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ORIGINAL ARTICLE

Epidermal growth factor receptor and STAT3 signal through KRAS have mutually opposite effects on CTEN

Saleh AlGhamdi[1,2*], Salih Ibrahim[1,3], Kanwal Balloch[1], Darryl Jackson[1], Mohammad Ilyas[1]

ABSTRACT

Background: C-terminal tensin-like (CTEN) is a protein located at focal adhesions and has been reported to be an oncogene in the colon, breast, lung, and gastric cancer. In this study, we investigated whether two other proposed mechanisms, i.e., epidermal growth factor receptor (EGFR) and Signal transducer and activator of transcription 3 (STAT3) signaling were involved in regulating CTEN expression.

Methodology: Initially, we manipulated EGFR signaling by (i) stimulation with epidermal growth factor (EGF) and (ii) inhibition by the PD153035 in the colorectal cancer cell lines SW620 and C32. In C32, EGF stimulation resulted in the upregulation of KRAS and CTEN, whereas exposure to PD153035 resulted in the downregulation of both KRAS and CTEN. EGFR activation and inhibition were reflected by, respectively, increased and decreased cell motility although the effect of EGFR activation was lost by CTEN knockdown. In SW620, which harbors a KRAS mutation, modulating EGFR activity in this way did not affect either KRAS or CTEN. STAT3 signaling has also been reported to positively regulate CTEN. We tested this in SW620 by directly knocking down STAT3 and exposing cells to interleukin-6 (an activator of STAT3). STAT3 knockdown resulted in increased CTEN, whereas STAT3 activation resulted in the downregulation of CTEN.

Results: Testing for KRAS expression showed that STAT3 was negatively regulating KRAS, and this was reflected in the CTEN expression. Functional analysis, however, showed that the inhibition of STAT3 resulted in a reduction of cell motility in a KRAS and CTEN-independent manner.

Conclusion: We conclude that both EGFR signals through KRAS to modulate CTEN (and consequently integrin-linked kinase/focal adhesion kinase) and stimulates cell motility. STAT3, however, negatively regulates KRAS and consequently CTEN although its net effect is to stimulate motility through an alternative mechanism.

Keywords: EGFR, CTEN, KRAS, STAT3, colon cancer, breast cancer.

Introduction

CTEN is a protein located at focal adhesions and has been reported to be an oncogene in the colon, breast, lung, and gastric cancer (1). In colorectal cancer (CRC), CTEN has also been found to be upregulated and is localized to both cytoplasm and nucleus (2,3). It is a member of the Tensin family of proteins that interact with several structural and signaling molecules such as vinculin, paxillin, Src, focal adhesion kinase, phosphatidylinositol-3-kinase (PI3-K), and Crk-associated substrate p130CAS, actin as well as integrins (4-6). Interaction with these molecules would suggest that CTEN may play a role in regulating cell motility. However, colony-forming assay data also suggest that CTEN may be involved in conferring some features of "stemness," and in lung cancers, data suggest an additional role in the regulation of cell proliferation through modulating the cell cycle (7,8). CTEN may have several functions but, since it is a recently described gene, data about its regulation are sparse. In breast cancer, it has been shown that CTEN is positively regulated by c-Erb-B2 protein which is overexpressed in a specific subset of breast cancers due to gene amplification (7). The c-Erb-B2

Correspondence to: Mohammad Ilyas
*Department of Pathology, College of Medicine, University of Nottingham, Nottingham, United Kingdom.
Email: mohammad.ilyas@nottingham. ac.uk
Full list of author information is available at the end of the article.
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protein is a part of the epidermal growth factor receptor (EGFR) signaling pathway (9) which signals through Kras to the Braf/ Motogen-Activated Protein Kinase (MAPK) pathway. Furthermore, circumstantial support for the role of EGFR signaling in the regulation of CTEN comes from studies, showing high levels of CTEN expression in lung cancer (10); tumors in this organ have a high frequency of disrupted EGFR/Kras signaling due to either KRAS or EGFR mutation. The frequency of EGFR/c-Erb-B2 amplifications in CRC is low although this is because gain-of-function mutations in KRAS/ murine sarcoma viral oncogene homolog (BRAF) are extremely common and are seen in up to 60% of tumors (11,12). We previously studied a series of CRC cell lines for both expressions of CTE and somatic mutation in several known oncogenes/ tumor suppressors (3,11). A combined evaluation of these data showed a significant association between high CTEN expression and KRAS/BRAF mutation (p = 0.03) (7). These data, together with the published data in breast cancers, led us to hypothesize that CTEN is a target of the EGFR/KRAS/BRAF signaling pathway.

In this work, we sought to test this hypothesis first in CRC cell lines and then, for validation, in pancreatic and lung cancer cell lines. These tumors also show a high frequency of the activation of the EGFR signaling pathway , often through KRAS mutation but also c-Erb-B2 amplification (in pancreatic cancers, especially) and EGFR kinase-activation mutations (in lung cancers, especially) (7). Besides EGFR signaling, published data have implicated Signal transducer and activator of transcription 3 (STAT3) as an upstream positive regulator of CTEN (13). On the contrary, others have reported that CTEN affects the STAT3 activity and showed that CTEN inhibits STAT3 activity (14). Given these inconsistencies, we sought to evaluate the role of Stat3 in the regulation of CTEN.

While CTEN is found to form a physical complex with the cytoplasmic tails of integrins, there are several molecules also present in focal adhesions that may regulate integrin activity. One of these is CD24 which is a small heavily glycosylated glycosylphosphatidylinositol (GPI)-anchored cell membrane protein that has been reported to be involved in trafficking integrins into lipid rafts (15). Many of the changes induced by modulating CTEN levels in CRC cells were also induced by the modulation of CD24. Evaluation of the phosphokinase array following CD24 knockdown in CRC cell lines (16) showed a similar pattern of kinase alteration as was seen following CTEN knockdown. These similarities between CD24 and CTEN led us to the hypothesis that CD24 may represent another mechanism of regulating CTEN expression.

Subjects and Methods

Cell treatments

Cells were stimulated with growth factors and interleukin 6 (IL-6) and signaling pathway inhibitors (PD153035 to inhibit the EGFR). For these experiments, cells were seeded in the appropriate plate according to the experimental setting. To stimulate the EGFR signaling pathway, cells were treated with recombinant human epidermal growth factor (EGF) (at a final concentration of 10 ng/ml, Invitrogen, UK) 24 hours before analysis. Controls for this experiment were treated with buffer solution with fetal bovine serum (FBS). To inhibit the EGFR signaling, cells were treated with PD153035 (at a final concentration of 10 μM, BioScience, UK) 24 hours before analysis. The controls for this experiment were treated with dimethyl sulphoxide (DMSO). The STAT3 signaling pathway was activated by stimulation with recombinant IL-6 (Immuntools, UK) at a concentration of 20 ng/ml. Cells were stimulated 24 hours before analysis. Following treatment with the appropriate growth factors, cells were then used either in protein extraction or in functional assays such as the migration assay.

RNA extraction and quantitative Reverse transcription polymerase chain reaction (RT-PCR) Quantitative Reverse transcription polymerase chain reaction (Q-RT-PCR)

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocols and quantified on a NanoDrop ND-1000 UV-Vis Spectrophotometer (LabTech International Ltd, Ringmer, UK) (16). Complementary DNA (cDNA) was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) in accordance with the manufacturer's instructions. The quantification of CTEN and KRAS was performed using the standard curve method. All experiments were conducted in triplicate, and test gene values were normalized to the housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase (HPRT). Each Polymerase chain reaction (PCR) was of a final volume of 25 μl and contained 10 ng of cDNA template, 16SYBR Green Master Mix (Stratagene), and 250 nM primers. PCR was performed on an MX3005P real-time PCR machine (Stratagene, UK), and cycling conditions were 5 minutes' denaturation at 95ºC followed by 40 cycles of 30 seconds' denaturation at 95ºC/30 seconds annealing (60ºC for Cten/59ºC for HPRT/50ºC for KRAS/30 seconds extension at 72ºC and a single final extension for 10 minutes). The data for Q-PCR were analyzed using the MxPro-QPCR software for the Mx3005P QPCR system.

Western blotting

Whole-cell extracts were prepared using lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 1 mM Ethylenediaminetetraacetic acid (EDTA), 0.1% SDS], supplemented with protease and phosphatase inhibitors (Sigma, Gillingham, UK). 30 mg of protein was loaded on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes by semi-dry transfer. After blocking, membranes were incubated overnight at room temperature with the indicated primary antibody [anti-CTEN (Sigma, WH0084951M1, 1:1000), mouse anti-KRAS (Abcam, ab16795, 1:250), mouse anti-b-actin (Sigma, 1:2000), and mouse anti-CD24 (SWA11, supernatant specific for an N-terminal epitope, a kind gift from Prof Altevogt)]. After three washes in TBS/Tween-20 (0.05%), blots were incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-linked secondary

antibody. After three further washes, the detection was performed using the Enhanced Chemiluminescence Kit (Pierce). Bands were visualized using X-ray films (Kodak) and quantified using ImageJ software.

Cell migration

Transwell cell migration assays were performed using a Boyden chamber containing a polycarbonate filter with an 8-µm pore size (Costar) as described earlier (17). In brief, culture medium (600 µl) supplemented with 20% FBS was added to the lower chamber, and 2.5×10^5 cells of the control and treated cells were added into the upper chamber (in 100 µl of culture medium supplemented with 10% FBS). The number of cells migrating through the membrane was manually counted after 24 hours. Assays were performed in triplicate and on two separate occasions.

The wound-healing assay (also known as the scratch assay) was used as an alternative means of measuring cell motility. Cell wounding assays were performed in 6-well plates. Cells were grown to 90% confluence and then serum-deprived for 24 hours in a serum-free medium.

A sterile 200-µl pipette tip was used to create three separate parallel scratches, and migration of the cells across the wound line was assessed after 24 hours. Photographs were taken using a charge-coupled device camera (Canon, Japan) attached to an inverted phase-contrast microscope (Zeiss, Germany) using a 20× objective lens at time 0 and 24 hours. The distance between the edges was measured and compared visually. Assays were done in triplicate and repeated at least in three different settings.

Results

CTEN is targeted by EGFR-Kras signaling

KRAS and CTEN expression

First, Kras was knocked down in SW620. This cell line contains a KRAS mutation and is a high expresser of CTEN. The knockdown of Kras in SW620 (annotated as SW620Kras-) resulted in the downregulation of CTEN (Figure 1a) when compared with scrambled controls (SW620ssc). This effect was validated in the cell line DLD1, which also contains a KRAS mutation and is a high expression of CTEN (DLD1Kras- *vs.* DLD1ssc). To further test the association between KRAS and CTEN, KRAS was knocked down in the cell line Colo205 (containing a mutation in BRAF but wild type for KRAS). If KRAS was signaling through BRAF to alter CTEN, then the knockdown of KRAS in Colo205 would not affect. In this case, the level of CTEN was

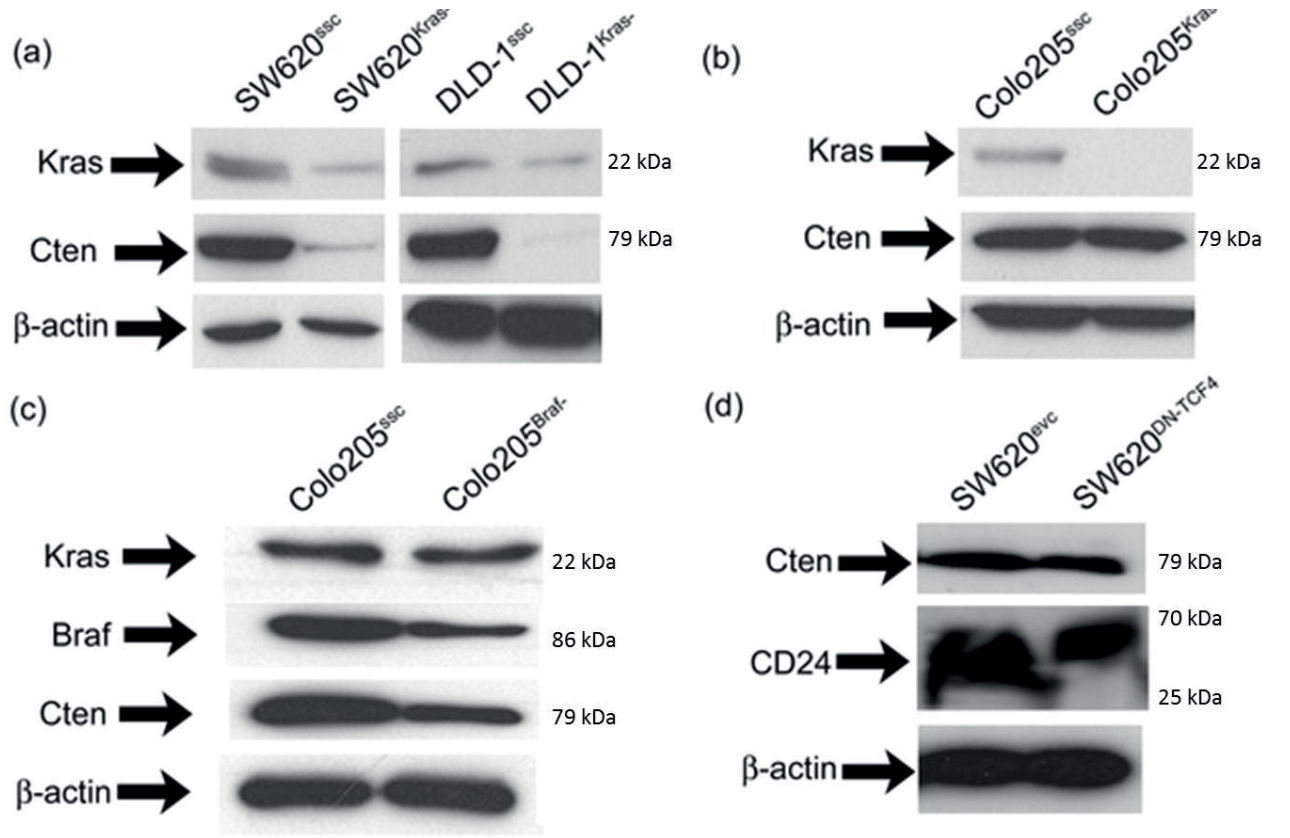

Figure 1. *Functional relationship between KRAS and CTEN. (a) Knockdown of KRAS in SW620 and DLD1 (both mutants for KRAS) resulted in a downregulation of CTEN levels (SW620Kras- vs. SW620ssc and DLD1 KRAS vs. DLD1ssc). On the contrary, (b) shows that KRAS knockdown in Colo205 (containing a BRAF V600E mutation) did not affect Cten expression (Colo205Kras- vs. Colo205ssc), whereas (c) shows that knockdown of BRAF in Colo205 did cause downregulation of Cten (Colo205Braf- vs. Colo205ssc). (d) shows that inhibition of Wnt signaling in SW620 with a dominant-negative TCF4 expression vector (SW620DN-TCF4) did not alter CTEN levels compared with the empty vector (SW620evc), whereas the levels of CD24 (a known target of Wnt signaling) were reduced.*

unaltered following KRAS knockdown (Colo205KRAS- *vs.* Colo205ssc, Figure 1b). When BRAF was knocked down in Colo205, a 52% reduction in BRAF protein was mirrored by a 50% reduction in CTEN expression (Colo205Braf- *vs.* Colo205ssc, Figure 1c), suggesting that KRAS influences CTEN through BRAF.

KRAS regulates CTEN at the transcriptional level

The data showed that CTEN is regulated, in part at least, by KRAS/BRAF signaling although it was uncertain whether this occurs through direct upregulation of transcription or inhibition of degradation. To test this, CTEN mRNA levels were measured by quantitative RT-PCR following KRAS knockdown. This showed that there was a 43% reduction in normalized CTEN mRNA levels (Figure 2a), suggesting that there was transcriptional regulation. To further validate this, cells were exposed to a proteasomal inhibitor following KRAS knockdown. This would be expected to prevent protein degradation, but it did not prevent the reduction in the levels of CTEN (Figure 2b), thus supporting the quantitative PCR results which suggested that KRAS/BRAF signaling alters the transcription of Cten to regulate Cten protein levels.

Functional interaction between KRAS and CTEN

KRAS was shown to positively regulate CTEN but, since KRAS has several hundred downstream targets, this may be a reproducible observation without any direct functional relevance to cell biology. To test whether this relationship had any functional effect, we compared the conditions of KRAS knockdown with KRAS knockdown and concomitant ectopically expressed CTEN protein. If the ectopically expressed CTEN could rescue the effects induced by KRAS knockdown, it would suggest that there was functional relevance. SW620 cells were thus cotransfected with Kras-specific siRNA duplexes and a construct causing ectopic expression of GFP-Cten (SW620Kras-/GFP-Cten) and compared with cells cotransfected with KRAS-specific siRNA and GFP expressing empty vector controls (EVCs) (SW620Kras-/evc). Experimental controls were cells co-transfected with scrambled siRNA duplexes and GFP empty vector (SW620ssc/evc) creating a condition, in which neither KRAS nor CTEN were altered (Figure 3a). Knockdown of KRAS with cotransfection of GFP empty vector significantly reduced cell motility in transwell migration assay (Figure 3b, SW620ssc/evc *vs.* SW620 KRAS −/evc, $p < 0.001$). However, ectopic expression of GFP-Cten restored the cell motility (SW620 KRAS-/evc *vs.*SW620Kras-/GFP-Cten, $p < 0.001$).

Cell wounding assays using the same transfection protocol demonstrated the same effect and validated the data (Figure 3c, $p < 0.001$). Thus, the data show that the effect of KRAS knockdown on cell motility can be rescued by the ectopic expression of CTEN, thereby confirming the functional nature of the relationship between KRAS and CTEN.

Functional KRAS/CTEN interaction in pancreatic and lung cancers

The current data have shown that, in CRC, KRAS appears to regulate CTEN and, through this, to regulate cell motility. To test whether this was a colon-specific relationship or whether it also occurred in other tumor

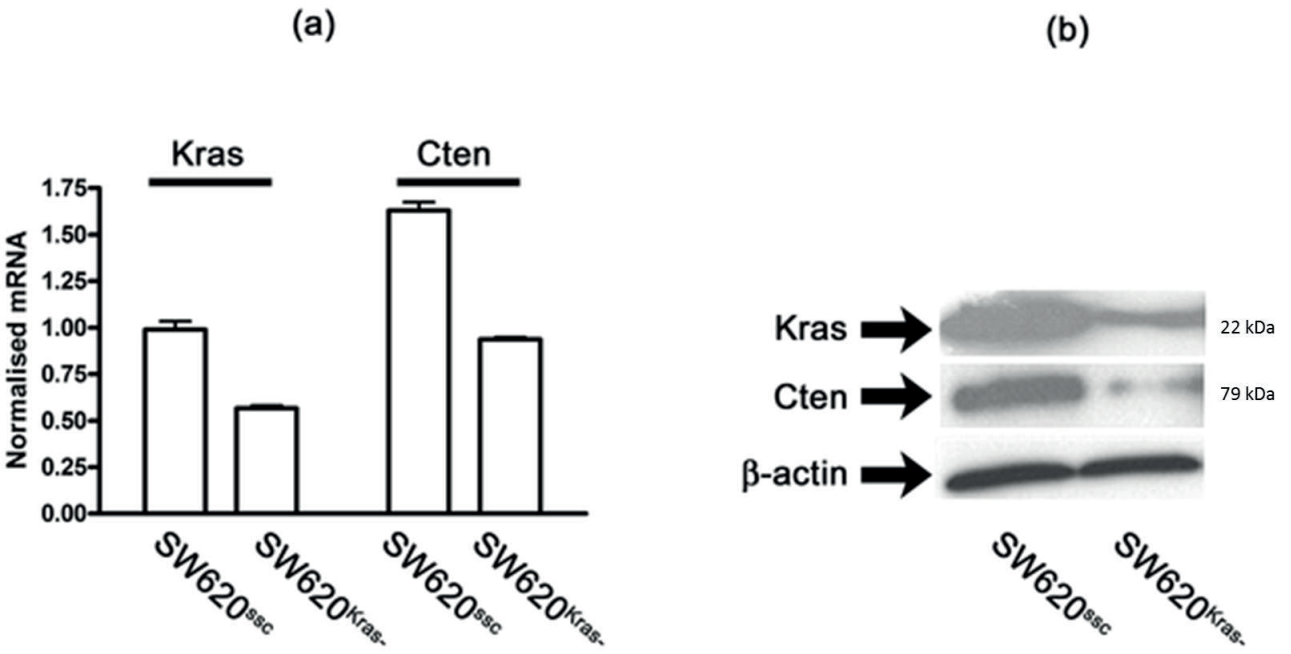

Figure 2. *KRAS regulates CTEN through transcriptional control. (a) Following KRAS knockdown in SW620, mRNA levels of both KRAS and CTEN were quantified and normalized to the housekeeping gene HPRT. There was a reduction in the level of both mRNAs (data shown from three replicates). (b) To further support this, cells were exposed to a proteasome inhibitor, and the levels of KRAS and CTEN proteins were quantified. There was a reduction in the levels of both proteins suggesting that the changes in CTEN levels following KRAS knockdown were not due to altered degradation. Controls consisting of DMSO carrier alone did not affect protein levels.*

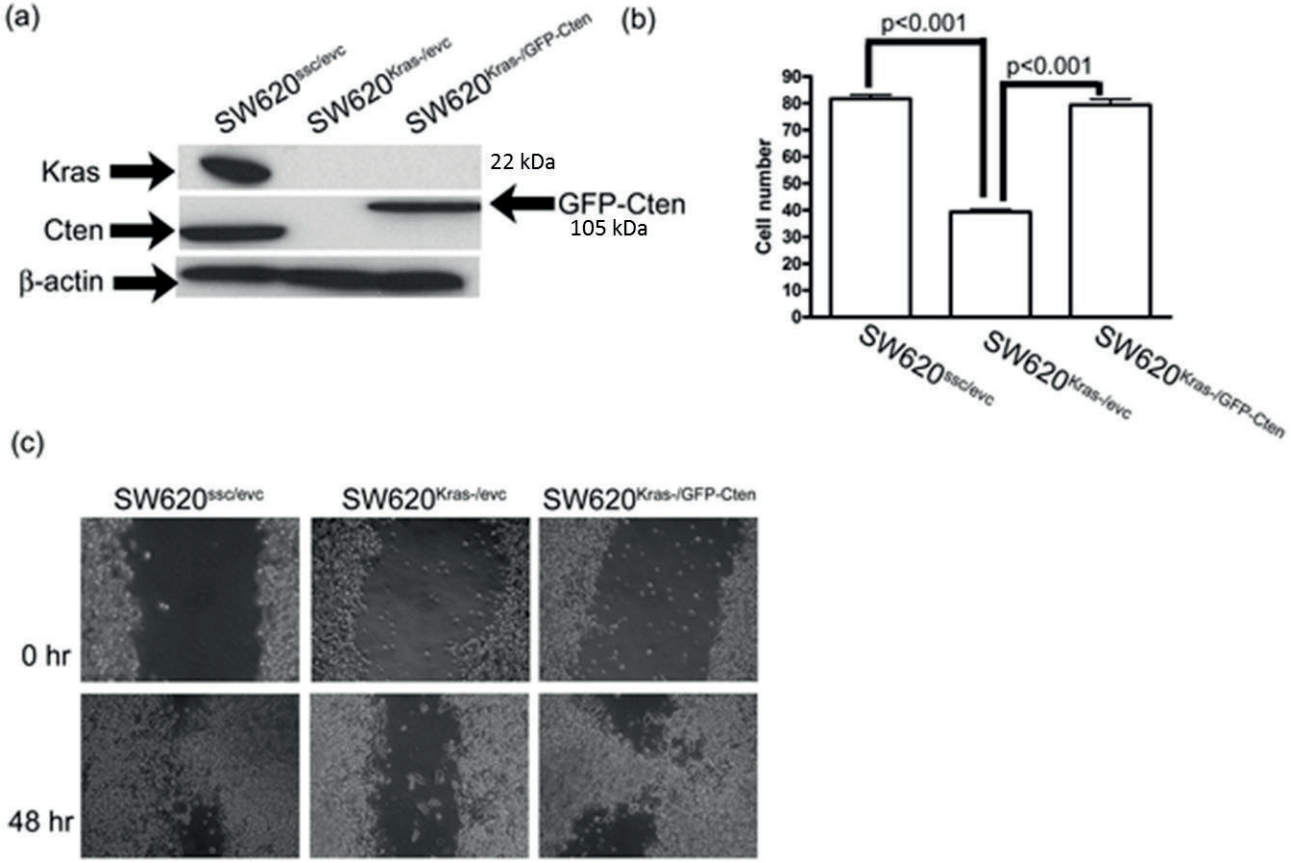

Figure 3. *The relationship between KRAS and CTEN in the colon. (a) SW620 cells were transfected with Kras-specific siRNA and cells were either co-transfected with GFP-Cten expression vector (SW620 KRAS-/GFP-Cten) to restore CTEN or GFP empty vector (SW620 KRAS −/evc). Control cells were co-transfected with scrambled controls siRNA duplexes and GFP empty vector (SW620ssc/evc). The ectopically expressed CTEN has a larger size due to the GFP tag. (b) Transwell migration assays and (c) wounding assays showing that in SW620, knockdown of KRAS inhibited cell motility which was rescued by the ectopic expression of CTEN. (EVC = empty vector control, GFP = Green fluorescent protein, SSC = sequence scrambled controls).*

types, the experiments were repeated in cell lines derived from pancreatic and lung cancers. Colo357 and PSN1 are both pancreatic cancer cell lines that show high CTEN expression and are mutant for KRAS. The knockdown of KRAS in both cell lines resulted in the downregulation of CTEN compared with scrambled controls (Figure 4a). A second siRNA duplex targeted to KRAS was also tested to preclude "off-target" effects, and this also showed inhibition of CTEN expression (Figure 4b). Functional studies to further test this relationship were performed in PSN1, and once again, cotransfections were used to create the conditions, whereby KRAS alone was knocked down (PSN1Kras−/evc), KRAS was knocked and CTEN restored (PSN1 KRAS-/GFP-CTEN), and neither was altered (PSN1ssc/evc). The data paralleled those in the CRC cell lines and KRAS knockdown were demonstrated to inhibit cell motility (PSN1 KRAS-/evc *vs.* PSN1ssc/evc, $p < 0.001$), whereas this could be rescued by the restoration of CTEN expression (PSN1KRAS-/evc *vs.*PSN1KRAS-/GFP-CTEN, $p < 0.001$, Figure 4c). The same findings were obtained in the third model of which further supports the proposed relationship between KRAS and CTEN in cancer.

The cell lines A549 and H226, both are derived from non-small cell lung cancer, and both show high CTEN expression and are mutant for KRAS. The knockdown of KRAS in both cell lines resulted in the downregulation of CTEN compared with scrambled controls (Figure 5a). Functional studies were performed to further test this relationship, and once again, cotransfections were used to create the conditions, whereby KRAS alone was knocked down (A549KRAS−/evc and H226KRAS-/evc), KRAS was knocked and CTEN restored (A549KRAS-/GFP-CTEN and H226KRAS-/GFP-CTEN), and neither was altered (A549ssc/evc and H226ssc/evc). The data paralleled those in the CRC and pancreatic cancer cell lines and KRAS knockdown were demonstrated to inhibit cell motility (A549KRAS-/evc *vs.*A549ssc/evc, $p < 0.001$ and H226KRAS-/evc *vs.*H226ssc/evc, $p = 0.001$), whereas this could be rescued by restoration of CTEN expression (A549KRAS-/evc *vs.*A549KRAS-/GFP-CTEN, $p < 0.001$, and H226KRAS-/evc *vs.*H226KRAS-/GFP-CTEN, $p = 0.002$) (Figure 5b).

Regulation of CTEN expression by the EGFR signaling pathway

To test a hypothesis that CTEN is regulated by the EGFR-KRAS-Braf axis in CRC, we sought to investigate the effect of EGFR on CTEN in CRC cell lines. For this experiment, the C32 CRC cell line was selected as it

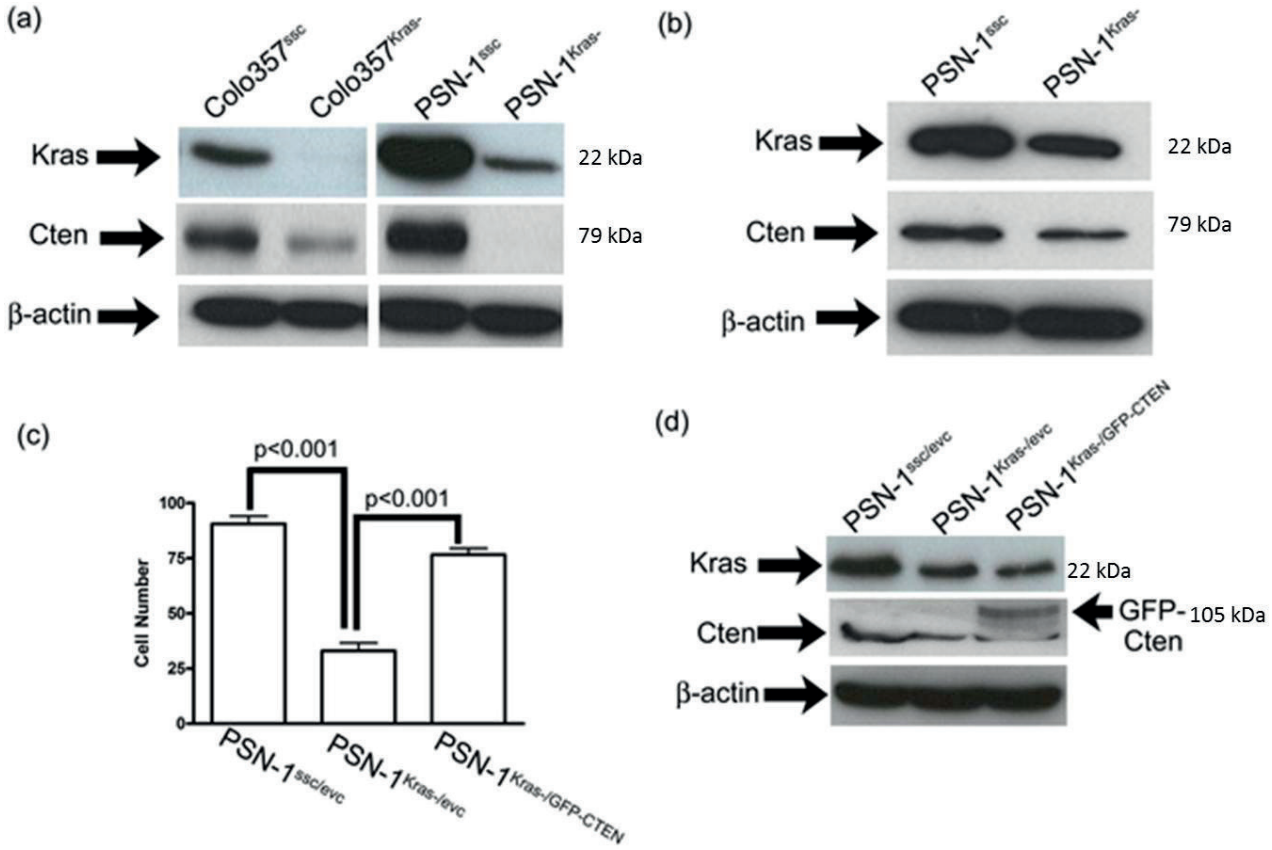

Figure 4. *The relationship between KRAS and CTEN in the pancreas. Experiments to test the relationship between KRAS and CTEN were repeated in pancreatic cancer cell lines. (a) shows that when KRAS was knocked down in Colo357 and PSN1, this caused downregulation of CTEN (Colo357KRAS- vs.Colo357ssc and PSN-1KRAS- vs.PSN-1ssc). To obviate any confounding off-target effects, a second anti-KRAS siRNA duplex was used. (b) shows that the second KRAS-specific duplex also resulted in a reduction in both KRAS levels and CTEN levels. (c) shows that, as observed in the colon, in pancreatic cell lines, knockdown of KRAS inhibited motility (PSN-1KRAS-/evc vs.PSN-1ssc/evc), and this could be rescued by transfection of GFP-tagged CTEN (PSN-1KRAS-/evc vs. PSN-1KRAS-/GFP-CTEN). (d) is confirmation of the changes induced by the gene knockdown/forced expression in the rescue experiments.*

is wild type for KRAS; the experiments would require stimulation and inhibition of the EGFR and this would not be possible in cell lines which contained mutant KRAS. The C32 cells were stimulated with recombinant EGF (10 ng/ml) or DMSO control. Stimulation using EGF resulted in upregulated KRAS as well as CTEN when compared to DMSO. The upregulation of CTEN was accompanied by a downregulation of tensin 3, the "tensin switch."

As an extra control, experiments were also undertaken in the SW620 cell line which is mutant for KRAS and thus would not be expected to respond to stimulation with EGF. No effect in either CTEN or KRAS was seen in SW620 following stimulation with EGF (Figure 6) or with DMSO.

To further validate these data, the converse experiment was performed by treating C32 cells with an EGFR inhibitor, PD153035. The stimulation of C32 cells with PD153035 (10 μM) resulted in the downregulation of both KRAS and CTEN although there was no change in Tensin 3 (Figure 6a). These experiments were also performed on SW620, and no effect was found following stimulation with PD153035. To conclude these experiments, it was necessary to show a functional effect of EGF stimulation on cell motility and confirm that it could be negated by the knockdown of CTEN. Thus, C32 cells underwent knockdown of CTEN, and cells were harvested after 48 hours.

These were then tested using the transwell migration assay in two sets, with and without EGF mixed with the media in the upper chamber with a concentration of 20 ng/ml. C32 cells transfected with anti-CTEN siRNA (C32CTEN-) had reduced cell motility following EGF stimulation compared to cells transfected with scrambled control (C32ssc). Other controls showed that, as expected, EGF did stimulate cell motility (C32ssc/EGF *vs.*C32ssc/DMSO) and knockdown of CTEN did cause a reduction in cell motility (C32ssc /DMSO *vs.*C32CTEN-/DMSO) (Figure 7).

Wnt signaling does not regulate CTEN

We have previously shown that there is early upregulation of CTEN, i.e., during the adenomatous phase of tumor development, in CRC. This raises the possibility that CTEN may be a target of Wnt signaling. To extend the observations, we tested the role of Wnt signaling in regulating CTEN by transfecting a construct expressing dominant-negative DN-TCF4 into SW620. To confirm the efficacy of the DN-TCF4 expression, we evaluated its effect on CD24 expression by

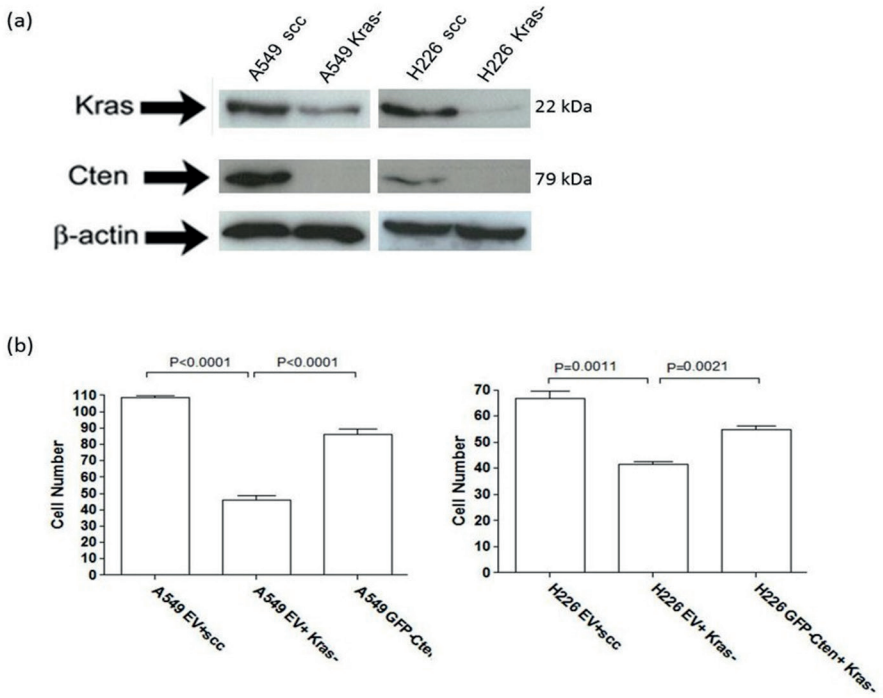

Figure 5. *The relationship between KRAS and CTEN in the lung. Sam experiments were repeated in lung cancer cell lines. (a) shows that when KRAS was knocked down in A549 and H226, this caused downregulation of CTEN (A549KRAS- vs.A549ssc and H226KRAS- vs.H226ssc). (b) shows that, as observed in the colon and pancreases, in lung cell lines, knockdown of KRAS inhibited motility (A549KRAS-/evc vs.A549ssc/evc), and this could be rescued by transfection of GFP-tagged CTEN (A549KRAS-/evc vs.A549KRAS-/GFP-CTEN). This was also true in the H226 cell line too.*

quantitative Western blot. CD24 is a well-described target of Wnt signaling (18,19), and transfection of DN-TCF4 into SW620 resulted in a 40% reduction in the expression of CD24 (18,19). However, the transfection of DN-TCF4 into SW620 did not influence CTEN expression (Figure 1d), suggesting that Wnt signaling does not play a role in regulating CTEN expression.

The negative Effect of STAT3 on CTEN expression

To investigate whether STAT3 was a regulator of CTEN, we modulated the levels of STAT3 and measured the effects on levels of CTEN. Once again, we used a dual approach: STAT3 was knocked down in cell lines with targeted siRNA and STAT3 was activated by stimulating cell lines with IL-6. The knockdown of STAT3 increased the levels of CTEN protein expression in the CRC cell lines DLD1 and SW620. This suggests that STAT3 is an inhibitor of CTEN. Since activated STAT3 acts as a transcription factor, the levels of CTEN mRNA were measured following the STAT3 knockdown. Consistent with the Western blot data, CTEN mRNA levels were found to be increased, suggesting that STAT3 may act as a transcriptional repressor of CTEN. IL-6 is a cytokine which is known to stimulate STAT3 expression and activation (20). SW620 was stimulated with IL-6, and STAT3 levels were shown to be increased.

Consistent with the STAT3 knockdown experiments, stimulation with IL-6 resulted in the downregulation of CTEN protein (Figure 8). Taking these data together, we can conclude that, on the contrary to the data published by Barbieri et al. (13) STAT3 is a negative regulator of CTEN.

CD24 is an upstream regulator of CTEN

We hypothesized that CD24 may be a direct regulator of CTEN. To test this hypothesis, we used a dual approach of forced expression and gene knockdown of CD24 in CRC cell lines and measured the effect of modulation of CD24 levels on CTEN expression. The CRC cell lines DLD1 and SW620 have a high expression of both CD24 and CTEN. Knockdown of CD24 resulted in the downregulation of CTEN expression in both cell lines (Figure 9a). The reciprocal experiment of CD24 forced expression was performed in the CRC cell line HCT116, which expresses neither CD24 nor CTEN. The CD24 expression construct, CD24-pcDNA3.1was transfected into HCT116, and CTEN was found to be induced (Figure 9b). These data suggest that CD24 is a positive regulator of CD24 although, interestingly, the alterations in CTEN were not accompanied by a tensin switch as was seen following activation of the EGFR pathway. To further investigate the relationship between CD24 and CTEN, both genes were knocked down individually in DLD1 and SW620, and the effects on transwell migration were measured. Cell motility was found to be reduced after knockdown of both CTEN and CD24. The effect of CTEN knockdown was found to be greater than the effects of CD24 knockdown, which suggests that CTEN activity is not dependent on CD24 (Figure 9c).

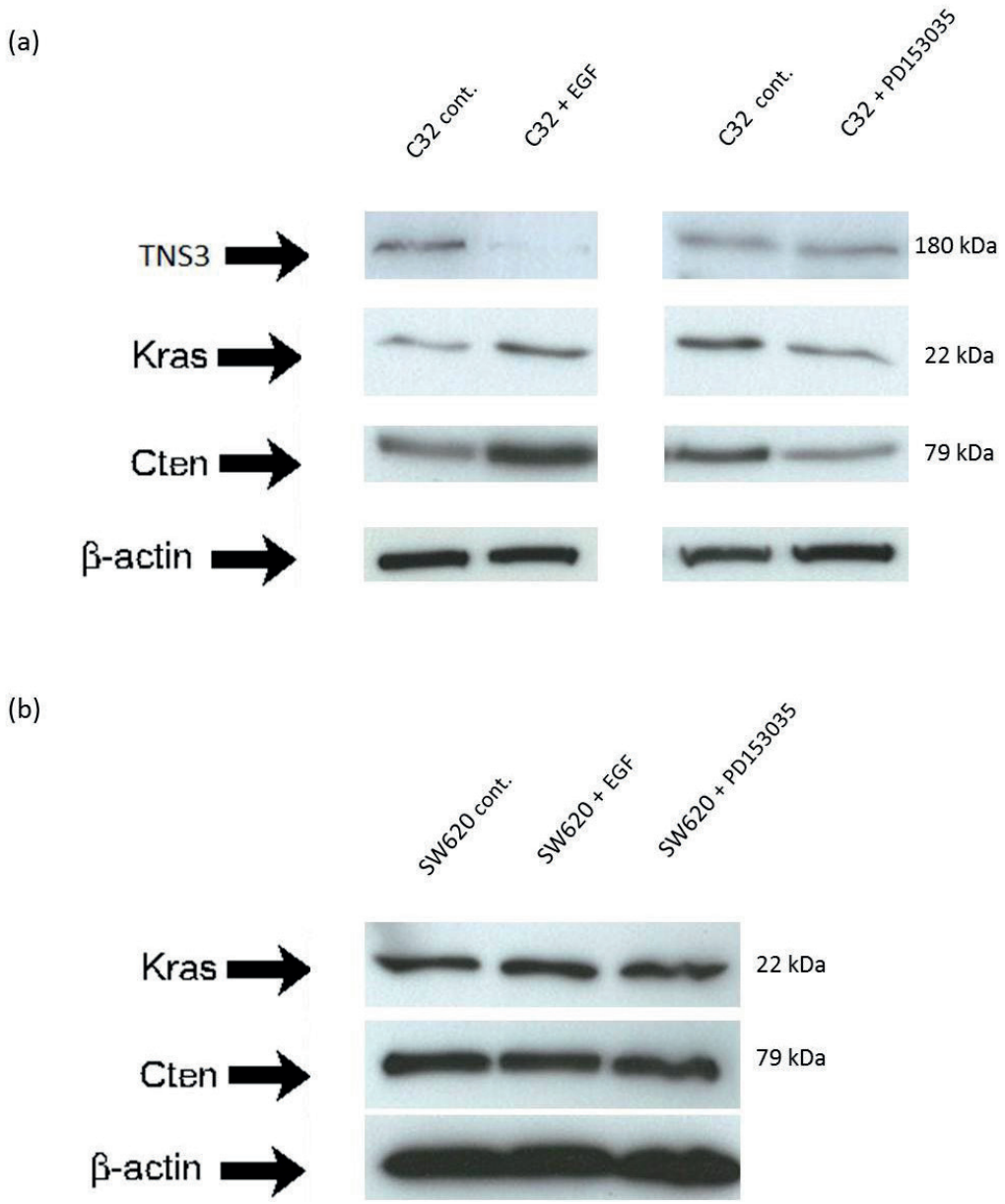

Figure 6. *The relationship between EGFR and CTEN.(a) Stimulation of C32 using EGF resulted in a downregulation of TNS3 and upregulation in KRAS and CTEN levels, whereas inhibition using PD153035 shows downregulation of KRAS and CTEN with no effect on TNS3. (b) shows that neither EGF nor PD153035 affects KRAS and CTEN in Colo205 (containing a KRAS mutation).*

Discussion

Little is known about the mechanism of CTEN regulation, and in this study, we investigated several potential regulatory pathways. One of the working hypotheses was that of an EGFR/KRAS/Braf/CTEN signaling pathway. The data have demonstrated first that CTEN is a true target of KRAS/Braf signaling since (i) knockdown of KRAS results in the downregulation of CTEN in two cell lines which are mutant for KRAS and (ii) knockdown of KRAS in a cell line mutant for BRAF has no effect on CTEN expression, whereas knockdown of Braf in this cell line does result in the downregulation of CTEN. Furthermore, the quantification of CTEN mRNA and the use of proteasomal inhibitors to prevent protein degradation suggested that the level of control lays at CTEN transcription. Since there are a large number of reported targets of KRAS and it is unlikely that they will all be functionally relevant (21), we have shown that the relationship between KRAS and CTEN is functionally important since inhibition of motility following KRAS knockdown can be rescued by the ectopic expression of CTEN. Furthermore, we have demonstrated that the interaction between KRAS and CTEN is similar in pancreatic and lung cancer, suggesting that this is a generic relationship that is not limited to CRC. We can conclude that CTEN is a target of KRAS/Braf signaling although it is clear that other mechanisms are controlling CTEN expression since we have identified occasional cell lines that are mutant for KRAS but which show low

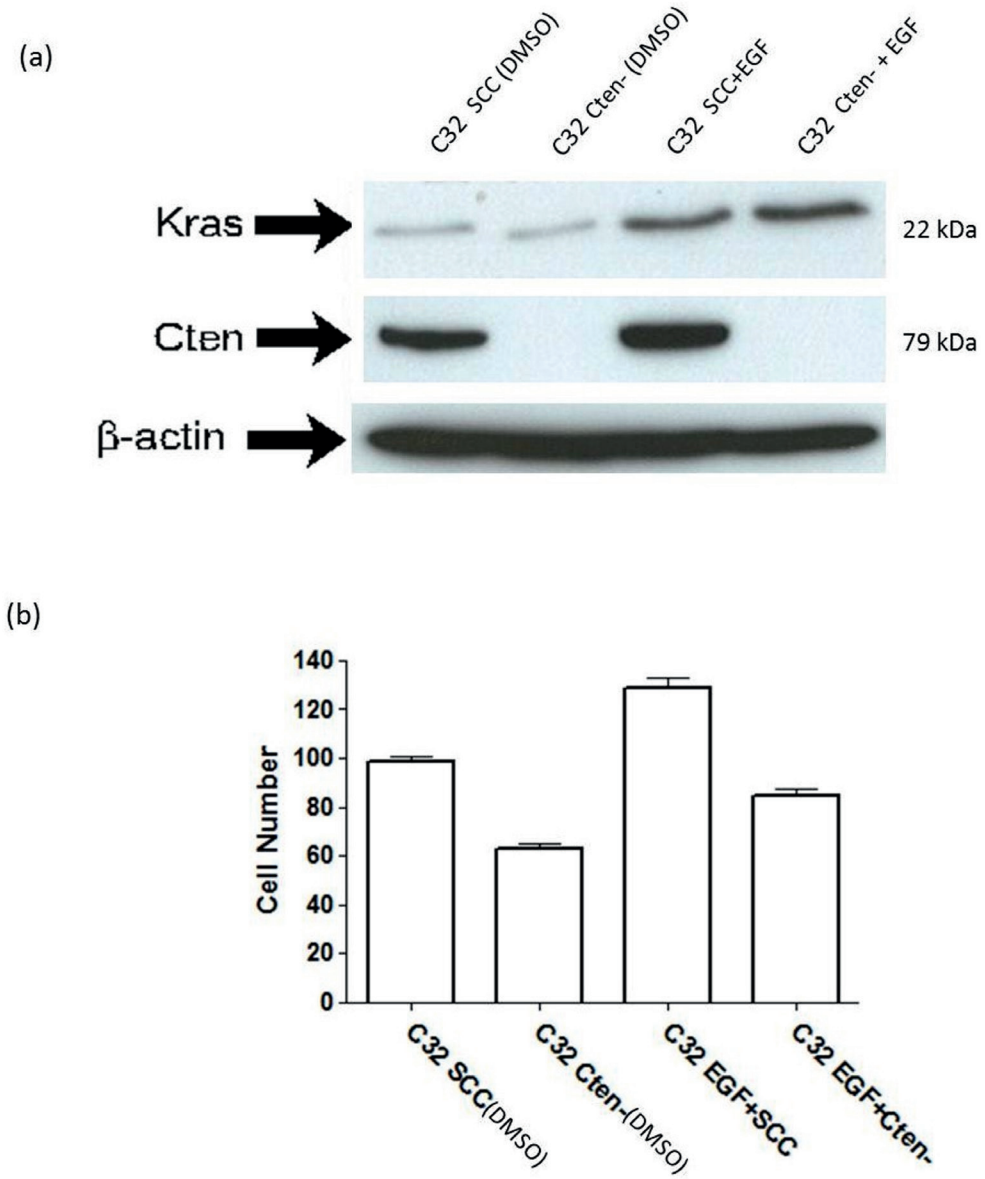

Figure 7. *Functional relationship between EGFR and CTEN. (a) Stimulation of C32 using EGF in combination with CTEN knockdown resulted in upregulation in KRAS and CTEN levels if it is there. (b) shows that the cell motility affected by both EGF and CTEN siRNA.*

levels or non-expression of CTEN. Conversely, there are cell lines that are wild type for KRAS/BRAF but which have high levels of CTEN. Currently, we can speculate that when CTEN expression is elevated in a tumor with KRAS/BRAF mutation, the association is likely to be causal. We tested whether the EGFR signaling pathway was specifically involved in regulating CTEN by either stimulating CRC cell lines with EGF or inhibiting EGFR signaling with the inhibitor PD153035. These experiments had to be conducted in cell lines which were wild type for KRAS, but the inclusion of cell lines that are mutant for KRAS allowed extra control to be used as these cell lines would be expected to be resistant to the effects of altered EGFR signaling. The data confirmed that CTEN was indeed a specific target of EGFR signaling and that, as with breast cancer, EGFR mediated activation of CTEN was accompanied by a "tensin switch," whereby the upregulation of CTEN is accompanied by downregulation of tensin 3. KRAS acts as a secondary messenger for a large number of receptor tyrosine kinases in addition to EGFR, and many of these, on ligand binding, can stimulate cell motility (22). As well as raising some fascinating questions about the role of CTEN in regulating cell motility, the data also have some therapeutic implications. The use of biologics targeted to the EGFR can have a dramatic effect on cancer, resulting in a marked reduction in tumor size and clinical downstaging (23). However, as would be expected, tumors containing KRAS/BRAF mutations are refractory to the therapeutic effects of anti-EGFR antibodies. If future studies confirm that CTEN is a part of the EGFR-KRAS signaling pathway, it may represent a new therapeutic option for the significant number of cancers containing KRAS/BRAF mutation in the colon,

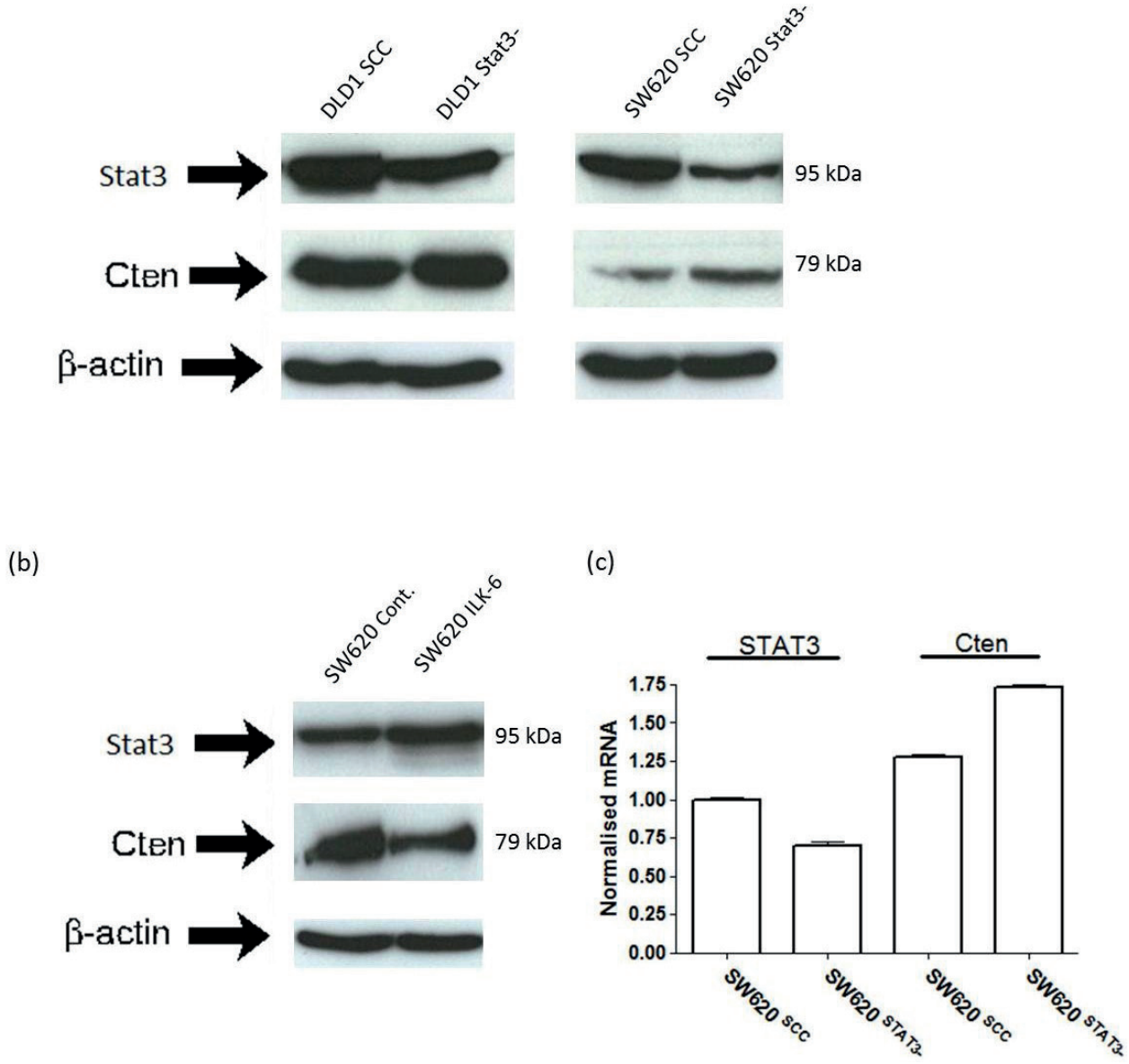

Figure 8. *STAT3 and CTEN expression. (a) STAT3 knockdown in DLD1 and SW620 results in the upregulation of CTEN expression, and (b) stimulation of STAT3 using IL-6 results in the downregulation of CTEN expression compared to the control. (c) Following STAT3 knockdown in SW620, mRNA levels of both STAT3 and CTEN were quantified and normalized to the housekeeping gene HPRT. There was increasing in the level of both CTEN mRNAs associated with the reduction of the STAT3 level.*

pancreas, and lung, and therefore, ineligible for anti-EGFR therapy. Published studies have suggested that STAT3 may also be an upstream regulator of CTEN (13). The findings, using both STAT3 knockdown and IL-6 stimulation (to activate STAT3), showed that it is probably a negative regulator of CTEN. Part of this effect could be due to STAT3 mediated inhibition of KRAS. The data contradict the finding of Barbieri et al. (13) which may just be a reflection of different experimental systems. Barbieri's work was done in mice and breast tumor cells (MCF-10). The relationship between CTEN and STAT3 is complex since Kwon et al. have shown that mutations in the SH2 domain of CTEN increased the expression of the phospho-STAT3 (14), suggesting that CTEN has a negative downstream effect on STAT3 activation. This is supported by the findings in phosphokinase array which showed increases in the expression of phospho-STAT3 in response to knockdown of CTEN. This may represent a possible feedback loop in the pathway linking STAT3 and CTEN, and the complexity of this pathway is further shown by the contradictory role of STAT3 as it is reported as a tumor suppressor and promoter (24). The findings have suggested that CD24 may positively regulate CTEN expression although the precise mechanism for this is still unknown although it is a strong possibility that it would be mediated through integrins. On the contrary to EGFR signaling, the CD24 activation of CTEN is not associated with the tensin switch, and thus, it may represent an alternative pathway. We have identified three possible pathways of CTEN regulation. All three will likely interact, possibly with other regulators, and the net effect of these interactions will dictate the levels of CTEN expression.

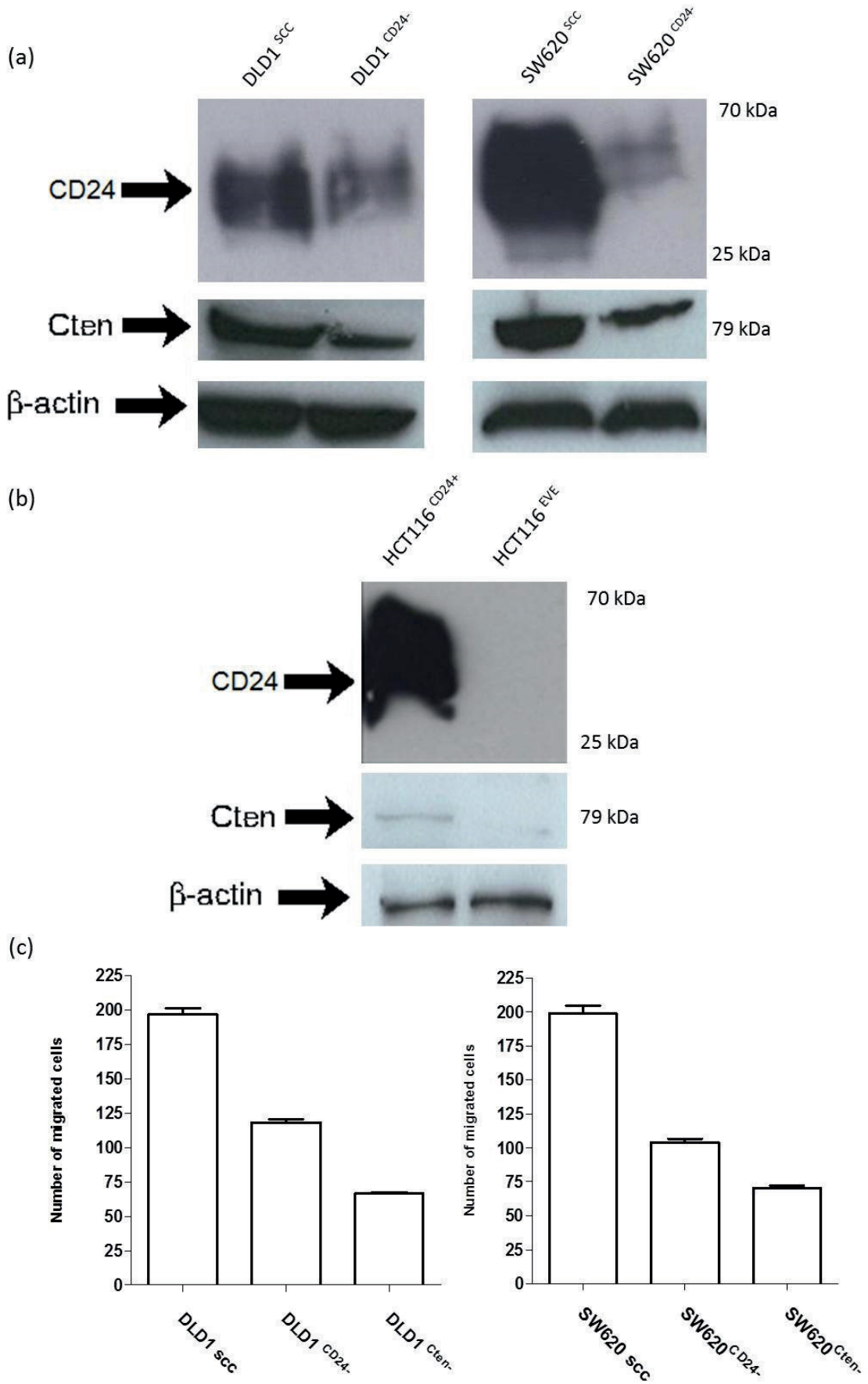

Figure 9. *CD24 and CTEN expression. (a) CD24 knockdown in DLD1 and SW620 results in the downregulation of CTEN expression, and (b) ectopic transfection CD24 plasmid results in the upregulation of CTEN expression compared to the SCC in HCT116 which negative for CTEN.(c) shows that both CTEN and CD24 knockdown on the cell motility with greater effect for CTEN*

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Ethical approval
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Consent for publication
Informed consent was obtained from the patients.

Author details
Saleh AlGhamdi[1,2], Salih Ibrahim[1,3], Kanwal Balloch[1], Darryl Jackson[1], Mohammad Ilyas[1]
1. Department of Pathology, College of Medicine, University of Nottingham, Nottingham, United Kingdom
2. Clinical Research Department, ACRA, Research Center, King Fahad Medical City, Riyadh, Saudi Arabia
3. Department of Biochemistry, College of Medicine, Kirkuk University, Iraq

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