






Transcriptional Enhancer Factor Domain Family member 4 Exerts an Oncogenic Role in Hepatocellular Carcinoma by Hippo-Independent Regulation of Heat Shock Protein 70 Family Members

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Transcriptional enhancer factor domain family member 4 (TEAD4) is a downstream effector of the conserved Hippo signaling pathway, regulating the expression of genes involved in cell proliferation and differentiation. It is up-regulated in several cancer types and is associated with metastasis and poor prognosis. However, its role in hepatocellular carcinoma (HCC) remains largely unexplored. Using data from The Cancer Genome Atlas, we found that TEAD4 was overexpressed in HCC and was associated with aggressive HCC features and worse outcome. Overexpression of TEAD4 significantly increased proliferation and migration rates in HCC cells *in vitro* as well as tumor growth *in vivo*. Additionally, RNA sequencing analysis of *TEAD4*-overexpressing HCC cells demonstrated that *TEAD4* overexpression was associated with the up-regulation of genes involved in epithelial-to-mesenchymal transition, proliferation, and protein-folding pathways. Among the most up-regulated genes following *TEAD4* overexpression were the 70-kDa heat shock protein (HSP70) family members *HSPA6* and *HSPA1A*. Chromatin immunoprecipitation-quantitative real-time polymerase chain reaction experiments demonstrated that TEAD4 regulates *HSPA6* and *HSPA1A* expression by directly binding to their promoter and enhancer regions. The pharmacologic inhibition of HSP70 expression in *TEAD4*-overexpressing cells reduced the effect of TEAD4 on cell proliferation. Finally, by overexpressing *TEAD4* in yes-associated protein (YAP)/transcriptional coactivator with PDZ binding motif (*TAZ*)-knockdown HCC cells, we showed that the effect of TEAD4 on cell proliferation and its regulation of HSP70 expression does not require YAP and TAZ, the main effectors of the Hippo signaling pathway. **Conclusion:** A novel Hippo-independent mechanism for TEAD4 promotes cell proliferation and tumor growth in HCC by directly regulating HSP70 family members. (*Hepatology Communications* 2021;5:661-674).

Transcriptional enhancer factor domain (TEAD) proteins are a family of transcription factors that bind the consensus 5'-CATTCCA/T-3' sequence through their shared TEA DNA binding domain.⁽¹⁾ TEAD-regulated transcription strictly depends on the binding of TEAD transcription factors with various coactivators,^(2,3) among which are the yes-associated protein (YAP) and transcriptional

Abbreviations: CAM, chorioallantoic membrane; ChIP, chromatin Immunoprecipitation; CREAM, Cognitive Reliability and Error Analysis Method; CTGF, connective tissue growth factor; CTR, control; DMSO, dimethyl sulfoxide; EMT, epithelial-to-mesenchymal transition; GO, Gene Ontology; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; IgG, immunoglobulin; IHC, immunochemistry; kb, kilobase; KNK437, heat shock protein family A inhibitor 1; qPCR, quantitative real-time polymerase chain reaction; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; seq, sequencing; TAZ, transcriptional coactivator with PDZ binding motif; TCGA, The Cancer Genome Atlas; TEAD, transcriptional enhancer factor domain; TEAD4, transcriptional enhancer factor domain family member 4; YAP, yes-associated protein.

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coactivator with PDZ binding motif (TAZ), two major effectors of the Hippo signaling pathway,^(4,5) and the vestigial-like protein 1 (VGLL1).⁽⁶⁾ In mammals, TEADs are highly conserved and widely expressed^(7,8) and play a pivotal role in development by mediating cell proliferation and organ size control through the Hippo signaling pathway.⁽⁹⁾ In cancer cells, TEADs regulate proliferation, migration, differentiation, epithelial-to-mesenchymal transition (EMT), apoptosis, and invasion.⁽¹⁰⁾ In particular, TEAD factors regulate the expression of progrowth factors, such as connective tissue growth factor (CTGF),^(10,11) cysteine-rich angiogenic inducer 61 (Cyr61),⁽¹¹⁾ AXL receptor tyrosine kinase (AXL),⁽¹²⁾ MYC proto-oncogene bHLH transcription factor (Myc),⁽¹³⁾ baculoviral inhibitor of apoptosis repeat-containing 5 (survivin),⁽¹³⁾ and insulin-like growth factor binding protein 5 (IGFBP5).⁽⁶⁾ Furthermore, overexpression of TEADs and their association with a poor clinical outcome have been reported in many cancer types, including breast, lung, prostate, colon, and gastric cancers as well as melanoma and glioblastoma.⁽¹⁴⁻¹⁶⁾

TEAD family member 4 (TEAD4), the gene encoding for the transcriptional enhancer factor

(TEF)-3, is overexpressed in several tumor types, such as breast and gastric cancers,⁽¹⁶⁾ as well as hepatoblastoma, the most common type of pediatric liver cancer.⁽¹⁷⁾ Notably, TEAD4 acts as an oncogene through Hippo signaling-dependent⁽¹⁸⁾ and signaling-independent mechanisms.^(6,15) In hepatocellular carcinoma (HCC), it is known that TEAD4 acts through the Hippo signaling pathway, wherein the TEAD4/YAP complex cooperates with forkhead box M1 (FOXM1) in inducing chromosome instability.⁽¹⁹⁾ It has also been shown that up-regulation of the YAP2/TEAD4 axis by sirtuin 1 (SIRT1) deacetylation of YAP2 promotes HCC cell proliferation.⁽²⁰⁾ Furthermore, TEAD4/YAP and hepatocyte nuclear factor 4 α (HNF4 α) regulate hepatocarcinogenesis by reciprocal repression in mice and rats.⁽²¹⁾ Thus, until now, TEAD4 was thought to play a YAP-dependent role in HCC oncogenesis.

In this study, we show that TEAD4 is overexpressed in a subset of HCC and that it promotes cell proliferation in HCC cells both *in vitro* and *in vivo*. We further show that TEAD4 directly regulates HSP70 in a Hippo-independent mechanism.

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Materials and Methods

CELL LINES

HCC-derived cell lines (HepG2, SNU449, HLE, and Huh7) were maintained in a 5% CO₂-humidified atmosphere at 37°C and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Bio-Concept, Allschwil, Switzerland), and 1% minimal essential medium–nonessential amino acids (ThermoFisher Scientific, Basel, Switzerland). Stable *YAP*- and *TAZ*-knockdown Huh7 and SNU449 cell lines (described in Tang et al.⁽²²⁾) and their respective controls were maintained in complete medium supplemented with puromycin (1 µg/mL). All cell lines were confirmed negative for mycoplasma infection using the polymerase chain reaction (PCR)-based Universal Mycoplasma Detection kit (American Type Culture Collection, Manassas, VA). For HSP70 inhibitor 1 (KNK437; CAS 218924-25-5; Calbiochem, Sigma-Aldrich, St. Louis, MO), cells were incubated with 100 µM of the inhibitor and the corresponding dimethyl sulfoxide (DMSO) control.

HUMAN SAMPLES

Ten human, unselected, nonconsecutive HCCs retrieved from the archives of the Institute of Medical Genetics and Pathology at the University Hospital Basel (Basel, Switzerland) were included in this study. These 10 samples had already been prescreened for HSP70 protein expression. The study was performed in accordance with the Helsinki Declaration and approved by the Ethics Committee of Basel. Data were collected retrospectively in a nonstratified and nonmatched manner, including patient age, tumor diameter, location, prognostic tumor/prognostic node stage, grade, histologic subtype, and vascular invasion.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) was performed as described.⁽²³⁾ Details are presented in the Supporting Information.

PLASMIDS AND TRANSFECTION

For *TEAD4* overexpression, pLV[Exp]-enhanced green fluorescent protein (EGFP)/Neo-EF1A>hTEAD4 was designed and ordered on the vector

builder website (<https://en.vectorbuilder.com/>), and the empty control vector was plasmid cytomegalovirus (pCMV)-mir EGFP. For *TEAD4* silencing, pSuper-retro-puro empty vector and pSuper-retro-puro sh*TEAD1/3/4* were adapted from Zhao et al.⁽¹¹⁾ The expression vectors were transiently transfected using the jetPRIME transfection reagent (Polyplus, Illkirch, France) following the manufacturer's instructions. The expression of the plasmids was evaluated by western blot and quantitative reverse-transcription (qRT)-PCR analysis. Cells were harvested 48 hours after transfection for further experiments.

PROTEIN EXTRACTION AND WESTERN BLOT RNA EXTRACTION AND qRT-PCR AND RNA SEQUENCING

Protein extraction and western blot RNA extraction along with qRT-PCR and RNA sequencing (RNA-seq) are detailed in the Supporting Information.

PROLIFERATION AND MIGRATION ASSAYS

Proliferation and cell migration assays were performed using the xCELLigence Real-Time Cell Analysis-dual purpose (ACEA Biosciences, San Diego, CA) system. All experiments were performed in triplicate. Results are shown as mean ± SD. Statistical significance was assessed by the *t* test.

WOUND-HEALING ASSAY

Twenty-four hours after transfection, a 100-µL micropipette tip was used to create a scratch in the cell monolayer in each well of the six-well plate. To monitor the wound closure, representative phase-contrast images of each well were taken at 0, 8, 12, and 24 hours after the scratch was made. Each experimental condition was evaluated in triplicate. Statistical significance was assessed by the *t* test.

CHORIOALLANTOIC MEMBRANE ASSAY

Fertilized chicken eggs were obtained from a local hatchery (Gepro Geflügelzucht AG, Flawil,

Switzerland) at day 1 of gestation and were incubated at 37°C with 60% humidity for 10 days. The cells were harvested 24 hours after transfection, suspended (2×10^6 cells per chorioallantoic membrane [CAM] assay) in 10 μ L of medium (DMEM), mixed 1:1 with matrigel (Matrigel Matrix; Ref. 354234; Corning, Tewksbury, MA), and grafted onto the CAM of 9-day-old chicken embryos. The chicken embryos were maintained *in ovo* at 37°C for another 4 days, followed by removal of the tumors. Pictures of each tumor were taken with a Canon EOS 1100D digital camera. Tumor size measurements were performed by averaging the volume (height \times width \times width) of each tumor, using ImageJ as described.⁽²⁴⁾

CELLTITER-GLO CELL VIABILITY ASSAY

CellTiter-Glo (G7573; Promega, Dübendorf, Switzerland) was used to determine the number of viable cells based on adenosine triphosphate content. Twenty-four hours after transfection, cells were seeded in a 96-well plate. After 8 hours, drug treatment was added to the cells and cell viability was measured by adding 100 μ L of CellTiter-Glo/well at 24, 48, and 72 hours posttreatment. Statistical significance was assessed by multiple *t* test. In sorafenib experiments, results were normalized to DMSO. Curve fitting was performed using Prism software (GraphPad Software, San Diego, CA) and the nonlinear regression equation.

ANALYSIS OF CHROMATIN IMMUNOPRECIPITATION SEQUENCING DATA

Chromatin immunoprecipitation (ChIP)-seq data for TEAD4 in the HepG2 human liver cell line were produced using mouse monoclonal immunoglobulin G (IgG) raised against recombinant protein TEAD4 and were obtained from the Gene Expression Omnibus (accession number GSM1010875).⁽²⁵⁾ Processed data as an hg19 bigWig file were loaded into Integrative Genomics Viewer,⁽²⁶⁾ and the “Find Motif” tool was used to search for the most conserved bases of *TEAD4* binding motif within regions of *TEAD4* ChIP-seq peaks. TEAD4 binding motif was obtained from the JASPAR 2018 database.⁽²⁷⁾ TEAD4 enhancers were called from the *TEAD4* broadPeak BED file using the Cognitive Reliability

and Error Analysis Method (CREAM),⁽²⁸⁾ with the options WScutoff = 1.5, Minlength = 1000, and peakNumMin = 2. CREAM calls enhancers based on clusters of peaks, taking into account the distribution of distances between peaks in ChIP-seq data from a given sample.

ChIP

Cells from four 10-cm Petri dishes at 70%-80% confluence were crosslinked in 1% formaldehyde for 10 minutes with continuous shaking. The crosslinking was stopped by adding 0.15 M glycine while continuing shaking. After collecting the cells by scraping, the pellet was washed 3 times with cold phosphate-buffered saline. The nuclei of the cells were isolated and lysed. Chromatin shearing was performed using Bioruptor Pico (Diagenode, Liège, Belgium). The number of cycles and the settings were as described.⁽²⁹⁾ At the same time, the antibody was coupled with magnetic protein G beads (100-03D; Invitrogen, Carlsbad, CA) by incubating 75 μ L of protein G beads with 10 μ g of TEAD4 antibody (TEF-3, sc-101184; Santa Cruz Biotechnology, Dallas, TX) or 10 μ g of mouse IgG (sc-2025; Santa Cruz Biotechnology) as a negative control for 1 hour with constant rotation. At the end of the sonication process, an aliquot of the chromatin was kept as input control for every sample and an equal amount of sonicated chromatin was incubated with the previously produced antibody-coupled magnetic beads at 4°C overnight while rotating. The samples were washed and then eluted (all buffers are as described in the original protocol from Blecher-Gonen et al.⁽³⁰⁾). Ribonuclease treatment and then proteinase K treatment were performed on all samples, followed by overnight reverse crosslinking at 65°C with continuous shaking. DNA purification was performed using Agencourt AMPure XP (A63880; Beckman Coulter, Brea, CA). TEAD4 abundance on specific target gene promoters was quantified by qRT-PCR compared to IgG negative control. Primer sequences are listed in Supporting Table S1.

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 6.0 and R. For *in vitro* and *in vivo* studies, statistical significance was determined by the

two-tailed unpaired Student *t* test. Differences were considered statistically significant at $P < 0.05$. All experiments were performed at least twice. The statistical parameters (i.e., the exact value of *n*, *P* values) are noted in the figures and figure legends. Results are shown as mean \pm SD.

Analysis of The Cancer Genome Atlas Liver Data Set

Analysis of The Cancer Genome Atlas (TCGA) liver data set is described in the Supporting Information.

Results

TEAD4 OVEREXPRESSION PROMOTES HCC TUMOR GROWTH *IN VITRO* AND *IN VIVO*

As *TEAD4* overexpression has been associated with poor clinical outcome in a number of cancer entities, we asked whether *TEAD4* overexpression plays a similar role in HCC. We first confirmed that *TEAD4* was overexpressed in HCC using the TCGA data set. Compared to the nontumoral livers, HCCs showed *TEAD4* overexpression ($P < 0.001$, Mann-Whitney U test) in 21% ($n = 73/371$) of cases (Supporting Fig. S1A,B). Additionally, we observed that *TEAD4* expression was associated with aggressive HCC features, such as high Edmondson grade, high stage, and histologic changes, more frequently observed in severe diseases, such as the presence of necrosis, pleomorphic, and multinucleated cells ($P < 0.05$; Supporting Fig. S1C). Additionally, *TEAD4*-overexpressing tumors were more enriched in Hoshida molecular subtype S1, which is associated with higher proliferation and worse outcome (Supporting Fig. S1C). Furthermore, we observed that *TEAD4* overexpression was associated with worse overall survival and was independently prognostic in patients with HCC in univariate and multivariate models (hazard ratio, 1.52; 95% confidence interval, 1.00–2.29) (Supporting Fig. S1D; Supporting Table S2).

Having determined that *TEAD4* is indeed overexpressed in HCC, we modulated the expression of *TEAD4* in HCC cell lines, examined its effect on cell growth, and further exploited these models to dissect the possible oncogenic role of *TEAD4*. Using a

complementary DNA construct, we overexpressed *TEAD4* in two HCC cell lines with low endogenous levels of *TEAD4* expression (Huh7 and SNU449; Supporting Fig. S2A,B). We further used short-hairpin RNAs to silence *TEAD4* in two HCC cell lines with high endogenous levels of *TEAD4* expression (HLE and HepG2; Supporting Fig. S2A,B). We observed that *TEAD4* overexpression significantly increased proliferation as well as cell migration in both Huh7 and SNU449 cells (Fig. 1A,B; Supporting Fig. S2C,D). On the contrary, *TEAD4*-silenced HLE and HepG2 cells significantly proliferated and migrated less compared to control cells (Fig. 1A,B; Supporting Fig. S2C,D). The wound-healing assay further confirmed the role of *TEAD4* in regulating the migration potential of HCC cells. Indeed, forced expression of *TEAD4* in Huh7 led to an increase in the gap closure rate, while *TEAD4* silencing slowed the speed of gap closure in HLE cells ($P < 0.01$, Mann-Whitney U test) (Fig. 1C). Finally, because sorafenib is still the primary choice for first-line treatment of patients with HCC, we tested if the modulation of *TEAD4* expression might affect the response to sorafenib. Our results showed that overexpression or silencing of *TEAD4* did not alter the sensitivity of the cells to sorafenib (Supporting Fig. S2E).

To further demonstrate the oncogenic role of *TEAD4* in HCC, we xenotransplanted *TEAD4*-overexpressing and control Huh7 cells into the chicken CAM and assessed tumor growth *in vivo* (Fig. 2A). Engraftment of tumor cells in the CAM has been successfully used as a model of tumorigenesis.⁽³¹⁾ Indeed, it has been shown that this densely vascularized extraembryonic tissue enables a fast, reproducible, and precise analysis of the principal steps of tumor progression and is a powerful tool for preclinical *in vivo* studies.⁽³²⁾ Briefly, 24 hours post-transfection, cells were harvested and resuspended in matrigel before being seeded into the CAMs. After 4 days, eggs were screened for tumor formation and tumors were harvested for quantification and further analysis. In accordance with the results obtained *in vitro*, *TEAD4*-overexpressing engrafted cells formed significantly larger tumors compared to control cells (Fig. 2B,C; $P < 0.05$, Mann-Whitney U test). IHC and western blot analysis confirmed that the resected tumors were indeed of human origin and that *TEAD4* overexpression could still be detected 5 days posttransfection (Fig. 2D).

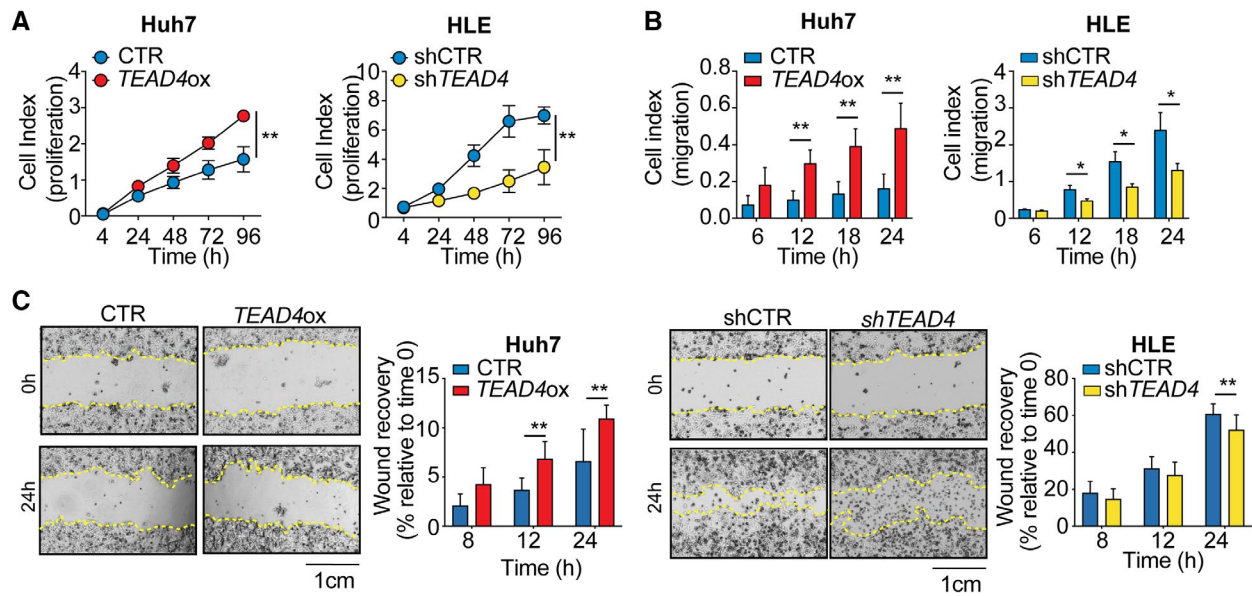


FIG. 1. Modulation of *TEAD4* expression level impacts cell proliferation and migration. (A) Proliferation and (B) migration kinetics of *TEAD4*-overexpressing (*TEAD4ox*) Huh7 and *TEAD4*-silenced (*shTEAD4*) HLE cells compared to their respective controls. (C) Wound-healing assay in *TEAD4*-overexpressing Huh7 and *TEAD4*-silenced HLE cells compared to their respective controls at specific time points. Representative pictures at time points 0 and 24 hours after scratch are shown for each condition. Scale bar in (C), 1 cm. Statistical significance was determined by the *t* test; **P* < 0.05, ***P* < 0.01. Data are mean ± SD. Abbreviations: h, hours; sh, short hairpin.

Taken together, our *in vitro* and *in vivo* data provide compelling evidence of the oncogenic role of *TEAD4* in HCC. In particular, we demonstrate the role of *TEAD4* in promoting proliferation and migration of liver cancer cells.

TEAD4 REGULATES CELL DIFFERENTIATION AND PROLIFERATION AND THE EXPRESSION OF HSP70 FAMILY GENES

To define the transcriptional changes induced by *TEAD4* overexpression, we performed RNA-seq on Huh7 cells overexpressing *TEAD4* (n = 4) or control vector (n = 4; Fig. 3A). Differential expression analysis identified 569 up-regulated and 316 down-regulated genes following *TEAD4* overexpression (quasi-likelihood F test, adjusted *P* < 0.05) (Fig. 3B; Supporting Table S3). Gene set enrichment analysis (GSEA)⁽³³⁾ revealed the up-regulated genes were involved in pathways, such as myogenesis, EMT, and the P53 pathway, all associated with cell differentiation and proliferation (Fig. 3C). Conversely, we observed a down-regulation of gene sets

involved in metabolism, such as protein secretion and bile acid metabolism (Fig. 3C). These results are consistent with the aggressive phenotype we observed in our *in vitro* and *in vivo* models following *TEAD4* overexpression as well as with the well-known role of *TEAD* proteins in regulating EMT and proliferation.⁽¹⁰⁾

In-depth analysis of the RNA-seq data revealed altered expression of the HSP family members, including some small HSPs (*HSPB9* and *HSPB1*), HSP40 (*DNAJA4*), and HSP70 (*HSPA1A*, *HSPA1B*, and *HSPA6*). Furthermore, the set of HSP70 genes defined by Klimczak et al.⁽³⁴⁾ was found to be significantly enriched in the *TEAD4*-overexpressing Huh7 cells (*P* < 0.05, GSEA; Fig. 3D), while other HSP gene sets (HSP40 and HSP90) were not significantly altered. In line with this, GSEA using Gene Ontology (GO) pathways showed enrichment of gene sets downstream of the HSP genes, such as those involved in HSP-binding protein-folding chaperones and unfolded protein binding (Fig. 3E). Surprisingly, the canonical Hippo targets seem not to be up-regulated in *TEAD4*-overexpressing cells, suggesting a potential Hippo-independent role of *TEAD4* in regulating HSP70 family members.

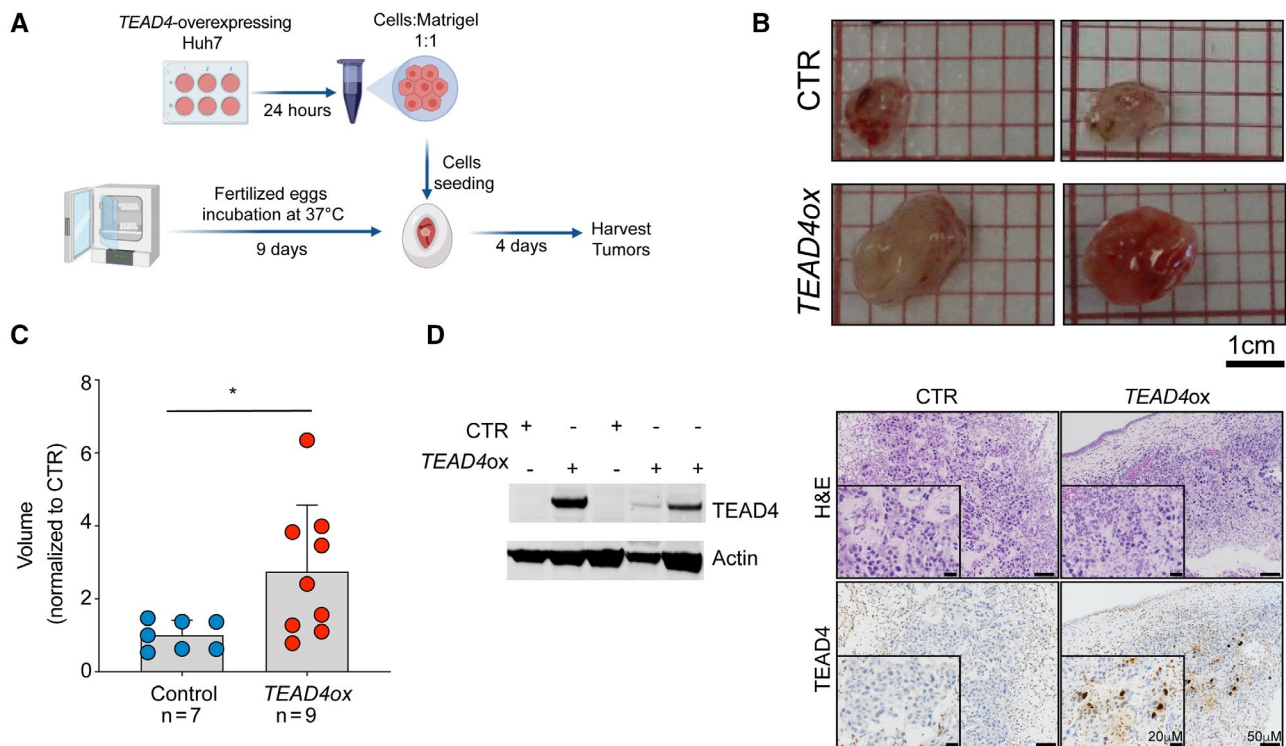


FIG. 2. *TEAD4* overexpression increases tumor growth *in vivo*. (A) Schematic representation of the CAM assay. (B) Photographs of *TEAD4*-overexpressing or control Huh7 cells implanted in CAMs and grown for 4 days postimplantation. (C) Volume of tumors derived from the CAM experiment ($n \geq 7$ tumors over two independent experiments). Values are normalized to the mean volume of control. (D) *TEAD4* expression in Huh7 tumors extracted 4 days postimplantation analyzed by western blot (left) and IHC (right). Tumoral cells were immunostained with the *TEAD4* antibody. Scale bars, (B) 1 cm and (D) 50 μm and 20 μm . Statistical significance was determined by the Mann-Whitney U test; * $P < 0.05$. Data are mean \pm SD. Abbreviations: H&E, hematoxylin and eosin; ox, overexpression.

Among the HSP70 genes, *HSPA1A* and *HSPA6* were the most significantly up-regulated genes following *TEAD4* overexpression (Fig. 3B). Using qPCR, we confirmed that overexpression of *TEAD4* led to a significant increase in *HSPA6* and *HSPA1A* expression in our *in vitro* models (Huh7 and SNU449; Fig. 3F). Our results suggested that HSP70 is a novel family of *TEAD4* target genes in HCC.

TEAD4 DIRECTLY REGULATES THE TRANSCRIPTION OF HSP70 FAMILY GENES

The HSP70 family comprises 13 gene products that differ from each other by expression level, subcellular location, and amino acid composition. Following exposure to different stressors, HSP70s bind to misfolded proteins and prevent their aggregation. As HSP70 family members and their overexpression have been shown

to play an oncogenic role in different cancer types,^(35,36) including HCC,^(37,38) we asked if HSP70 family genes were direct transcriptional targets of *TEAD4*. Because *HSPA6* and *HSPA1A* were the most up-regulated genes following *TEAD4* overexpression (Fig. 3B), we focused on these two members of the family. We analyzed a set of peak calls from *TEAD4* ChIP-seq performed on the HepG2 cell line.⁽²⁵⁾ Interestingly, we observed a *TEAD4* peak within the *HSPA6* promoter region, ~100 base pairs (bp) upstream of the transcription starting site (TSS), that contained two regions with the *TEAD4* binding motif (chr1:161493541-161493545 and chr1:161493648-161493652; Fig. 4A). Additional analysis of the peak calls using CREAM⁽²⁸⁾ identified a cluster of *TEAD4* peaks ~50 kilobases (kb) upstream of the *HSPA6* promoter (i.e., a *TEAD4* enhancer, chr1:31,776,368-31,832,367; Fig. 4A) and another enhancer ~47 kb downstream of the *HSPA1A* promoter (chr6:31,830,291-38,133,978; Fig. 4A), both well

within the 100-kb range of most promoter-enhancer interactions.⁽³⁹⁾ Peaks in both enhancers and at the gene promoters were also confirmed to contain the TEAD4 binding motif (Fig. 4A).

To confirm that TEAD4 directly binds to the promoter region of the *HSPA6* gene and the enhancer region of *HSPA1A*, we performed ChIP in *TEAD4*-overexpressing Huh7 cells, using anti-TEAD4

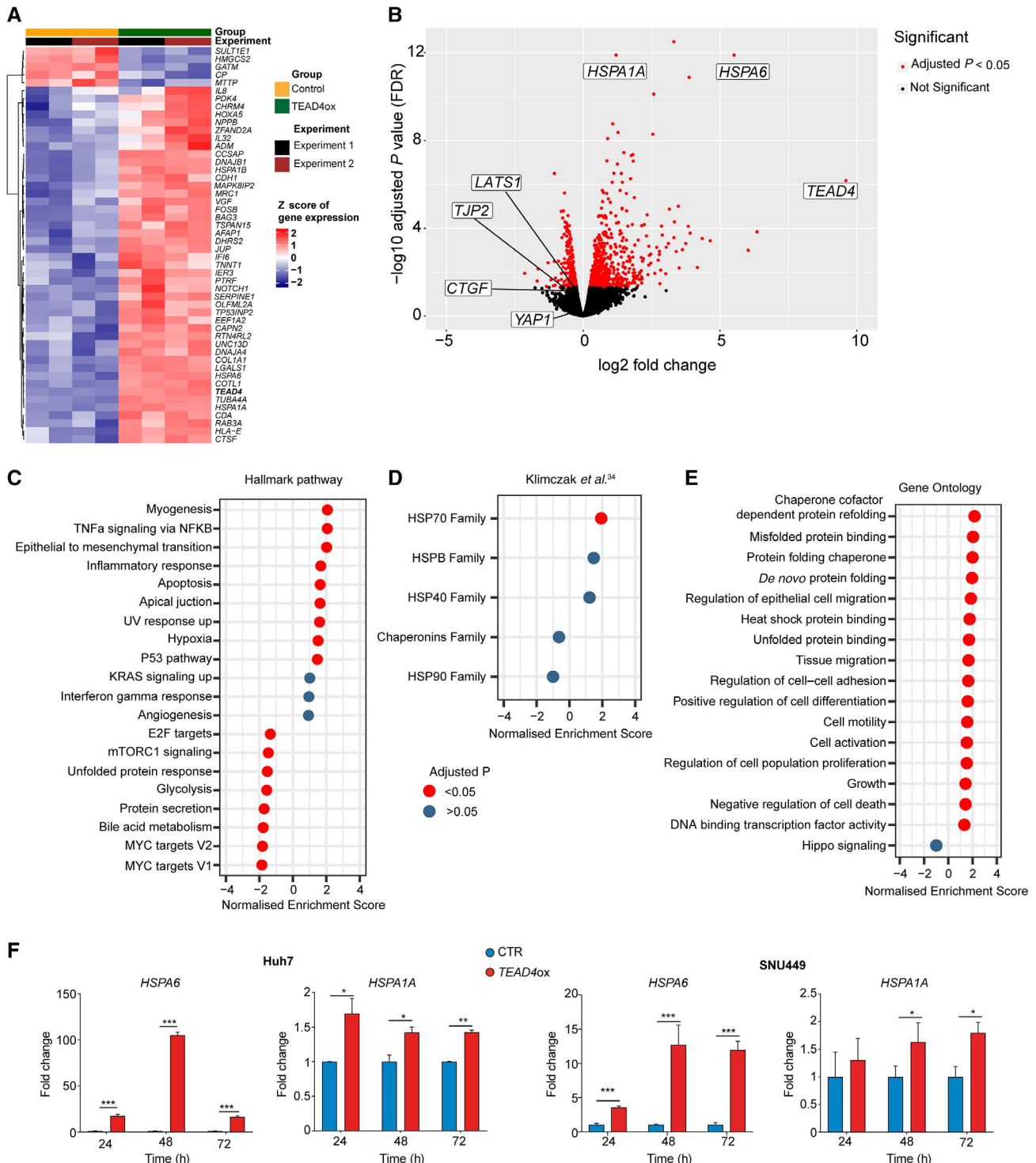


FIG. 3. TEAD4 regulates cell differentiation and proliferation and the expression of HSP70 family genes. (A) Heatmap showing the top 50 differentially expressed genes in *TEAD4*-overexpressing Huh7 cells. (B) Volcano plot of $-\log_{10}$ adjusted *P* value against \log_2 fold change to illustrate the differentially expressed genes between *TEAD4*-overexpressing Huh7 cells and control cells. Red dots indicate differentially expressed genes (adjusted $P < 0.05$). (C-E) GSEA plots show selected significantly enriched gene sets in *TEAD4*-overexpressing cells: Hallmark gene sets from (C) MSigDB database, (D) HSP family gene set, and (E) GO gene sets from the MSigDB database. (F) The Huh7 cell line was transiently transfected with a vector overexpressing *TEAD4* and the corresponding control. RNA was extracted at different time points. mRNA expression levels of *HSPA1A* (left) and *HSPA6* (right) were measured by qRT-PCR using *GAPDH* as the internal control. Data are shown as fold change relative to control. Results represent three independent experiments. Statistical significance was determined by the Mann-Whitney U test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are mean \pm SD. Abbreviations: E2F, E2 factor; FDR, false discovery rate; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; LATS1, large tumor suppressor kinase 1; mRNA, messenger RNA; mTORC1, mammalian target of rapamycin complex 1; NF κ B, nuclear factor kappa B; ox, overexpression; TJP2, tight junction protein 2; TNF α , tumor necrosis factor alpha; UV, ultraviolet.

antibody or IgG followed by qPCR. Using primers spanning the predicted TEAD4 binding region on the *HSPA6* promoter, the *HSPA1A* enhancer region, and the known TEAD4 target *CTGF* promoter,⁽⁷⁾ we confirmed that TEAD4 indeed binds to the *HSPA6* promoter and *HSPA1A* enhancer region (Fig. 4B).

To corroborate the results obtained *in vitro* we performed IHC staining on 10 HCC samples using TEAD4 and HSP70 antibodies. Analysis of this cohort revealed that samples positive for TEAD4 expression were also positive for HSP70 and vice versa ($P = 0.06$, Spearman correlation test; Fig. 4C). Of these 10 samples, eight were positive for HSP70 and two were negative. Six out of eight HCCs positive for HSP70 were also positive for TEAD4 (Fig. 4C), while the two HCCs negative for HSP70 were also negative for TEAD4.

Taken together, our results demonstrate that *HSPA6* and *HSPA1A* are transcriptional targets of TEAD4 in HCC through both TEAD4-promoter and TEAD4-enhancer interactions.

ONCOGENIC EFFECTS OF TEAD4 ARE MEDIATED THROUGH ITS UP-REGULATION OF HSP70

Given that the HSP70 family genes are direct transcriptional targets of TEAD4 and that HSP70s are known to play an oncogenic role in various cancer types, including HCC,⁽³⁷⁾ we hypothesized that the oncogenic effects of TEAD4 may be at least partially mediated by its up-regulation of HSP70. We therefore inhibited HSP70 pharmacologically in *TEAD4*-overexpressing cells with the benzylidene lactam compound KNK473 (Fig. 4C). KNK473 has been shown to inhibit the induction of HSPs, including HSP70, HSP40, and HSP105, *in vitro*.⁽⁴⁰⁾

Additionally, KNK437 was shown to revert E2 factor (E2F) transcription factor 1-mediated *HSP40* induction, which plays a role in promoting colorectal cancer tumor growth and metastasis *in vitro* and *in vivo*.⁽⁴¹⁾ Supporting our hypothesis, treatment of *TEAD4*-overexpressing Huh7 cells with 100 μ M of KNK437 abolished the effect of *TEAD4* overexpression on cell proliferation at 48 and 72 hours posttreatment (Fig. 4D). No significant reduction on cell proliferation was observed in control cells following treatment with KNK437.

Taken together, our results demonstrate that TEAD4 promotes tumor growth in HCC at least partially through direct regulation of the HSP70 family members. We further showed that the pharmacologic inhibition of HSP70 induction reverted the increased cell proliferation phenotype induced by TEAD4 overexpression.

TEAD4 REGULATES HSP70 AND CELL PROLIFERATION INDEPENDENT OF HIPPO SIGNALING

The transcriptional activity of TEAD proteins is highly dependent on the binding of their C-terminal protein interaction domain to several coactivators.^(42,43) Among those cofactors, YAP and TAZ,^(44,45) two major Hippo signaling pathway transcriptional coactivators, are the most well studied. When the Hippo signaling pathway is active, YAP and TAZ proteins are located in the nucleus, thus binding to TEADs and inducing transcription of TEAD downstream targets (Fig. 5A). Conversely, when the Hippo pathway is inactive, phosphorylation of YAP and TAZ allows TEADs to bind to other cofactors and activate alternative transcriptional targets (Fig. 5A).⁽⁴⁶⁾ The role of TEAD4 in HCC

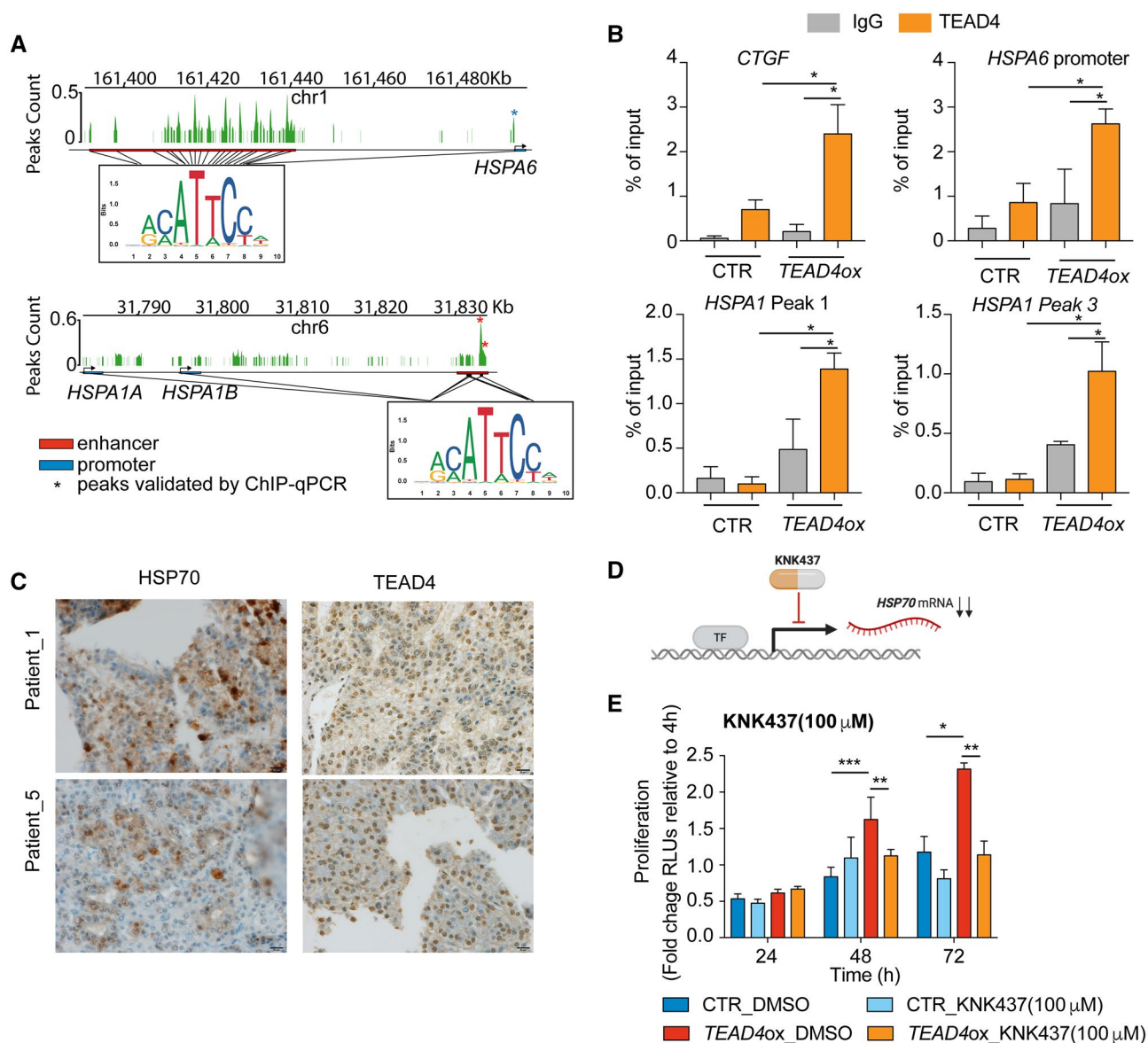


FIG. 4. TEAD4 impacts cell proliferation by directly binding HSP70 promoters and associated enhancers. (A) TEAD4 ChIP-seq peaks at the promoters of *HSPA6* and *HSPA1A*, and regions called as TEAD4 enhancers within 100 kb of *HSPA6* and *HSPA1A*, with TEAD4 binding motifs labeled. Enhancers and promoters are labeled as red and blue blocks, respectively. The TEAD4 binding motif logos are shown below the track, and positions in the *HSPA6* and in *HSPA1A* enhancers harboring this motif are indicated (this motif was also found at the promoter of all three genes). Asterisks indicate peaks validated by ChIP-qPCR. (B) ChIP-qPCR showing enrichment for TEAD4 binding at the *HSPA6* promoter and the *HSPA1A* enhancer. *CTGF* was used as a positive control. (C) Representative immunostain of TEAD4 and HSP70 on human HCCs. Magnification $\times 40$; scale bar 20 μm . (D) Schematic representation of the mechanisms of action of KNK437 on HSP70. (E) Proliferation of *TEAD4*-overexpressing Huh7 cells treated with 100 μM of KNK437 at various time points, relative to proliferation at 4 hours. DMSO was used as control. Results represent three independent experiments. Statistical significance was determined by the *t* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are mean \pm SD. Abbreviations: chr, chromosome; h, hours; mRNA, messenger RNA; ox, overexpression; RLU, relative light unit; TF, transcription factor.

carcinogenesis has so far been linked to the Hippo signaling pathway.^(19,21) Interestingly, analysis of RNA-seq data from *TEAD4*-overexpressing Huh7 cells revealed no significant change in well-known targets and/or

effectors of the Hippo signaling pathway (e.g., *YAP1*, large tumor suppressor kinase 1 [*LATS1*], tight junction protein 2 [*TJP2*], and *CTGF*; Fig. 3B) or in the Hippo signaling pathway as a whole ($q > 0.01$ GO; Fig. 3E),

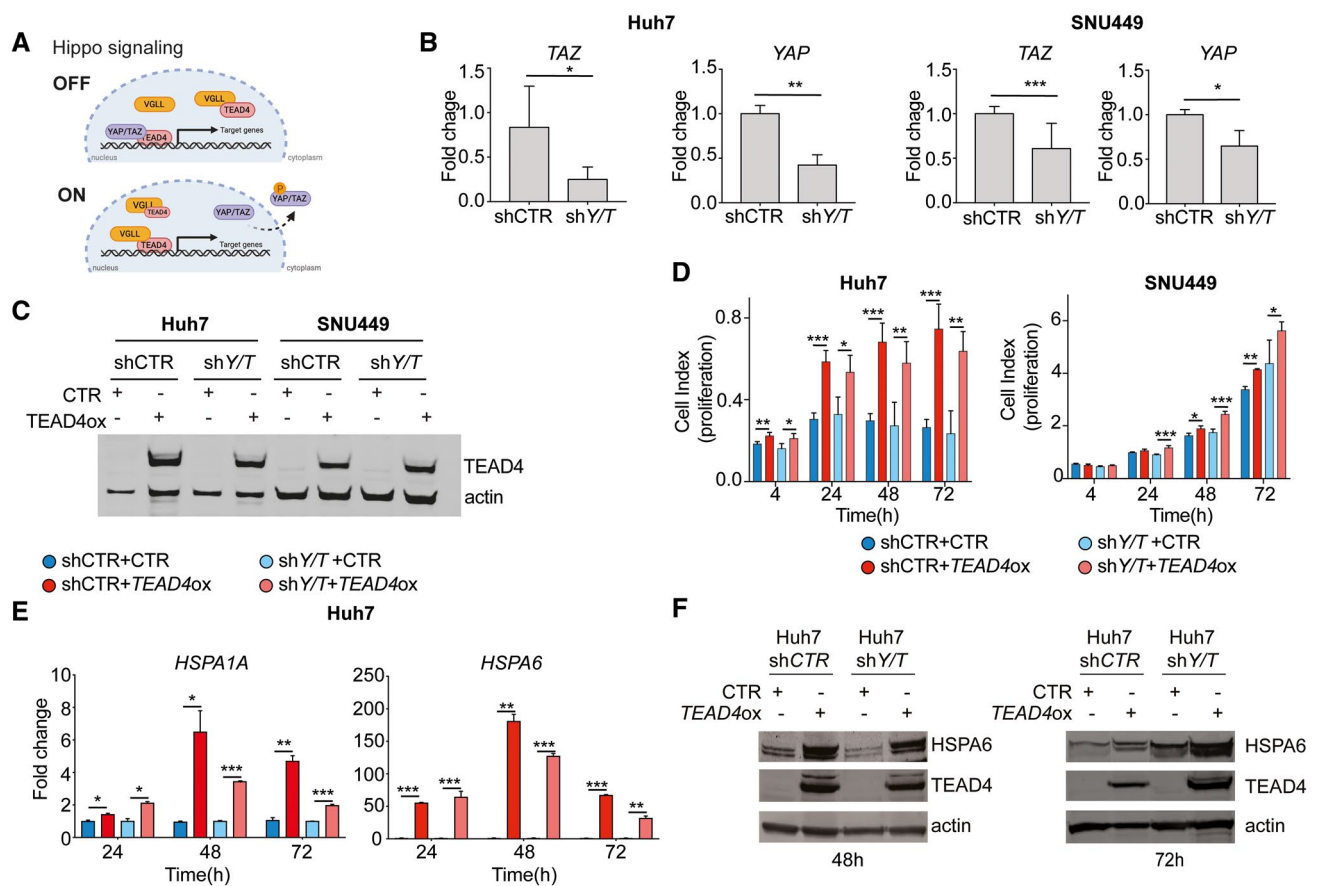


FIG. 5. The effect of *TEAD4* on cell proliferation is independent of expression of cofactors YAP and TAZ. (A) Schematic representation of *TEAD4* transcriptional activity associated with YAP/TAZ cofactor expression. (B) Expression levels of YAP and TAZ were measured in stable YAP/TAZ-knockdown (shY/T) and control (shCTR) Huh7 and SNU449 cell lines by qRT-PCR normalized to *GAPDH* as internal control. (C) TEAD4 expression in stably *TEAD4*-overexpressing and control Huh7 and SNU449 cell lines with knockdown of YAP/TAZ or control (shCTR). (D) Proliferation kinetics of stable *TEAD4*-overexpressing and control Huh7 cell lines with YAP/TAZ knockdown or control (shCTR) (left) and in *TEAD4*-overexpressing and control SNU449 stable cell lines with YAP/TAZ knockdown and control (shCTR) (right). (E) *HSPA6* and *HSPA1A* expression levels in *TEAD4*-overexpressing and control Huh7 cell lines with knockdown of YAP/TAZ or control (shCTR) were measured by qRT-PCR using *GAPDH* as reference. (F) Expression levels of *HSPA6* in *TEAD4*-overexpressing and control Huh7 cell lines with knockdown of YAP/TAZ or control (shCTR) were measured by western blot at 48 and 72 hours posttransfection. Data are shown as fold change to control. Results represent three independent experiments. Statistical significance was determined by *t* test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Data are mean \pm SD. Abbreviations: *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; h, hours; ox, overexpression; sh, short hairpin; VGLL, vestigial-like protein 1.

suggesting that the oncogenic properties of *TEAD4* in HCC are independent of the Hippo pathway.

To test the hypothesis that *TEAD4* does not require Hippo signaling to promote cell proliferation in liver cancer cells, we overexpressed *TEAD4* in stable YAP/TAZ-knockdown and control liver cancer cell lines (Supporting Fig. S3A,B; Fig. 5B,C). We first confirmed that the stable YAP/TAZ-knockdown cells had impaired Hippo signaling, as indicated by reduced *Cyr61*, *CTGF*, and vimentin expression (Supporting Fig. S3A,B). When *TEAD4* was overexpressed

(Supporting Fig. S3C), we observed that the proliferation rate was significantly increased in both stable YAP/TAZ-knockdown and control Huh7 and SNU449 cells (*P* < 0.001 and *P* < 0.05, respectively; Fig. 5D), suggesting that the presence or absence of the cofactors YAP and TAZ did not alter how *TEAD4* modulates cell proliferation *in vitro*.

Finally, to confirm that *TEAD4* regulates HSP70 expression independent of Hippo signaling, we measured *HSPA1A* and *HSPA6* expression in the stable YAP/TAZ-knockdown and control cells. In line with

our RNA-seq analysis, *HSPA1A* and *HSPA6* were up-regulated following *TEAD4* overexpression in both the stable *YAP/TAZ*-knockdown and the control cells (Fig. 5E; Supporting Fig. S3D), demonstrating that *TEAD4* regulates HSP70 expression independent of its *YAP* and *TAZ* coactivators. Additionally, we also performed western blot analysis to evaluate if the changes observed at the RNA level were also observed at the protein level. Indeed, we observed that the overexpression of *TEAD4* in cells with knockdown of *YAP/TAZ* led to an increased level of HSP70 at the protein level (Fig. 5F).

Our results suggest that the oncogenic effects of *TEAD4* overexpression in HCC are, at least in part, independent of the expression of the Hippo signaling *YAP* and *TAZ* coactivators.

Discussion

TEAD4 is a member of the *TEAD* transcriptional enhancer factor family composed of four members (*TEAD1-4*). Deregulation of *TEAD4* expression, as with other members of the family, has been extensively reported in several tumor types.^(16,47,48) In the present study, we showed that *TEAD4* promotes cell proliferation and tumor growth in HCC by directly regulating HSP70 family members through, at least in part, a Hippo-independent mechanism.

After confirming the overexpression of *TEAD4* in a subset of HCC in the TCGA data set, we evaluated the functional relevance of *TEAD4* up-regulation in HCCs by performing a series of *in vitro* and *in vivo* experiments. We demonstrated that *TEAD4* overexpression promoted tumor growth, cell proliferation, and migration in liver cancer cells while *TEAD4* silencing had the opposite effect. Our observations are consistent with the oncogenic role played by *TEAD4* in other tumor types. Indeed, it has been shown that down-regulation of *TEAD4* expression hampers cancer cell proliferation and invasiveness in *in vitro* and *in vivo* models of colorectal and gastric cancers.^(48,49) Our findings demonstrate that *TEAD4* also acts as an oncogene in HCC.

As a transcription factor, *TEAD4* regulates the transcription of many genes, activating or repressing several downstream pathways, such as cell growth, differentiation, and apoptosis.⁽¹¹⁾ Consistent with the role of *TEAD4* in cell differentiation, the gene

expression profile of *TEAD4*-overexpressing Huh7 cells showed up-regulation of pathways, such as spermatogenesis, EMT, and myogenesis. Surprisingly, chaperone folding and HSP pathways were also found to be significantly enriched in *TEAD4*-overexpressing cells. Specifically, we identified two members of the HSP70 family (*HSPA6* and *HSPA1A*) as among the most up-regulated genes. HSP70B and HSP70-1, the protein products of *HSPA6* and *HSPA1A*, respectively, are members of the ubiquitous and highly conserved HSP70 family of molecular chaperones. In normal cell conditions, chaperones are expressed at very low levels, ensuring the correct folding and transport of newly synthesized proteins.⁽⁵⁰⁾ However, chaperone levels increase during cell cycle and development⁽⁵¹⁾ as well as in response to cellular stress as occurs, for example, in tumors. Overexpression of HSP70 family members has been shown to play an oncogenic role in different cancer types,^(35,36) including HCC.^(37,38) In HCC, HSP70 has been reported to drive cell migration,⁽⁵²⁾ and genetic ablation of HSP70 was able to markedly impair chemically induced liver tumorigenesis and tumor progression.⁽³⁸⁾ Notably, HSP70 is a clinically important marker in HCC diagnosis; HSP70 expression, together with that of glutamine synthetase and glypican 3, is commonly used to differentiate early and low-grade tumors from dysplastic nodules.⁽⁵³⁾

Our reanalysis of the ChIP-seq data from the HepG2 cell line⁽²⁵⁾ revealed two mechanisms by which *TEAD4* can regulate the expression of HSP70 family genes; first by binding sites mapped less than 1 kb from the TSS of *HSPA6* and second through *TEAD4* enhancer regions ~50 kb upstream of the *HSPA6* promoter and ~47 kb downstream of *HSPA1A*. Both putative *TEAD4* enhancers and the HSP70 promoter regions were found to harbor *TEAD4* binding motif, and we confirmed the direct binding of *TEAD4* at these loci by ChIP-qPCR in *TEAD4*-overexpressing Huh7 cells. In fact, we demonstrated that the increased cell proliferation resulting from *TEAD4* overexpression was reversed by the pharmacologic inhibition of HSP70. We note that although KNK437 is a nonspecific HSP70 inhibitor, it has been shown to inhibit only inducible HSPs but has no effect on the expression of constitutively expressed HSP family members, including HSC70 and HSP90.⁽⁴⁰⁾ Therefore, although KNK437 is not specific to HSP70, it would only inhibit HSPs induced following *TEAD4*

overexpression. Our results thus indicate that the oncogenic effect of TEAD4 in HCC acts through its regulation of HSP70.

The transcription factor TEAD4 and its coactivators YAP and TAZ are considered major downstream effectors of the conserved Hippo signaling pathway.⁽¹⁸⁾ However, in colorectal and prostate cancers,^(6,15) TEAD4 has been found to play a role in EMT and cell proliferation independent of YAP/TAZ expression. Here, we demonstrated that *TEAD4* overexpression increased liver cancer cell proliferation independent of the expression of YAP/TAZ. Supporting our YAP/TAZ-knockdown experiments, our RNA-seq data from *TEAD4*-overexpressing cells revealed no significant changes on Hippo signaling following *TEAD4* overexpression. Furthermore, we showed that *TEAD4* overexpression induced *HSPA1A* and *HSPA6* expression in the context of YAP/TAZ knockdown. Together, our results indicate that TEAD4 regulates HSP70 expression and liver cancer cell proliferation independent of Hippo signaling.

In conclusion, we showed that TEAD4 plays an oncogenic role in HCC and that its oncogenic effect is mediated in part by its regulation of HSP70 genes. More importantly, our results unveil a novel role of TEAD4 outside its canonical Hippo-dependent mechanism.

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