The role of the non-canonical NF-κB pathway in colorectal cancer

Meera Patel

MBChB, MRCS

Submitted in fulfilment of the requirements for the degree of PhD

Institute of Cancer Sciences

College of Medical, Veterinary and Life Sciences

University of Glasgow

August 2019

The work presented in this thesis was performed entirely by the author except as acknowledged. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Meera Patel

August 2019

Acknowledgements

To my primary supervisor Professor Joanne Edwards, a heartfelt thank you for welcoming me into the lab and for the opportunity to work on this project. I am incredibly grateful for her ongoing support and encouragement.

A special thanks to my secondary supervisor Dr Jean Quinn and fellow PhD student Milly McAllister for their overwhelming support and patience whilst I carried out this work. Thank you to each member of the Edwards team- your advice and humour have helped me to navigate the last few years far better than I ever could without them. Thank you also to Dr Antonia Roseweir, Professor Donald McMillan and Professor Paul Horgan for their valuable contributions. I would also like to thank Dr Andrew Paul at the University of Strathclyde for allowing me to spend such important time in his lab.

Finally, I especially thank Mum, Dad, Parul, Michael and Fergus, for their unlimited support during the course of these studies and beyond.

Summary

Colorectal cancer (CRC) is the fourth most common cancer in the UK and despite earlier detection and improved treatments, it remains the second most common cause of cancer death. Currently, TNM staging is widely used to determine patient prognosis and the need for adjuvant therapies however, it is well recognised that CRC is a heterogeneous disease and patients with the same stage of disease can have very different survival outcomes. The molecular mechanisms underpinning these differences, and difficulties associated with treatment resistance have yet to be fully elucidated. This requires attention. Understanding molecular signalling pathways allows the discovery of new therapeutic targets and identification of biomarkers to enable treatments to be directed towards those patients who will benefit the most.

Nuclear Factor kappa B (NF-κB) has key roles in tumourigenic processes described as the hallmarks of cancer. However, the majority of this evidence is based on investigation of the canonical NF-κB pathway. The aims of the present study were to understand the role of the non-canonical NF-κB pathway in the development and progression of CRC, establish if expression of non-canonical pathway members could be employed as prognostic biomarkers and assess the viability of inhibiting key non-canonical kinase IKKα as a potential therapeutic strategy in CRC.

Using a tissue microarray (TMA), immunohistochemistry was used to assess expression of key members of the non-canonical NF-κB pathway in patients who had undergone surgery for stage I-III CRC. High cytoplasmic expression of IKKα was associated with adverse pathological tumour features including increasing T stage, poor tumour differentiation, tumour necrosis and low proliferation status. Key pathway members were also investigated in a cohort of patients who underwent treatment for screen-detected T1/2 CRC. Even in this cohort of early-stage disease, IKKα was associated with a more invasive phenotype. An unfamiliar ‘punctate’ pattern of IKKα expression was observed and this was associated with significantly inferior survival outcome in patients who had undergone surgery for stage I-III CRC, this was potentiated in patients with BRAF wild-type status. Using immunochemistry the present study was able to demonstrate the pattern of punctate IKKα expression was not associated with two IKKα phosphorylation sites. To investigate the distribution and localisation of IKKα further, markers of cellular transport were investigated using immunofluorescence. IKKα was co-located with a marker of the Golgi

apparatus. This observation raises a number of possible hypotheses that require further investigation.

Expression of the non-canonical NF-κB pathway was investigated in two colon cancer cell lines with contrasting mutational landscapes. Using western blot the present study was able to demonstrate the non-canonical NF-κB pathway can be inhibited with first-in-class IKKα inhibitors. This data, together with results from tissue studies suggest investigation of IKKα in CRC should be pursued further. Additional studies are required to investigate the predictive capacity of IKKα in the context of existing therapies used in the treatment of CRC.

Publications and presentations

Publications

Patel M, Hood H, Quinn J, Carlin LM, Andersen DK, Roseweir AK, Powell AGMT, Horgan PG, McMillan DC, Edwards J. The role of IKKα in colorectal cancer. (In preparation)

Patel M, Horgan PG, McMillan DC, Edwards JE. NF-κB pathways in the development and progression of colorectal cancer. *Translational Research.* 2018; *197:43-56.*

Patel M, McSorley ST, Park JH, Roxburgh CSD, Edwards J, Horgan PG, McMillan DC. The relationship between right-sided tumour location, tumour microenvironment, systemic inflammation, adjuvant therapy and survival in patients undergoing surgery for colon and rectal cancer. *British Journal of Cancer.* 2018; 118(5):705-712.

Presentations

The role of IKKα in colorectal cancer (**Poster**). Patel M, Hood H, Quinn J, Roseweir AK, Powell A, Horgan PG, McMillan DC, Edwards J. National Cancer Research Institute Conference 2018, Glasgow.

The relationship between the non-canonical NF-κB pathway, tumour microenvironment, systemic inflammation and survival in patients undergoing surgery for colorectal cancer (**Poster**). Patel M, Bennett L, Quinn JA, van Wyk HC, Horgan PG, McMillan DC, Park JH, Edwards J. ASCO GI - American Society of Clinical Oncology Gastrointestinal Cancers Symposium 2017, San Francisco. **Published abstract:** J Clin Oncol 35, suppl 4, 631-631.

The relationship between tumour location, tumour microenvironment, systemic inflammation and cancer-specific survival in patients undergoing surgery for colon cancer. (**Poster**) Patel M, Park JH, van Wyk HC, Edwards J, Horgan PG, McMillan DC. ASCO GI - American Society of Clinical Oncology Gastrointestinal Cancers Symposium 2017, San Francisco. **Published abstract:** J Clin Oncol 35, suppl 4, 689-689.

The relationship between the non-canonical NF-κB pathway, tumour microenvironment, systemic inflammation and survival in patients undergoing surgery for colorectal cancer (**Oral presentation**). Patel M, Bennett L, Quinn JA, van Wyk HC, Horgan PG, McMillan

D C, Park JH, Edwards J. Association of Surgeons Great Britain & Ireland 2017, Glasgow. **Published abstract:** Br J Surg 104, suppl 6, 131-131

The relationship between the non-canonical NF-κB pathway, tumour microenvironment, systemic inflammation and survival in patients undergoing surgery for colorectal cancer (**Oral presentation**). Patel M, Bennett L, Quinn JA, van Wyk HC, Horgan PG, McMillan DC, Park JH, Edwards J. UK & Ireland NF-κB Workshop 2017, Newcastle.

Contents

List of figures

List of tables

Abbreviations

AJCC	American joint committee on cancer
Akt	Protein kinase B
BRAF	B-Raf
BSA	Bovine serum albumin
CI	Confidence interval
CIMP	CpG island methylation
CRC	Colorectal cancer
CSS	Cancer-specific survival
CT	Computed tomography
DMEM	Dulbecco's Modified Eagle's Medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMR	Endoscopic mucosal resection
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FIT	Faecal immunochemical test
gFOBT	Guaiac faecal occult blood test
Groα	growth-regulated alpha protein
HDAC	Histone deacetylases

HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia inducible factor
HR	Hazard ratio
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IBD	Inflammatory bowel disease
ICCC	Interclass correlation coefficient
IHC	Immunohistochemistry
IκB	Inhibitor of kappa B
IKK	IκB kinase
IKKα	Inhibitor of nuclear factor kappa-B kinase alpha
IKK-β	Inhibitor of nuclear factor kappa-B kinase beta
IKKγ	Inhibitor of nuclear factor kappa-B kinase gamma
IQR	Interquartile range
IL-1	Interleukin1
IL-6	Interleukin 6
IL-8	Interleukin 8
KM	Klintrup-Mäkinen
LPS	Lipopolysaccharide
LTβR	Lymphotoxin β-receptor
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant 1
mGPS	modified Glasgow Prognostic Score

miRNA	MicroRNA
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSS	Microsatellite stable
mTOR	Mammalian target of rapamycin
NEMO	NF-kappa-B essential modulator
NIBP	NIK and IKKβ –binding protein
NIK	Nuclear factor kappa-B inducing kinase
NLR	Neutrophil lymphocyte ratio
NLS	Nuclear localisation sequence
NF-κB	Nuclear factor kappa B
OLFM	Olfactomedin proteins
Phospho-p100	Phosphorylation of p100
PI3K	Phosphoinositide 3-kinase
RHD	Rel homology domain
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SIGN	Scottish Intercollegiate Guidelines Network
siRNA	Small interfering RNA
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
STAT3	Signal transduction activator of transcription-3

STWS	Scott's tap water substitute
TAD	Transcriptional activation domain
TAK-1	Transforming growth factor-β-activated kinase 1
TAMS	Tumour-associated macrophages
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween®
TEMS	Transanal endoscopic microsurgery
TLR	Toll-like receptor
TMA	Tissue microarray
TNF	Tumour necrosis factor
TNFα	Tumour necrosis factor alpha
TNM	Tumour size, lymph node involvement, metastasis
TRAF	Tumour necrosis factor receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
UICC	Union for international cancer control

Chapter 1:

Introduction

1.1 Colorectal cancer epidemiology, pathology and prognostic factors

1.1.1 Colorectal cancer incidence, mortality and survival

Worldwide there are 1.2 million new cases of colorectal cancer (CRC) per year; it is the second most common cancer in women and the third most common in men, accounting for 9% and 10% of all cancer cases respectively. There is wide geographical variation in incidence with highest incidence rates in Australia, New Zealand, Europe, North America, Micronesia and Eastern Asia and much lower incidence rates in countries such as Korea and Slovakia (1). CRC is the fourth most common cancer in the United Kingdom (UK) (2014) and the third most common in males and females separately. In the UK, there are over 40,000 new cases of CRC each year. CRC is more common in men (55%) than women (45%) and incidence rates increase from the age of 50 with highest rates in those aged 85-89 for both sexes (2). Incidence rates for CRC are projected to fall by 11% in the UK between 2014 and 2035. The bowel screening programme was introduced in England in 2006 and thereafter rolled out in a staged process across the UK. Patients between the ages of 50-74 in Scotland, England and Wales are invited to a two-yearly screening process. The screening tool in the UK is a faecal immunochemical test, also known as a FIT test. The aim of the CRC screening programme is to detect early, asymptomatic CRC and pre-malignant polyps that have the potential to undergo invasive transformation.

Since the 1970s CRC mortality rates have decreased by 42% in the UK and at present is the second most common cause of cancer death, accounting for 10% of all cancer deaths in both sexes. European age-standardised mortality rates are significantly higher in Scotland and Wales compared to England and Northern Ireland. In England, the age-standardised mortality rate is 27 cases per 100,000 and 31 cases per 100,00 population in Scotland. Mortality is also influenced by deprivation, with 30% higher mortality rates for males and 15% higher mortality for females in most deprived areas when compared to least deprived. Cancer Research UK (CRUK) report mortality rates from CRC are projected to fall by 23% between 2014 and 2035, with the largest decrease for males (2). The trends in mortality are often attributed to screening however, Welch et al point out that no clinical trials of CRC screening demonstrate 50% reduction in mortality and given the low uptake of screening and indolent nature of CRC development, such reduction in mortality is very likely the result of surgical and chemotherapeutic advances i.e. centralisation of services,

improved surgical technique, standardisation of preoperative and postoperative care and addition of adjuvant chemotherapy (3).

Survival for CRC is strongly related to the stage of disease at diagnosis. In England and Wales, 57% of patients will survive five years or longer (2010-2011). When stratified by disease stage, patients with stage I disease have a 5-year survival of 95%, this drops to 7% in patients diagnosed with stage IV disease (2).

1.1.2 Colorectal cancer aetiology

Most cases of CRC are sporadic (80%). The remaining cases are hereditary; approximately 5 % of these are associated with a highly penetrant inherited mutation. The aetiologies of the remaining inherited CRCs are unclear but thought to be the result of single, less penetrant genes (4).

There are environmental and host factors associated with an increased risk of developing CRC. Some of these are modifiable and others are non-modifiable risk factors.

1.1.2.1 Environmental factors

There is a clear role for environmental factors in the development of CRC. Specifically, poor diets and sedentary lifestyles are reasons why CRC is considered a disease of western society (5). This is evidenced by studies that have shown that westward migration is associated with a higher risk of CRC, similar to that found in the host country (6). With respect to diet, high consumption of fat (7), alcohol (8) and red/processed meat (9) and low consumption of fibre (10) have been associated with an increased risk of CRC.

In the UK, 13% of CRC cases have been attributed to being overweight or obese (2). An inverse association between physical activity and risk of CRC is well-established (11). Unsurprisingly, long-term cigarette smoking is associated with increased risk of CRC in both sexes with evidence that early smoking cessation can reduced this risk (12). There is also evidence from non-randomised studies that suggest regular physical activity, diet limited in refined carbohydrates and red/processed meats and smoking cessation are important in reducing risk of recurrence and mortality in patients treated for CRC (13,14).

1.1.2.2 Host factors

CRC risk is strongly related to age and in the UK 95% of cases are diagnosed in those over the age of 50, with a peak incidence in those aged 85-89 (2).

Patients with inflammatory bowel disease (IBD), such as ulcerative colitis or Crohn's disease are at increased risk of developing CRC. It is well recognised that extent of disease and duration are important risk factors. Years of chronic colonic inflammation and subsequent alterations to genetic and epigenetic stability, oxidative stress and changes in the microbiome are all factors considered to be important in the pathogenesis of CRC in the context of inflammatory bowel disease (15). A meta-analysis of patients with ulcerative colitis reported the risk of CRC is 2% after 10 years disease duration and 18% after 30 years (16).

Patients with cardiovascular disease also have increased risk of colorectal neoplasia. One study reported that in patients undergoing coronary angiography for suspected coronary artery disease, the prevalence of colorectal neoplasia including cancer was significantly higher in those patients with coronary artery disease (17).

1.1.2.3 Hereditary colorectal cancer

Familial adenomatous polyposis

As discussed, 5% of all CRC cases are associated with a highly penetrant inherited syndrome (4). Less than 1% of CRC cases are the result of familial adenomatous polyposis (FAP) (18). This condition is associated with a germ-line mutation of the APC gene, which is inherited in an autosomal dominant pattern. Penetrance of the mutation can vary, however, the classical form of the condition is associated with 100% penetrance (19), which results in hundreds to thousands of adenomatous polyps forming within the colon with a certain risk of developing invasive cancer by the age of 40-50 years if they do not undergo a prophylactic colectomy. There are variants of FAP which include attenuated FAP, Gardner syndrome and Turcot syndrome, all with a variation of APC mutation (20).

Hereditary Non-polyposis Colorectal Cancer

Hereditary Non-polyposis Colorectal Cancer (HNPCC) or Lynch syndrome is the most common form of hereditary cancer accounting for 1-6% of all CRCs. It is often diagnosed

in the fourth decade of life, is associated with right-sided tumour location, synchronous and metachronous CRC as well as extracolonic malignancies (19). HNPCC results from a germ-line mutation in any of the mismatch-repair genes and therefore high microsatellite instability is a hallmark in HNPCC (21,22). Penetration varies according to the underlying mutation i.e. mutations in MLH1, MLH2, MSH6, PMS1 or PMS2 genes (23).

1.1.3 Host inflammation, tumour microenvironment and colorectal cancer prognosis

It is well recognised that tumour progression is not a cell-autonomous process and immune factors relating to the host and the tumour itself are important in the prognosis of patients with CRC. The host response to a tumour is evidenced by that which constitutes its microenvironment; tumour-infiltrating cells, extracellular matrix, vasculature, and the presence of immune cells with tumour-promoting and tumour-inhibiting properties. This tumour microenvironment is maintained by a complex crosstalk between tumour cells, tumour and host derived cytokines and immune cells. As such, tumour progression can be considered an imbalance between tumour invasiveness and the host immune response (24).

1.1.3.1 Cancer associated inflammation

Tumour promoting inflammation and evasion of immune destruction are both described in Hanahan and Weinberg's more recent review on the hallmarks of cancer (25). The first evidence linking inflammation and CRC was in the context of chronic inflammation and IBD-associated CRC. Sustained activation of NF-κB pathway in the tumour locale represents one mechanism whereby tumours can inactivate immune cells thus creating a pro-tumour environment and escape from the host immune system (26). NF-κB is a key regulator of inflammation and is active in both cancer cells and tumour-associated inflammatory cells (27).

1.1.3.2 Local inflammatory response in colorectal cancer

A number of studies have demonstrated the presence of an adaptive host immune response evidenced by the presence of tumour infiltrating lymphocytes (TILs), is associated with improved survival outcomes, independent of TNM (tumour, node, metastasis) stage (28) and microsatellite instability status (29), suggesting there are variations in immune contexture even in groups that are considered as homogenous. Several methods that aim to quantify the local inflammatory response have been proposed. For example, the Klintrup-

Mäkinen (KM) grade (30) is a semi-quantitative score which can be used to assess generalised inflammatory cell infiltrate at the invasive margin of CRC. High KM grade is independently associated with favourable survival outcomes in patients with primary operable CRC (31). Others have gone further to analyse the sub-populations of adaptive immune cells within the tumour microenvironment. The Immunoscore® proposed by Galon (32) aims to quantify lymphocyte populations and has been shown to have prognostic value in CRC, this is now under the scrutiny of an international validation study (33).

1.1.3.3 Systemic inflammatory response in colorectal cancer

There is substantial evidence which indicates the systemic inflammatory response is useful in predicting outcomes in patients with operable cancers (34). In CRC, inflammation-based prognostic scores (based on clinical laboratory measures of differential white cells and acute phase proteins) that evaluate the systemic inflammatory response have yielded prognostic value independent of the widely used TNM staging system (35,36). Specifically, scores such as modified Glasgow Prognostic Score (mGPS) and neutrophil lymphocyte ratio (NLR) have been consistently reported to have prognostic value in CRC (35,37–39).

1.1.4 Colorectal cancer pathogenesis

CRC is now well recognised as a heterogeneous disease arising as a result of genetic and molecular alterations. There are three pathways which result in genetic instability implicated in colorectal carcinogenesis. Vogelstein proposed the chromosomal instability pathway that accounts for 80% of CRC. The remaining proportion of CRC arises as a result of the microsatellite instability (MSI) and CpG island methylation (CIMP) pathways.

1.1.4.1 Chromosomal instability pathway

The majority of CRCs, whether sporadic or inherited, arise as a result of transformation of adenomatous polyps through the adenoma-carcinoma sequence. Sequential genetic mutations affecting oncogenes and tumour suppressor genes accompany the pathological transformation of normal colonic mucosa to dysplastic adenoma and finally to invasive carcinoma (40). This model proposed that the total accumulation of genetic changes rather than the order in which they occurred was responsible for the development of invasive carcinoma (41). Such changes include loss of tumour suppressor APC and subsequent

accumulation of β-catenin, KRAS mutation, loss of chromosome 18q and finally, loss of tumour suppressor p53 (42).

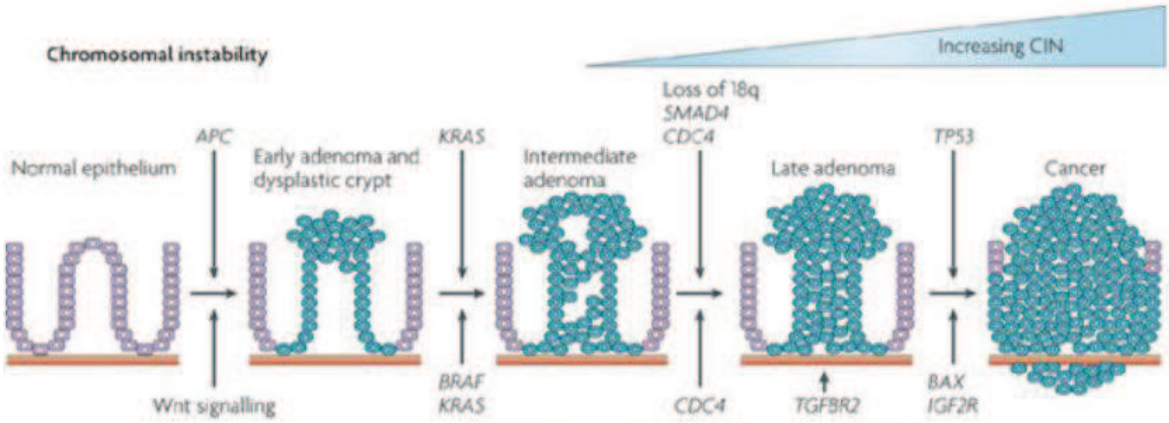


Figure 1: *Adenoma-carcinoma sequence in sporadic chromosomal instability CRC, demonstrating key mutational events that occur during the transition from non-invasive lesion to invasive cancer. Adapted from Walther, Johnstone et al.* (42)

1.1.4.2 Microsatellite instability pathway

15-18% of CRCs arise via the MSI pathway, which is characterised by mutational inactivation of mismatch repair genes responsible for correcting errors in DNA replication (MLH1, MSH2, MSH6 and PMS2). Approximately 15% of sporadic CRCs are microsatellite unstable and arise as a result of epigenetic silencing of MLH1. Whilst the majority of MSI CRCs arise as a result of somatic inactivation of this mismatch repair system, 3% of patients with MSI are the result of germline mutations resulting in HNPCC or Lynch syndrome; MLH1 and MLH2 being the most commonly affected genes (43,44). Cancers arising through the MSI pathway have distinct phenotypic features; they are often right-sided, with poor or mucinous differentiation, a tumour microenvironment dense in lymphocytes and are associated with superior survival outcomes (45–47). MSI status is determined by genetic sequencing however, immunohistochemical detection of mismatch repair markers are sensitive and specific for microsatellite instability (48).

1.1.4.3 CpG island methylation

DNA methylation is a widely studied epigenetic marker (49). Many genes contain cytosine and quinine-rich regions or CpG islands. Hypermethylation of these regions results in repression of tumour suppressor genes. One such example is inactivation of mismatch repair gene MLH1 by promoter methylation resulting in MSI. There is considerable

overlap between MSI, CIMP and BRAF in sporadic CRC (44,50,51). CIMP positivity is more frequently observed in the proximal colon independent of MSI status (50,52,53).

1.1.5 Pathological tumour assessment

Pathological assessment of the resected surgical specimen provides information that determines which patients require adjuvant therapy and ultimately prognosis. Analysis of the resected surgical specimen provides the following information: tumour and nodal stage, histological subtype, tumour differentiation, vascular invasion, perineural invasion, tumour perforation and involvement of surgical resection margins. The American Joint Committee on Cancer (AJCC) TNM classification is widely used to stage CRC. The T stage is defined by the invasiveness of the tumour i.e. extent of spread through the bowel wall. The N stage is defined by the number of lymph nodes with metastasis. The M stage (distant metastasis) is determined by radiological cross-sectional imaging i.e. computed tomography (CT) scan.

1.1.6 Consensus Molecular subtypes

CRC is a heterogeneous disease. This is reflected in how it presents, variation in molecular characteristics, response to treatment and ultimately patient survival. In 2015, an international consortium proposed four consensus molecular subtypes of CRC with distinguishing genomic features (54). The genes or pathways active are unique to each subtype. Whilst these subtypes are not independently prognostic, currently have no predictive value, nor are they readily translated into routine clinical practice, they provide a backbone for research into CRC. The consensus molecular subtypes are being explored in clinical trials as prognostic or predictive markers. More recently, a phenotypic subtyping method of CRC based on immune infiltrate, stromal invasion and proliferation rate has been proposed with the specific aim to make the consensus molecular subtypes more translatable into clinical practice (55).

1.2 Treatment of colorectal cancer

1.2.1 Surgery

Currently, if a patient does not have widespread metastatic disease, surgical resection is the primary treatment option for CRC. The principles of surgical management of colon and rectal cancer are very similar, that is, removal of the affected length of bowel with its vascular pedicle and accompanying lymphatics, with minimum 5cm margin of normal bowel either side of the tumour, en bloc resection of any structures attached to the tumour, a minimum of 12 lymph nodes examined to declare node negative disease and a tension-free anastomosis (56,57). For rectal cancer there is good evidence that performing total mesorectal excision (TME) with clear circumferential resection margins reduces the risk of local recurrence, distant metastases and improves survival after rectal cancer surgery (58–60).

1.2.2 Polypectomy and TEMS

The majority of patients undergoing investigations for CRC will undergo colonoscopy at some stage. Polypectomy or endoscopic mucosal resection (EMR) is the removal of an abnormal area of tissue and can be carried out at the time of colonoscopy. The aim is to remove pre-malignant lesions in order to prevent them developing into cancers.

Transanal endoscopic microsurgery (TEMS) utilises a microscope and instruments designed to perform surgery through the anus inside the rectum. This type of surgery can be used to remove polyps and early cancers from the rectum, avoiding major surgery as described in section 1.2.1.

1.2.3 Chemotherapy

The aim of systemic chemotherapy is to target occult systemic metastases that are already present at the time of diagnosis/surgery. This therapy can be administered in a neoadjuvant (prior to surgery) or adjuvant setting (after surgery). TNM staging determines which patients are offered chemotherapy. All patients with stage III CRC are considered for adjuvant chemotherapy. The benefit of fluorouracil-based therapy following surgery, in patients with stage III disease, was first reported by Sargent et al (61). This landmark study highlighted a 7% overall survival benefit conferred by such therapy when compared to untreated patients. Later, Andrè et al reported the addition of oxaliplatin to a regimen of

fluorouracil and leucovorin improved the adjuvant treatment of colon cancer (62,63). More recent studies have reported the lack of benefit conferred by adjuvant therapy in the context of patients with tumours demonstrating mismatch repair deficiency and may in fact be harmful (64). Whilst the use of adjuvant therapy in stage III patients is widely practiced in the United Kingdom, the use of mismatch repair (MMR) deficiency as a predictive marker is yet to be adopted into routine practice. Patients with stage II disease with additional risk factors are also considered for adjuvant therapy; however, a substantial proportion of these patients do not benefit from chemotherapy. Patients with resectable lung/liver metastases are offered neoadjuvant chemotherapy prior to surgical resection of the metastases. Those patients with unresectable lung/liver metastases are considered for cytoreductive or 'downstaging' chemotherapy. Patients with a significant response may be considered for surgical resection of the primary tumour and accompanying metastases.

1.2.4 Radiotherapy

In rectal cancer, magnetic resonance imaging (MRI) has been shown to have high diagnostic accuracy with respect to predicting whether the circumferential resection margin will be involved with tumour (65). Patients with rectal cancer are offered neoadjuvant radiotherapy (with or without chemotherapy) based on their risk of local recurrence as predicted by staging MRI. Currently, the Scottish Intercollegiate Guidelines Network (SIGN) guidelines on CRC advise patients with high risk of recurrence i.e. patients who require cytoreduction because of tumour encroachment on the mesorectal fascia should receive preoperative chemoradiotherapy followed by surgery. Those with moderate risk of local recurrence i.e. if the circumferential resection margin is threatened but not breached should be offered short course radiotherapy alone (66).

1.2.5 Biological therapy

Biological therapies are used in patients with metastatic CRC. KRAS testing is an important molecular marker that has entered routine clinical practice to identify patients with metastatic disease who may benefit from treatment with epidermal growth factor receptor (EGFR) monoclonal antibody e.g. Cetuximab or Panitumumab. Efficacy of EGFR monoclonocal antibody is confined to patients with KRAS wild-type tumours (67). Vascular endothelial growth factor antibody (VEGF), Bevacizumab, has also been associated with promising outcomes in patients with metastatic CRC.

1.3 NF-κB

The initial discovery of the Nuclear Factor kappa B (NF-κB) transcription factor was made three decades ago. Its identification was made in the DNA-binding activity of B lymphocytes where it was found to specifically recognise the gene encoding the immunoglobulin-κ light chain (68). Later Sen and Baltimore reported the inducible nature of NF-κB and its activity outwith the B cell family (69). There are two arms of the pathway: canonical and non-canonical, each has key roles in innate and adaptive immune responses, cell survival and inflammation (70). NF-κB is surrogate for a family of five related Rel-family proteins: NF-κB1 (p50, p105 or p50/p105), NF-κB 2 (p52, p100 or p52/100), p65 (Rel A), c-Rel (Rel) and RelB. These proteins have a common 300 amino acid Rel homology domain (RHD) (71) therefore, once activated, their structural similarities allow formation of homo- and heterodimers, nuclear localisation, DNA binding and association with inhibitor of NF-κB (IκB). There are three structural regions within the Rel homology domain these include the N-terminal domain, dimerisation domain (DD) and nuclear localisation signal (NLS). RelA, c-Rel and RelB possess a C-terminal transcriptional activation domain (TAD), which enables them to activate target gene expression. In comparison, p50 and p52 (derived from p105 and p100 respectively) lack this TAD and are only active once bound to a protein containing a TAD. Following cell surface receptor ligand binding, stimulation of a cell leads to recruitment of adaptors e.g. TRAF (tumour necrosis factor receptor-associated factor), to the cytoplasmic domain of the receptor. In turn, recruitment of IKK complex leads to phosphorylation of the IκB. In the latent cell IκB inhibitory proteins (IκBα, IκBβ, IκBε, IκBγ, IκBζ p100 and p105) remain bound to the DD and NLS of NF-κB proteins which are rendered inactive within the cytoplasm (72–76). IκBs contain multiple copies of a 30-33 amino acid sequence called ankyrin repeats which mediate their interaction with the RHD of NF-κB proteins, masking their NLS and thereby holding them bound inactive within the cytoplasm. Key steps in both arms of the pathway include activation by IκB kinase (IKK) complex resulting in the phosphorylation-induced proteasomal degradation of IκB proteins, allowing dimers to form, enter the nucleus and bind to κB sites in promotor or enhancer regions of target genes (71,77).

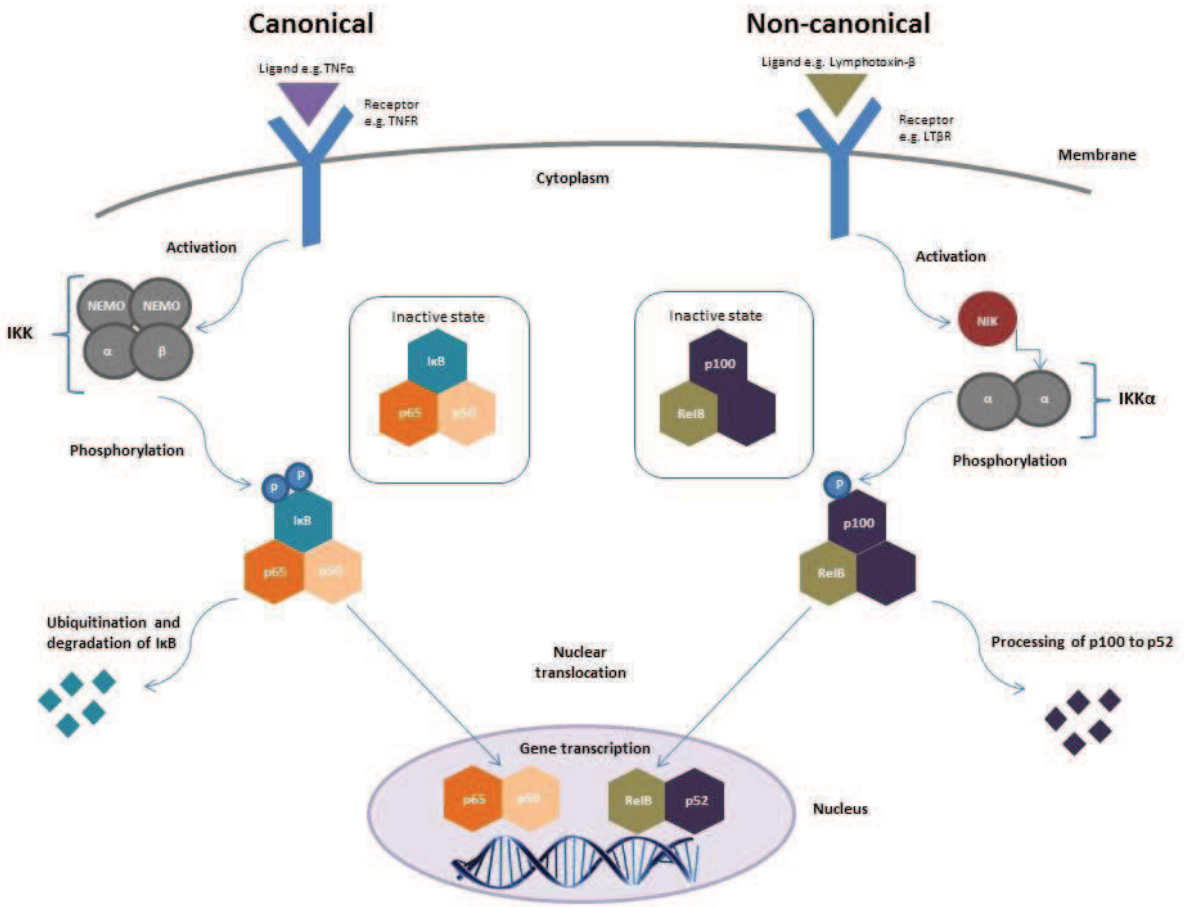


Figure 2: The canonical and non-canonical NF-κB pathways

1.3.1 IKK biology

Activation and regulation of the inhibitor of NF-κB kinase (IKK) complex is tightly regulated and an integral component of NF-κB homeostasis. In the canonical sense, IKKs are responsible for the degradation of IκB inhibitory proteins bound to NF-κB subunits such as p65 allowing movement into the nucleus. IKKα and IKKβ share a 50% identical amino acid sequence (78). The IKKs are regulated via their phosphorylation sites, once phosphorylated, conformational change of the kinase results in subsequent activation. IKKβ is phosphorylated at serines 177 and 181 (79,80). Crosstalk of IKKβ with Src kinase family is demonstrated by the c-Src dependent phosphorylation of Tyr^{188} and Tyr^{199} near the activation loop of IKKβ (81). With respect to the non-canonical NF-κB pathway IKKα is phosphorylated at serine 176 and 180. Autophosphorylation occurs at these sites when IKKα is part of the IKK-β/NEMO complex. NIK phosphorylates IKKα at serine 176 (82). Other proteins are also regulated via IKKα activation. For example, one mechanism of crosstalk between PI3K-Akt and NF-κB pathways is via phosphorylation of Thr23 on IKKα. Both IKKα and IKKβ have an NLS which renders them able to directly phosphorylate proteins at the nuclear level (83). For example, IKKα has been implicated in the induction of NF-κB gene expression via phosphorylation of Ser 10 in Histone 3 (84).

1.3.2 The canonical NF-κB pathway

Canonical NF-κB pathway activity results from stimulation of Toll-like receptors (TLRs), antigen receptors and proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), resulting in the recruitment of the IKK complex composed of subunits IKKα, IKKβ and IKKγ/NEMO (NF-κB essential modulator). The IKK complex is activated by either autophosphorylation (85) or via the IKK kinase, transforming growth factor-β-activated kinase 1 (TAK1) (86). The polyubiquitination of upstream kinase TAK1 results in activation of the IKK complex (86,87) however, the role of TAK1 for IKK activation varies amongst cell types; *in vivo*, TAK1-deficient mice embryonic fibroblasts show diminished levels of NF-κB activity (in response to TLRs, TNF and IL-1) whereas TAK1-deficient B-cells do not (88). IKKβ and NEMO are critical for canonical activity, as demonstrated by early embryonic death in knockout mice (89,90). IKKβ phosphorylates IκB (predominantly IκBα) and unmasks the NLS of p65 bound to p50:p65 (20).Removal of IκBα reveals this NLS, activating p50:p65 dimer translocation to the nucleus.

1.3.3 The non-canonical NF-κB pathway

Non-canonical NF-κB pathway activity results from the stimulation of a specific group of TNF superfamily receptors: lymphotoxin β-receptor (LTβR), BAFFR, RANKL or CD40 (92–96). These receptors are closely associated with TRAF family of proteins which act as mediators in both arms of the NF-κB pathway and are responsible for signal transduction from members of the TNF superfamily receptors, and subsequent activation of downstream kinases. Non-canonical activity arises as a result of NF-κB inducing kinase (NIK) stabalisation. NIK phosphorylates IKKα at serine 176 (82) which induces the phosphorylation dependent ubiquitination of NF-κB2 precursor protein, p100, at its C-terminal region (97). This is a crucial step in the pathway as induction of p100 results not only in the maturation of p52 but also acts as an inhibitor, preventing the translocation of RelB to the cell nucleus (98–100). Within the cytoplasm, p52 forms heterodimers with RelB, which then translocate to the nucleus. The non-canonical NF-κB pathway has key roles in regulating processes including production of lymphoid organs (responsible for B and T lymphocyte production), B-cell development and survival, dendritic cell function and bone metabolism (73,98).

1.3.4 NF-κB homeostasis

NF-κB is an inducible transcription factor and a pivotal regulator of key physiological functions. One such function is regulation of immune and inflammatory responses with NF-κB binding sites in the promoter regions of most genes encoding cytokines and chemokines (101). NF-κB has roles in epithelial tissues such as coordinating antimicrobial immunity and maintaining integrity of the gastrointestinal barrier (102,103). NF-κB also has roles in lymphocyte differentiation (104), cellular responses (105–107) and cell specialisation (108,109). Due to its very nature, NF-κB is tightly controlled by a negative feedback mechanism and therefore has anti-inflammatory as well as pro-inflammatory activity (110). Canonical NF-κB activity is very much dependent on the degradation of IκB, equally, IκB genes (α and ε) are under the influence of NF-κB activity and function as negative regulators that suspend the NF-κB response (111–113). Other regulatory mechanisms are upstream of the IKKs and each immunoreceptor uses specific complexes composed of adaptor molecules, ubiquitin ligases and protein kinases for context-dependent control of IKK. For example, TNF induced canonical activity is mediated via recruitment of TRADD, TRAF2, cIAP1 and RIP1 to the receptor and once IKK phosphorylates IκBα, this triggers K48-linked polyubiquitination and degradation of the proteasome (114).

NIK has a key role in the regulation on the non-canonical pathway. In the unstimulated cell, constant degradation of NIK occurs as a results of NIK/TRAF3 binding and thus preventing the processing of p100 (115). Furthermore, knockout of TRAF3 leads to the accumulation of NIK and constitutive processing of p100, demonstrating the important regulatory role of TRAF3 in non-canonical NF-κB activity (116). In the resting state, NIK stability is regulated by TRAF3, TRAF2, cIAP1 and cIAP2. In addition to IKKα induced phosphorylation of p100, IKKα also phosphorylates NIK to promote its destabilisation as a negative feedback mechanism (114).

1.3.5 NF-κB crosstalk

There is evidence of crosstalk between the canonical and non-canonical arms of NF-κB signalling. This is demonstrated by evidence of raised NIK levels in NEMO-deficient cells and similarly in IKKβ and p65 deficient mouse embryonic fibroblasts (MEFs) (117). Further studies demonstrate this crosstalk via the formation of a RelB:p50 dimer in the regulation of dendritic cell maturation (118). The complexity of this crosstalk is demonstrated by evidence which suggests IKKα inhibits the canonical NF-κB pathway by phosphorylating the scaffold protein TAX1BP1 and, subsequent activity of deubiquitinase A20 and termination of canonical NF-κB signalling (119). NF-κB interacts with other signalling pathways such as signal transduction activator of transcription-3 (STAT3) and hypoxia-inducible factor (HIF) pathway. There is also crosstalk between NF-κB, PI3K-Akt pathway (120) and mitogen activated protein kinase (MAPK) signalling pathway (121). In addition, IKK kinases have a number of roles independent of NF-κB signalling (11) e.g. Histone H3 phosphorylation (84), SMRT (83), suppression of tumour suppressor/anti-metastasis gene maspin (122) and destabilising p53 (123).

1.3.6 NF-κB and cancer

The link between NF-κB and cancer initially came from the idea that many cancers arise at sites of infection or chronic inflammation. NF-κB activation is implicated in each of the described hallmarks of cancer: proliferation, evasion of apoptosis and anti-growth signals, angiogenesis, invasion and metastasis, self-sufficiency in growth signals and immune responses within the tumour microenvironment (25,124).

NF-κB bridges the link between inflammation, immunity and cancer. c-Rel is a homolog of the retroviral oncogene v-Rel, it codes for an NF-κB subunit and provided one of the first links between NF-κB and carcinogenesis (71). NF-κB signalling influences a large number of genes which means it is involved in most aspects of tumorigenesis (124). Haematological malignancies are associated with mutations affecting components of the NF-κB signalling pathway (125–128) leading to the constitutive activation of both canonical and non-canonical arms however, these mutations are extremely rare in epithelial carcinomas. The protumourigenic activity of NF-κB can occur via canonical or non-canonical signalling (129,130). NF-κB influences important tumour supportive processes such as insensitivity to growth inhibitory signals, evading apoptosis, proliferation, angiogenesis and metastasis (131,132). It has also been implicated in chemo- and radio-resistance (133,134). Cellular proliferation is a hallmark of cancer (132), NF-κB target genes regulating promotion of cell growth include cyclin D1, cyclin E, CDK2 and c-Myc as well as growth signals GM-CSF and IL-6. NF-κB can inhibit apoptosis and thus prevent elimination of neoplastic cells. Such genes include Bcl2 members such as Bcl-x_L and IAPs (inhibitors of apoptosis) (124,135). NF-κB regulates angiogenesis through its target VEGF, as well as through NF-κB regulated chemokines such as IL-8 (136), CXCL 1 and 8 (137). Activation of the canonical pathway has been observed in a number of cancers including colorectal, breast, lung, pancreatic, oesophageal cancer and melanoma. The non-canonical pathway has been investigated in the context of epithelial cancers albeit to a lesser extent.

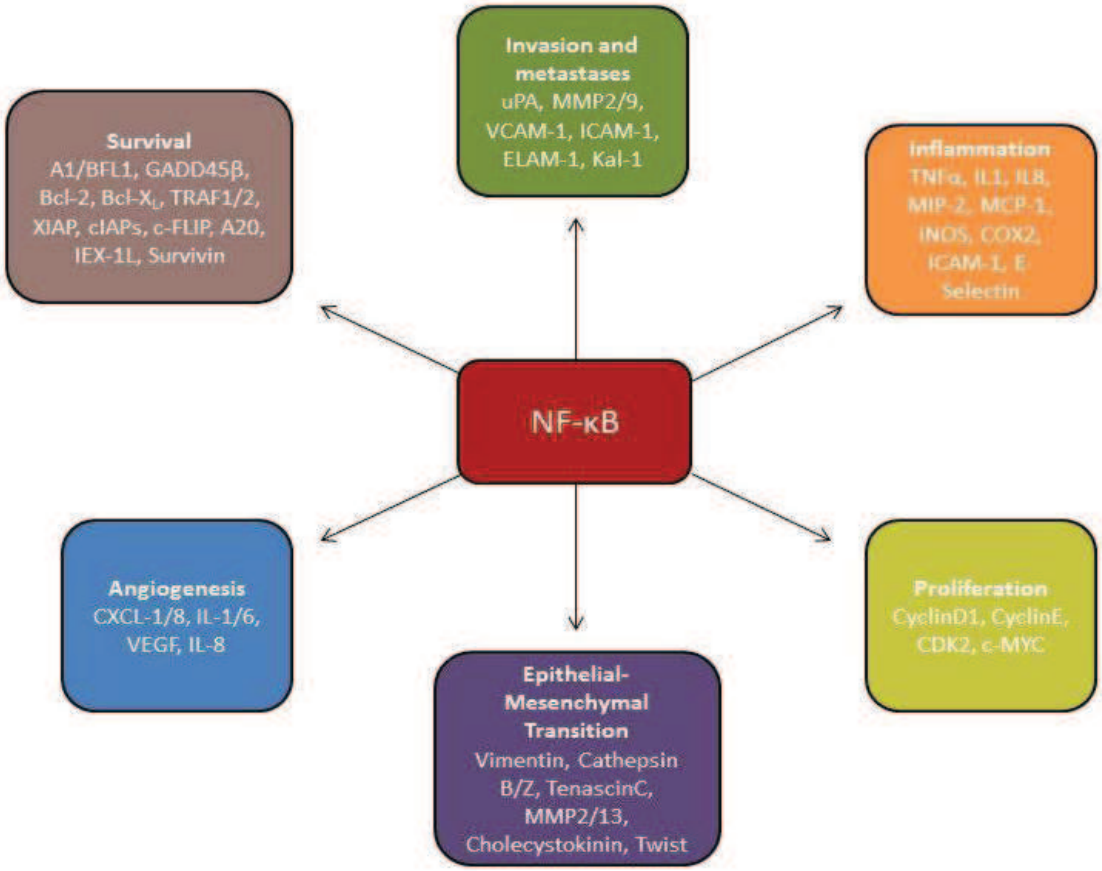


Figure 3. NF-κB-dependent target genes involved in different aspects of tumourigenesis. *Adapted from Bassères & Baldwin* (137).

1.4 NF-κB and colorectal cancer

Unlike haematological malignancies, no activating mutations of NF-κB in CRC have been reported (138) however, constitutive activation of NF-κB has been observed (139–142) and is associated with higher tumour stage (141,143), treatment resistance (133,134,140,144,145) and poor survival outcomes (146). As activation of NF-κB pathways has been associated with poor prognosis it is possible that members of the NF-κB pathways could be employed as prognostic markers or indeed novel therapeutic targets for CRC. The role of NF-κB in the transition of pre-malignant polyp to invasive carcinoma is unclear.

The relationship between the NF-κB pathway, inflammation and cancer has been widely reviewed (124,138,147). Much of the initial research linking NF-κB and CRC focused on chronic inflammation, was performed in mouse models of colitis and importantly demonstrated NF-κB functions are cell-type and tumour-type specific (129,148).

1.4.1 Proliferation and evading apoptosis/antigrowth signalling

1.4.1.1 Colorectal polys

Polyps are recognised as precursors to CRC. They are classified into either adenomas or serrated/hyperplastic polyps, both with malignant potential. Vogelstein and colleagues have detailed the stepwise accumulation of molecular alterations that accompany the adenoma-carcinoma sequence (chromosomal instability pathway) which sees transformation through increasing grades of adenomatous dysplasia to the development of invasive carcinoma (40). The role of NF-κB in adenoma formation in the context of colitis-associated cancer has been studied in mouse models that suggest NF-κB is implicated in the early stages of adenoma formation. However, there are no known studies that have mapped NF-κB expression in human tissue with respect to adenoma-carcinoma transformation. Nonetheless, a hybrid mouse model with intestinal epithelial cell-specific allelic deletion of APC and constitutive expression of IKKβ, displayed increase colonic adenoma formation than their APC allele deleted only counterpart. This study reported the hybrid model expressing IKKβ mostly increased adenoma number but did not increase the size of the adenoma suggesting IKKβ is implicated in adenoma initiation or early establishment (149). Serrated/hyperplastic polyps are considered as developing via distinct mechanism to that described by Vogelstein and it would be of interest to study NF-κB in

this context. If confirmed in human studies NF-κB could be employed to clinically identify which polyps have malignant potential.

The anti-apoptotic activity of NF-κB is mediated via Bcl2, Bcl-x_L, cFLIP, cIAP2, amongst other genes (124). Anti-apoptotic protein BAG-1 (Bcl-2-assocaited athanogene-1) is involved in key processes such as proliferation, cell signalling, transcription and apoptosis (150). Overexpression of BAG-1 has been reported in colorectal adenomas and carcinomas. The same study reported knockdown of BAG-1 in colon cancer cell lines inhibited NF-κB transcriptional activity (151). A separate study reported this process is regulated by antigrowth protein/tumour suppressor Rb (retinoblastoma) (152). Inhibition of NF-κB with BAG-1 siRNA/inhibitor of NF-κB suppressed cell yield and induced apoptosis. This study concluded inhibition of NF-κB and therefore suppression of BAG-1 represents a novel therapeutic strategy in CRC (151).

1.4.1.2 Colitis-associated cancer

The first genetic evidence linking canonical NF-κB activity, inflammation and CRC was from a mouse model of colitis-associated cancer where deletion of IKKβ in intestinal epithelial cells (IECs) and myeloid cells had distinct outcomes; deletion of IKKβ in IECs showed reduced adenoma incidence which was not related to levels of inflammation but had a direct effect on tumour promotion, as demonstrated by reduction in anti-apoptotic Bcl-2 protein, Bcl-xL. In contrast, deletion of IKKβ in myeloid cells was associated with a less marked reduction in adenoma incidence but was associated with reduced adenoma size, with reduced expression of genes encoding pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα, with no effect on apoptosis (129). These results suggest IKKβ mediated NF-κB activity has cell-specific roles in the development of colitis-associated cancer. Importantly, this study demonstrates the early role of NF-κB in the development of pre-cancerous adenomas which could be exploited clinically as a potential target. In mouse models of colitis, IKKβ inhibitors have been observed to lead to smaller and less frequent tumour formation and may offer a novel mode of early intervention (153).

When reviewed as a whole this evidence supports the hypothesis that there is a relationship between inflammation, NF-κB activity and tumourigenesis and indeed that NF-κB has a role in directly promoting tumourigenesis in colitis-associated cancer.

1.4.1.3 Metastatic CRC

The relationship between NF-κB and inflammation induced tumourigenesis was investigated in a metastatic colon cancer mouse model where it was observed that injection with bacterial lipopolysaccharide (LPS) resulted in metastatic tumour growth via inflammatory mediator TNFα. Intraperitoneal injection with colon cells transfected with mutant IκBα reduced tumour burden and improved murine survival. *In vitro*, this inhibition of NF-κB resulted in a cytocidal effect mediated by TRAIL (TNF-related apoptosis-inducing ligand) (154). Observations from this study further support the link between NF-κB induced inflammation and growth of malignant cells in colon cancer.

Human colon cancer stem cells have been identified (155,156) and can promote tumour formation and metastases *in vivo* (157,158). Signalling pathways implicated in this process include IL-6/STAT3 (157), activation of AMPK/mTOR and NF-κB (158). In a genetic model with restricted WNT-activation, ablation of NF-κB (p65) restricted intestinal crypt stem cell expansion. Moreover, enhanced NF-κB activity increased Wnt activation and induced dedifferentiation of non-stem cells that acquire tumour-initiating capacity (159). Altogether, these studies support the role of NF-κB and indeed other closely linked signalling pathways such as STAT3 in colon cancer stem cells implicated in tumourigenesis. Moreover, they highlight the need for further studies to investigate the effect of inhibiting NF-κB/STAT3 on tumour growth in the context of cancer stem cells to fully understand how these pathways could be exploited clinically as therapeutic targets.

Evasion of apoptosis is a hallmark of cancer. When proliferating cancer cells encounter hostile conditions e.g. hypoxia or insufficiency in nutrients, there are regulatory mechanisms that kick in. These include cell cycle arrest, apoptosis and autophagy. These processes are under the influence of tumour suppressor genes such as p53 (160). The p53 gene is mutated in 40-60% of CRCs (161). Whilst mutation of p53 is considered an important step in colorectal pathogenesis, studies investigating its prognostic and predictive capacity have reported conflicting results (42). However, the relationship between NF-κB and p53 and its importance in the development and progression of cancer is well documented (132). A drug- and cytokine-induced p53/NF-κB crosstalk has been reported in colorectal cancer cells. Furthermore, TNFα induced NF-κB target genes in colorectal cancer cells are dependent on this p53/NF-κB interaction (162). NF-κB is essential in p53-mediated cell death (163,164) and mutant p53 prolonged activation of NF-κB in cultured cells and was associated with increased susceptibility to colitis-associated

CRC *in vivo* (165). Histone deacetylases (HDACs) are a family of proteins, characterised into four classes and dictate epigenetic modulation and gene expression. HDAC upregulation has been observed in CRC and is associated with cell proliferation, survival and inhibition of cell differentiation. HDAC inhibitors have shown promising results in the context of CRC both *in vitro* and *in vivo*. The chemotherapeutic hydroxyurea co-induced p53 and NF-κB-dependent gene expression in colon cancer cell lines; using a specific class I HDAC inhibitor, this study also reported class I HDACs are required for the hydroxyurea-induced crosstalk between p53 and NF-κB (166). A further study has reported that following genotoxic stress, HDAC2 regulates NF-κB gene expression and apoptosis. Moreover, knockdown of p53 diminished induction of NF-κB gene expression in colon cancer cells. Inhibition of NF-κB/p53 regulated protein survivin, significantly sensitised colon cancer cells expressing wild-type HDAC2 to doxorubicin induced apoptosis (167). Altogether this work highlights the importance of NF-κB/p53 crosstalk in colon cancer and more recently, the role of HDAC proteins in mediating this relationship and revealing itself as a potential therapeutic strategy in colon cancer.

Fibronectin is an extracellular glycoprotein which plays a role in cell adhesion, growth, migration, differentiation, inflammatory cell activity and tumour angiogenesis (168–171) and is highly expressed in CRC cell lines. Silencing of fibronectin increased apoptosis-related gene products caspase-3, p53, PARP, Bax and cytochrome *c in vivo* but decreased levels of NF-κB suggesting a relationship between fibronectin and the NF-κB/p53 signalling pathway (172). This provides further evidence that NF-κB plays a central role in regulating cell death in the development and progression of CRC. Altogether, there is substantial evidence which shows NF-κB plays a diverse role in processes relating to proliferation, apoptosis and antigrowth signalling at all stages of CRC development i.e. from adenoma through to metastatic carcinoma and therefore offers exciting opportunities that could be exploited clinically.

1.4.2 Angiogenesis

Angiogenesis is a hallmark of cancer driven by VEGF. In addition to VEGF, CXCL1, CXCL8, IL-8 and COX-2 are also angiogenic regulators under the influence of NF-κB (137). Monoclonal antibodies against VEGF have been used to treat metastatic CRC for some time. VEGF is also inducible under hypoxic conditions by hypoxia-inducible factor (HIF). There are three HIF-α proteins: HIF-1α, HIF-2α and HIF-3α (regulator). The HIF pathway regulates cellular responses to hypoxia and also has a role in regulating

inflammation and immune responses via cross-talk with NF-κB (173). The importance of this crosstalk was demonstrated by a group who confirmed HIF-1α inhibition by the NF-κB inhibitor parthenolide lead to downregulation of hypoxia-dependent angiogenesis in HUVECs (human umbilical vein endothelial cells), reduction in HIF-1α target gene proteins and inhibition of hypoxia induced epithelial-mesenchymal transition (EMT). Furthermore, parthenolide treatment inhibited tumour growth, angiogenesis and progression in CRC xenograft models (174). Knock down of NEMO in CRC cell lines resulted in greater TNFα induced apoptosis and reduced expression of angiogenic factors IL-8, growth-regulated alpha protein (Groα) and monocyte chemoattractant protein 1 (MCP-1). *In vivo*, this knockdown resulted in reduced angiogenesis, tumour volumes, serum and tumour IL-8 expression and improved tumour regression when treated with fluorouracil (139,175). In CRC tissue, expression of NF-κB (p65) associates directly with expression of HIF-1α, VEGF and histological evidence of vascular invasion (142,176).

These studies support the role of NF-κB, angiogenesis and tumour progression in CRC and highlight the importance of understanding crosstalk with other pathways to develop targeted therapies, particularly in those patients who may not respond to or experience toxicity with the available anti-VEGF therapy.

1.4.3 Metastasis and self-sufficiency in growth signals

Unsurprisingly, NF-κB is involved in EMT of tumour cells. EMT results in epithelial cells acquiring the invasive and metastatic properties of mesenchymal cells and thus are important in promoting cancer metastasis (177).

Chemokines have been implicated in tumour metastases. Colon cells transfected with chemokine CXCL8 DNA displayed higher migration rate and EMT-like phenotype with increased expression of EMT markers N-cadherin, Vimentin and α-SMA as well as activation of PI3K/Akt/NF-κB pathway. CXCL8 transfected mice displayed more rapid tumour growth compared to controls (178).

Olfactomedins are a family of proteins that mediate development of the nervous system and haematopoiesis. Olfactomedin 4 (OLFM4) is an intestinal stem cell marker and a target of the Wnt/β-catenin pathway. A recent study reported OLFM4 as a negative regulator of Wnt/β-catenin and NF-κB pathways with inhibition of colon cancer development in APC mutated mice (179). Olfactomedin 1 (OLFM1) also has a tumour

suppressive role and is able to suppress growth, migration and invasion of CRC cells *in vitro*. Additionally, OLFM1 negatively regulated non-canonical NF-κB activity of NIK. Knockdown of OLFM1 promoted growth and metastasis of CRC cells *in vivo*. Low expression of OLFM1 in CRC tissue was associated with lymph node involvement, distant metastases and poor overall survival (180).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that have a physiological role in tissue remodelling and as such are able to degrade the extracellular matrix and facilitate tumour invasion. Overexpression of MMP 1, 2, 3, 7, 9 and 13 has been observed in human CRC (181) and was associated with poor prognosis and metastasis (182). The expression of MMP-9 is regulated by several transcription factors including NF-κB (183,184). The mechanistic link between NF-κB and MMP-9 was studied in CRC cells lacking the β subunit of the IKK complex and this showed NF-κB (IKK activity and p65) was required for TNFα induced MMP-9 gene expression (185).

NIK- and IKKβ-binding protein (NIBP) have been implicated in the regulation of cytokine-induced canonical NF-κB signalling. NIBP was over expressed in CRC tissue and was associated with metastasis (186,187). Nude mice injected with NIBP knockdown cells demonstrated less tumour formation, with no detectable tumour at 3 months (188). Expression of NIBP in 114 patient tissue samples of CRC was associated with tumour metastasis. The same study demonstrated NIBP overexpression induces activation of the canonical NF-κB pathway *in vitro.* Additionally, xenografts of NIBP-overexpressing cells generated liver metastases with increased expression of p65, MMP-2 and MMP-9 suggesting NIBP may increase CRC metastases via canonical NF-κB activity and upregulation of MMP-2 and MMP-9 (189).

Cytoskeletal proteins Fascin and Ezrin have also been implicated in EMT and metastasis via NF-κB activity (190,191).

Whilst the benefits of an adaptive immune response in the CRC tumour microenvironment has been widely demonstrated and now underpins therapeutic manipulation, the innate immune response is less understood. Specifically, the role of tumour-associated macrophages (TAMs) in CRC is controversial and the mechanisms behind phenotypical skewing towards either M1-like (pro-inflammatory/anti-tumour Th1 response) or M2-like (anti-inflammatory/pro-tumour Th2 response) response is incompletely understood (192). In a study of knock in mice expressing a dominant negative form of IKKα, treatment with

carcinogen resulted in markedly reduced adenoma formation at 20 weeks, with smaller tumours and slower proliferation rates. This was associated with increased recruitment of M1 (tumouricidal) -like myeloid cells into the tumour, which was not cell-autonomous but depended on interaction between mutant IKKα epithelial and immune cells (193). In an interesting study using a model of peritoneal metastasis in immune-competent mice, intraperitoneal injection with IκBα-suppressed colon cells induced an M1-like macrophage phenotype, with reduced liver and peritoneal metastases *in vivo*. This was associated with increased intratumoural activated $CD4^+$ and $CD8^+$ T cells and reduced angiogenesis (194). This study suggests targeting NF-κB could induce a phenotypic switch from M2-like immunosuppressive to an M1-anti-tumour macrophage in CRC.

Activin A is a member of the TGFβ superfamily and has been reported to have a role in inflammatory responses (195). Activin A is overexpressed in human colorectal tumours, especially in stage IV disease suggesting activin A may have a role in advanced CRC (196). Signalling pathways for activin and TGFβ are frequently disrupted in CRC (197). Activin downregulates p21 and increases migration and invasion of colon cancer cells (198). The link between NF-κB signalling and activin ligand expression was investigated by the same group who reported activin, but not TGFβ, induced NF-κB activation with subsequent increased MDM2 ubiquitin ligase and degradation of p21 via PI3K dependent mechanism. Further to this, a functional role for NF-κB in activin-induced colon cancer cell migration was reported (199).

Altogether, these findings further implicate NF-κB in important cancer processes such as EMT, autonomous growth signalling and regulation of the tumour microenvironment.

1.4.4 NF-κB crosstalk with other signalling pathways in CRC

There is evidence of cross talk between canonical and non-canonical NF-κB pathways (200,201), NF-κB and a number of other pathways, as well as studies demonstrating the ability of IKK kinases to directly target substrates in an NF-κB independent manner.

IL-1 can stimulate PI3K/Akt-dependent activation of NF-κB (p65), independent of the classical NF-κB pathway (202,203). In non-colon cells, oncogenic Ras required P13K and Akt to stimulate NF-κB-dependent transcription by targeting the transactivation domain of p65 rather than via classical degradation of IκB and subsequent translocation of p65 to the nucleus, with resulting reduced apoptosis (204). Loss of APC function and derangement of

Wnt/β-catenin signalling have been firmly implicated in the development of CRC (205). It has been reported that IKKα and IKKβ interacted with and were able to phosphorylate β-catenin, with IKKα specifically able to increase β-catenin-dependent gene expression in colon cancer cell lines (206). A separate study reported constitutive IKKβ expression in mouse IECs induced spontaneous tumour formation, enhanced chemical- and APC mutation-mediated carcinogenesis and contrary to previous reports was associated with increased expression of β-catenin (207). Furthermore, in human CRC tissue, it has been reported that PI3K/Akt/IKKα pathway regulates NF-κB and β-catenin with the ability to influence transcription of genes implicated in angiogenesis and metastasis (208). When this evidence is considered as a whole it strongly supports the hypothesis that there is an interaction between the β-catenin, PI3/AKT and NF-κB pathways in CRC development and progression. However, it is unclear whether IKKβ interacts with Wnt/β-catenin signalling in an NF-κB-dependent or independent fashion (209).

The NF-κB and STAT3 interaction is important in orchestrating the immune and inflammatory response within the tumour microenvironment and each has distinct functions in immune and cancer cells (210). NF-κB and STAT3 regulate common processes and share regulatory binding sites of anti-apoptotic, cell cycle and proliferation, tissue resistance and repair genes, in addition to genes regulating angiogenesis/responses to hypoxia, chemokine and cytokine expression (211). As well as having distinct and overlapping target genes, as demonstrated by the pattern of their respective gene binding sites (212,213), NF-κB and STAT3 have been shown to physically interact (212,214) with STAT3 causing the nuclear retention of NF-κB; STAT3 can prolong nuclear retention of phosphorylated p65 (215). Co-localisation of STAT3 and NF-κB has been observed in CRC cell lines (216). MicroRNAs (miRNAs) function to suppress gene expression and have been reported to regulate NF-κB activity (217,218). In CRC cell lines transfected with microRNA mimics/inhibitors, miRNA-221 and miRNA-222 activated NF-κB (p65) and STAT3 with associated increase in miRNA-221 and miRNA-222 suggesting these mRNAs function in a positive feedback regulatory loop to increase NF-κB and STAT3 activity. STAT3 activation is mediated by the tyrosine kinase JAK1 as a result of cytokine activity e.g. Interluekin-6 (IL-6). IL-6 is produced in an NF-κB dependent manner within myeloid cells in a model of colitis-associated cancer. The pro-tumourigenic effects of IL-6 were mediated by STAT3 (219). STAT3 activity is found in epithelial and lymphocytic cells; inhibition of STAT3 in colon cancer cells curbs tumour cell proliferation in xenograft models of CRC (220). In CRC tissue, expression of STAT3 is associated with downregulation of the local adaptive immune response and decreased cancer-specific

survival (221). No activating mutations in NF-κB or STAT3 have been detected in CRC suggesting they are secreted in a paracrine or autocrine fashion (222).

Adaptive immunity is important in cancer immunosurveillance (223) and in CRC, the presence of tumour infiltrating immune cells (Th1 derivatives) have shown prognostic value independent of the widely used UICC-TNM classification (28). In contrast, high expression of T helper cell 17 (Th17) cells in CRC was associated with poor prognosis (224). There are a number of studies based on inflammation-associated colon cancer models that implicate inflammatory cell-derived cytokines (IL-17A, IL-22, IL-6) in tumourigenesis (219,225,226). A study investigating the immune/inflammatory infiltrate and cytokine response in sporadic CRC found no differences in immune cell composition between uninvolved mucosa and tumour but did observe that this transition is marked by a functional switch in T cells leading to the accumulation of Th17-related cytokines, TNFα and IL-6. Additionally, tumour-infiltrating lymphocyte-derived supernatant induced proliferation of colon cancer cell lines with activation and co-localisation of NF-κB and STAT3. These findings were confirmed in a mouse model of CRC. Interestingly, this study also observed that administration of a compound targeting STAT3/NF-κB activation and crosstalk, reduced levels of STAT3/NF-κB-activating cytokines and tumour growth (216). This study highlights the importance of understanding the relationship between immune cell subtypes and cytokines and their influence on intracellular signalling pathways in sporadic CRC as well as highlighting STAT3/NF-κB crosstalk as a novel therapeutic target in CRC. Moreover, it highlights a deficiency in studies of NF-κB in models of sporadic CRC.

ERK5 and MEK5 overexpression has been reported in human adenomas and carcinomas and ERK5 expression correlated with NF-κB. Colon cells with over activated ERK5 displayed increased NF-κB nuclear translocation and transcriptional activity; orthoptically implanted tumours with over activated MEK5/ERK5 had greater lymph node metastasis, altogether suggesting MEK5/ERK/NF-κB signalling is implicated in tumourigenesis and metastasis (227).

As discussed previously the HIF pathways regulate cellular response to hypoxia and also have a role in regulating inflammation and immune responses via crosstalk with NF-κB. HIF and NF-κB share common activating stimuli, regulators and targets (173,228); Both are activated in a TAK1-dependent manner (229) and IKKβ deficient mice demonstrate defective HIF expression and induction of target genes including VEGF (228). High

expression of HIF-2α in CRC tissue is associated with increasing tumour stage, poor tumour differentiation, lymphovascular invasion, COX-2 expression and worse overall survival (230).

1.4.5 NF-κB independent roles of IKKα

The study of IKKα in CRC has provided insights into its NF-κB independent functions and highlight it as an important player in colorectal carcinogenesis. Constitutive activation of IKKα resulted in phosphorylation of SMRT at serine 2410 in CRC tissue. Furthermore, IKKα binds to Notch-dependent gene promoters with resultant release of chromatin-bound SMRT and upregulation of Notch-dependent gene and anti-apoptotic *cIAP2* upregulation (231). Inhibition of IKKα restored SMRT chromatin binding with inhibition of Notch-dependent gene transcription and subsequent reduction in tumour size in a CRC xenograft model (231). The same group have reported a truncated isoform of IKKα with the predicted molecular weight of 45kda generated by cathepsin-mediated cleavage of full length IKKα (FL-IKKα) within early endosomes. Nuclear truncated IKKα (p45-IKKα) forms a complex with full length IKKα and NEMO and is responsible for regulating phosphorylation of SMRT and histone H3. This study provides evidence that p45-IKKα resulted in diminished apoptosis *in vitro* and was required for CRC tumour growth *in vivo* (232). More recently, the same group demonstrated that KRAS and mutant $BRAF^{V600E}$ cells are different; whilst KRAS was able to induce canonical NF-κB signalling, $BRAF^{V600E}$ did not have the same effect. $BRAF^{V600E}$ was required for p45-IKKα phosphorylation in a TAK1 dependent but NF-κB independent fashion. Additionally, endosomal inhibition reduced proliferation of $BRAF^{V600E}$ cells in culture as well as tumour growth and metastasis in xenograft models of CRC. The addition of an endosomal inhibitor appeared to potentiate the effect of Irinotecan in this model. FL-IKKα, NEMO, TAK1 and BRAF were associated with the endosomal compartment however the nature of this association is unclear (233).

Whilst there is sufficient evidence gained from cell line and animal work relating to crosstalk between IKKα and other signalling pathways in the development and progression of CRC, there is limited evidence that this is clinically important due to lack of studies investigating NF-κB in patient specimens and how this may relate to phenotypic tumour characteristics and patient outcomes.

1.4.6 NF-κB and therapeutics in colorectal cancer

In 2005, Gilmore and Hersovitch reported over 750 inhibitors of the NF-κB pathway including a variety of natural and synthetic molecules (77).

COX-2 is a target gene of NF-κB (234) and is responsible for prostaglandin synthesis during inflammation. COX-2 is overexpressed in CRC (235), has been directly linked to colorectal tumourigenesis (236,237) and underpins the basis of numerous studies investigating the chemoprophylactic role of non-steroidal anti-inflammatory drugs (NSAIDs) such as Aspirin and selective COX-2 inhibitors (238). The mechanism of COX-2 induction is not fully understood however it has been reported that *in vitro* inhibition of NF-κB can reduce COX-2 expression in colorectal cancer cells. Additionally, it has been reported that upregulation of COX-2 is associated with increased expression of p65, p50 and IKKα in human colorectal cancer tissue, altogether suggesting a relationship between NF-κB signalling and COX-2 expression (239–241). Epidemiological data has demonstrated the protection conferred by Aspirin against CRC (242). Further studies have reported reduced risk of colorectal adenomas with regular Aspirin use (243–246). Use of daily Aspirin was associated with reduction in incidence of colorectal adenomas in patients with history of adenomas or previous CRC (247). There are a number of active clinical trials aimed at determining the risk-benefit profile of Aspirin as a chemopreventive agent. NF-κB plays a central role in anti-apoptotic NSAID activity in CRC cells *in vitro* (248–250). Both Aspirin and Sulindac inhibit IKKβ (248,251) and Aspirin, specifically, is able to induce phosphorylation and proteosomal-mediated degradation of IκBα and nuclear translocation of p65 (250). Although Aspirin is known as an inhibitor of NF-κB, in xenograft models of human CRC, Aspirin increased levels of phosphorylated IκBα and nuclear p65 with associated apoptotic effect (252). These apoptotic effects were also reported in colon cells *in vitro*, independent of p53 or MMR status (253). It is evident that part of the chemoprophylactic nature of Aspirin in CRC can be attributed to NF-κB activity.

Curcumin is a polyphenol derived from the spice turmeric and is active against a number of cell signalling pathways including NF-κB. In TNF stimulated cells treated with curcumin, one study reported absence of NF-κB activity by inhibition of IκBα phosphorylation and translocation of p65 to the nucleus (254). *In vitro*, curcumin inhibited COX-2 by inhibition of NIK/IKK signalling and thus prevented phosphorylation of IκB in human colon cells (239). Curcumin has been and continues to be studied extensively. It has been reported to reduced colonic tumourigenesis in animal models (255,256) and to favour

polarisation of macrophages toward an anti-tumour MI-like phenotype in xenograft models of colon cancer (256). NF-κB activity has been implicated in chemo- and radio-resistance (133,134) and curcumin has been reported to overcome this by blocking NF-κB activity (144). The combination of curcumin and chemotherapeutic agent capecitabine effectively reduced tumour volume, proliferation and microvessel density *in vivo* when compared to controls, with associated suppression of NF-κB-regulated gene products (145). In a separate study, curcumin was able to overcome oxaliplatin resistance via inhibition of NF-κB, *in vitro* (239). Later, the efficacious combination of curcumin and oxaliplatin was observed in xenograft models of CRC (257). The same group are currently investigating the combination of curcumin with FOLFOX chemotherapy within a phase II clinical trial of patients with metastatic CRC (258).

For classical NF-κB activity, the proteasome plays an important role in degradation of IκB inhibitory protein. Proteosome inhibitor Bortezomib is used to treat multiple myeloma and has been investigated in CRC. Leucovorin is often given in combination with 5-fluorouracil (5-FU) to treat metastatic CRC. Treatment of CRC cells with leucovorin and Bortezomib enhanced caspase activity and apoptosis more effectively than either agent alone; these findings were confirmed in mouse CRC xenografts (259). Bortezomib has demonstrated limited efficacy in combination with standard treatment in clinical trials of patients with metastatic CRC (260,261).

IKKα inhibitors

NF-κB activity interferes with the efficacy of chemotherapeutic agents through induction of anti-apoptotic genes. There is no doubt that inhibiting IKK kinases suppresses CRC tumour growth *in vivo* as well as enhancing sensitivity to 5-FU (153,262,263). A number of IKK inhibitors have been reported in the literature but none have made it into clinical practice. Until now tools to unpick the exact nature of NF-κB inhibition have been limited due to the broad spectrum and non-specific nature of inhibitors. However, it has now been reported that first-in-class IKKα specific inhibitors are available (264). These IKKα-selective inhibitors have been developed by a team at the University of Strathclyde and have shown promising results in prostate and pancreatic cancer, they will be investigated in colon cancer cell lines later in this thesis.

1.4.7 NF-κB as a biomarker in CRC

Activation of NF-κB has been observed in response to chemo- and radiotherapy (265–267). Much of the data relating to NF-κB as a predictor of treatment resistance in CRC is preclinical (268–273). In these studies, aberrant NF-κB activity has been implicated in resistance to drugs used in the treatment of CRC such as 5-FU, oxaliplatin and irinotecan. Irinotecan is used for the treatment of metastatic CRC. However, many patients will develop treatment resistance and disease progression which has been attributed to irinotecan-mediated NF-κB activation (272). The addition of an inhibitor of IKKα to mouse xenograft models of colon cancer potentiated the antitumoural effect of irinotecan and increased the sensitivity of colon cells to 5-FU *in vitro* (263). Within a clinical trial of patients with irinotecan-refractory metastatic CRC treated with cetuximab and irinotecan, patients with tumours that expressed NF-κB (p65) had inferior response rates and overall survival compared to those patients with tumours that did not express NF-κB (133). This is one of the only clinical studies that has evaluated NF-κB in a predictive capacity.

Overexpression of p65 in CRC tissue was associated with increasing tumour stage (141,143) and poor overall survival (274). A study investigating the prognostic significance of NF-κB, HIF-1α and VEGF expression in 148 patients who had undergone potentially curative resection for stage III CRC found that NF-κB expression was an independent predictor of overall survival. Additionally, the 56 patients who relapsed had inferior response rates (and overall survival) to palliative chemotherapy (176).

Patients with metastatic CRC undergo testing to determine KRAS status; this practice is widely established and harbouring the KRAS mutation precludes treatment with anti-EGFR monoclonal antibody. NF-κB can be activated through the RAS-RAF signalling pathway. Expression of oncogenic KRAS has been reported to result in NF-κB activation (275–277). Knockdown of KRAS reduces expression of p65 and phosphorylated-IκBα in CRC cell lines (278). Studies have reported higher expression of NF-κB (p65) in CRC tissue of patients with KRAS mutation than those without. NF-κB activation in the presence of KRAS mutation was associated with inferior overall survival in stage I-IV disease and reduced response to chemotherapy in patients with metastatic disease (279). However, in a study of patients with KRAS wild-type metastatic CRC treated with irinotecan and cetuximab, 65% had tumours expressing p65 and this was associated with poorer progression-free and overall survival than those patients with NF-κB negative

tumours, suggesting NF-κB expression is prognostic irrespective of KRAS mutational status.

A study of 22 patients investigating the relationship between radiotherapy for rectal cancer, NF-κB activation and treatment response reported that NF-κB target genes were upregulated in response to a single fraction of radiotherapy however, whilst expression of NF-κB subunit p50 was prognostic for overall survival, it was not predictive of pathological response to radiotherapy (280).

1.5 Chapter conclusions

This chapter summarises evidence from pre-clinical, translational and clinical studies that have reported on NF-κB with respect to the development and progression of CRC. There is substantial evidence implicating NF-κB in all stages of CRC development, from early adenoma to invasive cancer and metastasis. The role of NF-κB in CRC is undoubtedly complex and this complexity is enhanced by the crosstalk of NF-κB with a multitude of signalling pathways and regulators. Therefore, together with its essential role in normal cellular physiology it is unsurprising that inhibitors of NF-κB have yet to successfully emerge into clinical practice.

There is a growing body of evidence from studies in cell lines and animals implicating NF-κB activity in the development, progression and treatment resistance of CRC. Many studies focus on the measurement of p65 activity/expression as a measure of NF-κB activity which reflects the canonical pathway. Non-canonical NF-κB activity is largely underexplored. Importantly, there is a lack of studies in human tissue and therefore in understanding the relationship between NF-κB and phenotypic tumour characteristics. It would also be prudent to understand the role of NF-κB activity in the development of pre-malignant polyps and their transformation into invasive cancers. Finally, molecular and phenotypic subtypes of CRC have been proposed (54,281) and the study of NF-κB in the context of these subtypes would help to further decipher the biology of CRC.

1.6 Research aims and hypothesis

This aim of this thesis was to investigate the role of the non-canonical NF-κB pathway in CRC. The expression of key components of each pathway was investigated using archival tissue from patients who had undergone surgical resection for CRC as well as colon cancer cell lines.

This was performed in order to test the hypothesis that high expression of the non-canonical NF-κB pathway is associated with poorer outcome in patients with CRC. The aim of this study was to understand the association between members of the non-canonical NF-κB pathway, patient and tumour factors, survival and whether this pathway was a potential therapeutic target in CRC. A further aim was to understand if IKKα inhibition was a viable therapeutic strategy in CRC. To address this and test the hypotheses, the main objectives were as follows:

1. Understand the relationship between expression of non-canonical NF-κB pathway members, clinicopathological characteristics, features of the tumour microenvironment and survival.

2. Establish if expression of non-canonical pathway members could be employed as prognostic (or predictive) biomarkers in CRC.

3. Perform preliminary experiments to assess the viability of inhibiting IKKα as a potential therapeutic strategy using colon cancer cell lines.

Chapter 2:

Materials and methods

2.1 Tissue studies

Expression of members of the non-canonical NF-κB pathway was assessed using IHC (immunohistochemistry) on a tissue microarray (TMA) of patients who had undergone surgical resection for colorectal cancer.

2.1.1 Antibody validation

IKKα and RelB had previously been used in the laboratory and had been validated by Mr Lewis McKenzie and Dr Antonia Roseweir.

Protein	Antibody	Validation method
NIK	Bio-Techne, MAB6888	Validated in colon cancer cell line Single band on Western blot – 150kDa
IKKα	Genway, GWB-662250	Validated in prostate cancer cell lines Single band on Western blot – 85kDa Western blot of IKKα silenced lysates IHC on IKKα silenced cell pellets
RelB	Cell Signalling, 4954S	Validated in prostate cancer cell lines Single band on western blot – 70kDa Cellular location in cell pellets after lymphotoxin treatment

Table 1: Antibody validation. *Protein of interest, antibody used and validation method are detailed, as well as tumour types that antibodies were validated in.*

2.1.2 Patient TMA

Previously constructed TMAs were used in this thesis. The TMAs were constructed with formalin-fixed paraffin-embedded tissue (FFPE) blocks retrieved from Pathology archives. Haematoxylin and eosin stained sections were used to identify tumour rich areas from each block, marked slides were matched to the FFPE blocks and four 0.6mm cores were lifted from each donor block and placed into four separate recipient paraffin blocks. Two separate TMAs were used during investigation.

TMA	Number of patients	Stage and year of diagnosis	Purpose
1	1030	Stage I-IV patients, 1997-2007	To investigate expression of non-canonical NF-κB pathway in stage I-III symptomatic CRC
2	159	Screen-detected stage I-III, 2009-2011	To investigate expression of non-canonical NF-κB pathway in early stage screen-detected CRC patients

Table 2: Patient TMAs. *Number of patients, stage at diagnosis, year of diagnosis and purpose for investigation.*

2.1.2.1 Cohort 1: Glasgow cohort TMA

This retrospective TMA of patients undergoing surgery for stage I-IV CRC was constructed by Dr Johnathan Platt and Dr Arfon Powell. This cohort included 1030 patients who were identified from across the Glasgow hospitals (Glasgow Royal Infirmary, Glasgow Western Infirmary, Gartnavel General Hospital and Stobhill Hospital). A database was available with corresponding clinicopathological characteristics, measurements relating to the tumour microenvironment and patient survival.

Clinical follow up was updated using NHS Greater Glasgow & Clyde Safe Haven data. Median follow up time for survivors was 139 months (Interquartile range (IQR) = 120 – 166 months). At last follow up, 324 patients had died of their disease and 332 had died as a result of other causes. Survival data was missing for 21 patients. Clinical endpoints used were cancer-specific survival (CSS), death due to loco-regional or distant recurrences and overall survival (OS), death due to any other cause.

2.1.2.2 Cohort 2: DM-CRC-TMA (screening cohort)

This retrospective TMA of patients who had undergone either polypectomy or surgical resection for stage I-III screen-detected CRC was constructed by Ms Clare Orange (TMA and Image Analysis Unit Manager, University Department of Pathology, Queen Elizabeth University Hospital, Glasgow).

The patients within the TMA cohort came from original data for the first round of gFOBT (guaiac faecal occult blood test) screening conducted within the Greater Glasgow and Clyde health board. Of the 398 patients who were identified as having screen-detected

CRC, 370 received procedures with a curative intent. Patients with T3/4 disease (n=157) and those who received neoadjuvant chemotherapy (n=31) were excluded. The slide for 1 patient could not be obtained at the time of TMA construction. This left a cohort of 181 patients identified as having T1/2 disease who did not receive neoadjuvant chemotherapy. A further 13 patients were excluded as there was uncertainty over their diagnosis following pathological examination. 5 patients who received local rectal resection and 4 patients with no residual tumour were also excluded. This left a cohort of 159 patients whose tissue was used to construct a TMA.

Miss Hannah Hood updated clinical follow up. Median follow up time for survivors was 91 months (IQR = 83 – 95 months). At last follow up, 16 patients had died of their disease and 13 had died because of other causes. Clinical endpoints used were OS.

2.1.2.3 Control tissue

Full colorectal tissue sections and practice colorectal cancer TMAs without linked clinical data were available for optimisation of antibodies. These were used as positive and negative controls when performing IHC. Negative controls were exposed to the exact same conditions as the positive controls with the exception of primary antibody incubation at which time the negative control was incubated in antibody diluent alone.

2.1.3 Immunohistochemistry

IHC was performed on TMAs to assess the expression of members of the non-canonical NF-κB pathway. The aim of IHC is to attach a visible secondary antibody to a primary antibody that recognises a specific antigen, the antigen being the molecule of interest. EnVision™ is used as a secondary marker; it has a polymer backbone to which a large number of horseradish peroxidase (HRP) molecules and secondary antibody molecules have been coupled. The secondary antibody reacts with rabbit and mouse immunoglobulins. After incubation with EnVision™, DAB (3, 3'-diaminobenzidine) is applied, HRP molecules on the EnVision™ interact with the DAB/hydrogen peroxide substrate solution resulting in the deposition of brown precipitate at the site of antigen/antibody interaction. This brown staining can be visualised using a light microscope.

2.1.3.1 Preparation of slides

Once antibodies had been validated and optimised, TMAs were requested from the NHS Research Scotland Greater Glasgow and Clyde Biorepositry and were stored at 4°C. Prior to performing IHC, slides were baked for 15 minutes at 55°C to reduce the risk of lost cores.

2.1.3.2 Dewaxing and rehydration

Slides were immersed in solvents and graded alcohols in order to remove the wax-embedding matrix, allowing access to tissues by aqueous solutions. De-waxing was performed by immersing slides in xylene for 5 minutes (x2) and rehydrated through a series of graded alcohols (2x 100% for 3 minutes, 1x 90% alcohol for 2 minutes and finally 1x 70% for 2 minutes) and then rinsed in running water.

2.1.3.3 Antigen retrieval

The aim of antigen retrieval is to break the protein cross-links formed by formalin fixation thereby unmasking the epitope to allow antibody binding. This was performed using heat-induced antigen retrieval with a solution of either pH 6 citrate buffer or pH 9 Tris/EDTA buffer (Table 3). The citrate buffer was prepared using 2mM tri-Sodium citrate dehydrate (S/3320/53, Fisher Scientific, Loughborough, UK) and 8mM Citric acid (27109, Sigma-Aldrich, Poole, UK) in 1L of distilled water. The Tris/EDTA buffer was prepared using 5mM Tris Base (BP152-1, Fisher Scientific, Loughborough, UK) and 1mM Diaminoethanetetra-acetic acid disodium salt dehydrate (EDTA) (D/0700/60, Fisher Scientific, Loughborough, UK) in IL of distilled water.

The buffer solutions were preheated in a microwave for 13.5 minutes in an open pressure cooker. Once heated, slides were placed inside the pressure cooker and the lid was secured. The slides were heated for approximately 2 minutes until under pressure followed by a further 5 minutes of heating under pressure. Slides were left to cool in the antigen retrieval buffer for 30 minutes. Once cooled, slides were rinsed in running water for 10 minutes.

2.1.3.4 Blocking endogenous peroxidase activity

When using HRP coupled antibodies for detection, false positive staining or excessive background staining can occur. Therefore, it is important to quench endogenous peroxidase

to prevent this. This was done by incubating tissues in a 3% hydrogen peroxide solution for 10 minutes. Thereafter slides were rinsed in running water.

2.1.3.5 Blocking non-specific binding

Insufficient blocking can result in a high level of non-specific background staining to a variety of proteins within the tissue. This step aims to block all potential non-specific antibody (or indeed any other detection reagent) binding; it occludes antibody binding except where the antibody has competitive advantage, at its target epitope.

Firstly, tissue was circled with a Dako pen (S2002, Dako, Agilent Technologies, Stockport, UK) to create a hydrophobic barrier ensuring tissues remain covered by the applied solutions in the endeavour to produce a uniform stain and also to use reagents economically. Blocking was performed with either 5% horse serum (S200, Vector Laboratories, Peterborough, UK) or with 1 x Casein (SP-5020, Vector Laboratories, Peterborough, UK) at 25 ºC for 30 minutes. Both blocking agents were diluted in Tris Buffer Saline (TBS). The optimal blocking solution varied depending on the antibody in use (Table 3).

Protein of interest	Buffer solution	Blocking conditions	Antibody dilution & incubation conditions
NIK	Citrate pH6	5% horse serum 60 mins	1:750 dilution, overnight 4 ºC (Biotechne, MAB6888, mouse)
IKKα	Citrate pH6	5% horse serum 30 mins	1:1000 dilution, overnight 4 ºC (Genway, GWB-662250, rabbit)
IKKα S176	Tris-EDTA pH 9	1 x casein 30 mins	1:100 dilution, overnight 4 ºC (Abcam, ab138426, rabbit)
IKKα T23	Citrate pH6	1 x casein 30 mins	1:100 dilution, overnight 4 ºC (Abcam ab38515, rabbit)
RelB	Tris-EDTA pH9	5% horse serum 30 mins	1:80 dilution, overnight 4ºC (Cell Signalling, 4954S, rabbit)

Table 3: Optimal antibody conditions for immunohistochemistry. *The antigen retrieval buffer, blocking solution and antibody incubation conditions for each protein*

2.1.3.6 Incubation with primary antibody

Excess blocking solution was tapped off and tissue was immediately incubated in primary antibody, with the exception of negative control tissue. Antibodies were diluted in antibody diluent (S0809, Dako, Agilent Technologies, Stockport, UK) and applied to the slides. Tissues were incubated at 4 ºC overnight.

2.1.3.7 Incubation with secondary antibody

Slides were washed in TBS for 5 minutes (x2). Thereafter slides were incubated in EnVision™ (K5007, Dako, Agilent Technologies, Stockport, UK) which detects both mouse and rabbit primary antibodies for 30 mins at 25 °C. Slides were washed again in TBS for 5 minutes (x2).

2.1.3.8 Detection and visualisation

A DAB peroxidase kit was used (SK-4100, Vector Laboratories, Peterborough, UK). 1 drop of DAB buffer solution, 2 drops of DAB substrate solution and 1 drop of hydrogen peroxide solution was added to 5ml of distilled water. This was applied to the tissue for 5 minutes; slides were then washed in running water for 10 minutes.

2.1.3.9 Counterstaining

Counterstaining was performed by immersing slides in Harris Haematoxylin (LAMB/230-D, Raymond A Lamb Ltd, Eastbourne, UK) for 1 minute followed by a quick dip in 1% acid alcohol to remove excess haematoxylin. Tissue was blued in Scott's Tap Water Substitute (STWS) for 45 seconds. Acid alcohol was prepared using 396ml 70% ethanol and 4ml hydrochloric acid. STWS contained 80mM Magnesium sulphate (230391, Sigma, Poole, UK) and 40mM sodium hydrogen carbonate (102475W, VWR Poole, UK) in 2L of distilled water

2.1.3.10 Dehydration and mounting of slides

Following counterstaining, tissues were dehydrated in a series of graded alcohols: 1 x 70% for 1 minute, 1 x 90% for 1 minute, 2 x 100% for 1 minute and finally 2 x Xylene for 1 minute. Slides were mounted with coverslips using histological mounting medium, Omnimount (HS-110, SLS, Nottingham, UK).

2.1.3.11 Scanning and visualisation of slides

Stained TMA slides were scanned with a 20x objective lens and NA (numerical aperture) 0.75 which gives a scanning resolution of 0.46μm/pixel. Tissues were visualised on a Slidepath Digital Image Hub (Leica Biosystems, Newcastle, UK). Cores that were missing or contained less than 10% tumour were excluded from analysis.

2.1.4 Scoring of IHC

2.1.4.1 Weighted histoscore

Evaluation of staining intensity was performed by observers blinded to clinicopathological details and survival outcomes. The weighted histoscore previously described by Kirkegaard *et al* (282) was used to quantify protein expression. This method grades staining as absent (0), weak (1), moderate (2) or strong (3), multiplied by the percentage area of tumour cells in each category to give a score between 0 - 300. One observer blindly scored all cores; a second observer then independently scored 10% of cores. All observers were blinded to clinicopathological details, patient outcomes and the other observer's scores. Statistical analysis, specifically, interclass correlation coefficient (ICCC) was performed to evaluate inter-observer reliability. An ICCC>0.7 was considered as having scores with good correlation.

2.1.5 Statistical Analysis

Thresholds for protein expression were calculated using receiver operating characteristic (ROC) curve analysis. The relationship between clinicopathological characteristics and cytoplasmic and nuclear expression was assessed using the Chi-square test. The relationship between protein expression and 5- and 10-year CSS was assessed using Kaplan-Meier log-rank analysis and displayed as percentage surviving (standard error). The relationship between protein expression, clinicopathological characteristics and CSS was examined using Cox proportional hazards regression with 95% confidence intervals (CI). Variables with a p-value ≤ 0.05 on univariate analysis were entered into a multivariate model using a backwards conditional method to calculate hazard ratios (HR) and 95% CI. A p-value ≤ 0.05 was considered statistically significant. SPSS version 22.0 (IBM SPSS) was used for all statistical analysis. All statistical analysis was performed by myself and repeated by a second (and senior) investigator (Joanne Edwards) in order to validate the results obtained.

2.1.6 Immunofluorescence

Immunofluorescence was used to investigate a distinct pattern of staining observed with IKKα. Dual immunofluorescence was used to investigate whether IKKα was localised to a specific cellular structure. The aim of immunofluorescence is to use antibodies to label a specific antigen with a fluorescent dye (fluorophore). Immunofluorescence can be performed with a primary or secondary technique. The primary or direct technique employs the use of a single antibody that is chemically linked to a fluorophore. The fluorophore can then be detected with fluorescent microscopy. Secondary, or indirect immunofluorescence uses two antibodies; firstly an unlabelled primary antibody followed by a secondary antibody that is conjugated to the flurophore, recognises the primary antibody and binds to it. Dual immunofluorescence employs the use of primary antibodies raised in different host species with matching secondary antibody that allows assessment of co-distribution of two (or more) different antigens within the same sample.

2.1.6.1 Dewaxing and rehydration

Tissues were dewaxed and rehydrated as described in section 2.1.3.2 with the following exceptions:

-De-waxing of tissues in xylene was increased to 10 minutes (x3).

2.1.6.2 Protease step

Optimisation of the Golgi antibody required a protease step prior to heat-induced antigen retrieval. Following rehydration, tissues were incubated in Protease Plus (322331, Advanced Cell Diagnostics, Bio-Techne, Abingdon, UK) for 30 minutes at 40ºC in a temperature-controlled humidifying chamber (HybEz II Oven™, Advanced Cell Diagnostics, Bio-Techne, Abingdon, UK). Slides were cooled for 30 minutes before washing in running water for 10 minutes.

2.1.6.3 Antigen retrieval

In addition to the antigen retrieval methods described in section 2.1.3.3, a water bath was also used. Dako target antigen retrieval solution pH9 (10x) (S2367, Dako, Agilent Technologies, Stockport, UK) was diluted with distilled water to give a 1:10 dilution. The solution was pre-heated in a water bath and once a temperature of 92-95ºC was reached, slides were immersed into the antigen retrieval solution and incubated for 40 minutes.

Slides were left to cool in the antigen retrieval solution for 30 minutes. Once cooled slides were washed in running water for 10 minutes.

2.1.6.4 Blocking non-specific binding

Tissues were circled with Dako pen (S2002, Agilent Technologies, Stockport, UK). To block non-specific binding, slides were incubated in either 5% horse serum (S200, Vector Laboratories, Peterborough, UK), or 2% fetal bovine serum (FBS) (10270106, InVitrogen, Paisley, UK). Blocking agents were diluted in TBS.

2.1.6.5 Incubation with primary antibody

Primary antibody was diluted in the blocking solution (as used in 2.1.6.4) or TBS to produce the appropriate concentrations. Excess blocking solution was tapped off and tissues were incubated in the primary antibody overnight at 4 ^{o}C. When performing dual fluorescence, primary antibodies were prepared as a mixture and applied to tissues.

2.1.6.6 Incubation with secondary antibody

The following day, slides were rinsed in TBS for 10 minutes (x3). Thereafter, tissues were incubated in synthetic fluorescent dye (diluted in the appropriate blocking solution or TBS) that is conjugated to an antibody. Application of the secondary antibody and the remainder of the experiment was performed in a darker area out of direct light. This is because extensive exposure to light can result in photobleaching of the dye and thus affect secondary antibody conjugate performance. When performing dual fluorescence, secondary antibodies were prepared as a mixture and applied to tissues.

Protein of interest	Buffer solution	Blocking conditions	Primary antibody concentration (diluent) & incubation conditions	Secondary antibody
IKKα	Tris-EDTA pH9 (no protease step)	5% horse serum 20 mins	1:1000 (blocking solution), overnight 4 °C (Genway, GWB-662250, anti-rabbit)	1:500 dilution, 60 minutes 25 °C in the dark (Alexa Fluor® 555 goat anti-rabbit, ThermoFisher, A21428)
Golgi	1) Tris-EDTA pH9 (no protease step) 2) Dako antigen retrieval solution (1x) (with protease step) 3) Tris-EDTA pH9 (with protease step)	5% Horse serum 20 minutes	1:100 (TBS), overnight 4°C (Abcam, ab27043, anti-mouse)	1:500 dilution, 60 minutes 25 °C in the dark (Alexa Fluor® 488 goat anti-mouse, ThermoFisher, A11029)
Rab 5	Tris-EDTA pH9	5% horse serum 20 minutes	1:100 (blocking solution), overnight 4 °C (Abcam, ab66746, anti-mouse)	1:500 dilution, 60 minutes 25 °C in the dark (Alexa Fluor® 488 goat anti-mouse, ThermoFisher, A11029)
Rab 7	Tris-EDTA pH9	2% Fetal bovine serum 20 minutes	1:1000 (blocking solution), overnight 4 °C (Abcam, ab50533, anti-mouse)	1:500 dilution, 60 minutes 25 °C in the dark (Alexa Fluor® 488 goat anti-mouse, ThermoFisher, A11029)

Table 4: Optimal antibody conditions for immunofluorescence. *The antigen retrieval buffer, blocking solution, primary and secondary antibody incubation conditions for each protein.*

2.1.6.7 Counterstaining of nuclear DNA material and mounting

Slides were rinsed in TBS for 10 minutes (x3) and mounted using VECTASHIELD Mounting Medium which contained the nuclear counterstain DAPI (H-1200, Vector Laboratories, Peterborough, UK). DAPI is a nucleic acid stain that preferentially stains double-stranded DNA and RNA. Edges of coverslips were sealed with nail varnish and slides were wrapped in tinfoil and stored at 4^{o}C until they were visualised with a confocal microscope

2.1.6.8 Visualisation

A Zeiss LSM 780 confocal microscope was used to obtain immunofluorescent images from TMAs. Using a 10x objective lens, images were taken with a 40x oil immersion lens where a small drop of immersion oil was placed in the centre of the coverslip prior to imaging. Images were saved using ZEN 2 software (Zeiss, Germany).

Microscopes at the CRUK Beatson Institute Imaging Facility, Glasgow, were also used. For immunohistochemistry, an Olympus BX51 100x 1.3 NA oil lens with a Zeiss Axiocam 105 colour camera was used. For fluorescence, a Zeiss LSM880 Airyscan microscope using a 40x 1.3 NA Zeiss Plan apochromat oil lens was used in Airyscan ‘super-resolution’ mode with sequential scanning through a different emission filter for the green and red channels to minimise cross talk with default processing settings. The images were displayed using Imaris (Bitplane).

2.2 *In vitro* studies

2.2.1 Culturing of colon cancer cell lines

HT29, and T84 colon cancer cells were cultured. Cells were cultured in medium with FBS and 50 Units/ml Penicillin/Streptomycin (15070-063, Life Technologies, Paisley, UK). Cells were grown in T-75 flasks and maintained in 5% CO_2 at 37˚C, with media changed twice per week and cells passaged once a confluency of 70% was reached. To passage cells, flasks were washed once with PBS without magnesium and calcium, 1.5ml of trypsin was then added to remove traces of serum. After this was removed, 1ml of trypsin was added to detach cells from the flask and then incubated at 37˚C in 5% CO_2 until cells detached. Once the cells had detached, medium was added to inactivate trypsin. Cells were carefully pipetted on the side of the flask to free any clusters before being split 1:6 into new T-75 flasks with fresh medium. Cells were routinely tested for mycoplasma twice a year.

Cell line	Characteristics	Medium	Serum
HT-29 (ATCC® HTB-38™)	Colon origin KRAS wild-type BRAF V600E Microsatellite stable (MSS)	McCoy's 5A (Modified) Medium, GlutaMAX™ Supplement (36600021, Life Technologies, Paisley, UK)	10% FBS
T84 (ATCC® CCL-248™)	Colon origin (derived from metastatic site: lung) KRAS mutated BRAF wild-type MSS	Dulbecco's Modified Eagle Medium (DMEM)/F12 (Ham) (11320033, Life Technologies, Paisley, UK)	5% FBS

Table 5: Cell lines. *Cell line name, associated molecular characteristics* (283–285)*, incubation conditions.*

2.2.2 Stimulation of the non-canonical NF-κB pathway in colon cancer cells

To activate the non-canonical NF-κB pathway and stimulate expression of pathway members, HT-29 and T84 cells were stimulated with ligands over a number of time points and extraction of proteins was performed to observe expression of pathway members. The following ligands were used:

- Lymphotoxin $\alpha_1\beta_2$:

  20ng/ml lymphotoxin $\alpha_1\beta_2$ (L5162, Sigma-Aldrich, Poole, UK) was used for 1, 2, 4, 8, and 24-hour time points.

- TNFα:

  20ng/ml TNFα (SRP3177, Sigma-Aldrich, Poole, UK) was used for 1, 2, 4, 8 and 24-hour time points.

For western blot, cells were seeded in 12 well plates at $1x10^5$ cells/well, grown to 70% confluency before being serum starved in media with no FBS for 24 hours followed by stimulation with ligand at the appropriate concentration and time point before protein was lysed (section 2.3.1).

2.2.3 Inhibition of the non-canonical NF-κB pathway in colon cancer cells

Cells were seeded in 12 well plates at $1x10^5$ cells/well, grown to 70% confluency before being serum starved in media with no FBS for 24 hours. Cells were then pre-treated with IKKα inhibitor (SU 1433) with serial dilutions of 20mM stock before addition of stimulating ligand (lymphotoxin $\alpha_1\beta_2$ 15ng/ml).

2.3 Western blotting

2.3.1 Lysis of protein

Cells were exposed to the appropriate agonist and length of time and then placed on ice to halt further reactions and prevent protein degradation or dephosphorylation. Cells were washed twice with 300µl ice cold PBS and then 250µl of pre-heated Laemmli's sample buffer. Cells were scraped and pushed through a 21-gauge needle to shear chromosomal DNA. The samples were then transferred to Eppendorf tubes, boiled for 5 minutes to denature proteins, and stored at -20°C until required.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gels were prepared containing 0.4% SDS (sodium dodecyl sulphate), 1.5M Tris base (pH 8.4), 3% glycerol, 10% Ammonium persulfate (APS), 30% acrylamide/bisacrylamide, and distilled water to make up 12ml in total. Most proteins were suitable to be separated on 10% gels (4ml 30% acrylamide in 12ml total volume), but due to size and lack of specificity of the phospho-p100 antibody, a 7.5% (3ml 30% acrylamide in 12ml total volume) gel was required to allow separation of proteins. 0.05% tetramethylethylenediamine (TEMED) was added to begin the polymerisation process. Glass plates were set up on a Mini-PROTEAN Tetra cell casting module and the solution was poured between two plates with 200µl of 0.1% SDS added on top. Once gel polymerisation had occurred the SDS solution was removed and stacking gel containing 10% acrylamide, 0.1% SDS, 10% APS and 0.05% TEMED was poured on top of the resolving gel and a comb was inserted. Once polymerisation was complete, the comb was carefully removed and gels were assembled in a Bio-Rad Mini-PROTEAN electrophoresis tank filled with electrophoresis running buffer (25mM Tris, 129mM glycine, 0.1%SDS). 10-15µl (depending on the protein being investigated) of sample was loaded per well using a Hamilton microsyringe. A pre-stained marker of known molecular weights (161-0374, Bio-Rad, Watford, UK) was run on the same gel in order to identify the protein of interest by size and ensure successful transfer onto the membrane. Electrophoresis was performed at a constant voltage of 145V for usually 90 minutes, until bromophenol blue dye had reached the bottom of the gel.

2.3.3 Protein transfer

Transfer of proteins from the gel to nitrocellulose membrane (IPVH00010, Millipore, Watford, UK) was performed by electrophoretic blotting in wet conditions. Using a Bio-Rad cassette, a sandwich of sponge, filter paper, gel, membrane, filter paper and sponge was immersed in transfer buffer (25mM Tris, 19mM glycine, 20% methanol) and slotted into a Bio-Rad Mini-PROTEAN electrophoresis tank and a constant current of 400mA was applied for 135 minutes, an ice pack was also included to cool the tank. The presence of SDS gives the protein a negative charge, so the cassette was orientated with the membrane towards the anode meaning protein would move towards the positive electrode and bind to the nitrocellulose membrane.

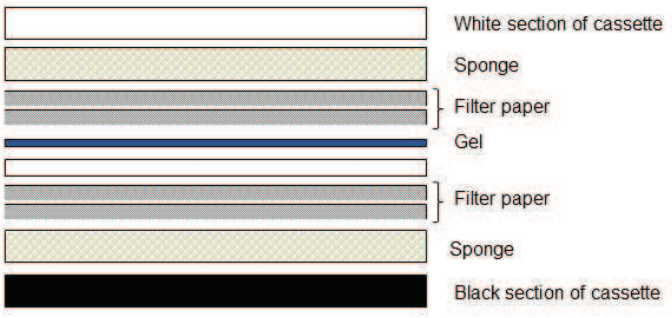


Figure 4: Assembly of sandwich for western blot transfer. *Diagram shows how the sandwich of sponge, filter paper, nitrocellulose membrane and gel were stacked for transfer.*

2.3.4 Blocking, staining and visualisation

Following transfer of proteins from gel to membrane, the sandwich was dismantled, and the membrane was removed. Non-specific binding was blocked by incubation in a solution of 1% milk in TBS for 60 minutes or 2% BSA (bovine serum albumin) in 0.1% or 0.03% TBST (TBS with Tween®) for 2 hours on a platform shaker. Membranes were washed and then incubated overnight in primary antibody diluted to optimal concentration. Conditions for each antibody are listed in Table 6. The following day membranes were washed in 0.1% or 0.03% TBST. Thereafter, secondary (either rabbit or mouse, depending on primary antibody used) HRP-conjugated antibody was diluted to 2:15000 and membranes were incubated at room temperature for the appropriate length of time. Membranes were washed again in TBST. Chemiluminescence was used to detect presence of protein. Membranes were incubated in 10ml of enhanced chemiluminescence (ECL) reagent (1:1 mixture of solution 1[1M Tris pH8.5, 250mM luminol, 250mM p-cymuric acid and water]

and solution 2 [1M Tris pH8.5, 0.19% H_2O_2 and water) for 1 minute 45 seconds on a platform shaker. Membranes were then placed into a cassette, covered with cling film and exposed to X-ray film for the required time and developed using a JP-33 Film Processor.

2.3.5 Stripping of membrane

To compare the amount of protein in each sample and allow assessment of the blotting, β-tubulin expression, a housekeeping gene independent of NF-κB, was used as a loading control. Nitrocellulose membranes were reprobed for β-tubulin. Membranes were stripped using a stripping buffer containing 0.05M Tris-HCl and 2% SDS. The membrane was incubated in 15ml of stripping buffer with 0.1M (105µl) β-mercaptoethanol for 60 minutes on a shaker. The membrane was washed in NaTT buffer for 15 minutes 3 times to remove all stripping buffer. Membranes were then reprobed, using the protocol described in section 2.3.4 for levels of β-tubulin (1:10000, ab21058, Abcam) as a loading control.

2.3.6 Quantification of expression levels

ImageJ (National Institute of Health, USA) was used to calculate expression levels of proteins observed via western blots. Levels were normalised to β-tubulin and fold change to control calculated using a one-way ANOVA with Bonferroni correction and Dunnett's test used to compare to control. Changes were considered significant if $p<0.05$ and highly significant if $p<0.001$.

Protein of interest	Antibody	Species	Amount of protein loaded per well	Blocking conditions	Wash after block	Primary antibody conditions	Wash after primary	Secondary antibody	Wash after secondary
p100/p52	05-361, Millipore	Mouse	10 µl	1% milk in TBS 60 mins	3 x 10 mins TBS	0.50:15000 (3% BSA 0.1% TBST), overnight room temperature	3 x 10 mins TBST	2:15000 (1% milk TBST)	3 x 10 mins 0.1%TBST
phospho-p100	4810, Cell Signalling	Rabbit	15 µl	1% milk in TBS 60 mins	3 x 10 mins TBS	2:3000 (0.5% BSA 0.1% TBST), overnight 4ºC	3 x 10 mins TBST	2:15000 (1% milk TBST)	3 x 10 mins 0.1% TBST
NIK	MAB 6888, R&D systems	Mouse	15 µl	2% BSA in 0.03% TBST 120 mins	-	2:3000 (0.2% BSA 0.03% TBST), overnight 4ºC	6 x 15 mins 0.03% TBST	2:15000 (0.2% BSA TBST)	6 x 15 mins 0.03% TBST
IKKα	14A231, Calbiochem	Mouse	15 µl	2% BSA in 0.03% TBST 120 mins	-	2:3000 (0.2% BSA 0.03% TBST), overnight 4ºC	6 x 15 min 0.03% TBST	2:15000 (0.2% BSA TBST)	6 x 15 mins 0.03% TBST

Table 6: Antibodies used for western blot and optimal conditions. *Table showing the company, catalogue number and species raised in for each antibody and the optimal conditions for western blotting.*

Chapter 3:

Expression of members of the non-canonical NF-κB pathway in colorectal cancer specimens (Glasgow cohort)

3.1 Introduction

There is a large body of evidence (mostly cell line and animal) implicating NF-κB in the development and progression of CRC (286). However, the non-canonical NF-κB pathway in CRC is largely under-investigated with limited studies in human tissue. To our knowledge there are currently no studies with large numbers of patients exploring the role of different pathway members. We aimed to investigate the expression of key members of the non-canonical NF-κB pathway (NIK, IKKα and RelB) in a CRC patient tissue microarray and assess the clinical significance of each member.

3.2 Antibody validation of members of the non-canonical pathway

3.2.1 Validation of anti-NIK antibody

Specificity of the anti-NIK antibody was confirmed in HT-29 and T84 colon cells using western blotting. This showed a single band of the predicted molecular weight (150kDa). HUVEC cells overexpressing NIK were used as a positive control.

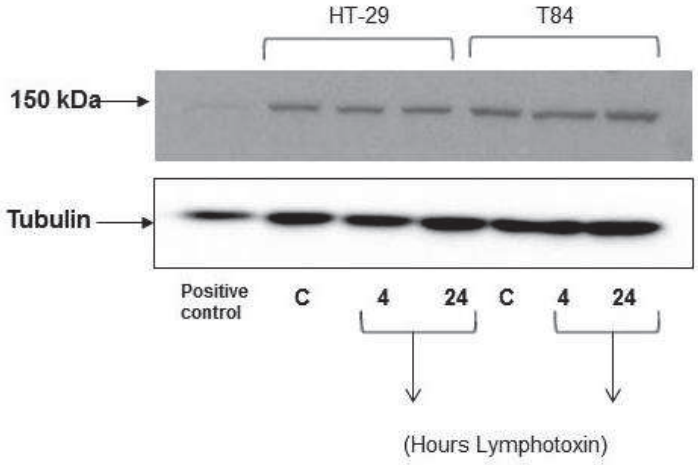


Figure 5: Validation of anti-NIK antibody. *Western blot showed a clear band of appropriate size (150 kDa) in HT-29 and T84 colon cancer cells.*

3.2.2 Validation of anti-IKKα antibody

Specificity of the anti-IKKα antibody was previously confirmed in our laboratory. Western blotting using the anti-IKKα antibody showed a single band of the predicted molecular weight (85 kDa). LNCap cells were pre-treated with 200nM siRNA against either IKKα or IKKβ to check specificity for IKKα. A reduction in expression was observed in the IKKα-silenced but not IKKβ-silenced cells. LNCap cell pellets were made and IHC was performed, IKKα-silenced cells showed a decrease in expression, this was not observed in IKKβ-silenced or control cells (287).

3.2.3 Validation of anti-RelB antibody

Specificity of the anti-RelB antibody was previously confirmed in our laboratory. Western blotting showed a single band of the predicted molecular weight (70 kDa). Cell pellets that were treated with 20ng/ml lymphotoxin for 24 hours showed an increase in nuclear localisation of RelB compared with untreated controls (287).

3.3 Expression and clinical outcome of members of the non-canonical pathway

3.3.1 Study group

In total there were 1030 patients who underwent surgery for symptomatic CRC between 1997 and 2007; 48% were female and approximately two-thirds were over the age of 65. In all, 138 patients (13%) had TNM stage I disease, 483 (47%) had stage II disease, 388 (38%) had stage III disease and 21 (2%) had stage IV disease. Overall, 430 (42%) patients had tumours located within the right colon, 340 (33%) within the left colon and 253 (25%) had rectal cancer. The majority of patients (62%) with stage IV disease had right-sided primary tumours. Information regarding adjuvant therapy was available in 350 (34%) patients; of these 350 only 128 (37%) received adjuvant therapy. Median follow-up of survivors was 139 months (IQR 120 – 166 months).

3.3.2 Expression of NIK

NIK expression was investigated in the full cohort of 1030 patients. 262 patients had cores missing or insufficient tumour for analysis and following exclusions outlined in Figure 6, there were 693 patients included for analysis. Exclusion criteria included mortality within 30 days of surgery, patients with metastatic disease (stage IV) and administration of neoadjuvant chemotherapy due to potential impact on the immunological tumour microenvironment. Expression of NIK was observed at the cytoplasmic and nuclear level. The histoscore range for cytoplasmic NIK ranged from 0 to 107. Expression of cytoplasmic NIK was graded as either low or high. Thresholds for analysis were calculated using ROC curve analysis; a cytoplasmic histoscore > 17 was considered as high expression. Only 70 patients displayed expression of NIK at the nuclear level with a histoscore range from 0 to 20. For the purposes of analysis, nuclear expression of NIK was considered as either absent or present. Examples of low and high cytoplasmic and nuclear NIK expression are displayed in Figure 7. On Chi-square analysis, there was no association between cytoplasmic and nuclear expression of NIK ($p= 0.534$). There was good correlation of scores between observers with ICCC scores of 0.86 for cytoplasmic expression and 1.00 for nuclear expression of NIK.

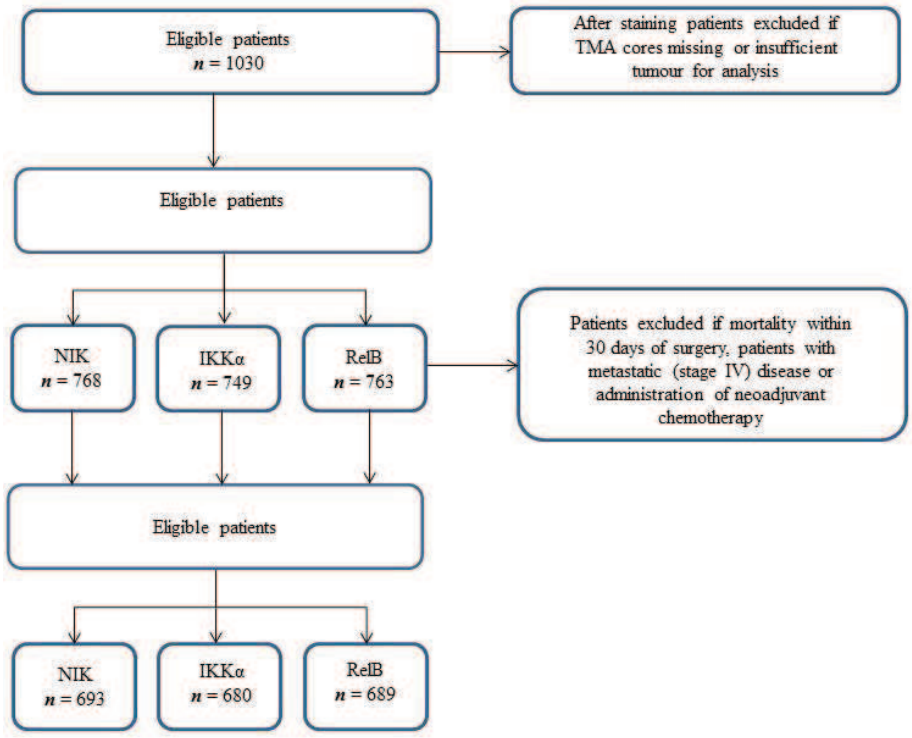


Figure 6: Flow diagram demonstrating patient exclusions. *Patients with missing cores or insufficient tumour for analysis were excluded. Thereafter patients who had either died within 30 days of surgery, had stage IV disease or had received neoadjuvant chemotherapy were excluded from final analysis.*

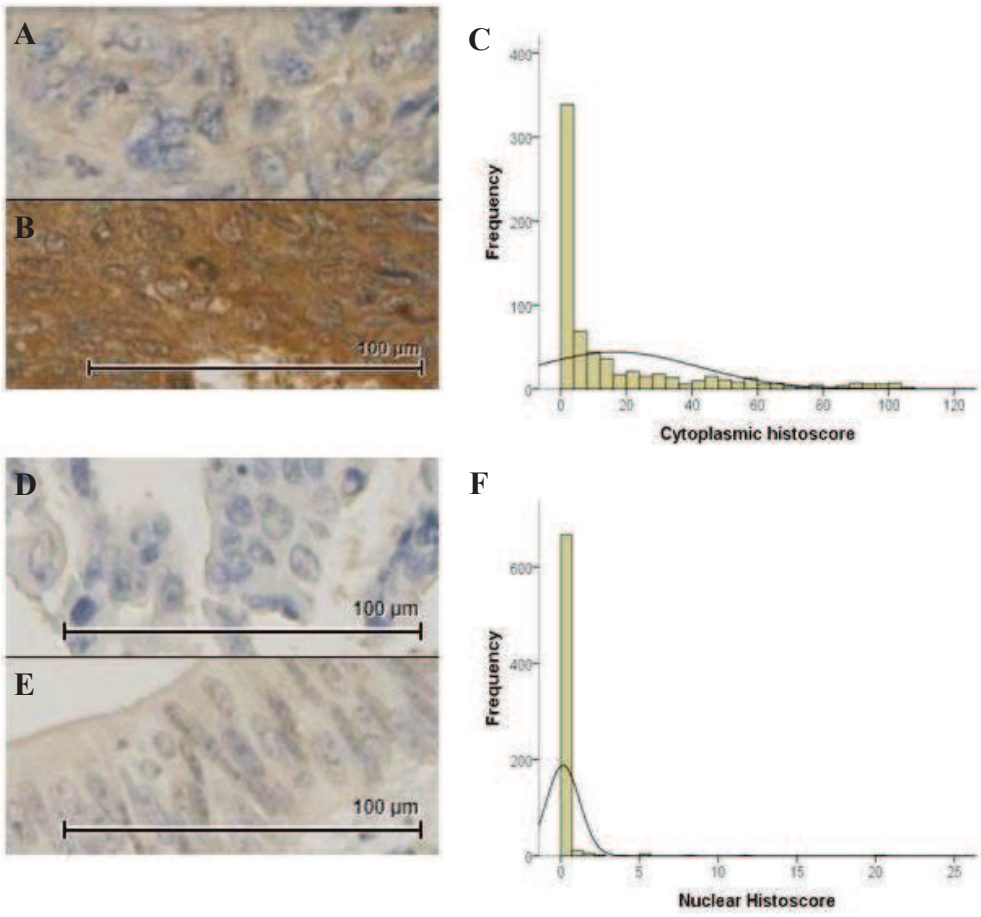


Figure 7: Expression of NIK. (*A) Examples of low and (B) high cytoplasmic, (D) low and (E) high nuclear NIK expression in epithelial colorectal cancer tissue, brown represents the protein of interest (images taken from TMA at 20x20 magnification, scale bar represents 100µm). Histograms show distribution of average histoscores across three tumour cores for cytoplasmic (C) and nuclear NIK (F).*

3.3.3 NIK, clinicopathological characteristics and the tumour microenvironment

Chi-squared analysis was performed to assess the association with clinicopathological factors of the cohort including age, sex, type of surgery (elective/emergency), tumour location (right colon/left colon/rectum), adjuvant therapy, TNM stage, tumour differentiation, venous invasion, margin involvement, necrosis, proliferation, MMR status, BRAF status, Klintrup-Mäkinen grade and tumour stroma percentage. Expression of cytoplasmic NIK was not associated with patient age (p=0.322), sex (p=0.631), type of surgery (p=0.175), tumour location (p=0.342) or administration of adjuvant chemotherapy (p=0.474). In terms of tumour characteristics, high expression of cytoplasmic NIK was associated with low tumour proliferation (p=0.050) but was not associated with TNM stage (p=0.708), tumour differentiation (p=0.356), venous invasion (p=0.681), margin involvement (p=0.307), tumour necrosis (p=0.381), MMR status (p=0.379) or BRAF status (p=0.431). With respect to the tumour microenvironment, high expression of cytoplasmic NIK was associated with an adverse Klintrup-Mäkinen grade (p=0.026) but was not associated with tumour stroma percentage (p=1.000).

High expression of nuclear NIK was associated with emergency surgery (p=0.024) but was not associated with patient age (p=0.208), sex (p=0.802), tumour location (p=0.752) or administration of adjuvant chemotherapy (p=0.484). Nuclear NIK expression was not associated with TNM stage (p=0.497), tumour differentiation (p=0.871), venous invasion (p=0.909), margin involvement (p=0.732), tumour necrosis (p=0.167), proliferation (p=0.221), MMR status (p=0.743) or BRAF status (p=0.693). With respect to the tumour microenvironment, high expression of nuclear NIK was associated with an adverse Klintrup-Mäkinen grade (p=0.005) but was not associated with tumour stroma percentage (p=0.389).

3.3.4 NIK expression and survival

To determine whether NIK expression was significantly associated with clinical outcome, Kaplan-Meier survival curves were plotted, low and high expression was compared using the log rank test. Expression of cytoplasmic NIK was not associated with CSS (p=0.106) (Figure 8A). However, when patients who had undergone elective surgery were considered alone (n=554), those with high expression of cytoplasmic NIK had inferior CSS when compared to those patients with low expression (HR 1.52 95% CI 1.08-2.14, p=0.016) (Figure 8C), with a reduction in 5-year survival from 80% to 71% and a reduction in 10-year survival from 75% to 64%. When stratified by tumour site, this reduction in CSS was potentiated in those patients with right-sided tumours (HR 2.23 95% CI 1.29-3.82, p=0.003). There was no association between NIK expression and survival in patients with left-sided (p=0.782) or rectal (p=0.511) tumours (Figure 9). In patients who were MMR deficient, high expression of cytoplasmic NIK was associated with reduced CSS (HR 2.77 95% CI 1.09-7.01, p=0.025) compared to those patients who were MMR proficient (p=0.074) (Figures 10A &10B). In patients harbouring the BRAF V600E mutation, high expression of cytoplasmic NIK was associated with reduced CSS (HR 2.86 95% CI 1.38-5.97, p=0.003) compared to those patients with BRAF wild-type status (p=0.224) (Figures 11A &11B).

Expression of nuclear NIK was not associated with CSS in the full cohort (p=0.732), in elective patients (p=0.211) (Figure 8) or when considered in the context of tumour location (right colon p=0.553; left colon p=0.800; rectum p=0.178). However, in MMR proficient patients, high expression of nuclear NIK was associated with reduced CSS (HR 1.74 95% CI 1.02-2.99, p=0.040) (Figure 10C). In patients with BRAF wild-type status, there was a reduction in survival in those patients with high nuclear expression of NIK however, this was not significant on log-rank analysis (HR 1.75 95% CI 0.98-3.12, p=0.054 (Figure 11C).

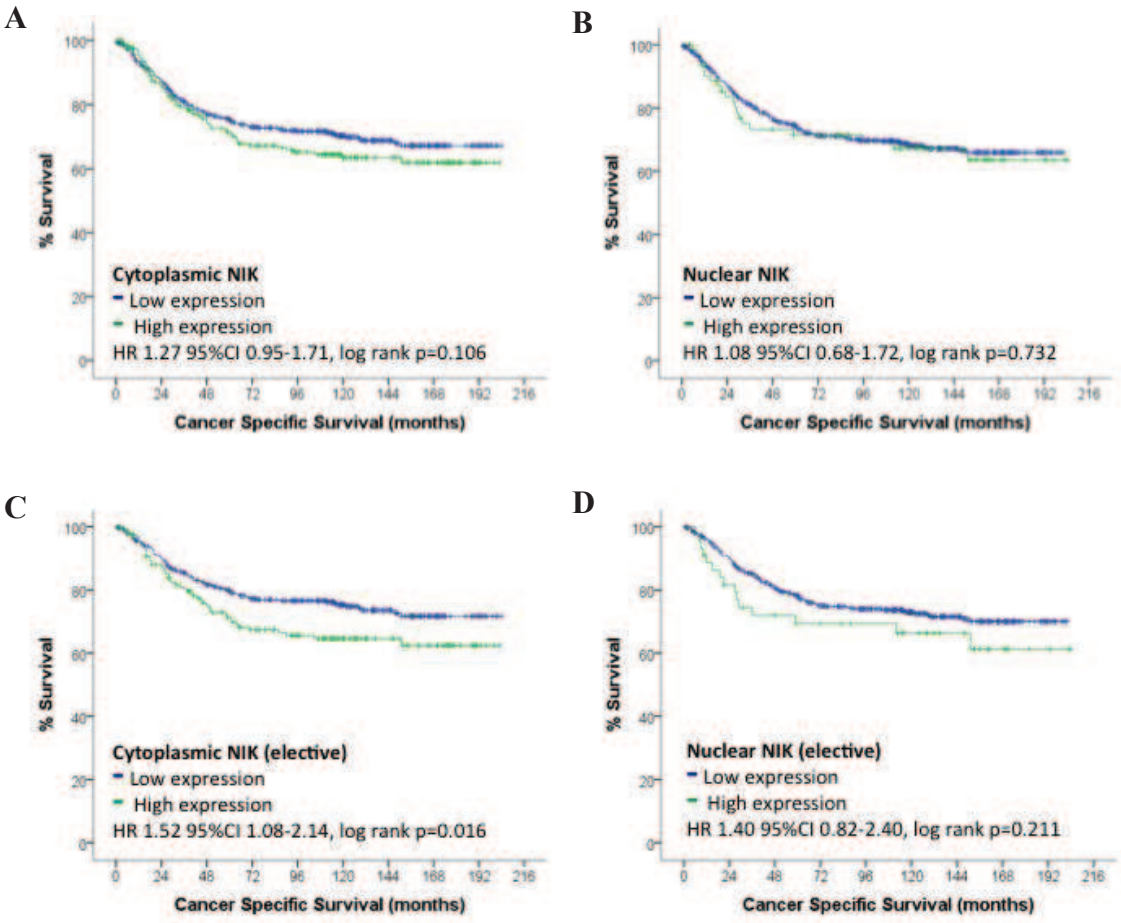


Figure 8: NIK expression and survival. *Expression of NIK in the cytoplasm (A) or nucleus (B) was not associated with CSS. Cytoplasmic NIK expression was associated with CSS in patients who underwent elective surgery (C). Nuclear expression of NIK was not associated with CSS in patients who underwent elective surgery (D).*

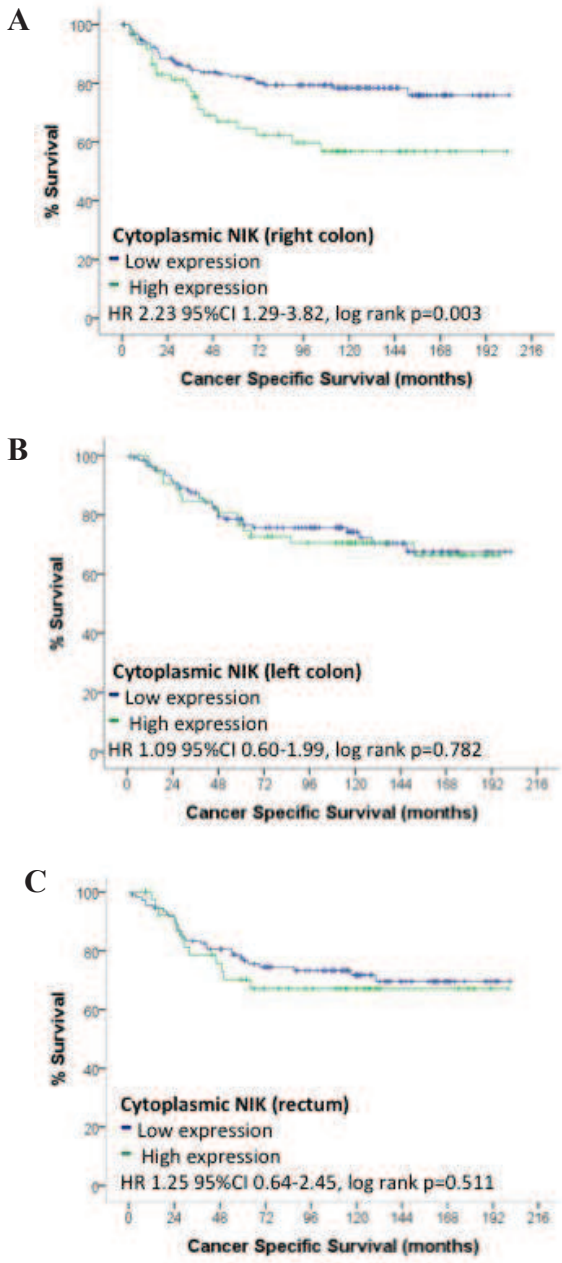


Figure 9: The association between cytoplasmic NIK expression, survival and tumour location in elective patients. *Cytoplasmic NIK expression was associated with CSS in patients with right-sided cancers (A) but not in those with left-sided (B) or rectal cancer (C).*

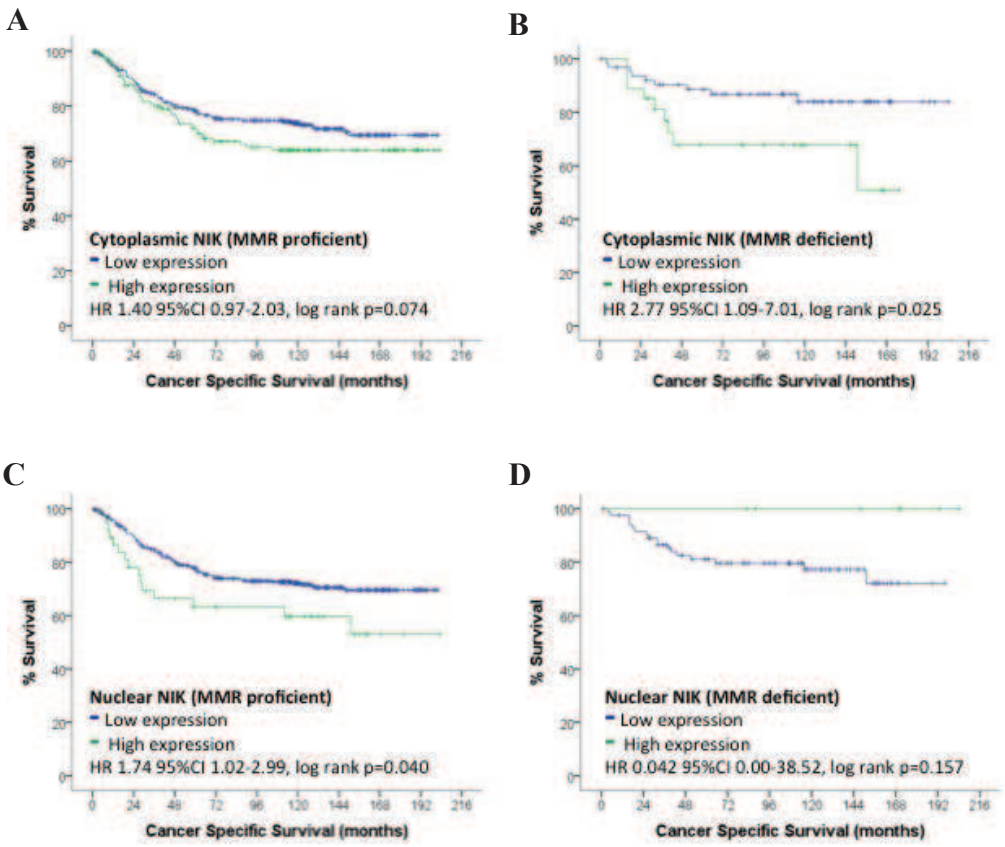


Figure 10: The association between NIK expression, survival and MMR status in elective patients. *Cytoplasmic NIK expression was not associated with CSS in MMR proficient patients (A) but was associated with CSS in MMR deficient patients (B). Nuclear expression of NIK was associated with CSS in MMR proficient patients (C) but not in MMR deficient patients (D).*

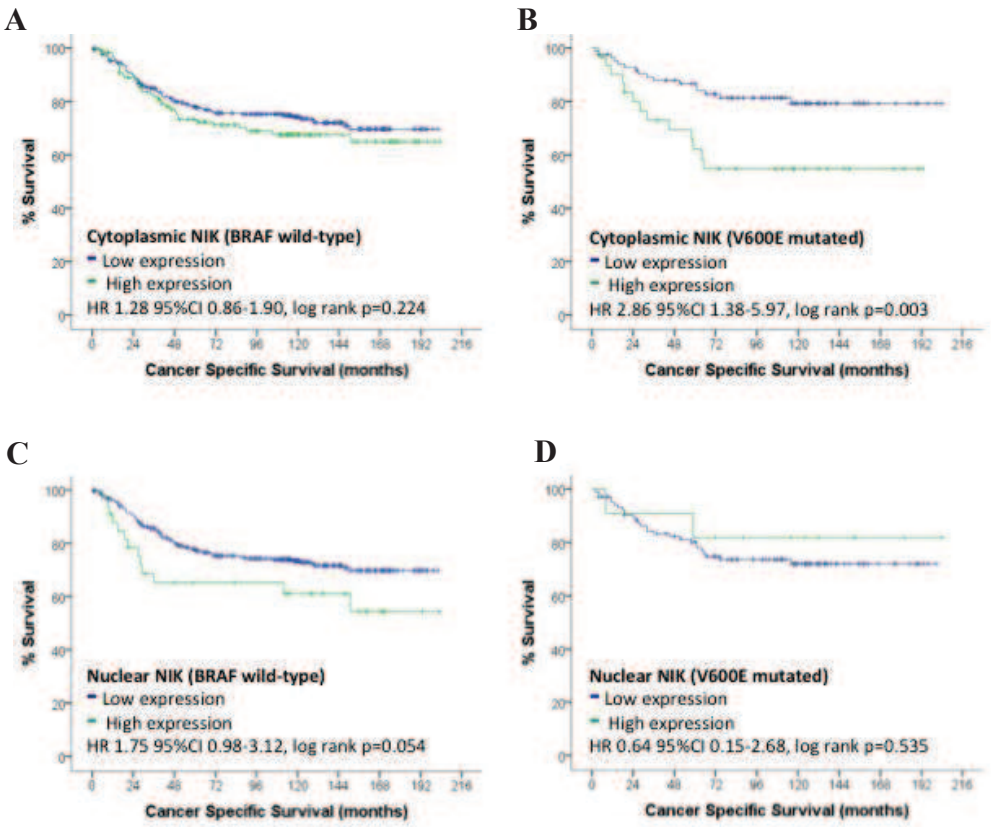


Figure 11: The association between NIK expression, survival and BRAF status in elective patients. *Cytoplasmic NIK expression was not associated with CSS in BRAF wild-type patients but was associated with CSS in BRAF V600E mutated patients (B). A trend towards reduced CSS was observed in BRAF wild-type patients with high nuclear expression of NIK (C) but not in BRAF mutated patients (D).*

3.3.5 Expression of IKKα

IKKα expression was investigated in 1030 patients. 281 patients had cores missing or insufficient tumour for analysis and following exclusions outlined in Figure 6, there were 680 patients included for analysis. Expression of IKKα was observed at the cytoplasmic and nuclear level. In addition, a proportion of patients demonstrated a distinct pattern of juxtanuclear punctate staining within the cytoplasm. Results will be discussed with respect to these three sites of IKKα expression. The histoscore range for cytoplasmic and nuclear IKKα ranged from 0 to 200 and from 0 to 137, respectively. Expression of cytoplasmic and nuclear IKKα was graded as either low or high. Thresholds were calculated using ROC curve analysis; >90 for cytoplasmic IKKα and >13 for nuclear IKKα was considered as high expression. Examples of low and high cytoplasmic and nuclear IKKα expression are displayed in Figure 12. Punctate expression of IKKα could not be assessed using the weighted histoscore and was therefore graded as absent, weak, moderate or high expression depending on the number and size of these discrete areas (Figures 13A-D). Cytoplasmic expression of IKKα negatively correlated with nuclear expression of IKKα (Pearson's $r=-0.126$, $p<0.001$). As punctate expression was not a continuous variable Chi-square test was used to compare it with cytoplasmic/nuclear expression. Punctate expression of IKKα was not associated with cytoplasmic expression ($p=0.294$), however, punctate expression of IKKα was inversely associated with nuclear IKKα ($p=<0.001$). There was good correlation of scores between observers with ICCC scores of 0.77 for cytoplasmic, 0.91 for nuclear expression of IKKα and 0.70 for punctate expression of IKKα.

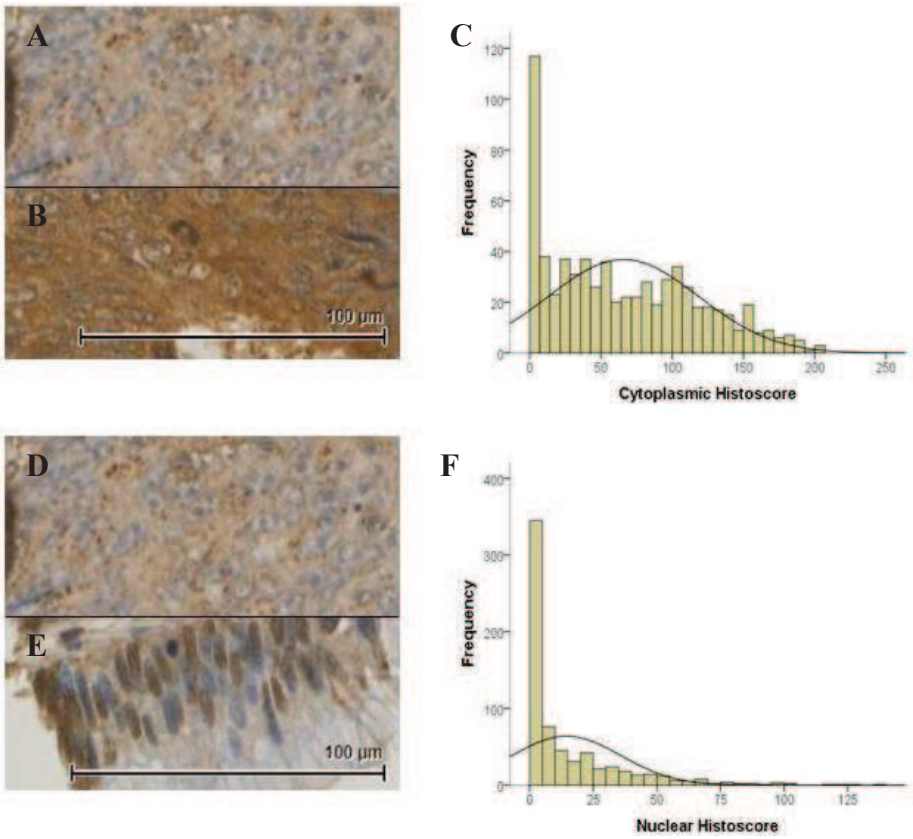
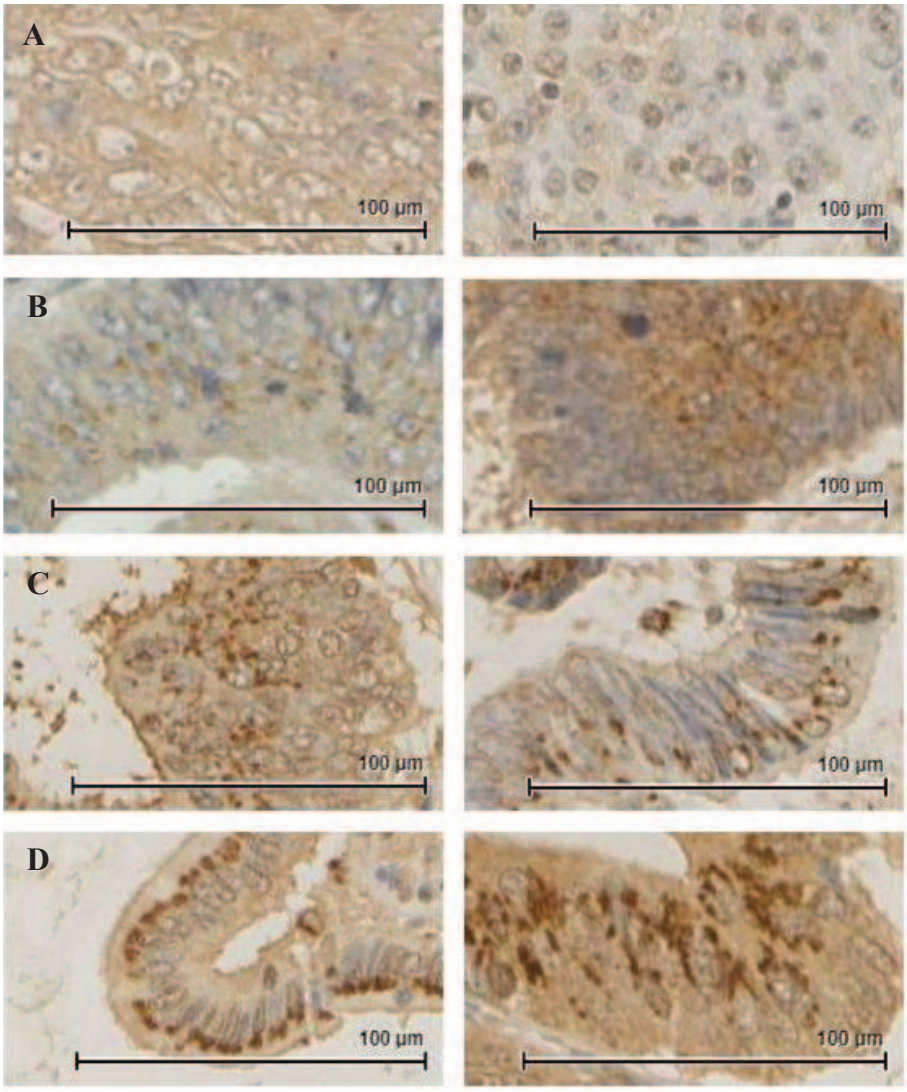


Figure 12: Expression of IKKα. *A) Examples of low and (B) high cytoplasmic, (D) low and (E) high nuclear IKKα expression in epithelial colorectal cancer tissue, brown represents the protein of interest (images taken from TMA at 20x20 magnification, scale bar represents 100µm). Histograms show distribution of average histoscores across three tumour cores for cytoplasmic (C) and nuclear IKKα (F).*



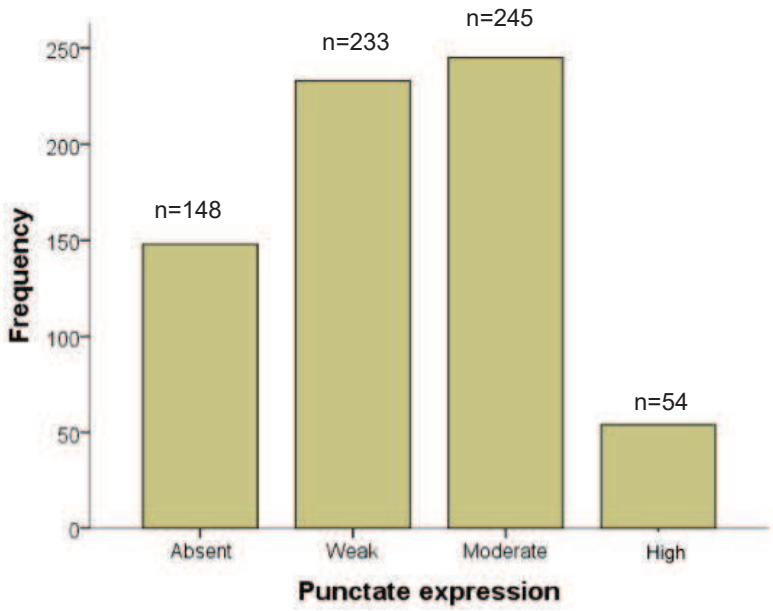


Figure 13: Punctate expression of IKKα. *Examples of (A) absent (B) low (C) moderate and (D) high punctate IKKα expression in colorectal cancer tissue. (images taken at 20x20 magnification, scale bar represents 100µm). Bar chart (E) shows frequency of expression categories.*

3.3.6 IKKα, clinicopathological characteristics and the tumour microenvironment

Expression of cytoplasmic IKKα was not associated with patient age (p=0.641), sex (p=0.806), type of surgery (elective/emergency) (p=0.878), tumour location (right colon/left colon/rectum) (p=0.164) or administration of adjuvant chemotherapy (p=0.813). In terms of tumour characteristics, high expression of cytoplasmic IKKα was associated with increasing T stage (p=0.012), poor tumour differentiation (p=0.010), tumour necrosis (p=0.013), low proliferation (p=0.013) and BRAF wild-type status (p=0.011) but not with N stage (p=0.175), TNM stage (p=0.231), venous invasion (p=0.093), margin involvement (p=0.501) or MMR status (p=0.378). With respect to the tumour microenvironment, expression of cytoplasmic IKKα was not associated with Klintrup-Mäkinen grade (p=0.396) or tumour stroma percentage (p=0.759). Associations between cytoplasmic IKKα, clinicopathological characteristics and the tumour microenvironment are displayed in Table 7.

Expression of nuclear IKKα was not associated with patient age (p=0.751), sex (p=0.087), type of surgery (p=0.254), tumour location (p=0.562) or administration of adjuvant chemotherapy (p=0.192). In terms of tumour characteristics, high expression of nuclear IKKα was associated with decreasing T stage (p=0.001), decreasing TNM stage (p=0.001), more favourable tumour differentiation (0.039) and high proliferation (p=0.025) but not with N stage (p=0.276), venous invasion (p=0.586), margin involvement (p=0.603), tumour necrosis (p=0.074), MMR status (p=0.356) or BRAF status (p=0.699). With respect to the tumour microenvironment, expression of nuclear IKKα was not associated with Klintrup-Mäkinen grade (p=0.216) or tumour stroma percentage (p=0.248). Associations between nuclear IKKα, clinicopathological characteristics and the tumour microenvironment are displayed in Table 7.

Expression of punctate IKKα was associated with male sex (p=0.028) but not with patient age (p=0.499), type of surgery (p=0.471) or tumour location (p=0.317). In terms of tumour characteristics, high expression of punctate IKKα was associated with more favourable tumour differentiation (p=0.001), absence of necrosis (p=0.004), low proliferation (p=0.044) and MMR competence (p<0.001). There was no association between expression of punctate IKKα and TNM stage (p=0.087), venous invasion (p=0.571), margin involvement (p=0.231) or BRAF status (p=0.317). With respect to the tumour microenvironment, expression of punctate IKKα was not associated with Klintrup-

Mäkinen grade (p=0.598) or tumour stroma percentage (p=0.809). Associations between punctate IKKα expression, clinicopathological characteristics and the tumour microenvironment are displayed in Table 8. Although expression of punctate IKKα was not associated with administration of adjuvant chemotherapy (p=0.732), it was of interest that of the 31 cancer deaths amongst patients who had received adjuvant chemotherapy, 7 patients had no expression of punctate IKKα, 6 patients had low expression of punctate IKKα, 14 patients had moderate expression of punctate IKKα and 3 patients had high expression of punctate IKKα. These results were an observation and not the result of any statistical test.

		All *n*= 680	Low cytoplasmic IKKα expression *n*=449	High cytoplasmic IKKα expression *n*=231	*p*	Low nuclear IKKα expression *n*=457	High nuclear IKKα expression *n*=223	*p*
Host Characteristics								
Age (n=680)	**<65**	208 (31)	140 (31)	68 (29)	0.641	138 (30)	70 (31)	0.751
	>65	472 (69)	309 (69)	163 (71)		319 (70)	153 (69)	
Sex (n=680)	**Female**	337 (50)	221 (49)	116 (50)	0.806	216 (47)	121 (54)	0.087
	Male	343 (50)	228 (51)	115 (50)		241 (53)	102 (46)	
Type of surgery (n=679)	**Elective**	546 (80)	361 (81)	185 (80)	0.878	362 (79)	184 (83)	0.254
	Emergency	133 (20)	87 (19)	46 (20)		95 (21)	38 (17)	
Tumour location (n=676)	**Right**	284 (42)	192 (43)	92 (40)	0.164	197 (43)	87 (39)	0.562
	Left	243 (36)	150 (34)	93 (41)		162 (36)	81 (37)	
	Rectum	149 (22)	105 (23)	44 (19)		96 (21)	53 (24)	
Tumour Characteristics								
T stage (n=680)	**1-2**	115 (17)	82 (19)	33 (15)	**0.012**	59 (13)	56 (25)	**0.001**
	3	377 (55)	256 (57)	121 (52)		265 (58)	112 (50)	
	4	188 (28)	111 (24)	77 (33)		133 (29)	55 (25)	

N stage (n=678)	**0** **1** **2**	429 (63) 179 (27) 70 (10)	287 (64) 121 (27) 39 (9)	142 (62) 58 (25) 31 (13)	0.175	279 (61) 132 (29) 46 (10)	150 (68) 47 (21) 24 (11)	0.276
TNM stage (n=680)	**I** **II** **III**	94 (14) 333 (49) 253 (37)	67 (15) 220 (49) 162 (36)	27 (12) 113 (49) 91 (39)	0.231	47 (10) 229 (50) 181 (40)	47 (21) 104 (47) 72 (32)	**0.001**
Tumour differentiation (n=680)	**Mod/well** **Poor**	611 (90) 69 (10)	413 (92) 36 (8)	198 (86) 33 (14)	**0.010**	403 (88) 54 (12)	208 (93) 15 (7)	**0.039**
Venous invasion (n=680)	**No** **Yes**	457 (67) 223 (33)	292 (65) 157 (35)	165 (71) 66 (29)	0.093	304 (67) 153 (33)	153 (69) 70 (31)	0.586
Margin involvement (n=680)	**No** **Yes**	642 (94) 38 (6)	422 (94) 27 (6)	220 (95) 11 (5)	0.501	430 (94) 27 (6)	212 (95) 11 (5)	0.603
Necrosis (n=668)	**Absent** **Present**	405 (61) 263 (39)	284 (64) 160 (36)	121 (54) 103 (46)	**0.013**	261 (58) 187 (42)	144 (66) 76 (34)	0.074
Proliferation (n=675)	**Low** **High**	273 (40) 402 (60)	165 (37) 280 (63)	108 (47) 122 (53)	**0.013**	197 (43) 257 (57)	76 (34) 145 (66)	**0.025**
MMR status (n=676)	**Proficient** **Deficient**	564 (83) 112 (17)	377 (84) 70 (16)	187 (82) 42 (18)	0.378	383 (84) 71 (16)	181 (82) 41 (18)	0.356
BRAF status (n=668)	**Wild-type**	518 (78)	330 (75)	188 (83)	**0.011**	347 (77)	171 (78)	0.699

	V600E	150 (22)	112 (25)	38 (17)		103 (23)	47 (22)	
Tumour microenvironment								
Klintrup-Mäkinen grade (n=670)	**Weak** **Strong**	447 (67) 223 (33)	292 (66) 153 (34)	155 (69) 70 (31)	0.396	306 (68) 142 (32)	141 (64) 81 (36)	0.216
Tumour stroma percentage (n=660)	**Low** **High**	514 (78) 146 (22)	338 (78) 98 (22)	176 (79) 48 (21)	0.759	340 (77) 104 (23)	174 (81) 42 (19)	0.248
Adjuvant therapy (n=261)	**No** **Yes**	166 (64) 95 (36)	109 (64) 61 (36)	57 (63) 34 (37)	0.813	119 (61) 75 (39)	47 (70) 20 (30)	0.192

Table 7: IKKα expression, associations with clinicopathological characteristics and the tumour microenvironment in patients undergoing surgery (elective and emergency) for stage I-III colorectal cancer

		All *n*=680	Absent punctate IKKα expression n=148	Low punctate IKKα expression *n*=233	Moderate IKKα punctate expression *n*=245	High punctate IKKα expression *n*=54	*p*
Host Characteristics							
Age (n=680)	**<65**	208 (31)	45 (30)	71 (30)	69 (28)	23 (43)	0.499
	>65	472 (69)	103 (70)	162 (70)	176 (72)	31 (57)	
Sex (n=680)	**Female**	337 (50)	86 (58)	114 (49)	118 (48)	19 (35)	**0.028**
	Male	343 (50)	62 (42)	119 (51)	127 (52)	35 (65)	
Type of surgery (n=679)	**Elective**	546 (80)	118 (78)	195 (84)	193 (79)	43 (80)	0.471
	Emergency	133 (20)	32 (22)	38 (16)	52 (21)	11 (20)	
Tumour location (n=676)	**Right**	284 (42)	68 (46)	99 (43)	90 (37)	27 (50)	0.317
	Left	243 (36)	54 (37)	81 (35)	92 (38)	16 (30)	
	Rectum	149 (22)	25 (17)	51 (22)	62 (25)	11 (20)	
Tumour Characteristics							
T stage (n=680)	**1-2**	115 (17)	25 (17)	42 (18)	39 (16)	9 (17)	0.561
	3	377 (55)	83 (56)	130 (56)	134 (55)	30 (55)	
	4	188 (28)	40 (27)	61 (36)	72 (29)	15 (28)	

N stage (n=678)	**0** **1** **2**	429 (63) 179 (27) 70 (10)	97 (66) 27 (18) 23 (16)	154 (66) 60 (26) 19 (8)	152 (62) 69 (28)	26 (48) 23 (43) 5 (9)	0.464
TNM stage (n=680)	**I** **II** **III**	94 (14) 333 (49) 253 (37)	22 (15) 76 (51) 50 (34)	33 (14) 118 (51) 82 (35)	32 (13) 120 (49) 93 (38)	7 (13) 19 (35) 28 (52)	0.087
Tumour differentiation (n=680)	**Mod/well** **Poor**	611 (90) 69 (10)	120 (81) 28 (19)	213 (91) 20 (9)	228 (93) 17 (7)	50 (93) 4 (7)	**0.001**
Venous invasion (n=680)	**No** **Yes**	457 (67) 223 (33)	96 (65) 52 (35)	158 (68) 75 (32)	166 (68) 79 (32)	37 (69) 17 (31)	0.571
Margin involvement (n=680)	**No** **Yes**	642 (94) 38 (6)	137 (93) 11 (7)	219 (94) 14 (6)	235 (96) 10 (4)	51 (94) 3 (6)	0.231
Necrosis (n=668)	**Absent** **Present**	405 (61) 263 (39)	81 (56) 63 (44)	125 (55) 104 (45)	161 (67) 81 (33)	38 (72) 15 (28)	**0.004**
Proliferation (n=675)	**Low** **High**	273 (40) 402 (60)	55 (38) 91 (62)	82 (35) 150 (65)	111 (46) 132 (54)	25 (46) 29 (54)	**0.044**
MMR status (n=676)	**Proficient** **Deficient**	564 (83) 112 (17)	105 (71) 42 (29)	199 (86) 32 (14)	214 (87) 31 (13)	46 (87) 7 (13)	**<0.001**
BRAF status (n=668)	**Wild-type**	518 (78)	111 (76)	173 (77)	196 (81)	38 (70)	0.317

	V600E	150 (22)	35 (24)	53 (23)	46 (19)	16 (30)	
Tumour microenvironment							
Klintrup-Mäkinen grade (n=670)	**Weak**	447 (67)	93 (64)	164 (72)	158 (65)	32 (60)	0.598
	Strong	223 (33)	53 (36)	65 (28)	84 (35)	21 (40)	
Tumour stroma percentage (n=660)	**Low**	514 (78)	112 (77)	178 (78)	188 (79)	36 (72)	0.809
	High	146 (22)	33 (23)	49 (22)	50 (21)	14 (28)	
Adjuvant therapy (n=261)	**No**	166 (64)	32 (64)	55 (63)	56 (61)	23 (72)	0.732
	Yes	95 (36)	18 (36)	32 (37)	36 (39)	9 (28)	

Table 8: Punctate expression of IKK*α*, associations with clinicopathological characteristics and the tumour microenvironment in patients undergoing surgery (elective and emergency) for stage I-III colorectal cancer

3.3.7 IKKα expression and survival

To determine whether IKKα expression was significantly associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic, nuclear and punctate expression were plotted; levels of expression were compared using the log rank test. Expression of cytoplasmic IKKα was not associated with CSS (Figure 14A), however, when patients who had undergone elective surgery were considered alone (n=546), there was a non-significant association between high expression of IKKα and reduced CSS (p=0.064) (Figure 14C). There was no association between cytoplasmic IKKα expression, CSS and tumour location. There was no association between cytoplasmic IKKα expression, CSS and MMR status (Figure 15). There was no association between cytoplasmic IKKα expression, CSS and BRAF status.

Expression of nuclear IKKα was not associated with CSS in the full cohort (p=0.993) (Figure 14B), in elective patients (p=0.959) or when considered in the context of tumour location (right colon p=0.739; left colon p=0.774; rectum p=0.554). There was no association between nuclear expression of IKKα, CSS and MMR status. There was no association between nuclear IKKα expression, CSS and BRAF status.

High expression of punctate IKKα was associated with significantly reduced CSS compared to those patients with low expression (HR 1.20 95% CI 1.02-1.42, p<0.001) (Figure 14D), with a reduction in 5-year survival from 83% to 61% and a reduction in 10-year survival from 78% to 49%. In the context of tumour location expression of punctate IKKα was significantly associated with CSS in patients with rectal cancer (HR 1.51 95% CI 1.02-2.24, p=0.030) when compared to patients with tumours in the right colon (HR 1.12 95% CI 0.88-1.43, p=0.011) and left colon (HR 1.18 95% CI 0.89-1.56, p=0.017). There was no association between punctate IKKα, CSS and MMR status (Figure 16). In patients with BRAF wild-type status, high expression of punctate IKKα was associated with reduced CSS (HR 1.38 95% CI 1.10-1.72, p=0.005) when compared to patients harbouring the BRAF V600E mutation (HR 1.04 95% CI 0.69-1.56, p=0.014) (Figure 17).

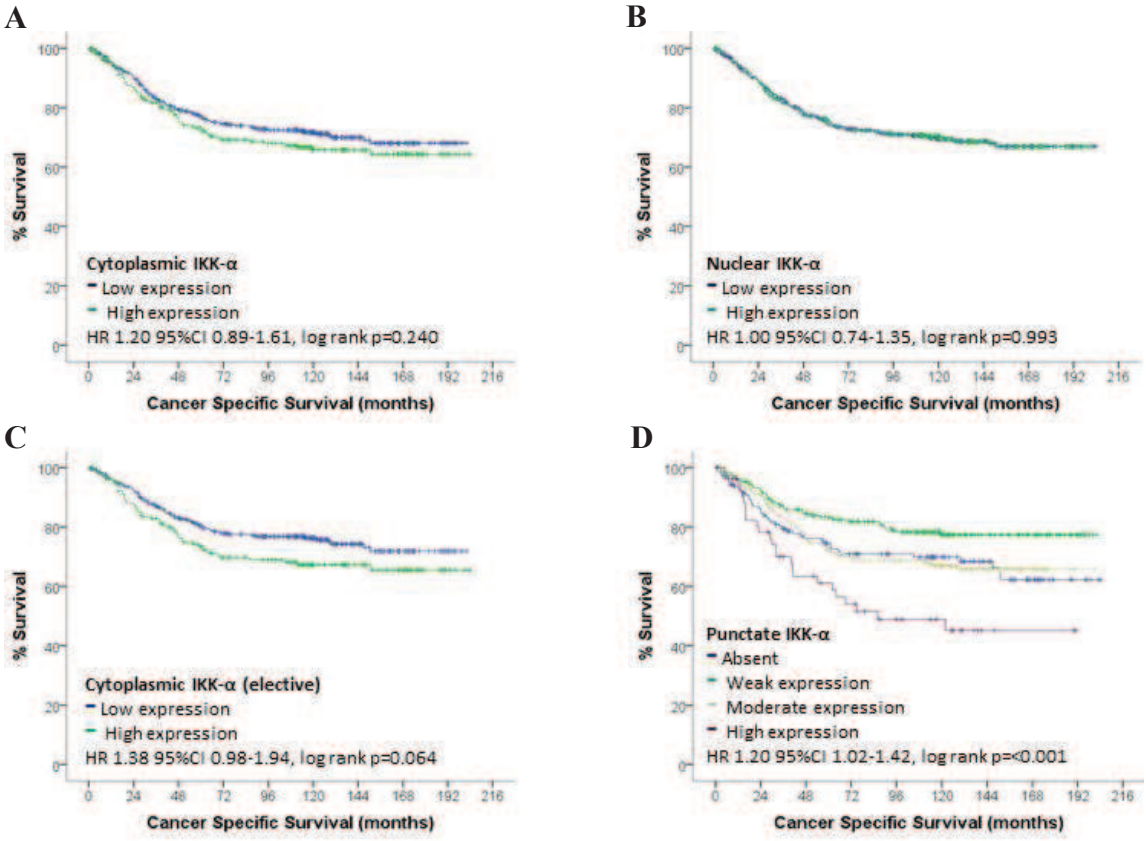


Figure 14: IKKα expression and survival. *Expression of IKKα in the cytoplasm (A) or nucleus (B) was not associated with CSS in the full cohort. There was a trend towards reduced CSS in patients with high expression of cytoplasmic IKKα who had undergone elective surgery (C). Punctate expression of IKKα was associated with CSS in the full cohort (D).*

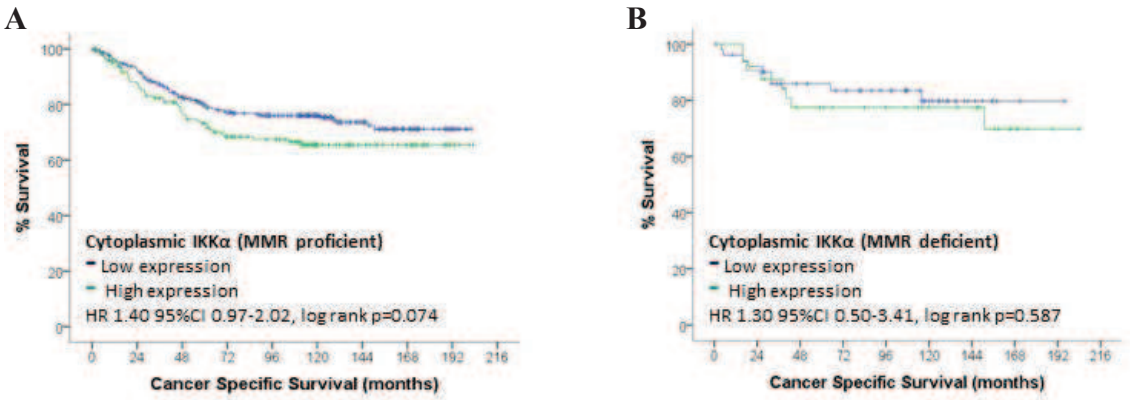


Figure 15: The association between cytoplasmic IKKα expression, survival and MMR status in elective patients. *Expression of IKKα in the cytoplasm was not associated with CSS in MMR proficient (A) or MMR deficient (B) patients.*

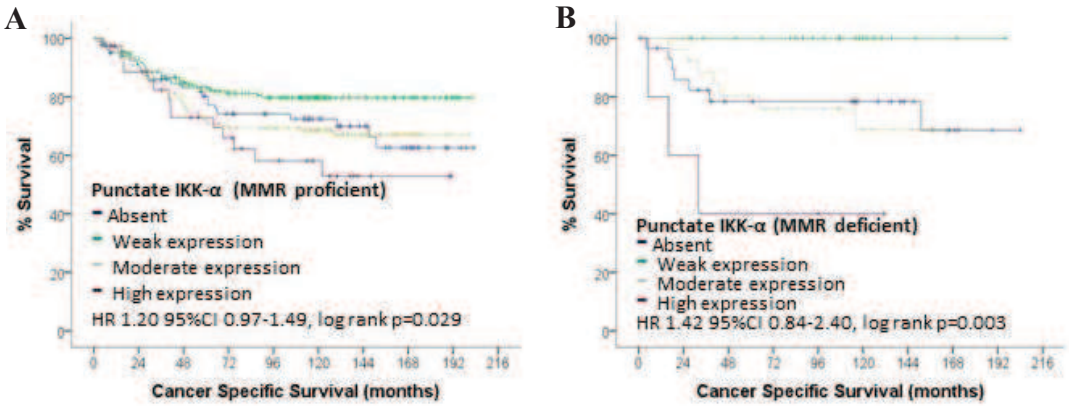


Figure 16: The association between punctate expression of IKKα, survival and MMR status in elective patients. *There was no association between punctate IKKα and CSS in MMR proficient (A) or MMR deficient (B) patients.*

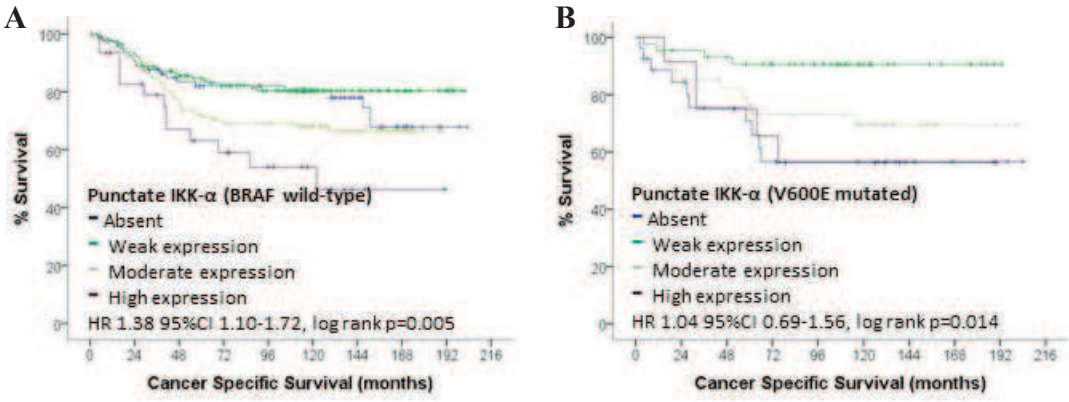


Figure 17: The association between punctate IKKα expression, survival and BRAF status in elective patients. *Punctate IKKα expression was associated with CSS in BRAF wild-type patients (A) but was not associated with CSS in BRAF V600E mutated patients (B).*

3.3.8 Expression of RelB

RelB expression was investigated in 1030 patients. 267 patients had cores missing or insufficient tumour for analysis and following exclusions outlined in Figure 6, there were 689 patients included for analysis. Expression of RelB was observed at the cytoplasmic and nuclear level. The histoscore range for cytoplasmic and nuclear RelB was 0 to 253 and 0 to 132, respectively. Expression of RelB was graded as either low or high. Thresholds were calculated using ROC curve analysis; >66 for cytoplasmic was considered as high expression. Due to a low number of patients with expression of nuclear RelB, for the purposes of analysis, nuclear expression of RelB was considered as either absent or present. Examples of low and high cytoplasmic and nuclear expression are displayed in Figure 18. On Chi-square analysis, there was an inverse association between cytoplasmic and nuclear expression of RelB ($p<0.001$). There was good correlation of scores between observers with ICCC scores of 0.74 for cytoplasmic expression and 0.97 for nuclear expression of RelB.

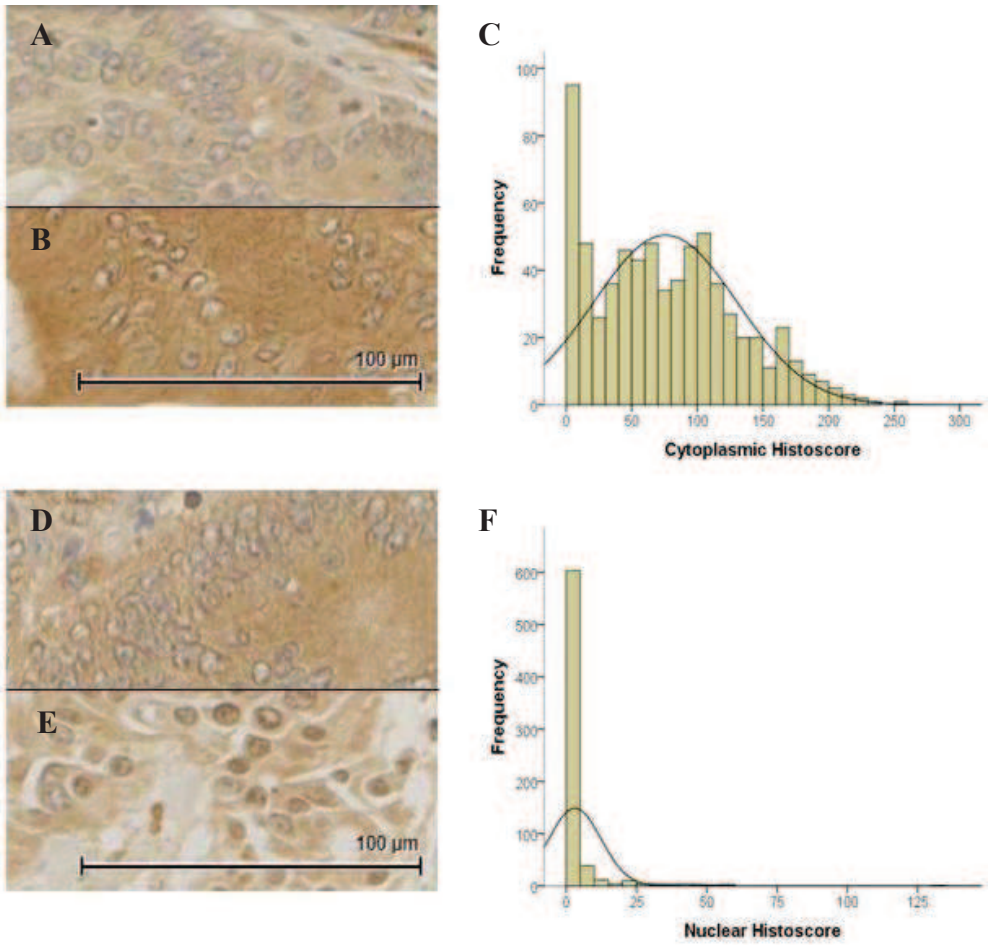


Figure 18: Expression of RelB. *A) Examples of low and (B) high cytoplasmic, (D) low and (E) high nuclear RelB expression in epithelial colorectal cancer tissue, brown represents the protein of interest (images taken from TMA at 20x20 magnification, scale*

bar represent 100μm). Histograms show distribution of average histoscores across three tumour cores for cytoplasmic (C) and nuclear RelB (F).

3.3.9 RelB, clinicopathological characteristics and the tumour microenvironment

Expression of cytoplasmic RelB was not associated with patient age (p=0.643), sex (p=0.941), type of surgery (elective/emergency) (p=0.486), tumour location (right colon/left colon/rectum) (p=0.060) or administration of adjuvant chemotherapy (p=0.122). In terms of tumour characteristics, high expression of cytoplasmic RelB was associated with increased tumour proliferation (p=0.029) but was not associated with TNM stage (p=0.516), tumour differentiation (p=0.925), venous invasion (p=0.973), margin involvement (p=0.324), tumour necrosis (p=0.166), BRAF status (p=0.199) or MMR status (p=0.355). With respect to the tumour microenvironment, expression of cytoplasmic RelB was not associated with Klintrup-Mäkinen grade (p=0.887) or tumour stroma percentage (p=0.316).

Expression of nuclear RelB was not associated with patient age (p=0.083), sex (p=0.446), type of surgery (elective/emergency) (p=0.139), tumour location (p=0.208) or administration of adjuvant chemotherapy (p=0.056). In terms of tumour characteristics, high expression of nuclear RelB was associated with high tumour proliferation (p=0.032) but was not associated with TNM stage (p=0.502), tumour differentiation (p=0.123), venous invasion (p=0.966), margin involvement (p=0.674), tumour necrosis (p=0.208), BRAF status (p=0.778) or MMR status (p=0.153). With respect to the tumour microenvironment, expression of nuclear RelB was not associated with Klintrup-Mäkinen grade (p=0.256) or tumour stroma percentage (p=0.860).

3.3.10 RelB expression and survival

To determine whether RelB expression was significantly associated with clinical outcome, Kaplan-Meier survival curves were plotted, low and high expression was compared using the log rank test. Expression of cytoplasmic RelB was not associated with CSS in the full cohort (p=0.748), in elective patients (p=0.726) or when considered in the context of tumour location (right colon p=0.481; left colon p=0.148; rectum p=0.814). There was no association between cytoplasmic RelB expression, CSS and MMR status. There was no association between cytoplasmic RelB expression, CSS and BRAF status.

Expression of nuclear RelB was not associated with CSS in the full cohort (p=0.520), in elective patients (p=0.349) or when considered in the context of tumour location (right colon p=0.170; left colon p=0.479; rectum p=0.260). There was no association between nuclear RelB expression, CSS and MMR status. There was no association between nuclear RelB expression, CSS and BRAF status.

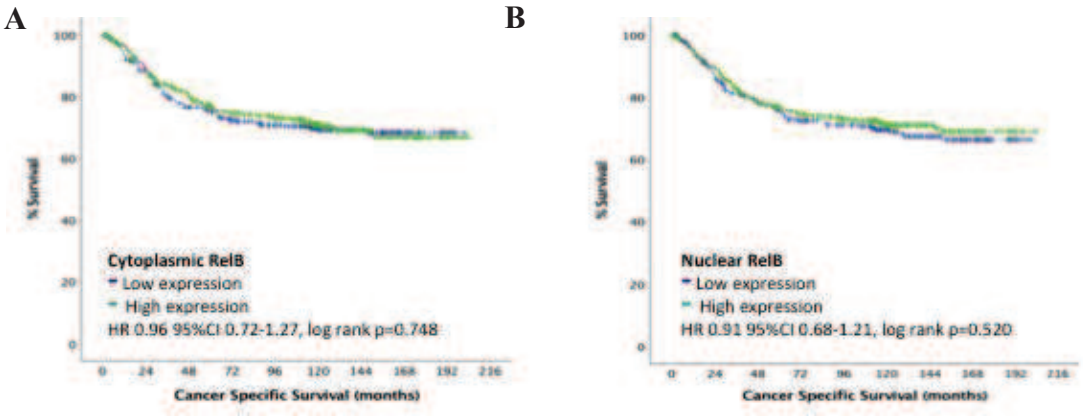


Figure 19: RelB expression and survival. *Expression of RelB in the (A) cytoplasm and (B) nucleus was not associated with CSS.*

RelB should be expressed in the cytoplasm and nucleus. A low number of patients with expression of nuclear RelB suggested a lack of non-canonical pathway activation. Alternatively, it may reflect an issue with detection of the protein. It is possible that the protein undergoes conformational change as it translocates to the nucleus and therefore my not be detected by the antibody in use. Previous studies in the host lab have also reported low levels of nuclear RelB in breast cancer (Lindsay Bennett) and so future studies should consider using a different RelB antibody.

3.3.11 Multivariate survival analysis

Univariate and multivariate survival analysis was performed in the full cohort. On univariate analysis, type of surgery (elective/emergency) ($p<0.001$), TNM stage ($p<0.001$), tumour differentiation ($p<0.001$), venous invasion ($p<0.001$), margin involvement ($p<0.001$), tumour necrosis ($p=0.039$), proliferation index ($p<0.001$), Klintrup-Mäkinen grade ($p<0.001$), tumour stroma percentage ($p<0.001$) and punctate IKKα expression ($p<0.001$) were associated with reduced CSS. However, age ($p=0.815$), sex ($p=0.331$), tumour location ($p=0.918$), MMR status ($p=0.272$), BRAF status ($p=0.997$), adjuvant therapy ($p=0.957$), cytoplasmic NIK ($p=0.106$), nuclear NIK ($p=0.732$), cytoplasmic IKKα ($p=0.240$), nuclear IKKα ($p=0.993$), cytoplasmic RelB ($p=0.748$) and nuclear RelB ($p=0.520$) were not associated with CSS (Table 9).

Variables that were significant on univariate analysis ($p<0.05$) were entered into a multivariate model with backward conditional method; type of surgery (elective/emergency) (HR 1.61 95% CI 1.15-2.25, $p=0.006$), TNM stage (HR 2.21 95% CI 1.68-2.90, $p<0.001$), venous invasion (HR 1.51 95% CI 1.11-2.06, $p=0.008$), margin involvement (HR 2.55 95% CI 1.57-4.17, $p=<0.001$), Klintrup-Mäkinen grade (HR 0.51 95% CI 0.35-0.74, $p<0.001$), tumour stroma percentage (HR 1.54 95% CI 1.12-2.13, $p=0.008$) and punctate IKKα (HR 1.22 95% CI 1.02-1.45, $p=0.027$) were independently associated with reduced CSS (Table 9).

Clinicopathological characteristics	Univariate HR (95% CI)	*P*	Multivariate analysis	*P*
Age (<65/>65)	1.00 (0.99 – 1.01)	0.815		
Sex (Female/Male)	1.13 (0.88 – 1.44)	0.331		
Type of surgery (Elective/Emergency)	1.96 (1.50 – 2.56)	<0.001	1.61 (1.15 – 2.25)	**0.006**
Tumour location (Right/Left/Rectum)	0.97 (0.83 – 1.14)	0.918		
TNM stage (I/II/III)	2.48 (2.01 – 3.06)	<0.001	2.21 (1.68 – 2.90)	**<0.001**
Tumour differentiation (Mod-well/poor)	2.02 (1.45 – 2.82)	<0.001		
Venous invasion (No/Yes)	2.14 (1.68 – 2.73)	<0.001	1.51 (1.11 – 2.06)	**0.008**
Margin involvement (No/Yes)	3.44 (2.35 – 5.04)	<0.001	2.55 (1.57 – 4.17)	**<0.001**
Necrosis (Absent/Present)	1.30 (1.01 – 1.66)	0.039		
Proliferation (High/Low)	0.64 (0.50 – 0.81)	<0.001		
MMR status (Proficient/Deficient)	0.83 (0.59 – 1.16)	0.272		
BRAF status (Wild-type/V600E)	1.00 (0.74 – 1.35)	0.997		
Tumour microenvironment				
Klintrup-Mäkinen grade (Weak/Strong)	0.40 (0.29 – 0.55)	<0.001	0.51 (0.35-0.74)	**<0.001**
Tumour stroma percentage (Low/High)	1.95 (1.50 – 2.52)	<0.001	1.54 (1.12-2.13)	**0.008**

Adjuvant therapy (No/Yes)	1.01 (0.68 – 1.50)	0.957		
Cytoplasmic NIK (Low/High)	1.27 (0.95-1.71)	0.106		
Nuclear NIK (Low/High)	1.08 (0.68-1.72)	0.732		
Cytoplasmic IKKα (Low/High)	1.19 (0.89 – 1.61)	0.240		
Nuclear IKKα (Low/High)	1.00 (0.74 – 1.35)	0.993		
Punctate IKKα (Absent/Low/Mod/High)	1.23 (1.02 – 1.42)	<0.001	1.22 (1.02 – 1.45)	**0.027**
Cytoplasmic RelB (Low/High)	0.96 (0.72-1.27)	0.748		
Nuclear RelB (Absent/Present)	0.91 (0.68-1.21)	0.520		

Table 9: Univariate and multivariate analysis in patients undergoing surgery (elective and emergency) for stage I-III colorectal cancer

3.4 Associations between members of the non-canonical NF-κB pathway

To understand the relationship between expression of different pathway members, Pearson's correlation coefficient was used to analyse continuous variables and Chi-square test was used to analyse expression between non-continuous variables. Cytoplasmic NIK expression correlated with cytoplasmic IKKα (Pearson's r=0.437, p<0.001) and cytoplasmic expression of RelB (Pearson's r=0.359, p<0.001). As nuclear NIK and nuclear RelB were non-continuous variables, Chi-squared analysis was used to assess their associations with the other proteins. Nuclear expression of NIK was not associated with cytoplasmic IKKα (p=0.483) or nuclear IKKα (p=0.200) but was inversely associated with cytoplasmic RelB (p=0.050) and directly associated with nuclear RelB (p=0.003). Cytoplasmic IKKα correlated with cytoplasmic RelB (Pearson's r=0.821, p<0.001) but was inversely associated with nuclear RelB (p<0.001). Nuclear IKKα was inversely associated with cytoplasmic RelB (p=0.003) but was not associated with nuclear RelB (p=0.354). See Figure 20 for diagrammatic representation of protein associations.

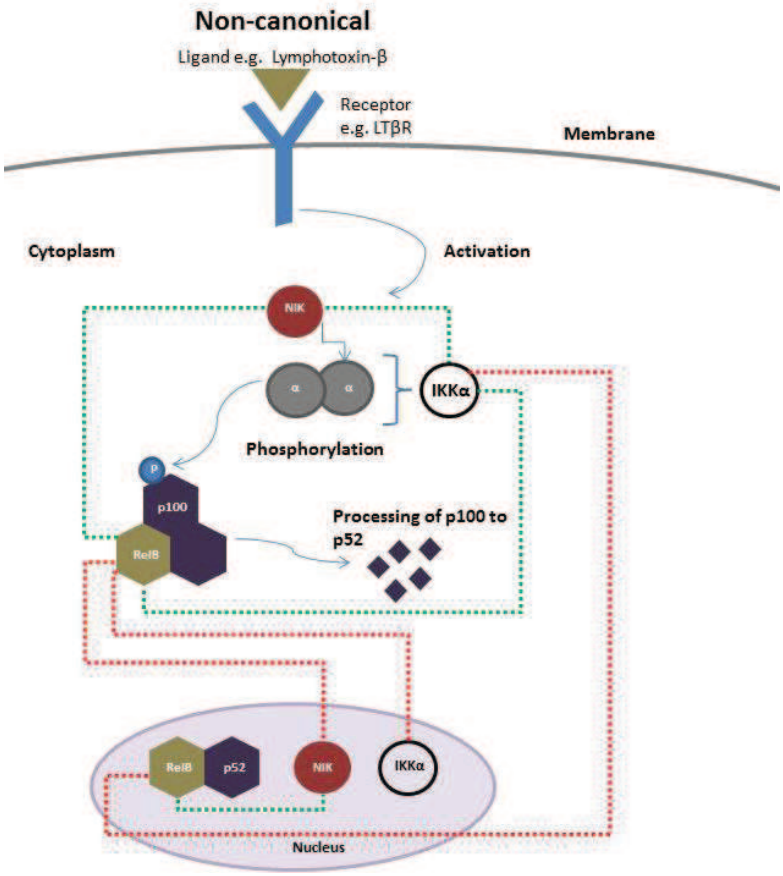


Figure 20: Associations between members of the non-canonical NF-κB pathway. *Green dashed line represents positive correlation/direct association and red dashed line represents negative correlation/indirect associations. Adapted from Patel et al* (286).

3.4.1 Combined cytoplasmic protein expression and survival

As there was a positive correlation between cytoplasmic NIK, IKKα and RelB, expression levels were combined to create two groups 1) low expression of NIK, IKKα and RelB (n=182) and 2) patients with high expression of one or more pathway members (n=381). In the full cohort, there was no association between these groups and survival (p=0.227). When patients who underwent elective surgery were considered alone, those patients with high expression of one or more proteins had significantly reduced CSS (HR 1.59 95% CI 1.06-2.40, p=0.025) (Figure 21). There was no association between combined cytoplasmic proteins, CSS and tumour location (right colon p=0.313; left colon p=0.232; rectum p=0.106) (Figure 22). However, high expression of combined cytoplasmic proteins was associated with reduced CSS in MMR proficient patients (HR 1.89 95% CI 1.19-3.00, p=0.030) but not in MMR deficient patients (p=0.590). There was no association between expression of combined cytoplasmic proteins, CSS and BRAF status (Figure 23).

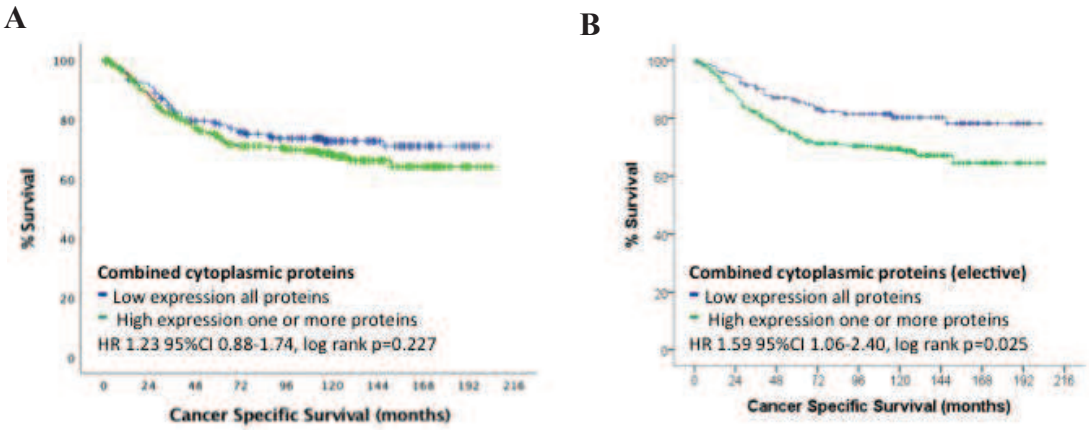


Figure 21: Combined cytoplasmic non-canonical proteins (NIK, IKKα and RelB) and survival. *There was no association between combined cytoplasmic proteins and CSS in the full cohort (A). High expression of one or more cytoplasmic proteins was associated with reduced CSS in patients who underwent elective surgery (B).*

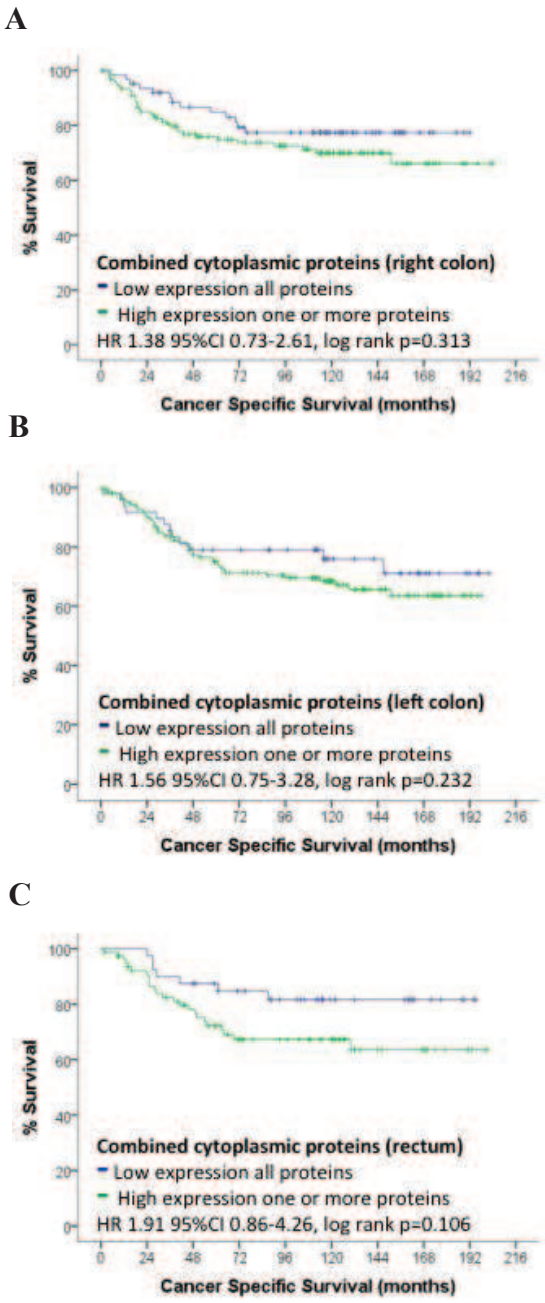


Figure 22: Combined cytoplasmic non-canonical proteins (NIK, IKKα and RelB), survival and tumour location in elective patients. *Expression of combined cytoplasmic proteins was not associated with tumour location (A-C).*

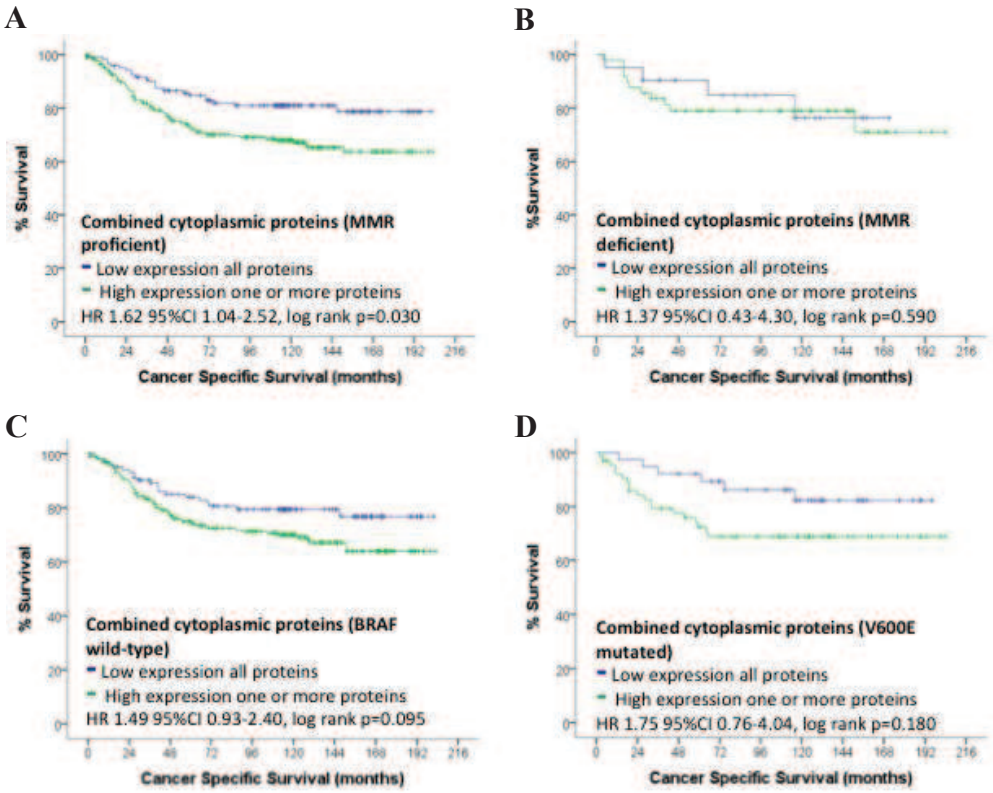


Figure 23: Combined cytoplasmic non-canonical proteins (NIK, IKKα and RelB), survival and MMR/BRAF status in elective patients. *Expression of combined cytoplasmic proteins was associated with CSS in MMR proficient patients (A) but not in patients with MMR deficient status (B). There was no association between expression of combined cytoplasmic proteins, CSS and BRAF status, (C) and (D).*

3.5 Chapter discussion

The results of this chapter demonstrate that in patients who have undergone surgery for stage I-III CRC, upregulation of the non-canonical NF-κB pathway was associated with adverse phenotypic tumour features and with inferior CSS. Some of the adverse features include increasing T stage, poor tumour differentiation, tumour necrosis, reduced tumour proliferation and reduced inflammatory cell infiltrate. There appears to be a relationship between cytoplasmic NIK expression and survival in patients with right-sided colon cancer. This relationship is further demonstrated by the association between cytoplasmic NIK expression and survival in patients who are MMR deficient and BRAF V600E mutated, two molecular characteristics that are well-established features of right-sided colon cancer. Recent retrospective analyses of large clinical trials of patients with metastatic CRC suggested patients with right-sided primary tumours have an inferior survival outcome when compared to patients with left-sided primary tumours. Therefore, it may be of interest to study the expression of NIK in a cohort of patients with metastatic disease. MMR deficient tumours are associated with a higher lymphocytic infiltrate and better survival outcome. Although it was not within the scope of these studies to characterise the immunological response within the tumour microenvironment, this work is currently being carried out in our lab and once complete, it would be of interest to study the association between innate/adaptive immune cells and non-canonical NF-κB proteins as the pathway is known to have essential roles in regulating these immune responses.

Expression of IKKα at the cytoplasmic level was associated with a number of adverse phenotypic tumour features. Conversely, high expression of IKKα at the nuclear level was associated with more a more favourable tumour phenotype. The explanation for this is unclear. It was of particular interest that an unfamiliar pattern of punctate IKKα expression was observed, the nature of which is uncertain. Moreover, this pattern was associated with inferior survival outcome in the full cohort and this association was potentiated in patients with rectal cancer and those who had BRAF wild-type status. It was noted that patients with low expression of punctate IKKα had improved survival outcome compared to those with absent/moderate/high expression. This low expression group may reflect a proportion of patients in who IKKα production and activity is still within normal homeostatic limits. Therefore, the other groups of expression may reflect those with aberrant IKKα production and activity outwith normal homeostatic functions which is responsible for the observed inferior survival outcomes. It was of particular interest that although this punctate pattern of IKKα was associated with inferior survival outcome, it was also associated with

favourable phenotypic features such as better tumour differentiation and absence of necrosis as well as less favourable features such as low proliferation. The explanation for this is unclear and it may reflect the extent of heterogeneity within the disease.

To understand if the proteins as a “panel” had more prognostic value, cytoplasmic proteins were considered together, high expression of one or more non-canonical NF-κB pathway members was associated with inferior CSS. The combined cytoplasmic protein expression score was not subjected to multivariate survival analysis, future studies should consider this to assess if it is independently associated with survival.

IHC is a widely used technique to selectively identify antigens. It is a multistep process that is prone to error and inconsistency at the experimental stage and during interpretation. Automated staining systems have the advantage of standardising the experimental technique and enabling run-to-run consistency, however, using machines has its own inherent problems and still requires knowledge and skill to optimise antibody staining. In the present study, protein expression was performed manually using the weighted histoscore, it has been suggested that automated image analysis software may be used as a more consistent method of measuring staining intensity. These systems measure staining intensity by measuring absorption. However, it is well known that one issue with using DAB as a chromogen is that there is only a linear relationship between the amount of antigen and staining intensity at lower levels of staining and for automated systems that rely on measuring absorption, the non-linear relationship between the amount of antigen and staining intensity at higher levels can be a source of inaccuracy.

As mentioned above, there are a number of factors that can impact on IHC staining outcomes. These include but are not limited to, age of the specimens, fixation methods, storage, duration and type of antigen retrieval and antibody specificity. It is well known that proteins are stable when tissues are preserved in wax blocks however; there is evidence of deterioration once paraffin sections have been cut. In the present study, low numbers of patients had expression of RelB at the nuclear level with a median histoscore of 0.33. This may reflect an issue with the antibody and subsequent detection of the protein. Whilst the antibody has previously been validated in prostate cancer cells, further validation may be required in CRC cells. Alternative methods of protein detection such as mass spectrometry could be used to assess protein expression however such techniques lack the practical properties and ease of clinical applicability that IHC offers.

Chapter 4:

Expression of members of the non-canonical NF-κB pathway in screen-detected T1/2 colorectal cancer specimens

4.1 Introduction

The results from Chapter 3 demonstrate that the non-canonical NF-κB pathway is upregulated in patients undergoing surgery for symptomatic stage I-III CRC and this was associated with tumour invasiveness and poor CSS. The bowel cancer screening programme captures a unique population of patients, many with early stage CRC. It is therefore of interest to understand whether or not the non-canonical NF-κB pathway is expressed in early stage disease and whether associations with phenotypic tumour characteristics or features of the tumour microenvironment are observed. We aimed to investigate the expression of key members of the non-canonical NF-κB pathway (NIK, IKKα and RelB) in a screen-detected T1/2 colorectal cancer patient tissue microarray and assess the clinical significance of each member.

4.2 Antibody validation

Antibody validation for NIK, IKKα and RelB was performed as described in results Chapter 3, section 3.2.

4.3 Expression and clinical outcome of members of the non-canonical pathway in screen-detected CRC

4.3.1 Study group

In total there were 159 patients who underwent removal (either local resection i.e. polypectomy/TEMS or, segmental bowel resection) for screen-detected CRC from the first round of gFOBT conducted within Greater Glasgow & Clyde health board. 69% were male and approximately two-thirds were over the age of 65. In all, 142 (89%) had TNM stage I disease and 17 (11%) had stage III disease; there were no patients with stage II disease. Overall, 21 (13%) patients had tumours located within the right colon, 95 (60%) within the left colon and 43 (27%) had rectal cancer. 40 (25%) patients underwent polypectomy, 4 (3%) underwent TEMS and 115 (72%) underwent segmental bowel resection. In total, 16 (10%) patients underwent adjuvant chemotherapy. Median follow-up of survivors was 91 months (IQR 83-95 months).

4.3.2 Expression of NIK

NIK expression was investigated in the full cohort of 159 patients. 43 patients had missing cores or insufficient tumour for analysis and following exclusions, 116 patients were included for analysis. Expression of NIK was observed at the cytoplasmic and nuclear level. The histoscore range for cytoplasmic NIK ranged from 40 to 200. Expression of cytoplasmic NIK was graded as either low or high. Thresholds for analysis were calculated using median values; a histoscore >148 was considered as high expression. Only 7 patients displayed expression of NIK at the nuclear level with a histoscore range from 0 to 15. For the purposes of analysis, nuclear expression of NIK was considered as either absent or present. On Chi-square analysis, there was no association between cytoplasmic and nuclear expression of NIK (p= 0.244). There was good correlation of scores between observers with ICCC scores of 0.94 for cytoplasmic expression and 0.99 for nuclear expression of NIK.

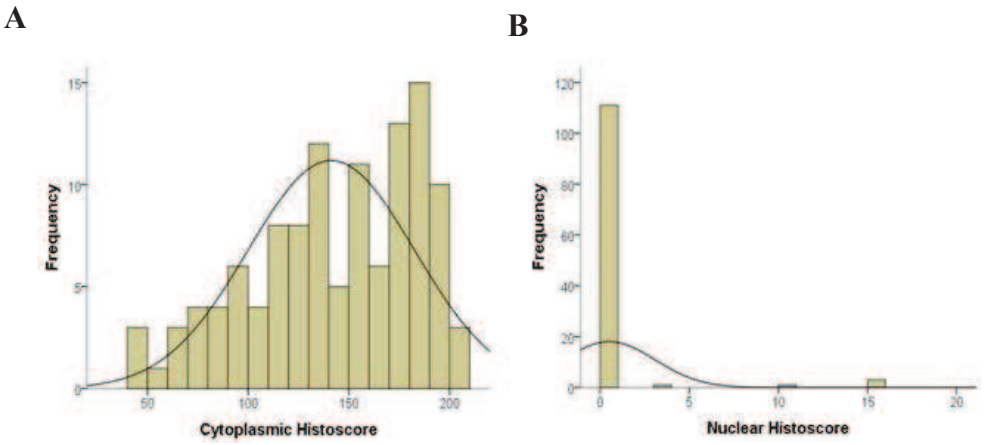


Figure 24: Expression of NIK. *Histograms show distribution of average histoscores across three tumour cores for cytoplasmic (A) and nuclear NIK (B).*

4.3.3 NIK, clinicopathological characteristics and the tumour microenvironment

Chi-square analysis was performed to assess the association with clinicopathological factors of the cohort including age, sex, type of surgery (polpectomy/TEMS or segmental bowel resection), tumour location (right colon/left colon/rectum), adjuvant therapy, TNM stage, tumour differentiation, venous invasion, proliferation, Klintrup-Mäkinen grade and tumour stroma percentage. Expression of cytoplasmic NIK was associated with greater likelihood of undergoing segmental bowel resection as opposed to polypectomy/TEMS

alone (p=0.020). However, there was no association with patient age (p=0.455), sex (p=1.000), tumour location (p=0.100) or administration of adjuvant chemotherapy (p=0.768). Cytoplasmic NIK was not associated with TNM stage (p=0.408), tumour differentiation (p=0.081), venous invasion (p=0.351) or proliferation (p=0.260). With respect to the tumour microenvironment, cytoplasmic NIK expression was not associated with Klintrup-Mäkinen grade (p=0.883) or tumour stroma percentage (p=0.639).

Expression of nuclear NIK was not associated with patient age (p=0.425), sex (p=0.113), type of surgery (p=0.342), tumour location (p=0.258) or administration of adjuvant chemotherapy (p=0.190). Nuclear NIK was not associated with TNM stage (p=0.295), tumour differentiation (p=0.658), venous invasion (p=0.975) or proliferation (p=0.384). With respect to the tumour microenvironment, nuclear NIK expression was not associated with Klintrup-Mäkinen grade (p=0.774) or tumour stroma percentage (p=0.429).

4.3.4 NIK expression and survival

To determine whether NIK expression was significantly associated with clinical outcome in this screen-detected cohort of T1/T2 CRC, Kaplan-Meier survival curves were plotted, low and high expression was compared using the log rank test. Expression of cytoplasmic NIK (p=0.051) or nuclear NIK was not associated with OS (p=0.788) (Figure 25).

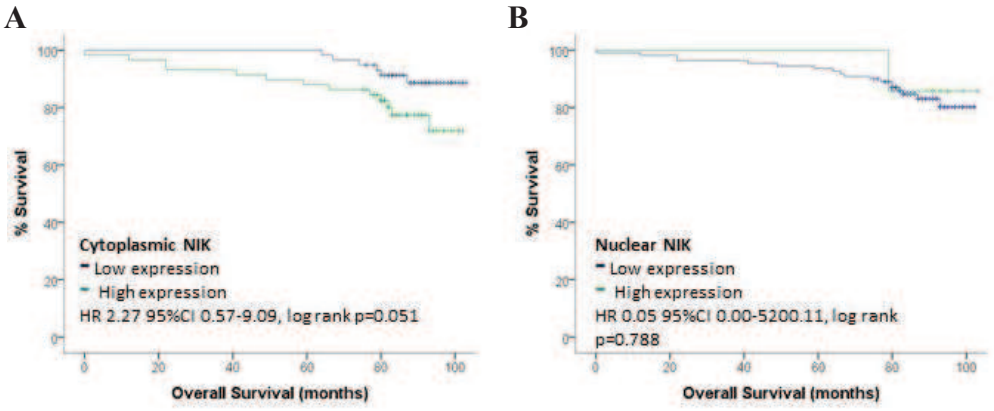


Figure 25: NIK expression and survival. *Cytoplasmic (A) and nuclear (B) NIK expression was not associated with OS.*

4.3.5 Expression of IKKα

IKKα expression was investigated in the full cohort of 159 patients. 25 patients had cores missing or insufficient tumour for analysis and following exclusions, 134 patients were included for analysis. Expression of IKKα was observed at the cytoplasmic and nuclear level. As described in Chapter 3, a proportion of patients demonstrated a distinct pattern of juxtanuclear punctate staining within the cytoplasm. Results will be discussed with respect to these three sites of IKKα expression. The histoscore range for cytoplasmic IKKα ranged from 15 to 230. Expression of cytoplasmic IKKα was graded as either low or high. Thresholds for analysis were calculated using median values; a histoscore >115 was considered as high expression. The histoscore range for nuclear IKKα ranged from 5 to 180. Expression of nuclear IKKα was graded as either low or high. Thresholds for analysis were calculated using median values; a histoscore >78 was considered as high expression. Expression of cytoplasmic IKKα correlated with expression of nuclear IKKα (Pearson's $r=0.264$, $p=0.002$). As punctate expression was not a continuous variable Chi-squared test was used to compare it with cytoplasmic/nuclear expression. Punctate expression of IKKα was directly associated with cytoplasmic expression ($p=<0.001$), however, was not associated with nuclear IKKα ($p=0.162$). There was good correlation of scores between observers with ICCC scores of 0.93 for cytoplasmic, 0.91 for nuclear and 0.70 for punctate expression of IKKα.

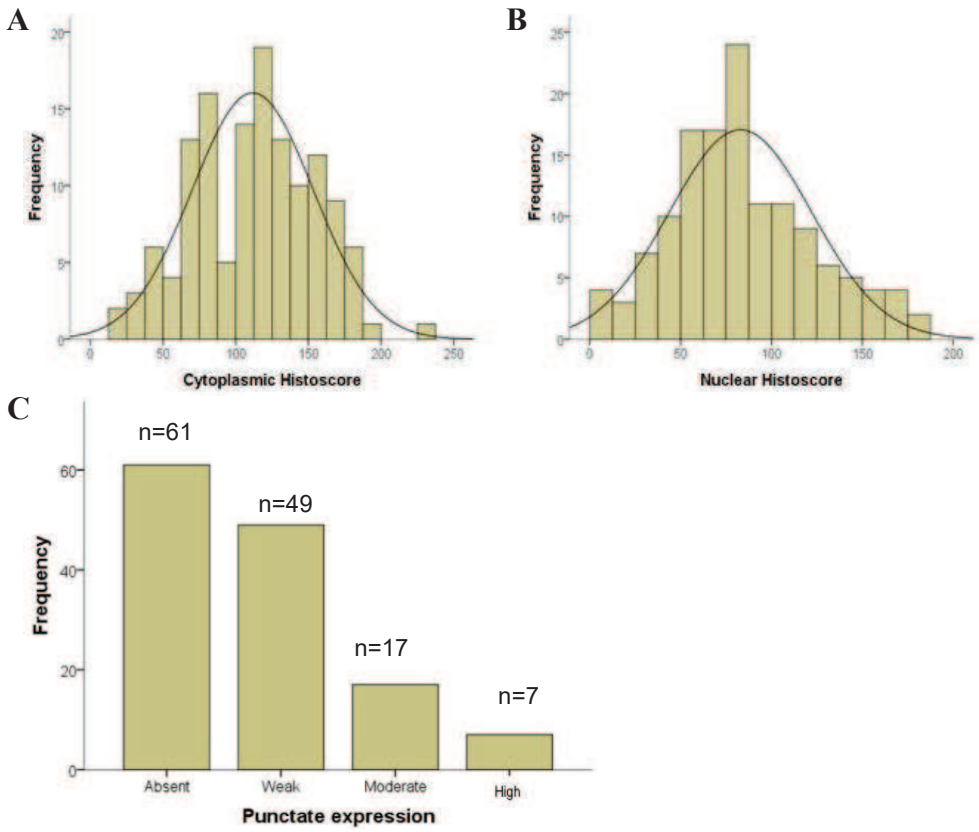


Figure 26: Expression of IKKα. *Histograms show distribution of average histoscores across three tumour cores for cytoplasmic (A) and nuclear IKKα (B). Bar chart (C) shows frequency of punctate expression categories.*

4.3.6 IKKα, clinicopathological characteristics and the tumour microenvironment

Expression of cytoplasmic IKKα was associated with greater likelihood of undergoing segmental bowel resection as opposed to polypectomy/TEMS alone (p=0.032) but was not associated with patient age (p=0.644), sex (p=0.326) tumour location (right colon/left colon/rectum) (p=0.078) or administration of adjuvant chemotherapy (p=0.066). In terms of tumour characteristics, high expression of cytoplasmic IKKα was associated with increasing T stage (p=0.014), N stage (p=0.012) and TNM stage (p=0.005) but was not associated with tumour differentiation (p=0.284), venous invasion (p=0.579) or proliferation (p=0.066). With respect to the tumour microenvironment, expression of cytoplasmic IKKα was directly associated with Klintrup-Mäkinen grade (p=0.006) but was not associated with tumour stroma percentage (p=1.000). Associations between cytoplasmic IKKα, clinicopathological characteristics and the tumour microenvironment are displayed in Table 10.

Expression of nuclear IKKα was associated with right-sided tumour location (p=0.032) but was not associated with patient age (p=0.861), sex (p=0.450), type of surgery (p=0.697) or administration of adjuvant chemotherapy (p=0.572). Nuclear NIK was not associated with TNM stage (p=1.000), tumour differentiation (p=0.312), venous invasion (p=0.549) or proliferation (p=0.099). With respect to the tumour microenvironment, nuclear IKKα expression was not associated with Klintrup-Mäkinen grade (p=0.484) or tumour stroma percentage (p=0.382).

Expression of punctate IKKα was associated with older age (p=0.049) and administration of adjuvant chemotherapy (p=0.042) but not with patient sex (p=0.439), type of surgery (p=0.172) or tumour location (p=0.377). In terms of tumour characteristics, higher expression of punctate IKKα was directly associated with N stage (p=0.038) but not with T stage (p=0.343), TNM stage (p=0.158), differentiation (p=0.516), venous invasion (p=0.225) or proliferation (p=0.193). With respect to the tumour microenvironment, expression of punctate IKKα was not associated with Klintrup-Mäkinen grade (p=0.848) or tumour stroma percentage (p=0.369). Associations between punctate IKKα expression, clinicopathological characteristics and the tumour microenvironment are displayed in Table 11.

		All *n*= 134	Low cytoplasmic IKKα expression *n*= 69	High cytoplasmic IKKα expression *n*=65	*p*	Low nuclear IKKα expression *n*= 67	High nuclear IKKα expression *n=67*	*p*
Host Characteristics								
Age (n=134)	**<65**	55 (41)	27 (39)	28 (43)	0.644	27 (40)	28 (42)	0.861
	>65	79 (59)	42 (61)	37 (57)		40 (60)	39 (58)	
Sex (n=134)	**Female**	55 (30)	18 (26)	22 (34)	0.326	18 (27)	22 (33)	0.450
	Male	127 (70)	51 (73)	43 (66)		49 (73)	45 (67)	
Type of surgery (n=134)	**No Surgery**	36 (27)	24 (35)	12 (19)	**0.032**	17 (25)	19 (28)	0.697
	Resection	98 (73)	45 (65)	53 (81)		50 (75)	48 (72)	
Tumour location (n=134)	**Right**	18 (14)	5 (7)	17 (26)	0.078	4 (6)	14 (21)	**0.032**
	Left	81 (60)	46 (67)	35 (54)		43 (64)	38 (57)	
	Rectum	35 (26)	18 (26)	13 (20)		20 (30)	15 (22)	
Tumour Characteristics								
T stage (n=134)	**1**	92 (69)	54 (78)	38 (59)	**0.014**	45 (67)	47 (70)	0.711
	2	42 (31)	15 (22)	27 (41)		22 (33)	20 (30)	

N stage (n=98)	**0** **1** **2**	82 (84) 12 (12) 4 (4)	42 (93) 3 (7) 0 (0)	40 (76) 9 (17) 4 (7)	**0.012**	42 (84) 6 (12) 2 (4)	40 (83) 6 (13) 2 (4)	0.934
TNM stage (n=134)	**I** **II** **III**	118 (88) 0 (0) 16 (12)	66 (96) 0 (0) 3 (4)	52 (80) 0 (0) 13 (20)	**0.005**	59 (88) 0 (0) 8 (12)	59 (88) 0 (0) 8 (12)	1.000
Tumour differentiation (n=134)	**Mod/well** **Poor**	130 (97) 4 (3)	68 (99) 1 (1)	62 (95) 3 (5)	0.284	66 (99) 1 (1)	64 (95) 3 (5)	0.312
Venous invasion (n=129)	**No** **Yes**	91 (71) 38 (29)	48 (73) 18 (27)	43 (68) 20 (32)	0.579	46 (73) 17 (27)	45 (68) 21 (32)	0.549
Proliferation (n=133)	**Low** **High**	71 (53) 62 (47)	31 (46) 37 (54)	40 (62) 25 (38)	0.066	40 (61) 26 (39)	31 (46) 36 (54)	0.099
Tumour microenvironment								
Klintrup-Mäkinen grade (n=106)	**Weak** **Strong**	66 (62) 40 (38)	38 (76) 12 (24)	28 (50) 28 (50)	**0.006**	36 (66) 19 (34)	30 (59) 21 (41)	0.484
Tumour stroma percentage (n=108)	**Low** **High**	84 (78) 24 (22)	42 (78) 12 (22)	42 (78) 12 (22)	1.000	44 (75) 15 (25)	40 (82) 9 (18)	0.382

Adjuvant therapy (n=134)	**No**	120 (90)	65 (94)	55 (85)	0.066	61 (91)	59 (88)	0.572
	Yes	14 (10)	4 (6)	10 (15)		6 (9)	8 (12)	

Table 10: IKKα, associations with clinicopathological characteristics and the tumour microenvironment in screen-detected T1/2 colorectal cancer

		All *n*= 134	Absent punctate IKKα expression *n*=61	Low punctate IKKα expression *n*=49	Moderate punctate IKKα expression *n*=17	High punctate IKKα expression *n*=7	*p*
Host Characteristics							
Age (n=134)	**<65** **>65**	55 (41) 79 (59)	32 (53) 29 (47)	16 (33) 33 (67)	4 (24) 13 (77)	3 (43) 4 (57)	**0.049**
Sex (n=134)	**Female** **Male**	40 (30) 94 (70)	17 (28) 44 (72)	15 (31) 34 (69)	4 (24) 13 (76)	4 (57) 3 (43)	0.439
Type of surgery (n=134)	**No Surgery** **Resection**	36 (27) 98 (73)	20 (33) 41 (67)	8 (16) 41 (84)	5 (29) 12 (71)	3 (43) 4 (57)	0.172
Tumour location (n=134)	**Right** **Left** **Rectum**	18 (13) 81 (61) 35 (26)	4 (7) 38 (62) 19 (31)	10 (20) 27 (55) 12 (25)	3 (18) 11 (64) 3 (18)	1 (14) 5 (72) 1 (14)	0.377
Tumour Characteristics							
T stage (n=134)	**1** **3**	92 (69) 42 (31)	45 (74) 16 (26)	31 (63) 18 (37)	12 (71) 5 (29)	4 (57) 3 (43)	0.343

N stage (n=98)	**0** **1** **2**	82 (84) 12 (12) 4 (4)	37 (90) 3 (7) 1 (3)	32 (78) 8 (20) 1 (2)	11 (92) 1 (8) 0 (0)	2 (50) 0 (0) 2 (50)	**0.038**
TNM stage (n=134)	**I** **II** **III**	118 (88) 0 (0) 16 (12)	57 (93) 0 (0) 4 (7)	40 (82) 0 (0) 9 (18)	16 (94) 0 (0) 1 (6)	5 (71) 0 (0) 2 (29)	0.158
Tumour differentiation (n=134)	**Mod/well** **Poor**	130 (97) 4 (3)	59 (97) 2 (3)	47 (96) 2 (4)	17 (100) 0 (0)	7 (100) 0 (0)	0.516
Venous invasion (n=129)	**No** **Yes**	91 (71) 38 (29)	39 (66) 20 (34)	34 (72) 13 (28)	12 (75) 4 (25)	6 (86) 1 (14)	0.225
Proliferation (n=133)	**Low** **High**	71 (53) 62 (47)	28 (47) 32 (53)	28 (57) 21 (43)	11 (65) 6 (35)	4 (57) 3 (43)	0.193
Tumour microenvironment							
Klintrup-Mäkinen grade (n=106)	**Weak** **Strong**	66 (62) 40 (38)	30 (65) 16 (35)	21 (55) 17 (45)	11 (65) 6 (35)	4 (80) 1 (20)	0.848
Tumour stroma percentage (n=108)	**Low** **High**	84 (78) 24 (22)	35 (75) 12 (25)	32 (80) 8 (20)	12 (75) 4 (25)	5 (100) 0 (0)	0.369

Adjuvant therapy (n=134)	**No**	120 (90)	57 (93)	41 (84)	17 (100)	5 (71)	**0.042**
	Yes	14 (10)	4 (7)	8 (16)	0 (0)	2 (29)	

Table 11: Punctate expression of IKKα, associations with clinicopathological characteristics and the tumour microenvironment in screen-detected T1/2 colorectal cancer

4.3.7 IKKα expression and survival

To determine whether IKKα expression was significantly associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic, nuclear and punctate expression were plotted; levels of expression were compared using the log rank test. Expression of cytoplasmic IKKα (p=0.355), nuclear IKKα (p=0.266) and punctate IKKα (p=0.402) was not associated with OS.

4.3.8 Expression of RelB

RelB expression was investigated in the full cohort of 159 patients. 13 patients had missing cores or insufficient tumour for analysis and following exclusions, 146 patients were included for analysis. Expression of RelB was only observed at the cytoplasmic level. The histoscore range for cytoplasmic RelB ranged from 15 to 167. Expression of cytoplasmic RelB was graded as either low or high. Thresholds for analysis were calculated using median values; a histoscore >94 was considered as high expression. There was good correlation of scores between observers with ICCC scores of 0.93 for cytoplasmic expression and 1.00 for nuclear expression of RelB.

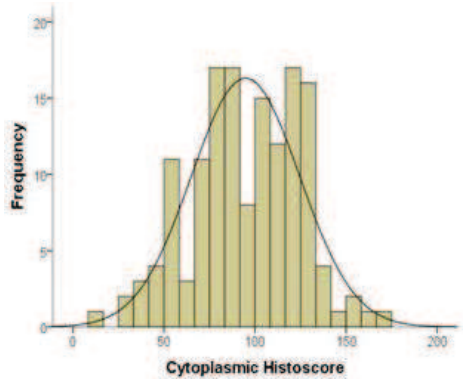


Figure 27: Expression of RelB. *Histogram shows distribution of average histoscores across three tumour cores for cytoplasmic RelB (A).*

4.3.9 RelB, clinicopathological characteristics and the tumour microenvironment

Expression of cytoplasmic RelB was not associated with patient age (p=1.00), sex (p=0.591), type of surgery (p=0.458), tumour location (right colon/left colon/rectum) (p=0.621) or administration of adjuvant chemotherapy (p= 0.287). Low RelB expression was associated with poor tumour differentiation (p=0.043) however, was not associated with TNM stage (p=0.797), venous invasion (p=0.673) or proliferation (p=0.273). With respect to the tumour microenvironment, RelB was not associated with Klintrup-Mäkinen grade (p=0.847) or tumour stroma percentage (p=0.636).

4.3.10 RelB expression and survival

To determine whether cytoplasmic RelB expression was significantly associated with clinical outcome, a Kaplan-Meier survival curve was plotted; levels of expression were compared using the log rank test. Expression of cytoplasmic RelB was not associated with OS (p=0.695).

4.4 Associations between members of the non-canonical NF-κB pathway

To understand the relationship between expression of different pathway members, Pearson's correlation coefficient was used to analyse continuous variables and Chi-square test was used to analyse expression between non-continuous variables. Cytoplasmic NIK expression correlated with cytoplasmic IKKα (Pearson's r=0.372, p<0.001) but did not correlate with cytoplasmic expression of RelB (Pearson's r=0.026, p=0.787). As nuclear NIK was a non-continuous variable, Chi-square analysis was used to assess the associations with the other proteins. Nuclear expression of NIK was not associated with cytoplasmic IKKα (p=0.538), nuclear IKKα (p=0.114) or cytoplasmic RelB (p=0.753). Cytoplasmic IKKα did not correlate with cytoplasmic RelB (Pearson's r=0.016, p=0.851). Nuclear IKKα was not associated with cytoplasmic RelB (p=0.082).

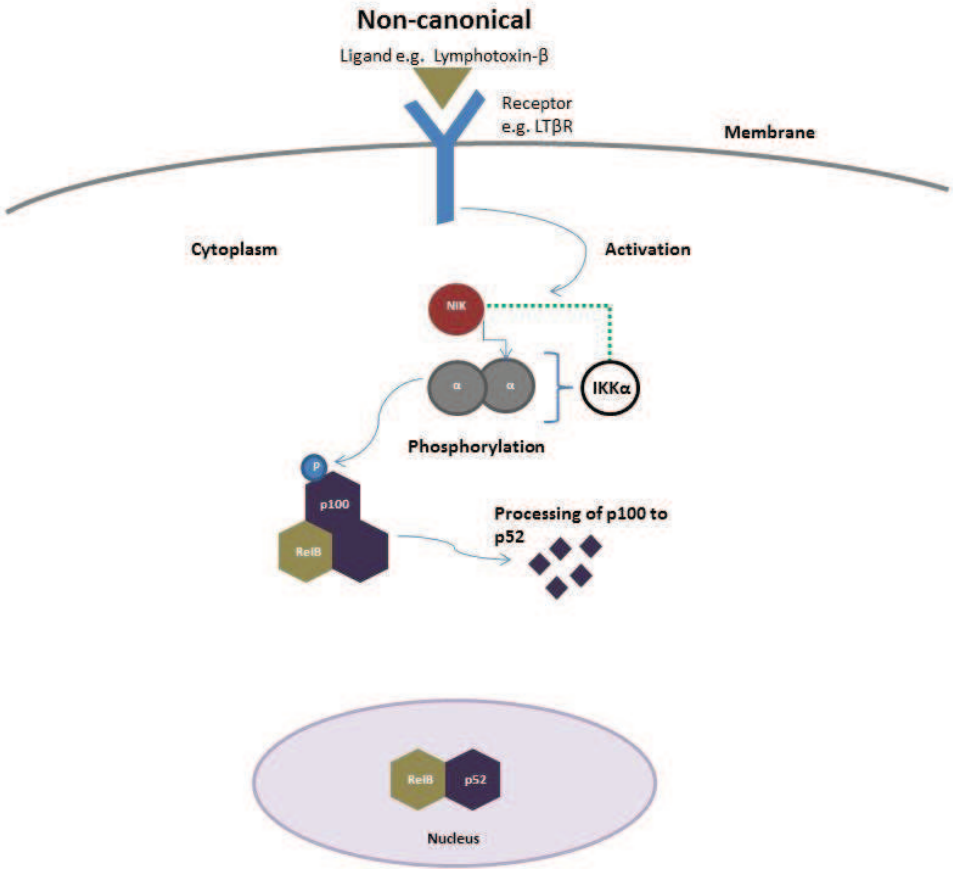


Figure 28: Associations between members of the non-canonical NF-κB pathway. *Green dashed line represents positive correlation between proteins. Adapted from Patel et al* (286).

4.4.1 Combined cytoplasmic protein expression and survival

As there was positive correlation between expression of cytoplasmic NIK and IKKα, expression levels were combined to create two groups 1) low expression of NIK and IKKα (n=28) and 2) patients with high expression of either NIK, IKKα, or both (n=89). Although there was no significant association between combined cytoplasmic proteins and OS it was of interest that there were 2 deaths in the low expression group and 18 in the high expression group (p=0.088).

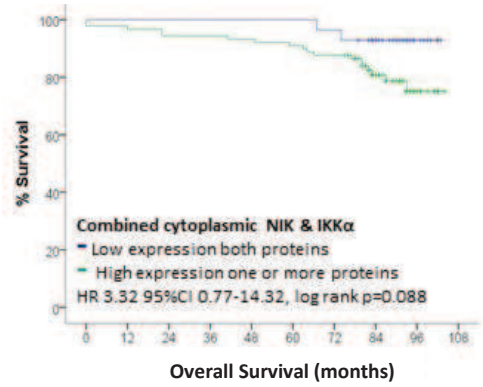


Figure 29: Combined cytoplasmic proteins (NIK and IKKα) and survival. *Combined NIK and IKKα expression was not associated with OS.*

4.5 Chapter discussion

The results of the present study demonstrate that in patients with screen-detected T1/T2 CRC, upregulation of the non-canonical NF-κB pathway was associated with an adverse tumour phenotype. Specifically, high expression of IKKα was associated with tumour invasiveness and therefore a greater likelihood of patients undergoing formal segmental bowel resection rather than local resection alone i.e. polypectomy or TEMS. Although low patient numbers and events limited a statistically meaningful survival analysis it was of interest that a greater number of deaths in patients with high expression of non-canonical proteins were observed. The results from this chapter demonstrate that IKKα was expressed in early stage disease and therefore non-canonical NF-κB upregulation is likely to be an important early event in the tumourigenic process. For example, Colomer and co-workers reported deficiency of IKKα in APC mutated mice reduced tumour initiation and proliferation but was not required for normal tissue homeostasis (301). The benefits of the CRC screening programme have been under scrutiny, as discussed earlier in this thesis, results from clinical trials of patients enrolled in a CRC screening programme have failed to explain the decline in CRC mortality. Nonetheless, these patients offer a unique opportunity to study early tumours before patients become symptomatic. The results presented in this chapter support the notion that IKKα has an important role in the early stages of CRC development.

Colorectal polyps are benign precursor lesions with malignant potential. Over time polyps can transform into invasive cancers. Patients with colorectal polyps are often placed on surveillance programmes which require colonoscopy at regular intervals. Polyps can be low- or high-risk and at present polyp size, number and histological features determine the frequency of colonoscopy. It would be of interest for future studies to investigate the expression of IKKα in pre-malignant colorectal polyps with varying grades of dysplasia. This may offer more targeted surveillance for patients at high-risk of developing new polyps and reduced the number of unnecessary colonoscopies which would have both health and economic impact.

Chapter 5:

Investigation of IKKα distribution and localisation in colorectal cancer specimens

5.1 Introduction

The results from Chapters 3 and 4 report a distinct pattern of punctate juxtanuclear IKKα staining (Figure 31). In the Glasgow cohort, this was associated with significant reduction in CSS in patients who had undergone surgery for stage I-III CRC. Antibody specificity experiments for IKKα have been performed previously in the host laboratory and it has been established that the antibody is specific for IKKα. Western blotting using the anti-IKKα antibody shows a single band of the appropriate size (85 kDa) and expression changes in response to siRNA silencing of IKKα, whilst siRNA silencing of IKKβ does not alter expression. In addition, when the cells were fixed and paraffin embedded, a similar change in expression was observed using IHC (Figure 30). The figure below has been included to demonstrate IKKα antibody specificity has been confirmed and we are confident that the protein that was detected is indeed IKKα.

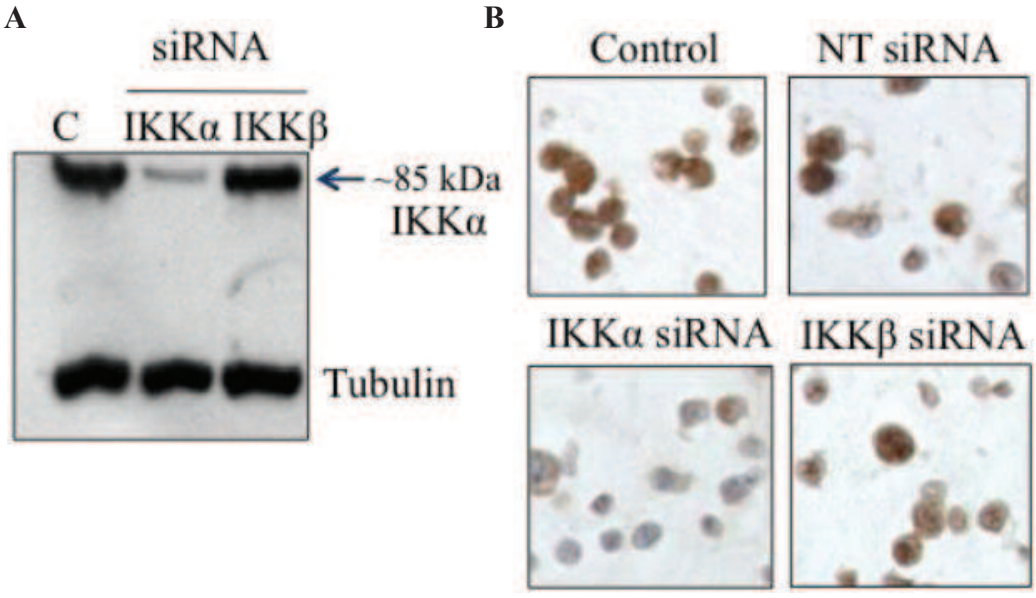


Figure 30: Validation of the anti-IKKα antibody. *(A) Western blot showing a single band of appropriate size (85 kDa). A reduction in expression was observed in lysates from IKKα-silenced but not in IKKβ-silenced LNCaP cells and (B) paraffin embedded cell pellets of LNCaP cells treated with siRNA showed decrease in expression in IKKα-silenced but not IKKβ-silenced, compared to control cells.*

As the antibody was specific for IKKα it was hypothesised that this pattern of staining may be representative of IKKα phosphorylation sites or the accumulation of IKKα within a specific cellular structure located in the perinuclear region. The figure below is a higher resolution image of the punctate IKKα staining, captured by Dr Leo Carlin at the CRUK Beatson Institute Imaging Facility, Glasgow.

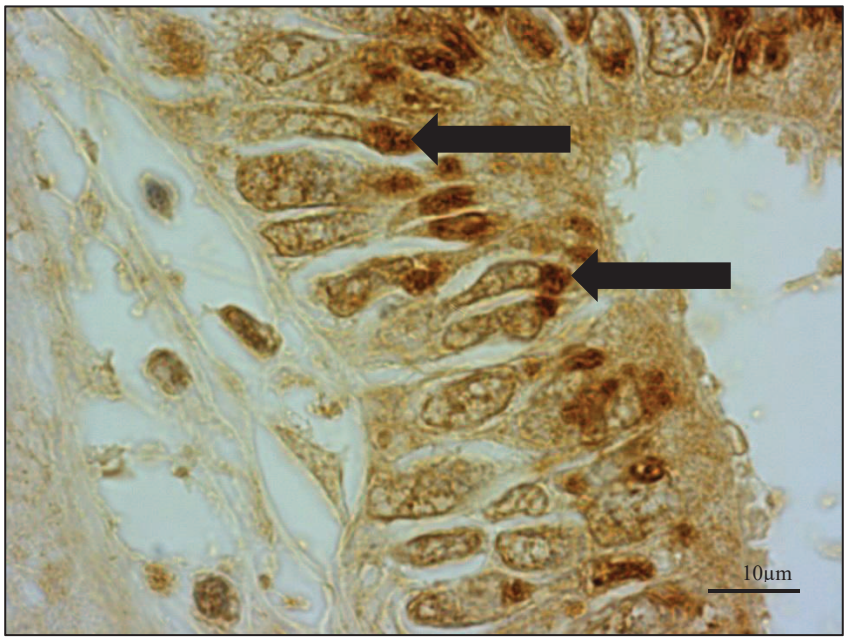


Figure 31: **Localisation of IKKα in the perinuclear space.** *IKKα is identified in a juxtanuclear position in epithelial colorectal cancer tissue (black arrows) (images acquired on an Olympus BX51 100x 1.3 NA oil lens with a Zeiss Axiocam 105 colour camera, scale bar represents 10µm).*

5.2 Immunohistochemical expression of IKKα in other cancers

To understand whether punctate IKKα expression reported in Chapters 3 and 4 was observed in other solid cancers, tissue images from studies within the host lab that have previously investigated IKKα using the same antibody, were reviewed on Slidepath Digital Image Hub (Leica Biosystems, Newcastle, UK). IKKα expression in breast (Dr Lindsay Bennett) and prostate cancer (Mr Lewis Mckenzie) was observed at the cytoplasmic and nuclear level, however, punctate IKKα expression was not observed in any of the 362 breast cancer specimens or 163 prostate cancer specimens that were available for assessment.

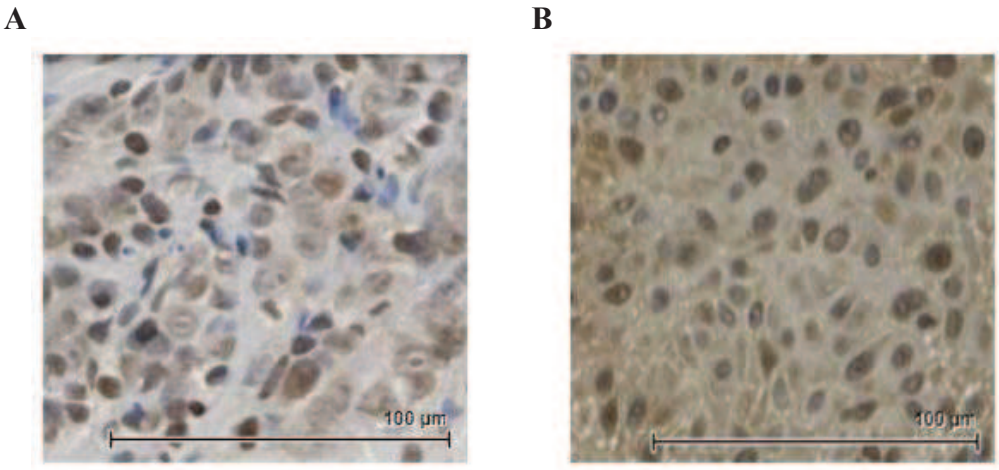


Figure 32: IKKα in other solid cancers. *Localisation of IKKα in the perinuclear space was not observed in patients with breast (A) or prostate cancer (B) (images taken at 20x20 magnification, scale bar represents 100µm).*

5.3 Investigation of IKKα phosphorylation sites

Phosphorylation is key to the regulatory processes that control NF-κB. We hypothesised that punctate IKKα expression represented sites of IKKα phosphorylation. To understand whether the staining pattern of interest was representative of non-canonical IKKα expression or indeed behaviour of IKKα independent of traditional non-canonical signalling, phosphorylation sites of IKKα were investigated. Two phosphorylation sites were investigated; serine 176, the site where NIK phosphorylates IKKα (82) and therefore representative of non-canonical IKKα expression and threonine 23, the site of Akt-mediated IKKα phosphorylation (120). Immunohistochemistry was used to stain colorectal TMAs with the relevant antibodies. Punctate IKKα expression was not observed with either of the phosphorylation sites that were investigated, suggesting that this is not associated with phosphorylation and might represent the protein before or after phosphorylation has occurred.

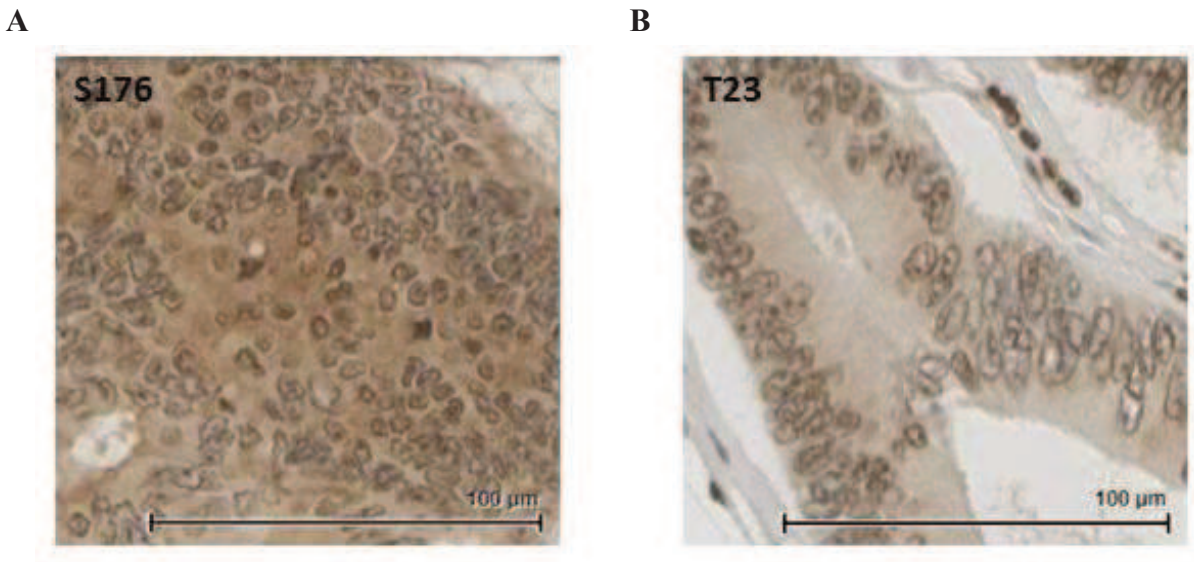


Figure 33: IKKα phosphorylation sites. *Punctate IKKα expression was not observed with IKKα phosphorylation sites S176 (A) or T23 (B) in epithelial colorectal cancer tissue (images taken at 20x20 magnification, scale bar represents 100µm).*

5.4 Immunofluorescence

5.4.1 IKKα immunofluorescence

IHC results from Chapters 3 and 4 demonstrated localisation of IKKα in the perinuclear space. In the Glasgow cohort, this was associated with significantly reduced CSS and so it was of interest to investigate this further with immunofluorescence to enable higher resolution of the staining pattern. Anti-IKKα (Genway, GWB-66250) used for IHC in Chapters 3 and 4 was optimised on CRC TMAs. A fluorescent staining pattern comparable to that observed with IHC was identified in a number of patients. As with IHC, there was a variation in the number and size of these discrete areas.

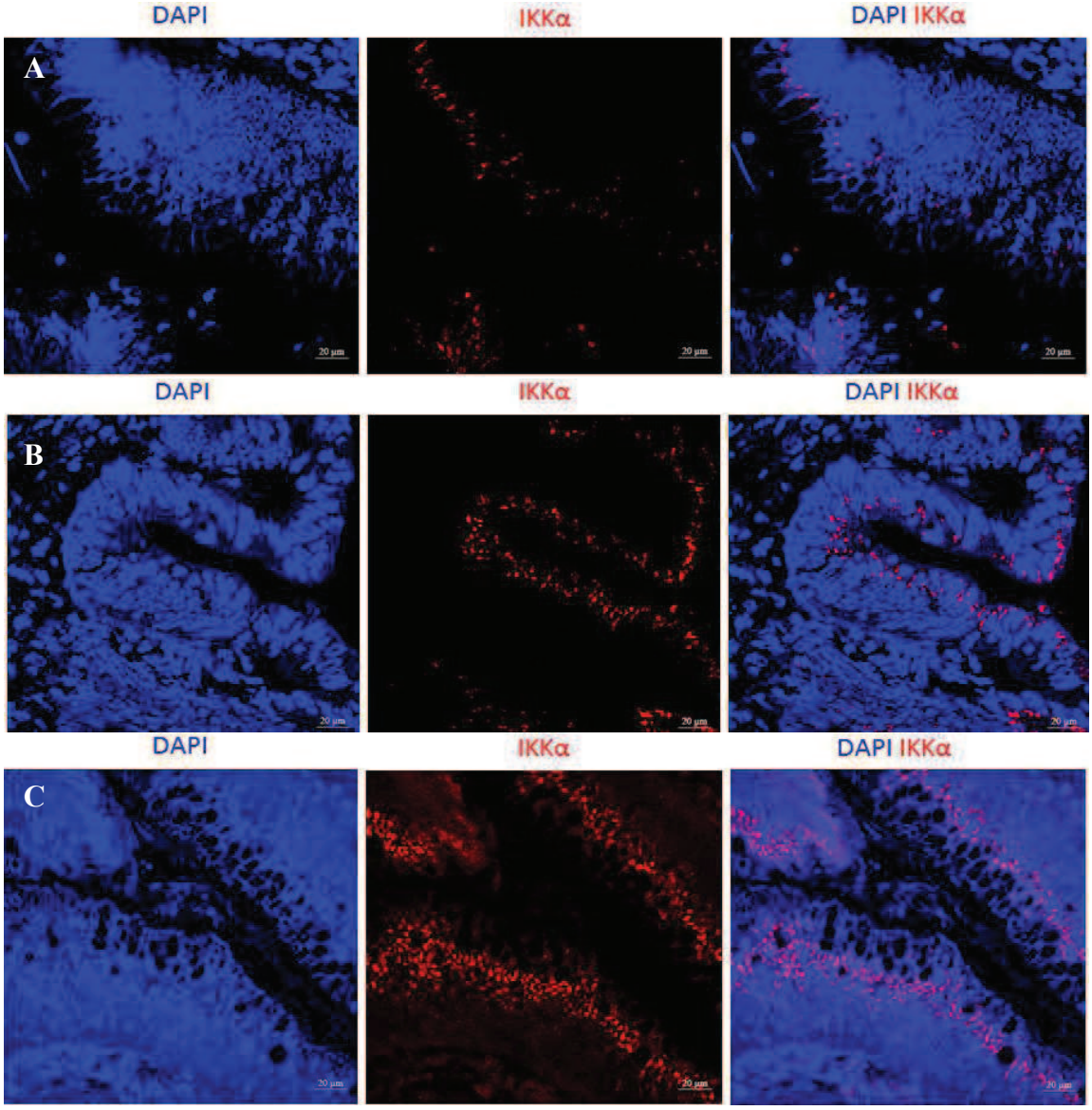


Figure 34: IKKα immunofluorescence. *Punctate expression of IKKα (red) in the perinuclear space was observed. DAPI (blue) represents nuclear counterstain. Examples of low (A), moderate (B) and high (C) expression of punctate IKKα (images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20µm).*

It was of interest that expression of punctate IKKα was also observed in normal colorectal tissue found adjacent to tumour samples. Figure 35 demonstrates punctate staining within normal colonic epithelial cells.

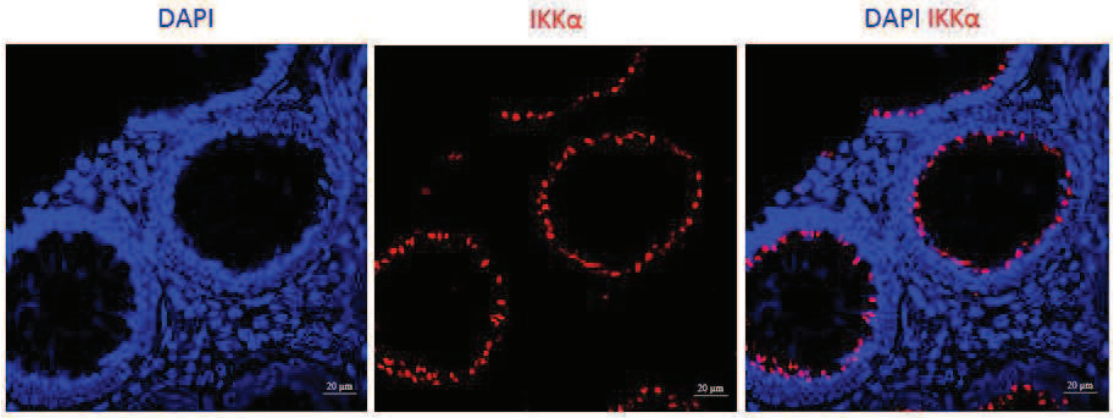


Figure 35: IKKα expression in normal colorectal tissue. *Punctate IKKα (red) expression was also observed in normal colorectal epithelial tissue adjacent to tumour. DAPI (blue) represents nuclear counterstain. (Images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20µm).*

5.4.2 Dual immunofluorescence – IKKα and markers of cellular transport

5.4.2.1 Introduction

We hypothesised that punctate expression of IKKα was representative of IKKα localised within a specific cellular compartment, e.g. endo-lysosomal compartment or the Golgi. Using primary antibodies raised in different host species, markers of these cellular structures were investigated using dual fluorescence with IKKα. Staining was optimised on CRC tissue specimens. Exchange of substances between compartments of a cell is mediated by vesicular transport. The endocytic compartment has a key role in sorting cellular cargo between the plasma membrane, the Golgi apparatus and lysosome for recycling or degradation (288). Endocytic vesicles that bud from the plasma membrane fuse to form the early endosome which subsequently mature to late endosomes. The early endosome is a diverging point between the degradative and recycling pathways, and is also essential for transport of proteins to the trans-Golgi network (289). Rab proteins belong to a family of Ras-like GTPases and cycle between a GDP-bound 'off' state and GTP-bound 'on' state which results in binding to effectors. Rab5 regulates fusion of the plasma membrane to the early endosome, where it is primarily localised (290). The late endosome acts as a 'sorting hub' where cellular cargo can either be recycled to the Golgi or plasma membrane, or can be transported to the lysosome for degradation. Rab7 is also a member of RAS-related GTP-binding proteins and is important in regulation of vesicular transport. Rab7 controls the aggregation and fusion of late endocytic/lysosome structures and is essential for maintenance of the perinuclear lysosome (291).

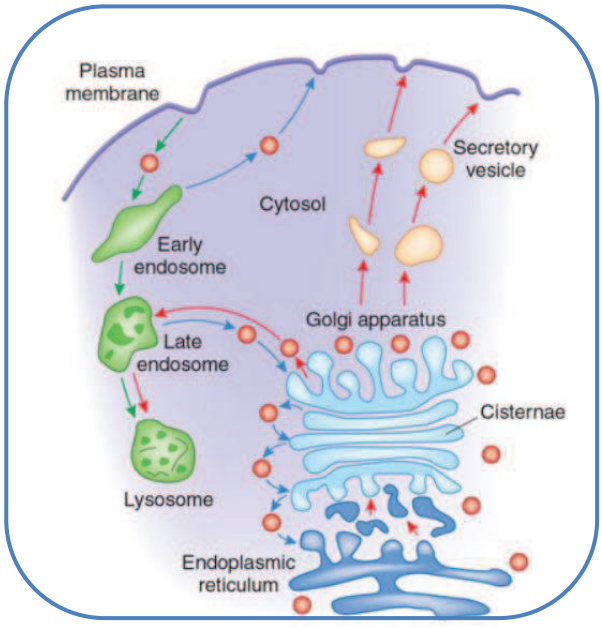


Figure 36: Intracellular transport pathways. *Adapted from Xu et al* (292).

5.4.2.2 Endosomal markers - Rab5 and Rab7

Rab5 and Rab7 are well-established markers of the early and late endosome, respectively. Anti-Rab5 and anti-Rab7 antibodies were used to perform dual fluorescence with IKKα in order to determine whether IKKα was localised to either of the endosomal compartments. Confocal images are shown below (Figures 37 and 38). There was no evidence of co-localisation between IKKα and markers of the endosomal compartment.

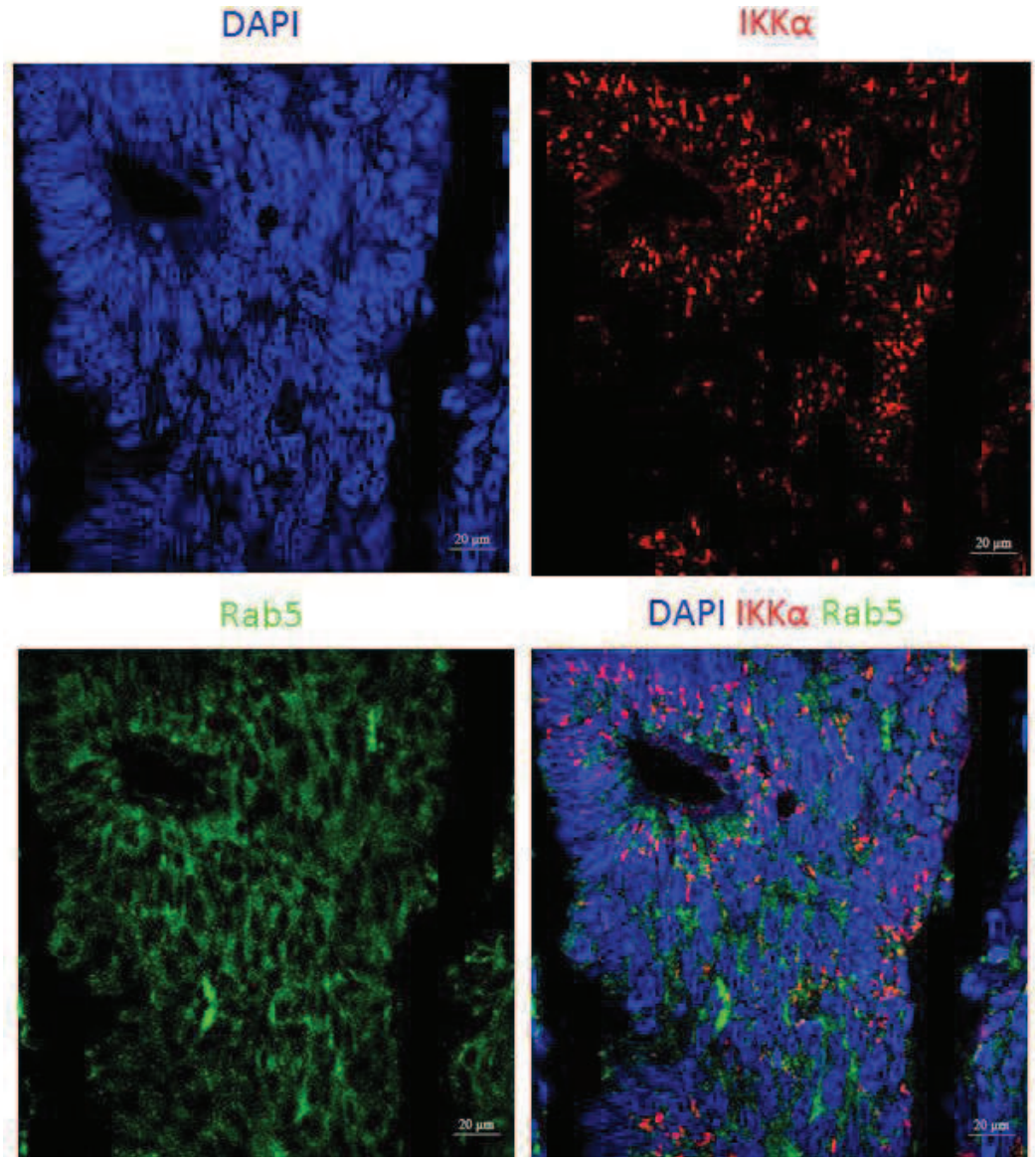


Figure 37: IKKα and Rab5 dual fluorescence. *Co-localisation between IKKα (red) and Rab5 (green) was not observed in colorectal cancer tissue. DAPI (blue) represents nuclear counterstain. (Images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20μm).*

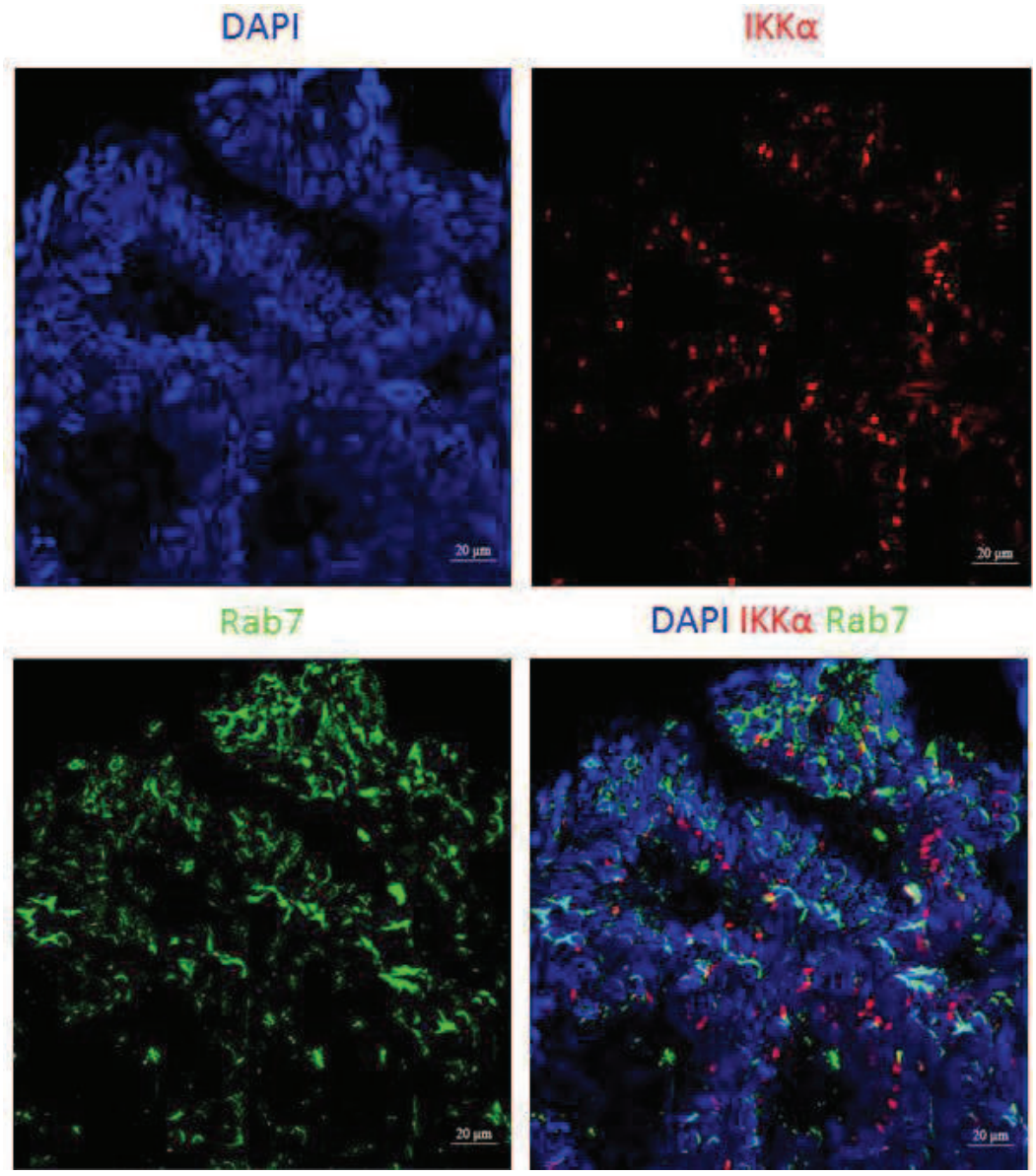


Figure 38: IKKα and Rab7 dual fluorescence. *Co-localisation between IKKα (red) and Rab7 (green) was not observed in colorectal cancer tissue. DAPI (blue) represents nuclear counterstain. (Images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20µm).*

5.4.2.3 Golgi marker – Golgi 58

The Golgi has key roles in trafficking and post-translational modifications of proteins and lipids. To identify the Golgi apparatus, dual immunofluorescent staining with IKKα and a well-established Golgi marker, Golgi 58K, was optimised in CRC tissue. A variety of antigen retrieval methods were used during optimisation of the Golgi antibody. The images below demonstrate that IKKα was co-located with the Golgi compartment (Figures 39-41), where the golgi marker appears to closely surround IKKα punctate expression. More detailed imaging was performed at the CRUK Beatson Institute Imaging Facility, Glasgow (Figure 42).

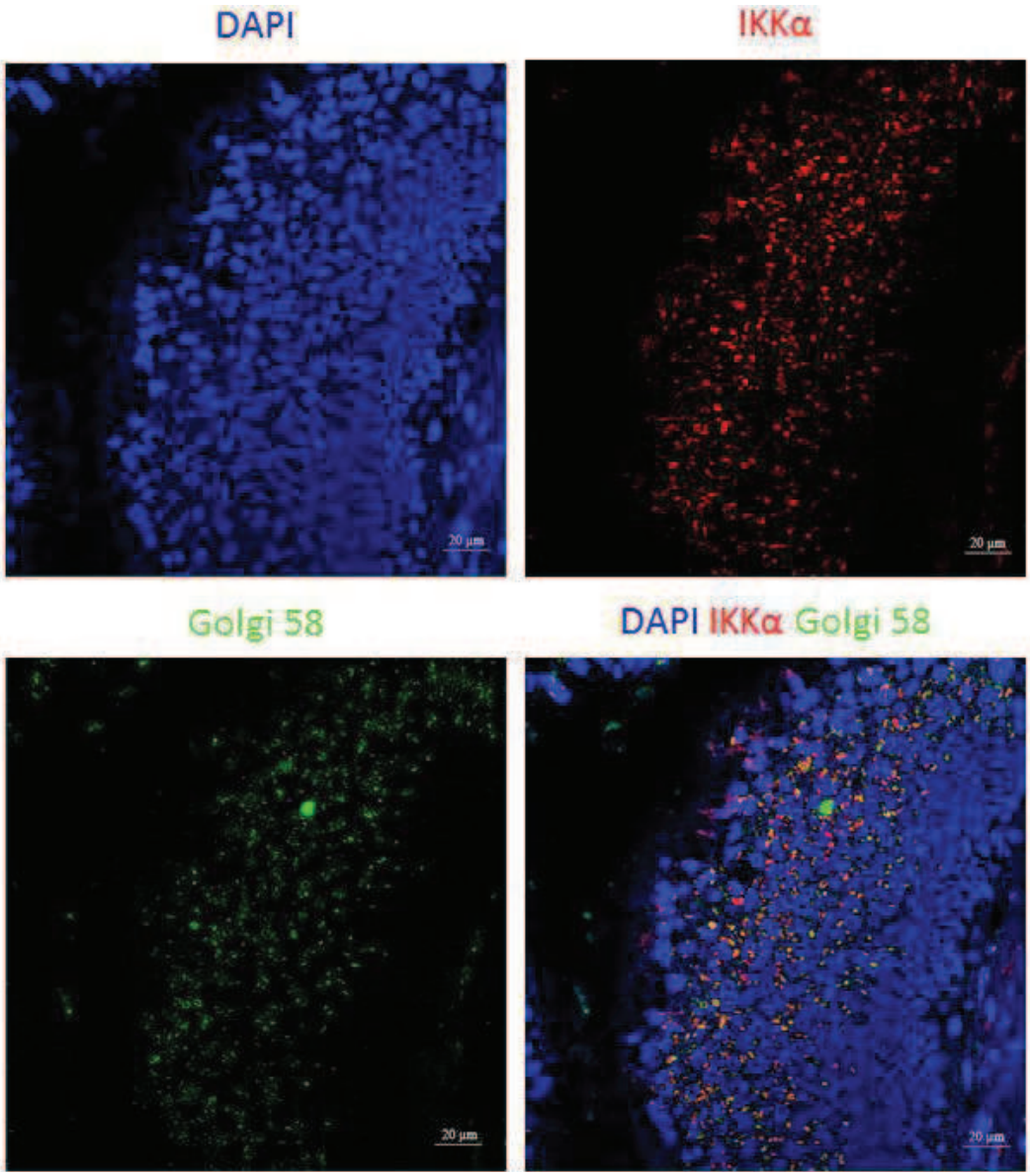


Figure 39: IKKα and Golgi 58 dual fluorescence. *IKKα co-located with a Golgi marker in colorectal cancer tissue. Antigen retrieval was performed with TRIS/EDTA buffer pH9 in a microwave (images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20µm).*

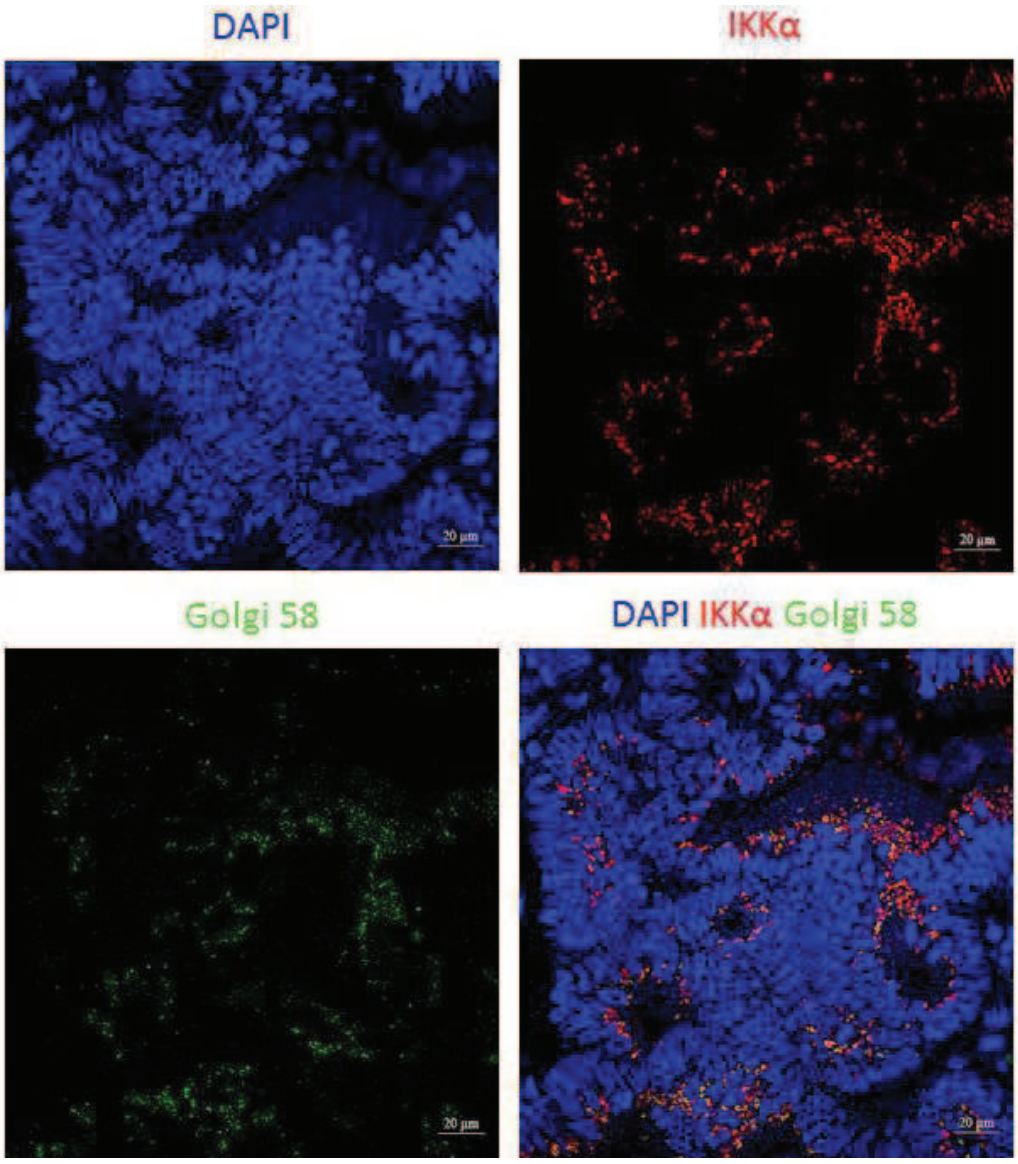


Figure 40: IKKα and Golgi 58 dual fluorescence. *IKKα co-located with a Golgi marker in colorectal cancer tissue. Two-part antigen retrieval was performed: protease step at 40˚C for 30 minutes followed by heated induced retrieval in antigen retrieval buffer for 40 minutes in a water bath at 92-95°C (images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20µm).*

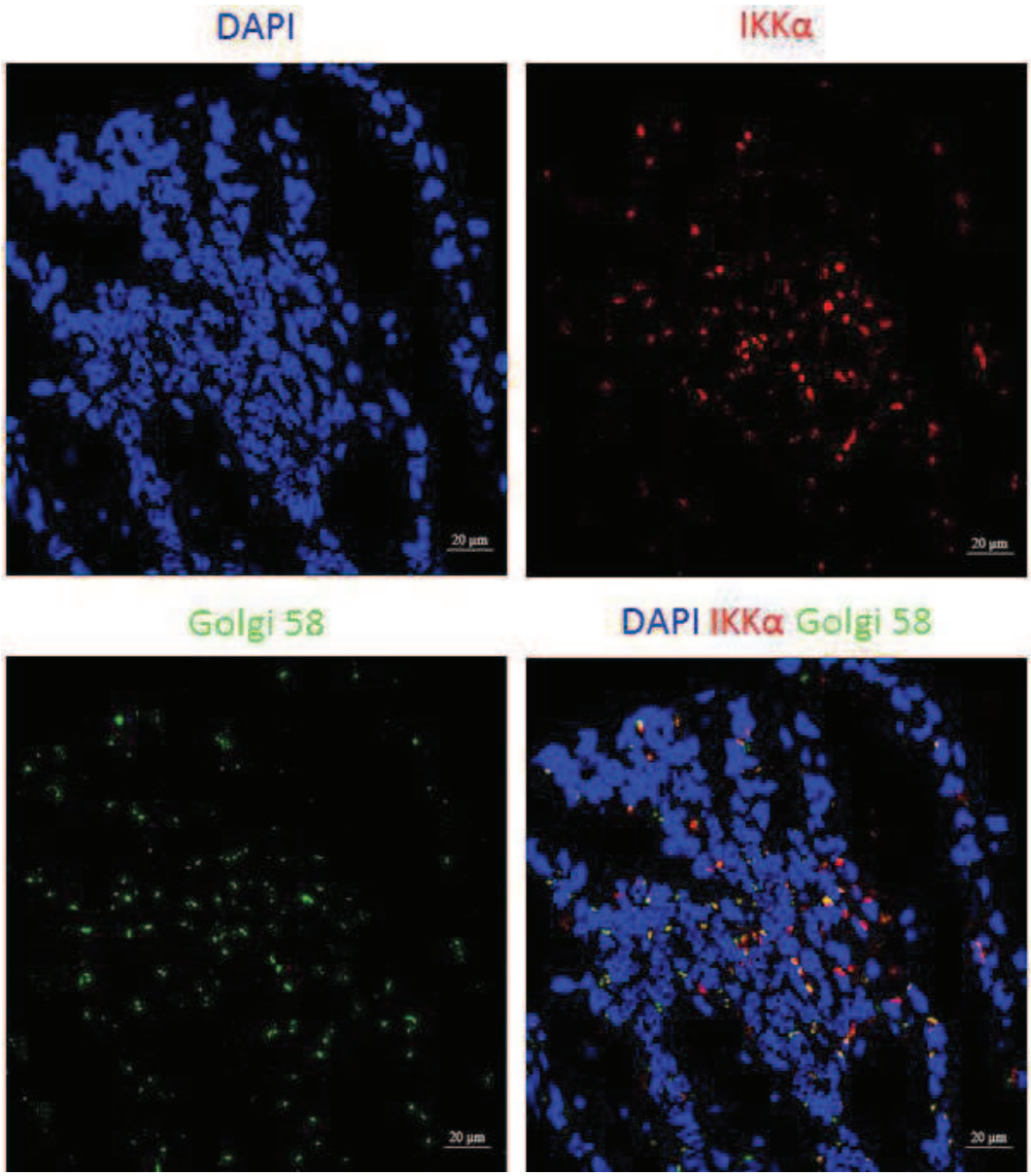


Figure 41: IKKα and Golgi 58 dual fluorescence. *IKKα co-located with a Golgi marker in colorectal cancer tissue. Two-part antigen retrieval was performed: protease step at 40˚C for 30 minutes followed by heated induced retrieval with TRIS/EDTA buffer pH9 in a microwave (images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20µm).*

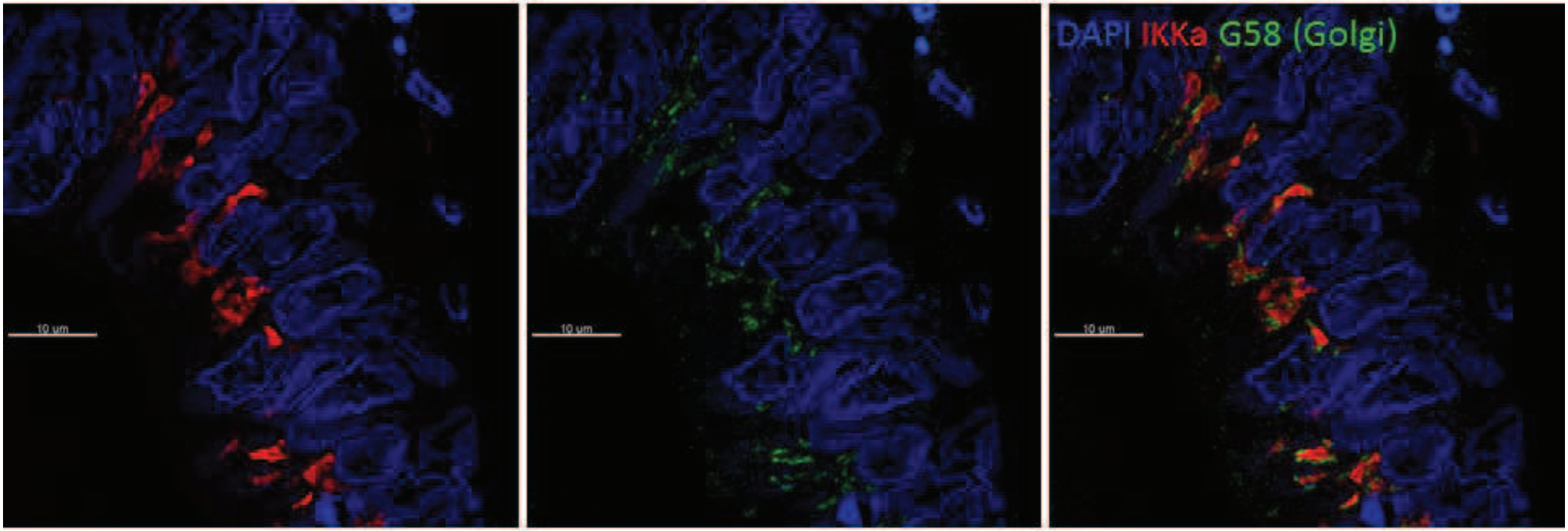


Figure 42: IKKα and Golgi 58 dual fluorescence. *IKKα co-located with a Golgi marker in colorectal cancer tissue. Two-part antigen retrieval was performed: protease step at 40˚C for 30 minutes followed by heated induced retrieval with TRIS/EDTA buffer pH9 in a microwave (images were acquired using a 40x 1.3 NA Zeiss Plan apochromat oil lens on a Zeiss LSM880 Airyscan microscope. The image is a single slice acquired in Airyscan 'super-resolution' mode with sequential scanning through a different emission filter for the green and red channels to minimise crosstalk with default processing settings. The image was displayed using Imaris (Bitplane), scale bar represents 10µm).*

Primary antibody non-specific binding can result in background staining. This can be due to binding to Fc receptors on target cells, by non-specific interactions with cellular proteins or by cell autofluorescence. To help differentiate non-specific background signal from specific antibody signal, an isotype control primary antibody was used as a negative control. An isotype control antibody lacks specificity to the target but matches the class and type of antibody used in the application. To match the primary antibody's (Golgi 58) host species and class, including light chains, a mouse IgG1 kappa isotype control antibody (14-4714-82, ThermoFisher, UK) at the same concentration (1:100) was used to assess for non-specific background staining. There was no evidence of non-specific background staining with the isotype control antibody (Figure 43).

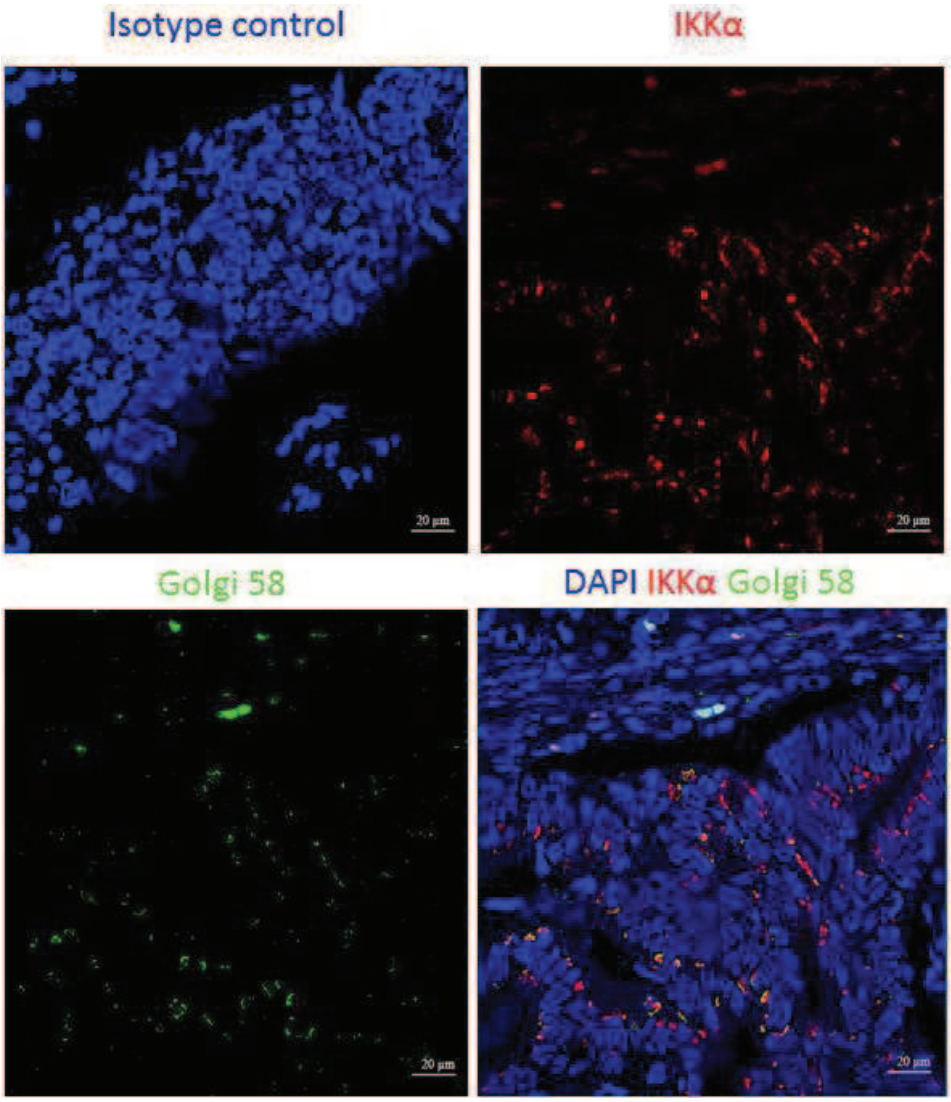


Figure 43: IKKα and Golgi 58 dual fluorescence with isotype control antibody as negative control. *Non-specific background staining was not observed. Two-part antigen retrieval was performed: protease step at 40˚C for 30 minutes followed by heated induced retrieval performed with TRIS/EDTA buffer pH10 in a microwave (images taken at x400 magnification using a ZEISS LSM 780 confocal microscope, scale bar represents 20µm).*

5.5 Chapter discussion

The results of this chapter demonstrate the distinct pattern of punctate IKKα expression in colorectal cancer patient tissues observed in Chapters 3 and 4 is not an artefact of IHC. The antibody is specific for IKKα. Moreover, the staining pattern has been demonstrated again using immunofluorescence and a variety of antigen retrieval methods. The variation in the amount of punctate expression i.e. absent, low, moderate or high was also observed with immunofluorescence. The expression of punctate IKKα was only assessed using a TMA. It would be of interest to understand if the distribution of punctate IKKα varies in a full tumour section and if there is any variation in the expression of IKKα in relation to the invasive tumour margin. Results presented in this chapter also demonstrate the presence of punctate IKKα expression in normal colonic tissue adjacent to tumour, this raises questions about the expression of IKKα in normal colonic tissue. We have to consider that IKKα may have an important homeostatic function in normal colonic epithelial cells and therefore future studies should investigate the expression of IKKα in normal colonic tissue adjacent to invasive tumour as well as in healthy non-cancerous colonic tissue. Metastatic sites such as liver, lung and peritoneum will also provide a platform to study IKKα expression in more advanced disease, this will be an important group to study in the context of emerging IKKα inhibitors. These inhibitors will be investigated in colon cancer cell lines in the next chapter of this thesis.

In addition, the results from this chapter suggest IKKα is co-located with a marker of the Golgi apparatus. Whilst investigation of this relationship was beyond the scope of this thesis, it raises an important area for future investigation. For example, why does IKKα co-locate with the Golgi apparatus/Golgi-related structure? Does this represent dysregulation of the non-canonical NF-κB pathway? Does the accumulation of IKKα represent defunct intracellular transport machinery? As the Golgi also has a role in processes such as autophagy, future work will involve investigation of IKKα with autophagy markers such as p62 and LC3 along with lysosomal markers, LAMP. The use of multiplex immunostaining offers the opportunity to investigate multiple markers in the same tissue specimen, it is an exciting technique which allows for examination of spatial arrangement of proteins of interest as well as protein interaction/co-localisation. Altogether, results from this chapter provide an interesting area for future study.

Chapter 6:

Expression of the non-canonical NF-κB pathway in colon cancer cell lines

6.1 Introduction

Activation of the canonical NF-κB pathway in colon cancer cell lines has been reported in previous studies (286). However, studies investigating the non-canonical pathway in colon cell lines are limited. Results from tissue studies in patient cohorts (chapters 3 and 4) suggest high expression of non-canonical proteins was associated with an adverse tumour phenotype and poor clinical outcome. Therefore, it was of interest to investigate the expression of the non-canonical NF-κB pathway in colon cancer cell lines. It is well known that the development of CRC is a multistep process that results from the accumulation of genetic mutations in tumour suppressor genes and proto-oncogenes. Mutations in KRAS and BRAF are two of the primary alterations in colorectal tumourigenesis and are established markers of resistance to anti-EGFR monoclonal antibodies in the treatment of CRC. Therefore, two cell lines were investigated; HT-29 (KRAS wild-type, BRAF V600E mutated) and T84 (KRAS mutated, BRAF wild-type) (chapter 2, Table 5). Expression of non-canonical proteins was assessed in cell lines after exposure to ligands.

6.2 Activation of the non-canonical NF-κB pathway in colon cancer cell lines

Lymphotoxin α1β2 and TNFα have been reported to drive activation of the non-canonical and canonical NF-κB pathway, respectively. These ligands were selected to investigate pathway activation in colon cancer cell lines. After cells were rendered quiescent by incubation in serum free media for 24 hours, they were then exposed to either lymphotoxin α1β2 or TNFα, both at 20ng/ml for various incubation periods. Proteins were extracted from cells and separated by SDS-PAGE and transferred to a nitrocellulose membrane before visualisation (as described in chapter 2, section 2.3). To understand whether the non-canonical NF-κB pathway was expressed in the cellular setting, key markers of pathway activation including p100/52 and phosphorylation of p100 (phospho-p100) at serine residues 866/870 were measured. Western blotting was performed using protein extracts prepared from HT-29 and T84 cells.

6.2.1 Activation of the non-canonical NF-κB pathway in HT-29 and T84 cells

6.2.1.1 IKKα expression

To confirm IKKα was expressed in both cell lines investigated, HT-29 and T84 cells were stimulated with lymphotoxin α1β2 over a time course of 4 and 24 hours. This experiment was performed once. Expression of IKKα was detected in HT-29 and T84 cells. In HT-29 cells this increased at the 24-hour time point. In T84 cells, a higher basal level of IKKα was observed and this was unchanged with lymphotoxin α1β2 stimulation. To confirm this observation, future experiments should aim to repeat this with a loading control.

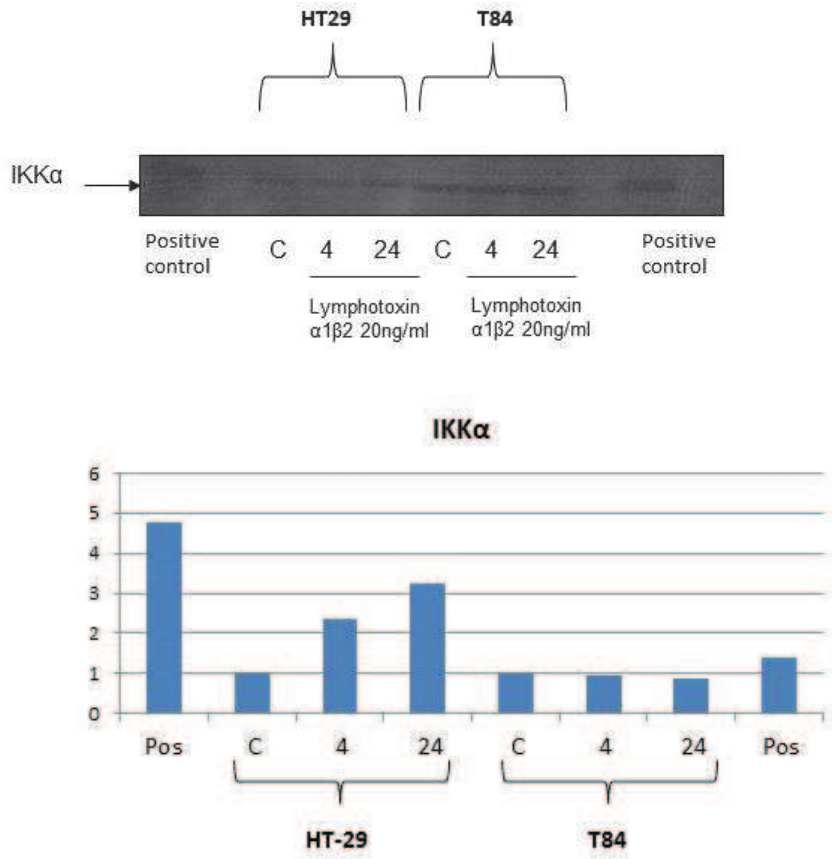
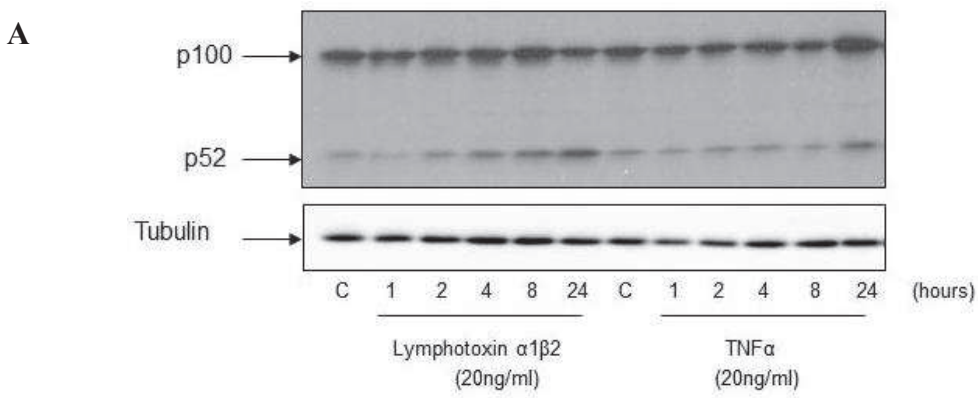


Figure 44: Expression of IKKα in HT-29 and T84 colon cancer cell lines following lymphotoxin α1β2. *HT-29 and T84 cells were exposed to 20ng/ml of lymphotoxin α1β2 over 4 and 24 hour time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of IKKα (n=1). Using ImageJ, blots were quantified by scanning densitometry. Expression levels of IKKα in cells exposed to lymphotoxin α1β2 and TNFα were compared to untreated cells and displayed as fold change to control in the bar graphs. Further statistical analysis was not performed. The results are representative of one independent experiment.*

6.2.1.2 p100/52 expression

HT-29 and T84 cells were stimulated with lymphotoxin α1β2 and TNFα in 3 separate experiments to allow quantification of p100/52 expression. Images were quantified using ImageJ and protein expression was normalised to β-tubulin loading control. In order to compare the amount of protein in each sample and allow proper assessment of the blotting, β-tubulin expression, a housekeeping gene independent of NF-κB, was used as a loading control. Differences in expression upon lymphotoxin α1β2 stimulation were calculated using fold change to untreated cells and graphs of expression were drawn. For p100/52, statistical analysis using a one-way ANOVA with Bonferroni correction and Dunnett's test was performed to compare to control levels.

Figure 45A and 45B show a representative image of lymphotoxin α1β2 and TNFα stimulation in HT-29 and T84 cells, respectively. The average fold change compared to untreated cells from three independent experiments over a time course of 1 to 24 hours was calculated. In HT-29 cells, p52 expression increased non-significantly at 4 hours with a peak at 24 hours (p=0.256), compared to control cells. TNFα exposure increased p52 expression at 24 hours where a 4.6 average fold increase was observed (p=0.035), compared to control cells. This observation requires further investigation; expression of nuclear p52 may achieve more reliable results. In T84 cells, high basal levels of p52 were observed at all time points (indicative of constitutive activation of the non-canonical pathway) with a non-significant peak at 24 hours in response to lymphotoxin α1β2 (p=0.146).



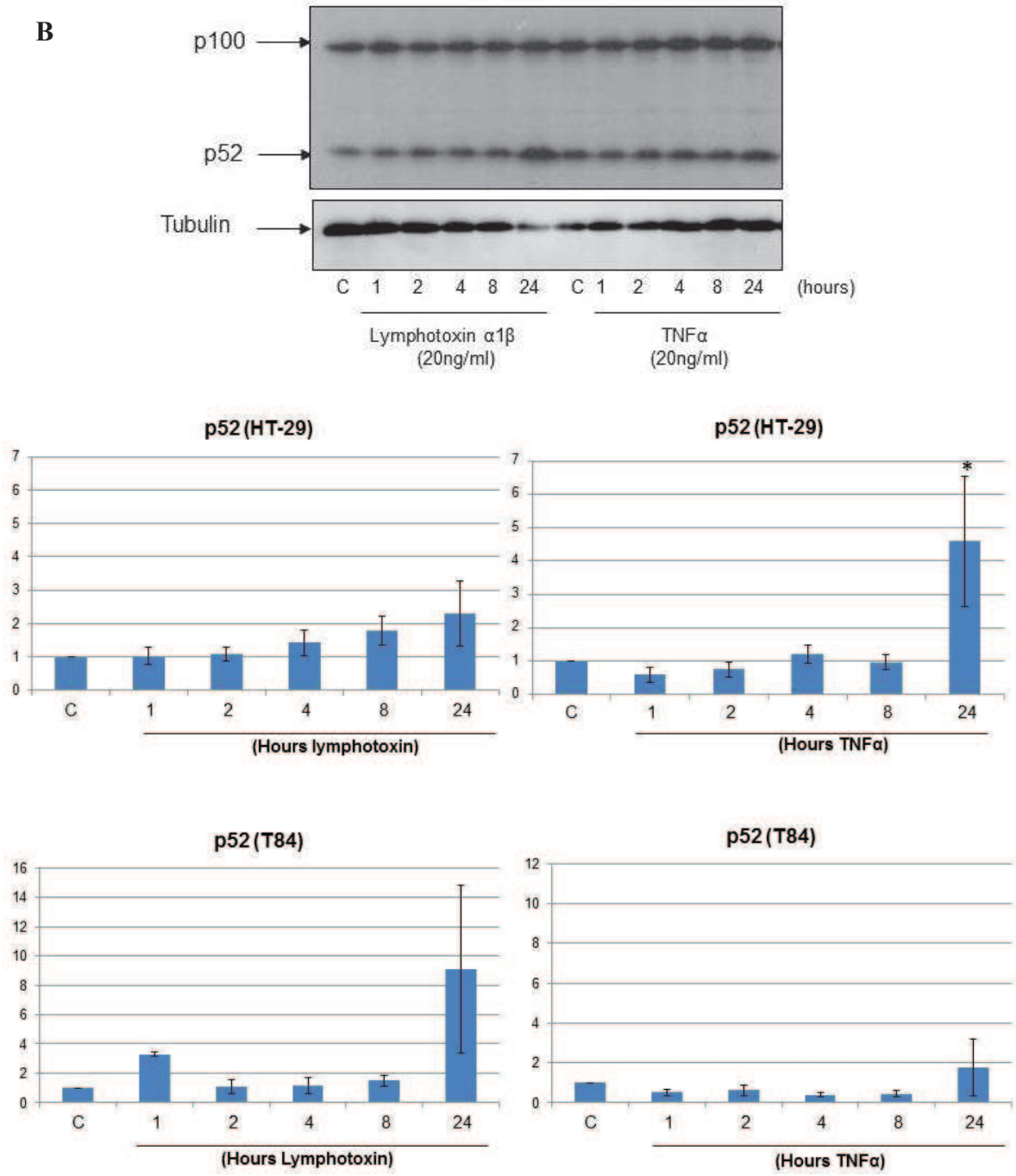


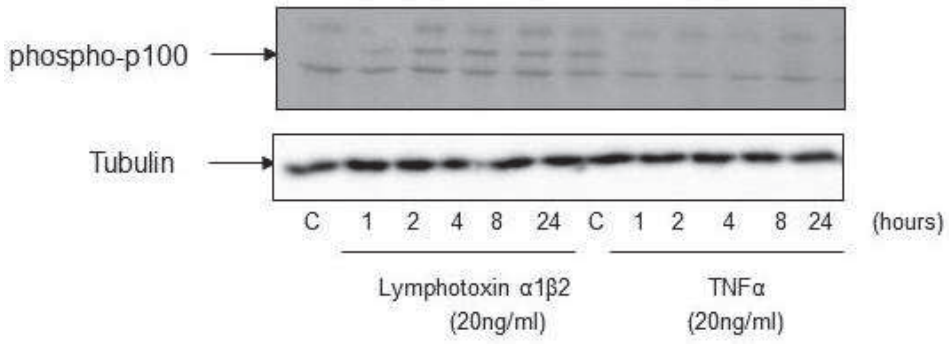
Figure 45: Expression of p100/52 in HT-29 and T84 colon cancer cell lines following lymphotoxin α1β2 and TNFα exposure. *(A) HT-29 and (B) T84 cells were exposed to 20ng/ml of lymphotoxin α1β2 or TNFα over various time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of p100/52(n=3). β-tubulin was used as a loading control. Using ImageJ, blots were quantified by scanning densitometry. Expression levels of p52 in cells exposed to lymphotoxin α1β2 and TNFα were compared to untreated cells and displayed as fold change to control in the bar graphs. Error bars represent standard error. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. * shows significant difference of $p<0.05$. The results are representative of three independent experiments.*

6.2.1.3 Phospho-p100 expression

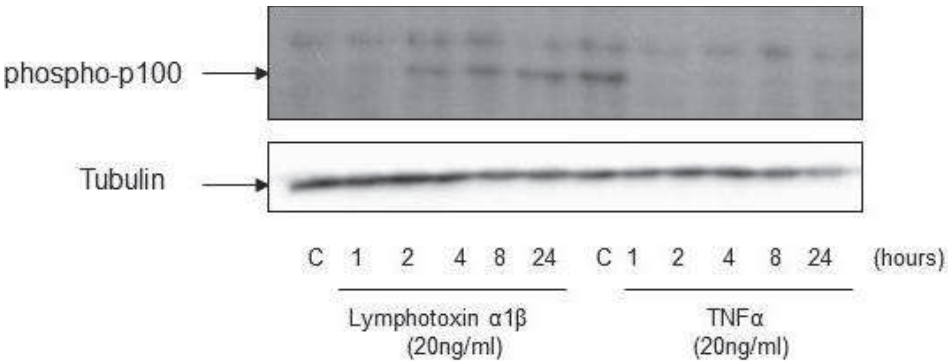
IKKα induced phosphorylation of p100 at serine 866/870 is a key step in non-canonical NF-κB pathway activation (293). Phospho-p100 expression was investigated in HT-29 and T84 cells. Due to time restrictions this experiment was only performed twice. Again, images were quantified using ImageJ and protein expression was normalised to β-tubulin loading control. Differences in expression upon lymphotoxin α1β2 and TNFα stimulation were calculated using fold change to untreated cells and graphs of expression were drawn. As this experiment was only performed twice statistical analysis was not performed.

Figure 46A and 46B show a representative image of lymphotoxin α1β2 and TNFα stimulation in HT-29 and T84 cells over a time course of 1 to 24 hours. In HT-29 and T84 cells, phospho-p100 expression increased over a 24-hour period, this peaked at 4 hours in HT-29 cells and at 24 hours in T84 cells. Expression of phospho-p100 was not observed with TNFα stimulation.

A



B



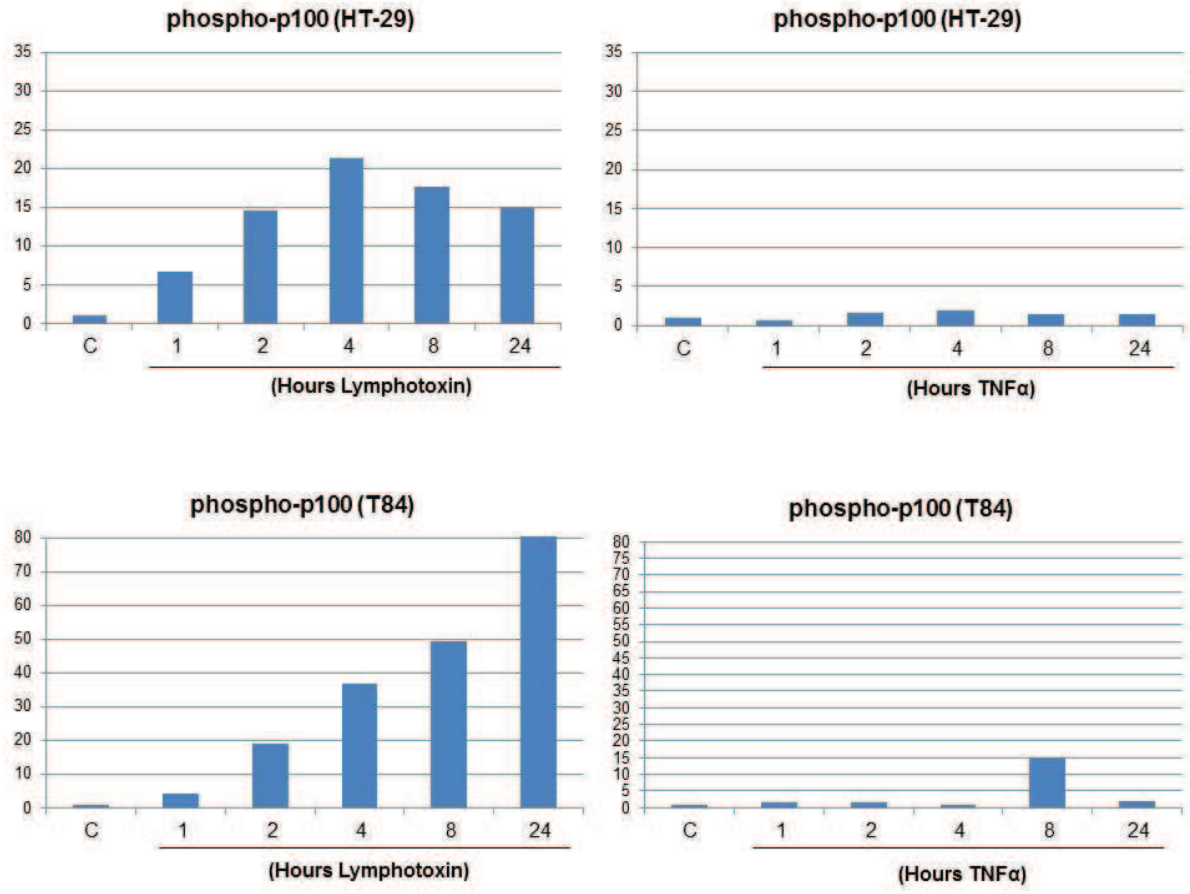


Figure 46: Expression of phospho-p100 in HT-29 and T84 colon cancer cell lines following lymphotoxin α1β2 and TNFα exposure. *(A) HT-29 and (B) T84 cells were exposed to 20ng/ml of lymphotoxin α1β2 or TNFα over various time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of phospho-p100 (n=2). β-tubulin was used as a loading control. Using ImageJ, blots were quantified by scanning densitometry. Expression levels of phospho-p100 in cells exposed to lymphotoxin α1β2 and TNFα were compared to untreated cells and displayed as fold change to control in the bar graphs. The results are representative of two independent experiments.*

6.3 Pharmacological inhibitory effect of selective IKKα inhibitor (SU1433) on the non-canonical NF-κB pathway in colon cancer cell lines

Collaborators from the Strathclyde Institute of Pharmacy and Biomedical Sciences at the University of Strathclyde have developed first-in-class IKKα selective inhibitors (SU compounds). These compounds have been investigated in the context of prostate and pancreatic cancer and have been taken forward into the *in vivo* setting. It is therefore of interest to understand the potency of these compounds on IKKα at the cellular level in colon cancer cells. Due to previous antibody issues with respect to detecting phosphorylated IKKα, p100/52 and phospho-p100 expression were used as measures of non-canonical NF-κB pathway inhibition.

6.3.1 The effect of SU1433 on lymphotoxin a1β2-, TNFα- and RANKL-induced non-canonical NF-kB signalling in HT-29 and T84 colon cancer cells

A preliminary experiment (n=1) was constructed to identify activation of the pathway following exposure to different ligands. HT-29 and T84 cells were pre-treated for 30 minutes with 3μM SU1433 followed by stimulation for 24 hours with either lymphotoxin α1β2 or TNFα. RANKL, an activator of the non-canonical NF-κB pathway was also investigated. All ligands were used at a concentration of 20ng/ml. Statistical analysis was not performed for this experiment.

Figure 47 shows a representative image of cells treated with 3μm SU1433 and stimulation with either lymphotoxin α1β2, TNFα or RANKL. All three ligands stimulated p52 expression, with highest levels observed with lymphotoxin α1β2. This was observed in both HT-29 and T84 cells, therefore, lymphotoxin α1β2 was chosen as the ligand of choice for future experiments.

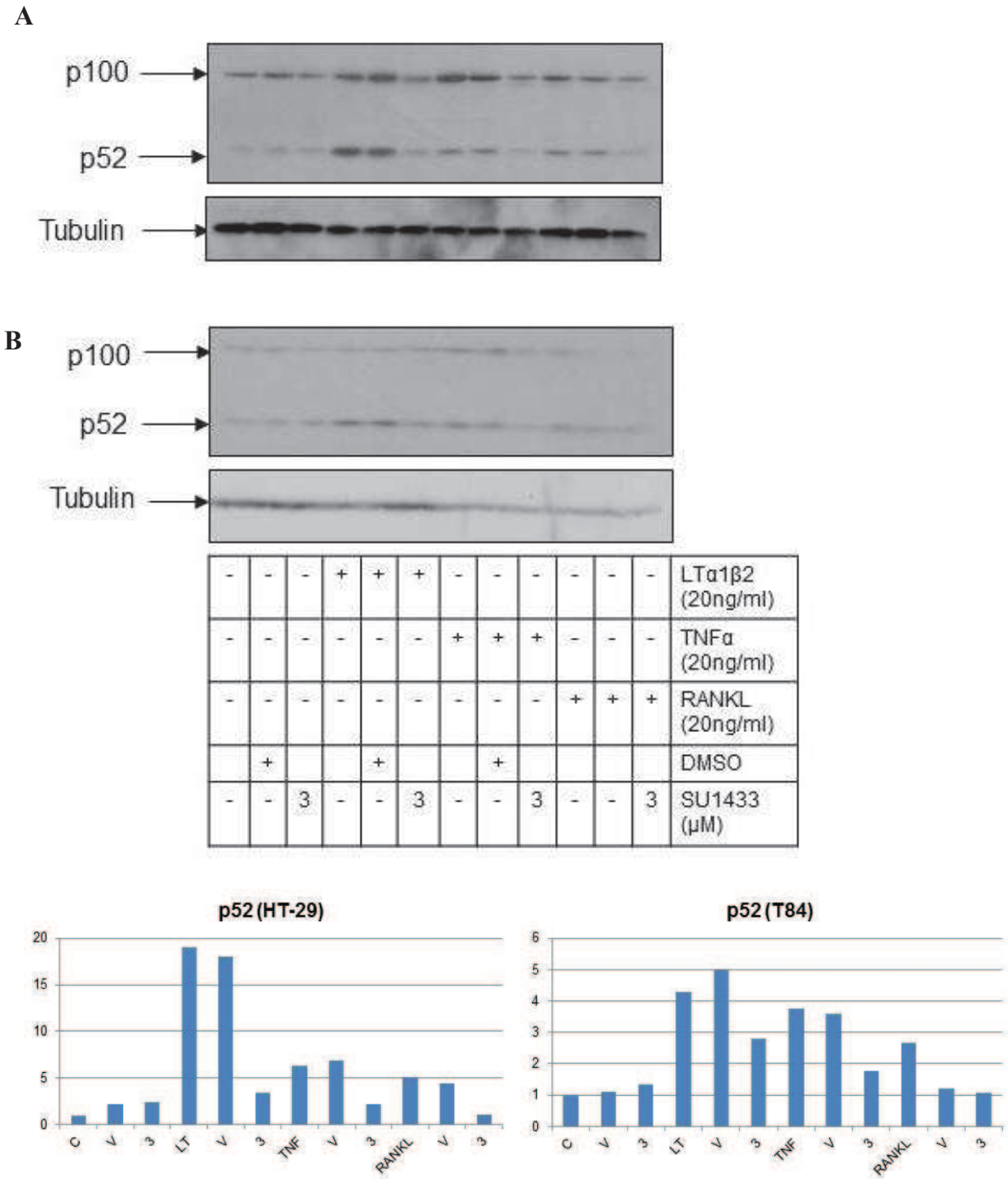


Figure 47: The effect of SU1433 on lymphotoxin a1β2-, TNFα- and RANKL-induced non-canonical NF-kB signalling in HT-29 and T84 colon cancer cells. *(A) HT-29 and (B) T84 cells were pre-treated with SU1433 for 30 minutes and then exposed to either 20ng/ml of lymphotoxin α1β2, TNFα or RANKL for 24 hours. Protein was then extracted, separated by SDS-PAGE and assessed for expression of p100/52 (n=1). Using ImageJ, blots were quantified by scanning densitometry. Expression levels of p52 in treated cells were compared to untreated cells across the three agonists and displayed as fold change to control in the bar graphs. The results are representative of one independent experiment.*

6.3.2 The effect of SU1433 on lymphotoxin a1β2 induced non-canonical NF-κB pathway in HT-29 colon cancer cells

In order to assess the inhibitory effects of SU1433 on the non-canonical NF-κB pathway, cells were treated with a dose range of 0.1-10μM of SU1433 followed by stimulation with lymphotoxin α1β2 (15ng/ml) for 4 hours. Expression of p100/52 and phospho-p100 were then assessed by western blotting. Due to time restrictions the following experiments were only carried out in HT-29 colon cancer cells.

6.3.2.1 p100/52 expression

Figure 48 demonstrates the impact of a dose range of SU1433 (0.1-10μM) on lymphotoxin a1β2 induced p52 formation in HT-29 colon cancer cells. A decrease in p52 expression was observed with dose range of SU1433 ≥ 1μM, however, this did not reach statistical significance across three independent experiments (p=0.141). Future experiments may wish to consider a longer pre-treatment with SU1433 and/or assess the effects of inhibition over a longer time period and to study inhibition of the pathway in T84 colon cancer cells.

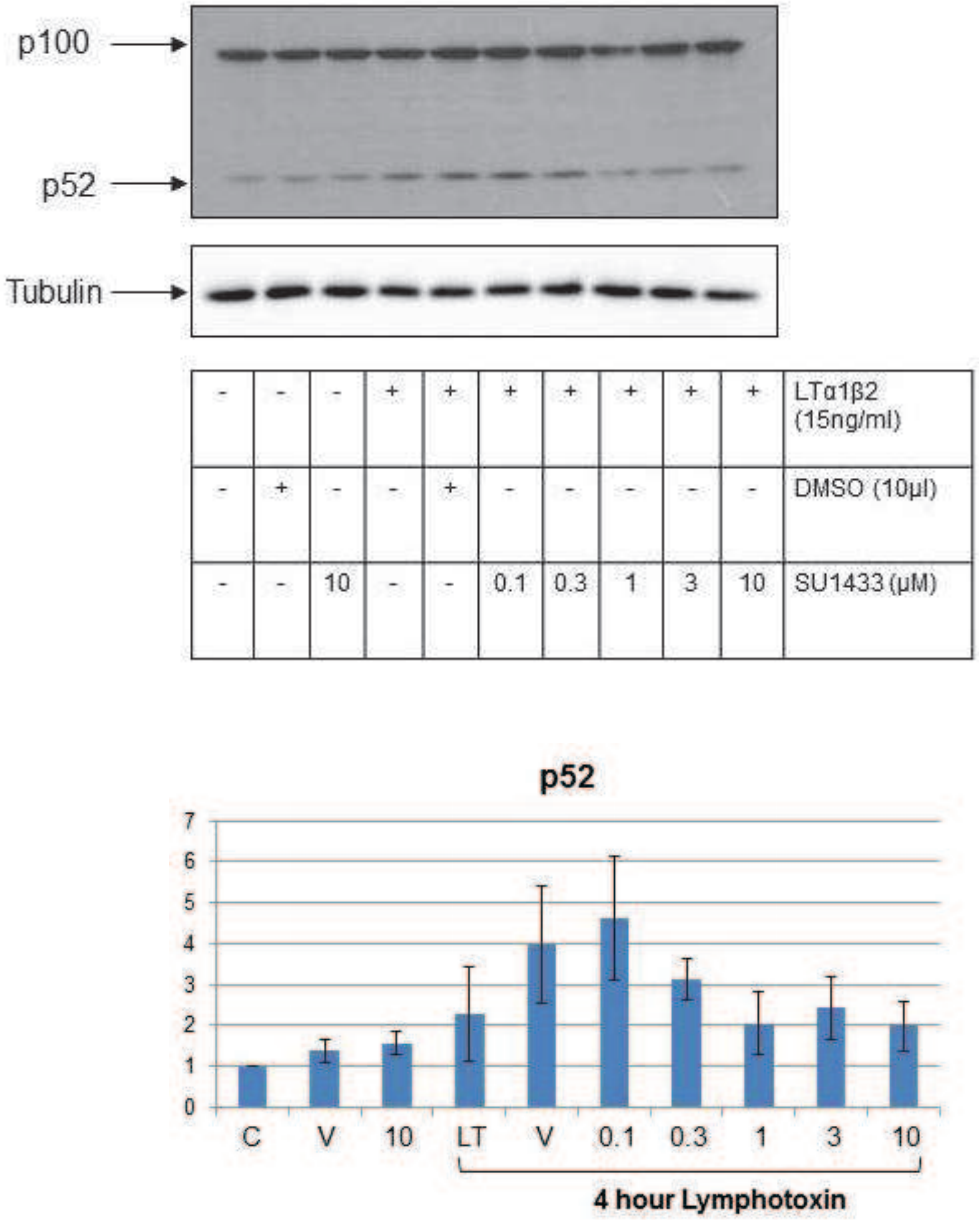
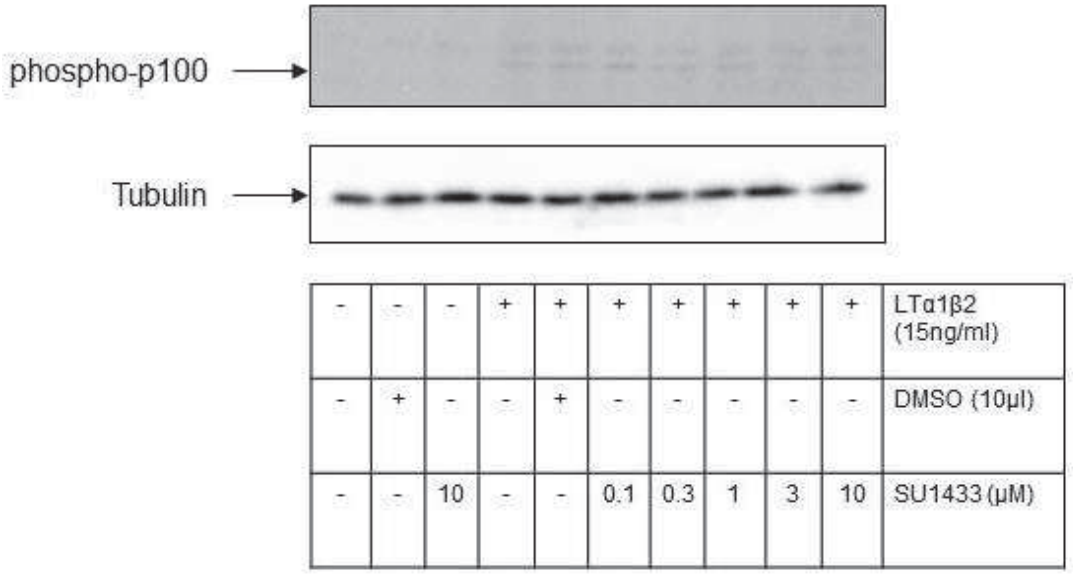


Figure 48: The effect of SU1433 on lymphotoxin a1β2-induced non-canonical NF-kB signalling in HT-29 colon cancer cells. *HT-29 cells were pre-treated with a dose range of SU1433 (0.1-10µM) for 30 minutes and then exposed to 15ng/ml of lymphotoxin α1β2 for 4 hours. Protein was then extracted, separated by SDS-PAGE and assessed for expression of p100/52 (n=3). Using ImageJ, blots were quantified by scanning densitometry. Expression levels of p52 in treated cells were compared to control and displayed as fold change to control in the bar graphs. Error bars represent standard error. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. The results are representative of three independent experiments.*

6.3.2.2 Phospho-p100 expression

HT-29 cells treated with a dose range of 0.1-10μM of SU1433 followed by stimulation with lymphotoxin α1β2 (15ng/ml) for 4 hours were assessed for expression of phospho-p100. A decrease in phospho-p100 expression was observed with a dose range ≥1μM, however this did not reach statistical significance across three independent experiments (p=0.298) (Figure 49). Again, future experiments may wish to consider a longer pre-treatment with SU1433 and/or assess the effects of inhibition over a longer time period and to study inhibition of the pathway in T84 colon cancer



cells.

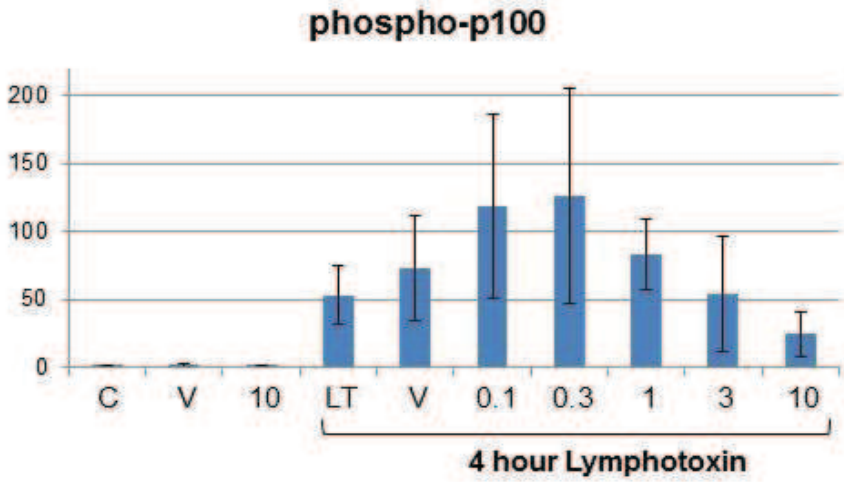


Figure 49: The effect of SU1433 on lymphotoxin a1β2-induced non-canonical NF-kB signalling in HT-29 colon cancer cells. *HT-29 cells were pre-treated with a dose range of SU1433 (0.1-10μM) for 30 minutes and then exposed to 15ng/ml of lymphotoxin α1β2 for 4 hours. Protein was then extracted, separated by SDS-PAGE and assessed for expression of phospho-p100 (n=3). Using ImageJ, blots were quantified by scanning densitometry. Expression levels of phospho-p100 in treated cells were compared to control and displayed*

as fold change to control in the bar graphs. Error bars represent standard error. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. The results are representative of three independent experiments.

6.4 Chapter discussion

The results from this chapter are from preliminary experiments that show the effect of ligand stimulation and IKKα inhibition on non-canonical NF-κB pathway activation using two colon cancer cell lines with contrasting mutational landscapes. In these preliminary experiments, reduction in pathway expression was observed in cells treated with the IKKα inhibitor, although this did not reach statistical significance. One explanation for this could be due to inadequate pre-treatment with inhibitor and future studies should aim for pre-treatment for as long is it takes for the cells to progress through two cell cycles. There is further experimental limitation as not all experiments were performed in triplicate due to time constraints and therefore future studies will aim to carry out more robust investigation to ensure reproducibility of results and enable protein quantification. Additionally, experiments using the IKKα inhibitor were limited to HT29 cells, higher basal levels of pathway proteins were observed in T84 cells which harbour a BRAF mutation, future experiments should also investigate the compound in T84 cells. Induction of the non-canonical NF-κB pathway results in phosphorylation of p100 and this was used as a measure of pathway activation. It is important to measure the quantity of phosphorylated protein, which represents the amount of active protein rather than measuring just the total amount of the protein present in a sample. However, as with many commercially available phospho-antibodies, it had many non-specific bands. Alternative markers of pathway activation have been considered. For example, phosphorylation of IKKα could be used as a marker of pathway activation however available antibodies are unable to distinguish between phosphorylated IKKα and IKKβ.

Future studies should also pursue investigation of IKKα inhibitors in a complete panel of colon cancer cell lines, this will allow some insight into the effectiveness of the inhibitors in the context of different mutational landscapes. IKKα inhibitors developed at the University of Strathclyde have shown promising results in prostate and pancreatic cancer cell lines. Additionally, results in metastatic prostate cancer xenografts have shown good efficacy with reduction in tumour volume, however, their activity has been limited by the bioavailability of the compound. Until this issue has been addressed, our lab will pursue investigation of the inhibitors in murine- and patient-derived organoids.

Chapter 7:
Discussion

Tissue studies

The canonical NF-κB pathway has been under scrutiny in the context of solid and haematological cancers for some years. Specifically in the context of CRC there is a mounting body of evidence relating to its role in tumourigenic processes. However, evidence from murine studies has demonstrated that inhibition of the canonical NF-κB pathway (IKKβ inhibition) is incompatible with life and so cannot be pursued as a viable therapeutic strategy. Therefore, the non-canonical arm of NF-κB presents itself for investigation in CRC and indeed other solid malignancies. The purpose of studying key members of the non-canonical NF-κB pathway in human CRC tissue was to understand how these proteins are expressed and their association with clinical and pathological features and patient outcomes.

Results from the Glasgow cohort (chapter 3), that is, patients who underwent surgery for stage I-III CRC were investigated using IHC for the expression of NIK, IKKα and RelB. High expression of cytoplasmic NIK was associated with adverse features including low proliferation and reduced inflammatory infiltrate at the invasive margin, both tumour features are known to be associated with poor prognosis in CRC (30,294). It is well recognised that patients who present as an emergency with CRC due to colonic obstruction, perforation or bleeding, have worse survival outcomes. This is due to a number of reasons; patients are older, often present with more advanced disease, experience the morbidity associated with emergency surgery and have a prolonged admission to hospital. Patients undergoing elective surgery represent a more heterogeneous group and when they were considered alone, those patients with high cytoplasmic expression of NIK had significantly worse CSS than those with low expression. This association was potentiated in patients with right-sided cancers and in patients with two molecular features typically associated with right-sided cancers, MMR deficiency and BRAF V600E mutation (47,295). In current practice, emergency presentation with colorectal cancer is uncommon. The difference observed in emergency and elective patients in the present study may be a historical feature associated with age of the cohort. There has been a growing interest in understanding the role of tumour location in CRC, and more recently some institutions have recommended treatment strategies based on tumour location and associated molecular characteristics (296). Therefore, it would be of interest to study the expression of cytoplasmic NIK in a larger number of patients with MMR deficient tumours and/or BRAF mutation, particularly in light of emerging NIK inhibitors that have demonstrated encouraging results in the context of autoimmune conditions (297). The role of tumour location has been under

scrutiny for some time. Colon and rectal cancer are managed as clinically distinct entities with different therapeutic and surgical strategies. Some studies suggest these tumour sites should be considered as clinically and biologically distinct disease processes based on differences in anatomic sites, embryological origin, function, treatment options and metastatic patterns. In the present study, analyses were performed with colon and rectal cancer patients combined, this strategy risks overlooking important associations between pathway expression, clinicopathological features and survival specific to tumour site (colon/rectum). To address this, future work will perform subgroup analyses to investigate these tumour sites separately. Approximately 10% of patients displayed expression of NIK at the nuclear level and this was associated with increased likelihood of patients undergoing emergency surgery and reduced inflammatory infiltrate at the invasive tumour margin. Patients who present as an emergency typically have more advanced tumours, the non-canonical NF-κB pathway has functions in regulating immune responses and so this observation may reflect dysfunction of the pathway in tumours with higher TNM stage and associated loss of normal immunological responses. There is substantial evidence that illustrates the importance of the immunological response within the tumour microenvironment of CRC. More recently in an international validation study, the Immunoscore® measuring the adaptive immunological tumour response has been shown to reliably estimate the risk of colon cancer recurrence (33). Although it was not within the scope of these studies to assess the immune contexture, it would be of interest to investigate the association between innate/adaptive immune cells and non-canonical NF-κB proteins as the pathway is known to have essential roles in regulating these immune responses. However, in contrast to cytoplasmic NIK, expression of NIK at the nuclear level was associated with significantly worse CSS in patients who were MMR proficient. Previous *in vitro* studies have demonstrated that NIK undergoes shuttling between the cytoplasm, nucleoplasm and nucleoli and has suggested the subcellular distribution of NIK to different compartments might be a means of regulating its function (298). Altogether, this raises questions about the role of NIK in CRC particularly in the context of MMR status. It should be noted that only 70 patients displayed expression of NIK at the nuclear level with a low histoscore range from 0 to 20. Although the NIK antibody had specificity confirmed using western blot, further validation studies should be performed. In addition, the investigation of NIK expression should be undertaken in external patient cohorts to see if results from the present study are reproduced.

Results from the Glasgow cohort (chapter 3), that is, patients who underwent surgery for stage I-III CRC showed high expression of cytoplasmic IKKα was associated with

phenotypic features associated with poor prognostic outcome; higher T stage, poor tumour differentiation, tumour necrosis and low proliferation. In contrast, high expression of nuclear IKKα was associated with more favourable tumour characteristics such as lower TNM stage, better tumour differentiation and high proliferation. The explanation for this is unclear. However, in contrast to results of the present study, there are a number of studies that suggest nuclear IKKα has a tumourigenic role. For example, nuclear functions of IKKα in CRC, independent of NF-κB have been described. The role of Notch signalling in cancer has been extensively reported and increased expression of Notch-target genes is a common feature in many cancers (299). Fernández-Majada and co-workers reported the aberrant activation of IKKα and subsequent recruitment to the chromatin in a variety of Notch targets, lead to the release of SMRT and transcriptional activation of these genes in colon cell lines (231). Activation of IKKα has also been implicated in the development of castrate-resistant prostate cancer. Luo and co-workers reported nuclear translocation of IKKα inhibited metastasis suppressor Maspin, and induced a metastatic phenotype *in vivo*, a process that is a consequence of tumour infiltration by RANKL-expressing inflammatory cells that activate IKKα in the nuclei of carcinoma cells to repress Maspin transcription. This study reported the amount of active nuclear IKKα in mouse and human prostate cancer correlated with metastatic progression (122,300). Altogether, with respect to results of the present study, it is unclear why high expression of nuclear IKKα was associated with favourable phenotypic tumour features and this warrants further investigation and validation in external patient cohorts.

In chapter 3, levels of cytoplasmic protein expression were combined to create two groups (low expression of NIK, IKKα and RelB vs. high expression of one or more proteins). Results showed that patients who had tumours expressing high levels of just one of the three non-canonical NF-κB proteins under investigation, had significantly reduced CSS with a reduction in 5-year survival from 85% to 75% and a reduction in 10-year survival from 79% to 70%. This reduction in survival was potentiated in patients who were MMR proficient. Overall, results from IHC suggest expression of cytoplasmic non-canonical NF-κB pathway members was associated with adverse phenotypic tumour features and reduction in CSS. The combined cytoplasmic protein expression score was not subjected to multivariate survival analysis, future studies should consider this to assess if it is independently associated with survival.

The aim of the CRC screening programme is to detect cancers at an early stage to enable curative treatment and to remove pre-cancerous polyps that have malignant potential.

Results from chapter 4 demonstrate that even in early stage screen-detected T1/2 CRC, the expression of cytoplasmic IKKα was associated with a more invasive tumour phenotype. High expression of cytoplasmic IKKα was associated with higher TNM stage and as such a greater likelihood of undergoing bowel resection rather than local resection i.e. polypectomy/TEMS. Low patient numbers and events limited statistically meaningful survival analysis in the screening cohort however; it was of interest that a large number of deaths were observed in patients highly expressing one or more non-canonical NF-κB proteins. Altogether, results from the screening cohort demonstrate that IKKα is important at the early stages of tumour invasiveness and therefore non-canonical NF-κB upregulation is likely to be important in the early stages of tumour development and progression rather than a phenomenon of advanced disease. For example, Colomer and co-workers reported deficiency of IKKα in APC mutated mice reduced tumour initiation and proliferation but was not required for normal tissue homeostasis. Using organoid-spheroid models, this group also demonstrated deficiency of IKKα resulted in a decrease in levels of genes essential for maintaining intestinal stem cell function (301). The results of this study suggest IKKα is essential for tumour initiation but is non-essential for maintaining normal homeostasis thereby making IKKα a clinically exploitable target.

Whilst expression of IKKα at the nuclear and cytoplasmic level was expected, a third pattern of staining was also observed. This 'punctate' pattern of IKKα expression was observed in the perinuclear space in a number of patients. The size and number of the puncta were variable. Expression of IKKα at the nuclear or cytoplasmic level was not associated with CSS however, patients with increasing expression of punctate IKKα had significantly worse CSS at 5- and 10-years following surgery, this was potentiated in patients with BRAF wild-type status. High expression of punctate IKKα was independently associated with CSS in the full cohort suggesting it is an important prognostic factor in patients undergoing surgery for CRC. This pattern of IKKα accumulation was considered a possible staining artefact however, the anti-IKKα antibody used in the present study has been extensively validated and is specific for IKKα as detailed in chapter 5, section 5.1. Additionally, a CRC tissue sample was always used as a negative control and the punctate staining was not observed in absence of the primary antibody. To this end, future studies should aim to use a different anti-IKKα antibody, with confirmed IKKα specificity, to further validate results of the present study. As the punctate pattern of IKKα was a novel finding, a qualitative method of assessment was used. However, we have acknowledged that to enable consistent and reproducible interpretation

of staining, attempts to quantify this staining pattern should be the scrutiny of future work, for example, using automated image analysis.

Bowen and co-workers have also reported a juxtanuclear, dot-like pattern of staining during investigation of collagen expression with IHC in normal and malignant colorectal tissue specimens. This study found the dot-like, juxtanuclear pattern of collagen expression mirrored staining observed with a 58K Golgi marker (302). Bowen and co-workers did not employ immunofluorescence in the study. Results from chapter 5 demonstrate a pattern of immunofluorescent IKKα staining that was comparable to the punctate staining observed with IHC. To understand the distribution of IKKα further, in the present study, markers of cellular transport were investigated using dual immunofluorescence. Using an antibody raised against a resident Golgi enzyme (58K, formiminotransferase cyclodeaminase), results of the present study showed IKKα was co-located with the Golgi apparatus or indeed a Golgi-related structure, where the Golgi marker appeared to closely surround IKKα punctate expression. Although the explanation for this is unclear, there are a number of possible hypotheses:

1. **Co-location of IKKα with the Golgi represents an NF-κB independent pathway involving IKKα**. For example, Margalef and co-workers reported full-length IKKα undergoes cathepsin-dependent processing to produce a truncated isoform (p45-IKKα) in cytoplasmic vesicles associated with the early endosomal marker Rab5, and that this truncated isoform of IKKα is implicated in CRC tumourigenesis *in vivo* (232). In an attempt to understand if the pattern of punctate expression observed in the present study corresponded to the 'vesicles' reported by Margalef and co-workers, two different anti-p45-IKKα antibodies were employed however, staining was not achieved with either IHC or immunofluorescence despite multiple attempts at optimisation. Additionally, results of the present study do not lend support to this groups work as punctate IKKα did not locate with the endosomal marker Rab5. This could be explained by variations in IHC technique as well as factors impacting tissue quality as discussed earlier in this thesis.

2. **Co-location of IKKα with the Golgi represents dysfunction of Golgi-related processes.** Glycosylation is a key post-translational modification that takes places in the endoplasmic reticulum/Golgi network. The process of glycosylation involves the addition of carbohydrate molecules to proteins to regulate their activity. Such modifications ensure correct protein folding, stability and direction of the new

protein to distinct cellular compartments (e.g. nucleus, membrane) (303). Abnormal glycosylation has been associated with many human cancers. Specifically in CRC, Kellokumpu and co-workers reported a change in Golgi structure. They observed that in normal colorectal tissue the Golgi has a 'horseshoe'-shaped configuration however, in cancer cells, this organisation is absent and the Golgi appear as small punctate structures around, or close to the nuclei. The same study was able to demonstrate structural and functional changes of the Golgi apparatus in CRC cells *in vivo* and *in vitro* can be attributed to alterations in Golgi pH (304) and this leads to lowered glycosylation potential.

3. **Co-location of IKKα with the Golgi represents a localised perinuclear signalling pathway.** Upregulation of key receptors involved in stimulation of the non-canonical NF-κB pathway in the perinuclear area may be responsible for a localised pathway stimulating IKKα production and activity. To investigate this further, distribution of those receptors that have an NLS should be studied.

4. **Co-location of IKKα with the Golgi may represent involvement with cGAS/STING pathway of cytosolic DNA sensing.** The cGAS/STING (Cyclic GMP-AMP synthase/Stimulator of interferon genes) pathway was initially described with respect to the antimicrobial immune response but is also under scrutiny for its role in detecting tumour-derived DNA. Cytoplasmic sensor STING is located on the endoplasmic reticulum membrane and once activated is trafficked to the Golgi. STING activates IKKβ and TBK1, TBK1 phosphorylates STING resulting in the recruitment of IRF3 for phosphorylation. IRF3 enters the nucleus where it works with canonical NF-κB proteins (p50/p65) to regulate expression of type I interferon and other pro-inflammatory cytokines (305). The trafficking of STING to the perinuclear compartment where it forms a large punctate structure has been described (306). Therefore, a possible hypothesis is that IKKα is implicated the regulation of the cGAS/STING pathway by recruitment to the perinuclear compartment.

In the pursuit of a personalised medicine approach to cancer care, the discovery of predictive biomarkers will allow new and existing treatments to be directed to those patients in who they will be most effective. It is well recognised that two patients with the same stage of CRC can have differing responses to treatment and survival outcomes. A limitation of the present study was that information regarding adjuvant therapy was only

available in 34% of patients and therefore the predictive capacity of non-canonical NF-κB proteins was not examined. This is a significant drawback of the present study and has an impact on the ability to draw conclusions about the predictive capacity of IKKα in this cohort. This incomplete data may also explain why adjuvant therapy was not associated with survival in the present study. Archival data regarding adjuvant therapy was not accessible via the ChemoCare database due to the age of the cohort used in chapter 3 (patients underwent surgery between 1997-2007). It would be important for future tissue studies using a newer patient cohort to investigate the predictive capacity of both NIK and IKKα with respect to chemo- and radiotherapy. For example in other cancers, such as oestrogen receptor-positive breast cancer, high expression of IKKα was associated with reduced time to recurrence and inferior CSS (287) and in a separate study investigating the predictive capacity of IKKα, negative expression was an independent predictive biomarker for lower breast cancer recurrence on sequential therapy (307).

***In vitro* studies**

Results from tissue studies suggested a role for the non-canonical NF-κB pathway in CRC tumourigenesis. Furthermore, differences in survival were observed in patients with BRAF wild-type status and BRAF V600E mutation. *In vitro* studies were therefore performed to investigate the non-canonical NF-κB pathway in two colon cancer cells lines with contrasting mutational landscapes; KRAS wild-type and BRAF mutated HT-29 cells and KRAS mutated and BRAF wild-type T84 cells.

In chapter 6, markers of non-canonical NF-κB pathway activation (p100/52 and phospho-p100) were evaluated using western blotting. Stimulation of HT-29 and T84 colon cancer cells resulted in increased expression of p100/52. Although this was not statistically significant, future work should consider assessing for nuclear p52 using nuclear fractions; this may provide more confirmative results. Phospho-p100 was also detected in HT-29 and T84 cells following exposure to lymphotoxin α1β2 however, this result could not be quantified as the experiment was only performed twice due to time limitations. With this drawback in mind, future studies should aim to confirm these initial observations to ensure reliable and reproducible results. T84 cells displayed higher basal levels of pathway proteins even before stimulation. Mutations in the non-canonical NF-κB pathway have been reported in multiple myeloma (308). Although there are no reported mutations associated with non-canonical NF-κB signalling in CRC, constitutive activation of the pathway has been reported in prostate cancer cell lines (309) and in pancreatic cancer cells

where NIK is constitutively involved in the processing of p100 and nuclear transport of p52 and RelB (310).

Margalef and co-workers reported differences in KRAS- and BRAF-mutant CRC cells. They reported only mutant KRAS cells were capable of inducing the NF-κB pathway and BRAF activated an IKKα independent process which resulted in the phosphorylation of a proteolytic fragment of IKKα (p45-IKKα), an abundance of p45-IKKα was observed in BRAF mutated colon cancer tissue. Treatment of cells with a RAF inhibitor resulted in reduced p45-IKKα detection but did not alter expression of IKKα/β, IκBα or p100/52 (311). Altogether this study suggested that in BRAF mutated colon cancer cells, the production of p45-IKKα is independent of the non-canonical NF-κB pathway. In the present study, lymphotoxin α1β2 stimulated samples of HT-29 and T84 cells were assessed for expression of IKKα. A band at 85kDa was indicative of full-length IKKα however, a band at 45kDa, reflecting truncated IKKα reported by Margalef and co-workers, was not detected. An antibody raised against p45-IKKα was also used (results not shown) however, there were no findings to lend support to the work of Margalef and co-workers.

As discussed, IKKα is upregulated in prostate and pancreatic cancer cells. Unpublished results have shown that the combination of IKKα inhibitor with existing treatments has a synergistic effect, with cancer cells becoming more sensitised to current standard treatments for both castrate-resistant prostate cancer and pancreatic cancer. Encouragingly, preliminary results from *in vivo* studies in mice with metastatic prostate cancer xenografts have shown reduction in tumour volume. Results from chapter 6 showed that in HT-29 cells treated with a dose range of IKKα inhibitor, SU1433, inhibition of non-canonical NF-κB activity was observed as reflected by diminished expression of p52 and phosphorylated-p100. Although this did not reach statistical significance over three independent experiments, future experiments should consider repeating this work with different conditions such as a longer pre-treatment with inhibitor and/or ligand exposure over a longer time period. Due to time limitations this experiment was only performed in HT-29 cells, future studies should aim to examine the effect of IKKα inhibitors in T84 colon cancer cells, which displayed a higher basal level of non-canonical NF-κB activity.

Targeting specific signalling pathways that drive tumourigenesis offer the potential of a more specific therapy with reduced systemic toxicity. One of the most successful examples of this is the anti-HER2 antibody in HER2-positive breast cancer. Selecting a molecular target or pathway will depend on how the pathway is dysregulated. NF-κB dysregulation

has been observed in a number of cancers, and specifically in CRC, is associated with tumour promoting processes and resistance to existing therapies (286). Therefore, inhibiting NF-κB presents itself as potential therapeutic strategy. The proteasome inhibitor Bortezomib has been used successfully in multiple myeloma however, this success has not translated into solid malignancies including CRC. NF-κB has essential functions in tissue homeostasis and immunity. To date, non-selective IKK inhibitors or IKKβ-specific inhibitors have been reported however, they have been associated with significant side effects. Genetic and pharmacological inhibition of IKKβ results in extreme toxicity (110), effects of inhibiting the canonical NF-κB pathway include inflammatory skin disease and a compromising effect on colonic epithelium (312). In addition, murine studies show IKKβ knockout results in extensive liver damage (313). Further to this, there are number of reasons why IKKα appears to be a good therapeutic strategy in general. Firstly, previous studies have shown that IKKα is dispensable for normal tissue function as well as normal canonical NF-κB activity. Secondly, IKKα inhibitors have demonstrated good efficacy in prostate and pancreatic cancer cells. Until more recently, there had been no reports of IKKα selective inhibitors. Through collaboration with the University of Strathclyde, our laboratory has been involved in testing first-in-class IKKα selective inhibitors. These inhibitors have shown promising early results and progress into pre-clinical testing is awaited. The present study has not been able to generate sufficient data regarding the effectiveness of these compounds *in vitro*, more rigorous testing of these compounds in a complete panel of colon cancer cell lines, patient-derived organoids and murine models of CRC will be pursued.

Future work

The results presented in this thesis lend support to previous studies that have investigated expression of the non-canonical NF-κB pathway in breast and prostate cancer tissue specimens. Specifically, expression of IKKα has once again been highlighted as an important prognostic marker for survival. In the context of CRC, the canonical arm of NF-κB has been implicated in chemo- and radio-resistance however, the non-canonical arm is under investigated. It would be important for future studies to investigate the predictive capacity of IKKα with respect to existing chemotherapy agents and responses to radiotherapy. Although patients with metastatic disease were excluded from analysis in the present study, future studies should explore expression of the pathway in more advanced disease. There is substantial evidence that illustrates the importance of the immunological response within the tumour microenvironment of CRC. Our lab is carrying out work to

assess the immune contexture in the Glasgow cohort used in this thesis. This work will generate essential information that will allow us to understand the association between non-canonical NF-κB pathway expression and the innate and adaptive immune responses within the tumour microenvironment. How the results presented in this thesis relate to the consensus molecular subtypes of CRC will also provide an interesting area for future investigation.

We have also started carrying out work to understand the genomic and transcriptomic differences in patients with low and high punctate IKKα expression. These results will help us to identify upstream drivers and their relationship with the pathway in CRC.

Whilst preliminary experiments were carried out in colon cancer cells lines using IKKα inhibitors, more rigorous testing should be the focus of future studies. To understand the role of IKKα in more detail, mechanistic studies should aim to assess the impact of IKKα activation/silencing on cell viability/apoptosis as well as gene expression. In addition, we plan to assess the efficacy of these inhibitors in murine- and patient-derived organoids. In the present study, localisation of IKKα in the perinuclear space was investigated in tissue; however, this was not extended into the cellular setting. It would be of interest to investigate the distribution of IKKα in colon cancer cells and organoids using fluorescence and in particular any effect on IKKα localisation following treatment with selective IKKα inhibitors.

We hypothesised that high expression of the non-canonical NF-κB pathway was associated with poorer outcome in patients undergoing surgery for stage I-III CRC. In conclusion, results presented in this thesis have shown the non-canonical NF-κB pathway and in particular, IKKα, has a possible role in progression of CRC resulting in a more invasive tumour phenotype and significantly reduced survival in patients undergoing surgery for stage I-III CRC. The perinuclear accumulation of IKKα has raised a number of questions regarding its potentially tumourigenic role. Together with preliminary cell line work, results from this thesis provide sufficient evidence to pursue additional tissue studies and mechanistic work to fully elucidate the role of IKKα in CRC and the potential therapeutic impact of selective IKKα inhibitors *in vitro*.

References

1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.1. Cancer Incidence and Mortality Worldwide: IARC CancerBase. 01/08/2018. Available from: http://globocan.iarc.fr/Default.aspx

2. Cancer Research UK. Bowel Cancer Statistics. 01/08/2018. Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/incidence

3. Welch HG, Robertson DJ. Colorectal cancer on the decline - why screening can't explain it all. *N Engl J Med*. 2016; 374(17):1605–7

4. Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer. *Gastroenterology*. 2010; 138(6):2044–58

5. Wynder EL, Kajitani T, Ishikawa S, Dodo H, Takano A. Environmental factors of cancer of the colon and rectum II. Japanese epidemiological data. *Cancer*. 1969; 23(5):1210–20

6. Haggar FA, Boushey RP. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clin Colon Rectal Surg*. 2009; 22(4):191–7

7. Reddy BS. Types and amount of dietary fat and colon cancer risk: Prevention by omega-3 fatty acid-rich diets. *Environ Health Prev Med*. 2002; 7(3):95–102

8. Wang Y, Yang H, Shen C-J, Ge J-N, Lin J. Association between alcohol consumption and colorectal cancer risk: a case-control study in the Han Chinese population. *Eur J Cancer Prev.* 2018; 27(5):433-437

9. Aykan NF. Red meat and colorectal cancer. *Oncol Rev*. 2015; 9(1):288

10. Aune D, Chan DSM, Lau R, Vieira R, Greenwood DC, Kampman E, et al. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *BMJ*. 2011; 343:6617–6617

11. Wolin KY, Yan Y, Colditz GA, Lee I-M. Physical activity and colon cancer prevention: a meta-analysis. *Br J Cancer*. 2009; 100(4):611–6

12. Chao A, Thun MJ, Jacobs EJ, Henley SJ, Rodriguez C, Calle EE. Cigarette smoking and colorectal cancer mortality in the cancer prevention study II. *J Natl Cancer Inst* 2000; 92(23):1888–96

13. Van Blarigan EL, Meyerhardt JA. Role of physical activity and diet after colorectal cancer diagnosis. *J Clin Onc.* 2015; 33(16):1825–34

14. Yang B, Jacobs EJ, Gapstur SM, Stevens V, Campbell PT. Active smoking and mortality among colorectal cancer survivors: The cancer prevention study II nutrition cohort. *J Clin Oncol*. 2015; 33(8):885–93

15. Kim ER, Chang DK. Colorectal cancer in inflammatory bowel disease: The risk, pathogenesis, prevention and diagnosis. *World J Gastroenterol.* 2014; 20(29):9872–81

16. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: A meta-analysis. *Gut*. 2001; 48(4):526–35

17. Chan AOO, Man HJ, Kwok FL, Morris JS, Siu DCW, Tong T, et al. Prevalence of colorectal neoplasm among patients with newly diagnosed coronary artery disease. *J Am Med Assoc*. 2007; 298(12):1412–9

18. Mishra N, Hall J. Identification of patients at risk for hereditary colorectal cancer. *Clin Colon Rectal Surg*. 2012; 25(2):67–82

19. Lynch HT, de la Chapelle A. Hereditary Colorectal Cancer. *N Engl J Med*. 2003; 348(10):919–32

20. Varesco L. Familial adenomatous polyposis: Genetics and epidemiology. *Tech Coloproctol.* 2004; 8(2):S305-S308

21. Aaltonen L, Peltomaki P, Leach F, Sistonen P, Pylkkanen L, Mecklin J, et al. Clues to the pathogenesis of familial colorectal cancer. *Science*. 1993; 260(5109):812–6

22. Peltomaki P, Lothe RA, Aaltonen LA, Pylkkanen L, Nystrom-Lahti M, Seruca R, et al. Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome. *Cancer Res*. 1993; 53(24):5853–5

23. Cohen SA, Leininger A. The genetic basis of Lynch syndrome and its implications for clinical practice and risk management. *Appl Clin Genet.* 2014; 7:147–58

24. Ferrone C, Dranoff G. Dual roles for immunity in gastrointestinal cancers. *J Clin Onc.* 2010; 28(26):4045–51

25. Hanahan D, Weinberg RA. Hallmarks of Cancer : The Next Generation. *Cell*. 2011; 144(5):646–74

26. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene.* 2008; 27(45): 5904–12

27. Balkwill F, Coussens LM. Cancer: An inflammatory link. *Nature.* 2004; 431(7007):405–6

28. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*. 2006; 313(5795):1960–4

29. Mlecnik B, Bindea G, Angell HK, Maby P, Angelova M, Tougeron D, et al. Integrative analyses of colorectal cancer show immunoscore is a stronger predictor of patient survival than microsatellite instability. *Immunity*. 2016; 44(3):698–711

30. Klintrup K, Mäkinen JM, Kauppila S, Väre PO, Melkko J, Tuominen H, et al. Inflammation and prognosis in colorectal cancer. *Eur J Cancer*. 2005; 41(17):2645–54

31. Richards CH, Flegg KM, Roxburgh CSD, Going JJ, Mohammed Z, Horgan PG, et al. The relationships between cellular components of the peritumoural inflammatory response, clinicopathological characteristics and survival in patients with primary operable colorectal cancer. *Br J Cancer*. 2012; 106(12):2010–5

32. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, et al. Towards the introduction of the “Immunoscore” in the classification of malignant tumours. *J Pathol*. 2014; 232(2):199–209

33. Pagès F, Mlecnik B, Bindea G, et al. International validation of the consensus Immunoscore for classification of colon cancer: a prognostic and accuracy study. *Lancet.* 2018;391(10135):2128-2139

34. Dolan RD, McSorley ST, Horgan PG, Laird B, McMillan DC. The role of the systemic inflammatory response in predicting outcomes in patients with advanced inoperable cancer: Systematic review and meta-analysis. *Crit Rev Oncol Hematol.* 2017; 116:34–46

35. Li M-X, Liu X-M, Zhang X-F, Zhang J-F, Wang W-L, Zhu Y, et al. Prognostic role of neutrophil- to- lymphocyte ratio in colorectal cancer: A systematic review and meta- analysis. *Int J Cancer*. 2014; 134(10):2403–13

36. Park JH, Watt DG, Roxburgh CSD, Horgan PG, McMillan DC. Colorectal Cancer, Systemic Inflammation, and Outcome: Staging the Tumor and Staging the Host. *Ann Surg*. 2016; 263(2):326–36

37. Roxburgh CS, McMillan DC. Role of systemic inflammatory response in predicting survival in patients with primary operable cancer. *Futur Oncol*. 2010; 6(1):149–63

38. Guthrie GJK, Charles KA, Roxburgh CSD, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: Experience in patients with cancer. *Crit Rev Oncol Hematol.* 2013; 88(1):218–30

39. McMillan DC. The systemic inflammation-based Glasgow Prognostic Score: A decade of experience in patients with cancer. *Cancer Treat Rev*. 2013; 39(5): 534–40

40. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med*. 1988; 319(9): 525–32

41. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* 1990;

61(5):759–67

42. Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. *Nat Rev Cancer.* 2009; 9(7): 489–99

43. Worthley DL, Whitehall VL, Spring KJ, Leggett BA. Colorectal carcinogenesis: Road maps to cancer. *World J Gastroenterol*. 2007; 13(28):3784–91

44. Boland CR, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology*. 2010; 138(6)

45. Ward R, Meagher A, Tomlinson I, O'Connor T, Norrie M, Wu R, et al. Microsatellite instability and the clinicopathological features of sporadic colorectal cancer. *Gut*. 2001; 48(6):821–9

46. Popat S, Hubner RA, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol*. 2005; 23(3):609–18

47. Gelsomino F, Barbolini M, Spallanzani A, Pugliese G, Cascinu S. The evolving role of microsatellite instability in colorectal cancer: A review. *Cancer Treat Rev.* 2016; 51:19-26

48. Lindor NM, Burgart LJ, Leontovich O, Goldberg RM, Cunningham JM, Sargent DJ, et al. Immunohistochemistry versus microsatellite instability testing in phenotyping coloreetal tumors. *J Clin Oncol*. 2002; 20(4):1043–8

49. Esteller M. Molecular Origins of Cancer: Epigenetics in Cancer. *N Engl J Med*. 2008; 358:1148–59

50. Curtin K, Slattery ML, Samowitz WS. CpG Island Methylation in Colorectal Cancer: Past, Present and Future. *Patholog Res Int*. 2011; 2011:1–8

51. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet*. 2006; 38(7):787–93

52. Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, Murtaugh MA, et al. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. *Gastroenterology*. 2005; 129(3):837–45

53. Slattery ML, Curtin K, Wolff RK, Boucher KM, Sweeney C, Edwards S, et al. A comparison of colon and rectal somatic DNA alterations. *Dis Colon Rectum*. 2009; 52(7):1304–11

54. Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med*. 2015; 21(11):1350–6

55. Roseweir AK, McMillan DC, Horgan PG, Edwards J. Colorectal cancer subtypes: Translation to routine clinical pathology. *Cancer Treat Rev*. 2017; 57:1–7

56. Nelson H, Petrelli N, Carlin A, Couture J, Fleshman J, Guillem J, et al. Guidelines 2000 for colon and rectal cancer surgery. *J Natl Cancer Inst*. 2001; 93(8):583–96

57. Hortobagyi GN, Esserman L BT. Neoplasms of the small intestine, vermiform appendix, and peritoneum, and carcinoma of the colon and rectum. In: Holland-Frei *Cancer Medicine*. 2006; 1151–1176

58. Heald RJ, Moran BJ, Ryall RDH, Sexton R, MacFarlane JK. Rectal Cancer: The Basingstoke experience of total mesorectal excision, 1978-1997. *Arch Surg*. 1998; 133(8):894–9

59. Martling AL, Holm T, Rutqvist L-E, Moran B, Heald R, Cedermark B. Effect of a surgical training programme on outcome of rectal cancer in the County of Stockholm. *Lancet*. 2000; 356(9224):93–6

60. Wibe A, Rendedal PR, Svensson E, Norstein J, Eide TJ, Myrvold HE, et al. Prognostic significance of the circumferential resection margin following total mesorectal excision for rectal cancer. *Br J Surg*. 2002; 89(3):327–34

61. Sargent DJ, Goldberg RM, Jacobson SD, Macdonald JS, Labianca R, Haller DG, et al. A pooled analysis of adjuvant chemotherapy for resected colon cancer in elderly patients. *N Engl J Med*. 2001; 345(15):1091–7

62. André T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med*. 2004; 350(23):2343–51

63. André T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol*. 2009; 27(19):3109–16

64. Sargent DJ, Marsoni S, Monges G, Thibodeau SN, Labianca R, Hamilton SR, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol*. 2010; 28(20):3219–26

65. Group MS. Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: prospective observational study. *BMJ*. 2006; 333(7572):779–0

66. Scottish Intercollegiate Guidelines Network (SIGN). Diagnosis and management of colorectal cancer. 03/06/2019. Available from: https://www.sign.ac.uk/assets/sign126.pdf

67. Van Cutsem E, Köhne C-H, Hitre E, Zaluski J, Chang Chien C-R, Makhson A, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med*. 2009; 360(14):1408–17

68. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*. 1986; 46(5):705–16

69. Sen R, Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. *Cell*. 1986;47(6):921–8

70. Karin M, Cao Y, Greten FR, Li Z-W. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer*. 2002; 2(4):301–10

71. Gilmore TD. The Rel/NF-kappaB signal transduction pathway: introduction. *Oncogene*. 1999; 18(49):6842–4

72. Ghosh G, Wang VYF, Huang D Bin, Fusco A. NF-kB regulation: Lessons from structures. *Immunol Rev*. 2012; 246(1):36–58

73. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-κB. *Annu Rev Cell Biol*. 1994; 10:405–55

74. Gilmore TD. Introduction to NF-κB: players, pathways, perspectives. *Oncogene*. 2006; 25(51):6680–4

75. Rice NR, MacKichan ML, Israël A. The precursor of NF-kappa B p50 has I kappa B-like functions. *Cell*. 1992; 71(2):243–53

76. Naumann M, Nieters A, Hatada EN, Scheidereit C. NF-kappa B precursor p100 inhibits nuclear translocation and DNA binding of NF-kappa B/rel-factors. *Oncogene*. 1993; 8(8):2275–81

77. Gilmore TD, Herscovitch M. Inhibitors of NF-κB signaling: 785 and counting. *Oncogene*. 2006; 25(51):6887–99

78. Woronicz JD, Gao X, Cao Z, Rothe M, Goeddel D V. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science*. 1997; 278(5339):866–9

79. Mercurio F. IKK-1 and IKK-2: Cytokine-activated IB kinases essential for NF-κB activation. *Science*. 1997; 278(5339):860–6

80. Régnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. Identification and characterization of an IkappaB kinase. *Cell*. 1997; 90(2):373–83

81. Huang W-C. c-Src-dependent tyrosine phosphorylation of IKKbeta is involved in tumor necrosis factor-alpha -induced intercellular adhesion molecule-1 expression. *J Biol Chem*. 2003; 278(11):9944–52

82. Ling L, Cao Z, Goeddel DV. NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. *Proc Natl Acad Sci U S A*. 1998; 95(7):3792–7

83. Chariot A. The NF-κB-independent functions of IKK subunits in immunity and

cancer. *Trends Cell Biol*. 2009; 19(8):404–13

84. Yamamoto Y, Verma UN, Prajapati S, Kwak Y-T, Gaynor RB. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature*. 2003; 423(6940):655–9

85. Liu F, Xia Y, Parker AS, Verma IM. IKK biology. I*mmunol Rev*. 2012; 246(1):239–53

86. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*. 2001; 412(6844):346–51

87. Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB. TAK1 is critical for IkB kinase-mediated activation of the NF-kB pathway. *J Mol Biol*. 2003; 326(1):105–15

88. Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, et al. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol*. 2005; 6(11):1087–95

89. Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, et al. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med*. 1999; 189(11):1839–45

90. Rudolph D, Yeh WC, Wakeham A, Rudolph B, Nallainathan D, Potter J, et al. Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev*. 2000; 14(7):854–62

91. Ghosh S, Baltimore D. Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* 1990; 334(6267):678–82

92. Coope HJ, Atkinson PGP, Huhse B, Belich M, Janzen J, Holman MJ, et al. CD40 regulates the processing of NF-κB2 p100 to p52. *EMBO J*. 2002; 21(20):5375–85

93. Kayagaki N, Yan M, Seshasayee D, Wang H, Lee W, French DM, et al. BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-κB2. *Immunity*. 2002;

17(4):515–24

94. Claudio E, Brown K, Park S, Wang H, Siebenlist U. BAFF-induced NEMO-independent processing of NF-κB2 in maturing B cells. *Nat Immunol*. 2002; 3(10):958–65

95. Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, et al. The lymphotoxin-β receptor induces different patterns of gene expression via two NF-κB pathways. *Immunity*. 2002; 17(4):525–35

96. Novack DV, Yin L, Hagen-Stapleton A, Schreiber RD, Goeddel D V, Ross FP, et al. The IkappaB function of NF-kappaB2 p100 controls stimulated osteoclastogenesis. *J Exp Med*. 2003; 198(5):771–81

97. Senftleben U, Cao Y, Xiao G, Greten FR, Krähn G, Bonizzi G, et al. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science*. 2001; 293(5534):1495–9

98. Xiao G, Harhaj EW, Sun SC. NF-κB-inducing kinase regulates the processing of NF-κB2 p100. *Mol Cell*. 2001; 7(2):401–9

99. Sun SC. The noncanonical NF-κB pathway. *Immunol Rev*. 2012; 246(1):125–40

100. Solan NJ, Miyoshi H, Carmona EM, Bren GD, Paya CV. RelB cellular regulation and transcriptional activity are regulated by p100. *J Biol Chem*. 2002; 277(2):1405–18

101. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med*. 1997; 336(15):1066–71

102. Gewirtz AT, Rao AS, Simon PO, Merlin D, Carnes D, Madara JL, et al. Salmonella typhimurium induces epithelial IL-8 expression via Ca(2+)-mediated activation of the NF-kappaB pathway. *J Clin Invest*. 2000; 105(1):79–92

103. Lavon I, Goldberg I, Amit S, Landsman L, Jung S, Tsuberi BZ, et al. High susceptibility to bacterial infection, but no liver dysfunction, in mice compromised for hepatocyte NF-kappaB activation. *Nat Med*. 2000; 6(5):573–7

104. Snapper CM, Zelazowski P, Rosas FR, Kehry MR, Tian M, Baltimore D, et al. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J Immunol*. 1996; 156(1):183–91

105. Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS. Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. *Oncogene*. 2000; 19(9):1123–31

106. Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS. NF-κB Controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol*. 1999; 19(8):5785–99

107. La Rosa FA, Pierce JW, Sonenshein GE. Differential regulation of the c-myc oncogene promoter by the NF-kappa B rel family of transcription factors. *Mol Cell Biol*. 1994; 14(2):1039–44

108. Nickols JC, Valentine W, Kanwal S, Carter BD. Activation of the transcription factor NF-kappaB in Schwann cells is required for peripheral myelin formation. *Nat Neurosci*. 2003; 6(2):161–7

109. Cao Y, Karin M. NF-κB in Mammary Gland Development and Breast Cancer. *J Mammary Gland Biol Neoplasia*. 2003; 8(2)

110. Greten FR, Arkan MC, Bollrath J, Hsu L-C, Goode J, Miething C, et al. NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell*. 2007; 130(5):918–31

111. Sun SC, Ganchi PA, Ballard DW, Greene WC. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science*. 1993; 259(5103):1912–5

112. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science*. 2002; 298(5596):1241–5

113. Kearns JD, Basak S, Werner SL, Huang CS, Hoffmann A. IkappaBepsilon provides

negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. *J Cell Biol*. 2006; 173(5):659–64

114. Ruland J. Return to homeostasis: Downregulation of NF-κB responses. *Nature Immunology*. 2011; 12(8):709-14

115. Liao G, Zhang M, Harhaj EW, Sun SC. Regulation of the NF-kB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *J Biol Chem*. 2004; 279(25):26243–50

116. He JQ, Zarnegar B, Oganesyan G, Saha SK, Yamazaki S, Doyle SE, et al. Rescue of TRAF3-null mice by p100 NF-κB deficiency. *J Exp Med*. 2006; 203(11):2413–8

117. Gray CM, Remouchamps C, McCorkell KA, Solt LA, Dejardin E, Orange JS, et al. Noncanonical NF-kappaB signaling is limited by classical NF-kappaB activity. *Sci Signal*. 2014; 7(311):ra13

118. Davis-Turak J, Macal M, Huang JQ, Kearns JD, Yu T, Fagerlund R, et al. Control of RelB during dendritic cell activation intergrates canonical and non-canonical NF-kB pathways. *Nat Immunol.* 2013; 13(12):1162–70

119. Shembade N, Pujari R, Harhaj NS, Abbott DW, Harhaj EW. The kinase IKKα inhibits activation of the transcription factor NF-κB by phosphorylating the regulatory molecule TAX1BP1. *Nat Immunol*. 2011; 12(9):834–43

120. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires Akt serine-threonine kinase. *Nature*. 1999; 401(6748):82–5

121. Beinke S, Robinson MJ, Hugunin M, Ley SC. Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IκB kinase-induced proteolysis of NF-κB1 p105. *Mol Cell Biol*. 2004; 24(21):9658–67

122. Luo J-L, Tan W, Ricono JM, Korchynskyi O, Zhang M, Gonias SL, et al. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature*. 2007; 446(7136):690–4

123. Xia Y, Padre RC, De Mendoza TH, Bottero V, Tergaonkar VB, Verma IM. Phosphorylation of p53 by IkappaB kinase 2 promotes its degradation by beta-TrCP. *Proc Natl Acad Sci U S A*. 2009; 106(8):2629–34

124. Naugler WE, Karin M. NF-kB and cancer - identifying targets and mechanisms. *Curr Opin Genet Dev*. 2008; 18(1):19–26

125. Willis TG, Jadayel DM, Du MQ, Peng H, Perry AR, Abdul-Rauf M, et al. Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. *Cell*. 1999; 96(1):35–45

126. Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, et al. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell*. 2000; 6(4):961–7

127. Lenz G, Davis RE, Ngo VN, Lam L, George TC, Wright GW, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science*. 2008; 319(5870):1676–9

128. Annunziata CM, Davis RE, Demchenko Y, Bellamy W, Gabrea A, Zhan F, et al. Frequent engagement of the classical and alternative NF-κB pathways by diverse genetic abnormalities in multiple myeloma. *Cancer Cell*. 2007; 12(2):115–30

129. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, et al. IKK-beta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell*. 2004; 118(3):285–96

130. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature*. 2009; 462(7269):108–12

131. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol*. 2005; 5(10):749–59

132. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000; 100(1):57–70

133. Scartozzi M, Bearzi I, Pierantoni C, Mandolesi A, Loupakis F, Zaniboni A, et al. Nuclear factor-kB tumor expression predicts response and survival in irinotecan-refractory metastatic colorectal cancer treated with cetuximab-irinotecan therapy. *J Clin Oncol*. 2007; 25(25):3930–5

134. Wang C-Y, Mayo MW, Baldwin AS. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science*. 1996; 274(5288):784–7

135. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*. 1999; 18(49):6853–66

136. Huang S, Robinson JB, DeGuzman A, Bucana CD, Fidler IJ. Blockade of Nuclear Factor-κB Signaling Inhibits Angiogenesis and Tumorigenicity of Human Ovarian Cancer Cells by Suppressing Expression of Vascular Endothelial Growth Factor and Interleukin 8. *Cancer Res*. 2000; 60(19):5334–9

137. Bassères DS, Baldwin AS. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene*. 2006; 25(51):6817–30

138. Ben-Neriah Y, Karin M. Inflammation meets cancer, with NF-κB as the matchmaker. *Nat Immunol*. 2011;12(8):715–23

139. Sakamoto K, Maeda S, Hikiba Y, Nakagawa H, Hayakawa Y, Shibata W, et al. Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. *Clin Cancer Res*. 2009; 15(7):2248–58

140. Lind DS, Hochwald SN, Malaty J, Rekkas S, Hebig P, Mishra G, et al. Nuclear factor-κB is upregulated in colorectal cancer. *Surgery*. 2001; 130(2):363–9

141. Kojima M, Morisaki T, Sasaki N, Nakano K, Mibu R, Tanaka M, et al. Increased Nuclear Factor-kB activation in human colorectal carcinoma and its correlation with tumor progression. *Anticancer Res*. 2004; 24(2 B):675–81

142. Yu H-G, Zhong X, Yang Y-N, Luo H-S, Yu J-P, Meier JJ, et al. Increased expression of nuclear factor-kappaB/RelA is correlated with tumor angiogenesis in human colorectal cancer. *Int J Colorectal Dis*. 2004 ;19(1):18–22

143. Moorchung N, Kunwar S, Ahmed KW. An evaluation of nuclear factor kappa B expression in colorectal carcinoma: an analysis of 50 cases. *J Cancer Res Ther*. 2014; 10(3):631–5

144. Sandur SK, Deorukhkar A, Pandey MK, Pabón AM, Shentu S, Guha S, et al. Curcumin modulates the radiosensitivity of colorectal cancer cells by suppressing constitutive and inducible NF-kappaB activity. *Int J Radiat Oncol Biol Phys*. 2009; 75(2):534–42

145. Kunnumakkara AB, Diagaradjane P, Anand P, Kuzhuvelil HB, Deorukhkar A, Gelovani J, et al. Curcumin sensitizes human colorectal cancer to capecitabine by modulation of cyclin D1, COX-2, MMP-9, VEGF and CXCR4 expression in an orthotopic mouse model. *Int J Cancer*. 2009; 125(9):2187–97

146. Wu D, Wu P, Zhao L, Huang L, Zhang Z, Zhao S, et al. NF-κB expression and outcomes in solid tumors: A systematic review and meta-analysis. *Medicine (Baltimore)*. 2015; 94(40):e1687

147. Didonato JA, Mercurio F, Karin M. NF-κB and the link between inflammation and cancer. *Immunol Rev*. 2012; 246(1):379–400

148. Eckmann L, Nebelsiek T, Fingerle AA, Dann SM, Mages J, Lang R, et al. Opposing functions of IKKbeta during acute and chronic intestinal inflammation. *Proc Natl Acad Sci U S A*. 2008; 105(39):15058–63

149. Shaked H, Hofseth LJ, Chumanevich A, Chumanevich AA, Wang J, Wang Y, et al. Chronic epithelial NF-κB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. *Proc Natl Acad Sci U S A*. 2012; 109(35):14007–12

150. Townsend PA, Cutress RI, Sharp A, Brimmell M, Packham G. BAG-1: a multifunctional regulator of cell growth and survival. *Biochim Biophys Acta - Rev Cancer*. 2003; 1603(2):83–98

151. Clemo NK, Collard TJ, Southern SL, Edwards KD, Moorghen M, Packham G, et al. BAG-1 is up-regulated in colorectal tumour progression and promotes colorectal tumour cell survival through increased NF-κB activity. *Carcinogenesis*. 2008;

29(4):849–57

152. Collard TJ, Urban BC, Patsos HA, Hague A, Townsend PA, Paraskeva C, et al. The retinoblastoma protein (Rb) as an anti-apoptotic factor: Expression of Rb is required for the antiapoptotic function of BAG-1 protein in colorectal tumour cells. *Cell Death Dis*. 2012; 3(10):e408

153. Hayakawa Y, Maeda S, Nakagawa H, Hikiba Y, Shibata W, Sakamoto K, et al. Effectiveness of IkB kinase inhibitors in murine colitis-associated tumorigenesis. *J Gastroenterol*. 2009; 44(9):935–43

154. Luo J-L, Maeda S, Hsu L-C, Yagita H, Karin M. Inhibition of NF-kappaB in cancer cells converts inflammation- induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. *Cancer Cell*. 2004; 6(3):297–305

155. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*. 2007; 445(7123):106–10

156. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature*. 2007; 445(7123):111–5

157. Tsai K, Yang S, Lei Y, Tsai C, Chen H, Hsu C, et al. Mesenchymal stem cells promote formation of colorectal tumors in mice. *Gastroenterology*. 2011; 141(3):1046–56

158. Wu X-B, Liu Y, Wang G-H, Xu X, Cai Y, Wang H-Y, et al. Mesenchymal stem cells promote colorectal cancer progression through AMPK/mTOR-mediated NF-kappaB activation. *Sci Rep*. 2016; 6(71):21420

159. Schwitalla S, Fingerle AA, Cammareri P, Nebelsiek T, Göktuna SI, Ziegler PK, et al. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell*. 2013; 152(1–2):25–38

160. Amin ARMR, Karpowicz PA, Carey TE, Arbiser J, Nahta R, Chen ZG, et al. Evasion of anti-growth signaling: A key step in tumorigenesis and potential target for treatment and prophylaxis by natural compounds. *Seminars in Cancer Biology*.

2015; 35:S55–77

161. Russo A, Bazan V, Iacopetta B, Kerr D, Soussi T, Gebbia N. The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: Influence of tumor site, type of mutation, and adjuvant treatment. *J Clin Oncol*. 2005; 23(30):7518–28

162. Schneider G, Henrich A, Greiner G, Wolf V, Lovas A, Wieczorek M, et al. Cross talk between stimulated NF-kappaB and the tumor suppressor p53. *Oncogene*. 2010; 29(19):2795–806

163. Ryan KM, Ernst MK, Rice NR, Vousden KH. Role of NF-kappaB in p53-mediated programmed cell death. *Nature*. 2000; 404(1992):892–7

164. Webster GA, Perkins ND. Transcriptional cross talk between NF-κB and p53. *Mol Cell Biol*. 1999; 19(5):3485–95

165. Cooks T, Pateras IS, Tarcic O, Solomon H, Schetter AJ, Wilder S, et al. Mutant p53 prolongs NF-κB activation and promotes chronic inflammation and inflammation-associated colorectal cancer. *Cancer Cell*. 2013; 23(5):634–46

166. Schäfer C, Göder A, Beyer M, Kiweler N, Mahendrarajah N, Rauch A, et al. Class I histone deacetylases regulate p53/NF-κB crosstalk in cancer cells. *Cell Signal*. 2017; 29:218–25

167. Wagner T, Kiweler N, Wolff K, Knauer SK, Brandl A, Hemmerich P, et al. Sumoylation of HDAC2 promotes NF-κB-dependent gene expression. *Oncotarget*. 2015; 6(9):7123–35

168. Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci*. 2002; 115(Pt 20):3861–3

169. Rybak JN, Roesli C, Kaspar M, Villa A, Neri D. The extra-domain A of fibronectin is a vascular marker of solid tumors and metastases. *Cancer Res*. 2007; 67(22):10948–57

170. Fleur M La, Beaulieu AD, Kreiss C. Fibronectin gene expression in

polymorphonuclear leukocytes. *J Biol Chem*. 1987; 262(5):2111–5

171. Hines KL, Kulkarni AB, McCarthy JB, Tian H, Ward JM, Christ M, et al. Synthetic fibronectin peptides interrupt inflammatory cell infiltration in transforming growth factor beta 1 knockout mice. *Proc Natl Acad Sci U S A*. 1994; 91(11):5187–91

172. Yi W, Xiao E, Ding R, Luo P, Yang Y. High expression of fibronectin is associated with poor prognosis, cell proliferation and malignancy via the NF-B/p53-apoptosis signaling pathway in colorectal cancer. *Oncol Rep*. 2016; 36(6):3145–53

173. D'Ignazio L, Bandarra D, Rocha S. NF-kappa B and HIF crosstalk in immune responses. *Febs J*. 2016; 283(3):413–24

174. Kim SL, Kim SW, Park YR. P-134 Parthenolide suppresses hypoxia induced angiogenesis and epithelial-mesenchymal transition by regulating hypoxia inducible factor 1 α signaling in colorectal cancer. *Ann Oncol*. 2016; 27(suppl 2):ii39-ii39

175. Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell*. 2004; 6(5):447–58

176. Kwon HC, Kim SH, Oh SY, Lee S, Kwon KA, Lee JH, et al. Clinicopathological significance of nuclear factor-kappa B, HIF-1 alpha, and vascular endothelial growth factor expression in stage III colorectal cancer. *Cancer Sci*. 2010;101(6):1557–61

177. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009; 139(5): 871–90

178. Shen T, Yang Z, Cheng X, Xiao Y, Yu K, Cai X, et al. CXCL8 induces epithelial-mesenchymal transition in colon cancer cells via the PI3K/Akt/NF-kappaB signaling pathway. *Oncol Rep*. 2017; 37(4):2095-2100

179. Liu W, Li H, Hong S-H, Piszczek GP, Chen W, Rodgers GP. Olfactomedin 4 deletion induces colon adenocarcinoma in Apc(Min/+) mice. *Oncogene*. 2016; 35(40):5237-5247

180. Shi W, Ye Z, Zhuang L, Li Y, Shuai W, Zuo Z, et al. Olfactomedin 1 negatively

regulates NF-κB signalling and suppresses the growth and metastasis of colorectal cancer cells. *J Pathol.* 2016; 240(3):352–65

181. Zucker S, Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Rev*. 2004; 23(1-2):101–17

182. Chu D, Zhao Z, Zhou Y, Li Y, Li J, Zheng J, et al. Matrix metalloproteinase-9 is associated with relapse and prognosis of patients with colorectal cancer. *Ann Surg Oncol*. 2012; 19(1):318–25

183. Bond M, Fabunmi RP, Baker AH, Newby AC. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: An absolute requirement for transcription factor NF-κB. *FEBS Lett*. 1998; 435(1):29–34

184. Takahra T, Smart DE, Oakley F, Mann DA. Induction of myofibroblast MMP-9 transcription in three-dimensional collagen I gel cultures: Regulation by NF-κB, AP-1 and Sp1. *Int J Biochem Cell Biol*. 2004; 36(2):353–63

185. Fukuyama R, Kwok PN, Cicek M, Kelleher C, Niculaita R, Casey G, et al. Role of IKK and oscillatory NFkB kinetics in MMP-9 gene expression and chemoresistance to 5-fluorouracil in RKO colorectal cancer cells. *Mol Carcinog*. 2007; 46(5):402–13

186. Hu W-H, Pendergast JS, Mo X-M, Brambilla R, Bracchi-Ricard V, Li F, et al. NIBP, a novel NIK and IKK(beta)-binding protein that enhances NF-(kappa)B activation. *J Biol Chem*. 2005; 280(32):29233–41

187. Xu C, Qin M, Tan L, Liu S, Huang J. NIBP impacts on the expression of E-cadherin, CD44 and vimentin in colon cancer via the NF-κB pathway. *Mol Med Rep*. 2016;5379–85

188. Zhang Y, Liu S, Wang H, Yang W, Li F, Yang F, et al. Elevated NIBP/TRAPPC9 mediates tumorigenesis of cancer cells through NFκB signaling. *Oncotarget*. 2015; 6(8):6160–78

189. Qin M, Liu S, Li A, Xu C, Tan L, Huang J, et al. NIK- and IKKbeta-binding protein promotes colon cancer metastasis by activating the classical NF-kappaB pathway and MMPs. *Tumour Biol*. 2016; 37(5):5979–90

190. Li J, Wei K, Yu H, Jin D, Wang G, Yu B. Prognostic value of ezrin in various cancers: A systematic review and updated meta-analysis. *Sci Rep*. 2015; 5:17903

191. Gavert N, Ben-Shmuel A, Lemmon V, Brabletz T, Ben-Ze'ev A. Nuclear factor-kappaB signaling and ezrin are essential for L1-mediated metastasis of colon cancer cells. *J Cell Sci*. 2010; 123(Pt 12):2135–43

192. Erreni M, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) and inflammation in colorectal cancer. *Cancer Microenviron*. 2011; 4(2):141–54

193. Göktuna SI, Canli O, Bollrath J, Fingerle AA, Horst D, Diamanti MA, et al. IKKα promotes intestinal tumorigenesis by limiting recruitment of M1-like polarized myeloid cells. *Cell Rep*. 2014; 7(6):1914–25

194. Ryan AE, Colleran A, O'Gorman A, O'Flynn L, Pindjacova J, Lohan P, et al. Targeting colon cancer cell NF-κB promotes an anti-tumour M1-like macrophage phenotype and inhibits peritoneal metastasis. *Oncogene*. 2014; 34:1563–74

195. Jones KL, Mansell A, Patella S, Scott BJ, Hedger MP, de Kretser DM, et al. Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia. *Proc Natl Acad Sci U S A*. 2007; 104(41):16239–44

196. Wildi S, Kleeff J, Maruyama H, Maurer CA, Büchler MW, Korc M. Overexpression of activin A in stage IV colorectal cancer. *Gut*. 2001; 49(3):409–17

197. Watanabe T, Wu TT, Catalano PJ, Ueki T, Satriano R, Haller DG, et al. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N Engl J Med*. 2001; 344(16):1196–206

198. Bauer J, Ozden O, Akagi N, Carroll T, Principe DR, Staudacher JJ, et al. Activin and TGFβ use diverging mitogenic signaling in advanced colon cancer. *Mol Cancer*. 2015; 14:182

199. Jana A, Krett NL, Guzman G, Khalid A, Ozden O, Staudacher JJ, et al. NFkB is essential for activin-induced colorectal cancer migration via upregulation of PI3K-MDM2 pathway. *Oncotarget*. 2017; 8(23):37377-37393

200. Zarnegar B, Yamazaki S, He JQ, Cheng G. Control of canonical NF-kappaB activation through the NIK-IKK complex pathway. *Proc Natl Acad Sci*. 2008; 105(9):3503–8

201. Perkins ND. Integrating cell-signalling pathways with NF-κB and IKK function. *Nat Rev Mol Cell Biol*. 2007; 8(1):49–62

202. Sizemore N, Leung S, Stark GR. Activation of Phosphatidylinositol 3-Kinase in Response to Interleukin-1 Leads to Phosphorylation and Activation of the NF-κB p65/RelA Subunit. *Mol Cell Biol*. 1999; 19(7):4798–805

203. Reddy SA, Huang JH, Liao WS. Phosphatidylinositol 3-kinase in interleukin 1 signaling. Physical interaction with the interleukin 1 receptor and requirement in NFkappaB and AP-1 activation. *J Biol Chem*. 1997; 272(46):29167–73

204. Madrid L V, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS, Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol Cell Biol*. 2000; 20(5):1626–38

205. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science*. 1997; 275(5307):1784–7

206. Lamberti C, Lin KM, Yamamoto Y, Verma U, Verma IM, Byers S, et al. Regulation of beta-catenin function by the IkappaB kinases. *J Biol Chem*. 2001; 276(45):42276–86

207. Vlantis K, Wullaert A, Sasaki Y, Schmidt-Supprian M, Rajewsky K, Roskams T, et al. Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice. *J Clin Invest*. 2011; 121(7):2781–93

208. Agarwal A, Das K, Lerner N, Sathe S, Cicek M, Casey G, et al. The AKT/I kappa B kinase pathway promotes angiogenic/metastatic gene expression in colorectal cancer by activating nuclear factor-kappa B and beta-catenin. *Oncogene*. 2005; 24(6):1021–31

209. Sunami Y, Wirth T. Intestinal carcinogenesis: IKK can go all the way. *J Clin Invest*.

2011; 121(7):2551–3

210. Bollrath J, Greten FR. IKK/NF-kappaB and STAT3 pathways: central signalling hubs in inflammation-mediated tumour promotion and metastasis. *EMBO Rep*. 2009; 10(12):1314–9

211. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev.* 2010; 21(1):11–9

212. Yang J, Liao X, Agarwal MK, Barnes L, Auron PE, Stark GR. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NF-kB. *Genes Dev*. 2007; 21(11):1396–408

213. Dauer DJ, Ferraro B, Song L, Yu B, Mora L, Buettner R, et al. Stat3 regulates genes common to both wound healing and cancer. *Oncogene*. 2005; 24(21):3397–408

214. Wu Z, Zhang X, Yang J, Wu G, Zhang Y, Yuan Y, et al. Nuclear protein IkappaB-zeta inhibits the activity of STAT3. *Biochem Biophys Res Commun*. 2009; 387(2):348–52

215. Lee H, Herrmann A, Deng JH, Kujawski M, Niu G, Li Z, et al. Persistently activated Stat3 maintains constitutive NF-κB activity in tumors. *Cancer Cell*. 2009; 15(4):283–93

216. De Simone V, Franze E, Ronchetti G, Colantoni A, Fantini MC, Di Fusco D, et al. Th17-type cytokines, IL-6 and TNF-alpha synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth. *Oncogene*. 2015; 34(27):3493–503

217. Li T, Morgan MJ, Choksi S, Zhang Y, Kim Y-S, Liu Z. MicroRNAs modulate the noncanonical transcription factor NF-kappaB pathway by regulating expression of the kinase IKKalpha during macrophage differentiation. *Nat Immunol*. 2010; 11(9):799–805

218. Hai Ping P, Feng Bo T, Li L, Nan Hui Y, Hong Z. IL-1β/NF-kb signaling promotes colorectal cancer cell growth through miR-181a/PTEN axis. *Arch Biochem Biophys*. 2016; 604:20–6

219. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, et al. IL-6 and Stat3 Are Required for Survival of Intestinal Epithelial Cells and Development of Colitis-Associated Cancer. *Cancer Cell*. 2009; 15(2):103–13

220. Corvinus FM, Orth C, Moriggl R, Tsareva SA, Wagner S, Pfitzner EB, et al. Persistent STAT3 activation in colon cancer is associated with enhanced cell proliferation and tumor growth 1. *Neoplasia*. 2005; 7(6):545–55

221. Park JH, Van Wyk H, McMillan DC, Quinn J, Clark J, Roxburgh CSD, et al. Signal transduction and activator of transcription-3 (STAT3) in patients with colorectal cancer: Associations with the phenotypic features of the tumor and host. *Clin Cancer Res*. 2017; 23(7):1698–709

222. Terzić J, Grivennikov S, Karin E, Karin M. Inflammation and Colon Cancer. *Gastroenterology*. 2010; 138(6):2101–14

223. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. 2002; 3(11):991–8

224. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, Berger A, Bruneval P, Fridman WH, Pagès F GJ. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res*. 2011; 15;71(4):1

225. Hyun YS, Han DS, Lee AR, Eun CS, Youn J, Kim HY. Role of IL-17A in the development of colitis-associated cancer. *Carcinogenesis*. 2012; 33(4):931–6

226. Kirchberger S, Royston DJ, Boulard O, Thornton E, Franchini F, Szabady RL, et al. Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J Exp Med*. 2013; 210(5):917–31

227. Simões AES, Pereira DM, Gomes SE, Brito H, Carvalho T, French A, et al. Aberrant MEK5/ERK5 signalling contributes to human colon cancer progression via NF-κB activation. *Cell Death Dis*. 2015; 6(4):e1718

228. Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, et al. NF-kappaB links innate immunity to the hypoxic response through transcriptional

regulation of HIF-1alpha. *Nature*. 2008; 453(7196):807–11

229. Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D, Rocha S. Mechanism of hypoxia-induced NF-kappaB. *Mol Cell Biol*. 2010; 30(20):4901–21

230. Yoshimura H, Dhar DK, Kohno H, Kubota H, Fujii T, Ueda S, et al. Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. *Clin Cancer Res*. 2004; 10(24):8554–60

231. Fernández-Majada V, Aguilera C, Villanueva a, Vilardell F, Robert-Moreno a, Aytés a, et al. Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. *Proc Natl Acad Sci U S A*. 2007; 104(1):276–81

232. Margalef P, Fernández-Majada V, Villanueva A, Garcia-Carbonell R, Iglesias M, López L, et al. A truncated form of IKKα is responsible for specific nuclear IKK activity in colorectal cancer. *Cell Rep*. 2012; 2(4):840–54

233. Margalef P, Colomer C, Villanueva A, Montagut C, Iglesias M, Bellosillo B, et al. BRAF-induced tumorigenesis is IKKalpha-dependent but NF-kappaB-independent. *Sci Signal*. 2015;8(373):ra38

234. Schmedtje JF, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem*. 1997; 272(1):601–8

235. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, Dubois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*. 1994; 107(4):1183–8

236. Taketo MM. COX-2 and colon cancer. *Inflamm Res*. 1998; 47(Suppl 2):S112-6

237. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, et al. Suppression of intestinal polyposis in Apc(delta716) knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*. 1996; 87(5):803–9

238. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, et al.

Celecoxib for the Prevention of Sporadic Colorectal Adenomas. *N Engl J Med.* 2006; 355(9):873–84

239. Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S, et al. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. *Oncogene*. 1999; 18(44):6013–20

240. Charalambous MP, Maihöfner C, Bhambra U, Lightfoot T, Gooderham NJ. Upregulation of cyclooxygenase-2 is accompanied by increased expression of nuclear factor-kappa B and I kappa B kinase-alpha in human colorectal cancer epithelial cells. *Br J Cancer*. 2003; 88(10):1598–604

241. Charalambous MP, Lightfoot T, Speirs V, Horgan K, Gooderham NJ. Expression of COX-2, NF-kappaB-p65, NF-kappaB-p50 and IKKalpha in malignant and adjacent normal human colorectal tissue. *Br J Cancer*. 2009; 101(1):106–15

242. Thun MJ, Namboodiri MM, Heath Jr. CW. Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med*. 1991; 325(23):1593–6

243. Chan AT, Giovannucci EL, Meyerhardt JA, Schernhammer ES, Curhan GC, Fuchs CS. Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. *JAMA*. 2005; 294(8):914

244. Chan AT, Giovannucci EL, Schernhammer ES, Colditz GA, Hunter DJ, Willett WC, et al. A prospective study of aspirin use and the risk for colorectal adenoma. *Ann Intern Med*. 2004; 140(3):157–66

245. Garcia Rodriguez LA, Huerta-Alvarez C. Reduced incidence of colorectal adenoma among long-term users of nonsteroidal antiinflammatory drugs: a pooled analysis of published studies and a new population-based study. *Epidemiology*. 2000; 11(1044–3983):376–81

246. Greenberg ER, Baron JA, Freeman DH, Mandel JS, Haile R. Reduced risk of large-bowel adenomas among aspirin users. *J Natl Cancer Inst*. 1993; 85(11):912–5

247. Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, et al. A

randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med*. 2003; 348(10):883–90

248. Yamamoto Y, Yin MJ, Lin KM, Gaynor RB. Sulindac inhibits activation of the NF-kappaB pathway. *J Biol Chem*. 1999; 274(38):27307–14

249. Shao J, Fujiwara T, Kadowaki Y, Fukazawa T, Waku T, Itoshima T, et al. Overexpression of the wild-type p53 gene inhibits NF-kappaB activity and synergizes with aspirin to induce apoptosis in human colon cancer cells. *Oncogene*. 2000; 19(6):726–36

250. Stark LA, Dunlop MG. Nucleolar Sequestration of RelA (p65) Regulates NF- B-Driven Transcription and Apoptosis. *Mol Cell Biol*. 2005; 25(14):5985–6004

251. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature*. 1998; 396(6706):77–80

252. Stark LA, Reid K, Sansom OJ, Din F V, Guichard S, Mayer I, et al. Aspirin activates the NF-kappaB signalling pathway and induces apoptosis in intestinal neoplasia in two in vivo models of human colorectal cancer. *Carcinogenesis*. 2007; 28(5):968–76

253. Din FV, Stark LA, Dunlop MG. Aspirin-induced nuclear translocation of NFkappaB and apoptosis in colorectal cancer is independent of p53 status and DNA mismatch repair proficiency. *Br J Cancer*. 2005; 92(6):1137–43

254. Singh S, Aggarwal BB. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane). *J Biol Chem*. 1995; 270(42):24995–5000

255. Rao CV, Rivenson A, Simi B, Reddy BS. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res*. 1995; 55(2):259–66

256. Tu SP, Jin H, Shi JD, Zhu LM, Suo Y, Lu G, et al. Curcumin induces the differentiation of myeloid-derived suppressor cells and inhibits their interaction with cancer cells and related tumor growth. *Cancer Prev Res*. 2012; 5(2):205–15

257. Howells LM, Sale S, Sriramareddy SN, Irving GRB, Jones DJL, Ottley CJ, et al. Curcumin ameliorates oxaliplatin-induced chemoresistance in HCT116 colorectal cancer cells in vitro and in vivo. *Int J Cancer*. 2011; 129(2):476–86

258. Irving GR, Iwuji CO, Morgan B, Berry DP, Steward WP, Thomas A, et al. Combining curcumin (C3-complex, Sabinsa) with standard care FOLFOX chemotherapy in patients with inoperable colorectal cancer (CUFOX): study protocol for a randomised control trial. *Trials*. 2015; 16(1):110

259. Wang S, Wang L, Zhou Z, Deng Q, Li L, Zhang M, et al. Leucovorin enhances the anti-cancer Effect of Bortezomib in colorectal cancer cells. *Sci Rep*. 2017; 7(1):682

260. Kozuch PS, Rocha-Lima CM, Dragovich T, Hochster H, O'Neil BH, Atiq OT, et al. Bortezomib with or without irinotecan in relapsed or refractory colorectal cancer: Results from a randomized phase II study. *J Clin Oncol*. 2008; 26(14):2320–6

261. Caponigro F, Lacombe D, Twelves C, Bauer J, Govaerts AS, Marréaud S, et al. An EORTC phase I study of Bortezomib in combination with oxaliplatin, leucovorin and 5-fluorouracil in patients with advanced colorectal cancer. *Eur J Cancer*. 2009; 45(1):48–55

262. Becker C, Fantini MC, Schramm C, Lehr HA, Wirtz S, Nikolaev A, et al. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity*. 2004; 21(4):491–501

263. Lagadec P, Griessinger E, Nawrot MP, Fenouille N, Colosetti P, Imbert V, et al. Pharmacological targeting of NF-kappaB potentiates the effect of the topoisomerase inhibitor CPT-11 on colon cancer cells. *Br J Cancer*. 2008; 98(2):335–44

264. Anthony NG, Baiget J, Berretta G, Boyd M, Breen D, Edwards J, et al. Inhibitory Kappa B kinse α (IKKα) inhibitors that recapitulate their selectivity in cells against isoform-related biomarkers. *J Med Chem*. 2017; 60(16):7043-7066

265. Wang CY, Cusack JC, Liu R, Baldwin AS. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. *Nat Med*. 1999; 5(4):412–7

266. Holley AK, Xu Y, Clair DKS, Clair WHS. RelB regulates manganese superoxide dismutase gene and resistance to ionizing radiation of prostate cancer cells. *Ann N Y Acad Sci*. 2010; 1201:129–36

267. McCool KW, Miyamoto S. DNA damage-dependent NF-κB activation: NEMO turns nuclear signaling inside out. *Immunol Rev*. 2012; 246(1):311–26

268. Rakitina TV, Vasilevskaya IA, O'Dwyer PJ. Additive Interaction of Oxaliplatin and 17-Allylamino-17-demethoxygeldanamycin in Colon Cancer Cell Lines Results from Inhibition of Nuclear Factor κB Signaling. *Cancer Res*. 2003; 63(24):8600–5

269. V. Almendro, J. Maurel, J. Augé, G. Laus, J. Domingo-Domenech, E. Fernández and PG. Role of metalloproteinase-7 in oxaliplatin acquired resistance in colorectal cancer cell lines. *J Clin Oncol*. 2006; 24:18(supp):20042–72

270. Samuel T, Fadlalla K, Gales DN, Putcha BDK, Manne U. Variable NF-κB pathway responses in colon cancer cells treated with chemotherapeutic drugs. *BMC Cancer*. 2014; 14:599

271. Zhao C, Zhao Q, Zhang C, Wang G, Yao Y, Huang X, et al. miR-15b-5p resensitizes colon cancer cells to 5-fluorouracil by promoting apoptosis via the NF-κB/XIAP axis. *Sci Rep*. 2017; 7(1):4194

272. Xu Y, Villalona-Calero MA. Irinotecan: Mechanisms of tumor resistance and novel strategies for modulating its activity. *Ann Oncol*. 2002; 13(12):1841–51

273. Voboril R, Weberova-Voborilova J. Constitutive NF-kappaB activity in colorectal cancer cells: impact on radiation-induced NF-kappaB activity, radiosensitivity, and apoptosis. *Neoplasma*. 2006; 53(6):518–23

274. Berardi R, Maccaroni E, Mandolesi A, Mantello G, Onofri A, Biscotti T, et al. Nuclear factor-κB predicts outcome in locally advanced rectal cancer patients receiving neoadjuvant radio-chemotherapy. *Dig Liver Dis*. 2012; 44(7):617–22

275. Hanson JL, Hawke NA, Kashatus D, Baldwin AS. The nuclear factor κB subunits RelA/p65 and c-Rel potentiate but are not required for Ras-induced cellular transformation. *Cancer Res*. 2004; 64(20):7248–55

276. Finco TS, Westwick JK, Norris JL, Beg AA, Der CJ, Baldwin AS. Oncogenic Ha-Ras-induced signaling activates NF-kappaB transcriptional activity, which is required for cellular transformation. *J Biol Chem*. 1997; 272(39):24113–6

277. Meylan E, Dooley AL, Feldser DM, Shen L, Turk E, Ouyang C, et al. Requirement for NF-kappaB signalling in a mouse model of lung adenocarcinoma. *Nature*. 2009; 462(7269):104–7

278. Lin G, Tang Z, Ye YB, Chen Q. NF-kB activity is downregulated by KRAS knockdown in SW620 cells via the RAS-ERK-IkBa pathway. *Oncol Rep*. 2012; 27(5):1527–34

279. Lin G, Zheng XW, Li C, Chen Q, Ye Y Bin. KRAS mutation and NF-kB activation indicates tolerance of chemotherapy and poor prognosis in colorectal cancer. *Dig Dis Sci.* 2012; 57(9):2325–33

280. O'Neil BH, Funkhouser WK, Calvo BF, Meyers MO, Kim HJ, Goldberg RM, et al. Nuclear factor κ-Light chain-enhancer of activated b cells is activated by radiotherapy and is prognostic for overall survival in patients with rectal cancer treated with preoperative fluorouracil-based chemoradiotherapy. *Int J Radiat Oncol Biol Phys*. 2011; 80(3):705-11

281. Roseweir AK, McMillan DC, Horgan PG, Edwards J. Colorectal cancer subtypes: Translation to routine clinical pathology. *Cancer Treat Rev*. 2017; 57:1-7

282. Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, et al. Observer variation in immunohistochemical analysis of protein expression, time for a change? *Histopathology*. 2006; 48(7):787–94

283. Ahmed D, Eide PW, Eilertsen IA, Danielsen SA, Eknæs M, Hektoen M, et al. Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis*. 2013; 2:e71

284. Medico E, Russo M, Picco G, Cancelliere C, Valtorta E, Corti G, et al. The molecular landscape of colorectal cancer cell lines unveils clinically actionable kinase targets. *Nat Commun*. 2015; 6:7002

285. ATCC® Cell lines by Gene Mutation. 02/08/2018. Available from: https://www.atcc.org/~/media/pdfs/culture%20guides/cell_lines_by_gene_mutation.ashx

286. Patel M, Horgan PG, McMillan DC, Edwards J. NF-κB pathways in the development and progression of colorectal cancer. *Transl Res*. 2018; 197:43-56

287. Bennett L, Quinn J, McCall P, Mallon EA, Horgan PG, McMillan DC, et al. High IKKα expression is associated with reduced time to recurrence and cancer specific survival in oestrogen receptor (ER)-positive breast cancer. *Int J Cancer*. 2017; 140(7):1633–44

288. Kiontke S, Langemeyer L, Kuhlee A, Schuback S, Raunser S, Ungermann C, et al. Architecture and mechanism of the late endosomal Rab7-like Ypt7 guanine nucleotide exchange factor complex Mon1-Ccz1. *Nat Commun*. 2017;8:14034

289. Jovic M, Sharma M, Rahajeng J, Caplan S. The early endosome: A busy sorting station for proteins at the crossroads. *Histol and Histopathol*. 2010; 25(1):99–112

290. Simonsen A, Lippé R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, et al. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature*. 1998; 394(6692):494–8

291. Bucci C, Thomsen P, Nicoziani P, Mccarthy J, Deurs B Van, Califano ML, et al. Rab7 : A Key to Lysosome Biogenesis .*Mol Cell Biol*. 2000; 11(2):467–80

292. Xu D, Esko JD. A Golgi-on-a-chip for glycan synthesis. *Nat Chem Biol*. 2009; 5(9):612-3

293. Xiao G, Fong A, Sun S-C. Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation. *J Biol Chem*. 2004; 279(29):30099–105

294. Melling N, Kowitz CM, Simon R, Bokemeyer C, Terracciano L, Sauter G, et al. High Ki67 expression is an independent good prognostic marker in colorectal cancer. *J Clin Pathol*. 2016; 69(3):209–14

295. Clarke CN, Kopetz ES. BRAF mutant colorectal cancer as a distinct subset of colorectal cancer: clinical characteristics, clinical behavior, and response to targeted therapies. *J Gastrointest Oncol*. 2015; 6(6):660–7

296. Abrahao ABK, Karim S, Colwell B, Berry S, Biagi J. The predictive effect of primary tumour location in the treatment of metastatic colorectal cancer : a Canadian consensus statement. *Cur Oncol*. 2017; 24(6):390–400

297. Brightbill HD, Suto E, Blaquiere N, Ramamoorthi N, Sujatha-Bhaskar S, Gogol EB, et al. NF-κB inducing kinase is a therapeutic target for systemic lupus erythematosus. *Nat Commun*. 2018; 9(1):1–14

298. Birbach A, Bailey ST, Ghosh S, Schmid J a. Cytosolic, nuclear and nucleolar localization signals determine subcellular distribution and activity of the NF-kappaB inducing kinase NIK. *J Cell Sci*. 2004; 117(Pt 16):3615–24

299. Leong K, Karsan A. Recent insights into the role of Notch signaling in tumorigenesis. *Blood*. 2006; 107(6):2223–33

300. Ammirante M, Luo JL, Grivennikov S, Nedospasov S, Karin M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature*. 2010; 464(7286):302–5

301. Colomer C, Margalef P, Gonzalez J, Vert A, Bigas A, Espinosa L. IKKa is required in the intestinal epithelial cells for tumour stemness. *Br J Cancer*. 2018; 118(6):839–46

302. Bowen KB, Reimers AP, Luman S, Kronz JD, Fyffe WE, Oxford JT. Immunohistochemical localization of collagen type XI alpha1 and alpha2 chains in human colon tissue. *J Histochem Cytochem*. 2008; 56(3):275–83

303. Stanley P. Golgi glycosylation. *Cold Spring Harb Perspect Biol*. 2011; 3(4):1–13

304. Kellokumpu S, Sormunen R, Kellokumpu I. Abnormal glycosylation and altered Golgi structure in colorectal cancer: Dependence on intra-Golgi pH. *FEBS Lett*. 2002; 516(1–3):217–24

305. Chen Q, Sun L, Chen ZJ. Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. *Nat Immunol*. 2016; 17(10):1142–9

306. Saitoh T, Fujita N, Hayashi T, Takahara K, Satoh T, Lee H, et al. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Proc Natl Acad Sci U S A*. 2009; 106(49):20842–6

307. Roseweir AK, Bennett L, Dickson A, Cheng K, Quintayo M-A, Bayani J, et al. Predictive Biomarkers for Endocrine Therapy: Retrospective Study in Tamoxifen and Exemestane Adjuvant Multinational (TEAM) Trial. J *Natl Cancer Inst*. 2017; 110(6):616–627

308. Keats JJ, Fonseca R, Chesi M, Schop R, Baker A, Chng W-J, et al. Promiscuous mutations activate the noncanonical NF-κB pathway in multiple myeloma. *Cancer Cell*. 2007; 12(2):131–44

309. Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IκB kinase α and NF-κB in prostate cancer cells is inhibited by ibuprofen. *Oncogene*. 1999; 18(51):7389-94

310. Nishina T, Yamaguchi N, Gohda J, Semba K, Inoue J ichiro. NIK is involved in constitutive activation of the alternative NF-κB pathway and proliferation of pancreatic cancer cells. *Biochem Biophys Res Commun*. 2009; 388(1):96–101

311. Margalef P, Colomer C, Villanueva A, Montagut C, Iglesias M, Bellosillo B, et al. BRAF-induced tumorigenesis is IKKa-dependent but NF-kB–independent. *Sci Signal*. 2015; 8(373):1–13

312. Pasparakis M. Regulation of tissue homeostasis by NF-B signalling: Implications for inflammatory diseases. *Nat Rev Immunol*. 2009; 9(11):778–88

313. Li Q, Van Antwerp D, Mercurio F, Lee KF, Verma IM. Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science*. 1999; 284(5412):321-5