The Long Non-coding RNA TMPO-AS1 Promotes Bladder Cancer Growth and Progression via OTUB1-induced E2F1 Deubiquitination

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Research

Keywords: Long non-coding RNA, bladder cancer, TMPO-AS1, E2F1 , OTUB1, loop

Posted Date: September 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-74122/v1

The long non-coding RNA TMPO-AS1 promotes bladder cancer growth and progression via OTUB1-induced E2F1 deubiquitination

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Abstract

Background

Bladder cancer (BC) is the most common malignant tumor of the urinary system. Increasing evidence indicates long non-coding RNAs (lncRNAs) play crucial roles in cancer tumorigenesis, development, and progression. However, the role of TMPO antisense RNA 1 (TMPO-AS1) is still need to be explored in BC.

Methods

The lncRNA TMPO-AS1 expression was evaluated by bioinformatics analysis and further validated by qRT-PCR. Loss- and gain-of- function assays were performed to determine the biological functions of TMPO-AS1 in BC proliferation, migration, and invasion. Chromatin immunoprecipitation, luciferase reporter assays, western blotting, RNA pull-down, RNA immunoprecipitation assays, and fluorescence in situ hybridization were conducted to explore the molecular mechanisms of TMPO-AS1/E2F transcription factor 1 (E2F1) loop.

Results

TMPO-AS1 is upregulated in bladder cancer and is associated with BC patients' poor prognoses. Functional experiments demonstrated that

TMPO-AS1 promotes bladder cancer cell proliferation, migration, invasion, and inhibits cell apoptosis *in vivo* and *in vitro*. Mechanically, E2F1 is responsible for the TMPO-AS1 upregulation. Additionally, TMPO-AS1 facilitates the interaction of E2F1 with OTU domain-containing ubiquitin aldehyde binding 1 (OTUB1), leading to E2F1 deubiquitination and stabilization, thereby promotes BC malignant phenotypes. Furthermore, rescue experiments showed that TMPO-AS1 promotes BC growth in an E2F1-dependent manner.

Conclusions

Our study is the first to uncover a novel positive regulatory loop of TMPO-AS1/E2F1 important for the promotion of BC malignant behaviors. The TMPO-AS1/E2F1 loop should be considered in the quest for new BC therapeutic options.

Keywords: Long non-coding RNA, bladder cancer, TMPO-AS1, E2F1 , OTUB1, loop

Background

Bladder cancer (BC) is the most common malignant tumor of the urinary system worldwide [1], 549,393 new cases and 199,922 cancer-related deaths were reported in 2018 [2]. The majority of BCs are urothelial cell carcinomas. Urothelial BC can be categorized into non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC). Approximately, 75% of BC patients exhibit NMIBC with high recurrence and progression, while the remaining 25% of BC patients present with MIBC and have a poor prognosis [3]. Although therapies including transurethral resection tumor, cystectomy, chemotherapy, radiation, and immunotherapy have contributed to the reduction of BC-associated morbidity/mortality, the five-year survival rates of BC patients have hardly improved, causing a heavy burden on the health care systems worldwide [4]. Therefore, it is urgent to explore the molecular mechanisms and biomarkers of BC to develop better diagnostic, monitoring, and therapeutic approaches and reduce disease burden.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs, longer than 200 nucleotides with limited protein-coding potential [5]. Accumulating pieces of evidence have revealed that lncRNAs influence several biological/pathological processes, such as cell proliferation, metastasis, drug resistance, and metabolism, and are involved in multiple

diseases, particularly in cancer [6, 7, 8]. LncRNAs may be oncogenes or tumor suppressors via the regulation of gene expression (e.g. epigenetic regulation, transcriptional activation or repression, post-transcriptional modulation), or even via protein modification [9, 10, 11]. Previous studies have suggested that numerous lncRNAs are involved in BC [12, 13, 14]. For instance, MALAT1 promotes BC metastasis by associating with suz12 [15]. FOXD2-AS1 facilitates bladder tumor growth and recurrence via a positive feedback loop with Akt and E2F1 [16]. Moreover, recent studies have demonstrated that upregulated TMPO antisense RNA 1 (TMPO-AS1) serves as competing endogenous RNA to sponge microRNAs (miRNAs) in multiple carcinomas including hepatocellular carcinoma, thyroid cancer, lung adenocarcinoma, and breast cancer [17, 18, 19, 20]. However, whether TMPO-AS1 plays a role by interacting with other molecules in BC is an interesting question requiring further investigation.

Besides regulating mRNA transcription, transcription factors (TFs) are also involved in the regulation of lncRNA transcription [21]. E2F1, a member of the E2F transcription factor family consisting of eight proteins, is a transcription activator essential for the regulation of cell cycle, apoptosis, cell proliferation, and DNA damage response [22]. Studies have demonstrated that E2F1 can modulate the expression of

lncRNAs and coding genes [23, 24]. However, little is known on the E2F1-mediated lncRNAs regulation in BC. Protein stabilization can be modulated by ubiquitination and deubiquitination [25]. E2F1 was reported to be stabilized via preventing its ubiquitination [26]. POH1, a deubiquitinase, was reported to promote the development of liver cancer via E2F1 deubiquitylation [27]. However, no lncRNAs have been linked to E2F1 ubiquitination/deubiquitination.

Here, we focus on the roles of TMPO-AS1 in BC carcinogenesis and investigate the upstream and downstream regulations of TMPO-AS1. We demonstrate that E2F1 activates TMPO-AS1 transcription, on the other hand, TMPO-AS1 facilitates the interaction of E2F1 with OTUB1, a deubiquitinase, and consequently increases E2F1 protein levels via stabilization, promoting BC malignant phenotypes. Therefore, the TMPO-AS1/E2F1 positive feedback loop should be considered as a novel target for BC.

Materials and methods

Bioinformatics analysis

The lncRNA TMPO-AS1 expression data in 33 types of human cancers was obtained from the Gene Expression Display Server (GEDS) [28]. TMPO-AS1 expression in BC tissues and normal tissues were

analyzed using The Cancer Genome Atlas (TCGA) BLCA RNA-seq data retrieved from UCSC XENA (https://xena.ucsc.edu), TANRIC [29], Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov, GSE133624 and GSE120736 datasets) [30], The prognostic value of TMPO-AS1 was evaluated using GEPIA 2 (http://gepia2.cancer-pku.cn/#index) [31]. hTFtarget and ChIPBase v2.0 were used to find out the potential TFs of TMPO-AS1 [32, 33]; JASPAR 2018 database was utilized to identify the E2F1-TMPO-AS1 binding profile [34]. Genes co-expressed with TMPO-AS1 (TCGA-BLCA dataset) were defined as those with correlation coefficients $\geqslant 0.6$ and p values <0.01 using Co-LncRNA [35]. Pathway enrichment analysis was conducted using Metascape [36]. The interaction of E2F1, OTUB1, and TMPO-AS1 was predicted via catRAPID [37] and PRIdictor [38]. The interaction between E2F1 and OTUB1 proteins was predicted using HDOCK [39]. The ubiquitination sites of E2F1 were predicted using UbPred [40].

Clinical samples

Resected BC and normal adjacent specimens were collected from BC patients admitted to the Third Xiangya Hospital from 2016 to 2018; all patients were provided with written informed consent. Six pairs of BC and paired adjacent normal tissues were stored in liquid nitrogen at -80

°C. This study was approved by the ethics committee of the Third Xiangya Hospital, Central South University.

Cell culture and treatment

The human bladder cell lines BIU87, 5637, T24, EJ, and RT4 were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1 mmol/L glutamine and 100 U/mL penicillin/, at 37°C in an incubator with 5% CO_2. Protein synthesis inhibitor cycloheximide (10 μg/ml, Millipore, Sigma, C1998) and proteasome inhibitor MG132 (20 μM, Selleck, S2619) were used to examine ubiquitin proteasome-related protein degradation.

Quantitative real-time PCR

Total RNA was extracted from the BC tumor tissues, the paired adjacent normal tissues, and BC cells (T24 and RT4) using TRIzol (Invitrogen, , Carlsbad, CA, USA) according to the manufacturer's instructions. The PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) was used to synthesize complementary DNAs (cDNAs). qRT-PCR was performed using the SYBR Green PCR Master Mix (Toyobo, Osaka,

Japan), as per the manufacturer's instructions. β-actin and GAPDH served as the internal references. The $2^{-\Delta\Delta CT}$ method(Ct, cycle threshold) was applied to determine the relative gene expression. All experiments were performed in triplicate. The primer sequences used in this study are listed in Table 1.

Table 1. Primers used for qRT-PCR, siRNAs oligonucleotides, and ChIP

Primers used for qRT-PCR		
	TMPO-AS1-F	AGAGCCGAACTACGAACCAA
	TMPO-AS1-R	CTGTCCCTTATCGGCGTCT
	E2F1-F	ACGTGACGTGTCAGGACCT
	E2F1-R	GATCGGGCCTTGTTTGCTCTT
	β-actin-F	CATGTACGTTGCTATCCAGGC
	β-actin-R	CTCCTTAATGTCACGCACGAT
	U1-F	GGGAGATACCATGATCACGAAGGT
	U1-R	CCACAAATTATGCAGTCGAGTTTCCC
siRNAs oligonucleotides		
	TMPO-AS1-F	GAGCCGAACUACGAACCAACU
	TMPO-AS1-R	UUGGUUCGUAGUUCGGCUCUG
	E2F1	ACCTCTTCGACTGTGACTTTG
	OTUB1-F	AGCGACUCCGAAGGUGUUAAC
	OTUB1-R	GUUAACACCUUCGGAGUCGCU
	Negative control-F	UUCUCCGAACGUGUCACGUTT
	Negative control-R	ACGUGACACGUUCGGAGAATT
ChIP		
	TMPO-AS1-F	CAACAAGTGCGACACTCCAT
	TMPO-AS1-R	GTGTGGAGGGCTTTTTGAAC
	GAPDH-F	TACTAGCGGTTTTACGGGCG
	GAPDH-R	TCGAACAGGAGGAGCAGAGAGCGA

Cell transfection

For *in vitro* function assays, TMPO-AS1-targeted, E2F1-targeted, and OTUB1-targeted small interfering RNAs(si-TMPO-AS1, si-E2F1, si-OTUB1), and overexpression plasmids for TMPO-AS1 and E2F1(OE-

TMPO-AS1, OE-E2F1) as well as the empty vectors were purchased from GeneChem Co. (Shanghai, China) and transfected into T24 and RT4 cells using the Lipofectamine 3000 Reagent (Invitrogen Carlsbad, CA). For *in vivo* xenograft experiments, RT4 cells were stably transfected with the empty lentiviral vectors, sh-TMPO-AS1(designed according to si-TMPO-AS1), or sh-TMPO-AS1 together with E2F1 expressing lentiviral vectors purchased from GeneChem (Shanghai, China) according to manufacturers' protocol. The empty vector was used as the negative control. The transfection efficiency was determined via qRT-PCR.

Methyl thiazolyl tetrazolium (MTT) assay

The MTT assay was conducted according to the manufacturer's instructions. The BC cells were inoculated in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. Next, 10 μl of MTT solution (Sigma Chemicals, St. Louis, MO, USA) was added to each well and cells were cultured at 37°C for 4 h. Next, cell viability/proliferation was estimated via measuring the absorbance at 570 nm with the Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA).

Colony formation assay

Colony formation assay was performed as previously described [41]. BC cell proliferation was evaluated using colony formation assay. Briefly, T24 and RT4 cells, treated as described above, were seeded into 6-well plates at a density of 1000 cells/well and cultured for 2-3 weeks. Then, cells were washed with FBS, fixed with 4% paraformaldehyde, stained with 1% crystal violet, and counted (more than 50 cells).

Cell apoptosis analysis

Cell apoptosis in BC cells (T24 and RT4 cells) was investigated via flow cytometry using the Annexin V-PE/7-AAD kit (KA3809,Abnova,Wuhang, China), according to the manufacture's protocol.

Wound healing assay

A wound healing assay was conducted as previously described [42]. Briefly, cells were seeded into 6-well plates at a density at 1×10^5 cells/well. Then, a sterile 200 μl pipette tip was used to scratch a straight line in the cell monolayer. Next, cells were washed with FBS and cultured for 48 h. The scratch width was measured 48 h later.

Transwell assay

Transwell assay was conducted to evaluate cell migration and invasion. 5×10^4 BC cells (T24 and RT4) were suspended in serum-free medium and seeded into the upper chamber of 8 μm pores (Corning, Corning, NY, USA) with Matrigel (BD Biosciences, San Jose, CA, USA) while complete medium with 10% FBS was added to the lower chamber. After 48-h incubation, the migrating cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA), photographed under a microscope (DMB5-2231P1, Hong Kong, China).

Western blotting

Total protein was extracted using RIPA buffer (Beyotime, Shanghai, China) with a protease inhibitor cocktail (Roche, Basel Switzerland). Protein concentration was measured using a BCA kit (Thermo Fischer Scientific, Waltham, MA, USA). Protein samples were resolved via SDS-PAGE and transferred onto PVDF membranes. Next, the membranes were blocked in PBS containing 5% skim milk powder at room temperature for 1 h, and incubated with the primary antibodies at 4°C overnight, followed by the secondary antibodies. The protein bands were visualized using the Pierce® ECL Western Blotting Substrate kit

(32106;Thermo Scientific), and normalized by β-actin as reference. The antibodies used in this study were listed in Additional file 1: Table S1

Luciferase reporter assay

The wild-type or mutant-type of E2F1 binding sites to the promoter of TMPO-AS1 were synthesized and inserted into pGL3 vector (Promega, Madison, WI, USA), respectively. T24 and RT4 cells were seeded into 48-well plates and co-transfected with the above vectors along with E2F1 expression or control plasmids. Forty-eight hours later, the luciferase activity was measured and analyzed using the Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the EZ Magna ChIP™ kit (Millipore, Burlington, MA, USA) according to the manufacturers' instructions. Briefly, 1×10^7 cells (T24 and RT4) were fixed with 1% formaldehyde and treated with 10% glycine. Next, the cross-linked chromatin was broken into small DNA fragments via sonication. The sonicated DNA was immunoprecipitated using antibodies against E2F1 and control rabbit IgG (Bioss Antibodies Inc., Woburn,

MA, USA). qRT-PCR was performed to quantify the precipitated chromatin using the specific primers. The primers are listed in Table1.

RNA pull-down assay

TMPO-AS1 was transcribed *in vitro* and labeled with 3' end biotinylation. The RNA pull-down assay was performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fischer Scientific, Waltham, MA, USA). Briefly, the lysates of control or TMPO-AS1 overexpressing T24 and RT4 cells were incubated with control or biotinylated TMPO-AS1 at room temperature for 4h, followed by addition with streptavidin magnetic beads (Thermo Fisher, USA) at 4°C for 60 min with rotation. After three washing steps with washing buffer, the RNA-binding proteins were eluted using 50 μl elution buffer and analyzed via western blotting.

RNA immunoprecipitation (RIP)

RIP was performed using the EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Burlington, MA, USA) based on the manufacturer's instructions. Cell extracts were incubated with magnetic beads conjugated with antibodies against SNRNP70 (Cat.#CS203216), normal rabbit IgG(Cat.#PP64B), and anti-E2F1

(Cat.#OM250777). were used as positive and negative controls. Anti-SNRNP70 antibody was used as positive control, whereas normal rabbit IgG was used as a negative control. The relative abundance of TMPO-AS1 was normalized to the amount of enriched U1snRNA via qRT-PCR.

Co-immunoprecipitation (Co-IP)

Cell lysates were incubated with primary antibody against E2F1 (1:80, OM250777, Omnimabs) at 4℃ overnight. The rabbit IgG antibody (1:150, Bioss Antibodies Inc., Woburn, MA, USA) was used as a negative control. Next, cell lysates were mixed with protein A/G agarose (Cat. #P1012, Beyotime Biotechnology) at 4℃ for 2 h, followed by centrifugation and washing steps. The precipitated complex was separated using SDA-PAGE and analyzed via western blotting.

Fluorescence in situ hybridization (FISH)

FISH assay was performed using Ribo™ Fluorescent in Situ Hybridization (RiboBio, Guangzhou, China) following the manufacturer's instructions. TMPO-AS1 and 18S probes were synthesized and labeled with Cy3 fluorescent dye. Fluorescence was detected under a confocal laser microscope (Leica, SP5).

Immunohistochemistry (IHC)

IHC staining was performed as previously described[43]. Tissues sections obtained from paraffin-embedded tissues were dewaxed in xylene and rehydrated in an ethanol gradient. Next, tissues were incubated in 1 % hydrogen peroxide and boiled in citrate buffer (10 mM, pH=6.0) for 15 min. Subsequently, tissues were incubated with the primary antibodies against Ki-67 (1:1000;27309-1-AP, Proteintech, Chicago, IL, USA), E2F1 (1:200, OM250777, Omnimabs), and caspase-3(1:200,19677-1-AP, Proteintech) at 4℃ overnight, followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody. (SP-9000, zsbio, Beijing, China) at room temperature for 30 min. Diaminobenzidine was used for chromogen; hematoxylin was used as the nuclear counterstain.

Xenograft mouse model

The experiments were approved by the Animal Care and Use Committee of the Central South University. 1×10^6 RT4 cells transfected with the empty, TMPO-AS1 knockout, or depletion of TMPO-AS1 coupled with E2F1 lentiviral vectors were injected subcutaneously into the flanks of male BALB/c nude mice (4- to 6-week-old, n=4 per group) obtained from the Shanghai Experimental Laboratory Animal Center

(Shanghai, China). Tumor volumes were measured every 3 days and calculated as follows: tumor volume = $(D \times d^2)/2$, where D and d refer to the longest and shorter diameters, respectively. Mice were euthanized after twenty-five days.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software, version 8 (GraphPad Software, San Diego, CA, USA). Data are presented as the mean ± SD of at least three independent experiments. The relationship between E2F1 and TMPO-AS1 was analyzed using the Pearson correlation coefficient. Significant differences were analyzed using Student's t-test or the one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

TMPO-AS1 is upregulated in BC tissues and is associated with Poor Prognosis

To evaluate TMPO-AS1 expression in tumor and normal tissues, we used the online database GEDS and found that TMPO-AS1 expression is upregulated in multiple tumor tissues compared with that in normal tissues (Fig. 1a). Consistently, the expression of TMPO-AS1 was

upregulated in BC tissues compared with the normal tissues according to TCGA (Fig. 1b) and TANRIC databases (Fig. 1c). We further confirmed that TMPO-AS1 expression was higher in six paired BC tissues compared with the corresponding normal tissues via qRT-PCR (Fig. 1d). Moreover, the integrative analysis of GSE133624 and GSE120736 showed that TMPO-AS1 was not only highly expressed in BC tissues (Fig. 1e) , but also exhibited higher levels in MIBC *versus* NMIBC samples (Fig. 1f). Of note, higher expression of TMPO-AS1 was associated with BC recurrence (Fig. 1g), and advanced tumor stage (Additional file 2: Figure S1). Furthermore, BC patients with higher TMPO-AS1 expression levels were associated with shorter disease-free survival time (Fig. 1h). Taken together, these data suggest that TMPO-AS1 is highly expressed in BC tissues and is associated with poor prognosis.

TMPO-AS1 promotes cell proliferation, migration, and invasion of BC cells, and survival *in vitro*.

Since TMPO-AS1 expression was the highest in T24 and RT4 cells among five BC cell lines (BIU87, 5637, T24, EJ, and RT4) using qRT-PCR (Fig. 2a), they were selected for following experiments. To investigate the functions of TMPO-AS1, si-RNA targeting TMPO-AS1

and expression plasmid of TMPO-AS1 were transfected into T24 and RT4 cells. As shown in Fig. 2b, the transfection efficacy was examined using qRT-PCR. Next, loss- and gain-of-function assays were performed. The results showed that knockdown of TMPO-AS1 significantly inhibited cell viability/proliferation (Fig. 2c, d) , migration (Fig. 2e), and invasion (Fig. 2f), while TMPO-AS1 overexpression resulted in the opposite effects. Additionally, cell apoptosis was induced in TMPO-AS1-silenced T24 and RT4 cells, whereas overexpression of TMPO-AS1 inhibited cell apoptosis (Fig. 2g). Overall, these findings suggest that TMPO-AS1 plays an oncogenic role in BC cells, promoting their proliferation, migration, invasion, and survival *in vitro*.

E2F1 activates the transcription of TMPO-AS1 in BC cells

To figure out the underlying mechanism of TMPO-AS1-mediated carcinogenic properties in BC, we investigate the upstream and downstream genes of TMPO-AS1. Previous studies have revealed that TFs can serve as the upstream genes of lncRNAs and regulate their transcription [21, 44]. Therefore, we screened out the potential TFs of TMPO-AS1 using hTF target and ChIP base V2.0 (Fig. 3a, Additional file 3: Table S2). We obtained 36 candidate TFs for TMPO-AS1, Of note, E2F1 had the highest positive correlation with TMPO-AS1 among 36

TFs. (Additional file 3: Table S2). Importantly, a previous study reported that the overexpression of E2F3 induced the promoter activity of TMPO-AS1/LAP2, an antisense transcript [45]. Meanwhile, we analyzed genes co-expressed with TMPO-AS1 (correlation coefficient ≥0.6 and *p* value <0.01) in BC using Co-LncRNA (Additional file 4: Table S3). Additionally, we performed pathway analysis of TMPO-AS1-associated genes via Metascape and found that TMPO-AS1 is involved in the E2F pathway (Fig. 3b). Thus, we focused on E2F1 in the following research. E2F1 was positively correlated with TMPO-AS1 in BC as evidenced by TCGA and GSE133624 datasets (Fig. 3c, d). Furthermore, the expression of TMPO-AS1 was downregulated after E2F1 silencing, while upregulated in E2F1 overexpressing BC cells (Fig. 3e). The putative binding sites between TMPO-AS1 and E2F1 were at -945 ~ -935 bp upstream transcription start site as predicted by JASPAR(Fig. 3f). As expected, overexpression of E2F1 dramatically enhanced the luciferase activity of wild-type TMPO-AS1 promoter, whereas E2F1 failed to alter the transcriptional activity of mutant TMPO-AS1 promoter (Fig. 3g). Moreover, the results of ChIP assay demonstrated that E2F1 was remarkably enriched in TMPO-AS1 promoter region relative to the control IgG (Fig. 3h). Overall, these results indicate that E2F1 can bind to the promoter of TMPO-AS1 and activate its transcription.

TMPO-AS1 regulates E2F1 protein levels via protein stabilization

Given that lncRNAs have been reported to interact with E2F1 [46, 47], we then investigate whether TMPO-AS1 could interact with E2F1. As shown in Fig. 4a, a possible interaction between TMPO-AS1 and E2F1 was predicted by catRAPID. Consequently, we conducted RNA pull-down assays to validate the prediction. The results showed that E2F1 was abundantly enriched by TMPO-AS1 probe compared with the control oligo, especially in TMPO-AS1 overexpressing RT4 and T24 cells (Fig. 4b). Similarly, RIP results demonstrated that E2F1 remarkably immunoprecipitated TMPO-AS1, which was enhanced upon TMPO-AS1 overexpression in RT4 and T24 cells (Fig 4c). The interaction between TMPO-AS1 and E2F1 prompted us to investigate whether TMPO-AS1 influenced the expression of E2F1. Interestingly, overexpression or knockdown of TMPO-AS1 had no significant impact on *E2F1* mRNA expression (Fig. 4d), while notably increased or decreased E2F1 protein expression in RT4 and T24 cells (Fig. 4e), respectively, suggesting TMPO-AS1-mediated E2F1 regulation occurs at the post-transcriptional level. Then, we found that proteasome inhibitor MG132 markedly increased the stability of E2F1 protein in cycloheximide-treated RT4 and T24 cells (Fig. 4f), implying the turnover of E2F1 was subjected to

ubiquitin proteasome system. Importantly, silencing TMPO-AS1 significantly shortened the half-life of E2F1 (Fig. 4g) Altogether, our data demonstrate that TMPO-AS1 directly interacts with E2F1 and regulates its protein levels via protein stabilization.

TMPO-AS1 stabilizes E2F1 via OTUB1-mediated deubiquitination

To investigate the underlying mechanism of TMPO-AS1-mediated E2F1 stabilization. Firstly, we performed FISH assay and found that TMPO-AS1 is predominantly distributed in the cytoplasm in both T24 and RT4 cells (Fig. 5a), indicating TMPO-AS1 might involve in translational regulation. It has been proposed that POH1 (PSMD14), a deubiquitinase, binds to and deubiquitilates E2F1 protein, which contributes to E2F1 stabilization, thereby promotes liver cancer development[27]. Moreover, it was predicted that E2F1 has four potential ubiquitination sites by UbPred (Additional File 5: Fig S2A). Therefore, we hypothesized that TMPO-AS1 may associate with a specific ubiquitinase/deubiquitinase to regulate E2F1 ubiquitination. Since we have studied a group of deubiquitinases (OTUB1, UCHL5, USP5, COPS6, and PSMD14) in BC (unpublished data), we concentrated on

deubiquitinases in the following studies. We evaluated the binding potential of the above deubiquitinases to TMPO-AS1 using PRIdictor. The results highlighted OTUB1, as the deubiquitinase that most likely associates with TMPO-AS1(Additional File 5: Fig S2 B). To validate the prediction, we conducted RNA pull-down assays and found that TMPO-AS1 precipitated more OTUB1 than the other deubiquitinases (Fig. 5b). Similarly, RIP assays showed that TMPO-AS1 was obviously immunoprecipitated by the anti-OTUB1 antibody compared with IgG (Fig. 5c). Furthermore, CatRAPID showed that the region of TMPO-AS1 at 76-127 nucleotides exhibits a high potential of interaction with the OTUB1 amino acid residues at 51-152 (Additional File 5: Fig S2C), further supporting the association between TMPO-AS1 and OTUB1. Despite the direct association between TMPO-AS1 and OTUB1, TMPO-AS1 failed to alter OTUB1 protein expression (Fig. 5d). Obviously, OTUB1 knockdown led to a decrease in E2F1 protein levels in RT4 and T24 cells(Fig. 5e). In addition, as per HDock prediction, OTUB1 was very likely to bind to E2F1 (Fig. 5f). Importantly, the results of Co-IP assays showed that silencing TMPO-AS1 not only increased E2F1 ubiquitination, but also mitigated the interaction between OTUB1 and E2F1(Fig. 5g), which could be rescued by overexpression of TMPO-AS1. Furthermore, knockdown of OTUB1 significantly reversed the decreased

ubiquitination of E2F1 induced by overexpression of TMPO-AS1 in RT4 and T24 cells(Fig. 5h). Taken together, these results indicate that TMPO-AS1 upregulates E2F1 protein levels via OTUB1-mediated deubiquitination and the consequent protein stabilization.

E2F1 promotes cell proliferation, migration, and invasion of BC cells, and inhibits cell apoptosis *in vitro*

E2F1 is associated with cell proliferation, cell cycle progression, apoptosis, cancer metastasis, and invasiveness [48]. Hence, we speculated that E2F1 would promote BC tumorigenesis and development. According to the data retrieved from TCGA and the GEO (GSE133624) databases, the expression of E2F1 was remarkably higher in BC tissues than that in normal tissues (Fig. 6a,b). Furthermore, western blotting and IHC analyses showed that the expression of E2F1 was upregulated in six paired BC tissues compared with the adjacent normal tissues (Fig. 6c,d). As anticipated, further function experiments demonstrated that E2F1 knockdown significantly inhibited cell proliferation (Fig. 6e, f), migration(Fig. 6g), invasion (Fig. 6h) , and induced apoptosis (Fig. 6i), whereas the overexpression of E2F1 led to the opposite effects. Altogether, these results reveal that E2F1 promotes malignant phenotypes of BC cells.

TMPO-AS1 regulates malignant phenotypes of BC via E2F1 *in vitro*

Since overexpression of either E2F1 or TMPO-AS1 enhanced the proliferation, migration, and invasion of BC cells, and TMPO-AS1 upregulated E2F1 protein levels, we hypothesized that TMPO-AS1 would promote BC progression via E2F1. To prove this theory, we restored E2F1 expression in TMPO-AS1-silenced BC cells (Fig. 7a) and performed MTT, colony formation, wound healing , and transwell assays As shown, E2F1 restoration in BC cells significantly reversed the inhibition on cell proliferation (Fig. 7b, c), migration (Fig. 7d) and invasion (Fig. 7e) induced by silencing TMPO-AS1. Furthermore, TMPO-AS1 silencing-mediated promotion of apoptosis could be abrogated by E2F1 overexpression (Fig. 7f). Therefore, our data demonstrate that TMPO-AS1 promotes BC cell proliferation, migration, invasion, and survival via E2F1 *in vitro*.

TMPO-AS1 regulates BC growth via E2F1 *in vivo*

To investigate whether TMPO-AS1 would promote BC growth via E2F1 in vivo, we performed xenograft experiments. RT4 cells with the negative control, stably knockdown of TMPO-AS1, or co-treatment of TMPO-AS1 depletion and E2F1 were subcutaneously injected into the

flanks of nude mice, respectively. Every 5 days, the tumor volumes were measured. Results showed that TMPO-AS1 knockout significantly suppressed tumor growth compared to the control group, whereas E2F1 overexpression abolished the inhibitory effect on tumor growth induced by TMPO-AS1 knockout (Fig. 8a,b). Furthermore, IHC staining showed that TMPO-AS1 depletion led to a substantial decrease of Ki-67 and E2F1 protein levels, and to a notable increase in the expression of caspase-3. Of note, this phenotype was reversed by the overexpression of E2F1 (Fig. 8c). Collectively, the above findings demonstrate that TMPO-AS1 regulates tumor growth of BC via E2F1 *in vivo*.

Discussion

Bladder cancer is the most common urinary system malignancy, causing heavy burden worldwide. Therefore, it is imperative to elucidate the carcinogenesis of BC. In our study, we show that E2F1 stimulates TMPO-AS1 transcription. In turn, TMPO-AS1 facilitates the interaction of E2F1 with OTUB1, thereby upregulates E2F1 protein levels. Altogether, our study highlights TMPO-AS1 forms a positive feedback loop with E2F1 in BC, and suggests that both TMPO-AS1 and E2F1 are potential therapeutic targets for BC patients.

An increasing number of studies have demonstrated lncRNAs play a role in tumor initiation, development, and progression in numerous cancers, including BC [13, 16, 49]. Here, we found that TMPO-AS1 is highly expressed in BC, and correlates with poor prognoses. We performed *in vitro* experiment and proved that TMPO-AS1 could function as an oncogene, consistent with previous studies [17, 50, 51, 52]. Our studies suggests TMPO-AS1 may be a potential therapeutic target in BC patients.

LncRNAs are regulated by multiple transcription factors. E2F1 is a potent transcription factor, already demonstrated to regulate the expression of lncRNAs [47, 51, 53]. Thousands of abnormally expressed lncRNAs have been reported in BC [14]. However, little is known about the upstream regulation of these lncRNAs. For instance, how E2F1 regulates the expression of lncRNAs is yet to be fully understood. In the present study, we found that TMPO-AS1 positively correlates with E2F1 and that E2F1 activates the transcription of TMPO-AS1. Therefore, our data, showing that TMPO-AS1 is upregulated in BC due to E2F1, contribute to a better understanding of the regulatory mechanisms upstream of lncRNAs.

The function of a lncRNA depends largely on its subcellular location.

549 LncRNAs enriched in nucleus are involved in transcriptional regulation,
550 chromosomal interactions, and RNA processing, while cytoplasmic-
551 enriched lncRNAs participate in the post-transcriptional and translational
552 regulation [54]. In the present study, TMPO-AS1 was identified to be
553 enriched in both the nucleus and cytoplasm of BC cells based on FISH
554 assay. Of note, we found TMPO-AS1 directly binds to E2F1, increasing
555 its stability. The knockdown of TMPO-AS1 resulted in higher E2F1
556 ubiquitination levels. In the current study, we reveal that TMPO-AS1
557 facilitates the interaction of E2F1 with OTUB1, a deubiquitinase, and
558 therefore is indirectly responsible for the deubiquitination-mediated
559 increase in E2F1 protein stability. While the specific ubiquitinating
560 linkage of E2F1 by OTUB1 is of interest that should be identified in
561 future investigation. Numerous studies have revealed that TMPO-AS1
562 contributes to tumorigenesis. However, most of them suggest that this is
563 mediated *via* a TMPO-AS1-miRNA-mRNA axis [17, 18, 51, 52], while
564 others propose that TMPO-AS1 forms RNA-RNA complexes to regulate
565 gene expression [19, 55]. Very recently, a study has shown that TMPO-
566 AS1 promotes BC cell growth via a TMPO-AS1/miR-98-5p/EBF1
567 positive feedback loop [51]. However, this study only elucidated the
568 function of TMPO-AS1 as a miRNA "sponge" for *EBF1* mRNA and only
569 *in vitro*; the role of TMPO-AS1 in BC, *in vivo*, is still unknown. Our

study provide the first evidence that TMPO-AS1 could interaction with proteins, and thereby influences protein-protein interactions, revealing a novel regulatory pattern of TMPO-AS1.

Transcription factor E2F1 has been reported to play a vital role in cancer stemness, progression, and chemoresistance [46, 56, 57]. It is well known that E2F1 is overexpressed in several cancers, and upregulated E2F1 is a strong predictor of BC progression, from the superficial to the invasive [48]. FOXD2-AS1 was reported to promote BC progression and recurrence via the activation of the Akt/E2F1 signaling pathway [16]. In our study, we found that the overexpression of either E2F1 or TMPO-AS1 boosted cell proliferation, migration, invasion, and inhibits the apoptosis of BC cells; their knockdown obviously led to the opposite. However, up to now, it was not clear whether TMPO-AS1 could function as an oncogene in an E2F1-mediated manner. Importantly, function recovery assays *in vitro* and rescue experiments *in vivo* indeed showed that TMPO-AS1 promotes BC growth in an E2F1-dependent manner. Our results, along with previous studies, demonstrate that E2F1 promotes cancer carcinogenesis, development, and progression. Several studies indicated that positive feedback loops are extremely important for the regulation of cancer carcinogenesis and progression [16, 51, 58]. Here, we disclose a new positive feedback loop that contributes to BC

tumorigenesis, involving TMPO-AS1 and E2F1. Altogether, our data support a mutual regulatory pattern between lncRNAs and transcription factors, which may lead to increased oncogenic activity in cancer development.

Despite we indeed reveal novel mechanisms for BC carcinogenesis, it is undeniable that our work has limitations as follows: our study lacks of sufficient clinical BC samples, thus we validate the clinical significance of TMPO-AS1 using online software rather than our own data. In addition, the specific sequence and structure of TMPO-AS1 contributing to OTUB1-mediated E2F1 deubiquitination remain unknown. And the downstream oncogenic molecules of E2F1 in BC are still unclear. We will investigate those unknown but interesting questions in future study.

In summary, our study propose a novel mechanism of tumorigenesis in BC: TMPO-AS1 exerts oncogenic functions in an E2F1-dependent manner. Therefore, the TMPO-AS1/E2F1 should be considered in the development of new therapeutic approaches for BC.

Conclusions

Collectively, our study uncovered the upstream and downstream regulation of TMPO-AS1, revealing a novel mechanism of BC tumorigenesis, development and progression. The TMPO-AS1/E2F1

positive feedback loop may be a potential new target for the treatment of BC patients.

List of abbreviations

BC: bladder cancer

lnRNAs: long non-coding RNAs

TMPO-AS1:TMPO antisense RNA 1

E2F1: E2F transcription factor 1

OTUB1: OTU domain-containing ubiquitin aldehyde binding 1

NMIBC: non-muscle-invasive bladder cancer

MIBC: muscle-invasive bladder cancer

miRNA: microRNA

TF: transcription factor

TCGA: The Cancer Genome Atlas

ChIP: Chromatin immunoprecipitation

FISH: Fluorescence in situ hybridization

IHC: Immunohistochemistry

qRT-PCR: quantitative Real-time polymerase chain reaction

RIP: RNA immunoprecipitation

Co-IP: Co-immunoprecipitation

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of The Third Xiangya Hospital, and written informed consent was obtained from each patient involved in the study.

Consent for publication

Written informed consent for publication was obtained from all participants involved in our study.

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was financially supported by the National Natural Science Foundation of China (81874137), the Outstanding Youth Foundation of Hunan Province (2018JJ1047), the Huxiang Young Talent Project (2016RS3022), the Hunan Province Science and Technology Talent Promotion Project (2019TJ-Q10), and the Independent Exploration and Innovation Project of Central South University, Grant/Award Number: 2019zzts825.

Authors' contributions

KC and YY Z designed this study. JY L and MQ X collected the clinical samples. JY L and YY Z contributed to the experiments implementation. YX C, DH, and LX analyzed the data. KC, YY Z, and YX Zhu drafted the manuscript. LG, ZW W, and LP D edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figure legends

Figure 1. The upregulation of TMPO-AS1 predicts the poor prognosis of BC patients. a TMPO-AS1 is highly expressed in the majority of cancers according to GEDS. **b, c** The expression of TMPO-AS1 in BC *versus* normal tissues was verified by analyzing TCGA and TANRIC data. **d** qRT-PCR was conducted to validate TMPO-AS1 expression in six pairs of BC tissues and adjacent normal tissues collected in this study(replicate=3). **e-g** The expression profile of TMPO-AS1 in BC, as per two datasets retrieved for the GEO: GSE133624 and GSE120736. **h** The disease-free survival curve showed high levels of TMPO-AS1 expression were associated with poor prognosis of BC patients using GEPIA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 2. TMPO-AS1 promotes cell proliferation, migration, and invasion, and the survival of BC cells *in vitro*. a qRT-PCR showing the mRNA levels of TMPO-AS1 in five bladder cell lines (BIU87, 5637, T24, EJ, and RT4). **b** The efficiencies of TMPO-AS1 knockdown or

overexpression in RT4 and T24 cells were examined by qRT-PCR. Mock, the negative control; Si-TMPO-AS1, siRNA targeting TMPO-AS1; OE-TMPO-AS1, ectopic expression of TMPO-AS1. **c, d** The effect of TMPO-AS1 knockdown and overexpression on cell proliferation was measured using MTT and colony formation assays**. e, f** The effects of TMPO-AS1 knockdown or overexpression on RT4 and T24 cells' migration and invasion were evaluated via wound healing and transwell assays. **g** Cell apoptosis was analyzed by flow cytometry in RT4 and T24 cells silencing or overexpressing TMPO-AS1, stained with Annexin V-PE/7-AAD. Error bars represent the mean ± S.D. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* Mock group (negative control).

Figure 3. E2F1 binds to TMPO-AS1 promoter and activates its transcription. a The potential transcription factors of TMPO-AS1 were predicted using ChIPBase v2.0 and hTFtarget. **b** The pathway enrichment analysis of genes co-expressed with TMPO-AS1 using Metascape. **c, d** The correlation between TMPO-AS1 and E2F1 mRNA expression in BC tissues was assessed using the Pearson's correlation analysis in datasets retrieved from TCGA and the GEO (GSE133624). **e** The expression of TMPO-AS1 was evaluated using qRT-PCR in RT4 and T24 cells

893 silencing or overexpressing E2F1. Mock, the negative control; Si-E2F1,
894 siRNA targeting E2F1; OE-E2F1, ectopic expression of E2F1. **f** The
895 binding sites between E2F1 and TMPO-AS1 promoter region were
896 predicted using JASPAR. **g** Luciferase reporter assays were conducted to
897 measure the luciferase activity of TMPO-AS1 promoter in RT4 and T24
898 cells overexpressing E2F1. Vector, the negative control. **h** ChIP assay
899 demonstrated E2F1 bound to the TMPO-AS1 promoter. Error bars
900 represent the mean ± S.D. from three independent experiments. * $P <$
901 0.05, ** $P < 0.01$ *versus* negative controls.
902
903 **Figure 4. TMPO-AS1 upregulates E2F1 protein levels via the**
904 **promotion of protein stability. a** The interaction between TMPO-AS1
905 and E2F1 was predicted using catRAPID. **b** RNA pull-down assay was
906 performed to validate the potential interaction between TMPO-AS1 and
907 E2F1 in RT4 and T24 cells overexpressing TMPO-AS1. **c** RIP assay was
908 performed to further confirm the interaction between E2F1 and TMPO-
909 AS1, in TMPO-AS1 overexpressing RT4 and T24 cells; IgG was used as
910 a RIP negative control. Error bars represent the mean ± S.D. from three
911 independent experiments. *** $P < 0.001$ *versus* negative control. **d** *E2F1*
912 mRNA expression was assessed using qRT-PCR in TMPO-AS1 silencing
913 or overexpressing BC cells. **e** E2F1 protein expression was evaluated via

western blotting in TMPO-AS1 silencing or overexpressing RT4 and T24 cells**. f** Western blotting showing proteasome inhibitor MG132 protein increased E2F1 stability in cycloheximide-treated RT4 and T24 cells. CHX, cycloheximide. **g** Western blotting showing decreased E2F1 stability was observed in TMPO-AS1 knockdown RT4 and T24 cells.

Figure 5. TMPO-AS1 stabilizes E2F1 via OTUB1-mediated deubiquitination

a TMPO-AS1 cellular localization was evaluated using FISH. **b** RNA pull-down assays demonstrated that OTUB1 was the most likely deubiquitinase binding to TMPO-AS among a group of deubiquitinases (OTUB1,UCHL5, USP5, COPS6, and PSMD14). **c** RIP assays were performed to validate the interaction between TMPO-AS1 and OTUB1, in the context of TMPO-AS1 overexpressing RT4 and T24 cells; IgG was used as a RIP negative control. Error bars represent the mean ± S.D. from three independent experiments. $^{***}P < 0.001$ *versus* negative control. **d** Western blotting showing TMPO-AS1 silencing had no effect on OTUB1 protein levels in RT4 and T24 cells. **e** Western blotting showing knockdown of OTUB1 decreased E2F1 protein levels in RT4 and T24 cells. Si- OTUB1, siRNA targeting OTUB1. **f** HDOCK showing E2F1(yellow) and OTUB1(brown) were very likely to bind to each other.

g Co-IP assays were performed in control or si-TMPO-AS1 treated RT4 and T24 cells using an anti-E2F1 antibody, followed by western blotting to analyze the ubiquitin levels of E2F1. **h** RT4 and T24 cells transfected with the empty vector, TMPO-AS1 overexpressing, or along with si-OTUB1 were subjected to immunoprecipitation with anti-E2F1 antibody, followed by western blotting with an anti-ubiquitin antibody.

Figure 6. E2F1 promotes the proliferation, migration, and invasion, and inhibits the apoptosis of BC cells *in vitro*. a, b The transcriptional expression of *E2F1* was upregulated in BC tissues compared with that in normal tissues based on data retrieved from TCGA and GSE133624 . **c** E2F1 protein expression was assessed via western blotting in six paired BC, and adjacent normal tissue samples. **d** E2F1 protein expression was also assessed using immunohistochemistry in six paired BC, and adjacent normal tissue samples. **e** The MTT was performed to determine the viability/proliferation of E2F1 knockdown and overexpressing RT4 and T24 cells. **f** The effects of E2F1 knockdown and overexpression on BC cell proliferation were assessed via colony formation assays. **g, h** The migratory and invasive capacities of E2F1 knockdown and overexpressing RT4 and T24 cells were evaluated via wound healing and transwell assays. **i** Cell apoptosis was analyzed by flow cytometry. Error

bars represent the mean ± S.D. from three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 *versus* Mock group (negative control).

Figure 7. TMPO-AS1 promotes BC malignant phenotypes via E2F1 *in vitro*. **a** TMPO-AS1 expression was evaluated using qRT-PCR in RT4 and T24 cells transfected with the empty vector, sh-TMPO-AS1, or co-transfected with sh-TMPO-AS1 and E2F1. **b, c** MTT and colony formation assays were employed to assess the proliferative ability of transfected cells. **d, e** Migration and invasion were estimated using wound healing and transwell assays. **f** The apoptosis in transfected BC cells stained with Annexin V-PE/7-AAD was evaluated via flow cytometry analysis. Error bars represent the mean ± S.D. from three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 8. TMPO-AS1 regulates BC growth *in vivo* via E2F1.

a Representative images of the tumor xenografts 25 days after the subcutaneous injection of RT4 cells transfected with the empty vector, sh-TMPO-AS1, and sh-TMPO-AS1 along with E2F1 into the flanks of nude mice. **b** The tumor volumes were measured every 3 days in the three groups. Three independent experiments were performed. * P < 0.05. **c** The expression of E2F1, Ki-67 and caspase-3 in xenografts was evaluated

using immunohistochemistry. Representative images are shown. **d** A schematic diagram illustrating the role and mechanism of TMPO-AS1 in BC tumorigenesis and progression.

Table 1:

The primer sequences used in this study.

Additional files

Additional file 1:

Table S1. The antibodies used in this study.

Additional file 2:

Figure S1. The expression of TMPO-AS1 in different clinical stages of BC samples.

Additional file 3:

Table S2. 36 overlapping TFs, obtained from hTFtarget and ChIP Base v2.0, and the correlation analysis of these TFs with TMPO-AS1.

Additional file 4:

Table S3. Genes that co-expressed with TMPO-AS1 retrieved from Co-LncRNA.

Additional file 5:

Figure S2. E2F1 ubiquitination sites and the interactions between TMPO-AS1 and five deubiquitinases. A The potential ubiquitination sites in E2F1. B The interaction between TMPO-AS1 and five deubiquitinases(UCHL5, USP5, COPS6, PSMD14, and OTUB1) predicted by PRIdictor. C CatRAPID was used to predict the interaction pattern between TMPO-AS1 and OTUB1.

Figures

Fig.1

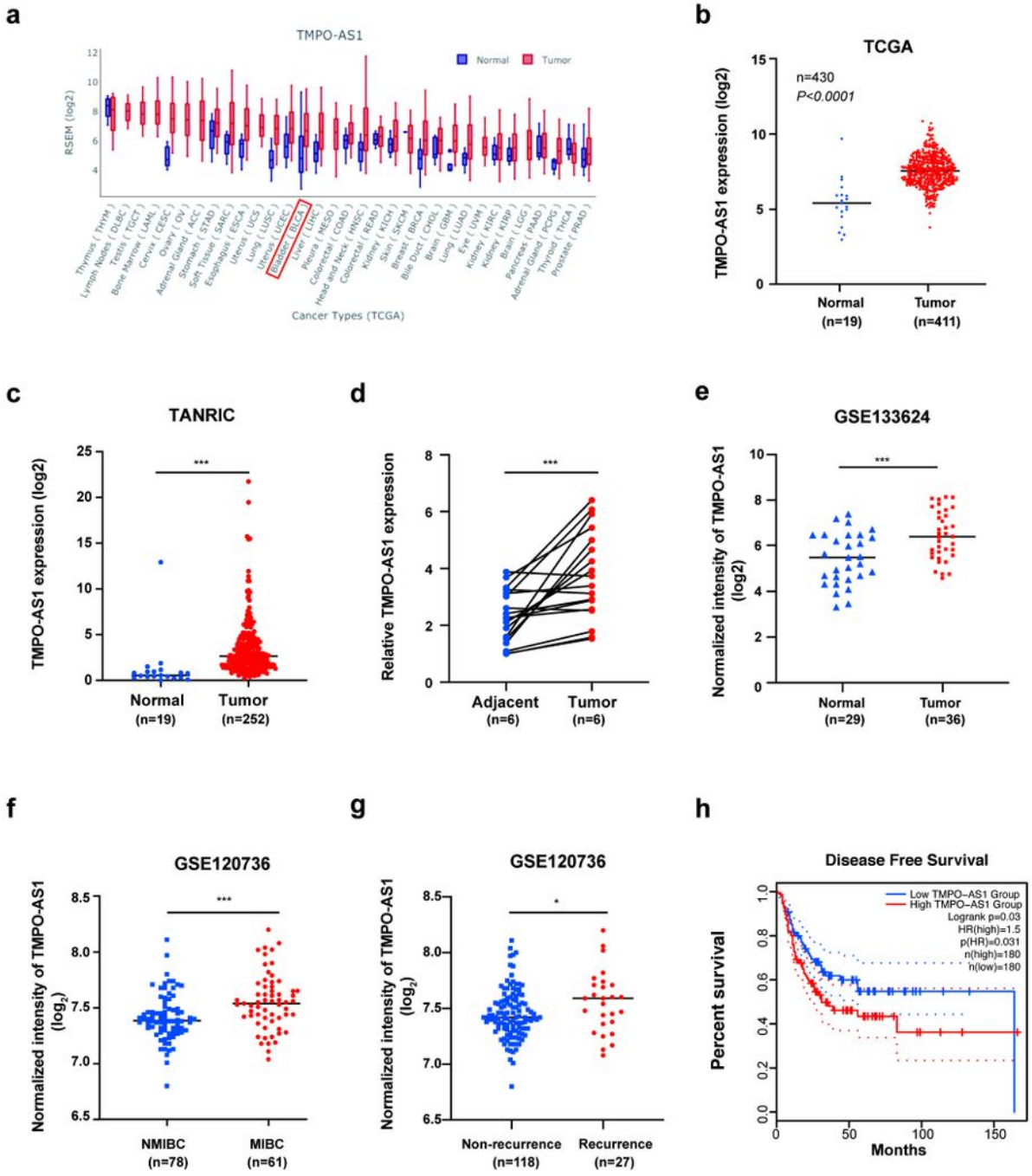

Figure 1

The upregulation of TMPO-AS1 predicts the poor prognosis of BC patients. a TMPO-AS1 is highly expressed in the majority of cancers according to GEDS. b, c The expression of TMPO-AS1 in BC versus normal tissues was verified by analyzing TCGA and TANRIC data. d qRT-PCR was conducted to validate

TMPO-AS1 expression in six pairs of BC tissues and adjacent normal tissues collected in this study(replicate=3). e-g The expression profile of TMPO-AS1 in BC, as per two datasets retrieved for the GEO: GSE133624 and GSE120736. h The disease-free survival curve showed high levels of TMPO-AS1 expression were associated with poor prognosis of BC patients using GEPIA. * P < 0.05, **P < 0.01, ***P < 0.001.

Fig.2

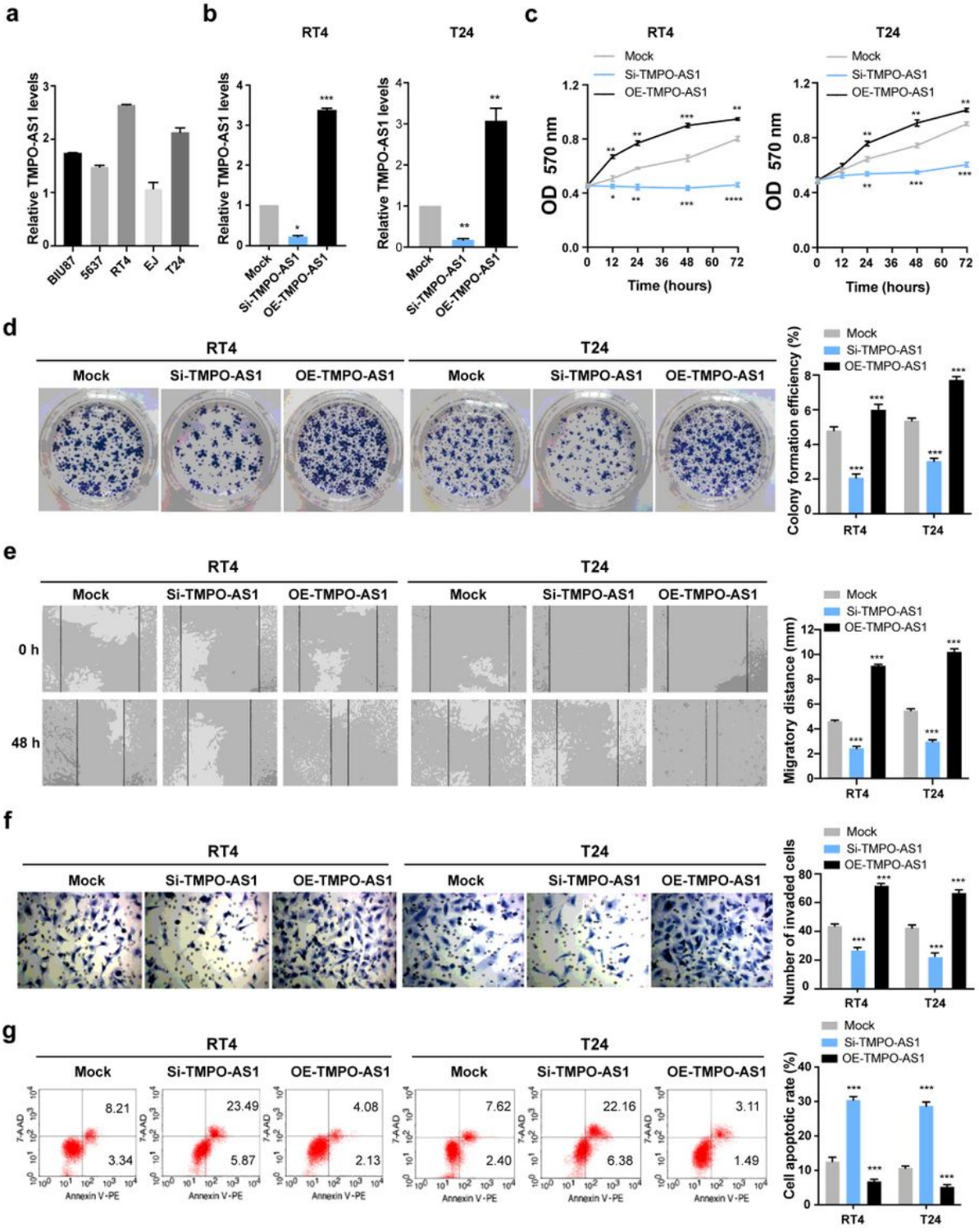

Figure 2

TMPO-AS1 promotes cell proliferation, migration, and invasion, and the survival of BC cells in vitro. a qRT-PCR showing the mRNA levels of TMPO-AS1 in five bladder cell lines (BIU87, 5637, T24, EJ, and RT4). b The efficiencies of TMPO-AS1 knockdown or overexpression in RT4 and T24 cells were examined by qRT-PCR. Mock, the negative control; Si-TMPO-AS1, siRNA targeting TMPO-AS1; OE-TMPO-AS1, ectopic expression of TMPO-AS1. c, d The effect of TMPO-AS1 knockdown and overexpression on cell proliferation was measured using MTT and colony formation assays. e, f The effects of TMPO-AS1 knockdown or overexpression on RT4 and T24 cells' migration and invasion were evaluated via wound healing and transwell assays. g Cell apoptosis was analyzed by flow cytometry in RT4 and T24 cells silencing or overexpressing TMPO-AS1, stained with Annexin V-PE/7-AAD. Error bars represent the mean ± S.D. from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Mock group (negative control).

Fig.3

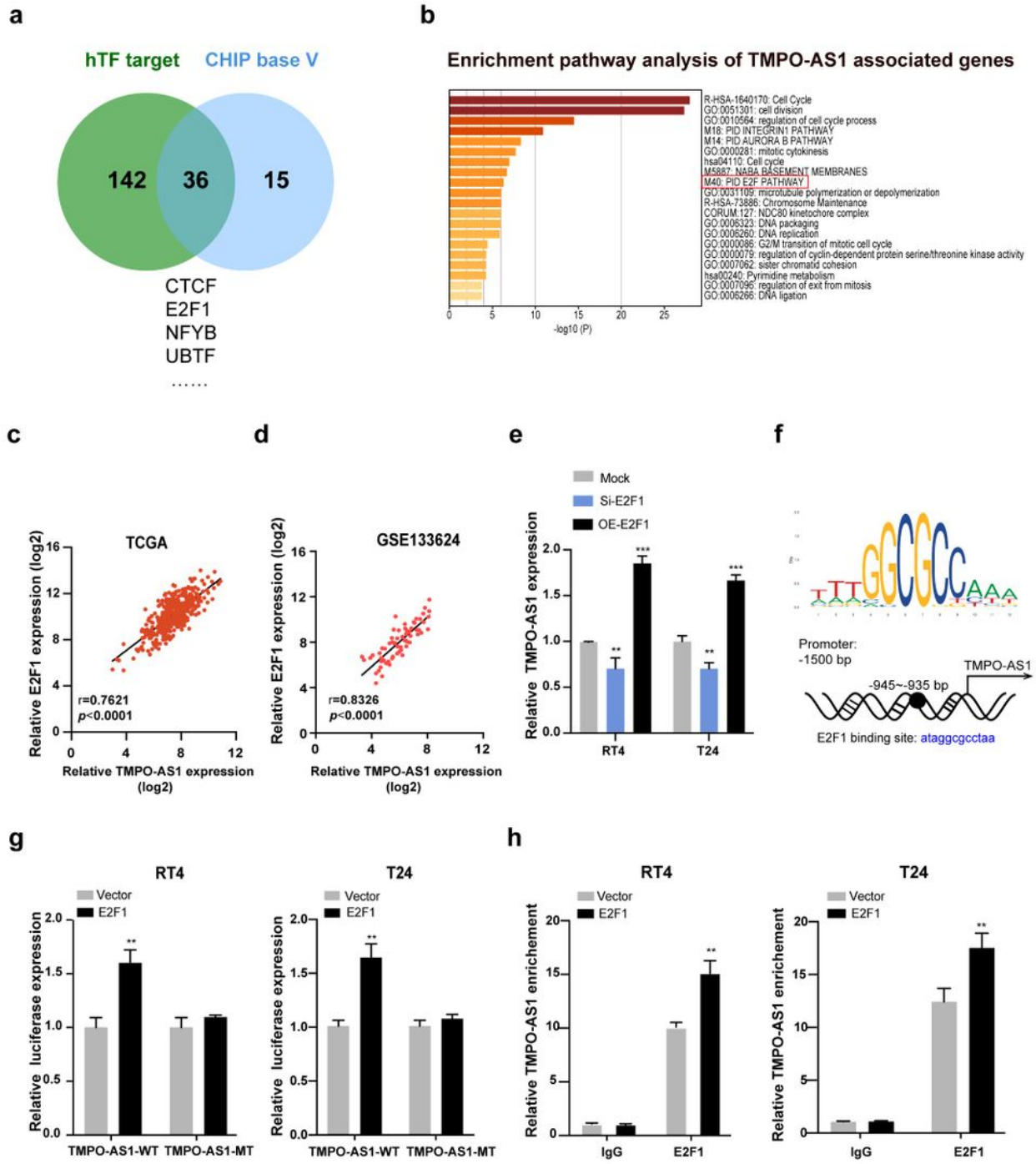

Figure 3

E2F1 binds to TMPO-AS1 promoter and activates its transcription. a The potential transcription factors of TMPO-AS1 were predicted using ChIPBase v2.0 and hTFtarget. b The pathway enrichment analysis of genes co-expressed with TMPO-AS1 using Metascape. c, d The correlation between TMPO-AS1 and E2F1 mRNA expression in BC tissues was assessed using the Pearson's correlation analysis in datasets retrieved from TCGA and the GEO (GSE133624). e The expression of TMPO-AS1 was evaluated using

qRT-PCR in RT4 and T24 cells silencing or overexpressing E2F1. Mock, the negative control; Si-E2F1, siRNA targeting E2F1; OE-E2F1, ectopic expression of E2F1. f The binding sites between E2F1 and TMPO-AS1 promoter region were predicted using JASPAR. g Luciferase reporter assays were conducted to measure the luciferase activity of TMPO-AS1 promoter in RT4 and T24 cells overexpressing E2F1. Vector, the negative control. h ChIP assay demonstrated E2F1 bound to the TMPO-AS1 promoter. Error bars represent the mean ± S.D. from three independent experiments. * P < 0.05, **P < 0.01 versus negative controls.

Fig.4

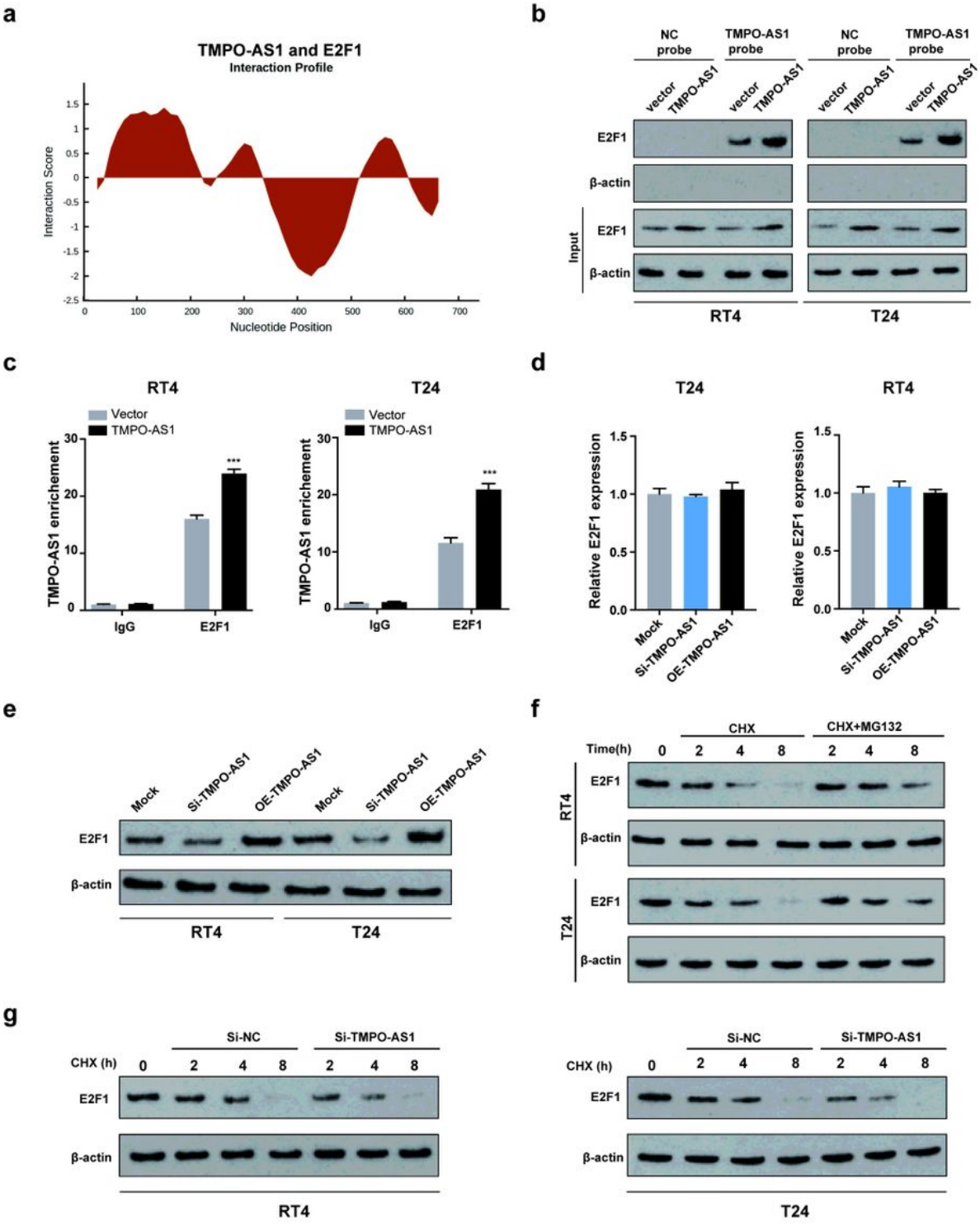

Figure 4

TMPO-AS1 upregulates E2F1 protein levels via the promotion of protein stability. a The interaction between TMPO-AS1 and E2F1 was predicted using catRAPID. b RNA pull-down assay was performed to validate the potential interaction between TMPO-AS1 and E2F1 in RT4 and T24 cells overexpressing TMPO-AS1. c RIP assay was performed to further confirm the interaction between E2F1 and TMPO-AS1, in TMPO-AS1 overexpressing RT4 and T24 cells; IgG was used as a RIP negative control. Error bars represent the mean ± S.D. from three independent experiments. ***P < 0.001 versus negative control. d E2F1 mRNA expression was assessed using qRT-PCR in TMPO-AS1 silencing or overexpressing BC cells. e E2F1 protein expression was evaluated via western blotting in TMPO-AS1 silencing or overexpressing RT4 and T24 cells. f Western blotting showing proteasome inhibitor MG132 protein increased E2F1 stability in cycloheximide-treated RT4 and T24 cells. CHX, cycloheximide. g Western blotting showing decreased E2F1 stability was observed in TMPO-AS1 knockdown RT4 and T24 cells.

Fig.5

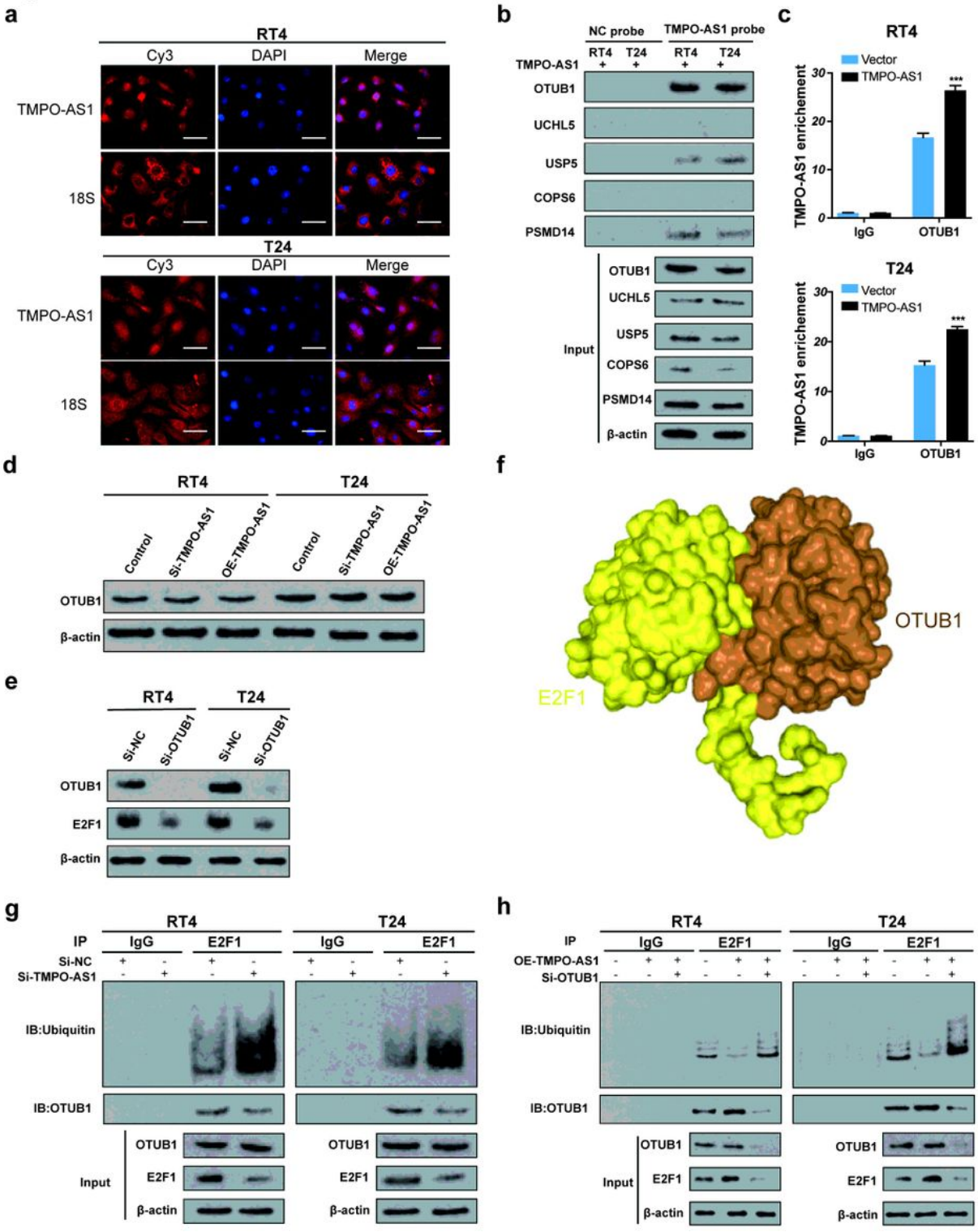

Figure 5

TMPO-AS1 stabilizes E2F1 via OTUB1-mediated deubiquitination a TMPO-AS1 cellular localization was evaluated using FISH. b RNA pull-down assays demonstrated that OTUB1 was the most likely deubiquitinase binding to TMPO-AS among a group of deubiquitinases (OTUB1,UCHL5, USP5, COPS6, and PSMD14). c RIP assays were performed to validate the interaction between TMPO-AS1 and OTUB1, in the context of TMPO-AS1 overexpressing RT4 and T24 cells; IgG was used as a RIP negative control.

Error bars represent the mean ± S.D. from three independent experiments. ***P < 0.001 versus negative control. d Western blotting showing TMPO-AS1 silencing had no effect on OTUB1 protein levels in RT4 and T24 cells. e Western blotting showing knockdown of OTUB1 decreased E2F1 protein levels in RT4 and T24 cells. Si- OTUB1, siRNA targeting OTUB1. f HDOCK showing E2F1(yellow) and OTUB1(brown) were very likely to bind to each other. g Co-IP assays were performed in control or si-TMPO-AS1 treated RT4 and T24 cells using an anti-E2F1 antibody, followed by western blotting to analyze the ubiquitin levels of E2F1. h RT4 and T24 cells transfected with the empty vector, TMPO-AS1 overexpressing, or along with si-OTUB1 were subjected to immunoprecipitation with anti-E2F1 antibody, followed by western blotting with an anti-ubiquitin antibody.

Fig.6

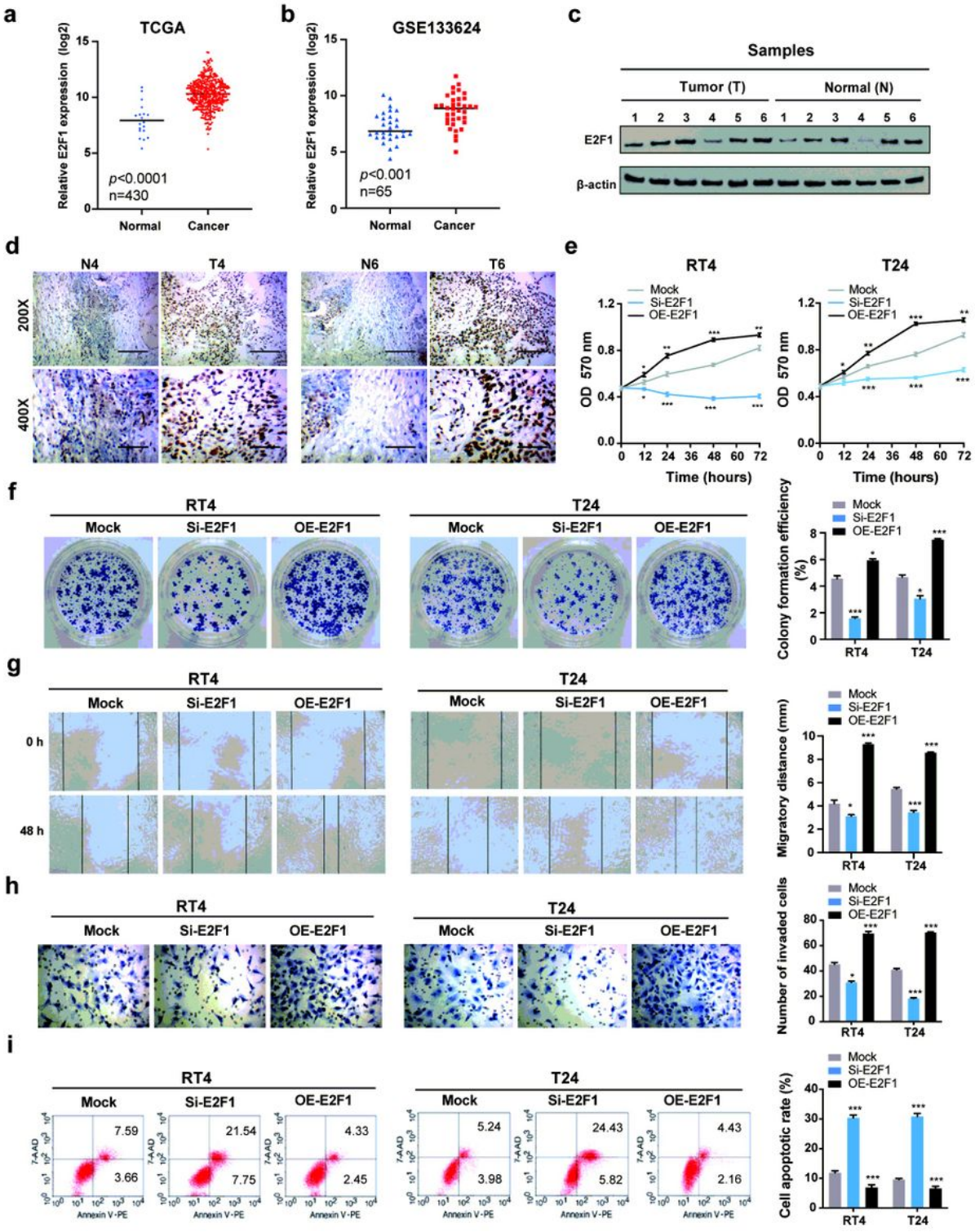

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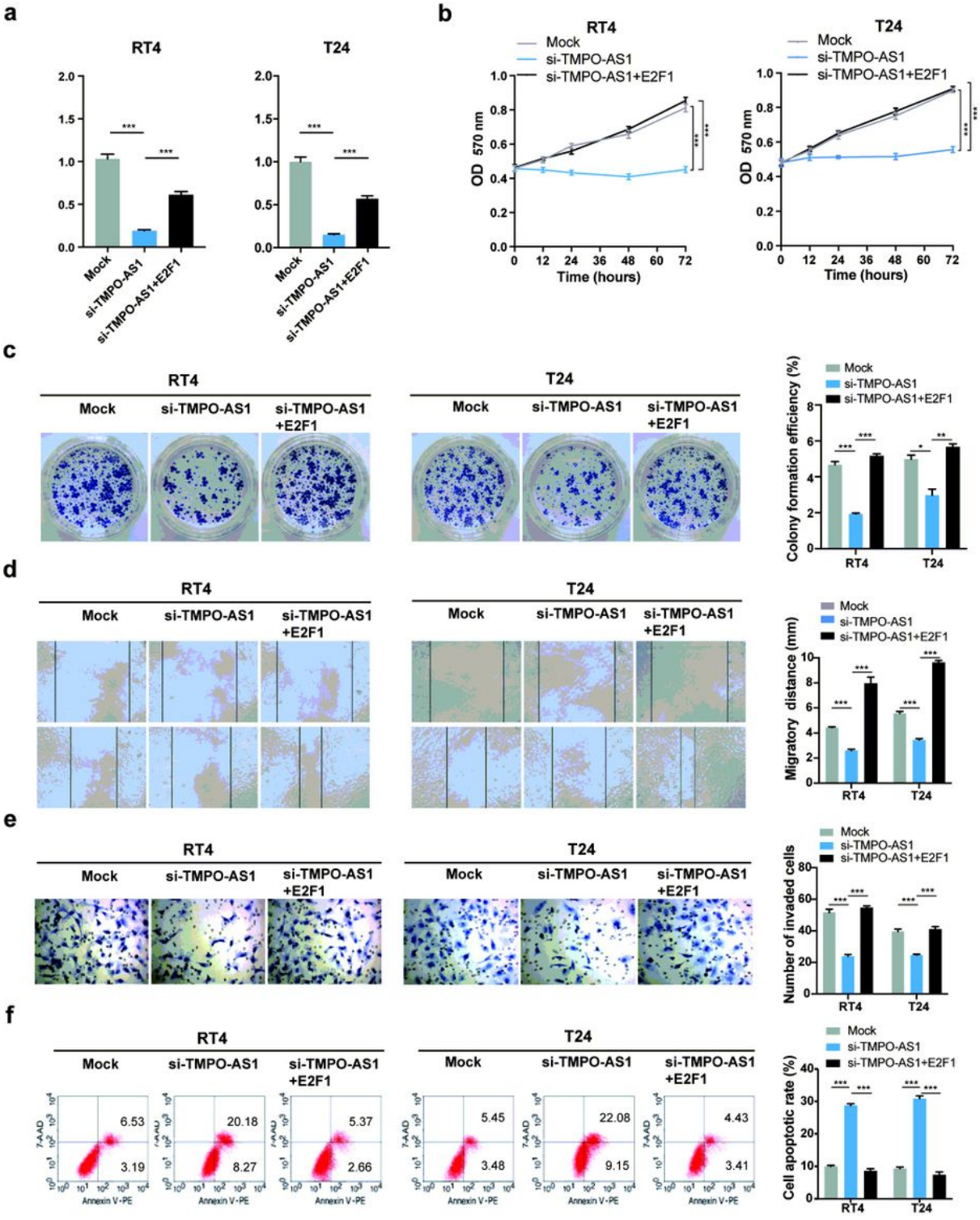
Fig.7



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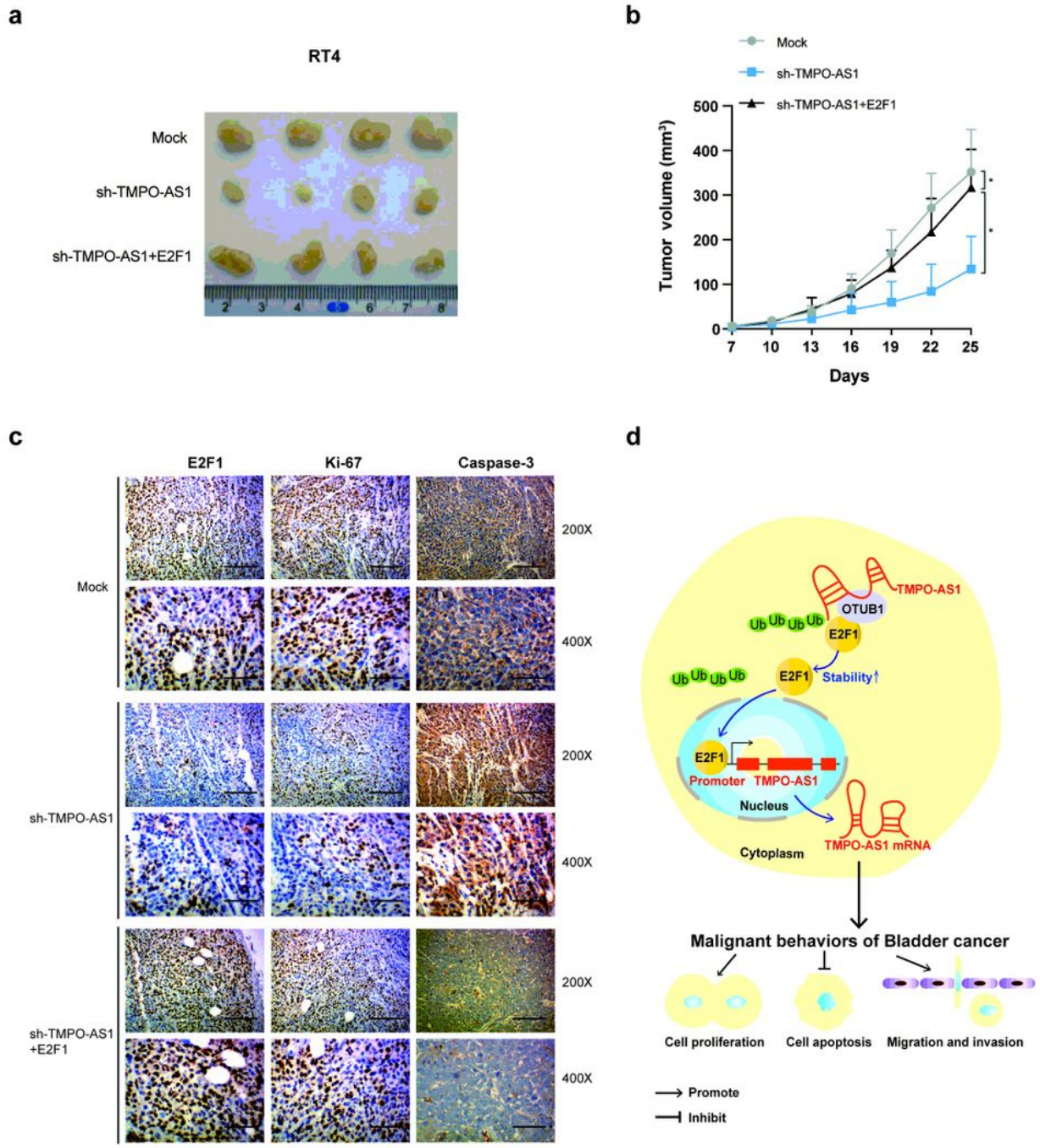

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Supplementary Files

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