhanced eye roughness. (E) Reciprocally, suppression was observed after restoring FAK56 expression. (G-H) Similar enhancement was observed by halving the dose of *FAK56* gene after expression of $dRET^{CA}$. (I) Doubling $dRET^{CA}$ dose caused a very rough, small eye; comparable to (D), which was partially suppressed when FAK56 was co-expressed (J). Scale bars, 100 µm. (K) Quantification of the relative eye sizes from the indicated genotypes, shown in A, B, D and E. Note that FAK loss resulted in decreased eye size in RET^{WT} expressing animals, which was rescued by FAK re-expression.

To further test this hypothesis, we performed a rescue experiment using co-expression of FAK56 together with dRET in a fully $FAK^{-/-}$ background (*GMR-Gal4/+*; $FAK^{-/-}$; *GMR-*$dRET^{WT}$*/UAS-FAK*). Importantly, GMR-driven FAK56 expression caused no detectable adult eye phenotype and had minor retinal patterning defects (Figure 3.4A), but it did permit restoration of normal eye size and reversal of the patterning defects observed in the $FAK^{-/-}$; *GMR-*$dRET^{WT}$ flies (Figure 3.6E). Relative eye sizes of the abovementioned genotypes are displayed in Figure 3.6K. This indicates that FAK56 re-expression is able to overcome both FAK56 loss and ectopic dRET signalling.

When we expressed a gain-of-function form of RET ($dRET^{CA}$), similar phenotypes were observed. One copy of *GMR-*$dRET^{CA}$ also led to a rough

phenotype (Figure 3.6G), which was enhanced after losing one copy of FAK56 (Figure 3.6H; *FAK^{CG1}/GMR-dRET^{CA}*). Interestingly, a double dose of *GMR-dRET^{CA}* (*2X dRET^{CA}*) resulted in a much rougher eye phenotype, resembling *FAK$^{-/-}$; GMR-dRET^{WT}* eyes (Figure 3.6I). Co-expression of FAK56 partially suppressed this phenotype (Figure 3.6J; *GMR-Gal4/+; GMR-RET^{CA}; UAS-FAK/+*), providing further evidence that FAK can even suppress a constitutive active isoform of RET. Overall, these genetic experiments highlight the influence that the ratio between RET and FAK have on the *Drosophila* eye tissue. Thus, different RET/FAK ratios produce variable effects on the patterning and size of the eye epithelium, which presumably are linked with the cellular composition of the eye tissue.

3.4.5 Moderate RET/FAK ratios supress apoptosis

To gain further insights on how different RET/FAK ratios influence tissue cell fate *in vivo*, we examined the cell composition of the patterning retina. Each ommatidial unit consists of eight photoreceptor and six supporting cells (four cone cells and two primary pigment cells) (Figure 3.7A) (Cordero et al., 2004). A hexagonal lattice surrounds the units (white coloured in Figure 3.7A'-C'). Photoreceptor cell clusters are specified first; they constitute 'organizing centres' that instruct neighbouring cells to differentiate into cone cells and primary pigment cells. The hexagonal lattice patterns by local cell reorganization and elimination of surplus cells via a wave of developmental programmed cell death (PCD).

We carried out immunofluorescence assays in pupal retina tissues to visualize the final pattern of cells at 42hs after puparium formation. *FAK56* mutant retinas were indistinguishable from their wild type counterparts (Figure 3.7A and B). When dRET^{WT} was expressed within a *FAK56* wild type background, the array of ommatidia was altered, displaying supernumerary interommatidial cells (Figure 3.7C). Nevertheless, the ommatidial cores remained normal, with four cone cells surrounded by two primary pigment cells. Previously, it has been reported that RET signalling affects different processes such as proliferation, cell death and differentiation (Read et al., 2005). In the case of *FAK$^{+/+}$*; *GMR-dRET^{WT}* flies, the major cause of the adult rough eye phenotype was a greater number of interommatidial cells, which suggested that developmental

programmed cell death might have been suppressed during eye development. We examined this possibility by analysing pupal retinas at 28h after puparium formation, a time in development where there is a very high level of apoptotic cell death. Indeed, while wild type or *FAK56*$^{-/-}$ retinas displayed a large number of apoptotic cells (Figure 3.7D and E), there were virtually no cells in *GMR-dRET*^{WT} retinas that were undergoing programmed cell death (Figure 3.7F).

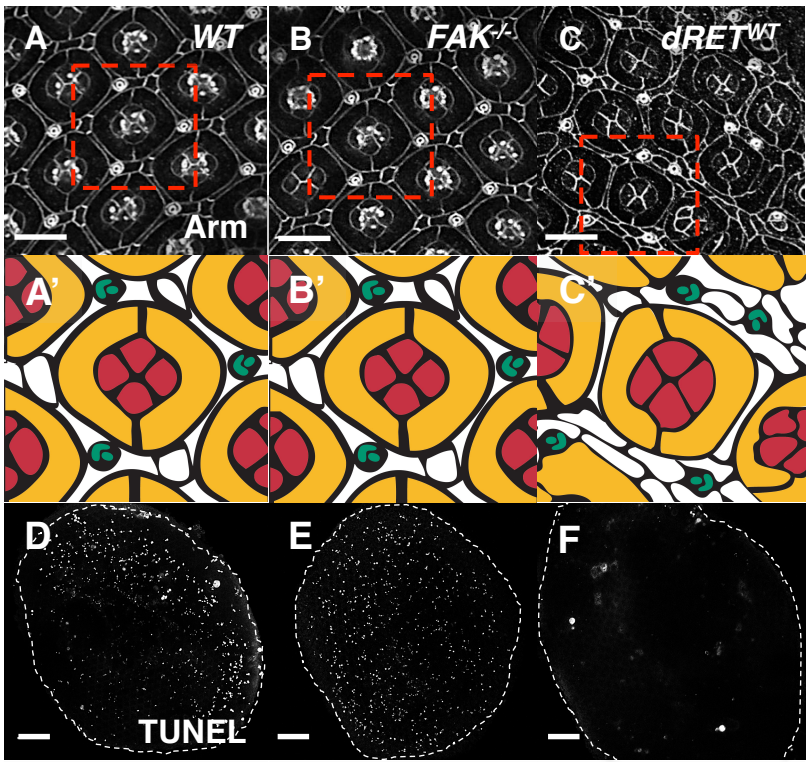

Figure 3.7 Moderate RET/FAK relative levels lead to PCD inhibition
(A-C) Armadillo immunostaining revealed cell outlines of *Wild type*, *FAK*$^{-/-}$, and *dRET*^{WT} retinas at 42hs after puparium formation (APF). The boxed areas were traced to highlight their cellular composition. Each ommatidium is composed of 4 cone cells (red), 2 primary pigments cells (yellow), 6 secondary and three tertiary cells (white), and three-bristle cells (green) make the hexagonal lattice. Note that *FAK*$^{-/-}$ eyes display normal patterning (B). *RET*^{WT} retinas displayed normal ommatidial cores but additional interommatidial cells (white cells in C'). Scale bars, 10 µm. (D-F) TUNEL images of retinas at 28h APF. Note that the normal levels of developmental programmed cell death observed in *wild type* and *FAK*$^{-/-}$ retinas were suppressed in *RET* retinas. Scale bar, 50 µm.

Programmed cell death during eye development is mainly dependent on the pro-apoptotic protein Hid (Head Involution Defective) (Sawamoto et al., 1998, Bergmann et al., 1998, Kurada and White, 1998). Hid over-expression in the developing eye (*GMR-hid*) triggers apoptotic cell death throughout the tissue leading to a small eye phenotype (Figure 3.8A) (Grether et al., 1995). When dRET^{WT} was simultaneously expressed with Hid, the eye size increased significantly (Figure 3.8B), indicating that RET signalling could block Hid or its downstream effectors. RET's ability to suppress Hid-induced apoptosis depended on its effectors Src and Ras: down-regulation of Ras85D or Src42A by RNA

interference prevented RET-mediated suppression of *GMR-hid* small eye phenotype (Figure 3.8E-F). In contrast, while FAK expression by itself has minimal effects on the *GMR-hid* eye phenotype (Figure 3.8C), it did suppress RET inhibitory effect (Figure 3.8D).

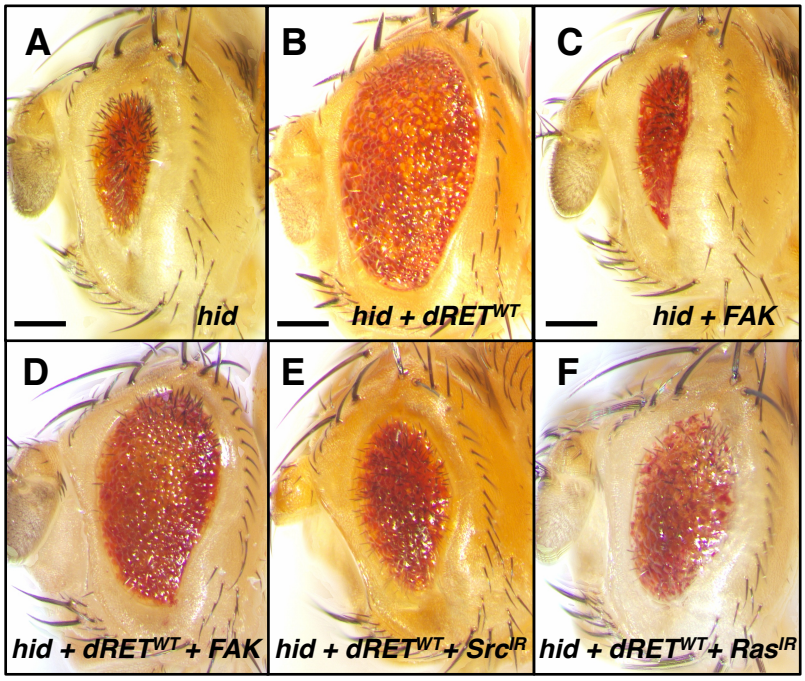

Figure 3.8 Src and Ras act downstream of RET to inhibit Hid-induced apoptosis
(A) Hid overexpression (*GMR-hid*) gave a small eye phenotype, which was suppressed by dRET^{WT} co-expression (B). This role of dRET was also suppressed by FAK co-expression (D). (C) FAK56 expression in the eye does not prevent Hid-killing effects (*GMR-Gal4/+; GMR-hid/+; UAS-FAK/+*). *Src42A*^{RNAi} and *Ras85D*^{RNAi} expression in the eye also suppresses the dRET^{WT}-dependent inhibition of Hid-induced apoptosis (*GMR-Gal4/+; GMR-hid/+; GMR-dRET*^{WT}*/UAS-Src42D*^{RNAi} *or Ras85D*^{RNAi}), proving this anti-apoptotic role of dRET was dependent on its effectors Src and Ras. Scale bar, 100 μm.

Taken together, our data suggest that dRET expression suppresses both Hid-induced ectopic cell death and developmental cell death via its known effectors Src42A and Ras85D, which have been already shown to inhibit developmental cell death and Hid in the *Drosophila* eye (Sawamoto et al., 1998, Bergmann et al., 1998, Kurada and White, 1998, Vidal et al., 2007). Therefore, in the retina, the output of RET overexpression in a *FAK56*$^{+/+}$ background— genetically defined here as a moderate RET/FAK ratio— is the inhibition of cell death; and given the dependency on Ras, we also speculate this genetic scenario determines a moderate level of RAS/MAPK signalling (Please see section 3.4.7).

3.4.6 High RET/FAK ratios drive ectopic differentiation

While the output of moderate RET/FAK ratios resulted in suppression of cell death, the small eye phenotypes observed under conditions of higher

RET/FAK ratios (Figure 3.6D and I) suggested different cell fate outcomes. We then further analysed two different experimental conditions expected to produce high RET to FAK ratios, namely (i) the expression of one copy of $dRET^{WT}$ in a *FAK56* mutant background (*$FAK^{-/-}$; GMR-$dRET^{WT}$*), and (ii) the expression of two copies of $dRET^{CA}$ in a *FAK56* wild type background (*2X $dRET^{CA}$*), as these displayed the roughest, reduced eye size phenotypes (Figure 3.9B and C, similar to Figure 3.6D and I, respectively). *$FAK^{-/-}$; GMR-$dRET^{WT}$* pupal retinas lacked the hexagonal array and identifiable ommatidial units (Figure 3.9F-F'). In this case, there were clusters of numerous cone-like cells. Some bristle cells and a few cells recognisable as primary pigment-like remained, but there were no detectable cells with the appearance of normal interommatidial cells (Figure 3.9F'). In order to confirm the identity of those cells, we stained for the transcription factor Cut, a well-known cone cell marker (Blochlinger et al., 1993). In control retinas, Cut localised constitutively to the nucleus of the four cone cells from each ommatidium (Figure 3.9E'') (Blochlinger et al., 1993). In contrast, in *$FAK^{-/-}$; GMR-$dRET^{WT}$* retinas (Figure 3.9F''), the cell clusters were indeed made of numerous Cut-expressing cone-like cells. Thus, in these experimental conditions, RET signalling drives ectopic differentiation into the cone cell fate.

Similarly to *$FAK^{-/-}$; GMR-$dRET^{WT}$*, *2X GMR-$dRET^{CA}$* pupal retinas displayed a mesh of cone-like cells as shown by Cut staining (Figure 3.9C and G-G''), where the few remaining cells displayed bristle cell morphology. The expression of FAK56 within this context rescued the small eye size phenotype (Figure 3.9D). Most remarkably, it also reduced the number of ectopic cone cells and resulted in the re-appearance of normal ommatidial cores and surrounding interommatidial cells in the pupal retinas (Figure 3.9H-H'').

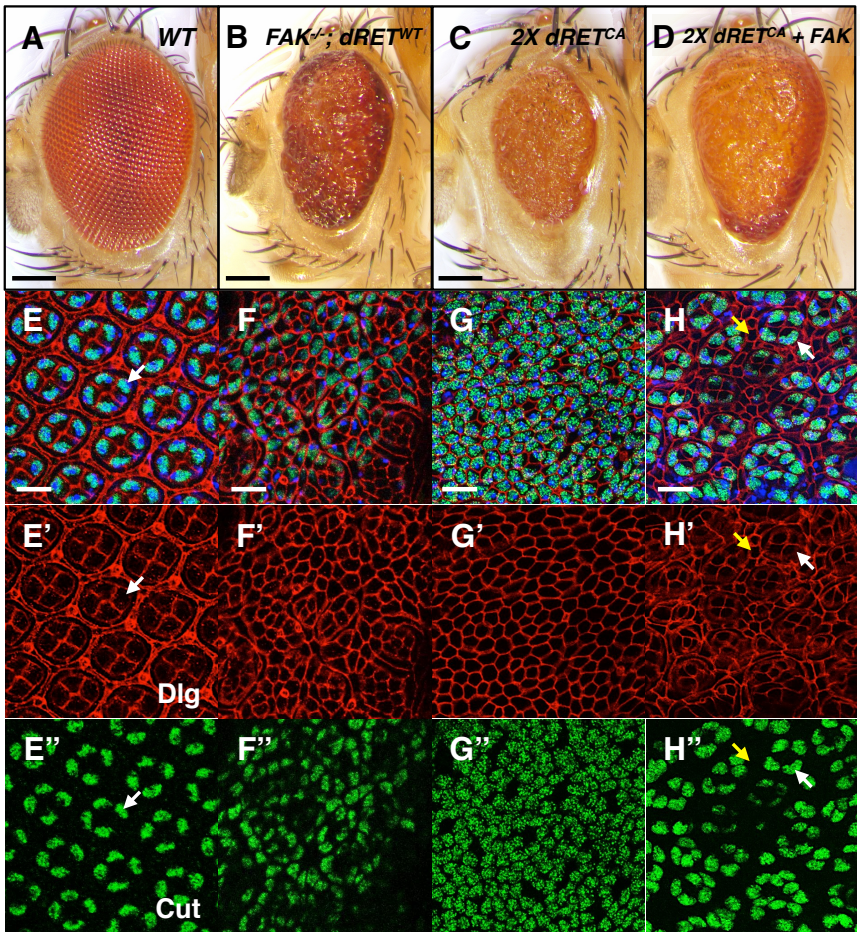

Figure 3.9 High relative levels between RET and FAK induce ectopic cone cell differentiation in the eye

We examined the cellular composition of the pupal retinas in correspondence to the adult eye phenotypes shown in panels A-D. Scale bars, 100 μm. (E-H) Merged imaged from stainings for nuclei (DAPI, blue), Dlg (cell outlines, Red) and Cut (cone cells, green), from retinas at 42h APF. Bottom panels show Dlg (E'-H') and Cut (E''-H'') stainings individually. (E) Note the symmetric hexagonal array, and four Cut-positive cone cells per ommatidium (white arrows) in control retinas. (F, G) Note the change in cellular composition of these retinas with high RET/FAK levels, primarily composed of Cut-positive cone-like cells. (H) FAK56 expression within a *2X dRET^{CA}* background suppressed this phenotype; some normal four-cone cell clusters (white arrows) and interommatidial cells reappeared (yellow arrows) can be identified. Scale bars, 10 μm.

Moreover, similarly to the RET-mediated suppression of cell death (Figure 3.7 and 3.8), the down-regulation of Ras85D also prevented this ectopic differentiation and restored the eye size and patterning back to a normal array of ommatidia (Figure 3.10).

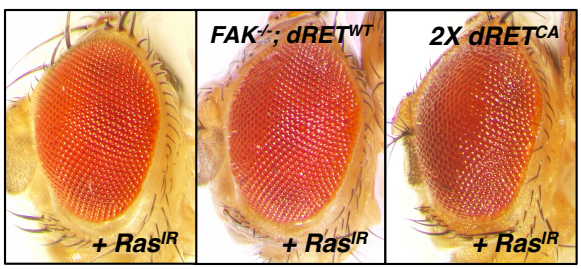

Figure 3.10 The RET/Ras axis drives cone cell differentiation

Ras85D^{RNAi} expression was able to rescue back to normal the highly rough and small eye phenotypes of *FAK*$^{-/-}$*; GMR-dRET*^{WT} (Figure 3.9C) and 2X *GMR-dRET*^{CA} flies (Figure 3.9C), proving Ras signalling is a central driving force of ectopic cone cell differentiation.

We also tested whether the supernumerary cone cells could be a consequence of ectopic differentiation during the larval stage. Since photoreceptor cells induce cone cell differentiation, one possibility is that aberrant photoreceptor cell numbers trigger ectopic cone cell differentiation. However, we observed normal clusters of eight photoreceptors in *FAK*$^{-/-}$*; GMR-dRET*^{WT} eye discs, albeit some clusters had rotation defects (Figure 3.11A-C).

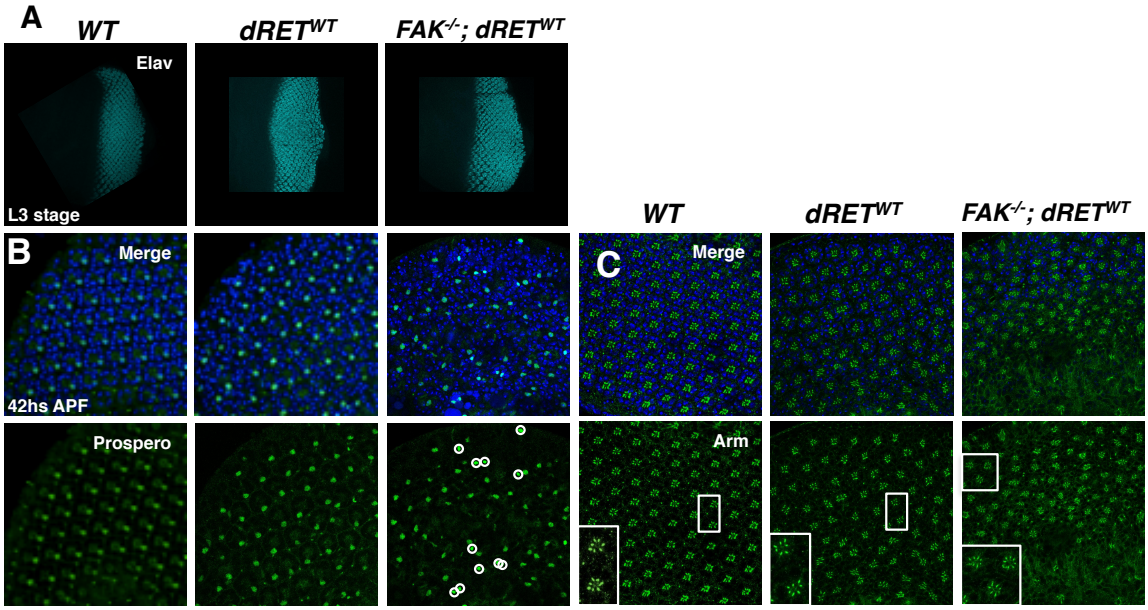

Figure 3.11 RET expression and/or *FAK* deletion does not affect the development of photoreceptor cells
(A) Immunofluorescence staining against the pan-neuronal marker ELAV revealed that photoreceptor differentiation was not altered in *GMR-dRET*^{WT} or *FAK*$^{-/-}$*; GMR-dRET*^{WT} eye discs. (B) Staining against the R7-photoreceptor marker, Prospero, at later stages of eye development (42hs APF) showed one single R7 photoreceptor nuclei per cluster in all genotypes. Circles indicate bristle cell nuclei, which also express Prospero. Co-visualization of R7 and bristle nuclei is due to misfolding of *FAK*$^{-/-}$*; GMR-dRET*^{WT} retinas. (C) Armadillo staining further demonstrated the normal clusters of photoreceptor cells. All the clusters showed seven photoreceptors cells at a given focal plane although planar polarity rotation problems were observed in *FAK*$^{-/-}$*; GMR-dRET*^{WT} retinas.

Together, these results indicate that in a genetically defined high RET/FAK ratio, which could be alternatively interpreted as an enhanced Ras/MAPK signalling, most of non-neuronal eye cell types ectopically differentiate into cone cells.

3.4.7 FAK impairs MAPK downstream of RET signalling

Next, in order to gain insights into the RET signalling inhibition mechanism we assessed the role of FAK in influencing the RET-signalling effectors.

dRET was reported to activate the PI3K/Akt pathway (Dar et al., 2012), therefore we assessed whether there was also an ectopic activation of *Drosophila* Akt1 (v-Akt murine thymoma viral oncogene homologue 1) in our experimental conditions. However, immunostaining assays against phosphorylated-Akt1 showed no difference in Akt activation after expression of dRET^{CA} (Figure 3.12A). This suggests that RET signals independently of PI3K/Akt in the imaginal disc domains we utilized.

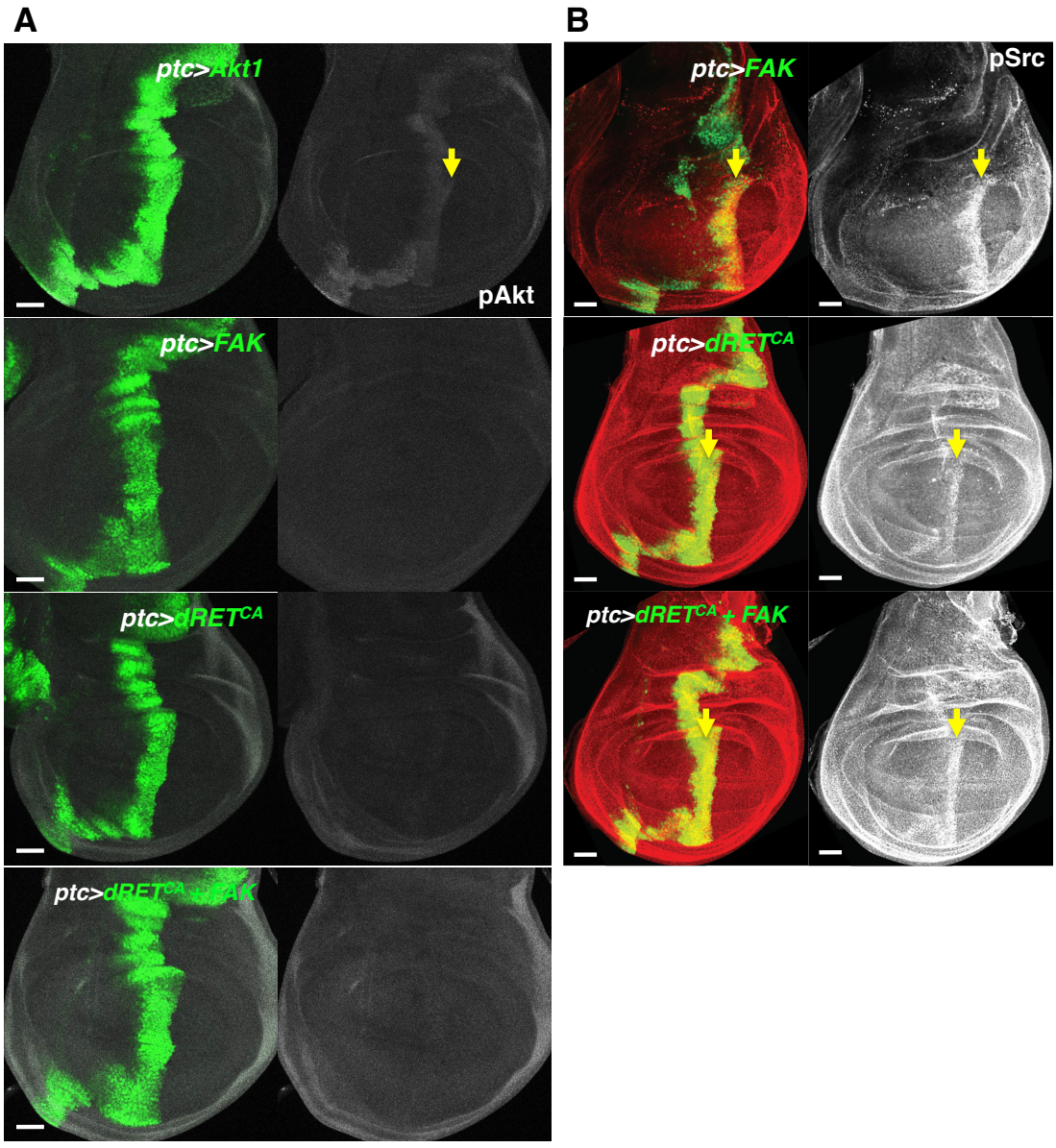

Figure 3.12 Ret induces Src but not AKT activation
(A) Over-expression of Akt resulted in increased pAkt staining within the *ptc* stripe (arrow). In contrast, Akt pathway is not activated by RET^{CA}, FAK56 or simultaneous expression of both proteins when expressed in the *ptc* domain of the wing discs. Scale bars, 50 μm. (B) FAK56 expression by itself (*ptc-Gal4, UAS-GFP/+; UAS-FAK/+*) or alongside *dRET^{CA}* (*ptc-Gal4, UAS-GFP/+; UAS-FAK/UAS-dRET^{CA}*) caused an activation of Src kinase in the ptc compartment of the wing disc (arrows). Scale bars, 50 μm.

We showed before that Src and MAPK were activated upon RET expression (Figure 3.1D''-E). Src phosphorylation was seen after FAK56 expression itself and remained unchanged upon co-expression with dRET^{CA} (Figure 3.12B). Regarding MAPK, FAK56 did not modulate its phosphorylation on the cell population where it was expressed (*ptc>FAK*) but it did activate MAPK in the immediate neighbouring cells in a non-cell autonomous manner (Figure 3.13A and yellow arrows on 3.13B). Strikingly, MAPK phosphorylation within dRET^{CA}-overexpressing cells (Figure 3.13C-D) was reduced after FAK56 co-expression (Figure 3.13E-F). This indicates that FAK is able to block RET signalling by inhibiting specifically the MAPK pathway.

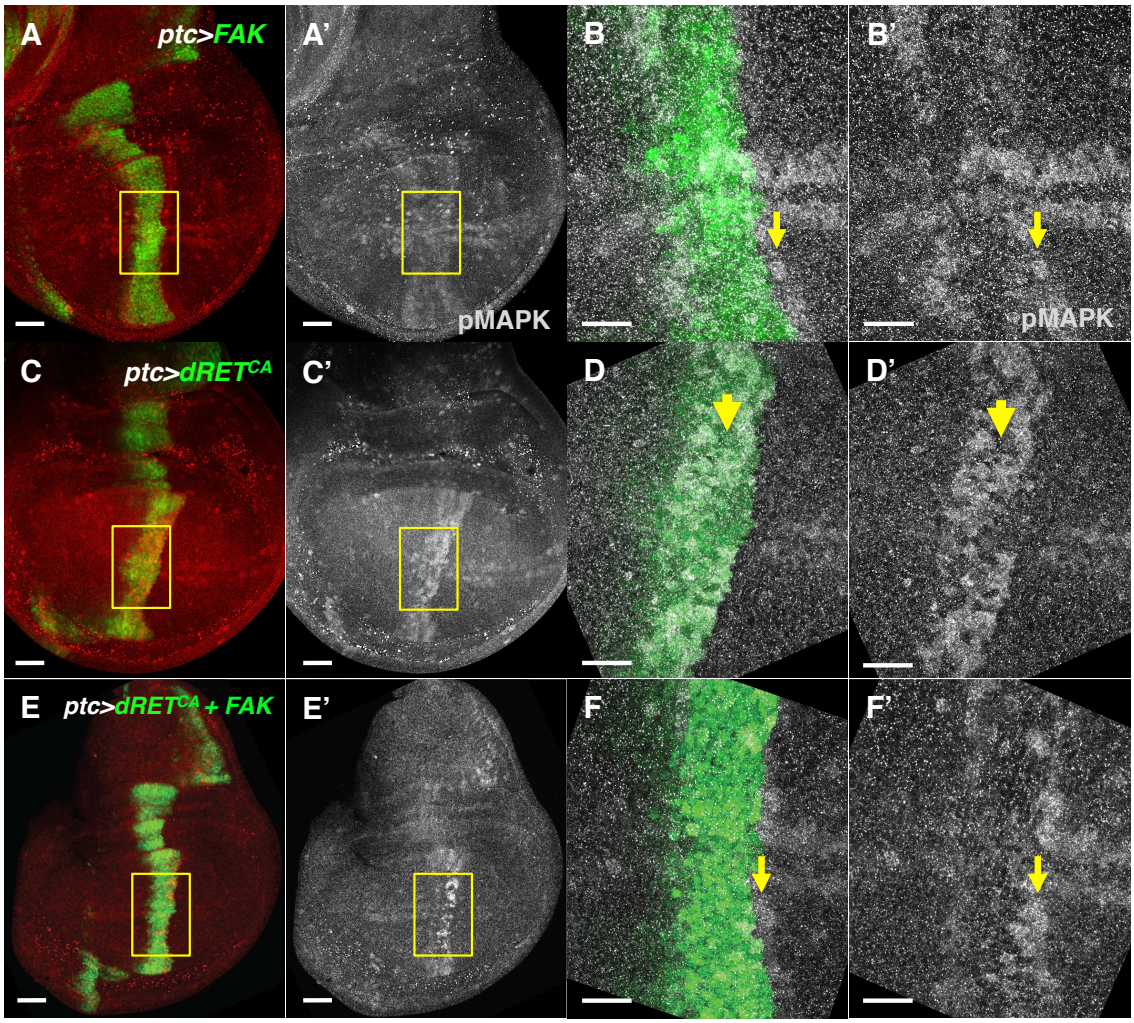

Figure 3.13 FAK inhibits RTK signalling by impairing Ras/MAPK pathway
(A-F) Phosphorylated (active) MAPK staining images from wing discs with the indicated genotypes. Panels in the right are high magnification views from the boxed areas in the left panels. Please note that Figure 3.1B is a control panel for these experiments showing GFP expression alone in the *ptc* stripe does not cause phosphorylation of MAPK. (A-B) FAK56 was expressed in the *ptc*-compartment. Note that pMAPK staining was unchanged within *ptc*-expressing cells (green), while cells abutting the *ptc* domain displayed increased levels of pMAPK staining (arrows). (C-D) dRET^{CA} expression increased pMAPK staining in cells within the *ptc* domain (arrow in D'). (E-F) Co-expression of FAK56 suppressed the dRET^{CA}-induced increase in

pMAPK within the *ptc* domain. Panels A, C and E: Scale bars, 50 μm (left) and B, D, F 25 μm (right).

3.4.8 FAK suppresses EGFR signalling

Next, we evaluated whether the ability of FAK56 to inhibit receptor tyrosine kinase signalling to MAPK was specific for RET. The epithelial growth factor receptor (EGFR) is known to bind to FAK in mammals (Sieg et al., 2000, Long et al., 2010) and to activate MAPK in *Drosophila* (Diaz-Benjumea and Hafen, 1994). Therefore, we took a similar approach and co-expressed *Drosophila* EGFR (dEFGR) and FAK56 in the eye or in the wing disc *ptc* domain with the *GMR-Gal4* and *ptc-gal4* drivers, respectively. As it happened with dRET, dEGFR also induced FAK (Figure 3.14A) but not Akt activation (Figure 3.14B).

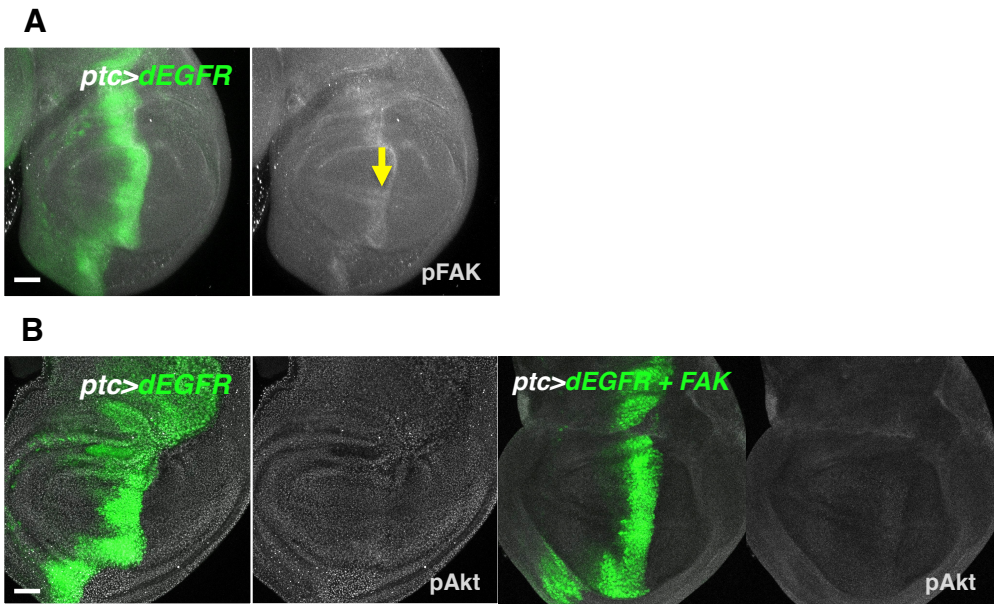

Figure 3.14 FAK is also activated by dEGFR
(A) Immunostaining assays showed increase phosphorylation of FAK upon dEGFR expression in the *ptc* stripe of the wind disc (arrow). Genotype: *UAS-dEGFR/+; ptc-gal4, UAS-GFP/+*. (B) dEGFR did not activate Akt phosphorylation significantly in the ptc stripe; FAK co-expression made no difference either. Scale bar, 50 μm.

Interestingly, co-expression with FAK also resulted in a significant rescue of the *GMR>dEGFR* phenotype (Figure 3.15A and B) and inhibition of MAPK activation (Figure 3.15C-D'). Moreover, FAK allowed a remarkable increase in survival of *ptc>dEGFR* flies (Figure 3.15E).

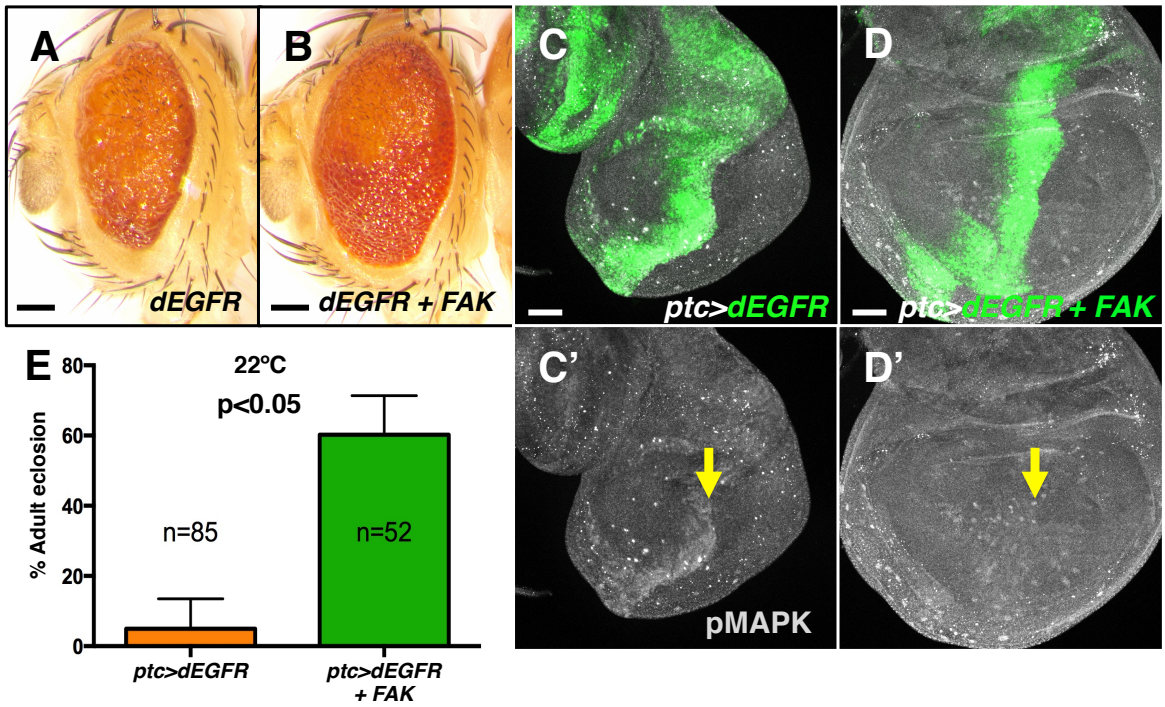

Figure 3.15 FAK56 suppresses the EGFR/MAPK axis in *Drosophila* epithelia
(A-B) Adult eyes expressed *Drosophila* EGFR alone or in combination with FAK56. Note that FAK56 co-expression suppressed the rough, small eye phenotype driven by EGFR. Scale bar, 100 µm. (C-C') Expression of dEGFR within the *ptc* domain resulted in increased MAPK phosphorylation (arrow in bottom panel). (D-D') Co-expression of FAK56 rescued the ectopic pMAPK staining within the *ptc* stripe. Scale bar, 50 µm. (E) Quantification of the penetrance on adult eclosion for the indicated genotypes. Note that FAK56 co-expression significantly rescued the developmental lethality associated to *ptc*-driven dEGFR expression.

Mechanistically, FAK seems to act in a similar fashion with both RTKs as the mutant isoforms of FAK showed the same pattern of suppression when expressed within a *GMR>dEGFR* context: the autophosphorylation-site mutant was still capable of partially suppressing the EGFR-induced small eye phenotype (Figure 3.16), while the amino-terminal domain mutant failed to modify it significantly. Again, as discussed in section 3.4.3, a quantification of each transgene's expression level has to be performed in order to validate the biological significance of these observations.

Thus, FAK56 suppressed not only dRET but also other RTKs, namely dEGFR and its FERM domain appears essential to initiate its negative regulation.

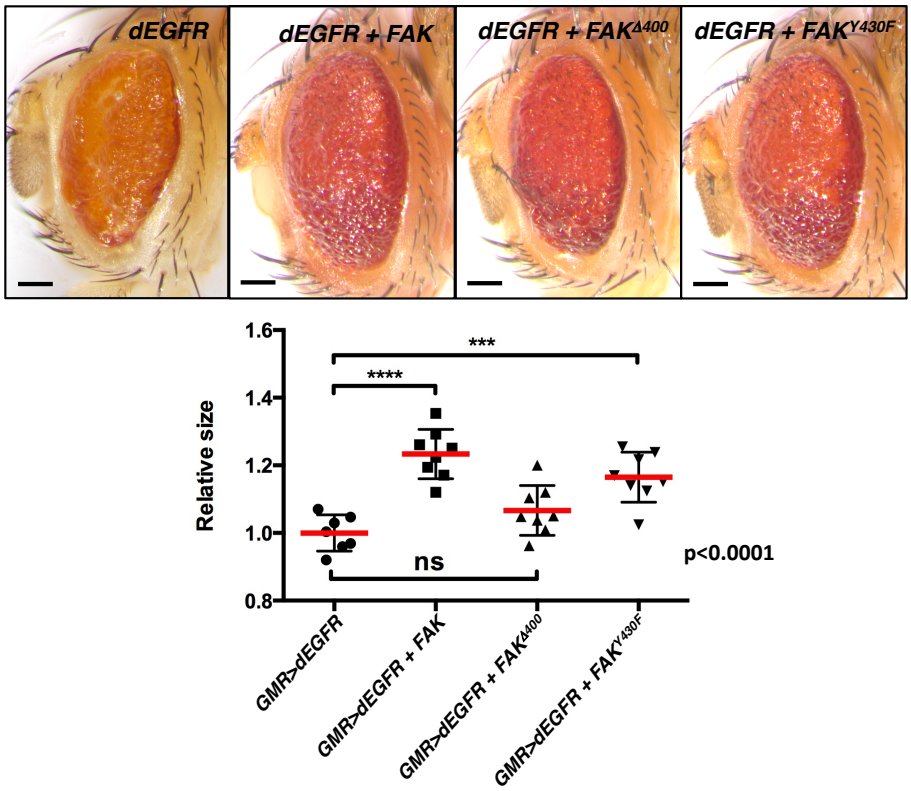

Figure 3.16 FAK56 N-terminal domain scaffolding functions mediate suppression of EGFR
Eye adult images showing expression of different FAK mutant isoforms and their effects on the dEGFR overexpression phenotype. Note that similar to the case of RET, the N-terminus domain mutant ($FAK^{\Delta 400}$) did not suppress dEGFR's reduced eye size, while wild type FAK and a kinase mutant FAK isoform (FAK^{Y430F}) did. Scale bars, 100 μm. The graph shows a quantification of relative eye sizes from the different genotypes. *n.s.*: not statistically different; **** = $p<0.0001$.

3.4.9 FAK role is conserved role downstream of RTK signalling

We next tested whether the negative role of FAK downstream of RTK signalling was conserved between flies and humans. Since FAK56 inhibited dEGFR signalling, we chose the human breast adenocarcinoma MDA-MB-231 cell line, which express high levels of EGFR and FAK (Corkery et al., 2009, Price et al., 1999, Owens et al., 1995, Agochiya et al., 1999), as a system to explore how ERK (MAPK) signalling reacts to changes in EGFR/FAK ratios. An efficient knockdown of the FAK protein was achieved using small interfering RNAs for 48 hours after transfection (Figure 3.17A). Remarkably, when cells were grown in presence of serum we observed an increase of ERK1/2 phosphorylation (pERK1/2) after FAK knockdown, while the total ERK1/2 and EGFR levels remained constant. We observed a similar increase in ERK1/2 phosphorylation, although to a lesser degree, at 72hs after transfection (Figure 3.17A). Moreover, we also observed a similar increase in pERK1/2 phosphorylation upon FAK knockdown in H1299 cells (Figure 3.17B), a human lung adenocarcinoma that also express high levels of wild type EGFR (Rusch et al., 1993, Amann et al., 2005).

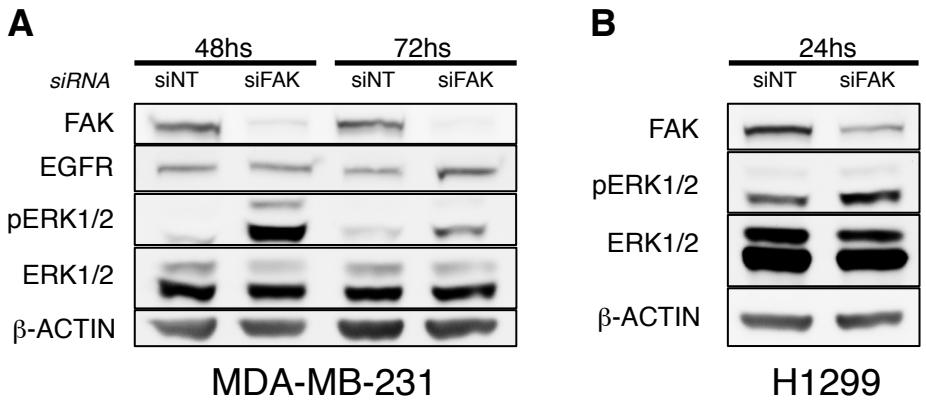

Figure 3.17 FAK suppression of EGFR signalling is conserved in human breast and lung cancer cell lines
(A) Western blots from protein extracts from MDA-MB-231 cells (left panel) at 48 or 72 h after transfection with FAK siRNA. FAK protein levels were effectively knocked down. Total levels of EGFR and ERK were not changed, whereas there was a marked upregulation in phosphorylated ERK1/2 upon FAK knockdown, which was more apparent at 48 h after siRNA transfection. Actin levels were probed as an additional loading control. (B) Western blot from protein extracts from H1299 human non-small cell lung carcinoma cells after 24 hours transfection with FAK siRNA. An increase of activated ERK1/2 was also observed after FAK knockdown, while levels of ERK did not changed. Actin levels were probed as an additional loading control.

When serum-starved FAK-siRNA MDA-MB-231 cells where treated with EGF in order to selectively activate the EGFR receptor, the same dramatic increase of ERK (MAPK) activation was observed (Figure 3.18). These results indicate that indeed, the suppressive role of FAK on RTK/MAPK signalling is conserved between *D. melanogaster* and humans as it also applies to EGF/EGFR/ERK signalling in human cancer cell lines.

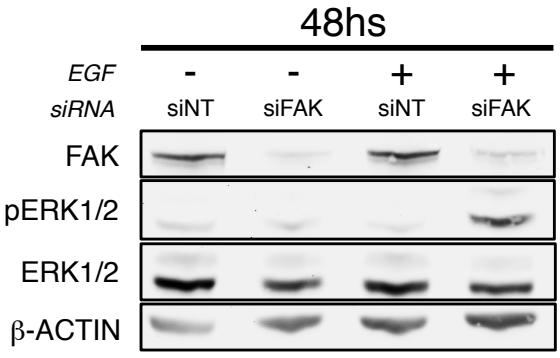

Figure 3.18 FAK knockdown enhances ERK1/2 activation after specific induction of EGFR
MDA-MB-231 cells were transfected with either non-targeting (siNT) or FAK-specific siRNA (siFAK) and serum starved prior to addition of EGF. Note that FAK knockdown (48hs after transfection) resulted in increased phosphorylation of ERK1/2 in response to EGF treatment (30 μM, 15 minutes).

3.4.10 FAK reduces EGFR content at the cell surface

To gain mechanistic insights into how FAK suppresses RTK/MAPK signalling, we next examined the cellular distribution of EGFR in MDA-MB-231 cells. Previous work indicated that growth factor receptors regulate cell

signalling differently depending on its localization at the plasma membrane or at internalized vesicles (Sigismund et al., 2008, Miaczynska et al., 2004, Sadowski et al., 2009). Since FAK knockdown did not affect total levels of EGFR (Figure 3.17A), we hypothesised that it could be a change of receptor subcellular localization what explains the enhanced ERK signalling.

Immunofluorescence staining for EGFR in control or FAK knockdown cells demonstrated a change of receptor levels at the cell surface: in FAK-siRNA cells, EGFR was increased at the plasma membrane at the expense of the intracellular pool (Figure 3.19A-B). We quantified these data using ImageJ (Fiji) software (see materials and methods) and observed a 27% increase in the fraction of total EGFR located at the cell surface (Figure 3.19C). This increase could account for the increased ERK (MAPK) signalling in cells with reduced FAK levels.

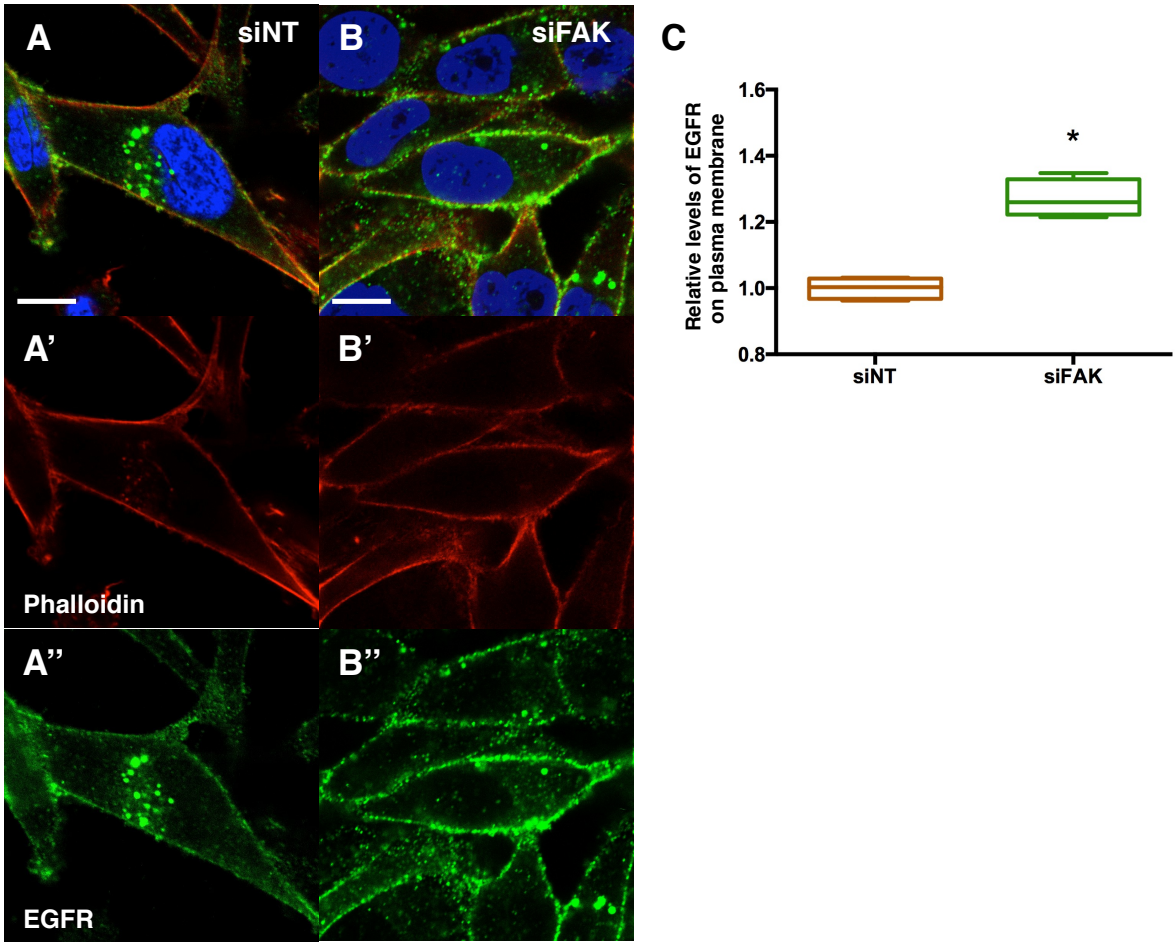

Figure 3.19 FAK retains EGFR at the plasma membrane
(A-B) MDA-MB-231 cells transfected with non-targeting (NT) siRNA or FAK siRNA were immunostained with anti-EGFR antibody and rhodamine phalloidin. Note the differential localisation of EGFR. While in siNT cells the receptor is distributed in plasma membrane and internal vesicles, FAK downregulation leads to an increase of EGFR levels at the cell membrane. Scale bar, 10 μm. (C) This difference is statistically significant as assessed by Mann-Whitney test;

p=0.0286. The values are expressed as relative levels of the receptor against the mean value of siNT cells; four fields for each condition were analysed: n=347 (siNT), n=414 (siFAK). Immunofluorescence staining and quantifications were done by Jesica Diaz Vera.

To test this hypothesis, we experimentally increased the fraction of EGFR at the plasma membrane using the Dynamin GTPase inhibitor Dynasore, widely used to retain receptors at the cell surface by inhibition of dynamin-dependent endocytosis (Macia et al., 2006, Mesaki et al., 2011, Henriksen et al., 2013, Rizzolio et al., 2012). This treatment phenocopied FAK knockdown as *siNT*-cells treated with Dynasore showed higher ERK1/2 activation than non-treated *siNT*-cells, without affecting ERK1/2 or EGFR total levels (Figure 3.20). Moreover, simultaneous treatment of Dynasore and FAK-siRNA did not further increase pERK1/2 levels (Figure 3.20) suggesting that these two factors converge in a similar effect.

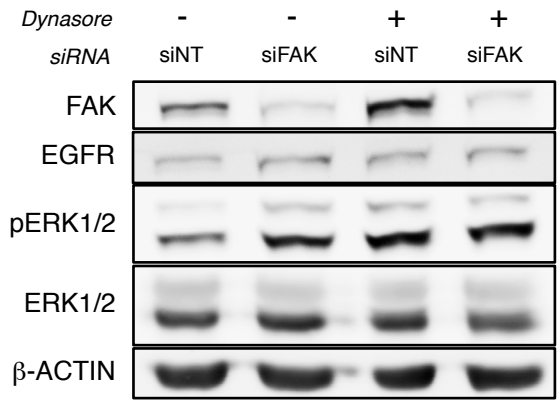

Figure 3.20 Inhibition of dynamin-dependent endocytosis retains EGFR at the plasma membrane

MDA-MB-231 cells were transfected with either non-targeting (siNT) or FAK-specific siRNA (siFAK) and deprived of serum prior to addition of 80μM Dynasore hydrate (Sigma Aldrich). siNT-transfected cells showed an increased pERK1/2 level in response to Dynasore treatment (80 μM, 30 minutes) although no change was observed in siFAK cells. Total levels of EGFR and ERK were not changed and actin levels were probed as an additional loading control.

In numerous contexts, EGFR signals to ERK preferentially when located at the cell surface (Marshall, 1995, Irwin et al., 2011). Our results indicate that FAK prevents EGFR localisation at the plasma membrane by a yet unknown mechanism. Moreover, the inhibition of receptor internalisation showed EGFR signals through ERK when located at the cell surface. Overall, these data suggest that down-regulation of FAK results in a more abundant EGFR membrane pool and a consequent enhanced ERK phosphorylation.

3.5 Discussion

Our research provides evidence that *Drosophila* FAK (FAK56) plays a suppressive role downstream of RTK signalling, particularly within an environment of oncogenic stress induced by RTK over-expression/hyper-activation. We found that ectopic dRET and dEGFR signalling were able to activate FAK56 and the MAPK pathway. Nevertheless, FAK56 negatively regulated RTK-induced signalling to the MAPK pathway. The highly conserved FERM domain of FAK56 was necessary for its functional suppression of *Drosophila* RTK signalling, which is consistent with the physical interactions between FAK and several RTKs described in mammals (Chen et al., 2011, Chen and Chen, 2006, Plaza-Menacho et al., 2011). This negative feedback regulation indicates that the balance between RTK and FAK is what determines the dosage of MAPK pathway activity, which ultimately dictates the final cellular fate (Figure 3.21).

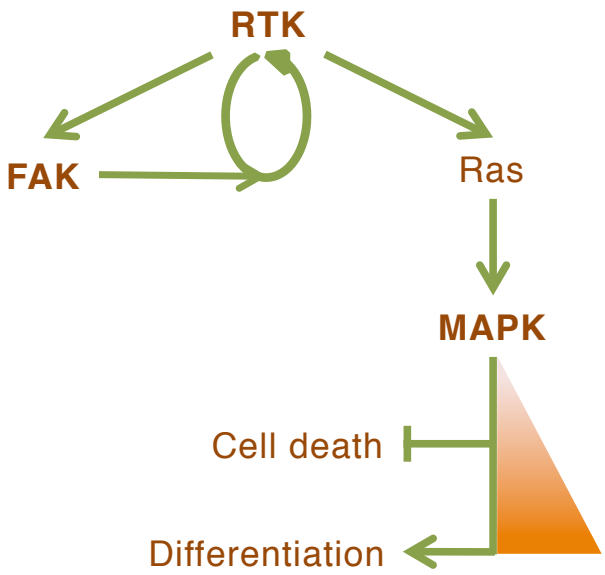

Figure 3.21 Model of RTK negative regulation by FAK
This diagram illustrates the FAK regulatory mechanism over RTK signalling. RTKs activate FAK and Ras/MAPK signalling pathway while FAK regulates RTKs trafficking between the cytosolic and cell surface pool, possibly to keep a balanced signalling. In our system, when FAK is reduced or absent, RTKs accumulates at the plasma membrane, and thus enabling a higher flux of signalling through Ras/MAPK pathway.

We characterized cell outcomes in detail in the patterning eye anlage. In this tissue, it is well known that different levels of MAPK pathway result in different outcomes: moderate levels of activation direct survival of cells during the wave of developmental apoptosis (Halfar et al., 2001), whereas high levels of activation result in ectopic differentiation into photoreceptors (Halfar et al., 2001) or the cone cell fate (Matsuo et al., 1997). Correspondingly, we observed

that experimental genetic manipulations expected to produce moderate RET/FAK ratios —such as the expression of one copy of wild type dRET in a *FAK56*$^{+/+}$ background— resulted in reduced developmental apoptosis and supernumerary interommatidial cells. On the other hand, genetically defined high RET/FAK activity ratios —such as the expression of one copy of wild type dRET in a *FAK56*$^{-/-}$ background, or the expression of two copies of constitutively activated dRET in a *FAK56*$^{+/+}$ background— resulted in ectopic differentiation into cone cells. Importantly, all these RET-driven phenotypes were suppressed by the co-overexpression of FAK56 that lowered RET/FAK ratios.

The initial characterization of the RET-driven eye model identified several components of the Src and Ras/MAPK pathways (Read et al., 2005). These authors further observed that high levels of RET signalling, achieved by the expression of two copies of activated RET, resulted in non-patterned retinas composed of identical cells with cuboidal morphology, which were proposed to be undifferentiated precursor cells. This led to the conclusion that high RET signalling could block differentiation in this tissue. Contrarily, under similar experimental conditions, experiments presented here showed such cuboidal cells express the cone cell marker Cut. Thus, we conclude that high relative levels between RET and FAK likely result in a proportional activation of the MAPK pathway, which force differentiation into the cone cell fate.

Previous work reported that in *FAK56* mutant embryos, the activity of the MAPK pathway is normal during development (Tsai et al., 2008). This indicates that endogenous MAPK signalling does not normally became hyper-activated simply as a result of FAK loss and is in sharp contrast to the case of over-expression of RET or EGFR as reported here. We postulate that in imaginal disc epithelia, FAK56 constitutes a signalling “fuse” that can act as a signalling negative feedback for RET and other RTKs, specifically in conditions of oncogenic stress induced by ectopic RTK activation. Relevant to this, we recently reported mammalian FAK can ‘protect’ epithelial cells from un-regulated active RET or Src signalling; when FAK is absent, some epithelial cancer cells specifically respond by targeting these promiscuous oncogenic kinases for autophagic degradation (Sandilands et al., 2012a, Sandilands et al., 2012b). It therefore seems that a common feature of FAK’s regulatory function is to ‘buffer’ potentially hazardous oncogenic signalling of deregulated tyrosine

kinases, whether by restraining their signalling to downstream effector pathways as shown here, or by promoting their rapid clearance by degradation (Sandilands et al., 2012a, Sandilands et al., 2012b).

In *Drosophila*, negative regulation of MAPK by FAK56 has been previously observed in neuromuscular junction (NMJ) growth (Tsai et al., 2008); in fact, this was one of the few developmental defects detectable in *FAK56* mutant animals. Interestingly, it was suggested that this 'non-canonical' negative regulation of MAPK by FAK56 was specific to the process of integrin-dependent NMJ growth (Tsai et al., 2008). Importantly, our data imply instead that this is a more commonly used mechanism that occurs also in epithelial tissues, downstream of ectopic RET and EGFR signalling. Thus, the FAK56-mediated negative regulation of MAPK signalling is widespread across *Drosophila* tissues, and we show that this has important consequences for cell and tissue fate. Most importantly, we observed that FAK's ability to restrain signalling through the MAPK pathway is conserved in human breast carcinoma cells downstream of EGFR signalling. We demonstrate that this novel role of FAK relies on its ability to affect receptor sub-cellular localisation. RTKs normally reside at the plasma membrane or within internalised cytosolic vesicles. There is a constant transport of vesicles between these two pools, which allow cells to keep a healthy RTK signalling by degrading old receptor molecules or resetting their activity and sending them back to the cell surface . Depending on the cell type, receptor type and downstream signalling pathways, RTKs can activate a certain signalling pathway either from the plasma membrane or endocytic vesicles; we showed that EGFR signals to Ras/MAPK pathway preferentially from the cell surface, and FAK is able to regulate EGFR/Ras/MAPK signalling by controlling receptor localisation. We speculate that FAK may favour receptor internalisation or reduce receptor recycling, *i.e.* the transport of vesicles from the internal pool towards the membrane. These possible mechanisms would explain why loss of FAK leads to more receptor at the membrane and a consequent upregulation of MAPK signalling pathway as seen in MDA-MB-231 cells.

The attenuation of the MAPK signalling transduction pathway by FAK is in stark contrast to FAK's well-established role linking integrin engagement to the activation of Ras/MAPK (Schlaepfer et al., 1994). Therefore, the regulation of MAPK by FAK may be context-dependent. It is worth noting that previous studies

reporting the activation of MAPK by FAK utilized immortalized cultured fibroblasts (Schlaepfer et al., 1994); it is possible that FAK-mediated negative regulation of MAPK applies to epithelial cells *in situ* and acts downstream of RTKs but integrins.

Ectopic activation of FAK in a range of human carcinomas (Owens et al., 1995) and its role promoting migration and survival of malignant cells make it an attractive therapeutic target. While most of the research has focused on cell culture systems, the availability of conditional *FAK* knockout alleles allowed gene deletion in specific tissues and within the context of cancer models. This led to the demonstration that APC-driven colorectal tumours (Ashton et al., 2010), skin papilloma (McLean et al., 2004) and breast tumours depend on FAK to progress to carcinoma (Lahlou et al., 2007). Many small molecule inhibitors have been developed to target FAK kinase activity, and clinical trials are in progress (Infante et al., 2012). On the other hand, EGFR has been shown to inhibit FAK activity leading to increased cell motility and invasion (Lu et al., 2001) and in some tumors FAK downregulation has also been related with malignancy (Gabriel et al., 2006, Ayaki et al., 2001, Ohta et al., 2006, Lu et al., 2001). Moreover, it has been shown that Ras activation, via the MEK/ERK pathway, results in serine phosphorylation of FAK, and dephosphorylation of specific FAK tyrosine residues usually associated with activation (Zheng et al., 2009, Antonyak and Cerione, 2009). Therefore, the signaling crosstalk from FAK and RTK/Ras/MAPK is complex and context-dependent (Zheng and Lu, 2009), but we propose that FAK's emerging tumour suppressor functions need to be understood as FAK inhibitors move towards potential clinical use.

We also present evidence that the N-terminal FERM domain may prove essential in the regulation of RTKs but not its kinase function. We showed that a FERM domain deletion isoform of FAK56 was not able to suppress RET and EGFR effects in the *Drosophila* eye. On the other hand, a point-mutant FAK56 isoform that lack the key auto-phosphorylation site, considered necessary for kinase activity, did suppress those effects. These results highlighted the important regulatory roles of the FERM domain by likely mediating interactions with RET and EGFR. Additionally, the data suggested the kinase domain was dispensable in the regulation of RTK signaling. However, it is important to note that although this protein lack the canonical tyrosine residue involved in the initiation of the

kinase activity, other tyrosine residues were shown sufficient to trigger FAK kinase activity upon interaction with RTKs, independently of the main auto-phosphorylation site (Chen and Chen, 2006). Therefore, our results do not rule out completely the participation of FAK kinase activity, as there may be other tyrosine residues along the sequence that can play a similar role to the auto-phosphorylation site.

The clear involvement of the FERM domain, which mediates interactions with proteins and lipids, is interesting as we move towards more specific chemotherapies that avoid off-targets and do not affect severely healthy cells. In fact, new drug design programs aim to tackle the scaffolding functions of FAK rather than its kinase activity (Cance et al., 2013). Drugs that inhibit kinase activities are generally unspecific given the high residue conservation of the kinase domains, specifically the ATP-biding pocket. Therefore, exploiting the specificities of protein-protein interactions appears as a promising future for drug discovery programs. Although the making of new small molecules highly specific for a certain interaction is a big challenge for scientists, further characterization and identification of regions and residues involved in those interactions will help to progress towards the design of more specific drugs and efficient therapeutic protocols against FAK.

Chapter 4 – FAK as a tumour promoter

4.1 Summary

Human FAK has been shown to regulate cell survival through distinct mechanisms: by activating the PI3K/Akt signalling pathway (Khwaja et al., 1997); by preventing RIP interaction with the DISC complex (Kurenova et al., 2004); and by facilitating p53 ubiquitination and degradation (Ilic et al., 1998) (Lim et al., 2008).

Given the high expression of FAK in the CNS of flies and humans, we looked at whether FAK56 regulates survival of neurons and glia and which mechanisms participate in this context. We found FAK56 does prevent caspase-dependent cell death in nervous tissues such as the developing eye, larval brain lobes and nerve cord. Recently, a pro-apoptotic function of the N-terminal domain of Relish, the *Drosophila* homologue of human NF-κB1 and NF-κB2 (Karin and Ben-Neriah, 2000), was described in a model of retinal degeneration in *Drosophila* (Chinchore et al., 2012). In fact, double mutants for *FAK56* and *Relish* inhibited cell death in CNS and eye discs. Thus, FAK56 flies may become a good model to study neurodegenerative diseases and might help to obtain information about the role of human FAK (or Pyk2) in tumours of the nervous system.

The data presented here corresponds to the second main project I run during my PhD work. At the time of the preparation of this thesis, the story was still developing and some of the data here presented is preliminary.

4.2 Brief introduction

Apoptosis is an evolutionary conserved form of programmed cell death that eliminates cells that are in excess or damaged. Caspases (Cystein-Aspartic acid specific proteases) exist as zymogens that are proteolytically activated to then cleave and inactivate target proteins, and eventually kill the cell (Kurokawa and Kornbluth, 2009, Crawford and Wells, 2011). In *Drosophila*, there are two types of caspases; "initiator" caspases primarily activate "effector" caspases, which ultimately destroy the cellular components during apoptosis

(Steller, 1995). In *Drosophila* there are seven caspases; three initiator caspases: *Dronc* (Drosophila Nedd-2-like caspase), *Dredd* (Death-related ced-3/Nedd2-like), and *Strica/Dream*, and four effector caspases: *Dcp-1* (Death caspase-1), *Drice* (Drosophila ice), *Damm*, and *Decay* (Death executioner caspase related to Apopain/Yama) (Salvesen and Abrams, 2004).

The genes *reaper* (*rpr*) (White et al., 1994), *head involution defective* (*hid*) (Grether et al., 1995), *grim* (Chen et al., 1996) and sickle (Srinivasula et al., 2002, Christich et al., 2002, Wing et al., 2002) form the 'RHG group' and represent the 'central pathway' in the initiation of programmed cell death (PCD) and caspase activation in flies (Figure 4.1). They are antagonists of the *Drosophila* Inhibitor-of-Apoptosis Protein DIAP1 (Yoo et al., 2002, Wang et al., 1999) and similarly to mammalian Smac/DIABLO proteins (Verhagen et al., 2000) (Chai et al., 2000), they regulate caspase activation by disrupting caspase/IAP interaction (Silke et al., 2000). In mammals, the 'intrinsic' pathway of cell death is initiated at the mitochondrion; in flies, the RHG group constitutes the intrinsic pathway but the role of mitochondria in regulation of cell death remains still far from clear (Bender et al., 2012). Moreover, the Apaf-1 homolog, Dark (D-Apaf-1/HAC-1) (Rodriguez et al., 1999), and two Bcl-2 family proteins (Drob-1/Debcl/dBorg-1/dBok and Buffy/dBorg-2) (Quinn et al., 2003) (Colussi et al., 2000) are also regulators of the intrinsic apoptotic pathway in *Drosophila*.

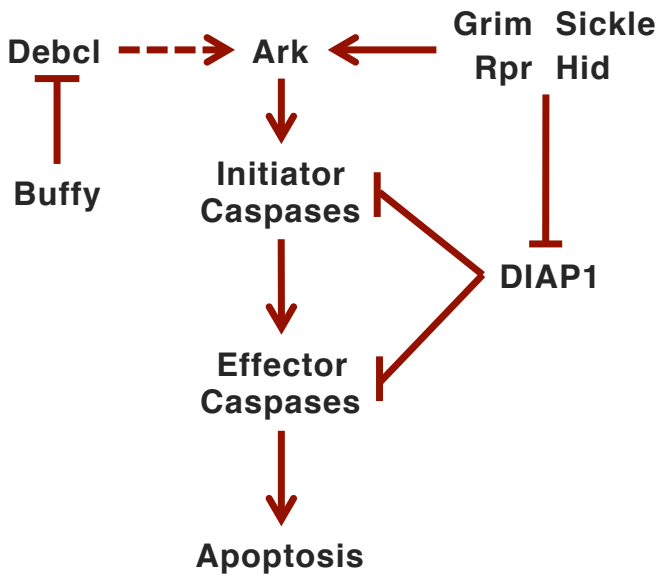

Figure 4.1 The canonical pathway of cell death in *Drosophila melanogaster*
Diagram shows the main intermediaries of apoptotic cell death in flies. DIAP1 is an important site where death signals are integrated and it plays a major role in preventing caspase activation. Several IAP-antagonist factors such as Grim, Sickle, Reaper and Hid, inhibit DIAP1 by

different mechanisms and release caspase activity. Ark is essential for the activation of the initiator caspase Dronc, which in turn activates effector caspases such as Drice and Dcp-1 that ultimately kill the cell by successive cleavage of essential target proteins. The pro-apoptotic Bcl-2 homologue Debcl and the anti-apoptotic Bcl-2 homologue Buffy also regulate Dronc activity through mechanisms that may also involve mitochondria and outer membrane permeabilization, as it occurs in mammals, although they are still poorly characterised in flies.

In mammalian cells, the "extrinsic" apoptotic pathway is mediated by the tumour necrosis factor (TNF) receptor superfamily (also called death receptors (DRs)) (Oberst and Green, 2011). However, these receptors can trigger either apoptotic or survival signalling into the cell. When the receptor binds its ligand, it recruits the death domain (DD)-containing adaptor proteins such as TRADD (TNFR-associated death domain protein), FADD (Fas-associated death domain protein) or TRAF2 (TRADD-associated factor 2), RIP and caspase-8. Either signalling requires caspase-8 activity but what determines one outcome or the other is the cleavage of RIP (Lin et al., 1999).

Extrinsic apoptosis initiates after RIP cleavage. It causes a more stable interaction between TRADD and FADD, which leads to increased caspase-8 activation and the formation of the death inducing signalling complex (DISC) (Lin et al., 1999). This complex triggers apoptosis through c-Jun N-terminal kinase (JNK) pathway activation among other signals (Deng et al., 2003). Conversely, if RIP is not cleaved, the complex promotes survival signalling through the activation of NF-κB pathway and subsequent expression of the caspase-8 inhibitor FLIP (FLICE-like inhibitory protein), which competes with caspase-8 for FADD binding site and prevents its activation (Oberst and Green, 2011).

NF-κB is a family of transcription factors that form homo- or hetero-dimmers. In absence of stimulus, the NF-κB dimmers are retained in the cytoplasm by the inhibitor protein IκB (α/β/ε). Upon stimulus, the IκB kinase IKK phosphorylates and promotes degradation of IκBα, thus releasing NF-κB inactivation and allowing its translocation to the nucleus and transcriptional activation of their target genes. NF-κB transcription factors have been involved in several contexts such us immune responses, survival, proliferation, differentiation and cell death (Oeckinghaus et al., 2011), which implies also a dual role in tumourigenesis (Ben-Neriah and Karin, 2011).

On the other hand, the pro-apoptotic properties of the tumour suppressor p53 respond to DNA damage (Ryan et al., 2001). Genotoxic stress leads to

activation of DNA damage repair systems, cycle arrest or apoptosis (Sionov and Haupt, 1999). If the damage is too detrimental for the cell, the transcription factor p53 regulates the expression of pro-apoptotic genes and kills the cell. In fact, the majority of cancers have either mutations in *p53* gene or inability to activate it that impede cell death (Vousden and Lu, 2002).

Human FAK was shown to bind RIP and p53 and prevents their pro-apoptotic functions. In the first case, FAK impedes RIP association with DISC (Kurenova et al, 2004) (Kamarajan et al., 2010). In the nucleus, FAK binds p53 and Mdm2 and favours its ubiquitination and degradation (Lim et al., 2008) (Golubovskaya et al., 2005).

In flies, Immune Deficiency (IMD) is the homologue of RIP and *Drosophila* p53 (dp53) is the homologue of mammalian p53. IMD was discovered as key component of the fly immune system against bacteria. IMD activates the *Drosophila* NF-κB homologue *relish* and induces expression of antimicrobial peptides (Lemaitre et al., 1995). On the other hand, d*p53* was involved in the cell death response induced after UV-irradiation (Brodsky et al., 2000) (Jassim et al., 2003). Independent overexpression of both proteins has shown to induce expression of the *reaper* (*rpr*) gene (Georgel et al., 2001) (Brodsky et al, 2000), thus connecting them to canonical death pathway in flies.

FAK56 is highly expressed in the developing nervous system and eye discs of larvae (Murakami et al., 2007). This high expression may be suggesting that FAK56 plays important roles in these tissues. In fact, FAK56 was shown to play supporting roles in processes such as action potential conduction along axons (Ueda et al., 2008) and maintenance of the glial tubular structure around photoreceptor neurons (Murakami et al., 2007). Additionally, FAK56 was involved in the regulation of neuromuscular growth through inhibition of Ras/MAPK signalling (Tsai et al., 2008).

4.3 Objective

Besides the previously described functions, our aim was to investigate whether FAK56 had also anti-cell death properties in *Drosophila* nervous tissues

and we were particularly interested in exploring a possible interaction between FAK56 and dp53 and/or IMD.

4.4 Results

4.4.1 FAK56 overexpression alters number of scutellar bristles

A simple observation suggested FAK56 might have caspase-inhibitory properties. Expression of FAK56 with the *ptc-Gal4* driver produced no defect in the adult wing (see Figure 3.4D) but extra macrochaetae in the scutellar region of the notum (90% penetrance, n=120) (Figure 4.2A). Inhibition of caspase activity by the baculovirus caspase-inhibitor protein p35 also gave the same phenotype (McEwen and Peifer, 2005).

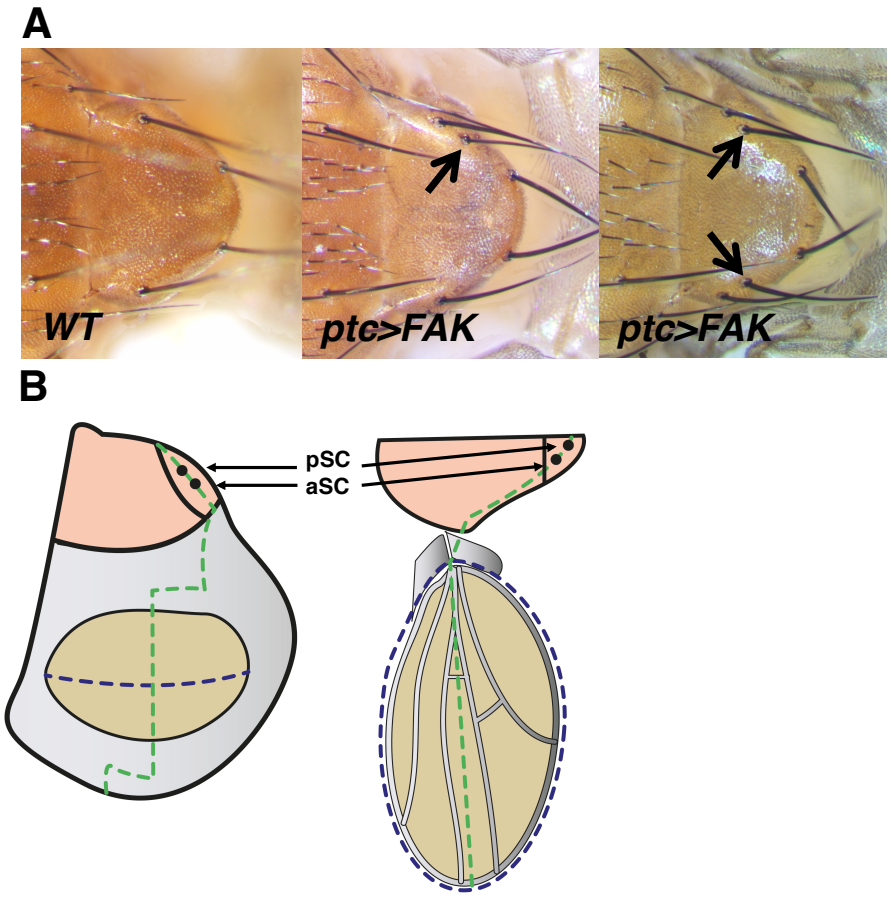

Figure 4.2 Ectopic expression of FAK56 produces extra scutellar bristles
(A) Images of the adult fly scutellum. Wild type (*WT*) flies have two pairs of scutellar macrochaetae: two anterior (aSC) and two posterior (pSC) (see B). Expression of FAK56 within the *patched (ptc)* domain of the wing disc (green line in B) produces extra scutellar bristles, with one or two extra macrochaetae per notum. Animals genotype: *ptc-Gal4, UAS-GFP/+; UAS-FAK/+*. (B) Drawing depicting the correlation between the larval wing imaginal disc and the adult wing: the heminotum of the fly (pink), the hinge (silver) and the wing itself (light brown) (see Figure 1.7 for more details). Scutellar bristles are shown as black dots in the heminotum. The *ptc* domain (green line) in the wing disc includes the region where posterior and anterior scutellar SOPs differentiate. Note that these bristle precursors rearrange in the adult notum.

Macrochaetae are sensory organs of the *Drosophila* peripheral nervous system (PNS). They are composed of a neuron, a glial cell and three non-neuronal supporting cells, which originate from neuronal precursor cells called sensory organ precursors (SOPs). There are two pairs of SOPs per wing disc, which reside in the proximal region of each disc and give origin to the anterior (aSC) and posterior (pSC) macrochaetae observed in the adult scutellum (Figure 4.2A and B). During the differentiation process that gives rise to SOPs and then bristles, caspase activity but apoptosis is required (Kanuka et al., 2005), suggesting a non-apoptotic role of caspases in differentiation.

As shown in Figure 4.1B, the *patched* stripe covers the region that will give rise to the scutellum of the adult fly. Ectopic expression of FAK56 within this domain was able to impair the caspase-dependent differentiation process and caused supernumerary bristles, suggesting a caspase-inhibitory function of FAK56. Notably, this extra bristle phenotype also occurs in *Dark*, *Dredd*, *Dronc*, *Dcp-1*, and *Debcl* mutant adults (Rodriguez et al., 1999, Galindo et al., 2009, Laundrie et al., 2003, Xu et al., 2005, Chen et al., 1998), all of which are intermediaries of cell death in *Drosophila*.

4.4.2 FAK56 is highly expressed in larval neuronal tissues

Previous reports showed FAK56 is ubiquitously and highly expressed in CNS and imaginal discs of third instar larvae (Murakami et al., 2007). Information we collected from FlyAtlas (Chintapalli et al., 2007) confirmed this and also indicated FAK56 is expressed in nervous tissues of the adult (Figure 4.3).

Tissue	mRNA Signal	Present Call	Enrichment	Affy Call
Brain	96 ± 2	4 of 4	3.70	Up
Head	31 ± 1	4 of 4	1.20	None
Eye	37 ± 1	4 of 4	1.41	Up
Thoracicoabdominal ganglion	85 ± 4	4 of 4	3.20	Up
Salivary gland	79 ± 7	4 of 4	3.03	Up
Crop	48 ± 5	4 of 4	1.90	Up
Midgut	21 ± 2	2 of 4	0.80	None
Tubule	63 ± 0	4 of 4	2.40	Up
Hindgut	33 ± 2	4 of 4	1.30	None
Heart	24 ± 4	4 of 4	0.94	None
Fat body	34 ± 6	4 of 4	1.30	None
Ovary	72 ± 3	4 of 4	2.80	Up
Testis	16 ± 0	4 of 4	0.60	Down
Male accessory glands	31 ± 2	4 of 4	1.20	None
Virgin spermatheca	51 ± 6	4 of 4	1.95	Up
Mated spermatheca	44 ± 6	4 of 4	1.68	None
Adult carcass	27 ± 3	4 of 4	1.00	None
Larval CNS	133 ± 7	4 of 4	5.08	Up
Larval Salivary gland	134 ± 16	4 of 4	5.11	Up
Larval midgut	38 ± 1	4 of 4	1.47	Up
Larval tubule	61 ± 3	4 of 4	2.40	Up
Larval hindgut	40 ± 4	4 of 4	1.54	None
Larval fat body	58 ± 6	4 of 4	2.20	Up
Larval trachea	31 ± 5	4 of 4	1.22	None
Larval carcass	34 ± 2	4 of 4	1.30	None
S2 cells (growing)	49 ± 2	4 of 4	1.88	Up
Whole fly	26 ± 2	4 of 4		

Figure 4.3 FlyAtlas expression pattern for FAK56
FAK56 expression in several larval and adult tissues. 'Larval' refers to feeding larvae grown on standard *Drosophila* diet at 23˚C; 'Adult' refers to 7 days old flies grown on the same conditions. 'mRNA signal' expresses how abundant is FAK56 in each tissue; 'Present call' shows how many of 4 independent arrays for each tissue gave a detectable expression; 'Enrichment' and 'Affinity Call' summarise the relative levels of FAK56 expression in each tissue versus the level in the whole fly. Red rectangles highlight the high expression of FAK56 in adult brain and eye plus the larval central nervous system. Source: FlyAtlas.org

Potentially, the observation that *FAK56* mutant flies have greater sensitivity to mechanical and electrical stress (Ueda et al., 2008) could be explained by the defects in the tubular structure of nerves (Murakami et al., 2007) and weakened action potential conduction (Ueda et al, 2008). However, given caspases can be inhibited by FAK56 in neuronal precursors of the bristles and FAK56 is highly expressed in neurons and glia, we decided to test whether FAK56 could regulate cell death and caspase activation in fly nervous tissues.

4.4.3 FAK56 regulates cell death in developing CNS and eye discs

Indeed, TUNEL staining showed a striking amount of cell death in *FAK56* eye discs and larval brains compared to wild type tissues (Figure 4.4A and B).

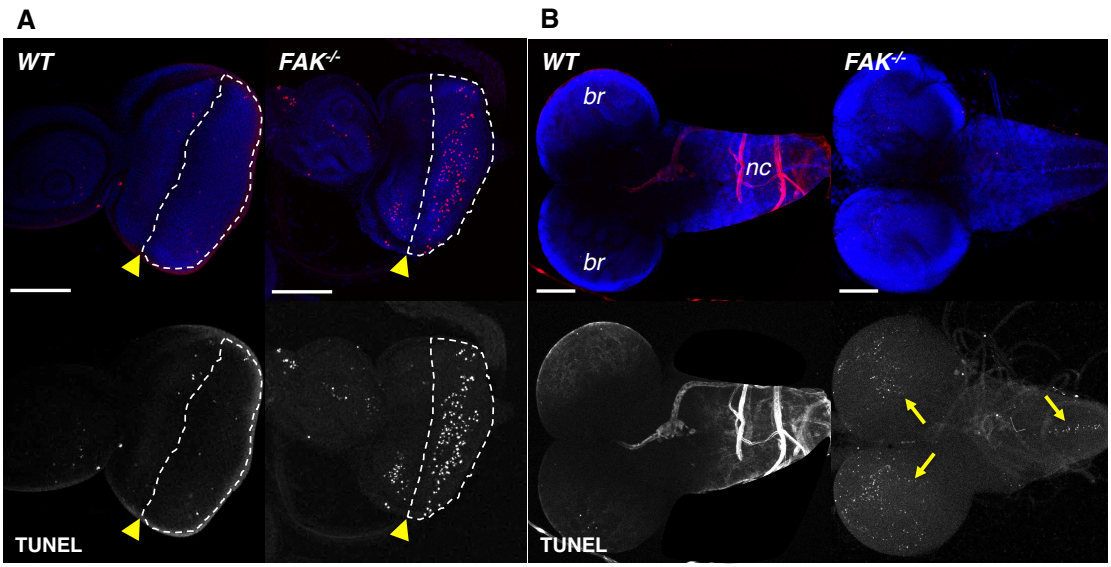

Figure 4.4 Absence of FAK56 induces cell death
Cell death occurs after FAK56 loss in *Drosophila* larval nervous tissues. (A) The region posterior to the morphogenetic furrow (yellow arrowheads) is a differentiating neuroepithelium (white dashed lines). The anterior region is a proliferating tissue. TUNEL staining (red and grey) of *FAK56*$^{-/-}$ eye discs shows massive cell death only in the neuroepithelium. DAPI staining (blue) labels nuclei. (B) Cell death is observed in the brain lobes (*br)* and ventral nerve cord (*nc*) (yellow arrows) of *FAK*$^{-/-}$ CNS but is absent in *wild type* (*WT*) tissues. Red tubules attached to the nerve cord shown is unspecific background staining. Scale bar: 100um.

This result was interesting and surprising at the same time. First, it was exciting to see that FAK56 was able to regulate cell death only in the neural differentiating region of the eye disc (posterior to the morphogenetic furrow) (Figure 4.4A). Secondly, because post-mitotic cells are generally more resistant to apoptotic stimuli than their undifferentiated precursors (Fixemer et al., 2002, Feinstein-Rotkopf and Arama, 2009), it was curious to observe that FAK56 loss alone was enough to trigger death in a tissue composed mainly of normally death-resistant cell types such as neurons and cone cells.

Consistently, when we measured the eye sizes of *wild type* and *FAK56* flies we also observed a significant difference in eye area (Figure 4.5). Overall, these results prove FAK56 has a physiological anti-apoptotic role in the eye disc and central nervous system of *Drosophila* larvae.

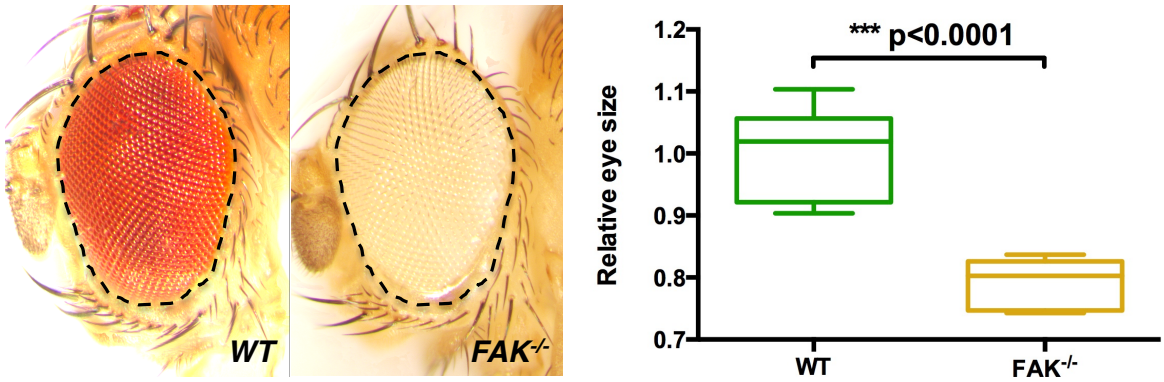

Figure 4.5 *FAK56* eyes are smaller than *wild type*.
Adult eye images of *wild type* and *FAK*$^{-/-}$ animals and their corresponding area measurements. Eye size quantification is expressed in relative levels to the average wild type area, depicted in black dashed lines. The difference in size was statistically significant (***); n=10 for each genotype.

4.4.4 Absence of FAK56 triggers caspase-dependent cell death

Next, we assessed if caspases were active after loss of *FAK56*. We expressed the caspase inhibitor p35 (Hay et al., 1994) and the *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1) (Hay et al, 1995), which is the natural inhibitor of *Drosophila* initiator and effector caspases, with the *GMR-Gal4* promoter. GMR stands for Glass Multimer Reporter and consists of a tandem of sequences recognised by the transcription factor Glass (Moses and Rubin, 1991). The *glass* gene is expressed in all cells posterior to the morphogenetic furrow (Moses and Rubin, 1991); therefore, Gal4 is expressed specifically in the differentiating region of the eye disc (Figure 4.6). We observed a clear suppression of death in p35 and DIAP1 expressing discs indicating that activation of caspases is leading to apoptosis in *FAK56* deficient eye imaginal discs (Figure 4.6).

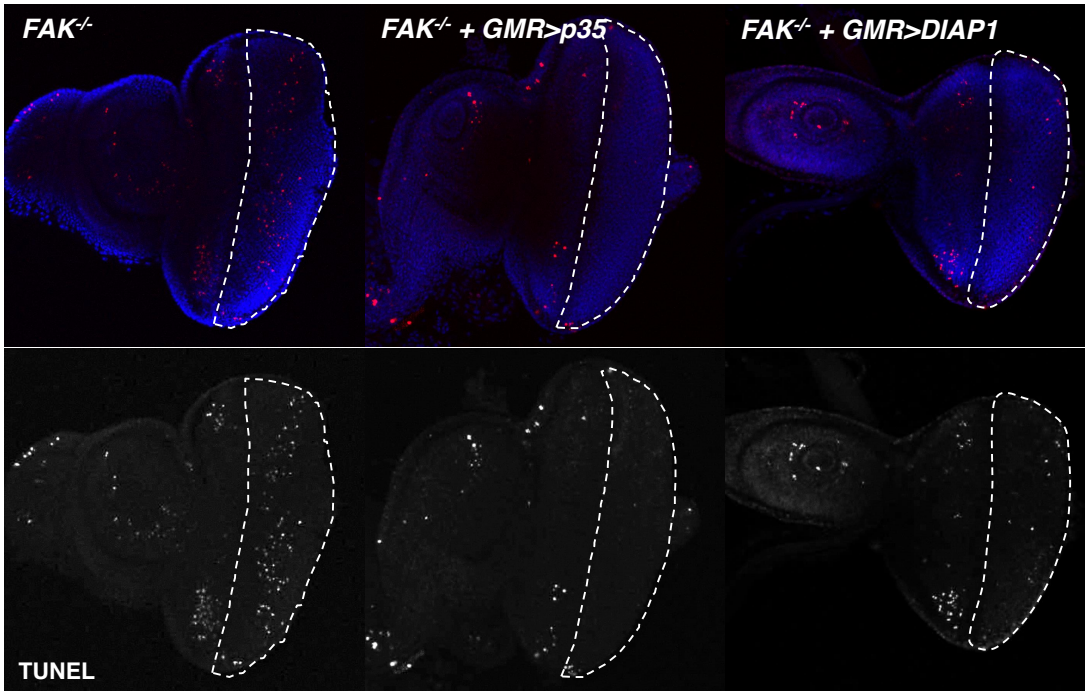

Figure 4.6 Caspase-dependent cell death occurs after *FAK56* loss
Lack of *FAK56* led to caspase-dependent apoptosis in the eye disc, which was suppressed by the GMR-driven expression of caspase inhibitors p35 and DIAP1. White dashed lines illustrate the *GMR* domain. TUNEL staining is shown in red and DAPI (nuclei) staining in blue.

Additionally, we demonstrated that re-expression of FAK56 was sufficient to prevent cell death (Figure 4.7A) and to restore the eye back to a normal size (Figure 4.7B).

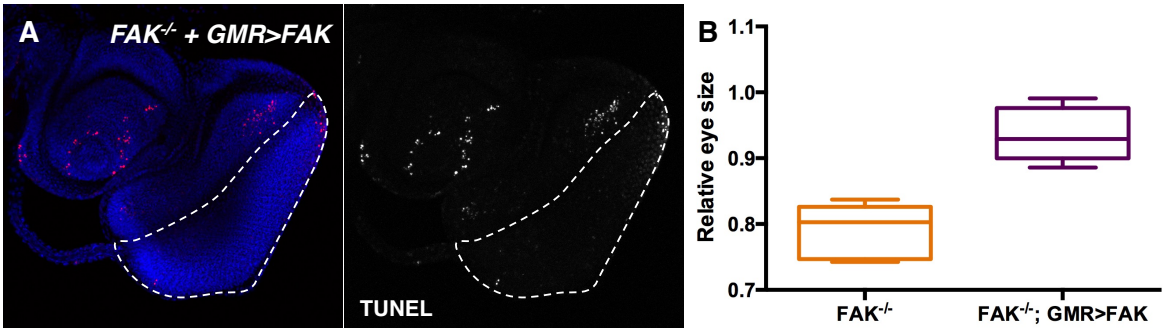

Figure 4.7 FAK56 expression prevents cell death in *FAK*$^{-/-}$ eye imaginal discs
(A) GMR-dependent FAK56 re-expression was enough to rescue apoptosis in *FAK*$^{-/-}$ eye neuroepithelium. This observation demonstrated that cells die exclusively as a consequence of FAK56 loss. White dashed lines illustrate the *GMR* domain. (B) Eye size quantification is expressed in relative levels to the average wild type area (value=1.0). The difference in eye size between *FAK*$^{-/-}$ and *FAK*$^{-/-}$; *GMR-Gal4/UAS-FAK* flies was statistically significant (***); n=10 for each genotype.

4.4.5 Reaper induces apoptosis after FAK56 loss

In a search for cell death regulators downstream of FAK56, we began testing the pro-apoptotic RHG genes (*reaper (rpr)*, *hid* and *grim*). The expression of these genes in the eye showed different grades of cell death (Figure 4.8). We co-expressed FAK56 simultaneously with RHG genes to look for genetic

interactions and apoptosis suppression. Interestingly, FAK56 suppressed partially but significantly *rpr*-induced apoptosis, while hid and grim effects remain unchanged.

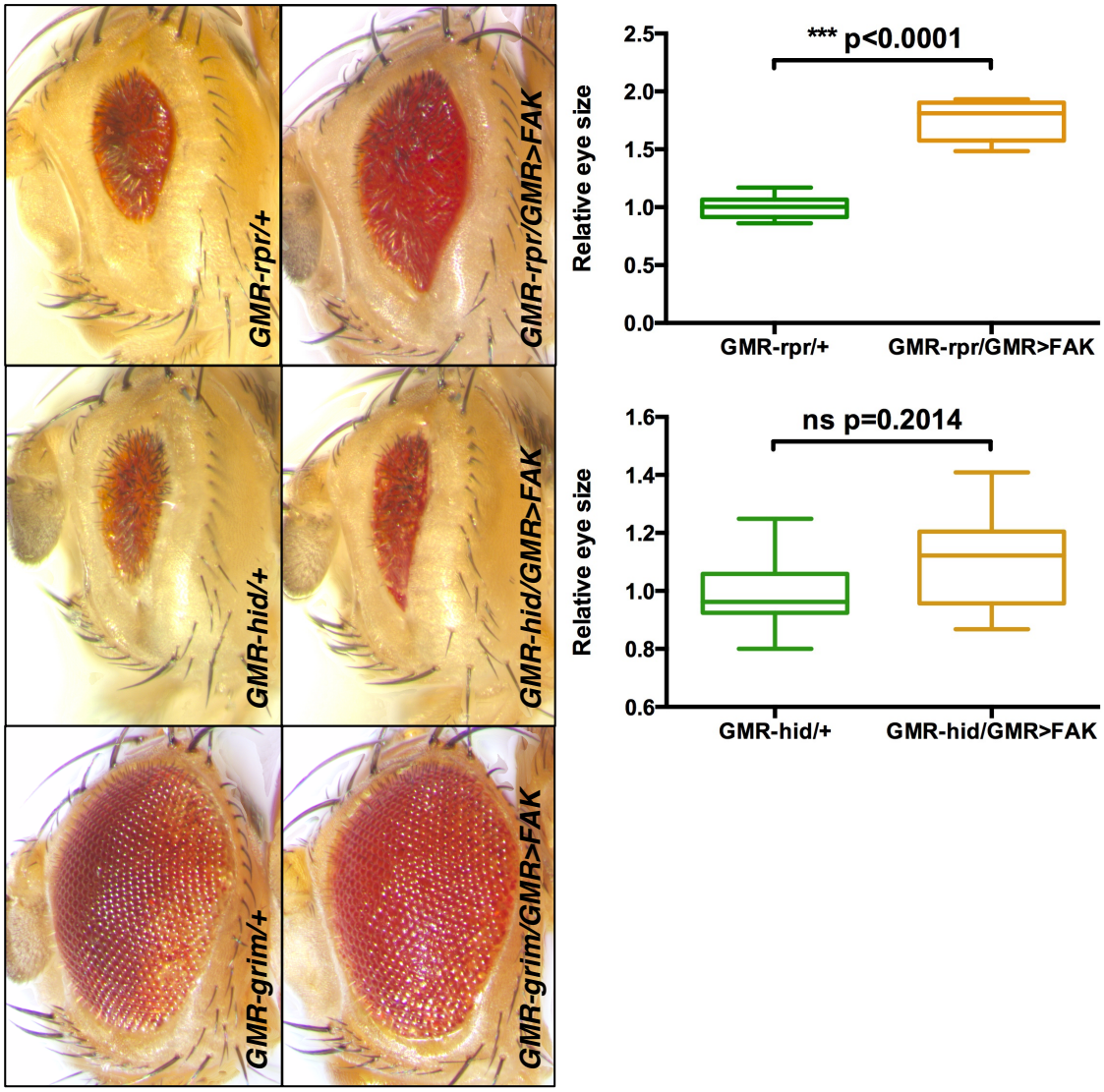

Figure 4.8 FAK56 partially blocks *reaper*-induced cell death in the eye.
Recombinant constructs between the *GMR* promoter and the *RHG* genes give variable apoptotic phenotypes in the adult eye. Co-expression with FAK56 resulted in suppression of the *reaper (rpr)*-induced phenotype only. This was confirmed by measuring the eye area of these animals (n=10 per genotype). A statistically significant difference ($p<0.05$) was observed in *reaper* but *hid*- or *grim*-induced phenotypes.

To confirm this genetic interaction, we made use of genetic deficiencies that span the *RHG* region on the third chromosome of *Drosophila* genome. The genetic deficiency *Df(3L)H99* holds a deletion of the whole cassette including *rpr*, *hid* and *grim*, while *Df(3L)XR38* only lacks the *rpr* gene (White et al., 1994). Thus, by combining *FAK56*, *Df(3L)H99* and *Df(3L)XR38* flies we created *FAK56; rpr* double mutants, which were viable. TUNEL staining of eye discs and larval CNS of these animals showed a complete suppression of cell death (Figure 4.9).

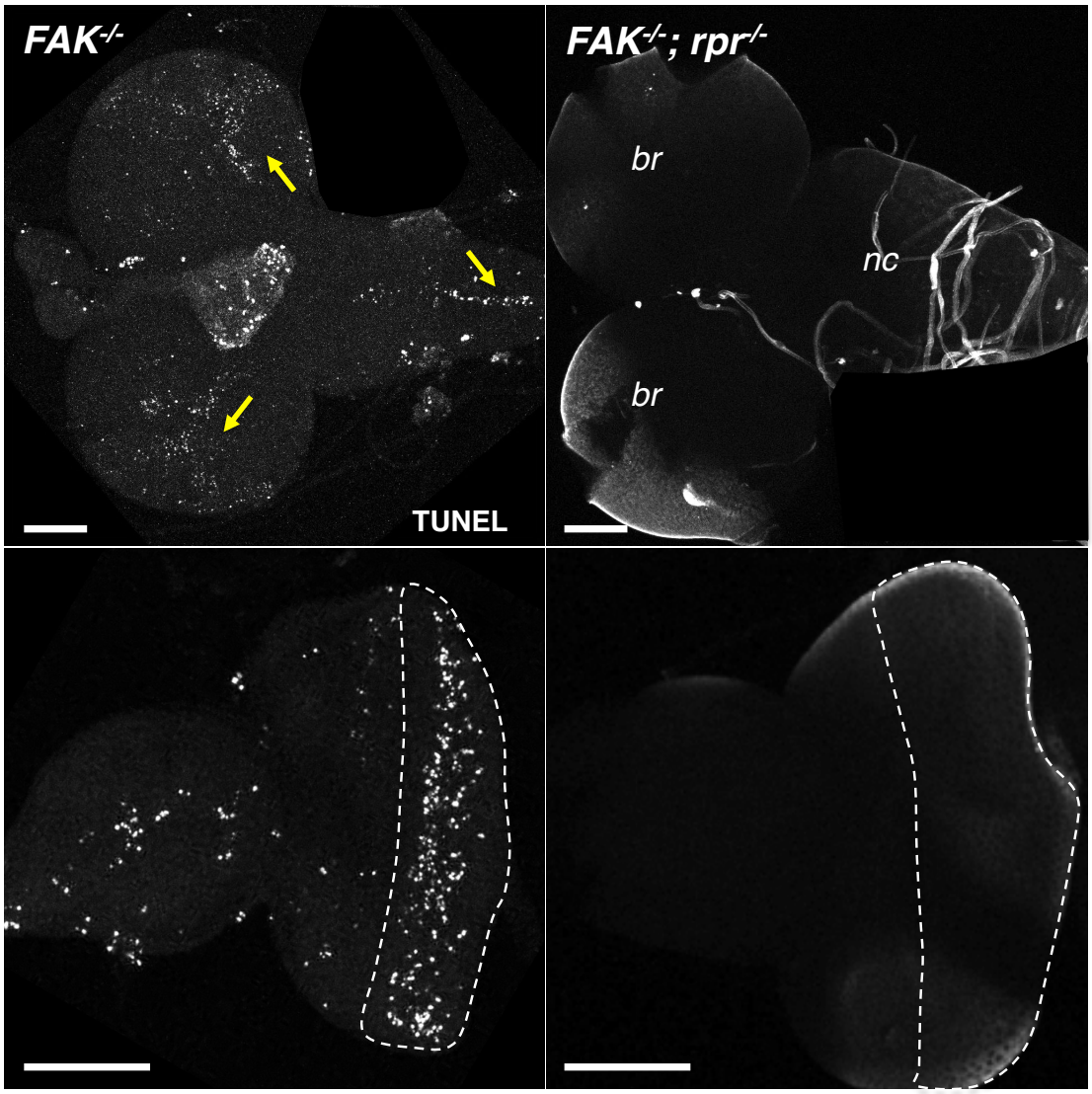

Figure 4.9 Deletion of *reaper* locus blocks FAK56 loss-induced cell death
Apoptosis in CNS and eye discs of FAK$^{-/-}$ larvae is fully suppressed by a deletion of the *reaper* gene. Animal genotypes: *FAK^{CG1}* and *FAK^{CG1}; Df(3L)H99/Df(3L)XR38*. White dashed lines illustrate the *GMR* domain. Brain lobes (*br)* and ventral nerve cord (*nc*) are shown.

We also screen several known pro-apoptotic factors in *Drosophila* that induce cell death by different mechanisms: *debcl* is a Bcl-2 (B-cell lymphoma 2) family homologue that activates caspase-dependent cell death independently of RHG genes (Colussi et al., 2000), and *eiger* (*egr*) is the *Drosophila* homologue of the TNF ligand superfamily that induces JNK-dependent cell death in flies (Igaki et al., 2002, Moreno et al., 2002). FAK56 co-expression did not prevent their apoptotic effects in the adult eye (Figure 4.10A), neither their inactivation suppressed apoptosis induced by the loss of *FAK56* in the eye disc (Figure 4.10B).

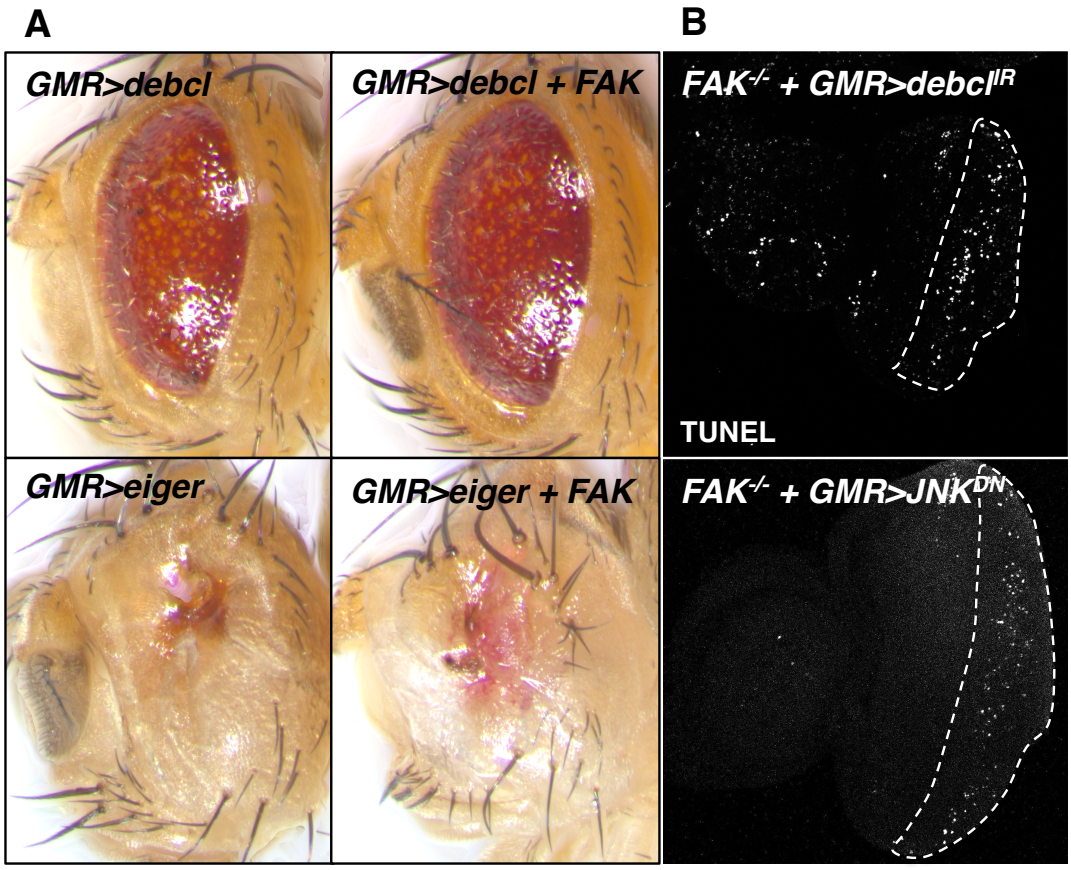

Figure 4.10 FAK56 does not prevent *debcl* and *eiger* pro-apoptotic effects.
(A) The Bcl-2 homologue Debcl triggers an intrinsic apoptotic pathway through mitochondria, which is not blocked by simultaneous expression of FAK. Similarly, Eiger-induced JNK-dependent apoptosis was not suppressed by FAK56 expression. (B) Knockdown of Debcl by RNA interference or expression of a JNK-dominant negative allele did not prevent apoptosis in *FAK56* eye discs. White dashed lines illustrate the *GMR* domain. Genotypes from left to right: *GMR-Gal4/+; UAS-debcl/+ // GMR-Gal4/+; UAS-debcl/+; UAS-FAK/+ // GMR-Gal4/+; FAK$^{-/-}$; UAS-debcl-IR/+ // GMR-Gal4/+; UAS-eiger/+ // GMR-Gal4/+; UAS-eiger/+; UAS-FAK/+ // GMR-Gal4/+; FAK$^{-/-}$; UAS-JNK^{DN}/+* .

Summarising, our results indicated FAK56 was able to prevent *reaper* expression or activation while other pro-apoptotic proteins, such as *hid, grim, debcl* or *eiger,* were not involved in apoptotic cell death induced by FAK56 loss.

4.4.6 Do FAK56 and dp53 interact in flies?

In our search for intermediaries between FAK56 and Reaper, the first candidate we looked at was dp53. This protein is the *Drosophila* homologue of mammalian tumour suppressor p53 and it has been shown to regulate *reaper* expression by direct binding on its promoter region (Sogame et al., 2003, Brodsky et al., 2000). Also, a direct interaction between p53 and FAK has been reported in mammals, where FAK, together with Mdm2, helps the degradation of p53 through protein-protein interaction between the N-terminal FERM domain of FAK and the TAD domain of p53 (Lim et al., 2008). The amino acids involved in

this interaction have been mapped in p53 (Golubovskaya et al., 2008b) (Figure 4.11). In *Drosophila*, there are 3 isoforms of p53 (Figure 4.11), which share some functional and structural characteristics of mammalian p53 family members (Marcel et al., 2011, Fan et al., 2010). For instance, the dΔNp53 isoform shares functional properties with human ΔNp63 and ΔNp73 as they are able to inhibit cell differentiation. Structurally, dp53 sequence shows higher similarity to mammalian p63 and p73 although it lacks the sterile alpha motif (SAM) involved in oligomerisation that is present in mammalian p53 (Fan et al., 2010). Sequence alignments between *Drosophila* and mammalian TAD domains of p53 proteins showed a putative stretch of 7 residues similar to the FAK-interacting sequence found in mouse p53 (Figure 4.11).

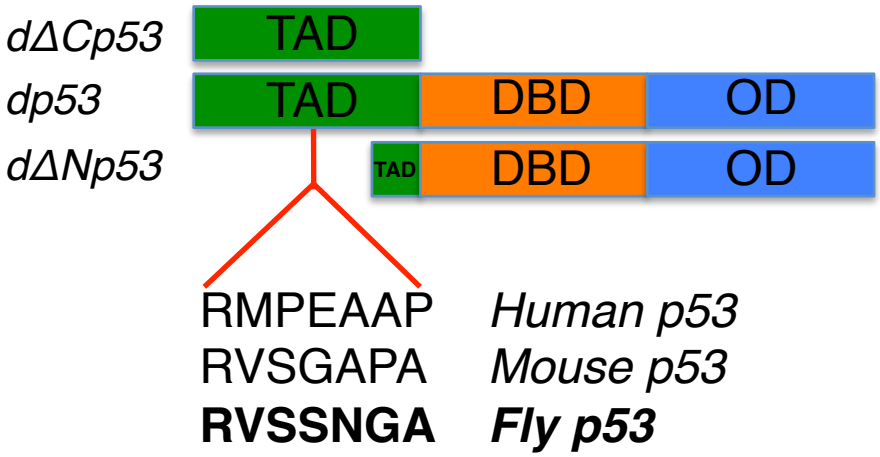

Figure 4.11 Full-length *Drosophila* p53 contains a putative FAK-interacting sequence
Drosophila p53 isoforms: dΔCp53 is a short isoform bearing only the transactivation domain (TAD) that has no mammalian counterpart; dp53 is the full-length isoform including also the DNA-binding domain (DBD) and oligomerisation domain (OD); and dΔNp53 is the TAD-truncated isoform, encoded from an internal promoter similarly to two N-terminal p53 isoforms in humans (Δ40p53 and Δ133p53) (Marcel et al., 2011). We found the sequence RVSSNGA in the transactivation domain (TAD) of dp53, which resembles the mouse p53 sequence.

Therefore, we became interested in testing if this interaction occurs in flies as well. The expression of the N-terminal truncated isoform dΔNp53, as expected, caused cell death in the eye that could not be rescued by the simultaneous expression of FAK56 (Figure 4.12A). Intriguingly, the effects triggered by the full-length isoform of p53 were not suppressed either (Figure 4.12A).

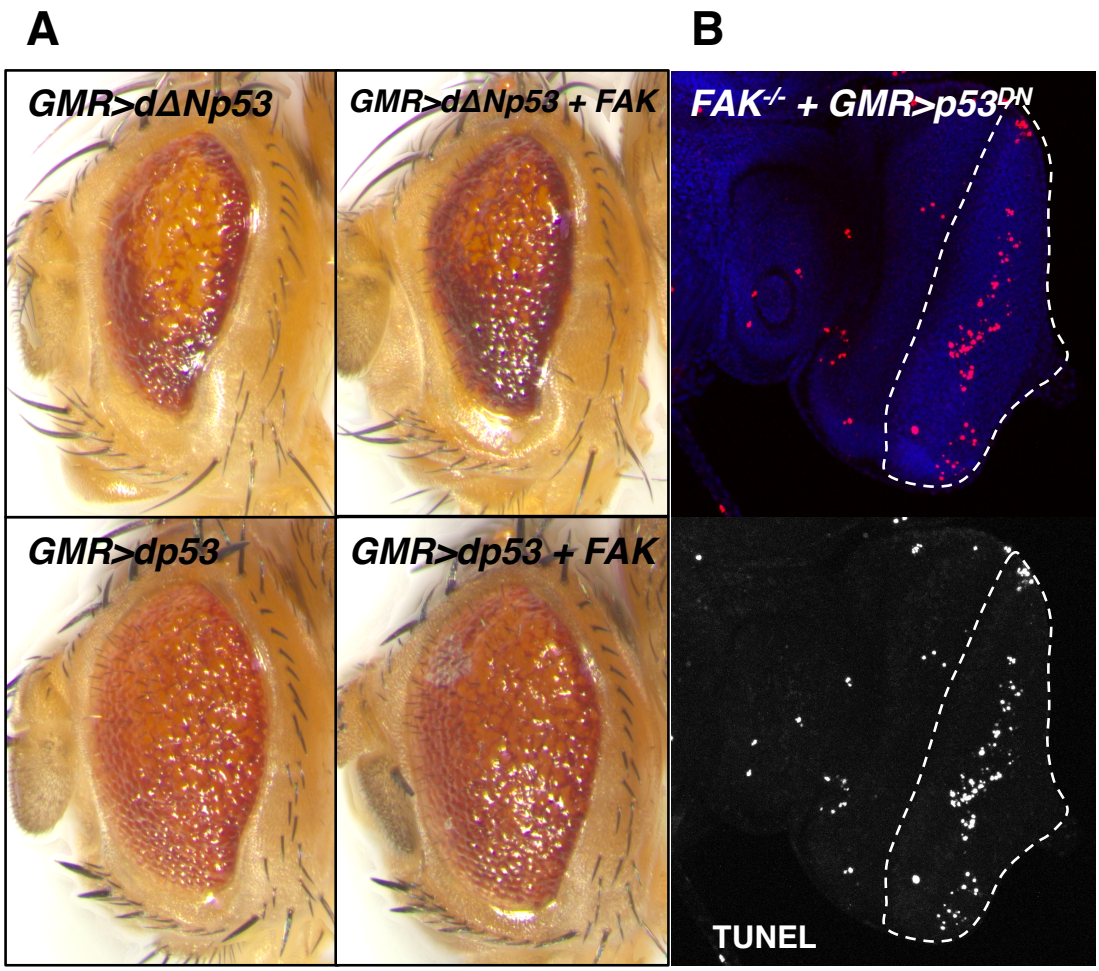

Figure 4.12 ***Drosophila*** **p53 does not interact genetically with FAK56.**
(A) Expression of two isoforms of dp53 in the eye gave slightly different phenotypes. The dΔNp53 isoform causes a smaller eye than dp53 but neither phenotype is suppressed by FAK56 co-expression. (B) Alternatively, inhibition of p53 activity does not prevent FAK56 loss-induced cell death in the eye imaginal disc. White dashed lines illustrate the *GMR* domain.

Additionally, the expression of a dominant negative isoform of dp53 (Brodsky et al., 2000) did not suppress the high levels of apoptosis triggered after loss of FAK56 (Figure 4.12B). Overall, these results suggested dp53 was not involved downstream of FAK56 in the regulation of *reaper* expression.

4.4.7 Relish is involved in FAK56 loss-induced cell death

We next investigated another candidate that could link FAK56 and Reaper: *immune deficiency* (*imd*), the *Drosophila* homologue of human RIP. IMD is involved in host defense against bacteria but also contains a death domain (DD). It participates in the so-called 'IMD pathway', which interestingly, is the *Drosophila* homologue to the mammalian extrinsic pathway although was only described in the context of bacterial infection. This immune response pathway consists of the Peptidoglycan recognition protein (PGRP-LC), which recognises mainly the Gram negative bacteria diaminopimelic acid-type (DAP)

peptidoglycan; IMD; dFADD; the caspase-8 orthologue Dredd; the NF-κB family member Relish; and the NF-κB activator IκB kinase (IKK), which consists of two subunits Kenny and IRD5) (De Gregorio et al., 2002).

In response to infection by Gram-negative bacteria, the IMD pathway allows the activation of NF-κB and consequent expression of antimicrobial peptides. When the pathway is active, IKK and Dredd phosphorylate and cleave Relish, respectively, to allow nuclear translocation of its N-terminal domain (NTD) and transcriptional activation (De Gregorio et al, 2002). Interestingly, IMD overexpression has been shown to activate *rpr* expression as well as to induce apoptosis in the fat body (Georgel et al., 2001) and the NTD domain was recently shown to have pro-apoptotic properties when overexpressed in the adult eye with *GMR-Gal4* (Chinchore et al., 2012). Moreover, in a retinal degeneration model in flies, Dredd, IKK and Relish were involved in the death of photoreceptors (Chinchore et al., 2012). As shown in Figure 4.4A, absence of FAK56 led to cell death of eye cell types. Hence, we hypothesised that Relish and its pro-apoptotic functions could be involved in this scenario.

To test that, we created double mutant flies for *FAK56* and *Relish* and carried out a TUNEL assay in larval CNS and eye discs. Figure 4.13 shows that indeed, Relish mutation rescues apoptosis in *FAK56* tissues.

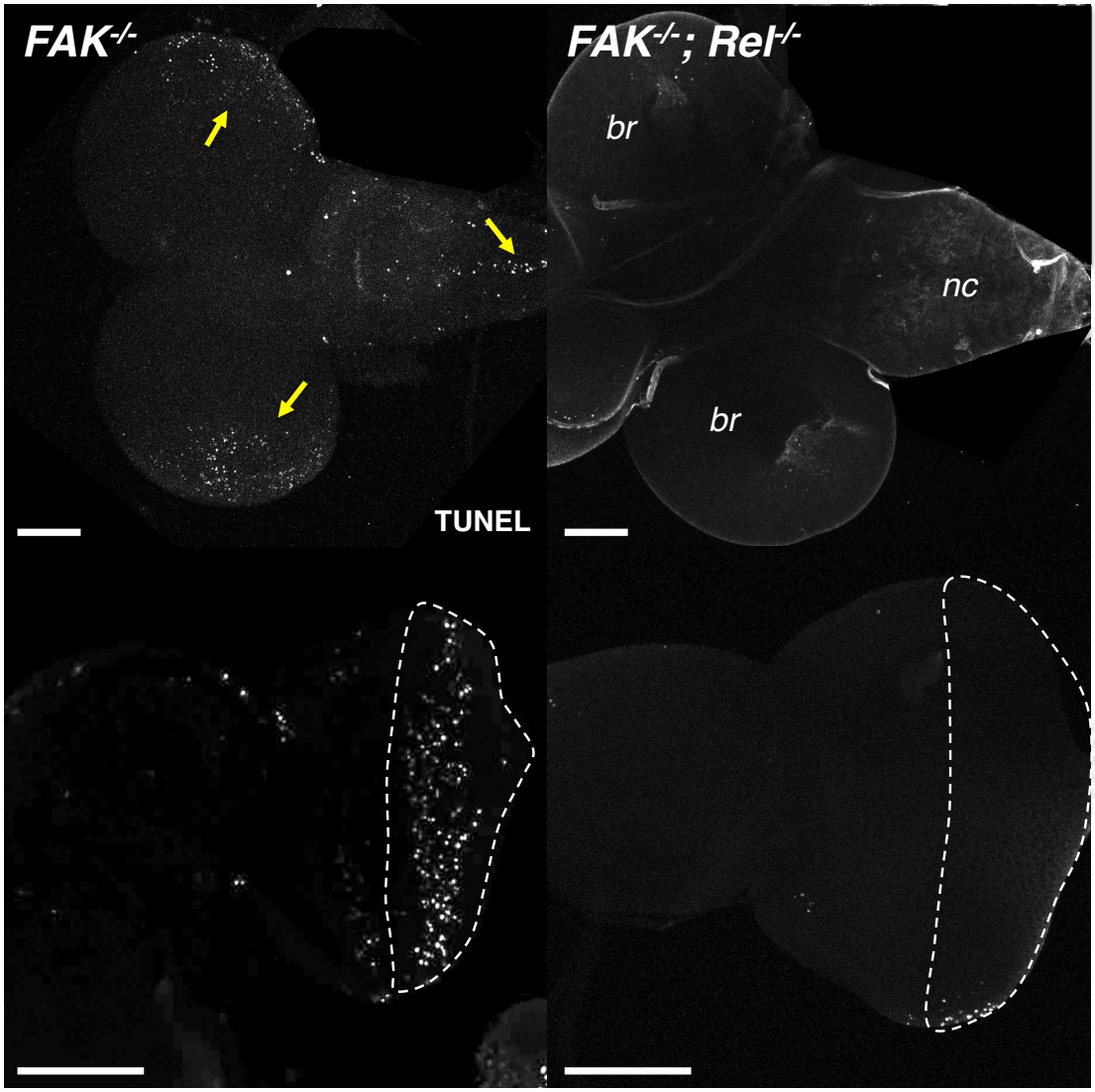

Figure 4.13 Lack of *Relish* also blocks FAK56 loss-induced cell death
Apoptosis in CNS and eye discs of *FAK*$^{-/-}$ larvae is fully suppressed in *FAK; Relish* double mutants. Two independent mutant alleles were utilised in order to inactivate *Relish*: *Relish*^{E20} and *Relish*^{E38} (Hedengren et al., 1999). White dashed lines illustrate the *GMR* domain. Brain lobes (*br)* and ventral nerve cord (*nc*) are shown.

Summarising, our results suggest a novel connection FAK56-Relish-Reaper in the context of cell death of neuronal cells.

4.5 Discussion

The data obtained up to date and presented here uncovers a clear survival role of FAK56 in the larval CNS and eye disc. Also, it provides a link between FAK56, NF-κB Relish and the pro-apoptotic protein Reaper in neuronal tissues. However, further investigation is needed in order to address many questions still unanswered.

4.5.1 Which cell types are dying?

It still remains unclear which cell types are affected by *FAK56* deletion. Given that larval CNS is mainly composed of neurons and glial cells, these cell types are the main candidates; however, the cellular composition of the eye is more diverse. We may assume photoreceptor neuronal cells are dying, as GMR-driven expression has been considered to be specific in this cell type and GMR-driven FAK re-expression in the eye disc inhibited cell death. However, several reports (Moses and Rubin, 1991); (Read et al., 2005) and my own data (Figure 3.7C) indicate this is not entirely correct as not only photoreceptor cells express Glass but all cells after the morphogenetic furrow (Moses and Rubin, 1991); therefore, GMR promoter is likely active also in all those cells. Additionally, GMR-driven RET expression led to a blockade of developmental apoptosis of interommatidial precursor cells (Figure 3.7C), implying GMR promoter is active in these cells. Furthermore, loss of photoreceptors is expected to alter the ommatidial structure due to differentiation problems (Fan et al., 2010); nevertheless, we had observed that the hexagonal pattern of *FAK56* retinas did not look abnormal, which means that number and differentiation of photoreceptor clusters and its accessory cells were unaltered (Figure 3.7B and 4.4).

In order to justify the smaller eye size without affecting the eye architecture, another possible candidate to consider are undifferentiated neuronal precursors. As it happens in the wing disc, where FAK56 expression was able to block caspase activity in neuronal precursors and produce extra bristles (Figure 4.2), FAK56 could be regulating caspase activity of undifferentiated neuronal precursor cells in the eye, which after FAK56 loss may die as a consequence of excessive caspase activity.

During the end of larval life, the undifferentiated and proliferating epithelial cells of the eye disc transform into a well-patterned neuroepithelium through a series of ordered differentiation steps. Photoreceptor 8 (R8) cells becomes specified first right after the morphogenetic furrow. They constitute ‘organizing centers’ that instruct neighbouring undifferentiated cells to differentiate sequentially into the other seven photoreceptor cells, cone cells and primary pigment cells.

We hypothesised that if some undifferentiated neuronal precursors die after FAK56 loss, other precursors from the surroundings will replace them and become part of the ommatidial units in order to maintain the normal architecture of the eye. Consequently, there will be less undifferentiated neuronal precursors and fewer ommatidia overall. This might explain why there are no differentiation problems or structural ommatidia defects but only a reduced eye size. Then, a careful quantification of the number of ommatidia of *FAK56* eyes should be done in order to test this hypothesis and to confirm the identity of the dying cells.

4.5.2 FAK56 and dp53

Most of research about dp53 has been done with dΔNp53 and little information has been reported about dp53 (Marcel et al., 2011). For instance, expression of dΔNp53 in the eye was reported to produce Hid-dependent apoptosis (Fan et al., 2010) while dp53 induces Rpr-dependent apoptosis in the wing disc (Shlevkov and Morata, 2012) (Dichtel-Danjoy et al., 2013). We observed no genetic interaction between Hid and FAK56 but a significant suppression of Rpr effects in the eye, which led us to think that FAK56 and dp53 may be linked, as it occurs in mammals (Golubovskaya et al., 2005, Lim et al., 2008).

Although we found a putative FAK-interacting sequence in dp53, homologous to the FAK binding site of mouse p53 (Figure 4.11), we have not observed a genetic interaction between them in the *Drosophila* eye. This suggests that the 7-amino acid stretch may simply have no such a function in dp53 or that FAK56-dp53 interaction does no occur under these circumstances and should be tested in different tissue contexts. As mentioned before, dp53 induces *reaper* expression in the wing disc (Shlevkov and Morata, 2012) (Dichtel-Danjoy et al., 2013); therefore, it may be more appropriate to study FAK56-dp53 interaction in the wing imaginal disc.

Besides apoptosis, *Drosophila* ΔNp53 also caused differentiation problems. Inhibition of apoptosis by p35 did not rescue completely the *GMR-dΔNp53* eye phenotype but a reduced number of photoreceptor and cone cells was still observed (Fan et al., 2010). *GMR-dp53* eye size and rough pattern suggest there

are also apoptosis and differentiation problems, although in a lesser extent than dΔNp53, as also shown in the wing disc (Dichtel-Danjoy et al., 2013). Further characterisation of this phenotype is required in order to understand why GMR-driven expression of FAK56 did not rescue completely dp53-induced eye phenotype. It may be due to a lack of interaction/regulation between FAK56 and dp53, or FAK56 may be suppressing dp53-dependent apoptotic effects without affecting differentiation problems. This latter hypothesis should be further confirmed by a quantification of the corresponding eye sizes (Figure 4.12A) and a detailed characterization of the cellular composition of those eyes.

4.5.3 *FAK56*, *reaper* and *relish*

Our results suggest a connection between FAK56 and NF-κB Relish, and FAK56 and Reaper (Rpr) within a cell death scenario. The connection between Relish and Rpr remains unclear in our system but there is reported evidence that suggests they may be linked. Relish is activated through the IMD pathway as a consequence of bacterial infection (Hedengren et al., 1999) and IMD has been shown to induce *rpr* expression and cell death (Georgel et al., 2001). This connection encourages further investigation to clarify the mechanisms that may activate IMD pathway or *reaper* expression after loss of FAK56 in nervous tissues.

IMD pathway is the *Drosophila* homologue of the extrinsic apoptotic pathway triggered by TNF in mammals. However, it has not been related to cell death in physiological conditions but it was shown to induce *reaper* expression and apoptosis when overexpressed in the fat body (Georgel et al., 2001). Consequently, *FAK56* mutants might become a good system to study this property, which in turn could highlight a new extrinsic pathway in flies, besides the well-characterised Eiger (TNF)-induced JNK-dependent cell death pathway (Igaki et al., 2002) (Moreno et al., 2002). Interestingly, a model of neuromuscular junction (NMJ) degeneration in *Drosophila* displayed JNK-independent neuronal cell death (Keller et al., 2011). FAK56 function in NMJ and glia cells has been deeply characterised in flies (Murakami et al., 2007, Tsai et al., 2008, Tsai et al., 2012a, Tsai et al., 2012b, Ueda et al., 2008) and according to our results, *FAK56* loss triggers a Relish-dependent apoptotic pathway that does not require JNK signalling; therefore, the role of Relish and the IMD pathway becomes increasingly attractive to explore in these systems.

Furthermore, although the well-characterised apoptotic role of *Drosophila* RHG genes (Hay and Guo, 2006) many death stimuli that trigger their activation still remain elusive; these results suggest FAK56 might sense some of these deadly signals, comparably to anoikis, which is triggered upon integrin detachment from extracellular matrix.

4.5.4 Pro-apoptotic role of NF-κB

Since its discovery (Sen and Baltimore, 1986), the NF-κB superfamily has been involved in diverse processes such as immunity, inflammation, memory and, importantly, cancer initiation and progression (Ben-Neriah and Karin, 2011). The survival role of NF-κB has been well characterised after TNF-treatment (Beg and Baltimore, 1996, Van Antwerp et al., 1996, Wang et al., 1996). After stimulation of TNF receptor, the TRADD/TRAF2/Caspase-8/RIP complex gets active and RIP phosphorylates and activates IκB Kinase (IKK), which in turn releases NF-κB from the inhibitor IκB (α/β/ε). Then, NF-κB enters the nucleus and induces expression of survival factors (Foo and Nolan, 1999).

Conversely, if RIP is cleaved by caspase-8, the outcome is the opposite. NF-κB pathway cannot become active, thereby potentiating apoptosis (Lin et al., 1999). Several reports have demonstrated NF-κB also favours cell death in mammals (Ryan et al., 2000, Ho et al., 2011, Campbell et al., 2004, Lin et al., 1998, Liu et al., 2006), although NF-κB pro-death mechanisms are still unclear. Interestingly, *Drosophila* Relish has a cleavage site between its amino- and carboxy-terminal domains (Erturk-Hasdemir et al., 2009), and a recent report in *Drosophila* has shown that the expression of the Relish N-terminal domain alone has pro-apoptotic effects (Chinchore et al., 2012).

It is tempting to speculate that the pro-apoptotic signals triggered after loss of *FAK56* might lead to IMD activation and NF-κB Relish cleavage; thus, unlocking the transcriptional activity of its N-terminal domain and the expression of pro-death target genes. Reaper participation in this context suggests there may be a crosstalk between the extrinsic and intrinsic apoptotic pathways in *Drosophila,* similarly to what was reported in mammals (Deng et al., 2003). In this report, the TNFR-RIP signalling induced Smac/DIABLO-dependent

apoptosis and in *FAK56* flies, IMD pathway might activate *reaper* (a *Drosophila* Smac/DIABLO orthologue)-dependent apoptosis.

NF-κB activation and survival function are essential to tumour progression and cancer resistance to chemotherapy (Foo and Nolan, 1999, Wang et al., 1996). Therefore, the growing evidence supporting pro-apoptotic roles of NF-κB may be relevant to cancer treatment strategies; as it happens with FAK (discussed in Chapter 3). Thus, targeting NF-κB pathway in those tumour types where it is acting as a tumour suppressor pathway might be counterproductive and detrimental for the host.

Chapter 5 – Dual roles of TNF/Eiger in *Drosophila* models of cancer

5.1 Summary

During the first five months of my PhD I took part in a project that was already running in the lab. Its main objective was to study aspects of the interplay between the immune system and tumour initiation, focusing particularly in the role of the *D. melanogaster* TNF-homologue Eiger (Egr). The system used was a *Drosophila* model of cancer consisting of genetically designed tumours expressing the Ras^{V12} oncoprotein and deficient for the cell polarity regulator gene *scribble*.

My participation in this project contributed to the understanding of the behaviour of tumour-associated macrophages (TAMs). We showed that circulating TAMs are recruited by tumour cells and react by expressing Egr. We were able to see this recruitment thanks to the development of a novel technique, which allowed the injection of RFP-expressing macrophages into tumour-bearing larvae. The results of this work were published in 2010 (Cordero et al., 2010).

5.2 Introduction

5.2.1 TNF and its dual roles in mammals

The TNF superfamily comprises cytokines with the ability to stimulate survival or death signals (reviewed in (Bertazza and Mocellin, 2010)). In fact, there is evidence supporting a dual role of mammalian TNF, as an anti- and pro-tumour factor. Treatment with TNF has been shown to induce necrotic death of subcutaneous murine tumours (Balkwill et al., 1986) while TNF deficient mice displayed higher resistance to developing tumours than wild type mice (Moore et al., 1999). Although it is known that the TNF receptors superfamily are able to trigger survival or death signalling under certain circumstances (Thakar et al., 2006), the detailed molecular mechanisms determining such opposing roles of TNF remain still incomplete, being the unknown genetic composition of the tumour models utilized one of the main sources of uncertainty.

5.2.2 TNF in Drosophila

Eiger (eda-like cell death trigger) is the only member of the TNF cytokine superfamily in *Drosophila* (Igaki et al., 2002, Moreno et al., 2002). It is a protein of 409 amino acids that contains a short cytoplasmic tail (37 amino acids), a transmembrane domain, and an extracellular region consisting of a 'stalk' and a C-terminal TNF homology domain (THD) (Narasimamurthy et al., 2009). THD sequence presents highest similarity with human ligand EDA-A2 (Ectodysplasin-A2), hence its name, but shares significant homology with all mammalian TNF superfamily members.

Eiger (Egr) pro-death functions were uncovered by its mis-expression in imaginal discs, leading to JNK activation and cell death (Igaki et al., 2002, Moreno et al., 2002). Additionally, *egr* mutant flies were shown sensitive to extracellular pathogens (Schneider et al., 2007) and Egr-induced cell death was observed upon bacterial infection (Shlevkov and Morata, 2012), demonstrating the role of Egr in cell death and immunity is conserved between flies and humans (Vidal, 2010). On the other hand, although Egr was recently shown to regulate glial cell proliferation after neuronal cell death (Kato et al., 2009), more examples of physiological roles of Egr in cell proliferation are lacking.

Every epithelium has an intrinsic tumour suppressor capacity to eliminate cells that lose their apico-basal polarity. However, if this process fails it may lead to tumour formation as non-polarised cells accumulate and become more motile; in fact, most human tumours derive from epithelial cells that have lost regulation of cell polarity (Lowe et al., 2004). Similarly, fully mutant flies for cell polarity regulators genes such as *scribble* (*scrib*), *lethal giant larvae* (*lgl*) and *disc large* (*dlg*) develop imaginal discs with a tumour-like appearance (Perrimon, 1988, Bilder and Perrimon, 2000, Humbert et al., 2008). Contrarily, *scrib* mutant cells within an epithelium of wild type cells are eliminated by cell death triggered by the Egr/JNK pathway (Brumby and Richardson, 2003); (Igaki et al., 2009), indicating that the killing effect of Egr is also important in cancer-related scenarios in flies.

Moreover, the expression of the oncoprotein Ras^{V12} in those $scrib^{-/-}$ clones (Ras^{V12}; $scrib^{-/-}$) prevents cell death and produces an invasive tumour-like growth

(Pagliarini and Xu, 2003, Brumby and Richardson, 2005) (Figure 5.1A and A'), which opened new interrogations about the role of Egr in tumour growth.

5.2.3 The fly immune system in the Ras-scribble cooperation

Vertebrate immunity consists of innate and adaptive immunity, the latter being the youngest, in terms of evolution. Invertebrates, as *Drosophila*, only possess innate immunity as the only defense against invaders. Fly innate immunity has two 'weapons', the humoral response and the cellular response. The first consists in the expression of anti-microbial peptides initiated by the IMD and Toll pathways to defend from bacteria and fungi. The cellular response requires the 'blood cells', or hemocytes, to work (reviewed in (Bertazza and Mocellin, 2010)).

There are three blood cell types: lamellocytes, crystal cells and plasmatocytes. Lamellocytes encapsulate invasor bodies and with the help of crystal cells, responsible for humoral melanisation and wound healing, and kill the invader. Plasmatocytes, are the mammalian macrophages counterpart. They are professional phagocytes that kill microbes or clean apoptotic corpses and also participate in wound healing by secreting extracellular matrix components (Sandu et al., 2010).

Despite its normal role in defending the host from external threats, healing wounds or eliminating dangerous cell, extensive evidence link the immune cells with cancer development as well. In fact, tumour associated macrophages (TAMs) promote cancer progression through induction of tumour proliferation, increasing tumour cell migration, invasion and generation of blood vessels that help metastasis (Siveen and Kuttan, 2009).

Recent work suggests that the fly immune system also plays similar roles in *Drosophila* as plasmatocytes (hereafter called haemocytes) were shown to recognise wounds, to prevent tumour growth in $scrib^{-/-}$ animals and interestingly, to associate with Ras^{V12}; $scrib^{-/-}$ tumours (Pastor-Pareja et al., 2008).

5.3 Objectives

We aim to dissect the role of Egr in *Ras^{V12}; scrib$^{-/-}$* tumour formation and to characterize the contribution of tumour-associated haemocytes (TAHs).

5.4 Results

5.4.1 What is the source of Egr?

Ras^{V12}; scrib$^{-/-}$ tumours were malignant as they grew and invaded the brain lobes and ventral nerve cord (VNC) of the central nervous system (Pagliarini and Xu, 2003, Igaki et al., 2006, Uhlirova and Bohmann, 2006) and kill the animal, which died as oversized larvae (Figure 5.1A and A'). On the other hand, *Ras^{V12}; scrib$^{-/-}$; egr$^{-/-}$* clones displayed non-invasive overgrowth and remained contained at their sites of origin. Strikingly, these animals reached the pupal stage (Figure 5.1B and B') (Cordero et al., 2010). This demonstrated for the first time that Eiger also favours tumour progression as its mammalian counterpart.

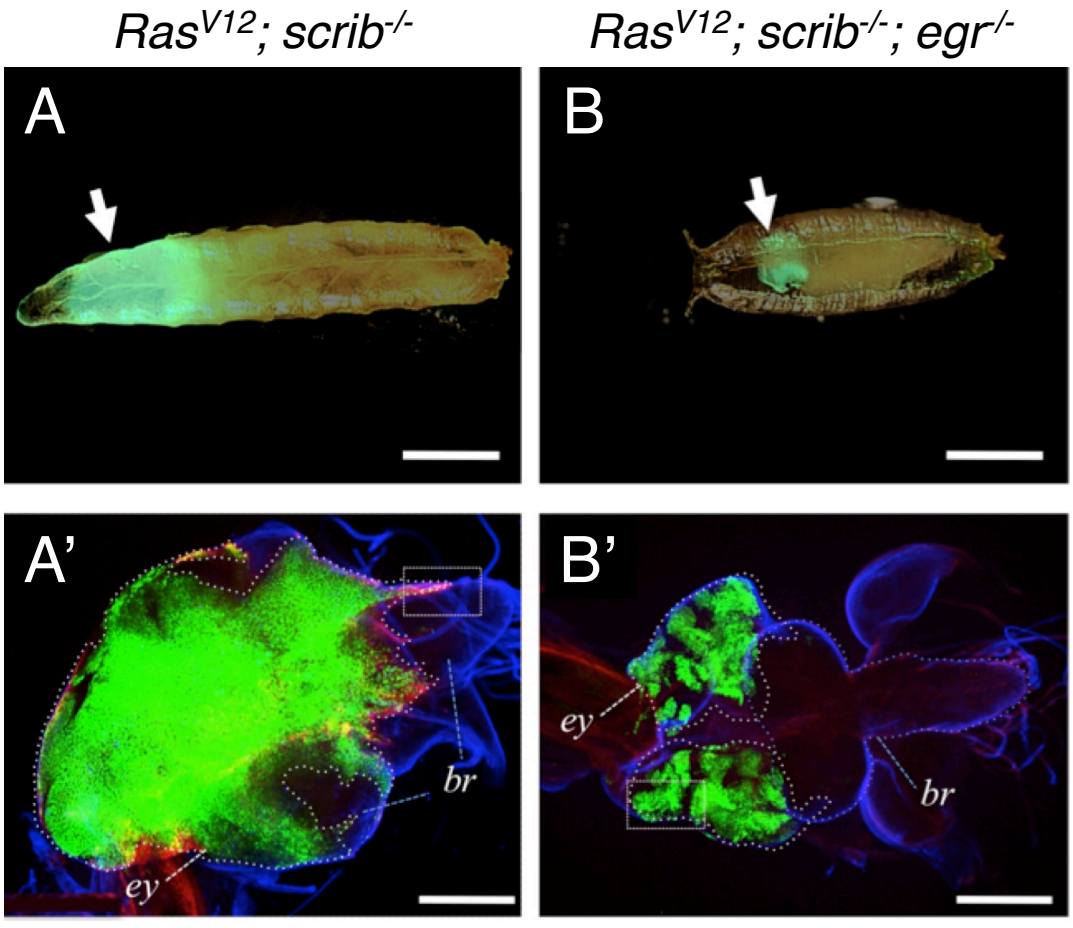

Figure 5.1 Egr is a tumour promoter in Ras-scribble oncogenic cooperation
GFP-labelled clones of cells with the following relevant genotypes were created in developing eye-antennae discs: (A, A') *Ras^{V12}; $scrib^{-/-}$* clones created by MARCM in an *$egr^{+/+}$* host. Animal genotype: *eyFLP/+; act>y^{+}>GFP/UAS-Ras^{V12}; FRT^{82B}, $scrib^{1}$/FRT^{82B}, tub-Gal80.* (B, B') *Ras^{V12}; $scrib^{-/-}$* clones created by MARCM in an *$egr^{-/-}$* host. Animal genotype: *eyFLP/+; egr^{1} act>y^{+}>GFP/egr^{1}, UAS-Ras^{V12}; FRT^{82B}, $scrib^{1}$/FRT^{82B}, tub-Gal80.* A and B display the most frequent whole-animal phenotypes for the indicated genotypes. A' and B' display larval cephalic complexes labelled for GFP (green), dMMP1 (red) and Laminin B1 (blue); eye-antennae discs (*ey*) and brains (*br*) were outlined with white and blue dotted lines, respectively. Scale bars: 150 mm (A-B), 100 mm (A'-B'). These observations were made by Marcos Vidal.

The source of the endogenous Egr in *Ras^{V12}; $scrib^{-/-}$* tumours was then examined in order to understand the mechanisms mediating this pro-tumour role. In mammals, the association of immune cells to tumours is well characterized (Siveen and Kuttan, 2009). Recent work in *Drosophila* had demonstrated that haemocytes associate to *Ras^{V12}; $scrib^{-/-}$* tumours (Pastor-Pareja et al., 2008). Therefore, haemocytes were good candidates to be the Egr-expressing cells associated to these tumours.

With this hypothesis in mind, different immunofluorescence experiments carried out in the lab indicated that (see (Cordero et al., 2010) for more results):

- Haemocytes associate to cells deficient for 'scribble' group genes and induces JNK-signalling;
- Egr is at least in part expressed by Tumour Associated Haemocytes (TAHs);
- Egr expression in haemocytes and signalling activation are likely triggered upon interaction of haemocytes with tumour cells.

5.4.2 Hemolymph transfusion technique in larvae

As hemocytes were found associated to imaginal discs of normal animals (Pastor-Pareja et al., 2008) a key open question was whether TAHs originate from a population of residing hemocytes, or instead if they are recruited to the tumours from the population of circulating cells. To answer this question, we devised a technique of hemolymph transfusion, which consists of injecting donor RFP-labelled hemocytes into larvae carrying tumours (Figure 5.2). Hemolymph

transfusion and tissue transplantation techniques are common practice in adults (for instance, (Gateff, 1978); however, here we created larvae expressing RFP in hemocytes and transfused their hemolymph to animals bearing GFP-labelled, eye-antennae clones of *Ras^{V12}; scrib$^{-/-}$* cells. After a 24h recovery period the transfused larvae were dissected and imaged by confocal microscopy.

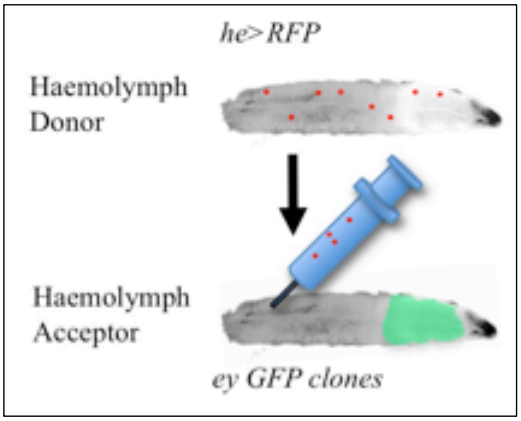

Figure 5.2 Hemolymph microinjection
Schematic representation of the hemolymph transfusion experiment. Red Fluorescent Protein (RFP) is expressed through *hemesee-Gal4* in hemocytes of donor larvae. Hemolymph is collected from several donors and injected on the rear of larvae containing GFP-labelled tumours. See Materials and Methods, section 1.3.4.

Our initial experiments indicated that transfused haemocytes associated to GFP-labelled tumours. Given that haemocytes stick to any organic surface we could not discount that haemocytes were associating to tumour cells non-specifically. To address these concerns, we made careful quantifications of transfused haemocytes associated with imaginal discs with clones of the following genotypes: wild type (GFP-expressing only), *Ras^{V12}*, *scrib$^{-/-}$* and *Ras^{V12}; scrib$^{-/-}$* (Figure 5.3A-D). We found that while transfused haemocytes attached to all discs at a low frequency, a significant increase in attachment was observed in with *Ras^{V12}; scrib$^{-/-}$* clones, even after normalisation against clone size (Figure 5.3E).

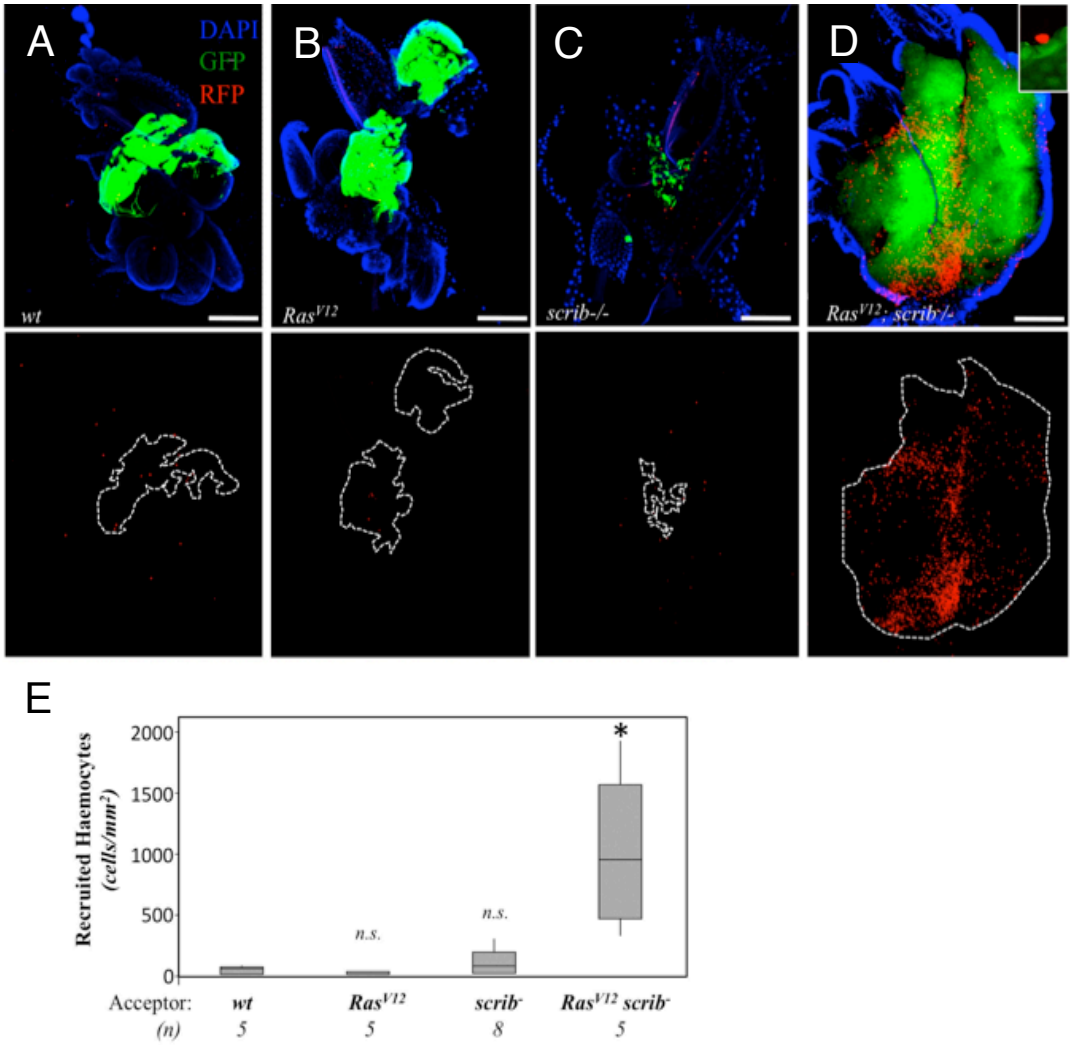

Figure 5.3 ***Ras^{V12}; scrib$^{-/-}$*** **proliferative clones recruit circulating hemocytes**
(A-D) Representative images from the transfusion experiment. The bottom panels display the transfused haemocytes in red and white dotted lines the outlines of the clones. Scale bars: 100 mm. (E) Box plot quantification for the number of associated transfused haemocytes for the indicated acceptor genotypes. The number of animals analysed is indicated. The asterisk (*) means statistical significance (*p=0.0122*) in a non-parametric Mann-Whitney test; *n.s.* means non statistically significant.

Collectively, these results indicate that *Ras^{V12}; scrib$^{-/-}$* tumours preferentially attract and/or retain haemocytes from the circulation.

5.5 Discussion

Egr provides a switch from non-invasive to invasive scribble tumour growth in the presence of oncogenic Ras. Thus, JNK and Ras/MAPK signalling are both active and hence cooperate to promote proliferation in *Ras^{V12}; scrib$^{-/-}$* cells. The transcription factors NF-κB and AP-1 (activating-protein 1) are activated by mammalian TNF when it stimulates survival (Choi et al., 2013). Interestingly, JNK and p38 (MAPK) signalling regulate AP-1 to promote cell proliferation (Shi et al., 2011). It is tempting to speculate that a similar situation is occurring in

Ras^{V12}; scrib$^{-/-}$ cells. Here, Ras/MAPK signalling overcomes the death-effects of JNK but they also cooperate to induce tumour proliferation. Little is known about the role of Relish in this context, the fly homologue of NF-κB. Therefore, assuming Eiger is triggering the extrinsic apoptotic pathway in *Drosophila*, as TNF does in mammals, it would be interesting to explore the role of IMD pathway (see Chapter 4) and the NF-κB Relish in the progression of *Ras^{V12}; scrib$^{-/-}$* tumours.

Consistently to what we observed in flies, human tumours also attract macrophages from the circulation (Siveen and Kuttan, 2009). These attractants, such as chemokines, or those that play similar roles in flies remain to be found. Also, the tumour microenvironment has been shown to regulate the behaviour of TAMs; for instance, making them stimulate the tumour growth or contrarily, killing the damage-mutant cells. *Drosophila* models of cancer can be very useful in the contribution to the understanding of TAHs (TAMs) reactions toward the tumour, as we can engineer the genetic content of the cancer cells and have a finer control of the tumour variables. Additionally, we can use the transfusion technique presented here to inject genetically controlled haemocytes and thus, study and control genetic variables of the immune system. Thus, further studies of the tumour microenvironment in *Drosophila,* i.e. tumour cells/surrounding tissue/immune system, might provide useful data that can be relevant to human cancer biology.

Chapter 6 – Final discussion and Conclusions

6.1 Context-dependent behaviours of FAK

6.1.1 Tumour suppressor

The study of Focal Adhesion Kinase during my PhD allowed me to witness different functions of this protein in different contexts: FAK inhibits RTK/Ras signalling when receptor expression is upregulated in fly epithelia and human cancer cells; FAK blocks cell death of neuronal tissues in flies; and FAK buffers Src signalling in order to maintain cell viability of mouse skin cancer cells (Sandilands et al., 2012a).

This is logical based on FAK ubiquitous expression, at tissue and cellular level, and its diverse range of interactions. FAK is a complex and important protein for the cell. It is mainly a scaffolding protein that interacts with a still growing list of binding partners and many of them regulate its kinase activity in response to diverse stimuli. Therefore, it is reasonable that FAK can regulate many cellular processes and can adapt its behaviour to what its partners and cell/tissue environment require.

Observations made in fly epithelia *in vivo* and human cell culture indicate FAK can work as a 'fuse', as a signalling buffer. In other words, FAK is able to regulate the dose of the signal delivered from the ECM/plasma membrane to the nucleus and cytoplasm. Depending on the cell capabilities and stimuli, those signals will lead to inhibition of cell death, growth, differentiation, proliferation or migration.

Also, we described a new role of FAK in the regulation of RTKs, such as EGFR and RET. Certain stressful situations lead to unexpected behaviours and here, we demonstrated that within a stressful context driven by RTK overexpression, FAK can negatively regulate RTK signalling. Specifically, FAK inhibits the RTK/Ras/MAPK axis, opposite to its well-described role downstream of integrin receptors, where it favours Ras/MAPK signalling. In human cancer cells with high levels of EGFR, FAK regulates the traffic of the receptor between the plasma membrane and the cytoplasm, maintaining a balanced signalling. In

the absence of FAK, there are more RTK molecules at the membrane and therefore increased signalling. This can be a consequence of reduced internalisation or increased recycling of the receptor, both of which would lead to a new steady state with more receptors at the membrane.

We also described how cell fates of the eye tissue are affected by different RET/FAK ratios, which ultimately determine the dose of Ras/MAPK signalling pathway. Genetically defined ratios between RET and FAK that corresponded with a 'moderate' (higher than normal) MAPK signalling produced a blockade of apoptosis during eye development. This caused supernumerary interommatidial cells and a mispatterned eye. Another set of genetically defined RET/FAK ratios created higher levels of MAPK signalling that forced eye interommatidial precursor cells to differentiate into cone cells. Moreover, in mitotic tissues such as the wing disc, the effects of two distinct RET/FAK ratios were: a blockade of differentiation of cross veins (moderate MAPK signalling) and proliferation (high MAPK signalling). Therefore, we can conclude that for a given signalling pathway and dose, the final outcome will depend exclusively on the cell/tissue type.

6.1.2 Tumour promoter

The high level of expression of FAK56 in the *Drosophila* CNS and eye discs might suggest it plays an important role in those tissues. Indeed, data presented in this thesis suggest FAK56 plays an anti-apoptotic role in the nervous system of *Drosophila*. Massive levels of apoptosis were observed in the FAK-deficient larval eye imaginal discs and central nervous system without affecting animal viability. Consequently, it can be speculated that neurons and glial cells are being affected but this still remains unclear.

Also, we uncovered a link between FAK56 and Relish (NF-κB), and FAK56 and Reaper (Rpr) in these tissues. The connection between Relish and Rpr remains elusive but there is reported evidence that suggests they may be linked. Relish is activated through the IMD pathway as a consequence of bacterial infection (Hedengren et al., 1999). IMD has also pro-apoptotic capabilities as its over-expression has been shown to induce *rpr* expression and cell death in the fat body (Georgel et al., 2001). This connection opens up a possibility to study

the role of IMD pathway in nervous tissues, such as FAK56 loss-induced cell death.

Overall, these results, which need further investigation, might have implications in the study of neurobiology and cancers of the nervous system.

6.2 Implications of the discovery in the FAK/cancer field

The mechanism characterised in chapter 3 of this thesis adds up to a growing body of evidence supporting non-canonical roles of FAK. Also, the negative regulation that FAK exerts over RTK/Ras/MAPK signalling might have important implications in cancer biology and therapy.

Many human tumours are initiated or driven by oncogenic mutations that alter the normal functioning of RTKs (Lemmon and Schlessinger, 2010). For instance, breast, lung, ovarian, bladder and brain cancers express higher levels or mutated versions of a receptor (Blume-Jensen and Hunter, 2001). FAK is considered a good target for cancer therapy as it has been shown to inhibit apoptosis and favour growth and migration of tumour cells (Cance et al., 2013). In fact, it is highly expressed in many tumours, particularly in invasive ones (McLean et al., 2005). However, FAK might also have tumour suppressor properties such as the negative regulation over RTK signalling described in this thesis. So, in those RTK-driven cancer scenarios, FAK targeting could be detrimental for the host. Then, it will be necessary to identify the actual role of FAK before designing therapeutic strategies against it.

It is possible to speculate that FAK upregulation could be a consequence of an RTK gain-of-function mutation. Thus, the cell would prevent aberrant signalling and a stressful situation. However, with the later accumulation of mutations during tumour progression, it may turn out that high expression of FAK becomes beneficial for the tumour cell. Thus, it would help cell survival by blocking cell death pathways, and stimulate cell migration by accelerating the turnover of focal adhesions. This view is supported by the facts that FAK gain-of-function mutations has not been found; FAK overexpression is not sufficient to initiate tumour development; and the vast majority of FAK-expressing tumours

have acquired oncogenic mutations before and present highly invasive and metastatic behaviour.

Additionally, we showed that this oncogenic stress response was conserved in both flies and mammals. The fact that FAK mutant flies have no developmental defects (Grabbe et al., 2004) but FAK deficient mice are embryonic lethal (Ilic et al., 1995) suggests that FAK's function in development was acquired later in evolution and the above mentioned stress response could be FAK's ancestral role.

Regarding the anti-apoptotic role of FAK described in chapter 4, which correlates with most of literature available about this protein, there are two facts that I would like to highlight: the context-dependency of this role and the relevance of its scaffolding functions versus its kinase activity.

In *Drosophila*, FAK prevents cell death physiologically in neuronal or pro-neuronal cells. It is tempting to think this role also occurs in mammals, given its high levels of expression in analogous tissues (Menegon et al., 1999). Therefore, FAK may become an interesting target to look at in brain tumours. Secondly, considering that FAK scaffolding functions exceed its kinase-dependent effects, it would be more appropriate to target protein-protein interactions related to cancer such as FAK-p53 (Golubovskaya et al., 2005, Lim et al., 2008), FAK-RTKs (Sieg et al., 2000, Plaza-Menacho et al., 2011) and FAK-RIP (Kurenova et al., 2004). For instance, RIP is involved in TNF-induced apoptosis (Lin et al., 1999) and its interaction with the FERM domain of FAK was shown to prevent this outcome (Kamarajan et al., 2010); hence, drug targeting against FAK/RIP interaction would be the path to follow in cancer scenarios where FAK has clear anti-apoptotic roles. Related to this, we also present data suggesting a pro-apoptotic role of the NF-κB family member Relish in fly CNS and eye imaginal discs. Relish is activated downstream of IMD (Hedengren et al., 1999), which is the *Drosophila* homologue of RIP. Therefore, we can speculate that a similar situation may happen in these tissues, where FAK and IMD might regulate cell death. Further work has to be done in order to confirm this hypothesis.

As described throughout this thesis, FAK is a multifunctional protein that interacts with a still growing list of partners. *Drosophila* has only one homologue

of this cytoplasmic protein tyrosine kinase family and there is evidence it can behave similarly to mammalian FAK or Pyk2 (Fox et al, 1999)(Ueda et al., 2008). Therefore, besides the many more functions that remain to be seen in *Drosophila*, FAK56 can be used as a good model to study context-dependent reactions of FAK protein family members and extrapolate the results to human FAK or Pyk2 accordingly.

6.3 Future directions

Given the two independent roles of FAK56 described in this thesis, which are linked to cancer, and taking into account the fact that most of research about FAK56 has been done in neurobiology-related topics, it is clear to see that there is still a wide open field to explore with *Drosophila* FAK: particularly, its role in epithelial tissues and the here-proven role in tumourigenesis. For instance, it would be fascinating to take advantage of *Drosophila* powerful genetic tools to model FAK properties related to cancer-linked disciplines such as cell migration and invasion.

Last but not least, a comment about IMD/Relish pathway in *Drosophila*. Its participation in the immune system has been extensively described and it will continue this way (Ramet and Hultmark, 2013). However, the mammalian orthologue NF-κB pathway has been involved in several contexts different than immunity and vastly related to survival, proliferation and also cell death (Oeckinghaus et al., 2011). Recently, the pro-apoptotic functions of NF-κB Relish began to be uncovered (Chinchore et al., 2012). However, many questions remain unanswered about IMD pathway out of the immunity field in *Drosophila*; for instance, will IMD/Relish pathway share the survival roles with its mammalian homologue in *Drosophila* cancer-related scenarios?

A good system to look at this is the $scrib^{-/-}$, Ras^{V12} tumour model. The mammalian TNF/TNFR-induced pathway activates JNK through a mitogen-activated protein (MAP) kinases cascade of phosphorylation (Deng et al., 2003). NF-κB is also involved in that response, although generally activated to promote survival (Beg and Baltimore, 1996, Van Antwerp et al., 1996, Wang et al., 1996). In flies, Eiger, the TNF homologue, also activates JNK through a MAP kinases phosphorylation cascade (Igaki et al., 2002); and the IMD pathway, the

Drosophila homologue of the TNF-induced signalling pathway, can activate both JNK and NF-κB Relish, which in turn regulate each other as in mammals (Kim et al., 2005) (Park et al., 2004, Tang et al., 2001, De Smaele et al., 2001). Besides the clear evolutionary conservation of these pathways, the connection between Eiger and IMD pathway is still poorly characterised in flies, especially in cancer-like scenarios (Schneider, 2007, Brandt et al., 2004, Schneider et al., 2007). Thus, the *scrib*$^{-/-}$, *Ras*^{V12} tumour model provides a system to study this possible interaction. Eiger promotes proliferation and invasion through an evident cooperation between JNK and Ras/MAPK signalling, which was shown to drive proliferation in part by overcoming the killing effects of JNK activity (Brumby and Richardson, 2005). Therefore, it would be very interesting to explore whether Relish becomes activated after JNK inhibition and contributes to tumour growth in this genetic context.

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