Cten promotes epithelial-mesenchymal transition through post-transcriptional stabilization of Snail

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ABBREVIATIONS

EMT	Epithelial to mesenchymal transition
CHX	Cycloheximide
ECM	Extracellular matrix
ABD	Actin binding
SH2	Src homology 2
PTB	Phosphotyrosine binding
CRC	Colorectal cancer
gDNA	Genomic DNA
GFP-Cten^{R474A}	Cten construct with a mutated SH2 domain
siRNA	Small interfering RNA
GFP-EV	Empty vector control
SW620$^{\Delta Cten}$	Cten knockout SW620 cell line

ABBREVIATED TITLE

Cten stabilises Snail during EMT

KEY WORDS

Cten, Snail, migration, colorectal cancer, EMT

ABSTRACT

Cten promotes cell migration however the knowledge of underlying signalling pathways is sparse. We have shown that Cten downregulates E-cadherin, a feature of epithelial to mesenchymal transition (EMT). This prompted us to investigate whether Cten further contributed to EMT processes to regulate cell motility.

The regulation of Snail by Cten was investigated following overexpression, knockdown (by RNA-interference) or knockout of Cten in HCT116, Caco-2 and SW620 colorectal cancer (CRC) cell lines. Subsequently, the cycloheximide (CHX) pulse chase assay was used to investigate changes in Snail protein stability and the functional relevance of Cten-Snail signalling was investigated.

Snail was identified as a downstream target of Cten signalling using multiple approaches of Cten expression manipulation. Furthermore, this activity was mediated through the SH2 domain of Cten. The CHX assay confirmed that Cten was regulating Snail at a post transcriptional level and this was through the prevention of Snail degradation. Cell migration, invasion and colony formation efficiency were increased following forced expression of GFP-Cten but subsequently lost when Snail was knocked down, demonstrating a functional Cten-Snail signalling axis.

In conclusion, we have described a novel Cten-Snail signaling pathway that contributes to cell motility in CRC, mediated by the stabilization of Snail protein. This finding potentially furthers the understanding of EMT regulatory networks in cancer metastasis.

INTRODUCTION

Cten (also known as Tensin 4), is a member of the tensin protein family which localise to focal adhesions [1]. The tensins share a common domain structure comprising an N-terminal actin binding domain (ABD), a C-terminal Src homology 2 (SH2) domain and a phosphotyrosine binding (PTB) domain. The PTB domain is known to bind β-integrins at focal adhesions and thus these proteins act as both mechanical and signalling platforms linking the cytoskeleton to the cell exterior [2]. Cten however is a more distantly related protein which lacks the N-terminus ABD but still contains the C-terminus signal transduction components [3].

Cten expression is mainly upregulated in tumour tissues including the colon, pancreas, breast and melanoma and is generally associated with metastasis [3-6]. Experimentally, Cten has been shown to induce cell motility (fitting with a role in metastasis) however the underlying signalling mechanisms are not well established [7]. In non-tumour breast epithelial cell lines, Cten was shown to regulate cell migration by a 'tensin switch' mechanism whereby the upregulation of Cten was associated with a decrease in Tensin 3 expression. Downstream, differential binding to DLC1 induced cell motility through RhoA-ROCK signalling [2,8]. In CRC, and possibly cancer tissue in general, it is likely that alternative signaling mechanisms exist as firstly, we have not found no evidence of a tensin switch in CRC [9]. Secondly, we found that DLC1 expression is often lost in CRC, suggesting that DLC1 is not a major player in Cten signaling. Despite the mechanistic differences, there is a common functional activity of Cten in stimulating cell migration and invasion, consequently alternative signalling mechanisms must be present [10,11].

EMT is a process whereby epithelial cells acquire a mesenchymal phenotype to enhance cell migration. This is a process native to physiological events such as wound healing and embryogenesis and it is also likely to play a role in metastasis [12-14]. The loss of E-cadherin at adherens junctions causes disruption of cell to cell adhesion thereby allowing invasion and migration away from the primary tumour. However this is also accompanied by additional molecular changes which induce cell motility [15-18]. Snail is a transcription factor central to the regulation of EMT through the downregulation of E-cadherin but is also known to regulate other genes associated with EMT [12-14]. Interestingly, we have previously shown that Cten represses E-cadherin and therefore may induce EMT [10].

Considering the data that both we and others have published, we hypothesised that Cten may induce cell motility through the regulation of additional biomarkers of EMT. Here we demonstrate that Cten regulates the protein stability of Snail which is mediated via Cten's SH2 domain. Furthermore we show that Cten signals through Snail to increase cell migration and invasion in CRC cell lines.

MATERIALS AND METHODS

Cell culture

HCT116, Caco-2 and SW620 colorectal cell lines, a kind gift from Prof Ian Tomlinson, were cultured in DMEM (GlutaMAX™ supplement, Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Cell line identities were verified by high resolution melting (HRM) mutation analysis as described previously [19].

Cten deletion in SW620

CRISPR/Cas9 gene technology allows editing of the genome of cell lines. We reasoned that by knocking out both copies of Cten in SW620, we would be able to carry out both gene knockdown and gene over expression experiments in isogenic cell lines. A plasmid construct expressing GFP-tagged Cas9 and guide RNA targeting exon 3 of Cten (CCGCCAGATCAAGGTGCCACGA) (Sigma, St Louis, MO) was transfected into SW620 cells. Ten µg CRISPR-Cas9 construct was transfected with 10 µl Lipofectamine according to the manufacturer's instructions. Forty eight hours post transfection, GFP expressing cells were sorted into 96 well plates using the Astrios Cell Sorter (Beckman Coulter, High Wycombe, UK) and single cells were expanded to form clonal cell lines. Genomic DNA (gDNA) was extracted from the resulting clones using the Genelute Mammalian Genomic DNA Extraction Kit (Sigma) and the region around the CRISPR target site amplified (Supplementary table 1). Clones were screened for mutation by HRM and those revealing a shift in melting temperature from the wild type cell line were selected. The amplicons were cloned into a TOPO vector using the TOPO TA cloning kit (Thermo Fisher Scientific) and recombinants purified using the Genelute Plasmid Miniprep Kit (Sigma) according to the manufacturer's protocol. In order to obtain homozygous knockout, a clone showing mutation of one allele was then expanded and put through another round of gene editing to mutate the second allele.

Site-directed mutagenesis

We have recently shown that the conserved arginine at position 474 is essential for the functioning of the SH2 domain in Cten (manuscript in preparation). In brief, the Phusion site

directed mutagenesis kit (Thermo Fisher Scientific) was used to convert the arginine to an alanine residue (GFP-Cten^{R474A}) according to the manufacturer's protocol. The presence of the mutation was confirmed by sequencing.

Cell transfection

Lipofectamine transfection reagent (Thermo Fisher Scientific) was used to transfect plasmids (for over expression) and small interfering RNA (siRNA) duplexes (for gene knockdown) into cells. Cells were grown in 6 well plates to 60-70% confluency. For over expression experiments, 5 µg GFP-Cten expression construct, GFP-Cten^{R474A} or an empty vector control expressing GFP only (GFP-EV), were transfected with 10 µl Lipofectamine in Opti-MEM media (Thermo Fisher Scientific) according to the manufacturer's protocol. Transfection reagents were replaced with DMEM after 6 hours and the cells were harvested 24 hours post transfection. For knockdown experiments, cells were grown to 50% confluency and 100nM siRNA duplexes targeting Cten, Snail or Luciferase (Supplementary table 2) were transfected together with 10 µl Lipofectamine. The transfection reagents were replaced with DMEM after 6 hours and the cells were harvested 48 hours post transfection. For co-transfection, 10 µl Lipofectamine was transfected together with 5 µg plasmid and 100 nM siRNA in Opti-MEM media according to the manufacturer's instructions. The cells were incubated with the transfection reagents for 6 hours and experimentation performed 48 hours post transfection.

Western blot

Cell lysates were obtained using RIPA buffer (Thermo Fisher Scientific) supplemented with phosphatase and protease inhibitor (Thermo Fisher Scientific). Fifty µg protein was heated

at 95^{o}C for 5 minutes then cooled on ice for 5 minutes. The protein was fractionated on a 4-12% NUPAGE Bis-Tris gel with NUPAGE MOPS running buffer (Thermo Fisher Scientific) using the NUPAGE gel electrophoresis system (Thermo Fisher Scientific). Proteins were transferred onto a PVDF membrane (GE Life Science, Chicago, IL) using the Trans Blot semi-dry transfer system (Biorad). Following blocking in 5% milk 0.01% tween PBS or 5% BSA 0.01% tween TBS, membranes were incubated with optimally diluted primary antibodies; Cten 1:10,000 (Sigma) and Actin 1:50,000 (Sigma) diluted in 5% milk 0.01% tween and PBS, Snail 1:1,000 (Cell Signaling, Danvers, MA) diluted in 5% BSA 0.01% tween PBS overnight. Following washing, membranes were incubated with the appropriate anti-mouse or anti-rabbit secondary antibody 1:10,000 (Sigma) for 1 hour at room temperature. The ECL prime detection kit (GE Life Sciences) was used for protein band visualisation using x-ray film (GE Life Sciences) or the C-DiGiT Blot Scanner (LI-COR, Lincoln, NE).

Cycloheximide chase assay

Cycloheximide inhibits translation allowing the rates of protein degradation to be evaluated. HCT116 cells were transfected with either GFP-Cten or GFP-EV and 24 hours post transfection were treated with 100 µg/ml CHX (Sigma). Protein lysates were collected following 0, 1, 2 or 4 hours exposure to CHX. Western blotting was performed as described above.

Co-immunoprecipitation

Cell lysates were pre-cleared by incubating with 20 µl protein G/A agarose beads (Thermo Fisher Scientific) at 4°C with rotation for 30 minutes. Lysates were centrifuged at 4°C at 13,000 rpm for 5 minutes and the supernatant retained. Two µg Cten antibody was added

to 500 µg pre-cleared lysate and incubated rotating overnight at 4°C. Thirty µl Protein A/G beads were added to the IP reactions and left rotating overnight at 4°C. Separately, 500 µg of pre-cleared lysate (without antibody) was also incubated with Protein A/G beads as a negative control. The beads were pelleted by centrifugation at 13,000 rpm at 4°C for 5 minutes and washed twice in ice cold PBS. Beads were re-suspended in 10 µl NUPAGE loading Buffer (Thermo Fisher Scientific) and heated at 95° for 5 minutes, kept on ice for 5 minutes and centrifuged for 2 minutes at 13,000 rpm before loading onto an SDS gel for western blot analysis. Fifty µg lysate was loaded for the input.

Quantitative Reverse Transcription-PCR (qRT-PCR)

qRT-PCR was used to quantify mRNA expression. RNA was extracted using the Total RNA Extraction Kit (Sigma) according to manufacturer's protocol. For cDNA synthesis, 1 µg RNA together with 0.5 µg Random hexamers (Thermo Fisher Scientific) were heated for 5 minutes at 70^{o}C and then cooled for 5 minutes at 4^{o}C. This was reverse transcribed using 200 U M-MLV Reverse Transcriptase (Promega) and 0.5 mM dNTP's (Promega), heated at 37^{o}C for 1 hour followed by 95^{o}C for 10 minutes. Gene quantification was performed using Go Taq Mastermix (Promega, Madison, WI) and 250 nM each primer (Supplementary table 1). The run cycle comprised 95°C for 2 minutes, 40x (95°C for 3 seconds, annealing temperature for 30 seconds) and a melt curve stage 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds 95°C for 15 seconds and 60°C for 15 seconds. HPRT was used as the endogenous control and PCR efficiency of the primer pairs was determined using the standard curve method. Since primer pairs had similar efficiency the $2^{-\Delta\Delta Ct}$ method was used for gene quantification.

PrestoBlue proliferation assay

PrestoBlue (Thermo Fisher Scientific) was used as an indirect method to measure the total number of live cells. A total of $1x10^5$ cells were seeded in a 24 well plate and allowed to adhere for 3 hours. Cells were treated with 1 ml PrestoBlue for 1 hour and the absorbance measured using the Fluorstar plate reader (560/590 nm). Further readings were taken at 24, 48 and 72 hour time points. The blank fluorescence reading (PrestoBlue with no cells) was subtracted from each experimental reading. The blank corrected values were normalised to the 0 hour time point.

Transwell cell migration and invasion assays

The Transwell system (Corning, Corning, NY) was used to assess changes in cell migration. The Transwell inserts (6.5 mm diameter; 8 µm pore size) were incubated in DMEM at 37°C for 1 hour prior to use. Following this, 600 µl of DMEM (20% FBS) was added to the outer wells of the Transwell plate and the Transwell inserts placed inside. A total of $1x10^5$ cells in DMEM (10% FBS) were seeded onto the Transwell insert. The plate was incubated at 37°C for 24 hours. Following this, the cells that had migrated through to the bottom of the outside well, using the higher FBS concentration as a chemoattractant, were manually counted. Triplicate wells were seeded for each experimental condition. The Transwell invasion assay was performed according to this protocol with the exception that $2x10^5$ cells were seeded onto a Transwell insert coated in Basement membrane extract (3 mg/ml, Corning) and cells allowed to migrate for 48 hours prior to counting.

Colony formation assay

Colony formation in soft agar was used to assess anchorage independent cell growth. One ml 1% agar layer (Sigma) containing DMEM was plated in 6 well plates. Overlaying this was 1 ml 0.7% agarose layer (Sigma) containing 2,500 cells in DMEM. Plates were incubated at 37°C for 21 days and fed with 0.5 ml of DMEM. Following this, the plates were stained with 0.005% crystal violet 4% formaldehyde for 1 hour. The number of colonies of approximately >50 cells in size were manually counted and the colony formation efficiency determined (number of colonies counted/number of cells seeded x 100).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (v6). The Shapiro-Wilk test was used to test for normality. The unpaired T test or ANOVA statistical tests were applied following for experiments with 2 or more than 2 treatment groups respectively.

RESULTS

Creation of a Cten knockout SW620 cell line

The manipulation of Cten expression can be used to study its biological effects through over expression in cell lines expressing low endogenous levels of Cten, or by the depletion of Cten expression in high endogenously expressing cell lines. We have previously reported the endogenous expression of Cten across a panel of normal and colorectal cancer cell lines [10]. SW620 normally expresses high levels of Cten. CRISPR-Cas9 technology was used to create a Cten knockout SW620 cell line. A clone was obtained in which both alleles of Cten had undergone frameshift mutations resulting in a truncated protein ($SW620^{\Delta Cten}$). The presence of the mutations was confirmed by sequencing and the complete lack of Cten protein expression was confirmed by Western blotting (Figure 1a and 1b). The resulting sequences

were predicted to encode truncated proteins of length 333 and 191 amino acids (ORF Finder, NCBI) (Supplementary table 3). Functional evaluation of SW620$^{\Delta Cten}$ revealed that, consistent with previously published data, loss of Cten was associated with a reduction in both cell migration and invasion (Figure 1c and 1d) [7]. The creation of isogenic cell lines with both the presence and absence of the full length Cten gene provides suitable a model to study Cten biology.

Cten is a positive regulator of Snail Expression

Since we found little evidence of DLC1 expression in colorectal cancer (Supplementary figure 1), we investigated alternative Cten signalling mechanisms in this tumour type. We have shown that Cten is a regulator of E-cadherin expression, which suggests it may be involved in the regulation of EMT [10]. We investigated whether Cten may also regulate Snail expression in colorectal cancer. The colorectal cell line HCT116, which expresses very low endogenous levels of Cten, was transfected with either GFP-Cten or GFP-EV expression constructs and the changes in Snail protein expression investigated by western blot (Figure 2a and supplementary figure 2). Over expression of GFP-Cten in HCT116 cells resulted in an increase in Snail expression compared to the lysate of those cells transfected with GFP-EV. This suggests that Cten positively regulates Snail expression. To demonstrate that this effect was not cell line specific, Cten was also over expressed in Caco-2 cells, also a low Cten expressing cell line. Consistent with this, Cten was shown to upregulate Snail protein expression. To further validate this result with another methodology, Cten was knocked down using siRNA duplexes in SW620, a cell line which expresses high levels of endogenous Cten. Knockdown of Cten was associated with a decrease in Snail protein expression. Finally, this was further validated in SW620$^{\Delta cten}$ cells. Constitutive depletion of Cten in this way also

resulted in reduction of Snail levels. The data, in combination, confirm that Cten does indeed positively regulate Snail protein expression.

Cten regulates Snail in an SH2 dependent manner

Cten contains an SH2 and a PTB domain at its C-terminus. SH2 domains partake in signal transduction events via tyrosine phosphorylation and this domain in Cten and other Tensin family members has previously been shown to be critical for its activity [20,21]. We sought to determine whether the SH2 domain of Cten was required for the upregulation of Snail expression. HCT116 cells were transfected with GFP-Cten, GFP-Cten^{R474A} or GFP-EV expression constructs and the changes in Snail protein expression were assessed by western blot (Figure 2b and supplementary figure 2). Both GFP-Cten and GFP-Cten^{R474A} were expressed to a similar level which allowed for comparison of Snail protein expression between the different treatment conditions. Over expression of GFP-Cten led to an increase in Snail protein expression. Over expression of GFP-Cten^{R474A} also led to an increase in Snail expression compared to GFP-EV but this was less than the induction by GFP-Cten indicating that the SH2 domain of Cten could be important for the upregulation of Snail expression.

Cten increases Snail protein stability

To further investigate the mechanism of Snail upregulation by Cten we next performed qRT-PCR to determine whether this was occurring at a transcriptional or post-transcriptional level. Cten was over expressed in HCT116 and knocked down in SW620 cells. In both experiments there was no change in Snail mRNA expression compared to the control (Figure 3a and 3b). This suggests that the regulation of Snail by Cten is occurring at a post-transcriptional level.

Expression may be regulated post-transcriptionally either by increased protein synthesis or reduced protein degradation. The CHX chase assay was used to determine whether Cten stabilised Snail protein preventing its degradation (Figure 3c). HCT116 cells following transfection of GFP-Cten or the empty vector control were treated with 100 μg/ml CHX to inhibit protein synthesis. This allowed for the tracking of protein degradation by western blot. Our data showed that when Cten was present, Snail protein degradation was markedly delayed. In the cells transfected with GFP-Cten, Snail protein expression was still highly expressed 2 hours after treatment whereas, in the control cells, Snail protein had mostly been degraded at 1 hour.

Cten has been shown to form a complex with β-catenin in the nucleus. As both Cten and Snail translocate between the nucleus and cytoplasm, it was hypothesised that they could form a physical complex and once in complex, Cten could prevent the degradation of Snail protein. To investigate protein binding interactions, a co-immunoprecipitation experiment was performed however, this revealed that Snail and Cten proteins did not bind to each other using this assay (Figure 3d). Together, these results show that Cten regulates Snail protein stability but as they do not form a physical complex, this is probably due to signaling downstream of Cten mediated by the SH2 domain.

The regulation of Snail by Cten is functionally active

Having shown that Cten regulates Snail, we next wanted to investigate whether this interaction was functionally relevant. Both Cten and Snail regulate cell invasion and migration and since Cten regulates Snail expression, it is possible that Cten may regulate

these activities through Snail signaling. We have previously shown that Cten has no effect on cell proliferation but before performing further assays, it was necessary to determine whether Snail had any effect on cell number. Snail was knocked down using siRNA in HCT116 and following this, the PrestoBlue assay was performed to assess cell proliferation (Figure 4a and 4b). The PrestoBlue assay showed no change in activity when Snail was knocked down compared to the control. This suggests that Snail does not have any effect on cell proliferation in HCT116 cells. Next, we over expressed GFP-Cten in HCT116 and simultaneously knocked down Snail (Figure 4c and supplementary figure 2). Transwell migration, invasion and colony formation assays were then performed (Figure 4d-4f). Over expression of GFP-Cten was associated with an increase in cell migration and invasion which was subsequently lost when Snail was knocked down. Similarly, over expression of GFP-Cten was associated with an increase in colony formation efficiency which was reduced on depletion of Snail expression.

The effect of Snail on Cten cell function was investigated in an additional cell line using an alternative methodology. In agreement with the findings in HCT116 cells, over expression of Cten in SW620$^{\Delta Cten}$ cells increased both cell migration and invasion and both cell functions were decreased on Snail knockdown (Figure 5 and supplementary figure 2). Together, the data implies that Cten regulates cell migration, invasion and colony formation through the upregulation of Snail protein.

DISCUSSION

EMT is considered to play a critical role in cancer metastasis endowing a cell with greater migratory capabilities as well as properties of "stemness". Although progress has been made in recent years to try and elucidate the underlying signaling mechanisms, there is still a lot about the regulation of this process that remains unknown. We have previously shown that Cten is a regulator of EMT and that it stimulates cell motility in tumour cells [10]. To our knowledge, this is the first time that it has been shown that Cten may also mediate EMT through the positive regulation of Snail expression and that this regulation is achieved through enhancing the stability of Snail protein (summarized in figure 6).

Using multiple approaches to modulate Cten expression, we have shown that any induced changes were followed by similar changes in expression of Snail protein. Multiple approaches were used to eliminate artefacts of methodology and this was validated in multiple cell lines to eliminate cell line specific effects. These data firmly establish the relationship between Cten and Snail. The SH2 domain of Cten is essential for several of the functions of Cten [20]. Using a construct in which the SH2 domain in Cten was inactivated ($Cten^{R474A}$) we were able to show reduced levels of induction with this construct. This shows that Cten signaling to Snail is mediated via the SH2 domain.

Our data show that Cten regulation of Snail is through post-transcriptional mechanisms and most probably due to stabilization of Snail protein. We were unable to observe any change in Snail mRNA levels following modulation of Cten but the CHX chase experiments unequivocally showed delayed degradation in the presence of Cten. Snail is a transcription factor which requires tight regulation to ensure appropriate expression of downstream targets. It is often regulated at the protein level to ensure signals can be promptly switched

off upon external stimuli and this fits with our data [22]. The exact mechanism by which this stabilization occurs is unclear. We were unable to demonstrate the formation of a complex between Cten and Snail and thus an alternative explanation must be sought.

Snail nuclear localisation is essential for its transcriptional activity. The phosphorylation of Snail regulates its export from the nucleus and subsequent degradation via the ubiquitin proteasome pathway [22]. It would be of interest to determine whether Cten signaling is involved in this process. Also of interest, is to determine whether Cten regulates the expression of Snail downstream targets. Cten has been shown to regulate E-cadherin, a known target of Snail signaling however this regulation was at a post-transcriptional level and since Snail is a transcriptional regulator of E-cadherin, it is unlikely that these signal in the same pathway [10]. Snail controls the transcriptional activity of a number of other genes involved in EMT in addition to genes involved in other cellular processes which could possibly also be targets of Cten [23].

We are confident that Cten can be added to the list of genes which can regulate Snail and that this relationship is important to the functional activity of Cten. We over expressed Cten whilst at the same time knocking down Snail to create a situation where Cten was present but there was no Snail present. This resulted in an abrogation of the effect of Cten on cell migration, cell invasion and colony formation. These data confirm that the induction of Snail by Cten is not just a bystander phenomenon. We have previously found that Cten upregulates ILK and FAK to increase cell motility and both of these proteins are now known to play a role in EMT [6,11]. It would be of interest to determine whether ILK or FAK are signaling intermediates in the Cten-Snail pathway. The role of EMT in cancer metastasis has

been well documented however most of this work has been performed *in vitro*. These studies were performed in cell lines and validation of these experiments in animal models is required to confirm that these effects also occur *in vivo*. There is accumulating *in vitro* evidence for the contribution of EMT to cancer metastasis however, the occurrence and relevance of EMT *in vivo* is debated. In mouse models of pancreatic and lung cancer, although EMT contributed to chemoresistance, it was not required by metastasising cells [24,25]. However, EMT is complex and at present is not fully understood and consequently these mouse models used may not fully recapitulate EMT processes in human cancer.

In conclusion, we have uncovered a novel mechanism of Snail regulation in CRC which increases cell motility and colony formation. Knowledge of mechanisms regulating cell migration may help to identify novel markers for therapeutic targeting of cancer metastasis.

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STATEMENT OF AUTHOR CONTRIBUTIONS

HT: Performed the majority of the experimental work including experimental design and analysis of the results and prepared the manuscript. AA: Assisted with the creation of the SW620$^{\Delta Cten}$ cell line and performed the functional assays involving this cell line. MA: Created

the GFP-Cten^{R474A} construct. MI: Conceived the idea of the project and reviewed the manuscript. The manuscript was approved by all authors.

LIST OF SUPPLEMENTARY MATERIAL

Supplementary figure 1: DLC1 expression in CRC cell lines.

Supplementary figure 2: Quantification of Snail protein expression

Supplementary table 1: Primer sequences.

Supplementary table 2: siRNA sequences.

Supplementary table 3: Predicted amino acid sequence at both Cten alleles following CRISPR-Cas9 editing (ORF Finder, NCBI).

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TABLE AND FIGURE LEGENDS

Figure 1: Cten knockout abrogates cell motility. A) Sequencing of both Cten alleles following CRISPR-Cas9 genome editing of SW620 cells. B) Western blot analysis of Cten expression in $SW620^{\Delta Cten}$ and $SW620^{\Delta control}$ cells. C) Transwell migration over 24 hours in $SW620^{\Delta Cten}$ and $SW620^{\Delta control}$ cells. D) Transwell invasion over 48 hours in $SW620^{\Delta Cten}$ and $SW620^{\Delta control}$ cells.

Figure 2: Cten regulates Snail expression through SH2 domain signaling. A) Snail protein expression following over expression of GFP-Cten in HCT116 and Caco-2 cells or knockdown and knockout of Cten in SW620 cells. B) Over expression of GFP-Cten and mutant GFP-$Cten^{R474A}$ in HCT116 and resultant changes in Snail protein expression.

Figure 3: Cten stabilises Snail protein. A) qPCR of Snail expression following siRNA knockdown of Cten in SW620 cells. Control SW620 cells transfected with Luciferase targeting siRNA as a negative control (n=3). B) Snail mRNA expression following over expression of GFP-Cten in HCT116 (n=3). C) Treatment of GFP-Cten transfected HCT116 cells with CHX (100 μg/ml) for 0-4 hours and resultant changes in Snail protein expression. D) Co-immunoprecipitation of Cten and Snail in HCT116 cells. Results are representative of at least 3 experimental replicates.

Figure 4: Cten signals through Snail to regulate cell functional activity in HCT116 cells. A) siRNA knockdown of Snail in HCT116 cells. siRNA targeting luciferase was used as a negative control. B) Treatment of HCT116 cells with PrestoBlue, assessed over 0-72 hours following Snail knockdown. C) Co-transfection of GFP-Cten or GFP-EV together with either Snail of Luciferase targeting siRNA. D) Transwell migration assay following manipulation of Cten and

Snail expression. E) Transwell invasion assay in HCT116 cells. F) Colony formation efficiency in soft agar over 21 days. Results are representative of at least 3 experimental replicates (* = $p<0.05$, **** = $p<0.0001$).

Figure 5: Cten signals through Snail to regulate cell functional activity in SW620$^{\Delta Cten}$ cells. A) GFP-Cten or GFP-EV over expressed in SW620$^{\Delta Cten}$ cells and co-transfected with either Snail of Luciferase targeting siRNA. B) Transwell migration assay in in SW620$^{\Delta Cten}$ cells following manipulation of Cten expression. C) Transwell invasion assay in SW620$^{\Delta Cten}$ cells. Results representative of at least 3 experimental replicates (** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

Figure 6: The regulation of Snail protein stability by Cten. Cten signaling via the SH2 domain stabilizes Snail expression by inhibiting protein degradation. Once stabilized, Snail signaling promotes cell migration and invasion in colorectal cancer cell lines.

Supplementary figure 1: DLC1 expression in CRC cell lines. A) DLC1 mRNA expression across a panel of CRC cell lines. B) DLC1 expression by western blot in the top expressing and additional CRC cell lines.

Supplementary figure 2: Quantification of Snail protein expression. A) Quantification in Caco-2 cells following GFP-Cten over expression. B) Quantification of Snail following overexpression of GFP-Cten^{R474A} or GFP-Cten in HCT116 cells. C) Quantification of Snail protein following manipulation of Cten and Snail expression in HCT116 cells. D) Quantification in Cten knockout SW620 cells.

Supplementary table 1: Primer sequences.

Supplementary table 2: siRNA sequences.

Supplementary table 3: Predicted amino acid sequence at both Cten alleles following CRISPR-Cas9 editing (ORF Finder, NCBI).