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OMICs based identification of the mechanisms that underpin FAK's regulation of gene expression

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Doctor of Philosophy

The University of Edinburgh

2020

Declaration

I declare that this thesis has been composed by myself and details my own research unless stated otherwise in the text. No part of this thesis has been submitted elsewhere for any other degree or personal qualification.

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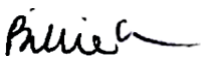

January 2020

Abstract

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase, commonly over-expressed in cancer that is well known to be a key regulator of cancer cell proliferation, survival, migration and invasion. It has recently been shown to modulate the anti-tumour immune response via transcriptional regulation of chemokines and cytokines. These findings have fuelled interest in FAK as a plausible therapeutic target in cancer and FAK kinase inhibitors are currently in Phase I and II clinical trials.

To date, much of the research on FAK has focused on its role at focal adhesions, detailing its major functions in cell motility and cancer cell invasion. However, 16 years ago, FAK was shown to translocate to the nucleus and it is now well established that this occurs under conditions of cellular stress such as oncogenic transformation. In the nucleus, FAK binds to a number of transcription factors such as P53, MDM2, IL33, RUNX1 and SP1. These nuclear interactions have been linked to proliferation, survival, differentiation and regulation of the anti-tumour immune response and cell cycle. However, despite these findings, the mechanism by which FAK regulates gene expression in the nucleus has yet to be defined. With FAK kinase inhibitors currently in clinical trials, it is important to understand the molecular mechanisms associated with FAK's transcriptional regulation, the role of the kinase activity, and the potential therapeutic benefits of targeting FAK's nuclear function.

We therefore investigated the mechanism by which FAK regulates transcription by integration of a number of 'omics' approaches to define the nuclear FAK interactome and FAK-dependent gene programmes, as well as FAK's role in regulating chromatin accessibility and transcription factor binding. Integration of these datasets predicted that FAK regulates the binding of AP-1 and ETS transcription factors to chromatin. Furthermore, our findings indicate that a subset of FAK-regulated genes display changes in chromatin accessibility. Chromatin ImmunoPrecipitation (ChIP) validation experiments on the enhancer region of one of these genes, *IL33*, show reduced binding of JUN upon loss of FAK's kinase activity. This predicts that FAK is regulating chromatin accessibility at this region of the *IL33* gene.

The work presented in this thesis therefore identifies a novel mechanism by which FAK regulates transcription; further experiments will be required to define the epigenetic complexes responsible for FAK-dependent chromatin accessibility changes. This work adds to our understanding of how an integrin adhesion protein can control cancer cell behaviour via transcription in the nucleus.

Lay abstract

Focal adhesion kinase (FAK) is a protein that is used by cells when they grow and stick to their surroundings. When someone has cancer, one of the main risks is that the cancer may spread (or metastasise) causing cancers elsewhere in the body that are more difficult to treat. It has been shown in laboratory experiments, that when FAK is blocked, the risk of the cancer spreading can be reduced. More recently FAK has also been shown to move to the cell compartment where the regulation of genetic information (genes) occur – called the nucleus. Here FAK has been shown to interact with proteins called transcription factors that bind to regions next to genes (genomic regions) to turn genes on or off. By binding these transcription factors in the nucleus, FAK can regulate inflammation which has long been linked to the development of cancer. Therapies that target the body's immune system to boost its own tumour killing activity offer a new form of cancer treatment with fewer negative side effects than chemotherapy. My work has investigated the mechanism by which FAK regulates the expression of genes that are important in cancer, in particular inflammatory genes. To do this, I have integrated a number of datasets defining 1) the proteins that FAK binds in the nucleus, 2) the genomic regions that FAK is regulating and 3) whether this turns on or off these genes that are FAK-regulated. My work has shown that FAK does indeed regulate a transcription factor to enhance the expression of a gene involved in cancer-inflammation. This work shows that FAK in the nucleus is an important regulator of the expression of genes in cancer by impacting a number of key transcription factors in the nucleus.

Acknowledgements

Firstly, I would like to thank my supervisor Val Brunton – who has taken over overseeing the final year of my PhD and has helped me greatly with the challenging task of writing a thesis. I would also like to thank Margaret Frame who provided good advice on thesis writing and in lab meetings.

Bryan Serrels for being my supervisor for most of my PhD, who provided immense support in experiments and contributed significantly to this work.

Niamh McGivern for being a huge support during my PhD and for generating the FAK BirA* SCC cell lines.

I would like to thank Rosanna Upstill-Goddard for performing the ATAC-seq bioinformatic analysis and therefore has contributed significantly to this work.

Niall Quinn and Adam Bryon for running the mass spectrometry samples and for assistance with the data analysis, respectively.

Graeme Grimes for the raw sequence alignment and differential expression analysis for the mRNA-seq data.

I would like to thank Ben – I wouldn't have been able to do this without you!

Last but not least I would like to thank my mum, dad, stepmum and little Ava for being a tremendous emotional support in this difficult time.

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Table of abbreviations

Abbreviaton	Meaning
ANOVA	Analysis of variance
AP-1	Activator protein
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
BSA	Bovine serum albumin
CCL5	C-C Motif Chemokine Ligand 5
ChIP	Chromatin immunoprecipitation
CST	Cell signalling technologies
CXCL10	C-X-C Motif Chemokine Ligand 10
DAPI	4′,6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EMT	Epithelial–mesenchymal transition
ETS	E26 transformation-specific or E-twenty-six
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and drug administration
FU	Fluorescence intensity
GO	Gene ontology
GRB2	Growth factor receptor-bound protein 2
IF	Immunofluorescence
IGFBP3	Insulin-like growth-factor binding protein 3
IL33	Interleukin-33
IP	Immunoprecipitation
IPA	Ingenuity pathway analysis
KD	Kinase-deficient
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LFQ	Label free quantification
LPP	Lipoma-preferred partner
MAPK	Mitogen activated protein kinase
MMTV	Mouse mammary tumour virus
mRNA-seq	messenger RNA-sequencing
MS	Mass spectrometry
NES	Nuclear export sequence
NLS	Nuclear localisation sequence
NP-50	Nonyl phenoxypolyethoxylethanol
NS	Not significant
PBS	Phosphate buffer saline
PBS.T	Phosphate buffer saline + Tween
PCR	Polymerase chain reaction

PI3K	Phosphoinositide 3-kinases
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RBMS3	RNA Binding Motif Single Stranded Interacting Protein 3
RT	Room temperature
RTK	Receptor tyrosine kinase
RUNX1	Runt-related transcription factor 1
s	seconds
SCC	Squamous cell carcinoma
SEM	Standard error of mean
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering RNA
SRC	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase
TAF9	TATA-Box Binding Protein Associated Factor 9
TBP	TATA-Box Binding Protein
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TE	Tris-EDTA
TF	Transcription factor
TFA	Trifluoroacetic Acid
TME	Tumour microenvironment
WB	Western blot
WWOX	WW Domain Containing Oxidoreductase

Nomenclature

For simplicity and consistency, all abbreviated gene and protein names are reported in capital letters throughout this thesis.

Chapter 1 Introduction

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is a key signal integrator regulating adhesion, migration and survival pathways. It is known to be upregulated in cancer and as such, is now a focus of cancer therapeutics as an alternative therapy to conventional toxic chemotherapy. It has recently been described to be a key regulator of gene expression of immunosuppressive cytokines by binding to transcriptional complexes in the nucleus: this drives cancer progression by suppressing anti-tumour immunity, making it a key cancer target to enhance the effectiveness of cancer immunotherapy. That said, the mechanisms by which FAK regulates transcription have never been defined and this was the basis of my project. In order to introduce my project, I will firstly describe the hallmarks of cancer, as FAK has been shown to be a key regulator of many of these. This will be followed by a description of the approaches used to target cancer. Then I will describe the structure and physiological functions of FAK, before describing its cancer-associated functions. This introduction is followed by a description of the studies showing that FAK in the nucleus is a key regulator of gene expression in cancer, identifying it is an important cancer target, and discussion of clinical trials that use FAK inhibitors. This introduction will conclude by expanding on the Aims of my project.

1.1 The hallmarks of cancer

Cancer is a multi-step disease characterised by aberrant cell proliferation resulting in the growth of a tumour, which may spread from the primary site to other organs of the body. Following an initiating event, cancer is driven by a number of features - 1) sustaining proliferation advantage, 2) deregulated cellular energetics, 3) resisting cell death, 4) genome instability and mutation, 5) induction of angiogenesis, 6) activating invasion and metastasis, 7) tumour promoting inflammation, 8) enabling replicative immortality, 9) avoiding immune destruction and 10) evading growth suppressors. These were described as the hallmarks of cancer in the seminal review by Hanahan and Weinberg in 2011 (**Figure 1.1**).

1.1.1 Sustaining proliferative advantage

Cancer cells acquire these characteristics by genetic reprograming of key signalling pathways (i.e. MAPK, WNT, P53) which are widely known to regulate a number of

cellular processes important in cancer development and survival such as proliferation, cell migration and differentiation. Activation of cancer-causing 'oncogenes' cause loss of proliferation control and ultimately cancer by disrupting the aforementioned signalling pathways amongst others. Key examples of oncogenes include BRAF which is mutated in 60% of melanomas which is a key regulator of the RAS-RAF-MEK-ERK-MAPK pathway, driving cancer-associated processes including proliferation (Davies *et al*., 2002).

1.1.2 Deregulated cell energetics

In addition to acquiring proliferative signals, tumours require sufficient oxygen and nutrients to survive and proliferate (reviewed in Gentric *et al*., 2016). Lack of availability of these essential components can often lead to nutrient deprivation and regions of hypoxia (i.e. deprivation of oxygen in tissues) (reviewed in Muz *et al*., 2015, reviewed in Gentric *et al*., 2016). To combat this, tumours often undergo a 'metabolic switch' termed the Warburg effect, where tumour cells favour glycolytic metabolism over the more efficient oxidative phosphorylation (reviewed in Gentric *et al*., 2016). This "metabolic switch" arises due to changes in expression of metabolic gene programs associated with mitochondrial regulation. For example, it has been shown that P53 induces the expression of PUMA, which inhibits the function of mitochondrial pyruvate carrier (MPC) resulting in decreased mitochondrial pyruvate uptake and increased glycolysis (Kim *et al*., 2019).

1.1.3 Resisting cell death

Apoptosis is a process by which the cell undergoes programmed cell death following insults such as DNA damage, infection, oncogenesis or exposure to cytotoxic agents in cancer treatment. When a cell undergoes apoptosis, it exhibits morphological changes including detachment of extracellular matrix (ECM) and loss of cell-cell interactions (reviewed in Elmore *et al*., 2007). This is controlled by pro-apoptotic proteins such as FAS, BAX, NOXA and PUMA that stimulate apoptosis in both adherent and suspension cells via the activation of caspases (reviewed in Harris and Levine, 2005). When caspases are activated they cleave proteins such as those that

maintain membrane integrity (reviewed in Elmore *et al*., 2007). The most notable DNA damage sensor and inducer of apoptosis is P53 which is a key inducer of FAS, BAX, NOXA and PUMA-induced apoptosis (reviewed in Harris and Levine, 2005). Resisting cell death is a key hallmark of cancer and is one of the contributing factors allowing cells to grow indefinitely (reviewed in Hanahan and Weinberg, 2000). Indeed, cancer cells often have mutation or deletion of P53 that contributes to resistance to apoptosis. For example, a study has shown that mutations in the *P53* gene results in reduced *BAX* mRNA expression and resistance to apoptosis mediated by the chemotherapy agent, cisplatin, in human ovarian cancer cells (Perego *et al*., 1996).

1.1.4 Genome instability and mutation

Acquisition of a multitude of hallmarks is dependent on the genetic changes in neoplastic cells. Particular mutations allow genetic advantage of subclones of cells, which enable their outgrowth and overpopulation in the tissue in the local environment (reviewed in Hanahan and Weinberg, 2011). Tumour progression can be depicted as a series of clonal expansions, each brought about by the random acquisition of a mutant genotype that provides a growth advantage (reviewed in Hanahan and Weinberg, 2011). Cancer cells increase mutational burden by breaking down the genomic surveillance machinery which prevent cells from undergoing apoptosis when cells acquire DNA damage (reviewed in Hanahan and Weinberg, 2011): for example, mutations in the core DNA repair machinery i.e. BRCA1 which repairs DNA via non-homologous end-joining and homologous recombination DNA repair pathways (reviewed in Gorodetska *et al*., 2019). A meta-analysis study found that women with a germline BRCA1 mutation have a 55% risk of developing breast cancer and a 39% risk of developing ovarian cancer by the time they are 70 (Chen *et al*., 2007).

1.1.5 Inducing angiogenesis

Angiogenesis is the formation of new blood vessels and is essential for maintaining tumour growth. Tumours alter the ratio of pro- and anti-angiogenic factors, a process termed the ‘angiogenic switch’ (reviewed in Baeriswyl and Christofori, 2009). Well established angiogenesis stimulators include vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1) (reviewed in Hanahan and Weinberg,

2011). Hypoxia is believed to be the main driver of angiogenesis, as drops in oxygen levels induce the activity of the transcription factor hypoxia-inducible factor 1-α (HIF-1-α), which ultimately drives the expression of VEGF and its receptor (VEGFR) (reviewed in Nishida et al., 2006). Endothelial cells activated by VEGF produce matrix metalloproteases (MMPs) which break down the ECM allowing endothelial cells to migrate (reviewed in Nishida *et al*., 2006). The endothelial cells also divide into hollow tubes that can mature slowly into blood vessels (reviewed in Nishida *et al*., 2006). Furthermore, blood vessel formation allows tumour cells to break away from the tumour to spread to a different area of the body in a process called metastasis (reviewed in Nishida *et al*., 2006).

1.1.6 Induction of invasion and metastasis

Metastasis is responsible for approximately 90% of cancer-associated deaths (reviewed in Lambert *et al*., 2017). The dissemination of primary tumour cells to distant sites is termed the 'invasion-metastasis cascade' which is a process consisting of a multitude of steps (reviewed in Jiang et al., 2015). These include 1) acquisition of invasive potential, 2) accelerated growth and invasion of the basement membrane into surrounding tissues, 3) angiogenesis, blood and/or lymphatic vessel invasion and release into the circulation, followed by 4) colonisation and invasion of secondary sites and formation of micro- or marco-metastasis (reviewed in Jiang *et al*., 2015). A process called epithelial-mesenchymal transition (EMT) is associated with invasion and metastasis (reviewed in Fouad *et al*., 2017). EMT is a normal process utilised in wound healing and embryogenesis that is hijacked by cancer cells to allow loss of differentiated epithelial characteristics and acquisition of an undifferentiated, stem-like mesenchymal phenotype (reviewed in Fouad *et al*., 2017). Cells that have undergone EMT are more invasive but it has been reported that EMT is non-essential for all steps of metastasis, yet may be important for chemoresistance (Fisher *et al*., 2015). The EMT process is driven by a set of master transcription factors – SLUG, TWIST, SNAIL, ZEB1 (reviewed in Lambert *et al*., 2017). For example, TWIST1 drives the expression of matrix metalloprotease 2 (MMP2) and its expression is associated with invasion in breast cancer cells (Zhu *et al*., 2019).

1.1.7 Tumour promoting inflammation

The 'seed and soil' hypothesis was proposed by Paget in 1889, who suggested that cancer cell 'seeds' need the correct environment 'soil' to grow and metastasise. As a tumour grows, it becomes deprived of oxygen and nutrients. Therefore, the tumour scavenges oxygen and nutrients by stimulating angiogenesis (reviewed in Balkwill, Capasso and Hagemann, 2012). Cancer cells can cause abnormal secretion of inflammatory mediators called cytokines, which kick start blood vessel formation and drive tumour growth by generating an immuno-suppressive environment to protect the tumour from attack by cytotoxic $CD8^+$ T cells (reviewed in Jiang and Zhu, 2015). Thus, inflammation is key for cancer progression by creating an immunosuppressive tumour microenvironment (TME) which supports tumour growth.

The TME is complex, comprised of non-malignant cells, stromal cells and a diverse range of immune cells (reviewed in Balkwill, Capasso and Hagemann, 2012). The immune populations in the TME include tumour-killing cells such as cytotoxic $CD8^+$ memory T cells, natural (NK) killer cells and immuno-suppressive cells including $CD4^+$ T helper cells, regulatory $CD4^+$ T-cells (Treg) and tumour associated macrophages (TAMs) (reviewed in Balkwill, Capasso and Hagemann, 2012). Furthermore, there are dendritic cells which become damaged due to the hypoxic and inflammatory TME resulting in them not being able to stimulate an immune response to tumour-associated antigens (Tachibana *et al*., 2005). Additionally, the TME is also comprised of non-malignant cells or stroma such as cancer associated fibroblasts (CAFs), vascular endothelial cells and pericytes, as well as ECM proteins (i.e. collagen, elastin and fibronectin) (reviewed in Balkwill, Capasso and Hagemann, 2012). CAFs arise when fibroblasts become activated through wound healing and fibrosis, promoting the production of cytokines, chemokines and other immune modulators (reviewed in Bremnes, Capasso and Hagemann, 2011). CAFs inhibit tumour growth in the early stages of tumourigenesis, but promote tumour growth in the late stages of cancer (reviewed in Cirri and Chiarugi, 2011). The immune and non-immune population will be described in more detail in **section 1.1.9**.

1.1.8 Enabling replicative immortality

As cells go through replication cycles, they often acquire mutations and stop dividing (senescence) (reviewed in Hanahan and Weinberg, 2000). Mechanistically this occurs because the ends of chromosomes (telomeres) contain thousands of repeats of a 6p sequence element (TTAGGG), which occurs because the eukaryotic DNA replication machinery cannot efficiently replicate the 3' end of chromosomes during S phase (reviewed in Hanahan and Weinberg, 2000). As cells go through successive cell cycles, they lose their ability to protect the end of chromosome DNA and the TTAGGG nucleotide repeats reduce in size after each cell division due to the 'end replication problem' (reviewed in Hanahan and Weinberg, 2000). Telomeric DNA is maintained by the telomerase enzyme which adds hexanucleotide DNA repeats to the end of telemetric DNA, preventing cell senescence and enabling replicative mortality of cancer cells (reviewed in Shay, 2016). Telomerase is often upregulated in malignant cells and its importance for tumour growth is exemplified by studies showing that inhibition of telomerase perturbs growth in human breast and colon cancer cells (Hahn *et al*., 1999).

1.1.9 Avoiding immune destruction

Although tumour-killing $CD8^+$ T cells enter the TME, a complex network of immunosuppressive cells and cytokines impair their tumour killing activity and lead to them becoming exhausted (reviewed in Jiang and Zhu, 2015). Exhausted T cells are characterized by the expression of a number of inhibitory cell surface receptors including programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4), where binding of these receptors to their ligands perturbs T cell activity (reviewed in Jiang and Zhu, 2015). Tumours over-express the PD-1 receptor ligand, PD-1L, which is a predictor of poor prognosis (Gao *et al*., 2009). Thus, the PD-L1/PD-1 axis is a key regulator of T cell exhaustion in cancer.

$CD8^+$ T cells are assisted in their tumour killing function by $CD4^+$ T helper 1 cells, which are known to secrete interleukin-2 (IL-2) and interferon-gamma (IFN-γ). High numbers of $CD8^+$ T cells correlate with good prognosis (Fridman *et al*., 2012).

Tumours can avoid immune destruction by inhibiting the activity of $CD8^+$ T cells by attracting $CD4^+$ $FoxP3^+$ $CD25^+$ T cells (Tregs) into the TME (reviewed in Vignali *et al*., 2008). Tregs are important for suppressing the immune system to prohibit aberrant inflammation preventing autoimmune and chronic inflammatory conditions, as well as suppressing the anti-tumour immune response (reviewed in Vignali *et al*., 2008). For example, Tregs can release inhibitory cytokines such as IL-10 and transforming growth factor β (TGF-β) which suppress the tumor killing activity of effector T cells (reviewed in Jiang and Zhu, 2015). Additionally, Tregs can be recruited to the tumour by the tumour stroma secreting the cytokine CXCL10, which leads to an immunosuppressive and pro-tumourigenic microenvironment (Lunardi *et al*., 2014). Thus, Tregs can be modulate $CD8^+$ T cells by multiple mechanisms to promote an immunosuppressive TME.

Further immune cells that contribute to avoiding immune destruction include tumour associated macrophages (TAMs) and myeloid-suppressor cells. TAMs are macrophages that collect in the TME and are stimulated to enter tumours by a number of chemical signals including M-CSF, CCL2, VEGF and angiopoietin-2 (reviewed in Jiang and Zhu, 2015). The latter recruits blood monocytes which travel through tumour vessels and encourage polarisation of macrophages into the TME (reviewed in Jiang and Zhu, 2015). TAMs are pro-tumourigenic and are correlated with poor prognosis in patients (reviewed in Lewis and Pollard, 2006). The build-up of myeloid-suppressor cells is an established mechanism of stimulating tumour formation by suppressing T cell activation and inducing T cell exhaustion (reviewed in Jiang and Zhu, 2015).

Tumours can display a desmoplastic reaction that is characterised by a surplus of connective tissue surrounding an invasive tumour (reviewed in DeClerk, 2012). Desmoplasia is caused by an increased synthesis of ECM proteins, including collagen, secreted by cells such as myofibroblasts (reviewed in Barksy *et al*., 1984). It is believed that this provides a barrier for $CD8^+$ T cell infiltration, prohibiting anti-tumour immune responses (Jiang *et al*., 2016). Thus, the immune cells and ECM in the TME can contribute to avoiding immune destruction.

1.1.10 Evading growth suppressors

In healthy cells there are many anti-proliferative signals that can stop unnecessary and uncontrolled proliferation which can lead to cancer. Anti-growth signals can cause proliferation block by being forced into the quiescent state (reviewed in Hanahan and Weinberg, 2000). In this state the proliferation cycle can be reactivated if a cell is stimulated by certain extracellular stimuli. The RB gene is often mutated in human cancer especially retinoblastoma, osteosarcoma and small cell lung cancer (SCLC) (reviewed in Engel *et al*., 2015) and is a key growth suppressor in its phosphorylated form (pRB). pRB perturbs proliferation by sequestration and modulation of the function of the E2F transcription factor which controls genes that regulate the G_1 to S

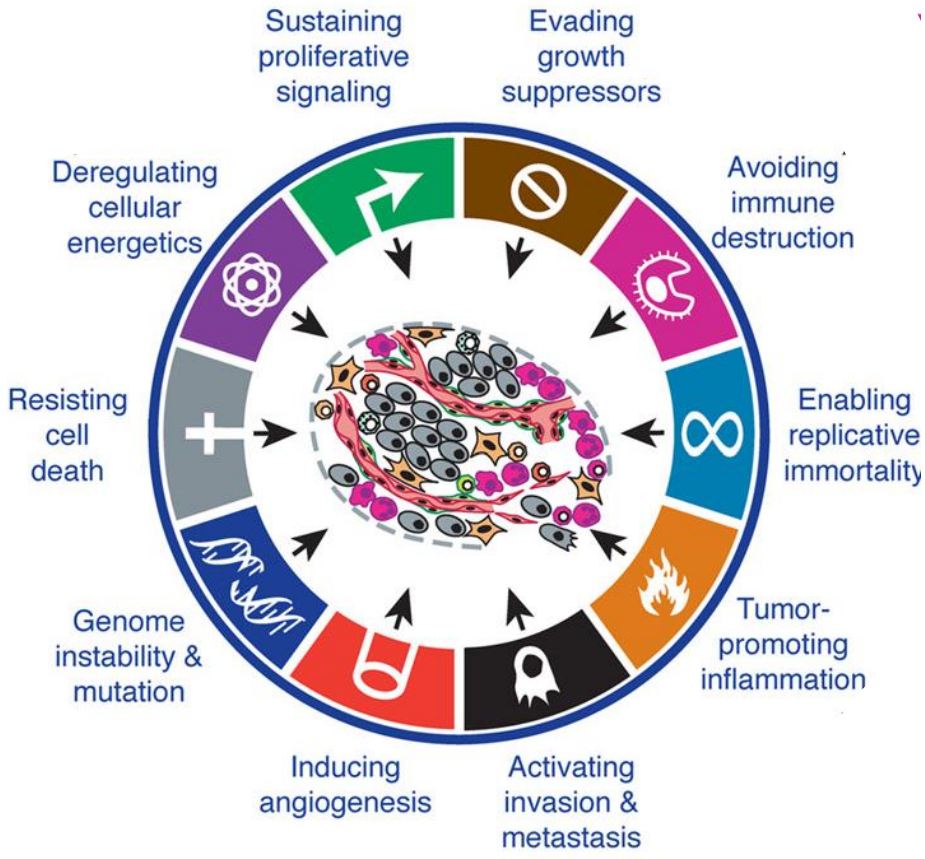


Figure 1.1 | The hallmarks of cancer. Tumours use a multitude of mechanisms to survive including resisting cell death, enabling replicative mortality, inducing invasion/metastasis, evasion of growth suppressors, induction of angiogenesis and sustainment of proliferative advantage. Emerging hallmarks of cancer include establishing tumour-promoting inflammation and aversions of immune destruction. Dysregulated epigenetics and genome instability can drive these pro-tumour processes. Figure from Hanahan and Weinberg, 2011.

phase of the cell cycle (reviewed in Hanahan and Weinberg, 2000). Another example, is P53 which can stimulate G_1 arrest by regulating the expression of P21, that is known to inhibit cyclin E-CDK2 (reviewed in Harris and Levine, 2005). Cyclin E-CDK2 modulates the ability of the RB protein to repress E2F1 activity and this promotes the transcription of genes involved in preparation of the cell to progress from G_1 to S phase of the cell cycle (reviewed in Harris and Levine, 2005).

1.2 Targeting cancer

For reasons mentioned above, cancer is a complex disease that is difficult to treat due to the heterogeneity of tumours (consisting of a complex mixture of stromal and cancer cells), acquisition of drug resistance mechanisms and the requirement for combination therapies that may target non-cancer cell components of the tumour (reviewed in Hanahan and Weinberg, 2011). Radiotherapy and surgery are ultimately the most effective treatments. Whether a patient gets surgery depends on the stage of their cancer, whether it has metastasised, their general health and the location of the tumour (Cancerresearchuk.org, 2019a). Surgery involves the excision of the tumour and is often used in combination with radiotherapy or chemotherapy (reviewed in Wyld, Audisio and Poston, 2015). Radiotherapy uses ionising radiation that can directly kill cancer cells or result in genetic changes in the cell that leads to cell death. Although there is some damage to healthy tissues, cancer cells do not repair themselves as efficiently after radiation damage as healthy cells (reviewed in Baskar *et al*., 2012). There are different options for delivery of radiation to the tumour such as delivering it externally or in the form of radioactive capsules inserted near the tumour site (reviewed in Baskar *et al*., 2012). There are also different types of radiation beams used including x-rays, gamma rays, electron beams, or proton beam therapies (reviewed in Baskar *et al*., 2012). Proton beam therapy has received much attention and promise as a superior form of radiation therapy as it can deliver more targeted high energy particles to the tumour site, with minimal damage of surrounding tissue (reviewed in Tian *et al*., 2018).

However, radiation or surgery may not eradicate the tumour, especially in the case of late stage tumours. In such cases, systemic treatments might be required alone or in

combination with radiotherapy or surgery. Chemotherapy is the most common systemic treatment for cancer and most work in a similar manner (reviewed in Palumbo *et al*., 2013). Its success is based on the fact that cancer cells have a higher mitotic rate and constant requirement for nutrients compared to non-malignant cells (reviewed in Palumbo *et al*., 2013). These drugs are often non-specific so can kill healthy cells as well as cancer cells and therefore have a number of toxic side effects that may be difficult to manage (e.g. nausea, vomiting, hair loss, fatigue). Conventional chemotherapeutics include alkylating agents (e.g. cisplatin, oxaplatin), antimetabolics (e.g. methodextrate, fluorourcil), anti-tumour antibiotics (e.g. doxorubicin, bleomycin), topoisomerase inhibitors (e.g. etoposide) and microtubule stabilizers (e.g. paclitaxel, docetaxel) (reviewed in Palumbo *et al*., 2013). However, chemotherapy and many new cancer therapies can exhibit problems with intrinsic (i.e. characteristics already present in the tumours that confer resistance to drug prior to treatment) and acquired resistance (i.e. resistance to a drug that develops during treatment). There are many mechanisms by which resistance can occur, including mutations in genes controlling apoptosis (i.e. P53), activation of molecules downstream of the target molecules, and activation of proteins on the plasma membrane that control efflux of chemicals from the cell (reviewed in Choi, 2005).

Although conventional chemotherapy generally can be successful, some patients do not respond to cytotoxic drugs or refuse to take them due to the toxic side effects. New therapies are being increasingly developed that are more targeted, including those that target certain pathways dysregulated in cancer. These include drugs targeting the RAS-RAF-MEK-MAPK pathway, which is a major pathway that mediates growth factor-induced proliferation. The *BRAF* gene encodes an upstream effector kinase, and has an activating mutation in 60% of melanoma and a lower frequency of mutation in a number of different cancers (Davies *et al*., 2002). Since this discovery there has been much focus in designing inhibitors to target BRAF in melanoma, but this has unfortunately been met with problems of resistance due to re-activation of the MAPK pathway (reviewed in Aroznewna and Wellbrock, 2017).

As previously described, inflammation is a key driver of cancer growth (see **section 1.1.7**), and there has been much interest in manipulating the immune system in order to stimulate an anti-tumour immune response. Cancer immunotherapies are a form of treatment that attempts to trigger the immune system's own natural defences to fight cancer (reviewed in Mellaman *et al*., 2011). Immunotherapies can come in various forms including targeted antibodies, cancer vaccines, adoptive cell transfer and checkpoint inhibitors (reviewed in Mellaman *et al*., 2011).

Adoptive cell therapy is where immune cells taken from the patients are engineered to stimulate anti-tumour activity of the T cells and then inserted into the patient (reviewed in Sharpe and Mount, 2015). For example, CAR T cell therapy is where T cells are genetically engineered to express chimeric antigen receptor (CAR), which combines antibody specificity with T-cell activator function (reviewed in Sharpe and Mount, 2015). CARs are composed of an antigen-binding domain (usually directed to a protein on the tumour that is over expressed), an anchor to attach the CAR to the T cell and an internal signalling domain to mediate signal transduction into the T cells (reviewed in Sharpe and Mount, 2015). CD19 is expressed during B cell development and therefore is expressed in virtually all B-cell cancers and CAR targeting the CD19 protein on B cells is the most investigated CAR-T cell therapy thus far (reviewed in Maude *et al*., 2015). CD19-CAR T cells have been reported to cause complete remission in 90% of acute lymphoblastic leukaemia patients in one ground-breaking study (Maude *et al*., 2014). However, CD19-CAR T cell therapy causes patients to develop cytokine release syndrome (CRS) which is an inflammatory disorder caused by excessive release of cytokines (reviewed in Maude *et al*., 2015). This has been shown to be reversed with an anti-IL-6 receptor antibody tocilizumab in patients with severe side effects (Maude *et al*., 2014).

The use of vaccines to prevent cancers of viral origin such as hepatitis B virus and human papillomavirus (HPV) have had considerable success (reviewed in Mellaman *et al*., 2011). However, developing therapeutic vaccines for existing cancers are more problematic for multiple reasons: cancer cells suppress the immune system; large tumours do not respond well to vaccines; when patients are old or sick they have a

weakened immune system (Cancer.net, 2018). There is one FDA approved therapeutic vaccine for prostate cancer, Sipuleucel-T, which is where antigen presenting cells (APCs) which stimulate the $CD4^+$ helper T cell response and $CD8^+$ T cells, are taken from the patient and incubated with a recombinant fusion protein containing prostatic acid phosphatase (PAP), which is commonly expressed on prostate tumours and granuloycte-macrophage colony stimulating factor (GM-CSF), an immune activator (reviewed in Anassi *et al*., 2011). This process activates the APCs and stimulates them to attack PAP-expressing prostate cancer cells when implanted back into the patients (reviewed in Anassi *et al*., 2011). Sipuleucel-T has been shown to prolong overall survival in patients with metastatic castration-resistant prostate cancer, with manageable side effects including chills, fever and headache (Kantoff *et al*., 2010).

As previously described, tumours use multiple mechanisms to evade immune mediated tumour clearance such as expression of the PDL1 receptor on tumours which binds to the PD1 receptor in cancer killing T cells 'exhausting' the immune response. As previously mentioned, overexpression of PDL1 on tumours is a predictor of poor prognosis (Gao *et al*., 2009) and antibodies that target the PD1/PDL1 axis is a valid therapeutic option in cancer therapy (reviewed in Mellaman *et al*., 2011). Indeed, this therapy has been shown to induce tumour regression and stabilisation of a number of advanced cancers (Brahmer *et al*., 2012). Antibodies against the immune checkpoint molecule CTLA4, that is expressed on T cells, and when activated dampens down the immune response, has shown to improve overall survival in patients with metastatic melanoma (Hodi *et al*., 2010). Side effects of both medications were immune-related conditions that were reported to be manageable (Hodi *et al*., 2010, Brahmer *et al*., 2012). That said, many cancers do not respond well to immuno-therapy such as pancreatic ductal adenocarcinoma (PDAC), which is at least in part cause by a strong 'desmoplastic reaction' (see **section 1.2.9**) (Jiang *et al*., 2019). Thus, immunotherapy could potentially be an effective treatment for cancer with manageable side effects. However, much work will be required to understand the mechanism of resistance in some cancers to immunotherapies and how to overcome this.

1.3 Introduction to FAK

FAK is a non-receptor kinase that has been associated with almost all of the hallmarks of cancer and as such, in this next section I will focus on providing some background to the structure and function of FAK. Then I will proceed to present the data identifying FAK as a key regulator of cancer-related hallmarks.

1.3.1 FAK discovery and early studies

FAK is a ubiquitously expressed 125kD protein which was discovered in 1992 as a substrate of the viral SRC oncogene and as a highly tyrosine-phosphorylated protein that localised to cell adhesion sites called focal adhesions (Schaller *et al*., 1995). Focal adhesions are large macromolecular sub-cellular structures formed when the ECM contacts cell-adhesion molecule heterodimeric receptors called integrins (reviewed in Mitra *et al*., 2005). Integrins are a major family of plasma membrane receptors that consist of heterodimers of non-covalently associated single pass-type I transmembrane protein α and β subunits (Harburger and Calderwood, 2009). These cell adhesion receptors can sense the surrounding environment thereby providing information about the location, cell adhesion state and surrounding ECM molecules (outside-in signalling) or control the affinity for ECM ligands by changing the conformation of their extracellular domains (inside-out signalling) (reviewed in Hynes, 2002). Both modes of signalling require focal adhesion protein complex formation around the cytoplasmic tails of integrins to generate large macromolecular assemblies that can consist of over 100 proteins, and FAK is a crucial focal adhesion kinase component operating downstream of integrins (reviewed in Mitra *et al*., 2005).

The first mouse model to study FAK function was made in 1995 by Ilic and colleagues who found that FAK-deficient mice generated by gene targeting died due to mesoderm defects during development. These embryos displayed reduced motility *in vitro*, which could explain developmental defects, as cell motility is important for embryonic development (Ilic *et al*., 1995). The mesodermal developmental defect could also be due to loss of FAK mediated suppression of P53-mediated apoptosis (Ilic *et al*., 1998). The FAK-deficient phenotype was very similar to that of a fibronectin-deficient

phenotype, suggesting that FAK is a key player in mediating fibronectin-integrin signalling at this stage of development (IIic *et al*., 1995).

This work was followed by studies identifying that FAK binds to a number of focal adhesion complex proteins including talin (Chen *et al*., 1995), Paxillin (Hildebrand *et al*., 1995), HIC-5 (Nishiya *et al*., 2001), ezrin (Poullet *et al*., 2001) and p130CAS (Polte and Hanks, 1995). FAK has was shown to interact with kinases such as the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K), which was shown to be enhanced by stimulation with growth factors such as VEGF (Qi *et al*., 2001) and platelet derived growth factor (PDGF) (Chen and Guan, 1994). These findings along with other studies showing that FAK functions upstream of GRB2/RAS/MAPK signalling indicated a key role of FAK in growth factor signalling (Schlaepfer *et al*., 1994). This work fuelled many structural and functional studies in the next two decades using advanced techniques such a proteomics, X-ray crystallography and fluorescence resonance energy transfer (FRET) to gain detailed mechanistic insight into the structure and function of FAK.

1.3.2 Proteomics identified that FAK is a key component of integrin adhesion complexes (IACs)

IACs are highly complex and dynamic structures that are functional links to the cytoskeleton and other cell compartments and cellular processes. The use of proteomics has been highly informative in determining the composition and core components of IACs (reviewed in Byron and Frame, 2016). Proteomics is the study of the entire set of proteins expressed in a cell at a particular time. Interactome proteomics is the study of proteins binding to a target protein that uses antibodies that bind to the target protein, which allows purification of any potential interactors with the target protein. Furthermore, cells can be fractionated which allows the study of the interactome of a particular protein in a particular cell compartment (e.g. the nucleus). Bioinformatic analysis, using programs such as STRING or Ingenuity Pathway analysis (IPA) integrates a number of proteomics datasets to identify all known interactions within the proteome to allow network construction.

Interactome proteomics is a very informative approach to study interaction partners and functions of a particular protein, but it can be difficult to determine the key components of these networks as many interactome data have combined interaction information from datasets performed under different conditions and cell types (reviewed in Byron, Humphries and Humphries, 2012). With this in mind, Horton *et al*., 2015 used bioinformatics to determine the common protein components in 5 different IAC proteomic datasets to define the core components of IACs (**Figure 1.2A**). This resulted in a consensus adhesome of 60 proteins, with 4 clear structural nodules – α-actinin-zyxin-VASP, talin-vinculin, FAK-paxillin and ILK-PINCH-Kindlin (Horton *et al*., 2015, **Figure 1.2B**). Thus, integration of different proteomic

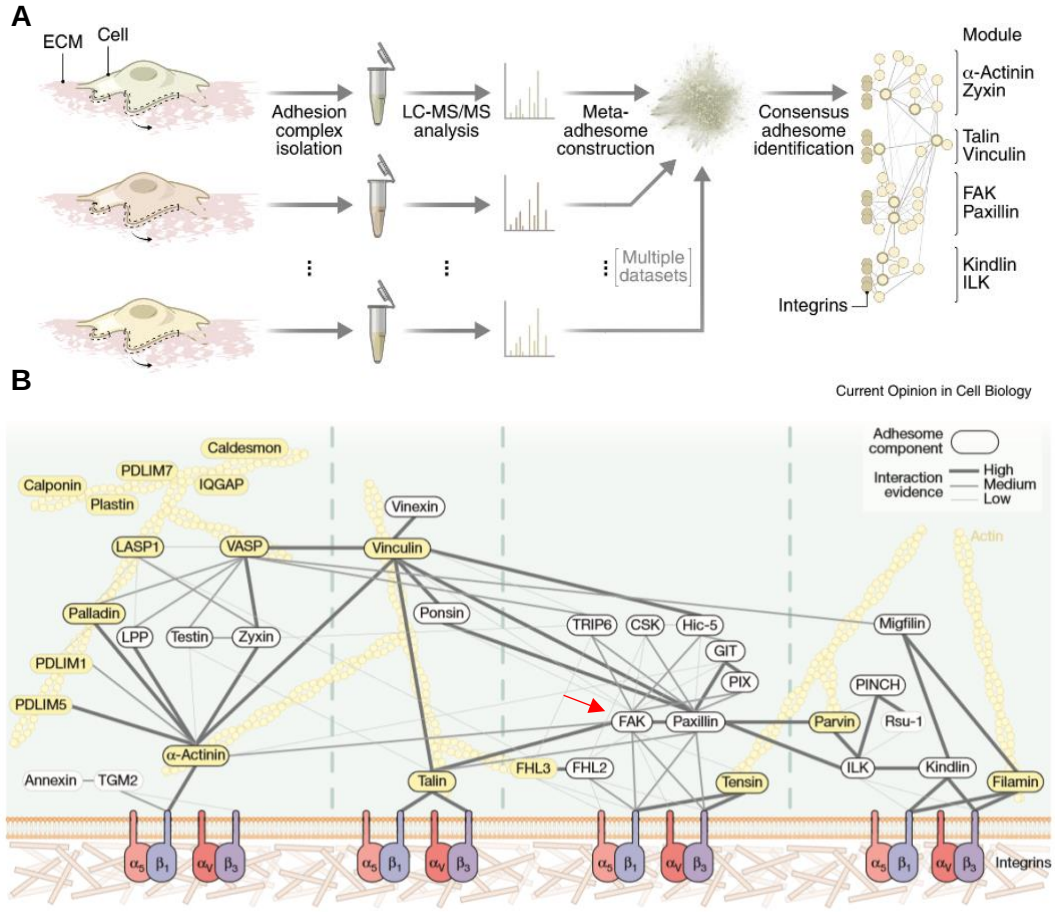


Figure 1.2 | The consensus adhesome. A) Experimental design used by Horton *et al*., 2015 in order to define the core IAC components and 4 nodules of the 'consensus adhesome' on the far right. B) Protein-protein interactions between core components of the consensus adhesome are scored based on experimental evidence of that interaction, indicated by the thickness of the lines. Thick black border indicates components of the consensus adhesome. Yellow nodes indicate proteins present in the consensus adhesome as defined in (Horton *et al*., 2015) that bind to actin, while white nodes indicate consensus adhesome components that do not bind actin. Actin (unlabelled yellow circles) is depicted for illustrative purposes but is not a component of the consensus adhesome. Arrow points to FAK. Figure in A from Byron and Frame, 2016. Figure B edited from Horton *et al*., 2015.

datasets has robustly identified that FAK is a key component of IACs and is temporally regulated in these complexes.

1.3.3 FAK general structure

FAKs structure facilitates its scaffolding, kinase and nuclear localisation, which in turn regulates physiological and cancer-associated processes. FAK is composed of a N-terminal FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domain, C-terminal focal adhesion targeting domain (FAT), three proline rich regions and a central kinase domain (reviewed in Frame *et al*., 2010, **Figure 1.3**). In this section, I will discuss the FAK structural domains individually with particular focus on key publications using advanced techniques such as fluorescence energy resonance energy transfer (FRET), NMR, and X-ray crystallography that were used to study the structure of FAK.

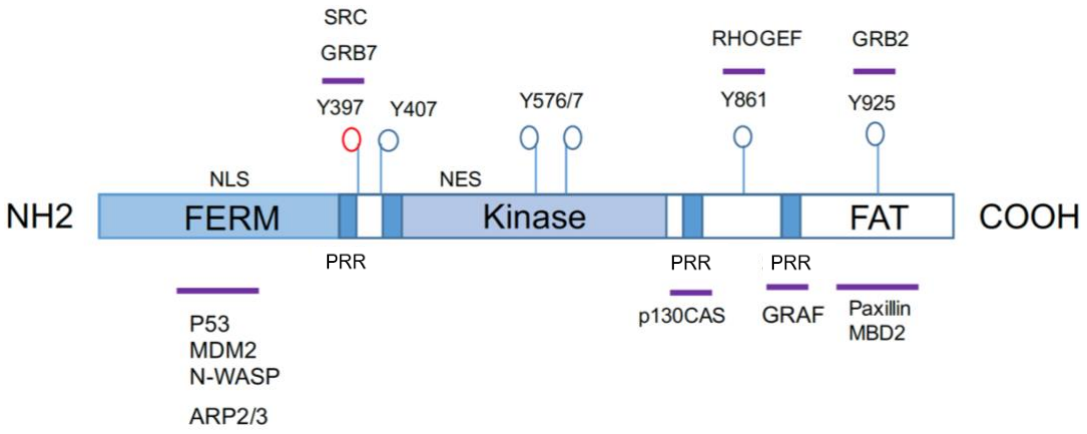


Figure 1.3 | FAK general structure. FAK consists of an N-terminal FERM domain, a central kinase domain and a C-terminal focal adhesion targeting (FAT) domain. It has a number of phosphorylation sites within its structure which regulate its binding to various protein partners as depicted in the figure. Kinase activation of FAK requires autophosphorylation site at tyrosine 397 (red). Proline rich regions (denoted as PRR) which bind to SRC homology 3 Domain (SH3)-domain containing proteins such as p130CAS are indicated. Regions of FAK where protein interaction partners bind are indicated.

1.3.4 FAT domain

Early studies showed that the C-terminal portion of FAK is required for FAKs localisation at focal adhesions, while abolition of the N-terminal or FAK's kinase activity had no effect on its focal adhesion localisation (Hildebrand *et al*., 1993). Crystallography and NMR studies showed that the FAT domain consists of 4 helices that form a right-turn elongated bundle that is stabilised by hydrophobic interactions (Lui *et al*., 2002a, Hayashi *et al*., 2002). This domain binds scaffolding proteins such as Paxillin, potentially targeting it to focal adhesions (Hidlebrand *et al*., 1995). It was shown by mutagenesis that Paxillin binding involves two hydrophobic patches on opposite faces of the bundle and the authors proposed a model in which two LD motifs of Paxillin adopt amphipathic helices that insert into the hydrophobic core of the FAT domain creating a six helix bundle (Hayashi *et al*., 2002). The FAT domain is regulated by phosphorylation of Y926 that binds the SH2 domain of GRB2 (Schlaepfer *et al*., 1994). The solution structure showed it is not possible for GRB2 and Paxillin to bind at the same time as the binding SH2 (SRC homology 2) and leucine-aspartate repeat (LD) motifs overlap (Lui *et al*., 2002a). Therefore, GRB2 binding may cause release of FAK from focal adhesions, allowing it to participate in downstream signaling (Liu *et al*., 2002a). Interestingly, HIC-5 has been shown to bind FAK at Paxillin-binding motifs (Fujita *et al*., 1998) and further studies showed that HIC-5 inhibited integrin-induced cell spreading on fibronectin by competitive binding of FAK binding with paxillin (Nishiya *et al*., 2001). This would prevent downstream integrin-mediated signal transduction and it is interesting to speculate whether this could modulate differential pathways that are not dependent on FAK-Paxillin binding.

It has been controversial whether Paxillin actually recruits FAK to focal adhesions. FAK has been shown to bind to β-integrin tails (Schaller *et al*., 1995) but the validity of these findings *in vivo* has been questioned. FAK binds the integrin binding protein talin (Chen *et al*., 1995) and FAK is necessary for talin recruitment to nascent and spreading but not mature focal adhesions (Lawson *et al*., 2012). However, talin is required for FAK localisation to mature adhesions, suggesting that talin and FAK may be required for recruitment of each other to different focal adhesions under different

circumstances (Serrels and Frame, 2012). Thus, the protein partner that targets FAK to focal adhesions remains to be determined.

The C-terminal of FAK can be expressed as a truncated FAK protein called FRNK, which acts as an endogenous inhibitor of FAK function by blocking focal adhesion formation on fibronectin (Richardson and Parsons, 1996). A study found that FRNK expression was restricted to the post-natal period but then decreased dramatically in adult smooth muscle cells (Taylor *et al*., 2001). Interestingly, FRNK expression was upregulated during balloon-induced carotid artery injury (Taylor *et al*., 2001). Together these results suggest expression of FRNK may be important in regulating vascular development and remodelling (Taylor *et al*., 2001).

1.3.5 FERM domain

Four-point-one ezrin radixin moesin (FERM) domains are present in a number of mammalian proteins (reviewed in Frame *et al*., 2010). The functions of FERM domain containing proteins suggest they link the plasma membrane with the cytoskeleton by direct protein binding or association with phosphoinositides (reviewed in Frame *et al*., 2010). Structural and cell biology findings have made the FAK FERM domain one of the most well studied FERM domain containing proteins (reviewed in Frame *et al*., 2010). It has been identified that the FERM domain of FAK contains three structural modules reminiscent of a clover leaf (denoted F1, F2 and F3) (Ceccarelli *et al*., 2006). The authors noted that the FERM domain of FAK is distantly related to other FERM proteins and the divergent structure may be indicative of a different function (Ceccarelli *et al*., 2006). Prior to this study, it was shown that FAK's FERM domain could interact with the kinase domain of FAK and inhibit its function. The N-terminus of FAK acts in *trans* (intermolecular) to inhibit FAK phosphorylation when expressed *in vivo* or *in vitro* (Cooper *et al*., 2003). The FERM domain is essential for the regulation of FAK kinase activity (see below) but also has established roles as a scaffold in protein-protein interaction complexes (reviewed in Frame *et al*., 2010). It was shown that the FERM domain is likely important for growth factor signalling as it can bind the hepatocyte growth factor receptor MET, epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) (Chen *et al*.,

2011). MET was shown to phosphorylate FAK on Y194, which was predicted to cause a conformational change in the FERM domain which disrupts the intramolecular inhibitory interaction between the FERM and kinase domain, leading to FAK activation (Chen *et al*., 2011).

1.3.6 FAK kinase domain

The FAK kinase structure has a bilobal structure (denoted the N-terminal and C-terminal lobes) reminiscent of other protein kinases. The N- terminal lobe contains 5 stranded anti-parallel β-sheets, while the larger C-terminal lobe is mostly α-helical and contains an activation loop (Nowakowski *et al*., 2002). When integrin and growth factor receptors are activated, FAK becomes rapidly phosphorylated at Y397, a site of autophosphorylation, enhancing its catalytic activity (Schaller *et al*., 1994). This creates a SH2 (Src homology 2) binding site for SRC (Calalb *et al*., 1995) and SRC then phosphorylates Y576 and Y577 and this stimulates maximal catalytic activity of FAK (reviewed in Mitra *et al*., 2005). Detailed structural insight into the mechanism by which FAK is regulated came from a study by Lietha and colleagues where they determined the mechanism by which FAK auto-regulates its kinase activity (Lietha *et al*., 2008). They determined the autoinhibited structure of the FAK FERM domain, kinase linker and kinase domain, finding that residues 567-573 in the activation loop extend near the FERM domain, partially blocking the catalytic cleft (**Figure 1.4A**). Furthermore, the FERM domain makes extensive interactions with the kinase C- lobe when FAK is autoinhibited impeding access to the catalytic active site and phosphorylation by the tyrosine kinase SRC (**Figure 1.4A**). Finally, it was found that the autophosphorylation of Y397 is inhibited, as the kinase linker region where it resides is incorporated into the β-sheet in the FERM domain (**Figure 1.4A**). They later found that in the structure of the active kinase that the activation loop is allowed free access to the active site (Lietha et al., 2008). Overlaying the active structure over the inactive structure of the kinase domain indicated that the pY576 in the phosphorylated activation loop collides with the FERM domain determined in the inactive FERM-linker-kinase structure (**Figure 1.4B**). This suggests that the active form does not conform to the position of the FAK FERM domain in the inactive state and therefore

the FERM domain is not excluding the kinase domain in the active conformation (Lietha *et al*., 2008).

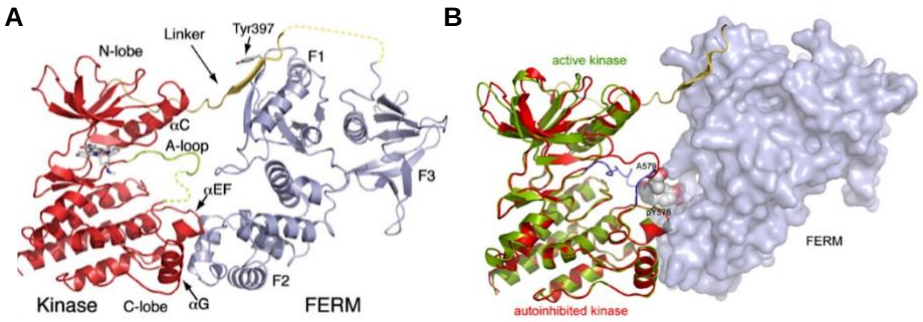


Figure 1.4 | Structure of autoinhibited FAK. A) The structure of the autoinhibited kinase domain with FERM, linker and kinase regions in ribbon representation. The FERM domain (blue) binds the kinase domain (red). Main interaction is between the F2 FERM domain binds the kinase C-domain. The Y397 of the kinase domain is located between the kinase N-lobe and the FERM F1 lobe in the autoinhibited structure. Dashed lines indicate the disordered regions in the kinase linker. B) Overlay of inactive and active FAK kinase structures. The structure of the autoinhibited structure is depicted with the FERM domain in surface representation. The kinase and linker structures are in ribbon representation. The inactive and active kinase were imposed based on their C-lobe. The pY576 and A579 in the activated kinase are represented by space filling representation. Figures taken from Lietha *et al*., 2008.

Based on these findings, a model of FAK kinase autoinhibition and activation was proposed. Lietha and colleagues suggested that in the autoinhibited structure, the FERM domain blocks the FAK active site and sequesters the Y397 and activation loop (**Figure 1.5**, left). Then, an activating molecule which is thought to be a lipid (see below) binds to the F2 FERM domain and then disrupts the kinase domain interaction with the FERM domain (**Figure 1.5**, middle) (Lietha *et al*., 2008). Disassembly of the autoinhibited conformation allows FAK to autophosphorylate Y397, which then allows docking of the SRC kinase SH2 domain to the pY397 and SRC SH3 domain to the Proline rich (PxxP) in the linker regions (Lietha *et al*., 2008). SRC then phosphorylates Y576 and Y577 in the activation loop of FAK, thereby stimulating full catalytic activity (**Figure 1.5**, right) (Lietha *et al*., 2008).

The SRC-FAK complex then phosphorylates and recruits a number of other signalling proteins including p130CAS, p85 regulatory subunit of PI3K and GRB2 (reviewed in Frame *et al*., 2010). FAK can be phosphorylated at many other sites including serine (722, 732, 843, 910) and tyrosine (397, 407, 576, 577, 861, 925) residues. However, it has been shown my mass spectrometry that there are an additional 25 uncharacterised phosphorylation sites (15 serine, 5 threonine and 5 tyrosine residues) in the FAK structure, suggesting there may be many more mechanisms of FAK regulation yet to be identified (Grigera *et al*., 2005).

In a revolutionary study, Goni and colleagues used a combination of FRET and molecular dynamic simulation to study the link between integrin signalling and FAK activation. Their findings concluded that FAK interacts with phosphatidylinositol 4,5-bisphosphate (PIP_2) in a basic patch (KAKTLRK) in the FERM domain and binding of PIP_2 mediates FAK autophosphorylation (Goni *et al*., 2014). This work also showed by electron microscopy that addition of PIP_2 induces FAK clustering, but mutation of the basic patch in the FERM domain perturbs cluster formation (Goni *et al.*, 2014). Furthermore, FRET studies showed that PIP_2 stimulates partial opening, while addition of SRC was required to induce full domain opening of the FAK FRET sensor (Goni *et al*., 2014). They proposed a model in which PIP_2 levels generated by plasma membrane-recruited PIP5Kγ induces partial opening of the FAK structure exposing

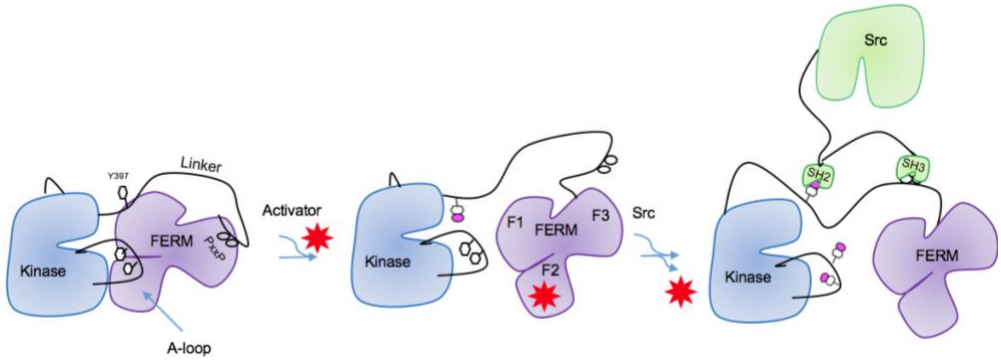


Figure 1.5 | Model of FAK kinase activation. On the left, the FERM domain blocks the active site of the kinase and sequesters the activation loop and Y397 phosphorylation sites. Lietha and colleagues believe the PxxP motif present in the linker binds to the F3 lobe of the FERM domain, based on previous findings (Ceccarelli *et al*., 2006). An uncharacterised activating stimulus, later inidicated to be a lipid (Goni *et al*., 2014), is required to displace the FERM domain by competitive binding to the F2 FERM surface. FAK's kinase autophophorylates Y397 and allows access for Src kinase to the linkers. Src then phosphorylates the Y576 and Y577 of the activation loop residues, which stimulates full catalytic activity of FAK kinase. Figure is based on the findings presented in Lietha *et al*., 2008.

the auto-phosphorylation site of FAK and allowing auto-phosphorylation. SRC then binds pY397 and phosphorylates Y576/Y577 in the activation loop that stimulates full FAK catalytic activation (Goni *et al.*, 2014). The kinase domain is then released from the membrane clustered FERM domain (Goni *et al*., 2014).

Despite these structural studies elucidating the mechanism of FAK activation very few substrates of FAK have been identified. However, FAK's kinase activity is very important for its role in physiological and cancer-related processes and as such, FAK kinase inhibitors are in clinical trials (sees **section 1.6.1)**. It is likely that based on the studies presented in Lietha *et al*., 2008 and Goni *at al*., 2014 that FAK's kinase activity is required for a conformational change that differentially regulates its signalling functions and interaction with protein partners that can regulate key cancer processes.

1.4 FAK conventional functions

In this section, I will speak about FAK's biological functions before proceeding to discussing its role in cancer.

1.4.1 Motility

FAK has been shown to be a key regulator of cell motility and this is controlled by various pathways (reviewed in Mitra *et al*., 2005). The best characterised is via the binding and phosphorylation of p130CAS by SRC/FAK (Cary *et al.*, 1998). Phosphorylation of p130CAS allows it to associate with the adaptor protein CRK and this complex regulates membrane ruffling and cell migration by regulating DOCK180 and the small GTPase RAC1 (Cho *et al*., 2000, Cheresh, 1999). FAK can also stimulate cell migration by binding PI3K (Reiske *et al*., 1999) and the adaptor molecular, GRB7 (Han *et al*., 2000). Thus, FAK is a key signal integrator functioning downstream of integrins to regulate cell migration.

Assembly and disassembly of actin-based structures such as lamellipodia and invadopodia require the Rho family of small guanosine triphosphatases (GTPase) such as RHOA, CDC42 and RAC1 (reviewed in Lawson and Ridley, 2018). FAK has been shown to suppress RHO activity in order to stimulate focal adhesion turnover (Ren *et*

al., 2000). FAK activates RHO GTPases by binding guanine nucleotide exchange factors (GEFs) such as p190RHOGEF (Zhai *et al*., 2003) and this interaction is important for regulation of focal adhesion formation during fibronectin-stimulated cell motility (Lim *et al*., 2008a). FAK also binds to GTPase activating proteins (GAPs), including GRAF which is a GAP for RHO and CDC42 (Hildebrand *et al*., 1996) and ASAP1 which is a GAP for the ARF subfamily of GTPases (Liu *et al*., 2002b). Thus, FAK is involved in the coupling regulation of RHO GTPases.

FAK has been shown to be involved in formation of actin-based structures by directly binding to ARP2/3 to enhance ARP2/3 dependent actin polymerization, leading to lamellipodia formation (Serrels *et al*., 2007). Furthermore, FAK can phosphorylate N-WASP, a CDC42 downstream effector, which allows its cytoplasmic translocation where it activates the ARP2/3 complex and facilitates actin polymerization at the leading edge of migrating cells (Wu *et al*., 2004). FAK can also stimulate directional migration by complexing with a scaffolding protein, RACK1 and c-AMP degrading phosphodiesterase PDE4D5 at nascent adhesions (Serrels *et al*., 2011). Thus, FAK is a key regulator of cell migration by binding to adaptor proteins and actin-regulators.

1.4.2 Angiogenesis

Cell-ECM adhesion promotes migration of endothelial cells (EC) at various stages of the angiogenic process (reviewed in Rizzo, 2004). FAK is important for EC migration *in vitro* and *in vivo* (Peng *et al*., 2004). It has been shown to do this by regulating the activity of VEGF which induces the formation of new blood vessels (Peng *et al*., 2004). Studies have shown VEGF-induced activation of PI3K was dependent on FAK and this led to VEGF-A-induced migration of porcine aortic endothelial cells expressing VEGF receptor-2 (VEGFR-2) (Qi and Claesson-Welsh, 2001). Additionally, FAKs nuclear localisation (see **section 1.4**) and its kinase activity have shown to direct VEGFR2 gene transcription to regulate endothelial cell proliferation and migration (Sun *et al*., 2018). An additional regulator of angiogenesis, angiopoietin-1, has been shown to increase FAK phosphorylation during angiogenesis *in vitro* (Kim *et al*., 2000) and a SRC/FAK/integrin αvβ5 complex has been shown to be essential in VEGF-stimulated angiogenesis *in vivo* (Eliceiri *et al*., 2002).

1.4.3 Proliferation

FAK has been linked to regulation of cell proliferation in many contexts (Zhao *et al*., 1998, Lim *et al*., 2008b, Sun *et al*., 2018). FAK has been shown to regulate expression of cyclin D1 levels to promote G_1 cell cycle progression (Zhao, Reiske and Guan, 1998) by modulating the activities of key transcription factors. For example, FAK/integrin signalling regulates the binding of the transcription factor EtsB on the cyclin D1 promoter (Zhao, Pestell and Guan, 2001). Thus, FAK regulates proliferation, at least in part, by regulating transcription factor function to regulate the expression of genes involved in the regulation of proliferation, such as cyclin D1.

1.5 FAK is overexpressed in cancer and is a key regulator of cancer-associated processes

It is well established that FAK is highly expressed in a number of human tumours including squamous cell carcinoma (SCC) (Agochiya *et al*., 1999), breast and colorectal (Owens *et al*., 1995), PDAC (Jiang *et al*., 2016) and this can be through increases in gene copy number and/or protein expression (Sulzmaier *et al*., 2014, Agochiya *et al*., 1999). FAK has also shown to drive tumourigenesis *in vivo* in various mouse models such as in breast (Lahlou *et al*., 2007), SCC (Maclean *et al*., 2004), and colorectal cancer (Ashton *et al*., 2010). Furthermore, increased FAK expression correlates with invasive potential (Owens *et al*., 1995) and poor patient outcome (Golubovskaya *et al*., 2014). As such, a large body of evidence supporting FAK's importance in cancer has led to the initiation of several Phase I and Phase II clinical trials using FAK kinase inhibitors (see **section 1.6**).

FAK has been shown to control and co-ordinate many cancer-associated cellular processes including cell invasion (Owens *et al*.,1995), cell proliferation and survival (Lim *et al*., 2008b), angiogenesis (Tavora *et al*., 2010), resisting apoptotic signals (Zhang *et al*., 2004) and avoiding immune destruction (Serrels *et al*., 2015). Hence, FAK is an important mediator of many cellular processes that are linked to the hallmarks of cancer. Therefore, I will describe each hallmark associated with FAK and the role of FAK in this hallmark in detail below.

1.5.1 FAK is a key regulator of invasion and metastasis

Early studies identified that FAK expression was correlated with the invasive capacity of tumours (Owens *et al*., 1995), linking it to the cancer hallmark activating invasion and metastasis. Mechanistic studies have found that FAK regulates focal adhesion dynamic assembly and turnover to promote invasion in breast cancer by regulating the turnover of invadopodia which are actin rich cell membrane protrusions essential for invasion (Chan *et al*., 2009). Studies have found expression of FRNK inhibits cell invasion in v-SRC-transformed NIH 3T3, which was shown largely to be due to a reduction in MMP2 expression and secretion (Hauck *et al*., 2002). This suggests the SRC-FAK signalling complex at focal adhesions regulates the expression of MMP2, and matrix degradation.

EMT is a process linked to cancer cell invasion. One driver of this process called TGF-β is a cytokine that induces the expression of the transcription factor SNAIL, which is important in EMT induction. TGF- β has also been shown to cause delocalisation and reduced expression of E-cadherin, an important component of adherens junctions, whose loss of at cell-cell junctions results in an invasive, mesenchymal-like cell phenotype, which is a characteristic marker of EMT (Cicchini *et al*., 2006). SRC-dependent activation of FAK is required for TGF-β-induced induction of mesenchymal and invasiveness markers in hepatocytes (Cicchini *et al*., 2008). FAK may also mediate TGF-β-induced EMT via induction of the AKT pathway (Deng *et al*., 2010). It has also been shown that FAK/PI3K/AKT signalling is required for EMT and resistance to the drug sorafenib in hepatocellular carcinoma cells (HCC) (Zhang *et al*., 2016). Together these results suggest that FAK is a regulator of EMT by signalling via SNAIL and the AKT pathway.

The WNT pathway is also an important regulator of EMT and it is commonly dysregulated in cancer (reviewed in Beachy, Karhadkar and Berman, 2004). Studies have shown that upon APC loss in the mouse, intestinal expression of FAK is increased in a c-MYC-dependent manner and is required to activate downstream effectors of the

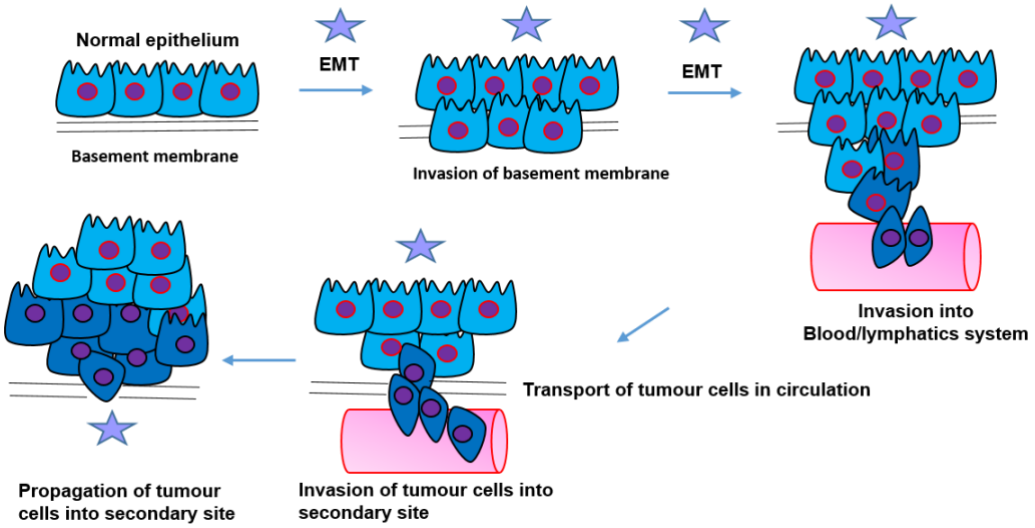


Figure 1.6 | FAK is required for multiple steps in the invasion-metastasis cascade. The dissemination of primary tumour cells to distant sites is termed the 'invasion-metastasis cascade' which is a process consisting of a number of steps. These include acquisition of invasive potential, accelerated growth and invasion of the basement membrane into surrounding tissues, angiogenesis, blood and/or lymphatic vessel invasion and release into the circulation, followed by colonisation and invasion of secondary sites and formation of micro- or marco-metastasis (reviewed in Jiang *et al*., 2015). A process called epithelial-mesenchymal transition (EMT) is a process associated with increasing the invasive capacity of cancer cells. All steps marked by a star are stages of invasion that FAK has been shown to be involved in.

WNT pathway and AKT/mTOR signalling, showing that FAK is an important driver of intestinal tumourigenesis (Ashton *et al*., 2010). Further studies have found FAK can also regulate the WNT/β-catenin pathway and intestinal tumourigenesis by phosphorylating GSKβ, which prevents β-catenin proteolytic degradation, thus causing aberrant activation of the WNT pathway (Gao *et al*., 2015). Together the above studies suggest FAK is an important mediator of tumour initiation and invasion by regulating WNT signalling.

In the case of solid epithelial cancers, metastasis is responsible for approximately 90% of cancer-associated deaths and cancer cell invasion into the basement membrane is an essential step in metastatic cancer progression (reviewed in Lambert *et al*., 2017). This process requires changes in the focal adhesion and cytoskeleton dynamics of tumour cells, as well as changes in the expression of MMPs, which facilitate invasion by degrading the ECM. High FAK expression has been correlated with

lymphovascular invasion in breast cancer and correlated with poor patient prognosis and reduced survival (Golubovskaya *et al*., 2014). Indeed, integrin β1-FAK signalling is important for proliferation of mouse mammary carcinoma cells that have spread to the lungs (Shibue *et al*., 2009). FAK is also required for angiogenesis in tumours (Tavora *et al*., 2016), another key requirement for metastasis (see **section 1.4.4**). Thus, FAK is a key regulator of metastasis by regulating EMT, invasion, matrix degradation, entry into the blood/lymphatics system and proliferation at the secondary site as summarised in **Figure 1.6**.

1.5.2 FAK is required for sustaining proliferation and resisting growth suppressors

FAK is required for SCC tumour cell proliferation in the absence of integrin adhesions in 3D but not in 2D cultures (Serrels *et al*., 2012), suggesting that FAK is required for proliferation when cells have lost attachment to the ECM. Furthermore, FAK has also been shown to regulate proliferation in a SCC model via downregulation of a key inhibitor of proliferation, IGFBP3 (Canel *et al*., 2017), also linking it to the cancer hallmark 'evading growth suppression'.

1.5.3 Inducing (tumour) angiogenesis

FAK regulates angiogenesis linking it to the cancer hallmark 'inducing angiogenesis'. Deletion of FAK from ECs *in vivo* inhibited tumour growth and angiogenesis, thus suggesting EC FAK is essential for tumour angiogenesis (Tavora *et al*., 2016). Furthermore, FAK/GRB2/MAPK signalling is important in promoting tumour angiogenesis (Mitra *et al*., 2006). Thus, these findings implicate the importance of FAK signalling complexes in the regulation of angiogenesis in physiological and tumour contexts.

1.5.4 Regulation of cancer stem cells (CSCs)

FAK has been shown to regulate CSCs in the tumour microenvironment. CSCs are considered to be immortal and can sell-renew, differentiate and are capable of causing cancer recurrence (reviewed in Wang *et al*., 2015). Several reports have shown that

FAK plays a role in self-renewal and tumour-initiating abilities of CSCs (Kolev *et al*., 2017, Luo *et al*., 2009a). Furthermore, it was shown that inhibition of FAK's kinase activity with either the VS-4718 or VS-6063 FAK kinase inhibitors significantly reduced the population of CSCs in xenograft models of triple-negative breast cancer (Kolev *et al*., 2017). The WNT/β-catenin pathway is also implicated in stem cell renewal and regeneration (reviewed in Beachy, Karhadkar and Berman, 2004) and interestingly it was shown that a constitutively active form of β-catenin could reverse the targeting of CSCs by FAK inhibition, while deletion of FAK *in vitro* shows defects in self-renewal and migration (Kolev *et al*., 2017). Deletion of FAK in the mouse MMTV-PyMT breast cancer model *in vivo* reduces the population of CSCs in tumours, suppressing mammary tumourigenesis (Luo *et al*., 2009a). Together these data suggest that FAK can modulate stem cell function by the regulation of WNT/β-catenin signalling.

1.5.5 Resistance to (programmed) cell death

FAK has well documented anti-apoptosis functions in a number of cancers such as SCC (Zhang *et al*., 2004), leukemia (Sonoda *et al*., 2000), colon cancer (Golubovskaya *et al*., 2003) and glioblastoma (Sonoda *et al*., 1999), linking it to the hallmark 'resisting cell death'. In particular, FAK has been shown to suppress anoikis (Zhang *et al*., 2004), where the cell undergoes apoptosis as a result of detachment between healthy cells and the underlying ECM (Frisch and Francis, 1994). This process is often disrupted in cancer and can lead to metastasis (reviewed in Hanahan and Weinberg, 2011). As mentioned above, FAK is required for proliferation of SCC cells in 3D but not 2D (Serrels *et al*., 2012). Additionally, FAK has been shown to suppress apoptosis of cells upon treatment with agents such as hydrogen peroxide (Sonoda *et al*., 1999) and the cytotoxic agents, etoposide (Sonoda *et al*., 2000) and staurosporine (Golubovskaya *et al*., 2003).

Studies have found that FAK regulates cell survival by signalling through pathways such as the PI3K/AKT (Sonoda *et al*., 1999), P53 (Zhang *et al*., 2004) and MAPK (Subauste *et al*., 2004). Indeed, FAK has been shown to directly interact with key components of the aforementioned pathways such as the p85 regulatory subunit of

PI3K (Qi *et al*., 2001) and P53 (Golubovskaya *et al*., 2005, Lim *et al*., 2008b). Interestingly, in the case of P53, the N-terminal of FAK was shown to directly interact with the transactivation domain of P53 and this was associated with a reduction of the transcriptional activity of P53 (Golubovskaya *et al*., 2005). FAK can suppresses P53-mediated apoptosis (IIic *et al*., 1998) most likely by directly affecting the transcriptional activity of P53.

1.5.6 Avoiding immune destruction

As previously described, the tumour microenvironment is the environment surrounding the tumour supporting growth, which is characterised by the presence of immune cells and non-malignant cells that support tumour growth (reviewed in Balkwill, Capasso and Hagemann, 2012). The cells of the TME are modulated by cytokines secreted by the tumour, fibroblasts and from immune cells populating the microenvironment. These cytokines include chemokines such as CCL5 and CXCL10 that attract regulatory T cells and thereby generate an immunosuppressive TME (Lunardi *et al*., 2014, Wang *et al*., 2017). FAK regulates the expression these immunosuppressive chemokines in the tumour microenvironment (Serrels *et al*., 2015, see **section 1.7**), linking it to the cancer hallmark 'avoiding immune destruction'.

A subset of tumours display a desmoplastic reaction where there is a growth of fibrous tissue around the tumour, which can act as a barrier to cytotoxic T cell infiltration (Jiang *et al*., 2016). This is a characteristic of PDAC for example which has a 3% 5-year survival rate (Cancer Research UK, 2019b). This makes these tumours very difficult to treat with immunotherapies, as evidenced by trials showing that these tumours are not generally responsive to anti-PD-1 and anti-CTL4 checkpoint blockage therapies (Kunk *et al*., 2016). Jiang and colleagues found that FAK activity correlates with enhanced fibrosis and low $CD8^+$ T cell infiltration (Jiang *et al*., 2016). Furthermore, they found treatment with a FAK kinase inhibitor (VS-4718) limited tumour progression in the KPC mouse model, which is a mouse model of PDAC (Jiang *et al*., 2016). The perturbed tumour progression was associated with reduced tumour fibrosis and a reduction of immunosuppressive cells in the TME, rendering these tumours more responsive to T cell immunotherapy and PD-1 antagonists (Jiang *et al*.,

2016). This suggests FAK is a key regulator of the TME by 1) influencing the expression and secretion of immuno-suppressive cytokines and chemokines and 2) creating a fibrotic TME, perturbing $CD8^+$ T cell infiltration and immune-mediated anti-tumour effects.

1.6 Nuclear FAK, a novel regulator of gene expression

Next, I will discuss novel functions of FAK in the nucleus that have emerged recently and which is associated with a number of the hallmarks of cancer and is the focus of my PhD project.

1.6.1 FAK binds to transcription factors in the nucleus to regulate gene expression

To date, much of the research on FAK has focused on its role at focal adhesions, delineating its major functions in cell motility and cancer cell invasion. However, more than a decade ago, FAK was described to translocate to the nucleus in rat myocardium subjected to hypertonic stress (Yi *et al*., 2003). Since this initial observation, nuclear FAK has been shown to interact with a limited number of transcriptional regulators, which include P53 (Lim *et al*., 2008b), GATA4 (Lim *et al*., 2012), MBD2 (Luo *et al*., 2009b) MEF2 (Cardoso *et al*., 2016), TAF9 (Serrels *et al*., 2015), IL33 (Serrels *et al*., 2017), RUNX1 (Canel *et al*., 2017) and nucleophosmin (NS) (Tancioni *et al*., 2015). These nuclear interactions have been linked to regulation of proliferation (Lim *et al*., 2008b, Canel *et al*., 2017), inflammation (Lim *et al*., 2012, Serrels *et al*., 2015), myoblast differentiation (Luo *et al*., 2009b) and tumour growth (Serrels *et al*., 2015, Tancioni *et al*., 2015). Thus, FAK seems to be able to regulate transcription of target genes by binding to transcription factors, yet, thus far, this has not been extensively studied and its mechanisms are not well understood.

1.6.2 FAK translocates to the nucleus when cells are exposed to stress

In 2008, it was shown that FAK contained a nuclear localisation sequence within its N-terminal FERM domain (Lim *et al*., 2008b) and a nuclear export sequence (NES) in its kinase domain (Ossovskaya *et al*., 2008). From the relatively small body of literature published on nuclear FAK it is evident that FAK translocates to the nucleus under conditions of cellular stress. For example, treatment of cells with cytotoxic agents like staurosporine (Lim *et al*., 2008b) or hydrogen peroxide (Luo *et al*., 2009b), cytokines like TNFα (Lim *et al*., 2012), or oncogenic transformation (Serrels *et al*., 2015), have all been shown to increase the levels of nuclear FAK. Interestingly, other focal adhesion proteins including HIC-5 (Shibanuma *et al*., 2003) and Paxillin (Sathe *et al*., 2016) have also been shown to translocate to the nucleus in response to stress induced by hydrogen peroxide or disruption of focal adhesion dynamics, respectively. Additionally, the receptor tyrosine kinase PDGFRβ in response to platelet derived growth factor (PDGFBB) was shown to translocate to the nucleus to regulate chromatin remodeling and subsequent P21 expression (Papadopoulos *et al*., 2018). Together this suggests that nuclear translocation of FAK, and potentially focal adhesion components and receptors, could be a mechanism of signalling from the cell surface to the nucleus.

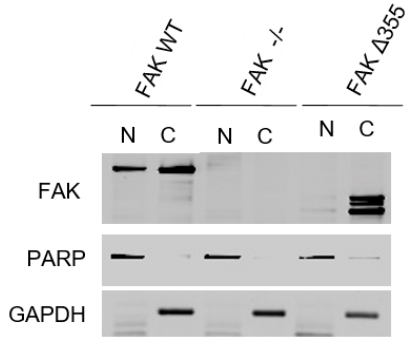


Figure 1.7 | FAK-FERM domain is essential for FAK nuclear translocation. FAK WT SCC, FAK -/- SCC and FAK Δ355 SCC (FAK-FERM domain mutant) cells were fractionated and subjected to SDS-PAGE analysis. Membrane was probed for FAK, PARP (nuclear marker) and GAPDH (cytoplasmic marker). N=nuclear fraction and C=cytoplasmic fraction. Courtesy of B. Serrels/M. Frame.

Despite this interest in nuclear FAK function, the mechanism has not yet been determined by which FAK translocates to the nucleus. Furthermore, it is not known whether FAK kinase activity regulates its nuclear translocation. Recent studies have shown that FAK kinase activity stimulated by Myo1E binding to the FAK proline rich region 1 results in pY397 FAK enrichment in the nucleus in Myo1E-reconstitued cells

(Heima *et al*., 2017). Additionally, nuclear FAK was reduced by treating cells with the FAK inhibitor PF-562271 and SRC nuclear levels were also decreased (Heima *et al*., 2017). In contrast, it has been reported that FAK inhibition increases nuclear translocation (Lim *et al*., 2012). Further work from the Frame group has shown that FAK's FERM domain is essential for nuclear translocation, as a FERM domain mutant resides within the cytoplasm (**Figure 1.7**). Whether FAK's kinase activity regulates its nuclear translocation and the mechanism by which FAK translocates to the nucleus remains to be determined.

1.6.3 Nuclear FAK regulates inflammation

The first study showing that nuclear FAK regulates inflammation was by Lim and colleagues showing that nuclear FAK enhances GATA4 E3 ligase dependent degradation, perturbing VCAM1 expression and suppressing inflammation (Lim *et al*., 2012, **Figure 1.9A**). This was followed by work from our laboratory and others that have highlighted the importance of FAK in regulation of the pro-tumorigenic microenvironment, linking nuclear FAK function to the cancer hallmark – avoiding immune destruction. For example, the Frame group recently showed a requirement for nuclear FAK in the transcriptional regulation of chemokines and cytokines required to support tumour growth through evasion of the anti-tumour immune response which will be discussed in more detail in **section 1.7** (Serrels *et al*., 2015, Serrels *et al*., 2017, **Figure 1.9B**). Furthermore, work from the Kairbaan Hodivala-Dilke group demonstrated that endothelial-FAK is required for NF-κB mediated production of cytokines in response to DNA-damaging agents such as doxorubicin (Tavora *et al*., 2014). This group also noted an increase in nuclear FAK levels under these conditions, but did not test directly whether nuclear FAK was mediating the chemokine response (personal communication, B. Serrels). Thus, FAK is important for the establishment of a pro-tumorigenic micro-environment, likely through transcriptional regulation of chemokines and cytokines.

1.6.4 Nuclear FAK regulates proliferation and apoptosis

As previously described, P53 controls proliferation and apoptotic signals and thus is considered to be a tumour suppressor (reviewed in Hanahan and Weinberg, 2011). Early studies into nuclear FAK signalling suggested that FAK can translocate to the nucleus in response to the cytotoxic agent staurosporine and binds P53, enhancing P53 degradation via the modulating the interaction between P53 and the E3 ubiquitin protein ligase, MDM2 (Lim *et al*., 2008b). This enhanced P53 degradation, results in increased proliferation and survival (Lim *et al*., 2008b, **Figure 1.9C**). Recent data suggests that Merlin may mediate the interaction between the P53-FAK and MDM2-P53 complex in mesothelioma cells (Ou *et al*, 2016). Additionally, FAK was also shown to regulate the nuclear localisation of enhancer of zeste homologue 2 (EZH2) and its tri-methylation activity on H3K27Me3 mark in hepatocellular carcinoma (HCC) to regulate proliferation and tumour growth (Gnani *et al*., 2017). Furthermore, Canel and colleagues showed that FAK suppresses the mRNA expression of the proliferation-inhibitor, *IGFBP3*, in SCC cells suppressing the transcriptional activity of RUNX1 by forming a repressive complex with SIN3A (Canel *et al*., 2017, **Figure 1.9D**). Together these data link nuclear FAK to the cancer hallmarks 'sustaining proliferative advantage and resisting cell death'.

1.6.5 Nuclear FAK regulates angiogenesis

Multiple studies have shown that FAK is important in the regulation of angiogenesis (see **section 1.3.3** and **1.4.6**) and nuclear FAK has recently been shown to be a key regulator of angiogenesis linking it to the cancer hallmark 'inducing angiogenesis'. Vascular endothelial growth factor receptor 2 (VEGFR2) is a key regulator of angiogenesis as it can bind VEGF-C and VEGF-D to regulate blood vessel formation (Zhou *et al*., 2019), and FAK regulates VEGFR2 by associating with the RNA Pol II complex at the VEGFR2 promoter, resulting in stimulation of EC proliferation and migration (Sun *et al*., 2018). Thus, nuclear FAK is a key regulator of angiogenesis by regulating transcriptional machinery at the VEGFR2 promoter.

1.6.6 Nuclear FAK regulates muscle cell function

As described in **section 1.5.2**, nuclear FAK translocates to the nucleus in rat myocardium subjected to hypertonic stress (Yi *et al*., 2003). Following on from this work, it was shown that nuclear FAK is a key regulator of muscle cells via complexing with transcriptional regulators (Luo *et al*., 2009b, Cardoso *et al*., 2016). Indeed, FAK accumulates in the nucleus in myotubes in response to hydrogen peroxide and binds MBD2 to regulate the expression of myogenin, a key regulator of muscle differentiation (Luo *et al*., 2009b). MBD2 is a component of the transcriptional repressor NuRD chromatin remodelling complex (Lai and Wade, 2011) and the authors proposed that FAK sequesters MDB2 thereby dissociating the NuRD complex, relieving transcriptional repression at the myogenin promoter (Luo *et al*., 2009b). Further studies showed that FAK binds to the transcription factor, myocyte enhancer factor-2 (MEF2) via the FAK-FAT domain, in response to mechanical stress in cardiomyocytes. ChIP experiments showed that the FAK:MEF2 complex was bound

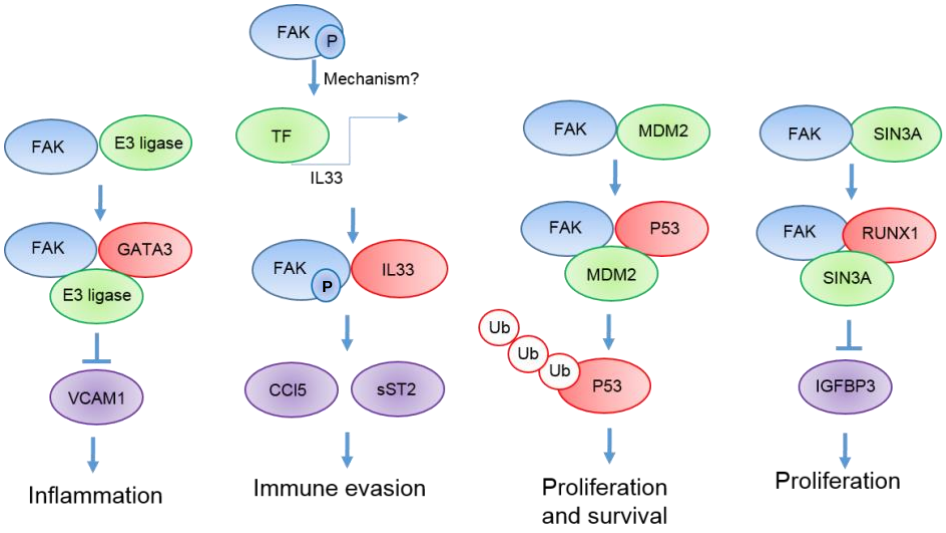


Figure 1.8 | Summary of nuclear FAK signalling pathways. FAK-regulates multiple transcription factors to regulate expression of a subset of genes. FAK also regulates the degradation of transcription factors such as C) P53 (Lim *et al*., 2008b) and A) GATA3 (Lim *et al*., 2012) by kinase-independent mechanisms. B) FAK regulates the anti-tumour immune response via kinase dependent mechanisms (Serrels et al., 2015, 2017); however it is not established whether FAK binding to IL33 is kinase dependent or independent. Secondly it has not been determined the mechanism in which FAK regulates the expression of IL33. C) FAK enhances P53 degradation by scaffolding P53 to the MDM2. D) FAK binds RUNX1 and SIN3A to form a repressive complex and thereby suppress IGFBP3 expression.

to the promoter of the stress responsive gene *JUN* to enhance its expression (Cardoso *et al*., 2016). Thus, FAK is a key regulator of muscle cell function by interacting with components of chromatin remodifying complexes and transcription factors.

1.6.7 Role of FAKs kinase activity in the nucleus?

Although, FAK has been proposed to bind a number of transcription factors in the nucleus, it has not yet been shown to phosphorylate any of these nuclear binding partners and therefore may primarily have an adaptor function in the nucleus (reviewed in Schoenherr *et al*., 2018). However, FAK kinase activity is important for its nuclear function as a regulator of gene expression in SCC cells (Serrels *et al*., 2015, see **section 1.7**) and findings have indicated that when FAK kinase is activated, it has an alternative conformation (Lietha *et al*., 2008). Therefore, it remains possible that FAKs auto-inhibited conformation may be important for scaffolding functions in the nucleus as FAK kinase-independent interactions have been reported with key transcription factors (i.e. P53, Lim *et al*., 2008b). A summary of what is known of FAK's role in the nucleus is presented in **Figure 1.8**.

1.7 Targeting FAK in cancer

As described above, both of FAK's canonical adhesion and nuclear functions are relevant to the hallmarks of cancer. There has been much interest in targeting FAK in human cancer and as such clinical trials testing FAK inhibitors in cancer patients are ongoing.

Many pharmaceutical companies have generated small molecule inhibitors of FAK which are ATP mimetics, and directly inhibit FAKs kinase activity (reviewed in Yoon *et al*., 2015). There are a number of FAK inhibitors available to date including PF-573,228, GSK2256098, VS-4718 and VS-6063 (reviewed in Yoon *et al*., 2015). Studies have shown that inhibition of FAK's kinase activity is useful in reducing malignancy in a number of cancer cell lines and *in vivo* (Golubovskaya *et al*., 2003, Serrels *et al*., 2015). These *in vitro* and *in vivo* findings have stimulated the induction of a number of clinical trials many of which have been completed showing good

tolerability of FAK kinase inhibitors (clinicalTrials.gov, NCT01951690, NCT01138033, NCT00666926, NCT01905111, NCT01335269).

Studies have tried to establish biomarkers that render tumours more sensitive to FAK inhibitor treatment. It was proposed that mesothelioma tumours that display loss of the tumour suppressor protein, merlin, have greater sensitivity to FAK inhibition by VS-6063 (Shapiro *et al*., 2014). Merlin is involved in adherens junction maturation and therefore loss of merlin results in unstable cell-cell adhesions, rendering cells more dependent on FAK/integrin signalling (Shapiro *et al*., 2014). This study led to a phase II trial (clinicalTrials.gov, NCT01870609), combining cisplatin and VS-6063 in mesothelioma patients that display merlin loss. However, the study was terminated due to lack of efficacy of the drug.

FAK kinase inhibition may not be effective as a monotherapy as it results in growth suppression rather than cell death. Indeed, it has been reported that FAK inhibition alone results in disease stabilisation rather than tumour regression (Soria *et al*., 2016). Furthermore, acquired resistance to FAK kinase inhibitors has been observed when treating PDAC mouse tumours (Jiang *et al*., 2019). Thus, targeting multiple pathways in addition to FAK signalling may be more effective in preventing resistance in tumours and providing more durable responses.

Recent *in vivo* studies have identified that combinatorial therapies including FAK kinase inhibitors are showing promising results. For example, our laboratory has used a multi-parametric, high content phenotypic screening approach to determine compounds that enhance FAK inhibition (unpublished, J. Dawson). This led to the identification that HDAC inhibitors when combined with FAK kinase inhibition perturbed proliferation of a number of cancer cell lines (Unpublished, J. Dawson). Furthermore, a study by Hirata and colleagues showed in a mouse melanoma model, that the ERK/MAPK pathway is reactivated upon treatment with the BRAF inhibitor PLX4720, and this is linked to increased stromal density (Hirata *et al.,* 2015). They found that the stromal cells, melanoma associated fibroblasts (MAFs), proximal to the cancer cells, remodel the matrix which reactivates the ERK/MAPK pathway through

the ß1-integrin-FAK-SRC pathway (Hirata *et al*., 2015). They showed that treating mice with a combination of PLX4720 and a FAK inhibitor resulted in a reduction in tumour volume in patient derived xenografts (Hirata *et al*., 2015). These findings suggested that this drug combination might benefit melanoma patients who have increased MAF infiltration, elevated fibrous ECM and activated SRC/FAK signalling (reviewed in Frame and Serrels, 2015). Trials are currently recruiting combining the dual RAF/MEK inhibitor, RO5126766 and the VS-6063 (clinicalTrials.gov, NCT03875820) in patients with non-small cell lung cancer (NSCLC). Furthermore, an active trial is ongoing combining the MEK inhibitor trametinib with GSK2256098 in patients with advanced pancreatic cancer to determine the anti-tumour effect of this drug combination (clinicalTrials.gov, NCT02428270).

The findings that FAK kinase inhibition using the VS-4719 FAK inhibitor causes a reduction in Treg cells and an increase in $CD8^+$ T cells in tumours (Serrels et al., 2015 – see **section 1.7.1**) provides a strong rationale for a combination treatment of a FAK kinase inhibitor with anti-PD-1 or anti-PD-1L antibodies. Trials are currently ongoing combining FAK inhibitors (VS-6063) with anti-PD-1 antibodies in a number of cancers including breast and mesothelioma (clinicalTrials.gov, NCT02758587). Furthermore, a US Phase I trial is recruiting in patients who have advanced pancreatic tumours, where the FAK kinase inhibitor (VS-6063) is given in combination with both an anti-PD-1 antibody and the chemotherapeutic agent gemcitabine (clinicalTrials.gov, NCT02546531). Results from animal studies combining FAK kinase inhibitors, PD-1 antagonists and gemcitabine yielded very encouraging results (Jiang *et al*., 2016), and the results of all combination of FAK inhibitors with immunotherapy are eagerly awaited.

As with all immunotherapy, and molecularly targeted therapies, it is difficult to predict which patients will benefit from combination of anti-PD-1 and FAK kinase inhibitor therapy combinations. However, work from our colleague Alan Serrels has identified tumours that express the co-stimulatory molecule CD80 are more receptive to inhibition of FAKs kinase activity using a FAK kinase inhibitor BI 853520 (personal communication, M. Frame). Furthermore, the authors identified that the co-expression

of CD80 and FAK preferentially occurs in cancer cell lines from patients with Burkitt's lymphoma, chronic myeloid leukemia, diffuse large B-cell lymphoma and Hodgkin's lymphoma. FAK inhibitors have not been tested in patients with these cancer types, but this work suggests that this could be an effective treatment for these patients (personal communication, M. Frame).

1.8 Change in thought; FAK may primarily work via transcription and immune evasion

As presented, it is clear that FAK is an important regulator of cancer. A number of findings from our laboratory has identified that FAK regulates gene expression in cancer cells via control of cytokines and chemokines that suppress the anti-tumour immune response (Serrels *et al*., 2015). Following work identified that FAK regulates the transcription factor IL33 to control the expression of immunosuppressive cytokines such as CCL5 (Serrels *et al*., 2017). IL33 is a nuclear and secreted cytokine (Lu *et al*., 2016). In its secreted form it functions as an alarmin which is a molecule that is released from cells that have been injured or infected to stimulate an immune response (Lu *et al*., 2016). Secreted IL33 alerts immune cells that express its receptor ST2 and has been shown to be important for stimulating the $CD8_+$ T cell response (Lu *et al*., 2016). The function of IL33 in the nucleus is poorly defined. It has been shown to regulate the mRNA and protein expression of the transcription factor NF-κB P65 subunit by binding to the P65 promoter and this is associated with the expression of the NF-κB-target genes, ICAM1 and VCAM1 (Choi *et al*., 2012). The finding that IL33 functions downstream of FAK in the regulation of immunosuppressive cytokines suggested a potential mechanism in which nuclear FAK signalling regulates the anti-tumour immune response (Serrels *et al*., 2017). Furthermore, our laboratory found that FAK regulates the transcription factor RUNX1 to regulate proliferation by suppressing the expression of *IGFBP3* mRNA (Canel *et al*., 2017). This work further added to knowledge on the mechanism by which nuclear FAK signalling can regulate cancer-associated functions. In the following section, I will describe the findings published in these papers, as these form the basis for my PhD project. This will be followed by

the aims of my PhD project. Firstly, I will describe the cell lines used in these studies and my thesis.

Our laboratory deleted FAK in SCC cells using a Cre-Lox recombination system in 2012 (described in **materials and methods**). SCCs were induced in K14CreER $FAK^{flox/flox}$ FVB mice by performing the dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol 13-acetate two-stage cutaneous chemical carcinogenesis protocol (McLean *et al*., 2004). SCCs were excised and cells isolated and cultured in the presence of 4-hydroxytamoxifen to induce FAK deletion (Serrels *et al*., 2012). FAK WT, FAK-nuclear localisation defective (FAK NLS) and FAK-kinase defective (FAK KD) proteins were re-expressed in the FAK -/- cell line using standard retroviral induction. A summary of the SCC cell lines used by our laboratory and in my thesis can be found in **Table 1.1**.

1.8.1 FAK has been shown to translocate to the nucleus in response to cellular stress such as oncogenic transformation

As described in **section 1.5**, FAK translocates to the nucleus in response to cellular stress such as treatment with cytotoxic agents (Lim *et al*., 2008b) or hydrogen peroxide (Cardoso *et al*., 2016), suggesting that nuclear FAK signalling could be a response to cellular stress. FAK is present in the nucleus of FAK WT SCC cells, whereas there was no detectable amount of FAK in the nuclear compartment of the normal keratinocytes from which SCCs are derived suggesting that nuclear FAK translocation is also regulated by oncogenic transformation. Nuclear FAK is important for tumour

Cell line name	Genotype	SCC	Re-expression
FAK WT	K14CreER $FAK^{flox/flox}$	FAK -/-	FAK WT
FAK -/-	K14CreER $FAK^{flox/flox}$	FAK -/-	N/A
FAK NLS	K14CreER $FAK^{flox/flox}$	FAK -/-	FAK NLS
FAK KD	K14CreER $FAK^{flox/flox}$	FAK -/-	FAK KD

Table 1.1 | Mouse SCC cell lines generated by Frame group. SCC lines were previously generated (McLean *et al*., 2004, Serrels *et al*., 2012) as described in material and methods using a Cre-Lox recombination system. KD = Kinase-defective, NLS = Nuclear localisation defective.

growth, as expression of a nuclear localisation impaired mutant of FAK (FAK NLS) results in tumour regression in syngeneic immune competent FVB mice (Serrels *et al*., 2015). However, the mechanisms and physiological reasons by which FAK is translocated to the nucleus are yet to be defined.

1.8.2 Nuclear FAK controls transcription of immunosuppressive cytokines and chemokines

As mentioned our laboratory has found that nuclear FAK regulates the anti-tumour immune response via the transcriptional regulation of chemokines and cytokines (Serrels *et al*., 2015). Specifically, it was found that FAK is enriched in the chromatin fraction where it interacts with regulators of chemokine expression, such as the basal transcription factor complex component TAF9. Expression of CCL5 and other cytokines was shown to protect SCC tumours from immune destruction by $CD8^+$ T cells in immune competent FVB mice via attraction of Tregs into the TME. The authors further identified a paracrine signalling axis between CCL5 and its receptors CCR1,2,3 in the FAK WT tumours and tumour infiltrating Tregs. Considering these findings, the authors proposed a model by which nuclear FAK regulates transcription of immunosuppressive chemokines (such as *CCL5* and *CXCL10*) to promote tumour growth via interaction with transcriptional regulators such as TAF9 in the nucleus. This work had strong clinical implications as treatment of SCC tumours with the FAK kinase inhibitor VS-4718 resulted in a decrease of Treg infiltration into the tumour and complete tumour regression, which suggested that use of FAK inhibitors in clinical trials may promote immune-mediated tumour regression. It was not determined whether FAK kinase activity can regulate binding to these chromatin modifying complexes/transcriptional regulators and whether this was due to structural changes imposed by the kinase-activated FAK structural conformation (Serrels *et al*., 2015).

1.8.3 FAK interacts with a network of chromatin modifiers and transcriptional regulators

The mechanism by which FAK-dependent transcription factors regulate the anti-tumour immune response was further investigated by performing FAK nuclear

proteomics identifying that FAK interacts with a complex network of transcription factors in the nucleus which included the nuclear cytokine IL33 (Serrels *et al*., 2015).

Nuclear FAK was shown to control the expression of the cytokine *IL33* by the interaction of transcription factors and transcriptional regulators (Serrels *et al*., 2017). Further, nuclear FAK and IL33 were confirmed to bind in the nucleus of SCC cells which regulates the expression of *CCL5* and the IL33 soluble decoy receptor ST2 (*sST*), through the interaction with transcription factors and transcriptional regulators. sST is the soluble form of the IL33 receptor which inhibits the activity of secreted IL33, and thereby perturbs immune responses mediated by secreted IL33 (reviewed in Milovanovic *et al*., 2012). The authors proposed a model based on their findings that FAK/IL33 regulated expression and secretion of sST and CCL5 into the TME results in suppression of the anti-tumour immune response by suppressing the activing of secreted IL33 and promoting Treg infiltration (Serrels *et al*., 2017).

Further work provided insight into FAK's role in the nucleus as a key regulator of transcription in cancer by the finding that nuclear FAK regulates the expression of the secreted protein IGFBP3, which was shown to be a negative regulator of the cell cycle and tumour growth in SCC tumours (Canel *et al*., 2017). Nuclear FAK was shown to bind to a transcription factor, RUNX1 which regulates IGFBP3 expression and a suppressor of RUNX1 transcriptional activity, SIN3A. Further investigation found that FAK controlled the nuclear levels of SIN3A expression and ultimately thereby controlled SIN3A interaction with RUNX1. The authors proposed a mechanism by which FAK regulates the expression of IGFBP3, namely by changing the transcriptional activity of RUNX1, which in turn regulated the cell cycle and tumour growth. This work showed that FAK can modulate transcriptional complexes in the nucleus to regulate the expression of growth suppressors to drive tumour growth (Canel *et al*., 2017).

1.9 Aims

These studies provided insight into the function of FAK in the nucleus and led to the hypothesis that FAK regulates transcription of particular genes by binding to

transcription factors and chromatin modifying complexes. Building on these hypotheses, I set out to investigate 1) FAK binding partners in the nucleus using **proximal proteomics**, 2) FAK-dependent transcription factor promoter/enhancer recruitment to chromatin and chromatin accessibility changes at particular genes by use of **ATAC-seq** and 3) to determine the FAK-dependent transcriptome in SCC cells using **mRNA-seq**. I then integrated these datasets in order to understand which transcription factors are responsible for FAK-dependent gene expression changes and the nuclear proteins that FAK binds which may scaffold to transcription factor complexes. By using these approaches, I aimed to gain increased information on the mechanisms by which FAK is regulating the expression of specific genes – in particular those associated with FAKs cancer-associated functions.

The techniques I will use to tackle each of these aims are;

1) **Proximal proteomics** to define novel FAK-binders in the nucleus. Specifically, I will use published protocols RIME (Mohammed *et al*., 2016) and BioID (Roux *et al*., 2013). RIME is a protocol that uses formaldehyde cross-linking to capture protein-protein complexes and protein-DNA complexes in the nucleus. It only captures interactions in close proximity to the protein of interest as formaldehyde has a short spacer arm of 2Å. BioID is a biochemical labelling approach in which a Biotin ligase tag (BirA*) is added to your protein of interest which biotinylates any protein in close proximity when biotin is added to cell culture media. All proteomics datasets will be generated using nuclear lysates to enrich for transcription factor complexes.

2) **ATAC-seq** (Buenrostro *et al*., 2015) will be performed to define FAK, FAK kinase and FAK nuclear localisation dependent changes in transcription factor recruitment to chromatin and chromatin accessibility changes at gene promoters of interest. This protocol uses a hyperactive Tn5 transposase which digests only at open regions of chromatin, followed by seqeucing of the DNA within the exposed chromatin. Accessible regions determined by ATAC-seq display increased coverage at a particular region of the genome. The sequence

within the ATAC-seq peaks will be analysed and if it contains a motif sequence that a transcription factor is known to bind; the transcription factor which binds to that genomic region can be predicted. Any predicted FAK-dependent transcription factor binding changes will be determined by performing **chromatin immunoprecipitation-PCR (ChIP-PCR)**, which utilises formaldehyde cross-linking to capture protein-DNA interactions. The DNA in which the protein is binding to can be analysed by qPCR by designing primers to capture the transcription factor motif on the genomic region of interest. This will confirm whether a transcription factor displays FAK-dependent binding to a particular genomic region either as a result of FAK-dependent chromatin accessibility changes in that region or FAK-regulated transcription factor recruitment.

3) **mRNA-seq** will be used to determine FAK and FAK-kinase dependent genes programs. mRNA-seq is a widely used technique in which RNA samples are converted to cDNA and sequenced to analyse changes in transcript levels between different cell lines and conditions. This is building on previous microarray work presented in Serrels *et al*., 2015, which identified FAK-dependent gene programs, but stopped short of identifying FAK kinase dependent gene programs.

Chapter 2 Materials and Methods

2.1 Materials

Table 2.1 Reagent list

Reagent	Supplier	Cat. no.
1 x MEM	Gibco	11090081
100x NEAA	Sigma-Aldrich	6895
Agencourt AMPure XP magnetic beads	Beckman Coulter	A63880
Agilent High Sensitivity DNA Bioanalysis kit	Agilent	5067-4626
Agilent Bioanalyzer RNA 6000 pico assay	Agilent	5067-1513
Amersham™ ECL™ Rainbow™ Marker - Full range	Sigma-Aldrich	GERPN800E
Ampicillin sodium salt	Sigma-Aldrich	A9518
BamHI	New England Bio Labs (NEB)	R0136S
Biotin powder	Sigma-Aldrich	B4639
Calcein	Invitrogen	1430
cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail	Roche	5892970001
DMEM, High Glucose	Sigma-Aldrich	D6429
Dynabeads Protein A	Invitrogen	10001D
Dynabeads Protein G beads	Invitrogen	10004D
Hygromycin	Millipore	400052
G418	Sigma-Aldrich	A1720
Growth Factor reduced Matrigel	Corning	356231
L-Glutamine 200mM	Gibco	2503081
Lipofectamine 2000	Qiagen	11668019
Lipofectamine RNAiMax transfection reagent	Thermo Fisher	13778030
MEM vitamin solution	Sigma-Aldrich	M6895
4–15% Mini-PROTEAN® TGX Stain-Free™ Protein Gels, 12 well, 20 µl	Bio-Rad laboratories	456-1085
Nextera DNA Flex Library Prep kit	Illumina	20018703
NotI	NEB	R0189S
NEBNext High-Fidelity 2x PCR master mix	NEB	M0541
One Shot™ MAX Efficiency™ DH5α-T1R Competent Cells	Thermo Fisher Scientific	12297016
Opti-MEM Reduced Serum Medium, GlutaMAX	Thermo Fisher Scientific	51985026
PhosSTOP™	Roche	4906845001

Pierce BCA Protein Assay kit	Thermo Fisher Scientific	23225
Pierce ECL Western blotting substrate	Thermo Fisher Scientific	32106
Polybrene	Millipore	1003-G
Proteinase K	Ambion	AM2544
Qiagen MinElute PCR purification kit	Qiagen	28004
QIAGEN Plasmid Maxi kit	Qiagen	12162
QIAquick Gel Extraction kit	Qiagen	28704
QIAquick PCR purification kit	Qiagen	28104
Qubit dsDNA HS Assay kit	Thermo Fisher Scientific	Q32851
RNA-easy kit	Qiagen	74104
RNaseA/T1	Ambion	AM2286
Sodium Pyruvate	Gibco	11360039
Streptavidin MyOne C1 beads	Invitrogen	65002
SuperScript™ First-strand Synthesis System	Invitrogen	11904018
SYBR Select Master Mix	Thermo Fisher scientific	4472908
T4 DNA ligase	New England Bio Labs	M0202S
10x Tris/Glycine/SDS	Bio-Rad laboratories	1610772
Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs	Bio-Rad laboratories	1704159
Trypsin 0.05 % EDTA	Gibco	25300054
Vectasheild Antifade mounting medium with DAPI	Vector laboratories	H1500

Table 2.2 Buffers

Buffer	**Composition**
RIPA	150 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris [pH 8]
Cyto	10 mM Tris [pH 7.5], 0.05% NP-40, 3 mM $MgCl_2$, 1 mM EGTA,
MS Elution buffer 1	2 M Urea, 50 mM Tris-HCl [pH 7.5], Trypsin 1µg/µl
MS Elution buffer 2	2 M Urea, 50 mM Tris-HCl [pH 7.5], 1 mM DTT
ATAC-seq lysis buffer	10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3mM $MgCl_2$, 0.1% IGEPAL CA-630
ChiP lysis buffer	10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 0.5% NP-40
ChIP 1xRIPA 150 mM NaCl	50 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, 0.2% SDS, 0.2% Sodium Deoxycholate, 1% Triton X-100
ChIP 1xRIPA 500 mM NaCl	50 mM Tris-HCl [pH 8], 500 mM NaCl, 1 mM EDTA, 0.2% SDS, 0.2% Sodium Deoxycholate, 1% Triton X-100

SDS lysis buffer	50 mM Tris-HCl [pH 7.5], 1% SDS, 10 mM EDTA
ChIP dilution buffer	50 mM Tris-HCl [pH 7.5], 167 mM NaCl, 1.1% Triton X-100, 0.11% Sodium Deoxycholate
Tris-EDTA	10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8]
ChIP direct elution buffer	10 mM Tris-HCl [pH 8], 300 mM NaCl, 5 mM EDTA [pH 8], 0.5% SDS
High salt solubilisation buffer	50 mM Tris-HCl [pH 8.0], 2.5 M NaCl, 0.05% NP-40
Chromatin extraction buffer with NP40	10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.2% NP-40
Chromatin extraction buffer without NP40	10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol
No-Salt buffer	10 mM HEPES [pH 7.9], 3 mM EDTA, 0.2 mM EGTA
Laemmli sample buffer (2x)	4% SDS 20% , Glycerol, 120 mM Tris-Cl [pH 6.8], 0.2% Bromophenol blue

Table 2.3 Antibody list

Antibody	**Supplier**	**Cat. no.**	**WB**	**ChIP**	**IP**	**IF**
JUN	CST	9165	1/1000	0.48 µg	N/A	N/A
JUNB	CST	3753	1/1000	N/A	N/A	N/A
IgG	CST	2729	N/A	0.48 µg	1/100	N/A
pFAK (Y397)	CST	3283	1/1000	N/A	N/A	N/A
FAK	BD	610087	1/1000	N/A	N/A	N/A
HIC-5	BD	611165	1/1000	N/A	N/A	N/A
Paxillin	CST	2542	1/1000	N/A	N/A	N/A
WWOX	Abcam	ab137726	1/2000	N/A	N/A	N/A
LPP	CST	33895	1/1000	N/A	N/A	N/A
GAPDH	CST	5174	1/1000	N/A	N/A	N/A
Histone H3	CST	4499	1/1000	N/A	N/A	N/A
Streptavidin-HRP	CST	3999	1/1000	N/A	N/A	1/200
Alexa fluor 488 anti-mouse	Invitrogen	A-11094	N/A	N/A	N/A	1/200

Streptavidin Fluor 488	Invitrogen	S11223	N/A	N/A	N/A	1/200
Anti-FAK Antibody, clone 4.47, agarose conjugate	Sigma-Aldrich	16-173	N/A	N/A	10µg	N/A
Anti-rabbit IgG, HRP-linked Antibody	CST	7074	1/10000	N/A	N/A	N/A
Anti-mouse IgG, HRP-linked Antibody	CST	7044	1/5000	N/A	N/A	N/A

Table 2.4 In-house designed primers

mRNA/genomic region	**Primer sequence**
CXCL10	F:CCCACGTGTTGAGATCATTG R:CACTGGGTAAAGGGGAGTGA
GAPDH	F:CTGCAGTACTGTGGGGAGGT R:CAAAGGCGGAGTTACCAGAG
IL33	F:GGATCCGATTTTCGAGACTTAAACAT R:GCGGCCGCATGAGACCTAGAATGAAGT
RBMS3	F:GCCAACTAACATCGTGGGTC R:GTCTGGGGAGAGGTATCAGC
IL33 enhancer	F:ACCCTGGAGTGTTCTTTGCA R:TGCCTTCTGAAGCTTACTCGA
IL33 negative control region	F:ATGTGTGCTGTGTGTATGCC R:ACATTAAGGGCAGGAGACGT

Table 2.5 Pre-designed primers

Product name	**mRNA**	**Supplier**	**Catalogue no.**
Mm_Jun_1_SG QuantiTect Primer Assay	*JUN*	Qiagen	QT00296541

Mm_Junb_1_SG QuantiTect Primer Assay	*JUNB*	Qiagen	QT00241892
Mm_Sfrp1_1_SG QuantiTect Primer Assay	*SFRP1*	Qiagen	QT00167153
Mm_Wnt7b_vb.1_SG QuantiTect Primer Assay	*WNT7B*	Qiagen	QT02416127

Table 2.6 shRNA/siRNAs used for knockdown experiments

siRNA/shRNA	**Cat. No.**	**Supplier**
Non-targeting siRNA #2	D-001210-02-05	Dharmacon
siGenome JUN set of 4 siRNAs	MQ-043776-01-0002	Dharmacon
siGenome JUN Smartpool	M-043776-01-00005	Dharmacon
TRC lentivoral pLKO.1 Empty vector	RHS4080	Dharmacon
TRC lentiviral RBMS3 shRNA #1 (clone ID: TRCN0000096889)	RMM3981-201814942	Dharmacon
TRC lentiviral RBMS3 shRNA #2 (clone ID:TRCN0000096891)	RMM3981-201805340	Dharmacon

Table 2.7 Reference list for bioinformatics programs

Program	**Reference**
Bcbio-next gen pipline	Bcbio-nextgen.readthedocs.io. (2020). Pipelines — bcbio-nextgen 1.1.5 documentation. [online] Available at: https://bcbio-nextgen.readthedocs.io/en/latest/contents/pipelines.html [Accessed 10 Jan. 2020].
Diffbind	Ross-Innes, C.S., Stark, R., Teschendorff, A.E., Holmes, K.A., Ali, H.R., Dunning, M.J., Brown, G.D., Gojis, O., Ellis, I.O., Green, A.R., Ali, S., Chin, S., Palmieri, C., Caldas, C., Carroll, J.S. (2012). "Differential oestrogen receptor binding is associated with clinical outcome in breast cancer." Nature, 481(7381), pp. 389-93
ChIPseeker	Yu, G., Wang, L., He, Q. (2015). 'ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization.' Bioinformatics, 31(14), pp. 2382-2383.

MACS2	Zhang, Y.,Tao Liu, Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W. and Liu, X.S. (2008). 'Model-based Analysis of ChIP-Seq (MACS).' Genome Biology, 9, R137.
Kallisto	Bray, N.L., Pimentel, H., Melsted, P. and Pachter, L. (2016). 'Near-optimal probabilistic RNA-seq quantification', Nature Biotechnology, 34, pp. 525–527.
DESeq2	Love, M.I., Huber, W. and Anders, S. (2014). 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.' Genome Biology, 15, pp. 550.
Tximport	Soneson C, Love MI, Robinson MD (2015). "Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences." F1000Research, 4. doi: 10.12688/f1000research.7563.1.
Cluster 3	Hoon, M. J. L. de., Imoto, S., Nolan, J., and Miyano, S. (2004). 'Open Source Clustering Software.' Bioinformatics, 20(9), pp. 1453—1454.
TreeView	Saldanha, A.J. (2004). 'Java Treeview—extensible visualization of microarray data', Bioinformatics, 20(17), pp. 3246–3248.
MaxQuant	1. Cox, J. and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnology, 26(12), pp. 1367–1372. 2.Tyanova, S., Temu, T., Carlson, A., Sinitcyn, P., Mann, M., and Cox, J.. (2015). 'Visualization of LC-MS/MS proteomics data in MaxQuant'. Proteomics, 15(8), pp. 1453–1456.
Perseus	Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, MY., Geiger, T., Mann, M. and Cox, J. (2016). 'The Perseus computational platform for comprehensive analysis of (prote)omics data.' Nature Methods. 13(9), pp. 731-40.

2.2 Methods

2.2.1 Cell culture

Extraction and generation of FAK SCC cell model is depicted in Serrels *et al*., 2012. SCC cells were induced in K14CreER *FAK*$_{flox/flox}$ in FVB mice using the dimethylbenz[a]anthracene/12-*O*-tetradecanoylphorbol 13-acetate two-stage cutaneous chemical carcinogenesis protocol. Cells were extracted and cultured with 4-hydroxytamoxifen to induce FAK deletion. Standard Retroviral induction was used to stably express FAK WT and FAK mutant constructs with either a mutation in FAK's kinase linker (FAK KD, K454R) or its nuclear localization sequence (FAK NLS, R177A/R178A/R190A/R191A/R216A/R218A) into a FAK -/- clone (further details of the retroviral transduction protocol can be found in Serrels *et al*., 2012). Cells expressing the FAK WT and FAK mutant constructs were maintained in 0.25 mg/ml Hygromycin. A schematic of the mutants used in this thesis can be found in **Figure 2.1**.

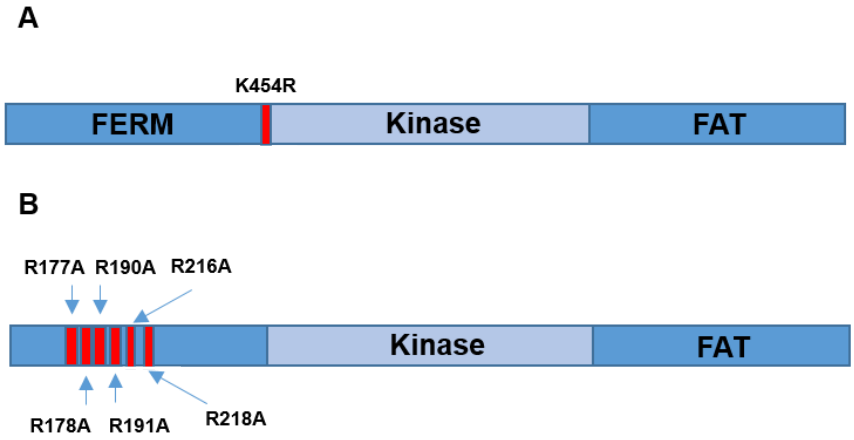


Figure 2.1 | Schematic of FAK mutants used in thesis. A) FAK kinase-dead) and B) FAK nuclear-localisation mutants were generated in Serrels *et al.*, 2012 and Serrels *et al*., 2015 by site-directed mutagenesis. A) The FAK KD mutant has a K454R mutation in its kinase domain linker region which ablates FAK kinase activity. B) The FAK NLS mutant contains mutations in multiple regions of FAK NLS in the FERM domain, which perturbs FAK translocation to the nucleus. Region of mutations are indicated by red stripes.

FAK WT SCC, FAK -/- SCC, FAK NLS SCC, FAK KD SCC, FAK WT BirA, FAK KD BirA*, HIC-5 BirA* FAK WT, HIC-5 BirA* FAK-/-, FAK-/- BirA* EV and FAK WT BirA* EV were grown in minimum essential media (1 x MEM) supplemented with 10% FBS, 1% MEM Vitamin solution, 1% MEM Non-essential amino acids, 1% Sodium Pyruvate, and 1% L-Glutamine (all from Gibco). Human embryonic kidney 293T cells were maintained in DMEM, high glucose containing 10% FBS. Cells were cultured at 37$_o$C in 5% Carbon Dioxide and passaged with trypsin 0.05% EDTA (Gibco) every other day. When setting up experiments, all cells were plated on a 10cm dish (Corning) except for when performing inverted invasion and immunofluorescence assays, in which cells were set up in 12 well plates (Corning).

2.2.2 Generation of BirA* expressing lines

HIC-5 was amplified using the following primers F:GCGGCCGCATGGAGGACCTGGATGCCCTGC and R: GGATCCTCAGCCGAAGAGCTTCAGGAAAGC in a standard PCR reaction under the following cycling conditions: – 98 $_o$C for 10 seconds, 30x (98 $_o$C for 10 seconds, 60 $_o$C for 10 seconds and 72 $_o$C for 4 minutes). For subcloning into pQXCIN-BirA*-Myc vector, the PCR product was run on an agarose gel and gel purified used Qiagen Gel purification kit. The PCR product and pQXCIN-BirA*-Myc vector were digested with 1 µl of NotI, 1 µl BamHI, 5 µl of buffer 3.1 (all from NEB), made up to a total of 20 µl with dd$H_2$0 and then incubated at 37 $_o$C for 2 hours. The digests were then PCR purified using PCR purification kit (Qiagen). Then the PCR products were ligated into the digested pQCXIN-BirA*-Myc vector using T4 DNA ligase (NEB), before the ligation products being transformed into DH5α competent cells (Thermo Fisher scientific) following manufacturer's protocol. The bacteria were then plated onto ampicillin plates and incubated overnight at 37 $_o$C. Colonies were then picked and grown in 5 ml of LB broth with 100 µg/ml ampicillin. After a diagnostic digest, the colonies which contained the HIC-5 insert were grown in 200 ml LB broth with 100 µg/ml ampicillin and the plasmids were purified using a MaxiPrep kit (Qiagen) following manufactures guidelines.

The BirA empty vector pQCXIN-BirA*-Myc or HIC-5 pQCXIN-BirA*-Myc were transfected into Phoenix Ecotropic cells using Lipofectamine 2000 (Thermo Fisher scientific) according to manufacturer's guideline. Two days post transfection the medium was passed through a 0.45 μm Millex-AC filter (Millipore), before mixing the virus titre containing media and SCC MEM media 50% FBS with polybrene (Millipore) to reach a final concentration of 5 μg/ml. This was added to FAK WT and FAK -/- SCC cells and left for 24 hours, prior to selection using G418 (400 μg/ml). Dr N. McGivern generated the FAK WT BirA*, FAK KD BirA* and FAK -/- BirA* EV SCC lines in a manner identical to as described above.

2.2.3 shRNA-mediated depletion of *RBMS3*

To generate lentiviral particles for knockdown of *RBMS3*, human embryonic kidney 293T cells were transfected with 2 μg of *RBMS3* shRNA or pLKO.1 empty vector (all from Dharmacon, TRC lentiviral) using Lipofectamine 2000 (Thermo Fisher scientific), following the manufacturers protocol. Two days after transfection the medium was passed through a 0.45 μm Millex-AC filter (Millipore), before mixing the virus titre containing media and SCC MEM media 50% FBS with polybrene (Millipore) to reach a final concentration of 5 μg/ml. This mixture was added to FAK -/- SCC cells, which were left for 24 hours and cells were then selected using puromycin (2 μg/ml).

2.2.4 siRNA-mediated depletion of *JUN*

FAK WT SCC cells were plated so they would be 60 - 80% confluent the following day. *JUN* or non-targeting control siRNA (all from Dharmacon, siGenome) was mixed with 500 μl Opti-MEM Reduced Serum Medium with GlutaMAX to a final concentration of 33 μM. Then 12 μl of RNAiMax Lipofectamine reagent (Invitrogen) was combined with 500 μl of Opti-MEM Reduced Serum Medium, GlutaMAX and incubated at room temperature for 10 minutes, before being mixed with the siRNA/Opti-MEM Reduced Serum Medium with GlutaMAX mix and incubated for 15 minutes. This mixture was then added to FAK WT SCC cells in 4 ml Opti-MEM Reduced Serum Medium with GlutaMAX and incubated for 24 hours at 37°C, prior to generating whole cell lysates or RNA extracts.

2.2.5 Whole cell lysate preparation

To prepare whole cell lysates, cells washed in ice-cold PBS and were lysed in RIPA buffer (containing cOmplete Ultra protease and PhosSTOP phosphatase inhibitor cocktail tablets, both from Roche) and incubated on ice for 15 minutes, scraped and centrifuged at 4 °C maximum speed for 15 minutes to clear lysates. Samples were stored at -20 ^{o}C for future use.

2.2.6 Biochemical fractionation

For fractionation experiments, cytoplasmic fractions were prepared by lysing cells in Cyto buffer (containing cOmplete Ultra protease and PhosSTOP phosphatase inhibitor cocktail, both from Roche) for 5 minutes at 4 °C, collected and centrifuged at 1000g for 5 minutes at 4°C. Supernatant was collected as the cytoplasmic fraction and the nuclear pellet was washed in Cyto buffer, centrifuged at 1000g for 5 minutes at 4 °C. The pellet was resuspended in RIPA buffer (containing cOmplete Ultra protease and PhosSTOP phosphatase inhibitor cocktail, both from Roche), incubated for 15 minutes at 4°C, and cleared by centrifuging at maximum speed for 15 minutes at 4 °C. Nuclear lysates were stored at -20 ^{o}C for future use.

2.2.7 Immunoprecipitation

The protein concentration of FAK WT SCC and FAK -/- SCC nuclear lysates was quantified using micro BCA protein assay kit (Thermo Scientific) as per manufacturer's instructions. 1 mg of nuclear lysates was used for immunoprecipitations experiments along with 2 µg of target antibody and the same amount of IgG. Immunoprecipitations were incubated overnight at 4 ^{o}C with rotation. The following day, the immunoprecipitation samples were washed 3x with RIPA and 2x with PBS before being boiled in 20 µl of 2x Laemmli sample buffer for 10 minutes. The samples were then analysed by western blot analysis as described in this section.

2.2.8 Western blotting

Western blotting was performed using BioRAD system following manufacturer's instructions. Protein concentration was determined using a micro BCA protein assay

kit (Thermo Fisher Scientific) as per manufacturer's instructions. In summary, samples were prepared for immunoblotting by boiling lysates for 5 minutes in 10 µl of Laemmli sample buffer (2x). For protein lysates, between 5-40 µg was always loaded. Immunoprecipitations were prepared by boiling for 10 minutes in 20 µl of Laemmli sample buffer (2x), in which the whole volume was loaded into gel wells. One well was always loaded with 5 µl of Amersham™ ECL™ Rainbow™ Marker - Full range (Sigma-Aldrich) to determine the molecular weight of the protein bands of the samples during imaging. Samples were ran on 5-14% gradient MINI-protein gel (Bio-Rad laboratories) at 100 V for 50 minutes in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad laboratories). Then the gel was transferred to a nitrocellulose membrane using Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs (Bio-Rad laboratories) in a Transblot turbo Bio-Rad Power Pack (Bio-Rad laboratories) following the manufactures instructions. Specifically, the transfer pack was placed in a Bio-Rand Power Pack and transferred at 25 V for 10 minutes. The membrane was then blocked in 5% BSA or 5% milk in PBS + 0.1% Tween for one hour, with shaking. The primary antibody was put on at the concentration specified in **Table 2.3** for that particular antibody primary in 5% BSA or 5% Milk with 0.1% Tween, overnight at 4 ^{o}C with shaking. The following day the membranes were washed three times in PBS + 0.1% Tween, with 15 minutes incubation during washes. A mouse or rabbit secondary antibody was applied (dependent on the species of the primary antibody) in 1/5000 or 1/10000 dilution, respectively, in blocking buffer (same buffer used for primary antibody incubation). The blots were then washed three times with PBS + 0.1% Tween, with 15 minutes incubation during washes. The blots were then developed using Pierce ECL Western blotting substrate (Thermo Fisher Scientific) following the manufacturer's instructions and blots were imaged using the Universal Hood 3 Bio-Rad Imager (Bio-Rad Laboratories). Chemiluminescence images were taken at various exposures dependent on the intensity of the antibody staining on the membrane. Additionally, a colourimetric image to visualise protein markers, which was then overlaid onto the chemiluminescence image to confirm the protein bands on the gel match the molecular weight of the protein of interest. Immunoblots were quantified for densitometry analysis using Image J.

2.2.9 Rapid immunoprecipitation of endogenous proteins

FAK WT and FAK -/- SCC cells were incubated in 1% Formaldehyde-supplemented 1 x MEM for 8 minutes with gentle agitation before quenching in 0.2 M Glycine. Cells were then collected in PBS and fractionated in LB1, LB2 and LB3 buffers (buffer compositions can be found in Mohammed *et al*., 2016). Nuclei were sonicated with 5x1 minutes pulses at high power with 2 minutes rest in between on ice using the Diagenode Biorupter (Diagenode). Lysates were cleared and FAK was immunoprecipitated using an anti-FAK 4.47 agarose conjugated antibody. After extensive washing (10x) in RIPA and 2x in 100 mM Ammonium Bicarbonate (AMBIC) solution, beads were snap frozen and submitted for LC-MS/MS analysis.

2.2.10 Streptavidin pull down experiments using BirA* system

FAK WT BirA*, FAK KD BirA* or FAK -/- BirA* EV SCC cells were incubated with 50 µM Biotin overnight prior to lysis in RIPA buffer. For the nuclear interactome analysis, FAK WT BirA*, FAK KD BirA*, BirA* EV SCCs or HIC-5 BirA* FAK WT, HIC-5 BirA* FAK -/-, FAK-/-BirA EV control SCC cells were incubated with 50 µM biotin prior to fractionation following the 'Biochemical fractionation protocol' outlined in this section. Whole cell lysate or nuclear lysate samples were sonicated using a Diagenode BioRuptor (Diagenode), 3x1 minute pulses at high power with 2 minutes rest in between on ice and cleared by centrifugation at maximum speed for 15 minutes at 4°C. Protein concentration was determined using a micro BCA kit and up to 2 mg of protein lysate was incubated with MyOne C1 Streptavidin Dynabeads beads (Life Technologies) overnight at 4$_{o}$C with rotation. Beads were washed 3x in RIPA and 2x in PBS, snap frozen and submitted for LC-MS/MS analysis. For validation experiments the beads were washed 3x in RIPA and 2x in PBS and then boiled in 2x Laemmli sample buffer for 10 minutes before immunoblot analysis.

2.2.11 Proteomics – In bead digestion

In bead digestion of RIME and BirA* proteomic samples was performed by Dr N. Quinn (Dr A. Von Kriegsheim group, IGMM, University of Edinburgh). The prepared samples following the 'RIME' or 'Streptavidin pull down experiments using BirA*

system' protocol detailed in this chapter were subjected to in bead digestion for preparation MS analysis. First, the peptides were eluted from the beads by adding 60µl MS Elution buffer 1 (containing 1 µg/µl trypsin) to the beads, which were incubated on the thermomixer for 30 minutes at 27 ^{o}C. The samples were centrifuged at 14000 rpm for 30 seconds at 4 ^{o}C and the supernatant was then transferred to a new Eppendorf tube. Then 25 µl of MS Elution buffer 2 was added to the sample before centrifuging at 14000 rpm at 4 ^{o}C for 30 seconds. The supernatant was then collected into a new tube and incubated overnight at 4 ^{o}C. The following day, 20 µl of 5 mg/ml iodoacetamide was added to the samples and then the samples were incubated in the dark for 30 minutes at room temperature. The digestion was stopped by adding 1 µl of 100% Trifluoroacetic Acid to the samples. The C18 column was activated by loading 30 µl of 80% Acetonitrile/Trifluoroacetic Acid 0.1% followed by 30 µl 0.1% Trifluoroacetic Acid. The sample was loaded onto the activated column, which was then washed 2x with 25 µl of 0.1% Trifluoroacetic Acid. The peptides were eluted with 25 µl of 0.1% Trifluoroacetic Acid into fresh tube and dried in a SpeedVac. The pellets were then resuspended in 12 µl of 0.1% TFA for MS analysis. MS analysis was performed in the Edinburgh Cancer Research Centre Proteomics facility using the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific).

Dr A. Bryon carried out the label-free quantification (LFQ) of mass spectrometry data (MS) using MaxQuant (v 1.5.7.4). Peptide lists were searched against the UniProt mouse database (downloaded on 5 July 2017) and the Andromeda search engine for common contaminants. Modification settings were – carbamidomethylation (fixed), methionine oxidation (variable), N-terminal glutamine cyclisation (variable) and protein N-terminal acetylation (variable). For BioID experiments, in addition to the aforementioned modification settings, had lysine biotinylation set as a variable modification (up to five modifications per peptide). The minimum length of a peptide used for downstream analyses was seven amino acids and one peptide ratio or more was required for LFQ. Proteins that matched to common contaminants or to the reversed peptide were excluded.

Statistical analysis was performed using Perseus (version 1.5.2.6) in which all the test vs control sample LFQ intensities were compared using two-tailed Student's t-test or one-way ANOVA with multiple comparisons, depending on the number of groups to be compared.

2.2.12 Immunofluorescence

FAK WT BirA*, FAK KD BirA*, and FAK -/- BirA* EV SCC cells were plated on coverslips and left to adhere for 24 h prior to fixation in 3.7% formaldehyde in PBS. Coverslips were washed 3x in TBS, blocked in 5% BSA/PBS for 30 minutes, washed 3x in PBS and incubated with primary antibody diluted in block overnight. Coverslips were then washed a further 3x in TBS, incubated with Streptavidin Alexa Fluor 488 or anti-rabbit Alexa Fluor 488 (both from Invitrogen), diluted in block for 30 minutes, washed and mounted in Vectashield Antifade mounting medium with DAPI (Vector laboratories). Slides were imaged using an Olympus FV-1000 confocal microscope (Olympus).

2.2.13 Assay for Transposase-Accessible chromatin using sequencing (ATAC-seq)

For ATAC-seq sample preparation, 200,000 FAK WT, FAK -/-, FAK KD cells and 100,000 FAK NLS SCC cells were harvested by trypsinisation and centrifuged at 600 g at 4 ^{o}C for 5 minutes. The cell pellet was gently washed with 50 µl of PBS and centrifuged at 600 g for 5 minutes. The cell pellet was gently resuspended with ATAC-seq lysis buffer and then centrifuged immediately for 10 minutes at 600 g. The supernatant was discarded and the transposition reaction set up using the Nextera DNA Flex library Prep kit (Illumina). Specifically, the reaction mix was set up using 25 µl of TD reaction buffer, 4.7 µl of 2x TDE1 in the FAK WT SCCs, FAK-/- and FAK KD SCCs or 2 µl of 1x TDE1 of FAK NLS, before making up the remainder of the reaction mixtures up to 50 µl with nuclease-free H_20. The nuclei pellet was resuspended in the transposition reaction mix and incubated for 30 minutes on the Eppendorf thermal mixer, before purification using the Qiagen MinElute PCR purification kit (Qiagen) and DNA elution using 10 µl of buffer EB. PCR was then amplified using 10 µl transposed DNA, 10 µl nuclease free water, 2.5 µl of barcoded PCR primer 1, 25 µl of

barcoded PCR primer 2 and 25 µl of NEBNext High-Fidelity 2x PCR master mix (NEB). The following cycling conditions were used: 1x cycle (5 minutes at 72 ^{o}C, 30 seconds at 98 ^{o}C), followed by 5x cycles (10 seconds at 98 ^{o}C, 30 seconds at 63 ^{o}C, 1 minute at 72 ^{o}C). This was followed by a qPCR to determine the amount of cycles needed to amplify library. A qPCR was set up using 5 µl of previously amplified DNA, 0.4 µM of barcode primer 1, 0.4 µM primer 2, 5 µl of 2x SYBR green and 5 µl of NEB PCR master mix (New England BioLabs). The following cycling conditions were used for the qPCR: 1x cycle 30 seconds at 98 ^{o}C, 20x cycles (98 ^{o}C for 10 seconds, 63 ^{o}C for 30 seconds and 1 minute at 72 ^{o}C.). In order to calculate the additional cycles numbers required, the linear Rn was plotted versus cycle number before determining the cycle number that corresponds to one-third of the maximum fluorescent intensity of each sample. Then the remaining 45 µl PCR reaction was PCR amplified following the cycling template as follows: 1x cycle 30 seconds at 98 ^{o}C followed by the N cycles of 10 seconds at 98 ^{o}C, 30 seconds at 63^{o}C, 1 minute at 72 ^{o}C. The amplified library was then purified using Qiagen Minelute PCR purification kit (Qiagen) and DNA was eluted in 20 µl EB buffer.

In order to remove large DNA fragments Agencourt AMPure XP magnetic beads (Beckman Coulter) (0.55x of the sample volume) was mixed with the DNA sample and incubated for 5 minutes at room temperature. Then the samples were added to the magnetic stand and the supernatant was collected. In order to remove excess adapter sequences, Agencourt AMPure XP magnetic beads (1.25x of the sample volume) were incubated for 5 minutes with the DNA sample before being put on to the magnetic stand. When the sample went clear the supernatant was discarded and the beads were kept. The beads were then washed twice with 500 µl of 80% ethanol with a 1 minute incubation time. Then the ethanol was completely removed and the Eppendorf tubes were removed from the magnetic rack and resuspended in 23 µl of nuclease-free H_20. The samples were then incubated for 1 minute before being put back on the magnetic stand and the eluted DNA from the beads was stored in DNA lo-binding Eppendorfs at 4 ^{o}C. The DNA samples were quantified using Qubit (Thermo Fisher Scientific) and run on the Agilent Bioanalyser 2100 (Agilent) following the manufacturer's instructions. The deep sequencing was performed at the University of Glasgow's

sequencing service by Dr M. Bailey using a HiSeq 4000 Paired-end sequencing was performed on all samples. All read lengths were 100 bp.

Dr R. Upstill-Goddard performed the adapter trimming and alignment of the ATAC-seq data to the *mus musculus* reference genome mm10 using the bcbio-nextgen ATAC-seq pipeline. The quality control was performed using FastQC and adapter sequences trimmed from sequencing reads using atropos. Furthermore, sequence alignment was performed using bowtie, while greylisting was performing using chipseq-greylist. Accessible regions (i.e. ATAC-seq peaks) were called from the BAM files using MACS2 (v. 2.1.1.20160309), implementing the following parameters: -B -broad -q 0.05 –nomodel -shift -100 -extsize 200 -g 1.87e9. Details of read alignment and number of ATAC-seq peaks identified per sample are included in **Table 2.8**.

Table 2.8 ATAC-seq general statistics

Sample Name	**% Duplications**	**Error rate**	**Reads Mapped (x10$_6$)**	**% Mapped**	**Total sequences (x10$_6$)**	**% GC**	**Total ATAC-seq peaks**
FAK WT1	32.80%	1.92%	82.9	63.40%	130.9	46%	53728
FAK WT2	35.60%	1.78%	77.6	69.60%	111.5	44%	39056
FAK -/-1	31.80%	1.94%	82.2	62.80%	131	44%	43153
FAK -/-2	32.90%	1.94%	81.6	61.00%	133.8	44%	35256
FAK NLS1	36.70%	2.05%	65	57.60%	112.9	46%	24402
FAK NLS2	46.10%	1.80%	77.7	64.80%	120	44%	24402
FAK KD1	20.20%	2.14%	57.7	56.80%	101.5	46%	32961
FAK KD2	26.20%	2.01%	55.4	60.50%	91.6	46%	45956

Differentially accessible regions between the FAK WT and that of the FAK -/-, FAK NLS and KD SCCs were identified using the R/Bioconductor package DiffBind. Differential peak calling was performed for each pairwise comparison and significantly differential peaks were defined as those with a FDR (false discovery rate) equal or below 0.05. Default parameters were used which can be found at https://bioconductor.org/packages/release/bioc/manuals/DiffBind/man/DiffBind.pdf.

Dr R. Upstill-Goddard performed the motif enrichment within differentially accessible ATAC-seq peaks (motif enrichment analysis) using HOMER (v.5.9), which identifies motif binding sites (i.e. genomic regions that matches known transcription factor motifs) in the differentially accessible peaks. The default parameters were used for the motif enrichment analysis using HOMER (genome masked -mask, region size -200, motif length -12, mismatches -2, -p1, number of motifs to find –s 25).

ATAC-seq peaks were assigned to genes using ChIPseeker, where each peak was assigned to the gene with the closest transcriptional start site (TSS). The parameters used for ChIPseeker were tssRegion=c(-500, 2000), annoDb = "org.Mm.eg.db", TxDb = TxDb.Mmusculus.UCSC.mm10.knownGene.

2.2.14 Chromatin immunoprecipitation

For chromatin immunoprecipitation experiments, $4x10_6$ FAK WT SCC and FAK KD SCC cells were plated on 10cm dishes (Corning). The following day, the medium was aspirated and the cells were incubated for 5 minutes in 7 ml of 1% formaldehyde in 1x MEM (no supplements or FBS added). The formaldehyde containing medium was then aspirated and the cells were incubated for 5 minutes in 200 mM Glycine in 1x MEM (no supplements or FBS added). The cells were then washed with 10 ml of PBS prior to incubation for 10 minutes in 7 ml of ChIP lysis buffer with PhosSTOP™ and cOmplete™ ULTRA Tablets, Mini EASYpack Protease Inhibitor Cocktail tablets added (both from Roche). The cells were then scraped in 1 ml of ChIP lysis buffer and centrifuged at 3000 rpm for 3 minutes at 4 ^oC. The pellet was then resuspended in 100 µl of SDS lysis buffer and 400 µl of ChIP dilution buffer. Samples were sonicated using the Diagenode BioRupter at high power for 10 minutes with 30 seconds on/off,

which causes shearing of DNA fragments to 1000-200 bp in optimisation studies. The samples were then centrifuged at 13000 rpm for 15 minutes at 4^{o}C. The supernatant was collected in a new tube and 50 µl taken for an input sample. A mixture of Dynabeads Protein A (Invitrogen) and Protein G (Invitrogen) Dynabeads were washed three times with 500 µl of cold 1xRIPA 150mM NaCl with PhosSTOP^{TM} and cOmplete™ ULTRA and Mini EASYpack Protease Inhibitor Cocktail tablets (both from Roche). Then 50 µl of the bead mixture was added to the sonicated lysate along with 0.48 µg of JUN antibody or equal amount of rabbit IgG (both from CST). The immunoprecipitations were incubated with rotation at 4 ^{o}C overnight. The following day the beads were put on a magnetic stand and washed with 1000 µl cold 1x RIPA 150 mM NaCl with PhosSTOP^{TM} and cOmplete™ ULTRA and Mini EASYpack Protease Inhibitor Cocktail tablets (both from Roche), 1000 µl of cold RIPA 500 mM NaCl and twice with cold Tris-EDTA (TE) buffer. On the last Tris-EDTA wash, the beads were transferred to a new tube, the TE buffer was removed and resuspended in 200 µl of ChIP direct elution buffer. Then 150 µl 150 mM RIPA NaCl buffer and 8 µl of 10% SDS solution was added to the input sample. Both the input and immunoprecipitation samples were incubated overnight at 65 ^{o}C. The following day the input and immunoprecipitation samples were incubated with 2 µl of RNaseA/T1 (Ambion) for 30 minutes at 37 ^{o}C. Then the input and immunoprecipitation samples were incubated with 5 µl of 10 mg/ml proteinase K (Ambion) for 1 hour at 55 ^{o}C. The DNA was then purified using the QIAquick PCR purification kit (Qiagen) following manufacturer's instructions. ChIP and input DNA were eluted in 30 µl EB and subjected to qPCR analysis using primers capturing the JUN motif region on the *IL33* enhancer and those direct against upstream of the *IL33* enhancer with no known AP-1 binding motif to correct for background binding. Left over DNA from FAK WT SCC lines ChIP input sample was used to generate a standard curve to check and normalise results to primer efficiencies. The ChIP-PCR data was analysed by the standard % input method.

2.2.15 Chromatin extraction

The chromatin extraction protocol was taken from McAndrew *et al.*, 2016. Briefly, 1.5x10^{6} of FAK WT SCC and FAK -/- SCCs were plated on 10 cm dishes overnight.

The following day, the cells were washed twice with cold PBS prior to being harvested in 400 µl of Chromatin extraction buffer containing NP-40. The lysates were then centrifuged for 5 minutes at 6500 g and the nuclear pellet was washed with 400 µl with Chromatin extraction buffer without NP-40. The lysate was centrifuged for 5 minutes at 6500 g and the pellet was resuspended in 400 µl of No-salt buffer and was incubated with rotation at 4 ^{o}C for 30 minutes. Then the samples were centrifuged for 5 minutes at 6500g and the pellet resuspended with 160 µl of high salt solubilisation buffer and was incubated with rotation at 4 ^{o}C for 30 minutes before being centrifuged for 10 minutes at 21000 g. Proteins were precipitated by adding 16 µl TCA. After incubating on ice for 15 minutes the samples were then centrifuged for 15 minutes at 21000 g and the resulting pellet was washed twice with 500 µl of ice cold acetone. The pellets were air-dried and resuspended in 20 µl of 2x Laemmli sample buffer.

2.2.16 RNA extraction

RNA extraction was performed using RNeasy Mini kit (Qiagen) following manufactures instructions. Cells were always collected in 350 µl of RLT buffer with 0.1% β-mercaptoethanol added.

2.2.17 cDNA synthesis

cDNA synthesis was performed using the SuperScript™ First-strand Synthesis System (Invitrogen) following the random hexamers protocol in the manufacturer's instructions (1 µl of Random hexamers was used in step 1). 2-5 µg of RNA was converted to cDNA on an assumed 1:1 ratio.

2.2.18 qRT-PCR and ChIP-qPCR

qRT-PCR and ChIP-qPCR analysis was performed using SYBR green mastermix (Thermo Scientific) following manufacturer's instructions. The reaction volume in each well was always a total of 10 µl and 10 µM of primers were used. The following cycling conditions were used: 98^{o}C for 10 seconds, 30x (98 ^{o}C for 10 seconds, 60 ^{o}C for 1 minute and 72 ^{o}C for 4 minutes) and 72 ^{o}C for 5 minutes.

2.2.19 mRNA-sequencing (mRNA-seq)

RNA was extracted from FAK WT, FAK -/-, FAK NLS and FAK KD SCC cells using RNA-easy kit (Qiagen) following manufacturer's instructions, whereby 350µl of buffer RLT with 0.1% β-mercaptoethanol was used for the initial step of the protocol. To check the samples for purity, the samples were run on the Agilent 2100 Bioanalyser using the Bioanalyzer RNA 6000 pico assay (Agilent). Samples that achieved a RNA integrity number (RIN) of 8 or above were considered a suitable quality for sequencing. The RIN value obtained from each sample are listed in **Table 2.9**.

Table 2.9 RIN values for mRNA-seq samples

Sample	RIN value
FAK WT 1	9.6
FAK WT 2	9.6
FAK WT 3	9.7
FAK -/- 1	9.7
FAK -/- 2	9.6
FAK -/- 3	9.7
FAK KD 1	9.6
FAK KD 2	9.7
FAK KD 3	9.6

The samples were then shipped to BGI Hong Kong for paired-end sequencing using the Illumina HiSeq4000. BGI prepared the RNA for sequencing using the TruSeq RNA Library Prep Kit v2 (Illumina) following Low Sample (LS) protocol. In short, the protocol consisted of purification and fragmentation of the mRNA, first and second strand cDNA synthesis, repairing and acetylation of the 3' cDNA ends, adapter ligation, PCR amplification, validation using the Agilent 2100 DNA High Sensitivity kit (Agilent) and normalisation and pooling of libraries for sequencing. All read lengths were 100 bp. General statistics of the mRNA-seq data is reported in **Table 2.10**.

Table 2.10 mRNA-seq general statistics

Sample Name	Clean Reads ($x10^6$)	Clean bases ($x10^8$)	Q20(%)	GC(%)
FAK WT1	70.3	70.3	98.20%	50.90%
FAK WT2	84.7	84.7	98.10%	51.00%
FAK WT3	78.1	78.1	98.20%	50.80%
FAK -/-1	75.9	75.9	98.10%	51.10%
FAK -/-2	57.6	57.6	98.10%	51.00%
FAK -/-3	72.9	72.9	98.00%	53.10%
FAK KD1	77	77	97.10%	50.90%
FAK KD2	75.5	75.5	98.20%	50.80%
FAK KD3	78	78	98.00%	51.00%

Alignment to the *Mus musculus* GRCm38 reference transcriptome was performed using the pseudoalignment software kallisto (v.043.1). Default parameters were used which can be located at https://pachterlab.github.io/kallisto/manual. Transcript abundance was summarised to gene level (EnsDb.Mmusculus.v,79) and imported into differential expression analysis package R package DESeq2 (2.1.24.0) for analysis using the R package tximport (1.2.0). Genes which had zero read counts were removed prior to differential expression analysis.

Differential expression analysis was performed using DESeq2 (2.1.24.0) using default parameters, where FAK WT vs FAK -/- SCC cells, as well as FAK WT vs FAK -/- SCC cell lines read counts were compared. Default parameters can be found at https://bioconductor.org/packages/release/bioc/manuals/DESeq2/man/DESeq2.pdf.

The Wald test was used for hypothesis testing in DESeq2 and all P-values were corrected for multiple testing using the Benjamini-Hochberg method. Transcripts which acquired a corrected P-values below 0.05 were considered statistically significant between cell lines tested in the differentially expression analysis. Furthermore, genes that also required a fold change of below or above – 1 (halved) or +1 (doubled) were taken for further gene ontology analysis using IPA as described in section 2.2.22 and hierarchical clustering analysis.

For hierarchical clustering analysis (**Figure 4.2**), the differential expression data was first z-scored before performing Pearson correlation with an average-linkage matrix using Cluster 3 (v.1.59) and data was visualised in Java TreeView (v.1.1.6).

2.2.20 Inverted invasion assays

To prepare the invasion assays, 100 µl of growth-factor reduced Matrigel/PBS (1:1) mix was added inside a transwell (corning) which was inserted into a 12 -well plate (Corning) and incubated for 30 minutes at 37 ^{o}C. The plate was turned upside down and 4x10^{4} FAK WT SCC, FAK -/-PLKO and the FAK -/- RBMS3 shRNA1-2 SCCs were placed on the bottom of the transwell, the bottom of the plate was carefully put on top and was incubated for 4 hours at 37 ^{o}C. The transwells were then washed three times in PBS. The transwells were transferred to a new 12-well plate (Corning) with 1 ml of serum free MEM media (containing 1% MEM Vitamin solution, 1% MEM Non-essential amino acids, 1% Sodium Pyruvate, and 1% L-Glutamine) at the bottom of the wells and 100 µl of complete SCC MEM media (with 10% FBS as the chemoattractant) inside the transwell. Following a 3 day incubation at 37 ^{o}C, 500µl of calcein/PBS (1:1000) was added to the bottom of the well and to the transwell. After a 1 hour incubation at 37 ^{o}C, the cells were imaged on an Olympus FV1000 confocal microscope (Olympus). Z-stack images were taken with 10 µm sections up to 260 µm of the matrigel and the % of total area of cells migrating beyond 80 µm through the matrigel were summed and expressed as relative to the FAK -/- PLKO results.

2.2.21 Network analysis

Network analysis was performed using the Qiagen Ingenuity pathway analysis (IPA). In Chapter 3, the following parameters were used in all network construction unless otherwise stated: Database sources (Ingenuity expert information, BioGrid, InAct), direct interaction, experimentally observed, protein-protein, all species. In Chapter 5, the same settings as just described above were used except only mammalian interactions were shown and functional interactions (i.e. protein-DNA interactions) were also indicated in the network. All networks were exported in the program Cytoscape for final figure presentation.

2.2.22 Functional annotation analysis

Functional annotation analysis was performed either by using IPA and DAVID gene ontology (https://david.ncifcrf.gov/). For the IPA GO analysis the protein list as determined by MS was uploaded to the IPA software and a disease and functions core analysis was performed. For the DAVID gene ontology analysis, the protein list was uploaded to the functional annotation tool using the *mus musculus* as the background reference. All terms with a Benjamini-Hochberg corrected P-value above 0.05 was considered statistically significant.

2.2.23 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 (GraphPad Software, CA, USA). All P-values above 0.05 were considered statistically significant.

Chapter 3 FAK interacts with a network of focal adhesion proteins and transcriptional regulators

3.1 Introduction

As described in the Introduction, nuclear FAK regulates expression of genes and is present in the chromatin fraction isolated from SCC cells (Serrels *et al*., 2015, **Figure 3.1**). However, the mechanism by which FAK regulates gene expression has yet to be established. In order to investigate the function of FAK in the nucleus, I performed nuclear interactome proteomics in order to identify the range of interaction partners of FAK in the nucleus.

Use of standard proteomic techniques has shown that FAK binds to a putative network of over 1500 proteins in the nucleus (Serrels *et al*., 2015). With such a large dataset, it is very difficult to identify true and direct interaction partners of FAK. In order to isolate interactions that are more likely to be direct in comparison to standard proteomics techniques, I used proximity-based proteomics approaches - biotin-ligase tagging (BioID) and rapid immunoprecipitation of endogenous proteins (RIME). In **section 3.2.1**, I follow-up a novel nuclear interaction partner of FAK, namely HIC-5, a protein that shuttles between the cytoplasm and the nucleus, which I detected in FAK nuclear proximity-based datasets. To determine the function of this novel FAK interaction partner in the nucleus, I also performed BioID proteomics to identify proximal HIC-5 interaction partners in the nucleus in FAK WT and FAK -/- SCC cells.

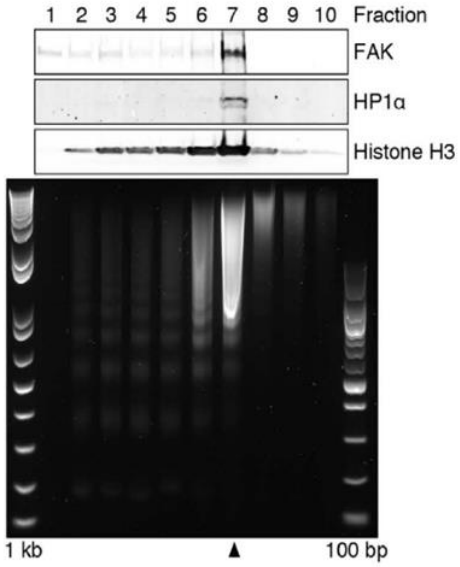


Figure 3.1 | FAK is present in the chromatin fraction. FAK WT SCC nuclei were digested with MNase and soluble chromatin ran on a sucrose step gradient. The DNA from each fraction was analysed by agarose gel electrophoresis. Fraction 7 (indicated by arrow) is the chromatin containing fraction (Figure from Serrels et al., 2015).

3.2 Aims

- To apply proximity-dependent proteomics approaches to define novel nuclear interaction partners of FAK.
- To identify key interaction partners by integrating the proximity-based proteomics datasets with that of the nuclear FAK interactome from the same cells (published in Serrels *et al*., 2015).
- To use proximity-dependent biotin labelling to investigate the potential nuclear function of a nuclear FAK interaction partner identified in my proteomics datasets, namely HIC-5.

3.3 Results

The aim of this section was to apply proteomics to identify FAK binding proteins in the nucleus. Specifically, I made use of two published protocols, RIME and BioID. These protocols have been selected as they are specifically designed for isolating proteins that are in close proximity with the target protein. In section 3.3.3, I will integrate these proximity-based proteomics datasets with the nuclear FAK interactome dataset (published in Serrels *et al*., 2015) to determine which are the most robust interaction partners of FAK in the nucleus.

3.3.1 Application of RIME to identify FAK nuclear interacting proteins

RIME is a recently developed proteomics protocol that uses some of the principles that underpin ChIP, e.g. formaldehyde cross-linking and sonication, to enrich for transcription factor protein complexes (Mohammed *et al*., 2016). The use of formaldehyde results in more efficient crosslinking of protein:DNA complexes, but also crosslinks protein:protein complexes within a space of 2Å. The cells are first fractionated to enrich for nuclear proteins. Furthermore, the samples are then sonicated which breaks apart residual non-crosslinked complexes and fragments chromatin, to ease the purification of protein complexes associated with DNA. The samples are then subjected to co-immunoprecipitation using an antibody directed against the target

protein and submitted for mass spectrometry (MS) analysis in order to define the range of proteins that are interacting with the target protein.

Firstly, I applied RIME to identify FAK protein complexes in FAK WT cells and also performed RIME in the FAK -/- SCC cells to control for background binding. Three biological replicates of protein lysates extracted from FAK WT and FAK -/- cells were nuclear fractionated, formaldehyde cross-linked, sonicated and immunoprecipitation/mass spectrometry was performed following the published RIME protocol (Mohammed *et al*., 2016). Label-free quantification (LFQ) intensities identified by mass spectrometry in the FAK WT and the FAK -/- SCC lines were statistically compared using a two-tailed unpaired t-test, in which all Benjamini-Hochberg corrected P-values below 0.05 were considered statistically significant. Benjamini-Hochberg correction controls for the fact that sometimes small P-values can be obtained by chance in large datasets, resulting in incorrect rejection of the null hypothesis. This analysis identified 398 proteins that were significantly enriched in the FAK WT SCC cells in comparison to the FAK -/- SCC cells (listed in **Table A1**), from now on denoted as the FAK RIME interactome dataset. With mass spectrometry datasets, there are many computational tools to understand how the proteins in datasets may interact physically or functionally. I have mainly used Ingenuity pathway analysis (IPA) for protein interaction network construction, which is a program that integrates a number of protein interaction databases. Due to the large size of the FAK RIME interactome dataset it was not possible to present the full network. Instead I present subnetworks of FAK's 'nearest neighbours' (i.e. known direct interaction partners of FAK), which highlighted several known FAK protein interactions, e.g. SRC, paxillin and GRB2 (Mitra *et al*., 2005) (**Figure 3.2A**). Furthermore, filtering for 'transcriptional regulator' in the FAK WT RIME dataset using IPA indicated there were a number of proteins associated with the regulation of transcription that FAK interacts with, including PFDN-1, MEG-3 and NAA15 (**Figure 3.2B**). However, the dataset did not reveal any known FAK:transcriptional regulator complexes (e.g. P53; Lim *et al*., 2008b). Next, I performed Gene Ontology (GO) enrichment analysis on the whole FAK RIME dataset in order to determine if any of the proteins identified are involved in any cancer processes known to be regulated by FAK. GO enrichment

analysis indicated that the nuclear FAK-associated proteins identified by RIME were involved in various cellular processes that FAK has been shown to regulate, including formation of the MAPKKK cascade ($P < 0.00001$), invasion of cells ($P < 0.00001$) and cell survival ($P < 0.00001$) (**Figure 3.3A**). The RIME protocol therefore identified many FAK-binding proteins involved in several FAK-regulated processes and identified 12 novel transcriptional regulators.

A more in-depth analysis of the proteins in the entire dataset using GO enrichment analysis, demonstrated that a significant proportion of the proteins identified were enriched in the cytoskeleton ($P < 0.00001$) and the ribosome ($P < 0.00001$) (**Figure 3.3B**). This suggests that the subcellular fractionation within the RIME protocol may not be efficient.

To address this, I looked at nuclear fractions of cells obtained using the RIME protocol to determine if the fractionation protocol within the RIME protocol was working efficiently. This identified that treatment of FAK NLS SCC cells with 1% formaldehyde, as per the standard published RIME protocol, results in a high level of

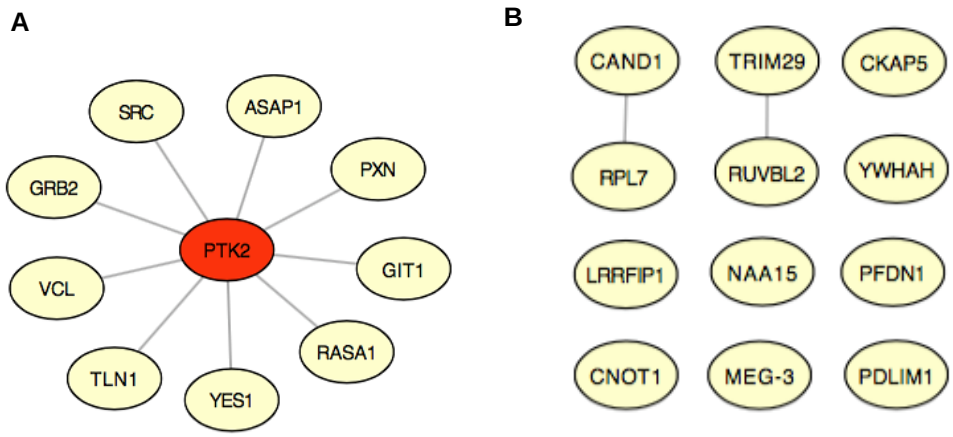


Figure 3.2 | RIME experiments pull down known direct interaction partners of FAK and novel interactions with proteins associated with transcription. FAK WT SCC and FAK -/- SCC cells were fractionated following the published RIME protocol (Mohammed *et al.*, 2016). FAK immunoprecipitations were submitted for mass spectrometry analysis and data was filtered using IPA software to enrich for A) direct interaction partners of FAK and B) transcriptional regulators. Non-specific streptavidin binding was controlled by performing a two-tailed unpaired t-test between the FAK WT SCC from that of FAK -/- SCC cell line data. Network files were imported into Cytoscape for final figure presentation. A) All known direct protein-protein interactions of FAK (red) are coloured yellow. Lines indicate protein-protein interactions. N=3 biological replicates.

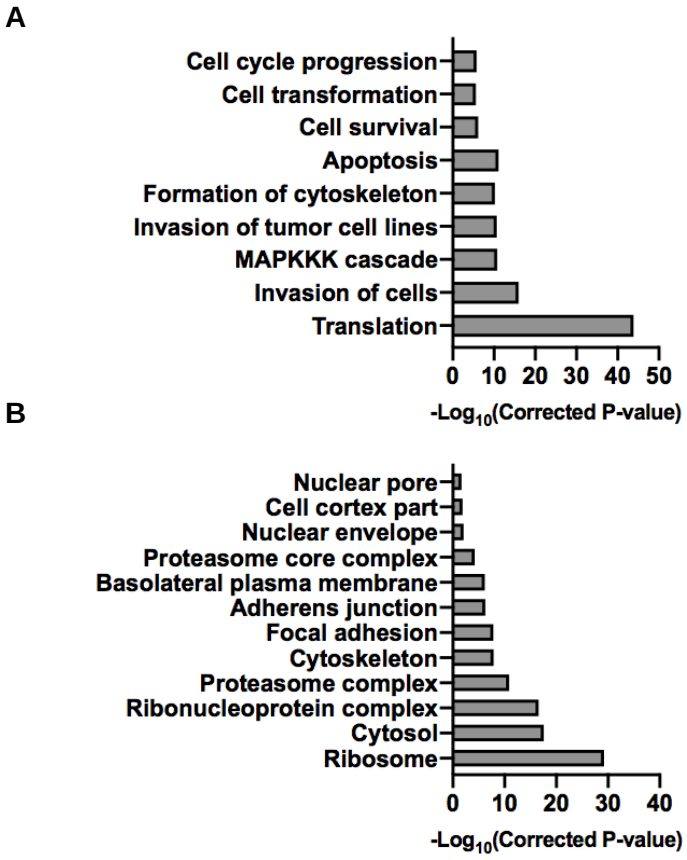


Figure 3.3 | GO analysis of FAK RIME interactome dataset. A) Subcellular localisation analysis was carried out by performing a functional annotation clustering analysis using the DAVID GO database. B) Functional analysis was carried out by performing Disease and Functions core analysis using IPA software. The –log10 was taken of all P-values.

contamination of the known cytoplasmic protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the nuclear fraction (**Figure 3.4**, lane 5). Therefore, I hypothesised that reducing the formaldehyde concentrations might reduce this contamination, as monitored by blotting for GAPDH in the nuclear fraction. Treatment of FAK NLS SCC cells with a series of formaldehyde concentrations ranging from 0.2%-0.8% showed a higher amount of the nuclear protein Poly (ADP-ribose) polymerase (PARP) when crosslinked with 0.2% formaldehyde and lower protein levels of the cytoplasmic protein GAPDH (**Figure 3.4**, first lane). However, there was still a visible amount of GAPDH present in the nuclear fractions, even at lower formaldehyde concentrations, suggesting that formaldehyde cross-linking significantly reduced the effectiveness of fractionation to purify nuclear proteins.

3.3.2 Use of BioID to determine proximal nuclear FAK interactome

A second approach that I used for the identification of nuclear FAK protein complexes was BioID. BioID is a technology designed for the identification of proteins in close proximity to the target protein, and it utilises biochemical labelling to "tag" interaction partners (Roux *et al*., 2013). The BioID technology uses a Bifunctional ligase/repressor (BirA) isolated from *E.coli* which is genetically engineered to have an active site mutation (R118G) that destabilises the retention of the activated biotin molecules (biotinyl-5'-AMP), named BirA* (Roux *et al*., 2013). When tagged to a target protein using standard cloning techniques, the BirA* enzyme can react with primary amines of proteins in close proximity to the target protein when the cells are incubated with biotin (Roux *et al*., 2013). FAK WT and FAK KD proteins that contain a BirA* tag at the C-terminus were expressed via a vector in FAK -/- SCC cells. Additionally, a vector expressing just the BirA* enzyme that is not tagged to a protein was expressed in the FAK -/- SCC cells to control for spontaneous biotinylation. Before performing any proteomics experiments, I first validated the constructs for use by checking multiple parameters, including phosphorylation, protein localisation and the ability to bind to known protein complexes, e.g. SRC, Paxillin.

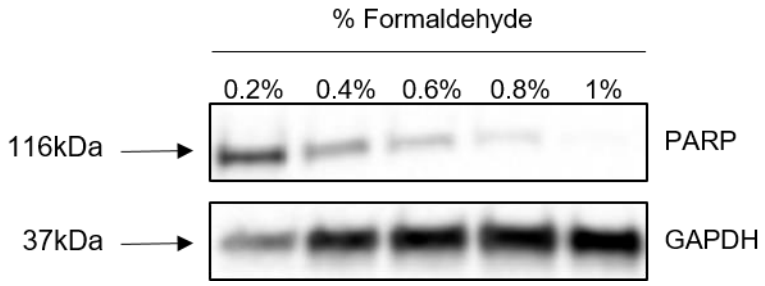


Figure 3.4 | Optimisation of RIME protocol to increase effectiveness of fractionation. FAK NLS samples were treated with a range of formaldehyde concentrations ranging from 0.2%-1%. Cells were fractionated following the published RIME protocol (Mohammed *et al*., 2016) and subjected to SDS-PAGE analysis. The blot was probed for PARP (upper panel) and GAPDH (lower panel) to determine the quality of nuclear fractionation.

To check that the BirA* tag did not disrupt the subcellular localisation of FAK, FAK WT BirA*, FAK KD BirA* and FAK -/- BirA* empty vector (EV) control cells were plated on coverslips and stained with an N-terminal FAK antibody, followed by an Alexa Fluor 488 secondary antibody. Another set of FAK WT BirA*, FAK KD BirA* and FAK -/- BirA* EV control cells were stained with a streptavidin Alexa Fluor 488 antibody. If the BirA* tag does not disrupt the localisation of the FAK proteins, I would expect to see clear focal adhesion staining of FAK. As can be seen from **Figure 3.5**, FAK WT and FAK KD BirA*-tagged proteins localise to focal adhesions. FAK is not detected in the nucleus of SCC cells, as it is not clustered by membrane lipids there and therefore is more difficult to detect by immunofluorescence (**Figure 3.5**). Yet biochemical fractionation does clearly show that FAK is present in the nucleus and increased levels of FAK have been exhibited in cancer (Serrels *et al*., 2015), as discussed in the introduction to this thesis. Another set of FAK WT BirA*, FAK KD BirA* and FAK -/- BirA* EV control cells were plated on coverslips and stained with a streptavidin Alexa Fluor 488 antibody. FAK WT BirA*- and FAK KD BirA*-expressing cells were also plated onto coverslips and stained with the streptavidin

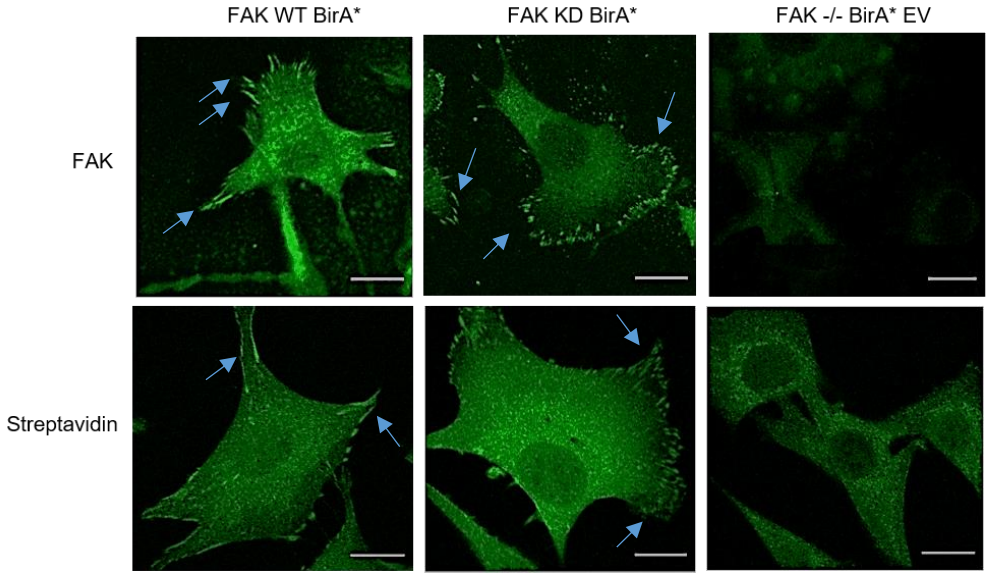


Figure 3.5 | BirA* tagged FAK localises to focal adhesions. FAK WT BirA*, FAK KD BirA* and FAK -/- BirA* EV control cells were plated onto coverslips. For FAK staining, cells were stained with an N-terminal FAK antibody, while cells stained with Streptavidin were fed with biotin before staining with a Streptavidin Alexa Fluor 488 antibody. Upper panel shows coverslips stained with N-terminal FAK antibody Alexa Fluor 488, while lower panel shows coverslips stained with Streptavidin Alexa Fluor 488. Scale bar represents 15µm. Locations of focal adhesions are indicated by blue arrows. It must be noted that there are many more focal adhesions present in the FAK WT BirA* and FAK KD BirA* images and that arrows are simply used in this case to indicate typical focal adhesion staining for non-specialists. N=1

Alexa Fluor 488 antibody, which showed all the proteins in the cells that are biotinylated by the FAK WT BirA* or FAK KD BirA* proteins (**Figure 3.5**). The FAK WT BirA* and the FAK KD BirA* predominantly biotinylated focal adhesion and cytoplasmic proteins (**Figure 3.5**). The FAK -/- BirA* empty vector cells were stained with the streptavidin Alexa Fluor 488, which indicated non-specific biotinylation carried out by the untagged-BirA* protein expressed in the FAK -/- cells. As described in the Introduction to this thesis, FAK autophosphorylates itself on Y397 to regulate its binding to SRC and activate its full catalytic activity (reviewed in Frame *et al*., 2010). Western blotting with an anti-FAK-pY397 antibody demonstrated that the kinase activity of FAK also has the capacity to auto-phosphorylate in the presence of a BirA* tag (**Figure 3.6**). Analysis of well-established FAK protein complexes demonstrated that addition of a BirA* tag does not interfere with either N-terminal Src or C-terminal Paxillin protein interactions (**Figure 3.7**). Furthermore, as expected, the interaction of FAK with SRC was kinase dependent, whereas its interaction with Paxillin was not (**Figure 3.7**, reviewed in Mitra *et al*., 2005). Collectively, these results suggest that the addition of a 30kDa BirA* tag to the C-terminus of FAK does not disrupt its sub-cellular localisation, Y397 phosphorylation and protein partner binding.

Building on these observations, we prepared three biological replicates of biotinylated whole cell lysates extracted from FAK WT and FAK -/- cell lines for MS analysis. This was done to confirm that using the BirA* system, expected FAK complexes are identified to validate the approach. LFQ intensities in the FAK WT BirA* and the FAK -/- BirA* EV control SCC lines were statistically compared using a two-tailed unpaired t-test, in which all P-values that are below 0.05 (without multiple testing

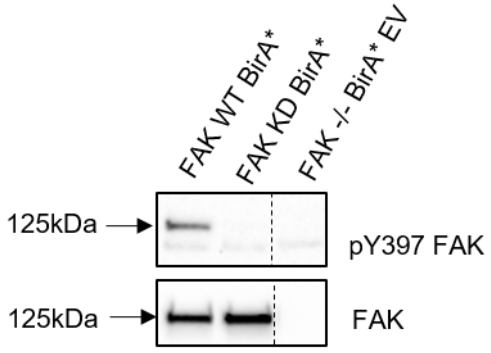


Figure 3.6 | FAK WT BirA* is phosphorylated on Y397. Whole cell lysates from FAK WT BirA*, FAK KD BirA* and FAK -/- BirA* EV control cell lines were subjected to SDS-PAGE and probed for pY397 FAK (upper panel) and FAK (lower panel). Dotted line indicates a sample in the middle of the blot was omitted due to lack of relevance. N=1 Courtesy of B. Serrels

correction) were considered statistically significant. This identified 57 significantly enriched proteins in the FAK WT BirA* dataset (listed in **Table A2**). MS analysis of whole cell lysates from FAK WT BirA* and FAK -/- BirA* EV control cells identified 9 known FAK interacting proteins, including SRC, paxillin, CRK, ASAP1 and cortactin (**Figure 3.8** – shown in pink). Furthermore, GO enrichment analysis of the reported localisation of proteins using DAVID GO analysis online software highlighted a significant overrepresentation of proteins associated with focal adhesions ($P = 0.00144$) and the cytoskeleton ($P = 0.00104$) (**Figure 3.9A**), subcellular locations at which FAK is known to be enriched. Additional GO analysis of the FAK BioID MS data using IPA indicated that identified proteins were implicated in processes known to be regulated by FAK, including cell spreading ($P < 0.00001$), invasion ($P < 0.00001$), apoptosis ($P = 0.000378$), endocytosis ($P = 0.00126$) and formation of focal adhesions ($P < 0.00001$) (**Figure 3.9B**).

To define the proximity-based nuclear interactome of the FAK WT BirA* cells, I generated three biological replicates of biotinylated nuclear lysates extracted from FAK WT and FAK -/- cell lines as described in Material and Methods. LFQ intensities

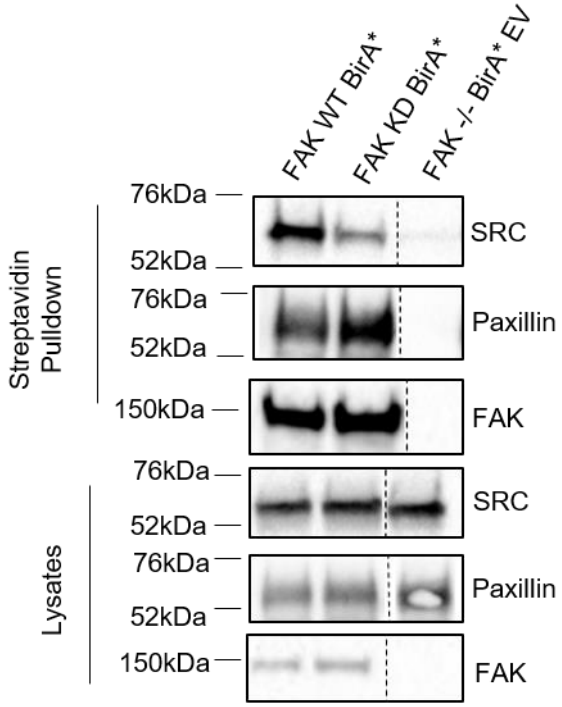


Figure 3.7 | FAK-BirA binds to known focal adhesion proteins. FAK WT BirA*, FAK KD BirA*, and FAK -/- BirA* EV (control) SCC cell lines were treated overnight with biotin, before lysis. The resulting biotinylated proteins were captured using Streptavidin C1 Dynabeads magnetic beads, separated by SDS-PAGE and probed for FAK (lower panel), SRC (upper panel) and paxillin (middle panel). Due to presence of the BirA tag, FAK molecular weight is approximately 150 kDa. N=1

in the FAK WT BirA* and the FAK -/- BirA* EV control cell lines were statistically compared using a two-tailed unpaired t-test, in which all Benjamini-Hochberg corrected P-values below 0.05 were considered statistically significant. This identified 42 significantly enriched proteins in the nuclear FAK WT BirA* dataset (listed in **Table A3**), denoted from now on as the nuclear FAK BioID interactome dataset. Interactome analysis using IPA suggested that FAK associates with a network of focal adhesion proteins in the nucleus, including HIC-5, SRC and GRB2 (focal adhesion proteins constituted 11% of the nuclear FAK WT BirA* dataset) (**Figure 3.10**). Additionally, 13 transcriptional regulators were identified as putative FAK nuclear interaction partners in this dataset, including LPP, WWOX and TOX4. To identify FAK-kinase dependent interactions, LFQ intensities were statistically compared between the FAK WT BirA* cells and FAK KD BirA* cells nuclear interactome datasets using a two-tailed unpaired t-test. All interactions which had corrected P-values of 0.05 were considered statistically significant. We found that 13 potential FAK interactors were found to be significantly enriched in the FAK WT BirA* in comparison to the FAK KD BirA* SCC cell line (**Figure 3.10** - pink and **Figure 3.11A-B**), suggesting that these are FAK kinase-dependent. These include known kinase-dependent interactors, such as SRC, CRK and ASAP1, as well as transcriptional regulators such as TOX4 and WWOX. Novel kinase-dependent interactions included PRKCDBP and MURC. The caveolae-associated protein FLOT2 was the only FAK interaction partner that was enriched in the FAK KD BirA* cells with respect to FAK WT BirA* (**Figure 3.11A-B**), suggesting this interaction may be specific in the auto-inhibited conformation.

To validate interaction partners in the nucleus that were identified in the FAK BioID interactome dataset, I performed streptavidin pulldowns using nuclear lysates from the FAK BioID cell lines, followed by western blotting. These showed that WWOX and LPP all bound to FAK KD BirA* and FAK WT BirA*, with only background binding in the FAK BirA* EV control cells (**Figure 3.12A-B**). Based on the MS analysis, WWOX is predicted to be a kinase-dependent interaction partner of nuclear FAK; however, the pulldown experiments analysed by western blotting showed that it bound to FAK independently of FAK's kinase activity (**Figure 3.12B**).

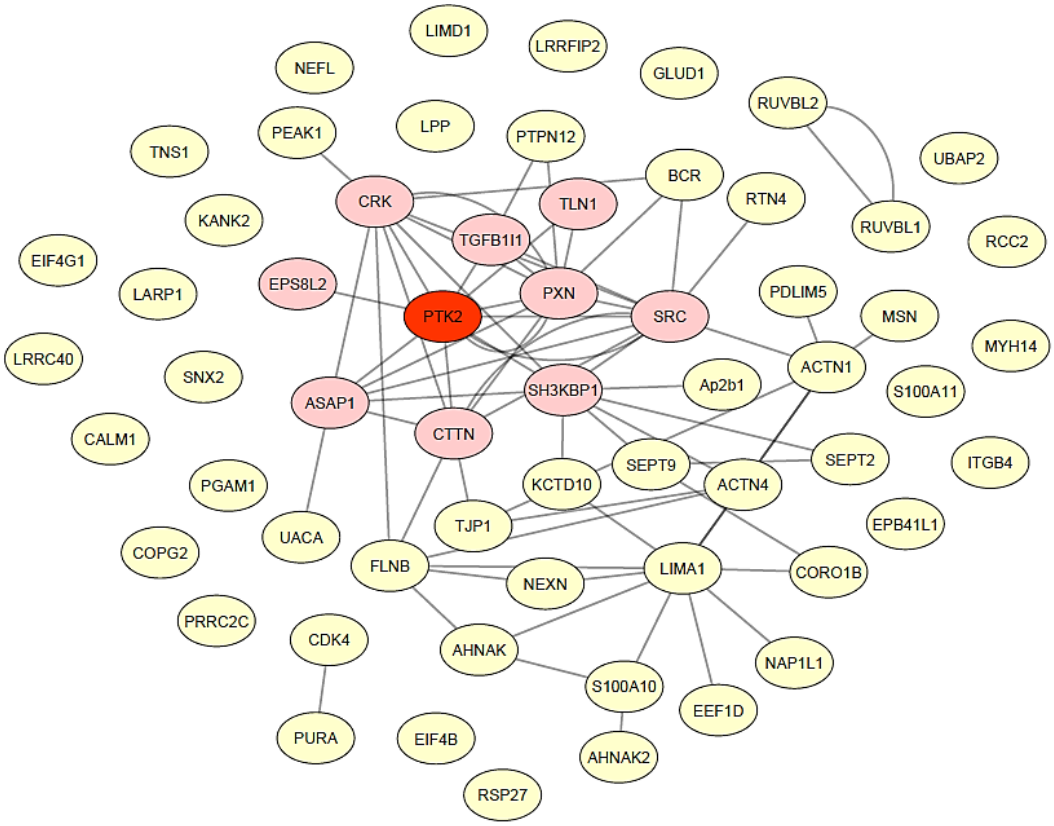


Figure 3.8 | Identification of FAK interactors in FAK WT BirA* SCC whole cell lysates using BioID. FAK WT BirA* and FAK -/- BirA* EV control cells were treated overnight with biotin before lysis. The resulting biotinylated proteins were isolated using Streptavidin C1 Dynabeads and submitted for MS analysis. Data was filtered using IPA software. Non-specific streptavidin binding was controlled by determining performing a two-tailed unpaired t-test between FAK WT BirA* data and FAK-/- BirA* EV datasets. Network files were imported into Cytoscape for final figure presentation. All known direct protein-protein interactions of FAK (red) as identified by mining BioGrid, InAct and Ingenuity Experit knowledge protein-protein interactions databases using IPA are coloured pink. Lines indicate protein-protein interactions. N=3 biological replicates.

To determine the subcellular distribution of the FAK binders in this dataset, I analysed the FAK BioID interactome protein list using the DAVID database, which showed that the identified proteins were significantly enriched for proteins reported in focal adhesion complexes ($P < 0.00001$) and cell junctions ($P < 0.00001$) (**Figure 3.13A**). Although the search did not identify an enrichment of nuclear proteins, many of the focal adhesion proteins enriched in the 'focal adhesion complexes' term have dual roles in the cytoplasm and nucleus such as Paxillin (Sauthe *et al*., 2016) and HIC-5 (Shibanuma *et al*., 2003). Furthermore, functional enrichment analysis in IPA indicated transcription was a significantly enriched term ($P = 0.000234$) (**Figure**

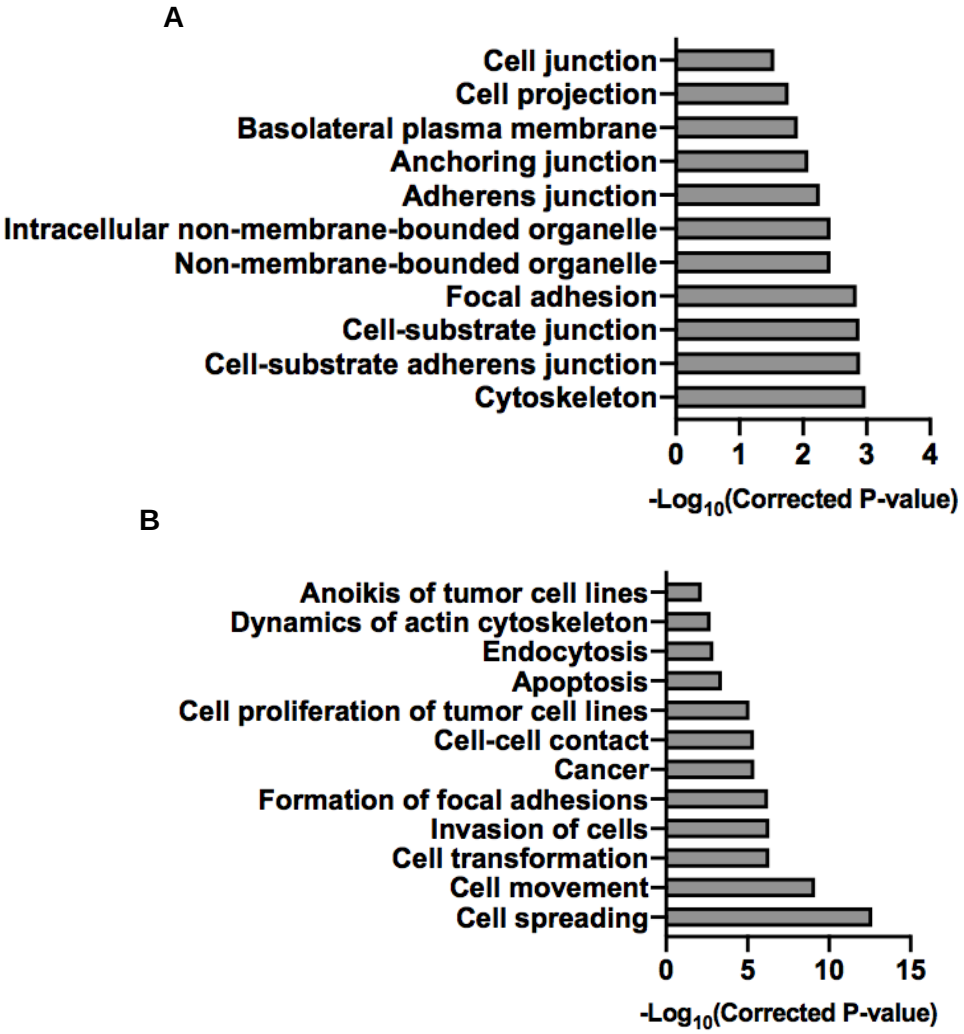


Figure 3.9 | GO analysis of FAK WT BirA* whole cell lysate dataset. A) Subcellular localisation analysis was carried out by performing a functional annotation clustering analysis using the DAVID GO database. B) Functional analysis was carried out by performing Disease and Functions core analysis using IPA software. The –log10 was taken of all P values.

3.13B), which was not the case in the FAK BioID interactome dataset prepared with whole lysates (**Figure 3.9B**). Proteins included under this term encompassed many focal adhesion proteins with known roles in transcription, such as HIC-5 (Chodankar *et al*., 2014, Li *et al*., 2011), as well as regulators of transcription which FAK has not been previously shown to bind, such as WWOX (Gaudio *et al*., 2008). Findings from the functional enrichment analysis indicated these FAK nuclear interaction proteins are implicated in a number of FAK-regulated processes including endocytosis (P = 0.00728) and anoikis of tumour cell lines (P = 0.00225). Thus, the BioID approach has

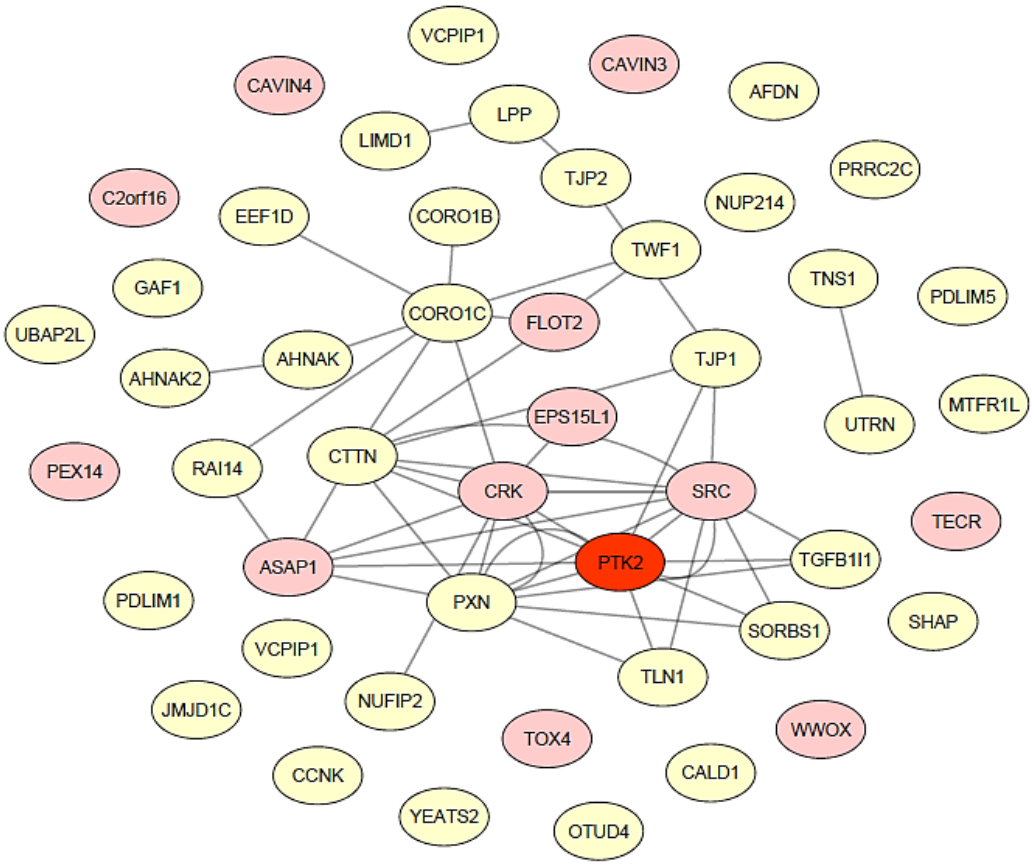


Figure 3.10 | Identification of FAK interactors in FAK WT BirA* SCC nuclear lysates using BioID. FAK WT BirA*, FAK KD BirA* and FAK -/- BirA* EV control cells were treated overnight with biotin before lysis. The resulting biotinylated proteins were isolated using Streptavidin C1 Dynabeads and submitted for MS analysis. Data was filtered using IPA software. Non-specific streptavidin binding was controlled by performing a two-tailed unpaired t-test between FAK WT BirA* data and FAK-/- BirA* EV datasets. Kinase-dependent interactions were determined performing a two-tailed unpaired t-test between FAK WT BirA* data and FAK-/- BirA* EV control datasets. Network files were imported into Cytoscape for final figure presentation. All novel kinase-dependent interactions of FAK (red), as identified by determining significantly enriched (via t-test, Benjamini-Hochberg P-value < 0.05) interaction partners in FAK WT BirA* over that of the FAK KD BirA* dataset, are coloured pink. Lines indicate protein-protein interactions. N=3 biological replicates.

successfully identified both known and novel proximal interaction partners of FAK in the sub-cellular nuclear fraction and some of these regulate transcription and other cancer-associated processes.

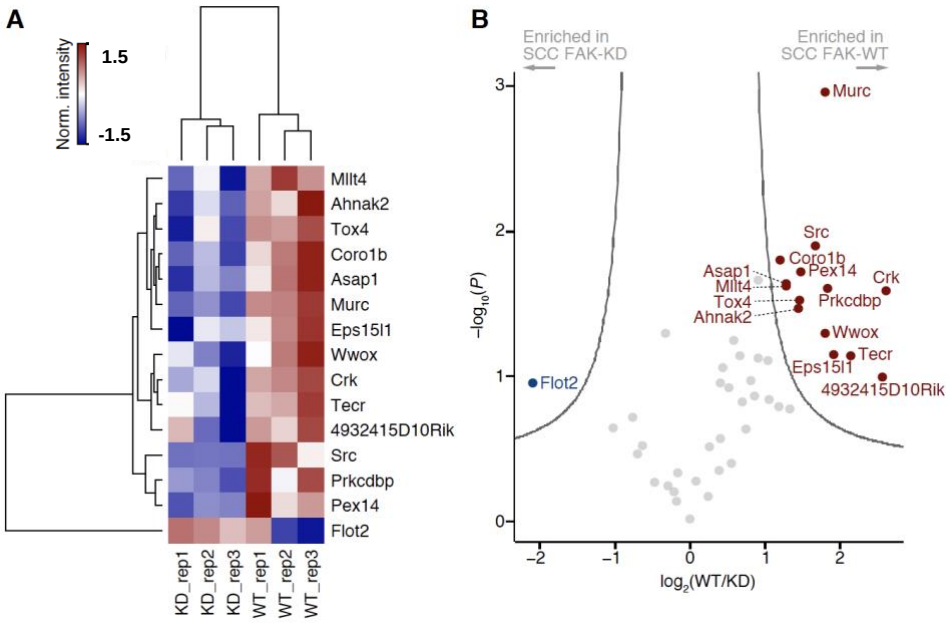


Figure 3.11 | Determination of kinase-dependent nuclear interactions of FAK. LFQ intensities between FAK WT BirA* and FAK KD BirA* dataset were tested for statistical significance using a one-way ANOVA test. A) A heatmap of the normalised LFQ intensities of the FAK-kinase-dependent interactions in FAK WT BirA* and FAK KD BirA* replicates. Normalised LFQ intensities were standardised using the *Z*-score. Scale is 1.5 to -1.5, as indicated in the key. B) A volcano plot depicting the significance of the FAK WT BirA*/FAK KD BirA* analysis on the *y*-axis and the logarithm-transformed fold change of LFQ values from FAK WT BirA*/FAK KD BirA* (WT/KD) on the *x*-axis. N=3 biological replicates. Courtesy of A. Byron.

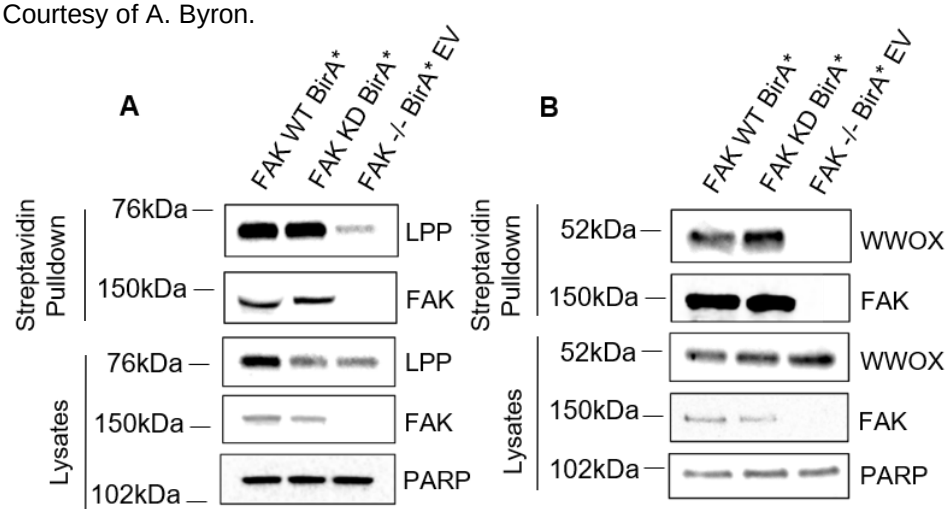


Figure 3.12 | Validation of nuclear FAK WT BirA* interactors. FAK WT BirA*, FAK KD BirA*, and FAK -/- BirA* EV SCC cell lines were treated with biotin overnight, before nuclear fractionation. The resulting biotinylated proteins were captured using Streptavidin C1 Dynabeads magnetic beads, separated by SDS-Page and probed for FAK and the nuclear marker PARP (lower panel) and A) LPP (upper panel) or B) WWOX (upper panel). Due to presence of the BirA* tag, FAK molecular weight is approximately 150kDa. N=1

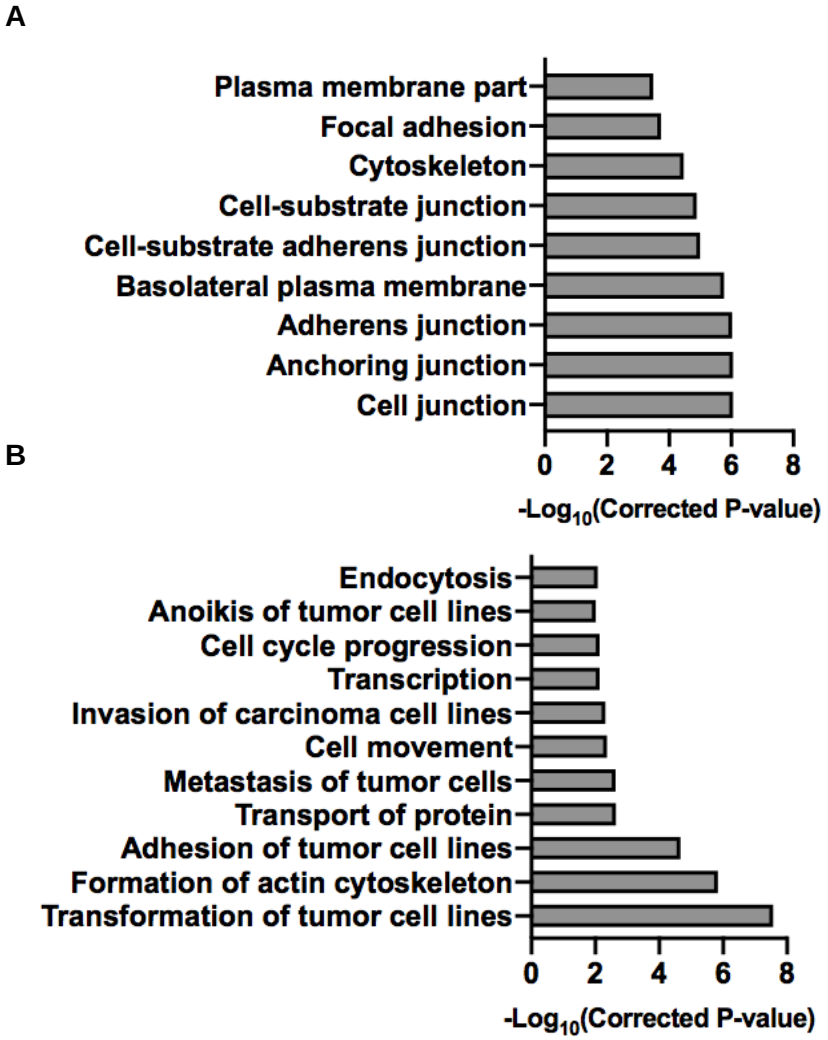


Figure 3.13 | GO analysis of nuclear FAK WT BirA* dataset. A) Subcellular localisation analysis was carried out by performing a functional annotation clustering analysis using the DAVID GO database. B) Functional analysis was carried out by performing Disease and Functions core analysis using IPA software. The –log10 was taken of all P values.

3.3.3 Common FAK nuclear interaction partners using different proteomic approaches

In order to identify robust interaction partners of FAK in the nucleus, I compared the proximity-dependent proteomics dataset with the nuclear FAK interactome dataset published in Serrels *et al.*, 2015 (**Figure 3.14**). Due to the problems with fractionation

described above, the FAK RIME interactome dataset was not included. Comparisons between the nuclear FAK interactome and nuclear FAK BioID interactome MS datasets showed that there were 16 interaction partners in common, including UTRN, RAB11FIP5, NUP214, NUFIP2, RAI14, TECR, MTFR1l, OTUD4 and notably HIC-5 (**Figure 3.14**). HIC-5 is a protein that shuttles from the cytoplasm to the nucleus (Shibanuma *et al.*, 2003) and is a known FAK binder at focal adhesions (Nishiya *et al.*, 2001). Like FAK, it has been shown to translocate to the nucleus when cells are exposed to stress signals such as hydrogen peroxide (Shibanuma *et al.*, 2003). In the nucleus, HIC-5 functions as a co-regulator for glucocorticoid receptor target genes and the C-MYC gene by recruiting P300 or sequestering β-catenin from target promoters, respectively (Chodankar *et al.*, 2014, Li *et al.*, 2011). Therefore, HIC-5 was an interesting protein interaction partner to validate by co-immunoprecipitation experiments. These showed that FAK and HIC-5 robustly interact in the nucleus (**Figure 3.15**). Thus, HIC-5 is a robust interaction partner of FAK in the nucleus, validating the approach that identified that interaction.

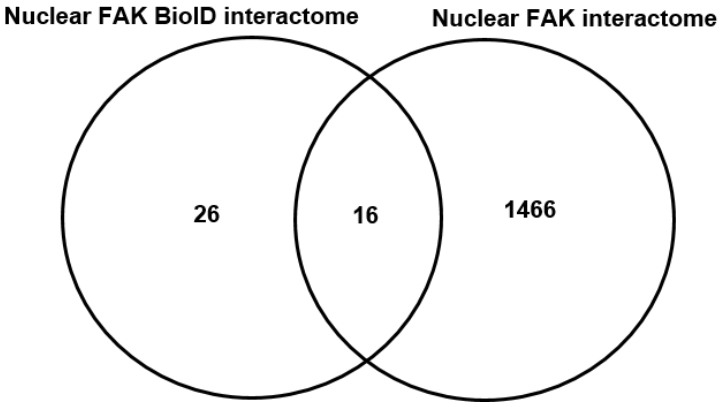


Figure 3.14 | Overlay between different nuclear FAK interactome datasets. The nuclear FAK BioID dataset and nuclear FAK interactome dataset (Serrels *et al.*, 2015) were overlaid using VENNy online software to determine common interaction partners of FAK between the datasets. N=3 for both proteomics datasets.

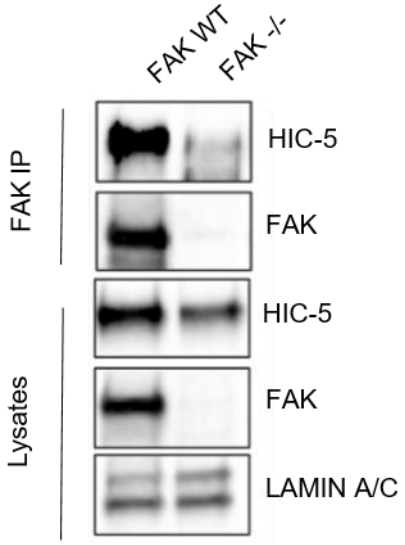


Figure 3.15 | FAK interacts with HIC-5 in the nucleus. Nuclear fractions were prepared from both FAK WT and FAK -/- SCC cells prior to immunoprecipitation with FAK antibody. Samples were subjected to SDS-PAGE and probed with antibodies to HIC-5 and FAK. Lamin A/C served as a marker for the quality of nuclear fractionation. Courtesy of B. Serrels. N=1

3.3.4 Use of proximal proteomics to determine the nuclear function of a novel interaction partner of FAK in the nucleus

Our analyses in the previous section identified HIC-5 as a nuclear interaction partner of FAK in both the FAK BioID nuclear interactome and FAK nuclear interactome datasets (Serrels *et al*., 2015).

To investigate the nuclear function of HIC-5, and to understand whether the presence of FAK can modulate the nuclear interactome of HIC-5, I generated a HIC-5 BirA* fusion protein and expressed this in FAK WT cells (denoted HIC-5 BirA* FAK WT) and in FAK -/- cells (denoted HIC-5 BirA* FAK -/-). The BirA* EV control was expressed in the FAK WT and FAK -/- SCC cells as background controls, denoted as FAK WT BirA* EV and FAK -/- BirA* EV control, respectively. To determine the nuclear HIC-5 interactome, I performed streptavidin pulldowns with three replicates of nuclear lysates extracted from each of the described cell lines and submitted these for mass spectrometry. LFQ intensities of the HIC-5 BirA* FAK WT and the FAK WT BirA* EV control SCC lines were statistically compared using a two-tailed unpaired t-test, in which all Benjamini-Hochberg corrected P-values below 0.05 were considered statistically significant. This identified 76 significantly enriched proteins

in the HIC-5 BioID nuclear interactome dataset (Listed in **Table A4**). GO enrichment analysis of the nuclear HIC-5 BirA* FAK WT MS dataset indicated there was a significant proportion of proteins reported to localise to the nuclear lumen (P = 0.0319) and the nucleoplasm (P = 0.0357) (**Figure 3.16A**), suggesting that the fractionation used here was valid reflecting the function of HIC-5 as a transcriptional regulator in the nucleus (Chodankar *et al*., 2014, Li *et al*., 2011). Furthermore, GO enrichment analysis of functional terms showed these potential HIC-5 binding partners play roles in cancer-related processes such as invasion (P < 0.00001), metastasis of cells (P = 0.000299) and EMT (P = 0.000164) (**Figure 3.16B**). Additionally, the data showed a significant enrichment of proteins that regulate transcription of DNA (P = 0.00307), including β-catenin, JMJD1C, EHMT1, MTA3, WWOX and SUMO1. Interaction network analysis showed that HIC-5 associated with a putative network of transcriptional regulators in the nucleus, with β-catenin forming the most connected node in the network (**Figure 3.17**). Furthermore, the HIC-5 BirA* FAK WT dataset indicated that 24 (31% of the nuclear HIC-5 BirA* FAK WT dataset) proximal interaction partners are in common with the FAK WT BirA* dataset, including the transcriptional regulators WWOX, JMJD1C and TOX4 (**Figure 3.17**).

Finally, comparison of the HIC-5-BirA* FAK WT and HIC-5-BirA* FAK -/- interactome proteomics datasets showed that deletion of FAK did not result in a significant loss of putative HIC-5 interactors in the nucleus (except for FAK itself) (**Figure 3.18**). Interestingly, loss of FAK in FAK -/- cells enriched for HIC-5 association with the proline-rich coiled-coil protein (PRRC2B) and the scaffolding protein Synaptopodin-2 (**Figure 3.18**).

Together, these data show that HIC-5 interacts with a network containing focal adhesion proteins and transcriptional regulators in the nucleus, many of which overlap with the FAK BioID nuclear interactome. Furthermore, comparison of the HIC-5 BioID interactome in the FAK WT vs FAK -/- background identified that loss of FAK is associated with significantly enriched association of HIC-5 with the scaffolding protein synaptopodin-2 and proline-rich coiled-coil protein PRRC2B.

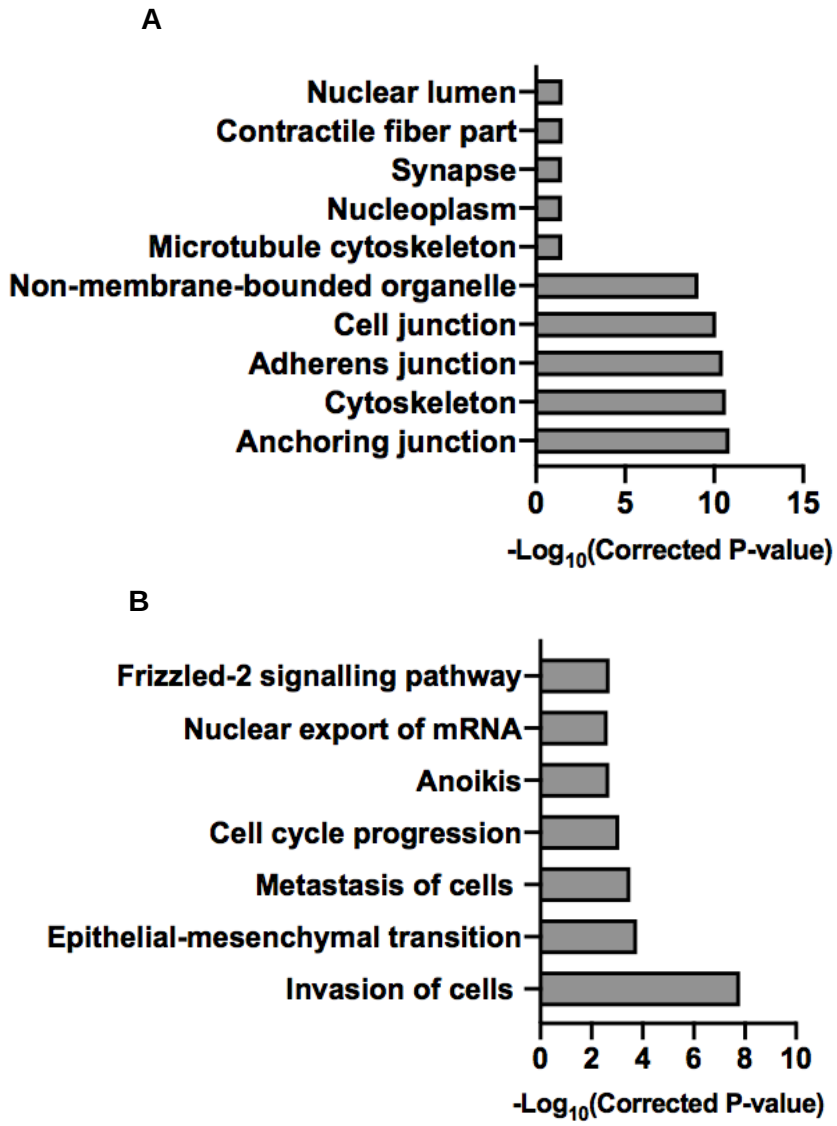


Figure 3.16 | GO enrichment analysis of nuclear HIC-5 BirA* FAK WT MS dataset. A) Subcellular localization analysis was carried out by performing a functional annotation clustering analysis using the DAVID GO database. B) Functional analysis was carried out by performing Disease and Functions core analysis using the IPA software. The –log10 was taken of all P values.

Figure 3.17 | Identification of HIC-5 interactors in FAK WT cell lines. FAK WT, HIC-5 BirA* FAK WT cells and BirA* EV control FAK WT cells were treated overnight with biotin before nuclear fractionation. The resulting nuclear biotinylated proteins were isolated using Streptavidin C1 Dynabeads and submitted for MS analysis. Data was filtered using IPA software. Non-specific streptavidin binding was controlled by statistically testing using a two-tailed unpaired t-test BirA* EV FAK WT data and HIC-5 BirA* FAK WT LFQ values. Network files were imported into Cytoscape for final figure presentation. Network files were imported into Cytoscape for final figure presentation. All significantly enriched FAK-kinase-dependent interactions are indicated in pink generated. HIC-5 (Tgfb1I1) is coloured red. Lines indicate protein-protein interactions. N=3 biological replicates for both proteomic datasets

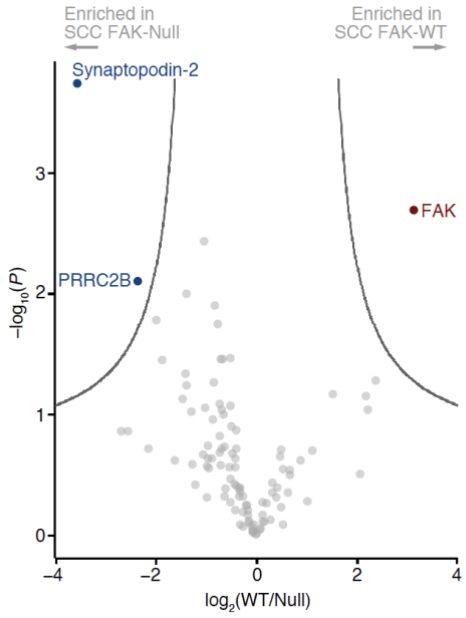


Figure 3.18 | Determination of FAK-dependent nuclear interactions of HIC-5. LFQ intensities between HIC-5 BirA* FAK WT and HIC-5 BirA* FAK -/- dataset were tested for statistical significance using a one-way ANOVA test. Shows a volcano plot reporting the statistical significance of the HIC-5 BirA* FAK WT and HIC-5 BirA* FAK -/- analysis on the *y*-axis and logarithm-transformed fold change of LFQ values from HIC-5 BirA* FAK WT and HIC-5 BirA* FAK -/- (WT/Null) analysis on the *x*-axis. Courtesy of A. Byron. N=3 biological replicates.

3.4 Discussion

Previous studies have shown FAK can translocate to the nucleus and bind to a number of transcriptional regulators to control the expression of specific genes (Lim *et al*., 2008b, Lim *et al*., 2012, Serrels *et al*., 2015, Cardoso *et al*., 2016, Serrels *et al*., 2017, Canel *et al*., 2017). Using proximity-based proteomics, I identified that FAK has multiple putative interactions with novel transcriptional regulators in the nucleus, focal adhesion proteins that are known to translocate to the nucleus and a number of proteins involved in endocytosis. Characterisation of the function of a FAK nuclear interaction partner, HIC-5, indicated that it interacts with transcriptional regulators and focal adhesion proteins in the nucleus.

3.4.1 FAK interacts with a network of focal adhesion proteins and transcriptional regulators in the nucleus

As outlined above, I used two proteomics approaches, namely RIME and BioID, to define nuclear FAK protein interaction networks. My initial work applying RIME identified several known FAK interacting proteins, e.g. SRC, paxillin and cortactin (**Figure 3.2A**). However, the large number of adhesion/cytoplasmic proteins present

in the final interaction networks suggested that the sub-cellular fractionation associated with RIME may not be efficient after crosslinking (**Figure 3.3A**), which was confirmed by checking the amount of cytoplasmic proteins in the 'nuclear' fractions prepared using the fractionation protocol within the RIME (**Figure 3.4**). The RIME protocol (Mohammed *et al*., 2016) is designed to enrich for chromatin complexes as it uses formaldehyde, which can both cross-link protein-protein and protein-DNA interactions. However, I found that cross-linking abolishes the ability to fractionate the cells effectively, and therefore this protocol may be best suited for the study of transcription factors that are located in the nucleus.

More promising data was obtained using the BioID system, whereby nuclear FAK BioID streptavidin pulldown-MS experiments showed that FAK associates with a network of transcriptional regulators, including HIC-5, LPP, WWOX and TJP1 (**Figure 3.10**). Further, validation by streptavidin pulldown-western blot experiments showed that FAK binds to both LPP and WWOX in the nucleus (**Figure 3.12**), indicating this approach is effective in determining novel nuclear interaction partners of FAK.

As previously described, BioID is a proximity-dependent labelling approach which means that any protein in close proximity to any target protein (10 nm) is labelled with a biotin tag (reviewed in Trinkle-Mulcahy, 2019). BioID proximity-dependent labelling is a powerful approach to determine novel protein-protein interactions that are more likely to be direct than standard affinity purification/MS approaches. However, it does have some caveats. For example, it has been reported that the size of the tag can cause mislocalisation of the target protein (reviewed in Trinkle-Mulcahy, 2019), but this was not a problem for FAK (125kDa) as the BirA tag did not impair its focal adhesion localisation (**Figure 3.5**). An additional caveat is that the BirA* tag can ablate the function of the protein (reviewed in Trinkle-Mulcahy, 2019). This did not seem to be the case with the FAK WT BirA* fusion protein as it was regulated normally (**Figure 3.6**) and bound to SRC (reviewed in Mitra *et al*., 2005, **Figure 3.7**).

Another disadvantage of BioID is the time required to generate enough biotinylated proteins sufficient for proteomics analysis (18-24 hours), making it unsuitable for studying a dynamic process which may occur over a time scale of minutes (Branon *et al*., 2018). Other similar proximity-dependent labelling approaches are available, such as APEX, a different tag of 27kDa which is a monomeric ascorbate peroxidase which catalyses biotin-phenol oxidation to a radical that is short lived in the presence of hydrogen peroxide (reviewed in Trinkle-Mulcahy, 2019). Subsequent reaction with amino-acids that are electron rich results in biotinylation of proteins in close proximity (reviewed in Trinkle-Mulcahy, 2019). APEX only takes around 1 minute to label proteins, which means it can provide a snapshot of the interactome changes over a short time. This is not possible with BioID due to the slow kinetics, which results in the identification of all proteins proximal to the BirA* tag over a period of many hours (Branon *et al*., 2018, reviewed in Trinkle-Mulcahy, 2019). However, APEX requires the use of hydrogen peroxide, which is toxic to the cells and thus may cause artefacts in the interactome dataset. BioID only requires the use of biotin, which is non-toxic, and despite the slow kinetics of biotinylation, there have been over 100 applications of BioID since the protocol was published 6 years ago in Roux *et al*., 2013 (reviewed in Trinkle-Mulcahy, 2019).

Recent efforts have tried to combined the fast labelling kinetics of APEX with the non-toxicity of the BioID approach – whereby investigators changed the amino acid in the BirA* catalytic site mutation from R118G to R118S and introduced an additional 15 mutations in the BirA* sequence (Branon *et al*., 2018). This approach, namely TurboID, results in the generation of sufficient biotinylated material for proteomics in 10 minutes (Branon *et al*., 2018). This may be used in future studies to define the FAK interactome over a very short period of time. To understand FAK function in the nucleus, I believe the BioID approach I used here was informative.

A key advantage of the BioID approach is the potential to enrich for direct interaction partners as the BirA*-tagged protein will attach a biotin moiety to a protein in close proximity. However, as the potential interaction partner does not need to bind the BirA*-tagged protein, this does not exclude the possibility that a scaffolding protein

could mediate the binding of FAK protein partners. Thus, experiments such as glutathione S-transferase (GST) pulldowns using recombinant proteins of potential FAK-binding partners could be used to determine if the interaction partners I identified are binding directly.

I found 42 proteins that were identified to be significant FAK binding partners in the nucleus. Other reports studying the BioID interactome of predominantly nuclear proteins, such as Separase have yielded larger datasets identifying approximately 200 interactors (Agircan *et al*., 2016). Reasons for this could be that a small proportion of FAK is in the nucleus relative to total FAK protein levels in the cell so it is more difficult to capture interactions in the nucleus that are significantly enriched over the BirA* EV control-expressing cells.

In the FAK WT BioID nuclear interactome dataset, a number of focal adhesion proteins were identified as FAK-interacting proteins in the nucleus. Specifically, a number of proteins present in the consensus adhesome (Horton *et al*., 2015) have been shown to translocate to the nucleus, i.e., Paxillin (Sathe *et al*., 2016), LPP (Guo *et al*., 2006), Zyxin (Nix and Beckerle, 1997) and HIC-5 (Shibanuma *et al*., 2003), the latter being in response to cellular stress. Interestingly, not all proteins in the consensus adhesome have NLS sequences. For example, although Paxillin and Zyxin have NES sequences they do not have a NLS sequence (reviewed in Lim *et al*., 2013), while FAK has been shown to have an NLS within its N-terminal FERM domain (Lim *et al*., 2008b). Therefore, it is unknown whether other focal adhesion proteins containing NLS sequences can mediate nuclear translocation of other proteins in the consensus adhesome. Work from the Frame laboratory has identified that 70-80% of the consensus adhesome can be detected in the nuclear fraction of SCC cells (A. Byron, unpublished). Studying the nuclear functions of canonical focal adhesion proteins and the mechanism by which they translocate to the nucleus are of active interest to the Frame group, as this represents a mechanism by which signals are transmitted from focal adhesions to regulate the expression of genes. As FAK is upregulated in the nucleus of cancer cells, where it regulates the expression of genes that regulate the anti-tumour immune response to support tumour growth (Serrels *et al*., 2015), FAK

and potentially other focal adhesion proteins in the nucleus could be part of a mechanism to regulate cancer-associated processes.

It is possible that FAK-interacting partners in the nucleus that are focal adhesion proteins could be scaffolding FAK to the novel transcriptional regulators identified (or vice-versa), especially since many of these proteins are known direct interaction partners of FAK (i.e. Paxillin, HIC-5, SRC, ASAP1). Thus, it is plausible that these focal adhesion proteins could have a role in FAK-mediated transcription. This could be tested by firstly confirming these interactions with focal adhesion proteins in the nucleus, and secondly, confirming whether focal adhesion proteins can bind to transcriptional regulators that FAK regulates and whether binding to chromatin and/or gene promoters is impacted by FAK depletion. Furthermore, it would be interesting to determine whether focal adhesion protein nuclear localisation mutants impact on FAK-target gene expression (e.g. *CCL5*).

However, it must be noted that it is possible that the proteins identified as proximal to FAK could be biotinylated at focal adhesions before translocating to the nucleus where they may not bind FAK. Thus, this stresses the need to use different approaches (such as co-immunoprecipitation) in order to validate that the nuclear interaction partners identified in the FAK BioID nuclear interactome dataset are in fact nuclear interactors. However, as presented in **Figure 3.15**, a novel interactor of FAK in the nucleus, HIC-5, has been robustly validated by performing co-immunoprecipitation experiments.

Several proteins identified in the FAK BioID nuclear interactome dataset have known importance in cancer. For example, HIC-5, a regulator of steroid receptor and canonical WNT signalling (Ghogomu *et al*., 2006), is linked to prostate cancer progression (Li *et al*., 2014). Furthermore, WWOX has been shown to interact with BCL9-2, to increase the activity of the β-catenin-TCF/LEF (T cell factor/lymphoid enhancers factors family) complex to regulate the WNT pathway in breast cancer (El-Hage *et al*., 2015). Additionally, LPP has been shown to regulate the activity of the transcription factor ETV4 to regulate the mRNA expression of the invasion regulator

MMP1 (Guo *et al*., 2006). Thus, these proximal proteomic datasets have identified several FAK-transcriptional regulator interactions with notable importance in cancer.

3.4.2 BioID identified FAK kinase-dependent nuclear interactions

As described in the Introduction of this thesis, FAK's kinase activity and nuclear function is important for its role in mediating cancer-associated gene expression changes (Serrels *et al*., 2015, 2017), which is mediated by its interactions with transcription factors such as IL33 (Serrels *et al*., 2017). The exact role of FAK's kinase activity in the nucleus is not fully understood as no *bona fide* substrates of FAK have been identified in this cell compartment. However, it could be important in regulating the function of its binding partners and this could ultimately impact on its biological consequences. FAK kinase activity regulates its binding to protein partners such as SRC at focal adhesions (reviewed in Mitra *et al*., 2005), and therefore I set out to determine the importance of FAK kinase activity in potential nuclear protein-protein interactions.

I therefore incorporated a FAK kinase-defective mutant (FAK KD BirA* cells) in the BioID nuclear pulldown MS experiments. A number of statistically significant potential kinase-dependent interactions were identified, including SRC, CORO1B, PEX14, CRK, PKCDBP, TECR, EPS15L1, 4932415D10RIK, MII1T4, TOX4, ASAP1, AKNAK2, MURC and WWOX (**Figure 3.11**). It could be that the enrichment of binding may be due to increased expression of some of these proteins at the protein or mRNA level, which requires further experimental data to investigate.

Highly enriched potential kinase-dependent interactions included members of the cavin family of proteins, PKCDBP and MURC, which are important mediators of caveolae-dependent endocytosis (reviewed in Chidlow and Sessa, 2010). Caveolae are invaginations of the cell membrane that mediate uptake of molecules outside the cell (reviewed in Chidlow and Sessa, 2010). There is also evidence that these small invaginations can enhance signalling of some pathways that are important in cancer. For example, sustained activation of AKT in early endosomes is mediated by

PKCDBP and this stimulates pro-survival signalling in lung cancer cells (Yamaguchi *et al*., 2019).

PKCDBP and MURC are associated with caveolae (reviewed in Chidlow and Sessa, 2010), but their role in caveolae-dependent endocytosis has not been fully established. However, it has been shown that PKCDBP is important for regulating caveolae dynamics by regulating the amount of time caveolae spend on the plasma membrane, suggesting it may regulate the turnover of caveolae on the plasma membrane to regulate signalling, membrane tension or lipid homeostasis (Mohan *et al*., 2015). It is surprising that I detected caveolae-associated proteins in the nucleus, as caveolae-dependent endocytosis is a cytoplasmic mechanism. However, a study has identified that a caveolin-1/TIE2 complex translocates to the nucleus in response to ionising radiation to facilitate the DNA repair in response to genotoxic stress in glioma cells (Hossain *et al*., 2017). Thus, components of the caveolae-dependent endocytosis pathway have been detected in the nucleus previously in the context of cellular stress, and this may promote cell survival in cancer.

The role of FAK binding to these proteins and the function of cavins in the nucleus remains to be determined. Interestingly, the protein FLOT-2 is enriched in the FAK KD BirA* vs FAK WT BirA* analysis; FLOT-2, like cavins, is associated with caveolae membranes (Bickel *et al*., 1997). Furthermore, FLOT-2 expression has been reported to be associated with metastasis of nasopharyngeal carcinoma by activating the AKT and NF-κB pathways (Liu *et al*., 2015) and its expression has been correlated with poor prognosis in non-small cell lung cancer (Wen *et al*., 2015). Together, these findings suggest that FAK binding to members of the caveolae-dependent endocytosis pathway may be important for survival in SCC cells. Interestingly, FAK has already been shown to bind to caveolin-1 (Swaney *et al*., 2006) and it is a key mediator of caveolin-1 expression during EMT (Bailey and Lui, 2008). However, FAK binding to the caveolin components of the endocytic machinery in the nuclear compartment has not been reported before. This suggests that FAK kinase activity may be a regulator of the endocytic machinery in the nucleus, although the role of the latter in the nucleus is

not known. Further experiments will be required to confirm this and also to establish by what mechanism FAK kinase activity specifically contributes to this.

As yet, we do not know the mechanism by which FAK binds to proteins in the nucleus. As mentioned, no substrates of FAK in the nucleus have been identified. Further work will be required to understand the significance of FAK kinase activity in regulating the binding to its protein partners in the nucleus and to address whether there are direct substrates or if it is a FAK kinase-dependent conformational change that alters its binding properties.

3.4.3 HIC-5 interacts with an extensive network of transcriptional regulators in the nucleus

HIC-5 was present in both of the FAK nuclear proteomic datasets and experimental validation indicated that FAK binds HIC-5 in the nucleus (**Figure 3.15**). Although FAK is shown to interact with HIC-5 at focal adhesions (Nishiya *et al.*, 2001), binding in the nucleus had not been reported previously.

Proximal proteomics using a HIC-5 BirA* protein expressed in a FAK WT SCC cell background showed that HIC-5 binds to a network of focal adhesion proteins and transcriptional regulators in the nucleus. 'Transcription of DNA' was a highly enriched GO term (**Figure 3.16B**), and proteins included under this term are implicated in cancer, such as β-catenin, which is a co-activator of WNT pathway genes (reviewed in MacDonald *et al*., 2009). HIC-5 has been shown to repress β-catenin-mediated MYC expression by competitive binding with T-cell factor-driven member 4 (TCF-4) on the *MYC* gene (Li *et al*., 2011). HIC-5 has also been shown to negatively regulate Lymphoid Enhancer Factor (LEF) target gene expression (Ghogomu *et al*., 2006), another β-catenin-regulated transcription factor. Another protein included under this GO term was the histone demethylase JMJD1C, which is a positive regulator of invasion and metastasis by regulating the expression of the transcription factor ATF2 (Chen *et al*., 2018). Thus, the HIC-5 BioID interactome dataset has identified many transcriptional regulators with notable importance in transcription and cancer.

Interestingly, this dataset predicted that HIC-5 binds to the protein SUMO1 and RANBP2, which both regulate SUMOylation of proteins, a post-translational modification which regulates the structure and subcellular localisation of proteins, as well as transcription factor function (Pichler *et al*., 2002, reviewed in Lyst and Stancheval, 2007). RANBP2 is an E3 Sumo ligase that regulates SUMO1 conjugation to target proteins. Interestingly, transcriptional regulators in the HIC-5 nuclear interactome are SUMOylated with the SUMO1 moiety added by RANBP2 activity (Pichler *et al*., 2002). SUMOylation is essential for promyelocytic leukemia protein (PML) function by promoting P53 transcriptional activity (Satow *et al*., 2012). Furthermore, SUMOylated FAK has been reported to be enriched in the nucleus (Kadaré *et al*., 2003). However, the significance of this modification in FAK-mediated transcription has not been identified. Thus, the interactome data suggests that HIC-5 can also potentially be SUMOylated or mediate SUMOylation (i.e. by scaffolding complexes) and it is possible this may impact on its own transcriptional activity or that of other transcriptional regulators, such as has been shown for PML (Satow *et al*., 2012).

Comparison of the HIC-5 BirA* FAK WT SCC and Hic5 BirA* FAK -/- SCC datasets showed that there were no interaction partners enriched in the nucleus in the FAK WT vs FAK -/- analysis (except for FAK itself) (**Figure 3.18**). However, in the FAK -/- vs FAK WT analysis, the proteins synaptopodin-2 and PRRC2B were enriched in the HIC-5-BirA* FAK -/- dataset. Synaptopodin-2 is a scaffolding protein that induces actin filament assembly (Kai, Fawcett and Duncan, 2015) and it is known to translocate to the nucleus in myotubules and myoblasts where it is hypothesised to potentially regulate mRNA transport in times of cellular stress (Weins *et al*., 2001). It is interesting to speculate whether HIC-5 could be binding to synaptopodin-2 to compensate for a potential scaffolding function FAK is providing in the nucleus. These data imply that FAK could play a scaffolding role in HIC-5-mediated transcription by scaffolding HIC-5 to nuclear complexes. I did not determine whether enriched proteins in the HIC-5 BirA* FAK -/- vs HIC-5 BirA* FAK WT SCC datasets are differentially expressed at the protein level, which could contribute to the data presented here.

Together, these data suggest that HIC-5 binds to a network of transcriptional regulators in the nucleus. However, further experiments will be required to determine the significance of these interactions to HIC-5-mediated transcription and biological functions.

3.5 Conclusions

The data presented in this chapter have shown that FAK interacts with focal adhesion proteins and transcriptional regulators in the nucleus. Many of these interaction partners both have functions in transcription and are important in cancer. Furthermore, I set out to determine the interactome of FAK's nuclear interaction partner, HIC-5, to gain insight into its nuclear function, which showed that HIC-5 also interacts with transcriptional regulators in the nucleus. Thus, FAK and HIC-5 in the nucleus can independently bind to transcriptional regulators which could potentially regulate gene expression. Further characterisation of these individual interaction partners is required to determine the importance of these interactions for transcription in this cancer cell model.

Chapter 4 FAK regulates AP-1 and ETS transcription factor motif enrichment in accessible regions of chromatin

4.1 Introduction

Nuclear FAK is an important regulator of gene expression in cancer (Serrels *et al*., 2015). However, FAK does not contain a DNA binding sequence and therefore likely regulates transcription indirectly, potentially mediated through interactions with transcription factors and co-factors. Studies have shown FAK binds the transcription factors GATA4 (Lim *et al*., 2012), MBD2 (Luo *et al*., 2009b), MEF2 (Cardoso *et al*., 2016) and IL33 (Serrels *et al*., 2017) to regulate the expression of VCAM-1, Myogenin, JUN and CCL5, respectively. To identify which transcription factors are the key drivers of FAK-dependent gene expression, I have used ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) to determine FAK-dependent, FAK nuclear localisation-dependent and FAK kinase-dependent alterations in chromatin accessibility and transcription factor motif enrichment.

ATAC-seq is a method for assaying chromatin accessibility, which uses a hyperactive Tn5 transposase to simultaneously digest and tag open chromatin regions of the genome with sequencing adapters (tagmentation). Regions of the genome where DNA is accessible will contain significantly more sequencing reads and form peaks in the ATAC-seq signal that are detectible by peak calling tools (Buenrostro *et al*., 2013). These regions can be further categorised into various regulatory element types (promoters, enhancers, etc.) by taking into account their distance from a transcriptional start site.

ATAC-seq data also enables a computational method called motif enrichment analysis, which identifies transcription factor binding sites within accessible chromatin regions. Each ATAC-seq signal will contain more sequencing reads than less exposed regions of the genome and often the sequence within these peaks will contain motif sequences for particular transcription factors (**Figure 4.1**). Analysis of the sequence within these ATAC-seq peaks can identify binding sites for particular transcription factors and if the transcription factor motifs are differentially exposed on the chromatin between different cell lines and conditions. This technique can yield a list of transcription factors that have binding capability to these motifs sequences. However, this technique does not confirm that the transcription factor is bound to the chromatin and therefore

techniques need to be employed (i.e. ChIP) in order to confirm that the predicted transcription factor identified from motif enrichment analysis is in fact bound to the DNA in accessible regions of chromatin.

In this section, I will define FAK-regulated chromatin accessibility changes and transcription factor motif enrichment in order to identify key genomic regions and transcription factors regulated by FAK. Specifically, I will use ATAC-seq to define global chromatin accessibility changes between FAK WT, FAK -/-, FAK NLS and FAK KD SCC cell lines. Furthermore, motif enrichment analysis will be performed to identify potential FAK-regulated transcription factors. I will then gain mechanistic insight into how FAK may be regulating these predicted transcription factors by integrating the FAK-regulated transcription factors identified by motif enrichment analysis with the FAK WT BioID and nuclear FAK interactome proteomics datasets. Finally, I use the same approach as outlined above to investigate the mechanism by which FAK regulates a known FAK-regulated gene, *IL33*. To validate my motif enrichment findings, I will confirm that the predicted FAK-regulated transcription factors are differentially regulated on the chromatin between FAK SCC cell lines by performing chromatin preparations and ChIP.

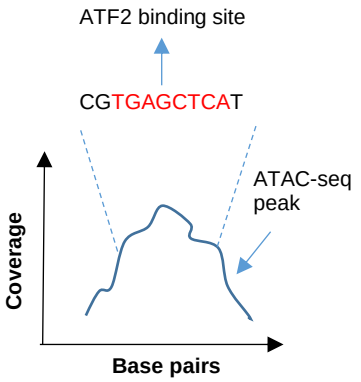


Figure 4.1 | Motif enrichment analysis. Schematic of the logic underpinning motif enrichment analysis. The sequence within ATAC-seq peaks will contain binding sites for particular transcription factors which can be predicted by HOMER. Therefore, it is possible to predict which transcription factor is potentially binding to that particular region and whether this is differentially regulated between cell lines and conditions. The motif shown here is that of the AP-1 member, ATF2.

4.2 Aims

- Identify FAK-dependent global chromatin accessibility changes.
- Predict global FAK-regulated transcription factors.

- Integrate the FAK-regulated transcription factor dataset with nuclear FAK proteomic datasets to gain insight into the mechanism by which FAK regulates these transcription factors.
- Investigate the mechanism by which FAK regulates the expression of the *IL33* gene.

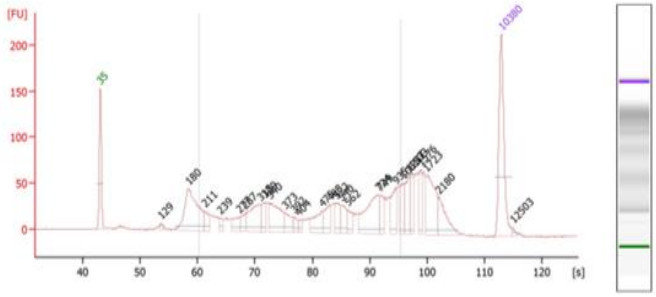
4.3 Results

4.3.1 Optimisation of ATAC-seq conditions in the FAK SCC model

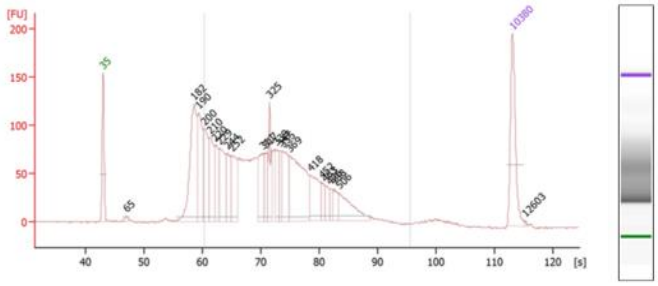
ATAC-seq is generally considered to be a straightforward protocol. However, the main challenge of this approach is obtaining a sample of high yield and good DNA fragment profile. Too many small fragments in the sample (overtagmentation) cluster more easily in sequencing and therefore will create a huge bias for these fragments (some of which will simply be excess sequencing adapters). Additionally, it is important not to have too many large fragments as this disrupts the sequencing process by folding over and binding at both ends in the flow cell. As such, all ATAC-seq samples are analysed on an Agilent Bioanalyser, which essentially performs capillary electrophoresis to determine the distributions of DNA fragment size in the samples. Good and poor examples of DNA fragment disruptions are shown in **Figure 4.2**.

ATAC-seq samples were prepared from FAK WT, FAK -/-, FAK NLS and FAK KD SCC lines. The FAK WT, -/- and KD SCC samples were prepared with 200,000 cells and 2x Tn5 transposase enzyme, which yielded good fragment profiles. However, the FAK NLS samples all showed overtagmentation in these conditions. Therefore, I reduced the cell number to 100,000 cells and enzyme concentration to 1x, which yielded good quality DNA fragment profiles for sequencing. The final conditions used for each of the SCC lines are summarised in **Figure 4.3A** and representative DNA fragment profiles are depicted in **Figure 4.3B**. The sequencing of the ATAC-seq samples was carried out by the University of Glasgow sequencing service.

A Example of a good spectrum



B Example of a bad spectrum = too many small fragments



C Example of a bad spectrum = too many large fragments

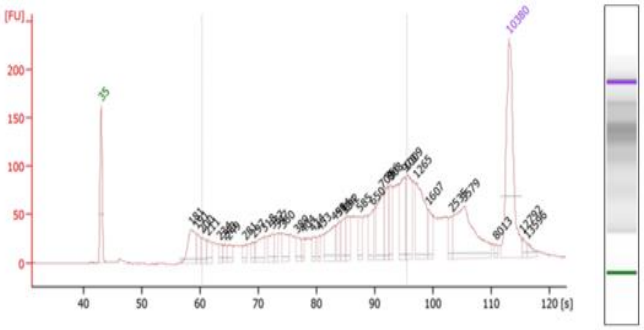


Figure 4.2 | Example of DNA size fragment spectra. DNA samples prepared following the ATAC-seq protocol (as described in Materials and Methods) were run on the Agilent Bioanalyser, along with high and low molecular weight markers. Virtual DNA gel shown to the right of the profiles. Arbitrary fluorescence units (FU) are plotted by time in seconds (s). A) is an example of a good example of a DNA fragment protocol. B) is an example of an overtagmented sample and C) is an example of a profile with too many large fragments.

A

Cell line	Cell No. Input	Enzyme
FAK WT	200,000	2x
FAK -/-	200,000	2x
FAK NLS	100,000	1x
FAK KD	200,000	2x

B

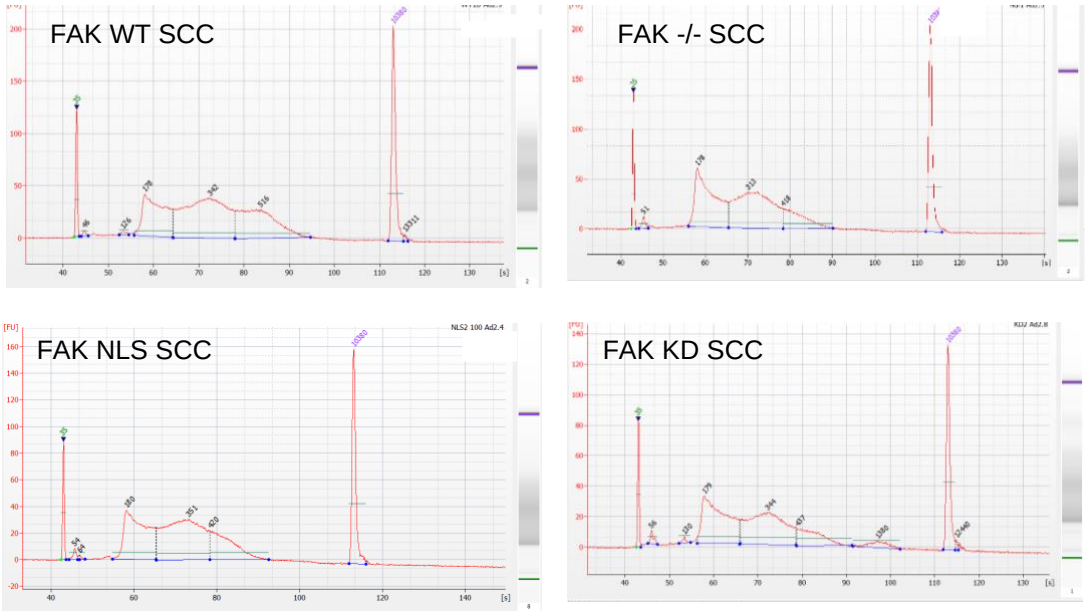


Figure 4.3 | Samples submitted for ATAC-seq A) Final conditions used for preparation of ATAC-seq samples in the indicated cell lines. B) Representative DNA fragment profiles of FAK SCC samples submitted for ATAC-seq analysis. DNA samples prepared following the ATAC-seq protocol (as described in Materials and Methods) were run on the Agilent Bioanalyser, along with high and low molecular weight markers. Virtual DNA gel shown to the right of the profiles. Arbitrary fluorescence units (FU) are plotted by time in seconds (s).

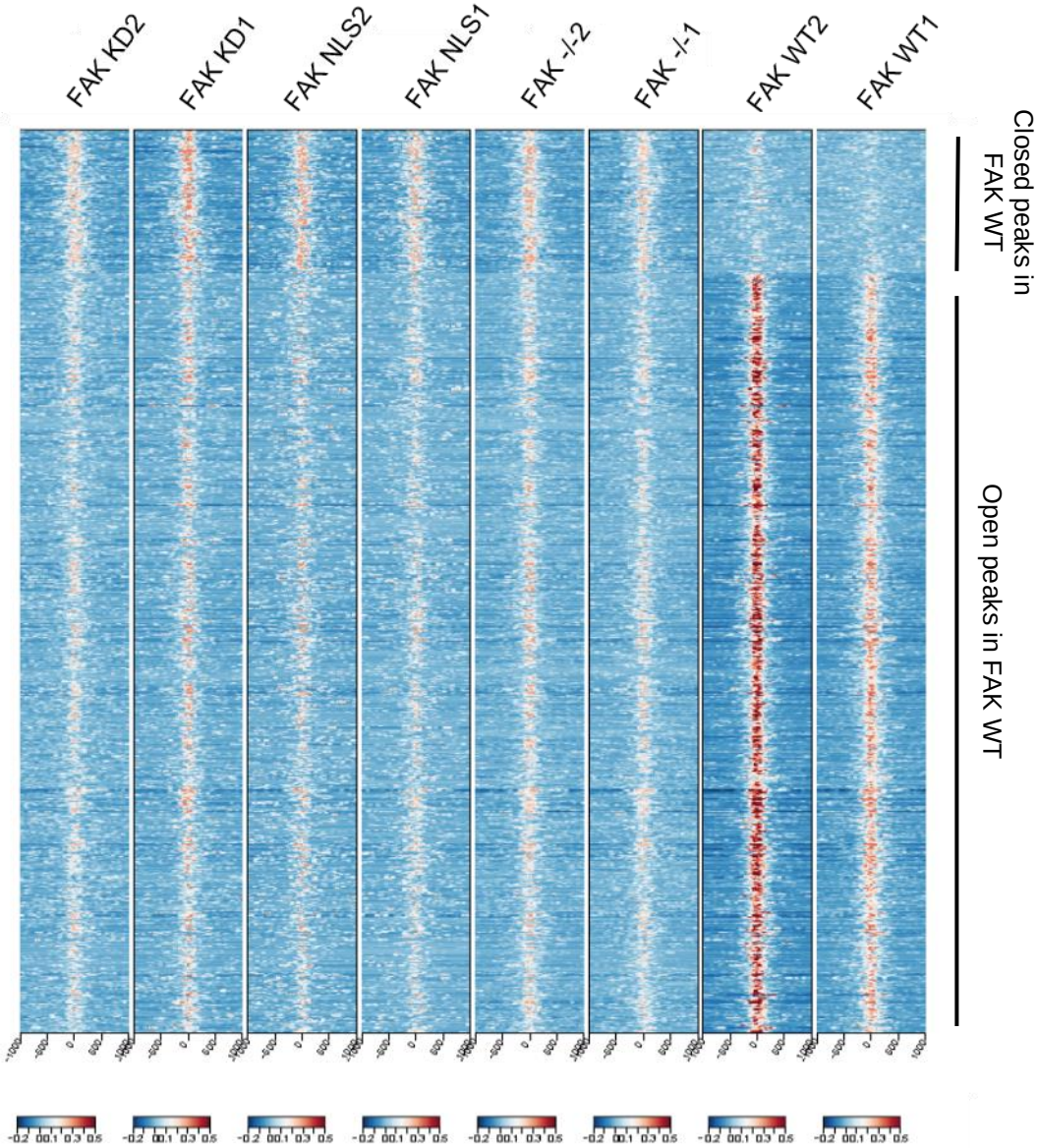


Figure 4.4 | Global chromatin accessibility changes observed upon FAK loss. Heatmap representation of significantly different chromatin accessibility changes (FDR ≤ 0.05) between FAK WT SCC, FAK -/- SCC, FAK NLS an FAK KD SCC samples, as determined by Diffbind. To generate the heatmap, the data was z-scored (dark red representing a z-score of 0.5 and dark blue indicating a z-score of -0.2). Only peaks in the FAK -/-, FAK NLS and FAK KD that have differential accessibility with respect to FAK WT are shown. Peaks are not shown in any particular order. In the FAK WT cells 595 peaks were open, while 112 were closed with respect to FAK -/-, NLS and KD cell lines (indicated by black lines to right of heatmap). Red indicates highly accessible regions (centre of peak), whereas blue indicates less accessible regions. Numbers indicate the biological replicate of the sample. Numbers at bottom of the heatmap represent distance from the centre of the ATAC-seq peak (0 is the centre of the peak). N=2 biological replicates. Courtesy of R. Upstill-Goddard

4.3.2 ATAC-seq chromatin accessibility results

We aligned the sequencing results to the mm10 (*Mus musculus*) reference genome, before using the bcbio ATAC-seq pipeline to perform the accessibility analysis. Peaks (accessible regions) were called from the BAM files using the MACS2 algorithm. The number of peaks per sample can be found in **Table 2.7**. The distribution of peaks were similar in the FAK WT, -/-, NLS and KD cells; with the majority of peaks located 5 to 100kb from the TSS. Differentially accessible regions were identified using the R/Bioconductor package DiffBind. Differential peak calling was performed for each pairwise comparision and significantly, differential peaks were defined as those with a FDR of below or equal to 0.05. Results from the chromatin accessibility analyses indicated changes in a subset of genes between FAK WT, FAK -/-, FAK NLS and FAK KD SCC cell lines, as shown in the chromatin accessibility heatmap in **Figure 4.4**. Specifically, there appeared to be a difference in chromatin accessibility in a subset of genes between FAK WT vs FAK -/-, FAK NLS and FAK KD SCC cell lines (**Figure 4.4**). Further investigation of the genes that are regulated by chromatin accessibility was performed in Chapter 5, where I have integrated the subset of genes that display FAK-dependent chromatin accessibility changes with FAK-regulated gene expression data to determine which FAK-dependent chromatin accessibility changes are associated with differential gene expression.

		FAK WT vs -/-		FAK WT vs NLS		FAK WT vs KD	
TF family	TF	P-value	q-value	P-value	q-value	P-value	q-value
AP-1	JUN	1E-244	0	1E-244	0	1E-23	0
AP-1	JUNB	1E-76	0	1E-109	0	1E-12	0
AP-1	ATF3	1E-31	0	1E-19	0	1E-08	0
AP-1	FRA1	1E-85	0	1E-32	0	1E-14	0
AP-1	FRA2	1E-69	0	1E-78	0	1E-14	0
ETS	ELF1	1E-73	0	1E-68	0	1E-13	0
ETS	SPDEF	1E-36	0	1E-152	0	1E-22	0
ETS	ETV1	1E-36	0	1E-85	0	1E-28	0
ETS	ETS1	1E-32	0	1E-23	0	1E-31	0
ETS	ETV4	1E-30	0	1E-50	0	1E-17	0

Table 4.1 | FAK regulates AP-1 and ETS motif enrichment in accessible chromatin. Motif enrichment analysis was performed in FAK WT, FAK -/-, FAK NLS and FAK KD cell lines by searching the motif sequences within the ATAC-seq peaks which have predicted transcription factor binding. Most highly enriched AP-1 and ETS motifs are shown for each FAK WT vs -/-, NLS or KD SCC analysis. TF = Transcription factor known to have binding capabilities to FAK-regulated motifs. N=2 biological replicates.

4.3.3 Motif enrichment analysis to determine potential FAK-regulated transcription factors

As previously described, motif enrichment analysis allows one to predict if transcription factor motifs are differentially exposed on the chromatin between different cell lines and conditions based on enrichment of particular transcription factor motifs in accessible regions. We performed the motif enrichment analysis using the motif database HOMER, which identifies genomic sequences that matches the motifs (motif binding sites) in the differentially accessible peaks. All transcription factor motifs present in differentially accessible regions in FAK WT cells compared with that of FAK -/-, FAK NLS and FAK KD cells. This analysis detected multiple statistically significant (q-value ≤ 0.05) changes in motif enrichment in differentially accessible peaks in the FAK WT vs -/- SCC lines (196 transcription factor motifs), FAK WT vs KD SCC lines (118 transcription factor motifs) and FAK WT vs NLS SCC lines (205 transcription factor motifs) by comparative analysis. In particular, there were many highly enriched transcription factor motifs in the FAK WT cells that are known to be bound by members of the AP-1 and ETS domain transcription factor family. Some of the most highly significantly enriched transcription factor motifs known to be bound by the AP-1 and ETS domain transcription factor family

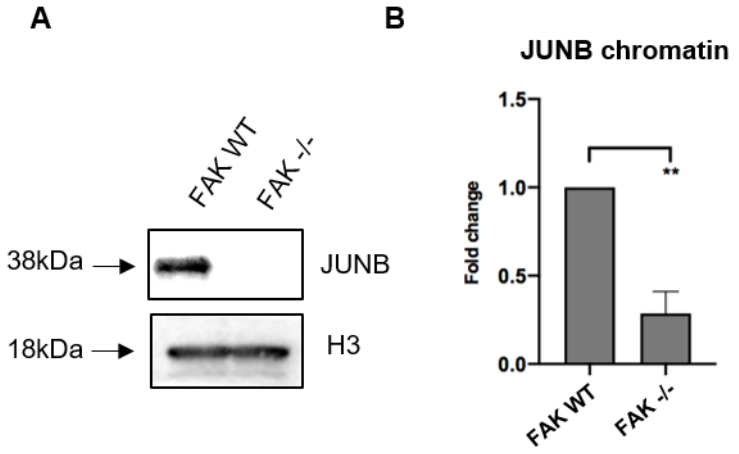


Figure 4.5 | FAK regulates JUNB levels in the chromatin fraction. A) Chromatin was harvested and subjected to SDS-PAGE analysis. The blot was probed with a JunB antibody and a histone H3 antibody to control for protein loading. B) JunB protein levels in the chromatin fraction were quantified using ImageJ and values normalised to histone H3 levels. A two-tailed unpaired t-test was used for statistical analysis. ** = P ≤ 0.01, N=3 biological replicates, mean+/-SEM.

determined by motif enrichment analysis in common between the FAK WT vs FAK -/-, FAK NLS and FAK KD analysis are shown in **Table 4.1**. The transcription factors predicted from the motif enrichment analysis are herein referred to as 'predicted transcription factors'.

To investigate my global motif enrichment findings, I made chromatin preparations from FAK WT and FAK -/- cells to determine if any of the transcription factors known to bind to the FAK-regulated motifs in differentially accessible regions display increased protein levels in the chromatin fraction of FAK WT cells. The results showed a statistically significant enrichment of the AP-1 factor JUNB (A.k.a JunB) in the chromatin fraction of the FAK WT compared to FAK -/- cells (**Figure 4.5A-B**, P = 0.0045). Further investigation showed reduced expression of JUNB in whole cell extracts from the FAK -/-, FAK NLS and FAK KD cells compared with the FAK WT cells (**Figure 4.6**), while there was no difference in *JUNB* mRNA expression between the cell lines (**Figure 4.7**, P > 0.05). Follow up of another highly enriched AP-1 member, JUN (A.k.a. c-Jun), showed increased expression in the chromatin fraction in the FAK-/- cells compared to the FAK WT cells (**Figure 4.8**), suggesting FAK does not globally regulate the occupancy of this transcription factor to chromatin in the manner predicted from the motif enrichment data.

As stated above, multiple transcription factor motifs in differentially accessible regions were significantly enriched between the FAK WT vs FAK -/-, FAK NLS and FAK KD cells lines according to the motif enrichment analysis (173 transcription factor motifs on average between the analyses). Use of HOMER also determined the transcription factors known to bind these FAK-regulated transcription factor motifs. Due to the large number identified by these analyses, I wanted to create a refined list in order to make it more manageable for further interactome downstream analysis, so as to establish how these transcription factors predicted from the motif enrichment

analysis are connected to one another and to the nuclear FAK interactome regulating these transcription factors. In order to do this, I filtered all the predicted transcription factors that are only enriched in the FAK WT cells (**Figure 4.9A**) and then constructed

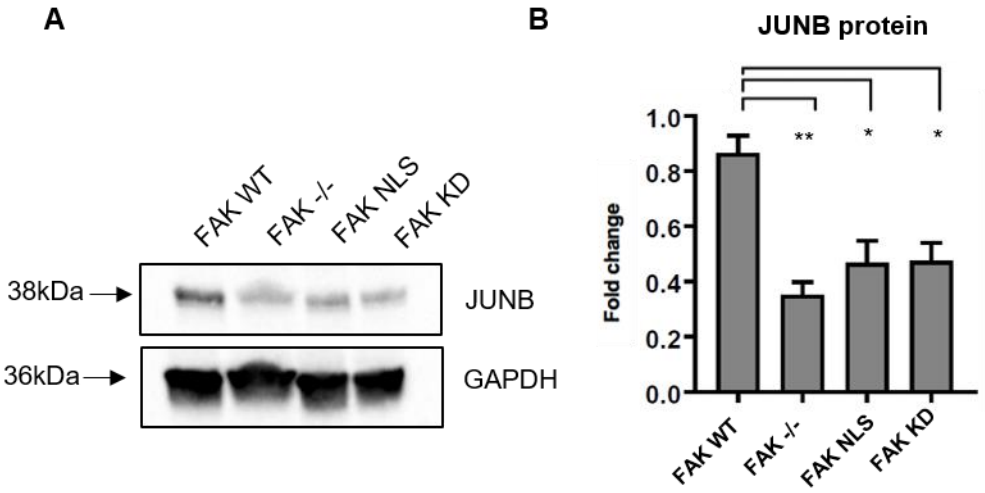


Figure 4.6 | FAK regulates JUNB protein levels. A) Whole cell lysates were harvested and protein was quantified prior to SDS-PAGE analysis, as described in Materials and Methods. The blot was probed with a JUNB antibody and a GAPDH antibody to control for protein loading. B) JUNB protein level in all replicates was quantified using ImageJ and then values normalised to GAPDH levels. A one-way ANOVA test was used for statistical analysis. * = P ≤ 0.05, ** = P ≤ 0.01, N=3 biological replicates, mean+/-SEM.

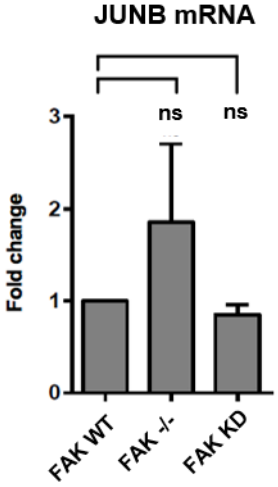


Figure 4.7 | FAK does not regulate *JUNB* mRNA expression. RNA extraction and cDNA synthesis was carried out in FAK WT, -/- and KD cell lines as outlined in Materials and Methods. qRT-PCR was performed using *JUNB* primers. CT values were normalised to each samples respective *GAPDH* CT and then each replicate. A one-way ANOVA test was used for statistical analysis. ns = P > 0.05, N=3 biological replicates, mean+/-SEM

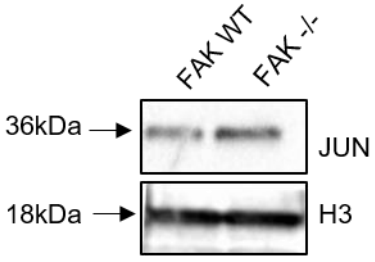


Figure 4.8 | FAK does not regulate JUN levels in the chromatin fraction consistent with the motif enrichment findings. Chromatin was harvested and subjected to SDS-PAGE analysis. The blot was probed with a JUN antibody and a histone H3 antibody to control for protein loading. N=1

a protein-protein and functional (i.e. protein-DNA interactions) interaction network with this list of transcription factors using IPA (**Figure 4.9B**). The resulting network indicated that these FAK WT enriched transcription factors are well-connected, i.e. many regulate the expression of the other transcription factors in the network. To gain mechanistic insight into how FAK may regulate these transcription factors, I integrated the nuclear FAK interactome datasets (described in Chapter 3) with that of FAK interactors and the FAK WT enriched transcription factor list (**Figure 4.10A-B**). Interestingly, in **Figure 4.10B** I have highlighted the established FAK interactors SP1 (Serrels *et al*., 2017) and RUNX1 (Canel *et al*., 2017) as the motifs for these transcription factors are enriched in the FAK WT motif enrichment analysis.

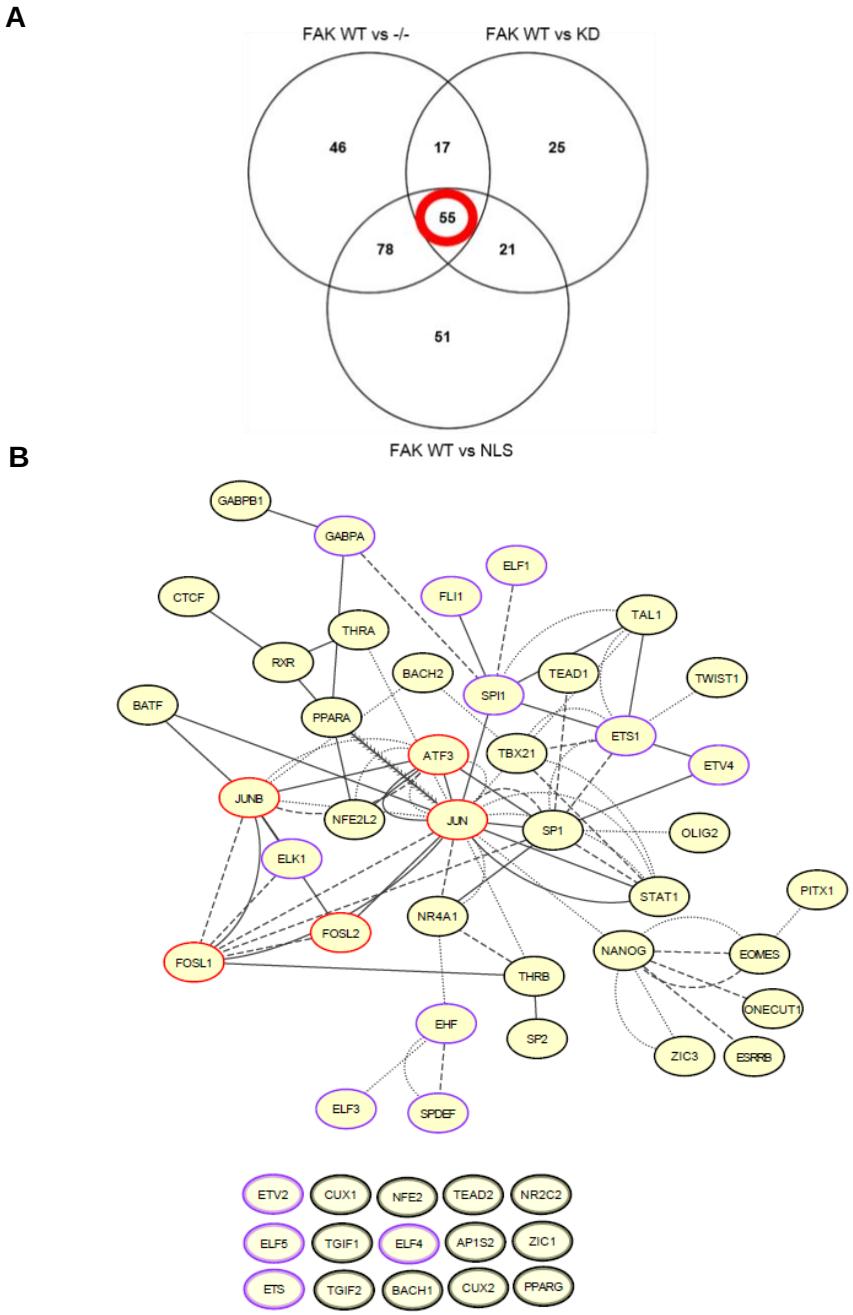


Figure 4.9 | Identification of transcription factor networks regulated by FAK. (A) VENN diagram displaying overlap in the predicted FAK-regulated transcription factors identified by motif enrichment analysis. Samples from FAK WT, FAK-/-, FAK NLS and FAK KD SCC were prepared for ATAC-seq analysis as described in Materials and Methods. All predicted FAK WT transcription factors are circled in red. B) The FAK WT SCC list created in A) (circled in red) was used to seed a network using Ingenuity pathway analysis and then exported into Cytoscape for final figure presentation. Only direct, mammalian interactions shown between transcription factors. Protein-protein and functional connections are detailed in the key. AP-1 transcription factors members outlined in red and ETS members outlined in purple. N=2 biological replicates.

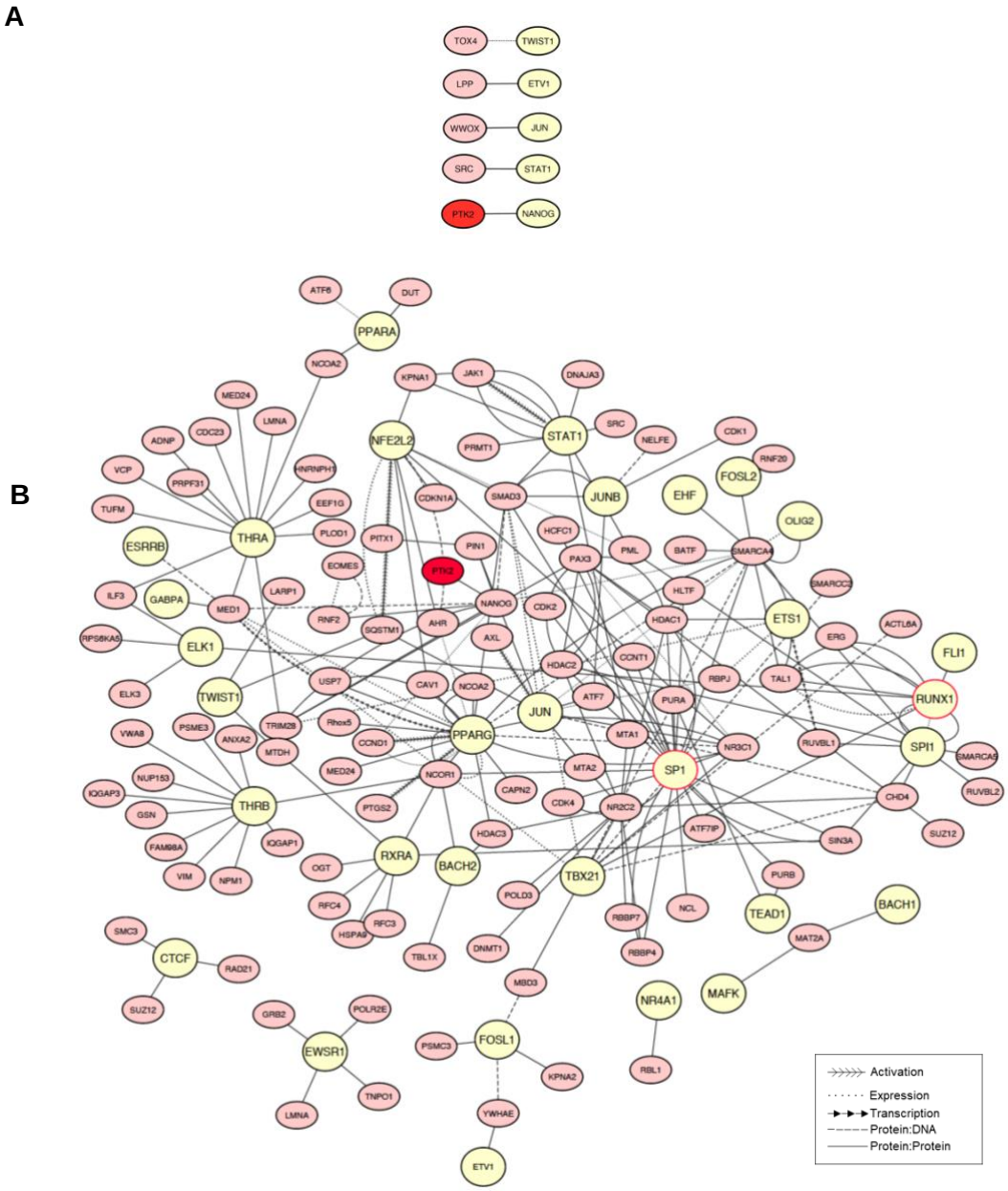


Figure 4.10 | Integration of predicted FAK WT enriched transcription factors with nuclear FAK interactome datasets show potential key nodes. Network detailing upstream, direct mammalian connections between A) FAK nuclear BioID (section 3.3.3) and B) FAK nuclear interactome (Serrels *et al*., 2015) and global predicted FAK WT enriched transcription factors were constructed using Ingenuity pathway analysis. Symbols for protein-protein and functional connections are shown as detailed in the key. Proteins highlighted in pink are present in A) nuclear FAK WT BioID and B) Nuclear FAK interactome datasets. Proteins in yellow are the global FAK WT enriched transcription factors, as predicted by motif enrichment analysis. Proteins in B) circled in red are proteins both present in the nuclear FAK interactome dataset (Serrels *et al*., 2015) and are FAK WT enriched transcription factors predicted by motif enrichment analysis. N=3 Biological replicates for proteomics datasets and N=2 biological replicates for ATAC-seq dataset.

4.3.4 FAK-dependent changes in accessibility and transcription factor binding on *IL33* gene

In addition to looking at changes in transcription factor binding on a global scale, I have also used the ATAC-seq results to analyse the regulation of a specific gene, *IL33*. Previous work from the Frame group has shown FAK binds and regulates the nuclear cytokine IL33, and this functions downstream of FAK in regulating the expression of immunosuppressive cytokines, thereby supporting tumour growth (Serrels *et al*., 2017). The mechanism by which FAK regulates *IL33* mRNA expression has not been established which is what I will investigate in this next section. The mouse *IL33* gene is located on chromosome 19 and consists of 8 exons and 7 introns with two overlapping transcripts – transcript 1 and transcript 2 (**Figure 4.11**). Transcript 2 may have an alternative transcriptional start site (TSS) to transcript 1 as transcript 2 has a large first intron, spanning over 20kb upstream of the IL33 transcript 1 TSS. Inspection of the chromatin accessibility traces on the *IL33* gene from the FAK SCC cell lines indicated that there was a loss of chromatin accessibility on the *IL33* promoter/enhancer regions upon loss of FAK or FAK function (**Figure 4.12A**). This was particularly striking in the FAK NLS and FAK KD cells, which showed almost complete loss of chromatin accessibility at the *IL33* promoter/enhancer regions (**Figure 4.12A**). We performed a motif enrichment analysis on the ATAC-seq peaks proximal to the *IL33* promoter in the FAK WT cells, identifying 25 FAK-dependent transcription factor motifs such as motifs known to bind AP-1 components JUN, ATF2 and ATF7. Interactome analysis using the Ingenuity pathway analysis software indicated that the transcription factors known to bind the FAK-regulated motifs on the *IL33* promoter/enhancer are well connected and regulate the expression of other FAK WT enriched predicted transcription factors on the *IL33* promoter (**Figure 4.12B**). Locations of the motifs within the ATAC-seq peaks for a number of the predicted FAK-regulated transcription factors on the *IL33* gene are indicated in **Figure 4.12C**. Integration of the FAK BioID nuclear proteomics dataset with the transcription factors known to bind FAK-regulated motifs on the FAK WT *IL33* promoter/enhancer regions showed a modest number of potential upstream connections between the proteomics data and the transcription factor list – namely WWOX:JUN and YEATS2:TBP (**Figure 4.13A**). Integration of the FAK nuclear interactome data (Serrels *et al*., 2015),

with that of the transcription factors on the FAK WT *IL33* promoter/enhancer regions, indicated that there were many upstream connections between the FAK proteomics dataset and the FAK WT transcription factors on the *IL33* promoter/enhancer regions (**Figure 4.13B**). The network formed 5 subnetworks all connected by the AP-1 factor, JUN, which formed the central node of the network.

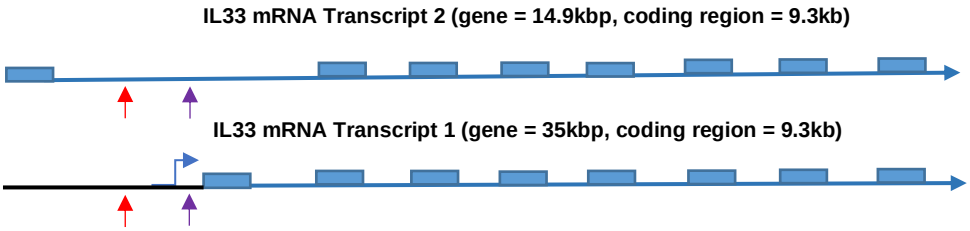


Figure 4.11 | Schematic of the IL33 gene. Schematic of the IL33 gene transcripts 1 and 2. Both the IL33 transcripts have a total of 8 exons and are both identical except that the IL33 transcript 2 has a larger first intron than the IL33 transcript 1. Boxes indicate exons and blue indicate regions that are part of the IL33 transcripts. The black line upstream of IL33 transcript 1 schematic indicates an upstream genomic region. Red arrows indicate regions where there is an ATAC-seq peak of a probable enhancer, whereas purple arrows indicate regions where there is a possible promoter region.

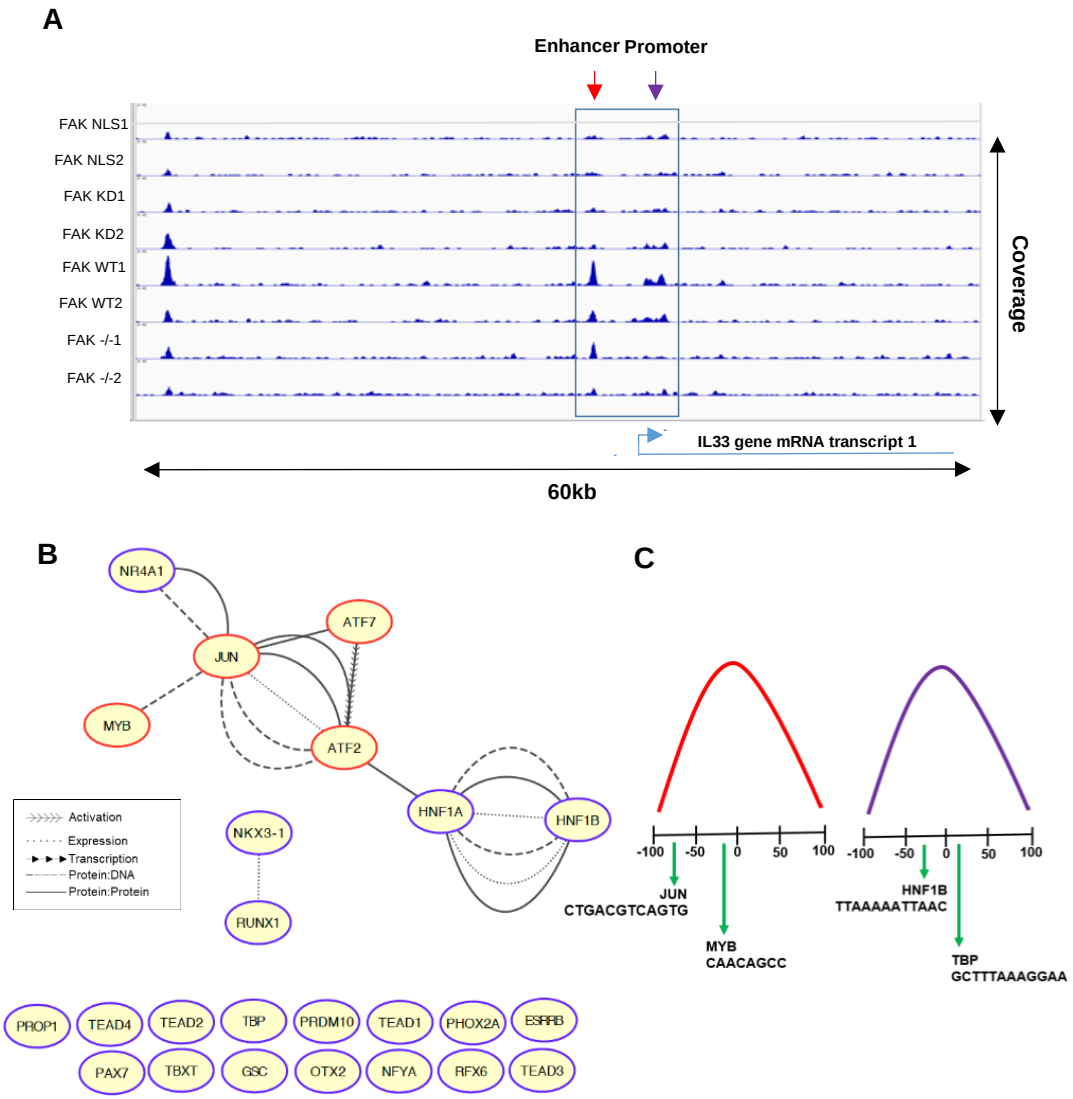


Figure 4.12 | FAK regulates chromatin accessibility and transcription factor motif enrichment at the *IL33* promoter and enhancer regions. A) Shows chromatin accessibility traces obtained from two replicates of each cell line used in the ATAC-seq experiment. Outlined is the region of interest. Y axis is coverage whereas x axis is kb. A schematic of the location of the *IL33* gene (transcript 1 shown only) with respect to the chromatin accessibility trace is shown below. Courtesy of R. Upstill-Goddard. B) The list of transcription factors known to bind FAK-regulated motifs on the *IL33* gene enhancer/promoter regions was used to seed a network using Ingenuity Pathway Analysis and then exported into Cytoscape for final figure presentation. Only direct, mammalian connections are shown between transcription factors known to bind FAK-regulated motifs. Protein-Protein and functional connections are detailed in the key. Transcription factors known to bind FAK-regulated motifs on the enhancer regions are circled in red and FAK-regulted motifs on the promoter region are circled in purple. N=2 Biological replicates. C) Schematic showing locations of the transcription factor binding sites within the enhancer (red) and promoter (purple) ATAC-seq peaks on IL33 gene. Motif sequences reported underneath transcription factor name. 0 indicates the centre of the ATAC-seq peak.

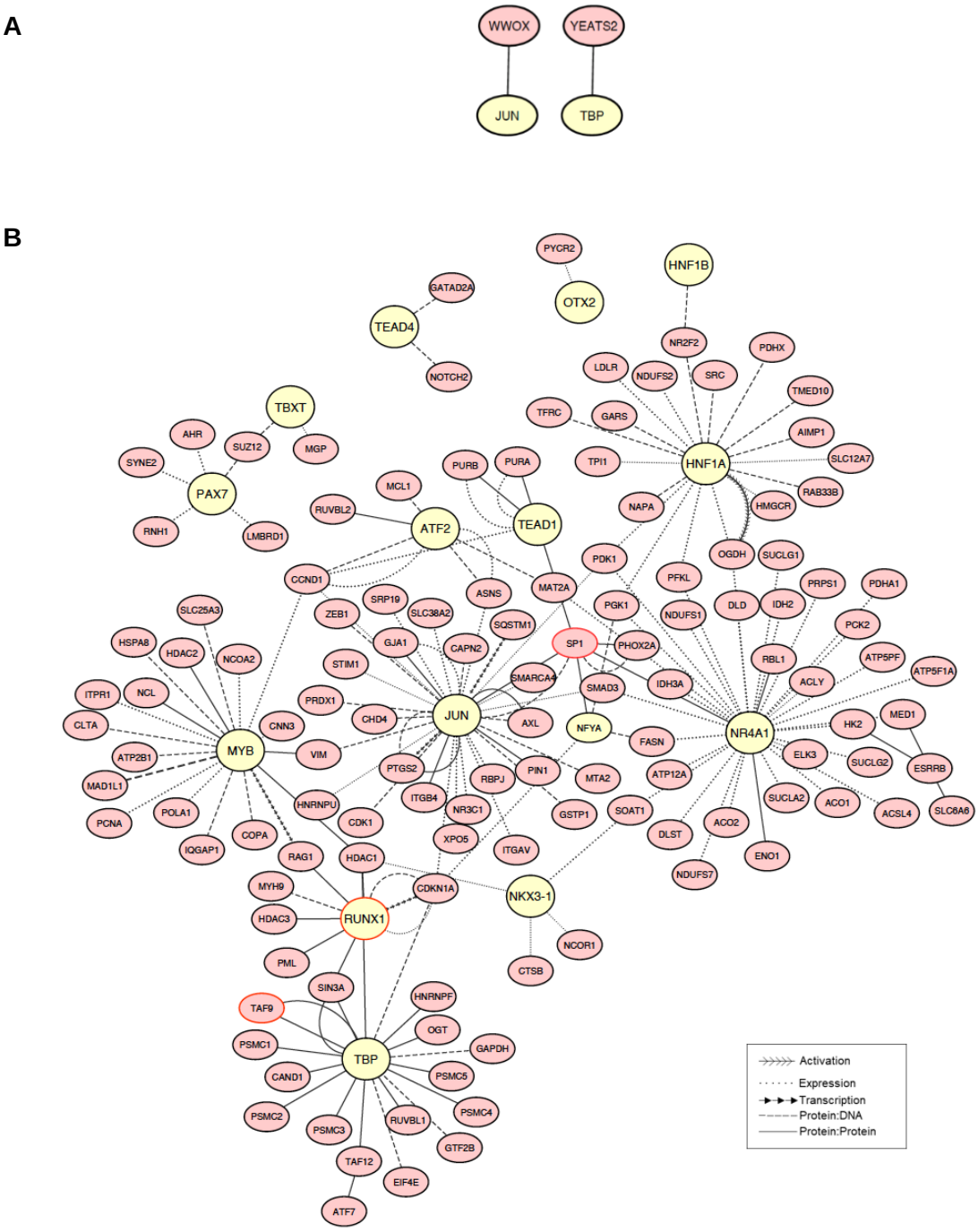


Figure 4.13 | Integration of the predicted FAK-regulated transcription factors on the *IL33* promoter/enhancer with nuclear FAK interactome datasets show potential key nodes. Network detailing upstream, direct mammalian connections between A) nuclear FAK BioID and B) FAK nuclear interactome (Serrels *et al*., 2015) and predicted FAK WT-enriched transcription factors on *IL33* gene were constructed using Ingenuity pathway analysis. Symbols for protein-protein and functional connections are detailed in the key. Proteins highlighted in pink are present in A) Nuclear FAK BioID and B) Nuclear FAK interactome datasets. Proteins in yellow are the transcription factors predicted to be bound on the *IL33* promoter/enhancer regions in the FAK WT cell lines, as determined by motif enrichment analysis. Proteins circled in red are those identified as FAK interactors by the Frame lab. N=3 biological replicates for proteomics datasets and N=2 biological replicates for ATAC-seq dataset.

JUN is a member of the AP-1 family which is an important regulator of skin inflammation (Schonthaler *et al*., 2011). In order to investigate the potential mechanism by which FAK regulates the expression of *IL33*, I performed siRNA mediated depletion of *JUN* mRNA (**Figure 4.14A**), which lead to a significant downregulation of *IL33* mRNA (**Figure 4.14B**, $P < 0.001$). Furthermore, *JUN* mRNA knockdown also resulted in a reduction in IL33 protein expression (**Figure 4.15A-B**). Lastly, the FAK and *IL33* target gene, *CXCL10* also showed reduced mRNA levels as a result of *JUN* knockdown (**Figure 4.16**, $P = 0.0024$). This implies that JUN may be a key regulator of *IL33* expression.

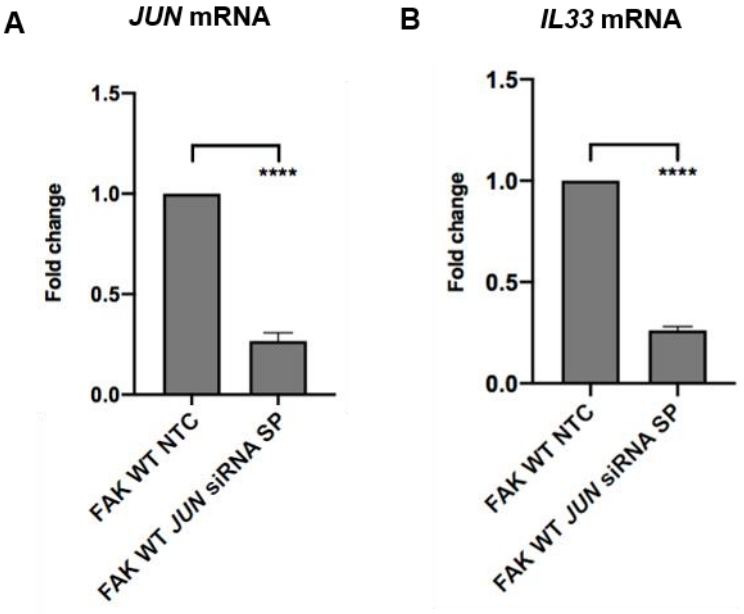


Figure 4.14 | *IL33* expression is disrupted upon *JUN* knockdown. FAK WT cells were transfected with a non-targeting control (NTC) or *JUN* smartpool siRNA prior to RNA extraction and cDNA synthesis as described in Materials and Methods. A) *JUN* and B) *IL33* qRT-PCRs were carried out using *JUN* and *IL33* primers, respectively. CT values were normalised to each samples respective *GAPDH* CT and then each replicate FAK WT NTC to gain a fold change in gene expression. SP = smartpool. N=3 biological replicates. Unpaired two-tailed t-test used for statistical analysis *** = $P \leq 0.001$.

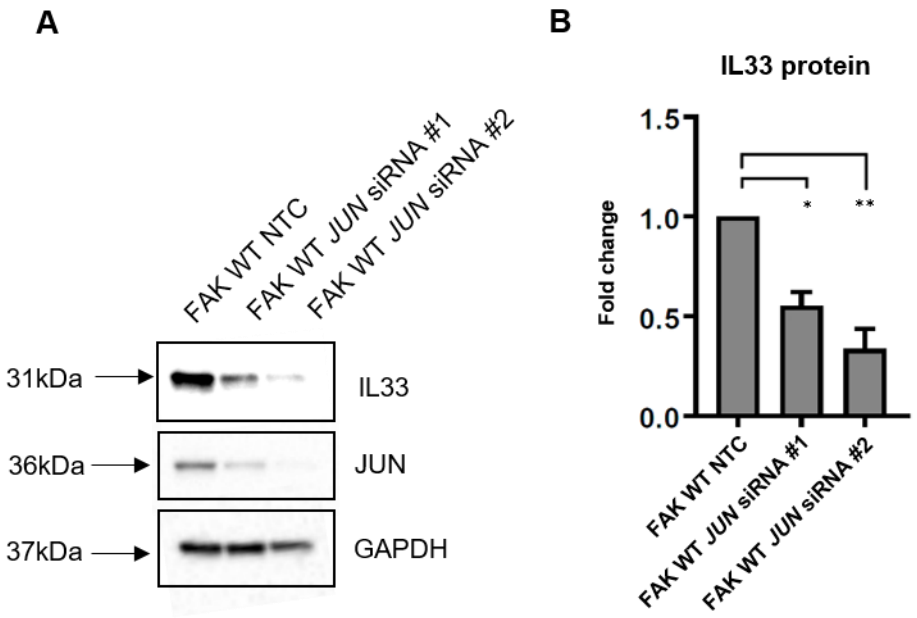


Figure 4.15 | Reduced IL33 protein expression upon *JUN* knockdown. A) FAK WT cells were transfected with NTC or individual JUN siRNAs as described in Materials and Methods. Whole cell lysates were prepared and quantified prior to SDS-PAGE analysis. Blots were probed with IL33, JUN and GAPDH antibodies as indicated to the right of the blot. B) IL33 protein expression was quantified using ImageJ and then values were normalised to GAPDH levels. N=3 biological replicates. One-way ANOVA used for statistical analysis. * = P ≤ 0.05, ** = P ≤ 0.01.

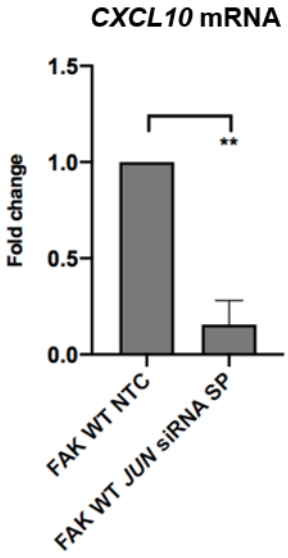


Figure 4.16 | *IL33* target gene expression is disrupted upon *JUN* knockdown. FAK WT cells were transfected with NTC or *JUN* smartpool siRNA prior to RNA extraction and cDNA synthesis as described in Materials and Methods. qRT-PCR was performed using *CXCL10* primers. N=3 biological replicates. SP = smartpool. Unpaired two-tailed t-test used for statistical analysis, ** = P ≤ 0.01.

4.3.5 ChIP analysis indicated loss of JUN binding to the IL33 enhancer in FAK-kinase defective mutant

I performed ChIP analysis to determine if JUN binds to the JUN/AP-1 motif sequences on the *IL33* enhancer and if there is FAK-dependent chromatin accessibility changes on the *IL33* enhancer that may perturb JUN binding. ChIP is a widely used protocol in molecular genetic research which utilises formaldehyde cross-linking to stabilise protein-DNA complexes allowing one to identify genomic regions in which a target protein binds. The genomic DNA that a target protein binds can be analysed by q-PCR with primers to the region of interest and a region upstream of this site to control for background binding.

Primers were designed for the region containing the JUN motif sequences on the *IL33* enhancer and a region upstream of this site to control for background binding (**Figure 4.17A**). I performed the JUN ChIP experiments in the FAK WT and the FAK KD cells as the FAK KD cells had a striking loss of chromatin accessibility on the enhancer, whereas the FAK -/- cells still displayed a small peak at this region (**Figure 4.12A**). According to the ChIP experiment data JUN binds the *IL33* enhancer in the FAK WT cells (**Figure 4.17B**, P = 0.0009). However, JUN did not significantly bind on the *IL33* enhancer in the FAK KD cells, when compared to the respective IgG control samples (**Figure 4.17B**). These data confirm the predictive motif enrichment findings presented in **section 4.3.4** and indicates that FAK regulates chromatin accessibility of the enhancer region of the *IL33* gene.

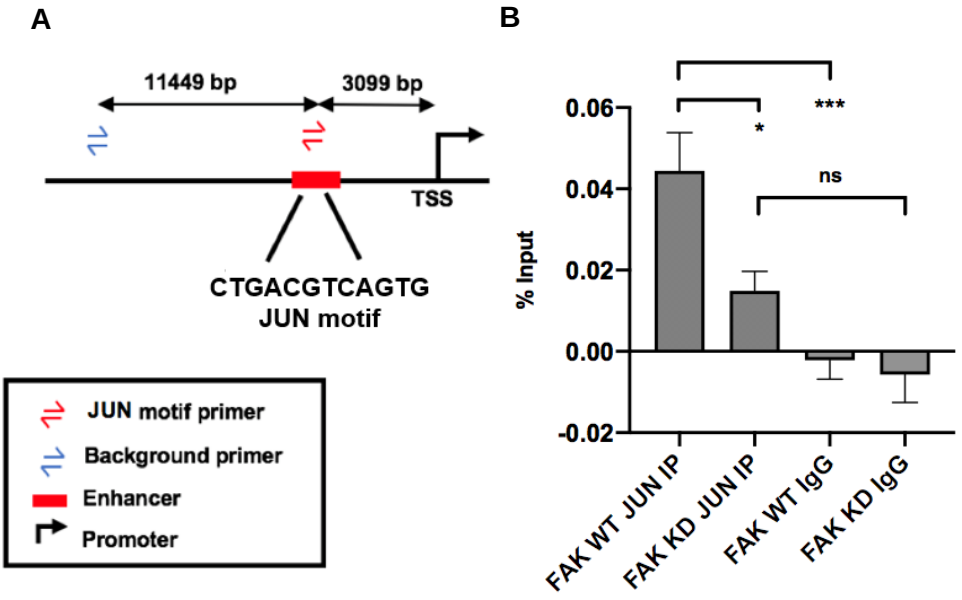


Figure 4.17 | JUN ChIP-qPCR analysis on *IL33* enhancer shows loss of JUN binding in FAK-kinase deficient cells. A) Schematic showing location of primers used. B) ChIP-qPCR analysis was performed on FAK WT and FAK KD cells as described in Materials and Methods. Primers were designed around the JUN motif identified in HOMER (JUN motif primer). To control for non-specific binding, primers were designed to an upstream region of the *IL33* gene where JUN is not predicted to bind (background primer). Pull down efficiency is reported as % Input, which was first normalised to the readings from the non-specific region and then the resulting value was compared to the IgG. N=5 biological replicates, mean +/- SEM. One-way ANOVA test was used for statistical analysis. ns = P > 0.05, * = P ≤ 0.05, *** = P ≤ 0.001.

4.4 Discussion

4.4.1 FAK is a potential regulator of AP-1 and ETS domain transcription factor binding to chromatin

The motif enrichment results presented here suggest that FAK is a potential regulator of AP-1 and ETS motif enrichment in accessible regions of chromatin. Interestingly, another ATAC-seq study comparing healthy cells with cancer cells found that genomic regions containing AP-1 and ETS motifs were more open in oesophageal adenocarcinoma versus healthy oesophageal cells (Britton *et al.*, 2017). However, it was not determined what the consequence of this was for oesophageal adenocarcinoma cancer phenotypes. AP-1 and ETS proteins are key transcriptional regulators of many cancer-related process such as proliferation (reviewed in Sharrocks, 2001, Eferl and

Wagner, 2003). This indicates that enrichment of available AP-1 and ETS binding sites could be important in driving cancer phenotypes. Further work will be required to understand whether these changes in AP-1 and ETS motif enrichment in accessible chromatin regions specifically drive FAK-dependent phenotypes.

Transcription factor motif sequences predicted by HOMER identifies regions in the genome that are known DNA binding motifs for particular transcription factors, based on ChIP-seq data for particular transcription factors. However, many transcription factor family members will bind to the same motif sequence, and therefore this analysis does not rule out the possibility that another transcription factor from the same family can bind to the exact same sequence. The motif enrichment analysis indicated that in some cases (i.e. AP-1 family) up to 5 transcription factor family members can bind the same motif sequence. It is therefore difficult to determine whether any one particular transcription factor is significantly enriched without performing the necessary ChIP-seq experiments on the individual family members. Overlap in motif binding sites is exemplified in validation experiments which showed JUN was upregulated in the FAK -/- SCC cells compared to the FAK WT SCC cells (**Figure 4.8**), which is opposite to the predictions from the FAK WT motif enrichment data. Therefore, the motif enrichment analysis likely reflects the possibility that another AP-1 factor could potentially be enriched on the chromatin in the FAK WT SCC cells. However, the global analysis is a good indicator of the transcription factor family motifs that are enriched across the genome between cell lines and analysis of the functions of these transcription families can give an indication of the consequence of this enrichment of transcription factor binding motifs based on the general function of those transcription factor families. That said, members of the same transcription family display divergent functions. For example, JUN has oncogenic transformation activity, while JUND has no oncogenic transformation capabilities (reviewed in Eferl and Wagner, 2003).

As previously described, FAK is a key regulator of inflammation in cancer which the Frame group has studied in detail using the K14 CreER $FAK^{flox/flox}$ cutaneous skin SCC mouse model (Serrels *et al*., 2015, 2017). Interestingly, JUNB is a key regulator of inflammation in the skin (reviewed in Schonthaler, Guinea-Viniegra and Wagner,

2011). This implies that JUNB could be a potential downstream effector in regulating FAK-dependent inflammation. The mechanism by which FAK downregulates JUNB protein expression and the significance of JUNB in these cells remains to be determined. The identification that one of the AP-1 members JUNB, had increased protein expression in the FAK WT cells compared to the mutant FAK -/-, NLS and KD cells is a reassuring result as it supports our predictive motif enrichment findings. However, FAK does not regulate the chromatin recruitment of JUNB *per se*.

FAK has been shown previously to regulate the protein stability of P53 by binding to MDM2 and scaffolding its interaction with P53 (Lim *et al*., 2008b). Thus, it is a possibility that FAK could regulate the protein stability of JUNB. JUNB is known to be constantly turned over by the ubiquitin-dependent degradation, which is facilitated by JUNB binding the E3 ligase ITCH (Li *et al*., 2016). It would be interesting to determine whether FAK can facilitate JUNB turnover. However, it is not established whether JUNB is predominantly turned over inside the cytoplasm or nucleus, which is a point to consider since the ubiquitin-dependent degradation generally occurs in the cytoplasm. Interestingly, evidence showed that FAK's nuclear localisation was important for regulating JUNB protein expression (**Figure 4.6**) suggesting that FAK may play a role in the nucleus to regulate its turnover. This work suggests FAK could potentially regulate transcription factor function by regulating protein expression. It would have been ideal to have validated the transcription factor levels in the chromatin fraction for more FAK-regulated transcription factors predicted by the motif enrichment analysis; however due to the lack of effective antibodies this was not possible.

FAK is present in the chromatin fraction isolated from SCC cells (Serrels *et al*., 2015) consistent with it potentially regulating gene expression via direct binding of transcription factors at target genes. Integration of the nuclear FAK interactome and nuclear FAK BioID dataset with the FAK WT motif enrichment data indicated some possible mechanisms by which FAK could regulate AP-1 and ETS proteins. For example, WWOX was identified as a FAK interacting protein and it binds to JUN suppressing its transcriptional activity by sequestering it in the cytoplasm (Gaudio *et*

al., 2008). This report found the majority of WWOX and JUN co-localised in the cytoplasm and the significance of the WWOX:JUN interaction in the nucleus is unknown. A number of established FAK binding partners in the nucleus (Serrels *et al*., 2015) identified previously have direct connections to transcription factors such as SP1 (Serrels *et al*., 2017) and NANOG (Ho *et al*., 2012). SP1 has been reported to bind to JUN to regulate the expression of human 12(S)-lipooxygenase expression (Chen and Chang, 2000), while FAK directly binds NANOG in embryonic stem cells (ES) cells to regulate invasion (Ho *et al*., 2012). Additionally, the FAK nuclear BioID interactome data (Chapter 3) suggested that FAK binds to co-activators such as LPP which has been shown to regulate the ETS family member ETV4 in MDA-MB-321 human breast cancer cells (Guo *et al*., 2006). Thus, there are a number of interesting upstream and direct connections in the nuclear FAK interactome and that of the predicted FAK WT enriched transcription factor binding proteins. This interactome analysis suggests that FAK could regulate recruitment of the predicted transcription factors to chromatin by binding co-activators such as LPP or by direct binding of the transcription factor such as NANOG. As discussed in **section 4.3.4**, it is possible that FAK can regulate the chromatin accessibility within target genes and this could potentially recruit transcription factor recruitment to that genomic region.

The motif enrichment data suggests that FAK may be important for regulating transcription factor recruitment/function to specific gene promoters. However, we cannot distinguish between a direct effect of FAK mediating transcription factor recruitment to chromatin or FAK signalling that feeds down to regulate transcription factor chromatin recruitment. For example, it is known that MAPK phosphorylates ETS1 and ETS2 transcription factors, enhancing transactivation activity (Foulds *et al*., 2004). Interestingly, FAK has been shown to function upstream of the GRB2/RAS/MAPK signalling pathway (Schlaepfer *et al*., 1994). It has also been shown that the Toll-like receptor 2 (TLR2) /FAK/PI3K/AKT signalling axis is important for downstream JUN activity in response to peptidoglycan that stimulates *IL-6* expression (Chiu *et al*., 2009). Thus, it is possible that FAK-dependent transcription factor recruitment could in fact be due to loss of upstream signalling functions of FAK in the cytoplasm.

By incorporating the FAK NLS mutant into the motif enrichment analysis, we found that 205 predicted transcription factors show FAK nuclear localisation dependent motif enrichment. Furthermore, the FAK WT vs KD cell line motif enrichment analyses indicate that there are 149 predicted transcription factors whose motif enrichment is regulated by FAK's kinase activity, implying its importance for regulating transcription factor function. FAK's kinase activity and FAK's nuclear localisation are both important for regulating the anti-tumour immune response (Serrels *et al*, 2015). In this study, it was not established whether FAK's kinase activity in the nucleus was important for this function. There are reported FAK kinase independent interaction partners with transcription factors in the nucleus i.e. P53 (Lim *et al*., 2008b) and RUNX1 (Canel *et al*., 2017). Thus, it remains to be established whether FAK's kinase activity is important for transcriptional responses or is solely important for signalling upstream that regulate transcription factor function downstream.

An important point to discuss is that this study has been performed in one cell model. No other research group has performed an analysis of FAK-dependent transcription factor motif enrichment and chromatin accessibility changes using ATAC-seq before. Therefore, to understand the significance of these results to other cell types and cancers, these analyses need to be expanded to other cell types. What this study has indicated is that FAK could have a more direct ability to regulate transcription factor function, especially apparent from FAK WT vs FAK NLS cell line motif enrichment analysis. Although in other cell types the particular transcription factors that FAK is regulating may change to account for cell- and tissue-specific gene expression.

4.4.2 FAK regulation of IL33 enhancer chromatin accessibility

FAK binds to the IL33 protein and also regulates its mRNA and protein expression (Serrels *et al*., 2017). Furthermore, it was shown that IL33 functions downstream of FAK in SCC cells regulating the expression of immuno-suppressive cytokines such as *CXCL10* and *CCL5*, which promote tumour immune evasion (Serrels *et al*., 2017). I

next investigated the mechanism by which FAK regulates *IL33* expression. Analysis of the chromatin accessibility traces showed that there was a reduction in accessible regions at the enhancer and promoter regions of the *IL33* gene (**Figure 4.12A**).

Building on these observations, I next identified which transcription factor motifs were enriched in accessible regions upstream of the *IL33* gene by performing motif enrichment analysis, to predict the key transcription factors responsible for regulating *IL33* expression. Previous work had used an *in silico* analysis to predict the transcription factors that regulate *IL33* expression, which identified RUNX1 and SP1 as potential regulators. However, knockdown studies found no significant reduction of IL33 protein level when RUNX1 or SP1 was suppressed individually (Serrels *et al*., 2017), suggesting these transcription factors are not independently essential for regulating *IL33* expression. Motif enrichment analysis in the FAK WT SCC cells detected AP-1 and MYB motifs on the *IL33* enhancer regions, while the promoter mainly displayed enrichment of ETS and RUNX transcription factor motifs (**Figure 4.12B**). Therefore, I focused on the predicted FAK-regulated AP-1 member, JUN as the interactome analyses indicated it may be a central node in the network (**Figure 4.13B**) and it is highly implicated in oncogenesis (reviewed in Eferl and Wagner, 2003). Knockdown experiments showed that JUN indeed controls *IL33* expression (**Figure 4.14B**, **Figure 4.15A-B**) and that of its target gene, *CXCL10* (**Figure 4.16**). JUN ChIP experiments indicated that JUN binds to the enhancer region of *IL33* and this is disrupted upon loss of FAK's kinase activity (**Figure 4.17B**). Furthermore, our JUN ChIP results indicated a 0.04% enrichment over input which is similar to that reported in previous JUN ChIP studies (Veluscek *et al*., 2016). Thus, JUN can directly regulate *IL33* expression by binding to the *IL33* enhancer region.

From this work, I hypothesise that FAK regulates chromatin accessibility at the enhancer/promoter region of the *IL33* gene. Access to DNA is a highly regulated process which is dependent on a number of factors, including external stimuli and development cues (reviewed in Klemm *et al*., 2019. Mechanistically, chromatin accessibility is mediated by transcription factors and chromatin modifying complexes (reviewed in Klemm *et al*., 2019). AP-1 has been shown to recruit the BAF chromatin

remodelling complex to enhancers of genes to establish accessible chromatin (Vierbuchen *et al*., 2017). BAF is an ATP-dependent chromatin remodeling complex essential for the regulation of gene expression via modulating chromatin accessibility (reviewed in Alfert, Moreno and Kerl, 2019). Others have found that AP-1 binding is essential for maintaining chromatin accessibility at glucocorticoid target genes (Biddie *et al*., 2011). Taken together these data suggest that AP-1 can regulate chromatin accessibility at target genes by recruiting chromatin modifying complexes such as BAF.

Finally, previous work using IL33-BirA*-proteomics found that IL33 interacts with multiple members of the BAF complex, along with a number of other chromatin modifying complexes (Serrels *et al*., 2017). Since FAK binds and regulates the expression of *IL33* (Serrels *et al*., 2017), it is plausible that IL33, in complex with FAK, can regulate the expression of itself by recruiting chromatin modifying complexes to promoter/enhancers regions. Thus, there are a number of possible mechanisms by which FAK could be regulating chromatin accessibility at the *IL33* gene and these warrant further study in different cellular contexts.

4.5 Conclusions

The work presented here has indicated that FAK regulates AP-1 and ETS motif enrichment in accessible regions on chromatin and chromatin accessibility of a subset of genes. Chromatin accessibility data of an established FAK regulated gene, *IL33*, indicated FAK-dependent changes in chromatin accessibility in the promoter and enhancer region of this gene. Further experiments indicated that JUN can potentially regulate the expression of *IL33* by binding to its enhancer, which is lost when FAK-kinase activity is disrupted. Thus, these data implicate a potential mechanism by which FAK regulates *IL33* expression.

Chapter 5 FAK and FAK's kinase activity is important in regulating the transcription of genes associated with inflammation and invasion

5.1 Introduction

FAK dependent gene programs were previously determined using Affymetrix microarrays (Serrels *et al*., 2015, **Figure 5.1**). Although this work identified FAK as a regulator of chemokines and cytokines, it stopped short of addressing FAK kinase dependent gene programs. In this section, I used mRNA-seq in the FAK WT, FAK -/- and FAK KD SCC cell lines to define FAK- and FAK-kinase regulated gene sets, in order to determine the range of genes regulated by FAK and the role of its kinase activity in transcription.

In **section 4**, I identified that a known FAK-regulated gene, *IL33*, exhibits FAK-regulated changes in chromatin accessibility at the *IL33* promoter/enhancer regions. Building on these observations, I decided to investigate whether other FAK-regulated genes also exhibit FAK-dependent changes in chromatin accessibility at promoter/enhancer of genes (**section 4.2.1**). By doing this, I aimed to determine if regulation of chromatin accessibility is one potential mechanism by which FAK regulates transcription. I also investigated whether any of the identified FAK-regulated genes which display FAK-dependent chromatin accessibility changes at promoter/enhancer regions could potentially have roles in established FAK functions (i.e. invasion).

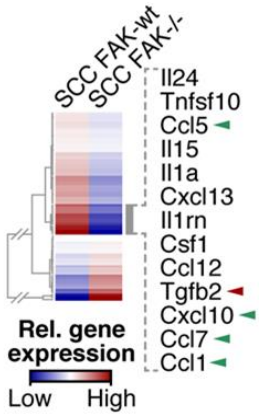


Figure 5.1 | FAK is a key regulator of the expression of inflammatory genes. Chemokine and cytokine expression in FAK WT and FAK -/- SCC cell lines were analysed using Affymetrix microarrays. Gray bar indicates cluster of inflammatory genes upregulated in FAK WT cell line. Green arrowheads highlight genes known to function in Treg recruitment and the red arrowhead indicates genes involved in peripheral Treg induction. Figure taken from Serrels *et al*., 2015

5.2 Aims

- Define the range of FAK- and FAK-kinase regulated genes.
- Identify the importance of FAK kinase activity in FAK-regulated transcription.
- Integrate mRNA-seq data with the ATAC-seq chromatin accessibility findings to determine if FAK-regulated differentially expressed genes also display FAK-dependent changes in chromatin accessibility.
- Establish whether novel FAK-regulated genes which display FAK-dependent chromatin accessibility changes at promoter/enhancer regions are biologically important for regulating FAK function.

5.3 Results

5.3.1 Identification of FAK- and FAK-kinase dependent gene sets using mRNA-seq

To identify FAK and FAK-kinase dependent genes sets, I prepared three biological replicates of RNA from FAK WT, FAK -/- and FAK KD cell lines and submitted the samples to the transcriptomic company BGI (Hong Kong) for sequencing. RNA-seq analyses the presence and amount of RNA in a specific sample, allowing one to compare the amount of a particular RNA between cell lines and conditions (Finotello and Camillo 2014). Specifically, mRNA-seq was performed which analyses only mRNA transcripts and excludes non-coding RNA. We aligned transcripts to the GRCm38.cDNA mouse reference transcriptome and differential expression analysis was performed between FAK WT, FAK -/- and FAK KD cell lines results using the DESeq2 software. All compared transcripts that were differentially expressed in the FAK WT vs FAK -/- and FAK WT vs FAK KD cell lines that displayed above or below -1 (halved) or 1-fold (doubled) change, respectively and acquired a corrected P-value of 0.05 were considered statistically significant in the differential expression analysis.

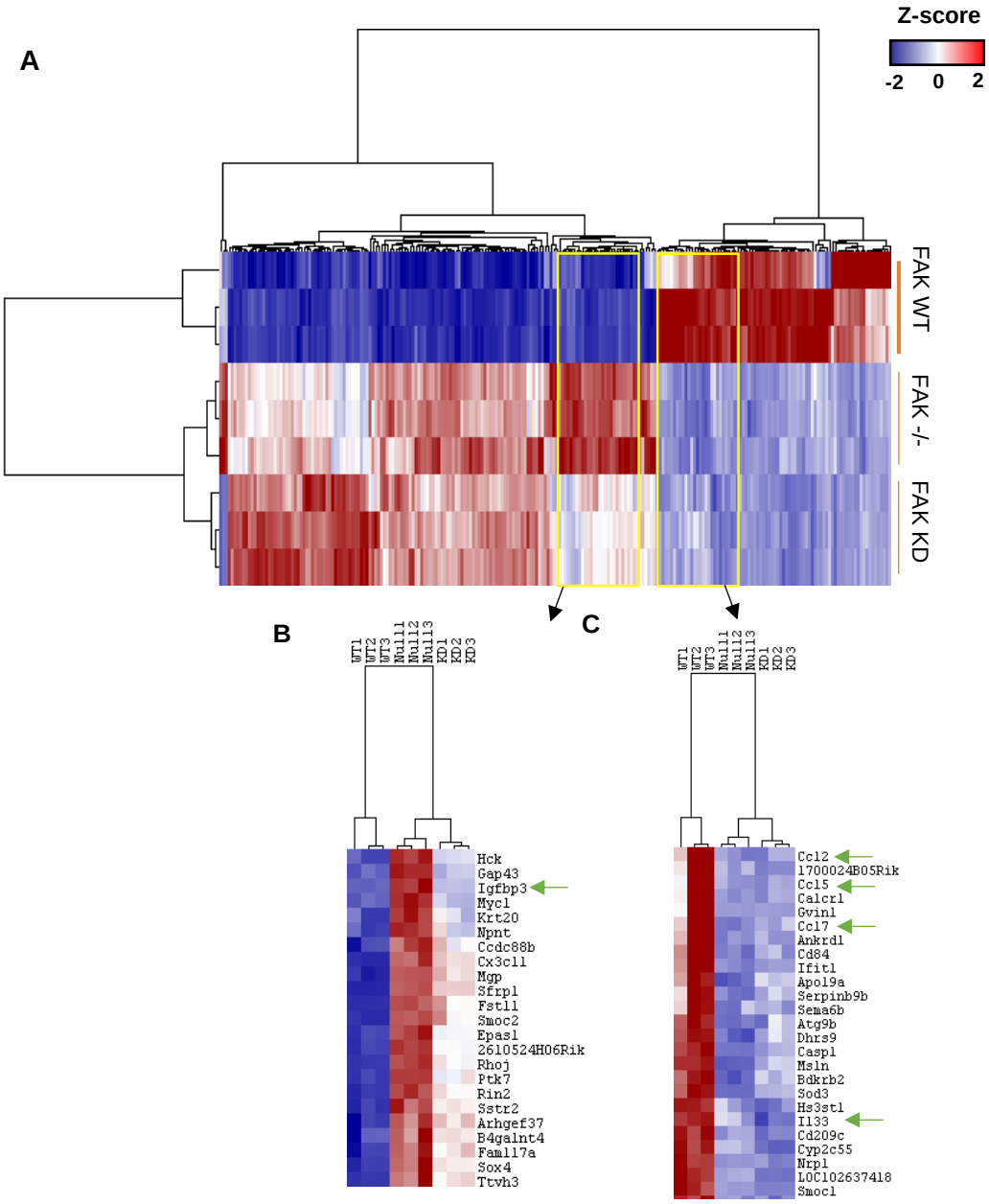


Figure 5.2 | FAK mRNA-seq analysis identifies many known FAK-regulated genes. RNA from FAK WT, FAK -/- and FAK KD cell lines were submitted for mRNA-seq analysis and differential expression analysis performed as described in Material and Methods. A list of common differentially expressed genes between the FAK WT vs FAK -/- and FAK WT vs KD analyses was constructed using the VENNy online software. The data was Z-scored prior to hierarchical clustering using Cluster 3.0 software and visualisation in TreeView. A) Shows clustered heatmap representing the transcriptome of all common FAK- and FAK-kinase regulated genes. B) & C) Show heatmaps from Figure A), which include known FAK-regulated genes (indicated by green arrows). Yellow rectangles in A) represent the zoomed areas of the heatmap in B) and C). N=3 biological replicates.

The data showed approximately 400 differentially expressed genes in the FAK WT vs FAK -/- and the FAK WT vs FAK KD analyses. Hierarchical clustering analysis indicated that the biological replicates in each cell line showed consistent levels of expression for the majority of differentially expressed transcripts (**Figure 5.2A**). Furthermore, hierarchical clustering indicated that the FAK -/- and FAK KD cell line gene expression data showed similar changes in gene expression changes between the two cell lines (**Figure 5.2A**). Additionally, this analysis indicated there was a clear subset of genes that were either up or downregulated in both the FAK WT vs FAK -/- and FAK WT vs FAK KD cell lines (**Figure 5.2A**). These included a number of known FAK-regulated genes that regulate proliferation such as *IGFBP3* (Canel *et al*., 2017, **Figure 5.2B**) and inflammation which included *IL33* (Serrels *et al*., 2017), *CCL5* and *CCL2* (Serrels *et al*., 2015, **Figure 5.2B-C**). In summary, this analysis indicated that the expression changes were consistent in the differentially expressed genes between

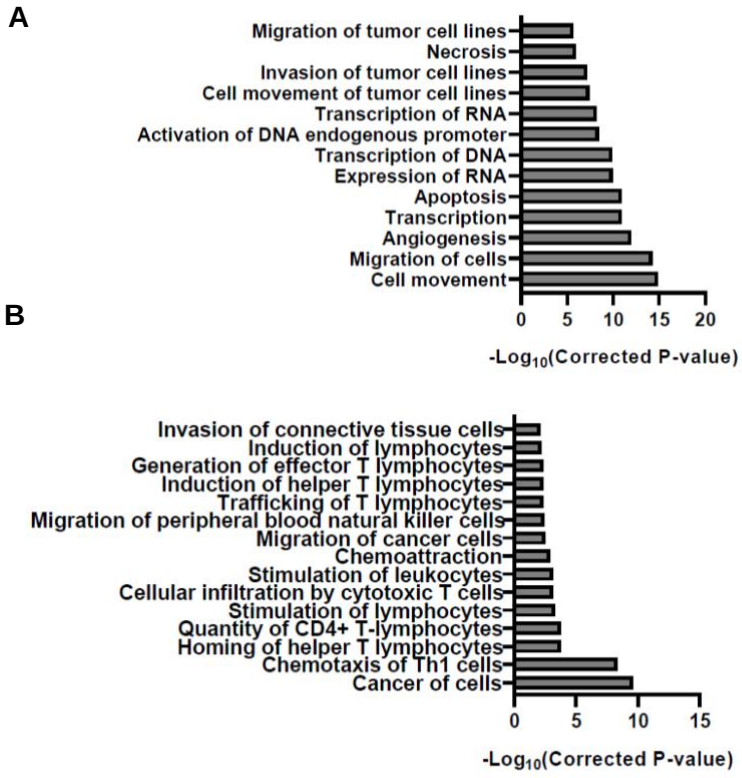


Figure 5.3 | GO enrichment analysis of FAK WT vs FAK -/- differential expression analysis. Functional analysis was carried out by performing a Disease and Functions core analysis using Ingenuity pathway analysis software. A) Indicates GO enrichment analysis of upregulated genes and B) shows GO enrichment analysis of all downregulated genes in the FAK WT vs FAK -/- differential expression dataset. –Log10 was calculated for all Benjamini-Hochberg corrected P-values.

the replicates within the replicates in each cell type, as well as identifying multiple known FAK-regulated genes. Together, these findings confirm the validity of this dataset.

To establish the functions of the FAK- and FAK-kinase dependent genes identified in the whole dataset, I performed GO enrichment analysis on the differentially expressed genes identified in both the FAK WT vs FAK -/- and FAK WT vs FAK KD cell line differential expression analyses. This indicated that loss of FAK was associated with an increase in genes associated with invasion ($P < 0.0001$), migration of tumour cell lines ($P < 0.0001$) and angiogenesis ($P = 0.00578$) (**Figure 5.3A**). It should be noted that there were overlapping terms in the GO enrichment analysis i.e. 'migration' and 'migration of tumour cells'. Loss of FAK was also associated with a downregulation of inflammatory genes specifically associated with the regulating T lymphocytes including $CD4_+$ T cells ($P = 0.000197$) and Th_1 cells ($P = 0.000194$) (**Figure 5.3B**).

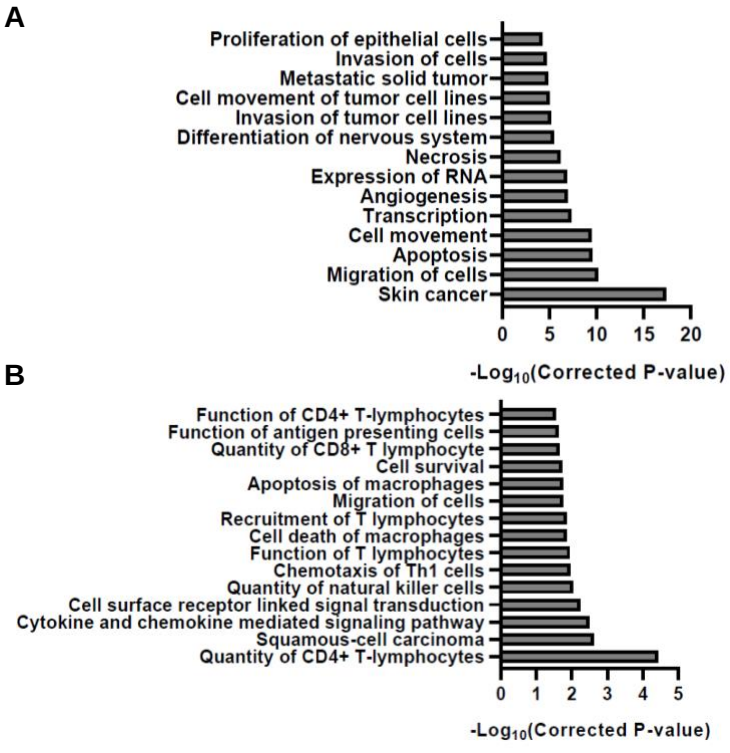


Figure 5.4 | GO enrichment analysis of FAK WT vs FAK KD cell line differential expression analysis. Functional analysis was carried out by performing a Disease and Functions core analysis using Ingenuity pathway analysis software. A) Reports GO enrichment analysis of upregulated genes and B) shows GO enrichment analysis of all downregulated genes in the FAK WT vs FAK KD cell lines differential expression analysis. –Log10 was calculated for all Benjamini-Hochberg corrected P-values.

Genes included under these terms included known FAK-regulated genes such as *CCL2* and *CCL5* (Serrels *et al*., 2015). Similarly, the FAK WT vs FAK KD cell line differential expression analysis indicated loss of FAK's kinase activity was also associated with an increase in the expression of genes associated with invasion ($P < 0.0001$), migration ($P < 0.0001$), angiogenesis ($P < 0.0001$) and transcription ($P < 0.0001$) (**Figure 5.4A**). Additionally, loss of FAK's kinase activity was associated with downregulation of genes associated with inflammation, in particular genes regulating the quantity of $CD4_+$ T lymphocytes ($P < 0.0001$), which was the most highly enriched inflammation term in the FAK WT vs FAK KD cell line differential expression analysis (**Figure 5.4B**). Together, the mRNA-seq results broadly align with well-known functions of FAK.

Validation of the mRNA-seq data by qRT-PCR analysis confirmed that the mRNA expression of the invasion-regulator *SFRP1*, not previously been shown to be a FAK-regulated gene, was significantly upregulated in the FAK -/- and FAK KD cells with respect to FAK WT cells (**Figure 5.5A**). The invasion-associated *WNT7B* gene was also upregulated in the FAK KD but was not significantly upregulated in FAK -/- with

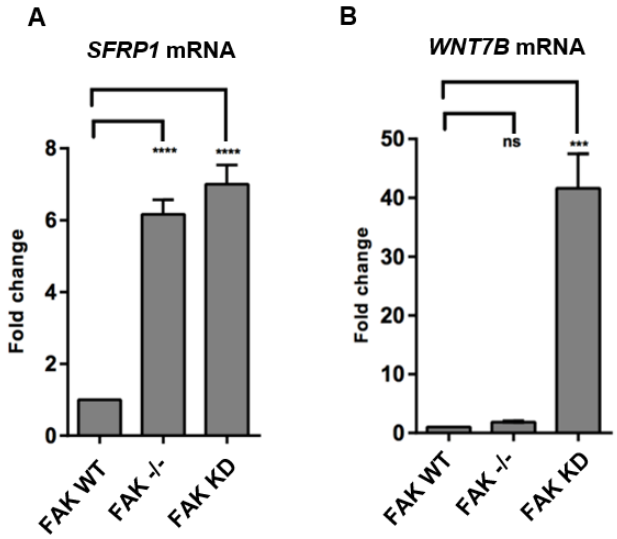


Figure 5.5 | Validation of FAK mRNA-seq data. FAK WT, FAK -/- and FAK KD cells were harvested for RNA extraction and cDNA synthesis as described in Materials and Methods. qRT-PCR was carried out using A) *SFRP1* or B) *WNT7B* primers, including *GAPDH* primers to control for loading. All CT values were normalised to *GAPDH* and respective FAK WT samples. Mean +/- SEM, N=3 biological replicates, *** = $P < 0.001$, **** = $P < 0.0001$.

respect to FAK WT cells (**Figure 5.5B**). Therefore, *WNT7B* expression in the FAK -/- with respect to FAK WT cells is not consistent with the mRNA-seq findings.

Next I decided to investigate the contribution of FAK's kinase activity to FAK-dependent transcription as it is clear that there was similar functional term enrichment in the FAK WT vs FAK -/- and the FAK WT vs FAK KD differentially expressed genes GO enrichment analysis (**Figure 5.3A-B**, **Figure 5.4A-B**). All the differentially expressed genes present in the FAK WT vs FAK -/- and the FAK WT vs FAK KD differential expression analysis were compared using Venny online software. This indicated that approximately 40% of FAK-regulated genes are kinase-dependent (**Figure 5.6**). Further inspection established that many of the genes that were common between the two datasets were in fact genes associated with inflammation (i.e. *CCL2, CCL5, CCL7, CCL9, IL33*) and invasion (i.e. *WNT7b, WNT10B, SFRP1*). Thus, the comparative analysis presented here suggests that FAK's kinase activity is important for the transcriptional regulation of genes associated with inflammation and invasion.

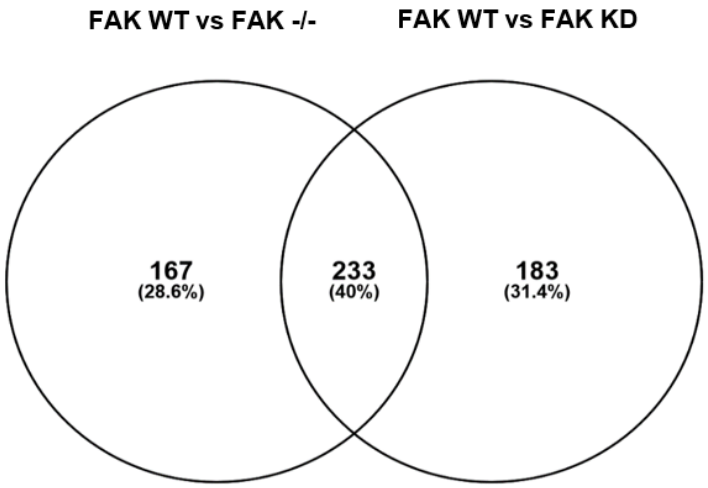


Figure 5.6 | Large proportion of FAK-regulated genes are kinase dependent. Differentially expressed genes identified in the FAK WT vs FAK -/- differential expression analysis were compared to the FAK WT vs FAK KD cell line differential expression analysis using Venny online software to determine common genes between the two differential expression analyses.

5.3.2 Integration of mRNA-seq and ATAC-seq FAK datasets identifies differentially expressed genes regulated by chromatin accessibility

In section 4, I identified that a known FAK-regulated gene, *IL33*, is regulated by FAK-dependent chromatin accessibility changes at its promoter/enhancer regions. In this section, I next investigated whether FAK-regulated genes identified by mRNA-seq in section 5.1.2 are also regulated by chromatin accessibility changes at promoter and enhancer regions. To do this, we filtered genes that display FAK-, FAK-nuclear localisation and FAK-kinase dependent changes in chromatin accessibility by analysing the FAK WT, FAK -/-, FAK NLS and FAK KD ATAC-seq datasets. Peaks were assigned to genes using ChIPseeker, where each peak was matched to the gene with the closest TSS. This list of genes displaying FAK- and FAK-kinase regulated chromatin accessibility changes were then compared with differentially expressed genes identified in the FAK WT vs FAK -/- and FAK WT vs FAK KD differential expression analyses (**Figure 5.7**, upper and middle panels). All genes that displayed significantly decreased chromatin accessibility (Benjamini-Hochberg corrected P-value < 0.05) at promoter/enhancer regions in the ATAC-seq dataset were matched with genes that exhibit significantly (Benjamini-Hochberg corrected P-value < 0.05) downregulated expression with respect the FAK WT in the mRNA-seq differential expression analysis (**Figure 5.7**, upper panel) and vice versa (**Figure 5.7**, middle panel), using Venny online software. Differentially expressed genes in common between the FAK WT vs FAK -/- and FAK WT vs KD differential expression analyses that display both FAK and FAK-kinase dependent changes in chromatin accessibility dataset were filtered (**Figure 5.7,** lower panel). This analysis included *IL33* as a downregulated gene that displays reduced chromatin accessibility at its promoter/enhancer regions, which I investigated in Chapter 4. An upregulated gene in FAK -/- and FAK KD cells which displays changes in accessibility upstream of its gene is *RBMS3*, which is a negative regulator of invasion in breast cancer (Yang, Quan and Ling, 2018). The FAK NLS cells chromatin accessibility dataset was not compared with gene expression data as the FAK NLS was not included in the mRNA-seq analysis. However, comparison of the FAK nuclear localisation dependent chromatin accessibility changes to this subset of genes identified that most of these genes are also

regulated by FAK nuclear localisation dependent changes in chromatin accessibility at promoter/enhancer regions (**Figure 5.7**, lower panel). Thus, in this section I have identified a subset of FAK-regulated genes that display FAK-dependent changes in chromatin accessibility.

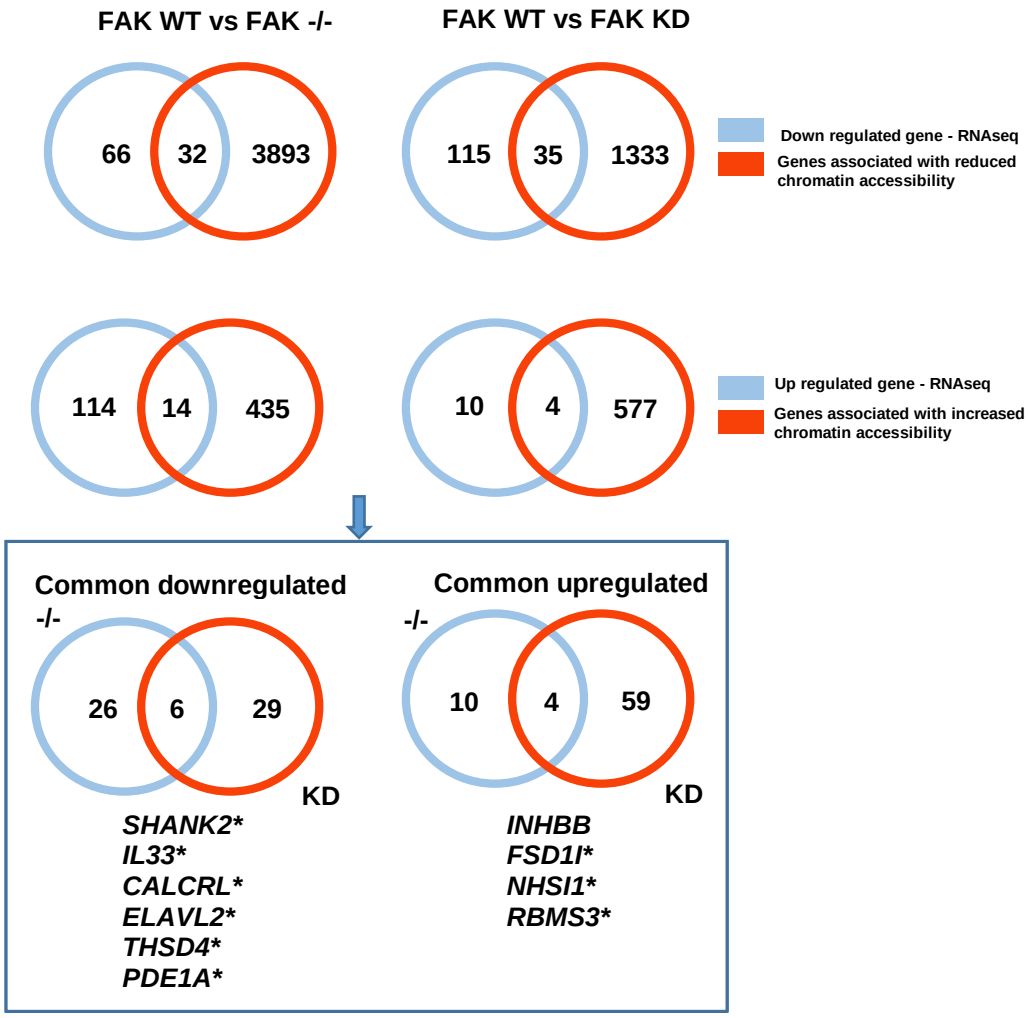


Figure 5.7 | Identification of differentially expressed genes regulated by chromatin accessibility. FAK WT vs FAK -/- and FAK WT vs FAK KD differentially expressed genes (right) were integrated with the FAK (left) and FAK kinase-dependent chromatin accessibility data (right), respectively. Upregulated genes were compared with genes that show increased chromatin accessibility at promoter/enhancer regions (upper panel) and vice versa (middle panel). Lower panel is where common down regulated genes (left) and common upregulated genes (right) in the FAK -/- and FAK KD (with respect to FAK WT) according to mRNA-seq analysis, which show changes in chromatin accessibility were identified in common between FAK WT vs KD and FAK WT vs KD analyses. In the lower panel, starred genes are those that display chromatin accessibility changes in FAK NLS cells and genes identified to have FAK and FAK-kinase dependent differential expression. -/- = FAK -/- and KD = FAK KD cell line. All data was filtered using Venny online software. Courtesy of B. Serrels

5.3.3 Knockdown of the FAK-regulated gene *RBMS3* mRNA stimulates invasion in FAK -/- cells

In this next section, I decided to investigate the function of *RBMS3*, a FAK-regulated differentially expressed gene which is also regulated by FAK-dependent changes in chromatin accessibility. RBMS3 is an RNA-binding protein proposed to inhibit the invasion of breast cancer cells (Yang, Quan and Ling, 2018) and its expression has been correlated with favourable prognosis in lung SCC (Liang *et al*., 2015). Interestingly, FAK -/- cells display perturbed invasive capacity due to loss of FAK-dependent invasion (Schoenherr *et al*., 2017) and therefore I investigated whether downregulation of *RBMS3* mRNA was important. Firstly, we confirmed that RBMS3 is in fact upregulated in the FAK -/- cells with respect to FAK WT cells on the protein level (**Figure 5.8**). To study whether RBMS3 controls invasion in this cell line, I generated *RBMS3* knockdown cells by transfecting cells with two separate shRNAs against *RBMS3* into FAK -/- cells: cells were also transfected with empty pLKO vector to generate a control cell line. This generated a stable knockdown of the *RBMS3* transcript in the FAK -/- cells, which was confirmed by *RBMS3* qRT-PCR as shown in **Figure 5.9**. To determine whether knockdown of RBMS3 increases invasion in FAK -/- cells, I performed inverted invasion assays using the RBMS3 knockdown cells. The results showed that there was an increase in invasive capacity of FAK -/- cells upon *RBMS3* knockdown (**Figure 5.10A**), however this was not statistically significant (**Figure 5.10B**). Analysis of the invasion levels of the three biological replicates showed there was a large variation in invasive capacity between the *RBMS3* knockdown samples, which could explain the lack of a significant result (**Figure 5.10B**). In conclusion, knockdown of *RBMS3* stimulates the invasion of FAK -/- cells in an *in vitro* assay; however, this was not shown to be a statistically significant increase. This could not be repeated due to time constraints.

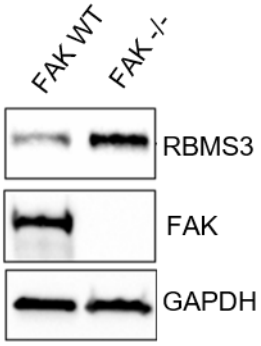


Figure 5.8 | RBMS3 protein is upregulated in FAK -/- cells. Western blotting and whole cell lysates prepared from FAK WT and FAK -/- cell lines as outlined in Material and Methods. The proteins were separated by SDS-PAGE and blots were probed for RBMS3 (upper panel), FAK (middle panel) and GAPDH as a loading control (lower panel). N=1. Courtesy of B. Serrels.

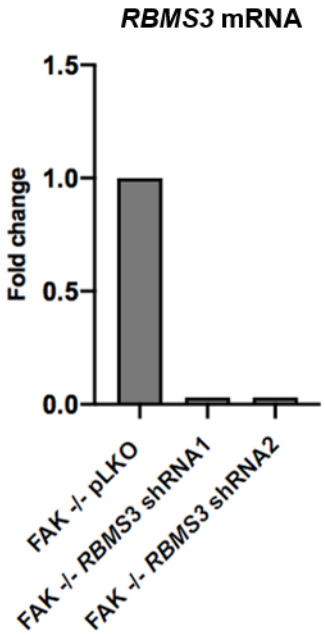


Figure 5.9 | Confirmation of *RBMS3* knockdown in FAK -/- cells. *RBMS3* was depleted using shRNA and qRT-PCR was carried out using *RBMS3* primers as described in Materials and Methods. CT values were normalised to *GAPDH* and set relative to FAK -/- pLKO samples. N=1

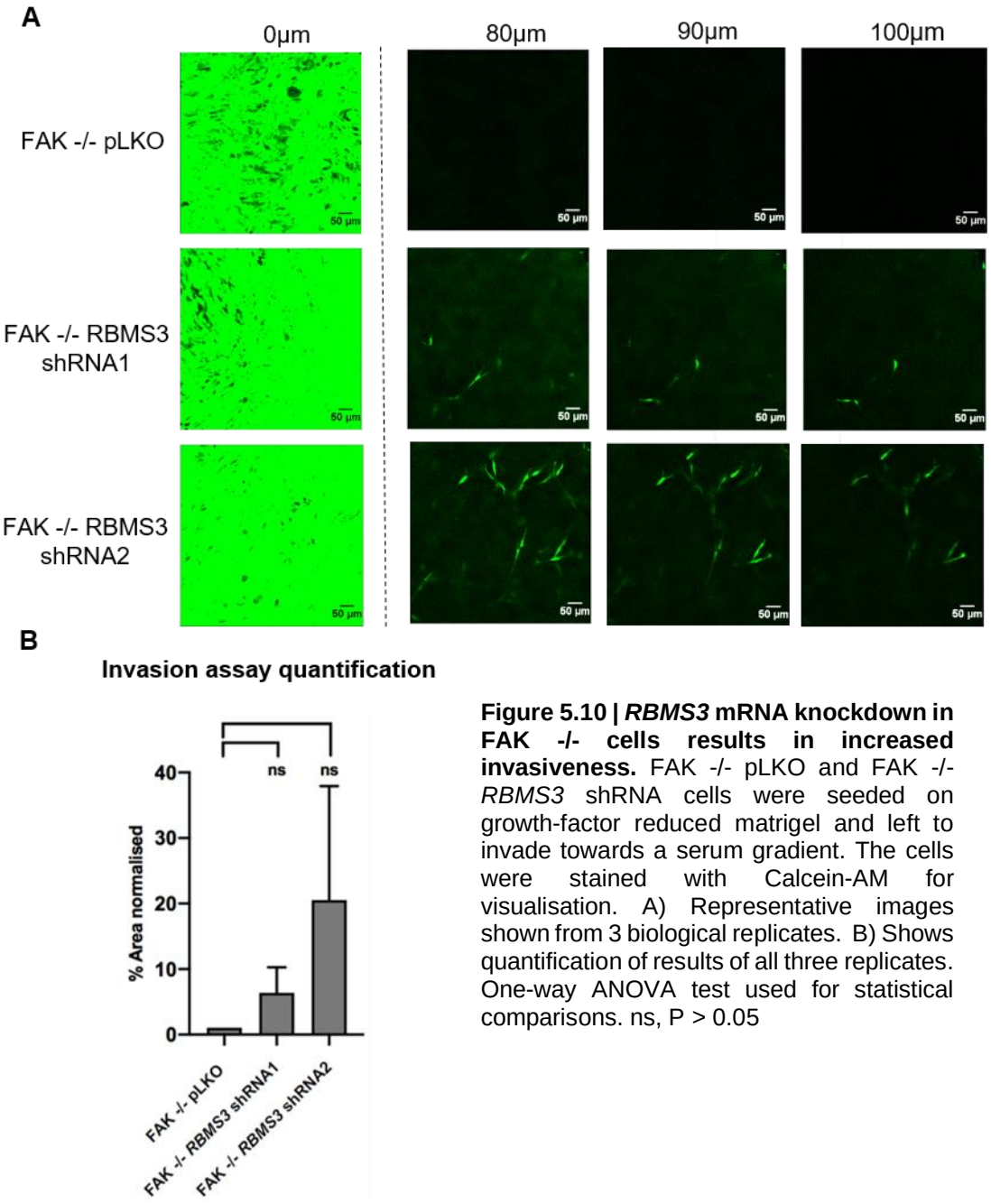


Figure 5.10 | *RBMS3* mRNA knockdown in FAK -/- cells results in increased invasiveness. FAK -/- pLKO and FAK -/- *RBMS3* shRNA cells were seeded on growth-factor reduced matrigel and left to invade towards a serum gradient. The cells were stained with Calcein-AM for visualisation. A) Representative images shown from 3 biological replicates. B) Shows quantification of results of all three replicates. One-way ANOVA test used for statistical comparisons. ns, $P > 0.05$

5.4 Discussion

5.4.1 FAK's kinase activity is important for regulating genes associated with inflammation and invasion

In this section, I identified that approximately 400 genes were differentially expressed between the FAK WT vs the FAK -/- and FAK WT vs FAK KD cells using mRNA-seq. GO enrichment analysis implied that FAK and FAK's kinase activity regulates a number of gene sets important in various pathways associated with invasion and inflammation (**Figure 5.3A-B**, **Figure 5.4A-B**, respectively). It is established that FAK is a key regulator of FAK cancer-associated inflammation (Serrels *et al*., 2015) and indeed many of the inflammation-associated genes that were shown to be FAK-regulated in the Affymetrix microarray analysis published previously (i.e. *CCL2, CCL5, IL33*, **Figure 5.1**, Serrels *et al*., 2015) were also shown to be regulated by FAK in the mRNA-seq results presented in this chapter, thereby confirming the validity of the mRNA-seq findings.

Comparison of FAK WT vs FAK -/- and the FAK WT vs FAK KD differential expression analyses showed that approximately 30% of genes were specific to the FAK WT vs FAK KD differential expression analysis (**Figure 5.6**). In the FAK -/- cells, all of FAK's functions i.e. nuclear, kinase, scaffolding will be deleted. Therefore, one would expect that most genes that require FAK kinase activity change in expression in the FAK WT vs FAK -/- differential expression analysis. Comparison between the differentially expressed genes in the FAK WT vs the FAK -/- cells and the FAK WT vs FAK KD cells indicated that 40% of FAK dependent genes were also FAK kinase dependent (**Figure 5.6**). As the GO analysis for the FAK WT vs FAK KD cell line differential expression datasets enriched for terms associated with invasion and inflammation (**Figure 5.3A-B**, **Figure 5.4A-B**), this indicates that FAK kinase activity is important for the transcription of genes that regulate these processes. The reason for determining whether certain genes are regulated by FAK's kinase activity is because of the incomplete understanding of its function as mentioned in the Introduction. I therefore used mRNA-seq to define FAK-regulated genes in order to identify whether the FAK kinase activity is important for transcription. FAK kinase inhibitors are in

clinical use and therefore gaining insight into the role of FAK's kinase activity will ultimately allow us to understand the reasons FAK's kinase is important in cancer and to potentially optimizing the sue of these inhibitors. Interestingly the most highly enriched inflammation term in the FAK WT vs KD differential expression analysis was 'Quantity of $CD4^+$ T lymphocytes' which included *CCL2* and *CCL5*. Certain types of $CD4^+$ T cells can aid the anti-tumour immune response (Th_1 cells), but a subset of $CD4^+$ T cells which express $FoxP3^+$ $CD25^+$ (Tregs) suppress the anti-tumour immune response (reviewed in Balkwill, Capasso and Hagemann, 2012). The Frame group has identified that treatment of FAK WT SCC tumours with the FAK kinase inhibitor, VS-4718, reduces the quantity of the tumour infiltrating Tregs (Serrels *et al*., 2015). This paper also identified that FAK regulates CCL5 expression and knockdown studies indicated that CCL5 was highly important in regulating the amount of tumour infiltrating Tregs in FAK WT tumours (Serrels *et al*., 2015). Thus, the FAK mRNA-seq data presented in this section supports previous data identifying that FAK's kinase activity is highly important for establishing an immuno-suppressive TME by regulating the transcription of chemokines (Serrels *et al*., 2015).

To my knowledge, no group has carried out mRNA-seq analysis to analyse the FAK-dependent transcriptome. However, a previous study has identified that treatment of mouse PDAC tumours with a FAK kinase inhibitor resulted in downregulation of a number of genes involved in regulating fibrosis such as *LOXL4* (Jiang *et al*., 2016). *LOXL4* was also shown to be downregulated as a result of loss of FAK or FAK's kinase activity in SCC cells in my mRNA-seq analysis aligning with previous findings. This implies that my mRNA-seq findings may also be relevant in other cell and tissue types.

In addition, the differential expression analysis indicated that genes associated with 'apoptosis of cardiomyocytes' and 'apoptosis of macrophages' were downregulated in FAK WT vs FAK -/- and FAK KD. This included *CASP1* which is a key regulator of apoptosis. FAK has shown to be a negative regulator of apoptosis (Lim *et al*., 2008b), thus the increase of *CASP1* expression in FAK WT SCCs is counterintuitive. That said, caspases require cleavage to be activated (reviewed in Elmore *et al*., 2007) and therefore changes in their expression alone won't strictly lead to apoptosis. Therefore,

further work will be required to establish if there was biological significance of *CASP1* mRNA being downregulated in the FAK -/- and FAK KD cell lines.

5.4.2 A subset of FAK-regulated differentially expressed genes display FAK-dependent changes in chromatin accessibility

The sequence of events that result in chromatin accessibility changes are not fully understood (reviewed in Klemm *et al*., 2019). Furthermore, external stimuli, developmental cues and the cell cycle, can all impact on chromatin accessibility (reviewed in Klemm *et al*., 2019). To determine FAK-regulated genes that display changes in chromatin accessibility, I integrated the FAK WT vs FAK -/- and FAK WT vs FAK KD chromatin accessibility dataset with that of the FAK WT vs FAK -/- and FAK WT vs FAK KD mRNA-seq dataset (**Figure 5.7**). This identified a small subset of FAK-dependent and FAK-kinase dependent genes that displayed FAK and FAK kinase-dependent changes in chromatin accessibility. Far more genes displayed only changes in chromatin accessibility than those that were also differentially expressed. Therefore, chromatin accessibility *per se* is not sufficient to permit FAK-dependent transcription. For transcriptional activation to occur, this may require a stimulus from a particular growth factor or cytokine for the full complement of transcription factors to bind and stimulate the expression of any particular gene. All these experiments presented have been performed under basal conditions and further experiments will be required to understand FAK-dependent gene expression changes in response to different cytokines and growth factors in order to recapitulate a more realistic i.e. *in vivo* representation of FAK-dependent transcription.

The data here suggest that FAK may regulate transcription, in part by regulating chromatin accessibility at promoter/enhancer regions of target genes. As discussed in the previous chapter, interaction partners of FAK can bind to complexes that regulate chromatin accessibility i.e. IL33 binds to components of the BAF complex (Serrels *et al*., 2017), which could link FAK to the regulation of chromatin accessibility. However, this does not rule out other potential mechanisms by which FAK can regulate transcription, for example by enhancing transcriptional activity which has

been demonstrated previously in the case of the transcription factor, MEF2 (Cardoso *et al*., 2016). Further analysis of the FAK ATAC-seq motif enrichment dataset and validation studies would be required to investigate alternative mechanisms by which FAK regulates transcription.

5.4.3 FAK regulates the chromatin-accessibility and expression of the *RBMS3* gene

To determine whether any of the FAK-regulated genes display changes in chromatin accessibility, I integrated the ATAC-seq chromatin accessibility and mRNA-seq data. This identified a list of novel genes that are FAK- and FAK-kinase regulated, that also exhibit FAK- and FAK kinase changes in chromatin accessibility (**Figure 5.7**). These included a number of genes known to regulate FAK-regulated processes, such as regulation of migration, pluripotency and anoikis resistance. These genes included *SHANK2* which was shown to be downregulated in FAK -/- and FAK KD cell lines. *SHANK2* is a major component of post-synaptic density that has been shown to be important for inhibiting differentiation of stem cells from apical papilla (Guo *et al*., 2017). *NHSI1B* has been reported to be important for regulating neuronal migration by regulating the planar cell polarity pathway (PCP), is also regulated by FAK (Walsh *et al*., 2011). In addition, *INHBB*, which is a negative regulator of anoikis resistance in nasopharyngeal carcinoma via suppression of TGF-β signalling, was upregulated in the FAK -/- and FAK KD cell lines with respect to FAK WT (Zou *et al*., 2018). In conclusion, many FAK-regulated genes that are also regulated by FAK-mediated changes in chromatin accessibility are involved in canonical FAK processes.

Integration of FAK-dependent chromatin accessibility with FAK-dependent gene expression data also identified *RBMS3* as a FAK-regulated gene that displays changes in chromatin accessibility at its promoter/enhancer region upon loss of FAK or FAK kinase activity (**Figure 5.7**). RBMS3 is an RNA-binding protein which, despite being discovered nearly 20 years ago (Penkov *et al*., 2000), has been understudied. The *RBMS3* gene is an established tumour suppressor (Liang *et al*., 2015, Chen *et al*., 2012) and low expression of *RBMS3* has been associated with poor prognosis in patients with gastric cancer (Zhang *et al*., 2016) and oesophageal SCC (Li *et al*., 2011). In human

breast cancer cell lines, RBMS3 has been shown to be an inhibitor of invasion (Yang, Quan and Ling, 2018). It functions by binding to mRNA and decreases mRNA stability, which was shown for the transcription factor TWIST: this results in reduced expression of the TWIST1 target gene, *MMP2* and a decrease in invasion *in vivo* (Zhu *et al*., 2019).

FAK -/- cells are known to be defective in invasive capacity due to loss of FAK-dependent invasion (Schoenherr *et al*., 2017). Therefore, I investigated whether downregulating *RBMS3* expression by shRNA could increase the invasive capacity in FAK -/- SCC lines. Loss of *RBMS3* was associated with an increased invasive capacity of FAK -/- cells (**Figure 5.10A**), however, this was not significant due to large variations in the increased invasion of *RBMS3* shRNA FAK -/- cells between biological replicates (**Figure 5.10B**). Further work will involve optimising the conditions of this experiment in order to decrease variability between the biological replicates. However, the data do indicate that *RBMS3* could be an important regulator of invasion in the FAK - /- SCC cell line.

One unaddressed question is which transcription factors are responsible for driving *RBMS3* expression. This could be determined by performing motif enrichment analysis, as performed in Chapter 4 for the *IL33* gene. The mechanism by which *RBMS3* expression is regulated is unknown. However, the human *RBMS3* gene is predicted to have binding sites for AMEF-2, C-MYC, EVI-1, AREDB6, GATA-1, RP58, MAX1, and MEF2A (Genecards, 2020). I was unable to perform motif enrichment analysis to identify the specific transcription factors that regulate mouse *RBMS3* mRNA expression due to time constraints but this will be the focus of future work to confirm which transcription factors are responsible for driving the expression of the *RBMS3* gene and determining the functional importance. Future motif enrichment analysis may also provide insight into the mechanism by which FAK-regulates chromatin accessibility at the *RBMS3* promoter/enhancer region, as there is evidence that particular transcription factors can interact with epigenetic complexes to regulate chromatin accessibility e.g. AP-1 interacts with the BAF complex to regulate chromatin accessibility in mouse embryonic fibroblasts (Vierbuchen *et al*., 2017).

5.5 Conclusions

The mRNA-seq data presented in this chapter indicates that FAK kinase activity is important to drive expression of genes that regulate inflammation and invasion. Integration of FAK mRNA-seq data with chromatin accessibility data showed that a small subset of FAK-dependent and FAK-kinase dependent genes display changes in chromatin accessibility. Further studies indicated that one of these genes, *RBMS3* is potentially important in negatively regulating FAK-dependent invasion. Together, these findings implicate chromatin accessibility as a mechanism by which FAK regulates transcription of a subset of target genes.

Chapter 6 Discussion, future directions and conclusions

FAK has been shown to be a key regulator of gene expression in cancer (Serrels *et al*., 2015, 2017). However, the mechanism by which FAK regulates transcription remains to be determined. With this in mind, I addressed the potential mechanisms by which FAK regulates transcription. To study this, I used a three-tiered OMICs approach in order to understand potential mechanisms by which FAK regulates transcription.

Specially, I aimed to define;

- Novel nuclear interaction partners of FAK using proximity-based proteomics to identify key proteins that FAK can directly bind that have roles in transcription
- FAK-dependent chromatin accessibility changes and transcription factor motif enrichment using ATAC-seq
- The full scope of FAK and FAK-kinase regulated genes using mRNA-seq

6.1 FAK regulates chromatin accessibility changes in SCC cell lines

Chromatin accessibility is a key mechanism to regulate gene expression. Increases in chromatin accessibility allows RNA Pol II complexes to bind to DNA and initiate transcription. Therefore, I aimed to determine whether FAK regulates chromatin accessibility using ATAC-seq. This identified a subset of genes that are regulated by chromatin accessibility changes in a FAK-, FAK-kinase and a FAK-nuclear localisation-dependent manner (Chapter 4). However, I have not established the mechanism by which FAK regulates chromatin accessibility. This is a complex question to answer, as the sequence of steps required to stimulate chromatin accessibility changes are not known (reviewed in Klemm *et al*., 2019).

One factor potentially contributing to changes in chromatin accessibility includes CpG DNA methylation, which has been shown to be associated with reduced chromatin accessibility (Thurman *et al*., 2012). Interestingly, the FAK-regulated gene, *SFRP1*,

which is upregulated in FAK -/- and FAK KD SCC lines when compared with the FAK WT SCC line in mRNA-seq analysis presented in Chapter 5, has aberrant cytosine promoter methylation in renal cell carcinoma in comparison to normal kidney tissues (Dahl *et al*., 2007). Furthermore, loss of *SFRP1* expression by gene methylation contributes to tumour phenotypes (Gumz *et al*., 2007). Therefore, it would be interesting to determine whether there are FAK-dependent DNA methylation changes which contribute to tumour phenotypes in SCC cells by performing bisulphite sequencing and phenotypic assays on candidate FAK-regulated genes that show FAK-dependent alterations in methylation. This dataset could be integrated with the ATAC-seq chromatin accessibility dataset in order to understand whether FAK-dependent changes in methylation correspond to alterations in chromatin accessibility. If FAK-regulated changes in DNA methylation are observed, it would be of interest to determine the mechanism by which FAK regulates DNA methylation and whether this results in the transcriptional repression of genes. FAK binds to the methyl-CpG binding protein with demethylase activity, MBD2, to induce myogenin expression and muscle differentiation (Luo *et al*., 2009b). This study suggested that FAK can potentially modulate regulators of DNA methylation.

Transcription factors are also thought to play a key role in regulating chromatin accessibility. This is because chromatin accessibility is regulated differentially across the genome and chromatin remodelling complexes do not have DNA sequence specificity, while transcription factors can bind to specific DNA motifs (reviewed in Klemm *et al*., 2019). There are a number of models by which transcription factors can regulate chromatin accessibility, including via their ability to initiate accessibility by co-operating with chromatin remodellers to establish accessible chromatin (reviewed in Klemm *et al*., 2019). Motif enrichment predicted that FAK regulates transcription factor motif enrichment both globally and at the level of specific genes such as *IL33*. Interestingly, JUN (c-Jun), a predicted FAK-regulated transcription factor on the *IL33* enhancer, has been shown to recruit the BAF complex to target enhancers that regulate chromatin accessibility (Vierbuchen *et al*., 2017). Thus, it is possible that FAK could regulate accessibility by regulating transcription factor function. However, the mechanism by which FAK does this remains to be established (discussed below). It

must be noted that many transcription factors (including JUN, GATA1) have been shown to not be able to bind inaccessible chromatin (Thurman *et al*., 2012). However, 'pioneer transcription factors' (transcription factors that can bind to inaccessible chromatin) such as ZFN274, KAP1 and SETDB1 have been implicated in regulating chromatin accessibility (Thurman *et al*., 2012). However, none of the aforementioned pioneer transcription factors were identified to display FAK-dependent motif enrichment in my work presented here. I conclude that regulation of chromatin accessibility is highly complex and further experiments will be required to understand the key FAK-dependent transcription factors driving chromatin accessibility in the SCC cell model used in these studies.

Integration of the chromatin accessibility and mRNA-seq datasets indicated that there were many more FAK-dependent changes in chromatin accessibility than there were FAK-regulated genes. This suggests that changes in chromatin accessibility at one or multiple regions in promoter or enhancer regions is not sufficient to drive gene expression. This finding is in agreement with previous studies where authors integrated ATAC-seq and mRNA-seq data to study chromatin accessibility and gene expression changes during differentiation of mouse lens fibers (Zhao *et al*., 2019). These authors found that only 9-28% of differentially expressed genes exhibited corresponding changes in chromatin accessibility (Zhao *et al*., 2019). This could be due to the fact a gene has multiple regulatory genomic regions and changes in chromatin accessibility in one or multiple regulatory genomic regions (i.e. enhancer or promoter) is not sufficient to stimulate gene expression (Zhao *et al*., 2019). Furthermore, it is also likely that transcription factors in the differentially accessible regions require a particular extracellular stimulus and/or presence of additional activators (Zhao *et al*., 2019). For example, it has been shown that *IL6* mRNA is induced by stimulation of IFN-β and/or IL1 in human mammary epithelial cells (Nan *et al*., 2018). Furthermore, recruitment of STAT2, IRF-9 and P65 to the IL6 promoter is essential for maximal activation of *IL6* mRNA expression (Nan *et al*., 2018). Together, these findings emphasise the complexity of control of gene expression, which requires the correct combination of transcription factors, chromatin accessibility changes and external stimuli. Further studies will be required to determine the effects of cytokine or growth factor

stimulation on chromatin accessibly and gene expression in order to recapitulate a more realistic representation of FAK-regulated chromatin accessibility and gene expression changes. **Figure 6.1** depicts the mechanism by which I hypothesise FAK regulates chromatin accessibility.

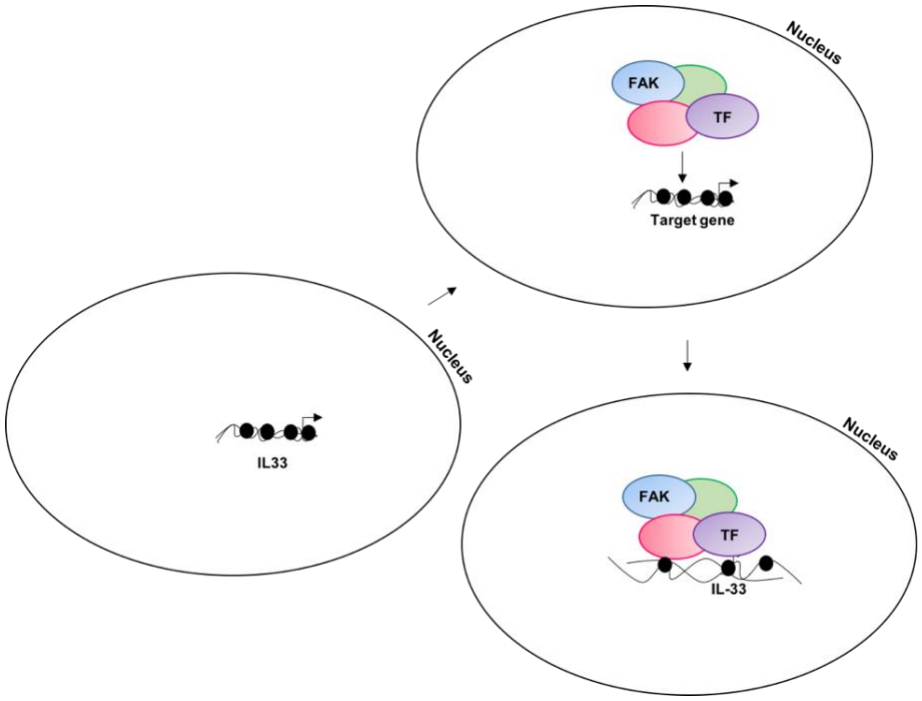


Figure 6.1 | FAK regulates chromatin accessibility at target genes. FAK likely co-operates with transcription factors (TF) and other regulators of transcription (green and red circles) to control chromatin accessibility changes at target genes.

6.2 FAK regulates transcription factor binding to chromatin

The motif enrichment analysis presented in this thesis suggested that FAK is a regulator of transcription factor motif enrichment in accessible regions of chromatin. However, I have not addressed the mechanism by which FAK does so. Transcription factors can be regulated by a number of mechanisms including sequestration in the cytoplasm, protein turnover and post-translational modifications. The AP-1 member, JUNB, displays reduced protein expression but unaltered mRNA expression in FAK -

/- SCC cells in comparison to FAK WT SCC cells, demonstrating that FAK regulates JUNB at the protein level. The levels of JUNB were checked in whole cell lysates and further work will be required to understand if the protein levels are changed in the nucleus relative to the cytoplasm, as this would indicate FAK regulates the translocation of JUNB to the nucleus. All the other transcription factors that were regulated by FAK and FAK function did not show changes in the mRNA expression according to the FAK mRNA-seq data, suggesting that FAK could potentially regulate transcription factors at the protein level. JUNB turnover is controlled by ubiquitin-dependent degradation that is mediated by binding to an E3 ligase called ITCH (Li *et al*., 2016): the nuclear FAK interactome dataset did indicate that FAK could bind a number of E3 ubiquitin ligases including ITCH (Serrels *et al*., 2015). It is also relevant that, in response to staurosporine or loss of cell adhesion, FAK translocates to the nucleus in a FERM-dependent manner, where the FAK FERM domain scaffolds the MDM2:P53 interaction and enhances MDM2-mediated P53 degradation (Lim *et al*., 2008b). Furthermore, Merlin may mediate the FAK mediated interaction between P53:FAK and P53:MDM2 in mesothelioma cells (Ou *et al*,. 2016). Future experiments will focus on confirming whether the protein levels of other FAK-regulated transcription factors are changed and understanding the mechanism by which FAK may regulate the protein levels of these transcription factors (i.e. proteasome-mediated turnover). It is possible that FAK may regulate these transcription factors by scaffolding E3 ligases to target proteins and thereby mediate ubiquitin-dependent degradation. It must be noted that FAK binding to E3 ligases may regulate stability of proteins in general, rather than just transcription factors. Indeed, ITCH has been shown to ubiquitinate the receptor CXCR4, in addition to transcription factors such as JUNB and SMAD2 (reviewed in Melino *et al*., 2008). Further work would be needed to confirm whether FAK can bind to E3 ligases such as ITCH, and what effect this has on protein degradation of transcription factors.

Nuclear FAK binds to the transcription factor, RUNX1, which regulates *IGFBP3* expression and a suppressor of RUNX1 transcriptional activity, SIN3A. Further studies found that FAK controlled the nuclear levels of SIN3A expression and thereby controlled SIN3A interaction with RUNX1 (Canel *et al*., 2017). This indicates that

FAK can regulate transcription factor function by modulating the nuclear levels of transcription factor regulators in the nucleus. This could be tested by determining if FAK-regulated transcription factors can still bind to canonical interaction partners in the absence of FAK and by determining the nuclear levels of known regulators of transcription factor function. That said, it cannot be excluded that FAK-regulated transcription factors may, in fact, be regulated by FAK function at focal adhesions. As previously mentioned, FAK functions upstream of GRB2/RAS/MAPK signalling (Schlaepfer *et al*., 1994) and the MAPK pathway leads to phosphorylation of ETS1 and ETS2 leading to their transcriptional activation (Foulds *et al*., 2004). Furthermore, the TLR2 receptor/FAK/PI3K/AKT signalling axis is important for downstream JUN activation in response to peptidoglycan and this results in stimulation of *IL6* mRNA expression (Chiu *et al*., 2009). Thus, there are multiple reported connections between the upstream function of FAK in the cytoplasm or at focal adhesions and FAK-regulated transcription factors identified in the ATAC-seq analysis. This emphasises the importance of the use of the FAK NLS SCC cell lines in the FAK ATAC-seq analysis, which indicated that 205 transcription factors are regulated by nuclear FAK, implying that FAK could have a more direct role by regulating transcription factors in the nucleus. Equally FAK could also bind to, and potentially phosphorylate, modulators of transcription factor function and thereby regulate the activity of transcription factors indirectly in the nucleus.

To investigate whether FAK regulates transcription factors in a direct manner (i.e. protein binding), I analysed upstream connections between the nuclear FAK BioID proteomics dataset or the FAK nuclear interactome proteomics dataset (Serrels *et al*., 2015) and the genome-wide FAK WT-enriched ATAC-seq data/motif enrichment dataset, using Ingeuity pathway analysis (IPA). This identified a number of upstream connections between nuclear FAK interactors and the FAK WT enriched genome-wide predicted transcription factors, including the FAK interactor, YEATS2 binds to TBP (TATA-box binding protein).Additionally, the FAK interactors SP1 and WWOX both bind to the FAK-regulated transcription factor, JUN, respectively. The interactome analysis suggested that there are many links between the FAK ATAC-seq data and the FAK nuclear interactome, implying that FAK can potentially modulate

transcription factor function by binding to known transcription factor binding partners. The mechanisms by which FAK may potentially mediate transcription factor function are depicted in **Figure 6.2**.

It is possible FAK could regulate transcription by acting as a transcription factor via direct binding to DNA. FAK ChIP-seq has been performed in WM858 melanoma cell lines, indicating that FAK binds to the consensus sequence GCGC[AC]TGCGC, which is similar to the motif sequence for the nuclear respiratory factor 1 (NRF1) , (T/C)GCGCA(C/T)GCGC(A/G) (Heim *et al*., 2017). Although this suggests that FAK could potentially bind to DNA directly, no DNA-binding domain of FAK has been discovered to date. Furthermore, since formaldehyde can also cross-link protein-protein interactions in addition to protein-DNA (Hoffman *et al*., 2015), it is possible that the work by Heim and colleagues could reflect FAK complexing with transcriptional complexes that are associated with DNA, without FAK binding to DNA directly.

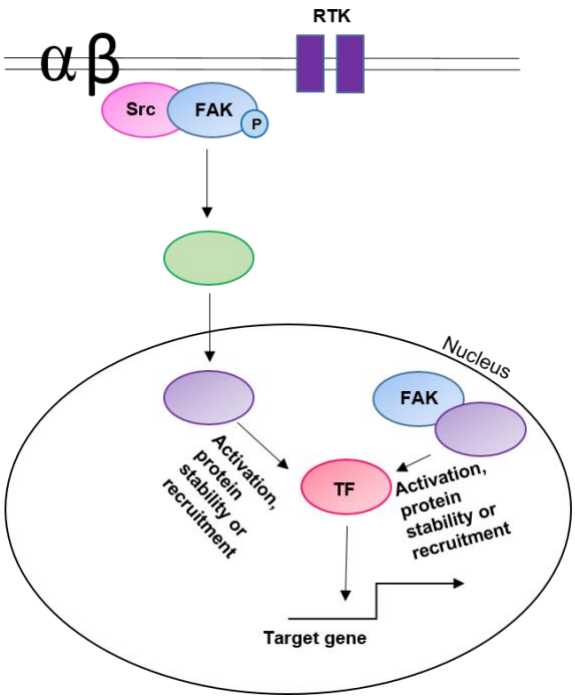


Figure 6.2 | Mechanisms that FAK may regulate transcription factor function. FAK can regulate transcription factor function by signalling downstream of integrins and growth factor receptors. Alternatively, FAK can signal in the nucleus to regulate activation, protein stability or recruitment of transcription factors to the chromatin. αβ = integrin αβ heterodimer, RTK = Receptor tyrosine kinase and TF = transcription factor.

6.3 FAK regulates *IL33* expression via the regulation of JUN

As outlined above, FAK-dependent chromatin accessibility changes were found on the *IL33* promoter/enhancer regions. IL33 is a cytokine secreted form cells when they are damaged (alarmin), which alerts immune cells that express the ST2 receptor and this stimulates $CD8^+$ T cell recruitment (Lu *et al*., 2016). It can also be present in the nucleus, where IL33 function is largely undefined. Nuclear IL33 is reported to regulate the expression of the P65 subunit of NF-κB by binding to its promoter and thereby regulating ICAM1 and VCAM1 expression (Choi *et al*., 2012). Work from our laboratory found that FAK controls the expression of *IL33* mRNA and the IL33 protein functions downstream of FAK in the regulation of an anti-tumour response (Serrels *et al*., 2017). This work implicated IL33 as a key mediator of nuclear FAK signalling; however, the mechanism by which FAK regulates IL33 expression was unknown. Therefore, I here addressed the mechanism by which FAK regulates the expression of the *IL33* gene as a platform for understanding the role of FAK in the nucleus.

As previously mentioned, there was reduced chromatin accessibility on the *IL33* promoter/enhancer regions upon loss of FAK, FAK-kinase activity and FAK nuclear localisation. Motif enrichment analysis of the *IL33* promoter/enhancer identified peaks in the FAK WT SCC cell line, predicted that AP-1, RUNX and MYB transcription factor family members bind to the promoter/enhancer regions of the *IL33* gene. Interactome analysis identified JUN as a central node in the network, implicating it as a signal integrator between the other transcription factors predicted by the motif enrichment analysis. Knockdown experiments indicated that JUN regulates *IL33* mRNA expression and IL33 protein expression. Furthermore, ChIP validated that JUN directly binds to the *IL33* enhancer region in the FAK WT SCC cells and this is lost in the FAK kinase-defective mutant-expressing cells. Together this suggests that JUN regulates the expression of *IL33* by binding to the *IL33* enhancer and that FAK can regulate the binding of this transcription factor, in turn, by regulating changes in chromatin accessibility at specific sequences in a kinase-dependent manner. As outlined above, it is not known if some of the transcription factors identified in the

motif enrichment analysis (i.e. JUN/AP-1) can regulate chromatin accessibility which has been implicated previously (Vierbuchen *et al*., 2017). Therefore, it is possible that FAK mediates changes in chromatin accessibility by modulating transcription factor's ability to recruit chromatin modifying complexes to promoters/enhancers. Future work will aim to define the mechanism by which FAK can directly regulate JUN function recruitment to promoters/enhancers and whether this is important for regulating accessibility.

Motif enrichment data also found that the motifs for the TATA-box binding protein (TBP) was lost from the promoter region of the *IL33* gene upon loss of FAK, its FAK-kinase activity or its nuclear localisation. Integration of the nuclear FAK interactome dataset published previously (Serrels *et al*., 2015) with the motif enrichment results, i.e. the predicted FAK WT enriched transcription factors on the *IL33* promoter, revealed a number of upstream connections between TBP and the FAK interactome. Indeed, cells require the basal transcription factor complex TFIID (composed of TBP and TAF components) to scaffold RNA Polymerase II to promoter regions to initiate transcription of many genes (reviewed in Butler and Kadonaga, 2002). TAF9 is a key component of the basal transcription factor complex, which is a FAK interacting protein, as shown by co-immunoprecipitation experiments (Serrels *et al*., 2015). FAK is thought to regulate the anti-tumour immune response by binding to the basal transcription factor complex component, TAF9, which regulates of *CCL5* expression (Serrels *et al*., 2015). This represents a direct link between FAK and the basal transcription complex that regulates gene expression. However, whether FAK regulates TAF9 remains to be determined. Further experiments would include ChIP of the TBP or TAF9 protein on the TATA-box of the *IL33* promoter to establish whether FAK-dependent changes in chromatin accessibility modulates binding of the basal transcription factor machinery at the *IL33* promoter.

The biological consequences of JUN regulating *IL33* expression has not been defined yet. As previously described, FAK's regulation of IL33 expression is important for the anti-tumour immune evasion (Serrels *et al*., 2017). It may be that JUN regulation of *IL33* mRNA expression is responsible. Indeed, JUN is a key regulator of inflammation

(reviewed in Schonthaler, Guinea-Viniegra and Wagner, 2011). It would be interesting to perform animal studies to understand the role of FAK-regulated JUN binding on the *IL33* enhancer in the anti-tumour immune response (as performed in Serrels *et al.*, 2015, 2017). However, JUN is an important transcription factor in many biological processes that are essential for the cell, such as proliferation (reviewed in Eferl and Wagner, 2003); therefore, performing animal experiments with JUN knockdown cells would not be informative to address the question posed above.

6.4 Nuclear FAK function regulates FAK-dependent phenotypes

FAK is involved in a number of cellular processes including invasion, angiogenesis and inflammation. In my thesis, I used proteomics in order to investigate the function of nuclear FAK by identifying novel interaction partners of FAK in the nucleus. Use of BioID, a proximity-based biotin ligation approach (Roux *et al*., 2013), identified that FAK can potentially interact with a number of proteins involved in regulating transcription factors (i.e. WWOX, LPP) in the nucleus (Chapter 3). GO enrichment analysis indicated that FAK protein binding partners in the nucleus, identified by the FAK BioID nuclear interactome, are involved in FAK-regulated processes, such as invasion, migration and anoikis. Thus, the ability of FAK to bind these nuclear interaction partners may control established FAK-regulated processes from the nucleus, likely via modulating gene expression.

Interestingly, analysis of the nuclear FAK BioID interactome dataset also predicted that FAK binds to a number of other focal adhesion proteins in the nucleus. These proteins included SRC, Paxillin and HIC-5 and, as expected, GO enrichment analysis indicated these were associated with regulating invasion and metastasis. Many focal adhesion proteins present in the nuclear FAK BioID dataset have been shown to translocate to the nucleus before, as is the case for LPP (Guo *et al*., 2006), Paxillin (Sathe *et al*., 2016) and HIC-5 (Shibanuma *et al*., 2003). Interestingly, 70-80% proteins present in the consensus adhesome, have been shown to translocate to the nucleus (A. Byron, unpublished), even though some do not have nuclear localisation sequences i.e.

Paxillin and Zyxin (reviewed in Lim *et al*., 2013). FAK does have more than one potential NLS in its FERM domain (Lim *et al*., 2008b, Serrels *et al*., 2015) and therefore FAK could potentially mediate trafficking of other focal adhesion proteins to the nucleus as complexes. This suggests that nuclear translocation of focal adhesion proteins could be a mechanism by which the cells transmit signals from focal adhesions to the nucleus to regulate cellular processes. It would also be of interest to determine whether other focal adhesion proteins could assist in the transcriptional roles of FAK in the nucleus. This may be addressed by knockdown of focal adhesion proteins known to bind FAK and determining whether they regulate FAK-dependent gene expression i.e. *CCL5, IL33*. Furthermore, it would be interesting to determine whether other focal adhesion proteins influence binding of FAK to transcription factors or transcriptional regulators. Ultimately, validation using co-immunoprecipitation from nuclear lysates prepared using a recently developed fractionation protocol in the Frame/Brunton groups that separates the peri-nuclear from the nuclear fraction would be valuable.

Integration of FAK ATAC-seq chromatin accessibility data with mRNA-seq data indicated that a subset of FAK and FAK-kinase regulated genes specifically display changes in chromatin accessibility. *RBMS3* was one of the genes that showed FAK-dependent changes in chromatin accessibility in its promoter region. This gene is upregulated in the FAK KD and FAK -/- SCC cells when compared to FAK WT SCC cells in mRNA-seq analyses, and it is known to be an inhibitor of breast cancer cell invasion (Yang, Quan and Ling, 2018). Therefore, I performed an initial invasion assay using *RBMS3* knockdown FAK -/- SCC cells to determine whether RBMS3 could be inhibiting FAK-dependent invasion in the FAK -/- SCC cells, in which FAK is required for invasion (Schoenherr *et al*., 2017). Although the results were not significant in this first experiment (time did not permit repetition), they did show that knockdown of *RBMS3* mRNA resulted in a trend towards increased invasion, suggesting RBMS3 may indeed negatively regulate invasion in these cells. I was also not able to address the nature of the transcription factors that regulate *RBMS3* expression due to time constraints. Future work would define this by performing motif enrichment analysis upstream of the *RBMS3* gene. Furthermore, siRNA-mediated

knockdown experiments would ideally be carried-out on potential *RBMS3*-regulated transcription factors to determine if they do in fact regulate *RBMS3* expression. After identifying transcriptional regulators of *RBMS*, ChIP analysis could be performed to establish whether they regulate *RBMS3* expression by binding to its promoter/enhancer regions. Although I did not perform mRNA-seq analysis on the FAK NLS SCC cells, FAK NLS SCC cells showed increased accessibility on the *RBMS3* gene promoter when compared to FAK WT SCC cells. This suggests that FAK could regulate chromatin accessibility at the *RBMS3* gene, potentially by interaction with chromatin modifying complexes and transcription factors identified in the FAK nuclear interactome. This work suggests a potential transcriptional mechanism by which FAK regulates invasion from activities within the nucleus, which could function in addition to, or independently of, FAK-regulation of invadopodia formation at the plasma membrane (Chan *et al*., 2009).

6.5 Role of FAK kinase activity in the nucleus

With FAK kinase inhibitors in clinical trials, it is important to understand the role of FAK's kinase activity in the regulation of cancer-associated processes. The work presented here indicates that FAK's kinase activity is important for mediating its chromatin accessibility changes, transcription binding and FAK nuclear protein interactions. However, the mechanism by which FAK kinase activity can regulate these functions has not been established, e.g. whether it phosphorylates exogenous substrates or simply alters its own conformation. Previous reports on nuclear FAK signalling have reported that FAK's kinase activity is not important for interacting with transcription factors in the nucleus (Lim *et al*., 2008b, Lim *et al*., 2012). However, FAK's kinase activity regulates its structure allowing it to adopt an 'open conformation' whereby its kinase domain and linker regions are exposed resulting in its autophosphorylation, SRC binding and full catalytic activation (Lietha *et al*., 2008). As the active FAK kinase is in a different conformation to the autoinhibited state, this could potentially allow binding of alternative interaction partners. FAK has also been shown to phosphorylate proteins including Paxillin (Bellis *et al*., 1995), and p130CAS (Tachibana *et al*., 1997). However, it is uncertain whether this can occur in the nuclear compartment and whether there are any nuclear substrates. FAK has been shown to

phosphorylate the transcription factor NANOG to regulate cancer cell invasion (Ho *et al*., 2012). NANOG and FAK co-localise in the nucleus and perinuclear regions, suggesting that FAK could potentially phosphorylate NANOG in the nuclear compartment (Ho *et al*., 2012). That said, no other potential substrates of FAK have been found in the nucleus and it may be that FAK primarily has an adaptor function in that cell compartment.

In Chapter 5, I showed that 40% of FAK-regulated genes are FAK kinase-dependent, suggesting FAK's catalytic activity is important in regulating gene expression. It is possible that many of the FAK- and FAK kinase-regulated genes are, in fact, regulated by upstream pathways into which FAK feeds (e.g. MAPK). Thus, future work on this aspect should focus on defining the importance of FAK kinase activity in the nucleus for transcriptional events, and whether or not there are exogenous substrates.

6.6 Clinical implications of findings

There is a desperate need for new cancer therapies that are more effective and are less toxic than chemotherapy or irradiation. FAK is a highly attractive target in cancer therapy due to its involvement in a number of cancer processes such as invasion, angiogenesis and regulation of the anti-tumour immune response. That said, FAK kinase inhibitors are not effective as a monotherapy likely because many proteins are able to compensate for FAK loss in promoting cell survival. There has been a number of trials that examine the efficacy of FAK kinase inhibitors for the treatment of cancer in combination with other drugs such as MEK/RAF inhibitors (clinicalTrials.gov, NCT02428270, NCT03875820) and PD-1 antibodies (clinicalTrials.gov, NCT02546531, NCT02758587) for a range of cancers, such as pancreatic cancer, NSCLC, breast and mesothelioma.

Drugs that target epigenetic regulators are of active interest as a mechanism to 're-program' the cancer genome to a 'healthy' epigenome (reviewed in Ahuja, Sharma and Baylin, 2016). Interestingly, *in vitro* studies have found co-targeting of MYC signalling and FAK kinase activity using JQ1, a bromodomain/Extra-Terminal motif (BET) family protein inhibitor and the FAK inhibitor VS-6063, respectively, results

in arrest of ovarian cancer cells at G_2/M and a decrease in cell survival (Xu *et al*., 2017). Interestingly, recent work from our laboratory has used multi-parametric phenotypic screening to identify drugs that may synergise with FAK inhibition (J. Dawson, unpublished). This work found that FAK kinase and HDAC co-inhibition resulted in proliferation arrest in SCC, lung cancer and oesophageal adenocarcinoma cells, as well as a reduction of tumour growth of these *in vivo* (J. Dawson, unpublished). Thus, it appears that co-targeting epigenetic regulators and FAK kinase activity may be effective in treating cancer. Whether this targeting of epigenetic regulators affects nuclear FAK signalling remains to be established.

In Chapter 4, I showed that FAK regulates the chromatin binding of AP-1 factors to chromatin across the genome and, specifically at the *IL33* enhancer region. AP-1 transcription factors are commonly dysregulated in cancer and are also of interest as potential targets for cancer therapeutics (Ye *et al*., 2014). Even although there has been interest in using AP-1 inhibitors in the treatment of cancer and inflammatory conditions, no drugs targeting AP-1 are approved for clinical use (Ye *et al*., 2014). AP-1 inhibitors such as SR11302 have been shown to reduce metastatic lesions in an *ex vivo* 4D model of lung cancer, without affecting tumour growth (Mishra and Kim, 2017). However, use of this inhibitor in an immune competent mouse model will be required to understand whether there is an effect of the tumour micro-environment on the anti-tumour effect of this drug. In my thesis presented here, I have shown that FAK can modulate JUN/AP-1 chromatin binding in a kinase-dependent manner. Therefore, it is possible that a combination of an AP-1 inhibitor and FAK kinase inhibitors may enhance the anti-tumour effect of FAK kinase inhibitors. Other ATAC-seq studies have identified that there is an increase in accessibility at AP-1 and ETS motif regions of promoters in oesophageal adenocarcinoma cells versus healthy oesophageal cells (Britton *et al*., 2017). It has not yet been determined what increased AP-1 and ETS transcription factor binding actually contributes to cancer biology. Further work would be required to establish the biological consequence of increases in accessibility at AP-1 and ETS motifs, and whether these contribute to oncogenesis or tumour progression.

6.7 Conclusions and future experiments required for publication

In conclusion, the work presented here suggests that FAK regulates transcription factor motif enrichment in accessible regions of chromatin, potentially via mediating chromatin accessibility changes. This work has stimulated questions regarding the importance of chromatin accessibility in controlling the expression of genes, and emphasises the complexity of transcriptional regulation. These results are intriguing given that FAK is generally regarded as a focal adhesion protein; it clearly has interesting molecular functions in the nucleus that regulate biological outcomes.

Based on my findings, **my conclusions** are that;

1) FAK regulates transcription factor motif enrichment globally and also potentially connects (directly and/or indirectly) to the transcription factors known to bind to these FAK-regulated motifs.
2) FAK regulates the chromatin accessibility and expression of a subset of genes
3) FAK regulates chromatin accessibility at the promoter and enhancer regions of the *IL33* gene and thereby modulates JUN transcription factor binding to the *IL33* enhancer region.

I will now describe the key experiments required for publication in a high impact journal, as we intend to publish this work. This work will form two papers: 1) detailing the mechanism by which FAK regulates transcription using FAK-regulation of IL33 expression as a model and 2) defining the functional role of RBMS3 in FAK-dependent invasion. These papers will inform on mechanisms by which FAK can regulate gene expression by modulating chromatin accessibility and how this, in turn, controls FAK-dependent phenotypes (i.e. inflammation and invasion). The experiments that remain to be performed include:

1) i) Firstly, I would define the key chromatin remodelling complexes that JUN binds to in the FAK SCC cells and whether this is impacted upon by loss of FAK, FAK

catalytic activity or nuclear localisation. I would also determine if the nuclear level of these chromatin modifying complex components is altered as FAK has previously been shown to regulate nuclear levels of chromatin modifying component SIN3A (Canel *et al*., 2017). I will perform IPA and literature searches to identify which are the best candidate chromatin modifying complexes that have connections to JUN.

ii) Perform ChIP to analyse JUN binding chromatin modifying complex components on the *IL33* enhancer using antibodies directed against the chromatin modifying component.

iii) Perform genome-editing on the AP-1 site on the *IL33* enhancer in FAK WT SCC cells in culture. This would generate FAK WT SCC cells with a mutation in the AP-1 motif in the *IL33* enhancer. I will then use ChIP to define if the chromatin modifying component binds to the AP-1 site in the *IL33* enhancer – if it does not bind this would imply that AP-1 may regulate the recruitment of this chromatin remodelling complex component.

2) i) Confirm that RBMS3 is inhibiting invasion in the SCC FAK SCC cells by optimising the inverted invasion assays presented in Chapter 5.

ii) Perform motif enrichment analysis on the *RBMS3* promoter/enhancer regions to predict which transcription factors are driving RBMS3 expression.

iii) Knockdown candidate RBMS3-transcription factors and determine whether they regulate the expression of *RBMS3*. This will confirm whether *RBMS3* expression is regulated by FAK-dependent chromatin accessibility changes at its promoter/enhancer regions.

iv) Since reduced expression of RBMS3 has been associated with metastasis (Zhu *et al*., 2019), I would like to determine whether knockdown of RBMS3 in the FAK WT cells can perturb metastasis in the SCC model by performing a tail-vein metastasis assay.

Overall, I hope to show that FAK in the nucleus is an important regulator of cancer-associated phenotypes and that this occurs via the regulation of transcription factor function and chromatin accessibility at target genes.

Appendix

A1 FAK RIME proteomics hits

Protein IDs	Protein names	Gene names
P34152	Focal adhesion kinase 1	PTK2
P63017	Heat shock cognate 71 kDa protein	HSPA8
P16858	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
P11499	Heat shock protein HSP 90-beta	HSP90AB1
P10126	Elongation factor 1-alpha 1	EEF1A1
E9Q616	Neuroblast differentiation-associated protein AHNAK	AHNAK
P58252	Elongation factor 2	EEF2
Q9QXS1	Plectin	PLEC
P17182	Alpha-enolase	ENO1
Q8BTM8	Filamin-A	FLNA
P05064	Fructose-bisphosphate aldolase A	ALDOA
P09411	Phosphoglycerate kinase 1	PGK1
P19096	Fatty acid synthase	FASN
P62702	40S ribosomal protein S4, X isoform	RPS4X
P47963	60S ribosomal protein L13	RPL13
P27659	60S ribosomal protein L3	RPL3
P26041	Moesin	MSN
Q6ZWN5	40S ribosomal protein S9	RPS9
P14148	60S ribosomal protein L7	RPL7
D3Z2H9	Tropomyosin 3, related sequence 7	TPM3-RS7
E9QAZ2	Ribosomal protein L15	RPL15
P26039	Talin-1	TLN1
P12970	60S ribosomal protein L7a	RPL7A
P62918	60S ribosomal protein L8	RPL8
P47962	60S ribosomal protein L5	RPL5
Q9D8N0	Elongation factor 1-gamma	EEF1G
P62965	Cellular retinoic acid-binding protein 1	CRABP1
P42932	T-complex protein 1 subunit theta	CCT8
P14131	40S ribosomal protein S16	RPS16
F6YVP7	40S ribosomal protein S18	RPS18
P14069	Protein S100-A6	S100A6
P07901	Heat shock protein HSP 90-alpha	HSP90AA1
E9PZF0	Nucleoside diphosphate kinase	NME2
P80317	T-complex protein 1 subunit zeta	CCT6A
P25444	40S ribosomal protein S2	RPS2

P62242	40S ribosomal protein S8	RPS8
E9PYL9	60S ribosomal protein L11	RPL11
Q6ZWV3	60S ribosomal protein L10	RPL10
P62717	60S ribosomal protein L18a	RPL18A
P62754	40S ribosomal protein S6	RPS6
P41105	60S ribosomal protein L28	RPL28
P57776	Elongation factor 1-delta	EEF1D
P61255	60S ribosomal protein L26	RPL26
P80315	T-complex protein 1 subunit delta	CCT4
P62281	40S ribosomal protein S11	RPS11
O55142	60S ribosomal protein L35a	RPL35A
O09167	60S ribosomal protein L21	RPL21
P80316	T-complex protein 1 subunit epsilon	CCT5
P53026	60S ribosomal protein L10a	RPL10A
Q9CY58	Plasminogen activator inhibitor 1 RNA-binding protein	SERBP1
Q60864	Stress-induced-phosphoprotein 1	STIP1
P11983	T-complex protein 1 subunit alpha	TCP1
P62301	40S ribosomal protein S13	RPS13
P63101	14-3-3 protein zeta/delta	YWHAZ
Q9JHU4	Cytoplasmic dynein 1 heavy chain 1	DYNC1H1
P62900	60S ribosomal protein L31	RPL31
P68134	Actin, alpha skeletal muscle	ACTA1
Q9CPR4	60S ribosomal protein L17	RPL17
Q61753	D-3-phosphoglycerate dehydrogenase	PHGDH
Q8CGC7	Bifunctional glutamate/proline--tRNA ligase	EPRS
P08207	Protein S100-A10	S100A10
P62264	40S ribosomal protein S14	RPS14
P80313	T-complex protein 1 subunit eta	CCT7
P62911	60S ribosomal protein L32	RPL32
P14115	60S ribosomal protein L27a	RPL27A
P23116	Eukaryotic translation initiation factor 3 subunit A	EIF3A
O08807	Peroxiredoxin-4	PRDX4
Q9D1R9	60S ribosomal protein L34	RPL34
P17751	Triosephosphate isomerase	TPI1
Q64727	Vinculin	VCL
O55131	Septin-7	SEPT7
P60867	40S ribosomal protein S20	RPS20
P08113	Endoplasmin	HSP90B1

P61514	60S ribosomal protein L37a	RPL37A
Q91VI7	Ribonuclease inhibitor	RNH1
Q9CQV8	14-3-3 protein beta/alpha	YWHAB
Q922D8	C-1-tetrahydrofolate synthase, cytoplasmic	MTHFD1
Q8VI36	Paxillin	PXN
P16110	Galectin-3	LGALS3
Q9CZX8	40S ribosomal protein S19	RPS19
P62827	GTP-binding nuclear protein Ran	RAN
Q60598	Src substrate cortactin	CTTN
Q9D0I9	Arginine--tRNA ligase, cytoplasmic	RARS
P63323	40S ribosomal protein S12	RPS12
Q80UG5	Septin-9	SEPT9
Q9DCL9	Multifunctional protein ADE2	PAICS
Q8C1B7	Septin-11	SEPT11
Q8BGD9	Eukaryotic translation initiation factor 4B	EIF4B
Q99MN1	Lysine--tRNA ligase	KARS
P13020	Gelsolin	GSN
Q8BMJ2	Leucine--tRNA ligase, cytoplasmic	LARS
O70456	14-3-3 protein sigma	SFN
Q9Z1Q9	Valine--tRNA ligase	VARS
P46935	E3 ubiquitin-protein ligase NEDD4	NEDD4
P62259	14-3-3 protein epsilon	YWHAE
Q6NZJ6	Eukaryotic translation initiation factor 4 gamma 1	EIF4G1
Q01853	Transitional endoplasmic reticulum ATPase	VCP
Q922B2	Aspartate--tRNA ligase, cytoplasmic	DARS
O88342	WD repeat-containing protein 1	WDR1
P60229	Eukaryotic translation initiation factor 3 subunit E	EIF3E
P97461	40S ribosomal protein S5	RPS5
Q91W50	Cold shock domain-containing protein E1	CSDE1
Q9Z0N1	Eukaryotic translation initiation factor 2 subunit 3, X-linked	EIF2S3X
Q8R1B4	Eukaryotic translation initiation factor 3 subunit C	EIF3C
P34022	Ran-specific GTPase-activating protein	RANBP1
Q61316	Heat shock 70 kDa protein 4	HSPA4
P28656	Nucleosome assembly protein 1-like 1	NAP1L1
P21107	Tropomyosin alpha-3 chain	TPM3
Q8BGQ7	Alanine--tRNA ligase, cytoplasmic	AARS
P61982	14-3-3 protein gamma	YWHAG

Q8CI51	PDZ and LIM domain protein 5	PDLIM5
Q6ZWU9	40S ribosomal protein S27	RPS27
P31230	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	AIMP1
Q9WU78	Programmed cell death 6-interacting protein	PDCD6IP
Q9QZD9	Eukaryotic translation initiation factor 3 subunit I	EIF3I
P50247	Adenosylhomocysteinase	AHCY
Q922F4	Tubulin beta-6 chain	TUBB6
Q9CR16	Peptidyl-prolyl cis-trans isomerase D	PPID
Q8BMK4	Cytoskeleton-associated protein 4	CKAP4
Q9WV32	Actin-related protein 2/3 complex subunit 1B	ARPC1B
Q8BU30	Isoleucine--tRNA ligase, cytoplasmic	IARS
Q08509	Epidermal growth factor receptor kinase substrate 8	EPS8
Q6ZWX6	Eukaryotic translation initiation factor 2 subunit 1	EIF2S1
Q61171	Peroxiredoxin-2	PRDX2
Q68FL6	Methionine--tRNA ligase, cytoplasmic	MARS
Q8QZY1	Eukaryotic translation initiation factor 3 subunit L	EIF3L
P62889	60S ribosomal protein L30	RPL30
P67984	60S ribosomal protein L22	RPL22
P47226	Testin	TES
Q61699	Heat shock protein 105 kDa	HSPH1
P11440	Cyclin-dependent kinase 1	CDK1
P34884	Macrophage migration inhibitory factor	MIF
P10639	Thioredoxin	TXN
P83882	60S ribosomal protein L36a	RPL36A
P50543	Protein S100-A11	S100A11
Q6PHZ2	Calcium/calmodulin-dependent protein kinase type II subunit delta	CAMK2D
Q61768	Kinesin-1 heavy chain	KIF5B
Q9CQC6	Basic leucine zipper and W2 domain-containing protein 1	BZW1
P63037	DnaJ homolog subfamily A member 1	DNAJA1
P05977	Myosin light chain 1/3, skeletal muscle isoform	MYL1
Q920E5	Farnesyl pyrophosphate synthase	FDPS
P30416	Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4
O70400	PDZ and LIM domain protein 1	PDLIM1
Q6ZQ38	Cullin-associated NEDD8-dissociated protein 1	CAND1

Q61792	LIM and SH3 domain protein 1	LASP1
Q99PT1	Rho GDP-dissociation inhibitor 1	ARHGDIA
P39447	Tight junction protein ZO-1	TJP1
Q99LF4	tRNA-splicing ligase RtcB homolog	RTCB
P61222	ATP-binding cassette sub-family E member 1	ABCE1
Q9CXW3	Calcyclin-binding protein	CACYBP
O70194	Eukaryotic translation initiation factor 3 subunit D	EIF3D
P16546	Spectrin alpha chain, non-erythrocytic 1	SPTAN1
P62874	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1
O55222	Integrin-linked protein kinase	ILK
P61161	Actin-related protein 2	ACTR2
Q6ZWY3	40S ribosomal protein S27-like	RPS27L
Q99L47	Hsc70-interacting protein	ST13
P14685	26S proteasome non-ATPase regulatory subunit 3	PSMD3
Q8BG32	26S proteasome non-ATPase regulatory subunit 11	PSMD11
Q8R050	Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	GSPT1
O70251	Elongation factor 1-beta	EEF1B
Q61024	Asparagine synthetase [glutamine-hydrolyzing]	ASNS
Q61035	Histidine--tRNA ligase, cytoplasmic	HARS
Q9QYJ0	DnaJ homolog subfamily A member 2	DNAJA2
Q5XJY5	Coatomer subunit delta	ARCN1
Q9Z1D1	Eukaryotic translation initiation factor 3 subunit G	EIF3G
Q9ER72	Cysteine--tRNA ligase, cytoplasmic	CARS
Q99JY9	Actin-related protein 3	ACTR3
P58771	Tropomyosin alpha-1 chain	TPM1
P40142	Transketolase	TKT
Q9QUM9	Proteasome subunit alpha type-6	PSMA6
Q8VDM4	26S proteasome non-ATPase regulatory subunit 2	PSMD2
P24547	Inosine-5-monophosphate dehydrogenase 2	IMPDH2
Q9DBJ1	Phosphoglycerate mutase 1;Phosphoglycerate mutase 2	PGAM1
P05480	Neuronal proto-oncogene tyrosine-protein kinase Src	SRC
Q9WTM5	RuvB-like 2	RUVBL2
Q99K51	Plastin-3	PLS3
O35685	Nuclear migration protein nudC	NUDC

P19157	Glutathione S-transferase P 1	GSTP1
Q61696	Heat shock 70 kDa protein 1A	HSPA1A
Q04736	Tyrosine-protein kinase Yes	YES1
P63001	Ras-related C3 botulinum toxin substrate 1	RAC1
Q9DBG3	AP-2 complex subunit beta	AP2B1
Q62523	Zyxin	ZYX
Q9D823	60S ribosomal protein L37	RPL37
Q9CWJ9	Bifunctional purine biosynthesis protein PURH	ATIC
Q8CIE6	Coatomer subunit alpha;Xenin;Proxenin	COPA
Q9WVK4	EH domain-containing protein 1	EHD1
P70677	Caspase-3;Caspase-3 subunit p17;Caspase-3 subunit p12	CASP3
Q8BP47	Asparagine--tRNA ligase, cytoplasmic	NARS
Q9D0R2	Threonine--tRNA ligase, cytoplasmic	TARS
Q61553	Fascin	FSCN1
Q99L45	Eukaryotic translation initiation factor 2 subunit 2	EIF2S2
Q8CDN6	Thioredoxin-like protein 1	TXNL1
Q8BJW6	Eukaryotic translation initiation factor 2A	EIF2A
O55029	Coatomer subunit beta	COPB2
Q8BFY9	Transportin-1;Transportin-2	TNPO1
Q99LE6	ATP-binding cassette sub-family F member 2	ABCF2
Q64737	Trifunctional purine biosynthetic protein adenosine-3	GART
P50516	V-type proton ATPase catalytic subunit A	ATP6V1A
Q9WUA2	Phenylalanine--tRNA ligase beta subunit	FARSB
P63254	Cysteine-rich protein 1	CRIP1
O35226	26S proteasome non-ATPase regulatory subunit 4	PSMD4
P62274	40S ribosomal protein S29	RPS29
Q8CAQ8	MICOS complex subunit Mic60	IMMT
P26638	Serine--tRNA ligase, cytoplasmic	SARS
Q61166	Microtubule-associated protein RP/EB family member 1	MAPRE1
P27546	Microtubule-associated protein 4	MAP4
Q62448	Eukaryotic translation initiation factor 4 gamma 2	EIF4G2
Q8R1F1	Niban-like protein 1	FAM129B
P68510	14-3-3 protein eta	YWHAH
Q3UZ39	Leucine-rich repeat flightless-interacting protein 1	LRRFIP1
Q99K85	Phosphoserine aminotransferase	PSAT1

Q6A028	Switch-associated protein 70	SWAP70
Q9QYI3	DnaJ homolog subfamily C member 7	DNAJC7
Q9Z2U1	Proteasome subunit alpha type-5	PSMA5
Q9JLV1	BAG family molecular chaperone regulator 3	BAG3
Q9EPC1	Alpha-parvin	PARVA
P55821	Stathmin-2	STMN2
O88487	Cytoplasmic dynein 1 intermediate chain 2	DYNC1I2
Q9ERK4	Exportin-2	CSE1L
Q91WQ3	Tyrosine--tRNA ligase, cytoplasmic	YARS
P54227	Stathmin	STMN1
Q9WTI7	Unconventional myosin-Ic	MYO1C
Q9R1P4	Proteasome subunit alpha type-1	PSMA1
Q76MZ3	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A
P46471	26S protease regulatory subunit 7	PSMC2
Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDHB
Q62418	Drebrin-like protein	DBNL
Q9WVJ2	26S proteasome non-ATPase regulatory subunit 13	PSMD13
Q60692	Proteasome subunit beta type-6	PSMB6
P84091	AP-2 complex subunit mu	AP2M1
P26043	Radixin;Ezrin	RDX
Q9R059	Four and a half LIM domains protein 3	FHL3
Q3UPL0	Protein transport protein Sec31A	SEC31A
Q60930	Voltage-dependent anion-selective channel protein 2	VDAC2
Q62261	Spectrin beta chain, non-erythrocytic 1	SPTBN1
Q8C0C7	Phenylalanine--tRNA ligase alpha subunit	FARSA
Q80W21	Glutathione S-transferase Mu 7	GSTM7
Q99K30	Epidermal growth factor receptor kinase substrate 8-like protein 2	EPS8L2
Q80UM3	N-alpha-acetyltransferase 15, NatA auxiliary subunit	NAA15
P28658	Ataxin-10	ATXN10
Q6PB66	Leucine-rich PPR motif-containing protein, mitochondrial	LRPPRC
Q91WK2	Eukaryotic translation initiation factor 3 subunit H	EIF3H
Q9EQH3	Vacuolar protein sorting-associated protein 35	VPS35
P52293	Importin subunit alpha-1	KPNA2
P26883	Peptidyl-prolyl cis-trans isomerase FKBP1A	FKBP1A

Q9DBG6	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	RPN2
Q8R2Q0	Tripartite motif-containing protein 29	TRIM29
P63073	Eukaryotic translation initiation factor 4E	EIF4E
P70336	Rho-associated protein kinase 2	ROCK2
Q61598	Rab GDP dissociation inhibitor beta	GDI2
P62334	26S protease regulatory subunit 10B	PSMC6
P62892	60S ribosomal protein L39	RPL39
O70435	Proteasome subunit alpha type-3	PSMA3
Q8R010	Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	AIMP2
P68181	cAMP-dependent protein kinase catalytic subunit beta	PRKACB
Q7TMB8	Cytoplasmic FMR1-interacting protein 1	CYFIP1
Q6P542	ATP-binding cassette sub-family F member 1	ABCF1
Q99JI4	26S proteasome non-ATPase regulatory subunit 6	PSMD6
Q08093	Calponin-2	CNN2
P46735	Unconventional myosin-Ib	MYO1B
Q99JX4	Eukaryotic translation initiation factor 3 subunit M	EIF3M
P59325	Eukaryotic translation initiation factor 5	EIF5
Q7TT37	Elongator complex protein 1	IKBKAP
Q5SWU9	Acetyl-CoA carboxylase 1;Biotin carboxylase	ACACA
P62880	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2
P15532	Nucleoside diphosphate kinase A	NME1
Q4VAA2	Protein CDV3	CDV3
A2AGT5	Cytoskeleton-associated protein 5	CKAP5
Q9DB05	Alpha-soluble NSF attachment protein	NAPA
P97355	Spermine synthase	SMS
Q9Z2U0	Proteasome subunit alpha type-7	PSMA7
Q9DCT8	Cysteine-rich protein 2	CRIP2
Q8CG47	Structural maintenance of chromosomes protein 4	SMC4
Q8CIB5	Fermitin family homolog 2	FERMT2
Q8VDN2	Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1
P54775	26S protease regulatory subunit 6B	PSMC4
Q9D358	Low molecular weight phosphotyrosine protein phosphatase	ACP1
Q9QUI0	Transforming protein RhoA	RHOA
Q3TLH4	Protein PRRC2C	PRRC2C

Q99JW4	LIM and senescent cell antigen-like-containing domain protein 1	LIMS1
O70503	Very-long-chain 3-oxoacyl-CoA reductase	HSD17B12
Q8BML9	Glutaminyl-TRNA Synthetase 1	QARS
E9QA15	Caldesmon 1	CALD1
Q62095	ATP-dependent RNA helicase DDX3Y	DDX3Y
Q60631	Growth factor receptor-bound protein 2	GRB2
Q8R1Q8	Cytoplasmic dynein 1 light intermediate chain 1	DYNC1LI1
P17809	Solute carrier family 2, facilitated glucose transporter member 1	SLC2A1
E9PVA8	GCN1 Activator Of EIF2AK4	GCN1L1
P30999	Catenin delta-1	CTNND1
Q9Z108	Double-stranded RNA-binding protein Staufen homolog 1	STAU1
Q6ZQ58	La-related protein 1	LARP1
O70591	Prefoldin subunit 2	PFDN2
O08585	Clathrin light chain A	CLTA
P99029	Peroxiredoxin-5, mitochondrial	PRDX5
P07742	Ribonucleoside-diphosphate reductase large subunit	RRM1
P63005	Platelet-activating factor acetylhydrolase IB subunit alpha	PAFAH1B1
P48024	Eukaryotic translation initiation factor 1	EIF1
Q3UHX2	28 kDa heat- and acid-stable phosphoprotein	PDAP1
Q9JI10	Serine/threonine-protein kinase 3	STK3
Q5SXY1	Cytospin-B	SPECC1
Q8BFR5	Elongation factor Tu, mitochondrial	TUFM
Q3THK7	GMP synthase [glutamine-hydrolyzing]	GMPS
P02468	Laminin subunit gamma-1	LAMC1
Q9DCF9	Translocon-associated protein subunit gamma	SSR3
E9Q0S6	Tensin-2;Tensin-3	TNS1
P24527	Leukotriene A-4 hydrolase	LTA4H
Q61033	Lamina-associated polypeptide 2, isoforms alpha/zeta	TMPO
Q9D2R0	Acetoacetyl-CoA synthetase	AACS
Q68FF6	ARF GTPase-activating protein GIT1	GIT1
P45376	Aldose reductase	AKR1B1
Q9QZ88	Vacuolar protein sorting-associated protein 29	VPS29
O54988	STE20-like serine/threonine-protein kinase	SLK
Q9QZQ1	Afadin	MLLT4

O88544	COP9 signalosome complex subunit 4	COPS4
E9PYG6	Ras GTPase-activating protein 1	RASA1
P27612	Phospholipase A-2-activating protein	PLAA
P51881	ADP/ATP translocase 2	SLC25A5
Q9ES46	Beta-parvin	PARVB
P49722	Proteasome subunit alpha type-2	PSMA2
P28740	Kinesin-like protein KIF2A	KIF2A
Q9EPL8	Importin-7	IPO7
Q8BT07	Centrosomal protein of 55 kDa	CEP55
Q8CG48	Structural maintenance of chromosomes protein 2	SMC2
P97379	Ras GTPase-activating protein-binding protein 2	G3BP2
Q9QYB1	Chloride intracellular channel protein 4	CLIC4
E9Q634	Unconventional myosin-Ie	MYO1E
Q9DBR7	Protein phosphatase 1 regulatory subunit 12A	PPP1R12A
P61089	Ubiquitin-conjugating enzyme E2 N	UBE2N
Q9Z0U1	Tight junction protein ZO-2	TJP2
G5E8K5	Ankyrin-3	ANK3
Q9D7S7	60S ribosomal protein L22-like 1	RPL22L1
Q9D0T1	NHP2-like protein 1	NHP2L1
Q9DBG7	Signal recognition particle receptor subunit alpha	SRPR
Q9QWY8	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1	ASAP1
O35344	Importin subunit alpha-4;Importin subunit alpha-3	KPNA3
Q8BZ98	Dynamin-3	DNM3
P02469	Laminin subunit beta-1	LAMB1
P70296	Phosphatidylethanolamine-binding protein 1	PEBP1
P63087	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	PPP1CC
Q3THS6	S-adenosylmethionine synthase isoform type-2	MAT2A
Q9DBZ5	Eukaryotic translation initiation factor 3 subunit K	EIF3K
Q9WVE8	Protein kinase C and casein kinase substrate in neurons protein 2	PACSIN2
Q3U9G9	Lamin-B receptor	LBR
Q9R0Y5	Adenylate kinase isoenzyme 1	AK1
Q8K1M6	Dynamin-1-like protein	DNM1L
P70460	Vasodilator-stimulated phosphoprotein	VASP
P35278	Ras-related protein Rab-5C	RAB5C
Q8BJ71	Nuclear pore complex protein Nup93	NUP93

O09131	Glutathione S-transferase omega-1	GSTO1
Q8K2Z4	Condensin complex subunit 1	NCAPD2
Q91VK1	Basic leucine zipper and W2 domain-containing protein 2	BZW2
Q9CZW5	Mitochondrial import receptor subunit TOM70	TOMM70A
Q9CWM4	Prefoldin subunit 1	PFDN1
Q9JIF7	Coatomer subunit beta	COPB1
Q8QZT1	Acetyl-CoA acetyltransferase, mitochondrial	ACAT1
Q63932	Dual specificity mitogen-activated protein kinase kinase 2	MAP2K2
Q9DCN2	NADH-cytochrome b5 reductase 3	CYB5R3
Q8BJY1	26S proteasome non-ATPase regulatory subunit 5	PSMD5
P46460	Vesicle-fusing ATPase	NSF
Q9JMA1	Ubiquitin carboxyl-terminal hydrolase 14	USP14
Q9JMH6	Thioredoxin reductase 1, cytoplasmic	TXNRD1
Q9D1E6	Tubulin-folding cofactor B	TBCB
E9Q3T0	60S acidic ribosomal protein P1	RPLP1
Q01730	Ras suppressor protein 1	RSU1
P63330	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	PPP2CA
Q9DBS9	Oxysterol-binding protein-related protein 3	OSBPL3
P83940	Transcription elongation factor B polypeptide 1	TCEB1
P63168	Dynein light chain 1, cytoplasmic	DYNLL1
Q8K297	Procollagen galactosyltransferase 1	COLGALT1
Q9CQM5	Thioredoxin domain-containing protein 17	TXNDC17
Q9JK48	Endophilin-B1;Endophilin-B2	SH3GLB1
P50518	V-type proton ATPase subunit E 1	ATP6V1E1
O88811	Signal transducing adapter molecule 2	STAM2
Q922W5	Pyrroline-5-carboxylate reductase 1, mitochondrial	PYCR1
Q7M6Y3	Phosphatidylinositol-binding clathrin assembly protein	PICALM
Q9EQP2	EH domain-containing protein 4	EHD4
P08003	Protein disulfide-isomerase A4	PDIA4
O08749	Dihydrolipoyl dehydrogenase, mitochondrial	DLD
Q9DBC7	cAMP-dependent protein kinase type I-alpha regulatory subunit	PRKAR1A
Q505F5	Leucine-rich repeat-containing protein 47	LRRC47
Q8C5L3	CCR4-NOT transcription complex subunit 2	CNOT2
Q61335	B-cell receptor-associated protein 31	BCAP31

Q6P2B1	Transportin-3;Fanconi anemia group I protein homolog	TNPO3
Q8VEM8	Phosphate carrier protein, mitochondrial	SLC25A3
Q61595	Kinectin	KTN1
Q9D8S3	ADP-ribosylation factor GTPase-activating protein 3	ARFGAP3
Q9D819	Inorganic pyrophosphatase	PPA1
Q9R0Q7	Prostaglandin E synthase 3	PTGES3
P97429	Annexin A4	ANXA4
P97807	Fumarate hydratase, mitochondrial	FH
E9PWG6	Condensin complex subunit 3	NCAPG
Q9R0P5	Destrin	DSTN
P46061	Ran GTPase-activating protein 1	RANGAP1
Q3UWL8	Prefoldin subunit 4	PFDN4
Q6PAM1	Alpha-taxilin	TXLNA
Q3UZ24	Apolipoprotein L 7e	APOL7E

A2 FAK BioID whole cell lysate proteomics hits

Protein IDs	**Protein names**	**Gene names**
E9Q616	Neuroblast differentiation-associated protein AHNAK	AHNAK
P34152	Focal adhesion kinase 1	PTK2
Q6URW6	Myosin-14	MYH14
P26443	Glutamate dehydrogenase 1, mitochondrial	GLUD1
A2AGL3	Ryanodine receptor 3	RYR3
E9PYB0	AHNAK Nucleoprotein 2	AHNAK2
Q91WK0	Leucine-rich repeat flightless-interacting protein 2	LRRFIP2
P20029	78 kDa glucose-regulated protein	HSPA5
Q8VI36	Paxillin	PXN
Q8BTM8	Filamin-A	FLNA
Q60598	Src substrate cortactin	CTTN
P61161	Actin-related protein 2	ACTR2
P14069	Protein S100-A6	S100A6
P62204;Q9D6P8	Calmodulin	CALM1
Q80X90	Filamin-B	FLNB
Q8BFW7	Lipoma-preferred partner homolog	LPP
Q6ZWU9	40S ribosomal protein S27	RPS27
Q9Z2H5	Band 4.1-like protein 1	EPB41L1
Q9QWY8	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1	ASAP1

P57776	Elongation factor 1-delta	EEF1D
Q8BX02	KN motif and ankyrin repeat domain-containing protein 2	KANK2
P42208	Septin-2	SEPT2
Q9WTI7	Unconventional myosin-Ic	MYO1C
Q60930	Voltage-dependent anion-selective channel protein 2	VDAC2
Q80UG5	Septin-9	SEPT9
Q6NZJ6	Eukaryotic translation initiation factor 4 gamma 1	EIF4G1
Q9WUM3	Coronin-1B	CORO1B
Q62219	Transforming growth factor beta-1-induced transcript 1 protein	TGFB1I1
Q8CI51	PDZ and LIM domain protein 5	PDLIM5
P58252	Elongation factor 2	EEF2
Q99P72	Reticulon-4	RTN4
O55131	Septin-7	SEPT7
Q69Z38	Pseudopodium-enriched atypical kinase 1	PEAK1
Q99K30	Epidermal growth factor receptor kinase substrate 8-like protein 2	EPS8L2
P60122	RuvB-like 1	RUVBL1
Q61081	Hsp90 co-chaperone Cdc37	CDC37
P50543	Protein S100-A11	S100A11
Q3TLH4	Protein PRRC2C	PRRC2C
Q8BGD9	Eukaryotic translation initiation factor 4B	EIF4B
Q9QXD8	LIM domain-containing protein 1	LIMD1
P08207	Protein S100-A10	S100A10
Q9QXT8	Calsenilin	KCNIP3
E9Q0S6	Tensin-1	TNS1
Q9QYJ0	DnaJ homolog subfamily A member 2	DNAJA2
P39061	Collagen alpha-1(XVIII) chain;Endostatin	COL18A1
Q8BH59	Calcium-binding mitochondrial carrier protein Aralar1	SLC25A12
Q6ZQ58	La-related protein 1	LARP1
P05480	Neuronal proto-oncogene tyrosine-protein kinase Src	SRC
Q6PAJ1	Breakpoint cluster region protein	BCR
Q9CWK8	Sorting nexin-2	SNX2
Q8BK67	Protein RCC2	RCC2
P42669	Transcriptional activator protein Pur-alpha	PURA
Q64010	Adapter molecule crk	CRK
O88342	WD repeat-containing protein 1	WDR1
Q9DBJ1	Phosphoglycerate mutase 1	PGAM1
Q8CGB3	Uveal autoantigen with coiled-coil domains and ankyrin repeats	UACA
Q9DBG3	AP-2 complex subunit beta	AP2B1

A3 FAK BioID nuclear proteomics hits

Protein IDs	Protein names	Gene names
E9Q616	Neuroblast differentiation-associated protein AHNAK	AHNAK
P34152	Focal adhesion kinase 1	PTK2
Q9WUM3	Coronin-1B	CORO1B
Q60598	Src substrate cortactin	CTTN
E9PYB0	Protein AHNAK2	AHNAK2
Q9WUM4	Coronin-1C	CORO1C
P39447	Tight junction protein ZO-1	TJP1
P57776	Elongation factor 1-delta	EEF1D
Q3TLH4	Protein PRRC2C	PRRC2C
E9QA15	Caldesmon	CALD1
O70400	PDZ and LIM domain protein 1	PDLIM1
Q8VI36	Paxillin	PXN
E9Q0S6	Tensin-1	TNS1
P26039	Talin-1	TLN1
Q80X50	Ubiquitin-associated protein 2-like	UBAP2L
Q9QZQ1	Afadin	MLLT4
E9Q6R7	Utrophin	UTRN
Q91WL8	WW domain-containing oxidoreductase	WWOX
Q62417	Sorbin and SH3 domain-containing protein 1	SORBS1
Q9QWY8	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1	ASAP1
Q8R361	Rab11 family-interacting protein 5	RAB11FIP5
Q80U93	Nuclear pore complex protein Nup214	NUP214
Q9Z0U1	Tight junction protein ZO-2	TJP2
Q3TUF7	YEATS domain-containing protein 2	YEATS2
Q5F2E7	Nuclear fragile X mental retardation-interacting protein 2	NUFIP2
Q8BU11	TOX high mobility group box family member 4	TOX4
Q8BFW7	Lipoma-preferred partner homolog	LPP
P05480	Neuronal proto-oncogene tyrosine-protein kinase Src	SRC
Q9EP71	Ankycorbin	RAI14

Q69ZK6	Probable JmjC domain-containing histone demethylation protein 2C	JMJD1C
Q8CI51	PDZ and LIM domain protein 5	PDLIM5
Q91VJ2	Protein kinase C delta-binding protein	PRKCDBP
Q64010	Adapter molecule crk	CRK
A0A1W2P6U8	RIKEN cDNA 4932415D10 gene	4932415D10RIK
Q62219	Transforming growth factor beta-1-induced transcript 1 protein	TGFB1I1
Q1W617	Protein Shroom4	SHROOM4
Q8CDG3	Deubiquitinating protein VCIP135	VCPIP1
Q60902	Epidermal growth factor receptor substrate 15-like 1	EPS15L1
A2AMM0	Muscle-related coiled-coil protein	MURC
Q9CY27	Very-long-chain enoyl-CoA reductase	TECR
Q60634	Flotillin-2	FLOT2
Q9R0A0	Peroxisomal membrane protein PEX14	PEX14
Q91YR1	Twinfilin-1	TWF1
Q9CWE0	Mitochondrial fission regulator 1-like	MTFR1L
B2RRE7	OTU domain-containing protein 4	OTUD4
Q9QXD8	LIM domain-containing protein 1	LIMD1
O88874	Cyclin-K	CCNK

A4 HIC-5 BioID nuclear proteomics hits

Protein IDs	**Protein names**	**Gene names**
E9Q616	AHNAK nucleoprotein (desmoyokin)	AHNAK
Q69Z38	Pseudopodium-enriched atypical kinase 1	PEAK1
Q9ERU9	E3 SUMO-protein ligase RanBP2	RANBP2
Q60598	Src substrate cortactin	CTTN
Q1W617	Protein Shroom4	SHROOM4
P39447	Tight junction protein ZO-1	TJP1
E9Q0S6	Tensin 1	TNS1

Q8C0T5	Signal-induced proliferation-associated 1-like protein 1	SIPA1L1
Q9WUM3	Coronin-1B	CORO1B
E9Q6R7	Utrophin	UTRN
E9PYB0	AHNAK nucleoprotein 2	AHNAK2
Q62417	Sorbin and SH3 domain-containing protein 1	SORBS1
Q3TLH4	Protein PRRC2C	PRRC2C
P26039	Talin-1	TLN1
E9PYF4	LIM domain only 7	LMO7
Q9Z0U1	Tight junction protein ZO-2	TJP2
Q80U93	Nuclear pore complex protein Nup214	NUP214
O35691	Pinin	PNN
Q80U72	Protein scribble homolog	SCRIB
O70400	PDZ and LIM domain protein 1	PDLIM1
H3BKP8	RIKEN cDNA 9930021J03 gene	9930021J03RIK
Q8BX02	KN motif and ankyrin repeat domain-containing protein 2	KANK2
Q91YE8	Synaptopodin-2	SYNPO2
Q9QZQ1	Afadin	MLLT4
E9Q3T0	60S acidic ribosomal protein P1	RPLP1
Q5F2E7	Nuclear fragile X mental retardation-interacting protein 2	NUFIP2
G3X9J0	Signal-induced proliferation-associated 1-like protein 3	SIPA1L3
Q61235	Beta-2-syntrophin	SNTB2
Q69ZK6	Probable JmjC domain-containing histone demethylation protein 2C	JMJD1C
Q61263	Sterol O-acyltransferase 1	SOAT1
Q9EP71	Ankycorbin	RAI14
Q80X50	Ubiquitin-associated protein 2-like	UBAP2L
Q8BU11	TOX high mobility group box family member 4	TOX4
Q9DBR7	Protein phosphatase 1 regulatory subunit 12A	PPP1R12A
Q80UG5	Septin-9	SEPT9
Q91VX2	Ubiquitin-associated protein 2	UBAP2
Q3TAQ9	WD repeat domain 36	WDR36
P46062	Signal-induced proliferation-associated protein 1	SIPA1
A2AQ25	Sickle tail protein	SKT

Q62219	Transforming growth factor beta-1-induced transcript 1 protein	TGFB1I1
Q8R361	Rab11 family-interacting protein 5	RAB11FIP5
Q8R4U7	Leucine zipper protein 1	LUZP1
Q9R0L6	Pericentriolar material 1 protein	PCM1
P62259	14-3-3 protein epsilon	YWHAE
Q810A7	ATP-dependent RNA helicase DDX42	DDX42
Q8C0D9	Centrosomal protein of 68 kDa	CEP68
P62141	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PPP1CB
E9Q6E5	Serine/arginine-rich-splicing factor 11	SRSF11
Q9D666	SUN domain-containing protein 1	SUN1
P63166	Small ubiquitin-related modifier 1	SUMO1
Q6Y7W8	PERQ amino acid-rich with GYF domain-containing protein 2	GIGYF2
Q8BKI2	Trinucleotide repeat-containing gene 6B protein	TNRC6B
Q02257	Junction plakoglobin	JUP
Q9R0E1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	PLOD3
P35831	Tyrosine-protein phosphatase non-receptor type 12	PTPN12
Q921L3	Transmembrane and coiled-coil domain-containing protein 1	TMCO1
Q8C1B7	Septin-11	SEPT11
Q924K8	Metastasis-associated protein MTA3	MTA3
Q60953	Protein PML	PML
G5E8K5	Ankyrin-3	ANK3
O54833	Casein kinase II subunit alpha	CSNK2A2
O55201	Transcription elongation factor SPT5	SUPT5H
Q99KW3	TRIO and F-actin-binding protein	TRIOBP
P70399	Tumor suppressor p53-binding protein 1	TP53BP1
Q8C115	Pleckstrin homology domain-containing family H member 2	PLEKHH2
Q8BG15	CTD small phosphatase-like protein 2	CTDSPL2
Q9EPE9	Manganese-transporting ATPase 13A1	ATP13A1
Q8R3N6	THO complex subunit 1	THOC1

P05977;P09542	Myosin light chain 1/3, skeletal muscle isoform;Myosin light chain 3	MYL1
Q6R891	Neurabin-2	PPP1R9B
Q02248	Catenin beta-1	CTNNB1
Q9Z108	Double-stranded RNA-binding protein Staufen homolog 1	STAU1
Q91WL8	WW domain-containing oxidoreductase	WWOX
Q5DW34	Histone-lysine N-methyltransferase EHMT1	EHMT1
Q9D0F3	Protein ERGIC-53	LMAN1
O55131	Septin-7	SEPT7
Q7TSC1	Protein PRRC2A	PRRC2A
Q6RHR9	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1	MAGI1
Q3UJB9	Enhancer of mRNA-decapping protein 4	EDC4
Q6A065	Centrosomal protein of 170 kDa	CEP170
Q8CI51	PDZ and LIM domain protein 5	PDLIM5
Q8CJG0	Protein argonaute-2	AGO2
Q8R2U0	Nucleoporin SEH1	SEH1L
Q4VBE8	WD repeat-containing protein 18	WDR18
Q8CG79	Apoptosis-stimulating of p53 protein 2	TP53BP2
Q6DFV3	Rho GTPase-activating protein 21	ARHGAP21
Q91ZX7	Prolow-density lipoprotein receptor-related protein 1	LRP1
Q60974	Nuclear receptor corepressor 1	NCOR1
Q99NH2	Partitioning defective 3 homolog	PARD3
Q9DB85	Ribosomal RNA-processing protein 8	RRP8
Q99PP7	E3 ubiquitin-protein ligase TRIM33	TRIM33
Q9ES28	Rho guanine nucleotide exchange factor 7	ARHGEF7
Q69ZL1	FYVE, RhoGEF and PH domain-containing protein 6	FGD6
Q4FZF3	Probable ATP-dependent RNA helicase DDX49	DDX49
E9Q9R9	Disks large homolog 5	DLG5
Q8R2N2	Cirhin	CIRH1A
Q61103	Zinc finger protein ubi-d4	DPF2
Q9D2N4;O70585	Dystrobrevin alpha	DTNA

Q9R0A0	Peroxisomal membrane protein PEX14	PEX14
Q9D0L8	mRNA cap guanine-N7 methyltransferase	RNMT
Q9Z2H5	Band 4.1-like protein 1	EPB41L1
Q7TPM1	Protein PRRC2B	PRRC2B
Q9D0M0	Exosome complex exonuclease RRP42	EXOSC7
P34152	Focal adhesion kinase 1	PTK2

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