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Investigation of Cten signalling and regulation in colorectal cancer

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Declaration

I declare that this thesis is the result of my own work during my period of study at the University of Nottingham, and it has not been submitted in any form to this, or to any other University, for a degree other than for which I am now a candidate.

MahamAkhlaq

Acknowledgement

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Dedication

This thesis is dedicated to my loving daughter Aaman, who has been a source of support and motivation throughout this process.

Abbreviations

ABD	Actin-binding domain
BSA	Bovine serum albumin
Bp	Base pairs
CFE	Colonies formation efficiency
CRC	Colorectal cancer
CMAC,Tboc-leu-Met	7-amino-4-chloromethylcoumarin, t-BOC-L-leucyl-L-methionine amide
Cten	C-terminal tensin-like
DAB	3, 3’-Diaminobenzidine tetrahydrochloride
DAPI	4', 6-Diamidino-2-phenylindole
DLC1	Deleted in liver cancer 1
DMSO	Dimethylsulfoxide
Dok2	Downstream of tyrosine kinase 2
DNA	Deoxyribonucleic acid
Ecad	E-cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EV	Empty vector
FAB	Focal adhesion-binding
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
HPRT	Hypoxanthine phosphoribosyltransferase
IHC	Immunohistochemistry
ILK	Integrin-linked kinase
JAK	Janus kinase
Kb	Kilo bases

kDa	Kilo dalton
LB	Luria broth
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
MDCK	Madin Darby Canine Kidney
MLCSV40	Immortalised human prostatic epithelial cell line
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
Ncad	N-cadherin
NLS	Nuclear localisation signal
NTC	No template control
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide dependent kinase 1
PI3K	phosphatidylinositol 3 kinase
PIK3CA	phosphatidylinositol kinase 3 catalytic subunit
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog
Rac	Ras-related C3 botulinum toxin 1
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
QPCR	Quantitative PCR
SDS	Sodium dodecyl sulfate
SH2	SRC Homology 2
SMAD4	Mothers against decapentaplegic homolog 4

STAT3	Signal transduction and activators of transcription 3
TBE	Tris/Borate/EDTA
TNS1	Tensin1
TNS2	Tensin2
TNS3	Tensin3
TNS4	Tensin4

Abstract

Cten (also known as Tensin4) is the fourth member of the Tensin gene family. It lacks the N terminal actin binding domain while retaining the C terminal SH2 and PTB domains. This helps to bind Cten to the intracytoplasmic tail of β1 integrin and puts it at the heart of focal adhesions. It is reported to be a tumour suppressor in kidney and prostate cancer where normal tissues show high expression. However in a number of tumours, including colorectal cancer, Cten has been labelled as an oncogene. Cten which normally is a cytoplasmic protein gives nuclear staining in colorectal metastatic deposits. It increases motility, invasion and colony formation in colorectal cancer cells. In this study we have tried toexplore the mechanism of functional activity and regulation of Cten. We looked at Cten in the nucleus in vitro and identified new downstream binding targets. In addition we investigated the role of the SH2 domain of Cten concentrating on its downstream signalling molecules and binding partners. Furthermore, we explored regulators of Cten.

In this study we have forced nuclear localisation of Cten by tagging it with a nuclear localisation sequence (NLS) and found a significant increase in cell motility. In order to investigate the SH2 domain we used site directed mutagenesis to change potentially important amino acids namely Arginine at 474 to Alanine (R474A), which is important for binding tyrosine phosphorylated proteins. Moreover, we displayed that Cten underwent tyrosine phosphorylation and additionally changed three tyrosine residues i.e. Y449F, Y479F and Y530F via site directed mutagenesis. We found R474 and Y479 to be important in regulating cell motility and that known downstream targets such as ILK and FAK are dependent on an intact SH2 domain. Furthermore we have identified Cten to be physically bound to FAK in the cytoplasm and nucleus and new downstream targets identified such as Src and Paxillin.

Regarding possible regulators of Cten, we found that Cten might be a possible substrate for calpain. Another regulator considered was CD24 due to its role in movement of integrins into lipid rafts and we found it was a positive regulator of Cten.

In conclusion localisation of Cten into the nucleus causes an augmentation of its motility enhancing functions. Cten regulates cell motility via its SH2 domain. Arginine 474 and Tyrosine 479 are important for its function. Cten regulates levels of ILK, FAK, Src and Paxillin through its SH2 domain and binds to FAK in both cytoplasm and nucleus. Calpain and CD24 were found to possible regulators of Cten in colorectal cancer. Future studies are needed to define its role in signalling at focal adhesions and these studies should be validated in other cancer cell models as well to establish Cten as regulator of cell motility in cancer.

Thesis related publications

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Multiple pathways regulate Cten in colorectal cancer but tensin switch does not occur.

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Src Homology 2 (SH2) domain of Cten important for cell motility and downstream signalling through Snail, Integrin linked kinase (ILK) and binding with Focal adhesion kinase (FAK).

M.Akhlaq, H.thorpe, D.Jackson and M Ilyas.

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1 Introduction

1. 1 Colorectal cancer

Colorectal cancer (CRC) is the fourth commonest form of cancer worldwide. It effects men and women equally(1) and is a major cause of morbidity and mortality in the world. CRC usually develops after the age of 40 years and likelihood of this cancer rises sharply above the age of 50.The pathogenesis of colorectal cancer consists of three main steps: initiation, promotion and progression. Modification in the sequence or structure of DNA is the first step, followed by uncontrolled expansion of tumour cells and then the progression depends on alternations in various oncogenes and tumour suppressor genes(2).

1.1.1 Staging

Prognosis depends on the stage of the disease at the time of diagnosis. The stage of the disease depends on the localisation of the cancer to the site of origin or extension to surrounding tissues and distant metastasis(3). In 1926 Lockhart-Mummery proposed a staging system based on depth of invasion and lymph node involvement. It was in 1932 when Dukes proposed a staging system which is still used today with addition of a few modifications. The latest modified Dukes staging system is called Modified Astler-Coller classification (MAC) given in detail in Table 1-1: The 5 year survival is directly proportional to the depth of invasion and decreases drastically with involvement of lymph nodes (4).Usually 5 year survival rate is 90% for localised stage, 70% for regional and 10% for distant metastasis (4).

1.1.2 Molecular pathogenesis

Colorectal cancer is a complex disease and multiple pathways exist in its tumourigenesis. The classical pathogenesis for CRC was composed of Vogelstein model of adenoma to carcinoma; although still relevant there are

limitations which were highlighted by molecular analysis of colorectal cancer tissues.

Table 1-1.Modified Astler-Coller classification/Staging of Colorectal Cancer.(4)

	Modified Astler-Coller Staging
Stage A	Lesions limited to the mucosa
Stage B1	Lesions extending into the muscularispropria, but not penetrating it, with negative nodes
Stage B2	Lesions penetrating the muscularispropria, with negative nodes
Stage C1	Lesions extending into the muscularispropria, but not penetrating it, with positive nodes
Stage C2	Lesions penetrating the muscularispropria with positive nodes
Stage D	Distant metastasis

Genetic instability is the key factor in development of CRC. Genetic instability can be of two main types; one occurs at the level of chromosome, chromosomal instability (CIN) and the other at DNA level called microsatellite instability (MSI). CIN is characterised by loss of heterozygosity (LOH) i.e. the loss of either maternal or paternal allele of a gene. LOH is seen on 5q, 17p and 18q classically in CRC. MSI occurs due to loss of activity of DNA mismatch repair genes (MMR) through mutations. Moreover, another pathway in CRC pathogenesis is CpG island methylator phenotype (CIMP) which causes methylation or silencing of tumour suppressor genes presenting an alternate to LOH and MMR mutations(5).

There are a number of genes involved in CRC pathogenesisincluding the classic tumour suppressor genes like APC (adenomatous polyposis coli), p53 and oncogenes like K-ras (Kirsten rat sarcoma). Among the oncogenes displaying increased expression, Cten is a novel addition. It is increased in colorectal tumour tissues as compared to normal mucosa and its expression is associated with advanced stage of the tumour and distant metastasis and consequently poor prognosis(6,7).

Cten belongs to a gene family called Tensin gene family, having members with similar genomic structure and proteins with nearly identical sequence. It has 4 members, namely Tensin 1(TNS1), Tensin 2(TNS2), Tensin 3(TNS3) and Tensin 4(TNS4) more commonly known as Cten(8).

1.2 The Tensin gene family

1.2.1 Genomic structure

Based on genomic structure and protein sequence, the Tensin gene family is now thought to be made up of 4 members: Tensin 1, 2, 3 and Cten. The protein structure is highly homologous among all of them (Figure 1-1). There is a conserved N and C terminus in Tensin 1, 2 and 3. However, Cten is a smaller molecule in comparison, lacking actin-binding domains which are found in the N terminus in other tensins. Although the rest of the Tensins have similar N and C terminal ends, they vary in the middle regions, which suggests their different roles in cell biology(8).

Tensin (also known as Tensin1) was first isolated in 1991 from chicken cardiac muscle. It is a 220kDa protein and is localised to focal adhesions(9). It binds to actin filaments and has a role in signal transduction. Human Tensin2 was

discovered shortly after in 2000. TNS2 cDNA codes for a 1285 amino acids and has a molecular weight of 170kDa. It was seen to have a strong homology to Tensin1 at the C and N terminus(10).

The length of gene Tensin 1(TNS1) (sometimes referred to as Tensin) is 1.5 kilo bases (kb) and the gene maps to chromosome 2q35-q36 (OMIM ref. no. 600076). It contains 33 exons and codes for a protein containing 1735 amino acids. The start codon is in exon 6 and the stop codon is located in exon 33(10). Tensin 2 (TNS2) gene maps to chromosome 12(OMIM ref. no. 607717).It is 12.5 kb in length and codes for a protein of 1285 amino acids. It has 28 exons; the presumed start codon is in exon 6, whereas stop codon is in exon 28(10).The Tensin 3(TNS3) gene maps to 7p12.3 (OMIM ref. no. 606825). Its cDNA is composed of 4415 base pairs (bp) which codes for 1445 amino acids, and has 31 exons. The protein comes out to be 180 kDa in weight when run on SDS-Page gel(11). The exons 5-15 in TNS2 are identical in size to same exons in TNS1 and code for the actin-binding domain (ABD). In addition, exons 21-28 in TNS2 are similar in size to exons 26-33 in TNS1 which encode the Src-homology 2 (SH2) and phosphotyrosine-binding (PTB) domains respectively. Similarly exons 5-15 (encode the ABD) and exons 23-30 (encode the SH2 and PTB domains) in TNS3, supporting the idea that all these Tensins are related and can be grouped in one gene family(10,11)

The COOH-terminal Tensin-like protein Cten, also known as Tensin4 (TNS4) gene (OMIM ref. no. 608385) is 21 kb in length and maps to 17q21.2. It has 13 exons and the cDNA comprises of 4,015 bp which encode a protein of 715 amino acids and runs at 90kDa on a SDS gel. Exon 1 has the start codon stop exon is in Exon 12. Exon 12 is the largest exonwhile the smallest is exon 11,

comprising of 1883 bp and 27 bp respectively. The SH2 and PTB domains are encoded by exons 4–11 which are identical to other Tensin genes, making it the fourth and smallest member of the Tensin gene family (12).

1.2.3 Basic protein structure

Tensins encode for a complex protein which has multiple domains capable of binding several different molecules which play important roles in cell structure and signalling. All Tensins with exception of Cten have an Actin binding domain (ABD) at the N terminus that binds to actin filaments as seen in Figure 1-1. Tensins not only have the capability to bind to actin fibres but also cross link them by binding to the barbed ends, thus creating a network of actin filaments (13). The C terminus has SH2 domain and the PTB domain. Binding with tyrosine phosphorylated proteins like Focal adhesion Kinase(FAK), p130Cas and P13 kinase is mediated through SH2 domain (9). The PTB can bind tyrosine phosphorylated and non-phosphorylated peptides and in Tensin proteins, PTB binds to the NPXY motif of β integrin's cytoplasmic tail within the focal adhesions(14,15). Tensins are reported to be phosphorylated at tyrosine, serine, and threonine residues whereas in Cten six potential tyrosine phosphorylation sites have been described (12,16).

1.3 Tensin gene family; a new component of cell adhesion and motility

The focal adhesion binding domain (FAB) either at the N terminal or C terminal is a constant feature of the proteins encoded by the Tensin family. This renders them an ideal partner of focal adhesions and suggests a role in regulating cellular adhesions and migration/motility. As the list of proteins present in focal adhesions continues to grow, they are divided according to

their location: extracellular, cytoplasmic and transmembrane. Tensins and their related proteins are major contributors to the focal adhesion family that are located in the cytoplasm(17). To understand the role of Cten and other Tensins in cell motility and migration, it is important to have a concept of function of cell adhesion and its components.

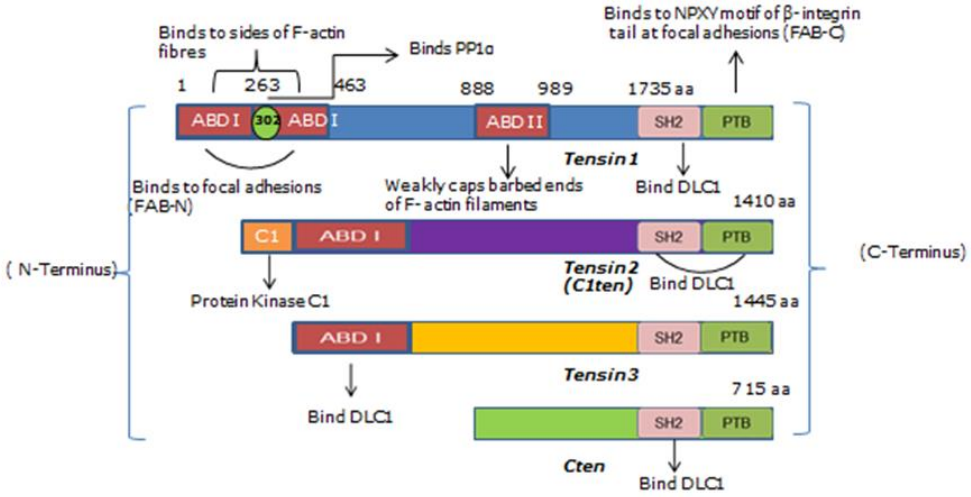


Figure 1-1 Functional and structural domains of Tensins.Tensin can be divided into three regions The N-terminus contains actin-binding domain (ABD) I and II that bind with actin filaments. ABD1also binds to focal adhesions in Tensin 1. The ABD II in the center of Tensin 1 retards the actin polymerization. The C-terminus contains a SH2 domain, PTB domain. This terminal also has a focal adhesion binding site at PTB domain. The SH2 domain binds to tyrosine phosphorylated proteins like PI3 kinase, FAK, and p130Cas.Tensin 1, tensin2, tensin3, and Cten are highly conserved at N- and C-terminal regions. An additional C1 domain (protein kinase C conserved region 1) is found in tensin2 only. Most of the tensins bind to DLC1 (deleted in liver cancer gene 1) but the binding sites vary a little bit.[Derived from (8)]

1.4 Key concepts of cell motility and migration

Cell migration and motility is a multistep process which includes the following stages:protrusion of the front of the cell, adhesion formation, stabilization at the leading edge followed by cell body translocation and release of adhesions and detachment of the cell's tail end(18).

Cell adhesion to the extracellular matrix (ECM) is a vital regulator of cell motility, migration and morphology. It is mediated through multi protein complexes comprising three broad types of proteins; the extracellular matrix proteins, the cell membrane proteins and inside the cell, the adhesion molecules. The cell adhesion molecules are usually transmembrane glycoproteins that mediate binding interactions at the extracellular (EC) surface and determine the specificity of cell–cell and cell–ECM recognition. They include mainly members of the integrin and cadherin family(19). The integrins are heterodimerproteins and they form thebasic structure of a focal adhesion complex. Integrins bind ECM to the cell and serve as a connection for communication between the two.Cadherins are important for inter cellular adhesion in epithelial cells and form bonds in between cells as shown in Figure 1-2(20).In case of cancer cell motility integrins and cadherin act antagonistically. This influences the outcome of whether the cells stay attached to their adjoining cells or migrate(21).

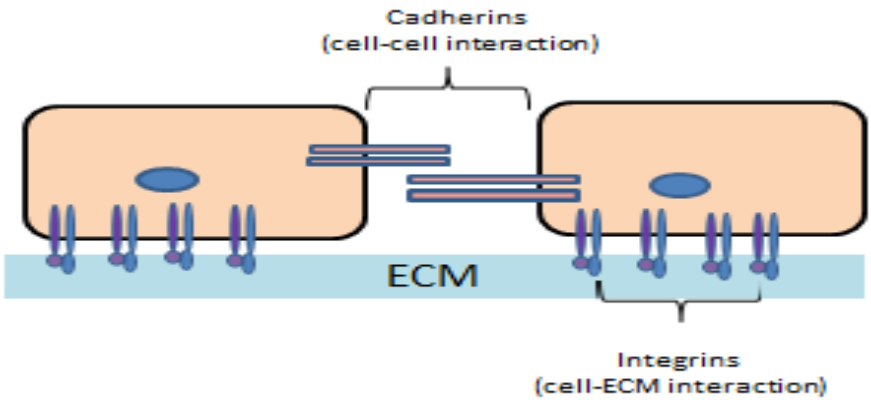


Figure 1-2: Schematic diagram of cadherin and integrin junctions.Cadherins form junction between adjacent cells. Integrins form connections with extracellular matrix proteins. Derived from (22)

1.4.1 Integrin signalling

Integrins are a family of cell surface adhesion receptors which play a role in cell anchorage. In addition, they transmit chemical signals into the cell (outside-in signalling), providing information on its location, local environment, adhesive state and surrounding matrix. These signals in turn determine cellular activities like migration and motility(23).

Integrins are formed by hetrodimers composed of non-covalently linked α and β subunits. Each subunit is a type I transmembrane glycoprotein that has relatively large extracellular domains and a short cytoplasmic tail (24). The β tails of integrins are more conserved than the more diverse alpha tails and their interactions more generalised.(23). Integrins also submit signals from within the cell to the outside (inside-out signalling), in response to signals that impinge on their cytoplasmic tails, regulating their affinity for extracellular ligands(25). Because of this dynamic function a number of protein complexes form around the cytoplasmic tails of integrin. To date a network of 156 components have been described, that make up integrin adhesome (26). This adhesome is given the name of focal contacts or focal adhesions (Figure 1-3).

1.4.2 Focal Adhesions

Focal Adhesions (FA) are comprised of a number of different protein (Figure 1-3) which act together and result is cell adhesion and assembly of stress fibres thus contributing to cell motility and migration(27,28). There are two types of proteins that assemble at FA, adaptor and signalling proteins. The adaptor proteins include talin, paxillin, Tensin, p130Cas and alpha-actinin. They help to alter cell morphology and create a traction force to move the cell during migration. They do so by providing strong linkages to the actin cytoskeleton

and connecting them firmly to ECM. The signalling proteins include kinases and phosphatases; these transmit signals from ECM to the cellular pathways controlling proliferation and migration. As integrins do not have an intrinsic enzymatic activity, these kinases, in particular FAK (focal adhesion kinase) and Src transmit signals from FA to the cellular apparatus (27,29,30).

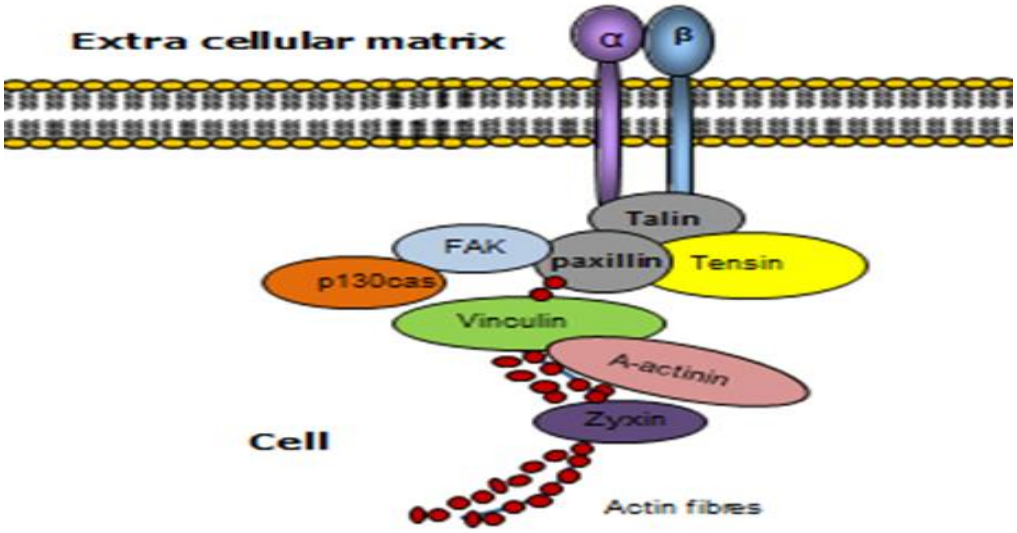


Figure 1-3:.Basic structure of a focal adhesion.The α and β integrins connect the extracellular matrix (ECM) to the intracytoplasmic adaptor proteins and actin fibres. Derived from (18)

Focal adhesions are complex structures so in order to understand their function in cell motility better;they have been studied in cultured fibroblasts and divided into 2 types; focal contacts/adhesions and fibrillar adhesions

Focal contacts are said to be made up of αvβ3 integrin which are peripheral oval structures with their set of proteins present at the edge of lamelopodia (leading edge of cell). Fibrillar adhesions are elongated central structures containing α5β1 integrins with their associated proteins attached to fibronectin

fibrils. In addition to their composition they also vary in the substrates they bind in ECM as seen in Figure 1-4 (31,32).

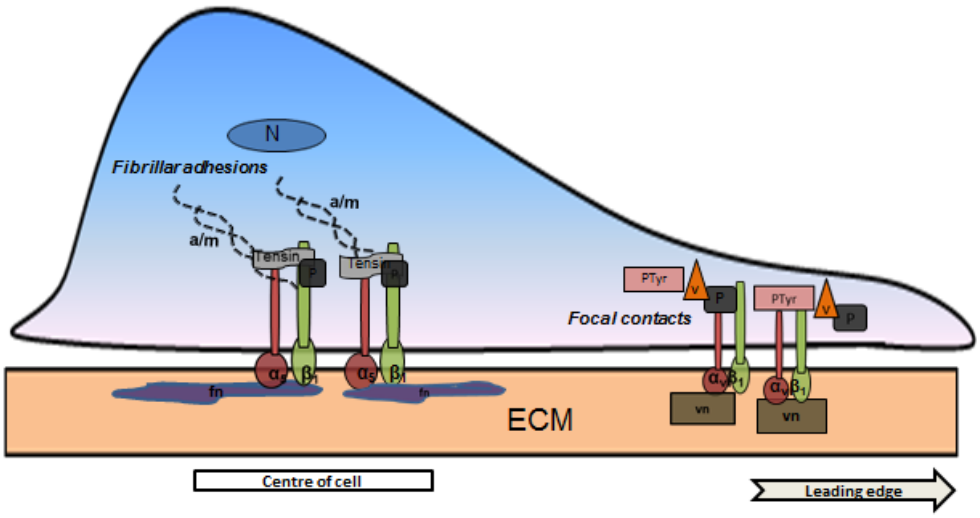


Figure1-4:.Difference between Fibrillar adhesions and focal contacts.Focal contacts are usually present at the leading edge of cells. The integrins involved αvβ3 are mainly attached to Vitronectin (Vn) in the ECM. Intracellularly they contain vinculin (v), paxillin (p) and other tyrosine phosphorylated protein (PTyr). Fibrillar adhesions α5β1 on the other hand can be found in the center of the cell due to binding with flexible fibronectin (fn) in the ECM and consist of Tensins, paxillin and contracting actin and myosin fibrils(a/m) which move them centripetally during cell motility.

1.4.3 Cadherin signalling

Cadherin is a transmembrane superfamily comprising of more than 100 proteins. The members of this family are diverse in their structure and function. They influence cell growth and invasion in a variety of ways.(33)Cadherins play a very important role in cell–cell adhesion. They are transmembrane glycoproteins family, and regulate cell to cell interaction in a calcium dependent manner. Its members include E (epithelial), N (neural), VE (vascular-endothelial), P (placental), R (retinal), and K (kidney) cadherins(34).E-cadherin is a vital and most well-known member of this

family. It is located in the adherens junctions of epithelial cells. E-cadherin is linked to actin filaments through catenins and this stabilises these junctions. β-catenin links E-cadherin to α catenin, which in turn links it to actin fibres either directly or indirectly(35). E-cadherin is considered a tumour suppressor protein. It can contribute to tumour suppression either by physically joining cells or by facilitating other signalling events. Its tumour suppressor activity could also result from binding and antagonizing the nuclear signalling function of β-catenin(36). N-cadherin is another cadherin known to be involved in cell motility. It replaces E-cadherin in cancer tissues and thought to help in movement of cancer cells. Although there is some controversy regarding the strict categorisation of N-cadherin as a positive regulator of tumour cell motility (33)Disruption of E-cadherin mediated adhesion is regarded as an important step towards cancer cell migration. Studies have shown a positive relation of down regulation of E-cadherin and nuclear localisation of β-catenin in colorectal cancer and Cten has been linked to both of these proteins (37).

1.4.4 Tumour cell microenvironment

In addition to the cell-cell adhesions cell-matrix adhesion also paly important role in the cell migration/invasion process. The microenvironment surrounding the tumour include the stromal cells, fibroblasts and macrophages. These cells influence the cell-matrix adhesions, once activated they alter cytokines and growth factors within the surrounding matrix which affect the function of the cell adhesion apparatus including the focal adhesions and consequently affect the cell migration and invasion process(38,39). For cells to achieve invasion and migration proteases are also very important which alter the surrounding microenvironment making way for the cell. These proteases not only alter the

ECM structurally but also activate other growth factors. These include Matrix MetalloProteases, cathepsins, the serine protease urokinase plasminogen activator (uPA)(38).

1.5 Cten

As mentioned before, Cten (C-terminal Tensin like) is a smallest member of Tensin gene family lacking the actin binding N terminal (38). Its features and functions are discussed in detail below.

1.5.1 Location (normal and cancer tissues)

According to Western Blot analyses carried out by Lo et al., in 2002 Cten was found in prostate and placenta out of the 15 normal human tissues examined. This is in contrast to tensin1 and 2 which are widely expressed in human body(12).However, Cten has been found to be highly expressed in a number of human tissue tumours in which its expression is minimal in the normal state (12). It is up regulated in advanced stage tumours of lung, gastric carcinomas, thymomas, melanomas, pancreatic carcinomas and colorectal cancers (39-44). In contrast it shows down-regulation in prostate cancer and cell lines where it is found normally (10 101). Detailed gene expression profile analysis is given in Table1-2 and Table1-3.

1.5.2 Function

There are a number of publications regarding the role of Cten in various tumours which show that its activity varies in tumours of different origins. It has been labelled as tumour suppressor in a few like prostrate and oncogenic in others (12,39-42). However its role as an oncogene is advancingly described and studied repeatedly. Cten's expression has been shown to be proportional to

advanced stage of tumours. This has established its role in enhancing cell motility and colony formation (7,45).

1.5.3 Role in cell motility/migration

Being positioned at the focal adhesions and due to the presence of an integrin binding PTB domain, Cten is at an ideal setting to affect motility of cells. In colorectal cancer cell lines, forced expression of Cten causes significant increase in cell migration and invasion, but does not alter cell numbers which further strengthens its role in cell migration(45). Similarly in breast cancer, lung and pancreatic cancer cell lines Cten augments cell motility (46-48). In order to better understand its function, a number of studies have been conducted, regarding Cten and its signalling partners which help in piecing together its precise role in cell motility.

Table 1-2 Expression of Cten by Expressed sequence tag (EST) in normal body tissues.

Tissue	Transcript per million(TPM)	Spot intensity based on TPM	Gene EST/Total EST in the pool
adipose tissue	0		0 / 12866
adrenal gland	0		0 / 32940
ascites	50		2 / 39834
bladder	133		4 / 29860
blood	0		0 / 122252
bone	0		0 / 71618
bone marrow	0		0 / 48737
brain	7		8 / 1092688
cervix	0		0 / 48486
connective tissue	0		0 / 149072
ear	0		0 / 16100
embryonic tissue	0		0 / 212896
esophagus	49		1 / 20154
eye	23		5 / 208840
heart	0		0 / 89524
intestine	68		16 / 231981
kidney	14		3 / 210778
larynx	42		1 / 23466
liver	0		0 / 205291
lung	41		14 / 334815
lymph	0		0 / 44302
lymph node	0		0 / 89748
mammary gland	19		3 / 151230
mouth	105		7 / 66150
muscle	9		1 / 106371
nerve	0		0 / 15535
ovary	0		0 / 101488
pancreas	37		8 / 213440
parathyroid	0		0 / 20594
pharynx	0		0 / 40725
pituitary gland	0		0 / 16526
placenta	84		24 / 283019
prostate	31		6 / 189536
salivary gland	0		0 / 20265
skin	56		12 / 210759
spleen	0		0 / 53397
stomach	41		4 / 95679
testis	0		0 / 435204
thymus	0		0 / 79697
thyroid	85		4 / 46583
tonsil	0		0 / 17021
trachea	19		1 / 51780
umbilical cord	0		0 / 13764
uterus	51		12 / 232093

Table 1-3Gene expression of Cten by Expressed sequence tag (EST) in tumours (derived from http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.438292)

Tumour	Transcript per million(TPM)	Spot intensity based on TPM	Gene EST/Total EST in the pool
adrenal tumor	0		0 / 12655
bladder carcinoma	170		3 / 17584
breast (mammary gland) tumor	21		2 / 93090
cervical tumor	0		0 / 34484
chondrosarcoma	0		0 / 82838
colorectal tumor	106		12 / 112517
esophagealtumor	57		1 / 17245
gastrointestinal tumor	42		5 / 118498
germ cell tumor	3		1 / 263230
glioma	65		7 / 107194
head and neck tumor	82		11 / 133826
kidney tumor	0		0 / 68872
leukemia	0		0 / 94479
liver tumor	0		0 / 96023
lung tumor	58		6 / 102765
lymphoma	0		0 / 72196
non-neoplasia	31		3 / 96623
normal	15		52 / 3328811
ovarian tumor	0		0 / 76185
pancreatic tumor	76		8 / 105004
primitiveneuroectodermaltumor...	0		0 / 127001
prostate cancer	0		0 / 103844
retinoblastoma	0		0 / 46439
skin tumor	7		1 / 125373
soft tissue/muscle tissue tumor	0		0 / 125265
uterine tumor	122		11 / 90107

1.5.4 Tensin 3-Cten switch

Tensin proteins are generally known to affect cell motility(10) and Cten is no exception. As we know that Cten lacks an ABD which is present in the rest of Tensin members and functions to bind and link actin filaments. Studies have shown that Cten can displace actin filaments bound by the other tensins at the focal adhesion site and thus help in augmenting cell motility.

Cten specifically replaces Tensin 3 at focal adhesions, and thus stimulates migration through displacement of actin fibres. This mechanism of switch from Tensin 3 to Cten is regulated by epidermal growth factor (EGF) in breast cancers and also seen in the normal mammary cell line, MCF10A (49). Treatment with EGF down regulates Tensin3 mRNA and up regulates Cten expression. However, EGF does not have the similar inhibitory effect on Tensin1 or Tensin 2 (49,50). A study has been conducted in colorectal cancer cell lines to replicate this effect of Tensin switch but hasn't found it as significant as in breast cancer(51) suggesting there might not be a Tensin switch in colorectal cancer but further studies are awaited to establish this.

1.5.4 E-cadherin interaction

Cten is known to cause an epithelial-mesenchymal transition (also known as EMT) in cells. In colorectal cancer cells lines, transient Cten over expression leads to elongated spindle like cell formation. This property is acquired when cellsstart to move or migrate. Ctencarries this out by negatively regulating the expression of E-cadherin in colorectal cancer. We already know that E-cadherin plays a role in the development of EMT as it regulates the cell-cell adhesion. Cten when expressed forcefully in colorectal cancer cell lines lowers levels of E- cadherin protein but does not affect it at transcription level. The exact mechanism by which Cten down regulates E-cadherin is still not clearly understood. It is possible that Cten causes increased destruction of E-cadherin instead of inhibiting its expression. Whatever the mechanism involved, this is an indication that Cten may well be a mediator between cadherins and integrins during cell motility(52).

1.5.5 Role of SH2 domain

Localization of Tensins to focal adhesions is a critical step in regulation of cell migration. The SH2 domain of Cten is crucial for binding to focal adhesions as it binds all the phosphotyrosine related proteins like FAK, ILK, and PI3 kinase. Mutations in the SH2 domain abolishes migration induced by Tensin family proteins(14). Similarly as Cten is highly homologous to Tensins at the C-terminal, a mutation in SH2 domain prevents invasive migration as is seen in Canine Kidney cell (MDCK) three dimensional culture studies(53). Not only does SH2 domain bind phosphorylated tyrosine proteins, it also binds tumour suppressor proteins like Deleted in Liver cancer 1 (DLC1) and localises it to focal adhesions (54).

Mutations in the SH2 domain of Cten has been shown to have a negative impact on increased basal activation of Stat3 in a model of tubulogenesis in dog kidney cell line. This mutation in Cten leads to decreased migration which can be reversed by expression of a constitutively active Stat3 alone.This Cten-Stat3 partnership which has been revealed is likely to play an important role in tubulogenesis and metastasis and this effect might be SH2 domain dependent(53).

1.5.6 Regulation of Deleted in liver cancer (DLC1)

DLC1 is a well know tumour suppressor gene. It has been seen to negatively regulate Rho GTPases and thus prevents tumour proliferation, cell survival and motility. It is seen that in order for DLC1 to perform its function as a tumour suppressor gene, it needs to be localized in the focal adhesions. This is done by interacting with the SH2 domain of Cten, as seen in prostatic cancer cell lines.

If there is a mutation in the SH2 domain of Cten, DLC1 fails to perform its function as a tumour suppressor and increases tumour cell migration and motility. This character of Cten in DLC1 activity suggests its role as tumour suppressor. But it is important to highlight here that this phenomenon is seen in prostatic cancer, which lacks Cten expressioncompared to normal prostate(54). Where Cten is not present normally and is only up regulated by EGF stimulation, it has been noted that Cten helps keep DLC1 in an inactive state with its intact SH2 domain and helps promote motility whereas Tensin 3 activates DLC1 function (Figure 1-5)(50).

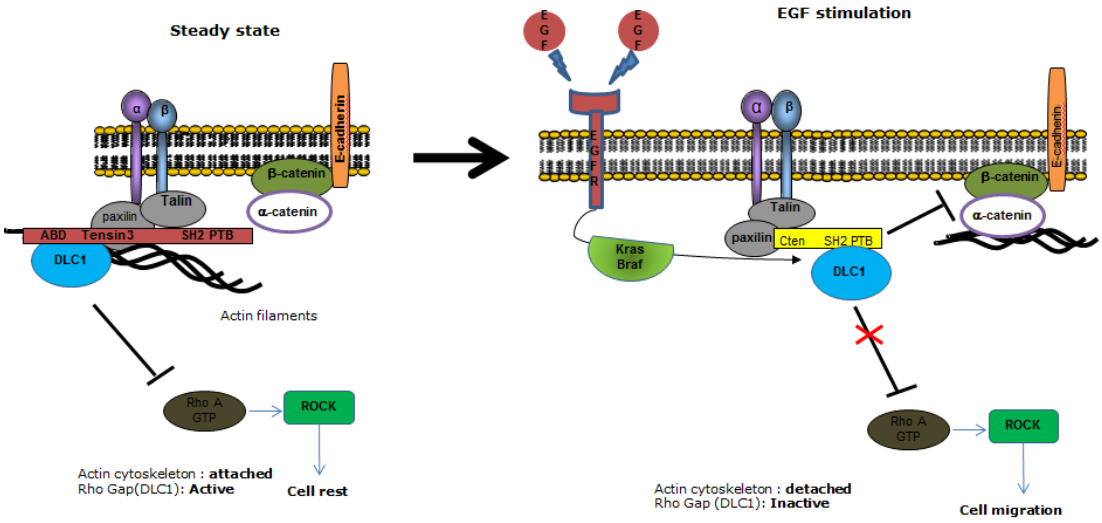


Figure 1-5: EGF stimulation causes switch from Tensin3 to Ctenand promotes cell migration by inactivation DLC1 resulting in absence of its inhibitory effect of Rho A activated ROCK pathway. Cten also increases motility by detachment of cell-cell E-cadherin junctions. Derived from (50)

1.5.6 Potential upstream and downstream partners

EGF, K-Ras and other growth factors

EGF pathway positively regulates Cten in breast cancer as well as colorectal cancer. EGF pathway signals through K-Ras/Braf, which are commonly mutated in colorectal carcinoma. Knockdown of mutant K-Ras has a negative

regulatoryeffect on Cten, which results in loss of cell motility but this loss can be rescued by addition of Cten by forced expression. Demonstrating that Cten is a downstream target of K-Ras/Braf signalling and is shown to be controlled at the transcription level(43). Not only is Cten up regulated by EGF signalling but it also sustains an increase in EGF receptor (EGFR) protein by inhibiting its degradation by c-Cbl. In this way it creates a positive feedback loop for EGF stimulation (55). In addition to EGF, there are a number of growth factors which have been added to the list of up regulators of Cten and thus cell motility including Fibroblast growth factor (FGF2), Nerve growth factor (NGF), Platelet derived growth factor (PDGF), Transforming growth factor(TGF-β) , Insulin like growth factor (IGF-1) , Interleukin 6 (IL-6), and IL-13(51).

Stat3

There has been a link recently discovered between Stat 3 and Cten. The latent signal transducer and activator of transcription 3(Stat3) is activated by a number of factors, like oncogenes, cytokines, growth factors, EGFR and HER2. This activation of Stat3 then leads to tumourogenesis, enhanced cell survival and growth; promote angiogenesis and epithelial to mesenchymal transition. Over-expression of Stat3 has been reported in a number of tumours(48). Stat3 may well be one of the upstream interacting partners of Cten leading to its role in cancer development. A recent study in human lung cancer cell lines put Stat3 as a positive regulator of Cten via its up regulation through EGF(47). Interaction of Cten with Stat3 has also been reported in rat breast cancer model, showing a functional loop of inflammation/Stat3-migration and metastasis by Cten. Interleukin 6 causes increased Cten induction through Stat3 activation. This model is well reflected by the fact that

there are increased Cten levels in inflammatory breast cancers (48). In contradiction, mutations in the SH2 domain of Cten have been shown to have negative impact on Stat3 activation in a model of tubulogenesis in dog kidney cell line. Cten in the normal state is seen to inhibit activation of Stat3 and mutation in the SH2 domain augments the activation of Stat3. Exactly how this takes place is still unknown. However, an unexpected Cten-Stat3 axis has been uncovered, which is likely to play an important role in tubulogenesis and cancer metastasis(53).More studies are required to actually determine the Cten's effect on Stat3 or vice versa as there still remains some confusion as to whether stat3 is an upstream or a downstream target or whether Cten has a feedback mechanism to control the activity of Stat3.

Integrin linked kinase (ILK)

To further establish the role of Cten in motility, its association with other focal adhesion related proteins needs to be studied in detail. One such partner of Cten is integrin linked kinase (ILK). Cten positively regulates levels of ILK. Forced expression and knockdown of Cten in colorectal cell lines correlates proportionally with ILK levels. The inhibition of ILK after forced expression of Cten nullifies the motility enhancing effects of Cten (7). This establishes ILK as a downstream target of Cten. It can be assumed that Cten acts as a regulator of kinases in the focal adhesion apparatus.

1.5.7 Role in apoptosis

Phosphotyrosine binding domain (PTB)

Cten also has a proposed role in apoptosis in the prostate. Cten is found to be cleaved during apoptosis induced by staurosporin in normal prostate epithelial

cells. Caspase-3 is the major protease that cleaves Cten at DSTD570 site, which falls just before the PTB domain. The resultant Cten fragment (571-715) which is the PTB domain reduces growth rate and promotes apoptosis. As the PTB domain binds to cytoplasmic tails of β integrins, this might disrupt association among integrins, actin fibres and other focal adhesion molecules and may lead to or form part of the process of focal adhesion breakdown, cell detachment and cell death due to apoptosis(56).

1.5.8. Role in the nucleus

Although Cten is considered as a focal adhesion molecule located within the cytoplasm. It has been seen that it localises in the nucleus as well. The interesting observation is that nuclear Cten expression correlates with advanced tumour stage and metastasis. This suggests a role of Cten beyond cellular adhesion foci(7). In the nucleus Cten is bound to β-catenin, thus linking a it to the well-known Wnt-pathway. It has been seen that a number of focal adhesion molecules like FAK, Zyxin and paxillin also travel to the nucleus and act as regulators by either binding to transcription factors or directly affecting the mRNAs (57,58). The exact role of Cten in β-catenin pathway however still needs investigating(6).

1.6 Possible medical applications

Effective treatment options are continually needed for patients with cancer. Cten, because of its up regulation in a number of cancers and especially in advanced stage tumours and metastatic tumours has an application for potential treatment or prognostic marker.

Cten correlates positively with EGFR and HER2 in breast cancer samples. It has been seen that using Lapatinib, which inhibits EGFR/HER2 in breast

cancer patients causes decrease in Cten levels. Therefore, Cten might act as predictor of patients response to EGFR-targeted therapy(49). If role of Cten is confirmed as part of EGFR/K-Ras signalling pathway in colorectal and pancreatic cancer cell lines, then anti-Cten therapy may present a novel treatment for colorectal cancer patients in future(43).Up-regulated Cten regulates cell migration induced by FGF2 and a variety of other growth factors like PDGF, NGF, TGF-β and IGF-1, strongly suggesting that Cten could be a potential downstream therapeutic target for treating cancers associated with abnormal signalling of these growth factors as well(51).

1.7 Hypothesis and Aims

In the light of the above discussion, we can appreciate that Cten is a novel regulator of cellular adhesion, migration and metastasis owing to its close interaction with integrins, focal adhesions and related proteins. It has been associated with many integral pathways of tumour development and progression as seen in breast, colorectal and a number of other cancers. There are still a lot of unanswered questions regarding its place in the focal adhesions and its binding partners and the exact mechanism through which is stimulates motility.

The aim of this study is to study in detail the functional activity of Cten. To achieve this aim the objectives are study the effect of Cten in nucleus, to study the role of SH2 domain and its downstream targets and to study the regulators of Cten.

2 Materials and Methods

2.1 Cell Culture

For colorectal cancer (CRC), the cell lines used were: HCT116, RKO, and SW620. MCF7 (breast cancer line) and Lovo (colorectal cancer cell line) was used as positive control for some proteins. The cell lines were a gift from Prof. Ian Tomlinson, the Molecular and Population Genetics Laboratory, London Research Institute, Cancer Research UK, London. All these cells lines grow as adherent cultures.

The clinical information and tissue culture conditions for each of the cell lines used were obtained from ATCC (www.atcc.org) and are summarised in Table 2-1. These cell lines were validated for mutation specific for them by colleagues in our laboratory. All cell lines were mycoplasma tested.

Tissue culture treated flasks (75 cm^2) and 6 well tissue culture plates were obtained from Costar, UK.

All cell lines were grown in culture medium made up ofDulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies) supplemented with 10 % Foetal calf serum (FBS) (Sigma, UK), 100 units/ml penicillin, and 100µg/ml streptomycin (Invitrogen, UK). Cells were grown in humidified 5% CO_2 atmosphere at 37^oC in an incubator.

Table 2-1 List of cell lines used in the study

Cell Line	Age	Sex	Duke stage	Differentiation	Site
HCT116	64	Male	D	Well	Colon
RKO	64	Male	C	Poor	Colon
SW620	51	Male	C	Moderate	Colon Lymph node
Lovo	56	Male	C		Colon (left supraclavicular region)
MCF7	69	Female	x	Adenocarcinoma	Breast(pleural effusion)

2.1.1 Cell Passaging

Cells were passaged on average once a week depending on their growth rate but usually when they would grow to 90-100% confluence. The cells were passaged by aspirating the previous media off using by a sterile aspirator. Sterile Phosphate buffered saline (PBS) by Sigma (D8537), Trypsin EDTA 1x (Sigma T4174) and DMEM were all kept in a water bath at 37^{o}C to bring their temperature down as they are stored at 4^{o}C. After aspirating the old media, cells were washed with 5ml of PBS, and then 2ml of Trypsin EDTA was added to cells and left in the incubator for 2 minutes for the cells to become detached. After that 8ml of DMEM was added to the cells, mixed thoroughly by pipetting up and down. 1ml of this mixture was added to a new flask with 9ml of fresh DMEM. Cells of passage number up to15 were used in the experiments. Cells with passage number above 15 were discarded by adding Trigene.

2.1.2 Freezing and Thawing

At the start of the project, the cell lines were grown in a large 225cm^2 flask (Corning) and multiple aliquots were frozen. Approximately 500,000 cells were mixed with 1ml of Freezing Mixture [90% Foetal Bovine Serum (FBS) and 5% Dimethyl sulphoxide (DMSO)Sigma D2650]. Cells were slowly frozen to -80^{o}C by placing in a box with ethanol to slowly bring down the temperature in order to avoid damage to the cells. With the aim of reducing the effect of prolonged passages on cells function, cells with passage number above 15 were discarded and replaced with cells frozen in -80^{o}C. After taking cells out of the freezer they were first grown in 25cm^{2}flask (Corning) and once confluent, the whole volume of cells was moved into a T25 cm^2 and grown as usual. The cells had different passage numbers varying from 2-4 passages at defrosting time.

2.1.3 Cell counting

50µl of cell suspension was mixed with 50µl of trypan blue (Sigma) and incubated for 5 minutes at room temperature. 10µl of Cells were filled onto the hemocytometer and counted under the microscope. Cells having taken up the blue dye were considered non-viable and were not counted. Viable cells in 3 big squares were counted (C1, C2, and C3) and divided by 3 to take an average of cells. The following formula was used to calculate number of cells in 1 ml.

C1+C2+C3/3 x 2(dilution factor of adding trypan blue) x 10^4 = cells/ ml of media.

For easier, quicker and reliable cell counting theBioRad Tc20 automated cell counter serial no. 508BR1790.was used.BioRad counting slides dual chamber for cell counter (145-0011) were charged with a cell suspension of 10µl of 50% cells with 50% Trypan blue The cell numbers counted by the counter were tested by manual counting and was found to be comparable.

2.2 Construction of Nuclear localisation sequence (NLS) tagged-Cten-GFP plasmid

Cten tagged with NLS was inserted into pEGFP-N1 plasmid from CLONETECH laboratories, Inc. Catalogue no. 6085-1. The plasmid map is shown in Figure 2-1. Detailed map of the plasmid obtained from Clonetech can be found in Appendix 10.1.

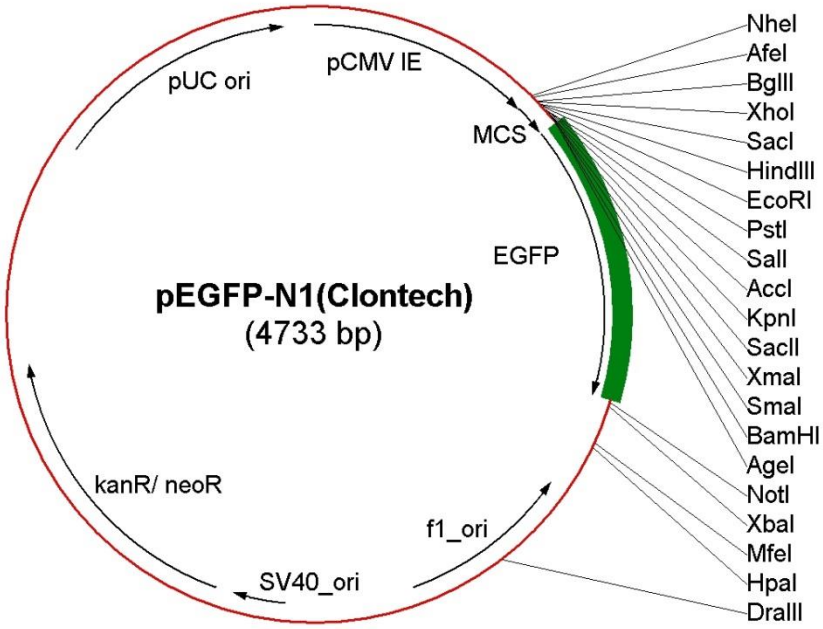


Figure 2-1: Map of pEGFP N1 plasmid used for cloning NLS tagged Cten.

Primers were designed using the primer 3 website (http://frodo.wi.mit.edu/primer3/input.htm)

The primers were designed in a way that the NLS sequence (received from F. Falcone)(59) was kept in frame with the start of Cten sequence (NCBI Reference Sequence: NM_032865.5).

Forward primer was designed as follows: HindIII site (AAG CTT), ATG NLS CTEN

Reverse primer: Cten sequence, BamH1 site (GGA TCC), and Start of GFP plasmid.

Primers were ordered through MWG-EUROFINS, UK.

Forward primer: 5'-**AAG CTT** ATG CCG AAA CGG AGA AGG ACA TAC ATG TCC CAG GTG ATG TCC-3'

Reverse primer: 5'-GT**G GAT CCA** TCC TTT CTG CGT CCT-3'

In the primer sequence, the bold letters represent enzyme binding site and red letters represent sequence for NLS.

A PCR reaction was run using proof reading pfuTaq polymerase enzyme (Promega). The product was amplified using a template plasmid of GFP-Cten (a kind gift from Dr. Su Hao Lo, Department of Biochemistry and Molecular Medicine, University of California-Davis,USA). The Cten is of Human origin.

The PCR was done to amplify NLS tagged with Cten. PCR was performed using Syber safe dye, 10ng of DNA template and 250nM of primers each, in a final reaction volume of 25µl. Thermal profile was set as: denaturation at 95^{o}C for 1 min, annealing at 68oC for 1 min: 30 sec and extension at 72^{o}C 1 min for 35 cycles. Products were run on 1% agarose gel at 100V for 30 minutes and visualized under UV light and results recorded using a Polaroid camera.

The product was cut from the gel and extracted using QIAquick gel extraction kit (QIAGEN, UK). This time the gel was not exposed to UV light as it would damage the DNA.

2.2.1 Agarose gel electrophoresis

100ml of a 1% w/v agarose (Gibco-BR Life Technologies, USA) gel was prepared in 1% Tris acetic acid and EDTA (TAE) buffer (Sigma, USA) and heated in a microwave for ~60 seconds. After the agarose had fully dissolved with intermittent agitation, the mixture was then cooled to room temperature.

Once the mixture had cooled, 10µl of Sybersafe dye (1000x) (Sigma, USA) was added and the gel was then poured into the gel apparatus and an appropriate comb was inserted. The gel was then left at room temperature to allow the agarose to set. Gels were run using 1x TAE solution as running buffer. 10µl PCR product / DNA samples were mixed with 2µl loading dye and loaded into the gel wells. Gels were run at ~100 Volts for 45 minutes. Samples were assessed by viewing on an ultraviolet transilluminator (UVP Inc., USA) and sized according to the 1kb DNA ladder (New England BioLabs, UK). If further band separation was required, the gel was placed in the electrophoresis equipment and electrophoresed for the desired time.

2.2.2 DNA extraction from agarose gel

PCR products or plasmid DNA were run on a gel to check the size of the PCR product. They were excised from the gel, under blue light, using a clean scalpel. The DNA was isolated from the gel using Gel Extraction Kit (Sigma NA1111) following the manufacturer's standard protocol. After weighing the gel fragments containing the band of interest, the fragment were dissolved in 3x volumes of solubilsation solution (supplied by the kit), then incubated for 10 minutes at 50^{o}C. After the gel slice had been dissolved completely, 1x gel volume of isopropanol (Sigma, USA) was added and mixed with the samples which were then washed with 750µl of wash solution (supplied with the Kit) to remove all traces of agarose and finally the DNA was eluted with 50µl of elution buffer.

2.2.3 DNA quantification by spectrophotometery

The final extracted DNA was quantified on a Nano Drop ND-1000 UV-Vis Spectrophotometer (LabTech International Ltd, Ringmer, UK). Using a spectrophotometer, a 'blank' measurement was obtained reading Elute solution used to extract DNA at λ260nm. Samples were analysed at λ260nm compared to the 'blank' result and all readings were measured by the ratio of λ260/λ280nm to check the purity of the samples. A ratio of 1.8 – 2 was considered for an indication of relative purity.

2.2.4 TOPO® cloning

The TOPO® TA cloning kit (Invitrogen K4500-01) was used for cloning PCR products. The kit contains a linearized plasmid vector withsingle 3´-thymidine (T) overhangs for TA Cloning® and Topoisomerase I enzyme covalently bound to the vector. If the template has an Adenosine (A) overhangs at the 3' prime end then it can efficiently ligate with the vector. Topoisomerase I enzyme binds to duplex DNA at specific sites and cleaves it at 5'-CCCTT in one strand. Then a covalent bond is formed between 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I (60,61).

Adenosine overhangs were added to the 3' end of the PCR product by incubating with adenosine (ATP) and taq polymerase enzyme (Promega) for 20 mins at 72^{o}C, placing on ice and using immediately for TOPO® cloning. 4µl of PCR product was mixed with 1µl of salt solution and 1 µl of Topovector and 5µl of water. The mixture was incubated at room temperature for 5 minutes and then transformed into competent E-coli cells using the heat shock method. Cells were plated onto LB agar plates with Kanamycin at

50µg/mlconcentration and coated with X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Promega, UK). Only the white/light blue colonies were picked as they contained the desired plasmid.

2.2.5 Enzyme restriction digest digestion

Plasmid and PCR products were digested using restriction enzymes by New England BioLabs (NEB) Inc. UK. The enzymes used were HindIII and BamH1, which both give 100% efficiency in NEB 2 buffer. Different concentration of DNA, enzyme and buffer were used. A 30µl reaction was set up containing 5µg of DNA and 3µl of 10x reaction buffer, 5µl of restriction enzyme and making up the remaining volume with distilled water. For BamH1 digestion BSA was also added in a concentration of 1µg/10µl reaction and specific BamH1 buffer (NEB, UK) was used for increased efficiency. The mixture was incubated at 37^{o}C for 4 hours and then run on 1% agarose gel.

2.2.6 Cloning NLS-Cten into the pEGFP-N1 expression vector

Sequential 5'-BamH1 and 3'-HindIII enzyme digestion of the NLS-Cten PCR product from the TOPO vector and pEGFP N1 vector was done for shuttling the NLS-Cten insert from TOPO vector plasmid into the GFP expression vector. The PCR products were viewed and excised under Ultraviolet light and gel-purified using QIAquick Gel Extraction Kit according to the manufacturer's protocol as described above. The isolated NLS-Cten insert was ligated into the pEGFP N1 vector at molar ratio 1:1, 1:3 vector to insert. Calculated as follows:

$$\frac{\text{ng of vector} \times \text{kb size of insert} \times \text{molar ratio of insert}}{\text{kb size of vector} \times \text{vector}}$$

Can also be calculated online at:

http://www.insilico.uni-duesseldorf.de/Lig_Input.html.

Two negative controls (digested pEGFP N1 plasmid only and competent cells only) were also set up to make sure that pEGFP N1 was completely digested and did not re-ligate again. Enzyme digestion and purification of NLS-Cten PCR product and pEGFPN1 plasmid DNA, and were done according to the manufacturer's protocol as described before.

2.2.7 Ligation and Transformation

Ligation of NLS-Cten and pEGFP N1 plasmid was done using T4 Ligase enzymeInvitrogen #15224-017) and 10x ligase buffer (Invitrogen P/NY90001). Usually 0.5µl of ligase enzyme was used in a 30µl reaction. The mixture was kept overnight at 4^{o}C.

Plasmid was transformed in Gold efficiency competent E coli cells by Bioline BIO 85047 (kept at -80^{o}C). For 50µl of cells, 5µl of plasmid ligation mix was used. Transformation was done by the heat shock method. In this method cells, cells were mixed with plasmid and incubated on ice for 30 minutes, then heated in water bath at 42^{o}C for 45 seconds and again kept on ice for 2 minutes. 900µl

of LB broth (Sigma, USA) was then added to the cells and kept on a shaker at 37^{o}C for 1 hour. After that, cells were centrifuged for 1 minute at maximum speed to make a soft pellet. 700µl of supernatant was discarded and rest (250µl) was mixed with cell pellet and plated on 10cm petri dishes (costar, UK) containing 20ml of LB agar (L2897 Sigma) and Kanamycin (Sigma) at 50µg/ml concentration. The plates were incubated at 37^{o}C overnight and colonies picked up the next day.

2.2.8 Midiprep and miniprep

In order to generate plasmid DNA, the Gen Elute Plasmid Miniprep and Midiprep Kits were utilized following the standard manufacturer's protocol. For 'Miniprep' preparations, white positive colonies containing the desired plasmid were picked with pipette tips and used to inoculate 5ml of LB broth (L3022, Sigma), containing 5µl of 50mg/ml stock kanamycin solution (Sigma, 1377) at a final concentration of 50µg/ml. For 'Midiprep', 1ml of the Miniprep growth was added to 100ml growth medium containing 50µg/ml kanamycin. The media was then incubated in the shaking incubator for overnight at 37ºC to allow culture of the selected bacteria.

Miniprep was performed using GenElute plasmid miniprep kit by Sigma Aldrich (PLN350). For 'Miniprep' preparations, the culture (3-5ml) was centrifuged for 1 minute at 13,000 rpm and the cell pellet was resuspended in 200µl of resuspension solution. This was then followed by lysis of the cell pellet through adding 200µl of Lysis solution. Then 350µl of Neutralizing/Binding solution was added to precipitate the cell debris and samples were centrifuged at 13,000rpm for 10 minutes, and then the

supernatant was passed through a Gen Elute Miniprep Binding column (Sigma). The columns were then washed by 750µl of washing solution and the DNA, which binds to the columns, was eluted by 50µl of Elution buffer.

For 'Midiprep' preparations, 100ml of bacterial culture was used to obtain DNA plasmids using GenElute using Plasmid midiprep kit (Sigma, NA0200-1KT). The solutions used in were the same as in the miniprepwith slightly modified protocol in order to prepare greater volumes of plasmid DNASequencing.

The plasmids grown were sent for sequencing to a sequencing Lab in Queens Medical Centre, Nottingham (https://www.nottingham.ac.uk/life-sciences/facilities/dna-sequencing/index.aspx).

Samples were prepared according to the lab's requirement of template concentration between 70-150ng/µl. Primers of 10pmol/µl in concentration were used. The sequencing results were analysed by ChromasLite software available at http://technelysium.com.au/. These sequences were then put on NCBI blast website to look for matches with the desired sequence.

2.2.9 Transfection optimization

Transfection optimization was done as by varying the confluence of cells in 6 well plates, amount of plasmid added, amount of lipofectamine and dilution media (Optimem).

In the beginning the yield was quite poor as apparent from FACS analysis in **Error! Reference source not found.** (A) By increasing the amount of plasmid o 8ug and adding 24ul Lipofectamine in a total of 1.5ml of Optimem media and starving cells in 1.5ml of Optimem media before transfection for 4 hours,

greatly increased the transfection efficiency for all plasmids as shown in Figure 2-2.

2.2.10 Flow cytometry

The cells were harvested from the 6 well, cell culture plates using Trypsin EDTA and then mixed with PBS and centrifuged for 3 minutes at 1000 rpm to extract Trypsin EDTA. The cells were then mixed with PBS again and viewed using Epics Altra Flow cytometer (Beckman Coulter) with the GFP filter (533/30). The data was analysed using Weasel software. Flow cytometery was used to measure the efficiency for transfection of the new NLS Cten and Cten as compared to GFP to achieve the highest efficiency possible close to 50% in both cell line RKO and HCT116.

2.2.11 Fluorescence microscopy

Cells were grown on sterile cover slips placed in 6 well plates and transfected as described before. After transfection cells were fixed in 4% formaldehyde while still inside the 6 well plates and stained with DAPI solution in PBS for 5 minutes. Cells were then mounted onto glass slides and coverslips using mounting media and sealed using nail varnish. Cells were then viewed using a DMRB Fluorescence Microscope and pictures were taken at highest magnification.

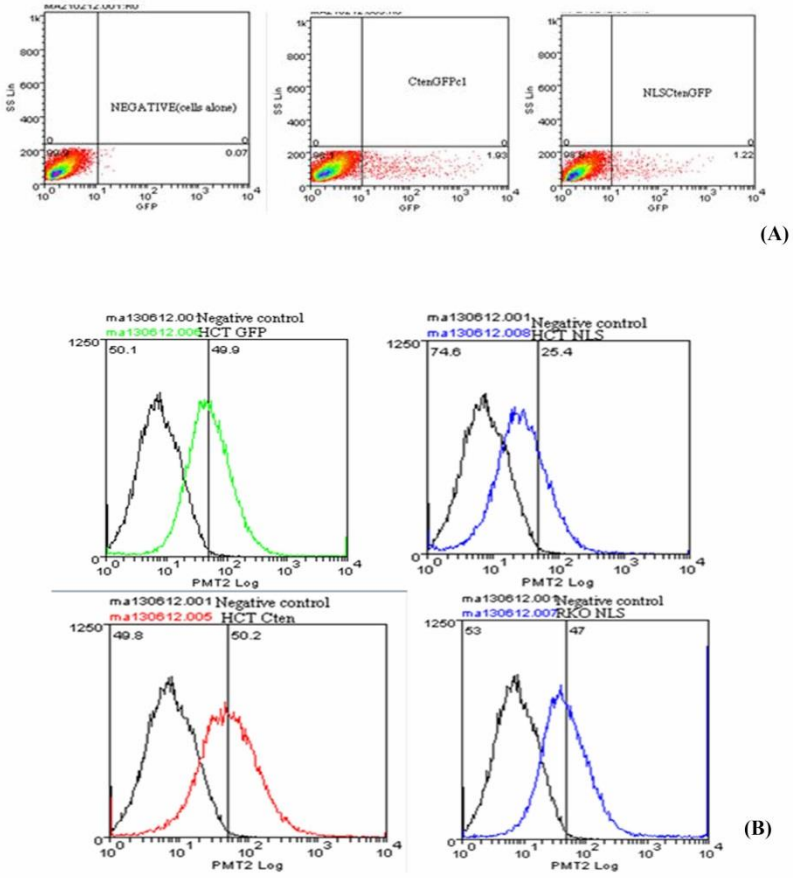


Figure 2-2: (A) Dot plot of the GFP florescence from the live cells viewed in Altra FACS machine.First one is the negative control, the second plot shows only 1.93% of cells emitting florescence when transfected with Cten. Whereas only 1.22% cells transfected with NLS-Cten-GFP show florescence. (B) The histogram shows different coloured peaks for different transfection conditions of GFP-Cten , NLS-Cten-GFP and pEGFP alone. Showing maximum of 50.2% efficiency with red curve for GFP-Cten transfected in HCT116 cells where blue curve in the bottom histogram showing 47% transfection efficiency in RKO cell line. Black curve is the negative control. PMT2 Log represents GFP florescence on X-axis.

2.3 Forced expression by transfection

300,000 were seeded in 6 well plates (Costar, UK) with 2ml of DMEM. When the cells reached to 60% confluence, after 24-hours, transfection with pEGFP (empty vector) and GFP-Cten and NLS-Cten-GFPplasmids was performed. Four hours before adding plasmid cells are starved by adding 1.5ml of Optimem media in each well and incubated at 37 ^{o}C with 5% CO2. For the 6-

well plate, 8µg of the plasmid and 24µl of the lipofectamine 2000 (Invitrogen, UK) was separately diluted in 250 µl of Optimemmedia (Invitrogen) respectively in eppendrof tubes and the dilutions were allowed 5 minutes to settle. The diluted plasmid was added to the diluted lipofectamine and allowed 30 minutes for the plasmid to bind to the lipofectamine at room temperature. Dilutions were added to the 1.5 ml Optimemmedia cell-containing wells. The Optimem media was changed after 6 hours with fresh 2ml 10% FBS DMEM. The cells were allowed to grow for another 24 hours. After that, the cells were either trypsinized for transwell assay or harvested for protein assay.

2.4 Knockdown and Co-Transfection

RNA interference is an intrinsic method employed by eukaryotic cells to silence specific proteins. Small interfering RNAs (siRNA) are usually 21-22bp long have characteristic 2 nucleotide 3 prime overhangs that helps them to recognised by enzymatic machinery which leads to degradation of the target mRNA. In mammalian cells siRNAs are produced from cleavage of longer double stranded RNA precursors by the RNaseIII endonuclease Dicer. This dicer binds with RNA binding protein and hands siRNA to RNA induced silencing complex (RISC). This complex contains a protein Argonaute 2 protein which cleaves the target RNA(62).This mechanism can be employed in knockdown of specific proteins in vitro settings when specific siRNA is integrated in a vector artificially. This vector is then transfected into the target cell lines by the usual process of transfection as explained earlier with a few modifications.

Knock down of a specific gene was performed in a 6 well plate with cells seeded so that they become 40% confluent the next day. The siRNA received

were diluted in 1000µl of RNAse free water to make a stock solution of 20µM. 10ul of this was used in a 2ml reaction making the final concentration of 100nM. Similar quantity of negative control Luciferase siRNAwas used as a control as a check for any off target/ nonspecific effects of the siRNA vector. The choice of negative control varies within laboratories, some prefer to use their own scrambled sequence control; others use luciferase and eGFP siRNA. We used Luciferase in our experiments. The advantage with the luciferase (or other marker genes) is you can use these siRNAs also as positive controls when co-transfected with the respective marker gene.

Control and experimental siRNA were mixed in 10-15µl of Lipofectamine and Optimem and kept for 30 minutes at room temperature. Afterwards they were added to the cells which were already in Optimem media. Media was changed to DMEM after 6-8hours. Proteins were harvested after 48 hours. The siRNA list used in this study is given in Table 2-2.

Table 2-2: Detailed sequences for the siRNA used in the study.

Gene	Sequences
Cten NM_032865	L GCCCAGAGCACCAUGUCAAUGAGAA R UUCUCAUUGACAUGGUGCUCUGGGC
ILK NM_004517.2	L UUCUCCGUGUUGUCCAGCCACAGGC R GCCUGUGGCUGGACAACACGGGAGAA
FAK NM_005607	L GAUGUUGGUUUAAAGCGAU R AUCGCUUUAAACCAACAUC
Universal non targeting siRNA (Luciferase specific)	L CAGUGUAGUAGUCGUUUC R GAAACGACUACUACACUG

For Co-Transfection of plasmid and siRNA, cells at 50% confluence were used. Same procedure as given above was used. 100nM of siRNA and 8µg of plasmid mixed with optimem and 24µl lipofectaminewas added to cells after 30 minutes at room temperature. Media was changed from Optimem to DMEM with 10% FBS after 6-8hrs. Proteins harvested after 48-72 hours.

2.5 Protein Extraction and quantification

The cells were treated with ice cold RIPA lysis buffer (Sigma) (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and Halt protease, phosphatase inhibitor cocktail 100x, PPI (Thermoscientific) at a concentration ratio of 1:100 to lyse cells and extract proteins. PPI was added to prevent protein degradation. Cells were left in ice for 20 minutes after which cells were centrifuged at 4^{o}C at 13000 rpm for 30 minutes. Supernatant was collected and stored in freezer at -20 ^{o}C. For the purpose of equal loading in western blotting experiments, the amount of proteins in each of the cell lysates was quantified using the BCA Protein Assay Kit (Thermos Scientific, Pierce BCA protein kit, 23225) according to the manufacturer's instructions using the microplate procedure. A series of Bovine Serum Albumin (BSA) solutions in the concentration range of 25–2000 µg/ml was used as a standard reference range. 12.5µl of each sample, standard and blank was pipetted into the 96 microplate wells (Costar, UK) as triplicates. Then 100µl of a 1:50 dilution of Reagent B: Reagent A was added to each well and the plate was shaken for 10 seconds and then incubated at 37°C for 30 minutes. The absorbance at 550nm was measured on original Multiscan lab systems Ex. A graph was plotted of optical density and concentration of the standards (Figure 2-3). The concentration of the sample protein was calculated by using the

following formula: **x=y-b/a**. Where y is the absorbance of the sample and x is the concentration of protein µg/ml. For cytoplasm and nuclear fractionation Biovision nuclear cytosol fractionation kit (K266-25) was used. Following the manufacturer's instructions the proteins were extracted and quantified as above.

2.5Western blotting

3.3.1 Homemade gels

The SDS-polyacrylamide gel used in western blotting (WB) consisted of two parts: a lower resolving gel and an upper stacking gel. The lower resolving gel consisted of 10% polyacrylamide (Bio-Rad Laboratories, UK) in resolving buffer solution (1.5M Tris-base pH 8.8, Bio-Rad Laboratories, UK 161-0798).mixed with 10% SDS (Bio-Rad 161-0416), distilled water, 10% APS (ammonium per sulphate) and TEMED (Sigma T8133) The upper stacking gel consisted of 5% polyacrylamide in stacking buffer solution (1 M Tris-base / pH 6.8, Bio-Rad 161-0799) mixed with 10% SDS, 10% APS, distilled water and TEMED The gel was then left at room temperature for 30 minutes to be polymerised and then transferred to the electrophoresis unit containing 500ml 1x Tris-Glycine/SDS electrophoresis buffer (25mM Tris base, 192mM Glycine, 0.1% SDS, pH 8.3) (Bio-Rad Laboratories,UK, 161-0772).

Fifty micrograms of proteins lysate were mixed with a loading buffer in a ratio (1:3). Eppendrof tubes (1.5ml) were labelled according to the various wells contents. Appropriate volumes of protein were added to a homemade 4x SDS loading buffer containing 5% β-mercaptoethanol (Sigma M7154). The samples were incubated at 90ºC on a heat block for 5 minutes to denature the protein and subsequently transferred to ice for another 5 minutes after denaturation.

After centrifuging for 2 minutes at 13,000g, the samples were loaded into the wells of the 10% SDS PAGE running gel and ran for 60 minutes at 60 mA for two gels in 1x Tris-Glycine/SDS electrophoresis buffer as running buffer (BioRad).

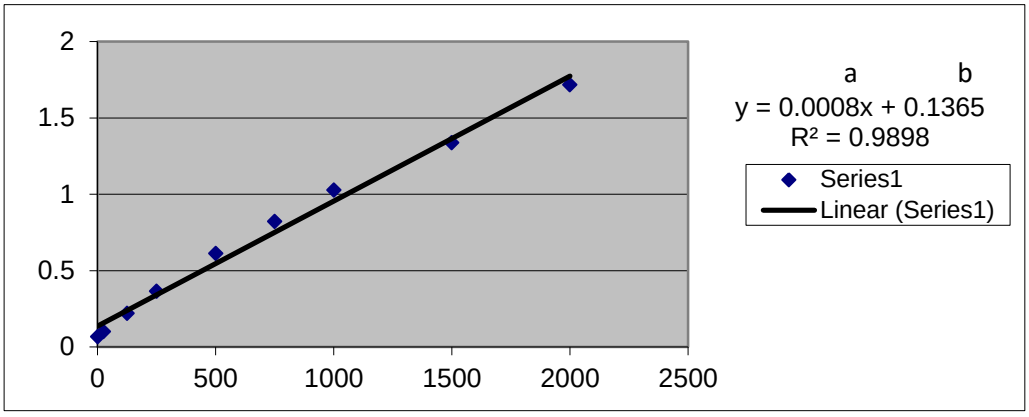


Figure 2-3 Protein assay: This graph is blotted according to the standards given in the kit. Y axis represents the optical density of the protein standards. X axis gives the concentration of protein in µg. Series 1 is the optical density of the serial dilution of protein standard. R2 gives precision of pipetting nearer to 1 is better. Using this graph X can be calculated.

An appropriate sized piece of Polyvinylidenediflouride (PVDF)membrane (hybond P Amersham) was soaked in methanol for 15 seconds then hydrated in transfer buffer (100ml of 10x Tris-Glycine+ 200ml of Methanol+700ml of distilled water) for 2 minutes. The gel was transferred onto nitrocellulose membranes by semi dry transfer. The membranes were incubated, at room temperature, in a blocking solution (5% milk powder or Bovine serum albumin dissolved in 0.1% Tween PBS) for one hour. After that, membranes were incubated overnight with primary antibodies. The antibodies used were diluted in specific diluents details given in Table 2-3. After overnight incubation, membranes were washed and incubated for one hour in an appropriate horseradish peroxidase-linked secondary antibody (Sigma Aldrich). Mostly anti rabbit (A6154) and antimouse (A4416) were used. The bands were

detected using enhanced Chemiluminescence kit (Amersham Bioscience). The membrane was exposed to the x-ray film in the dark room. The x-ray film (Kodak, UK) was revealed in developer and fixer and then washed by water.

2.5.1 Invitrogen western blot equipment

The homemade westerns and the BioRad solutions being used were giving variable results and the performance was poor as well. So we decided to change over to readymade gels and also change the solutions for running and transfer of the gels. We switched to using NovexNuPAGE SDS gel system (Invitrogen X cell sure lock Novex mini cell) NuPAGEBisTris gels (NP03335) of 1.5mm thickness and percentage of 4-12% were used. Proteins were loaded with NuPAGE 4x LDS sample buffer (NP0007) with 2 beta-mercaptoethanol. 20x NuPAGE MOPS SDS running buffer (NP0001-02) was used by diluting to 1x with distilled water. Semidry transfer method was employed to transfer proteins to PVDF membrane (hybond P Amersham) using BioradTransblot turbo transfer system. Bio Rad 10x Tris Glycine buffer was used mixed with methanol for transfer. ECL prime westernblot detection system (Amersham) was used for chemilumenescence. Hyper Films (Amersham GE healthcare # 28906835) were developed using Kodac developer and fixer. The films were scanned after drying. The bands were quantified using image J software (http://imagej.nih.gov/ij/). Table 2-3 Details of the antibodies used in the study including company purchased from and conditions used in western blot. Table 2-3 gives the details of all the primary antibodies used in this study

Table 2-3 Details of the antibodies used in the study including company purchased from and conditions used in western blot.

Antibody	Company	Band size kDa	Dilution buffer	Blocking solution	Dilution factor	Secondary antibody Dilution
Cten/TNS4	Sigma Aldrich WH0084951M1	Endogenous 77 Plasmid 110	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1:10,000	Mouse (1:5000)
ILK	Cell Signaling 3862s	50	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:5000	Rabbit (1:10,000)
FAK	Cell Signaling 3285s	125	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:5000	Rabbit (1:10,000)
pFAK (Tyr397)	Cell Signaling 3283s	125	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:5000	Rabbit (1:10,000)
E-cadherin	Cell Signaling 3195s	135	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:6000	Rabbit (1:10,000)
N-Cadherin	Abcam 98952	100	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:5000	Rabbit (1:10,000)
Snail	Cell Signaling 3895s	29	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1:1000	Mouse (1:5000)
CD24 (SWA11)	Gift from Peter Altevogt Group	20-70	3% milk 0.1% tween PBS	3% milk 0.1% tween PBS	1:1	Mouse (1:1000)

Paxillin	LSBio LS-C49487	68	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:5000	Rabbit (1:10,000)
Vincullin	Cell Signaling 4650	124	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:5000	Rabbit (1:10,000)
β-Actin	Sigma A1978	42	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1`:50,000	Mouse (1:25,000)
β-Catenin	Enzo Life Sciences 7D11	89	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	10ug/ml	Mouse (1:5000)
pY20	Sigma Aldrich P4110	variable	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1:10,000	Mouse (1:10,000)
β1 integrin	Thermo scientific MEM-101A	120	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	2ug/ml	Mouse (1:5000)
Src	Cell Signaling 2108s	60	5% BSA 0.1% tween PBS	5% BSA 0.1% tween PBS	1:5000	Rabbit (1:10,000)
Tensin 3	Sigma Aldrich SAB4200204	135	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1:1000	Mouse(:10,000)
Lamin	Abcam 9622	68kDa	5%BSA 0.1% tween TBS	5%BSA 0.1% tween TBS	1:1000	Rabbit(1:10,000)
Calpain I	Millipore MAB3104	80kDa	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1:1000	Mouse (1:10,000)

Calpain II	Abcam 39165	76kDa	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1:1000	Rabbit (1:10,000)
Calpain IV	Chemicon International MAB3083	30kDa	5% milk 0.1% tween PBS	5% milk 0.1% tween PBS	1:1000	Mouse (1:10,000)

2.6 Immunoprecipitation

Protein G agarose beads (Gammabind G sepharose, GE healthcare # 71-7057-00 AH) were coated with the desired antibody to precipitate particular protein from the sample. 1ml of all proteins was pre-cleared 20µl of beads by incubating for 30 minutes at 4^{o}C on a rotator. Protein were incubated overnight with antibody first then overnight with beads while rotating at 4^{o}C. After this 30µg of protein was incubated with 1 µg of antibody overnight and then with 30µl of beads overnight at 4^{o}C. Samples were then centrifuged at highest speed for 10 minutes at 4^{o}C. Washed twice with ice cold PBS (Sigma D8537). Supernatant was discarded and the beads were treated as protein and western blot was performed as described. Protein incubated with beads but without antibody was used as negative control. For input late 50µg of protein was loaded. A control IP was performed to check the method used and reliability. For this control IP a well-known interaction between E-cadherin and β catenin was studied and found to be successful as seen in Figure 2-4.

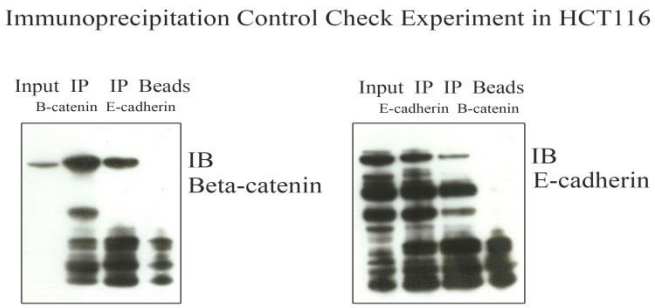


Figure 2-4:.IP of E-cadherin and β-catenin.Show a positive binding between E-cadherin and β-catenin in done in SW620. Left blot is blotted with Beta-catenin and right is blotted with E-cadherin. The negative control of beads alone gives no band in both cases showing true binding.

2.7 Transwell migration assay

Cells grown in 6 well plates (Corning) were harvested with 1x trypsin/EDTA (Sigma T4174) and 500 µl of DMEM growth media was added before transferring to a 1.5 ml tube and centrifuged for 5 minutes at 1000 RPM. The supernatant was discarded and pellets were re-suspended in 1 ml growth media. Cell migration assay was performed using 24 transwell chambers (8 um pore size, 6.5mm insert, 24 well plate, Costrar#3422, Corning USA). The lower chambers were filled with 600 µl of 20% FBS DMEM media. In the upper chambers, 1.0 $x10^5$ from the suspension was added mixed with 10% FBS DMEM media so that the total solution in the upper chamber is 100 µl. The cells were allowed to migrate at 37 ºC and 5% CO_2 in humid conditions for 48 hours. Migrated cells attached tothe bottom of the well were viewed through inverted microscope and counted.

2.8 Wound healing assay

Cells grown in 6 well plates were transfected with plasmids as mentioned before and then allowed to grow to 100% confluence. Before making scratches the base of the 6 well plated were marked with a blue line going through the middle. Three wounds/scratches were made using a 200µl tip. Then the cells were washed with PBS two times to get rid of the cells that had come off. 2ml of 10% FBS media was added to the wells and pictures were taken just above and below the marked line at the beginning i.e., T=0, taking care that the line is not visible in the picture. After 24 hrs pictures were again taken on the same spots above and below the marked line defined as T=24. The pictures were analyzed using the software T-Scratch (CSElab) to calculate the area of migration of cells using the following formula: Area of migration = 100 x (open wound area at 0 hour –open wound area at 24 hour).

2.9 Colony formation assay

Colony formation assay was done in 6 well plates in triplicate for each condition. A base layer and a top layer were laid down on 6 well-plates. The base layer was prepared using 1% agar (Sigma A1296-100G)mixed in cell culture grade water. It was mixed by heating in microwave and cooled to 40 degrees centigrade in water bath. Then equal amount of 2x DMEM (Sigma) with 20% FBS (Sigma) was added so that it comes out to be 1x DMEM and 10% FBS in total mixture.1ml of this mixture was added to each well and leave to set at room temperature. For the top layer 0.7% agarose (Sigma A6877-10G) solution was made using cell culture grade water, heated in microwave and cooled to 40 degrees. Equal amount of 2x DMEM with 20% FBS was added to agarose mixture. Transfected cells were harvested, counted and suspended in the top agar mixture so that each ml has 2500 cells in total that goes in each well on top of base layer. All the experiment was conducted in triplicates for each condition. Every step was done in sterile conditions. Cells were incubated at 37^{o}C with 5% CO2 incubator for 21 days. They were fed with 10% FBS 1xDMEM (Sigma) media two times a week. After 21 days the colonies were fixed and stained with 500µl of 0.005% crystal violet (Sigma C3886) mixed with 4% Formaldehyde solution (Sigma HT5011280) for 1 hour and counted under the microscope using grid pattern made under the 6 well plates to help in counting. Colony forming efficiency was calculated by dividing number of colonies counted by the total number of cells seeded. The resulting figures were plotted on graph using Prism 5.0 software and significance calculated.

2.10 Methylene blue proliferation assay

After transfection in 6 well plates, cells were harvested and counted. 20,000 cells were added to each well of a 24 well plate, each well containing 600µl of

1xDMEM with 10% FBS. Each condition was done is triplicate. Cells were seeded in such a way that each transfection condition has 9 wells in total. Three wells for Day0, Day 1 and Day2 respectively. After seeding cells on Day0, they were allowed to settle for 2 hours. Media was aspirated carefully, and 200µl of Methanol was added and incubated at room temperature for 30 minutes and extra methanol was aspirated. After fixing cells on all 3 days, 200ul of 1 % methylene blue (Sigma M4159) mixture was added and incubated for 30 minutes at room temperature. Then wells were washed with distilled water 3 times to get rid of excess dye. Afterwards, 100µl of ethanol and 1molar HCL 1:1 mixture is added per well and absorbance was measured at 650nm on Original multiscan lab systems plate reader. The values were plotted using Prism 5.0 software.

2.11 Presto Blue Proliferation assay

A new technique of measuring proliferation using cell viability reagent Presto (Invitrogen A13262) blue dye which isn't toxic to cells can monitor live cells over time and doesn't require different sets of wells for each day. Instead cells can be incubated with the dye and florescence can be measured using a plate reader which is much more precise as the same cells are then allowed to proliferate over another 24 hours and the chances of error is reduced plus this dye is more sensitive. Presto blue is a Resazurin based solution that works by changing from a blue non-florescent to a red highly florescent dye in the reducing environment of a metabolically active cell which can easily be measured using a plate reader.

Validation and optimisation of presto blue experiments was done before proceeding. The main purpose was to check if cell adherence to the bottom of

the glass plate is affected after exposure with presto blue. Secondly to note whether the time of exposure to presto blue has any effect on cell proliferation itself. As seen in, Figure 2-5(A), multiple exposures to presto blue and multiple washings affects the cell count slightly. Prolonged exposure of cells to presto blue also causes an inhibitory effect on proliferation Figure 2-5 (B). Keeping these findings in mind a method was devised to minimal washings and exposure of cells with dye for 1 hour only to have minimized effects of presto blue dye itself.

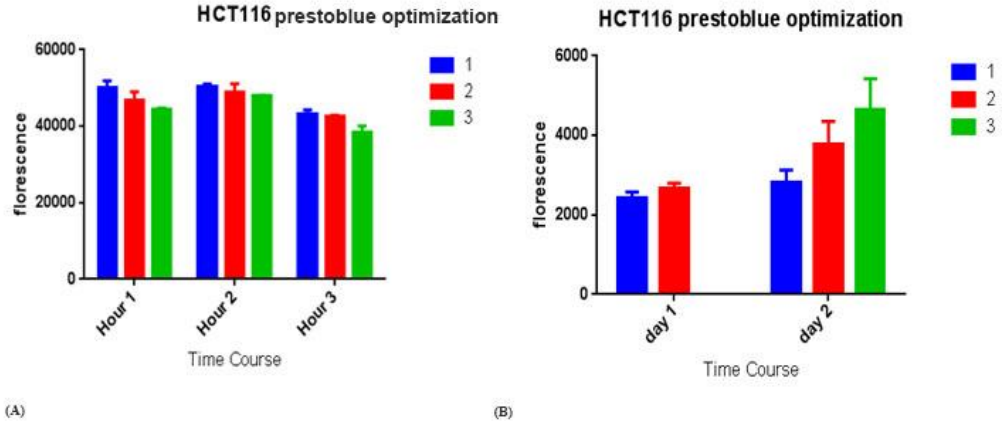
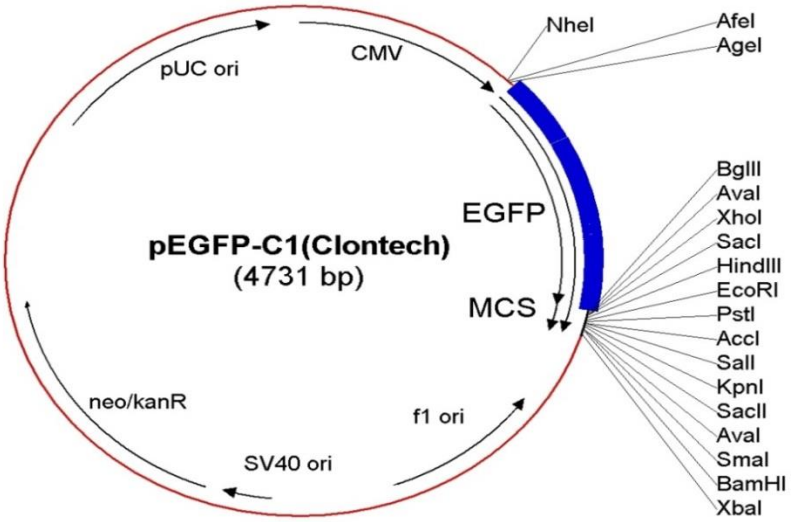


Figure 2-5: Presto blue optimization in HCT116. (A) Cells were grown to 100% confluence in a 24 well plate. 300µl of 10% prestoblue DMEM was added per well. Reading was taken after 1hour. Cells washed with PBS and incubated for another 1 hour and the process repeated again. Red, Blue and Green graph bars represent triplicates of wells. It us evident that after 3 hours of repeated washing and presto blue exposure has slightly affected the readings. (B) 20,000 HCT116 cells were seeded in 24 well plate, incubated for 8 hours. Condition 1 was exposed to presto blue overnight (blue). The red bar is the condition in which cells were exposed to prestoblue for 1 hour just before reading was taken (red). Green is the control which was not exposed to prestoblue on day 1 at all but exposed to presto blue on day 2. It is apparent that exposure to presto blue for prolonged period has a negative effect on proliferation reading.20,000 cells were seeded similarly to methylene blue assay in 24 well plates in triplicate for each condition. They were left to settle for 12 hrs. After 12 hrs DMEM media was aspirated carefully and a mixture of 1:100 of Prestoblue in DMEM was added to each well 400µl per well. This was left to incubate at 37^{o}C, 5%

CO_2incubator for 1 hour. After this time presto blue mixture was aspirated and added as 100µl/well of a 96 well plate Nunc (Invitrogen). Fluorescence was read on Omega Fluostar at excitation 550nmand emission 620 nm band widths. Gain was set up at 1000.All measurements done in triplicate. One well with no cells was treated the same as the others and kept as a baseline correction for the experiment.

2.12 Site directed mutagenesis

Site-directed mutagenesis is a method to create specific, targeted changes in double stranded plasmid DNA. These changes can either be insertions, deletions or substitutions of bases in the DNA sequence. In our study it has been used to introduce a point mutation by substituting 1 or 2 bases in the sequence leading to change in one amino acid in the resulting protein.The Phusion site directed mutagenesis kit (Finnzymes, Thermo Fisher Scientific) was used to introduce mutations into the Cten to test the functions of the domains. Following the manufacturers protocol, primers were designed having mutation in the middle. Tm was calculated by the Tm calculator (www.finnzymes.com). As the primer was more than 20 bases in length, annealing temperature greater than 3^oC higher than the Tm of the lower Tm primer given by the calculator was used. A template concentration of 10pg to 500ng was used. The reaction volume used was 50µl, with final concentration of 200µM each for dNTPs, 0.5µM for each primer and 0.02U/µl for the Phusion Hot start II DNA polymerase given with the kit. The reaction was of 25 -35 cycles with extension at 30s/kb of the product expected details in Table 2-4. GFP-Cten plasmid was used as template. The plasmid map in which Cten was inserted is given in Figure 2-6.



.

Figure 2-6: Map of pEGFP C1 plasmid in which Cten was inserted at the multiple cloning site (MCS) provided by Su Hao Lo group.

2.12.1 Primer design for point mutation

Primer design for point mutation in the SH2 domain of human Cten was designed using the primer X website (http://www.bioinformatics.org/primerx/). Primers sequences for all the constructs can be found in Table 2-5. At first primers were designed keeping the mutation in the middle of both forward and reverse primers and these primers were over lapping each other. But when a PCR reaction was run there was no product generated. Then primers were designed to keep the point mutation in the centre of the forward primer, while the reverse primer was designed just abutting the forward sequence but not overlapping it and going in the opposite direction as to cover the whole sequence of the template in the final PCR product as shown in Figure 2-7. There were some problems with 2 of the mutants regarding generation of the product after PCR containing the mutation. For those primers, changes were made in the primer design by increasing the length of the primers and moving

the mutation a bit further along the middle of the forward primer to achieve desired results. The final primers sequences used for generating the mutants constructs is given in Table 2-5.

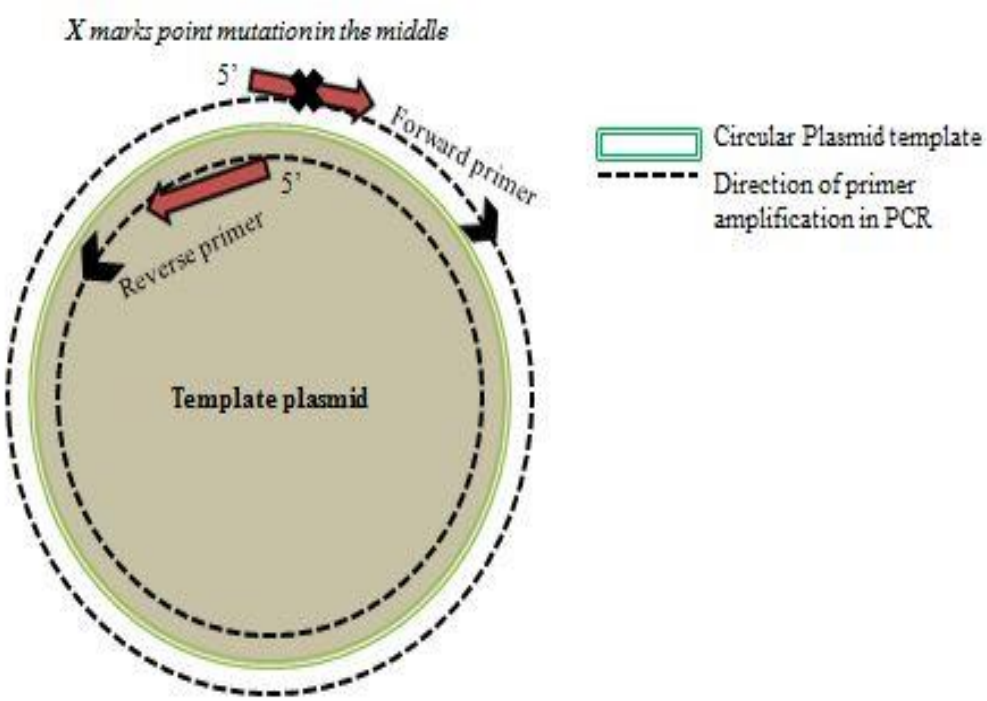


Figure Error! No text of specified style in document.**-1 A schematic diagram showing the design of primers pairs for site directed mutagenesis.**

2.1.1 Gel extraction, ligation, transformation and miniprep of PCR product obtained

After obtaining the PCR product, it was run on an electrophoresis gel to look for the right band size comparing with the template. Next the product was gel extracted (as explained in 2.2.1), ligated using T4 ligase enzyme over night at 4^{o}C. The ligated product was then transformed into NEB 5α competent E-coli high efficiency cells (C29871) (as explained in 2.2.7) keeping untransformed E-coli cells and unligated product as negative control. A few colonies were picked out of the total obtained Miniprep was performed (as explained 2.2.8) and sent for sequencing.

Table 2-4Cycle steps used in PCR run for site directed mutagenesisfor introducing point mutations.

Cycle step	Temp	Time	Number of cycles
Initial denaturation	98	30s	1
Denaturation	98	10s	25
Annealing	71	30s	
Extension	72	30s/kb 3:40 s	
Final extension	72	10 min	1
	4	hold	

Table 2-5 Sequence of primers designed and used for site directed muatagenesis. The red letter represents the point mutation added.

Primer name	Sequence
GFP-Cten^{R474A}	F:GGGGCTTTTGTCATAGCGGACAGCTCTTCATAC R: TGG CTC CTC CTT CCT CAG CAG CTC
GFP-Cten^{Y449F}	F: ACA TCT AAA TTC TGG TTT AAG CCA AAC RGTC CAT CAC GAA CTT CAT GGT
GFP-Cten^{Y479F}	F:AGC TCT TCA TTC CGA GGC TCC RGTC CCT TAT GAC AAA AGC CCC
GFP-Cten^{Y530F}	F:CAT CTC AAA GGA GCA GAT GAG GAG CCC TTC TTT GG R:CAC TCC TTT GGC AGA CGA CTC GAT GAG GAA

2.12.2 Sequencing

All the minipreps for the colonies grown for the constructs were sent for sequences to the DNA Sequencing Facilty based in the School of Life Sciences for accurate and comprehensive DNA sequence analysis service. Latest dye terminator chemistry (BigDye version 3.1) was used and samples were run on 3130xl ABI PRISM Genetic Analyzer (Life Technologies).

2.12.3 Comparison of sequences

Comparison of DNA sequence obtained was done by Blast tool on NCBI website (http://blast.ncbi.nlm.nih.gov/Blast).

Table 2-6 gives a detailed sequence of primers sent for sequencing. Table 2-7 gives the length of clean sequence obtained from each set of primers covering the length of the Cten sequence [TNS4 (NM_032865) Human cDNA ORF Clone] present inside the constructed vectors using the original plasmid received from Su Hao Lo. See appendix 2 for detail.

Table 2-6Primer sequences used for sequencing the whole length of the constructs made.

Primer names	Sequence 5'-3'
Cten new Forward	TGG AAA GTC CAG CCA ACT CT
Cten new Reverse	CAC AAT GGC TAT GTC CAC CA-
GFPN1 Forward	AAC TCC GCC CCA TTG ACG CAA ATG
GFP N1 Reverse	CGT CGC CGT CCA GCT CGA CCA G
GFP C1 Forward	CAT GGT CCT GCT GGA GTT
GFP C1 Reverse	CAA ATG TGG TAT GGC TGA TT

Table 2-7Gives details of the clean uninterrupted sequence obtained from the sequencing reactions that matches completely to the template plasmid.

Construct name	Primer used	Clean sequence matching TNS4 (NM_032865)
NLS-Cten-GFP	Cten new Forward	1140-2019
	Cten new Reverse	434-1240
	GFP N1 Forward	164-830
	GFP N1 Reverse	1464-2309
GFP-Cten	Cten new Forward	1178-2017
	Cten new Reverse	534-1198
	GFP C1 Forward	165-1064
	GFP C1 Reverse	1420-2313
GFP-Cten ^{R474A}	Cten new Forward	1123-2022
	Cten new Reverse	179-1230
	GFP C1 Forward	165-1086
	GFP C1 Reverse	1370-2314
GFP-Cten ^{Y449F}	Cten new Forward	1178-2018
	Cten new Reverse	220-1200
	GFP C1 Forward	165-1082
	GFP C1 Reverse	1360-2313
GFP-Cten ^{Y479F}	Cten new Forward	1125-2024
	Cten new Reverse	500-1224
	GFP C1 Forward	165-944
	GFP C1 Reverse	1420-2313
GFP-Cten ^{Y530F}	Cten new Forward	1132-2027
	Cten new Reverse	360-1252
	GFP C1 Forward	165-900
	GFP C1 Reverse	224-2313

2.13 Messenger RNA preparation and Reverse transcriptase QPCR

Total cellular RNA was extracted using kit by total RNA miniprep kit by Sigma (MRN70). Cells were lysed to extract RNA by adding 250µl of Lysis buffer with βmercaptoethanol per 1 well in a 6 well plate. All steps were performed at room temperature according to the prortocol with provided with the kit. RNA was quantified using nanodrop and stored at -80^{o}C.cDNA was generated from 1µg of RNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega 200U/µl). 1µg of RNA and 0.5 µg of random hexmaers (Thermo Scientific 50142 200ng/ml) was mixed together with RNAse free water to make 15µl volume incubated at 70^{o}C for 5 minutes and then on ice for 5 minutes After this a mixture of Reverse transcriptase (RT) enzyme, M-MLV RT buffer 5x (Promega) and dNTP 10mM (Thermo

Scientific) and water added to each sample and incubated at 37^{o}C for 1 hour followed by 95^{o}C for 10 mins. Samples without RT enzyme added served as negative control. Samples were then diluted 1:10 and stored at -80^{o}C.

Real time PCR was conducted by using Gotaqmastermix (Promega A6002). The reaction volume used was 30µl including Forward and reverse primers (6.25 µM stock) and cDNA, Gotaqmastermix and dH_2O. The quantities for 1 reaction are as follows:

Master mix	15µl
Forward primer (250 nM)	1.2µl
Reverse primer (250 nM)	1.2µl
dH_2O	9.6µl
cDNA	3µl

This was run on 7500 fast real time PCR system machine serial no. 275012368 (Applied Biosystems life technologies). The cycle set up was as follows:

Holding stage 95°C for 2 minutes, Cycling stage 40x (95°C for 3 seconds, annealing temperature for 30 seconds), Melt curve stage (95°C for 15 seconds, 60°C for 1 min, 95°C for 15 seconds, 60°C for 15 seconds).

QPCR primers were designed using of Universal Probe library Roche (https://lifescience.roche.com/webapp). Sequences are given in Table 2-8.

Delta deltaCT method was employed keeping HPRT as a house keeping gene and loading control using ABI 7500 software v 2.0.1 quantify results. Primer efficiency was calculated using serial dilution of primers in multiples of ten.

Efficiency of sample primers compared to HPRT graphs are shown in .All reactions were carried out in triplicates (Figure 2-8).

Table 2-8 Sequences for primers used for RT QPCR reaction

Reference sequence number	Primer sequence	Amplicon size (bp)	Annealing Temperature (^{o}C)
HPRT NM-_00194	L AAATTCTTTGCTGACCTGCTG R TCCCCTGTTGACTGGTCATT	122	60
Cten NM_032865.5	L GCGTCTGCTCAGA TCGAC R GATGAGGAAGTGTCGGATGG	110	56
E-Cadherin NM_004360.	L- TGGAGGAATTCTTGCTTTGC R- CGCTCTCCTCCGAAGAAAC	64	60
Src NM_005417.4	L-GGCGGCTTCTACATCACCT R-AGGGATCTCCCAGGCATC	92	56
Snail NM_005985.3	L-GCTGCAGGACTCTAATCCAGA r-ATCTCCGGAGGTGGGATG	84	60

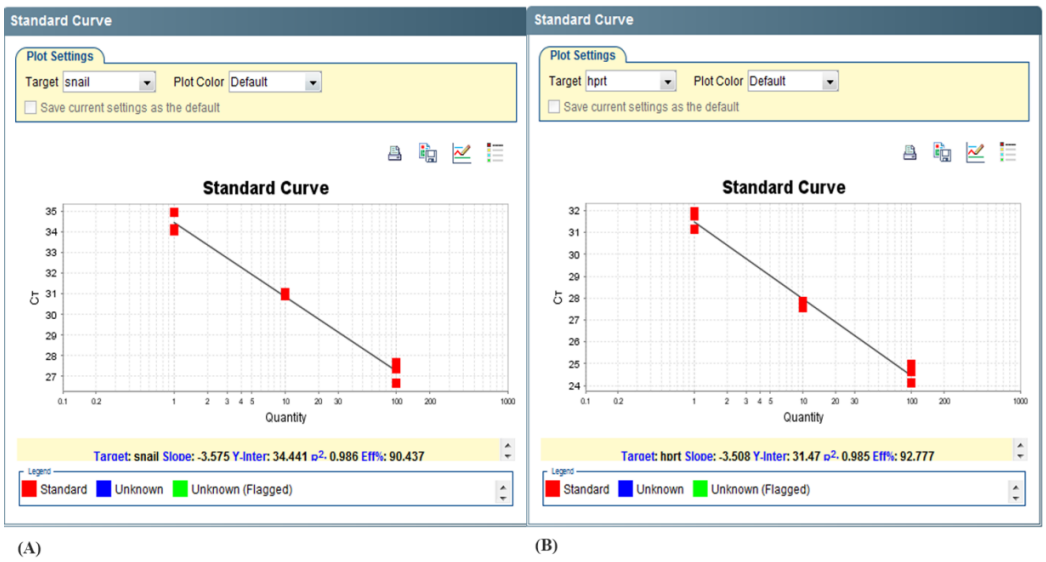


Figure 2-8: Standard curve for snail primers (A) and housekeeping gene HPRT (B).

2.14 Calpain activity assay

Calpain, a cytosolic calcium-dependent cysteine protease whose activity is involved in a number of biological processes including integrin mediated cell migration and apoptosis(63).This experiment was carried out to discover possible link between Cten and calpain The Calpain Activity Assay determines the catalytic activity of calpain by detection of cleavage of its substrate which

emits fluorescence upon cleavage. This florescence is detected by a fluorescent plate reader Comparison of the fluorescence of sample treated with calpain with a normal control allows determination of the fold increase in calpain activity.

Cells were plated as $1x10^5$/ml of DMEM with 10% FBS in T25cm^2flasks (Corning Costar UK). The cellswere left to settle and adhere overnight. Next day media was changed to serum free DMEM. Each condition was done in triplicate. To one flask 100µM Calpeptin (Calbiochem 03-34-0051) diluted in DMSO (Sigma D2650) was added. To another equal volume of DMSO diluent was added. All this was done in dark conditions. The flasks were incubated at 37^oC 5% CO_2. After that cells were tyrpsinised and transferred to eppendrof and spinned for 5 minutes at 2500rpm. Supernatant was aspirated. Cells were resuspended in 500µl of serum free DMEM. This volume was divided into 2 eppendroffs having 250µl each. To one tube 2µl of CMAC, tboc-L-leucyl-l-methionine-amide (10nM stock) was added and to another 2µl DMSO was added. 100µl of each in duplicate was added in a 96 well plate with optical bottom. Fluorescence was measured at 37^oC in BMG Floustar omega (excitation 350/emission 430 and gain 2000) for 1 hour at 10 minutes intervals. It was seen that fluorescence increases uptil 1 hour and then reaches a plateau. The difference in fluorescence was measured and plotted after 60 minutes.

2.15 Statistical Analysis

For the results of functional studies, data were displayed as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis using the unpaired two tailed Student's T-test was performed by the

GraphPad software. Differences were considered significant if P-values were < 0.05.

3 Effect of location on Cten Function

3.1 Introduction

In addition to Cten's location within the cytoplasm, an increase in its nuclear staining has been seen in advanced stage tumours and metastatic deposits(7). This led us to hypothesize that localisation of Cten to the nucleus may enhance Cten activities such as cell motility and stemness.

As Cten lacks an endogenous NLS sequence within it, we aimed to construct a plasmid tagging Cten with a nuclear localisation sequence (NLS), which would target Cten to the nucleus. This would allow us to study its effects in human colorectal cell lines compared to Cten located within the cytoplasm. In this study we report the role of Cten within the nucleus and its effects on cell motility and tumourigenicity of colon cancer cell lines.

3.2 Aims

1 Tag GFP-Cten with NLS.

2 Ensure that NLS-Cten-GFP construct goes into the nucleus.

3 To study the effect of NLS-Cten-GFP on functional aspects of Cten.

3.3 Results

3.3.1 Construction of Cten tagged with NLS

A PCR was run using proof reading Taq polymerase and the primers (given in material and methods) with GFP-Cten as template. The product obtained was run on an agarose gel. The product of the PCR came out to be around 2200 base pairs (bp), which is the right size for Cten with the NLS tag as shown in Figure 3-1 (A).This product was extracted from the gel and used for cloning in a topovector. The TA kit relies on A-overhangs in the PCR product pairing with T-overhangs in the plasmid. Since a proof reading Taq was used, the PCR products were blunt ended. A-overhangs were added as described in material

and methods. For cloning in the Topo vector, the TA cloning kit was used. Map of the Topo vector used is given in Figure 3-2. After cloning of NLS-Cten in Topo vector, the construct was called Topo-NLS-Cten and was transformed in competent E-coli cells as explained in Materials and methods (M&M), colonies were grown on LB agar. Five light blue colonies were picked. A miniprep was done for all five colonies. The 5 Topo-NLS-Cten constructs were digested with BamH1 enzyme and run onto a gel shown in Figure 3-1(B) and compared withTopovector alone cut with BamH1. The resultant gel showed that all 5 colonies had NLS-Cten in them. The orientation of Topo-NLS-Cten 1 and 4 was reversed (explained in Figure 3-3).They were digested again with both HindIII and BamH1 as seen in Figure 3-4(A). As you can see in Figure 3-4(A) that pEGFPN1 was not completely digested with BamH1, this reaction was then optimised to ensure complete digestion with BamH1 and HindIII Figure 3-4(B).

One of the Topo-NLS-Cten constructs was then double digested using HindIII and BamH1 to excise the product out of Topo vector. The product was run on a gel and extracted from it under blue light (explained in detail in Materials and Methods). The extracted DNA was then ligated with pEGFPN1, which was alsodigested with HindIII and BamH1 in 1:1 and 1:3 molar ratio. and transformed into E-coli. The result was: Bacterial cells alone=no growth, pEGFPN1 self-ligation= 57 colonies (probably because of incomplete digestion), NLS-Cten-GFP 1:1=no growth, NLS-Cten-GFP 1:3= 200 colonies.

As there were four times more colonies in the NLS-Cten-GFP 1:3 ligation than self-ligated pEGFP although the amount of pEGFPN1 in the ligation was just

twice more , so there was a chance that NLS-Cten was present in one of them. Ten random colonies were selected and grown as minipreps and run on a gel.

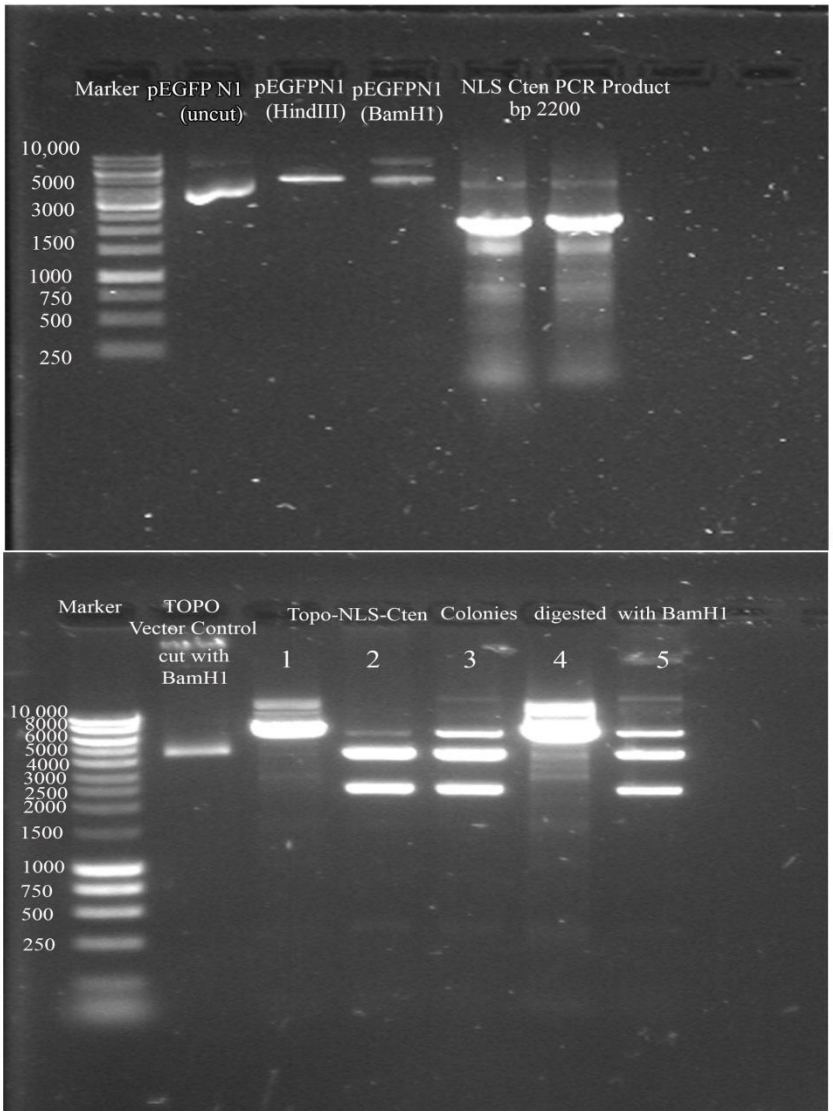


Figure 3-1:Topo-NLS-Cten construction. (A) The NLS tagged Cten PCR product falls in the range of 2200 base pairs where as pEGFPN1 is 5000kb. Circular plasmid always runs faster so pEGFPN1 was digested with HindIII and BamH1.Note that the pEGFPN1 digested with BamH1 enzyme is not completely digested having a faint band on top. (B) The first lane is Topovector control digested with BamH1 (5000kb). The lanes labelled 1-5 are Topo-NLS-Cten digested with BamH1. All clones have NLS-Cten in them as they are falling in 7000bp range. However due to different position of the BamH1 digestion sites NLS-Cten has been digested out in only Topo-NLS-Cten 2, 3 and 5.

As seen in Figure 3 4(C), NLS-Cten-GFP clones 1 and 5 fall in the right size of 7000 bpcompared to pEGFPN1 alone which is 5000bp. NLS-Cten-GFP 1 and 5 were further digested with BamH1 and Hind III to check that the insert of NLS-Cten falls in the right size of 2200bp (Figure 3-5 A). Then these 2 clones

were then sent for sequencing to confirm the addition of NLS sequence as shown in Figure 3-5(B).

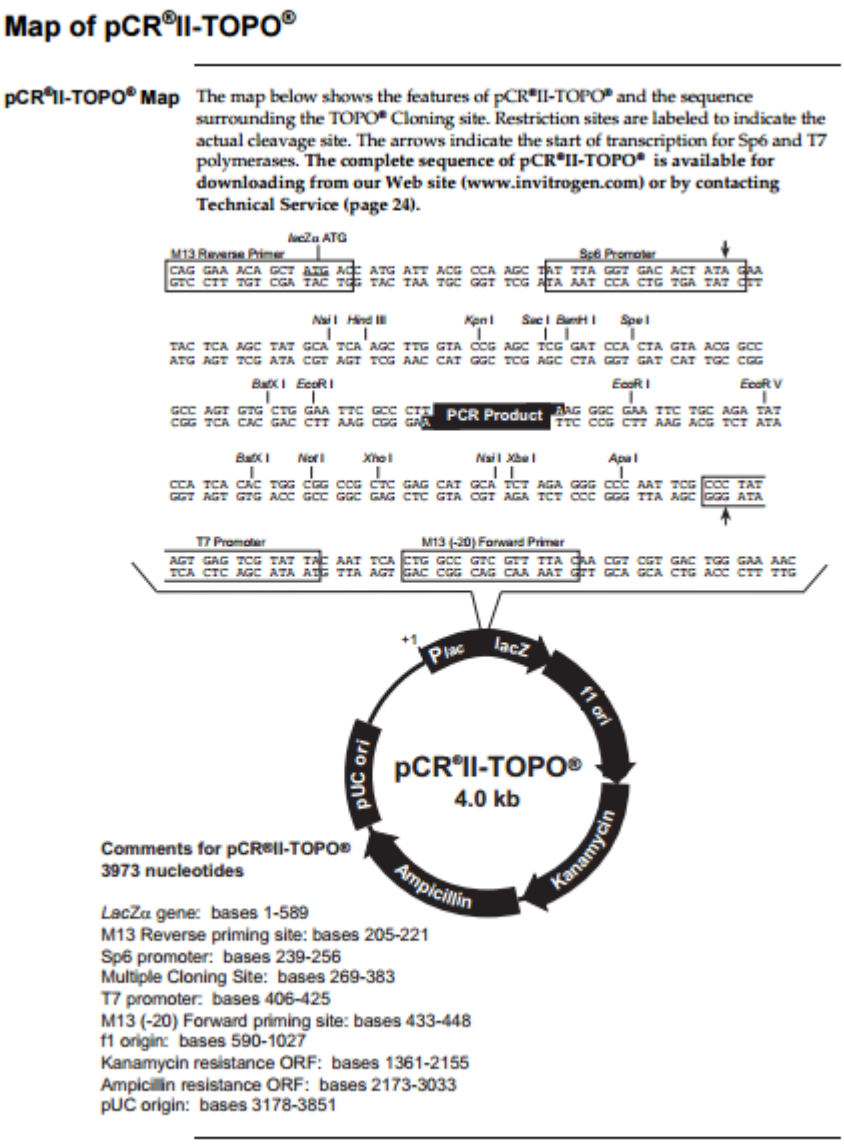


Figure 3-2 Map of Topo Vector used. There is a site in the middle of the restriction enzymes site at which the PCR product binds. The direction of the PCR product attached and the enzymes site determine the size of the cleavage product.

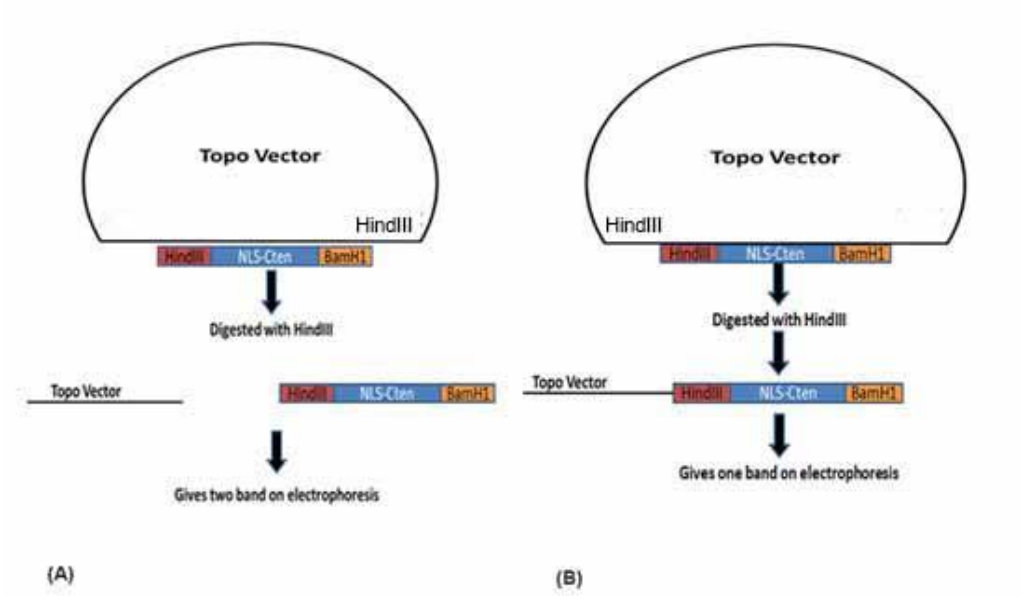
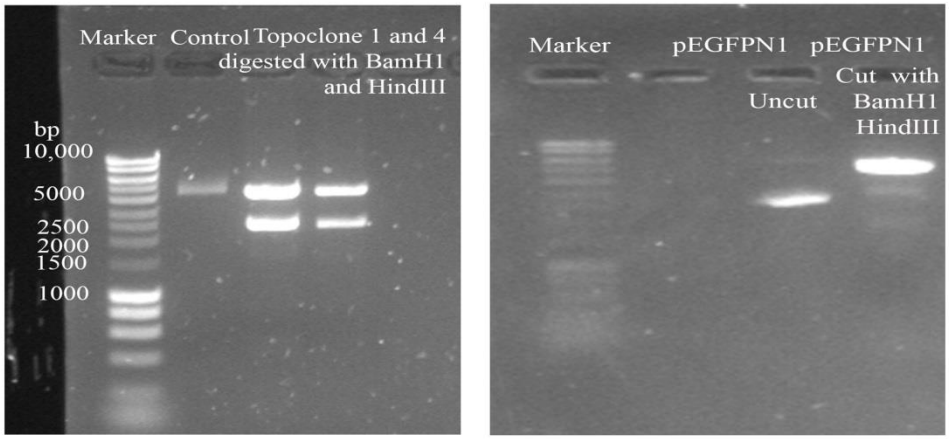
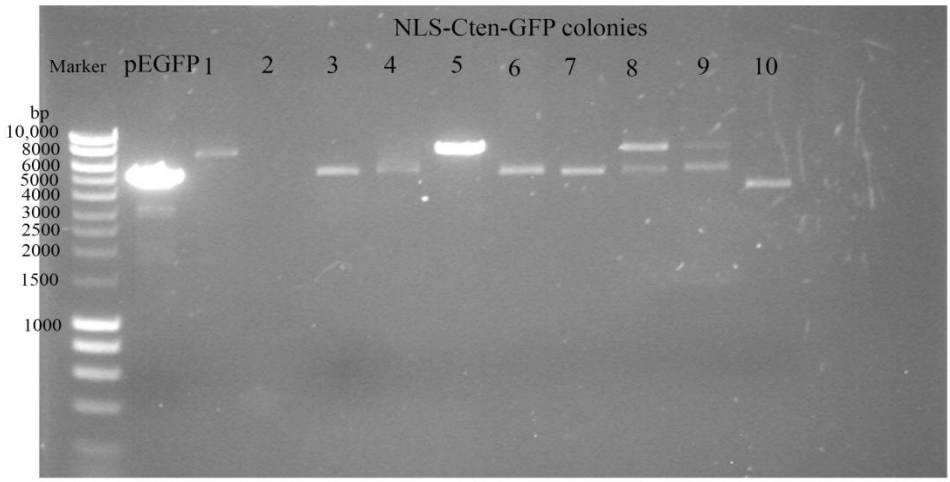


Figure 3-3:Diagrammatic representation of the NLS-Cten product as it binds with the Topovector. This binding is TA dependent and not restriction enzyme dependent. If the HindIII site of NLS-Cten and Topo vector are at the same side. The digestion with HindIII will give a linear product. If the HindIII site of the NLS-Cten product and Topovector are on the opposite sides, then digestion with HindIII will digest the PCR product (NLS-Cten) out and give 2 bands, one for NLS-Cten and other for Topovector.



(A)

(B)



(C)

Figure 3-4:Double digest of Topo-NLS-Cten.(A) Shows the gel run of double digest of Topo-NLS-Cten 1 and 4, having the NLS-Cten inside. (B) Shows complete linearization of pEGFPN1 after optimization of double digest with BamH1 and HindII having no secondary bands. (C) Gel run of the 10 colonies chosen after transformation of the NLS-Cten-GFP. Colony 1 and 5 show bands at the right size of 7000bp which is 2000 bp more that pEGFPN1 plasmid run as a control.

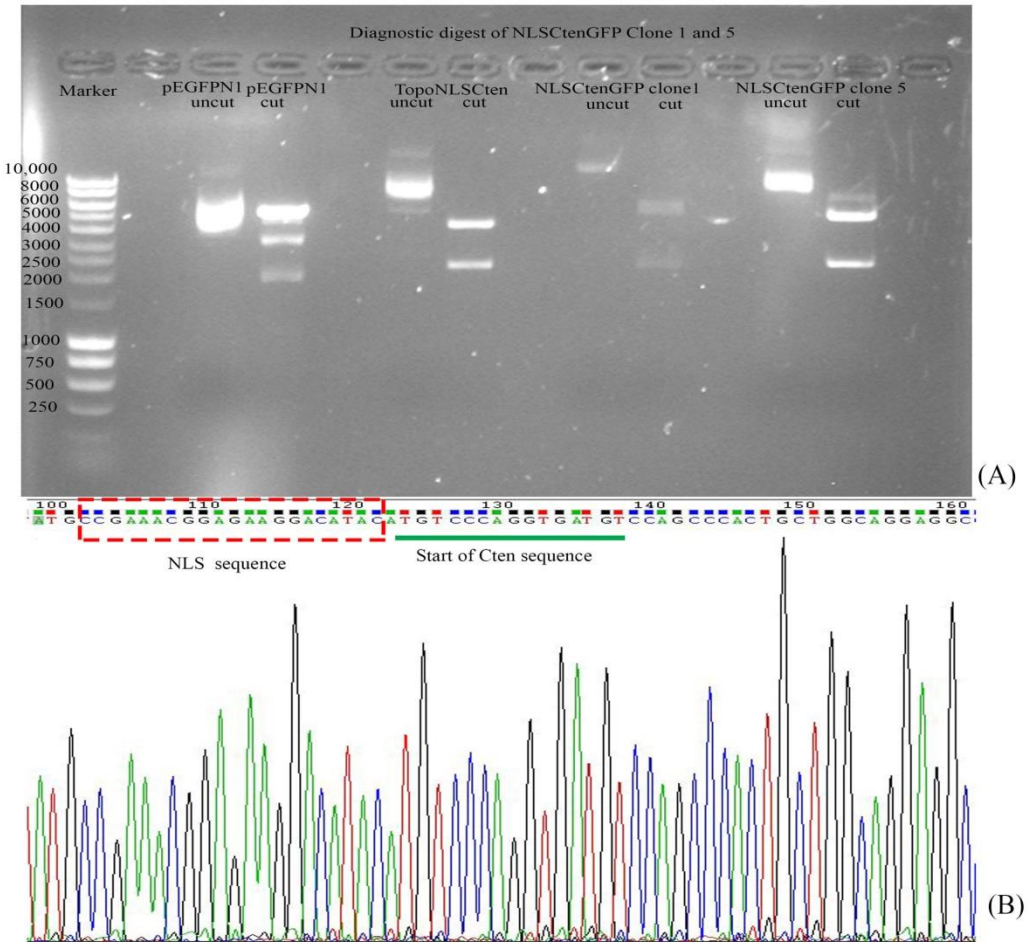


Figure 3-5:Diagnostic digest of NLS-Cten-GFP.(A) NLS-Cten-GFP Clone 1 and 5, pEGFPN1 and Topo-NLS-Cten vector were digested with HindIII and BamH1 to separate the insert from the vector. The insert in both clone 1 and 5 is 2200 bp same length as the NLS-CTEN in Topo vector. (B) Shows the sequence from one of the clones displaying the presence of complete NLS sequence at the right just before the beginning of Cten.

3.3.2 NLS-Cten-GFP goes to the nucleus

After successfully constructing NLS-Cten and forming anexpression construct of NLS-Cten-GFP, the next step was to confirm that tagging NLS to Cten actually takes it to the nucleus. As our construct was inserted in pEGFP, it was easy to follow its location by studying the GFP signal under a fluorescent microscope. NLS-Cten-GFP, GFP-Cten and pEGFP (or referred to as empty vectors in some cases) were all transfected in colorectal cell lines lacking endogenous Cten. It was seen that pEGFP stays in the cytoplasm only, while

GFP-Cten goes to nucleus as well as cytoplasm but mostly in the cytoplasm. However, the NLS-Cten-GFP construct goes into the nucleus only Figure 3-6.

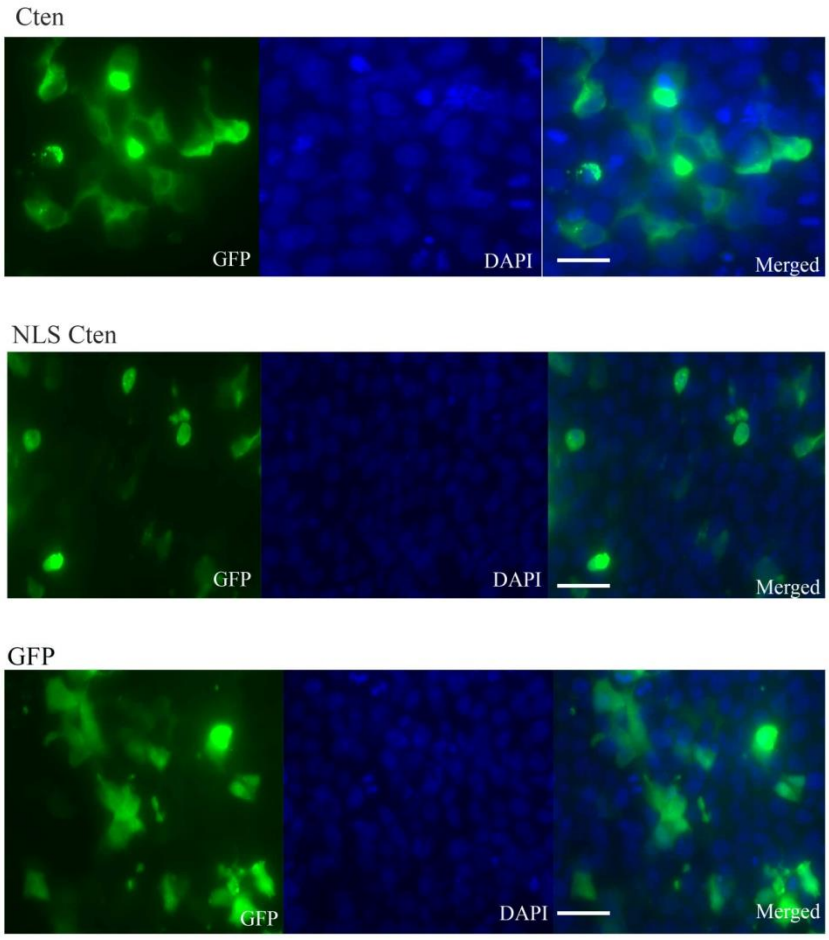


Figure 3-6: Pictures from the fluorescent microscope showing cells transfected with GFP-Cten, NLS-Cten-GFP and pEGFP.DAPI dye was used to stain the nuclei. Pictures were taken with separate filters for GFP and DAPI at the same magnification and then merged to show the location of the GFP stain compared with location of the nuclei in the cells. Scale bars represent 20µm.

3.3.3 NLS-Cten-GFP leads to increase in cell motility but does not affect cell proliferation

To study the effect of NLS-Cten-GFP, on cell motility and proliferation, we transfected the cells with NLS-Cten-GFP along with GFP-Cten and pEGFP or empty vector. Transfection efficiency was optimised as explained in materials

and methods to get the best possible efficiency for transfection. Migration was tested by 2 methods, transwell migration and wound healing assay. It was seen that in wound healing assay NLS-Cten-GFP increased cell motility and migration significantly as compared to GFP-Cten (p=0.01 and p=0.003) and pEGFP(p=0.009 and p=0.008) in HCT116 and RKO respectively. Similar results were seen on transwell migration assay and a significantly increased motility was observed in NLS-Cten-GFP as compared to GFP-Cten (HCT116 p=0.01) and (RKO p=0.02). This is evident in Figure 3-7 and Figure 3-8 B for HCT116 and in Figure 3-9 for RKO.

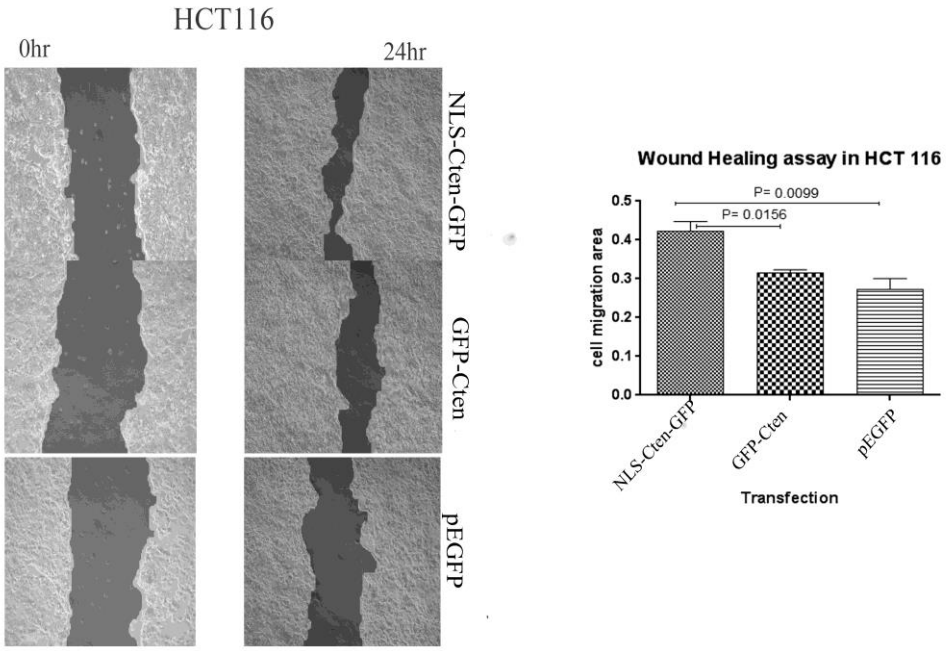


Figure 3-7 Pictures of wound healing assay in HCT116 at 0hour and after 24 hours as analysed by T-scratch software. The distance travelled by the edges of the wound was calculated as area of cell migration and put in Graph pad prism to give graphical depiction of the assay (shown on the right). Unpaired t test was used to study the difference between two conditions at a time and there was a significant difference seen between NLS-Cten-GFP, GFP-Cten and pEGFP. The error bars represent the standard deviation from the mean. Experiments were repeated three times with each condition run in triplicate n=3.

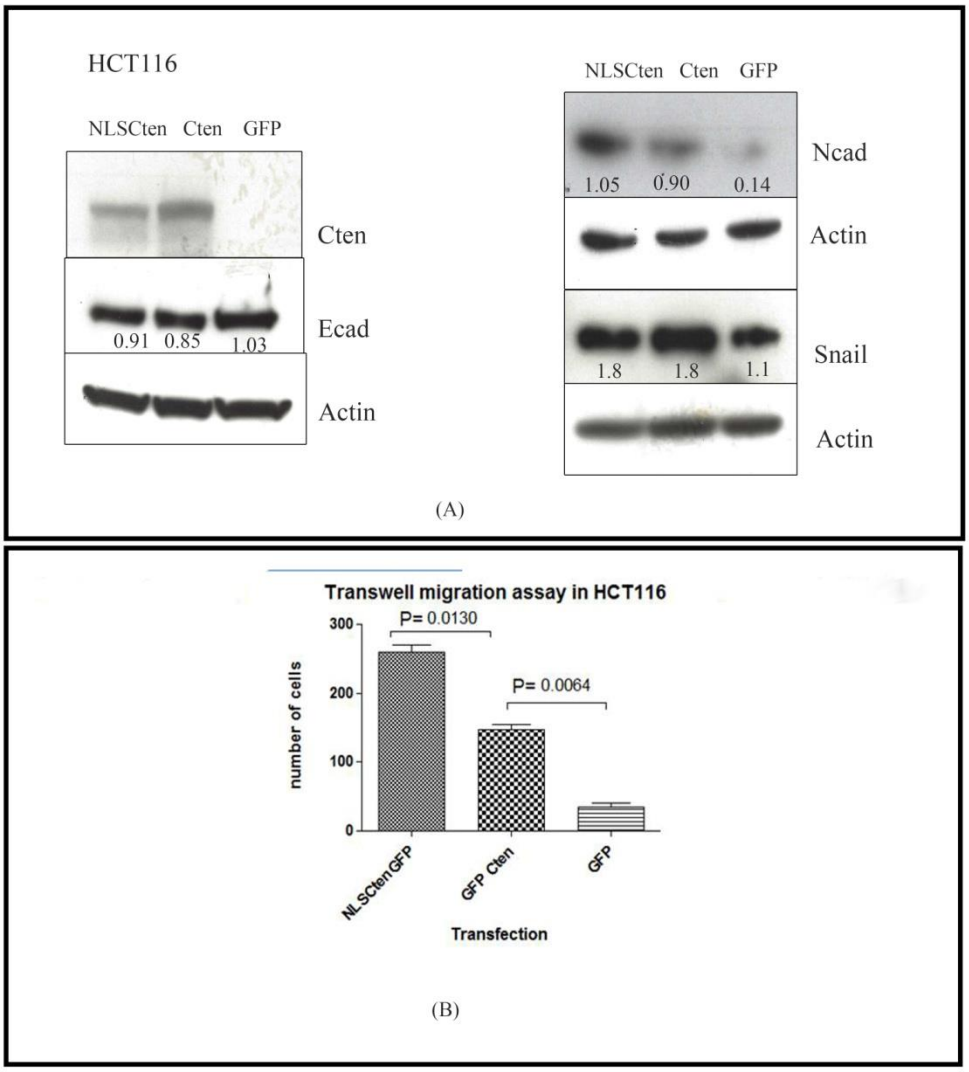


Figure 3-8:Western Blot and transwell migration assay with NLS-Cten-GFP in HCT116. (A) Shows images of western blot in HCT116 for expression of Cten and markers of EMT. The levels of E-cadherin go down for NLS-Cten-GFP as compared to pEGFP but not less than GFP-Cten (right). N-cadherin levels go up as compared to GFP-Cten and pEGFP but Snail levels are not affected by NLS-Cten-GFP when compared to GFP-Cten (left). β-actin is the loading control and all the bands are normalised to it. The figures under the western bands give their density normalised to the loading control. (B) Graphical representation of transwell migration assay in HCT116 showing a significant difference between NLS-Cten-GFP and GFP-Cten (unpaired t test). The error bars represent the standard deviation from the mean. Experiments were repeated three times with each condition run in triplicate n=3.

Western blot was performed to confirm the presence of Cten protein in all conditions before performing any functional assay. It was seen that the protein expression of NLS-Cten-GFP was always less than GFP-Cten in all experiments. One of the reasons could be that the protein was extracted as a

whole from the cells and not fractionated into different compartments like nuclear and cytoplasmic and since NLS-Cten-GFP is taken into the nucleus it is possible that it isn't extracted well enough to be seen in equal quantities on western blot.

Markers of EMT were also used to explore the reasons of increase in cell motility and migration in NLS-Cten-GFP. The markers include E-cadherin, N-cadherin and Snail. These proteins however are only expressed in HCT116 and not in RKO so western blot was performed only in HCT116 cells. As seen in Figure 3-8(A), N-cadherin goes up with NLS-Cten-GFP as compared to GFP-Cten and pEGFP as would be expected in a shift towards mesenchymal properties. However with Snail levels the increase was the same as with GFP-Cten but still more thatpEGFP. With E-cadherin, as was expected levels went down with NLS-Cten-GFP and GFP-Cten as compared to pEGFP. However, the levels of E-cadherin did not go down below the level seen in GFP-Cten transfected cells Figure 3-8(A).

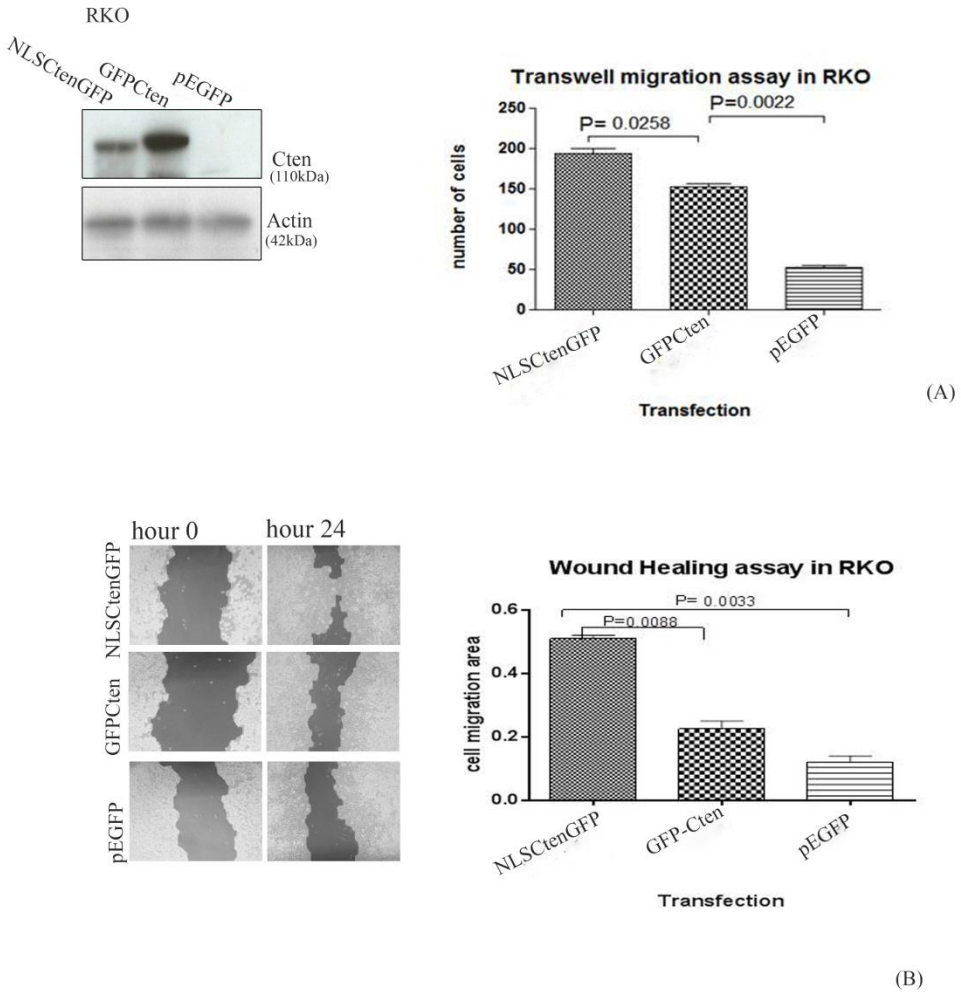


Figure 3-9:Wound healing assay and transwell migration assay with NLS-Cten-GFP in RKO.(A) Shows western blot pictures of protein extracted from transfection with NLS-Cten-GFP, GFP-Cten and pEGFP blotted with Cten antibody and graphical representation of number of cells migrating after 24 hours through a transwell in RKO cell lines. Unpaired t test was performed to compare the difference between each condition and found to be significant. It is important to note her that the transfection efficiency of the NLS-Cten-GFP expression vector is less than GFP-Cten but it still causes an increase in cell motility function (B) Pictures of wound healing assay in RKO at 0hour and after 24 hours as analysed by T-scratch software. The distance travelled by the edges of the wound was calculated as area of cell migration and put in Graph pad prism to give graphical depiction of the assay (shown on the right). Unpaired t test was used to study the difference between two conditions at a time and there was a significant difference seen between NLS-Cten-GFP, GFP-Cten and pEGFP. The error bars represent the standard deviation from the mean. Experiments were repeated three times with each condition run in triplicate n=3.

To check the effect of NLS-Cten-GFP on proliferation methylene blue proliferation assay was performed in both cells comparing with GFP-Cten and

empty vector and it showed no significant difference in between either of the three plasmids transfected as shown in Figure 3-10.

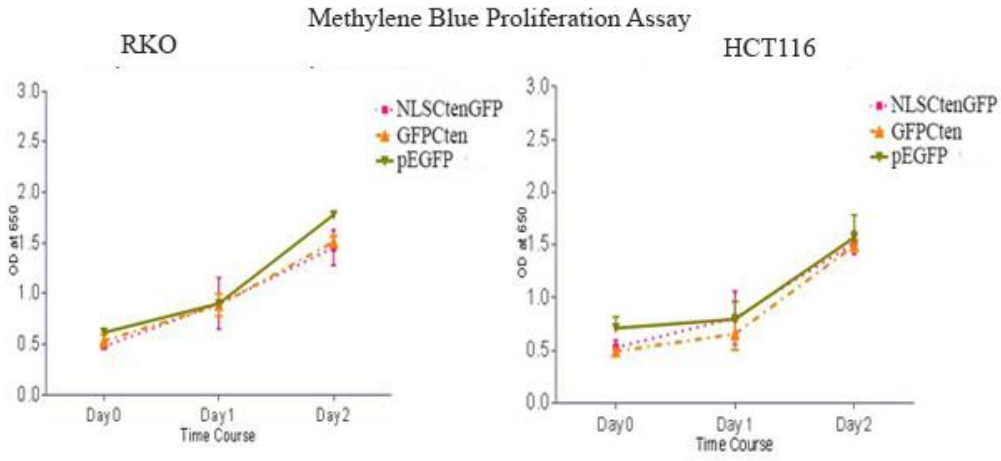


Figure 3-10 Graphical representation of methylene blue proliferation assay done in HCT116 and RKOafter transfection with NLS-Cten-GFP, GFPCten and pEGFP empty vector. 2way Annova was performed and showed no significant difference between the three conditions. The error bars represent the standard deviation from the mean. Experiments were repeated three times with each condition run in triplicate n=3.

3.4 Discussion

Colorectal cancer is one of the tumours in which Cten shows increased expression in advancing tumour stage and this has been seen by immunohistochemical staining of tumour samples taken from primary as well as metastatic tumours. Being a focal adhesion molecule it's staining should be cytoplasmic but it's also seen to present in the nucleus. Nuclear staining was seen especially in advanced stage cases and metastatic deposits in a study by our group in 2011(7). In another study by Lo et al, finds a high population of Cten in the nucleus in colorectal cancer.They have linked Cten to β-catenin in the nucleus and predict that this interaction maybe a contributing factor of Cten's role in enhancing invasion of cells(6). In our study, we have tagged Cten with NLS (nuclear localising sequence). The mechanism of NLS is quite

simple. It usually carries its cargo protein, in this case Cten, and helps it bind to a nuclear import receptor protein called importin and thus facilitates its move into the nucleus (64).

In our study we see that Cten when tagged with NLS (NLS-Cten-GFP) significantly increases cell motility compared to wild type Cten in our GFP-Cten expression construct. There could be a number of possibilities why there is an increase in cell motility; one of them may be that addition of NLS has an effect on Cten's action. There can also be a possibility that the signal peptide of Cten does not get cleaved off after it is taken to the cell membrane. The signal peptide of Cten starts from base pair 165 to 218, which has been included in the NLS-Cten-GFP expression construct but comes after the NLS tag. Maybe the NLS tag does not let it cleave off and that enhances its action of cell migration. Another possibility is that the GFP in our construct has been moved to the C terminal end of Cten, whereas in the GFP-Cten expression construct provided by Prof.Lo it is present at the N-terminus, which is our control of normal Cten gene function. But there are several studies in which GFP tag has been used in expression vectors and its position does not alter the gene function. It might be that this increase in cell motility is actually because Cten is affecting transcription of other cell motility proteins like ILK, E-cadherin and FAK in the nucleus or by binding and increasing the activity of other proteins like β-catenin, CD24 or EGFR. Cten has been linked to β-catenin in the nucleus. It would be interesting to find other binding partners of Cten in the nucleus, which might give us insight into its role in the nucleus. We hope to do this in future.

Another thing to acknowledge her is that the forward primer for NLS-Cten-GFP construction PCR has three ATGs (which is a start codon). One is before the NLS sequence but the others come after the NLS sequence which could result in some protein being expressed with NLS tag but some without it. This could be a possible reason for lower transcription efficiency we get with NLSCtenGFP and also for slightly less than ideal results on fluorescence microscopy.

As far as we know, this is the first time Cten has been tagged with an NLS and forcefully taken into the nucleus. It gives an opportunity to study the effects of Cten localisation on colony formation, invasion and proliferation. Animal model tests will be a good next step to take this project further. It can be performed by intrasplenic injection of CRC cells stably transfected with a NLS-Cten-GFP expression construct into nude mice and compare it with GFP-Cten transfected cells. As was done by our group to test the tumourigenicity of GFP-Cten in mouse models which revealed GFP-Cten transfected mouse had increased tumour load and liver metastasis as well(7).

In future we hope to elucidate the downstream partners of Cten in the cytoplasm and in the nucleus. Tagging Cten with NLS gave us a unique opportunity to see the effects of Cten localisation upon its role in cell migration and proliferation. It is also a new way to find out about the binding partners of Cten within the nucleus giving insight into its role in cancer metastasis.

4 Importance of Arginine 474 in SH2 domain of Cten

4.1 Introduction

Tyrosine phosphorylation has always been the key regulator of important cellular processes involved in cell signalling, cell cycling. Any disruption or dysfunction in this process can lead to various diseases including cancer. Binding of these phosphotyrosine residues leads to cascade of events important for these cellular processes. This binding is generally carried out by the SH2 and PTB domains. It was first thought that both SH2 and PTB binding is strictly tyrosine phosphorylation dependent but now SH2 domain has been shown to adhere strictly to phosphorylated tyrosines while PTB binds nonphosphorylated tyrosines mostly(65). In general, an SH2 domain consists of about 100 amino acid residues and is important in signal transduction. SH2 domains were first discovered in Src and fps (66) and an SH2 domain is present in almost all cytoplasmic tyrosine kinases. The SH2 domains contain a pocket which allows physical docking of proteins containing phosphorylated tyrosine residues(67). The structure of an SH2 domain consists of two α-helices (αA and αB) sandwiching a β-sheet consisting of seven anti-parallel strands (βA to βG). The phosphotyrosine peptide binds perpendicularly to the central β-sheet and the specific residues towards the N-terminal form a positively charged pocket for the phosphotyrosine residue(68).

The SH2 domain of Tensins has been linked to a number of key molecules for cell motility. SH2 domain of TNS1 is shown to be bound with FAK, p130Cas, PDK1 and Dok2. (69). In the SH2 domain a critical Arginine is said to regulate its interaction with other elements (53,54,70). The SH2 domain of Tensin is crucial for binding to focal adhesions as it binds all the phosphotyrosine related proteins like FAK, ILK, and PI3 kinase. Mutations in the SH2 domain

abolishes migration induced by Tensin family proteins as seen in canine kidney cell lines(14). As Cten is highly homologous to other Tensins at the C-terminal, a mutation in SH2 domain is shown to prevent invasive migration in one study(53).Figure 4-1 gives the sequence homology of the SH2 domain of all the Tensins.

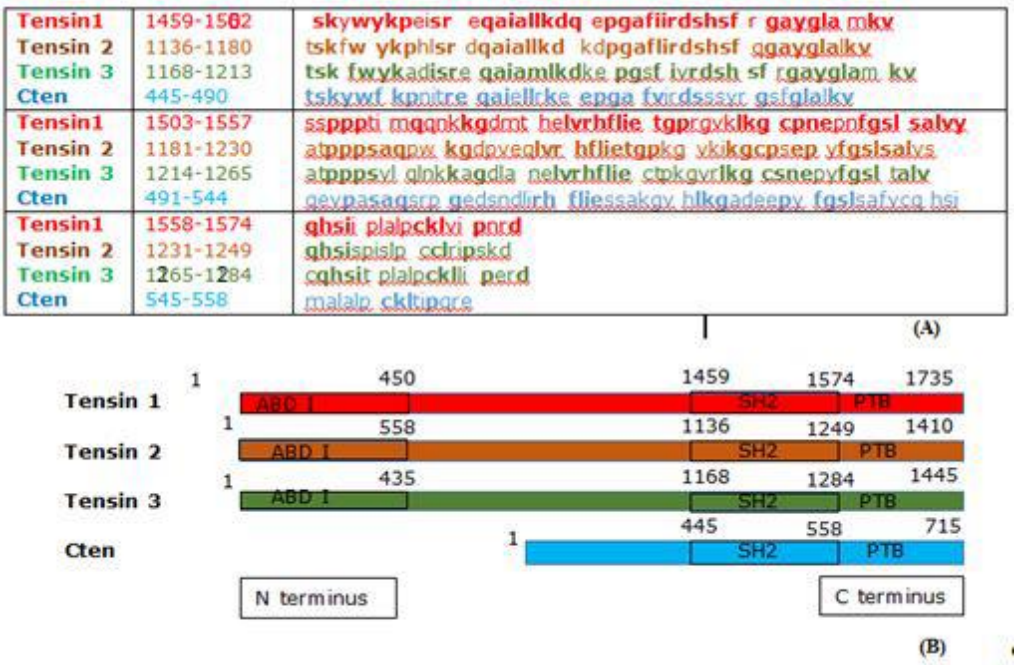


Figure 4-1:Protein sequence homology of the SH2 domain of all 4 human tensins.(A)The bold letters show the common amino acids in the SH2 domain of tensins. (B) The number of amino acid corresponding to the domain structure of all 4 tensins which show homology. The middle section of these proteins do not show any homology.

To determine whether Arginine at site 474 in the SH2 domain of Cten affects its motility augmenting effects and interaction with downstream targets like ILK and FAK, we introduced a point mutation at this position using site directed mutagenesis.

4.2Aim

I. To create a mutation the SH2 domain of Cten by changing the critical Arginine at position 474 to Alanine.

II. To study the effect of this mutation on functional activity of Cten.

III. To the study the effect of this mutation on the levels of downstream signalling partners of Cten.

4.3Results

4.3.1 GFP-Cten^{R474A} mutant constructed using site directed mutagenesis

As explained in the materials and methods, site directed mutagenesis was used to construct the GFP-Cten^{R474A} expression vector. The primer pair for the GFP-Cten^{R474A} mutant is given in material and methods. A temperature gradient PCR was run and temperature of 75^{o}C was chosen. To get the lowest concentration of template, a varied template concentration PCR was run and 500ng concentration was used. A 50ul reaction PCR was run with 500ng and the entire product volume was run on an electrophoresis gel and extracted from the gel using Blue light to view the product instead of UV light in order to avoid damage to the DNA in the product. Ligation and transformation was done as explained in material and methods. 5 colonies were sent for sequencing and the ones with the desired mutation were grown up as a midiprep (Figure 4-2).

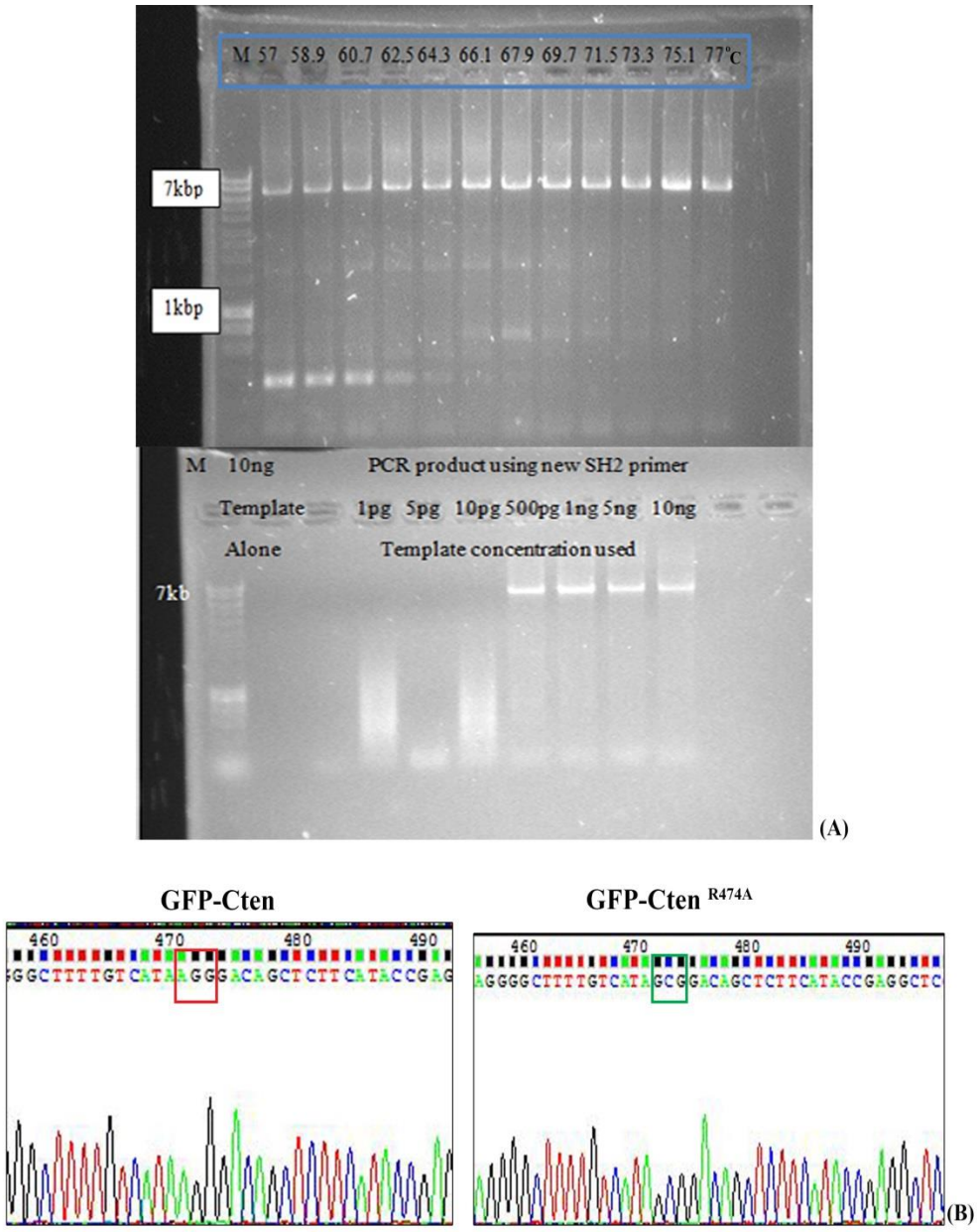


Figure 4-2:(A) Picture of the electrophoresis gel for temperature gradient PCR for the GFP-Cten^{R474A} site directed mutagenesis primers.and Gel run foroptimizing template concentration ranging from 10ng to 1pg. Minimum concentration of 500pg gave a visible product. (B) Sequence result showed the GFP-Cten^{R474A} varied from GFP-Cten in 2 bases from AG to GC.

4.3.2Mutation of critical Arginine to Alanine at 474 reduces cell migration but does not affect cell proliferation

Site directed mutagenesis was employed to change Arginine at the 474 position of Cten which falls in the SH2 domain of Cten to Alanine. It is well known fact

and shown by our group previously that Cten expression in colorectal cancer increases cell motility without affecting cell proliferation (7,43). These functions were tested in the CRC cell lines HCT116 and RKO (both lacking Cten expression). Cells were transfected with Cten wild type i.e. GFP-Cten expression vector, GFP-Cten^{R474A}and pEGFP as empty vector (EV) control (which will referred to as pEGFP for the rest of the study). Two cell motility assays were employed to determine the effect of GFP-Cten^{R474A}. First Transwell migration assay (Figure 4-3A) was done. Compared to pEGFP, transfection with GFP-Cten resulted in increased cell motility in both HCT116 and RKO (p=0.013 and p=0.003 respectively). Transfection with GFP-Cten^{R474A}) resulted in reduced motility compared to the wild type protein expressed by GFP-Cten (p=0.02 for both cell lines) Experiments using the wound healing assay validated these results (Figure 4-4) with enhanced cell motility across the wound for GFP-Cten versus pEGFP in both HCT116 and RKO (p=0.037 and p=0.011 respectively) whilst there was reduced some motility for transfection with GFP-Cten^{R474A} versus GFP-Cten (p=0.008 for both cell lines). It was seen that mutating Arginine caused a significant reduction in cell motility using both assays as compared to GFP-Cten.

The next step was to study the effect of Cten and GFP-Cten^{R474A}on cell proliferation to rule out the possibility of inhibitory effect of the mutant on cell proliferation causing decrease in cell motility. It was found that there was no effect on cell proliferation by GFP-Cten, GFP-Cten^{R474A}or pEGFP (Figure 4-3B). This was done is both cells lines HCT116 and RKO.

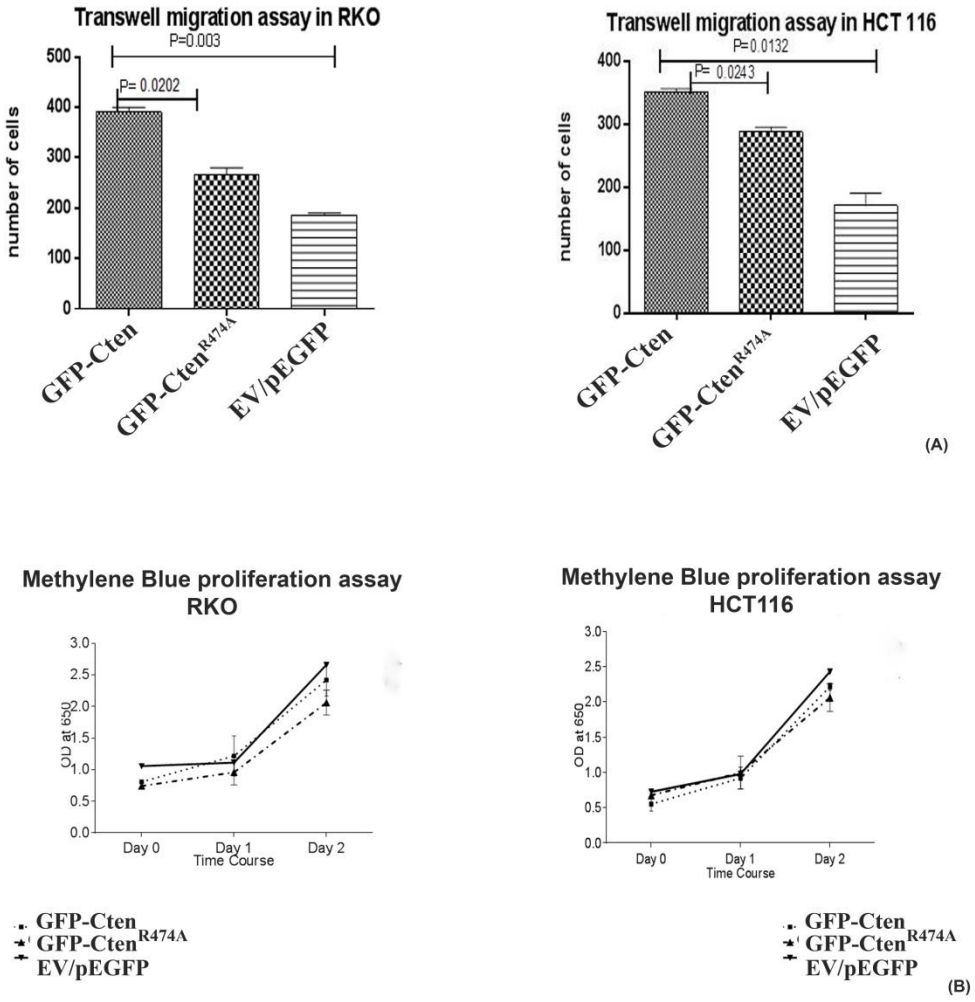


Figure 4-3:Graphical representation of number of cells migrating through a transwell after forced expression of GFP-Cten, GFP-Cten^{R474A} and EV/pEGFP in RKO and HCT116(A) showing significant decrease in cell numbers in GFP-Cten^{R474A} mutant expression but it is still more as compared to EV/pEGFP suggesting role of other domains of Cten motility enhancing function. The Methylene blue proliferation assay shows no significant change in cell proliferation between the three transfection conditions (B). The error bars represent the standard deviation from the mean. Experiments were repeated three times with each condition run in triplicate n=3.

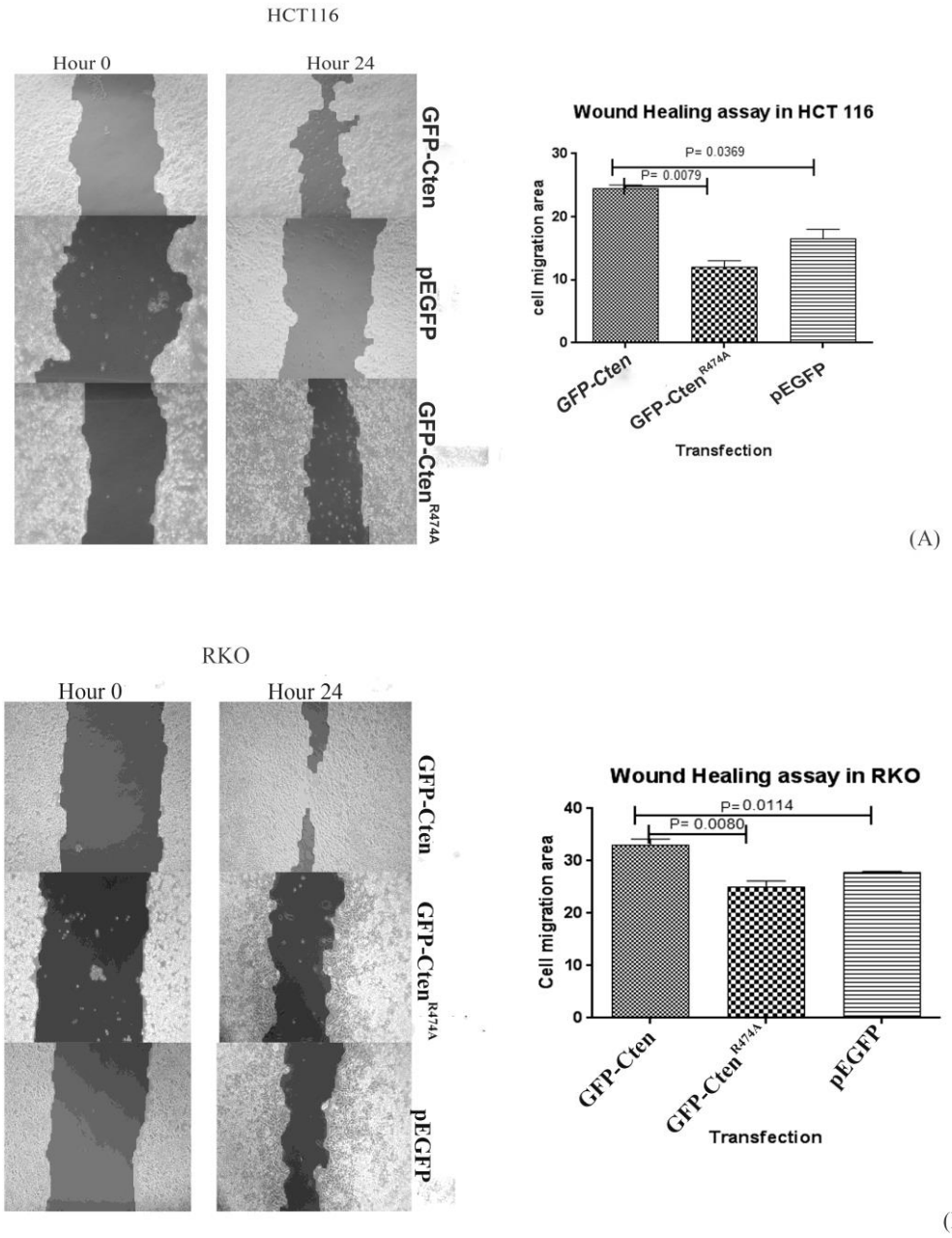


Figure 4-4:Showing the actual images of wound healing assay in HCT116 (A) and RKO (B) with graphical representation of area of cell migration after forced expression of GFP-Cten, GFP-Cten^{R474A}and pEGFP. Area of cell migration was calculated by subtracting the initial area of wound at hour0 to area of wound on hour 24. Unpaired student t test was used to calculate significance between Cten and R474A which shows a significant decrease in area travelled by the cells in HCT116 and RKO. The error bars represent the standard deviation from the mean. Experiments were repeated three times with each condition run in triplicate n=3.

4.3.4 Colony formation activity of GFP-Cten is also reduced in the GFP-Cten^{R474A}

Colony formation assay was performed using the mutant and comparing it to GFP-Cten and pEGFP. Just as the results of cell motility assays, it was seen that there was enhanced colony forming efficiency for GFP-Cten versus pEGFP in both HCT116 and RKO (p=0.001 for both cell lines) and mutation of the Arginine in GFP-Cten^{R474A}caused a significant decrease in colony formation in both HCT116 (p=0.008) and RKO (p=0.03) cell lines (Figure 4-5).

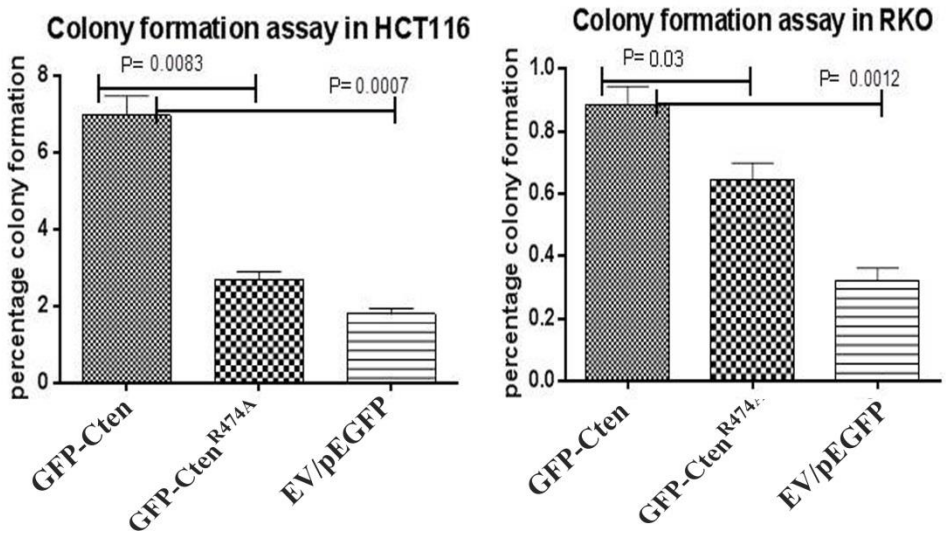


Figure 4-5:Graphs showing the percentage of colony formation in HCT116 and RKO.20,000 cells were seeded in each 6 well plate. Each condition was performed in triplicates. Two wells without cells were kept as negative control. Percentage was calculated by dividing number of colonies by number of cells seeded in total. Unpaired student t test was performed and showed a significant decrease in colony formation by the GFP-Cten^{R474A}in both cell lines. The error bars represent the standard deviation from the mean. Experiments were repeated three times with each condition run in triplicate n=3.

4.3.5 SH2 domain-dependent Cten signalling through Integrin-linked Kinase (ILK) and Focal Adhesion Kinase (FAK)

The evidence provided by migration assays and colony formation assay prove that mutation of this Arginine has a significant effect on the function of Cten. It was important to study the cause of these effects. We have previously shown that Cten causes up-regulation of and may signal through both ILK and FAK(7,46). We decided to look at these downstream targets of Cten. Protein was extracted after forced transfection of GFP-Cten, GFP-Cten^{R474A}and pEGFP in HCT116 and RKO. Western blot was performed for ILK and FAK and pFAK which has phosphorylated Tyrosine residue 397(as known as activated form of FAK). The protein levels of ILK, pFAK and FAK went up with GFP-Cten overexpression but these levels were reduced when GFP-Cten^{R474A} was transfected as compared to GFP-Cten, as shown in Figure 4-6.

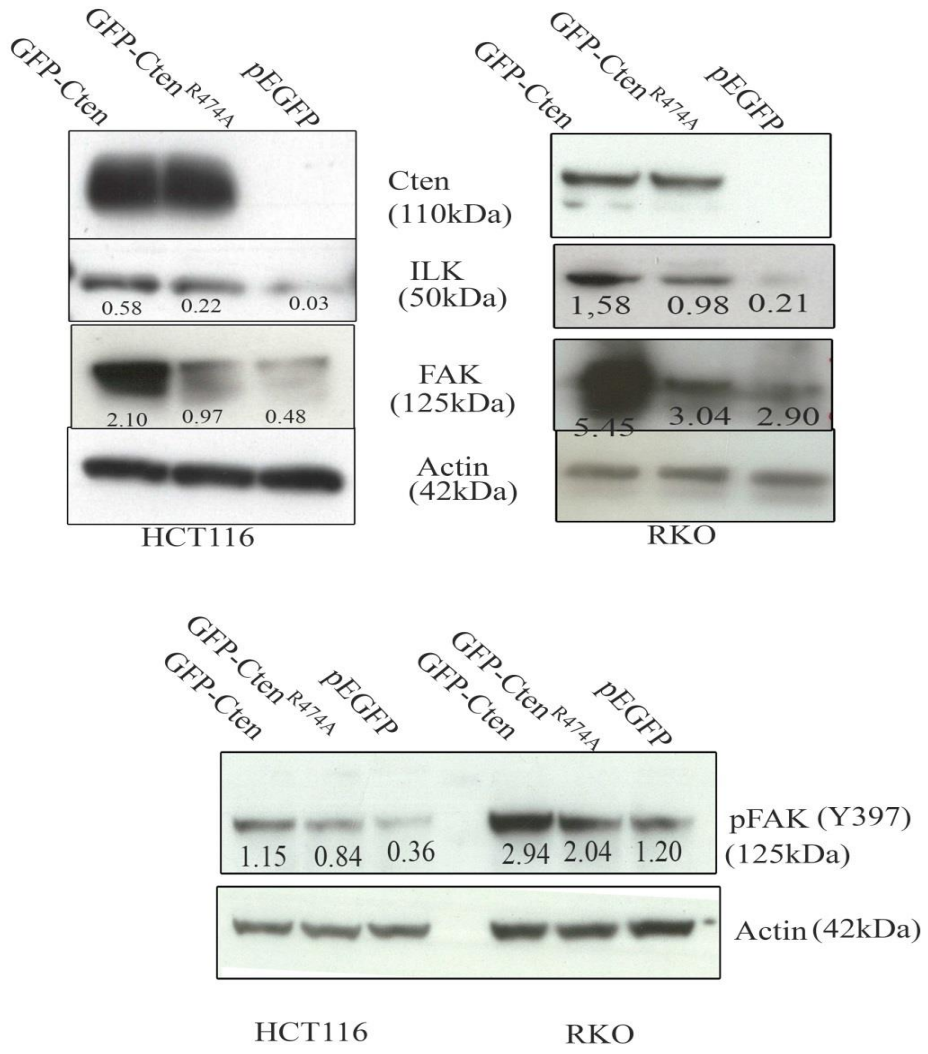


Figure 4-6:Western blot images of protein levels of ILK, FAK, and pFAK in HCT116 and RKO. Actin (β-actin) was used as loading and densitometry was done using image J software. A ratio of density of band of protein of interest and respective loading actin control was calculated. Levels of ILK, FAK and pFAK (Tyr397) went up with expression of GFP-Cten but went down with the GFP-Cten^{R474A}. Numbers on the bottom of each band represent the densitometry values calculated for each protein normalised to the loading control.

4.3.2 ILK and FAK levels are dependent on each other

Cotransfection with forced expression of GFP-Cten and knock down of ILK and FAK separately was done and levels were compared to GFP-Cten expression and pEGFP. It was seen that knocking down ILK was associated with a decrease in FAK levels and vice versa (Figure 4-7). Therefore it was concluded that levels of FAK and ILK may be interlinked and appear to have a stabilising effect on each other

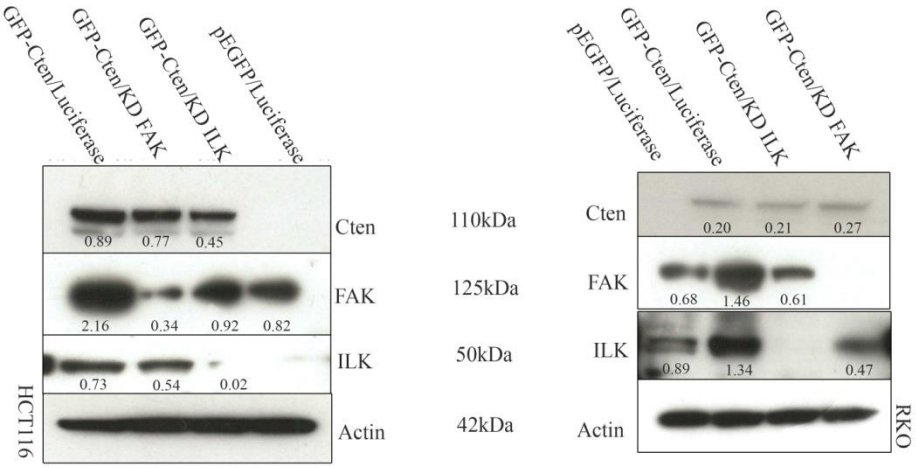


Figure 4-7:Cotransfection of Cten and knockdown of ILK and FAK was performed separately in HCT116 and RKOto look at the levels of each of these proteins in case of knock down of the other. As it can be seen from the western blot pictures knocking down of ILK causes a decrease in levels of FAK and vice versa.As previously shown Cten expression causes increase in levels of ILK and FAK. Numbers at the bottom of the bands represent the densitometry values for each band normalised to β-actin.

4.3.6 Markers of epithelial mesenchymal transition are affected by GFP-Cten

A panel of markers of epithelial mesenchymal switch (EMT) were used to ascertain whether they may be changed in association with cell function. It is shown that increase in cell motility in epithelial cells occurs as a result of decrease in E-cadherin at the junctions between cells. We have previously shown that Cten induces epithelial-mesenchymal transition(45) In addition to E-cadherin, N-Cadherin and Snail were also tested in the panel. As shown in Figure 4-8, the levels of E cadherin went down with GFP-Cten over expression as was expected but this loss was not seen withGFP-Cten^{R474A}. However pEGFP showed higher levels of E Cadherin as expected. N Cadherin levels went up with GFP-Cten expression and shown here for the first time it was seen that Snail levels also went up with Cten expression. This increase was reduced when GFP-Cten^{R474A}protein was transfected. It is interesting to note that although levels of these markers are reduced when the R474A mutant was

transfected, they were still higher than the levels seen with transfection of the GFP empty vector suggesting that there may be other residues controlling the EMT-inducing activity of Cten.

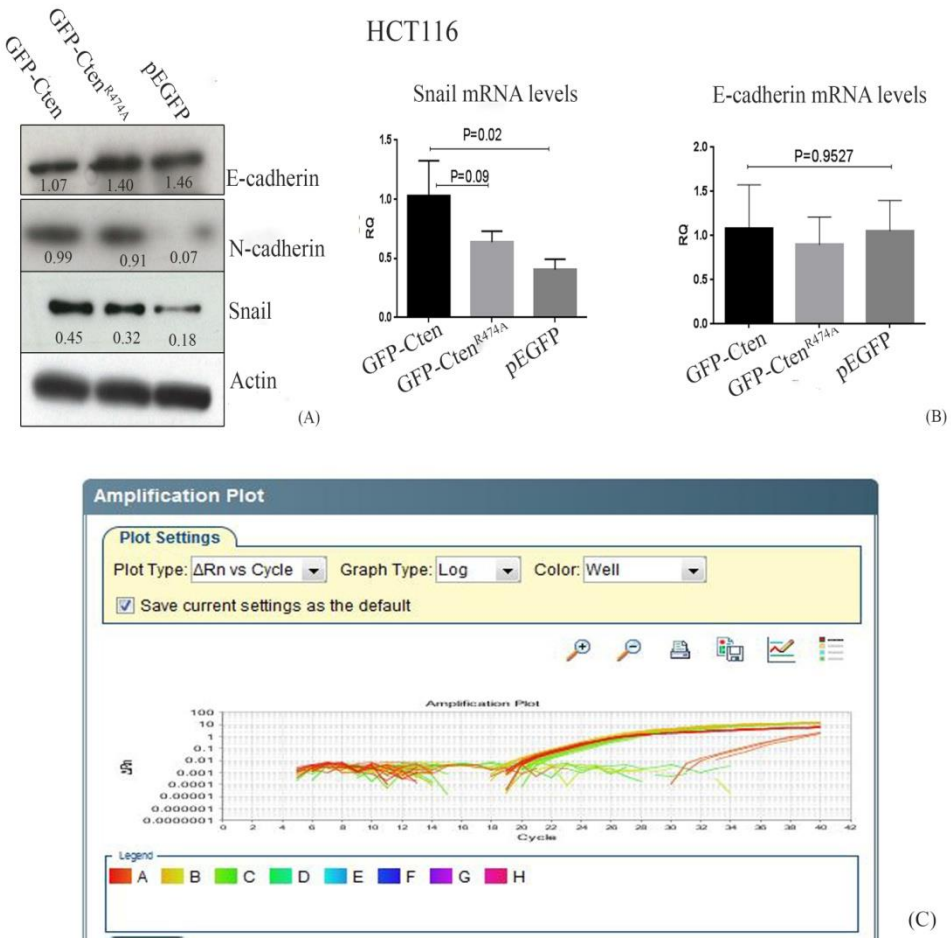


Figure 4-8:Western blot images for EMT markers in HCT116Which reflect the trend in cell motility.(A) E-cadherin levels go down with GFP-Cten, N-cadherin levels go up and levels of Snail go up as well. With GFP-Cten^{R474A}expression E cadherin levels went up and N-cadherin and snail levels went down.(B) mRNA levels of E-cadherin did not any change on GFP-Cten expression, whereas mRNA levels of Snail went up with GFP-Cten and went down with GFP-Cten^{R474A} and more still with empty vector pEGFP, however it wasn't statistically significant.(C) Represents the amplification plot for Snail, E-cadherin and HPRT. The colour red (A) is for HPRT, Green (B) for E-cadherin and Orange(C) for Snail.

Quantitative PCR was done to measure E-Cadherin mRNA and this did not show the changes seen in the protein levels. QPCR was done for Snail as well which showed that Snail mRNA levels mirrored the protein levels in western blot as shown in Figure 4-8.

4.3.7 Cten forms an SH2 domain dependent complex with FAK but not ILK or Snail

Looking at the effect of Cten on levels of ILK, FAK and Snail, we decided to see whether Cten physically binds to ILK, Snail or FAK. To find this out we used immunoprecipitation (IP) to determine the physical interaction between them. GFP-Cten, GFP-Cten^{R474A} and pEGFP were forcefully transfected in HCT116. IP was done both ways for FAK i.e. first Cten was immunoprecipitated out of the sample and blotted for FAK and then FAK was immunoprecipitated out and was blotted by Cten. However due to limitation of the procedure IP with ILK and Snail could only be done one way i.e. ILK and Snail were immunoprecipitated out and blotted with Cten. As ILK has a molecular weight of 50 kDa and Snail of 29 kDa, it is not possible to view it on the western blot because the beads employed for IP are coated with immunoglobulin which gives 2 bands one of heavy weight chain 50 kDa and the other at 25 kDa. IP for ILK and Snail showed no physical binding with Cten. This however, was not the case with FAK. When immunoprecipitated for FAK and blotted with Cten, it was seen that Cten and FAK bind together in HCT116. This binding was lost partially with GFP-Cten ^{R474A}(Figure 4-9). However, this interaction only worked one way and not the other as seen in Figure. To explore this further we explored whether this interaction occurs in nucleus or cytoplasm. Nuclear and cytoplasmic protein fractionation was performed and IP done. It was seen that Cten binds to FAK in both nucleus and cytoplasm (Figure 4-11)

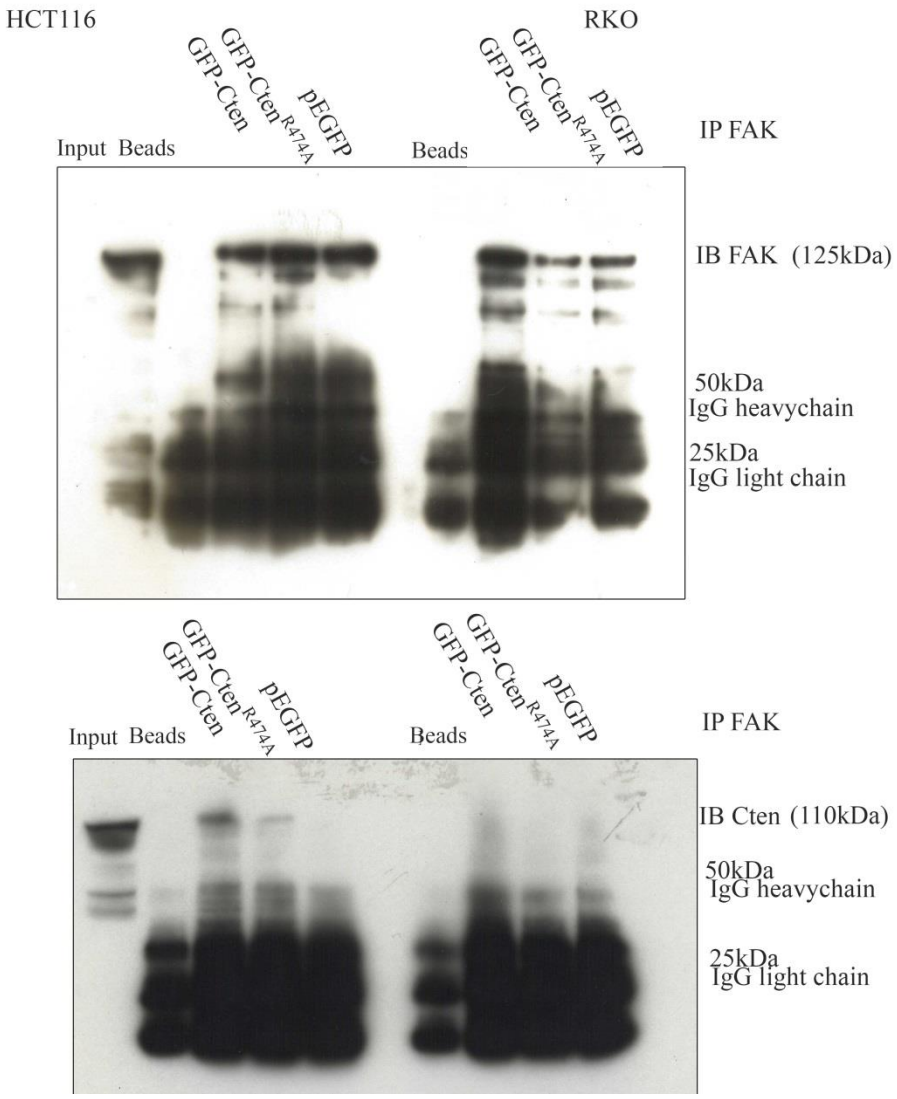


Figure 4-9:Immunopreciptation was done with FAK precipitated out after GFP-Cten, GFP-Cten ^{R474A}and empty vectorpEGFP was over expressed in both HCT116 and RKO. Samples were precleared with beads and in addition to input sample , a negative control of beads with sample and no antibody was run to check for unwanted binding.IP was confirmed by blotting with FAK and then blotted with Cten. In HCT116 it is apparent that FAK binds to Cten in the GFP-Cten overexpressed lane. This binding becomes weak with the GFP-Cten ^{R474A}and there is no binding in pEGFP. However it only occurred in HCT116 cell line and not RKO. The background staining at the bottom of the blots are bands from the heavy (50kDa) and light chains (25kDa) of the IgG sepharose beads, but it does not disturb the experiments as our antibodies of interest are both above 100kDa.

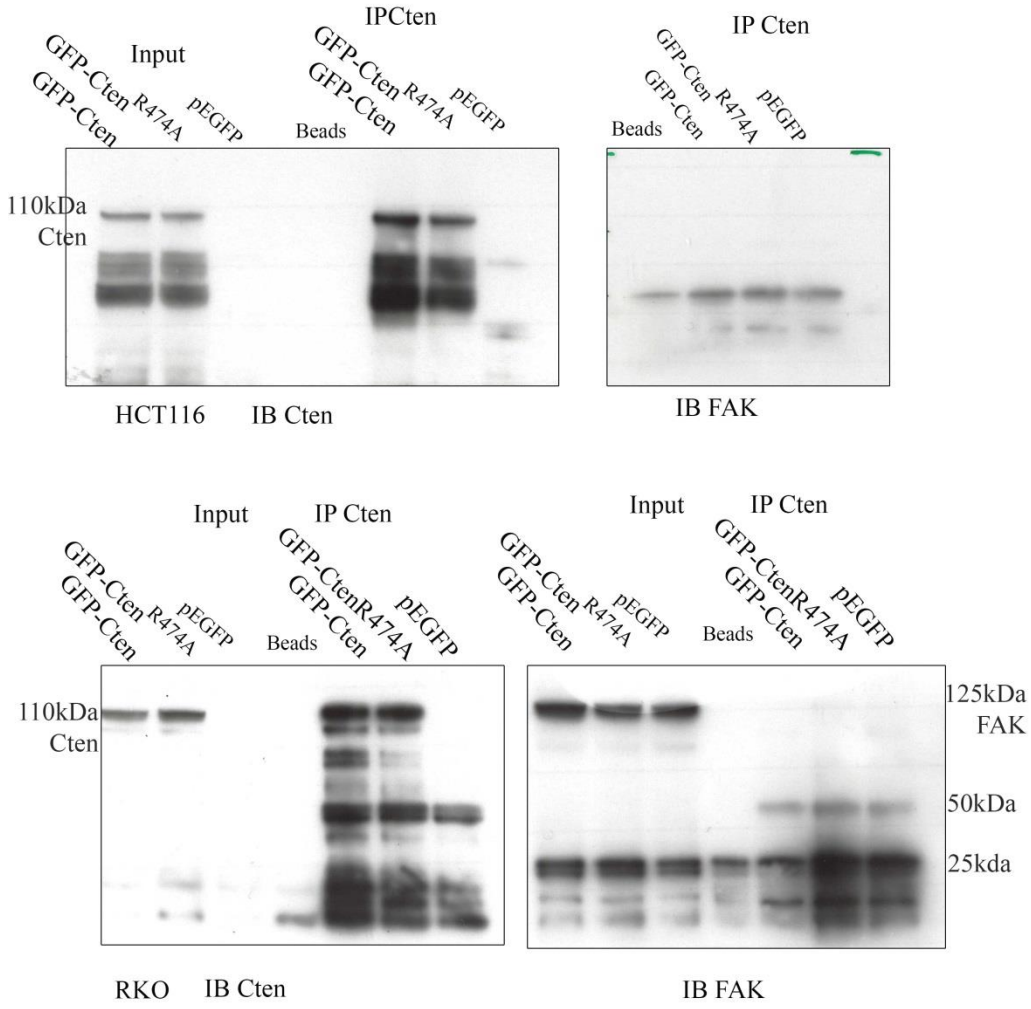


Figure 4-10:Blots for IP with Cten and blotted with FAK in HCT116 and RKO. Input lane acts as positive control Although the IP with Cten worked very well when blotted with Cten antibody (band of 110kDa), there were no bands seen on immunoblotting with FAK. The multiple bands at the bottom of each blot represent the heavy (band of 50kDa) and light chain (band of 25kDa)of IgGsepharose beads.

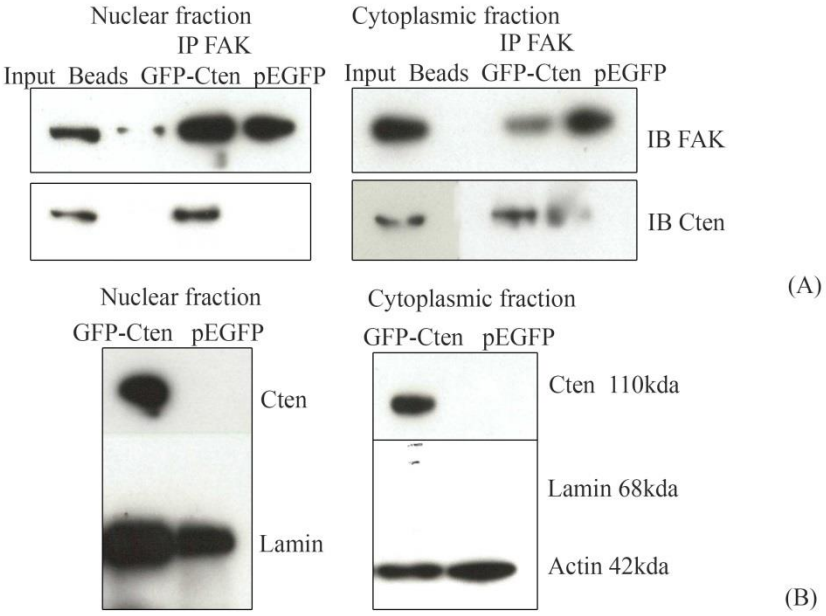


Figure 4-11. The top blot shows the IP of FAK in HCT116 nuclear and cytoplasmic protein fractions which have Cten and empty vector expressed respectively.Input acts as a positive control whereas, beads serve as negative control. When immunoblotted with Cten it is seen that FAK binds to Cten in the nucleus as well as in the cytoplasm. The fractionation was confirmed by keeping Lamin as a nuclear fraction loading control which is not found in the cytoplasmic fraction. Beta actin served as a loading control in the cytoplasmic fraction.

4.4Discussion

In this study we have successfully made a new construct containing Cten with a point mutation at residue 474 changing the Arginine to Alanine. This provides an ideal condition for studying the role of the SH2 domain of Cten in its interaction with other molecules and its effect on cell motility. Previous studies have shown that Cten promotes cell migration, invasion and colony formation in a number of tumours including colorectal, breast thymomaand pancreas (40,43,52,54,71)Our aim was to discover whether SH2 domain is important in the delivery of these functions. We first tested the functional activity of the SH2 domain in HCT116 and RKO, CRC cell lines which do not show expression of Cten normally.When GFP-Cten, GFP-Cten^{R474A} and pEGFP were transfected into these cell lines, there was no difference in cell proliferation. However both GFP-Cten and GFP-Cten^{R474A} induced cell

motility (as measured by transwell migration) when compared to pEGFP. Similarly, both GFP-Cten and GFP-Cten^{R474A} promoted colony formation in soft agar when compared to pEGFP. However, the induction of motility and colony formation was significantly greater when GFP-Cten was transfected compared to GFP-Cten^{R474A}. This data show that the SH2 domain is important for both the motility-inducing and the stemness-inducing activities of Cten. However, the fact that the mutant protein still induced these activities when compared to the empty vector indicates that there are other SH2-independent mechanisms of Cten activity.

The mutated Arginine at the site corresponds to the same at strand βB5 in Src kinase which was shown by titration calorimetry as critical for binding of SH2 domain with tyrosine phosphorylated proteins(70). This critical Arginine has since then been targeted and mutated in a number of studies which have researched its role in human as well as dog Cten. This arginine was considered important in interaction of Cten with DLC1 in MLCSV40 (immortalised human prostatic epithelial cell line) although, this interaction was independent of tyrosine phosphorylation of DLC1(54). In Madin-Darby canine kidney (MDCK) cells this arginine when mutated to Alanine prevented invasive migration and tubule formation(53).

Recent studies have shown that both the oncogenic and tumour-suppressive roles of Cten appear to be mediated through its SH2 domain (55). In both instances the mechanism is reported to be through modulation of the activity of the tumour suppressor gene Deleted in Liver Cancer (DLC)-1(50,72). In colorectal cancers (CRCs), DLC-1 is rarely expressed and we have been unable to demonstrate its expression in any of our CRC cell lines (data not shown).

We therefore consider that a DLC-1/Cten interaction is not likely to be important in Cten function in CRC. We thus hypothesized that, in CRC at least, the SH2 domain may be acting through FAK and ILK as these are known downstream targets of Cten(7,46,73). Transfection of GFP-Cten induced ILK, FAKas we have previously shown(46) and in our study phosphorylated-FAK at Y397 (the activated form of FAK) was also increased following GFP-Cten forced expression.

Mirroring the observations on cell function, we found that the GFP-Cten^{R474A} protein also induced FAK and ILK when compared to the empty vector but this was much less than the induction seen with the wild type GFP-Cten. The R474A mutation similarly abrogated the effects of Cten on further downstream markers of EMT (i.e. E-cadherin and N-cadherin).

What is interesting here is that for the first time we have shown that FAK binds physically to Cten and this binding goes down but is not completely obliterated by the R474A mutation. FAK overexpression is seen in several tumours and leads to increased cell survival, motility and proliferation in culture (74). Its aggregation within the focal adhesions is said to lead to its activation and autophosphorylation via interaction with focal adhesion molecules like talin. Phosphorylation at Y397 which is an autophosphorylation site in FAK leads to its linkage with Src. This complex is then believed to interact with molecules at focal adhesions like Tensins and p130cas. Activation and binding of these molecules may prompt activation of MAP-kinase and PI3-kinase which may be the basis of its cell motility enhancing properties(75). Tensin1(TNS1) is also seen to bind tyrosine phosphorylated proteins like p130Cas and FAK (76).

FAK has been co-immunoprecipitated with TNS1 as well as vinculin suggesting that TNS1 might mediate signal transduction through this interaction (77). Phosphorylation of FAK at Y397 has been proposed to be the result of reactive oxygen species (ROS) produced by integrin ligation and Rac1 so that it can bind to other phosphorylated proteins like Tensins, p130cas etc. and it also modifies FAK to respond to EGF signalling. The interaction with EGF causes phosphorylation of FAK at Y925 which in turn leads to focal adhesion disassembly and thus causes cell spreading in cell motility(78).This is quite interesting as Cten is induced by EGF and also negatively regulates its degradation(55). FAK is also known to locate in the nucleus (79) and so does Cten ((6). In our study we have shown that Cten binds to FAK not only in the cytoplasm but in the nucleus too. Cten might function to move FAK from nucleus into focal adhesions followed by its interaction with Src. The interaction with Cten might be one of the first steps in FAK activation and localisation and other events might follow leading to increased motility.

ILK is another molecule which goes up with Cten expression. It can be derived from our results that ILK might be linked to Cten via its SH2 domain.Both Cten and ILK are located at focal adhesions and regulate cell motility. Investigation of their functional relationship has also been shown previously(7).But ILK was not found to be physically bound to Cten in our study. ILK is a relatively new addition to the effector molecules of the integrin and growth factor signalling(80).

ILK and FAK are activated at different times when plated on fibronectin, ILK activity goes down at the time when FAK's activity levels go up. They might serve as effectors of motility at different points in time. (81). But this is not the

case in our results. We see thatILK and FAK appear to stabilise each other. ILK over expression leads to dramatic increase in cell motility by loss of cell-cell due to reduction in levels of E-cadherin. The decrease in E-cadherin is the result of movement of β-catenin to the nucleus and an increase in its binding partner Lef1, which causes down regulation of the gene encoding E-cadherin(82). ILK also inhibits GSK3 which can also lead to localisation of β-catenin to nucleus(80). The end result of an ILK increase can be seen in the decreased of E-cadherin levels. E-cadherin is one of the markers used in our study to show EMT on expression of Cten. The other markers used were N-cadherin and Snail. Decrease in E-cadherin and increase in N-cadherin known as the cadherin switch is a hall mark of EMT and is shown to be of significant importance in prostate cancer progression(83). A shift from Epithelial (E) cadherin to Neural (N) cadherin is usually seen in tumour metastasis. N cadherin is very important for pro-migratory signalling of tumour cells and is a link between invading cells (84). It was indeed seen in our results that N-cadherin levels go up with Cten expression although this interaction might be SH2 domain dependent as R474A mutation causes decrease in N cadherin levels. However, how N-cadherin fits into the Cten functioning circuits remains to be seen. It is said that Fibroblast growth factor receptor FGFR is important for N-cadherin signalling and they both act synergistically and lead to increased FGFR signalling and in turn Mitogen activated protein kinase-extracellular signal regulated kinase (MAPK-ERK) activation(85).

Snail was also induced with GFP-Cten and GFP-Cten^{R474A}. But as with the other markers like FAK and ILK the increase in Snail by GFP-Cten ^{R474A}was still less than that with GFP-Cten. To our knowledge this is first time anyone

has shown a relationship between Cten and Snail. Over expression of snail is seen in surgical specimens of colorectal cancer and leads to increased cell motility in colorectal cell lines(86). Snail is transcription factor which induces EMT by repressing levels of E-cadherin at transcriptional level. (87). Snail activity might be induced by Ctenthus leading to loss of E-cadherin. However we have not seen a loss of E-cadherin at mRNA levels as described previously(52).

By the results of our study it can be assumed that Cten acts as a regulator of FAK in the focal adhesion apparatus. Cten might act as an important mediator of integrin signalling by linking with molecules ILK, FAK, and Snail possibly via its SH2 domain. Possessing an SH2 domain puts Cten at the helm to conduct important mediation between the extra and intracellular environment as it also binds to integrins.

In future it would be interesting to look at other residues in the SH2 domain of Cten and target them to study their effect on Cten function. Specific pharmaceutical agents can be designed to target the SH2 domain of Cten and their effects can be studies on mouse models of colorectal cancer, so that someday these agents may possibly be used as treatment option for colorectal cancer.

5 Regulation of Cten in colorectal cancer by Calpain

5.1Introduction

Calpains belong to a family of cytoplasmic cysteine proteases. There are two major isoforms known as µ-calpain and m-calpain. Calpain is present ubiquitously in all organisms ranging from yeast to humans (88,89). The two isoforms are named as such because one requires micromolar and other requires millimolar concentrations of Ca^{2+} to carry out their activities. These isoforms are referred to as ubiquitous/typical or conventional calpains. Both µ-calpain and m-calpain share a 55-65% sequence homology and identical 28kDa and 80kDa subunits. The human genome is said to contain 14 genes which encode molecules belonging to the calpain gene family. Five of the calpains discovered seem to be tissue dependent like calpain 3 in smooth muscles, calpain 8 in skeletal muscles, calpain 6 is in placenta, calpain 11 in testes and calpain 12 in skin. Calpain 5, 7, 9, and 10 expression is more generalised whereas calpain 13 is found in lung and testes (90). Calpain is involved in a number of processes including cell motility, proliferation, cell cycle progression and differentiation to name a few. Calpain has been implicated in a number of diseases including cancers like gastric, pancreatic, schwannomas, meningiomas and colorectal cancer as well (91).M-calpain expression is increased in colorectal adenocarcinomas(92) and calpain 6 expression is increased in uterine sarcomas (93).

Calpain cleaves of a number of proteins involved in signal transduction and especially target molecules at and near focal adhesions such as talin, paxillin, vinculin, FAK, Src etc. Most work done on calpain and its effect on cell motility is done in platelets and endothelial cells (94,95). Calpain also regulates cell adhesion disassembly and turnover by targeting α-actinin and its

localisation into focal contacts(96).It would be interesting to find if calpain regulate motility through degradation or alteration of Cten or if Cten induces motility through modulation of calpain. To study this we need a process to alter the calpain activity, which can be possible by inhibition of calpain. There are many inhibitors of Calpain discovered so far. The first one was a natural inhibitor of calpain identified in skeletal muscles called Calpastatin that can bind to calpain and block its site of activity(97). In addition to this in vivo inhibitor, there is another one called Calpeptin which can be used to control calpain activity in vitro.It works by binding to the critical cysteine residue in the active site of calpain, preventing binding of its substrates. This property renders it ideal for experimental purposes (98-100)

Considering the role of calpain in cell motility and involvement in cell spreading and its overexpression in cancer cases, we decided to study its role in colorectal cancer lines and look for a possible link between Cten and calpain. As Cten is also part of focal adhesion signalling machinery we thought it would be worthwhile to explore this facet of cell motility involving focal adhesions.

5.2Aim

I. To see whether Cten expression has an effect on calpain levels in colorectal cancer cell lines.

II. To test the effect of calpain inhibition by Calpeptin on Cten levels.

5.3Results

5.3.1 Knockdown of Cten did not have any effect on calpain levels

To look at the effect of Cten on calpain levels we knocked down Cten in colorectal cell line SW620, having endogenous high levels of Cten using

siRNA already tested by our group. We looked at the levels of calpain I (80kDa), II (76kDa) and IV (30kDa). Unfortunately the calpain II antibody did not work. So we looked at the levels of calpain I and IV only and as shown in Figure 5-1(A) did not get a visible difference on western blot. When densitometry was performed the levels also didn't show a significant difference either.

5.3.2 Inhibition of calpain causes a decrease in levels of Cten and Tensin 3

The next step was to look at the activity of calpain and its effect of the levels of Cten. To inhibit calpains, a calpain inhibitor called Calpeptin was used. Calpain activity assay was employed to ensure that Calpeptin addition to SW620 cells inhibited calpain activity. To look at the activity of calpains a fluorescent substrate called CMAC Tboc-Leu-Met was used which emits fluorescence on cleavage by calpains. The fluorescence emitted reached a plateau after 60 minutes so at this point fluorescence was read. The presence of Calpeptin lead >50% reduction in florescence as compared to thenormal control DMSO as shown in Figure 5-2.

SW620 was incubated with Calpeptin for 60 minutes and then protein was extracted and western blot for Cten(endogenous 77kDa) and Tensin 3 (135kDa) was performed. It was seen that inhibition of calpains causes an increase in Cten levels. Although it wasn't visible at first but after densitometry it was apparent that not only does calpain inhibition increase Cten levels it also increases levels of Tensin 3 as shown in Figure 5-1(D).

5.3.3 Inhibition of calpain increases the levels of Src and paxillin but does not affect vinculin levels

Calpain inhibition not only increase levels of Cten (endogenous 77kDa), but also increases levels of Src (60kDa) and paxillin (68kda)as shown in Figure 5-1(C).The other molecule in our panel is vinculin (124kDa), which is also part of the focal adhesion molecules connecting integrins to actin cytoskeleton. There was no change in levels of vinculin seen after inhibition of calpain.5.3.4

5.3.4 Knockdown of Cten causes decreases in levels of Src and increase levels of paxillin

To check whether the effect of Src and paxillin levels after calpain inhibition was actually due to lack of activity of calpains or Cten was the mediator of these effect we decided to knock down Cten in SW620 and look at levels of paxillin and Src. Src showed the same effect as with the calpain inhibition assay and went down with Cten knock down and vice versa. Surprisingly paxillin went the opposite way. With knock down of Cten paxillin levels went up and didn’t follow the pattern shown with calpain inhibition assay Figure 5-4(C). Vinculin levels followed the pattern as shown in calpain inhibition assay and didn’t show any drastic change.

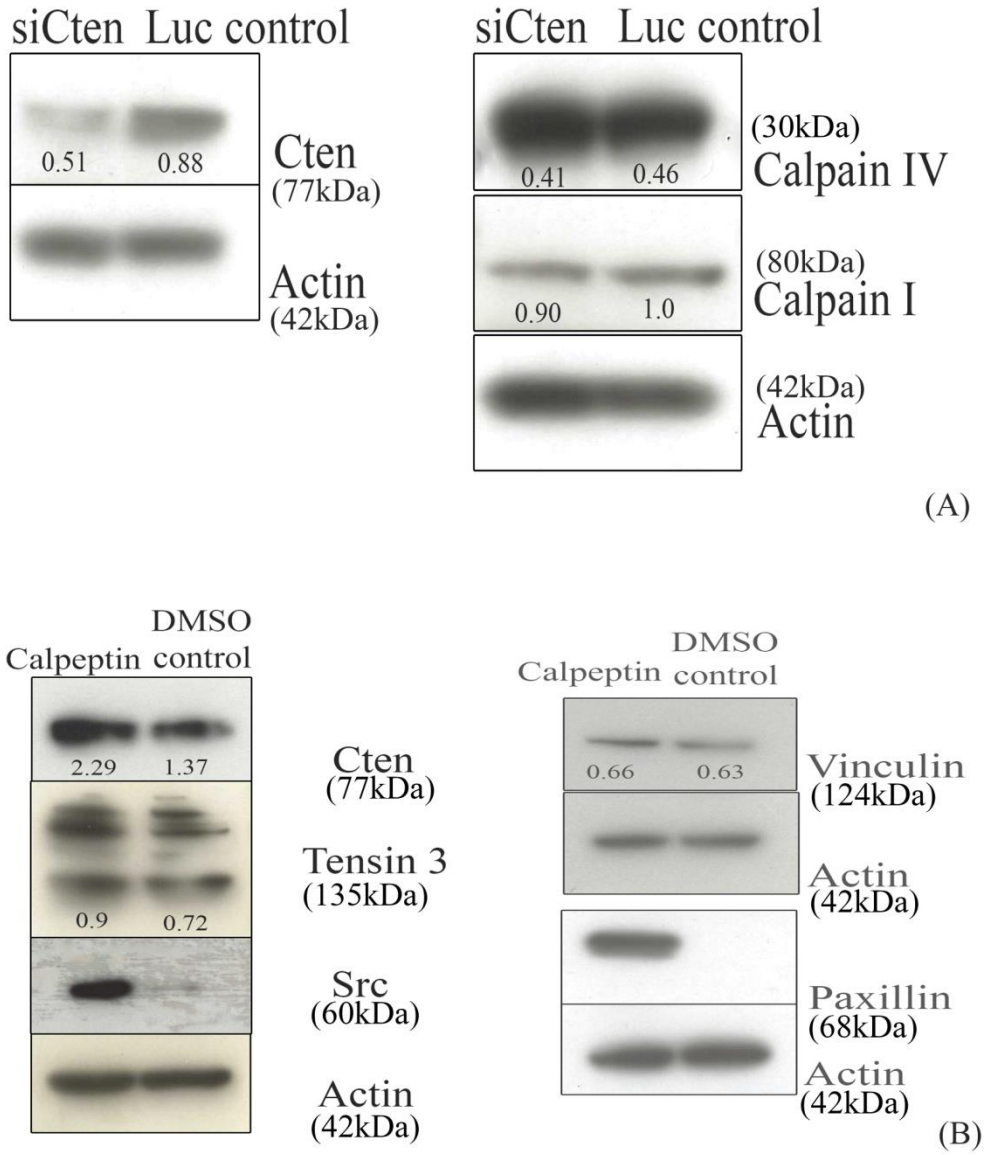


Figure 5-1.Relation between Cten and Calpain(A) Cten was knocked down using a siRNA already proved to give good knock down. Levels of calpain I and IV did not change on knock down of Cten. (B) Western blots showing increase in level of Cten, Src and Tensin 3 after calpain inhibition by Calpeptin compared to control. Levels of vinculin don't show any change and paxillin levels go up after addition of Calpeptin. The numbers at the bottom of the bands represent the density calculations normalised to the β-actin levels. As levels of Src and paxillin are quite apparent by the western blotcalculations have not been done.

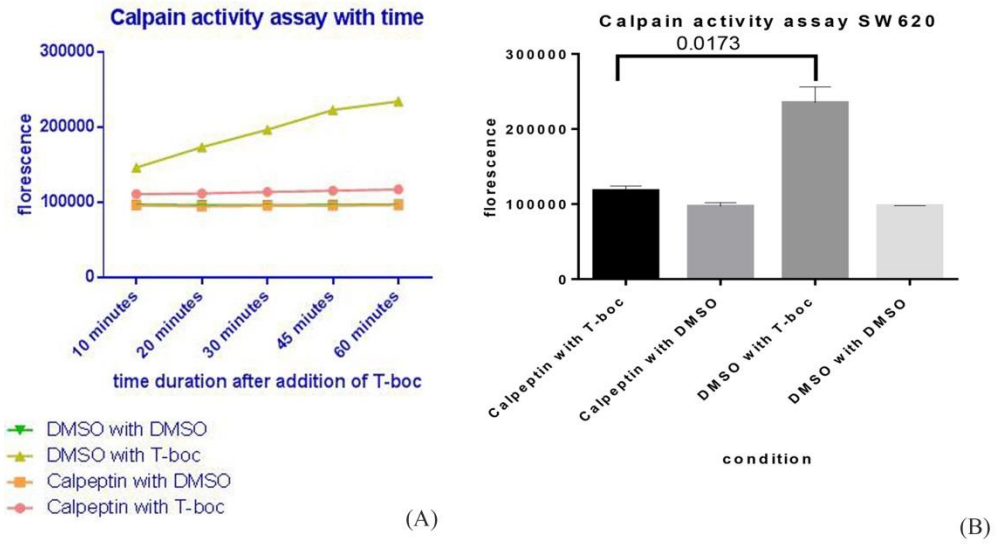


Figure 5-2.Calpain activity assay(A) Graphs showing inhibition of calpain after addition of Calpeptin. The levels of florescence after cleavage of Tboc reached a plateau after 60 minutes. (B) There is a significant decrease in the activity of calpain after addition of Calpeptin. DMSO was added as negative control as it was used to prepare Calpeptin.

5.3.5 Knocking down Cten causes a reverse in EMT

Cten is known to have a positive effect on cell migration in colorectal cancer cell lines in addition to a number of others. It is also know to affect epithelial mesenchymal transition markers including E-cadherin (E-cad), N-cadherin (N-cad) and recently discovered by our group snail. To check whether knock down of Cten reverses these effects on EMT. We knock down Cten and looked at levels of E-cadherin, N-cadherin and snail and found results supporting this claim as shown in Figure 5-4(B).Snail and N-cadherin levels go down, where E-cadherin levels go up after knock down of Cten. Cten knock down was confirmed by QPCR as well and show almost complete knock down at mRNA level (Figure 5-3). Snail and Src mRNA levels were also looked at (Figure 5-3 C&D). Although snail shows a difference in its levels after knocking down of Cten its not significant when repeated at 3 separate occasions. Src showed a difference in mRNA levels but in order to check whether it was true or not

Cten was forcefully expressed and Src levels were noted and didn't show any difference. So this was not considered to be a true effect.

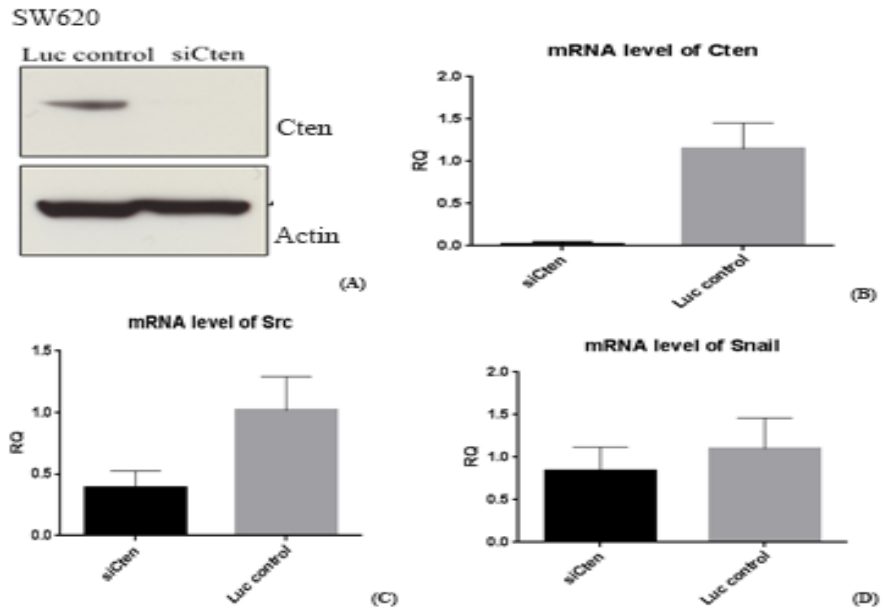


Figure 5-3.Effect of Cten knockdown on mRNA levels (A) western blot for Cten knockdown after transfection with siRNA. (B) mRNA levels of Cten show almost complete knock at transcriptional level. (C) (D) Src and Snail show a decrease in mRNA levels with Cten but results were not consistent or significant. siCten=knockdown of Cten using siRNA and Luc control=luciferase negative control.

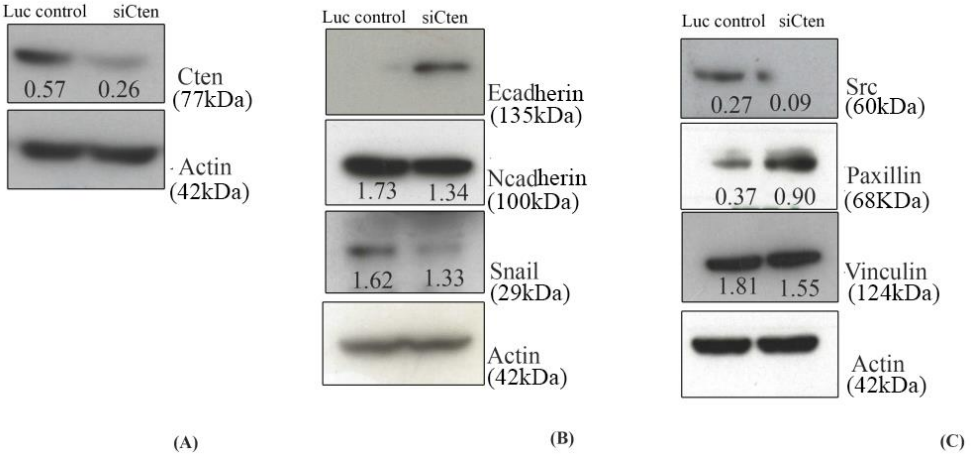


Figure 5-4. Effect of Cten knockdown on various proteins(A)(B)Western blot image after knockdown of Cten and control siRNA against luciferase shows Src levels go down when Cten levels go down, paxillin shows the opposite effect and goes up with Cten knockdown. Vinculin doesn't show a major difference. (C) After knock down of Cten levels of Ecad levels go up and Ncad levels go down. Snail levels also go own after Cten knockdown. Blot with Cten shows good knockdown. Numbers at the bottom of the bands represent the density of each band normalised to actin control. siCten=knockdown of Cten using siRNA and Luc control=luciferase negative control.

5.4Discussion

Calpain are essential elements to the process of cell motility whether it is myofibril organisation within muscle cells or at the level of focal adhesions(101). Calpain cleave a number of proteins which are vital in the focal adhesion machinery and thus act as a regulator to the function and signalling through these molecules. There are a number of studies showing the substrates of calpains within the focal adhesions(102) but so far no one has looked at Cten which is a vital part of these adhesions and plays a significant role in colorectal cancer.

In our study we have used antibodies against calpain I, II and IV. Calpain I, also called μ-calpain which is one of the primary isoforms found in cells(103) Calpain II is generally regarded as m-calpain. Both are also known as typical calpains. Calpain IV consists of only the 28kDa subunit of calpain(90).

In our experiments we have seen that although levels of Cten do not have an effect on the levels of calpain I and IV but it might be possible that the levels do not change but their activity does. The levels of Cten, however, were affected with calpain activity. Using the calpain inhibitor, Calpeptin, we observed that Cten levels went up after calpain inhibition. This suggests that Cten is a substrate of calpains in colorectal cancer cells. In a studyon the discovery of Tensin 1, it was also observed that calpain II acts as an enzyme and cleaves Tensin at multiple levels. The inhibitor used in this study was MDL which is a specific inhibitor of calpain II. Inhibition of calpain cleavage of Tensin 1 resulted in abnormal organization of actin cytoskeleton leading to morphological changes in cells leaving to inability to spread(104).Calpeptinused in our study has been known to have off target

effects besides calpain inhibition, so to say that Cten levels are being affected entirely by calpain inactivity might not be entirely the case. Calpeptinhas been shown to inhibit certain types of tyrosine phosphatases mainly upstream of small RhoGTPases which effect assembly of stress fibres and focal adhesion(99). It can also effect levels of IL6 and TGFβ to name a few all suggesting that it might affect other pathways independently of calpain(105).

One of the other substrate of calpain considered in our study is paxillin. Paxillin is part of the focal adhesion adaptor protein which is tyrosine phosphorylated and is responsible for signalling and regulating cell motility(106) Calpain cleavage of paxillin has been implicated in negative regulation of cell motility at focal adhesions(107). In our study we have confirmed that paxillin is a substrate of calpain but interestingly this cleavage of calpain in not mediated through Cten. In fact Cten regulates paxillin levels in the opposite direction. Which would fit the theory of paxillin acting as a negative regulator of motility as Cten is known to increase cell motility. However they are conflicting reports in the literature on the role of paxillin at focal adhesions and in cell spreading. Vinculin is also a known substrate of calpain(108) but in our study there wasn't a stark difference in its levels after calpain inhibition. The discrepancy could be down to different cell types used, epithelial in our study verses platelets in others.

Srcis another substrate of calpain in our study and its levels go up with calpain inhibition after addition of Calpeptin. Its levels also seem to be mirror levels of Cten. There are a number of studies in the literature about Src and its relationship with calpain with some conflicting results mainly because they have been performed in different cell types. Src is said to be a substrate of

calpain in neurons and its cleavage leads to cell death(109) In chick embryo fibroblasts Src appears to increase proteolysis of FAK by calpain (110) and FAK and calpain II can physically associate with each other(111). In other studies Src and FAK are also said to link with each other(30) and promote cell motility. A study on HEK and rat breast cancer cells showed that calpain 2 mediates enhanced Src activity by cleaving PTP1B which leads to increased phosphatase activity and removes an inhibitory phosphate from Src leading to formation of lamellopodia(112).All these studies suggest a link between calpain and Src and this link has been highlighted in our finding as well. Since FAK levels not only are regulated by Cten but there also a physical binding between the two.in our study, it can be hypothesized that Cten, FAK and Src interaction might regulate cell motility in colorectal cancer.

Cten is known to cause a shift towards EMT. This has already been shown in a number of papers from our group. This includes E-cadherin whose levels are only affected at protein and not transcriptional levels(45). In this chapter we have just reiterated the previous findings. Snail has been identified the first time as a downstream target of Cten at protein levels. Snail has been known to induce stemness characteristics and is overexpressed in colorectal colonospeheres before (113) The mRNA levels of Snail haven't been too convincing on repeat experimentswhen measured after knockdown of Cten in SW620.However in our previous findings (given in chapter 4), have shown Snail mRNA to significantly increase after Cten overexpression.

In conclusion, in this study we have shown for the first time in that Cten is a possible substrate of calpains. Src,a known target of calpain machinery hasbeen shown to be regulated through Cten. In addition paxillin has also been added to

the list of possible targets of Cten. A novel interaction has been brought to light which may serve as a foundation for further studies and may one day be used as a therapeutic target in colorectal cancer.

6 Role of Tyrosines in the SH2 domain of Cten

6.1 Introduction

So far we have identified the SH2 domain of Cten to be critically important for its functional activity. However when we mutated the Arginine to Alanine the construct did not cause reduction in cell motility to the level of empty vector pEGFP leading us to hypothesise that there may be other motility inducing residues located at other sites. Since Cten is proposed to be phosphorylated at a few sites(12). We choose to look at its tyrosine phosphorylation status and ventured further to identify additional residues within the SH2 domain which might be responsible for its function.

SH2 domain of Cten is important for binding phosphotyrosine. According to the basic structure of an SH2 domain it comprises of a conserved Arginine residue which provides a positively charged pocket for binding with phosphotyrosines. But the C-terminal recognition pocket of SH2 domain is much more divergent, and accounts for the differences among SH2 domains in recognition of specific phosphotyrosine-bearing proteins and is equally important. This binding is made even more specific by diversity of the residues, C-terminal to the phosphotyrosines of the binding protein as well, making each interaction between an SH2 domain and phosphotyrosine a unique one (114-117). All this diversity is important for determining the binding specificity of the SH2 domain. A single change in these residues can lead loss of binding specificity. A good example is the SH2 domain of Src where a mutation of the Threonine residue leads to affinity change(118).

As tyrosine phosphorylation is the first step in signal transduction, (119)and since Cten is a vital part of the focal adhesion complex and may function in

signal transduction we decided to mutate three tyrosines in and around its SH2 domain.We mutated 3 tyrosines at position 449, 479 and 530 to phenylalanine and studied its effect on cell motility and explored other binding partners and downstream signalling partners including Src, paxillin and vinculin.

6.2 Aims

I. To look at tyrosine phosphorylation of Cten and find out if Tyrosines within and near the SH2 domain are being phosphorylated.

II. Create three constructs consisting of Tyrosine at 449,479 and 530 changed into Phenylalanine.

III. Look at the effect of these constructs on functional aspects of Cten.

IV. Look at effect of these mutants on the downstream targets of Cten identified so far.

6.3 Results

6.3.1 Cten is tyrosine phosphorylated in endogenous state

To study the functioning of Cten closely we decided to look at whether Cten is being tyrosine phosphorylated. This is a vital step in signal transduction and Cten is considered part of the adaptor proteins at focal adhesions. To test this hypothesis we employed the process of immunoprecipitation (IP). We immunoprecipitated all phosphotyrosines (using PY20 antibody) out of a colorectal cell line SW620 which has high levels of Cten endogenously. After IP we blotted it for Cten and vice versa. We found out that there was a clear band at the same size as endogenous Cten(77kDa) after IP with PY20 (Figure 6-3). The IP was done in the reverse direction as well with Cten IP and blotting with PY20. This time there were multiple bands but there was a faint band at 77kDa when blotted with Cten (Figure 6-1).

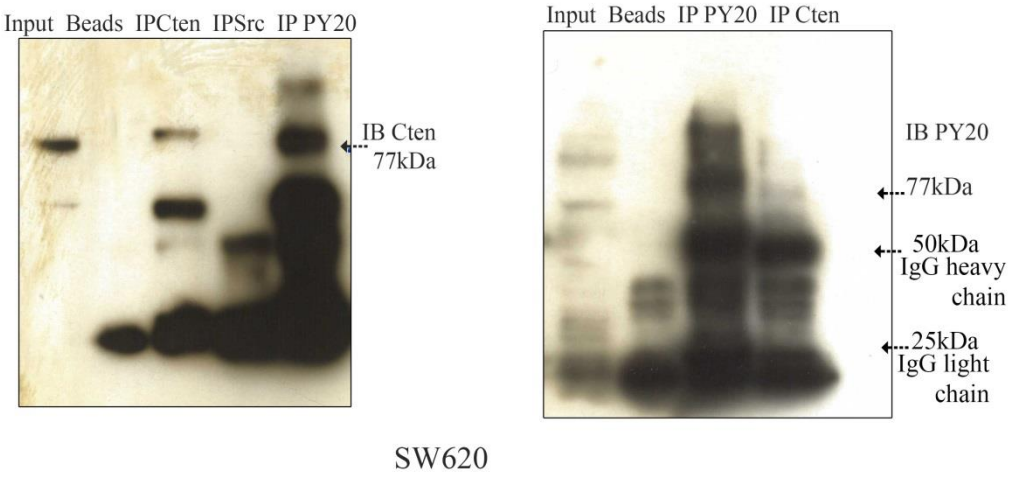
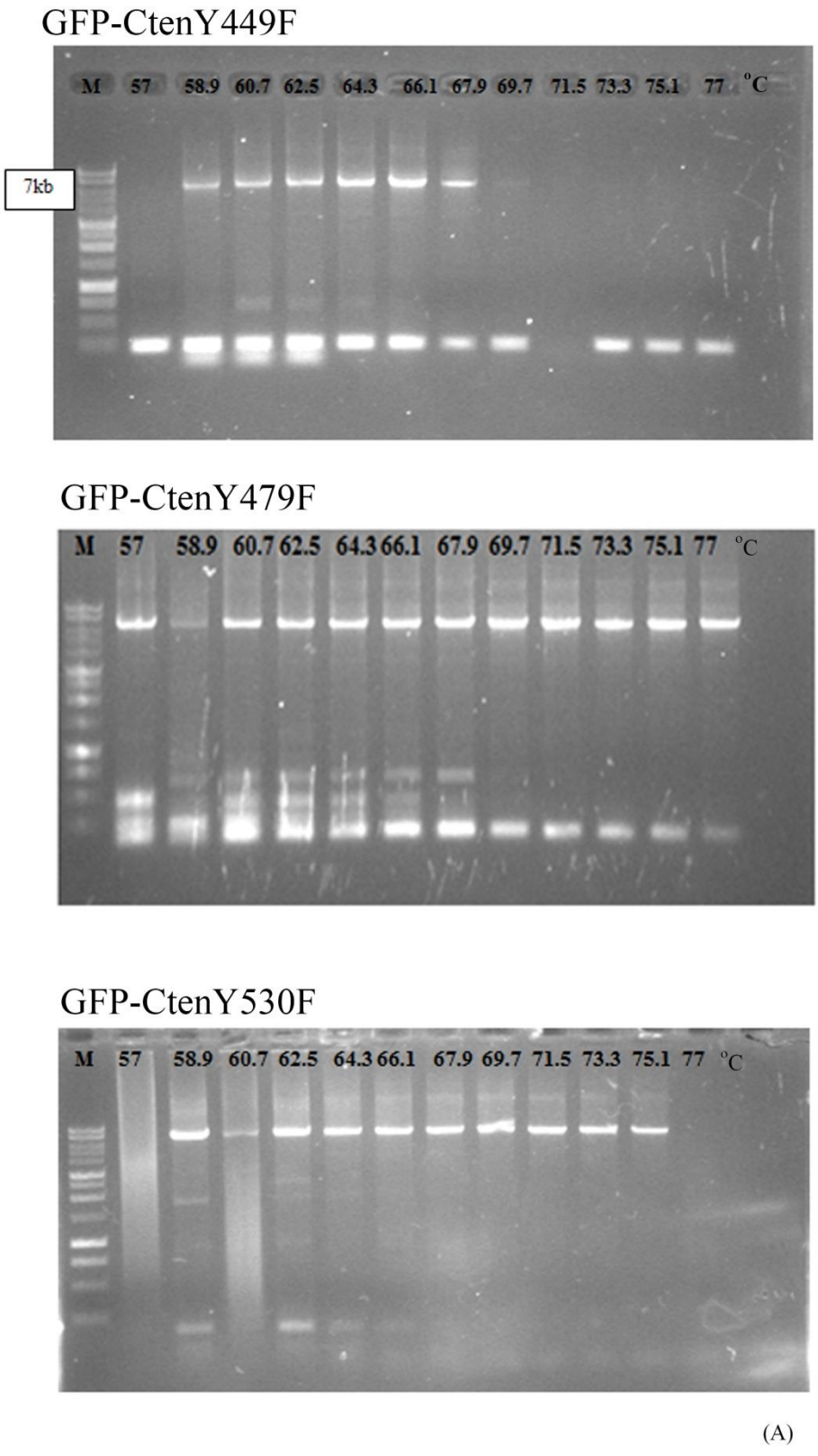


Figure 6-1.Shows western blot of IP Cten and IP PY20 blotted with Cten in SW620. Input serves as positive control and beads represent negative control..The required band of Cten is at 77kDa because of endogenous Cten in SW620. With phosphotyrosine precipitated out and blotted with Cten there is a clear band at 77kDa.

6.3.2 Tyrosine mutant expression vector construction

To find out which Tyrosines were being phosphorylated, we chose three Tyrosines in and around the SH2 domain of Cten. This was a conscious effort to find out if other amino acids besides the critical arginine inside the SH2 domain were responsible for the functional activity of Cten. To make the constructs the same method of site directed mutagenesis was used. The primers were designed with mutations in the middle of the forward primer. For this mutation only one base was changed A→T. Initially there were some problems with the cloning step with random deletions occurring and unexpected amplification of parts of the wild type sequence. We hypothesised that inappropriate homologous recombination was occurring and so two of the primer pairs were changed to make them longer and the mutation site moved away from the middle. A temperature gradient was run as shown in Figure 6-2(A). After this the whole procedure was followed and constructs were sent for sequencing and made sure that all sequence was correct and in the right order.

Figure 6-2(B) shows the comparison between Cten wild type and mutant constructs.



(A)

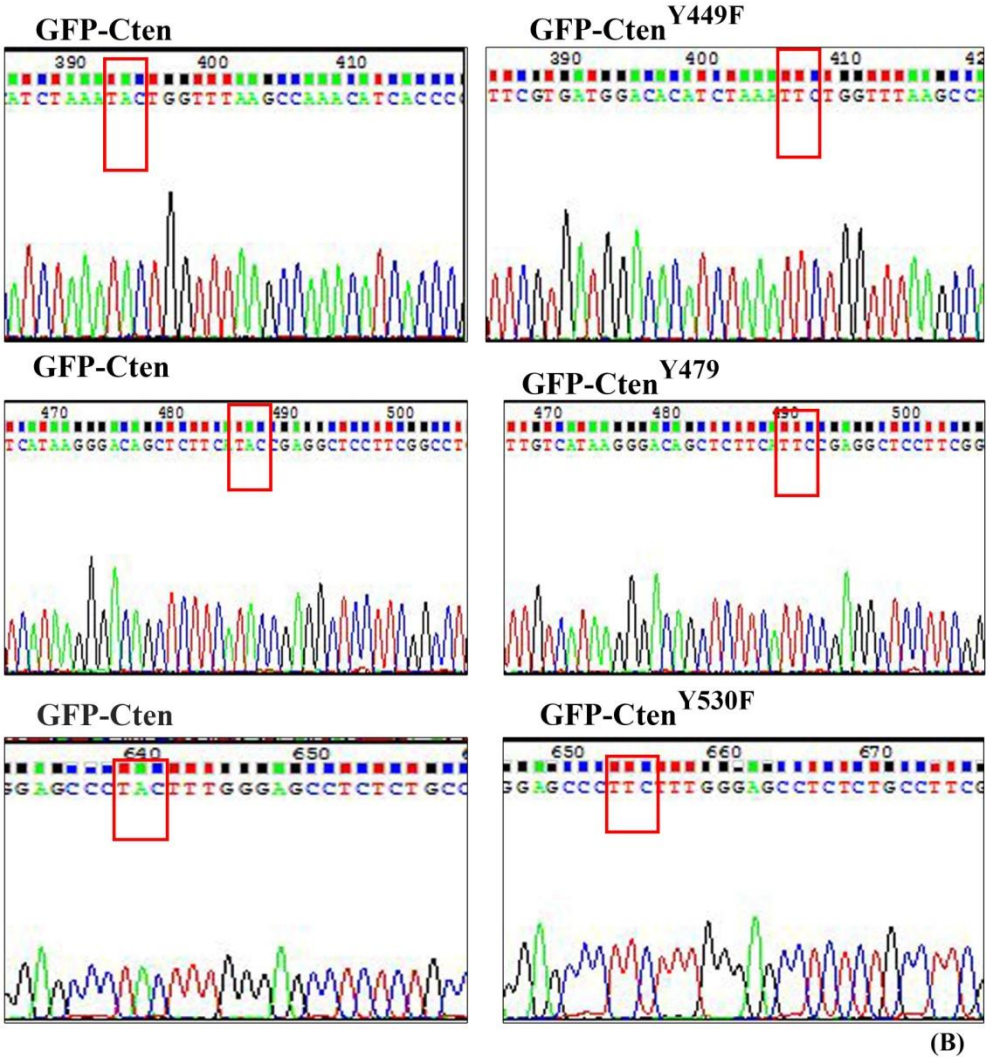


Figure 6-2(A) Pictures of electrophoresis gel run for all three primer pairs for construction of tyrosine to phenylealanine mutants.Gel was run after temperature gradient PCR as the expected product matching the template DNA was 7kb.(B) snapshots of the actual sequencing results obtained from sequencing of all the three mutant constructs.Left side shows GFP-Cten with TAC (Tyrosine). The right side shows these tyrosines changed to Phenylalanine TTC in respective mutant expression constructs.

6.3.3 Tyrosine 449,479,530 are not sole phosphorylation sites in Cten

Once the expression constructs had been validated, they were transfected into HCT116 and lysate was collected. IP was performed with Cten and blotted with antibody against all phosphotyrosines (PY20) and vice versa to see if the mutant expression constructs fail to phosphorylate. Unfortunately all the mutants showed tyrosine phosphorylation. The GFP-Cten^{Y449} didn't show a

high efficiency of transfection as seen in Figure 6-7. It gives a false impression that there is no phosphorylation but there is still a weak band which shows that it is also being tyrosine phosphorylated (Figure 6-3).

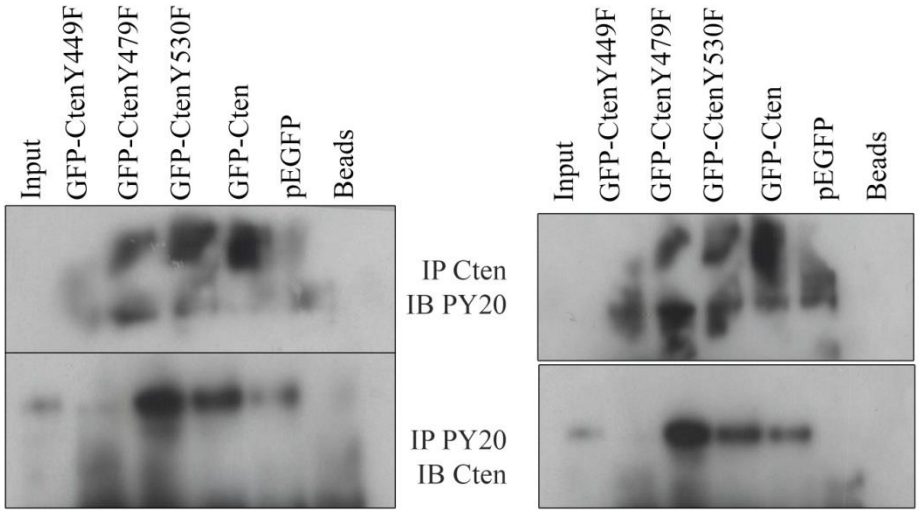


Figure 6-3.Shows IP with Cten and PY20 in HCT116 with forced expression of GFP-Cten and tyrosine mutant constructs. Blotted with PY20 gives smeared pattern of the antibody (top).When blotted with Cten show distinct bands at 110kDa except for a faint band with Y449F (bottom).

There are still a number of other tyrosines with in the 715 amino acid protein of Cten and it would be interesting to identify which tyrosines are being phosphorylated. Furthermore there maybe multiple tyrosines being phosphorylated and mutating just one might not show loss of phosphorylation. In-vitro kinase assay can be used to determine Cten phosphorylation status as well.

6.3.4 GFP-Cten^{Y479F}causes reduction of Cten's effect on motility

To check if any of these tyrosines were important for the cell motility function of Cten we performed functional assays of motility i.e, wound healing and transwell migration. It was interesting to see that GFP-Cten^{Y479F} caused a significant decrease in cell motility when compared with GFP-Cten in both cell

lines RKO (p=0.005) (Figure 6-4) and HCT116 (p=0.009) (Figure 6-5) in wound healing. Similar results were seen in transwell migration assay in HCT116 (p=0.0029) and RKO (p= 0.02)(Figure 6-6 A). To rule out an effect of cell proliferation by GFP-Cten^{Y479F} we performed presto blue assay to measure cell numbers. It was clear from the presto blue assay that there was no effect on proliferation (Figure 6-6B).

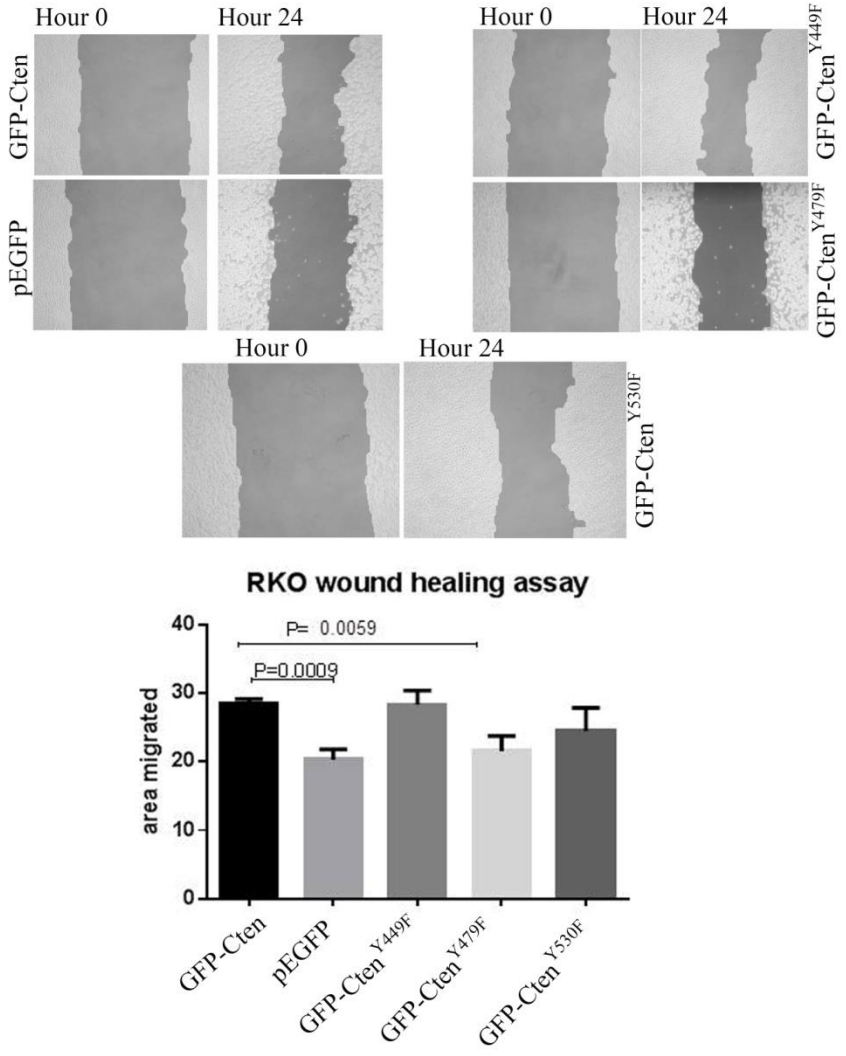


Figure 6-4.Wound healing assay in RKO with all three tyrosine mutansts, GFP-Cten and pEGFP (empty vector). The top shows actual pictures of the wounds scraches made in 6 well plate at 0 hour and same area after 24 hours to look at the distance (area) travelled by the cells. Bottom is the graphical represenation of the area travelled by cells in triplicate showing a significant difference between GFP-Cten and GFP-Cten^{Y479F} construct as well as GFP-Cten and pEGFP.

Unexpectedly GFP-Cten^{Y449F} showed a paradoxical effect of increase in cell motility more than GFP-Cten itself. This warrants further work up by optimising its transfection efficiency and studying effect on cell motility.

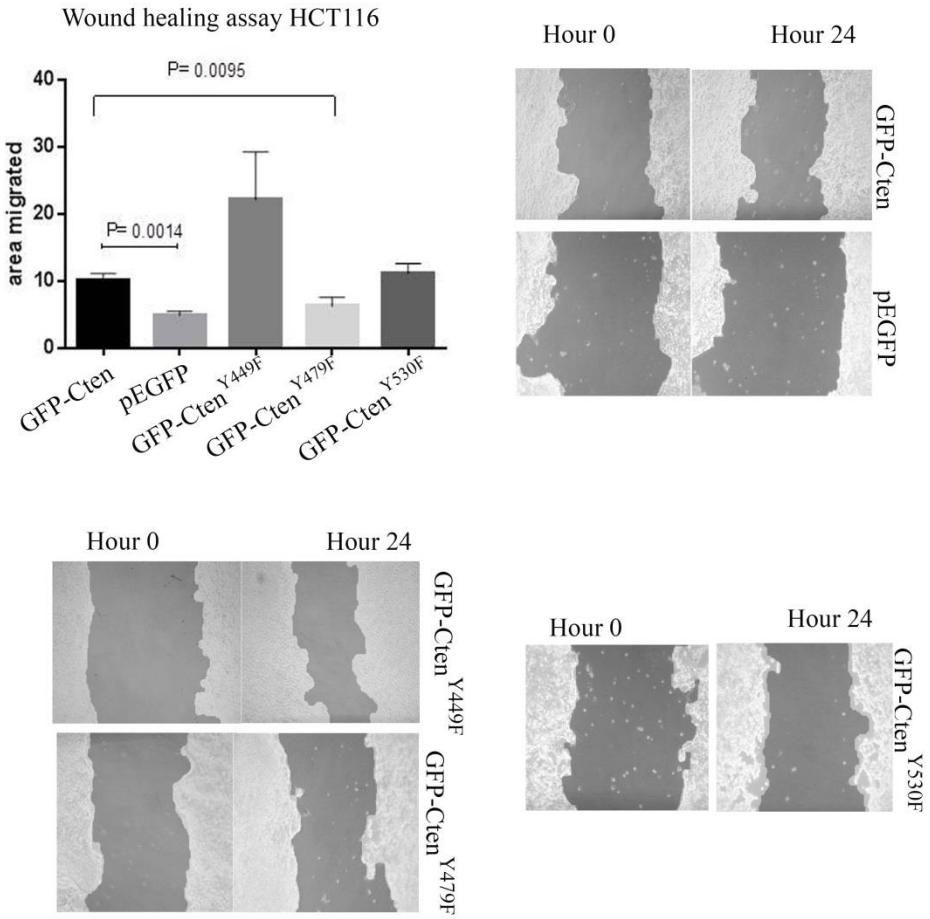


Figure 6-5.Wound healing assay in HCT116 with all three tyrosine mutansts, GFP-Cten and pEGFP (empty vector).This figure shows actual pictures of the wounds scraches made in 6 well plate at 0 hour and same area after 24 hours to look at the distance (area) travelled by the cells and graphical represenation of the area travelled by cells in triplicate showing a significant difference between GFP-Cten and GFP-Cten ^{Y479F}

construct as well as GFP-Cten and pEGFP.

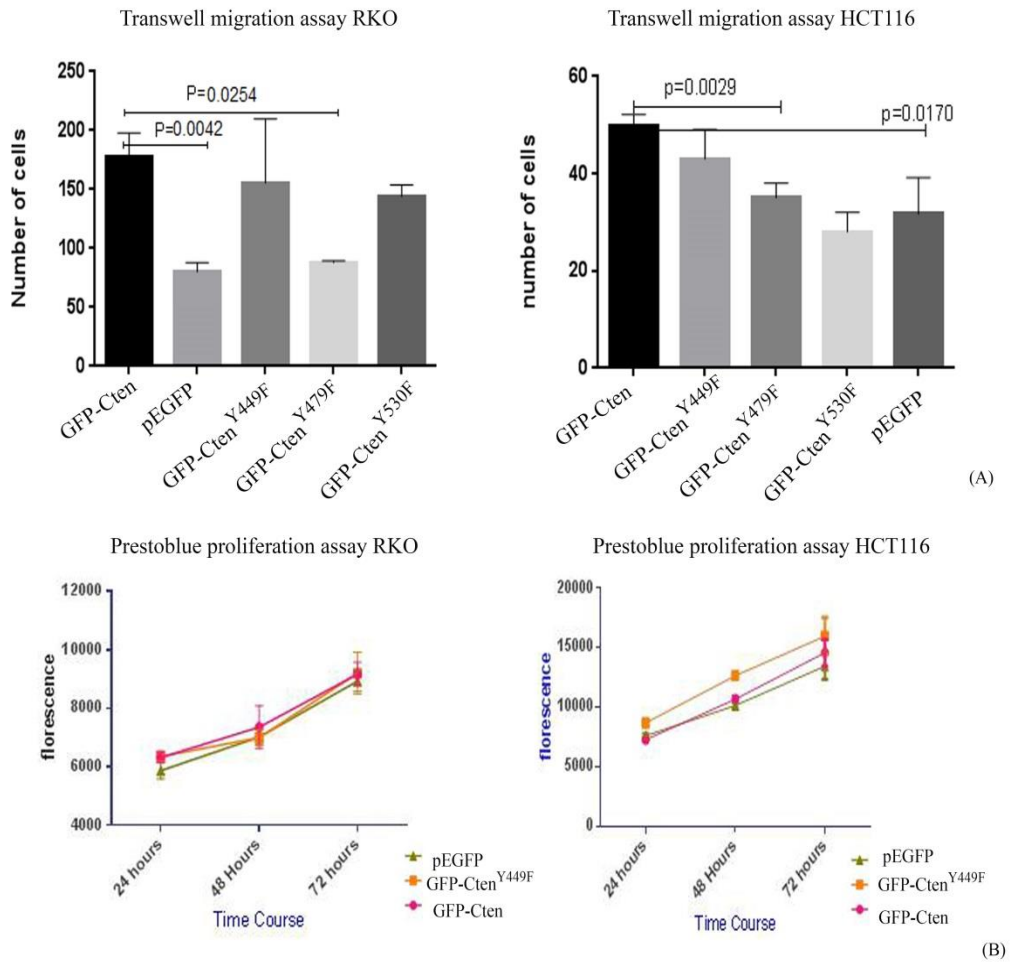


Figure 6-6.(A)Shows graphical representation of the number of cell migrated after 24 hours in RKO and HCT116 after forced expression of GFP-Ctenand all three mutant constructs and pEGFP. It There is a significant difference between GFP-Cten and GFP-Cten^{Y479F} as GFP-Cten^{Y479F}causes a significant decrease in cell motility compared to GFP-Cten in both cell lines. (B) Presto blue proliferation assay in HCT116 and RKO showing no significant effect on cell proliferation byGFP-Cten^{Y479F}.

6.3.5 Tyrosine mutant constructs effect Src and Paxillin levels

To see whether the Tyrosine mutants were the same weight as the template GFP-Cten, we ran a western blot with all three mutants, GFP-Cten and pEGFP empty vector blotted for Cten antibody. As seen in Figure 6-7 all mutants run at the same levels as GFP-Cten (110 kDa). GFP-Cten^{Y449F} was seen as a faint band as compared to others that could be because of poor transfection efficiency. As Src was identified as a possible target of Cten in the previously we looked at its levels in both HCT116 and RKO. It was seen that GFP-Cten

expression increases Src levels as compared to pEGFP which is in accordance with our previous findings in SW620. The mutant GFP-Cten^{Y449F} and GFP-Cten^{Y479F}caused a decrease in the Src levels as compared to GFP-Cten. Whereas there wasn't a very major difference in levels of Src in GFP-Cten^{Y530F} compared with GFP-Cten. It was apparent from the functional assays that GFP-Cten^{Y449F} and GFP-Cten^{Y530F} do not affect the cell motility function of GFP-Cten, it was decided to studyGFP-Cten^{Y479F}expression construct only for further western blots of target proteins.

Src levels were induced with GFP-Cten but they went down with GFP-Cten^{Y479F} mutant in HCT116 and RKO (Figure 6-8). GFP-Cten causedreduction in Paxillin levels which is similar to the effect seen in SW620. But this wasn't the case inRKO, where there was no change in paxillin levels (Figure 6-9). GFP-Cten^{Y479F} did not have any effect on Paxillin levels.

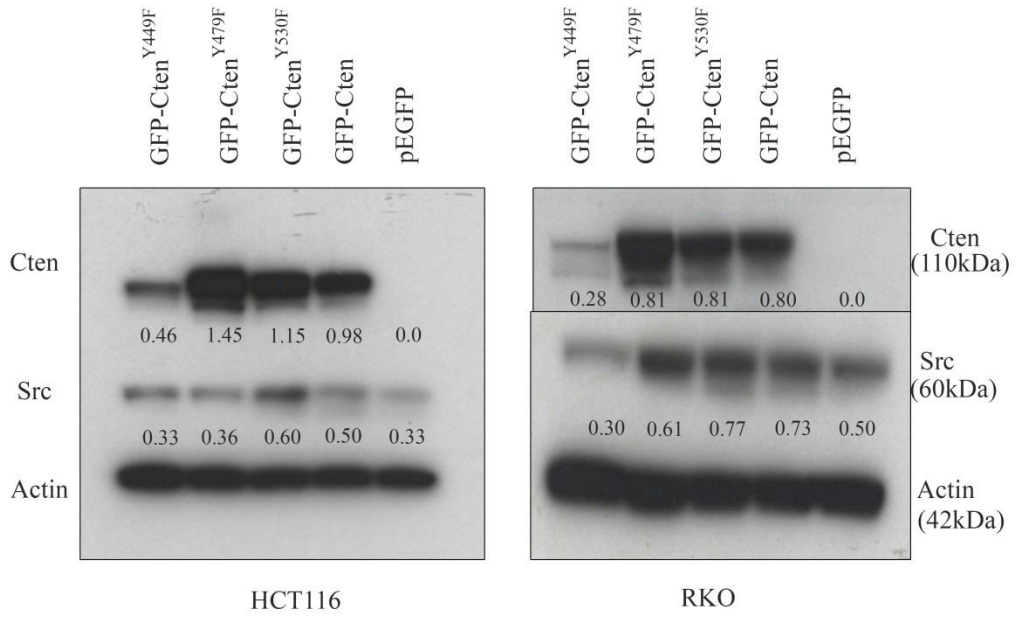


Figure 6-7.Western blot for Src levels. Western blot of all tyrosine mutants, GFP-Cten and pEGFP with Cten antibody showing bands at the right size of 110kDa Blot for Src for all mutants, GFP-Cten and pEGFP show a difference in levels. After densitometry and normalisation to Actin loading control, Src levels show an increase in levels with

GFP-Cten, GFP-Cten^{Y530F} as compared to pEGFP in both HCT116 and RKO. Whereas GFP-Cten^{Y479F}andGFP-Cten^{Y449F} cause a reduction in Src levels comapred to GFP-Cten.

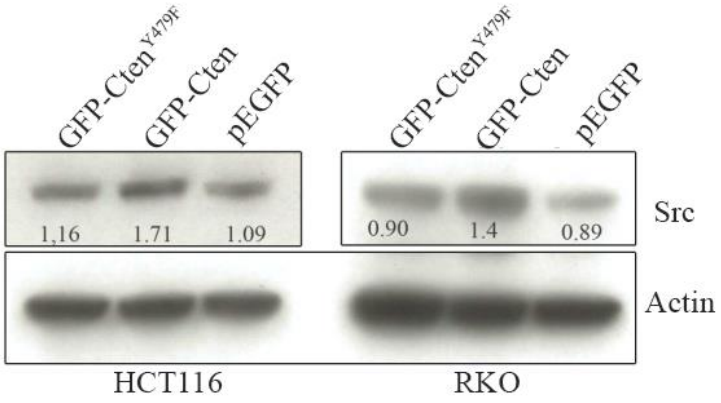


Figure 6-8.Western blot for Src and paxillin in HCT116 and RKOA repeat western blot for Src levels in HCT116 and RKO using forced expression of GFP-Cten^{Y479F}, GFP-Cten and pEGFP only shows an increase in levels of Src with GFP-Cten which is reversed by GFP-Cten^{Y479F} in both HCT116 and RKO. The numbers at the bottom of the blot are the calculations of densitometry after normalisation to actin control.

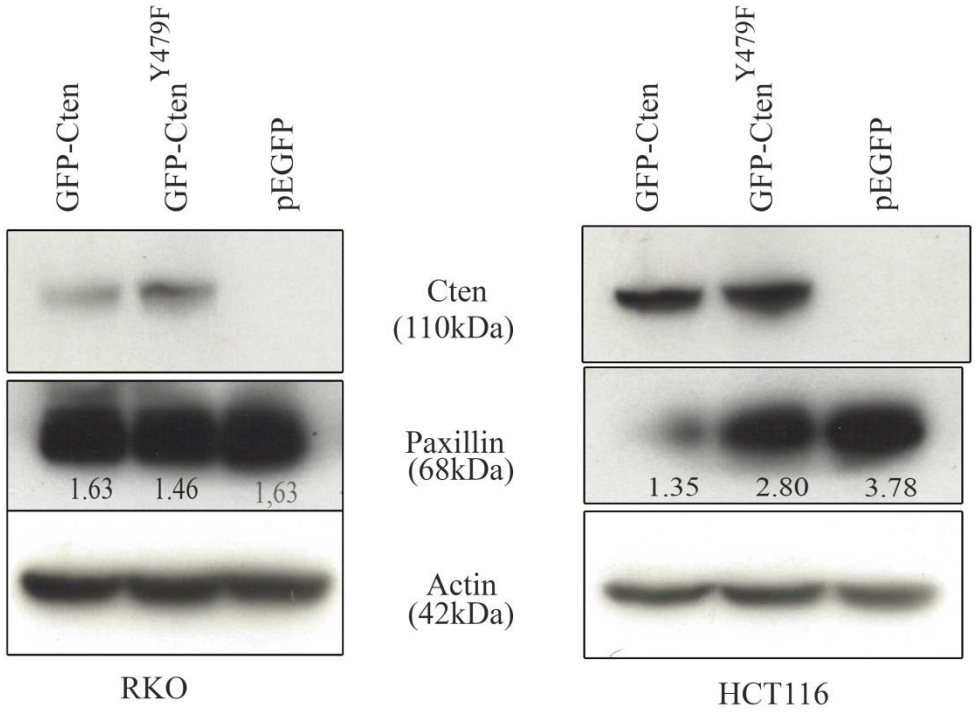


Figure 6-9.Western blot for Paxillin in HCT116 and RKOafter forced expression of GFP-Cten, GFP-CtenY479F and pEGFP. Numbers at the bottom of the bands show the levels

of Paxillin after densitometry. RKO shows no considerable differnce in levels whereas in HCT116 there is a decrease in Paxillin after GFP-Cten expression which is lost with GFP-CtenY479F and pEGFP.

6.3.6 GFP-Cten^{R474A} causes more effect on Src, Paxillin and β-catenin levels than the GFP-Cten^{Y479F} mutant

To investigate the mechanism through which GFP-Cten^{Y479F} was impeding the activity of Cten on motility, we looked at the new downstream targets identified in the previous chapter e.g. Src and paxillin. We saw in HCT116 and RKO there was a slight decrease in Src levels with GFP-Cten^{Y479F}as compared to GFP-Cten. Paxillin however, showed varying results. In HCT116 it followed the pattern shown in SW620 but in RKO there was an increase in paxillin levels. To check these levels against the GFP-Cten^{R474A} we repeated the western blots with Src, paxillin and we included β-catenin this time. β-catenin is only present in HCT116 out of the three cell lines being used. So it was only tested in HCT116. It is a known target and binding partner of Cten in nucleus. It was seen that Src, paxillin and β-catenin levels drastically went down with GFP-Cten^{R474A} in HCT116 as seen in Figure 6-10. Similar effect was seen in RKO but the only discrepancy was in levels of paxillin protein which went the opposite way (Figure 6-10).

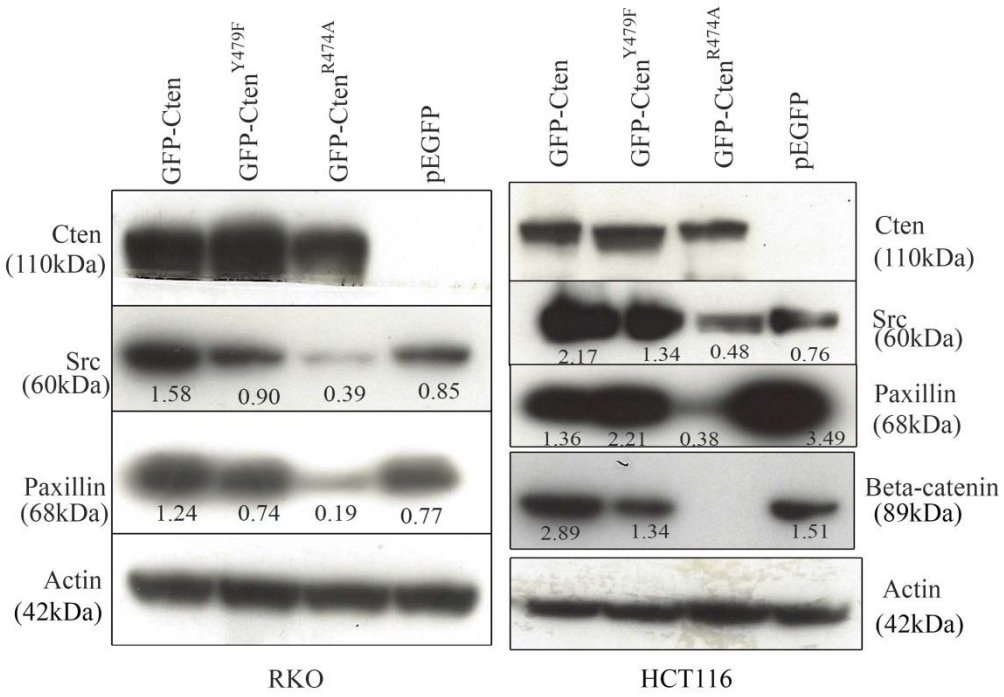


Figure 6-10.western blot for Src, Paxillin and β-catenin levels in HCT116 and RKO(there is no β-catenin expression in RKO) after expression of GFP-Cten, GFP-Cten^{R474A}, GFP-Cten^{Y479F} and pEGFP. Numbers at the bottom of the bands reflect the levels of respective proteins after densitometry calculations. In HCT116 and RKO Src levels were induced with GFP-Cten expression and reduced with GFP-Cten^{Y479F}and GFP-Cten ^{R474A}. However, GFP-Cten ^{R474A}causes a more dramatic reduction in levels. β-catenin levels in HCT116 go up with GFP-Cten expression but fall drastically withGFP-Cten ^{R474A}. Paxillin levels go down with GFP-Cten expression in HCT116 while not in RKO. GFP-Cten ^{R474A}expression causes a decrease in Paxillin levels in both cell lines.

6.3.7 FAK has a negative effect on Src levels

In previous chapter we have shown that Cten positively regulates FAK and here we show that Cten positively regulates Src. There is also a link between FAK and Src reported in literature(120,121). We decided to investigate whether Cten induces Src through FAK. Cten was forcibly expressed and FAK knocked down simultaneously. Unexpectedly FAK seems to have a negative effect on Src. Knocking down FAK caused levels of Src to go up in HCT116 and RKO when co-transfected with Cten as shown in Figure 6-11. It was more pronounced in RKO then HCT116. The FAK knockdown doesn't look convincing enough but it is probably due to GFP-Cten causing an increase in its levels.

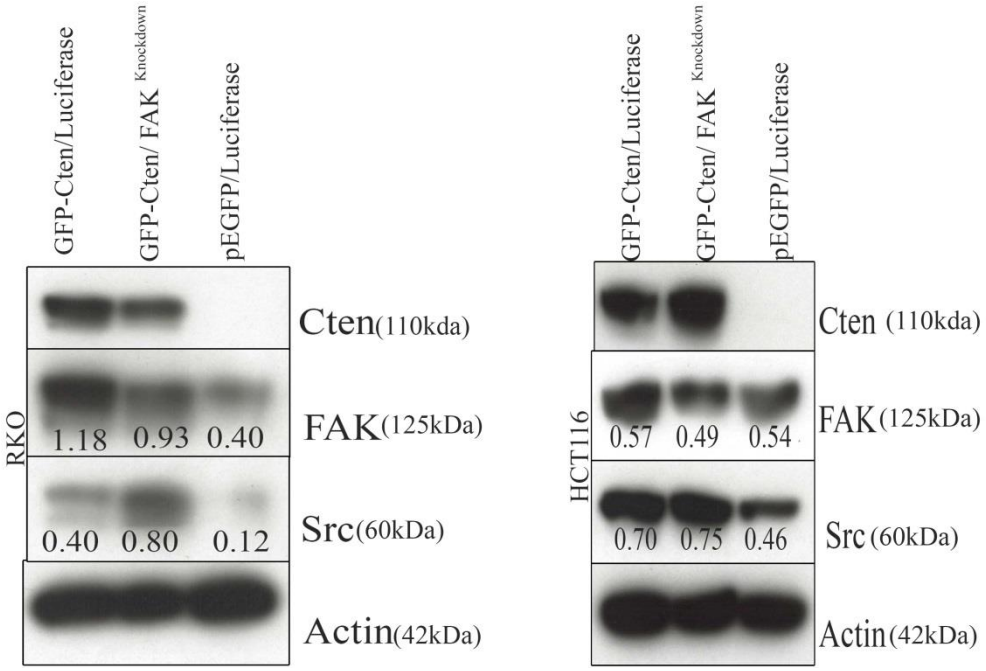


Figure 6-11.Western blot of cotransfection of GFP-Cten with knock down of FAK to look at levels of Src in HCT116 and RKO.Numbers at the bottom of the bands show levels of FAK and Src after density calculations. Src levels go up drastically in RKO and slightly in HCT116 after knockdown of FAK.

6.3.8 Cten does not bind to Src, while FAK binds to Src

We have seen that Src levels seem to go up with GFP-Cten expression and go down with mutations in the SH2 domain. We decided to look at the physical interaction between the two. On whole cell extracts it was seen that there was no interaction between the two after repeating IP in both directions i.e., IP with Src and blotting with Cten and vice versa (Figure 6-12). We looked at IP of Cten and Src in SW620 as well which yielded negative result (Figure 6-13)

As we have seen that FAK binds to Cten. We decided to see if FAK binds to Src because there is a positive physical interaction reported between the two in literature(30). It was seen that FAK does bind to Src (Figure 6-14) but we cannot be certain whether there is a tri- molecular binding among Cten, FAK and Src, as we didn't demonstrate a binding between Src and Cten but it is a possibility. As it is already known that Cten binds β-catenin by Liao etal (6) so

an additional IP of FAK and β-catenin was also performed to see whether FAK and β-catenin bind together and may be Cten, FAK and β-catenin form a tri-molecular bond. But FAK and β-catenin didn't show any binding (Figure 6-14).

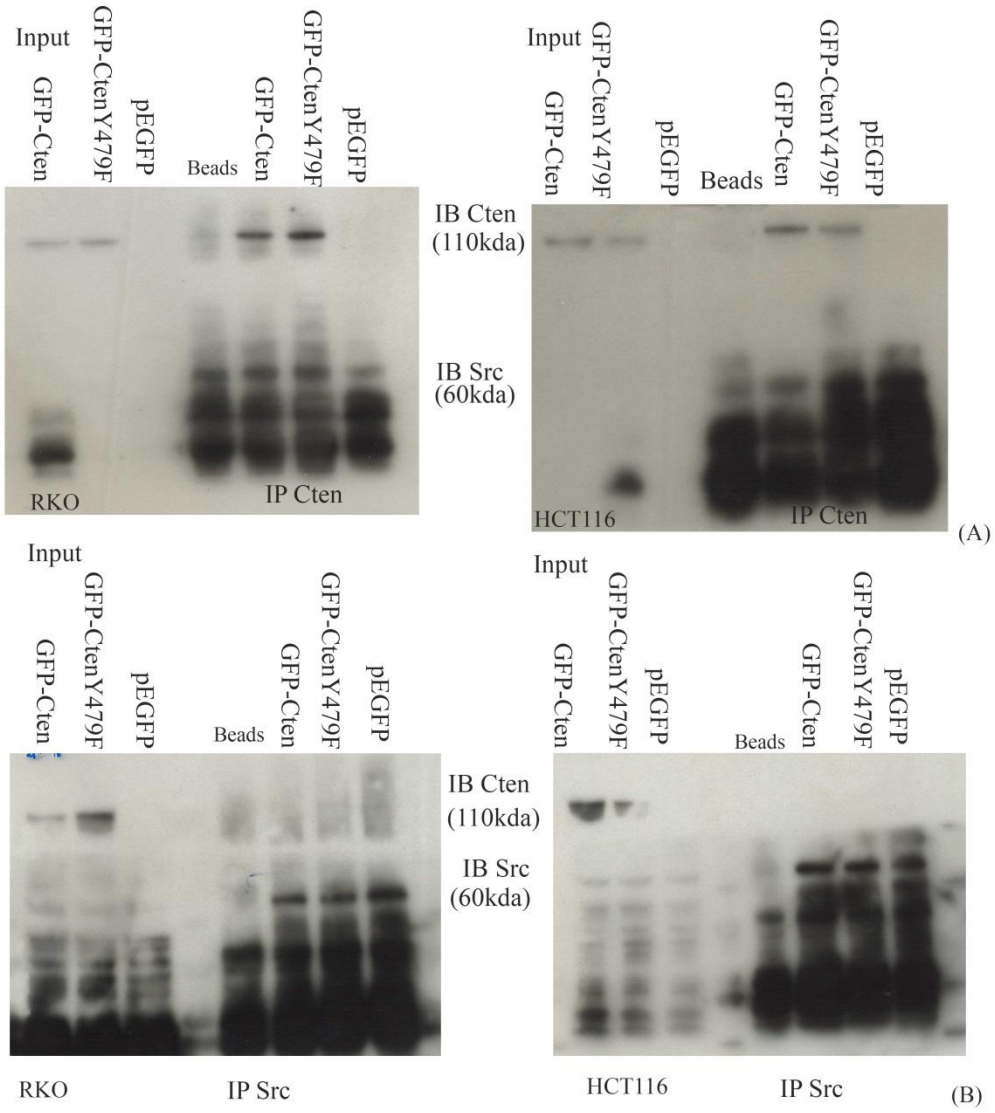


Figure 6-12.IP of Cten and Src(A) shows westernblot in RKO and HCT116 with IP of Cten blotted with Cten and Src after transfection with GFP-Cten, GFP-Cten ^{Y479F} and pEGFP. Cten IP gives bands at right size of 110kDa. But no visible bands for Src at 60kDa. Multiple bands at the bottom of the plot represent heavy and light chain of IgG coated on to the beads. (B) western blot for IP with Src in RKO and HCT116 blotted with Src and Cten. Input is positive for both antibodies however faint with Src. There are no bands when blotted with Cten in IP of Src.

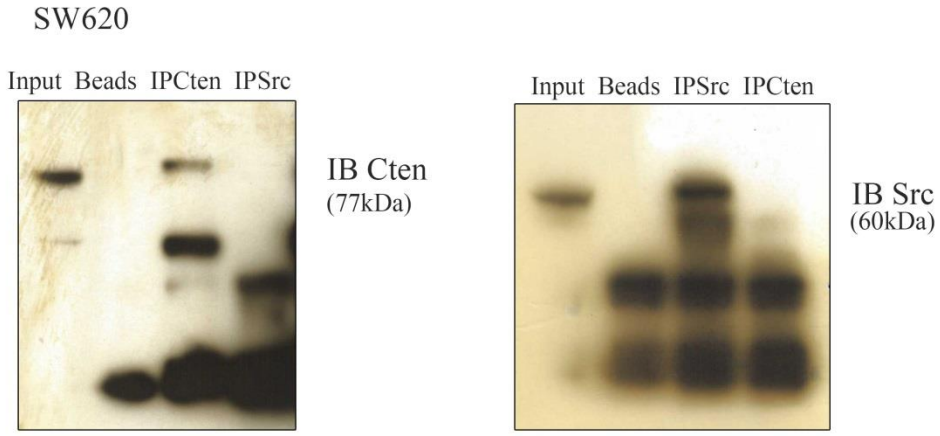


Figure 6-13.Immunoblot of Cten and Src in SW620 with IP of Cten and Src blotted with Cten and Src antibodies respectively. Input gives bands for both Cten and Src, the beads (negative control) lane is empty. There is no band visible in IP Src blotted with Cten and IP Cten blotted with Src showing no physical interaction endogenously.

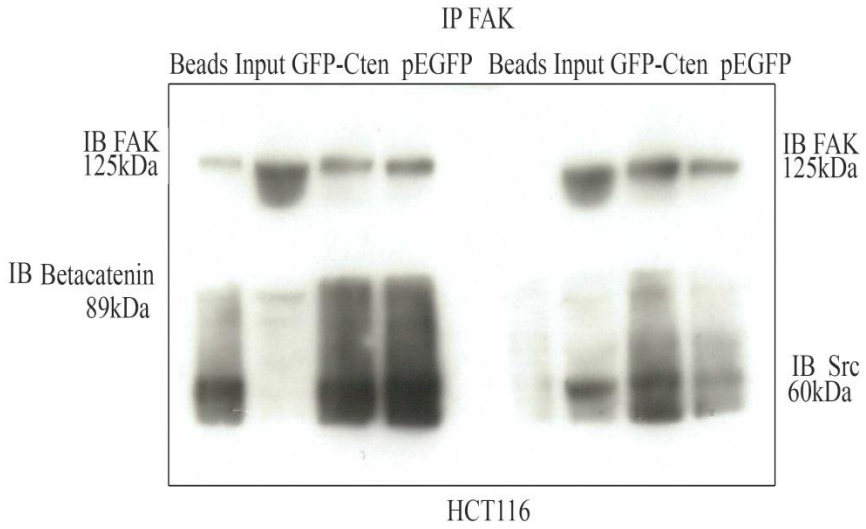


Figure 6-14.western blot for immunoprecipiation of FAK in HCT116 after transfection with GFP-Cten and pEGFP.Same blot is botted for FAK to check efficency of IP and blotted for β-catenin (left) giving a band at 89kDa in input but no vibily clear bands with GFP-Cten and pEGFP expession and IP with FAK. (Right) immunoblotting with Src gives bands at 60kDA in input and IP with FAK in both GFP-Cten and pEGFP expression.

6.4 Discussion

In this study we have shown for the first time that Cten is being tyrosine phosphorylated in colorectal cancer cells. However we could not find the exact tyrosines which are phosphorylated. As there are multiple proposed phosphorylation sites in Cten which are independently phosphorylated(12).

Thus even if one is knocked out the others still get phosphorylated. This could be one reason why we were unable to show which tyrosine was getting phosphorylated. So the tyrosines within the SH2 domain of Cten are either not being phosphorylated or are one of the many which are phosphorylated as their mutation did not affect tyrosine phosphorylation of Cten. This is in contrast to a study done in tensin 3 where residues Y1173, Y1206, and Y1256, corresponding to our Tyrosine 449, 479 and 530 through mutational analysis were shown to be necessary for SH2-PTB domain phosphorylation and critical for binding FAK, p130cas, Src, sam68, ILK and DLC1 although DLC1 and ILK were less tyrosine phosphorylation dependant (122). In our study we did not find a physical interaction between ILK and Cten as previously shown. In this chapter we looked at Src and Cten immunoprecipitation and found no interaction. However, we did find that GFP-Cten increases levels of Src and GFP-Cten^{Y479F} causes a decrease in its levels. In the tensin3 study they also claimed that these residues are important in regulating adhesion and migration. In our study we found only the GFP-Cten^{Y479F} to be responsible for regulating cell motility function of Cten. In the same study mentioned earlier it was seen that Tensin 3 was phosphorylated by Src which is something we didn't look at in our study and is something that should be explored further(122). There are other studies about growth factor EGF causing tyrosine phosphorylation of Tensin 3 and Tensin 1 as well (11,123). However the tyrosine phosphorylation status of Cten had not been studied till now.

We also looked at levels of Paxillin and β-catenin. There are two cell lines in which Cten appears to inhibit paxillin (HCT116) using different. However in RKO cell line there was no effect seen. It can be concluded that Cten is a

negative regulator of paxillin. β-catenin is only expressed in HCT116, where its levels were inducedwith GFP-Cten expression and reduced with GFP-Cten^{Y479F}and completely vanish with GFP-Cten^{R474A}. It could be that GFP-Cten^{R474A} is causing a degradation of β-catenin. β-catenin is known to be physically bound to Cten in HCT116 in the nucleus but its levels haven't been reported upon(6). Here it is noteworthy that levels of Src, β-catenin and Paxillin were maximally affected with GFP-Cten^{R474A}rendering it more important in the signalling function of Cten as compared GFP-Cten^{Y479F}. But the effect on motility was significant with both these mutations. It could be argued that GFP-Cten^{Y479F} might be important for signalling through downstream targets just as GFP-Cten^{R474A} but because of its position in the SH2 domain may affect its specificity of binding leading to change in its function. This has been described for Threonine residue in Src(118). In other studies it has been shown that some amino acids which might be irreplaceable in certain SH2 domains may not be as important in others for their function(124).

As we know that FAK binds to Src, which we have also shown through IP in our study and FAK is bound to Cten. We have explored binding of Cten with Src but this interaction hasn't emerged. It is known that FAK after its auto phosphorylation into an active state causes activation of Src at the focal adhesions and also its phosphorylation(30). Cten has emerged as upstream to FAK in our study previously and increasing the level of FAK as well as the activated FAK. Here we show it also induces level of Src leading us to believe that it is effecting Src levels via FAK. But when FAK was knocked down after

Cten overexpression Src levels went up suggesting a negative effect of FAK on Src which hasn't been reported in literature.

A contrasting effect of Cten on paxillin in HCT116 and RKO was seen in this study. However action is consistent between HCT116 and SW620 using two different methods i.e. Cten inhibits levels of paxillin. Paxillin is a part of focal adhesion and said to bind to FAK. There are contrasting studies about the function of paxillin in motility(125) and we haven't seem to identified any concrete evidence of paxillin in colorectal cancer cell lines either.

In summary we have identified that Cten is tyrosine phosphorylated and Y479 in the SH2 domain is important for cell motility, however, it isn't as effective on the downstream targets as R474. Further experiments are warranted to pinpoint the exact nature of its role on Cten's function.

7 CD24 and other molecules interacting with Cten

7.1 Introduction

One of the interesting things to come out in recent years regarding signal transduction is the concept of lipid rafts(126). Lipid rafts consist of dynamic microdomainsin the cell membrane, enriched in sphingolipids and cholesterol.(127). The most important property of lipid rafts is that they can include or exclude proteins, acting as signaltransduction platforms. The proteinswith raft affinity include, glycosylphosphatidylinositol (GPI) anchored proteins, Src- family kinases, cholesterol binding protein, like caveolins and hetrodimeric G proteins etc. (126-128).Among the (GPI) anchored proteins with raft affinity, CD24 is an emerging new molecule.

CD24 has been identified as a marker for poor prognosis in several tumours, like ovarian cancer (129), breast (130), pancreas(131), non-small cell lung cancer(132) and colorectal cancer(133).It has been suggested that CD24 acts as a gate keeper for lipid rafts. It exerts its function by regulating the entry of different proteins in lipid rafts, which serve as signalling platforms. CD24 is a stimulator of transmigration and invasion, as is seen by CD24- mediated increase in cell migration, by controlling the lateral localisation of β1 integrin within lipid rafts (134).CD24 was discovered in mice by Springer (135).In humans it's found in a variety of haematopoietic and non-haematopoietic cells including cancer cells(136,137). It islocated on chromosome 6q21.And the gene codes for a heavily glycosylated cell surface protein that is linked to membrane by a GPI anchor (138,139).CD24 protein has a molecular weight ranging from 20-70 kDa, owing to its variable glycosylation in different cell types as reviewed in (136).

CD24 mediates signal transduction by recruiting Src family kinases through the lipid rafts and activates mitogen activated protein kinase pathway in B and T cells(136).It is also involved in cell motility, tumour progression and metastasis. Several Phosphorylated proteins have been seen to co-immunoprecipitate with CD24 (140). It is also seen that CD24 causes increased activity of Src and recruits β1 integrins into lipid rafts (141). CD24 also stabilises and phosphorylates FAK, which been associated with cell adhesion, motility and migration (125,142). CD24 is a GPI anchored protein and therefore does not extend into the cytoplasm although there is evidence that these molecules can transduce signals to the nucleus (140). In our group we have also shown that CD24 shows immunostaining in the nucleus in colorectal cancer(143). Given the effect of CD24 on cell motility, there may be a link between CD24 and the intracytoplasmic actin cytoskeleton machinery. Since Cten attaches to β1 integrin and there is a link between Cten, FAK and Src shown in our study. We there may be a link between Cten and CD24. Given the similarities between CD24 and Cten we hypothesised that Cten might mediate its function through Cten. In addition in this chapter we look at various molecules within focal adhesion apparatus and their binding with Cten.

7.2 Aims

I. To look at the effect of Cten on CD24 and vice versa

II. To look at binding of Cten with proteins identified as targets in this study in endogenous state.

7.3 Results

7.3.1 CD24 is a positive regulator of Cten

Our group has preliminary data showing CD24 may be a regulator of Cten. In order to confirm this and ascertain whether CD24 mediated its function through Cten, we sought to forcibly express CD24 and concomitantly knock down Cten. Cten levels were seen to go up in HCT116 following CD24 transfection thus confirming that it is a regulator of Cten Figure 7-1. However, no Cten protein was seen on western blot in RKO. The reason could be that RKO has absolutely no endogenous Cten. HCT116 however does express very low levels of Cten. As Src is found to be a potential downstream target of Cten we decided to look at its levels as well. Here we see that Src levels are almost similar in CD24/GFP-Cten- and CD24/GFP-Cten. In RKO although there was no Cten expression seen the Src levels still show an increase with CD24 levels (Figure 7-1). CD24 plasmid and its empty vector has been used before by our group and shows good transfection efficiency(144).

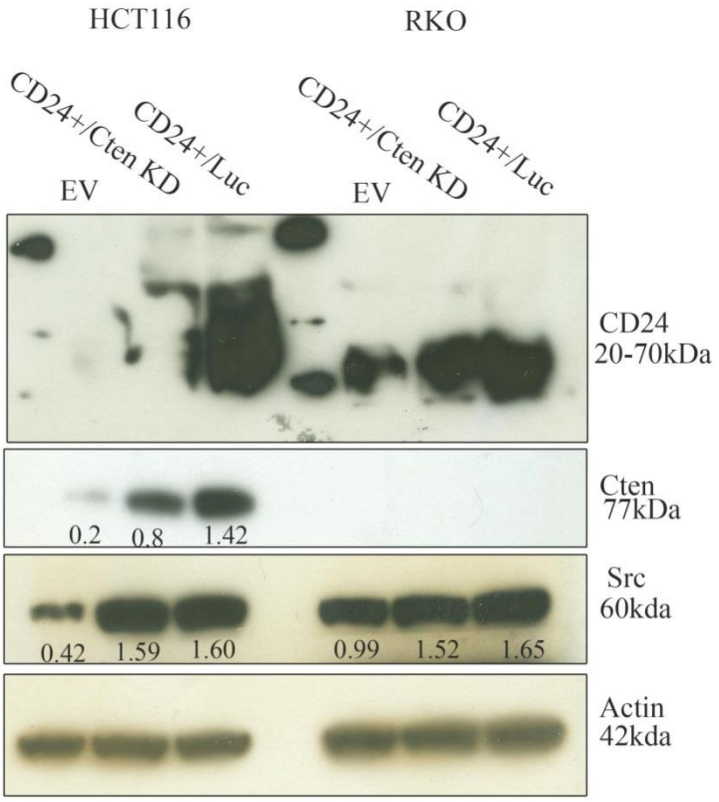


Figure 7-1.Western blot in HCT116 and RKO. Cotransfection of CD24and knock down Ctenwas perfomed with EV(empty vector) and Luciferase (Luc) as controls. Cten expression go up with CD24 forced expression. Src levls also mimick levels of CD24. Note a big airbubble in the CD+/CtenKD lane in HCT116.Cten knockdown is not complete but this cab be the result of upregulation of Cten byCD24. CD24 levels in the top blot are variable due to inconsistent transfection effieciency during cotransfection.

7.3.2 Cten physically binds with β-integrin in the endogenous state in SW620

A variety of molecules were identified and studied as potential targets of Cten in this study. To explore whether they physically bind to Cten we used a colorectal cell line SW620 with high levels of Cten endogenously. A number of immunoprecipitation experiments were performed with one protein being precipitated out and blotted with antibody against the same protein to confirm IP and blotted against another antibody/protein of interest to confirm their physical binding together. Out of all the experiments performed a few are

shown in Figure 7-2 and Figure 7-3. As it is commonly known that tensin gene family genes bind to the intracytoplasmic tail of β1-integrin making them part of the focal adhesion complex. We decided to do an IP with β1-integrin. It only worked when Cten was precipitated out and blotted with β1-integrin and not the other way around and that too gave a faint band as shown in 7-2(A). FAK is seen to bind Cten when it's over expressed by transfection with Cten GFP plasmid. However when IP was performed in SW620 there was no interaction seen. IP was performed both ways by blotting with FAK and Cten after both proteins were successfully precipitated out (Figure 7-2 B).

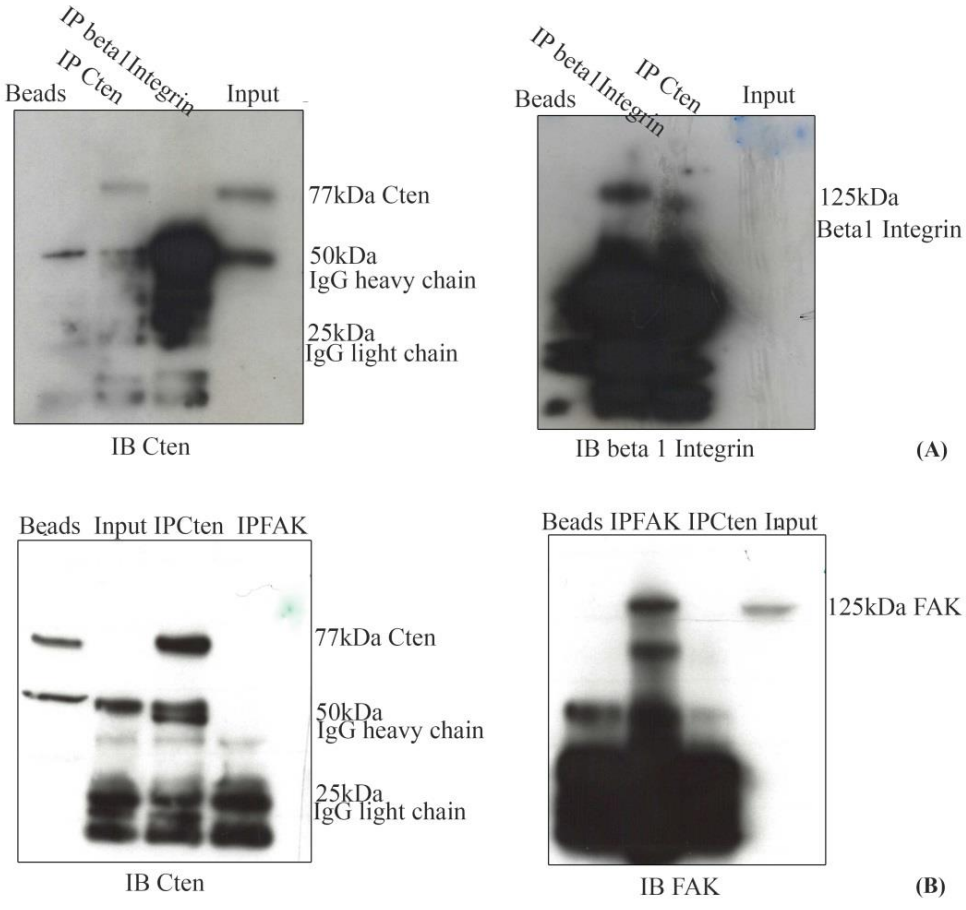


Figure 7-2.IP of Cten with β1-integrin and FAK in SW620. (A) IP with Cten and β1-integrin showed physical binding only in one direction when Cten was immunopreciptated out and blotted with β1-integrin (faint band was seen which is of suboptimal quality and needs further work). (B) IP with FAK and Cten blotted with same antibodies did not show any physical binding.

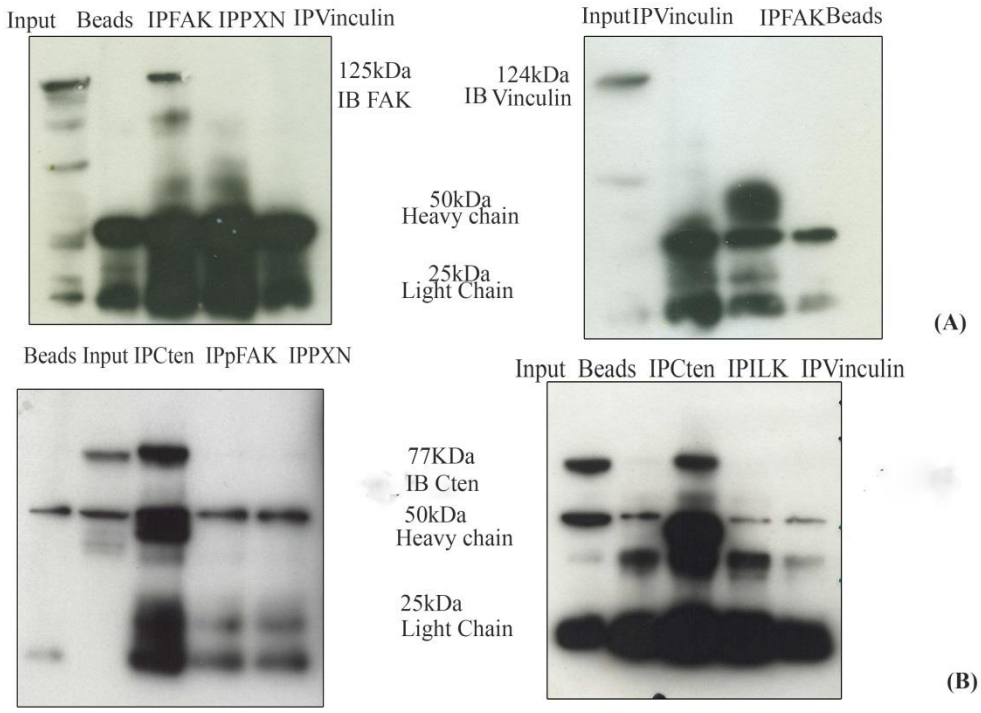


Figure 7-3.IP of Cten and FAK with Paxillin (PXN) and Vincullin in SW620.(A) Western blot of IPFAK, IPPXN and IPVinculinblotted with FAK shows no physical binding. Similarly IP of FAK and immunoblotted with Vincullin shows no binding (B) IP of Cten, PXN, pFAK, ILK and Vinculin blotted with Cten shows no physical interaction.

IP of Cten with Paxillin, ILK, Vinculin and pFAK(Tyr 397) as also performed but there was no physical interaction identified as shown in Figure 7-3 (B). IP of FAK with Paxillin was also performed, as it is a well-known fact that FAK and Paxillin bind together at the focal adhesions but this interaction was not found in our set of experiments (Figure 7-3). It could very well be because levels of endogenous Cten and other proteins are not enough to elicit a positive binding which is not a problem when Cten is being forcefully expressed by transfection. This reason can be backed by the fact that the concentration of antibodies required to get a positive band in western blot for Cten in SW620 is higher as compared to HCT116 where Cten is overexpressed by transfection.

7.4 Discussion

CD24 is known to increase cell migration, invasion and colony formation in colorectal cell lines (144,145). It has been shown in to effect proliferation, cell

adhesion and migration in a number of other tumour cell lines including ovarian, lung cancer, pancreatic and glioblastoma (146). As the positive effect of CD24 on cell motility has already been established by our group and other studies we looked at the relationship between Cten and CD24. Cten is also a positive regulator of cell motility, colony formation and invasion in colorectal cancer. Cten is seen to be a downstream target of Kras and CD24 and Cten form a part of the same signalling pathway(147).

In our study we have shown that Cten is a downstream target of CD24 in HCT116. This is a just a reiteration further establishing the fact that Cten is affected by CD24. Cten is also shown to bind to β1 integrin. As CD24 is known as a "gatekeeper" of lipid rafts and it has been shown that it recruits β1 integrin to lipid rafts (134,139). It could be a mechanism by which Cten is also recruited within the rafts and could be a mechanism by which Cten levels are increased via CD24. In our study we have also shown that CD24 causes increase in Src levels which also correlates to findings by other studies (146,148,149). But we have reported for the first time that these levels are dependent on Cten in HCT116 but not in RKO as the levels of Cten could not be shown on western blot. Although CD24 has been shown to increase phosphorylation of Src(146) but it has never been shown to be dependent on Cten. Cten as we know by our previous experiments causes increase in not only levels of Src but also FAK and pFAK(Tyr397) which are also effected by Src and CD24 (30,146) This further connects CD24 and Cten with the Src, Src and integrin pathway of cell motility.

Another novel finding in our study is that not only does CD24 regulate levels of Cten but Cten in turn regulates levels of CD24. Knocking down of Cten and

forced expression of Cten in HCT116 and RKO show that Cten levels cause an increase in CD24 levels. This indicates a regulatory feedback loop between Cten and CD24 which has not been discovered before. It means that Cten and CD24 are important for keeping each other levels elevated and one is dependent on the other. This fits CD24 into the focal adhesion cascade. To fit Cten in the puzzle of focal adhesion machinery we looked at the binding of Cten with other members like paxillin, vinculin, FAK, ILK but there was no evidence of a physical binding among them, including FAK and paxillin which is a well-established linking(150). However we have used only one cell line that is SW620 which is a shortfall of this study. In future we hope to use a panel of cell lines to establish linkage between these molecules in endogenous state within colorectal cancer.

In summary our study shows a novel relationship between CD24 and Cten. Cten and CD24 levels are shown for the first time to be dependent on each other. CD24 is shown to regulate Src levels which might be dependent on Cten. Our novel insight into CD24/Cten pathway may lead to development of new therapeutic agents and further investigation may lead to better understanding of the motility pathway within the lipid rafts.

8 General Discussion

8.1 Introduction

Cten is part of Tensin gene family. It is the smallest members which lacks the N terminal actin binding domain (8,73) and is an integral part of focal adhesions. It binds to NPXY of β1-integrin via its PTB domain (49) independent of tyrosine phosphorylation. Its expression in the tissue varies. Normally it is present in prostate and placenta. In prostate it is considered as a tumour suppressor(12). In other tissues including colon, stomach, breast, skin, pancreas, lung and ovaries, it is considered as an oncogene (6,7,39,41,49,71,151). This discrepancy can be due to the fact that it's not normally present in those tissues.Despite low levels of Cten in kidney it is still down regulated in tumours of kidney(152).

In our study we have looked at role of Cten in colorectal cancer motility in detail. Cten is considered as an oncogene in colorectal cancer whose levels are up regulated in advanced Duke staging and metastasis. Not only do Cten levels increase in metastasis but there is also a shift from cytoplasmic to nuclear localisation in metastatic deposits (7). Continuing from all the previous findings of our group we decided to further investigate role of Cten in cell motility focussing on its 1) localisation in the nucleus; 2) Its downstream signalling via SH2 domain and binding partners; 3) Its possible up stream regulating by Calpain and CD24.

8.2 Cten signalling

Downstream signalling via Cten is an important for cell motility. Its downstream targets include ILK and E-cadherin which was discovered by our group (7,52). In this study we identified a number of downstream targets including the previously mentioned ILK, FAK, Snail, Src, β-catenin and

paxillin. We discovered that SH2 domain of Cten is not only important for its function in cell motility and colony formation. It's also important for interacting with its downstream targets. The Arginine at 474 to Alanine is a known mutation for SH2 domain dormancy. It has been employed in a number of studies(53,54). Similar effect was seen in a study by Hong et al.(55)In addition to its effect on reduction of Cten function it also diminished the positive effect of Cten on its targets like ILK, FAK, Src and β-catenin levels. Besides the critical Arginine at 474 we identified another important residue Tyrosine 479 within the SH2 domain of Cten which is essential for its function. We have also for the first time revealed that Cten is tyrosine phosphorylated in colorectal cancer cell lines in endogenous as well as forcibly transfected state.

β-catenin levels were shown to go up with Cten. It is already known that Cten binds to β-catenin in the nucleus(6) and nuclear expression of Cten is increased in metastatic deposits of colorectal cancer. Since in the cytoplasm β-catenin binds to E-cadherin it could be a mechanism by which Cten reduces cytoplasmic interaction of β-catenin and E-cadherin and causes an EMT switch. As our lysates were of whole cell extracted protein this increase could be in the nuclear instead of the cytoplasm. Further experiments are needed to determine whether this increase is localised to the nucleus. In this study we have shown for the first time binding of Cten with FAK and this binding was partly lost with the GFP-Cten^{R474A}mutant. FAK is known to locate in the nucleus (79) as does Cten ((6). In our study we have shown that Cten binds to FAK not only in the cytoplasm but in the nucleus too. This would provide an explanation for our recent data showing association of nuclear FAK and

nuclear Cten in metastatic CRC (153). The functional significance of this nuclear localization remains uncertain.

We have shown that not only does Cten up regulate FAK levels but also increases pFAK (the activated form of FAK). Src is known to bind FAK at the focal adhesions and promotes its further phosphorylation(30). This occurs at the focal adhesion site with FAK bound to the integrins. Since we know that Cten binds to integrin and FAK it is possible that it binds to Src too in a trimolecular complex. However we were not able to show a physical interaction between Src and Cten but FAK and Src binding was apparent in our experiments. It is known that integrin stimulated FAK phosphorylation at Tyrosine 397 causes binding with Src family kinases (120,154). Cten may play a role here by increasing the levels pf phosphorylated FAK which may in turn cause increase in Src levels which was seen in our study(120). Integrins although are part of focal adhesions but they do not possess intrinsic signalling capabilities. They require the help of kinases like FAK and Src which transmit the messages form the ECM to the cell machinery.(27) It is interesting to note here that Tensin are considered as part of the scaffold/adaptor proteins at focal adhesions like talin and paxillin whose function is to alter cell morphology by controlling the actin cytoskeleton with the cell by changing its shape and generating traction force which helps propel the cells. In our study for the first time Cten has emerged as a potential signalling molecule inducing levels of FAK and Src (kinases involved in cell motility, at focal adhesions). This discovery renders Cten as a signalling as well as scaffolding protein important for cell motility and survival.

Src family kinases also affect E-cadherin internalisation via integrin activation as well as by endocytosis which brings us to the role of Cten in decreasing levels of E-cadherin which could be through Src, ILK, FAK and Snail All of the mentioned are downstream targets of Cten which are involved in E-cadherin regulation. However Snail is known to represses E-cadherin levels at transcriptional level(155) which isn't the case with Cten in our study. Snail levels were on the other hand affected on mRNA level with Cten overexpression but not with knockdown. This over expression in Snail levels was also SH2 domain dependent.

Trying to piece together the puzzle of functioning of Cten at the focal adhesions we have uncovered a number of signalling partners for Cten in the nucleus and the cytoplasm in context of cell motility. A summary can be found in Figure 8-1.

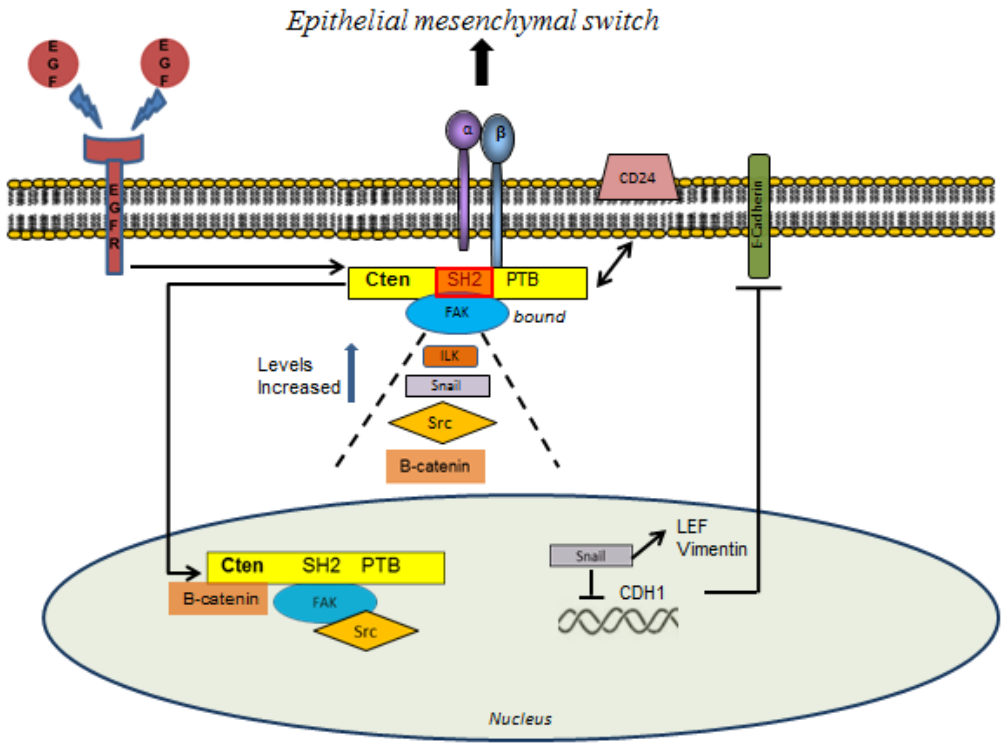


Figure 8-1.Summary of the signaling partners of Cten discovered in this study.EGF stimulation causes increase in Cten levels. Increased Cten results in increase in levels of FAK, ILK, Snail, Src and B-catenin. This increase is dependent on the SH2 domain of Cten. Cten is bound to FAK and goes in the nucleus where it binds to B-catenin and FAK. CD24 causes increase in Cten levels and vice versa. Snail causes increase in LEF (lymphocyte enhancer binding factor 1) and Vimentin levels and also causes a decrease in E-cadherin. All this leads to EMT and increase cell motility.

Cten regulation

Cten is said to be regulated by a number of different pathways. One of which has been identified by our group as the Epidermal growth factor (EGF) through Kras and Braf in colorectal cancer(43). In addition our group has shown that Stat3 negatively regulates Cten (156) Since then a number of Cten regulators have been discovered like FGF2 (fibroblast growth factor 2), PDGF (platelet derived growth factor), NGF (nerve growth factor), TGFβ (transforming growth factor beta), IL6 (interleukin 6), IL13, IGF1 (insulin growth factor 1) (51). In this study we have found 2 new regulators that are CD24 and calpain. CD24 is seen to up regulate levels of Cten in HCT116 cell line. It is a well-known stem cell marker which is located in the lipid rafts in the cell membrane and has no intracytoplasmic connections (136). It increases cell motility in colorectal cancer(144) through Akt pathway but its downstream targets are not very well known and Cten can be a possible target. However this warrants further study.

Cten level was shown to be affected by Calpain inhibition in this study. This could mean that Cten is a Calpain substrate directly or it is affected indirectly. Calpains have been an important part of cell motility in the literature and their substrates include most of the important focal adhesion molecules involved in cell motility including Src, Paxillin, FAK, vinculin and talin.(90,95,107). Calpains are known to cleave β1-integrin as well leading to disruption of focal adhesions and causing rounding of cells and leading to increased cell motility(157,158). However Cten cleavage should lead to decrease motility.

This raises a debate about Cten and Calpain and its role in cell motility. Our study mainly focuses on Cten in colorectal cancer setup whereas the studies about Calpain have mostly been conducted in muscles and endothelial cells which could be a cause of discrepancy. Another interesting study in murine fibroblasts shows that EGF activates m-Calpain (159) which would suggest that Cten levels should go up as EGF is a positive regular of Cten. This also contrasts with our findings but again the settings of the experiments differ in this case as well. On the other hand another study describes Tensin1 as a substrate of calpain II which is also located in the focal adhesions leading to rounding of cells and inhibition of cell spreading. Tensin1 has also been described to be cleaved by caspases another calcium dependent enzyme (104,160). It will be interesting to further study the role of Calpain in colorectal cancers and investigate effect of EGF on Calpain levels and Cten and effect on motility. Calpeptin, the inhibitor used in our study has also been shown to have independent effecton tyrosine phosphatases and other molecules like TGF-β, so we cannot rule out the possibility of a calpain independent effect here either(99,105).One more unusual finding in our study was increase in Tensin3 levels with rise in Cten levels after Calpain inhibition, which was surprising since it has been established that Cten expression causes a Tensin switch shifting Tensin3 away from focal adhesion giving way to Cten. These findings was reported in breast cell lines (49) and a recent study by Hung et.al refutes this idea in colorectal cancer cells(51) suggesting that this mechanism might be variable depending on cell context. These findings provide a new dimension in regulation of Cten in colorectal cancer and its role in cell motility and raises

new questions which could lead to further investigations into its function in future.

Cten has been implicated as oncogene in a number of tumours like pancreatic tumours, ovarian, breast, lung to name a few. It increases motility in pancreatic and lung cancer cell lines (46). Where there are some similarities in Cten function in these tumours and colorectal cancer, there lie some discrepancies as well. Cten's been shown to be induced by EGF stimulation through Kras in pancreas as well as colon(43) and Stat3 plays a positive effector in lung and breast and serves as a negative regulator in colon (47,48,156). It is also known to negatively affect DLC1 in breast cell lines(50) which in ase of colon couldn't be seen as DLC1 was not expressed in the colorectal cell lines(data not shown). It would be interesting to look at the downstream effectors identified in our study in other tumour cell lines and find a common role of Cten in all tumours in which Cten plays a role of oncogene.

8.4 Conclusions

(All conclusions are valid in colorectal cancer cell lines only)

1) Cten when localised to nucleus induces cell motility significantly.

2) Cten function depends on its SH2 domain. The intact Arginine at 474 is important for its function and its effect on downstream targets like FAK, ILK and Snail.

3) Cten binds FAK in HCT116 in the nucleus and cytoplasm.

4) Cten binds to β1-integrin. (needs further work)

5) Cten is being tyrosine phosphorylated in colorectal cancer cell lines.

6) GFP-Cten^{Y479F}leads to significant reduction in Cten motility enhancing function.

7) Calpain inhibition by Calpeptin leads to increase in Cten and Tensin 3 levels.
8) New downstream targets of Cten identified are Snail, Src, β-catenin and paxillin.
9) CD24 expression induces levels of Cten.

8.5 Strengths of the study

1) Experiments are done in 2 cell lines mostly.
2) Most of the experiments are done using 2 techniques i.e., knockdown and forced expression.
3) This is the first study that explores the role of Cten into the nucleus by constructing a vector tagging it to the nucleus.
4) In this study FAK has been shown to bind to Cten for the first time in the cytoplasm as well as the nucleus.
5) Cten has been shown to be a substrate for Calpains.
6) Four separate mutations have been successfully introduced in Cten and their roles explored individually.
7) Tyrosine phosphorylation of Cten has been reported for the first time.

8.6 Limitations of the study

1) All of the work is done in only colorectal cancer cell lines.
2) Calpain inhibition study is only done in SW620.
3) Only one inhibitor was employed for calpain inhibition.
4) Effect of calpain on Cten levels should also have been explored by its effect on cell motility function.
5) NLSCten construct should have been shown to express in the nucleus in more than one way.
6) The function of NLSCten construct in increasing cell motility should have been studied in more detail.

7) Transfection efficiency of GFP-Cten^{Y449F} constructs should have been improved upon to rule out its augmenting effect of cell motility.
8) Slight changes in protein expression can be due to transcription efficiency which should be accounted for.

8.7 Future directions and suggestions

1) The role of Cten in the nucleus and its targets should be explored in detail.
2) The mutations identified to be important for functioning of SH2 domain of Cten should be combined together in one construct to see whether their pooled effect completely reverses functions in Cten on motility and colony formation and the levels of its downstream targets.
3) Role of Snail should be explored further and its effect on ILK, E-cadherin and Src studied in detail.
4) All downstream targets of Cten like ILK, FAK, Snail and Src should be studied as immunohistochemical markers for patient samples and their levels should be compared to Cten levels. Their expression should also be correlated to the grading and staging of the tumour. This will help to identify potential markers for tumour progression and metastasis.
5) To look for potential therapeutic molecules which could targets and inhibit SH2 domain of Cten and study its effect on cell motility caused by Cten overexpression in colorectal cancer lines and possibly develop drugs which could eventually be used for therapeutic purposes.
6) Study the effects of all modified Cten expression vector in mouse tumour models.
7) Stable cell lines should be generated to study the effects on constructs in detail and study effect in mouse model.

8) It will be interesting to link all this data with other tumour cell lines (lung cancer, ovary and pancreatic cancer) by overexpression of Cten mutant expression vectors and effect on upstream regulators and downstream targets of Cten identified in this study.

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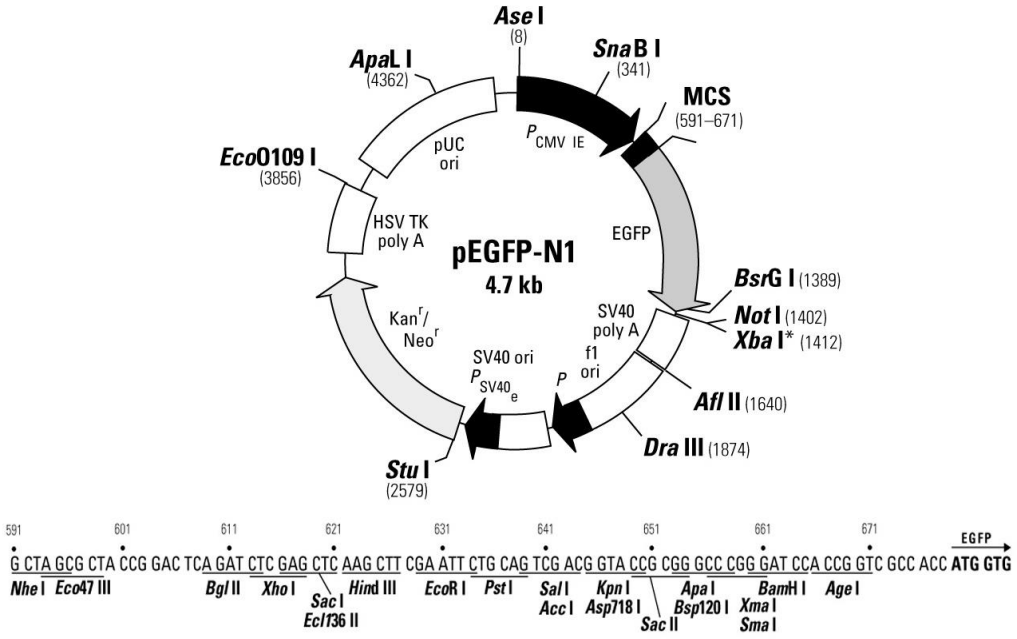
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10 Appendix

10.1 Appendix 1



10.2 Appendix 2

