The Role of Heat Shock Proteins, Amyloid Precursor Protein and B-cell CLL/lymphoma 3 and their Co-chaperones in Colorectal Cancer

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DECLARATION

The work presented in this thesis is entirely my own, except where clearly stated, and had not been submitted for a degree or comparable award to this or any other university institution.

ABSTRACT

The elevated expression of Heat shock proteins (HSPs) has been implicated in CRC prognosis and tumour cells may require the expression of HSPs and BCL2-associated athanogene (BAG1), the HSP co-chaperone, to survive. BAG1 is a regulator of amyloid precursor protein (APP); both are important in cellular proliferation and cancer. B-cell CLL/lymphoma 3 (BCL3) is an agonist in the tumour-associated NF-κB pathway. This study aims to establish the mutational status of *TP53, KRAS* and *PIK3CA*, and the activation status of AKT, in primary CRCs and then investigate the role of HSPs, BAG1, APP, and BCL3.

Oncogene induced senescence (OIS) is a potential roadblock to CRC. Recent cell culture studies suggest that OIS mediated by PI3K/AKT activation can be circumvented by high expression of HSPs in the absence of *TP53* mutation. While PI3K/AKT activation and *KRAS* mutations are independent inducers of OIS, PI3K/AKT activation can suppress KRAS-induced OIS when both are present in cultured cells. *KRAS* mutations, PI3K/AKT activation and *TP53* mutations are all common features of CRCs. For HSP to inhibit OIS in CRC may be dependent on the tumour's mutation spectrum. CRCs with oncogenic activation of the AKT pathway were associated with increased HSP27 expression, which may represent an important mechanism in suppressing p53 dependent senescence. No association was found between HSP27 or HSP72 expression with *TP53* mutation status, but HSP27 expression was strongly associated with the co-presence of wildtype *KRAS* and activated PI3K/AKT, indicating a possible

role of HSP27 in overcoming PI3K/AKT induced OIS in tumours. There was no correlation between BAG1 and APP co-expression in CRCs. BCL3 expression was heterogeneous, and elevated at the invasive edge. The expression of HSPs, APP, BCL3 was not correlated with any CRC clinicopathological features. Our studies suggest a role for using archival tissues in validating hypotheses generated from cell culture based investigations.

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STATEMENT OF INDEPENDENT WORK ATTRIBUTABLE TO CANDIDATE AND COLLABORATORS

Chapter 1

This chapter is entirely my own work.

Chapter 2

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Chapter 3

Nirosha Suraweera and Neel Sengupta for performing the KRAS characterisation. The Pathology core facility (Queen Mary's University) there assistance in the immunohistochemistry. Otherwise all this chapter is entirely my own work.

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Anne Williams (Bristol University) performed the BAG1 overexpression experiment. Otherwise all this chapter is entirely my own work.

Chapter 5

This chapter is entirely my own work.

Chapter 6

This chapter is entirely my own work.

References and Appendices

This chapter is entirely my own work.

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LIST OF ABBREVIATIONS

18q	Long arm of chromosome 18
AD	Alzheimer's disease
ADAM	Disintegrin And Metalloprotease
AICD	Amino-terminal APP intracellular domain
APP	Amyloid precursor protein
APC	Adenomatous Polyposis Coli
BAG1	Bcl-2 associated athogene 1
βAPP	Amyloid β-peptide
BCL3	B-cell CLL/lymphoma 3
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CHRPE	Congentital hypertrophy of the retinal pigment epithelium
CIMP	CpG island methylator phenotype
CIN	Chromosome Instability
CRC	Colorectal cancer
CT	Computer Tomography
CTF	C-terminal fragments
CTNNB1	Cadherin-associated protein
DCIS	Ductal carcinoma in situ
dH_20	Deionised water
DNEM	Dulbeccos Modified Eagle medium
Dsh	Dishevelled
EpCAM	Epithelial cell adhesion molecule

ER	Endoplasmic reticulum
FAP	Familial adenomatous Polyposis
FBS	Foetal Bovine serum
FFPE	Formalin-fixed, paraffin wax-embedded
FOB	Faecal occult blood
Fz	Frizzled
G-	Guanosine
GAP	GTPase-activating protein
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GF	Growth factors
HRAS	Harvey- RAS
KRAS	Kristen-RAS
Hi Di	Highly deionised
HSP	Heat shock protein
HSP27	Heat shock protein 27
HSP70	Heat shock protein 70
HSP72	Heat shock protein 70
HNPCC	Hereditary Non-polyposis Colorectal Cancer
IKB	Inhibitor of NF-κB
IKK	Inhibitors of kappa kinases
IMS	Industrial methylated spirit
LRP	Low-density lipoprotein receptor related protein family
LOH	Loss of heterozygosity

LS	Lynch Syndrome
MACS	Microsatellite Stable and Chromosomal stable
MAP	Mitogen activated protein
MAP	MutY Homolog adenamtous polyposis
mTOR	Mammalian target of rapamycin
MDM2	Mouse double minute 2 homolog
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1
MSH6	MutS homolog 6
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite Instability
MUTHY	MutY Homolog
NHR	Nuclear hormone receptor
NRAS	Neuroblastoma RAS
NF-κB	Nuclear factor kappa B
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PIK	Phosphoinositide kinases
PI3K	Phosphoinositide 3-kinases
PI4K	Phosphoinositide 4-kinases
PI5K	Phosphoinositide 5-kinases
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha
PIP2	Phosphatidylinositol-4,5-bisphosphate

PIP3	Phosphatidylinositol-3,4,5-triphosphate
PML	Promyleocytic leukaemia
PMS1	Postmeiotic segregation increased 1
PMS2	Postmeiotic segregation increased 2
Pol ε	Polymerase ε
Pol δ	Polymerase δ
PPAP	Polymerase Proofreading-associated Polyposis
PTEN	phosphatase and tensin homolog
RHD	REL homology domain
Rheb	RAS homolog enriched in brain
RT	Room temperature
sAPP	Secreted APP
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4
SRB	Sulforhodamine B
TBE	Tris base, 89 mM boric acid and 2mM EDTA
TCA	Trichloroacetic acid
TCF	T-Cell factor
TGF-BRII	Transforming growth factor β receptor II
TP53	Tumour supressor 53
TSC2	Tuberous sclerosis complex 2
TME	Total Mesorectal Excision
TMN	Tumour-Nodes-Metastasis
TSG	Tumour suppressor gene
VPA	Valproic acid

WB	Wash buffer

CHAPTER 1

Introduction

1.1 Incidence of Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer worldwide after lung and breast with 1.23 million people being diagnosed each year. It is the fourth most common cause of cancer related death accounting for 8% of all cancer deaths (608,000 deaths from CRC per annum). Almost 60% of CRC cases occur in developed countries with the lowest rates in Africa and Asia and the highest in Europe, North America and Australasia (Ferlay, Shin et al. 2010). In the UK CRC is the third most common cause of cancer again after lung and breast, with approximately 38,000 new cases diagnosed each year, and the second most common cause of death from cancer in the UK (Cancer Research UK. 2010).

1.2 Age, Sex and Site of Colorectal Cancer

The development of CRC is strongly associated with age and is therefore considered a disease of old age with 84% of cases arising in people aged 60 years or older (Cancer Research UK. 2010). The prevalence of adenomas, the precursor lesion to CRC (see Section 1.6), increases with age in the general population; with approximately 25% at age 50 and 50% by age 70 having an adenoma (Rex, Lehman et al. 1993). Both men and women have similar rates for CRC until age 50 years when there are more male cases of

CRC until the age of 80 when there are more female cases due to the larger proportion of women in this elderly population. Incidence rates are substantially higher in men then in women (1.4:1) with the life-time risk of being diagnosed with CRC in the UK estimated to be 1 in 16 for men and 1 in 20 for women. CRC is the third most common cancer in men after prostate and lung whilst in women it is the second most common after breast cancer. Two thirds of large CRC is in the colon and one third in the rectum with preponderance towards the left side of the bowel (Cancer Research UK. 2010).

1.3 Survival and Mortality

In the UK there are approximately 16,000 deaths per year as a result of CRC (Cancer Research UK. 2010). The 5-year survival in patients diagnosed with CRC has shown a gradual improvement over the past 30 years with significant improvement over the last years particularly during the Nineties with male and female relative survival rising from approximately 22% to over 52% (Morris, Sandin et al. 2011). These improvements are a result of public education, earlier diagnosis and better treatment. Patients presenting at a young age have a better prognosis compared to older patients (Rachet, Maringe et al. 2009). Also patients diagnosed at an early stage have a much better prognosis compared to those presenting at a later stage with more extensive disease (National Cancer Intelligence Network). There is a survival advantage of 15.5-20.5% in affluent patients with colonic or rectal cancer compared with the most deprived (Ellis, Coleman et al. 2012). The relative survival rate for patients at 10 years is only slightly lower than at 5

years suggesting that patients who survive 5 years are likely to have been cured (Rachet, Maringe et al. 2009).

1.4 Risk Factors

Most cases of CRC arise sporadically although both genetic and environmental influences contribute to the development of CRC. These we will consider below.

1.4.1 Family History and Genetics

Family history influences an individual's life time risk of developing CRC. The lifetime risk of developing CRC with no family history of CRC is 2% and this increases to 6% in a person with one first degree relative with CRC, and to 10% if this relative was diagnosed before 45 years old. In an individual with two first degree relatives there is a 17% life time risk of developing CRC (Kim 2009).

An estimated 30% of all CRC have a familial component. About 5% of these cases are associated with highly penetrant inherited mutations which have been well characterised clinically into hereditary syndromes and these discussed more fully below (see Section 1.11). The remaining 20-30% of inherited CRC is not completely understood and are likely to be caused by more frequent but less penetrant mutations effecting multiple CRC susceptibility loci that have a cumulative effect on risk (Houlston and

Tomlinson 2001; Penegar, Wood et al. 2007; Houlston, Cheadle et al. 2010; Jasperson, Tuohy et al. 2010).

1.4.2 Diet and Obesity

The continuous exposure of colorectal mucosa to intra-luminal contents allows dietary carcinogens to have potentially significant effects on epithelial cell growth and apoptosis. There has been an increase in the incidence of CRC in migrants moving from low risk to high-risk countries. In countries such as Japan where there has been an increase in Westernised diet there has also been a rapid rise in CRC (Marchand 1999; Zhang, Dhakal et al. 2012). A wide range of dietary factors have all been linked with CRC, but none have been conclusively linked to CRC induction (Chan and Giovannucci 2010).

A diet high in fibre and fruit and vegetables, has been associated with a reduced risk of CRC. The EPIC study, showed a 40% reduction of CRC cancer in individuals with a high intake of fibre (Gonzalez 2006). A high fibre diet could have a protective effect by diluting or absorbing faecal carcinogens, modulating colonic transit time, altering bile acid metabolism and lowering colonic pH. There is an increased risk of 20-30% in relation to the amount of red meat consumed as it is thought to stimulate endogenous insulin, which is a mitogen (Chan and Giovannucci 2010). There is a 30% reduction in risk if a higher intake of fish is consumed (Norat, Bingham et al. 2005).

Increased body weight is a recognised risk factor for colorectal adenomas (Okabayashi, Ashrafian et al. 2012) and CRC (Harriss, Atkinson et al. 2009) with 10% of colon cancers in the UK occurring in overweight or obese individuals (Bergstrom, Pisani et al. 2001). The risk of colon cancer increases by an estimated 25% in overweight and 50% in obese men (Moghaddam, Woodward et al. 2007). In both sexes a larger waist size and waist-to-hip ratio has been associated with an increase in colon cancer risk (Larsson and Wolk 2007).

1.5 Grading Colorectal Tumours

CRC is graded by the degree of tubular differentiation within the tumour. Well-differentiated or low grade tumours are composed of regular tubules lined by columnar epithelium with uniform nuclei and are comparable to that of normal adenomatous epithelium. Moderately differentiated or average grade tumours are composed of slightly irregular tubules. High grade or poorly differentiated tumours are formed by highly irregular or even absent tubules, replaced by single cells, clumps or sheets of undifferentiated cells. Tumours are often heterogeneous with areas of different grades and therefore the grade of the tumour is assigned according to the area of poorest differentiation (Day 2003).

1.6 Tumour Spread and Staging

CRC spreads by local extension, invasion into the lymphatic, venous, nervous system and to a lesser extent through transcoelomic spread. The

prime targets for metastasising cancer cells are the local lymph nodes. Due to the few lymphatics in the muscularis mucosa tumours confined to this plane are unlikely to metastasis. However, once a tumour has invaded into the muscularis propria, lymphatic spread is more likely to lead to progression from node to node and eventual systemic involvement of the liver, lungs, bone and brain. Tumours may also achieve distant metastasis without lymph node involvement by directly invading the vascular system, or by extensive local invasion into the peritoneum cavity enabling transcoelomic spread (Day 2003).

Staging CRC provides a prognostic stratification of patients who have undergone surgery. CRC is classically staged by the Dukes' classification (Jass and Morson 1987). Dukes described three stages as follows (Dukes 1932):

A. Tumour breaching the muscularis mucosae but not beyond the muscularis propria and no lymph node involvement

B. Tumour invading through muscularis propria into pericolic or perirectal tissue but no lymph node involvement

C. Tumour involving local lymph nodes.

The Dukes' classification was originally devised for rectal cancer and was based on the extent of disease determined by the degree of infiltration of the tumour through the bowel wall and lymph node involvement (Dukes and

Bussey 1958), but has undergone subsequent modifications with a stage C further subdivided into C1 if the highest lymph nodes are tumour free and C2 if they are diseased and the addition of stage D for disease with distant metastasises. It remains a reliable predictor of CRC disease outcome. Over 93% of patients with Dukes' stage A survive five years compared with 7% of patients with Dukes' D (Cancer Research UK. 2010). More recently the Tumour-Nodes-Metastasis (TNM) staging system has been adopted for CRC (Puppa, Sonzogni et al. 2010). The TNM system again relies on describing the anatomical extent of disease but fully stratified by bowel wall involvement (T1 to T4 according to the involved layers of intestinal wall), the number of lymph nodes involved (N1 to N2 according to the number of metastatic lymph nodes) and presence of metastasises (M1).

Although many factors have been proposed as independent prognostic indicators, tumour stage plays a fundamental role in the management of patients and remains the most powerful and reliable predictor of prognosis, and basis for provision of appropriate therapeutic treatment (Zlobec and Lugli 2008).

1.7 Pathology of Colorectal Tumours

Adenomatous polyps or adenomas, which arise from the glandular epithelium, are the major precursor lesion to CRC (Bujanda, Cosme et al. 2010).

The evidence of the development of carcinoma from adenoma comes from the following observations: 1) Adenomas are found in CRC specimens six times more frequently than in matched non-cancer specimens; 2) Additional adenomas are found in 75% of patients with adenocarcinoma; 3) Developing a metachronous cancer is twice as likely in patients with carcinoma and adenoma(s) found in the resected specimen compared with carcinoma alone; 4) Patients affected with multiple adenomas such as in hereditary syndromes, for example, familial adenomatous Polyposis (FAP), or with large, villous or severely dysplastic adenomas are likely to develop carcinoma; 5) Adenomas demonstrate all grades of dysplasia ranging from minor changes to severe dysplasia and carcinoma *in situ*; 6) Removing adenomas reduces the risk of developing CRC; 7) *In vitro* adenoma cells can transform into carcinoma cells; and 8) In adenomas a foci of carcinoma can often be detected and often residual adenomatous epithelium can be found in CRC specimens (Day 2003; Fearon 2010)

CRC develops following a stepwise series of events beginning with the transformation of normal colonic epithelium to a benign adenomatous polyp which develops into an advanced adenoma with high grade dysplasia and then finally into an invasive cancer, this is the ‘adenoma-carcinoma’ sequence (Fearon and Vogelstein 1990) (Figure 1).

1.8 Diagnosis of Colorectal Cancer

CRC is diagnosed on the basis of tumour biopsy at colonoscopy or sigmoidscopy. Staging is completed by computer tomography (CT) of the

chest, abdomen and pelvis to ascertain the extent of the disease and to ascertain if there are any metastases. The extent of the rectal disease is measured by Magnetic resonance imaging (MRI), which assesses the spread of the tumour in the mesorectum. Patients with suspected liver metastases are further investigated with ultrasound, CT, MRI or Positron emission tomography (PET) scan of the liver (Cunningham, Atkin et al. 2010; Patel, Floyd et al. 2012).

1.9 Screening for Colorectal Cancer in the General Population

Three quarters of patients with CRC have no apparent risk factor other than old age. The survival rates of patients with CRC have improved substantially over the last few years as a result of public education, early diagnosis and improved treatment. The use of population screening will be vital in reducing mortality rates further. The aim of screening is to detect cancers at an early stage or even premalignant stage and therefore prevent the development of advanced cancers (Pawa, Arulampalam et al. 2011).

Faecal occult blood (FOB) testing is the most widely used screening test in which two samples are collected from three consecutive stools before being analysed in the laboratory. Offered every two years it has been shown to potentially reduce CRC mortality rates by 25% although it is the least sensitive screening tool. As two-thirds of CRCs and adenomas develop in the rectum and sigmoid colon an examination by flexible sigmoidscopy has been proven to be a safe and practical test. By using a one-off flexible sigmoidscopy between the ages of 55 and 64 years old mortality was

reduced by 43% (Atkin, Edwards et al. 2010; Littlejohn, Hilton et al. 2012) in the screened population. The major benefit of flexible sigmoidscopy compared to FOB testing is that it is both diagnostic and can be therapeutic as adenomas can be removed at the same time. However, flexible sigmoidscopy is limited examination as it can only access part of the large bowel, and the procedure also carries the risk of intestinal perforation.

Alternatively, full colonoscopy assessing the whole large bowel, requires 48 hours of bowel preparation, sedation during the procedure and is also associated with greater risks of higher complications (Young and Womeldorph 2013). However, it is not considered a viable screening tool in the UK because of cost, although high quality programmes are available in Germany and Poland, and in the USA (Pox, Schmiegel et al. 2007). CT colonography is as sensitive as colonoscopy for the detection of large adenomas and cancers, but it also requires a full bowel preparation and exposes the patient to radiation (Atkin, Dadswell et al. 2013). Furthermore, any lesions identified by this means would need a colonoscopy to confirm and remove them. However, colonography is useful in patients who may not be able to tolerate colonoscopy.

Novel molecular DNA based blood or stool test have been developed that detect abnormalities in DNA extracted from epithelial cell found in faeces or blood. However, these tests are expensive and have yet to be optimised and validated and their reliability assessed fully (Cunningham, Atkin et al. 2010).

1.10 Treatment of Colorectal Cancer

For CRC complete resection of the tumour remains the only curative treatment, although treatment strategy is guided by appropriate staging of the disease. Total resection (R0) is imperative for optimal prognostic outcome. Patients with residual disease, either microscopic (R1) or macroscopic (R2) fare poorly if treated with surgery alone. Hence, resection of the tumour needs to be done with adequate margins with a distal margin of at least 5cm and lymph node clearance of at least 12 lymph nodes otherwise histological assessment may result in a downstage of the tumour (Smith, Driman et al. 2010). Colectomy is carried out for colon cancer either open or laparoscopically. Laparoscopic surgery is technically more demanding, however it has the advantage of reduced pain and ileus, shorter recovery period and length of stay in hospital (Dalton, Ghosh et al. 2011). For rectal cancer the technique of Total Mesorectal Excision (TME) (Heald, Moran et al. 1998) is used. TME helps ensure adequate circumferential and distal margins, and mesenteric lymphadenectomy, thereby reducing the risk of disease reoccurrence when used in conjunction with neo or adjunct chemotherapy (Heald and Ryall 1986). Sphincter preserving surgery may be carried out in patients with mid and low rectal cancers. Otherwise abdominoperineal resection is carried out in those with very low rectal tumours.

Although surgery is the foundation for cure, adjunct therapies such as chemotherapy and radiotherapy before or after surgery are used to counteract disease particularly in stage III (any T, N1 -2, and M0) and IV

disease (any T, any N and M1) (Cunningham, Atkin et al. 2010). In the UK patients with stage III disease are routinely offered adjunct chemotherapy as treatment has been shown to reduce death by 30% or increase survival by 10-15%. For patients with stage II disease, chemotherapy has been controversial due to the small gains versus treatment risks, high risk patients (T4 tumours, obstructing at presentation, poor differentiation, extramural venous spread or positive margins) are often offered treatment. The QUASAR study, treating stage II disease with fluorouracil did show a significant improvement in overall survival of 3.6% in both colon and rectal cancers (Gray, Barnwell et al. 2007).

In patients with metastatic CRC and disease reoccurrence, new chemotherapy agents in addition to Fluorouracil such as Irinotecan, Oxaliplatin and targeted antibody-based drugs have increase survival from 12 months to 2 years (Cunningham, Atkin et al. 2010).

1.11 Hereditary And Familial Colon Cancer Syndromes Predisposing to Colorectal Cancer

Hereditary or familial colon cancer accounts for less than 6% of cases of CRC. Here, we outline two of the most common syndromes.

1.11.1 Hereditary Nonpolyposis Colorectal Cancer (Lynch Syndrome) and Familial Adenomatous Polyposis

Hereditary Nonpolyposis Colorectal Cancer (HNPCC) or Lynch Syndrome (LS) is the most common hereditary syndrome accounting for 2-4% or approximately one in 300 of all CRC cases (Jasperson, Tuohy et al. 2010) with a life time risk of CRC in these patients of about 50-80%. LS is caused by a germ line mutation in one of the genes of the DNA nucleotide mismatch repair system, most commonly human DNA mismatch repair (MMR) gene mutS homolog 2, colon cancer, nonpolyposis type 1 (*MSH2*) and mutL homolog 1, colon cancer, nonpolyposis type 2 (*MLH1*) accounting for 90% of LS, whilst less commonly MutS homolog 6 (*MSH6)*, postmeiotic segregation increased 1 (*PMS1)* and postmeiotic segregation increased 2 (*PMS2*) (Rustgi 2007). However, a further subset has been described with a germ line deletion of epithelial cell adhesion molecule (*EpCAM)* with no MMR gene mutation but displaying LS phenotype (Kovacs, Papp et al. 2009). Therefore, almost all CRC associated with LS have microsatellite instability (MSI) a result of MMR loss (Pino, Mino-Kenudson et al. 2009) (see Section 1.13.2). In these patients CRC and polyps arise at an earlier age and are more proximal in location compared to sporadic tumours. They are poorly

differentiated, mucinous and have a large number of tumour infiltrating lymphocytes. Other malignancies are associated with LS and include, in particular, endometrial cancer, which is associated with an increase lifetime risk of 40-60%, occurs in LS patients; the syndrome is responsible for 2% of all endometrial cancers (Watson, Vasen et al. 2008). Other LS-associated cancers include gastric, ovarian, biliary, urinary tract, small bowel, brain and pancreatic cancer (Jasperson, Tuohy et al. 2010).

FAP is the second most frequent hereditary syndrome arising in approximately one in 7000 people affected by CRC. It is caused by a germline mutation in the Adenomatous Polyposis coli (*APC*) tumour suppressor gene. The inherited mutation results in the development of hundreds and thousands of bowel adenomas beginning in early adolescence with CRC inevitable in nearly all patients by age 50 unless the colon is removed (for further details see Section 1.14.1).

1.11.2 Other Colorectal Cancer Syndromes

MutY Homolog (MUTYH) adenamtous polyposis (MAP) is caused by homozygous mutation in the base excision repair pathway gene *MUTYH* resulting in an autosomal recessive adenomatous polyposis of the colorectum with an increased risk of CRC. MUTYH protects against transversions caused by oxidative DNA damage. Colonic polyposis usually occurs by the time an individual reaches their forties. MAP patients have a propensity for proximal colonic neoplasms. In addition, *MUTYH* mutations have also been found in early onset CRC with few or no polyps (Jasperson,

Tuohy et al. 2010; Nielsen, Morreau et al. 2011).

Recently whole genome sequencing of germline DNA from patients with CRC and polyps has revealed mutations in two related DNA polymerase genes: *POLE* and *POLD1* (Briggs and Tomlinson 2013). POLE is the catalytic subunit of polymerase ε (Pol ε) and POLD1 encodes the catalytic and proofreading subunit of polymerase δ (Pol δ); both are responsible for proof-reading repair during DNA replication. *POLE* or *POLD1* germline mutation also predisposes in some individuals to multiple colorectal adenomas and carcinomas. This particular syndrome has been called Polymerase Proofreading-associated Polyposis (PPAP). Similarly other individuals with PPAP have been found to have large adenomas or early onset CRC, which is also seen in LS and MAP (Briggs and Tomlinson 2013; Palles, Cazier et al. 2013).

1.12 Colorectal Cancer Genetics

CRC develops as a consequence of a series of acquired genetic mutations, resulting in cell proliferation, reduced programmed cell death/apoptosis together with increased angiogenesis, cellular metabolism and stromal invasion. CRC is initiated following mutation in a single precursor cell conferring growth advantage – clonal selection, leading to clonal expansion and the acquisition of further mutations resulting in additional growth advantage (Fearon and Vogelstein 1990).

The mutations occur in two broad categories of genes: proto-oncogenes and tumour suppressor genes (TSGs). When proto-oncogenes are mutated to become oncogenes they are activated leading to a ‘gain in function’; oncogenes encode for growth factors or their receptors, molecular signalling, cell cycle regulators, proliferation and survival factors. In contrast, TSGs are inactivated when mutated leading to a ‘loss in function’ (Kim 2009). As postulated by Knudson’s two-hit hypothesis this loss of function requires ‘two hits’ or two mutational events. Just a single event, loss of heterozygosity (LOH) or a point mutation will result in loss of function of only one gene, but when combined both gene copies in the normal diploid cell can be hit and no working gene left intact. TSGs restrain growth and proliferation, progression of cell cycle, mobility, and other functions related to stable differentiation.

In colonic epithelium, a single sheet of columnar epithelial cells underlined by the connective tissue of the lamina propria form finger-like invaginations or crypts. The interstinal crypts are clonal populations formed at the base of the crypts by stem cells, proliferating in the mid-crypt zone, before differentiating, dying and sloughing off in the gut lumen. The stem cell niche at the base of the crypt is surrounded by mesenchymal myofibroblasts that produce WNT ligands that activate Frizzled receptors on the stem cells. A stem cell that acquires a mutation that provides a selective advantage can result in a clonal expansion as it migrates up the crypt and subsequent colonisation with the mutant stem cell leading to clonal conversion of the stem cell niche. Mutated intestinal crypts expand by a process of crypt fission spreading the mutation within the epithelium or field cancerisation

(reviewed Humphries and Wright 2008)

Fearon and Vogelstein proposed that the adenoma-carcinoma sequence is determined by a stepwise accumulation of multiple mutations activating pro-oncogene genes and inactivation of TSGs (Fearon and Vogelstein 1990). This model predicts that at least 7 distinct mutations are required and genome wide sequencing has so far identified up to 80 mutated genes per CRC with less than 15 considered to be true 'drivers' of tumourgenesis (Fearon 2010). Those genes that are mutated frequently are considered ‘gene mountains’. Members of ‘gene mountains’ include oncogenes such as Kristen-Ras (*KRAS*) which promote cancer development when mutated and TSGs such as *APC*, the MMR genes and Tumour Protein 53 (*TP53)*, which fail to inhibit cancer development when mutated. However, amongst these are ‘gene hills’ which are genes mutated in less than 5% of tumours, although it has been suggested that it is the ‘gene hills’ that are more important as the majority of cancers do not have mutations in these gene mountains (Wood, Parsons et al. 2007). More recently the Cancer Genome Atlas project using whole-genome sequencing of CRC identified 32 recurrent somatic mutations. The study showed ubiquitous mutation in one or more members of the WNT signalling pathway and also identified novel and frequent mutations in WNT signalling genes *ARIDIA*, *SOX9* and *FAM123B.* Although the predominate WNT signalling mutation occurred in *APC* the sequencing also demonstrated that in the non-hypermutated (mutation rate <8.24 per 10^6) CRCs *APC* and *TP53* were significantly more frequently mutated compared to the hypermutated (mutation rate >12 per 10^6) group of

tumours. This suggested that these two groups progress through different sequences of genetic events (Cancer Genome Atlas Network 2012).

The initial appearance of an adenoma from normal colonic mucosa follows early inactivation of *APC*; mutation in *KRAS* then results in growth of the adenoma and progression to adenomatous intermediates, LOH of chromosome 18q results in adenoma growth and progression and inactivation of *TP53* occur concurrently with the transition from adenoma to carcinoma. Mutational activation of Phosphoinositide-3-kinase, catalytic, alpha (*PIK3CA*) occurs late in the adenoma-carcinoma sequence in only a small proportion of CRC (Figure 1).

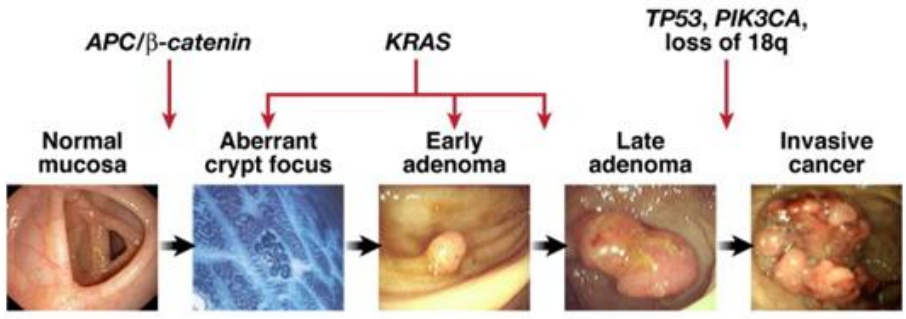


Figure 1. The 'adenoma-carcinoma' sequence model of carcinogenesis is a result of multiple stepwise genetic mutations. The formation of an aberrant crypt focus is the initial step in colorectal tumourgenesis. Activation of the Wnt signalling pathway occurs due to mutation in the APC gene or β-catenin. Progression from adenoma to carcinoma results from mutations in KRAS, TP53, LOH 18q, while mutation in PIK3CA is a late step in some CRC's (Adapted from Takayama et al, 1998).

1.13 Chromosomal and Microsatellite Instability in CRC

The frequency of naturally occurring baseline mutations is insufficient to account for the multiple mutations required for cancer to develop. A mutator phenotype must be acquired that increases the rate of new mutations, therefore providing an intrinsic genomic instability (Loeb, Loeb et al. 2003). It is considered that three distinct but not necessary mutually exclusive pathways: Chromosome Instability (CIN), Microsatellite Instability (MSI) and CpG island methylator phenotype (CIMP) (Issa 2004). Finally a fourth pathway has been suggested which has neither, the Microsatellite Stable and Chromosomal stable (MACS) CRC's (Cai, Xu et al. 2008; Silver, Sengupta et al. 2012).

1.13.1 Chromosome Instability

CIN is the most common pathway, which has been observed in 65-70% of sporadic CRC (Al-Sohaily, Biankin et al. 2012). CIN describes the accelerated rate of allelic gain or loss of entire or part of chromosomes (Lengauer, Kinzler et al. 1998) resulting in an imbalance in chromosome number (aneuploidy), genomic amplification and a high frequency of LOH (Pino and Chung 2010). CIN can be as a result of DNA replication stress and defective chromosome segregation (Burrell, McClelland et al. 2013); Telomere dysfunction; response to DNA damage and LOH (Lengauer, Kinzler et al. 1998; Wang, Cummins et al. 2004; Pino and Chung 2010). Therefore, this particular pathway is characterised by tumours with APC mutations following allelic loses on chromosome 5q, TP53 from 17p, KRAS

from 12p and losses on chromosome 18q resulting in SMAD4 mutation (Pino and Chung 2010). CIN tumours occur more frequently in the left and distal colon and are highly differentiated with no lymphocytic infiltration and are more sensitive to fluorouracil chemotherapy, although CIN tumours are associated with a poorer survival compared to MSI tumours (Soreide, Janssen et al. 2006).

1.13.2 Microsatellite Instability

MSI is characterised by a defective mismatch repair system that causes accumulation of errors in the DNA sequence. Microsatellites are simple sequence repeats of 1 to 6 nucleotides most commonly a dinucleotide repeat of cytosine and adenine (Thibodeau, Bren et al. 1993). Due to their repetitive nature microsatellites have a much higher mutation rate as DNA polymerases are prone to 'stall or slip' at these sequences resulting in a somatic change in sequence length and frameshift mutations through a gain or loss of repeat units (Boland and Goel 2010). Such mismatches are corrected by the mismatch repair pathway (MMR), however in MSI tumours there is a defect in the MMR pathway. In CRC arising in LS patients (see Section 1.11), MSI is caused by a germline mutation in a mismatch repair enzyme (e.g. MSH2). Whilst in approximately 15% of sporadic CRC, the MSI phenotype is due to epigenetic silencing of a mismatch repair gene most often MLH1 (Hughes, Khalid-de Bakker et al. 2012). Epigenetic silencing is commonly associated with the CpG island methylator phenotype (CIMP) and about 70-80% of sporadic MSI are attributed to CIMP (Toyota, Ohe-Toyota et al. 2000; Issa 2004). MSI tumours commonly have frameshift mutations in

β-catenin, particularly in tumours from LS patients (Johnson, Volikos et al. 2005), and transforming growth factor B receptor II (TGF-BRII) (Watanabe, Wu et al. 2001). MSI CRCs have a higher frequency of BRAF mutations, although have fewer mutations in KRAS, and TP53 (Samowitz, Holden et al. 2001; Oliveira, Westra et al. 2004). MSI tumours frequently occur in the proximal and right colon, they are poorly differentiated with lymphocytic infiltration which may associate with their better survival compared to CIN tumours (Soreide, Janssen et al. 2006).

1.13.3 CPG Island Methylator Phenotype

CIMP is associated with a subset of CRCs that are thought to be etiologically and clinically distinct, and characterised by methylation of a panel of specific CpG loci. CIMP is closely associated with MSI in patients without germline mutations in MMR genes and with frequent BRAF mutation (Walther, Johnstone et al. 2009). CIMP tumours have distinctly different histology compared to tumours developed from the traditional adenoma-carcinoma pathway and instead are thought to arise via the serrated neoplastic pathway (Noffsinger 2009; Snover 2011); and clinically there is evidence to suggest that CIMP is associated with prognosis (Ogino, Nosho et al. 2009; Dahlin, Palmqvist et al. 2010). Promoter CpG island methylation of TSGs are a common feature of CIMP. These CpG islands are clusters of cytosine-guanosine residues with gene promoters of nearly half the human genome embedded into them that can be methylated (Issa 2004). Methylation of the promoter region of a gene causes transcriptional repression and therefore silencing of the gene. An early event in CIMP tumours is the mutation of the

BRAF proto-oncogene, which inhibits normal apoptosis of colonic cells (Snover 2011), as well as methylation of the MMR gene MLH1, resulting in its transcriptional inactivation and causing MSI (Herman, Umar et al. 1998). Methylation of CIMP-related markers originally included MINT1, MINT2, MINT31, p16INK4α, p14ARF, and hMLH1, which are frequently used to identify tumours with this phenotype (Issa 2004). Other markers of this phenotype have since been added and the best choice of markers is in dispute. Weisenberger and colleagues introduced a panel of five genes including CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS, while Ogino and colleagues (2007) proposed that a panel of (at least) four markers including RUNX3, CACNA1G, IGF2, and MLH1 should constitute a sensitive and specific CIMP panel for the purpose of research and clinical use (Ogino, Kawasaki et al. 2007). At present, no set criteria for defining an ideal panel of CIMP markers exists (Hughes, Khalid-de Bakker et al. 2012).

1.13.4 Microsatellite Stable and Chromosome Stable

MACS are a subset of CRC that are both microsatellite stable and chromosome stable (MSI-CSI-/MIN-CIN-), that account for up to 30% of all sporadic CRCs with distinct molecular characteristics (Silver, Sengupta et al. 2012; Sengupta, Yau et al. 2013). MACS have been reported to be present in CRCs arising in younger patients (Chan, Curtis et al. 2001) and located preferentially in the proximal colon with poor differentiation (Cai, Xu et al. 2008). Our Laboratory has found that MACS are more likely to be left- than right-sided and that those patients with MACS cancers have a better survival

and lower hypermethylation status (see Section 1.21.3) compared with other CRC instability phenotypes (Silver, Sengupta et al. 2012).

1.14 Adenomatous Polyposis Coli, B-Catenin and WNT Signalling Pathway

1.14.1 Familial Adenomatous Polyposis Syndrome And The Adenomatous Polposis Coli Tumour Suppressor Gene

FAP has a prevalence of 1 in 10,000 individuals; it results in the development of hundreds and thousands of bowel adenomas beginning in early adolescence. Colonic cancer is inevitable with nearly all patients being diagnosed with CRC by age 50, with the average age of diagnosis 39 years, although 7% will develop CRC by age 21 (Jasperson, Tuohy et al. 2010). Therefore, these patients at diagnosis are offered prophylactic colectomy usually towards the end of the second decade of life before cancer is likely to develop. A number of varying phenotypic FAP related syndromes have been proposed, including attenuated FAP a less-severe form of FAP developing fewer polyps (less than 100) at a later age with a lifetime risk of developing CRC 69% (Burt, Leppert et al. 2004). FAP is also characterised by extracolonic manifestations including epidermoid cysts, osteomas, desmoids tumours and dental anomalies and a characteristic retinal lesion called congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Half, Bercovich et al. 2009). Turcot's syndrome is FAP associated with brain

tumours including medulloblastoma and glioblastoma (Hamilton, Liu et al. 1995).

The gene responsible for these syndromes is the APC TSG. The gene encodes a 312 kDa cytoplasmic protein consisting of 2,843 amino acids with multiple functional domains, transcribed by 15 exons (Fearon 2010). The APC protein normally binds to β-catenin the downstream effector of the Wnt signalling pathway (see Section 1.13.2). APC regulates β-catenin and is vital in Wnt signalling, it regulates differentiation, adhesions, migration, development, apoptosis and chromosomal segregation. The largest exon, number 15 accounts for 75% of the coding sequence and is the most common site of somatic and germline mutation (Goss and Groden 2000). Most mutations almost always result in a truncated protein as a result of premature protein translation termination.

Mutation of APC is the earliest step in colorectal tumourgenesis and therefore considered to be the 'gatekeeper gene'. Mutations of APC are found in three quarters of sporadic CRCs with mutations also found in 5% of dysplastic aberrant crypt foci and 63% of large adenomas suggesting that functional loss of APC is an early carcinogenic event (Powell, Zilz et al. 1992; Gupta and Schoen 2009). APC serves a vital role in the Wnt/β-catenin signalling pathway, which is crucial in regulating cell proliferation, differentiation and apoptosis (see Section 1.13.2). APC also regulates F-actin and microtubules both cytoskeletal proteins involved in cell adhesion, migration and mitosis (Aoki and Taketo 2007). Therefore, loss of APC from

normal colonic tissue may lead to chromosomal instability (Alberici and Fodde 2006) (see Section 1.13.1).

1.14.2 B-Catenin and the WNT Signalling Pathway

β-catenin is encoded by the catenin (cadherin-associated protein), beta 1 (CTNNB1) gene. CTNNB1 is a transcription factor that activates genes involved in cell cycle progression. β-catenin activity is the important down-stream effector of the Wnt signalling pathway (Segditsas and Tomlinson 2006). The Wnt/β-catenin signalling pathway or the canonical Wnt pathway is essential for controlling intestinal epithelial cell proliferation, and therefore important in malignant transformation (Gavert and Ben-Ze'ev 2007; Chien, Conrad et al. 2009). Wnt is a secreted glycoprotein that interacts with two cell surface receptors, Frizzled (Fz) seven transmembrane molecules and low-density lipoprotein receptor related protein family 5 and 6 (LRP 5 and LRP6) (Fearon 2010; Saif and Chu 2010). In the absence of Wnt, β-catenin is sequestered in the cytoplasm in a 'destruction complex', which consists of a subcellular trimeric complex containing the APC protein, the scaffolding protein axin, and glycogen synthase kinase-3B, which is subject to degradation following phosphorylation via the ubiqutin-proteasome pathway. This regulates the amount of β-catenin and thereby controls entry into the cell division cycle. Binding of the Wnt ligand to its receptors recruits the cytoplasmic phosphoprotein Dishevelled (Dsh) and this inactivates the 'destruction complex' leading to the inhibition of the Glycogen synthase kinase 3β (GSK3B) -mediated phosphorylation of β-catenin (Zeng, Tamai et al. 2005; Kimelman and Xu 2006). This allows β-catenin to escape

degradation and translocate to the nucleus. β-catenin enters the nucleus where it frees T-cell factor (TCF) from its repressors CtBP and Groucho, activating the transcription and expression of a variety of target genes responsible for cell cycle progression and proliferation, for example c-Myc and cyclin D. The binding of β-catenin and APC is necessary for APC to function as a TSG. With loss of APC function, β-catenin is able to accumulate and activate transcription genes which promote proliferation (White, Chien et al. 2012) (Figure 2).

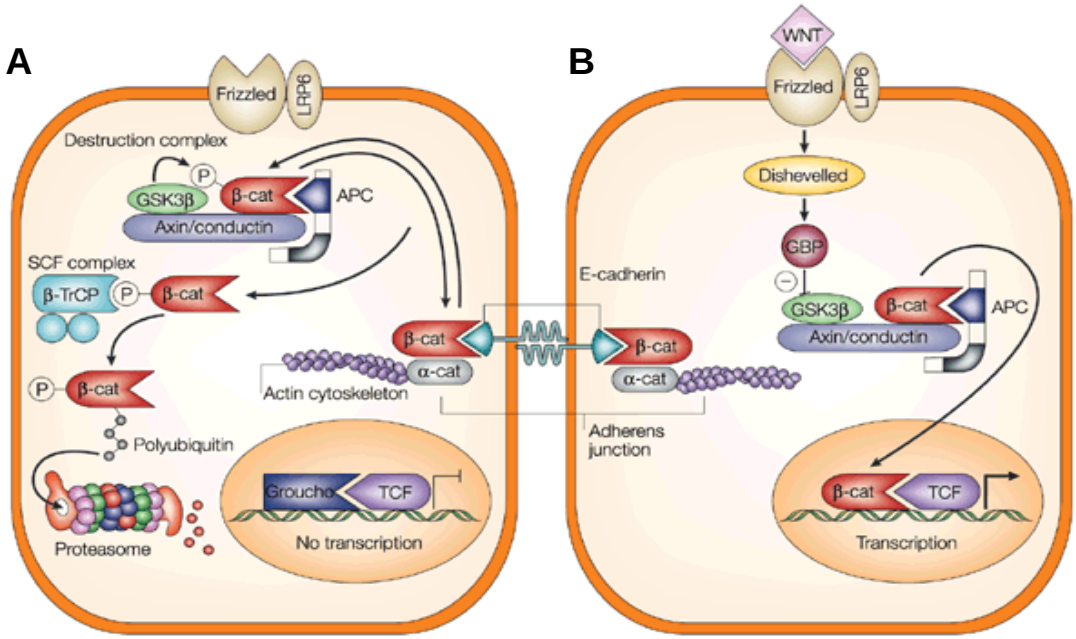


Figure 2. Modulation of β-catenin in the presence and absence of a Wnt signal. A. In the absence of a WNT signal, free intracellular β-catenin is kept low by proteasomal degradation. Free cytoplasmic β-catenin is recruited to a 'destruction complex' containing APC, axin/conductin and glycogen synthase kinase 3β (GSK3β). However, β-catenin is phosphorylated by GSK3β, allowing it to be recognized by an SCF complex containing β-TrCP, resulting in the addition of a polyubiquitin chain to β-catenin, and therefore making it recognizable for proteasome degradation. Hence, β-catenin cannot reach the nucleus, to co-activate TCF-responsive genes. B. In the presence of WNT, its receptor, Frizzled, in complex with LRP6, is activated. This leads to activation of GBP - an inhibitor of GSK3β. Consequently, β-catenin cannot be targeted for destruction and is free to diffuse into the nucleus, where it acts as a co-activator for TCF-responsive genes (Adapted Fodde et al, 2001).

1.15 The RAS Proto Oncogene

Mutations in the RAS family of proto-oncogenes are common and found in 20% to 30% of all tumours (Lau and Haigis 2009). The RAS superfamily of proteins which also include Harvey-RAS (HRAS), neuroblastoma-RAS (NRAS) and Kristen-RAS (KRAS) are a group of small guanosine triphosphate (GTP) binding proteins located on the inner plasma membrane characterised by the presence of a catalytic guanosine domain (G-domain), through which they may be activated (GTP-bound) or inactivated (guanosine diphosphate-bound (GDP) by extracellular stimuli (i.e. growth factors, cytokines, adhesion signals) (Bos 1989; Marshall 1996).

The RAS proteins are involved in intracellular signal transduction, and remain inactive until they bind to GTP. Once the GTP is bound to the RAS protein, this activates it leading to a conformational change in the RAS protein affecting its interactions with its downstream transducers, GTPase-activating proteins (GAPs), which amplify the effect of the activated RAS protein. This in turn also has the effect of further reducing the intrinsic GTPase activity, stimulated by GAPs to convert GTP to GDP and down regulating the activity of the RAS protein (Ahearn, Haigis et al. 2012). Oncogenic mutations of RAS are located in the active, GTP-bound state activating their downstream effectors (Castellano and Downward 2011).

RAS activates several pathways including the mitogen-activated protein (MAP) kinase cascade via the serine-threonine kinases of the RAF family, transmitting signals downstream resulting in the transcription of genes

involved in cell growth and division, as well as the PI3K/AKT pathway that inhibits apoptosis (Janssen, Abal et al. 2005; Castellano and Downward 2011) (Figure 3).

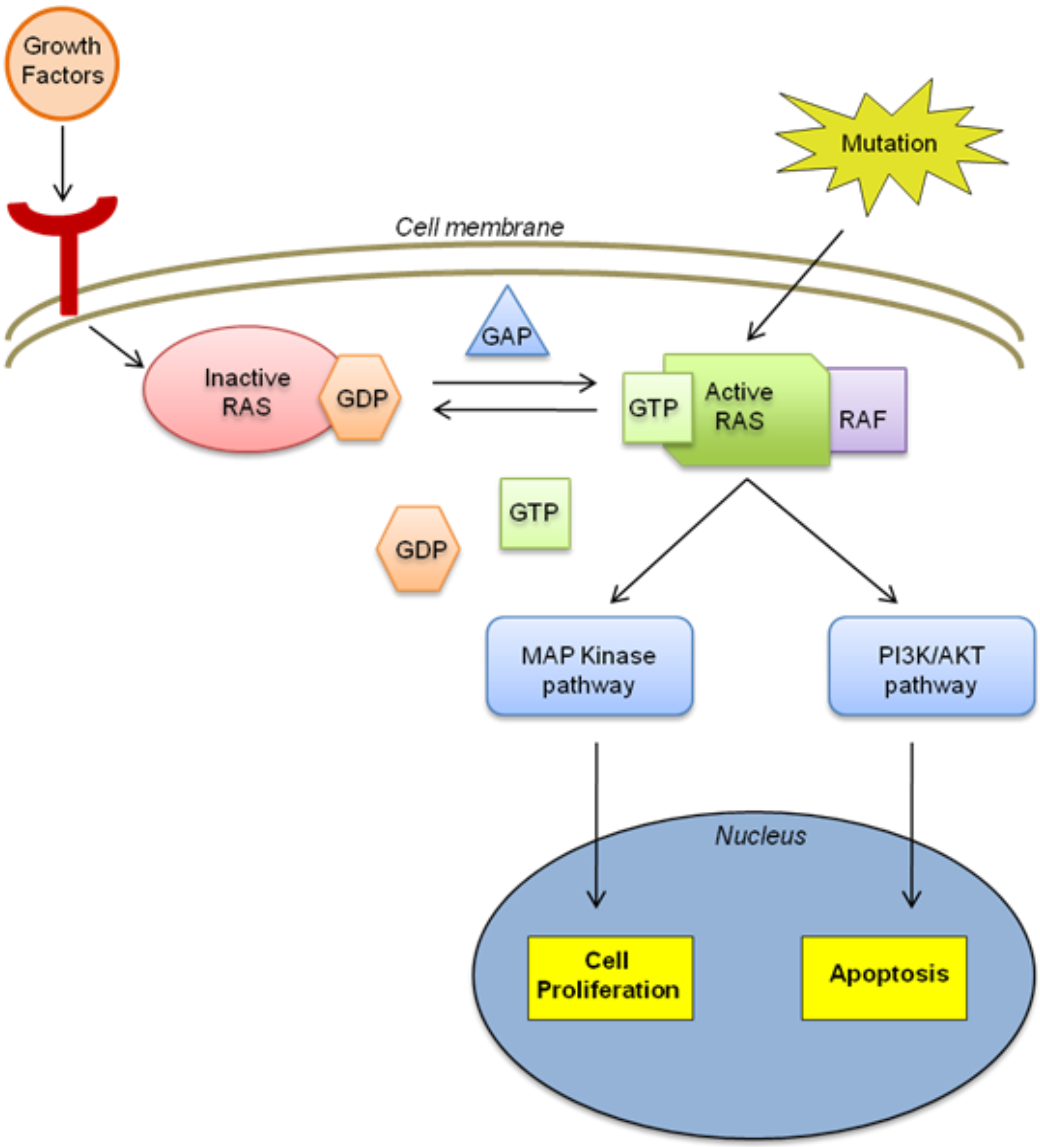


Figure 3. RAS Signalling. Binding of growth factors to the receptor tyrosine kinases results in the displacement of GDP from inactive RAS. RAS is activated following the binding of GTP, which recruits and activates RAF via MAP kinase pathways, stimulating cell proliferation and PI3K/AKT pathways, suppressing apoptosis. Inactivation of RAS occurs when GAP proteins hydrolyse GTP back to GDP. Oncogenic mutation of K-RAS reduces intrinsic GTPase activity and therefore prevents the binding of GAP proteins, causing constitutive signalling (Castellano and Downward 2011).

1.16 Mutation of the Kirsten-RAS Gene in Cancer

The most common mutation in the *KRAS* gene is a point mutation in exon 2 at codons 12 or 13 or less commonly in codon 61 of exon 3. These mutations result in activation of a number of effector pathways, including RAF/MAPK, JNK and phosphatidylinositol 3-kinase (PI3K) signalling pathways leading to prevention of apoptosis and growth promotion (Janssen, Abal et al. 2005). *KRAS* mutations are found with the highest prevalence in pancreatic carcinomas (>80%), followed by colon carcinoma and lung carcinoma (30-50%), and in other cancers including biliary tract, endometrial, cervical, bladder, liver, myeloid and breast cancer (Jancik, Drabek et al. 2010). *KRAS* mutations are present in 40-50% of CRC, and in 15-68% of colorectal adenomas (McLellan, Owen et al. 1993; Takayama, Ohi et al. 2001; Takayama, Miyanishi et al. 2006) of which only 10% of adenomas less than 1cm in diameter suggesting *KRAS* mutation is a relatively late event.

1.17 The *TP53* Tumour Suppressor Gene – 'Guardian Of The Genome'

TP53 is a TSG located on the short arm of chromosome 17. *TP53* encodes for a 393 amino acid protein consisting of four functional domains each responsible for its actions as a transcriptional regulator of genes that encode proteins involved in DNA repair, cell cycle regulation, cellular senescence and apoptosis (Levine and Oren 2009). TP53 has, therefore, been described as the 'guardian of the genome' (Lane 1992).

TP53 is activated following cellular stress: DNA damage, nucleoside depletion, and hypoxia and oncogene activation (Levine and Oren 2009). The regulation of TP53 protein is complex, involves multiple proteins (Figure 4) and is negatively regulated by Mouse double minute 2 homolog (MDM2) a ubiqutin ligase (Momand, Zambetti et al. 1992). When protein kinases are activated (such as ATM, DNA-PK, or CHK2) they phosphorylate TP53 at one of three residues (Ser15, Thr18 or Ser20) disrupting MDM2 binding, resulting in a proportional increase in TP53 levels. MDM2 expression itself is activated by TP53 and, therefore the increase of TP53 also increases MDM2. However, this has no effect while TP53 is phosphorylated (Moll and Petrenko 2003). Once the DNA damage is repaired, the protein kinases are no longer active. Therefore TP53 is quickly dephosphorylated and destroyed by the accumulated MDM2 via the ubiquitin system. In addition, TP53 can also be modified by acetylation, methylation and sumoylation (Levine 1997). When DNA is damaged TP53 activates DNA repair proteins such as the ribonucleotide reductase p53R2 involved in DNA replication repair (Tanaka, Arakawa et al. 2000).

Once TP53 is activated it initiates transcription of one of its downstream genes reflecting the nature of the stress, although the choice of which pathway is activated is not well understood. TP53 inhibits cell cycle transition at DNA damage checkpoints at G1/S and G2/M (Taylor and Stark 2001). The transcription of the cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediates G1 arrest (el-Deiry, Tokino et al. 1993). While TP53 induced transcription of Growth Arrest and DNA Damage (GADD45), and 14-3-3s

inhibit the Cyclin B/CDC2 complex which is essential for G2/M phase (Hermeking, Lengauer et al. 1997).

Apoptosis is initiated if DNA damage is irreparable and TP53 regulates apoptosis by inducing multiple apoptotic target genes acting at different levels of both the intrinsic and extrinsic death pathways. Within the intrinsic apoptosis pathway, TP53 induces the activation of Bc1-2 - BAX, NOXA and PUMA 'activator' genes which enhance secretion of cytochrome c from the mitochondria into the cytoplasm, leading to activation of caspases and subsequent apoptosis (Fridman and Lowe 2003). Within the extrinsic apoptosis pathway, TP53 induces the expression of Fas ligands (e.g. CD95) and killer Dr Receptors (e.g. TRAIL receptor 2) regulating apoptosis (Igney and Krammer 2002; Meulmeester and Jochemsen 2008).

TP53 is also involved in cell senescence, although the exact mechanisms that regulate TP53-induced senescence are poorly understood. A wide spectrum of stimuli can trigger TP53-dependent senescence, including the abnormal activation of oncogenes e.g. RAS. TP53-induced senescence is primarily driven via the p53-p21 pathway (Zuckerman, Wolyniec et al. 2009). It is possible that TP53-induced senescence is regulated by tumour suppressors that are unregulated in cell senescence inhibiting MDM2 negative feedback loop (e.g. ARF) or stabilizing TP53 through acetylation (e.g. Promyleocytic leukaemia - PML) (Campisi 2001; Zuckerman, Wolyniec et al. 2009) (Figure 4). In cancer cells TP53-dependent senescence is activated once HSP72 is depleted in vivo (Yaglom, Gabai et al. 2007; Gabai, Yaglom et al. 2009) (see Section 1.21.1)

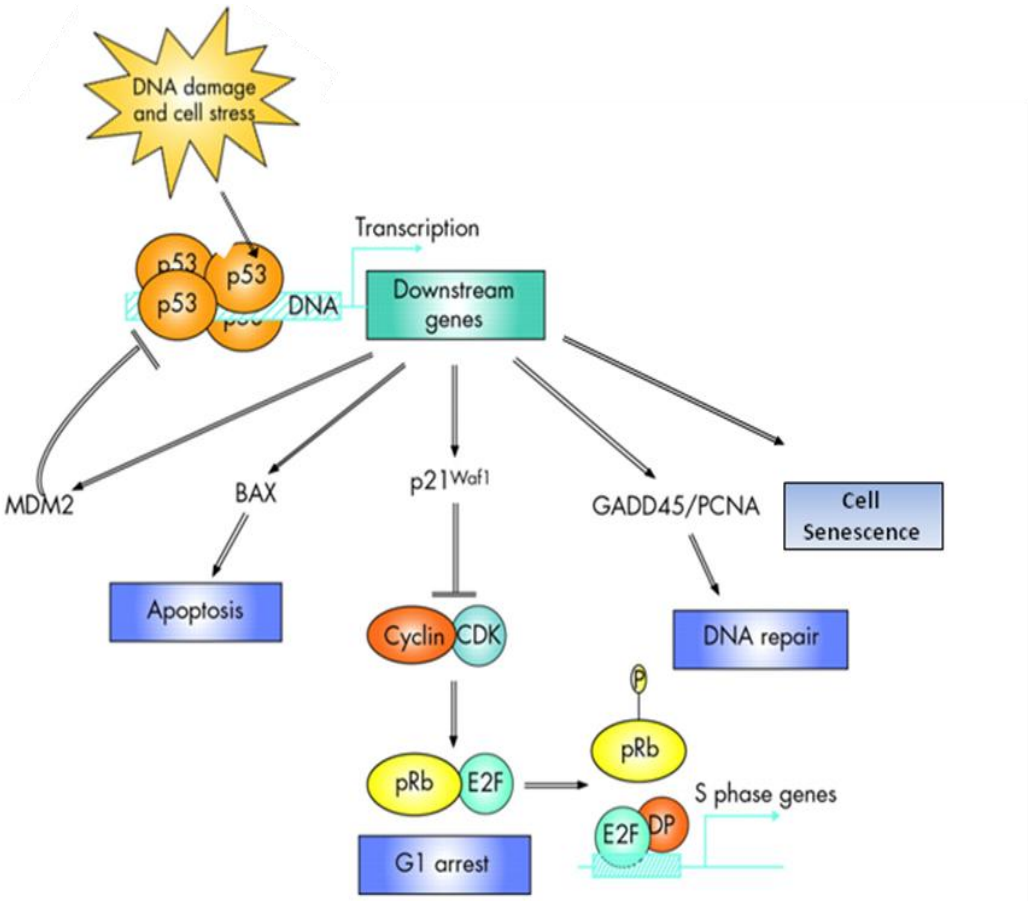


Figure 4. The role of TP53 in cellular response to stress. P53 is plays a vital role in the cellular response to stress and DNA strand breaks. Ultraviolet radiation, and oxidative stress all result in a rapid increase in TP53 concentration. This is normally tightly regulated by MDM2, a negative regulatory partner. MDM2 is an E3 ubiquitin ligase, which mediates both ubiquitination and proteasome dependent degradation of TP53. TP53 induction results in the transcription of numerous genes such as the Cyclin-dependent kinase (CDK) inhibitor protein p21WAF1, which mediates cellular senescence and G1/S growth arrest by blocking cyclin E-CDK 2 mediated phosphorylation of retinoblastoma protein (pRb). BAX involved in mitochondrial apoptosis; and Growth Arrest and DNA Damage (GADD45), which is involved in DNA repair (Adapted from Boland et al. 2005).

1.18 Mutation of the *TP53* Tumour Suppressor Gene

TP53 gene mutations are present in 50-55% of all cancers making it an almost universal hallmark of human tumours (Levine 1997). Most of the *TP53* mutations occur in exons 5 to 8 and 85% of these are missense mutations that occur in 5 codon hotspots (codons 175, 245, 248, 273 and 282) resulting in a stable protein that loses the ability to bind DNA and activate target genes (Vidaurreta, Maestro et al. 2008; Leroy, Fournier et al. 2013).

In 70% of CRC there is 17p LOH with *TP53* thought to be the main target TSG. *TP53* mutation is observed in 4-26% of adenomas, 50% of adenoma with invasive foci and in 50-70% of CRC defining its role in the transition from adenoma to carcinoma (Fearon 2010).

1.19 The Phosphoinositide Kinases and the AKT Pathway

Phosphoinositide kinases (PIKs) are lipid kinases categorised into three families: Phosphoinositide 3-kinases (PI3Ks), Phosphoinositide 4-kinases (PI4Ks) and Phosphoinositide 5-kinases (PI5Ks) (Samuels and Velculescu 2004). These act as signal transducers through phosphorylation of phosphoinositides. PI3Ks are further subdivided into class I, II and III (Katso, Okkenhaug et al. 2001). The class I PI3Ks are heterodimers consisting of a catalytic subunit and regulatory subunit activated by growth factor receptor tyrosine kinases (Vivanco and Sawyers 2002). These have been found to

have cancer-associated mutations within their catalytic subunit p110α encoded by the PIK3CA gene (Karakas, Bachman et al. 2006).

PIK3CA phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) forming Phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 is an important second messenger involved in cell growth, proliferation and survival and migration through it activation of the AKT pathway and by serving as an anchor for the serine kinases AKT1. PIP3 is in turn tightly regulated by phosphatase and tensin homolog (PTEN) dephosphorylating PIP3 to PIP2 (Chalhoub and Baker 2009). Somatic mutations of the *PTEN* TSG have been found in approximately 10% of CRCs (Wood, Parsons et al. 2007). Cowden syndrome is a rare hereditary autosomal dominant disorder associated with a germline mutation of *PTEN* in 80% of patients. Phenotypically the syndrome is characterised by multiple hamartomas, but not by an increased risk of CRC (Marsh, Dahia et al. 1998; Hobert and Eng 2009).

PIK3CA is mutated in 32% of CRCs (Samuels and Velculescu 2004) as well as 25% of gastric cancers, 36% hepatocellular carcinomas and 18-40% of breast cancers (Samuels, Wang et al. 2004). Mutation in *PIK3CA* leads to increase in PI3K activity resulting in an accumulation of PIP3 and activation of the AKT pathway. AKT activation is important in cancer as it regulates cell survival, proliferation and growth. Activation of AKT functions as an anti-apoptotic pathway. AKT inhibits activity of pro-apoptotic proteins such as BAD, capase-9 and MDM2, the negative regulator of TP53, enhancing its degradation (see Section 1.16). AKT prevents the degradation of CDKs such

as cyclin D1 GSK3β (see Section 1.13.2), the expression of CDK inhibitors, and regulates protein synthesis by regulating mammalian target of rapamycin (mTOR) thereby enhancing proliferation and cell growth (Vivanco and Sawyers 2002; Liao and Hung 2010) (Figure 5).

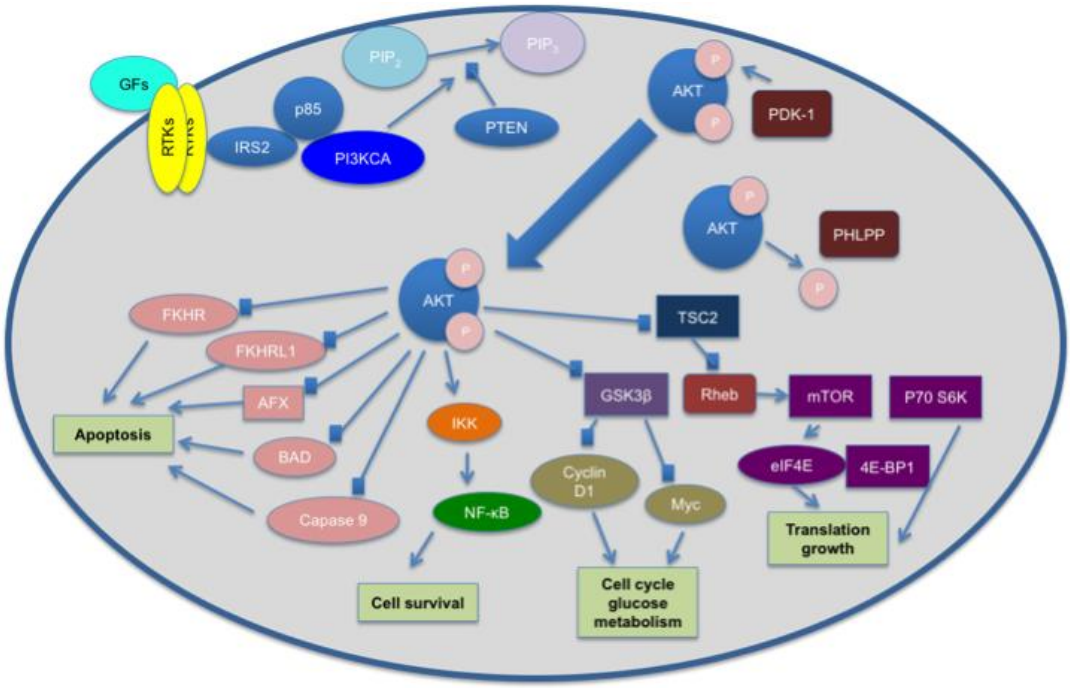


Figure 5. Phosphatidylinositol 3-kinase (PI3Ks) are activated by stimulation of receptor tyrosine kinases (RTKs). This triggers activation of PI3KCA and conversion by its catalytic domain of phosphatidylinositol (3,4)-bis-phosphate (PIP2) to phosphatidylinositol (3,4,5)-tris-phosphate (PIP3), PIP3 brings PDK1 and AKT to the membrane where PDK1 activates AKT phosphorylation. PIP3 is in turn tightly regulated by phosphatase and tensin homolog (PTEN) dephosphorylating PIP3 to PIP2. Similarly, the phosphatases in the PHLPP family directly dephosphorylate and therefore inactivate AKT.

AKT mediates the activation and inhibition of several targets through phosphorylation, resulting in cell survival, proliferation and growth. AKT promotes survival by inhibiting pro-apoptotic proteins such BAD, Caspase 9 and fork-head transcription factors (e.g. FKHER, FKHRL1 and AFX) inhibiting the transcription of pro-apoptotic genes. AKT phosphorylates inhibitors of kappa kinases (IKK), which indirectly increases the activity of nuclear factor kappa B (NF-κB) stimulating the transcription of pro-survival genes. Inhibition of GSK3β by AKT stimulates cell cycle progression by stabilizing cyclin D1 expression. AKT can directly phosphorylate and activate the mammalian target of rapamycin (mTOR), as well as cause indirect activation of mTOR by phosphorylating and inactivating (TSC2) tuberous sclerosis complex 2, which normally inhibits mTOR through the GTP-binding protein (Rheb) RAS homolog enriched in brain. When TSC2 is inactivated by phosphorylation, the Rheb is maintained in its GTP-bound state, allowing for increased activation of mTOR. mTOR signals to its downstream effectors S6 kinase/ribosomal protein S6 and 4EBP-1/eIF-4E to control protein translation. (Growth factors, GFs). (Adapted from Samuels and Ericson 2006).

1.20 Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Sen and Baltimore 1986) is a protein complex that controls transcription of a diverse array of genes involved in inflammatory response, regulating cell survival, proliferation, cell adhesion and the cellular microenviroment (Pahl 1999; Karin 2006; Fan, Dutta et al. 2008; Iliopoulos, Hirsch et al. 2009). Therefore, aberrant NF-κB activation can result in the induction of anti-apoptotic genes, cell proliferation via increased expression of proto-ocogenes and cyclins; promotion of metastasis by regulating the expression of metalloproteinase and cell adhesion genes; and angiogenesis by regulating genes controlling growth of new blood vessels. Thus, aberrant NF-κB activation plays a pivotal role in the generation and maintenance of malignancies (Karin 2006; Kim, Hawke et al. 2006; Fan, Dutta et al. 2008). NF-κB can be activated by cellular stress, DNA damage, and by activation of various oncogeneic pathways (Perkins 2012) (Figure 6).

The NF-κB transcription factor family is made up of five proteins which all share a conserved N-terminus domain called the REL homology domain (RHD) which is required for dimer formation, DNA binding and interaction with NF-κB inhibitor proteins. These consist of two subfamilies of proteins which form hetero or homodimer complexes: p65 (RelA), c-Rel, RelB, which are collectively referred to as the Rel subfamily each containing a transactivation domain for transcription; and the NF-κB subfamily NF-κB1 (p100) and NF-κB2 (p105) (Perkins 2012). The NF-κB encode for precursor proteins that are further processed into their active DNA-binding forms p50

and p52 during translation or phosphorylation-induced partial proteolysis (Hayden and Ghosh 2008). These lack the transactivation domain but are able to induce transcription when bound to other co-activating proteins, for example BAG1 (Southern, Collard et al. 2012).

NF-κB complexes are present in an inactive or pre-synthesized form not requiring any further protein synthesis as they are responsible for rapid cellular activation. Therefore, to maintain their inactive state NF-κB proteins are sequestered in the cytoplasm by the precursor proteins p100 and p105 or one of three typical NF-κB inhibitor proteins (IKB) Iκ-Bά, Iκ-Bβ and Iκ-Bε. All IKB proteins are characterised by multiple ankyrin sequence repeats (Hayden and Ghosh 2008). They bind to NF-κB complexes inhibiting their DNA binding while keeping them in cytoplasmic form. Activation results in signal induced degradation of the IκB proteins via IκB kinase (IKK) which phosphorylates IκBs resulting in ubiquitylation and protesome-mediated degradation with subsequent NF-κB nuclear translocation (Kanarek, London et al. 2010) to activate its target genes (Figure 6). NF-κB response is regulated through a negative feedback loops with NF-κB turning on expression of its IKB proteins which then re-inhibits NF-κB forming an auto feedback loop (Brasier, Lu et al. 2001). There are also two atypical which function differently IκB proteins BCL3 (see Section 6.1) and Iκζ.

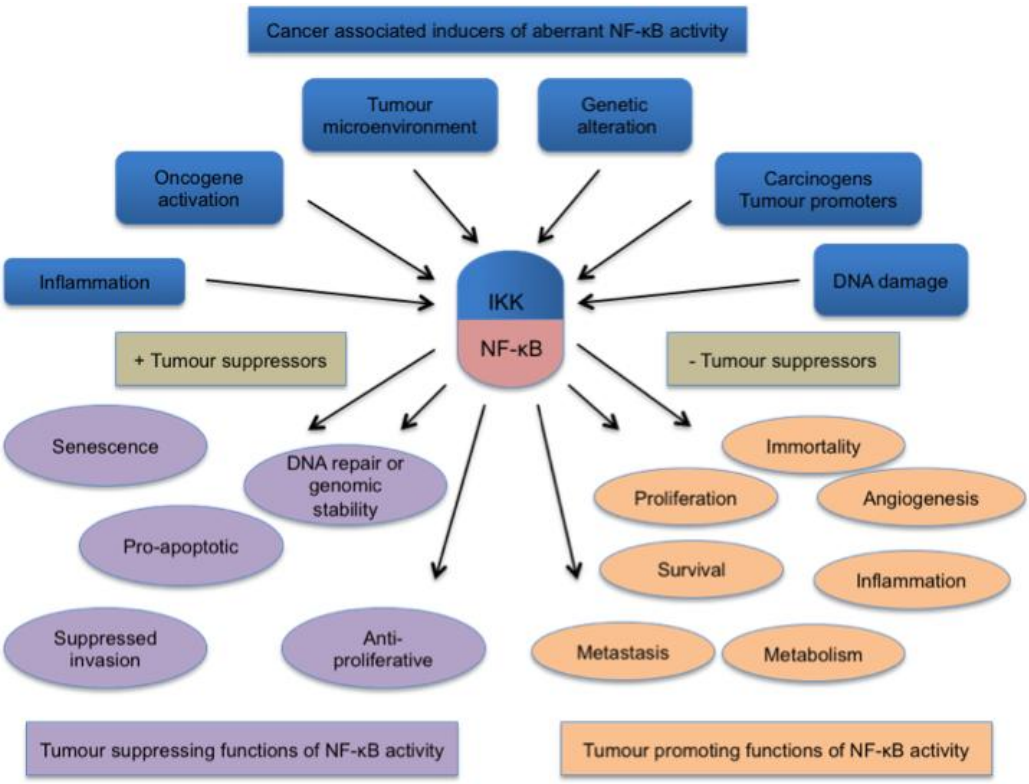


Figure 6. The diverse consequences of nuclear factor kappa B activation. A wide variety of pathways activate nuclear factor kappa B (NF-κB) and the inhibitor of NF-κB kinase (IKK). The NF-κB response is pleiotropic and its activation is context dependent. NF-κB activity is integrated with multiple tumour suppressor pathways. The tumour suppressor status of the cell is a key in determining the resulting action of NF-κB. The loss of tumour suppressors (e.g. TP53 or PTEN) leads to the tumour promoting functions of NF-κB activity, with increased proliferation and survival. While NF-κB activity can be pro-apoptotic, inhibiting proliferation and inducing cell senescence and DNA repair in the presence of tumour suppressors. (Adapted from Perkins 2012).

1.21 Loss of the Long Arm of Chromosome 18 in Colorectal Cancer

Allelic deletion of the long arm (18q) of chromosome 18 has been observed in 10% of early stage adenomas, 50% of late stage adenomas and 70% of CRC (Fearon 2010). This mutation event is the second most common region of chromosome loss in CRC. (Fearon and Vogelstein 1990) There are five TSGs found on chromosome 18, known as SMAD family member 2 (*SMAD2*), SMAD family member 4 (*SMAD4*), *PIGN*, *MEX3C* and *ZNF516* and mutations of these are associated with CRC. SMAD genes encode for intracellular mediators that respond to transforming growth factor B (TGFβ) signalling (Bellam and Pasche 2010). TGFβ is a cytokine that normally inhibits cell growth, induces differentiation and apoptosis in intestinal epithelial cells. Germline mutations of *SMAD4* are associated with the rare inherited syndrome, Juvenile Polyposis (Bevan, Woodford-Richens et al. 1999). While somatic mutations are found in 10-15% of CRCs and *SMAD2* mutations are found in 5% of CRCs (Fearon 2010). *PIGN*, *MEX3C* and *ZNF516* are cancer chromosomal instability suppressor genes preventing DNA damage as a result of DNA replication stress (Burrell, McClelland et al. 2013).

1.22 Molecular Chaperones and Co-Chaperones

Molecular chaperones and co-chaperones are proteins that maintain the normal physiological function of proteins. However, in cancer they may act independently or in combination to aid tumourgenesis through their effect on cell proliferation, apoptotic and cellular senescence pathways.

1.22.1 Heat Shock Proteins

Heat shock proteins (HSPs) are a highly conserved group of molecular chaperones that are induced by heat shock and other chemical and physical stresses. HSPs have the common property of modifying the structures and interactions of other proteins and are classified according to their molecular weight: HSP100; HSP90; HSP70; HSP60 and small HSPs (15-30kDa) including HSP27 (Garrido, Brunet et al. 2006).

Their transcription is regulated by transcription factors belonging to the heat shock factor (HSF) family; heat shock transcription factor-1 (HSF1) ensures transcriptional activation in stress. In tumourgenesis, mouse models have shown HSF1 to be important as *HSF1* knockout in *TP53* knock-in mice showed increased survival and delayed development of tumours (Dai, Whitesell et al. 2007). Similarly, in crossing *TP53* knockout and *HSF1* knockout mice lymphoma development was prevented (Min, Huang et al. 2007), indicating the importance of HSP in tumour development. The cytoprotective properties of HSPs reflect their primary function as molecular chaperones (Khalil, Kabapy et al. 2011; Ciocca, Arrigo et al. 2013). The

physiopathological features of the tumour microenvironment or a response elicited by oncoproteins during carcinogenesis could induce the expression of these HSPs.

The HSPs serve a variety of biological functions including the regulation of synthesis, folding or refolding denatured proteins or mis-folded proteins, the formation of multi-protein complexes and protein transport across cellular membranes (Lanneau, de Thonel et al. 2007), assembly, and degradation of different proteins (Beckmann, Mizzen et al. 1990; Khalil, Kabapy et al. 2011). The oncogenic potential of cells is highly dependent on their ability to survive insult. Elevated expression of HSPs has been reported for nearly all tumour types (Ciocca and Calderwood 2005). HSPs are crucial for the stability and function of many oncogenic proteins required for tumour development including TK receptors, signal transduction proteins, cell cycle regulatory proteins, antiapoptotic proteins.

HSP90 inhibitors are the only HSP inhibitors to have progressed into advanced stages of clinical development. HSP90 are ATPases that exert their chaperone role through a complex cycle regulated by ATP hydrolysis and co-chaperones such as HSP70 (Garcia-Carbonero et al. 2013). Inhibition of HSP90 causes client protein degradation via the ubiquitin-proteasome pathway (Hatakeyama et al. 2004). HSP inhibitors have been shown to selectively induce mutant rather than wild-type protein degradation as well as having the greatest effect in tumours addicted to particular driver oncogene products that are sensitive HSP90 clients. The first HSP90 inhibitor that was entered into clinical trials was Tanespimycin a

Geldanamycin analogue. Geldanamycin from *Streptomyces higroscopicus var geldanus* and Radicicol from the fungus *Monosporium bonorden* are both naturally occurring compounds that were found to inhibit HSP90 (Whitesell et al. 1994; Jhaveri et al. 2012). Although structurally unrelated both bind to the N-terminal nucleotide-binding domain of HSP90 with higher affinity than ATP, therefore preventing ATP binding and hydrolysis and ubiquitin-mediated protesomal degradation of client proteins (Neckers et al. 2012). As a result of poor solubility, stability or toxicity at therapeutic doses, a number of synthetic analogues have been further developed from these compounds. HSP90 inhibition results activation of HSF1 as well as upregulation of co-chaperones HSP70 and HSP27 which maybe counteractive (Garcia-Carbonero et al. 2013). Furthermore, both HSP70 and HSP27 inhibition was demonstrated to significantly increase the cytotoxicity of HSP90 inhibitors (Lee at al. 2012; Powers et al. 2008). Massey and colleagues (2010) found that inhibition of HSP70 induced degradation of HSP90 client proteins. Therefore HSP27 and HSP70 are considered to be critical co-chaperones for HSP90.

HSP27 and HSP70 are the most strongly induced chaperones (Garrido, Brunet et al. 2006). HSP27 is ubiquitously expressed at low levels until induced by stress and acts in an ATP independent manner. Its main function is to prevent protein aggregation (Lanneau, de Thonel et al. 2007). The HSP70 family in contrast are ATP-dependent, consisting of four major members (Brocchieri, Conway de Macario et al. 2008): HSP70 or HSP72 (72kDa) which is present at low levels in the absence of stress, until its

expression is induced by cellular stress; the generally ubiquitously expressed HSC70 (73 kDa); the glucose-regulated protein 78 (GRP78, 78 kDa) located mainly in the endoplasmic reticulum; and HSP75 (75 kDa), or mortalin and mtHSP70, which is located in the mitochondrial (Liu, Daniels et al. 2012). The HSP70 family of chaperones also interact as large multi protein complexes with various other co-chaperones including Bcl-2 associated athogene 1 (BAG1; see Section 1.22.2), Hsp70 interacting protein (Hip) or carboxy terminus of Hsc70 interacting protein (CHIP). HSP70 modulates chaperone function by increasing or decreasing affinity for substrates (McDonough and Patterson 2003; Sharp, Crabb et al. 2004; Garrido, Brunet et al. 2006; Shi, Zhang et al. 2007).

HSP27 has anti-apoptotic properties, (Garrido, Brunet et al. 2006). In cancer, cells depleted of HSP27 leads to spontaneous apoptosis (Nylandsted, Rohde et al. 2000) as this HSP can interfere with all the main apoptotic pathways (Lanneau, de Thonel et al. 2007; Dudeja, Mujumdar et al. 2009). Similarly HSP27 blocks caspase-dependent apoptotic pathways and depletion of this protein leads to apoptosis (Rocchi, Jugpal et al. 2006). HSP72 also has been shown to inhibit apoptotic pathways. Therefore, overexpression of HPS72 can result tumour survival advantage, as HSP72 is able to inhibit both intrinsic and extrinsic apoptosis pathways, by binding directly to the pro-apoptotic such as BAX preventing mitochondrial translocation (Stankiewicz et al. 2005) and to DR4 and DR5 preventing the assembly of the death inducing signalling complex (Guo et al. 2005).

HSP72 plays a major role in the regulation of the senescence program (Yaglom, Gabai et al. 2007). Cellular senescence is a complex programme with multiple end points that limits the number of division that a normal cell can undergo. Replicative senescence in normal cells results from telomere shortening and the accumulation of cell cycle inhibitors triggered following DNA damage. In cancer cells, unlike normal cells, the senescence program is also activated by certain active oncogenes (e.g. RAS or RAF), which trigger senescence via TP53-dependent and TP53-independent pathways (Braig and Schmitt 2006; Sherman, Gabai et al. 2007) (Figure 7). Senescence represents, therefore an important process for tumourgenesis as cancer cells proliferate without a limit and must acquire mechanisms to escape replicative senescence (Saretzki 2010). In cancer cells there is both inactivation of cell cycle inhibitors and reactivation of telomerase activity (Kim, Piatyszek et al. 1994). However, the senescence programme continues to remain functional in tumours, as activation of senescence is the mechanism of action of many anticancer drugs and therapies (Sherman 2010). HSP72 suppresses the senescence signalling pathways regulated by activated oncogenes allowing cancer cells to escape senescence and proliferate. HSP72 selectively suppress in breast and CRC cells (MCF7 and HCT116) and there are TP53-dependent and TP53-independent mechanisms of triggering senescence (Gabai, Yaglom et al. 2009). Likewise, HSP27 regulates cellular senescence by modulating the TP53-pathway (O'Callaghan-Sunol, Gabai et al. 2007).

Therefore, cancer cells may need HSP expression to suppress the senescence programme in order to survive and to proliferate. In accord with this both HSP72 and HSP27 are highly expressed in breast, endometrial, lung, gastric, liver, prostate cancer and their expression has been correlated with increased cell proliferation, metastases, poor response to chemotherapy and poor survival (Sherman and Multhoff 2007). In CRC cells high expression of HSP72 has been associated with poor survival, metastasis and resistance to chemotherapy (Grivicich, Regner et al.), and has been correlated with poor outcome when used as a prognostic marker in CRC patients (Kocsis, Madaras et al. 2010).

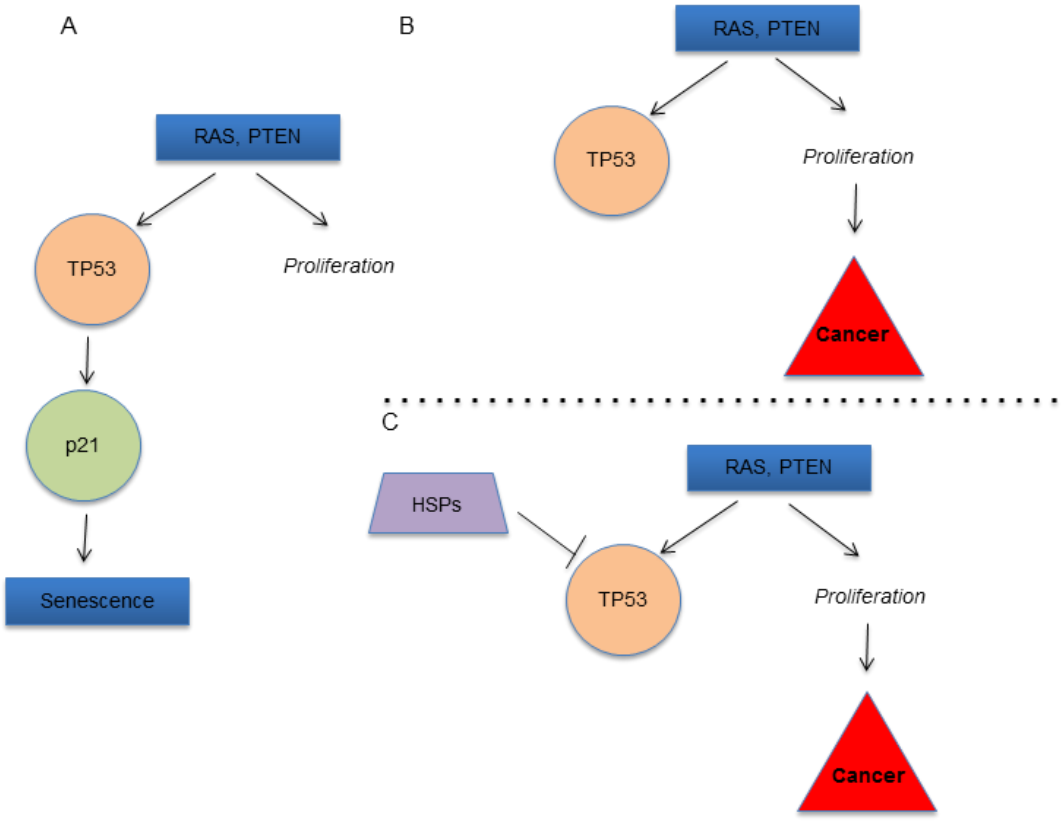


Figure 7. Heat shock proteins can inhibit TP53-dependent senescence. A. Activation of oncogenes leads to activation of TP53-dependent senescence following the induction of p21 causing inhibition of proliferation and senescence. B. Proliferating cells can acquire a mutation in the TP53 inhibitory pathway resulting in uncontrolled proliferation as in cancer. C. Oncogene activation of TP53-dependent senescence can be inhibited by Heat shock proteins (HSPs) allowing the cells to proliferate. (Adapted from Sherman et al, 2007).

1.22.2 BCL-2 Associated Athogene 1

BAG1 was discovered in a search for Bcl-2 interacting proteins (Takayama, Sato et al. 1995) and was the first BAG gene from an evolutionary conserved family of genes to be found. There are six BAG family genes in humans all characterised by a 70 amino acid commonly conserved region near the C-terminus called the BAG domain. This contains 110-124 amino acids forming three anti parallel helices of 30-40 amino acids, the second and third of which interact with HSP70 (see Section 1.22.1) (Briknarova, Takayama et al. 2001). BAG1, BAG2, BAG3, BAG4, and BAG6 all share a single BAG domain with the exception of BAG5 that has four such domains, but which differ in their N-terminus regions (Sharp, Crabb et al. 2004). They modulate apoptosis, tumorigenesis, neuronal differentiation, stress response and the cell cycle (Kabbage and Dickman 2008).

The BAG1 gene is located on chromosome 9p12 and comprises of seven exons. There are four functionally distinct BAG1 isoforms with differing actions all expressed by alternative translation initiation from a single mRNA. The most abundant is BAG1S (36 kDa), which is found in the cytoplasm, then nuclear BAG1L (50 kDa) and BAG1M (46 kDa) which is localised in the cytoplasm but translocates into the nucleus, and the finally the minor isoforms BAG1 (29 kDa).

BAG1 acts as a co-chaperone due to its direct binding of the BAG domain to the ATPase domain of Hsc70/HSP72 which allows further peptide binding domain interactions with other proteins substrates thereby acting as a

scaffold molecule. Many of the functions of BAG1 are as a result of its ability to bind and modulate the activity of Hsc70/HSP70.

HSPs direct refolding of denatured proteins (Takayama, Bimston et al. 1997). BAG1 isoforms have been shown to regulate HSP70 both negatively and positively reflecting some of its anti-apoptotic properties (Sharp, Crabb et al. 2004). BAG1 also contains an ubiqutin like domain that maybe vital for some of its actions. Ubiqutin is a small protein that normally binds to target proteins for ATP-dependent degradation by a proteolytic complex. As BAG1 is very stable this may be required to link proteosome activity with the chaperone molecules (Tang 2002; Townsend, Stephanou et al. 2005). BAG1 also interacts with RAF1 a serine/threonine protein kinase important in cell growth signalling in competition with HSP70. Nuclear hormone receptors (NHR) are involved in cell survival and are themselves controlled by HSPs and, therefore, BAG1 may offer a common mechanism of modulation. BAG1 also interacts with cell cycle control (anti-apoptotic Bcl-1 protein), translation regulators (GADD34), cell-surface receptors (PDGF and HGF) and growth factors (Sharp, Crabb et al. 2004). These binding interactions reflect BAG1 multiple functions effecting cell growth, apoptosis, proliferation, transcription and metastasis (Figure 8).

The ability of BAG1 to suppress apoptosis through its wide variety of interactions reflects the importance of BAG1 in cancer. BAG1 is minimally expressed in normal tissues but commonly expressed in tumours (Cutress, Townsend et al. 2002). Elevated levels of BAG1 are observed in pre-invasive DCIS precluding to breast cancer and therefore BAG1 may be

important at an early stage of tumourgenesis (Sharp, Crabb et al. 2004). BAG1 expression has been positively associated with tumour cell proliferation, infiltration and metastasis (Bai, Yi et al. 2007). There is increased BAG1 expression seen in breast (Brimmell, Burns et al. 1999; Millar, Anderson et al. 2009), lung (Rorke, Murphy et al. 2001), laryngeal (Yamauchi, Adachi et al. 2001), thyroid (Ito, Yoshida et al. 2003), endometrial (Moriyama, Littell et al. 2004) and oral squamous carcinoma (Shindoh, Adachi et al. 2000; Wood, Lee et al. 2009) with expression possibly correlating with clinicopathological outcome. BAG1 has also been shown to be a putative target of amyloid precursor protein (APP) binding to its intracellular C-terminal, with BAG1M overexpression leading to the accumulation of APP (see section 1.21.3) (Elliott, Laufer et al. 2009). In CRC nuclear BAG1 expression is a predictive factor for distant metastasis and poor prognosis (Kikuchi, Noguchi et al. 2002). Similarly, advanced Duke Stage, poor tumour differentiation and lymph node metastasis, distant metastasis, and malignant level, and poor prognosis is associated with increased total BAG1 expression. (Bai, Yi et al. 2007; Sun, Meng et al. 2010).

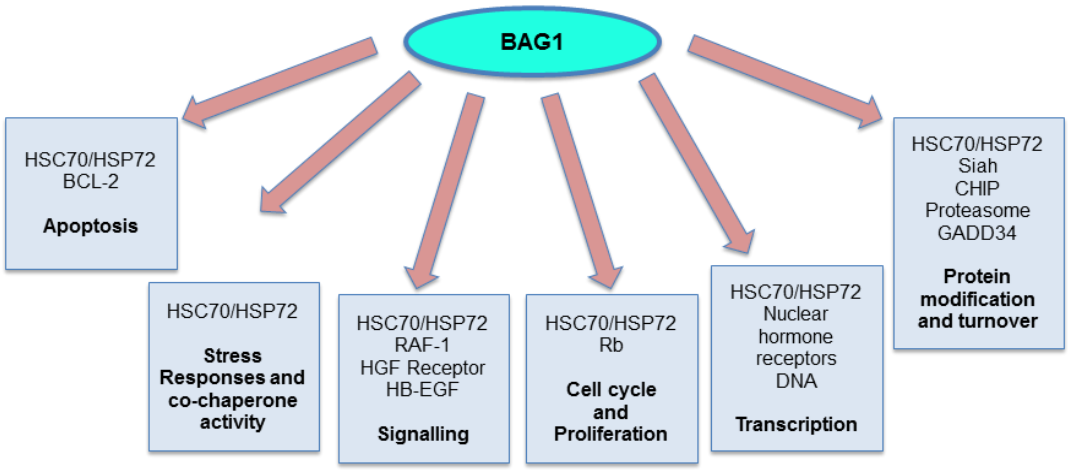


Figure 8. BAG1 is involved in cell signalling, apoptosis, stress responses/protein degradation, proliferation and transcription. BAG1 has a wide variety of cellular functions that are mediated through its direct interactions as well as being mediated through its interaction with the molecular chaperone HSP72. (Adapted Townsend et al, 2007).

1.23.3 Amyloid Precursor Protein

APP is a large type I membrane protein that is ubiquitously expressed throughout the body (Mattson 1997). The APP gene is located on the long arm of chromosome 21 and contains at least 18 exons (Yoshikai, Sasaki et al. 1990). Several isoforms of APP are generated by alternative splicing ranging from 363-770 amino acids, the major of which are 695, 751 and 770 amino acids long. The most common isoform APP695 is expressed in the central nervous system (Sisodia, Koo et al. 1993), while APP751 and APP770 isoforms are expressed in non-neuronal tissues, although the functional significance of this is not understood (Zheng and Koo 2006).

APP is synthesised on membrane-bound ribosomes and is post-translationally modified by N- and O-glycosylation, sulfation and phosphorylation. Immature APP is localised in the cis-Golgi and endoplasmic reticulum (ER), whilst mature APP localises in the trans-Golgi network and at the plasma membrane. Full length APP undergoes a process of ‘ectodomain shedding’ being sequentially cleaved by three proteinases. Firstly by α-secretase or β-secretase, resulting in secreted APP (sAPP) or sAPPα and sAPPβ, along with carboxy terminal fragments CTF83 or CTF99/CTF8. Following this γ-secretase cleavage of CTF83 produces p3, and of CTF99/CTF89, which results in amyloid β-peptide (βAPP) as well leaving the amino-terminal APP intracellular domains (AICDs) (Chow, Mattson et al. 2010) (Figure 9).

The various APP fragments have a number of functions and effects, although much of this remains to be elucidated particularly in non-neuronal cells. βAPP is the most well known isoform as excessive deposition is a pathological characteristic, along with the accumulation of neurofibrillary tangles and loss of neurons, observed in Alzheimer's disease (AD) (Chow, Mattson et al. 2010). sAPPα has been shown to protect neurons from oxygen-glucose deprivation. While sAPPβ lacks the neuroprotective effect it is critical in the development of central and peripheral neurons (Furukawa, Barger et al. 1996; Nikolaev, McLaughlin et al. 2009). AICD's may contribute to early pathophysiological mechanisms in AD. (Muller, Meyer et al. 2008) There is no established biological function for p3 (Chow, Mattson et al. 2010).

The role of APP remains poorly understood. APP and have been shown to be involved in cell growth, migration, neurite outgrowth and cell adhesion (Zheng and Koo 2006; Thinakaran and Koo 2008; Zheng and Koo 2011). APP and sAPP have been associated with the proliferation of a variety of cells and linked to the malignant progression of colorectal, pancreatic and melanoma cells lines (Meng, Kataoka et al. 2001; Hansel, Rahman et al. 2003; Botelho, Wang et al. 2010; Venkataramani, Rossner et al. 2010). The constitutive shedding of APP by α-secretase, a metalloprotease of the A Disintegrin And Metalloprotease (ADAM) family in particular ADAM10 (Kuhn, Wang et al. 2010), has been shown to be overexpressed in numerous cancers (Saftig and Reiss 2010) including CRC (Gavert, Conacci-Sorrell et

al. 2005). APP and ADAM10 have been suggested as novel targets for inhibition in the treatment of cancer (Duffy, McKiernan et al. 2009).

It has been suggested that AD is associated with a reduced risk of cancer and that cancer was associated with a reduced risk of AD (Roe, Fitzpatrick et al. 2010). Increased levels of APP have been associated with reduced survival and poor prognosis in prostate and oral squamous cell carcinoma (Ko, Lin et al. 2004; Takayama, Tsutsumi et al. 2009). APP has been shown to be selectively over expressed in colon and pancreatic carcinoma but not in normal tissues (Venkataramani, Rossner et al. 2010). Therefore, APP may have a vital role in tumour cells being involved in cell growth, differentiation and tumourgenesis, which require exploration.

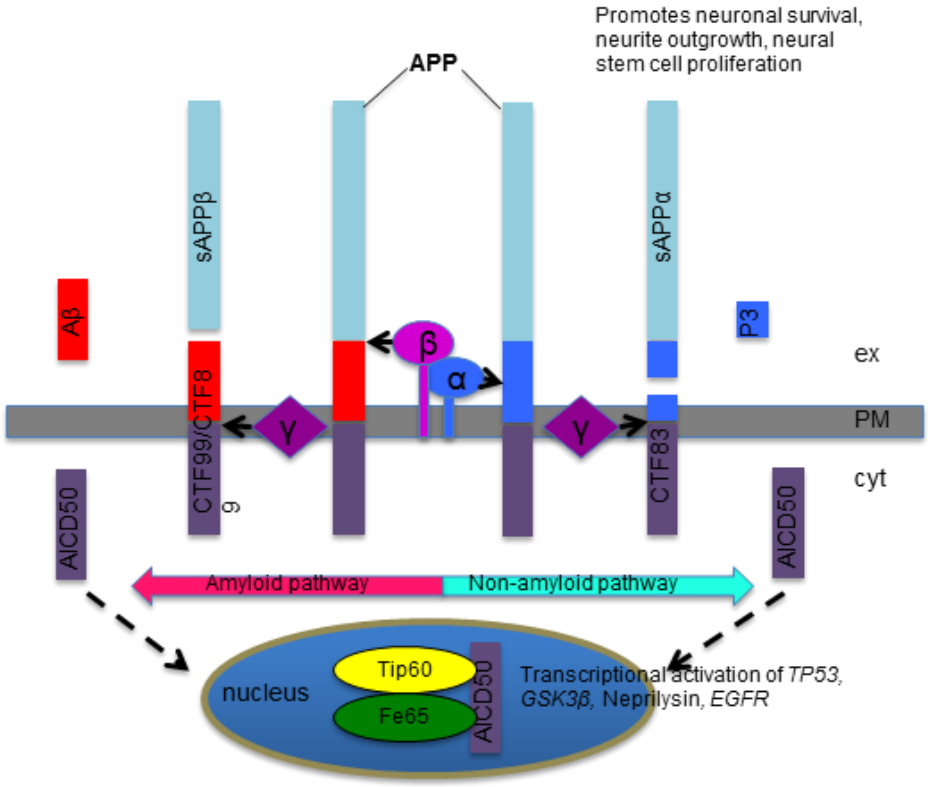


Figure 9. Amyloid precursor protein processing and cleavage products. Amyloid precursor protein is either initially cleaved by α- or β-secretase, the latter results in the Amyloid precursor protein (APP) processing pathway (left). Cleavage by α-secretase results in the generation of sAPPα (right). Following this cleavage by γ-secretase forms C-terminal fragments (CTF 88, p3 and AICD50). APP cleavage by β- and γ-secretase results in sAPPβ and then C-terminal fragments (CTF00 and CTF89) and β-amyloid (Adapted Chow et al, 2010).

1.24 Aims

Investigations into the mutational pathways and associated changes in gene expression may lead to identification of novel targets for therapeutic interventions in CRC. These mechanisms may vary between CRC phenotypes referring distinct clinicopathological features, which may affect their response to treatment.

In CRC there is elevated expression of HSPs. It has been proposed that CRC requires the expression of HSPs and BAG1, its co-chaperone, to survive. HSPs have been shown *in vitro* to suppress oncogenic activated cellular senescence, which maybe effected by different CRC mutational phenotype. Furthermore, BAG1 has been shown to be a possible regulator of APP. Although APP is more commonly associated with the pathogenesis of Alzheimer Disease, it has been suggested that APP is an important protein involved in both cellular proliferation and tumourgenesis, although this remains to be elucidated fully. Finally, BCL3 is an important IKB involved in the NF-κB pathway which has been shown to be important in CRC tumourgenesis.

This study aimed to investigate the role of HSPs, APP, BCL3 and their co-chaperones in CRC, in particular. Each chapter presents the relevant hypotheses to be tested and the specific aims at the start of the chapter. The relevant overarching aims are given below:

To evaluate the role of HSP27 and HSP72, and their involvement in OIS depending on the genetic background of the tumour and the relationship with clinicopathological features (Chapter 3).

To elucidate the role of APP in tumourgenesis and investigate a possible link to malignant progression and migration in CRC. Furthermore, to investigate any relationship with APP expression and clinicopathological features (Chapter 4).

To investigate the relationship between NF-κB IKK BCL3 and the PI3K/AKT pathway as well as BAG1 in CRC with clinicopathological features (Chapter 5).

CHAPTER 2

Materials and methods

2.1 Patients and Sample Collection

The collection and use of tissue was approved by the local Research Ethics Committee (East London and City Research Ethics committee 1) in September 2006. The work contained within this thesis is covered by the following ethics committee reference: REC06/Q0603/65. Informed consent was obtained from patients before their operation. Tissue was collected from consecutive patients undergoing operations for CRC at the Royal London Hospital. The CRC specimens formed part of a historical cohort of clinically well-characterised tumour specimens, which have been previously investigated in other studies within the group (Appendix I; Silver, A., N. Sengupta, et al. 2012). A total sample size of 66 was calculated to give 80% power at p=0.05 to detect a difference in a given event occurring in 35% of positive against 65% negative cancers.

Specimens were collected within 20 minutes of surgical resection. Tumour material was dissected, avoiding any grossly necrotic area, and normal mucosal tissue was taken at least 10 cm proximal to the cancer. The tissue was immediately snap-frozen in liquid nitrogen and stored at -80^{o}C. The corresponding pathology block sections of formalin-fixed, paraffin wax-embedded colorectal tumour samples were identified and retrieved from the Archive of Pathology at the Royal London Hospital.

2.2 Molecular Methods

2.2.1 DNA Extraction from Tissue

Genomic DNA was prepared from tumour and mucosal specimens using the QIAamp® DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Snap-frozen tissue (25 mg) was cut into small pieces and left in 180μl of genotyping buffer (ATL) overnight at 56^{o}C for protein degradation. If there was inadequate digestion of the sample following this incubation a further 20μl of Proteinase K (Sigma, UK) was added to the sample and incubation continued for a further 2 hours. AL buffer (200μl) was added to lyse the cells and mixed by vortexing before incubating for 10 minutes at 70^{o}C to yield a homogenous solution. 100% ethanol (200μl) was then added to each sample and after mixing by vortexing, samples were added to the QIAmp® Mini spin column placed in a 2ml collection microcentrifuge tube. The column, sample and tube were then centrifuged at 8000rpm for 1 minute. The filtrate was discarded and AW1 wash buffer (500μl) added to the spin column, which was placed in a new collection tube before centrifuging at 8000rpm for 1 minute. The filtrate was discarded again and AW2 wash buffer (500μl) added to the spin column and placed in a new collection tube before centrifuging at 13,000rpm for 3 minutes. This was then centrifuged again at 13,000rpm for 1 minute to ensure all traces of buffer are removed. The spin column was then placed into a new eppendorf tube and 100μl of buffer AE added to the column and incubated at room temperature for 1 minute before centrifuging at 8000 for 1

minute. The elute containing the purified DNA was collected in the eppendorf, which was then stored at -20^{o}C for future use.

2.2.2 Total RNA Extraction from Human Adenocarcinoma Cells

Genomic RNA was prepared from cell lines once at 70-80% confluence using the QiagenRNeasy Mini kit (Qiagen, Crawley, UK), according to the manufacturer's protocol. The culture media was drawn off the cells and cells washed in PBS twice. Then TRIzol (1ml) was added for homogenisation and cells returned to the cell incubator for 5-10 minutes (see Section 2.4). The homogenate was then drawn off and pipetted into a 2ml round-bottom microcentrifuge tube. Chloroform (200µl) was then added and the sample mixed vigorously for 15 seconds, before incubating at RT for 5 minutes. The sample was then centrifuged at 12,000 g for 15 minutes at 4^{o}C. The aqueous upper phase of sample was then transferred into a 2ml microcentrifuge tube and Isopropanol (500µl) was added and then mixed. The mixture was incubated for 10 minutes at RT before centrifuging at 12,000g for 15 minutes at 4^{o}C. The supernatant was removed leaving the RNA pellet and washed with 75% ethanol (1ml). This was then centrifuged at 7,500g for 5 minutes at 4^{o}C and the RNA pellet air-dried.

The RNA pellet was re-suspended in RNase-free water (100µl) and RLT buffer (350µl) and 100% ethanol (250µl) added, mixed well, and then pipetted into an RNeasy mini spin column placed in a 2ml collection tube. This was centrifuged at 8,000g for 15 seconds. The RNeasy spin column

was removed and placed into a new collection tube. RW1 buffer (350μl) was then added and this was centrifuged at 8,000g for 15 seconds. The flow through in the collection tube was discarded and 80μl of DNase I (10μl) plus RDD buffer (70μl) added and incubated for 15 minutes at RT to digest any remaining DNA. Following incubation RW1 buffer (350μl) was added to the RNeasy spin column and centrifuged at 8,000g for 15 seconds, discarding the flow through. RPE buffer (500μL) was then added to the spin column and centrifuged at 8,000g for 15 seconds and the flow through discarded. RPE buffer (500μl) was then added again to the spin column and centrifuged at 8,000g for 2 minutes, discarding the collection tube and flow through. The RNeasy spin column was then placed in a new collection tube and centrifuged at 12,000g for 1 minute to ensure all traces of buffer are removed and once again the collection tube and flow through were discarded. The RNeasy column was then placed in a new collection tube and RNase-free water (50μl) added to the spin column and centrifuged for 1 minute at 8,000g. The purified RNA present in the elute was captured in the collection tube. This was then nano dropped to establish concentration and the sample then stored at -80 for future use.

2.2.2.1 RNA Product Examination

3μl of 2x loading dye (ABgene, Surrey) was combined with 1μl aliquot of each sample of extracted RNA and compared against 5μl of DNA ladder (ABgene) on a 1% w/v agarose gel with ethidium bromide (0.5ug/ml) run for 35 minutes in 1 x TBE buffer (89mM Tris base, 89 mM boric acid and 2mM

EDTA) at 140 volts. Gels were examined under UV light in a GelDoc imaging system (BIO-RAD, Hertfordshire).

2.2.2.2 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesised using total RNA and used for real time PCR (see Section 2.2.5) using the High Capacity RNA-to- cDNA kit (Applied Biosystems, Carlsbad, California) according to the manufactured protocol. Each RNA to cDNA reaction consisted of: 2ng of template total RNA made up in 9µL RNase-free water, 10µL of 20X RT buffer, and 1µL 20X RT enzyme mix into a single well of a 96 well plate (Applied Biosystems, Carlsbad, California). Then the samples were incubated at 37^0C for 60 minutes and 95^0C for 5 minutes in a MJ Research Tetrad Thermal Cycler. This was then nano dropped to establish concentration and stored at -20 for future use.

2.2.3 Quantification of DNA and RNA

DNA and RNA were quantified using NanoDrop® ND-1000 (Fisher Scientific, Loughborough, UK). 1µl of stock DNA or RNA was loaded onto the optical pedestal. For DNA the double stranded DNA quantification was selected and for RNA, the single-stranded DNA option was selected.

2.2.4 DNA amplification by polymerase chain reaction

2.2.4.1 Oligonucleotide primer design

Oligonucleotide primers for polymerase chain reaction (PCR) were designed by using the human sequences available on Biotools with the package Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Rozen and Skaletsky 2000). Modifications made to the standard parameters in the package include a minimum 40% and maximum 64% primer GC content. All primers were obtained from Sigma-Aldrich (St. Louis, MO, USA). These were resuspended in distilled deionised water (dH_20) to a concentration of 100mM stock solution and diluted further to 10mM for the working stock solution.

A full list of all oligonucleotides primers used along with specific PCR conditions are provided in Appendix I.

PCR was performed on genomic tumour DNA in order to characterise activating mutations in the oncogenes TP53, KRAS and PIK3CA, concentrating on the known mutational hotspots in these genes. Mutation hot spots analysed were: TP53 exons 5, 6, 7 and 8 (Vidaurreta, Maestro et al. 2008); KRAS codon 12 and 13 (Andreyev, Norman et al. 2001); PIK3CA exons 9 and 20 (Samuels and Velculescu 2004)

2.2.4.2 PCR Protocol

Each PCR mix consisted of: 1μl template DNA and 24μl master mix pipette into a single well on a 96-well plate (Applied Biosystems, Carlsbad, California). The master mix consisted of 0.2μl dNTP's (0.05uL dATP, dCTP, dGTP, dTTP – 100mM each, ABgene, Surrey), 1μl of both forward and reverse primers (10mM), 19.175 μl dH_20, 0.125μl HotStarTaq DNA polymerase (Qiagen, UK), 2.5μl 10 x buffer (Qiagen, UK) A negative control was included in each reaction, containing 24μl master mix and 1μl dH_2O. PCR reactions were carried out on a MJ Research Tetrad Thermal Cycler with the heated lid option selected to prevent evaporation of reaction products.

Typically, the PCR conditions for all the PCRs using a heated lid, were:

1 x cycle	- denaturation/hotstart for 15 minutes at 95°C
35 x cycles	- denaturation for 30 seconds at 94°C
	- annealing for 45 seconds at 55-60°C
	- extension for 60 seconds at 72°C
1 x cycle	- final extension for 5 minutes at 72°C

2.2.4.3 PCR Product Examination

The PCR products were examined using 2-3% agarose gel electrophoresis to ensure that the anticipated DNA product was generated from the PCR. A 2μl aliquot of each PCR product was combined to 3μl of 2x loading dye

(ABgene, Surrey) and compared against 5μl of 1KB DNA ladder (ABgene, Surrey) on a 4% w/v agarose gel with ethidium bromide (0.5ug/ml) run for 35 minutes in 1 x TBE buffer (89mM Tris base, 89 mM boric acid and 2mM EDTA) at 140 volts. Gels were examined under UV light in a GelDoc imaging system (BIO-RAD, Hertfordshire).

2.2.4.4 PCR Product Purification

PCR products were then purified to remove residual reaction components such as Taq enzyme, nucleotides and primers. ExoSAP-IT® (USB Corporation, Cleveland Ohio) treats PCR products with no sample loss by removing primers and nucleotides. 5μl of post PCR reaction product was mixed with 2μl of ExoSAP-IT® for a combined 7μl reaction volume. This was incubated at 37^{o}C for 15 minutes to degrade remaining primers and nucleotides and then incubated at 80^{o}C for 15 minutes to inactivate ExoSAP-IT®, resulting in purified PCR product with which 15μl of dH_2O was added.

2.2.4.5 Sequencing Reaction

Each sequencing reaction consisted of: 0.5μl primer (10mM), 3.5μl purified PCR product, 4μl of 2.5x sequencing buffer, 1μl dH_20 water and 1μl BigDye® Terminator V3.1 (ABI PRISM, PE Applied Biosystems). Sequencing reactions were run in an MJ Research Tetrad Thermal Cycler,

with the following parameters: 95^{0}C denaturation for 10 seconds, 57^{0}C annealing for 5 seconds and a 60^{0}C extension for 3 minutes, for 25 cycles.

Sequencing products were then purified with the DyeEx 96 well kit (Qiagen, UK) using a prehydrated gel-filtration resin that binds to Dye bound ddNTPs, but not to the sequencing reaction products. The DyeEx plate is placed upon a collection plate and centrifuged for 3 minutes at 2,000rpm. After discarding the flow-through, the plate is placed on a 96 well plate. 10μl of the sequencing product is applied to each well, and then this is centrifuged for 3 minutes at 2,000 rpm. The resulting elute contained the purified sequencing reaction.

2.2.4.6 Sequencing Product Analysis

The ABO-Prism 3100 Genetic Analyser is calibrated using highly deionised (Hi Di) Formamide (Applied Biosystems, Carlsbad, California). Following DyeEx clean up, 1μl of sequencing product was added to 9μl of Hi Di Formamide, which was then denatured by heating at 95^{o}C for 5 minutes in a MJ Research Tetrad Thermal Cycler. This was then placed immediately in an ABO-Prism 3100 Genetic Analyser.

All templates were sequenced in their entirety on both forward and reverse strands and were edited and aligned using Sequencher 4.9 available on genocides (http://www.genecodes.com).

2.2.5 Real Time-PCR

Real time PCR was carried out using the specific gene expression assay probes (Applied Biosystems, UK) for each gene of interest (Appendix II). Each RT-PCR reaction consisted of: 2μl cDNA (30ng/l) (see Section 2.2.3), 10μl TaqMan Gene expression master mix, 1μl of the specific gene expression assay (Applied Biosystems, Carlsbad, California) and 7μl RNase-free water. Four replicates of each sample were used. GAPDH was used as the endogenous control.

RT-PCR reactions were carried out on cDNA from our chosen cell lines (see Section 2.4).

2.2.6 Quantitative Real Time PCR

PIK3CA copy number was investigated using the TaqMan® Copy number assay system (Applied Biosystems, Carlsbad, California). This is a duplex real-time polymerase chain reaction with both the target gene and reference gene probes in the same reaction. The copy number assay detects the target gene, and the reference assay detects a reference gene RNase P (Applied Biosystems, Carlsbad, California) that has two copies in a diploid genome. Therefore, the number of copies of the target gene in each sample is compared by relative quantification with the reference gene.

Each copy number quantification reaction was carried out on a 96 well plate, which consisted of: 4μl of DNA (5ng/μl) and 16μl Master mix. Master mix

consisted of 10μl 2x TaqMan genotyping master mix, 1μl TaqMan Copy number reference assay, 1μl PIK3CA TaqMan copy number assay and 2μl dH_20. Each sample was repeated 4 times and a negative control was included in each reaction, containing 16μl Master mix and 4μl dH_20 (Appendix II).

Copy number expression reaction was carried out on Applied Biosystems7500 Real-time PCR system (Applied Biosystems, Carlsbad, California). Post-PCR data analysis of copy number quantification was carried out using the Applied Biosystems CopyCaller™ software (Applied Biosystems, Carlsbad, California).

2.3 Histochemistry

2.3.1 Immunohistochemistry

Indirect immunohistochemical staining was performed using a kit-based (Vectastain Universal Elite ABC: Vector Laboratories, Burlingame, CA, USA) avidin-biotin-peroxidase complex method with commercially available antibodies (ABCAM, USA) for each protein of interest.

The conditions used for each antibody are detailed in Appendix III.

2.3.1.1 Clinical Material and Control Tissue

Block sections of formalin-fixed, paraffin wax-embedded (FFPE) colorectal samples were cut into 3 μm sections before being collected on coated slides (VWR International, Leicestershire, UK) for immnunostaining.

2.3.1.2 Antigen Retrieval and Unmasking

Tissue sections were de-waxed in two changes of xylene for 2 minutes and dehydrated in two changes of industrial methylated spirit (IMS) for 2 minutes. After washing in running tap water for 5 minutes thermal antigen retrieval was carried out by microwaving or waterbathing sections in unmasking buffer. Antigen unmasking solutions and antigen retrival optimisation are described fully in Appendix III.

2.3.1.3 Immunostaining

Sections were allowed to cool for 5 minutes and washed again in running tap water for 5 minutes. Endogenous peroxidase activity was blocked by placing sections in endogenous peroxidise blocking solution (Hydrogen peroxide 6ml to 194ml dH_2O) for 15 minutes. After further washing in running tap water for 5 minutes and soaking in wash buffer (WB; Dako, Cambridge, UK) for 5 minutes, sections were then incubated in normal blocking serum (normal horse serum) for 20 minutes at room temperature (RT). Excess blocking serum was tipped off and sections were incubated with the primary antibodies raised in its appropriate antibody diluent for 60 minutes at RT.

Sections incubated with antibody diluent only served as negative controls. Primary antibodies were washed off with WB and sections incubated with corresponding secondary anti-antibody diluted in WB, for 30 minutes at RT.

Secondary antibody was washed off with WB and sections incubated in avidin complex solution (in kit) for 30 minutes at RT, washed again with WB and incubated in diaminobenzidine solution (Bio-Genex-Laboratories, San Ramon, CA, USA) for 5 minutes. After further washing in running tap water for 5 minutes, sections were counter-stained in Gill's haematoxylin for 2 minutes, washed in running tap water for 5 minutes, dehydrated twice in IMS for 2 minutes, cleared twice in xylene for 2 min and mounted in Canada balsam (VWR International, Leicestershire, UK).

2.3.1.4 Light Microscopy and Qualitative Analysis

Sections were analysed at magnifications of x10, x20 and x40 using a light microscope (LeitzDialux 20, Leica Microsystems UK Ltd., Milton Keynes, UK). The presence and distribution of immunostaining was noted (nuclear vs cytoplasmic) and scored by two independent observers.

2.3.2 Immunofluroscence

2.3.2.1 Cell Preparation

Cover slips pre-treated with Poly-lysine (Sigma, UK) were placed in a 24 well plate to which 500μl of media containing 20-30,000 cells were seeded. This was then incubated overnight (see Section 2.4).

2.3.2.2 Fixation

The following day the media was removed and the cells rinsed with PBS (0.01M Phosphate buffered saline). The cells were then fixed in 4% Paraformaldehyde (Sigma, UK) adding 300μl per well and leaving for 20 minutes at RT. This was then taken off and washed off with PBS, three times for 5 minutes whilst being agitated on a plate shaker.

2.3.2.3 Staining

Blocking buffer was then added (5% FBS, PBS and 0.1%Triton X-100) and incubated for 60 minutes at RT. This was removed and 100μl of primary antibody, APP (1:500) (Millipore, Billerica, MA, USA) diluted in blocking buffer was added. This was left overnight at 4^{o}C on the plate shaker.

Primary antibody was washed off with PBS, three times for 5 minutes on the plate shaker at RT. The secondary antibody, 100μl anti-mouse 488 (1:1000) was then added and incubated for 60 minutes with the plate wrapped in foil

on the plate shaker for 60 minutes at RT. The secondary antibody was then washed off with PBS, three times for 5 minutes and finally left in ddH_2O prior to mounting of the cover slips. The cover slips were mounted onto slides using 90% Glycerol with the DNA counter stain 4',6-diamidino-2-phenylindole (DAPI; ABCAM, USA).

2.3.2.4 Light microscopy

Slides were analysed at magnifications of x10, x20 and x40 using a light microscope (Leica DM5000B, Leica Microsystems UK Ltd, Milton Keynes, UK) (Table 1). The presence and distribution of immunofluroscent staining was noted (nuclear versus cytoplasmic).

Table 1. Configuration of epifluroscence microscope.

Flurophore	**Excitation λ (nm)**	**Emission λ (nm)**	**Filter (nm)**
FITC	488	520	Band Pass 490nM +/- 20nM
DAPI	350	470	Band Pass 400nM +/- 20nM

2.3.2.5 Confocal Microscopy and Nuclear Colocalisation

Images were generated with a Zeiss LSM510 confocal laser-scanning microscope, using an oil immersion 40x/1.30 Numerical Aperture (N.A.) plan neofluar objective and the accompanying Zeiss software. Table 2. describes the settings of the confocal for each flourophore used.

Table 2. Configuration of confocal microscope filters.

Flurophore	Laser used	Excitation λ (nm)	Emission λ (nm)	Filter (nm)
FITC	Argon	488	520	Band Pass 505-550
TRITC/ Rhodamine	Helium/Neon	543	570/590	Long Pass 560
Cy5	Helium/Neon	650	670	Long Pass 680

Image files were collected as a matrix of 1024x1024 pixels that described the average of 8 frames scanned at 0.062Hz; these files were then exported into 16 bit.tif files, which were used for image analysis.

Images were analysed using MetaMorph® (Molecular Devices, Downington, PA, USA) using the multi wavelength cell scoring application, a flexible segmentation tool for multiple stain wavelengths, which can be used in the analysis of nuclear stain and cytoplasmic staining of a particular protein of interest. Each image was converted into a 16 bit monochrome tiff. file from the stack images for each field. For each image a monochrome image was made for the nuclear staining using DAPI and for the stained protein of interest (e.g. APP antibody) staining both nucleus and cytoplasm. An appropriate minimum and maximum width field was set by calibrating with one of the narrowest nuclei and one of the widest nuclei for nuclear staining and likewise for cytoplasmic and nuclei staining with one of the narrowest and one of the widest cells. Using the DAPI stain to identify the nucleus, the

results are overlaid to determine the cytoplasmic staining and nuclear staining of the protein stained (Figure 10).

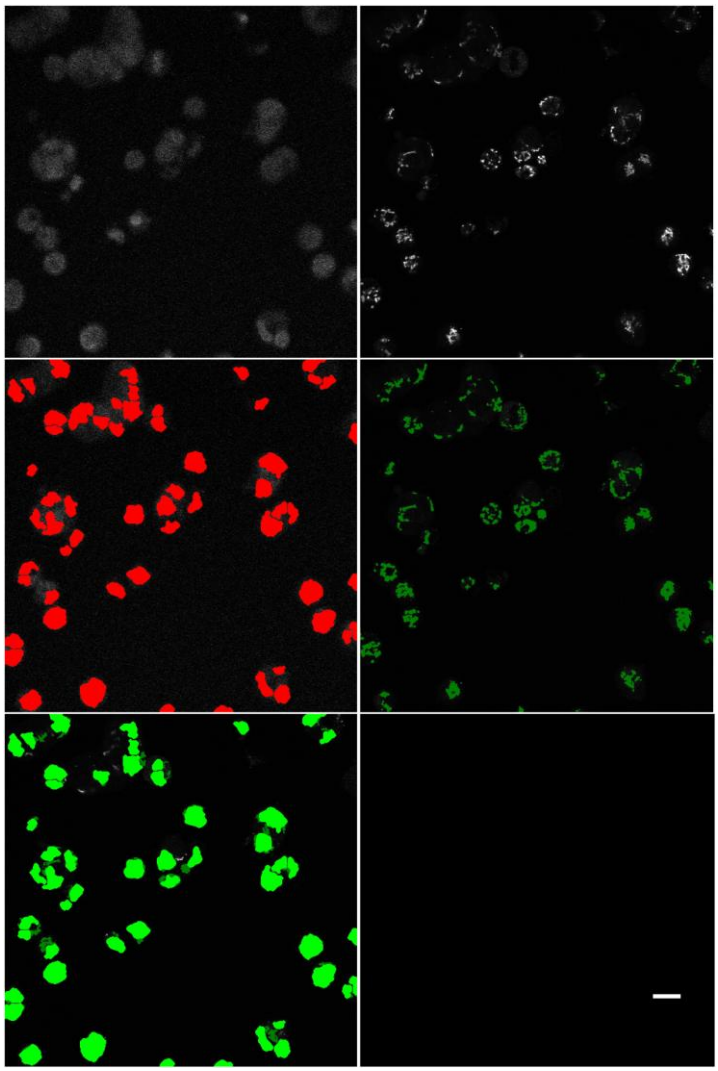


Figure 10. Representation images from MetaMorph® showing a monochrome image of DAPI and monochrome image of Amyloid precursor protein. These are images overlaid and counted; DAPI is used to identify all the nuclei in the cells and another overlay image counting all the Amyloid precursor protein (APP) cell staining. These are then overlapped on each other. The localisation of APP can be measured by counting the staining overlapped on the DAPI nuclear stain and subtracting this from the total staining to provide a measurement of cytoplasmic APP staining.

2.4 Cell Culture

Cell culture experiments were carried out on the following colorectal adenocarcinoma cell lines: HCT116, HT55, C80, C99, SW837 were obtained from Cancer Research UK Cell Services and LOVO, LS174T and HCT15 cell lines were obtained from Ian Tomlinson (Wellcome Institute of Human Genetics, Oxford). These were chosen, as they were well-characterised colon and rectal adenocarcinoma cell lines for which we had detailed mutational data for verification purposes. The lines were cultured in Dulbeccos Modified Eagle medium (DNEM, PAA, UK) with 10% Foetal Bovine serum (PAA, UK) and 1% Penicillin-Streptomycin (PAA, UK). Cells were incubated at 37°C in 5% carbon dioxide and in 100% relative humidity, the growth media was changed approximately every two to three days.

Cells were propagated until approximately 70-80% confluent and then subcultured by diluting (splitting) the cell population depending on approximate cell growth rates between 1:5 and 1:10. The culture medium was removed and adherent cells rinsed in sterile 1x Phosphate buffered saline (PBS) (Sigma, UK), washing off any dead cells and medium. Then 10% Trypsin EDTA (PAA, UK) sufficient to cover the cells was added and incubated at 37°C for 5 minutes detaching the cells from the flask. The detached cells were then diluted accordingly and culture medium added.

2.4.1 Amyloid Precursor Protein Inhibition with Valproic Drug Treatment

2.4.1.1 Cell Preparation

Valproic acid (VPA) (Sigma, UK) drug treatment was carried out on cell lines that were 70-80% confluent. The culture media was drawn off the cells, then 5ml 10% Trypsin EDTA (PAA, UK) was added to the flask to detach the cells. This was incubated for five minutes at 37°C, before 10ml of media was added to neutralize the Trypsin. The cell suspension was then counted using a haemocytometer, by drawing 10µl of cell elute into the cell counter compartment. 80,000 cells in 1ml were added to each well of a 24 well plate.

2.4.1.2 Measurement of Cell Response to Valproic Acid

After 48 hours of drug treatment. Cell viability was measured using a CellTitre-Blue® assay (Promega, UK) (see Section 2.4.4.1).

2.4.2 Cell Transfection

2.4.2.1 Transfection Optimisation

The cell number and concentration of transfection agent Lipofectamine 2000 (Invitrogen, Grand Island, USA) were optimised during test transfection using PLK-1 killing control (Qiagen, UK.) and scramble siRNA (AllStars Negative Control, Qiagen, UK). The siRNA targeting PLK-1 was used as a

transfection control ‘killing’ transfected cells with cell viability measured using Sulforhodamine B colorimetric assay (see Section 2.4.4.2).

2.4.2.2 Cell Preparation

Transfection was carried out on HCT116 at 70% confluency. The culture media was drawn off the cells, and then 5ml 10% Trypsin EDTA (PAA, UK) was added to the flask to detach the cells. This was incubated for five minutes at 37°C, before 10ml of media was added to neutralize the Trypsin. The cell suspension was then counted using a haemocytometer, by drawing 10µl of cell elute into the cell counter compartment. 50,000 cells in 1ml were added to each well of a six well plate.

2.4.2.3 Knockdown siRNA Transfection

Transfection was carried out using 500µl DMEM (PAA, UK) mixed with Lipofectamine 3/4/5 µl (Qiagen, UK) per well. This mixture was left for five minutes before adding 45µl (1Mm) of siRNA of either a scrambled siRNA (AllStars Negative Control, Qiagen, UK), PLK-1 siRNA, or a knockout siRNA for the gene of interest (Qiagen, UK). A full list of all siRNA target sequences are provided in Appendix II.

The siRNA mixture was left for 30 minutes to bind to the Lipofectamine complex before being added to the cells. Cell were incubated at 37 °C for 72 hours, at 24 hours the media was replaced with complete cell culture media, thus removing the transfection agent that would be toxic to the cells following

prolonged exposure. At 72 hours the cell were harvested for protein or RNA extraction.

2.4.4 Cell Viability Assays

Cell viability to assess cell growth was performed in two ways:

2.4.4.1 CellTiter-Blue® Assay

The CellTiter-Blue® Assay (Promega, UK) was used when cell were seeded on 96 well plates (VWR, UK). It relies on the ability of viable cells to convert a resazurin, a redox dye, into resorufin a fluorescent end product. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. The media was removed and replaced with complete media and CellTitre-Blue® assay (Promega, UK). This was mixed for ten minutes on a plate shaker, before being incubated for a further three hours at 37°C. Finally, fluorescence was recorded on a plate reader at $560_{Ex}/590_{Em}$ (FluSTAR Optima, BMG Labtech, UK).

2.4.4.2 Sulforhodamine B Colorimetric Assay

The sulforhodamine B (SRB) assay (Skehan et al. 1990) was used to determine cell density following transfection (see Section 2.4.2) or drug treatment (see Section 2.4.1) in 6 well plates (VWR, UK). The SRB colorimetric assay relies on SRB a bright-pink aminoxanthene dye that binds to amino-acid residues to cell fixed with trichloroacetic acid (TCA). SRB

binds stoichiometrically to the protein components of cells and therefore the amount of dye extracted from stained cells is directly proportional to the cell mass.

The culture media was carefully drawn off ensuring that the cell monolayer is not disrupted. Cold PBS was used to wash the cells 3 times. Then 700µl of cold 10% TCA added to each well, and the plate incubated at 4^{o}C for 1 hour. The wells are then gently washed 3 times with cold ddH_2O and the plate allowed to air-dry overnight fixing the cell monolayer. The fixed cell monolayer is then stained with 700µl of 0.057% SRB solution in each well. This is left to incubate at room temperature for 30 minutes before being washed with 1% acetic acid. The wells are washed 4 times removing any unbound dye. Then 500µl of 10mM Tris base solution is added to each well and the plate is placed on a gyratory shaker for 5 minutes to solubilise the protein bound dye. Then 100µl of the soluble protein dye solution was pipetted as four replicates into a 96 well plate (VWR, UK). Finally, absorbance is recorded on a plate reader at $490_{Ex}/530_{Em}$ (FluSTAR Optima, BMG Labtech, UK).

2.4.5 Cell Migration Study

Cell migration was measured using the ThinCert™ (Greiner Bio-one, Germany), which involves cell culture inserts in a multiwall cell culture plate. This assay uses a two-compartment system where cells are induced to migrate from an upper compartment through a porous PET membrane into a lower compartment, following a chemoattractant gradient.

Cell migration was carried out on cell lines that were at 70-80% confluency following seeding in 6 well plates (VWR, UK). The culture media was drawn off the cells, then 500µl 10% Trypsin EDTA was added to the flask to detach the cells. This was incubated for five minutes at 37°Cand 5% CO_2, before 1.5ml of DNEM serum free media was added to neutralize the Trypsin. The cell suspension was then counted using a haemocytometer, by drawing 10µl of cell elute into the cell counter compartment. To compare the migration of different cells following APP siRNA knockdown (see Section 2.4.2), the same concentration of cells was used. This suspension was then centrifuged at 3,000rpm for five minutes. The supernatant was drawn off and the cell pellet re-suspended in 200µl of DNEM serum free media, which was then pipetted into the centre of the cell culture inserts forming the top compartment. The inserts are then placed into a 24 well plate forming the lower compartment with 500µl of media as the chemoattractant for the cells to migrate. One well is left empty and acts as the control to measure the amount of cells that migrate through the porous PET membrane. The plate is then left to incubate for 24 hours at 37 °C and 5% $CO_2$for 24 hours. Cells seeded on the PET membrane in DNEM serum free media are induced to actively migrate through the PET membrane into the lower compartment with media containing media. After 24 hours the cell culture medium from each well of the cell culture is removed and replaced with DNEM serum free media with 8µM Calcein-AM (Sigma, UK), and incubated for 45 minutes in the cell culture incubator.

Calcein-AM is used to label the viable cells. The culture media is drawn off from the cell culture inserts and the inserts transferred to a new 24 well plate with 500µl of prewarmed Trypsin EDTA per well. This was incubated for ten minutes in the cell incubator to detach the cells. The cell culture inserts were then discarded and 200µl of the Trypsin EDTA solution containing the cells transferred into a well of a black bottom 96 well plate (VWR, UK). Finally, absorbance is recorded on a plate reader at $485_{Ex}/520_{Em}$ (FluSTAR Optima, BMG Labtech, UK). The fluorescence reading that quantified the number of migratory cells is then compared with that of scram and the APP siRNA knockdown cell lines.

2.5 Protein Methods

2.5.1 Protein Extraction

Cell lines were harvested for protein once 70-80% confluent in T75 cell culture flasks (TWR, UK). The culture media was drawn off the cells washed in cold PBS 3 times. A cell scrapper was used to dislodge adherent cells from the flask and 2ml cold PBS added. This was transferred into a 15ml Falcon tube and centrifuged at 1,500rpm for 5 minutes at 4^{o}C. The supernatant was drawn off and the cell pellet re-suspended in 2ml cold PBS, and centrifuged at 1,500rpm for 5 minutes at 4^{o}C. The supernatant was again drawn off and the cell pellet was re-suspended in 100-200µl Cell lytic buffer (Sigma, UK) with Protein K inhibitor cocktail added (1:100; Sigma, UK) and left for 10 minutes for cell lysis to occur whilst keeping the mixture cold on ice. Following this the cell homogenate was further homogenised by

sonicating the sample for 15 seconds, 4 times on ice. Finally the sample was passed through a fine bore needle 3 times, and stored at -20 for future use.

2.5.2 Western Blot

2.5.2.1 Protein Quantification and Preparation

Native protein samples were quantified so equal amounts were used thereby allowing the comparison of relative amounts of specific protein between samples. Standards were made using serial dilutions (1:2) from BSA 10mg/ml (Promega, UK). Quadruplicates of each standard and sample were pipetted into a U-bottomed plate (VWR, UK) adding 5µl of native protein or protein standard to 95 µl of Bradford reagent (Sigma, UK) and incubating for 15 minutes. This was then quantified using a 595nm absorption on the plate reader. 15ng of each sample were added to 15µl of 2x sample buffer (Sigma, UK) and made up to 30µl with dd. This was placed on a hot block at 90 for 10 minutes to be denatured.

Electrophoresis and transfer of proteins were carried out using the XCell Western Blot system (Invitrogen, UK).

2.5.2.2 Electrophoresis

A NuPAGE® Bis-Tris 4-10% gel (Invitrogen Life Sciences, Grand Island, NY, USA) was used for electrophoresis. The stack was assembled with MOPS running buffer (760ml dH_20 and 40ml MOPS SDS Nupage buffer) added to it

ensuring the gel was fully immersed.5μl of protein marker (HiMark™ Pre-stained Protein Standard; Invitrogen Life Sciences, Grand Island, NY, USA) was loaded into the gel and then 30μl of each prepared protein sample. The gel was run at 180V for 60 minutes.

2.5.2.3 Transfer of Proteins

A wet transfer was carried out. The transfer membrane was first activated in 100% Methanol for 10 seconds, and filter paper and sponges soaked in transfer buffer (25ml NuPage transfer buffer (Invitrogen Life Technologies, Grand Island, NY, USA), 50ml Methanol and 425ml dH_20). The gel was taken out of its case and placed in a stack formed by sandwiching sponge and filter papers (sponge/paper/gel/membrane/paper/sponge) before clamping this tightly together to ensures no air bubbles form between the gel and membrane. This was placed into the tank and topped up with transfer buffer, which was then placed into an ice bucket to run for 2 hours at 175Amps.

2.5.2.4 Staining and Detection

Following transfer, the membrane was incubated in blocking buffer (5g milk powder in 20ml PBS and 0.1% Tween® (Sigma, UK)) for 1 hour under agitation. The membrane was rinsed with TBST after incubation before the primary antibody diluted in blocking buffer was added. This was left overnight at 4^oC on the shaker. A full list of primary antibodies used and conditions are provided in Appendix III.

Primary antibodies were washed off with TBST and the membrane incubated with the secondary (the appropriate anti-body) for 1 hour on the shaker at room temperature. The secondary antibody was washed off with TBST, three times for 10 minutes and finally PBS for 5 minutes whilst being agitated. The membrane was placed on Clingfilm (protein side up) and 1.5ml of detection buffer added. The membrane was then incubated for 5 minutes at RT. Any excess was blotted off before the membrane was wrapped and viewed in the dark room after exposure to x-ray film.

2.5.2.5 Western Blot Densitometry

Western blot densitometry was carried out using image-processing tool ImageJ (http://rsb.info.nih.gov/ij/).

2.6 Statistical Methods

Statistical analyses were primarily performed using the statistical software program GraphPad Prism (GraphPad Software, CA, USA) and the statistical calculator VassarStats (http://www.vassarstats.net). A two-sided p value less than 0.05 was considered statistically significant.

2.6.1 Summary Statistics

Differences between the distribution of categorical variables (e.g. protein expression) were assessed by means of either the Chi-squared test or

Fishers exact test. Students T test was used for the comparison of continuous variables (e.g. Western blot densitometry).

Survival was assessed by using Kaplan-Meier and survival curves were compared using the Log rank test. Death from cancer was defined as the end point; if the patient died of another cause or was lost to follow up they were censored.

CHAPTER 3

Evaluating the role of Heat shock proteins and their co-chaperones in oncogene induced cell senescence in colorectal cancer

3.1 Overview and Rationale

Major inducible HSPs, such as HSP27 and HSP72 are upregulated in cancers cells, where they facilitate multiple cellular processes pertinent to tumourigenesis (Ciocca and Calderwood 2005; Ciocca, Arrigo et al. 2013). Due to their involvement in cancer development, the inhibition of HSPs has been proposed as a potential cancer treatment strategy (Evans, Chang et al. 2010; Massey, Williamson et al. 2010). Clinically, expression of HSP27 and HSP72 has been associated with advanced CRC disease (Hwang, Han et al. 2003; Wang, Qiu et al. 2005; Milicevic, Petkovic et al. 2007), distant metastasis (Zhao, Liu et al. 2007; Pei, Ge et al. 2010) and poor survival (Lazaris, Theodoropoulos et al. 1995; Sun, Zhang et al. 1997; Kocsis, Madaras et al. 2010; Tweedle, Khattak et al. 2010; Yu, Zhi et al. 2010). However, a number of other studies have failed to show any association of HSP overexpression with clinical pathological characteristics (Kanazawa, Isomoto et al. 2003; Shotar 2005; Tuna, Sokmen et al. 2006; Zhang, Gao et al. 2009; Tweedle, Khattak et al. 2010). In cell culture based studies, HSPs have been shown to promote cancer development by increasing cellular migration (Rousseau, Houle et al. 2000), differentiation (Kindas-Mugge and Trautinger 1994), and drug resistance (Tsuruta, Nishibori et al. 2008). HSPs

have also been shown to promote cell survival through the inhibition of apoptosis (Charette, Lavoie et al. 2000) and cell senescence (Sherman 2010).

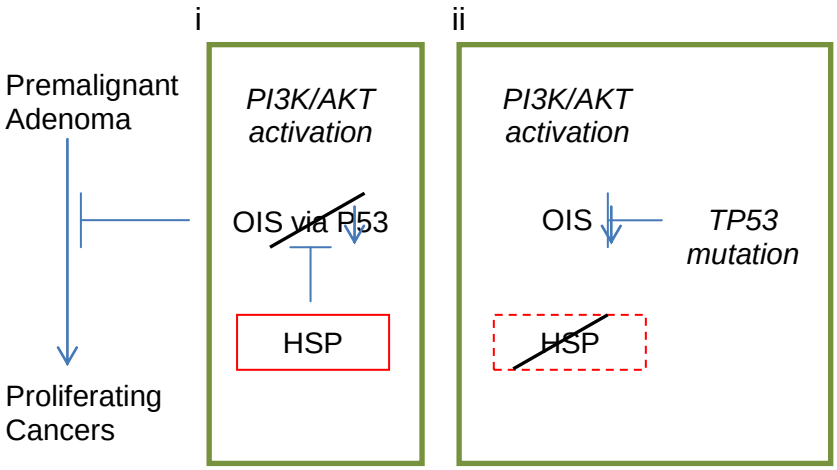
The constitutive activation of RAS signalling and its wide range of downstream effector pathways, including the PI3K/AKT pathway (Castellano and Downward 2011) is commonly seen in CRC with both of these genes shown to induce OIS. OIS is a major obstacle to cancer progression. As senescent cells are incapable of further proliferation, OIS has to be overcome for cancers to grow and progress. One way of bypassing OIS is by acquiring mutations in genes involved in the response to senescence, such as the *TP53* gene (Halazonetis, Gorgoulis et al. 2008). HSP27 and HSP72 have been proposed to assist in evasion of OIS *in vitro* (O'Callaghan-Sunol, Gabai et al. 2007; Yaglom, Gabai et al. 2007).

To evaluate whether HSPs play different roles in OIS depending on the genetic background of the tumour and the relationship with clinicopathological features. A group of unselected primary CRCs with clinicopathological data were screened for *TP53, KRAS*, and *PIK3CA* mutations and the protein expression of HSP27, HSP72, and AKT determined *in vivo*. The potential role of HSPs in TP53 dependent OIS was examined by associating HSP expression with *TP53* mutation status (Figure 11A). The requirement for HSP expression is likely dependent on the combination of *KRAS* mutation and *PI3K/AKT* mutation status (Figure 11B). First, the clinicopathological features of the patients are detailed.

A

Background: In the absence of a *TP53* mutation, OIS mediated by PI3K can be circumvented by high expression of HSP72 in breast cancer cell lines (Gabai et al., 2009).

My hypothesis: In CRC, HSP expression will more likely be present in tumours with functional P53 pathways to help overcoming OIS (i). HSP expression will not be necessary in tumours with *TP53* mutations (ii).



B

Background: it was reported that while PI3K/AKT activation alone acts as a moderate inducer of OIS, it could suppress senescence induced by an activated RAS oncogene to promote tumorigenesis (Kennedy et al., 2011).

My hypothesis: In CRC, HSP expression will more likely to be present in tumours with *KRAS* (i) or PI3K/AKT activation (ii). HSP expression will not be necessary in tumours with both, as PI3K/AKT activation will help to suppress *KRAS* induced OIS (iii).

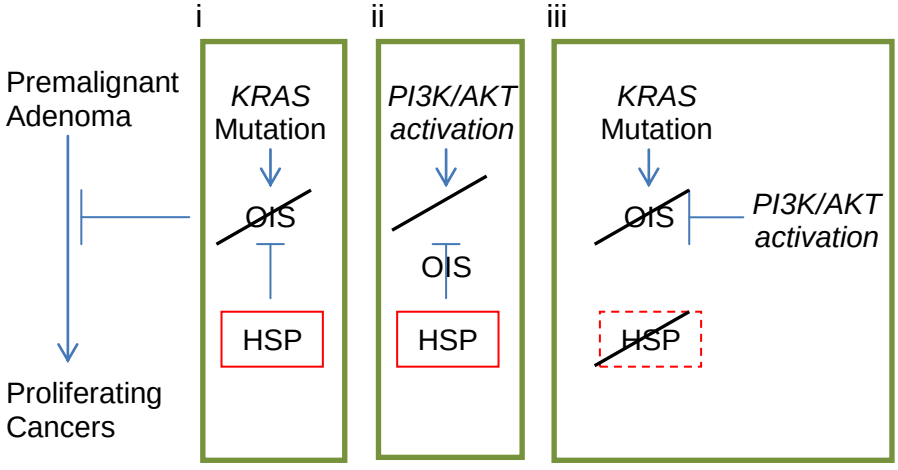


Figure 11. Background on findings from cell-cultured based studies and our hypotheses for testing them in CRC. (A) Association of HSP expression with *TP53* mutation status; (B) HSP expression dependence on a combination of *KRAS* mutation and *PI3K/AKT* mutation status.

3.2 Results

3.2.1 Clinicopathological Features of Patient Cohort

A total of 74 unselected primary CRC tumours (45 colon, 29 rectal) from 46 males and 28 females were investigated (Table 3). The age at diagnosis for this group ranged from 35 – 89 years old, with a median age, 75.8. The majority (64%) were left sided tumours (47/74). The cohort contained, 10 Duke's stage A (14%), 26 stage B (34%), 32 stage C (44%) and 6 stage D (8%) CRCs. The median follow-up was 24.1 months (range 1- 55.2) and 26 deaths from any cause were reported, of which 17 deaths were known to be due to CRC.

Table 3. Summary of clinicopathological features of colorectal cancer cohort.

Clinical parameter	n (%)	Clinical parameter	n (%)
Gender		**Depth of invasion**	
Male	46 (62)	T1	6 (8)
Female	28 (38)	T2	5 (7)
Age		T3	46 (62)
<70 years	26 (35)	T4	17 (23)
>70 years	48 (65)	**Nodal status**	
Site		N0	41 (55)
Left	47 (64)	N1	16 (22)
Right	27 (36)	N2	17 (23)
Resection margins		**Metastasis status**	
R0	60 (82)	M0	64 (86)
R1	7 (9)	M1	10 (14)
R2	7 (9)	**Duke's stage**	
Differentiation		A	10 (14)
Well	4 (5)	B	26 (34)
Moderate	56 (76)	C1	22 (30)
Poor	14 (19)	C2	10 (14)
		D	6 (8)

3.2.2 No Association of HSP27 or HSP72 Expression with Clinicopathological Parameters and Patient Survival

The expression pattern of HSP27 and HSP72 in our cohort of CRCs was determined by IHC (Figure 12 and 13; Table 4). No HSPs expression was observed in any normal tissues, but HSP27 staining was found in 74% of tumour samples (48/65) while HSP72 staining was present in 87% (60/69). The protein expression data was then examined in relation to a number of clinicopathological parameters: tumour differentiation; depth of invasion (T stage); nodal metastases (N stage) and distant metastases (M stage). No significant association was shown between either HSP27 or HSP72 and any of these features (Table 5). HSP27 or HSP72 was not associated with patient survival (Figure 14).

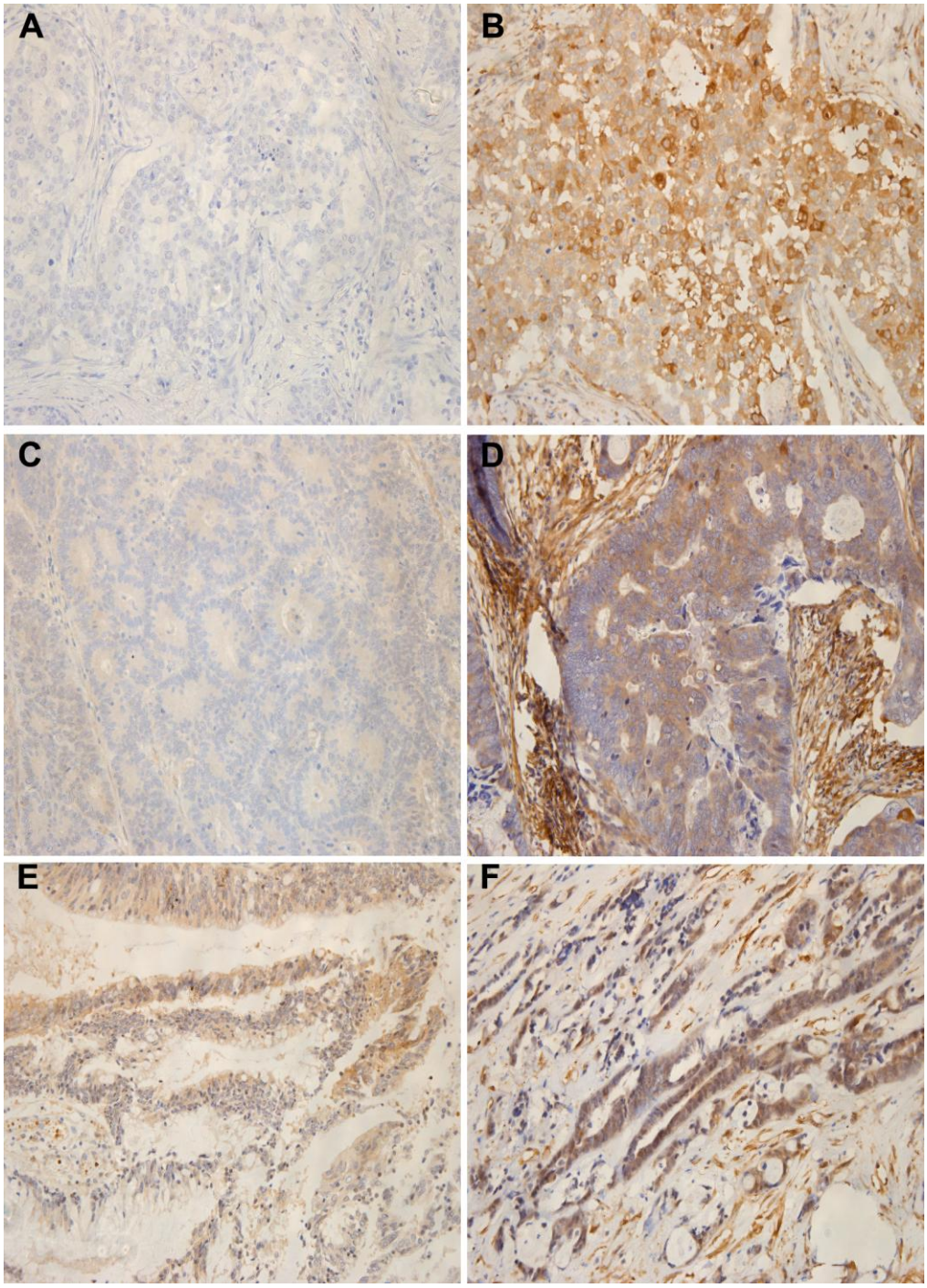


Figure 12. Representative examples of HSP27 Immunohistochemistry of colorectal tumour sample at x10 magnification. (A) HSP27 Breast carcinoma negative control; (B) HSP27 Breast carcinoma positive control; (C) HSP27, no expression detected; (D) HSP27, cytoplasmic positive; (E) HSP27, nuclear positive; (F) HSP27 positive. Samples were scored positive if there was nuclear/cytoplasmic staining in more than 20% of cells (Patel, Polanco-Echeverry et al. 2007).

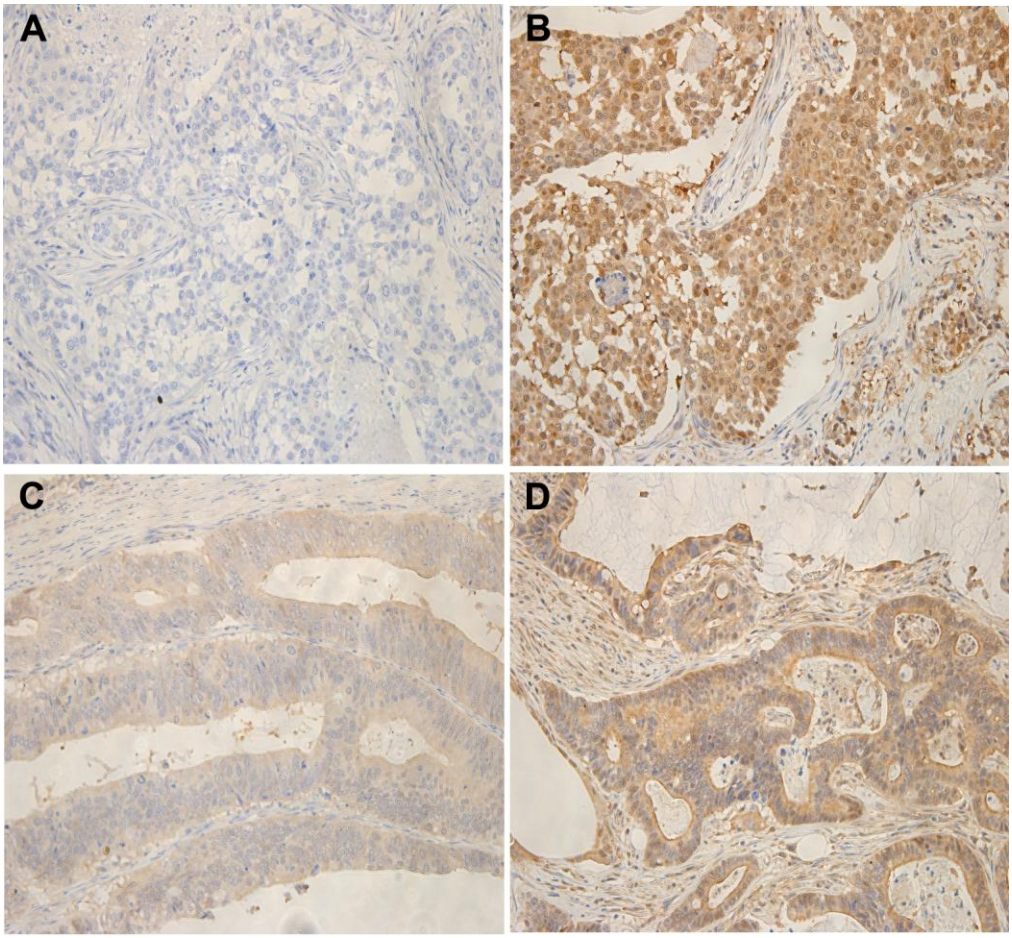


Figure 13. Representative examples of HSP72 Immunohistochemistry of colorectal tumour sample at x10 magnification. (A) HSP72 Breast carcinoma negative control; (B) HSP72 Breast carcinoma positive control; (C) HSP72, no expression detected; (D) HSP72, positive. Samples were scored positive if there was nuclear/cytoplasmic staining in more than 20% of cells (Patel, Polanco-Echeverry et al. 2007).

Table 4. Summary of cytoplasmic and nuclear staining of HSP27 and HSP72. HSP27 immunopositivity was seen in the majority of tumours (72%, 47/65) and most exhibited discrete cytoplasmic staining with no nuclear staining (58%, 38/65) while 26% (17/65) showed no immunostaining to HSP27. (E) Most CRCs (87%, 60/69) showed HSP72 immunopositivity, with both cytoplasmic and nuclear staining (62%, 43/69). HSP72 did not show immunopositivity in 13% of tumour samples (9/69).

HSP27[1]	**Nuclear +**	**Nuclear -**	**HSP72**[2]	**Nuclear +**	**Nuclear +**
Cytoplasmic +	9	38	Cytoplasmic +	43	16
Cytoplasmic -	1	17	Cytoplasmic -	1	9

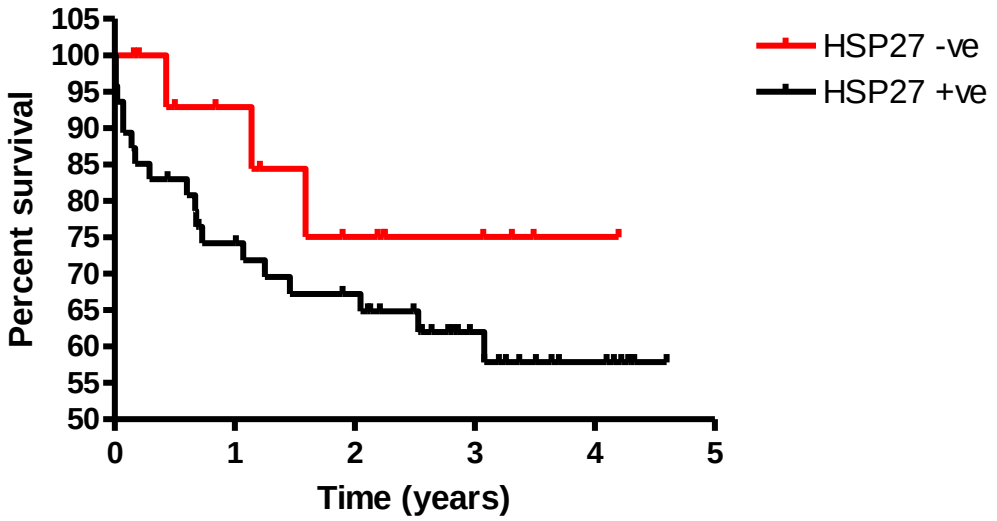
[1] IHC on 74 tumour samples; however 9 tumour slides not be scored due to poor staining or lack of tumour.

[2] IHC on 74 tumour samples, but 9 tumour slides could not be scored due to poor staining or lack of tumour.

Table 5. Expression of HSP27 and HSP72 in relation to clinicopathological features of colorectal cancers.

Clinical parameter	HSP27 - n (%)	HSP27 + n (%)	p value	HSP72 - n (%)	HSP72 + n (%)	p value
Resection margins						
R0	16 (21.6)	39 (52.7)	0.61	9 (12.2)	48 (64.9)	0.48
R1	0	4 (5.4)		0	6 (8.1)	
R2	4 (5.4)	5 (6.8)		0	6 (8.1)	
Differentiation						
Well	0	3 (5)	0.75	0	3 (4)	0.79
Moderate	14 (22)	38 (58)		8 (12)	45 (65)	
Poor	3 (5)	7 (11)		1 (1)	12 (17)	
Depth of invasion						
T1	1 (2)	4 (6)	0.79	0	5 (7)	0.18
T2	1 (2)	4 (6)		1 (1)	4 (6)	
T3	13 (20)	29 (45)		8 (12)	35 (51)	
T4	2 (3)	11 (17)		0	16 (23)	
Nodal status						
N0	10 (15)	26 (40)	1	8 (12)	31 (45)	1
N1	4 (6)	11 (17)		1 (1)	14 (20)	
N2	3 (5)	11 (17)		0	15 (22)	
Metastasis status						
M0	15 (23)	42 (65)	0.73	8 (12)	53 (77)	0.61
M1	2 (3)	6 (9)		1 (1)	7 (10)	

A



B

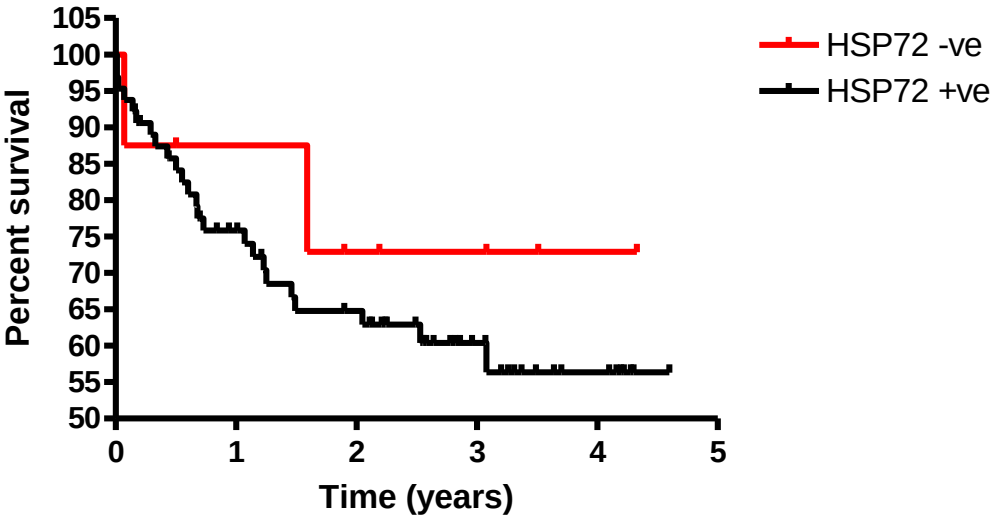


Figure 14. Kaplan-Meier plots correlating patient survival with protein expression of (A) HSP27 and (B) HSP72. There is no statistically significant difference (HSP27 p=0.28; HSP72 p=0.49) in survival between patients with tumour expressing the heat shock proteins and the patients with tumours negative for heat shock proteins expression by log rank test.

3.2.3 Stratification of CRCs According to TP53 and KRAS Mutation and PI3K/AKT Activation Status

Known regions of high mutation frequency were sequenced to detect mutations in *TP53* (exon 5-8), *KRAS* (codon 12 and 13) and *PIK3CA* genes (exon 9 and 20) (Table 3). Fifty one percent of the tumour samples contained *TP53* mutations (36/70) and most mutations were in exon 8 (18/36, 50%) whilst exon 5 had seven mutations (19%), exon 6 had six mutations (17%) and exon 7 had four mutations (11%) (Table 3). No sequence data was obtained for four samples. *KRAS* mutations were identified in 27% of samples (20/74) (Table 3), with codon 12 accounting for 85% (17/20) of mutations. Ten out of 74 CRCs harboured *PIK3CA* mutations (14%) and 80% of these mutations were found on exon 20 (8/10). *KRAS* and *PIK3CA* mutations were found together in 4 tumour samples (Table 6). The mutation rates of *TP53*, *KRAS* and *PIK3CA* are in line with data from the literature, confirming that this cohort is representative of a general, unselected group of CRCs (Levine 1997; Samuels and Velculescu 2004; Velho, Oliveira et al. 2005; Abubaker, Bavi et al. 2008).

To assess the activation status of PI3K/AKT pathway in our samples, IHC for phosphorylated AKT (pAKT) was performed (Patel, Polanco-Echeverry et al. 2007). pAKT staining was detected in 80% of our tumour samples (57/71). All of these tumours exhibited cytoplasmic localisation and 32% showed additional nuclear staining (18/57) (Figure 15, Table 7). As expected, most tumours with *KRAS* or *PIK3CA* (21/24) mutations were positive for pAKT staining, confirming PI3K/AKT activation.

Table 6. Gene mutations identified in colorectal cancer cohort. Four tumours with co presence: *KRAS* C12D with *PIK3CA* mutation P539S, H1047R or C1049S; C12V with S1008P. Mutations shown are in accord those presented in the COSMIC database (Forbes, Bindal et al. 2011).

Gene	Exon	Mutation and number of times observed					
		n=1	n=2	n=3	n=4	n=5	n=10
TP53	5	A138V	H179Y	R175H			
		C141R					
	6	Q192K	R213X				
		R196X					
		V218G					
		Y220L					
	7	Y234D	R248Q				
		G245S					
		M246T					
	8	G266E	R273C		C275Y		
		E271K	R273H		D281N		
		R273L					
		R280K					
		R282W					
		R306X					
PIK3CA	9	P539S					
		E575K					
	20	E1012G	S1008P	H1047R			
			G1049S				
RAS	1		G12A	G13D		G12V	G12D

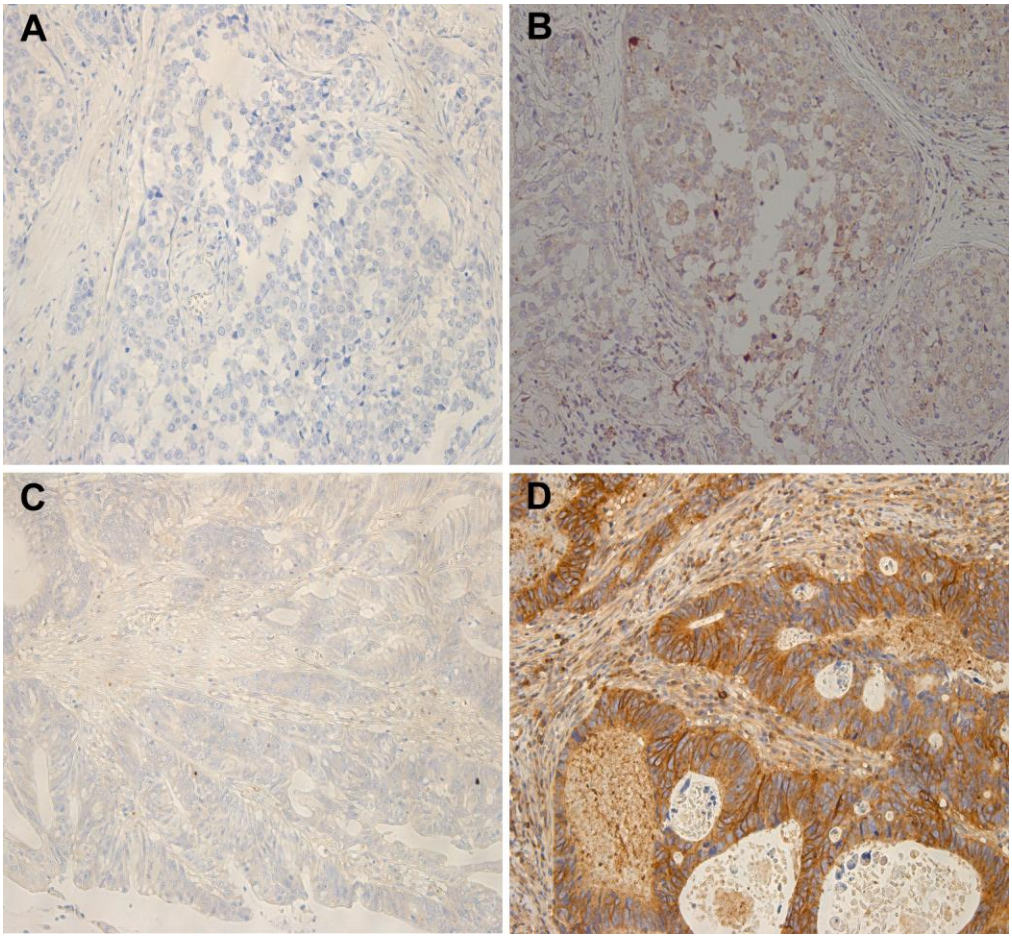


Figure 15. Representative examples of pAKT Immunohistochemistry of colorectal tumour sample at x10 magnification. (A) pAKT Breast carcinoma negative control; (B) pAKT Breast carcinoma positive control; (C) pAKT, nuclear positive; (D) pAKT, cytoplasmic positive. Samples were scored positive if there was nuclear/cytoplasmic staining in more than 20% of cells (Patel, Polanco-Echeverry et al. 2007).

Table 7. Summary of cytoplasmic and nuclear staining of pAKT. AKT immunopositivity was exhibited in 80% tumour samples (57/71) and all showed cytoplasmic immunostaining.

AKT[1]	Nuclear +	Nuclear -
Cytoplasmic +	18	39
Cytoplasmic -	0	14

[1] IHC carried out on 74 CRCs, scored on 71 (96%), 3 samples not scored due to poor staining or lack of tumour.

PIK3CA copy number variation is common in CRC and QRT-PCR was carried out to analyse the *PIK3CA* copy number of each tumour sample. Two normal controls (samples 334N and 339N) were used on each plate (n=4) to permit inter-plate comparison and to control for any variation between plates. *PIK3CA* amplification was defined as a copy number increase greater than two. Overall, *PIK3CA* copy number change was measured successfully in 85% of cases (63/74) and amplification was seen in 67% (42/63) of the tumour samples (Table 8). 40% of tumours that had an amplification of *PIK3CA* had a copy number of three, whilst 6% had a copy number greater than four.

PIK3CA amplification was seen in 67% (42/63) of the tumour samples and AKT protein expression was identified in just over half the cases (52%, 33/63). However amplification of *PIK3CA* and AKT expression was not significantly association (p=0.3, Table 9). The analysis of *PIK3CA* mutations on exons 9 and 20 found an overall mutation rate of 14% (8/74). This was associated with AKT expression in the majority of tumour that were found to have a *PIK3CA* mutation (7/8, 88%). Therefore, in subsequent analyses PIK3CA oncogenic pathway activation was considered to have occurred when either *PIK3CA* was mutated or AKT expressed in a tumour. As already noted, almost all tumours with *KRAS* or *PIK3CA* (21/24) mutations were positive for pAKT staining, confirming PI3K/AKT activation.

Table 8. *PIK3CA* copy number frequency in a cohort of sporadic colorectal cancers. A total of 63 out of 74 tumours were analysed successfully using QPCR and amplification was seen in most CRCs.

Copy Number	1&2	3	4	5&6
n=63	21	25	13	4
%	33	40	21	6

Table 9. *PIK3CA* Copy number amplification and AKT expression.

Copy Number	AKT -	AKT +	p value
≤ 2	3	17	0.3
> 2	7	33	

3.2.4 HSP27 Expression is Associated with Mutated TP53

Studies of cancer cell lines have suggested that HSPs are selectively up-regulated to suppress OIS in the absence of *TP53* mutations, thereby allowing uncontrolled proliferation (Figure 11A) (Sherman 2010). To examine this phenomenon *in vivo,* the expression results for HSP27 and HSP72 in CRCs were compared with or without *TP53* mutation. The expression of HSP27 was not significantly associated with the mutation status of *TP53* ($p = 0.08$), although there was a trend (Table 10A). Of the 34 tumours with a *TP53* mutation, the majority of these (28/34, 82%) expressed HSP27 indicating that selective upregulation of HSP27 does not occur preferentially in CRCs with wild type *TP53* (Table 6A). Similarly, no positive association was found between HSP72 expression and *TP53* ($p = 0.29$) (Table 10B).

Table 10. Association of HSP27 (A) and HSP72 (B) expression with *TP53* mutation status in all PI3K/AKT activated colorectal cancers. P value calculated using Fisher's exact test.

A

PI3K/AKT activated CRCs (n = 61)	**HSP27 -**	**HSP27 +**	**p value**
Mutated TP53	6	28	0.08
WT TP53	11	16	

B

PI3K/AKT activated CRCs (n = 65)	**HSP72 -**	**HSP72 +**	**p value**
Mutated TP53	3	31	0.29
WT TP53	6	25	

3.2.5 HSP27 Expression is Associated in PI3K/AKT Active Tumours with Wild Type KRAS, Independent of TP53 Mutation Status

Recent in vitro evidence suggests that activation of the PI3K/AKT pathway can induce or inhibit OIS depending on the presence or absence of activated *KRAS* (Kennedy, Morton et al. 2011) (Figure 11B). Whether the presence of *KRAS* mutation in a tumour influenced the relationship between PI3K/AKT activation and HSP expression was investigated. The CRC samples were divided into three groups: PI3K/AKT active tumours with mutated *KRAS*; PI3K/AKT active tumours with wild type *KRAS*; and PI3K/AKT inactive tumours. HSP27 was highly associated with PI3K/AKT-active CRCs with wild type *KRAS* ($p = 0.004$, Table 11A). In fact, over 90% of the tumours in this group (32/35) show HSP27 expression. Increased HSP27 expression in PI3K/AKT active tumours with mutated *KRAS*, was not observed suggesting HSP27 may play a different and possibly less critical role in this group of tumours. There was possibly a trend for higher expression of HSP72 in PI3K/AKT active CRCs with wild type *KRAS*, but the association was not statistically significant ($p = 0.08$, Table 11B).

Table 11. Association of HSP27 (A) and HSP72 (B) expression with *KRAS* mutation and PI3K/AKT activation status in the complete colorectal cancer cohort. There were no tumours expressing either HSP27 or HSP72 that had inactivated PI3K/AKT and mutant *KRAS.* (*Fisher's exact test p<0.05). WT, wild type.

A

	HSP27 –	**HSP27 +**	**p value**
PI3K/AKT + & *Mutated KRAS*	8	9	0.004*
PI3K/AKT + & *WT KRAS*	3	32	
PI3K/AKT – & *WT KRAS*	4	7	

B

	HSP72 –	**HSP72 +**	**p value**
PI3K/AKT + & *Mutated KRAS*	5	12	0.080
PI3K/AKT + & *WT KRAS*	3	36	
PI3K/AKT – & *WT KRAS*	1	11	

3.2.6 Protein Expression of Heat Shock Transcription Factor-1 is Common in Colorectal Cancer, but Does Not Associate with TP53 or KRAS Mutation

HSF1 is a transcription factor that controls the cellular response to disturbances in protein homeostasis and protects the proteome from physiologic stress. HSF1 determines the production of the HSPs such as HSP27 and HSP72. Given the link between HSP27 expression and *KRAS* mutation and PI3K/AKT activation status, a possible role for HSF1 in this process was investigated.

3.2.7 Colorectal Cancers are Mostly Positive for Heat Shock Factor-1, But Expression is Not Associated with Clinicopathological Features

The majority of the CRCs (73%) showed HSF1 immunopositivity (44/60). HSF1 exhibited strong staining in the nucleus with 44 tumours (73%, 44/60) and 23% (14/60) with cytoplasmic staining. The majority of tumours exhibited discrete nuclear staining and no cytoplasmic staining (35/60, 57%). In ten tumours (16.7%, 10/60) both cytoplasmic and nuclear staining was observed. Four tumours (1.7%) showed discrete cytoplasmic staining with no nuclear staining. About one fifth of CRC (20%, 12/60) did not show immunopositivity for HSF1 (Figure 16; Table 12).

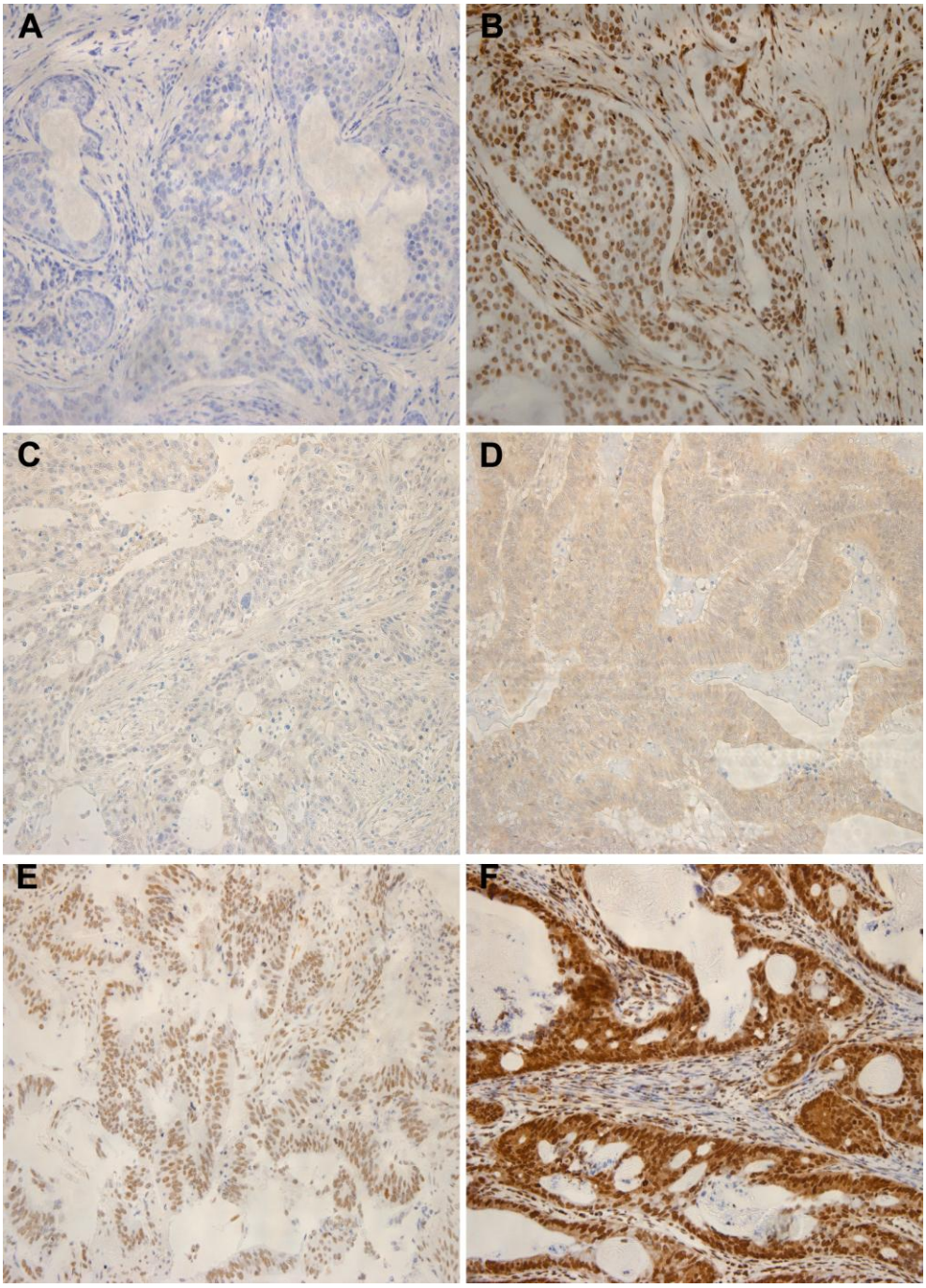


Figure 16. Representative examples of HSF1 Immunohistochemistry of colorectal tumour sample at x10 magnification. (A) HSF1 Breast carcinoma negative control; (B) HSF1 Breast carcinoma positive control; (C) HSF1, no expression detected; (D) HSF1, cytoplasmic positive; (E) HSF1, nuclear positive; (F) HSF1 positive. Samples were scored positive if there was nuclear/cytoplasmic staining in more than 20% of cells (Patel, Polanco-Echeverry et al. 2007).

Table 12. Summary of cytoplasmic and nuclear staining of HSF1. Most tumour samples showed discrete nuclear staining with no cytoplasmic staining.

HSF1[1]	Nuclear +	Nuclear -
Cytoplasmic +	10	4
Cytoplasmic -	34	12

[1] IHC on 74 tumour samples, 14 slides could not be scored due to poor staining or lack of tumour.

We then examined the protein expression data in relation to a number of clinicopathological parameters: tumour differentiation; Duke's stage; depth of invasion (T stage); nodal metastases (N stage) and distant metastases (M stage). No significant association was shown between HSF1 expression and any of these features (Table 13).

Table 13. Expression of HSF1 in relation to clinicopathological features of colorectal cancers. No significant association was shown between HSF1 expression and any clinicopathological features.

Clinical parameter	HSF1 -	HSF1 +	p value
Resection margins			
R0	11 (14.9)	39 (52.7)	0.68
R1	1 (1.4)	4 (5.4)	
R2	0	5 (6.8)	
Differentiation			
Well	0	3 (4.1)	0.32
Moderate	12 (16.2)	37 (50)	
Poor	0	8 (10.8)	
Duke's stage			
A	1 (1.4)	8 (10.8)	0.471
B	7 (9.5)	16 (21.6)	
C1	3 (4.1)	15 (20.3)	
C2	1 (1.4)	6 (8.1)	
D	0	3 (4.1)	
Depth of invasion			
T1	1 (1.4)	4 (5.4)	0.26
T2	0	5 (6.8)	
T3	10 (13.5)	26 (35.1)	
T4	1 (1.4)	13 (17.6)	
Nodal Status			
N0	9 (12.2)	25 (33.8)	0.34
N1	1 (1.4)	12 (16.2)	
N2	2 (2.7)	11 (14.9)	
Metastasis Status			
MO	12 (16.2)	41 (55.4)	0.32
M1	0	7 (9.5)	

3.2.8 Heat Shock Transcription Factor-1 Expression is Not Associated with TP53 or KRAS Mutation

HSF1 expression and *TP53* mutation were characterised in 58 tumours, of which 55% (32/58) had a *TP53* mutation. There was no significant association with *TP53* and total HSF1 expression (Table 9). HSF1 expression and *KRAS* mutation were characterised in 62 tumours, of which 29% (29/62) had a *KRAS* mutation. There was no significant association with *KRAS* and total HSF1 expression (Table 14).

Table 14. Association of HSF1 expression with *TP53* and *KRAS* mutation status. There was no significant association between total HSF1 with *TP53* or *KRAS* mutation status (p value, Fisher's Exact test). WT, wild type.

***TP53* status**	**HSF1 -**	**HSF1 +**	**p value**	***KRAS* Status**	**HSF1 -**	**HSF1 +**	**p value**
Mutated TP53	6	25	0.74	***Mutated KRAS***	5	11	0.27
WT TP53	6	19		***WT KRAS***	7	37	

3.2.9 BAG1 is Expressed in a Majority of Sporadic Colorectal Cancers and is Associated with TP53 Mutation

BAG1 is an important co-chaperone to HSP72 and is expressed in CRC (Clemo, Collard et al. 2008; Sun, Meng et al. 2011). Accordingly we assessed BAG1 expression and the relationship to clinicopathological features, including survival, and *TP53* and *KRAS* mutations status.

3.2.10 BAG1 is Expressed in the Cytoplasm and Nucleus of a Significant Proportion of Colorectal Cancers

A total of 60 tumour samples were stained and scored according to the strength of staining as published previously (Kikuchi, Noguchi et al. 2002; Sun, Meng et al. 2011). BAG1 immunopositivity was exhibited in nearly all the tumour samples (86%, 52/60), with only 8 tumour samples (13%) exhibiting no immunostaining (Figure 17). As expected most of the tumour samples had BAG1 staining of the cytoplasm (86%, 52/60), although 62% of tumours (37/60) were found to also show nuclear BAG1 immunopositivity. No tumours that showed nuclear staining without cytoplasmic immunostaining. The differences in strength of staining allowed for a scoring system to reflect this variation as noted by others (Kikuchi, Noguchi et al. 2002; Sun, Meng et al. 2011). A significant proportion (51%, 19/37) of tumours with nuclear staining showed particularly strong staining for BAG1 in the nucleus. There was no significant difference in the proportion of tumours showing strong or moderate nuclear or cytoplasmic immunostaining

(43%, 26/60, 43%), compared to those with weak or no nuclear or cytoplasmic staining (38%, 23/60) (Table 15).

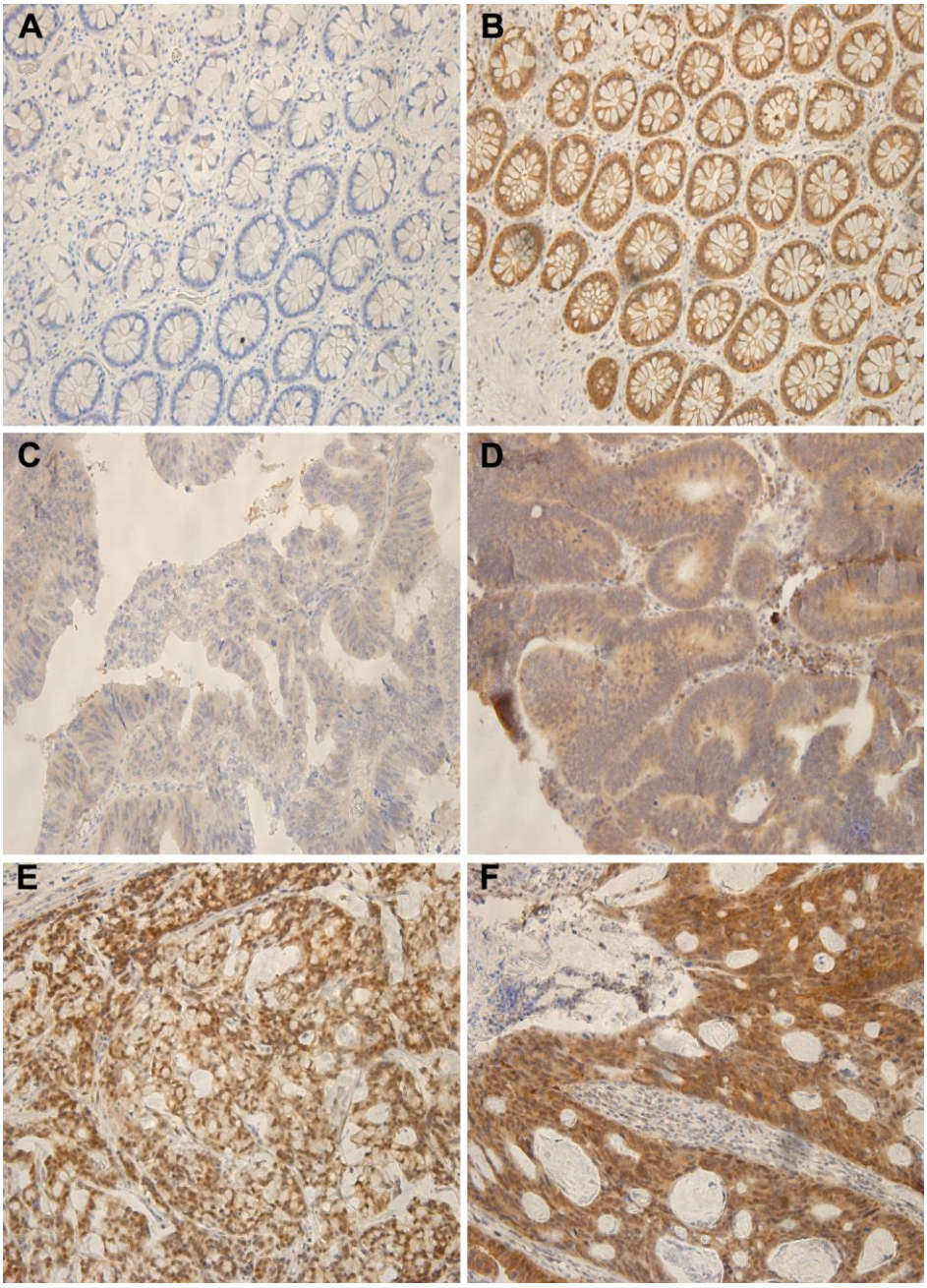


Figure 17. Representative examples of BAG1 immunostaining of tumour samples at x10 magnification. (A) BAG1 Appendix negative control; (B) BAG1 Appendix positive control; (C) BAG1, no expression detected; (D) BAG1, cytoplasmic positive; (E) BAG1, nuclear positive; (F) BAG1 positive. Samples were scored positive if there was nuclear/cytoplasmic staining in more than 20% of cells (Patel, Polanco-Echeverry et al. 2007). BAG1 showed easily differentiated staining and therefore was scored weakly positive to strongly positive in cytoplasmic and nuclear compartments. BAG1 was seen more strongly in the nuclear compartment of tumour samples.

Table 15. Summary of cytoplasmic and nuclear staining of BAG1. BAG1 was expressed in 86% of tumour samples (52/60) and the protein was seen in both nuclear and cytoplasmic staining in most samples (61.7%, 37/60). BAG1 is expressed in a large proportion of CRCs.

BAG1[1]	**Cytoplasmic +++**	**Cytoplasmic ++**	**Cytoplasmic +**	**Cytoplasmic -**
Nuclear +++	7	8	4	0
Nuclear ++	0	3	1	0
Nuclear +	3	5	6	0
Nuclear -	0	6	9	8

1 Immunostaining was carried out on 74 samples, 14 samples not be scored due to poor staining or lack of tumour.

3.2.11 BAG1 Expression was Associated with Increase Depth of Tumour Invasion, But Not Patient Survival

BAG1 expression was significantly associated with increased depth of invasion (p=0.02, Table 16). However, if an adjustment for multiple testing using Bonferroni correction is applied (r=6; lowers p from <0.05 to <0.008) then significance is lost. No other parameters were significant.

Previously, BAG1 has been associated with prognostic outcome (Kikuchi, Noguchi et al. 2002). To explore this finding in our cohort survival data was plotted against the expression of BAG1, but no significant association was found (Figure 18, Appendix VI).

Table 16. Expression of BAG1 in relation to clinicopathological features of colorectal cancers. Expression was significantly associated with increased depth of invasion, but if an adjustment for multiple testing applied (Bonferroni correction: r=6; lowers p from <0.05 to <0.008) then significance is lost.

Clinical parameter	BAG1 -	BAG1 +	p value
Resection margins			
R0	8 (10.8)	44 (59.5)	1
R1	0	3 (4.1)	
R2	0	5 (6.8)	
Differentiation			
Well	0	3 (4.1)	0.73
Moderate	8 (10.8)	41 (55.4)	
Poor	0	8 (10.8)	
Duke's stage			
A	3 (4.1)	6 (8.1)	0.38
B	2 (2.7	19 (25.7)	
C1	2 (2.7)	15 (20.3)	
C2	1 (1.4)	7 (9.5)	
D	0	5 (6.8)	
Depth of invasion			
T1	3 (4.1)	2 (2.7)	0.022*
T2	0	5 (6.8)	
T3	3 (4.1)	36 (48.6)	
T4	2 (2.7)	9 (12.2)	
Nodal Status			
N0	5 (6.8)	29 (39.2)	1
N1	2 (2.7)	12 (16.2)	
N2	1 (1.4)	11 (14.9)	
Metastasis Status			
MO	7 (9.5)	45 (60.8)	1
M1	1 (1.4)	7 (9.5)	

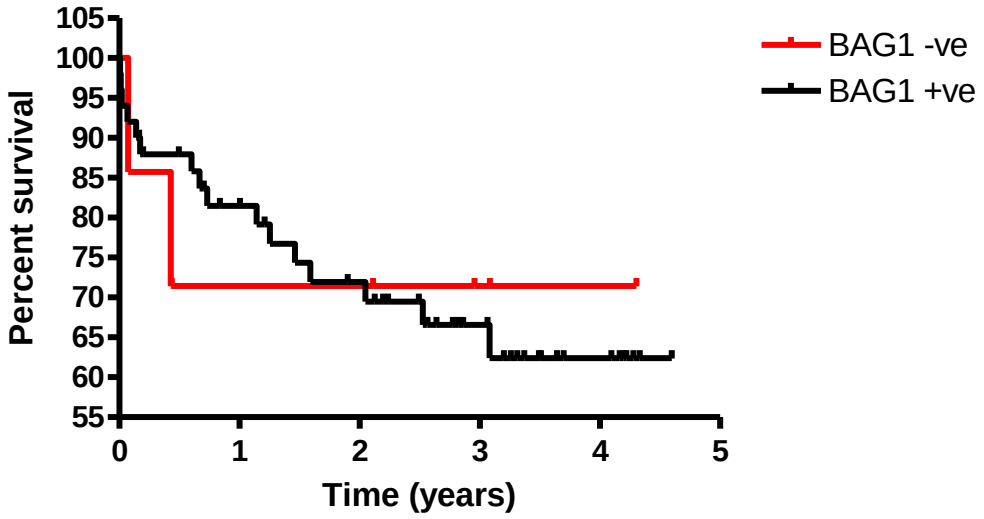


Figure 18. Kaplan-Meier plots correlating patient survival with protein expression of BAG1. There is no statistically significant difference (p=0.97) in survival between patients with tumour expressing BAG1 and the patients with tumours negative for BAG1 expression by log rank test.

3.2.12 Association of BAG1 Protein Expression with TP53 and KRAS Mutational Status

There were 57 tumour samples that were scored for BAG1 immunopositivity and characterised for *TP53* mutation status of these 56% (32/57) had a *TP53* mutation. A significant association between *TP53* mutation and those tumours that were BAG1 expression negative in cytoplasm (p=0.01), or BAG1 negative in either compartment (p=0.01) was found (Table 17). *KRAS* mutation status was known for 60 tumour samples that were scored for BAG1 immunopositivity, of these 88% (16/18) had a *KRAS* mutation. A similar proportion of WT *KRAS* (86%, 36/42), hence there was no significant association with *KRAS* and BAG1 expression. BAG1 immunopositivity was also scored in 60 tumour samples characterised for PI3/AKT activation. BAG1 was expressed in 79% (41/52) of tumour samples. However there was no significant association with PI3/AKT activation.

Table 17. Lack of BAG1 expression is associated with *TP53* mutation status. There is a significant association between *TP53* mutation and those tumours that were BAG1 expression negative (p=0.01). There was no significant association with *KRAS* or PI3/AKT activation (p=1 and p=0.33 respectively).

***TP53*[1] Status**	**BAG1 -**	**BAG1 +**	**p value**	***KRAS*[2] status**	**BAG1 -**	**BAG1 +**	**p value**
WT *TP53*	0	25	0.01*	**WT *KRAS***	6	36	1
Mutated *TP53*	7	25		**Mutated *KRAS***	2	16	

***PI3K/AKT*[3] Status**	**BAG1 -**	**BAG1 +**	**p value**
PI3K/AKT -	0	11	0.33
PI3K/AKT +	8	41	

[1] Immunostaining was carried out on 70 samples characterised for *TP53*, 13 samples not be scored for BAG1 due to poor staining or lack of tumour.

[2] Immunostaining was carried out on 74 samples characterised for *KRAS*, 14 samples not be scored for BAG1 due to poor staining or lack of tumour.

[3] Immunostaining was carried out on 74 samples characterised for PI3K/AKT and *KRAS*, 14 samples not be scored for BAG1 due to poor staining or lack of tumour.

3.3 Discussion

3.3.1 Clinicopathological Features of Patient Cohort

The study cohort used here consisted of more male then female patients (46:28), which is in keeping with the higher incidence of CRC in men and the greater lifetime risk of CRC in men (1:16) compared to women (1:20) (Cancer Research UK. 2010). The mean age of the patients in the study was 73 years old and this reflects the strong age related occurrence of CRC, with 84% of cases affecting people who are 60 years or older (Cancer Research UK, 2010). CRC predominantly affects the left side of bowel with over half of cases arising at this site compared to the right side of bowel; tumours occur most frequently in the sigmoid colon, rectosigmoid junction and rectum (Cancer Research UK, 2010). In my study cohort, most of the tumours were left sided rather than right sided and although there has been the suggestion that the distribution of CRC has shifted more proximally over time (Cucino, Buchner et al. 2002), this remains to be confirmed (Gomez, Dalal et al. 2004). The distribution reported here is a likely consequence of the random reselection of patients and. the distribution in my cohort is not significantly different ($p=0.058$) from that reported previously (Cancer Research UK. 2010), but does show a trend towards a higher frequency of right sided CRCs. Duke's staging for the tumour samples reflected expected incidence (Cancer Research UK, 2010) with Duke's stage B and C accounting for the majority of cases and pathological findings of vascular invasion and mucin were similarly in line with expectations.

3.3.2 No Association of HSP27 or HSP72 Expression with Clinicopathological Parameters and Patient Survival

HSP27 expression has been associated with good prognosis in endometrial adenocarcinoma (Geisler, Geisler et al. 1999), oesophageal squamous cell carcinoma (Kawanishi, Shiozaki et al. 1999), malignant fibrous histiocytoma (Tetu, Lacasse et al. 1992) and pancreatic cancer (Schafer, Seeliger et al. 2012). In contrast, to poor prognosis in osteosarcoma (Uozaki, Ishida et al. 2000), hepatocellular carcinoma (King, Li et al. 2000) and prostate cancer (Cornford, Dodson et al. 2000). Although, HSP27 expression and prognosis remains inconclusive in oral squamous cell carcinoma (Ito, Kawabe et al. 1998; Kapranos, Kominea et al. 2002; Lo Muzio, Campisi et al. 2006; Wang, Liu et al. 2009), gastric cancer (Kapranos, Kominea et al. 2002; Giaginis, Daskalopoulou et al. 2009) and ovarian cancer (Elpek, Karaveli et al. 2003; Elstrand, Kleinberg et al. 2009). HSP27 expression has also been reported to have no effect on prognosis in head and neck squamous cell carcinoma (Gandour-Edwards, Trock et al. 1998), bladder cancer (Storm, Mahvi et al. 1993) or renal cell carcinoma (Erkizan, Kirkali et al. 2004). In contrast, HSP72 differential pattern of expression in nasopharyngeal carcinomas has been associated with survival (Cai, Wang et al. 2012). In oesophageal carcinoma the expression intensity of HSP72 is related to the differentiation (Wang, Liu et al. 2005) and correlated with depth of invasion, pathological stage and blood vessel invasion (Nakajima, Kato et al. 2009).

In CRC, elevated expression of HSP27 has been associated with nodal status (Pei, Zhu et al. 2007), TMN staging (Yu, Zhi et al. 2010), poor

prognosis and survival (Garrido, Brunet et al. 2006; Yu, Zhi et al. 2010; Wang, Zhang et al. 2012). In contrast, Zhao et al. (2007) did not demonstrate any significant correlation between HSP27 expression and sex, age, site, histological grade, Duke's stage, and lymph node metastasis. However, the largest study to date by Tweedle and colleagues (2010) found that increased HSP27 expression was associated with poor survival in rectal cancers, but not colon cancer. This is also in line with findings by Bauer and colleagues (2012) found a significant association between high expression of HSP27 and poor prognosis in the cohort of patients with left sided CRC. This is interesting as increased expression of HSP27 have been associated with 5-FU chemotherapy resistance *in vivo* (Tsuruta, Nishibori et al. 2008), which is an important factor in the neoadjunct treatment of rectal cancer. Furthermore, as seen in the recent study by Wang and colleagues (2012) they also demonstrated that the overall survival of patients who had undergone 5-FU-based chemotherapy which had elevated expression of HSP27 was significantly shorter than patients with decreased HSP27 expression.

HSP72 expression is elevated in CRC (Kanazawa, Isomoto et al. 2003) with an association of high HSP72 expression *in vivo* with aggressive tumour behaviour (Hwang, Han et al. 2003; Kanazawa, Isomoto et al. 2003). In a large study by Bauer and colleagues (2012), there was no correlation found between HSP72 expression and histological grade, Duke's stage, and lymph node metastasis. However high HSP72 expression was associated with worse survival of the patients. Interestingly, Pfister and colleagues (2007)

using flow cytometry to show that in the subset of patients with colon carcinoma, HSP72 membrane expression correlated significantly with an improved overall survival; compared to a negative association with survival seen in lower rectal carcinoma. In another study, using ELISA high serum HSP72 levels were associated with poor clinical outcome of colon cancer patients (Kocsis, Madaras et al. 2010; Kocsis, Meszaros et al. 2011).

In my study both HSPs were expressed in the majority of CRC samples, with no expression observed in any normal tissues. When expressed, HSP27 was found to be located mainly in the cytoplasm and this corresponds with our findings, namely HSP27 was discretely expressed in the cytoplasm in 58% of the tumour samples. HSP72 also exhibited staining in both the cytoplasm and nucleus, although staining was primarily cytoplasmic (62%) reflecting the proteins anti-apoptotic role and its effects on senescence (Sherman 2010). However, I did not demonstrate any association of HSP27 or HSP72 expression with clinicopathological parameters and patient survival.

3.3.3 Stratification of CRCs according to TP53 and KRAS mutation and PI3K/AKT activation status

TP53 was mutated in 53% of the tumours, which was similar to previous studies (Levine 1997). Although, the frequency of mutations on each exon of *TP53* in our study cohort marginally differed from a small study of 31 patients, which was previously reported in the literature (Roa et al. 2000).

PIK3CA was found to be mutated in 16% of the tumour samples used here, which was lower than that reported (30%) previously by Samuels et al. (2004), but was similar to other reports of 13.6% (Velho, Oliveira et al. 2005) and 12% (Abubaker, Bavi et al. 2008). Overall, *PIK3CA* amplification was seen in 67% (42/63) of tumour samples and this was slightly higher compared to the frequency of *PIK3CA* amplification in 38% of CRC seen by Jehan and colleagues (2009) in their study of 448 CRC tumour samples, which used both Fluorescence *in situ* hybridization (FISH) and qRT-PCR for validation of copy number change. Similarly, I used qRT-PCR for the detection of gene copy number changes (Kubista, Andrade et al. 2006) as this technique allows for simultaneous amplification and detection of specific DNA sequences. Therefore, by measuring the accumulation of PCR product as it occurs (real time) and by knowing the number of amplification cycles, it is possible to quantify rapidly by extrapolation the number of DNA target molecules present in the initial sample. However, the efficiency and accuracy of real-time PCR is highly dependent on the primers and probes used. Here, the TaqMan Copy number assay measured a duplex real-time PCR reaction in which both the assay for the target gene and the reference assay are run simultaneously in the same reaction reducing the total amount of sample DNA used. The assay has the further advantage of using a pre-designed and validated assay for the target gene, *PIK3CA.*

AKT expression was seen in 96% of the tumour samples reflecting the activation of this important tumourgenesis pathway in CRC. However, just over half of the tumour samples with AKT expression were found to have

either *PIK3CA* mutation or a copy number amplification possibly suggesting a high false negative rate. The qRT-PCR in my study was performed on DNA extracted from fresh frozen tissue, which should provide DNA of good quality. However, it is possible that the quality of DNA maybe influenced by inadequate sample storage, preparation and hence there maybe variation in nucleic acid quality. This would results in variable results, particularly with an assay as challenging as copy number analysis. Furthermore, biological samples are complex and may contain inhibitory substances that can reduce the PCR efficiency (Kubista, Andrade et al. 2006); variable degrees of contamination by normal issue may interfere with estimation of copy number. However, the most likely explanation for any inherent inconsistency concerns the use of controls to limit interplate variability. I used two controls between the PCR plates for comparison, but unfortunately the use of both controls resulted in a wide variation of copy number causing a skewing result. As a consequence it was decided to use 339N only as the control for inter-plate comparison as this reduced apparent variation and corresponded move closer to activated AKT expression as would be expected. However, this may not have been an ideal compromise as D'haene and colleagues (2010) have suggested using as many controls as possible to improve the accuracy and precision of the calculated copy number. However, this needs to be closely monitored for inter-control variability. A more accurate means of determining copy number variations could be achieved by single nucleotide polymorphisms (SNP) analysis although this may be limited by cost (Sengupta, Yau et al. 2013).

3.3.4 HSP27 Expression was Associated with Mutated TP53

Previous studies have shown that premalignant human adenomas display features of senescence, but these are lost upon progression to carcinoma (Collado and Serrano 2005) indicating that OIS could be an important aspect of tumour suppression in CRC *in vivo*. Gabai and colleagues (2009) demonstrated that in cancer cells, HSP72 selectively suppresses TP53-dependent and TP53-independent mechanisms of triggering senescence. Likewise, HSP27 regulates cellular senescence by modulating the TP53 pathway (O'Callaghan-Sunol, Gabai et al. 2007) and, therefore, cancer cells may need or become 'addicted' to HSP expression to suppress the senescence programme in order to survive and to proliferate (Gabai, Yaglom et al. 2009). Consequently, I hypothesised that in cancers with a functional TP53 pathway, cancer cells would be addicted to high levels of HSP expression, whereas in cancers with *TP53* mutations, OIS can be circumvented and their proliferation or survival would not be dependent on HSP expression (Figure 11).

When *TP53* mutation status was compared with HSP27 and HSP72 expression, no significant association was found. I could not confirm increased expression of HSP with wild type *TP53* in primary tumours. In fact, HSP27 expression appears to be expressed more frequently in samples with *TP53* mutations, although this observation did not reach statistical significance. As HSP expression is likely to be involved in multiple pathways associated with tumourgenesis these results may not be unexpected if HSPs

are expressed in the tumour environment as a consequence of genetic alterations other than *TP53*.

3.3.5 HSP27 Expression was Associated in PI3K/AKT Active Tumours with Wild Type KRAS, Independent of TP53 Mutation Status

In tumours with oncogenic activation of the AKT pathway there was an association with HSP27 expression, which may represent an important mechanism in suppressing TP53 dependent senescence allowing cell proliferation and tumourgenesis. In direct contrast, however, HSP72 expression was not found to be associated with activation of the AKT pathway and this may be due to its complex interactions with BAG1 and other pathways that are independent of TP53.

PI3K/AKT activation and *KRAS* mutations are two common oncogenic events in CRC (Barault, Veyrie et al. 2008). Recent evidence has shown that while both the PI3K/AKT pathway and *KRAS* act as moderate and strong inducers of OIS respectively in immortalised fibroblasts, the concurrent activation of these two signalling modules inhibits senescence and accelerates cancer progression *in vitro* (Kennedy, Morton et al. 2011). I proceeded therefore to examine the *in vivo* data on HSP expression in relation to *KRAS* mutation and PI3K/AKT activation. HSP27 expression was associated with PI3K/AKT active, *KRAS* wild type tumours ($p = 0.004$), with more than 90% of these cases showing positive staining. In contrast, only 53% of the tumours with both *KRAS* mutations and PI3K/AKT activation showed HSP27 staining. A possible explanation for this phenomenon could

be that in these tumours, as opposed to cultured cells, PI3K/AKT activation may inhibit RAS induced OIS rendering other mechanisms such as expression of HSP27 redundant (Figure 11B). There is evidence from *in vitro* studies of functional interaction between HSP27 and AKT. For instance, HSP27 can be modified post-translationally through phosphorylation by AKT and other kinases (Kanagasabai, Karthikeyan et al. 2010). In addition, HSP27 chaperones AKT, prevents its dephosphorylation and degradation, hence promoting its stability and activation (Mearow, Dodge et al. 2002). However, the association between these genes and non-TP53 mediated OIS is unclear. Furthermore, HSP expression could not be related to wild type *TP53* in any of our subgroups so it is unlikely that HSP circumvents TP53 mediated OIS in these tumours.

The results presented here show clearly that CRCs have different requirements for HSP expression depending on their *KRAS* mutation and the PI3K/AKT activation status.

In preliminary experiments conducted by Dr J Adam at the Wellcome Institute for Human Genetics a pBabe-PURO SV40 large T construct (provided by P. Jat (UCL)) was used for retroviral infections of CRC cell lines carrying or not carrying the KRAS mutation. A Human G12V KRAS was amplified by PCR from template DNA and cloned into pWZL Hygro vector by restriction enzyme digestion (BamHI and SalI sites) and ligation. Colorectal cell lines, stably infected with KRAS, were generated using amphotropic helper-free phoenix cells that had been transfected with the G12V KRAS construct described above and then placed under Hygromycin selection. It

was consistently observed that cell lines infected successfully with KRAS showed altered, more spindle like morphology and cell doubling time was increased at least approximately two-fold, compared to control cells. Successful KRAS infection was confirmed by western blotting. These transfected CRC cell lines will provide *in vitro* cell culture models to investigate expression of HSP27 and HSP72 and assess the role of KRAS in modulating HSP expression relative to mutation status. These experiments are planned for the future (Appendix VII).

3.3.6 Colorectal Cancers are Mostly Positive for Heat-Shock Factor 1, but Expression was not Associated with Clinicopathological Features

HSF1 expression has been shown *in vivo* to be are elevated in number of cancer cell lines and tumour tissues, including prostate cancer cells (Hoang, Huang et al. 2000) and breast cancer (Khaleque, Bharti et al. 2008). HSF1 expression was seen in the majority of the tumour samples mainly exhibiting strong staining in the nucleus. This is not unsurprising as, when activated by stressors HSF1 accumulates within the nucleus where it binds to DNA containing heat shock elements that induce HSPs (Collado and Serrano 2005). In breast cancer HSF1 expression has been shown to increase and correlate with histological grade: HSF1 expression was highest in high grade breast cancer and advanced clinical stage; and HSF1 expression was associated with reduced survival (Santagata, Hu et al. 2011). In contrast, when the expression of HSF1 was examined in relation to: Duke's stage; depth of invasion (T stage); nodal metastases (N stage) and distant

metastases (M stage), no significant association was shown between HSF1 expression and any of these features.

3.3.7 Heat Shock Transcription Factor 1 Expression was not Associated with TP53 or KRAS Mutation

As HSPs are thought to be selectively up-regulated to suppress OIS in the absence of *TP53* mutations, thereby allowing uncontrolled proliferation, a possible role for HSF1 in this process was investigated (Sherman 2010). Interestingly, in fibroblasts the activation of TP53 is mediated through a pathway that is regulated by HSF1 and that this results in the transportation of TP53 into the nucleus (Li, Feldman et al. 2008). Furthermore, following on from the correlation observed here between HSP27 expression and *KRAS* mutation and PI3K/AKT activation status, a possible role for HSF1 in this process was also investigated. There was no significant association observed between *KRAS*, *TP53* mutation status and HSF1 expression. This may not be unexpected from studies suggesting a more complicated interplay between HSF1 and these oncogenic pathways. Min and colleagues (2007) in their study using *TP53*$^{-/-}$ mice that usually develop lymphomas found that loss of HSF1 altered the spectrum of tumours that arose in *TP53*$^{-/-}$ mice instead of prolonging expected tumour free survival. In *HSF1*$^{-/-}$*TP53*$^{-/-}$ mice, other tumour types developed including testicular carcinomas and soft tissue sarcomas, although there was no significant effect either on overall tumour incidence or on tumour-free survival (Min et al., 2007). In contrast, Dai and colleagues (2007) using *HSF1*$^{-/-}$ mice carrying a mutant *TP53* allele, found that tumour-free survival was prolonged dramatically.

3.3.8 BAG1 was Expressed in the Cytoplasm and Nucleus of a Significant Proportion of Colorectal Cancers

Overexpression of BAG1 protects cells from various apoptotic stimuli, enhanced proliferation and metastasis, and modulated the transcriptional activity of a variety of nuclear hormone receptors (Cutress, Townsend et al. 2002). The function of BAG1 in tumourgenesis has been attributed to its relationship with HSP72 as a co-chaperone and mediator of BAG1 functions (Townsend, Stephanou et al. 2005). BAG1 was expressed in 86% of tumour samples and this was also reflected by the expression of HSP72 (87%). The overall rate expression of BAG1 matched the expression seen in other IHC studies on CRC (Bai, Yi et al. 2007). Interestingly, all of the tumour samples that expressed BAG1 showed cytoplasmic staining with either strong or discrete cytoplasmic staining, but none showed exclusive nuclear staining. This was in contrast to the large study by Sun and colleagues (2011) which used a different antibody for BAG1 for their IHC finding that most of their tumours expressed BAG1 in the nucleus, and only a few expressed BAG1 immunopositivity in the cytoplasm.

A significantly higher expression of nuclear (62%) and cytoplasmic BAG1 expression (86%) was observed here in comparison to Kikuchi and colleagues (2002). These workers had reported previously a BAG1 expression of only 5% using a similar technique of standard avidin-biotin-peroxidase complex IHC technique. However when they used a Catalyzed Signal Amplification system (DAKO Corp), nuclear BAG1 was seen in 24% and cytoplasmic BAG1 in 65.1% of tumour samples respectively.

3.3.9 BAG1 Expression was Associated with Increase Depth of Tumour Invasion, But Not Patient Survival

Sun and colleagues (2011) in their study of 320 colon cancer samples demonstrated a significant association with increased BAG1 expression and pathologic grade, distant metastasis, Dukes stage, and prognosis, but expression was not correlated with the pathologic type, tumour diameter, depth of invasion, and lymph node metastasis. Bai and colleagues (2007) also found a significant association with degree of tumor differentiation, Dukes staging, metastasis and survival. Here, BAG1 expression was significantly associated with increased depth of invasion. However, if Bonferroni correction is applied (r=6; lowers p from <0.05 to <0.008) then significance is lost. No significant correlation with BAG1 expression and survival was found in the cohort of CRCs examined here. Multivariate logistical regression to adjust for Duke's stage might reveal an association as these two studies had randomly even proportions of samples at each stage compared to the study cohort used here, which had 70% Duke's stage B or C. Nuclear BAG1 expression has been also demonstrated as a useful predictive factor for metastasis and poor prognosis of CRC (Kikuchi, Noguchi et al. 2002). When comparing the present groups of tumour samples with or without nuclear expression I did not find a significant association with survival. The increased frequency of nuclear and cytoplasmic BAG1 expression in my study might explain the differences that were found in survival between previous studies.

3.3.10 Lack of BAG1 Expression is Associated with TP53 Mutation Status

It has been suggested that BAG1 overexpression may in fact be due to the up regulation of the BAG1 promoter by mutated *TP53* (Yang, Pater et al. 1999). Furthermore Wang and colleagues (2009) showed that BAG1 inhibited the transcriptional activating functions of TP73, and to a lesser extent TP63 and TP53. Therefore, BAG1 may interfere with TP53 growth arrest and stress-induced apoptosis in tumourgenesis. However, *TP53* mutation was associated with negative expression of BAG1 in the study presented here. Therefore, in CRCs with WT *TP53*, BAG1 was most likely to be expressed, whilst in those cancers with *TP53* mutations, BAG1 was not likely to be expressed.

3.3.11 Summary

In summary, our data suggests that HSP expression does not play a role in circumventing TP53 mediated OIS in CRC. CRCs may have different requirements for HSP expression that are dependent on *KRAS* mutation and PI3K/AKT activation status. Further *in vivo* investigations on a larger number of cancers will be necessary to complement investigations that have been conducted *in vitro* into oncogenic modulation of OIS. The work presented in the chapter pertinent to OIS has been published in Ghosh and colleagues (2012).

CHAPTER 4

Evaluating the role of Amyloid Precursor Protein and BCL2-associated Athogene in colorectal cancer

4.1 Overview and Rationale

APP expression has been shown to be dysregulated in glioblastoma, melanoma, and in oral squamous cell carcinoma, thyroid, prostate, and pancreatic cancer (Hansel, Rahman et al. 2003; Ko, Lin et al. 2004; Takayama, Tsutsumi et al. 2009; Botelho, Wang et al. 2010; Yang, Fan et al. 2012). Loss-of-function studies of these cancers have suggested an important role of APP in tumourgenesis through its involvement in cellular proliferation (Meng, Kataoka et al. 2001; Hansel, Rahman et al. 2003; Ko, Lin et al. 2004; Takayama, Tsutsumi et al. 2009; Botelho, Wang et al. 2010; Venkataramani, Rossner et al. 2010; Yang, Fan et al. 2012). Furthermore, APP expression has been correlated to melanoma and thyroid carcinoma tumour stage ((Botelho, Wang et al. 2010; Yang, Fan et al. 2012) and survival in oral squamous cell and prostate carcinoma. These studies highlight APP as a prognostic marker and potential therapeutic target (Ko, Lin et al. 2004; Takayama, Tsutsumi et al. 2009).

Only two studies have investigated the role of APP in CRC. These have used a small number of CRC cell lines (SW480, LOVO, CaCo-2, T84 and SW837) to investigate the role of APP (Meng, Kataoka et al. 2001; Venkataramani, Rossner et al. 2010). All have suggested that APP has an

essential role in cellular proliferation and growth, but only SW837 has been used for APP inhibition with antisense clones to inhibit expression. This resulted in reduced proliferative potential and colony forming efficiency, which was rescued with the conditioned medium of parent cells or with purified sAPP (Meng, Kataoka et al. 2001). Furthermore, reduced tumour proliferation *in vivo* was exhibited in the antisense clones using nude mice (Meng, Kataoka et al. 2001). More recently, Venkatarami and colleagues (2010) using siRNA knockdown of APP on a single line, SW480, demonstrated reduced cell proliferation. Valproic acid (VPA, 2-propylpentanoic acid), a histone deacetylase inhibitor known to down regulate APP and sAPP through its effects on APP maturation via its upregulation of GRP78, was used on SW480, LOVO, CaCo-2, T84 to demonstrate reduced cell proliferation when APP was inhibited by the drug (Venkataramani, Rossner et al. 2010).

Interestingly, in the brain tissue of AD patients, APP is co-localised with BAG1, the tumour co-chaperone, (Elliott, Laufer et al. 2009). Furthermore, the over expression of BAG1 was associated with increased amount of intracellular APP suggesting both a strong physical and a functional relationship between BAG1 and APP (Elliott, Laufer et al. 2009).

To date, only one study has used primary CRC samples to examine APP protein expression and IHC was reported for a very small sample size (n=3) (Venkataramani, Rossner et al. 2010). These workers reported strong expression of APP in colon carcinoma cells whereas no expression was seen in normal epithelial cells. However, the presented images showed very

high expression of APP that might be a consequence of overstaining caused by an overnight incubation (Venkataramani, Rossner et al. 2010).

To elucidate the role of APP in tumourgenesis and investigate a possible link to malignant progression in CRC, a larger panel of previously unstudied colonic and rectal adenocarcinoma cell lines and primary CRC samples was investigated (Figure 19A). IF was used to determine the location of APP in CRC cells. The effect of APP knockdown was evaluated, along with inhibition by VPA, to investigate the potential use of this novel drug in colon cancer therapy. Furthermore as APP and its secreted forms have been shown to promote migration (Thinakaran and Koo 2008), the effect of APP on cell migration was also studied. In addition, the potential role of combined BAG1 and APP expression in tumourgenesis was examined. We hypothesised that BAG1 inhibition or overexpression will result in reduced expression or overexpression of APP (Figure 19 B and C). Finally, as has been suggested in other cancers, the relationship of APP expression and clinicopathological features and survival of CRC patients was evaluated. First, the expression of APP and BAG1 was determined at mRNA and protein level using QTRT PCR and Western Blot in a panel of colonic and rectal adenocarcinoma cell lines: C80; C99; HCT15; HCT116; HT55; LOVO; LS174T and SW837.

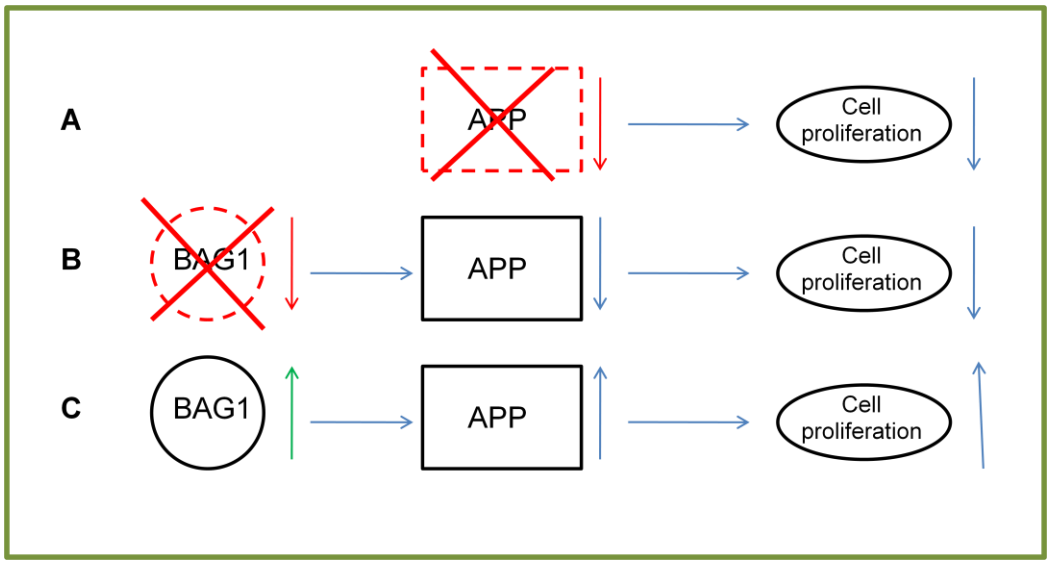


Figure 19. Background on Amyloid precursor protein findings from cell culture based studies and our hypothesis for testing in colorectal cancer. A: In CRC only two studies have shown that loss-of-function experiments of APP leads to reduced cell proliferation (Ventaramani et al. 2010; Meng et al. 2001). Our hypothesis: In a novel panel of CRC cell lines there will be a decrease in cell proliferation when APP expression is inhibited which will also correlate with findings for APP alterations in other cancers. B and C. Background: In AD in which there has been shown to be a physical relationship between BAG1 overexpression and APP expression, BAG1 expression has been shown to be correlated with APP expression (Elliott et al 2009). Our hypothesis: BAG1 inhibition or overexpression will result in reduced expression or overexpression of APP.

4.2 Results

4.2.1 Expression of BCL2-associated Athanogene and Amyloid Precursor Protein Varies in Colorectal Cancer Cell Lines

The expression of both *BAG1* and *APP* was measured at mRNA level using QRT PCR with quadruplicate samples of each cell line (see Section 2.2.6). Data was analysed relative to LOVO because this cell line showed the lowest level of expression compared to the other cell lines. QRT PCR demonstrated a seven-fold increase in expression of BAG1 in C99 and nearly a five-fold change of C80 relative to the LOVO cell line. SW837, HCT116 and LS174T had approximately a two and a half-fold increase in expression, while there was only a small fold change difference in HT55 and HCT15 (Figure 20). Western Blot showed expression of all the isoforms of BAG1 with the cytoplasmic BAG1 isoforms (33kDa) the most abundant. The cytoplasmic/nuclear BAG1 isoform (46kDa) was expressed the least in all cell lines. In HT55 and C80 the nuclear BAG1 isoform (50kDa) was expressed at low levels. Western blot quantification was undertaken for overall BAG1 gene expression. This was plotted relative to LOVO to allow comparison with mRNA BAG1 expression. Western blot quantification found that HCT116 had the highest expression of BAG1, whilst HCT15 and HT55 had the lowest expression of BAG1 relative to LOVO expression. C80 and C99 (0.8 relative expression to LOVO both respectively) had a lower expression of BAG1 at protein level compared to LOVO when compared to mRNA level (Figure 21). Overall, there was a difference in the level of

expression of BAG1 at mRNA and protein level, with BAG1 expressed with a greater variance at mRNA level than at protein level.

QRT PCR for mRNA expression of *APP* was also characterised relative to LOVO because this cell line showed the lowest level of expression compared to the other cell line. APP was increased nearly four-fold in SW837 and had a threefold change in C99 and HCT15. The rest of the cell lines C80, HT55, HCT116 and LS174T had an approximately two-fold increase (Figure 22). Western Blot showed expression of both the immature and mature isoforms of APP (110kDa and 130kDa). Western blot quantification was calculated for overall APP gene expression. This again was plotted relative to LOVO to allow comparison with mRNA *APP* expression. Protein expression of APP was highest expressed in HT55, whilst LS174T and SW847 had the lowest expression of APP relative to LOVO expression. This was in contrast to mRNA expression in which SW837 had the highest relative expression of *APP* (Figure 23).

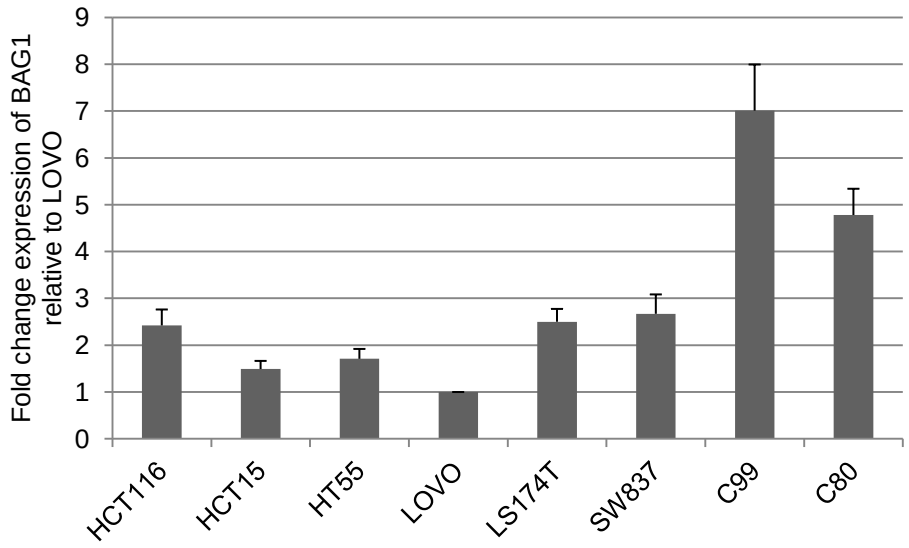


Figure 20. Variable expression of BAG1 in a panel of colorectal cancer cell lines. Expression assessed at mRNA level by QRT-PCR from mean of four experimental replicates.

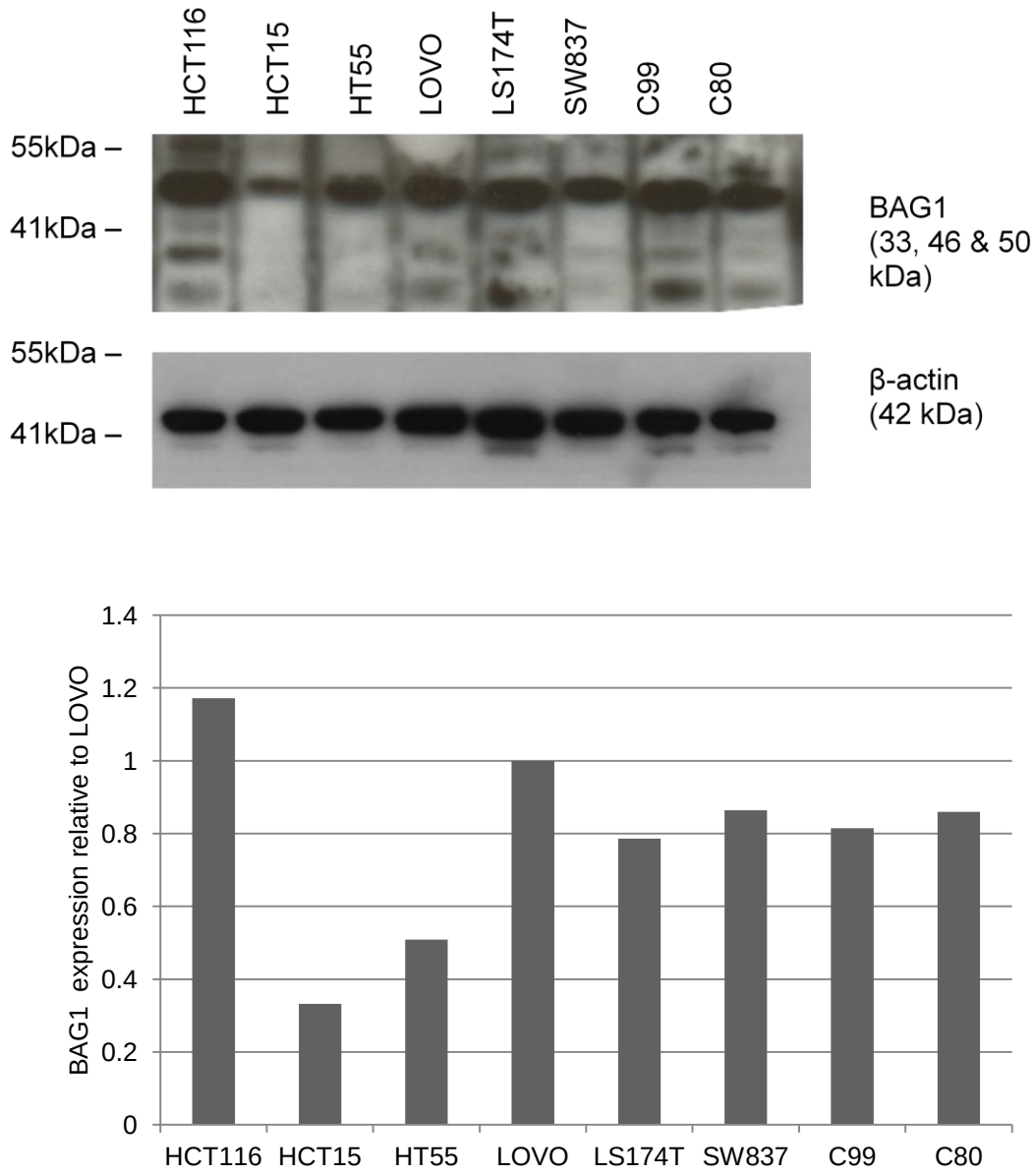


Figure 21. Variable expression of BAG1 in a panel of colorectal cancer cell lines. Expression assessed at the protein level by Western Blot. QRT-PCR for *BAG1* showed it to be expressed most highly in C80 and C99 with a fold change of 4.78 and 7 respectively. Western Blot showed expression of all the isoforms of BAG1 with the cytoplasmic BAG1 isoforms (33kDa) the most abundant and the cytoplasmic/nuclear BAG1 isoform (46kDa) expressed the least in all cell lines. In HT55 and C80 the nuclear BAG1 isoform (50kDa) was expressed at low levels. Western blot quantification was for overall BAG1 gene expression. This again was plotted relative to LOVO to allow comparison with mRNA *BAG1* expression. HCT116 had the highest expression of BAG1, whilst HCT15 had the lowest expression of BAG1 relative to LOVO expression. C80 and C99 had a lower expression of BAG1 at protein level compared to LOVO when compared to mRNA level.

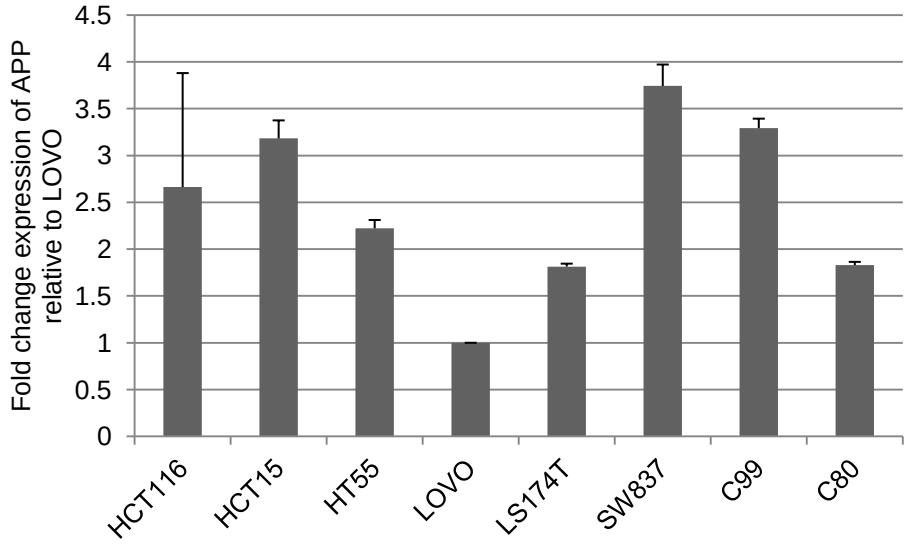


Figure 22. Variable expression of Amyloid precursor protein in a panel of colorectal cancer cell lines. Expression assessed at mRNA level by QRT-PCR from mean of four experimental replicates.

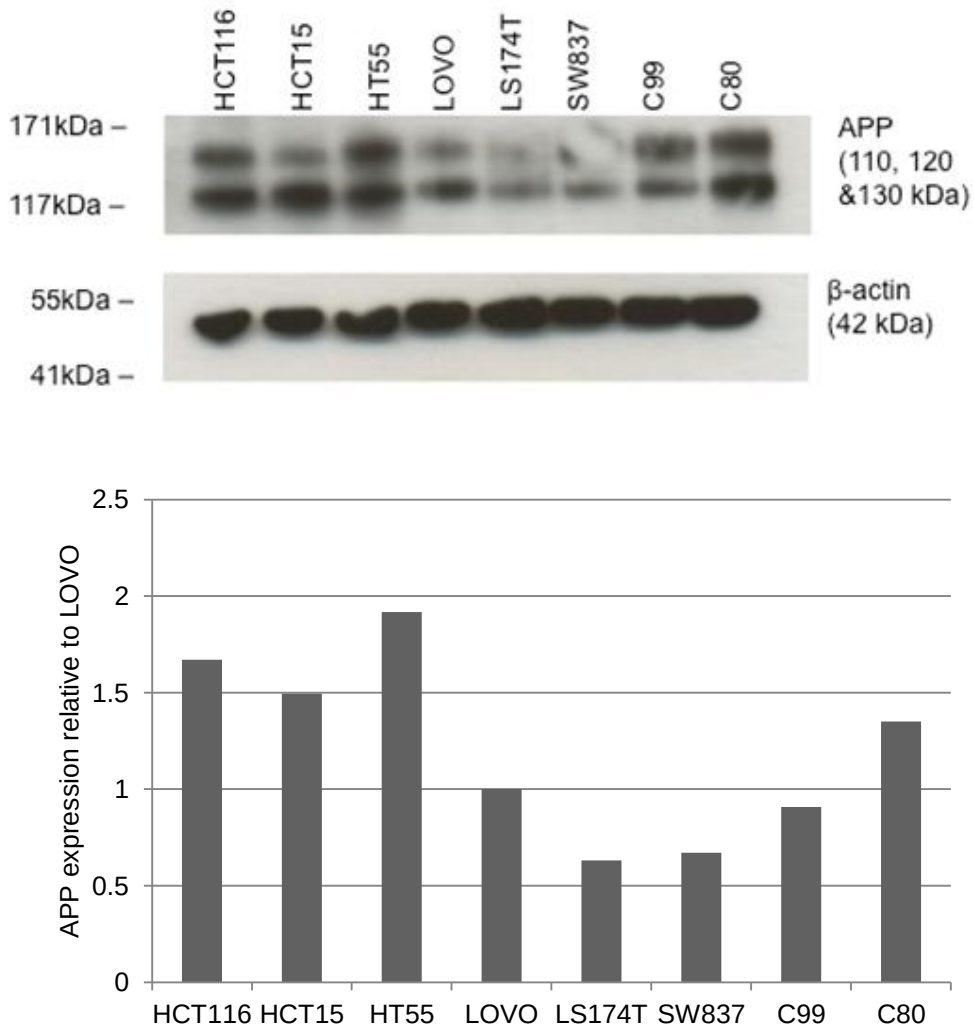


Figure 23. Variable expression of Amyloid precursor protein in a panel of colorectal cancer cell lines. Expression assessed at mRNA level by QRT-PCR from mean of four experimental replicates (A) and at protein level by Western Blot (B). QRT PCR for APP showed it to be expressed most highly in SW837 with nearly a four-fold increase in expression in SW837 relative to LOVO. There was a threefold change in C99 and HCT15. The rest of the cell lines C80, HT55, HCT116 and LS174T had an approximately two-fold increase expression of APP relative to LOVO. Western Blot showed expression of both the immature and mature isoforms of APP. Western blot quantification (C) was for overall APP gene expression. This again was plotted relative to LOVO to allow comparison with mRNA APP expression. HT55 had the highest expression of APP, whilst LS174T and SW847 had the lowest expression of APP relative to LOVO expression. This was in contrast to mRNA expression in which SW837 had the highest relative expression of APP. Overall there was a difference in the level of expression of BAG-1 at mRNA and protein level, with APP expressed with a greater variation of expression at mRNA level than at protein level.

4.2.2 Expression and Location of Amyloid Precursor Protein in Colorectal Tumour Cells

IF was performed on the cell lines to identify the location of different length APP strands in CRC cell lines. Understanding the location of a protein within the cell is a key part of determining its functional role. IF was carried out with the 22C11 antibody (Millipore, Billerica, MA, USA) which recognises amino acids 66-81 of the N-terminus of APP (Hilbich, Monning et al. 1993) and therefore full length APP plus all its N-terminal cleavage products. 22C11 recognises all three isoforms of APP: immature ~110kDa, sAPP ~120kDa, and mature ~130kDa after O-glycosylation (Hoffmann, Twiesselmann et al. 2000). By using the APP antibody (Millipore, Billerica, MA, USA) IF was expected to demonstrate a cytoplasmic distribution (Thinakaran and Koo 2008). However, in HCT116, C80, C99 and HT55 some nuclear staining was observed (Figure 24 -31).

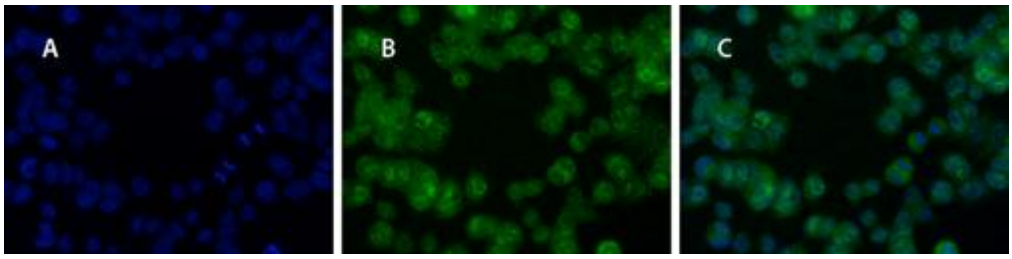


Figure 24. Representative example of fluorescent immunohistochemistry on HCT116 at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is observed in both cytoplasmic and nuclear compartments, although cytoplasmic staining is stronger.

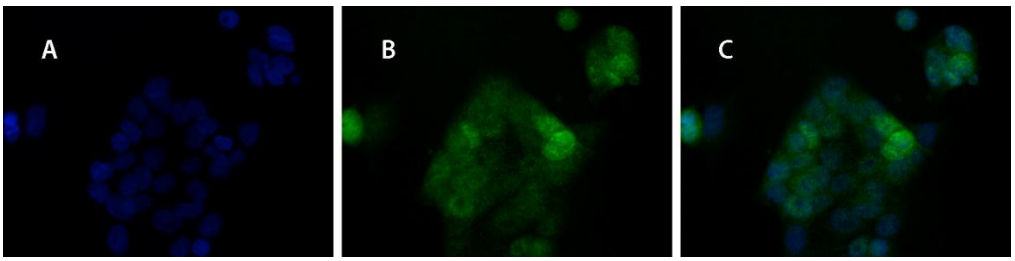


Figure 25. Representative example of fluorescent immunohistochemistry on HCT15 at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is cytoplasmic.

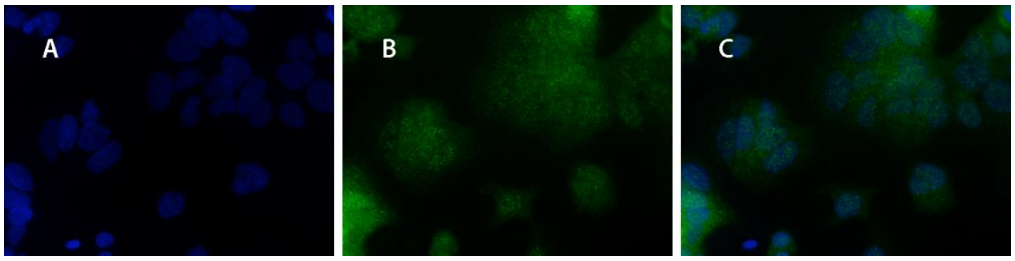


Figure 26. Representative example of fluorescent immunohistochemistry on HT55 at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is observed in both cytoplasmic and nuclear compartments, although cytoplasmic staining is stronger.

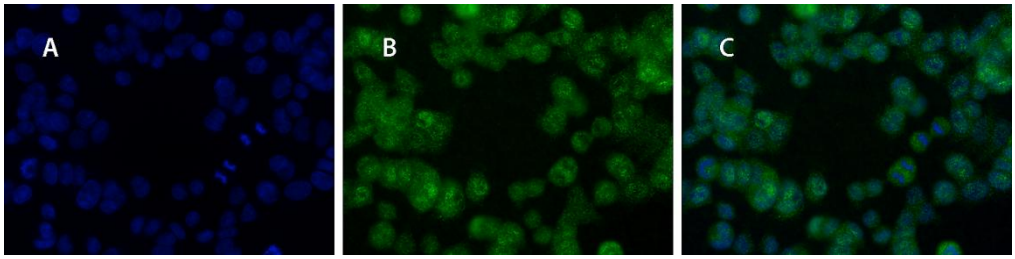


Figure 27. Representative example of fluorescent immunohistochemistry on LOVO at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is cytoplasmic.

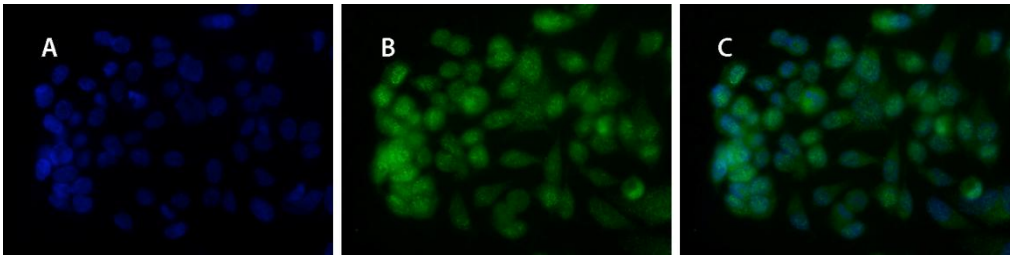


Figure 28. Representative example of fluorescent immunohistochemistry on LS174T at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is cytoplasmic.

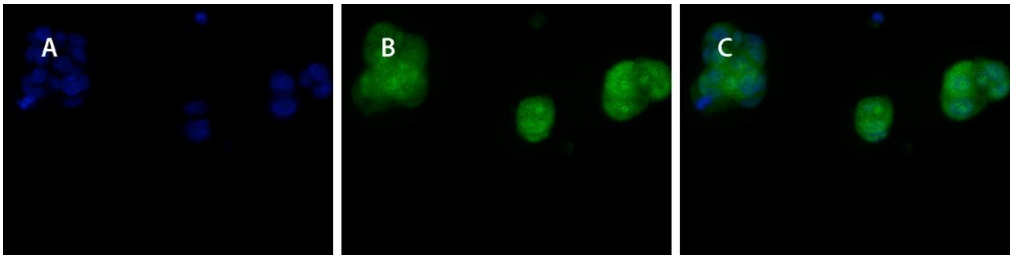


Figure 29. Representative example of fluorescent immunohistochemistry on SW837 at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is cytoplasmic.

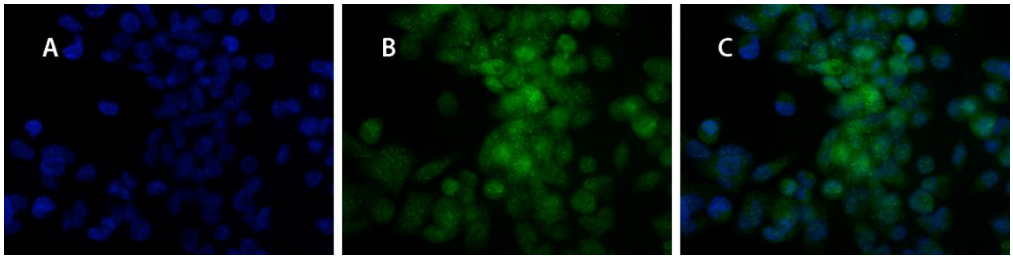


Figure 30. Representative example of fluorescent immunohistochemistry on C99 at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is observed in both cytoplasmic and nuclear compartments, although cytoplasmic staining is stronger.

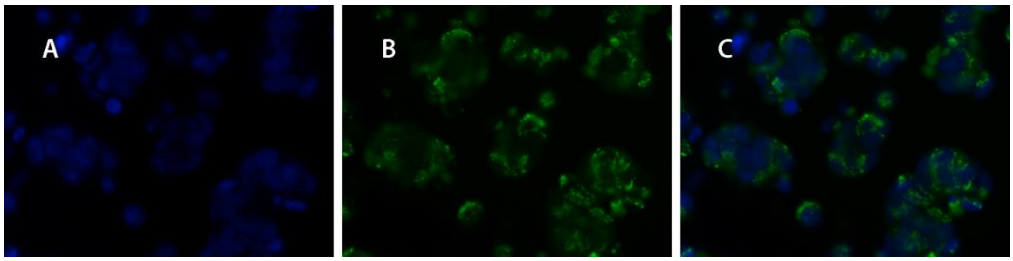


Figure 31. Representative example of fluorescent immunohistochemistry on C80 at 20x magnification. (A) DAPI staining of the nucleus is shown in blue; (B) APP staining in green; (C) Merged. APP expression is observed in both cytoplasmic and nuclear compartments, although cytoplasmic staining is stronger.

4.2.3 Nuclear and Cytoplasmic Expression of Amyloid Precursor Protein is Observed in Colorectal Cancer Cell Lines

To further evaluate the precise distribution of full length APP and its N-terminal cleavage products in CRC cell lines, confocal microscopy images for HCT116, C80, C99 and HT55 were analysed using MetaMorph® (see Section 2.3.2.4). APP expression was analysed from five different fields. This served to determine the expression of full length APP and N-terminal cleavage products in cytoplasmic or nuclear compartments of tumour cell lines. APP was expressed to a greater extent in the cytoplasmic compartment of all the cell lines. When comparing cytoplasmic with nuclear expression, full length APP was significantly expressed (all p values >0.01, Student's t-test) in the cytoplasmic relative to the nuclear compartment for C80, C99 and HT55 had the greatest proportion of APP expressed in the cytoplasm compared to the nucleus, with mean of 89% of APP expressed in the cytoplasm (p=0.000007, Student's t-test). HCT116 showed a trend towards significance for cytoplasmic expression of APP with 56% of APP expressed in the cytoplasm compared to the nucleus (Figure 32; p=0.09, Student's t-test). Therefore, the full length APP and N-terminal cleavage products were cytoplasmic in distribution.

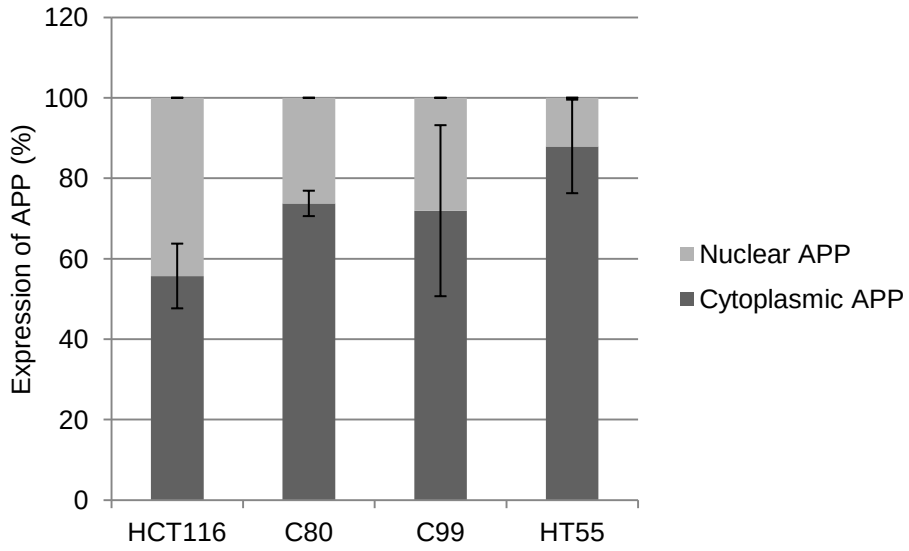


Figure 32. The distribution of full length Amyloid precursor protein in colorectal cancer cell lines. Data was obtained and analysed for five different fields images per line providing a mean and standard deviation between experiments The distribution of full length APP is mostly cytoplasmic in colorectal cancer cell lines, APP was seen to be more highly expressed in the cytoplasmic compartment of cells lines: HCT116; C80; C99 and HT55 (all p values >0.01, Student's t-test).

4.2.4 Knockdown of Amyloid Precursor Protein Results in an Inhibition in Tumour Cell Proliferation

To investigate the functional relevance of APP in CRC loss of function studies using siRNA knockdown was used. HCT116 was used as it proliferated rapidly, and was found to be easiest to transfect for loss-of-function studies using siRNA knockdown (see Section 2.4.2). The optimisation of the cell transfection is crucial to the efficiency of the experiment. Therefore, the amount of Lipofectamine 2000 for transfection was optimised first, using a specific siRNA that targets PLK-1. This results in the 'killing' of cells when successfully transfected (Strebhardt and Ullrich 2006) and can be used, therefore, as a marker of transfection efficiency. A volume of 5 µL of Lipofectamine resulted in significant cell death (70%) as quantified by the SRB cell proliferation assay and was used henceforth as the optimum condition for transfection (Figure 33).

Next, HCT116 was transfected with 4 siRNA's APP2, APP8, APP9 and APP10 (details of specific siRNAs given in Appendix II) to establish knockdown of APP expression. Western blot showed that transfection with siRNA's APP2, APP9 and APP10 resulted in knockdown of APP expression when compared to those cells transfected with scrambled siRNA (Figure 34). However, the siRNA APP8 did not knockdown APP expression. Analysing the blot intensity relative to the scrambled siRNA (see Section 2.6.2.5) demonstrated a significant knockdown of APP by siRNA APP2, APP9 and APP10 (all p values>0.001, Student's T test). siRNA APP10 resulted in the most significant knockdown of APP expression (92% knockdown). APP2

resulted in a 76% and APP9 85% knockdown of APP expression (Figure 34). In contrast, the siRNA APP8 caused an unexpected significant increase in APP expression ($p>0.005$). The expression of *APP* was suppressed efficiently by treatment of HCT116 cells with siRNAs APP2, APP9 and APP10 (Figure 34).

The effect of APP knockdown on cell proliferation was measured using SRB cell proliferation assay with each experiment carried out in triplicate (see Section 2.4.4.2). The transfection of HCT116 with siRNA's APP 2, APP 9 and APP 10 resulted in inhibition of cell proliferation. siRNA APP 10 resulted in the most significant inhibition in cell proliferation with tumour cell proliferation reduced by 75% compared to cell transfected with scrambled siRNA ($p=0.0005$, Student's t-test). In contrast, siRNA APP 9 40% and APP 2 caused 25% inhibition of tumour cell proliferation ($p=0.001$ and 0.01 respectively, Student's t-test) (Figure 35).

Therefore, in the CRC cell line HCT116, siRNA knockdown leading to a loss of function of APP results in an inhibition of cell proliferation.

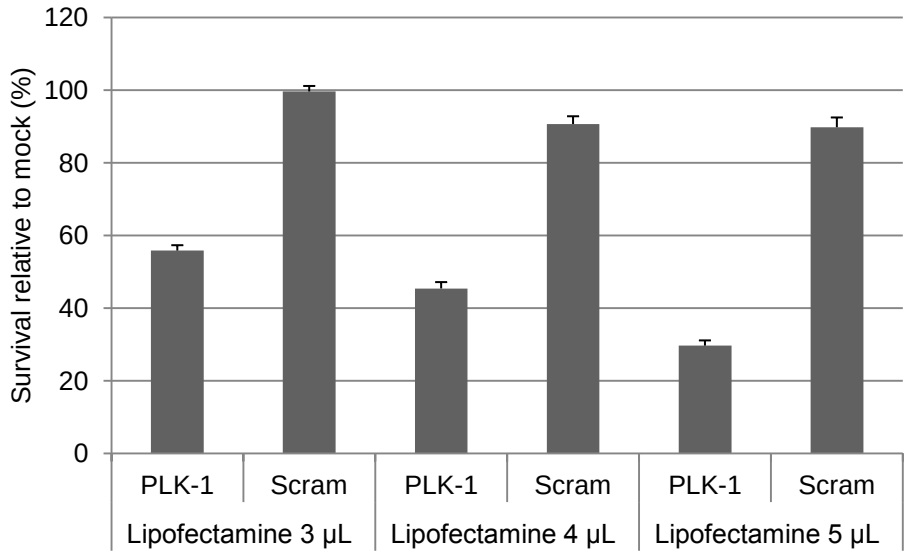
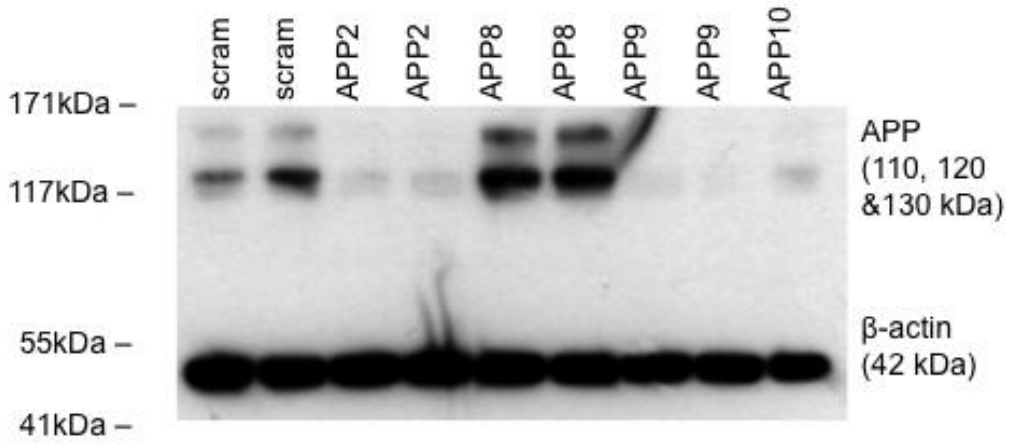


Figure 33. Transfection optimisation of HCT116 cells identifies the most appropriate Lipofectamine volume. Transfection of HCT116 cells with different concentrations of Lipofectamine was undertaken and cell death measured using SRB cell proliferation assay and compared to mock transfected cells. Using 5 µL of Lipofectamine resulted in most significant cell death (70%) as quantified by the SRB cell proliferation assay and was used, thereafter, as the optimum condition for transfection. Three experimental replicates were undertaken and each SRB assay was carried out in quadruplicate. The graph shows the mean average survival relative to the mock transfection. The error bars showing the standard deviation between replicates.

A



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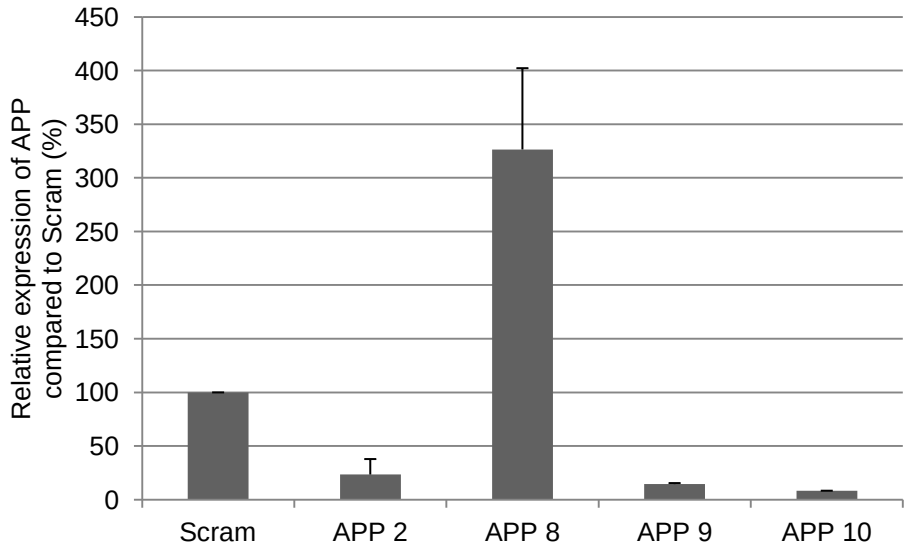


Figure 34. Most siRNAs targeted against Amyloid precursor protein cause efficient knockdown of gene expression. Western Blot images (A) of HCT116 cell line transfections with scrambled (Scram) siRNA; knockout siRNA APP 2; APP 8; APP 9 and APP 10. This shows effective knockout of APP with siRNA APP 2, APP 9 and APP 10, but upregulation with APP 8 B. Western blot quantification was calculated relative to scram expression of APP. Average blot density from two replicates with error bar showing the standard deviation. This figure shows a significant knockdown of APP by siRNA APP 2, APP 9, APP 10 ($p>0.001$, Student t-test). siRNA APP 8 caused a significant increase in APP expression ($p>0.005$, Student's t-test).

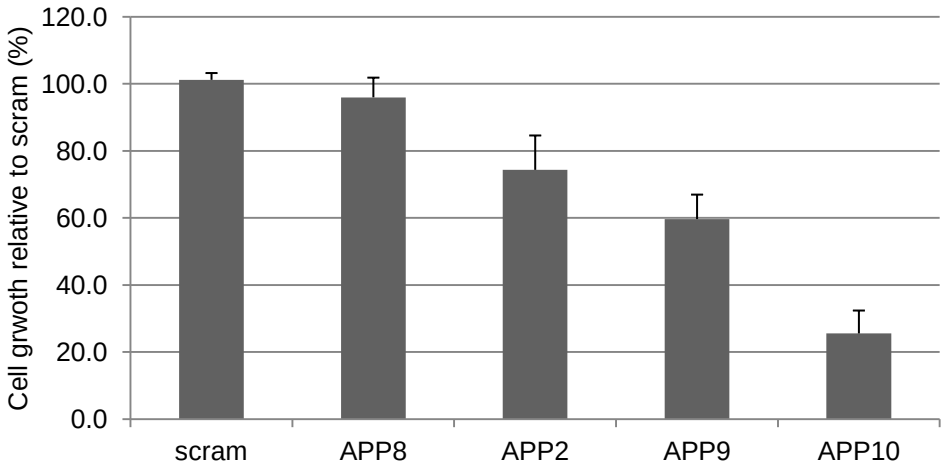


Figure 35. The effect of Amyloid precursor protein knockdown on cell proliferation. Using Sulforhodamine B Colorimetric cell proliferation assay with each experiment carried out in triplicate and a mean calculated for each siRNA with error bars showing the standard deviation. Knockdown of APP using siRNA resulted in a significant reduction in cell proliferation. HCT116 cell line transfected with: scrambled (scram) siRNA; knockout siRNA APP 2; APP 8; APP 9 and APP 10. Cell proliferation compared to scram using SRB cell proliferation assay after 72 hours post transfection. A significant reduction in cell proliferation as a result of APP knockdown for siRNA's APP 2, APP 9 and APP 10 was found ($p>0.01$, Student's t-test). The most significant reduction in cell proliferation was observed with siRNA APP 10 ($p=0.00005$, Student's t-test).

4.2.5 Valproic Acid Reduces Cell Proliferation in Colorectal Cancer Cell Lines

VPA is a histone deacetylase inhibitor which has been demonstrated to specifically inhibit APP maturation through its upregulation of an endoplasmic reticulum chaperone immunoglobulin-binding protein GRP79 and thereby down-regulate APP and sAPPα (Venkataramani, Rossner et al. 2010). It has been suggested as a novel drug for use in the treatment of CRC. Consequently, VPA was used to further investigate the effect of APP on cell proliferation and hence its effects on cellular tumourgenesis pathways in our full panel of colonic and rectal adenocarcinoma cell lines: C80; C99; HCT15; HCT116; HT55; LOVO; LS174T and SW837.

VPA drug inhibition was carried out initially at the lower concentrations (0mM, 1mM, 2.5mM, 5mM and 10mM) used by Venkataramani et al. (2010) on the single colon cancer cell line SW480 and the pancreatic cell line BxPC3. VPA at these low concentrations demonstrated a down regulation of APP (Venkataramani, Rossner et al. 2010). Cell lines were treated for 48 hours with VPA and the effect of VPA on cell proliferation was measured using Cell Titre Blue (see Section 2.4.4.1). When VPA exposed cell lines were compared with non treated controls one line, HCT116, demonstrated an overall significant inhibitory effect on cell proliferation, but only at the higher doses used (5mM and 10mM inhibited proliferation 27% and 42% compared to non treated cells, $p=0.004$ and $p=0.003$, respectively, Students's t-test). At the lower doses, there was no significant difference in cell proliferation between treated and non-treated HCT116 cells (1mM and

2.5mM VPA reduced cell proliferation 4% and 6%, respectively, p=0.765 and p=0.269, Student's t-test). The other cell lines showed variable effects at different concentrations of VPA, although none showed any significant difference when comparing non treated cells with drug treated cells (all p values >0.5) (Figure 36). Therefore, VPA did not demonstrate a stepwise inhibition in cell proliferation in this panel of CRC cell lines at these concentrations with the exception of HCT116, which showed inhibition at the highest two doses used. These results contradict the findings of Venkataramani and colleagues for the single cell line they used, SW480.

Although the results presented above for a panel of CRC cell lines did not confirm Venkataramani et al. (2010) findings at doses between 1 and 10 mM, these workers did also show a stepwise inhibition of cell proliferation when VPA was used at a ten-fold higher concentrations (12.5mM, 25mM, 50mM and 100mM). At these higher concentrations of VPA there was a significant concentration-dependent inhibition of cell proliferation observed in all our CRC cell lines (p>0.000007, Student's t-test). HCT116, LOVO and LS174T showed the most significant concentration-dependent effects on cell proliferation following treatment of VPA with more than 75% cell proliferation inhibition at 100mM. In contrast, C80 showed the least concentration-dependent effect on cell proliferation, but still showed a 62% cell proliferation inhibition at the same VPA dose (Figure 37).

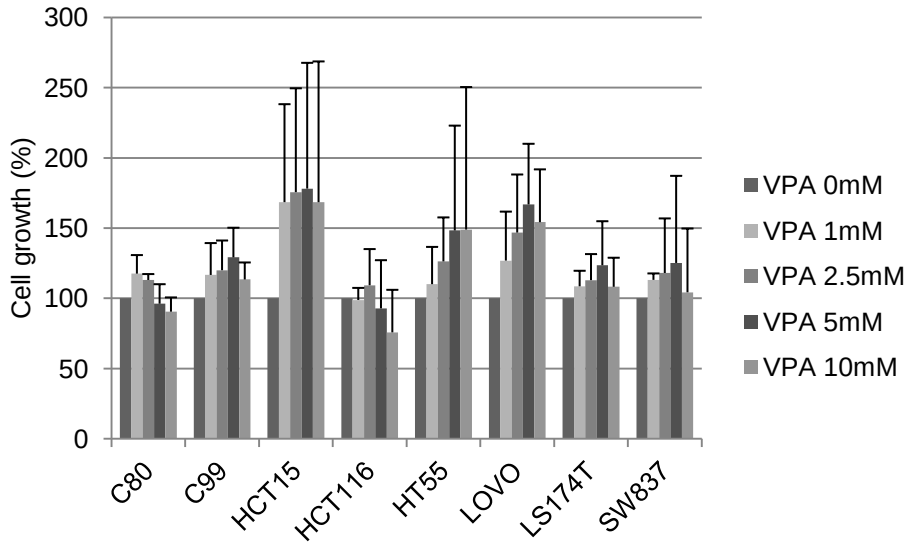


Figure 36. Valproic acid has a concentration-dependent influence on cell proliferation only in HCT116. Proliferation of treated cells was compared to non treated cells following 48 hours of VPA treatment at 1.25mM, 2.5mM, 5mM and 10mM on cell lines: C80; C99, HCT15, HCT116, HT55, LOVO, LS174T and SW837. Results are the mean of three experiments and the error bars represent the standard deviation. A significant inhibition of cell proliferation in HCT116 was observed at the higher doses of 5 and 10 mM ($p = 0.003$, Student's t-test). There was a variable effect on cell proliferation for the other cells lines, although this not significant.

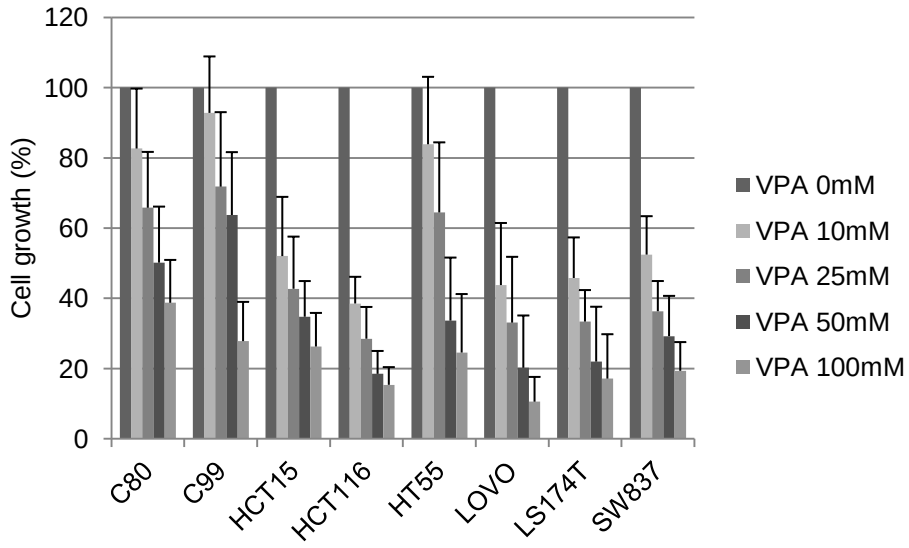
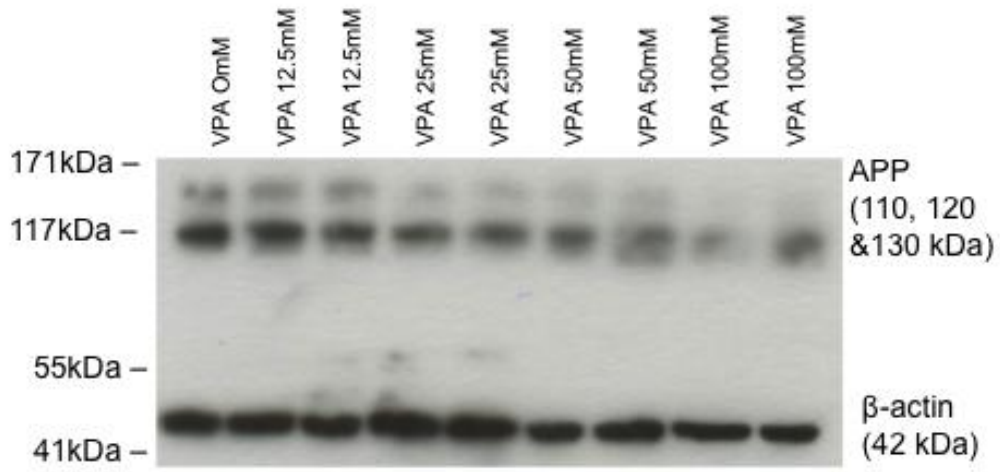


Figure 37. Valproic acid has a significant concentration-dependent influence on cell proliferation at high doses. Results shown are the mean of three experiments with error bars representing the standard deviation. Concentration-dependent effect on cell proliferation was demonstrated following 48 hours of VPA treatment at 12.5mM, 25mM, 50mM and 100mM on all cell lines (C80; C99, HCT15, HCT116, HT55, LOVO, LS174T and SW837). HCT116, LOVO and LS174T showed the most significant dose dependent effect ($p>0.000007$, Student's t-test).

4.2.6 Valproic Acid Inhibits Expression of Amyloid Precursor Protein in Colorectal Cancer Cell Lines

The effect of VPA on the expression of APP was therefore investigated in HCT116 as it was the most sensitive to VPA drug treatment and exhibited the most significant dose dependent effect ($p = 1.0x10^{-12}$) when treated at the highest dose of VPA (100mM). HCT116 was treated with a range of VPA doses (12.5mM, 25mM, 50mM and 100 mM) for 48 hours and the expression of APP examined using Western blot. HCT116 treated at the lowest dose of VPA (12.5mM) showed a mean decrease in APP expression of 13% although this was not significant when compared with non-drug treated cells ($p = 0.6$, Student's t-test). However, there was a significant decrease in APP expression following VPA drug treatment at the three higher doses (25mM, 50mM and 100mM, $p = 0.04$; 0.01 and 0.0004, respectively). HCT116 cell treated with 25 mM VPA had a 14% decrease in APP expression, those treated with 50 mM VPA a 40% reduction and 100mM a 72% decrease in APP expression. Therefore, VPA at high concentrations causes a stepwise inhibition of cell proliferation in this panel of CRC cell lines. Furthermore, in HCT116, which demonstrated the most significant dose dependent effect to VPA cell proliferation inhibition, the expression of APP was also reduced in similar dose dependent effect (Figure 38).

A



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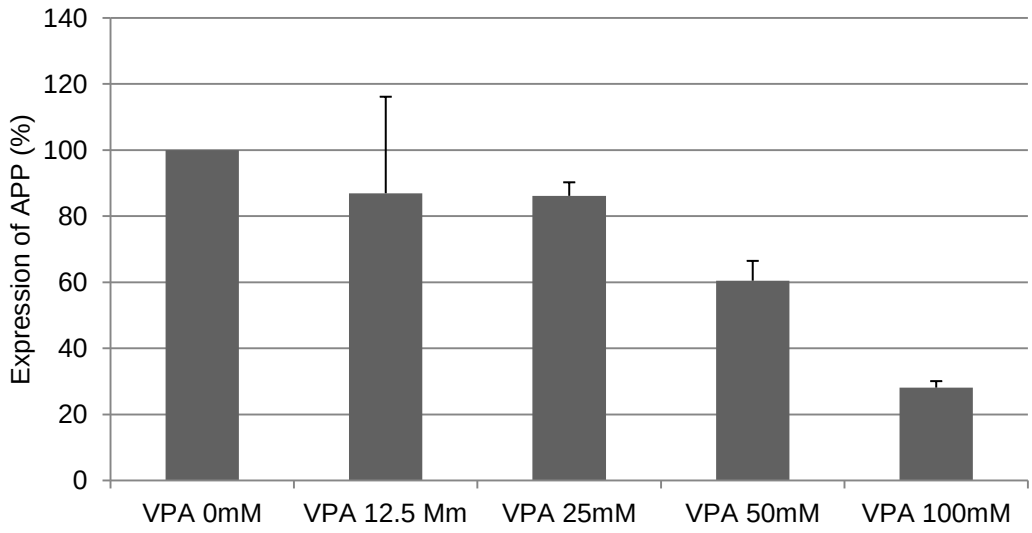


Figure 38. Valproic acid reduces Amyloid precursor protein expression in the HCT116 colorectal cancer cell line. Western Blot images (A) of HCT116 cell line treated with VPA 0mM, 12.5mM, 25mM, 50mM and 100 mM. B. Western Blot quantification of the concentration- dependent effect of 0mM, 12.5mM, 25mM, 50mM and 100 mM VPA on APP expression in HCT116. Densitometry analysis of two repeat blots provided a mean average and error bars indicate the standard deviation. This showed a significant decrease in expression of APP in HCT116 cells treated with VPA (25mM, 50mM and 100 mM VPA, $p = 0.04$, 0.01 and 0.0004, respectively, Student's t-test).

4.2.7 Amyloid Precursor Protein Expression is Not Significantly Altered by BAG1 Expression

APP is more commonly associated with the pathogenesis of AD. The accumulation of amyloid beta peptides derived from APP is a major histopathological hallmark of the disease (Anand, Kaushal et al. 2012). BAG1 isoforms are increased in the brain of AD patients (Elliott, Tsvetkov et al. 2007; Elliott, Laufer et al. 2009) and, furthermore, BAG1 has been found to co-localise with APP. Overexpression of BAG1 also increases the amount of intracellular full length APP suggesting both a strong physical and functional relationship between BAG1 and APP in the brain of AD patients (Elliott, Laufer et al. 2009).

In CRC BAG1 is an important co-chaperone involved in tumourgenesis and an increase in BAG1 expression could have a role in APP upregulation. To investigate the functional influence of BAG1 on APP expression and its possible role in tumourgenesis, a loss-of-function study of BAG1 using siRNA knockdown and BAG1 overexpression was performed and then APP expression determined.

HCT116 cells were transfected with 4 siRNAs BAG 4, BAG 10, BAG 11 and BAG 12 (details of specific siRNAs are given in Appendix IV) to knockdown BAG1 expression. To confirm that the expression of *BAG1* was efficiently suppressed by treatment of HCT116 with siRNA BAG 4, BAG 10, BAG 11 and BAG 12, knockdown was also investigated at mRNA level in transfected cells using QRT-PCR (see Section 2.2.6). All the siRNA's resulted in a

reduced expression of *BAG1* (Figure 39). The most significant knockdown of BAG1 was caused by siRNA BAG 4 with a 94% knockdown of *BAG1* expression (p=0.003, Student's t-test). BAG 11 AND BAG 12 caused a 92% and 85% knockdown in *BAG1* expression (p=0.005 and 0.02 respectively, Student's t- test).

Then, Western blot was carried out on HCT116 cells transfected with 4 siRNA's BAG 4, BAG 10, BAG 11 and BAG 12 that had already been shown to knockdown BAG1 mRNA expression. Western blot showed that transfection with siRNA's BAG 11 and BAG 12 resulted in knockdown of BAG1 protein expression when compared to those cells transfected scrambled siRNA (Figure 40). Analysing the blot intensity relative to the scrambled siRNA (see Section 2.6.2.5) demonstrated a significant knockdown of BAG1 by siRNA BAG 11 and BAG12. siRNA BAG 11 resulted in the most significant knockdown of BAG1 expression, 70% knockdown (p=0.01, Student's t-test). BAG 12 resulted in a 45% knockdown, although this only approached significance (Figure 40. p=0.08, Student's t-test).

As BAG1 expression was knockdown effectively by siRNA transfection, its effect on APP expression was then investigated. The expression of APP from HCT116 cell protein lysate following BAG1 siRNA knockdown transfection was measured using western blot (Figure 41). Analysis of the gel density of the western blot shows that APP expression is not significantly altered by BAG1 knockdown (BAG 10; BAG 11, and BAG 12, all p values p>0.2, Student's t-test,). Furthermore, when BAG1 was overexpressed in HCT116, the expression of APP from HCT116 cell protein lysate following

BAG1 overexpression transfection was measured using western blot (Figure 42). This demonstrated that APP expression is not altered by BAG-1 overexpression.

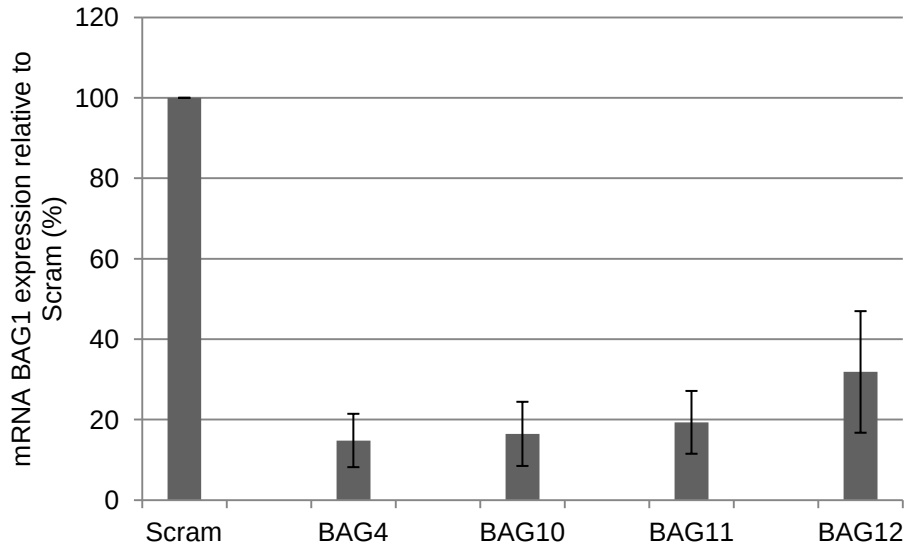
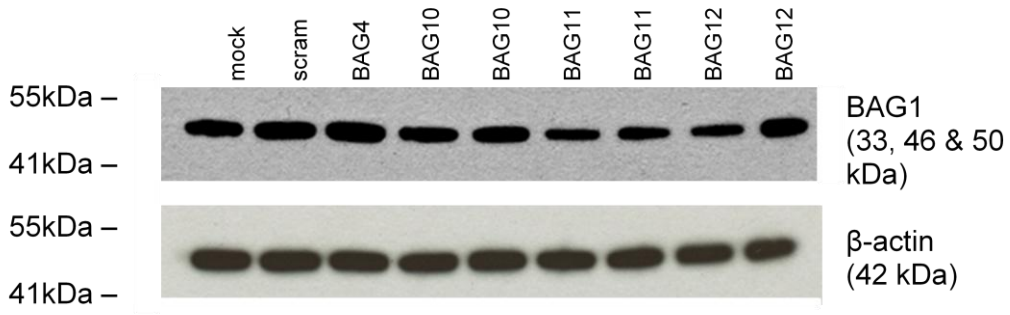


Figure 39. Efficient down regulation of mRNA BAG1 using siRNA. Expression of BAG1 relative to Scram following siRNA BAG1 knockdown demonstrated by QRT-PCR. Graph shows the mean average with error bars showing the standard deviation from two experimental replicates. A significant knockdown in expression of BAG1 for siRNA's BAG 4, BAG 10, BAG 11 and BAG 12 (all p values>0.05, Student's t-test). The most significant knockdown of BAG1 was caused by siRNA BAG 4 (p=0.003, Student's t-test).

A



B

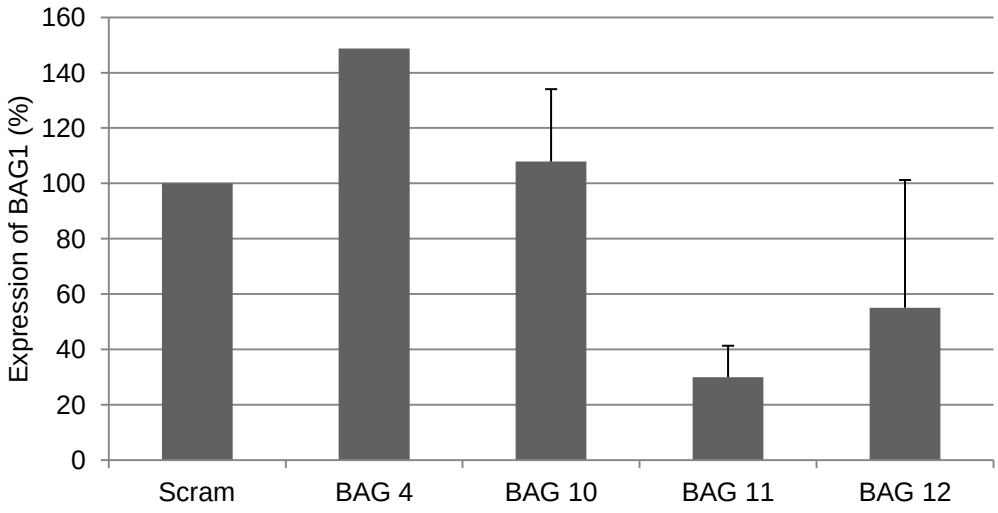
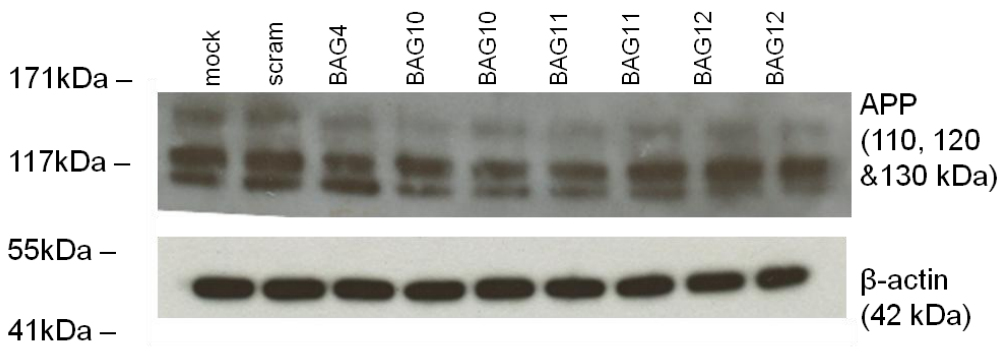


Figure 40. BAG1 protein expression is down regulated by targeted siRNAs. Western Blot images (A) of HCT116 cell line transfections with: scrambled (S) siRNA; knockout siRNA BAG 4; BAG 10; BAG 11 and BAG 1210. Densitometry results are the mean of two replicates and error bars represent standard deviation. Analysis of the gel density of the western blot shows that there is a knockdown of BAG1 expression by siRNA BAG 10, BAG 11 and BAG 12. siRNA BAG 11 caused a significant knockdown of BAG1 expression ($p = 0.01$, Student's t-test).

A



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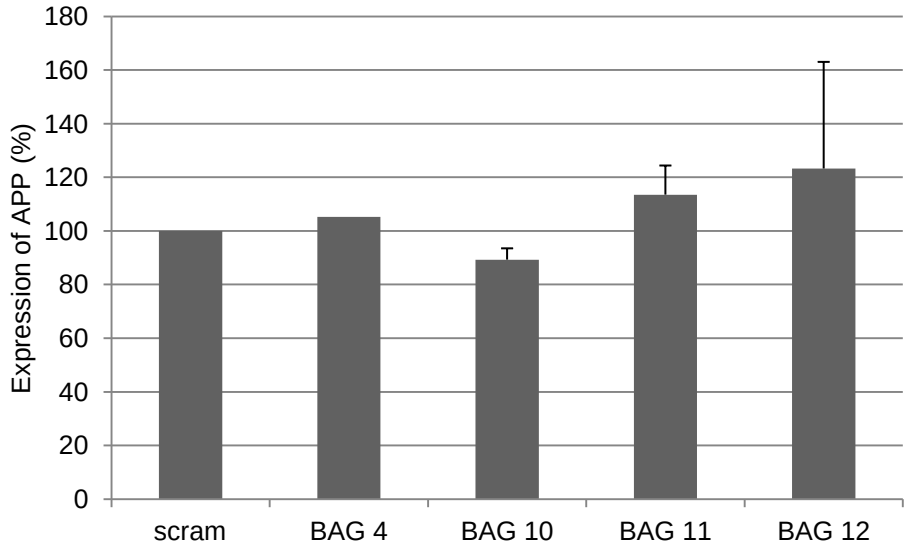


Figure 41. Knockdown of BAG1 does not alter Amyloid precursor protein expression in HTC116 colorectal tumour cells. A. Western blot images of APP expression following siRNA knockdown of BAG1. B. Analysis of the gel density of the western blot shows that APP expression is not significantly altered (all p values >0.2, Student's t-test). Data shown is the mean of two experimental replicates and error bars represent the standard deviation.

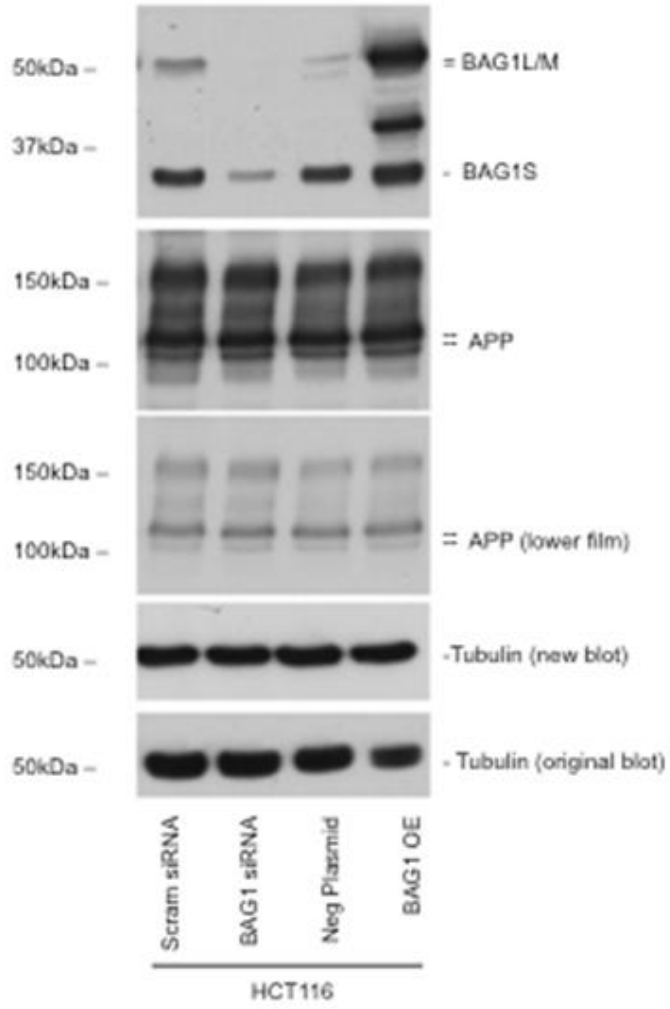


Figure 42. Overexpression of BAG1 does not alter Amyloid precursor protein expression in HCT116. Western blot images of APP expression following siRNA knockdown down and overexpression of BAG1 (BAG1 OE) are shown. The two exposures of APP and two different Tubulin blots used as a loading control. In stripping the BAG1 antibody off to reprobe with Tubulin the high exposure of BAG1 in the overexpressed cell line takes some of the Tubulin protein off with it, hence the lower Tubulin expression. Hence, the blot was re-run and a fresh probe used.

4.2.8 Amyloid Precursor Protein Knockdown Does Not Effect Cell Migration

As APP and its cleavage products have been shown to be involved in migration as well as cell proliferation (Meng, Kataoka et al. 2001; Hansel, Rahman et al. 2003; Ko, Lin et al. 2004; Takayama, Tsutsumi et al. 2009; Botelho, Wang et al. 2010; Venkataramani, Rossner et al. 2010; Jiang, Yu et al. 2012; Yang, Fan et al. 2012), the effect of APP knockdown on cell migration was measured using a migration assay (see Section 2.4.5). Transfection of HCT116 with siRNA's APP 2, APP 9 and APP 10 resulted in significant APP knockdown and inhibition of tumour cell proliferation with siRNA APP 10 the most significant inhibition in terms of tumour cell proliferation (Figure 34). Therefore, HCT1116 cells transfected with siRNA APP 9 and APP 2 were used to investigate the effect of APP knockdown on cell migration as APP 10 inhibited cell proliferation so much that too few cells would be viable to assess the effect of APP knockdown on migration. Fluorescent labelling and quantification of the migratory cells did not demonstrate a significant difference in migration of cells compared to scram (both p values >0.1, Student's t-test; Figure 43).

Therefore, APP knockdown does not effect cell migration in HCTT16, although it has been demonstrated to inhibit cell proliferation.

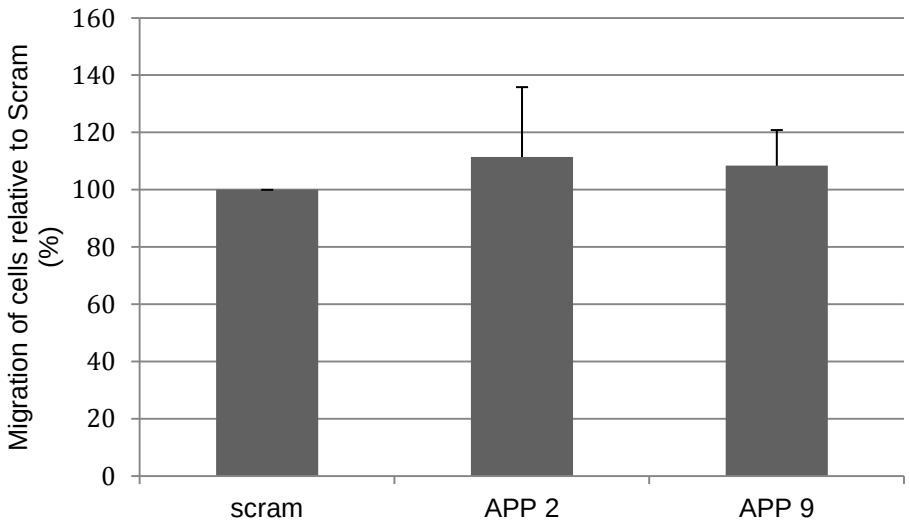


Figure 43. Cell migration was not observed in HCT116 cells transfected with Amyloid precursor protein siRNAs previously shown to knockdown Amyloid precursor protein. Fluorescent quantification did not demonstrate any significant difference in cell migration compared to scram. Data shown is the mean average of three experimental replicates and error bars represent the standard deviation (APP 2, p=0.3; APP 9, p=0.1, Student's T test).

4.2.9 Amyloid Precursor Protein Was Expressed in Both Nuclear and Cytoplasmic Compartments in the Majority of CRC Tumours

Previously, the expression of APP in CRC *in vivo* was described (Venkatarami et al, 2010). A strong expression of APP in colon carcinoma cells was shown, whereas no expression of APP was found in normal epithelial cells of the colon. However, this investigation appears to have been done on a small sample size (n=3) and concern was expressed that there was evidence of very significant overstaining. Similarly, Venkatarami and colleagues described distinct expression of APP in pancreatic cancer cells, but not in normal pancreatic cells. Again overstaining was evident (Venkatarami et al, 2010). APP expression was associated with increased tumour stage of disease (Takayama, Tsutsumi et al. 2009; Venkataramani, Rossner et al. 2010) and survival (Ko, Lin et al. 2004), although the quality of APP staining could not be ascertained as no representative IHC sections were shown. This is a disappointing omission from these publications. Therefore, APP expression in our cohort of primary CRC tumours was determined and associations sought with clinicopathological parameters and patient survival.

The expression of APP in our cohort of CRCs was determined by IHC (Figure 44; Table 18). APP was expressed only in the CRC cells, with no staining of normal colonic cells. Nearly half of the CRCs (49%, 29/59) showed some APP immunopositivity, with the majority exhibiting exclusive nuclear staining (76%, 22/29). The majority of these tumour samples were weakly positive had <5% of the tumour cells expressing APP (55%, 12/22),

with equal numbers showing moderate or strong expression ≥5 to <10% or ≥10% of tumour cells expressing APP (23%, 5/22). There were only a few tumours showing some cytoplasmic staining <5% in 7 tumour samples (12%, 7/59). There were no tumours that had both nuclear and cytoplasmic staining. Overall, APP was expressed, but at low levels, within the cohort of CRC samples with most CRC tumours that expressed APP only having <5% expression and a minority expressing >10% of tumour cells expressing APP.

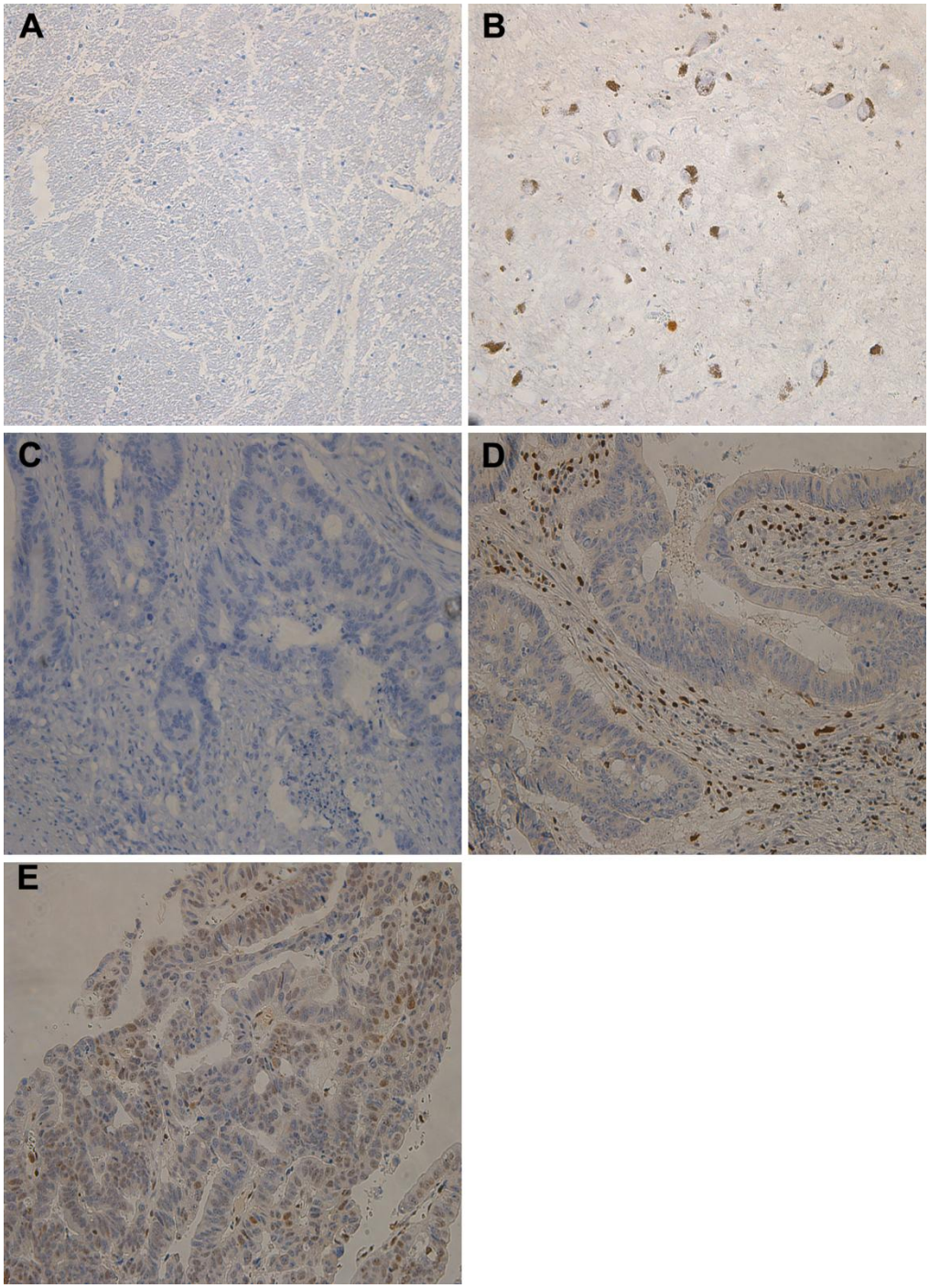


Figure 44. Representative examples of Amyloid precursor protein immunostaining of tumour samples at x10 magnification. (A) APP Brain negative control; (B) APP Brain positive control; (C) APP, no expression detected; (D) APP detected cytoplasmic positive and showing peritumoral lymphocyte staining of APP; (E) APP, nuclear positive. Samples were scored positive if there was nuclear/cytoplasmic staining in more than 10% of cells. Nearly half of the tumour samples exhibited APP nuclear staining.

Table 18. Immunohistochemitry on colorectal cancer tissue section tumour samples. 15 tumour slides could not be scored due to poor or lack of differentiation of the immunostaining or lack of tumour on the slide. Nuclear staining of APP is a consistent feature in a cohort of colorectal cancer specimens. Summary of cytoplasmic and nuclear staining of APP in a cohort of sporadic CRC. +++ = strongly positive; ++ = moderately positive; + = weakly positive.

	Nuclear +++	**Nuclear ++**	**Nuclear +**	**Nuclear -**
Cytoplasm +	0	0	0	7
Cytoplasm -	5	5	12	30

4.2.10 No Association of Amyloid Precursor Protein Expression with Clinicopathological Parameters and Patient Survival

As tumour stage and survival had been associated with APP in various cancers (Ko, Lin et al. 2004; Botelho, Wang et al. 2010; Yang, Fan et al. 2012), the protein expression of nuclear APP and total APP expression was then examined in relation to a number of clinicopathological parameters: tumour differentiation; Duke's stage; depth of invasion (T stage); nodal metastases (N stage) and distant metastases (M stage).

A total of 59 unselected primary CRC tumours from 21 males and 38 females were investigated (Table 1). The age at diagnosis for this group ranged from 35 – 89 years old, with a median age, 78.8. The majority (64%) were left sided tumours (38/57). The cohort contained, 9 Duke's stage A (15%), 23 stage B (39%), 24 stage C (41%) and 3 stage D (5%) CRCs. The median follow-up was 25.8 months (range 1- 55.2) and 21 deaths from any cause were reported, of which 13 deaths were known to be due to CRC (Table 19).

Cytoplasmic APP was not examined as it was only expressed in a few tumour samples. No significant association was shown between APP and any of these features (Table 20). APP expression was not associated with patient survival (Figure 45, Appendix VIII).

Table 19. Summary of clinicopathological features of Amyloid precursor protein immunohistochemistry tumour cohort.

Clinical Parameter	n (%)	Clinical Parameter	n (%)
Gender		**Depth of invasion**	
Male	21 (36)	T1	5 (8)
Female	38 (64)	T2	5 (8)
Age		T3	34 (58)
<70 years	18 (31)	T4	15 (26)
>70 years	41 (69)	**Nodal status**	
Site		N0	33 (56)
Left	41 (70)	N1	25 (42)
Right	18 (30)	N2	1 (2)
Resection margins		**Metastasis status**	
R0	47 (80)	M0	52 (88)
R1	6 (10)	M1	7 (12)
R2	6 (10)	**Duke's stage**	
Differentiation		A	9 (15)
Well	3 (5)	B	23 (39)
Moderate	47 (80)	C1	17 (29)
Poor	9 (15)	C2	7 (12)
		D	3 (5)

Table 20. Expression of Amyloid precursor protein in relation to clinicopathological features of colorectal cancers.

Clinical parameter	APP N-	APP N+	P value	APP -ve	APP +ve	p value
Resection margins						
R0	27 (47.4)	18 (31.6)	1	24 (42.1)	21 (36.8)	0.45
R1	4 (7)	2 (3.5)		2 (3.5)	4 (7)	
R2	4 (7)	2 (3.5)		2 (3.5)	4 (7)	
Differentiation						
Well	1 (1.8)	2 (3.5)	0.69	1 (1.8)	2 (3.5)	0.35
Moderate	29 (50.9)	17 (29.8)		25 (43.9)	21 (36.8)	
Poor	5 (8.8)	3 (5.3)		2 (3.5)	6 (10.5)	
Duke's stage						
A	2 (3.5)	4 (7)	0.34	2 (3.5)	4 (7)	0.79
B	13 (22.8)	9 (15.8)		10 (17.5)	12 (21.1)	
C1	10 (17.5)	7 (12.3)		9 (15.8)	8 (14)	
C2	7 (12.3)	1 (1.8)		4 (7)	4 (7)	
D	3 (5.3)	1 (1.8)		3 (5.3)	1 (1.8)	
Depth of invasion						
T1	0	3 (5.3)	0.04*	0	3 (5.3)	0.3
T2	2 (3.5)	2 (3.5)		2 (3.5)	2 (3.5)	
T3	20 (28.1)	14 (24.6)		16 (28.1)	18 (31.6)	
T4	13 (17.5)	3 (5.3)		10 (17.5)	6 (10.5)	
Nodal Status						
N0	17 (29.8)	15 (26.3)	0.19	14 (24.6)	18 (31.6)	0.59
N1	6 (10.5)	4 (7)		5 (8.8)	5 (8.8)	
N2	12 (21.1)	3 (5.3)		9 (15.8)	6 (10.5)	
Metastasis Status						
MO	28 (49.1)	21(36.8)	0.14	22 (38.6)	27 (47.4)	0.14
M1	7 (12.3)	1 (1.8)		6 (10.5)	2 (3.5)	

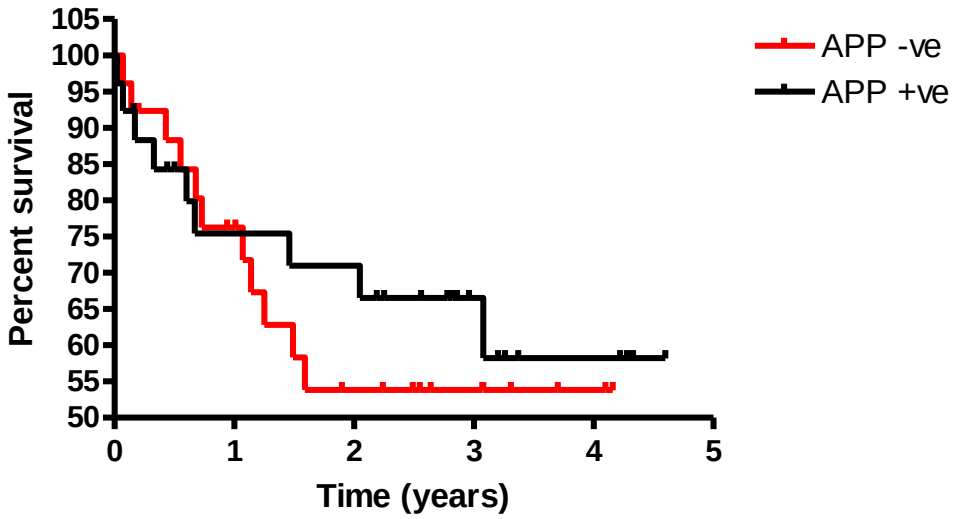


Figure 45. Kaplan-Meier plots correlating patient survival with protein expression of Amyloid precursor protein. There is no statistically significant difference in survival between patients with tumour expressing APP and the patients with tumours negative for APP expression by log rank test (p = 0.61).

4.3 Discussion

4.3.1 Differential Expression of Amyloid Precursor Protein and BAG1 in Cell Lines

A novel panel of cell lines much larger than previous CRC studies was investigated and characterised for the expression of APP and BAG1. All the cell lines expressed APP and BAG1, although there was a differential expression observed between the different cells lines. This would be consistent with other studies in other cancers that demonstrate the up regulation of APP (Botelho et al. 2009; Ko et al. 2004; Yang et al. 2012; Takayama et al. 2009; Hansel et al. 2003) and BAG1 (Brimmell, Burns et al. 1999; Shindoh, Adachi et al. 2000; Rorke, Murphy et al. 2001; Yamauchi, Adachi et al. 2001; Ito, Yoshida et al. 2003; Moriyama, Littell et al. 2004; Millar, Anderson et al. 2009). Interestingly, there was a difference in the level of expression of APP and BAG1 at mRNA compared to protein level expression. HCT15 was the only cell line that expressed mRNA APP and protein expression of APP at the same relative level of expression when comparison was made between the other cell lines. BAG1 mRNA and protein relative expression compared between the other cell lines was also the same for SW837. However comparing the relative abundance of APP or BAG1 relative between the different cell lines at mRNA and protein expression level does not necessarily provide accurate comparison and only provides a relative comparison (Vogel and Marcotte 2012). RT-QPCR has been shown to be an accurate means of mRNA quantification although can it too be limited by the design of experiment (Cikos, Bukovska et al. 2007) and

in our study we used a relative quantification comparing the different cell lines rather than absolute quantification study. Western blot quantification by Western blot densitometry has also considerable limitations leading in inaccuracy, that can be minimised by reducing operator bias (Gassmann, Grenacher et al. 2009). A more accurate analysis of protein expression would have been determined if Mass Spectrometric Immunoassay (MSIA) (Wong, Chan et al. 2009) was used, although this is expensive, or tag-based quantifications of proteins, which is labour-intensive and the tag may interfere with the protein expression (Maier, Guell et al. 2009). Relatively little is known about the regulatory mechanisms controlling the complex patterns of protein abundance and post-translational modification in tumors. Most reports on mRNA and protein abundances find only a weak correlation between the respective abundances of these two classes of biological molecules (Vogel and Marcotte 2012). Several biological factors were identified which influence this correlation, but also methodological constraints play a role when comparing mRNA to protein levels (Celis, Kruhoffer et al. 2000; Maier, Guell et al. 2009)

4.3.2 Amyloid Precursor Protein is Expressed in the Cytoplasmic Compartment of Cells Lines

IF showed unexpected expression of APP in the nucleus in HCT116, C80, C99 and HT55. However, further confocal analysis of the nuclear and cytoplasmic distribution of APP confirmed the expected localisation of APP in the cytoplasm. This would be in keeping with the APP antibody we used,

which recognised amino acids 66-81 of the N-terminus on the pre-A4 molecule (Hilbich, Monning et al. 1993).

4.3.3 Knockdown of Amyloid Precursor Protein Results in an Inhibition in Tumour Cell Proliferation

Meng and colleagues inhibited APP with antisense clones in SW837 and observed reduced proliferation and colony forming efficiency. Similarly, APP inhibition using siRNA knockdown of APP on SW480 demonstrated reduced cell proliferation (Venkatarami et al, 2010). Consistent with these two studies, the experiments detailed here showed that siRNA knockdown of APP on a third CRC cell line, HCT116, caused a decrease in cell proliferation. Inhibition of APP clearly reduces CRC cell proliferation *in vitro*.

The mechanism by which APP promotes tumour growth remains to be fully elucidated. APP expression itself can be induced by the proinflammatory and oncogenic RAS-MAP kinase signalling pathway (Villa, Latasa et al. 2001). APP is a proliferation factor in neural progenitor cells and mesenchymal stem cells, and is associated with ERK/MAP kinase signalling (Demars, Bartholomew et al. 2011). sAPPα has been shown to exhibit neurotrophic and proliferative properties in fibroblasts (Saitoh, Sundsmo et al. 1989), in thyroid epithelial cells inducing cell proliferation by acting as an autocrine growth factors downstream to TSH (Pietrzik, Hoffmann et al. 1998). The highly charged surface of the N-terminal domain of APP (residues 28−123) possess a disulfide-bonded, β-hairpin loops implicated in heparin sulfate proteoglycan binding (Corrigan, Pham et al. 2011). These

have structural similarities with cysteine-rich growth factors (such as epidermal growth factor, tumour necrosis factor and nerve growth factor) and therefore *in vivo* could function as a potential ligand for growth factor receptors with the growth-promoting activity of APP expressed after it is released from membranes through the action of secretases. (Rossjohn, Cappai et al. 1999). This complements other loss-of-function studies in other cancers, which have suggested an important role of APP in tumourgenesis through its role in cellular proliferation (Botelho et al. 2009; Ko et al. 2004; Yang et al. 2012; Takayama et al. 2009; Hansel et al. 2003). All these studies support a role for APP in the pathogenesis of cancer.

4.3.4 Valproic Acid Reduces Cell Proliferation and Inhibits Amyloid Precursor Protein in Colorectal Cancer Cell Lines

VPA has been shown to affect cell growth in different types of cancer *in vitro* and *in vivo* (Cinatl, Cinatl et al. 1997; Gottlicher, Minucci et al. 2001; Blaheta and Cinatl 2002; Xia, Sung et al. 2006; Stettner, Kaulfuss et al. 2007). Venkatarami and colleagues demonstrated it to be an effective and highly specific inhibitor of APP maturation and secretion, therefore suggesting it as a possible pharmacological agent with a useful therapeutic role in the treatment of cancer. We, therefore, evaluated further the role of VPA on cell proliferation and it effect on APP to understand the mechanism of action. Venkatarami and colleagues, in their study using the colorectal cell lines SW480, LOVO, CaCo-2, and T84. They showed that VPA ranging from 0 to 100mM (0, 1, 2.5, 5, 7.5, 10, 25, 50, 75 and 100mM VPA) resulted in a dose-dependent inhibition in proliferation in all their cell lines. These workers then

evaluated the effect of VPA on APP expression on a single line, SW480 using the lower concentrations of VPA (0, 1, 2.5, 5, and 10mM VPA), which also showed that APP expression decreased at lower doses of VPA. In this study we used a larger panel of colorectal cell lines (C80; C99, HCT15, HCT116, HT55, LOVO, LS174T and SW837) not investigated for APP inhibition. Here, the effect of VPA was evaluated at the lower concentrations used by Venkatarami and colleagues first to show the VPA dose dependent effect on APP expression. However, at these lower concentrations HCT116 demonstrated a dose dependent effect on cell proliferation at the two higher doses of 5 and 10 mM ($p = 0.003$, Student's t-test). VPA at the lowest concentrations (1 and 2.5 mM) did not affect cell proliferation (C80; C99; LS174T and SW837), and had the unexpected effect of increasing cell growth in HCT15; HT55; and LOVO, although this effect was not dose dependent. However, the data shown here, in comparison with that of Venkatarami and colleagues, would suggest that SW480 is particularly sensitive to VPA relative to the other lines I tested. This highlights the need to use extensive panels of well documented and mutation profiled cell line when testing for drug response.

The higher concentrations Venkatarami and colleagues used to demonstrate their dose dependent effect of VPA on cell proliferation did cause a dose dependent effect on our cell lines with HCT116, LOVO and LS174T showing the most significant VPA dose dependent effect ($p>0.000007$, Student's t-test). The effect of VPA on APP expression was further evaluated on HCT116, which showed that APP expression was also decreased in a dose

dependent manner. Therefore, the finding given here are mostly consistent with those of Venkatarami and colleagues namely that VPA at high concentrations has a dose dependent effect on cell proliferation. Combining this study with that of Venkatarami and colleagues clearly demonstrates the potential of VPA to inhibit CRC cell proliferation across a large panel of cell lines, albeit HCT116 was the only cell line to exhibit a dose dependent decrease in cell proliferation at the lowest concentrations of VPA and SW480 maybe particularly sensitive to he drug. Therefore, as the dose dependent effect of VPA was only observed consistently at higher concentrations of VPA, the other possibility of VPA cell toxicity should be considered. It is always possible that toxicity may be the consequence of VPA exposure that influences cellular proliferation rather than a direct effect of APP associated inhibition.

4.3.5 Amyloid Precursor Protein Expression is Not Significantly Altered by BAG1 Expression

In AD, there is a pathological accumulation of intraneuronal of Tau protein (Claeysen, Cochet et al. 2012) as a result of inhibited protein degradation. BAG1 associates with Tau protein in an HSP70 dependent manner with overexpression of BAG1 inducing an increase in Tau protein levels as a result of inhibition of Tau protein degradation (Elliott, Tsvetkov et al. 2007). In the study by Elliott et al. (2009) using immunoprecipitation experiments BAG1 was found to co-localise and bind to both tau tangles and intracellular APP further supporting the role of BAG1 in the pathogenesis of AD. In CRC, BAG1 has been shown to be over expressed and play an important role in

tumourgenesis (Sharp, Crabb et al. 2004). Therefore, as a result of this physical relationship it was hypothesised that BAG1 expression may similarly positively affect APP expression. BAG1 was knocked down successfully in HCT116, although there was no alteration in the expression of APP. Furthermore, overexpression of BAG1 in HCT116 did not result in an alteration in the expression of APP. Therefore, it can be concluded that BAG1 expression does not alter APP expression in HCT116.

4.3.6 Amyloid precursor protein knockdown does not effect cell migration

APP was effectively knocked down with siRNA's APP 2, APP 9 and APP 10 and this was associated with a significant inhibition of cell proliferation. In our migration study we used APP 2 and APP 9, as there were too few cells following inhibition of cell proliferation after APP knockdown with APP 10. There was no effect on cell migration in HCT116 siRNA APP knockdown cell lines. This was in contrast to the recent study by Jiang et al (2012) in acute myeloid leukemia where they found that siRNA knockdown of APP inhibited the migration of leukemic cell line Kasumi-1 when compared with controls (Jiang, Yu et al. 2012). This was also associated with a decrease in matrix metalloproteinase protein MMP 2; this protein has been linked with tumour metastasis (Gialeli, Theocharis et al. 2011). Gialeli and colleagues used a similar filter assay migration study method using Transwell© plates (Costar, Tewksbury, MA, USA) with Millipore membranes (Millipore, Billerica, MA, USA). However, they did not demonstrate any inhibition in cell proliferation in siRNA/APP inhibited cell lines in contrast to the findings presented here,

and compared to other previous studies (Meng, Kataoka et al. 2001; Hansel, Rahman et al. 2003; Takayama, Tsutsumi et al. 2009; Botelho, Wang et al. 2010; Venkataramani, Rossner et al. 2010).

4.3.7 Amyloid Precursor Protein was Expressed in Both Nuclear and Cytoplasmic Compartments in the Majority of CRC Tumours

The expression of APP in our cohort of CRCs was determined by IHC (Figure 32). Nearly half of the CRCs (49%, 29/59) showed some APP immunopositivity, with the majority exhibiting exclusive nuclear staining (76%, 22/29). The majority of these tumour samples were weakly positive had <5% of the tumour cells expressing APP (55%, 12/22), with equal numbers showing moderate or strong expression ≥5 to <10% or ≥10% of tumour cells expressing APP (23%, 5/22). There were only a few tumours showing some cytoplasmic staining <5% in 7 tumour samples (12%, 7/59). There were no tumours that had both nuclear and cytoplasmic staining. Overall, APP was expressed, but at low levels, within the cohort of CRC samples with most CRC tumours that expressed APP only having <5% expression and a minority expressing >10% of tumour cells expressing APP.

4.3.8 No Association of Amyloid Precursor Protein Expression with Clinicopathological Parameters and Patient Survival

APP was found to be expressed in nearly half of CRC tumour samples. The majority of these tumour samples were weakly positive had <5% of the tumour cells expressing APP (55%, 12/22), with equal numbers showing

moderate or strong expression ≥5 to <10% or ≥10% of tumour cells expressing APP (23%, 5/22). This is in contrast to the only study on CRC by Venkataramani and colleagues that showed a strong expression of APP, although in their study they only describe immunohistochemistry staining in three samples. Interestingly, we showed that nuclear staining of APP was a consistent feature in a cohort of CRC specimens. This was in contrast to our cell line characterisation and expected cytoplasmic staining of APP.

We also found that there was no expression of APP in normal epithelial cell of the colon. APP also demonstrates differential expression in a number of other cancers. In normal pancreatic tissue APP is expressed in the both acinar and islet cells, in contrast to pancreatic cancer tissue in which APP is also highly expressed in the ductal epithelium. This is significant, as it is the ductal epithelium that is thought to undergo carcinogenic change to give rise to pancreatic adenocarcinoma (Hansel, Rahman et al. 2003; Venkataramani, Rossner et al. 2010). In papillary thyroid carcinoma immunohistochemistry of specimens showed that APP expression was significantly increased in papillary thyroid carcinoma samples compared with normal thyroid tissue (Yang, Fan et al. 2012).

Expression of APP has been suggested to be a useful prognostic marker in a number of cancers. In melanoma there was a correlation with advancement of disease and APP expression with APP expression high in vertical growth phase melanomas, in visceral and subcutaneous metastases, and in melanoma-infiltrated lymph nodes, compared with melanoma *in situ*. These observations suggested that the progression from

melanoma *in situ* to advanced melanoma is accompanied by upregulation of APP (Botelho, Wang et al. 2010). This progression from early to advanced melanoma would also follow worsening prognosis and decreased patient survival (Wisco and Sober 2012). Similarly, in the study by Ko and colleagues (2004) in patients with oral squamous cell carcinoma these workers demonstrated an increase in APP mRNA expression correlated with worse prognosis and survival. This was correlated with protein expression of APP and also further validated with immunohistochemistry, which showed a significant increase of APP expression in oral squamous cell carcinoma tissue relative to non cancer matched pairs. Furthermore, in papillary thyroid carcinoma APP expression was also correlated with clinicopathological factors including tumour size, extracapsular invasion and lymph node metastasis (Yang, Fan et al. 2012). However, we did not demonstrate any significant association between APP expression and any clinicopathological features or patient survival, although the sample size used was small.

4.3.9 Summary

In summary, our data supports the role of APP in proliferation in CRC cell lines. In contrast to AD, the observation that BAG1 expression alters the express of APP was not demonstrated in CRC. APP clearly has a role in cell proliferation and VPA sensitivity varies markedly across cell lines highlighted the need to use substantial panels of well-characterised lines in testing drug response. The relatively small CRC tumour cohort used here did not demonstrate any association between APP expression with clinicopathological parameters or patient survival as has been previously

suggested. However, my study has raised concerns about the overstaining of APP in other studies, particularly where overnight incubation has been used. Further *in vivo* and *in vitro* investigations are required to further elucidate the role of APP in tumourgenesis.

CHAPTER 5

Evaluating the Role of B-cell CLL/Lymphoma 3 in Colorectal Cancer

5.1 Overview and Rationale

B-cell CLL/lymphoma 3 (*BCL3*) was first identified as a gene in the recurring chromosomal translocation t (14;19) in patients with chronic lymphocytic leukemia (McKeithan 1987; Ohno, Takimoto et al. 1990; Bhatia, Huppi et al. 1991). BCL3 is involved in regulating the NF-κB signal transduction pathway (Palmer and Chen 2008). NF-κB can be activated by cellular stress, DNA damage, and by activation of various oncogenic pathways, and it regulates inflammation, apoptosis and cell proliferation (Courtois and Gilmore 2006; Gilmore 2006). BCL3 activation results in up regulation of anti-apoptotic genes and is therefore an important component in the tumour promoting machinery playing a pivotal role in the generation and maintenance of malignancies (Courtois and Gilmore 2006; Perkins 2012).

The primary regulation of the NF-κB pathway is through the association of NF-κB complexes with inhibitor of κB proteins (IKB) (Hayden and Ghosh 2008). Aberrant activation of NF-κB has been reported in numerous solid tumours and cell lines (Baud and Karin 2009; Perkins 2012). In general, deregulation most often results from defects in the pathways regulating NF-κB instead of mutations in the NF-κB genes. Deregulation may be advantageous for cancer as NF-κB is a major activator of anti-apoptotic

gene expression thus having a positive effect on cell survival. NF-κB can be activated by cellular stress, DNA damage, and by activation of various oncogenic pathways (Karin 2006; Kim, Hawke et al. 2006; Fan, Dutta et al. 2008; Perkins 2012).

BCL3 proto-oncogene is an atypical member of the IKB family of proteins (Wulczyn, Naumann et al. 1992; Palmer and Chen 2008). These proteins normally repress the activation of the NF-κB signaling cascade complexes in the cytoplasm by directly binding to these diametric transcription factors (Maldonado and Melendez-Zajgla 2011) (see Section 1.19). BCL3 is atypical as it is involved in nuclear activation and repression of NF-κB signaling mediated by the formation of heterocomplexes with NF-κB1 (p50) or NF-κB2 (p52) homodimers converting them from transcriptional repressors into activators (Franzoso, Bours et al. 1993; Fujita, Nolan et al. 1993). Therefore, BCL3 could be important in the promotion of tumour cell survival, as it is an activator of the NF-κB pathway resulting in inhibition of apoptosis and increase in cell proliferation.

BCL3 was initially thought to enhance NF-κB mediated transactivation by removing inhibitory p50 and p52 homodimers allowing the binding of active NF-κB dimers resulting in NF-κB transactivation (Franzoso, Bours et al. 1992; Franzoso, Bours et al. 1993). However, BCL3 can also act as coactivator p50 or p52 without inhibiting their binding (Bours, Franzoso et al. 1993; Fujita, Nolan et al. 1993) by associating with p50 and p52 dimers and by providing a transactivating domain to the NF-κB complex.

BCL3 has also been shown to interact with stably bound NF-κB homodimers thereby reducing DNA binding and allowing transcriptionally active NF-κB complexes to induce gene expression (Palmer and Chen 2008). In contrast, BCL3 can increase p50 binding to NF-κB occupancy without coactivation, therefore indirectly repressing NF-κB target gene transcription (Watanabe, Iwamura et al. 1997; Palmer and Chen 2008) by delaying degradation of the DNA-bound p50 homodimers (Carmody, Ruan et al. 2007).

BCL3 is upregulated by several cytokines, including TNF alpha, IL4, IL1, IL6, IL10, adioponectin and IL-12 (Heissmeyer, Krappmann et al. 1999; Rebollo, Dumoutier et al. 2000; Kuwata, Watanabe et al. 2003; Hu, Nesic-Taylor et al. 2005; Valenzuela, Hammerbeck et al. 2005; Brocke-Heidrich, Ge et al. 2006; Brenne, Fagerli et al. 2009; Folco, Rocha et al. 2009) and downregulated by TP53 (Rocha, Martin et al. 2003), whilst also being terminated by p50 in an autoregulatory loop (Brasier, Lu et al. 2001). Interestingly, DNA damage upregulates BCL3 inducing the expression of HDM2 and suppressing the TP53 TSG (Kashatus, Cogswell et al. 2006). BCL3 phosphorylation by GSK3 regulates BCL3 turnover and transcriptional activity by limiting the transcription of BCL3 target genes (Viatour, Dejardin et al. 2004).

The exact role of BCL3 in oncogenesis remains to be fully elucidated. However, BCL3 has been shown to be involved in both oncogenic proliferation and apoptosis. Cellular proliferation can be increased by BCL3 acting as a coactivator of p52 dimers inducing the expression of the cyclin D1 and increasing the transition at the G1/S phase of the cell cycle (Westerheide, Mayo et al. 2001). In contrast, TP53 represses BCL3

induction of cyclin D1 changing the p52/BCL3 complexes on the promoter to p52/HDAC complexes inhibiting cyclin expression (Rocha, Martin et al. 2003). In breast cancer cells BCL3 has been correlated with CtBP1 expression. BCL3 is thought to stabilise CtBP1 in breast cancer cells, inhibiting its degradation and therefore also inhibiting apoptosis through repression of pro-apoptotic gene expression (Choi, Lee et al. 2010). Furthermore, BCL3 can inhibit apoptosis, by the transactivation of the anti-apoptotic gene BCL2 in MCF7AZ breast cancer cells (Viatour, Bentires-Alj et al. 2003).

BCL3 deregulation is observed in a number of cancers. In haematological malignancies BCL3 deregulation is well established and the BCL3 locus has been found to be translocated in small lymphocytic lymphomas, Burkitt-like lymphoma, diffuse large cell lymphomas and in B-cell chronic leukemias; overexpressed was found in non-Hodgkin and Hodgkin lymphomas (Au, Horsman et al. 2002; Canoz, Rassidakis et al. 2004; Mathas, Johrens et al. 2005; Schlette, Rassidakis et al. 2005).

Although not widely studied in carcinomas, overexpression of BCL3 is observed with activated overexpression of BCL3 seen in breast (Cogswell, Guttridge et al. 2000), nasopharyngeal(Thornburg, Pathmanathan et al. 2003), and endometrial cancer (Pallares, Martinez-Guitarte et al. 2004). Overexpression of BCL3 mRNA and protein occurred in breast tumours (Cogswell, Guttridge et al. 2000) and was shown to increase the growth of breast cancer xenografts (Pratt, Bishop et al. 2003). Furthermore, BCL3 is strongly overexpressed in a mouse model of skin carcinoma in late

papilloma and squamous cell carcinoma (Budunova, Perez et al. 1999). To date, the expression of BCL3 in CRC has only been has been described by a single study (Puvvada, Funkhouser et al. 2010) using a relatively small cohort of 20 tumour samples in which they found a trend towards an association (p= 0.07) between nuclear BCL3 expression and survival. Genome wide studies have failed to identify BCL3 as a susceptibility locus for CRC (Zanke, Greenwood et al. 2007; Tenesa, Farrington et al. 2008; Peters, Jiao et al. 2012).

AKT is a central mediator of cellular survival that is activated by PI3K and is negatively regulated by the PTEN tumour suppressor (see Section 1.18). AKT has been shown to activate IKB and hence NF-κB in several settings (Ozes, Mayo et al. 1999; Factor, Oliver et al. 2001). Furthermore, NF-κB homodimeric binding protein BAG1 has been shown to be a key survival protein in colorectal tumour cells, modulating homodimeric activity of p50 suggesting a potentially important role in CRC carcinogenesis by regulating key cell survival pathways (Southern, Collard et al. 2012). Therefore, BCL3 could also be important in the promotion of tumour cell survival in CRC and might act in combination with BAG1.

We therefore decided to investigate the relationship between BCL3 and the PI3K/AKT pathway as well as BAG1 in our cohort of primary CRCs with clinicopathological data (Figure 46). First, the clinicopathological features of the patients are detailed.

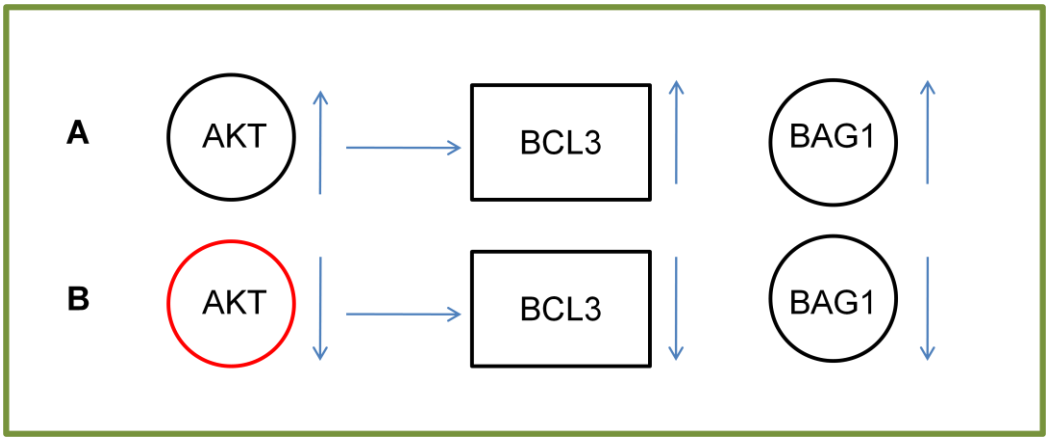


Figure 46. Background on BCL3 findings from cell culture based studies and our hypothesis for testing in colorectal cancer. AKT has been shown to activate IKB and hence NF-κB in several settings (Ozes, Mayo et al. 1999; Factor, Oliver et al. 2001) and BAG1 has been shown to be a key survival protein in colorectal tumour cells, modulating homodimeric activity of p50 suggesting a potentially important role in CRC carcinogenesis by regulating key cell survival pathways (Southern, Collard et al. 2012). Our hypothesis: In our cohort of primary colorectal cancer tumours (A) Increased expression of AKT will be positively associated with expression of BCL3 and BAG1; (B) Decreased expression of AKT will be negatively associated with BCL3 and BAG1 expression.

5.2 Results

5.2.1 BCL3 was Expressed in Both Nuclear and Cytoplasmic Compartments in the Majority of Colorectal Cancers

BCL3 expression in our cohort of primary CRC tumours was determined using IHC and then correlated with clinicopathological parameters and patient survival. IHC was carried out for BCL3 on 50 tumour samples; however, 2 tumour slides (4%) could not be scored due to poor immunostaining or lack of tumour on the slide. Therefore, a total of 48 tumour samples were scored with representative examples given in Figure 47.

Tumour samples were scored positive for BCL3 if there was nuclear/cytoplasmic staining in more than 10% of cells. BCL3 expression was found both in normal mucosa and carcinoma. The expression of BCL3 was heterogenous in the carcinoma tissue, with higher expression of BCL3 associated with the invasive edge of the tumour (Figure 48A and 48B). Interestingly, BCL3 antibody also incidentally specifically stained ‘tuft’ cells or enteroendocrine cells in adjacent normal tissue (Figure 48C and 48D). BCL3 staining of tuft cells has not been reported in the literature before. The majority of the CRCs (77%, 36/47) showed BCL3 immunopositivity with the majority of these exhibiting cytoplasmic staining (66%, 31/47). Mostly the cytoplasmic staining was moderate in intensity (10-30%; 43%, 20/47) with only 35% (11/31) of cytoplasmic staining showing strong intensity (<30%). While, the nuclear staining was observed in equal intensities in the tumours

(23%, 11/47 respectively), similar proportions of tumours exhibited discrete cytoplasmic staining or both cytoplasmic and nuclear staining (30%, 14/47 and 36%, 17/47 respectively). In five tumours (11%, 5/47) discrete nuclear staining with no cytoplasmic staining was observed (Table 21).

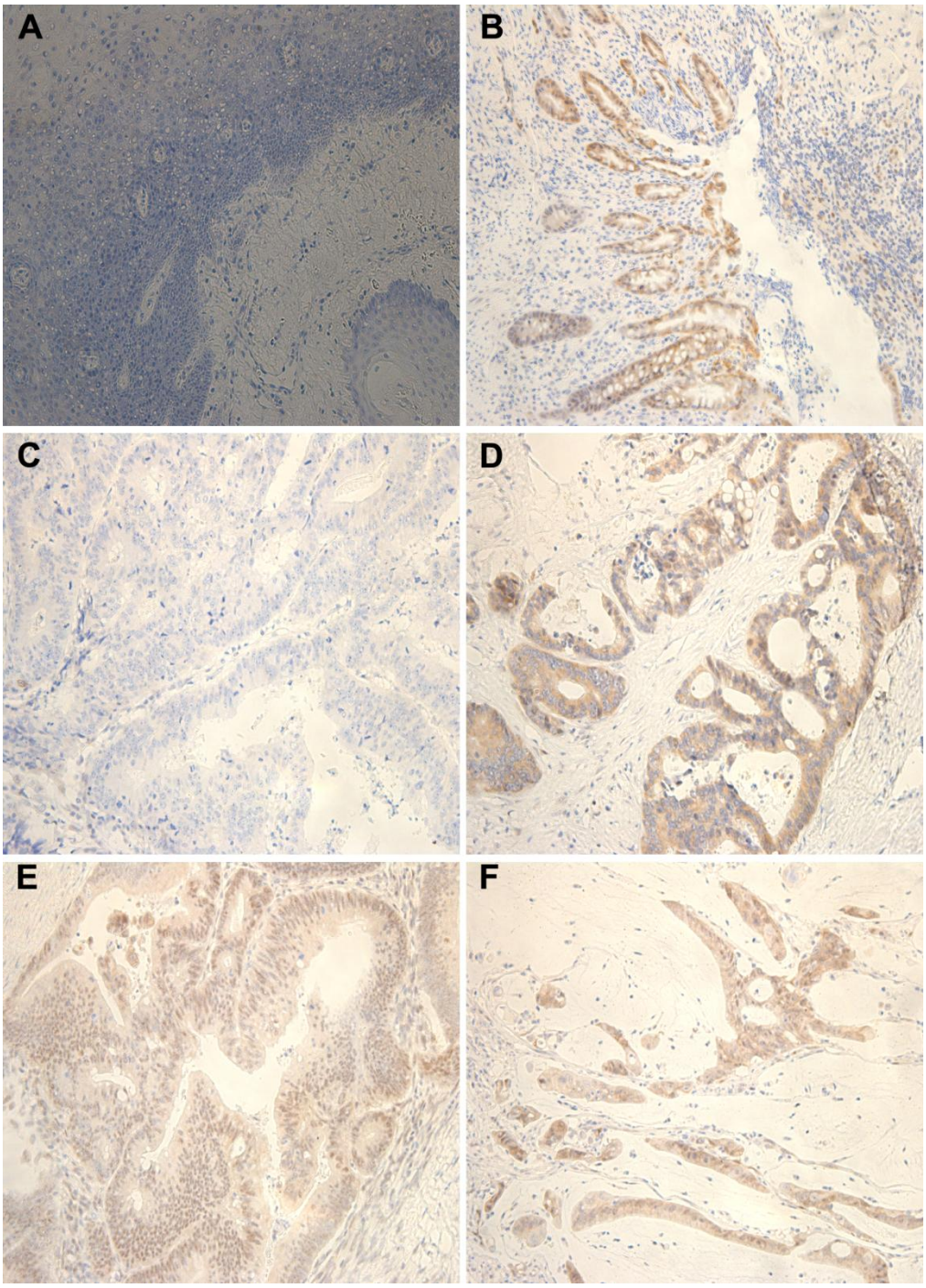


Figure 47. Representative examples of BCL3 immunohistochemistry of colorectal sample at x10 magnification. (A) BCL3 Tonsil negative control; (B) BCL3 Tonsil positive control; (C) BCL3, no expression detected; (D) BCL3 detected cytoplasmic positive; (E) BCL3, nuclear positive; (F) BCL3 positive. Samples were scored positive if there was nuclear/cytoplasmic staining in more than 10% of cells. The majority of the tumour samples exhibited BCL3 staining, with most exhibiting cytoplasmic staining.

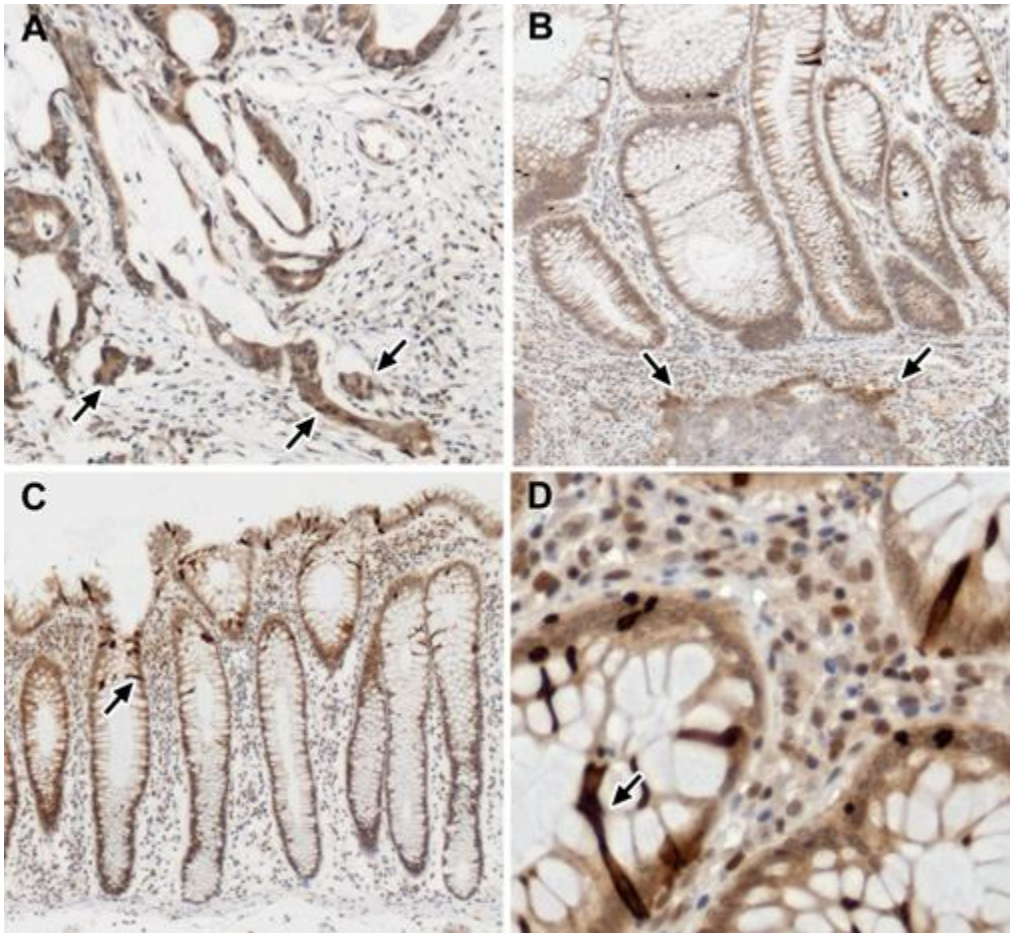


Figure 48. Representative examples of BCL3 immunohistochemistry of colorectal sample. Showing (A) Weak/absent nuclear and weak/moderate cytoplasmic staining of BCL3 strong at the invasion front of the carcinoma highlighted by arrows (x20 magnification); (B) Invasive edge of carcinoma has strong cytoplasmic and nuclear BCL3 staining highlighted by arrows (x20 magnification); (C) A normal region of mucosa showing weak/absent nuclear and weak/moderate cytoplasmic staining for BCL3 in crypt epithelium other than the tuft enteroendocrine cells highlighted by arrows (x10 magnification); (D) Intensely positive cytoplasmic staining of putative tuft enteroendocrine cells highlighted by arrows (x50 magnification).

Table 21. Summary of cytoplasmic and nuclear staining of BCL3. **I**mmunohistochemistry was performed on 50 tumour samples; however two tumour slides could not be scored due to poor or lack of differentiation of the immunostaining or lack of tumour on the slide.

BCL3	Nuclear-	Nuclear +	Nuclear ++
Cytoplasm -	11	4	1
Cytoplasm+	11	3	6
Cytoplasm ++	3	4	4

5.2.2 No Association of BCL3 Expression with Clinicopathological Parameters and Patient Survival

The protein expression of nuclear, cytoplasmic and total BCL3 expression was then examined in relation to a number of clinicopathological parameters: tumour differentiation; Duke's stage; depth of invasion (T stage); nodal metastases (N stage) and distant metastases (M stage).

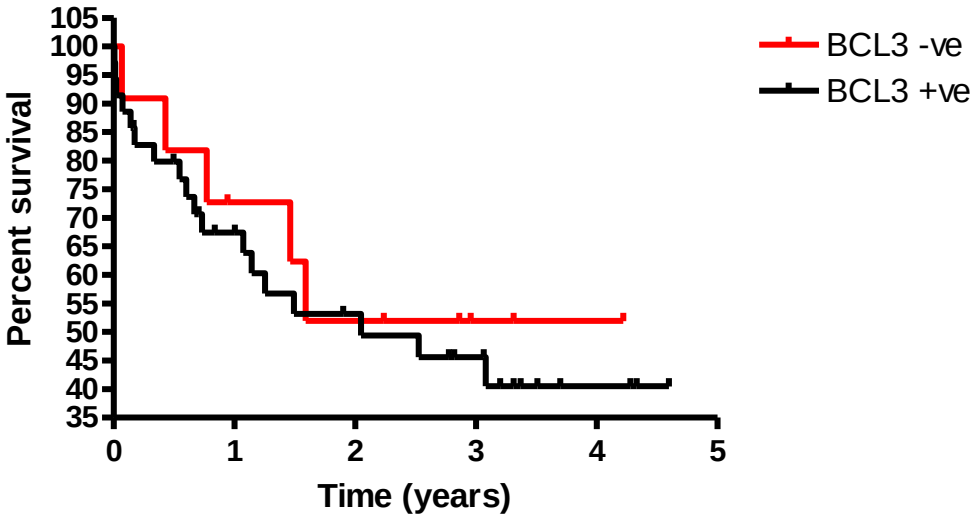
A total of 47 unselected primary CRC tumours from 17 males and 30 females were investigated. Although still relatively small, this cohort represents a >2-fold increase in patients numbers in comparison to the only other study in the literature that found a trend towards an association between nuclear BCL3 expression and survival (Puvvada, Funkhouser et al. 2010). The age at diagnosis for my group ranged from 35 – 89 years old, with a median age, 77.1. The majority (60%) were left sided tumours (28/47). The cohort contained, 5 Duke's stage A (11%), 15 stage B (32%), 23 stage C (49%) and 4 stage D (9%) CRCs. The median follow-up was 16.3 months (range 1- 55.2) and 22 deaths from any cause were reported, of which 14 deaths were known to be due to CRC (Table 22).

No significant association was shown between BCL3 and any of these features (Tables 23). In particular, neither nuclear nor total BCL3 expression was associated with patient survival and no trend was observed (Figure 49. p=0.27 and p=0.62 respectively). Furthermore, when strong nuclear expression of BCL3 (<30%) was tested for a correlation with survival there was no association demonstrated with patient survival (Appendix VII).

Table 22. Summary of clinicopathological features of BCL3 immunohistochemistry tumour cohort.

Clinical parameter	n (%)	Clinical parameter	n (%)
Gender		**Depth of invasion**	
Male	17 (36)	T1	1 (2)
Female	30 (64)	T2	4 (9)
Age		T3	30 (64)
<70 years	16 (34)	T4	12 (26)
>70 years	30 (66)	**Nodal status**	
Site		N0	23 (49)
Left	28 (60)	N1	23 (49)
Right	19 (40)	N2	1 (2)
Resection margins		**Metastasis status**	
R0	37 (79)	M0	39 (83)
R1	5 (11)	M1	8 (17)
R2	5 (11)	**Duke's stage**	
Differentiation		A	5 (11)
Well	1 (2)	B	15 (32)
Moderate	39 (83)	C1	16 (34)
Poor	7 (15)	C2	7 (15)
		D	4 (9)

A



B

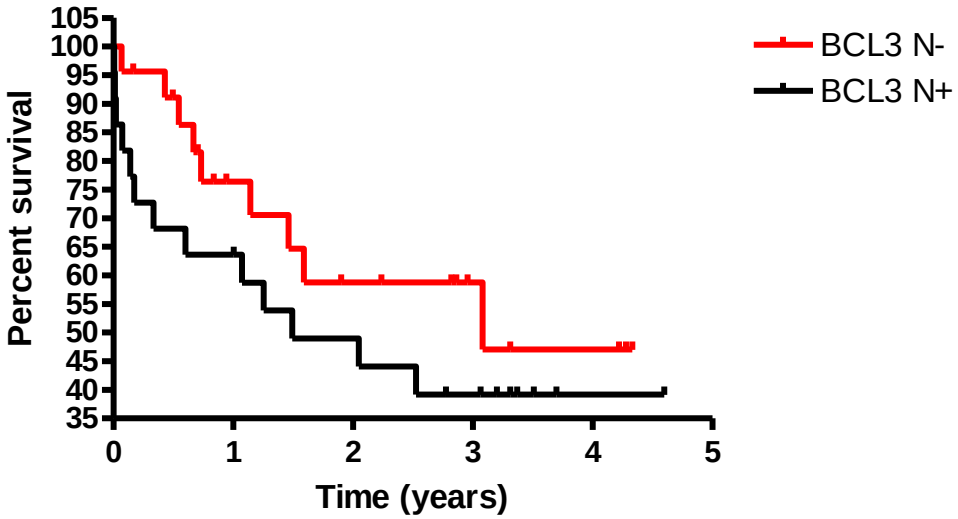


Figure 49. Kaplan-Meier plots correlating patient survival with protein expression of (A) BCL3 and (B) Nuclear BCL3. There is no statistically significant difference (BCL3 p=0.61; Nuclear BCL3 p=0.21) in survival between patients with tumour expressing BCL3 and the patients with tumours negative for BCL3 expression by log rank test.

Table 23. Expression of BCL3 in relation to clinicopathological features of colorectal cancers.

Clinical parameter	BCL3 C-	BCL3 C+	p value	BCL3 N-	BCL3 N+	P value	BCL3 -ve	BCL3 +ve	p value
Resection margins									
R0	14 (29.8)	23 (48.9)	0.30	20 (42.6)	17 (36.2)	0.88	10 (21.3)	27 (57.4)	0.60
R1	0	5 (10.6)		2 (4.3)	3 (6.2)		0	5 (10.6)	
R2	2 (4.3)	3 (6.4)		3 (6.4)	2 (4.3)		1 (2.1)	4 (8.5)	
Differentiation									
Well	0	1 (2.1)	0.79	0	1 (2.1)	0.15	0	1 (2.1)	1
Moderate	13 (27.7)	26 (55.3)		23 (48.9)	16 (34)		10 (21.3)	29 (61.7)	
Poor	3 (6.4)	4 (8.5)		2 (4.3)	5 (10.6)		1 (2.1)	6 (12.8)	
Duke's stage									
A	4 (8.5)	1(2.1)	0.24	2 (4.3)	3 (6.4)	0.91	2 (4.3)	3 (6.4)	0.38
B	4 (8.5)	8 (23.4)		8 (17)	7 (14.9)		4 (8.5)	11 (23.4)	
C1	5 (10.6)	8 (23.4)		8 (17)	8 (17)		2 (4.3)	14 (29.8)	
C2	1 (2.1)	6 (12.8)		4 (8.5)	3 (6.4)		1 (2.1)	6 (12.8)	
D	2 (4.3)	2 (4.3)		3 (6.4)	1 (2.1)		2 (4.3)	2 (4.3)	
Depth of invasion									
T1	1 (2.1)	0	0.12	1 (2.1)	0	0.66	1 (2.1)	0	0.42
T2	3 (6.4)	1 (2.1)		1 (2.1)	3(6.4)		1 (2.1)	3 (6.4)	
T3	9 (19.1)	21 (44.7)		16 (34)	14 (29.8)		7 (14.9)	23 (48.9)	
T4	3 (6.4)	9 (19.1)		7 (14.9)	5 (10.6)		2 (4.3)	10 (21.3)	
Nodal Status									
N0	10 (21.3)	13 (27.7)	0.09	12 (25.5)	11 (23.4)	1	7 (14.9)	16 (34)	0.07
N1	5 (10.6)	18 (38.3)		12 (25.5)	11 (23.4)		3 (6.4)	20 (42.6)	
N2	1(2.1)	0		1 (2.1)	0		1 (2.1)	0	
Metastasis Status									
MO	13 (27.7)	26 (55.3)	0.56	20 (42.6)	19 (40.4)	0.43	8 (17)	31 (66)	0.27
M1	3 (6.4)	5 (10.6)		5 (10.6)	3 (6.4)		3 (6.4)	5 (10.6)	

5.2.3 BCL3 Expression is not Associated with AKT or BAG1 Expression

Over half the tumours expressed both AKT and BCL3 (57%, 27/47), with only two tumours expressing either AKT or BCL3 (2/47, 4%). However, AKT was expressed in similar proportions of tumours that either expressed BCL3 or were negative for BCL3 expression and no significant difference was observed (Table 24).

BAG1 was expressed in the majority of tumours that expressed BCL3 (94%, 31/33). However, there were a similar proportion of tumours that did not express BCL3 but expressed BAG1 (80%, 8/10). Overall BCL3 expression was not significantly associated with BAG1 expression (p=0.22). There was no significant association seen between cytoplasmic or nuclear BCL3 expression with BAG-1 (Table 25; p=0.61 and 1 respectively).

Table 24. No association of total BCL3 expression with AKT. There was no significant correlation found between AKT expression and BCL3 expression. P value calculated using Fisher's exact test.

	BCL3 C-	BCL3 C+	p value	BCL3 N-	BCL3 N+	p value
AKT-	3	8	0.72	4	7	0.30
AKT +	13	23		21	15	

	BCL3-	BCL3 +	p value
AKT-	2	9	0.71
AKT+	9	27	

Table 25. Association of total BCL3 expression with BAG1. BAG1 immunohistochemistry was carried out on all the tumour samples stained for BCL3. Four of 47 could not be scored due to lack of tumour on the slide or poor staining. Expression of cytoplasmic, nuclear and total BCL3 was not significantly associated with BAG1 expression. P value calculated using Fisher's exact test.

	BCL3 C-	BCL3 C+	p value	BCL3 N-	BCL3 N+	p value
BAG1-	2	2	0.61	2	2	1
BAG1 +	13	26		21	18	

	BCL3-	BCL3 +	p value
BAG1 -	2	2	0.22
BAG1+	8	31	

5.3 Discussion

5.3.1 BCL3 was Expressed in Both Nuclear and Cytoplasmic Compartments in the Majority of Colorectal Cancers, Although Expression was not Associated with Clinicopathological Parameters and Patient Survival

BCL3 was found in both normal mucosa and tumour tissue. This was similar to the only study on CRC and BCL3 by Puvvada and colleagues (2010) who also found that BCL3 was observed in both normal mucosa and in tumour tissue, although a greater proportion of their tumour samples exhibited BCL3 (20/23, 87%) than reported here (77% and nuclear BCL3 23%). Puvvada and colleagues (2010) specifically scored for nuclear staining using a score multiplier with each sample scored in triplicate and the mean calculated from multiplier score based on the percentage of cell that were positive to any degree (0-3+, scoring 0-300); unfortunately, these workers do not describe clearly their intensity score. Here, a tumour sample was scored positive if BCL3 was seen in 10-30% of the tumour. Disappointingly, Puvvada and colleagues did not describe the pattern of BCL3 cytoplasmic staining. Here, the majority if the CRC tumour samples exhibited cytoplasmic staining with equal numbers of tumours exhibiting cytoplasmic or cytoplasmic and nuclear staining, and only five tumours were found to have only discrete nuclear staining. Interestingly, although the expression of BCL3 was heterogenous in the carcinoma tissue there was a higher expression observed at the invasive edge of the tumour.

BCL3 is an atypical IKB, which are usually cytoplasmic in activity, but which regulates nuclear NF-κB activity. BCL3 in the cytoplasm inhibits the nuclear translocation of the NF-κB p50 subunit (Naumann, Wulczyn et al. 1993; Watanabe, Iwamura et al. 1997). In the nucleus, BCL3 acts as transcriptional activator that promotes transcription of NF-κB target genes. Puvvada and colleagues (2010) found there to be a significant association between survival and nuclear BCL3 expression. This is unlikely to be correct given the expression distribution reported here and the known function of BCL3 in the cytoplasm. Puvvada and colleagues (2010) did not describe any correlation with other clinicopathological variables outlined in their study and which we also analysed, again identifying no associations.

BCL3 also specifically stained cells that morphologically are suggestive of tuft cells. Tuft cells are characterised by long and blunt microvilli with prominent rootlets, and by a well developed tubulovesicular system in the supranuclear cytoplasm (Sato 2007). Tuft cells have been proposed to represent a distinct subset of enteroendocrine cells though originating from the same Lgr5+ stem cell as enterocytes, goblet, Paneth, and entero-endocrine cells (Formeister, Sionas et al. 2009; Kokrashvili, Rodriguez et al. 2009). They have a unique marker signature and can be defined by the coexpression of SOX9, Cyclooxygenase-1 (COX1) and Cyclooxygenase-2 (COX2), hematopoietic prostaglandin-D synthase (HPGDS), and doublecortin-like kinase 1 (DCLK1) and require different transcription factors for their differentiation (Gerbe, van Es et al. 2011). As tuft cells express HPGDS and are the only epithelial cells expressing the COX1 and COX2

enzymes in the healthy intestinal epithelium, this maybe important given the central role played by inflammation and tumorigenesis in the intestinal epithelium (Stenson 2008; Wang and Dubois 2010). In mouse studies the presence of tuft cell clusters in tumors from *APC* or *KRAS* mutated mice suggested a possible contribution of tuft cells during tumorigenesis. (Janssen, el-Marjou et al. 2002; Colnot, Niwa-Kawakita et al. 2004). However, in humans tuft cells are found in adenomas, but rarely in adenocarcinoma suggesting that the tuft cell differentiation is conserved in early tumour tissue, but not in their more malignant counterpart, raising the question of the potential roles played by tuft cells during tumorigenesis (Gerbe, van Es et al. 2011). The specific staining of BCL3 would require serial or dual labelling for tuft cell specific markers to confirm BCL3 tuft cell staining, before further investigation to elucidate the novel role of BCL3 in these cells.

5.3.2 BCL3 Expression is not Associated with AKT or BAG1 Expression

AKT has been shown to activate IKB and hence NFK-B in several settings (Ozes, Mayo et al. 1999; Factor, Oliver et al. 2001) and activation of PI3K/AKT signaling pathway is known to inhibit BCL3 protein degradation (Viatour, Dejardin et al. 2004). Hence, it was hypothesised that AKT expression would be correlated with BCL3 expression. However, there was no significant correlation found between AKT expression and BCL3 expression. This may not be unexpected as the PI3K/AKT pathway is activated by plethora of stimuli in the tumour microenvironment (Vivanco and

Sawyers 2002) and therefore a simple comparison of expression may not be suitable for such a complex pathway. A correlation between AKT expression and BCL3 expression may exist in specific areas in the tumour such as the invasive edge of the tumour in which BCL3 was most highly expressed (Figure 47 -B). Co-expression could be investigated using dual labelling to investigate the simultaneous expression of both proteins.

BAG1 has been shown to promote tumour survival through regulation of NF-κB activity (Clemo, Collard et al. 2008). Southern et al. (2011) demonstrated that BAG1 interacts with p50 and, furthermore, it has been suggested that the BAG1-p50-NF-κB complexes have an important role in the colorectal carcinogenesis (Southern, Collard et al. 2012). It is for this reason that the relationship between BAG1 expression and BCL3 was also investigated.

Both BAG1 and BCL3 were not significantly associated with each other, although they were both highly expressed and their joint expression maybe as a result of the tumour microenvironment. Both BAG1 and BCL3 have important roles in tumourgenic pathways. BCL3 expression is increased by cytokines (Heissmeyer, Krappmann et al. 1999; Rebollo, Dumoutier et al. 2000; Kuwata, Watanabe et al. 2003; Hu, Nesic-Taylor et al. 2005; Valenzuela, Hammerbeck et al. 2005; Brocke-Heidrich, Ge et al. 2006; Brenne, Fagerli et al. 2009; Folco, Rocha et al. 2009), although not by BAG1. Exactly, how these proteins interact remains to be elucidated, as neither BAG1 nor BCL3 are currently considered to regulate each other. Furthermore, it is difficult to explain this mechanistically based on current knowledge through a possible interaction with p50 homodimeric binding and

the suggested role of BAG1-p50-NF-κB complexes may be linked (Southern, Collard et al. 2012). Perhaps, BCL3 is more pro-proliferative (Westerheide, Mayo et al. 2001) whilst BAG1 can potentiate cell survival. BAG1 has been shown *in vivo* to possibly promote the pro-survival function of the p50-p50 homodimeric NF-κB complexes in CRC cells (Southern, Collard et al. 2012), while paradoxically suppressing EGFR signaling which promotes cell survival through the KRAS and PI3K-AKT pathways (Berg and Soreide 2012). However BCL3 has also pro-survival effects under these conditions (Kashatus, Cogswell et al. 2006; Choi, Lee et al. 2010), it might therefore be useful to investigate the effect of BAG1 and BCL3 on the cell cycle.

5.3.3 Summary

In summary, our data suggests that BCL3 is highly expressed in CRC, although the expression of BCL3 was heterogenous in the carcinoma tissue there was a higher expression observed at the invasive edge of the tumour. BCL3 appeared to stain tuft cells the reason for this is unclear and further specific immunostaining and investigation is required.

CHAPTER 6

General Discussion

6.1 Summary of Work

The aim of the work reported in this thesis was to gain further insight into the role of HSP27, HSP72, BAG1, APP and BCL3 in CRC tumorigenesis. These proteins have important roles in CRC as a result of their involvement in apoptosis, cell senescence and proliferation, which have been demonstrated *in vitro* (Sharp, Crabb et al. 2004; Sherman and Multhoff 2007; Palmer and Chen 2008; Chow, Mattson et al. 2010). The studies reported here used a hypothesis driven and combinatorial approach that used both *in vivo* and *in vitro* based experimentation. Using a cohort of archived clinically well-defined CRC tumours, the role of CRC-relevant genes was characterised using a number of methodologies including gene sequencing analyses and IHC. Also, *in vitro* cell culture studies further elucidated the role of these proteins in CRC growth, proliferation and migration using both loss of function, drug inhibition and overexpression experiments.

A cohort of CRC tumours was used to evaluate whether HSP27 and HSP72 play different roles in OIS depending on the genetic background of the tumour and the relationship with clinicopathological features. *TP53, KRAS*, and *PIK3CA* mutations and the expression of HSP27, HSP72, BAG1 and AKT was characterised and analysed. There was no association of HSP expression with clinicopathological parameters and patient survival. BAG1

was expressed in a majority of CRC and was associated with *TP53* mutation. BAG1 expression was associated with increased depth of tumour invasion, but not patient survival. The potential role of HSPs in TP53 dependent OIS was examined by associating HSP expression with *TP53* mutation status, as well as HSP expression, when combined with *KRAS* mutation and *PI3K/AKT* mutation status. HSP27, but not HSP72 expression was found to be associated with mutated *TP53*. Furthermore, HSP27 expression was found to be associated in PI3K/AKT pathway active tumours with wild type *KRAS*, although this was independent of *TP53* mutation status (Ghosh, Lai et al. 2013). Collectively, these findings suggest that *in vivo* TP53 mediated OIS is not circumvented through the expression of HSP as has been observed in previous *in vitro* studies (O'Callaghan-Sunol, Gabai et al. 2007; Gabai, Yaglom et al. 2009). Furthermore, CRC cells in culture may have different requirements for HSP expression dependent on *KRAS* mutation and PI3K/AKT activation status (Ghosh, Lai et al. 2013).

Previous loss-of-function studies of various cancers have suggested an important novel role of APP in tumourgenesis through its involvement in cell proliferation and migration (Meng, Kataoka et al. 2001; Hansel, Rahman et al. 2003; Ko, Lin et al. 2004; Takayama, Tsutsumi et al. 2009; Botelho, Wang et al. 2010; Venkataramani, Rossner et al. 2010; Yang, Fan et al. 2012). Furthermore, as observed in AD, APP expression has been shown to be associated with the oncogenic protein BAG1 (Elliott, Laufer et al. 2009).

The novel role of APP in CRC tumourgenesis was evaluated in CRC cell lines and in our CRC tumour cohort. The knockdown or inhibition of APP demonstrated an inhibition in CRC cell proliferation. However, APP expression was significantly altered by BAG1 knockdown and overexpression cell culture studies. APP was expressed in both nuclear and cytoplasmic compartments in the majority of CRC tumours; however, there was no association of APP expression with clinicopathological parameters and patient survival. Therefore, although APP clearly has a role in cell proliferation, our *in vitro* investigation of our CRC tumour cohort does not demonstrate any significant association between APP expression with clinicopathological parameters or patient survival as has been previously suggested in other cancers.

BCL3 an atypical IKK was found to be involved in both oncogenic proliferation and apoptosis. AKT has been shown to activate IKK and hence NF-κB in several settings (Ozes, Mayo et al. 1999; Factor, Oliver et al. 2001). BCL3 expression was characterised to evaluate the relationship between BCL3 expression and clinicopathological features. The potential role of BCL3 and the PI3K/AKT pathway and BAG1 was investigated by associating BCL3 expression with AKT and BAG1 expression. BCL3 was heterogeneously expressed in both nuclear and cytoplasmic compartments in the majority of the cohort CRC tumours, with a higher expression observed at the invasive edge of the tumour. There was no association of HSP expression with clinicopathological parameters and patient survival.

Furthermore BCL3 expression was not associated with AKT or BAG1 expression.

6.2 Discussion of results and future work

In contrast to other larger studies (Tweedle, Khattak et al. 2010; Bauer, Nitsche et al. 2012) (Sun, Meng et al. 2011), I found that HSP and BAG1 expression was not associated with clinicopathological features or patient survival, which could be explained by differences in the numbers of left- or right-sided CRCs between my study and those of others. There was a left sided predominance of my CRC cohort. Interestingly, there are suggested differences in pathology and prognosis between right and left sided tumours possibly as a result of the different embryological development of the mid gut and hindgut from right and left side of the colon. Yamauchi and colleagues (2012) in the largest study investigating anatomical variation of CRC molecular features found that the highest frequency of KRAS mutation was found in the caecum (52%), as well as a linear increase of MSI, CIMP and BRAF mutations moving from the rectum (<2.3%) to the ascending colon (36-40%). Furthermore, DNA mismatch mutations were seen predominately in cancers from the right side of the colon, whereas mutations were rare in cancers of the descending, sigmoid colon and rectum (Hutchins et al. 2011). Multiple studies have found that patients with MSI positive tumours have a better overall prognosis and that MSI status is an independent favourable predictor of survival (Hemminki et al. 2000). While KRAS mutation more common in cancers from the right colon is associated with a significantly

poorer prognosis (Phipps et al. 2013). Most studies found an overall poorer survival in right compared to left sided CRC (Hansen and Jess 2013). Although, patients with right-sided cancer were more likely to be older, to be women, of a more advanced stage at diagnosis than left-sided colon cancers, and have more poorly differentiated tumours (Hansen and Jess 2013). With regard to metastases, Benedix and colleagues (2010) found that left sided CRC more frequently spread to the liver and pulmonary systems than RCC, whereas RCC more often spread to the peritoneum. Right-sided colon cancers are often in a more advanced stage at diagnosis than left-sided colon cancers and have different molecular biological patterns. However, when analysis was adjusted for stage, comorbidity, and treatment variables, no overall difference in 5-year mortality was seen between right- and left-sided colon cancers. Therefore, I feel that the differences in tumour molecular biology of left and right CRC, particularly in regards to KRAS may effect the expression and role of HSP. Indeed, Pei and colleagues (2011) found that HSP27 expression was greater in right sided CRC compared to left sided CRC, although this is contrary to what our findings would expect as WT KRAS was associated with HSP27 expression.

There are significant clinical and therapeutic consequences because of the heterogenous nature of CRC and the presence or absence of mutations to key oncogenic and tumour suppressor genes. This mandates a more targeted approach to the selection of therapies for an individual's tumour than currently practiced, In this regard, I found that wildtype *KRAS* and activated PI3K/AKT, was significantly associated with HSP27 expression,

therefore indicating a possible role of HSP27 in overcoming PI3K/AKT induced OIS in tumours. Therefore, KRAS and PI3K/AKT characterisation should be undertaken as part of tailored medical therapy with possible HSP inhibitors. To develop *in vitro* cell culture-based drug screening models I collaborated to obtain stably infected colorectal cell lines with mutant KRAS. These transfected CRC cell lines will also permit further investigations into the expression of HSP27 and HSP72 and the role of KRAS plays in modulating HSP expression relative to mutation status. This will be important to further evaluate the *in vitro* findings concluded from this study. Furthermore, these results could be further extended to develop a clinical trial to evaluate HSP-specific inhibitors tailored on the basis of molecular genetic characterisation. Therefore using the frequency of KRAS mutation and PI3K/AKT activation from my study we can estimate that during an accrual period of 12 months, follow-up for outcome (survival) assessed at 24 and 60 months and with power set at 80% and the false positive rate at 5% then the patient numbers required assuming a hazard ratio of 2.0 is 86 and for 1.2 the number is 1,231. This total number of patients also assumes that they are divided up in proportion to the ratios (mutation frequencies) obtained from my pilot data.

As stated HSP have a definite role in the circumvention of OIS. As the biology of the tumour develops the role of HSP in tumourgenesis could alter with the changing tumour milieu. In the early stages, HSP may act to overcome restraint on uncontrolled proliferation inhibiting OIS and then later on to aid in tumour survival preventing apoptosis and permit further growth

and metastasis. The action of many chemotherapy and radiation seems to involve the activation of the senescence program, with favourable outcome having been associated with senescent positive CRC tumours (Haugstetter et al. 2010). It is interesting that HSP72 and HPS27 have been associated with early stage I/II left sided CRC's and prognosis (Bauer et al. 2012). Therefore, the relationship between HSP expressions might be dependent on the progress of the tumour with differential roles for HSP between expanding clones with KRAS mutation and those with the WT copy. It would be interesting to study the functional role of HSP with a larger patients cohort and consider tumour development from dysplastic polyps to early and then late stage CRC with tumour location as a variable as the role of HSP seems to be more complex than just the causal associations may demonstrate.

I, and others, have shown a functional relationship between APP expression and proliferation that demonstrates the importance of APP in the pathogenesis of CRC (Meng, Kataoka et al. 2001; Venkataramani, Rossner et al. 2010). However, the precise mechanism underlying the tumour growth-promoting effect of APP remains to be clarified. APP has been shown to function as a growth factor and as a docking molecule in the membrane proximal signaling events (Chow, Mattson et al. 2010). The N-terminal domain of APP contains cysteine-rich regions and heparin-binding sites that are similar to growth factors and APP can therefore be classified as a member of the cysteine-rich growth factor superfamily (Rossjohn, Cappai et al. 1999). The extracellular domain can interact with matrix proteins and heparan sulfate proteoglycans reflecting the role of APP in migration,

adhesion, and cell-matrix and cell-cell interactions (Saitoh, Sundsmo et al. 1989; Ninomiya, Roch et al. 1993; Small, Clarris et al. 1999). My study is the largest to date investigating the expression of APP in CRC and the association with clinicopathological outcome. I showed that APP is expressed in only CRC tumour cells but not in normal cells, an observation that was in good agreement with albeit small study by Venkataramani and colleagues. In IHC studies by others on CRC (Venkataramani, Rossner et al. 2010), prostate cancer (Takayama, Tsutsumi et al. 2009), and oral squamous cell carcinoma (Ko, Lin et al. 2004), and in brain tissue APP staining was mainly cytoplasmic. In contrast, my study found strong nuclear staining with some cytoplasmic staining. The APP antibody recognises amino acids 66-81 of the N-terminus, which would be expected to demonstrate a cytoplasmic pattern of staining. However, the APP IHC in this study is much more specific compared to Venkataramani and colleagues whose published images are that appeared overstained and non-specific on CRC tissue possibly due to prolonged incubation. Recently, Okamoto and colleagues showed reactivity of N-terminal APP antibodies in the nucleus and nucleolus SK-N-SH human neuroblastoma cells suggesting that APP might translocate via nuclear shuttling proteins and remain in the nucleus or nucleolus (Okamoto 2012). However, using the same APP antibody (Milipore, Billerica, MA, USA) as used in my study cytoplasmic staining only was observed and this discrepancy might be the result of the ganglioside GM1 competing for the same APP antibody epitope (Zhang, Ding et al. 2009). Hence, further studies using different APP antibodies under standardised conditions would be beneficial particularly as so very few

studies have been reported to date on whether specific nuclear expression of APP is reproducible. APP expression was not shown to be associated with any clinicopathological features or patient survival, unlike that observed in various other cancers (Ko, Lin et al. 2004; Botelho, Wang et al. 2010; Yang, Fan et al. 2012), although a larger cohort would be more considered more robust. The numbers of patients required can be estimated from my data: 2,440 patients would be needed to demonstrate significance at p=0.05 with power set at 80% and false discovery at 5%. The numbers assume an accrual period of 12 months and that outcome survival is assessed at 60 months. That so many patients would be needed to show that APP expression has a significant associated with outcome (survival) suggests there is unlikely to be an association in CRC.

As APP was observed from the IHC to be nuclear in expression, this suggests that APP could be acting as a transcription factor or co-factor. The use of DNA microarray initially overexpressing APP would be a useful first step in investigating the specific molecular pathways that APP effects to inhibit proliferation. Furthermore, immunoprecipitation with mass spectrometry or a protein microarray could provide interesting findings particularly concerning the protein-protein interaction of APP and its various cleavage products that may or all be involved in proliferation. Understanding the mechanism underlying the effect of APP on the growth of carcinoma cells would also shed light on the undetermined physiological function of this ubiquitous protein in non-neural cells and its potential role in tumourgenesis.

Furthermore, it would be interesting to investigate the role of the ADAM family of proteases which have a role in tumourgenesis and cell proliferation (Duffy, Mullooly et al. 2011), and their interaction with APP using inhibitors. In particular, ADAM 10 which have been shown physiologically relevant, and the constitutive α-secretase of APP (Kuhn, Wang et al. 2010). ADAM10 has been found to be upregulated in pancreatic cancer and shown to promote migration and invasion of cancer cells.

The high expression of BCL3 in CRC, in particular the invasive edge of the tumour, does support the possibility of a potential role for BCL3 in CRC tumourgenesis. I did not find any association with BCL3 expression and clinicopathological features or patient survival, alhough the overall study cohort used was small, it was >2-fold larger than the only previous investigation into BCL3 and CRC ((Puvvada, Funkhouser et al. 20100 The analysis of more CRC tumours, particularly with regard to expression at the invasive tumour edge, might reveal an association between BCL3 and survival. In this regard, Puvvada and colleagues considered BCL3 activation prognostic in metastatic CRC. I did not demonstrate *in vivo* any association with BCL3 expression and AKT, but this might reflect the heterogeneous nature of the tumour. Dual IHC labeling would provide a more exacting method to investigate this specific association. It would also be interesting to investigate BCL3 and AKT signalling pathway as well as the activation of BCL3 and NF-κB pathway using CRC cell lines and also use similar loss- and gain of function studies with siRNA knockdown and overexpression of BCL3 as I used for APP and BAG1.

6.3 Study Limitations

Studies with tumour samples with well-defined molecular features and detailed and comprehensive clinicopathological data is a very necessary first step that permits important association to be identified. The next major limitation is sample size and the costs and time needed to put together extensive collections of tumours (particularly fresh frozen tissue) and their requisite datasets. My CRC cohort was extensively investigated for molecular features and had a linked comprehensive clinicopathological database. At the onset of the project power calculation suggested that we would have a sufficient cohort size using tumour samples for the HSP study and indeed significant associations were made. However, as the project progressed and further relevant proteins were investigated there was a decrease in the availability of archival FFPE blocks from molecular genotypes tissue. This served to reduced the cohort size of subsequent protein analysis, but valuable data was obtained that can direct the power calculations for future studies. This will be useful in case where published investigations were more limited in terms of cohort numbers. It is possible that expression of multiple proteins by IHC could have been more efficiency undertaken using a tissue microarray (TMA), although this facility was not available in the laboratory until the end of my PhD. However, tissue punches required for a TMA can poise issues if the tumour FFPE samples have come from patients who are currently being followed up. Sub group analysis to reduce cohort heterogeneity clearly requires larger cohorts and it is often useful to investigate particular CRC sub groups for association with protein

expression. Again, my work can be used to inform power calculation for the numbers required and identify from the p value instances where significance is very unlikely whatever the cohort size; a larger cohort would not have necessarily changed the overall outcome. In the instance of APP, you would need significant numbers of patients (estimated above at 2,440) to show that APP expression does have a significant associated with outcome (survival).

6.4 Conclusion

The use of cancer cell lines allow manipulations that are essential for dissecting molecular mechanisms, but cancers are more genetically heterogeneous (Lee, Endesfelder et al. 2011) and studies using primary tumour specimens from patients are essential to provide a comprehensive view of cancer cell and molecular biology. The shortcomings of cell culture experimentation in terms of their direct relevance to resected tumours are often overlooked, and my view is that it is preferable to run *in vivo* and *in vitro* experimental protocols in parallel. In this thesis, I have highlighted a role for HSP27 in overcoming PI3K/AKT induced OIS in resected tumours through its co-presence of wildtype *KRAS* and activated PI3K/AKT. My work indicates that therapeutic inhibition of the cellular mechanisms that suppress OIS in tumours is an attractive proposition, but that the precise mechanism may be specific to tumours with particular genetic backgrounds. This needs to be taken into consideration, particularly when testing compounds on CRC cells grown in culture, and especially where only a very limited number of lines are being assessed for drug efficacy as is often the case. CRC lines need to reflect primary CRCs in terms of mutational profiles, which will be problematic, but researchers need to be aware of the problems when drawing inference from the results of drugs tested in cell lines. CRC is both heterogeneous and highly complex and hence there are likely to be multiple factors that are as yet unknown. Interestingly, parallels on the complexities of CRC can be drawn from the experiences gained from the manipulation EGFR, a transmembrane receptor tyrosine kinase. EGFR is overexpressed

in 25%–75% of CRCs (Goldstein and Armin 2001), but anti-EGFR chemotherapeutic agents such as Cetuximab have had limited success (Bardelli and Siena 2010). Importantly, *KRAS* mutational status has been shown to be a critical factor in determining response rates to anti-EGFR chemotherapeutic agents (Lievre, Bachet et al. 2006). Clinical trials such as OPUS and CRYSTAL, which examined the use of anti-EGFR agents with first line chemotherapy agents, did not show an improvement in response in patients with mutant *KRAS,* but instead found that these agents may be detrimental (Bokemeyer, Bondarenko et al. 2009; Van Cutsem, Kohne et al. 2009; Bokemeyer, Bondarenko et al. 2011; Van Cutsem, Kohne et al. 2011) to patient outcome. Furthermore, other genetic alterations in oncoproteins modulating EGFR signalling (e.g. BRAF, PIK3CA and PTEN) and acquired *EGFR* mutation have been found to be determinants of resistance (Bardelli and Siena 2010; Montagut, Dalmases et al. 2012). Further *in vivo* investigations on a large numbers of primary cancers or more homogenous subgroups of CRC are necessary to complement investigations that have been conducted *in vitro* using cell lines. In particular, my findings emphasise a role for using archival tissues in validating hypotheses generated from cell culture based investigations.

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APPENDIX I – Molecular characterisation of colorectal cancer cohort

Microsatellite and chromosomal instability and BRAF mutation status in colorectal cancer cohort. This cohort of CRC's has been previously been well characterised. This cohort therefore included a total of 74 were analysed for CSI and 67 for MSI. CRC patients was tested for MSI using two well-established mononucleotide markers, BAT25 and BAT26 CIN was determined by investigating the ploidy status by mechanically extracting nuclei from tumour tissue and corresponding normal tissues and then by establishing nuclear DNA content and ploidy status using flow cytometric analysis. Those cancers not categorised as CIN and/or MSI were recorded as MACS. BRAF mutation (V599E) was observed in 12% of patients, the majority of these were MSI+. Four tumours were found to be MSI+ and CSI+ (Silver, A., N. Sengupta, et al. 2012).

MSI/CSI/MACS status	n (%)	BRAF mutation n (%)
MSI+	13 (19)	5 (7)
CSI+	31 (42)	2 (3)
MACS	28 (42)	2 (3)

APPENDIX II - PCR Primers and Conditions

PCR Primers (Chapter 3)

	5'-3' sequence
TP53	
Exon 5F	CACTTGTGCCCTGACTTTCA
Exon 5R	AACCAGCCCTGTCGTCTCT
Exon 6F	CAGGGCTGGTTGCCCA
Exon 6R	ACTGACAACCACCCTTAACCCC
Exon 7F	GAGCTTGCAGTGAGCTGAGA
Exon 7R	GGGTCAGAGGCAAGCAGA
Exon 8F	GGGACAGGTAGGACCTGATT
Exon 8R	GAGGCATAACTGCACCCTTG
KRAS	
Codon 13F	TTTGATAGTGTATTAACCTTATG
Codon 13R	TATTAAACAAGATTTACCTC
PIK3CA	
Exon 9F	GGGGAAAAATATGACAAAGAAA
Exon 9R	GAGAATCTCCATTTTAGCACTTAC
Exon 20F	CATTTGCTCCAAACTGACCA CTGGAATGCCAGAACTACAATC
Exon 20R	TGTGCATCATTCATTTGTTTCA TGTGGAATCCAGAGTGAGCTTTC

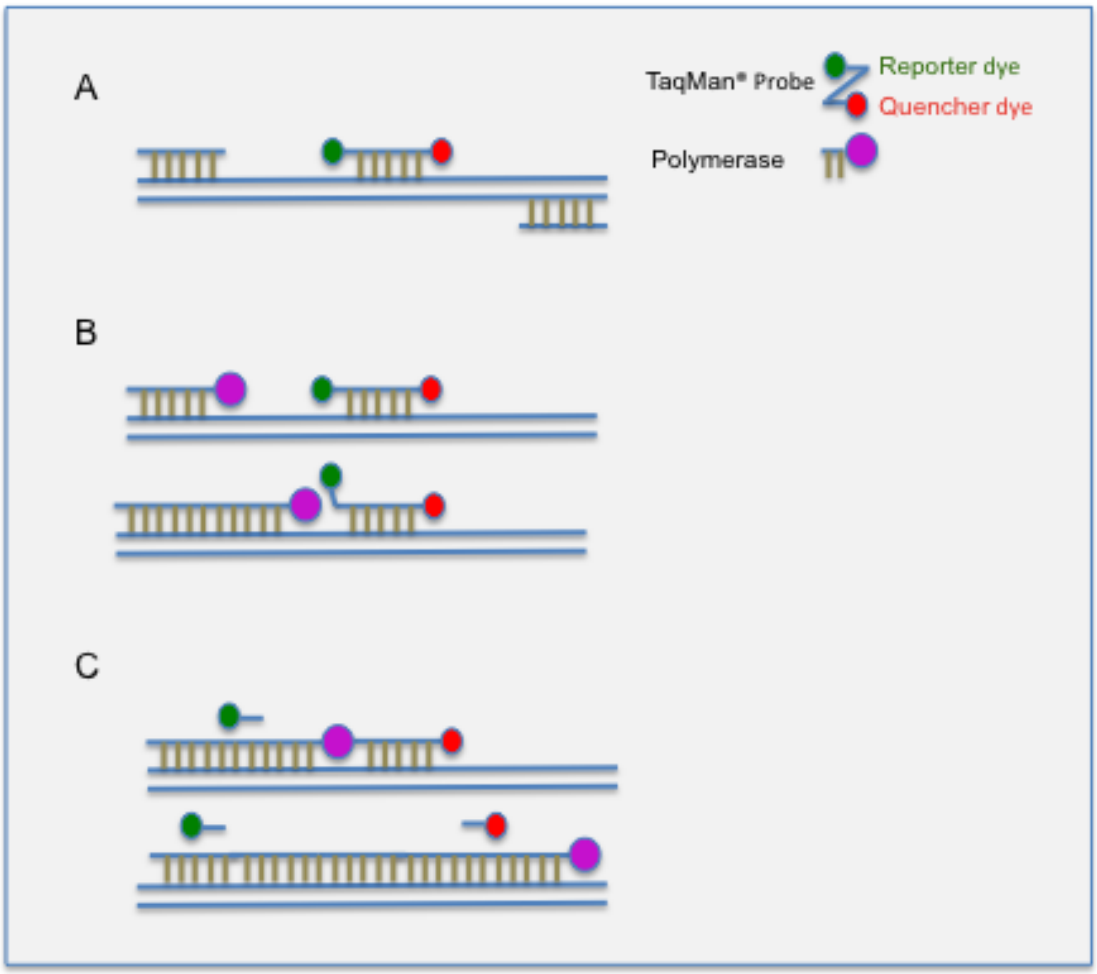
PCR Conditions for Tumour Sample Gene Mutation Characterisation (Chapter 3)

		TP53			
		Exon 5	Exon 6	Exon 7	Exon 8
Denaturation for 15 minutes	1 x cycle	95^0C	95^0C	95^0C	95^0C
Denaturation for 30 seconds	35 x cycle	94^0C	94^0C	94^0C	94^0C
Annealing for 45 seconds		56^0C	60^0C	63^0C	56^0C
Extension for 30 seconds		72^0C	72^0C	72^0C	72^0C
Final extension for 5 minutes	1 x cycle	72^0C	72^0C	72^0C	72^0C

		KRAS	***PIK3CA***	
		Codon 13	Exon 9	Exon 20
Denaturation for 15 minutes	1 x cycle	95^0C	95^0C	95^0C
Denaturation for 10 seconds	37 x cycle	95^0C	95^0C	95^0C
Annealing for 30 seconds		50^0C	58^0C	58^0C
Extension for 45 seconds		72^0C	72^0C	72^0C
Final extension for 10 minutes	1 x cycle	72^0C	72^0C	72^0C

APPENDIX III – Real time PCR

The TaqMan® Probe is an oligonucleotide that anneals to a specific sequence on the template between the forward and reverse primers (A). It has a high-energy Reporter dye at the 5' end which when intact is suppressed by a low-energy Quencher dye on the 3' end, as the close proximity of the dyes results in transfer of energy from high to low. During Real-Time PCR the accumulation of amplicon is detected during the reaction. The detection amplicon product is dependent on the exo-nuclease activity of Taq polymerase that cleaves off the TaqMan® Probe which is subsequently detected to quantify the exponential phase of the PCR reaction (B). Following cleavage of the probe, the Quencher no longer is able to inhibit the fluorescent emissions of the Reporter. Therefore the reporter signal increase is proportional to the amount of product being produced for a given sample (C).



APPENDIX IV - Probes

Probes

Target Sequences for siRNA Knockdown Transfection (Chapter 4)

	5'-3' sequence
APP	
APP 2	TTGGCCAACATGATTAGTGAA
APP 8	AAGGATGACTACAGACATTAA
APP 9	ACCCAATTAAGTCCTACTTTA
APP 10	CTGGTCTTCAATTACCAAGAA
BAG1	
BAG 4	CCGGGTCATGTTAATTGGGAA
BAG 10	CACCGTTGTCAGCACTTGGAA
BAG 11	CCCAAGGATTTGCAAGCTGAA
BAG 12	CTGAGCGGCTGCAGTCTACAA

TaqMan Gene Expression Probes (Chapter 3 and 4)

	PIK3CA	TP53	HSP72	HSP27	BAG1
Supplier	Applied Biosystems	Applied Biosystems	Applied Biosystems	Applied Biosystems	Applied Biosystems
Supplier code	Hs00503678	Hs99999147	Hs00359147	Hs03044127	Hs00185390

APPENDIX V - Immunohistochemistry and Western Blot

Immunohistochemistry Antigen Unmasking Solutions

Vector Antigen Unmasking Solution pH 6

Vector Antigen Unmasking solution pH 6 (Vector Laboratories, Burlingame, CA, USA). This solution is prepared by mixing 15ml of the stock antigen unmasking solution with 1600ml of distilled water.

Tris-EDTA-Citrate Unmasking Solution pH 8.1

A 10x strength stock solution is prepared from a mixture of Ethylendiaminetetra acetic acid (EDTA) (BDH, Germany) 15g; Tris (hydroxmethyl) methylamine (Tris Base) (BDH, Germany) 7.5g; sodium citrate (BDH, Germany) 9.6g and distilled water 3000ml. Sodium Hydroxide (BDH, Germany) is then added to this to pH 8.1.

Tris-EDTA Unmasking Solution pH 9.0

A 10 x strength stock solution is prepared from a mixture of EDTA (BDH, Germany) 11.1g; Tris Base (BDH, Germany) and distilled water 3000ml. Sodium Hydroxide (BDH, Germany) is then added to this to pH 9.0.

pH Meter Procedure

pH is measured using a Hanna pH 210 connected to an Expoxy Bodied Sealed Reference Combination electrode and Automatic temperature Compensation (ATC) probe. Firstly, the pH meter is calibrated. The probe is rinsed with distilled water and calibrated by placing it into a pH 7 buffer. To measure pH, the probe is placed into the solution to be measured allowing the pH reading to stabilise before adjusting the pH of the solution as required.

Antigen Retrieval Optimisation

Schematic of antigen retrieval combinations with microwave and water bath at 10 minutes and 40 minutes at pH 6.0, 8.1 and 9.0.

Antigen retrieval was optimised for each antibody by carrying out the immunocytochemistry method as described on 12 control tissue sections using a combination of either microwave or waterbath for 10 minutes and 40 minutes with antigen retrieval solutions at pH 6.0, 8.1 and 9.0 (figure).

pH	Microwave		Waterbath	
	10 mins	35 mins	10 mins	40 mins
6.0	1	2	3	4
8.1	5	6	7	8
9.0	9	10	11	12

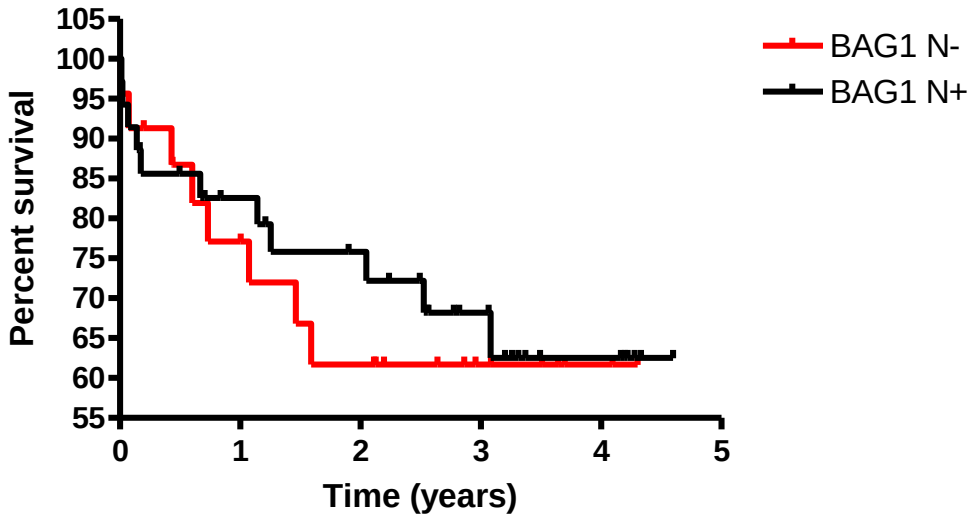
Antibody Conditions for Immunohistochemistry (Chapter 3, 4 and 5)

	HSF1	**AKT**	**BAG1**	**HSP27**	**HSP72**	**APP**	**BCL3**
Supplier	Abcam	Abcam	Abcam	Abcam	Abcam	Millipore	Abcam
Supplier code	ab52757	ab8932	ab32109	ab5579	ab31010	IHCR1002-6	ab49470
Clonality	Rabbit Monoclonal (EP1710Y)	Rabbit Polyclonal	Rabbit Monoclonal (Y166)	Rabbit Polyclonal	Rabbit Polyclonal	Mouse Monoclonal	Mouse Monoclonal
Control Tissue	Breast Ca	Breast Ca	Appendix/ colon	Breast Ca	Breast Ca	Brain	Tonsil
Pre-treatment	Microwave 35 minutes	Water bath 40 minutes	Microwave 35 minutes	Microwave 35 minutes	Microwave 35 minutes	Microwave 10 minutes	Microwave 35 minutes
Buffer	pH 6.0	pH 6.0	pH 8.1	pH 6.0	pH 6.0	pH 9.0	pH 8.1
Dilution	1:150	1:100	1:100	1:200	1:400	Prediluted	1:25

Antibody Conditions for Western Blot (Chapter 3 and 4)

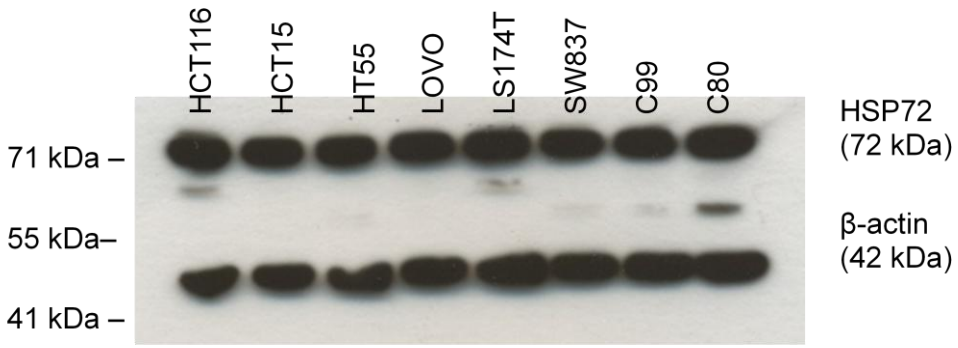
	BAG1	HSP27	HSP72	APP	B-catenin	B-actin
Supplier	Abcam	Abcam	Abcam	Millipore	Abcam	Abcam
Supplier code	ab32109	ab5579	ab31010	MAB348	ab8226	ab8226
Clonality	Rabbit Monoclonal (Y166)	Rabbit Polyclonal	Rabbit Polyclonal	Mouse monoclonal	Rabbit monoclonal	Mouse monoclonal
Dilution	1:250	1:1000	1:1000	1:250	1:1000	1:15000

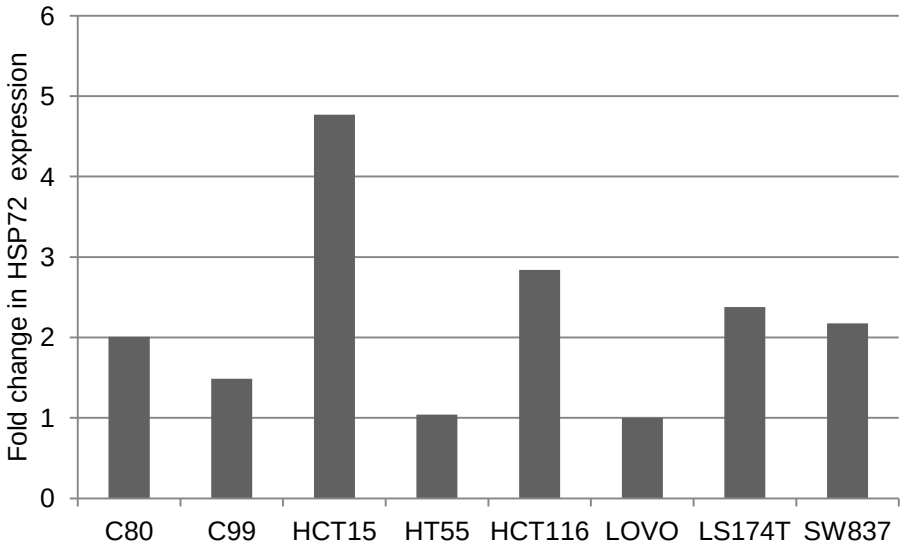
APPENDIX VI - Supplementary Nuclear BAG1 Survival Analysis



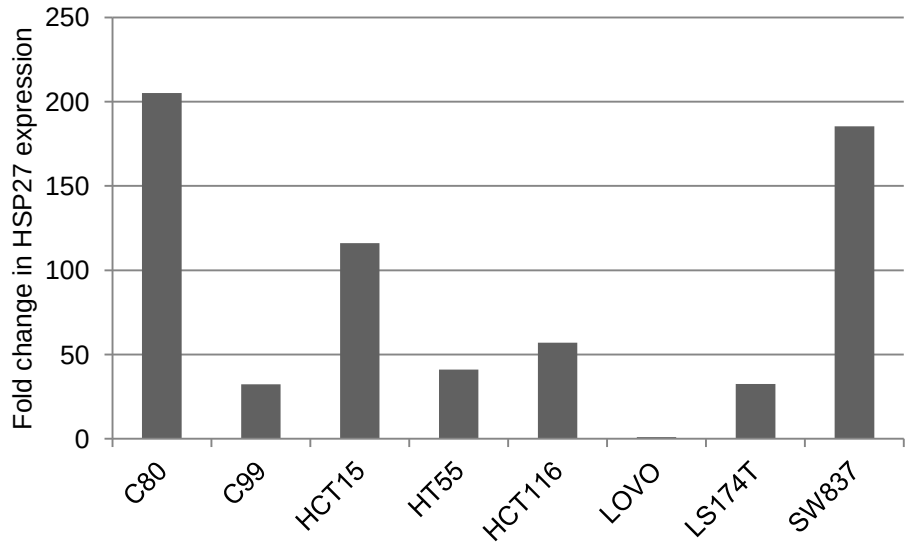
Kaplan-Meier plots correlating patient survival with protein expression of BAG1. There is no statistically significant difference (p=0.73) in survival between patients with tumour expressing nuclear BAG1 and the patients with tumours negative for nuclear BAG1 expression by log rank test.

APPENDIX VII - Preliminary Characterisation of Colorectal Cells Lines

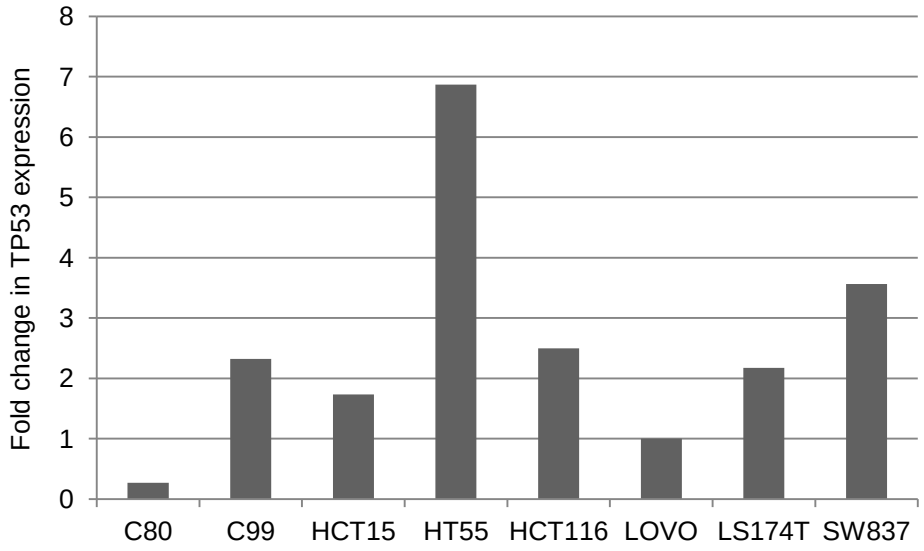




Expression of HSP72 demonstrated by Western Blot and Real-time PCR. HSP72 gene expression was plotted relative to LOVO. HT55 had a similar expression of HSP70 to LOVO, while the rest of the cell lines had increase expression of HSP72 compared to LOVO. Western Blot determined HSP72 protein expression in all the cell lines.

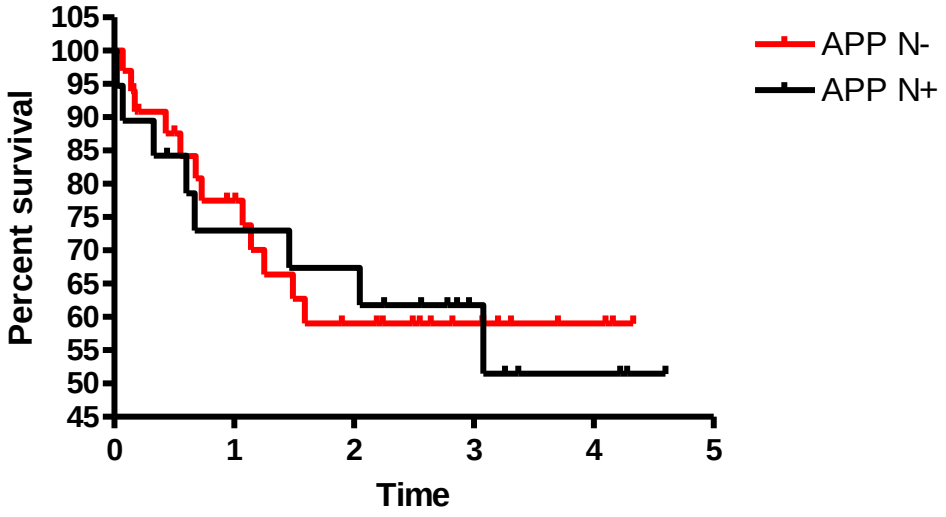


Expression of HSP27 relative to the endogenous control GAPDH in cell lines compared to LOVO. In all cell lines compared to LOVO there was a significant fold increase in expression of HSP27.

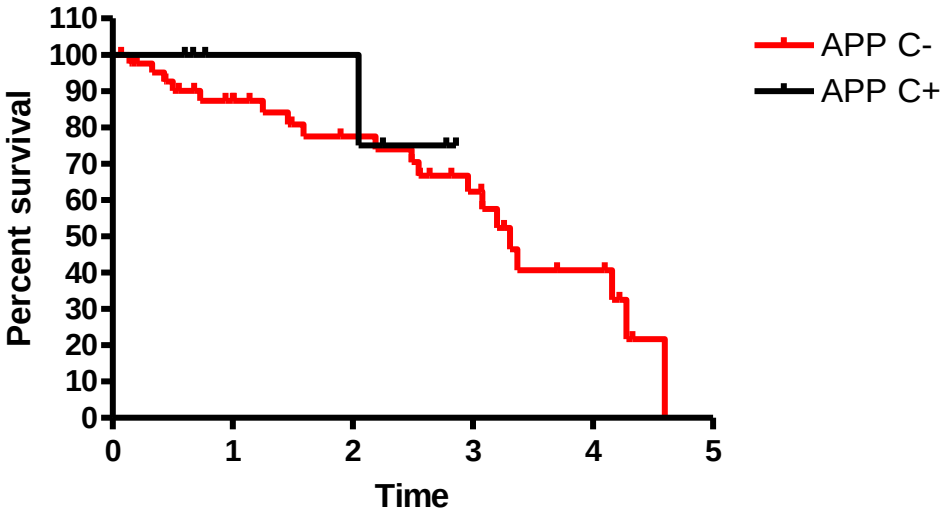


Expression of *p53* relative to the endogenous control GAPDH in cell lines compared to LOVO. All cell lines except C80 had increased expression of *p53* compared to LOVO.

APPENDIX VIII - Supplementary Amyloid Precursor Protein Survival Analysis

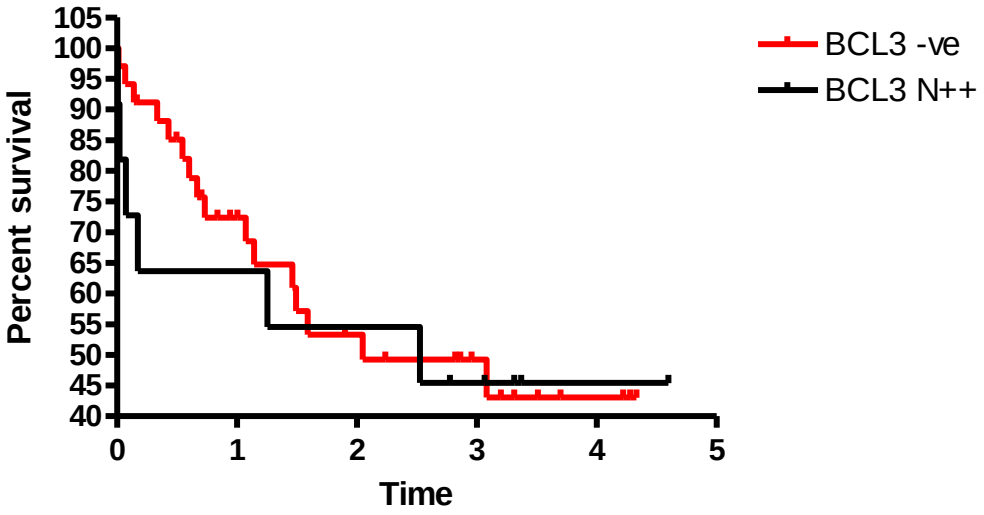


Kaplan-Meier plots correlating patient survival with protein expression of nuclear APP. There is no statistically significant difference in survival between patients with tumour expressing nuclear APP and the patients with tumours negative for nuclear APP expression by log rank test (p = 0.87).



Kaplan-Meier plots correlating patient survival with protein expression of cytoplasmic APP. There is no statistically significant difference in survival between patients with tumour expressing cytoplasmic APP and the patients with tumours negative for cytoplasmic APP expression by log rank test (p = 0.61).

APPENDIX XI - Supplementary BCL3 Survival Analysis



Kaplan-Meier plots correlating patient survival with protein expression of strong nuclear expression of BCL3. There is no statistically significant difference (p=0.76) in survival between patients with tumour expressing BCL3 and the patients with tumours negative for BCL3 expression by log rank test.