MONASH INSTITUTE OF MEDICAL RESEARCH

Deregulated gp130 signalling contributes to lung tumourigenesis and the development of cancer-related cachexia

A thesis submitted for the degree of
Doctor of Philosophy

By

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Abstract

Lung cancer is the leading cause of cancer death in Australia and worldwide, and is frequently associated with the devastating paraneoplastic syndrome of cachexia. While recent discoveries in the molecular foundations of lung cancer have produced specific, directed therapies with substantial benefit, these are effective in only a small proportion of patients with adenocarcinomas that demonstrate specific molecular characteristics. New therapies are desperately needed and the search for new molecular targets requires models with good translational potential. The potent immunomodulatory cytokine interleukin (IL)-6 has been linked with the development of lung cancer as well as cachexia, and presents a promising specific target in anti-cancer therapy.

This thesis addresses the question of the role of IL-6 by utilising the FF mouse which, by virtue of a "knock in" substitution within the IL-6 family co-receptor gp130, displays endogenously elevated IL-6. As a consequence of this mutation, the FF mouse displays hyper-activation of the latent transcription factor STAT3 in the absence of gp130-driven PI3K/Akt and MAPK signalling. Importantly, STAT3, PI3K/Akt and MAPK have all been implicated in lung cancer development and the development of cachexia

Initial characterisation of the FF mouse lung demonstrated substantial pulmonary inflammation that is largely composed of $B220^{+}$ cells. This inflammation is driven by IL-6 through STAT3 but not STAT1. Following this characterisation the FF mouse was used in two separate well-established lung cancer models; a cigarette carcinogen (NNK) model and a genetic ($Kras^{G12D}$) model. In the cigarette carcinogen model there was a significant reduction in tumour number and size in FF mice, which appeared to be largely independent of STAT3. The difference seen is contributed to by the loss of gp130-driven PI3K/Akt and/or MAPK signalling as evidenced by upregulation of tumour suppressor genes and down-regulation of oncogenes in the FF mouse, downstream of both PI3K/Akt and MAPK.

In the genetic model the key finding was of weight loss and early mortality in the absence of a significant change in tumour load in the FF:$Kras^{G12D}$ mice. Both the weight loss and early mortality were abrogated by genetically normalising STAT3 activation and ablating IL-6. Importantly, we also identified IL-6 trans-signalling as the signalling modality responsible for the cachexia by rescuing the weight loss using a monoclonal Ab targeting in the sIL6R.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Abbreviations and Symbols

AAH	Atypical Adenomatous Hyperplasia
AchR	Acetylcholine receptor
Akt	v-akt murine thymoma viral oncogene homolog 1
ANOVA	Analysis of Variance
AOM	Azoxymethane
Apc	Adenomatous Polyposis Coli
BALF	Bronchoalveolar Lavage Fluid
BASC	Bronchoalveolar Stem Cells
Bcl-2	B-cell CLL/lymphoma-2
β-ME	β-Mercaptoethanol
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
CBD	Cytokine Binding Domain
CCL	Chemokine (C-C motif) Ligand
Ccnb1	Cyclin B1
CD	Cluster of Differentiation
Cdc42	Cell division cycle 42 homolog (S. cerevisiae)
Cdk2	Cell division-stimulating protein 2
Cdkn1b	Cyclin-dependent kinase inhibitor 1B
CIA	Collagen-Induced Arthritis
CLC	Cardiotrophin-Like Cytokine
CNTF	Ciliary Neurotrophic Factor
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
Creb1	CAMP responsive element binding protein 1
CRP	C-reactive protein
CS	Cigarette Smoke
CSC	Cigarette Smoke Condensate
CSF	Colony Stimulating Factor
Ct	Cycle threshold
CT-1	Cardiotrophin-1
CTL	Cytotoxic T Lymphocytes
CXCL	Chemokine (C-X-C motif) Ligand
CYP450	Cytochrome P450
DAB	Diaminobenzidine chromogen
DepC	Diethylpyrocarbonate
DEXA	Dual-Energy Xray Absorptiometry
dH2O	Distilled/Reverse osmosis water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNMT1	cytosine-DNA methyltransferase 1
dnSTAT3	Dominant negative STAT3
DPBS	Dulbecco's Phosphate Buffered Saline

DSS	Dextran Sodium Sulphate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra-Acetic acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
EML4-ALK	Echinoderm Microtubule-associated protein-Like 4 - Anaplastic Lymphoma Kinase fusion gene
ERK	Extracellular Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FF	gp130F/F
FFPE	Formalin-Fixed, Paraffin-Embedded
Fos	FBJ osteosarcoma oncogene
Fox	Forkhead Box
Gab1	GRB2-associated protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
gp130	glycoprotein 130
GRB2	Growth factor Receptor Bound protein 2
H&E	Haematoxylin and Eosin
HBSS	Hanks Buffered Salt Solution
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
IBD	Inflammatory Bowel Disease
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IL-6R	Il-6 receptor
IP	Intra-peritoneal
JAK	Janus Kinase
kD	Kilodaltons
K-ras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LIF	Leukaemia Inhibitory Factor
LIFR	LIF receptor
LLC	Lewis Lung Carcinoma
LPS	Lipopolysaccharide
LSL	LoxP-STOP-LoxP
LT	Leukotriene
MAPK	Mitogen Activated Protein Kinases
MEF	Murine Embryonic Fibroblasts
MEM	Minimum Essential Eagle Medium
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
c-Myc	v-myc myelocytomatosis viral oncogene homolog
NF-kB	Nuclear Factor-Kappa Beta
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

NNK	Nicotine-derived Nitrosamine Ketone
NSAIDS	Non-Steroidal Anti-Inflammatory Drug
NSCLC	Non-Small Cell Lung Cancer
NTHi	Non-typeable Haemophilus influenzae
O6MG	Oxygen6-methylguanine
OBB	Odyssey Blocking Buffer
OSM	Oncostatin-M
OSMR	OSM receptor
p53/trp53	Tumour protein 53
pAkt	Phosphorylated Akt (Serine 473)
PAS	Pulmonary Adenoma Susceptibility
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
pERK	Phosphorylated ERK (Threonine 202/Tyrosine 204)
PFU	Plaque Forming Units
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PIP3	phosphatidyl-inositol-3,4,5-triphosphate
PRR	Pattern-Recognition Receptors
pSTAT3	Phosphorylated STAT3 (Tyrosine 705)
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
pY	Phospho-tyrosine
RA	Rheumatoid Arthritis
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute media
-RT	Without Reverse Transcriptase
RT	Room temperature
RTK	Receptor Tyrosine Kinases
SCC	Squamous Cell Carcinoma
SCLC	Small Cell Lung Cancer
SEM	Standard error of the mean
sgp130	Soluble gp130
SHP2	Src Homology tyrosine Phosphatase
shRNA	short hairpin RNA
sIL6-R	Soluble IL-6 Receptor
SOCS	Suppressor of Cytokine Signalling
SOS	Son of Sevenless
SPF	Specific Pathogen Free
STAT	Signal Transducer and Activator of Transcription
STAT3-C	Constitutively active STAT3
TACE	Tumour necrosis factor-α Converting Enzyme
TAM	Tumour Associated Macrophage
TGF	Transforming Growth Factor
TH	T helper cell
TKI	Tyrosine Kinase Inhibitor

TLR	Toll-like Receptors
TMB	Tetramethylbenzidine
TNF	Tumour Necrosis Factor
Treg	T regulatory cells
Tsc2	Tuberous sclerosis 2
TTF-1	thyroid transcription factor-1
TYK	Tyrosine Kinase
VEGF	Vascular Endothelial Growth Factor
WSXWS	tryptophan-serine-X-tryptophan-serine
WT	Wild-type
α	Alpha
β	Beta
γ	Gamma
δ	Delta
μm	Micron/Micrometer
μ	Micro

List of Presentations

Oral Presentations

2012: TSANZ Annual Scientific Meeting, Canberra, Australia.
Deregulated Interleukin-6 Signalling Suppresses Lung Tumourigenesis In Mice Induced By Tobacco Carcinogens
A Miller (Presenting author), S Ruwanpura, L McLeod, P Bardin, DN Watkins, BJ Jenkins.
Winner "Best Lung Cancer Oral Presentation"

2012: MIMR Scientific Review; Student Representative for CIIID, Clayton, Australia.
The role of deregulated gp130 signalling in the development of lung cancer
A Miller (Presenting author)

2012: MIMR Post-graduate Symposium, Clayton, Australia.
Deregulated Interleukin-6 Signalling Contributes To Lung Tumourigenesis In Mice Induced By Cigarette Carcinogens.
A Miller (Presenting author)

2012: MIMR-WEHI Collaborative Seminar, Clayton, Australia.
Deregulated Interleukin-6 Signalling Contributes To Lung Tumourigenesis In Mice Induced By Cigarette Carcinogens
A Miller (Presenting author)

Poster Presentations

2012: ATS International Conference, San Francisco, USA.
Deregulated Interleukin-6 Signalling Suppresses Lung Tumorigenesis in Mice Induced By The Tobacco-specific Carcinogen Nicotine-derived Nitrosamine Ketone.
A Miller (presenting author), S Ruwanpura, L McLeod, P Bardin, DN Watkins,

2010: TSANZ Annual Scientific Meeting, Brisbane, Australia.
Deregulated Gp130 Signalling In Mice Leads To Emphysema Independent Of Inflammation.
A Miller (Presenting author), S Ruwanpura, L McLeod, J Jones, P Bardin, G Anderson, B Jenkins.
Winner "Best Overall Poster"

2010: VIIIN Student Symposium
Deregulated Gp130 Signalling In Mice Leads To Emphysema Independent Of Inflammation.
A Miller (Presenting author).

CHAPTER 1

Literature Review

1.1 Lung cancer

1.1.1 Epidemiology

Lung cancer is the leading cause of cancer death in Australia and worldwide. In 2007 there were 9703 new cases of lung cancer and 7626 deaths, which equates to nearly 20% of all cancer related mortality (AIHW, 2008). It is the fourth most commonly diagnosed cancer in both men and women, and while the age adjusted incidence is falling in men, between 1986 and 2007 the incidence in women rose by 72% reflecting changes in smoking behaviour. In Australia, the relative 5 year survival for people diagnosed with lung cancer is only 13%, with women doing considerably better than men (15% vs. 11%) (AIHW, 2011). Whilst there have been some gains in survival outcomes for patients, they remain relatively minor; when comparing lung cancers diagnosed between 2000 and 2007 to those diagnosed between 1982 and 1987, five year relative survival has only improved from 10 to 15% for males and 8 to 11% for females (AIHW, 2011).

Unfortunately the high mortality rate of lung cancer it is not a problem isolated to the Western world. For instance, lung cancer is now the leading cause of cancer death in men in developing world countries (including China and much of Africa (Lam et al., 2004)), and the third highest in women after breast and cervix. It has been estimated that 56% of the burden of disease from lung cancer occurs in the developing world (Jemal et al., 2011). As will be discussed later, a larger proportion of lung cancers in the developing world is a result of environmental exposures other than cigarette smoke, but as the tobacco market in the Western world contracts it would be expected that the burden of disease will increase before it begins to fall (Jemal et al., 2011, Slama, 2008, Chaturvedi et al., 2002).

Lung cancer is broadly divided into two sub-types: Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC). SCLC is an aggressive tumour of neuro-endocrine origin that is aetiologically very closely linked to cigarette smoking (D'Angelo and Pietanza, 2010, van Meerbeeck et al., 2011). NSCLC accounts for approximately 85% of all lung cancer diagnoses, and itself is further divided into three main groups according to histology: adenocarcinoma, Squamous Cell Carcinoma (SCC) and large cell carcinoma. NSCLC, and more particularly adenocarcinoma, will be the focus of this thesis. There are a number of

reasons to focus on adenocarcinoma: it is the most common form of lung cancer, it is the most frequent in non-smokers, and the most significant advances in therapy have been attained in sub-classes of adenocarcinoma (Li et al., 2011, Hickinson et al., 2010).

Over the last 30 years there has been a shift in the histological subtypes of NSCLC diagnosed. While SCC dominated the NSCLC disease burden in the 1970s and 1980s, adenocarcinoma is now the most frequently diagnosed lung cancer (Gabrielson, 2006, Alberg et al., 2007). Contrary to the general trend, and that seen in the other subtypes, the incidence of adenocarcinoma of the lung is at best stable or in some populations (particularly women and certain ethnicities) continues to increase (Jemal et al., 2011, Wahbah et al., 2007). It is postulated that the change seen is a result of changes in cigarettes: introduction of filters leading to a change in depth of inhalation, reduction in tar content, reduction in poly-aromatic hydrocarbons and increase in the known stimulants of adenocarcinomas, N-nitrosamines (Devesa et al., 2005, Gabrielson, 2006).

Like many other cancers, a significant proportion of people with advanced lung cancer develop cachexia as a complication of their disease: in fact, even at diagnosis around 60% of people with lung cancer will report loss of weight (Bruera, 1997). While a definitive definition of cachexia has remained elusive, it is generally accepted to be the loss of skeletal tissue, and to a lesser degree adipose tissue, that is somewhat resistant to correction with traditional measures (Fearon et al., 2011). Along with pain and breathlessness it contributes significantly to the burden of disease, increased complications and reduced survival (Bruera, 1997). Better treatments are desperately needed.

1.1.2 Risk factors

1.1.2 (i) Cigarette smoke

While the earliest suggestions of a link between cigarette smoke and lung cancer in the 20th century (Fritz Linkint in 1929) are tinged with the political fervour of the time (Proctor, 2001a), his conclusions have nonetheless been consistently reproduced. Richard Doll and colleagues first published an examination of smoking rates in subjects with lung cancer in 1950 (Doll and Hill, 1950), demonstrating that there was a steep increase in cancer rates in smokers, particularly heavy smokers. They subsequently published prospectively gathered data in the British Doctors Study in 1954 and 1956 and every 10 years hence (Doll and Hill, 1954, Doll and Hill, 1956, Doll et al., 2004) confirming this association. The Surgeon General of the United States of America also identified the link, first in 1957 and subsequently in "The 1964 Report on Smoking and Health" that led to changes in packaging and advertising standards for cigarettes. The risk of lung cancer is closely linked to both the duration of smoking and the age smoking commenced. As an example, a 65-year-old man

smoking one pack per day and quitting within 25 years has a <1% risk of developing lung cancer in the following 10 year period. This is contrasted by a 65-year-old continuing to smoke two packs per day for more than 50 years with a 10-year risk of 14% (Bach et al., 2003). Lifetime non-smokers at 75 years of age have a 0.2% cumulative risk of developing lung cancer, rising to 15.9% in light smokers and 24.4% in heavy smokers (Brennan et al., 2006). However, why only around one in six smokers will develop lung cancer (Proctor, 2001b) remains poorly understood, but points to other environmental and/or genetic factors at play.

Cigarette smoke is a complex mixture with both gaseous and particulate components. The gaseous phase, largely nitrogen, makes up more than 85% by weight of the mixture, with the particulate phase, also referred to as 'tar', making up the remainder (Borgerding and Klus, 2005). In addition to carcinogens, co-carcinogens and mutagens (Bhalla et al., 2009), cigarette smoke is known to both contain and induce free radicals and oxidant species (IARC, 2012, Churg et al., 2008), and induce airway inflammation in all smokers (Bhalla et al., 2009, Adcock et al., 2011). The induction of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is an essential component of the acute inflammatory response (Medzhitov, 2008). Equally, if unresolved, oxidative stress contributes to chronic inflammation and, amongst other problems, an increased risk of cancer (Maeda and Akaike, 1998). Persistent high levels of ROS and RNS leads to DNA damage that outstrips DNA repair mechanisms (Cook et al., 2004). In support of the pathogenic role of ROS/RNS in lung cancer, is the association between increasing levels of oxidative stress and reduced anti-oxidant levels with advancing lung cancer stage (Esme et al., 2008).

Of the 4000 chemicals and over 60 carcinogens present in commercial cigarettes (Hecht, 2003), the two main carcinogens are the polyaromatic hydrocarbons and the N-nitrosamines. The N-nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone (Nicotine-derived Nitrosamine Ketone - NNK), have the most potent carcinogenic effect, and preferentially induce adenocarcinoma (Hoffmann and Hecht, 1985, Hoffmann et al., 1996). They are formed in the curing and processing of the tobacco when alkaloid amines, including nicotine, are nitrosated. In addition, there is a suggestion that these nitrosamines can be formed endogenously in cigarette smokers through the action of inhaled NO_x species on nitrosatable amines found in both diet and cigarette smoke (Hoffmann and Hecht, 1985). Interestingly, there is *in vitro* evidence that endogenous nitrosamine production may be enhanced by asbestos particles (Hoffmann and Hecht, 1985), providing some explanation for the synergistic effect of cigarette smoke and asbestos on lung cancer risk (Case, 2006, Nelson and Kelsey, 2002).

NNK is activated *in vivo* by both reductive and oxidative metabolism to the same methylating intermediate, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Alpha-hydroxylation is perhaps the most prominent means of activation and is facilitated by microsomal cytochrome P450 (CYP450) enzymes (Proulx et al., 2005). The α-hydroxylase with most activity is CYP2A13 (He et al., 2004b), which is found specifically in the respiratory epithelium and less so in the liver (Su et al., 2000), explaining the exposure route independence and organ specificity of its carcinogenesis (Akopyan and Bonavida, 2006). *In vivo* studies have shown that pulmonary specific activation of NNK is critical for lung tumour development while liver activity of the same enzyme is important in the clearance of NNK (Weng et al., 2007). The importance of this enzyme has been further illustrated by the work of Wang et al. showing that a polymorphism within the CYP2A13 allele leading to a reduction in function was associated with a substantial reduction in risk of adenocarcinomas in smokers (Wang et al., 2003).

The metabolite produced by α-hydroxylation, NNAL, is a reliable measure of NNK exposure, and as further evidence of NNK's role in lung carcinogenesis has been proposed as a biomarker of lung cancer risk (Church et al., 2009). This relationship is further strengthened by the correlation of lung cancer risk in those exposed to second-hand smoke with increased NNAL levels (Hecht, 2004) as well as those smoking 'light' cigarettes (Hecht et al., 2005). Both Cigarette Smoke Condensate (CSC) and NNK lead to transformation of normal lung epithelial cells (Klein-Szanto et al., 1992), and the addition of CSC to NNK treatment of susceptible mice does not augment tumour formation (Finch et al., 1996), suggesting that NNK is both adequate to induce malignant change and the likely effector substance in cigarette smoke. NNK administration has been shown to lead to DNA adducts including O^6-methylguanine (O^6MG) and 7-methylguanine (Belinsky et al., 1986), resulting in miscoding and tumourigenesis (Pegg, 1983). In animal models, DNA adducts occur rapidly, peaking at four hours following administration (Peterson and Hecht, 1991): levels of O^6MG correlate with tumour number (Belinsky et al., 1990) and their persistence determines the likelihood of tumour formation. Whilst the genetics of lung cancer will be discussed in more detail below, it is clear that the effect of the alkylating intermediates is DNA sequence-specific (Sendowski and Rajewsky, 1991), with increased levels of O^6MG leading to high rates of oncogenic base transition within codon 12 of the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene (Peterson and Hecht, 1991), which goes some way to explain the mutagenicity of the change. Additionally, there appears to be a high frequency of mutations within the tumour suppressor gene Tumour Protein 53 (*p53*/*TP53*) resulting in gene silencing (Cloutier et al., 2001). Interestingly, it has been suggested that repair of these DNA adducts is impaired by cigarette smoke by reducing the ability of cells to transfer the methyl group to an acceptor

protein (Cao et al., 1985). An alternate method of tumour suppressor gene silencing is through epigenetic events, particularly DNA methylation of the promoter sequences (Jones and Laird, 1999). NNK is known to lead to DNA methylation (Akopyan and Bonavida, 2006), at least in part by increasing accumulation of cytosine-DNA methyltransferase 1 (DNMT1) and this may contribute to its role as a carcinogen (Lin et al., 2010). The interest around methylation has been on two fronts: firstly, DNA methylation status in sputum has been proposed as an early biomarker of lung cancer, and secondly, there is a possibility of reversing the methylation with demethylating agents, providing another avenue of potential treatment (Risch and Plass, 2008). DNMT1 accumulation within lung cancers is strongly associated with smoking and correlates with poor prognosis (Lin et al., 2010), and a panel of DNA methylation markers has been associated with recurrence in early stage disease (Brock et al., 2008), suggesting methylation is both an early event and is important in malignant progression. This idea was examined *in vivo* by Belinsky et al., who showed that both genetically reducing DNMT1 and treating WT mice with a demethylating agent, substantially reduced the occurrence of NNK induced lung tumours (Belinsky et al., 2003).

Once activated, NNK can also alter regulation of a number of genes involved in cell cycle progression, proliferation and apoptosis (Figure 1.1). The Phosphoinositide 3-kinase/v-akt murine thymoma viral oncogene homolog 1 (PI3K/Akt) pathway is dysregulated in a number of human cancers including NSCLC (Vivanco and Sawyers, 2002) (Massion et al., 2002), and is likely to be an early initiating event (Tsao et al., 2003, Brognard et al., 2001). Both nicotine and NNK have been shown to increase activation of Akt by direct stimulation of both Acetylcholine (AchR) and β-adrenergic receptors (Schuller et al., 1999), leading to increased cellular proliferation and reduced apoptosis (Tsurutani et al., 2005, West et al., 2004a). B-cell CLL/lymphoma (Bcl)-2 and v-myc myelocytomatosis viral oncogene homolog (c-Myc), which are known to act co-operatively to enhance tumourigenesis (Reed et al., 1990), are both activated via NNK-induced phosphorylation, leading to both enhanced proliferation and cell survival (Jin et al., 2004). Cyclin D1, which is over-expressed and thought to play a key role in development of NSCLC (Ratschiller et al., 2003, Gautschi et al., 2007), is highly upregulated by NNK (Ho et al., 2005), again through activation of the AchR.

There is considerable evidence that inflammation plays a significant role in the development of NNK-induced tumours. Many authors have shown a reduction in tumour number with the use of both non-steroidal anti-inflammatory drugs (NSAIDs) and other anti-oxidants (Jalbert and Castonguay, 1992, Castonguay et al., 1998, Rioux and Castonguay, 1998, Bauer et al., 2004, Witschi et al., 1998). Interestingly, the aforementioned findings and other work (Barbie et al., 2009) suggests a crucial role of Nuclear Factor-Kappa Beta (NF-κB) in the activation of

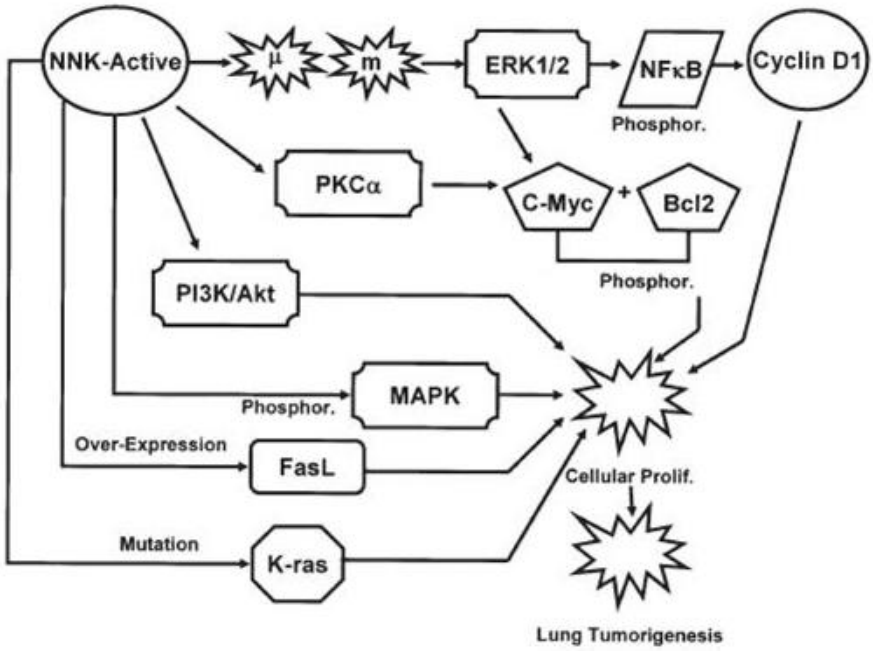


Figure 1.1: Signalling pathways affected by NNK. (From Akopyan, 2006).
In its active form NNK leads to signalling through a number of pathways involved in cellular proliferation and anti-apoptosis, leading ultimately to tumourigenesis.

these NNK-associated pathways, providing further evidence of the importance of inflammation which will be discussed in more detail below.

1.1.2 (ii) Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is characterised by progressive and irreversible loss of lung function, associated with airflow limitation and persistent pulmonary (and systemic) inflammation (Vestbo et al., 2012). In 1990, COPD was the 6th leading cause of death worldwide and ranked 12th in terms of Disability-Adjusted Life Years, with a prediction that by 2020 this will rise to 3rd and 5th respectively (Murray and Lopez, 1997). COPD is a multi-componential condition, consisting of obstruction of the large and small airways and destruction of the alveolar walls and enlargement of the air-spaces, or emphysema. Like lung cancer, cigarette smoking is the major risk factor for COPD; while cigarette smoke will cause physiological changes in the lungs of all exposed (Niewoehner et al., 1974), only 10-20% will develop clinical chronic lung disease (Young and Hopkins, 2010). This clearly points to genetic and environmental factors at play (Cho et al., 2010, Yang and Francis, 2009, Cohen et al., 1977). Given the major shared risk factor it is not surprising to see an increased risk of lung cancer associated with airflow limitation (Skillrud et al., 1986, Lange et al., 1990, Congleton and Muers, 1995, Young et al., 2009), but an understanding of mechanisms beyond this has remained elusive. More recent evidence suggests that subjects with emphysema are at an increased risk of lung cancer independent of both airflow obstruction and pack year smoking history (de Torres et al., 2007, Wilson et al., 2008), further suggesting a more complex relationship. Recent genome-wide studies have shown that COPD and lung cancer share a number of genetic risk factors (reviewed in (Young and Hopkins, 2011)) strengthening the proposed relationship. Many of the proposed genes relate to regulation of inflammatory pathways, and given both emphysema and lung cancer have prominent pulmonary inflammation this has been suggested as a mechanistic link between the two diseases (Barnes, 2004, Coussens and Werb, 2002, Lee et al., 2009), albeit unsubstantiated in the available literature to date.

In an inducible activated *Kras* model of adenocarcinoma, *LoxP*-STOP-*LoxP*Kras^{G12D} (*LSL*Kras^{G12D}), COPD-like inflammation induced by inhalation of Non-Typeable Haemophilus influenzae (NTHi), has been shown to increase the development of lung tumours (Moghaddam et al., 2009). Similarly, in two separate carcinogen-induced models, namely injection of Butylated Hydroxytoluene (BHT) and NNK, tumours were only induced in the presence of pulmonary inflammation, and reduced with administration of the NSAID

sulindac (Malkinson et al., 2000). Two alternative hypotheses have been proposed to link these functionally different diseases (Houghton et al., 2008):

1) Exposure to smoke or other environmental irritants leads to the recruitment and activation of inflammatory cells (e.g., neutrophils), which then release proteinases and ROS that degrade alveoli, leading to emphysema. The subsequent release of cytokines and other factors from inflammatory cells then facilitates tumour initiation and growth.

2) Lung progenitor cells, namely Bronchoalveolar Stem Cells (BASCs), attempt to replace damaged alveolar cells and maintain alveolar integrity caused by emphysema. However, repeated induction of BASC proliferation in the context of chronic inflammation and carcinogens predisposes these cells to become malignant.

While these hypotheses remain largely unproven, inflammation is inherent in both and therefore warrants further scrutiny.

1.1.3 Inflammation

Lung cancer is among a growing number of cancers linked to chronic inflammation (Coussens and Werb, 2002, Engels, 2008, Franks and Slansky, 2012), and in fact leukocyte infiltration is now considered a hallmark of malignancy (Colotta et al., 2009). Inflammation represents the body's first line of defence to exogenous insults, and the lung's vast and open interaction with the environment makes control of this inflammation all important. Acute inflammation is self-limiting and an essential homeostatic function (Medzhitov, 2008), whereas dysregulated and chronic inflammation is associated with conditions as diverse as obesity and diabetes (Bastard et al., 2006), anaemia (Ferrucci et al., 2010), and heart disease and stroke (Kaptoge et al., 2010). In addition to cigarette smoke, other generic inflammatory insults including domestic cooking smoke exposure (Lam et al., 2004), and infections by human papilloma virus and tuberculosis have been associated with an increased lung cancer risk (Zheng et al., 1987, Chen et al., 2004). In further support for a role for the deregulated immune system in promoting lung cancer, the rates of many cancers, including lung cancer, are increased in those individuals with chronic auto-immune inflammatory conditions including Systemic Sclerosis and Rheumatoid Arthritis (Adzic et al., 2008).

Macrophages

Macrophages are phagocytic cells that contribute to tissue homeostasis (Geissmann et al., 2010). They are divided into subsets depending on whether they are circulating or resident within tissue in the unstimulated state (Geissmann et al., 2010). Macrophages can be further divided into M1 and M2 by their method of activation (Misharin et al., 2011): the classical pathway leading to a robust T_H1 response (M1) defined by the production of Interferon (IFN)-

γ and to a lesser extent Tumour Necrosis Factor (TNF)-α, or an alternative pathway (M2) leading to a T_H2 response involving a lower production of the T_H1-type inflammatory cytokines and conversely high levels of the anti-inflammatory cytokine interleukin (IL)-10, as well as IL-4 and IL-5 (Mosser, 2003, Mills et al., 2000). Within the lung macrophages perform a crucial function, processing the multitude of inhaled microorganisms and particles, without unduly activating either the innate or adaptive immune systems (Holt, 1978).

In NSCLC there is a marked increase in macrophages, with a predominance of Tumour-Associated Macrophages (TAMs) (Duluc et al., 2007). These are largely polarised M2 macrophages with immunosuppressive properties, although populations of anti-tumour M1 TAMs have been described (Ma et al., 2010). They develop under the influence of the tumour micro-environment, with contributions from tumour cells, resident macrophages and other inflammatory cells, particularly regulatory T cells (Hagemann et al., 2006, Mantovani et al., 2008). Macrophages are recruited and activated to their M2 state under the influence of a variety of chemokines and inflammatory mediators, and numbers are maintained by ongoing monocyte recruitment rather than simply retention and prolonged survival (Mantovani et al., 1992). TAM-derived IL-10, Colony Stimulating Factor (CSF)-1, Transforming Growth Factor (TGF)-β (Mantovani et al., 2008) and Vascular Endothelial Growth Factor (VEGF) all contribute to this process, providing a self-sustaining system (Burke et al., 2003). In fact, in a lung tumour xenograft model, macrophage recruitment and tumour growth were significantly reduced in CSF-1-deficient mice (Nowicki et al., 1996). IL-10 is also secreted by various tumour cells including NSCLC cell lines (Gastl et al., 1993, Huang et al., 1995), and monocyte/macrophage production of IL-10 is further augmented by tumour cell production of Prostaglandin E2 (PGE2) (Hidalgo et al., 2002, Huang et al., 1996) and Cyclooxygenase 2 (COX-2) (Patel et al., 2012). The importance of IL-10 in the development and progression of lung cancer is evidenced by its association with late stage disease (Wang et al., 2011b), metastasis (Zeng et al., 2010) and poor patient survival (Wang et al., 2012, Zeni et al., 2007, Neuner et al., 2001). Other tumour-derived factors, including IL-6 and Leukaemia Inhibitory Factor (LIF) (Duluc et al., 2007), contribute to the predominance of the M2 phenotype by enhancing the effect of CSF-1.

In addition to the cytokines and growth factors mentioned above there are a number of other influences on the tumour micro-environment and macrophage behaviour, including transcription factors and chemokines. Forkhead Box m1 (Foxm1) is a transcriptional regulator and is expressed in proliferating cells including epithelial cells and macrophages (Korver et al., 1997, Yao et al., 1997). It has been shown to be highly expressed in numerous cancers including lung cancer (reviewed in (Myatt and Lam, 2007)) where it is associated

with a poor prognosis (Balli et al., 2011). It is important in lung tumour initiation (Kim et al., 2006, Wang et al., 2009), at least partly due to its role in monocyte migration to the tumour. *In vivo*, a mouse line with a conditional knockout of Foxm1 within macrophages showed not only a reduction in tumour size but also a reduction in TAMs following exposure to the chemical carcinogen BHT (Balli et al., 2011), suggesting a role for Foxm1 in macrophage trafficking to tumours and further reinforcing the importance of TAMS in tumour growth. Many chemokines, but particularly Chemokine (C-C motif) Ligand (CCL)-2 and CCL-5, produced by both tumour cells and resident lymphocytes, also contribute to macrophage recruitment (Murdoch et al., 2004). CCL-2 is highly expressed on lung tumour cells (Mantovani et al., 2004a) and is known to strongly recruit TAMs to the site of tumour formation (Ueno et al., 2000, Arenberg et al., 2000). The final stimulus for TAM accumulation is tissue damage, particularly in response to hypoxia (Lal et al., 2001), which in the context of lung cancer is again likely to occur through upregulation of VEGF (Kim et al., 2001).

Once *in situ*, TAMs produce a number of positive drivers of tumour growth and survival. In addition to enabling immune escape by the tumour (Huang et al., 1996), TAMs are known to enhance angiogenesis and lymphangiogenesis (Schoppmann et al., 2002), promote tumour growth and lead to tissue remodelling. Angiogenesis is promoted both through VEGF-dependent (Murdoch et al., 2004, Kimura et al., 2007) and -independent means (Sierra et al., 2008, Shojaei et al., 2007) leading to an increase in new vessel growth which is further enhanced by the degradation and remodelling of extracellular matrix proteins by matrix metalloproteinases (MMPs) (Kimura et al., 2007), amongst other proteinases. TAMs express a number of MMPs, including MMP-9 and MMP-12 (Qu et al., 2009a), augmenting the inflammatory environment and enabling increased tumour growth (Woessner, 1991) and metastasis (Westermarck and Kahari, 1999) by disruption of basement membranes. The importance of the MMPs is highlighted by the association of increased MMP-9 expression with metastasis (Liu et al., 2010) and reduced survival (Sienel et al., 2003) in human NSCLC.

Neutrophils

Neutrophils are a crucial part of the innate immune system (Mantovani et al., 2011) and have an important role in both the activation of the immune response and clearance of pathogens. There are a large number of neutrophils within the lung, although these are largely marginated within the vascular compartment, with only around 4% being within lung tissue (Gee and Albertine, 1993). In the event of an acute insult, interaction with constitutively and inducibly expressed molecules on the endothelial surface (selectins), retards the migration of circulating

neutrophils allowing chemokines and interleukins to activate the neutrophil, leading to emigration to the site of inflammation (Springer, 1995). Recruitment of neutrophils to tumours occurs in the same way, and is upregulated by pro-inflammatory mediators including TNF-α, IL-1β (Di Carlo et al., 2001) and Chemokine (C-X-C motif) Ligand (CXCL)-2 (Tazzyman et al., 2011). Similar to macrophages, there is also evidence for a role of IL-10 in promoting neutrophil accumulation within tumours (Di Carlo et al., 1998). Despite clear evidence of the importance of neutrophils in the normal inflammatory response of the lung and the demonstrated presence of neutrophils within tumours, their role in lung cancer remains unclear.

In vitro, when exposed to neutrophil conditioned media, human non-small cell carcinoma cells (of the adenocarcinoma type), displayed somewhat contradictory suppression of proliferation and IL-8 release, but an increase in invasiveness (Zelvyte et al., 2004). Pekarek et al. (Pekarek et al., 1995) showed that while growth of xenografts of UV-induced tumours required T-cell depletion, elimination of neutrophils *in vivo* actually lead to a reduction in tumour growth. Others (Tazzyman et al., 2011) have shown the same effect *in vitro* and *in vivo* using A549 cells, a human lung adenocarcinoma cell line. The findings of Houghton et al. (Houghton et al., 2010) have further strengthened the relationship by showing that tumour load was markedly reduced in the *LSL*Kras^{G12D} model of lung cancer in mice lacking the gene for neutrophil elastase. However, contrary to this is the suggestion that neutrophils play a role in anti-tumour immunity (Midorikawa et al., 1990, Di Carlo et al., 2001, Matsumoto et al., 1991), although the ability of circulating neutrophils to produce superoxide anion, which has been proposed to be involved in tumour lysis (Hafeman and Lucas, 1979), is substantially reduced in subjects with lung cancer (Hara et al., 1992).

Clinical evidence points toward a pro-tumour effect of neutrophils, with a number of studies suggesting peripheral blood (Watine and Charet, 1998) and sputum (Carpagnano et al., 2011) neutrophil levels have prognostic significance in NSCLC. Using IL-8 and Leukotriene B-4 (LTB-4) in exhaled breath condensate as a measure of sputum neutrophils, Carpagnano et al. showed a positive correlation between rising neutrophil numbers and increasing NSCLC stage (Carpagnano et al., 2011). Others have shown similar results by direct counting of peripheral blood neutrophil numbers. A rising pre-operative absolute number (Teramukai et al., 2009) or ratio of neutrophils to lymphocytes is associated with increased disease stage, and also predicts poor post-operative survival, including in early stage disease (Sarraf et al., 2009). While these studies did not examine the role of smoking on the inflammatory state, Tanaka et al. showed that increased blood neutrophils remained a significant determinant of poor prognosis in never smokers (Tanaka et al., 2011).

T cells

Derived from a common progenitor and developing in the thymus, T cells are divided into $CD8^+$ Cytotoxic T Lymphocytes (CTL) and $CD4^+$ T Helper (T_H) cells. The classical division of T_H cells according to their expression of TNF-α and IFN-γ (T_H1) or IL-4, IL-5 and IL-13 (T_H2) has been added to by the description of IL-17-expressing T_H cells (T_H17) (Miossec et al., 2009) and $CD4^+CD25^+Foxp3^+$ T regulatory cells (T_{reg})(Su et al., 2007), amongst others (Wilson et al., 2009). Lung tumours are infiltrated with both CTLs (Mami-Chouaib et al., 2002) and T_H cells (Ishibashi et al., 2006), suggesting the possibility of both a suppressive and promoting effect.

CTLs are postulated to have an anti-tumour effect as evidenced by an increased rate of solid tumours, including lung cancer, in those patients with impaired T cell immunity such as post organ transplantation (Herrero et al., 2005, Pham et al., 1995) and in HIV/AIDS (Hakimian et al., 2007, Shiels et al., 2010). This finding is supported by the increased incidence of spontaneous lung adenocarcinomas in mice lacking perforin (Street et al., 2002), a crucial component of the lymphocyte-dependent killing mechanism (Russell and Ley, 2002). While a number of groups have reported the discovery of populations of CTLs recognising lung tumour-specific antigens (Mami-Chouaib et al., 2002, So et al., 2005), suggesting a degree of immunogenicity, complete immune clearance is rare (Zou, 2005). In the absence of a T cell defect this would suggest interference in the presentation of this antigen or a tolerising effect. Certainly, it appears there is a proportional increase in T_{reg} cells within the tumour microenvironment (Zou, 2005), both in human tumours (Ishibashi et al., 2006) and mouse models (Sakaguchi et al., 2001), pushing the balance away from effective T cell activation. T_{reg} cells, which are double positive for surface markers CD4 and CD25 (Hori et al., 2003b), and uniquely the transcription factor Foxp3 (Hori et al., 2003a), act to suppress the immune activation of other cells (Zou, 2005). Whilst generally less numerous than TAMs in the tumour microenvironment, T_{reg} cells are also implicated in suppressing immune reaction to lung tumour antigens (Ishibashi et al., 2006) and allowing immune evasion. Interestingly, T_{reg} cells are shown to be elevated in Bronchoalveolar Lavage Fluid (BALF) of both smokers and subjects with COPD (Roos-Engstrand et al., 2009), adding further weight to the link between COPD and the development of lung cancer. Increased proportions of T_{reg} cells have also been identified in NSCLC samples compared with adjacent normal lung (Ishibashi et al., 2006) and populations of T_{reg} cells in draining lymph nodes have been correlated with progression in NSCLC (Su et al., 2007). T_{reg} cells are closely associated with tumour development in both carcinogen (NNK) and genetically driven (KrasLA2 (Johnson et al., 2001)) mouse models of lung cancer (Granville et al., 2009). Granville et al. showed that T_{reg} cell numbers are increased in mouse lungs in response to NNK treatment prior to tumour development, and

depleting T_{reg} cells with the chemotherapeutic agent rapamycin reduced lung tumours (Granville et al., 2009). To clarify the importance of T_{reg} cells they also showed that genetically depleting T_{reg} cells in mice bearing a genetically-activated oncogenic *Kras*, resulted in a significant reduction in lung tumour number.

B cells

Like those seen in COPD (Curtis et al., 2007, van der Strate et al., 2006), lymphoid follicles containing B cells have been identified in human NSCLC (Gottlin et al., 2011) although the role of B cells is unclear. In support of an anti-tumour role, studies have shown an association between infiltrating B cells and improvement in patient survival in NSCLC (Al-Shibli et al., 2008, Riemann et al., 1997). Further strengthening this idea, a number of studies examining B cell populations in human tumours show the presence of specific anti-tumour antibodies (Ichiki et al., 2004, Yasuda et al., 2006), and a reduction in tumour growth in response to auto-antibodies in a xenograft model of human lung cancer (Mizukami et al., 2006). In contrast, others have shown a reduction in tumour load in murine cancer models with antibody-derived B cell depletion (Kim et al., 2008), and an increase in haematological metastasis in patients with high expression of the B cell marker CD24 (Kristiansen et al., 2003). Additionally, there appears to be a reduction in circulating B cell numbers (Wesselius et al., 1987) and a reduced ability to produce antibodies in response to mitogen challenge (Venkataraman et al., 1989, Venkataraman et al., 1985) in B cells from patients with NSCLC.

1.1.4 Cytokines

A number of inflammatory cytokines and chemokines have also been implicated in lung carcinogenesis. IL-1β is linked to the development of a number of cancers (Li et al., 1992, Yoshida et al., 2002), including lung cancer (De Vita et al., 1998), and polymorphisms of the IL-1β gene are both associated with an increased risk of lung cancer (Engels et al., 2007, Landvik et al., 2009) and correlate with poor patient survival (Jatoi et al., 2009). IL-1β is known to activate COX-2 (Dinarello, 2002), which is responsible in part for the production of PGE2 and nitric oxide (Ermert et al., 2003), and are associated in the pathogenesis of lung cancer (Brown and DuBois, 2004), with higher expression correlating with poor prognosis (Dohadwala et al., 2006).

TNF-α release is strongly stimulated by cigarette smoke both *in vitro* (Ryder et al., 2002) and *in vivo* (Churg et al., 2002). It is released from macrophages, T cells and epithelial cells in response to a variety of stimuli, and, amongst other mechanisms, amplifies the inflammatory response by inducing IL-6 production and neutrophil attraction and activation (Barnes, 2004). Like IL-1β, serum levels of TNF-α have been correlated with disease stage (De Vita et al.,

1998) and is elevated in the BALF of subjects with NSCLC (Chyczewska et al., 1997). Higher levels are also associated with poor prognosis (Mostertz et al.). Adding weight to the importance of TNF-α, there appears to be a significant association between specific genetic polymorphisms leading to increased TNF-α levels and increased risk of lung cancer (Shih et al., 2006, Van Dyke et al., 2009). However, how TNF-α may be exerting its effect is unclear. While TNF-α acting alone leads to programmed cell death (Dinarello, 2000), it also activates NF-κB which counteracts this effect and promotes cell survival (Wang et al., 1998, Wu et al., 1998), further underlining the complexity of the interplay of inflammatory mediators. Berman et al. illustrated this point well by showing that blocking NF-κB with a selective COX-2 inhibitor (sulindac) enhanced TNF-α induced apoptosis of NSCLC cell lines *in vitro* (Berman et al., 2002). Contrary to the clinical data, a number of groups have shown that TNF-α release from alveolar macrophages and TNF-α-induced cytotoxicity is reduced by NNK (Proulx et al., 2007, Rioux and Castonguay, 2001), suggesting an anti-tumour effect of TNF-α. This is corroborated by the association between elevated TNF-α and reduction of the lung proto-oncogene thyroid transcription factor (TTF)-1 (Boggaram, 2009).

IL-17 has been associated with generic pulmonary inflammation (Ivanov et al., 2007, Park et al., 2005), as well as in the pathogenesis of emphysema (Harrison et al., 2008), at least in a smoke-induced mouse model. Unlike other tumours (Kryczek et al., 2009), it also appears to have a stimulatory effect on the development and progression of lung cancer. Levels of IL-17 in NSCLC correlate positively with the degree of neo-angiogenesis and tumour growth (Numasaki et al., 2005), lymphangiogenesis and decreased survival (Chen et al., 2009), suggesting perhaps a role in metastasis.

Due to its known immunomodulatory effect, IFN-γ has been suggested to have anti-tumour effects with therapeutic potential. Pre-treating A549 (human adenocarcinoma) cells with IFN-γ reduced IL-1β-induced IL-8 release (Boost et al., 2008), a chemokine produced by NSCLC cells (Zhang et al., 2012b) and a promoter of angiogenesis (Boldrini et al., 2005). Mice with both a single IFN-γ knock-out (Street et al., 2002) and a triple knock-out (IFN-γ, IL-3 and GM-CSF) develop tumours with high frequency in the context of significant pulmonary inflammation (Dougan et al., 2011). In further support of a role of IFN-γ and looking towards a therapeutic intervention, Wilderman et al. showed that IFN-γ gene-therapy substantially reduced lung tumours and increased survival in a *LSL*$Kras^{G12D}$ model of lung adenocarcinoma (Wilderman et al., 2005).

IL-6 also has a suggested role both in the development of lung cancer (Dowlati et al., 1999, Haura et al., 2006) and its progression and complications (Bayliss et al., 2011). This will be discussed in more detail in a later section.

1.1.5 Genetics

To address the short-coming in our current understanding of the molecular pathogenesis of lung cancer, gene expression profiling studies in human airway epithelial cells have identified a complex array of genes of diverse molecular function; these include inflammation, oxidant stress and glutathione metabolism, as well as oncogenes and tumour suppressors, whose expression is altered by cigarette smoking (Spira et al., 2004). Interestingly, many of the tumour suppressors and oncogenes within airway epithelial cells obtained at bronchoscopy failed to return to normal expression levels two years after smoking cessation. Further work identified an expression signature of approximately 80 genes implicated in inflammation, cell cycle progression, antioxidant defence, ubiquitination and DNA repair, has been assigned as a putative biomarker for lung cancer (Spira et al., 2007). These and other large scale genome-wide analytical approaches have also revealed numerous chromosomal abnormalities present in human lung adenocarcinomas (Figure 1.2) (Ding et al., 2008), only some of which are linked to known genetic mutations in lung cancer (Weir et al., 2007). More recently, using both whole genome and whole exome sequencing of paired tumour and normal tissue samples, Imielinski et al. have more extensively annotated somatic mutations present in human lung adenocarcinoma (Imielinski et al., 2012). While mutational frequencies in the most commonly mutated genes (including *TP53, KRAS, EGFR* and *STK11)* were similar to previously reported, the large number of samples and superior technology used allowed both a clarification of driver status in previously reported genes and the identification of new putative driver mutations. Thus, while such studies have started to unravel the complex genetic and epigenetic processes which contribute to the initiation and progression of lung cancer, they also highlight that many genes involved in lung carcinogenesis remain to be discovered.

Kras

One of the most extensively studied genes implicated in lung carcinogenesis is the K-ras proto-oncogene which has also been linked with inflammation. K-ras, part of the ras family of membrane bound GTPases, has low intrinsic activity and is involved in the control of cellular growth, differentiation and apoptosis (Campbell et al., 1998). Oncogenic *KRAS* has been identified in around 30% of human lung cancers (Anderson et al., 1991), particularly adenocarcinoma, and is strongly associated with cigarette smoking (Ahrendt et al., 2001): *K-ras* is activated by cigarette carcinogens including nitrosamines (Lantuejoul et al., 2009) and promotes an intense inflammatory infiltrate (Ji et al., 2006). Furthermore, recent studies have shown another link between *kras* and inflammation with exacerbation of lung tumour development in mice with an activating *kras* mutation and co-existent pulmonary

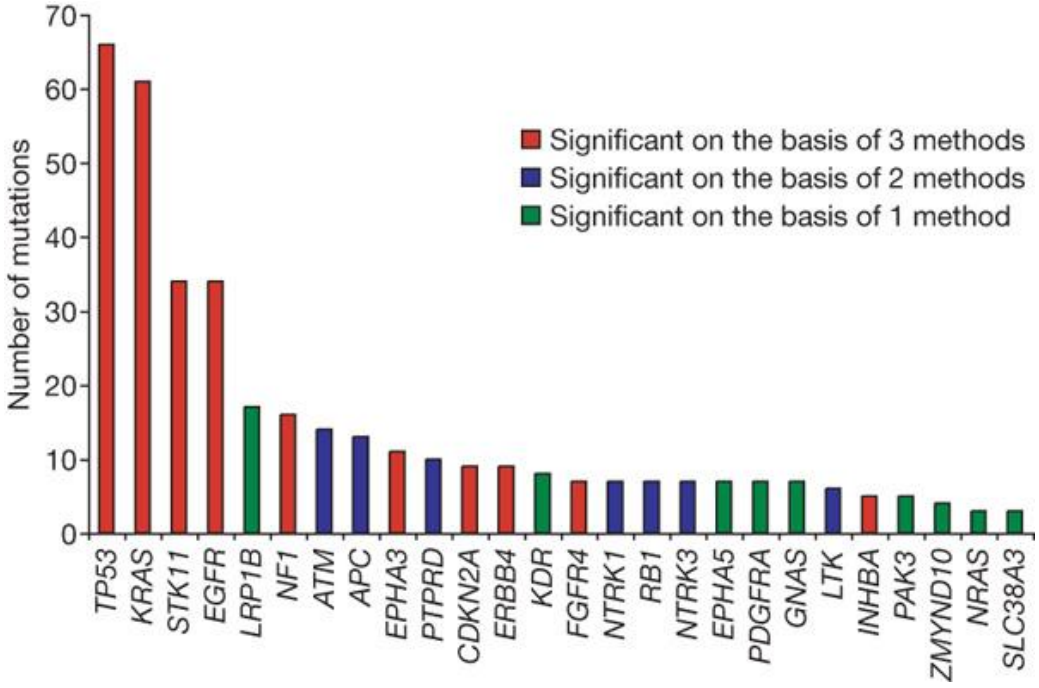


Figure 1.2. Human lung adenocarcinoma mutations. From Ding et al., 2008. Occurrence of significantly mutated genes in adenocarcinoma of the lung.

inflammation (Moghaddam et al., 2009). An activating mutation in *kras* has been shown to be an early initiating event in murine models of lung cancer (Johnson et al., 2001) and is present in up to 80% of spontaneous and all experimentally-induced murine lung cancers (Wakamatsu et al., 2007). Confirming the importance of K-ras, Fisher et al. (Fisher et al., 2001) and others (Tran et al., 2008) have shown that NSCLC has an "...oncogene-addiction", with tumours regressing once *kras* activation is turned off, even in the presence of silenced p53 or activated Myc. The most common mutation seen is a point mutation in codon 12 (Rodenhuis and Slebos, 1990), frequently a change from glycine to aspartic acid, therefore *kras* G12D. These point mutations result in a conformational change leading to constitutive transduction of a growth promoting signal (Tong et al., 1989). Downstream mediators of *kras* signalling include Extracellular-Regulated Kinases (ERK)1/2, PI3K (Engelman et al., 2008b) and interestingly also Signal Transducer and Activator of Transcription (STAT)3 (Gough et al., 2009, Li et al., 2007b).

EGFR

The Epidermal Growth Factor Receptor (EGFR), also known as HER1 and ErbB1, is one of a larger group of glycoprotein trans-membrane receptor tyrosine kinases (ErbB1-4). It is involved in cell cycle progression and proliferation and has been associated with innate immune responses (Burgel and Nadel, 2008). In addition to EGF, EGFR is activated by other members of the EGF family including TGF-α, amphiregulin and epiregulin (Linggi and Carpenter, 2006). Ligand binding leads to homo-dimerisation, and, as the intra-cellular domain has intrinsic kinase activity (Cohen, 1983), subsequent autophosphorylation of the cytoplasmic tyrosine residues. Down-stream mediators of EGFR activation include K-ras, Mitogen Activated Protein Kinases (MAPK), PI3K/Akt, and STAT3 (Akca et al., 2006, Ganti).

EGFR displays activating mutations in NSCLC: between 10% and 40% in adenocarcinoma (Engelman and Janne, 2008) but infrequently in other NSCLC or SCLC (Paez et al., 2004, Marchetti et al., 2005). These mutations consist of amino acid substitutions and deletions largely located within the kinase domain in exons 18, 19 and 21 (Lynch et al., 2004) and are predicted to enhance activation of downstream signalling cascades. Interestingly, mutations of the EGFR appear to be mutually exclusive of mutations of K-ras in lung adenocarcinoma (Yoshida et al., 2005, Marchetti et al., 2005). There appears to be an epidemiological phenotype of subjects with EGFR mutations. Mutation are far more common in subjects from East Asia (Paez et al., 2004, Engelman and Janne, 2008), in women (Shigematsu et al., 2005) and in never smokers (Pao et al., 2004), and predicts response to Tyrosine Kinase Inhibitors (TKIs) (Lynch et al., 2004, Paez et al., 2004).

Akca et al. showed that cells transfected with a mutant EGFR had prolonged survival in a serum free environment by increased activation of both Akt and STAT3 (Akca et al., 2006). The importance of STAT3 in the oncogenic signalling has been confirmed by others. Human lung adenocarcinoma cell lines expressing a mutant EGFR grow more slowly and undergo apoptosis when STAT3 is knocked down with a short hairpin (sh)-RNA (Alvarez et al., 2006) or anti-sense oligonucleotides (Haura et al., 2005b), but TKIs targeting the EGFR do little to reduce activation of STAT3 (Haura et al., 2005b). Shedding more light on the complex signalling pathways downstream of mutant EGFR, Gao et al. (Gao et al., 2007) confirmed the correlation between activated STAT3 and EGFR mutations in human adenocarcinoma, and show that in fact this increased STAT3 activation is through upregulated IL-6 expression.

STAT3

STAT3 is a known oncogene and has been shown to be upregulated in a variety of haematological and epithelial cancers (Gong et al., 2005, Bromberg, 2002), including persistent activation in up to 50% of human lung adenocarcinomas (Gao et al., 2007). STAT3 itself, and a subset of target genes, have been shown to be upregulated in COPD and lung cancer tissue regardless of smoking history (Qu et al., 2009b), suggesting that increased STAT3 activity, at least in part, may play a role in the COPD/emphysema to adenocarcinoma transition. Furthermore, STAT3 is involved in cell proliferation and survival, exerting this effect by regulation of genes in the Bcl-2 family, along with Cyclin D1 and VEGF (Yeh et al., 2006), which are frequently over-expressed in human lung cancer (Han et al., 2001, Gautschi et al., 2007). Tumour progression is further enhanced by promotion of tumour angiogenesis and suppression of anti-tumour immunity (Yu and Jove, 2004), by both reducing inflammatory mediators and maturation of effector cells (Wang et al., 2004). The importance of STAT3 in lung cancer is further supported by the known requirement of STAT3 for ras-mediated oncogenesis in lung cancer cells (Tran et al., 2008, Yeh et al., 2009) and the observation that dephosphorylation of STAT3 is associated with the regression of tumours in a *k-ras*-related murine lung adenocarcinoma model (Tran et al., 2008). Elevated STAT3 activity is a critical downstream regulator of tumour cell survival (Alvarez et al., 2006, Haura et al., 2005b) triggered by EGFR mutants in a subset of lung adenocarcinomas (predominantly never smokers) (Lynch et al., 2004, Pao et al., 2004). More recently, in these tumours, it has been proposed that increased activation of STAT3 is mediated by up-regulated expression (in an EGFR-dependent manner) of IL-6 (Gao et al., 2007).

Consistent with STAT3s known pro-inflammatory activity, over-expression of a hyper-activated variant of STAT3, STAT3-C, in pulmonary epithelium leads to inflammation and adenocarcinoma formation (Qu et al., 2009b, Li et al., 2007b). Using an MMP-12 over-

expression model, Qu et al. showed that both IL-6 levels in BALF and activation of STAT3 in epithelial cells increased with progressive transition from emphysema to adenocarcinoma in the presence of significant pulmonary inflammation (Qu et al., 2009a), thereby strengthening the link between IL-6/STAT3 signalling, inflammation and lung adenocarcinoma.

PI3K/Akt

The Phosphoinositide 3-Kinases are a family of lipid kinases capable of phosphorylating the D-3 position of inositide rings (Whitman et al., 1988). There are a number of isoforms but only the 1A isoform has been associated with cancer (Yuan and Cantley, 2008), so all discussion of PI3K will refer to this isoform. The PI3K pathway is involved in cellular metabolism, survival, proliferation and migration (Cantley, 2002). It is activated downstream of Receptor Tyrosine Kinases (RTKs) (Engelman et al., 2006), signalling via the production of phosphatidyl-inositol-3,4,5-triphosphate (PIP_3) leading to recruitment of Akt (Cantley, 2002). PI3K can also be activated directly by cytokine receptors such as glycoprotein (gp)130 (Johnson et al., 2012), through the interaction with Growth factor Receptor Bound protein(GRB)2-associated protein (Gab)1 (Takahashi-Tezuka et al., 1998). PTEN (Phosphatase and tensin homolog deleted on chromosome ten) is a tumour suppressor that is the primary brake on PI3K/Akt signalling (Chu and Tarnawski, 2004) by dephosphorylating PIP_3. While there are multiple points in the PI3K/Akt signalling cascade that have been implicated in other cancers, PTEN is the only one identified in NSCLC (Vivanco and Sawyers, 2002). Its importance is highlighted by the frequent loss of PTEN protein in NSCLC (Noro et al., 2007, Soria et al., 2002), which may be by homozygous deletion of the gene, loss of heterozygosity or hypermethylation of the promoter, and the association of TKI resistance in lung cancer with PTEN loss (Sos et al., 2009). This is supported experimentally by *in vivo* data showing the spontaneous development of lung adenocarcinoma in PTEN null mice (Yanagi et al., 2007a). Akt, a serine/threonine kinase, is the primary downstream signalling molecule of PI3K, doing so through a wide array of pathways. It is recruited to the plasma membrane by PIP_3 where it is activated by phosphorylation initially at threonine 308 and finally serine 473. Akt is constitutively active in many NSCLC cells, promotes cell survival, and is PI3K-dependent, as evidenced by reduced pAkt and apoptosis in response to the PI3K inhibitors LY294002 and wortmannin (Brognard et al., 2001). There is substantial evidence to suggest that over-expression of Akt is an early event in the development of lung cancer, with activation of Akt detected in dysplastic bronchial epithelial cells (Tsao et al., 2003), a precursor to malignant change.

p53

The p53 protein is an essential defence against uncontrolled cellular proliferation, providing tight regulation in the case of DNA damage leading to growth arrest, DNA repair and ultimately programmed cell death (apoptosis) if required. In response to cellular stress p53 binds DNA, leading to the complexing of p21 and cdk2 (cell division-stimulating protein 2) resulting in a halt to the cell cycle (NCBI, 1998-). Germ line mutations are rare but result in the dominant inherited familial cancer syndrome Li-Fraumeni Syndrome (Varley et al., 1997), which includes lung cancers. Conversely, p53 is known to be the most frequently altered gene in human malignancy (Hollstein et al., 1991), largely taking the form of missense mutations that result in loss of its downstream activation function (Hollstein et al., 1996) and accumulation of p53 protein. Accumulation of defective protein in lung cancer is associated with more aggressive disease (Soini et al., 1992) and has an adverse prognostic significance (Langendijk et al., 1995), as extensively reviewed in (Steels et al., 2001). Mutations within p53 appear to be related to smoking, with higher rates seen in heavy smokers as compared with non-smokers (Le Calvez et al., 2005), and the typical mutation (G-to-C transversion) is the most frequent associated with smoking (Vahakangas et al., 1992). Although p53 mutations are less common in animal models of lung cancer than seen in human disease (Hegi et al., 1993), the importance of p53 mutations in lung cancer development was demonstrated by the work of De Flora et al. who showed that tumour numbers were significantly increased in p53 mutant A/J mice when exposed to cigarette smoke (De Flora et al., 2003). Similarly, inactivation of p53 in the *LSL*K-ras^{G12D} mouse model of adenocarcinoma substantially increases tumour progression and metastasis, and shortens survival (DuPage et al., 2009, Fisher et al., 2001).

1.2 IL-6

1.2.1 Gp130 and the IL-6 family of cytokines

Interleukin-6 was first described as a B cell growth and differentiation factor (Kishimoto et al., 1995) but is now firmly established to be a cytokine with a multitude of functions (Akira et al., 1993), involved in haematopoiesis, generation of the acute phase response and host defence (Taga and Kishimoto, 1997). It is also thought to have a role in haematological and solid organ malignancy, chronic inflammation and auto-immune disease (Hirano and Kishimoto, 1992, Chalaris et al., 2011, Neurath and Finotto, 2011, Nishimoto et al., 2008, Silver and Hunter, 2010, Bataille et al., 1995).

IL-6 is part of a family of cytokines that share structural, signalling and functional similarities. The IL-6 family of cytokines includes IL-11, Leukaemia Inhibitory Factor (LIF),

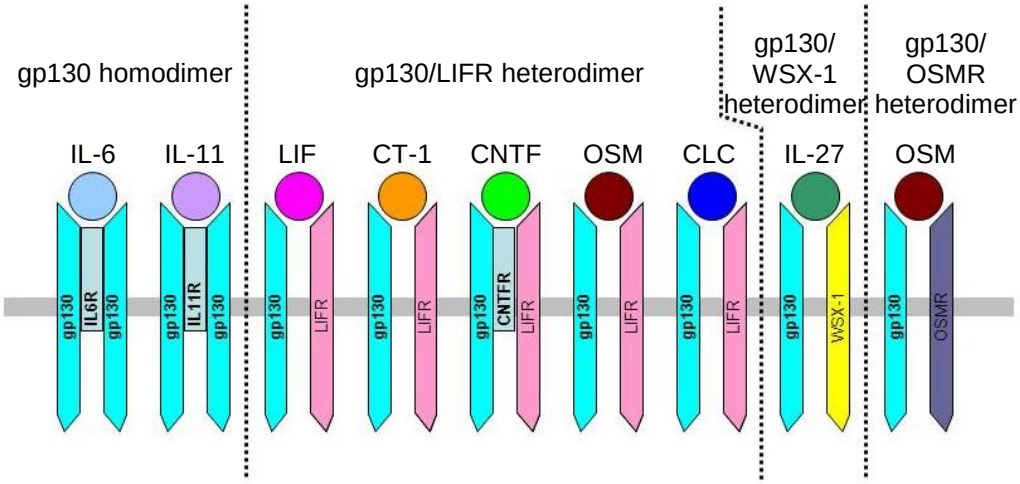
Oncostatin-M (OSM), Ciliary Neurotrophic Factor (CNTF), Cardiotrophin-1 (CT-1) Cardiotrophin-Like Cytokine (CLC) (Taga and Kishimoto, 1997) and IL-27 (Kastelein et al., 2007). They are all composed of four anti-parallel long α-helices, which are either straight (in the case of IL-6 and IL-11) or kinked. There is redundancy of function within the IL-6 family of cytokines at least in part because they all signal through the common β-subunit gp130 as described below (Taga and Kishimoto, 1997).

IL-6 is produced by monocytes/macrophages, T and B-cells, fibroblasts and endothelial cells (Ward, 2006), and effects a wide range of cells, including hepatocytes, lymphocytes and bone marrow progenitor cells (Kishimoto et al., 1995). The final protein is ~26kD, but varies in size in part according to the cells from which it is derived, depending upon the degree of glycosylation and phosphorylation, ranging from 17-84kD (Hirano and Kishimoto, 1992, Ward, 2006). Gene expression is readily inducible by a number of stimuli. Inflammatory cytokines IL-1β, TNF-α and the type-1 IFNs all lead to an upregulation of IL-6 gene expression (Content et al., 1985, Benveniste et al., 1990, Maruyama et al., 1995, Usui et al., 2004). Additionally, IL-6 is produced in response to activation of Toll-like Receptors (TLRs) and other Pattern-Recognition Receptors (PRRs) by lipopolysaccharide (LPS) and other microbial and endogenous ligands (Palsson-McDermott and O'Neill, 2004, Frost et al., 2006). Conversely, IL-6 production can be blocked by the action of glucocorticoids (Benveniste et al., 1990) and anti-inflammatory cytokines including IL-10 (Takeshita et al., 1996).

1.2.2 IL-6 signalling: Classical and trans-signalling

The IL-6 family of cytokines are grouped in part because of their common use of the gp130 receptor. IL-6 and IL-11 form a homodimer with gp130 and their individual α-subunit, in the case of IL-6 the IL-6 receptor (IL-6R). All the other members form a heterodimer with gp130 and either LIFR (LIF, CT-1, CNTF, OSM, CLC), OSMR (OSM) or WSX-1 (IL-27) (Figure 1.3). Signal transduction for all IL-6 family cytokines occurs in a similar way; for simplicity, and because it is the main focus of this dissertation, I will discuss the events involved in IL-6 signal transduction.

Gp130 is a 130kD trans-membrane glycoprotein that is ubiquitously expressed on cells (Rose-John et al., 2006) and has no inherent affinity for IL-6 (Taga and Kishimoto, 1997). It is a classic type I cytokine receptor and consists of 6 extra-cellular modules (Muller-Newen, 2003), a trans-membrane domain and a cytoplasmic domain (Heinrich et al., 1998). The N-terminal component is an Immunoglobulin (Ig)-like domain, with modules 2 and 3 making up the Cytokine Binding Domain (CBD) followed by three further fibronectin type-III modules (Heinrich et al., 2003) (Figure 1.4.). Module 2 contains four conserved cysteine residues and



<u>Figure 1.3.</u> IL-6 family of cytokines and receptors
IL-6 family of cytokines utilise the β-subunit gp130 in homodimers or heterodimers.

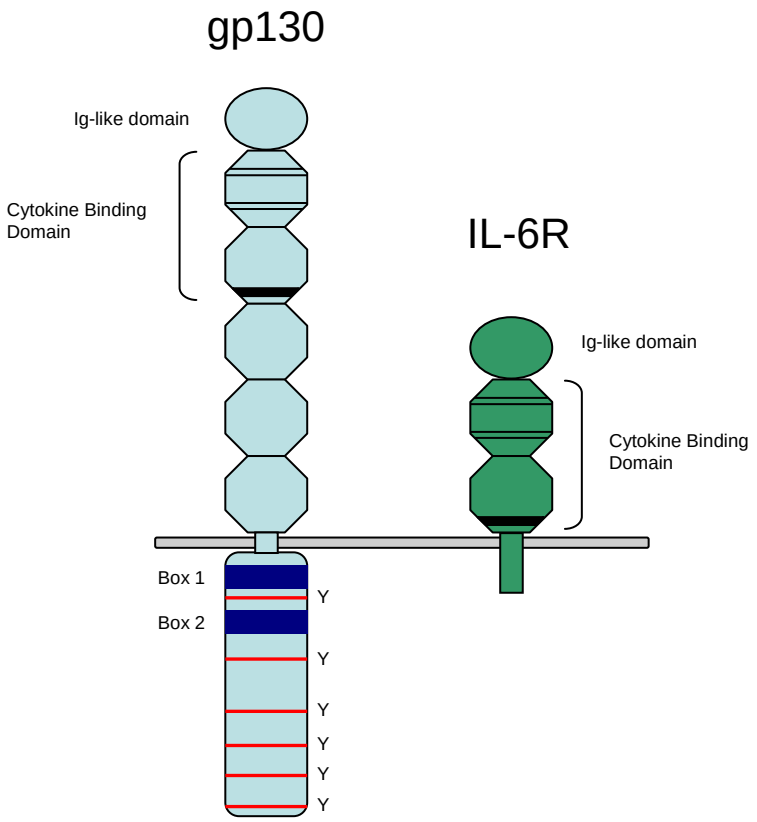
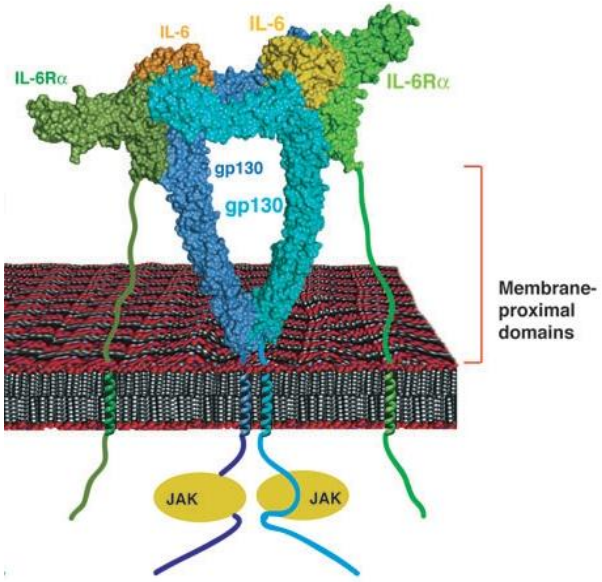


Figure 1.4: Gp130 and IL-6R structures (modified from Kishimoto, 1995 and Heinrich, 2003).
Gp130 consists of 6 extra-cellular domains including a Cytokine Binding Domain (CBD) characterised by conserved cysteine residues (black lines) and a WSXWS motif (black box). The intra-cellular component contains 2 conserved Box motifs and a number of tyrosine residues essential for signal transduction. The IL-6R is made up of an N-terminal Ig-like domain and a CBD. The intra-cellular component is short and plays no part in signal transduction.

module 3 a tryptophan-serine-X-tryptophan-serine (WSXWS) motif (Muller-Newen, 2003). The intracellular module does not have any intrinsic kinase activity (Taga and Kishimoto, 1997), but is constitutively associated with membrane proximal Janus Kinases (JAKs) (Muller-Newen, 2003) which bind conserved box 1 and box 2 motifs (Tanner et al., 1995, Heinrich et al., 2003). Crucially, the cytoplasmic domain also contains 5 tyrosine residues that are essential for signal transduction. The IL-6R consists of 3 extra-cellular domains; an N terminus Ig-like domain and two fibronectin type-III domains making up the CBD. Like gp130, within the fibronectin domains (Figure 1.4) are 4 conserved cysteine residues, a WSXWS motif adjacent to the membrane and a proline-rich hinge region (Kishimoto et al., 1995). The short cytoplasmic region of the IL-6R is not known to associate with any signal transduction molecules, and neither it nor the trans-membrane domain are required for signal transduction (Yawata et al., 1993). While gp130 is ubiquitously expressed, the IL-6R is expressed largely on hematopoietic cells and hepatocytes, limiting the effect of IL-6.

IL-6 binding to the IL-6R leads to recruitment of gp130 to form a hexameric complex of two each of IL-6, IL-6R and gp130 (Varghese et al., 2002), the structure of which is supported with crystallography data (Skiniotis et al., 2005) (Figure 1.5). Only with dimerisation of gp130 in this context is signal transduced to the cell. Following formation of this complex, gp130 binds to and activates JAK family tyrosine kinases. While JAK1, JAK2 and TYK2 (Tyrosine Kinase 2) (Stahl et al., 1994) all bind gp130, JAK 1 appears to be most important for IL-6 signalling (Rodig et al., 1998). On binding, the JAKs dimerise, are phosphorylated, and subsequently phosphorylate tyrosine residues within the cytoplasmic tail of gp130, providing activation sites for factors containing SH2 domains (Heinrich et al., 1998, Hirano et al., 1997, Stahl et al., 1994). Amongst these factors are the ubiquitously expressed latent transcription factors STAT1 and STAT3, which bind to the C-terminal tyrosine residues, are phosphorylated, form homo- or heterodimers and translocate to the nucleus where they regulate gene transcription (Heinrich et al., 1998, Heinrich et al., 2003) (Figure 1.6). It would appear that both STAT1 and 3 are also serine phosphorylated within the nucleus, increasing the transcription of target genes (Schuringa et al., 2001, Uddin et al., 2002). Many of the classical functions associated with IL-6 are regulated via STAT3; anti-apoptosis, the acute-phase response and B and T cell proliferation (Levy and Darnell, 2002).

In addition to recruitment of the STATs, tyrosine phosphorylation of the cytoplasmic tyrosine residues on gp130 leads to activation of the Src Homology tyrosine Phosphatase (SHP2). In this case it is the membrane proximal pY^{757} (in mice, pY^{759} in humans) which recruits SHP2 (Tebbutt et al., 2002, Cunnick et al., 2002) leading to activation of the PI3K/Akt and RAS/MAPK pathways (Heinrich et al., 1998, Taga and Kishimoto, 1997). While the precise mechanism of activation for these pathways is less well defined than the JAK/STAT pathway,



<u>Figure 1.5.</u> IL-6 signalling molecule crystal structure (from Skiniotis, 2005.) Crystal structure confirming the hexameric structure of the gp130/IL6 signalling complex. Each complex contains 2 molecules of gp130 (shown in blue), 2 molecules of IL-6R (shown in green) and 2 molecules of the IL-6 ligand (shown in yellow and orange).

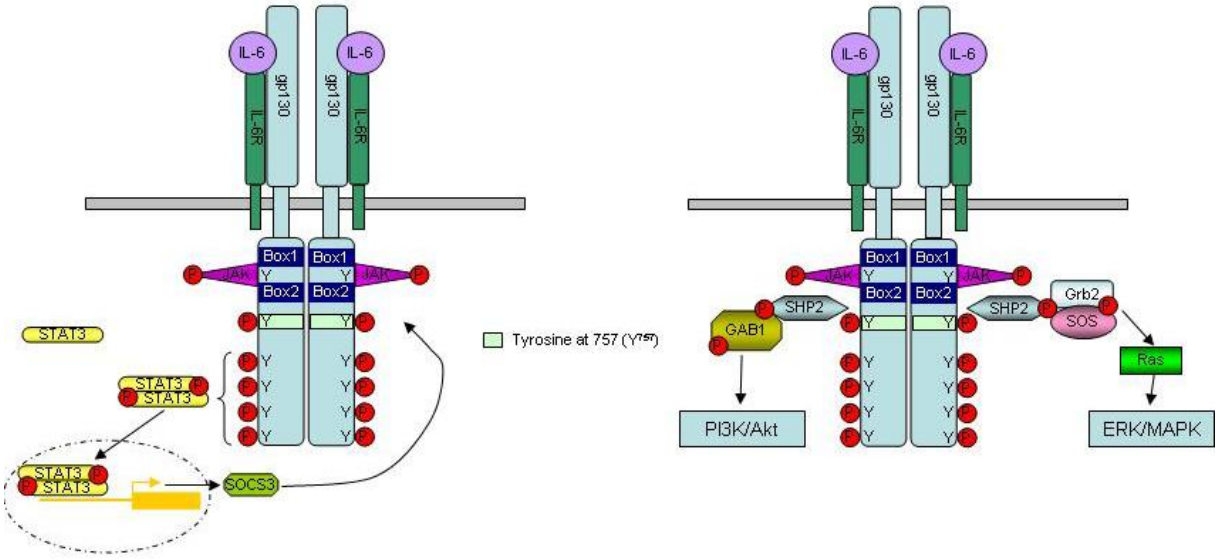


Figure 1.6. IL-6 signalling cascades
Following activation of the constitutively associated JAKs, tyrosine residues within the cytoplasmic tail of gp130 are auto-phosphorylated and lead to signal transduction through both the STATs (a) and SHP2 (b). SOCS3 (a) competes for binding at pY^{757}, blocking the activity of the JAKs and targeting the receptor complex for degradation by the proteosome.

it is clear that there is a SHP2-dependent, STAT-independent pathway downstream of gp130. Once activated by JAK1, SHP2 interacts with both Gab1 and the GRB2-SOS (Son Of Sevenless) complex. Tyrosine phosphorylation of the GRB2-SOS complex leads to activation of Ras, and subsequent ERK/MAPK signalling. On the other hand, phosphorylated Gab1 associates with PI3K, leading to the PI3K/Akt signalling cascade as described above. Interestingly, Gab1 also appears to lead to activation of the Ras/MAPK pathway (Takahashi-Tezuka et al., 1998), but is not essential (Cunnick et al., 2002).

In addition to the membrane bound IL-6R, a soluble form of the extra-cellular portion of the receptor (sIL-6R) exists (Narazaki et al., 1993) with comparable affinity for IL-6 as the membrane-bound receptor (Chalaris et al., 2011). The sIL-6R is able to bind with IL-6, and subsequently associate with gp130 leading to signal transduction. This is termed trans-signalling (cf. classical/cis-signalling) enabling IL-6 to act on all cells expressing gp130, even those lacking the membrane-bound IL-6R. The sIL-6R receptor can be formed in two ways, either through alternate splicing of the *Il6r* gene (Lust et al., 1992) or through the action of the metalloproteinase ADAM17/TACE (/A Disintergrin And Metalloproteinase/Tumour necrosis factor-α Converting Enzyme) which leads to shedding of the receptor from the cell surface (Chalaris et al., 2010a) (Figure 1.7). This shedding is inducible in response to inflammatory stimuli, including TNF-α, IL-1β and elevated C-reactive protein (CRP) (Chalaris et al., 2011, Kyriakou et al., 1997). Shedding from apoptosing cells, particularly neutrophils, may act to augment the inflammatory process by allowing IL-6 to act on epithelial cells, leading to the release of more pro-inflammatory chemokines and cytokines (Chalaris et al., 2007, Rabe et al., 2008). There is increasing evidence that trans-signalling is important in a number of diseases attributed to IL-6. This includes sepsis (Greenhill et al., 2011), osteoporosis, Crohn's Disease, Rheumatoid Arthritis (RA) and other auto-immune diseases (reviewed in (Kallen, 2002, Nowell et al., 2003)). It has also been associated with malignancy including myeloma, breast, prostate, bowel and ovarian cancers (Knupfer and Preiss, 2010, Lo et al., 2010, Becker et al., 2004, Kallen, 2002, Kyriakou et al., 1997).

A naturally-occurring antagonist exists for the sIL-6R in the form of soluble (s)gp130 (Narazaki et al., 1993). It binds to the sIL-6R/IL-6 complex thereby stopping interaction with membrane-bound gp130 (Jostock et al., 2001), targeting trans-signalling (Figure 1.7). Recently, to further muddy the signalling waters, Garbers et al. have shown that contrary to previous findings, sgp130 may inhibit classical signalling as well as trans-signalling (Garbers et al., 2011). These authors proposed that this is dependent on the concentration of sgp130 and the ratio of IL-6 to sIL-6R, suggesting that sgp130 was able to hold IL-6 in IL-6/sIL-6R complexes thereby diminishing the amount of IL-6 available for classical signalling. Alternate splicing of gp130 mRNA results in a stop codon prior to the trans-membrane coding

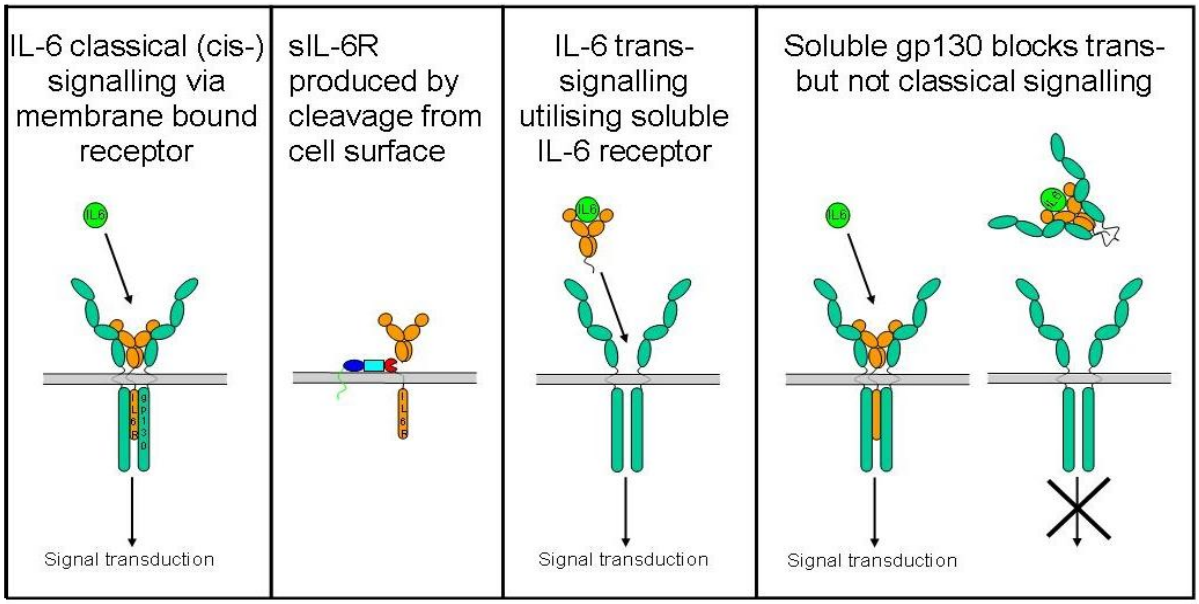


Figure 1.7. IL-6 trans-signalling
Trans-signalling occurs with the sIL-6R which is produced through alternate splicing or cleavage from the cell surface. Trans-signalling is opposed by sgp130.

region, leading to the production of sgp130 (Diamant et al., 1997). It has been detected at substantial levels in human serum (Narazaki et al., 1993), as well as in pleural fluid (Dore et al., 1997). Interestingly, rising levels are associated with increasing progression of a number of cancers (Kovacs, 2001, Kovacs, 2005). It has been proposed that sIL-6R and sgp130 work cooperatively to modulate the action of IL-6 (Heinrich et al., 1998).

As has been mentioned and will be discussed further in later sections, uncontrolled gp130 signalling is associated with a variety of pathological states. The importance of tight regulatory control of gp130 signalling is highlighted by our own studies and those of others, showing that gp130-dependent STAT3 hyper-activation promotes gastric and colorectal inflammation-associated tumourigenesis in mice (Jenkins et al., 2005a, Becker C, 2005, Bollrath et al., 2009). One of the most important regulatory mechanisms is performed by the SOCS (Suppressor of Cytokine Signalling) proteins, particularly SOCS3 (Heinrich et al., 2003), itself a target of IL-6/STAT3 signalling. SOCS3 contains a SH domain and binds to the pY^{757} domain, competing with SHP2. Here it acts directly on the JAKs to inhibit phosphorylation of gp130 (Endo et al., 1997) thereby stopping signal transduction (Figure 1.6). Regulation of IL-6/gp130 signalling involves internalisation of the receptor complex and degradation by the proteasome. As with signal transduction, it would seem that the IL-6 receptor is not important in the internalisation process, with a di-leucine motif within gp130 being the crucial region (Dittrich et al., 1994). In addition to stopping signal transduction, SOCS3 also interacts with proteins of the elongin family via the conserved 'SOCS box' region, targeting SOCS3 and gp130 for degradation by the proteasome (Zhang et al., 1999).

1.2.3 IL-6 cytokine family in lung cancer

IL-6 plays an important role in immune defence in the lung and in generation of the acute inflammatory response (Ward, 2006), but is also implicated in a range of pulmonary pathologies, not least lung cancer. Elevated levels of IL-6 and CRP (an acute phase protein induced by IL-6) have been associated with an increased risk of lung cancer (Pine et al., 2011, Zhou et al., 2012). IL-6 is elevated in BALF, serum and tumour tissue from patients with NSCLC (Chyczewska et al., 1997, Gao and Ward, 2007, Yanagawa et al., 1995) Elevated levels are also associated with cachexia, one of the most devastating complications of many cancers, including lung cancer (Baltgalvis et al., 2008, Bonetto et al., 2012b, Ohe et al., 1993, Zhang et al., 2008), and higher serum IL-6 levels in patients with advanced NSCLC correlate with worse prognosis (Songur et al., 2004). It is noteworthy that IL-6 is produced in human lung epithelial cells in response to cigarette smoke and protects against apoptosis triggered by smoke-induced DNA damage in a STAT3-dependent manner (Liu, 2007). Yeh et al. showed that constitutive STAT3 activity promotes the development of lung adenocarcinomas through

autocrine IL-6 stimulation and also contributes to another complication of lung cancer, malignant pleural effusions (Yeh et al., 2006).

A number of animal models of lung cancer have reinforced the clinical data showing the importance of IL-6 in lung cancer. In an inducible transgenic mouse model for lung epithelial-specific MMP12 over-expression, a step wise progression of pulmonary inflammation, emphysema and adenocarcinoma was associated with elevated BALF IL-6 levels and epithelial cell STAT3 activity (Qu et al., 2009a). Similarly, in a triple knockout (IFN-γ, GM-CSF and IL-3) mouse model of spontaneous lung cancer development, IL-6 was elevated in BALF and serum, and was further elevated as tumours progressed (Dougan et al., 2011). Increased IL-6 levels were also demonstrated in an *LSL*K-ras^{G12D} model with COPD-like pulmonary inflammation, which was associated with increased tumour load (Moghaddam et al., 2009). However, in this latter model it is unknown whether IL-6 contributed to cancer development or was merely a consequence of the inflammatory process. To help answer this question, Ochoa et al. genetically ablated IL-6 in a *LSL*K-ras^{G12D} mutant mouse, which led to attenuation of the tumour load both with and without the presence of COPD-like inflammation (Ochoa et al., 2011).

As mentioned above, IL-6 has also been implicated in complications of cancer, namely cachexia. IL-6 production is elevated (Okada et al., 1998) and polymorphisms of the IL-6 promoter leading to increased IL-6 expression are associated with a greater risk of cachexia and shorter survival in subjects with pancreatic cancer (Zhang et al., 2008). Similarly, in a systematic review of polymorphisms associated with cancer cachexia from 92 candidate genes, IL-6 was one of four identified that linked key pathways in the development of cachexia (Tan et al., 2011). With regards to lung cancer specifically, elevated IL-6 levels are associated with weight loss (Scott et al., 1996, Staal-van den Brekel et al., 1995), malnutrition and reduced survival (Songur et al., 2004). This human evidence is supported by animal models. For example, a Lewis Lung Carcinoma cell (LLC) xenograft model demonstrated that transfection of human IL-6 cDNA led to a markedly reduced survival without change in tumour size (Ohira et al., 1992). These authors followed up this finding by showing that both body weight and nutritional status (as measured by serum albumin levels) were reduced and associated with elevated IL-6 levels, but not TNF-α or IL-1α levels (Ohe et al., 1993).

1.2.4 Targeting IL-6 in Inflammatory Diseases and Cancer

In light of the above observational and experimental data, focus has also fallen on IL-6 as a therapeutic target in lung cancer. Antibodies against IL-6 and the IL-6R are used clinically and have been investigated in a number of other cancers and chronic inflammatory conditions, including RA and Inflammatory Bowel Disease (IBD), and brain (Kudo et al., 2009) and

bowel cancer (Neurath and Finotto, 2011). Interestingly, much of this pathological effect of IL-6 is attributed to trans- rather than classical-signalling (Atreya et al., 2000, Chalaris et al., 2011).

It is well established that IL-6 has a role in RA (Hirano et al., 1988, Chalaris et al., 2011, Sack et al., 1993). This is supported by experimental evidence showing that IL-6 knock-out mice are resistant to Collagen-Induced Arthritis (CIA) (Alonzi et al., 1998), a frequently used mouse model of arthritis. Taking this idea into the therapeutic realm, Takagi et al. showed that administration of an antibody targeting the IL-6R reduces the development of joint disease in the CIA model (Takagi et al., 1998). Following the development of a human recombinant IL-6R antibody, subjects with established RA showed a marked improvement in both symptoms and inflammatory markers following a single dose (Choy et al., 2002). This finding has been repeated on a number of occasions with similar results (Maini et al., 2006, Nishimoto et al., 2003, Nishimoto et al., 2004), is more effective than standard therapy (Jones et al., 2010, C. Gabay, 2012), and is now widely used in clinical practice (Mariette et al., 2011).

Crohn's Disease and Ulcerative Colitis are chronic inflammatory diseases of the bowel (together termed IBD) which are not only devastating diseases in their own right but also predispose sufferers to colon cancer (Weedon et al., 1973). Interestingly, IL-6 has been linked not only to the development of IBD (Mitsuyama et al., 2006b, Mudter and Neurath, 2007) but also to the transition to bowel cancer (Grivennikov et al., 2009, Bollrath et al., 2009). In a T-cell adoptive transfer murine model of colitis, blockade of IL-6 signalling by either an IL-6R antibody or the sgp130-Fc fusion protein led to a marked reduction in intestinal inflammation (Atreya et al., 2000). Building on this and other work, Ito et al. undertook a study of safety and efficacy of an IL-6R antibody in a small number of subjects with Crohn's Disease (Ito et al., 2004). Those on the active treatment had a higher clinical response and greater reduction in inflammatory markers, all suggesting a beneficial clinical effect. Given the high rates of colon cancer associated with the marked inflammation seen in IBD, investigation of the pathological role of IL-6 in this progression is only natural. Grivennikov et al. showed that in IL-6 knock-out mice, colonic inflammation and tumour numbers were reduced in the Dextran Sodium Sulphate/Azoxymethane (DSS/AOM) model of inflammation-induced colon cancer (Grivennikov et al., 2009), suggesting a possible role for IL-6 blockade in preventing this transition. Becker et al. (Becker et al., 2005) provided further evidence for this possibility by showing that in the same DSS/AOM model, a neutralising antibody to the IL-6R similarly reduced tumour load; this finding strengthened the case for anti-IL-6 therapy as a therapeutic option and provided a framework for reducing the progression of IL-6-driven chronic inflammation to cancer.

In lung cancer, a xenograft model using H1650 (lung adenocarcinoma) cells showed that a neutralising antibody to IL-6 substantially slowed tumour growth (Song et al., 2011). Somewhat contrary to these findings, Ohe et al. found no differences in tumour growth when LLC xenografts were transfected with IL-6 mRNA leading to increased production of IL-6 (Ohe et al., 1993); interestingly, these authors observed substantially more weight loss and a reduction in albumin levels, consistent with cancer related cachexia. Following on from this, Ohira et al. showed that blocking IL-6 with anti-inflammatory eicosanoids in the same model lead to prevention of this weight loss (Ohira et al., 1996). A humanised monoclonal antibody to IL-6 has been used in phase II trials in subjects with advanced NSCLC, which showed that blocking IL-6 was effective at reversing anaemia, improving nutritional parameters and reducing weight loss (cachexia), but had no effect on disease progression (Bayliss et al., 2011).

1.3 Animal models of lung cancer

1.3.1 Genetic models

1.3.1 (i) K-ras

K-ras mutations occur with high frequency in human adenocarcinoma (Anderson et al., 1991, Rodenhuis and Slebos, 1992) and thus many mouse models have been developed to reflect this. Initial efforts focused on another Ras family member, H-ras, with activated forms under the control of lung specific promoters leading to a high incidence of lung tumours (Tuveson and Jacks, 1999). To better model human lung cancers, Johnson et al. (Johnson et al., 2001) used a gene targeting method to generate mouse strains carrying mutant oncogenic alleles that relied on somatic recombination for expression. This resulted in a high lung tumour burden seen from as early as one week of age as well as a number of other solid organ tumours. Further refinement of the model was made by a number of laboratories (Jackson et al., 2001, Meuwissen et al., 2001) with organ specific sporadic activation of oncogenic K-ras using adenoviral based delivery of a Cre-recombinase (Figure 1.8). This has become the one of the most frequently used methods, both as a single mutation and with any number of other conditionally-activated proto-oncogenes or silenced tumour suppressor genes (DuPage et al., 2009, Ahn et al., 2011, Dovey et al., 2008). Inhalation of the virus leads to rapid and reproducible development of Atypical Adenomatous Hyperplasia (AAH), adenomas and adenocarcinoma of the bronchoalveolar type (Johnson et al., 2001). There is a clear dose response to virus units so both titration and administration must be done with care (Johnson et al., 2001, DuPage et al., 2009).

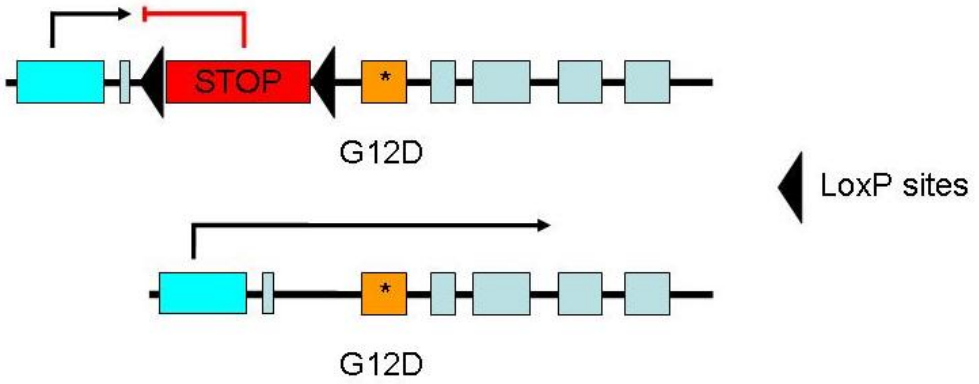


Figure 1.8. Inducible oncogenic Kras mouse model (*LSL*Kras^{G12D})
A stop codon is flanked by LoxP sites, upstream of oncogenic *Kras* (a). Inhalation of a Cre-recombinase results in excision of the stop codon and transcription of the oncogenic element (b).

1.3.1 (ii) STAT3-C

As inappropriate and persistent activation of STAT3 occurs in at least 50% of human lung adenocarcinoma (Haura et al., 2005b), a mouse model of persistent STAT3 activation was developed in an effort to better understand the pathways leading to tumourigenesis. STAT3C, which contains a substitution of two cysteine residues within the SH2 domain, dimerises spontaneously and translocates to the nucleus leading to gene transcription, mimicking persistent activation (Bromberg et al., 1999). Li et al. demonstrated that over-expression of STAT3C within the pulmonary epithelium of mice led not only to marked pulmonary inflammation but also the development of lung adenocarcinoma (Li et al., 2007b). While this model certainly supports the observation of increased STAT3 activation in human lung cancer, the artificial nature of the over-expression leaves room for a model displaying endogenous hyper-activation of STAT3.

1.3.2 Carcinogen model - NNK

A number of carcinogens have been used in animal models of lung cancer including urethane and the polycyclic hydrocarbons benzo(a)pyrene (Yao et al., 2004, Wattenberg, 1980) and 3-methylcholanthrene (Wattenberg, 1985, Wattenberg et al., 1994). A significant amount of work has been performed with urethane (ethyl carbamate) (extensively reviewed in (IARC, 2010)) including delineating differences in mouse susceptibility to lung tumourigenesis (Demant, 2003). However, there is little evidence in humans to suggest it is a carcinogen (IARC, 1972-present, Hagmar et al., 1986). In contrast, NNK is well established as a human carcinogen (Hoffmann et al., 1996) and is specific to cigarette smoke (Hecht and Hoffmann, 1988), making it the most translationally-relevant carcinogen model. NNK displays remarkable organ specificity, with lung tumours developing in mice regardless of the route of administration (Jalbert and Castonguay, 1992). As mentioned earlier, this specificity exists because NNK requires metabolism to its active derivative NNAL (Hecht, 1998) which occurs most efficiently with Microsomal CYPP450 enzymes that are found predominantly in the lung (CYP 2A13) and the liver (Su et al., 2000). Whilst hepatocellular carcinomas also occur with high frequency (Belinsky et al., 1990), tumours of other organs occur infrequently and appear to be dependent on the route of administration of the NNK. Illustrating this point, stomach tumours have only been reported when NNK was delivered in drinking water (Jalbert and Castonguay, 1992).

The susceptibility of mice to the oncogenic effects of carcinogens is strain specific. To explain this difference, a large number of susceptibility genes have been proposed (Demant, 2003, Festing et al., 1998, Devereux and Kaplan, 1998), with three designated as Pulmonary Adenoma Susceptibility (PAS) genes thought to be the most important in NNK-induced

tumourigenesis (Malkinson, 1989, Karasaki et al., 1997). *Pas1* (proposed to be *Kras2* (Lin et al., 1998)) is thought to explain the majority of the difference (Matzinger et al., 1997), as all the mouse strains that develop tumours in response to NNK, but none of those resistant carry this allele (Ryan et al., 1987).

There have been many protocols for inducing lung tumours in mice with NNK. These range from single (McClelland et al., 2007, Hollander et al., 2008) or multiple (Yang et al., 2004, Razani-Boroujerdi and Sopori, 2007) intra-peritoneal (IP) injections, continuous IP infusions (Igarashi et al., 2009), administration via drinking water (Jalbert and Castonguay, 1992) or by gavage (Conaway et al., 2005). There is clearly a dose response (Belinsky et al., 1990), and like the range of administration protocols, there is a wide range of doses. Even in those studies using A/J mice doses ranged significantly, from 2mg/mouse IP (McClelland et al., 2007) to 100mg/kg/dose IP (Razani-Boroujerdi and Sopori, 2007) (the equivalent of 2mg/dose for a 20mg mouse) to 9.1mg by gavage weekly for eight weeks (Jalbert and Castonguay, 1992). Finally, the length of exposure ranges from as short as 16 weeks (Jalbert and Castonguay, 1992, Lee et al., 2005, Hollander et al., 2008), to as long as 42 weeks (Conaway et al., 2005) in mice, or 72 weeks in other rodents (Hecht et al., 1983). Interestingly, addition of NNK to mainstream Cigarette Smoke (CS) has not been shown to alter tumour development. Finch et al. (Finch et al., 1996) compared mice exposed to CS to air exposure, and NNK to NNK plus CS. Mice were exposed to CS/air over 26 weeks following a single IP injection of NNK, and showed no difference in tumour incidence between those with NNK+CS and NNK alone. In contrast, Barta et al. showed that NNK-induced tumourigenesis was enhanced by COPD-like inflammation induced by bacterial infection (Barta et al., 2012).

1.3.3 SOCS3

As mentioned earlier the SOCS proteins are crucial in controlling signalling through the JAK/STAT pathway (Heinrich et al., 2003). Because of the prevalence of JAK/STAT deregulation in lung cancer, a number of investigators have examined the role of SOCS3 in the development of lung tumours. SOCS3 is frequently silenced in human lung cancer samples and cell lines through hypermethylation of the promoter (Baltayiannis et al., 2008, He et al., 2003b, He et al., 2004a). A549 (human adenocarcinoma) cells, which are deficient in SOCS3, display an increase in apoptosis and a reduction in proliferation when SOCS3 levels are restored (Yu et al., 2009b, Zhang et al., 2012a). Interestingly, those cell lines (NCI-H838) that display reduced SOCS3 expression also show constitutively expressed STAT3 (He et al., 2003a, He et al., 2003b).

1.4 The gp130$^{F/F}$ mouse

In an attempt to address the lack of animal models of deregulated endogenous gp130 signalling, the gp130$^{F/F}$ mouse was developed.

1.4.1 Genetics and signalling

The gp130$^{F/F}$ (FF) mouse has a knock-in substitution of phenylalanine for tyrosine at the Y^{757} residue within the cytoplasmic tail of gp130. This abolishes binding of both SOCS3 and SHP2 which simultaneously leads to exaggerated STAT3 activation and impaired signalling through the SHP2/Ras/MAPK and SHP2/GAB1/PI3K/Akt pathways (Tebbutt et al., 2002) (Figure 1.9).

1.4.2 Phenotype

The mice develop spontaneous multi-organ inflammation driven by hyper-activated STAT3, along with splenomegaly, lymphadenopathy and thrombocytosis (Jenkins et al., 2005a), and are hyper-sensitive to the effects of the bacterial wall component LPS (Greenhill et al., 2011). As a consequence of chronic inflammation in the stomach, the mice develop gastric tumours by 3 months of age (Tebbutt et al., 2002) which is both IL-11 and STAT3 dependent (Ernst et al., 2008).

1.4.3 Lung disease

The mice develop enlarged airspaces by 6 months of age and are noted to have inflammatory infiltrates. Further examination confirms the airspace enlargement is consistent with emphysema (Ruwanpura et al., 2011) rather than hypoplasia. However, the nature of the inflammatory infiltrates and the role of the inflammation in the development of the emphysema remains to be elucidated.

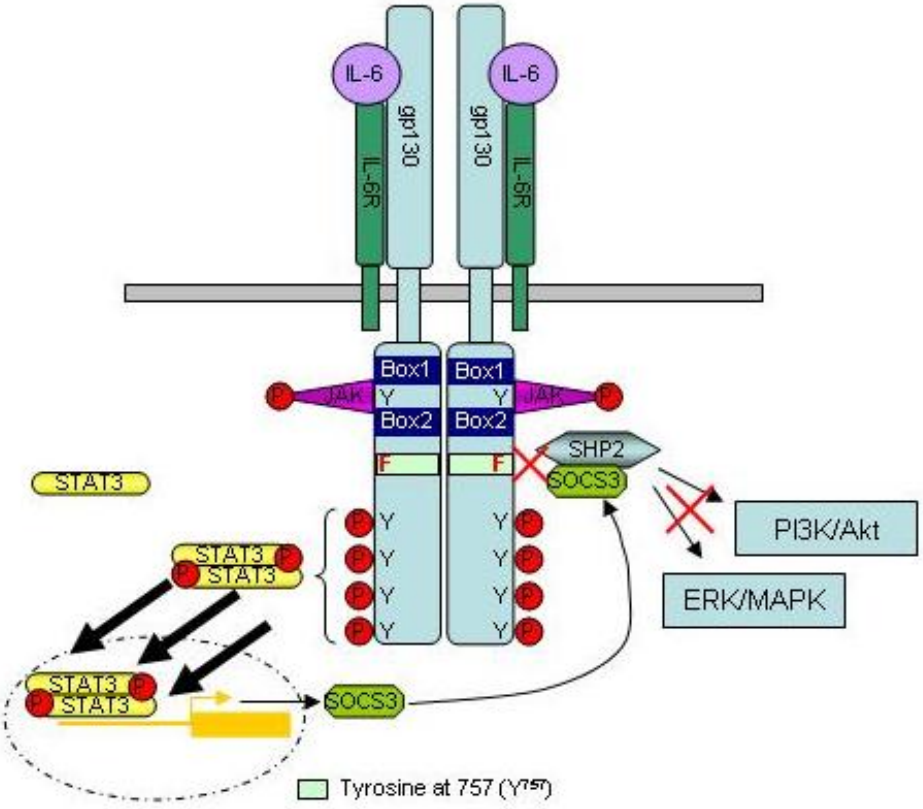


Figure 1.9. The gp130$^{F/F}$ model.
A knock-in substitution of phenylalanine for tyrosine abolishes the binding of SOCS3 and SHP2 leading to exaggerated STAT3 signalling and loss of PI3K/Akt and Ras/MAPK signalling.

1.5 Hypothesis and Aims

Given all the evidence linking IL-6 and lung cancer, I hypothesised that deregulated gp130/IL6 signalling in the FF mouse would influence carcinogenesis induced in both chemical (cigarette carcinogens) and genetic (*LSL*Kras^{G12D}) lung cancer models. I also hypothesised that IL-6 trans-signalling would be implicated in the development of lung cancer and its complications.

The overall aim was to investigate the role IL-6-driven gp130 signalling plays in the development of lung tumours, using both genetic and carcinogen induced models. Further, I aimed to examine which pathways downstream of gp130 were important in lung tumour development.

The specific Aims of the project were:

1. To characterise the pulmonary inflammation in mice displaying deregulated gp130 signalling.

2. To investigate the role of gp130/STAT3 activation in airway-epithelial responses to cigarette carcinogens.

3. To demonstrate that deregulated gp130/STAT3 signalling contributes to lung cancer susceptibility in response to exogenous cigarette carcinogens.

4. To examine the role of IL-6 classical and trans-signalling in the promotion of carcinogenesis and cachexia in an inducible activated *k-ras* model of lung cancer.

CHAPTER 2

Materials and Methods

2.1 Animal work

2.1.1 Mouse generation and housing

The gp130$^{F/F}$ (FF) mice homozygous for the gp130Y757F knock in mutation, along with FF compound mutant mice heterozygous for *Stat3* null (FF:St3-/+), and homozygous for *Il-6* null (FF:IL6-/-) or *Stat1* null (FF:St1-/-), were generated on a mixed 129Sv x C57BL/6 background as described previously (Tebbutt et al., 2002, Jenkins et al., 2005b). KrasG12D mice carrying an activated oncogenic *Kras* mutation preceded by a Lox-P flanked stop codon were generated (Jackson et al., 2001), and generously provided by D. Neil Watkins (Monash Institute of Medical Research, Melbourne). These *LSL*Kras^{G12D} mice were crossed with FF mice to generate FF:Kras^{G12D} mice, FF:St3-/+:Kras^{G12D} mice and FF:IL6-/-:Kras^{G12D} mice.

To produce mice susceptible to the carcinogenic effects of NNK, A/J mice (Ryan et al., 1987) were purchased from Walter and Eliza Hall Institute Animal Services (Kew, Victoria, Australia). FF and FF:St3-/+ mice (as well as wild-type (WT) control mice) were then back-crossed for 3 generations to produce Pseudo-A/J mice according to the protocol of Hollander et al. (Hollander et al., 2008). All experiments were performed following ethics approval from the Monash University Monash Medical Centre 'A' committee and included genetic- and age-matched wild-type (WT) mice where appropriate. Mice were housed in a Specific Pathogen Free (SPF) facility with a 12 hour light/dark cycle and *ad libitum* food and water.

2.1.2 Genotyping Polymerase Chain Reaction (PCR)

Specific regions of genomic DNA were amplified by PCR to determine the genotype of mice. DNA was extracted from tail clips and mice were genotyped for *gp130, Stat3, Stat1, IL6* and *Kras* as appropriate. For the Pseudo-A/J background, mice were also genotyped for the *Kras2_37* allele (*Pas1*), which has been shown to confer much of the strain-specific susceptibility to NNK, and mice homozygous for the susceptible A/J *Kras2_37* allele were used for breeding and experimentation.

DNA extraction and PCRs were performed by me or other members of the Cytokine Signalling Laboratory (see acknowledgements). Tail clips were incubated overnight in tail buffer (Appendix II) and Proteinase K (Roche, Mannheim, Germany) at 55°C. The following day 5M NaCl was added and the resultant mix incubated at Room Temperature (RT) before

centrifugation. The aqueous phase was removed to a clean eppendorf tube and mixed with isopropanol and centrifuged to pellet the DNA. The DNA pellet was washed in 70% ethanol, re-pelleted before drying for 2 hours at 37°C. The dried DNA pellet was resuspended in MilliQ water (Millipore, Bedford, MA, USA) overnight at 4°C. DNA was subsequently purified using a vacuum manifold utilising a UNIFILTER ® 800 plate (Whatman, UK), and PB binding buffer and PE wash buffer (Qiagen, Germany). DNA was finally eluted from the filter plate with warmed 0.25% TE buffer (Appendix II).

PCRs were performed in a standardised manner with protocols optimised by members of the Cytokine Signalling Laboratory for all genotyping except *Kras2_37*, which was received from C. Hollander (personal communication). Each reaction included the following noted at final concentrations utilising the GoTaq Flexi DNA polymerase kit (Promega, WI, USA): 100-250ng of DNA to a volume of 5µl, dNTPs at 0.2mM, MgCl at 1.5mM, primer A and B at 1µM (Sequences see Appendix III), colourless buffer at 1x, Dimethyl Sulfoxide (DMSO) at 5% (Sigma-Aldrich, Saint Louis, MO, USA), 0.5U Taq, and MQH_2O to a total of 50µl. Each PCR was run on a Biorad (Richmond, CA, USA) under the following conditions:

Initial denaturing	94°C	2 min	x1
Further denaturing	94°C	30 sec	
Annealing	54°C	30 sec	x40
Extension	72°C	15 sec	
Final extension	72°C	1 min	x1

All PCR products were run out on 2% agarose gels (Appendix II) made up with 1xTAE and SYBRSafe (Invitrogen) to visualise bands. A 1 kb Plus DNA Ladder (Invitrogen) was used to identify band size. DNA loading dye (5µl) (Appendix II) was added to samples only in the case of the Kras2_37 PCR, otherwise the PCRs were loaded neat into wells. Gels were run at 100V for 20-40 min in 1xTAE buffer and visualised under UV light in a gel dock (SafeImage, Invitrogen).

2.1.3 Administration of NNK to mice

NNK (Toronto Research Chemicals, North York, Canada) was dissolved in sterile Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen, Auckland, NZ) at a concentration of 20mg/ml. Mice aged between 6 and 8 weeks of age received 3 intra-peritoneal (IP) injections on alternate days, with each dose at 100mg/kg (Razani-Boroujerdi and Sopori, 2007). IP injections of sterile DPBS at equivalent volumes were used as the injection control. Mice were observed over 16 weeks.

2.1.4 Inhalation of Cre recombinase expressing adenovirus

Inhalation of AdenoCre (University of Iowa, Gene Transfer Vector Core, Iowa city, USA) was performed as previously described (DuPage et al., 2009). Mice at 6-8 weeks of age were anesthetised with Avertin ((2-2-2 Tribromoethanol), Sigma-Aldrich) at a working concentration of 20mg/ml in DPBS. Once deeply anaesthetised, mice inhaled a total of 125µl in two aliquots, made up as below in Minimum Essential Eagle Medium (MEM, Sigma-Aldrich) and Calcium Chloride ($CaCl_2$, Fluka, Steinheim, Germany). The second aliquot was delivered once the mouse had recovered to a normal breathing pattern. Following the second inhalation, mice were allowed to fully recover on a heated mat. Mice were observed over 6 weeks.

Component	**Volume inhaled**
AdCre	8.3ul ($5x10^8$PFU)
MEM	115.5µl
CaCl	1.2µl
Total	**125µl**

2.1.5 Administration of therapeutics to mice

Two anti-IL-6R monoclonal antibodies (2B10 and 25F10) were a kind gift from Dr Walter Ferlin (NovImmune, Geneva, Switzerland). All antibodies were given at 1mg/mouse by IP injection once weekly for the 6 weeks of observation, with the initial injection given on recovery from Adeno-Cre inhalation.

2.1.6 BALF, blood and tissue collection

Euthanasia of mice was performed by IP injection of Sodium pentobarbitone (100mg/kg; Troy Laboratories, NSW, Australia). BALF was collected following cannulation of the trachea. Lungs from each mouse were lavaged *in situ* with one 400µl and three 300µl aliquots of ice cold PBS. Blood was collected via closed cardiac puncture or cannulation of the inferior vena cava following death. Collected blood was placed into 1.5ml eppendorf tubes for serum preparation and/or microvette tubes (500KE SARSTEDT, Germany) for cell counts on an automated cell counter (Sysmex KX-21N, Sysmex Corporation, Kobe, Japan).

Lungs were collected for histology and/or protein and RNA extraction, and if both histology and protein/lung extracts were required, the left main lobe was first isolated by securing a suture around the hilum before the lobe was removed. For protein and RNA extraction, the lobe or lobes were placed in 1.5ml eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C. For histology, the trachea was cannulated and secured in place. The lungs were

perfused with 4% Normal Buffered Formalin (Sigma-Aldrich) at a pressure of 20cmH_2O for at least 2 min, before the trachea was tied off and the lungs and mediastinum removed *en bloc*. After 16 hours in formalin, tissues were transferred to 70% ethanol for storage.

2.1.7 Cytocentrifugation and staining

The total cell number in BALF was determined using an automated haematology analyser (Sysmex KX-21N). Cytospins were prepared from 50000 cells/sample and centrifuged at 91 x*g* for 7 min on a Cytospin III (Shandon Scientific, Cheshire, UK). Cytospin preparations were stained with DiffQuick (Dade Baxter, DE, USA) and cell differentials established by standard morphological criteria. A minimum of 200 cells per slide were counted.

2.1.8 Dual-Energy Xray Absorptiometry (DEXA)

Following euthanasia as above, mice were stored whole at -20°C. On the day of assessment, the mouse bodies were first brought to room temperature before scanning with the Lunar PIXImus (GE-Lunar Corp, Madison, WI). Analysis was performed with the proprietary software to yield body area (cm^2), bone mineral content (g), bone mineral density (g/cm^2), lean tissue mass (g), fat tissue mass (g) and total tissue mass (g).

2.2 Histology

2.2.1 Lung tissue preparation, processing, embedding and sectioning

Lungs were separated from heart and other mediastinal structures before being placed as a whole into a cassette. Tissue samples were processed using a standard procedure with one hour in each solution by the Monash Institute of Medical Research Histology Facility. Following processing, samples were embedded in low melting point paraffin (Paraplast Plus, Leica Microsystems, Peterborough, UK) and sectioned at 5μm in near-serial sections with two sections per slide.

2.2.2 Dewaxing and rehydration

Following dewaxing in Xylene (Merck, Kilsyth, Australia) slides were rehydrated in 100% then 70% ethanol and finally tap water.

2.2.3 Haematoxylin and Eosin staining

Following dewaxing and rehydration, slides were placed in Harris Haematoxylin (Amber Scientific, Midvale, WA, Australia) for 7 min before being washed in tap water. Differentiation was achieved with a single dip in acid alcohol (Appendix II) and 30 sec in a

weak ammonia solution (Appendix II) with a wash in tap water between each step. Intensity of staining was checked before being placed in Eosin (1% aqueous, Amber Scientific) for 4 min. After washing off excess stain, sections were dehydrated with increasing concentrations of ethanol and cleared with xylene before being cover-slipped with DePex mounting media (BDH, Nottingham, UK).

2.2.4 Immunohistochemistry procedure

Proteins of interest and cell phenotypic markers were identified in paraffin-embedded lung sections with antibodies to Proliferating Cell Nuclear Antigen (PCNA, Cell Signalling Technologies [CST], MA, USA), CD45 (BD Biosciences, MA USA), B220 (BD Biosciences) and TTF-1 (Novus Biologicals, CO, USA). Heat activated antigen retrieval was performed in citrate buffer (Appendix II) before staining. Endogenous peroxidise activity was quenched with 3% hydrogen peroxide (Merck). Non-specific binding was blocked with CAS block (Invitrogen) for CD45, B220 and TTF-1, and MOM blocking reagent for PCNA (Vector Laboratories, CA, USA). Antibody labelling was detected with a biotinylated secondary antibody (Vector Laboratories) and amplified with the Vector ABC Kit (Vector Laboratories) and diaminobenzidine chromogen (DAB, Dako, CA, USA). Nuclei were counterstained with Harris Haematoxylin before being dehydrated, cleared and cover-slipped as above.

2.2.5 Scoring immunohistochemistry staining

PCNA staining was scored as an index of stained to unstained cells within NNK-induced tumours. Photomicrographs were taken of tumours at 20x magnification. Images were viewed in Image J (v. 1.46d, NIH, USA) and positive and negative staining nuclei counted manually within a grid that was placed over photomicrographs with a random offset. A minimum of 100 cells / tumour were counted.

2.3 Tissue Culture

2.3.1 Thawing cells

Frozen cryotubes (Greiner, Frickenhausen, Germany) of cell stocks were removed from liquid nitrogen and after thawing in a water bath at 37°C were gently mixed with pre-warmed Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) or Roswell Park Memorial Institute media (RPMI, Invitrogen) supplemented with 10% v/v heat-inactivated Foetal Calf Serum (FCS) (JRH Biosciences, Lenexa, USA), 50U/ml Penicillin and 50U/ml Streptomycin (Invitrogen) and 2mM L-glutamine (Invitrogen) by repeated up and down pipetting. Resuspended cells were placed in a 15ml conical tube (Falcon tube; BD Biosciences,

Bedford, MA, USA) and centrifuged at 190 *xg* for 5 min to pellet cells. Cells were resuspended in fresh complete media as above and plated into a 175cm^2 flask (BD Biosciences). Human adenocarcinoma cell lines A549, NCI-H1395, NCI-H1993, NCI-H2228, NCI-H23 and NCI-H838 were a gift from Professor D. Neil Watkins.

2.3.2 Cell culture maintenance

Cells were maintained in a humidified atmosphere at 37°C with 5%CO_2 v/v in 25, 75 or 175cm^2 canted neck polystyrene flasks (BD Biosciences) and grown to 80-90% confluence before splitting 1:10 for all cells, except NCI-H2228 which were split at 1:2. Cells were harvested by removing the media, washing with RT sterile DPBS and incubating with 0.25% (w/v) Trypsin (Invitrogen, Lohne, Germany)/ 0.4% (w/v) Ethylene Diamine Tetra-Acetic Acid (EDTA, Amresco, Solon, USA) until cells began to lift off the flask. The trypsin activity was terminated with double the volume of complete media, and after cells were pelleted by centrifugation at 190 *xg* for 5 min they were resuspended in complete media and transferred back in to flasks.

2.3.3 Freezing cells

After cells were harvested as above, they were resuspended in freezing media (5% DMSO in FCS) and transferred to 1ml cryotubes for freezing. Tubes were frozen overnight at -80°C before being transferred to liquid nitrogen for long term storage.

2.3.4 Viable cell counting

After passaging cells as described in section 2.3.2, 10µl of cell suspension was added to an equal volume of 0.4% trypan blue (Sigma-Aldrich). Viable cells were identified by trypan blue dye exclusion and counted using a haemocytometer (Precicolor HBG, Germany).

2.3.5 MTT Cell Viability Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich) assays were performed in both 96 and 48 well formats with either 0.5 x 10^3 cells/well (96 well) or 1 x 10^4 cells/well (48 well) that had been allowed to adhere overnight. Fresh media containing the appropriate agonists and/or antagonists was then added and cells were incubated up to 6 days. After test media was removed and cells were gently washed with warm DPBS, 100µl of MTT at 0.2mg/ml in complete media was added. Cells were incubated for 4 hr before the MTT solution was carefully removed and 100µl DMSO was added to solubilise crystals. Plates were incubated at RT for 15 min on a shaker before intensity was

determined on a FLUROstar Optima plate-reader (BMG Labtech, Offenburg, Germany) at 560nm.

2.3.6 Quantification of cell supernatant IL-6 and sIL-6R levels

Cells ($1x10^6$) were plated in 6 well plates in 500ul complete media and incubated overnight. Supernatants were removed, spun for 5 min at 190 *xg* to pellet cellular debris and frozen at -20°C until required. Protein levels were determined by ELISA as described below (2.5.6).

2.3.7 *In vitro* stimulation and inhibition

Cells were stimulated with NNK and IL-6. NNK was dissolved in media and used at 100nM, 1μM, 10μM and 500μM for MTT assays. IL-6 and Hyper-IL-6 were used at 100ng/ml and shIL-6R at 1:100. Inhibition of IL-6 signalling was achieved with a human anti-IL-6R monoclonal antibody (MRA/Tocilizumab) used at 10ug/ml, which was a kind gift from Dr Walter Ferlin (NovImmune).

2.4 RNA preparation and analysis

2.4.1 Cell harvesting for RNA extraction

Following stimulations, 350μl cold RLT (Qiagen) / β-Mercaptoethanol (β-ME, Sigma-Aldrich) buffer was added to the cells (10μl β-ME /1ml RLT), which were scraped and passed through a blunt 25 gauge needle 5 times. Cell lysates were frozen down and kept at -20°C, or RNA purification was continued as below.

2.4.2 Total RNA extraction from tissues

50μg of tissue was homogenised in 1mL of Trisure (Bioline, Alexandria, NSW, Australia) in a flat bottomed tube and transferred to a 15ml Falcon tube. Tubes were centrifuged for 30min at 1930 *xg* at 4°C to pellet insoluble material. The supernatant was transferred to a clean 15ml Falcon and 200μl Chloroform (Ajax Finechem, Seven Hills, NSW, Australia) was added, mixed, incubated at RT for 3 min and centrifuged for 45min at 1930 *xg*. The upper aqueous phase was transferred to a clean 15ml Falcon tube, 500μl Isopropanol was added, mixed, incubated at RT for 10min and centrifuged for 45min at 1930 *xg* to pellet the RNA. After discarding the supernatant, the pellet was washed in 500μl 75% ethanol/Diethylpyrocarbonate (DepC) treated H_2O (Appendix II) and centrifuged for 15min at 3000rpm. The supernatant was again discarded and the pellet dried for 30 min at RT. After resuspension in 50μl DepC H_2O, the RNA concentration was determined by assay on a

Nanodrop (Thermo Scientific, Wilmington, USA). RNA quality was determined by assessing 18S to 28S ratio on a 1% agarose/TAE gel.

2.4.3 RNA extraction from Formalin-Fixed, Paraffin-Embedded (FFPE) tissue

RNA extraction was performed with the Qiagen FFPE RNeasy® kit according to the manufacturer's instructions. Briefly, after trimming the block of excess paraffin, 4-6 20µm sections were placed into a 2mL eppendorf tube after the first 2 sections were discarded. 1ml of xylene was added, and after vigorous vortexing for 10 sec, the tube was centrifuged at full speed for 2 min to pellet the tissue. Once the supernatant was removed, 1mL 100% ethanol was added to the pellet, vortex mixed and centrifuged at full speed for 2 min. The ethanol was carefully removed and the pellet air-dried. Buffer PKD and Proteinase K were added to the pellet, mixed and then incubated at 56°C for 15min and subsequently 80°C for 15min. Buffer RPE was added to adjust binding conditions, mixed and transferred to the gDNA Eliminator® spin column for removal of contaminating genomic DNA. The column was centrifuged for 30sec at 8000 *xg*. Absolute ethanol was added to the flow through and this was then transferred to an RNeasy MinElute® spin column and centrifuged at 8000 *xg* for 15sec. The membrane was washed with two aliquots of RPE buffer before the RNA was eluted with 30µl RNase free H_2O by centrifugation at full speed for 1 min.

2.4.4 RNA purification

RNA purification and DNase treatment were performed "on column" with the QIAGEN RNeasy® kit according to the manufacturer's instructions. Briefly, 100µg of RNA determined by Nanodrop was added to RLT buffer containing 100% ethanol and 1% β-ME. After thorough mixing by pipetting up and down, the entire volume was added to an RNeasy column and centrifuged for 30sec at 8000 *xg*. The flow through was discarded, RW1 buffer added before centrifugation for 30sec at 8000 *xg*. 10µg DNase/70µl RDD buffer was applied directly to the membrane and incubated for 15min at RT. The membrane was then washed with RW1 buffer and then twice with RPE buffer, with centrifugation for 30sec at 8000 *xg* between each wash. The flow through was discarded and the membrane dried by centrifugation at maximum speed for 1min. RNA was eluted with 2 x 30µl aliquots of RNase free H_2O and the concentration and quality determined as before.

2.4.5 Reverse transcription reaction

RNA extracted from frozen tissue was reverse transcribed with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Briefly, 1µg of RNA made up to 9.4µl with DepC H_2O was added to 2µl of random hexamers and denatured

by being incubated at 65°C for 10 min. Once on ice, 4µl 5x Transcriptase Reaction Buffer, 0.5µl RNase inhibitor, 2µl dNTPs, 1µl Dithiothreitol (DTT), and 1.1µl High Fidelity Reverse Transcriptase were added to samples. A control reaction without the reverse transcriptase (-RT) was performed to confirm no contaminating genomic DNA was present. Samples were incubated at 55°C for 60 min before the reactions were heat inactivated by incubating at 85°C for 5 min.

RNA from cells and FFPE tissue was reverse transcribed using the Superscript III cDNA Synthesis Kit (Invitrogen) as I showed this to produce higher quality cDNA. Briefly, 1µl of 10mM dNTPs and 1µl of random hexamers was added to 500-1000ng of RNA made up to 8µl with DepC H_2O and incubated at 65°C for 5 min. After cooling on ice for 1 min, 2µl 10xRT buffer, 4µl 25mM magnesium chloride, 2µl 0.1M DTT, 1ul RNase out and 1µl Superscript III was added. A control reaction without the reverse transcriptase (-RT) was performed to confirm no contaminating genomic DNA was present. Samples were incubated at 25°C for 10 min then 50°C for 50 min before the reactions were heat inactivated by being incubated at 85°C for 5 min, pulsed down and placed on ice. Finally, 1µl RNase H was added to samples before they were incubated at 37°C for 20 min to degrade any remaining RNA.

Resulting cDNA was used immediately in qPCR or stored at -20°C until used.

2.4.6 Quantitative Real-Time PCR (qPCR)

An Applied Biosystems 7900HT Fast Real-Time PCR system (Forster City, USA) and SYBR Green (Invitrogen) were used for qPCR. All primer sequences are shown in Appendix III. cDNA was added to SYBR Green, 0.2µM forward primer, 0.2µM reverse primer and DepC H_2O to produce a reaction volume of 10µl. Samples were plated in triplicate in MicroAmp Optical 384-well plates (Applied Biosystems). The qPCR cycling protocol involved 40 amplification cycles and an additional dissociation step to confirm PCR product purity. Data acquisition and analysis was performed with the SDS Version 2.4 software (Applied Biosystems). Genes of interest were normalised to 18S and fold change determined using the delta-delta cycle threshold (Ct) method.

2.5 Protein preparation and analysis

2.5.1 Lysate preparation from tissue and cells

Protein lysates were prepared from snap frozen lung tissue by homogenising approximately 50mg tissue in 750µl ice cold protein lysis buffer (Appendix II) supplemented by 0.1mmol sodium fluoride (Sigma), 16µM sodium orthovanadate (Sigma), 0.1% protease inhibitor (Roche) and 0.1% phosphatase inhibitor (Roche). After agitation for 30 min at 4°C,

homogenates were centrifuged for 5 min at 8000 *xg* to pellet debris. Supernatants were transferred to clean 1.5ml eppendorf tubes and pre-cleared of non-specific immunoglobulins by the addition of 20µl Protein G sepharose beads (GE Healthcare, Uppsala, Sweden) and further agitated for 30 min at 4°C. The beads were pelleted by centrifugation for 3 min at 3000rpm and the supernatant transferred to a clean eppendorf tube and stored at -80°C until required.

Cells for preparation of protein lysates were seeded in a 6 or 24 well plate containing either 0.5 x 10^6 cells (6 well) or 1 x 10^5 cells (24 well). After removal of media and a wash with warmed DPBS, 300 or 100µl of ice cold lysis buffer supplemented as above was added. Cells were scraped within the wells before the buffer was transferred to eppendorf tubes and agitated for 30 min at 4°C, after which homogenates were centrifuged for 5 min at 8000 *xg* to pellet debris. Supernatants were transferred to a clean eppendorf tube and stored at -80°C until required.

2.5.2 Lowry Protein Assay

Protein concentrations were determined using a Lowry assay (Lowry et al., 1951). Standards were prepared from stock solutions of Bovine Serum Albumin (BSA) at concentrations of 1mg/ml, 5mg/ml and 10mg/ml (Table 2.1). Working Reagent was prepared by adding 20µl Reagent S (Biorad) to each 1ml of Reagent A (Biorad). Standards and 5µl of protein lysates were pipetted into wells of a flat-bottomed 96 well plate (Becton, Franklin Lakes, USA) in duplicate. 25µl of the Working Reagent was added to each well followed by 200µl of Reagent B (Biorad). After mixing, the plate was incubated at RT for 15min and absorbance was determines at 450nm using the FLUROstar Optima plate reader (BMG Labtech, Ortenberg, Germany).

Table 2.1: Preparation of protein standards for Lowry assay.

BSA stocks	-	1mg/ml	1mg/ml	1mg/ml	5mg/ml	10mg/ml	10mg/ml
Standard Conc.	**-**	**1µg**	**2µg**	**4µg**	**8µg**	**16µg**	**32µg**
Stock vol.	0µl	1µl	2µl	4µl	1.6µl	1.6µl	3.2µl
dH20 vol.	5µl	4µl	3µl	1µl	3.4µl	3.4µl	3.4µl
Final vol.	5µl	5µl	5µl	5µl	5µl	5µl	5µl

2.5.3 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to determine protein electrophoretic mobility/size. 1.5mm polyacrylamide gels (10%) were prepared (Appendix II) using Mini-PROTEAN Tetra Electrophoresis casting plates (Biorad). Gels were transferred to a Mini-PROTEAN Tetra Electrophoresis system tank and adequate Running Buffer (Appendix II) to cover wells was added. An equal volume of sample buffer (Appendix II) was added to 20µg of protein before samples were denatured by being incubated at 95°C for 5 min. Samples were added to wells alongside a standard molecular weight marker (New England Biolabs, Ipswich, USA). The gels were run at 100V until the leading protein band reached the bottom of the gel. The gel was transferred onto nitrocellulose Hybond-C extra membranes (Amersham, Buckinghamshire, UK) using the wet transfer method. Briefly, after all components were equilibrated in Transfer Buffer (Appendix II) a sandwich was made of two fibre-pads, two filter papers, the nitrocellulose membrane and the gel and transferred to a Mini Transfer-Blot Electrophoretic Transfer Cell apparatus (Biorad) and covered with transfer buffer. Proteins were transferred for 90 min at 100V.

2.5.4 Western Blotting

Non-specific binding was blocked by incubating membranes with Odyssey Blocking Buffer (OBB, Li-Cor, NE, USA) supplemented with 0.2% (w/v) BSA and 0.01% Tween-20 (Sigma-Aldrich) for 1 hour at RT. Membranes were subsequently probed with the appropriate dilution of primary antibody (Appendix II) in OBB supplemented as above, overnight at 4°C. After washing the membrane three times for 5 min in PBS / 0.01% Tween-20 to remove unbound antibody, the membranes were incubated with the appropriate fluorescent labelled secondary antibody (AlexaFluor 680 (Invitrogen) or IRDye800CW (Li-Cor)) diluted 1:3000 in OBB supplemented with 0.01% Tween-20 for 1 hour at RT. Membranes were rewashed three times for 5 min in PBS / 0.01% Tween-20. Target proteins were visualised on the Odyssey Fluorimager (Li-Cor).

2.5.5 Stripping Western blot membranes

Membranes requiring probing for additional proteins were stripped in Stripping Buffer (Appendix II) for 5 min at 55°C. After washing the membrane three times for 5 min in PBS / 0.01% Tween-20, it was reblocked in OBB supplemented with BSA and Tween-20 as above, for 30 min at RT. Probing with primary and secondary antibodies was then performed as described in Section 2.5.4.

2.5.6 Enzyme-linked Immunosorbent Assay (ELISA)

Human IL-6 (BD Biosciences) and sIL6-R (R&D Systems, MN, USA) were quantified using commercial ELISA kits according to the manufacturer's instructions. Briefly, flat-bottomed 96-well f96 maxisorp plates (Nunc, Roskilde, Denmark) were coated with 100µl Capture antibody diluted in coating buffer (Appendix II) and incubated overnight at 4°C. The plate was brought back to RT and washed three times with PBS/ 0.05% Tween-20 before non-specific binding was blocked by incubating with 200µl blocking buffer for 1 hour at RT. The plate was then washed as described above and serial dilutions of standards added and 100µl of samples added in triplicate. A top standard of 500pg/ml and bottom standard of 3.91pg/ml was used, diluted in blocking buffer. Samples and standards were incubated for 2 hours at RT before the plate was again washed three times as above. For the IL-6 ELISA, detection antibody and Streptavidin-HRP were then added together (Appendix II) and incubated for 2 hours at RT. For the sIL-6R ELISA first the detection antibody was added and incubated for 2 hours at RT then after 3 washes the Streptavidin-HRP added and incubated for 20 min at RT. Following washes as described above 100µl 3,3',5,5' tetramethylbenzidine (TMB) substrate (Thermo Scientific, IL, USA) reagent was added and incubated for 20 min at RT. The reaction was terminated by addition of 50µl stop solution (Appendix II) and absorbance measured at 450nm on the FLUROstar Optima plate reader. Protein levels were determined by comparison to the standard curve.

2.6 Fluorescence-Activated Cell Sorting (FACS)

2.6.1 Preparation of lung single cell suspensions

Following BALF collection, heart and lungs were excised and the pulmonary circulation was cleared by perfusion with 10ml PBS via the right ventricle. The lung tissue was placed into 1ml of 0.2% BSA in Hanks Buffered Salt Solution (HBSS, Invitrogen) and mechanically dissociated before being incubated in HBSS and collagenase A (Roche Applied Sciences) at a concentration of 1.5mg/ml at 37ºC for 60 min. 4mg/ml Deoxyribonuclease I (Sigma-Aldrich) was added for 30 min at 37ºC. Lungs were further disaggregated by aspiration through a 21 gauge needle. Resultant single cells were washed twice with DMEM before red blood cells were depleted with buffered ammonium chloride (Appendix II). Cells were washed with PBS, filtered through 200µm mesh and counted $1x10^6$ cells were resuspended in 1% Fc block (eBioscience, San Diego, CA) in FACS buffer (Appendix II).

2.6.2 Cell staining

Each sample was labelled in triplicate; a myeloid, B cell and T cell tube with directly conjugated antibodies to CD45, Gr1, Mac1, B220, CD3e and/or CD4 and CD8 for 30min on ice (Appendix II). Samples were counted on a FACSCANTOII cell analyser (Becton Dickinson). Live cells were identified by propidium iodide exclusion. The percentage of leukocytes was determined as the proportion of CD45 positive live cells, and this population was further differentiated according to other surface markers.

2.7 Statistical analysis

Statistical analyses were performed using Prism 5 for Windows (v.5.04; GraphPad Software, Inc. La Jolla, USA). Differences between two groups were determined using t-tests and Analysis of variance (ANOVA) was used for groups of three or more. Corrections for the population characteristics (including distribution) was made as appropriate. Data is expressed as mean ± Standard Error of the Mean (SEM) unless otherwise stated. Significance was set at $p < 0.05$.

CHAPTER 3

Characterisation of the pulmonary inflammatory profile in mice with deregulated gp130 signalling

3.1 The gp130$^{F/F}$ (FF) mouse as a model for pulmonary inflammation and emphysema; implications for the development of NSCLC

There is abundant evidence that inflammation plays a role in the development of lung cancer (Coussens and Werb, 2002, Engels, 2008), and that it is likely to be a crucial co-factor in the transition from COPD/emphysema to lung cancer (Houghton et al., 2008, Moghaddam et al., 2009). Both lung cancer and emphysema are aetiologically linked to cigarette smoke but there appears to be a link between the two independent of the amount of smoke exposure (de Torres et al., 2007). It is well known that cigarette smoke induces pulmonary inflammation in all smokers but only a small percentage develop clinical disease (Niewoehner et al., 1974, Young and Hopkins, 2010), suggesting environmental or genetic modifiers. Both IL-6 and STAT3 (Haura et al., 2005b, Qu et al., 2009b, Chyczewska et al., 1997) are intimately associated with lung cancer and emphysema but their precise roles remain to be fully elucidated, due in part to a paucity of informative genetically-defined animal models. One such model shows that when a persistently-activated STAT3 mutant (STAT3C) is over-expressed in the mouse pulmonary epithelium, lung tumours develop (Li et al., 2007b). Whilst this adds weight to the evidence of a role for STAT3 in the development of lung cancer, this artificial over-expression model does not mimic endogenous elevations in STAT3 activation or delineate upstream pathways or ligands that may lead to this activation. Conversely, whilst IL-6 knock-out mice show a reduction in tumour development in a *LSL*Kras^{G12D} model of lung cancer (Ochoa et al., 2011), it is unclear whether it is STAT3, PI3K/Akt or Ras/MAPK downstream of gp130 that is important for development of tumours.

To better define the role deregulated gp130 signalling plays in the development of lung cancer and emphysema, I utilised the gp130$^{F/F}$ (FF) mouse which displays elevated expression levels of IL-6, hyperactivation of gp130/STAT3 signalling and loss of gp130 driven PI3K/Akt and Ras/MAPK activation (Tebbutt et al., 2002), as described in Chapter 1. The FF mouse is known to develop spontaneous multi-organ inflammation (gastritis, peritonitis) as well as a

number of hematopoietic derangements including neutrophilia, thrombocytosis, splenomegaly and lymphadenopathy (Jenkins et al., 2007). In addition, FF mice spontaneously develop gastric adenomas as a consequence of the STAT3 driven gastric inflammation (Tebbutt et al., 2002), thus clearly demonstrating the transition to malignant disease as a result of chronic inflammation. The extra-pulmonary abnormalities seen in the FF mice are substantial but there does seem to be a degree of separation of the driving pathways, that is it is IL-11 driving gastric tumourigenesis but IL-6 driving the pulmonary pathology. This compartmentalisation does allow for examination of lung cancer pathogenesis in the presence of the systemic abnormalities without these significantly confounding our findings.

Before engaging these mice in well-defined models of lung cancer, the lung phenotype needed to be defined. In aging these mice, it was noted that the lungs of 6 month old FF mice showed enlarged airspaces consistent with emphysema (Ruwanpura et al., 2011), and further analysis was undertaken. The aim of this section of my project was therefore to characterise the inflammatory infiltrate in FF mice displaying deregulated gp130 signalling. In addition, I also examined a number of compound mutant FF mice to genetically dissect the gp130-associated ligand and transcription factors involved in the pulmonary inflammation. It was expected, as has been shown previously in other organs (Greenhill et al., 2011, Jenkins et al., 2005a), that STAT3,but not STAT1, would be the driver of the pulmonary inflammation, so I used FF mice with genetically normalised STAT3 levels, $gp130^{F/F}$:STAT3-/+ (FF:St3-/+) and ablation of STAT1 levels (FF:St1:-/-). Finally, to examine the role of IL-6 specifically, I used FF mice crossed with IL-6 knock-out mice (FF:IL6-/-). Importantly, FF:St3-/+ and FF:St1-/- mice, but not FF:IL6-/- mice, develop emphysema (Ruwanpura et al., 2011, Ruwanpura et al., 2012). This work forms part of the overall phenotypic characterisation and the above cited two published manuscripts detail this (see Appendix I).

3.2 Augmented pulmonary gene expression of key inflammatory mediators in FF mice

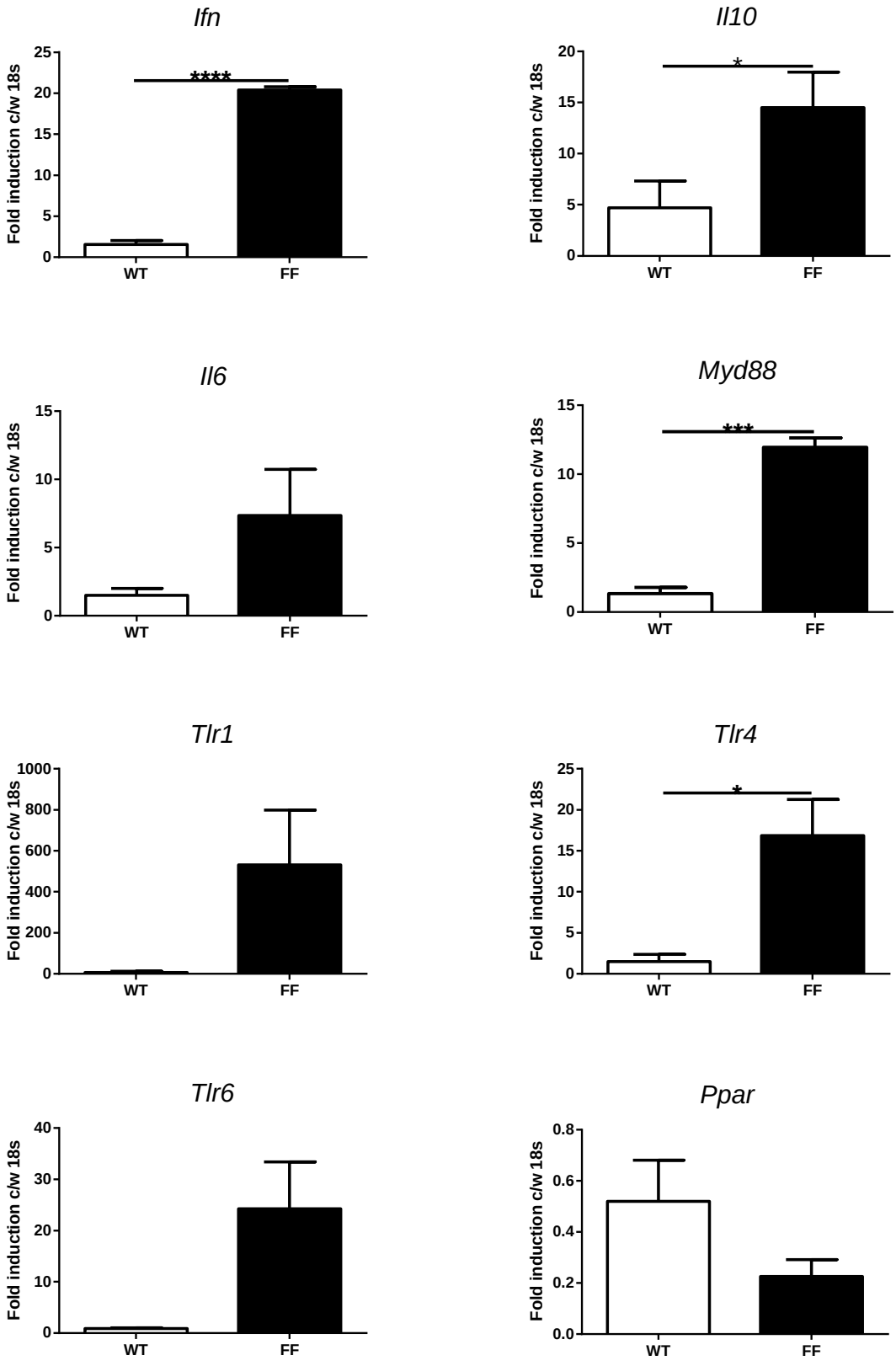
The first step of my characterisation was to assess for alterations in the pulmonary expression of genes encoding key inflammatory cytokines and chemokines previously associated with lung disease, and particularly lung cancer (Barnes, 2004, Coussens and Werb, 2002, De Vita et al., 1998, He et al., 2007, Lappalainen et al., 2005, Pinto et al., 2011). To gain a wider view of the changes in inflammatory gene expression, and considering the proposed role of the Toll-like receptor (TLR) signalling pathway in cancer (Bauer et al., 2009, He et al., 2007, Pinto et al., 2011), and its importance in the regulation of inflammation (Beutler, 2009), I undertook a TLR-specific PCR array (Appendix III). For this purpose, I compared the relative expression in 6 month old FF mice with age-matched WT controls, and considered any change of greater than 1.5-fold to be significant. Using these parameters I identified 8

genes consistently elevated (*Ifng*, *Il10*, *Il1β*, *Il6*, *Myd88*, *Tlr1*, *Tlr4* and *Tlr6*) and 1 gene reduced (*Pparα*) in FF mice (Appendix III).

Interestingly, all the upregulated genes are pro-inflammatory with the exception of IL-10 which is known to be a potent immunosuppressive cytokine (de Waal Malefyt et al., 1992). Despite the generally pro-inflammatory tumour micro-environment, increased expression of IL-10 has been implicated in the pathogenesis of lung cancer (Wang et al., 2011a, Zeng et al., 2010, Zeni et al., 2007). Ppar-α (Peroxisome proliferator–activated receptor-α) is also anti-inflammatory and plays a major role in lipid metabolism and perhaps in epithelial proliferation (Yasui et al., 2008). While Ppar-γ has been shown to be expressed in a number of cancer cells including lung cancer (Segawa et al., 2002), the role Ppar-α plays in the pathogenesis of lung cancer is less clear. It has been shown to be increased in NNK-induced mouse lung tumours (Li et al., 2010) but somewhat contradictory to this agonists appear to have no effect on apoptosis or proliferation (Tsubouchi et al., 2000).

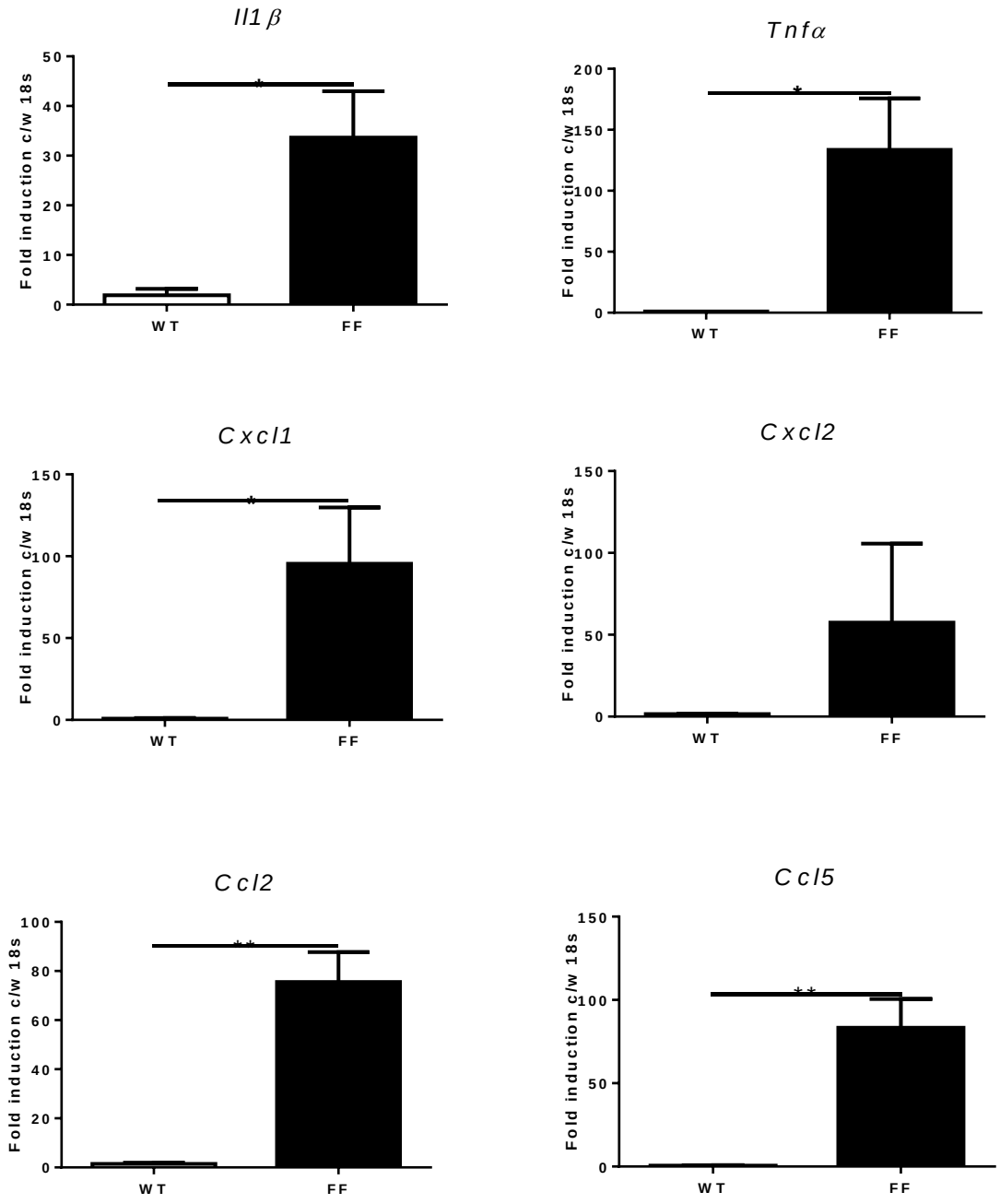
To validate these gene expression findings, qRTPCR was performed on 6 month old WT and FF mice (n=3 per genotype) with the exception of *Il1β*, results for which are included below. However as shown in Figure 3.1, while all genes showed a trend in the same direction as was suggested by the PCR array, only *Ifng*, *Il10*, *Myd88* and *Tlr4* were found to have a significant difference ($p<0.0001$, $p=0.0432$, $p=0.0005$ and $p=0.0385$ respectively; unpaired t-test with Welch's correction). *Il6* expression has been previously shown to be significantly elevated in other organs in the FF mice (Jenkins et al., 2007), and in fact, when analysing more mice (thereby reducing type-1 error) was significantly upregulated in the lung also (Ruwanpura et al., 2011). Even without the other 3 genes showing significance it is clear the FF mice show an inflammatory phenotype, so while increasing mouse numbers may have produced a significant result these genes were not pursued further in additional mice of either genotype.

To further examine the inflammatory profile, subsequent gene expression analyses were performed on those mediators known to play a role in lung cancer. As discussed in chapter 1, TNF-α and IL-1β are known to play an important role in the development and progression of lung cancer (De Vita et al., 1998), as well as pulmonary inflammation and COPD (Barnes, 2004). CXCL-1 and -2 are chemotactic for macrophages and neutrophils, are elevated in the airways of subjects with COPD and on tumour cells (Mantovani et al., 2004b), and are stimulated by TNF-α (Barnes, 2004) and IL-1β (Lappalainen et al., 2005). CCL-2 and -5 are important in chemotaxis of macrophages and T lymphocytes, respectively, and are implicated in inflammation, emphysema (Barnes, 2004, Bracke et al., 2007) and lung cancer (Mantovani et al., 2004a). At 6 months of age, all examined cytokines (*Il1b* ($p=0.0217$) and *Tnfa* ($p=0.0256$)) and chemokines (*Cxcl1* ($p=0.0354$), *Cxcl2*, *Ccl2* ($p=0.0044$) and *Ccl5*



<u>Figure 3.1:</u> Genes identified in the PCR array with differential regulation were validated with qRTPCR.

qRTPCR expression analyses of *Ifnγ*, *Il10*, *Il6*, *Myd88*, *Tlr1*, *Tlr4*, *Tlr6* and P*parα*, were performed on cDNA derived from the lungs of 6 month old WT and FF mice. Relative expression data (n = 3 per genotype) following normalisation for *18S* expression are shown, and are presented from triplicate analysis as the mean ± SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001, versus expression in age-matched WT mice.



<u>Figure 3.2:</u> FF mice show upregulated gene expression of inflammatory cytokines and chemokines.

qRTPCR expression analyses of *Il11β*, *Tnf-α*, *Cxcl*, Cxcl*2*, *Ccl2* and *Ccl5* were performed on cDNA derived from the lungs of 6 month old WT and FF mice. Relative expression data (n = 4 per genotype) following normalisation for *18S* expression are shown, and are presented from triplicate analysis as the mean ± SEM. *p < 0.05, **p < 0.01 versus expression in age-matched WT mice (unpaired t-test with Welch's correction).

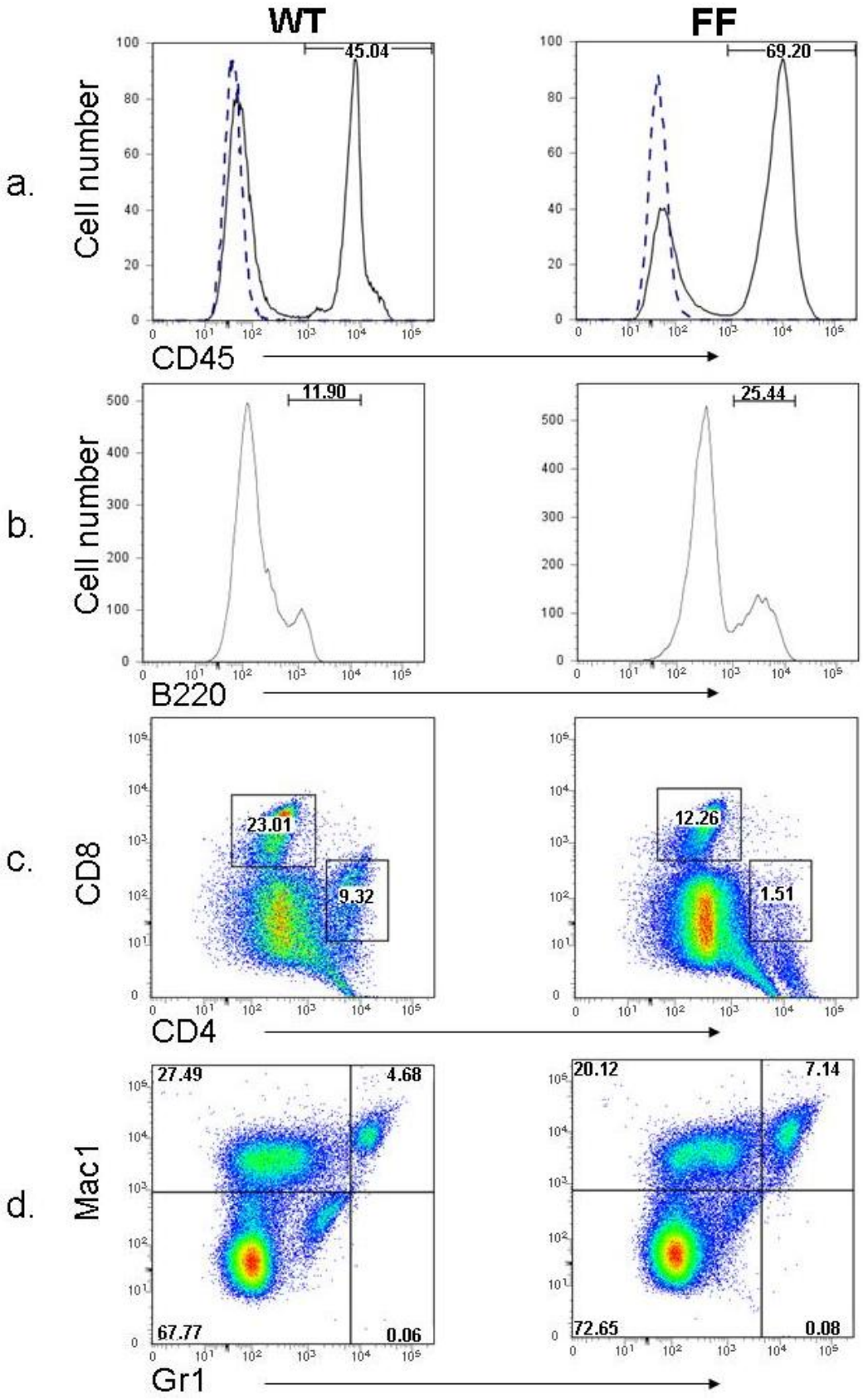
(p=0.0085)) expression levels were increased in FF mice when compared to WT mice (Figure 3.2).

3.3 FF mice have an increase in lung tissue inflammatory cells without a change in the intra-alveolar component

It has been previously shown that there are a number of distinct inflammatory cell populations within the lung, and at least 2 of these (parenchymal and intra-alveolar) have been suggested to play a role in the pathogenesis of lung cancer (Anselmo et al., 2005, Coussens and Werb, 2002). Accordingly, I next examined the inflammatory cells resident within the lung, both within the tissue compartment by flow cytometry, and the intra-alveolar compartment by examining bronchoalveolar lavage fluid (BALF).

The lungs of 6 month old WT and FF mice were collected as described in Chapter 2, and to minimise contamination by cells within the vascular compartment, lungs were perfused free of blood with ice-cold PBS via the right ventricle prior to harvest. Single cell suspensions stained for myeloid and lymphoid (i.e. B and T cell) markers were initially gated on CD45, a pan-leukocyte marker. As shown by the representative plots in Figure 3.3a, there was a clear increase in the proportion of $CD45^+$ inflammatory cells within the lung of FF mice compared to WT mice (see also Table 3.1). Further differentiation of the $CD45^+$ cell populations revealed that $B220^+$ cells accounted for the majority of this increase. There was a degree of individual variability with some mice showing line specific changes, but overall there was no significant change in T cell proportions ($CD4^+$ or $CD8^+$ cells) or within the myeloid lineage ($Mac1^+$ or $Mac1^+/Gr1^+$ cells) (Figure 3.3b-d and Table 3.1).

Substantial evidence exists that the inflammatory cell population within the alveolar compartment changes when challenged with exogenous stimuli, most commonly cigarette smoke (Gualano et al., 2008). Furthermore, there is some suggestion that this population of inflammatory cells contributes to the pathogenesis of both COPD (Barnes, 2004) and lung cancer (Anselmo et al., 2005), although there is also data suggesting such inflammatory cell populations are unchanged in lung cancer (Staal-van den Brekel et al., 1998). With this in mind, it was important to examine the intra-alveolar cell population as part of the description of the FF lung phenotype. Following euthanasia, BALF was obtained (as per Chapter 2), and subjected to cytocentrifugation, following which differential cell counts were attained by manual counting. As expected in the non-stimulated lung, the majority of the cells were macrophages (Takaishi et al., 1991), with small proportions of neutrophils and lymphocytes, and infrequent other (e.g. basophils, eosinophils) inflammatory cells (Table 3.2). Contrary to what was observed within the tissue compartment, there was no difference in the proportion



<u>Figure 3.3:</u> FF mice have an increase in pulmonary B220$^+$ cells.

Shown are representative histograms (a and b) and dot plots (c and d) of whole lung single cell suspensions from 6 month old WT and FF mice that were subjected to multi-colour FACS. Cells were gated on CD45 to delineate leukocytes, and subsequently on cell type specific markers.

Table 3.1. The lungs of FF mice display an increased proportion of inflammatory cells with a predominance of B220$^+$ cells.

	CD45$^+$	Mac1$^+$ / Gr1$^-$	Mac1$^+$ / Gr1$^+$	B220$^+$	CD4$^+$	CD8$^+$
WT	48.1% ±9.5%	11.5% ±3.8%	2.2% ±1.5%	6.2% ±4.1%	3.7% ±2.4%	8.5% ±6.3%
FF	69.7% ±5.5% **	15.5% ±7.7%	4.8% ±5.8%	16.2% ±6.5% **	2.9% ±3.6%	8.1% ±3.4%

Whole lung single cell suspensions from WT and FF mice that were subjected to multi-colour FACS. Cells were gated on CD45 to delineate leukocytes, and subsequently on cell type specific markers. Inflammatory cell number and differential as a proportion of all counted cells, displayed as mean ± SD from n=7 individual mice per genotype. **$p < 0.01$ (unpaired t-test with Welch's correction).

Table 3.2. There is no difference between WT and FF mice in the inflammatory cell populations in BALF.

	Macrophages	**Neutrophils**	**Lymphocytes**	**Other**
WT	98.3 ±1.2	0	1.7 ±1.2	0
FF	97.1 ±1.9	0	2.9 ±1.9	0

Percentage of inflammatory cells in BALF determined by manual counting of at least 200 cells from cytocentrifuge preparations. Represented at mean ± SD from n=3 mice per genotype.

of cells within the BALF between WT and FF mice. This is perhaps unsurprising given the mice were not exposed to any exogenous stimuli.

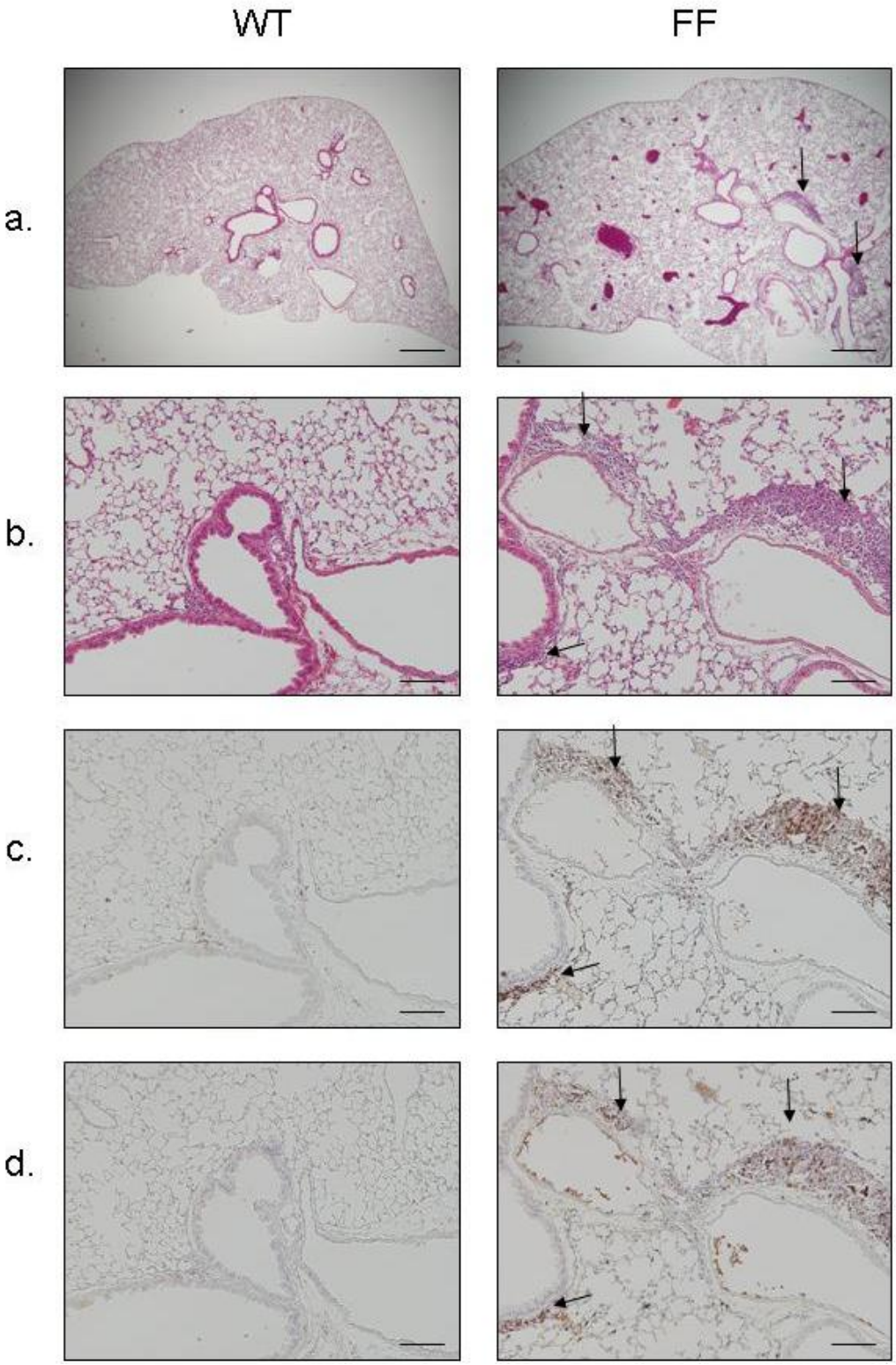
3.4 The increase in inflammatory cells in the FF lung is not uniform

Having established an increase in both the total number of inflammatory cells, and particularly $B220^+$ cells, in the lungs of FF mice, I set out to determine if the cells were uniformly distributed throughout the lungs. Histopathological examination of formalin-fixed, paraffin-embedded sections of lungs stained with H&E revealed that there were peri-bronchovascular infiltrates in the lungs of FF mice that were not seen in WT mice (Figure 3.4a and b). By contrast, there was no clear change in parenchymal inflammatory cells. To confirm these infiltrates were in fact inflammatory cells, and more particularly the $B220^+$ cells identified by FACS, the sections were stained with CD45 and B220. As seen in Figure 3.4c and d, the peri-bronchovascular infiltrates stained strongly for both CD45 and B220 confirming this was the same population identified by flow cytometry.

3.5 IL-6 signalling via STAT3, but not STAT1, appears to drive lung inflammation in FF mice

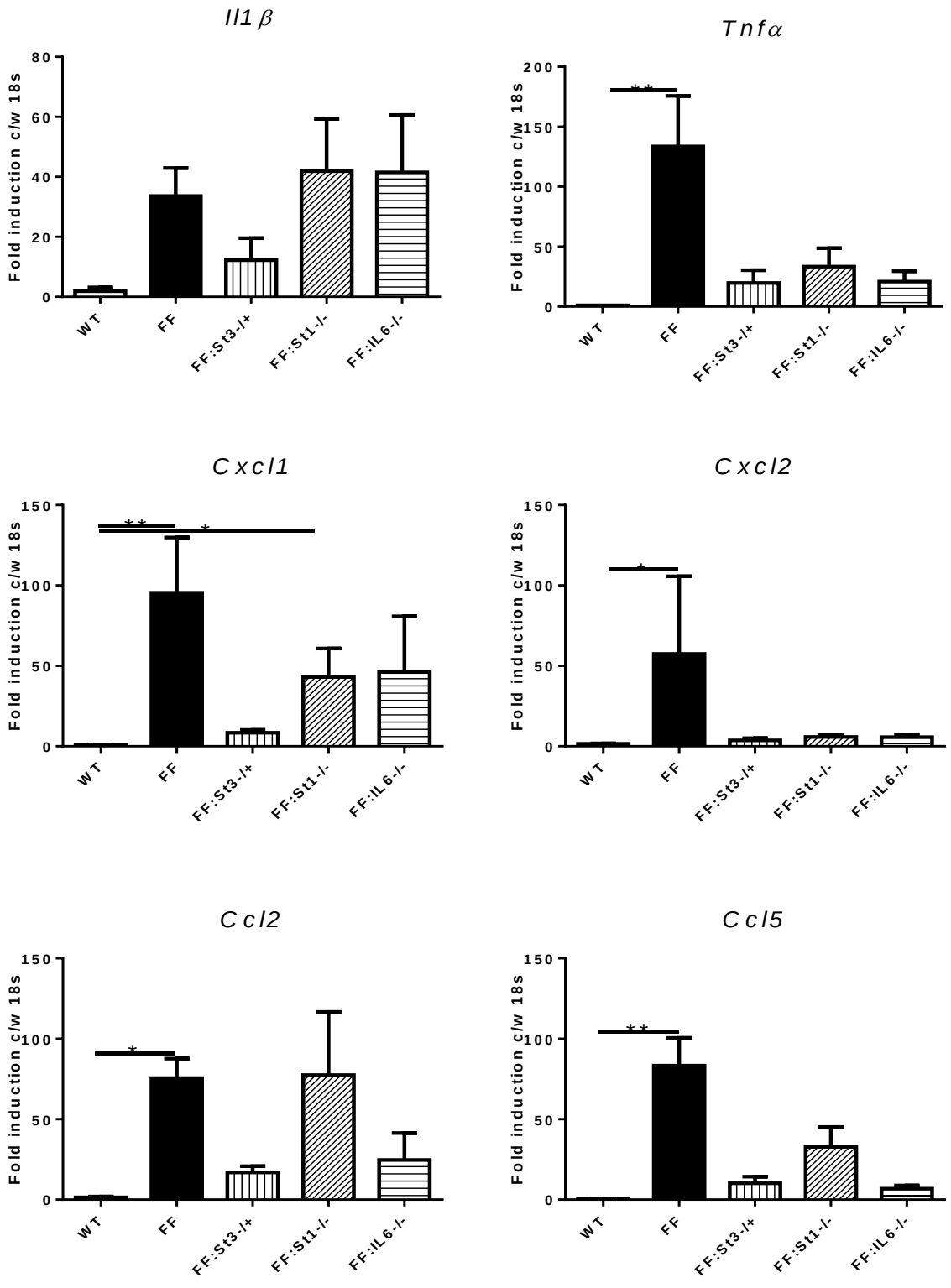
While other gp130 ligands have been linked with lung disease (Fritz et al., 2011), IL-6 is the main pro-inflammatory cytokine implicated in lung inflammation, emphysema and lung cancer (Ochoa et al., 2011, Yanagawa et al., 1995, Bhowmik et al., 2000). Jak/Stat signalling downstream of gp130 has similarly been linked to lung cancer pathogenesis, predominantly through STAT3 (Qu et al., 2009b, Achcar Rde et al., 2007, Ai et al., 2012, Bowman et al., 2000). STAT1 is also pro-inflammatory and does signal downstream of gp130, although the majority of its actions are effected downstream of the IFNs (Imada and Leonard, 2000), but a role in lung cancer is not clearly defined (Li et al., 2007a). Therefore, to genetically elucidate the role of IL-6 and STAT1/3 in promoting pulmonary inflammation in FF mice, I next examined gene expression and inflammatory cell populations as described above in the following compound mutant mice: FF:St3-/+ and FF:St1-/- mice which develop emphysema, and FF:IL6-/- mice that do not develop emphysema. In addition to defining a causal role for the STATs and IL-6 in the development of the pulmonary inflammation, this work also assisted in the delineation of the role of inflammation in the development of emphysema, as described in the published manuscripts (see Appendix I).

Gene expression of all the inflammatory mediators was comparable between 6 month old WT and FF:St3-/+ mice (Figure 3.5). While some upregulation was seen (e.g. *Tnfα* and *Ccl2*) this change was not significant when the group was analysed as a whole. Similarly, there is no significant difference between 6 month old FF and FF:St3-/+ mice despite an apparent large



<u>Figure 3.4.</u> FF lungs have peri-bronchovascular infiltrates which are made up predominantly of B220^{+} cells.

Shown are representative photomicrographs of lungs from 6 month old WT and FF mice stained with H&E (a, b), CD45 (c), and B220 (d). Scale bars = 500μm in row a. and 100μm in rows b. - d. Arrows indicate inflammatory infiltrates.



<u>Figure 3.5.</u> Gene expression of inflammatory mediators is variably elevated in FF compound mutant mice.

qRTPCR expression analyses of *Il1β*, *Tnfα*, *Cxcl1*, *Cxcl2*, *Ccl2* and *Ccl5* were performed on cDNA derived from the lungs of 6 month old mice from the noted genotypes. Relative expression data (n=4 per genotype) following normalisation for *18S* expression are shown, and are presented from triplicate analysis as the mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus expression in age-matched WT mice (Kruskall-Wallace test).

difference for a number of genes (*Tnfα*, *Cxcl1*). Conversely, whilst not as consistently or significantly elevated as in the FF, *IL1b*, *Cxc1*, *Ccl2* and to a lesser degree *Ccl5*, all remained elevated in the lungs of FF:St1-/- mice, suggesting STAT3 but not STAT1 was driving the increased gene expression of inflammatory mediators.

In four of the genes examined, expression levels in the lungs of FF:IL6-/- mice were similar to that demonstrated in FF:St3-/+ mice (*Tnfα*, Cxcl*2*, *Ccl2* and *Ccl5*), while the other two (*Il1β* and *Cxcl1*) showed expression more similar to the FF:St1-/- mice. However, like the FF:St3-/+ mice there was no significant difference between WT and FF:IL6-/- mice when the group was analysed as a whole. Likewise, while *Tnfα*, *Cxcl2* and *Ccl5* appear substantially down in the FF:IL6-/- compared with the FF, this difference does not reach statistical significance at least in part due to wide intra-group variation in gene expression. There is a definite trend in many of the genes studied which may be proven to be significant when repeated with larger numbers.

At the cellular level, flow cytometry revealed a profile in all three compound mutant FF mice (FF:St3-/+, FF:St1-/- and FF:IL6-/-) more similar to the FF mouse than the WT mouse (see Table 3.3). There is a trend towards an increase in all inflammatory cells and $B220^+$ cells, but there is no significant difference between any of the compound mutant mice and WT mice when the group is analysed as a whole.

Like the BALF profiles of the WT and FF mice, there was no significant difference in the inflammatory cell differential profiles in any of the compound mutant mice (Table 3.4), with macrophages again being the predominant cell type present.

The inflammatory status of the lungs of these mice was also examined by comparing H&E stained lung sections from 6 month old mice of each genotype. These analyses revealed that the lungs of both the FF:St3-/+ and FF:IL6-/- mice did not contain the peri-bronchovascular infiltrates seen in the FF lung, whereas they persisted in the FF:St1-/- mouse lung (Figure 3.6).

Taken together, these data suggest that FF mice spontaneously develop pulmonary inflammation by the age of 6 months that is driven by IL6 in a STAT3 dependent, but STAT1 independent, manner.

3.6 Discussion

The main finding from this section of my project was that the FF mice have substantial pulmonary inflammation that is largely comprised of $B220^+$ cells, and that is driven by IL-6 through the STAT3, but not STAT1 signalling pathway. These data are consistent with published data showing marked systemic and multi-organ inflammation in the FF mouse (Tebbutt et al., 2002, Jenkins et al., 2005a, Greenhill et al., 2011). This is in the context of

Table 3.3. The inflammatory cell profile in lungs of FF compound mutant mice is not significantly different from WT mice.

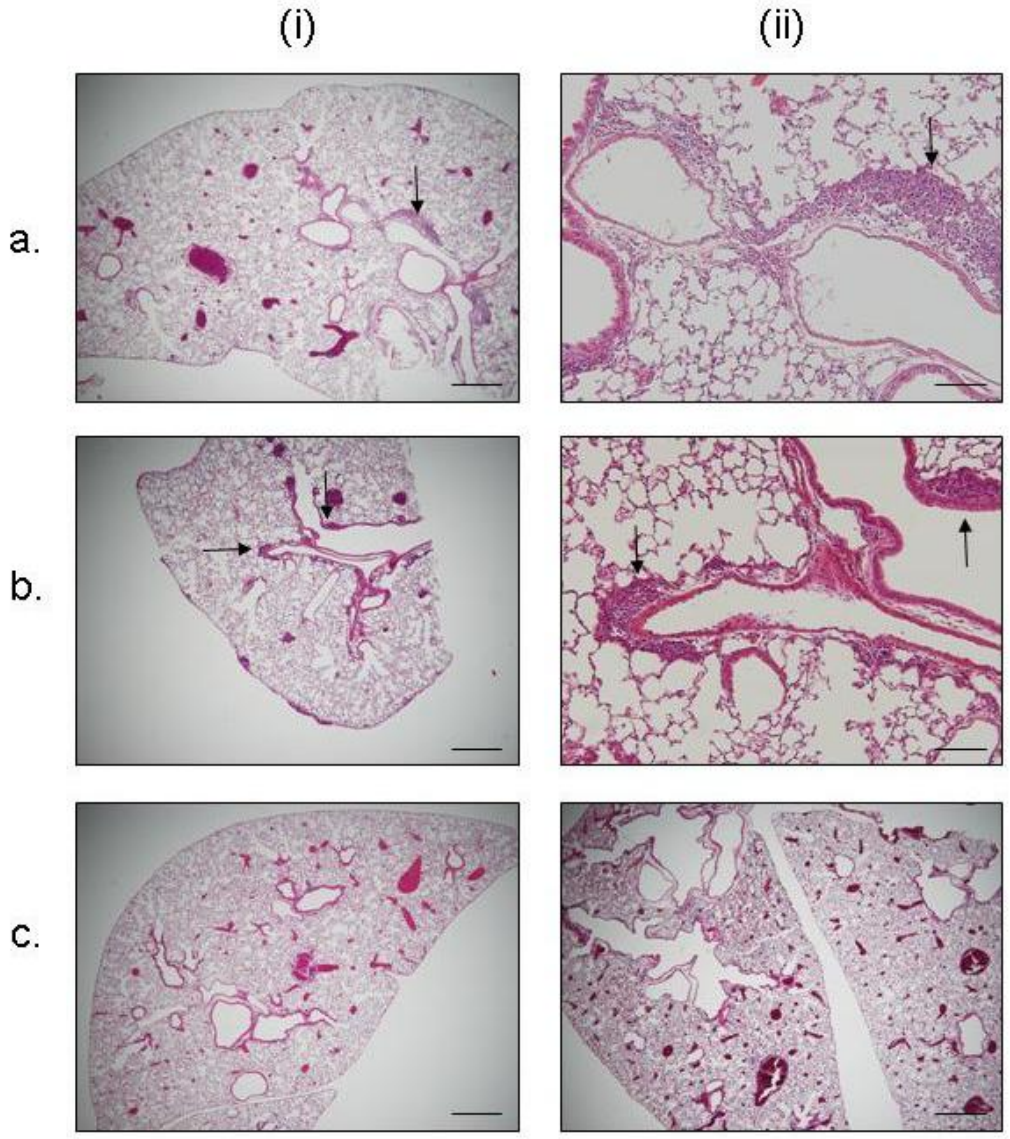
	$CD45^+$	$Mac1^+$ / $Gr1^-$	$Mac1^+$ / $Gr1^+$	$B200^+$	$CD4^+$	$CD8^+$
WT	48.1% ±9.5%	11.5% ±3.8%	2.2% ±1.5%	6.2% ±4.1%	3.7% ±2.4%	8.5% ±6.3%
FF	69.7% ±5.5% **	15.5% ±7.7%	4.8% ±5.8%	16.2% ±6.5% **	2.9% ±3.6%	8.1% ±3.4%
FF:St3-/+	56.7% ±6.6%	15.3% ±6.0%	2.0% ±1.4%	14.0% ±4.0%	1.6% ±0.5%	4.6% ±2.0%
FF:St1-/-	52.5% ±4.3%	10.8% ±2.9%	0.4% ±0.2%	12.3% ±0.7%	1.0% ±0.3%	11.8% ±1.9%
FF:IL6-/-	55.0% ±3.5%	17.4% ±4.6%	1.8% ±1.3%	12.7% ±2.6%	1.8% ±1.7%	3.9% ±2.6%

Inflammatory cell number and differential as a proportion of all counted cells. Displayed as mean ± SD. ** = $p < 0.01$ (Kruskal-Wallace test).

Table 3.4. There was no significant difference in the profile of inflammatory cells in BALF among the mouse genotypes.

	Macrophages	**Neutrophils**	**Lymphocytes**	**Other**
WT	98.4 ±1.2	0	1.7 ±1.24	0
FF	97.1 ±1.86	0	2.9 ±1.9	0
FF:St3-/+	98.5 ±0.9	0	1.5 ±0.9	0
FF:St1-/-	97.8 ±1.2	0.5 ±0.5	1.7 ±1.6	0
FF:IL6-/-	97.1 ±2.5	0.5 ±0.6	2.3 ±2.5	0.1 ±0.2

Percentage of inflammatory cells in BALF determined by manual counting of at least 200 cells from cytocentrifuge preparations. Represented at mean ± SD from n=3 mice per genotype.



<u>Figure 3.6:</u> The peri-bronchovascular infiltrates seen in the FF mouse lung are absent in FF:ST3-/+ and FF:IL6-/- mice but persist in FF:ST1-/- mice

Shown are representative photomicrographs of lungs from 6 month old FF [a], FF:ST1-/- [b], FF:ST3-/+ [c(i)], and FF:IL6-/- [c(ii)] mice stained with H&E. Scale bars = 500μm in column (i) and c.(ii) and 100μm in panels a.(ii) and b.(ii). Arrows indicate inflammatory infiltrates.

other work from our laboratory showing for the first time the spontaneous development of pulmonary emphysema driven by endogenous over-expression of IL-6, but dissociated from STAT3 driven inflammation (Ruwanpura et al., 2011, Ruwanpura et al., 2012). Subsequent to our work, O'Donoghue et al. have also shown that the FF mouse is susceptible to the development of lung fibrosis in response to Bleomycin, in a STAT3 dependent manner (O'Donoghue et al., 2012).

Whilst our work is novel, others have shown that in a smoke induced model of emphysema, reducing numbers of $B220^{+}$ cells by chemically inducing the potent anti-inflammatory molecule heme oxygenase-1, had no effect on the development of emphysema (Brandsma et al., 2008). Interestingly, they showed this reduction in the absence of a change in IL-6 expression. In a transgenic MMP-12 over-expression model that develops marked pulmonary inflammation and emphysema, Qu et al. found that gene expression of both IL-6 and STAT3 increased as emphysema, and subsequently adenomatous hyperplasia, developed (Qu et al., 2009a). However, they did not go on to show that STAT3 was causative, only that it was elevated when the inflammation and emphysema was present.

B220 is a cell surface marker found on all murine B cells, but does not distinguish between immature and activated cells (Rodig et al., 2005). Accordingly, for the purposes of this discussion I equate B220 staining with being a B cell. As previously noted, IL-6 was originally described as a B cell maturation factor (Kishimoto et al., 1995), and the elevation of IL-6 in the FF mouse would explain the development of B cell lymphoid aggregates within the lung. Interestingly, lymphoid collections are seen in the lungs of subjects with severe COPD and are thought to contribute to the inflammatory process (Curtis et al., 2007, van der Strate et al., 2006, Hogg et al., 2004). Additionally, as mentioned in Chapter 1, B and T cells may also both play a role in the development and progression of lung cancer (Granville et al., 2009, Kim et al., 2008, Kristiansen et al., 2003). IL-6 is elevated in serum and BALF from subjects with emphysema/COPD (Bhowmik et al., 2000), higher still in those with NSCLC as well as in the tumour tissue itself (Chyczewska et al., 1997, Gao and Ward, 2007, Yanagawa et al., 1995).

Deregulated activation of STAT3 has been strongly implicated in the development of pulmonary and systemic inflammation, and in lung cancer (Haura et al., 2005b, Qu et al., 2009b). While primarily activated by IL-6, STAT3 is also activated by other type I cytokines including GM-CSF as well as type I IFNs, IL-10 and EGF (Platanias, 2005, Leonard, 2001). This means that a wide variety of upstream events result in activation of STAT3 illustrating the strength of the FF model; STAT3 driven events can be attributed to gp130 acting cytokines in the FF mouse. Downstream targets of STAT3 are inflammatory mediators including IL-6, IL-17, IL-23, IL-1β, CCL2 and COX-2, but also anti-apoptosis genes of the

Bcl family, pro-proliferative genes including myc and Cyclin-D1, and angiogenic genes such as VEGF (Yu et al., 2009a, Chatterjee et al., 2009, Gao and Ward, 2007). Work in our laboratory has examined the role of IL-17 and the IL-17/23 signalling axis using an IL-17 knock-out mouse, and found no correlation in this model with levels of these cytokines (data not shown). STAT3 hyper-activation in the FF mouse is further evidenced by the upregulation of these anti-apoptotic and proliferative genes in a STAT3 dependent manner (Jenkins et al., 2005a). Not surprisingly in this inflammatory and pro-survival environment, in addition to lung cancer, increased STAT3 activation is clearly associated with a number of inflammation associated cancers including stomach, prostate, colorectal and ovarian cancer (Sugar, 2006, Barton et al., 2004, Bollrath et al., 2009, Grivennikov et al., 2009, Klampfer, 2008, Wang et al., 2005, Li et al., 1992, Jenkins et al., 2005a).

By contrast, a role for STAT1 in pulmonary inflammation and subsequent lung disease progression is less clear. STAT1 is known to have pro-inflammatory properties and it is particularly important in IFN responses to infection (Katze et al., 2002). More recently it has become clear STAT1 has other functions, one being a role in pathways controlling cell death, likely downstream of IFN-γ (Kim and Lee, 2007). In this context it may also play a role in IFN driven anti-tumour effects (Engel and Neurath, 2010). The presence and activation status of STAT1 is inconsistently reported with regards to a variety of cancers, including that of the lung. Chen et al. showed that increased STAT1 gene expression in a five-gene signature positively correlated with improved survival in NSCLC (Chen et al., 2007), suggesting perhaps that STAT1 reduces proliferation or induces apoptosis, but others have shown lung tumours displaying high STAT1 expression increases risk of death, albeit in a population with more advanced disease (Suwinski et al., 2012). *In vitro* data has shown that IFN-γ induced apoptosis of A549 cells is not reduced by blocking STAT1, and constitutive expression of STAT1 alone has no effect on cellular proliferation (Li et al., 2007a, Kurdi and Booz, 2007). The role STAT1 plays in other cancers is equally contradictory. It has been reported that highly malignant tumours display reduced expression of STAT1 (Yoshimura, 2006) and that STAT1 expression is down regulated as breast cancer progresses (Chan et al., 2012). In contrast, increased expression has been associated with a number of malignancies including gastric adenocarcinoma, soft tissue sarcomas and leukaemia, and has also been associated with radiation resistance in squamous cell carcinoma cells (Bowman et al., 2000, Ernst et al., 2008, Khodarev et al., 2004, Zimmerman et al., 2012). Even in a well-known inflammation associated tumour, colorectal cancer, there appears to be contrary effects. One of the key regulators of STAT1, SOCS1, is frequently silenced in colorectal carcinoma through hypermethylation of the promoter (Fujitake et al., 2004). In SOCS1 knock-out mice, elevated STAT1 is associated with the development of colorectal cancer (Hanada et al., 2006), but *in*

vitro, knocking down STAT1 with a small interfering RNA leads to a reduction in chemotherapy induced apoptosis (McDermott et al., 2005), suggesting a role for STAT1 in regulating cell death. It is unclear why this disparity exists although there is a suggestion that it may play different roles in different cells types (epithelial versus immune cells) (Yoshimura, 2006) and expression may change during cancer progression (Chan et al., 2012, Alonso et al., 2004). Given the somewhat confusing literature that exists, the fact that it is STAT3 rather than STAT1 that predominantly signals downstream of gp130 in response to IL-6 , and the apparent lack of effect of STAT1 signalling on the lung phenotype of the FF mouse, no further work was done with the FF:St1-/- mice.

The pathways not examined in this section of my project are those mediated through the activation of SHP2, namely the PI3K/Akt and Ras/MAPK pathways, since 1) gp130-dependent signalling through these pathways is absent in the FF mouse, and 2) while increased activation of Akt and MAPK have been associated with inflammation in the lung (Bozinovski et al., 2002, Hellermann et al., 2002), it is unlikely that loss of these pathways would contribute to the inflammatory phenotype. Nonetheless, the FF mouse provides a unique *in vivo* model not only of elevated IL-6 expression and/or signalling in the presence of emphysema to study the development of lung cancer, but also dissociates downstream signal transduction. This allows separation of the JAK/STAT and SHP2-dependent (PI3K/Akt and Ras/MAPK) pathways, better defining the pathways important in lung cancer development, and ultimately providing insights into more specific therapeutic targets. This will be further investigated in subsequent chapters.

CHAPTER 4

The role of deregulated gp130 signalling in response to a cigarette carcinogen *in vitro* and *in vivo*.

4.1 Introduction

Historically, modelling lung cancer in mice has been difficult because of their relative resistance to the effects of cigarette smoke. While life-long exposure to cigarette smoke at high concentrations does lead to the development of tumours, the tumours have low incidence and are generally benign in nature (Henry and Kouri, 1986, Hutt et al., 2005). The use of more susceptible mouse strains, such as A/J mice, meant that exposure could be for shorter periods, but again tumour multiplicity and malignancy was low (D'Agostini et al., 2001, Witschi, 2005, Witschi et al., 2006). To combat this problem, the administration of carcinogens to mice has been successful in mimicking human cigarette exposure. As I mentioned in Chapter 1, urethane has been used extensively to delineate the differential susceptibility of mouse strains to the development of lung tumours (Manenti et al., 1995, Karasaki et al., 1997, Festing et al., 1998). Additionally, it has been used to investigate the role of genetic mutations in the development of tumours and in chemoprevention studies (Witschi, 2000, Kim et al., 2006, Yanagi et al., 2007b, Luo et al., 2010). However, while urethane is clearly an effective carcinogen in rodents, it is not a known human carcinogen, thus limiting the translation of this work (Hagmar et al., 1986, IARC, 1972-present, IARC, 2010).

Conversely, NNK is a tobacco specific carcinogen and is firmly established as a human carcinogen (Hecht and Hoffmann, 1988, Hoffmann et al., 1996, Church et al., 2009). In rodents it is effective across a wide range of doses with clear dose dependence (Belinsky et al., 1990), and has remarkable organ specificity due to the requirement for activation by CYP450 enzymes found specifically in the respiratory epithelium (Su et al., 2000). NNK is known to activate a multitude of pathways (Figure 1.1) and it has been consistently shown to activate Akt and ERK1/2 MAPK *in vitro* and *in vivo* (Tsurutani et al., 2005, West et al., 2004a, West et al., 2004b).

As discussed in Chapter 1, STAT3 is a potent pro-inflammatory and oncogenic transcription factor, and numerous mouse models and clinical studies have implicated deregulated STAT3 activation as a contributing component to lung carcinogenesis (Ai et al., 2012, Akca et al., 2006, Alvarez et al., 2006, Li et al., 2007b, Qu et al., 2009b). However, there are few

published works on the effect of NNK on STAT3 activation or on the activation of the immune (innate and adaptive) system, and what little literature exists is inconsistent. Such inconsistencies may be explained, at least *in vitro*, by cell type specific differences in the actions of NNK, since NNK appears to reduce the secretion of IL-6 and CCL2 in human macrophages (Rioux and Castonguay, 2001), but increases the production of these pro-inflammatory mediators in microglial cells (Ghosh et al., 2009). A similar differential responsiveness of different cell types to NNK is also seen regarding STAT3 activation. For instance, while Guo et al. showed activation of STAT3 in A549 cells that had been serum starved for 12 hours using a concentration of 1μM NNK (Guo et al., 2012a), Kalantari-Dehaghi and colleagues showed that NNK at the same concentration in HPV-18 immortalised human bronchial epithelial cells had no effect on the gene expression of *STAT3* (a transcriptional target of its itself upon activation), despite transcriptional upregulation of other oncogenic genes including *AKT* and *MYC* (Kalantari-Dehaghi et al.). In a study examining differential inhibitory response to MAPK/ERK kinase (MEK) inhibition of NSCLC cell lines harbouring either KRAS or KRAS/PTEN mutations, Yoon et al. showed that in cells resistant to MEK inhibition there was a significant activation of STAT3 (Yoon et al.). Furthermore, dual inhibition with a MEK inhibitor and either a JAK2 inhibitor or STAT3 gene-knockdown led to apoptosis and a reduction in proliferation, thus suggesting a role for STAT3 in the growth of these cells.

In vivo, gene expression of *Stat3* and *Nf-κb* (*p50*) is increased in the lungs of mice treated with urethane both prior to, and following, the development of tumours (Pandey and Gupta, 2012). Similarly, protein levels of STAT3 and NF-κB, as shown by immunoblotting, are increased, and this was associated with an increase of STAT3 downstream targets including IL-6 (Narayan and Kumar, 2012, Pandey and Gupta, 2012). Confirming this, in CCR5 deficient mice, urethane induced tumours are reduced and this correlates with reduced activation of NF-κB/STAT3 and anti-apoptotic genes downstream of STAT3 (Lee et al., 2012). To further examine how STAT3 may contribute to tumourigenesis, mice with epithelial specific knock-out of the *Stat3* gene were exposed to urethane and displayed a reduction in lung tumour formation and an increase in anti-tumour immunity, suggesting a role for STAT3 in suppressing, rather than promoting, an anti-tumour immune/inflammatory response (Ihara et al., 2012). However, such observations are yet to be validated with a *bone fide* cigarette carcinogen. Furthermore, the upstream mechanisms leading to STAT3 activation in the above *in vitro* and *in vivo* studies are unknown. Since gp130-acting cytokines are one of the main inducers of STAT3 activity, it is conceivable that such cytokines may facilitate the activation of STAT3 *in vivo* following exposure to NNK. Testing this notion, in this Chapter I used the FF mouse model which provides a key advantage to

attribute any actions of hyper-activated STAT3 to upstream gp130, and to also separate the roles of STAT3 and ERK/MAPK and PI3K/Akt downstream of gp130.

4.2 NSCLC cell lines display differing baseline STAT3 activation and response to NNK

A number of other groups have found that STAT3 activation varies markedly in unstimulated human NSCLC cell lines (Guo et al., 2012a). To examine both the baseline activation status of STAT3 and cellular response to NNK, I used two cell lines displaying high IL-6 expression (H2228 and H838) and one with low expression (A549) (described further in Chapter 5: Figure 5.1) that had been serum starved for 18 hours with NNK at 100nM. At baseline, the two IL-6^{High} cell lines showed high levels of pSTAT3 (Figure 4.1a), while the IL-6^{Low} A549 cells did not have detectable levels, suggesting a correlation between IL-6 expression and STAT3 activity levels. At this concentration of NNK there was no discernible activation of STAT3 in any of the cell lines, but an increase in both phosphorylated (p)Akt (Serine 473) and pERK 1/2 (Threonine 202/ Tyrosine 204) at 5 min in the H838 cells. There was also a more modest increase in pAkt in A549 and H2228 cells at 15 min, thus confirming that NNK was active in these cells. To examine whether the lack of stimulation was dose related, I also stimulated the A549 cells with NNK at the higher concentrations of 50μM and 500μM (Figure 4.1b). At both concentrations there was a very slight increase in pSTAT3 (Tyr705) at 15 min, and this time point also yielded a robust increase in pERK1/2 (Thr202/Tyr204), both of which were not seen at the lower concentration (Figure 4.1a). Importantly, the responsiveness of these cells to IL-6 stimulation was confirmed by the robust increase in pSTAT3 and pERK1/2 levels within 15 min of IL-6 stimulation (Figure 4.1b). Note for the IL-6 stimulation, the human soluble (hs)IL-6R was also used to ensure a response regardless of the differential expression of IL-6R in these cells. Finally, as others have shown an increase in IL-6 with the addition of FCS (Bihl et al., 1998), and there is a very clear increase in pSTAT3 with IL-6 stimulation (Figure 4.1b), I plated two cell lines (A549, low baseline IL-6 and pSTAT3; H838, high baseline IL-6 and pSTAT3) in low serum media (0.5% FCS) for at least 72 hours to see if I could reduce the baseline activation of STAT3, and whether this made any difference to the response to NNK. Following prolonged low serum conditions, baseline STAT3 activation was not detectable in H838 cells, but did show a response to 100nM NNK with a slight increase in pSTAT3 from 90 min (Figure 4.2). Interestingly, following this prolonged serum starvation these cells did not show an increase in pAkt as they had previously. Conversely, the A549 cells did not show any increase in pSTAT3 but did show a marked increase in pAkt at 5min. Nonetheless, the NNK-induced increase in levels of pAkt seen to some degree in all cell lines (Figure 4.1a) is consistent with the published literature

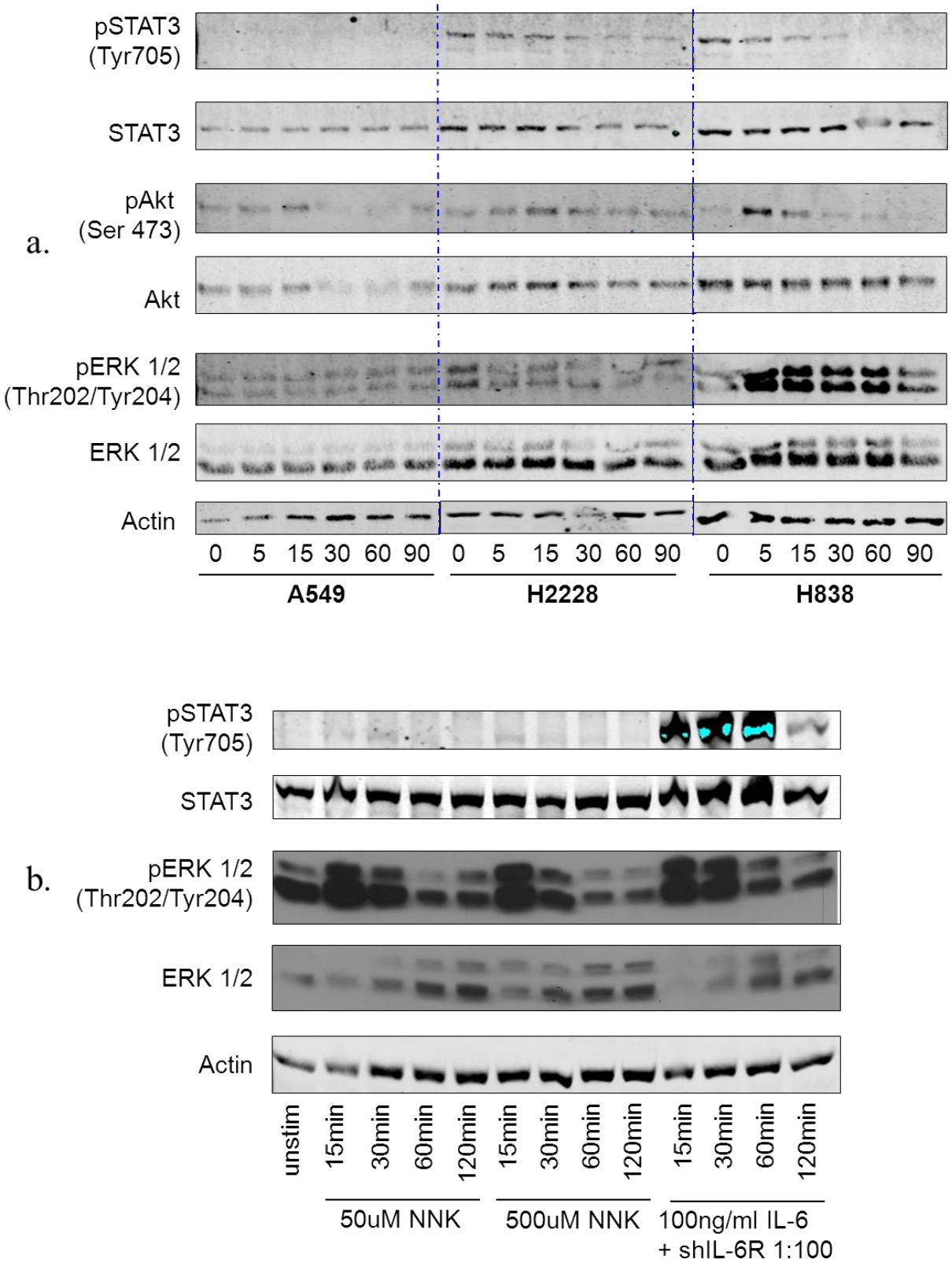
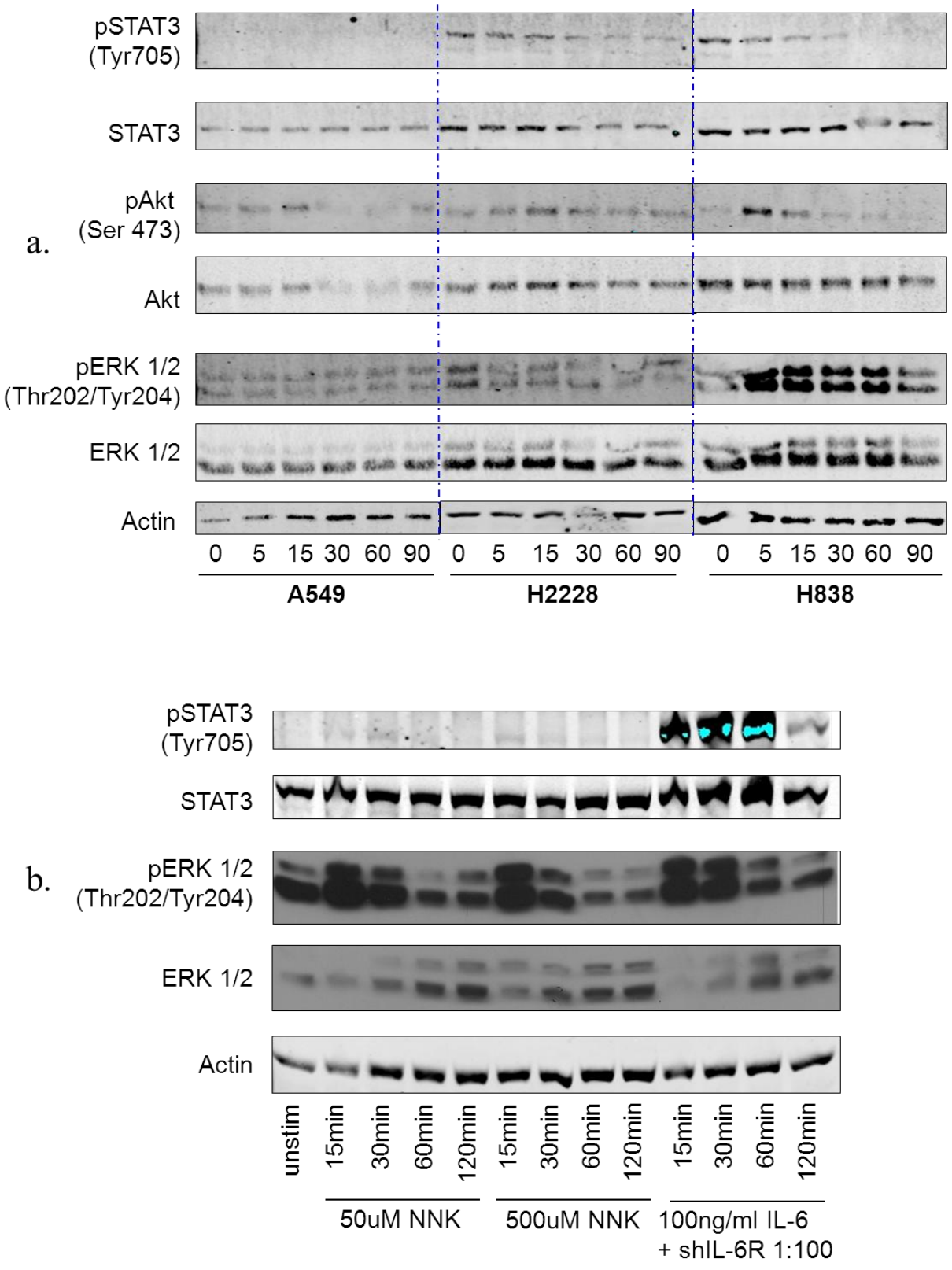


Figure 4.1. NSCLC cell lines show differential baseline levels of pYSTAT3 and variable responses to NNK.

Serum starved cells were stimulated with NNK at 100nM (a.) or 50μM and 500μM (b.). IL-6 was used as a STAT3 activation control. Blots are representative of n=2 individual stimulations.



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Serum starved cells were stimulated with NNK at 100nM (a.) or 50μM and 500μM (b.). IL-6 was used as a STAT3 activation control. Blots are representative of n=2 individual stimulations.

(Tsurutani et al., 2005, West et al., 2004b). However, why the H838 cells should lose this response when serum starved for a long period, and A549 cells show earlier and more prominent activation is unclear.

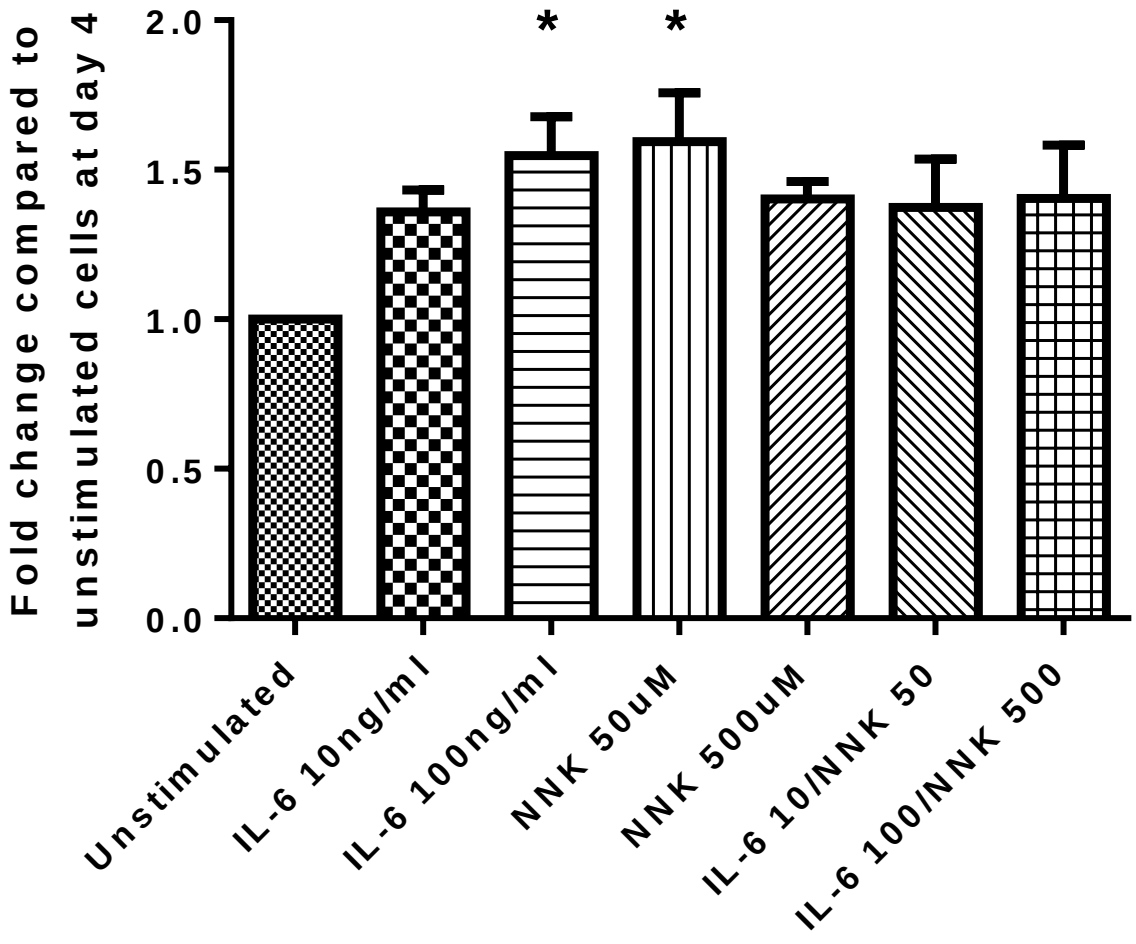
The above differences detected in STAT3 activation is consistent with the current literature showing variable expression of pSTAT3 at baseline in NSCLC cell lines (Guo et al., 2012a, Looyenga et al., 2012), and with clinical data showing constitutive activation of STAT3 in approximately 50% of NSCLC (Achcar Rde et al., 2007, Cortas et al., 2007, Haura et al., 2005b). Bihl et al. found that serum starvation of A549 cells for 24 hours lead to a significant reduction in IL-6 production, consistent with the observed reduction in pSTAT3 with prolonged serum starvation (Figure 4.2) but others have found the converse (Looyenga et al., 2012, Bihl et al., 1998). This difference is not explained by cell type or culture conditions, as both publications included A549 cells, showing different results, and all cells were grown in RPMI-1640 media. The only difference was the percentage of FCS/FBS used, but this would not explain such divergent changes when this serum was taken away.

4.3 Growth response of NSCLC cell lines to NNK

To confirm that NNK induces a growth response in NSCLC cell lines, I stimulated A549 cells with NNK at two concentrations. To assess whether there was an additive or synergistic effect between IL-6 (and shIL-6R) and NNK, I also stimulated cells with a combination of both reagents at a low and high concentration, as well as with IL-6/shIL-6R alone at two concentrations. At day 4 post-stimulation, an MTT assay showed a significant increase in growth in cells treated with either IL-6 at 100ng/ml or NNK at 50μM (Figure 4.3), and the magnitude of the response appeared comparable between IL-6 and NNK stimulation (~ 1.5-fold increase). Despite these increases to IL-6 and NNK individually, there does not appear to be an additive or synergistic effect when IL-6 and NNK are used in combination.

4.4 FF mice have a reduction in tumour formation in response to NNK

NNK reliably induces multiple lung tumours in rodents (reviewed in (Akopyan and Bonavida, 2006)), but this response is strain specific (Demant, 2003). A/J mice have a high susceptibility to the development of lung tumours due in large part to a specific *Kras* allele, identified as the *Pas1* gene (Lin et al., 1998, Matzinger et al., 1997, Ryan et al., 1987). However, some groups have shown the development of lung tumours in less susceptible strains including in C57/BL6 mice, albeit with low tumour multiplicity (Igarashi et al., 2009, Yang et al., 2004). We therefore initially treated 3 month old WT and FF mice on a mixed background (C57/BL6, 129) with NNK and observed them over 24 weeks. No tumours were



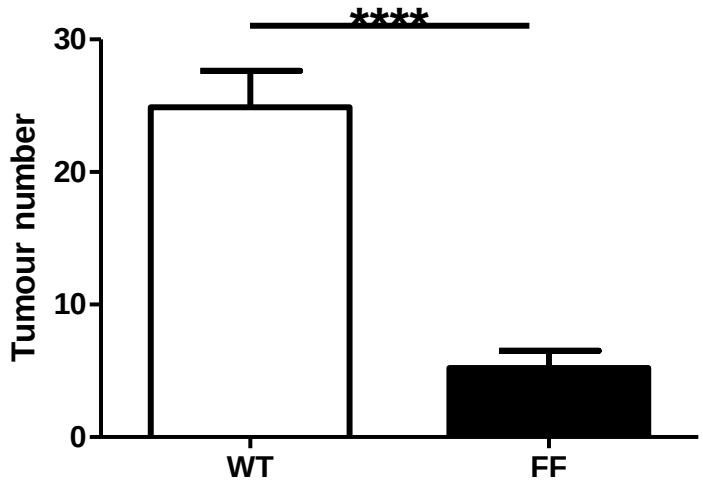
<u>Figure 4.3:</u> There is a small but significant increase in growth in response to NNK and IL-6 in NSCLC cells.
MTT assay shown as an average (mean + SD) of n=3 normalised to own unstimulated control. * = $p < 0.05$ compared to unstimulated cells based on 1-way ANOVA with Neuman-Keuls Multiple Comparison Test.

observed in either WT or FF mice at 24 weeks (data not shown). The inability to induce tumours in the mixed background mice may relate to the length of exposure as both the above studies used long observation times, up to 12 months post NNK treatment. Untreated FF mice have reduced survival and uncommonly survive past 10-12 months of age (Jenkins et al., 2005a), and for this reason a longer observation time was not possible. We subsequently undertook a limited (3 generation) backcross of the FF mouse onto an A/J background to produce pseudo-A/J mice (Hollander et al., 2008). While traditionally a backcross of at least 10 generations is required, Hollander et al. have shown it is possible to produce lung cancer susceptible mice through a targeted breeding program focussing on the specific susceptibility gene *Kras2_37*.

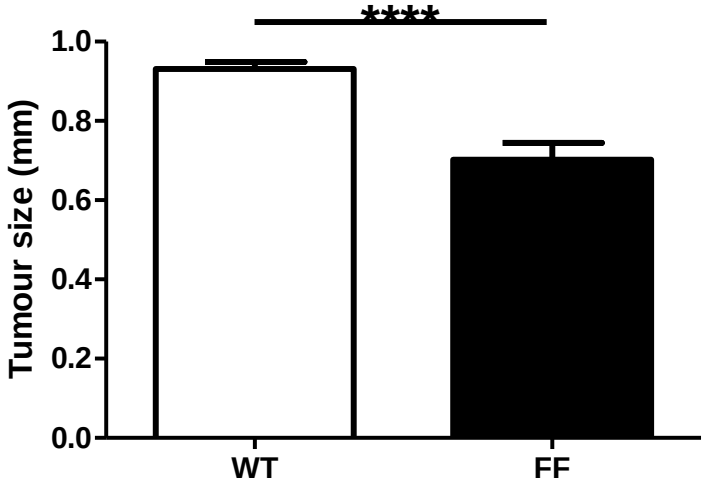
Following injection with NNK, FF and littermate WT mice on a pseudo-A/J background were closely monitored and weighed weekly over a period of 16 weeks. At the completion of the experiment, mice were euthanised and lungs were prepared as described in Chapter 2, and tumours were counted and measured. While no sporadic lung tumours were seen in untreated animals, all NNK treated animals developed tumours. Strikingly, there was a significant (~5-fold) reduction in the number of tumours in FF mice (5.22±1.29: mean±SEM) compared to WT mice (24.88±2.76, $p<0.001$) (Figure 4.4a). In addition, the size of the tumours was significantly smaller in the FF mice (0.70mm vs 0.93mm; $p<0.0001$) (Figure 4.4b). To examine whether the tumours were morphologically equivalent, sections were prepared and stained with H&E (Figure 4.5a. and b.). Although there is a clear difference in tumour number and size, the tumours in WT and FF mice appeared to be histologically similar. They display well-circumscribed spherical masses of cells completely obliterating alveolar architecture and compressing adjacent tissue consistent with adenomas, without features of adenocarcinoma (Conaway et al., 2005, Dixon et al., 1991, Nikitin et al., 2004). As described in Chapter 3, naïve FF mice have pronounced pulmonary inflammatory infiltrates from 12 weeks of age onwards, and given the role of inflammation in the development of lung cancer (Coussens and Werb, 2002, Cho et al., 2011), lung sections from NNK-treated WT and FF mice were stained with CD45 to investigate both the degree of inflammatory cell infiltrate and delineate any differences between NNK-treated WT and FF mice. As shown in Figure 4.5c, the tumours lack any significant inflammatory infiltrate and there was no appreciable difference between the number of inflammatory cells in the tumours of FF and WT mice. Furthermore, high power views of the tumour in WT and FF mice show only small infiltrates of mononuclear cells, with no difference between the two groups (Figure 4.5d).

To see whether the reduced lung tumourigenesis in the NNK-treated FF mice correlated with a lower proliferative potential in the lung, the Proliferative Index was measured by the percentage of PCNA-positive cells. Although there was a slight reduction (%) in the number

a.

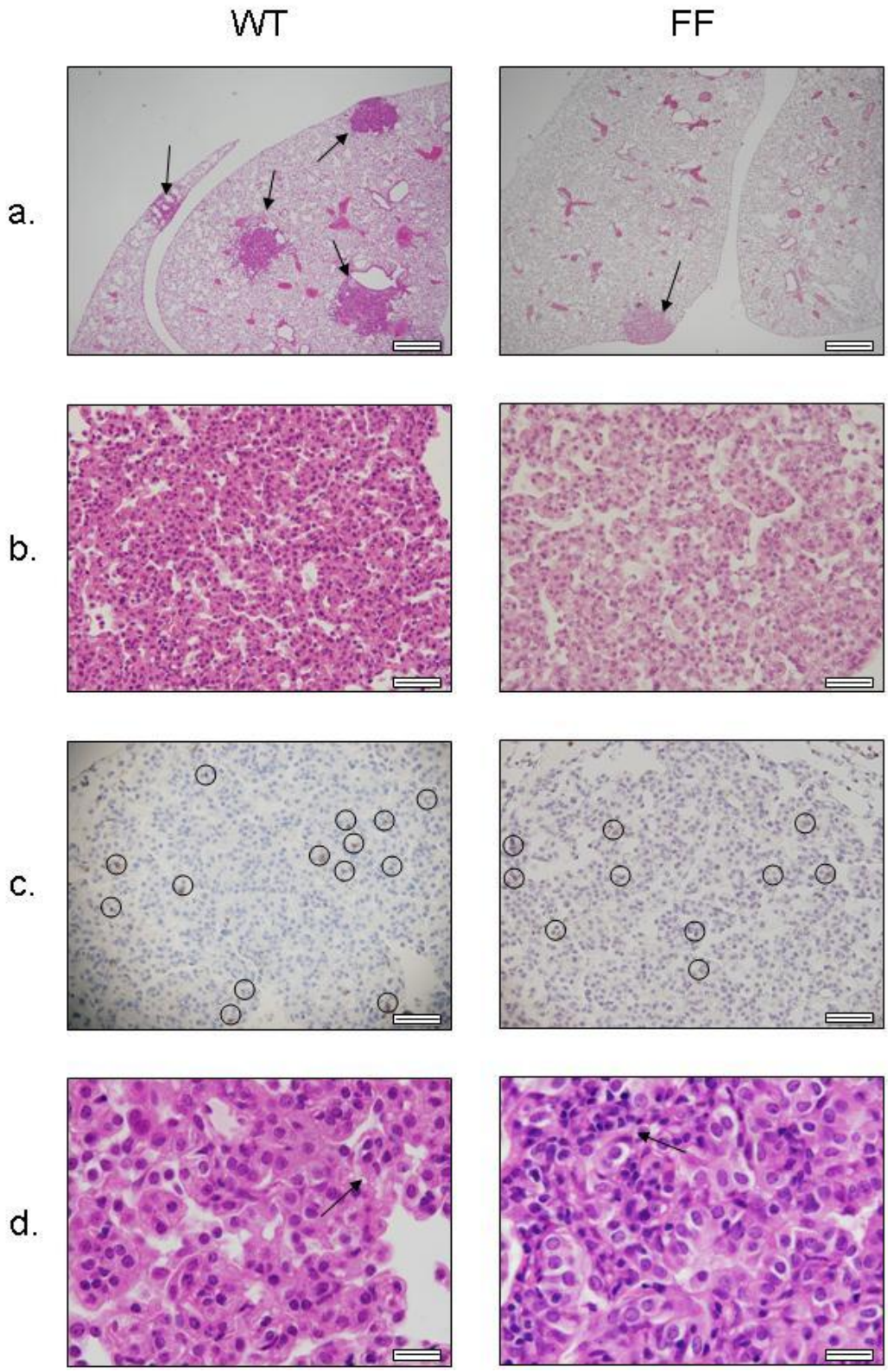


b.



<u>Figure 4.4.</u> FF mice have significantly fewer and smaller tumours than WT mice.

Tumours were counted manually by 2 independent observers. Represented as mean±SEM from n=8 individual animals. **** $p < 0.0001$, unpaired t-test.



<u>Figure 4.5.</u> NNK-induced lung tumours are histologically similar in WT and FF mice.

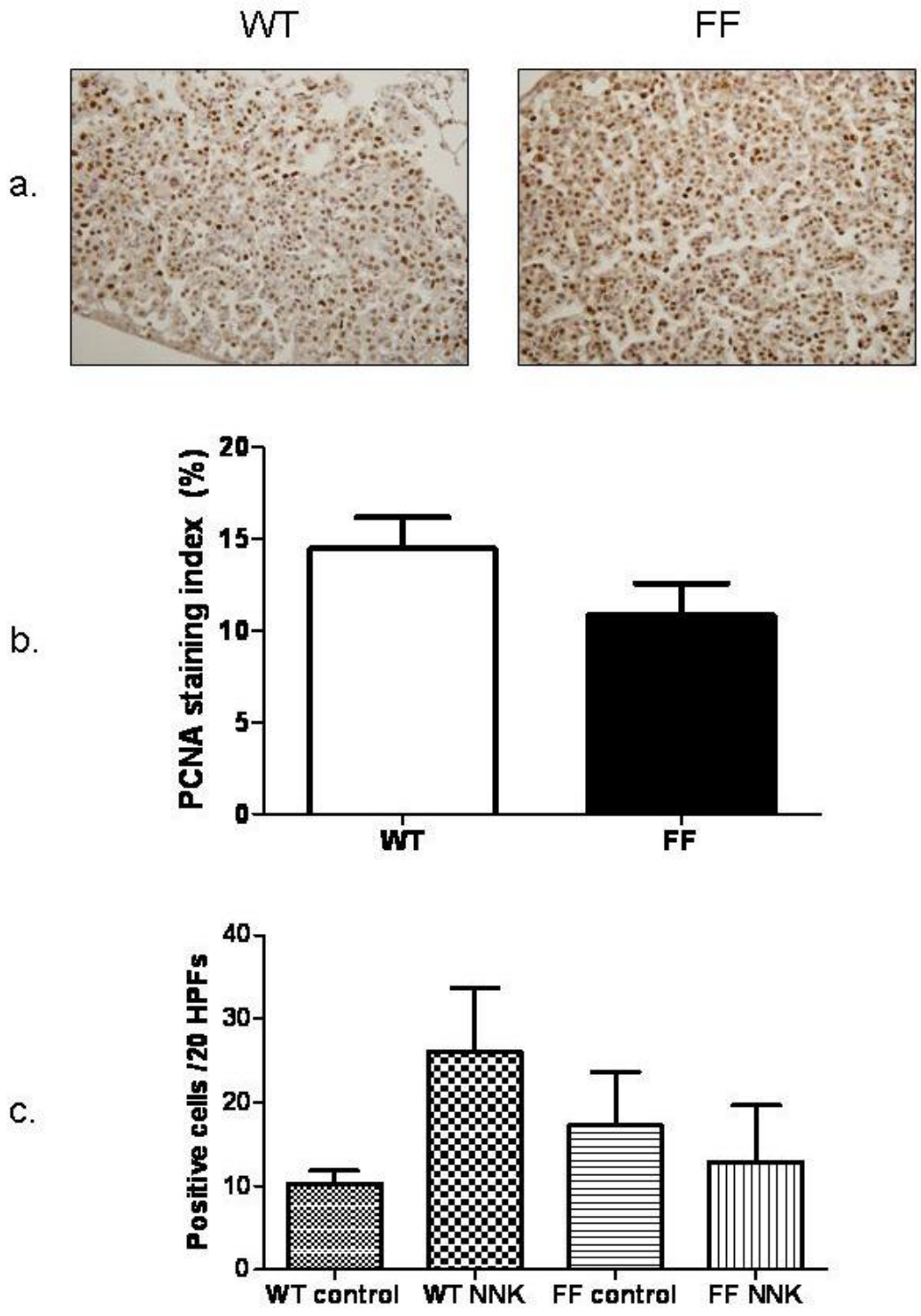
Whilst more numerous in the WT mouse, tumours share similar morphology histologically and lack any significant inflammatory infiltrate. Representative sections of WT and FF mice stained with H&E (a and b) and CD45 (c). Scale bars = 500μm in row a. and 50μm in rows b. and c. and 20μm in row d. Arrows indicate tumours in row a. Positive stained cells circled in row c. Clusters of lymphocytes marked with arrows in row d.

of PCNA-positive cells in tumours from FF compared to WT mice, this difference was not statistically significant (Figure 4.6a and b). To examine whether there was a difference in apoptosis to explain the reduced tumour formation (number and size) in FF mice, TUNEL staining was performed (by SR in the laboratory). While TUNEL positive cells were too rare to enumerate in tumours, there was no significant difference in the number of TUNEL positive cells in lung parenchyma between the two NNK-treated genotypes (Figure 4.6c).

4.5 STAT3 activation via gp130 does not contribute to NNK-induced lung carcinogenesis in FF mice

As stated previously, over-activation of the oncogenic transcription factor STAT3 has been implicated in numerous inflammatory tumours including stomach, bowel, prostate and lung (Ernst et al., 2008, Jenkins et al., 2005a, Grivennikov et al., 2009, Klampfer, 2008, Barton et al., 2004, Guo et al., 2012b, Achcar Rde et al., 2007, Ai et al., 2012). Conversely, in a urethane-induced lung tumourigenesis model, STAT3 has been assigned an anti-tumourigenic role (Ihara et al., 2012). In addition to these somewhat contradictory findings (which most likely reflect the complex pro- and anti-tumourigenic activities of STAT3 (Yu et al., 2009a), the upstream mechanisms leading to STAT3 hyper-activity in cancer are largely ill-defined, and little is known about the role of STAT3 in an NNK-induced model. Regarding the latter, my *in vitro* data in Figures 4.1 and 4.2 suggest that STAT3 is not strongly induced by NNK. Together with the reduced tumour formation in NNK-treated FF mice displaying gp130-mediated STAT3 hyper-activation (Tebbutt et al., 2002, Jenkins et al., 2005a), these observations would imply that STAT3 does not promote NNK-induced tumour formation in FF mice.

I initially investigated the *in vivo* activation status of NNK-induced STAT3 in the lungs of WT mice by Western blot analyses. Consistent with my *in vitro* data (Figure 4.1), NNK did not activate STAT3 in the lungs of tumour-bearing WT mice (Figure 4.7). Accordingly, to formally exlude a role for STAT3 in the development of NNK-induced tumours *in vivo*, NNK-induced tumourigenesis was compared between FF:St3-/+ mice displaying genetically-normalised activation of the gp130-STAT3 signalling axis (Jenkins et al., 2005a, Jenkins et al., 2007, Ernst et al., 2008), and FF mice. Following injection of FFSt3-/+ mice (as per the WT and FF mice using an established protocol (Razani-Boroujerdi and Sopori, 2007)), it was notable that FF:St3-/+ mice also had significantly fewer and smaller tumours than WT mice, and were not significantly different from FF mice in tumour number (Figure 4.8). However, the tumours in FF:St3-/+ mice were significantly larger than FF mice. Thus, while these data strongly suggest that STAT3 plays little role in tumour initiation (based on tumour number), the increase in the size of tumours upon hemizygous ablation of *Stat3* in FF:St3-/+ mice



<u>Figure 4.6:</u> There is no significant difference between WT and FF mice in tumour proliferative index or apoptosis.

Proliferative index describes the % of tumour cells staining positive with PCNA. Mean±SEM from n=3 mice, counting at least 100 cells / tumour. Apoptosis measured by number of cells positive for TUNEL staining per 20 high power fields. Mean±SEM from n=2 control mice and n=3 NNK treated mice.

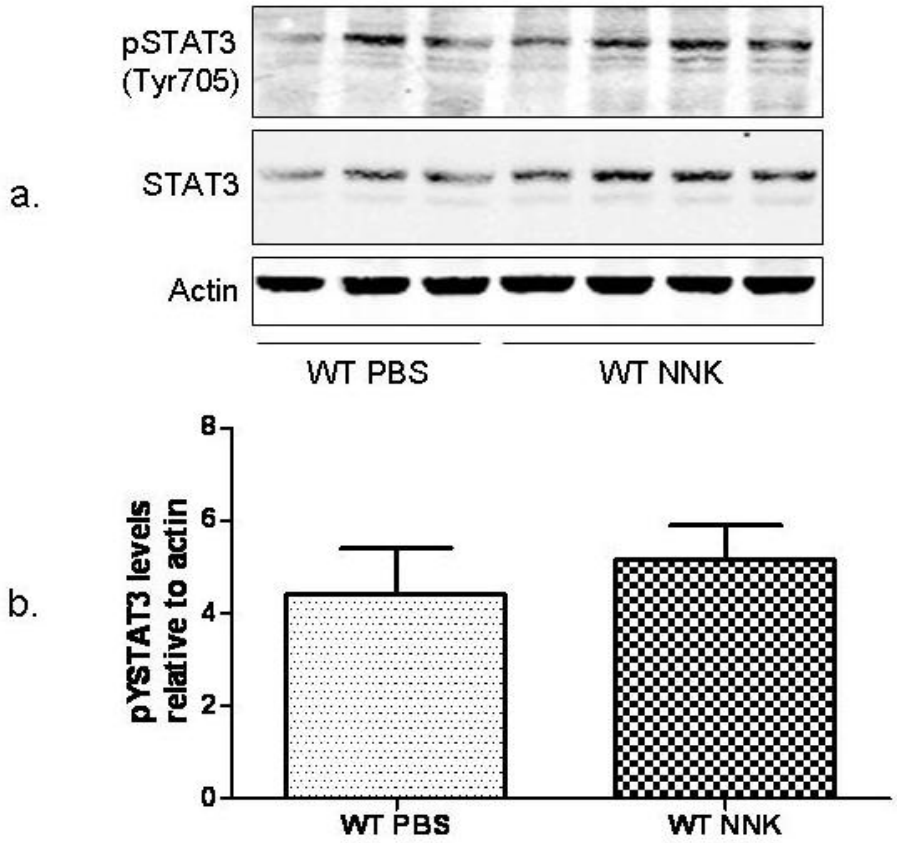


Figure 4.7: There is no activation of STAT3 in response to NNK treatment in WT mice.
Protein lysates from whole lung of WT mice treated with PBS and NNK and observed over 16 weeks blotted with antibodies against pYSTAT3, total STAT3 and actin as a loading control (a.). Densitometry performed using Image J (see Chapter 2) and expressed as mean ± SEM.

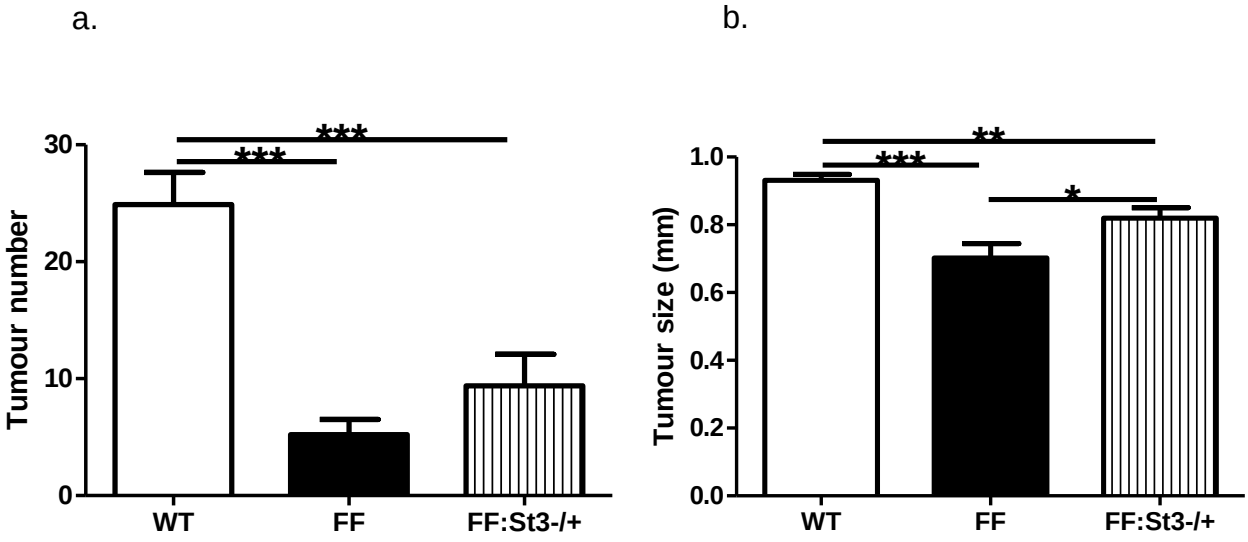


Figure 4.8. FF:St3-/+ mice have significantly fewer and smaller tumours than WT mice and are not significantly different in number from FF mice.
Tumours were counted manually by 2 independent observers. Represented as mean±SEM from n=8 individual animals.. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA with Tukey's Multiple Comparison Test.

(compared to FF mice) suggests STAT3 may play a role, yet to be defined, in suppressing the growth of NNK-induced tumours.

4.6 Key oncogenic pathways downstream of PI3K/Akt and MAPK are deregulated in FF mice

A possible explanation for the lower tumour formation in NNK-induced FF mice is that the loss of specific gp130 signalling pathways, as a consequence of the gp130Y757F mutation, is responsible for the reduced tumour load seen in the FF mouse. In this regard, the FF mouse lacks gp130/SHP2-dependent PI3K/Akt and MAPK signalling (Tebbutt et al., 2002), and the PI3K/Akt and ERK MAPK pathways are widely implicated in the development of lung cancer (Hollander et al., 2011, Yang et al., 2008, West et al., 2004a, West et al., 2004b), and are activated in response to NNK (Figure 4.1a and 4.1b, and (Tsurutani et al., 2005, Kalantari-Dehaghi et al., Yang et al., 2004)).

To further examine differences in these signalling pathways in response to NNK in the lungs of WT and FF mice, I extracted RNA from FFPE tissue as described in Chapter 2 and undertook pathway-specific PCR arrays targeting genes belonging to the PI3K/Akt and MAPK pathways (Gene lists in Appendix III). I compared gene expression in mice treated with NNK and observed over 16 weeks with untreated mice of the same genotype. A fold-change in expression between untreated and NNK-treated animal of 2-fold or greater was considered significant. For both pathways, there were more genes whose expression was deregulated (both up- and down-regulated) in FF mice than WT mice treated with NNK when compared to their untreated counterparts (Figure 4.9). Consistent with the hypothesis that deregulated gp130/PI3K and gp130/MAPK signalling contributes to the development of lung tumours, key oncogenes *Creb1, Fos* and *Kras* and regulators of cell cycle progression *Ccnb1* and *Cdc42* downstream of PI3K and MAPK were down-regulated in FF mice and unchanged in WT mice (Table 4.1 and 4.2). Conversely, tumour suppressor genes including *Trp53* and *Tsc2* and the inhibitor of cell cycle progression *Cdkn1b* were up-regulated in FF mice but down-regulated in WT mice. The alternate regulation of these genes in FF versus WT mice treated with NNK points to a crucial role of gp130/SHP2-dependent signalling through PI3K/Akt and Ras/MAPK in the development and progression of lung tumours induced by NNK.

4.7 Discussion

In this part of the project, I have provided evidence that FF mice develop significantly fewer tumours than WT mice in response to the cigarette carcinogen NNK. This reduction appears

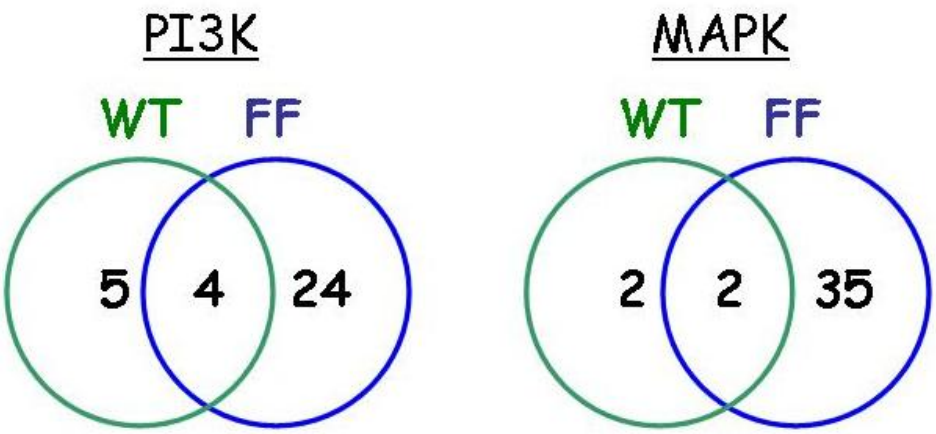


Figure 4.9. Venn diagram depicting the number of overlapping and distinct genes with altered (up and down) expression in the lungs of NNK-treated FF mice.
Number of genes showing ≥2 fold change (up and down) in NNK-treated vs. untreated WT and FF mice. Average of n=2 in each group.

Table 4.1. Key deregulated genes from MAPK pathway-specific PCR array.

Gene	Fold difference c/w untreated mice		Functional grouping
	WT	FF	
Ccnb1	0.62	0.10	Cell Cycle Proteins Regulated by the Erk1/2 Pathway
Cdc42	1.04	0.09	MEKK1 Interacting proteins
Creb1	0.78	0.16	Activated Transcription Factors
Fos	0.63	0.55	Genes with Induced Expression
Kras	0.68	0.07	Raf Regulating proteins
Trp53	0.94	2.45	Genes with Induced Expression

Gene expression analysis comparing NNK treated to untreated mice. Significance was set at 2-fold difference. Represents the average of two samples.

Table 4.2. Key deregulated genes from PI3K pathway-specific PCR array.

Gene	Fold difference c/w untreated mice		Functional grouping
	WT	FF	
Cdc42	0.75	0.09	PI3K Subunit p85 Genes and Regulation of Actin Organization and Cell Migration
Cdkn1b	1.01	3.23	PTEN Dependent Cell Cycle Arrest and Apoptosis
Fos	0.88	0.10	IGF-1 Signaling Pathway
Myd88	0.31	2.41	Inactivation of Gsk3 and the Accumulation of β-Catenin
Tsc2	0.49	3.80	Genes Involved in the mTOR Signaling Pathway

Gene expression analysis comparing NNK treated to untreated mice. Significance was set at 2-fold difference. Represents the average of two samples.

to be largely independent of STAT3, and is likely mediated through gp130/SHP2-dependent PI3K/Akt and/or MAPK pathway signalling.

A role for both the PI3K/Akt and MAPK pathways in the development of lung tumours is well established in cell culture, murine models and from clinical samples. Indeed, expression of pAkt is high in human bronchial dysplasia specimens, a precursor to cancer, suggesting activation of Akt is an early event in the development of human lung cancer (Tsao et al., 2003). In further support of this idea, Okudela et al. showed the frequency of activated Akt in pre- and early malignant lesions is similar to advanced adenocarcinoma (Okudela et al., 2004). Interestingly, they linked this increase in activation to Kras mutations. Given the experimental evidence linking IL-6 to tumour development in a *LSL*Kras^{G12D} model of lung cancer (Ochoa et al., 2011), it raises the possibility of a role of IL-6/gp130 signalling in lung carcinogenesis, which is also supported by the fact that gp130, IL-6R and IL-6 are all highly expressed in resected NSCLC specimens (Haura et al., 2006).

Akt is constitutively active in a majority of NSCLC cell lines, promoting cell survival (Brognard et al., 2001), and is robustly up-regulated by NNK *in vitro* in immortalised bronchial epithelial cells (Kalantari-Dehaghi et al.). West et al. have also shown that the PI3K/Akt pathway is up-regulated in tumourigenic bronchial epithelial cells, and expression of Akt is increased as NNK-induced tumours progress (West et al., 2004a, West et al., 2004b). These findings are consistent with my data showing high baseline expression of pAkt in the lung adenocarcinoma cell lines, and while the *in vivo* data provided by West and colleagues are consistent with my finding of the likely involvement of the PI3K/Akt pathway in tumour development, they do not investigate upstream signalling events to explain this. Based on my data generated from the NNK-treated FF mice (in which gp130/SHP2-mediated PI3K/Akt signalling has been abolished), it is tempting to speculate that NNK leads to upregulation of gp130 ligands (IL-6) resulting in gp130/SHP2-dependent activation of PI3K and subsequent signalling downstream through Akt. Such a scenario would provide a rationale for investigating small molecules or antibodies acting against IL-6/gp130 in the management of NSCLC, as has been described for other inflammatory cancers (Chalaris et al., 2011, Lo et al., 2010). While there are no definitive data linking IL-6/gp130 signalling with Akt in the pathogenesis of lung cancer, proliferation and survival of multiple myeloma cells *in vitro* was promoted by IL-6 via Akt (Hideshima et al., 2001). Perhaps more importantly with this in mind, an anti-IL6 antibody has been used with good clinical effect in early phase human trials in myeloma and showed inhibition of downstream Akt signalling (Fulciniti et al., 2009).

Similar to Akt, MAPK signals downstream of a number of receptors including EGFR (via Ras) and gp130, and at least in myeloma cell lines there appears to be cross-talk between the

gp130-dependent PI3K/Akt and ERK/MAPK pathways (Greenberg et al., 2002). ERK/MAPK signalling is important in the proliferation of NSCLC cell lines, as evidenced by its suppression by glucocorticoids leading to a reduction in cell growth (Greenberg et al., 2002), and is implicated in a number of cancers including lung cancer (Dhillon et al., 2007). *In vivo*, increased ERK/MAPK activation is associated with an increase in NNK-induced tumours in CC10 knock-out mice (Yang et al., 2004). Confirming that this finding is not carcinogen specific, Ramakrishna et al. showed a significant increase in activated ERK1/2 in lung tumours induced by N-nitrosodimethylamine in mice (Ramakrishna et al., 2002). Interestingly, the expression level of the *Kras2* wild-type allele, the most important tumour susceptibility gene in mice, is inversely related to activation of ERK/MAPK and tumour formation; when the *Kras2* wild-type allele is deficient or lost, ERK/MAPK activity is increased and tumour formation is exacerbated in response to chemical carcinogens, reinforcing the crucial role ERK/MAPK plays in tumourigenesis (Zhang et al., 2001). However, in none of these studies is there a thorough evaluation of upstream pathways leading to MAPK activation. The work I have presented further supports an important role for ERK/MAPK in the development of NNK-induced lung tumours, and furthermore add to this by identifying gp130 signalling as a likely crucial upstream pathway.

Finally, while STAT3 is constitutively activated in approximately 50% of human adenocarcinomas (Gao et al., 2007), there is little experimental evidence examining the interaction of STAT3 with *bona fide* cigarette carcinogens. The cell culture data I have presented is largely consistent with published literature, showing constitutive activation of STAT3 in many, but not all, NSCLC cells lines (Guo et al., 2012a), and minimal activation of STAT3 by NNK which appears to be cell line and dose dependent. The *in vivo* data shows for the first time that, at least in the context of loss of gp130/SHP2-dependent Akt and ERK signalling, NNK-induced tumour development is not STAT3 dependent. Given the proposed oncogenic role of STAT3 in many cancers including lung cancer (Li et al., 2007b, Qu et al., 2009b, Achcar Rde et al., 2007, Gao et al., 2009), it seems unlikely that an increase in STAT3 would lead to a reduction in tumour development. However, such a notion must also consider the reported anti-tumourigenic role for STAT3 in a urethane lung cancer model (Ihara et al., 2012). In addition, an alternate explanation could be provided from a study in mammary gland involution which showed that expression of PI3K regulatory subunits responsible for reducing levels of activated Akt is increased by STAT3. In STAT3-deficient cells, expression of the regulatory subunits fails to rise leading ultimately to an increase in apoptosis (Abell et al., 2005). With this in mind it is conceivable that in the context of hyperactivated STAT3 in the FF mouse (where gp130/SHP2-dependent PI3K/Akt and ERK/MAPK signalling is absent), the balance may be tipped towards apoptosis rather than proliferation. In another

study, the idea of STAT3 promoting an apoptotic signal was examined by generating SOCS3 knock-out MEFs. They showed that excessive STAT3 activation in the absence of SOCS3 led to apoptosis and growth arrest rather than proliferation (Lu et al., 2006). However, in A549 cells, which are deficient in SOCS3 (yet which from my data in Figure 4.1 do not display elevated basal levels of STAT3 activation), forced expression of SOCS3 lead to growth inhibition associated with a reduction in STAT3, Akt and ERK activation (Yu et al., 2009b) suggesting a complex, and perhaps cell dependent homeostatic process. On balance, given the lack of difference between NNK-treated FF and FF:St3-/+ mice, and the minimal activation of STAT3 by NNK in NSCLC cell lines, it is more likely that STAT3 plays little to no role in NNK-induced tumours.

In conclusion, the data presented in this Chapter suggest that gp130-dependent PI3K/Akt and ERK/MAPK signalling is important in NNK-induced lung tumours *in vivo*, independent of STAT3. Whilst delineation of precise signalling pathways downstream of SHP2 remains to be done, as well as identification of the specific IL-6 family cytokines involved, this work nonetheless provides new molecular targets for therapy.

CHAPTER 5

The role of IL-6 classical versus trans-signalling in the promotion of carcinogenesis and cachexia in an inducible activated *k-ras* model of lung cancer.

5.1 Introduction

IL-6 is increased in subjects with NSCLC regardless of whether BALF, serum or pleural fluid is examined (Chyczewska et al., 1997, De Vita et al., 1998, Dowlati et al., 1999, McKeown et al., 2004, Tas et al., 2005, Yanagawa et al., 1995, Yeh et al., 2006). Moreover, elevated levels of IL-6 are significantly associated with greater morbidity and worse survival (Enewold et al., 2009, Kasymjanova et al., 2010, Scott et al., 1996, Songur et al., 2004), suggesting that IL-6 plays a causal role in the disease rather than just being elevated as a function of the inflammation inherent in lung cancer. What is less clear is whether IL-6 is produced either systemically or locally by stromal cells or the cancer cells themselves. To address this question, Bihl et al. utilised an *in vitro* model examining the expression of IL-6 mRNA, levels of IL-6 protein in cell supernatant and the effect of exogenous IL-6 on proliferation of a number of human NSCLC cell lines (Bihl et al., 1998). They found that both gene expression (RT-PCR) and protein levels (ELISA) varied significantly between the cell lines; IL-6 mRNA was detectable in only three (A549, Calu3 and Calu6) of the 6 NSCLC cell lines tested which corresponded with detectable secretion of IL-6 into the supernatant, delineating potentially IL-6-dependent and IL-6-independent cell lines. The addition of FCS to the media of IL-6-dependent cells led to an increase in both IL-6 secretion and proliferation. This increase was blocked by the addition of IL-6 anti-sense oligonucleotides but not neutralising anti-IL-6 antibodies, and the addition of exogenous IL-6 did not increase proliferation. They comment that this further characterises the "IL-6-dependent proliferative pathway as intracellular", suggesting a local production of IL-6 rather than relying on infiltrating inflammatory cells. Another possibility, and one that they did not examine, relates to the IL-6R. While gp130 is ubiquitously expressed on cells, the IL-6R is largely expressed on immune and hepatic cells, meaning for IL-6 to act directly on the lung cancer cells the sIL-6R would need to be present. Stimulation of endogenous IL-6 production rather than the presence of exogenous IL-6 may also stimulate production of sIL-6R, thus enabling autocrine signalling.

While downstream signalling from gp130 is well studied in other cancers, including myeloma and gastric cancer (Hideshima et al., 2001, Ernst et al., 2008, Jenkins et al., 2005a), the precise gp130-dependent signalling cascade(s) and interactions with other known oncogenes is ill-defined in lung cancer. One key oncogene is K-ras, which has been extensively studied in the context of adenocarcinoma as activating mutations are present in around 30% of tumours (Anderson et al., 1991). Such mutations frequently occur in codon 12 of the *K-ras* gene, and occur almost exclusively in smokers (Rodenhuis and Slebos, 1990, Ahrendt et al., 2001). The *LSL*Kras^{G12D} mouse model has been used extensively and is considered the gold standard genetic tool to investigate the molecular and cellular basis (e.g. role of oncogenes, tumour suppressor genes and inflammation) of lung carcinogenesis (Johnson et al., 2001, Shaw et al., 2007, Barbie et al., 2009, Winslow et al., 2011, Moghaddam et al., 2009, Dovey et al., 2008, Engelman et al., 2008a); however, there is little literature defining interactions between K-ras and IL-6/gp130. Ochoa et al. have shown that genetic ablation of IL-6 inhibited tumour development in an *LSL*Kras^{G12D} model of adenocarcinoma whereby they examined the interaction of oncogenic K-ras with COPD-like inflammation induced by NTHi (Ochoa et al., 2011). However, they did not examine downstream IL-6 signalling targets, or whether IL-6 signalling involved the membrane bound or soluble IL-6R. Support for an interaction between IL-6/STAT3 and Ras signalling can be taken from other cancers, particularly in multiple myeloma, breast and pancreatic cancer. For instance, like lung cancer, Ras mutations are frequently seen in codon 12 in multiple myeloma and IL-6 levels correlate with Ras activation, proliferation and protection from dexamethasone-induced apoptosis *in vitro* (Rowley and Van Ness, 2002, Hoang et al., 2006). In Ras-transformed mammary epithelial cells, blockade of either IL-6 or STAT3 with specific shRNA or JAK inhibitors reduces tumourigenic potential and migration of the cells (Leslie et al., 2010). In pancreatic cells, while STAT3 appears to be non-essential for normal pancreatic growth, gp130/STAT3 signalling is frequently upregulated in pancreatic cancer, with constitutive STAT3 expression in a majority of human tumour samples and pancreatic cancer cell lines (Corcoran et al., 2011). Moreover, the conditional genetic inactivation of STAT3 in a K-ras (*Kras*^{G12D}) induced mouse model of pancreatic cancer showed that STAT3 was essential both in the induction and progression of tumours in a gp130 dependent manner. Lesina et al. also showed the requirement for activated STAT3 in the development of *Kras*^{G12D} induced pancreatic adenocarcinoma, and additionally examined upstream signalling leading to STAT3 phosphorylation (Lesina et al., 2011). They showed that infiltrating immune cells (largely macrophages) were responsible for the secretion of IL-6 leading to activation of STAT3 within pancreatic acinar cells thus promoting carcinogenesis. However, IL-6 itself was not enough to induce this activation, with only a complex of IL-6/sIL-6R (Hyper-IL-6) leading to

a robust phosphorylation response *in vitro*, implicating only trans- and not classical signalling. This literature supports the potential interaction between oncogenic K-ras and IL-6/gp130/STAT3 signalling with regards to lung cancer, as well as specifically IL-6 trans-signalling, and one of the aims of this section of my project is to further elucidate this.

In addition to a proposed role of IL-6 in the development and progression of lung cancer, it is also linked with the devastating paraneoplastic syndrome of cachexia. It has been estimated that approximately 20% of all cancer related deaths are secondary to cachexia (Muscaritoli et al., 2006), and in patients with lung cancer, weight loss is associated with poorer outcomes in response to chemotherapy (Ross et al., 2004). Accurately determining the prevalence of cachexia in lung cancer has proved difficult at least partly because of the changing incidence with progression of disease. As an example, in a group of patients with NSCLC presenting for surgery only 12% reported weight loss, while weight loss was reported in 58% of patients presenting for chemotherapy (Ross et al., 2004, Win et al., 2007). This can be explained at least in part due to differences in disease stage: the majority of patients in the surgical cohort had early stage disease (stage I, 62%, and stage 2, 22%) while those in the cohort receiving chemotherapy have advanced (stage III and IV) disease.

While definitions of cachexia vary it is generally accepted to be unintended weight loss, made up of varying degrees of adipose tissue and skeletal muscle (Tisdale, 2010), with or without symptoms, that is associated with poorer outcomes for the individual (Bozzetti and Mariani, 2009). While anorexia is frequently present, the degree of weight loss is generally out of proportion to the reduction in dietary intake, and dysregulation of a number of metabolic pathways has been implicated, including IL-6 (Blum et al., 2011, Simons et al., 1999, Bruera, 1997). CRP, which is frequently used as a surrogate marker for the activity of IL-6, and serum levels of IL-6 itself, has been correlated with weigh loss in cancer patients, including those with lung cancer (Kim et al., 2012, Songur et al., 2004, Okada et al., 1998). But a potential pathogenic role of IL-6 in cachexia is not confined to lung cancer, as evidenced by mice with a mutation within the Adenomatous Polyposis Coli tumour suppressor gene (Apc(Min/+) mice) which spontaneously develop bowel tumours associated with loss of both muscle and adipose tissue (Baltgalvis et al., 2008). Furthermore, genetic ablation of IL-6 in these mice resulted in no muscle and fat loss compared with C57BL/6 mice, as well as significantly reducing tumour load. Interestingly, in the same study, while over-expression of IL-6 increased cachexia in the Apc(Min/+) mice it did not induce cachexia in non-tumour-bearing mice. In regard to lung cancer, cachexia in the LLC cachexia model is associated with elevated IL-6 levels and an increase in STAT3 activation in skeletal muscle (Bonetto et al., 2012a). In addition, transfecting LLC cells with human IL-6 cDNA prior to xenograft formation leads to weight loss and reduced survival in the tumour-bearing mice, without

elevation of TNF-α or IL-1α, further suggesting an important role for IL-6 in cachexia (Ohe et al., 1993). However, these observations have yet to be shown in an orthotopic lung cancer model. By utilising the FF:Kras^{G12D} mouse I was able to examine the effect on body weight of lung cancer development in the context of endogenous hyper-activation of STAT3 and elevation of IL-6. Furthermore, access to a number of IL-6R antibodies which differentially target classical and trans-signalling allowed the investigation of the therapeutic utility of IL-6 blockade in experimental lung cancer-related cachexia.

5.2 Unstimulated NSCLC cell lines secrete variable amounts of IL-6 and sIL-6R

As described above, different human lung adenocarcinoma (LAC) and undifferentiated NSCLC cell lines secrete variable amounts of IL-6. To confirm this finding and to examine whether these cells also secreted the sIL-6R, supernatants were collected from cells that had been plated overnight in 6 well plates at a density of 1 x 10^6 cells/well in complete media (RPMI) with 10% FCS.

Consistent with previous data (Bihl et al., 1998), there was a substantial difference between cell lines in the amount of IL-6 detectable in the supernatant (Figure 5.1). Interestingly, this increase did not uniformly correlate with levels of shIL-6R in the supernatant (Figure 5.2). While the H838 cells display both high IL-6 and sIL-6R levels, the H2228 cells, which secrete the highest amount of IL-6, have one of the lowest productions of sIL-6R. This difference cannot be explained by variation in the cell characteristics, since all the cells are epithelial and derived from human lung adenocarcinoma. There are differences in the stage of the disease, the origin of the tissue (e.g. primary tumour or lymph node), the mutations present and the sex and smoking status of the donor (See Appendix IV), but these differences are not concordant with the variation seen in IL-6 and IL-6R secretion. For example, the two highest IL-6 secreting cell lines (H2228 and H838) differ in tissue of origin, sex and smoking status of the donor, and in one of two major mutations. In contrast, one of the highest IL-6 secreting lines (H838) and one of the lowest secreting lines (H1993) share tissue of origin, smoking status of the donor and two major mutations.

5.3 Gene expression of IL-6 signalling pathway components varied in unstimulated NSCLC cell lines

To further examine components of the IL-6 signalling cascade in the human LAC cell lines, I measured gene expression for *GP130*, *IL6*, *IL6R* and *TACE* (Figure 5.3). Gene expression levels were compared to those of A549 cells because 1) these human lung cells were originally cultured from explanted malignant tissue (Giard et al., 1973) but maintain characteristics of type II pneumocytes (Lieber et al., 1976), and 2) they are well characterised

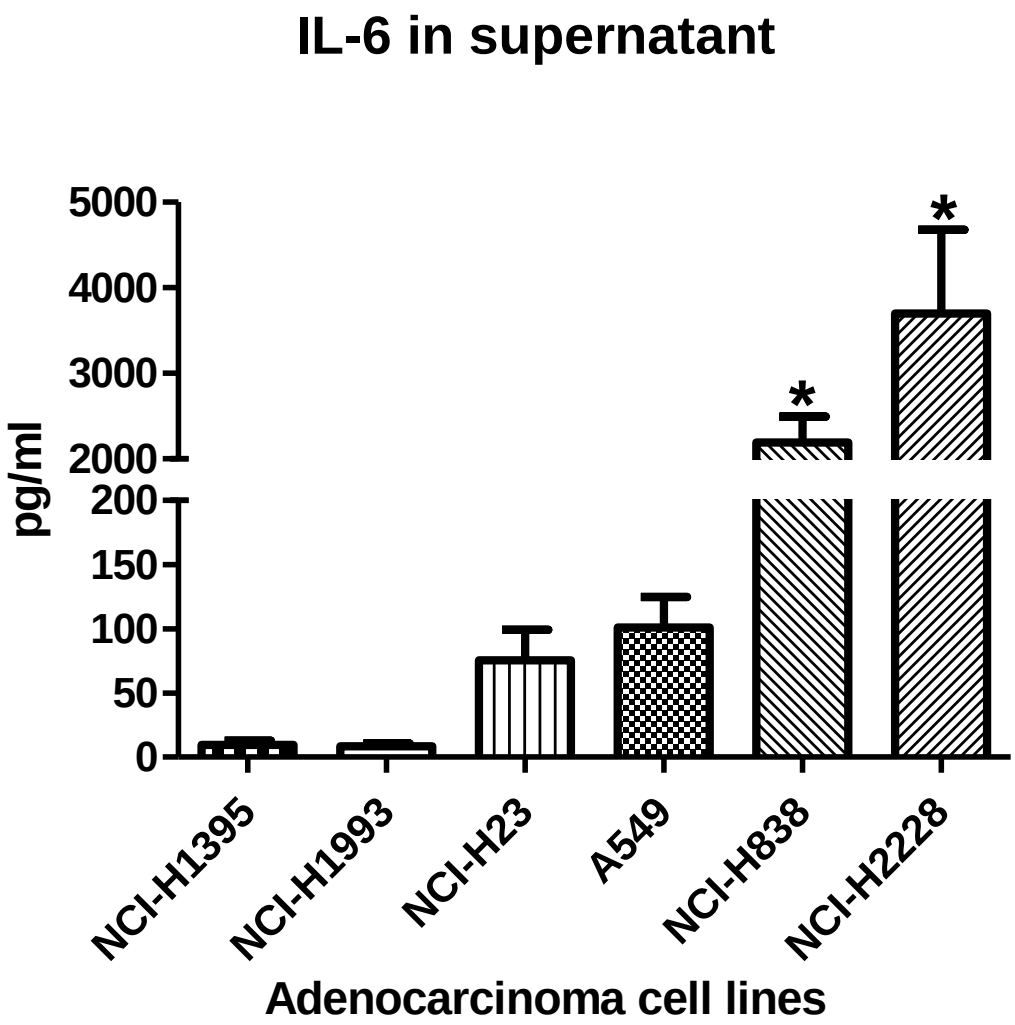
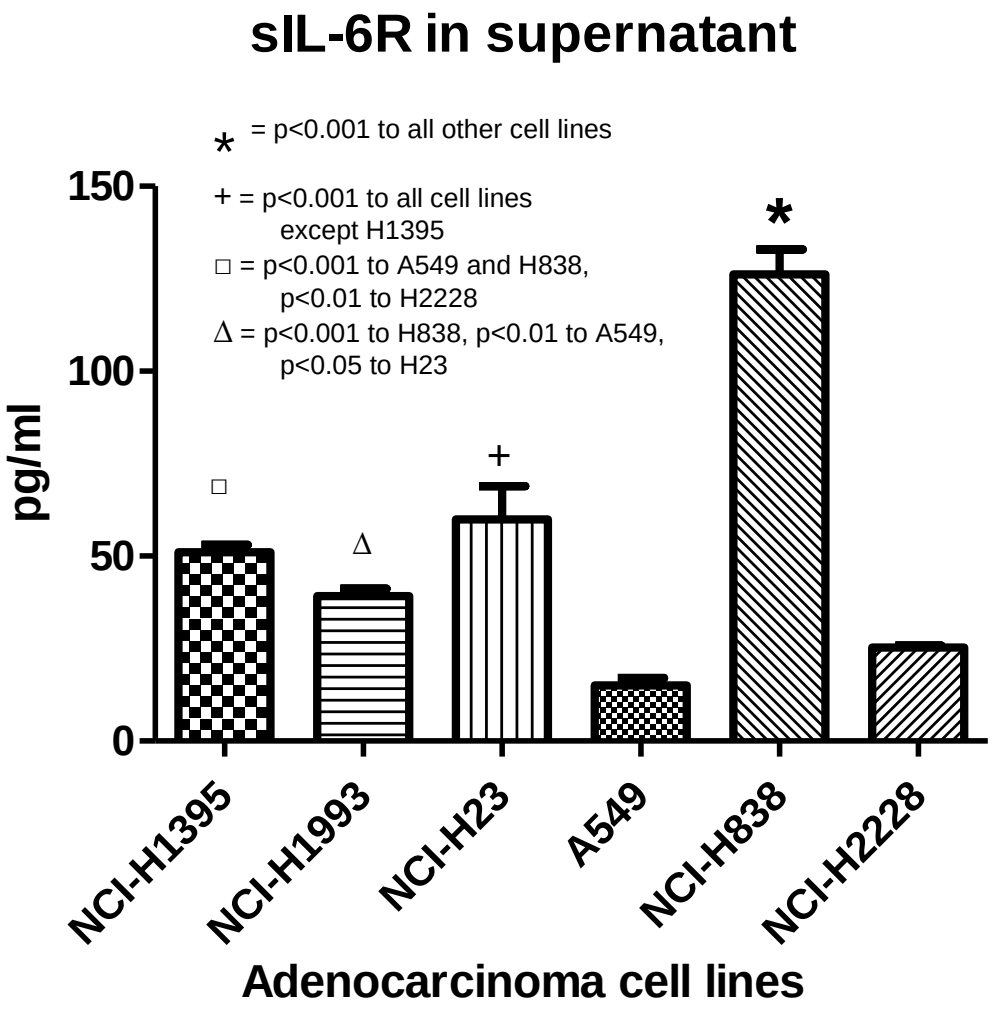
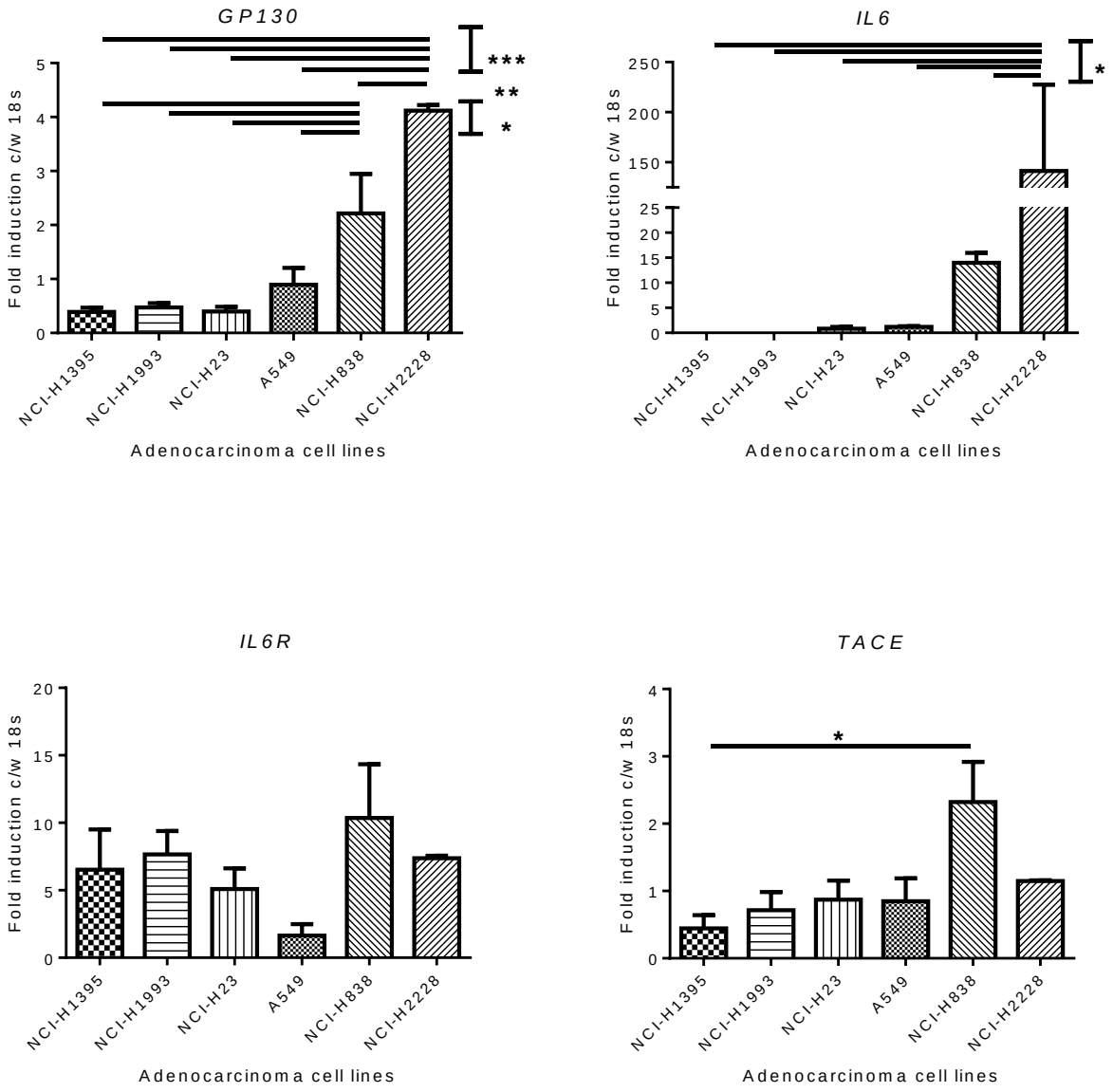


Figure 5.1. There was a significant difference in secreted IL-6 between high expressing cell lines (H2228 and H838) and the remaining cell lines.
Expressed as mean + SEM from at least 5 supernatant samples. * $p<0.001$ compared with all other cell lines. One-way ANOVA with Neuman-Keuls Multiple Comparison Test.



<u>Figure 5.2.</u> There was a significant difference in the amount of shIL-6R secreted in the supernatant.

Data are expressed as mean + SEM from 3 supernatant samples. p values as marked. One-way ANOVA with Neuman-Keuls Multiple Comparison Test.



<u>Figure 5.3.</u> Gene expression of IL-6 signalling pathway components varies significantly between NSCLC cell lines.

qRTPCR expression analyses of *GP130*, *IL6*, *IL6R* and *TACE* were performed on cDNA derived from NSCLC cell lines as marked. Relative expression data (n = 3 per cell line) following normalisation for *18S* expression are shown, and are presented from triplicate analysis as the mean ± SEM, compared to the 'standard' NSCLC cell line A549 cells. *p < 0.05, **p < 0.01, ***p < 0.001. One-way ANOVA with Neuman-Keuls Multiple Comparison Test.

and considered a standard in human lung cell lines (www.ott.nih.gov). Consistent with IL-6 secretion levels, both the H2228 and H838 cells had significantly higher gene expression of *GP130*, and in the case of H2228 also of *IL6*. While there were no significant differences in gene expression of the *IL6R* between cell lines, the H838 cells did show somewhat upregulated expression of *TACE*, which may explain the increased levels of sIL-6R detectable in the supernatant as TACE is responsible to cleaving the membrane bound receptor to produce sIL-6R (Chalaris et al., 2010b).

5.4 Blocking the IL-6R leads to a reduction in growth in H2228 and H838 cells

To examine whether differences in IL-6 secretion from the cell lines correlated with differential growth rates of these cells, I performed MTT assays on cells cultured in the presence of 1% FCS. However, as shown in Figure 5.4a, there was no significant difference in rates of growth among the cell lines. I next used the MRA antibody, which has previously been shown to suppress IL-6-induced responses (Nishimoto et al., 1994, Sato et al., 1993) to see if cell growth could be impaired upon blockade of IL-6 signals. MRA is able to bind both membrane bound and soluble IL-6R thus leading to global IL-6 blockade. Notably, blockade of IL-6R led to a significant reduction in growth of those cell lines with high IL-6 and sIL-6R secretion (H2228 and H838) compared to untreated controls (Figure 5.4b). This is consistent with data from Bihl and colleagues showing that NSCLC cell lines may exhibit either IL-6-dependent or -independent growth (Bihl et al., 1998). However, in contrast to their findings, I have shown a significant, albeit modest, reduction in growth by blocking IL-6 signals in two cell lines. A possible explanation for this discrepancy could be that the cell lines I used had higher expression of IL-6 and sIL-6R, since the two cell lines common to the two studies (A549 and H23) did not show a reduction in growth in my experiment. Aside from differences in the inherent binding affinity and avidity of the antibodies, and the reversibility of the interaction, the varying results may be explained by our differing strategies in blocking IL-6; I used an antibody against the IL-6R while they used one that directly targeted the IL-6 ligand, albeit both targeting global IL-6 signalling.

Having shown that at least a subset of human lung adenocarcinoma cell lines secrete both IL-6 and sIL-6R, and are dependent on it for proliferation, the findings were tested in an *in vivo* model of adenocarcinoma.

5.5 Deregulated gp130 signalling does not significantly increase tumour load in a *LSL*Kras^{G12D} model of lung cancer.

Since it was first developed, the *LSL*Kras^{G12D} model of lung cancer has been used extensively to further delineate molecular pathways involved in the pathogenesis of lung adenocarcinoma,

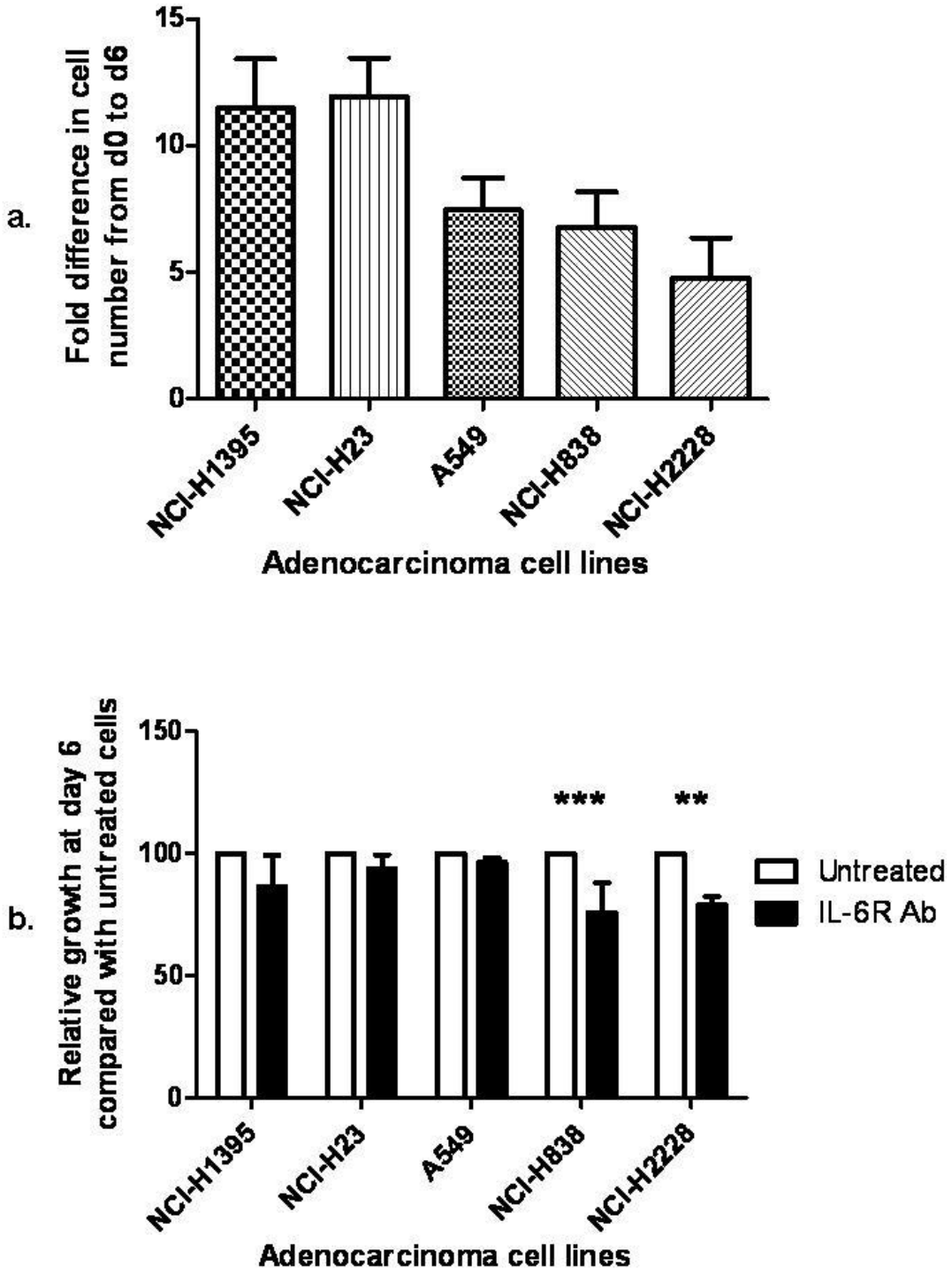


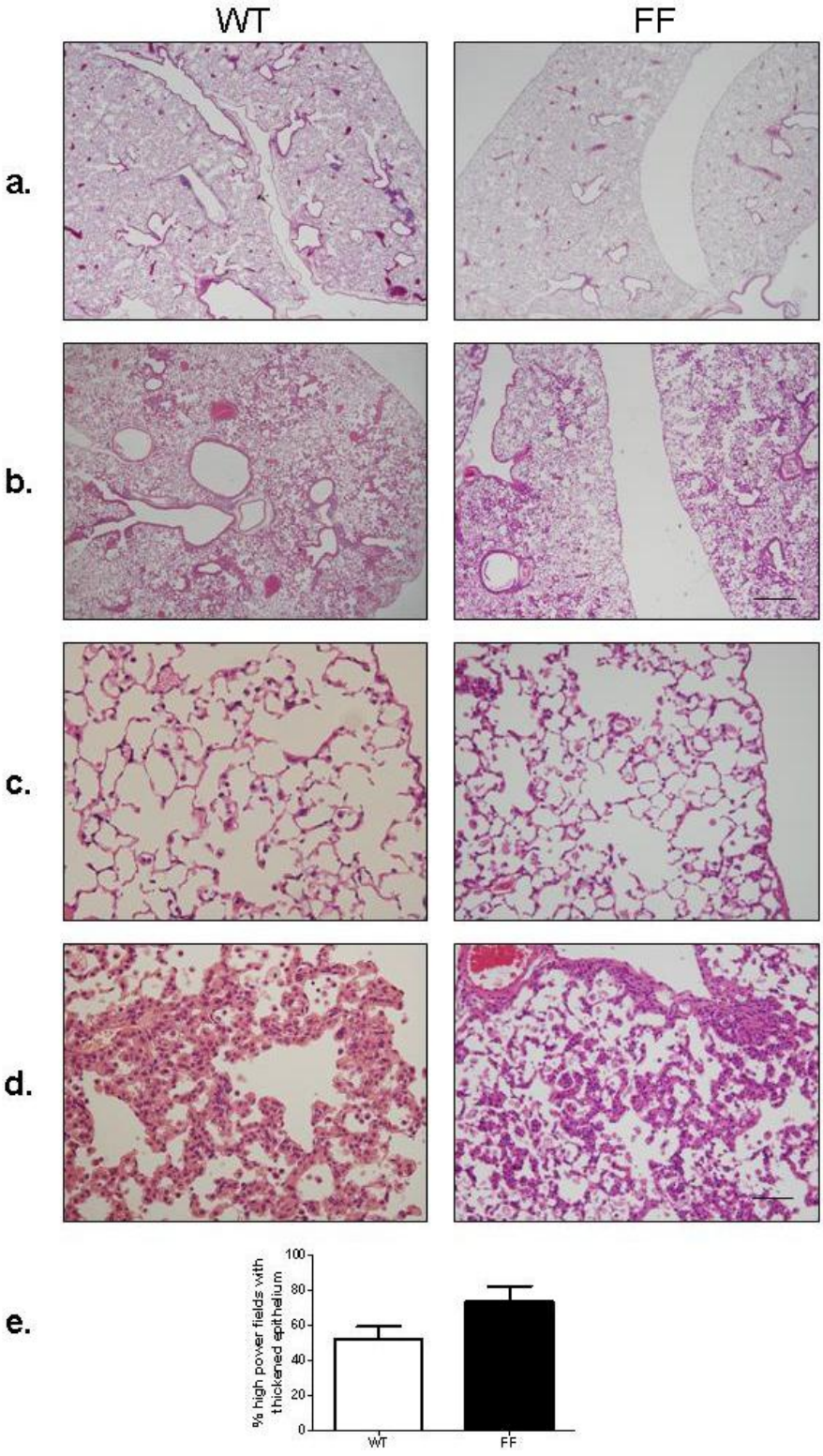
Figure 5.4. Antibody-mediated blockade of IL-6R reduces cell growth in the NSCLC cell lines that have high expression of IL-6 and sIL-6R.

a. No significant difference was seen between cell lines in the magnitude of cell growth (MTT assay) over 6 days; comparison of untreated cells from day 0 and day 6. Mean ± SEM from n=3 experiments. b. Comparison of cell growth (MTT assay) in cells treated with IL-6R Ab (MRA) at 10μg/ml relative to untreated cells, after 6 days of treatment. ** $p < 0.01$, *** $p < 0.001$; Two-way ANOVA with Bonferroni multiple comparisons test.

as well pre-clinical trials of anti-cancer therapy (Dovey et al., 2008, DuPage et al., 2009, Fisher et al., 2001, Granville et al., 2009, Houghton et al., 2010, Wilderman et al., 2005). Therefore, I utilised this model by crossing it with the FF mice to examine the interaction between activated K-ras and deregulated gp130 signalling characterised by elevated IL-6 and hyper-activated STAT3 (Jenkins et al., 2007, Ruwanpura et al., 2011). For this purpose, 6-8 week old mice inhaled a Cre-recombinase expressing, non-replicative adenovirus with a plan to observe over 12 weeks, a period shown long enough for mice to develop large adenomas (DuPage et al., 2009). The FF mice at this age have been shown to not have significant pulmonary inflammation or established emphysema (Ruwanpura et al., 2011). Unfortunately, contrary to published data (Houghton et al., 2010, DuPage et al., 2009) it was noted that a significant proportion of the FF mice were dying before the end of the 12 week observation period, so the study was limited to 6 weeks meaning the mice were 3 months old when the experiment was completed.

Initial examination of the lungs of FF:Kras^{G12D} and WT:Kras^{G12D} littermates revealed that while they did not develop defined adenomas following inhalation of Cre-recombinase, there was extensive alveolar membrane thickening (Figure 5.5b) not seen in PBS treated mice (Figure 5.5a). Additionally, the alveolar thickening was patchy with normal lung (Figure 5.5c) adjacent to abnormal areas (Figure 5.5d). Given a lack of defined lesions we quantified the change by counting the proportion of high power fields with thickened alveolar membranes in a representative section of the whole lung. While there was a slight increase in the proportion of the FF:Kras^{G12D} lung affected by this change compared to the WT:Kras^{G12D} lung, this was not significant (Figure 5.5e). To exclude any influence of the adenovirus on the development of the alveolar membrane thickening we included two separate controls for WT:Kras^{G12D} and FF:Kras^{G12D} mice: Kras^{WT} mice exposed to the virus and Kras^{G12D} mice which inhaled only the vehicle. None of these mice developed the epithelial thickening described above (Figure 5.5a).

As FF mice are known to have an increase in lung inflammatory cells (see Chapter 3 and Figure 3.3) we undertook further IHC staining to clarify what cell types were contributing to the epithelial thickening. To confirm the epithelial thickening seen was not due to infiltration with inflammatory cells I stained the sections with the CD45 antibody, a pan-lymphocyte marker. Whilst inflammatory infiltrates were still evident in the peri-bronchovascular region in the FF:Kras^{G12D} lungs (Figure 5.6a and b, arrow), CD45 cells did not make up the majority of the thickened areas (Figure 5.6b). In an attempt to further define the cell type involved we used an antibody against TTF-1 which is an epithelial marker with high specificity for lung adenocarcinoma (Yatabe et al., 2002, Reis-Filho et al., 2000). The thickened epithelium stained heavily for TTF-1 (Figure 5.6c), suggesting that the thickening seen is due to an



<u>Figure 5.5.</u> Histological assessment of FF:Kras^{G12D} mice and WT:Kras^{G12D} littermates following treatment with Cre-recombinase or PBS.

Representative low power photomicrographs of PBS treated (a) and Cre treated (b) lungs from WT:Kras^{G12D} and FF:Kras^{G12D} mice showing patchy alveolar membrane thickening in treated but not control lungs. Bar represents 500μm. Representative photomicrographs from WT:Kras^{G12D} and FF:Kras^{G12D} mice showing normal appearing (c) and thickened (d) alveolar membranes following activation of oncogenic *Kras* following Cre inhalation. Bar represents 100μm. (e) Graph showing the proportion of high power fields with abnormally thickened epithelium. 20 fields counted at 40x magnification on a representative section of whole lung. n=5/group. Represented as mean ± SEM.

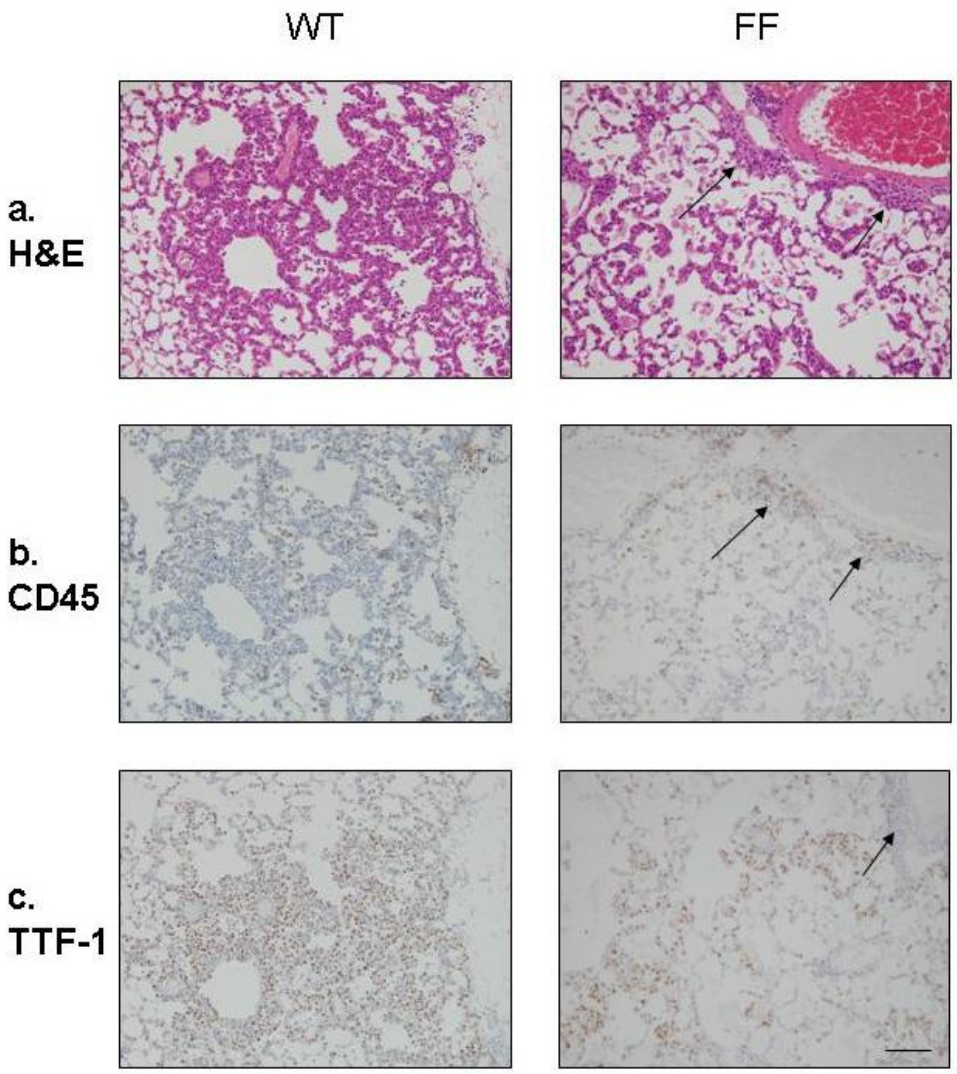


Figure 5.6 The alveolar membrane thickening is an increase in epithelial cells not inflammatory cell infiltrates.
Photomicrographs of representative sections from WT:Kras^{G12D} and FF:Kras^{G12D} mice 6 weeks post inhalation of Adenovirus expressing a Cre-recombinase. Sections stained with H&E and equivalent sections labelled with antibodies to CD45 and TTF-1. Arrows in b. and c. show peri-bronchovascular inflammatory infiltrates. Bar represents 100μm.

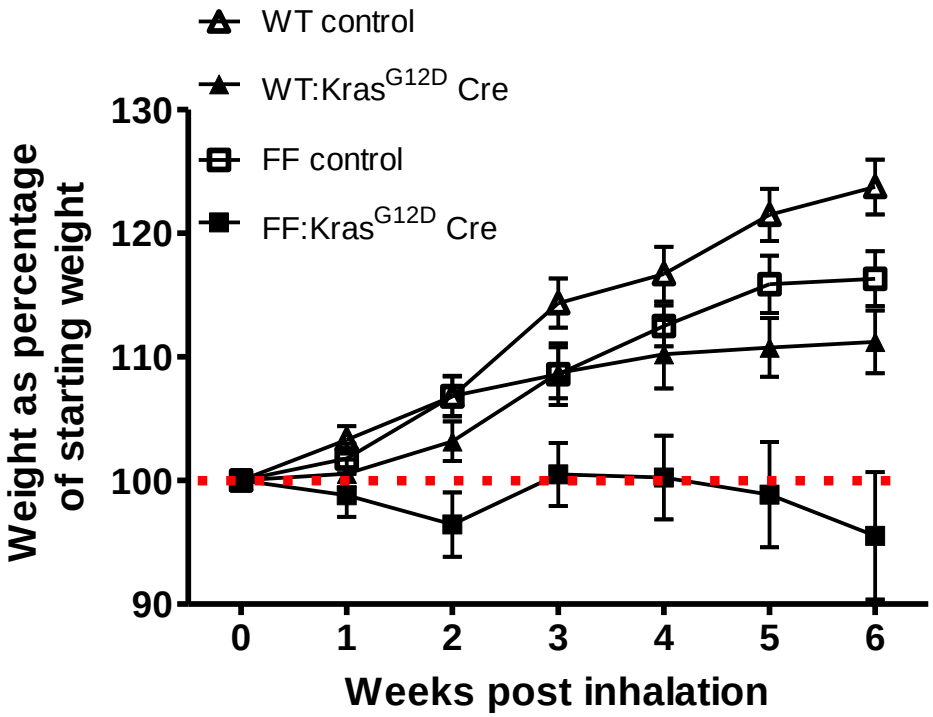
increase in epithelial cells rather than infiltration with inflammatory cells, and consistent with adenomatous hyperplasia.

5.6 FF mice lose weight following activation of oncogenic *Kras* which is abrogated by genetic reduction of STAT3 and IL-6

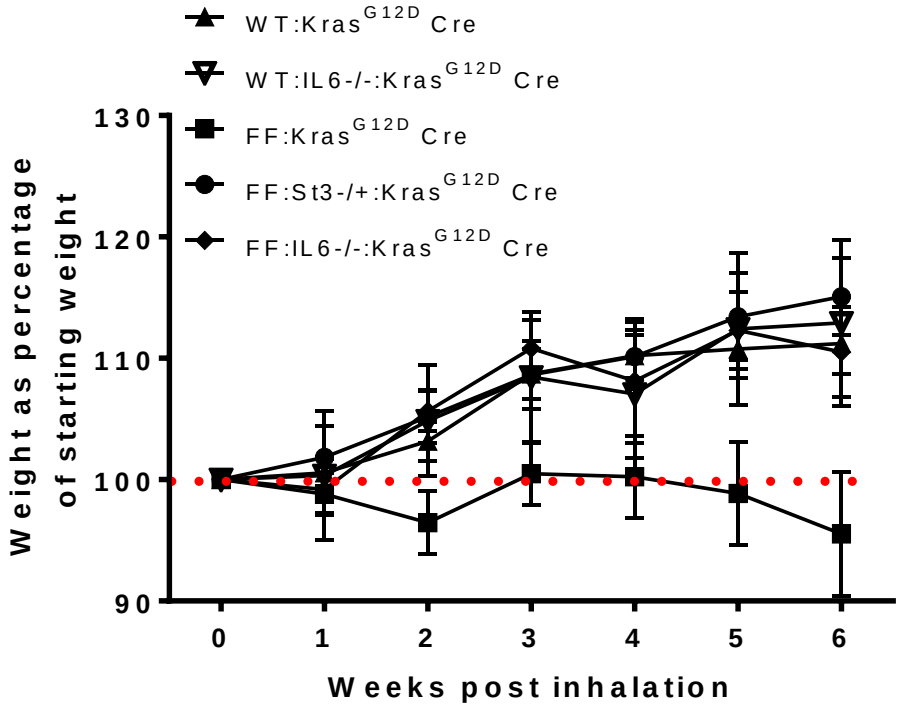
During the observation period it was noted that FF:Kras^{G12D} mice treated with Cre-recombinase failed to gain weight, and ultimately lost weight (Figure 5.7a). Importantly, this was not due to any difference seen between the starting weight of any of the genotypes utilised in these experiments (Figure 5.8). Similarly there was no difference in weights between FF:Kras^{WT} mice treated with Cre-recombinase and FF:Kras^{G12D} treated with vehicle over the 6 week observation period (Figure 5.9), so these groups are subsequently shown together as FF control (likewise the two corresponding groups for gp130^{WT} are displayed as WT control). While there was no difference in weight at the completion of the observation period in FF control mice compared with WT control mice, both WT:Kras^{G12D} and FF:Kras^{G12D} mice has significantly lower weight than their control littermates (Figure 5.7a: WT $p<0.01$ at 5 weeks, $p<0.001$ at 6 weeks; FF $p<0.05$ at 4 weeks, $p<0.001$ at 5 weeks, $p<0.0001$ at 6 weeks; 2way ANOVA with Bonferroni Multiple Comparison Test). Strikingly, there was also a significant difference in the weight of FF:Kras^{G12D} mice when compared to WT:Kras^{G12D} mice ($p<0.01$ at 5 weeks, $p<0.0001$ at 6 weeks).

To further elucidate the ligand and signalling molecules responsible for the weight loss in the FF mice, I went on to examine the effect of activation of oncogenic K-ras in FF compound mutant mice. For this we utilised FF:St3-/+:Kras^{G12D} which display genetically normalised STAT3 levels (Jenkins et al., 2005b) and FF:IL6-/-:Kras^{G12D} mice which have genetically absent IL-6. As a control for these experiments, and to examine the interaction of IL-6 and oncogenic K-ras without deregulated gp130 signalling, we also used WT:IL6-/-:Kras^{G12D} mice. Following the exact inhalation procedure described above (see also Chapter 2), we observed that there was no significant difference in the weight gain of control mice of any genotype (data not shown), or between WT:Kras^{G12D} and WT:IL6-/-:Kras^{G12D} mice (Figure 5.7b). In contrast to these observations, there was a complete rescue from weight loss in both FF:St3-/+:Kras^{G12D} and FF:IL6-/-:Kras^{G12D} mice treated with Cre-recombinase, with significantly greater weight gain when compared with FF:Kras^{G12D} mice (St3-/+ $p<0.05$ at 5 weeks, $p<0.01$ at 6 weeks; IL-6-/- $p<0.05$ at 6 weeks) (Figure 5.7b). Furthermore, neither the FF:St3-/+:Kras^{G12D} nor FF:IL6-/-:Kras^{G12D} mice were significantly different from WT:Kras^{G12D} mice in terms of weight change over the observation period, therefore suggesting that the weight loss seen in the FF:Kras^{G12D} mice is driven by IL-6 through STAT3.

a.



b.



<u>Figure 5.7.</u> FF mice lose weight over the 6 weeks following Cre inhalation in a STAT3 and IL-6 dependent manner.
Graphs showing change in weight over the 6 weeks of experiment expressed as a percent of the starting weight (mean ± SEM; n≥7/group).

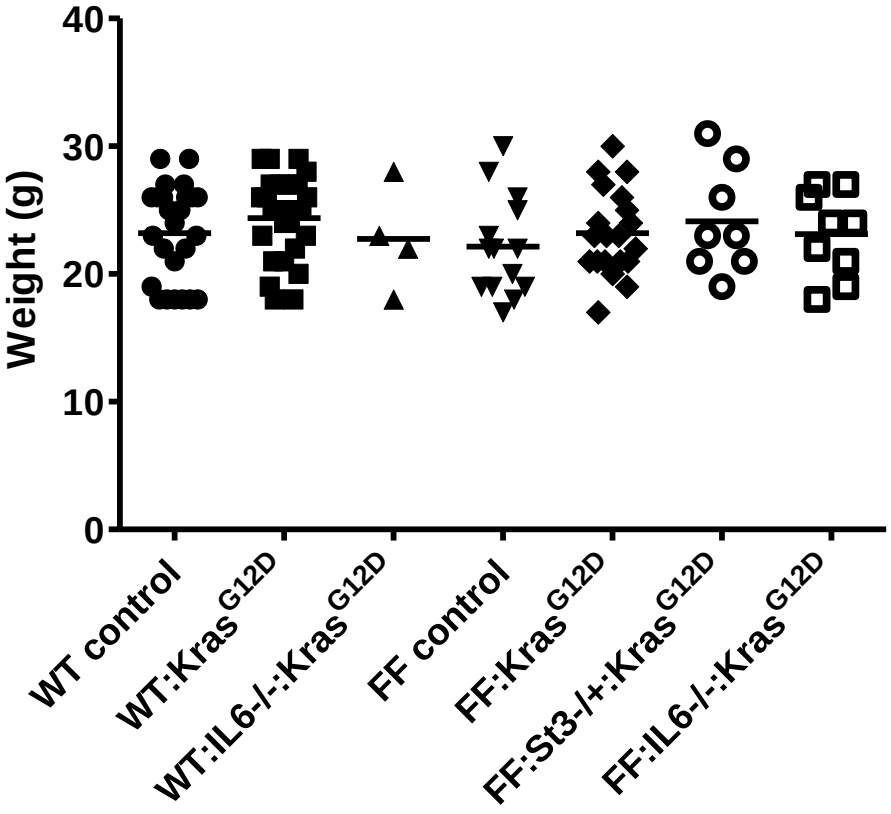
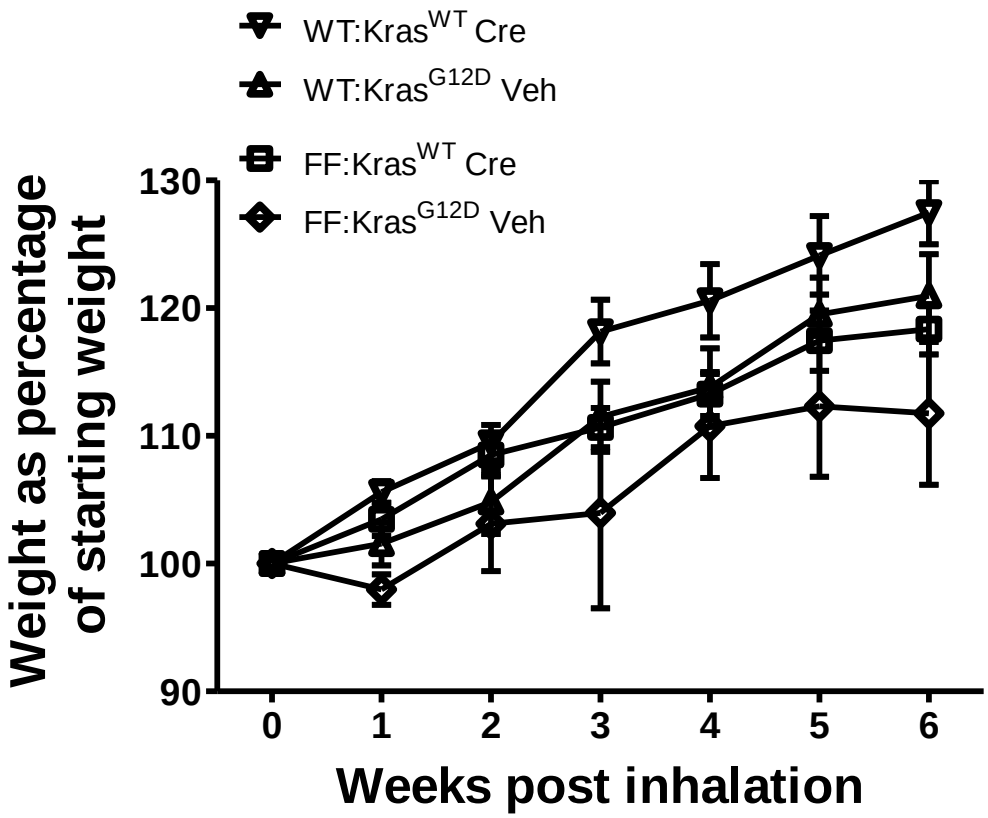


Figure 5.8. There was no difference in starting weight between experimental groups. Scatter dot-plot of all included experimental groups showing mean. There is no significant difference between the groups.



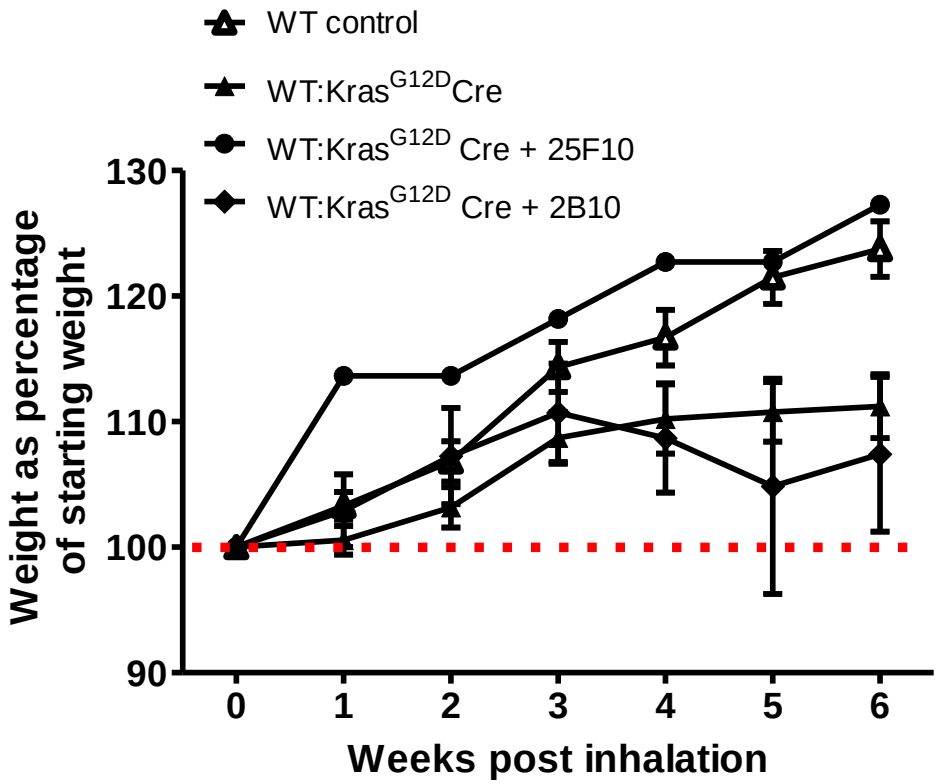
<u>Figure 5.9.</u> There is no significant difference in weight between control groups. Graph showing weight change over 6 weeks of observation, plotted as mean ± SEM. n≥4/group. There is no significant difference between the two WT or FF control pairs.

5.7 Specifically targeting IL-6 trans-signalling abrogates weight loss and improves survival of Cre-inhaled tumour-bearing FF mice

Having established a role for exaggerated IL-6/STAT3 signalling in the weight loss associated with oncogenic K-ras-induced tumourigenesis in FF mice, with a view to a therapeutic intervention, I next treated mice with antibodies directed against the IL-6R. For this purpose I used two monoclonal antibodies (obtained by our collaboration with NovImmune) which display different binding affinity for the IL-6R and have varying effects on IL-6 signalling. The first was 2B10 which binds directly to the IL-6R, blocking IL-6 docking and leads to efficient blockade of classical (membrane-bound) signalling, having a minimal effect on trans-signalling (Lissilaa et al., 2010). The second antibody (25F10) binds to a putative gp130-binding site on the IL-6R, thus primarily affecting trans-signalling (Lissilaa et al., 2010). The advantage of using both antibodies is that we can examine not only the effect of IL-6R blockade but also better delineate which signalling modality is important in the weight loss seen.

WT:Kras^{G12D} and FF:Kras^{G12D} mice were treated with Cre-recombinase as above and subsequently had IP injections of the IL-6R antibodies at a dose of 1mg weekly, with the first dose given on recovery from the anaesthetic used for inhalation. It was planned to include a minimum of 3 mice per treatment group but for two reasons not all the final groups had this number: first, a number of mice died or required euthanasia whilst under experiment, and second, the volume of antibody was limited so priority was given to the FF mice. In WT mice, while there was no significant difference in weight gain with either antibody treatment (2B10 n=3; 25F10 n=1), mice treated with 25F10 had a weight gain more similar to control mice (Figure 5.10a), albeit only in one mouse. In contrast, the weight gain in Cre-inhaled FF:Kras^{G12D} mice treated with 25F10 (n=4) was significantly greater when compared to Cre-inhaled untreated animals (Figure 5.10b: $p<0.01$ at 4 weeks, $p<0.0001$ at 5 weeks, $p<0.0001$ at 6 weeks), which was not seen in the single mouse that completed the 6 weeks with treatment with 2B10. In addition to the marked weight loss observed in the Cre-inhaled FF:Kras^{G12D} mice, it was also noted they either frequently died prior to the completion of the 6 week observation period or had weight loss in excess of acceptable limits and required early euthanising; 33.3% of Cre-inhaled FF:Kras^{G12D} mice died prior to the end of the 6 week period compared with 7.1% of control FF:Kras^{G12D} mice (Figure 5.11b). By contrast, this was not seen in the WT animals regardless of their genotype or treatment (Figure 5.11a), in which 100% survived for the 6 week experimental period. Most importantly, and consistent with the weight data from Figure 5.10, genetically reducing the levels of STAT3 (FF:St3-/+:Kras^{G12D}), and targeting IL-6 either genetically (FF:IL6-/-:Kras^{G12D}) or with the 25F10 mAb which efficiently blocks IL-6 trans-signalling dramatically improved the survival of these mice to

a.



b.

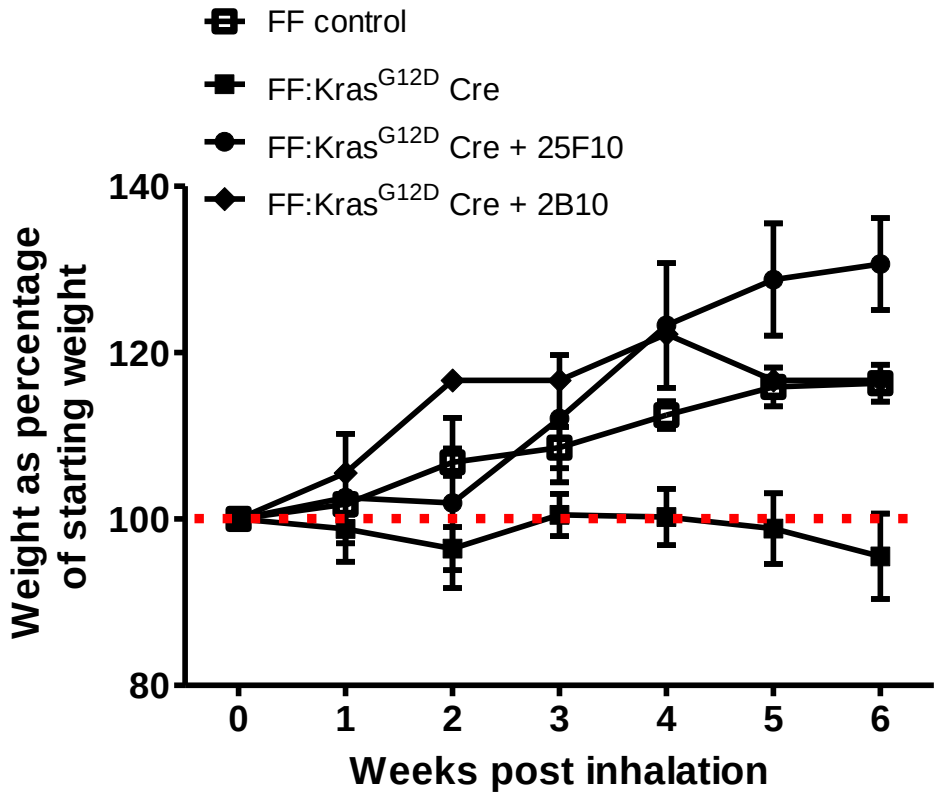
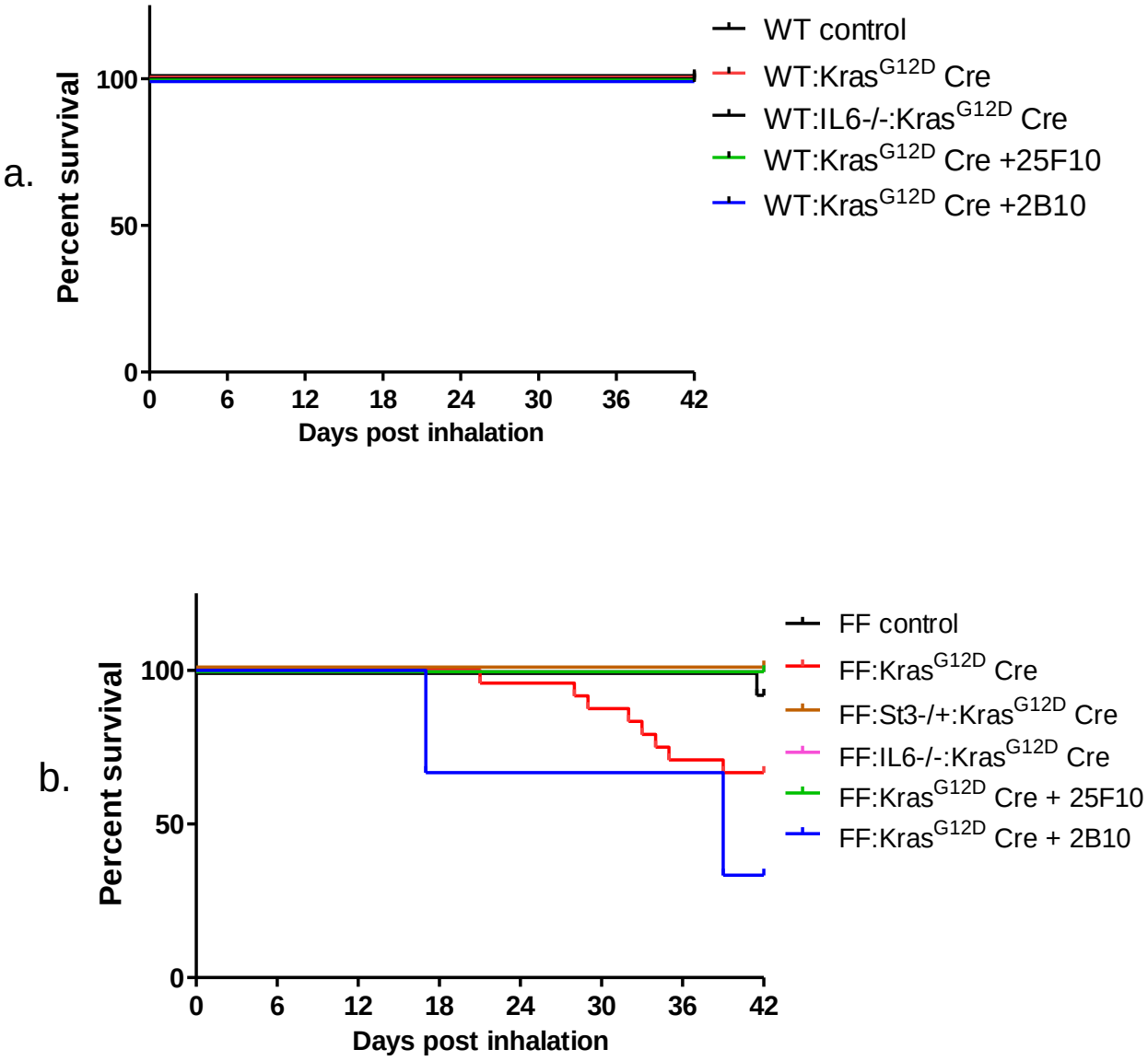


Figure 5.10. Targeting IL-6 trans-signalling but not classical signalling leads to restoration of weight gain in FF mice.
Graphs showing change in weight over the 6 weeks of experiment expressed as a percent of the starting weight (mean ± SEM; n≥3/group except WT:Kras^{G12D} + 25F10 and FF:Kras^{G12D} + 2B10 which are only n=1)). Both antibodies were injected IP weekly beginning at week 0, at a dose of 1mg/injection.



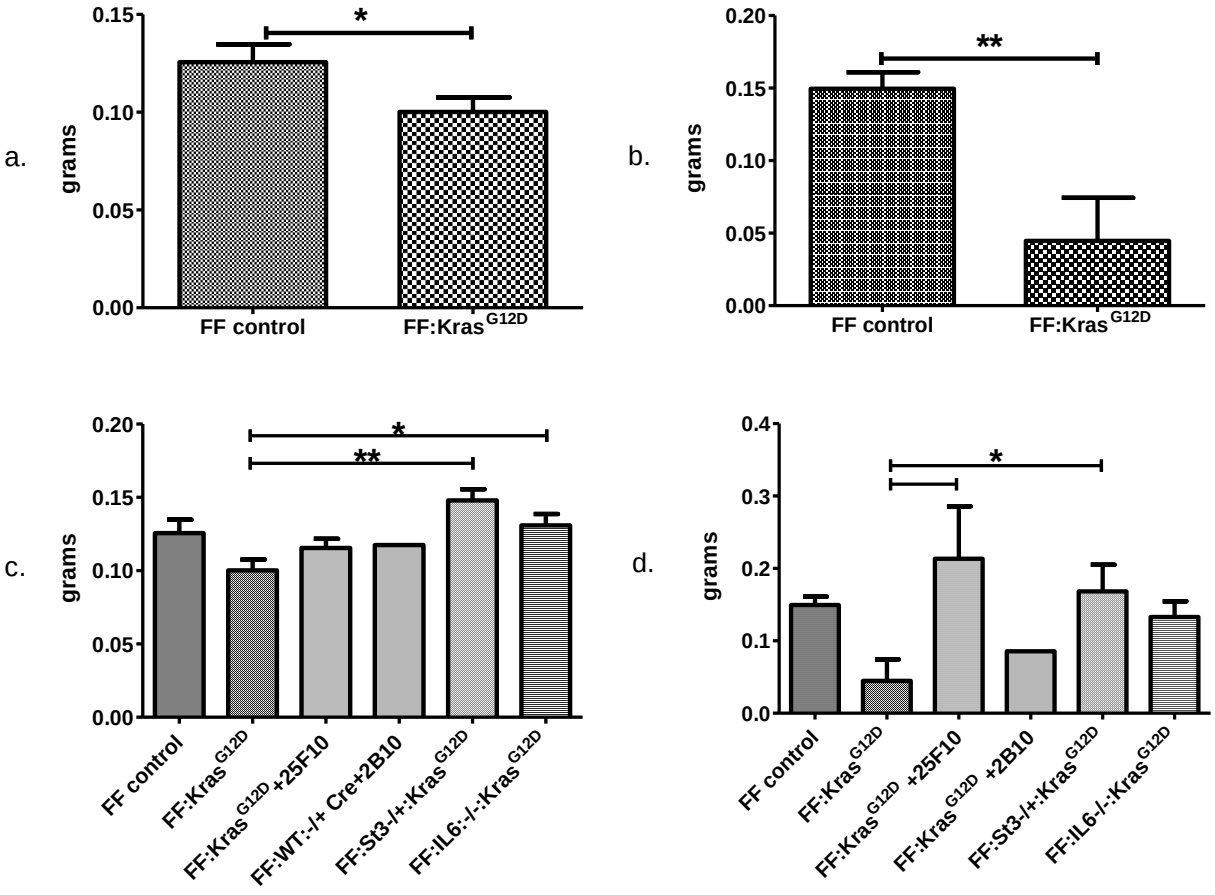
<u>Figure 5.11.</u> FF:Kras^{G12D} mice have reduced survival which appears to be mediated through IL-6 and STAT3 signalling.
Survival curves for WT and FF mice -/+ antibody treatment, and compound mutant mice as marked. n≥3 except WT:Kras^{G12D} Cre + 2B10 which is only n=1. There is a significant difference between the curves for the FF mice, $p<0.01$ (Log-rank Test).

100% (compared to 66.6% for Cre-inhaled FF:Kras^{G12D} mice; there was a significant difference between the survival curves ($p<0.01$) (Figure 5.11b). Interestingly, treatment with the IL-6R antibody 2B10 (which primarily blocks classical signalling) had no effect on mortality, adding further weight to the suggestion above that it is trans rather than classical signalling that is important. Of course, the number of mice in this study limits the veracity of any conclusions we can draw. This is preliminary work allowing for further hypothesis generation and directing additional studies. The strength of the study is the powerful genetic tools used but this comes at the cost of relying on the sometimes fickle breeding program to generate the appropriate genotypes.

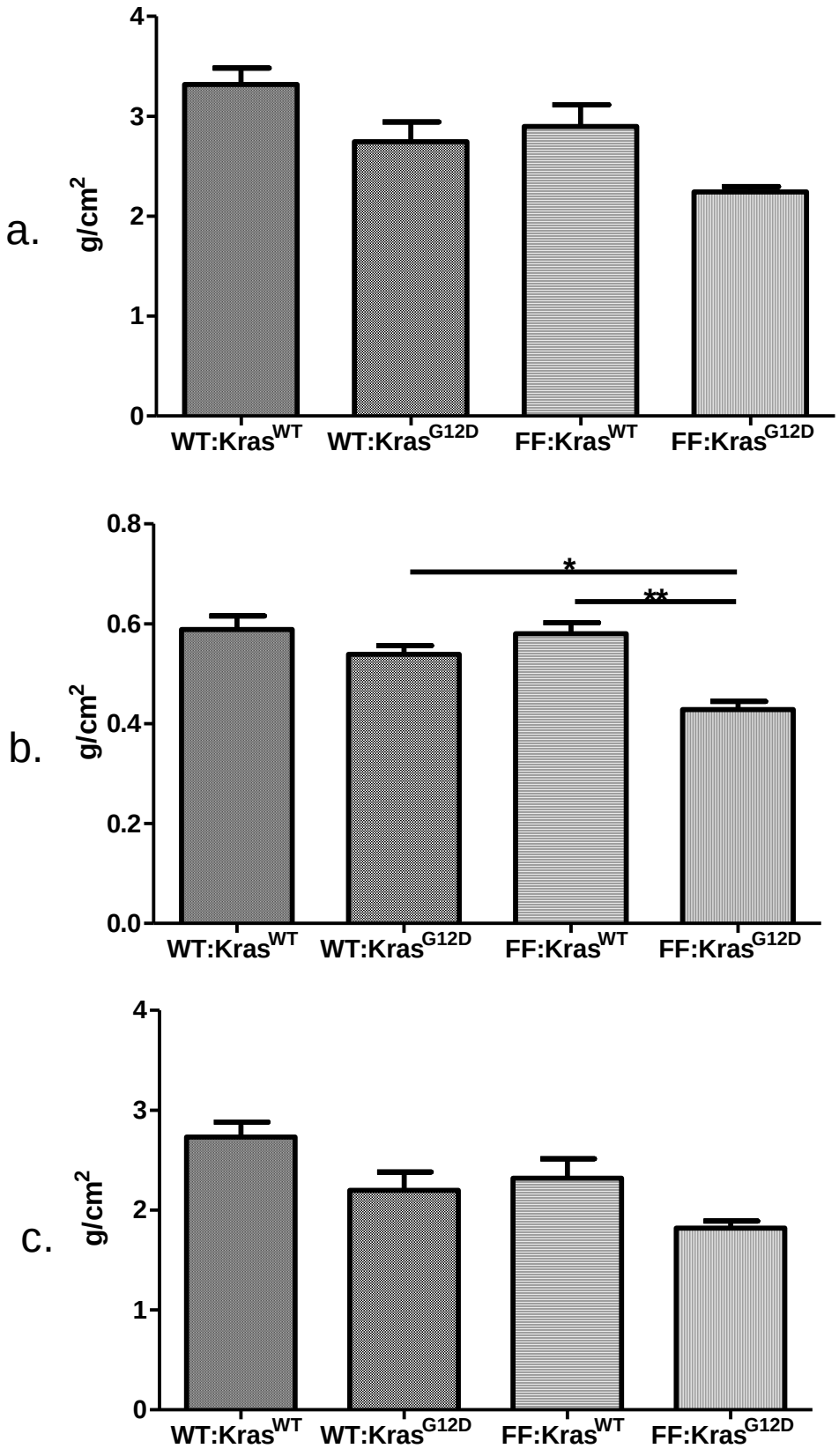
5.8 FF mice lose mass from both fat and lean compartments

The combination of weight loss and reduced survival is consistent with the syndrome of cancer-related cachexia (Bozzetti and Mariani, 2009, Tisdale, 2009), which is so common in human lung cancer (Ross et al., 2004, Win et al., 2007). To further examine whether the weight loss was confined to one body compartment, representative muscle (gastrocnemius) and fat tissue (peri-gonadal) was dissected from the mice and weighed (Baltgalvis et al., 2008). There was a significant reduction in the weight of both gastrocnemius (one-tailed $p = 0.0291$; unpaired t-test) and peri-gonadal fat ($p = 0.0069$) in the FF:Kras^{G12D} mice when compared to FF controls (Figure 5.12 a and b). I again utilised both genetic and therapeutic approaches to ascertain the influence of elevated IL-6 and STAT3 on the compartmental weight loss and what mode of IL-6 signalling is at play. When the group was analysed as a whole, the gastrocnemius weight in both FF:St3-/+:Kras^{G12D} and FF:IL6-/-Kras^{G12D} mice was significantly greater than FF:Kras^{G12D} mice (Figure 5.12c; St3-/+ $p<0.01$, IL6-/- $p<0.05$; 1way ANOVA with Bonferroni's multiple comparison test). Similarly, the adipose tissue weight was significantly greater in FF:St3-/+:Kras^{G12D} mice and FF:Kras^{G12D} treated with 25F10 when compared to FF:Kras^{G12D} mice (without antibody treatment) (Figure 5.12d; $p<0.05$). This confirms my earlier findings (Figure 5.7) showing that elevated IL-6 and STAT3 are responsible for the weight loss seen in FF:Kras^{G12D} mice, and clarifies that they contribute to both muscle and fat tissue loss. In addition, certainly in the case of fat tissue loss, it is clear that it is trans-signalling, and not classical signalling, that is responsible for the actions of IL-6.

In an effort to confirm these findings by another method, and also to provide a possible point of translation to observation of human disease, I collaborated with Dr Pablo Enriori in the Department of Physiology, Monash University, and undertook DEXA on a sub-group of these



<u>Figure 5.12.</u> FF mice lose both lean and fat tissue mass.
Weight of body length normalised gastrocnemius muscle (a,c) and peri-gonadal fat (b,d). Bar graphs show mean + SEM for the marked genotypes, with n=5 (a,b) or n=3 (c,d) with the exception of FF:Kras^{G12D} + 2B10 for which there is only n=1 in both graphs (and was excluded from the analysis). *p<0.05, **p<0.01; unpaired t-test graphs a and b, and 1way ANOVA with Bonferroni's multiple comparison test graphs c and d.



<u>Figure 5.13.</u> There is a significant reduction in fat tissue density in FF:Kras^{G12D} mice measure by DEXA.

DEXA performed on whole mice using the Lunar PIXImus and analysed with proprietary software. Bar graphs of total tissue density (a), fat tissue density (b) and lean tissue density (c). Showing mean + SEM; n ≥ 3/group. ** $p < 0.01$, * $p < 0.05$; 1way ANOVA with Bonferroni multiple comparison test.

mice (Pedroso et al., 2012, Chen et al., 2012, Nagy and Clair, 2000, Di Sebastiano and Mourtzakis, 2012) . Notably, DEXA analyses revealed a significant reduction in fat tissue density in the FF:Kras^{G12D} mice when compared to both control FF:Kras^{WT} mice ($p<0.01$) and WT:Kras^{G12D} mice ($p<0.05$), and while there was also a slight reduction in total tissue density (Figure 5.13a) and lean tissue density (Figure 5.13c) in the FF:Kras^{G12D} mice this difference did not reach significance. The predominance of fat loss may be explained by the length of the experiment, as others have shown fat loss to occur earlier and more profoundly than muscle loss, albeit in a ApcMin-/- model of bowel cancer (Baltgalvis et al., 2008).

Taken together these data suggest that the significant weight loss seen in the FF:Kras^{G12D} mice is made up of both fat and lean tissue loss, perhaps with a predominance of fat tissue. That I have not shown significance in all the sub-groups is not surprising given the relatively low numbers of mice analysed, but a trend certainly exists.

5.9 Discussion

In this section of my project I have shown that NSCLC cell lines secrete IL-6 and sIL-6R at variable levels, and that high expressing cells rely, at least in part, on autocrine IL-6 signalling for growth. I have also for the first time provided evidence that elevated IL-6 and increased activity of STAT3 downstream of gp130 correlates with a cachexia-like syndrome in an orthotopic lung cancer model, without significant increase in tumour load.

The *in vitro* work I performed confirms work from other laboratories that IL-6 secretion varies between NSCLC cell lines, suggesting that the growth of human NSCLC cells is likely to be governed by both IL-6 dependent and independent mechanisms (Bihl et al., 1998). My work also adds to this literature by showing that, at least in the H838 cells, both the IL-6 secretion and growth dependence correlate with sIL-6R production, suggesting that IL-6 trans-signalling is the modality implicated in cell growth. Finally, these data also suggest that TACE is likely to facilitate the production of sIL-6R in H838 cells, as while the gene expression of the IL-6R was similar between cell lines, TACE was markedly elevated in the H838 cells, correlating with sIL-6R protein levels in the cell supernatant.

Mutations of K-ras are frequent in human lung adenocarcinoma, and while elevation of IL-6 levels and increased expression of activated STAT3 are also often noted there is little literature examining the interaction between the two pathways (Anderson et al., 1991, Yanagawa et al., 1995, Ai et al., 2012, Qu et al., 2009b). Ras signals downstream of a number of receptor tyrosine kinases most notably EGFR, but IL-6-dependent K-ras signalling is seen in myeloma, breast and pancreatic cancers (Corcoran et al., 2011, Leslie et al., 2010, Rowley and Van Ness, 2002, Lesina et al., 2011) so an important interaction is plausible. Of particular relevance is the work in pancreatic cancer, not only showing the importance of both

local and systemic inflammation on the progression of the cancer in a *Kras*^{G12D} model, but also importantly demonstrating a role for IL-6 trans-signalling specifically (Lesina et al., 2011).

The data I have shown suggests that deregulated gp130 signalling has only a minor effect on tumourigenesis in a *LSL*Kras^{G12D} model of lung adenocarcinoma. Somewhat contrary to this Ochoa et al. have demonstrated a role for IL-6 signalling in the development of lung tumours in a *LSL*Kras^{G12D} model by showing a reduction in tumour load with the genetic ablation of IL-6 (Ochoa et al., 2011). This disparity can be explained to a degree by the different models we have utilised to examine the question. The FF model displays elevated levels of IL-6 and hyper-activated STAT3, while the model used by Ochoa et al. has a total absence of IL-6 signalling. The minor pro-tumourigenic influence seen in our model may suggest a threshold effect of IL-6, meaning that increases in IL-6 above a critical saturation point have little additional effect.

Interestingly, they found that exposing IL-6 KO mice to NTHi led to profound pulmonary inflammation comparable to IL-6 WT mice, but with substantially reduced tumour load. This is in agreement with my IHC data showing very little inflammation associated with the adenomatous hyperplasia (Figure 5.7) and suggests a pro-tumourigenic role for IL-6 independent of inflammation. Of course we have the genetic tools to further tease out the role of both IL-6 (FF:IL6-/-:Kras^{G12D}) and STAT3 (FF:St3-/+:Kras^{G12D}) and this forms part of ongoing work performed by others in our laboratory.

Drawing firm conclusions from this novel work is somewhat limited by the low numbers of mice ultimately included in analysis. That significant differences were seen despite these low numbers adds credence to the findings and serves to further refine our experimental hypothesis and leading to more definitive work. This early data largely confirms and extends current literature, but clearly studies including larger numbers are required to refute contrary findings.

While there is no literature associating Ras with cachexia in lung cancer, there is an association in malignant melanoma. Over-expression of mutant Ras in melanoma cells not only increases cell growth and invasiveness, but mice bearing xenografts of cells over-expressing mutant Ras develop cachexia (Fujita et al., 1999). However, there are no reports of cachexia in the *LSL*Kras^{G12D} model of lung cancer, suggesting that activation of oncogenic K-ras *in vivo* alone is not sufficient to induce cachexia. Lung cancer related cachexia has traditionally been modelled in mice using LLC cell xenografts, with little literature regarding orthotopic lung cancer models and cachexia (Diament et al., 1998). The FF:Kras^{G12D} mouse provides an orthotopic model of lung cancer with concomitant elevation of IL-6 mimicking that in a subset of patients who display weight loss, reduced survival and increased IL-6 levels

(Songur et al., 2004). The results of my work are consistent with xenograft studies showing that cachexia is associated with an increase in IL-6 levels and increased STAT3 expression, and increased weight loss and mortality in mice bearing xenografts transfected with hIL-6 cDNA (Bonetto et al., 2012a, Ohe et al., 1993). My studies extend this by showing that genetically normalising levels of either STAT3 or IL-6 is enough to abrogate the weight loss seen with tumour development, and return survival to WT levels.

Additionally, by virtue of the differential results seen with antibodies targeting either classical (2B10) or trans-signalling (25F10), my work, albeit still in its relative infancy, would suggest that therapeutically targeting specifically trans-signalling (rather than global (trans and classical) or classical IL-6 signalling) may provide relief from the devastating para-neoplastic syndrome of cachexia. The idea to use anti-IL-6 therapies is not new, in fact a humanised anti-IL-6 monoclonal antibody (ALD518) which blocks both classical and trans-signalling has been used in stage II clinical trials for subjects with cachexia secondary to NSCLC (Bayliss et al., 2011). ALD518 has been shown to be effective in reducing loss of lean body mass and improving symptoms without a clear increase in adverse effects over placebo. While the trials of ALD0518 in lung cancer showed few adverse events, dose finding and safety studies in RA have shown a significant rate of liver dysfunction, mild neutropaenia and thrombocytopaenia, and increases in cholesterol and triglyceride levels, although these rarely required withdrawal from the trial (Mease et al., 2012). Similarly, the clinical use of another antibody targeting global IL-6 signalling in RA (Tocilizumab) has been associated with a number of (non-serious) adverse events, including increases in serum cholesterol (Nishimoto et al., 2003). With the increasing evidence that classical signalling coordinates the homeostatic properties associated with IL-6 signalling (Heinrich et al., 2003, Kishimoto, 2010), and dysregulated trans-signalling is responsible for pathological inflammation and cancer (Greenhill et al., 2011, Jones et al., 2011, Rose-John et al., 2006, Lo et al., 2010, Mitsuyama et al., 2006a), there are obvious advantages of targeting trans-signalling alone at the receptor level rather than global IL-6 signalling. My data provides support for the idea that IL-6 trans-signalling may be responsible for the cachexia associated with lung cancer, and selective targeting of trans-signalling may be therapeutically useful.

CHAPTER 6

Final Discussion and Conclusion

My project aimed to elucidate signalling mechanisms leading to the development of lung cancer using a sophisticated animal model displaying deregulated gp130 signalling. Specifically, my first aim formed part of the larger characterisation of the FF mouse and involved defining the inflammatory profile of the FF mouse lung. Subsequent aims utilised the FF mouse in two well characterised lung cancer models to examine the role of deregulated gp130 signalling plays in the development of lung cancer and its complications, specifically cancer-related cachexia. Finally, I used human lung adenocarcinoma cell lines to examine the activation of key oncogenic signalling pathways. The main findings of the project are as follows:

1. the FF mouse has substantial IL-6- and STAT3-driven pulmonary inflammation, consisting largely of $B220^{+}$ cells;
2. deregulated gp130/SHP2-driven signalling through PI3K/Akt and MAPK pathways in the FF mouse leads to a reduction in cigarette carcinogen (NNK)-driven lung tumourigenesis independent of STAT3;
3. lung cancer-related cachexia is exacerbated by deregulated IL-6/STAT3 signalling, and rescued by blockade of IL-6 trans-signalling.

Lung cancer is the leading cause of cancer death worldwide with more than 7000 people dying each year in Australia (AIHW, 2011). Unfortunately the majority of tumours are discovered when they are inoperable, and therapeutic options are limited. Clearly new treatments are needed and this is only likely to be achieved with a better understanding of the pathobiology of the disease. In fact, two major advances in treatment have come through the development of agents targeting sub-classes of adenocarcinoma, specifically those displaying activating mutations of EGFR and the EML4-ALK fusion gene (Soda et al., 2007, Lynch et al., 2004, Nakajima et al., 2010, Paez et al., 2004, Kwak et al., 2010). However, whilst these breakthroughs have led to remarkable responses and prolonged remission, the development of resistance leads ultimately to treatment failure (Brugger and Thomas, 2012). Accordingly, further advances in identifying additional targets for therapeutic intervention are urgently needed, and this relies in part on informative animal models that reflect the diversity of the disease grouped as lung adenocarcinoma.

One aspect of tumourigenesis that is getting increasing recognition is inflammation, with many solid epithelial tumours, including lung cancer, now clearly linked with inflammation

(Colotta et al., 2009, Coussens and Werb, 2002, Heikkilä et al., 2007, Jenkins et al., 2005a, Weedon et al., 1973, Engels et al., 2007, Engels, 2008). The potent inflammatory mediator IL-6 has frequently been shown to be elevated in subjects with lung cancer and is associated with poor outcomes (Pine et al., 2011, Chyczewska et al., 1997, Ohe et al., 1993, Songur et al., 2004). While we are not the first to examine the interaction of IL-6 signalling *in vitro* or *in vivo* in the context of lung carcinogenesis, the advantage of the FF model is that is displays endogenously elevated IL-6 levels and STAT3 activation, rather than artificial (transgenic) over-expression of these signalling components. In this respect, this model enables clear attribution of any actions of hyper-activated STAT3 to gp130 and polarises downstream signalling from gp130 by lacking SHP2 driven PI3K/Akt and Ras/MAPK signalling.

Initial characterisation of the FF mouse demonstrated increased inflammation in the lungs of FF mice as I reported in Chapter 3. This is consistent with other published data from our laboratory showing multi-organ inflammation and augmented inflammatory responses in the FF mouse (Tebbutt et al., 2002, Jenkins et al., 2005b, Greenhill et al., 2011). Gene expression of key inflammatory cytokines implicated in both COPD and lung cancer, including TNF-α, IL-1β and IL-6, are all elevated in the FF mouse lung (Ruwanpura et al., 2011, Barnes, 2004, De Vita et al., 1998). Similarly, gene expression of granulocyte-attracting chemokines CXCL-1 and -2, and T cell-attracting chemokines CCL-2 and -5 are elevated in the FF lung, and have previously been associated with lung cancer (Mantovani et al., 2004a, Mantovani et al., 2004b). This appeared to be mediated by IL-6 through STAT3 but not STAT1 as evidenced by normalised expression levels of these genes in FF:IL6-/- and FF:St3-/+ mice. By contrast, these gene expression levels in FF:St1-/- mice remained elevated and were a similar to FF levels. To examine lung parenchymal inflammatory cell populations I subjected single cell suspensions of whole lung to multi-colour FACS, demonstrating an increase in inflammatory cells as marked by CD45 positivity, with the majority being $B220^{+}$ (B cells). This was confirmed with immunohistochemistry, showing peri-bronchovascular infiltrates of B cells in the FF, but not WT lungs, which is consistent with published work showing nodular peri-bronchovascular infiltrates in transgenic mice over-expressing IL-6 (Kuhn et al., 2000). None of the compound mutant mice had inflammatory cell populations significantly different to WT or FF mice when assessed by FACS, but in line with my gene expression findings, on immunohistochemical staining both FF:St3-/+ and FF:IL6-/- mice lacked the peri-bronchovascular infiltrates seen in the FF mouse, while they persisted in the FF:St1-/- mouse.

Taken together these findings describe a model of spontaneous IL-6- and STAT3-dependent pulmonary inflammation. The B cell predominance is readily explained by the role of IL-6 in the maturation of B cells (Kishimoto et al., 1995), and while not necessarily the dominant cell in the pathogenesis of either emphysema or lung cancer, lymphoid follicles have been noted

in both and are considered to have more than bystander significance (Granville et al., 2009, Curtis et al., 2007, Kim et al., 2008). The STATs are known to have wide-ranging and diverse effects on regulation of inflammation, cell growth and proliferation (Yeh et al., 2006, Bowman et al., 2000, Bromberg, 2002, Hodge et al., 2005). STAT3 but not STAT1 has consistently been associated with pulmonary inflammation (Haura et al., 2005a, Qu et al., 2009b), and this is concordant with my data showing the spontaneous inflammation is independent of STAT1, but reliant on STAT3. However, this work must be taken in context. It forms part of a larger project fully characterising the lung phenotype of the FF mouse, which develops as a consequence of deregulated gp130 signalling (Ruwanpura et al., 2012, Ruwanpura et al., 2011). The main finding of this characterisation was that the FF mouse spontaneously develops pulmonary emphysema, which, while as a result of elevated IL-6 levels, was in fact independent of STAT3 and the consequent inflammation. This disconnection further reinforces the complexity of gp130-driven signalling and the need for informative animal models. There is ample evidence associating inflammation with the development of emphysema (van der Strate et al., 2006, Barnes et al., 2003), but our data does support that of others who have shown that reducing B cell numbers had little effect on cigarette smoke induced emphysema (Brandsma et al., 2008). Similarly, while IL-6 is well known to be involved in the inflammatory response within the lung, the literature examining its role in the development of emphysema is somewhat unclear. Emphysema developed in mice in which gp130 had been post-natally deleted (Betz et al., 1998) but conversely Kuhn et al. showed the development of emphysema in a transgenic mouse over-expressing IL-6 (Kuhn et al., 2000). Our work is in support of the work of Kuhn et al. suggesting increased IL-6 contributes to emphysema, and extends it by suggesting this is independent of STAT3 and inflammation.

Having characterised the FF mouse lung inflammatory profile, Chapter 4 describes the use of the FF mouse in a cigarette-carcinogen model of lung cancer. To reiterate, the FF mouse provides a model of endogenous increased IL-6 and hyper-activated STAT3 signalling, in the absence of pathways downstream of SHP2, specifically PI3K/Akt and MAPK signalling. Increased Akt and MAPK activation has been associated with pulmonary inflammation (Bozinovski et al., 2002, Hellermann et al., 2002) and thus it seemed unlikely that the absence of these SHP2-dependent signalling pathways downstream of gp130 in the FF mouse would contribute to the inflammatory phenotype. Because of this they were not investigated as part of the characterisation. However, in the context of the development of lung cancer this absence allows the separation of STAT3 from PI3K/Akt and MAPK downstream of gp130, enabling the closer delineation of the roles they play in tumourigenesis. While STAT3 has a solid pedigree as a generic oncogene and is associated with lung cancer (Bromberg et al.,

1999, Gao et al., 2009, Levy and Inghirami, 2006), there is no work examining its role in NNK-induced lung cancer. On the other hand, Akt and ERK have consistently been shown to be upregulated by NNK *in vitro*, and *in vivo* their activation increases with NNK induced tumour progression (West et al., 2004a, West et al., 2004b, Kalantari-Dehaghi et al., Ho et al., 2005, Jin et al., 2004, Memmott et al., 2010, Yang et al., 2004). What is lacking however is an examination of upstream signalling pathways, something the FF mouse allowed me to do.

I showed that despite hyper-activated STAT3 in the FF mouse there was a reduction in both tumour number and size in response to NNK. While there are data suggesting a role for STAT3 in promoting apoptosis over proliferation in the context of mammary gland involution (Abell et al., 2005) it seems highly unlikely that the increase in STAT3 is responsible for the reduction given its credentials as an oncogene (Li et al., 2007b, Achcar Rde et al., 2007). This idea is supported by the data showing that in human NSCLC (A549) cells reduction in STAT3 expression through forced expression of a negative regulator (SOCS3) lead to reduced proliferation (Yu et al., 2009b). Furthermore, my *in vitro* data suggested minimal activation of STAT3 by NNK, and western blot analysis of WT mouse lungs chronically exposed to NNK showed no increase in pY-STAT3 levels, all suggesting a lack of involvement rather than a negative impact. Finally, the number of tumours that developed in FF:St3-/+ mice was not significantly different to FF mice, suggesting that, at least in the absence of gp130-dependent PI3K/Akt and MAPK signalling, STAT3 plays little role in NNK induced tumourigenesis.

If STAT3 is not playing a role the implication is that it is the loss of gp130/SHP2-dependent signalling that is responsible for the reduction in tumours seen. While the role of STAT1 was investigated in the initial characterisation of the FF mouse it was not investigated in either of the lung cancer models. Unlike STAT3, STAT1 appears to be down-regulated in response to cigarette smoke (Eddleston et al., 2011), and while STAT1 plays an important role in response to viral infection (Katze et al., 2002), its role in lung cancer is unclear; current literature associates increased STAT1 with both better and worse prognosis (Chen et al., 2007, Suwinski et al., 2012). Due to this uncertainty, and because STAT1 did not appear to play a role in either the inflammatory profile of the FF lung or the development of emphysema, to this point the FF:St1-/- mouse was not utilised in these experiments. However, given the reduction in lung tumours seen in the FF mouse, and the association of STAT1 with the induction of apoptosis (Kim and Lee, 2007), the FF:St1-/- mouse may provide a valuable genetic tool in future experiments to further clarify crucial signalling pathways.

There is evidence of IL-6-dependent Akt signalling in myeloma, and in fact IL-6 blocking antibodies both reduced Akt activation and malignant progression (Fulciniti et al., 2009,

Hideshima et al., 2001). I examined this possibility by extracting RNA from formalin-fixed lung and undertaking two pathway-specific PCR arrays. This showed the down regulation of key oncogenes in the FF mouse lung treated with NNK when compared to untreated FF controls, and conversely upregulation of tumour suppressor genes. These data suggest the possible involvement of both gp130/SHP2-dependent PI3K/Akt and MAPK pathways, and add to the extensive published data showing a role for both PI3K/Akt and MAPK in lung tumour development by suggesting gp130 dependence.

The final aspect of my project examined the interaction of elevated IL-6 and hyper-activated STAT3 with oncogenic *Kras* and the impact of this on the development of cancer-related cachexia. In Chapter 5 I examined this interaction, both *in vitro* and *in vivo*, in the context of different modalities of IL-6 signalling; classical versus trans-signalling. I showed *in vitro* that in addition to secreting variable amounts of IL-6 consistent with previous data (Bihl et al., 1998), the cells also produced sIL-6R without clear correlation to mutations present or characteristics of the donor tumour. This suggests that at least some of the cells utilise trans-signalling and by extension that trans-signalling may be important *in vivo*.

Secondly I showed *in vivo* that even in the absence of a significant increase in tumour load, FF:Kras^{G12D} mice gained significantly less weight than their WT litter-mates, and in fact lost weight over the 6 week experiment. Additionally, there was a significant difference in the survival of FF:Kras^{G12D} mice compared to FF controls (and all WT mice), correlating with the weight loss. The weight loss occurred in both lean and fat tissue compartments, with a 20% reduction in gastrocnemius weight and 70% reduction in peri-gonadal fat weight, consistent with the syndrome of cancer-related cachexia (Fearon et al., 2011), and, at least for fat tissue, was confirmed with DEXA. Following on from this I used both genetic and therapeutic approaches to clarify the signalling pathways and modalities (ie trans- vs. classical signalling) responsible for the cachexia.

The weight loss and increased mortality seen in the FF:Kras^{G12D} mice was rescued by both genetically reducing STAT3 (FF:St3-/+:Kras^{G12D}) and ablating IL-6 (FF:IL6-/-:Kras^{G12D}), or by targeting IL-6 trans-signalling with the IL-6R antibody 25F10. Importantly, this rescue was not observed with the IL-6R antibody 2B10, which targets primarily classical signalling. Taken together I have shown that while tumour load was not different between WT and FF mice, cancer-related cachexia developed in FF mice as a consequence of elevated IL-6 and hyper-activated STAT3. Moreover, this work defines IL-6 trans-signalling as the modality responsible for the cachexia. There is considerable evidence linking IL-6 to lung cancer-related cachexia in clinical series and xenograft models (Kim et al., 2012, Kosacka et al., 2008, Ohe et al., 1993, Ohira et al., 1996, Ohira et al., 1992) but this is the first time IL-

6/STAT3-dependent lung cancer-related cachexia has been shown in a $Kras^{G12D}$ orthotopic lung cancer model, and the first time it has been linked to IL-6 trans-signalling.

However, we are not the first to show the efficiency of targeting trans-signalling in avoiding cachexia in other cancer models. In mice with a colon adenocarcinoma (C-26) xenograft injected with a monoclonal antibody directed at the sIL-6R (MR16-1), Fujita et al. showed a significant reduction in gastrocnemius muscle loss compared with mice injected with IgG (Fujita et al., 1996). In line with the data I have presented this reduction in muscle loss was in the absence of any effect on tumour size. A similar effect on muscle loss was seen in the same model with the use of Suramin, a small molecule inhibitor that blocks IL-6 binding to the IL-6R (Strassmann et al., 1993). Similarly, in the APCMin-/- model of colon cancer, White et al. showed a cessation of body weight and muscle loss when the MR16-1 antibody was administered (White et al., 2011).

Likewise, a causal role for STAT3 is not a unique finding in the context of global IL-6 signalling. When STAT3 is genetically reduced by transfection with a tissue specific dominant-negative (dn)STAT3, muscle loss is significantly, but not completely, reduced in a C-26 xenograft model of cachexia (Bonetto et al., 2012b). They comment that the incomplete rescue points towards other mediators at play in what is clearly a multi-factorial syndrome, suggesting perhaps that Akt downstream of gp130 may play a promoting role. Similarly, others have pointed to ERK signalling as a contributor to cachexia in the same C-26 model (Penna et al., 2010). Whilst the FF model cannot specifically address these data, that we see complete rescue of weight loss in the absence of both pathways downstream of gp130 may add weight to a role for these signalling pathways in addition to STAT3.

The data I have presented showing a lack of effect of deregulated gp130 signalling on tumour load is consistent with other work. For instance, blockade of IL-6 in the C-26 xenograft model of colon cancer leads to a reduction in muscle loss without affecting tumour size (White et al., 2011). Similarly, in the LLC xenograft model of lung cancer, transfecting LLC cells with IL-6 cDNA lead to substantial weight loss and reduced survival without altering the tumour size (Ohe et al., 1993). To confirm the cachexia and mortality was secondary to IL-6, mice were injected with an α-IL-6 antibody resulting in survival equivalent to mice bearing non-IL-6-transfected LLC xenografts. By contrast, my and these above-mentioned data are at odds with some published data (Ochoa et al., 2011). For example, Ochoa et al. showed that genetic ablation of IL-6 lead to a reduction of tumour load both with and without pulmonary inflammation induced by NTHi.

The primary limitation of the work presented in chapter 5, and to a lesser degree parts of chapter 4, is that only very low numbers of mice are included. This resulted from a combination of early mortality in selected groups and a low volume of specific reagents. This

early and preliminary data does suggest a role for the IL-6 signalling pathway in the development of lung cancer associated cachexia, but clearly further work is required and is ongoing in our laboratory. There are significant differences between groups where adequate numbers are available for analysis providing valuable information to refine hypotheses and experimental procedures for this subsequent work.

The potential for clinical translation of the data showing protection from cachexia with the use of an antibody or small molecule inhibitor (e.g. sgp130Fc (Jones et al., 2011)) targeting IL-6 trans-signalling is clearly very exciting. While there is much pre-clinical work still to be done there is a dire need for new therapies for lung cancer-related cachexia that are both safe and effective. Antibodies directed against global IL-6 signalling have been shown to be effective in treating cachexia (Bayliss et al., 2011), and while serious adverse effects were low, given the evidence to show that it is trans-signalling responsible for both tumourigenesis and complications including cachexia (Fujita et al., 1996, White et al., 2011, Becker et al., 2004, Lo et al., 2010, Weidle et al., 2010), specific targeting of trans-signalling may make this therapy safer and better tolerated. But treatment of cachexia is not the only potential translational relevance of this work.

As mentioned there are two prominent new molecular therapies in sub-groups of lung adenocarcinoma. The first is TKIs for the treatment of individuals with tumours expressing activating mutations of EGFR. While for many the response is impressive (Lynch et al., 2004, Paez et al., 2004), there is a proportion of tumours with inherent TKI resistance and more in whom it develops (Engelman and Janne, 2008, Li et al., 2012). Trials examining inhibitors of PI3K/Akt in this context have shown positive results (Gadgeel and Wozniak, 2013, Li et al., 2012). Given the data I have presented here suggesting a role for gp130-dependent PI3K/Akt signalling in lung tumourigenesis, and the known importance of IL-6-dependent STAT3 signalling in the oncogenic potential of mutant EGFR (Gao et al., 2007, Alvarez et al., 2006), targeting IL-6/gp130 in the context of TKI resistance provides another therapeutic avenue.

The second new treatment is a TKI targeting EML4-ALK rearrangements, which are present in around 5% of human lung adenocarcinoma (Li et al., 2011). In an early phase clinical trial of Crizotinib a majority of patients with the EML4-ALK rearrangement experienced tumour shrinkage or stable disease (Kwak et al., 2010). However, like tumours expressing activated EGFR, resistance to therapy has been noted (Kim et al., 2013). The idea of using dual inhibition, specifically including inhibition of STAT3, has been shown to be effective in some human LAC lines expressing the EML4-ALK mutation (Tanizaki et al., 2012), including NCI-H2228 cells. My data showing high expression of IL-6 and growth restriction with an

antibody targeting the IL-6R in these cells would suggest IL-6 blockade provides an alternative strategy for targeting these tumours.

Therapeutic intervention is the primary area of recommended further study in both *in vivo* arms of this project, particularly as there are clinically available agents acting at the IL-6R. Not only would it give valuable preclinical data but it would also enable closer delineation of important signalling molecules and pathways. In the cigarette carcinogen model treating WT mice with an antibody targeting the IL-6R (classical and trans-signalling) or sIL-6R (trans-signalling only) could be used both to further explore mechanisms of tumour development (by treating mice from the time of NNK administration) and to assess therapeutic response (by treating mice once tumours had developed). In terms of further investigation of the development of cachexia, whilst not performed in this study, DEXA allows for repeat assessment of body composition in live mice, having the obvious advantage of producing longitudinal data. Using the $Kras^{G12D}$ model to observe the change of body composition over time and evaluate the impact of targeting IL-6 trans-signalling provides an exciting pre-clinical model of real consequence.

In summary, my results confirm a role for deregulated gp130 dependent PI3K/Akt and MAPK signalling in the development of lung adenocarcinoma, and confirm the role of IL-6/STAT3 in lung cancer related cachexia, establishing the FF:$Kras^{G12D}$ mouse as a new mouse model. The success of the therapeutic intervention performed, although very early in its development, both implicates IL-6 trans-signalling as the modality leading to cachexia and displays the exciting potential for ongoing therapeutic trials.

APPENDIX I

Manuscripts containing work generated for this thesis

Ruwanpura, S. M., McLeod, L., **Miller, A.,** Jones, J., Bozinovski, S., Vlahos, R., Ernst, M., Armes, J., Bardin, P. G., Anderson, G. P. and Jenkins, B. J. *"Interleukin-6 promotes pulmonary emphysema associated with apoptosis in mice."* Am J Respir Cell Mol Biol 2011; 45(4): 720-730.

Ruwanpura, S. M., McLeod, L., **Miller, A.,** Jones, J., Vlahos, R., Ramm, G., Longano, A., Bardin, P. G., Bozinovski, S., Anderson, G. P. and Jenkins, B. J. *"Deregulated Stat3 signaling dissociates pulmonary inflammation from emphysema in gp130 mutant mice."* Am J Physiol Lung Cell Mol Physiol 2012; 302(7): L627-639.

APPENDIX II

Buffers and Solutions

Tail Buffer (1L):

0.05M Tris, 0.025M EDTA, 0.05M NaCl, 0.005% SDS

50x TAE:

2 M Tris-HCl, 50 mM EDTA, 5.7% (v/v) glacial acetic acid, adjusted to pH 8.0.

T.E. buffer

10 mM Tris-HCl, 1 mM EDTA, adjusted to pH 8.0.

2% agarose

2 g of electrophoresis LE analytical grade agarose (Promega)

100ml of stock 1X TAE

Gently heat and agitate until agarose has dissolved.

DNA Loading Dye:

0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 10 mM EDTA, 20% Glycerol, adjusted to pH 8.0

Acid alcohol

5% HCl in 70% ethanol

Ammonia water

10 drops of ammonia in a staining trough of water

Citrate buffer

0.12g citric acid, 1.3g trisodium citrate in 500ml dH20 - pH to 6.0

Diethyl Pyrocarbonate (DEPC-treated water)

0.1% DEPC (Sigma).

Made up with 1L with MilliQ water. Incubated O/N at 37°C then autoclaved.

Protein lysis buffer

10ml 5M NaCl, 50ml 1M Tris-HCl (pH 7.5), 10ml glycerol, 10ml Triton X-100, 10ml 10% SDS and 2ml 0.5M EDTA

Make up to 500ml with MQH_2O

Table A1. Ingredients and volumes for SDS Polyacrylamide gels.

	10% lower gel	**Stacking gel**
MQ water	9ml	3.4ml
Acrylamide	6.6ml	800µl
Buffer	5.2ml	1.25ml
10% Ammonium persulfate	200µl	50µl
Temed	10µl	5µl

Lower Gel Buffer

2 M Tris-HCl and 10% SDS, adjusted to pH 8.8.

Stacking Gel Buffer

1 M Tris-HCl and 10% SDS, adjusted to pH 6.8.

Running buffer

25 mM Tris-HCl, 192 mM Glycine and 0.1% (w/v) SDS.

Sample buffer

2.5% (v/v) SDS, 25% Glycerol, 0.04% (w/v) Bromophenol blue, 0.125 M Tris-HCl, adjusted to pH 6.8.

Prior to use add 5% (v/v) β-Mercaptoethanol.

10x Transfer buffer

9.65g Tris-HCl, 45g glycine, made up to 500ml with MQH_2O, pH to 8.3

Table A.2. List of Primary antibodies for Western Blotting including the working concentration, secondary antibody and protein size.

Antibody	Concentration	Company	Secondary antibody	Size (kD)
Actin	1:500	Santa Cruz	Anti-mouse	44
Total ERK	1:1000	Santa Cruz	Anti-rabbit	42
pThr/TyrERK	1:1000	Cell Signalling Technologies	Anti-rabbit	42
Total Akt	1:1000	Cell Signalling Technologies	Anti-rabbit	61
pSerAkt	1:500	Cell Signalling Technologies	Anti-rabbit	61
Total STAT3	1:1000	Cell Signalling Technologies	Anti-rabbit	86
pTyrSTAT3	1:1000	Santa Cruz	Anti-rabbit	80

Stripping buffer

0.2 M Glycine, 0.05% (v/v) Tween 20 and 100 mM β-Mercaptoethanol, adjusted to pH 2.5.

10x Phosphate buffered saline (PBS; pH 7.4)

14 mM NaCl, 3 mM KCl, 10 mM Na2HPO4.12H2O, 1.8 mM KH2HPO4, adjusted to pH 7.4.

Table A3. Reagent ingredients and working concentrations of antibodies for ELISA kits.

	hIL-6	shIL-6R
Coating Buffer	142.6mg $NaHCO_3$ and 31.8 mg Na_2CO_3 made up to 20ml and pH 9.5	PBS
Blocking buffer	10% FCS in PBS	1% BSA in PBS
Capture antibody	1:250	2ug/ml
Detection antibody	1:500	100ng/ml
Streptavidin-HRP	1:200	1:250

ELISA Stop Solution

2M HCl

RBC lysis buffer

90ml 0.16M NH_4Cl mixed with 10ml 0.17M Tris (pH 7.65)

Adjust pH to 7.2 with concentrated HCl, filter sterilise.

FACS buffer

1% FCS in PBS

Table A4. List of fluorophore labelled antibodies and working concentrations used for FACS

	Fluorophore	Concentration	Company
CD45	APC	1:100	BD biosciences
Gr1 (Ly6G/Ly6C)	FITC	1:1600	BD biosciences
Mac1 (CD11b)	PE	1:1600	BD biosciences
B220	FITC	1:2000	BD biosciences
CD3e	PE	1:500	eBioscience
CD4	PB	1:1000	BD biosciences
CD8	PE	1:100	BD biosciences

APPENDIX III

Primer Sequences and PCR Array Gene Tables

***Kras2_37* Genotyping PCR:**

Primer A: CTGGGTGTTAGGGAACCATA

Primer B: CAAAGCACGGATGGCATCTT

Sequences of qRT primers

Table A5. List of Human qRT primers

Human primers	
Name	Primer sequences
18S	**F** 5'-CGGCTACCACATCCAAGGAA-3' **R** 5'-GCTGGAATTACCGCGGCT-3'
IL6	**F** 5'-CTCCAGGAGCCCAGCTCTGA-3' **R** 5'-CCCAGGGAGAAGGCAACTG-3'
IL6R	**F** 5'-AAAGCTGGGCAGGTTGGTG-3' **R** 5'-AGCTTGTGCAGAGGTGTTGAG-3'
GP130	**F** 5'-TCTGGGAGTGCTGTTCTGCTT-3' **R** 5'-TGTGCCTTGGAGGAGTGTGA-3'
TACE	**F** 5'-GAAGTGCCAAGGAGGCGATTA-3' **R** 5'-CGGGCACTCACTGCTATTACC-3'

Table A6. List of Mouse qRT primers

Mouse primers	
Name	Primer sequences
18S	**F** 5'-CGGCTACCACATCCAAGGAA-3' **R** 5'-GCTGGAATTACCGCGGCT-3'
Il6	**F** 5'-ATGGATGCTACCAAACTGGAT-3' **R** 5'-TGAAGGACTCTGGCTTTGTCT-3'
Ifnγ	**F** 5'-ACAATGAACGCTACACACTGCAT-3' **R** 5'-TGGCAGTAACAGCCAGAAACA-3'
Il10	**F** 5'-GGTTGCCAAGCCTTATCGGA-3' **R** 5'-ACCTGCTCCACTGCCTTGCT-3'
Myd88	**F** 5'-GTCCGACCGTGACGTCCTGC-3' **R** 5'-CCACCATGCGGCGACACCTT-3'
Tlr1	**F** 5'-GGACCTACCCTTGCAAACAA-3' **R** 5'-GGTGGCACAAGATCACCTTT-3'
Tlr4	**F** 5'-TCTGAGCTTCAACCCCTTG-3' **R** 5'-TGCCATGCCTTGTCTTCA-3'
Tlr6	**F** 5'-CCAAGAACAAAAGCCCTGAG-3' **R** 5'-TGTTTTGCAACCGATTGTGT-3'
Pparα	**F** 5'-TATTCGGCTGAAGCTGGTGTAC-3' **R** 5'-CTGGCATTTGTTCCGGTTCT-3'
Tnfα	**F** 5'-CAAATTCGAGTGACAAGCCTG-3' **R** 5'-GAGATCCATGCCGTTGGC-3'
Il1b	**F** 5'-CAACCAACAAGTGATATTCTCCATG-3' **R** 5'-GATCCACACTCTCCAGCTGCA-3'
Ccl2	**F** 5'-AGGTGTCCCAAAGAAGCTGTA-3' **R** 5'-ATGTCTGGACCCATTCCTTCT-3'
Ccl5	**F** 5'-ATATGGCTCGGACACCACTC-3' **R** 5'-GTGACAAACACGACTGCAAGA-3'
Cxcl1	**F** 5'-CCTTGACCCTGAAGCTCCCT-3' **R** 5'-CGGGTGCCATCAGAGCAGTCT-3'
Cxcl2	**F** 5'-AACATCCAGAGCTTGAGTGTGA-3' **R** 5'-TTCAGGGTCAAGGCAAACTT-3'

Table A7. Gene list for TLR signalling pathway PCR array

PCR Array Catalog #:			PAMM-018 - Toll-Like Receptor Signalling Pathway		
	Unigene	**Refseq**	**Symbol**	**Description**	**RT2 Catalog**
A01	Mm.4475	NM_013482	Btk	Bruton agammaglobulinemia tyrosine kinase	PPM06247A
A02	Mm.336851	NM_009812	Casp8	Caspase 8	PPM02923E
A03	Mm.290320	NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	PPM03151F
A04	Mm.3460	NM_009841	Cd14	CD14 antigen	PPM06249E
A05	Mm.89474	NM_009855	Cd80	CD80 antigen	PPM03227E
A06	Mm.1452	NM_019388	Cd86	CD86 antigen	PPM03228A
A07	Mm.439656	NM_009883	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	PPM03505B
A08	Mm.3996	NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase	PPM03197B
A09	Mm.248327	NM_019948	Clec4e	C-type lectin domain family 4, member e	PPM06261E
A10	Mm.4922	NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	PPM02990E
A11	Mm.1238	NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	PPM02989A
A12	Mm.877	NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	PPM02978D
B01	Mm.405823	NM_007922	Elk1	ELK1, member of ETS oncogene family	PPM03038E
B02	Mm.5126	NM_010175	Fadd	Fas (TNFRSF6)-associated via death domain	PPM03427F
B03	Mm.246513	NM_010234	Fos	FBJ osteosarcoma oncogene	PPM02940B
B04	Mm.313345	NM_010439	Hmgb1	High mobility group box 1	PPM05059E
B05	Mm.334313	NM_008284	Hras1	Harvey rat sarcoma virus oncogene 1	PPM03682B
B06	Mm.392569	NM_010472	Agfg1	ArfGAP with FG repeats 1	PPM25355A
B07	Mm.6388	NM_010479	Hspa1a	Heat shock protein 1A	PPM04801E
B08	Mm.1777	NM_010477	Hspd1	Heat shock protein 1 (chaperonin)	PPM04797A
B09	Mm.1245	NM_010510	Ifnb1	Interferon beta 1, fibroblast	PPM03594B
B10	Mm.240327	NM_008337	Ifng	Interferon gamma	PPM03121A
B11	Mm.277886	NM_010546	Ikbkb	Inhibitor of kappaB kinase beta	PPM03198B
B12	Mm.874	NM_010548	Il10	Interleukin 10	PPM03017B
C01	Mm.103783	NM_008351	Il12a	Interleukin 12A	PPM03019A
C02	Mm.15534	NM_010554	Il1a	Interleukin 1 alpha	PPM03010E
C03	Mm.222830	NM_008361	Il1b	Interleukin 1 beta	PPM03109E
C04	Mm.896	NM_008362	Il1r1	Interleukin 1 receptor, type I	PPM03011B
C05	Mm.14190	NM_008366	Il2	Interleukin 2	PPM02937B
C06	Mm.1019	NM_031168	Il6	Interleukin 6	PPM03015A
C07	Mm.2856	NM_010559	Il6ra	Interleukin 6 receptor, alpha	PPM03027E
C08	Mm.38241	NM_008363	Irak1	Interleukin-1 receptor-associated kinase 1	PPM03201E
C09	Mm.152142	NM_172161	Irak2	Interleukin-1 receptor-associated kinase 2	PPM04212B
C10	Mm.105218	NM_008390	Irf1	Interferon regulatory factor 1	PPM03203B
C11	Mm.3960	NM_016849	Irf3	Interferon regulatory factor 3	PPM04692B
C12	Mm.275071	NM_010591	Jun	Jun oncogene	PPM03037A
D01	Mm.87787	NM_010735	Lta	Lymphotoxin A	PPM03114A
D02	Mm.3177	NM_010739	Muc13	Mucin 13, epithelial transmembrane	PPM06250A
D03	Mm.2639	NM_010745	Ly86	Lymphocyte antigen 86	PPM06259A
D04	Mm.116844	NM_016923	Ly96	Lymphocyte antigen 96	PPM02951B
D05	Mm.18494	NM_008928	Map2k3	Mitogen-activated protein kinase kinase 3	PPM03572B
D06	Mm.412922	NM_009157	Map2k4	Mitogen-activated protein kinase kinase 4	PPM03575B
D07	Mm.15918	NM_011945	Map3k1	Mitogen-activated protein kinase kinase kinase 1	PPM03569F
D08	Mm.258589	NM_172688	Map3k7	Mitogen-activated protein kinase kinase kinase 7	PPM03071A
D09	Mm.21495	NM_016700	Mapk8	Mitogen-activated protein kinase 8	PPM03234B
D10	Mm.43081	NM_013931	Mapk8ip3	Mitogen-activated protein kinase 8 interacting protein 3	PPM04787A
D11	Mm.68933	NM_016961	Mapk9	Mitogen-activated protein kinase 9	PPM03584B
D12	Mm.213003	NM_010851	Myd88	Myeloid differentiation primary response gene 88	PPM03399A

E01	Mm.256765	NM_008689	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	PPM02930E
E02	Mm.102365	NM_019408	Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	PPM03204E
E03	Mm.170515	NM_010907	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	PPM02943B
E04	Mm.220333	NM_010908	Nfkbib	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	PPM04223E
E05	Mm.300795	NM_010909	Nfkbil1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	PPM04214A
E06	Mm.238146	NM_172766	Nfrkb	Nuclear factor related to kappa B binding protein	PPM34394A
E07	Mm.442385	NM_011630	Nr2c2	Nuclear receptor subfamily 2, group C, member 2	PPM06260E
E08	Mm.28957	NM_023324	Peli1	Pellino 1	PPM06256A
E09	Mm.21855	NM_009402	Pglyrp1	Peptidoglycan recognition protein 1	PPM06263E
E10	Mm.212789	NM_011144	Ppara	Peroxisome proliferator activated receptor alpha	PPM03307B
E11	Mm.378990	NM_011163	Eif2ak2	Eukaryotic translation initiation factor 2-alpha kinase 2	PPM05148B
E12	Mm.292547	NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2	PPM03647E
F01	Mm.4869	NM_009044	Rel	Reticuloendotheliosis oncogene	PPM03194E
F02	Mm.249966	NM_009045	Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	PPM04224E
F03	Mm.112765	NM_138952	Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2	PPM06268A
F04	Mm.34580	NM_019786	Tbk1	TANK-binding kinase 1	PPM04209A
F05	Mm.203952	NM_174989	Ticam1	Toll-like receptor adaptor molecule 1	PPM06265A
F06	Mm.149280	NM_173394	Ticam2	Toll-like receptor adaptor molecule 2	PPM31634A
F07	Mm.23987	NM_054096	Tirap	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	PPM06262B
F08	Mm.273024	NM_030682	Tlr1	Toll-like receptor 1	PPM04211B
F09	Mm.87596	NM_011905	Tlr2	Toll-like receptor 2	PPM04220B
F10	Mm.33874	NM_126166	Tlr3	Toll-like receptor 3	PPM04216B
F11	Mm.38049	NM_021297	Tlr4	Toll-like receptor 4	PPM04207F
F12	Mm.116894	NM_016928	Tlr5	Toll-like receptor 5	PPM04206E
G01	Mm.42146	NM_011604	Tlr6	Toll-like receptor 6	PPM04210B
G02	Mm.23979	NM_133211	Tlr7	Toll-like receptor 7	PPM04208A
G03	Mm.196676	NM_133212	Tlr8	Toll-like receptor 8	PPM04213E
G04	Mm.44889	NM_031178	Tlr9	Toll-like receptor 9	PPM04221A
G05	Mm.1293	NM_013693	Tnf	Tumor necrosis factor	PPM03113F
G06	Mm.116683	NM_009397	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	PPM03207A
G07	Mm.1258	NM_011609	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	PPM03087C
G08	Mm.103551	NM_023764	Tollip	Toll interacting protein	PPM06269B
G09	Mm.264255	NM_001033161	Tradd	TNFRSF1A-associated via death domain	PPM04474E
G10	Mm.292729	NM_009424	Traf6	Tnf receptor-associated factor 6	PPM03082A
G11	Mm.371667	NM_080560	Ube2n	Ubiquitin-conjugating enzyme E2N	PPM05418E
G12	Mm.278783	NM_023230	Ube2v1	Ubiquitin-conjugating enzyme E2 variant 1	PPM06258E
H01	Mm.3317	NM_010368	Gusb	Glucuronidase, beta	PPM05490B
H02	Mm.299381	NM_013556	Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1	PPM03559E
H03	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	PPM04803E
H04	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	PPM02946E
H05	Mm.328431	NM_007393	Actb	Actin, beta	PPM02945A
H06	N/A	SA_00106	MGDC	Mouse Genomic DNA Contamination	

Table A8. Gene list for PI3K/Akt signalling pathway PCR array

PCR Array Catalog #:			PAMM-058 - PI3K/Akt Signalling pathway		
	Unigene	**Refseq**	**Symbol**	**Description**	**RT2 Catalog**
A01	Mm.316628	NM_019655	Adar	Adenosine deaminase, RNA-specific	PPM06001A
A02	Mm.6645	NM_009652	Akt1	Thymoma viral proto-oncogene 1	PPM03377E
A03	Mm.177194	NM_007434	Akt2	Thymoma viral proto-oncogene 2	PPM03378B
A04	Mm.235194	NM_011785	Akt3	Thymoma viral proto-oncogene 3	PPM03291B
A05	Mm.384171	NM_007462	Apc	Adenomatosis polyposis coli	PPM05172E
A06	Mm.4387	NM_007522	Bad	BCL2-associated agonist of cell death	PPM02916E
A07	Mm.4475	NM_013482	Btk	Bruton agammaglobulinemia tyrosine kinase	PPM06247A
A08	Mm.88829	NM_015733	Casp9	Caspase 9	PPM03383E
A09	Mm.273049	NM_007631	Ccnd1	Cyclin D1	PPM02903E
A10	Mm.3460	NM_009841	Cd14	CD14 antigen	PPM06249E
A11	Mm.1022	NM_009861	Cdc42	Cell division cycle 42 homolog (S. cerevisiae)	PPM04527E
A12	Mm.2958	NM_009875	Cdkn1b	Cyclin-dependent kinase inhibitor 1B	PPM02909B
B01	Mm.3996	NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase	PPM03197B
B02	Mm.23692	NM_007788	Csnk2a1	Casein kinase 2, alpha 1 polypeptide	PPM04607B
B03	Mm.291928	NM_007614	Ctnnb1	Catenin (cadherin associated protein), beta 1	PPM03384A
B04	Mm.378990	NM_011163	Eif2ak2	Eukaryotic translation initiation factor 2-alpha kinase 2	PPM05148B
B05	Mm.290022	NM_145625	Eif4b	Eukaryotic translation initiation factor 4B	PPM37725A
B06	Mm.3941	NM_007917	Eif4e	Eukaryotic translation initiation factor 4E	PPM05111A
B07	Mm.6700	NM_007918	Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	PPM05118E
B08	Mm.260256	NM_001005331	Eif4g1	Eukaryotic translation initiation factor 4, gamma 1	PPM35743A
B09	Mm.405823	NM_007922	Elk1	ELK1, member of ETS oncogene family	PPM03038E
B10	Mm.3355	NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)	PPM02926E
B11	Mm.278458	NM_008019	Fkbp1a	FK506 binding protein 1a	PPM37127E
B12	Mm.246513	NM_010234	Fos	FBJ osteosarcoma oncogene	PPM02940B
C01	Mm.29891	NM_019739	Foxo1	Forkhead box O1	PPM03381B
C02	Mm.338613	NM_019740	Foxo3	Forkhead box O3	PPM03393E
C03	Mm.21158	NM_020009	Mtor	Mechanistic target of rapamycin (serine/threonine kinase)	PPM05092A
C04	Mm.378921	NM_010288	Gja1	Gap junction protein, alpha 1	PPM05460A
C05	Mm.273117	NM_010345	Grb10	Growth factor receptor bound protein 10	PPM36621E
C06	Mm.439649	NM_008163	Grb2	Growth factor receptor bound protein 2	PPM03710E
C07	Mm.394930	NM_019827	Gsk3b	Glycogen synthase kinase 3 beta	PPM03380B
C08	Mm.334313	NM_008284	Hras1	Harvey rat sarcoma virus oncogene 1	PPM03682B
C09	Mm.13849	NM_013560	Hspb1	Heat shock protein 1	PPM02936B
C10	Mm.268521	NM_010512	Igf1	Insulin-like growth factor 1	PPM03387E
C11	Mm.275742	NM_010513	Igf1r	Insulin-like growth factor I receptor	PPM04714E
C12	Mm.274846	NM_010562	Ilk	Integrin linked kinase	PPM05291A
D01	Mm.38241	NM_008363	Irak1	Interleukin-1 receptor-associated kinase 1	PPM03201E
D02	Mm.4952	NM_010570	Irs1	Insulin receptor substrate 1	PPM05117E
D03	Mm.263396	NM_010578	Itgb1	Integrin beta 1 (fibronectin receptor beta)	PPM03668B
D04	Mm.275071	NM_010591	Jun	Jun oncogene	PPM03037A
D05	Mm.248907	NM_008927	Map2k1	Mitogen-activated protein kinase kinase 1	PPM03565E
D06	Mm.196581	NM_011949	Mapk1	Mitogen-activated protein kinase 1	PPM03571E
D07	Mm.311337	NM_011951	Mapk14	Mitogen-activated protein kinase 14	PPM03578A
D08	Mm.8385	NM_011952	Mapk3	Mitogen-activated protein kinase 3	PPM03585E
D09	Mm.21495	NM_016700	Mapk8	Mitogen-activated protein kinase 8	PPM03234B
D10	Mm.16366	NM_010839	Mtcp1	Mature T-cell proliferation 1	PPM25835A
D11	Mm.213003	NM_010851	Myd88	Myeloid differentiation primary response gene 88	PPM03399A
D12	Mm.256765	NM_008689	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	PPM02930E

E01	Mm.170515	NM_010907	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	PPM02943B
E02	Mm.371570	NM_008774	Pabpc1	Poly(A) binding protein, cytoplasmic 1	PPM40357A
E03	Mm.260227	NM_011035	Pak1	P21 protein (Cdc42/Rac)-activated kinase 1	PPM04553E
E04	Mm.221403	NM_011058	Pdgfra	Platelet derived growth factor receptor, alpha polypeptide	PPM03640C
E05	Mm.34411	NM_172665	Pdk1	Pyruvate dehydrogenase kinase, isoenzyme 1	PPM28207E
E06	Mm.29768	NM_133667	Pdk2	Pyruvate dehydrogenase kinase, isoenzyme 2	PPM27629A
E07	Mm.10504	NM_011062	Pdpk1	3-phosphoinositide dependent protein kinase 1	PPM03376E
E08	Mm.260521	NM_008839	Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	PPM05112A
E09	Mm.101369	NM_020272	Pik3cg	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	PPM03469A
E10	Mm.259333	NM_001024955	Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	PPM03374F
E11	Mm.12945	NM_008841	Pik3r2	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	PPM05078A
E12	Mm.222178	NM_011101	Prkca	Protein kinase C, alpha	PPM03501B
F01	Mm.207496	NM_008855	Prkcb	Protein kinase C, beta	PPM03502E
F02	Mm.28561	NM_008860	Prkcz	Protein kinase C, zeta	PPM05103A
F03	Mm.245395	NM_008960	Pten	Phosphatase and tensin homolog	PPM03379A
F04	Mm.254494	NM_007982	Ptk2	PTK2 protein tyrosine kinase 2	PPM35305A
F05	Mm.8681	NM_011202	Ptpn11	Protein tyrosine phosphatase, non-receptor type 11	PPM05096A
F06	Mm.292510	NM_009007	Rac1	RAS-related C3 botulinum substrate 1	PPM03391E
F07	Mm.184163	NM_029780	Raf1	V-raf-leukemia viral oncogene 1	PPM03707E
F08	Mm.259653	NM_145452	Rasa1	RAS p21 protein activator 1	PPM02958E
F09	Mm.235580	NM_011250	Rbl2	Retinoblastoma-like 2	PPM02896B
F10	Mm.319175	NM_053075	Rheb	Ras homolog enriched in brain	PPM31802A
F11	Mm.757	NM_016802	Rhoa	Ras homolog gene family, member A	PPM05485A
F12	Mm.301827	NM_009097	Rps6ka1	Ribosomal protein S6 kinase polypeptide 1	PPM03385C
G01	Mm.394280	NM_028259	Rps6kb1	Ribosomal protein S6 kinase, polypeptide 1	PPM33288A
G02	Mm.86595	NM_011368	Shc1	Src homology 2 domain-containing transforming protein C1	PPM04024B
G03	Mm.434583	NM_009231	Sos1	Son of sevenless homolog 1 (Drosophila)	PPM04649E
G04	Mm.45044	NM_020493	Srf	Serum response factor	PPM03580E
G05	Mm.18154	NM_009337	Tcl1	T-cell lymphoma breakpoint 1	PPM25914A
G06	Mm.23987	NM_054096	Tirap	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	PPM06262B
G07	Mm.38049	NM_021297	Tlr4	Toll-like receptor 4	PPM04207F
G08	Mm.103551	NM_023764	Tollip	Toll interacting protein	PPM06269B
G09	Mm.224354	NM_022887	Tsc1	Tuberous sclerosis 1	PPM34003A
G10	Mm.30435	NM_011647	Tsc2	Tuberous sclerosis 2	PPM27785A
G11	Mm.1574	NM_028459	Wasl	Wiskott-Aldrich syndrome-like (human)	PPM24689A
G12	Mm.332314	NM_011738	Ywhah	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	PPM41052B
H01	Mm.3317	NM_010368	Gusb	Glucuronidase, beta	PPM05490B
H02	Mm.299381	NM_013556	Hprt	Hypoxanthine guanine phosphoribosyl transferase	PPM03559E
H03	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	PPM04803E
H04	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	PPM02946E
H05	Mm.328431	NM_007393	Actb	Actin, beta	PPM02945A
H06	N/A	SA_00106	MGDC	Mouse Genomic DNA Contamination	

Table A9. Gene list for MAPK signalling pathway PCR array

PCR Array Catalog #:			PAMM-061 - MAP Kinase Signalling Pathway		
	Unigene	**Refseq**	**Symbol**	**Description**	**RT2 Catalog**
A01	Mm.220946	NM_009703	Araf	V-raf murine sarcoma 3611 viral oncogene homolog	PPM04525A
A02	Mm.209903	NM_009715	Atf2	Activating transcription factor 2	PPM03036B
A03	Mm.4815	NM_007628	Ccna1	Cyclin A1	PPM03258B
A04	Mm.4189	NM_009828	Ccna2	Cyclin A2	PPM02913C
A05	Mm.260114	NM_172301	Ccnb1	Cyclin B1	PPM02894E
A06	Mm.22592	NM_007630	Ccnb2	Cyclin B2	PPM03259E
A07	Mm.273049	NM_007631	Ccnd1	Cyclin D1	PPM02903E
A08	Mm.333406	NM_009829	Ccnd2	Cyclin D2	PPM02900E
A09	Mm.246520	NM_007632	Ccnd3	Cyclin D3	PPM02908E
A10	Mm.16110	NM_007633	Ccne1	Cyclin E1	PPM02891B
A11	Mm.1022	NM_009861	Cdc42	Cell division cycle 42 homolog (S. cerevisiae)	PPM04527E
A12	Mm.111326	NM_016756	Cdk2	Cyclin-dependent kinase 2	PPM02902E
B01	Mm.6839	NM_009870	Cdk4	Cyclin-dependent kinase 4	PPM02911C
B02	Mm.31672	NM_009873	Cdk6	Cyclin-dependent kinase 6	PPM02912E
B03	Mm.195663	NM_007669	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	PPM02901A
B04	Mm.2958	NM_009875	Cdkn1b	Cyclin-dependent kinase inhibitor 1B	PPM02909B
B05	Mm.168789	NM_009876	Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)	PPM02895A
B06	Mm.4733	NM_009877	Cdkn2a	Cyclin-dependent kinase inhibitor 2A	PPM02906E
B07	Mm.423094	NM_007670	Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	PPM02910E
B08	Mm.1912	NM_007671	Cdkn2c	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	PPM02893C
B09	Mm.29020	NM_009878	Cdkn2d	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	PPM02897A
B10	Mm.3996	NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase	PPM03197B
B11	Mm.277735	NM_007742	Col1a1	Collagen, type I, alpha 1	PPM03845F
B12	Mm.453295	NM_133828	Creb1	CAMP responsive element binding protein 1	PPM03382E
C01	Mm.132238	NM_001025432	Crebbp	CREB binding protein	PPM04423B
C02	Mm.157069	NM_010052	Dlk1	Delta-like 1 homolog (Drosophila)	PPM04528A
C03	Mm.18036	NM_007891	E2f1	E2F transcription factor 1	PPM02892E
C04	Mm.8534	NM_007912	Egfr	Epidermal growth factor receptor	PPM03714F
C05	Mm.181959	NM_007913	Egr1	Early growth response 1	PPM02938B
C06	Mm.405823	NM_007922	Elk1	ELK1, member of ETS oncogene family	PPM03038E
C07	Mm.292415	NM_011808	Ets1	E26 avian leukemia oncogene 1, 5' domain	PPM03600A
C08	Mm.290207	NM_011809	Ets2	E26 avian leukemia oncogene 2, 3' domain	PPM03623A
C09	Mm.246513	NM_010234	Fos	FBJ osteosarcoma oncogene	PPM02940B
C10	Mm.439649	NM_008163	Grb2	Growth factor receptor bound protein 2	PPM03710E
C11	Mm.334313	NM_008284	Hras1	Harvey rat sarcoma virus oncogene 1	PPM03682B
C12	Mm.330160	NM_022310	Hspa5	Heat shock protein 5	PPM03586B
D01	Mm.13849	NM_013560	Hspb1	Heat shock protein 1	PPM02936B
D02	Mm.275071	NM_010591	Jun	Jun oncogene	PPM03037A
D03	Mm.32074	NM_032397	Kcnn1	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1	PPM04195A
D04	Mm.383182	NM_021284	Kras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	PPM04020E
D05	Mm.4745	NM_013571	Ksr1	Kinase suppressor of ras 1	PPM04532A
D06	Mm.248907	NM_008927	Map2k1	Mitogen-activated protein kinase kinase 1	PPM03565E
D07	Mm.331392	NM_019920	Lamtor3	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 3	PPM04533E
D08	Mm.275436	NM_023138	Map2k2	Mitogen-activated protein kinase kinase 2	PPM03564B
D09	Mm.18494	NM_008928	Map2k3	Mitogen-activated protein kinase kinase 3	PPM03572B
D10	Mm.412922	NM_009157	Map2k4	Mitogen-activated protein kinase kinase 4	PPM03575B
D11	Mm.325746	NM_011840	Map2k5	Mitogen-activated protein kinase kinase 5	PPM04534E
D12	Mm.14487	NM_011943	Map2k6	Mitogen-activated protein kinase kinase 6	PPM03568B

E01	Mm.3906	NM_011944	Map2k7	Mitogen-activated protein kinase kinase 7	PPM03235E
E02	Mm.15918	NM_011945	Map3k1	Mitogen-activated protein kinase kinase kinase 1	PPM03569F
E03	Mm.211762	NM_011946	Map3k2	Mitogen-activated protein kinase kinase kinase 2	PPM04215E
E04	Mm.27041	NM_011947	Map3k3	Mitogen-activated protein kinase kinase kinase 3	PPM03574E
E05	Mm.28587	NM_011948	Map3k4	Mitogen-activated protein kinase kinase kinase 4	PPM03576E
E06	Mm.148278	NM_008279	Map4k1	Mitogen-activated protein kinase kinase kinase kinase 1	PPM04536E
E07	Mm.196581	NM_011949	Mapk1	Mitogen-activated protein kinase 1	PPM03571E
E08	Mm.39253	NM_009158	Mapk10	Mitogen-activated protein kinase 10	PPM04539E
E09	Mm.91969	NM_011161	Mapk11	Mitogen-activated protein kinase 11	PPM04540A
E10	Mm.38343	NM_013871	Mapk12	Mitogen-activated protein kinase 12	PPM04541C
E11	Mm.27970	NM_011950	Mapk13	Mitogen-activated protein kinase 13	PPM04542B
E12	Mm.311337	NM_011951	Mapk14	Mitogen-activated protein kinase 14	PPM03578A
F01	Mm.8385	NM_011952	Mapk3	Mitogen-activated protein kinase 3	PPM03585E
F02	Mm.480076	NM_015806	Mapk6	Mitogen-activated protein kinase 6	PPM04543A
F03	Mm.38172	NM_011841	Mapk7	Mitogen-activated protein kinase 7	PPM04544B
F04	Mm.21495	NM_016700	Mapk8	Mitogen-activated protein kinase 8	PPM03234B
F05	Mm.2720	NM_011162	Mapk8ip1	Mitogen-activated protein kinase 8 interacting protein 1	PPM04785A
F06	Mm.173337	NM_021921	Mapk8ip2	Mitogen-activated protein kinase 8 interacting protein 2	PPM04545A
F07	Mm.43081	NM_013931	Mapk8ip3	Mitogen-activated protein kinase 8 interacting protein 3	PPM04787B
F08	Mm.68933	NM_016961	Mapk9	Mitogen-activated protein kinase 9	PPM03584B
F09	Mm.221235	NM_008551	Mapkapk2	MAP kinase-activated protein kinase 2	PPM04546B
F10	Mm.272206	NM_010765	Mapkapk5	MAP kinase-activated protein kinase 5	PPM04786B
F11	Mm.268548	NM_008558	Max	Max protein	PPM03577A
F12	Mm.24001	NM_025282	Mef2c	Myocyte enhancer factor 2C	PPM04548A
G01	Mm.209327	NM_021461	Mknk1	MAP kinase-interacting serine/threonine kinase 1	PPM04549B
G02	Mm.459300	NM_020021	Mos	Moloney sarcoma oncogene	PPM04550E
G03	Mm.2444	NM_010849	Myc	Myelocytomatosis oncogene	PPM02924E
G04	Mm.27908	NM_023699	Nfatc4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	PPM04552A
G05	Mm.400954	NM_010937	Nras	Neuroblastoma ras oncogene	PPM03238E
G06	Mm.260227	NM_011035	Pak1	P21 protein (Cdc42/Rac)-activated kinase 1	PPM04553E
G07	Mm.292510	NM_009007	Rac1	RAS-related C3 botulinum substrate 1	PPM03391E
G08	Mm.184163	NM_029780	Raf1	V-raf-leukemia viral oncogene 1	PPM03707E
G09	Mm.273862	NM_009029	Rb1	Retinoblastoma 1	PPM02899B
G10	Mm.44482	NM_018754	Sfn	Stratifin	PPM03467A
G11	Mm.100399	NM_008540	Smad4	MAD homolog 4 (Drosophila)	PPM04411A
G12	Mm.222	NM_011640	Trp53	Transformation related protein 53	PPM02931B
H01	Mm.3317	NM_010368	Gusb	Glucuronidase, beta	PPM05490B
H02	Mm.299381	NM_013556	Hprt	Hypoxanthine guanine phosphoribosyl transferase	PPM03559E
H03	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	PPM04803E
H04	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	PPM02946E
H05	Mm.328431	NM_007393	Actb	Actin, beta	PPM02945A
H06	N/A	SA_00106	MGDC	Mouse Genomic DNA Contamination	

APPENDIX IV

Human NSCLC cell line details

Line	Species	Type	EGFR	Kras	TP53	SMARCA4	ALK	BRAF	STK11/LKB1	MYC	PIK3CA	PTEN	Sex	Stage	Origin	Smoking	Other
A549	Human	Adenocarcinoma	N	Y	N	Y	N	N	Y	N	N	N	Male	?	Lung	?	
NCI-H1395	Human	Adenocarcinoma	N	N	N	N	N	Y	Y	N	N	N	female	stage 2	Lung	smoker	
NCI-H1993	Human	Adenocarcinoma	N	N	Y	N	N	N	Y	N	N	N	female	stage 3a	LN	smoker	
NCI-H2228	Human	Adenocarcinoma	N	N	Y	N	Fusion	N	N	N	N	N	female	?	Lung	non-smoker	
NCI-H23	Human	Adenocarcinoma	N	Y	Y	Y	N	N	Y	N	N	N	male	?	Lung	?	**
NCI-H838	Human	Adenocarcinoma	N	N	Y	N	N	N	Y	N	N	N	male	stage 3b	LN	smoker	

** C-myc, L-myc, v-src, v-abl, v-erb B, c-raf 1, Ha-ras, Ki-ras and N-ras RNAs

Table A10. Characteristics of Human adenocarcinoma cell lines.

Y = mutation identified; N = no mutation identified; Fusion = gene fusion identified; ? = No information available

APPENDIX V

Suppliers

Ajax Finechem	Seven Hills, NSW, Australia
Amber Scientific	Midvale, WA, Australia
Amersham	Buckinghamshire, UK
Amresco	Solon, USA
BD Biosciences	MA, USA
BDH	Nottingham, UK
Bioline	Alexandria, NSW, Australia
Cell Signalling Technologies	MA, USA
Dade Baxter	DE, USA
Dako	CA, USA
eBioscience	San Diego, CA
Fluka	Steinheim, Germany
GE Healthcare	Uppsala, Sweden
Greiner	Frickenhausen, Germany
Invitrogen	Auckland, NZ
Invitrogen	Lohne, Germany
JRH Biosciences	Lenexa, USA
Leica Microsystems	Peterborough, UK
Li-Cor	NE, USA
Merck	Kilsyth, Australia
Millipore	Bedford, MA, USA
NovImmune	Geneva, Switzerland
Novus Biologicals	CO, USA
Nunc	Roskilde, Denmark
Precicolor HBG	Giessen-Lützellinden, Germany
Promega	WI, USA
Qiagen	Hilden, Germany
R&D Systems	MN, USA
Roche	Mannheim, Germany
SARSTEDT	Nümbrecht, Germany
Sigma-Aldrich	Saint Louis, MO, USA
Thermo Scientific	Wilmington, IL, USA
Toronto Research Chemicals	North York, Canada
Troy Laboratories	NSW, Australia
University of Iowa	Gene Transfer Vector Core, Iowa city, USA
Vector Laboratories	CA, USA
Walter and Eliza Hall Institute Animal Services	Kew, Victoria, Australia
Whatman	Maidstone, UK

APPENDIX VI

Equipment

Agarose gel electrophoresis	Mini-sub Cell GT, Bio-Rad
Centrifuges	J2-21M/E, Beckman Biofuge stratos, Heraeus instruments
Cell incubator	HERA Cell, Heraeus instruments
Flow Cytometry	FACS Canto II Flow Cytometry, BD Pharmigen
Homogeniser	Ika Ultra Turrax T25, Crown Scientific
Plate Reader	Fluostar Optima, BMG Labtech, Offenburg, Germany
qRT-PCR cycler	7900HT Real-Time PCR System, Applied Biosystems, Forster City, USA
Spectrophotometer	ND-100, Nanodrop Technologies
Gel dock	SafeImage, Invitrogen, Lohne, Germany
Cell counter	Sysmex KX-21N, Sysmex Corporation, Kobe, Japan
DEXA	Lunar PIXImus, GE-Lunar Corp, Madison, WI
Western blot development	Odyssey Fluorimager, Li-Cor, NE, USA
Cytocentrifuge	Cytopin III, Shandon Scientific, Cheshire, UK
PCR machine, Biorad MyCycler Thermal Cycler	Biorad, Richmond, CA, USA

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