Carboxyl-terminal modulator protein positively regulates Akt phosphorylation and acts as an oncogenic driver in breast cancer

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Running Title: CTMP promotes Akt activation and tumorigenesis

Keywords: Akt phosphorylation, breast cancer, CTMP, oncogene, tumorigenesis

Precis: Results address some controversy in the field by corroborating the concept that CTMP promotes Akt phosphorylation and functions as an oncogenic molecule in breast cancer.

Conflict of interest: The authors declare no conflicts of interest.

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Abstract

Akt activation has been implicated broadly in tumorigenesis, but the basis for its dysregulation in cancer cells is incompletely understood. In this study, we sought to clarify a regulatory role for the Akt-binding carboxy-terminal modulator protein (CTMP), which has been controversial. In evaluating CTMP expression in paired normal-tumor specimens of 198 breast cancer patients, we found that CTMP was upregulated in breast tumors where it was associated with poor patient survival. Notably, CTMP expression also correlated positively with Akt phosphorylation in breast cancer clinical specimens and cell lines. Furthermore, ectopic expression of CTMP promoted cell proliferation and enhanced the tumorigenic properties of estrogen-dependent breast cancer cells. This effect was correlated with increased sensitivity to insulin-induced Akt phosphorylation, which is mediated primarily by the PI3K/Akt pathway. In contrast, shRNA-mediated silencing of endogenous CTMP decreased the proliferation of estrogen-dependent or estrogen-independent breast cancer cells. Mechanistic investigations defined the N-terminal domain of CTMP at amino acids 1-64 as responsible for Akt binding. Taken together, our results firmly corroborate the concept that CTMP promotes Akt phosphorylation and functions as an oncogenic molecule in breast cancer.

Keywords: Akt phosphorylation, breast cancer, CTMP, oncogene, tumorigenesis
Introduction

Akt serine/threonine kinase is one of the most important signaling molecules that respond to growth hormones. Akt activation through the secondary messengers generated by phosphatidylinositol 3-kinase (PI3K) regulates various downstream signaling molecules that modulate crucial biological responses, including proliferation, survival, protein synthesis, and metabolism (1-4). In recent decades, Akt activity dysregulation has frequently been discovered in a wide range of human cancers (5, 6). Moreover, evidence has shown that the combination of Ras and Akt constitutive activation induces tumor formation in mice, suggesting a vital role for Akt in oncogenesis (7). Therefore, understanding the regulatory mechanisms of Akt is important for developing therapeutic strategies against human cancers.

The structure of Akt consists of three conserved domains: an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal regulatory domain. Akt is activated through two sequential regulatory mechanisms. First, Akt translocates to the plasma membrane through the direct binding of its PH domain to phosphatidylinositols-(3,4,5)P3 (PIP3) generated by PI3K (8). At the plasma membrane, 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates Akt at the T308 residue in the catalytic domain (9). Although the phosphorylation of T308 residue is necessary and sufficient to activate Akt, additional phosphorylation at S473 by rapamycin complex 2 (mTORC2) is required to accomplish full activation (10).

In addition to the upstream regulators, Akt activity is regulated by its interacting proteins (11, 12). Through their interaction with the PH domain of Akt, Tc11 (13), Grb10 (14), JIP1 (15), and RasGAP (16) facilitate growth factor-induced Akt
activation. The CDC37/heat shock protein-90 complex binds to the kinase domain of Akt and prevents Akt degradation resulting from the ansamycin antibiotic-triggered proteasome pathway (17). TRB3 inhibits insulin-induced Akt phosphorylation at both the T308 and S473 residues and regulates cell growth and apoptosis (18). Another Akt-interacting protein, APPL1, also binds to the kinase domain of Akt and enhances Akt activation, although the functional role of this interaction remains unclear (19). Several regulatory proteins, including APE (also known as HKRP1), Arg-binding protein 2γ (ArgBP2γ), Src, and prohibitin 2 (PHB2), bind to the C-terminal domain of Akt and regulate Akt activity through different mechanisms.

Recently, the carboxy-terminal modulator protein (CTMP) was identified as a negative regulator of Akt that inhibits Akt phosphorylation and activation by binding to the C-terminus of Akt in a region including the hydrophobic motif (HM) and the COOH-terminal serine phosphorylation site at the plasma membrane (20). CTMP expression phenotypically and functionally modulates the cell morphology, growth rate, and tumorigenesis of v-Akt-transformed cells, suggesting a tumor-suppressive function of CTMP. In agreement with the inhibitory role of CTMP on Akt activation, the reduction of CTMP mRNA was observed in glioma cell lines through an epigenetic regulation (21). Furthermore, the RNA-interference depletion of CTMP prevents ischemia-induced neuronal cell death (22). However, a study by Ono et al. (23) provided evidence that CTMP might act as a positive regulator of Akt. Ono et al. showed that the overexpression of CTMP induced Akt phosphorylation, leading to increased Akt activity and facilitating anti-apoptosis and glucose metabolism (23, 24). The conflicting findings from Maira et al. (20) and Ono et al. (23) raise important questions. What is the correlation between CTMP and Akt phosphorylation in human cancers? Are CTMP protein levels correlated with tumor progression? At the
molecular level, it is also necessary to validate the CTMP and Akt interaction and the Akt-binding domain of CTMP in cancer cells.

In our study, CTMP up-regulation was found in human breast cancer cell lines and in breast cancer specimens, and its expression levels were positively correlated with Akt phosphorylation. Moreover, CTMP levels were inversely correlated with breast cancer patient survival. The enforced expression of CTMP increased basal and insulin-induced Akt phosphorylation, which facilitated cell proliferation, soft agar colony formation, and \textit{in vivo} tumorigenesis. CTMP knockdown using siRNA reduced breast cancer cell proliferation. In addition, we identified that the N-terminal domain of CTMP containing amino acids 1-64 was responsible for the Akt interaction. We conclude that CTMP functions as a positive regulator of Akt and plays an oncogenic role in breast cancer.
Materials and Methods

Tissue microarrays

In this study, two different sets of tissue microarrays were used. One set was the screening tissue microarray, TA30-A, which was used to evaluate CTMP expression in different cancers. TA30-A contains a total of 146 normal and tumor punches representing colon, breast, gastric, and liver cancer and lung adenocarcinoma. The other set was the validating breast cancer tissue microarray, TMA-BC, from 204 breast cancer patients diagnosed between 1995 and 2011. TMA-BC was created from Kaohsiung Veterans General Hospital archives with IRB approval (VGHKS12-CT9-057).

Immunohistochemistry (IHC)

Tissue sections (5 μm) were dewaxed and rehydrated. Antigen retrieval was done by incubating the slides in 10 mM citric buffer (pH6.0) and microwaved for 15 min. After blocking, the slides were incubated with primary antibody against CTMP or phospho-Akt-S473 followed by biotin-conjugated secondary antibody, polymer-HRP and diaminobenzidine tetrahydroxychloride (DAB) solution.

Cell culture and proliferation analysis

The MCF-10A, MCF-7, BT474, BT483, MDA-MB-231 and HeLa cell lines were cultured in the culture medium suggested by American Type Culture Collection (ATCC). For long-term culturing, mycoplasma test was performed every month for all cell lines. For cell proliferation analysis, $2 \times 10^4$ cells were infected with a lentivirus carrying CTMP shRNAs or transfected with GFP-CTMP plasmids and
seeded in six-well culture plates. The cells were harvested at different time points, and the cell numbers were counted using a hemocytometer.

**CTMP shRNA lentivirus preparation**

The pLKO.1-shCTMP-shRNAs were purchased from the National RNAi Core Facility, Taiwan (clone number #1, TRCN0000048718; #2, TRCN0000048720). The pLKO.1-shCTMP-shRNAs and pLKO.1 lentiviral vector-only lentivirus particles were prepared by co-transfection with the packaging plasmid SPAX2 and the envelope plasmid MD2G into HEK293T cells.

**Plasmid constructs, transfection, and stable clone selection**

The full-length cDNA of human CTMP was amplified using PCR from cDNA libraries with the primer set as follows: forward, ATGCTGAGGAGCTGC; reverse, TTATGTCAGACTTTTAGCAGGAT. Truncated CTMP fragments were generated from the full-length CTMP plasmid using the specific primers and were constructed into the pGEX-KG or pEGFP-C1 expression vectors.

**GST pull-down assay**

To study interaction of Akt and CTMP, GST or GST-CTMP fusion proteins were generated using E. coli BL21 (DE3) cells. Purification of GST-fused proteins for the GST pull-down assay was performed as reported previously with minor modifications (25).

**Soft agar assay**

A soft-agar assay was performed on six-well plates with a base layer of 0.5% agarose gel containing DMEM and a upper layer of 0.35% agarose gel with FBS.
Co-immunoprecipitation (co-IP) and Western blot analysis

To study the association of Akt and CTMP, HeLa cells were co-transfected with pCMV2-Flag-CTMP fragments and pcDNA3.1-HA-Akt1 constructs. The transfected cells were treated with 100 nM of insulin after a 12 h starvation period and then lysed with RIPA buffer containing proteinase inhibitors. Co-IP was performed using the Catch and Release Reversible Immunoprecipitation System (Upstate, Chandlers Ford, UK).

Immunofluorescence assays

To analyze CTMP and Akt subcellular localization, 2.5 × 10^4 HeLa cells were seeded on cover slips in 12-well dishes and allowed to attach and grow for 24 h. The following day, pEGFP-CTMP and pcDNA3.1-HA-Akt1 constructs were co-transfected into HeLa cells. After a 4-h incubation, the transfection medium was replaced by serum-free medium for 6 h of starvation. The cells were then stimulated with 100 nM of insulin for 10 min. After fixation and permeabilization, the cells were incubated with anti-HA primary antibody (Santa Cruz, 1:50 dilution) followed by rhodamine-conjugated secondary antibody (1:100 dilution).

In situ proximity ligation assay (PLA)

MCF-7 cells were seeded on cover slips and transfected with Flag-CTMP and HA-Akt constructs. After a 12 h starvation, the cells were stimulated with 10% serum for 30 min. In situ PLA was performed according to the Olink Bioscience protocol using Duolink™-PLA reagents with minor changes (Olink Bioscience, Uppsala, Sweden). The number of in situ proximity ligation signals was counted using ImageJ software.
Xenograft tumor growth

Nude mice were obtained from the National Laboratory Animal Center. The mice were maintained in a specific pathogen-free (SPF) environment at the Laboratory Animal Center of National Cheng-Kung University. For the animal experiment, each mouse was subcutaneously inoculated with $1 \times 10^5$ cells of GFP and GFP-CTMP stable clones of HeLa or MCF-7 cells in the left and right flank, respectively. The tumor size was measured every 2-3 days.

Bioinformatics

The associations of CTMP expression levels with the prognosis of patients with multiple myeloma, glioma, breast cancer, or melanoma in four other expression profile studies were obtained from the PrognoScan database (26).

Statistical analyses

All observations were confirmed by at least three independent experiments. The results are presented as the mean±SE. We used two-tailed, paired Student's t-tests for all pair-wise comparisons. The survival curves were analyzed using the log-rank Kaplan-Meier method.

Additional information was described in Supplementary Materials and Methods.
Results

CTMP is up-regulated in breast tumors, and high CTMP expression correlates with poor prognosis of breast cancer patients.

To clarify whether CTMP functions as an oncoprotein or a tumor suppressor, we first analyzed its expression through tissue microarrays consisting of breast, colon, gastric, liver, and lung cancer tissues (Supplementary Table 1). Among all the cancer types in the tissue microarray, 27 of 35 (77%) breast cancer tissues showed positive CTMP expression within the tumor regions; however, CTMP immunoreactivity was not found in normal breast tissues (0/4, 0%). For other cancer types, both the normal tissues and the tumors showed positive CTMP staining signals. To determine the CTMP expression pattern in the breast cancer patients, we collected clinically resected specimens of paired normal breast tissue and breast tumor for IHC staining with a CTMP antibody. Among the 204 patients, 127 (62.25%) patients showed higher CTMP expression in the tumor than in normal tissue (N<T; Fig. 1A), 44 patients (21.57%) showed equal levels of CTMP in the tumor and in normal tissue (N=T), and 33 patients (16.18%) showed lower CTMP expression in the tumor than in normal tissue (N>T). Generally, CTMP was up-regulated in most breast tumors compared with the normal tissues of breast cancer patients (Fig. 1B). To evaluate the prognostic significance of CTMP expression, we scored CTMP expression in each tumor sample on a scale from 0 (no expression) to 3 (high expression) based on the staining intensity (Fig. 1C). In a Kaplan-Meier log-rank analysis, a high CTMP expression (score 2 and 3) was significantly correlated with reduced overall or disease-free survival compared with patients with low CTMP expression (score 0 and 1; Fig. 1D). Among 88 patients with stage I or IIa breast cancer, 60 patients (68.18%) exhibited high CTMP expression, and 28 patients (31.82%) exhibited low CTMP
expression. This CTMP expression pattern was similar to the total enrolled patients, of whom 131 (66.16%) had high CTMP expression, and 67 (33.84%) had low CTMP expression. In addition, high CTMP expression was significantly correlated with worse overall or disease-free survival of patients with stage I or IIa breast cancer (Fig. 1E). These results indicate that CTMP expression was up-regulated in the early stages of breast cancer and inversely correlated with breast cancer patient prognosis.

The associations of gene expression with prognosis in various cancers are available from the PrognoScan database (26). Therefore, the associations of CTMP expression with the prognosis of patients with multiple myeloma, glioma, breast cancer, or melanoma were examined in four different cohorts (Supplementary Table 2). High CTMP expression was correlated with poor prognosis in all cohorts: Arkansas, UCLA (1996-2003), Stockholm (1994-1996), and NYU. Consistent with our findings, the results of bioinformatic analyses strongly indicated that CTMP expression could be a novel prognostic marker for patients with breast cancer and other cancer types.

**Up-regulation of CTMP enhances breast cancer cell tumorigenesis.**

Because CTMP was up-regulated in the tumors but not the normal tissues of breast cancer patients, we hypothesized that CTMP may be involved in breast cancer tumorigenesis. To test this hypothesis, CTMP-overexpressed MCF-7 cells, a benign breast cancer cell line, and HeLa cells, which have been used as a cellular model to study the molecular interaction of CTMP and Akt in previous contradictory reports published by Maira et al. (20) and Ono et al. (23), were used in a soft agar colony-forming assay to evaluate the function of CTMP in the *in vitro* tumorigenesis of breast cancer cells (Fig. 2C). After one to two weeks of culturing, the colonies
generated from GFP-CTMP-transfected cells were clearly visible (Fig. 2A). A representative image shows that high-GFP-CTMP-expressing cells formed larger colonies than the low-GFP-CTMP-expressing cells in the soft agar containing 3% serum (Fig. 2A, insets). A quantification analysis showed that the enforced expression of GFP-CTMP in MCF-7 and HeLa cells cultured in soft agar containing 10% serum significantly increased their colony-forming capacity (Fig. 2B), indicating that CTMP promotes anchorage-independent growth, a characteristic of cell transformation. In addition, increased colony numbers of CTMP-transfected MCF-7 and HeLa in low serum-containing (3% serum) soft agar suggested that CTMP overexpression supports growth factor-independent growth, which relies on Akt activity (Fig. 2B). To substantiate these in vitro observations, we examined whether CTMP up-regulation enhances the tumor formation ability of breast cancer cells in an animal model. Stable clones of MCF-7 and HeLa cells expressing GFP or GFP-CTMP were subcutaneously injected into nude mice. Four weeks after transplantation, the size of tumors generated from GFP-CTMP-overexpressing MCF-7 and HeLa cells was significantly larger than the tumors generated from GFP-transfected cells (Fig. 2D). In agreement with the results from clinical analyses of breast cancer specimens, these data demonstrate that the up-regulation of CTMP expression promotes breast cancer cell tumorigenesis.

**CTMP is positively correlated with Akt phosphorylation and proliferation of breast cancer cell lines.**

CTMP was identified as a regulatory protein of Akt, and Akt phosphorylation has been linked to cancer cell tumorigenesis. To evaluate the correlation of CTMP expression and Akt activation in breast cancer, we first examined CTMP levels and Akt phosphorylation in five breast cancer cell lines, including the normal breast cell line MCF-10A, the benign breast cancer cell line MCF-7, and the metastatic breast
cancer cell lines BT474, BT483, and MDA-MB-231. Compared with MCF-10A, elevated CTMP expression and Akt phosphorylation at S473 were detected in all tested breast cancer cell lines (Fig. 3A). Among the four breast cancer cell lines, BT474, BT483, and MDA-MB-231 expressed higher levels of CTMP and Akt phosphorylation than MCF-7 (Fig. 3A). To determine the correlation of CTMP and Akt phosphorylation in human breast cancer, we examined CTMP protein levels and Akt phosphorylation in human breast cancer tissues using Western blot and IHC assays. Consistent with the findings from the breast cancer cell lines, cancerous specimens exhibited increased levels of CTMP protein and Akt phosphorylation at S473 compared with the surrounding normal breast tissues (Fig. 3B and C). We further quantified the IHC signals of CTMP and phospho-Akt S473 from the serial sections of 45 breast cancer samples. The quantification data showed a remarkable positive correlation (correlation coefficient=0.7; p<0.001) between CTMP levels and Akt phosphorylation in human breast cancer tissues (Fig. 3D).

Akt activation has been shown to promote cell cycle progression and proliferation by modulating cell cycle inhibitors (27). We next evaluated the effect of CTMP on cell proliferation. MCF-7 cells were stably transfected with GFP or GFP-CTMP plasmids and cultured in medium containing 1% serum, which allows for cell growth while Akt is activated (28). The ectopic expression of CTMP facilitated MCF-7 cell proliferation compared with mock or vector-only control groups (Fig. 3E, left panel). We further stably transfected GFP or GFP-CTMP expression plasmids into HeLa cells. Different expression levels (low and high) of GFP-CTMP stable transfectants were selected, and the proliferation ability of these cells in low-serum culture conditions was analyzed. Both the low and high levels of GFP-CTMP overexpression significantly enhanced HeLa cell proliferation compared with control
cells or GFP transfectants (Fig. 3E, right panel). Next, we sought to determine whether CTMP is necessary for breast cancer cell proliferation. We introduced lentivirus-based shRNAs specific for CTMP into MCF-7 cells and analyzed the effect of CTMP knockdown on cell proliferation ability. Endogenous CTMP protein was efficiently depleted by two clones of CTMP shRNA but not by scrambled shRNA (scram; Fig. 3F, inset). CTMP knockdown significantly reduced the cell proliferation of MCF-7 cells compared with the scrambled control (Fig. 3F). The MDA-MB-231 breast cancer cell line is highly invasive and exhibits greater proliferation potential (29). Thus, we also analyzed the effect of CTMP silencing on the proliferation ability of MDA-MB-231, which showed prominent CTMP and Akt phosphorylation (Fig. 3A). CTMP silencing by two different clones of CTMP shRNA significantly reduced cell proliferation (Fig. 3G). Together, these data indicate that CTMP is overexpressed and shows a positive correlation with Akt phosphorylation in breast cancer cell lines and the tumor portions of clinical breast cancer specimens. In addition, a functional assay revealed that CTMP promotes breast cancer cell growth.

**CTMP facilitates insulin-induced Akt phosphorylation through PI3K signaling.**

To define the regulatory function of CTMP on Akt phosphorylation, we stably transfected GFP-CTMP into HeLa cells. CTMP overexpression enhanced Akt phosphorylation at both S473 and T308 without altering total Akt levels (Fig. 4A, left panel). It is noteworthy that low GFP-CTMP overexpression was sufficient to induce Akt phosphorylation at both S473 and T308 and showed no difference from high GFP-CTMP-expressed cells. This result may explain the similar effects of low and high GFP-CTMP levels on the proliferation and colony formation ability of transfected HeLa cells (Fig. 2A and B and Fig. 3E). Moreover, the phosphorylation of GSK3β, the downstream protein kinase of Akt, at S9 was increased in CTMP-
overexpressed cells, indicating the regulatory function of CTMP on Akt activity (Fig. 4A, left panel). In contrast, CTMP knockdown by shRNAs diminished Akt phosphorylation at S473 and T308 as well as GSK3β phosphorylation at S9 in MDA-MB-231 breast cancer cells (Fig. 4A, right panel). The co-transfection of dominant-negative Akt with GFP-CTMP in MCF7 cells blocked CTMP-induced GSK3β phosphorylation at S9 (Fig. 4B) and cell proliferation (Fig. 4C). These results demonstrate that the effect of CTMP on cell proliferation is mediated through Akt.

To exclude the possibility that the GFP tag may affect CTMP function, HeLa cells were transiently transfected with the HA vector or the HA-tagged CTMP (HA-CTMP), and the transfected cells were treated with insulin for different time durations. Insulin treatment rapidly increased Akt phosphorylation at T308 and S473 in vector-only control cells, and Akt phosphorylation (especially at T308) in response to insulin was markedly enhanced in the HA-CTMP-overexpressing cells (Fig. 4B). In addition, this enhancement of insulin-induced Akt phosphorylation by HA-CTMP persisted to 25 min after insulin treatment (Fig. 4B, lower panel). Subsequently, we tested the dose effects of insulin on GFP- or GFP-CTMP-stable transfected HeLa cells. As shown in Fig. 4C, Akt phosphorylation at T308 and S473 of GFP-expressed control cells increased in a dose-dependent manner, and CTMP-stable overexpression enhanced Akt phosphorylation after different insulin dose treatments. A quantification analysis revealed that CTMP overexpression not only promoted insulin-induced Akt phosphorylation levels but also increased the sensitivity of Akt to low-dose insulin stimulation (below 10 nM of insulin treatment; Fig. 4D and E). This enhancement of Akt phosphorylation by CTMP was further confirmed by incubating cells in medium containing different serum percentages (Supplementary Fig. S1). Consistent with a previous study indicating that PI3K is an upstream protein kinase that mediates Akt
phosphorylation, the insulin- and serum-induced Akt phosphorylation in either GFP-expressed or GFP-CTMP-expressed HeLa cells was inhibited in the presence of a PI3K inhibitor, LY294002, suggesting that CTMP-enhanced Akt phosphorylation depends on PI3K activity (Fig. 4F and Supplementary Fig. S2).

**CTMP binds to Akt through its N-terminal domain (1-64 a.a.).**

To examine the interaction between CTMP and Akt, we first analyzed the subcellular distribution of CTMP and Akt. GFP-CTMP and HA-Akt1 co-transfected HeLa cells were stimulated with insulin after a 12 h starvation. As shown in Fig. 5A, CTMP and Akt were distributed at the plasma membrane under basal conditions. Insulin stimulation induced Akt translocation to the plasma membrane, which increased its co-localization with CTMP at the plasma membrane (Fig. 5A). This observation is consistent with our findings showing that enforced CTMP expression elevated basal levels of Akt phosphorylation and promoted insulin-induced Akt phosphorylation (Fig. 4). Next, we co-transfected Flag-CTMP and HA-Akt plasmids into MCF-7 cells, and the interaction of CTMP and Akt was verified by an *in situ* proximity ligation assay (PLA) that enables the detection and quantification of physically interacting protein–protein complexes in native cells (30). Compared with the serum-starved cells, 10% serum stimulation markedly increased the number of PLA signals at the plasma membrane, which was visualized by E-cadherin labeling (Fig. 5B). The association of CTMP and Akt was further investigated by immunoprecipitation (IP). From the cell lysates of HeLa cells treated with or without insulin, the HA antibody specifically precipitated phosphorylated Akt at T308 and S473 as well as total Akt, which was identified by an HA antibody (Fig. 5C). Consistent with the findings shown in Fig. 4, the cells transfected with Flag-CTMP exhibited higher Akt phosphorylation levels. Most importantly, CTMP could be
detected in the HA-IP products, indicating the association between CTMP and Akt proteins (Fig. 5C).

To identify the Akt-binding domain of CTMP, a pull-down assay was performed using different CTMP deletion fragments (Fig. 5D). Consistent with the results from co-IP experiments, purified GST-fused full-length CTMP (1-240 a.a.) precipitated Akt from the cell lysate, indicating the direct interaction of CTMP and Akt (Fig. 5E, lane 3). However, only the GST-fused CTMP deletion fragments containing 1-64 a.a. and 1-183 a.a. precipitated Akt (Fig. 5E, lanes 4 and 5), which indicated that the 1-64 a.a. of CTMP are sufficient to bind to Akt. This interaction of CTMP N-terminal domain 1-64 a.a. with Akt was further confirmed using a co-IP assay, which showed that the Akt antibody precipitated the GFP-tagged CTMP deletion fragment containing 1-64 a.a. (Fig. 5F). These results indicate that the N-terminal domain of CTMP (1-64 a.a.) contributes to the protein-protein interaction with Akt. Next, we investigated whether 1-64 a.a. of the CTMP fragment interacts with Akt in cells by performing a PLA assay. As shown in Fig. 5G, both the full-length protein and the 1-64 a.a. CTMP fragment showed a striking increase in PLA signals at the plasma membrane in the presence of 10% serum, whereas the 63-240 a.a. CTMP fragment produced limited signals. The transfection of a plasmid expressing the 63-240 a.a. fragment of truncated CTMP did not induce Akt and GSK3β phosphorylation, soft agar colony formation, or cell proliferation of MCF7 cells, and the 1-64 a.a. truncated CTMP had little effect (Supplementary Fig. 3). These data suggest that the N-terminal domain 1-64 a.a. of CTMP is required but not sufficient to induce Akt activity and the associated biological events. Thus, we identified the N-terminal domain of CTMP containing 1-64 a.a. that directly binds to Akt at the plasma membrane and promotes Akt phosphorylation.
Taken together, our findings demonstrate that CTMP is up-regulated in breast tumors and is associated with the poor survival of breast cancer patients. In parallel, our *in vitro* and *in vivo* studies revealed that CTMP up-regulation promotes breast cancer cell tumorigenesis. In addition, CTMP interacts with Akt through its N-terminal domain containing 1-64 a.a. at the plasma membrane and facilitates Akt phosphorylation at T308 and S473 (Fig. 6). Thus, we conclude that CTMP functions as a positive regulator of Akt and promotes breast tumor growth, suggesting that CTMP may be an oncoprotein in human breast cancer.
Discussion

The over-activation of Akt has been identified in a wide range of cancers, including breast cancer (31). CTMP has been reported to bind to the carboxy terminus of Akt and to regulate its activity, although the regulatory role of CTMP on Akt remains controversial (20, 23). Here, we demonstrated that CTMP was up-regulated in breast tumors and was associated with poor survival of breast cancer patients. Our data are consistent with the expression profile of CTMP in the publicly available microarray datasets from Gene Expression Omnibus (GEO). From the GSE3744 database (32), the transcript levels of CTMP in 40 breast tumor samples and 7 normal breast tissues were compared, and CTMP expression was significantly up-regulated in breast tumors (Supplementary Fig. S4). In 2012, Curtis et al. released a transcriptome database, which consists of 2,000 breast cancer patients (33). We analyzed the open access data of the training set, including 980 patients, and found that the expression of CTMP mRNA was not correlated with the overall survival of these patients ($P=0.575$, data not shown), suggesting that the CTMP protein expression, but not mRNA, could be used to predict the prognosis of the breast cancer patients. In addition, we found that CTMP expression showed a positive correlation with Akt phosphorylation in breast cancer cell lines and tumor specimens. In vitro and in vivo studies indicated the oncogenic activity of CTMP in breast cancer. Thus, our data indicate that CTMP functions as a positive regulator of Akt and facilitates breast cancer tumorigenesis.

CTMP has been considered a tumor suppressor because it repressed in vivo tumorigenesis by inhibiting Akt activation and promoted apoptosis (20, 34). Hwang and colleagues suggested an anti-tumor activity of lentivirus-mediated CTMP gene therapy by using a K-ras null lung cancer mouse model (35). Simone et al. indicated the pro-apoptotic function of the polypeptides derived from CTMP N-terminal...
domain in pancreatic cancer cells (36). The anti-proliferation and apoptosis effects of CTMP have also been demonstrated in different *in vitro* cell models (22, 37, 38). However, the inhibitory function of CTMP on Akt phosphorylation and apoptosis was challenged by Ono et al., who provided evidence that CTMP facilitates Akt phosphorylation and enhances the anti-apoptotic activity of the tested cells (23). Notably, the studies from Maira et al. and Ono et al. both applied only biochemical and molecular approaches to investigate the consequence of CTMP-Akt interaction *in vitro* (24). To clarify the CTMP function on Akt phosphorylation and tumorigenesis, it is necessary to study their correlation in specific types of human cancers. In 2004, Knobbe et al. reported that reduction of CTMP mRNA levels could be found in 40% (37/93) of primary glioblastomas and in 67% (6/9) of glioma cell lines, and Akt phosphorylation at S473 could be detected in all glioma cell lines, suggesting that CTMP may function as a tumor suppressor by inhibiting Akt phosphorylation in human glioblastomas (21). In our study, we found that CTMP was up-regulated in 136 out of 204 (66.67%) breast cancer specimens and was significantly correlated with poor patient survival. Therefore, our data indicate that CTMP functions as an oncoprotein and enhances Akt phosphorylation in breast cancer. These findings suggest the possibility that CTMP may have different functions in different cancer types. Further studies are required to broadly investigate the CTMP functions in distinct human cancers.

Bioinformatic analysis suggests that CTMP consists of a functional uncharacterized N-terminal domain (residues 1-112) and a C-terminal domain (residues 113-240) with thioesterase activity (39). Here, we found that the N-terminal domain of CTMP containing 1-64 a.a. is responsible for its Akt-binding ability. A previous study showed that the first ~20 residues, containing a mitochondrial
localization signal (MLS), are responsible for the mitochondrial localization of CTMP (38). The N-terminal portion of CTMP is cleaved in the mitochondria by the mitochondrial protein peptidase to generate a truncated CTMP protein (36–240 a.a.). Upon apoptosis, the truncated CTMP is released into the cytosol and facilitates apoptosis through binding to Hsp70 but does not interact with Akt (38). The loss of the Akt-binding motif (1-36 residues) of the truncated CTMP may partially explain why the authors did not detect the binding of CTMP and Akt under apoptotic conditions. These observations suggest that posttranslational modification and subcellular localization may be important factors that modulate CTMP function on Akt activity and cellular physiology.

The PI3K/Akt signaling pathway mediates a variety of cellular processes, including cell proliferation, migration, gene expression, and survival. PI3K/Akt pathway dysregulation is frequently found in human breast cancer, such as in point mutations of PIK3CA (40, 41), a loss of PTEN activity (42-44), and mutations of Akt1 (44). A number of drugs have been developed to target the genes involved in the PI3K/Akt signaling pathway, such as PI3K and mTOR. Here, we demonstrated that CTMP was up-regulated in breast cancer and showed a positive correlation with Akt. We also provided evidence that CTMP binding to Akt through its N-terminal domain enhances Akt activation and promotes cell proliferation and tumorigenesis. These findings indicate that CTMP has the potential to serve as a diagnosis and prognosis marker and to be a therapeutic target for human breast cancer.
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**Figure legends**

**Fig. 1.** CTMP is up-regulated in breast tumor and is associated with poor survival in breast cancer patients. (A) Representative IHC images show the CTMP expression in the paired normal tissues and tumors of three breast cancer patients. (B) Immunohistochemistry was performed to examine CTMP expression in the tissue array and the CTMP staining intensity was analyzed using HistoQuest image analysis software. The mean intensity is presented as the black bar in the normal and tumor groups. (C) Representative images of breast tumor specimens showed the CTMP expression score from 0 to 3. (D) Kaplan-Meier plots of overall survival (upper) and disease-free survival (lower) of 198 patients with breast cancer, stratified by CTMP level. (E) Kaplan-Meier plots of overall survival (upper) and disease-free survival (lower) of 88 patients with stage I or stage IIa breast cancer, stratified by CTMP level.

**Fig. 2.** CTMP up-regulation enhances the *in vitro* and *in vivo* tumorigenesis of breast cancer cells. (A) The effect of CTMP on *in vitro* tumorigenesis was analyzed using a soft agar assay. Parental HeLa cells and CTMP-overexpressed transient transfectants were cultured in soft agar consisting of medium with 10% or 3% FBS for 1-2 weeks. (B) The quantification of colonies from the soft agar assay for MCF-7 (upper panel) and HeLa (lower panel) cell lines is presented. *p*<0.05, **p**<0.01, indicates a significant increase compared with relative vector-only control groups. (C) MCF-7 and HeLa cells were stably transfected with GFP-only vectors or GFP-CTMP plasmids. The CTMP expression in the cell lysate of transfectants was examined through Western blot assays (D). Nude mice were subcutaneously inoculated with vector-only or GFP-CTMP stable transfectants of MCF-7 (D) or HeLa (E) cells.
data are presented as the mean of tumor volume±SE; n=7, *p<0.05 compared with vector-only xenograft mice.

**Fig. 3.** CTMP positively correlates with Akt phosphorylation and promotes breast cancer cell proliferation. (A) The expression of CTMP and total or phosphorylated Akt at S473 of indicated cell lines was examined through Western blot assays. (B) CTMP and Akt phosphorylation levels in the total cell lysate acquired from N-T paired clinical specimens were examined through Western blot assays. (C) Immunohistochemistry was performed to examine the expression of CTMP and phospho-Akt (S473) in the serial sections of normal breast tissues or breast tumor samples. Scale bar=100 µm. (D) Quantification of the signal intensity of CTMP and phospho-Akt (S473) from 45 clinical breast cancer specimens. The correlation coefficient=0.7; p<0.001. (E) The proliferation ability of MCF-7 (left panel) and HeLa (right panel) transfectants was determined by cell counting. *, p<0.05 indicates a significant increase compared with mock control groups. (F) MCF-7 cells were infected with lentivirus carrying scrambled (scram) or CTMP-specific shRNA (clone #1 and clone #2); then, CTMP protein levels were examined through Western blot assays (inset). The effect of scramble and siCTMP on cell proliferation was determined by cell counting. (G) MDA-MB-231 cells were infected with lentivirus carrying scrambled (scram) or CTMP-specific shRNA (clone #1 and clone #2); then, CTMP protein levels were examined by Western blot assays (inset). The cell proliferation of scrambled and CTMP shRNA clone #1 and clone #2 infected cells was determined. #, p<0.05 indicates a significant decrease compared with scrambled controls.
**Fig. 4.** CTMP up-regulation enhances PI3K-mediated Akt phosphorylation. (A) HeLa cells were stably transfected with GFP-CTMP plasmid (left panel). MDA-MB-231 cells were infected with lentivirus carrying scramble and CTMP shRNA clone #1 and #2 (right panel). The phosphorylation of Akt at T308 and S473 and GSK3β at S9 was examined through Western blot assays. (B) MCF7 cells were transfected with GFP vector, GFP-CTMP, and/or dominant-negative Akt (DN-Akt). The expression of Akt, pGSK3β at S9, and GSK3β was analyzed through Western blot assays. (C) The proliferation of MCF7 cells transfected with GFP vector, GFP-CTMP, and/or DN-Akt was examined using an MTT assay. (D) HeLa cells were transiently transfected with HA-tagged CTMP plasmid. After 100 nM insulin for different times, the phosphorylation of Akt was analyzed through Western blot assays. Band quantification is presented as the fold increase of phospho-Akt at both T308 (open bars) and S473 (black bars). (E) Stable CTMP transfectants of HeLa cells were treated with 0, 5, 20, and 100 nM insulin for 10 min. The phosphorylation of Akt was determined through Western blot assays. For quantification, the ratio of phospho-Akt at S473 (F) and at T308 (G) to Akt was calculated. (H) Vector-only and CTMP stable transfectants of HeLa cells were starved for 12 h followed by treatment of 100 nM insulin with/without LY294002 for 10 min. Akt phosphorylation was determined through Western blot assays.

**Fig. 5.** CTMP interacts with Akt through its N-terminal domain. (A) HeLa cells were co-transfected with GFP-tagged CTMP and HA-tagged Akt plasmids. The cells were pre-starved for 12 h and stimulated with 100 nM insulin for 10 min. The co-
localization of CTMP and Akt at the plasma membrane is indicated by the arrow head. Scale bar=20 μm. (B) MCF-7 cells were co-transfected with HA-tagged Akt and Flag-tagged CTMP full-length constructs. The interaction of Akt and CTMP (red dots) was analyzed using an in situ PLA assay. The plasma membrane was visualized by immunostaining with an anti-E-cadherin antibody (green). Scale bar=20 μm. The quantification of the PLA signal is presented (n=7); p<0.05 compared with the starvation control. (C) The cell lysates from vehicle or insulin-stimulated cultures were immunoprecipitated with an anti-HA antibody. The IP product was analyzed through Western blot assays. (D) Different CTMP fragments were constructed into a GST-fused expression vector. (E) For the in vitro binding assay, full-length and deletion fragments of GST-CTMP fusion proteins were purified for the GST pull-down assay. The presence of Akt in the GST pull-down products was examined through Western blot assays. (F) HeLa cells were transfected with the GFP-tagged CTMP fragment (1-64 a.a.) plasmid. The cell lysate was immunoprecipitated with an anti-Akt antibody and then analyzed by Western blot assays. (G) MCF-7 cells were co-transfected with HA-tagged Akt and Flag-tagged CTMP full-length and mutation constructs. The interaction of HA-Akt and individual Flag-CTMP deletion fragments was examined by in situ PLA following the procedure described above.

Fig. 6. A diagram illustrating the possible mechanisms by which CTMP facilitates insulin-induced Akt phosphorylation in breast cancer cells.
Figure 1 (Liu et al.)

A

patient #1  patient #2  patient #3

Normal  Normal  Normal

Tumor  Tumor  Tumor

B

\[ p < 0.001 \]

CTMP intensity (pixels)

CTMP intensity (pixels)

CTMP intensity (pixels)

Normal  Tumor  Normal  Tumor  Normal  Tumor

C

0  1  2  3

D

overall survival  disease-free survival

overall survival  disease-free survival

E Stage I + IIa

overall survival  disease-free survival

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Figure 2 (Liu et al.)

Panel A: Images showing colony formation in MCF7 cells with different serum concentrations and CTMP expression levels.

Panel B: Bar graphs showing colony number/well for MCF7 and Hela cells with 10% or 3% serum.

Panel C: Western blots for MCF7 and Hela cells with different expression levels of CTMP and GFP.

Panel D: Graph showing tumor volume (cm³) over time (weeks) for MCF7 cells with CTMP compared to vector.

Panel E: Graph showing tumor volume (cm³) over time (weeks) for Hela cells with CTMP compared to vector.
Figure 3 (Liu et al.)

(A) Western blot analysis of Akt and CTMP in MCF-10A, MCF-7, BT474, BT483, and MDA-MB-231 cells. 

(B) Western blot analysis of pAkt(S473), Akt, CTMP, and Actin in normal and tumor samples.

(C) Immunohistochemical staining of CTMP and pAkt(S473) in normal and tumor tissues.

(D) Scatter plot showing the correlation between CTMP intensity and pAkt(S473) intensity.

(E) Growth curves of MCF7 and Hela cells transfected with CTMP, vector, or mock.

(F) Growth curves of MCF7 cells transfected with shRNAs targeting CTMP, scram, #1, and #2.

(G) Growth curves of MDA-MB-231 cells transfected with shRNAs targeting CTMP, scram, #1, and #2.
Figure 4 (Liu et al.)

A

B

C

D

E

F

G

H

Insulin (nM)

Insulin (nM)

Insulin (nM)

Insulin (nM)
Carboxyl-terminal modulator protein positively regulates Akt phosphorylation and acts as an oncogenic driver in breast cancer

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