Oncogene (2016) **35,** 3718–3728

www.nature.com/onc

ORIGINAL ARTICLE

CHIP-mediated degradation of transglutaminase 2 negatively regulates tumor growth and angiogenesis in renal cancer

B Min[1], H Park[2], S Lee[2], Y Li[3], J-M Choi[2], JY Lee[4], J Kim[5], YD Choi[4], Y-G Kwon[2], H-W Lee[2], S-C Bae[6], C-O Yun[3] and KC Chung[1]

The multifunctional enzyme transglutaminase 2 (TG2) primarily catalyzes cross-linking reactions of proteins via (γ-glutamyl) lysine bonds. Several recent findings indicate that altered regulation of intracellular TG2 levels affects renal cancer. Elevated TG2 expression is observed in renal cancer. However, the molecular mechanism underlying TG2 degradation is not completely understood. Carboxyl-terminus of Hsp70-interacting protein (CHIP) functions as an ubiquitin E3 ligase. Previous studies reveal that CHIP deficiency mice displayed a reduced life span with accelerated aging in kidney tissues. Here we show that CHIP promotes polyubiquitination of TG2 and its subsequent proteasomal degradation. In addition, TG2 upregulation contributes to enhanced kidney tumorigenesis. Furthermore, CHIP-mediated TG2 downregulation is critical for the suppression of kidney tumor growth and angiogenesis. Notably, our findings are further supported by decreased CHIP expression in human renal cancer tissues and renal cancer cells. The present work reveals that CHIP-mediated TG2 ubiquitination and proteasomal degradation represent a novel regulatory mechanism that controls intracellular TG2 levels. Alterations in this pathway result in TG2 hyperexpression and consequently contribute to renal cancer.

Oncogene (2016) **35,** 3718–3728; doi:10.1038/onc.2015.439; published online 16 November 2015

INTRODUCTION

Renal cell carcinoma (RCC) is a life-threatening disease with no effective clinical therapy. RCC primarily develops in people over 40 years of age. Metastatic RCC does not exhibit any particular symptoms before disease progression. Therefore, it is important to understand the molecular mechanisms of RCC progression in order to discover new therapy target.

Recently, several reports show that high expression level of transglutaminase 2 (TG2) contributes to the development of RCC. Thus, TG2 is proposed to be a potential diagnostic marker for RCC.[1–4] TG2 catalyzes a calcium-dependent transamidating reaction that results in cross-linking substrate proteins.[5] TG2 is a negative regulator of von Hippel-Lindau (VHL) tumor-suppressor protein. TG2 induces VHL polymerization through its cross-linking activity.[3] *VHL* is the first identified mutation gene for hereditary RCC and also affects most sporadic RCC case.[6,7] The VHL acts an E3 ubiquitin ligase and ubiquitinates HIF-1α. In renal cancer, inactivated VHL prevents HIF-1α degradation, and consequent dysregulation of HIF-1α activity results in transcriptional activation of hypoxia-inducible genes. HIF target genes include a variety of pro-tumorigenic genes, such as vascular endothelial growth factor (*VEGF*), platelet-derived growth factor (*PDGF*), multidrug resistance pump (*MDR-1*) and erythropoietin (*EPO*).[8] Elevated HIF-1α target genes elicit chemotherapy resistance, invasiveness and vascularity in RCC. TG2 also contributes to the occurrence of kidney diseases.[9–11] For example, increased TG2 activity may accelerate vascular calcification in chronic kidney failure.[10,11] In addition to RCC, altered TG2 expression has a role in certain types of cancers. For example, elevated TG2 expression is observed in pancreatic carcinoma,[12,13] breast carcinoma,[14] malignant melanoma,[15] ovarian carcinoma,[16] lung carcinoma,[17] glioblastomas[18] and renal cancer.[1,3,19] Despite the importance of TG2 function in cancer, the underlying mechanisms of TG2 regulation and degradation are not well studied.

Carboxyl-terminus of Hsp70-interacting protein (CHIP) was first identified as a co-chaperone protein because of its interaction with Hsp70.[20–23] CHIP promotes the ubiquitination of many tumor-related proteins, including p53,[24] HIF-1α,[25] PTEN,[26] c-Myc,[27] Smad3[28] and SRC-3,[29] which consequently promotes their degradation through proteasomal machinery. With regard to its effect on cell growth, there are several reports indicating a tumor-suppressive function of CHIP in gastric cancer,[30] prostate cancer,[31] hepatoma,[32] glioma[33] and breast cancer.[34,35] Previous study revealed that CHIP knockout mice display enhanced aging phenotypes. Especially, CHIP deficiency led to increased SA-β-Gal activity, a molecular marker for aging, in kidney tissues.[36] Nevertheless, the exact relationship between CHIP expression and RCC has not yet been clarified. In this study we found that CHIP expression is markedly decreased, whereas TG2 is highly expressed in RCC cells and patient tissues. In addition, we demonstrate that TG2 is a novel substrate of CHIP and altered TG2 degradation is closely linked to renal cancer.

RESULTS

TG2 accumulation, accompanied by CHIP downregulation, is observed in human renal cancer

We found that TG2 mRNA level is increased between age-matched control samples and kidney tissues from renal cancer patients.

[1]Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea; [2]Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea; [3]Department of Bioengineering, College of Engineering, Hanyang University, Seoul, Republic of Korea; [4]Department of Urology, Yonsei University College of Medicine, Seoul, Republic of Korea; [5]Department of Anatomy, School of Medicine, The Catholic University of Korea, Seoul, Republic of Korea and [6]Department of Biochemistry, College of Medicine, Chungbuk National University, Cheongju, Republic of Korea. Correspondence: Professor KC Chung, Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Yonsei-ro 50, Seodaemun-gu, Seoul 03722, Republic of Korea.
E-mail: kchung@yonsei.ac.kr
Received 20 March 2015; revised 3 October 2015; accepted 19 October 2015; published online 16 November 2015

Analyzes of three available ONCOMINE data sets from control tissues versus RCC tissues showed that TG2 levels are significantly upregulated in all renal cancer patients tested (Figure 1a). We also examined the expression pattern of TG2 and CHIP proteins in renal cancer cell lines. Analyzes of NIH Developmental Therapeutics Program indicated that the relative TG2 mRNA levels are upregulated in eight renal cancer lines, whereas CHIP is downregulated in six different renal cancer cells (Figure 1b). We also compared the protein levels of these two proteins in normal kidney cells and renal cancer cells. Western blot analyzes

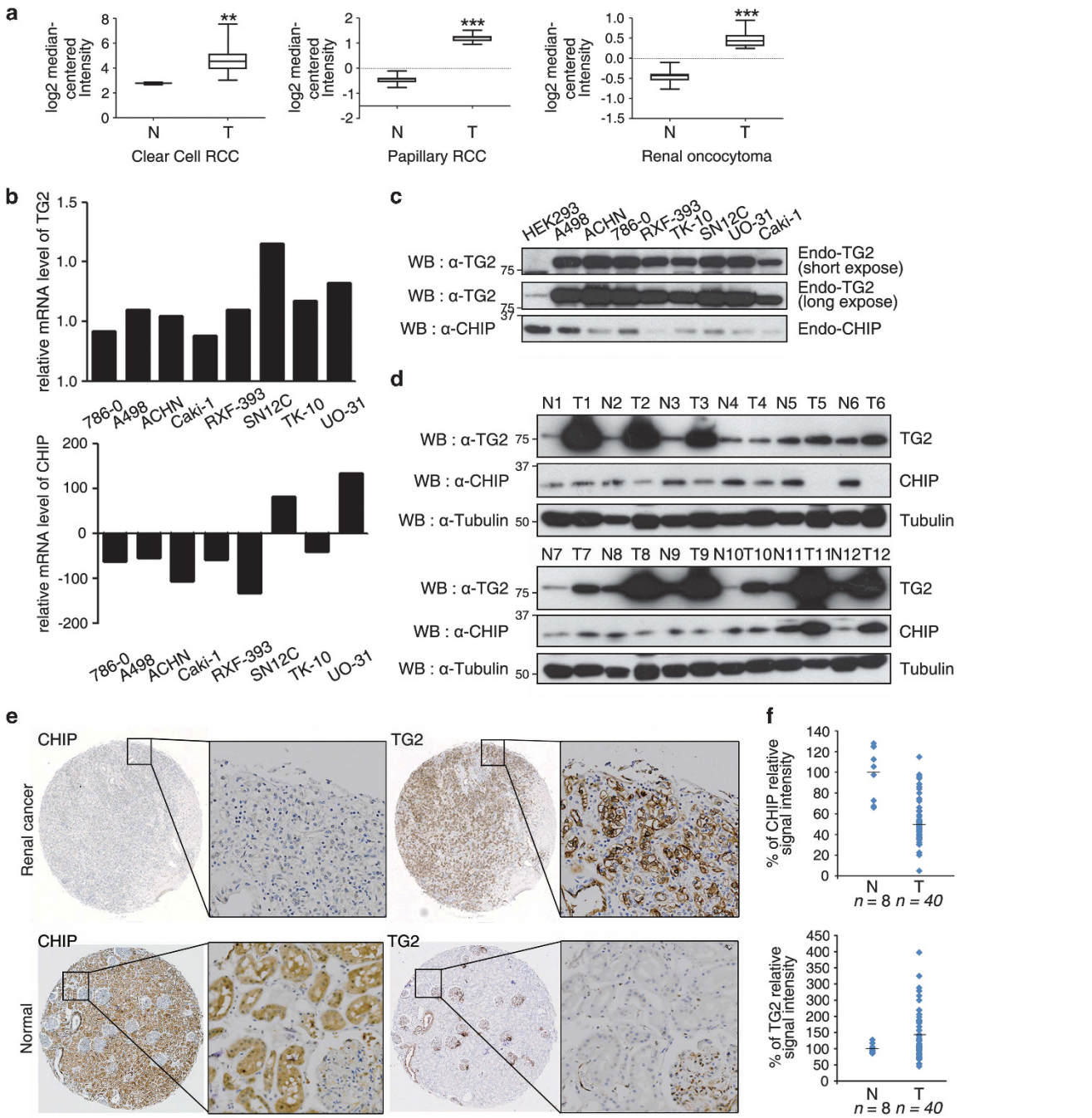

Figure 1. Increased TG2 protein levels accompany decreased CHIP levels in human renal cancer tissues. (**a**) TG2 levels in three different RCC types were analyzed using the ONCOMINE database (www.oncomine.org). Data sets were filtered to display upregulation in three types of renal cancer versus normal renal tissue (clear cell RCC: $^{**}P < 0.006$, papillary RCC: $^{***}P < 0.0001$, renal oncocytoma: $^{***}P < 0.0001$, based on the Student's *t*-test). (**b**) Where specified, the mRNA levels of *TG2* and *CHIP* in eight different RCC cell lines were analyzed using microarray data from the U.S. National Cancer Institute (NCI) (http://dtp.nih.dov/mtweb/targetdata). The graphs are shown in logarithmic scale (mRNA levels in the cell line/mRNA levels in the reference pool). (**c**) Immunoblot analyzes for TG2 and CHIP levels in a normal kidney cell line (HEK293) and eight renal cancer cell lines. (**d**) Immunoblot analyzes for TG2 and CHIP in non-cancerous normal ($n = 12$) or paired cancer tissues ($n = 12$) from renal cancer patients. (**e**) Representative immunohistochemically stained images of normal or RCC tissues using the anti-TG2 or anti-CHIP antibodies. Areas in the black squares are magnified in the right slide panels. (**f**) Immunohistochemical staining of tissue microarrays with anti-TG2 or anti-CHIP antibodies. The expression intensity profiles of TG2 and CHIP in renal cancer tissues ($n = 40$) and normal control tissues ($n = 8$) were detected with each antibody and visualized in the graph. N, normal tissues; T, tumor tissues.

of a normal kidney cell line (HEK293) and eight different renal cancer cell lines also showed that TG2 is highly expressed, accompanied by extensive CHIP downregulation (Figure 1c). Moreover, immunohistochemical analyzes of normal kidney tissue demonstrated that endogenous TG2 expression levels and localization are nearly opposite to the expression levels and localization of CHIP. Specifically, high TG2 levels were detected in endothelial cells underlying the arteries and veins of the renal cortex (Supplementary Figure S1). In contrast, CHIP was highly expressed in artery smooth-muscle cells of the renal cortex (Supplementary Figure S1). Similarly, endothelial cells within the inner stripe of outer medulla revealed high TG2 levels with reduced CHIP levels. High CHIP expression and low TG2 expression were also observed in the collecting duct and thick ascending limb (Supplementary Figure S1). Western blotting analyzes revealed that TG2 protein expression was significantly higher in 11 of 12 (91.6%) tissue samples from renal cancer patients (Figure 1d) compared to control samples. Consistent with previous findings, CHIP protein levels were considerably lower in 7 of 12 (58.3%) tissue samples from renal cancer patients (Figure 1d) and 7 of 7 (100%) renal cancer cell lines (Figure 1c). In addition, immunohistochemical staining of 40 types of renal cancer tissue and 8 types of normal renal tissue showed that TG2 levels are increased by 42% in renal cancer tissues ($P < 0.05$) compared with normal tissues. In contrast, CHIP levels in kidney cancer tissues are decreased by 53% ($P < 0.005$, Figures 1e and f) compared with normal tissues. These results confirmed that TG2 is upregulated and CHIP is downregulated in renal cancer. Moreover, the inverse correlation between intracellular TG2 and CHIP levels in normal and renal cancer suggests a putative role in kidney cancer occurrence and progression.

TG2 interacts with CHIP

Consistent with the previous reports,[1,3,19] our results showed that TG2 protein levels are significantly increased in renal cancer tissues (Figure 1). Moreover, TG2-mediated cancer cell migration is regulated by Hsp70.[37] Because Hsp70 is required for the ubiquitin E3 ligase activity of CHIP through proper substrate recognition, we speculated that CHIP might participate in the degradation of TG2. To test this hypothesis, we first examined whether TG2 binds to CHIP. Ectopically expressed TG2 binds to CHIP in HEK293 cells (Figure 2a). The interaction between endogenous TG2 and endogenous CHIP was also detected in HEK293 cells (Figure 2b) and human renal tissues (Figure 2c). Mapping of the mutual binding domains between CHIP and TG2 using their deletion mutants revealed that the catalytic core domain of TG2 associates with CHIP via the TPR domain (Supplementary Figure S2). *In vitro* pull-down assays confirmed the direct interaction of TG2 and CHIP (Figure 2d). Immunofluorescence analyzes demonstrated that TG2 and CHIP co-localize in the cytosolic area (Figure 2e). Thus, these findings suggest that CHIP and TG2 interact.

CHIP decrease TG2 protein stability

Next, we tested whether CHIP affects the stability of TG2. TG2 protein levels were greatly reduced by CHIP in a dose-dependent manner (Figures 3a and b, and Supplementary Figure S3a). In contrast, knockdown of *CHIP* enhanced the steady-state levels of TG2 (Figure 3c). Measuring the half-life of TG2 showed that CHIP attenuates TG2 stability by reducing the half-life of TG2 (Figures 3d and e). We then determined which region(s) of CHIP is critical for TG2 degradation. The CHIP-K30A mutant does not bind to chaperones and the CHIP-H260Q mutant is widely used as a dominant negative mutant. Compared with the wild-type CHIP, these two mutants do not affect TG2 protein levels (Supplementary Figure S3c). In addition, CHIP decreased the

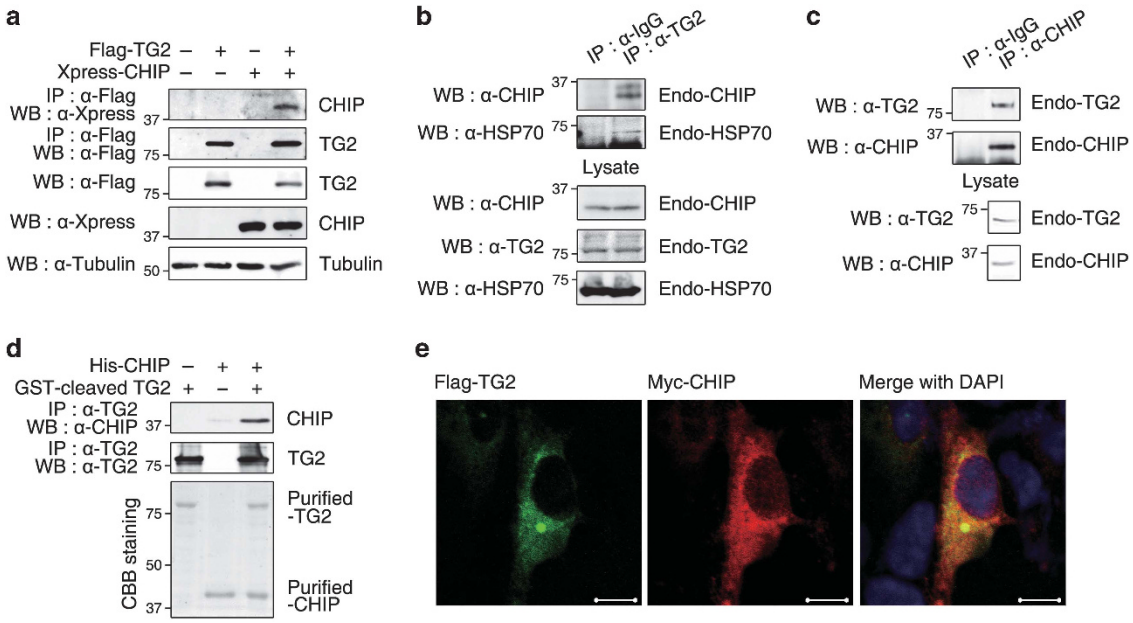

Figure 2. TG2 interacts with CHIP. (**a**) Physical interaction between Flag-tagged TG2 and Xpress-tagged CHIP in HEK293 cells. Proper expression of the transiently expressed proteins was verified by immunoblotting with indicated antibodies. (**b**) Endogenous interaction between TG2 and CHIP in HEK293 cells. (**c**) Endogenous interaction between TG2 and CHIP in kidney tissues. Normal kidney tissue extracts were immunoprecipitated with the mouse anti-CHIP antibody or mouse IgG (as a negative control), followed by immuoblotting with the anti-TG2 antibody. (**d**) *In vitro* interaction between purified GST-cleaved TG2 and His-tagged CHIP. Purified CHIP and TG2 proteins (1 μg) were incubated overnight at 4°C with the TG2 antibody, followed by immunoblotting with the indicated antibodies. Each purified protein is visualized using Coomassie brilliant blue staining. (**e**) Representative confocal images of immunostaining for Flag-tagged TG2 (green) and Myc-tagged CHIP (red) in HEK293 cells. Scale bar, 10 μm.

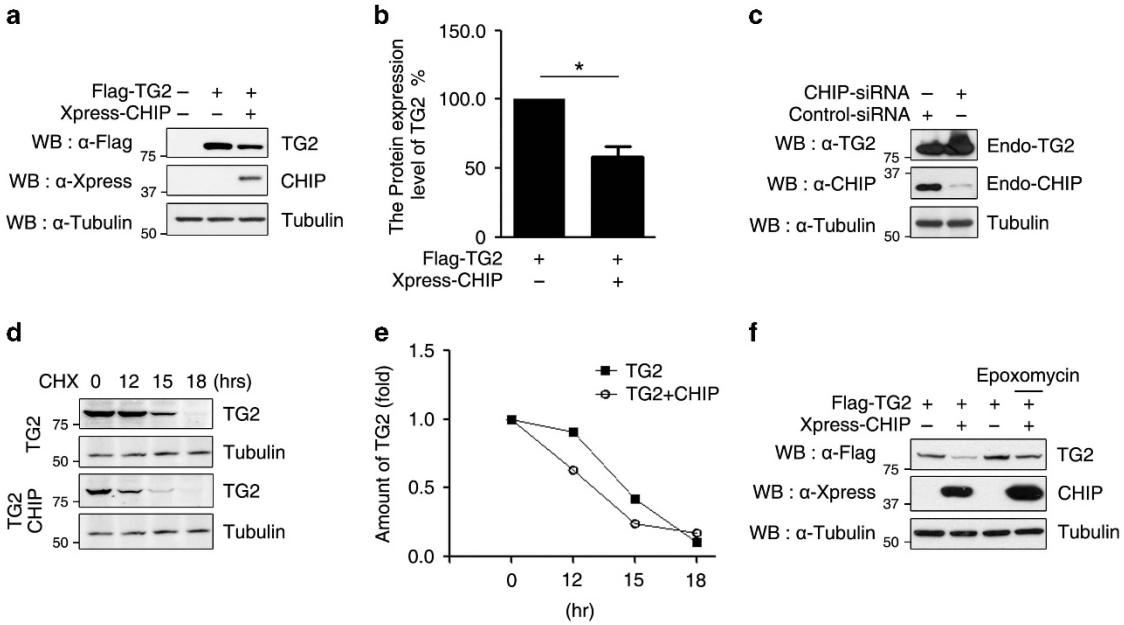

Figure 3. CHIP overexpression downregulates TG2 protein levels. (**a**, **b**) HEK293 cells were transiently transfected for 48 h with Flag-TG2 and/or Xpress-tagged CHIP cells, and cell lysates were immunoblotted with indicated antibodies (**a**). The band intensity of TG2 was quantified using Multi Gauge v3.1 software (Fuji Photo Film Co. Ltd., Tokyo, Japan) (**b**). The graph represents the mean ± s.d. (in arbitrary units) of three independent experiments (*$P < 0.05$, based on the Student's *t*-test). (**c**) ACHN cells were transfected for 24 h with control siRNA or CHIP-siRNA. Proper expression of TG2 and CHIP was verified with the respective antibodies. (**d**, **e**) Cells were treated with 40 μg/ml cycloheximide for the indicated times, and cell lysates were immunoblotted with the anti-Flag antibody. (**f**) Where indicated, HEK293 cells were transfected for 24 h with Flag-tagged TG2 alone or together with Xpress-tagged CHIP, and treated with 2 μM epoxomycin for 6 h. Cell lysates were analyzed by western blotting with the indicated antibodies.

levels of the catalytically inactive TG2 mutant as well as wild-type TG2 (Supplementary Figure S3d). Furthermore, when cells were pretreated with cystamine, a TG2 inhibitor, which binds to the catalytic core domain of TG2 and inhibits its enzymatic activity, there was no significant reduction of TG2 level in the presence of CHIP, accompanying with the blockade of interaction between TG2 and CHIP (Supplementary Figure S3e). The finding that CHIP-mediated downregulation of TG2 recovers to control levels upon treatment with epoxomycin, a proteasomal inhibitor, indicates that CHIP negatively regulates TG2 levels through proteasome-dependent degradation (Figure 3f). On the basis of the previous report that TG2 can be internalized and degraded by autophagy,[38] we additionally examined the effect of autophagy inhibitor, bafilomycin A1, on CHIP-mediated TG2 degradation. As shown in Supplementary Figure S4, the reduction of TG2 level by CHIP was unaffected by bafilomycin A1 treatment. Taken together, these results suggest that CHIP promotes TG2 degradation through proteasome pathway.

CHIP facilitates K48-linked polyubiquitination of TG2

Next, we tested whether CHIP-mediated TG2 degradation occurs via TG2 ubiquitination. Ectopically expressed CHIP promoted the ubiquitination of TG2 through the Lys-48 residue of ubiquitin, but not through Lys-63 (Figures 4a and b). Moreover, CHIP-mediated TG2 ubiquitination only occurred when the six other lysine residues, not K48, were replaced with conjugation-defective arginine. Overexpression of the ubiquitin-K48R point mutant abrogated this process (Figures 4c and d). In addition, TG2 interacts with and co-localizes with the CHIP-ΔU-box mutant as well as wild-type CHIP (Supplementary Figures S5a and b). However, the CHIP-ΔU-box and CHIP-H260Q mutants could not reduce TG2 protein levels (Figure 5g, Supplementary Figures S3c and S5a), because the C-terminal U-box domain is critical for CHIP ubiquitin ligase activity. TG2 ubiquitination was increased by overexpressed CHIP WT but not its mutants (Figure 5e and Supplementary Figure S5c). Moreover, *in vitro* ubiquitination assays with recombinant CHIP and TG2 showed that CHIP markedly enhances TG2 ubiquitination (Figure 4e), suggesting that CHIP directly ubiquitinates TG2. Overall, our results suggest that CHIP promotes TG2 ubiquitination via the Lys-48 residue of ubiquitin. Consistent with previous findings, western blot analyzes of ACHN cell extracts using ubiquitin-K48-specific antiserum showed that K48-linked ubiquitination of TG2 was significantly increased by CHIP overexpression (Supplementary Figure S6a). Analyzes of TG2 ubiquitination also revealed that renal cancer tissues had much lower ubiquitination compared with normal control samples (Supplementary Figure S6b).

Heat shock protein 70 promotes CHIP-mediated TG2 degradation

CHIP dually acts as an ubiquitin E3 ligase and Hsp70 co-chaperone, and Hsp70 is critical for CHIP ubiquitin E3 ligase activity. We next determined whether Hsp70 binding to CHIP affects TG2 ubiquitination. As shown in Figures 5a, b and g, TG2 levels were decreased in the presence of wild-type CHIP, but not in the presence of the CHIP-K30A or CHIP-ΔTPR mutants lacking chaperone-binding activity. These results suggest that CHIP binding to Hsp70 is important for its action toward TG2. These results were further supported by the finding that pretreatment with methylene blue, an inhibitor of Hsp70, inhibits CHIP-mediated TG2 degradation (Figure 5c). In contrast, overexpression of Hsp70 with CHIP-enhanced TG2 degradation (Figure 5d). In addition, endogenous Hsp70 and TG2 co-localized in HEK293 cells (Figure 5f). These data suggest that Hsp70 binding to CHIP promotes TG2 ubiquitination.

TG2 downregulation and CHIP upregulation suppress renal cancer cell growth

On the basis of a previous report that TG2 is highly expressed in RCC, we next tested whether the inverse relationship between

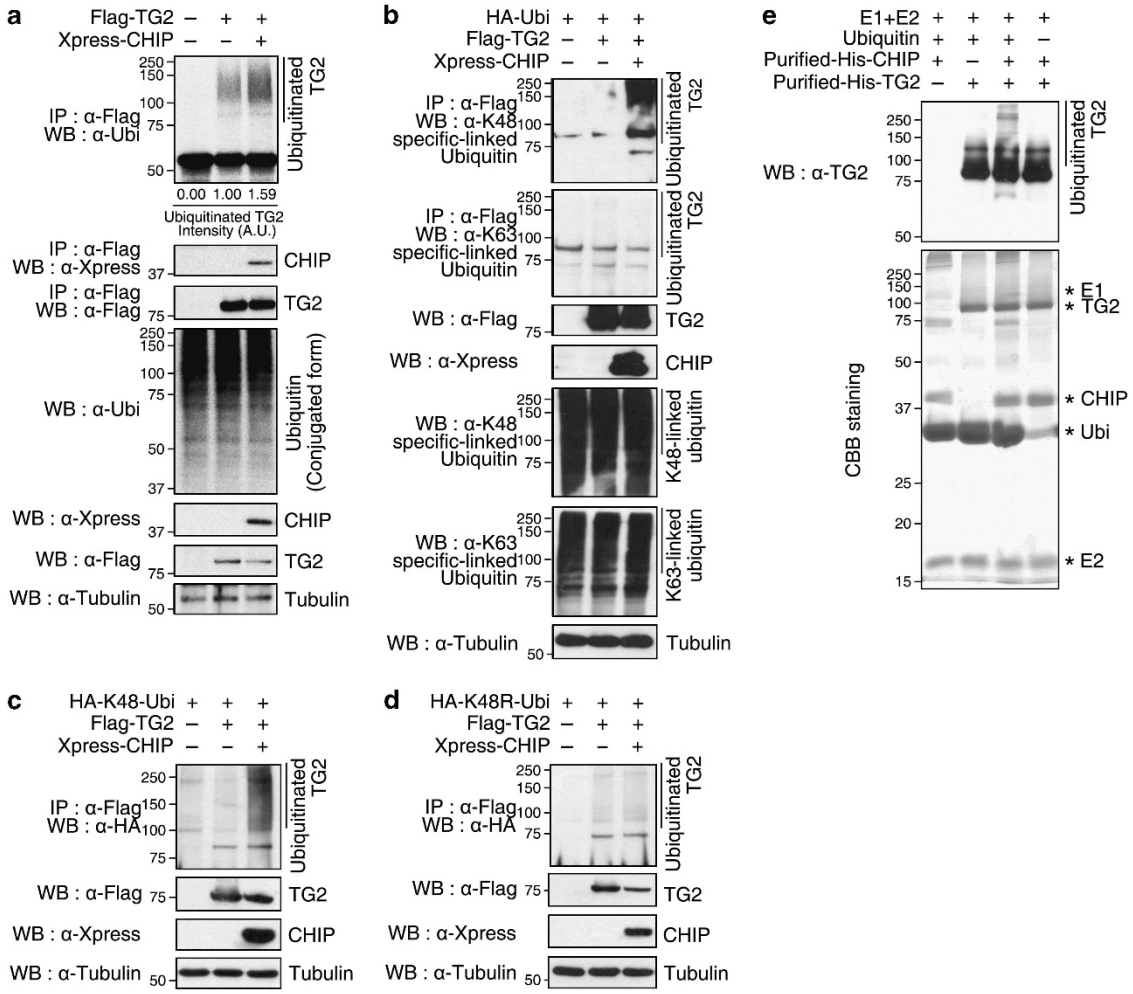

Figure 4. CHIP promotes Lys-48-linked polyubiquitination of TG2. (**a**) Cells were transfected with TG2 alone or together with CHIP, and treated for 6 h with 10 μM MG132. Cell extracts were immunoprecipitated with the anti-Flag antibody, followed by immunoblotting with the anti-Ubiquitin antibody. (**b**) Cell lysates were immunoprecipitated with the anti-Flag antibody, followed by immunoblotting with anti-K48- or anti-K63-specific ubiquitin antibodies. (**c**, **d**) Where specified, cells were transfected for 24 h with Flag-TG2, Xpress-CHIP, HA-K48-ubiquitin (**c**), or K48R-ubiquitin (**d**) alone or in combination, and treated for 6 h with 10 μM MG132. Cell extracts were immunoprecipitated with the anti-Flag antibody followed by immunoblotting with the anti-HA antibody. (**e**) Where specified, purified E1, E2, ubiquitin, CHIP and TG2 proteins were incubated with *in vitro* ubiquitination buffers. Reaction samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by CBB staining or immunoblotting with the indicated antibodies. Asterisks indicate the location of each recombinant protein.

TG2 and CHIP is linked to regulation of kidney cell growth. As shown in Figure 6a, renal cancer cell growth was significantly decreased by CHIP overexpression and/or *TG2* knockdown. Moreover, CHIP overexpression plus *TG2* knockdown inhibits cell growth synergistically. Second, we measured colony formation and demonstrated either CHIP overexpression or *TG2* knockdown considerably decreases colony formation (Figures 6b and c). Similar to cell proliferation, CHIP overexpression combined with *TG2* knockdown synergistically reduced the number of colonies. To further investigate the role of CHIP-mediated TG2 downregulation in *in vivo* renal tumorigenesis, ACHN, ACHN-CHIP-OE or ACHN-TG2-KD cells were injected subcutaneously into nude mice and tumor growth was monitored. As shown in Figures 6d and e, the size of tumors in mice injected with ACHN-CHIP-OE or CHIP-TG2-KD cells were smaller than control ACHN cells. We evaluated microvessel densities using CD31 immunohistochemical staining and also observed a significant reduction in tumor tissues from cells with CHIP overexpressed and *TG2* knocked down (Figure 6f).

Taken together, these results indicate that CHIP-mediated TG2 degradation negatively regulates renal cell growth and colony formation.

CHIP-mediated TG2 downregulation in renal cancer cell inhibits angiogenesis *in vivo*

Dysfunction of VHL inhibits HIF-1α ubiquitination and induces expression of multiple mitogenic growth factors, including *VEGF*, *PDGF* and *TGF*. Upregulated *VEGF*, *PDGF* and *TGF* then promoted tumor angiogenesis. So, we examined whether CHIP-mediated TG2 regulation affects renal cancer angiogenesis. Wound-healing assays revealed that both CHIP overexpression and *TG2* knockdown result in a much lower migration potential of ACHN cells compared with control cells (Figure 7a and Supplementary Figure S7a). We also examined the effect of CHIP overexpression and *TG2* knockdown on renal cancer angiogenesis by measuring *in vitro* human umbilical vein endothelial cell (HUVEC) tube formation. We noted that HUVECs have similar levels of CHIP and TG2 expression as ACHN cells (Supplementary Figure S7b). In addition, the average number of complete tubular structures formed by HUVECs was significantly decreased when conditioned media obtained from ACHN cells with CHIP overexpression or *TG2* knockdown were added. Furthermore, ACHN cells with CHIP overexpressed and *TG2* knocked down significantly decreased HUVEC tube formation compared with only overexpressed CHIP or

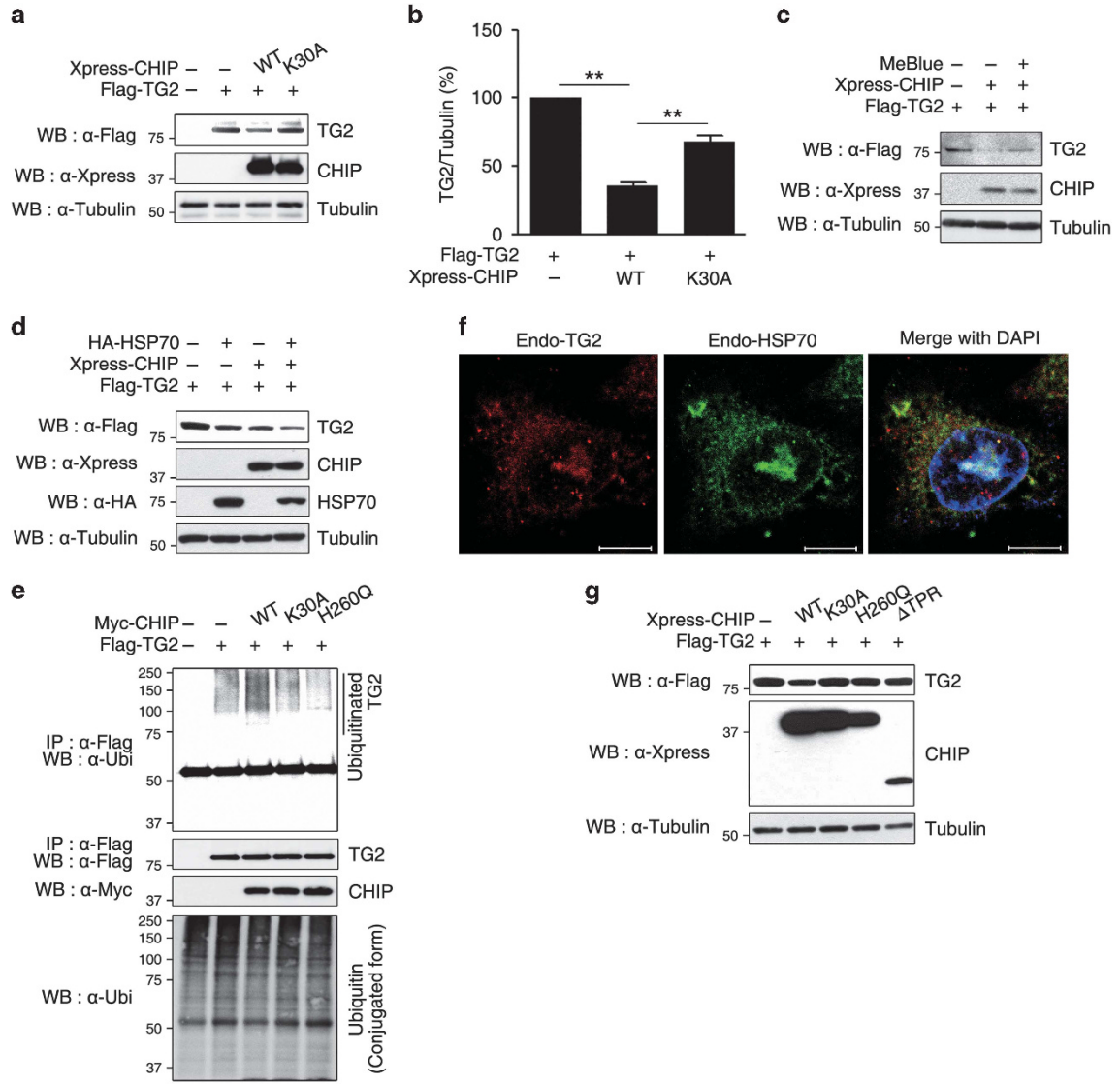

Figure 5. Hsp70 promotes CHIP-mediated TG2 ubiquitination and subsequent proteolysis. (**a**, **b**) HEK293 cells were transiently transfected for 48 h with Flag-TG2, Xpress-tagged wild-type CHIP, or the CHIP-K30A mutant alone or in combination, as indicated. Cell lysates were immunoblotted with their indicated antibodies (**a**). The band intensity of Flag-tagged TG2 was quantified using Multi Gauge v3.1 software (Fuji Photo Film Co. Ltd.) (**b**). The graph represents the mean ± s.d. (in arbitrary unit) of three independent experiments (**$P < 0.01$, based on the Student's *t*-test). (**c**) Where specified, HEK293 cells were transiently transfected for 24 h with Flag-TG2 alone or together with Xpress-CHIP. Cells were then treated for 6 h with 1 μM methylene blue. Cell lysates were immunoblotted with the indicated antibodies. (**d**) Immunoblot analyzes of the interaction between Flag-TG2 and Xpress-CHIP in the absence or presence of Hsp70. Proper expression of the transiently expressed proteins was verified by immunoblot analyzes of cell lysates with anti-Flag, anti-Myc and anti-Xpress antibodies. Tubulin was used as a protein-loading control. (**e**) HEK293 cells were transfected for 24 h with Flag-TG2 alone or together with Xpress-tagged wild-type CHIP, CHIP-K30A or CHIP-H260Q, and treated for 6 h with 10 μM MG132. Cell lysates were immunoprecipitated with the anti-Flag antibody, followed by immunoblotting with the anti-Ubiquitin antibody. Proper expression of Flag-TG2, Xpress-CHIP-WT, Xpress-CHIP-K30A, Xpress-CHIP-H260Q and ubiquitin was verified using the indicated antibodies. (**f**) Representative confocal images of immunostaining for endogenous TG2 (red) and Hsp70 (green) in HEK293 cells. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. (**g**) Immunoblot analyzes of TG2 expression in HEK293 cells transiently transfected with Flag-TG2 alone or together with Xpress-tagged wild-type CHIP, CHIP-K30A, CHIP-H260Q or CHIP-ΔTPR. Proper expression of Flag-TG2 was verified using the anti-Flag antibody.

TG2 knocked down (Figures 7b and c). We then examined the effect of CHIP overexpression or *TG2* knockdown on HUVEC tube length. Consistent with previous observations, these processes were remarkably diminished by CHIP-mediated TG2 downregulation (Supplementary Figure S7c). To further address the functional role of CHIP overexpression and *TG2* knockdown on the invasive and metastatic potential of renal cancer cells, we examined their effects on *in vivo* tumor angiogenesis using Matrigel plug assays. Four types of ACHN cells stably expressing vehicle, CHIP (ACHN-CHIP-OE), and/or TG2-shRNA (ACHN-TG2-KD) were prepared and used in assays to determine whether TG2 and CHIP regulate new blood vessel formation in mice. As shown in Figure 7d, vascular cell proliferation was decreased by CHIP overexpression or *TG2* knockdown. Interestingly, ACHN cells with CHIP overexpressed and *TG2* knockdown rarely formed blood vessel (Figure 7d and Supplementary Figure S8d). We quantified the extent of angiogenesis by measuring hemoglobin content in the plugs and found that either CHIP overexpression or *TG2* knockdown inhibits *in vivo* neovascularization. In addition, CHIP overexpression plus *TG2* knockdown additively decreased the hemoglobin

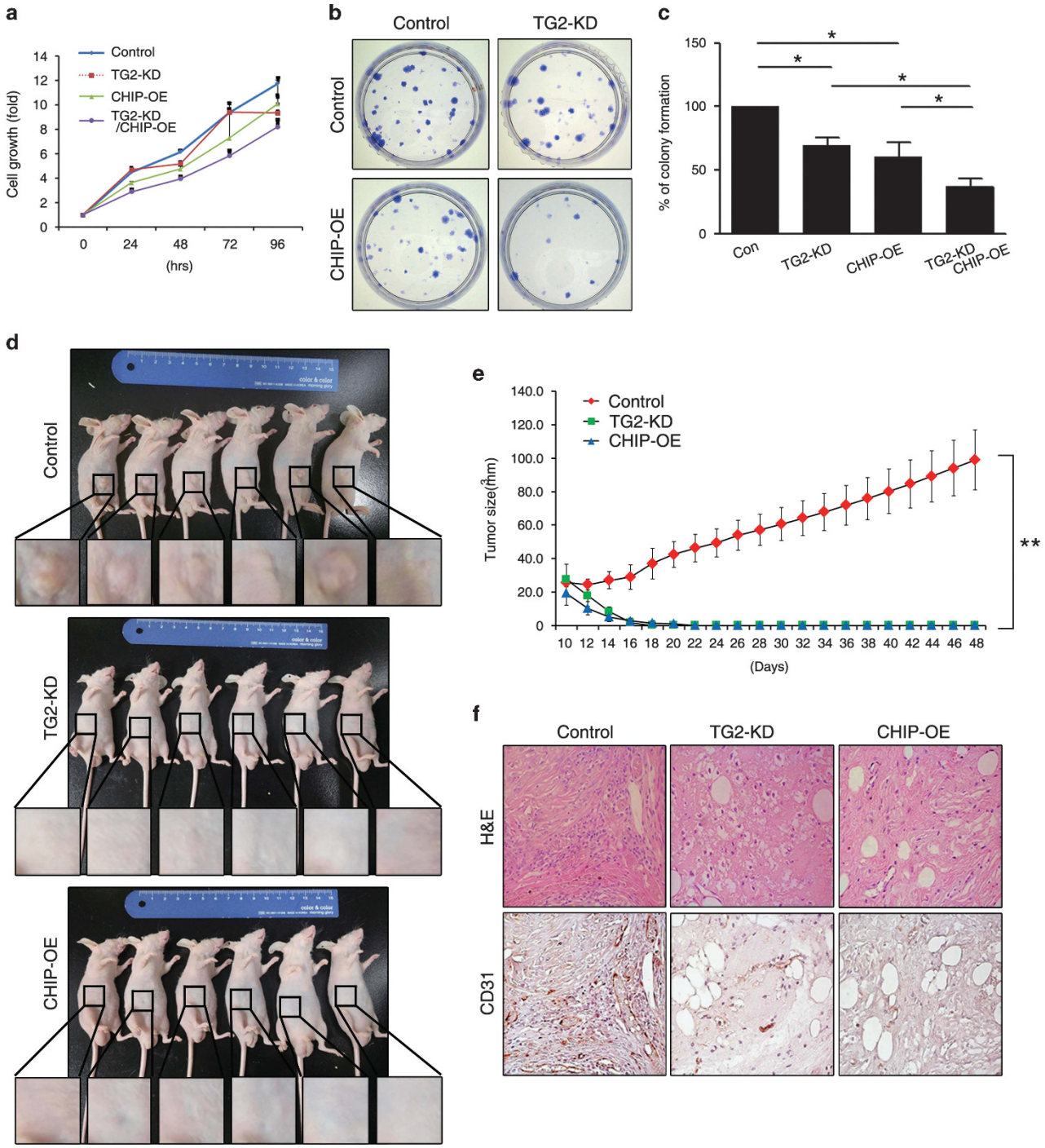

Figure 6. CHIP-mediated TG2 downregulation decreases renal cancer tumorigenesis. (**a**) The cell proliferation rate was measured using a MTT assay. Data are expressed in arbitary units ($n = 5$). (**b**, **c**) Same four ACHN cell lines were plated into culture dishes. After 3 weeks, colonies were photographed using crystal violet staining (**b**). The percentage of colony formation (± s.d.) was measured in three independent experiments (arbitary units; $^*P < 0.05$, based on the Student's *t*-test) (**c**). (**d**, **e**) Where specified, control ACHN, ACHN-CHIP-OE or ACHN-TG2-KD cells were injected into male athymic nu/nu mice (aged 6 to 8 weeks) via subcutaneous implantation of 1×10^7 cells in the abdomen (**d**). Tumor growth curves of mice xenografts cells are presented in **e**. Results are expressed as the mean ± s.e.m. ($n = 6$, $^{**}P < 0.01$). Tumors showing no growth were assigned a volume of zero. Tumor volume (V) was calculated as $V = 0.523 \times a^2 \times b$ (a is the smallest superficial diameter; b is the largest superficial diameter). (**f**) Sections of tumors from injected nude mice were stained with hemotoxylin and eosin and CD31 antibody.

content (Supplementary Figure S8a). Analysis of CD31 staining, another marker for new blood vessel formation, supported these results (Figure 7f and Supplementary Figure S8b). Furthermore, we investigated whether the absence of *TG2* gene in ACHN cells has also negative effect on the tumor vascularization. *TG2* knockout in ACHN cells was accomplished by using crisper/Cas9 system and confirmed by mismatch-sensitive T7E1 assay (Figure 7f) and western blotting (Figure 7g). Matrigel plug assays revealed that TG2 knockout greatly reduces *in vivo* neovascularization (Figure 7h and Supplementary Figure S8e). Moreover, the plugs from *TG2*

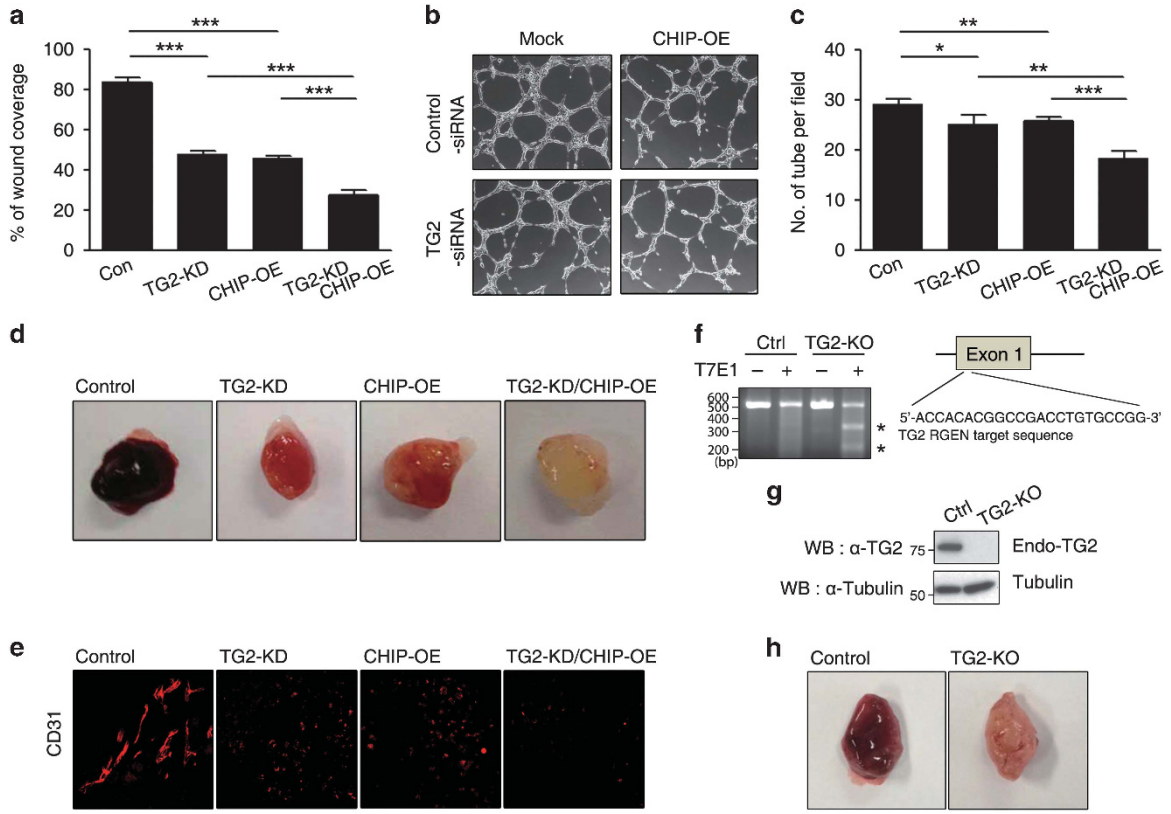

Figure 7. CHIP-mediated TG2 downregulation decreases renal cancer angiogenesis. (**a**) The cell monolayer was scratched. Cell migration rates were monitored using microscopy. The percentage of wound coverage per field was measured in 10 random fields for each group using Multi Gauge v3.1 software (Fuji Photo Film Co. Ltd.; $***P < 0.001$, based on the Student's *t*-test). (**b**, **c**) ACHN cells conditioned media were collected. HUVECs were plated onto BD Matrigel-coated plates at a concentration of 1.5×10^5 cells/well in conditioned media. Microphotographs were taken after 12 h (**b**). Tube networks were quantified using Multi Gauge v3.1 software (Fuji Photo Film Co. Ltd.; $n = 6$, $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$, based on the Student's *t*-test) (**c**). (**d**) Where indicated, unpolymerized Matrigel was mixed with VEGF (200 ng/ml) plus and injected subcutaneously into C57BL/6 mice. Matrigel plugs were excised from the mice and photographed after 5 days. (**e**) Sections of each Matrigel plug stained with CD31 were examined using confocal microscopy (blood vessels, red; DNA, blue, scale bar, 100 μm). (**f**, **g**) CRISPR/Cas9-mediated *TG2* gene knockout (*TG2*-KO) in ACHN cells was confirmed by T7E1 assay and western blotting with anti-TG2 antibodies. (**h**) Where indicated, unpolymerized Matrigel was mixed with VEGF (200 ng/ml) plus the control or *TG2*-KO ACHN cells and injected subcutaneously into C57BL/6 mice. Matrigel plugs were excised from the mice and photographed after 5 day.

knockout samples displayed much lower content of hemoglobin (Supplementary Figure S8c). The overall data suggest that CHIP-mediated TG2 downregulation in renal cancer cell inhibits angiogenesis *in vivo*.

DISCUSSION

Here we demonstrate that CHIP negatively regulates kidney cell tumorigenesis through TG2 ubiquitination and that dysfunction of CHIP with resultant TG2 accumulation triggers RCC. The present study also reveals a novel regulatory mechanism of tumor suppression by TG2. Oncogenic pathway resulting from high TG2 expression and low CHIP activity has been reported in several types of cancers. For example, TG2 expression in multiple cancer cell types has been shown to activate HIF-1, NF-κB and Akt. Although TG2 interacts with HIF-1β and attenuates the HIF-1-mediated hypoxic response pathway,[39] HIF-1α is ubiquitinated by CHIP.[25] In addition, the expression of TG2 is inversely correlated with PTEN, which then triggers the constitutive activation of FAK/Akt and downstream cell survival signaling.[40] Interestingly, Akt as another binding partner of CHIP is also ubiquitinated by CHIP.[41] Furthermore, TG2 activates non-canonical NF-κB signaling in multiple cancers.[42] In contrast, CHIP downregulates NF-κB-mediated signaling in colorectal cancer.[43] Furthermore, a previous study reported that increased TG2 levels cross-link the VHL tumor suppressor, thereby promoting VHL polymerization and decreasing VHL function. Although we did not assess the CHIP effect on VHL-mediated downstream signaling pathway, it is highly probable that TG2-mediated VHL polymerization would be affected by the action of CHIP ubiquitin E3 ligase.

Although not thoroughly explored, additional question arising from the present study is how TG2 expression is highly increased and CHIP expression is remarkably reduced in RCC. As we newly observed lower CHIP protein levels in renal cancer cell lines and human renal cancer tissues, genetic analyzes of patients with renal cancer were performed to assess the possible cause of low *CHIP* gene expression. However, searching for The Cancer Genome Atlas ($n = 540$ renal cancer patients) and COSMIC cancer databases ($n = 435$) revealed no 'loss-of-function' gene mutations. Additional analyzes of copy-number variation showed the loss of *CHIP* in 17.5% (76/435) of kidney cancer patient samples (data not shown). However, compared with other types of cancer (for example, 45% in ovarian cancer), this frequency is not sufficient to be a renal cancer-specific phenomenon. Alternatively, we suggest that low *CHIP* mRNA levels may result from epigenetic variations in the *CHIP* gene. In support of this hypothesis, patients with gastric cancer[30] and colorectal cancer[43] have significant hyper-DNA-methylation in the *CHIP* promoter, which eventually results in low

CHIP gene expression. From these results, we guess that suppression of CHIP-mediated TG2 ubiquitination would result in TG2 accumulation in RCC.

Unlike renal cancer, TG2 expression is greatly decreased in breast cancer or glioma. Interestingly, *TG2* gene is abnormally hyper-methylated in breast cancer or glioma.[44,45] In addition to CHIP-mediated degradation of TG2 via ubiquitin-proteasome system, TG2 can also be degraded by autophagy pathway.[38] On the basis of these reports and the present finding, it is highly probable that numerous types of regulatory systems may affect TG2 expression and stability, depending on the specific cellular environment and differential cancer type.

The present study also revealed that CHIP-mediated TG2 ubiquitination decrease angiogenesis *in vitro* and *in vivo*. These data suggest that TG2-HIF signaling pathway may affect tumor angiogenesis in RCC. Moreover, we showed that tube formation in HUVECs is significantly inhibited in the presence of conditioned media from renal cancer ACHN cells with CHIP overexpressed or *TG2* knocked down. Our speculation for this finding is that unidentified factors (for example, cytokines) released from ACHN cells upon the alteration of TG2/CHIP levels or their activities could modulate endothelial cell angiogenesis. Furthermore, we demonstrate that CHIP-mediated downregulation of TG2 reduces *in vivo* formation of vasculature structures, suggesting that TG2 negatively regulates tumor angiogenesis in renal cancer.

TG2 is a multifunctional enzyme whose altered regulation contributes to the development of a number of diseases. Regarding tumorigenesis, TG2 affects cell growth, cell migration, wound healing, angiogenesis, tumor growth and metastasis in many tumor types. So, besides RCC, CHIP-mediated TG2 degradation and its altered regulation give us valuable important for various kinds of tumor formation.

Several studies recently reported high TG2 levels in RCC patients. Supporting those reports, our current data showed increased TG2 mRNA and protein levels in eight renal cancer cell lines and kidney tissues from RCC patients. Although the causes of increased TG2 levels in RCC are poorly understood, our data suggest an underlying mechanism for this observation. We demonstrated that CHIP predominantly acts as an ubiquitin E3 ligase of TG2 under resting conditions, and CHIP-mediated TG2 ubiquitination and subsequent degradation might be hindered during RCC development. It is unclear why altered CHIP action toward TG2 occurs specifically in renal tissues; however, it could be possible that CHIP-mediated TG2 ubiquitination and/or the regulatory mechanisms of TG2 stability differ among tissues, and RCC may selectively and significantly affect CHIP system. Future studies should examine this hypothesis.

In conclusion, the present study suggests that tight regulation of TG2 via CHIP-mediated ubiquitination and proteasomal proteolysis could be important for normal kidney cell growth. Alterations in this balance result in decreased CHIP activity and abnormal TG2 accumulation, which likely causes RCC. Therefore, we propose that these two proteins could be potential new therapeutic targets of renal cancer.

MATERIALS AND METHODS

Cell culture and DNA transfection

Human embryonic kidney epithelial (HEK293) cells, the human renal cancer cell lines, ACHN cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were grown at 37 °C with 5% CO_2. DNA transfections were performed using Lipofectamine PLUS reagents (Invitrogen) according to the manufacturer's instructions

Co-immunoprecipitation and western blot analyzes

Cells were washed with ice-cold phosphate-buffered saline and whole-cell lysates were prepared in Tris-lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and a protease inhibitor cocktail). Cell lysates (~500 μg) were incubated overnight at 4 °C with 0.5 μg of the appropriate primary antibody. Samples were incubated for 2 h at 4 °C with protein A-sepharose beads and washed three times with lysis buffer. After centrifugation, samples were eluted with 2X sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). Membranes were blocked with 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA) in tris-buffered saline supplemented with 0.1% tween 20 (TBST), and incubated overnight with the primary antibody in TBST. Membranes were washed three times with TBST buffer and incubated with the HRP-conjugated secondary antibody in TBST. Protein bands were visualized using ECL reagent (PerkinElmer, Waltham, MA, USA) per the manufacturer's instructions.

In vitro ubiquitination assays

In vitro ubiquitination assays were performed in a reaction buffer composed of 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM ATP and 1 mM $CaCl_2$. The reaction mixture (40 μl) containing 25 μM ubiquitin, 0.1 μM rabbit E1 and 2.4 μM UbcH5a as ubiquitin E2 conjugating enzyme, 1 μM His-CHIP, and 1 μM His-TG2 in reaction buffer was incubated for 2 h at 30 °C. The reactions were terminated by the addition of 10 μl of 5× SDS sample buffer, and the samples were boiled for 5 min at 95 °C. Proteins were resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and blotted with the anti-TG2 antibody.

Cell growth analyzes

Control ACHN, ACHN-CHIP-OE and ACHN-TG2-KD cells were plated into 96-well plates (1×10^3 cells/well) and allowed to grow for 24, 48 and 72 h. Cell proliferation was assessed at each time point using MTT assays.

Colony formation assays

After DNA transfection, ACHN cells were plated at a density of 500 cells per 60 mm plate and allowed to grow for 14 days. Colonies were stained with crystal violet.

In vitro tube incorporation assays

HUVECs were seeded in growth factor-reduced BD Matrigel (BD Biosciences, Franklin Lakes, NJ, USA)-coated 24-well plates and incubated for 6 h in conditioned media from each stable ACHN cell line (control ACHN, ACHN-CHIP-OE, ACHN-TG2-KD and ACHN-CHIP-OE/TG2-KD). The number of cells incorporated into tubes was counted using optical microscopy and normalized based on tube area.

Mouse tumor xenograft assays

Male athymic nu/nu mice (aged 6–8 weeks; Charles River, Yokohama, Japan) were subcutaneously injected in the abdomen with ACHN cells (1×10^7 cells) transfected with vehicle (control), ACHN-CHIP-OE, or ACHN-TG2-KD. Tumor volume (V) was monitored every other day and calculated as $V = 0.52 \times a^2 \times b$ (a is the smallest superficial diameter; b is the largest superficial diameter).

Tumor histology and immunohistochemistry

Tumor tissues were fixed in 4% paraformaldehyde and embedded in paraffin for histologic examination and immunohistochemical staining. Representative sections were stained with hematoxylin and eosin, and examined using light microscopy. To quantify capillary density and TG2/CHIP expression, tumor sections were stained with anti-Mouse CD31 IgG (BD Biosciences), anti-Mouse TG2 IgG (Cell Signaling Technologies, Danvers, MA, USA) or anti-Mouse CHIP IgG (Cell Signaling Technologies). All slides were counterstained with Mayer's hematoxylin.

Renal cancer tissue sampling from patients

A total of 12 renal cancer tissue samples were obtained from patients diagnosed with renal cancer from September 2012 to November 2013 at Severance Hospital, Seoul, Korea. The ethical committee of the hospital and Yonsei University approved this study (IRB No.: 4-2012-0471 from

Yonsei University College of Medicine and YUIRB-2012-009-01 from Yonsei University).

Data deposition

The sequences reported in this study have been deposited in the GenBank database (Accession No. NP_005852.2 (human CHIP), No. NP_001497192.3 (mouse CHIP), No. NP_004604.2 (human TG2), and No. NP_033399.1 (mouse TG2)).

Statistical analyzes

The significance of differences between groups was validated by unpaired two-tailed *t*-test using Prism (version5.0; GraphPad). All values are expressed as mean ± s.d.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank SY Kim and DH Lee for providing plasmids and JJ Hwang for providing renal cancer cell line lysates. We also thank KH Chun, IK Chung and J Song for their technical assistances, critical comments and helpful discussions. This research was supported by grants from the National Research Foundation of Korea (NRF; 2014M3C7A1064545 to KCC) funded by the Ministry of Science, ICT & Future Planning (MSIP), Republic of Korea, and from the Korea Healthcare Technology R&D Project (HI14C0093 to KCC) through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea. This work was supported in part by NRF grants (2015R1A2A2A01003080 and 2007-0056092 to KCC).

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