

Microarray analysis reveals Tmub1 as a cell cycle-associated protein in rat hepatocytes

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Abstract. Transmembrane and ubiquitin-like domain containing protein 1 (Tmub1), formerly known as hepatocyte odd protein shuttling (HOPS) has been recognized as a ubiquitously expressed shuttling protein that moves between the nucleus and cytoplasm in hepatocytes. Tmub1 is involved in liver regeneration and functions as a bridging protein in tumor cell proliferation. To investigate the transcriptional profile and potential biological processes affected by Tmub1 expression in normal rat hepatocytes, microarray and bioinformatics experiments were used to identify 127 mRNAs differentially expressed between Tmub1-overexpression, Tmub1-knockdown and normal BRL-3A cells (fold-change ≥ 2.5). The expression levels of 17 key node genes associated with the cell cycle were confirmed by reverse transcription-quantitative polymerase chain reaction analysis. Flow cytometry, 5-Ethynyl-20-deoxyuridine, Cell Counting Kit-8 and western blotting experiments revealed the effects on the cell cycle and the inhibition of proliferation in BRL-3A cells overexpressing Tmub1. Further co-immunoprecipitation assays demonstrated that Tmub1 interacts with cyclin A2 during the cell cycle and

that the overexpression of Tmub1 may postpone cyclin A2 and cyclin B1 degradation in the M phase. The results of the present study indicated that Tmub1 functions as a cell proliferation inhibitor and cell cycle-associated protein.

Introduction

Recent studies in the field of liver regeneration have focused on how pattern recognition receptors and a variety of molecules are activated after partial hepatectomy (PH) (1,2). Transmembrane and ubiquitin-like domain containing protein 1 (Tmub1), also named hepatocyte odd protein shuttling (HOPS)/DULP, is a key factor regulating liver-specific biological events, such as protein synthesis during liver regeneration, by binding to elongation factor eEF-1A (3). Tmub1 contains 3 transmembrane domains, a ubiquitin-like (UBL) domain and a nuclear export signal (NLS) that are critical during hepatocyte proliferation (4). Tmub1 is upregulated in the regenerating liver (3) and is actively exported from the nucleus in dividing cells but predominantly located in the nucleus during growth arrest (5). Ubiquitylation is the major mechanism of protein degradation via the proteasome and can be regarded as a posttranslational modification (6), and some UBLs domain-containing proteins such as SUMO have demonstrated crosstalk with ubiquitylation (7). The functions of Tmub1 have been reported to be involved in a wide range of cellular processes not only in regenerating liver cells but also in other tissues. For instance, Tmub1 is involved in the IL-6-induced proliferation pathway in the liver (8), regulates locomotor activity and wakefulness by interacting with calcium modulating ligand (CAMLG) (9), and facilitates the recycling of the AMPAR subunit GluR2 to the cell surface in the mouse brain (10). Tmub1 is also an essential component of the centrosome assembly during the cell cycle (5). However, the physiological and molecular functions of Tmub1 are far from being clear at the whole-gene transcriptional profiling level. The aim of this study was to investigate the influence of Tmub1 expression on the transcriptional profile and its possible roles in the cell cycle in the rat hepatocyte cell line BRL-3A. Our data showed that Tmub1 is primarily a cell cycle-related regulatory protein in rat hepatocytes.

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Abbreviations: Tmub1, transmembrane and ubiquitin-like domain containing protein 1; Lv, lentiviral vectors; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; DAVID, database for annotation, visualization, and integrated discovery; IP, immunoprecipitation

Key words: transmembrane and ubiquitin-like domain containing protein 1, rat, microarray analysis, liver regeneration, cell cycle, cell proliferation, cyclin A2

Materials and methods

Antibodies. Specific rabbit anti-rat polyclonal antibodies to Tmub1 (no. ab180586), cyclin D1 (no. ab134175) and GAPDH

(no. ab181602) were provided by Abcam (Cambridge, MA, UK). The rabbit anti-rat polyclonal cyclin A2 antibody (no. GTX103042) was provided by GeneTex Inc. (Irvine, CA, US). Rabbit anti-rat polyclonal cyclin B1 (no. wl01760) and cyclin E1 (no. wl01072) antibodies were provided by Wanleibio Inc. (Shenyang, China). HRP-conjugated secondary goat anti-mouse (no. SA00001-1) and goat anti-rabbit (no. SA00001-2) antibodies were provided by Proteintech Inc. (Rosemont, IL, US). All the primary antibodies above were used at a 1:1,000 dilution with a secondary antibody at a 1:5,000 dilution for western blot analysis.

Cell culture and cell cycle synchronization. Normal rat hepatocyte cells (BRL-3A; Cell Bank of Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 μ g/ml streptomycin, and cultured in a humidified atmosphere of 5% CO₂ at 37°C. For G1 phase synchronization, cells were grown in FBS-free medium for 48 h. For S phase synchronization, cells were grown in the presence of 2 mM thymidine for 18 h, washed twice with PBS and released into thymidine-free media for 6–8 h, and finally grown again for 12 h in the presence of 2 mM thymidine. For M phase synchronization, cells were grown in the presence of 330 nM nocodazole for 18 h. Release from the arrest were accomplished by 2 washes with PBS followed by growth in fresh medium (11).

Construction of Tmub1 recombinant lentiviral vectors. The Tmub1 overexpression and knockdown recombinant lentiviral vectors were constructed and purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). Full length of rat Tmub1 complementary DNA was cloned into GV287 vector (GeneChem, Co., Ltd.) and the resulting vector was designated as flag-Tmub1. The knockdown lentiviral vector GV115 (GeneChem, Shanghai, Co., Ltd.) was constructed with the following shRNA sequence: GGTCTCAACACATACGAC TGA. Negative control lentiviral vectors were constructed in both the overexpression and knockdown lentiviral vectors.

Microarrays and computational analysis. BRL-3A cells were divided into five groups and cultured as follows: Tmub1 overexpression lentivirus-transduced [Lv-Tmub1(+)], Tmub1 knockdown lentivirus-transduced [Lv-Tmub1(-)], Lv-Tmub1(+)-Negative Control, Lv-Tmub1(-)-Negative Control and normal control. The cells were harvested 48 h post-infection and total RNA for microarray analysis was extracted using an extraction reagent (TRIzol; Invitrogen). Then, complementary DNA was synthesized and labeled before it was purified and hybridized to the microarray (Arraystar, Rockville, MD). The microarray scanning data were extracted using Agilent Feature Extraction software. The quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). Overlapped differentially expressed genes with at least a 2.5-fold-change in either direction were considered to be up- or downregulated. Hierarchical clustering was conducted based on differentially expressed

mRNAs using Multiple Experiment Viewer (MeV) v4.6 software. Gene Ontology (GO) (12) and pathway analyses were applied to determine the roles of these differentially expressed mRNAs in the biological GO terms or pathways. The functional analysis of the differentially expressed genes was performed using GO and the KEGG pathway database (13) with the online tool DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>) (14). The protein interaction network and nodes were determined based on the number of connections between differentially expressed mRNAs using STRING (<http://string.embl.de>) and the open source software platform Cytoscape_v3.2.1 (National Institute of General Medical Sciences, USA) (15). The microarray data were deposited in NCBI Gene Expression Omnibus and are accessible through GEO series accession number GSE97040.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using an extraction reagent (TRIzol; Invitrogen; Thermo Fisher Scientific, Inc.). The reverse transcription was performed with PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions, and PCR amplification reactions were performed with SYBR® Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Primers for qPCR are listed in Table I. The relative quantification of the mRNA levels was normalized to the rat GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels and calculated with the $\Delta\Delta$ Cq method (16).

Western blot analysis. Cells were collected and then lysed in 2X SDS (sodium dodecyl sulfate) sample buffer [100 mM Tris-HCl, pH 6.8, 10 mM EDTA (ethylene diamine tetraacetic acid), 4% SDS, and 10% glycine] and were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with 30 μ g of protein in each lane, at 70 V for 30 min and then 90 V for 90 min. Then, the proteins were transferred onto PVDF (polyvinylidene fluoride) membranes for 1.5 h at 300 mA. After blocking in 5% non-fat milk for 1 h at room temperature, the membranes were incubated with the indicated primary antibodies at 4°C overnight and horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. The GAPDH protein was used as a loading control.

Flow cytometry analysis. After 48 h of infection, cells were collected and then fixed in 75% ethanol. Then, the fixed cells were resuspended in propidium iodide/RNase/PBS buffer and incubated in the dark (37°C, 30 min). The cells were then passed through a flow cytometer (FACSCalibur; BD Biosciences, San Diego, CA, USA) equipped with a 488-nm argon laser to measure the DNA content. The data analysis was performed with the appropriate ModFit LT 2.0 software (Verity Software House, Topsham, ME, USA).

Co-immunoprecipitation. First, 10⁷ cells were collected and lysed with 500 μ l of cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl and 1% Triton X-100 with sodium pyrophosphate, β -glycerophosphate, EDTA, Na₃VO₄ and leupeptin) containing 1:50 protease inhibitor cocktail and 1:50 phosphatase inhibitor cocktail. Next, 60 μ l of SureBeads™ protein

Table I. Sequences of primers for RT-qPCR assays.

Gene	Forward primer	Reverse primer
AURKB	5'-CGGATGCATAATGAGATGGTAGAT-3'	5'-TCCCCACCATCAGTTCATAGC-3'
MCM5	5'-GTTCTGGGAACAGGGTAC-3'	5'-CATCTGGGAGCCAGAACCATC-3'
INCENP	5'-GGACTGGAATCGGAGTGGTC-3'	5'-TCTCCTCAACAACAGCACCC-3'
Ns5atp9	5'-GCAAAAAGGCATCGGGGAAT-3'	5'-TCAGGTTGCAAAGGACATGC-3'
TTK	5'-AGGCTGATAAAGAGTCACCACC-3'	5'-GCTTCTGGGGCCATGTAGTT-3'
STAT1	5'-AACGGTCCCAAAATGGAGGT-3'	5'-TGTAGGGCTCAACAGCATGG-3'
SERPINE	5'-GTGGTTCGGCACAATCCAAC-3'	5'-TGCTGAGTGAAGGCGTAGTG-3'
VEGFA	5'-TTCGTCCAATTCTGGGCTC-3'	5'-GCTTTCTGCTCCCCTTCTGT-3'
NOS2	5'-TGGTGAAAGCGGTGTTCTTTG-3'	5'-CTTATACTGTTCCATGCAGACAACCTT-3'
Pla2g2a	5'-CATGGCCTTTGGCTCAATTCAGGT-3'	5'-ACAGTCATGAGTCACACAGCACCA-3'
CCNA2	5'-GTCAACCCCGAAAAAGTGGC-3'	5'-GGGGTGATTCAAAACTACCATCC-3'
Rrm2	5'-TTTGTCCCCTTGCCATTA-3'	5'-GCAGTGACCATCAAGCAAG-3'
Sirt1	5'-GAATTCTTAACCAGCATTGGGAACCTTAGC-3'	5'-GGATCCTTGAGGAAGATAATCCAGTCA-3'
MCM3	5'-TGTCTCGGTTTGACCTGCTC-3'	5'-TCCAGTGTCCGTGCTGTAAC-3'
PLK4	5'-AGGGAAGCTAGGCACTTCATG-3'	5'-GGAAGACCACCTTTTGAC-3'
GAPDH	5'-GCCATCAACGACCCCTTCATT-3'	5'-CGCCTGCTTACCACCTTCTT-3'

G magnetic beads (no. 1614023; Bio-Rad Laboratories, Inc., Hercules, CA, USA) were incubated with 3 μ g of antibody on a rotating platform for 30 min at room temperature and then incubated with 500 μ l of (1 mg) cell lysate and rotated overnight at 4°C. The IP products were eluted by 40 μ l of 1X Laemmli buffer and incubated for 10 min at 95°C. Western blot analysis was used for the subsequent protein detection.

5-Ethynyl-20-deoxyuridine (EdU) assays. BRL-3A cells were seeded onto 24-well plates. Twenty-four h later, the BRL-3A cells were infected with either Lv-Tmub1 (+), Lv-Tmub1 (-) or NC vectors accordingly. Forty-eight h after infection, the cell proliferation was determined *in vitro* via the EdU DNA Proliferation in Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) based on the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8) assay. Cells were seeded at a concentration of 10³/ml with 5 replicates in a 96-well plate and cultured overnight. On the following day, the cell viability was measured by the CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). A volume of 10 μ l of CCK-8 solution was added to each well at 0, 24, 48, or 72 h after culture. The cells were incubated at 37°C for 2 h, and the absorbance values at 450 nm were measured using an enzyme-linked analyzer (Thermo Fisher Scientific, Inc.).

Statistical analysis. All experimental data were analyzed by GraphPad Prism 5.1 (GraphPad Software, Inc., La Jolla, CA, USA) or SPSS version 19 (IBM Corp., Armonk, NY, USA). Data were presented as the mean \pm SD of three independent experiments. Statistical analyses shown in the figures were performed using t-tests or one-way analysis of variance with least significant difference post hoc tests. All graphs were plotted by the use of GraphPad Prism 5.1 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Transcriptional profiling in Tmub1 overexpressed or knock-down BRL-3A cells. We analyzed the mRNA expression profiles of cells infected with lentivirus either overexpressing or knocking down Tmub1 and of normal control BRL-3A cells (Tmub1 expression were shown in Fig. 1D). The microarray analysis identified 836 differentially expressed genes that were either up- or downregulated, and 127 node genes were screened by STRING. The GO and KEGG pathway analysis using the DAVID database demonstrated that the top five regulated GO categories targeted by Tmub1 overexpression and knockdown were response to cellular process, biological regulation, regulation of biological process, response to stimulus, and regulation of cellular process. The most significant pathway of the differentially expressed genes was cell cycle pathway (Fig. 1C). The node gene network was screened by the number of interaction edges by Cytoscape software (Fig. 1B), and the clustering analysis showed distinct trends in the expression of node genes and key node genes among the 5 groups (Fig. 1A). Seventeen key node genes were identified, and RT-qPCR analysis confirmed the microarray data (Fig. 1E). These data demonstrated the close relation among Tmub1 and the cell cycle related genes.

Tmub1 is a negative regulator of the cell cycle and proliferation in hepatocyte cells. In order to investigate whether Tmub1 influences cell proliferation in BRL-3A cells, we conducted EdU and CCK-8 assays. The results showed that, compared to the normal control group, The cell proliferation rate of Lv-Tmub1 (-) cells was significantly higher and the cell proliferation rate of Lv-Tmub1 (+) cells was significantly lower (Fig. 2A-C). These findings demonstrated that Tmub1 has a negative impact on the BRL-3A cell proliferation.

In order to investigate how the cell cycle pathway was affected by Tmub1 expression, cell cycle analysis were

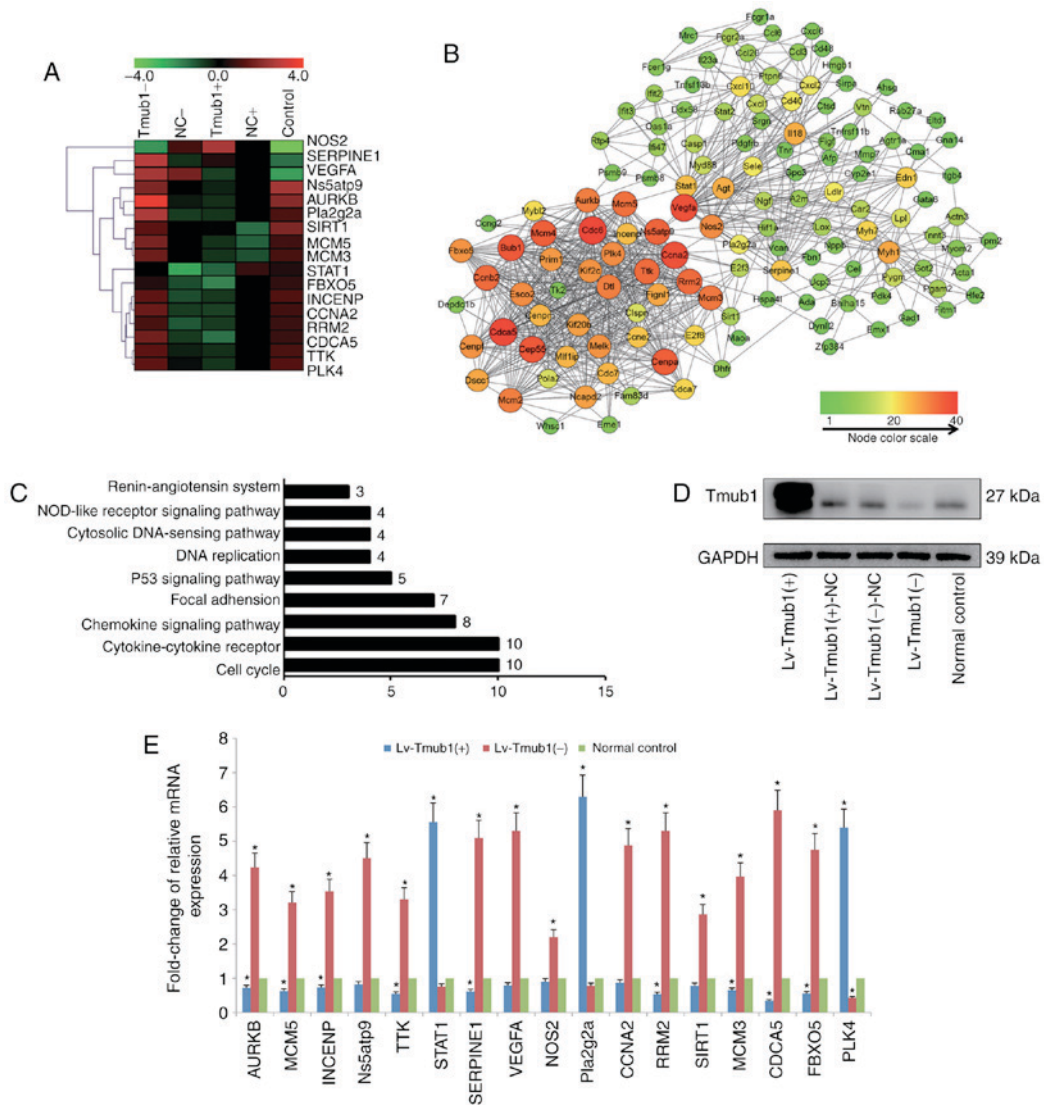


Figure 1. Differentially expressed genes after Tmub1 overexpression or knockdown. (A) Hierarchical clustering of Tmub1-, NC-, Tmub1+, NC+ and control BRL-3A cells (columns) and 17 key node genes (rows). Up-regulated genes were marked in red and down-regulated genes were marked in green. (B) Network of node genes. The differentially expressed genes after Tmub1 overexpression or knockdown were subjected to STRING (<http://string.embl.de>) to screen the node genes, network of node genes was demonstrated by software Cytoscape v3.2.1. The color brightness and shape size of nodes were determined by the number of interaction edges. (C) Counts of different genes in KEGG pathways analysis by the DAVID database. (D) Tmub1 protein expression by Western blotting assay. Cell lysates were collected 2 days after lentivirus vector infection. (E) Reverse transcription-quantitative polymerase chain reaction validation of 17 key node genes. The results were normalized to the GAPDH values for each gene, samples were normalized to the normal control. The fold-changes were shown as mean ± standard deviation in three independent experiments. Compared with control group, statistically significant differences were determined by one-way analysis of variance with least significant difference post hoc test, indicated as: *P<0.05 vs. the normal control. Tmub1, transmembrane and ubiquitin-like domain containing protein 1; NC, normal control; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization, and Integrated Discovery.

conducted. The proportion cells in S phase of the Lv-Tmub1 (-) group increased significantly compared to that of the Lv-Tmub1 (+) and normal control groups, while the proportion of cells in G1 phase decreased significantly; the proportion of Lv-Tmub1 (+) cells in G1 phase increased significantly, while the proportion of cells in S phase decreased significantly (Fig. 2D and E). These results indicated that Tmub1 may affect the G1/S phase transition.

Tmub1 interacts with cyclin A2 in the cell cycle. Cyclin A plays important roles in the G1/S and G2/M transitions. Cyclin A-CDK2 complexes are active through S phase, while cyclin A-CDC2 and cyclin B-CDC2 complexes are active

during the G2 and M phases (17). Cyclin A and cyclin B1 must be degraded in M phase for a proper G2/M transition (18). To investigate the possible relationship between Tmub1 and cell cycle cyclins in M phase, Lv-Tmub1 (+), Lv-Tmub1(-) and normal control BRL-3A cells were synchronized in M phase by nocodazole treatment (Fig. 3A, the synchronization efficiency of all cell groups were shown in Fig. 3B) and then lysed 0, 0.5, 1, 1.5 h after being released from the arrest. The expression of cyclin A2, cyclin B1, cyclin D1 and cyclin E1 was detected by Western blot analysis. In Tmub1-overexpressing BRL-3A cells, the level of cyclin A2 remained high at 1 h, while the cyclin A2 level in the negative control cells significantly dropped at 1 h. In contrast, the cyclin A2 level in the

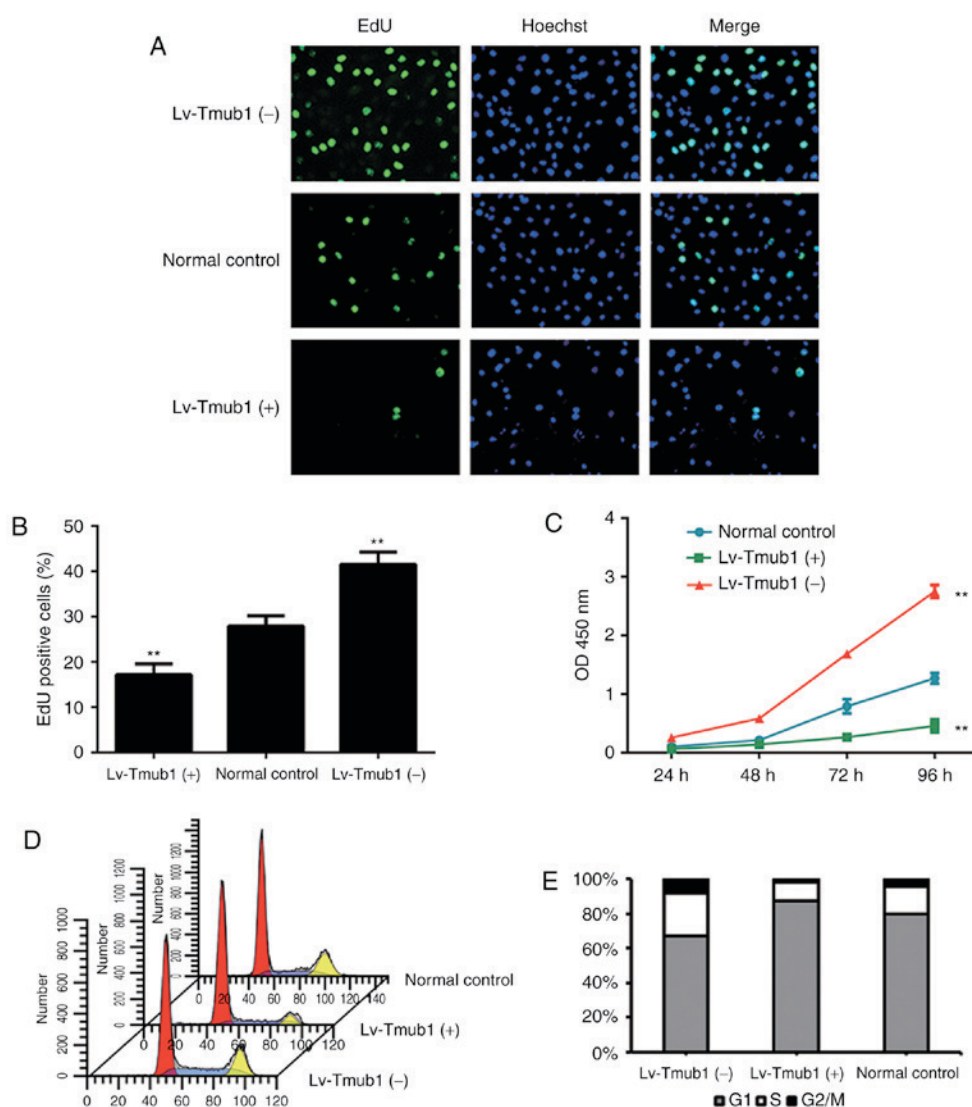


Figure 2. Tmub1 inhibits BRL-3A cell proliferation and the cell cycle progression. (A and B) Effects of Tmub1 on cell proliferation in Tmub1+, Tmub1- and normal control BRL-3A cells by EdU assay and (C) Cell Counting Kit-8 assay. Values represent the mean \pm standard deviation of three separate experiments. Compared with control group, statistically significant differences were determined by one-way analysis of variance with least significant difference post hoc test, indicated as: ** $P < 0.01$ vs. the normal control. (D and E) Detection of cell cycle in Tmub1+, Tmub1- and normal control BRL-3A cells by flow cytometry. Distribution of cells in G0/G1, S and G2/M phases were calculated. Tmub1, transmembrane and ubiquitin-like domain containing protein 1; OD, optical density; EdU, 5-Ethynyl-20-deoxyuridine.

Tmub1-knockdown BRL-3A cells dropped even earlier. The same result was observed for cyclin B1, which indicated that Tmub1 may inhibit cyclin A2 and cyclin B1 degradation in M phase (Fig. 3C, upper panel). The expression patterns of cyclin D1 and cyclin E1 showed no difference between the three groups. However, the results showed that the expression of cyclin D1 and cyclin E1 was negatively associated with that of Tmub1 (Fig. 3C, lower panel).

Next, we conducted co-immunoprecipitation assays to examine the possible interaction between Tmub1 and cyclins A2, B1, D1 and E1. Interestingly, Only cyclin A2 showed the possible interaction with Tmub1 (Fig. 4B). Further cell cycle synchronization and co-immunoprecipitation assays were conducted to explore the phases in which Tmub1 and cyclin A2 specifically interact. As shown in Fig. 4C-E, Tmub1 bound to cyclin A2 in G1, S and M phases. These results indicated that the interaction between Tmub1 and cyclin A2 may be close throughout the cell cycle, Tmub1 may interact with

Cyclin A2 for both G1/S transition and M phase progression. The results in Fig. 4A show that cyclin A2 expression was significantly lower in the Tmub1-knockdown BRL-3A cells, indicating that Tmub1 may function as a positive regulator of cyclin A2 during the cell cycle.

Discussion

Tmub1 was first described as a hepatocyte shuttling protein that is ubiquitously expressed and moves between the nucleus and cytoplasm. However, the studies on Tmub1 in the past decade since its discovery are scattered and the main biological function of Tmub1 still has not been revealed yet. Within the nucleus of resting cells, Tmub1 overexpression causes cell cycle arrest in G0/G1, and Tmub1 knockdown causes centrosome hyperamplification, leading to multinucleated cells and the formation of micronuclei (5). Recent studies have indicated that the Tmub1 gene was overexpressed in cultured primary

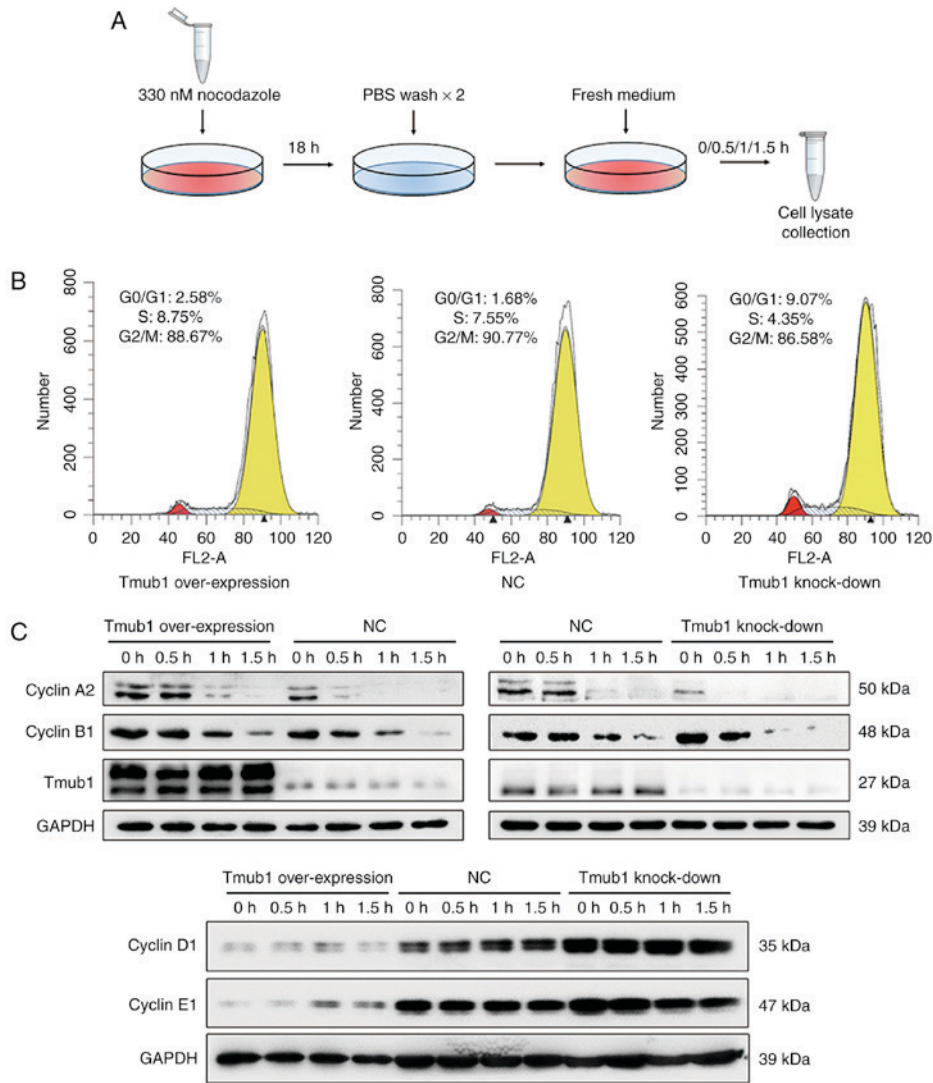


Figure 3. Tmub1 influences cyclins expression in M phase. (A) M phase synchronization method by nocodazole. Tmub1+, Tmub1- and NC BRL-3A cells were synchronized in M phase by 330 nM nocodazole treatment for 18 h, followed by replacement of fresh medium. Cell were harvest every 30 min for the following Western Blot analysis. (B) M phase synchronization efficiency by flow cytometry analysis. Tmub1+, Tmub1- and NC BRL-3A cells were collected at 0 h after the nocodazole treatment. (C) Cell cycle cyclins were detected by western blot analysis. GAPDH was used as the loading control. Tmub1, transmembrane and ubiquitin-like domain containing protein 1; NC, normal control.

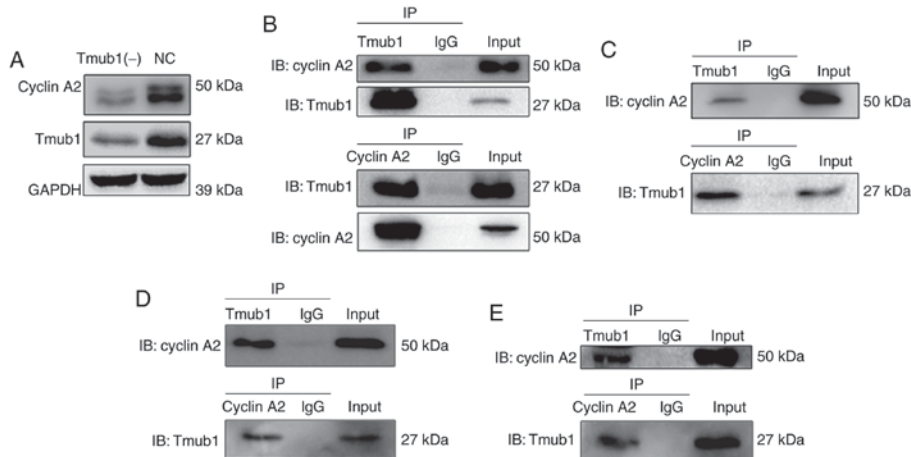


Figure 4. Tmub1 interacts with cyclin A2 in the cell cycle. (A) Tmub1- and NC-BRL-3A cell lysates were subjected to western blot analysis for cyclin A2, Tmub1 and GAPDH. (B) Co-immunoprecipitation revealed the interaction between the endogenous Tmub1 and cyclin A2 in rat normal BRL-3A cells. (C-E) Co-immunoprecipitation of Tmub1 and cyclin A2 in the cell cycle. BRL-3A cells were synchronized in (C) G1 phase, (D) S phase or (E) M phase by serum starvation, double thymidine block or nocodazole treatment, respectively. Tmub1, transmembrane and ubiquitin-like domain containing protein 1; IP, immunoprecipitation.

neurons (9). Tmub1 acts as a bridge in the NPM and p19Arf interaction, indicating the possible ability to oppose tumor cell proliferation (4). Our previous study shows that IL-6 is the upstream regulator of Tmub1, and Tmub1 knockdown synergizes with IL-6 in inducing hepatocyte proliferation (8). Moreover, C/EBP β is a key transcription factor involved in the regulation of Tmub1 expression (19). To investigate the role of Tmub1 in the cell cycle, whether Tmub1 functions as a key transcriptional regulatory molecule of the cell cycle-related genes is the first question to be answered.

In this study, stable liver cells (Tmub1 gene overexpression or knockdown) were used to study the influence of Tmub1 expression on the genome-wide transcriptional profile by mRNA microarray analysis. By comparing these gene expression profiles to that of the normal rat liver cell line BRL-3A, 836 differentially expressed genes (up- or downregulated) with 127 node genes were identified. These data demonstrated that overexpression or knockdown of Tmub1 may affect the expression of many genes with important functions in regenerating liver cells. Further pathway analysis identified pathways with important roles in the regulation of cellular proliferation and the cell cycle (20-24). Among the node genes, 17 key node genes (AURKB, MCM5, INCENP, Ns5atp9, TTK, STAT1, SERPINE1, VEGFA, NOS2, Pla2g2a, CCNA2, RRM2, SIRT1, MCM3, CDCA5, FBXO5, and PLK4) were screened and validated by RT-qPCR, and because most of these genes are related to the cell cycle (25-30), Tmub1 may be a crucial regulatory protein in the cell cycle-regulating network.

Many proteins regulating cell cycle transitions and progression through checkpoints have been studied in the past few decades (31). Among these proteins, cyclin-CDK complexes are basic regulators of cell cycle progression (32). Cyclins A and B play a central role in the control of mitosis, with cyclin A being degraded in prometaphase before cyclin B in metaphase by the ubiquitin-proteasome-system or autophagy (33). Mammalian cells have two types of cyclins As; cyclin A1 is specifically expressed in the testis, while cyclin A2 ubiquitously expressed. Accordingly, cyclin A2 is usually linked to cell proliferation and as such is often found expressed at a high level in human cancers (34). Cyclin A also mediates the progression through S phase by forming the CDK2-cyclin A complex (35). P21 binds and inactivates cyclin-CDK complexes that mediate G1/S progression, resulting in the lack of phosphorylation of Rb, E2F sequestration and cell cycle arrest at the G1/S transition (36). Our findings indicated that Tmub1 may participate in the G1/S transition and S phase progression by interacting with cyclin A2 and may delay cyclin A2 and cyclin B degradation in M phase, which strongly suggested that Tmub1 is a cell cycle-associated protein.

In addition to the canonical ubiquitylation pathway, proteins can also be modified through attachment to ubiquitin-like proteins (UBLs), which have conserved ubiquitin-like sequences and control different types of biological processes (37,38). Although UBLs are found to have diverse roles in various processes, the studies on the functions of many members in this family are only beginning. As a ubiquitin-like protein, Tmub1 was found to mediate the ubiquitylation and degradation of the HMG-CoA reductase HMGCR (39). In this process, Tmub1 bridges SPFH2 to a membrane-bound ubiquitin ligase gp78 in endoplasmic reticulum membranes.

Our results showed that Tmub1 may inhibit the degradation of cyclin A2 and B1, indicating that Tmub1 may play different roles in ubiquitylation by interacting with different proteins. Therefore, the question of how Tmub1 functions as an ubiquitin-like protein remains to be answered. The UBL domain of Tmub1 may contribute to the posttranslational modification of several cell cycle proteins, and Tmub1 may function as an 'effector' in the complicated and precise network of cell cycle regulation. In further studies, we intend to investigate how Tmub1 regulates cell cycle proteins, specifically focusing on the interaction between Tmub1 and cyclin A2, and the possible regulatory role of Tmub1 in cyclin ubiquitylation and degradation.

In conclusion, our study identified Tmub1 as a cell cycle-associated protein. Tmub1 regulates gene expression, inhibits hepatocyte proliferation and affects the cell cycle by interacting with cyclins.

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