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Original Article

tRF-20-M0NK5Y93-induced MALAT1 promotes colon cancer metastasis through alternative splicing of SMC1A

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Abstract: Recent studies have indicated that changes in the tumor microenvironment, such as hypoxia, result in the discrepant expression of noncoding small RNA tRNA-derived fragments (tRFs), affecting the phenotype of tumor metastasis. The biological function of tRFs in tumors has attracted increasing attention, but the mechanism by which tRFs mediate tumor metastasis has not been clarified. The direct regulatory relationship between tRFs and lncRNAs and the mechanism by which noncoding RNAs regulate alternative splicing are still unknown. In this study, the mechanism of tRF-mediated SMC1A alternative splicing and regulation of colon cancer metastasis was studied from multiple dimensions of cell, molecule, animal and clinical. Our present studies revealed that tRF-20-M0NK5Y93 inhibits colon cancer metastasis and that there is a significant correlation between the expression of tRFs, metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), and SRSF2 through complete transcriptional sequencing and bioinformatics. Mechanistic investigations indicated that tRFs could regulate the expression of MALAT-1 by binding to specific sites on MALAT-1. MALAT1, which is a long noncoding RNA (lncRNA), regulates alternative splicing of (structural maintenance of chromosomes 1A) SMC1A by interaction with SRSF2, resulting in discrepant expression of various isoforms, SMC1A001, SMC1A201, SMC1A005, and SMC1A003. Our findings revealed the interaction between different types of noncoding RNAs on alternative splicing, which is expected to be a novel potential therapeutic target.

Keywords: ncRNA, tRFs, MALAT1, SRSF2, SMC1A

Introduction

Tumor metastasis is a series of complex sequential processes involving multifactor involvement, multistep development and multigene regulation [1]. The cohesin protein complex is the key factor in chromosome mitosis and maintenance of genome stability [2] and consists of four proteins, namely, SMC1A, SMC3, and RAD21/SCC1stag/SCC3/SA. SMC1A, SMC3, and SA3 mutations in somatic cells have been shown to be characteristic of chromosomal instability (CIN) in colon cancer, which occurs in the early stages of tumor metastasis. In our previous study, we found that the SMC1A gene was highly expressed at the early stage of the formation of colon cancer micrometastases [3]. SMC1A promotes the growth of colon cancer cells by activating the Akt and ERK1/2 signaling pathways, upregulating CDK4 and PCNA expression, and inhibiting apoptosis by inhibiting PRAS40 signaling and PARP degradation [4].

The SMC1A gene is located on chromosome X, and six transcripts are produced by selective splicing: only SMC1A-001, SMC1A-201, SMC1A-003 and SMC1A-005 can be translated into protein products. SMC1A-004 has no open reading frame, and SMC1A-002 produces

nonsense-mediated degradation (https://www.ncbi.nlm.nih.gov/gene). By analyzing the expression relationships of different transcripts of the SMC1A gene, our previous study found that 003 had a ceRNA regulatory effect on the expression of 001 and 201, and endogenous competitive regulation among transcripts played a role through miR-let-7b, miR-4687 and miR-3653. This suggests a fine regulatory mechanism of selective splicing at the transcriptional level.

Alternative splicing is a process in which the precursor mRNA formed by genes through transcription is reconnected to exons through shear under the regulation of various transcription and posttranscription factors [5]. Disordered shearing regulation can affect the metabolism, apoptosis, angiogenesis, invasion and metastasis of tumor cells. The splicing process is finely regulated by the interaction of various cis-acting sequences and trans-acting factors, including the involvement of multiple splicing factors of SR and hnRNP family proteins. Tripathi et al. found that MALAT-1 can regulate selective variable shearing by affecting the number and phosphorylation level of SR proteins (SRSF) at the cellular level [6].

Small RNA fragments from tRNAs are a type of sncRNA derived from tRNAs or the precursors of tRNAs [7, 8]. Under stress such as hypoxia, mature tRNA or precursor tRNA can be specifically cleaved to produce tRNA-derived fragments (tRF) or tRNA half molecules (tiRNA) [9]. Differential expression of tRFs and tiRNAs has been found in a variety of tumor cell lines that are involved in cell proliferation, apoptosis and invasive metastasis including lymphocytic leukemia, colorectal cancer, lung cancer, and ovarian cancer [10-12]. Roughly, few studies have shown that abnormally expressed tRFs in tumors can be used as promising diagnostic markers or therapeutic targets. However, whether tRFs can directly bind to lncRNAs and further regulate the function of lncRNA protein chaperones in tRF-mediated selective shear regulation of tumor metastasis is largely unknown. tRFs are produced by precise biogenetic processes, with their specific nucleotide composition, physiological function, and biogenesis. Given that MALAT-1 forms a three-helical long transcript and a tRNA cloverleaf-like small transcript, whether tRNA and its derivative tRFs are related to MALAT-1 gene expression and the regulatory relationship between tRFs and MALAT-1 remain to be further explained.

In this study, we aimed to elucidate the mechanism by which tRFs mediate alternative splicing to regulate colon cancer metastasis. Our hypothesis is that tRF-20-M0NK5Y93 can directly bind to the long noncoding RNA MALAT1 and regulate colon cancer metastasis through SMC1A selective splicing involving the variable splicing factor SRSF2.

Materials and methods

Tissue samples

Four pairs of human colon cancer tissue samples and liver metastasis tissue samples were collected in the Department of Colorectal Surgery at the Zhejiang University Second Affiliated Hospital between 2019 and 2021. All patients were pathologically confirmed to have colon cancer with liver metastasis, and no patients received prior treatment including chemotherapy for their colon condition before surgery. All patients provided written informed consent. This study was approved by the Ethics Committee of the Zhejiang University Second Affiliated Hospital.

RNA sequencing

Total RNA was extracted from colon cancer cells using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA integrity was analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA concentrations were measured using the Qubit RNA assay kit and Qubit fluorescence meter (Invitrogen, Carlsbad, USA). The samples used in subsequent experiments met the following requirements: the RNA integrity number (RIN) of tRF-20-M0NK5Y93 was 2.0; the ratio of 28S to 18S was 1.5. The specific sequencing method is described in detail [9].

Cell culture

RKO and HCT116 cells were treated with 10% FBS (fetal bovine serum, Gibco, Life Technologies, USA), 0.1 mg/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, USA) in modified Eagle's medium (MEM) and Dulbecco's

modified Eagle's medium (DMEM) (Gibco, Life Technologies, USA). All cells were cultured in either normal oxygen in a CO_2 constant temperature incubator (37°C, 21% O_2, 5% CO_2) (Thermo Fisher Scientific, Rockford, IL, USA) or hypoxia in a hypoxia incubator (37°C, 1% O_2, 5% CO_2) for 24 h.

Cell transfection

RKO and HCT116 cells were used for the assay. The 6-well plate cells were transfected with plasmids, and the cells were transfected when the cell coverage rate reached 70-80%. Then 2 uL Lipofectamine 2000 (Invitrogen) was added to 50 uL Opti-MEM, gently whisked and mixed, stood for 5 min, and then 6 ug plasmid or siRNA was added to 50 uL Opti-MEM. The mixture was blown gently, mixed well, and then stood for 5 min. Then the two tubes of preparation liquid were mixed and stood for 20 min. The cells were removed and the culture medium was discarded, and then 3 mL new culture medium was replaced. The mixture was added to the cells drop by drop, gently mixed, placed in an incubator, and transfected for 24 h or 48 h for subsequent experiments.

By transfecting Dharmacon SMART Pool siRNA (Table S1) from the RNAiMax transfection reagent (Invitrogen) mixture into RKO and HCT-116 cells, si-MALAT1 was knocked down with a small interfering RNA (siRNA). SiGLO was used as a nontargeted siRNA control, and transfection efficiency was measured after 48 h.

Sequences for the tRF-20-M0NK5Y93 inhibitor (5'-ACCCACAAUCCCCAGCUCCG-3') and negative control (NC inhibitor, 5'-UUCCUCCGAACGAGUCACGUTT-3') were purchased from GenePharma (Shanghai, China).

Protein extract preparation and western blotting

An appropriate amount of tissue was cut from the ice in the EP tube, and the tissue was ground with a tissue grinder after adding an appropriate amount of protein lysate.

The reaction was placed on ice for 10 min and then centrifuged at 4°C 14000 rpm for 7 min. The supernatant was placed in another EP tube and centrifuged again under the same conditions as above. The supernatant was placed in another EP tube, and the volume of the supernatant was recorded. The subsequent experimental steps were consistent with the cell sample extraction method. The colon cancer cells were collected and lysed with RIPA buffer. The cells containing lysate were placed on a shaker platform at 4°C, violently shaken for 30 s, placed on ice for 4 min, and the process was repeated 5 times. Centrifuge 12000 r at 4°C for 5 min at 1000 r/min. Then, a BCA kit was used to determine the protein concentration of the lysate. After loading buffer was added, the loading buffer was heated at 90°C for 10 min to denature the protein. Equal amounts of total protein samples were added to a 12% polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes using a membrane transfer apparatus and then incubated in 5% skim milk at room temperature for 2 h. The PVDF membrane was incubated with the specific primary antibody at 4°C overnight. The primary antibodies included rabbit anti-SMC1A001 (CST-4802S), anti-SRSF2 (Proteintech-20371-1-AP), anti-Flag (Sigma-F1804), and β-actin (Sigma-A5441). The corresponding secondary antibody was incubated at room temperature for 1 h. The blots were exposed to X-ray film (Thermo Scientific) and processed in an X-Omat processor or imaged using an Odyssey LiCOR CLx infrared imaging system.

Quantitative real-time PCR (qRT-PCR)

Tissue extraction requires pretreatment. We removed the tumor tissues of the four groups of mice previously stored in a -80°C freezer. Under sterile conditions, a small part of the tissue was cut and placed into 2-mL Eppendorf tubes, and 500 μL of TRIzol solution was added. The tissue was ground with a tissue grinder, and the subsequent experiments were continued (note: pause every 15 s to prevent tube damage). The subsequent experimental steps were consistent with the cell sample extraction method. The cells (transfected cell lines including RKO and HCT116) were collected in a 1.5-ml RNase-free Eppendorf (EP) tube, and 500 μl of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added, mixed well and incubated for 5 min to allow the cells to fully lyse. Total RNA was extracted with TRI Reagent (Sigma), and 1 μg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega). PCR was performed on 1/10 volume (2 μl) of the cDNA using PCR Mix (Kapa Biosystem).

The tRFs, LncRNA MALAT-1, SRSF2 and SMC-1A isoforms were reverse-transcribed using murine leukemia virus (MMLV) (Promega, Madison, WI, USA). The forward and reverse primers were designed and synthesized (Table S2). qRT-PCR amplifications were performed using SYBR Green qPCR Master Mix (ABI, 4367659) and an ABI 7300 real-time PCR detection system.

Cellular migration and invasion assays

RKO and HCT116 cells transfected with plasmids were collected and starved in serum-free medium for 24 h. The cells were digested and centrifuged for resuspension counting. The cells were evenly distributed in the upper compartment of the 24-well Transwell plate. Serum-free medium was added to the upper chamber, and culture medium containing 10% fetal bovine serum was added to the lower chamber. In the invasion assay, 20 μl of Matrigel mixture (1:1 with Matrigel and DMEM) was spread on the upper layer of the chamber and left at 37°C for 30 min until the gel cured. This step was not required for the migration assay. After 24 h, the cells were fixed with 4% paraformaldehyde. Crystal violet was added to the orifice plate for 1 h and then washed with clean water. The upper compartment of the Transwell was removed, the excess cells in the upper compartment were erased, the cells were observed under a microscope, and the typical field of vision was recorded.

Cellular proliferation

MTT was used to detect cellular viability. RKO and HCT116 cells were transfected with si-MALAT1 and SMC1A overexpression plasmids and their corresponding NC controls for 48 and 24 h, respectively. The cells were collected in a 96-well plate (1 × 10 > L/well) with 6 multiple wells in each group. Under the conditions of 37°C and 5% CO_2, 10 μl of 5 mg/mL MTT solution was added after 12, 36 and 72 h. After continued incubation for 4 h, 96-well plates were removed, 100 μl of supernatant was slowly absorbed, and 1000 μl of dimethyl sulfoxide was added until the purple methylene jugeno crystal was completely dissolved and then used within 30 min. An enzyme labeling instrument (Bio-Rad, Hercules, CA, USA) was used to measure the optical density (OD) at 570 nm. The experiment was repeated three times.

RNA pulldown

Detection of tRF-20-M0NK5Y93 and tRF-20-M0NK5Y93 in RKO cells by RNA pulldown assay. Binding relationship of MALAT1, MALAT1 and SRSF2, SRSF2 and SMC1A001. A total of 3 × 10^6 cells were seeded on a 10-cm dish and cultured overnight, and the final concentration of 100 nmol/L biotin-labeled tRF-20-M0NK-5Y93 mimic and SRSF2 overexpression plasmid (the probe was customized by Ribobio) RKO cells were transfected, and the control group was transfected with biotin-labeled NC sequence. Forty-eight hours after transfection, the cells were collected, and the cell lysate was added on ice and incubated at 4°C for 4 h. Then, 50 μL of streptavidin magnetic beads was added to the abovementioned cell lysis solution, and the cells were rotated and equilibrated at 4°C for 30 min. The magnetic beads were collected by centrifugation and washing, and the magnetic beads were extracted by the TRIzol method. The total RNA was reverse-transcribed into cDNA, and the cDNA was used as a template for PCR. The total system (20 μL) included 10 μL of 2 × EasyPfu PCR SuperMix, upstream and downstream primers (1 μL of each compound), 1 μL of cDNA, and 7 μL of ddH_2O. The reaction program was as follows: 95°C for 5 min; 94°C for 20 s, 58°C for 20 s, and 72°C for 2 min for 35 cycles. The levels of MALAT1, SRSF2 and SMC1A001 were detected, with GAPDH as the internal reference. Fifty microliters of cell lysate without RNA pulldown were used as an input control.

Fish assay

Prepare the probes. Then, a larger wet box was used to place the slices crosswise, and 10-μL probes were dropped on the sliced tissue. Add a coverslip, cover the wet box and incubate at 37°C for 12-16 h. Washing after hybridization: carefully remove the coverslip and preheat 40 ml of posthybridization washing solution at 43°C to wash the sections for 15 min; wash twice with 2XSSC (37°C) for 10 min each time. Then, the sections were placed in 1 × PBS in a staining jar for detection. Do not allow sections to dry. Sections were removed from 1 × PBS to remove excess water and avoid specimen drying. Place the slices in a wet box and process 4 slices at the same time. Then, 30-60 μl of rhodamine anti-antibody or FITC avidin was used for each section and incubated at room tem-

perature for 20 min. Remove the plastic cover film and place the sections in a staining jar containing 1 × PBS. Wash 3 times at room temperature with 1 × PBS for 2 min each time. Take out the sections from 1 × PBS, tilt the sections to drain the liquid; drop 30-60 μl of anti-avidin antibody on each section, add a plastic cover film, and incubate at room temperature for 20 min. The plastic cover film was removed, and the sections were placed in a staining jar containing 1 × PBS. Wash 3 times at room temperature with 1 × PBS, 2 min each time; take out the slices from 1 × PBS, tilt the slices to drain the liquid; drop 30-60 μl of anti-avidin antibody on each slice, add plastic cover film, incubate at room temperature for 20 min; wash at room temperature with 1 × PBS 3 times, 2 min each time. The last step was nuclei staining. DAPI (10-20 μl) was added to each section, covered with a coverslip, incubated in 2-5 ml at room temperature and observed under a fluorescence microscope as soon as possible or stored in a closed box at -20°C. Sections were observed under a microscope within 1 h after staining.

Determination of the double luciferase reporter

RKO and HCT116 cells were inoculated in 24-well plates for 24 h (80% fusion rate). We constructed two plasmids containing luciferase reporter genes and wild-type (Wt) or mutant (Mut) LPAR1 3'-UTR. Then, each plasmid was cotransfected into RKO cells with the NC analog or tRF-20-M0NK5Y93 analog. The MALAT1 wild-type or mutant 3'-UTR plasmid was cotransfected with tRF-20-M0NK5Y93 mimic or NC mimic (Promega, USA) for 24 h. The luciferase assay was performed according to the instructions of the Dual luciferase Reporter Assay System (Promega, USA).

In vivo studies

Animal studies were performed under the experimental animal use guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals and the National Institutes of Health and Use Committee of Zhejiang University, China. Male athymic BALB white mice aged 5 weeks were used. For the in vivo lung metastasis model, a caudal vein of mice was injected with CT-26 cells transduced with NC inhibitor/si-NC, tRF-20-M0NK5Y93 inhibitor/si-NC, NC inhibitor/si-MALAT1, or tRF-20-M0NK5Y93 inhibitor/si-MALAT1 (1×10^5 cells per mouse, n = 8 for each group) diluted in 100 μl of a PBS/Matrigel (BD Biosciences) mixture (1:1). Eight weeks after injection, mice were killed, and the number of metastatic lung tumors was analyzed.

Statistics

Each assay was independently performed at least 3 times. Statistical analyses were performed using GraphPad Prism 8.0.3 software. Student's t-test (two tailed) and ANOVA were used to detect differences between two groups or among more than two groups, respectively. The Western blot results were quantified by ImageJ software (National Institutes of Health). All data are shown as the means ± standard deviations (SDs).

Results

Identification of significantly changed key tRFs and their roles in colon cancer metastasis under hypoxia

The Transwell assays showed that the number of migrated and invaded cells was significantly increased in RKO cells under hypoxia compared with normoxia (**Figure 1A**). Changes in the tumor microenvironment, such as hypoxia, nutrient deficiency, low pH, inflammatory factors, and TGF-β, play a key role in initiating tumor cell metastasis. Studies have confirmed that hypoxia also causes differences in the expression of tRFs [13]. In a previous study, we observed that the expression of 14 tRFs differed significantly in colon cancer cells under hypoxic conditions [14]. Among them, tRF-20-M0NK5Y93 showed the most significant difference, showing decreased expression compared with normoxia. The RKO and HCT116 cell lines were utilized for subsequent assays. Furthermore, to explore the biological role of tRF-20-M0NK5Y93 in colon cancer cells, we transfected tRF-20-M0NK5Y93 mimics into RKO and HCT116 cells for gain-of-function experiments. The transfection efficiency was tested by qRT-PCR (**Figure 1B**, **1C**). The analysis showed upregulated expression of tRF-20-M0NK5Y93 in the mimic group compared with the NC inhibitor group and a significant decline

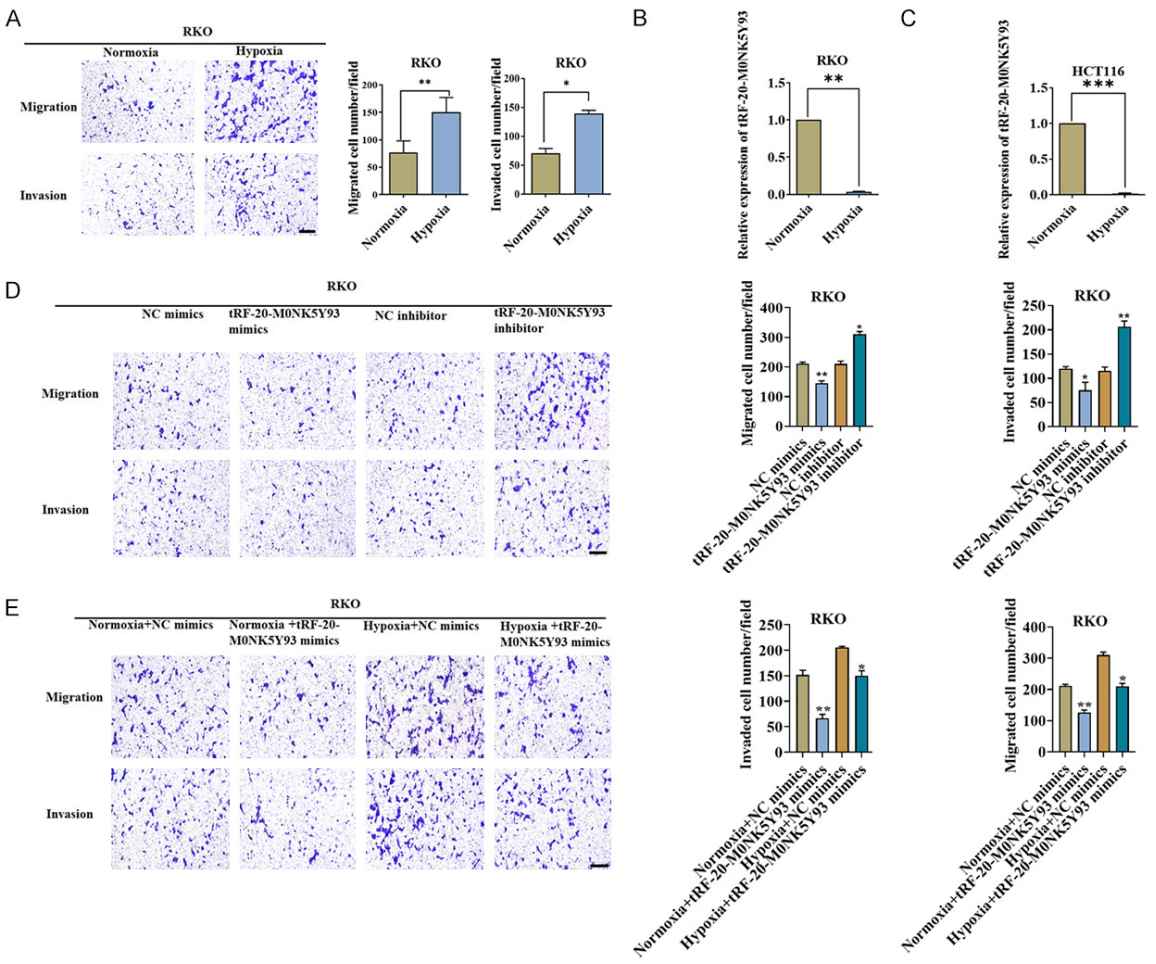


Figure 1. tRF-20-M0NK5Y93 regulates the malignant activities of colon cancer cells under hypoxia in vitro. A. The invasion and migration abilities of RKO cells were elevated under hypoxia. Scale bar = 120 μm. B, C. The transfection efficiency was tested by qRT-PCR. D. tRF-20-M0NK5Y93 overexpression suppressed RKO cell invasion and migration by Transwell assay compared to NC, while tRF-20-M0NK5Y93 knockdown promoted cell invasion and migration. Scale bar = 150 μm. E. Overexpression of tRF-20-M0NK5Y93, which inhibited the invasion and migration of RKO cells, effectively rescued the promoting role of hypoxia. Scale bar = 150 μm. Values are triplicate-replicated and displayed as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

in tRF-20-M0NK5Y93 expression in the inhibitor group compared with the NC inhibitor group (Figure S1A, S1B). For loss-of-function experiments, we transfected the tRF-20-M0NK5Y93 inhibitor into RKO and HCT116 cells to knock down tRF-20-M0NK5Y93. As expected, tRF-M0NK5Y93 knockdown promoted the migration of both RKO and HCT116 cells (**Figure 1D**). The cell migration and invasion of both RKO and HCT116 cells can be promoted by hypoxic conditions, while overexpression of tRF-20-M0NK5Y93, which inhibited the invasion and migration of RKO and HCT116 cells, effectively impaired the role of hypoxia (**Figure 1E**). The parallel experiments for HCT116 cells are shown in Figure S2.

tRF-20-M0NK5Y93 directly interacts with lncRNA MALAT1 and regulates MALAT1 expression in colon cancer cells

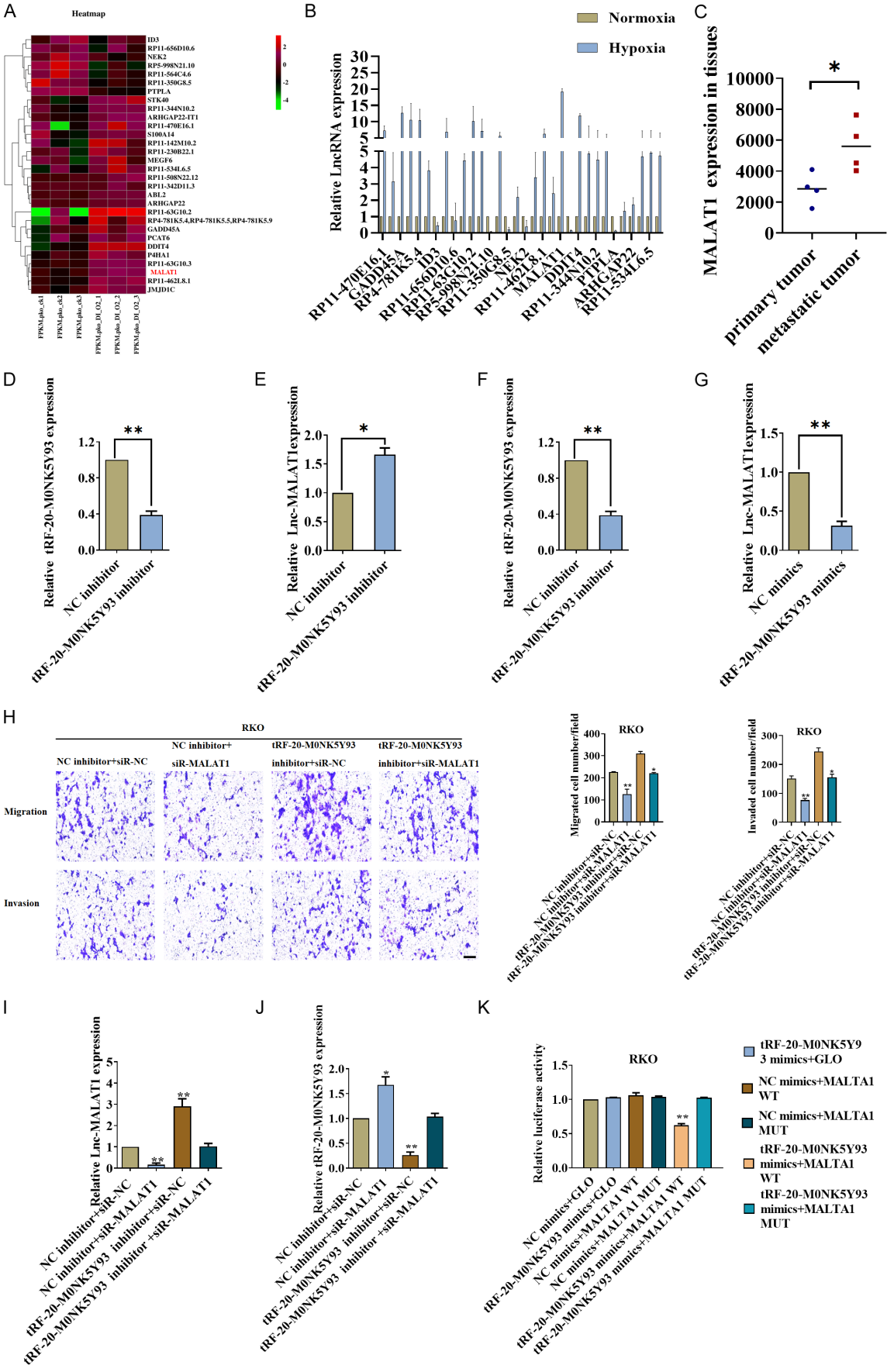
To explore the molecular mechanism underlying the role of tRF-20-M0NK5Y93 in colon cancer metastasis, we analyzed the results from whole transcript sequencing and later bioinformatics analysis and found that lncRNA MALAT1 had the highest upregulation among the upregulated lncRNAs. Then, qRT-PCR assays were performed to verify the result (**Figure 2A**, **2B**). Additionally, LncRNA MALAT1 expression in tumors with liver metastasis was higher than that in primary colon cancer tissues in 4 pairs of colon cancer patients without preoperative

treatment by full transcript sequencing (**Figure 2C**). To investigate further molecular mechanisms, we performed qRT-PCR assays to determine the regulatory relationship between tRF-20-M0NK5Y93 and MALAT1. The transfection efficiency of the tRF-20-M0NK5Y93 inhibitor was measured by qRT-PCR (**Figure 2D**). The assay results suggested that the MALAT1 RNA expression level was significantly higher in the tRF-20-M0NK5Y93 knockdown group than that in the negative control group (**Figure 2E**). Then, we transfected tRF-20-M0NK5Y93 mimics into RKO cells to upregulate the expression of tRF-20-M0NK5Y93, and the transfection efficiency of tRF-20-M0NK5Y93 mimics was measured by qRT-PCR (**Figure 2F**). qRT-PCR assay results suggested that the RNA expression level of MALAT1 was clearly lower in the tRF-20-M0NK-5Y93 mimic-transfected group than that in the negative control group (**Figure 2G**). To further investigate whether tRF-20-M0NK5Y93 influences the migration of colon cancer cells via its association with MALAT1, we performed a "rescue" experiment by transfecting a tRF-20-M0NK5Y93 inhibitor with or without MALAT1 knockdown. Knockdown of MALAT1 significantly attenuated the promoting effects of tRF-20-M0NK5Y93 on the metastasis of RKO cells (**Figure 2H**). When tRF-20-M0NK5Y93 was knocked down, the expression of MALAT-1 increased. After simultaneous knockdown of tRF-20-M0NK5Y93 and MALAT-1, the expression of MALAT-1 was higher than that of MALAT-1 knockdown alone (**Figure 2I**). This shows that tRF-20-M0NK5Y93 regulates the expression of MALAT-1. When MALAT-1 was knocked down, the expression of tRF-20-M0NK5Y93 increased. However, this result was rescued after tRF-20-M0NK5Y93 inhibitor transfection (**Figure 2J**). The abovementioned experiments further confirmed that the results of the invasion and migration experiments (**Figure 2H**) were based on the total effects of two molecular level changes. Then, we performed a dual-luciferase reporter assay to confirm that tRF-20-M0NK5Y93 could target MALAT1 directly. The results suggested that tRF-20-M0NK5Y-93 overexpression significantly decreased Wt MALAT1 luciferase activity; however, no obvious change occurred in Mut MALAT1 luciferase activity in RKO and HCT116 cells (**Figure 2K**). Then, we explored the intracellular localization of tRF-20-M0NK5Y93, and the results showed that tRF-20-M0NK5Y93 localized to the nucleus. tRF-20-M0NK5Y93 and MALAT1 both localized in the same cellular compartment, the RKO cell nucleus (**Figure 2L**). We speculated that the combined tRF-20-M0NK5Y93 and MALAT1 may be transported to the nucleus to play a role. To further demonstrate our hypothesis and explore the potential target of tRF-20-M0NK5Y93, we took the intersection of the results from the LncTarget database (http://www.cuilab.cn/lnctar). According to the prediction and analysis of gene sequence comparison, tRF-20-M0NK5Y93 has regions that can bind to MALAT1, and the binding sites are located in the 4957-4976 region of MALAT1 (**Figure 2M**). Herein, the above mentioned results suggested that tRF-20-M0NK5Y93 could directly target MALAT1 and negatively regulate MALAT1 expression. RNA pulldown assays further revealed that tRF-20-M0NK5Y93 could directly bind with lncRNA MALAT1 (**Figure 2N**).

Changes in SMC1A transcript expression under hypoxic conditions and their effects on the invasion and migration of colorectal cancer

We took advantage of qRT-PCR assays to determine SMC1A transcript expression in RKO and HCT116 cells. The expression of SMC1A transcript 001 increased under hypoxic conditions, while the expression of other transcripts 201, 005, and 003 decreased (**Figure 3A**). The results suggested significant overexpression of the SMC1A001 transcript in comparison with other transcripts in colon cancer cells. The results of Western blot assays also showed that SMC1A001 increased under hypoxia in comparison to normoxia (**Figure 3B**). Furthermore, we transfected SMC1A001 overexpression plasmids into RKO cells and performed cell proliferation assays and migration assays to study the influence of SMC1A001 on RKO cell growth and migration, respectively (**Figure 3C**, **3D**). The assay results showed that SMC-1A001 overexpression clearly promoted RKO and HCT116 cell migration and invasion in comparison to the negative control groups.

Then, tRF-20-M0NK5Y93 inhibitor and mimics were transfected into RKO and HCT116 cells to explore the regulatory relationship of tRF-20-M0NK5Y93 on SMC1A001 and SMC1A003. The mRNA expression of SMC1A001 and SMC1A003 and the protein expression of SMC1A001 were tested by qRT-PCR and western blot assays. The results suggested that the


A
Heatmap
ID3
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RP5-998N21.10
RP11-564C4.6
RP11-350G8.5
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RP11-344N10.2
ARHGAP22-IT1
RP11-470E16.1
S100A14
RP11-142M10.2
RP11-230B22.1
MEGF6
RP11-534L6.5
RP11-508N22.12
RP11-342D11.3
ABL2
ARHGAP22
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RP4-781K5.4,RP4-781K5.5,RP4-781K5.9
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NEK2
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MALAT1
DDIT4
RP11-344N10.2
PTPLA
ARHGAP22
RP11-534L6.5
C
MALAT1 expression in tissues
primary tumor
metastatic tumor
D
Relative tRF-20-M0NK5Y93 expression
NC inhibitor
tRF-20-M0NK5Y93 inhibitor
E
Relative Lnc-MALAT1expression
NC inhibitor
tRF-20-M0NK5Y93 inhibitor
F
Relative tRF-20-M0NK5Y93 expression
NC inhibitor
tRF-20-M0NK5Y93 inhibitor
G
Relative Lnc-MALAT1expression
NC mimics
tRF-20-M0NK5Y93 mimics
H
RKO
NC inhibitor+siR-NC
NC inhibitor+ siR-MALAT1
tRF-20-M0NK5Y93 inhibitor+siR-NC
tRF-20-M0NK5Y93 inhibitor+siR-MALAT1
Migration
Invasion
Migrated cell number/field
Invaded cell number/field
I
Relative Lnc-MALAT1 expression
J
Relative tRF-20-M0NK5Y93 expression
K
RKO
Relative luciferase activity
NC mimics+GLO
tRF-20-M0NK5Y93 mimics+GLO
NC mimics+MALTA1 WT
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tRF-20-M0NK5Y93 mimics+MALTA1 MUT

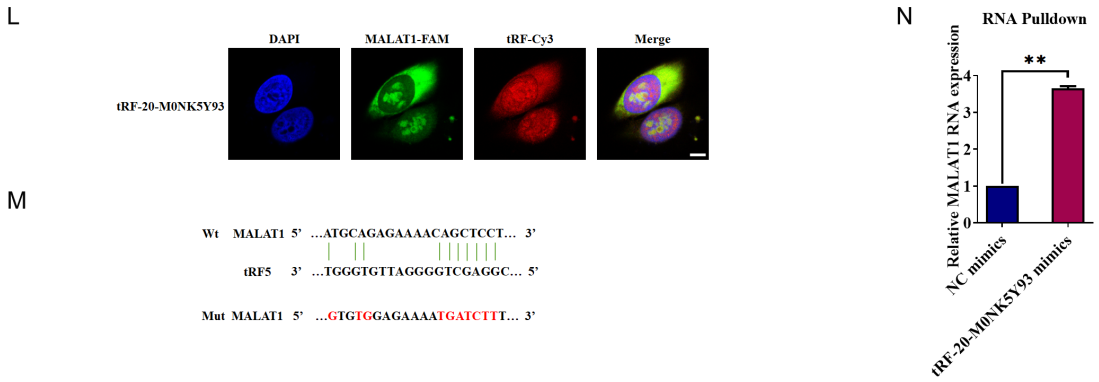


Figure 2. tRF-20-M0NK5Y93 directly interacts with lncRNA MALAT1 and regulates MALAT1 expression in colon cancer cells. A. The lncRNA MALAT1 had the highest upregulation among the upregulated lncRNAs. B. qRT-PCR assays were performed to verify the results. C. LncRNA MALAT1 expression in tumors with liver metastasis is higher than in primary colon cancer tissues in 4 pairs of colon cancer patients without preoperative treatment. D. The transfection efficiency of the tRF-20-M0NK5Y93 inhibitor was measured by qRT-PCR. E. MALAT1 expression was significantly higher in the tRF-20-M0NK5Y93 downregulation group than that in the negative control group. F. The transfection efficiency of tRF-20-M0NK5Y93 mimics was measured by qRT-PCR. G. The RNA expression level of MALAT1 was clearly lower in the tRF-20-M0NK5Y93 mimic-transfected group. H. Knockdown of MALAT1 significantly attenuated the promoting effects of tRF-20-M0NK5Y93 on the metastasis of RKO cells. Scale bar = 120 μm. I. The expression levels of tRF-20-M0NK5Y93 and MALAT1 in the cells were simultaneously knocked down, and the expression level of MALAT1 was increased compared with that of knockdown of MALAT1 only. J. The expression level of tRF-20-M0NK5Y93 was increased when both were knocked down at the same time compared with that when only tRF-20-M0NK5Y93 was knocked down. K. A dual-luciferase reporter assay was performed to confirm that tRF-20-M0NK5Y93 could target MALAT1 directly. L. tRF-20-M0NK5Y93 localized to the nucleus. tRF-20-M0NK5Y93 and MALAT1 both localize in the same cellular compartment, the cell nucleus. Scale bar = 20 μm. M. tRF-20-M0NK5Y93 has regions that can bind to MALAT1, and the binding sites are located in the 4957-4976 region of MALAT1. N. RNA pulldown assays further revealed that tRF-20-M0NK5Y93 could directly bind with lncRNA MALAT1.

SMC1A001 expression level in the tRF-20-M0NK5Y93 inhibitor group was clearly higher than that in the negative control inhibitor group, while its expression level in the tRF-20-M0NK5Y93 mimic group was significantly lower than that in the negative control mimic group. However, the change in the expression of SMC1A003 was the opposite (**Figure 3E-I**). When we took hypoxia into consideration, the overexpression of tRF-20-M0NK5Y93 weakened the effect of hypoxia in modulating the expression of SMC1A001 and SMC1A003 (**Figure 3J**, **3K**). Interestingly, tRF-20-M0NK5Y93 overexpression partially rescued the promotive role of SMC1A001 on the invasion and migration of colon cancer cells (**Figure 3L**). The parallel experiments for HCT116 cells are shown in Figure S3.

LncRNA MALAT1 regulates colon cancer cell metastasis by modulating SMC1A expression levels in colon cancer cells

To investigate whether siRNA-MALAT1 could efficiently knock down MALAT1-1, we transfected three parallel siRNA-MALAT1 constructs into cells. The transfection efficiency was measured by qRT-PCR in RKO cells. The results showed that MALAT1 expression significantly decreased after cells were transfected with siR-MALAT1-1 for 48 h (**Figure 4A**). Therefore, we used siR-MALAT1-1 to carry out follow-up assays. After we transfected MALAT1 siRNA into RKO cells, the invasion, migration and proliferation abilities of the cells all decreased (**Figure 4B-D**). Additionally, the mRNA and protein expression of SMC1A001 was downregulated (**Figure 4E**, **4G**), while the expression of SMC1A003 was upregulated (**Figure 4F**) after we transfected MALAT1 siRNA into RKO and HCT116 cells. Next, we set hypoxia as the experimental condition and further observed that hypoxia could partially rescue the metastasis inhibition caused by MALAT1 knockdown. Correspondingly, both the mRNA and protein levels of SMC1A001 decreased compared to those under hypoxia only, while the expression of SMC1A003 increased (**Figure 4H-J**). Furthermore, after we knocked down tRF-20-M0NK5Y93 and MALAT1 together, the results showed that the expres-

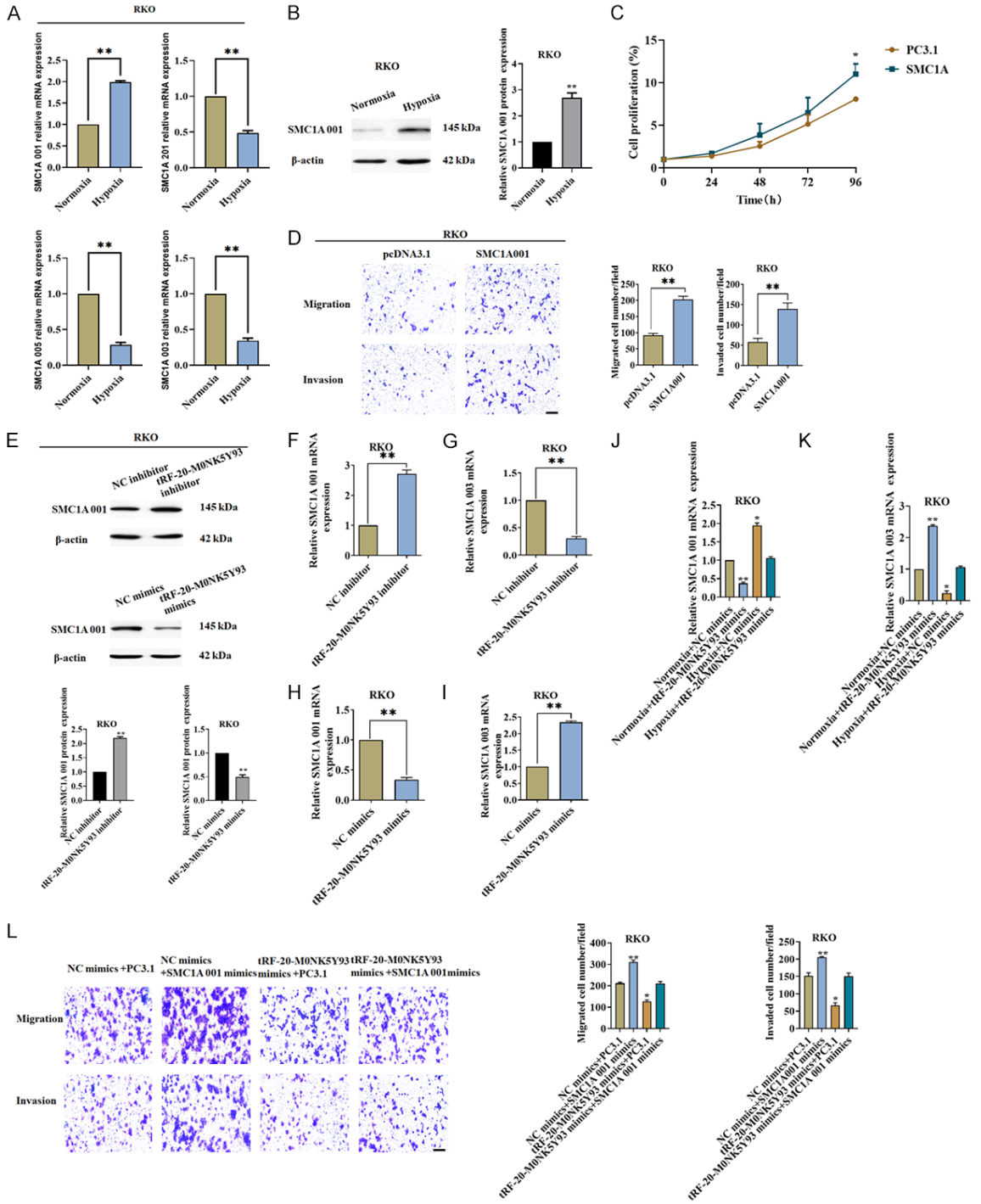


Figure 3. SMC1A001 promotes the proliferation and migration of colon cancer cells. A, B. SMC1A isoform expression in colon cancer cell lines by qRT-PCR and western blot. β-Actin was used as a control. C, D. SMC1A001 overexpression promoted the proliferation, invasion and migration of RKO cells, as determined by MTT assay and Transwell assay, compared to the vector group (NC). Scale bar = 120 μm. E-I. tRF-20-M0NK5Y93 knockdown promoted the mRNA and protein expression of SMC1A001, while tRF-20-M0NK5Y93 overexpression suppressed the expression of SMC1A001, as assessed by qRT-PCR and western blotting, respectively. However, the change in the expression of SMC1A003 was the opposite. J, K. When we take hypoxia into consideration, the overexpression of tRF-20-M0NK5Y93 weakens the effect of hypoxia in modulating the expression of SMC1A001 and SMC1A003. L. tRF-20-M0NK5Y93 overexpression could partially rescue the promotive role of SMC1A001 on invasion and migration of RKO cells by migration assay compared to vector group (NC). Scale bar = 120 μm. Values are triplicate-replicated and displayed as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

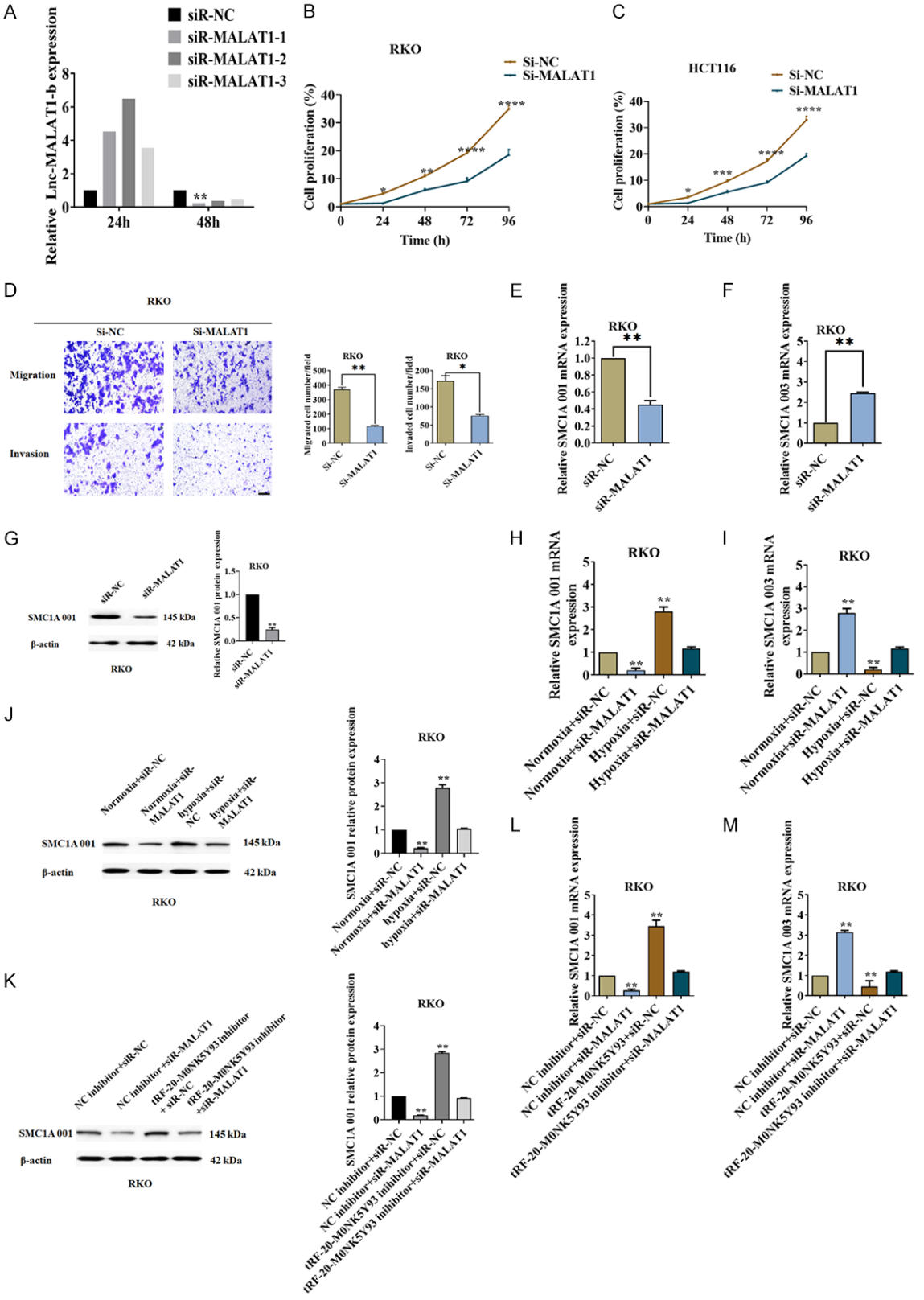


Figure 4. MALAT1 promotes colon cancer cell metastasis by modulating SMC1A expression levels in colon cancer cells. A. Knockdown efficiency of si-MALAT1-1, si-MALAT1-2 and si-MALAT1-3 was detected in RKO cells transfected with siRNA-MALAT1 after 24 h and 48 h by qRT-PCR with its quantification result. The results showed that MALAT1 expression significantly decreased after cells were transfected with siR-MALAT1-1 for 48 h. B-D. MALAT1 knockdown

suppressed the proliferation and migration of RKO cells, as determined by MTT assay and migration assay. Negative control (NC) indicates the control group. Scale bar = 120 μm. E-G. Knockdown of MALAT1 in RKO cells resulted in downregulated expression of SMC1A001 by qRT-PCR and western blot with its quantification result and upregulated expression of SMC1A001 by qRT-PCR. β-Actin was used as a control. H-J. Hypoxia promoted the expression of SMC1A001 and inhibited the expression of SMC1A003, rescuing the role of MALAT1 inhibition in the expression of SMC1A001. K-M. Knockdown of tRF-20-M0NK5Y93 promoted the expression of SMC1A001, which rescued the suppressive role of MALAT1 on the expression of SMC1A001, as measured by qRT-PCR and Western blot with quantification results, while knockdown of tRF-20-M0NK5Y93 inhibited the expression of SMC1A003, which rescued the promotive role of MALAT1 on the expression of SMC1A003, as measured by qRT-PCR. β-Actin was used as a control. Values are triplicate-replicated and displayed as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

sion level of SMC1A001 was increased and the expression level of SMC1A003 was decreased compared with knocking down MALAT1 only (**Figure 4K-M**). These results indicate that MALAT1 may be an oncogenic driver in the metastasis of colon cancer by modulating SMC1A001 expression levels in colon cancer cells. The parallel experiments for HCT116 cells are shown in Figure S4.

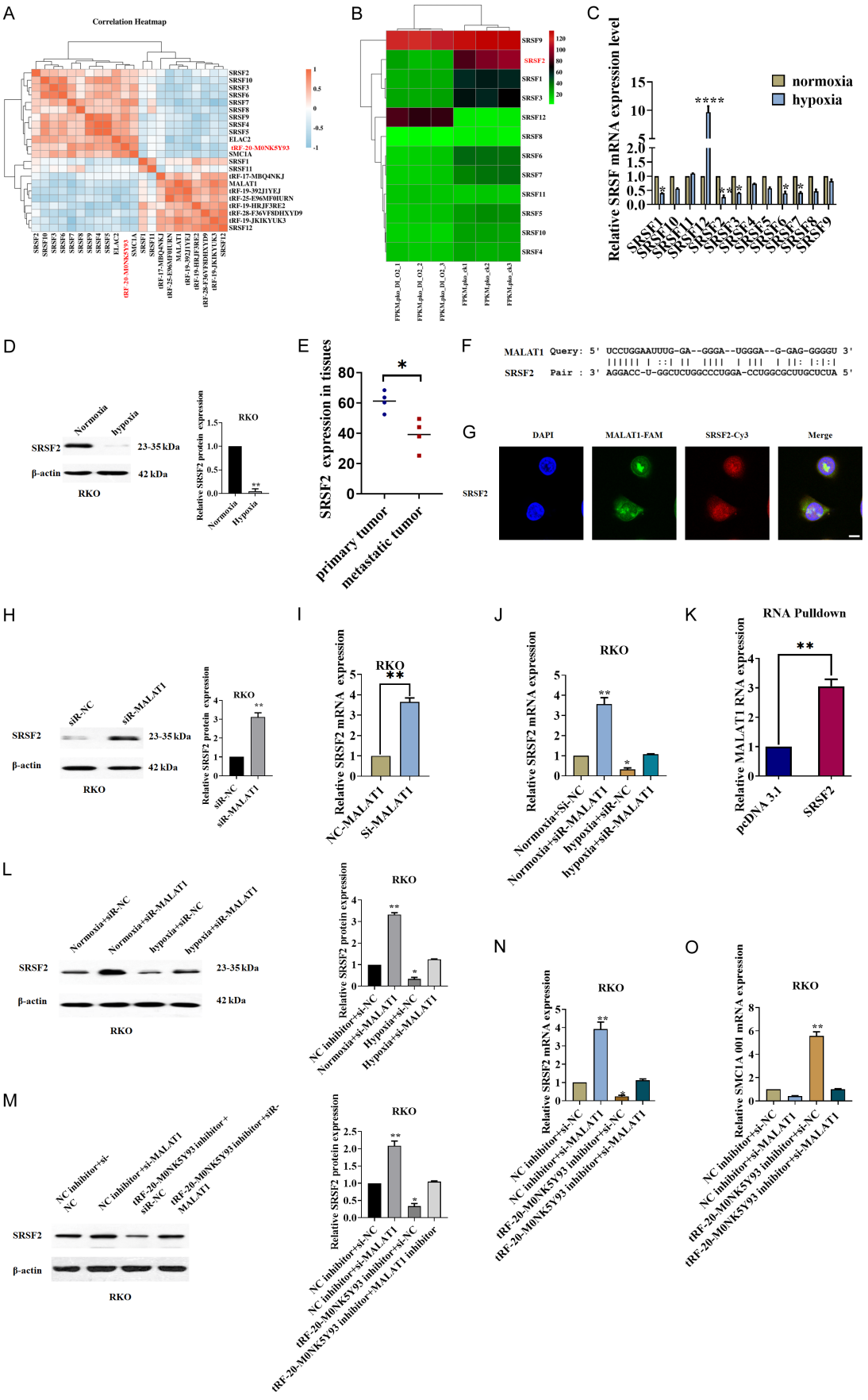
The tRF-20-M0NK5Y93-SRSF2 signaling pathway regulates colon cancer cell metastasis through RNA alternative splicing of SMC1A

Gene expression correlation analysis showed that tRF-20-M0NK5Y93, MALAT-1, and SRSF2 had significant expression correlations (**Figure 5A**). Whole transcript sequencing and bioinformatics analysis showed that the expression of proteins involved in transcript alternative splicing was almost uniformly suppressed, especially SRSF2, which was significantly suppressed at the mRNA and protein expression levels (**Figure 5B**). To confirm the results of whole transcript sequencing data, we investigated the expression levels of SRSF2 in colon cancer cells by qRT-PCR and western blotting (**Figure 5C**, **5D**). The results showed that SRSF2 was notably lower in liver metastasis tissues than in primary tumors (P < 0.05) (**Figure 5E**).

According to the prediction and analysis of gene sequence comparison, MALAT1 has regions that can bind to SRSF2. The binding position and sequences are shown in **Figure 5F**. The combined MALAT1 and SRSF2 MALAT1 and SRSF2 both localize in the same cellular compartment, the cell nucleus (**Figure 5G**). The results revealed that they may be transported to the nucleus to play a role. The results revealed that downregulation of MALAT1 expression significantly increased SRSF2 expression (**Figure 5H**, **5I**), but this phenomenon could be reversed by hypoxia (**Figure 5J**, **5L**). Moreover, we performed an RNA pulldown assay to study the direct interaction between SRSF2 and lncRNA MALAT1. The data suggested that SRSF2 enriched much more MALAT1 in RKO cells transfected with SRSF2 overexpression plasmids normalized to that in the pcDNA3.1 group, indicating that MALAT1 binds to SRSF2 (**Figure 5K**). Interestingly, cotransfection with the tRF-20-M0NK5Y93 inhibitor abolished the effect of si-MALAT1 on promoting SRSF2 expression (**Figure 5M**, **5N**) and decreased the expression of SMC1A001 (**Figure 5O**). Together, these results indicate that tRF-20-M0NK5Y93 regulates colon cancer metastasis under hypoxia through the correlation between MALAT1 and the alternative splicing protein SRSF2. The parallel experiments for HCT116 cells are shown in Figure S5.

SRSF2 promotes the migration and invasion of colon cancer cells and regulates alternative splicing of SMC1A

To study the biological role of SRSF2 in colon cancer cells, we carried out gain-of-function and loss-of-function experiments. For loss-of-function experiments, we transfected shRNA plasmids into RKO and HCT116 cells to knock down SRSF2. The sequences of shRNAs designed to knock down SRSF2 are shown in Table S3. qRT-PCR results pooled from ten independent experiments showed that shRNA (sh-SRSF2) significantly diminished SRSF2 in RKO and HCT116 cells (Figure S1C, S1D). As expected, sh-SRSF2 transfection promoted the migration and invasion of both RKO cell lines (**Figure 6A**). For gain-of-function experiments, we transfected SRSF2 overexpression plasmids into RKO and HCT116 cells. The transfection efficiency was tested by qRT-PCR (Figure S1E, S1F). Transwell assays revealed that SRSF2 inhibited the migration of RKO cells (**Figure 6B**). To explore the interaction between SRSF2 and SMC1A in the migration and inva-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Correlation Heatmap
B
C
Relative SRSF mRNA expression level
normoxia
hypoxia
D
Normoxia
hypoxia
SRSF2
23-35 kDa
β-actin
42 kDa
RKO
Relative SRSF2 protein expression
Normoxia
Hypoxia
E
SRSF2 expression in tissues
primary tumor
metastatic tumor
F
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SRSF2 Pair : 3' AGGACC-U-GGCUCUGGCCCUGGA-CCUGGCGCUUGCUCUA 5'
G
DAPI
MALAT1-FAM
SRSF2-Cy3
Merge
SRSF2
H
siR-NC
siR-MALAT1
Relative SRSF2 protein expression
I
Relative SRSF2 mRNA expression
NC-MALAT1
Si-MALAT1
J
Normoxia+Si-NC
Normoxia+siR-MALAT1
hypoxia+siR-NC
hypoxia+siR-MALAT1
K
RNA Pulldown
Relative MALAT1 RNA expression
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hypoxia+siR-NC
hypoxia+siR-MALAT1
Relative SRSF2 protein expression
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NC inhibitor+si-MALAT1
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Hypoxia+si-NC
Hypoxia+si-MALAT1
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NC inhibitor+si-MALAT1
tRF-20-M0NK5Y93 inhibitor+siR-NC
tRF-20-M0NK5Y93 inhibitor+siR-MALAT1
tRF-20-M0NK5Y93 inhibitor+si-NC
tRF-20-M0NK5Y93 inhibitor+si-MALAT1 inhibitor
N
Relative SRSF2 mRNA expression
O
Relative SMC1A 001 mRNA expression

Figure 5. tRF-20-M0NK5Y93 regulates alternative splicing of SMC1A001 mRNA via SRSF2. A. Gene expression correlation analysis showed that tRF-20-M0NK5Y93, MALAT-1, and SRSF2 had significant expression correlations. B. The expression of proteins involved in transcript alternative splicing was almost uniformly suppressed, especially SRSF2, which was significantly suppressed at the mRNA expression level. C, D. SRSF2 expression is downregulated under hypoxia as assessed by qRT-PCR and western blot assays. β-Actin was used as a control. E. SRSF2 was notably lower in metastatic tissues than that in primary tumors. F. MALAT1 has regions that can bind to SRSF2 according to the prediction and analysis of gene sequence comparison. G. MALAT1 and SRSF2 both localize in the same cellular compartment, the RKO cell nucleus. Scale bar = 20 μm. H, I. MALAT1 knockdown significantly increased SRSF2 expression. J, L. Rescue experiments show that the effect of si-MALAT1 on SRSF2 expression was partly compromised by hypoxia in RKO cells. K. RNA pulldown assay results showed that SRSF2 enriched MALAT1 in RKO cells transfected with SRSF2 overexpression plasmids normalized to that in the pcDNA3.1 group. M-O. Rescue experiments show that the effect of si-MALAT1 on SRSF2 and SMC1A001 expression was partly compromised by tRF-20-M0NK5Y93 knockdown in RKO cells. Values are triplicate-replicated and displayed as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

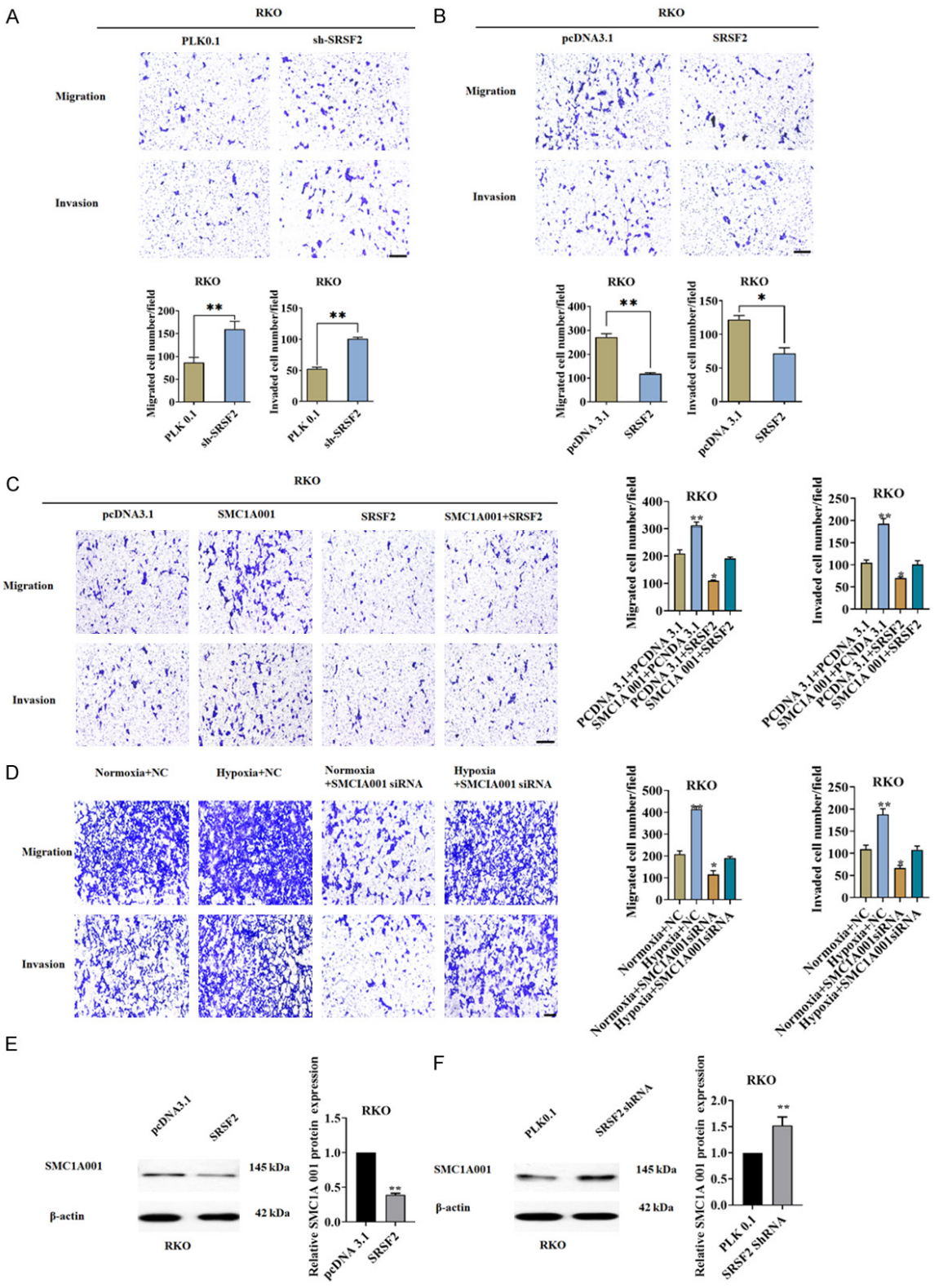
sion of colon cancer, we cotransfected SRSF2 and SMC1A001 overexpression plasmids. The assays showed that overexpression of SRSF2 could rescue the promoting role of SMC1A001 on the metastasis of colon cancer (**Figure 6C**). The results showed that SMC1A001 could also promote cell migration and invasion under hypoxia (**Figure 6D**). To explore the molecular mechanism underlying the role of SRSF2 in colon cancer metastasis, we transfected SRSF2 overexpression and shRNA plasmids to perform qRT-PCR and western blot assays. As expected, SMC1A001 expression was downregulated when SRSF2 was overexpressed, and vice versa (**Figure 6E**, **6F**). RNA pulldown assays also confirmed that SMC1A001 was enriched when SRSF2 enriched much more SMC1A001 in RKO cells transfected with SRSF2 overexpression plasmids normalized to that in the pcDNA3.1 group, indicating that SRSF2 binds to SMC1A001 directly (**Figure 6G**).

To further confirm these results in vivo, we subcutaneously injected CT-26 cells (1×10^5 cells per mouse, n = 8 for each group) diluted in 100 μl of a PBS/Matrigel (BD Biosciences) mixture (1:1) into nude mice. Data from in vivo assays also showed that the relative tumor amount increase caused by the tRF-20-M0NK5Y93 inhibitor was stronger than that caused by the tRF-20-M0NK5Y93 inhibitor with si-MALAT1 (**Figure 6H**). The parallel experiments for HCT-116 cells are shown in Figure S6. The diagram we made as followed. tRF-20-M0NK5Y93 binds the domain of MALAT1 and regulates its expression, giving rise to decreased SRSF2, which induces alternative splicing of SMC1A in colon cancer cells, facilitating colon cancer metastasis (**Figure 7**). The tumor tissues of four groups of mice were cut, and PCR and WB experiments were performed to check the mRNA and protein expression of SRSF2 and SMC1A in vivo. The protein expression of SRSF2 and SMC1A in the tumor tissues of the four groups of mice is shown in Figure S7A. The mRNA expression levels of the two molecules are shown in Figure S7B. Thus, the regulatory relationship between tRF-20-M0NK5Y93, MALAT-1, SRSF2 and SMC1A expression was further verified in vivo.

Discussion

In this study, functionally, tRF-20-M0NK5Y93, which was decreased under hypoxia, inhibited the migration and invasion of colon cancer cells. Interestingly, our study revealed that tRF-20-M0NK5Y93 could directly bind the special sequences of lncRNA MALAT1, negatively regulating the expression of MALAT1. Moreover, MALAT-1 could bind SRSF2 and regulate its expression, resulting in discrepant expression of various SMC1A isoforms. Our findings demonstrate for the first time a molecular mechanism involving tRFs, lncRNAs and splicing-related RNA-binding proteins, providing a novel explanation for the vital roles of tRFs in tumorigenicity.

Our previous study found that tRF-20-M0NK5Y93 was significantly downregulated under hypoxic conditions. In this study, we found that the expression of MALAT1 was elevated in liver metastatic tissue compared with colon cancer primary tissue. The expression of proteins involved in alternative splicing of transcripts was almost uniformly suppressed, especially the expression levels of SRSF2 at the mRNA and protein levels. The expression of the SMC1A001 transcript increased, and the expression of other short transcripts decreased. Gene expression correlation analysis suggests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RKO
PLK0.1
sh-SRSF2
Migration
Invasion
RKO
Migrated cell number/field
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PLK 0.1
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B
RKO
pcDNA3.1
SRSF2
Migration
Invasion
RKO
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pcDNA 3.1
SRSF2
C
RKO
pcDNA3.1
SMC1A001
SRSF2
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SMC1A 001+PCNDA 3.1
PCDNA 3.1+SRSF2
SMC1A 001+SRSF2
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Hypoxia+NC
Normoxia+SMC1A001siRNA
Hypoxia+SMC1A001siRNA
E
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145 kDa
β-actin
42 kDa
RKO
Relative SMC1A 001 protein expression
RKO
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F
PLK0.1
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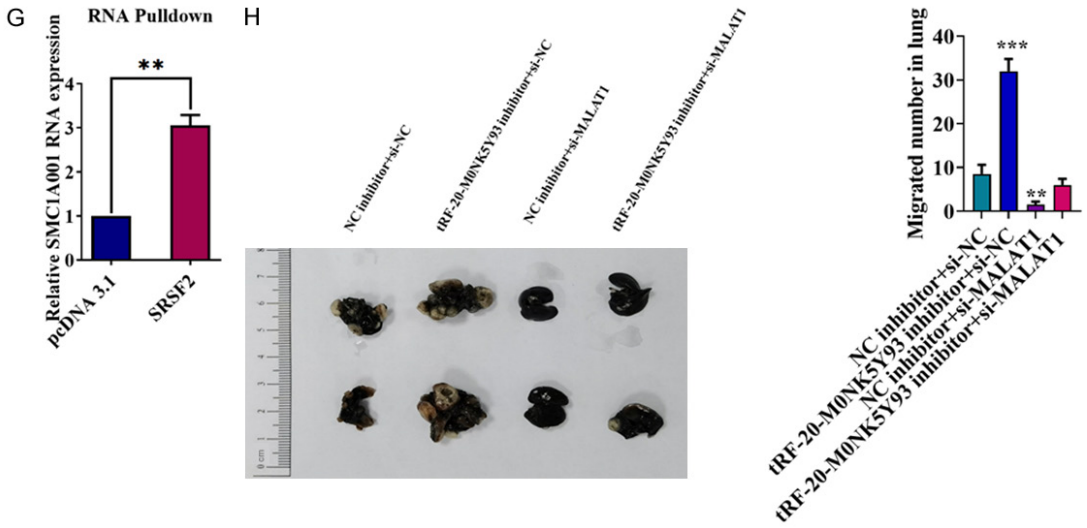

Figure 6. SRSF2 promotes the migration and invasion of colon cancer cells and regulates alternative splicing of SMC1. A. sh-SRSF2 transfection promoted the migration and invasion of both RKO cell lines. Scale bar = 120 μm. B. Transwell assays revealed that SRSF2 inhibited the migration of RKO cells. Scale bar = 120 μm. C. The assays showed that overexpression of SRSF2 could rescue the promoting role of SMC1A001 on the metastasis of colon cancer. Scale bar = 150 μm. D. SMC1A001 also promoted cell migration and invasion under hypoxia. Scale bar = 120 μm. E, F. SMC1A001 expression was downregulated when SRSF2 was overexpressed, and vice versa. G. RNA pulldown assays also confirmed that SMC1A001 was enriched when SRSF2 enriched much more SMC1A001 in RKO cells transfected with SRSF2 overexpression plasmids normalized to that in the pcDNA3.1 group, indicating that SRSF2 binds to SMC1A001 directly. H. The in vivo results showed that the tRF-20-M0NK5Y93 inhibitor promoted lung metastasis, while si-MALAT1 inhibited lung metastasis, and cotransfection of both inhibitors rescued the effect on the NC group.

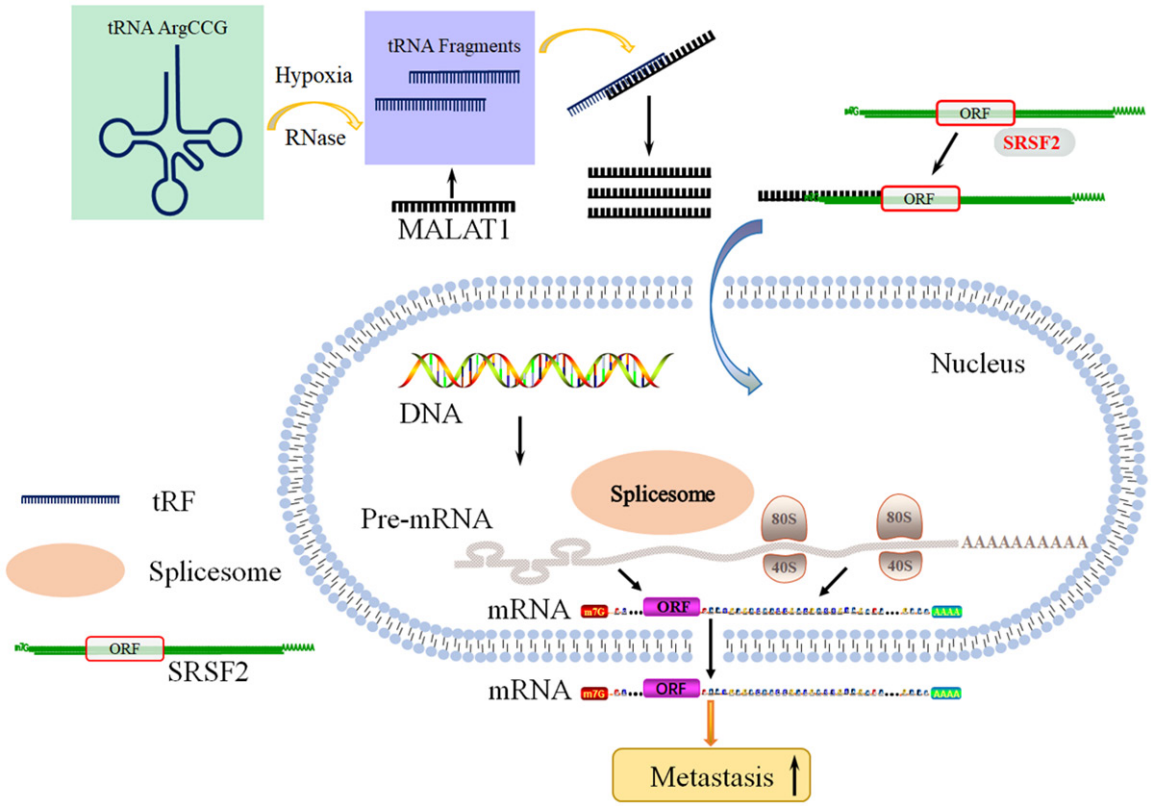

Figure 7. Working model. tRF-20-M0NK5Y93 binds the domain of MALAT1 and regulates its expression, giving rise to decreased SRSF2, which induces alternative splicing of SMC1A in colon cancer cells, facilitating colon cancer metastasis.

that tRF-20-M0NK5Y93, MALAT-1, and SRSF2 have significant expression correlations. The invasion and migration abilities of RKO and HCT116 cells were upregulated and downregulated, respectively, when transfected with the tRF-20-M0NK5Y93 inhibitor and mimics. Together, these data reveal that tRF-20-M0NK5Y93 may be a novel anticancer molecule in colon cancer and that tRF-20-M0NK5Y93 may be a promising treatment in colon cancer.

The SMC1A gene is a key molecule of the cohesin complex. The alteration or abnormal expression of cohesin will lead to abnormal separation of chromosomes during the synaptic process of mitosis, thereby initiating the occurrence and development of tumors [15]. Here, our study showed that the expression of the SMC1A001 transcript increased under hypoxia, and the expression of other short transcripts was decreased, as measured by qRT-PCR. This indicates that alternative splicing of SMC1A is related to the metastasis of colon cancer and partly explains the previous results that colon cancer cells have increased invasion and migration ability under hypoxia. In this study, we knocked down tRF-20-M0NK5Y93 to detect changes in the expression of SMC1A001 and found that SMC1A001 was upregulated at the RNA and protein levels, while other short isoforms were decreased.

tRFs are not well-understood, and their in-depth mechanisms have not been fully illustrated. In this study, a fully different model in which tRF-20-M0NK5Y93 could directly bind to the special sequence of lncRNA MALAT1 and negatively regulate its expression was found. *MALAT1* was originally discovered as a prognostic factor for metastasis in several human cancers, including lung cancer and breast cancer [16, 17]. Here, the results show that MALAT1 expression is higher in liver metastasis tissues than in primary tissues. We further show that overexpression of MALAT1 in CRC cells resulted in metastasis of these cells in vitro and in vivo. In contrast, knockdown of MALAT1 in CRC cells resulted in reduced proliferative capacity. The results indicate that MALAT1 is upregulated under hypoxia and acts as an oncogene. Interestingly, recent studies have shown that the MALAT1 gene is also mutated in other cancers, such as liver and breast cancers, and also functions as an oncogene [18-20]. Our data confirmed that MALAT1 played a strong oncogenic role by promoting the proliferation and migration of colon cancer cells.

Some tRFs exhibit a gene-silencing mechanism similar to that of typical microRNAs [21]. It has been demonstrated that all miRNAs are loaded into Argonaut proteins in the RNA-induced silencing complex (RISC) and act as posttranscriptional regulators by binding to the 3'-untranslated region (UTR) of mRNAs. This seed-dependent miRNA binding inhibits the translation and/or promotes the degradation of mRNA targets [22]. Our study demonstrated that there is a direct interaction between tRF-20-M0NK5Y93 and MALAT1 by dual-luciferase assays, and the downstream molecule MALAT1 is negatively regulated by tRF-20-M0NK5Y93. Thus, we speculated that tRF-20-M0NK5Y93 plays a role as microRNA, leading to the degradation of MALAT1.

Alternative splicing greatly improves the diversity and complexity of transcripts without changing the genome sequence and structure. However, abnormal alternative splicing may also lead to the occurrence and development of tumors. Many studies have shown that the disorder of alternative splicing directly affects the invasion, apoptosis, metabolism and other characteristics of cancer cells and is closely related to cancer. Multiple lncRNAs can be adjusted for alternative splicing. MALAT1, also known as nuclear enrichment abundance transcription 2 (Neat2), is one of these lncRNAs [23]. Selective snips are regulated by a variety of trans-acting protein factors, including a class of common RNA-binding proteins known as serine arginine (SR) family proteins [24, 25]. Although SR protein is widely expressed in cells and tissues, its cellular expression level is strictly regulated [26]. Tripathi et al. found that MALAT-1 can regulate alternative splicing by affecting the number and phosphorylation level

of SR proteins (SRSF1, SRSF2 and SRSF3) at the cellular level [6]. Furthermore, SRSF2 plays a critical role as an alternative splicing factor, suggesting that the oncogenic activity of MALAT1 may be based on SRSF2-dependent alternative splicing activities. Here, we found that the expression of SRSF2 was downregulated upon MALAT1 upregulation. Then, RIP assays further demonstrated that MALAT1 and SRSF2 directly interact. The rescue experiment results suggest that SRSF2 mediates the alternative splicing of tumor progressive targets and plays an essential role in MALAT1-mediated tumor metastasis. In conclusion, MALAT1 induces SRSF2 downregulation, activating a pro-oncogenic alternative splicing program.

It is well-known that isoforms of the SMC1A gene, only SMC1A001, SMC1A201, SMC1A-003, and SMC1A005, can be translated into mature protein products. Therefore, we explored the effects of MALAT1 on the alternative splicing activity of the well-established SRSF2 target SMC1A, which is known to be involved in invasion and migration. Accordingly, the results indicated that the downregulation of SRSF2, a splicing factor, can increase the activity of adjacent splice sites, resulting in increased splicing of the long transcript SMC1A001 and decreased expression of short transcripts. However, the reason why the expression of SMC1A001 is upregulated when SRSF2 expression is decreased remains obscure. Here is our hypothesis. One of the ways that SRSF works is to recognize and bind ESE (enhancer of exon splicing) or ISE (enhancer of intron splicing) to increase the activity of adjacent splice sites. Therefore, SRSF2 may play a role in promoting splicing by binding to the ESE of the SMC1A pre-mRNA. The downregulation of SRSF2 expression weakened the effect of promoting the splicing of exon X, resulting in increased transcripts such as SMC1A001, thereby promoting the metastasis of colon cancer.

Conclusion

In short, this study revealed that tRF-20-M0NK5Y93 acts as a tumor suppressor in colon cancer. tRF-20-M0NK5Y93 can bind the special sequences of MALAT1, resulting in the upregulation of MALAT1. MALAT1 exerts its oncogenic effect by inducing SRSF2-dependent SMC1A mRNA alternative splicing. These findings regarding tRF-20-M0NK5Y93 provide novel insight into the molecular interaction between tRNA fragments and lncRNA and may be helpful in developing precise approaches for tumor screening and treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

sncRNAs, Small noncoding RNAs; ncRNA, Noncoding RNA; tRFs, tRNA-derived fragments; lncRNA, Long noncoding RNA; MALAT-1, Metastasis-Associated Lung Adenocarcinoma Transcript 1; SMC1A, Structural Maintenance of Chromosomes 1A; RT-PCR, Reverse Transcription PCR; MTT, 3-(4,5)-dimethylthiahiazo(-z-yl)-3,5-diphenytetrazoliumromide; RIP, RNA immunoprecipitation; NC, Negative Control; SDS-PAGE, Sodium Dodecyl Polyacrylamide Gel Electrophoresis; PVDF, Polyvinylidene Fluoride.

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Table S1. Small interfering RNA (siRNA) of si-MALAT1 and its corresponding siRNA control

	Sequences
siR-NC sense	UUCUCCGAACGAGUCACGUTT
siR-NC antisense	ACGUGACUCGUUCGGAGAATT
hMALAT1 si-1 sense	GCUUAGUUGGUCUACUUUAAA
hMALAT1 si-1 antisense	UAAAGUAGACCAACUAAGCGA
hMALAT1 si-2 sense	GGAGAAUUGCGUCAUUUAAAG
hMALAT1 si-2 antisense	UUAAAUGACGCAAUUCUCCCT
hMALAT1 si-3 sense	CAAUAGAGGCCCUCUAAAUAA
hMALAT1 si-3 antisense	AUUUAGAGGGCCUCUAUUGCC

Table S2. The forward and reverse primers of Gene

	primer sequence
tRF-20-M0NK5Y93	F: CAGTGCAGGGTCCGAGGTAT
	R: CCTTCCAAGCAGT
SMC1A001	F: TGGTTGCCAATTACACTGCC
	R: TTGATACATGCCACCCCTGT
SMC1A201	F: CTCTCGCCTCGCATTGGTA
	R: GGGATCGACACCTCAAGCAT
SMC1A 001+003+002	F: GGGTGCTGTGGAATCTAT
	R: CTCTTTGGGGTTCTTCAT
SMC1A001+005	F: ACCGCTTCAATGCTTGTT
	R: GGCTCTTCAGGGTTCTCA
SMC1A001+201	F: ACCGCTTCAATGCTTGTT
	R: GGCTCTTCAGGGTTCTCA
MALAT1	F: CGGGTGTTGTAGGTT
	R: TCAGGGTGAGGAAGTA
SRSF2	F: CTGCGGGTGCAAATGG
	R: CTGGAGACCGACGAGGA

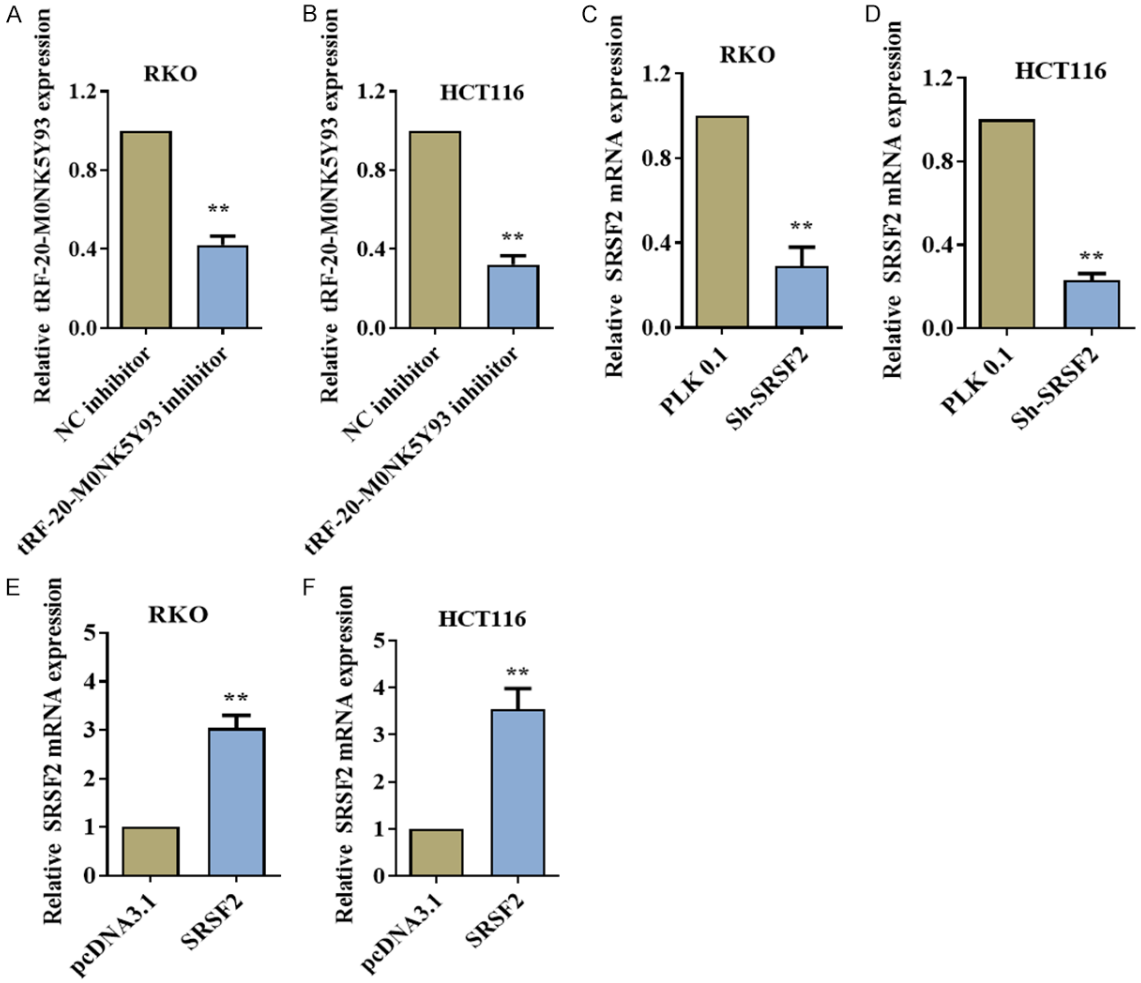
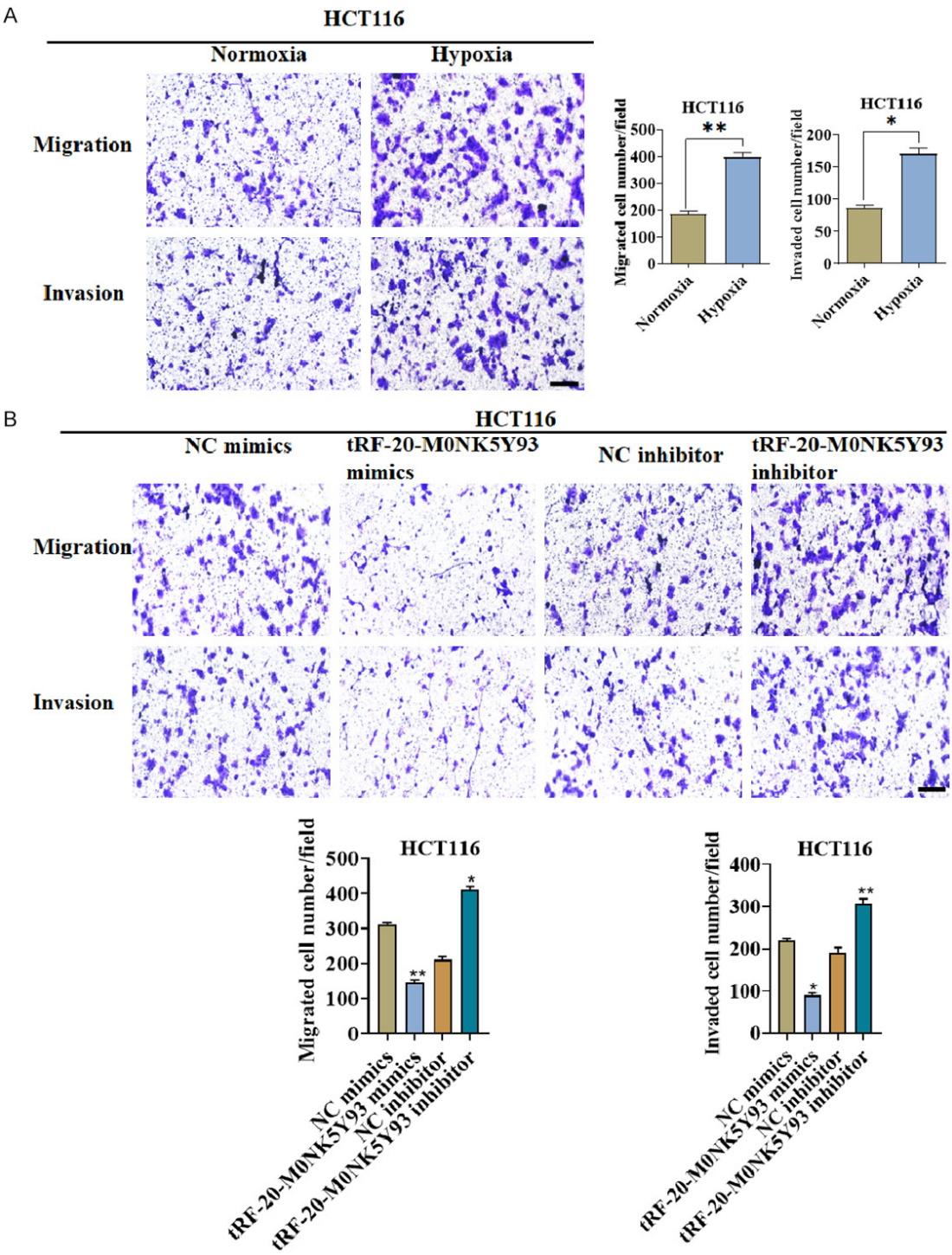


Figure S1. A, B. The analysis showed down-regulated expression of tRF-20-M0NK5Y93 a significant decline in tRF-20-M0NK5Y93 expression in the inhibitor group compared with the NC inhibitor group. C, D. qRT-PCR results pooled from ten independent experiments showed that shRNA (sh-SRSF2) significantly diminished SRSF2 in RKO and HCT116 cells. E, F. SRSF2 overexpression plasmids into RKO and HCT116 cells. The transfection efficiency was tested by qRT-PCR.


A
HCT116
Normoxia
Hypoxia
Migration
Invasion
HCT116
Migrated cell number/field
**
Normoxia
Hypoxia
HCT116
Invaded cell number/field
*
Normoxia
Hypoxia
B
HCT116
NC mimics
tRF-20-M0NK5Y93 mimics
NC inhibitor
tRF-20-M0NK5Y93 inhibitor
Migration
Invasion
HCT116
Migrated cell number/field
NC mimics
tRF-20-M0NK5Y93 mimics
NC inhibitor
tRF-20-M0NK5Y93 inhibitor
HCT116
Invaded cell number/field
NC mimics
tRF-20-M0NK5Y93 mimics
NC inhibitor
tRF-20-M0NK5Y93 inhibitor

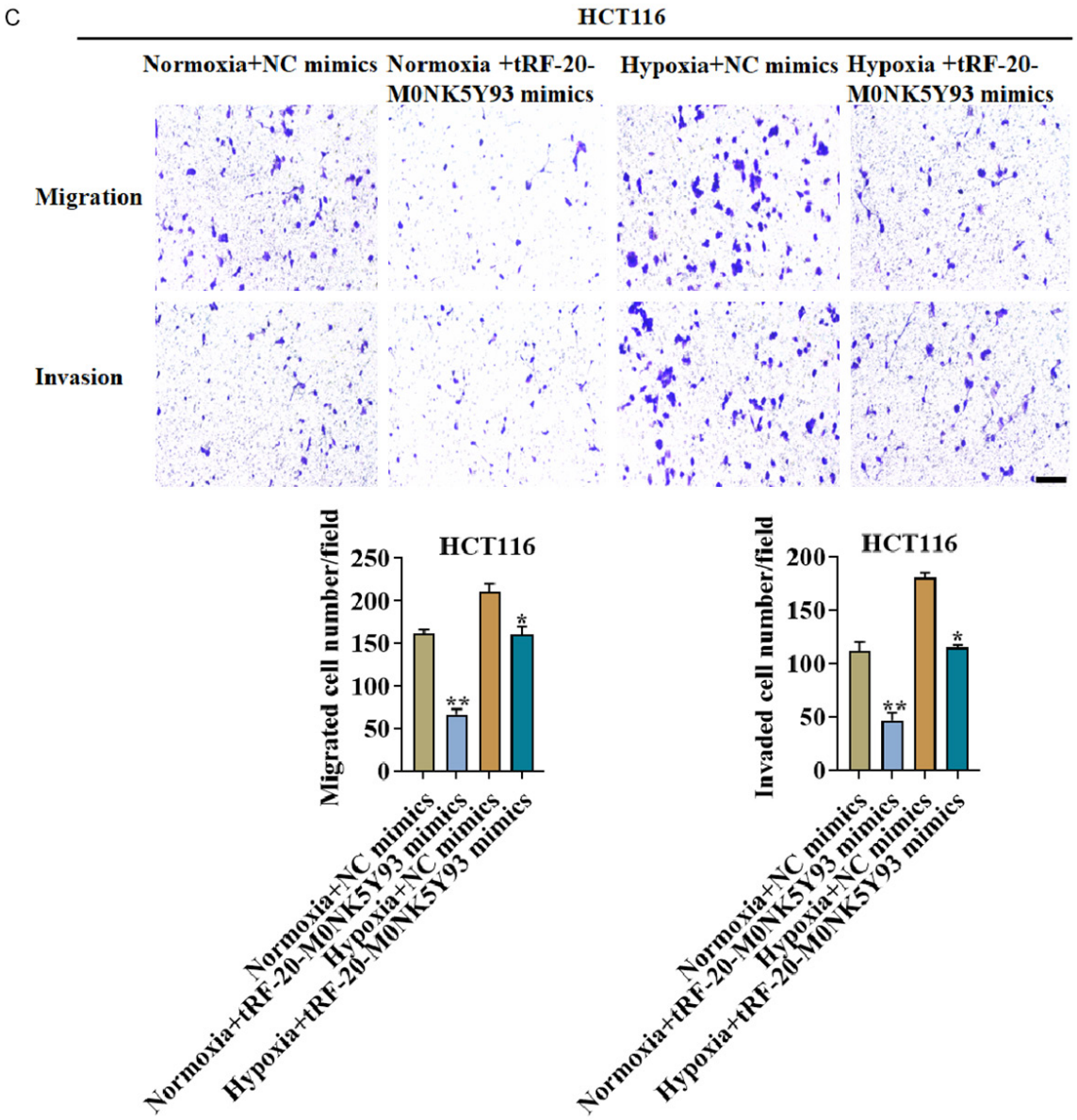
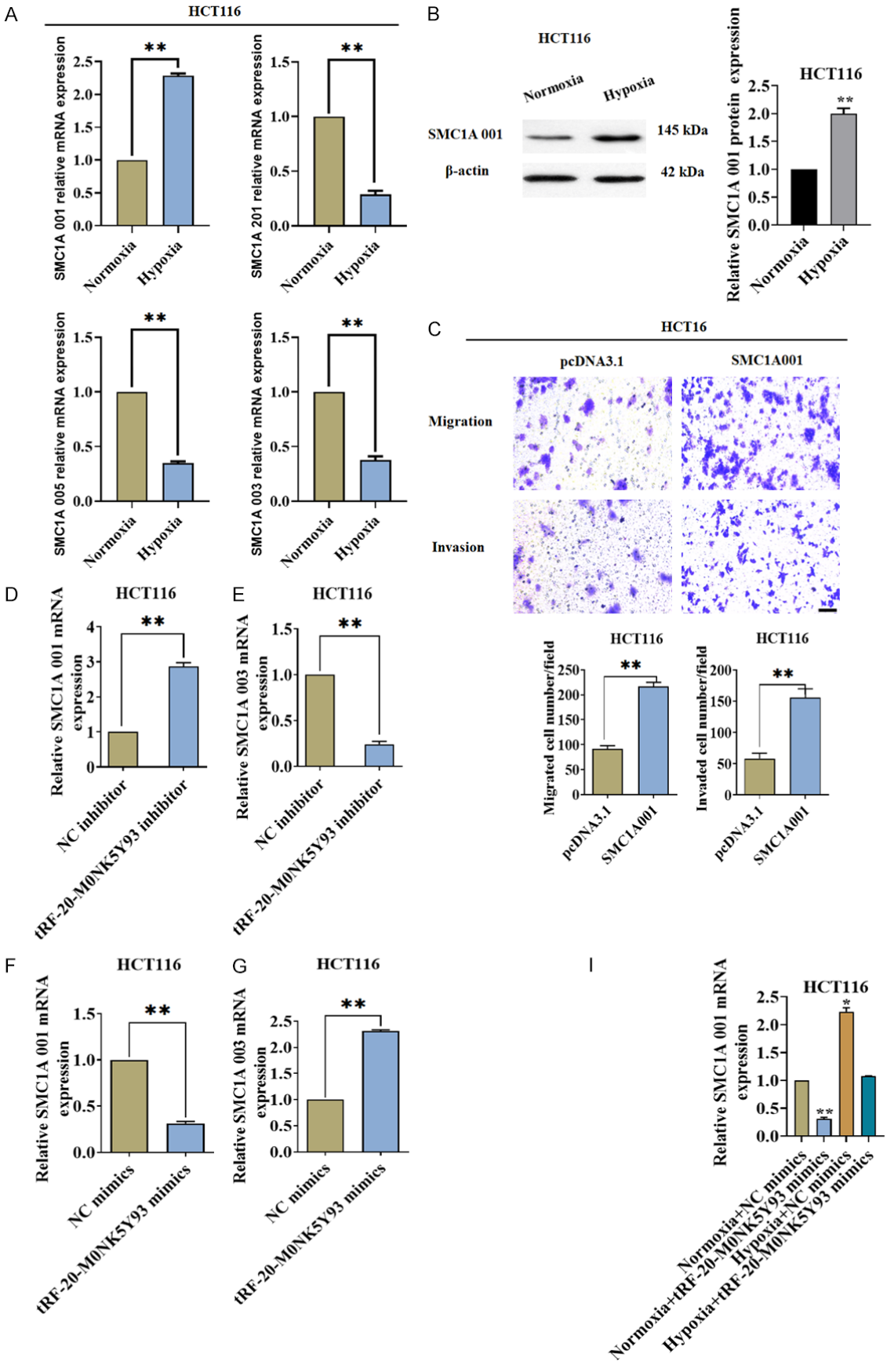


Figure S2. A. The Transwell assays showed that the number of migrated and invaded cells was significantly increased in HCT116 cells under hypoxia compared with normoxia. B. tRF-M0NK5Y93 knockdown promoted the migration of HCT116 cells. C. The cell migration and invasion of HCT116 cells can be promoted by hypoxic conditions, while overexpression of tRF-20-M0NK5Y93, which inhibited the invasion and migration of HCT116 cells, effectively impaired the role of hypoxia.


A
HCT116
SMC1A 001 relative mRNA expression
SMC1A 201 relative mRNA expression
SMC1A 005 relative mRNA expression
SMC1A 003 relative mRNA expression
Normoxia
Hypoxia
B
HCT116
Normoxia
Hypoxia
SMC1A 001
β-actin
145 kDa
42 kDa
Relative SMC1A 001 protein expression
C
HCT16
pcDNA3.1
SMC1A001
Migration
Invasion
Migrated cell number/field
Invaded cell number/field
D
Relative SMC1A 001 mRNA expression
NC inhibitor
tRF-20-M0NK5Y93 inhibitor
E
Relative SMC1A 003 mRNA expression
F
Relative SMC1A 001 mRNA expression
NC mimics
tRF-20-M0NK5Y93 mimics
G
Relative SMC1A 003 mRNA expression
I
Relative SMC1A 001 mRNA expression
Normoxia+NC mimics
Normoxia+tRF-20-M0NK5Y93 mimics
Hypoxia+NC mimics
Hypoxia+tRF-20-M0NK5Y93 mimics

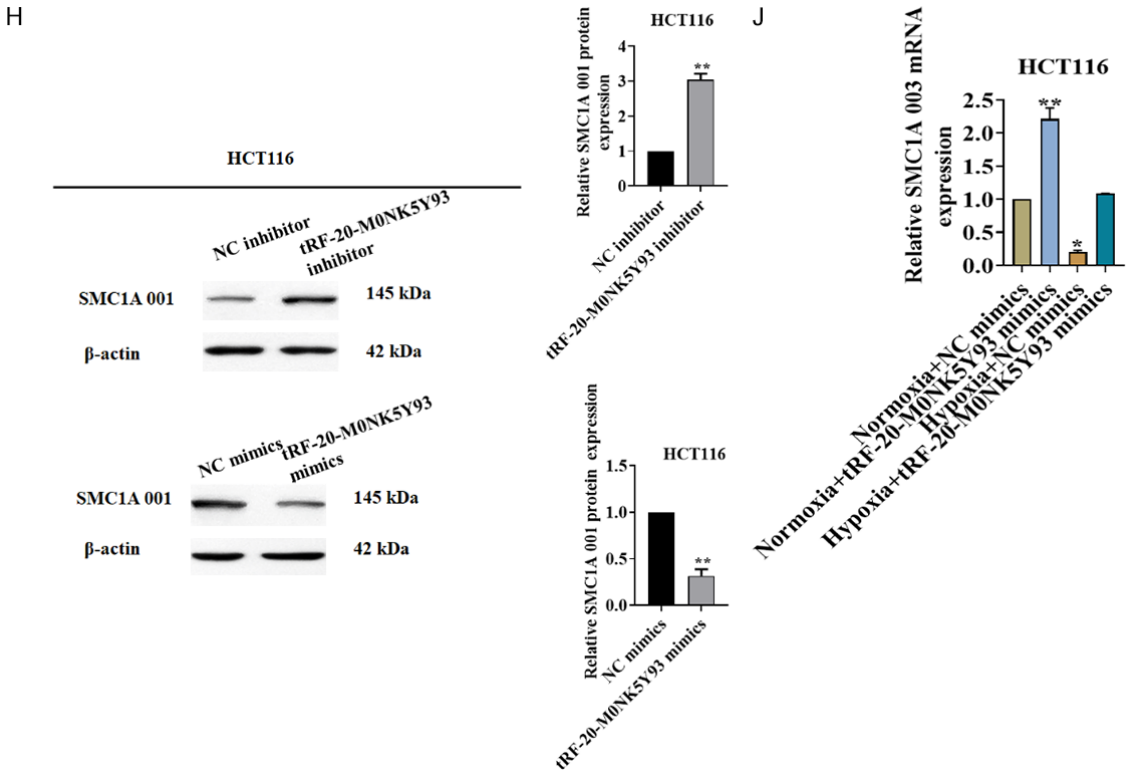


Figure S3. A. The expression of SMC1A transcript 001 increased under hypoxic conditions, while the expression of other transcripts 201, 005, and 003 decreased (**Figure 3A**). B. The results of Western blot assays also showed that SMC1A001 increased under hypoxia in comparison to normoxia. C. The assay results showed that SMC1A001 overexpression clearly promoted HCT116 cell migration and invasion in comparison to the negative control groups. D, F, H. The results suggested that the SMC1A001 expression level in the tRF-20-M0NK5Y93 inhibitor group was clearly higher than that in the negative control inhibitor group, while its expression level in the tRF-20-M0NK5Y93 mimic group was significantly lower than that in the negative control mimic group. E, G. However, the change in the expression of SMC1A003 was the opposite. I, J. When we took hypoxia into consideration, the overexpression of tRF-20-M0NK5Y93 weakened the effect of hypoxia in modulating the expression of SMC1A001 and SMC1A003.

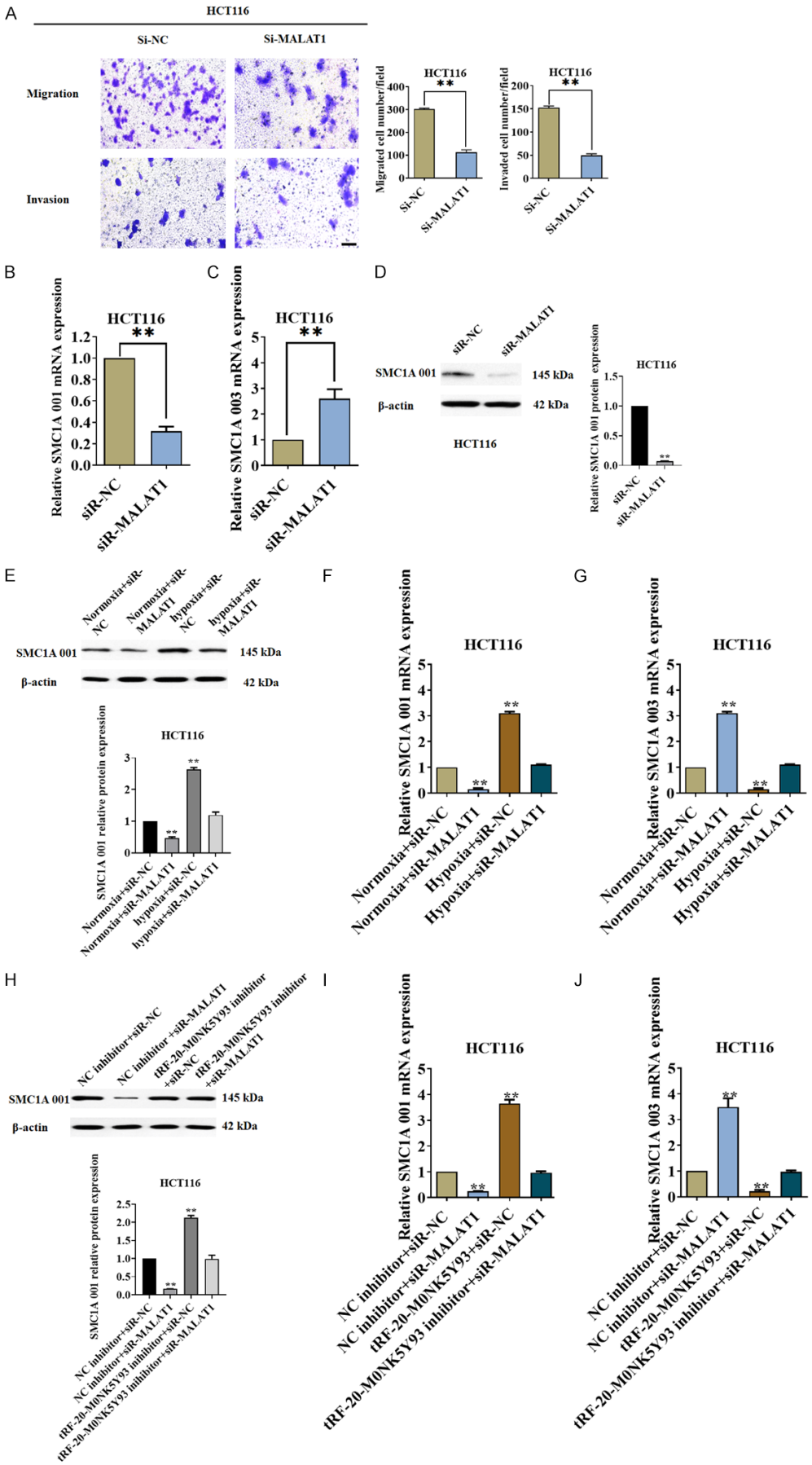

A
HCT116
Si-NC
Si-MALAT1
Migration
Invasion
Migrated cell number/field
Invaded cell number/field
B
Relative SMC1A 001 mRNA expression
siR-NC
siR-MALAT1
C
Relative SMC1A 003 mRNA expression
D
SMC1A 001
β-actin
145 kDa
42 kDa
Relative SMC1A 001 protein expression
E
Normoxia+siR-NC
Normoxia+siR-MALAT1
hypoxia+siR-NC
hypoxia+siR-MALAT1
SMC1A 001 relative protein expression
F
Relative SMC1A 001 mRNA expression
G
Relative SMC1A 003 mRNA expression
H
NC inhibitor+siR-NC
NC inhibitor+siR-MALAT1
tRF-20-M0NK5Y93 inhibitor +siR-NC
tRF-20-M0NK5Y93 inhibitor +siR-MALAT1
I
J

Figure S4. A. After we transfected MALAT1 siRNA into RKO cells, the invasion, migration and proliferation abilities of the cells all decreased. B, D. Additionally, the mRNA and protein expression of SMC1A001 was downregulated after we transfected MALAT1 siRNA into RKO cells. C. while the expression of SMC1A003 was upregulated after we transfected MALAT1 siRNA into HCT116 cells. E-G. both the mRNA and protein levels of SMC1A001 decreased compared to those under hypoxia only, while the expression of SMC1A003 increased. H-J. Furthermore, after we knocked down tRF-20-M0NK5Y93 and MALAT1 together, the results showed that the expression level of SMC1A001 was increased and the expression level of SMC1A003 was decreased compared with knocking down MALAT1 only.

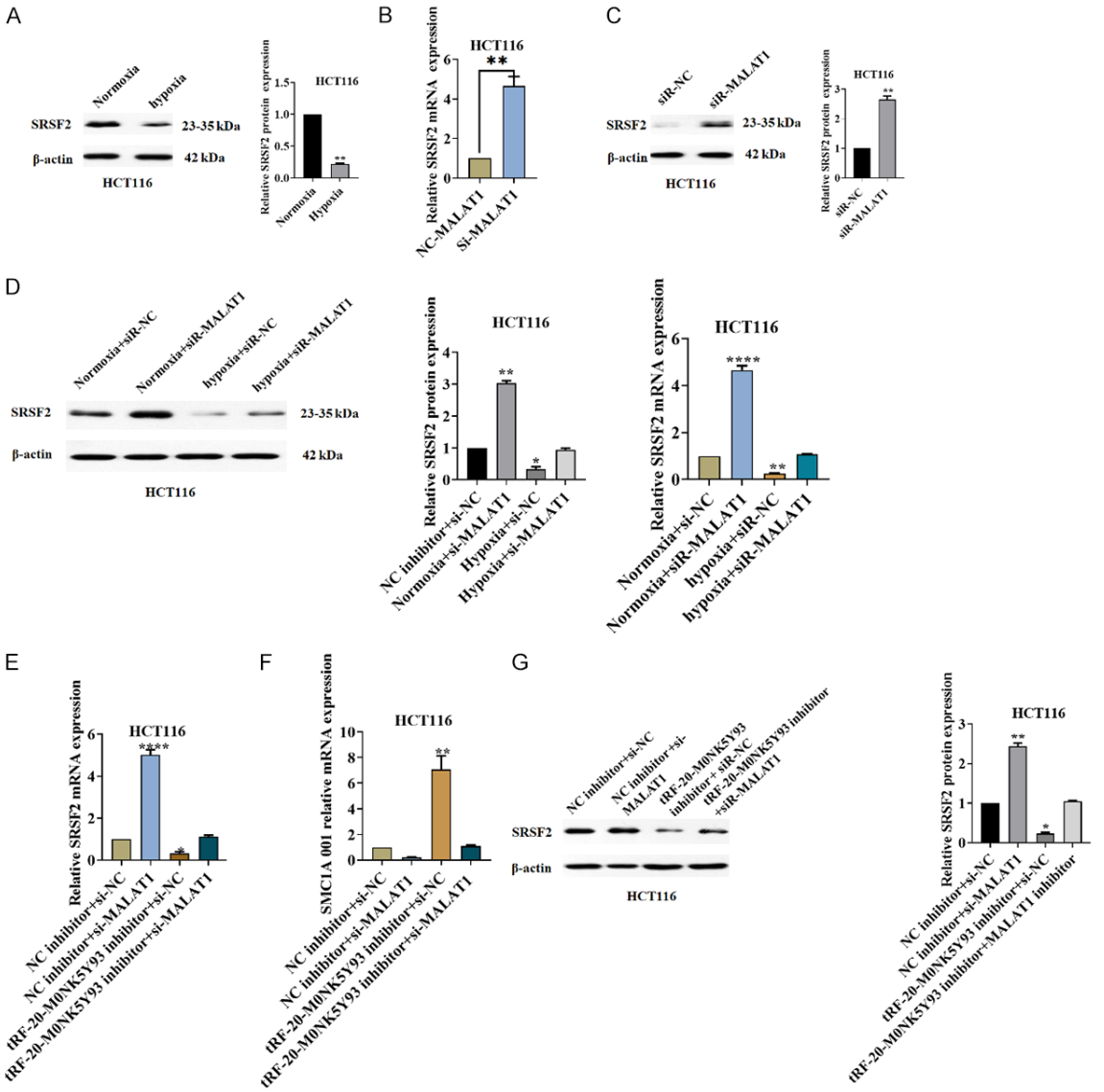


Figure S5. A. SRSF2 was down-regulated under hypoxia conditions in HCT116 cells. B, C. The results revealed that downregulation of MALAT1 expression significantly increased SRSF2 expression. D. This phenomenon could be reversed by hypoxia. E-G cotransfection with the tRF-20-M0NK5Y93 inhibitor abolished the effect of si-MALAT1 on promoting SRSF2 expression and decreased the expression of SMC1A001.

Table S3. sh-SRSF2 sequence

shRNA	sh-SRSF2 sequence
shRNA 1	CCGGCGACTTCAGATTTCATTATTGCTCGAGCAATAATGAAATCTGAAGTCGTTTTTG
shRNA 2	CCGGGTATCGGCAAGCAGTGTAAACCTCGAGGTTTACACTGCTTGCCGATACTTTTTG
shRNA 3	CCGGCGGGCCTTGCATATAAATAACCTCGAGGTTATTTATATGCAAGGCCCGTTTTTG

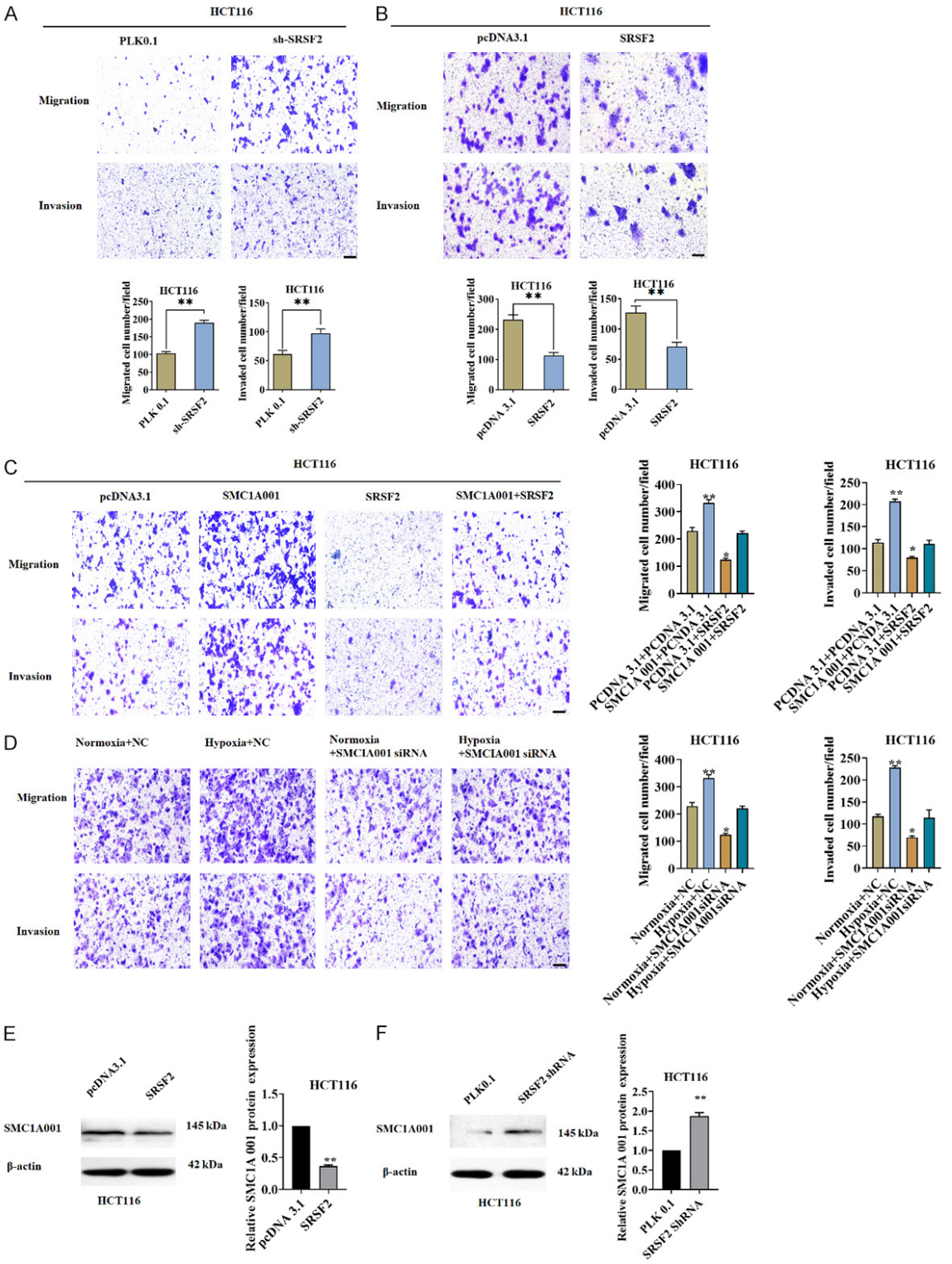


Figure S6. A. sh-SRSF2 transfection promoted the migration and invasion of both HCT116 cell lines. B. Transwell assays revealed that SRSF2 inhibited the migration of HCT116 cells. C. We cotransfected SRSF2 and SMC1A001 overexpression plasmids. The assays showed that overexpression of SRSF2 could rescue the promoting role of SMC1A001 on the metastasis of colon cancer. D. The results showed that SMC1A001 could also promote cell migration and invasion under hypoxia. E, F. SMC1A001 expression was downregulated when SRSF2 was overexpressed, and vice versa.

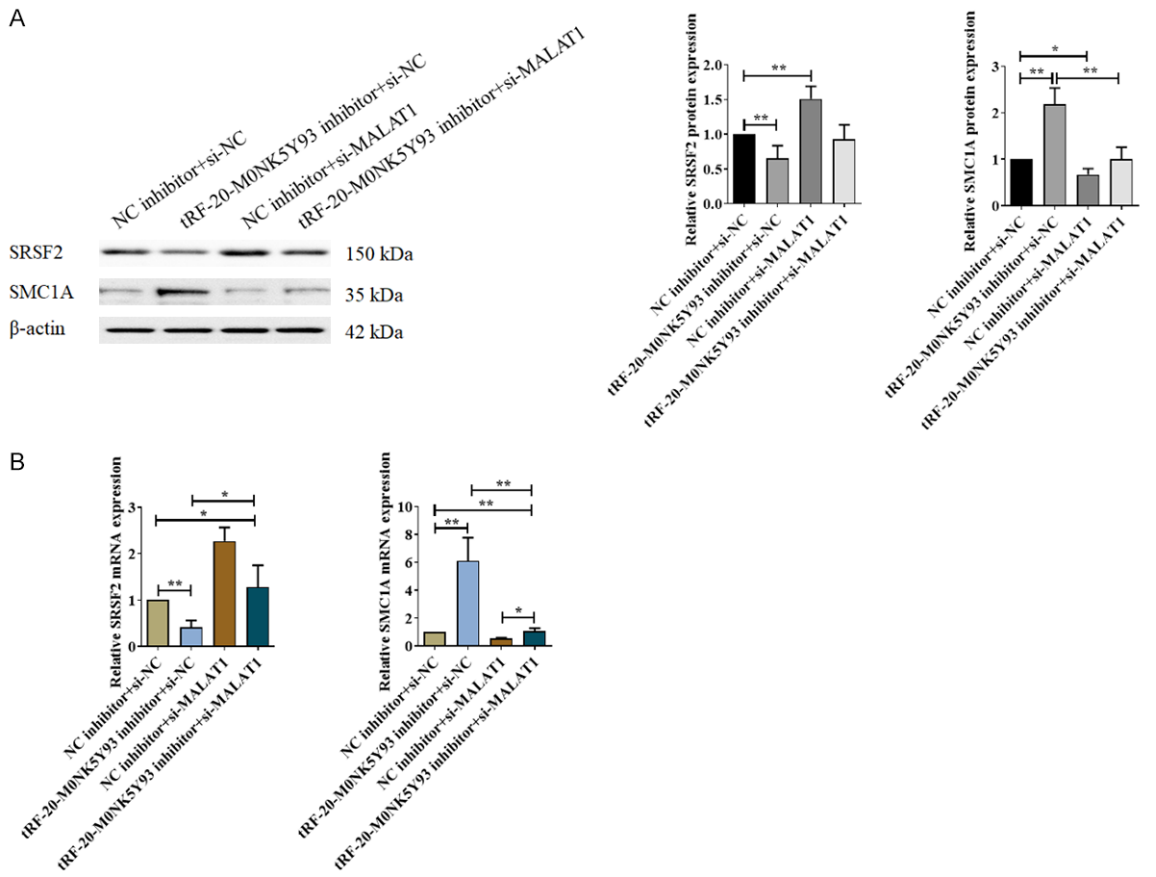


Figure S7. A. The protein expression of SRSF2 and SMC1A in the tumor tissues of the four groups of mice is shown. B. The mRNA expression levels of the two molecules are shown.