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CTEN prolongs signaling by EGFR through reducing its ligand-induced degradation

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Abstract

Activation of epidermal growth factor receptor (EGFR) triggers signaling pathways regulating various cellular events that contribute to tissue development and function. Aberrant activation of EGFR contributes to tumor progression as well as therapeutic resistance in cancer patients. CTEN (TNS4) is a focal adhesion molecule that is a member of the tensin family. Its expression is upregulated by EGF and elevated CTEN mediates EGF-induced cell migration. In the presence of CTEN, we found that EGF treatment elevated the level of EGFR protein but not mRNA. The extended half-life of activated EGFR sustained its signaling cascades. CTEN reduced ligand-induced EGFR degradation by binding to the E3 ubiquitin ligase c-Cbl and decreasing the ubiquitination of EGFR. The SH2 domain of CTEN is not only required for binding to the phosphorylated tyrosine residue at codon 774 of c-Cbl, but is also essential for the tumorigenicity observed in the presence of CTEN. Public database analyses indicated that CTEN mRNA levels are elevated in breast, colon, lung, and pancreas cancers, but not correlated with EGFR mRNA levels in these cancers. In contrast, immunohistochemistry analyses of lung cancer specimens showed that CTEN and EGFR protein levels were positively associated, in support of our finding that CTEN regulates EGFR protein levels through a posttranslational mechanism. Overall, this work defines a function for CTEN in prolonging signaling from EGFR by reducing its ligand-induced degradation.

Keywords

CTEN; TNS4; EGFR; c-Cbl; ubiquitination

INTRODUCTION

Epidermal growth factor receptor (EGFR) tyrosine kinase engages a vast array of signaling pathways to regulate tissue development and homeostasis (1, 2). EGFR signaling is normally induced by ligand binding (such as EGF), leading to receptor dimerization, autophosphorylation, activation of downstream signaling molecules and cellular events such

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as proliferation, migration, and differentiation. Given its importance, EGFR signaling is tightly regulated. Ligand binding not only induces receptor activation but also triggers suppression mechanisms to ensure precise control of EGFR signaling output (3). One of the negative regulations is governed through ubiquitin-dependent EGFR degradation, which is largely mediated by the RING finger E3 ubiquitin ligase c-Cbl. c-Cbl binds to tyrosine-phosphorylated EGFR through its tyrosine-kinase-binding (TKB) domain, allowing recruitment of ubiquitin conjugating enzymes and transfer of ubiquitin to phosphorylated EGFR. Ubiquitination of EGFR then facilitates its internalization and degradation in lysosomes (4). Disruption of this negative regulatory system could trigger cellular events that contribute to tumor formation.

Focal adhesions connect extracellular matrix to cytoskeletal networks and play critical roles in cell adhesion, migration, proliferation, and survival. They are also involved in crosstalk with growth factor receptors, such as EGFR, to elicit a wider range of cellular responses. CTEN (C-terminal tensin-like) is the smallest protein in the tensin focal adhesion family (5) known to regulate cell adhesion and migration (6). Little, if any, CTEN is expressed in normal tissues including breast, lung, ovary, and colon (7). However, its expression is profoundly increased when these tissues develop tumors. In colon cancer, 76% of patients' tumor samples exhibit significantly elevated CTEN protein levels (8), which is associated with poor prognosis (9). In invasive breast cancer, CTEN expression correlates with high EGFR and HER2 levels, as well as with metastasis to lymph nodes (6), and is associated with poor prognosis (10). Manipulation of CTEN protein levels alters cell invasion, epithelial mesenchymal transition (EMT), and colony formation capacities of colon cancer cells (8, 11). Activation of EGFR by EGF leads to an expressional switch from tensin 3 (TNS3) to CTEN, and this up-regulation of CTEN with reduced tensin 3 contributes to EGF-promoted mammary cell migration (6). MEK activity is required for EGF-induced CTEN overexpression (6). Consistently, it is reported that gain-of-function mutations in K-RAS and B-RAF (both upstream molecules of MEK) are associated with CTEN overexpression (12). Altogether, these findings closely link CTEN to EGFR signaling cascades.

In the present study, we have identified a novel role for CTEN in EGFR regulation. Our data indicate that CTEN suppresses ligand-induced EGFR degradation by reducing receptor ubiquitination, which is accomplished via the ability of CTEN to interact with c-Cbl in a phosphotyrosine-SH2 dependent manner. Overexpression of CTEN enhances cancer cell migration, invasion, as well as colony formation activities, which can be reversed by EGFR inhibitor. In addition, the SH2 domain of CTEN is essential for promoting these tumorigenic properties. The clinical relevance of our findings is supported by the up-regulation of CTEN mRNA in a variety of cancer types and the correlation of elevated CTEN and EGFR protein levels in lung cancer.

MATERIALS AND METHODS

Reagents and plasmid constructs

Antibodies against EGFR, c-Cbl, ubiquitin (EMD Millipore, Billerica, MA, USA), ERK1/2, pY845-EGFR, pY992-EGFR, pY1045-EGFR, pY1068-EGFR, pY1173-EGFR, and phospho-tyrosine (Cell signaling Technology, Danvers, MA, USA), Flag (Sigma-Aldrich, St Louis, MO, USA), phospho-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were purchased from indicated commercial suppliers. Recombinant human EGF was purchased from Promega Corporation (Madison, WI, USA). Lactacystin was purchased from Sigma-Aldrich. Cetuximab (Erbitux™) was from ImClone System Incorporated (NJ, USA). Flag-tagged CTEN full-length, N-terminal, C-terminal plasmids were constructed by PCR amplification of the GFP-CTEN (13). PCR products were then directionally cloned into *Eco*RI and *Bam*HI sites in the vector, pFLAG-CMV-2 (Sigma-Aldrich). HA-tagged c-

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Cbl mutants were constructed by site-directed mutagenesis with the HA-tagged wild-type c-Cbl.

Cell culture and DNA transfection

All cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and authenticated by ATCC by cytogenetic analysis this established provider. They were cultured and used within 6 months after resuscitation in appropriate media: Dulbecco's modified Eagle's medium (DMEM) 4.5 g/L glucose for HEK 293T, SW480 and A549 cells; RPMI-1640 for 5637 cells; McCoy's 5A medium for HCT116 cells supplemented with 10% FBS (Sigma-Aldrich), 1% penicillin/streptomycin, and 2 mM L-glutamine. All cells were maintained at 37 °C in humid air with 5% CO2 condition. For the EGF-treatment experiments, cells were starved in serum free media for 24h and then replaced with media containing EGF, prepared freshly from an aqueous 100 mg/ml recombinant human EGF solution. Transfections were performed using Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol.

RNA extraction and RT-qPCR

Total cellular RNAs were extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the instructions of the user manual. cDNAs were generated from 2 ug RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analyses were performed with Sybr Green PCR Master Mix using PCR primers on an ABI Prism HT7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). EGFR primer sequences were as follows: 5′-CGGGACATAGTCAGCAGTG-3′ (forward) and 5′-GCTGGGCACAGATGATTTTG-3′ (reverse). Relative EGFR mRNA expression was determined by dividing the threshold of each sample by the threshold of beta-actin, solving for 2^x and adjusting the relative value to the whole number.

Coimmunoprecipitation

Cell lysates of each 100-mm dish were prepared in 1 ml NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA) supplemented with 1X protease and phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) by gentle pipetting. The cell lysates were then incubated on ice for 20 min and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants were collected and precleared with protein A-agarose for 30 min at 4°C with agitation. The precleared aliquots were then immuneprecipitated with M2 Flag affinity beads or specific antibodies-conjugated agarose for 2–4h at 4°C with agitation. Beads were washed three times by using lysis buffer supplemented with 300 mM NaCl, and pellets were boiled for 5min in 10 μl 2X SDS sample buffer for the analysis.

GST-pull down assays

The GST fusion proteins containing the SH2 domain or PTB domains of CTEN have been previously described (14). GST fusion proteins, or GST alone, were expressed in *Escherichia coli* BL21 strain and affinity purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. For *in vitro* pull down assay, 750 ug of EGF-treated whole-cell extracts in NP-40 lysis buffer from HEK 293T cells were added to 25ug of affinity-purified fusion proteins bound to glutathione-Sepharose beads. The bound proteins were separated by electrophoresis and examined by immunoblotting. The peptide competition assay was performed by preincubating the GST fusion proteins, which bound to the glutathione-Sepharose, with 100 ug of indicated peptides or phosphopeptides before adding the cell

lysates. The peptides and phosphopeptides were commercially synthesized (GenScript, Piscataway, NJ, USA): c-Cbl Y700, CGEEDTEYMTPSSR (700XP), CGEEDTEpYMTPSSR (700P); c-Cbl Y774, CENEDDGpYDVPKPP (774XP), CENEDDGpYDVPKPP (774P). The purities of all peptides were>80% and their composition were verified by mass spectrometry.

Migration, invasion, and colony formation assays

HCT116 cells (5×10^4 cells) were transfected with EGFP-CTEN wild-type, R474A mutant, or control vector for 4–6h before seeding onto the upper chamber of a Transwell (8 um pore size, BD Bioscience, San Jose, CA, USA). The cells in the upper chamber were maintained in serum-free medium and the lower chamber was filled with culture medium supplemented with 10 % FBS. After 24h, the non-migrating cells were removed from the upper surface of the membrane by scrubbing and the migrated cells on the lower surface were stained with crystal violet (0.25% crystal violet, 3.7% formaldehyde, 80% methanol) and counted. For transwell invasion assay, the transfected cells were seeded onto the upper chamber of a Transwell. The experimental process is comparable to the transwell migration assay described above, except that the membrane of the Transwell was pre-coated with 2 ug per well serum-reduced matrigel (BD Bioscience), and invasion was assessed by cell counting after 48h incubation. For colony formation assay, cells (5×10^3 per well) were seeded in 6-well plates and subjected to drug selection for 2 to 3 weeks, with G418 (400ug/ml) selective medium refreshed every 3 days. The stable G418 resistant colonies were counted after staining with crystal violet, washed with water, and air-dried. For EGFR inhibition, 5 μg/ml of Cetuximab was added to both upper and lower chambers in migration and invasion assay. In colony formation assay, 5 μg/ml of Cetuximab was added to the selection medium to inhibit EGFR signaling.

Microarray Analysis

The gene expression datasets were downloaded from the NCBI Gene Expression Omnibus (GEO). We chose four different types of cancer datasets that have balanced number of disease and control samples in each dataset. Figure 5 showed the gene expression datasets that were used in this study, including breast cancer (GSE15852), colon cancer (GSE10950), non-small cell lung cancer (GSE12236), and pancreatic ductal adenocarcinoma (GSE28735). Partek Genomics Suite (Partek software, version 6.6 Beta) was used to analyze gene expression values and to generate dot plots. Expression differences between tumor and adjacent normal tissues were assessed by 1-way ANOVA. Statistical analysis was carried out using Student's t-test. A p-value <0.05 was considered significant. All reported p-values are two-tailed.

Immunohistochemistry and evaluation

Human lung cancer (8 small cell carcinomas, 13 adenocarcinomas, 18 Squamous cell carcinomas, 9 other type non-small cell carcinomas) and adjacent paired tissue microarray slides were purchased from Pantomics Inc (San Francisco, CA, USA). The formalin-fixed, paraffin-embedded tissue slides were first incubated at 65°C for 30 min. Then the samples were deparaffinized and placed in a pressure cooker containing 10mM buffered sodium citrate solution (pH 6.0) for antigen retrieval. The slides were immersed in 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase activity. After that, the slides were blocked before being incubated overnight at 4°C with 1:50 diluted rabbit monoclonal anti-CTEN antibody (Spring Bioscience, Fremont, CA, USA) or with mouse monoclonal anti-wild type EGFR antibody (DAK-H1-WT from Dako, Carpinteria, CA, USA). Detection was performed on next day with streptavidin-biotinylated peroxidase-conjugated reagents with 3-amino-9-ethyl carbazole (AEC) as the chromogen (Vector Laboratories Inc., Burlingame, CA, USA). The samples were observed using a Zeiss Axioplan2 optical microscope imaging

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system with a real-colour AxioCam high-resolution charge-coupled device camera (Carl Zeiss MicroImaging, Thornwood, NY, USA). Slides were evaluated and the staining intensity was ranked on a scale ranging from 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong intensity staining). The score of staining >1 was identified as up-regulated in CTEN or EGFR expression. Statistical analysis was carried out using GraphPad Prism (version 5.01, GraphPad Software Inc., La Jolla, CA, USA). The statistical relationships between CTEN and EGFR of IHC data were evaluated through the two-sided Fisher's exact test. *P* values less than 0.001 were considered statistically highly significant.

RESULTS

CTEN expression modulates EGFR protein levels during EGF treatment

It has previously been shown that EGF-induced CTEN up-regulation promotes cell migration (6). To further investigate the potential role of CTEN in EGFR signaling and its regulation mechanism, recombinant CTEN was expressed in HEK 293T cells, which did not express endogenous CTEN. Then cells were treated with various concentrations of EGF for 1 hr. As shown in Figure 1A, EGF-induced EGFR down-regulation was significantly reduced in the presence of CTEN. At this 1 hr time point, maximal suppression of EGFR down-regulation by CTEN was observed at 10 ng/ml EGF treatment. Consistently, the activation of downstream kinases, such as ERK1/2 (measured by its phosphorylation level), was markedly higher in the presence of CTEN (Fig. 1A). Time course experiments indicated that the half-life of EGFR was extended from 45 min to 90 min in the presence of EGF when CTEN was overexpressed (Fig. 1B). Because EGFR tyrosine phosphorylation is an immediate event upon ligand binding and also represents the activated form of EGFR, the levels of tyrosine phosphorylated EGFR were examined. As shown in Figure 1C, total tyrosine phosphorylation levels of EGFR (pEGFR) were significantly enhanced in the presence of CTEN. The increase of pEGFR appeared to be a reflection of EGFR protein level. Once the pEGFR levels were normalized to total protein levels (tEGFR), there were no differences between cells with or without CTEN. By using phospho-site specific antibodies, higher phospho-Y845 (pY845), pY992, pY1045, pY1068, and pY1173 levels together with increased EGFR amounts were detected in CTEN transfectants than in mock cells (Fig 1D). Altogether these results demonstrated that the presence of CTEN significantly diminished EGF-induced EGFR down-regulation, sustained activated receptor, and therefore prolonged EGFR signaling.

The effect of CTEN on EGFR protein and mRNA levels was also examined in colon (SW480), lung (A549) and bladder (5637) cancer cell lines that overexpress endogenous CTEN. Both basal and inducible down-regulation of EGFR protein levels were significantly enhanced when CTEN expression was silenced by siRNA (Fig. 1E). Furthermore, EGFR mRNA levels were not changed after CTEN was silenced in these cell lines (Fig. 1F), indicating that the effect is independent of transcriptional control.

CTEN reduces ligand-induced ubiquitination of EGFR and modulates c-Cbl-EGFR interaction

It is known that ubiquitination is a critical step for EGF-induced degradation of EGFR. To examine the effect of CTEN on EGFR ubiquitination, HEK 293T cells were transfected with CTEN and then treated with or without EGF. Equal amounts of EGFR were immunoprecipitated and their ubiquitination (Ub) levels were measured. As shown in Figure 2A, CTEN significantly reduced the ubiquitination of EGFR in the presence of EGF. This suggests that CTEN protects ligand-induced EGFR degradation through reducing its ubiquitination. c-Cbl is the primary E3 ubiquitin ligase that binds directly to pY1045 and indirectly to pY1068 sites of EGFR and then ubiquitinates the receptor upon ligand

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stimulation. Hence, the phosphorylation levels of both sites were examined to test whether CTEN might compromise c-Cbl binding sites on EGFR. Equal amounts of EGFR were immunoprecipitated and their pY1045 and pY1068 levels were measured in a time course experiment (Fig 2B). In agreement with our findings in Figure 1C, no remarkable phosphorylation difference at these sites between control and CTEN transfectants was observed. In the same experiment, the interaction between c-Cbl and EGFR was analyzed. Overall, increasing amounts of c-Cbl were interacted with EGFR in control cells, while c-Cbl-EGFR interaction levels were reducing in CTEN transfectants during the time course (Fig 2B). Nonetheless, significant more c-Cbl proteins were bound to EGFR in the present of CTEN before 15 min. After 15 min, more c-Cbl-EGFR interaction was detected in the control cells. These data strongly suggest that CTEN may regulate the dynamic of c-Cbl-EGFR interaction.

CTEN interacts with the E3 ubiquitin ligase c-Cbl in a SH2-phosphotyrosine dependent fashion

Since (a) EGF-induced EGFR ubiquitination level is compromised in the presence of CTEN, (b) CTEN modulates the c-Cbl-EGFR interaction without altering pY1045 and pY1068 levels, and (c) c-Cbl protein levels are not altered by CTEN expression (Fig. 3), we investigated whether CTEN could interact with c-Cbl and therefore affect EGFR ubiquitination. To demonstrate the interaction between CTEN and c-Cbl, Flag-tagged full-length, N-terminus, or C-terminus of CTEN were transfected into HEK 293T cells (Fig 3A). Recombinant CTEN and its associated proteins were immunoprecipitated with anti-Flag antibody. As shown in Figure 3B, c-Cbl was coimmunoprecipitated with Flag-tagged full-length CTEN and this interaction was markedly enhanced by EGF treatment, suggesting that the c-Cbl-CTEN interaction might be phosphorylation-dependent. Interestingly, the interaction was retained when the full-length CTEN was replaced with its C-terminal fragment (residues 438-715), but was almost abolished when the N-terminal region (1-438) of CTEN was employed (Fig. 3B). The C-terminal region of CTEN contains two phosphotyrosine-binding domains, the SH2 (Src Homology 2) and PTB (PhosphoTyrosine Binding) domains. We used CTEN loss-of-function mutations (R474A for the SH2 domain and R650A for the PTB domain) to identify the c-Cbl-binding domain. As shown in Figure 3C, SH2 mutant (R474A) of CTEN clearly lost the ability to interact with c-Cbl, whereas PTB mutant (R650A) sustained the binding to c-Cbl after EGF induction (Fig. 3C). These results indicate that the SH2 domain of CTEN is essential for ligand-induced binding to c-Cbl. We further confirmed the interaction between the CTEN SH2 domain and c-Cbl using a GST fusion pull-down assay. GST, GST-SH2, and GST-PTB fusion proteins were expressed in and purified from *E. Coli*. The fusion proteins were incubated with cell lysates from EGF-treated or untreated HEK 293T cells, and the pull-down assay was carried out as described in Materials and Methods. Indeed, CTEN GST-SH2, but not GST or GST-PTB, fusion proteins were able to pull down c-Cbl (Fig. 3D), suggesting that the SH2 domain of CTEN binds to tyrosine phosphorylated c-Cbl.

c-Cbl is a prominent substrate of EGFR tyrosine kinase and tyrosine 674 (AIY$_{674}$SLAAR), 700 (JEY$_{700}$MTPS), 731 (CTY$_{731}$EAMY), and 774 (DGY$_{774}$DVPK) residues are four major phosphorylation sites (15–17). To identify the potential phosphorylation-dependent binding sites on c-Cbl, we individually mutated tyrosine 674, 700, 731, and 774 to phenylalanine and examined their CTEN binding activities. c-Cbl Y774F mutant appeared to lose binding to CTEN after ligand stimulation (Fig. 3E), indicating that tyrosine 774 of c-Cbl could be a crucial residue for EGF-induced phosphorylaiton-dependent interaction with CTEN. To verify the role of Y774 residue in CTEN binding, synthetic peptides containing the Y700 or Y774 sites with or without phosphorylation were used to compete with c-Cbl binding to GST-SH2 (Fig. 3F). Only phosphorylated Y774 peptide (774P) was able to block

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the interaction between CTEN and c-Cbl, further confirming that Y774 of c-Cbl is a specific phosphotyrosine residue for binding to SH2 domain of CTEN.

A functional SH2 domain of CTEN is essential for reducing EGF-induced EGFR ubiquitination/degradation and promoting CTEN-mediated tumorigenicity

Our results showing c-Cbl-CTEN interaction suggest that the effect of CTEN on EGF-induced EGFR down-regulation may depend on CTEN SH2 domain binding to phosphorylated Y774 on c-Cbl. To test this possibility, EGFR ubiquitination in the presence of CTEN SH2 mutant (R474A) was analyzed. As shown in Figure 2A, R474A no longer exhibits the potential to reduce the ubiquitination of activated EGFR. We further examined the effect of CTEN R474A on EGFR protein and ERK1/2 activation levels in the presence of EGF. Down-regulation of EGFR in response to EGF was reduced in CTEN-transfected HEK 293T cells when compared to those of experimental groups that transfected with control vector and R474A mutant (Fig. 4A). Consistent with the diminished down-regulation of EGFR, phosphorylation of the EGFR effector ERK1/2 in response to EGF was markedly increased in the presence of wild-type CTEN, but not R474A mutant (Fig. 4A). These results demonstrate that the effect of CTEN on EGF-induced EGFR ubiquitination, degradation, and signaling is primarily mediated through the CTEN SH2 domain.

Accumulating reports have indicated that CTEN overexpression may be correlated to various types of cancer development (8, 10, 18, 19). Our results demonstrate that the interaction between the SH2 domain of CTEN and pY774 c-Cbl is important in attenuating EGFR degradation and prolonging EGFR signaling, which might play important roles in tumor progression. Thus, we set up a series of experiments to examine the potential role of CTEN SH2 domain in tumorigenic properties. Ectopic expression of CTEN in HCT116 (low CTEN) colon cancer cells significantly promoted cell migration, invasion, and colony formation activities (Fig. 4C–E). In contrast, no enhancement effects on these activities were observed in cells transfected with CTEN SH2 mutant (R474A), demonstrating the essential role of the SH2 domain on CTEN-mediated tumorigenicity in cancer cells. To further validate the involvement of EGFR signaling in these CTEN-mediated phenotypes, the experiments were repeated in the presence of cetuximab, an anti-EGFR antibody. While cetuximab had no effect on HCT116 cell migration and invasion, it suppressed HCT116 colony formation activity (Fig. 4C–E). More importantly, the effects of CTEN overexpression were reversed by cetuximab, implicating a direct link between CTEN and EGFR signaling in these processes.

CTEN mRNA levels are up-regulated in a variety of cancers and its protein expression is correlated with that of EGFR in human lung samples

Several lines of evidence have indicated that CTEN mRNA expression is up-regulated in various cancers (8, 10, 18, 19). To extend these findings, we took advantages of four microarray datasets available from GEO (Gene Expression Omnibus) and analyzed CTEN and EGFR expression profiles using the Partek Genomics Suite Software. Compared with paired normal tissues, we found that CTEN mRNA expression is significantly up-regulated in tumors from breast (GSE15852)(20), colon (GSE10950) (21), lung (GSE12236)(22), and pancreas (GSE28735)(23) (Fig. 5). Nonetheless, EGFR mRNA levels were down-regulated with statistical significance in breast cancer, but not in colon, lung and pancreas datasets. These results show that CTEN transcript was increased, but not correlated with EGFR mRNA levels in these cancers. Since our cell culture studies suggested that CTEN expression altered EGFR protein but not mRNA levels, we analyzed the relationship between CTEN and EGFR protein levels by immunohistochemical (IHC) staining in normal and lung cancer consecutive tissue section samples. To rule out the possibility that truncated variants of EGFR such as EGFR-vIII may escape from ligand-induced down-regulation, we

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limited our analysis to wild-type EGFR in these samples by using an anti-wild-type EGFR antibody (DAK-H1-WT). Figure 6A shows representative IHC staining images of CTEN and EGFR. In a total of 96 cases, CTEN and EGFR are up-regulated in 50 (52.1%) and 53 (55.2%) samples, respectively (Fig. 6B). Among them, 39 (40.6%) samples show both elevated CTEN and EGFR IHC staining, whereas 32 (33.3%) samples display no change or down-regulation on CTEN and EGFR together (Fig. 6B). The statistical analyses indicate a strongly positive correlation between CTEN and EGFR protein levels in these lung samples, supporting the cell culture finding that CTEN up-regulation increases EGFR protein level.

DISCUSSION

Previous studies have shown that CTEN expression is induced by EGF treatment in a variety of normal and cancer cells, including MCF10A, RWPE-1, HeLa, and SW480 cells (6, 24). Up-regulated CTEN disrupts the interactions between integrin receptors and actin cytoskeleton (6) and promotes cancer cell migration, invasion, and colony formation (6, 8, 9, 11, 24). In this report, we have identified a novel function of CTEN in negatively regulating c-Cbl-mediated EGFR degradation. By binding to activated c-Cbl, CTEN is able to decrease EGFR ubiquitination and degradation. At this point, we do not know exactly how c-Cbl-CTEN interaction leads to the reduction of EGFR ubiquitination. It is possible that, by binding to c-Cbl, either CTEN sequesters c-Cbl away from activated EGFR or/and reduces its E3 ligase activity. The former seems to be supported by our results showing that c-Cbl-EGFR interaction was markedly reduced in the presence of CTEN after 15 min EGF treatment. Since c-Cbl-CTEN interaction depends on c-Cbl tyrosine phosphorylation, which is relied on its binding to activated EGFR, this may explain the delay disruption of c-Cbl-EGFR interaction by CTEN. Nonetheless, CTEN could somehow transiently (15 min) but robustly enhance c-Cbl-EGFR interaction. How CTEN can accomplish these dynamic processes remains to be investigated. The function of c-Cbl in promoting EGFR degradation is known to be suppressed in several ways (25), including (a) dephosphorylation of crucial phosphotyrosine sites on c-Cbl by SH2-containing phosphatase-1 (SHP1), (b) targeting of c-Cbl for degradation by the E3 ligase atrophin-1-interating protein-4 (AIP4), (c) binding of c-Cbl to Sprouty 1/2 pTyr 55 sites that compete with the pTyr 1045 site on EGFR, and (d) the formation of a complex between c-Cbl and PAK-interacting exchange factor (βPIX), PAK1 and Cdc42. Our study presents a novel mechanism in modulating EGFR protein levels and provides additional layer of feedback control in EGFR signaling. In addition to EGFR, c-Cbl contributes to ubiquitination and down-regulation of receptors for platelet-derived growth factor, vascular endothelial growth factor, fibroblast growth factor, hepatocyte growth factor, macrophage colony-stimulating factor 1, neutrophin, and insulin, as well as members of non-receptor tyrosine kinase Src family (26). It will be interesting to examine whether CTEN may regulate the stabilities of these proteins through binding to c-Cbl.

We have demonstrated that a functional SH2 domain is essential for CTEN's role in promoting cancer cell migration, invasion, as well as colony formation, and that CTEN SH2 domain binds to c-Cbl and prolongs EGFR signaling. These results indicate that the prolonged EGFR signaling is responsible for transformation properties, which is supported by the fact that EGFR inhibitor could reverse CTEN-mediated phenotypes. Moreover, SH2 domains of tensin members also interact with PI3 kinase, p130Cas, focal adhesion kinase, phosphoinositide-dependent kinase-1 (PDK-1) and downstream of tyrosine kinase 2 (Dok-2) (27–29). These binding partners may all contribute to CTEN-enhanced cancer phenotypes. On the other hand, SH2 domains of CTEN and tensins bind to the tumor suppressor DLC1 (deleted in liver cancer 1) in a phosphotyrosine-independent manner, which is required for DLC1's tumor suppression activity (14). The function of recruiting DLC1 to focal adhesion for preventing cell transformation seems contradictory to the phenotypes observed in Figure 4. One likely reason is that DLC1 is down-regulated or absence in many cancer cell lines

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including HCT116 cells used in this study. Nonetheless, these findings also implicate the complexities of CTEN regulatory networks in normal cells.

Up-regulation and aberrant activation of EGFR are highly associated with tumor progression as well as therapeutic resistance in human cancer patients (30, 31). EGFR targeted therapies are employed to treat several types of cancers including colorectal cancer, breast cancer, and lung cancer. While showing promising effects in some patients, many other patients still suffer from side effects and resistance associated with anti-EGFR agents. The reasons that underline the anti-EGFR resistance have not yet been clarified, but may related to much higher EGFR levels/activities and RAS/RAF mutations in some patients (32). Considering that CTEN (a) regulates activated EGFR levels, (b) is a downstream of the RAS/RAF/MEK pathway, (c) plays roles in tumor migration/invasion, and (d) is expressed at very low levels in most normal tissues, targeting CTEN along with EGFR might be a more efficient approach for cancer therapies.

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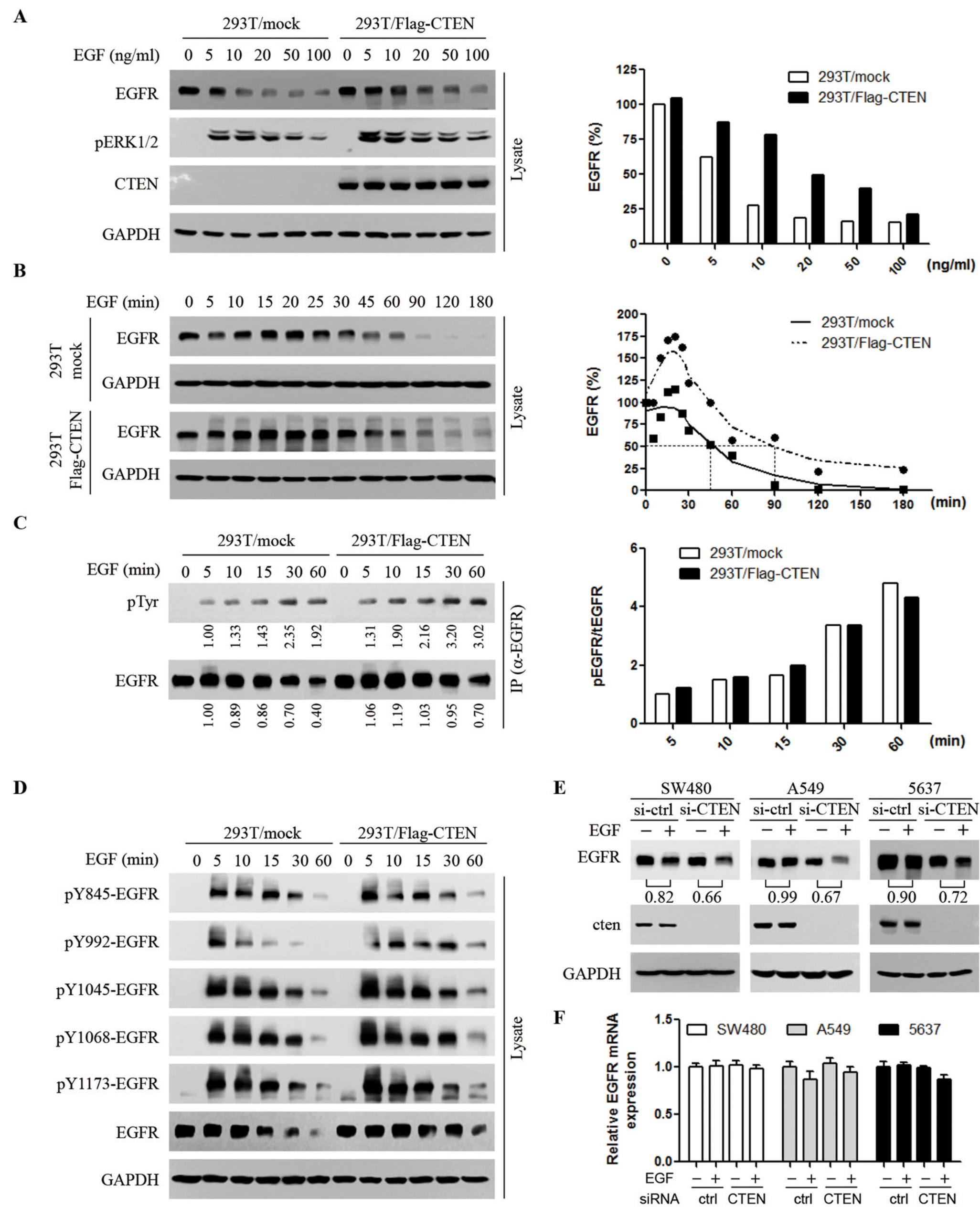

Figure 1. CTEN attenuates EGF-induced EGFR down-regulation in a transcription-independent manner

(A) Dose-response of EGFR down-regulation. Mock or Flag-CTEN-overexpressing HEK 293T cells were stimulated with indicated dosages of EGF for 1h. Left panel: representative immunoblot analyses; right panel: bar graphs showing the relative protein levels of EGFR. (B) Kinetics of EGFR protein levels. Mock or Flag-CTEN-overexpressing HEK 293T cells were treated with 10 ng/ml of EGF for indicated time. Left panel: immunoblot analyses with the relevant antibodies; right panel: quantification of the EGFR protein. The intensity of each band was quantified, and expressed as a percentage using the signal at dose 0 or time 0 as a reference. (C) Kinetics of EGFR tyrosine phosphorylation (pEGFR) levels. The mock or transfected cells were stimulated with 10 ng/ml of EGF for indicated time. Total EGFR (tEGFR) were immunoprecipitated (IP) from equal amounts of cell lysates with anti-EGFR

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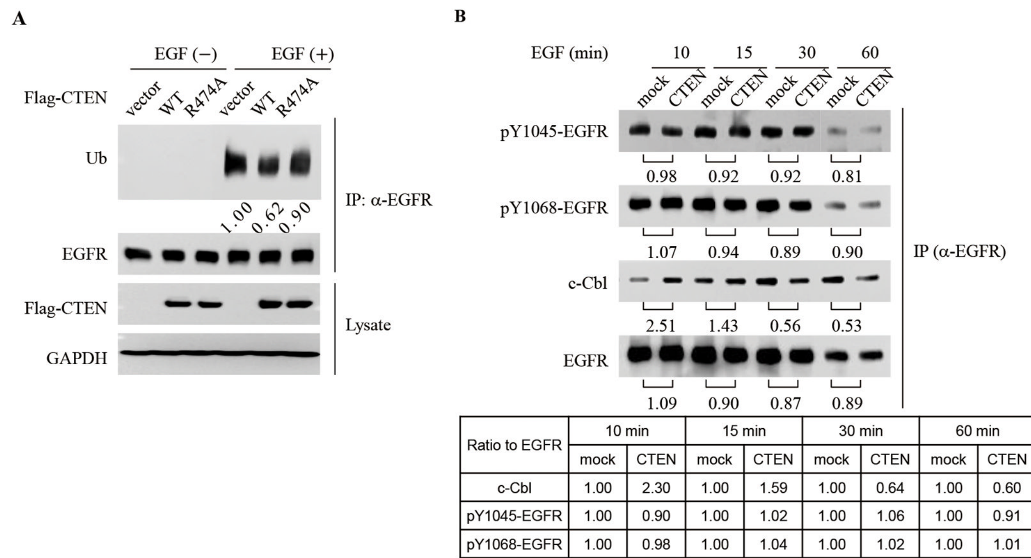
antibody, followed by immunoblotting with anti-phosphotyrosine antibodies. Left panel: representative immunoblot analyses; right panel: bar graphs showing the ratios of pEGFR/tEGFR at indicated time points. The intensity of each band was quantified, and shown as a fold change from the 5 min time point of mock cells. (D) Phosphorylation levels of specific tyrosine sites on EGFR. Equal amounts of cell lysates from mock cells and CTEN-transfecteants were immunoblotted with indicated antibodies. (E) Down-regulation of EGFR in the absence of CTEN. 50 ng/ml of EGF were treated in control (si-ctrl) or CTEN (si-CTEN) siRNA knockdown SW480, A549, and 5637 cells for 1h. Band intensities of EGFR were quantified and the degrees of EGFR down-regulation (EGF treated/untreated) were shown as ratios. (F) Real-time PCR analyses of EGFR mRNA levels. Control or CTEN siRNA knockdown SW480, A549, and 5637 cells were untreated or treated with EGF (50 ng/ml) for 1h. EGFR mRNA levels were analyzed by real-time PCR and normalized to the internal control, beta-actin mRNAs. Error bars represent mean ± SEM. Results for (A - E) are representative of 2 independent experiments each and that for (F) is the average of 3 independent experiments.



Ratio to EGFR	10 min		15 min		30 min		60 min	
	mock	CTEN	mock	CTEN	mock	CTEN	mock	CTEN
c-Cbl	1.00	2.30	1.00	1.59	1.00	0.64	1.00	0.60
pY1045-EGFR	1.00	0.90	1.00	1.02	1.00	1.06	1.00	0.91
pY1068-EGFR	1.00	0.98	1.00	1.04	1.00	1.02	1.00	1.01

Figure 2. CTEN reduces ubiquitination but not tyrosine phosphorylation levels of EGFR induced by EGF

(A) Ubiquitination of endogenous EGFR after EGF treatment. HEK 293T cells transiently transfected with control vector, Flag-CTEN wild-type (WT), or Flag-CTEN R474A mutant were preincubated with 10 uM of lactacystin for 1h. The cells were treated with or without 10 ng/ml of EGF for 15 min. Cell lysates were immunoprecipitated (IP) with antibodies specific for EGFR, followed by immunoblotting with anti-Ub (ubiquitin) or anti-EGFR antibodies. The intensities of the bands were compared to those in EGF treated control cells. Total cell lysates were immunoblotted with anti-Flag (for Flag-CTEN) or anti-GAPDH antibodies. (B) Kinetics of EGF-induced EGFR phosphorylation and c-Cbl-EGFR binding in the presence of CTEN. The mock or Flag-CTEN-transfected HEK 293T cells were stimulated with 10 ng/ml of EGF for indicated time. To pull down equal amount of EGFR from mock and CTEN-overexpressing cells at each time point, adjusted amounts of cell lysates were immunoprecipitated (IP) with antibodies specific for EGFR, followed by immunoblotting with anti-c-Cbl, pY1045-EGFR, pY1068-EGFR, and total EGFR. Upper panel: representative immunoblot analyses. The intensity of each band was quantified and the changes between CTEN and mock transfection samples were shown as ratios (CTEN-overexpressing/mock) before normalized to EGFR levels. Lower panel: analysis showing the fold change to mock sample at each time point after normalized to corresponding EGFR.

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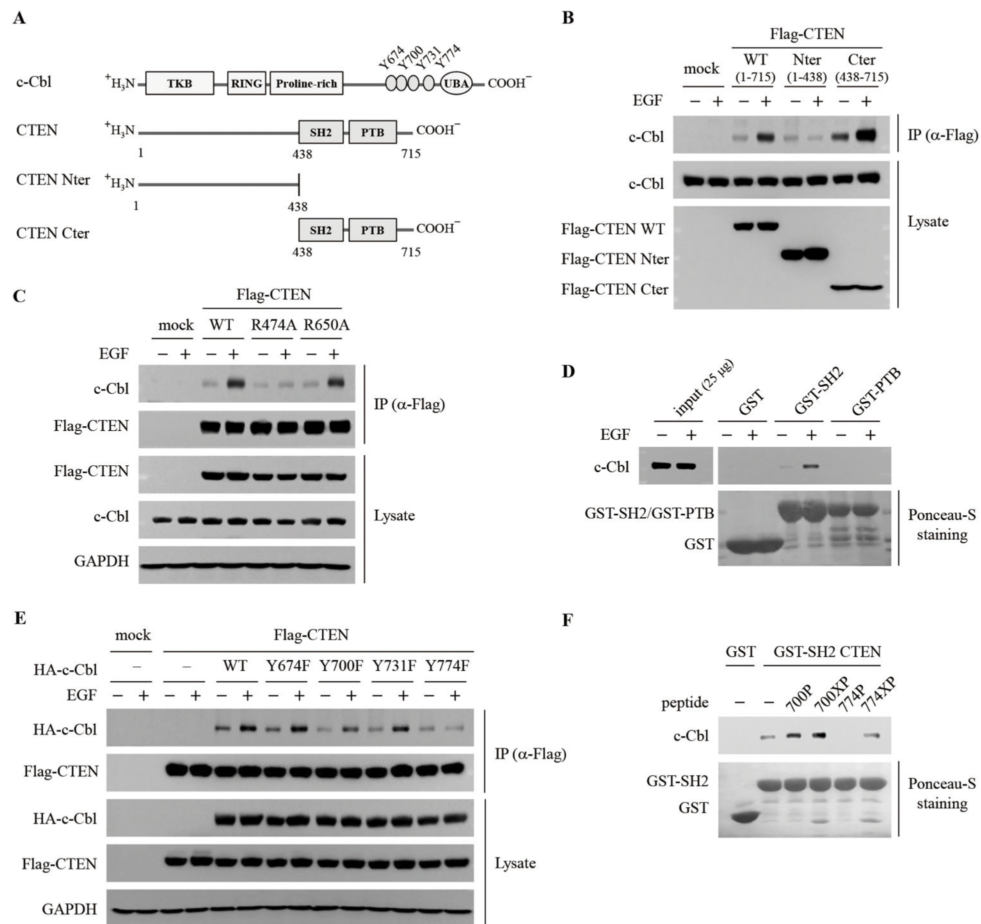

Figure 3. The SH2 domain of CTEN is essential for the interaction with tyrosine-phosphorylated c-Cbl

(A) The domain structures of CTEN and c-Cbl proteins. c-Cbl contains an N-terminal TKB (tyrosine-kinase-binding) region, a RING finger, a proline-rich region and a C-terminal UBA (ubiquitin-associated) domain. Four tyrosine phosphorylation residues on c-Cbl are listed. CTEN possesses an SH2 (Src homology 2) domain and a PTB (phosphotyrosine-binding) domain. Mutations of arginine 474 and 650 residues within CTEN abolish the binding activities of the SH2 and PTB domains, respectively. CTEN N-terminus (Nter) and C-terminus (Cter) fragment mutants are illustrated. (B) Coimmunoprecipitation with CTEN fragments. HEK 293T cells were transfected with Flag-tagged CTEN wild-type (WT), N-terminus (Nter), or C-terminus (Cter) fragments and then treated with or without 10 ng/ml EGF for 15 min. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody and immunoblotted with anti-c-Cbl antibody. Immunoblot analyses of c-Cbl and Flag-CTEN in total cell lysates are shown. (C) Same as in (B) except that Flag-tagged CTEN wild-type (WT), SH2 mutant (R474A), and PTB mutant (R650A) were employed. (D) GST pull-down assay. GST or GST fusion proteins were incubated with equal amounts of lysates from EGF-treated or untreated HEK 293T cells. Bound proteins were subjected to immunoblotting with anti-c-Cbl antibody (upper right panel). The bait proteins were detected by Ponceau-S staining (lower panel). (E) Coimmunoprecipitation of wild-type and various tyrosine mutants of HA-tagged c-Cbl. HA-c-Cbl WT, Y674F, Y700F, Y731F or Y774F were cotransfected with Flag-CTEN into HEK 293T cells. Lysates from untreated cells or cells treated for 15 min with 10 ng/ml of EGF were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-HA antibody. Immunoblot analyses of HA-c-Cbl and Flag-

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CTEN in total cell lysate are shown. (F) Phosphopeptide competition assay. GST or GST-SH2 CTEN proteins were preincubated with c-Cbl phosphopeptides (700P, 774P) or non-phosphorylated peptides (700XP, 774XP) for 30 min. Lysates from EGF-stimulated (10ng/ml, 15 min) HEK 293T cells were added and the incubation continued for another 90 min. GST or GST-SH2 bound proteins were separated by SDS-PAGE and identified by immunoblotting with anti-c-Cbl antibody (upper panel). The bait proteins were detected by Ponceau-S staining (lower panel).

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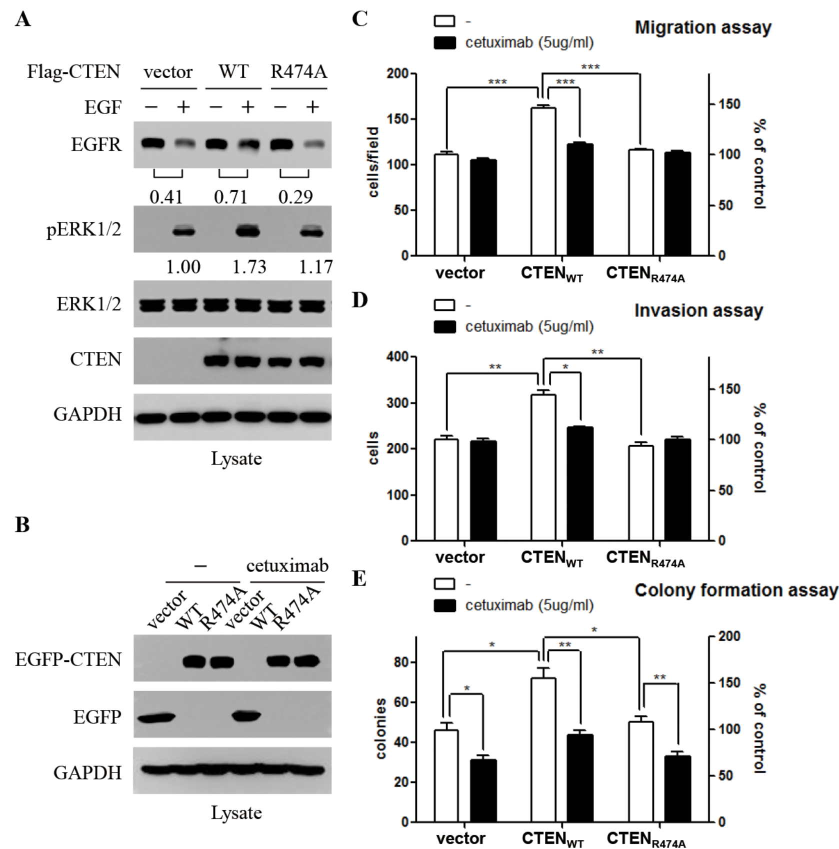

Figure 4. The SH2 domain of CTEN plays a critical role in regulating EGFR signaling and CTEN-mediated tumorigenic properties

(A) The potential of EGFR signaling by CTEN overexpression. HEK 293T cells transfected with control vector, Flag-CTEN WT or SH2 mutant (R474A) were treated with 10 ng/ml of EGF for 1h. Equal amounts of total cell lysates were analyzed by immunoblotting with the indicated antibodies. Band intensities of EGFR and pERK1/2 were quantified. The degrees of EGFR down-regulation (EGF treated/untreated) were shown as ratios. The changes of pERK1/2 in CTEN WT or R474A mutant transfectants were compared to control cells. (B) Representative immunoblot analyses showing equal recombinant protein levels expressed in cells used in (C-E). (C) Cell migration, (D) invasion, and (E) colony formation of HCT116-EGFP vector, HCT116-EGFP-CTEN-WT, and HCT116-EGFP-CTEN-R474A transfectants with or without EGFR inhibitor treatment. Numbers of cells or colonies were counted and compared to that of HCT116-EGFP vector cells. (C-E) are presented as the means and 95% confidence intervals (error bars) from three independent experiments. *P* values were calculated using two-sided Student t test.*$p<0.05$, **$p<0.01$.

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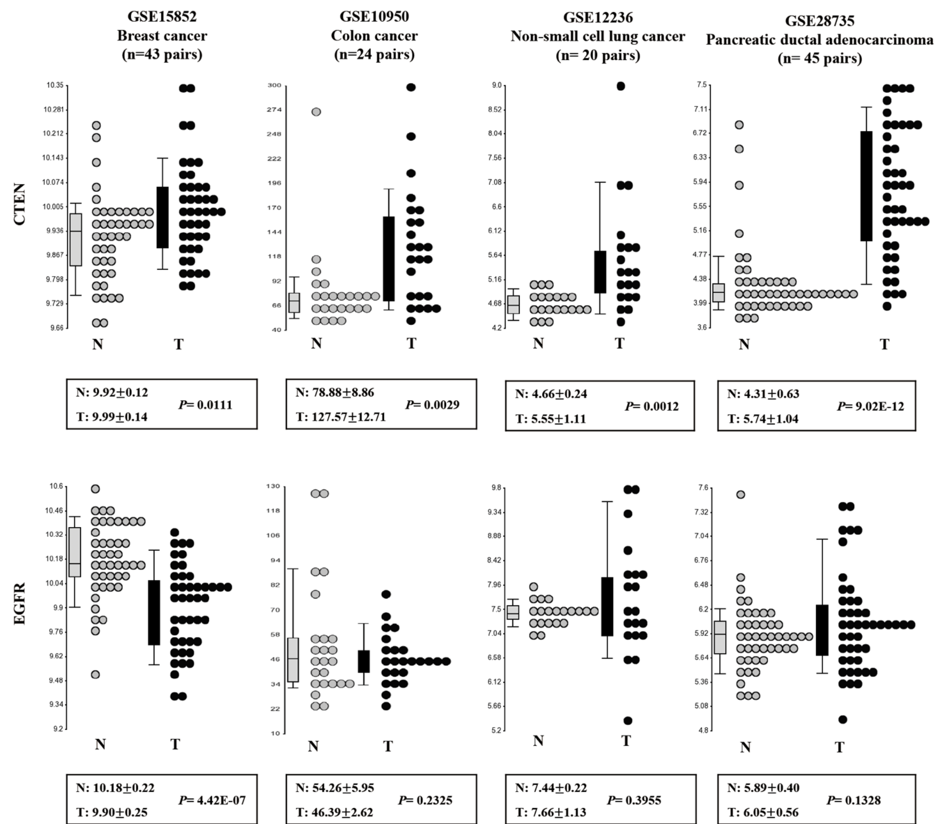

Figure 5. CTEN mRNA expression is up-regulated in human tumor samples

The gene expression data of 43 breast cancer (GSE15852), 24 colon cancer (GSE10950), 20 non-small cell lung cancer (GSE12236), and 45 pancreatic ductal adenocarcinoma (GSE28735) with matched normal samples were obtained from the National Center for Biotechnology Information Gene Expression Omnibus database. Dot plots for CTEN and EGFR mRNA expression were created from the indicated gene expression datasets. N: normal; T: tumor. The changes in CTEN and EGFR in four gene expression datasets were shown in boxes. Comparisons of mean values and standard deviations between the normal and tumor groups were analyzed using the Partek Genomics Suite Software. Statistical significance of the differences was analyzed using paired Student's t test.

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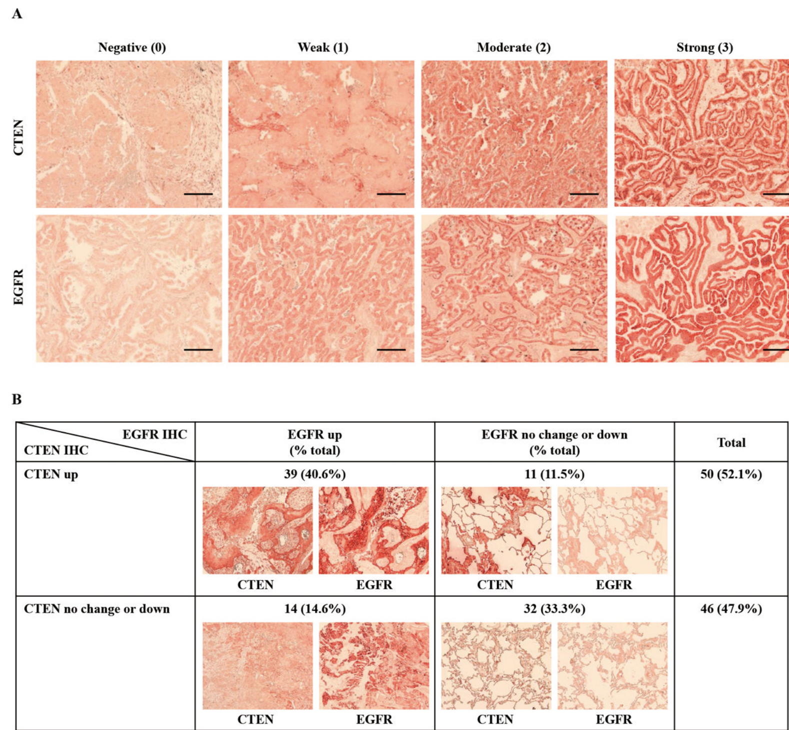

Figure 6. CTEN and EGFR protein levels show a positive correlation in human lung cancer and normal tissue microarray

(A) Representative images of CTEN and EGFR immunohistochemistry: negative (0), weak (1), moderate (2), and strong (3). Scale bars = 200 µm. (B) Statistical analysis and representative images for CTEN and EGFR correlation. Results were acquired from the IHC staining in 96 cases of human lung tissue microarray.