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Investigations into the Role of Cten Signalling in Colorectal Cancer

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Declaration

This work entitled 'Investigations into the Role of Cten Signalling in Colorectal Cancer' has been composed by Abdulaziz Mohammed Asiri, and I confirm that the result presented in this thesis is my own work during my period of study at the University of Nottingham, unless otherwise mentioned. No part of this thesis has been submitted for any degree, diploma or any other type of qualification at any other institution.

Abdulaziz Mohammed Asiri

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"Dedicated to the memory of my father"

I dedicate this work to my father who passed away shortly before the completion of this thesis.

Words cannot describe how grateful to you dear father, you have supported me in every possible way from day one, you were my backbone whenever I needed someone to rely on, you were my councillor whenever I needed advice, you were my bank whenever I needed financial help, you were my mentor whenever I needed mental and physical support, you were my life tutor whenever needed. You were everything a son could ask for and much more. I will be ever grateful for everything you did for me and am sorry you did not live to see me finish my PhD, but your beautiful memory will be with me eternally.

Abbreviations

5-FU	Fluorouracil
ABD	Actin binding domain
ACF	Aberrant crypt foci
AJCC	America Joint Committee on Cancer
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
C1	Protein kinase C conserved region 1
Cas9	CRISPR-associated nuclease 9
CHX	Cycloheximide
CIMP	CpG island methylator phenotype
CIN	Chromosome instability
Co-IP	Co-immunoprecipitation
CRC	Colorectal cancer
CRISPR	Clustered regularly interspaced short palindromic repeats
CSC	Cancer stem cell
CT	Computerised tomography
DAB	Diaminobenzidine
DLC1	Deleted in liver cancer 1
DMEM	Dulbecco's Modified Eagle's Medium
DSB	Double strand break
dsRNA	Short double stranded RNA
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
EV	Empty vector
FAK	Focal adhesion kinase
FAs	Focal adhesions
FAT	Focal adhesion-targeting domain
FBS	Foetal bovine serum
FERM	four-point-one, ezrin, radixin, moesin
FGF2	Fibroblast growth factor 2
GAK	Cyclin G associated kinase
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
gRNA	Guide RNA
GSK3β	Glycogen synthase kinase-3 beta
HDR	Homology directed repair
HER2	Human epidermal growth factor
HNPCC	Hereditary non-polyposis colorectal cancer
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HRM	High resolution melting
HRP	Horseradish peroxidase
IGF1	Insulin-like growth factor 1

IHC	Immunohistochemistry
IL-13	Interleukin 13
IL-6	Interleukin 6
ILK	Integrin-linked kinase
IMS	Industrial methylated spirits
Indel	Insertion or deletion
JAK	Janus kinase
Kras	Kirsten rat sarcoma viral oncogene homolog
LEF	Lymphoid enhancer factor
MAPK	Mitogen activated protein kinase
MDCK	Madin Darby Canine Kidney
MET	Mesenchymal to epithelial transition
MLH1	MutL Homolog 1
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MMP	Matrix metalloproteases
MMR	DNA mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
NGF	Nerve growth factor
NHEJ	Non-homologous end joining
NICE	National Institute for Health and Care Excellence
NLS	Nuclear localisation signal
NLS-Cten	NLS tagged Cten
PAK1	p21 activated kinase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-Kinase
PMS2	Postmeiotic segregation increased 2
PTB	Phosphotyrosine binding
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative reverse transcription PCR
RFA	Radiofrequency ablation
RhoA	Ras homolog family member A
RhoGAP	Ras homology-GTPase-activating
RIPA	Radio immunoprecipitation assay
RISC	RNAi induced silencing complex
RLC	Regulatory light chain
ROCK1	Rho-associated protein kinase1
ROS	Reactive oxygen species
RT-	Reverse transcriptase negative
RTK	Receptor tyrosine kinases
SAM	Sterile alpha motif
sgRNA	Short guide RNA
SH2	Src homology 2
siRNA	Small interfering RNA
START	Steroidogenic acute regulatory -related lipid transfer

Stat3	Signal transducer and activator of transcription factor 3
SW620$^{\Delta Cten}$	SW620 Cten knockout cell line
TACE	Transarterial chemoembolisation
TALENs	Transcription activator-like effector nucleases
TBS	Tris buffered saline
TBS-T	Tris buffered saline-tween
TCF	T-cell factor
TGFβ1	Transforming growth factor beta1
Tm	Melting temperature
TMA	Tissue microarray
TP53	Tumour protein p53
UICC	International Union Against Cancer
VEGF	Vascular endothelial growth factor
Wt.	Wild type
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
ZFNs	Zinc finger nucleases

Abstract

C-terminal tensin-like (Cten, also known as Tensin4) is the member of the tensin gene family. Cten functions as an oncogene in a variety of cancer types and its expression is commonly associated with poor prognosis and metastasis in colorectal cancer (CRC). Although several studies have shown that Cten has a critical role in the regulation of cell motility and invasion in different tumour tissues, the underlying signalling mechanisms have not been fully elucidated. This thesis investigated the biological activity of Cten in four different ways in order to further elucidate the mechanisms of Cten signalling in CRC cells.

Potential downstream targets of Cten signalling involved in the regulation of epithelial-to-mesenchymal transition (EMT) induced cell motility i.e. Rho-associated protein kinase1 (ROCK1), Src and Snail were investigated. Cten expression was manipulated in different cell lines using multiple approaches including forced expression, gene knockdown and constitutive depletion (through Crispr/Cas9 gene deletion) to eliminate artefacts of methodology and cell line specific effects. Snail, Src and ROCK1 were identified as novel downstream targets of Cten signalling and additionally, Cten was shown to increase the stabilisation of both Src and Snail proteins. The functional relevance of Cten-Snail, Cten-Src and Cten-ROCK1 signalling was assessed, and the overall findings demonstrated that Cten could promote cell motility and colony formation directly through the positive regulation of the Src/ROCK1/Snail dependent axis.

To gain a deeper insight into the mechanisms of Cten's biological function, mutations, at two important residues (i.e. arginine 474 and tyrosine 479) in the Src homology 2 (SH2) domain of Cten were introduced into one construct (GFP-Cten$^{R474A+Y479F}$) using site directed mutagenesis. These two residues in the SH2 domain of Cten were found to not only be important for interacting with Src, ROCK1, or Snail signalling, but also for regulating cell motility and colony formation efficiency.

Numerous Cten regulatory factors have been identified, however, little is known about how Cten is activated and regulated in cancer cells. The relationship between transforming growth factor beta 1 (TGFβ1) and Cten was investigated and stimulation of cells with TGFβ1 or knockdown of TGFβ1 resulted in changes in Cten expression as well as its downstream targets of ROCK1, Src, Snail, and N-cadherin. Furthermore, this positive interaction between TGFβ1 and Cten was functionally relevant and caused changes in cell motility. and the nuclear translocation of ROCK1, Src, and Snail protein increased by TGFβ1 is probably mediated via upregulation of the Cten signalling pathway

The biological function of Cten in the nucleus was further investigated and shown to increase nuclear localisation of Src, ROCK1, and Snail, further promoting the migratory capability and colony formation efficiency in CRC cells. Finally, Cten expression was shown to positively correlate with both ROCK1 and Src expression in a series of primary CRCs. This correlation was consistent with that observed following manipulation of Cten expression in CRC cell lines.

In conclusion, this study has revealed a number of novel findings regarding the biological function of Cten signalling in CRC. However, further validation of the findings may enhance the understanding of the role of Cten in the invasion-metastasis cascade in the future.

Thesis-related Publications and Conference Communications.

A. Peer-reviewed Publications

H Thorpe., **A Asiri**., M Akhlaq. and M Ilyas. 2017. Cten promotes epithelial-mesenchymal transition through post-transcriptional stabilization of Snail. Molecular Carcinogenesis.

B. In process publications

M Akhlaq, **A Asiri**, H Thorpe, D Jackson, and M Ilyas. Cten forms an SH2 domain-dependent complex with Focal Adhesion Kinase to induce cell motility in colorectal cancer (submitted for publication).

A Asiri, TP Raposo, A Alfahed, and M Ilyas. TGFβ1-induced cell motility is mediated through Cten in colorectal cancer (submitted for publication)

A Asiri, M Akhlaq, M S Toss, H Thorpe, TP Raposo, A Alfahed, and M Ilyas. Cten Increases Tumourigenicity and Cell Motility Through the Upregulation of Src/ROCK1/Snail Signalling Axis (manuscript in preparation).

C. Presentations and Conference Communications

A Asiri, H Thorpe, M Akhlaq, and M Ilyas. (2015), Cten regulates cell motility through Snail in colorectal cancer. 8th Saudi Student Conference in the UK - poster presentation.

A Asiri, M Akhlaq, H Thorpe, and M Ilyas. (2016), Cten regulates Snail through Src signalling pathway and may promote metastasis in colorectal cancer. 9th Saudi Student Conference in the UK - poster presentation.

A Asiri, M Akhlaq, H Thorpe, and M Ilyas. (2016), Cten stimulates cell motility through Src signalling in colorectal cancer. NCRI Cancer Conference - poster presentation.

A Asiri, and M Ilyas. (2017), Cten stimulates cell motility and tumour metastasis through ROCK1 signalling in colorectal cancer. 10th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland- poster presentation.

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1 General Introduction

1.1 Colorectal Cancer

1.1.1 Epidemiology and Aetiology

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both males and females in the UK. The number of cases diagnosed with the disease has gradually increased since the mid-1970s, mainly in the male population over the age of 50, with approximately 1.24 million cases resulting in over 600,000 cancer related deaths per year worldwide. Despite recent improvements in investigation, screening, detection methods and treatment options with CRC, outcomes remain poor, with approximately half of those suffering attempted curative resection dying from the disease and an overall 5-year survival of approximately 55% (Center et al., 2009).

Despite advancements in the understanding of the genetic, cellular and environmental factors involved in CRC, the aetiology of the disease remains unclear and is likely to be very complex. Indeed, few precise aetiological factors have been identified for most CRCs (Brenner et al., 2014). There is strong evidence that family history is a significant risk factor for developing CRC. This familial risk factor is mostly polygenic inheritance, although some inherited syndromes are also directly responsible for the occurrence of CRC (Burt, 2008, Johns and Houlston, 2001, Brenner et al., 2014). There is also strong evidence that life-style, poor diet (high red meat, high in fat, and low in fibre), obesity, a high intake of alcohol as well as some pre-existing disorders of the gut, such as inflammatory bowel diseases, are all significantly implicated in the development of CRC (Dai et al., 2007, Kim and Chang, 2014, Bagnardi et al., 2015)

1.1.2 Colorectal Carcinogenesis

CRC is epithelial in origin arising from uncontrolled proliferation of abnormal cells and it is believed to be caused by both internal and external factors. The majority of CRCs are adenocarcinomas which arise as a result of a stepwise accumulation of genetic and epigenetic mutations that convert a normal epithelial cell firstly into benign neoplasms

(adenomas). These then progress into a malignant cancer which can infiltrate through the normal structures of the bowel wall and into surrounding tissues such as pelvis and the abdomen. With further progression, this tumour cells may metastasise to the local lymph nodes and then to distant sites such as the liver (Hanahan and Weinberg, 2000, Ewing et al., 2014).

The stepwise development of CRC was first described in the Fearon and Vogelstein model (Fearon and Vogelstein, 1990) in which specific genetic changes were mapped onto the morphological changes seen during the evolution of a tumour from normal epithelium to invasive malignancy (Figure 1-1). Since the original description of the Fearon and Vogelstein model, a number of different genetic pathways leading to CRC have been described including: 1) chromosomal instability (CIN), 2) microsatellite instability (MSI), and 3) CpG island methylator phenotype (CIMP+), (Bagnardi et al., 2015).

Chromosomal instability (also known as aneuploidy) refers to alterations in the number of chromosomes (Fearon and Vogelstein, 1990, Armaghany et al., 2012). CIN is characterised by deletion or amplification of chromosomal regions in most cases (Worthley and Leggett, 2010). Fearon and Vogelstein reported that CIN can be a key part of the adenoma carcinoma sequence. This sequence describes a step-wise progression starting from the dysplastic aberrant crypt foci (ACF) to the formation of adenoma and advanced metastatic disease. It has been suggested that this 'traditional' pathway from normal epithelium through to malignant cancer arises as a result of the accumulation of genetic alterations, including loss or mutation of adenomatous polyposis coli (APC) gene function, KRAS mutation and tumour protein p53 (TP53), a major tumour suppressor gene (figure 1-1) (Grady, 2004, Ewing et al., 2014).

Microsatellites are nucleotide repeat sequences which are present across the entire genome. The microsatellite instability (MSI) is characterised by a high frequency of mutations at microsatellite sequences. It is caused by a failure in the function of the DNA

mismatch repair (MMR) system resulting in an increase in mutations during replication. Microsatellites are especially prone to mutations during replication and thus loss of MMR results in MSI. The proteins involved in MMR include MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MutS homolog 6 (MSH6) or PMS2 postmeiotic segregation 2 (PMS2). These proteins combine to from a complex which allows MMR and loss of any one of these four proteins can result in loss of MMR function.

MSI has been observed most commonly in hereditary non-polyposis colorectal cancer (HNPCC, also known as Lynch Syndrome), whilst approximately 15-20% sporadic tumours show MSI (Söreide et al., 2006, Hoeijmakers, 2001). The MSI pathway is commonly found to involve alteration in Wnt signalling followed by mutations in BRAF/KRAS. The status of MSI is induced by the positive selection of cancer cells with mutations affecting microsatellites in TGFβ receptor 2 (TGFβR2), insulin-like growth factor 2 receptor (IGF2R) and BAX to enable a TP53-independent mechanism of progression to carcinoma (figure 1-1), (Walther et al., 2009).

Cytosine and guanine frequently form a dinucleotide pair (CpG) and multiple CpGs (known as CpG islands) are frequently found in gene promoter regions. Expression of approximately 50% of human genes is regulated by methylation of promoter CpG islands (Ewing et al., 2014). Methylation of CpG islands in promoter regions usually occurs in normal cells to silence several genes, including tumour suppressor genes, however, unmethylated promoter sequences have been reported to be significant in cancer cells. The CIMP was found to have a strong association with the MSI and BRAF mutation (Illingworth and Bird, 2009). It has been proposed that CIMP linked to hypermethylation of the MMR gene MLH1 is the key transcriptional mechanism driving the development of sporadic CRC with MSI through inactivation of certain genes that may function as tumour suppressor genes, resulting in silencing of these genes and loss of genomic stability (Illingworth and Bird, 2009, Bariol et al., 2003, Mojarad et al., 2013).

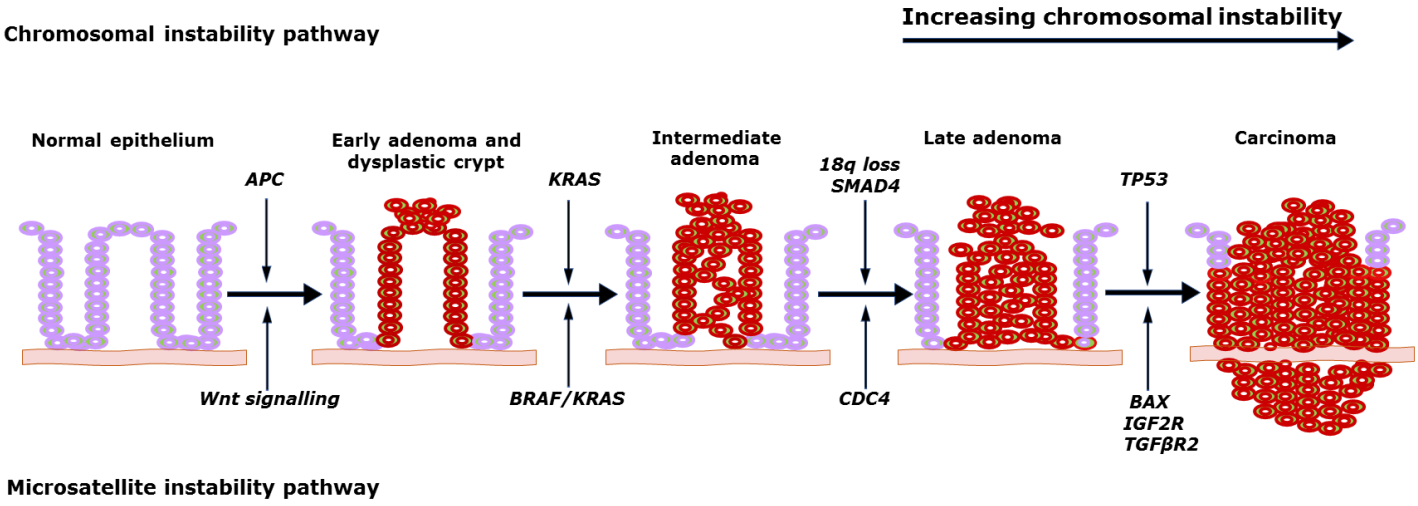

Figure 1–1: The adenoma-carcinoma sequence
The progression of colorectal adenocarcinoma from normal epithelium to carcinoma is driven by genomic alterations along specific pathways. The classical chromosomal instability (upper) and microsatellite instability (lower) pathways state that sequential genetic events including mutations disrupting APC/Wnt signalling, KRAS/BRAF signalling, loss of 18q/SMAD signalling and TP53 signalling.

1.1.3 Clinical Features

The majority of patients with early CRC are asymptomatic. However, symptoms when present commonly include abdominal pain, weight loss, rectal bleeding and change in bowel habits (Brenner et al., 2014). Two thirds of CRCs are found in the distal (left side) and the remaining third are present in the transverse and right colon. Symptoms of bowel obstruction are rare, but when present impart a poor prognosis (Hamilton et al., 2005, Thompson et al., 2007, Bass et al., 2009).

1.1.4 Diagnosis and Investigation

The diagnosis and investigation of CRC in the UK is achieved following the British Society of Gastroenterology (BSG) and the National Institute for Health and Care Excellence (NICE) guidelines (NICE, 2011, BSG, 2009). The primary diagnosis of CRC is performed by three main detection methods including colonoscopy, sigmoidoscopy or double contrast barium enema to detect any abnormalities in the rectal wall or colon and to allow for biopsies to be taken for further investigation. Following this, assessment of spread are required to identify the tumour stage and this can be carried out by computerised tomography (CT) scanning, x-ray, or magnetic resonance imaging (MRI)(Poston et al., 2011).

1.1.5 Staging and Prognosis

Staging is an assessment of the spread of a tumour throughout the body. Tumour stage is often assessed by two staging systems recommended by the RCPath for CRC in the UK such as Dukes' staging and TNM classification system (Haq et al., 2009, RCPath, 2014). The Dukes' staging system older than the TNM system but still preferred by some oncologists and surgeons (Haq et al., 2009). Dukes' staging was designed for the classification of CRC and most recently has been modified by Astler and Coller stages CRC into stage A to D (table 1-1).

Tumour stage is now commonly assessed by the TNM system of the America Joint Committee on Cancer (AJCC) and The International Union Against Cancer (UICC) (table 1-2). It is considered more accurate than the old system and offers greater patient sub-classification (Edge and Compton, 2010). The components of the system include size and depth of the tumour penetration (T), the extent of lymph nodes involvement (N) and the occurrence of metastatic disease (M) (figure 1-2).

Table 1–1: The modified Astler-Coller staging system of CRC.

Stage	Criteria
A	No penetration through the muscularis propria
B	Tumours invade into muscularis propria
B1	Tumours invade into serosa
C	Tumours with any level of penetration but have regional lymph nodes involvement
C1	1-4 regional lymph nodes involved
C2	4 or more regional lymph nodes involved
D	Distant metastases

Table 1–2: The Union for International Cancer Control 5th TNM classification of CRC.

Stage	Criteria
T	**Primary Tumour**
TX	Cannot be assessed
T0	No invasive tumour
T1	Tumours invade the submucosa
T2	Tumours invade into but not through muscularis propria
T3	Tumours penetrate through muscularis propria into serosa
T4a	Tumours invade other organs or structures
T4b	Tumours perforate the visceral peritoneum
N	**Regional Lymph Nodes**
NX	Cannot be assessed
N0	No regional lymph node metastases
N1	1-3 regional lymph nodes involved
N2	4 or more regional lymph nodes involved
M	**Distant Metastases**
MX	Cannot be assessed
M0	No distant metastases
M1	Distant metastases identified

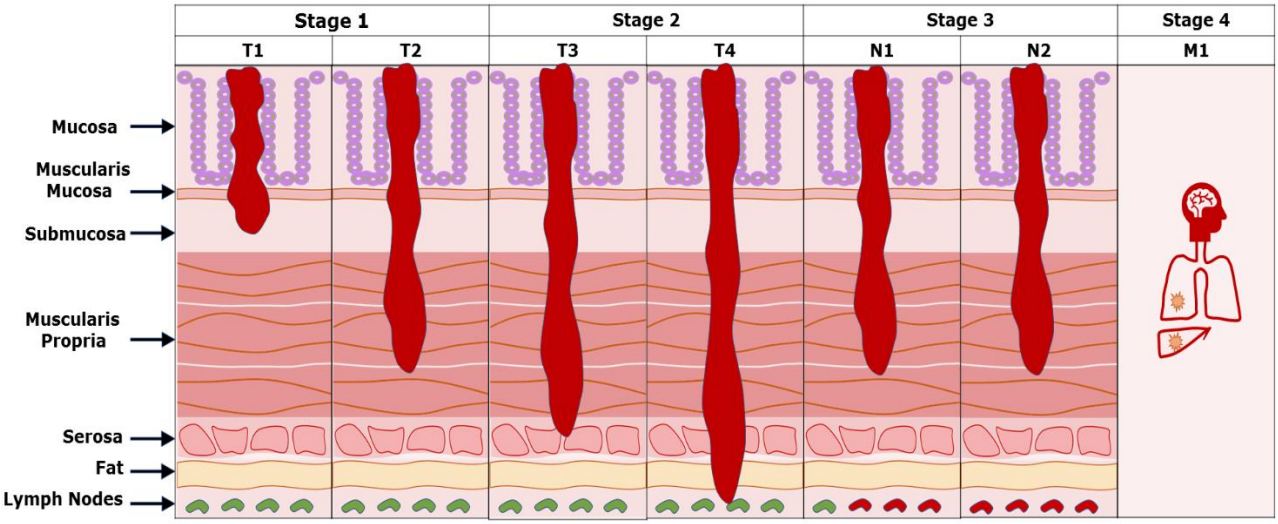

Figure 1–2: TNM staging system of CRC.
The TNM system consists of four stages which assess the size and depth of tumour penetration (T1-T4), the number of lymph nodes involved (N0-N2), and the presence and absence of distant metastasis (M0 or M1).

1.1.6 Therapeutic Options

The therapeutic options for CRC are based on the tumour stage at diagnosis as well as the patient's molecular phenotypes. Treatment for patients with CRC often involves different modalities including surgery, chemotherapy, or radiotherapy. Surgery is the standard treatment for primary CRC and has the greatest effect on survival. The aim of surgical treatment is to achieve local control of the disease and comprises complete resection of the tumour, its major vascular pedicle, and its lymphatic drainage which is necessary for precise tumour staging. The therapeutic options available for patients with advanced metastatic CRC are limited. A combination of surgery and neoadjuvant and/or adjuvant therapy can be used to eliminate the tumour (NICE, 2011).

The aim of neoadjuvant and/or adjuvant therapy is to augment the local control achieved by surgery, with complete disease control to stop the development of metastases and reduce the recurrence risk (Boland and Fakih, 2014). Adjuvant chemotherapy is commonly used for patients with CRC at stages of II and III (Carrato, 2008). Several studies have reported a documented risk reduction of death for patients with CRC at stage II, with an approximately 9% improvement for rectal cancer and 5% for colon cancer in 5-year survival. Several drugs may be used for chemotherapy including Five-Fluorourcail (5-FU), Irinotecan (Camptosar), Capecitabine (Xeloda),and Oxaliplatin (Eloxatin), (Meyerhardt and Mayer, 2005), with 5-FU commonly used in combination with oxaliplatin (FOLFOX protocol) (Des Guetz et al., 2009). Many trials have indicated that treatment with 5-FU improved recurrence free survival and 5-year survival in patients with CRC (Francini et al., 1994, Taal et al., 2001).

Targeted therapies, such as monoclonal antibodies (known as biological therapies) and molecular marker inhibitors, are used in the treatment of advanced CRC. These interfere with specific molecules to help prevent and reduce the development of tumour growth, unlike chemotherapeutic agents that non-specifically target proliferating cells.

Monoclonal antibodies such as Cetuximab and Panitumumab can be used to target the extracellular domain of EGFR - they competitively block the EGF receptors and consequently stop the activation of downstream pathways. However, these drugs are ineffective in patients with mutated downstream target genes, such as KRAS and BRAF, which are commonly mutated in CRC (Misale et al., 2012). Therefore, these anti-EGFR drugs are only recommended for those with advanced metastatic disease expressing wild type KRAS (Kopetz et al., 2009). Moreover, monoclonal antibodies, including Bevacizumab and Ramucirumab, are also used to target vascular endothelial growth factor (VEGF) to stop the growth of blood vessel in patients with advanced metastatic CRC (Ferrara et al., 2004). Other procedures such as radiofrequency ablation (RFA) and transarterial chemoembolisation (TACE) are indicated in certain circumstances (Brown et al., 2012). Therefore, the current therapeutic options available for patients with more advanced metastatic CRC are limited. Thus, more importantly, a better molecular understanding of advanced metastatic disease will allow for more precision medicine in the treatment of CRC to achieve better patient prognosis.

1.2 Metastasis

1.2.1 Multistep Cell-Biological Processes

Metastasis is complex multistep cell-biological process by which cancer cells first leave the primary tumour to enter the blood vessels or lymphatic vessels and then spread to distant sites where they proliferate and form a secondary tumour mass (figure 1-3), (Valastyan and Weinberg, 2011). Most cancer related deaths occur due to metastatic disease. Thus, it is important to understand the mechanisms of metastasis in order improve cancer treatment and patients' survival (Spano et al., 2012).

1.2.2 Cancer Cell Dissemination to Metastatic Colonisation

Cancer metastasis can occur in several different forms, but commonly there are multiple biological steps in metastasis (Brooks et al., 2010). Initially, new blood vessels develop at the primary tumour (known as angiogenesis) to support its metabolic needs before metastasis starts, followed by dissociation of cancer cells from the primary tumour tissue. The key process of this step is the downregulation of cell to cell adhesion in cancer cells through EMT. However, occasionally collective migration allows tumour cells to move and migrate in a group, without going through EMT and loosing cell-cell adhesions. following EMT, the detached tumour cells invade through the basement membrane and migrate through the extracellular matrix (ECM) surrounding the tumour epithelium. Next there is intravasation of tumour cells into blood vessels, which is characterised by increased proteolytic activity and ECM breakdown.

Cell invasion is an initial step in cancer metastasis, also known as the invasion-metastasis cascade. To achieve this, proteolytic enzymes such as matrix metallo-proteinases (MMPs) are needed to facilitate ECM degradation. Normal tissues have very low MMP activity which is carefully controlled through transcriptional and post-transcriptional processes (Brooks et al., 2010, Valastyan and Weinberg, 2011). However, during tumour progression, cancer cells downregulate matrix protease inhibitors. At the same time, cells induce the expression of integrin to mediate adhesion to the ECM. The

process of cell invasion into tumour-associated vasculature is called intravasation. Following intravasation, cancer cells migrate to secondary distant sites through the circulatory system (Brooks, 2010). On arrival at a target distant site, cancer cells form cell-cell adhesion to endothelial cells via integrins, selectins and members of the immunoglobulin superfamily (IgSF) (Guan, 2015), before penetrating the endothelial cell layer (extravasation) (Brooks et al., 2010). It has been reported that VEGF released by metastatic cells increases vascular permeability by activating the Src family kinase pathways in the endothelial cells, subsequently disturbing endothelial cell-cell junctions. This enables extravasation of circulating tumour cells and the establishment of metastatic colonies or micrometastasis. These can grow to form macro-metastases under these critical conditions before progression to clinically detectable tumour growth (Criscuoli et al., 2005). In the final step of metastasis, tumour cells proliferate and colonise as a secondary tumour at the target organ site (Valastyan and Weinberg, 2011).

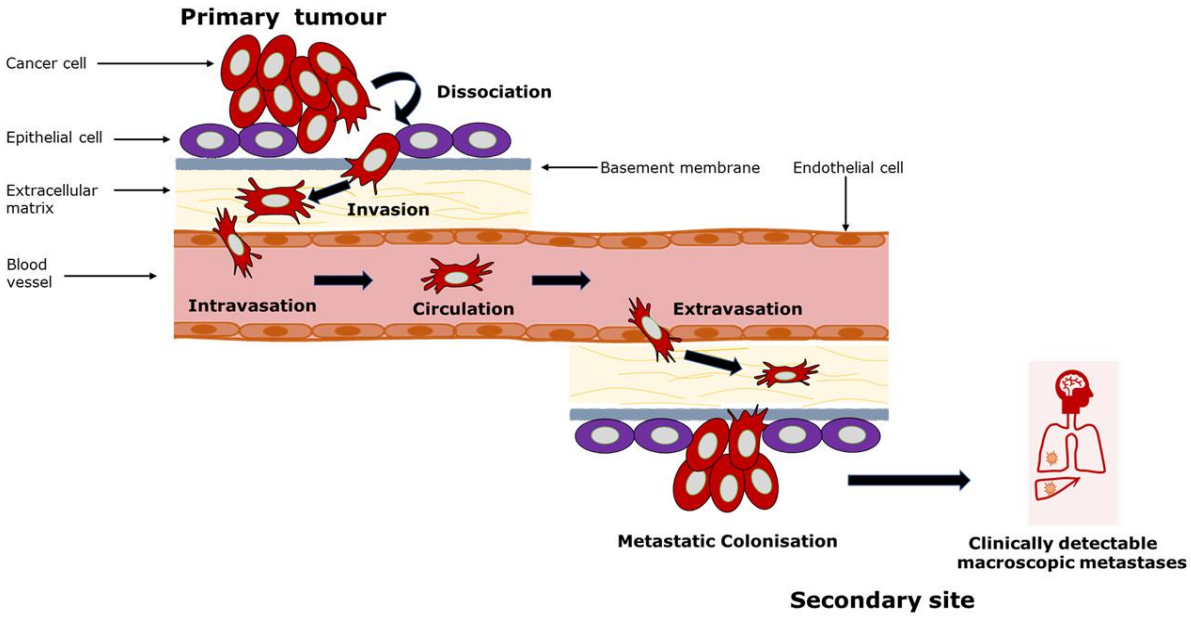

Figure 1–3: Progression of events during cancer metastasis.
Cancer cell dissociate from the primary tumour site into the blood circulation, extravasation at a distant secondary site, colonisation of a distant organ as micrometastasis and, finally outgrowth as macroscopic metastasis.

1.2.3 The Role of EMT in Metastasis

As described above, loss of cell to cell adhesion is essential for the dissociation of cancer cell from the primary tumour mass. During tumour progression, epithelial cells lose their cell to cell interaction, undergoing a developmental shift to very invasive cells which have a fibroblastoid or mesenchymal phenotype. This process is known as EMT, a highly regulated process in healthy cells. The protection of the epithelial structure requires strong cell to cell adhesion including cadherin based adherens, tight junctions, gap junctions and desmosomes. Epithelial cells also require cell-ECM adhesions mediated by integrins. Cell-ECM and cell-cell adhesion are disrupted to release the epithelial cells from the surrounding tissue mass. Hence, these cells lose their polarity and gain the ability to migrate to a new location by reorganisation of the action cytoskeleton (Mani et al., 2008, Polyak and Weinberg, 2009). In addition, degradation of the matrix by protease enzymes, such as MMPs, allow the cells to invade through a three-dimensional ECM. EMT is induced by several oncogenic signalling pathways and environmental factors including TGF-β/SMAD, integrin, receptor tyrosine kinases (RTK)/Ras signalling, Notch, Hedgehog, Wnt/β-catenin, NF-kB-dependent pathways, hyaluronan expression, MMP exposure, and reactive oxygen species (ROS). However, 'EMT' includes a broad range of changes in epithelial plasticity (Radisky, 2005, Radisky et al., 2005, Zoltan-Jones et al., 2003, Polyak and Weinberg, 2009).

The expression of E-cadherin plays a significant role in the structure of epithelial cells by allowing them to maintain position and polarity within the tissue, thus acting as a tumour suppressor. Inhibition of E-cadherin function to break the tight junctions is therefore a critical step driving EMT process (McCaffrey and Macara, 2011). The expression of E-cadherin can be functionally inactivated or downregulated by several mechanisms during tumour progression such as somatic mutation, post-transcriptional control, downregulation of gene expression through promoter hypermethylation, transcriptional repression and histone deacetylation (Huber et al., 2005, Onder et al.,

2008). The expression of N-cadherin is upregulated in mesenchymal cells, allowing cells to function independently and enabling migration (Onder et al., 2008). The P13K/Akt signalling axis is also involved in driving EMT through interacting with other important signalling pathways such as Snail. When the PI3K/Akt signalling axis is activated, phosphorylated activated Akt leads to the induction of transcription factor expression, for example, Slug and Snail that act to suppress the expression of E-cadherin at cell-cell junctions, enabling a cell to detach from its host tissue. Silencing or inhibition of Snail can reduce tumour growth and invasiveness, whereas upregulation of Snail can correlate with metastasis and poor prognosis. It has been reported that vimentin and fibronectin expression are also increased, as is the expression of MMPs allowing basement matrix degradation to take place (Huber et al., 2005, Xu et al., 2015). The reverse process of EMT known as mesenchymal-epithelial transition (MET) is thought to enable the seeding of cancer cells at a target distant site and they may develop into metastases under the right environmental conditions (Friedl and Wolf, 2003, Friedl and Gilmour, 2009).

1.2.4 Focal Adhesion and Metastasis

The initial steps of metastasis require the coordination of the microenvironment and intracellular signals to enable the cancer cells to be detached from each other and from the ECM at the primary site. In tumours, several cell adhesion molecules are involved in the cell-matrix linkage (Okegawa et al., 2004). Focal adhesions (FAs) are large protein assemblies that form a connection between the cell cytoskeleton and the ECM through integrins. The interactions between the cell cytoskeleton and the ECM play a critical role in regulating cell functions such as cell morphology, motility, proliferation differentiation and survival. Thus, any disruption in these functions can lead to cancer cell transformation (Hynes, 2002). FAs are located around membrane bound integrin receptors. Integrins are cell adhesion proteins that play a key role in converging signals from the cell membrane to inside the cells (Takada et al., 2007). They are heterodimers which consist of non-covalently to α and β transmembrane proteins. There are approximately 18 α and 8 β subunits that combine to give 24 different transmembrane

heterodimers in total (DeMali et al., 2003). The two key roles of these integrins are to convey external and internal signalling within the cell. Externally, these integrin heterodimers act as ECM receptors for proteins including thrombospondins, laminin, vitronectin and fibronectin. Internally, integrins bind to the actin cytoskeleton through large dynamic protein complexes containing four-point-one, ezrin, radixin, moesin (FERM) domains, such as kindlins and talin to activate conformational changes which consequently lead to integrin activation (Plow et al., 2000, Humphries et al., 2006, Moser et al., 2009). The FA proteins that bind to the β cytoplasmic tail of the integrin subunit often do so via phosphotyrosine binding (PTB) domains that bind to 1 of 2 conserved NXXY motifs in the cytoplasmic tail of integrin (figure 1-4), (Zaidel-Bar et al., 2007, Campbell and Humphries, 2011).

FAs functionally act as signalling platforms to regulate several cellular processes, in particular, cell migration by providing a mechanical linkage between the cell interior and the ECM (Lo, 2006). FAs are important for the cell to migrate by grasping the ECM to generate essential forces to pull the cell body forward, with continuous cycles of cell movement, adhesion formation, and attachment to the substratum at the leading edge, followed by cell body translocation and release of this attachment at the cell posterior (Wehrle-Haller, 2012, Maziveyi and Alahari, 2017). The adaptor FA proteins, such tensin, talin, paxillin, P130Cas, vincullin, and α-actinin, provide links between the actin cytoskeleton and integrins to connect cells to the ECM, whilst kinases including Src and FAK transmit downstream intracellular signals via tyrosine phosphorylation processes. This linkage allows generation of the tension necessary to promote changes in cell morphology and traction to move the cell body during migration (Westhoff et al., 2004, Legate and Fässler, 2009, Fukumoto et al., 2015). Although the protein kinases such as Integrin-linked kinase (ILK), PKC, and CSk are present in FAs, Src and FAK are the most well characterised and dominant tyrosine kinases in FA signalling pathways, playing key roles in integrin-mediated FA assembly (Maziveyi and Alahari, 2017). Since integrins have no intrinsic catalytic activity, these tyrosine kinases mediate signals to the cellular

machinery by auto-phosphorylating several integrin-associated proteins at position Y397 (Toutant et al., 2002). This allows the binding of Src, which in turn, stimulates the phosphorylation of FAK and the binding of additional proteins (Mitra and Schlaepfer, 2006). FAK induces the turnover and formation of FA dynamics by the further recruitment of talin protein (Lawson et al., 2012). Moreover, recruitment of paxillin, as well as vinculin, provides further stabilisation to these protein assemblies (Pasapera et al., 2010).

Maturation of FAs is a process initiated by activation of Ras homology family member A (RhoA) and its downstream signalling pathways, including Rho-associated protein kinase (ROCK), mDia, and myosin II regulatory light chain (RLC). Under increased traction conditions, this signalling results in a thick actin stress fibre formation and may recruit further FA proteins such as zyxin and tensin (Chrzanowska-Wodnicka and Burridge, 1996, Cao et al., 2012, Zaidel-Bar et al., 2007). The FA formation and rapid disassembly is a feature of actively migrating cells. In certain cases, further maturation of FAs to fibrillar adhesions maintains firm cell-ECM attachment and this stage is generally associated with non-motile cells (Yoshigi et al., 2005, Clark et al., 2010). Cell migration is commonly associated with the increased expression of many proteins in the FA complex such as Src, FAK and tensin family (Chen et al., 2000, Chan et al., 2010). Therefore, any alterations in these FA protein expressions can contribute towards changes in cell motility and consequently risk of cancer metastasis (Cao et al., 2012).

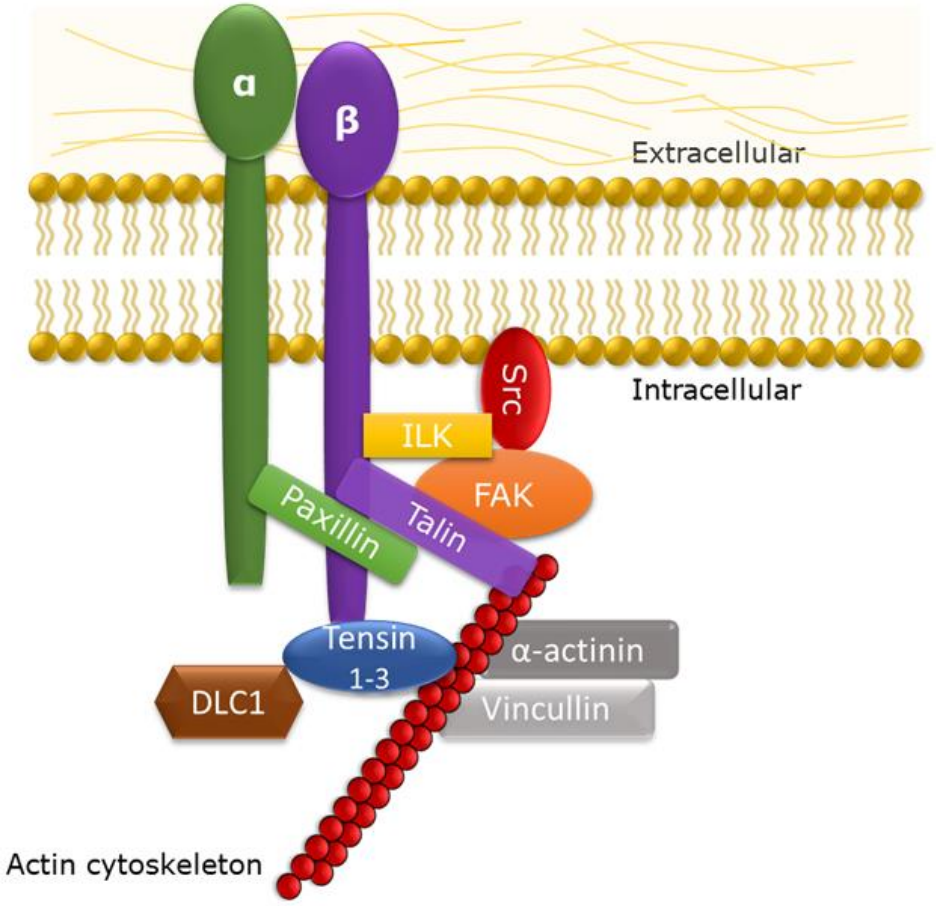

Figure 1–4: The structure of focal adhesion complexes.
Focal adhesions are located around membrane bound integrin receptors. They form the transmembrane linkages between the ECM components and actin cytoskeleton via large dynamic molecular protein complexes, including protein kinases, phosphatases and adaptor molecules. Tensin 1 to 3 bind to actin cytoskeleton.

1.3 The Human Tensin Family

1.3.1 Structure of Tensin Members

The human tensin family consists of four protein members, tensin 1, tensin 2 (C1-TEN), tensin 3, and tensin 4 (Cten), which are found complexed with the cytoplasmic tail of β integrin at focal adhesions. Tensin family members are homologs to each other at both protein and gene levels, with the exception of tensin 4 (Cten). Tensin 1-3 share common actin binding (ABD), Src homology 2 (SH2) and phosphorylation binding (PTB) domains yet with divergent central regions (figure 1-5). Tensin 1-3 also share sequence homology with the tumour suppressor protein PTEN (phosphatase and tensin homolog), which is often disrupted in cancer. Human tissue samples have been used to analyse the expression level of the four tensin genes by northern blot analysis, showing tensin 1, 2, and 3 are expressed in most tissues (Chen et al., 2000, Chen et al., 2002, Cui et al., 2004, Lo and Lo, 2002). Tensin1 and tensin 2 share similar expression profiles with higher expression in the kidney, heart, lung, liver and skeletal muscle, and lower expression in the thymus, brain, and peripheral blood leukocytes (Chen et al., 2000, Chen et al., 2002). The expression of tensin 3 was found to be very high in the kidney and placenta, and very low in the thymus, brain, and peripheral blood leukocytes, similar to tensin 1 and 2 (Cui et al., 2004). In contrast to other tensin protein members, Cten shows a much more restricted expression profile, with high expression in the prostate and placenta, and essentially no expression in all other tissues tested (Lo and Lo, 2002). However, Cten expression has since been observed in other tissues (Martuszewska et al., 2009).

Tensin 1 was first identified in 1985. It is located on the long arm of chromosome 2 (2q35-36) and composed of 33 exons, 28 of which are coding exons (the start codon is in exon 6, whereas the stop codon is in exon 33), and giving rise to a translated protein product of 1735 amino acids length, with a molecular mass of 185 kDa (Davis et al., 1991, Chen et al., 2000). Tensin 1 consists of 2 ABD located in the N-terminal region

that interact with actin filaments and an extra ABD located in the central region of the protein which has a high sequence homology to insertin, an actin capping protein which regulates the rate of actin polymerisation. The C-terminal end of the tensin 1 contains SH2 and PTB domains, and focal adhesion binding (FAB) site. Tensin 1 interacts with β integrin tails via its PTB domain, whereas it binds to tyrosine phosphorylated proteins such as FAK, PI3 kinase, and P130Cas through its SH2 domains (Lo et al., 1994, Chuang et al., 1995, Lo, 2004).

Tensin 2 was first identified in 2002 as a protein related to tensin 1 by sharing around 60% and 67% similarity respectively of homologous N- and C-terminal sequence regions. Tensin 2 is located on chromosome 12 and composed of 28 exons, of which 23 are coding exons (start codon is in exon 6, whereas stop codon is in exon 28). It maps to chromosome 12q13 and encodes a protein containing 1410 amino acids with a molecular weight of 170 kDa. Unlike tensin 1, tensin 2 has no insertin-like region in its centre and it may not play the same actin remodelling role as tensin 1 (Chen et al., 2002). Although tensin 2 shares common ABD, SH2 and PTB domains with other tensin members, it is the only member that has a protein kinase C conserved region 1 (C1) at the N-terminus. This C1 domain is known to bind to phospholipids (Johnson et al., 2000, Hafizi et al., 2002).

Tensin 3 was first identified in 2004 by Cui et al and is also believed to exist as different isoforms. Tensin 3 is located on the short arm of chromosome 7 (7p12) and has 31 exons. It contains 4415 base pairs (bp) that code for a protein of1445 amino acids with a molecular weight of 155 kDa. Tensin 3 shares similar domain structure with tensin 1 and tensin 2 and contains 32 tyrosine residues, 13 of which are predicted to be potential phosphorylation sites and possible candidates for signal transduction (Cui et al., 2004).

Cten was first identified in 2002 by Lo and Lo as the most recent member of tensin family which maps to chromosome 17q21 and translates into a 715-amino acid protein with a molecular mass of 77 kDa. It comprises 12 exons and, of these, exons 4-11 only share high sequence homology to the C-terminus of the other tensins and shares a common SH2 domain and PTB domain. Unlike other tensin protein members 1-3, Cten lacks the actin binding domain which could result in its inability to bind to the actin cytoskeleton and may therefore play a critical role in cellular processes (Lo and Lo, 2002). Interestingly, Cten localises not only to focal adhesions but also to the nucleus, suggesting that Cten might act as a transducer molecule probably involved in nuclear processes in addition to events on the surface of the cell (Liao et al., 2009).

1.3.2 Function of Tensin Members

The tensin family members play a vital role in regulating many cell functions including cell adhesion and migration, as well as other crucial biological events such as proliferation, differentiation and apoptosis (Lo and Lo, 2002). It is believed that tensins have a key role in linking the ECM to the actin cytoskeleton, thereby mediating the signalling for cell morphology and motility associated with cytoskeletal reorganisation (Lo et al., 1994, Cao et al., 2012, Clark et al., 2010). However, understanding of the tensin signalling pathways is lacking. Although members of the tensin family may, to some extent, have distinct roles due to their divergent regions, they share both the lack of embryonic lethality in mice and extensive sequence homology, which suggests functional redundancy of the tensins. It has been reported that tensins interact with other proteins localised at the focal adhesions complex such as FAK, ILK, Src, P130Cas, caplain, and Src (Albasri et al., 2011a, Al-Ghamdi et al., 2013, Thorpe et al., 2015, Zhao et al., 2016, Cao et al., 2012). This suggests that tensins possibly control cell function through the regulation of these proteins localised at the focal adhesion complexes and actin remodelling. In addition, some studies suggested that tensin 1 can accomplish this by either an actin capping role or actin monomer insertion (Lo et al., 1994, Chuang et al., 1995). It has been demonstrated that other members of the tensin family may promote

actin structural rearrangements, however the regulation of this and how this contributes to focal adhesion turnover remain incompletely understood (Cao et al., 2012). Tensin members 1-3 are commonly involved in maturation of focal adhesion processes. Therefore, both tensin 1 and 3 may play a key role in the maturation to fibrillary adhesions through the recruitment of mature focal adhesions, although they are absent from nascent adhesions (McCleverty et al., 2007, Zaidel-Bar et al., 2007). Tensins are localised to different areas during cell migration, for example, tensin 2 was found to be located mostly at the leading edge of the cell whereas, in contrast, tensin 3 was found to be located towards the cell rear (Chen et al., 2002, Clark et al., 2010). Furthermore, tensins are cleaved by proteases, such as caspase 3, which leads to loss of cell attachment during etoposide-induced apoptosis (Lo et al., 2005). This may be prevented by using a general caspase inhibitor or specific caspase-3 inhibitors (Kook et al., 2003). Cten was also found to be cleaved by caspase 3, specifically after screening some potential cleaving enzymes including other caspases. Moreover, it was shown that the site of a caspase-3 cleavage lay between the SH2 and PTB domains at the C-terminal end of the protein, with cleavage giving rise to two fragments, tensin 4 without the PTB domain and the PTB domain alone. Therefore, these investigations suggest that members of the tensin family could potentially be apoptotic signalling effectors through being cleavage substrates of caspases, and their cleavage products may translocate inside the cells with downstream consequences (Lo et al., 2005). The dysfunction of tensin signalling pathways has been found to be involved in carcinogenesis, however, the role of this family in disease progression is unclear and requires further investigation. The roles adopted by the tensins i.e. as oncogenes or tumour suppressors are dependent on the tensin member as well as the type of tissue or context (Qian et al., 2009, Martuszewska et al., 2009, Albasri et al., 2011a).

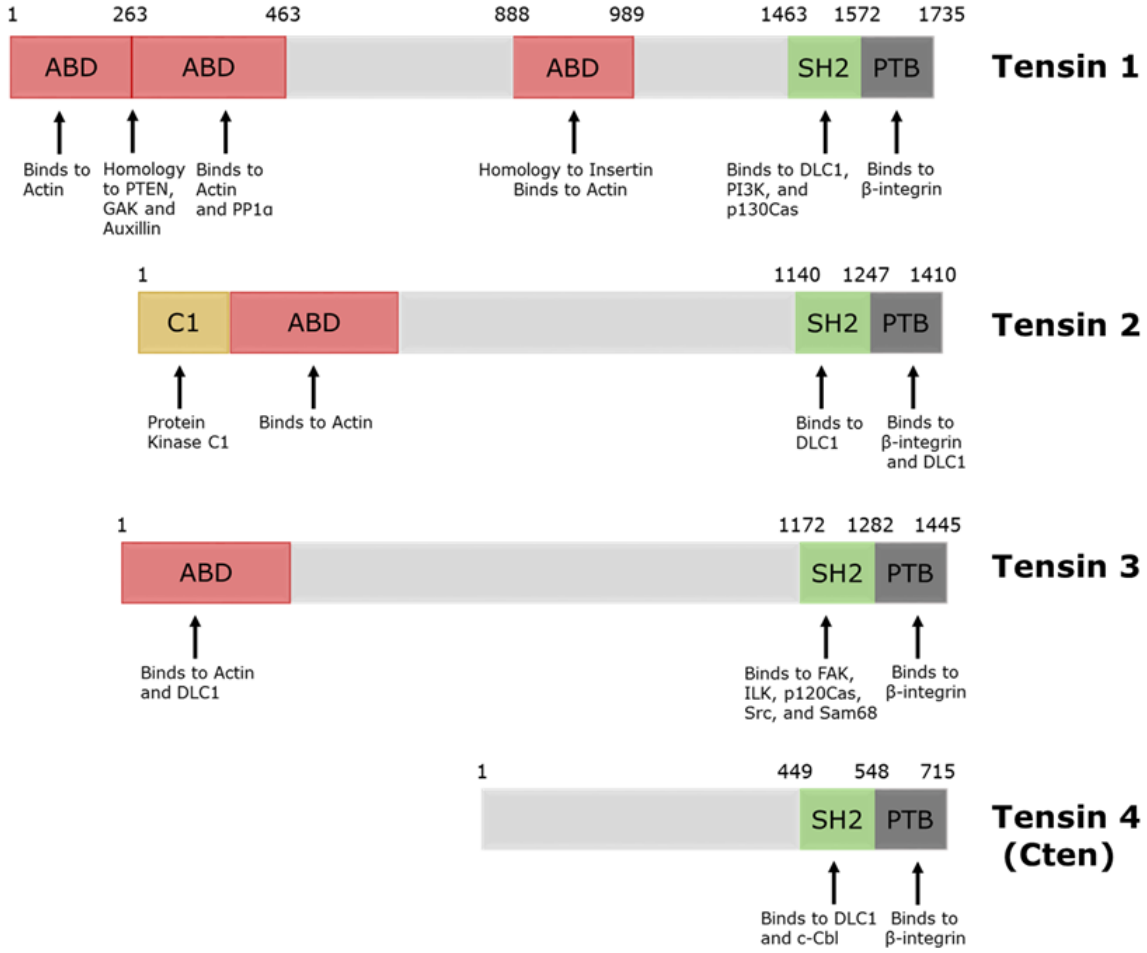

Figure 1–5: The structure of tensin family members.
Tensin 1-3 contain the N-terminus actin binding domain (ABD) in addition to the C-terminus Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains present in tensin 4 (Cten) enabling a connection to the actin cytoskeleton. The grey boxes in all tensin members represent similar amino acid sequences whose functions have not been discovered yet.

1.4 Current State of Cten

1.4.1 The Expression of Cten in Human Tissues

Although Cten is one of the most recently identified proteins amongst the four tensin family members, its role in human cancer has been more widely investigated. Cten was first identified as putative tumour suppressor in prostate cancer, where it was found to be downregulated in renal cell carcinomas, and patient tumour samples at both mRNA and protein levels tested by Western and Northern blots respectively. However, the role of Cten as oncogene in other tumour tissues is now becoming increasingly apparent (Martuszewska et al., 2009, Lo and Lo, 2002).

1.4.2 Cten As a Tumour Suppressor Gene

Cten is located on the short arm of chromosome 17q21 that is frequently deleted in prostate cancer (Lo and Lo, 2002), and was recently found to be cleaved specifically by caspase-3 during apoptosis (Lo et al., 2005). Despite this, how Cten exerts its tumour suppressor functions is largely unknown, it has been suggested that Cten functions by interacting with the well-known tumour suppressor, deleted in liver cancer-1 (DLC1), in the prostate through its SH2 domain, however, independent of tyrosine phosphorylation, leading to localisation of DLC1 to the focal adhesions (Liao et al., 2007). Furthermore, it has been found that the loss of Cten in prostate cancer is implicated in resistance to the chemotherapeutic agent paclitaxel, supporting its suggested role as a tumour suppressor protein, indicating that expression of Cten may serve as a potential prognostic indicator for prostate cancer (Li et al., 2010). Cten expression was also found to be downregulated in kidney cancer, although the correlation between the loss of Cten mRNA expression and high tumour grade was non-significant (Martuszewska et al., 2009).

1.4.3 Cten As an Oncogene

In contrast to its status in kidney and prostate cancer, the expression of Cten was shown to be elevated at both protein and mRNA level in cancer cells compared to normal cells (Liao et al., 2009). Although not normally present in most tissues, Cten expression has been reported to be increased and it is thought to function as oncogene in several types of tumours including colon, pancreas, breast, lung, and thymoma, with an association with late stage diseases in most tissues investigated (Sasaki et al., 2003a, Sasaki et al., 2003b, Albasri et al., 2011b, Albasri et al., 2009, Al-Ghamdi et al., 2013). Currently, how Cten is activated and regulated in these tumours remains unclear, however, there seems to be multiple pathways involved and it is largely dependent on tissue or context types.

Cten expression has mostly been investigated in CRC tissues and overall proposes that upregulation of its expression may play a role in metastasis. The mRNA expression was investigated in both tumours and normal mucosal cells, showing that most tumours experience upregulation, with more than a 35 fold increase in Cten observed in 5 cell lines derived from metastatic deposits (Albasri et al., 2009). Cten expression was found to be upregulated in all disease stages suggesting a key role for Cten in tumour progression and formation (Liao et al., 2009). Cten expression in 462 clinical samples demonstrated that Cten expression correlated with poor prognosis, advanced Duke's stage, and distant metastasis in CRC (Albasri et al., 2011a). This study also used a larger sized cohort and thus may be considered more representative than the former study. Furthermore, the expression of Cten was also assessed in paired cases of matched primary colorectal tumours and liver metastasis, revealing that Cten nuclear expression was extensively prevalent in metastatic deposits, indicating that Cten in the nucleus may be implicated in in metastatic disease (Albasri et al., 2011a).

Cten expression appears to be associated with an advanced stage disease in both CRC and breast cancer. Breast tumour samples analysed by immunohistochemistry showed that there was no association between Cten expression and tumour size, but there was for high EGFR and human epidermal growth factor receptor (HER2) expression, low oestrogen receptor expression, lymph node metastasis and tumour grade (Katz et al., 2007). Another study performed in 1,409 invasive breast tumours using immunohistochemistry showed that Cten staining was found in 90% of these tumours and associated with tumour grade, size, nodal involvement, and poor Nottingham prognostic index. Moreover, this study also found that tumours with a high level of Cten expression had a poorer prognosis compared to those with a low level of Cten expression and also had increased possibility of developing metastasis (Albasri et al., 2011b). Taken together, these findings implicate Cten in breast cancer metastasis.

In melanoma tissues, Cten expression was found to be associated with high grade disease, tumour size, and poor prognosis, and was shown to increase with tumour progression with approximately 7%, 24%, 41% and 46% positivity in normal, dysplastic, primary and metastatic melanoma tissues respectively (Sjoestroem et al., 2013). In lung cancer, a lack of a significant difference in the level of Cten expression between tumour and normal tissue was found, however the ratio of Cten expression in tumour to normal tissue was significantly greater for stage II-IV than stage I, proposing that the expression level of Cten is upregulated during the progression of established lung tumour (Sasaki et al., 2003a). Similarly, in thymomas, the expression level of Cten mRNA was significantly higher in tumour stage IV than stage I (Sasaki et al., 2003b).

Although the role of Cten in tumour formation and progression has not been fully elucidated, recent studies on Cten expression in most tissues suggest that it may play a key role in advanced metastatic disease. Since Cten expression was found to be associated with metastasis, understanding the mechanisms of signal transduction underlying this process requires further investigation.

1.4.4 Cten and Cell Motility

Cten has been reported to regulate cell migration and invasion following gene expression manipulation using *in vitro* models (Albasri et al., 2011a, Albasri et al., 2009, Liao et al., 2009). In addition, the role of Cten in metastasis has also been investigated by injecting CRC cells stably transfected with Cten into nude mice, showing that similar tumour numbers were formed in the spleen and liver, but that the tumour were larger than the tumours formed in the control mice with a shorter overall survival (Albasri et al., 2011a).

Although mechanisms underlying the role of Cten in cell motility in numerous cancer cell types have been described, the full understanding of the additional layers of complexity and signalling pathways that may cooperate with Cten to regulate both cell migration and invasion remain to be resolved.

1.4.5 Potential Downstream Targets

Localisation of Cten with other tensin members to focal adhesion dynamics is an important step in the regulation of cell motility. Cten binds to other focal adhesion localised proteins though its SH2 domain region, such as FAK, ILK, and PI3 kinase (Lo, 2004). FAK is a tyrosine kinase that plays a vital role in the regulation of proteins localised at focal adhesion complexes. FAK is recruited to focal adhesions, where it is phosphorylated and induces cell migration in complex with Src (Mitra and Schlaepfer, 2006). FAK has been found to co-immunoprecipitate with tensin 1, thus it is possible that this molecule directly binds Cten (McLean et al., 2000, Yamashita et al., 2004a). FAK expression is upregulated in several types of cancer such as CRC (Lark et al., 2003). Interestingly, in pancreatic cancer cell lines, Cten lies upstream of, and signals through FAK, thereby inducing both cell invasion and migration (Al-Ghamdi et al., 2013). Moreover, it has been reported that the expression of Cten was also associated with p-FAK expression, indicating that Cten may stimulate the activation of FAK in addition to expression level (Albasri et al., 2014).

ILK is non-receptor protein kinase that localises at focal adhesions dynamics and it also may function as both a scaffold and signal transduction molecule. ILK has been implicated in the regulation of several biological events, such as cell migration and invasion, and is frequently found to be upregulated in a number of cancers including CRC (Bravou et al., 2003, Yan et al., 2014). Furthermore, Cten was identified as an upstream regulator of, and signals through ILK, thus promoting cell migration and metastasis in CRC cell lines (Albasri et al., 2011a). This therefore suggests that Cten may act as a key regulator of protein kinases in the focal adhesion complexes.

DLC1 is known as a tumour suppressor that regulates actin fibre assembly and cell adhesion through downstream inactivation of Rho-associated, coiled-coil containing protein kinase (ROCK). DLC1 contains a Ras homology-GTPase activating protein (Rho-GAP) domain that is responsible for catalysing the hydrolysis of GTP bound to GDP, therefore rendering the Rho-GTPase inactive, resulting in the uncoupling of focal adhesions and stress fibres, and cell rest (Wong et al., 2003, Durkin et al., 2007, Lahoz and Hall, 2008, Healy et al., 2008, Liao and Lo, 2008, Cao et al., 2012). In addition to containing a Rho-GAP domain, DLC1 also comprises a sterile alpha motif (SAM) domain, a steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain, and a focal adhesion-targeting (FAT) domain. The FAT domain has been shown to increase the tumour suppressor activities of DLC1. The binding to DLC1 has been mapped to the PTB, SH2 and ABD of the tensin proteins (Qian et al., 2007, Cao et al., 2012). DLC1 was also found to be recruited to the focal adhesion dynamics via interactions between its FAT domain and an S/TIYXXI/V motif in the SH2 domains of the tensin proteins including Cten (Liao et al., 2007, Liao and Lo, 2008, Cao et al., 2012).

The GAP activity of DLC1 is believed to be regulated by its SAM domain which functions as an autoinhibitory switch that prevents Ras homology family member A (RhoA) suppression. It is suggested that in non-migrating cells, tensin 3 is highly expressed and binds to DLC1 through its actin binding domain via both the SAM domain and central

region of DLC1. This binding prevents DLC1's autoinhibitory mechanism, thereby inactivates DLC1 and inhibits RhoA signalling. However, as Cten lacks an actin binding domain at its N-terminus region, unlike tensin 3, the DLC1 SAM domain can bind and suppress the Rho-GAP domain and inactivates DLC1. This explains why Cten is unable to do the same, due to its lack of a similar region. It has been suggested that Cten expression is high, whereas tensin 3 expression is very low in migratory cells enabling Cten to displace tensin 3 from DLC1 binding. Upregulation of DLC1 leads to rapid hydrolysis of Rho-GTP bound to Rho-GAP, resulting in downregulation of ROCK expression, hence reducing actin stress fibre, uncoupling of focal adhesions, and cell rest/an immobile state (Cao et al., 2012). However, binding of Cten causes downregulation of Rho-GAP activity in DLC1, leading to an induction of focal adhesion turnover, actin stress fibre remodelling, and enhanced cell migration (Liao et al., 2007).

In addition to the focal adhesions protein turnover regulation, Cten has been found to be associated with the regulation of adherens junction proteins including E-cadherin (Albasri et al., 2009). E-cadherin is a transmembrane receptor protein that localises to the adherens junctions of epithelial cells and regulates homophilic cell to cell interactions in a calcium dependent manner. It normally functions as a tumour suppressor protein in most epithelial cells and downregulation of its expression can promote invasive and metastatic behaviour in tumour cells (McCaffrey and Macara, 2011). The cytoplasmic region of E-cadherin is linked to the actin cytoskeleton through β-catenin, p120-catenin, and α-catenin and forms adherens junctions. It is possible that Cten could be involved in the disruption of these adherens junctions by dysfunction of E-cadherin which results in the loss of cell-cell interactions, as well as loss of cell-ECM adhesion at focal adhesions. E-cadherin is considered a negative regulator of Wnt signalling as it sequesters cytoplasmic β-catenin at adherens junctions. It has been shown that loss of E-cadherin leads to the release of membrane bound β-catenin, enabling its nuclear translocation and Wnt signalling activation (Howard et al., 2011). Following Wnt signalling activation, β-catenin translocates to the nucleus and binds to T-cell factor/lymphoid enhancer factor

(TCF/LEF) promoting the transcription of target genes which could be involved with stem cell maintenance and crypt homeostasis in the gastrointestinal epithelium (Palacios et al., 2005). β-catenin had previously been found to co-immunoprecipitate with Cten only in the nucleus and not the cytoplasm, and increased colony formation and anchorage independent cell growth. This suggests that these proteins may collaborate to induce tumourigenicity and are also involved in the regulation of CSC networks (Barbieri et al., 2010, Liao et al., 2009, Dong et al., 2009); however, whether nuclear localised Cten regulates the Wnt signalling axis requires further investigation.

It has been reported that integrin signalling through the Src/FAK signalling pathway has been linked to a downregulation in E-cadherin expression in colon cancer (Avizienyte et al., 2002). ILK signalling has been shown to inhibit E-cadherin expression through the induction of the transcription factor Snail (Tan et al., 2001). In addition, ILK/Snail regulates E-cadherin expression at both protein and mRNA level (McPhee et al., 2008), however, Cten regulates E-cadherin expression at the protein level only (Albasri et al., 2009). This suggests that Cten possibly regulates the degradation of E-cadherin protein. Cten may also regulate E-cadherin through other focal adhesion localised proteins such as calpain, a protease shown to cleave E-cadherin and may be involved in regulating motility (Ye et al., 2013). However, the regulation of E-cadherin by Cten signalling remains unclear and requires further investigation.

1.4.6 Upstream Regulators of Cten

Numerous informative studies have shown that Cten expression is possibly regulated by several upstream signalling pathways. Some of these studies reported that the regulation of Cten expression appears to be under the control of the canonical EGFR-KRAS-mitogen activated protein kinase (MAPK) signalling pathway. A study by Katz et al. showed that stimulation with EGF led to an increase in expression levels of Cten in breast cell lines, which was prevented by inhibition of MAPK signalling. Activation of EGF promoted actin stress fibre reorganisation, however, despite the presence of an EGF

stimulator, downregulation of Cten led to a disruption in actin fibre dynamics, suggesting that EGF signals through the Cten pathway to regulate actin fibre networks (Katz et al., 2007). In addition, the regulation of Cten by EGF signalling was validated in MCF10A cells. These studies showed that stimulation with EGF induced Cten expression and resulted in auto-inhibition of the Rho-GAP activity of the known tumour suppressor DLC1 (Cao et al., 2012, Hung et al., 2014). These results were later confirmed by Chan et al, who further showed that the EGF-driven upregulation of Cten expression was abolished following the inhibition of MAPK signalling in hepatocellular carcinoma cells (Chan et al., 2015).

KRAS and BRAF mutations are common in CRC, with approximately 50% of tumours compared to EGFR mutations (Vaughn et al., 2011). Al-Ghamdi et al. showed that Cten may also be a potential downstream target of KRAS, the initiator of the proliferative ERK/MAPK signalling pathway, in CRC cell lines. Cten levels were suppressed after KRAS knockdown. Cten expression levels had no effect following KRAS knockdown in cells containing BRAF mutations, but Cten levels decreased after BRAF knockdown suggesting that Cten may be regulated through KRAS/BRAF signalling axis (Al-Ghamdi et al., 2011). Furthermore, the functional relevance between KRAS and Cten was also investigated in this study. It was shown that knockdown of KRAS in cells highly expressing Cten led to inhibition of the cell motility-inducing effects of Cten in both colon and pancreatic cancer cells, suggesting that Cten may be the mediator through which KRAS regulates cell motility (Al-Ghamdi et al., 2011).

One of the most informative studies on the regulation of Cten expression was published by Hung et al, and investigated the effects of activation of several cancer associated growth factors and cytokines on Cten expression in CRC and non-malignant prostate cell lines, including transforming growth factor beta (TGF-β), EGF, fibroblast growth factor 2 (FGF2), nerve growth factor (NGF), platelet-derived growth factor (PDGF), interleukin 13 (IL-13), insulin-like growth factor 1 (IGF-1), and interleukin 6 (IL-6). MAPK and PI3K/Akt

signalling were found to be the two main signalling pathways involved in growth factor mediated Cten expression. Activation, together with inhibition of PI3K or Mek inhibitors, blocked the induction of Cten expression mediated through all of these growth factors and cytokines, except through IGF-1 which may signal independently of the MAPK and PI3K/Akt signalling pathway (Hung et al., 2014).

Signal transducer and activator of transcription factor 3 (Stat3) is another main signalling pathway reported to be involved in inducing Cten expression. Cten expression was first shown to be a consistently upregulated downstream target of STAT3 signalling, mediated by EGFR activation or by the cytokine, IL-6, in breast cancer-derived cell lines. STAT3 signalling is constitutively activated in a subset of breast tumours, or is stimulated by the cytokine IL-6, suggesting a fundamental role of Cten in breast cancer progression and metastasis (Barbieri et al., 2010). These results were later confirmed by Bennett et al., who further showed in lung cancer cell lines that inhibition of STAT3 signalling abolished EGFR induced Cten expression. In addition, Cten expression found to be downregulated following Janus kinase (JAK) signalling inhibition. Cten may also be regulated by JAK through STAT3 signalling (Hung et al., 2014).

Although there is a lack of understanding of the mechanisms underlying the regulation of Cten, a number of Cten regulatory factors have been described, indicating that once Cten and its key role in tumour progression has better characterised, it has the potential to provide an attractive therapeutic target for treatment of several human cancers.

1.5 Aims and Hypothesis

The role of Cten signalling in CRC progression is, at present, largely unknown, and in part controversial, although, studies have clearly indicated that Cten plays a role in aggressive disease and in most tumour tissues investigated, expression is associated with metastasis. The main aim of this study was to further elucidate the mechanisms of Cten signalling and how they may contribute to CRC metastasis.

1. *Determine the underlying signalling mechanisms by which Cten promotes cell function though EMT pathways.*

It is possible that Cten increases cell motility through the upregulation of focal adhesions due to its localisation at focal adhesion complexes and interaction with other focal adhesion localised proteins. In addition, Cten is implicated in the regulation of EMT processes through the downregulation of E-cadherin expression. It was hypothesised that Cten could regulate Src, ROCK1 or Snail expression, thereby enhancing CRC progression.

2. *Ascertain the importance of the SH2 domain of Cten.*

Arginine and tyrosine mutations within the SH2 domain of Cten are important for its function in CRC cells, however, these mutations individually did not show complete reduction of its effects on colony formation and cell migration. The present study hypothesised that a combination of both mutations together in one construct may result in a complete abrogation of Cten's ability to interact with its downstream targets and induce cell motility and colony formation in CRC cells.

3. *Investigate whether TGFβ1 could signal upstream dependently of Cten to promote cell motility through the positive regulation of Src/ROCK1/Snail pathway CRC cells.*

TGFβ1 signals downstream to EMT and since Cten promotes cell motility through EMT signalling, it was hypothesised that Cten may be a signalling intermediate in the TGFβ1/EMT pathway to increase cell motility and that this could promote metastasis in CRC cells.

4. *Determine the importance of nuclear localised Cten*

Cten was found to be localised to the nucleus and this nuclear localisation was observed to be more predominant in the metastatic tumour than the primary tumour. Therefore, it was hypothesised that Cten in the nucleus could promote the functional activity of Cten in CRC cells. Proper subcellular localisation of proteins is important for them to function in the cells thus, it was further hypothesised that Cten, when localised to the nucleus, may increase nuclear localisation of its downstream targets including Src, ROCK1, or Snail protein.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Cell Maintenance

This work was carried out in CRC cell lines HCT116, RKO, and SW620, which were a kind gift from Prof Ian Tomlinson (table 2-1). The identify of these cell lines was authenticated by PCR-single-locus-technology (see appendix).

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) antibiotic free supplemented with 10% foetal bovine serum (FBS) (Sigma) and maintained at 37°C in a 5% CO_2 environment under sterile conditions. The cells were checked under the microscope to assess cell viability and ensure cultures remained free of contamination. Cells were regularly tested for mycoplasma. Cells were fed twice weekly by discarding the media and replacing with fresh DMEM (containing FBS).

2.1.2 Cell Passage

Cells were allowed to grow until they reached approximately 70-80% confluence and then passaged. Cells were detached from the flasks by washing with phosphate buffered saline (PBS) (Sigma), followed by incubation for 5 minutes at 37°C in 2 ml of trypsin/EDTA (0.5% trypsin, 1 mM EDTA, Sigma). Cells were resuspended in 8 ml of DMEM and pelleted by centrifugation at 1,500 rpm for 5 minutes. The media was discarded, and pelleted cells were then resuspended in a volume of media dependent upon the split ratio (i.e. 10 ml for a 1:10 split ratio). An aliquot of cell suspension (1 ml) was transferred to a new flask containing 10 ml of DMEM (containing FBS).

Table 2–1: characteristics of CRC cell lines.

Cell line	Gender	Age	Dukes' Stage	Differentiation	Origin	MSI Status	CIN	KRAS	BRAF	PIK3CA	PTEN	TP53
RKO	Male	64	C	Poor	Colon	MSI	NO	Wt.	V600E	H1047R	Wt.	Wt.
HCT116	Male	64	D	Well	Colon	MSI	NO	G13D.	Wt.	H1047R	Wt.	Wt.
SW620	Male	51	C	Moderate	Lymph node	MSS	Yes	G12V	Wt.	Wt.	Wt.	R273H;P309S

2.2 Plasmid Preparation

2.2.1 Plasmid Transformation

Plasmid expression vectors were used to forcibly express the Cten protein. The pEGFP-C1 plasmid (CLONETECH laboratories Inc.) inserted with Cten was kindly donated by Prof Su Hao Lo and the pEGFP-N1 plasmid containing nuclear localisation signal (NLS)-Cten and mutated Cten plasmids (R474A and Y479F) were kindly provided by Dr Maham Akhlaq. The Cten targeting CRISPR plasmid was designed and purchased from Sigma.

NEB 5-alph competent *E. coli* cells (New England Biolabs) were transfected with plasmid DNA using the heat shock method. In brief, 5 µl of plasmid was added to 50 µl of competent cells (*E. coli*) and placed on ice for 30 minutes. Following this, the cell/plasmid mix was placed in the water bath for 30 seconds at 42°C before being placed on ice for 2 minutes. Then, 950 µl of room temperature SOC Outgrowth Medium was added and the cells incubated on a shaker (200 rpm) for 1 hour at 37°C. The cells were then centrifuged at 13,000 rpm for 2 minutes, 900 µl of the supernatant was removed and the cells resuspended in the remainder. The cells were spread onto agar plates (20 ml LB agar/20 µl kanamycin) and colonies allowed to grow overnight at 37°C. The following day, 1 colony for each construct was picked and streaked onto a new plate and the remainder of the *E. coli* left on the tip was added to 5 ml LB broth supplemented with 5 µl kanamycin. The plates and mini prep were incubated overnight at 37°C (mini prep on a shaker). Following incubation, 5 ml of mixture was transferred into 100 ml LB broth medium supplemented with 100 µl kanamycin and incubated overnight at 37°C on a shaker (200 rpm). Cells were then harvested by centrifugation at 5,000 x g for 15 minutes.

2.2.2 Plasmid Purification

Plasmid purification was performed using the Genelute Plasmid Miniprep or Midiprep kit (Sigma) in accordance with the manufacturer's instructions. Briefly, for 'Miniprep' preparations, the culture (3-5 ml) was centrifuged at 13,000 rpm for 1 minute and the bacterial cell pellet was resuspended in 200 µl of chilled Resuspension/RNase A Solution, followed by lysis of the cell pellet by the addition of 200 µl of Lysis Solution. The contents were inverted 6 times and incubated for 4 minutes. Following this, 350 µl of Neutralising/Binding solution was added to precipitate the cell debris and samples were centrifuged for 10 minutes at 13,000 rpm, then the supernatant was passed through a Gen Elute Miniprep Binding column (Sigma). To prepare the mini prep column, 500 µl of Column Preparation Solution was added, centrifuged at 13,000 rpm for 1 minute and the eluate discarded. The columns were then washed by the addition of 750 µl of wash solution, followed by centrifugation at 13,000 rpm for 2 minutes. To elute the DNA plasmid, the binding column was transferred to a new collection tube, 100 µl of Elution Solution was added before centrifugation at 13,000 rpm for 1 minute. The DNA was quantified using the NanoDrop system (Thermo Fisher Scientific).

For 'Midiprep' preparations, 100 ml of bacterial culture was used to obtain DNA plasmids using the GenElute Plasmid midiprep kit (Sigma, NA0200-1KT). The solutions used in were the same as in the miniprep with a slightly modified protocol to generate larger volumes of plasmid DNA. Following quantification by the NanoDrop system, 0.5 µg of each DNA sample in a volume of 10 µl together with DNA loading buffer (Promega) were run alongside 5 µl of 1 kb DNA ladder (Promega) on a 1% agarose gel containing SYBR Safe at 100 V for 50 minutes. Products were viewed using a gel documentation viewing system. The DNA samples were also sent for sequencing to a sequencing Lab in Queens Medical Centre, Nottingham for further confirmation.

2.3 Transfection and Co-transfection

2.3.1 Gene Forced Expression

Lipofectamine 2000 (Thermo Fisher Scientific) was used to transfect the target cells with plasmid DNA following the standard manufacturer's protocol. To optimise the transfection efficiency, 5–20 µl of Lipofectamine 2000 was incubated together with the plasmid construct (2–8 µg). The transfection efficiency was confirmed by flow cytometry according to Green fluorescent protein (GFP) expression. Following flow cytometry assessment, the condition producing the greatest protein expression after transfection of plasmid was considered optimal. Usually 5 µg of plasmid DNA was added to cells together with 10 µl Lipofectamine 2000 for Cten mutant plasmids, NLS-Cten, Cten and empty vector constructs; 4 µg of plasmid added to 5 µl of Lipofectamine 2000 for the CRISPR-Cas9 construct.

The optimal plasmid concentration was added to 250 µl of Opti-MEM. Following this, the diluted Lipofectamine 2000 and plasmid mixes were combined to give a total volume of 500 µl. These were incubated for 20 minutes at room temperature and then added dropwise to the cells. The cells were incubated with the transfection reagents for 6 hours, the media was replaced, and experimentation performed 48 hours post transfection.

The transfection procedure was usually carried out in a six well plate (Costar) with a total volume of 2 ml per well. For plasmid forced expression, cells were seeded in the usual DMEM growth media and incubated for 24 hours at 37^0C. The following day, after cells reached 60-70% confluence, the media was replaced with 1.5 ml of Opti-MEM reduced serum media and cells were incubated at for 1 hour at 37^0C. The optimised volume of Lipofectamine 2000 for each condition was added to 250 µl of Opti-MEM and incubated for 5 minutes. The optimal concentration of plasmid was added to 250 µl of Opti-MEM. After 5 minutes, the diluted plasmid was mixed with the diluted Lipofectamine

2000 to give a total volume of 500 µl and incubated for 20 minutes at room temperature. The mixture was then added dropwise to the cells and incubated at 37^{0}C for 6 hours before replacing the Opti-MEM with fresh media (i.e. DMEM plus 10% FBS). The cells were allowed to grow for 48 hours before further experiments.

2.3.2 Gene Expression Knockdown

Small interfering RNA (siRNA) duplexes (Thermo Fisher) were used to inhibit gene expression and transfection of these siRNAs was performed according to the protocol for plasmid transfection. For optimisation, 50–200 nM of siRNA duplexes were added together with 10 µl of Lipofectamine 2000 (table 2-2) and incubated for 48 hours at 37^{0}C. The transfection efficiency was confirmed by western blot and the condition that gave the greatest reduction in protein, whilst using the minimal siRNA concentration, was considered optimal: 100 nM of siRNA for the knockdown of Cten and Snail; 200 nM of siRNA for the knockdown of ROCK1, Src, or TGFβ1. Luciferase targeting siRNA was used as a control and transfected at the same concentration as the target siRNA.

For gene knockdown, cells were seeded in the usual DMEM growth media in a six well plate (Costar) with a total volume of 2 ml per well and incubated for 24 hours at 37^{0}C. The following day, after cells reached 40-50% confluence, the media was replaced with 1.5 ml of Opti-MEM reduced serum media and cells were incubated for 1 hour at 37^{0}C. Then, 10 µl of Lipofectamine 2000 for each condition was added to 250 µl of Opti-MEM and incubated for 5 minutes. The optimal concentration of siRNA was added to 250 µl of Opti-MEM. After 5 minutes, the diluted siRNA was mixed with the diluted Lipofectamine 2000 to give a total volume of 500 µl and incubated for 20 minutes at room temperature. The mixture was then added dropwise to the cells and incubated at 37^{0}C for 6 hours before replacing the Opti-MEM with fresh media (i.e. DMEM plus 10% FBS). The cells were allowed to grow for 48 hours before performing further experiments.

2.3.3 Co-transfection

Some experiments required forced expression and gene knockdown at the same time. For these co-transfection experiments, cells were seeded in the usual DMEM growth media in a six well plate and incubated for 24 hours at 37^0C. Once cells reached 50% confluence, cells were transfected with the optimal plasmid and siRNA concentrations with 10 µl of Lipofectamine 2000 in 500 µl medium per well as described above. The cells were incubated with the transfection reagents for 6 hours and experimentation performed 48 hours post transfection.

Table 2–2: siRNA sequences

Target	siRNA Sequence
ROCK1	GAC AGA UGC GGG AGC UAC AAG AUC A
Cten	UUC UCA UUG ACA UGG UGC UCU GGG C
SRC	GAG CCC AAG CUG UUC GGA GGC UUC A
TGFβ1	CCA CCU GCA AGA CUA UCG ACA UGG A
SNAIL1	CCU UCU CUA GGC CCU GGC UGC UAC A
LUCIFERASE	CGU ACG CGG AAU ACU UCG A

2.4 Cell Treatment

2.4.1 TGFβ1 Stimulation

In order to stimulate TGFβ1 signalling, cells were seeded in a six well plate and starved in serum free DMEM for 24 hours at 37^0C prior to stimulation. Cells at 50-60% confluency were treated with 0–20 ng/ml Recombinant Human TGFβ1 (R&D Systems) in DMEM growth media (supplemented with 10% FBS), with a total volume of 2 ml per well. Cells were harvested after incubation for 48 hours.

2.4.2 Cycloheximide

Cycloheximide (CHX) is an inhibitor of protein synthesis activity and can be used to measure protein stability. From the point that cells are exposed to CHX, protein synthesis stops, and the degradation of the pre-existing protein can be measured. For the CHX assay, twenty-four hours post cell transfection, the media in all wells was replaced with fresh DMEM (supplemented with 10% FBS) containing 100 µg/ml CHX and incubated for different time points starting from 0–24 hours dependent upon the protein half-life. Following incubation, cells were harvested at the same time point and western blotting was performed.

2.5 Western Blotting

2.5.1 Protein Extraction

Western blotting was used to detect and analyse protein expression. To extract protein from cell lysates, the media was removed from the cells and they were washed with PBS. Following this, cell lysates were prepared using chilled radio immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) supplemented with phosphatase and protease inhibitors (Thermo Fisher Scientific), applied directly to the cells. The mixture was then incubated at an angle on ice for 15 minutes, then collected together using a cell scraper (Costar, UK), transferred to 1.5 ml Eppendorf tubes and centrifuged at 13,000 rpm at 4ºC for 30 minutes. Finally, the supernatant was collected, aliquoted into 0.5 ml Eppendorf tubes and stored at -20ºC.

2.5.2 Quantification

The bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) was used to quantify protein concentration according to manufacturer's protocol. Briefly, 12.5 µl of either albumin standard or protein was pipetted into the 96 microplate wells (Costar) in duplicate. Then, 100 µl of BCA reagent was added to each well and the plate was shaken for 30 seconds and incubated at 37°C for 30 minutes. Following incubation, the absorbance was measured at 550 nm using the Multiskan EX plate reader (Thermo Fisher Scientific). The concentrations of protein were then determined from a standard curve of protein standards of known concentrations.

2.5.3 SDS-PAGE Gel and Western Blot

Fifty micrograms of protein was added to NUPAGE LDS Sample Buffer (Thermo Fisher Scientific) containing 5% β-mercaptoethanol. The protein samples were heated at 90^0C on a heat block for 5 minutes to denature the protein, incubated on ice for another 5 minutes after denaturation, then centrifuged for 2 minutes at 13,000 rpm. Following this, the protein samples were electrophoresed on a pre-cast 4–12% NUPAGE Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel (Thermo Fisher Scientific) using the NUPAGE gel electrophoresis system with NUPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific) at 125 V for 90 minutes. Protein was transferred to a methanol activated PVDF membrane (GE Life Sciences) using the NUPAGE Transfer Buffer (Thermo Fisher Scientific) and the Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) at 20 V for 30 minutes. After the transfer, the membranes were blocked with 5% milk or 5% bovine serum albumin (BSA) in 0.1% tween PBS (PBS-T) or 0.1% tween TBS (TBS-T) (dependent on antibody diluents) for 40 minutes with gentle rotation at room temperature. Following this, the primary antibody was incubated with the membrane overnight with gentle rotation at 4°C (table 2-3). The membranes were then washed 3 times in PBS-T or TBS-T at room temperature for 5 minutes and incubated with the appropriate horseradish peroxidase (HRP) labelled anti-rabbit or anti-mouse secondary antibody (Sigma) for 60 minutes at room temperature. Subsequently, the membranes were washed twice in PBS-T or TBS-T and once in PBS or TBS each for 5 minutes at room temperature.

To enable detection of the bound antibody, the membrane was incubated with ECL Prime detection solution (GE Life Sciences) for 5 minutes at room temperature. The membrane was then imaged using the LiCor C-Digit Blot Scanner (LI-COR Biotechnology). Li-Cor Image Studio Lite software was used for densitometry data acquisition.

Table 2–3: Optimised antibody conditions for western blot

Antibody	Source	Supplier	Primary dilution	Secondary dilution	Diluent
ROCK1	Rabbit	Cell signalling	1:1,000	1:1,000	5% BSA+0.1% Tween TBS
N-Cadherin	Mouse	Abcam	1:500	1:5,000	5% BSA+0.1% Tween TBS
E-Cadherin	Rabbit	Cell signalling	1:1,000	1:5,000	5% BSA+0.1% Tween TBS
Cten	Mouse	Sigma	1:5,000	1:1,000	5% Milk+0.1% Tween PBS
Tubulin	Mouse	Abcam	1:5000	1:2000	5% Milk+0.1% Tween TBS
Lamin B1	Rabbit	Abcam	1:10,000	1:5000	5% BSA+0.1% Tween TBS
Src	Rabbit	Cell signalling	1:5,000	1:1,000	5% BSA+0.1% Tween PBS
TGFβ1	Rabbit	Cell signalling	1:5,000	1:1,000	5% BSA+0.1% Tween TBS
Actin	Mouse	Sigma	1:20,000	1:10,000	5% Milk+0.1% Tween PBS
Snail	Rabbit	Cell signalling	1:1,000	1:5,000	5% BSA+0.1% Tween PBS

2.6 Nuclear and Cytoplasmic Extraction

The nuclear and cytoplasmic fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, cells were harvested by trypsinisation 48 hours post transfection and pelleted by centrifuging at 500 x g for 5 minutes. The pellet was then resuspended in 500 µl of cold PBS and transferred to a 1.5 ml Eppendorf tube. This was centrifuged at 500 x g for 3 minutes and the supernatant was removed, leaving the pellet as dry as possible. Following this, the volume of cell pellet was estimated, and an appropriate volume of cold CER I (cytoplasmic extraction reagent I) with protease inhibitor was applied. This was then vortexed for 15 seconds and placed back on ice for 10 minutes. After incubation, an appropriate volume of CER II was added, followed by vortexing for 5 seconds, and incubated on ice for 1 minute. The Eppendorf tube was again vortexed, followed by centrifugation at 16,000 x g for 5 minutes at 4°C and the supernatant (cytoplasmic extract) was transferred to a clean, pre-chilled tube and incubated on ice. An appropriate volume of cold NER (nuclear extraction reagent) with added protease inhibitor was then added to the remaining pellet and vortexed for 15 seconds every 10 minutes for a total of 40 minutes. Following this, the Eppendorf tube was centrifuged at 16,000 x g for 5 minutes at 4°C. Next, the supernatant (nuclear extract) fraction was transferred to a clean, pre-chilled tube. Thereafter, western blotting was performed as previously described.

2.7 Co-immunoprecipitation

The co-immunoprecipitation (co-IP) was performed to investigate the binding interactions between proteins. To prepare the cell lysates, a pre-clearing stage was performed to reduce non-specific binding. First, 1 ml of protein was incubated with 20 µl of Protein G/A agarose beads (Calbiochem IP05) with gentle rotation at 4°C for 30 minutes. The lysate was then centrifuged at 4°C at 13,000 rpm for 5 minutes to pellet the beads and the supernatant retained. Subsequently, 1 µg of ROCK1 (10 µl), Snail (4 µl), Cten (2 µl), or Src (3 µl) antibody was added to 300 µg of the pre-cleared lysate and

incubated with gentle rotation overnight at 4°C. In addition, 300 µg of pre-cleared lysate (without antibody) was also incubated with the pre-cleared beads for the negative control. The following day, 30 µl of the Protein A/G beads were added to the reactions and incubated on a rotator for a further 24 hours at 4°C. The beads were pelleted by centrifugation at 13,000 rpm at 4°C for 5 minutes and washed twice in ice cold PBS. The sample was resuspended in 10 µl 3X NUPAGE loading Buffer and heated at 95° for 5 minutes, kept on ice for 5 minutes and centrifuged for 2 minutes at 13,000 rpm before loading (50 µg of protein) onto an SDS gel for western blot analysis.

2.8 Immunofluorescence

Cells were seeded in multi chamber slides and incubated at 37°C for 24 hours post transfection or treatment. Cells were washed in PBS for 5 minutes on a shaker and fixed with 1:1 acetone/methanol for 20 minutes at -20°C. Following this, cells were washed three times in PBS for 5 minutes each and blocked with 1% BSA and 22.52 mg/mL glycine in PBS-T (PBS+0.1% Tween 20) at room temperature for 60 minutes. After blocking, the primary antibody diluted in 1% BSA+PBS-T was incubated with cells overnight with gentle rotation at 4°C (table 2-4). Then, cells were washed three times in PBS for 5 minutes each and incubated with Alexa Fluor 488 (green) or Alexa Fluor 568 (red) secondary antibody (Thermo Fisher Scientific) diluted in 1% BSA+PBS-T with gentle rotation at room temperature in the dark for 60 minutes. Cells were then washed three times in PBS-T for 5 minutes in the dark and incubated with 1x DAPI (1:5000) (Sigma) in PBS with gentle rotation for 30 minutes. Following this, cells were again washed a further 2 times in PBS for 5 minutes in the dark and mounted with a drop of fluorescein mounting media (Sigma) on glass slides. The cells were viewed using the Leica Microsystems confocal microscope at x40 objective and Leica Application Suite X software was used for image acquisition.

Table 2–4: Optimised antibody conditions for immunofluorescence

Antibody	Source	Supplier	Primary dilution	Secondary dilution	Diluent
ROCK1	Rabbit	Abcam	1:100	1:200	1% BSA+0.1% Tween PBS
Cten	Mouse	Sigma	1:200	1:200	1% BSA+0.1% Tween PBS
Src	Rabbit	Cell signalling	1:200	1:200	1% BSA+0.1% Tween PBS
Snail	Rabbit	Abcam	1:100	1:200	1% BSA+0.1% Tween PBS
Lamin B1	Rabbit	Abcam	1:500	1:200	1% BSA+0.1% Tween PBS
Lamin B1	Mouse	Abcam	1:50	1:200	1% BSA+0.1% Tween PBS

2.9 Immunohistochemistry (IHC)

Immunohistochemical staining was carried out on tissue microarray (TMA) sections (4 µm thickness) mounted on glass slides using the Novolink Polymer Detection Kit (Leica) in accordance with the manufacture's recommended instructions. Briefly, TMA slides were heated at 60^0C for10 minutes, followed by dewaxing in xylene and dehydration in industrial methylated spirits (IMS) using the Autostainer XL (Leica). Antigen retrieval was accomplished by heating the samples at 95^0C in 10 mM sodium citrate buffer at pH 6.0 or in Tris/EDTA buffer at pH 9.0 for 20 minutes. Slides were then incubated with Peroxidase Block solution at room temperature for 5 minutes and washed with Tris-Buffered Saline (TBS) (pH 7.6) followed by Protein Block for 5 minutes and a TBS wash. Following blocking, the primary antibodies diluted in Antibody Diluent (Leica) were incubated with slides for 1 hour at room temperature. Slides were then incubated with Post Primary Block for 30 minutes at room temperature. Following this, slides were washed, and Novolink Polymer was applied for 30 minutes, followed by a further wash with TBS. Visualisation of bound antibody was achieved using diaminobenzidine (DAB) prepared from DAB Chromogen and DAB Substrate Buffer. This was incubated for 5 minutes, washed with TBS and following this, the slides were treated with haematoxylin for 6 minutes. Finally, the slides were cleared in xylene and rehydrated in IMS using the Autostainer XL before mounting in DPX.

Whole slides were scanned using the Nanozoomer digital slide scanner (Hamamatsu) and viewed using the associated NDP.view 2 viewing software (Hamamatsu). The TMAs were manually scored using the H score calculated using the formula (3 x percentage of strongly stained cells) + (2 x percentage of moderately stained cells) + (percentage of weakly stained cells), which assigned a score of 0-300 for each core.

2.10 Quantitative Real Time-PCR

2.10.1 Primer Design

Primers were designed to cover exon boundaries and amplify all protein coding transcript variants using either Universal Probe Library software (Roche, http://www.universalprobelibrary.com) or a Primer Basic local alignment search tool (BLAST) (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast) (table 2-5). Primer specificity was checked against the Refseq mRNA database using Primer BLAST including melting temperature (Tm), GC content and self-complementarity.

Table 2–5: RT qPCR primer sequences

Target Gene	Forward Primer Sequence	Reverse Primer Sequence
HPRT	AAATTCTTTGCTGACCTGCT	TCCCCTGTTGACTGGTCATT
SRC	GGCGGCTTCTACATCACCT	AGGGATCTCCCAGGCATC
SNAIL1	GCTGCAGGACTCTAATCCAGA	ATCTCCGGAGGTGGGATG

2.10.2 RNA Extraction

RNA extraction was performed using the Mammalian Total RNA Mini-prep kit (Sigma) according to the manufacturer's recommended protocol. To extract the RNA, 250 µl of Lysis buffer containing 1% β-mercaptoethanol was added to cells grown in a 6 well plate. The lysate was collected and centrifuged at 13,000 rpm for 2 minutes. Following this, 250 µl of 70% ethanol was added to the lysate and mixed by pipetting. The sample was transferred to a binding column, centrifuged at 13,000 rpm for 15 seconds, the flow through was discarded and the spin column placed back in the collection tube. Subsequently, 250 µl of Wash 1 buffer was added, centrifuged at 13,000 rpm for 15 seconds, the flow through was discarded and the collection tube replaced. Then, 80 µl of a master mix of DNase I (10 µl per column) and buffer (70 µl per column) was prepared and added directly to the column and incubated for 15 minutes at room temperature. Then, 250 of Wash 1 buffer was added to the column, centrifuged at 13,000 rpm for 15 seconds, the flow through was discarded and the collection tube replaced. Subsequently, 500 µl of Wash 2 buffer (containing ethanol) was added to the column, centrifuged at 13,000 rpm for 15 seconds and the flow through was discarded, the collection tube replaced and repeated with 500 µl of Wash 2 buffer and centrifuged for 2 minutes. The column was transferred to a new 1.5 ml tube before the addition of 50 µl of elution buffer directly to the spin column membrane and centrifugation at 13,000 rpm for 1 minute to elute the RNA. The RNA was quantified using the NanoDrop system.

2.10.3 Reverse Transcription

To generate complementary DNA (cDNA) from the RNA template, 0.5 µg of random hexamers (Thermo Fisher Scientific) and 1 µg of RNA was made up to a total volume of 15 µl with nuclease free H_2O. This was incubated at 70°C for 5 minutes to remove secondary structure and immediately placed on ice for a further 5 minutes. Following incubation, M-MLV RT Buffer (Thermo Fisher Scientific), dNTPs (0.5 mM) (Thermo Fisher Scientific), M-MLV Reverse Transcriptase (200 U) (Thermo Fisher Scientific), and nuclease free H_2O were added to each sample to make up to 25 µl of master mix. These samples were then incubated at 37°C for 1 hour to allow strand synthesis, followed by 95°C for 10 minutes. Samples without RT enzyme added served as negative controls. CDNA samples were then diluted 1:10 with nuclease free H_2O prior to gene quantification and stored at -80°C. Real time PCR was conducted using Gotaq (Promega) on a 7500 Fast Real-time PCR system machine serial no. 275012368 (Applied Biosystems life technologies).

2.10.4 QPCR

The annealing temperature optimisation for each primer was performed using gradient PCR. Go Taq DNA Polymerase (Promega), 250 nM of the forward and reverse primers (Eurofins) and 10% cDNA were added to each reaction with a total volume of 10 µl. Following preparation, sample reactions were run under cycle conditions of 95°C for 2 minutes, 40x (95°C for 30 seconds, 60°C +/- 10°C for 30 seconds, 72°C for 30 seconds) and 72°C for 10 minutes using the Primus 96 thermocycler (MWG Biotech Inc.). The products were run on a 2% agarose gel containing SYBR Safe along with a 100 bp ladder (Promega). The temperature that gave the greatest specific product together with an absence of non-specific products was considered optimal.

After optimising primer annealing temperature, the primer efficiency was determined using a standard curve. The PCR product was prepared as described before using serial dilutions of cDNA template. QPCR was run on the 7500 Fast Real-Time PCR System under cycle conditions of 95°C for 2 minutes, followed by 40 x (95°C for 3 seconds, optimised annealing temperature for 30 seconds). This was followed by the melt curve stage from 60–95^{0}C. PCR products with efficiencies of 90–110% were deemed acceptable. If reaction efficiencies were obtained outside of this range, magnesium (2–4 mM) and primer concentrations (50–350 nM) were optimised until the reactions were in this range of efficiency.

Following optimisation, a total volume of 30 µl was run on the 7500 Fast Real-Time PCR System under cycle conditions of 95°C for 2 minutes, followed by 40 x (95°C for 3 seconds, optimised annealing temperature for 30 seconds). Negative controls (no template) were run for each target gene and RT controls (RNA only) were run for each sample. Each sample was run in triplicate. The gene expression was quantified using the 2-ΔΔCt method, with the expression of hypoxanthine phosphoribosyl-transferase (HPRT) used for gene normalisation. This method was acceptable as the various pairs of primers had equivalent cycling efficiency.

2.11 Flow Cytometry

Flow cytometry was performed following transfection to quantify GFP expressing cells. Following transfection, cells were trypsinised and resuspended in 500 µl PBS. The quantification of Cten transfected cells was performed using the Coulter Altra Flow Cytometer (Beckman Coulter) with an argon laser (488 nm). The data obtained were analysed using Weasel software (version 3.0.2). Cells were first gated to exclude any cell debris and gates were applied to detect GFP expressing cells using untransfected cells as a negative control.

2.11.1 Cell Sorting

Cells transfected with either Cten or Cten targeting CRISPR-Cas9 were sorted for downstream experiments based on the GFP tag expression. Following this, cells were harvested by trypsinisation and resuspended in 500 µl of PBS. GFP expressing cells were sorted using the 488-nm argon laser of the MoFlo cell sorter (Beckman Coulter). As with standard flow cytometry, cells were gated to exclude any cell debris, then the expression of GFP in untransfected cells was used as a cut-off to gate the GFP expressing cells in the transfected samples. Weasel was used for data analysis.

2.12 CRISPR-Cas9 Gene Editing

2.12.1 Clonal Expansion of CRISPR Transfected Cells

CRISPR-Cas9 genome editing technique was performed to establish a Cten knockout SW620 cell line. The CRISPR-Cas9 construct targeted to exon 3 of Cten was designed and made by Sigma. Single GFP expressing cells were sorted into 96 well plates following transfection of the CRISPR-Cas9 construct (CCGCCAGATCAAGGTGCCACGA) in SW620 cell line. Cells were clonally expanded over a 28-day period until cell growth was sufficient for passage into a T75 flask.

2.12.2 Confirmation of Gene Knockout

2.12.2.1 DNA Extraction

To confirm the knockout of Cten, DNA was extracted from the CRISPR-Cas9 transfected cells using the Mammalian Genomic DNA Miniprep kit (Sigma) in accordance with the manufacturer's protocol. Briefly, 5 x 10^6 cells were harvested and resuspended in 200 µl of Resuspension solution, followed by RNase treatment with 20 µl of RNase A Solution at room temperature for 2 minutes. Following this, 20 µl of Proteinase K was added and cells were lysed by adding 200 µl of Lysis solution. The Genelute column was prepared by adding 500 µl of Column Preparation Solution and centrifuged at 12,000 x g for 1 minute and the flow through was discarded. Then, 200 µl of ethanol was added to the cell lysate and vortexed before loading onto the column. The sample was centrifuged at 12,000 x g for 1 minute and placed in a new collection tube. The sample was washed by adding 500 µl of Wash Solution and centrifuged at 12,000 x g for 1 minute and then placed in a new collection tube. This was followed by a second wash with centrifugation for 3 minutes. The column was then centrifuged at 12,000 x g for 1 minute and transferred to a new collection tube before the addition of 200 µl of elution buffer directly to the spin column membrane and centrifugation at 12,000 x g for 1 minute to elute the DNA. The DNA was quantified using the NanoDrop system.

2.12.2.2 PCR

PCR was performed to amplify the region of the Cten gene around the CRISPR-Cas9 target site using Hotshot Mastermix (Clent Life Science), 625 nM forward and reverse primers (table 2-6), 10% LC green (BioFire Defense) and 1 ng/µl of the DNA template. The PCR product was run under cycle conditions of 95^{0}C for 5 minutes, 45 x (95^{0}C for 20 seconds, 70^{0}C for 45 seconds, and 72^{0}C for 30 seconds), followed by an extension stage at 72^{0}C for 2 minutes using the Primus 96 thermocycler. The PCR products were run on a 2% agarose gel containing SYBR Safe and visualised using a gel documentation viewing system.

Table 2–6: PCR primer sequences used in the preparation of SW620$^{\Delta Cten}$ cell line

Target Gene	Primer Sequence
Cten	Forward: AGGGAGGGTGACTGGAACTG
	Reverse: GAGAGACACGAAGCAGCAGT

2.12.2.3 High Resolution Melting (HRM)

HRM was used to confirm the deletion at the target region. HRM works on the principle that DNA will melt when heated but the pattern of melting will depend on the DNA sequence. HRM can therefore be used to screen for differences between DNA sequence (e.g. between wild type and mutant sequence) due to differences in the melting profiles. Briefly, 10 µl of PCR product was added to 10 µl of mineral oil and melted over a range of 68–95^{0}C with a temperature increase of 0.3^{0}C/second using the LightScanner (BioFire

Defense) and HR1 High Resolution Melter (Idaho Technology Inc.) instruments. The melt curves were analysed using the HR1 or LightScanner Instrument and analysis software packages. Those cell populations displaying an altered melt curve profile compared to wild type SW620 cells (>4% change in fluorescence) were considered to contain a mutation.

2.12.2.4 TOPO TA cloning and Blue White Screening

In order to isolate each Cten allele for subsequent sequencing, the PCR product was directly inserted into a plasmid vector using the TOPO TA cloning kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Briefly, 4 µl of the PCR product was incubated with 1 µl of Salt Solution and 1 µl of the TOPO vector at room temperature for 5 minutes and then placed on ice.

The plasmid transformation was performed as described in section 2.2 however, to perform blue white screening, the agar plates were coated with 40 µl of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; Sigma) at a concentration of 20 mg/ml. The following day, white colonies (recombinants) were picked for miniprep preparation and purification as described in section.

2.12.2.5 Sanger Sequencing

To determine whether a mutation at the target region sequence of Cten had occurred, plasmids were sent off for sequencing by the University of Nottingham Sequencing Facility using the 3130xl ABI PRISM Genetic Analyser (Thermo Fisher Scientific). Results obtained from sequencing were then analysed using Chromas Lite and compared to the Cten gene reference sequence (NC_000017.11).

2.13 Site Directed Mutagenesis

2.13.1 PCR

The Phusion site directed mutagenesis kit (Thermo Fisher Scientific) was used to create point mutations in Cten plasmid DNA according to the manufacturer's protocol (figure 2-1). Briefly, the primers were designed to be phosphorylated at the 5' end, having mutations in the middle of the forward primer and purified with reverse phase high performance liquid chromatography (RP-HPLC) (table 2-7). The annealing temperature was optimised for each primer set using gradient PCR as described before. To optimise the template concentration, 10 pg to 500ng was used. The reaction volume used was 50 µl, with final concentration of 200 µM each for dNTP, 0.5 µM for each primer and 0.02 U/µl for the Phusion Hot start II DNA polymerase provided with the kit. The reaction cycle comprised an initial denaturation stage for 30 seconds at 98°C, followed by 25 x (98°C for 10 seconds, 66°C for 30 seconds and 72°C for 4 minutes) and a final extension stage at 72°C for 10 minutes. Products were run on a 1% agarose gel at 100 V for 50 minutes and bands visualised using a blue light transilluminator.

Table 2–7: PCR primer used in the preparation of mutated Cten plasmid. The red letter represents the point mutations added.

Primer	Sequence
$Cten^{R474A+Y479F}$	Forward: GTCATAGCGGACAGCTCTTCATTCCGA GGC Reverse: AAAAGCCCCTGGCTCCTCCTTCCT

2.13.2 Gel Extraction

After visualising the PCR product band, gel extraction was performed using the Genelute Gel Extraction Kit (Sigma) according to the manufacturer's standard protocol. The DNA band was excised from the gel using a clean scalpel and weighed. After weighing, 300 µl of solubilisation solution was added for every 100 mg of gel to the gel fragments containing the band of interest and incubated for 10 minutes at 50–60°C with occasional vortexing. Following this, the binding column was prepared by adding 500 µl of Column Preparation Solution and centrifuged for 1 minute at 13,000 x g and the flow through was discarded. After the gel fragments had been dissolved completely, 100 µl of isopropanol for every 100 mg of gel was added to the solubilised gel and mixed. The solubilised gel solution was loaded onto the binding column, centrifuged at 13,000 x g for 1 minute and the flow through was discarded. The column was washed with 700 µl of Wash solution, centrifuged at 13,000 xg for 1 minute and the flow through discarded. The tube was centrifuged for a further minute before transferring the column to a new collection tube. The DNA was eluted with 50 µl of Elution solution and centrifuged at 13,000 x g for 1 minute. The eluate was retained.

2.13.3 Ligation, Transformation, Miniprep, and Sequencing

Ligation of extracted plasmid DNA was preformed using T4 DNA Ligase kit according to the manufacturer's recommended protocol. In brief, 3 µl of DNA was incubated with Ligation Buffer (2 µl), nuclease free H_2O (4.5 µl) and T4 DNA ligase (0.5 µl) (Thermo Fisher Scientific) at room temperature for 5 minutes and then chilled overnight at 4°C. Nuclease free H_2O was used instead of T4 DNA ligase for the negative control. The ligated product was then transformed into NEB 5α competent *E-coli* high efficiency cells (as explained in 2.2.1), keeping untransformed *E-coli* cells and unligated product as negative controls. A few colonies were selected for sequencing as described in section 2.2.2.

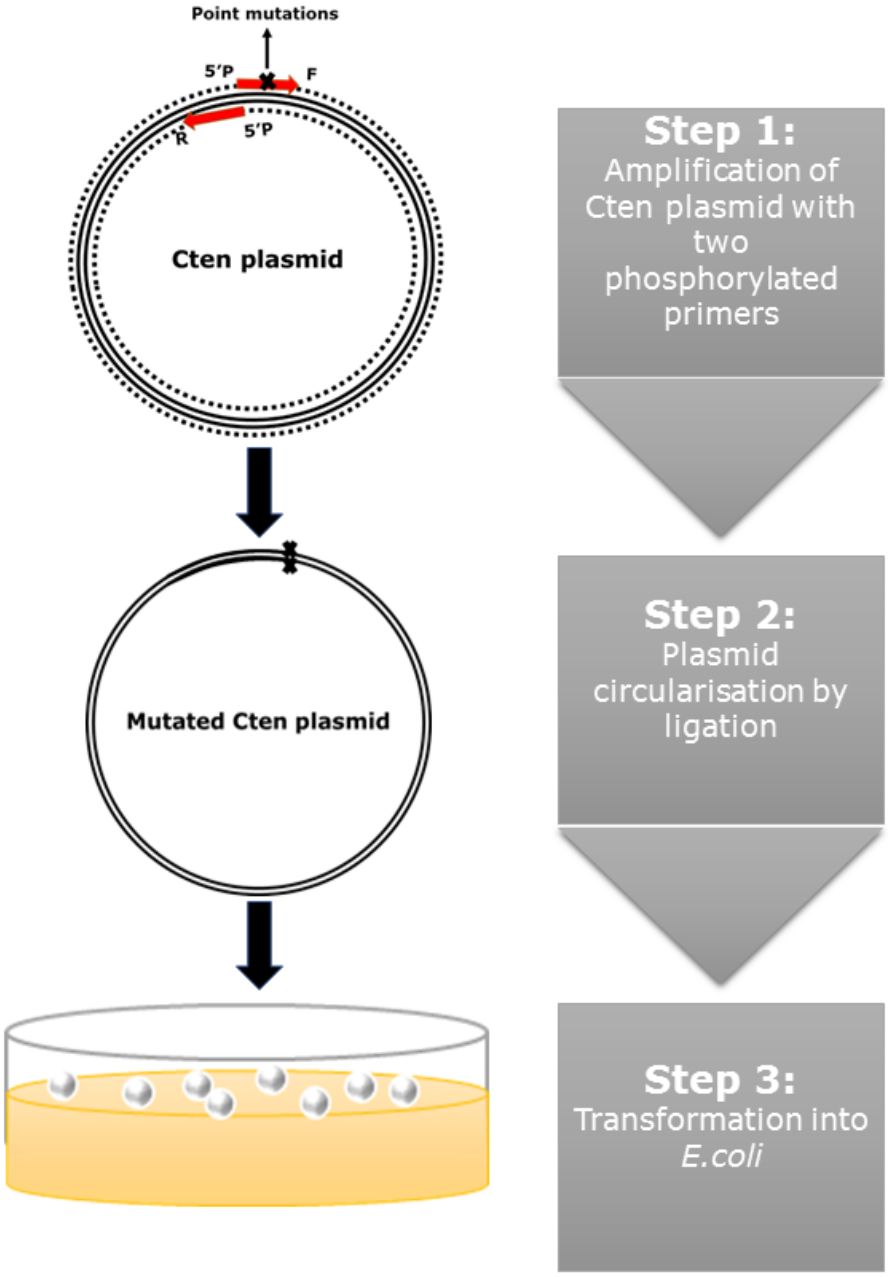

Figure 2–1: Diagrammatic representation of the Phusion site directed mutagenesis protocol used to create point mutations in the Cten plasmid.

2.14 Function Assays

2.14.1 Transwell Migration Assay

Cell migration was measured in 24 well plates using the Transwell migration assay (Corning) (figure 2-2). The Transwell migration assay uses a Boyden chamber containing a polycarbonate filter with an 8 µm pore size (Corning) and this was incubated in DMEM at 37°C for 1 hour prior to use. The lower chamber was filled with 200 µl of DMEM with 20% FBS; the upper chamber was filled with 100 µl of DMEM with 10% FBS and seeded with 1 X 10^5 cells. The cells migrated through the membrane towards the higher FBS concentration chemoattractant and cells on the bottom of the well were manually counted after 24–48 hours. Experiments were performed in triplicate and on at least on two separate occasions.

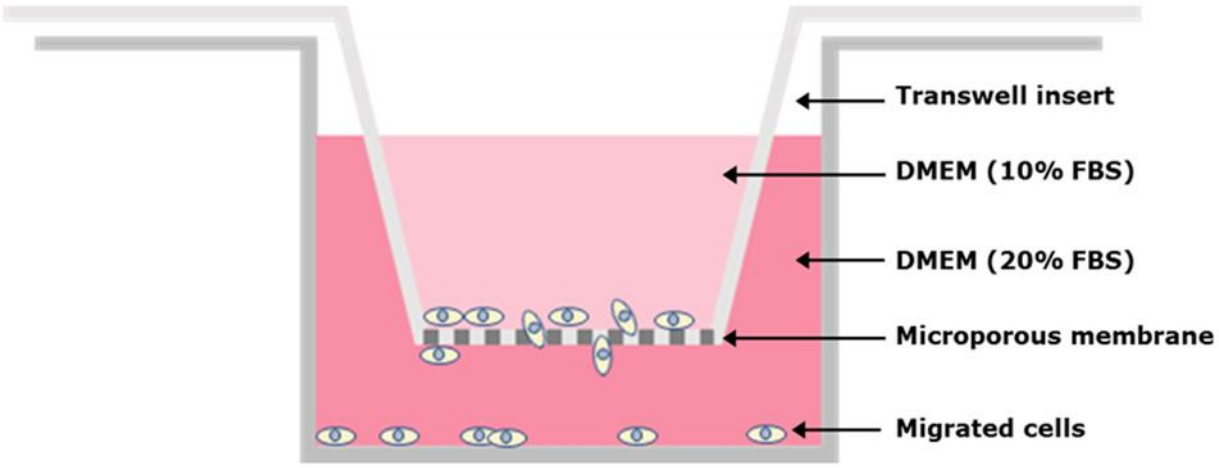

Figure 2–2: Diagrammatic representation of the Transwell assay system used to investigate cell migration and invasion, with 20% FBS used as a chemoattract for migrating or invading cells.

2.14.2 Transwell Invasion Assay

Cell invasion was measured using the Transwell migration assay as described above in the cell migration assay. However, the upper chamber was prepared by coating the filter membrane with 80 µl of Matrigel basement membrane extract (Corning) at a concentration of 3 mg/ml and incubated overnight at 37°C. The following day, the coated filter membranes were incubated in DMEM at 37°C for 1 hour prior to use before 2 x 10^5 cells suspended in 100 µl of DMEM with 10% FBS were added over the Matrigel in the upper chamber. After 48 to 72 hours, the cells at the bottom of the well were counted visually by microscope, averaged and compared between several groups. For each experiment, the assays were performed in triplicate.

2.14.3 Wound Healing Assay

"Wound healing" scratch assay was performed an alternative assay to assess cell migration (figure 2-3). Briefly, 5-7 x 10^5 cells /ml were seeded into culture-insert 2 well (Ibidi) attached in 6 well plates and incubated for 24 hours at 37°C until they reached confluency. After cell attachment, the cell free gap "scratch" was created by removing the culture inserts using sterile tweezers, followed by adding 2 ml of fresh media to the cells. The pictures were taken at time points 0 and 24 using an inverted microscope (Nikon) at 10x magnification. The width of the cell free gap was approximately 500 microns (+/- 50 microns) at time 0 hours. Experiments were performed in duplicate and on at least on two separate occasions. Cell migration was assessed by measuring the remaining open area of the wound by ImageJ software.

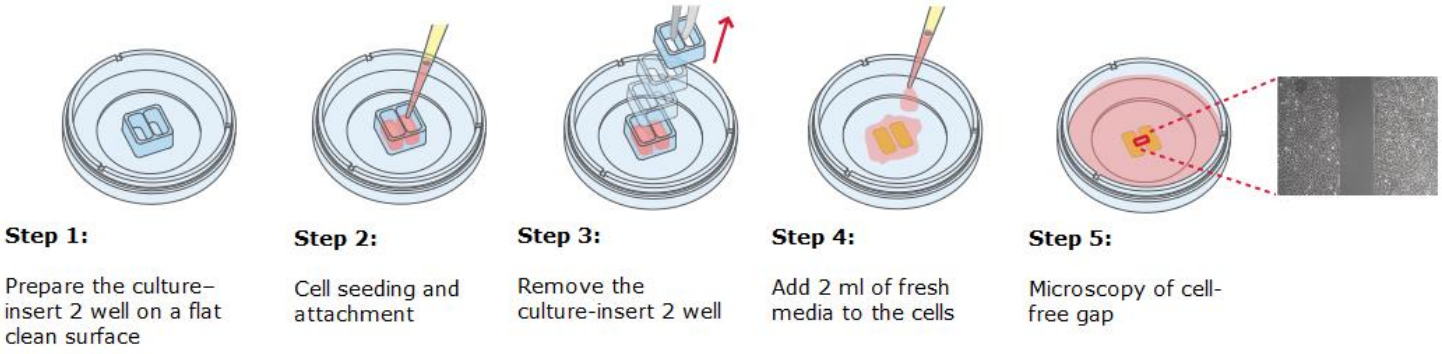

Figure 2–3: Diagrammatic representation of the wound healing assay protocol using ibidi culture-inset 2 well system.

2.14.4 Proliferation Assay

Cell proliferation was assessed using PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 5 x 10^3 cells were seeded in a 96 well plate and allowed to attach for 24 hours. Following this, 100 µl of PrestoBlue Cell Viability Reagent was added to the cells and without cells as control, and incubated for 1 hour at 37^0C. Following incubation, the fluorescent unit (OD) of each well of the 96 well plate was then measured using the BMG FLUOstar Optima Plate reader (540 nm/ 590 nm). Further readings were taken at 48 and 72-hour time points. The blank fluorescence reading was subtracted from each experimental fluorescence reading and the blank corrected values were then normalised to the 24 hour time point.

2.14.5 Colony Formation Assay

Colony formation in soft agar was performed to assess anchorage independent cell growth. Briefly, 1 ml of a 0.7% agarose (Sigma) containing 2,500 transfected cells in DMEM (supplemented with 10% FBS) was plated in 6 well plates on top of 1 ml of a 1% agar layer (Sigma) containing DMEM (supplemented with 10% FBS). Plates were incubated at 37°C for 3 weeks and fed with 0.5 ml of DMEM (supplemented with 10% FBS). Following this, the plates were stained with 0.005% crystal violet and 4% formaldehyde for 1 hour. The number of colonies were manually counted, and the colony formation efficiency determined (number of colonies counted/number of cells seeded x 100).

2.15 Statistical Analysis

2.15.1 Data Analysis for *in Vitro* Studies

All *in vitro* experiments were performed at least in duplicate and the data are presented as mean ± standard deviation (SD), unless stated otherwise. The Shapiro-Wilk statistical test was used to determine normality. If the data were normally distributed, either the unpaired t-test (for data sets containing 2 treatment groups) or the analysis of variance (one-way ANOVA) (for more than 2 treatment groups) statistical tests were applied. The two-way ANOVA test was used for data sets containing 2 treatment groups that have been split on two or more independent variables. GraphPad Prism software (version 6.0) was used for statistical analysis and a two-tailed p-value < 0.05 was considered significant.

2.15.2 Data Analysis for TMAs

IBM SPSS statistics software (v 22) was performed for all the statistical analysis of IHC TMA staining. The expression of protein was categorised into low and high based on the median. The Chi squared test was used to test for associations between marker expression and the clinicopathological parameters. The Wilcoxon signed ranks test was used to determine associations between the marker expression in the normal colon and tumour tissue for non-normally distributed, continuous data. The Spearman's rank test was performed to determine correlations between the expression of two different markers. This was applied to continuous data that did not follow a normal distribution pattern. For all statistical tests, a p-value < 0.05 was considered significant.

3 Establishment of a SW620$^{\Delta Cten}$ Cell Line Using the CRISPR/Cas9 System

3.1 Introduction

The clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR associated 9 (Cas9) system is a RNA-mediated adaptive immune system common in archaea and bacteria. It protects host cells against invading non-self-DNA elements such as virus and plasmids. Currently, the CRISPR/Cas9 system is the most efficient technique used for genome-engineering applications, including specific nucleotide editing, insertion or gene activation/repression (Dominguez et al., 2016). It has been reported that the CRISPR/Cas9 system induces targeted genomic deletion more precisely and efficiently than previously used zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) genome editing technologies (He et al., 2016).

The CRISPR system uses Cas9 nuclease, which is guided by a short guide RNA (sgRNA) to generate double stranded breaks (DSBs) in the target genomic sequences (Dominguez et al., 2016). Cells with DSBs use two major pathways to repair damage, including non-homologous end joining (NHEJ)-mediated error-prone DNA repair and homology-directed repair (HDR)-mediated error-free DNA repair (figure 3-1). HDR is a precise repair pathway that uses a donor plasmid to generate high fidelity repair. The use of a donor DNA sequence allows specific nucleotide editing in the genome using an exogenous DNA template with homology to promote DSB repair through the HDR pathway. If this DNA template is used during HDR repair, any modification in the DNA sequence will be incorporated into the genome, allowing for specific mutations to be made, including the repair of dysfunction mutations. In contrast, the repair of DSB introduced at the target sequence via the NHEJ pathway does not require a donor template like the HDR pathway, but can repair this break by joining the two broken ends together. This usually leads to small deletions at the target sequence region which can cause a frameshift and subsequent gene knockout either by changing the coding sequence or by the introduction of a premature stop codon (Wei et al., 2013).

Investigation of the role of Cten signalling has previously been accomplished by manipulating Cten expression in a different number of cancer cell lines, either by

knockdown of the expression of Cten using siRNA duplexes targeting Cten mRNA or by forced expression of Cten using plasmid vector (Albasri et al., 2009, Albasri et al., 2011a, Albasri et al., 2011b, Thorpe et al., 2017). To knockdown gene expression, short double stranded RNA (dsRNA) oligonucleotides are inserted into the cell and cleaved by a RNAse III endonuclease called Dicer. Following incorporation into the multiprotein, the RNA induced silencing complex (RISC) guides the siRNA to target mRNA containing a sequence homologous to the siRNA and cleaves the mRNA at the target region. Thus, gene expression is specifically inhibited and degraded at the post-transcriptional level. The disadvantage of using siRNA technique to knockdown Cten is that it is only transient as this method works at a post-transcriptional level. Furthermore, the level of protein expression on knockdown is often inconsistent between experiments (Barrangou et al., 2015). Therefore, to overcome such disadvantages, the CRISPR-Cas9 genome editing system was used to establish a Cten knockout cell line, producing an alternative and probably a more robust model to interrogate the role of Cten signalling in CRC.

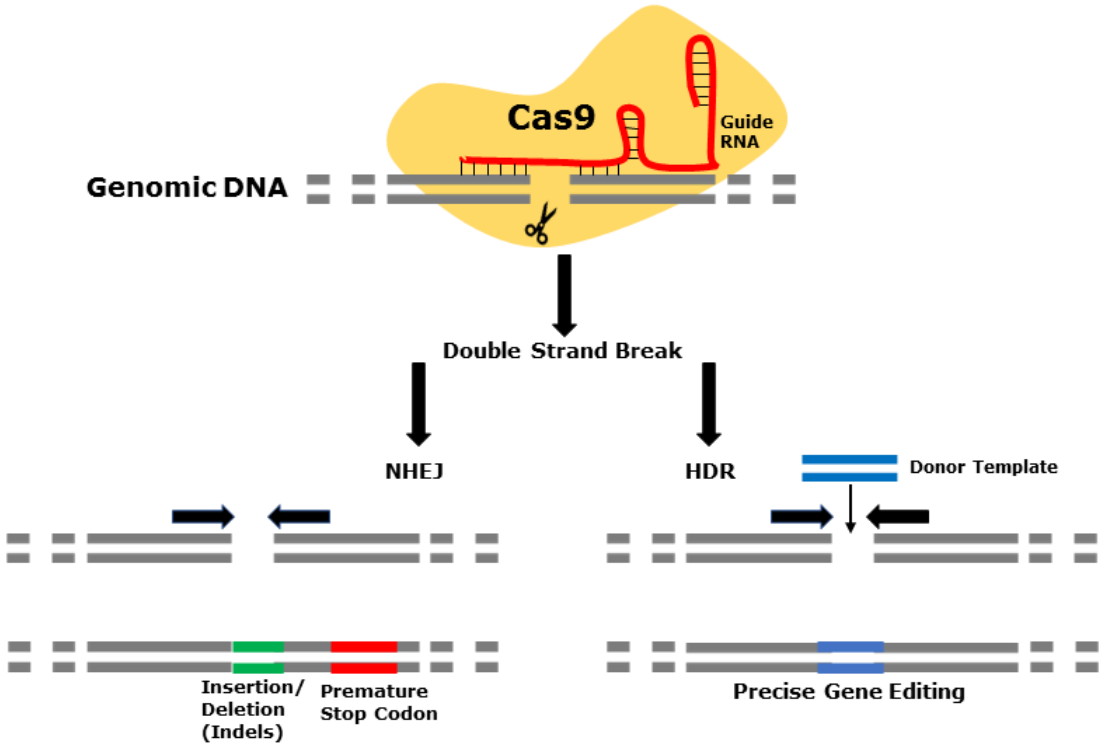

Figure 3–1: The principle of CRISPR/Cas9 system.
Cas9 recruitment to the target genomic DNA sequences is mediated by a short guide RNA (sgRNA). Cas9 induced double stranded breaks (DSBs) in the target genomic sequences are repaired either by non-homologous end joining (NHEJ) that induces random indel mutations at the site of the DSBs to generate products of variable length and sequence or by homology-directed repair (HDR) using a donor DNA template, which allows the introduction of desired sequence changes.

3.2 Results

3.2.1 CRISPR/Cas9-GFP Construct Transfection Efficiency Optimisation

The establishment of a SW620$^{\Delta Cten}$ cell line was achieved using a premade CRISPR/Cas9-GFP construct. The construct produces both the gRNA to target Cten at exon 3 (CCGCCAGATCAAGGTGCCACGA) and the Cas9 endonuclease to cleave the DNA at this target region (figure 3-2). It also encodes for Green Fluorescent Protein (GFP) which can be used to track the plasmid. The transfection efficiency of the CRISPR/Cas9-GFP plasmid was optimised in CRC cell line SW620 (which has high expression levels of endogenous Cten). The number of cells expressing GFP was determined by flow cytometry. The condition using 5 µl of Lipofectamine 2000 together with 4 µg of CRISPR/Cas9-GFP plasmid gave the optimal transfection efficiency (16.70%), (figure 3-3). These conditions were used for subsequent transfection experiments.

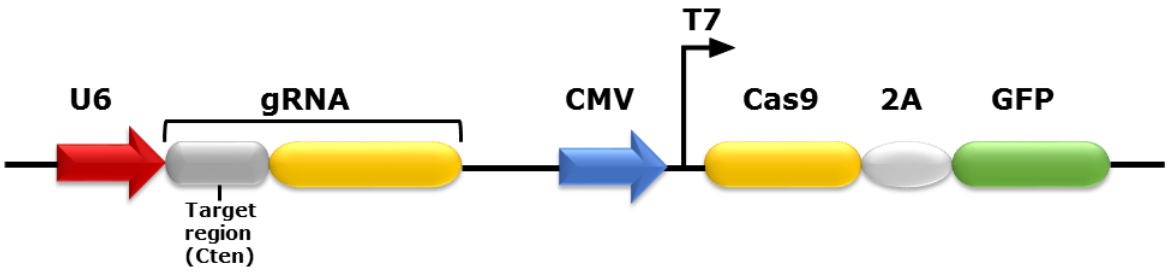

Figure 3–2: The CRISPR/Cas9 single vector format.
The CRISPR/Cas9 plasmid construct contained the gRNA sequence targeted to exon 3 of Cten downstream of the U6 promoter. The Cas9 gene was bonded to the GFP with a 2A peptide downstream of a CMV promoter.

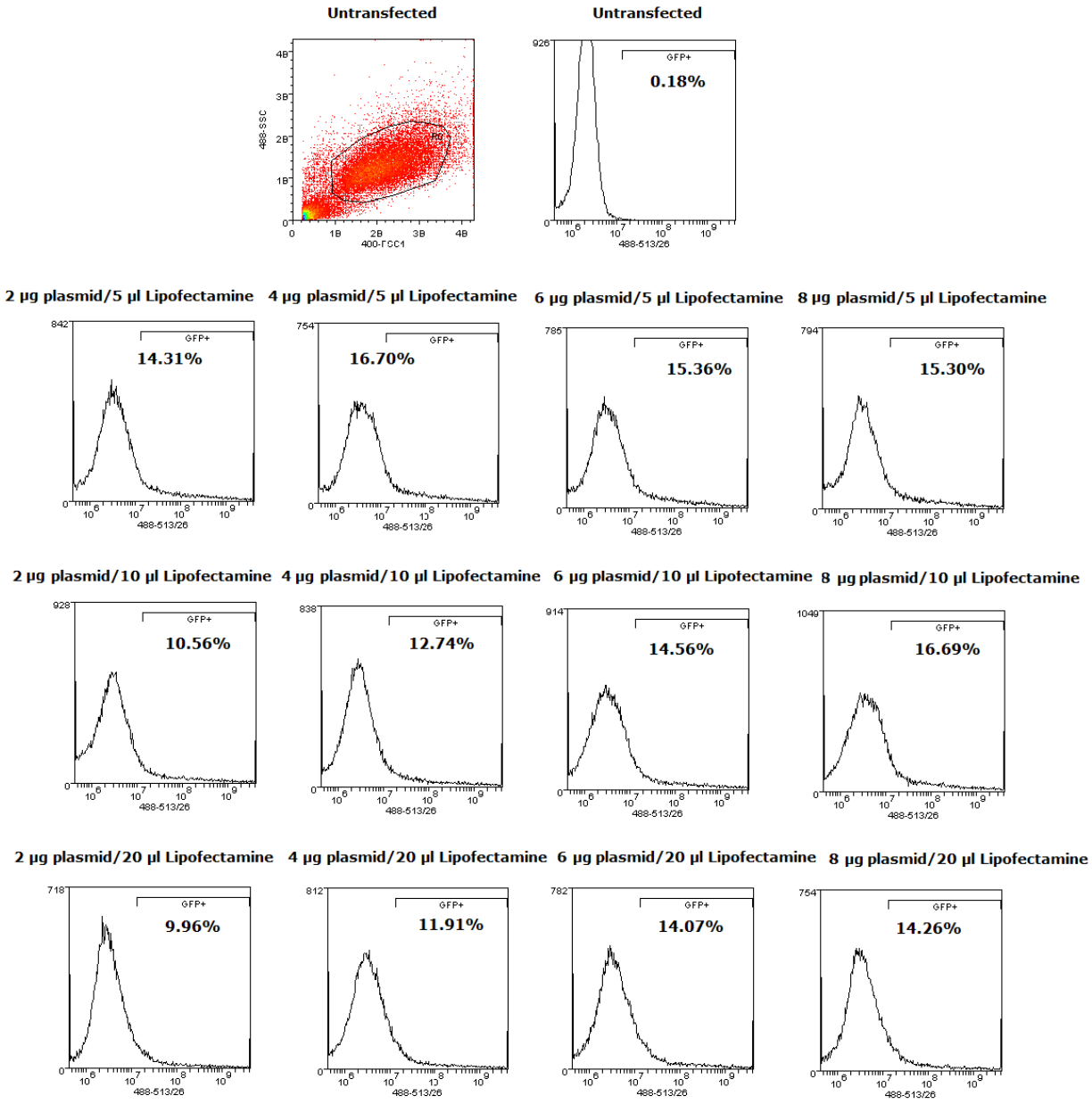

Figure 3–3: CRISPR/Cas9-GFP Construct Transfection Efficiency Optimisation
The percentage of GFP expressing SW620 cells was determined by flow cytometry. Transfection of 4 µg of CRISPR/Cas9-GFP plasmid together with 5 µl of Lipofectamine 2000 gave the greatest transfection efficiency (16.70%).

3.2.2 Confirmation of CRISPR/Cas9 Induced Mutations

To confirm whether CRISPR/Cas9 construct was able to induce mutations at the target region in exon 3 of Cten, the construct was transfected into SW620. DNA was extracted from pools of both untransfected and CRISPR transfected cells. PCR was used to amplify the region around the target site. Agarose gel electrophoresis showed bands corresponding to 333 bp as predicated for the amplification PCR product. HRM analysis was then performed on the PCR product and this showed a shift in the melting profile of the CRISPR transfected compared to the untransfected SW620 cells, indicating that mutations have been introduced into the Cten DNA sequence (figure 3-4).

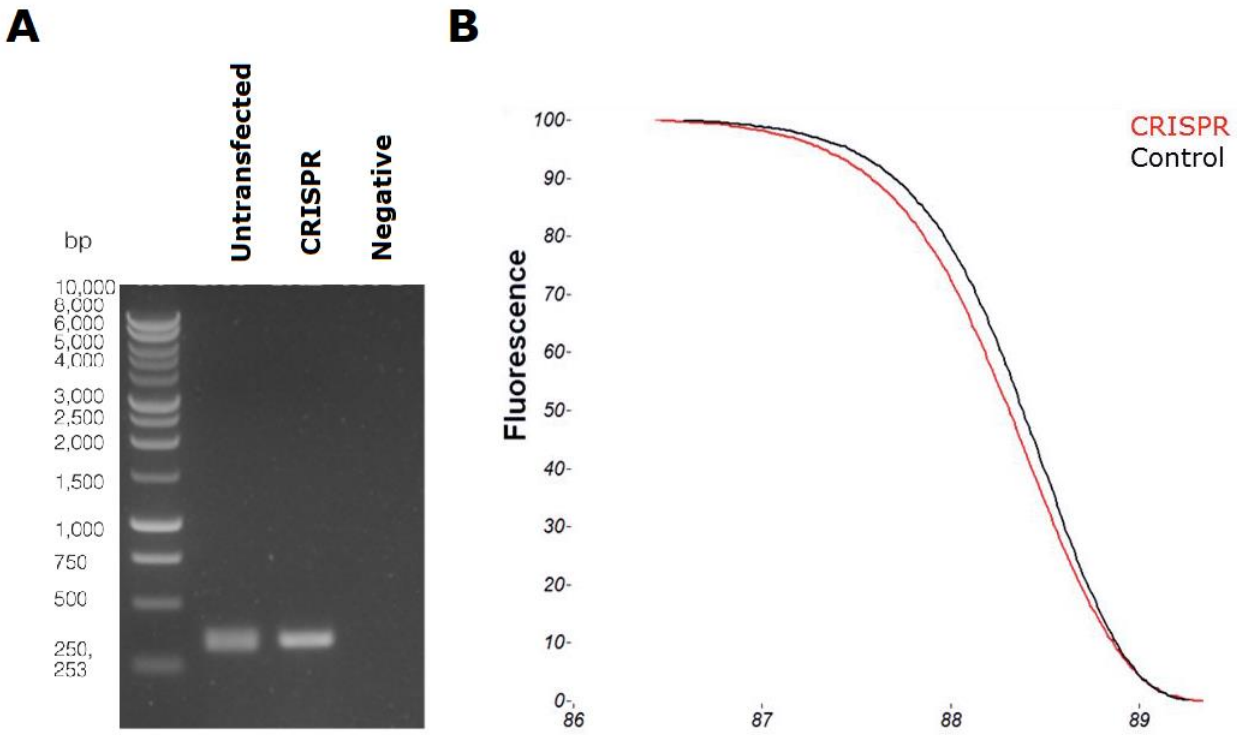

Figure 3–4: Confirmation of CRISPR/Cas9 induced mutation by HRM.
A) PCR amplification of the region around the target site in exon 3 of Cten (333bp) for both CRISPR/Cas9 transfected and untransfected SW620 cells. B) HRM analysis showed a shift in the melting profile of the CRISPR transfected (Red) compared to the untransfected SW620 cells (Black).

3.2.3 Generation of Homogenous Cell Populations

When the CRISPR/Cas9 vector is transfected into a pool of cells, the random nature of the indel mutations at the target region will result in cells with a variety of different mutations as well as some cells which do not have mutations. For experimental work, a single homogenous cell population, each containing the same mutation, is required. For this, cells transfected with the CRISPR/Cas9 construct underwent cell sorting based on expression of GFP. The sorted GFP expressing cells underwent a second round of sorting and single cells were dispensed in wells of a tissue culture plate (1 cell / well) and clonally expanded (figure 3-5). A total of 384 isolated single transfected cells with the CRISPR/Cas9 construct were seeded into 96 well plates. Of these, 20 of which generated colony growth and were expanded to the T75 flask stage.

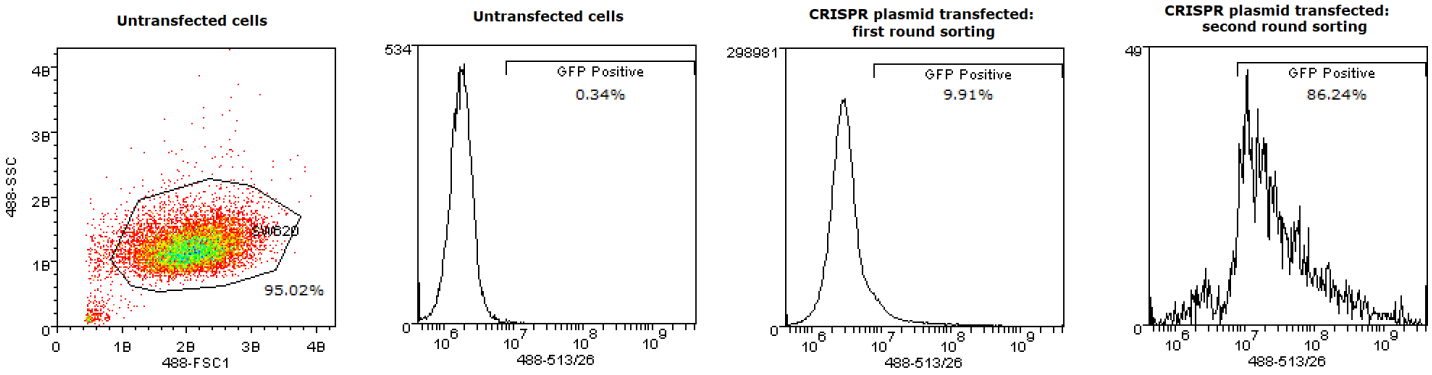

Figure 3–5: Isolation of single transfected cells with the CRISPR/Cas9 construct.
CRISPR/Cas9 transfected SW620 cells were sorted based on the GFP tag expression. Cells went through two rounds to confirm that only GFP positive cells were isolated.

3.2.4 CRISPR/Cas9 Induced Mutations of the Cten Gene

To generate a gene knockout from the introduction of indel mutations within the coding exon, a frameshift was required to create a premature stop codon by inducing a mutation in the downstream translated sequence. After the subsequent clonal expansion of transfected cells, DNA was extracted for PCR to amplify the region around the target site in exon 3 of Cten. To validate whether both alleles had deletion mutations, the PCR products were inserted into a vector by TA cloning and amplified in a bacterial host. Plasmid DNA extracted from different white colonies arising from each PCR product was sequenced and subjected to BLAST analysis (NCBI) to identify any difference in the DNA sequence between CRISPR/Cas9 targeting Cten and the wt. Cten sequence. One clone from the clonally expanded cell lines sequenced contained a deletion in both Cten alleles. The alleles from this cell population showed 19 and 20 bp deletions at the target region of Cten (figure 3-6), (figure3-7). The sequence analysis using ORF Finder (NCBI) showed that these sequences translated to 333 and 191 amino acid proteins respectively before a stop codon was reached (compared to 715 amino acids of the wild type Cten). These predicted proteins, however, bore no homology to Cten beyond the site of the deletion thus confirming that the Cten protein was knocked out. This cell line was labelled as SW620$^{\Delta Cten}$.

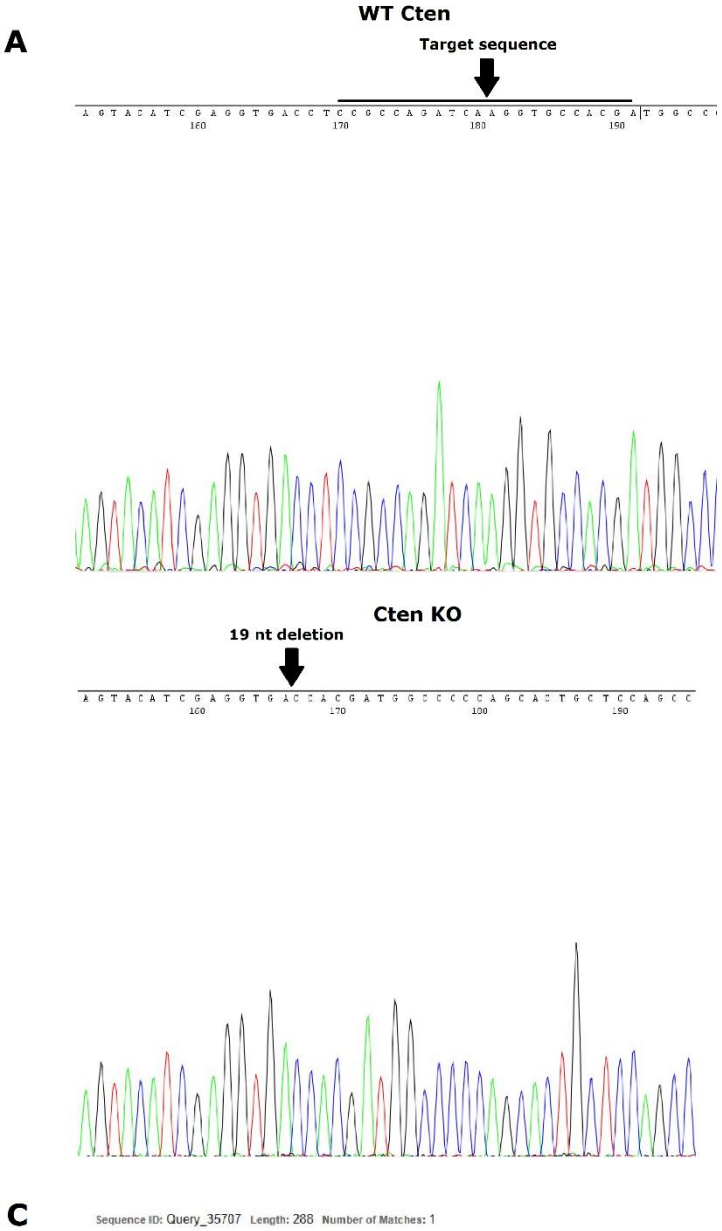

Figure 3–6: BLAST analysis of CRISPR /Cas9 induced mutations.
A) Sequencing of Cten allele following CRISPR/Cas9 genome editing of SW60 sequence of Cten. B) BLAST analysis showed a 19 nucleotides (nt) deletion in the DNA sequence derived from the CRISPR/Cas9 transfected SW620 cells compared to the wt. Cten sequence.

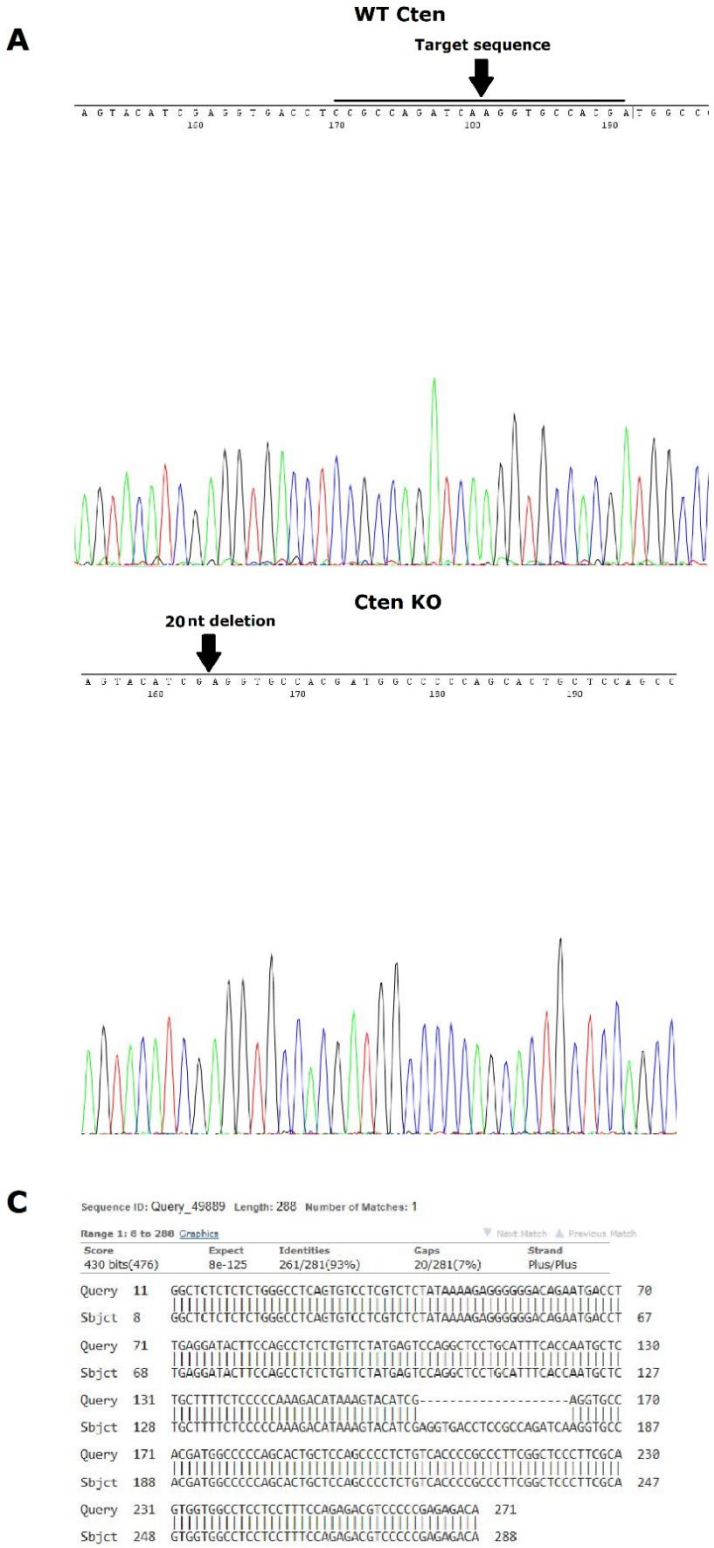

Figure 3–7: BLAST analysis of CRISPR /Cas9 induced mutations. *A) Sequencing of the second allele of Cten following CRISPR/Cas9 genome editing of SW60 sequence of Cten. B) BLAST analysis showed that the second allele contained a 20 nucleotides deletion of the Cten gene sequence.*

3.2.5 Cten Protein Expression Is Absent in the SW620$^{\Delta Cten}$ Cell Line

The deletions in Cten were predicted to induce frame shifts, which result in a premature stop codon in the amino acid sequence and subsequent loss of protein expression. To validate this, protein was extracted from the generated SW620$^{\Delta Cten}$ cell line and the control SW620 cell line for western blotting. The level of Cten protein expression was completely lost in the SW620$^{\Delta Cten}$ cell line even under long exposures, whilst still present in the control SW620 cell line (figure 3-8 A). This confirms that Cten was successfully knocked out in SW620$^{\Delta Cten}$ cell line using the CRISPR/Cas9 genome editing system.

3.2.6 Cten Knockout Decreases SW620 Cell Motility Capability

The effect of Cten knockout on SW620 cell motility was then investigated. Cten was shown previously to regulate cell motility in different cancer cell lines using a dual approach of gene manipulation. The functional evaluation of the SW620$^{\Delta Cten}$ cell line showed that knockout of Cten was associated with a significant reduction in both migration and invasion compared to the control SW620 cell line (figure 3-8 B, C). The generation of isogenic cell lines with both the absence and the presence of the full length Cten gene provides a robust model to interrogate Cten biology.

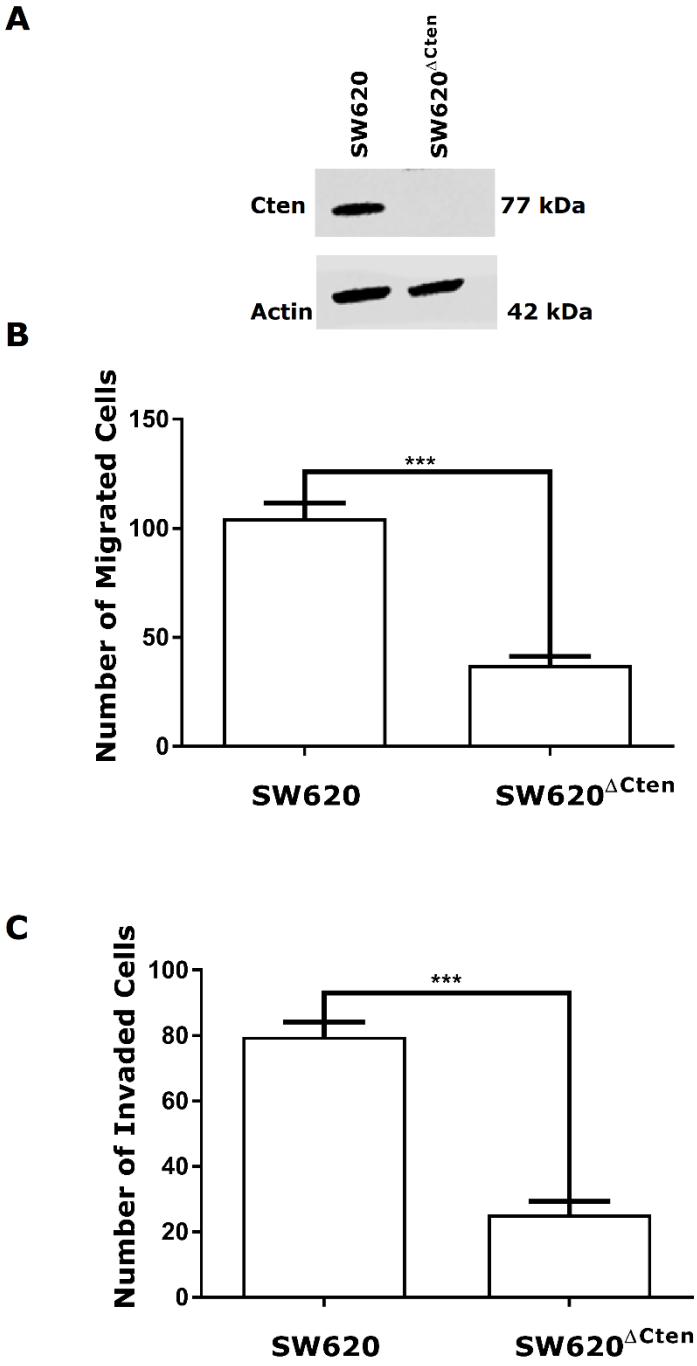

Figure 3–8: Cten knockout abrogates cell invasion and migration. *A) Western blot analysis revealed that Cten protein was knocked out in SW620ΔCten cells compared to the SW620 cells control. B) Cten knock out was associated with a significant reduction in SW620ΔCten cell migration compared to the control SW620 cell line (P ≤ 0.0001). C) Knockout of Cten decreased cell invasion in SW620ΔCten cells compared to the control SW620 cell line (P ≤ 0.0001) (unpaired t-test, n=3).*

3.2.7 Discussion

The CRISPR/Cas9 system is a powerful tool for precise genome editing. CRIPSR/Cas9 was successfully used to knockout Cten in a CRC cell line, SW620 (high expressing Cten cell line), to produce an alternative robust model to temporary siRNA knockdown for interrogating the role of Cten signalling in CRC.

The Cten knockout was confirmed at both DNA and protein levels. Induction of frame shift mutations resulted in a premature stop codon on each Cten allele. However, there is a possibility that truncated Cten protein is still expressed in the cell as the coding region of Cten starts in exon 2 and the CRISPR target sequence was in exon 3. This leaves a small section of the Cten coding sequence remaining before the frameshift and non-homologous sequence after the frameshift. The SW620$^{\Delta Cten}$ was used to validate the previous functional experiments. Knockout of Cten in SW620 using the CRISPR/Cas9 system was associated with a significant reduction in both cell migration and invasion as previously demonstrated using siRNA knockdown.

Although, the CRISPR/Cas9 system is the most powerful technique used for genome modifications, it is still a relatively new technique and more improvements are required as the extent of off-target effects is currently unclear. This is however also a disadvantage when using siRNA mediated knockdown as an alternative screening system. There have been a number of strategies described to mitigate the potentials of off-target effects including truncated gRNA, dual nickase and dCas9-FokI strategy (Wiles et al., 2015). Nonetheless, further improvement and development of the CRISPR/Cas9 system will deliver even greater achievements.

In conclusion, the CRISPR/Cas9 system has been successfully used to knockout Cten in the CRC cell line SW620, therefore this may enable the development of more robust *in vitro* models to investigate the role of Cten signalling in colorectal tumours.

4 Investigation of Novel Downstream Targets of Cten Signalling in Colorectal Cancer

4.1 Introduction

Cten has been shown to enhance cell migration and invasion. These are features which contribute to EMT and which may contribute to the development of metastasis. Cten was found to signal through focal adhesion localised protein such as ILK and FAK (Albasri et al., 2011a, Al-Ghamdi et al., 2013) and has shown to downregulate E-cadherin (Albasri et al., 2009). Although some mechanisms underlying the role of Cten in cell motility have been described, the full understanding of additional layers of complexity and signalling pathways that may cooperate with Cten to regulate both cell migration and invasion remain to be resolved.

EMT plays a critical role in normal development and oncogenesis as previously discussed. A major EMT regulator, Snail, is a zinc finger transcription factor that functions as a transcriptional repressor through binding to the sequence of E-boxes in the promoter region of the target genes (Lamouille et al., 2014, Wang et al., 2013). Snail has been shown to directly downregulate epithelial markers, such as E-cadherin (encoded by CHD1), which are thought to be metastatic suppressors during tumour progression and upregulate mesenchymal markers, mainly vimentin or fibronectin, thereby increasing cell migration and invasion as well as a fibroblast-like cell morphology (Medici et al., 2008).

Interestingly, previous studies carried out by our group showed that Cten downregulates E-cadherin (Albasri et al., 2009) and upregulates FAK and ILK (Albasri et al., 2011a, Al-Ghamdi et al., 2013) and has also been found to be associated with EMT processes (Yang et al., 2016). Therefore, it was hypothesised that Cten may signal through Snail induced EMT to regulate cell motility in CRC cells.

Rho-associated protein kinase1 (ROCK1), a member of the Rho-associated serine/threonine kinase family, is another interesting signalling pathway investigated in this chapter. ROCK1 functions as an oncogene and is overexpressed in several types of tumour tissues including CRC (Xi et al., 2015). ROCK1 has been found to be a direct

downstream effector of the RhoA signalling pathway, regulating a wide range of cellular functions such as contraction, proliferation, migration, invasion, adhesion, and metastasis (Wei et al., 2016). Previous studies have also shown that RhoA/ROCK1 and the tensin family pathways play important roles in signal transduction and actin cytoskeleton reorganisation during motion respectively (Lo, 2004, Wei et al., 2016). Cten has been shown to localise DLC1, known as tumour suppressor, to integrin-mediated focal adhesions via its SH2 domain (Liao et al., 2007). Activation of DLC1 Rho-GAP activity was found to be an essential for the downregulation of ROCK signalling, which results in dissociation of focal adhesions, actin stress fibres, and cell rest/an immobile state (Cao et al., 2012). However, the Rho-GAP activity in DLC1 has been found to be downregulated by the binding of Cten, leading to an induction in focal adhesions turnover, actin stress fibre remodelling, and enhanced cell migration (Liao et al., 2007). Therefore, taken together, it was hypothesised that Cten may signal through the ROCK1 pathway to increase cell motility and consequently promotes CRC metastasis.

Src is a member of the membrane-associated non-receptor cytoplasmic tyrosine kinase family. The proto oncogene Src has been found to be overexpressed in many tumour tissue types including CRC and plays a critical role in tumour angiogenesis and metastatic progression (Byun et al., 2017, Chen et al., 2014). Src is induced by cytokines, receptors of growth hormone factors, and adipokines, and is regulated by a vast range of signalling pathways, such as STAT3 and MAPK. Src shows both feedforward and feedback regulation mechanisms that stimulates and inhibits its functional activity. For instance, upregulation of Src expression induces Csk-binding protein for feedback inhibition, whereas upregulation of FAK by Src results in a feedforward regulation (Jiang et al., 2006). Src and FAK have been shown to functionally and physically interact to promote a wide range of biological processes including cell migration and invasion (Bolós et al., 2010). It has been reported that Src mediates phosphorylation of FAK at Y576 and Y577 sites and that FAK upregulates Src expression by binding and interacting with the SH2 domain (Thomas et al., 1998). Others have also shown a similar interaction

between Src and integrin; Src induces the expression of integrin (Kline et al., 2009) and is also upregulated by integrin (Desgrosellier et al., 2009). The Src/FAK complex has also been found to bind and phosphorylate a vast range of adaptor proteins such as P130Cas, which in turn promote actin polarisation signalling and induce focal adhesion turnover during cell migration through the activation of the Rac/GEF (guanine nucleotide exchange factor) pathway. Further studies have reported that upregulation of Src decreases RhoA activity through the phosphorylation of p190RhoGAP, resulting in the relaxation of cytoskeleton tension for membrane extension (Lee et al., 2010, Arthur et al., 2000). Src signalling has also been reported to be implicated in the regulation of EMT. It has been shown that the suppression of EMT by c-Src inhibitor or siRNA knockdown lead to alterations in the expression of transcription factors, as shown by an upregulation of E-cadherin and downregulation of vimentin expression in metastatic breast carcinoma cells (Liu and Feng, 2010). Although Src has not yet been linked to Cten, it is involved in the regulation of other tensin proteins (Davis et al., 1991).

Since Cten and Src are involved in the regulation of EMT, such as E-cadherin, and both regulate FAK signalling, it was hypothesised that Cten may increase cell motility and promote metastatic CRC progression through upregulation of Src signalling.

4.2 Results

4.2.1 Cten Is a Positive Regulator of ROCK1, Src, and Snail Expression in CRC Cells

To investigate whether Cten positively regulates Snail, Src, or ROCK1, CRC cell lines, HCT116 and RKO, which express very low endogenous levels of Cten, were transfected with either GFP-EV control (expressing GFP) or GFP-Cten (expressing GFP-tagged Cten) expression constructs. Changes in protein level of Snail, Src, and ROCK1 was determined by western blot (figure 4-1). Overexpression of GFP-Cten in both HCT116 and RKO cell lines resulted in upregulation of Snail, Src, and ROCK1 expression compared to cells transfected with GFP-EV control. To show that this effect was not only in HCT116 and RKO cell lines, Cten was also forcibly expressed in the Cten knockout cell line, $SW620^{\Delta Cten}$. Consistent with this, Cten was shown to induce Snail, Src, and ROCK1 protein expression. To further confirm these results with another methodology, Cten was knocked down using Cten targeting siRNA duplexes in SW620 which express high endogenous levels of Cten and western blotting was again performed to determine changes in the expression of Snail, Src, and ROCK1. Knockdown of Cten was associated with a downregulation of Snail, Src, and ROCK1 protein expression. Finally, knockout of Cten using the CRISPR method in $SW620^{\Delta Cten}$ cells also resulted in the reduction of Snail, Src, and ROCK1 protein levels compared to the lysates of SW620 cells. Taken together, it appears that Cten positively regulates Snail, Src, and ROCK1 protein levels.

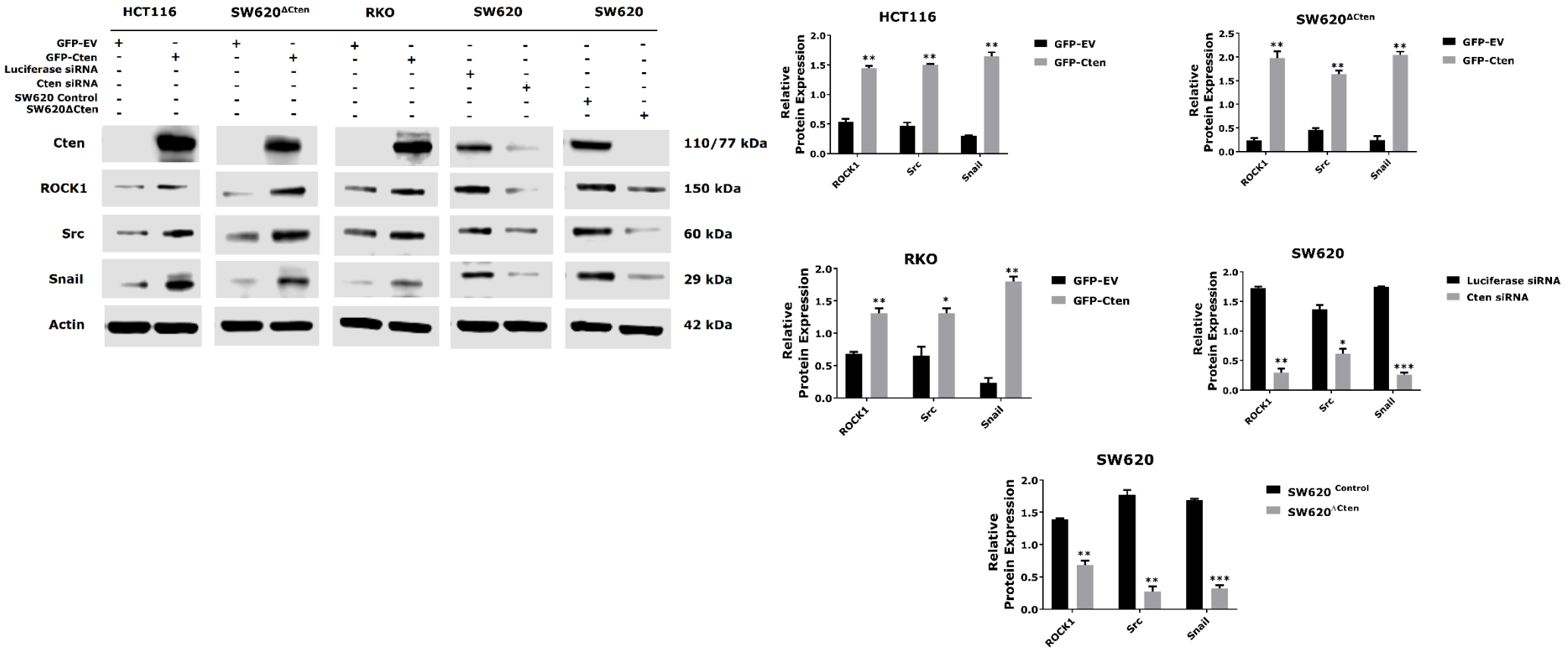

Figure 4–1: Cten regulates ROCK1, Src, and Snail protein expression.
Forced expression of Cten in HCT116, RKO, and SW620$^{\Delta Cten}$ was associated with an increase in ROCK1, Src, and Snail expression. Knockdown or knockout of Cten resulted in a decrease in ROCK1, Src, and Snail protein expression. Graphs on the right represent the densitometry values calculated for each protein band normalised to actin. Results are represented as the mean ±SEM of at least two independent experiments (= p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).*

4.2.2 Cten Promotes EMT Through Post-Transcriptional Stabilisation of Snail

4.2.2.1 Cten increases Snail protein stability

Having shown that Cten regulates Snail protein expression, further investigation of the mechanism of Snail expression upregulation by Cten signalling was next assessed using qRT-PCR to determine whether this was occurring at a transcriptional or post-transcriptional level. Cten was forcibly expressed in HCT116 cells and knocked down in SW620 cells. In both cell lines, there was no change in Snail mRNA level compared to the control, implying that Cten regulates Snail expression at a post-transcriptional only (figure 4-2 A, B).

Expression can be regulated post transcriptionally either by inducing protein synthesis or preventing protein degradation. To investigate this, a CHX Chase assay was performed to determine whether Cten stabilised Snail protein thereby preventing its degradation. GFP-Cten and empty vector (GFP-EV) control were transfected into HCT116 cells, then cells were treated with 100 µg/ml CHX to inhibit protein synthesis. Cells were analysed at different time points thus allowing the tracking of Snail protein degradation by western blot (figure 4-2 C, D). Snail protein had mostly been degraded at 1 hour in lysates of cells transfected with GFP-EV control, whereas Snail protein expression was still highly expressed 3 hours after treatment in cells transfected with GFP-Cten. However, it is possible that this is an artefact of visual analysis which simply reflects the different starting levels of Snail in GFP-Cten compared to Snail in GFP-EV control. To allow for this, the rate of protein decay was plotted. Densitometry was used to quantify the proteins and Snail levels were normalised to actin. The level at time 0 was set as 1 and the levels at the other time points were plotted as a ratio to time 0 levels. The slope of the curve represents the decay rate and was found to be much steeper (indicating faster decay) in the samples from GFP-EV control than were higher than in GFP-Cten. The data suggest that Cten increases Snail protein expression by stabilising the protein and preventing its degradation.

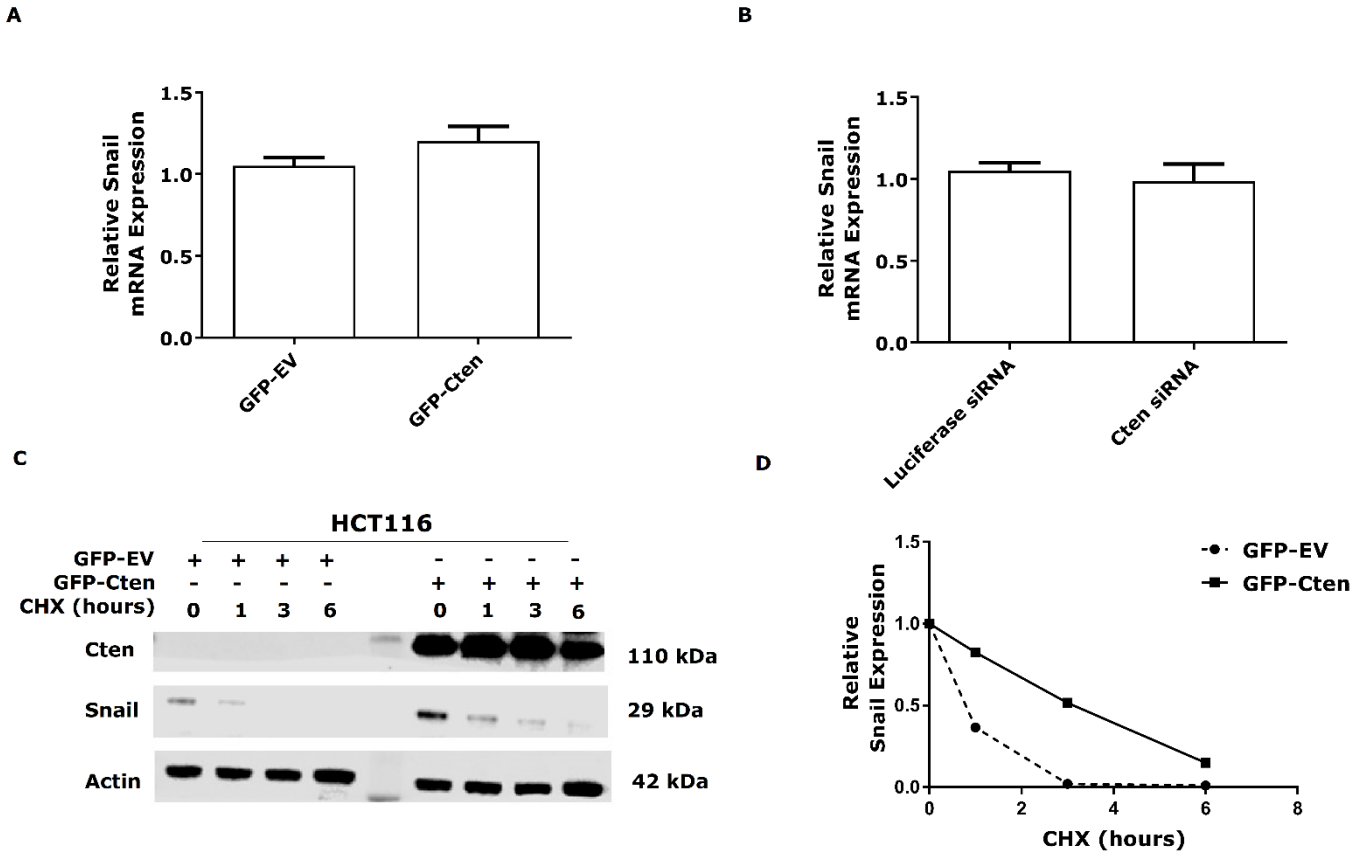

Figure 4–2: Cten stabilises Snail protein.
A) There was no change in Snail mRNA level following transfection of -GFP-Cten compared to GFP- empty vector control (EV) Control in HCT116 cells (P = 0.0645). B) Snail mRNA expression was unchanged following transfection of Cten targeting siRNA compared to luciferase siRNA control in SW620 cells (P = 0.3956) (unpaired t-test, n=3). C) Snail protein was stabilised for longer in GFP-Cten transfected cells compared to GFP-EV following the treatment of HCT116 cells with CHX (100 µg/ml) for 0-6 h. D) The Snail decay curve following treatment with CHX. The expression of Snail was normalised to actin and then normalised to the 0 h time point.

4.2.2.2 The regulation of Snail expression by Cten signalling is functionally relevant

Since Snail has been identified as a downstream target of Cten signalling and both Cten and Snail have been shown to play an important role in regulating cell migration and invasion, next it was of interest to investigate whether this interaction was functionally relevant. To test this hypothesis, GFP-Cten was overexpressed in HCT116 cells together with knockdown of Snail expression and the changes in cell function was assessed (figure 4-3). The capability of Cten to promote cell migration was evaluated using Transwell migration assays. Overexpression of GFP-Cten in HCT116 resulted in an increase in cell migration compared to those cells transfected with empty vector (GFP-EV) and luciferase siRNA control. However, this increase in cell migration was lost when Snail subsequently was knocked down. This suggests that Cten may signal through Snail to regulate cell migration. The ability of Cten to induce cell invasion using the Transwell invasion assay, which is similar method to Transwell migration assay, with the exception that cells must migrate through a layer of basement membrane extract was then investigated. Overexpression of GFP-Cten was associated with an increase in cell invasion compared to the GFP-EV and luciferase siRNA transfected cell control. However, this increase in the number of invading cells was significantly abrogated following Snail knock down, suggesting that Cten may directly regulate cell invasion via the Snail signalling pathway. The effect of Cten on cell tumourigenicity was next assessed using colony formation assay. Similarly, overexpression of GFP-Cten in HCT116 cells resulted in an increase in colony formation efficiency and this was reduced when GFP-Cten was overexpressed together with Snail siRNA. This implies that Cten may signal through the Snail pathway to regulate colony formation efficiency.

The functional association between Cten and Snail was further tested in an additional cell line using an alternative model (figure 4-4). The creation of $SW620^{\Delta Cten}$ as a cell line in which Cten has been deleted has been described. This was used as an alternative and the findings were in complete agreement with the findings in HCT116. Thus, forced

expression of GFP-Cten in SW620$^{\Delta Cten}$ was associated with induced cell migration, invasion, and colony formation efficiency and these cell functions were abrogated on the depletion of Snail expression. Taken together, the data suggests that Cten may regulate cell functions through the upregulation of Snail expression.

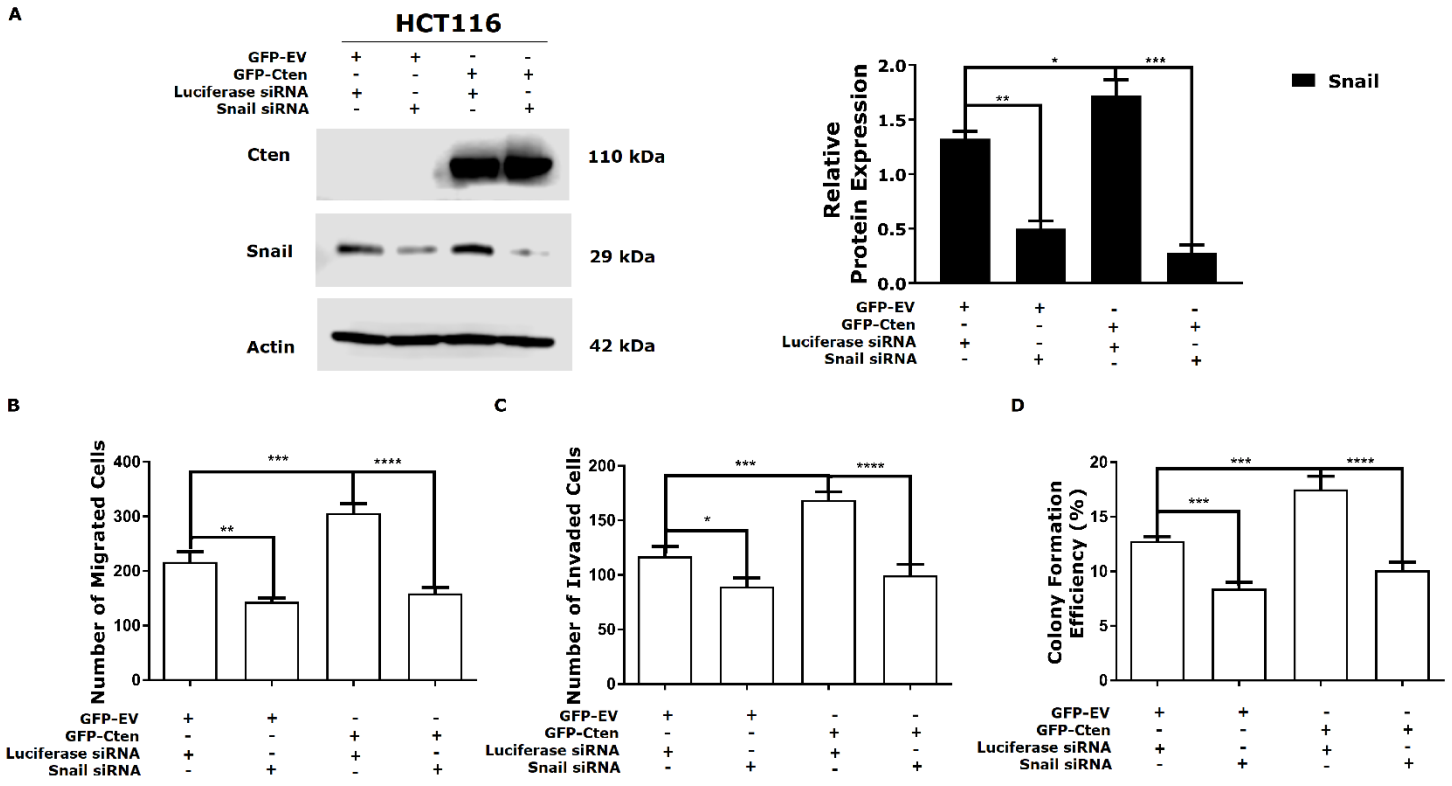

Figure 4–3: Cten signals through Snail to increase cell functional activity in HCT116 cells.
A) *HCT116 cells were co-transfected with GFP-EV or GFP-Cten together with either luciferase or Snail targeting siRNA and the changes in Snail protein expression were determined by western blot. Graph on the right represents the densitometry values calculated for Snail protein level normalised to actin (n=3). B) Overexpression of GFP-Cten increased cell migration in HCT116 (P=0.0003) which was lost with subsequent Snail knockdown (P ≤ 0.0001). C) Overexpression of GFP-Cten increased cell invasion in HCT116 (P= 0.004) which was lost on Snail knockdown (P ≤ 0.0001). D) Overexpression of GFP-Cten was associated with an increase in colony formation abilities in HCT116 (P= 0.0005) and this was decreased when Snail was subsequently knocked down (P ≤ 0.0001). Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).*

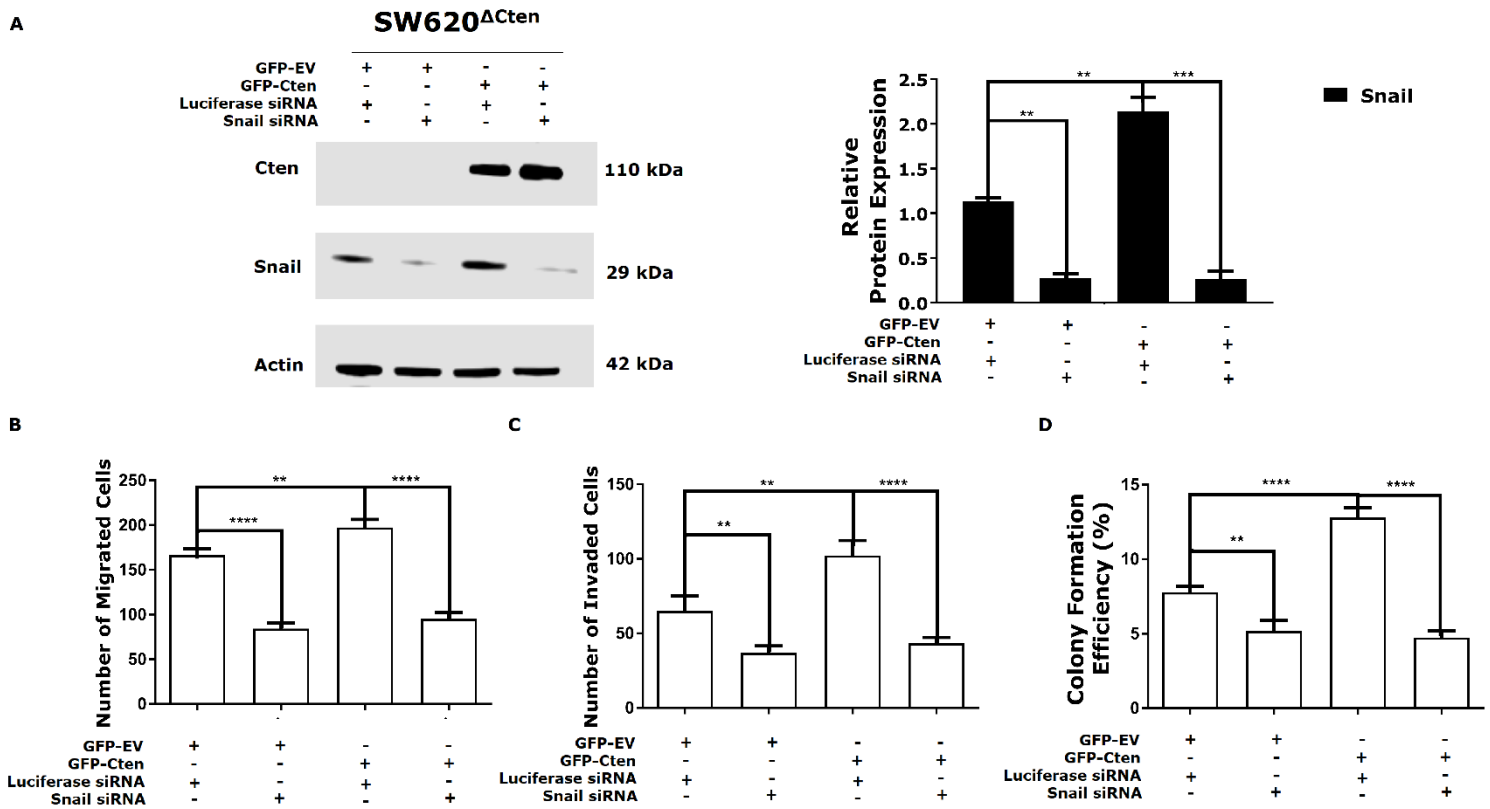

Figure 4–4: Cten signals through Snail to increase cell functional activity in SW620$^{\Delta Cten}$ cells.
A) *SW620$^{\Delta Cten}$ cells were co-transfected with GFP-EV or GFP-Cten together with either luciferase or Snail targeting siRNA and the changes in Snail protein expression were determined by western blot. Graph on the right represents the densitometry values calculated for Snail protein level normalised to actin (n=2). B) Overexpression of GFP-Cten increased cell migration in SW620$^{\Delta Cten}$ which (P= 0.0016) was lost with subsequent Snail knockdown (P ≤ 0.0001). C) Overexpression of GFP-Cten increased cell invasion in SW620$^{\Delta Cte}$ (P= 0.0012) which was lost on Snail knockdown (P ≤ 0.0001). D) Overexpression of GFP-Cten was associated with an increase in colony formation abilities in SW620$^{\Delta Cten}$ (P ≤ 0.0001) and this was decreased when Snail was subsequently knocked down (P ≤ 0.0001). Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).*

4.2.3 The Stabilisation of Src by Cten Signalling Promotes Metastatic CRC Progression

4.2.3.1 Cten stabilises Src protein expression

We have shown that Cten positively regulates Src expression. It is possible that Cten is regulating Src expression at the transcriptional or post-transcriptional level or both. To investigate this, Cten expression was manipulated using a dual approach of Cten forced expression in HCT116 and Cten knockdown in SW620 cells and qRT-PCR was performed to quantify changes in Src mRNA expression level. In both cell lines, there was no change in Src mRNA level compared to the control (figure 4-5 A, B). This suggests that Cten does not alter Src mRNA expression levels and that it may regulate Src expression at a post-transcriptional level. It is possible that Cten regulates Src expression post transcriptionally either by increased protein synthesis or blocked protein degradation. To interrogate this possibility, HCT116 cells following transfection of GFP-EV control and GFP-Cten were treated with 100 μg/ml of CHX, an inhibitor of protein synthesis, at different time points and Src protein degradation was then assessed by western blot (figure 4-5 C, D). The half-life of Src protein was ~6 h in the presence of GFP-EV control but was appreciably longer, ~12 h, when Src was expressed in the presence of GFP-Cten. Densitometry was used to quantify the proteins and Src levels were normalised to actin. The level at time 0 was set as 1 and the levels at the other time points were plotted as a ratio to time 0 levels. The Src decay curve was found to be much steeper (indicating faster decay) in the samples from GFP-EV control than the samples from GFP-Cten transfected cells following treatment with CHX. This indicates that Cten increases Src protein expression stability.

Cten has been shown to physically bind and interact with DLC1 through its SH2 domain. Cten has also been shown to physically bind and form a complex with β catenin in the nucleus. As Cten and its downstream targets, Snail, Src, and ROCK1 translocate between cytoplasm and nucleus, it was hypothesised that Cten could form a complex with these proteins and thereby block the degradation of Snail and Src proteins. To interrogate

protein binding interactions between Cten and these molecules, a co-immunoprecipitation experiment was performed in SW620 cells. Immunoprecipitation (IP) was performed using antibodies to Snail, Src, and ROCK1 and, as a positive control, Cten. The samples were then blotted with antibody to Cten. IP with Cten antibody pulled down Cten confirming that the system worked. However, IP with the other antibodies did not pull down Cten. This shows that Cten does not physically bind with its downstream targets, Snail, Src, and ROCK1 using this assay (figure 4-5 E). Together, these data indicate that Cten regulates Snail and Src stability, but as they do not form a physical complex, this is probably due to signalling downstream of Cten mediated by the SH2 domain.

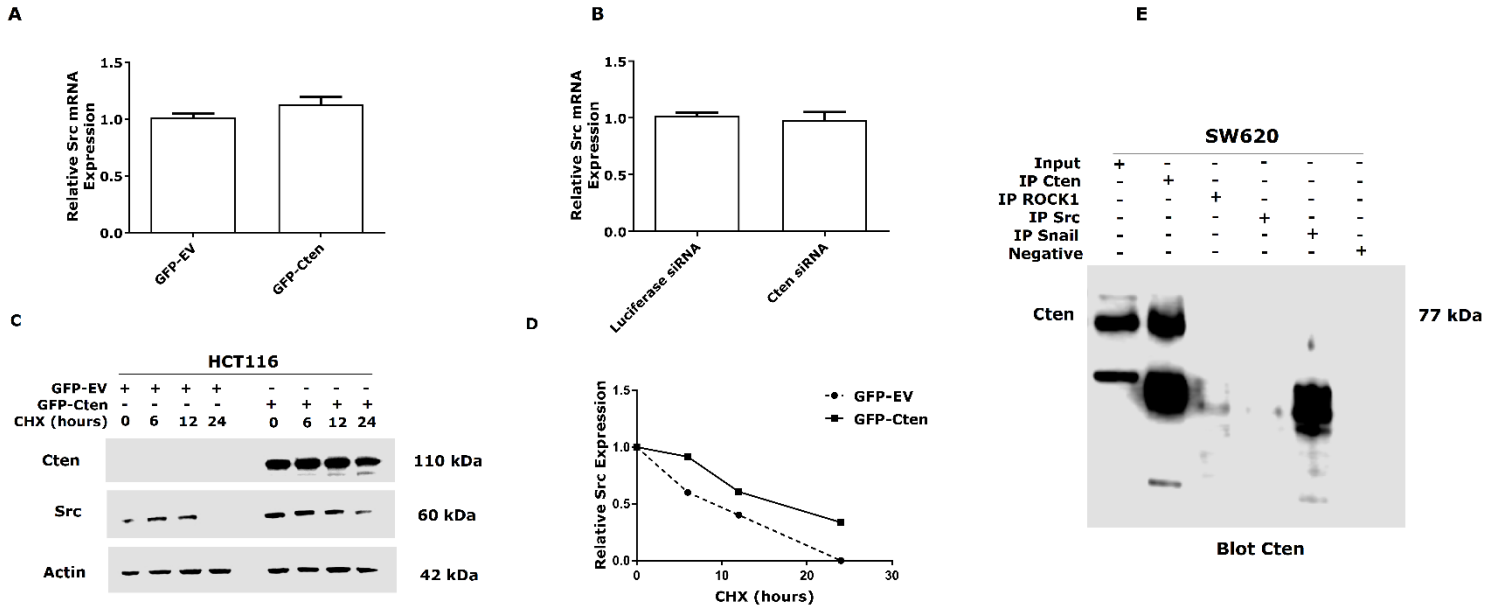

Figure 4–5: Cten increases Src protein stability.
A) Src mRNA expression level did not change following transfection of GFP-Cten in HCT116 cells compared to empty vector (EV) control transfected cells (P = 0.0589). B) The mRNA level of Src expression remined unchanged following transfection of Cten targeting siRNA in SW620 cells compared to luciferase siRNA control transfected cells (P = 0.4696) (unpaired t-test, n=4). C) Src protein was stabilised for much longer in HCT116 cells expressing GFP-Cten construct compared to GFP-EV control following treatment with CHX (100 µg/ml) for 0-24 h. D) The Src decay curve following treatment with CHX. Src protein expression was normalised to actin and then normalised to the 0 h time point. E) Endogenous full length Cten does not co-immunoprecipitate with either Src, ROCK1 or Snail. in SW620 cells.

4.2.3.2 Cten regulates Snail and ROCK1 through Src signalling

We have shown that Cten positively regulates Snail, Src, and ROCK1 protein expression. These are all linked to the EMT and thus we wondered whether there was any relationship between them or whether they were independent targets of Cten. Since Cten has an SH2 domain, it was hypothesised that Cten most likely mediates signalling through Src to regulate Snail and ROCK1 expression. To test this possibility, Cten was forcibly expressed in HCT116 and SW620$^{\Delta Cten}$ and Src was simultaneously knocked down. GFP-Cten and luciferase siRNA were co-transfected into HCT116 and overexpression of GFP-Cten was associated with an increase in the protein level of Src, Snail and ROCK1 expression compared to empty vector (GFP-EV) co-transfected with luciferase siRNA control. However, when GFP-Cten and Src siRNA were co-transfected (producing a condition of high Cten but low Src), this increase in Snail and ROCK protein expression was reduced suggesting that Cten may signal through Src to regulate both Snail and ROCK1 signalling (figure 4-6 A).

The Cten/Src/ROCK1/Snail signalling axis was further investigated in SW620ΔCten cells in exactly the same way. In agreement with the findings in HCT116 cells, overexpression of GFP-Cten was associated with an induction in Snail and ROCK1 protein expression. This was abrogated on the depletion of Src expression (figure 4-7 A). Taken together, the data implies that Cten may regulate Snail and ROCK1 signalling through the upregulation of Src expression.

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4.2.3.3 Cten may signal through Src/ROCK1/Snail pathway to increase cell function

Having shown that Src may be a signalling intermediate in a Cten/ROCK1/Snail pathway, we investigated whether Cten signals through the Src/ROCK1/Snail pathway to regulate cell function. To test this hypothesis, Src expression was knocked down using siRNA in HCT116 cells together with the forced expression of GFP-Cten and the changes in cell function were evaluated using several functional assays (figure 4-6).

The ability of Cten to regulate cell migration was evaluated using both wound healing and Transwell migration assays. Overexpression of GFP-Cten in HCT116 resulted in an increase in cell migration compared to the GFP-EV and luciferase siRNA control transfected cells. However, this increase in cell migration was appreciably lost when Src subsequently was knocked down, suggesting that Cten collaborates with Src to regulate cell migration through the ROCK1/Snail signalling axis.

The ability of Cten/Src signalling to regulate cell invasion using the Transwell invasion assay was next investigated. Similarly, overexpression of GFP-Cten was associated with an increase in cell invasion compared to those cells transfected with the GFP-EV and luciferase siRNA control. However, this induction in the number of invading cells was significantly reduced subsequent to Src knockdown, suggesting that Cten may also signal through the Src/ROCK1/Snail pathway to regulate cell invasion.

The capability of Cten/Src signalling on cell tumourigenicity was then investigated using a colony formation assay. The cell line HCT116 transfected with GFP-Cten resulted in an increase in colony formation efficiency and this was abrogated when GFP-Cten was forcibly expressed together with Src siRNA. This therefore suggests that Cten may signal through Src/ROCK1/Snail pathway to regulate colony formation efficiency.

The relationship between Cten and Src was further investigated in the SW620$^{\Delta Cten}$ cell line. Overexpression of GFP-Cten resulted in an induction of cell migration, invasion, and colony formation efficiency and these cell functions were abrogated on the depletion of Src expression (figure 4-7). Taken together, these findings imply that Src could be a signalling intermediate in the Cten/ROCK1/Snail pathway to regulate cell migration, invasion, and colony formation efficiency.

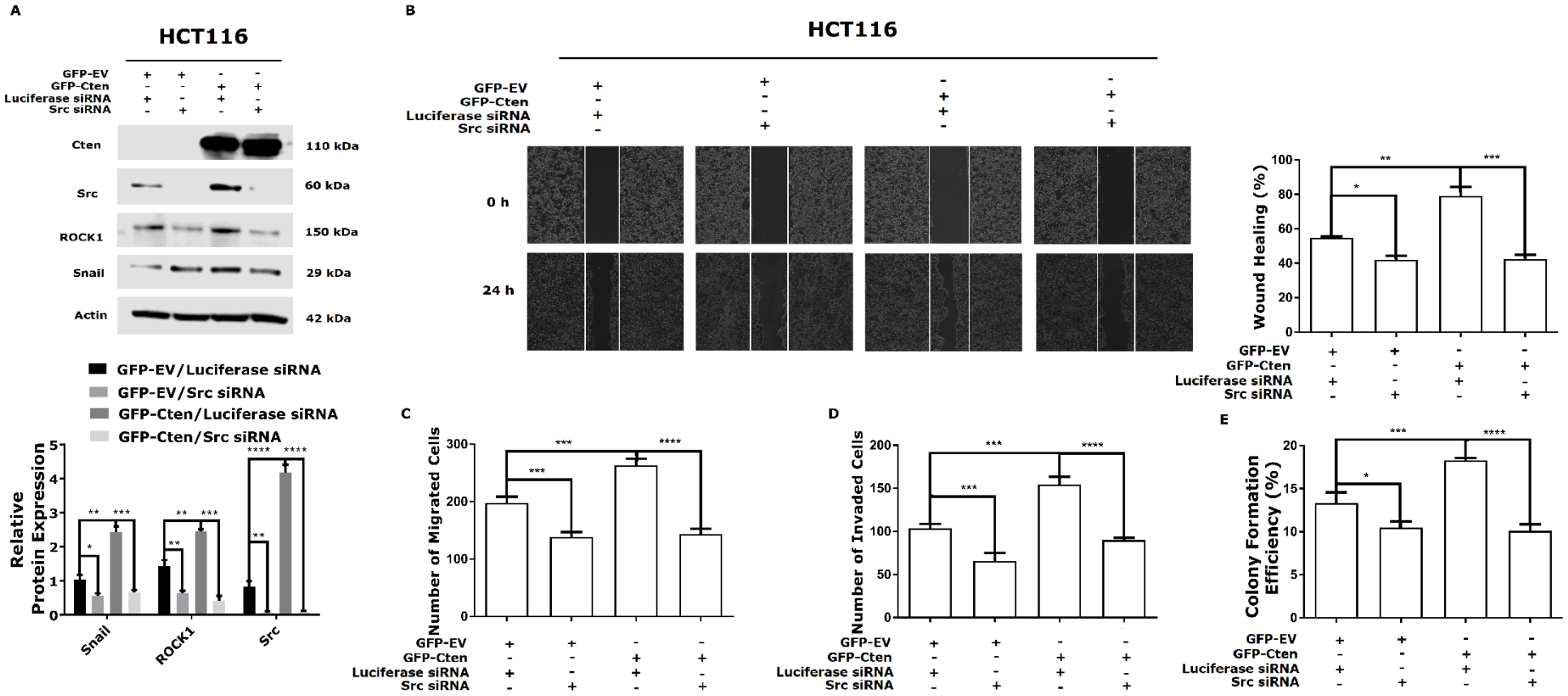


Figure 4–6: Cten regulates cell functions through Src signalling in HCT116 cells.
A) HCT116 cells were overexpressed with GFP-EV or GFP-Cten together with either luciferase or Src targeting siRNA and the changes in Src, ROCK1 and Snail protein expression were determined by western blot. Graph on the lower panel represents the densitometry values calculated for each protein band normalised to actin (n=3). B) Wound healing assay showed increased closure of wound following Cten forced expression (P = 0.0049) and this was inhibited when Src was subsequently knocked down (P = 0.0010). C) Overexpression of Cten was associated with an increase in cell migration (P = 0.0002) and this was reduced following Src knockdown (P ≤ 0.0001). D) Overexpression of Cten increased cell invasion (P = 0.0001) and this was lost with subsequent Src knockdown (P ≤ 0.0001). E) Overexpression of Cten induced colony formation efficiency (P = 0.0004) and this was lost on Src knockdown (P ≤ 0.0001). Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).

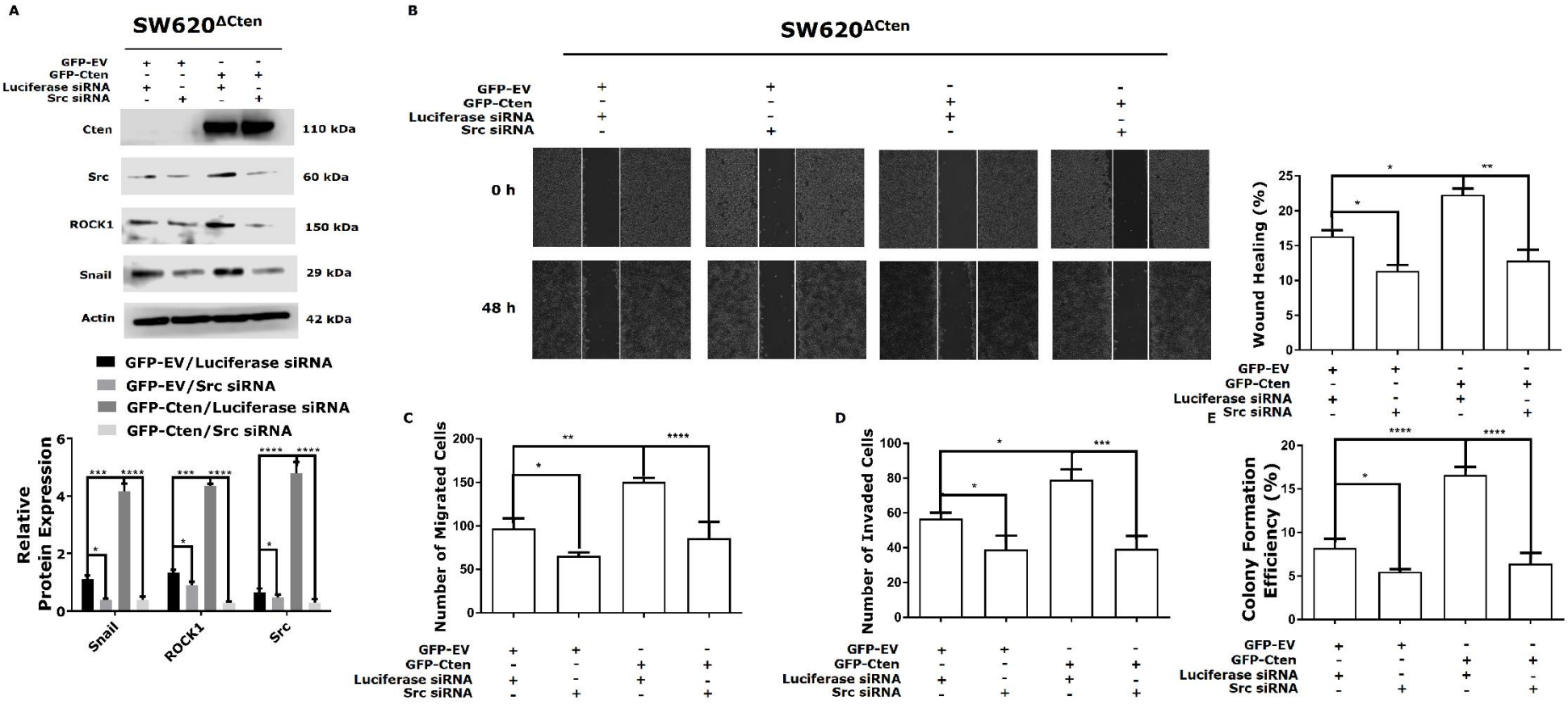


Figure 4–7: Cten regulates cell functions through Src signalling in SW620$^{\Delta Cten}$ cells.
A) SW620$^{\Delta Cten}$ cells were overexpressed with GFP-EV or GFP-Cten together with either luciferase or Src targeting siRNA and the changes in Src, ROCK1 and Snail protein expression were determined by western blot. Graph on the lower panel represents the densitometry values calculated for each protein band normalised to actin (n=3). B) Overexpression of Cten in SW620$^{\Delta Cten}$ increased closure of wound (P = 0.0182) which was lost with subsequent Src knockdown (P = 0.0033). C) Overexpression of Cten resulted in an induction of cell migration (P = 0.0021) and this was inhibited when Src subsequently knocked down (P = 0.0006). D) Overexpression of Cten increased cell invasion (P = 0.0124) and this was lost with subsequent Src knockdown (P = 0.0003). E) Overexpression of Cten was associated with an increase in colony formation efficiency (P ≤ 0.0001).and this was lost on Src knockdown (P ≤ 0.0001) Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).

4.2.4 Upregulation of Snail by Cten Promotes the Tumourigenicity of Colorectal Cancer Through ROCK1/Src Signalling Axis

4.2.4.1 Cten regulates Snail through ROCK1/Src axis

We have shown that Cten positively regulates ROCK1 expression and this may be achieved through Src signalling. Since biological pathways often have feedback loops, we wondered whether ROCK1 and Src can influence each other. To investigate this possibility, both HCT116 and SW620$^{\Delta Cten}$ cell lines underwent forced expression of Cten and simultaneous knockdown of ROCK1. GFP-Cten construct and ROCK1 siRNA knockdown to interrogate Src and Snail signalling. GFP-Cten was overexpressed in HCT116 and ROCK1 subsequently knocked down (figure 4-8 A). Overexpression of GFP-Cten and luciferase siRNA control resulted in an increase in Src, Snail and ROCK1 expression compared to the lysate of those cells co-transfected with empty vector (GFP-EV) and luciferase siRNA control. However, Overexpression of GFP-Cten and knockdown of ROCK1 (by simultaneous transfection of ROCK1 siRNA) resulted in a downregulation of both Src and Snail protein expression. The findings in HCT116 cells were also tested in the SW620$^{\Delta Cten}$ cell line (figure 4-9 A). Overexpression of GFP-Cten resulted in an increase in both Src and Snail protein expression and these increases were reduced with knockdown of ROCK1 signalling. Taken together, the data implies that upregulation of Snail by Cten is may be mediated through a ROCK1/Src pathway.

4.2.4.2 The regulation of cell functions by Cten signalling is probably through ROCK1/Src/Snail dependent pathways

Our data show that Cten upregulates Src, ROCK1 and Snail and that the regulation of Snail appears to be through Src and ROCK1. However, ROCK1 and Src also appear to have an effect on each other suggesting either a feedback loop or mutual stabilisation. Since all molecules are reported to play a role in cell migration, we next investigated whether the interaction between Cten and ROCK1 was functionally relevant. To test this hypothesis, GFP-Cten was overexpressed in HCT116 cells together with knockdown of ROCK1 expression and the changes in cell function assessed using several functional assays (figure 4-8).

The effect of Cten on cell migration was tested using both wound healing and Transwell migration assays. The cell line HCT116 was overexpressed using GFP-Cten construct and this was associated with an increase in cell migration, however, this increase was significantly reduced when ROCK1 was subsequently knocked down compared to the cells transfected with GFP-Cten and luciferase siRNA control. The effect was also investigated using the Transwell invasion assay through a layer of basement membrane extract (Matrigel) and the results were identical. This increases the possibility that ROCK1 may achieve this functional activity by intermediating a signalling in the Cten/Src/Snail pathway.

The ability of Cten to promote cell tumourigenicity was next tested using the colony formation assay. Similarly, overexpression of GFP-Cten in HCT116 cells was associated with an induction of colony formation efficiency and this was abrogated when GFP-Cten was overexpressed together with ROCK1 siRNA knockdown. This implies that Cten may signal through the ROCK1 pathway to regulate colony formation efficiency.

The functional relationship was further tested in SW620$^{\Delta Cten}$ cells by overexpressing GFP-Cten and knocking down ROCK1 together (figure 4-9). The overexpression of GFP-Cten and knockdown of ROCK1 produced similar levels of abrogation of cell migration, invasion, and colony formation efficiency compared to the cells co-transfected with GFP-Cten and luciferase siRNA control. Taken together, the findings in both cell lines suggest that the regulation of cell functions by Cten signalling could be via ROCK1/Src/Snail pathways.

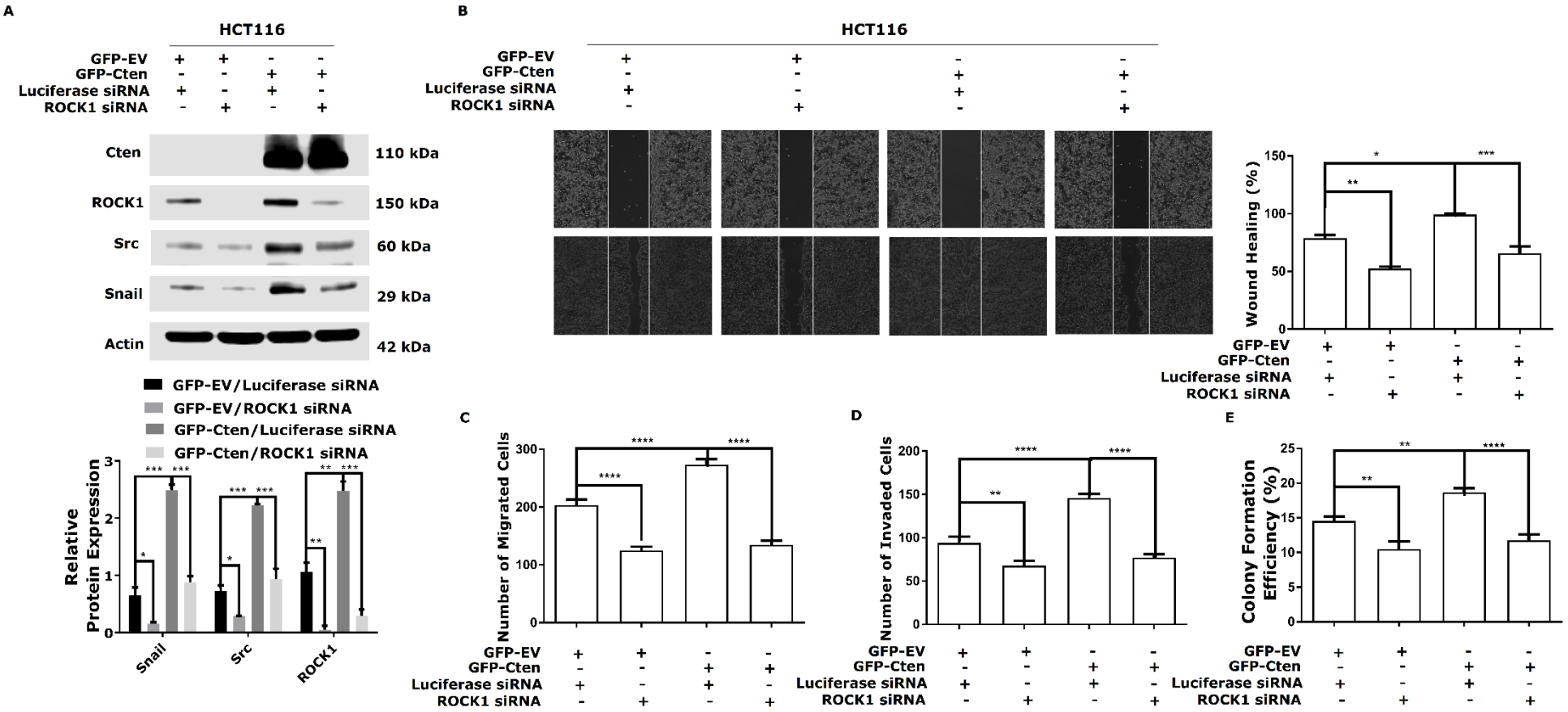


Figure 4–8: Cten promotes cell functional activity through ROCK1 signalling in HCT116 cells.
A) HCT116 cells were overexpressed with GFP-EV or GFP-Cten together with either luciferase or ROCK1 targeting siRNA and the changes in ROCK1, Src and Snail protein levels were determined by western blot. Graph on the lower panel represents the densitometry values calculated for each protein band normalised to actin (n=3). B) overexpression of Cten in HCT116 increased wound closure and induced cell migration (P = 0.0129) and this was significantly reduced when ROCK1 was subsequently knocked down (P = 0.0020). C) Overexpression of Cten in HCT116 resulted in an increase in cell migration (P ≤ 0.0001) which was lost on ROCK1 knockdown (P ≤ 0.0001). D) Overexpression of Cten increased cell invasion (P ≤ 0.0001) and this was lost following ROCK1 knockdown (P ≤ 0.0001). E) Overexpression of Cten in HCT116 increased colony formation efficiency (P = 0.0013) and this was reduced with subsequent ROCK1 knockdown (P ≤ 0.0001). Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).

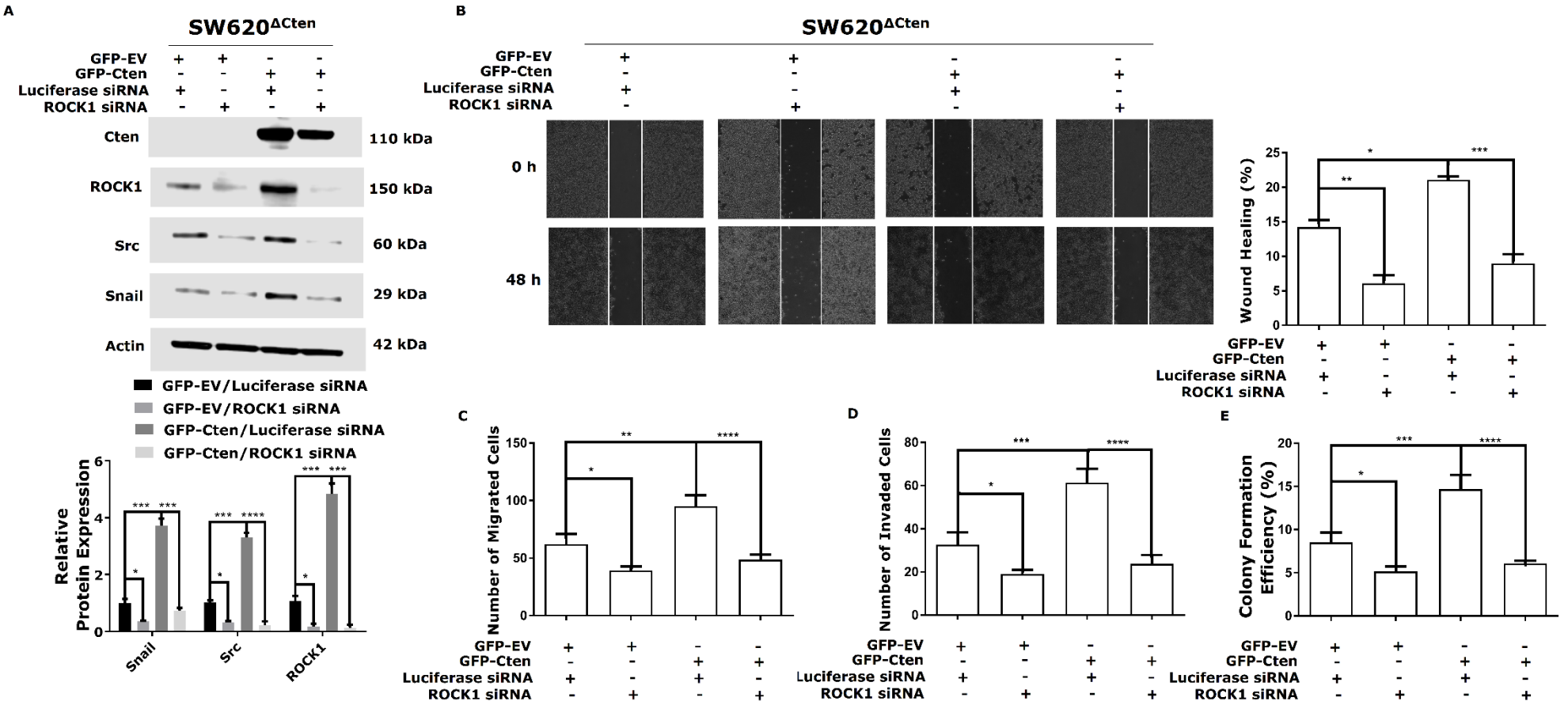


Figure 4–9: Cten promotes cell functional activity through ROCK1 signalling in SW620$^{\Delta Cten}$ cells.

A) SW620$^{\Delta Cten}$ cells were overexpressed with GFP-EV or GFP-Cten together with either luciferase or ROCK1 targeting siRNA and the changes in ROCK1, Src and Snail protein expression were determined by western blot. Graph on the lower panel represents the densitometry values calculated for each protein band normalised to actin (n=3). B) Overexpression of Cten in SW620$^{\Delta Cten}$ increased closure of wound (P = 0.0104) and this was decreased with subsequent ROCK1 knockdown (P = 0.0012). C) Overexpression of Cten was associated with an increase in cell migration (P = 0.0021) and this was significantly abrogated when ROCK1 was subsequently knocked down (P = 0.0002). D) Overexpression of Cten SW620$^{\Delta Cten}$ enhanced cell invasion (P = 0.0004) and this was lost with subsequent ROCK1 knockdown (P = 0.0005). E) Overexpression of Cten in SW620$^{\Delta Cten}$ increased colony formation efficiency (P ≤ 0.0001).and this was decreased on ROCK1 knockdown (P ≤ 0.0001). Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).

4.3 Discussion

Cten has been shown to increase cell motility and colony formation in most tumour tissues investigated, but the underlying signalling mechanisms that induce this functional activity remain unknown (Sasaki et al., 2003a, Sasaki et al., 2003b, Albasri et al., 2009, Albasri et al., 2011b, Al-Ghamdi et al., 2013). It would be of interest to interrogate how Cten regulates cell functions, since only a small number of Cten downstream targets have been identified so far. To reduce artefacts of methodology and cell line specific effects, Cten expression was modulated using multiple approaches including forced expression, gene knockdown, and gene deletion in different cell lines. The data revealed that any induced changes (raised or decreased expression) of Cten were followed by similar changes in expression of Snail, Src and ROCK1 protein. In this chapter, Snail, Src, and ROCK1 have been identified as novel downstream targets of Cten and signalling through a ROCK1/Src/Snail pathway is responsible for the induction of cell motility and colony formation mediated by Cten.

Cten did not induce any changes in the mRNA level of Snail following modulation of Cten expression, but the lysates of those cells treated with CHX Chase clearly revealed delayed degradation of Snail protein in the presence of Cten. These data suggest that Cten regulates Snail expression through post-transcriptional mechanisms and probably achieved this due to the stabilisation of Snail protein expression. Although, the mechanisms underlying the stabilisation of Snail protein is unclear, Snail is a transcription factor often regulated at the protein level and requires tight regulation of its transcriptional activity to ensure signals can be promptly switched off upon external stimuli. Snail nuclear localisation is important for the transcriptional activity of its downstream targets. The phosphorylation of Snail regulates its export from the nucleus and following degradation through the ubiquitin proteasome pathway (Domínguez et al., 2003). It is possible that Cten prevents the degradation of Snail by regulating this process. Therefore, further investigations are required to address how Cten is promoting the stabilisation of the Snail protein.

Previous studies have shown that Cten promotes cell motility and colony formation in tumour cells through the regulation of EMT processes (Albasri et al., 2009). This chapter has shown for the first time that Cten may achieve these properties through the positive regulation of the master marker of EMT, Snail. This is further evidence that Cten is involved in the regulation of EMT processes and probably through the Snail signalling pathway. However, downregulation of E-cadherin, an epithelial marker, is achieved by the upregulation of the mesenchymal marker N-cadherin, but this was not observed following the modulation of Cten expression in CRC cells (Thorpe et al., 2017). Snail regulates the expression of several other target genes implicated in the regulation of EMT process, such as occludin and claudins, in addition to genes involved in other cellular events which could also be downstream targets of Cten (Ikenouchi et al., 2003). Snail is also involved in the regulation of several stem cell pathways which are associated with EMT pathways (Fan et al., 2012, Hwang et al., 2014). Cten expression so far has not been associated with the regulation of stem cell pathways, but should be explored.

Since Src is one of the important kinases in focal adhesion dynamics, it was of interest to investigate the mechanistic details of the Cten/Src pathway. In addition to the stabilisation of Snail protein by Cten, it was also shown that upregulation of Src by Cten signalling is through post-transcriptional mechanisms and most likely due to Src protein stability. The Src mRNA levels were unaffected following the manipulation of Cten expression in both CRC cell lines, HCT116 and SW620. However, CHX Chase experiments revealed that Src protein in the presence of Cten exhibits a longer half-life than the lysates of those cells transfected with empty vector control. Thus, upregulation of Src by Cten prevents Src from degradation at a post-transcriptional level. This suggests that Cten may promote Snail protein stability through Src signalling. However, the precise mechanisms by which this stabilisation occurs remains unclear. Co-immunoprecipitation experiments revealed that Cten was unable to form a physical complex with any of its downstream targets, Snail, Src, and ROCK1, therefore an alternative explanation must

be sought. Src protein has previously been shown to be degraded through a proteasome-ubiquitin dependent pathway (Hakak and Martin, 1999). It is possible that Cten inhibits ubiquitin mediated degradation through either preventing ubiquitination or preventing proteasomal degradation. Both of these scenarios would seem unlikely as this would have effects on a wide range of proteins in addition to Src. Others have shown that Src protein synthesis is promoted by ErbB2 through the activation of Akt/mTOR/4E-BP1 protein translation pathways and stabilised by ErbB2 through the inhibition of calpain-mediated Src protein degradation (Tan et al., 2005). Cten has been shown to be induced by ErbB2 signalling (Katz et al., 2007) and it is possible that Cten could protects Src protein from degradation by regulating ErbB2 pathway or its downstream targets. Therefore, it would be of interest to investigate whether Cten signalling is involved in this process.

Both Cten and Src have been shown to supresses the transcription of E-cadherin, most probably through upregulation of the transcriptional repressor Snail (Guo and Giancotti, 2004, Thorpe et al., 2017, Nagathihalli and Merchant, 2012). Additionally, both Cten and Src have shown to regulate the non-receptor focal adhesion kinase FAK (Al-Ghamdi et al., 2013, Carragher et al., 2003). The Src dependent activation of FAK has shown to promote cell function to induce EMT whilst having no effect on the mRNA level of Snail (Cicchini et al., 2008). Since the regulation of Snail expression by both Cten and Src was at a post-transcriptional level only, it is possible that Cten regulates Snail expression through Src signalling. Interestingly, this report also showed that Cten could regulate ROCK through the upregulation of Src signalling pathway. It has previously been shown that ROCK is phosphorylated by Src in focal adhesion complexes and this phosphorylation was associated with an abrogation of RhoA binding activity of ROCK, which subsequently leads to continued myosin-mediated contractility and focal adhesion elongation during the stimulation of lysophosphatidic acid (Lee et al., 2010). The functional relationship between Cten and Src was also investigated in this study by overexpressing Cten and knocking down Src at the simultaneously. The overexpression

of Cten and knockdown of Src produced similar levels of abrogation of cell migration, invasion, and colony formation efficiency. These findings confirm that the induction of Src by Cten is not just a bystander phenomenon. Previous studies have shown that Cten promotes cell motility through the upregulation of FAK and ILK and both proteins are now known to play a role in the regulation of EMT processes (Albasri et al., 2011a, Al-Ghamdi et al., 2013). It would be of interest to determine whether FAK or ILK are signalling intermediates in the Cten/Src pathway, since both Src and FAK bind and form a physical complex at focal adhesions (Bolós et al., 2010).

ROCK1 has been shown to regulate focal adhesion turnover and actin stress fibre remodelling through the phosphorylation of FAK (Lock et al., 2012). ROCK1 has also been found to regulate the Snail pathway through post-transcriptional mechanisms (Zhang et al., 2016). This study revealed for the first time that ROCK1 is a potential downstream target of Cten and may be regulated by Cten through the Src pathway. However, Cten was shown to apparently regulate both Src and Snail protein expression through the ROCK1 signalling pathway. Further examination of our data showed that, in the conditions where empty vector control was co-transfected with siRNA targeted to either Src or ROCK1, each had an effect on the other. Thus, knockdown of Src resulted in a reduction of ROCK1 and knockdown of ROCK1 resulted in Src. The two proteins seem to mutually stabilise each other but, as with Src, ROCK1 was also found to be important in the functional activity of Cten. Overexpression of Cten and knocking down ROCK1 simultaneously created a situation where Cten was present, but there was an absence of ROCK1, resulting in an inhibition of cell migration, invasion and colony formation efficiency.

These findings suggest that Cten dependent upregulation of ROCK1/Src/Snail signalling pathway participates in the induction of cell motility and colony formation, consequently promoting CRC metastasis. The relationship between Src and ROCK1 remains of uncertain nature and significance. They could from a linear pathway with some degree of mutual interaction or some feedback loops or they could form independent signalling

pathways. Our lab has previously found that ILK and FAK are both downstream targets of Cten but are also mutually stabilising.

Despite several studies that have investigated the role of Cten in cancer metastasis, most of these studies have been performed *in vitro*. Further validation of these studies in animal models is required to confirm that these effects also occur *in vivo*. In conclusion, these results have revealed novel downstream targets of Cten in CRC which promote cell motility and colony formation. Elucidation of the underlying signalling mechanisms that regulate cell functions may help to identify novel biomarkers for therapeutic targeting of cancer metastasis.

5 The Role of SH2 Domain in Cten Function

5.1 Introduction

In all tensin proteins including Cten, the SH2 domain is located very close to the PTB domain at the C-terminal region (Lo, 2004). The SH2 domain of these family members is approximately 100 amino acids in length. The tensin SH2 domain is a distinct tertiary structure existing within several intracellular proteins to recognise and bind docking sites in other proteins that contain the phosphotyrosine amino acid residues, forming a physical complex between these two proteins (Liu et al., 2006). However, more recently, it has been found that some SH2 domains can bind and interact with other proteins in a phosphotyrosine independent manner (Dai et al., 2011).

Tyrosine phosphorylation is the first step in signal transduction and is critical for protein to protein interactions at focal adhesion complexes (Ullrich and Schlessinger, 1990, Wozniak et al., 2004). SH2 domains of all four tensin proteins are involved in signal transduction events via tyrosine phosphorylation, which has previously been shown to be important for its functional activity (Davis et al., 1991, Hong et al., 2013). The tensin SH2 domain is found to be bound with phosphotyrosine related proteins including FAK, ILK and P130Cas, which are involved in focal adhesion (Wavreille and Pei, 2007). Cten was found to bind and interact with DLC1 through its SH2 domain. Mutations in the Cten SH2 domain result in failure to recruit DLC1 to focal adhesion complexes and induces cell migration (Liao et al., 2007). The SH2 domain of tensin proteins has been shown to be involved in inducing cell motility and mutations in this domain inhibit cell migration induced by these members in canine kidney cancer cells (Huaiyang and LO, 2003).

Previous data from this lab have revealed that Cten signals through the SH2 domain to increase cell motility and colony formation (submitted for publication). Arginine mutated to alanine at the critical position, 474 in the SH2 domain of Cten, was found to be very important for its functional activity. It was seen that Cten's ability to induce cell motility and colony formation was significantly decreased. However, this Cten mutant ($Cten^{R474A}$)

construct did not cause a complete loss in the induction of cell motility and colony formation compared to the GFP empty vector control. Furthermore, Cten was shown to be tyrosine phosphorylated and tyrosine mutated to phenylalanine at position 479 in Cten SH2 domain was found to be important for its functional activity.

As both mutations are important for functioning of the Cten SH2 domain but do not individually cause complete reverse of its effect on cell motility and colony formation, it was hypothesised that a combination of both mutations in one construct will completely abolish Cten's ability to induce cell motility and colony formation.

5.2 Results

5.2.1 Generation of Cten$^{R474A+Y479F}$ Construct Using Site Directed Mutagenesis

The generation of the Cten$^{R474A+Y479F}$ construct was achieved using a site directed mutagenesis kit. The primers were designed to have both mutations, R474A and Y479F (shown in red colour), in the middle of the forward primer (5'P gtc ata gcg gac agc tct tca ttc cga ggc 3'). The annealing temperature was optimised using gradient PCR and 66°C was chosen (figure 5-1). To use the lowest template concentration as recommended by the kit, template concentrations were optimised and 250 pg was found to be optimal (figure 5-2). A 50 µl reaction volume was run with 250 pg DNA and 10 µl of PCR products were run on a 1% agarose gel and bands visualised using a blue light transilluminator instead of UV light to avoid damage to the DNA in the PCR product. Agarose gel electrophoresis showed bands corresponding to 7,000 bp as predicted for the amplification PCR product.

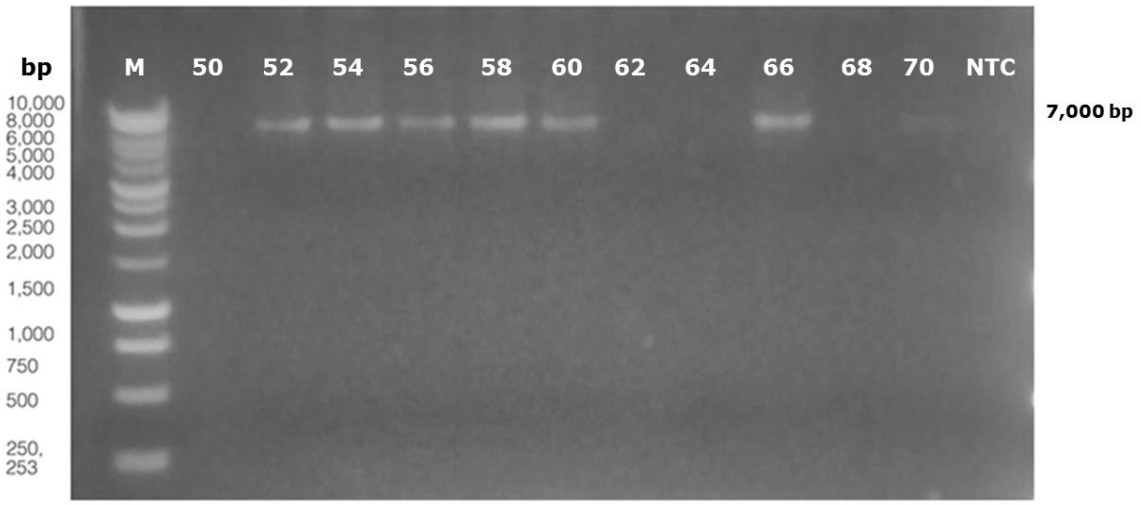


Figure 5–1: Annealing temperature optimisation.
Gradient PCR products for the Cten$^{R474A+Y479F}$ site directed mutagenesis primers were run on a 1% agarose gel and 66°C was chosen as the optimal temperature. No template control (NTC).

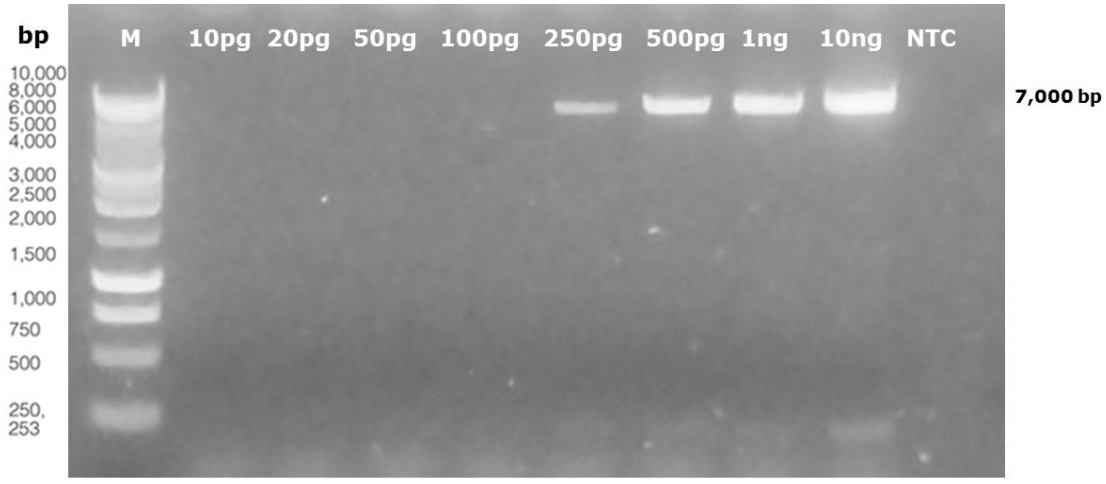


Figure 5–2: Agarose gel of template concentration optimisation.
PCR products of a varied template concentration ranging from 10 pg to 10 ng were run on a 1% agarose gel and 250 pg was selected as the optimal concentration. *No template control (NTC).*

5.2.2 Sequencing of the Cten$^{R474A+Y479F}$ Construct

The sequencing of the Cten$^{R474A+Y479F}$ construct was performed to ensure that both mutations had successfully induced in the wild type Cten plasmid. To confirm this, the DNA band was excised from the gel and ligated, then inserted into a vector by TA cloning and amplified in a bacterial host as previously described. Five colonies were sequenced and subjected to BLAST analysis (NCBI) to identify both mutations created in the DNA sequence between the Cten$^{R474A+Y479F}$ construct sequence and wild type Cten construct sequence (figure 5-3).

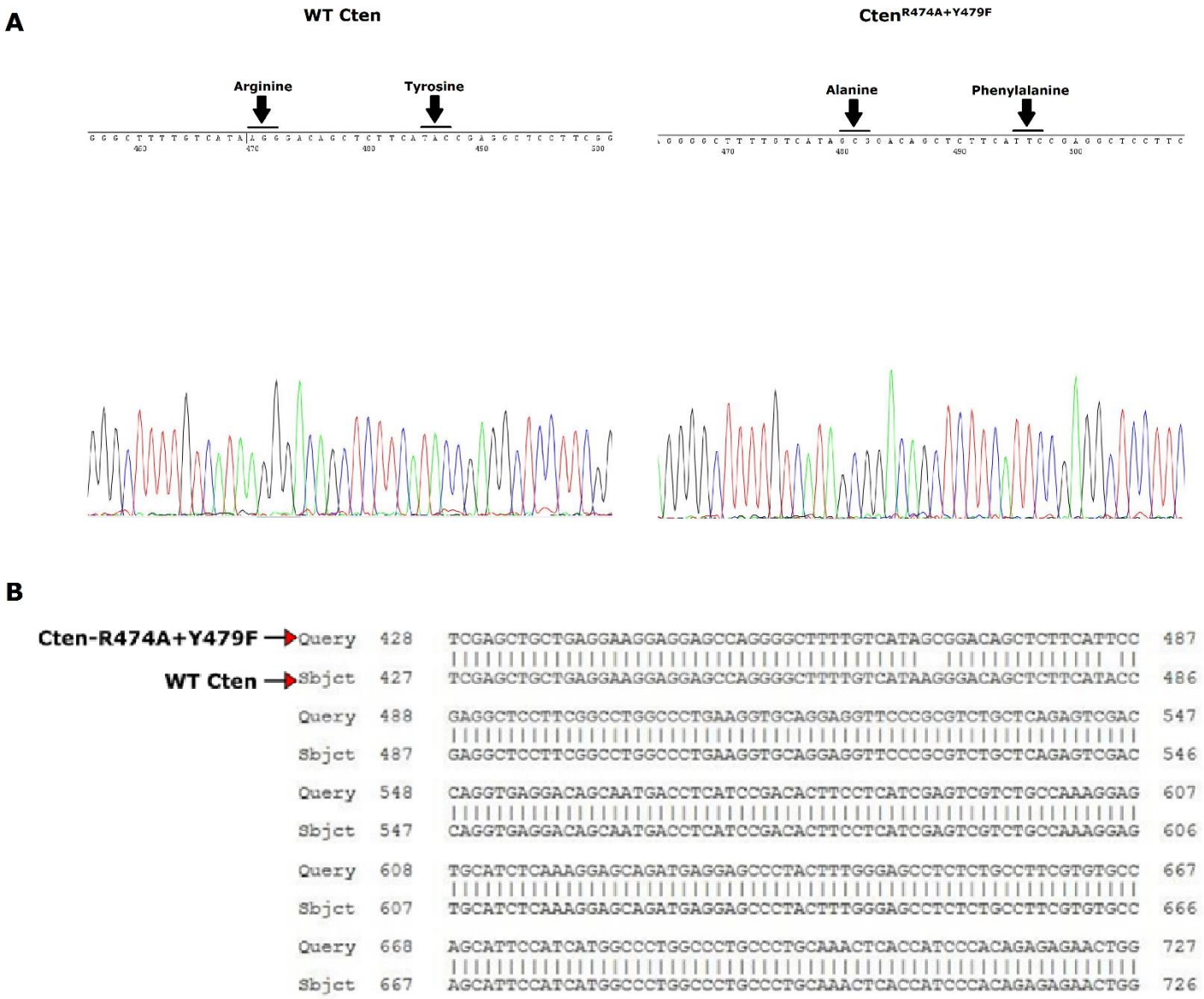


Figure 5–3: Sequencing of the Cten wild type and Cten$^{R474A+Y479F}$ plasmids.
A) Sequencing revealed that the final construct mutated from wild type Cten to R474A+Y479F (arginine to alanine and tyrosine to phenylalanine). B) BLAST analysis revealed that both mutations have successfully been created in the wild type Cten plasmid.

5.2.3 Cten$^{R474A+Y479F}$ Mutant Reduces Cell Motility and Colony Formation but Does Not Affect Cell Proliferation

Having shown that Cten regulates Src, ROCK1, and Snail through post-transcriptional mechanisms, next it was investigated whether the SH2 domain of Cten was required for the upregulation of Src/ROCK1/Snail dependent pathway to promote cell functions. To explore this, Cten was forcibly expressed using the GFP-Cten$^{R474A+Y479F}$ mutant, as well as the two premade constructs given by Dr Maham Akhlaq, GFP-Cten-^{R474A} (figure 10-1) and GFP-Cten^{Y479F} (figure 10-2), together Cten, and the GFP-empty vector as control in both HCT116 and SW620$^{\Delta Cten}$ CRC cell lines. Changes in protein expression were assessed by western blot (figure 5-4 A), (figure 5-5 A). All constructs including the wt. GFP-Cten, GFP-Cten^{R474A}, GFP-Cten^{Y479F}, and GFP-Cten$^{R474A+Y479F}$ were expressed to a similar level, which allowed for comparison of Cten's downstream targets, Src, ROCK1, and Snail protein expression between the different transfection conditions. Overexpression of the GFP-Cten construct led to an increase in the expression levels of ROCK1, Src, and Snail protein. Overexpression of GFP-Cten^{R474A} and GFP-Cten^{Y479F} constructs also led to an increase in ROCK1, Src, and Snail protein expression compared to the GFP-EV control, but this was less than the induction associated with the GFP-Cten construct. Overexpression of GFP-Cten$^{R474A+Y479F}$ decreased expression levels of ROCK1, Src, and Snail proteins compared to the wt, Cten and both mutant constructs separately. This indicates that both of these amino acid residues in the SH2 domain of Cten could be important for the upregulation of Src/ROCK1/Snail pathway.

Previous data from this lab have shown that both GFP-Cten^{R474A} (arginine to alanine) and GFP-Cten^{Y479F} (tyrosine to phenylalanine) mutations separately are important for functional activity of Cten SH2 domain. but do not cause complete abrogation of its effect on cell motility and colony formation (submitted for publication). Therefore, it was hypothesised that the combination of both mutations in one construct will completely abolish Cten' ability, subsequently reducing cell motility and colony formation. Both cell lines, HCT116 and SW620$^{\Delta Cten}$, were transfected with GFP-Cten-^{R474A}, GFP-Cten^{Y479F}, and

GFP-Cten$^{R474A+Y479F}$ constructs together GFP-Cten, and the GFP-empty vector control and the functional assays were performed (figure 5-4), (figure 5-5). The Transwell migration and wound healing assays showed that the GFP-Cten$^{R474A+Y479F}$ construct significantly decreased Cten's ability to induce cell migration compared to both GFP-Cten^{R474A} and GFP-Cten^{Y479F} constructs as well as the wt. GFP-Cten. Furthermore, in both cell lines, cell invasion was reduced when cells were transfected with the GFP-Cten$^{R474A+Y479F}$ construct compared to the wt. Cten as well as both GFP-Cten^{R474A} and GFP-Cten^{Y479F} constructs

The PrestoBlue assay was also performed to assess the effect of the wt. Cten and other mutant constructs on cell proliferation. This assay provides a measure of cell metabolic activity, thus provides an indirect measure of cell viability. In both cell lines, HCT116 and SW620$^{\Delta Cten}$, there was no significant difference in cell proliferation between the empty vector control construct and wt. GFP-Cten, GFP-Cten^{R474A}, GFP-Cten^{Y479F}, and GFP-Cten$^{R474A+Y479F}$ constructs transfected cells at 72 hours.

A three-dimensional colony formation assay was performed to assess the ability of the transformed cells to grow independently of a solid surface (anchorage independent growth) and it may be a vital feature in the generation of metastasis deposits. The ability of the Cten $^{R474A+Y479F}$ mutant to reduce colony formation in soft agar in CRC cell lines, HCT116 and SW620$^{\Delta Cten}$ was evaluated after 21 days of growth. The GFP-Cten$^{R474A+Y479F}$ construct transfected HCT116 cells showed a lower number of colonies than GFP-Cten-^{R474A}, GFP-Cten^{Y479F}, and wt. GFP-Cten transfected cells and similar colony formation efficiency with the empty vector control transfected cells. This was also validated in SW620$^{\Delta Cten}$ cells, which showed a similar colony formation efficiency pattern. This suggests that the combination of the two mutations, R474A and Y479F, in one construct would be important for the ability of Cten SH2 domain to increase colony formation efficiency compared to both mutation constructs and the wt. Cten.

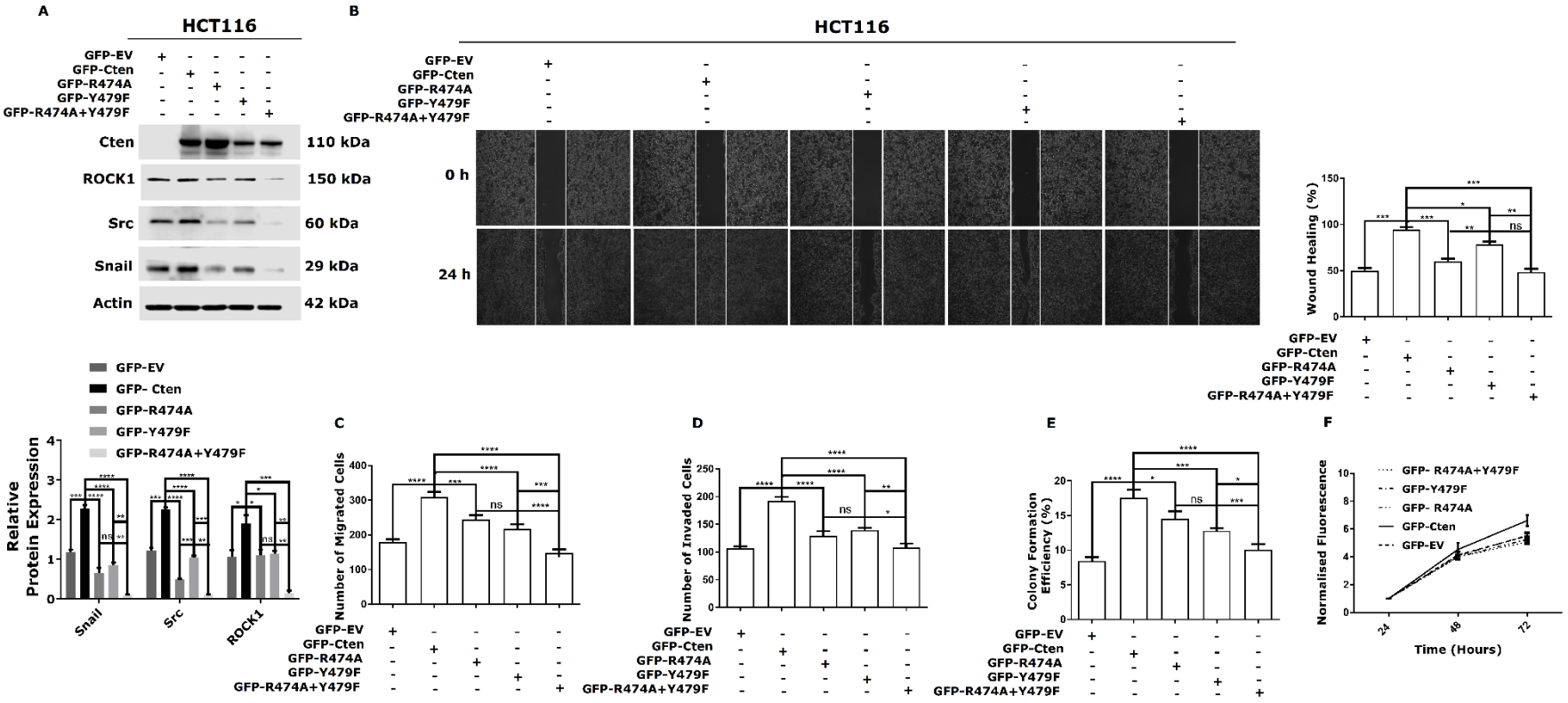


Figure 5–4: Cten regulates cell functional activity through SH2 domain in HCT116 cells.

A) Overexpression of GFP-Cten was associated with an increase in ROCK1, Src, and Snail protein expression and this was abrogated when HCT116 cells was overexpressed with mutant GFP-Cten^{R474A}, GFP-Cten^{Y479F}, and GFP-Cten$^{R474A+Y479F.}$ B) Overexpression of GFP-Cten in HCT116 increased wound closure and induced cell migration (P = 0.0001) and this was significantly reduced when cells were overexpressed with mutant GFP-Cten$^{R474A+Y479F}$ (P = 0.0001). C) Overexpression of GFP-Cten in HCT116 resulted in an increase in cell migration (P ≤ 0.0001) which was lost on mutant GFP-Cten$^{R474A+Y479F}$ construct (P ≤ 0.0001). D) Overexpression of GFP-Cten increased cell invasion (P ≤ 0.0001) and this was lost following GFP-Cten$^{R474A+Y479F}$ overexpression (P ≤ 0.0001). E) Overexpression of Cten in HCT116 increased colony formation efficiency (P ≤ 0.0001) and this induction was reduced with mutant GFP-Cten$^{R474A+Y479F}$ construct (P ≤ 0.0001). F) Cell viability assay showed that there was no significant difference between GFP-Cten construct and GFP-Cten^{R474A}, (P = 0.4886), GFP-Cten^{Y479F} (P = 0.4800), GFP-Cten$^{R474A+Y479F}$ (P = 0.5123), and GFP-EV control constructs (P = 0.5104). Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).

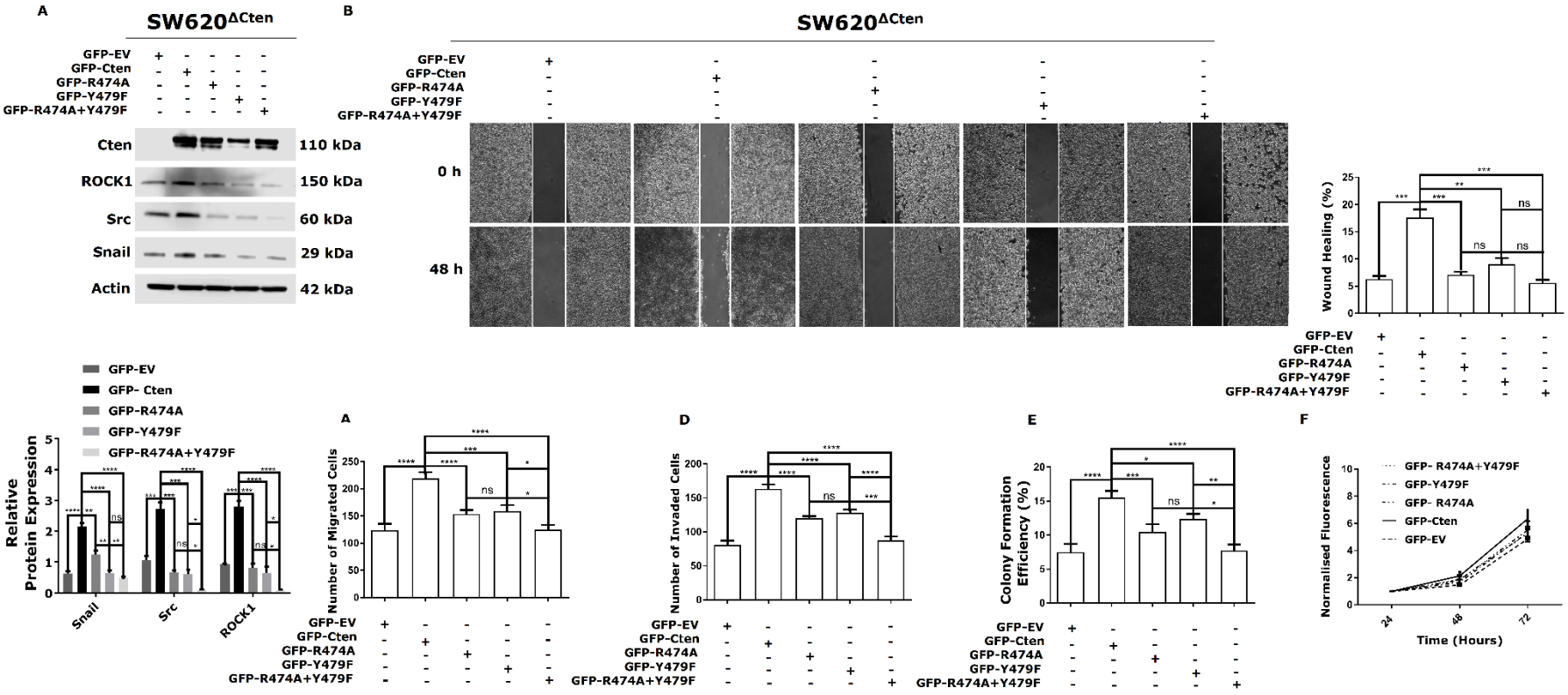


Figure 5–5: Cten regulates cell functional activity through SH2 domain in SW620$^{\Delta Cten}$ cells.

A) Overexpression of Cten was associated with an increase in ROCK1, Src, and Snail protein expression and this was abrogated when SW620$^{\Delta Cten}$ cells was overexpressed with mutant GFP-Cten^{R474A}, GFP-Cten^{Y479F}, and GFP-Cten$^{R474A+Y479F}$. B) Overexpression of GFP-Cten in SW620$^{\Delta Cten}$ increased closure of wound (P = 0.0004) and this was decreased with mutant GFP-Cten$^{R474A+Y479F}$ construct (P = 0.0003). C) Overexpression of GFP-Cten resulted in an increase in cell migration (P ≤ 0.0001) and this was significantly abrogated when cells were subsequently overexpressed with GFP-Cten$^{R474A+Y479F}$ construct (P ≤ 0.0001). D) Overexpression of GFP-Cten SW620$^{\Delta Cten}$ enhanced cell invasion (P ≤ 0.0001) and this was lost with mutant GFP-Cten$^{R474A+Y479F}$ construct (P ≤ 0.0001). E) Overexpression of GFP-Cten in SW620$^{\Delta Cten}$ induced colony formation efficiency (P ≤ 0.0001).and this was decreased on mutant GFP-Cten$^{R474A+Y479F}$ construct (P ≤ 0.0001). F) Cell viability assay showed that there was no significant difference between GFP-Cten construct and GFP-Cten^{R474A}, (P = 0.9996), GFP-Cten^{Y479F} (P = 0.9990), GFP-$^{R474A+Y479F}$ (P = 0.9993), and GFP-EV control constructs (P = 0.9955). Results are represented as the mean ±SD of three independent experiments (one-way ANOVA).

5.3 Discussion

Cten has been found to be involved in regulating several biological events including cell migration, invasion, and colony formation in numerous cancer cell types, however the understanding of the biological functions of Cten and the potential mechanisms involved are sparse. The SH2 domain of Cten is thought to be essential for its functional activity within the cell (Hong et al., 2013). The arginine at the 474 site corresponds to βB5 in Src kinase, which was found to be critical for binding of the SH2 domain with several tyrosine phosphorylated proteins such as FAK (Yeo et al., 2006). In prostate cancer, it was found that arginine in Cten is responsible for interacting with the known tumour suppressor DLC1 via its SH2 domain in a phosphorylated tyrosine independent manner, leading to localisation of DLC1 to the focal adhesion complexes (Liao et al., 2007). Others have shown that arginine when mutated to alanine in the SH2 domain of Cten prevented the transition from extension formation to invasive migration during tubule formation, resulting in increased basal STAT3 activation in Madin-Darby canine kidney (MDCK) cells (Kwon et al., 2011). More recently, unpublished data from our laboratory have shown that arginine at site 474, lying within the SH2 domain, has an impact on Cten's ability to increase both cell motility and colony formation efficiency in CRC cells, because when it is mutated to alanine, cell migratory capabilities and colony formation efficiency were significantly reduced. Besides the critical arginine at 474, our group also mutated tyrosine to phenylalanine at position 479 in the Cten SH2 domain. Our group further found that Cten is tyrosine phosphorylated and the Y479 mutation is also critical for its functional activity (submitted for publication). However, the individual mutations in the SH2 domain of Cten did not cause complete reduction of its effect on both colony formation and cell migration suggesting that there may be some additional elements that are essential for its functional activity. Therefore, in this study, it was hypothesised that the combination of both mutations in one construct will completely abolish Cten's ability to upregulate ROCK1, Src, and Snail protein expression and induce cell motility and colony formation in CRC cells.

This study has successfully created a new construct containing a point mutation at position 474 mutating arginine to alanine and tyrosine to phenylalanine at position 479 in the SH2 domain of Cten using site directed mutagenesis. Western blot analysis showed that previously created constructs, GFP-Cten^{R474A} containing arginine mutation or GFP-Cten^{Y479F} containing tyrosine mutation, did not cause strong reduction of its effect on ROCK1, Src, or Snail protein expression. However, the GFP-Cten$^{R474A+Y479F}$ construct expression has revealed very interesting findings, that induction of ROCK1, Src, or Snail protein levels by Cten was completely reduced when using this construct in which the SH2 domain in Cten was inactivated. As mentioned in the previous chapter, Cten regulates both Src and Snail protein stability but as they do not form a physical complex, this is most likely due to signalling downstream of Cten mediated by the SH2 domain. Taken together, these findings suggest that Cten signalling to Src/ROCK1/Snail pathway is mediated via the SH2 domain.

The effect of Cten mutant constructs on cell migration, invasion, and colony formation was also investigated in this chapter. The GFP-Cten$^{R474A+Y479F}$ construct showed a complete reduction of the effect of Cten on cell migration, invasion, colony formation abilities compared to the wt. Cten as well as both mutations individually and this fits with previous data generated from this lab (submitted for publication). This suggests that arginine at 474 and tyrosine at 479 in the SH2 domain of Cten play a critical role in promoting cell motility and colony formation efficiency in CRC cells. However, experiments on proliferation revealed no significant difference between EV control for either wt. Cten or other mutant constructs, GFP-Cten^{R474A}, GFP-Cten^{Y479F}, and GFP-Cten$^{R474A+Y479F}$, thus confirming the previous study indicating that Cten does not affect cell proliferation (Albasri et al., 2009).

In conclusion, this study has successfully combined both mutations together in one construct. Investigations have demonstrated that the SH2 domain of Cten is important for its functional activity. The findings revealed that the Cten SH2 domain lost its effects

on its downstream targets as well as cell migratory capabilities and colony formation efficiency when the mutated arginine and tyrosine were combined together in one construct, indicating that Cten signalling to promote cell migration, invasion and colony formation through the upregulation of Src/ROCK1/Snail dependent pathway is mediated by the SH2 domain.

6 TGFβ1 Mediated Cell Migration and Invasion Requires Cten Signalling in Colorectal Cancer

6.1 Introduction

Cten is a potential biomarker in many cancers, acting as oncogene in most tumour types, and is particularly associated with metastatic disease (Sasaki et al., 2003a, Sasaki et al., 2003b, Albasri et al., 2009, Albasri et al., 2011b, Al-Ghamdi et al., 2013). Cten expression is possibly upregulated through the activation of upstream signalling pathways since so far, no mutations or amplification of Cten in cancers have been documented. A study by Katz et al. showed that stimulation with EGF led to upregulated Cten expression at a post-transcriptional level only in breast cell lines, whereas others have shown that Cten is upregulated by the EGFR at both the transcriptional and post-transcriptional level (Katz et al., 2007, Cao et al., 2012). Further reports suggested that Cten is regulated by KRAS in both CRC and pancreatic cancer cells (Al-Ghamdi et al., 2011). Cten expression was also shown to be negatively regulated by STAT3 in CRC cell lines, whereas others have found that Cten is upregulated by STAT3 in human lung cancer cells (Thorpe et al., 2015, Bennett et al., 2015). One of the most informative studies have suggested that Cten could be under the regulation of several growth factors and cytokines including EGF, TGF-β, FGF2, NGF, PDGF, IL-13, IGF-1, and IL-6 (Hung et al., 2014). How Cten is activated and regulated in these tumours is unclear, nonetheless, there seems to be multiple pathways involved, and it appears to be largely dependent on tissue or context types.

TGF-β1 is a polypeptide member of the growth factor family that plays a physiological role in the regulation of wound healing, angiogenesis, differentiation, and proliferation. TGF-β 1 can function as a tumour suppressor in normal epithelial cells and in the early stage of cancer. However, the growth inhibitory function of TGF-β1 is selectively lost in late stage cancer which results in an induction of cell migration, invasion and metastasis (Araki et al., 2010, Nagaraj and Datta, 2010). Previous studies have shown that TGF-β1 is involved in the regulation of EMT processes through numerous downstream pathways, including Ras/MAPK (Janda et al., 2002), RhoA (Bhowmick et al., 2001), and Jagged

1/Noch (Zavadil et al., 2004). TGFβ1 has also been found to signal through FAK to upregulate EMT related mesenchymal and invasiveness markers and delocalise E-cadherin membrane (Cicchini et al., 2008). Furthermore, the TGF-β1 mediated Smad signalling pathway has been shown to play an important role in EMT associated with metastatic progression (Janda et al., 2002). However, a possible role of Cten in TGF-β1 mediated EMT and cell motility in CRC cells has not been investigated. Therefore, it was hypothesised that TGF-β may induce cell motility and promote EMT processes through the Cten signalling pathway.

6.2 Results

6.2.1 TGF-β1 Regulates Cten Expression

TGF-β1 signals downstream to EMT and since Cten is linked to EMT and probably achieves this through its downstream targets Src/ROCK1/Snail pathway, it was of interest to determine whether Cten expression is under the regulation of the TGF-β1 signalling pathway in CRC cells. To investigate this, SW620 cells were pretreated with different concentrations of TGF-β1 stimulator from 0 to 20 ng/ml for 48 hours and the changes in protein level of Cten and its downstream targets, ROCK1, N-cadherin, E-cadherin, Src, and Snail were evaluated by western blot. SW620 cells showed a dose-dependent increase in Cten, ROCK1, Src, Snail, and N-cadherin expression, whereas the protein expression level of E-cadherin was associated with decreased expression following stimulation with TGF-β1 (figure 6-1 A). The optimum concentration of TGF-β1 stimulator (20 ng/ml) was selected for subsequent TGF-β1 stimulation experiments.

The relationship between TGF-β1 and Cten was further investigated in an additional cell line, HCT116. In agreement with the findings in SW620 cells, stimulation of TGF-β1 was associated with an increase in the protein expression levels of Cten, ROCK1, Src, Snail, and N-cadherin, whereas E-cadherin expression was inhibited (figure 6-2 A). The ability of TGF-β1 to induce cell functions was then investigated using the Transwell migration assay, wound healing assay, Transwell Matrigel invasion assay, and PrestoBlue assay. In both SW620 and HCT116 cell lines, cell migration, invasion, and proliferation were increased when cells were treated with TGF-β1 stimulator compared to untreated control (figure 6-1), (figure 6-2).

The effect of TGF-β1 on Cten and its downstream targets was again investigated in SW620 cell but using an alternative methodology. Assuming that there was some endogenous production of TGF-β1 by the cell lines, this was directly knocked down using siRNA duplexes. Western blotting was again performed to look at the changes in protein

expression level of Cten, ROCK1, Src, Snail, E-cadherin and N-cadherin. Confirming the TGF-β1 stimulation results, knockdown of TGF-β1 resulted in a reduction of Cten, ROCK1, Src, Snail, N-cadherin protein expression levels. Additionally, TGF-β1 knock down was associated with an increase in the expression level of E-cadherin compared to luciferase targeting siRNA control (figure 6-3 A). The effect of TGF-β1 knockdown on cell functions was also tested in this study. Knockdown of TGF-β1 was associated with a significant decrease in cell migration, invasion and proliferation compared to the luciferase control (figure 6-3). Collectively, these findings suggest that TGF-β1 may promote both cell motility and proliferation through the upregulation of EMT processes in CRC cells.

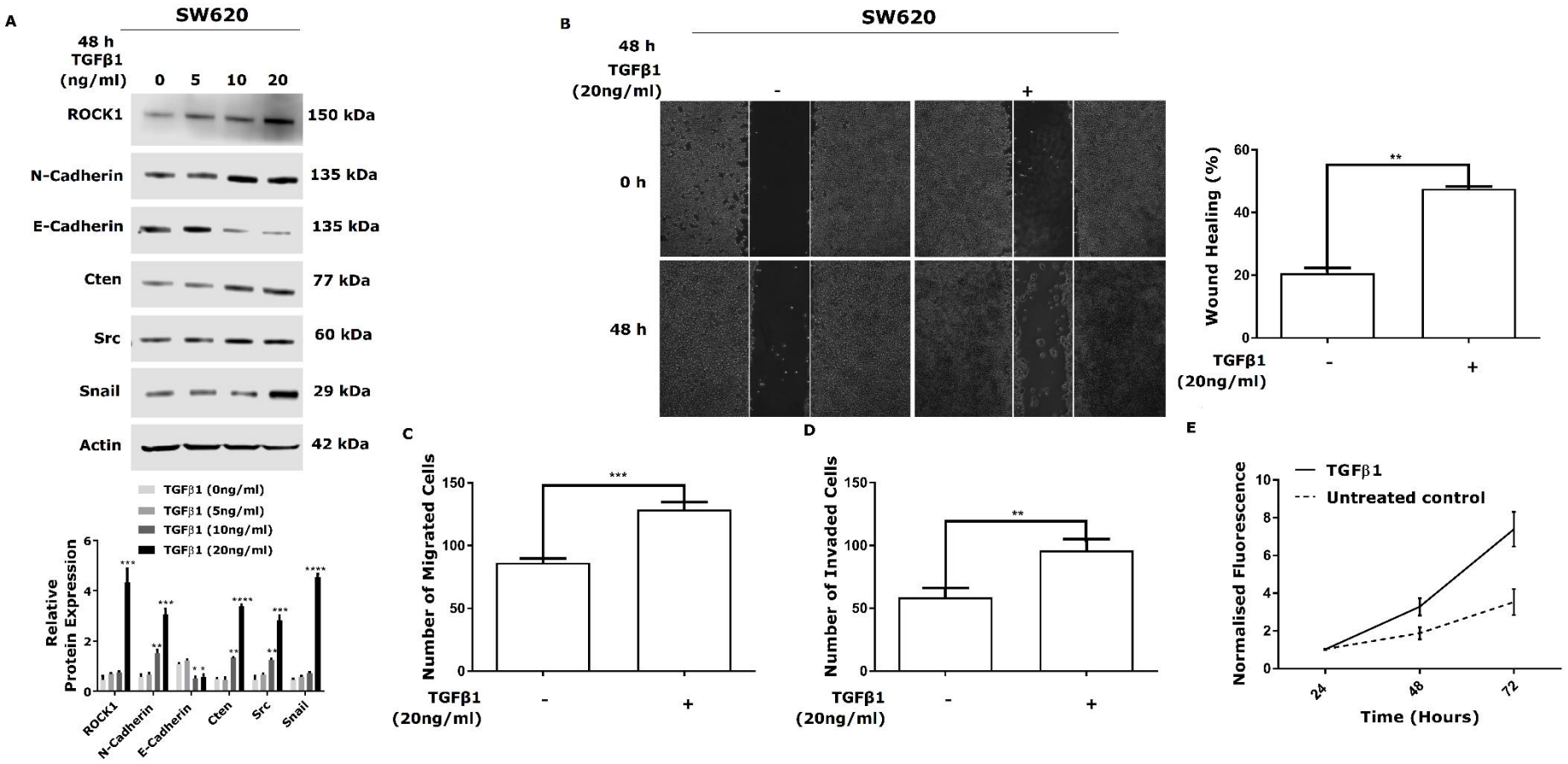


Figure 6–1: TGFβ1 increases Cten protein expression in a dose-dependent manner.
A) SW620 cells were stimulated with TGFβ1 (0-20 ng/ml) for 48 hours and the changes in Cten, ROCK1, N-cadherin, E-cadherin, Src, and Snail protein expression were determined by western blot. B) Stimulation of SW620 cells with TGFβ1 treatment (20 ng/ml for 48 hours) induced closure of wound compared to untreated control (P = 0.0024). C) Stimulation of TGFβ1 was associated with an increase in cell migration compared to untreated SW620 cells control (P = 0.0005). D) Treatment of SW620 cells with TGFβ1 stimulator enhanced cell invasion compared to untreated control (P = 0.0055), (unpaired t-test). E) Stimulation of TGFβ1 in SW620 cells was associated with an increase in cell proliferation compared to untreated control (P ≤ 0.0001), (two-way ANOVA). Results are represented as the mean ±SEM of three independent experiments.

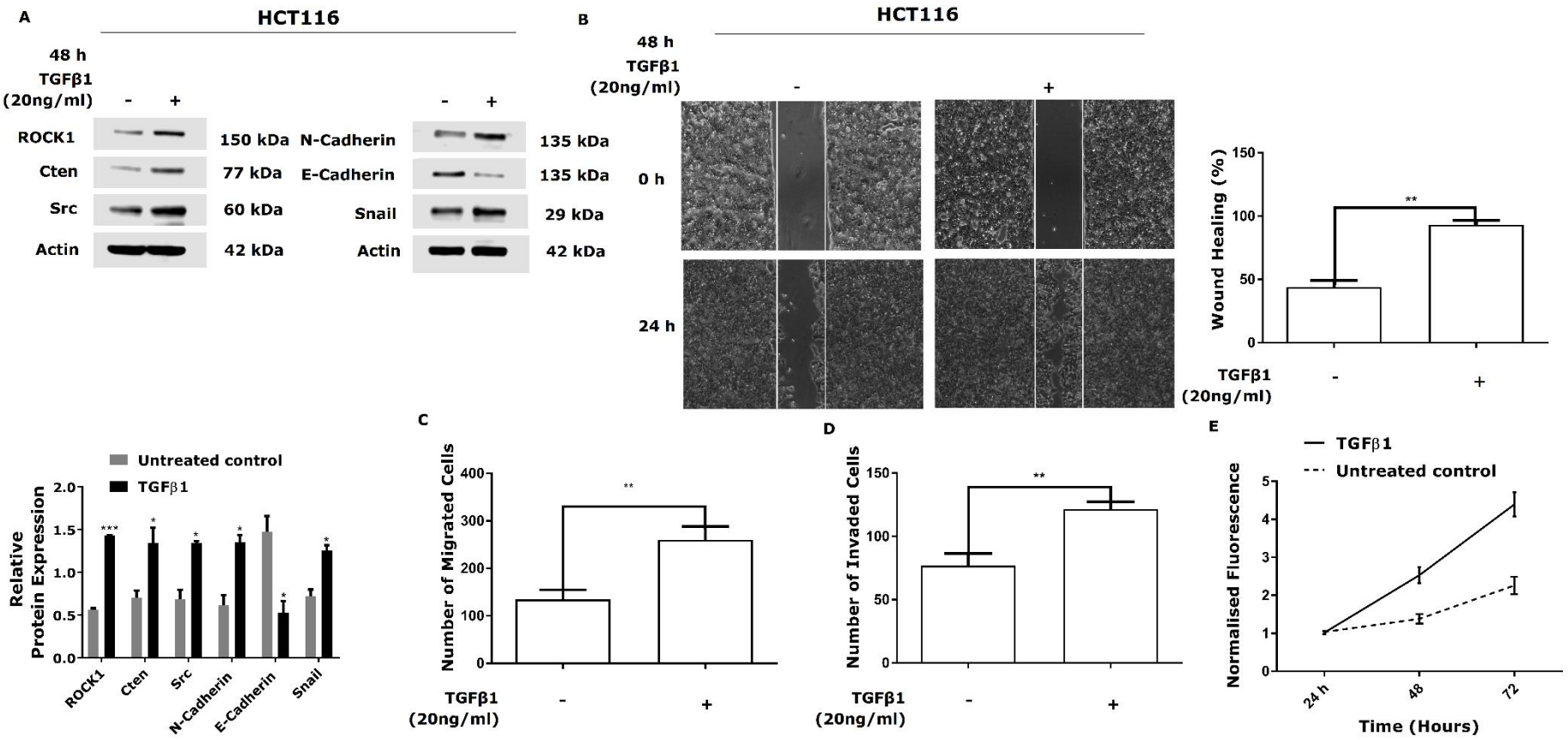


Figure 6–2:TGFβ1 stimulation increases Cten protein expression in HCT116 cells.
A) HCT116 cells were pre-treated with TGFβ1 stimulator (20 ng/ml for 48 hours) and the changes in Cten, ROCK1, N-cadherin, E-cadherin, Src, and Snail protein expression were determined by western blot. B) Treatment of HCT116 cells with TGFβ1 TGFβ1 stimulator (20 ng/ml for 48 hours) induced wound closure compared to untreated control (P = 0.0084). C) Stimulation of TGFβ1 was associated with an increase in cell migration compared to untreated HCT116 cells control (P = 0.0032). D) Stimulation of SW620 cells with TGFβ1 treatment increased cell invasion compared to untreated control (P = 0.0022), (unpaired t-test). E) Stimulation of TGFβ1 in HCT116 cells resulted in an increase in cell proliferation compared to untreated control (P ≤ 0.0001), (two-way ANOVA). Results are represented as the mean ±SEM of three independent experiments.

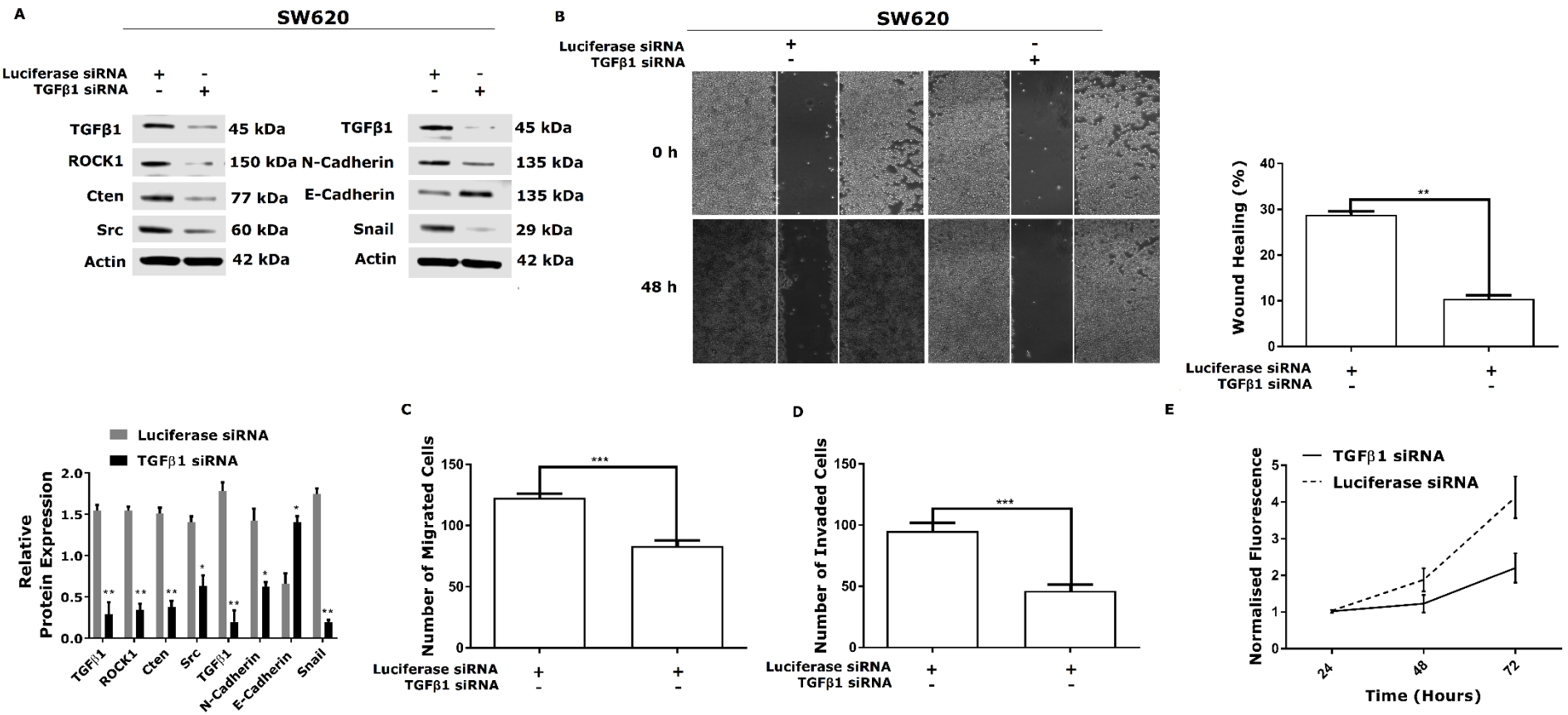


Figure 6–3: TGFβ1 knockdown decreases Cten protein expression in SW620 cells.
A) SW620 cells were transfected with TGFβ1 targeting siRNA duplexes (200 nM/ml for 48 hours) and the changes in Cten, ROCK1, N-cadherin, E-cadherin, Src, and Snail protein expression were determined by western blot. B) Knockdown of TGFβ1 in SW620 decreased wound closure compared to luciferase targeting siRNA control (P = 0.0016). C) Knockdown of TGFβ1 was associated with a decrease in cell migration compared to luciferase transfected HCT116 cells control (P = 0.0003). D) Knockdown of TGFβ1 in SW620 cells decreased cell invasion compared to luciferase siRNA control (P = 0.0005), (unpaired t-test). E) Knockdown of TGFβ1 in SW620 cells resulted in a reduction in cell proliferation compared to luciferase siRNA control (P ≤ 0.0001), (two-way ANOVA). Results are represented as the mean ±SEM of three independent experiments.

6.2.2 Cten Knockout Has No Effect on TGF-β1 Induced Cell Proliferation but Abrogates TGF-β1 Induced Cell Migration and Invasion.

Since both TGFβ1 and Cten are involved in the regulation of EMT processes and TGF-β1 also induces Cten expression, it was hypothesised that Cten may be a signalling intermediate in the TGF-β1/EMT pathway. To interrogate the role of Cten in the induction of EMT by TGF-β1 in CRC cells, the Cten knockout cell line previously created using CRISPR/Cas9 technology, SW620$^{\Delta Cten}$ (Thorpe et al., 2017) was stimulated with TGF-β1 and western blotting was performed. The results revealed that stimulation of SW620$^{\Delta Cten}$ with TGF-β1 was associated with a small increase in N-cadherin expression but ROCK1, Src, Snail, and E-cadherin protein expression level remained unchanged compared to the sham-treated cells control (figure 6-4 A). This implies that Cten may be responsible for TGF-β1 induced EMT.

Although both TGF-β1 and Cten play a key role in regulating cell migration and invasion, the role of Cten in TGF-β1 induced cell motility has not been highlighted. To determine that the upregulation of Cten by TGF-β1 was functionally active, SW620$^{\Delta Cten}$ cells were stimulated with TGF-β1 and a Transwell migration, wound healing, Matrigel invasion assay, and PrestoBlue assays were performed. Stimulation of SW620$^{\Delta Cten}$ cells. with TGF-β1 was associated with an increase in cell proliferation compared to the untreated cell control, however, the ability of TGF-β1 to induce cell migration and invasion was abrogated when Cten is absent in SW620$^{\Delta Cten}$ cells (figure 6-4). This suggests that Cten may not be involved in TGF-β1 induced cell proliferation, but it may be a signalling intermediate in the TGF-β1/EMT pathway regulating cell migration and invasion in CRC cells.

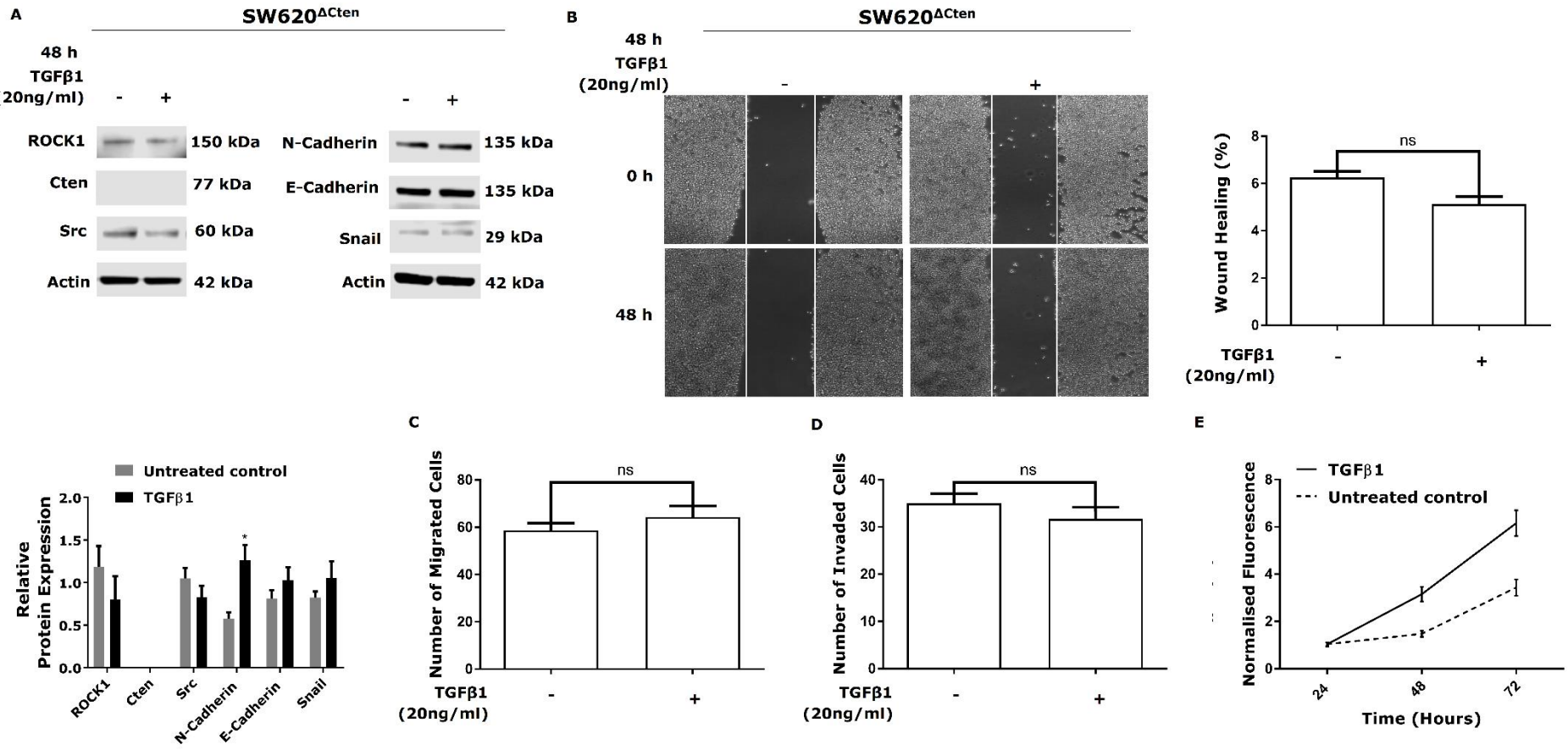
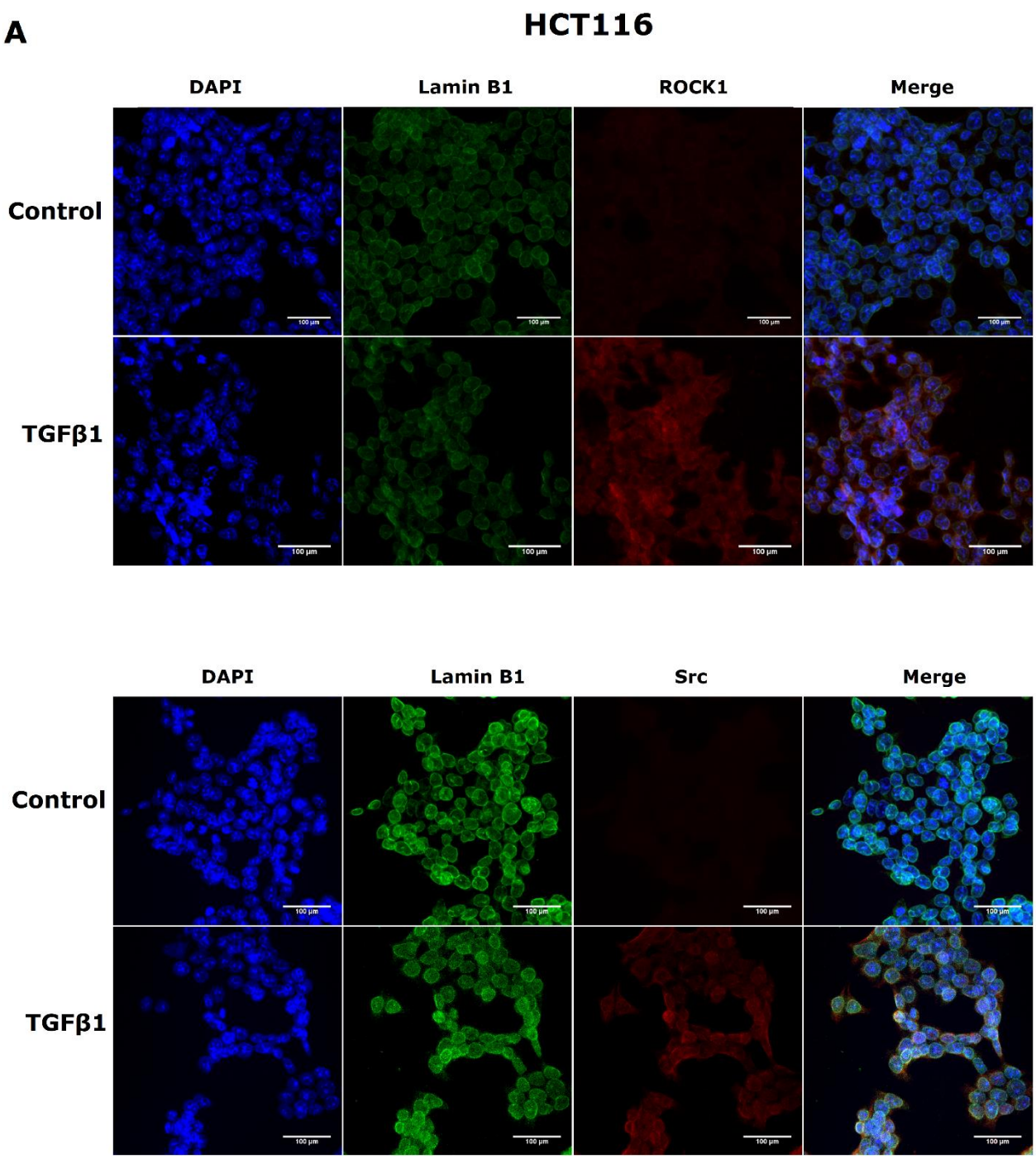


Figure 6–4: TGFβ1 signals through Cten to regulates EMT and promotes cell migration and invasion.
A) Stimulation of SW620$^{\Delta Cten}$ cells with TGFβ1 treatment (20 ng/ml for 48 hours) was associated with a small increase in N-cadherin expression and ROCK1, Src, Snail, and E-cadherin protein expression level remained unchanged from the untreated cells control. B) Wound healing assay showed no significant differences between TGFβ1 stimulation and untreated SW620$^{\Delta Cten}$ cells control (P = 0.0585). C) Stimulation of TGFβ1 in SW620$^{\Delta Cten}$ cells did not cause a significant increase in cell migration compared to untreated control (P = 0.1561). D) Treatment of SW620$^{\Delta Cten}$ cells with TGFβ1 stimulator did not enhance cell invasion compared to untreated control (P = 0.1469), (unpaired t-test). E) Stimulation of TGFβ1 in SW620$^{\Delta Cten}$ cells was associated with an increase in cell proliferation compared to untreated control (P ≤ 0.0001), (two-way ANOVA). Results are represented as the mean ±SD of three independent experiments.

6.2.3 TGFβ1 Induces Nuclear Localisation of Cten/Src/ROCK1/Snail.

Since TGF-β1 stimulation can induce EMT and upregulate Cten protein expression, it was of interest to determine whether TGF-β1 is capable of inducing Cten protein translocation to the nucleus. To gain a deeper insight into the mechanisms of TGF-β1 regulation of Cten functions, HCT116 cells were stimulated with TGF-β1 and the expression of Cten and its downstream targets in nucleus were observed by immunofluorescences staining. The results showed that stimulation of HCT116 cell with TGF-β1 for 48 h induced Cten, Src, ROCK, and Snail protein expression as well as translocation to the nucleus. By contrast, in untreated HCT116 cells, there was no Cten or its downstream target proteins detected in the nucleus (figure 6-5). To determine whether the ability of TGF-β1 stimulation to induce Cten nuclear translocation was not technique or cell line specific, TGF-β1 was stimulated in the SW620 cell line and both immunofluorescence staining, and nuclear and cytoplasmic extraction were performed to directly determine the subcellular localisation of Cten and its downstream targets, Src, ROCK1, and Snail proteins. Subcellular fractionation experiments showed that a fraction of Cten, Src, ROCK1, or Snail proteins was indeed mostly translocated in the nucleus following treatment with TGF-β1 (figure 6-6). Consistently, immunofluorescence imagining further indicated that stimulation of SW620 with TGF-β1 treatment also increased the expression and nuclear translocation of Cten, ROCK1, Src, and Snail compared to the untreated cell control (figure 6-7). To explore the mechanism by which TGF-β1 induces the nuclear translocation of Src, ROCK and Snail proteins, it was determined whether nuclear accumulation of Src, ROCK and Snail induced by TGF-β1 is dependent on the upregulation of Cten signalling. To probe this, TGF-β1 was stimulated in Cten knockout cell line, $SW620^{\Delta Cten}$, and the nuclear translocation was determined by immunofluorescence staining. The results revealed that knockout of Cten abrogates the expression and nuclear translocation of Src, ROCK and Snail induced by TGF-β1 stimulation (figure 6-7). Taken together, these data suggest that nuclear translocation of

ROCK1, Src, and Snail protein is probably mediated by TGF-β1 via the upregulation of the Cten signalling pathway.


A
HCT116
DAPI
Lamin B1
ROCK1
Merge
Control
TGFβ1
DAPI
Lamin B1
Src
Merge
Control
TGFβ1

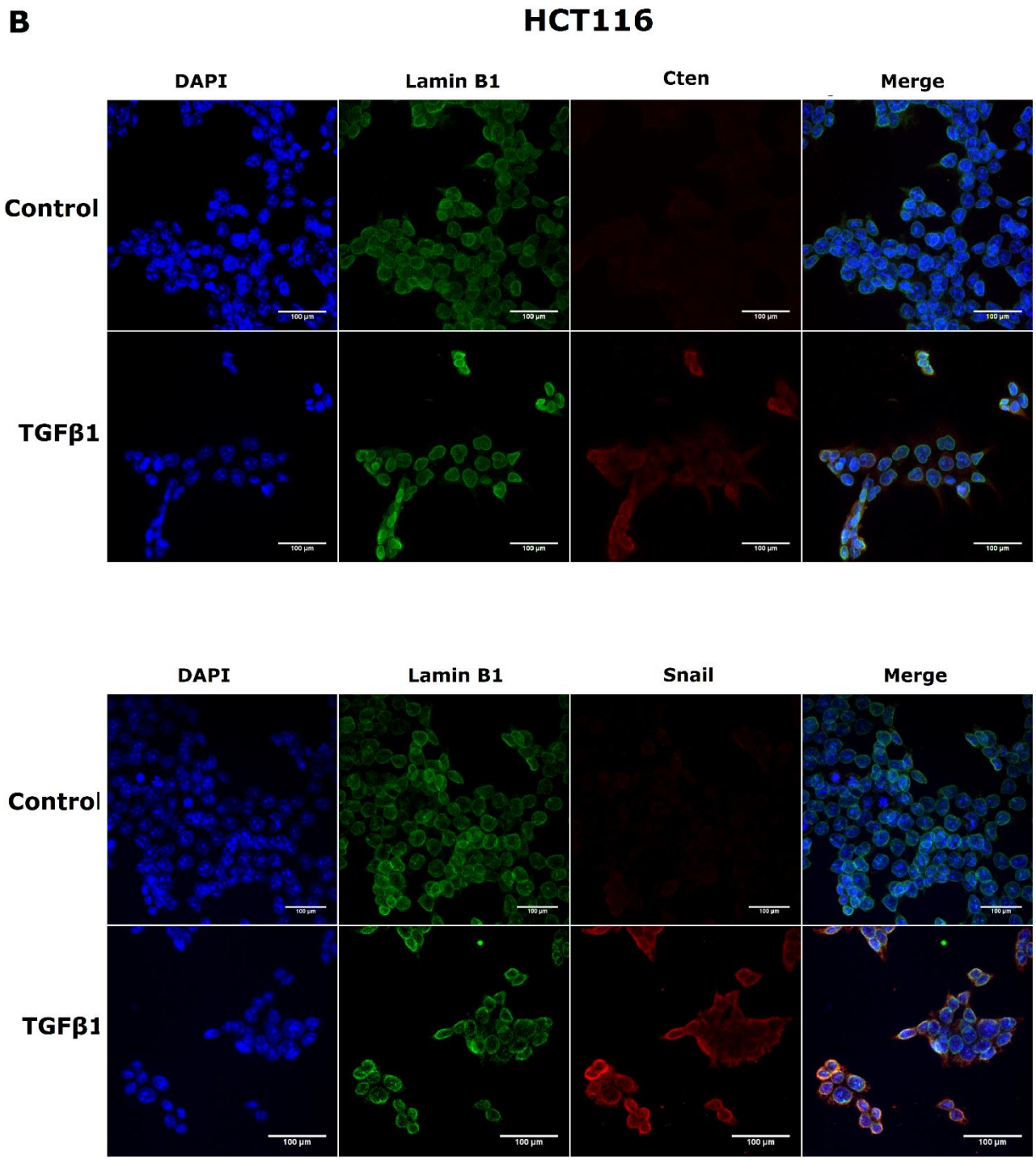


Figure 6–5: TGFβ1 increases nuclear translocation and expression of Cten and its downstream targets in HCT116 cells.
A and B). The subcellular localisation of ROCK1, Src, Cten, and Snail was examined by confocal microscopic images of DAPI (blue), nuclear envelope marker Lamin B (green) and ROCK1, Src, Cten or Snail protein (red) in untreated control and treated cells with TGFβ1 stimulation (20ng/ml) for 48 hours. (scale bar100 µm).

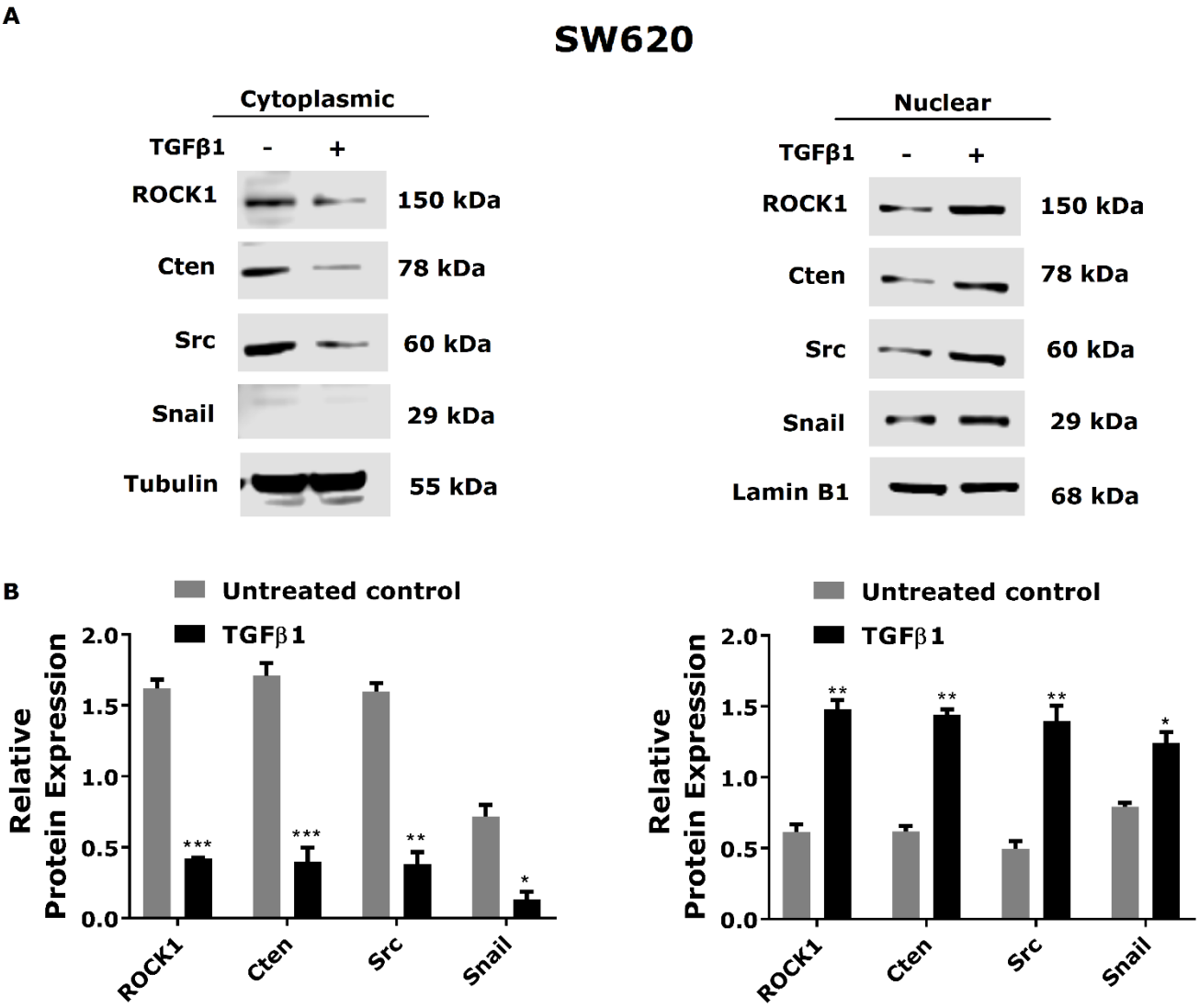
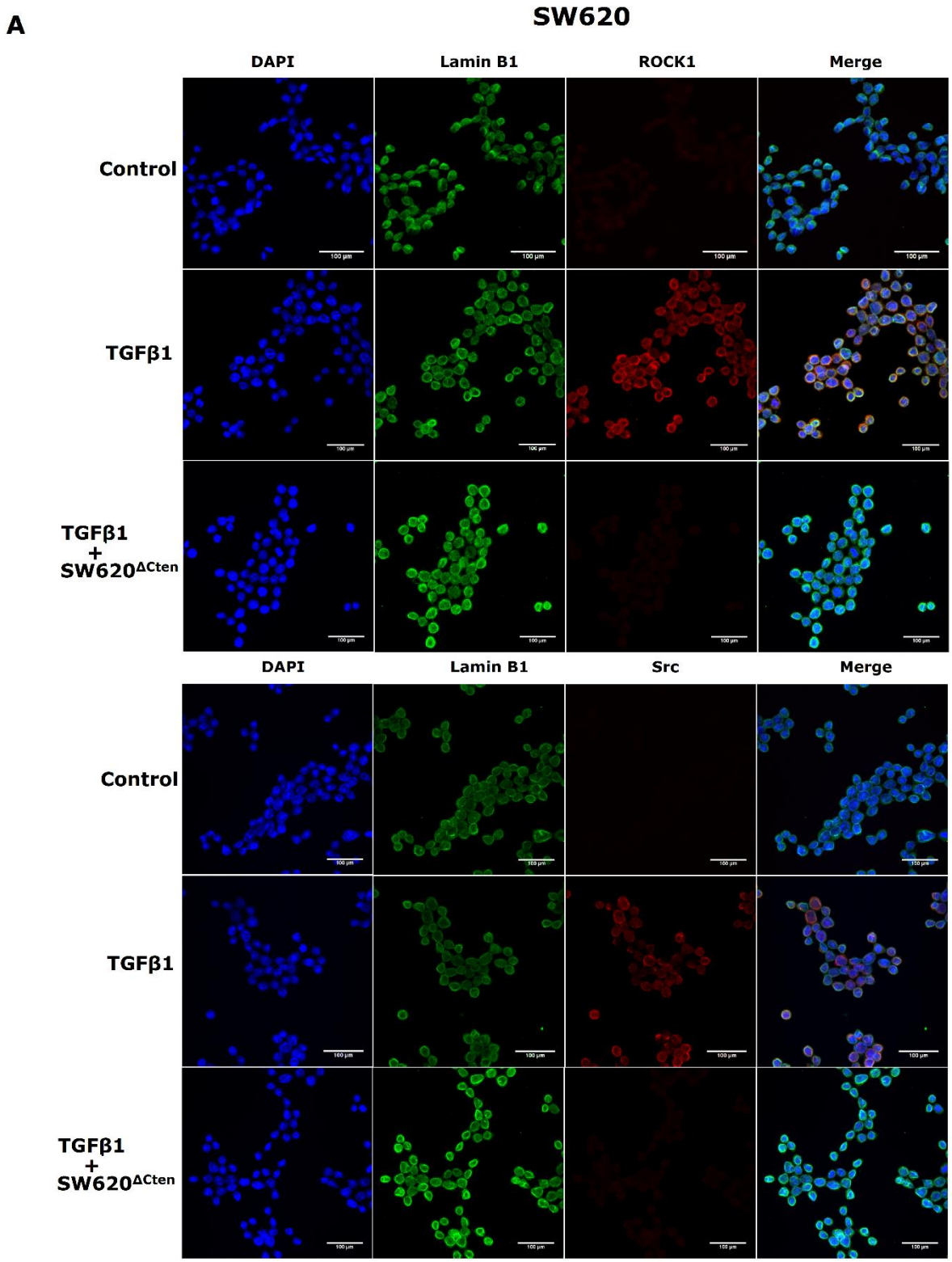


Figure 6–6: TGFβ1 induces nuclear translocation of Cten and its downstream targets in SW620 cells.

*A) Subcellular fractionation extraction was performed following the treatment of SW620 cell with or without 20 ng/ml of TGFβ1 stimulator for 48 hours and the lysates were assessed by western blot. Lamin B1 and tubulin were used as loading control for cytoplasmic and nuclear fractions. B) Quantitative determination of the relative expression of ROCK1, Cten, Src, and Snail protein fractions following treatment with TGFβ1. Results are represented as the mean ±SEM of two independent experiments (unpaired t-test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).*


A
SW620
DAPI
Lamin B1
ROCK1
Merge
Control
TGFβ1
TGFβ1 + SW620ΔCten
DAPI
Lamin B1
Src
Merge
Control
TGFβ1
TGFβ1 + SW620ΔCten

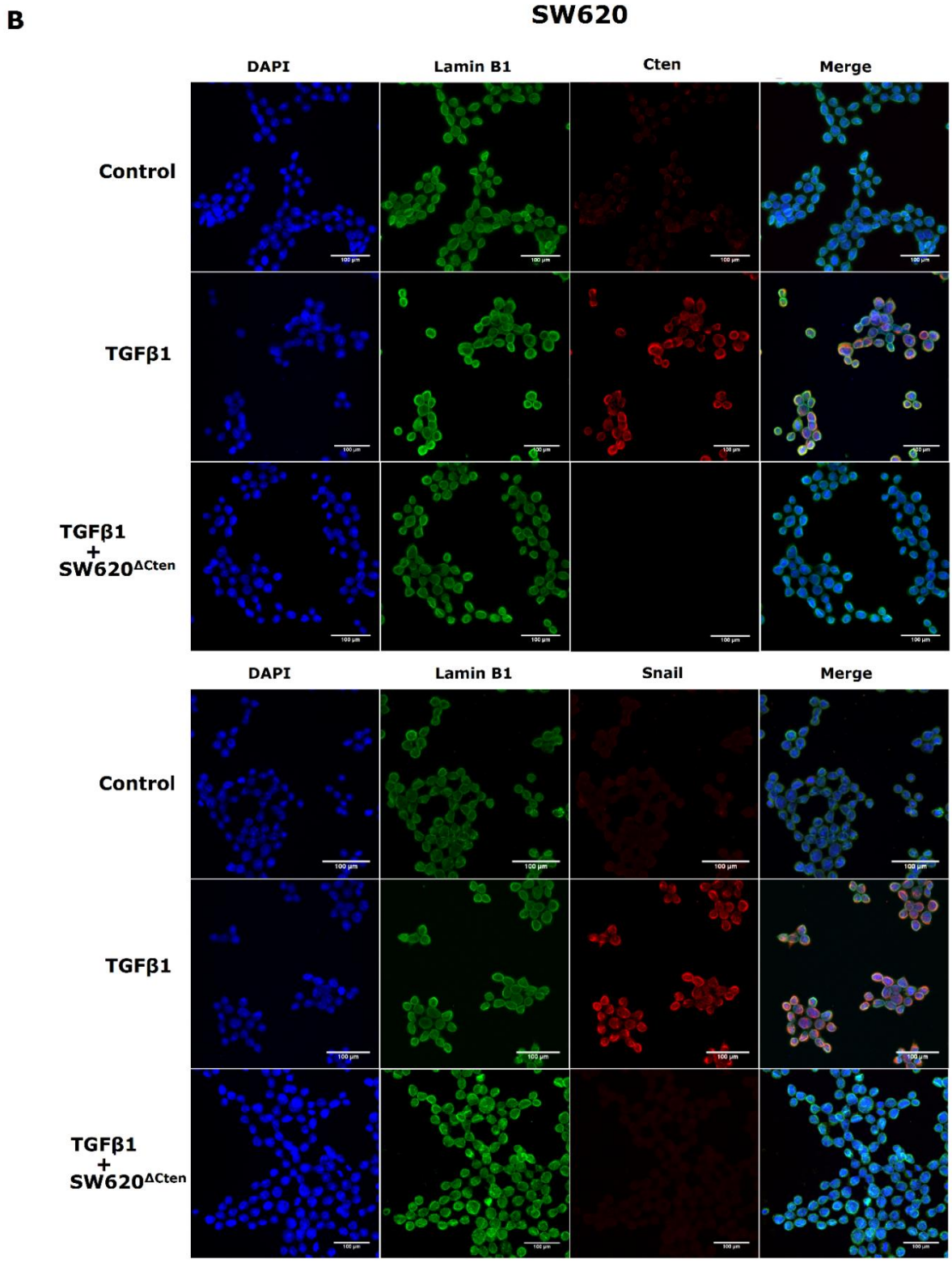


Figure 6–7: Knockout of Cten blocks ROCK1, Src, and Snail nuclear translocation and expression induced by TGFβ1 in SW620 cells.
A and B). The subcellular localisation of ROCK1, Src, Cten, and Snail was assessed by confocal microscopic images of DAPI (blue), nuclear envelope marker Lamin B (green) and ROCK1, Src, Cten or Snail protein (red) in untreated control and treated cells with 20ng/ml of TGFβ1 for 48 hours. (scale bar represents 100 μm).

6.3 Discussion

EMT is a critical process occurring during tumour metastasis. Cten has been shown to act as oncogene in most tumour types and involved in regulation of EMT processes, however, the mechanisms that upregulate the expression of Cten induced EMT have not been elucidated (Albasri et al., 2009, Albasri et al., 2011b, Al-Ghamdi et al., 2013, Thorpe et al., 2017). Previous research from our laboratory suggested that Cten expression is regulated by EGFR/KRAS signalling (Al-Ghamdi et al., 2011) and the study by Katz et al (Katz et al., 2007) showing the role of Her2 in up-regulating Cten would seem to validate this. We and others further have also found that Cten could be under the regulation of several cytokines (such as IL6/Stat3) and growth factors (Hung et al., 2014, Thorpe et al., 2015). The present study directly shows, for the first time, that Cten plays an essential role in TGF-β1 induced EMT (and possibly metastasis) in CRC cells and that this may be through upregulation of the Src/ROCK1/Snail signalling pathway.

TGF-β1 plays a key role in promoting EMT initiation and tumour metastasis. It has been documented that TGF-β1 induces the expression of several transcription factors including Twist, Zeb, Slug, and Snail (Peinado et al., 2007). The current study has shown that TGF-β1 may also mediate EMT through the positive regulation of Cten expression in CRC cell lines, HCT116 and SW620. This study has also shown that stimulation of TGF-β1 mediated EMT can cause upregulation of Cten's downstream pathways, Src, ROCK, and Snail expression. This study has also shown that stimulation of TGF-β1 mediated EMT can cause upregulation of Cten's downstream pathways, Src, ROCK, and Snail expression. These findings were further confirmed using an alternative methodology i.e. Knockdown of TGF-β1 resulted in downregulation of Cten expression and its downstream targets and upregulation of E-cadherin expression in SW620 cells. There are several downstream targets of TGF-β1 mediated EMT which may possibly be upregulating Cten and this may be further investigated including Ras/MAPK (Janda et al., 2002), RhoA (Bhowmick et al., 2001), and Jagged 1/Noch (Zavadil et al., 2004). As a functional

consequence of TGF-β1 signalling activation, increased abilities of proliferation, migration, and invasion were displayed in both HCT116 and SW620 cells. The ability of TGF-β1 to regulate these properties in CRC cells was also confirmed using siRNA duplexes. These results are in accordance with previous studies that TGF-β1 induced EMT can promote cell motility and proliferation in a vast range of different tumour cells (Pang et al., 2016, Bu and Chen, 2017).

Previous published data from our group have shown that Cten induced EMT and promotes cell motility through FAK, ILK or Snail signalling (Albasri et al., 2011a, Al-Ghamdi et al., 2013, Thorpe et al., 2017). Others have shown that FAK and/or ILK are required for TGF-β1 induced EMT and to promote cell motility (Cicchini et al., 2008, Lee et al., 2004). Snail also has been shown to act as a mediator of TGF-β1 induced EMT and Akt activation (Cho et al., 2007). Finally, TGF-β1 induces activation of integrin receptor and since Cten binds to integrin, Subsequently, it was of interest to investigate whether Cten is a signalling intermediate in the TGF-β1/EMT pathway, since both TGF-β1 and Cten are linked to EMT and promote cell motility. Interestingly, TGF-β1 was unable to induce EMT markers and other downstream targets, ROCK1 and Src without Cten, indicating involvement of Cten signalling in TGF-β1 induced EMT. The functional relevance of Cten upregulation is demonstrated in TGF-β1 induced cell motility. The data herein have pointed to an important role of Cten in regulating cell motility induced by TGF-β1, since Cten knockout shows an abrogation of the effect of TGF-β1 treatment on both cell migration and invasion in the SW620$^{\Delta Cten}$ cell line. However, Cten knockout failed to block the stimulatory effect of TGF-β1 treatment on cell proliferation. Previous reports have shown that Cten does not affect cell proliferation in CRC cells (Albasri et al., 2009). This therefore increases the possibility that Cten is not involved in TGF-β1 induced cell proliferation, but it may be acting as a mediator of TGF-β1 induced cell migration and invasion in CRC cells. In addition to Cten, there are other downstream pathways involved in TGF-β1 mediated cell migration, including JAK/STAT3 (Liu et al., 2014), PI3K-Akt (Bakin et al., 2000), and Reelin (Yuan et al., 2012), so it would be of

interest to determine whether Cten acts parallelly or synergistically with these pathways in future studies.

Cten has shown to localise to the nucleus, however, the mechanisms by which the Cten molecule is translocated to the nucleus is unknown (Albasri et al., 2011a). Cten needs to be translocated into the nucleus to exert its biological effects and the participation of TGF-β1 in inducing cytoplasmic to nuclear translocation of Cten was investigated in this study. This chapter showed that TGF-β1 induced nuclear translocation of Cten and that of downstream Src, ROCK1, and Snail in two CRC cell lines, HCT116 and SW620, with increased expression. A similar phenomenon of Src and Snail nuclear translocation was also observed when cells were exposed to TGF-β1 treatment (Takahashi et al., 2009, Cho et al., 2007). Intriguingly, the relevance of Cten upregulation is illustrated in EMT expression and nuclear localisation induced by TGF-β1. The present results show that Cten is essential for TGF-β1 induced Src, ROCK1, or Snail nuclear translocation, since TGF-β1 shows very limited effects in Cten knockout cell line. Although further investigations are required to demonstrate a causative relationship, these data suggest that increased expression and nuclear translocation of ROCK1, Src, and Snail protein is probably mediated by TGF-β1 via the upregulation of the Cten signalling pathway.

In summary, the data presented have indicated that TGFβ1 and Cten signalling may cooperate in promoting EMT and metastasis in CRC cells through the dependent upregulation of the Src/ROCK1/Snail signalling axis. If this is confirmed, then the TGF-β1/Cten pathway may become a potential therapeutic target for colorectal cancer.

7 Investigating the Role of the Nuclear Localisation of Cten in Colorectal Cancer

7.1 Introduction

Cten mainly localises to the cytoplasmic tails of integrins at focal adhesions and it is part of a large, dynamic complex and it can promote cell motility and colony formation. Cten is not confined only to focal adhesions but is also present in the nucleus, which could suggest that Cten might act as a transducer molecule involved in nuclear processes in addition to events on the surface of the cell (Liao et al., 2009, Albasri et al., 2011a). Nuclear localisation of Cten was found to be highly frequent in metastatic tumours compared to the primary tumour suggesting that Cten signalling in the nucleus may further promote tumour progression. Cten has been shown to physically bind and form a complex with β catenin in the nucleus only (Liao et al., 2009).

Src is normally maintained inactive in the cytoplasm when it is phosphorylated at the site of Y530. However, Src translocates to the membrane and becomes fully activated by autophosphorylation at the Y416 once dephosphorylated (Ingley, 2008, Bolós et al., 2010). The activated Src within cell membrane initiates intercellular transduction signalling pathways that promote cell function (Frame, 2002).

Although a subcellular localisation of ROCK1 has not been well characterised, there are several lines of evidence showing that ROCK1 is predominantly cytoplasmic in a variety of different cancer cells (Julian and Olson, 2014). Others have pointed to ROCK1 association with the cell membrane (Stroeken et al., 2006).

Snail expression is controlled at the transcriptional level by several signalling pathways, including TGF-β, FGF, Wnt, and BMPs (Barrallo-Gimeno and Nieto, 2005). More recently, published data from our laboratory revealed that the expression of Snail is also under the regulation of Cten signalling (Thorpe et al., 2017). Studies have also shown that the activity of Snail can be regulated through post translation modifications to control its subcellular localisation. It has been shown that the phosphorylation of Snail by p21-

activated kinase (PAK1) increases nuclear localisation of Snail (Yang et al., 2005). Furthermore, loss of the zinc transporter LIVI leads to cytoplasmic localisation of Snail (Yamashita et al., 2004b), suggesting that regulation of nucleocytoplasmic localisation of Snail is critical for its activity.

The previous chapter revealed that Cten signalling is required for increased expression and nuclear translocation of ROCK1, Src, and Snail proteins mediated by TGF-β1. Here, it was hypothesised that nuclear localised Cten may play an important role in inducing its functional activity and increasing its downstream target nuclear translocation in CRC cells.

7.2 Results

7.2.1 Cten Expression in Colorectal Cancer Tissue

The subcellular localisation and expression of Cten in 84 CRC cases was studied using immunohistochemical staining. The TMA consists of randomly selected tumour and was constructed for the specific purpose of studying tumour protein localisation. Cores in the TMA were taken from different areas of the tumour including the invasive, centre, and luminal edges. The normal colon adjacent to the tumour area was also sampled for each case.

Cten antibody was previously optimised using kidney tissues by Dr Hannah Thorpe and a concentration of 1:250 was chosen as it gave minimum background staining. This condition was used for subsequent colon TMA staining (figure 7-1). Cten expression was shown to localise mostly to the cytoplasm, which is consistent with its localisation at focal adhesions. Cten expression was categorised into low or high based on the median. The Wilcoxon signed ranks test was used to test for significant differences in Cten expression between the tumour and normal colon. There was no significant difference in Cten expression between normal colon and tumour tissues ($p= 0.095$). The Chi squared test was used to test for associations between Cten staining and the clinical features. Cten expression was not associated with clinical features including tumour grade, tumour stage, lymph node stage, vascular invasion, Dukes' stage resection margin (table 7-1).

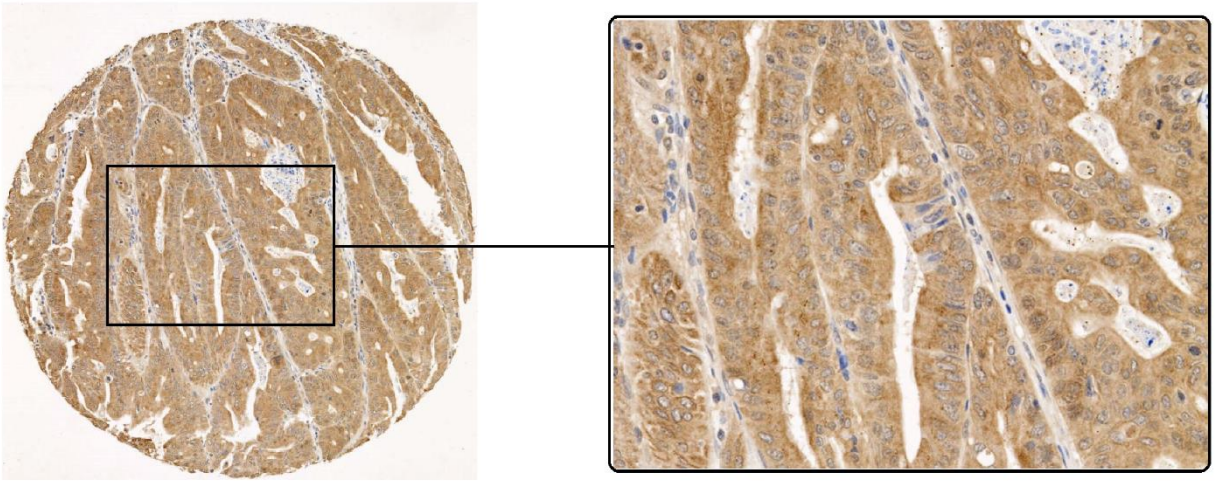


Figure 7–1: Cten staining of colorectal tumour tissues.
TMA cores of CRC revealed Cten staining in the cytoplasm.

Table 7–1: Association of Cten staining in the cytoplasmic localisation and the clinicopathological parameters. Significance was tested using the chi squared test.

	Cytoplasmic Staining		
Parameter	Low	High	P
Tumour Grade			0.100
1	1	1	
2	39	38	
3	5	0	
Tumour Stage			0.443
1	2	1	
2	4	8	
3	27	19	
4	12	11	
Lymph Node Stage			0.538
0	26	27	
1	15	9	
2	4	3	
Vascular Invasion			0.512
No	21	21	
Yes	24	18	
Dukes Stage			0.610
A	5	8	
B	21	17	
C1	17	11	
C2	2	2	
Resection Margins			0.354
Free	40	38	
Involved	3	1	
KRAS Mutation			0.377
No	27	19	
Yes	14	15	

7.2.2 Src Expression in Colorectal Cancer Tissue

The localisation of Cten was previously investigated and here, the localisation of Src was investigated in 84 cases of CRC following antibody optimisation. The antibody optimisation was performed in kidney tissue due to the lack of colon tissue availability for optimisation. An antibody dilution of 1:50 was selected as it gave a good staining intensity.

Following optimisation, the colon TMA was stained (figure 7-2). Src expression was localised to the cytoplasm in 37/84 (44%) and membranous Src staining was detected in 38/84 (45.2%) of the tumour cases. Src staining was not associated with the clinicopathological features including tumour grade, tumour stage, lymph node stage, vascular invasion, Dukes' stage, resection margin, or KRAS mutation as determined using the Chi squared statistical test (table 7-2). The Wilcoxon signed ranks test revealed that Src expression was significantly greater in the tumour compared to normal colon in both cytoplasmic ($p=0.001$) and membranous compartment ($p= 0.001$). Furthermore, Src expression was shown to positively correlate with Cten cytoplasmic staining as determined by the Spearman's rank statistical test (table 7-3), (figure 7-3).

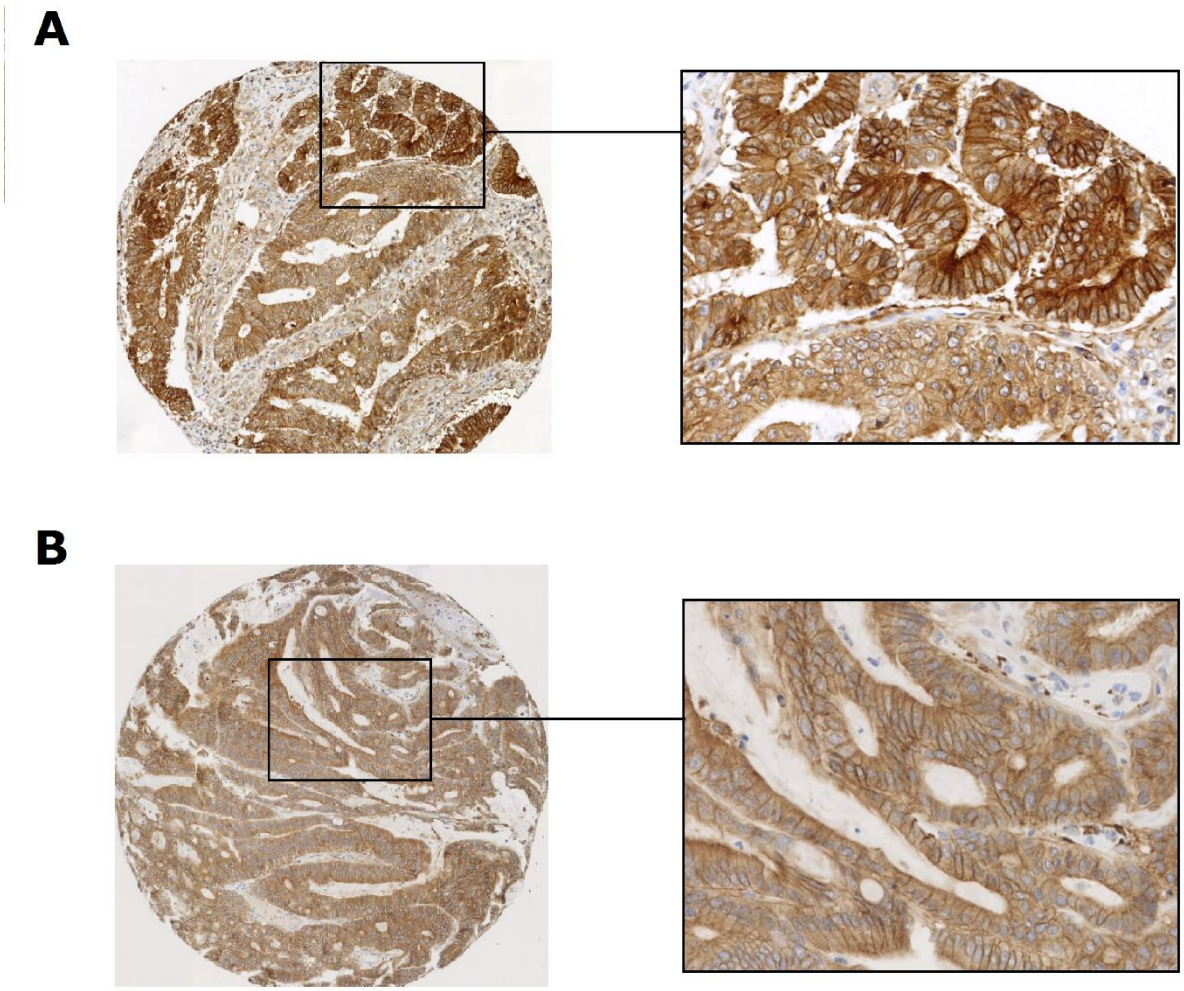


Figure 7–2 : Src staining of colorectal tumour tissues.
A) TMA cores of CRC revealed Src staining in the cytoplasm and membrane. B) TMA cores of CRC revealed Src staining in the membrane

Table 7–2: Association of Src staining of the colorectal tumours and the clinicopathological features (chi-squared statistical test applied).

	Cytoplasmic Staining			Membranous Staining		
Parameter	Low	High	P	Low	High	P
Tumour Grade			0.745			0.963
1	1	1		1	1	
2	44	33		42	35	
3	2	3		3	2	
Tumour Stage			0.809			0.967
1	2	1		2	1	
2	8	4		6	6	
3	24	22		25	21	
4	13	10		13	10	
Lymph Node Stage			0.689			0.104
0	29	24		25	28	
1	13	11		15	9	
2	5	2		6	1	
Vascular Invasion			0.510			0.079
No	22	20		19	23	
Yes	25	17		27	15	
Dukes Stage			0.184			0.123
A	10	3		6	7	
B	17	21		18	20	
C1	16	12		20	8	
C2	3	1		1	3	
Resection Margins			0.435			0.407
Free	43	35		42	36	
Involved	3	1		3	1	
KRAS Mutation			0.970			0.377
No	24	22		27	19	
Yes	15	14		14	15	

Table 7–3: The correlation of Cten and Src staining of colorectal tumours (Spearman's rank test).

	Cytoplasmic Cten Staining	
	r_s	p
Cytoplasmic Src Staining	0.176	0.114
Membranous Src Staining	0.347**	0.001

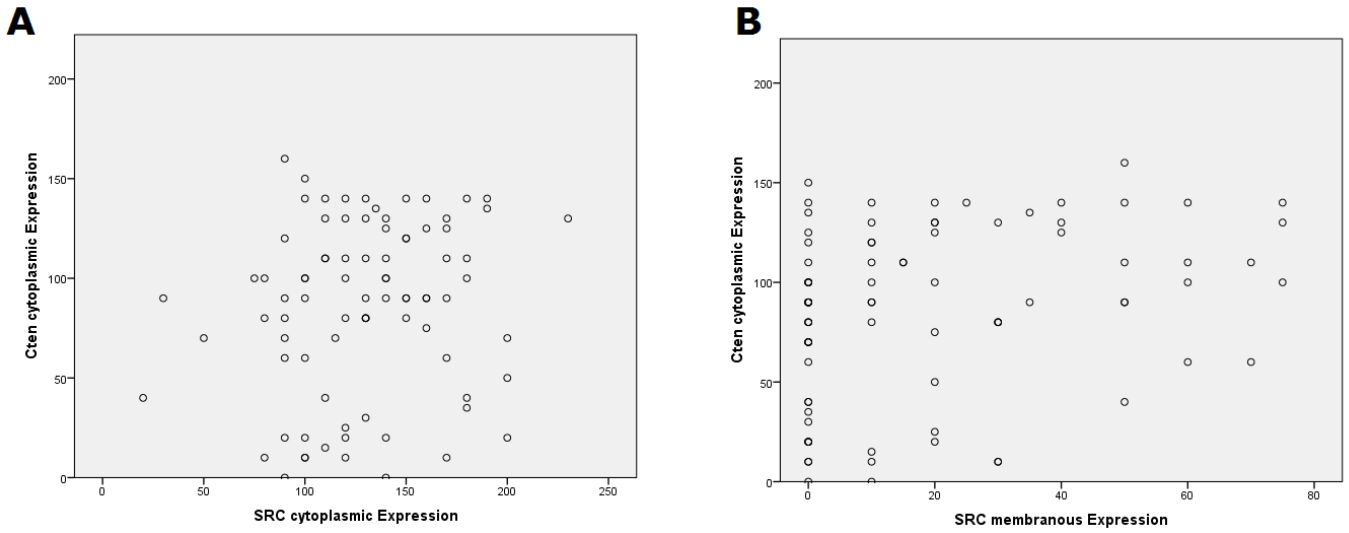


Figure 7–3: The correlation of Cten and Src staining.
A) The graphs show the correlations of Cten cytoplasmic and Src cytoplasmic, B) Cten cytoplasmic and Src membranous.

7.2.3 ROCK1 Expression in Colorectal Cancer Tissue

The role of ROCK1 in cancer is currently under debate and data on the expression of ROCK1 protein in CRC tissues are sparse. The localisation of ROCK1 was investigated in 82 cases of CRC (2 core cases could not be analysed) following antibody optimisation. ROCK1 was first optimised in appendix tissue, selected as it expresses ROCK1 protein and as the availability of colon tissue for optimisation was lacking. Antibody dilutions of 1:50, 1:100, and 1:250 were used for optimisation. A concentration of 1:50 was selected as it gave a greater staining intensity.

Following optimisation, the colon TMA was subjected to staining for ROCK1 (figure 7-4). ROCK1 was found to localise to the cytoplasm, with most of the tumour cases showing high cytoplasmic staining. The Wilcoxon signed ranks test was used to test for significant differences in ROCK1 expression in the normal colon and tumour. There was no significant difference in ROCK1 expression between normal colon and tumour tissues in cytoplasmic staining ($p= 0.463$). Membranous staining was however significantly greater in the tumour compared to normal colon ($p= 0.001$). The Chi squared test was used to test for associations between ROCK1 staining and the clinical features. ROCK1 staining was not associated with tumour grade, tumour stage, lymph node stage, vascular invasion, Dukes' stage, resection margin, or KRAS mutation (table 7-4). Interestingly, the Spearman's rank statistical test demonstrated that ROCK1 expression did positively correlate with Cten cytoplasmic staining (table 7-5), (figure 7-5) and with both Src cytoplasmic and membranous staining (table 7-6), (figure 7-6).

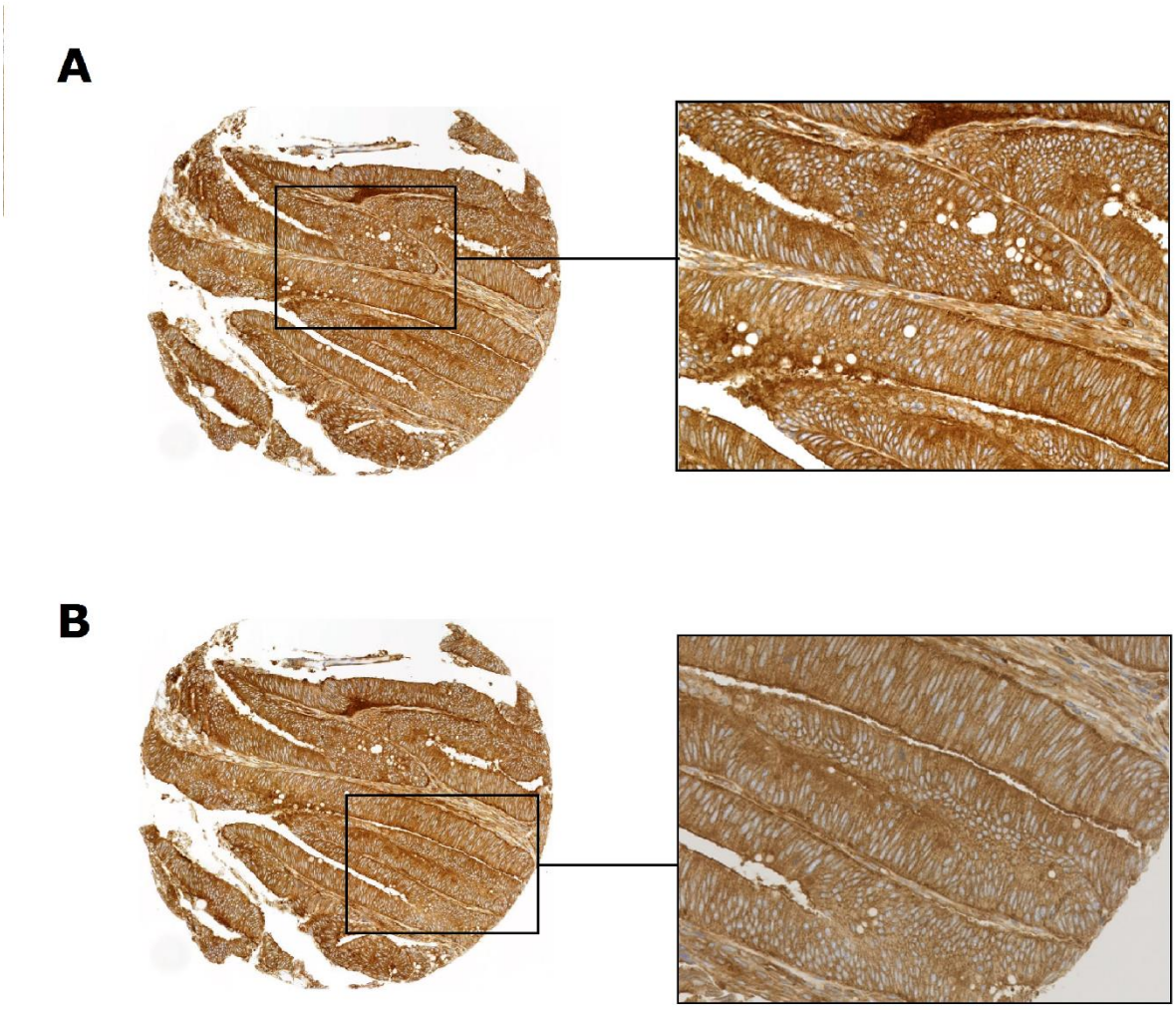


Figure 7–4: ROCK1 staining of colorectal tumours.
A) TMA cores of CRC revealed ROCK1 expression in the cytoplasm. B) ROCK1 staining was also localised to the membrane.

Table 7–4: The association between ROCK1 expression in colorectal tumours and the clinicopathological features using the chi-squared statistical test.

	Cytoplasmic Staining			Membranous Staining		
Parameter	Low	High	P	Low	High	P
Tumour Grade			0.444			0.382
1	1	1		2	0	
2	38	37		47	30	
3	4	1		4	1	
Tumour Stage			0.705			0.146
1	1	2		3	0	
2	5	7		5	7	
3	24	21		32	14	
4	13	9		13	10	
Lymph Node Stage			0.527			0.394
0	25	26		33	20	
1	13	11		17	7	
2	5	2		3	4	
Vascular Invasion			0.825			0.821
No	21	20		26	16	
Yes	22	19		27	15	
Dukes Stage			0.607			0.793
A	5	8		7	6	
B	19	17		24	14	
C1	15	13		19	9	
C2	3	1		2	2	
Resection Margins			0.330			0.588
Free	38	38		48	30	
Involved	3	1		3	1	
KRAS Mutation			0.197			0.918
No	19	25		28	18	
Yes	17	12		18	11	

Table 7–5: The correlation of Cten and ROCK1 staining of colorectal tumours (Spearman's rank test).

	Cytoplasmic Cten Staining	
	r_s	p
Cytoplasmic ROCK1 Staining	0.250*	0.024
Membranous ROCK1 Staining	0.165	0.138

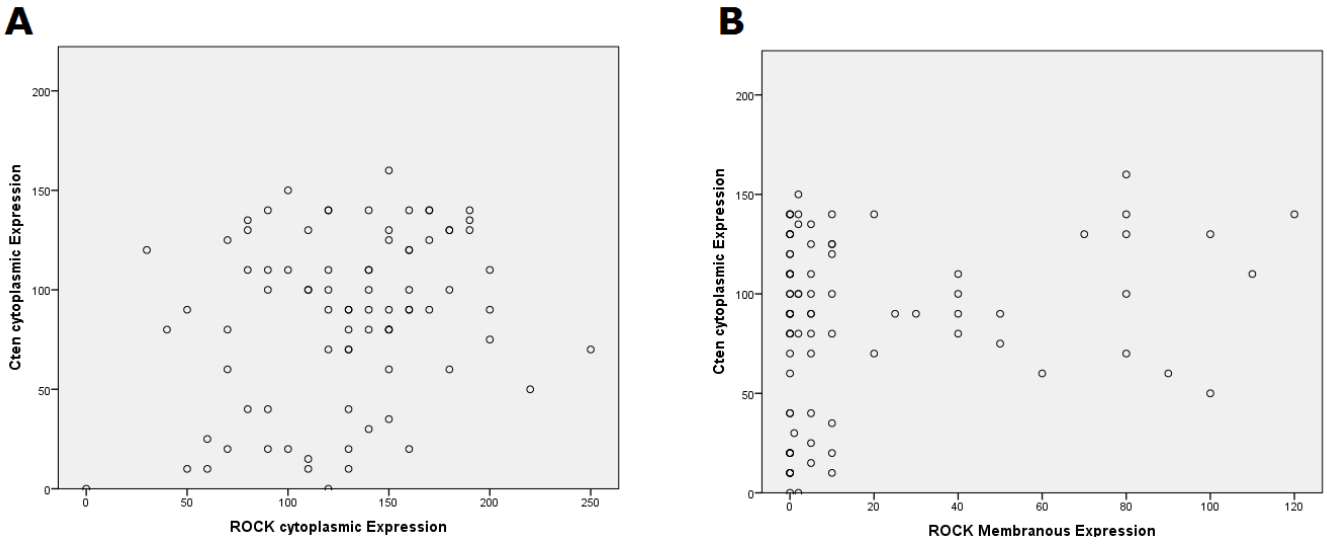


Figure 7–5: The correlation of Cten and ROCK1 staining
A) The graphs display the correlations of Cten cytoplasmic and ROCK1 cytoplasmic, B) Cten cytoplasmic and ROCK1 membranous.

Table 7–6: The correlation of ROCK1 and Src staining of colorectal tumours (Spearman's rank test).

	Cytoplasmic Src Staining		Membranous Src Staining	
	r_s	p	r_s	p
Cytoplasmic ROCK1 Staining	0.386**	0.000	0.222*	0.045
Membranous ROCK1 Staining	0.215*	0.050	0.245*	0.027

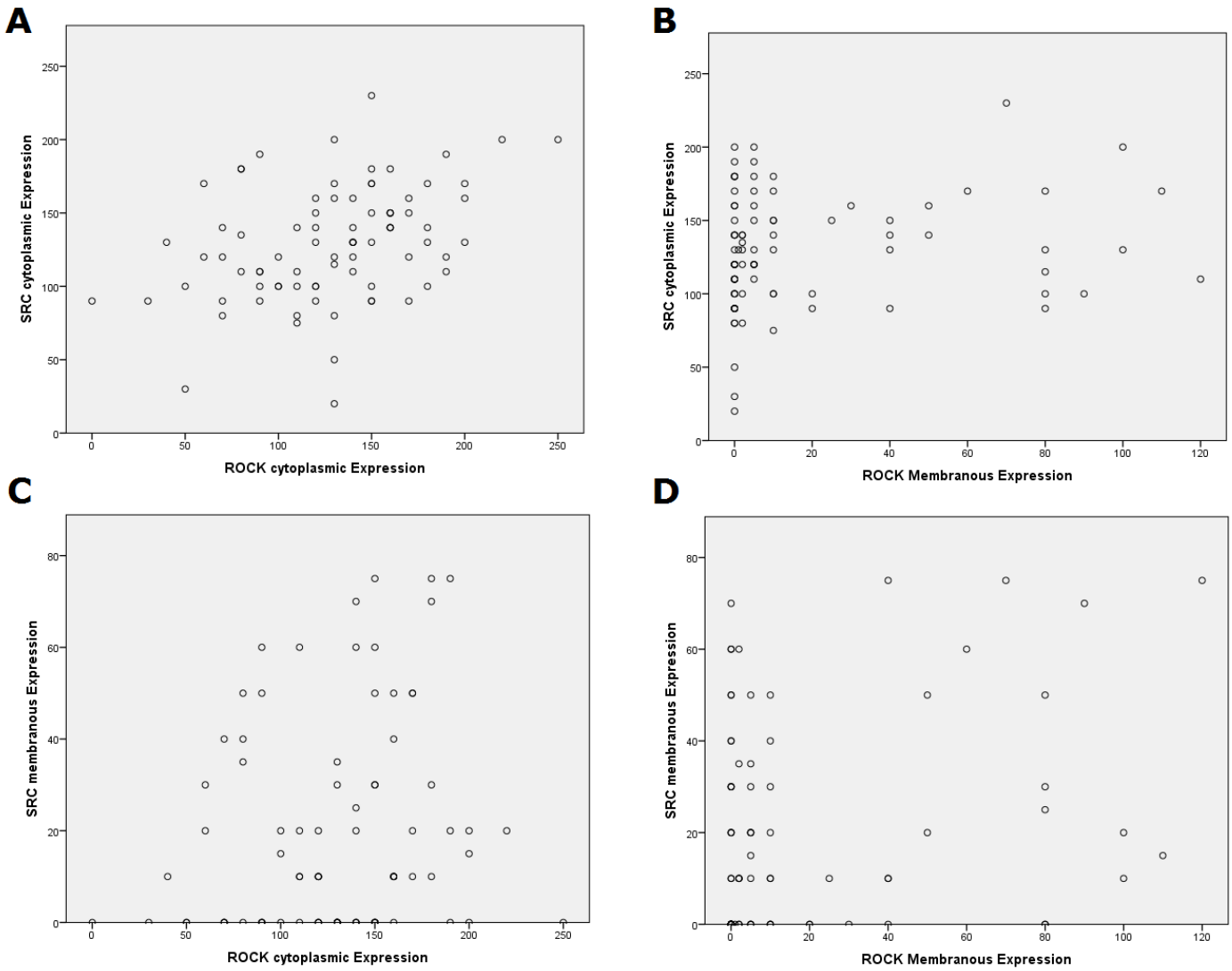


Figure 7–6: The correlation of Src and ROCK1 staining.
A) The graphs display the correlations of Src cytoplasmic and ROCK1 cytoplasmic, B) Src cytoplasmic and ROCK1 membranous, C) Src membranous and ROCK1 cytoplasmic, D) Src membranous and ROCK1 membranous.

7.2.4 Nuclear Localisation of Cten Increases Cell Motility and Colony Formation Abilities.

Cten was shown to bind to β-catenin in the nucleus, a component of the Wnt signalling pathway that is frequently upregulated in CRC (Liao et al., 2009). Additionally, Cten nuclear localisation was found to be associated with metastasis (Albasri et al., 2011a). The targeting of Cten to the nucleus was used in this study to investigate the role of Cten nuclear localisation in CRC cells. A construct containing GFP-Cten tagged with a nuclear localisation signal (GFP-Cten-NLS) was previously generated by Dr Maham Akhlaq. Since Cten was shown to positively regulate Snail, Src, and ROCK1 protein levels, it was of interest to determine whether targeting of Cten to the nucleus would also increases Cten's downstream signalling proteins. To investigate this, Cten was forcibly expressed using GFP-Cten-NLS construct, together with GFP-Cten, and the GFP-empty vector as control in both HCT116 and SW620$^{\Delta Cten}$ CRC cell lines and the changes in protein level of Snail, Src, and ROCK1 were determined by western blot. Overexpression of GFP-NLS was associated with a small increase in the expression of all downstream proteins compared to cells transfected with GFP-EV construct in both HCT116 and SW620$^{\Delta Cten}$ cell lines. However, the changes in protein level of Snail, Src, and ROCK1 following overexpression of GFP-Cten-NLS construct was always less than GFP-Cten expression (figure 7-4 A), (figure 7-5 A).

The functional activity of Cten in the nucleus is largely unknown. To investigate this, GFP-Cten-NLS was overexpressed in both HCT116 and SW620$^{\Delta Cten}$ cell lines together with GFP-Cten and GFP-EV control and the changes in cell function assessed using several functional assays (figure 7-4), (figure 7-5). The effect of Cten on cell migration was determined using both wound healing and Transwell migration assays. In both cell lines, cell migration was increased when cells were transfected with the GFP-Cten plasmid compared to the GFP-EV plasmid control. Overexpression of GFP-Cten-NLS construct was associated with an increase in cell migration further still, indicating that nuclear localisation of Cten acts to induce cell migration compared to the wild type Cten.

Furthermore, cell invasion was increased when cells were transfected with GFP-Cten-NLS construct compared to both wt. GFP-Cten and the GFP-EV control in both HCT116 and SW620$^{\Delta Cten}$ cell lines.

The soft agar assay was also performed to assess the effect of nuclear localised Cten on colony formation abilities. GFP-Cten transfected HCT116 cells were associated with an increase in colony number compared to the GFP-EV control and cells transfected with GFP-Cten-NLS gave a greater number of colonies than GFP-Cten transfected cells. This was further validated in the SW620$^{\Delta Cten}$ cell line which displayed a similar pattern of colony formation efficacy. This implies that localisation of Cten to the nucleus may increase colony formation abilities compared to the wt. Cten.

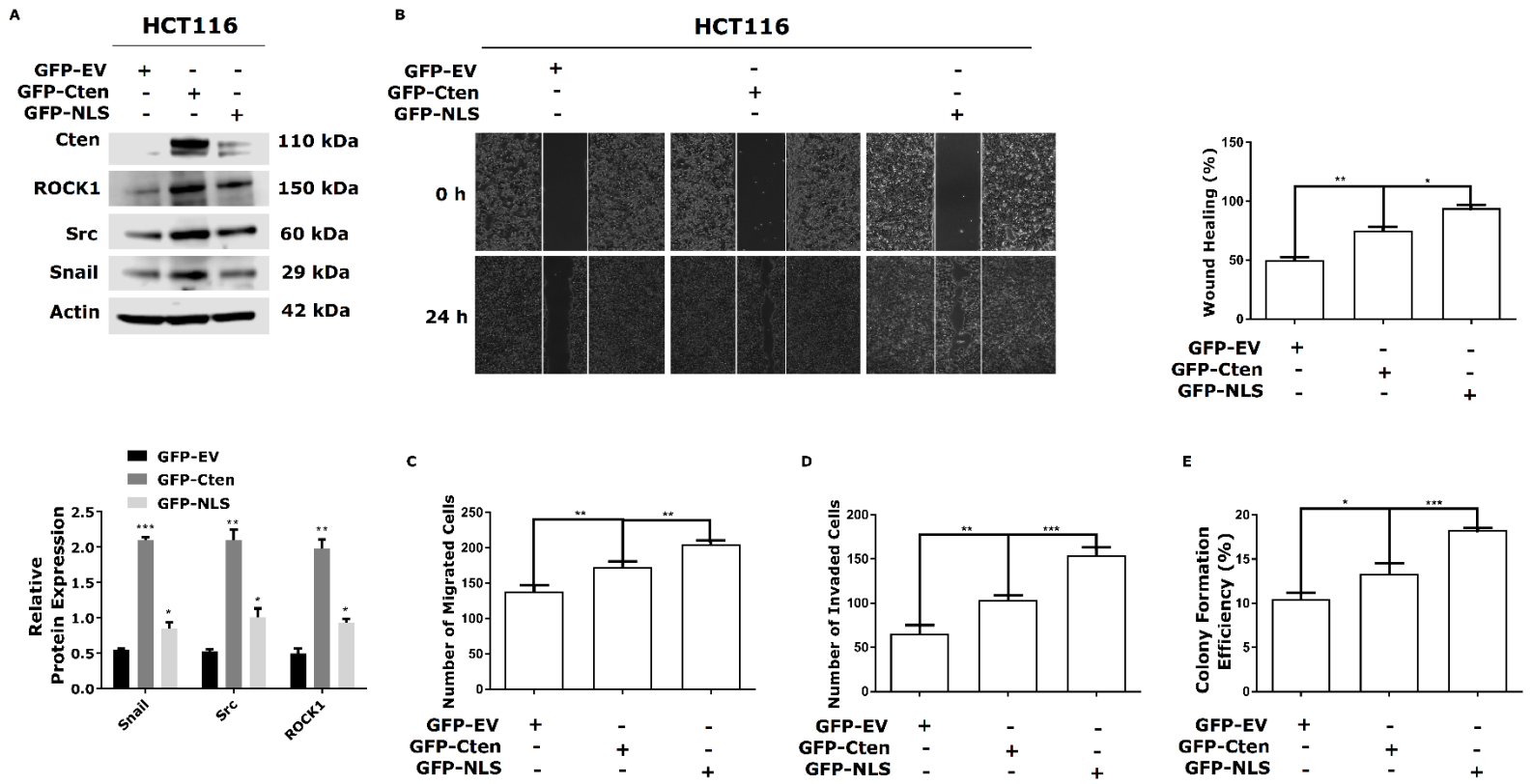


Figure 7–7: Nuclear Cten increases HCT116 cell functions.
A) HCT116 cells were overexpressed with GFP-Cten and GFP-Cten-NLS, and the changes in, ROCK1, Src, and Snail protein expression were determined by western blot. Graph on the lower panel represents the densitometry values quantified for each protein normalised to actin (n=3). B) Wound healing assay revealed increased closure of wound following GFP-Cten forced expression (P = 0.0052) and this was further increased when Cten was targeted to the nucleus (GFP-Cten-NLS), (P = 0.0119). C) Upon overexpression of GFP-Cten in HCT116 a greater number of cells migrated through the Transwell membrane (P = 0.0035) and this migratory effect was further increased upon the targeting of Cten to the nucleus (P = 0.0050). D) Overexpression of GFP-Cten increased cell invasion (P = 0.0030) and this was further induced with GFP-Cten-NLS construct (P = 0.0006). E) Overexpression of GFP-Cten in HCT116 gave higher colony formation efficiency than the empty vector control (GFP-EV) (P = 0.0144) and more colonies were formed when Cten was targeted to the nucleus (P = 0.0009). Results are represented as the mean ±SD of two independent experiments (one-way ANOVA).

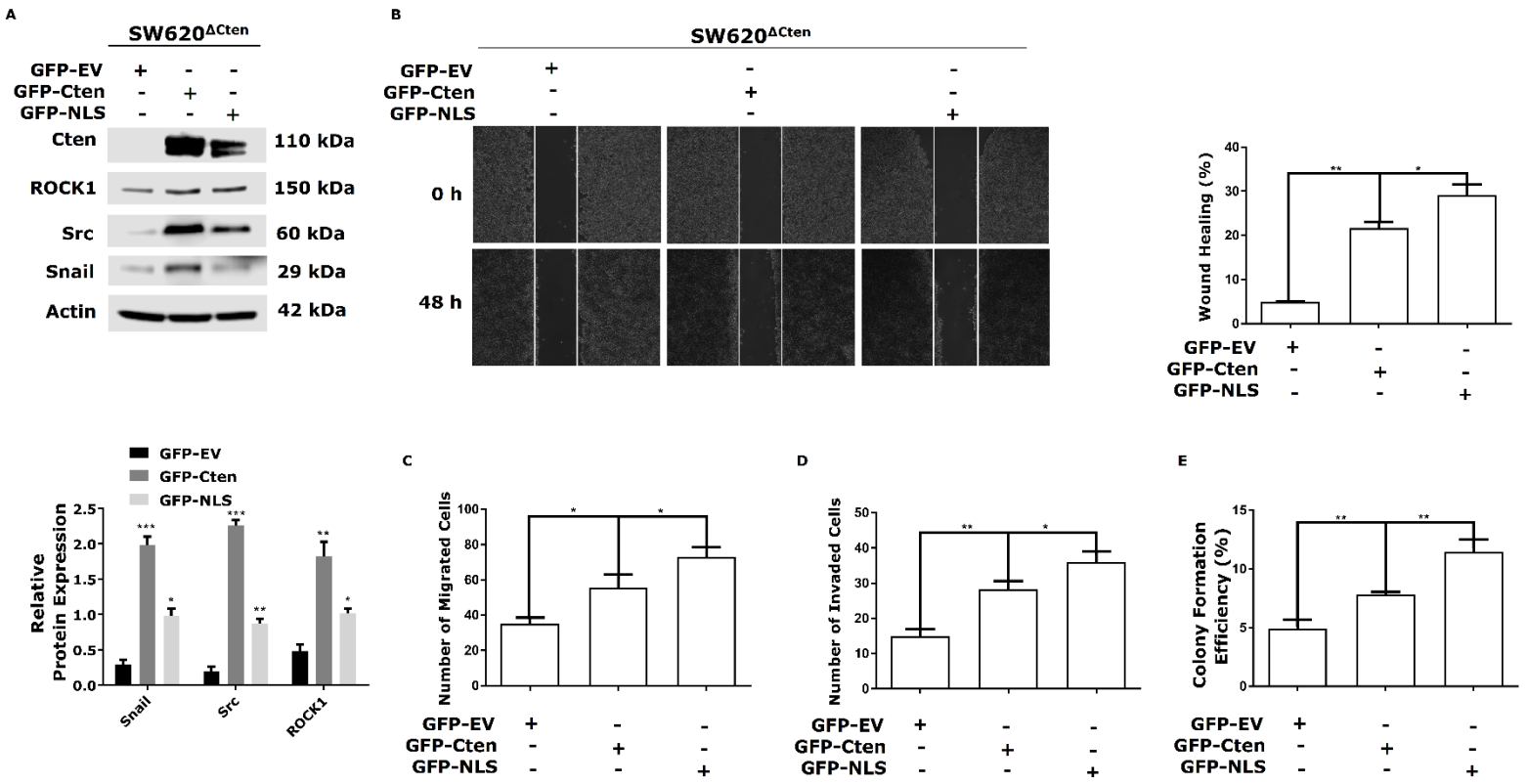


Figure 7–8: Nuclear Cten increases SW620$^{\Delta Cten}$ cell functions.
A) SW620$^{\Delta Cten}$ cells were overexpressed with GFP-Cten and GFP-NLS, and the changes in, ROCK1, Src, and Snail protein expression were determined by western blot. Graph on the lower panel represents the densitometry values quantified for each protein normalised to actin (n=3). B) Overexpression of GFP-Cten in HCT116 increased wound closure and induced cell migration (P = 0.0040) and this was further increased when Cten was subsequently targeted to the nucleus (GFP-NLS), (P = 0.0397). Overexpression of GFP-Cten in SW620$^{\Delta Cten}$ was associated with an increase in cell migration (P = 0.0114) and this migratory effect was further increased upon the targeting of Cten to the nucleus (P = 0.0248). D) Overexpression of GFP-Cten resulted in an induction of cell invasion (P = 0.0014) and this was further induced with GFP-NLS transfected SW620$^{\Delta Cten}$ cells (P = 0.0210). E) GFP-NLS transfected SW620$^{\Delta Cten}$ cells gave higher colony formation efficiency than GFP-Cten transfected cells (P =0.0023) which produced more colonies than the empty vector control (GFP-EV) (P = 0.0069). Results are represented as the mean ±SD of two independent experiments (one-way ANOVA).

7.2.5 Cten Increases Nuclear Translocation of Src, ROCK1, and Snail in Colorectal Cancer Cells

Since both wildtype Cten and nuclear localised Cten have shown to increase cellular levels of Src, ROCK1 and Snail protein expression, it was of interest to determine whether Cten can induce Src, ROCK1, or Snail protein translocation to the nucleus. To explore this, HCT116 cells were transfected with GFP-Cten-NLS together with GFP-Cten and GFP-EV control and the expression of Src, ROCK1, and Snail in nucleus were determined by nuclear and cytoplasmic fractionation. Subcellular fractionation experiments demonstrated that a fraction of Src, ROCK1, and Snail proteins were indeed mostly translocated in the nucleus when cells were transfected with either wt. Cten or nuclear localised Cten constructs. By contrast, in HCT116 cells transfected with the empty vector control, there were little or no proteins detected in the nucleus (figure 7-6). This suggests that Cten signalling is not only regulating Src, ROCK1, and Snail at the protein level, but is also responsible for their import or export from the nucleus.

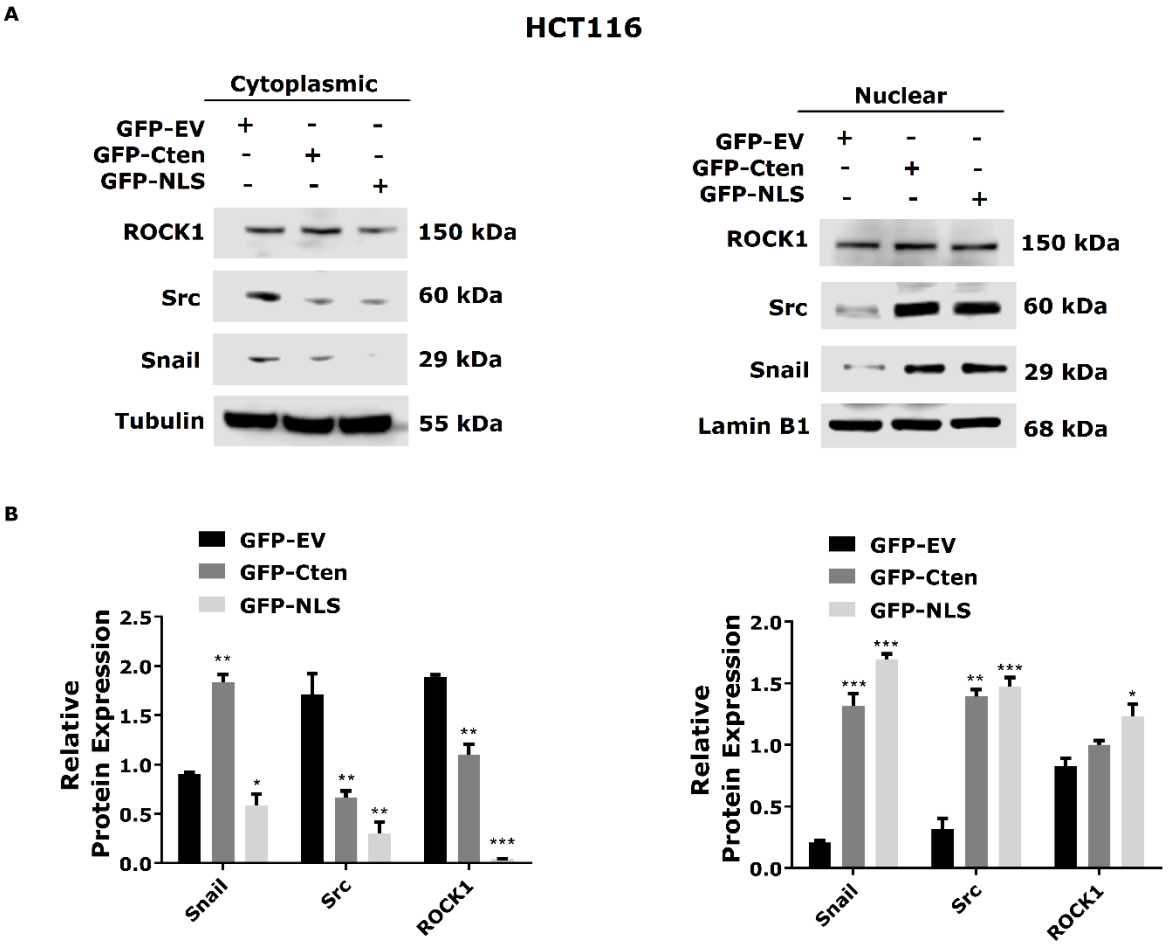


Figure 7–9: Cten and NLS increases ROCK1, Src, and Snail nuclear localisation in HCT116 cells.
*A) Subcellular fractionation experiment was performed following transfection of HCT116 cell with GFP-Cten and GFP-Cten-NLS and the lysates were assessed by western blot analysis. Lamin B1 and tubulin were used as loading control for cytoplasmic and nuclear fractions. B) Quantitative determination of the relative expression of ROCK1, Src, and Snail protein fractions following overexpression of GFP-Cten and GFP-Cten-NLS constructs in HCT116. Results are represented as the mean ±SD of two independent experiments (one-way ANOVA, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).*

7.3 Discussion

Cten has previously been found to localise to the nucleus, however, the relevance of nuclear localised Cten in terms of cell signalling and tumour progression is unknown (Albasri et al., 2011a). The transport of proteins between the cytoplasm and nucleus is a highly controlled process. The NLS usually carries its cargo protein and enables it to bind to nuclear import receptor protein known as importin which, helps its translocation into the nucleus (LaCasse and Lefebvre, 1995, Köhler et al., 1999).

Previous studies have determined the expression and localisation of Cten in CRC tissue and this was validated herein in a limited set of tumours (Albasri et al., 2011a). Cten staining was found to localise mainly to the cytoplasm with all cases showing high cytoplasmic staining. This chapter also investigated the role of nuclear localised Cten in CRC cells. Although the GFP-Cten-NLS construct has previously been optimised by our group, the expression of Cten using this construct was consistently lower than overexpression of the wt. Cten construct in both HCT116, and SW620$^{\Delta Cten}$ cell lines. This may be because of the presence of multiple start codons within the primer used to create the GFP-Cten-NLS plasmid, which may have led to reduced translation of the full NLS tag and protein expression. It is also possible that the nuclear localised Cten construct may have undergone a change reducing its expression levels or transfection efficiency in mammalian cells (unpublished data). Overexpression of nuclear localised Cten construct was associated with a small increase in Src, ROCK1, and Snail protein expression. However, as mentioned above, there were issues with the NLS construct expression which may compromise this due to Cten being more weakly expressed. Despite the low levels of protein expression, the GFP-Cten_NLS was highly efficient in inducing functional changes. The nucleus-targeted Cten was shown to further induce functional changes including cell migration, invasion and colony formation abilities compared to the GFP-Cten (which was expressed as a much higher level). The reason for such efficient induction of the functional activities is uncertain. Given the fact that

nuclear Cten has been shown to be in complex with β-catenin, one possible explanation is that the nuclear Cten enhances β-catenin/Wnt signalling.

Proper subcellular localisation of proteins is critical for their functional activity within the cells. Snail is an important transcriptional regulator of cell activity in response to extracellular signals and it needs be translocated from the cytoplasm to the nucleus to function (Barrallo-Gimeno and Nieto, 2005). Previous studies have shown that phosphorylation of Snail is essential for its post-transcriptional regulation, which in turn regulates its subcellular localisation (Domínguez et al., 2003, Zhou et al., 2004). One of the kinases that phosphorylates Snail is glycogen synthase kinase 3β (GSK3β), which not only induces nuclear export of Snail, but also its rapid degradation via the ubiquitin proteasome pathway (Zhou et al., 2004, Kim et al., 2012). It has been shown that PAK1 induced phosphorylation favours the nuclear localisation of Snail, thus enhancing its activity (Yang et al., 2005). The results herein showed that both wt. Cten and nuclear localised Cten increase nuclear localisation of Snail. This suggests that Cten not only regulates Snail protein expression through protein stabilisation, but also may be involved in the regulation of its shuttling into the nucleus and thus regulating its function. It is possible that Cten could prevent Snail protein from degradation by downregulating the GSK3β pathway. Therefore, it would be of interest to investigate whether Cten signalling is involved in this process.

The proto oncogene Src has been found to be overexpressed and activated in several types of tumour tissues and commonly associated with the development of tumours and progression to distant metastasis (Byun et al., 2017; Chen et al., 2014). The localisation of Src expression in colorectal tumours and normal colon was consistent with Src's role and function in cancer. Src membranous localisation was more prominent in the tumour where it can initiate intercellular signal transduction signalling pathways that promote cell migration and invasion, whereas Src cytoplasmic localisation is more prominent in normal colon tissue. Cten expression was shown to correlate with Src staining only in the

membranous compartment. This increases the possibility that Cten expression could be responsible for Src activation. However, Src expression was not associated with any of the clinical features of this data set.

Subcellular localisation of Src is essential for its functional activity. Src usually remains inactive in the cytoplasm, however, upon external stimuli, Src is translocated from the cytoplasm to the cell membrane, becoming fully activated by phosphorylation at the site of Y416 to exert its biological effects (Ingley, 2008; Bolós et al., 2010). The activated Src within the cell membrane has been shown to initiate intercellular signal transduction signalling pathways that promote cell function (Frame, 2002). The participation of Cten in inducing cytoplasmic to nuclear translocation of Src was investigated in this study. Interestingly, overexpression of wt. Cten resulted in an increase of the nuclear translocation of Src protein. The nuclear translocation of Src was further increased when Cten was localised to the nucleus, suggesting that co-localisation of Cten with Src is important for regulating Src function.

The expression of ROCK1 has been reported to be upregulated in most tumour tissues including CRC (Liu et al., 2011, Cai et al., 2015, Xi et al., 2015). Although a subcellular localisation of ROCK1 has not been well described, most of studies on ROCK1 subcellular localisation demonstrated that ROCK1 protein is predominantly expressed in the cytoplasm in several cancer cells types (Julian and Olson, 2014). ROCK1 may also be localised at the cell membrane (Stroeken et al., 2006). The findings herein revealed that ROCK1 expression is mainly localised to the cytoplasm with most of the CRC cases. A similar phenomenon of a prevalent stronger positivity for ROCK1 expression was observed in the cytoplasm of colorectal tumour from CRC patients (Sari et al., 2013). A significant correlation between ROCK1 and Cten expression was observed in cytoplasmic staining. Interestingly, cytoplasmic ROCK1 localisation was also positively correlated with Src expression in both the cytoplasmic and membranous compartments. This implies that there was a marked increase in Cten/Src/ROCK1 expression in CRC patients.

However, there was no significant associations between ROCK1 expression and the clinicopathological features of this data set. The biological activity of ROCK1 in cancer tissues can be regulated via alteration of its subcellular localisation. The findings herein showed that nuclear localised Cten can degrade ROCK1 protein from the cytoplasm and translocate it to the nucleus, suggesting that Cten may act as a co-regulator of ROCK1 translocation only in the nucleus.

In conclusion, although this study obtained very interesting findings, there are some disadvantages of these experiments that may account for discrepancies observed with previous studies, such as the limited size of the data set and the cores were taken only from three different areas of the tumour during TMA construction, which may weaken any overall effect if Cten, Src, or ROCK1 are only localised to specific regions of the tumour tissue which were not sampled in the cores. The current study demonstrated that Cten could increase nuclear translocation of Src, ROCK1 or Snail, promoting cell motility and colony formation abilities when targeted to the nucleus. Although wt. Cten was unable to form a physical complex with Snail, Src, and ROCK1 as mentioned in the previous chapter, it would be of interest to determine whether nuclear localised Cten forms part of a large complex in the nucleus and binds to any of its downstream targets Snail, Src, or ROCK1, which may give further clues to its role in this localisation. Furthermore, it would be interesting to determine whether Cten transports between the cytoplasmic and nuclear compartments by using inhibitors of the nuclear import and export translocation process.

8 General Discussion

8.1 Introduction

Cten is the smallest member of the tensin family which contains C-terminus homology, but lacks the N-terminus actin binding domain present in the other tensin protein members (Lo, 2004). Cten is gaining prominence as oncogene in a vast range of different cancers including CRC (Albasri et al., 2009, Al-Ghamdi et al., 2011, Thorpe et al., 2015). Although several recent studies have investigated the role of Cten in different tumour tissues, data about either its regulation and its downstream signalling pathways are lacking. The central aim of this thesis was to gain a deeper insight into the mechanisms of Cten function in CRC cells in order to further understand the signalling pathways involved in tumour metastasis. The downstream signalling of Cten and regulation were investigated in *in vitro* models by the manipulation of cell signalling pathways to determine its role in CRC cells. Having found the potential signalling targets and regulation of Cten, functional assays were then performed to evaluate the relevance of such interactions in CRC cells. This study has revealed a number of novel findings regarding Cten signalling in colorectal tumour, however, this data needs to be validated and extended in the future.

8.2 Project Overview and Summary of Findings

8.2.1 Cten Increases Tumourigenicity and Cell Motility Through the Upregulation of Src/ROCK1/Snail Signalling Axis

Cten is known to increase cell motility and colony formation abilities in a variety of different cancers (Sasaki et al., 2003a, Sasaki et al., 2003b, Albasri et al., 2009, Albasri et al., 2011b, Al-Ghamdi et al., 2013). However, to date, only a small number of Cten downstream targets have been identified. Since Cten is a recently described gene, the data about how Cten regulates cell functions is lacking. This study, for the first time, has identified Src, ROCK1, and Snail as potential novel downstream targets of Cten in CRC cells and signalling through Src/ROCK1/Snail pathways was responsible for the induction of cell motility and colony formation mediated by Cten.

EMT signalling is a critical process occurring during tumour metastasis. It has been shown to induce migration and invasion of cancer cells away from primary tumour (Radisky, 2005) Therefore, it was of interest to investigate whether Cten is involved in the induction of EMT processes through the upregulation of Snail expression, a master regulator of EMT. Cten expression was manipulated by multiple approaches including forced expression, gene knockdown, and constitutive deletion in different cell lines to eliminate artefacts of methodology and cell line specific effects. Manipulation of Cten expression was followed by similar changes in expression level of the Snail protein. This is the first study to identify Snail as a novel downstream target of Cten in CRC cells. In addition, Cten was shown to regulate Snail expression through post-transcriptional mechanisms, probably via stabilisation of the Snail protein. This positive interaction was also functionally relevant. An abrogation in cell motility and colony formation efficiency was noticed when Cten was forcibly expressed and Snail simultaneously knocked-down using targeted siRNA knockdown. This suggests that Cten regulates these cell functions in a Snail dependent manner.

The stabilisation of the Snail protein by Cten is a novel finding and provides further evidence that Cten is involved in the regulation of EMT processes and promotes tumour metastasis. However, downregulation of E-cadherin, an epithelial marker, is mediated through the upregulation of the mesenchymal marker, N-cadherin, but this was not observed following the modulation of Cten expression in CRC cells (Thorpe et al., 2017). This suggests that the Cten/Snail/N-Cadherin/E-cadherin dependent pathway is unlikely to be important in CRC cells. Although both E-cadherin and N-cadherin are well described downstream targets of Snail signalling, Snail also signals downstream to other proteins such as occludin and claudins which could also be downstream targets of Cten (Ikenouchi et al., 2003). The phosphorylation of Snail regulates its export from the nucleus following degradation through the ubiquitin proteasome pathway (Domínguez et al., 2003). Cten has been associated with the stabilisation of proteins through binding to c-Cbl via its SH2 domain. It has been shown that the SH2 domain of Cten can protect the EGFR protein from degradation by the ubiquitin proteasome (Hong et al., 2013). Therefore, it is possible that Cten prevents the degradation of Snail protein through the regulation of this process.

The proto oncogene Src was also investigated in this thesis. Src is a non-receptor cytoplasmic tyrosine and one of the important kinases in focal adhesion complexes. It has been shown to physically bind and interact with the known downstream pathway of Cten signalling, FAK protein at focal adhesion dynamics. Although the relationship between Cten and Src has not previously been interrogated, Src has been found to be linked with other tensin proteins (Davis et al., 1991). Therefore, this led us to hypothesise that Src expression may also be under the regulation of Cten signalling. This study has revealed for the first time that Cten is a positive regulator of Src protein expression. In addition to the stabilisation of Snail protein by Cten signalling, Cten also regulates the stability of Src protein and it may accomplish this stability through the upregulation of Src expression by Cten signalling, therefore preventing Src from degradation at a post-transcriptional level. It is possible that Cten may promote Snail

protein stability through Src signalling. However, the precise mechanisms by which this stabilisation occurs remains unclear. Earlier studies have suggested that Src protein could be degraded through the proteasome-ubiquitin dependent pathway (Hakak and Martin, 1999). Additionally, it has been suggested that Src protein stabilisation is most likely to be induced through the upregulation of Akt/mTOR/4E-BP1 pathways and inhibition of calpain-mediated Src protein degradation by ErbB2 signalling (Tan et al., 2005). It is possible that Cten could protect Src protein from degradation by regulating the ErbB2 pathway and/or Src subsequent degradation via the ubiquitin proteasome pathway. Therefore, it would be interesting to explore whether Cten signalling is involved in this process.

Having shown that Cten regulates Src, ROCK1, and Snail protein expression, next it was investigated whether Src is signalling intermediate in the Cten/ROCK1 or Cten/Snail pathway. Previous reports have shown that both Cten and Src downregulate E-cadherin expression level, most likely through the positive regulation of the well-known transcriptional repressor, Snail signalling (Guo and Giancotti, 2004, Nagathihalli and Merchant, 2012, Thorpe et al., 2017). Furthermore, both Cten and Src have been shown to regulate FAK, which is also implicated in the regulation of Snail signalling (Carragher et al., 2003, Al-Ghamdi et al., 2013). The present study has shown that Cten could signal to Snail downstream of Src. ROCK1 has been found to be phosphorylated by Src at focal adhesion complexes and this phosphorylation resulted in a downregulation of RhoA binding activity of ROCK1, which then leads to continued myosin-mediated contractility and focal adhesion elongation during lysophosphatidic acid activation (Lee et al., 2010). Interestingly, the current study also revealed that Cten could signal to ROCK1 through the positive regulation of Src signalling. The interactions between Cten and Src signalling was also functionally active. Overexpression of the Cten construct and knockdown of Src expression simultaneously was associated with similar levels of abrogation of cell migration, invasion, and colony formation efficiency. These findings confirm that the positive regulation of Src expression by Cten signalling is not just a

bystander phenomenon. Previous reports from this lab have shown that Cten promotes cell motility through the upregulation of FAK and ILK. Both proteins are now known to play a role in the regulation of EMT processes (Albasri et al., 2011a, Al-Ghamdi et al., 2013), therefore, it is possible that FAK or ILK could be signalling intermediates in the Cten/Src pathway. Investigations into signalling downstream of Cten to FAK or ILK to induce Src protein stability is also warranted.

ROCK1 has been shown to regulate focal adhesions turnover and actin stress fibre remodelling through the phosphorylation of FAK and is involved in the regulation of EMT processes through Snail signalling at a post-transcriptional level (Lock et al., 2012, Zhang et al., 2016). ROCK1 was identified as a novel target of Cten and is probably regulated by Cten through the Src signalling pathway via post-transcriptional mechanisms. Surprisingly, this study also revealed that Cten could signal downstream to regulate both Src and Snail protein expression through ROCK1. These novel findings imply that both Src and ROCK1 are dependent signalling intermediates in the Cten/Snail pathway. This study also confirmed that ROCK1 and Cten are functionally relevant in CRC cells. Overexpression of Cten and knockdown of ROCK1 simultaneously was associated with decreases in cell migration, invasion and colony formation efficiency. These novel findings suggest that Cten could promote cell motility and colony formation efficiency through the positive regulation of the ROCK1/Src/Snail signalling dependent pathway, consequently inducing CRC metastasis.

Considering these findings, it is most likely that Cten is implicated in the induction of EMT processes via the Src/ROCK1/Snail dependent pathway, thereby contributing to the influences of Cten mediated cell motility *in vitro*. Further validations of whether these pathways contribute to cell motility *in vivo* and colorectal tumour metastasis are required to confirm these findings. In conclusion, this study has identified novel downstream targets of Cten signalling which may cooperate with Cten to promote cell motility and

colony formation in CRC cells. Elucidation of the underlying signalling mechanisms that regulate cell functions may help to identify novel biomarkers for therapeutic targeting of cancer metastasis.

8.2.2 Cten Regulates Cell Motility and Colony Formation Through Src/ROCK1/Snail Pathway in an SH2 Dependent Manner

Cten has been found to play a role in the regulation of many cellular processes including cell migration, invasion, and colony formation in a variety of different tumour cells, however the understanding of the underlying mechanisms of how Cten functions and regulates these cell properties is lacking.

Cten contains the SH2 domain and PTB signalling component of the other tensins, but lacks the actin binding domain capabilities and consequently, may play a novel role in biological events. Several reports have shown that the SH2 domain of Cten is essential for its functional activity within the cell. The mutation of arginine at position 474 to alanine was found to be critical for the SH2 domain of Cten function. It was found that arginine in the SH2 domain of Cten is responsible for interacting with the DLC1 in a tyrosine phosphorylated independent manner, resulting in a localisation of DLC1 to the focal adhesion complexes (Liao et al., 2007). Others have shown that the mutation of arginine to alanine in the SH2 domain of Cten prevented the transition from extension formation to during tubule formation, which subsequently leads to an increase in the activation of basal STAT3 in MDCK cells (Kwon et al., 2011) Furthermore, previous reports from our laboratory have suggested that, in addition to the critical arginine at 474, tyrosine at position 479 within the SH2 domain of Cten is important for its function in CRC cells. However, when arginine 474 was mutated to alanine or tyrosine 479 to phenylalanine individually, it did not cause complete reduction of Cten's effects on colony formation and cell migration (submitted for publication). This led us to hypothesise that a combination of both mutations together in one construct may lead to inactivation of the functional activity of Cten signalling and abolish its ability to interact with its downstream targets to induce cell motility and colony formation in CRC cells. The combination of the two mutations together in one construct has revealed very interesting findings. Using a construct in which the SH2 domain of Cten was inactivated (GFP-Cten$^{R474A+Y479F}$), there were reduced expression levels of ROCK1, Src and Snail protein

with this construct. Cten was shown to increase the stabilisation of both Src and Snail protein, probably due to the signalling downstream of Cten mediated by the SH2 domain. The SH2 domain of Cten is not only important for interacting with its downstream pathways, but also important for its biological function in inducing cell motility and colony formation efficiency. The combined mutations have demonstrated a complete abrogation of Cten's abilities to promote migratory capabilities and colony formation efficiency in CRC cells. Taken together, these findings suggest that Cten signalling to Src/ROCK1/Snail pathway to promote cell motility and colony formation abilities is probably mediated via the SH2 domain.

In conclusion, this study has provided a novel dimension in the regulation of Cten's biological function and potential mechanisms involved in colorectal tumour, raising new questions which may lead to further investigations into its function in the future.

8.2.3 Upregulation of Cten Expression by TGFβ1 Increases the Cell Migration and Invasion of Colorectal Cancer Probably Through Src/ROCK1 Signalling Axis

It is of importance to investigate and understand the mechanisms regulating tumour metastasis. EMT is a critical process occurring during metastasis and Cten was found to be implicated in regulating this process, however, the mechanisms that upregulate Cten mediated EMT have not been fully elucidated (Albasri et al., 2009, Thorpe et al., 2017). Previous published data has suggested that Cten signalling could be regulated by KRAS, EGFR, or STAT3 signalling in several tumour types (Al-Ghamdi et al., 2011, Thorpe et al., 2015). It has also been shown that Cten is regulated by several cytokines and growth factors (Hung et al., 2014). Since EMT processes are regulated by Cten signalling in CRC, it was hypothesised that the TGF-β1 may signal upstream of this pathway. This hypothesis, in addition to providing further elucidation and understanding of signalling mechanisms leading to metastasis in colorectal tumour, would also potentiate the use of Cten as a therapeutic target, since it could be a signalling intermediate in the TGF-β1/EMT pathway.

Cten was identified as a novel downstream target of TGF-β1 signalling in CRC cells and the involvement of Cten in the regulation of EMT processes induced by TGF-β1 signalling was also confirmed. TGF-β1 and Cten were shown to signal in the same pathway, but it is possible that TGF-β1 and Cten signal independently with regard to N-cadherin expression. TGF-β1 signalling could regulate Cten expression via several downstream pathways including Ras/MAPK (Janda et al., 2002), RhoA (Bhowmick et al., 2001), and Jagged 1/Noch (Zavadil et al., 2004), which could be confirmed by manipulation of these signalling pathways. The ability of TGF to regulate cell migration, invasion, and proliferation in CRC cells was also confirmed in this thesis using multiple approaches, and is in keeping with previously published data (Pang et al., 2016, Bu and Chen, 2017). The interaction between TGF-β1 signalling and Cten was also functionally relevant. The knockout of Cten was shown to block the effect of TGF-β1 stimulation on both cell

migration and invasion in the SW620$^{\Delta Cten}$ cell line, but had no effect on TGF-β1 induced cell proliferation. Cten has previously been reported to regulate cell motility but not cell proliferation in CRC cells (Albasri et al., 2009). Together, this suggests that Cten is not involved in TGF-β1 mediated cell proliferation, but it could be acting as a mediator of TGF-β1 induced cell migration and invasion in CRC cells. It is likely that Cten is an intermediate signalling protein in TGF-β1 mediated cell migration via other downstream pathways, including JAK/STAT3 (Liu et al., 2014), PI3K-Akt (Bakin et al., 2000), and Reelin (Yuan et al., 2012).Therefore, it would be interesting to explore whether Cten acts parallelly or synergistically with these pathways in future investigations.

Despite that Cten was previously found to localise to the nucleus, the mechanisms by which the Cten protein is translocated to the nucleus to exert its biological effects is unknown (Albasri et al., 2011a). TGF-β1 was able to increase expression and nuclear translocation of Cten and other downstream pathways including ROCK1, Src and Snail in CRC cells. The nuclear translocation of Src and Snail protein induced by TGF-β1 has before been investigated in other tumour cells and this is in agreement with our data (Takahashi et al., 2009, Cho et al., 2007). Intriguingly, Cten was found to be important for TGF-β1 induced Src, ROCK1, or Snail nuclear translocation as TGF-β1 shows very limited effects in the Cten knockout cell line. This suggests that TGF-β1 directly increases expression and nuclear translocation of ROCK1, Src, and Snail protein through the positive regulation of the Cten signalling pathway.

In conclusion, these experiments have demonstrated that the TGF-β1 could signal upstream dependently of Cten to promote cell migration and invasion through the positive regulation of the Src/ROCK1/Snail pathway in CRC cells. A therapeutic inhibition of this pathway may prove useful for targeting of CRC metastasis, however further investigations are needed to confirm these findings.

8.2.4 Nuclear Localised Cten Promotes Oncogenic Function and Increases Nuclear Translocation of ROKC1, Src, and Snail in Colorectal Cancer

Cten was previously found to be localised to the nucleus and this nuclear localisation was observed to be more predominant in the metastatic tumour than the primary tumour (Albasri et al., 2011a). This work investigated the role of nuclear localised Cten in CRC cells, Cten, when forcibly expressed in the nucleus resulted in a small increase in Src, ROCK1, and Snail protein expression compared to the EV control but still lower than the wt. Cten construct expression. However, overexpression of Cten in the nucleus further increased cell motility and colony formation efficiency compared to the wt. Cten construct expression. This therefore suggests that nuclear localisation of Cten does strongly increase these cell properties, promoting tumour metastasis in CRC.

Proper subcellular localisation of proteins is critical for their functional activity within the cells. Snail has been found to translocate to the nucleus following phosphorylation by PAK1 to enhance its functional activity (Yang et al., 2005). The results herein showed that the nuclear localisation of Snail was increased following overexpression of both wt. Cten and nuclear localised Cten. This suggests that Cten not only regulates Snail protein expression, but may also be involved in the regulation of its subcellular localisation, thus regulates its function. Src has been found to be overexpressed and activated in a number of tumour tissues and commonly associated with tumour metastasis (Byun et al., 2017, Chen et al., 2014). The present study demonstrated for the first time that Cten expression was positively correlated with membranous localised Src in CRC patients. This increases the possibility that Cten expression could be responsible for the overexpression of Src in colorectal tumours. Src is a cytoplasmic localised protein, which following phosphorylation at the site of Y416, translocates to the membrane and becomes fully activated to exert its function (Ingley, 2008, Bolós et al., 2010). Interestingly, Cten was shown to induce the nuclear translocation of Src protein. Additionally, Src nuclear translocation was further increased when Cten was localised to

the nucleus. This suggests that Cten is important for Src post-transcriptional regulation, which in turn regulates its subcellular localisation.

ROCK1 expression in colorectal tumours has also been studied in this thesis. ROCK1 expression was found to be overexpressed in different tumour tissues including CRC (Liu et al., 2011, Cai et al., 2015, Xi et al., 2015). The findings herein revealed that ROCK1 is mainly expressed in the cytoplasm in most of the CRC cases and this was in agreement with previous published data (Sari et al., 2013). Furthermore, this study showed that there was a significant correlation between ROCK1 and Cten expression in the cytoplasmic compartment. Interestingly, cytoplasmic ROCK1 localisation was also positively correlated with Src expression in both the cytoplasmic and membranous staining. This thesis also revealed that nuclear localised Cten can induce ROCK1 nuclear translocation. Taken together, these findings imply that overexpression of both ROCK1 and Src protein is probably mediated by Cten signalling in CRC patients.

In summary, these findings indicate that when Cten is localised to the nucleus, it increases nuclear localisation of Src, ROCK1, and Snail, further increasing cell motility and colony formation efficiency in CRC cells. In addition to the subcellular localisation experiments, Cten expression was shown to be positively correlated with both ROCK1 and Src expression in most of the CRC cases investigated. However, further investigation into the molecular mechanisms involved is warranted.

8.2.5 Final Conclusions

This work addressed the role of Cten signalling in metastatic CRC cells. It is clear that Cten plays a role in colorectal tumour progression, probably through the promotion of metastasis. It has been shown that Cten promotes cell functions *in vitro* through the stabilisation of Src and Snail proteins. Further mechanisms as to how Cten protects Src or Snail proteins from degradation remain to be investigated. It has been confirmed that mutation of both arginine and tyrosine in the SH2 domain of Cten is important for Cten to function and interact with its downstream targets to regulate cell mobility and colony formation efficiency in CRC cells. This study also showed that TGF-β1 induced EMT processes regulate cell invasion and migration directly through Cten signalling and probably via the Src/ROCK/Snail axis, since TGF-β1 stimulation did not show an increase in either the expression or nuclear translocation of these proteins when Cten was absent in the SW620$^{\Delta Cten}$ cell line. It also confirmed that Cten in the nucleus does promote the functional activity of Cten in CRC cells and in this subcellular location, Cten shows increased nuclear localisation of its downstream targets, Src, ROCK1 and Snail (figure 8-1). Additionally, Cten expression showed a positive correlation with both ROCK1 and Src protein expression in most of the CRC cases studied and this was in keeping with the findings observed following Cten manipulation in CRC cell lines. However, these data are based on *in vitro* experiments using CRC cell models, which may not accurately recapitulate the complexity of the tumour *in vivo*. Therefore, further investigations are required to not only establish *in vivo* relevance, but also determine whether these findings could be further explored for therapeutic development.

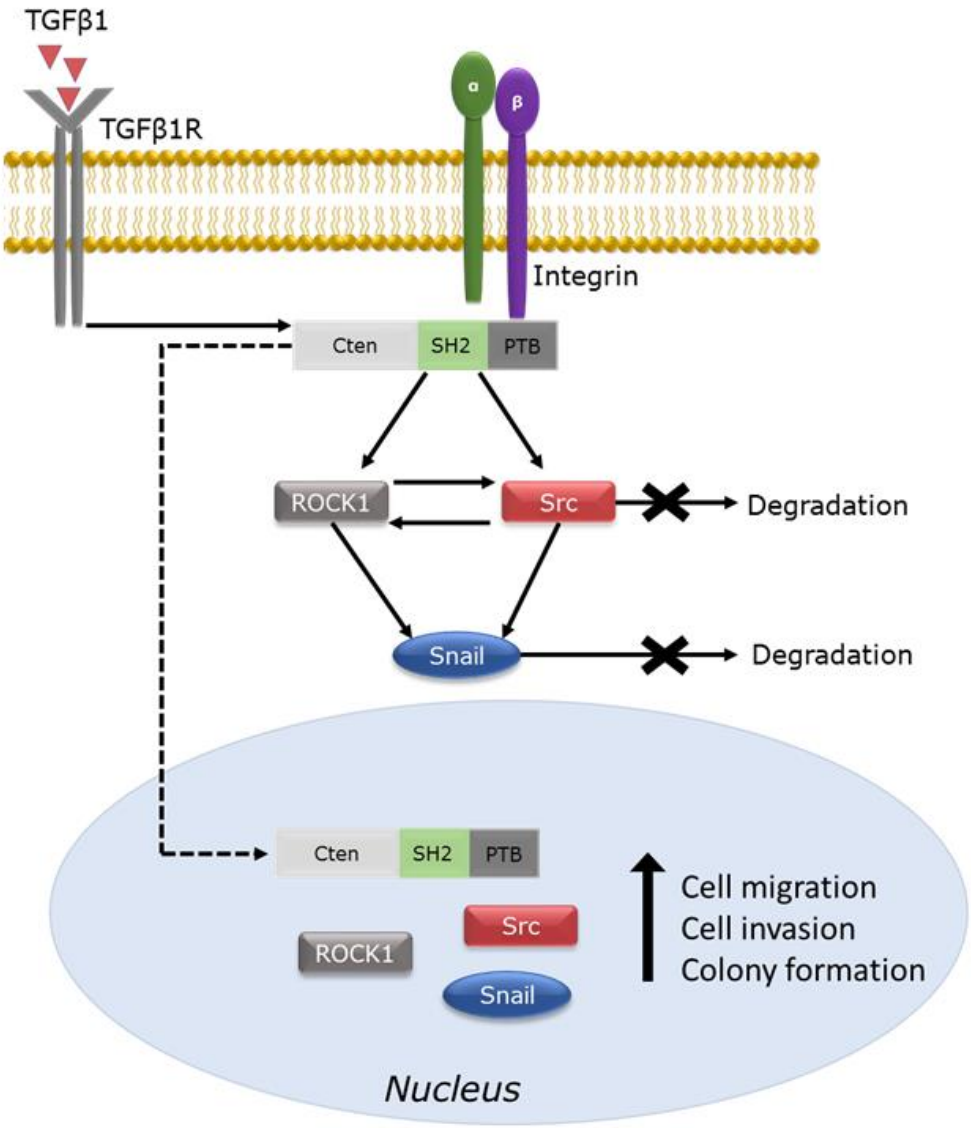


Figure 8–1: A concluding diagrammatic representation of Cten signalling, determined from this thesis.
Cten signals downstream to regulate ROCK1, Src and Snail protein expression through its SH2 domain. Downstream, Cten stabilises both Src and Snail proteins. Cten regulates Snail mediated cell motility and colony formation efficiency through Src/ROCK1 dependent pathway. TGFβ1 regulates ROCK1, Src, and Snail expression and promotes cell migration and invasion directly through Cten signalling. Cten in the nucleus increases nuclear localisation of its downstream targets.

8.2.6 Limitations of the study

In this study, the CRISPR /cas9 system was used to provide validation of an alternative to transient siRNA knockdown experiments. However, the deletion of Cten gene using CRISPR/cas9 system was performed in only one cell line, SW620. In addition to this, another possible disadvantage is the potential for off-target effects which may limit the applications of CRISPR/Cas9-mediated gene modification, especially in those mammalian genomes that contain similar DNA sequences such as other members of the tensin gene family.

The functional relationship between TGFβ1 and Cten was investigated in this study however, this was established only in one cell line, therefore additional studies are necessary to confirm this functional interaction for a holistic approach.

Another disadvantage of this study is that localisation of Cten and its downstream targets was only investigated in a small size of the data set and the cores were taken only from 3 different areas of the tumour during construction of the TMAs. This possibly will weaken any overall effect if Cten, Src or ROCK1 are only localised to specific regions of the tumour which were not sampled in the cores.

This work has investigated Cten signalling pathway using CRC cell lines grown *in vitro* using standard culture techniques, including the culture of cells directly onto plastic. However, this environment does not closely resemble those experienced *in vivo*. Also *in vitro* experiments and immmunohistochemical assays are only a snapshot at a particular time and do not reflect the temporal variation of molecular events.

8.2.7 Future Perspectives

This study identified potential novel downstream targets of Cten including Src, ROCK1 and Snail in CRC cells. Further validation of these downstream targets in different tumour types, such as lung and pancreatic cancer, are required. It would also be interesting to determine whether Cten is involved in the regulation of Src, ROCK1 or Snail downstream signalling pathways, in particular, whether Cten regulates the well-known downstream target of ROCK1, myosin light chin 2 (MLC 2) or Slug, which is in the same protein family as Snail.

The present study has demonstrated the role of Cten signalling EMT regulation, however, further investigations into the molecular mechanisms involved in this process are warranted. This should include the determination of how Cten prevents both Snail and Src proteins from degradation. Since Cten regulates Snail protein expression through Src signalling pathway, it is possible that Cten promotes the stabilisation of Snail through the regulation of the Src pathway. It could also be through the regulation of Snail phosphorylation by the GSK3β pathway or through the regulation of proteasome-ubiquitin dependent pathway. Cten could also be involved in the regulation of the ErbB2 pathway or ubiquitin proteasome pathway to protect Src protein from degradation. Therefore, further investigations are required to address how Cten promotes the stabilisation of both Src and Snail proteins.

This study has determined that the SH2 domain of Cten is critical for its functional activity, thus therapeutic inhibition of the Cten SH2 domain could be useful for the targeting of cell invasion and migration in future studies.

These investigations were established in cell lines and confirmation of these experiments in animal models is needed to determine that these influences also occur *in vivo*.

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10 Appendix

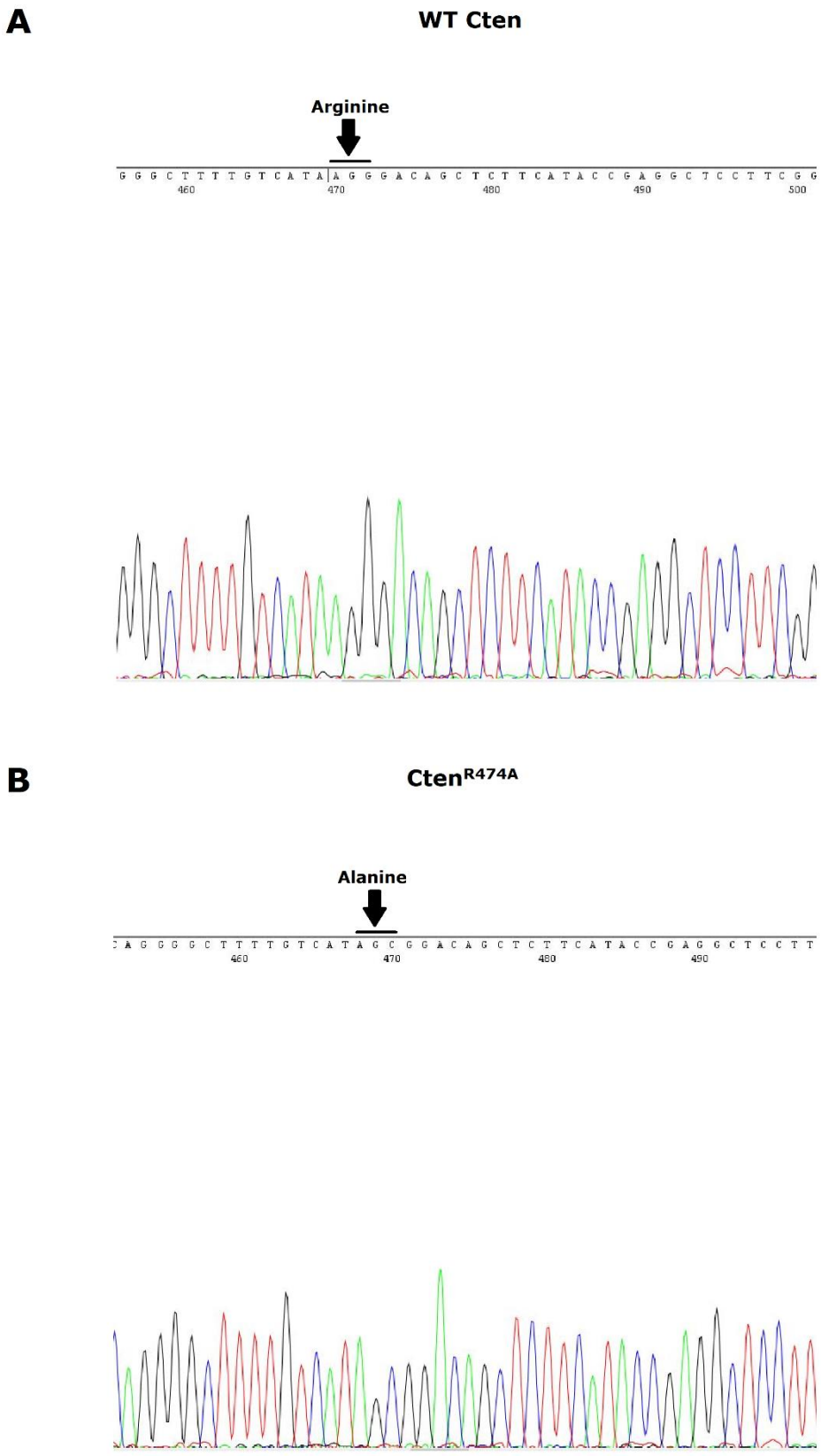

Figure 10–1: Confirmation of the Cten^{R474A} plasmid used in this study.
A and B) Sequencing revealed that the final construct mutated from wild type Cten to R474A (arginine to alanine).

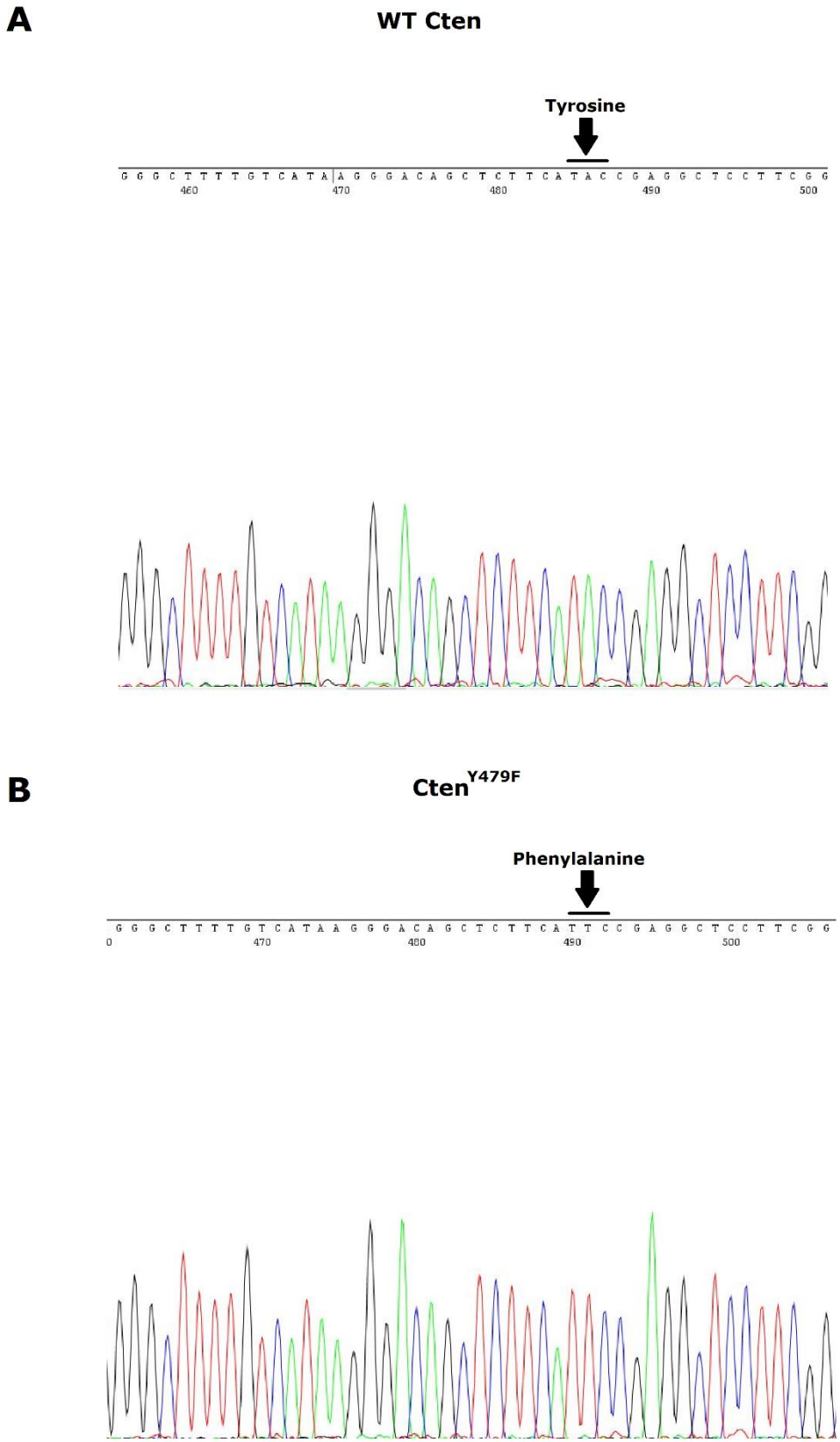

Figure 10–2: Confirmation of the Cten^{Y479F} plasmid used in this study.
A and B) Sequencing revealed that the final construct mutated from wild type Cten to Y479F (tyrosine to phenylalanine).

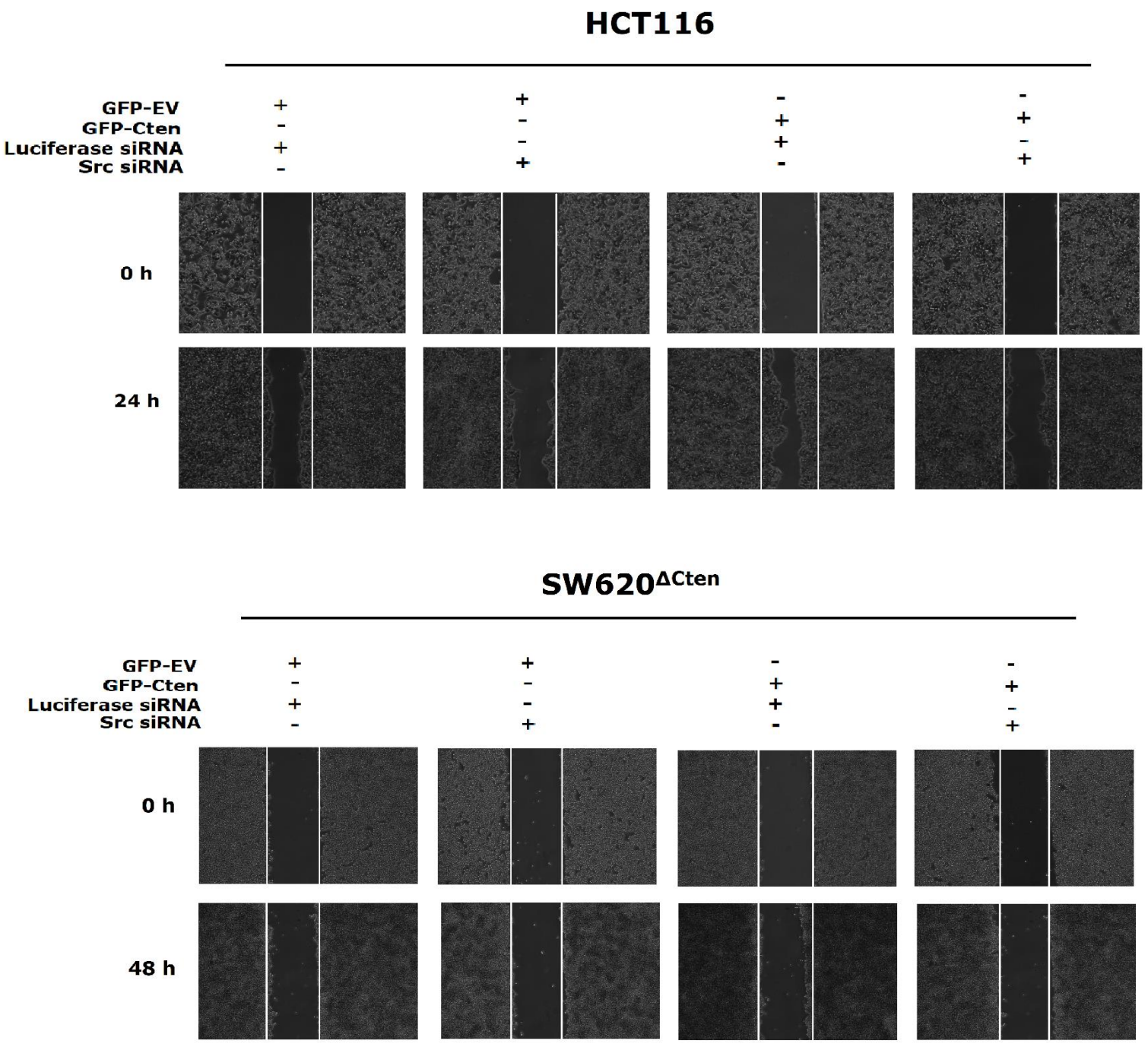

Figure 10–3: Representative magnified wound healing assay results corresponding to Fig 4-6 and 4-7 (chapter 4).

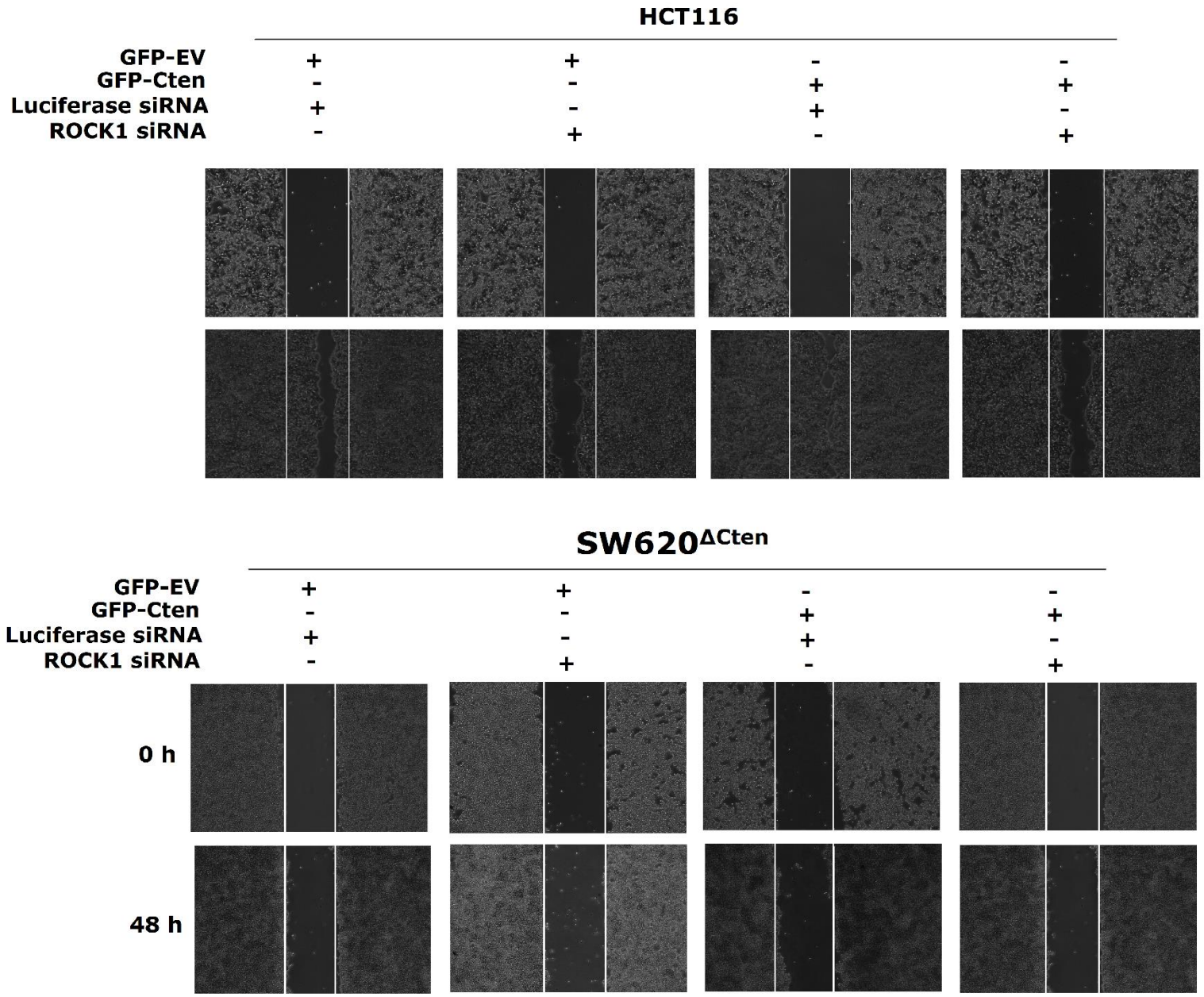

Figure 10–4: Representative magnified wound healing assay results corresponding to Fig 4-8 and 4-9 (chapter 4).

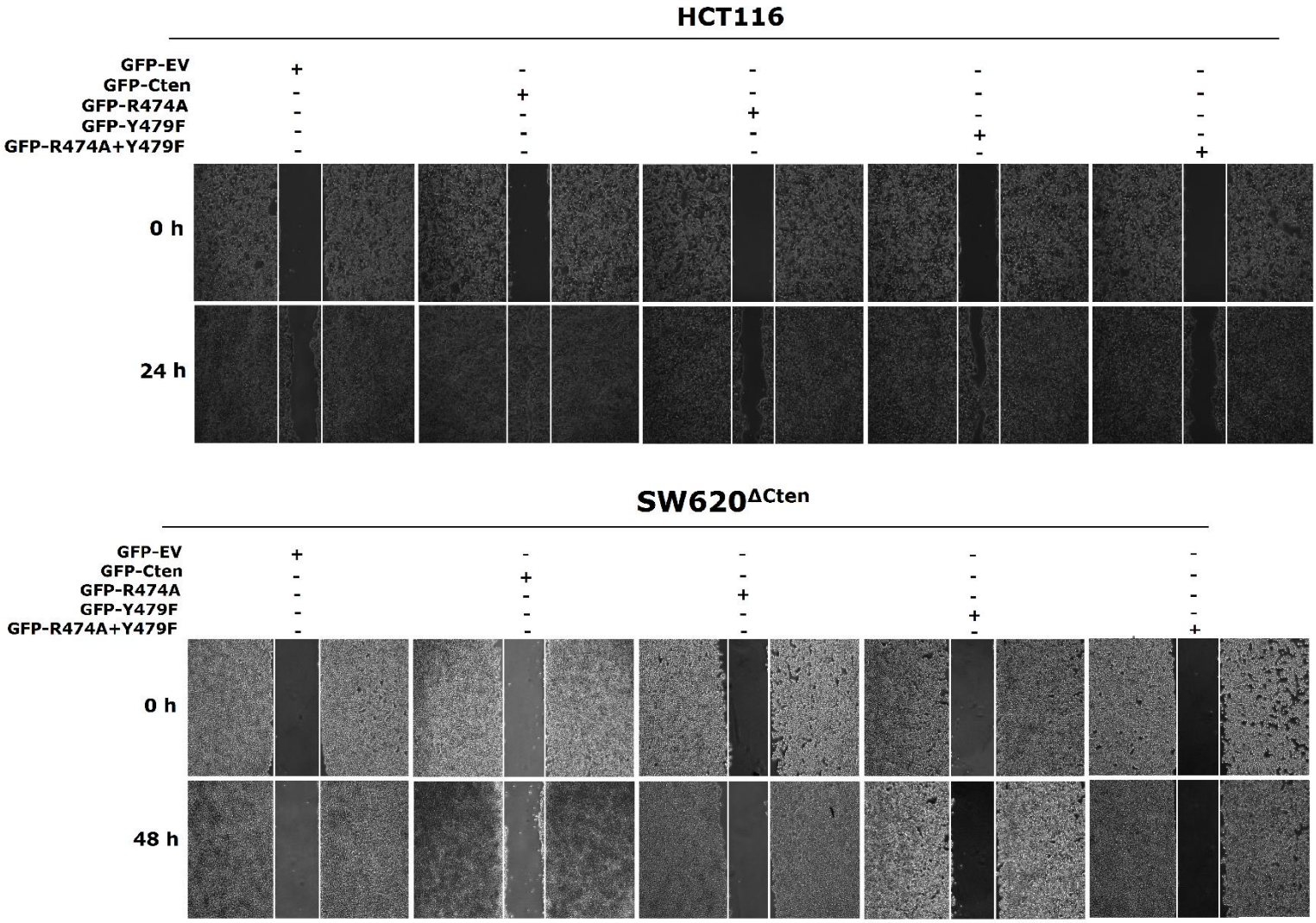

Figure 10–5: Representative magnified wound healing assay results corresponding to Fig 5-4 and 5-5 (chapter 5).

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School of Medicine
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Teresa Pereira Raposo
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06.02.2017

Certificate

Order
By order of Teresa Pereira Raposo (School of Medicine) we were requested to perform a cell line authentication test. Following samples were examined:

Our sample number	Client sample name	
CL170130_006	SW480	
CL170130_007	DLD1	
CL170130_008	SW620 (A)	A - OLD, SW620 Cten KO
CL170130_009	HCT116	
CL170130_010	RKO	
CL170130_011	SW620 (B)	B - SW620 WT

Method:
Genetic characteristics were determined by PCR-single-locus-technology. 21 independent PCR-systems Amelogenin, D3S1358, D1S1656, D6S1043, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433 and FGA were investigated (Promega, PowerPlex 21 PCR Kit).
In parallel, positive and negative controls were carried out yielding correct results.

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Ust-Id Nr. DE 285161076
Steuernummer 114/116/00159

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Results:

DNA-System	DNA-criteria SW480 CL170130_006	DNA-criteria DLD1 CL170130_007	DNA-criteria SW620 (A) CL170130_008	DNA-criteria HCT116 CL170130_009	DNA-criteria RKO CL170130_010
AM	X, X	X, Y	X, X	X, X	X, X
D3S1358	15, 15	17, 17	16, 16	12, 16, 17, 18, 19	12, 16, 17, 18, 19
D1S1656	13, 14	17.3, 19.3	13, 14	12, 13, 14, 15	12, 13, 14, 16.3
D6S1043	11, 12	11, 13	11, 12	12, 13, 14	12, 13, 14.1, 20
D13S317	12, 12	8, 11	12, 12	10, 12, 13	8, 10, 11, 12
Penta E	10, 10	7, 14	10, 10	13, 14	10, 13, 14
D16S539	13, 13	12, 13	9, 13	11, 12, 13, 14	11, 12, 13
D18S51	13, 13	11, 17	13, 13	16, 17, 18	11, 12, 16, 17
D2S1338	17, 24	17, 25	17, 24	16, 16	16, 16
CSF1PO	13, 14	11, 12	13, 14	7, 10	7, 8, 10
Penta D	9, 15	9, 14	9, 15	9, 13	9, 10, 13
TH01	8, 8	7, 9.3	8, 8	8, 9	8, 9, 10
vWA	16, 16	18, 19	16, 16	17, 22, 23	17, 21, 22, 23
D21S11	30, 30.2	29, 32.2	30, 30.2	29, 30	29, 30
D7S820	8, 8	10, 12	8, 9	11, 12	11, 12
D5S818	13, 13	13, 13	13, 13	10, 11	10, 11
TPOX	11, 11	8, 11	11, 11	8, 8	8, 8
D8S1179	13, 13	15, 15	13, 13	12, 13, 14	9, 12, 13, 14
D12S391	17, 17	19, 22	17, 17	17, 18, 20, 21, 22	15, 17, 18, 20, 21, 22
D19S433	13, 13	14, 16	13, 13	12, 13	12, 13, 14, 18.2
FGA	24, 24	22, 22	24, 24	18, 19, 21, 22, 23	18, 19, 22, 23

DNA-System	DNA-criteria SW620 (B) CL170130_011
AM	X, X
D3S1358	16, 16
D1S1656	13, 14
D6S1043	11, 12
D13S317	12, 12
Penta E	10, 10
D16S539	9, 13
D18S51	13, 13
D2S1338	17, 24
CSF1PO	13, 14
Penta D	9, 15
TH01	8, 8
vWA	16, 16
D21S11	30, 30.2
D7S820	8, 9
D5S818	13, 13
TPOX	11, 11
D8S1179	13, 13
D12S391	17, 17
D19S433	13, 13
FGA	24, 24

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Steuernummer 114/116/00159

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Summary:
The following cell lines could be detected in the online database of the DSMZ (http://www.dsmz.de/de/service/services-human-and-animal-cell-lines/online-str-analysis.html):

Our sample number	Client sample name	DSMZ name
CL170130_006	SW480	SW-480
CL170130_007	DLD1	DLD-1
CL170130_008	SW620 (A)	SW620 [SW-620]
CL170130_009	HCT116	HCT-116
CL170130_010	RKO	RKO can be present in the mixture but the main component is HCT-116
CL170130_011	SW620 (B)	SW620 [SW-620]

Dr. Burkhard Rolf
Director Forensic Services

Dr. Michaela Bosch
Project Manager DNA-Forensics

Cell_line-certificate_eng_V03_141211

Vorlage_Verwandtschaftsanalyse_EUROFINS_v02_121127

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