TTC22 promotes m6A-mediated WTAP expression and colon cancer metastasis in an RPL4 binding-dependent pattern

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Article

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Abstract

WTAP, an essential component of the RNA N-6-methyladenosine (m6A) modification complex, guides METTL3-METTL14 heteroduplexes to target RNAs in the nuclear speckles of mammalian cells. Here, we show that TTC22 is widely coexpressed with WTAP and FTO in many human tissues by mining Genotype-Tissue Expression (GTEx) datasets. Our results indicate that the direct interaction of TTC22 with 60S ribosomal protein L4 (RPL4) promotes the binding of WTAP mRNA to RPL4, enhances the stability and translation efficiency of WTAP mRNA, and consequently increases the level of WTAP protein. Also, WTAP mRNA itself is an m6A target and YTHDF1 is characterized as an essential m6A binding protein in WTAP mRNA. TTC22 triggers a positive feedback loop between WTAP expression and WTAP mRNA m6A modification, leading to an increased m6A level in total RNA. The knockdown of RPL4, WTAP, or YTHDF1 expression diminishes the TTC22-induced increase in the m6A level of total RNA. Thus, TTC22 caused dramatic expression changes in genes related to metabolic pathways, ribosomal biogenesis, the RNA spliceosome, and microorganism infections. Importantly, TTC22 upregulates the expression of SNAI1 by increasing m6A level and thus promotes lung metastases of colon cancer cells in mice. In conclusion, our study showed that TTC22 upregulates WTAP and SNAI1 expression, which contributes to TTC22-induced colon cancer metastasis.

Introduction

N-6-methyladenosine (m6A) is the most abundant modification in eukaryotic RNA, accounting for more than 80% of RNA modifications [1, 2]. In mammalian cells, WT1 associated protein (WTAP) is one of three essential components (METTL3, METTL14, and WTAP) of the m6A RNA methyltransferase complex in the nucleus [3, 4]. Although m6A is generally recognized as a rapid response of cells to environmental factors (i.e., microorganisms, hypoxia, drugs, and malnutrition) [5–7], m6A-mediated alternative RNA splicing may also play crucial roles in embryonic development, cell state maintenance/transition, and cancer development [8–10]. However, it has yet to be elucidated how cells sense these environmental factors and adaptively modulate the m6A level of total RNA.

Tetratricopeptide Repeat Domain 22 (TTC22) is rarely studied in the literature. In midbody interactome analyses, TTC22 was found to interact with the KIF23 protein [11]. Clinically, TTC22 has been reported to associate with diseases including dystonia 23 and posterior polymorphous corneal dystrophy (PPCD) [12, 13]. We recently reported that TTC22v1 mRNA is targeted by miR663a, an anti-inflammatory miRNA [14]. While TTC22 expression is significantly downregulated in human colon cancer cells, the level of TTC22 expression is positively associated with the risk of colon cancer metastasis. These observations prompt us to investigate whether TTC22 drives cancer metastasis and the involved molecular pathways. Here, we found for the first time that TTC22 increases the expression of WTAP and Snail family transcriptional repressor 1 (SNAI1) expression depending on RPL4 binding and m6A modification and promotes colon cancer metastasis in vitro and in vivo.

Results
**TTC22 maintains the m6A level of total RNA in colon cancer cells**

By mining Genotype-Tissue Expression (GTEx) transcriptome datasets [15, 16], we found that the level of **TTC22** mRNA was positively or reversely correlated with those of **WTAP** and **FTO** in most human tissues, with tissue/organ specificity (Figure S1), implying the possibility that **TTC22** modulates cellular RNA m6A homeostasis. Based on our previous finding that **TTC22** mRNA is a **miR663a** target in colon cancer cells [14], we determined the m6A level of total RNA in a set of colon cancer cell lines with enforced alteration of **TTC22**. The results of m6A dot blot analysis showed that **TTC22** overexpression (**TTC22-OE**) increased the m6A level of total RNA in HCT116 and SW480 cells (Fig. 1A), whereas knockdown of **TTC22** expression by siRNA (**TTC22-KD**) or knockout of the **TTC22** gene by CRISPR/Cas9 (**TTC22-KO**; Figure S2) in LoVo and Caco-2 cells, respectively, decreased the m6A level of total RNA (Fig. 1B and 1C). Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis confirmed the average ratio of m6A to total adenosine (m6A/rA) was increased by 25% in **TTC22-OE** HCT116 cells relative to vector control cells (Fig. 1D). These results indicate that **TTC22** upregulates m6A modification of total RNA in human cells.

To identify possible pathways by which **TTC22** increases the total m6A RNA level, we performed RNA sequencing using both **TTC22-OE** and **TTC22-KD** HCT116 cells and found that many differentially expressed genes were enriched in metabolic pathways, RNA degradation, viral/bacterial infections, and other cancer-related pathways (Fig. 1E-1H). The results of m6A immunoprecipitation combined with RNA sequencing (meRIP-seq) and KEGG enrichment analysis also showed that most m6A target genes were enriched in ribosome-related biological processes and RNA transportation (Figure S3A-S3D). m6A peaks were significantly enriched in the 5'UTR, CDS, and 3'UTR (Figure S4, p < 0.005), especially in the vicinity of stop codons, in Caco-2 cells with and without **TTC22-KO/KD**. No proportion change was found in various m6A-enriched peak segments among these Caco-2 cells. The diversity of significant m6A-containing motifs, especially the typical consensus 5'-DRACH-3', was also similar (Figure S5). These findings are consistent with our above observation that **TTC22** upregulates m6A modification of total RNA.

**TTC22 increases the stability and translation efficiency of WTAP mRNA**

To gain insight into the mechanisms by which **TTC22** modulates the m6A level of total RNA, we compared the expression status of m6A methyltransferase and demethylase genes in these cells with and without enforced changes in **TTC22**. The result of Western blot analysis showed that the amount of **WTAP** is markedly decreased in **TTC22-KO** and **TTC22-KD** Caco-2 cells but increased in **TTC22-OE** SW480 cells (Fig. 2A). Immunostaining coupled with confocal microscopy soundly confirmed that the endogenous **WTAP** co-localized with endogenous **TTC22** and the level of **WTAP** and **TTC22** are highly correlated (Fig. 2B). Furthermore, stable knockdown of **WTAP** expression by an shRNA expression vector (shWTAP) greatly decreases the m6A level of total RNA in HCT116 and RKO cells and completely reverses the **TTC22**-induced increase in the m6A level of total RNA in these cells (Fig. 2C). These results demonstrated that **WTAP** is responsible for the upregulation of m6A modification of total RNA by **TTC22**.
To explore whether TTC22 regulates the transcription of the WTAP gene, we tested the level of WTAP mRNA in these cell lines. No difference in the WTAP mRNA level was detected between HCT116 cells with and without TTC22-OE or between Caco-2 cells with and without TTC22-KD (Fig. 2D, left). However, when nascent transcription of the WTAP gene was blocked by treatment with the RNA polymerase II inhibitor actinomycin D (ACTD, final concentration 5 µg/mL), the level of WTAP mRNA was significantly increased in TTC22-OE HCT116 cells and significantly decreased in TTC22-KD Caco-2 cells relative to the corresponding controls (Fig. 2D, right), suggesting that TTC22 increases the stability of existing WTAP mRNA but does not affect the transcription of the WTAP gene. To further study whether TTC22 affects WTAP protein synthesis or degradation, we blocked the nascent synthesis of the WTAP protein using the ribosomal inhibitor cycloheximide (CHX, 50 µg/mL) and found that the amount of WTAP protein was lower in TTC22-KO Caco-2 cells than in TTC22-WT cells at baseline and that the half-life of WTAP protein was decreased in TTC22-KO (1.9 hr) compared to TTC22-WT cells (3.4 hr) after CHX treatment (Fig. 2E Figure S6). In addition, we blocked proteasome activity in Caco-2 cells with MG132 (10 µM) and found that MG132 treatment did not rescue the difference in the amount of WTAP protein (Fig. 2F).

Then, we examined the widespread impact of TTC22 on mRNA translation. We separated various RNA fractions, including nontranslating ribosomes (<40S), translation initiating ribosomes (40S to <80S) and monosomes (80S) as well as actively translating polysomes (>80S) from TTC22-OE and vector control HCT116 cells by ribosome profiling. Fewer monosomes and more polysomes were induced by TTC22-OE (Fig. 2G). Notably, the ratios of WTAP mRNA in paired polysome fractions were higher in TTC22-OE cells than in control cells (Fig. 2H). Together, the above results indicate that TTC22 increases WTAP expression by enhancing the stability and translation efficiency of WTAP mRNA.

To evaluate the relationship between the levels of TTC22 and WTAP expression in human tissues, we measured the amount of TTC22 and WTAP proteins in the colon, stomach, and esophageal cancer tissue and paired normal tissue samples from eight patients (Figure S6A). As expected, TTC22 and WTAP proteins were significantly correlated in both cancer and normal tissues (R = 0.57 and 0.50; Figure S6B), suggesting that TTC22 upregulates WTAP expression in vivo. Taken together, the above results indicate that TTC22 posttranscriptionally upregulates WTAP expression in human cells.

WTAP mRNA is a methyltransferase target via YTHDF1 as the m6A binding protein

The results of mining public cross-linking and immunoprecipitation (CLIP)-seq datasets [17–19] indicated that WTAP mRNA might bind to METTL3, WTAP, ALKBH5, and YTHDF1/2/3 proteins (Figure S7). By analyzing our meRIP-seq datasets for TTC22-KD and TTC22-KO Caco-2 cells, we found m6A-enriched reads in the 3'UTRs of the last exons of two transcript isoforms of WTAP mRNAs, and the ratio of short isoforms to long isoforms was markedly increased in TTC22-KD and TTC22-KO cells relative to scrambled RNA control and TTC22-WT cells, respectively (Fig. 3A, highlighted by light blue frames). Moreover, similar results were found in the RNA-seq datasets for the input RNA controls. In quantitative meRIP-PCR analysis, the level of total m6A-modified WTAP mRNA (including the short and long isoforms) was significantly decreased in TTC22-KO Caco-2 cells and increased in TTC22-OE HCT116 cells (Fig. 3B).
These phenomena reveal that WTAP mRNA itself is an m6A methyltransferase target and that TTC22 may induce alternative splicing of WTAP pre-mRNAs and increase WTAP expression.

YTHDF1/2/3 and YTHDC1 are typical m6A RNA binding proteins [20, 21]. To identify the binding protein(s) for m6A-modified WTAP mRNA, we knocked down the expression of these m6A RNA binding protein-encoding genes in HCT116 cells using a series of siRNA sets (Table S1). We found that YTHDF1 knockdown (down approximately 87%; siYTHDF1) significantly decreased the levels of WTAP protein expression and m6A modification of total RNA and that siYTHDF1 treatment completely reversed TTC22-mediated increases in WTAP expression and m6A modification of total RNA, while knockdown of YTHDF2/3 and YTHDC1 (down 33%/35% and 54%) did not (Fig. 3C, 3D). Furthermore, WTAP mRNA was immunoprecipitated with the anti-YTHDF1 antibody (RIP-PCR), and its level was decreased in TTC22-KO Caco-2 cells compared to the control cells (Fig. 3E). Collectively, the above results indicate that YTHDF1 is an essential binding protein for m6A-modified WTAP mRNA and contributes to TTC22-mediated upregulation of WTAP expression.

**TTC22 increases WTAP expression by interacting with RPL4**

To further investigate the detailed mechanisms by which TTC22 increases WTAP expression, we used LC-MS/MS to characterize TTC22-interacting proteins in the TTC22-flag pulldown complexes isolated from HCT116 cells. RPL4 (60S ribosomal protein subunit L4), translation elongation factor EF-1α1, and hnRNA-binding proteins hnRNPH and hnRNPF were identified in the complexes (Fig. 4A). Coimmunoprecipitation (CoIP) analysis showed that only endogenous RPL4 but not EF-1α1 or hnRNPH/F interacts with TTC22 in Caco-2 cells (Fig. 4B). Thus, the roles of RPL4 in TTC22-induced enhancement of WTAP expression were further studied.

We found that the RPL4 and TTC22 proteins were localized in both the cytoplasm and nucleus albeit with higher expression in the cytoplasm in Caco-2, HCT116, LoVo, and SW480 cells (Fig. 4C). Moreover, endogenous/exogenous TTC22 (labeled with anti-TTC22 or anti-Flag antibody) and endogenous RPL4 were densely colocalized in the nucleoli and cytoplasm as particles in HCT116, SW480, and Caco-2 cells with and without TTC22-OE or TTC22-KO as indicated (Fig. 4D, S8). To verify whether TTC22 upregulates WTAP expression via TTC22-RPL4 binding, we found that RPL4 knockdown completely reversed TTC22-induced upregulation of WTAP expression in both HCT116 and LoVo cells (Fig. 4E). In addition, cytoplasmic WTAP and RPL4 proteins were increased in these TTC22-OE cells compared to the corresponding vector control cells (Fig. 4F).

Notably, WTAP mRNA enriches in the anti-RPL4 immunoprecipitated RNA (RPL4-RIP) complexes and its level is increased in TTC22-OE HCT116 and SW480 cells compared to the control cells ($p = 0.013$ and 0.014, respectively; Fig. 4G). Furthermore, endogenous WTAP mRNA was detected not only in the TTC22-RIP and RPL4-RIP complexes, but also in the double RIP analysis (RPL4-RIP based on TTC22-RIP) in Caco-2 cells, which have high levels of endogenous TTC22 and RPL4 expression (Fig. 4H), suggesting the natural formation of WTAP mRNA-TTC22/RPL4 protein complexes. In addition, m6A-modified WTAP mRNA was also detected in the TTC22-RIP complexes in the meRIP-PCR based on TTC22-RIP analysis.
(Fig. 4H), suggesting the occurrence of m6A-modified WTAP mRNA in the TTC22/RPL4 protein complexes. In RNA-FISH analysis combined with the confocal assay, we found that WTAP mRNA and GFP-labeled TTC22 protein were detected in both the nucleus and cytoplasm of HCT116 cells. The subcellular distribution pattern of WTAP mRNA was similar in these cells with and without enforced TTC22 overexpression (Fig. 4I).

Using public GTEx and The Cancer Genome Atlas (TCGA) transcriptome datasets in the Gene Expression Profiling Interactive Analysis (GEPIA) server [15, 16], we further observed a strong positive association between the mRNA levels of RPL4 and WTAP in all normal human tissues from 570 subjects and 711 patients with cancer (R = 0.50 and 0.59, p < 0.001; Fig. 4J). The above results reveal that the TTC22-RPL4 interaction upregulates WTAP expression and that RPL4 is essential for the TTC22-mediated increases in WTAP expression and the m6A level of total RNA.

Furthermore, we did not find YTHDF1 binding to TTC22 in LoVo and HCT116 cells with and without TTC22-OE in Co-IP experiments, (Fig. 4K).

**Characterization of TTC22 and RPL4 binding domains**

TTC22 consists of 7 tetratricopeptide (TPR) repeats, which are found to mediate protein-protein interactions or chaperone activity [22]. To characterize the key domains in TTC22 for RPL4 binding, we constructed nine TTC22 truncation mutants via deletion of various TPR domains and found that four TTC22 mutants (ΔTPR1, ΔTPR12, TPR3, and TPR3-4) with deletion of TPR1 (66–99 aa) could not bind to RPL4, while the TPR1-2 mutant retained RPL4 binding affinity (Fig. 5A). Deletion of the TPR1 domain markedly reversed the TTC22-induced increases in WTAP expression and the m6A level of total RNA in HCT116 and SW480 cells (Fig. 5B). These results suggest that TPR1 is responsible for the TTC22-RPL4 interaction and for TTC22-mediated upregulation of WTAP expression.

Rpl4 contains a very conserved long internal loop (44–113 aa) and a prominent C-terminal eukaryote-specific extension (264–362 aa) that are essential for binding to its specific partner Acl4, nuclear import, and pre-60S ribosomal subunit assembly [24]. We used four RPL4 truncation mutants [25] and found that the RPL4-F4 mutant (354–427 aa) could not bind to TTC22 and that the RPL4-F3 mutant could bind weakly to TTC22. However, full-length RPL4 and the RPL4-F1 and RPL4-F2 mutants (containing the loop) could bind to TTC22 (Fig. 5C). Overexpression of the RPL4-F2 mutant increased the amount of WTAP in both LoVo and HCT116 cells. In contrast, overexpression of the RPL4-F4 mutant decreased the amount of WTAP in these cells (Fig. 5D). These results suggest that the C-terminus and N-terminus of RPL4 likely have opposite effects on TTC22 binding and TTC22-mediated upregulation of WTAP expression.

Furthermore, we found that shRNA stable knockdown of RPL4 expression fully reversed TTC22-induced SNAIL1 and WTAP m6A mRNA enrichment in HCT116 cells (Fig. 5E), implying a crucial impact of RPL4 on m6A modification of these mRNAs.

TTC22 upregulates SNAIL1 expression in colon cancer cells via m6A
We previously reported that high TTC22 expression was associated with a high risk of colon cancer metastasis and a short overall survival time of patients [14]. To identify possible mechanisms by which TTC22 promotes cancer metastasis, we analyzed the aforementioned RNA sequencing datasets for TTC22-OE and TTC22-KD HCT116 cells and found that many differentially expressed genes were enriched in epidermis development, cell junction assembly, and hemidesmosome assembly in gene ontology biological process analysis (Figure S9A and S9B). The results of mining public Cancer Cell Line Encyclopedia (CCLE) cDNA microarray datasets [26] also showed a positive relationship between TTC22 and epithelial-mesenchymal transition (EMT)-related genes such as CDH1 (coexpression coefficient, R = 0.42) and ZEB1 (R=-0.39). These results imply that TTC22 may affect cancer metastasis via EMT-related pathways.

To characterize genes accounting for TTC22-induced EMT, we determined the amounts of a set of EMT-related genes by Western blot analysis and found that the amount of SNAI1 was consistently increased in TTC22-OE HCT116 and SW480 cells, whereas the amounts of other EMT genes, including ZEB1, VIM, CTNNB1, and SNAI2, were not (Figure S10). In addition, no concordant change in the level of SNAI1 mRNA was observed between TTC22-OE and TTC22-KD HCT116 cells in RNA-seq datasets or quantitative RT-PCR analysis (Figure S9C and S9D). Therefore, the effect of TTC22 on SNAI1 expression at the posttranscriptional level was further investigated.

As expected, the amount of SNAI1 protein was increased in TTC22-OE SW480 and HCT116 cells (Fig. 6A) and was decreased in TTC22-KD cells, as determined by Western blot analysis (Fig. 6B). In the rescue assay, TTC22-OE almost fully reversed TTC22-KD-induced downregulation of SNAI1 and WTAP. When new protein synthesis was blocked by CHX, no difference in the amount of SNAI1 was detected between TTC22-OE and vector control HCT116 cells (half-life of SNAI1 protein, 3.7 vs. 2.9 hr). When protein degradation was blocked by MG132, the difference in the amount of SNAI1 was still maintained (Fig. 6C and Figure S11). In the immunoprecipitation ubiquitination assay, the amount of ubiquitinated SNAI1 did not differ between TTC22-OE HCT116 cells with or without MG132 treatment (Fig. 6D). Similar to WTAP mRNA, SNAI1 mRNA could be detected in TTC22-RIP complexes (Fig. 6E). Taken together, these results indicate that TTC22 may upregulate SNAI1 expression at the translational level.

It was recently reported that SNAI1 mRNA is also an m6A target [27]. Using the m6A-specific meRIP-PCR assay, we found that the level of SNAI1 mRNA in the anti-m6A antibody-precipitated RNA was significantly lower in TTC22-KO Caco-2 cells than in TTC22-WT cells, while it was significantly higher in TTC22-OE HCT116 cells than in vector control cells (Fig. 6F). Moreover, stable knockdown of WTAP expression by shRNA (shWTAP) decreased the amount of SNAI1 in both HCT116 and SW480 cells and markedly reversed the TTC22-induced upregulation of SNAI1 expression (Figure S12). These findings support the hypothesis that TTC22 upregulates SNAI1 expression through m6A RNA modification.

**TTC22 increases the migration and lung metastasis of colon cancer cells**

We previously reported that TTC22 can increase the risk of colon cancer metastasis [14]. Here, we found that stable TTC22-OE significantly increased the migration of HCT116 cells (Fig. 7A), while TTC22-KO...
significantly decreased the migration of Caco-2 cells (Fig. 7B). Consistent with the finding that TTC22-OE significantly promoted the migration of shNC control cells, individual shRNA stable knockdown of WTAP or RPL4 expression significantly inhibited the migration of HCT116 cells in the IncuCyte wound-healing analysis (Figure S13). However, TTC22-OE did not enhance shWTAP or shRPL4 HCT116 cell migration, implying that TTC22 might promote cell migration in a WTAP or RPL4 expression-dependent way. Furthermore, stable TTC22-OE significantly increased the experimental lung metastasis of HCT116 cells injected into the tail veins of BALB/c nude mice relative to that of vector control cells (12 mice/group; Fig. 7C). These results support the hypothesis that TTC22 promotes the metastasis of human colon cancer.

Discussion

RNA modification is an additional layer in the regulation of gene expression and plays crucial roles in host cellular adaptive responses and the development of diseases, including cancer and obesity [5–10, 28]. Although the identification of RNA modifier genes and the functions of downstream m6A targets have attracted the attention of many researchers, it is not clear how environmental factors trigger cellular adaptive responses and how cells coordinate the expression of these m6A modifier genes to maintain the m6A level of total RNA. TTC22 mRNA is a miR663a target that links inflammation with colon cancer development and metastasis [1]. WTAP, a mammalian splicing factor, is an essential component in the m6A methyltransferase complex that weakly binds and guides the METLL3-METLL14 heterodimer core complex to target hnRNAs [3, 4, 19, 29, 30]. Here, we demonstrate that WTAP mRNA itself is an m6A target and that TTC22 interacts with WTAP m6A mRNA, increases the m6A level in WTAP mRNA, induces alternative splicing of m6A-modified WTAP mRNA, and enhances the translational efficiency of WTAP mRNA using YTHDF1 as a binding protein. Moreover, TTC22 increases the total mRNA m6A level, including that of SNAIL mRNA, and promotes colon cancer metastasis.

Although we did not find a difference in the level of WTAP mRNA in cells with TTC22-OE or TTC22-KD, we indeed found that the level of WTAP mRNA was significantly increased by TTC22-OE and was significantly decreased by TTC22-KD in cells in which novel RNA transcription was blocked by ACTD treatment, suggesting that TTC22 may also increase the stability of WTAP mRNA. In addition, although the proteasome-mediated protein degradation might not contribute to TTC22-triggered the upregulation of WTAP, we could not exclude that TTC22 may prevent WTAP from degradation through other pathways.

According to CLIP-seq databases for protein-RNA interactions, WTAP mRNA binds to METTL3, WTAP, ALKBH5, YTHDF1, YTHDF2, and YTHDF3 proteins in HeLa, HEK293T, and Huh7 cells [17–19, 20, 31]. Our meRIP-seq datasets further showed that the last exons of both the long and short WTAP isoforms were enriched with m6A modification. TTC22 not only increased the m6A level in WTAP mRNA but also altered its splicing pattern and increased its stability and translation efficiency, consequently leading to the upregulation of WTAP expression. Our findings are consistent with a previous report that the WTAP complex may regulate alternative splicing of WTAP pre-mRNA by promoting the production of a shorter isoform, leading to a change in WTAP protein expression [4]. This shorter WTAP mRNA isoform encoding
KIAA0105 binds fewer supplemental components of the m6A methyltransferase complex (such as KIAA1429, ZC3H13, and RBM15) than the longer isoform [4, 32]. The increased level of the shorter WTAP mRNA isoform in TTC22-KD and TTC22-KO cells was also consistent with the upregulation of WTAP expression by TTC22 in the present study. Our work and those of others demonstrate that WTAP expression is regulated by a positive feedback mechanism via increased m6A level and that TTC22 could activate this WTAP feedback mechanism by interacting with RPL4.

Our study further showed that YTHDF1 is the m6A-modified WTAP mRNA-binding protein that is essential for TTC22-induced upregulation of WTAP expression. This is consistent with the given function of YTHDF1 in inhibiting YTHDF2-mediated m6A-modified RNA degradation [21, 32] and enhancing the YTHDF3-mediated translation efficiency of m6A-modified mRNA [33]. However, we found that siRNA-knockdown of YTHDF2 or YTHDF3 expression could not abolish TTC22-induced WTAP expression. One possible reason may be that while most YTHDF1 was downregulated by siRNA, YTHDF2 and YTHDF3 expression were only partially downregulated in our experiments. In other words, the remaining amounts of YTHDF2 or YTHDF3, if needed, may be enough to support TTC22 and YTHDF1-mediated upregulation of WTAP expression.

Previous studies have reported that hypoxia regulates the expression of ALKBH5, METTL14, and YTHDF3 and consequently alters the m6A modification and expression of target transcripts [6, 34]. Moreover, recent research findings have revealed that both METTL3 knockdown and overexpression result in the upregulation of WTAP expression [35], indicating that the homeostasis of WTAP expression is regulated by METTL3 expression. mir663a is an anti-inflammatory miRNA [36, 37], and TTC22 mRNA is one of the mir663a targets [14]. In this study, we demonstrated that TTC22 may alter the splicing pattern and increase the stability and translation efficiency of WTAP mRNA by increasing its m6A level. We also showed that the percent of m6A peaks was similar between Caco-2 cells with and without TTC22-KO/KD. This is consistent with the reported m6A methylome of HeLa cells with and without EMT [27]. This finding indicates that TTC22 may trigger the positive feedback regulation of WTAP expression.

RPL4 is widely expressed in various human tissues. The PRL4 N-terminus is important for Epstein-Barr virus (EBV) to establish persistent B-lymphoblastoid cell infection related to tumorigenesis [38]. We found that the TTC22 TPR1 and RPL4 N-termini are essential for the TTC22-RPL4 interaction. Both deletion of the TTC22 TPR1 domain and knockdown of RPL4 expression abolished the TTC22-induced increase in WTAP expression. Our RNA-seq analysis showed that TTC22 functional changes led to the differential expression of genes enriched in metabolic pathways, RNA degradation, viral/bacterial infections, and pathways in cancer. According to the results of GTEx and TCGA RNA-sequencing dataset mining [15], the mRNA levels of RPL4 and WTAP are strongly positively associated with each other in normal human tissues. These findings soundly indicate that RPL4 binding is essential for TTC22-mediated upregulation of WTAP expression and increased m6A level of total RNA. The fact that WTAP and SNAI1 mRNA were found in the TTC22-RIP and PRL4-RIP complexes suggests that the TTC22-RPL4 interaction may directly promote WTAP expression through protein-mRNA binding. Interestingly, we did not observe an interaction
between TTC22 and YTHDF1 in LoVo and HCT116 cells, suggesting that TTC22-WTAP (m6A) mRNA binding and YTHDF1-WTAP m6A mRNA binding may be two sequential events.

The present study concludes that TTC22 increases m6A RNA modification in an RPL4-WTAP-YTHDF1 axis-dependent manner. The TTC22 gene may promote colon cancer metastasis by inducing positive feedback regulation of WTAP expression and subsequently elevating SNAI1 expression through m6A modification.

**Methods**

**Human tissue samples**

Esophageal squamous cell carcinoma (n = 8), gastric carcinoma (n = 8), colon carcinoma (n = 8), and paired surgical margin samples were collected from patients in Beijing Cancer Hospital. The Institutional Review Board of Peking University Cancer Hospital & Institute approved this study, which was carried out per the principles outlined in the Declaration of Helsinki. Informed consent was obtained from each patient prior to inclusion in the study.

**Cell culture and transfection**

The human colorectal cancer cell lines HCT116, SW480, and RKO were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in RPMI 1640 medium. The human colorectal cancer cell lines Caco-2 and LoVo were purchased from the National Infrastructure of Cell Line Resource (Beijing, China). The Caco-2 cell line was grown in minimal essential medium (MEM) containing 1% nonessential amino acids (Thermo Fisher Scientific, USA). The LoVo cell line was cultured in F12/K medium. All the other cell lines were grown in the same medium that contained 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). The status of mycoplasma contamination was regularly monitored for these cell lines.

**Construction of the TTC22 expression vector**

The pCMV6-Myc/Flag-TTC22 expression vector was purchased from OriGene Tech Inc. (Rockville, MD, USA). The truncated TTC22 vectors were generated using mutation-PCR and cloned into the pCMV6-Myc/Flag vector with restriction enzymes AsiSI and MluI. To generate cells with transient TTC22 overexpression (TTC22-OE), HCT116 and SW480 cells were transfected with the empty control vector using XtremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). To generate cells with stable TTC22 overexpression, HCT116 cells were transduced with the lentiviral TTC22 expression vector or empty control vector (Gene Pharma, Shanghai, China).

**Knockdown of gene expression by siRNA/shRNA**

For knockdown of gene expression, sets of three synthesized duplex RNAi oligos targeting human TTC22, WTAP, YTHDF1, YTHDF2, YTHDF3 or YTHDC1 mRNA were purchased from Gene Pharma (Shanghai,
China; Table S1). Two siRNAs for RPL4 were purchased from RuiBo Bio (stB0001897A for siRPL4#1 and stB0001897B for siRPL4#2; Guangzhou, China). The LV3(H1/GFP& Puro)-shWTAP and empty vector plasmids were also constructed by GenePharma (Shanghai, China). Lentivirus particles of pLV-hU6-shRPL4 and the negative control were from SyngenTech Co., Ltd (pHS-ASR-1219 and pHS-ASR-LW429, Beijing, China). Cells at 70–80% confluence were transfected with siRNAs using X-tremeGENETM siRNA Transfection Reagent or with plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Successful knockdown of the target gene was confirmed by Western blot and qRT-PCR analyses. Scrambled siRNA sequences were used as negative controls.

**Gene editing for TTC22 knockout**

To generate TTC22-KO Caco-2 cells, the CRISPR/Cas9 editing system was used according to a published protocol [39]. Briefly, cells transfected with CRISPR/Cas9 and the guide RNA (Table S1) were selected with puromycin (5 µg/mL) for 2 weeks. The surviving cells were picked and seeded into 96-well plates for the formation of cell clones and further expansion. The expanded monoclonal cell populations were named TTC22-KO subclones. The status of gene knockout was defined by PCR sequencing.

**RNA extraction and quantitative RT-PCR**

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The quality and concentration of RNA samples were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Qualified RNA samples were used to synthesize cDNA using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Quantitative RT-PCR (qRT-PCR) was performed using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix reagent (FastStart Universal SYBR Green Master, Roche, Mannheim, Germany).

**RNA immunoprecipitation (RIP)-based assays**

RIP was conducted with a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17–700, Millipore, USA) according to the manufacturer's instructions. Briefly, cells seeded in a 10-cm dish at 70–80% confluence were harvested by trypsinization. Subsequently, 5 µg of an anti-m6A (ab151230, Abcam, USA) or anti-YTHDF1 (ab220162, Abcam, USA) antibody or the corresponding control IgG (rabbit IgG, 17–700, Millipore, USA) was conjugated to protein A/G magnetic beads (17–700, Millipore, USA) by incubation with rotation for 30 min at room temperature; the beads were then washed three times and incubated with cell lysates prepared in RIP immunoprecipitation buffer (860 µL of RIP Wash Buffer, 35 µL of 0.5 M EDTA and 5 µL of RNase Inhibitor) at 4°C overnight. After washing with RIP Wash Buffer six times, the beads were resuspended in 150 µL of proteinase K digestion buffer containing 117 µL of RIP Wash Buffer, 15 µL of 10% SDS, and 18 µL of 10 mg/mL proteinase K and incubated with rotation for 30 min at 55°C. Input and coimmunoprecipitated RNAs were purified for further m6A sequencing by Novogene (Beijing, China) or quantitative RIP-PCR.
Double RIP assays

The protocol for the double RIP assays was essentially the same as that for the primary IP assays. Briefly, 5 µg of an anti-TTC22 antibody or corresponding control IgG (rabbit IgG, 17–700, Millipore, USA) was conjugated to protein A/G magnetic beads (17–700, Millipore, USA) by incubation with rotation for 30 min at room temperature; the beads were then washed three times and incubated with cell lysates prepared in RIP immunoprecipitation buffer (860 µL of RIP Wash Buffer, 35 µL of 0.5 M EDTA and 5 µL of RNase inhibitor) at 4°C overnight. After washing with RIP Wash Buffer 6 times, 100 µL of elution buffer (0.1 M glycine, pH 3.5) was added to each sample and control resin. The samples and controls were incubated with gentle shaking for 5 min at room temperature. The resin was centrifuged for 30 sec at 5,000–8,200 x g. The supernatants were transferred to new test tubes containing 10 µl of 10 x Wash Buffer using a Hamilton syringe or equivalent device. Bead eluates from the first immunoprecipitation were then subjected to IP with an anti-RPL4 antibody.

m6A dot blot analysis

m6A dot blot analysis was performed following a previously reported protocol with a slight modification [40]. Briefly, the indicated amount of total cellular RNA was denatured in a 3-fold volume of RNA incubation buffer (46.2% formaldehyde and 53.8% 20 · SSC) at 95°C for 5 min and was then chilled on ice. Next, RNA samples (50–200 ng) were spotted directly onto a positively charged nylon membrane (RPN303B, GE Healthcare, USA) and air-dried for 5 min. The membrane was then UV cross-linked in an ultraviolet crosslinker, blocked with 1% Blocking Reagent (11096176001, Roche) in maleic acid (pH 7.5), and incubated with an anti-m6A antibody (ab151230, Abcam) overnight at 4°C. An HRP-conjugated anti-rabbit IgG secondary antibody was added to the membrane for 1 hr at room temperature with gentle shaking, and the membrane was then developed with enhanced chemiluminescence. Methylene blue staining was used to verify that equal amounts of RNA were spotted on the membrane.

LC-MS/MS measurement of the m6A/rA ratio

LC-MS/MS quantification of m6A was performed by Cloudseq Biotech Inc. (Shanghai, China) following the recommended protocol. Total RNA from stable TTC22-OE and vector control HCT116 cells was isolated using TRizol reagent (Invitrogen, MA, USA). One microgram of total RNA was digested with 4 µL of nuclease P1 (Sigma, St. Louis, MO) in 40 µL of buffer solution (10 mM Tris-HCl [pH 7.0], 100 mM NaCl, 2.5 mM ZnCl2) at 37°C for 12 hrs, and was then incubated with 1 µL of alkaline phosphatase (Sigma-Aldrich, Darmstadt, Germany) at 37°C for 2 hrs. The RNA suspension was divided into 100 µL aliquots and injected into the LC-MS/MS system. Nucleosides were separated by reversed-phase high-performance liquid chromatography on an Agilent C18 column (Agilent Technologies, San Diego, CA) and analyzed by mass spectrometry using an AB SCIEX QTRAP 5500 (AB Sciex LLC, Framingham, MA). Pure nucleosides were used to generate standard curves, from which the concentrations of adenosine (A) and m6A in the sample were calculated. The content of m6A was then calculated as a percentage of the total adenosine content in RNA (rA) [19].

Western blot analysis
For Western blot analysis, cells at approximately 80% confluence were lysed in 1x loading buffer (Sigma). To prepare cytoplasmic and nuclear extracts, cells were homogenized and separated with a Nuclear/Cytosol Fractionation Kit (Thermo Scientific Pierce, USA). The purities of the cytoplasmic and nuclear extracts were verified by immunoblotting with anti-β-Tubulin and anti-Lamin B antibodies, respectively. For the immunoprecipitation (IP) assay, cells were homogenized in lysis buffer (REF04693159001, Roche, Mannheim, Germany) for 20 min at 4°C. The supernatant was collected after centrifugation at 12,000 x g for 20 min at 4°C and was then incubated with the indicated antibodies conjugated to protein A or G-Sepharose (Roche, Mannheim, Germany). Cell lysates or immunoprecipitates were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Nonspecific binding was blocked with 5% nonfat milk in PBS overnight at 4°C, and the membranes were rinsed twice with PBST. Then, the membranes were incubated with the indicated primary antibodies at room temperature for 1.5 hr, washed six times with PBST, incubated with horseradish peroxidase-conjugated secondary antibodies for 45 min, and washed again as described above. Protein bands were visualized with an enhanced chemiluminescence system (Thermo Scientific, Rockford, IL).

The primary antibody specific for TTC22 was a polyclonal antibody produced in rabbits challenged with his-TTC22 protein purified from Escherichia coli (YouKe Biotech, Shanghai, China). Other indicated primary antibodies were as follows: anti-METTL3, anti-METTL14, anti-WTAP (#96391, #51104, and #56501; Cell Signaling Technology, USA); anti-FTO, anti-m6A, anti-YTHDF1, anti-YTHDF2, anti-YTHDF3, anti-YTHDC1, anti-hnRNPH (#ab126605, #ab151230, #ab220162, #ab220163, #ab220161, #ab122340, and #ab10374; Abcam, UK); anti-hnRNPF, anti-RPL4, anti-EF-1α1 (#sc-32309, #sc-100838, and #sc-21758; Santa Cruz, USA); and anti-GAPDH (#60004-1, Protein Tech, China).

RNA sequencing

For whole-genome transcriptome profiling, four libraries were generated from total RNA samples extracted from Flag-TTC22-OE, empty vector control, scramble RNA control, and TTC22-KD (siTTC#1&2&3) HCT116 cells using a TruSeq® RNA Sample Preparation Kit (Illumina Inc.) according to the manufacturer’s protocol and were then subjected to Illumina HiSeq PE150 sequencing (Genminix, Shanghai, China) using the 150-base pair single-end sequencing module. Hisat2 (version:2.0.4) was used to map the cleaned reads to the human hg38 reference genome. The mRNA data obtained from transcriptome sequencing were subjected to statistical analysis with a t-test and corrected with the RVM model. Significantly differentially expressed mRNAs (TwoClassDif) between TTC22-OE and vector control cells or between TTC22-KD and scramble RNA control cells were thus identified. Upregulated and downregulated genes (fold change > 1.5, calculated with the Ballgown algorithm) were analyzed to identify significant functions and significant signaling pathways based on the Gene Ontology (GO) database (GO-Analysis) or KEGG database. The differentially expressed genes are listed in Tables S2 and S3. Raw data were deposited to the Gene Expression Omnibus under accession number GSE189982.

meRIP sequencing
meRIP sequencing was carried out by Novogene (Beijing, China) according to the standard procedure [41] with minor changes. Briefly, total RNA was extracted from cells, and mRNAs were isolated from the total RNA using a Dynabeads mRNA DIRECT Purification Kit (61011, Thermo Fisher, USA). Fragmented mRNAs (~100 nt) were incubated with an anti-m6A antibody for immunoprecipitation. Purified mRNA fragments were used for meRIP sequencing library generation and were sequenced with an Illumina HiSeq 4000. The differentially expressed genes are listed in Tables S4 and S5. Raw data were submitted in Gene Expression Omnibus under accession number GSE188521.

**meRIP on RIP double assay**

The RNA samples were extracted from anti-TTC22 antibody immunoprecipitated protein-RNA complexes and reimmunoprecipitated with anti-m6A antibody as described above.

**Polysome profiling**

Polysome profiling was performed as previously reported with a slight modification [42]. Briefly, TTC22-OE and vector control HCT116 cells (four 150-mm culture dishes) were treated with 100 µg/mL CHX at 37°C for 10 min. Cells were then lysed with 0.5 mL hypotonic buffer (5 mM Tris–HCl, pH 7.5, 2.5 mM MgCl2, 1.5 mM KCl and 1x protease inhibitor cocktail) supplemented with 100 µg/ml CHX (Cell Signaling Technology 2112), 1 mM DTT, and 100 units of RNase inhibitor (Promega, N2111) and incubated on ice for 10 min with vortexing briefly every 2–3 min. Samples were centrifuged at 16,000 g for 8 min at 4°C. Supernatants (cytosolic cell extracts) were collected, and the absorbance at OD 260 nm was measured. The same amount of lysate (approximately 10–30 OD 260 nm) was loaded onto 10% – 50% cold sucrose gradients in buffer (20 mM HEPES-KOH, pH 7.4, 5 mM MgCl2, 100 mM KCl, 100 µg/ml CHX, 10 units/ml RNase inhibitor and 1x protease inhibitor cocktail) and centrifuged at 38,000 rpm in a Beckman SW41Ti rotor for 3 hrs at 4°C. After centrifugation, 15 equal-sized fractions (0.74 mL/fraction) were collected and analyzed using a UV detector (BIO-RAD, ECONO UV MONITOR) and a piston gradient fractionator (BIOCOMP, PGF). For RT-qPCR, fractions were mixed with TRIzol reagent.

**RNA-FISH assay**

Fluorescence-conjugated WTAP mRNA probes were generated according to protocols from RiboBio Co., Ltd (Guangzhou, China). Cells transiently transfected with GFP-TTC22 expression or empty control vector were pretreated with 4% paraformaldehyde, hybridized with RNA probe sets labeled with Cy3, and then stained with DAPI using the RiboTM Fluorescent In Situ Hybridization kit (C10910, Guangzhou RiboBio Co., Ltd., China) according to the manufacturer’s instructions. Images were obtained with a confocal microscope (Olympus, Japan). In addition, 18S rRNA and U6 RNA were used as cytoplasmic and nuclear RNA controls, respectively [43].

**Cell migration assays**

Wound healing and cell migration assays were used to evaluate cell motility. In the wound healing assay, HCT116 and Caco2 cells were seeded into 96-well plates at a density of 10,000 cells/well. When the cells were 90% confluent, a scratch was gently created through the cell monolayer with a scarificator, and loose
cells were removed by washing. Cells were cultured under standard conditions and photographed every 6 hrs in the long-term dynamic observation platform (IncuCyte, Essen, MI, USA). Both the relative wound density and wound width were analyzed using IncuCyte ZOOM software (Essen, Ann Arbor, MI, USA).

**Experimental lung metastasis assay**

Four-week-old female BALB/c nude mice were purchased from HFK Biotechnology (Beijing, China). An experimental lung cancer metastasis model was generated as we recently described [14]. Briefly, $1.0 \times 10^6$ HCT116 cells stably transfected with TTC22 or control vector were injected into the tail veins of randomly selected BALB/c nude mice (12 mice/group). Eight weeks after injection, the experiment was terminated, and the lungs were harvested and fixed in Bouin’s solution and 4% paraformaldehyde for hematoxylin and eosin (HE) staining. Metastatic nodules on the lung surfaces were counted. This study was approved by the institute’s animal ethics committee.

**Database sources**

Gene expression cDNA array datasets for 1037 cancer cell lines were downloaded from the publicly available database [https://portals.broadinstitute.org/ccle/home] of the Broad-Novartis Cancer Cell Line Encyclopedia [26].

**Statistical analysis**

Statistical analysis was carried out using SPSS 22.0 software (SPSS Inc., Chicago IL, USA). Data are presented as the mean ± SD of at least three independent experiments or as the median (25–75 percentiles). Statistical analysis methods included Student’s t-test, one-way analysis of variance (ANOVA), and two-way ANOVA. All tests are two-sided. A p-value < 0.05 was considered statistically significant.

**Data sharing plans**

The original contributions represented in the study are included in the article/supplemental tables S1-S5.

**Abbreviations**

CCLE, Cancer Cell Line Encyclopedia; CoIP, coimmunoprecipitation; CLIP, cross-linking and immunoprecipitation; GTEx, Genotype-Tissue Expression; m6A, N-6-methyladenosine; EMT, epithelial-mesenchymal transition; RIP, RNA immunoprecipitation; RPL4, 60S ribosomal protein L4; siRPL4, siRNA-knockdown of RPL4 expression; siYTHDF1, siRNA-knockdown of YTHDF1 expression; TCGA, The Cancer Genome Atlas; TTC22-KD, knockdown of TTC22 expression by siRNA; TTC22-KO, knockout of the TTC22 gene by CRISPR/Cas9; TTC22-OE, TTC22 overexpression

**Declarations**
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Author contributions

DJD was responsible for designing the study, analyzing various RNA-seq datasets, interpreting the results, obtaining the main financial support, and writing the manuscript. WT was responsible for performing part of the experiments, interpreting the results, obtaining partial financial support, and writing the manuscript. ABY was responsible for performing most experiments and interpreting the results. HFY and LKG contributed to the animal experiments. JZ analyzed the expression levels of the TTC22 and WTAP genes in human tissue samples.

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Figures

Figure 1
Effects of TTC22 on the total RNA m6A level in colon cancer cell lines. (A-C) The total RNA m6A level in colon cell lines with different TTC22 expression changes, as determined by m6A-specific dot blot analysis; (D) The ratio of total m6A to total adenosine in RNA (m6A/rA) in two independent biological replicates of HCT116 colon cancer cells with and without TTC22-OE, as determined by LC-MS/MS analysis. (E and F) Top 10 pathways of genes downregulated and upregulated by TTC22-OE in HCT116 cells in RNA-seq analysis; (G and H) Top 10 pathways of genes upregulated and downregulated by TTC22-KD in HCT116 cells in RNA-seq analysis.
Figure 2

Effects of WTAP on TTC22-induced total m6A RNA modification. (A) Effects of TTC22 expression changes on various m6A modifiers, including WTAP, in Caco-2, SW480, and RKO cells. (B) Confocal images of endogenous WTAP protein in Caco-2 cells with endogenous TTC22 expression. (C) TTC22-induced enhancement of m6A RNA modification was abolished by stable knockdown of WTAP expression by shRNA (shWTAP) in HCT116 and RKO cells. (D) Effects of blockade of RNA synthesis by actinomycin D (ACTD, final concentration 5 μg/mL, 18 hrs) on TTC22-induced WTAP expression changes in HCT116 and Caco-2 cells, as determined by quantitative RT-PCR analysis; (E and F) Effects of blockade of protein synthesis by CHX and of protein degradation by MG132 on TTC22-KO induced WTAP expression changes in Caco-2 cells, as determined by Western blot analysis. The equations of trend lines was used to calculate the half-life of WTAP protein: 3.4 and 1.9 hr for TTC22-WT and TTC22-KO cells. (G and H) Effect of TTC22-OE on the levels of total RNA and the WTAP mRNA polysome in HCT116 cells, as determined by polysome profiling analysis.
Figure 3

Effects of changes in *TTC22* and *YTHDF1* expression on the levels of m6A-modified *WTAP* mRNA and WTAP protein. (A) Graphical presentation of the last exon in the long and short isoforms of *WTAP* mRNA in TTC22-KO and TTC22-KD Caco-2 cells, as determined by meRIP-seq; (B) The levels of m6A-modified *WTAP* mRNA in Caco-2 cells with and without TTC22-KO and in HCT116 cells with and without TTC22-OE, as determined by quantitative RNA m6A immunoprecipitation combined with PCR (meRIP-PCR).
(C and D) Effects of siRNA-mediated transient knockdown of YTHDF1/2/3 and YTHDC1 expression for 72 hrs on TTC22-induced enhancement of WTAP expression (by Western blot analysis) and the m6A level of total RNA (by m6A dot blot analysis) in HCT116 cells. (E) The levels of WTAP mRNA enriched by the YTHDF1 protein in Caco-2 cells with and without TTC22-KO, as determined by quantitative RT-PCR analysis of RNA immunoprecipitated by anti-YTHDF1 antibody (RIP-PCR). *p<0.05; **p<0.01
Figure 4

Characterization of the TTC22-RPL4 interaction and its effect on WTAP expression in various cell lines. (A) Identification of TTC22-specific binding proteins in the LC-MS/MS proteomic analysis of the Flag-TTC22 purified protein complex; (B) Detection of the interaction of endogenous TTC22 with the RPL4 protein in Caco-2 cells in CoIP analysis; (C) Co-localization of endogenous TTC22 with the RPL4 protein in the cytoplasm (cyto) and nucleus (nucl) of various cell lines, as determined by Western blot analysis; (D) Merged confocal microscopy images of TTC22 and RPL4 proteins in various cell lines with and without TTC22-OE or TTC22-KO; (E) Effect of knockdown of PRL4 expression by siRPL4#1 or siRPL4#2 on TTC22-induced enhancement of WTAP expression; (F) Subcellular localization of endogenous RPL4 and WTAP in HCT116 and SW480 cells with or without TTC22-OE; (G) Detection of RPL4-bound WTAP mRNA through RPL4-RIP in HCT116 and SW480 cells with or without TTC22-OE; (H) Detection of WTAP mRNA in TTC22- and or RPL4-/m6A immunoprecipitated RNA (TTC22-RIP, RPL4-RIP, m6A-meRIP, TTC22-RPL4 and TTC22-m6A double RIP) by PCR; (I) Subcellular location of WTAP mRNA and GFP-TTC22 protein in HCT116 cells with and without TTC22-OE in RNA-FISH analysis; (J) The status of WTAP and RPL4 coexpression in RNA-seq datasets of normal human tissues from the GTEx and TCGA projects (these charts were adapted from images downloaded from the website [http://gepia.cancer-pku.cn] [15,16]). (K) Detection of YTHDF1 binding to endogenous TTC22 in LoVo cells and binding to Flag-TTC22 in HCT116 in Co-IP analysis.
Figure 5

Comparisons of the TTC22-RPL4 binding potential of various mutants of the TTC22 and RPL4 proteins. (A) The results of CoIP analysis of Flag-labeled TTC22 mutants with endogenous RPL4 in HCT116 cells; the various TTC22 truncations are shown graphically; (B) Influence of deletion of TTC22 TPR1 on WTAP expression, as determined by Western blot and m6A dot blot analyses; (C) The results of CoIP of Flag-labeled RPL4 mutants with endogenous TTC22 in LoVo cells; the various RPL4 truncations are shown...
graphically; (D) Influence of deletion of various RPL4 TPR1 fragments on WTAP expression in LoVo and HCT116 cells, as determined by Western blot analysis; (E) Influence of shRNA stable knockdown of RPL4 expression on TTC22-OE enhanced m6A modification of WTAP and SNAI1 mRNA in HCT116 cells, as determined by meRIP-qPCR analysis. AlphaFold-predicted 3D structures of the TTC22/Q5TAA0 and RPL4/P36578 proteins were adapted from images downloaded from the website (https://alphafold.ebi.ac.uk); pLDDT, and AlphaFold calculated a per-residue confidence score between 0 and 100 [24].

Figure 6
Figure 6

Effects of TTC22 on the level of SNAI1 expression in colon cancer cells. (A) SNAI1 expression changes in TTC22-OE colon cancer cell lines, as determined by Western blot analysis; (B) SNAI1 expression changes in TTC22-KD cell lines. The results of the rescue experiment are also presented on the right side. (C) SNAI1 expression changes in TTC22-OE cells cotreated with the ribosomal inhibitor CHX (final concentration, 50 mg/mL; top) or proteasome inhibitor MG132 (10 μM; middle), as determined by Western blot analysis; the average GAPDH-normalized band density (ratio) in the SNAI1 protein lane is also shown in the right charts. (D) The results of anti-Ubiquitin antibody immunoprecipitation analysis of TTC22-OE HCT116 cells with and without MG132 treatment. (E) The results of TTC22-RIP analysis. (F) The results of meRIP-PCR analysis of the enrichment of m6A-modified SNAI1 mRNA in Caco-2 cells with and without TTC22-KO (right) and HCT116 cells with and without TTC22-OE (left).
Figure 7

Effects of stable changes in *TTC22* expression on the migration and lung metastasis of colon cancer cells. (A) Results of the wound-healing migration assay of *TTC22*-overexpressing HCT116 cells using long-term monitoring with an IncuCyte ZOOM live-cell imaging system; the amount of TTC22 protein was also measured by Western blot analysis. (B) Results of the wound-healing migration assay of *TTC22*-knockout (KO) Caco-2 cells. (C) Results of experimental lung metastasis of *TTC22*-overexpressing...
HCT116 cells (1´10^6 cells per mouse) injected into the tail veins of BALB/c mice (12 mice/group). The number of lung nodules and lung weight are presented as the median (25-75 percentiles).

**Supplementary Files**

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- SupplementalfiguresR3.pdf
- TableS1Sequencesofoligonucleotidesandprimers.doc