Impaired Phosphorylation and Ubiquitination by p70 S6 Kinase (p70S6K) and Smad Ubiquitination Regulatory Factor 1 (Smurf1) Promote Tribbles Homolog 2 (TRIB2) Stability and Carcinogenic Property in Liver Cancer

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Background: TRIB2 is functionally important for liver cancer cell survival and transformation.

Results: Structure-function and biochemistry-based analysis revealed domains critical for TRIB2 protein stability.

Conclusion: Impaired phosphorylation and ubiquitination by p70S6K and Smurf1 increase protein stability of TRIB2 in liver cancer.

Significance: The uncovered mechanism underlying regulation of TRIB2 provides new therapeutic insights into TRIB2-dependent liver cancer.

Tribbles homolog 2 (TRIB2) is critical for both solid and non-solid malignancies. Recently, TRIB2 was identified as a liver cancer-specific Wnt/β-catenin signaling downstream target and is functionally important for liver cancer cell survival and transformation. TRIB2 functions as a protein that interacts with E3 ubiquitin ligases and thereby modulates protein stability of downstream effectors. However, the regulation underlying TRIB2 protein stability per se has not yet been reported. In this study, we found that TRIB2 was up-regulated and exhibited high stability in liver cancer cells compared with other cells. We performed a structure-function analysis of TRIB2 and identified a domain (amino acids 1–5) at the N terminus that interacted with the E3 ubiquitin ligase Smurf1 and was critical for protein stability. Deletion of this domain extended TRIB2 half-life time accompanied with a more significant malignant property compared with wild type TRIB2. Furthermore, Smurf1-mediated ubiquitination required phosphorylation of TRIB2 by p70 S6 kinase (p70S6K) via another domain (amino acids 69–85) that is also essential for correct TRIB2 subcellular localization. Mutation of Ser-83 diminished p70S6K-induced phosphorylation of TRIB2. Moreover, the high stability of TRIB2 may be due to the fact that both p70S6K and Smurf1 were down-regulated and negatively correlated with TRIB2 expression in both liver cancer tissues and established liver cancer cell lines. Taken together, impaired phosphorylation and ubiquitination by p70S6K and Smurf1 increase the protein stability of TRIB2 in liver cancer and thus may be helpful in the development of diagnosis and treatment strategies against this malignant disease.

TRIB2
t is a member of the tribbles family of pseudokinase proteins originally identified by their roles in Drosophila morphogenesis (1). Emerging evidence suggests a potential role of tribbles proteins in both solid and non-solid malignancies. High TRIB2 expression can contribute to the formation and progression of melanoma lesions (2). TRIB2 has also been identified as a potential driver of lung tumorigenesis through a mechanism that involves down-regulation of differentiation-inducing transcription factor CCAAT/enhancer-binding protein α (3). Through inhibition of CCAAT/enhancer-binding protein α, Trib2-reconstituted mice uniformly developed fatal transplantable acute myelogenous leukemia (4). Furthermore, TRIB2 has recently been found to be a NOTCH1 target gene that functions in the degradation of key proteins and modulation of MAPK signaling pathways and is thus implicated in hematopoietic tumorigenesis (5). By using a combination of genomic and cellular approaches, we recently identified TRIB2 as a critical downstream effector of Wnt signaling in liver cancer cells (6). Moreover, we found that TRIB2 is also functionally important for liver cancer cell survival and transformation and acts as a crucial signaling nexus to couple the Hippo/YAP

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4The abbreviations used are: TRIB2, tribbles homolog 2; p70S6K, p70 S6 kinase; Smurf1, Smad ubiquitination regulatory factor 1; YAP, Yes-associated protein; BTRC, β-transducin repeat-containing E3 ubiquitin protein ligase; CHX, cycloheximide; Ub, ubiquitin; IHC, immunohistochemistry; IF, immunofluorescence; TMA, tissue microarray; IP, immunoprecipitation; mTOR, mammalian target of rapamycin; TDD, TRIB2 degradation domain; PML, promyelocytic leukemia.
the E3s in several biological processes is emphasized. The relevance of specific substrates, and facilitating or directly catalyzing ubiquitin-sable role by recruiting ubiquitin-loaded E2s, recognizing spe-
some system plays an essential regulatory role in critical cellular
reporting TRIB2 functions as a protein that interacts with E3 ubiquitin ligases, such as BTRC and COP1, at the C terminus and thereby modulates the protein stability of downstream effectors (3–7). However, the regulatory relationship between TRIB2 and p70S6K.

Protein ubiquitination mediated by the ubiquitin-protea-
some system plays an essential regulatory role in critical cellular processes. In this system, E3 ubiquitin ligases play an indispensa-
ble role by recruiting ubiquitin-loaded E2s, recognizing specific substrates, and facilitating or directly catalyzing ubiquitin transfer to the respective molecular targets (8). The relevance of the E3s in several biological processes is emphasized in vivo and often accompanied by the occurrence of pathological disorders, including cancer (9, 10). A flurry of studies reported that the E3 ubiquitin ligase Smurf1 performs a dual role in cancer by functioning as both a tumor promoter and suppressor by controlling the stability of several important proteins with central roles in cell cycle progression, proliferation, differentiation, metastasis, genomic stability, and senescence (11–14). It is known that the substrate binding of E3 ligases is initialized by target protein modification, such as phosphorylation (15). However, potential kinases involved in Smurf1 ubiquitination/protein degradation signaling remain unclear.

In this study, we found that TRIB2 was highly up-regulated and much more stable in liver cancer cells than in other cells. We provide evidence to show that Smurf1 is involved in the ubiquitination and proteasomal degradation of TRIB2. Furthermore, phosphorylation of TRIB2 by p70S6K was found to promote Smurf1-induced degradation. Taken together, our study adds TRIB2 to the substrate list of Smurf1 and establishes the functional relationship between TRIB2 and p70S6K.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Expression Vectors**—HepG2, Bel-7402, SMMC-7721, HL-7702, QSG-7701, Chang liver, Hun6, HuH7, SNU-449, and HEK-293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells were treated with 50 μg/ml protein synthesis inhibitor cyclohexi-
mide (CHX; Sigma), 50 nm rapamycin (Cell Signaling Technol-
ogy, Boston, MA), 25 μM proteasome inhibitor MG132 (Cay-
man Chemical Co., Ann Arbor, MI), 50 μM INK inhibitor
SP600125 (Beyotime, Haimen, China), 20 μM p38 inhibitor SB203580 (Cell Signaling Technology), 10 μM MEK1/2 inhib-
tor U0126 (Cell Signaling Technology), 30 μM MAPK kinase inhibitor PD98059 (Cell Signaling Technology), 50 μM PKA/ cAMP-response element-binding protein stimulator forskolin (Beyotime), 50 μM JAK inhibitor AG490 (Beyotime), 0.5 mM AMP-activated protein kinase stimulator 5-aminoimidazole-4-carboxamide 1-β-d-ribofuranoside (Beyotime), 5 μM PKC in-
hibitor phorbol 12-myristate 13-acetate/12-O-tetradecano-
lyphorbol-13-acetate (Beyotime), 5 mM LKB1-AMP-activated protein kinase activator metformin (Beyotime) as well as 50 μM PI3K inhibitor wortmannin (Beyotime), or 5 μg/ml doxorubi-
 cin (Sigma) for the indicated times before harvest. shRNAs tar-

gating TRIB2, Smurf1, and p70S6K were cloned into pLKO.1 lentiviral vectors. The cDNA fragments encoding human TRIB2 were cloned into pCDNA3.1 (+) vector. The mutant TRIB2 S83A was constructed using overlapping PCR. p70S6K-Myc and Smurf1-FLAG expression plasmids were constructed as described previously (16, 17). Ub-HA was a gift from Jiabin Sun (Shanghai Jiaotong University, Shanghai, China). The primers used for construction of shRNAs and TRIB2 expres-
sion plasmids are listed in Tables 1 and 2, respectively.

**Immunohistochemistry (IHC), Immunofluorescence (IF), and Western Blotting**—For IHC, human liver cancer tissue microar-
ray (TMA) slides were purchased from US Biomax, Inc. (Rock-
ville, MD). Following deparaffinization and rehydration of the tissues sections, antigen retrieval was performed at 100 °C for 2 h with Tris-EDTA buffer, pH 6.0 (Beyotime). Endogenous
peroxidase was blocked with 3% peroxide for 20 min followed by three additional 5-min rinses in PBS. Sections were then blocked in a buffer containing 5% BSA and 0.1% Triton X-100 and incubated overnight in primary antibodies against TRIB2 (Abnova, Taipei, Taiwan, catalog number H00028951-M04), Smurf1 (Abnova, catalog number H00057154-M01), or p70S6K (Santa Cruz Biotechnology, Santa Cruz, CA, catalog number sc-9027). Signal detection was accomplished using the Vec-
tastain ABC kit (Vector Laboratories, Burlingame, CA). Sec-

**TABLE 1**

| Primer          | Sequence (5’–3’) |
|-----------------|------------------|
| TRIB2-shRNA1-F  | CCGGAAGAACAAAAAGGTTAAAACTTAACTCGAGTTAAGTATAATTTCTGCCCTTTTTTTTG |
| TRIB2-shRNA1-R  | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |
| TRIB2-shRNA2-F  | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |
| TRIB2-shRNA2-R  | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |

CCTGCCCTTTTTTTTG |
| Smurf1-shRNA1-F | CCGGAAGGTTCTTTTTTG |
| Smurf1-shRNA1-R | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |
| Smurf1-shRNA2-F | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |
| Smurf1-shRNA2-R | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |
| p70S6K-shRNA1-F | CCGGAAGGTTCTTTTTTG |
| p70S6K-shRNA1-R | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |
| p70S6K-shRNA2-F | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |
| p70S6K-shRNA2-R | AATTCAAAAAAAGGGCAGAAATTATACTTAACTCGAGTTAAGTATAATTTCTGCCCTT |

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Expression Vectors**—HepG2, Bel-7402, SMMC-7721, HL-7702, QSG-7701, Chang liver, Hun6, HuH7, SNU-449, and HEK-293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells were treated with 50 μg/ml protein synthesis inhibitor cycloheximide (CHX; Sigma), 50 nm rapamycin (Cell Signaling Technology, Boston, MA), 25 μM proteasome inhibitor MG132 (Cayman Chemical Co., Ann Arbor, MI), 50 μM INK inhibitor SP600125 (Beyotime, Haimen, China), 20 μM p38 inhibitor SB203580 (Cell Signaling Technology), 10 μM MEK1/2 inhibitor U0126 (Cell Signaling Technology), 30 μM MAPK kinase inhibitor PD98059 (Cell Signaling Technology), 50 μM PKA/cAMP-response element-binding protein stimulator forskolin (Beyotime), 50 μM JAK inhibitor AG490 (Beyotime), 0.5 mM AMP-activated protein kinase stimulator 5-aminoimidazole-4-carboxamide 1-β-d-ribofuranoside (Beyotime), 5 μM PKC inhibitor phorbol 12-myristate 13-acetate/12-O-tetradecano-lyphorbol-13-acetate (Beyotime), 5 mM LKB1-AMP-activated protein kinase activator metformin (Beyotime) as well as 50 μM PI3K inhibitor wortmannin (Beyotime), or 5 μg/ml doxorubicin (Sigma) for the indicated times before harvest. shRNAs targeting TRIB2, Smurf1, and p70S6K were cloned into pLKO.1 lentiviral vectors. The cDNA fragments encoding human TRIB2 were cloned into pCDNA3.1 (+) vector. The mutant TRIB2 S83A was constructed using overlapping PCR. p70S6K-Myc and Smurf1-FLAG expression plasmids were constructed as described previously (16, 17). Ub-HA was a gift from Jiabin Sun (Shanghai Jiaotong University, Shanghai, China). The primers used for construction of shRNAs and TRIB2 expression plasmids are listed in Tables 1 and 2, respectively.

**Immunohistochemistry (IHC), Immunofluorescence (IF), and Western Blotting**—For IHC, human liver cancer tissue microarray (TMA) slides were purchased from US Biomax, Inc. (Rockville, MD). Following deparaffinization and rehydration of the tissues sections, antigen retrieval was performed at 100 °C for 2 h with Tris-EDTA buffer, pH 6.0 (Beyotime). Endogenous peroxidase was blocked with 3% peroxide for 20 min followed by three additional 5-min rinses in PBS. Sections were then blocked in a buffer containing 5% BSA and 0.1% Triton X-100 and incubated overnight in primary antibodies against TRIB2 (Abnova, Taipei, Taiwan, catalog number H00028951-M04), Smurf1 (Abnova, catalog number H00057154-M01), or p70S6K (Santa Cruz Biotechnology, Santa Cruz, CA, catalog number sc-9027). Signal detection was accomplished using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were scored using a semiquantitative scale for each individual tumor tissue on the array slide with − for negative staining (<5% of cells staining), ± for very weak staining (i.e.
10–20% of cells showing weak staining in an appropriate subcellular distribution), + for weak staining (i.e., 20–40% of cells showing weak to intermediate intensity staining), ++ for strong staining (i.e., ≥10% of cells showing very intense staining or >50% of cells showing weak to moderately intense staining in an appropriate subcellular distribution), or +++ very strong staining (i.e., ≥30% of cells showing very intense staining or >80% of cells showing moderately intense staining). Scoring results were simplified into either negative (score of −) or positive (score of ± or +++) categories. Statistic analysis was done using χ² analysis, and a p value of <0.05 was considered statistically significant.

For IF, cells were fixed by 4% paraformaldehyde for 15 min, washed with PBS and blocking buffer (3% FBS, 1% heat-inactivated sheep serum, 0.1% Triton X-100), and then incubated overnight at 4 °C in primary antibodies against TRIB2 (Abnova, catalog number H00028951-M04), Ki67 (Abcam, catalog number ab15580), p70S6K (Abnova), and TRIB2-FLAG (expressed in HEK293T cells and purified by immunoprecipitation with an anti-FLAG antibody).

Cell Proliferation, Caspase-3/7 Activity, Soft Agar Assays, and Quantitative RT-PCR—Cell proliferation was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based proliferation assay as described before (6, 9). The caspase-3/7 activity was determined using the Caspase-Glo 3/7 assay system (Promega, Madison, WI). An anchorage-independent soft agar growth assay was performed as we described previously (6, 9). Quantitative RT-PCR was performed as described previously (9). The primers used for detection of TRIB2 mRNA were: forward, ggtgtgcaaggtgtttgatcag, and reverse, gaaggaatgcatgtccccatag.

**Immunoprecipitation**—For regular immunoprecipitation, cells were washed with PBS and subsequently lysed in Western/IP lysis buffer (Beyotime). Protein lysates were centrifuged at 14,000 × g for 10 min to pellet debris. After preclearing for 1 h with 50 µl of protein A/G-Sepharose (Invitrogen), the supernatants were incubated at 4 °C overnight with 3 µg of antibodies as indicated cross-linked to protein A/G-Sepharose beads. Beads were washed five times with lysis buffer, resuspended in SDS loading buffer, and analyzed by Western blotting analysis with antibodies as indicated. For in vitro reciprocal co-immunoprecipitation of purified proteins, TRIB2- or Smurf1-GST fusion proteins purchased from Abnova (catalog numbers H00028951-P01 and H00057154-P01) were resolved and mixed in Western/IP lysis buffer. The following procedures were the same as that for the regular immunoprecipitation.

**In Vivo and in Vitro Ubiquitination Assays**—For the in vivo ubiquitination assay, cells were treated with MG132 (25 µM) for 5 h before harvest to avoid the proteasome-mediated degradation. The cell lysate was prepared in HEPEs lysis buffer supplemented with protease inhibitors, and proteins were immunoprecipitated with the indicated antibody and detected by Western blotting with antibody as indicated. For the in vitro ubiquitination assay, E1, UbcH5c (E2), HA-Ub (provided by Dr. Lin Jiabei from Shanghai Jiaotong University), GST-Smurf1 (Abnova), and TRIB2-FLAG (expressed in HEK293T cells and purified by immunoprecipitation with an anti-FLAG antibody) were incubated at 30 °C for 2 h, and the assay was terminated with protein loading buffer as described by Guo et al. (18).

**In Vivo and in Vitro Phosphorylation of TRIB2**—For the in vivo phosphorylation assay, we immunoprecipitated TRIB2 or

### Table 2

| Primer     | Sequence (5′–3′)          |
|------------|---------------------------|
| C-WT-F     | ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| C-Fa-F     | ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| C-Fl-F     | ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| C-Fc-F     | ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| C-Fd-F     | ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| C-FLAG-R   | ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| C-HA-R     | ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| N-FLAG-WT-F| ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| N-FLAG-WT-R| ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| Del-C1-FLAG-R| ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| Del-C2-FLAG-R| ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| TRIB2-S83A-R| ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
| TRIB2-S83A-F| ATGCCGATCCATGAAATACTACAGCTACCAGTTACCCATCACGATAGCTTCTAGGATTAGCTGCAAGGTGTTTGAT |
TRIB2-FLAG from cells under different treatments as indicated with the anti-TRIB2 antibody or anti-FLAG antibody. The precipitated proteins were separated by SDS-PAGE containing 25 mM Phos-tag and subjected to regular Western blotting analysis.

For in vitro phosphorylation assay, immunoprecipitated TRIB2-FLAG and p70S6K-Myc (prepared by Dr. Jiafei Lin from Shanghai Jiaotong University) were incubated in kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl₂, 2 mM dithiothreitol, 20 mM ATP, 20 mM β-glycerophosphate, 20 mM disodium p-nitrophenyl phosphate, 0.1 mM sodium orthovanadate) at 30 °C for 30 min. The phosphorylated TRIB2-FLAG was separated by SDS-PAGE containing 25 mM Phos-tag and subjected to regular Western blotting analysis.

Xenograft Mouse Model—5 × 10⁶ Bel-7402 cells expressing shRNA against GFP (Control) or TRIB2 were subcutaneously injected into 8-week-old athymic nude mice (Bikai, Shanghai, China). Tumor size was measured every 6 days using a caliper, and the tumor volume was calculated as 0.5 × L × W² where L is length and W is width. The mice were euthanized at 30 days after injection.

RESULTS

TRIB2 Was Critical for Tumorigenesis in Liver Cancer Cells—First, we found that TRIB2 was highly up-regulated in liver cancer tissues compared with corresponding healthy tissues (Fig. 1A). We also examined TRIB2 expression in different cell lines, including three normal hepatic cell lines (QSG-7701, HL-7702, and Chang liver), human embryonic kidney cell line (HEK-293T), and six liver cancer cell lines (Huh7, Huh6, SMMC-7721, SNU-449, HepG2, and Bel-7402). We found that TRIB2 was more highly expressed in liver cancer cells compared with other cells and was highest in HepG2 cells (Fig. 1B). Thereby, we mainly focused on HepG2 cells for the following study.

As described in our previous study, TRIB2 is critical for maintenance of transformative phenotype in HepG2 cells (6). We examined here whether TRIB2 expression is also important for other liver cancer cell lines. By using the same shRNAs against TRIB2 as well as the treatment (including infecting cells with either separate shRNA2 or a combination of shRNA1 or shRNA2) from our previous study (6) (Fig. 1C), we confirmed that inhibition of TRIB2 decreased cell proliferation as measured by Ki67 immunostaining (Fig. 1D) and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based assay (Fig. 1E) and markedly impaired the ability to form colonies in soft agar in both SMMC-7721 and Bel-7402 cells (Fig. 1F). Furthermore, Bel-7402 cells with TRIB2 knocked down exhibited increased apoptosis (Fig. 1G) and reduced tumor volume and growth rate in xenograft mice models (Fig. 1H).
TRIB2 Was Stable in Liver Cancer Cells—An interesting question is how TRIB2 is up-regulated in liver cancer cells. We have found that transcription of TRIB2 is regulated by Wnt/β-catenin signaling (6); however, the post-transcriptional regulation of TRIB2 is not well understood. Here we tested TRIB2 stability by CHX chase experiments and found that exogenous TRIB2-FLAG proteins expressed in HEK-293T (Fig. 2A) and HL-7702 cells (Fig. 2B) were degraded quickly with half-life times less than 2 h. By comparison, TRIB2-FLAG proteins were relatively stable in both HepG2 (Fig. 2C) and Bel-7402 cells (Fig. 2D) with similar half-life times of ~5 h. Because the HepG2 cell line carries an active mutation of β-catenin (6, 19), whereas the Bel-7402 cell line contains wild type β-catenin, we suggest that the lower degradation rate leading to up-regulation of TRIB2 in liver cancer cells was independent of Wnt/β-catenin signaling.

Then we tested whether endogenous TRIB2 was also relatively stable in liver cancer cells. As shown in Fig. 2, E–H, endogenous TRIB2 was measured by Western blotting analysis after treating cells as indicated with CHX (50 μg/ml) for the indicated times. S, shorter exposure; L, longer exposure.

TRIB2 Is Regulated by Smurf1 via Ubiquitination—Then increased accumulation of ubiquitinated TRIB2-FLAG was detected by treatment with MG132, a proteasome inhibitor.
Compared with HepG2 cells, the ubiquitination level of TRIB2 was much higher in HL-7702 (Fig. 3A, lane 1 versus lane 3), suggesting that the proteasome may regulate TRIB2 degradation in normal hepatic cells but is impaired in liver cancer cells.

Because it is known that selective ubiquitination of the target protein relies on the binding of E3 ligase (20), we then explored the relationship between TRIB2 and its potential liver cancer-associated E3 ligase. Equal amounts of paired liver cancer and adjacent healthy tissues from 15 independent patients were mixed and tested for expression of a list of E3 ligases, including CDC4, URB5, RCHY1, MDM2, SKP2, UBE3A, CBL, and Smurf1. Only Smurf1 showed a significant down-regulation in liver cancer compared with the healthy tissues (Fig. 3B), indicating that the high stability of TRIB2 in liver cancer may be Smurf1-dependent. Gain and loss of function analysis demonstrated that Smurf1 knockdown induced TRIB2 protein expression (Fig. 3C), whereas Smurf1 overexpression dose-dependently reduced the TRIB2 protein level (Fig. 3D). We then ruled out the possibility that Smurf1 regulates TRIB2 expression at the transcriptional level because quantitative PCR detected no significant TRIB2 mRNA changes in cells with Smurf1 knocked down compared with the control (Fig. 3E).

We next asked whether Smurf1 functions as an E3 ligase to promote the ubiquitination of TRIB2. Overexpressed Smurf1 enhanced the ubiquitination of TRIB2 in cultured HepG2 cells (Fig. 3F). In addition, purified Smurf1 protein promoted the ubiquitination of TRIB2 directly in vitro (Fig. 3G). Taken together, these data indicate that Smurf1 acts as a biologically relevant E3 ligase for TRIB2.

Smurf1 Interacted and Was Negatively Associated with TRIB2—To confirm the interaction between TRIB2 and Smurf1, we performed co-immunoprecipitation (co-IP) experiments and found that TRIB2-HA could be readily pulled down by Smurf1-FLAG antibodies (A6), and co-immunoprecipitation with antibodies against TRIB2 or Smurf1 was performed in vitro using purified TRIB2 and Smurf1 proteins (all purchased from Abnova). A control IgG was used as the negative control for immunoprecipitation. F and G, TRIB2 negatively correlated with Smurf1. Representative IHC images of TRIB2 and Smurf1 staining from the TMA analysis are shown in F. Statistical analysis of the TMA data is shown in G. H, TRIB2 and Smurf1 expression in five cases of liver cancer and corresponding adjacent tissues as measured by Western blotting. N, normal; T, tumor.

Rapamycin Induced and Dephosphorylated TRIB2 through p70S6K—Substrate binding of E3 ligase is initialized by target protein modification, such as phosphorylation (21, 22).
TRIB2 is Regulated by p70S6K and Smurf1

FIGURE 5. Phosphorylation and regulation of TRIB2 by p70S6K. A, Western blots of TRIB2 proteins in HepG2 cells before and after treatment with chemical stimulators/inhibitors as indicated. B, proteins as indicated were tested by Western blotting in HepG2 treated with either DMSO or 50 nM rapamycin for 24 h. C and D, rapamycin decreased TRIB2 electrophoretic mobility. Cell lysates from HepG2 cells treated with rapamycin (50 nM) at different time points were resolved by SDS-PAGE using 25 mM Phos-tag-conjugated acrylamide followed by standard Western blotting with anti-TRIB2 antibody (C). Parallel protein samples with (+) or without (−) treatment with alkaline phosphatase for 8 h indicate the position of differentially phosphorylated forms of TRIB2 (D). E and F, p70S6K regulated TRIB2. Western blots of TRIB2 in control (treated with GFP shRNA) and HepG2 cells with p70S6K knocked down (E). HepG2 cells were transfected with increasing concentrations of p70S6K expression plasmids, and Western blotting analysis of TRIB2 was performed 24 h post-transfection (F). G, knockdown of p70S6K reduced phosphorylation of TRIB2. Cell lysates from control (infected with GFP shRNA) and HepG2 cells with p70S6K knocked down were resolved by SDS-PAGE using 25 mM Phos-tag-conjugated acrylamide followed by standard Western blotting with anti-TRIB2 antibody. H, knockdown of p70S6K reduced TRIB2 phosphorylation in vivo. HepG2 cells were transfected with TRIB2-FLAG expression plasmids. Whole lysates from HepG2 cells infected with shRNA against GFP (−) or p70S6K (+, sh1) were immunoprecipitated with the anti-FLAG antibody. The precipitated proteins were separated by SDS-PAGE containing 25 mM Phos-tag and subjected to regular Western blotting analysis. I, p70S6K promoted TRIB2 phosphorylation in vitro. TRIB2-FLAG and p70S6K-Myc were incubated in kinase buffer at 30 °C for 30 min. The phosphorylated TRIB2-FLAG was separated by SDS-PAGE containing 25 mM Phos-tag and then subjected to regular Western blotting analysis. J, transcription of TRIB2 was not affected by p70S6K knockdown. TRIB2 mRNA was evaluated by quantitative PCR in control (infected with shRNA against GFP) and HepG2 or HuH7 cells with p70S6K knocked down (infected with p70S6K-sh1). Data are shown as mean ± S.D. from three independent experiments. GAPDH mRNA was treated as a loading control. Error bars represent S.D. from three independent experiments.

Treating cells with chemical inhibitors or stimulators of several key signaling cascades, we found that only treatment with the mammalian target of rapamycin (mTOR) signaling inhibitor rapamycin led to up-regulation of TRIB2 (Fig. 5A). Also, treatment with rapamycin resulted in significant dephosphorylation of the mTOR signaling downstream effector p70S6K (Fig. 5B), suggesting that TRIB2 expression may be regulated by rapamycin-associated factors. To test whether rapamycin changes the phosphorylated forms of TRIB2, we incorporated Phos-tag and Mn$^{2+}$ in regular SDS-polyacrylamide gels to increase the resolution of the phosphorylated forms of TRIB2. Dramatically, nearly 100% of TRIB2 proteins were hypophosphorylated upon a 0.5-h treatment with rapamycin and slightly hyperphosphorylated after 8 h (Fig. 5C). The predominant band shift as a result of hyperphosphorylation of TRIB2 was confirmed as it could be reversed by calf intestine phosphatase treatment (Fig. 5D).

As both p70S6K and TRIB2 can be regulated by rapamycin (Fig. 5, A and B), we hypothesized that TRIB2 expression relies on p70S6K activity. S6K has two isoforms, 70 and 85 kDa, due to alternative translation initiation. We found that the p70 isoform had a much higher expression level compared with the p85 isoform in HepG2 cells (Fig. 5E). We then developed two independent shRNAs against S6K that could effectively inhibit both p70 and p85 isoforms and found that, similar to Smurf1, TRIB2 protein could be induced by knockdown of S6K (Fig. 5F). Because the p70 isoform is predominantly expressed, we mainly focused on this isoform (p70S6K) in the following study. We overexpressed p70S6K and found that TRIB2 was dose-dependently reduced (Fig. 5F). Furthermore, knockdown of p70S6K markedly reduced hyperphosphorylated forms of TRIB2 and induced hypophosphorylated forms (Fig. 5G), demonstrating that trans-phosphorylation and regulation of TRIB2 may be mediated by p70S6K.

To establish whether the phosphorylation of TRIB2 is p70S6K-dependent in vivo, we transfected TRIB2-FLAG into control and HepG2 cells with p70S6K knocked down. Knockdown of p70S6K caused a reduction of hyperphosphorylated TRIB2 but an induction of hypophosphorylated TRIB2 (Fig. 5H). We further tested whether TRIB2 can be directly phosphorylated by p70S6K in vitro. After electrophoretic separation of proteins by SDS-PAGE containing Phos-tag, hyperphosphorylated TRIB2 was observed only when co-incubated with purified p70S6K (Fig. 5I), indicating that TRIB2 is phosphorylated by p70S6K. Also, we found that the regulation of TRIB2 by p70S6K is independent of transcription because TRIB2 mRNA levels were similar between control and cells with p70S6K knocked down (Fig. 5).

p70S6K Interacted and Was Negatively Correlated with TRIB2—We then explored the relationship between TRIB2 and p70S6K. We performed reciprocal co-IP and found that TRIB2-FLAG could be pulled down by p70S6K-Myc and vice versa (Fig. 6A). It is reported that the p70 isoform of S6K is mostly cytoplasmic, whereas p85 has a nuclear localization signal (23,
24). However, we found that the co-localization signal of exogenous p70S6K-Myc and TRIB2-FLAG was very strong and mostly in the nucleus of HepG2 cells (Fig. 6B). Co-localization of TRIB2 and p70S6K was also detected in liver cancer tissues (Fig. 6C). Furthermore, co-IP for endogenous TRIB2 and p70S6K proteins in liver cancer tissues (the same sample used in Fig. 4C) revealed that these two proteins readily co-immunoprecipitated (Fig. 6D). Moreover, TMA and Western blotting data showed that TRIB2 expression is negatively correlated with p70S6K (Fig. 6E–G), thus confirming the hypothesis that p70S6K antagonizes TRIB2.

A Domain at N Terminus Was Required for TRIB2 Degradation—To map the functional domains responsible for TRIB2 stability, we created a variety of mutants containing truncated versions of TRIB2 protein. These mutants removed part of the N- or C-terminal region and were fused with a FLAG tag at either the N or C terminus (Fig. 7A and B). Protein analysis of these mutants confirmed that proteins of the expected sizes were expressed (Fig. 7C).

We first ruled out the possibility that tag position affects protein stability as there were no significant degradation difference between N- or C-terminal FLAG-fused wild type (WT) TRIB2 proteins under CHX treatment (Figs. 2A and 8A, panels b’ and e’–g’).

As the most proximal five amino acids (MNIHR) were the common domain deleted in all the N-terminally mutated constructs (Fig. 7B), we suggest that this domain (we named it the TRIB2 degradation domain (TDD)) is capable of inducing a significant enhanced degradation of TRIB2. Therefore, we examined the effects of TDD on TRIB2 ubiquitination. TRIB2 mutants lacking TDD (FW-a and F1) were also ubiquitinated but to a lesser extent than WT TRIB2 (Fig. 8B). Furthermore, compared with the WT, reduced co-localization of Smurf1 and TRIB2 was detected in TDD-deleted TRIB2 (FW-a and F1) (Fig. 8C). Taken together, these results suggest that TDD promoted ubiquitination of TRIB2, which ultimately resulted in proteasome-dependent degradation associated with Smurf1.

Fc-d Domain of TRIB2 Was Important for Phosphorylation and Correct Localization—Co-IP studies using various TRIB2 mutated constructs and p70S6K expression plasmid showed that deletion of the N terminus (amino acids 1–85) abolished binding of TRIB2 (Fd and F2) with p70S6K, whereas deletion of amino acids 1–51 (F1) had no such effect (Fig. 9A), suggesting that the region encompassing amino acids 52–85 contains the critical domain for the interaction between TRIB2 and p70S6K. Furthermore, the FLAG-tagged N-terminally deleted (without

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**FIGURE 6.** Relationships between TRIB2 and p70S6K. A, interaction between p70S6K and TRIB2. TRIB2-FLAG was co-transfected with p70S6K-Myc into HepG2 cells. TRIB2 and p70S6K associations were examined by reciprocal co-IP assay as indicated. B, co-localization of TRIB2-FLAG with p70S6K-Myc in HepG2 cells as measured by IF assays. Scale bar, 10 μm. C, representative immunostaining images of TRIB2 and p70S6K in liver cancer tissue. Scale bar, 150 μm. D, endogenous TRIB2 from liver cancer tissues (a mixture of samples from 15 patients) was immunoprecipitated with anti-TRIB2 antibodies (Ab), and co-immunoprecipitation of p70S6K was shown by anti-p70S6K Western blotting. A control IgG was used as the negative control for immunoprecipitation. E and F, TRIB2 negatively correlated with p70S6K. Representative IHC images of TRIB2 and p70S6K staining from the TMA analysis are shown in E. Statistical analysis of the TMA data is shown in F. G, TRIB2, Smurf1, and phosphorylated p70S6K (p-p70S6K) expression in five cases of liver cancer and corresponding adjacent tissues as measured by Western blotting. N, normal; T, tumor.
amino acids 1–85) TRIB2 (Fd and F2) showed a cytosolic enrichment and a speckle-like localization comparable with the Fc (without amino acids 1–68) protein. The co-localization signal was also reduced for Fd and F2 TRIB2 mutants and the p70S6K protein compared with other mutants (Fig. 9B), suggesting that the most proximal amino acids (amino acids 69–85; Fc-d domain) were required for correct localization of the TRIB2 protein and interaction with p70S6K.

Using Phos-tag-containing SDS-PAGE, we observed that p70S6K-induced hyperphosphorylation of TRIB2 was almost diminished (only when exposed for a very long time could a weak signal be detected) for protein (Fd) without the Fc-d domain compared with other mutant proteins (WT, F1, and Fc) (Fig. 9C, lane 7 versus lanes 1, 3, and 5), indicating a role for the Fc-d domain in TRIB2 phosphorylation.

To identify the exact phosphorylation site within domain Fc-d, we aligned the amino acids sequences of human, mouse, cattle, and frog and found the Ser-83 residue is conserved among these species (Fig. 9D). Moreover, by using a Phos-tag gel, we detected no mobility shift by overexpression of p70S6K.
when alanine was substituted by serine (S83A) (Fig. 9E), suggesting a role for this phosphorylation site of TRIB2 in the interaction with p70S6K.

Rapamycin Induced TRIB2 Translocation from Nucleus to Cytoplasm—To find out whether rapamycin affects TRIB2 cellular localization, we treated cells with rapamycin. We detected that translocation of TRIB2 from the nucleus to the cytoplasm happened when cells were treated with rapamycin (Fig. 10A). This observation was confirmed by fractionation studies, which revealed that rapamycin facilitated TRIB2 localization from the nuclear fraction to the cytosolic fraction (Fig. 10B). By IF assay, we also found that treatment with rapamycin led to cytosolic accumulation of TRIB2, whereas Smurf1 (Fig. 10C) and p70S6K (Fig. 10D) remained largely unaffected. Because p70S6K activity can be inhibited by rapamycin (Fig. 5B), we concluded that translocation of TRIB2 may be regulated by p70S6K.

Up-regulation of TRIB2 Caused Rapamycin Resistance—Then we tested TRIB2, Smurf1, and p70S6K expression patterns in different cell lines. We found that relatively higher TRIB2 expression correlated with lower p70S6K/Smurf1 expression in HepG2 cells compared with Huh7 cells (Fig. 11A). Also, compared with a normal hepatic cell line, HL-7702, phosphorylated p70S6K/p70S6K and Smurf1 were down-regulated in HepG2 and Bel-7402 cells, suggesting that lower p70S6K and Smurf1 expression levels led to the declining degradation rate of TRIB2 in liver cancer cells (Fig. 11B).

Next, we treated Huh7, HL-7702, and HepG2 cells with rapamycin. Compared with the significant induction of TRIB2 by rapamycin in HepG2 cells, only a slight up-regulation of TRIB2 was detected in Huh7 cells. However, rapamycin was unable to affect TRIB2 expression in HL-7702 cells (Fig. 11, C and D), suggesting that TRIB2 was much more sensitive to rapamycin induction in liver cancer cells, especially those with lower Smurf1/p70S6K expression. By contrast, rapamycin-induced growth inhibition was most obvious in HL-7702 cells, whereas it was least significant in HepG2 cells (Fig. 11E). Moreover, the effect by which rapamycin impaired the ability of cells to form colonies in soft agar was more obvious in Huh7 cells compared with that in HepG2 cells (Fig. 11F), suggesting that the reduced cell survival and transformative phenotype by rapamycin were negatively associated with the induction of TRIB2. Take together, up-regulation of TRIB2 may cause rapamycin resistance in liver cancer cells.

p70S6K Promoted Smurf1-induced Degradation of TRIB2 via TDD—To address the hypothesis that p70S6K promotes Smurf1-mediated degradation of TRIB2, we stably transfected WT or mutated TRIB2 constructs into HepG2 cells and treated these cells with rapamycin. We found that only WT TRIB2 was up-regulated compared with those mutants without TDD (FW-a and F-c) (Fig. 12A), suggesting that TDD was important for rapamycin-induced up-regulation of TRIB2. We also observed that deletion of TDD in TRIB2 protein resulted in reduced binding to Smurf1 (Fig. 12B), suggesting that TDD was also important for the interaction between TRIB2 and Smurf1.

Furthermore, rapamycin reduced Smurf1 binding to WT TRIB2; however, such effect was not observed when TDD was deleted (for FW-a mutant) (Fig. 12C), suggesting that rapamycin reduced Smurf1 binding to TRIB2 via TDD. Similarly,
knockdown of p70S6K markedly impaired the association between WT TRIB2 and Smurf1 but had no effects when TDD was deleted (Fig. 12D). In addition, we observed that the reduction of TRIB2 by overexpression of Smurf1 could be partially reversed by knockdown of p70S6K (Fig. 12E), which supported our hypothesis that the binding of Smurf1 to TRIB2 was p70S6K-dependent.

By performing CHX chase analysis, we found that knockdown of either p70S6K or Smurf1 could prolong the half-life time of endogenous TRIB2 (Fig. 12, F and G). Consistently, ubiquitination of TRIB2 was markedly reduced after knockdown of either p70S6K or Smurf1 (Fig. 12H), suggesting that both Smurf1 and p70S6K were important for the stability and ubiquitination of TRIB2.

We reported previously that the oncoprotein YAP was down-regulated after knockdown of TRIB2 (6). Consistently, in this study, we found that YAP was up-regulated before knockdown of TRIB2 was ectopically expressed (Fig. 12I). Furthermore, this event could be reversed by simultaneously expressing either Smurf1 or p70S6K, and a synergistic effect was observed when both proteins were overexpressed (Fig. 12I), suggesting that the regulation of YAP by TRIB2 was also controlled by Smurf1 and p70S6K.

**DISCUSSION**

In functional assays, we showed that suppression of TRIB2 protein reduced the transformative phenotype in liver cancer cells (Fig. 1, D–H). Consistently, overexpression of TRIB2 was capable of reversing doxorubicin-induced apoptosis in normal hepatic cells (Fig. 13C) and reinforcing malignant properties of liver cancer cells (Fig. 13, A and B). Thus, we concluded that high TRIB2 expression could contribute to the formation and progression of liver cancer.

There are three distinguishable regions in TRIB2 protein, including an N-terminal portion, a central serine/threonine kinase-like domain, and a C-terminal region that binds the E3 ligases COP1 and BTRC (7, 9). The C terminus is essential for inhibition of myeloid differentiation and induction of transformation in leukemia cells, whereas the N terminus, which is characterized by a high serine and proline content, is not required for these activities (7, 25). However, in the present study, we showed that a TDD within the N terminus (Fig. 7B) was critical for protein stability of TRIB2 (Fig. 8, A and B).
Deletion of the TDD extended the half-life time of TRIB2 (Fig. 8A) and gave it a more significant transformative property (Fig. 13, A–C). Smurf1 is known to recognize a “PY” motif that has the ability to interact with WW domains of the HECT family E3 ligases (26). Unfortunately, no such motif was found in TRIB2; however, Smurf1 did interact with TRIB2 via the TDD, which is composed of five amino acids (MNIHR) (Figs. 4, A–E, and 7B), and deletion of this domain reduced the association between these two proteins (Fig. 12B). We also revealed that TRIB2 expression was negatively associated with Smurf1 in liver cancers (Fig. 4, F–H) and provide strong evidence that Smurf1 binding was an important event for TRIB2 degradation (Figs. 3, C, D, F, and G, and 12, B–H).

Several lines of evidences suggest that Smurf1-mediated ubiquitination of target proteins requires phosphorylation (21, 22). Here we have demonstrated that treatment of rapamycin, an inhibitor of mTOR complex 1, up-regulated TRIB2 through dephosphorylation of this protein (Fig. 5, A–C). Gain and loss of function analysis revealed that mTOR downstream effector p70S6K negatively regulated TRIB2 (Fig. 5, E and F). p70S6K bound and phosphorylated TRIB2 via the Fc-d domain (amino acids 69–85) in the central region of TRIB2 (Fig. 13D, panel a’) as dephosphorylation of TRIB2 resulted from either depletion of p70S6K (Fig. 5G) or loss of the Fc-d domain (Fig. 9C). We also revealed that Ser-83 was the phosphorylation site of p70S6K in the TRIB2 protein (Fig. 9E), confirming the close relationship between these two proteins.

Here we observed that the predominant p70 isoform of S6K was nuclearly expressed in liver cancer cells (Figs. 5E and 6B). We also revealed that rapamycin-induced up-regulation of TRIB2 may occur through dephosphorylation of the protein.
Rapamycin facilitated TRIB2 translocation from the nucleus to the cytoplasm (Fig. 10, A and B), whereas the intracellular localization of p70S6K remained unchanged (Fig. 10 D), suggesting that p70S6K was spatially separated from its substrates and that the function of p70S6K (at least phosphorylation of TRIB2) was carried out in the nucleus. In addition, localization of Smurf1 was unaffected before and after rapamycin treatment as well (Fig. 10 C), indicating that cytosolic TRIB2 may survive Smurf1 binding and subsequent ubiquitination. In other words, nuclear retention of TRIB2 facilitated ubiquitination of itself by Smurf1, thus providing a novel therapeutic method to treat TRIB2-dependent tumors.

It is also reasonable to assume that Smurf1–induced TRIB2 degradation is p70S6K-dependent because reduction of p70S6K activity by either shRNA or rapamycin impaired Smurf1 binding to WT TRIB2. However, the effect was abolished after deletion of TDD (Fig. 12, C and D), clearly revealing that TDD was a functional motif linking TRIB2, Smurf1, and p70S6K. Moreover, we observed that degradation of TRIB2 required both p70S6K and Smurf1. Loss of p70S6K impaired Smurf1-mediated TRIB2 degradation (Fig. 12E), indicating that degradation of TRIB2 was initiated by phosphorylation and sequential recruitment of Smurf1.

mTOR complex 1 is known to be highly active in most tumors, including liver cancer. Inhibitors of mTOR complex 1, including rapamycin and its analogs, are being evaluated as antitumor agents. However, resistance to the growth inhibitory effects of rapamycin is common. Blocking of downstream mTOR signaling by rapamycin promotes PML gene expression in glioblastoma cells, leading to prevention of mTOR inhibitor-dependent cell death. Low doses of the PML inhibitor arsenic trioxide abrogate PML expression and reverse mTOR kinase inhibitor resistance (27). Totary-Jain et al. (28) reported that long term rapamycin treatment resulted in up-regulation of miR-17–92 cluster, an onco-microRNA, and down-regulation of tumor suppressor microRNAs. Inhibition of miR-17–92 or delivery of tumor suppressor microRNAs restored sensitivity to rapamycin. By profiling a total of 13 hepatic cell lines for rapamycin-induced growth inhibition, Jimenez et al. (29) described that the locus of rapamycin resistance was downstream from...
TRIB2 Is Regulated by p70S6K and Smurfi1

FIGURE 13. TDD played a negative role in TRIB2 carcinogenesis property. A–C, transformative phenotype was strengthened by deletion of TDD. Transformation activities were measured by anchorage-independent soft agar colony formation assays in Bel-7402 and HepG2 cells stably transfected with plasmids as indicated (A). TRIB2 promoted tumorigenesis in vivo. Tumor volumes were measured for 30 days after subcutaneous injection of Bel-7402 cells stably transfected with plasmids as indicated. n = 5 per group (B). TRIB2 mutant rescued doxorubicin-induced apoptosis in HL-7702 cells as measured by caspase-3/7 activity (C). *, p < 0.05; **, p < 0.01 (analyzed by Student's t test). Error bars represent S.D. from three independent experiments for A and C and from five independent experiments for B. D, illustration of TRIB2 protein structure (panel a') and possible mechanism underlying TRIB2 degradation by p70S6K and Smurfi1 (panel b').

inhibition of mTOR complex 1. TRIB2 is a potential oncogene and is up-regulated by both rapamycin and p70S6K knockdown (Fig. 5, B and E). Moreover, the reduced cell survival and transformative phenotype by rapamycin are negatively associated with the induction of TRIB2 (Fig. 11, C–F). Thereby, we hypothesized that TRIB2 may contribute to the mechanisms of rapamycin resistance in liver cancer. This will need to be investigated further.

Furthermore, we concluded that up-regulation of TRIB2 in liver cancer tissues as well as established liver cancer cell lines (Fig. 1, A and B) may result from relatively high protein stability (Fig. 2, G and H). TRIB2 promotes tumorigenesis as it can protect YAP from degradation (6). Here we found that YAP expression could also be regulated by Smurfl and p70S6K through their interaction with TRIB2 (Fig. 12), thereby adding two novel factors to the YAP regulatory network.

In summary, we have demonstrated that TRIB2 is a critical regulator in liver cancer tumorigenesis. Impaired phosphorylation and ubiquitination of TRIB2 by p70S6K and Smurfl increased its protein stability, which in turn enhanced carcinogenic properties of TRIB2 (Fig. 13D, panel b'). Thus, our study may provide new therapeutic insights into TRIB2-dependent liver cancer.

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