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ORIGINAL ARTICLE

TRAF6 regulates YAP signaling by promoting the ubiquitination and degradation of MST1 in pancreatic cancer

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Abstract

TNF receptor-associated factor 6 (TRAF6), a regulator of NF-κB signaling, has been reported to be associated with the oncogenesis of various tumors including pancreatic cancer, but the underlying mechanisms remain unknown. Here, we found that knocking down the expression of TRAF6 impaired YAP signaling. Moreover, TRAF6 promoted the migration and colony formation of pancreatic cancer cells through YAP. Then, we found that TRAF6 interacted with and promoted the ubiquitination and degradation of MST1, and the expression of TRAF6 and MST1 was negatively correlated in primary human pancreatic cancer samples. Our results reveal that TRAF6 regulates YAP signaling by promoting the ubiquitination and degradation of MST1 in pancreatic cancer, suggesting that TRAF6 could be a possible E3 ligase of MST1 and a potential therapeutic target.

Keywords Pancreatic cancer · Tumorigenicity · Ubiquitination · TRAF6 · MST1 · Hippo–YAP · NF-κB

Introduction

Pancreatic cancer is one of the deadliest malignancies worldwide, with an overall 5-year survival rate of 8% [1]. Multiple factors contribute to its poor prognosis, including an insidious onset, a tendency for early metastasis, resistance to conventional chemotherapies, and relatively poor response to targeted therapies [2]. Thus, further exploration of the underlying mechanisms and the identification of novel therapeutic targets for pancreatic cancer are needed.

TNF receptor-associated factor 6 (TRAF6) is a member of the tumor necrosis factor (TNF) receptor-associated factor family that lies downstream of the TNF receptor, the IL-1 family, and most Toll-like receptors (TLRs), and is a controlling node in the initial activation of nuclear factor-κB (NF-κB) and JNK signaling pathways in response to pro-inflammatory cytokines or endotoxin lipopolysaccharide (LPS) [3, 4]. TRAF6 is a RING domain E3 ligase that can ubiquitinate its substrates and itself when stimulated, and the Lys63-based polyubiquitin chains generated by self-ubiquitination function as recruitment adaptors to attract other substrates [5]. The known TRAF6 substrates include IκB kinase (IKK), Akt1, Beclin1, and hnRNPA1 [5–8]. In addition to its role in inflammatory cascades, TRAF6 is also involved in the oncogenic process [9–12], although the specific mechanisms remain elusive.

Hippo signaling is an evolutionarily conserved pathway that controls organ size through the regulation of cell proliferation and apoptosis [13]. Core to the Hippo pathway is a kinase cascade, wherein activated mammalian Ste20-like serine/threonine kinase 1/2 (MST1/2) phosphorylates and activates the large tumor-suppressor serine/threonine kinase 1/2 (LATS1/2), which in turn phosphorylates and inhibits Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), two major downstream effectors of the Hippo pathway [14]. Dephosphorylated YAP and TAZ translocate into the nucleus and induce the expression of genes that promote cell proliferation and inhibit apoptosis [15]. *YAP* is over-expressed in various human cancers [16], and sustained *YAP* expression promotes growth

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and tumor development, suggesting an important role for the Hippo pathway in tumorigenesis [17].

We previously showed that *TRAF6* is over-expressed in pancreatic cancer and promotes the tumorigenicity of pancreatic cancer cells [12]. In the present study, we showed that TRAF6 promoted the migration and colony formation of pancreatic cancer through YAP. We identified TRAF6 as a possible E3 ligase of MST1, as it interacted with and promoted the degradation of MST1. The results from the present study reveal the specific mechanism of TRAF6-induced tumorigenicity and indicate that TRAF6 may act as a bridge between inflammation and tumorigenesis.

Methods

Cell culture, plasmids, and transfection

Pancreatic cancer cell lines (HPAC and MIAPaca2) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai Institutes of Cell Biology, Chinese Academy of Sciences, Shanghai, China) and cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin-G, and 100 µg/ml streptomycin. Cells were incubated at 37 °C in humidified air containing 5% CO_2.

Pancreatic cancer cells were transfected with plasmids encoding Flag-YAP [18] and Myc-TRAF6 [12] constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's descriptions. FG12 lentiviral vector was used to produce siRNA as described previously [12]. The siRNA sequences used were as follows: TRAF6 1# 5′-GCCUAA UCAUUAUGAUCUATT-3′, TRAF6 2# 5′-GCCTGCATC ATCAAGTCAATA-3′, YAP 5′-CAGGTGATACTATCA ACCAAA-3′. Cells were sorted by flow cytometry 3 days after transfection.

YAP reporter assay

For the characterization of YAP activity in pancreatic cancer cells, the cells were transfected with 8xGTIIC-Luc (34615; Addgene, Watertown, MA, USA) and PRL-TK plasmids. Luciferase luminescence was measured using the Dual-Glo luciferase assay kit (Promega, Madison, WI, USA).

Boyden chamber assay

Boyden chambers (polycarbonate membranes with 8 µm pore size) were obtained from Neuroprobe Corporation, Bethesda, MD, USA. Cells (2×10^5) in 0.05 ml medium containing 1% FBS were placed in the upper chamber, and the lower chamber was loaded with 0.152 ml medium containing 10% FBS. After incubation for 12 h, the cells that migrated to the lower surface of the filter were detected by traditional hematoxylin and eosin (H&E) staining, and five fields of each well were counted. Three wells were examined for each cell type, and the experiments were repeated at least three times.

Antibodies

Anti-TRAF6 (sc-8409) and anti-YAP (sc-376830) were purchased from Santa Cruz, Shanghai, China. Anti-CYR61 (14479), anti-CTGF (86641), anti-CyclinE (20808), anti-Myc-Tag (2276), and anti-GST (2624) were from Cell Signaling Technology, Shanghai, China. Anti-MST1 (PA5-22015) and anti-GAPDH (GA1R) were from Invitrogen, and anti-Ubiquitin (ab7780) was from Abcam, Shanghai, China. Horseradish peroxidase-conjugated secondary antibodies against mouse (sc-2748) and rabbit (sc-2750) were obtained from Santa Cruz. Fluorescent dye-conjugated secondary antibodies (A-11001, A-11034, 31660, 31686) were obtained from Thermo Fisher Scientific, Shanghai, China.

Western blotting and immunoprecipitation

Cells were lysed in RIPA buffer supplemented with 1 mmol/L Na_3VO_4, protease inhibitor cocktail (P8340; Sigma, St Louis, MO, USA), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Lysates were sonicated and incubated for 30 min on ice followed by centrifugation at 13,200 rpm for 15 min at 4 °C. Equal volumes of sample supernatant were loaded onto SDS-PAGE, and the bands transferred onto PVDF (Millipore, Shanghai, China; 0.45 µm, IPVH00010) membranes. The membranes were blocked with 5% nonfat milk and incubated with primary antibody overnight at 4 °C. Then, the membranes were probed with HRP-conjugated secondary antibody for 1 h at room temperature. The protein bands were detected with ECL SuperSignal reagent (#34078; Thermo Fisher Scientific).

For immunoprecipitation, cells were lysed in lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH = 7.6), 1% NP-40, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF, and 0.1% protease inhibitor cocktail. Proteins were precleared using 1–2 µg normal IgG together with protein A/G PLUS- agarose beads (sc-2003; Santa Cruz) for 1 h and then incubated with primary antibody at 4 °C overnight, followed by further incubation with 20 µl beads at 4 °C for 2 h. The beads were then washed 5–6 times with lysis buffer, and the pellets were eluted with SDS loading buffer and resolved by SDS-PAGE.

Colony formation assay

For the cell growth assay, equal numbers of cells were seeded in triplicate in six-well plates and cultured in DMEM medium supplemented with 10% FBS for 10 days. The medium was changed every other day. After 10 days of incubation, the colonies were fixed with ice-cold methanol and stained with 0.05% crystal violet. Colonies containing > 50 cells were counted.

Immunofluorescence staining

Cells were seeded onto coverslips and fixed with 3% paraformaldehyde for 10 min, and then permeabilized in 0.5% Triton-X for 5 min at room temperature. Cells were blocked with 5% goat serum at room temperature for 1 h. Then, cells were incubated with primary antibody at 37 °C for 20 min, followed by incubation with Alexa Fluor 488 or rhodamine-conjugated secondary antibodies at 37 °C for 20 min. Cell nuclei were counterstained with DAPI. Slides were washed with PBS between incubations.

GST pull-down assay

The fusion protein of GST-TRAF6 and GST protein were prepared as described previously [19]. An aliquot (5 µg) glutathione (GST) or GST-TRAF6 protein was added to MiaPaca2 cell lysate, followed by overnight incubation with gentle rotation and addition of glutathione-Sepharose 4B beads (GE Healthcare, Shanghai, China) for 3 h. Beads were washed three times and eluted with 2 × SDS loading buffer.

Ubiquitination assay

For in vivo ubiquitination assays, HEK293T cells were transfected with plasmids for 48 h and then lysed in 1.5% SDS cell lysis buffer. Lysates were boiled for 10 min immediately followed by centrifugation for 10 min at 16,000×*g*. The supernatants were collected and diluted with 1% NP-40 Co-IP buffer, then immunoprecipitated and analyzed by SDS-PAGE.

Survival and correlation analysis

Gene expression profiling interactive analysis (GEPIA) [20], a web server for analyzing RNA sequencing expression data from the cancer genome atlas [21] and the Genotype-Tissue Expression projects [22], was used to study the impact of MST1 level on the survival of pancreatic cancer patients and the correlation between MST1 and TRAF6.

Results

Knocking down the expression of TRAF6 impairs YAP signaling

Our previous study [12] showed that *TRAF6* was up-regulated in pancreatic cancer. However, the molecular mechanisms are not fully understood. Here, we screened the signaling pathways downstream of *TRAF6* using the luciferase reporter assay. As shown in Fig. 1a, knocking down the expression of *TRAF6* inhibited the activity of YAP both at the basal level and upon stimulation of YAP. However, *TRAF6* expression had a minimal effect on Topflash (Wnt pathway), GLISB (hedgehog pathway), and the TGF-β pathway (data not shown). Knocking down the expression of *TRAF6* impaired the expression of cellular communication network factor 1 (*Cyr61*), connective tissue growth factor (*CTGF*), and *Cyclin E*, three target genes of YAP (Fig. 1b). Assessment of the effect of TRAF6 on the level of YAP protein showed that down-regulation of *TRAF6* decreased the protein level of YAP (Fig. 1c). In summary, these data suggested that TRAF6 regulated the transcriptional activity of *YAP* by down-regulating the concentration of YAP protein.

TRAF6 promotes the migration and colony formation of pancreatic cancer cells through YAP

We next examined whether TRAF6 promoted the migration and colony formation of pancreatic cancer cells through YAP. We first knocked down the expression of *YAP* in *TRAF6*-over-expressing cells (Fig. 2a). Knocking down the expression of *YAP* abolished the promoting effects of TRAF6 on the migration of pancreatic cancer cells (Fig. 2b, c). Moreover, TRAF6 promoted the anchorage-independent growth of cancer cells, which was impaired by knocking down the expression of *YAP* (Fig. 2d, e). Taken together, these data indicated that YAP mediated the functions of TRAF6.

TRAF6 interacts with MST

The decreased concentration of YAP protein in *TRAF6*-knockdown cells suggested that TRAF6 might regulate signaling upstream of YAP. To test this hypothesis, we examined the interaction between TRAF6 and major components of YAP signaling. Co-localization of TRAF6 and MST1 was observed in MiaPaca2 cells in the immunofluorescence assay (Fig. 3a). In the GST pull-down assay, GST-TRAF6 formed a complex with endogenous MST1 in MiaPaca2 cells (Fig. 3b). The

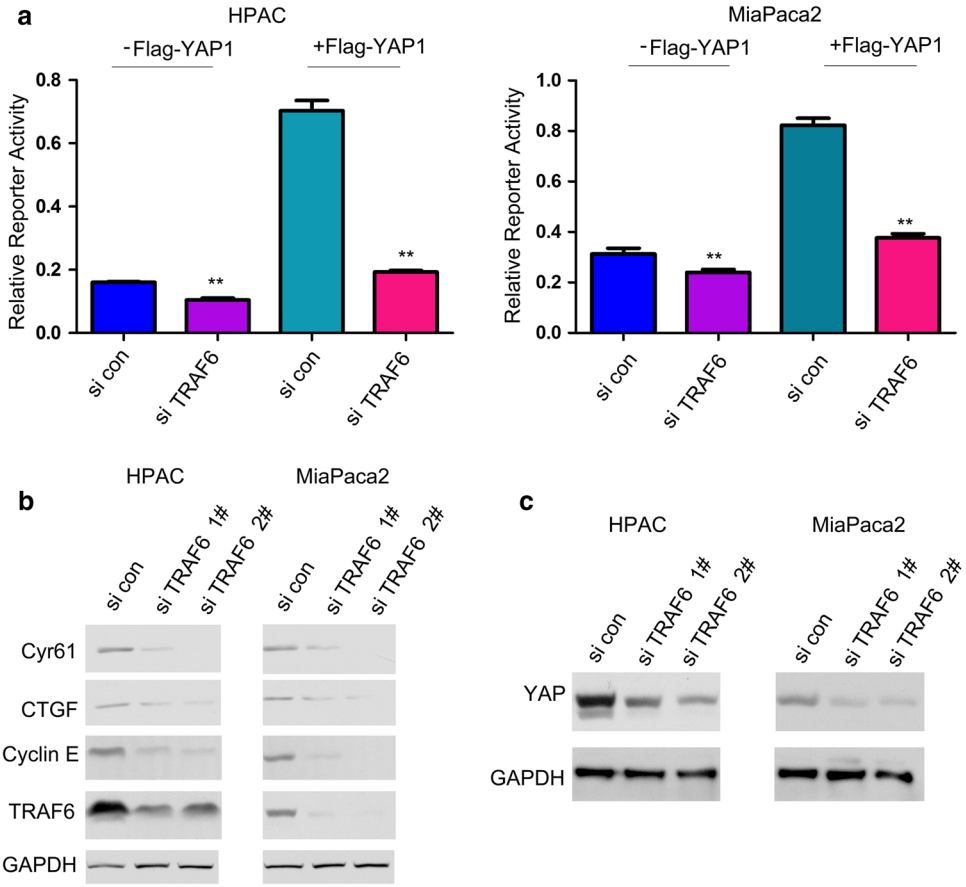


Fig. 1 Knocking down the expression of TRAF6 impaired YAP signaling. When TRAF6 was knocked down using RNAi, **a** the YAP activity of both endogenous and over-expressed YAP was measured using the YAP reporter assay. The experiments were performed in triplicates. **b** The level of Cyr61, CTGF, and Cyclin E, three target genes of YAP, was assessed by Western blot; The experiments were performed three times, and the representative data were shown. **c** The expression of YAP was assessed by Western blot with GAPDH as a loading control. The experiments were performed three times, and the representative data were shown

interaction between endogenous TRAF6 and MST1 was demonstrated in MiaPaca2 cells using the immunoprecipitation assay (Fig. 3c). Collectively, these data suggested that TRAF6 interacted with MST1.

TRAF6 promotes the degradation of MST

Because TRAF6 is an E3 ubiquitin ligase, we next examined whether TRAF6 regulated the level of MST1 protein. Over-expression of *TRAF6* in MiaPaca2 and HPAC cells decreased the level of MST1 protein in a dose-dependent manner (Fig. 4a), whereas knock-down of TRAF6 increased the level of MST1 protein (Fig. 4b). The E3-ligase-inactive form of TRAF6 (C70A) failed to down-regulate MST1, suggesting that the down-regulation of MST1 was dependent on the E3 ligase activity of TRAF6 (Fig. 4a, right). Consistent with these observations, TRAF6 promoted the ubiquitination of MST1 (Fig. 4c). Taken together, these results suggested that TRAF6 promoted the degradation of MST1.

The expression of TRAF6 and MST1 is negatively correlated in pancreatic cancer

We next examined whether the expression of *TRAF6* and *MST* was negatively correlated in pancreatic cancer samples by mining the GEPIA database. Consistent with previous findings [23], the expression of *MST1* was positively correlated with the survival of pancreatic cancer patients (Fig. 5a). Correlation analysis showed that the expression of *TRAF6* and *MST* was negatively correlated in pancreatic cancer samples (Fig. 5b). Therefore, the clinical data further supported the regulation of MST by TRAF6.

Discussion

Chronic inflammation can promote cancer development at all stages, from initiation through tumor promotion, all the way to metastatic progression [24]. Although various underlying

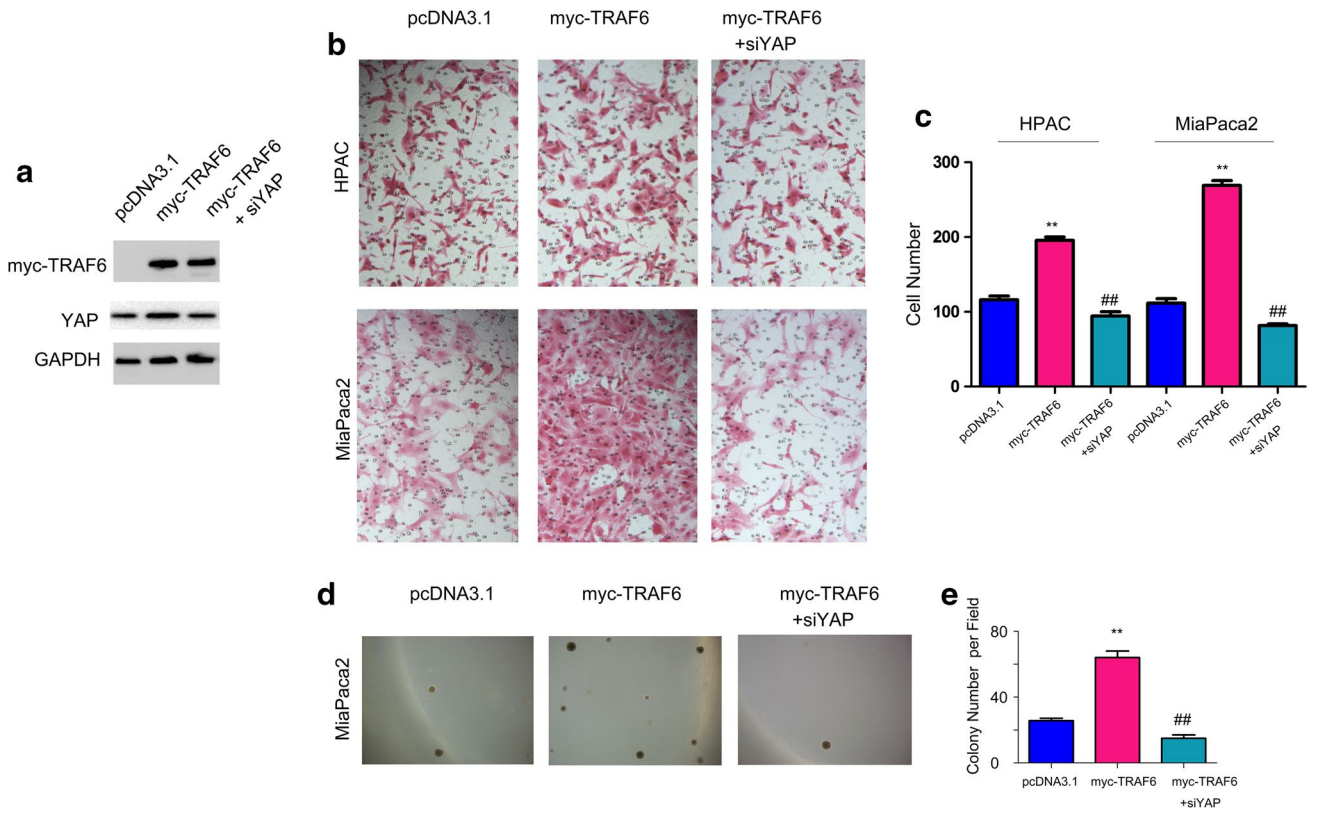


Fig. 2 TRAF6 promoted the migration and colony formation of pancreatic cancer cells through YAP. **a** Knocking down the expression of YAP in the TRAF6-over-expressing cells. **b** Cell migration of control cells, TRAF6-over-expressing cells, and TRAF6-over-expressing/YAP knock-down cells was measured by the Boyden chamber assay. Three independent experiments were conducted, and representative images of the results are shown. **c** The quantitative data for B were expressed as the mean ± SEM. $^{**}P < 0.05$; $^{\#\#}P > 0.05$. **d** The same amount of control, TRAF6-over-expressing, and TRAF6-over-expressing/YAP knock-down MiaPaca2 cells were seeded in triplicate in 6-well plates for 10 days, and representative images of the results are shown. **e** The quantitative data for **d** were expressed as the mean ± SEM. Colonies with more than 50 cells were counted. $^{**}P < 0.05$; $^{\#\#}P > 0.05$

pathways have been discovered, there are no relevant drugs targeting these pathways, especially for pancreatic cancer. We previously reported that *TRAF6,* which mainly functions in inflammation, is over-expressed in pancreatic cancer, promoting tumorigenicity [12]. Here, we showed that TRAF6 regulated the oncogenic Hippo–YAP pathway by interacting with and promoting the degradation of MST1, providing a further link between inflammation and tumorigenesis and suggesting a novel therapeutic target for the treatment of pancreatic cancer.

MST1 and MST2, homologs of the Drosophila Hpo, can be activated by oxidative stress and play central roles in the Hippo pathway, controlling cell proliferation, differentiation, and apoptosis during development [23]. Song et al. [25] reported that MST1 and MST2 act as tumor suppressors by restricting cell proliferation and survival. MST1 is regulated by phosphorylation (Thr183) and caspase-mediated cleavage [26]. The cleaved, constitutively active form of MST1/2 is necessary to maintain the quiescent state of hepatocellular cells, and combined MST1/2 deficiency results in hepatocellular carcinoma cells (HCC) [27]. Our results have shown for the first time that MST1 could be ubiquitinated and suggested that TRAF6 is the responsible E3 ligase. Over-expression of *TRAF6* led to the degradation of MST1 in pancreatic cancer cells, and a negative correlation between TRAF6 and MST1 was detected in clinical samples. Agents targeting TRAF6 may restore the level of MST1 and help suppress tumor progression.

Although the NF-κB signaling pathway plays a role in controlling the initiation and progression of human cancer [28], *YAP* knock-down almost completely counteracts the effects of simultaneous *TRAF6* over-expression, indicating that, from an oncogenic perspective, the main effector of *TRAF6* over-expression is *YAP*. However, further investigation is necessary in the setting of primary human tumors in an inflammatory microenvironment. Lv et al. [29] have recently reported that YAP interacts with TRAF6 and modulates its ubiquitination and degradation in endothelial cells, and this needs to be verified in tumor cells, which is beyond the scope of the present study.

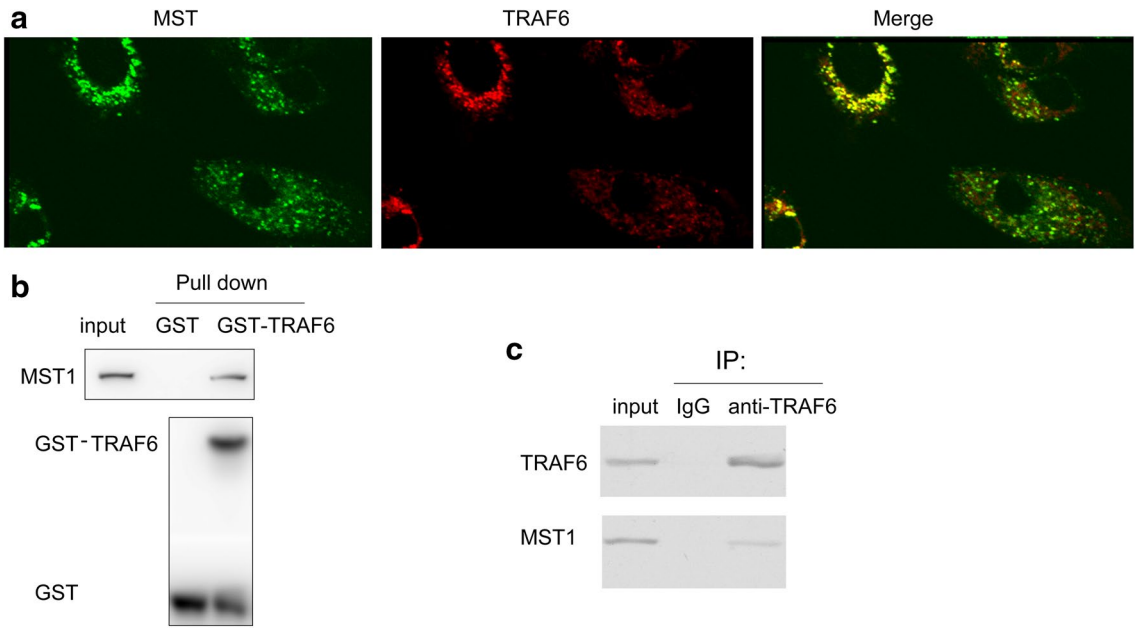
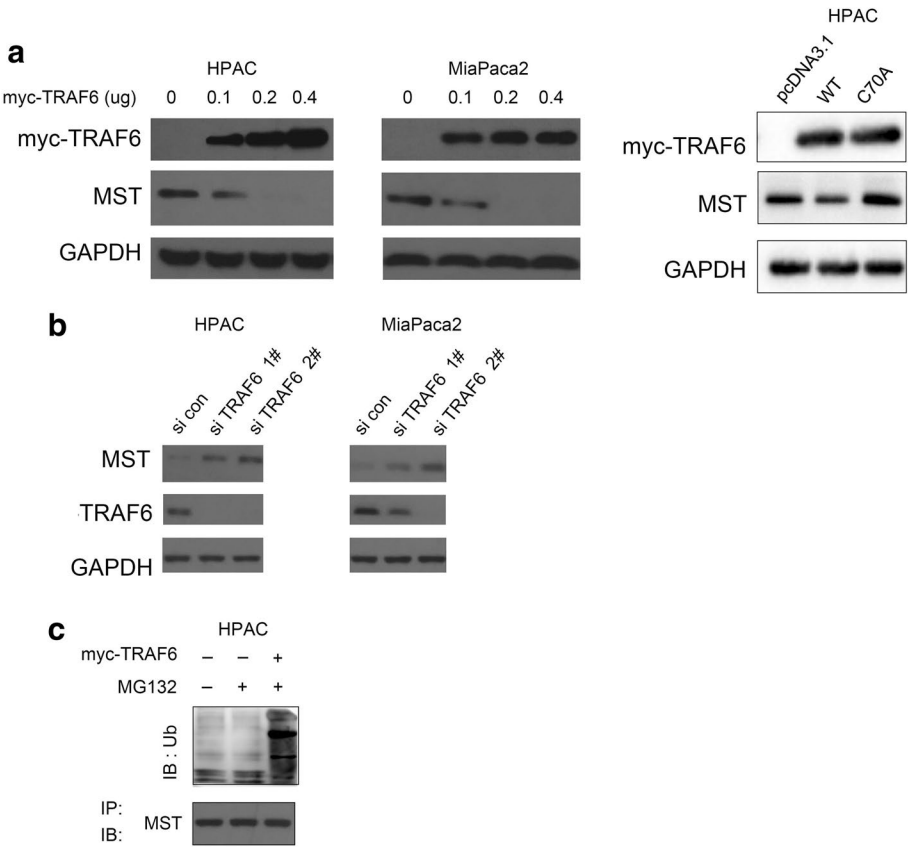


Fig. 3 TRAF6 interacted with MST1. **a** Immunofluorescence staining for MST1 and TRAF6 in MiaPaca2 cells. **b** Lysates from MiaPaca2 cells were incubated with GST-TRAF6 or GST fusion protein and glutathione-Sepharose 4B beads for 3 h before washing. The elution was subsequently analyzed by Western blot with anti-MST1 antibody. **c** MST1 was blotted on immunoprecipitated endogenous TRAF6 in lysates from MiaPaca2 cells

Fig. 4 TRAF6 promoted the degradation and ubiquitination of MST1. **a** MiaPaca2 and HPAC cells were transfected with increasing amounts of myc-TRAF6 plasmid. MST1 level was measured by Western blot 24 h later. For the right panel, the HPAC cells were transfected with TRAF6 (wt) or TRAF6 mutant (C70A), and the protein level of MST1 was examined. **b** MST1 level was measured in TRAF6 knock-down and control cells. **c** Ubiquitin was blotted on immunoprecipated MST1 in lysate from control cells and TRAF6 over-expressing cells treated with proteasome inhibitor MG132 or not



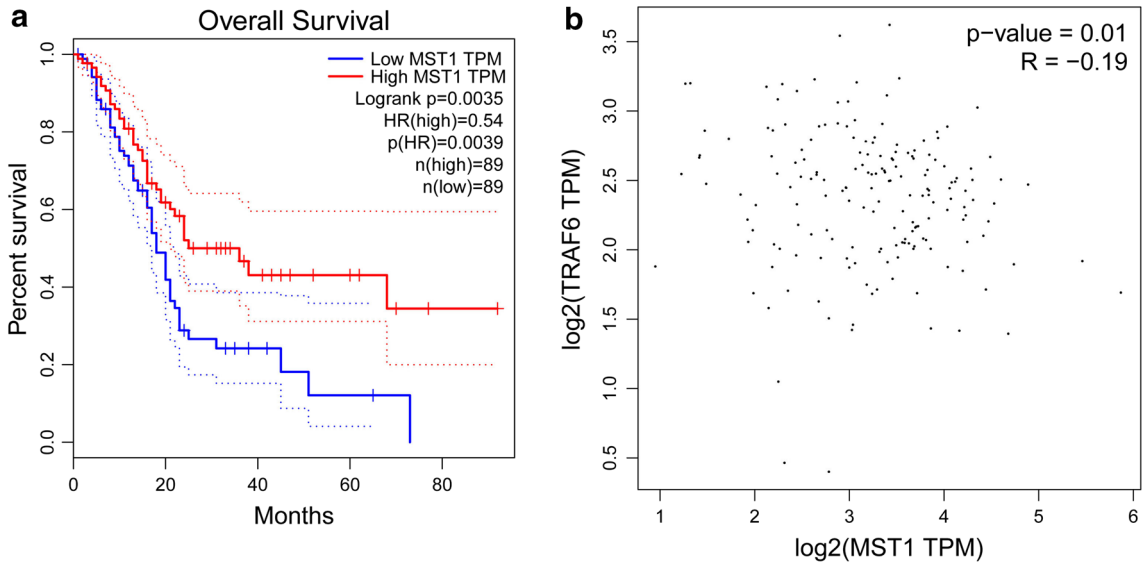


Fig. 5 The expression of TRAF6 and MST was negatively correlated in pancreatic cancer samples. **a** The overall survival of patients with pancreatic cancer of low or high MST1 transcripts per million (TPM) calculated using the GEPIA server. **b** The expression of TRAF6 and MST was negatively correlated in pancreatic cancer samples, as calculated using the GEPIA server

In summary, the present study showed that TRAF6 regulated YAP signaling and promoted the progression of pancreatic cancer through YAP. TRAF6 interacted with and promoted the ubiquitination and degradation of MST, suggesting that TRAF6 is an E3 ligase for MST1.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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