LncRNA TMPO-AS1 promotes esophageal squamous cell carcinoma progression by forming biomolecular condensates with FUS and p300 to regulate TMPO transcription

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Esophageal squamous cell carcinoma (ESCC) is one of the most life- and health-threatening malignant diseases worldwide, especially in China. Long noncoding RNAs (lncRNAs) have emerged as important regulators of tumorigenesis and tumor progression. However, the roles and mechanisms of lncRNAs in ESCC require further exploration. Here, in combination with a small interfering RNA (siRNA) library targeting specific lncRNAs, we performed MTS and Transwell assays to screen functional lncRNAs that were overexpressed in ESCC. TMPO-AS1 expression was significantly upregulated in ESCC tumor samples, with higher TMPO-AS1 expression positively correlated with shorter overall survival times. In vitro and in vivo functional experiments revealed that TMPO-AS1 promotes the proliferation and metastasis of ESCC cells. Mechanistically, TMPO-AS1 bound to fused in sarcoma (FUS) and recruited p300 to the TMPO promoter, forming biomolecular condensates in situ to activate TMPO transcription in cis by increasing the acetylation of histone H3 lysine 27 (H3K27ac). Targeting TMPO-AS1 led to impaired ESCC tumor growth in a patient-derived xenograft (PDX) model. We found that TMPO-AS1 is required for cell proliferation and metastasis in ESCC by promoting the expression of TMPO, and both TMPO-AS1 and TMPO might be potential biomarkers and therapeutic targets in ESCC.

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INTRODUCTION

Esophageal carcinoma (ESCA) is the 6th leading cause of cancer-related mortality worldwide. In China, the predominant histological subtype of ESCA is esophageal squamous cell carcinoma (ESCC), which ranks 4th in cancer-related mortality. Although the clinical community has achieved some diagnostic and therapeutic advances, patients with advanced ESCC have a poor prognosis due to recurrence and metastasis, leading to a 5-year survival rate of less than 20%. Genetic abnormalities and molecular alterations play essential roles in the progression of ESCC and are potential therapeutic targets. Therefore, a more comprehensive understanding of the molecular mechanism underlying ESCC progression is vital for the development of novel biomarkers and effective therapeutic targets for this disease.

Long noncoding RNAs (lncRNAs) are a class of transcripts with a length of more than 200 nucleotides and virtually no protein-coding potential. LncRNAs play extensive roles in various physiological and pathological processes, including tumor initiation and progression. Recent reports have revealed diverse functional mechanisms for lncRNAs, such as acting as microRNA sponges, endogenous small interfering RNA (siRNA) precursors, or molecular scaffolds to interact with proteins or other RNAs, and even encoding short peptides. Roles of lncRNAs in ESCC have been reported. For example, the lncRNA DNM3OS confers radio-resistance by regulating the DNA damage response, and AGPG regulates PFKFB3-mediated tumor glycolytic reprogramming. These studies indicate that targeting lncRNAs could be a novel approach for ESCC therapy. However, further investigations into more specific roles of lncRNAs in ESCC tumorigenesis and progression are still needed.

Natural antisense (NAT) lncRNAs are classified by their genomic location with respect to the cognate protein-coding genes. The sequences of NAT lncRNAs are often partially complementary to the transcripts of their neighboring genes, and NAT lncRNAs and their neighboring genes often exhibit concordant or discordant expression patterns. Recent studies have shown that NAT lncRNAs function as epigenetic regulators of the expression of their cognate genes.

In this study, we found that the upregulated NAT lncRNA TMPO-AS1 functions as an oncogenic regulator in ESCC. TMPO-AS1 promoted ESCC cell proliferation, G1/S progression and metastasis. Mechanistically, TAS1 recruited FUS and p300 to the TMPO promoter and formed condensates in situ, which upregulated TMPO expression by increasing the deposition of H3K27ac in the...
promoter and activating TMPO transcription in cis, subsequently regulating the expression of CyclinD1 and metastasis-associated protein 1 (MTA1) to promote ESCC progression. Overall, this study showed the biological roles and underlying mechanisms of the TMPO-AS1/TMPO axis in ESCC and suggested TMPO-AS1 as a promising prognostic indicator and therapeutic target in ESCC.

MATERIALS AND METHODS

Cell lines and cell culture

Het-1A and NE-1 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). HEC293T, KYSE30, KYSE150, KYSE180, KYSE410, KYSE510 and KYSE520 cells were obtained from the German Cell Culture Collection (DSMZ, Braunschweig, Germany). TE-1, TE-9, TE-11 and TE-15 cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Medical Sciences, Shanghai, China). Cells were grown in basic Dulbecco’s modified Eagle’s medium (DMEM) or RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (HyClone, Logan, UT, USA) at 37°C in 5% CO2. All cells were further verified via STR-PCR DNA profiling by Guangzhou Cellcook Biotech Co., Ltd. (Guangzhou, China) and tested negative for mycoplasma contamination before use.

Human tissue specimens

Clinical samples were collected from Sun Yat-sen University Cancer Center (SYSUCC; Guangzhou, China). All patients were histologically diagnosed with ESCC. Written informed consent was obtained from all patients. The study was approved by the Medical Ethics Committee of Sun Yat-sen University.

Cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) models

To establish CDX models, ESCC cells expressing control shRNA (shCtrl) or TAS1-targeting sh#1 or sh#2 were injected subcutaneously into the dorsal flanks of 4-week-old female BALB/c nu/nu mice (five mice per group). Tumor growth was monitored every 3 days after transplantation using calipers. Mice bearing xenografts were euthanized at the endpoint, and tumors were weighed. PDX models were established as described previously19 and were used to assess the in vivo therapeutic effects of TAS1 using ASOs. When the volume of the PDXs was ~500 mm^3, we began a total of 4 consecutive doses. The target sequence is provided in the supplementary methods.

RESULTS

Identification of TMPO-AS1 as an oncogenic natural antisense IncRNA

We previously designed a highly efficient and specific siRNA library targeting the 50 most highly expressed IncRNAs in ESCC tumor samples compared to paired normal adjacent tissues from The Cancer Genome Atlas (TCGA) database. Using this library, we previously identified the IncRNA AGPG, which affects cell proliferation and glycolysis20. We transfected the siRNA library into two human ESCC cell lines, KYSE150 and TE-11, and performed MTS cell viability assays and Transwell migration assays to identify the IncRNAs that play essential roles in ESCC tumorigenesis and progression (Fig. 1a). Fourteen IncRNAs were found to exert promotive effects on cell proliferation, and 12 were potentially involved in cell migration; 8 of the IncRNAs were shared between both groups and might thus be involved in both cell proliferation and migration (Fig. 1a). Among these 8 IncRNAs, silencing of TMPO-AS1 most potently attenuated ESCC cell proliferation and migration (Fig. 1b; the p values are shown in Supplementary Table 4). TMPO-AS1 is an antisense IncRNA located on chromosome 12q23.1 and is transcribed from the antisense strand in the opposite direction of TMPO and composed of 2 exons (Supplementary Fig. 1a). To check the coding potential, we performed the in silico analysis with the Coding Potential...
The IncRNA TMPO-AS1 (TAS1) is upregulated in ESCC and indicates poor prognosis. 

a) Schematic showing the design of the screen for IncRNAs potentially involved in both cell viability and migration in ESCA. Eight IncRNAs regulated both cell proliferation and migration in KYSE150 and TE-11 cells, including TAS1; \( n = 3 \) biologically independent samples. The \( p \) values for each group are shown in Supplementary Table 4. 

b) TAS1 expression in ESCA tissues from TCGA data. 

c) TAS1 expression and OS analysis in ESCC samples from the SYSUCC cohort. \( (n = 108, \text{survival analysis: log-rank test, two-sided}) \). 

d) Detection of TAS1 subcellular localization in KYSE150 cells by FISH. Scale bar: 5 \( \mu \)m. 

e) TAS1 expression in the nuclear and cytoplasmic fractions of KYSE150 cells and TE-11 cells, as detected using qPCR. 

f) Determination of the TAS1 copy number in ESCC cell lines and normal esophageal epithelial cell lines; \( n = 3 \), compared with NE1. The data are presented as the mean ± S.D. values. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \); ns, not significant.
Assessment Tool (CPAT) to calculate the score for TMPO-AS1. According to CPAT analysis, the coding probability of TMPO-AS1 is 0.001, which is lower than that of other well-characterized IncRNAs, such as nuclear paraspeckle assembly transcript 1 (NEAT1), colon cancer-associated transcript 1 (CCAT1), and NF-kB interacting RNA (NKILA) (Supplementary Fig. 1b). In addition, for in vitro validation of the peptide-coding potential, the TMPO-AS1 sequence was inserted upstream of 3x Flag-Tag cassette in a plasmid, transfected into HEK293T cells, and immunoblotted with the Flag antibody. Consistent with the very low coding probability calculated by CPAT, no peptide or protein was detected (Supplementary Fig. 1c).

**TMPO-AS1 expression is upregulated in ESCC and associated with poor prognosis in patients**

Analysis of TCGA data showed upregulated TMPO-AS1 expression in tumor samples compared to normal tissues in various types of cancer tissues (Supplementary Fig. 1d), especially in ESCA tissues (Fig. 1c). In addition, survival analysis showed that patients with high TMPO-AS1 expression had shorter overall survival (OS) times across the whole set of various types of cancers (Supplementary Fig. 1e), suggesting that TMPO-AS1 may be a pancancer oncogene. Specifically, high TMPO-AS1 expression was also correlated with an unfavorable outcome in TCGA-ESCA patients (Supplementary Fig. 1f, n = 74). Because ESCC is one of the most predominant subtypes of ESCA, we verified that the TMPO-AS1 expression level was significantly higher in ESCC tissues (Fig. 1d). We also performed survival analysis in our independent ESCC cohort (Sun Yat-sen University Cancer Center (SYSUCC), n = 108). We categorized the TMPO-AS1 expression level according to the median value: the expression level was defined as high if higher than the median value and as low otherwise. High TMPO-AS1 expression was associated with unfavorable OS in patients with ESCC (Fig. 1e). The clinical characteristics of this cohort are shown in Supplementary Table 5. In addition, multivariate analysis showed that TMPO-AS1 was an independent prognostic factor in patients with ESCC (Supplementary Table 6).

Then, we examined the distribution of TMPO-AS1 by performing fluorescence in situ hybridization (FISH) and subcellular fractionation assays followed by qPCR. Our results showed that TMPO-AS1 was localized predominantly in the nucleus, with a small amount localized in the cytoplasm, similar to the distribution pattern of the well-characterized nuclear IncRNA U6 (Fig. 1f, g; Supplementary Fig. 1g).

Next, we examined TMPO-AS1 expression in a panel of ESCC cell lines and two normal esophageal epithelial cell lines (Het1A and NE1) and found that the TMPO-AS1 level was significantly higher in the tumor cell lines than in normal cell lines (Supplementary Fig. 1h). We further determined the copy number of TMPO-AS1 and found that it was also increased in the ESCC cell lines compared to the normal cell lines (Fig. 1h). Together, these findings suggest that TMPO-AS1 upregulation might play a role in ESCC development.

**TMPO-AS1 promotes cell proliferation, migration, and invasion in vitro**

We further investigated the oncogenic function of TMPO-AS1 by customized antisense oligonucleotide (ASO)-induced knockdown and lentiviral-mediated overexpression of TMPO-AS1 in ESCC cells (Supplementary Fig. 2a-c). The target sequences are shown in Supplementary Table 7. Then, we performed MTS assays and found that TMPO-AS1 knockdown significantly reduced cell proliferation (Fig. 2a). In addition, BrdU incorporation assays revealed that silencing TMPO-AS1 reduced ESCC cell proliferation (Fig. 2b). Cell cycle analysis showed that TMPO-AS1 knockdown resulted in G1/S arrest (Fig. 2c). Furthermore, Transwell assays showed that TMPO-AS1 silencing inhibited the migration and invasion of ESCC cells (Fig. 2d, Supplementary Fig. 2d). Interestingly, ectopic overexpression of TMPO-AS1 had minimal effects on these parameters (Fig. 2e, f; Supplementary Fig. 2e).

Consistent with the effects of TMPO-AS1 on ESCC cell proliferation and migration, we also observed a positive yet non-significant association between TMPO-AS1 expression and ESCA pathological stage in the TCGA database (Supplementary Fig. 2f).

**TMPO-AS1 facilitates ESCC tumor growth and metastasis in vivo**

Next, we explored the role of TMPO-AS1 in tumorigenesis and tumor development in vivo. In the subcutaneous cell line-derived xenograft (CDX) model, TMPO-AS1 knockdown significantly inhibited tumor growth, as indicated by the decreased tumor volume and tumor weight (Fig. 3a–c). Then, we established a popliteal sentinel lymph node metastasis model in nude mice to evaluate the effects of TMPO-AS1 on ESCC lymph node metastasis. The popliteal lymph nodes were harvested 8 weeks after tumor cell injection (Fig. 3d). The lymph nodes weighed slightly less in the TMPO-AS1 knockdown group than in the control group (Supplementary Fig. 3a). The metastasis-positive lymph nodes were identified by examining H&E-stained serial sections of each inguinal lymph node for metastatic micrometastases. At least one locus of metastatic micrometastases was required for classification as a metastasis-positive lymph node. Representative pictures of metastatic micrometastases are shown and marked in Supplementary Fig. 3b. Our data revealed a significantly reduced metastasis ratio in the TMPO-AS1-silenced group (Fig. 3e), suggesting that TMPO-AS1 knockdown suppressed lymph node metastasis of ESCC. In addition, tail vein injection of TMPO-AS1 knockdown cells or control cells was performed to examine lung metastasis. In vivo bioluminescence imaging showed a decreased luminescence intensity in the lungs of mice injected with cells group compared to control cells (Fig. 3f). H&E staining of serial sections of lung tissues was performed to confirm metastasis and quantify metastatic nodules (Fig. 3f). The results showed significantly reduced numbers and volumes of metastatic nodules in the TMPO-AS1-silenced group (Fig. 3g), indicating that TMPO-AS1 knockdown suppressed hematogenous metastasis of ESCC.

**TMPO-AS1 performs its biological functions by regulating TMPO in ESCC**

TMPO is located on the opposite strand of TMPO-AS1 on chromosome 12q21.2 and is the cognate gene of TMPO-AS1. Evidence suggests that TMPO plays diverse roles in various cancers. Since some antisense IncRNAs perform their biological functions by regulating neighboring genes, we investigated the regulatory relationship between TMPO and TMPO-AS1 expression in ESCC tissues. We found that TMPO expression was positively correlated with TMPO-AS1 expression in the SYSUCC-ESCC dataset (Fig. 4a). Furthermore, TMPO silencing obviously reduced the expression of TMPO (Fig. 4b), whereas ectopic overexpression of TMPO-AS1 did not affect the TMPO level (Supplementary Fig. 4a). In contrast, TMPO silencing had no effect on TMPO-AS1 expression (Fig. 4c). The ASOs and siRNAs were designed to specifically target the nonoverlapping sequences of these two genes to exclude any off-target effects. Specific silencing of TMPO was confirmed by qPCR and WB analyses (Supplementary Fig. 4b). Similar to the TMPO-AS1 expression pattern in ESCC, the TMPO expression level was also increased in ESCC tissues, as confirmed by qPCR and immunohistochemistry (IHC) (Supplementary Fig. 4c, d). TMPO was also upregulated in most ESCC cells (Supplementary Fig. 4e). We next investigated the role of TMPO in ESCC. Consistent with the phenotypes we observed after TMPO-AS1 knockdown, the MTS assay showed that TMPO silencing reduced ESCC cell proliferation (Fig. 4d). Cell cycle analysis revealed induction of G1/S phase arrest after TMPO knockdown (Supplementary Fig. 4f). Transwell assays revealed that TMPO knockdown inhibited ESCC
Fig. 2  TAS1 promotes cell proliferation, migration and invasion in vitro. a MTS assays were performed to measure the proliferation (OD 490 nm) of KYSE150 and TE-11 cells with TAS1 knockdown (KD) compared with control cells (n = 3). b BrdU incorporation assays (OD 450 nm) of KYSE150 and TE-11 cells with TAS1 KD compared with control cells (n = 3). c Statistical analysis of the cell cycle distribution (%) of KYSE150 and TE-11 cells with TAS1 KD compared with control cells. d Statistical analysis of the migration and invasion rates (%) of KYSE150 and TE-11 cells with TAS1 KD compared with control cells. e Statistical analysis of the migration and invasion rates (%) of KYSE30 and TE-15 cells with TAS1 overexpression (OE) (n = 3). f MTS assays were performed to measure the proliferation of KYSE30 and TE-15 cells with TAS1 OE compared with control cells (n = 3). The data are presented as the mean±S.D. values. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
Fig. 3  TAS1 facilitates tumor growth and metastasis in vivo. a Image of subcutaneous xenograft tumors formed by KYSE150 and TE-11 cells transduced with shTAS1 #1, shTAS1 #2 or shCtrl in nude mice. (n = 5). b, c Subcutaneous tumor volume curve and statistical analysis of the weight of tumors formed by KYSE150 and TE-11 cells treated as indicated (n = 5). d Image of popliteal lymph nodes harvested 8 weeks after injection of KYSE150 and TE-11 cells with lentiviral shRNA vector-mediated TAS1 KD into the left footpads of nude mice (n = 6). e Statistical analysis of the incidence of popliteal lymph node metastasis in the indicated groups (chi-square test, two-sided). f Representative images of whole-body in vivo bioluminescence and H&E staining (scale bar, 100 μm) in lung sections from mice injected via the tail vein with KYSE150 and TE-11 cells with stable TAS1 (#1 and #2) knockdown or control (Ctrl) cells on Day 56 postinjection. g Statistical analysis of the metastatic lung nodules confirmed by H&E staining (n = 5). The data are presented as the mean±S.D. values. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
Fig. 4 TAS1 performs its biological functions by cis-activating TMPO transcription. a The correlation between TAS1 and TMPO mRNA expression in clinical ESCC tissues (SYSUCC, \( n = 97 \), Pearson correlation analysis). b Detection of TMPO expression by qPCR and WB in KYSE150 and TE-11 cells with TAS1 KD compared with control cells (\( n = 3 \)). c Detection of TAS1 expression by qPCR in KYSE150 and TE-11 cells with TMPO KD compared with control cells (\( n = 3 \)). d MTS assays were performed to evaluate the proliferation (OD 490 nm) of KYSE150 and TE-11 cells with TMPO KD (\( n = 3 \)). e Statistical analysis of the migration and invasion rates (%) of KYSE150 cells treated as indicated and the migration and invasion rates (\( n = 3 \)). f–h MTS assays and statistical analysis of the cell cycle distribution (%) of KYSE150 cells treated as indicated and the migration and invasion rates (\( n = 3 \)). i A schematic diagram of the NRO assay. The data are presented as the mean±S.D. values. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \); ns, not significant.
cell migration and invasion (Fig. 4e, Supplementary Fig. 4g). Thus, TMPO promotes cell proliferation, migration and invasion, mimicking the effects of TMPO-AS1, on ESCC cells.

We conducted a series of rescue experiments to investigate whether TMPO-AS1 performs its function in ESCC by regulating TMPO. Consistent with our prediction, MTS and Transwell assays showed that TMPO overexpression in TMPO-AS1-silenced cells decreased the inhibition of cell proliferation, G1/S progression, migration and invasion (Fig. 4f-h, Supplementary Fig. 4h). Collectively, these data suggest that TMPO-AS1 might promote
ESC 

**TMPO-AS1 regulates the transcription of its cognate sense gene TMPO in cis**

Numerous antisense IncRNAs have been reported to regulate the transcription of their cognate genes. TMPO-AS1 is a NAT IncRNA transcribed in the opposite direction starting from the first intron in the antisense strand of TMPO, and it includes the transcription start site (TSS) and the 5'UTR of TMPO. Therefore, we conducted an NRO assay to evaluate the regulation between TMPO-AS1 and TMPO. NRO assays can measure the transcription efficiency without the influence of degradation by labeling nascent transcripts with bromouridine. The results showed that TMPO-AS1 knockdown reduced the level of nascent TMPO mRNA transcripts. TMPO-AS1 did not affect the degradation rate of TMPO mRNA in the presence of the transcription inhibitor actinomycin D (ActD). Together, these results suggest that TMPO-AS1 regulates TMPO transcription instead of affecting TMPO mRNA stability. Combined with the observation that ectopic expression of TMPO-AS1 exerted minimal effects, our results indicate that TMPO-AS1 might act in cis but not in trans to activate TMPO expression.

**TMPO-AS1 increases the H3K27ac level in the TMPO promoter by recruiting FUS and p300 to form biomolecular condensates**

Next, we examined the gene loci of TMPO and TMPO-AS1 in the UCSC Genome Browser. We found that in different cell types, H3K27ac, which is the hallmark of open chromatin with active transcription, was enriched in the TSS-harboring regions of both genes. Next, we performed ChIP-qPCR using the anti-H3K27ac antibody in KYSE150 and TE-11 cells. Four pairs of primers (P1-P4) specific for the TMPO promoter region were designed, and their sequences are shown at the bottom of Supplemental Fig. 5d. The results of qPCR analysis using P3 revealed that the TMPO promoter region was enriched by the anti-H3K27ac antibody (Fig. 5a, b). Furthermore, TMPO-AS1 silencing significantly reduced the H3K27ac level in the TMPO promoter region (Fig. 5a, b). Therefore, H3K27ac enrichment in the promoter region might be the reason for the upregulated expression of TMPO in ESCC cells.

The molecular function of IncRNAs is closely associated with their subcellular localization. We already determined that TMPO-AS1 was localized predominantly in the nucleus (Fig. 1f, g). Nuclear IncRNAs have been reported to recruit chromatin-remodeling proteins to chromatin and thereby control transcriptional activity. NAT IncRNAs also perform their functions by interacting with RNA binding proteins (RBPs). To identify possible TMPO-AS1-interacting proteins, we performed a targeted screen of intranuclear RBPs and found that TMPO-AS1 was very likely to interact with the RBP FUS, with a probability of 0.9 (http://pridb.gdcb.iastate.edu/RPSSeq/). FUS is a well-characterized RNA binding protein with various roles in different cellular processes, such as transcriptional regulation, RNA splicing, RNA transport, DNA repair and the DNA damage response. FUS is able to phase separate and form biomolecular condensates with itself or other molecular partners, which drives aberrant chromatin looping and cancer development. Then, we performed RNA pulldown followed by immunoblot analysis on ESCC cell lysates. The results validated the interaction between TMPO-AS1 and FUS (Fig. 5c). We also performed MS2-tagged RNA affinity purification (MS2-TRAP) and immunoblot analysis to further characterize the interaction between TMPO-AS1 and TMPO in situ. Coexpression of MS2-TMPO-AS1 and Flag-tag MS2 coat protein (MCP) led to significant enrichment of FUS by the anti-Flag antibody compared with the isotype control, indicating that FUS specifically binds to TMPO-AS1 (Fig. 5d). This observation was further confirmed by a RIP assay, where TMPO-AS1 was successfully enriched by the anti-FUS antibody (Fig. 5e). However, FUS expression did not change after TMPO-AS1 knockdown (Supplemental Fig. 5e). Next, we performed a ChIRP assay, which is based on affinity capture of a target IncRNA-chromatin complex, with biotinylated ASO probes for TMPO-AS1 and subjected the precipitated products to qPCR and immunoblot analysis; the results indicated that TMPO-AS1 indeed bound to the promoter sequence of TMPO (Fig. 5f), and the immunoblot analysis further confirmed the direct binding between TMPO-AS1 and FUS (Fig. 5g). Taken together, these results indicate that the expression level of TMPO-AS1 does not affect the expression level of FUS in ESCC cells but influences FUS recruitment to the TMPO promoter.

FUS can form ribonucleoprotein complexes with IncRNAs and recruit the histone acetyltransferase complex to the TSS of target genes to regulate their transcription by interacting with HAT complex members, including p300, CBP, and TIP60. Therefore, we performed co-IP with both anti-FUS and anti-p300 antibodies in ESCC cells. We first confirmed the direction of the interaction between FUS and p300 (Supplemental Fig. 5f). Furthermore, ChIRP followed by immunoblotting showed that p300 was enriched in the TMPO-AS1 probe group compared to the scrambled probe group (Fig. 5g). IF and FISH colocalization analyses showed that TMPO-AS1, FUS, and p300 were colocalized in the nucleus, and they were observed as puncta, suggesting the formation of IncRNA-protein biomolecular condensates (Fig. 5h). Interestingly, TMPO-AS1 silencing evidently reduced the number of colocalized puncta (Fig. 5i), indicating that TMPO-AS1 is likely to facilitate the formation of biomolecular condensates with FUS and p300.

We intended to further identify the downstream factors of TMPO-AS1 and TMPO involved in ESCC progression. A qPCR array containing 12 genes associated with G1/S phase transition and 89 metastasis-related gene probes was used to compare the mRNA expression profiles between TMPO-AS1-knockdown cells and control cells as well as between TMPO-knockdown cells and control cells as an approach to further...
Fig. 6  TAS1 constitutes a potential therapeutic target in ESCC. a Images of ex vivo tumors from the ESCC PDX model (n = 5). b, c Tumor volume curve and statistical analysis of the tumor weight of the PDX tumors. d Representative images of H&E staining and immunohistochemical staining for Ki67, TMPO, CyclinD1 and MTA1 in randomly selected PDX tumors from each group. Scale bar, 100 μm. e Statistical analysis of the Ki67 proliferation index (n = 5). f Statistical analysis of the immunohistochemical scores for the indicated genes (n = 5). The data are presented as the mean±S.D. values. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
identify downstream factors of TMPO-AS1 and TMPO involved in ESCC cell proliferation and metastasis. Interestingly, the expression of CyclinD1 and MTA1 was downregulated after knockdown of either TMPO-AS1 or TMPO (Supplementary Fig. 5g). Immunoblot analysis showed reduced expression of CyclinD1 and MTA1 in TMPO-AS1-silenced cells (Supplementary Fig. 5h). Rescue experiments indicated that the downregulation of CyclinD1 and MTA1 expression induced by TMPO-AS1 silencing was reversed by TMPO overexpression (Supplementary Fig. 5i). Collectively, these results reveal that TMPO-AS1 recruits FUS/p300 to the TMPO promoter.
and forms biomolecular condensates by direct binding, promoting H3K27ac and facilitating the transcription of TMPO, resulting in subsequent upregulation of CyclinD1 and MTA1, ultimately leading to ESCC tumor development.

**Effects of TMPO-AS1 targeting on ESCC tumors in vivo**

To examine the therapeutic potential of targeting TMPO-AS1, we established PDX models derived from two patients diagnosed with ESCC at SYSUCC. We injected ASOs against TMPO-AS1 optimized in the in vitro study intratumorally into PDZ-bearing BALB/c nude mice, which resulted in marked decreases in the tumor volume and tumor weight (Fig. 6d), suggesting the promising therapeutic potential of targeting TMPO-AS1. I&F staining of the excised tumors showed no obvious morphological differences between the treatment group and the control group (Fig. 6d). Immunohistochemical staining showed that TMPO-AS1 knockdown significantly impaired tumor proliferation, as indicated by the reduced Ki67 index (Fig. 6d, e). Accordingly, the expression levels of TMPO and the downstream proteins CyclinD1 and MTA1 were also obviously reduced, consistent with the results described above (Fig. 6d, f).

**The TMPO-AS1/TMPO axis is associated with ESCC development**

We used a cohort of ESCC tissues (SYSUCC, n = 108; clinicopathological information is provided in Supplementary Table 9) to analyze TMPO-AS1 expression using qPCR and to analyze TMPO, Ki67, CyclinD1, and MTA1 expression using IHC in order to collectively evaluate whether the TMPO-AS1/TMPO axis is clinically and pathologically relevant in ESCC. TMPO, Ki67, CyclinD1 and MTA1 were expressed at higher levels in the TMPO-AS1-low group (Fig. 7a, b), confirming the promoting effects of TMPO-AS1 on TMPO expression and ESCC progression.

Furthermore, we analyzed the clinical relevance of TMPO to patient outcomes. The correlations between TMPO expression and clinicopathological features are shown in Supplementary Table 10. Kaplan–Meier analysis showed that high TMPO expression was associated with poor outcomes in patients with ESCC (Fig. 7c). Then, according to qPCR analysis of TMPO-AS1 and immunohistochemical staining of TMPO, the samples were classified into the TMPO-AS1/TMPO-high, TMPO-AS1/TMPO-intermediate, and TMPO-AS1/TMPO-low groups, and the patients in the TMPO-AS1/TMPO-high subgroup had the worst prognosis among the three groups (Fig. 7d). In summary, these data further indicated that TMPO-AS1/TMPO potentially constitute promising prognostic indicators and therapeutic targets in ESCC.

**DISCUSSION**

ESCC is a predominant histological subtype of esophageal malignancy, especially in Asia. More than 90% of esophageal cancer cases in the East Asian region are ESCC. With the development of cancer therapies, the survival of patients with ESCC has improved. However, the overall therapeutic effect is poor due to the lack of promising targets, with a 5-year survival rate of less than 10% for patients with advanced disease. Therefore, studies aiming to further elucidate the molecular mechanisms underlying the development of ESCC are urgently needed. Recently, IncRNAs have emerged as important epigenetic regulators that play essential roles in various physiological and pathological processes. The functions and mechanisms of IncRNAs have been increasingly appreciated in different cancers. For example, IncRNAs have been reported to be associated with diverse pathological functions, including tumor proliferation, metastasis, angiogenesis, metabolism, and microenvironmental remodeling. Therefore, we intended to identify functionally essential IncRNAs in ESCC by performing phenotypic screening of aberrantly expressed IncRNAs using a siRNA library based on TCGA transcriptomic data. TMPO-AS1, an antisense IncRNA of TMPO located on chromosome 12q23.1, was the candidate with the most potent suppressive effects in our screen. TMPO-AS1 expression was upregulated in ESCC, and high TMPO-AS1 expression indicated poor prognosis in patients with ESCC. Recent studies have reported that various IncRNAs are abnormally expressed and have crucial functions in ESCC. For example, Zhang et al. revealed that the IncRNA DNM3OS regulates the DNA damage response, which results in radioresistance during ESCC treatment.

A study by Li et al. showed that the long intergenic noncoding RNA POU3F3 promotes ESCC tumor growth by interacting with EZH2 to increase the methylation of POU3F3 and reduce POU3F3 expression. TMPO-AS1 expression has been reported to be upregulated in various cancers, including bladder cancer, pancreatic cancer, and lung adenocarcinoma. However, the role of TMPO-AS1 in ESCC is less understood. In this study, we reported that TMPO-AS1 promotes tumor progression through activation of TMPO transcription in cis in ESCC. Functionally, TMPO-AS1 promoted ESCC cell proliferation and migration both in vitro and in vivo (Figs. 2, 3). Mechanistically, TMPO-AS1 performed its function by activating TMPO transcription in cis (Figs. 4, 5). TMPO-AS1 promoted TMPO transcription by recruiting FUS and p300 and forming condensates in situ to acetylate lysine 27 of histone 3 in the promoter (Fig. 5). TMPO, also termed lamina-associated polypeptide 2 (LAP2), is the cognate neighboring gene of TMPO-AS1 located on chromosome 12q21.2, and 6 nuclear isoforms can be produced through alternative splicing. Evidence suggests important roles for TMPO in various cancers—TMPO expression is upregulated in non-small-cell lung cancer, glioblastoma, and digestive tract carcinomas, although little is known about its role in ESCC. Among the various types of IncRNAs, NAT IncRNAs are attracting increasing attention. NAT IncRNAs are widespread in the genomes of diverse species, including humans. These NATs and their cognate genes often show concordant or discordant expression patterns. Diverse transcriptional or post-transcriptional mechanisms have been associated with the ability of NATs to regulate the expression of their sense transcripts. Cis-acting NAT IncRNAs serve as scaffolds to recruit chromatin-modulating proteins to facilitate DNA methylation, histone modification, and chromatin remodeling, ultimately leading to activated transcription of the cognate gene. NAT IncRNAs may compete with their sense transcripts for binding of RNA
polymerase II (RNA Pol II) and regulatory transcription factors, resulting in transcriptional interference. 

For the first time, we reported the transcriptional activation of TMPO mediated by TMPO-AS1 (Fig. 7e). Li et al. reported that TMPO-AS1 promotes thyroid cancer cell proliferation by splicing mier-498 to increase TMPO expression45. Here, we found that TMPO-AS1 acts in cis to activate TMPO expression at the transcriptional level. The difference in the mechanism by which TMPO-AS1 regulates TMPO expression might be tissue specific. The model we proposed echoes the roles played by the IncRNA SATB homeobox 2 antisense RNA 1 (SATB2-AS1) in promoting SATB2 expression92, the IncRNA homeobox A cluster (HOXA) transcript at the distal tip (HOTTIP) in activating HOXA gene expression93, and the IncRNA HEAL in regulating HIV-1 replication94. However, the underlying mechanisms employed by these IncRNAs are different. For example, HOTTIP interacts with WDR5 and recruits the MLL complex to maintain H3K4me3 and activate HOXA gene transcriptions. However, HOTTIP requires chromosome looping to bring the HOTTIP locus spatially closer to its target genes for its cis-regulatory action95. The different mechanisms might be due to differences in the distances between the TSSs of NAT IncRNAs and their cognate genes. As exemplified by TMPO-AS1, the expression of some IncRNAs is correlated with that of their sense protein-coding genes (Fig. 4). This finding may reflect the observation that NAT IncRNAs are essential for regulating the expression of their paired genes, suggesting that this cis-regulatory mechanism might be universal for NAT IncRNAs.

LncRNAs are attracting increasing attention as novel therapeutic targets, especially in cancer45. Treatments targeting lncRNAs have been universal for NAT lncRNAs. As exemplified by TMPO-AS1, the expression of some IncRNAs is correlated with that of their sense protein-coding genes (Fig. 4). This finding may reflect the observation that NAT IncRNAs are essential for regulating the expression of their paired genes, suggesting that this cis-regulatory mechanism might be universal for NAT IncRNAs.

In summary, our current study showed that TMPO-AS1 expression was upregulated in ESCC and that high TMPO-AS1 expression was associated with poor prognosis. TMPO-AS1 promotes ESCC cell proliferation and metastasis by activating TMPO transcription in cis. These data suggest that TMPO-AS1 and TMPO may be novel biomarkers and promising diagnostic and therapeutic targets in ESCC. However, further studies must be performed to elucidate the precise molecular mechanisms by which TMPO might regulate cancer cell proliferation and metastasis in ESCC.

DATA AVAILABILITY

All data generated during this study are included in this published article and its supplementary files.

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**AUTHOR CONTRIBUTIONS**

Z.-L.Z., Z.-X.L., H.-Y.L. and X.-J.L. designed the study. X.-J.L., M.-M.H., J.L., and J.-B.Z. collected the data. X.-J.L., M.-M.H., J.L., J.-B.Z., Q.-N.W., Q.M., Z.-L.Z., Z.-X.L. and H.-Y.L. analyzed and interpreted the data. X.-J.L., Q.-N.W., Y.-X.C. and J.L. performed the statistical analysis. R.-H.X., K.-J.L., D.-L.C. and Z.-L.Z. provided administrative, technical, or material support. X.-J.L., Z.-L.Z., Z.-X.L. and H.-Y.L. wrote and revised the manuscript. All authors reviewed the manuscript and approved the final version.

**ETHICAL APPROVAL AND CONSENT TO PARTICIPATE**

The clinical ESCC specimens were used with permission from the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center, China. All animal experiments were performed in accordance with a protocol approved by the Ethics Committee of the Institutional Animal Care of Sun Yat-sen University Cancer Center, China.

**COMPETING INTERESTS**

The authors declare no competing interests.

**ADDITIONAL INFORMATION**

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