TRAF6 upregulates expression of HIF-1α and promotes tumor angiogenesis

Heng Sun1,*, Xue-Bing Li1,†,*, Ya Meng1, Li Fan1, Min Li 2, and Jing Fang 1,¶

1 The Key Lab of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031.
2 The Vivian L. Smith Department of Neurosurgery, Department of Integrative Biology & Pharmacology, University of Texas Medical School at Houston.

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¶ Correspondence: Jing Fang, the Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, SIBS, Chinese Academy of Sciences, Shanghai 200031. Fax: 86-21-54920291; Email: jfang@sibs.ac.cn.

* Equal contribution

# Present address: Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Lung Cancer Institute, Tianjin Medical University General Hospital, Tianjin 300052, China

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ABSTRACT

TNFR-associated factor TRAF6 is a key activator of NF-κB, playing a critical role in the regulation of innate immune responses and their connection to adaptive immune responses. TRAF6 interactions determine receptor-induced cell death versus survival. TRAF6 has been implicated in cancer but its contributions have not been investigated deeply. In this study, we demonstrate that TRAF6 upregulates expression of hypoxia-inducible factor 1α (HIF-1α). TRAF6 affects HIF-1α protein levels but has little effect on mRNA level. TRAF6 increases HIF-1α protein independent of oxygen. We found that TRAF6 binds HIF-1α and mediates its K63-linked polyubiquitination. The E3 ligase activity of TRAF6 was required to increase HIF-1α protein levels. Finally, we showed that TRAF6 promoted tumor angiogenesis and growth. Our results reveal how TRAF6 functions to upregulate HIF-1α expression and promote tumor angiogenesis.
INTRODUCTION

Hypoxia-inducible factor (HIF) 1 is a heterodimeric transcription factor composed of α and β subunits. HIF-1 is overexpressed in many human cancers and the level of its activity in cells correlates with tumorigenicity and angiogenesis (1-3). HIF-1α is induced by hypoxia, oncogenes and growth factors. HIF-1α is constitutively expressed but rapidly degraded by the ubiquitin-proteasome pathway under normoxia (1-3). The prolyl hydroxylation of HIF-1α by prolyl hydroxylase domain proteins (PHDs) at P402 and P564 residues is critical in the regulation of HIF-1α steady-state level (1-3). Under hypoxic condition, the absence of oxygen prevents PHDs from modifying HIF-1α, allowing HIF-1α to accumulate. Vascular endothelial growth factor (VEGF) is mainly regulated by HIF-1α at transcriptional level (1-3). VEGF plays a critical role in tumor angiogenesis (4). Angiogenesis is the formation of new blood vessels from preexisting ones and is required for tumor growth and metastasis (5).

TNF receptor associated factor 6 (TRAF6) is a key activator of NF-κB. It functions as a signal transducer in the NF-κB pathway that activates IκB kinase (IKK) in response to pro-inflammatory cytokines. TRAF6 is an E3 ubiquitin ligase and catalyzes K63 polyubiquitination of TAK1 that is required for IKK activation (6, 7). The interaction of TRAF6 with UBE2N/UBC13, and UBE2V1/UEV1A, which are ubiquitin conjugating enzymes catalyzing the formation of polyubiquitin chains, has been found to be required for IKK activation by this protein. TRAF6 mediates not only the signaling from the members of the TNF receptor superfamily, but also from Toll/IL-1 family (8, 9) and from receptors such as CD40 and RANK (10,11). It also interacts with
various protein kinases including IRAK1, SRC and PKCzeta, which provides a link between distinct signaling pathways.

Recent studies indicate that TRAF6 also plays an important role in cancer. Overexpression of TRAF6 in primary mouse marrow cells results in a myelodysplastic syndrome that develops into a fatal acute myeloid leukemia (12). TRAF6 protein levels are also higher in myelodysplastic syndrome patients (12). It was reported that amplification of the TRAF6 locus was a somatic and frequent event in several human cancer types (13). A recent study identifies TRAF6 as a commonly amplified oncogene bridging RAS and NF-κB in lung cancer (14). Inhibition of TRAF6 in lung cancer cells suppressed NF-κB activation, anchorage-independent growth, and tumor formation (14). Despite these findings, the precise role of TRAF6 protein in cancer has not been extensively investigated. Recent studies suggest that TRAF6 could be a crucial mediator of inflammation-induced tumor growth. Inflammation is closely related to tumor angiogenesis (15) and plays an important role in regulating expression of HIFα (16). Therefore we asked whether TRAF6 is involved in regulation of HIF-1α and tumor angiogenesis. In this manuscript, we demonstrate that TRAF6 can function to upregulate expression of HIF-1α and promote tumor angiogenesis. We find that TRAF6 binds HIF-1α and increases HIF-1α polyubiquitination. Interestingly, TRAF6 enhances HIF-1α protein independent of oxygen. Its E3 activity is required for increasing HIF-1α protein. TRAF6 promotes in vivo tumor angiogenesis. Our findings expand the TRAF6’s function to the HIF-1 signaling.
MATERIALS AND METHODS

Cell culture and reagents

Human colon cancer RKO cells, human cervix cancer HeLa cells, and human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were originally from the American Type Culture Collection. Short tandem repeat analysis was performed to authenticate the cells. All cells were passaged for less than 3 months after resuscitation in lab. TRAF6, HA, C-myc, His, GST, Hsp90 and CD31 antibodies were from Santa Cruz Biotechnology. HIF-1α and HIF-2α antibodies were from BD Biosciences and Novus Biologicals, respectively. IκBα antibody was from Cell Signaling Technology. RACK1 antibody was from BD Biosciences. Flag and β-actin antibodies, lipopolysaccharides (LPS) and protein synthesis inhibitor cycloheximide (CHX) were from Sigma (St. Louis, MO). Dimethyloxaloylglycine (DMOG) was from Frontier Scientific (Logan, UT). And the growth-factor-reduced Matrigel was from BD Biosciences (Bedford, MA). IL-1β was from R&D Systems (Minneapolis, MN).

Construction of vectors

HIF-1α expressing vector was constructed by PCR and coloned to pcDNA3.1-myc vector. Truncated HIF-1α(HIF-1α(Δ363-368)) was also cloned into the pcDNA3.1 vector. HIF-1α (P402A/P564A) was generated by site-directed mutagenesis. TRAF6 was constructed by PCR and cloned to pCMV-Tag2B vector. The dominant negative TRAF6 (TRAF6-DN) and TRAF6(C70A) were constructed as described (17,18).
Glutathione S-transferase (GST)-HIF-1α fusion proteins were constructed by inserting PCR-generated DNA fragments encoding regions of HIF-1α into pGEX4T1. The construct pET28a-TRAF6 for producing His-TRAF6 was generated by PCR. The vectors encoding HA-Ub(K63) and HA-Ub(K63R) were as described previously (19). The VEGF luciferase reporter (HIF-driven) and β-galactosidase plasmids were as described (20).

**Small interfering RNA (siRNA)**

siRNA oligos were purchased from Gene Pharma (Shanghai, China). The siRNA oligos were transfected into cells by using Lipofectine 2000 as per the manufacture’s instruction. The sense sequences of these oligos were as follows:

- Control, 5’UUCUCCGAACGUGUCACGUTT3’;
- siTRAF6-1, 5’CCCAGUCACACAAUGAGAAUTT3’;
- siTRAF6-2, 5’GCGCUGUGCAAACUAUAUATT3’.

**Construction of TRAF6-targeting recombinant adenovirus (Ad-shRNA)**

The TRAF6-targeting recombinant adenovirus Ad-shTRAF6 and control adenovirus Ad-shLacZ were generated with the BLOCK-iT™ U6 RNAi Entry Vector Kit and BLOCK-iT™ Adenoviral RNAi Expression System (Invirogen) according to the manufacturer’s instructions. Briefly, DNA fragments encoding shRNAs against TRAF6 or LacZ (as the negative control) were introduced into pENTR/U6 vector under the control of the human U6 promoter. Each adenoviral vector was propagated in 293A cells to produce the virus. The virus designed for lacZ or TRAF6 knockdown contained the following target sequences.

- shLacZ control:
Top, 5’CACCGCTACAAATCAGCGATTTTCGAACATGCATTTGTGTGTAG3’,
Bottom, 5’AAAACTCAAAATCAGCGATTTTTCGAACATGCATTTGTGTGTA3’.

shTRAF6-1:
Top, 5’CACCGGATATTCAGGGATGAGTTTACGAATAAACTCATCCCTGAATATCC3’,
Bottom, 5’AAAAGGATATTCAGGGATGAGTTTATTCGTAAACTCATCCCTGAATATCC3’.

shTRAF6-2:
Top, 5’CACCGCCACGGGAAATATGTAATATCGAAATATTACATATTTCCCGTGGC3’,
Bottom, 5’AAAAGCCACGGGAAATATGTAATATTTCGATATTACATATTTCCCGTGGC3’.

Immunoblotting and immunoprecipitation

Cells were lysed on ice for 30 min in RIPA buffer (100 mM Tris, 150 mM NaCl, 1% Triton, 0.1% deoxycholic acid, 1 mM EDTA, and 2 mM NaF) supplemented with 1 mM sodium vanadate, 1 mM leupeptin, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM pepstatin A. Equal amounts of protein were resolved on SDS-PAGE and transferred to a nitro-cellulose membrane. The proteins of interest were detected using specific antibodies. Immunoprecipitation was performed as follows: 500 μg of cellular proteins were incubated with 1 μg of primary antibody at 4°C for 3 hours. Twenty microliters of protein A/G PLUS agarose beads were added and the incubation continued overnight. The beads were washed with ice-cold cell lysis buffer and boiled in SDS-PAGE loading buffer for 5 minutes before electrophoresis.

Real-time PCR

Real-time PCR was performed as described (21). β-actin was as the internal control.

The primers are as follows.

β-actin: 5’GATCATGGCTCCTCCTGCAGC-3’ (F), 5’ACTCCTGCTGACTCCAC3’ (R);
VEGF: 5’CTCTACCTCCACCAGGATG3’ (F), 5’GCTGCGCTGATAGACATCCA3’ (R);
bFGF: 5’ CCAGTTTGGTATGCGA3’(F), 5’TCTTCTGCCAAGTGCTCTGTT3’(R);
Glut1: 5’GCGGTTTGGCTACTCAT3’(F), 5’ACTTCAAAGAAGGCCACAAAGC3’(R);
PDK1: 5’CGGATCAGAACCAGACACAA3’(F), 5’CAGGATCCACCCCCGACGCT3’(R);
PHD3: 5’ATCACTTCTCCTGTCG3’(F), 5’CAGCGACCACCCCTGTTG3’(R);
mCD31: 5’CTCATATGCGGTTGCTAT3’(F), 5’TCTTCTGGCGCTTGCTTT3’(R);
TRAF6: 5’CCACCCCCTGGGAAAAGCAAGT3’ (F), 5’ATGCAAGGCTTTGCAGACCT3’(R).
HIF-1α: 5’CGTCGAAAAGAAAAGTCTCGAGAT3’(F), 5’AGGCCTTATCAAGATGCGACT3’(R).

All primers are for human genes except mCD31 ones that are for mouse CD31 gene.

**GST Pull-Down Assay**

Bacterial cells were lysed using the following buffer: 20 mmol/L Tris-Cl, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% NP40, pH 7.5. Bacterial lysates containing GST fused indicated regions of HIF-1α protein were incubated with glutathione-Sepharose 4B beads at 4°C overnight. The beads were washed and then incubated with bacterial lysates containing His-TRAF6 for 6 h. After washing, the bound proteins were eluted from the beads and subjected to electrophoresis.

**Immunohistochemical staining with CD31 antibody**

Histochemical staining of tissues was performed as described (22). Tumor tissues were fixed and processed by conventional paraffin-embedded method. The tumor sections (5-μm thick) were heat-immobilized, deparaffinized, and rehydrated. Antigen retrieval was done by incubation in 10 mM citrate buffer (pH 6.0) for 10 min, followed
by the incubation with 5% block serum for 1 h. Sections were incubated with CD31 antibody at 4°C overnight. After washes, the sections were incubated for 1 h with goat anti-mouse secondary IgG, and detected by incubation with streptavidin–biotin–horseradish peroxidase complex. The tissue sections were stained with hematoxylin for a short time, and subsequently detected under a microscope.

**In vivo Matrigel plug angiogenesis assay and xenograft growth assay**

Matrigel plug assay was done as described previously (22). In brief, RKO cells ($3 \times 10^6$) were mixed with Matrigel (1:2, v/v). The mixture (0.3 mL) was injected s.c. into the flank sides of BALB/cA-nu/nu male nude mice (4 weeks old, from Shanghai Experimental Animal Center, Shanghai). On day 12th, the plugs were removed and trimmed of the surrounding tissues for hemoglobin assay. In vivo xenograft growth assay was performed as described (21). In brief, five-week-old male nude mice were separated into 2 groups and were subcutaneously injected with RKO cells ($5 \times 10^6$) infected with control or shTRAF6 virus at each flank. All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute of Nutritional Sciences.

**Statistical analysis**

The data represent mean ± s.e.m. from three independent experiments except those that are specifically indicated. Statistical analysis was performed using Student’s $t$-test.
RESULTS

TRAF6 increases HIF-1α protein level

Though recent studies have indicated that TRAF6 is involved in cancer, the precise role of TRAF6 in cancer is unclear. We were interested in whether TRAF6 was involved in regulation of HIF signaling. To this end, we determined the effects of TRAF6 on expression of HIF-1α. We found that knockdown of TRAF6 decreased the protein level of HIF-1α (Fig. 1A). And this had little effect on HIF-1α mRNA level (Fig. 1B). Consistent with the results, overexpression of TRAF6 enhanced the protein level of HIF-1α (Fig. 1C), without affecting the HIF-1α mRNA (Fig. 1D). These results suggest that TRAF6 upregulates HIF-1α at a post-transcriptional level. Interestingly, TRAF6 enhanced HIF-1α in the presence of DMOG (Fig. 1C). DMOG is an inhibitor of PHDs and upregulates expression of HIF-1α through increasing HIF-1α stability. The results suggest that TRAF6 and PHDs work independently to regulate HIF1α levels. And our following data do indicate that TRAF6 and PHDs work independently to regulate HIF1α. Knockdown of TRAF6 decreased HIF-1 transcriptional activities (Fig. 1E) and VEGF mRNA (Fig. 1F). Inhibition of TRAF6 also repressed transcription of other HIF-1 targets such as bFGF, Glut1, PDK1 and PHD3 (Fig. 1G). Taken together, these results suggest that TRAF6 is involved in regulation of HIF signaling.

One may ask whether or not TRAF6 influences the expression of HIF-2α, another member of HIF family (1, 2). To know this, we inhibited TRAF6 and determined HIF-2α. The results show that knockdown of TRAF6 resulted in a decrease of HIF-1α but did not led to a decrease of HIF-2α (Fig. 2A). Consistently, overexpression of
TRAF6 had little effect on protein level of HIF-2α, though it enhanced that of HIF-1α (Fig. 2B). These results suggest that TRAF6 does not regulate expression of HIF-2α. The other TRAF family members TRAF2 and TRAF5 have similar motifs as TRAF6 has and they all have been demonstrated to mediate activation of NF-κB (9). We therefore determined the effect of TRAF2 and TRAF5 on expression of HIF-1α and we found neither TRAF2 nor TRAF5 induced expression of HIF-1α (Fig. 2C).

TRAF6 is downstream of IL-1/IL-1R and LPS/TLR4 pathway (8, 9). Therefore, we determined whether IL-1β and LPS induced HIF-1α through TRAF6. We found that treatment of cells with IL-1β or LPS induced HIF-1α. And knockdown of TRAF6 prevented IL-1β and LPS from inducing HIF-1α (Fig. 2D). These results suggest that TRAF6 is involved in induction of HIF-1α expression by IL-1β and LPS.

**TRAF6 upregulates HIF-1α independent of oxygen**

Results of Fig. 1C show that TRAF6 enhances HIF-1α protein in the presence of DMOG, suggesting that TRAF6 regulates HIF-1α independent of oxygen. To confirm this, we determined the effect of TRAF6 on HIF-1α expression under hypoxia and found that TRAF6 enhanced HIF-1α even more in hypoxia (Fig. 3A). We constructed a vector encoding HIF-1α(P402A/P564A), a HIF-1α mutant that could not be hydroxylased by PHDs. As shown in figure 3B, overexpression of TRAF6 still enhanced the protein level of HIF-1α(P402A/P564A). These results imply that TRAF6 regulates HIF-1α expression independent of oxygen.

Hsp70 binds HIF-1α and mediates the HIF-1α ubiquitination and degradation
independent of oxygen (23). We found that overexpression of Hsp70 decreased HIF-1α protein, which could be reversed by co-expression of TRAF6 (Fig. 3C). And TRAF6 did not prevent the interaction between HIF-1α and Hsp70 (Fig. 3D). These results suggest that TRAF6 regulates HIF-1α not through Hsp70. It was known that the receptor of activated protein kinase C (RACK1) competes with Hsp90 for binding to HIF-1α and mediated O2-independent degradation of HIF-1α (24). We found that TRAF6 did not affect the interaction between Hsp90 and HIF-1α (Fig. 3E). And TRAF6 still enhanced HIF-1α in the presence of RACK1 (Fig. 3E). The results suggest that TRAF6 regulates HIF-1α not through RACK1. NF-κB is also known to regulate expression of HIF-1α (25, 26). TRAF6 is a critical regulator of NF-κB. We therefore determined whether TRAF6 regulates HIF-1α through NF-κB. The vector encoding IκBα-SR was employed. IκBα-SR, an IκBα mutant, is not phosphorylated by IKK complex and functions to inhibit NF-κB. We found that TRAF6 still upregulated HIF-1α protein in the presence of IκBα-SR (Fig. 3F), suggesting that TRAF6 regulates HIF-1α independent of NF-κB. Overexpression of TRAF6 did not induce HIF-1α in the presence of MG132, an inhibitor of proteasome (Fig. 3G). And knockdown of TRAF6 did not lead to a decrease of HIF-1α in the presence of MG132 (Fig. 3H). These data suggest that TRAF6 regulates HIF-1α in a proteasome-dependent pathway.

**TRAF6 associates with HIF-1α**

We analyzed the sequence of HIF-1α protein and found that it had a TRAF6-binding motif \( ^{363} \text{PVESSD}^{368} \) (PxExxAr/Ac) (Ar for aromatic residues; Ac for acidic residues)
(Fig. 4A). This gave us a hint that TRAF6 might bind HIF-1α. Immunoprecipitation assay indicated that the exogenous TRAF6 and HIF-1α bound each other (Fig. 4B). Moreover, the endogenous TRAF6 and HIF-1α were co-immunoprecipitated (Fig. 4C). The results suggest that TRAF6 associates with HIF-1α. To know whether PVESSD is required for TRAF6 binding HIF-1α, we constructed a vector encoding HIF-1α truncated mutant deleting PVESSD (HIF-1α(Δ363-368)). The immunoprecipitation assay indicates that TRAF6 had an interaction with HIF-1α but not HIF-1α(Δ363-368) (Fig. 4D). Our mapping data indicate that TRAF6 binds HIF-1α(330-530), but not HIF-1α(1-329) or HIF-1α(531-826) (Fig. 4E). These data imply that PVESSD is required for TRAF6 to bind HIF-1α.

We examined HIF-2α protein and it does not have the PxExxAr/Ac sequence. And HIF-2α expression is not regulated by TRAF6 (Fig. 2B). We therefore asked whether TRAF6-HIF interaction is required for increased HIF-1α expression. To this end, the vector encoding HIF-1α(Δ363-368) was employed in our work. The effect of TRAF6 on stability of HIF-1α and HIF-1α(Δ363-368) was determined. The results show that the stability of HIF-1α(Δ363-368) was reduced (Fig. 4F). The results suggest that binding to HIF-1α is required for TRAF6 to enhance this protein.

**TRAF6 mediates ubiquitination of HIF-1α**

TRAF6 is an E3 ligase that catalyzes K63-ubiquitination (6, 7). We therefore asked whether the E3 activity of TRAF6 was required for enhancing HIF-1α expression. Two constructs that encoded domain-negative TRAF6 (TRAF6-DN) and TRAF6 mutant
TRAF6(C70A) were employed. Both TRAF6-DN and TRAF6(C70A) are E3 activity
deficient (17,18). Neither TRAF6(C70A) nor TRAF6-DN enhanced HIF-1α protein (Fig.
5A), suggesting that TRAF6 activity is required for upregulating HIF-1α. We found that
TRAF6 enhanced dramatically the polyubiquitination of HIF-1α in the presence of
Ub(K63) (Fig. 5B) but not Ub(K63R) (Fig. 5C). Deletion of the TRAF6 binding
sequence (PVESSD) from HIF-1α reduced the polyubiquitination of HIF-1α in the
presence of TRAF6 (Fig. 5D). TRAF6-DN and TRAF6(C70A) had little effect on
polyubiquitination of HIF-1α (Fig. 5E). These results suggest that TRAF6 targets
HIF-1α for K63 ubiquitination. We found that expression of Ub(K63) enhanced HIF-1α
protein level by TRAF6 (Fig. 5F).

**TRAF6 promotes tumor angiogenesis**

It is known that HIF-1α plays a critical role in regulating angiogenesis through
inducing expression of VEGF. Our above results have shown that TRAF6 influences
HIF-1α protein, HIF-1 transcriptional activity and VEGF expression. We therefore
determined the effects of TRAF6 on tumor angiogenesis by means of Matrigel plug
assay. The RKO cells were infected with control or shTRAF6 adenovirus and the
infected cells mixed with Matrigel were injected into nude mice. We found that
inhibition of TRAF6 reduced the amount of hemoglobin in the plug (Fig. 6A), implying
that TRAF6 inhibits tumor angiogenesis. To verify this, we tested effect of TRAF6 on
xenografts growth. RKO cells were infected with control or shTRAF6 adenovirus and
the infected cells were inoculated into the nude mice. Knockdown of TRAF6
suppressed the xenograft growth of RKO cells (Fig. 6B and C). Knockdown of TRAF6 reduced protein level of HIF-1α, but not HIF-2α, in xenografts (Fig. 6D). Inhibition of TRAF6 reduced formation of blood vessels (Fig. 6E). Our real-time PCR assay indicates that knockdown of TRAF6 decreased the mRNA level of VEGF, bFGF and mouse CD31 in xenografts (Fig. 6F).

DISCUSSION

TRAF family members are intracellular proteins that transmit signals from the intracellular portion of TNFR superfamily members. As a unique member of TRAFs, TRAF6 has been shown to be a critical factor for the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) family (8, 9). Therefore, TRAF6 represents a central point of convergence for the signal transduction by the TNFR and the IL-1R/TLR superfamilies and thus plays a critical role in the regulation of innate immune responses, as well as the connection between the innate and adaptive immune responses. In addition, TRAF6 interacts with a variety of proteins that regulate receptor-induced cell death or survival. Thus, TRAF6-mediated signals may directly induce cell survival or interfere with the death receptor-induced apoptosis. An accumulation of evidence confirms TRAF6 as a regulator of cell fate including cancer cells (27). TRAF6 could be a crucial mediator of inflammation-induced tumor growth and progression, metastasis and tumor surveillance. Empirical differences in TRAF proteins expression in human cancer have been reported. TRAF6 exhibits the highest and most consistent
expression in human cancer cell lines (28, 29). Starczynowski et al (14) found that TRAF6 exhibited concomitant mRNA overexpression and gene amplification in NSCLC and SCLC tissues. And Starska et al (30) reported a positive relationship of TRAF6 in tumor margin cells with the histological grade and the mode of tumor invasion by RT-PCR in laryngeal carcinoma. In malignant lymphoma, TRAF6 is known to mediate oncogenesis of marginal zone B cell lymphoma of mucosa-associated lymphoid tissue, which is associated with *Heliobacter pylori*—induced antigenic stimulation and accompanying inflammation in tumorigenesis (31). Ma et al (32, 33) found TRAF6-siRNA significantly inhibited proliferation and enhanced apoptosis of EC109 esophageal cancer cells. Though these studies and others have shown that TRAF6 plays an important role in cancer, the precise role of TRAF6 in cancer is not well understood. In this manuscript we demonstrate that TRAF6 upregulates expression of HIF-1α, leading to increased tumor angiogenesis. Regulation of HIF-1α can be at either a transcriptional or a post-transcriptional level (1,2). Our results show that TRAF6 has little effect on mRNA level of HIF-1α (Fig. 1), indicating that TRAF6 regulates HIF-1α post-transcriptionally. Compared to HIF-1α, the stability of HIF-1α(Δ363-368) is reduced in the presence of TRAF6 (Fig. 4F). These results suggest that TRAF6 influences the protein stability of HIF-1α. Regulation of expression of HIF-1α may also be at a translational level (34). So, we cannot exclude the possibility that TRAF6 regulates HIF-1α through this pathway.

HIF-1α is constitutively expressed but rapidly degraded by the ubiquitin-proteasome pathway under normoxia. TRAF6 did not influence HIF-1α
expression in the presence of proteasome inhibitor MG132 (Fig. 3G and H), suggesting that TRAF6 enhances HIF-1α in a proteasome way. We found that TRAF6 enhanced HIF-1α in the presence of DMOG (Fig. 1) and it upregulated expression of HIF-1α (P402A/P564A) (Fig. 3A). These data imply that TRAF6 regulates HIF-1α independent of oxygen. Luo et al (23) demonstrated that Hsp70 mediated oxygen-independent degradation of HIF-1α. Our results show that TRAF6 still enhanced HIF-1α protein in the presence of Hsp70 (Fig. 3C) and it did not interfere with the interaction between Hsp70 and HIF-1α (Fig. 3D). Thus, TRAF6 may not regulate HIF-1α through Hsp70. Similarly, TRAF6 regulates HIF-1α independent of Hsp90 (Fig. 3E), a protein that mediates O2-independent degradation of HIF-1α (24).

NF-κB is also known to regulate expression of HIF-1α (25). We found that inhibition of NF-κB could not prevent TRAF6 from enhancing the protein level of HIF-1α (Fig. 3F), suggesting that TRAF6 regulates HIF-1α not through NF-κB.

We found that TFAR6 associated with HIF-1α, which is required for HIF-1α stability (Fig. 4). And the E3 ligase activity is required for TRAF6 to regulate HIF-1α (Fig. 5A). These results drove us to determine whether TRAF6 mediates polyubiquitination of HIF-1α. And our results indicate that TRAF6 enhanced ubiquitination of HIF-1α in the presence of Ub(K63) (Fig. 5B) and Ub(K63) enhance protein level of HIF-1α by TRAF6 (Fig. 5F). The results suggest that TRAF6 mediates ubiquitination of HIF-1α, which is involved in upregulation of HIF-1α. Further investigation is needed to confirm this, for example, to determine the lysine residues of HIF-1α that TRAF6 modifies by means of Mass-Spectra analysis. It will be also
interesting to determine the possible function of ubiquitination of the sites by means of site-directed mutation.

Our results suggest that TRAF6 enhances HIF-1α in a proteasome-dependent way, because TRAF6 could not influence HIF-1α protein level in the presence of MG132 (Fig. 3G and H). The mechanism by which a polyubiquitinated protein is targeted to the proteasome is not fully understood. Ubiquitin-receptor proteins may escort polyubiquitinated proteins to the proteasome (35), but the specifics of this interaction and its regulation are unclear. So, it will be interesting to determine whether TRAF6 blocks HIF-1α escort to proteasome, leading to HIF-1α upregulation. TRAF6 does not regulate expression of HIF-2α (Figs. 2A and B). We also analyzed HIF-2α protein and could not find any TRAF6-binding consensus in it. This is probably one reason that TRAF6 does not influence expression of HIF-2α.

TRAF6-mediated signaling involves in a variety of inflammatory, apoptotic, and gene regulatory pathways. Activation of some signaling mechanisms, including AKT (36) and NF-κB, has been suggested to be involved in TRAF6-mediated oncogenesis. Herein, we demonstrate that TRAF6 upregulates HIF-1α and promotes tumor angiogenesis. Our results may provide a new sight into the function of TRAF6 in tumorigenesis. A recent study demonstrates that TRAF6 is involved in TLR-induced angiogenesis, which provides more evidence (37). Peng et al., reports recently that TRAF6 is involved in the potentiation of proliferation of glioma cells (38). We also find in our work that knockdown of TRAF6 suppresses proliferation of RKO cells (Data not shown). These results suggest that TRAF6 may influence tumorigenesis through
multiple ways. The function of TRAF6 may be multiple, multifarious, and multifaceted and the precise mechanisms by which TRAF6 contributes to tumor progression needs extensive investigation. Nevertheless, TRAF6 is becoming an attractive target molecule for therapeutic strategies against cancer.

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FIGURE LEGENDS

Figure 1 TRAF6 enhances expression of HIF1α.

(A) RKO and Hela cells were transfected with control or TRAF6 siRNA oligos. After 48 h, the cells were harvested and the HIF-1α protein was determined. Relative HIF-1α protein level was determined by measuring the density of HIF-1α band and normalized to that of β-actin. The control HIF-1α level was designated as 1. (B) RKO and Hela cells were transfected with TRAF6 or control siRNA oligoes as described above. The mRNA level was determined by real-time PCR. (C) 293T and Hela cells were transfected with Flag-TRAF6 or control empty plasmid. After 24 h, the cells were treated with or without DMOG (1 mM) for 6 h. The cells were then harvested and the protein level of HIF-1α was determined. (D) The cells were transfected as described in Fig. 1C. HIF-1α mRNA was determined by real-time PCR. The data are mean±s.e.m. (n=3). *, p < 0.05 vs control. (E) Knockdown of TRAF6 inhibits HIF-1 transcriptional activity. The cells were transfected with VEGF promoter reporter and β-gal (β-galactosidase) plasmids, and siRNA oligos for 48 h. The cells were harvested and luciferase activity was determined. The ratio of firefly luciferase to β-gal activity was determined and normalized to that of control. The data are mean±s.e.m. (n=3). *, p < 0.05 vs control. (F) The cells were treated as described in Fig. 1E. real-time PCR was performed to determine the mRNA levels of TRAF6 and VEGF. The data are mean±s.e.m. (n=3). *, p < 0.05 vs control. (G) Inhibition of TRAF6 repressed transcription of bFGF, Glut1, PDK1 and PHD3. The data are mean±s.e.m. (n=3). *, p < 0.05 vs control.
Figure 2 TRAF6 does not regulate expression of HIF-2α.

(A) TRAF6 has little effect on HIF-2α. RKO cells were infected with shLacZ (control) or shTRAF6 adenovirus (MOI = 100). After 48 h, the cells were harvested for determination of HIF-2α. (B) RKO cells were transfected with control or Flag-TRAF6 plasmid as indicated. After 24 h, the cells were harvested for determination of HIF-2α. (C) TRAF2 and TRAF5 do not enhance the protein level of HIF-1α. 293T cells were transfected with TRAF2 or TARF5 plasmid as indicated. After 24 h, the cells were harvested for HIF-1α determination. (D) Knockdown of TRAF6 blocks IL-1β- and LPS-induced HIF-1α. The cells were transfected with control or TRAF6 siRNA oligos as indicated. After 48 h, 293T cells were treated with IL-1β (10 ng/ml) for 4 h and RKO cells were treated with LPS (1 μg/ml) for 8 h.

Figure 3 TRAF6 regulates HIF-1α independent of oxygen.

(A) TRAF6 increases HIF-1α under hypoxia (1% O₂). 293T cells were transfected with Flag-TRAF6 or control plasmid as indicated. After 24 h, the cells were subjected to 1% O₂ for 4 h. (B) TRAF6 enhances protein level of HIF-1α(P402A/P564A). 293T cells were transfected as indicated. After 24 h, the cells were harvested for determination of HIF-1α. (C) TRAF6 regulates HIF-1α independent of Hsp70. 293T cells were transfected as indicated. After 24 h, the cells were harvested for determination of Myc-HIF-1α. (D) TRAF6 does not influence the interaction of Hsp70 and HIF-1α. 293T cells were transfected as described in Fig. 3C. The interaction between HA-Hsp70 and Myc-HIF-1α was determined. (E) TRAF6 regulates HIF-1α independent of Hsp90. 293T cells were transfected with Flag-TRAF6 and Myc-HIF-1α
plasmids as indicated. After 24 h, the cells were harvested. (F) TRAF6 regulates expression of HIF-1α independent of NF-κB. 293T cells were transfected with Flag-TRAF6 and Flag-IκBα-SR plasmids as indicated. After 24 h, the cells were harvested. (G) TRAF6 does not enhance HIF-1α in the presence of MG132. 293T cells were transfected with Flag-TRAF6 or control plasmid. After 24 h, the cells were treated with or without MG132 (20 μM) for 4 h. (H) Knockdown of TRAF6 has little effect on expression of HIF-1α in the presence of MG132. RKO cells were transfected with control or TRAF6 siRNA oligos. After 48 h, the cells were treated with or without MG132 (20 μM) for 4 h.

**Figure 4 TRAF6 associates with HIF-1α.**

(A) HIF-1α has TRAF6 binding motif (PxExxAr/Ac). The amino acid sequence of HIF-1α (330–430) including conservative TRAF6 binding sites (with underline) is shown. (B) The interaction between exogenous TRAF6 and HIF-1α. 293T cells were transfected as indicated and interaction between Flag-TRAF6 and Myc-HIF-1α was determined. (C) The endogenous TRAF6 and HIF-1α binds each other. 293T cells exposed to hypoxia (1% O₂) for 16 h. The cells were harvested and the association between endogenous TRAF6 and HIF-1α was determined. (D) TRAF6 cannot bind HIF-1α(Δ363-368). 293T cells were transfected as indicated and protein-protein interaction was performed by immunoprecipitation. (E) TRAF6 binds HIF-1α(330-530). GST-pulldown assay was performed as described in Methods. (F) The stability of HIF-1α(Δ363-368) is weaker than that of HIF-1α. 293T cells were transfected with Myc-HIF-1α or Myc-HIF-1α(Δ363-368). After 24 h, the cells were treated with 100
μg/mL of CHX for different times as indicated. Relative Myc-HIF-1α protein level was determined by measuring the density of Myc-HIF-1α band and normalized to that of β-actin. The data are mean±s.e.m. (n=3). *, p < 0.05.

Figure 5 TRAF6 catalyzes ubiquitination of HIF-1α.

(A) The mutant TRAF6 has little effect on HIF-1α expression. 293T cells were transfected as indicated. After 24 h, the cells were harvested. (B) TRAF6 enhances polyubiquitination of HIF-1α in the presence of Ub(K63). 293T cells were transfected as indicated. After 24 h, the cells were harvested and ubiquitination of HIF-1α was determined. (C) TRAF6 has little effect on HIF-1α ubiquitination in the presence of Ub(K63R). 293T cells were transfected as indicated. After 24 h, the cells were harvested and HIF-1α ubiquitination was determined. (D) TRAF6 cannot mediate ubiquitination of HIF-1α(Δ363-368) in the presence of Ub(K63). 293T cells were transfected as indicated. After 24 h, the cells were harvested for determination of ubiquitination of HIF-1α and HIF-1α(Δ363-368). (E) The mutant TRAF6 cannot mediate ubiquitination of HIF-1α. 293T cells were transfected as indicated. After 24 h, the cells were harvested for determination of polyubiquitination of HIF-1α. (F) Overexpression of Ub(K63) increases HIF-1α. 293T cells were transfected as indicated. The cells were harvested after 24 h, and HIF-1α was determined.

Figure 6 TRAF6 promotes tumor angiogenesis.

(A) In vivo Matrigel plug gel assay. RKO cells were used in the experiment. The haemoglobin content in gel was determined and the data are mean ± s.e.m. **, p < 0.01 vs shLacZ control. (B) Knockdown of TRAF6 represses xenograft growth of RKO
cells. The data are mean ± s.e.m. * p < 0.05; ** p < 0.01; #, p < 0.001. (C) On day 19th, the mice were sacrificed and tumors were weighed. Box and whisker plots show the minimum, lower quartile, median, upper quartile, and maximum of the data. Representative tumors are shown below. (D) Expression of HIF-1α and HIF-2α in tumors. Six shLacZ and 6 shTRAF6 xenografts were randomly selected for determination of HIF-1α and HIF-2α. (E) Immunohistochemical staining and micro-vessel density (MVD) determination. The tumor tissues were stained using CD31 antibody as described in Methods. MVD was determined by counting the number of CD31-positive blood vessels per field at 400× magnification. Eight fields were randomly selected for the determination. The data are mean ± s.e.m (n=5). ** p < 0.01. (F) Knockdown of TRAF6 suppresses expression of VEGF, bFGF and CD31. Expression of VEGF, bFGF and CD31 was determined by real-time PCR. The data are mean ± s.e.m (n=6). ** p < 0.01.
Figure 2

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1α/actin: 1 0.6 0.5
2α/actin: 1 1.1 1.1

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1α/actin: 1 1.6
2α/actin: 1 0.9

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Figure 4

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330  SQPQCIVCVNYVSGI1QHDLIFSL
355  QQTECVLKDPESSDDMKMTQLFTKVE
380  SEDTSLLLLKKEPDALTTLAPAA
405  GDTIISLDGFNSDTETDDQQLEEVP

B

293T

Input  IP: Flag
Myc-HIF-1α  +  +  +  +
Flag-TRA6  -  -  -  +
Myc-HIF-1α  -  +  +  -
Flag-TRA6  +  +  +  -

Input  IP: Myc

Myc-HIF-1α  -  +  +  -
Flag-TRA6  +  +  +  -

C

IP

Input  IgG  TRAF6
HIF-1α  +  +  +
TRA6  +  +  +

130 kDa

D

Input  IP: Myc

1: Myc-HIF-1α + Flag-TRA6
2: Myc-HIF-1α(Δ363-368) + Flag-TRA6

E

GST-HIF-1α

Input  GST  1-329  330-550  531-626
His-TRA6  +  +  +  +
GST  +  +  +  +

F

CHX (100 ng/ml)

Time (min)  0  2  4  6  8  12
Myc-HIF-1α  +  +  +  +  +  +
β-Actin  +  +  +  +  +  +
Myc-HIF-1α(Δ363-368)  +  +  +  +  +  +
β-Actin  +  +  +  +  +  +

Relative HIF-1α

0  2  4  6  8  12

Myc-HIF-1α
Myc-HIF-1α(Δ363-368)
Figure 5

A

293T

Flag-TRAF6

HIF-1α

Flag

β-Actin

B

HA:Ub(K63)

Myc-HIF-1α

Flag-TRAF6

IP: HA

IB: HIF-1α

IB: Flag

Uniquitinated

1 3.5

C

HA:Ub(K63)

Myc-HIF-1α

Flag-TRAF6

IP: HA

IB: HIF-1α

IB: Flag

Uniquitinated

HIF-1α

D

Myc-HIF-1α

Flag-TRAF6

HA:Ub(K63)

IP: HA

IB: HIF-1α

IB: Flag

E

HA:Ub(K63)

Myc-HIF-1α

Flag-TRAF6

IP: HA

IB: HIF-1α

IB: Flag

Uniquitinated

HIF-1α

F

HA:Ub(K63)

Myc-HIF-1α

Flag-TRAF6

IP: HA

IB: HIF-1α

IB: Flag

β-Actin

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TRAF6 upregulates expression of HIF-1α and promotes tumor angiogenesis

Heng Sun, Xue-Bing Li, Ya Meng, et al.

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