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TRIB2 inhibits Wnt/β-Catenin/TCF4 signaling through its associated ubiquitin E3 ligases, β-TrCP, COP1 and Smurf1, in liver cancer cells

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ABSTRACT

Tribbles homolog 2 (TRIB2) is specifically regulated by Wnt signaling in liver cancer cells but not in colon cancer cells. However, whether and how TRIB2 regulates Wnt signaling in liver cancer cells remains unclear. Here, we report that TRIB2 negatively regulates Wnt activity through a reduction in protein stability of TCF4 and β-Catenin. Mechanistically, TRIB2 associated-ubiquitin E3 ligases beta-transducin repeat-containing E3 ubiquitin protein ligase (β-TrCP), COP1 and Smad ubiquitination regulatory factor 1 (Smurf1) reduced TCF4/β-Catenin expression, and these effects could be enhanced by TRIB2. Moreover, deletion of the binding regions of these E3-ligases within the TRIB2 protein decreased ubiquitination of TCF4/β-Catenin and reduced nuclear accumulation of β-TrCP, COP1 and Smurf1, which suggested that TRIB2 regulated-Wnt activity is closely correlated with its associated E3 ligases.

Structured summary of protein interactions:
TCF4 and **TRIB2** colocalize by fluorescence microscopy (1, 2, 3)
TRIB2 and **Beta-TrCP** colocalize by fluorescence microscopy (View interaction)
TRIB2 and **SMURF1** colocalize by fluorescence microscopy (View interaction)
TRIB2 and **COP1** colocalize by fluorescence microscopy (View interaction)
TRIB2 physically interacts with **COP1** and **Beta-TrCP** by anti bait coip (View interaction)
Catenin beta and **TRIB2** colocalize by fluorescence microscopy (1, 2)

1. Introduction

Tribbles homolog 2 (TRIB2) is a member of the Tribbles family of pseudokinase proteins that were originally identified by their roles in Drosophila morphogenesis [1]. Previous studies revealed that TRIB2 is critical during tumorigenesis of multiple cancer types. TRIB2 overexpression in melanoma contributes to the formation and progression of lesions [2]. TRIB2 in lung cancer is described as a potential tumor driver through the downregulation of differentiation-inducing transcription factor CEBPα [3]. TRIB2 in hematopoietic tumorigenesis acts as a downstream effecter of Notch signaling and modulates MAPK signaling [4].

We performed large-scale sequencing analysis in the highly Wnt signaling activated liver cancer line, HepG2, and the colon cancer cell line, LS174T, to identify a unique Wnt signaling controlled-target gene in liver cancer cells that contributes to liver tumorigenesis. We found that TRIB2 was specifically activated by TCF4/β-Catenin signaling in HepG2 cells but not LS174T cells, which suggests that TRIB2 is a liver cancer-specific Wnt signaling target [5]. Furthermore, we found that TRIB2 plays a critical role in liver cancer cell survival and transformation [5]. However, whether and how TRIB2 inversely regulates Wnt signaling remains unknown.

Abbreviations: TRIB2, Tribbles homolog 2; CHX, cycloheximide; Smurf1, Smad ubiquitination regulatory factor 1; β-TrCP, beta-transducin repeat-containing E3 ubiquitin protein ligase; Ub, ubiquitin; IF, immunofluorescence; WB, Western blotting

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Tribbles homologs, including TRIB2, are characterized by a central serine/threonine kinase-like domain (KD), which is the defining feature of the Tribbles proteins. However, these proteins are pseudokinases because they lack a catalytic core motif within the KD [6,7]. Other than the KD, our and previous studies suggest that TRIB2 functions as a protein that interacts with ubiquitin E3 ligases, such as beta-transducin repeat-containing E3 ubiquitin protein ligase (β-TrCP), COP1 and TRIM21, at the C terminus, which modulates the protein stability of downstream targets [3–5,8,9]. Recently, we discovered that TRIB2 up-regulation in liver cancer cell lines HepG2, SMMC-7721 and Bel-7402 is due to a relative lower protein degradation rate compared to the transformed hepatocyte line HL-7702 [10]. TRIB2 can be phosphorylated at S83 by p70S6K before further ubiquitination by the ubiquitin E3 ligase Smad ubiquitination regulatory factor 1 (Smurf1). Impaired phosphorylation and ubiquitination by p70S6K and Smurf1 promotes TRIB2 stability and carcinogenic properties in liver cancer cells [10]. We also described that ubiquitin E3 Smurf1 interacts with the N-terminus of the TRIB2 protein, and this event is essential for the post-translational regulation of TRIB2 [10]. This result adds another E3 ligase to the list of TRIB2 E3 ligase interactions. β-TrCP is one E3 ligase that mediates protein degradation of β-Catenin [11,12], but whether and how other TRIB2-associated E3 ligases regulate Wnt signaling is not clear.

The present study found TRIB2 inhibited Wnt signaling activity through a facilitation of the ubiquitination of its key factors, TCF4 and β-Catenin, in liver cancer cells. TRIB2 induced a nuclear accumulation of β-Catenin, but this did not lead to the induction of Wnt activity. However, nuclear accumulation of β-Catenin resulted in an increase in the opportunity to come into contact with the TRIB2-associated E3 ligases β-TrCP, COP1 and Smurf1. TRIB2 overexpression also induced nuclear expression of the above three E3 ligases. Deletion of the binding regions of these E3 ligases within the TRIB2 protein decreased the ubiquitination of TCF4/β-Catenin and reduced the nuclear accumulation of β-TrCP, COP1 and Smurf1, which indicates a close cooperation between TRIB2 and E3 ligases in the regulation of Wnt activity.

2. Materials and methods

2.1. Cell culture and vectors

Liver cancer cell lines, HepG2, Huh7, Bel-7402 and SMMC-7721, and a transformed hepatocyte line, HL-7702, were cultured in DMEM supplemented with 10% FBS. ShRNAs against β-TrCP, COP1 and Smurf1 were purchased from Genechem Biotech LTD (Shanghai, China). ShRNA against TRIB2 was described and verified in our previous studies [5,10]. Wild-type (WT) TRIB2-FLAG was cloned into either a pGIPZ-based lentiviral-expressing vector or pcDNA3.1(+) vectors. Mutant TRIB2-FLAGs were all cloned into pcDNA3.1(+) vectors, and the primers for cloning are described in our previous study [10]. The Del-Smurf1 (plasmid expressing mutant TRIB2 protein without the amino acid residues (a.a.) 1–5) is equivalent to the FW-a-TRIB2-FLAG (C) in our previous study, Del-COP1 (plasmid expressing mutant TRIB2 protein without the a.a. 324–343) is equivalent to the Del-C1-FLAG (C), and Del-COP1/β-TrCP (plasmid expressing mutant TRIB2 protein without a.a. 307–343) is equivalent to Del-C2-FLAG (C) in our previous work [10]. The Smurf1-FLAG- and β-TrCP-FLAG-expressing plasmids were obtained from our previous work [10,13]. The β-Catenin-Myc- and TCF4-Myc-expressing plasmids were gifts from Dr. Xiangfan Liu (Shanghai Jiaotong University). The COP1-FLAG-expressing plasmid was purchased from Origene (Beijing, China).

2.2. Immunofluorescence (IF) and Western blotting (WB)

Cells were fixed using 4% paraformaldehyde for 15 min for IF, washed with PBS and blocking buffer (3% FBS + 1% HISS + 0.1% Triton X-100), and incubated overnight at 4 °C in primary antibodies against β-Catenin (Epitomics, Burlingame, CA, USA, #1247), COP1 (Abcam, Ab56400), β-TrCP (Cell Signaling Technology (CST), Boston, MA, USA, #4394), Smurf1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-100616), TCF4 (CST, #5174), TRIB2 (Abcam, ab117981) or FLAG-Tag (CST, #8146 or #2368).

Subcellular extracts for WB were prepared using a kit from Active Motif (Carlsbad, CA, USA). Proteins were resolved on SDS–PAGE gels followed by standard WB. The following primary antibodies were used: GAPDH (CST, #5174), HA-Tag (CST, #3724), FLAG-tag (CST, #2368), TRIB2 (Abcam, Ab84683), β-Catenin (Epitomics, #1247), COP1 (Abcam, Ab56400), β-TrCP (CST, #4394), β-tubulin (CST, #2128), Histone (CST, 4499), Smurf1 (Santa Cruz Biotechnology, sc-100616), TCF4 (CST, #5174), Myc-tag (CST, #2278) or Ub (CST, #3933).

2.3. Immunoprecipitation (IP)

The details of these procedures were described previously [10]. Anti-TRIB2 (Abnova, Taipei, Taiwan, #H00028951-M04) antibodies were used for IP. IPs were washed at least five times and subjected to WB analysis.

2.4. Luciferase reporter analysis

The pTOP-FLASH firefly luciferase reporters were co-transfected with Renila luciferase expression plasmids (pRL-TK, Promega, Madison, WI, USA) into the cells under the different indicated treatments. The detection of luciferase activities was analyzed using a dual-luciferase reporter kit (Promega).

2.5. Cell proliferation, soft-agar assays and quantitative RT-PCR (qPCR)

Cell proliferation was measured using an MTT-based proliferation assay as described previously [5]. Anchorage-independent soft-agar growth assay and qRT-PCR were performed as previously described [5]. The following primers were used for qPCR: Axin2-F: ACAACAGCATTGTCTCCAAGCAGC, Axin2-R: GCGCCTGGTCAAACAT GATGGAAT, SP5-F: CCGATGCGGCTACAGGT, SP5-R: CTGCAGGCCT TTCTCCAG.

2.6. Ubiquitination assays

Cell lysates were prepared in Western/IP lysis buffer (Beyotime, Haimen, China) supplemented with protease inhibitors (Roche, Mannheim, Germany). Lysates were centrifuged at maximum speed for 15 min, the supernatant was retrieved, and the amount of protein was quantitated. Similar amounts of lysate were used for IP with an anti-Myc tag antibody (CST, #2276) and protein A/G beads (Novex, Oslo, Norway), which was performed at 4 °C overnight. The beads were washed in the same lysis buffer at least 5 times before boiling for 20 min in protein loading buffer (Beyotime). The eluted proteins were detected using anti-ubiquitin (Ub) antibodies (CST, #3933) and standard WB.

2.7. Xenograft mouse model

A total of 5×10^6 Bel-7402 cells infected with Empty or WT-TRIB2-expressing lentivirus were subcutaneously injected into athymic nude mice (Bikai, Shanghai, China). Tumor size was measured every 6 days using a caliper, and the tumor volume was

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