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The long noncoding RNA AATBC promotes breast cancer migration and invasion by interacting with YBX1 and activating the YAP1/Hippo signaling pathway

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Abstract

Background

Long noncoding RNAs (lncRNAs) play an important role in the regulation of gene expression and are involved in several pathological responses. However, many important lncRNAs in breast cancer have not been identified and their expression levels and functions in breast cancer remain unknown.

Methods

We used the microarray data to identify differentially expressed lncRNAs between breast cancer and adjacent breast epithelial tissues. *In vitro* and *in vivo* assays were used to explore the biological effects of the differentially expressed lncRNA Apoptosis-Associated Transcript in Bladder Cancer (AATBC) in breast cancer cells. The mass spectrometry and RNA pulldown were used to screen AATBC interacting proteins. Using the Kaplan-Meier method, survival analysis was performed.

Results

The expression of AATBC was significantly high in breast cancer samples, and this high AATBC level was tightly correlated with poor prognosis in breast cancer patients. *In vitro* and *in vivo* experiments indicated that AATBC promoted breast cancer cells migration and invasion. AATBC specifically interacted with Y-box binding protein 1 (YBX1), which activated the YAP1/Hippo signaling pathway by binding to macrophage stimulating 1 (MST1) and promoting the nuclear translocation of Yes associated protein 1 (YAP1), allowing its function as a nuclear transcriptional regulator.

Conclusions

AATBC is highly expressed in breast cancer and contributes to patients' progression, indicating that it could serve as a novel prognostic marker for the disease. Mechanistically, AATBC affects migration and invasion of breast cancer cells through an AATBC-YBX1-MST1 axis, resulting in activating the YAP1/Hippo signaling pathway. This is also an important supplement to the composition of the YAP1/Hippo signaling pathway. The model of "AATBC-YAP1" may bring a new dawn to the treatment of breast cancer.

Background

Breast cancer is a common malignancy with serious effects on the health of women around the world [1,2]. Surgery and chemotherapy are the primary modalities for breast cancer treatment. Moreover, the recurrence and progression rates of breast cancer remain high, and the 5-year cancer-specific survival for relapsed patients with metastatic disease is estimated to be 66-100%, depending on the stage at presentation. Unfortunately, the mechanism underlying metastasis has not been fully elucidated.

The onset and development of breast cancer are caused by changes in the expression of a series of protein-coding genes as well as a number of nonprotein-coding genes, including those expressing long noncoding RNAs (lncRNAs)[3-6]. lncRNAs participate in the formation of RNA-protein complexes, act as microRNA sponges[7], and regulate targeted gene transcription and splicing, thereby regulating the expression of downstream genes and signaling pathways. Increasing evidence demonstrates that they play important regulatory roles in cancer development [8-10].

In this study, we used microarray data to screen differentially expressed lncRNAs in breast cancer, and found that one lncRNA, apoptosis associated transcript in bladder cancer (AATBC) was highly expressed in breast cancer tissues and significantly associated with breast cancer patient poor prognosis. *In vitro* and *in vivo* experiments demonstrated that AATBC promoted migration and invasion of breast cancer cells by binding Y-box binding protein 1 (YBX1), inhibiting the phosphorylation of yes-associated protein 1 (YAP1), and resulting in Hippo signaling pathway activation.

Methods

Data analysis

Three breast cancer gene expression datasets (accession numbers: GSE65194, GSE42568, and GSE20711) were downloaded from the Gene Expression Omnibus (GEO) database (Supplementary Table 1). Datasets GSE65194 and GSE42568 were used to screen differentially expressed lncRNAs between breast cancer tissues and normal breast tissues using the Significant Analysis of Microarray (SAM) software (Fig. S1a).

Tissue samples

Paired paraffin-embedded breast cancer and adjacent breast tissue samples from 21 patients were used to assess the expression of AATBC (Table S2). Tissue samples were collected from the Second Xiangya Hospital of Central South University. All specimens were confirmed by histopathological examination, and the study was approved by the Research Ethics Board of Central South University. Signed informed consent was obtained from each participant before enrollment in this study.

In situ hybridization (ISH) and immunohistochemistry

In situ hybridization was performed to detect AATBC expression in tissue specimens using three 30-bp nucleotide probes from different AATBC regions. The AATBC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control probes were synthesized and labeled with DIG-dUTP at the 3' end using a kit from Invitrogen (Shanghai, China). The probes are listed in Table S3, and in situ hybridization was performed using a kit from Boster Bio (Lot No. 10F26C; Pleasanton, CA, USA) according to the manufacturers' instructions. Immunohistochemistry was performed using the Ultrasensitive SP KIT-9720 (1909309710D), according to the manufacturers' instructions. The slides were incubated with primary antibody (macrophage stimulating 1 (MST1) and YAP1, Cell Signaling, Danvers, MA, USA) at 4 °C

overnight. A semi-quantitative scoring criterion was used based on the staining intensity and number of positive cells. All sections were independently scored by two pathologists who were blinded to the clinicopathological features and clinical data (Table S2).

Cell lines, plasmids, small interfering RNA (siRNA), and transfection

The MDA-MB-231 cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Zeta-Life, USA). The MCF-7 cell line was cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS, penicillin, streptomycin, and insulin (Genview, USA). The ethical review of cell line was uploaded.

The AATBC full sequence, the AATBC Del1 sequence, and the *YAP1* full cDNA sequence were cloned into the pcDNA3.1 (+) plasmid, and the *YBX1* full cDNA sequence was cloned into the pCMV-HA plasmid. All constructs were confirmed by sequencing. The pGL3-CTGF promoter luciferase reporter plasmid was used to examine *YAP1* luciferase activity and the pRL-TK plasmid was used as a negative control **[10]**. The siRNAs targeting AATBC, *YBX1*, and *YAP1* were synthesized by GenePharm (Shanghai, China). All sequences of primers are presented in Table S3.

For siRNA knockdown, cells were seeded overnight and then transfected with 100 nM gene-specific siRNA or a scrambled control siRNA (Sangon, China) using Lipofectamine RNAiMAX Reagent (Invitrogen, Breda, The Netherlands). For plasmid transfection, the Lipofectamine 3000 Transfection Reagent Kit (Life Technologies, Carlsbad, CA, USA) was used with Opti-MEM (Invitrogen).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Life Technologies). Nuclear and cytoplasmic fractions were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA). Complementary DNA was synthesized using the PrimeScript RT Reagent Kit (Takara Bio, Dalian, China), and qRT-PCR was performed using a SYBR Premix ExTaq II Kit (Takara Bio) in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) to determine the relative expression levels of target genes. Three independent analyses were conducted for each gene, and the mean was calculated.

Wound healing and Transwell experiments

After cells were grown to 80-90% density, a 10 μL pipet tip was used to create a wound in the cell monolayer. Images were captured at regular time intervals until the wounds healed. Transwell Cell Culture Inserts (8 μm pore size, BD Biosciences, Franklin Lakes, NJ, USA) was used for Transwell experiments.

3D cell culture experiments

Matrigel (100 μL) was added to a 24-well plate and incubated for 45 min to allow coagulation. Cell pellets containing 2,000 cells were resuspended in 200 μL 10% Matrigel, and slowly and uniformly added to the Matrigel-coated plate. The plates were incubated for 30 min to allow the cells to sink, and 200 μL of cell

culture medium was slowly added. After 48 h, the upper medium was gently aspirated and replaced with 300 μL of complete medium, which was changed every 48 h. Using an inverted microscope, we acquired daily images of five randomly selected fields of view per sample to measure cell growth. The Image Pro Plus 6.0 software was used to count the cells and the filopodia/cell in each field of view.

Animal experiments

The mice were divided into three groups, and then MDA-MB-231 cells transfected with the AATBC overexpression vector, siAATBC, or the negative control were injected into mice via their tail veins. All mice were sacrificed 6 weeks after inoculation. The lungs were removed, imaged, embedded in 10% paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological examination and evaluation of metastasis. The number and area of lung-surface metastases in each mouse were recorded. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of Central South University (Changsha, China).

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

To screen the downstream proteins of AATBC, proteomic analysis of MDA-MB-231 cells was performed after AATBC knockdown using an UltiMate 3000 RSLCnano system coupled to an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Scientific, Bremen, Germany). An average fold change of ≥ 2.0 or ≤ 0.5 was used to define differentially expressed proteins. Statistical significance was determined by Student's *t*-tests ($p < 0.05$). Altered signaling pathways after AATBC knockdown were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Western blot analysis

Briefly, 50 μg of cell lysates was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4 °C with primary antibodies against YAP1, MST1, MST2, large tumor suppressor kinase 1 (LATS1), phospho-YAP1 (Ser127), YBX1, GAPDH, and nucleolin (C23) (Cell Signaling Technology, USA). The next day, membranes incubated with antibodies targeting total proteins were washed with PBS containing 5% TWEEN-20, whereas membranes incubated with antibodies targeting phosphorylated protein were washed with tris-buffered saline containing 5% TWEEN-20. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein signals were visualized using an electrochemiluminescent detection reagent and a MiniChemi Chemiluminescence imager (Sage Creation Science, Beijing, China).

Dual luciferase reporter experiments

Cells were cotransfected with a YAP1 luciferase reporter plasmid targeting the cellular communication network factor 2 (*CTGF*) promoter region, the AATBC overexpression plasmid or the empty plasmid, and the pRL-TK plasmid (which encodes *Renilla* luciferase as an internal control). After 48 h, the luciferase

activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was normalized to the *Renilla* luciferase activity. Three independent experiments were performed.

RNA pulldown experiments, RNA immunoprecipitation (RIP) and coimmunoprecipitation

Biotin-labeled sense or antisense AATBC RNA (4,622 nucleotides) and three deletion constructs (spanning nucleotides 1-2270, 2271-3767, and 3768-4622) were synthesized and transcribed *in vitro* using the Biotin RNA Labeling Mix (Roche, USA) and T7 RNA polymerase (Promega). Biotinylated RNA (3 μg) was immunoprecipitated according to the instructions. Associated proteins were resolved by gel electrophoresis and visualized by silver staining. RIP experiments were performed according to the instructions of the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). MDA-MB-231 cells were lysed and incubated with 3 μg of the indicated antibodies and protein A/G beads (Life Technologies, Oslo, Norway) overnight. Immunoprecipitates were washed five times with lysis buffer and analyzed by western blotting.

Immunofluorescence

MDA-MB-231 and MCF7 cells were fixed with 4% paraformaldehyde for 20 min at room temperature and blocked with 5% bovine serum albumin for 30 min. The cells were incubated at room temperature with YBX1 (1:200, Proteintech, USA) and MST1 antibodies (1:200, Cell Signaling Technologies) for 1 h and then with Alexa Fluor 488- and 568-conjugated secondary antibodies (Life Technologies) at room temperature for 1 h. After each step, the cells were washed twice with PBS for 5 min. The prepared specimens were counterstained with DAPI (Southern Biotechnology Associates, USA) for 2 min and observed with a confocal microscope (UltraVIEW VoX; Perkin-Elmer, Waltham, MA, USA).

Results

The lncRNA AATBC is highly expressed in breast cancer and associated with advanced tumor stages and poor prognosis

To screen for differentially expressed lncRNAs between breast cancer samples and adjacent breast epithelial tissues, three breast cancer microarray datasets were downloaded from the GEO database (Table S1). The GSE42568 and GSE65194 datasets were used for differential expression analysis between breast cancer samples and adjacent breast epithelial tissues[11]. After SAM, the differential expression probes from the two datasets were integrated and analyzed. There were 1,751 common differentially expressed probes including 1,642 that were highly representing 1,434 genes, and 109 that were relatively lowly expressed, representing 106 genes. The analysis process is shown in Fig.S1a. Based on NetAffx, RefSeq, and Ensembl annotations of noncoding RNAs, seven lncRNAs including AATBC, HOTAIR, LOC642852, taurine up-regulated 1 (TUG1), deleted in lymphocytic leukemia 2 (DLEU2), myocardial infarction associated transcript (MIAT), and uncharacterized LOC730101 were overexpressed, and uncharacterized LOC730101 were overexpressed and one lncRNA [LOC100133920,

methylenetetrahydrofolate dehydrogenase (NADP+dependent) 1 like pseudogene] was downregulated in breast cancer tissues, compared to in healthy breast epithelial tissues, in both datasets (Fig.S1b-c).

Among these lncRNAs, AATBC was highly expressed in breast cancer samples according to the GSE42568 and GSE65194 datasets (Fig.1a). Further analysis showed that AATBC overexpression was positively associated with poor overall survival and poor recurrent-free survival of breast cancer patients, according to the GSE20711 and GSE42568 datasets (Fig.1b**)**. *In situ* hybridization based on 21 pairs of paraffin-embedded breast cancer and adjacent breast epithelial tissue samples confirmed the high expression of AATBC in breast cancer sample (Fig.1c-d).

AATBC promoted breast cancer cell migration and invasion.

First, we verified the overexpression and knockdown effects of an AATBC overexpression vector and two siRNA sequences, respectively, in the MDA-MB-231 and MCF7 cell lines (Fig. S2a). Next, we performed wound healing and transwell experiments and our results showed that AATBC overexpression could promote the migration and invasion of MDA-MB-231 and MCF7 breast cancer cells (Fig. 2a-b**;** Fig. S2b). A Matrigel-based 3D cell culture model was also used to explore the role of AATBC in MDA-MB-231 cell adhesion and invasion. AATBC overexpression showed a significantly increased number of pseudopodia, whereas expression of the two AATBC siRNAs led to the opposite phenotype (Fig. 2c). We also detected a change in the metastasis-associated molecules in MDA-MB-231 and MCF7 cells after AATBC siRNA treatment or overexpression. AATBC was found to promote the expression of MMP2, MMP9, N-Cadherin, ZEB1, ZEB2, Slug, Snail, and Claudin-1 (Fig. 2d). These results suggested that AATBC promoted breast cancer cell migration and invasion *in vitro*. In addition, we also confirmed that AATBC affected breast cancer cell apoptosis (Fig. S2c).

AATBC enhanced breast cancer lung metastasis in nude mice

We next examined the *in vivo* effects of AATBC expression in breast cancer cells. Four-week-old nude mice were randomly divided into three groups (n = 7 mice/group), and were injected with 1×10^7 MDA-MB-231 cells transfected with siAATBC siRNA or the AATBC overexpression plasmid. After 6 weeks, mice were sacrificed. Their lung tissues were removed, fixed, and imaged, and the number of metastatic nodules was counted (Fig. 3a-c). Next, mouse lungs were paraffin-embedded for H&E staining (Fig. 3d). Nodules and cancer nests in nude mice injected with siAATBC-treated cells were smaller and significantly reduced in number than in the negative control mice. In contrast, nude mice injected with the AATBC overexpressing MDA-MB-231 cells had larger nodule numbers and cancer nests than the control mice.

AATBC promoted breast cancer cell migration and invasion by inhibiting the YAP1/Hippo signaling pathway

To determine the mechanism behind AATBC-driven breast cancer metastasis, we identified AATBC-regulated proteins in MDA-MB-231 cells transfected with siAATBC or scrambled siRNA by LC-MS/MS (Fig. S3a). We identified 690 differentially expressed proteins, including 298 that were downregulated by

AATBC and 392 that were upregulated by AATBC (Table S4). Enrichment analysis in DAVID revealed that several components of the Hippo signaling pathway were altered upon AATBC knockdown (Fig. S3b). Western blotting was used to confirm the expression of the main components in this pathway among different groups, and p-YAP1 (Ser127) was found to be altered after AATBC knockdown or overexpression (Fig. 4a). Despite the changes observed at the protein level, qRT-PCR analysis indicated that AATBC did not affect the mRNA levels of the main components of the Hippo signaling pathway in MDA-MB-231 cells (Fig. S4). It is well known that YAP1 acts as a transcriptional regulator in the Hippo signaling pathway and activates transcription of genes involved in cell proliferation; further, it suppresses apoptotic genes. Analysis of cytoplasmic and nuclear fractions revealed that the overexpression of AATBC increased YAP1 protein translocation to the nucleus in MDA-MB-231 and MCF7 cells, while siAATBC had the opposite effect (Fig. 4b).

As a nuclear co-transcription regulator, YAP1 can exert biological functions upon entering the nucleus. Connective tissue growth factor (*CTGF*) is a classical target gene of YAP1, and the transcriptional activity of YAP1 can be detected by the luciferase reporter gene vector harboring the *CTGF* promoter[12]. The regulatory effect of AATBC on YAP1 prompted us to study whether AATBC regulated the transcriptional activity of YAP1. Thus, we measured the luciferase activity of the *CTGF* promoter in MDA-MB-231 and MCF7 cells after overexpression or knockdown of AATBC. As shown in Fig. 4C, downregulation of AATBC impaired the transcriptional activity of YAP1 in the MDA-MB-231 and MCF7 cells, whereas forced expression of AATBC enhanced the transcriptional activity of YAP1 in these cells.

Next, we checked whether YAP1 regulated the effects of AATBC on migration and invasion in breast cancer through overexpression or knockdown of YAP1 (two siRNAs) (Supplementary Fig.5a). The results showed that the two siYAP1 and the YAP1 overexpression vector could modulate the function of AATBC with respect to migration and invasion *in vitro* (Supplementary Fig.5b-g). The wound healing and transwell experiments showed that the knockdown of AATBC or YAP1 inhibited the migration and invasion of MDA-MB-231 and MCF7 cells, whereas the overexpression of AATBC or YAP1 enhanced the migratory and invasive properties of MDA-MB-231 and MCF7 cells. Notably, overexpression of YAP1 reversed the inhibitory effect of siAATBC when the YAP1 overexpression vector and AATBC siRNAs were co-expressed in MDA-MB-231 and MCF7 cells. Moreover, knockdown of YAP1 also reversed the enhancing functions of AATBC when the AATBC overexpression vector and siYAP1 were co-expressed in MDA-MB-231 and MCF7 cells. These data suggested that YAP1 participated in the AATBC-modulated migration and invasion of MDA-MB-231 and MCF7 cells.

AATBC promoted breast cancer cells migration and invasion by interacting with YBX1

To explore the mechanism through which AATBC regulates YAP1/Hippo signaling in breast cancer, RNA pulldown experiments were performed. Biotinylated sense and antisense AATBC strands were transcribed *in vitro* and incubated with MDA-MB-231 lysates. The RNA-protein complexes were then captured on streptavidin affinity magnetic beads and subjected to mass spectrometry (Fig. 5a). However, the YAP1 protein was not identified, indicating that AATBC did not directly interact with YAP1.

We next screened the mass spectra data and found that YBX1, a Y-box protein, was among the identified potential interacting proteins (Supplementary Table 5); YBX1 was also identified as a potential AATBC-binding protein by the catRAPID algorithm [13](Fig.S6; Table S6). Therefore, we next sought to verify the interaction between YBX1 and AATBC. Western blot experiments showed that the AATBC sense strand could bind to YBX1, whereas the antisense strand displayed very weak binding (Fig. 5b). To confirm this interaction between AATBC and YBX1, RIP experiment were conducted. MDA-MB-231 and MCF7 cell lysates were incubated with YBX1 antibody, and co-precipitated RNA was analyzed by qRT-PCR using primers targeting AATBC, LOC284454 (another lncRNA; used as a negative control), and U1 small nuclear ribonucleoprotein subunit 70 (SNRP70; used as a positive control). The enrichment of AATBC, but not LOC284454 lncRNA, was observed in both cell lines, indicating a specific interaction between AATBC and YBX1 (Fig. 5c). MST1 was selected as a negative control, and it did not interact with AATBC. We next performed a deletion-mapping assay to determine the region of AATBC that interacts with YBX1. The AATBC sense strand was divided into three fragments. The data showed that the Del 1 fragment (nucleotides 1–2270) bound to YBX1 with higher affinity than the Del 2 (nucleotides 2271–3767) and Del 3 (nucleotides 3768–4622) fragments (Fig. 5d). Taken together, these observations indicate that AATBC binds YBX1 via the fragment containing nucleotides 1-2270. Fortunately, we have also proved AATBC mainly interacting with C-terminal fragment (Fig. 5e).

Next, we measured whether AATBC Del 1 promoted migration and invasion through the YAP1/Hippo pathway. The data of the wound healing assay and transwell assay with or without Matrigel showed that overexpression of AATBC Del 1 could induce migration and invasion of MDA-MB-231 and MCF7 cells through the Hippo signaling pathway (Fig. 5f-h).

YBX1 regulated the YAP1/Hippo signaling pathway by interacting with MST1.

Further, transwell experiments with or without Matrigel and wound healing experiments showed that YBX1 reversed the migrative and invasive phenotype of MDA-MB-231 and MCF7 cells mediated by AATBC (Fig.S7). These results suggested that AATBC promoted the migration and invasion of breast cancer cells by directly interacting with YBX1.

Immunofluorescence showed that YBX1 could colocalize with MST1, an upstream kinase of the YAP1/Hippo signaling pathway (Fig. 6a). This indicated further interaction between YBX1 and MST1. Accordingly, this interaction was confirmed by immunoprecipitation (Fig. 6b). Co-immunoprecipitation experiment was used to detect the interaction between MST1 and YBX1 mutants. We transfect YBX1 full length or YBX1 deletion mutants (C-terminal and △C) into MDA-MB-231 cells respectively. 48h later, collect cells for Co- IP experiment. WB detects the specific structural region of MST1 binding YBX1, through experimental results, we determined that the C-terminal of YBX1 is the structural basis for interaction with MST1(Fig. 6c).Moreover, as we predicted, there was an interaction between YBX1 and MST1 in MDA-MB-231 and MCF7 cells, and YBX1 was found to regulate the expression of many components of the YAP1/Hippo signaling pathway (Fig. 6d).

Further, we checked whether YBX1 participated in the regulatory effect of AATBC on the YAP1/Hippo signaling pathway. When AATBC was knocked down, the expression of MST1, MST2, LATS1, and pYAP1 proteins was markedly increased, and overexpression of YBX1 rescued this increase. In contrast, the overexpression of AATBC decreased the expression of MST1, MST2, LATS1, and pYAP1 proteins, and YBX1 depletion could rescue these changes (Fig. 6e and Fig.S7). These data suggested that AATBC regulated the YAP1/Hippo signaling pathway by binding to YBX1.

MST1, a serine/threonine kinase and core component of the mammalian YAP1/Hippo pathway, has two isoforms, a 59-kDa full-length protein and a truncated 36-kDa amino-terminal fragment, both of which have full catalytic activity [**14**]. To determine whether AATBC contributed to MST1 stability, AATBC was overexpressed in MDA-MB-231 or MCF7 cells, which were then treated with bortezomib to inhibit proteasome-mediated degradation (Fig. 6f; dimethyl sulfoxide [DMSO] was used as a control). In AATBC-overexpressing cells, compared to the negative control cells, levels of 36-kDa MST1 were increased and that of 58-kDa MST1 were decreased, suggesting that AATBC overexpression promoted MST1 cleavage and degradation. AATBC-modulated cleavage and degradation of MST1 could be rescued when YBX1 was knocked down. These results suggested that AATBC regulated MST1 expression by decreasing its physical stability and YBX1 acted as a bridge connecting AATBC and the YAP1/Hippo signaling pathway.

The expression and correlation between AATBC and YAP1, YBX1, and MST1 in clinical samples and mice tissues.

Next, we utilized 21 pairs of paraffin-embedded breast cancer and adjacent breast epithelial tissue samples to assess the potential clinical relationships among AATBC, YBX1, MST1, and YAP1 (Fig. S8a). Immunohistochemical staining showed that MST1 was mainly expressed in the cytoplasm of only 19.04% (4/21) of the breast cancer samples, in contrast to 71.43% (15/21) of the adjacent breast epithelial samples. Conversely, both YAP1 and YBX1 were highly expressed in breast cancer tissues, compared with in adjacent breast epithelial tissues (high expression rate: breast cancer, 100% [21/21] vs. adjacent breast epithelium, 9.5% [2/21]). As shown in Fig.S8b, we defined the patients who showed higher AATBC, YBX1, and YAP1 expression and lower MST1 expression in tumor tissues than in normal tissues as relevant patients. Conversely, we defined the patients who showed the opposite findings as irrelevant patients. We found that 95.24% of patients were relevant patients. We also found that 60% of these patients showed high AATBC, YBX1, and YAP1 expression and low MST1 expression in tumor tissues and low AATBC, YXB1, and YAP1 expression and high MST1 expression in normal tissues. We defined these patients as highly relevant patients. Both types of patients showed that AATBC expression was positively correlated with YAP1 expression and negatively correlated with MST1 expression in breast cancer tissues. Meanwhile, we also examined the expression of AATBC, YBX1, MST1, and YAP1 in the lung tissues of nude mice (Fig.S8c and Table S7). Compared to the negative control group, the AATBC group showed high YBX1 and YAP1 expression and low MST1 expression, whereas the siAATBC group showed the opposite findings. These data suggested that AATBC could negatively regulate MST1 expression and positively regulate YBX1 and YAP1 *in vivo*.

Discussion

Many studies have indicated that lncRNAs play important roles in tumors. However, the functions of most remain unclear. Large amounts of expression data regarding various tumors are stored in public databases, and screening differentially expressed lncRNAs from these databases is an effective way to discover new tumor-associated lncRNAs. In this study, we identified a lncRNA-AATBC, which was highly expressed in two separate breast cancer microarray datasets and associated with poor prognosis for breast cancer patients, indicating that it could be a promising target for breast cancer treatment.

Among these eight molecules, TUG1, DLEU2, MIAT, and HOTAIR have been reported to be associated with breast cancer progression or poor prognosis. As the aim of this study was to find new lncRNA molecules associated with poor prognosis of breast cancer and we hoped that we could find a potential prognostic factor and therapeutically targeted molecule for breast cancer, the study of these molecules did not contribute much to the manuscript. For LOC642852 and LOC100133920, their full names being sorting nexin 18 pseudogene 2 and methylenetetrahydrofolate dehydrogenase (NADP+dependent) 1 like pseudogene, respectively, there are also few research manuscripts on such pseudogenes and thus, we did not study these two molecules. As for LOC730101, it has been reported that the downstream molecular mechanism through which it promotes cancer development is by affecting the Wnt signaling pathway. Whereas there is only one literature report of AATBC currently, which found that it is related to cancer apoptosis-related in bladder cancer, but the molecular mechanism underlying its cancer-promoting effect has not been studied in depth, and thus, we chose AATBC for follow-up study.

To identify the mechanism through which AATBC affects breast cancer, we performed quantitative proteomics to analyze differentially expressed proteins in MDA-MB-231 cells transfected with and without siAATBC. Several molecules of the YAP1/Hippo signaling pathway were found to be modulated by AATBC. In the Hippo pathway, YAP1 is a crucial downstream effector and plays an important role in the development of various tumors; it is also a potential target for cancer therapy[15-20]. Normally, the upstream Hippo components MST1/MST2 and LATS1/LATS2 promote YAP1 phosphorylation, promoting the phosphodependent binding of 14-3-3 proteins, which sequester YAP1 in the cytoplasm. Thus, the majority of YAP1 is retained in the cytoplasm and cannot enter the nucleus, rendering the Hippo pathway inactivated. In some tumor cells, MST1/MST2 and LATS1/LATS2 can inhibit YAP1 phosphorylation, resulting in its translocation into the nucleus, where it acts as a cotranscription factor, activating transcriptional programs that result in a series of physiological changes, including proliferation, invasion, metastasis, and other processes advantageous to tumors [15-20]. In this study, we found that AATBC regulates the YAP1/Hippo signaling by binding YBX1. Further study will be required for deeper understanding of the molecular mechanism through which AATBC regulates the YAP1/Hippo pathway in breast cancer.

Pulldown experiments and RIP experiments showed that AATBC and YBX1 could interact with each other, and YBX1 was also found to interact with the MST1 protein, but there was no interaction between AATBC and MST1. Thus, AATBC might serve as a molecular guide to mediate the binding of YBX1 and MST1

and AATBC could participate in the YBX1-mediated inhibition of YAP1 phosphorylation through the formation of a YBX1/MST1 complex. Recent reports have demonstrated that YBX1 interacts with lncRNAs and participates in their functions [21-25]. For example, hepatocellular carcinoma up-regulated long noncoding RNA acts as a YBX1 scaffold, promoting the phosphorylation of YBX1 and extracellular signal-regulated kinase to promote hepatocarcinogenesis [26]. YBX1 also interacts with and activates HOXC cluster antisense RNA 3 to promote gastric cancer progression [23, 26]. As demonstrated for the lncRNA SWI/SNF complex antagonist associated with prostate cancer 1, lncRNAs act as molecular guides, binding proteins and delivering them to specific targets [27]. This is the first report suggesting that AATBC acts as a YBX1 scaffold, regulating the YAP1/Hippo signaling through an AATBC/YBX1/MST1 axis that inhibits YAP1 phosphorylation and promoting its translocation and transcriptional activity (Fig.6f).

Conclusion

In summary, we demonstrate that AATBC is a novel oncogene in breast cancer, which could promote-breast cancer cell migration and invasion *in vitro* and accelerate tumor metastasis *in vivo*. Furthermore, we identify an AATBC/YBX1/MST1 signaling axis that is pivotal for breast cell metastasis. This is also an important supplement to the composition of the YAP1/Hippo signaling pathway. These results indicate that AATBC is a potential prognostic factor and therapeutic target for breast cancer.

Abbreviations

AATBC: Apoptosis-Associated Transcript in Bladder Cancer

CTGF: Cellular Communication Network Factor 2

DAVID: Database for Annotation Visualization and Integrated Discovery

DLEU2: Deleted in Lymphocytic Leukemia 2

DMSO: Dimethyl Sulfoxide

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde 3-phosphate Dehydrogenase

GEO: Gene Expression Omnibus

LATS1: Large Tumor Suppressor Kinase 1

LC-MS/MS: Liquid Chromatography Coupled to Tandem Mass Spectrometry

lncRNAs: Long noncoding RNAs

MIAT: Myocardial Infarction Associated Transcript

MST1: Macrophage Stimulating 1

OS: Overall Survival

qRT-PCR: Quantitative Reverse Transcription-polymerase Chain Reaction

RFS: Recurrent-Free Survival

RIP: RNA Immunoprecipitation

SAM: Significant Analysis of Microarray

siRNA: Small interfering RNA

TUG1: Taurine up-regulated 1

YAP1: Yes Associated Protein 1

YBX1: Y-box Binding Protein 1

Declarations

Ethics approval and consent to participate

Tissue samples in this study were collected from the Second Xiangya Hospital of Central South University. All specimens were confirmed by histopathological examination, and the study was approved by the Research Ethics Board of Central South University. Signed informed consent was obtained from each participant before enrollment in this study. And all experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of Central South University (Changsha, China).

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

M.W., M.D., D.W. and C.G. were responsible for the experimental design. T.T., F.X., and B.X. performed the data analysis, and statistical analysis. M.Z., X.L., Y.L. and Z.Z. were involved in drafting and revision of the manuscript. W.X., G.L. and Z.Z. supervised this study. All authors discussed the results and commented on the manuscript.

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Figures

Figure 1

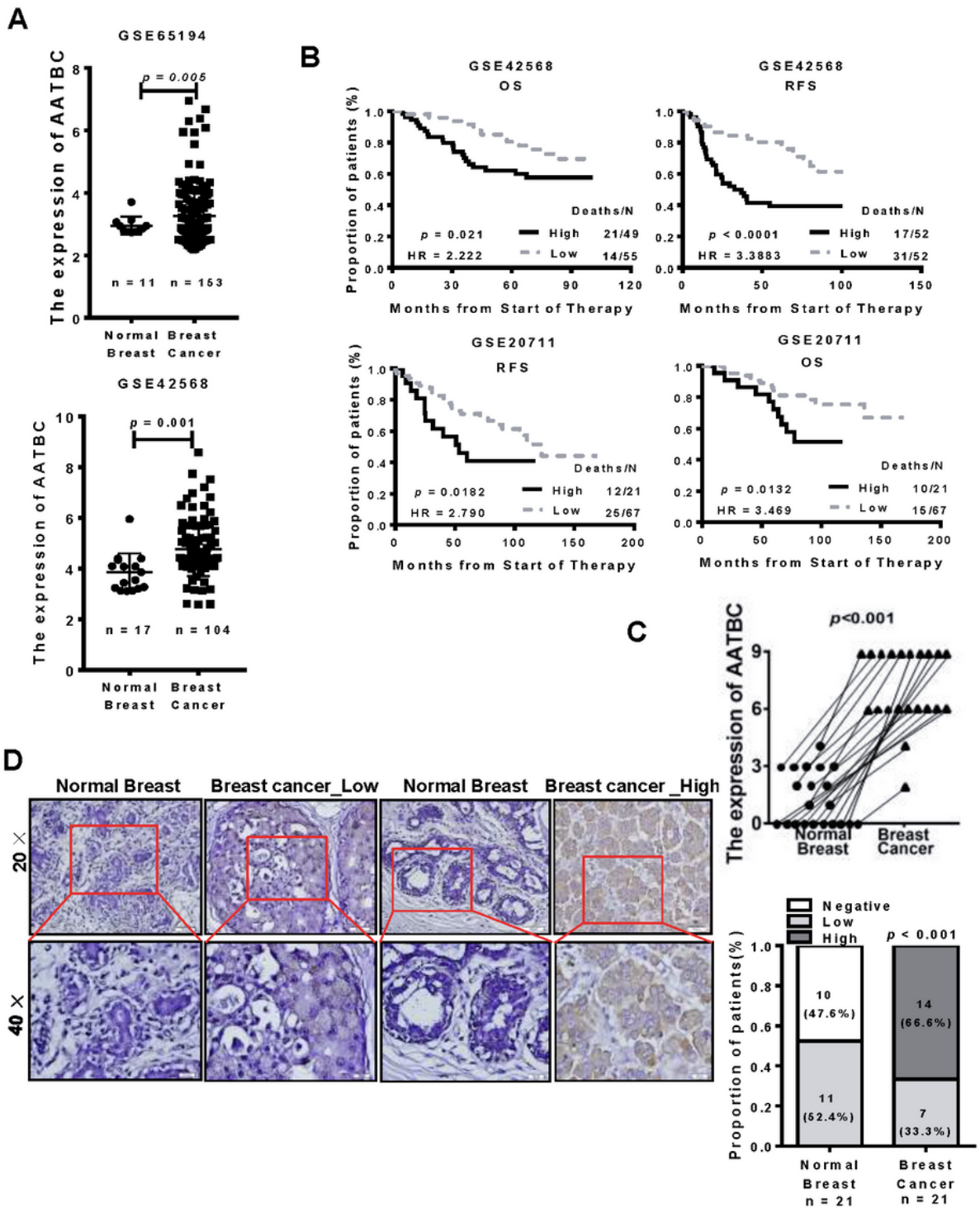

Figure 1

AATBC is overexpressed in breast cancer tissues, and is significantly associated with poor prognosis of breast cancer patients. a The expression of AATBC in normal breast tissue and breast cancer tissues according to the GSE65194 (normal breast, n = 11; breast cancer, n = 153) and GSE42568 (normal breast, n = 17; breast cancer, n = 104) two databases. b Kaplan Meier analysis of the impact of high AATBC expression on the overall survival and relapse free survival of breast cancer patients in the GSE42568 (n =

104) and GSE20711 cohort (n = 88). c-d Relative AATBC expression detected by in situ hybridization in 21 pairs of breast cancer and adjacent breast epithelial tissues. Representative field views in matched samples are shown. HR: hazard ratio; N: normal breast epithelial tissues; OS: overall survival; RFS: relapse free survival.

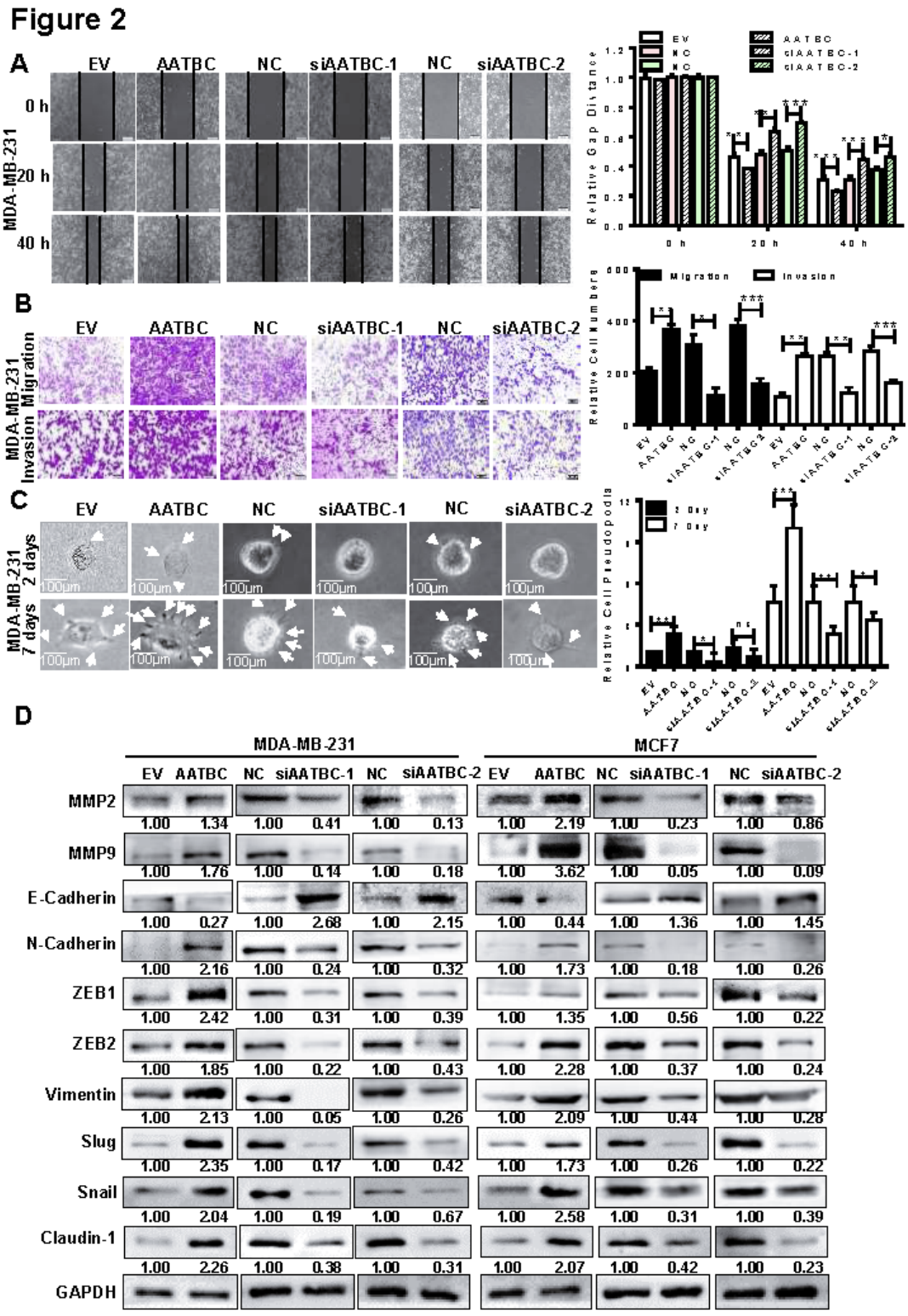

Figure 2

AATBC expression promotes the migration and invasion abilities of MDA-MB-231 cells. a Wound healing experiments were performed after AATBC overexpression and siRNA knockdown in MDA-MB-231 cells. Data represent the mean ± standard error of the mean. b Transwell experiments were performed without (migration) and with (invasion) Matrigel after AATBC overexpression and siRNA knockdown in MDA-MB-231 cells. c A 3D culture model of invasion using MDA-MB-231 cell spheroids formed in Matrigel. White arrows indicate scattered protrusions formed on the spheroid surfaces. Scale bars = 100 μm. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. d Western blotting showed that AATBC regulated the expression of some EMT and metastasis markers.AATBC: the AATBC overexpression vector; EV: the empty vector; NC: negative control siRNA; siAATBC-1: AATBC siRNA-1；siAATBC-2: AATBC siRNA-2.

Figure 3

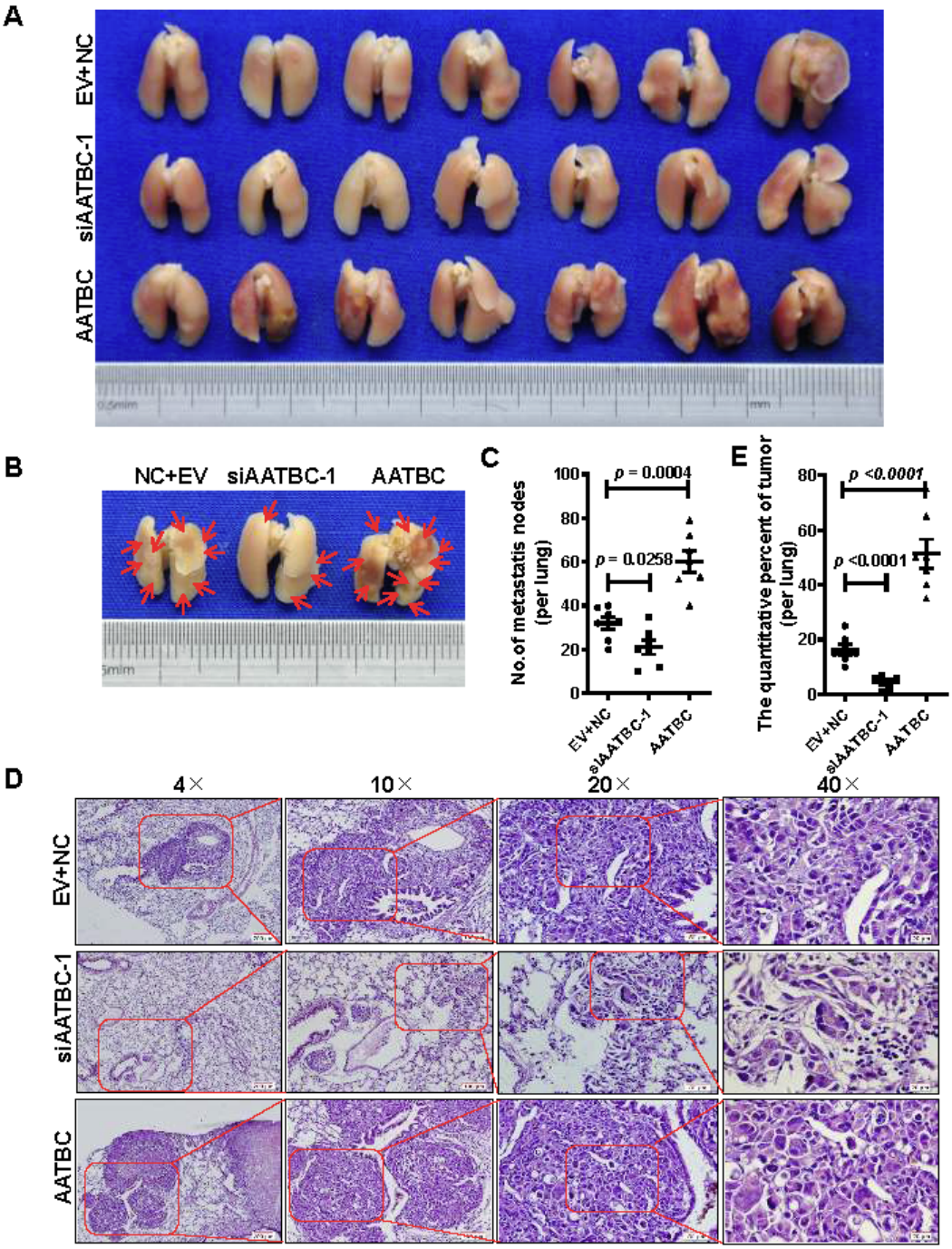

Figure 3

AATBC promotes lung metastasis of breast cancer cells in vivo. MDA-MB-231 cells transfected with AATBC siRNA, scrambled siRNA and the empty vector, or the AATBC overexpression plasmid were injected into the tail veins of nude mice (n = 7/group), which were sacrificed 6 weeks later. Representative images of lungs a and metastatic nodules b indicated by arrows. c Quantification of the number of metastatic

nodes in the lungs. d H&E staining of lung metastatic lesions from each group. (AATBC: AATBC overexpression; NC: scrambled control siRNA; siAATBC: AATBC siRNA-1).

Figure 4

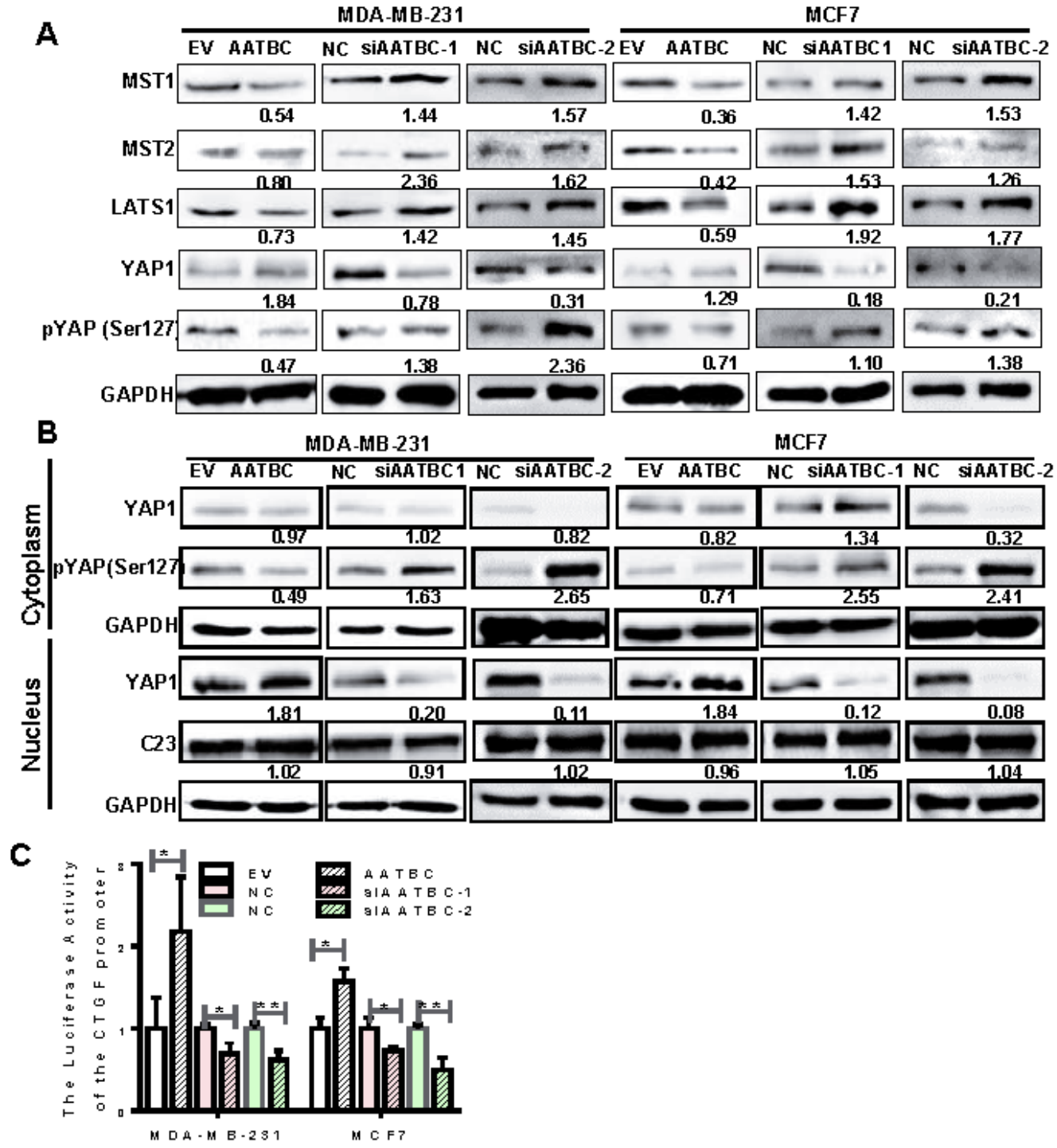

Figure 4

AATBC regulates the YAP1/Hippo signaling pathway. a Western blot analysis of the Hippo signaling pathway components in MDA-MB-231 cells after AATBC overexpression or knockdown. b Western blot analysis of YAP1 phosphorylation and nuclear translocation in MDA-MB-231. α-tubulin was used as a

cytoplasmic marker, and C23 was used as a nuclear marker. c Knocking down AATBC in MDA-MB-231 and MCF7 cells impaired the transcriptional activity of YAP1 based on the luciferase assays, whereas the overexpression of AATBC enhanced the activity of YAP1 in MDA-MB-231 and MCF7 cells. *, $p < 0.05$ *; $p < 0.01$. AATBC: the AATBC overexpression vector; EV: the empty vector; NC: negative control siRNA; siAATBC-1: AATBC siRNA-1; siAATBC-2: AATBC siRNA-2.

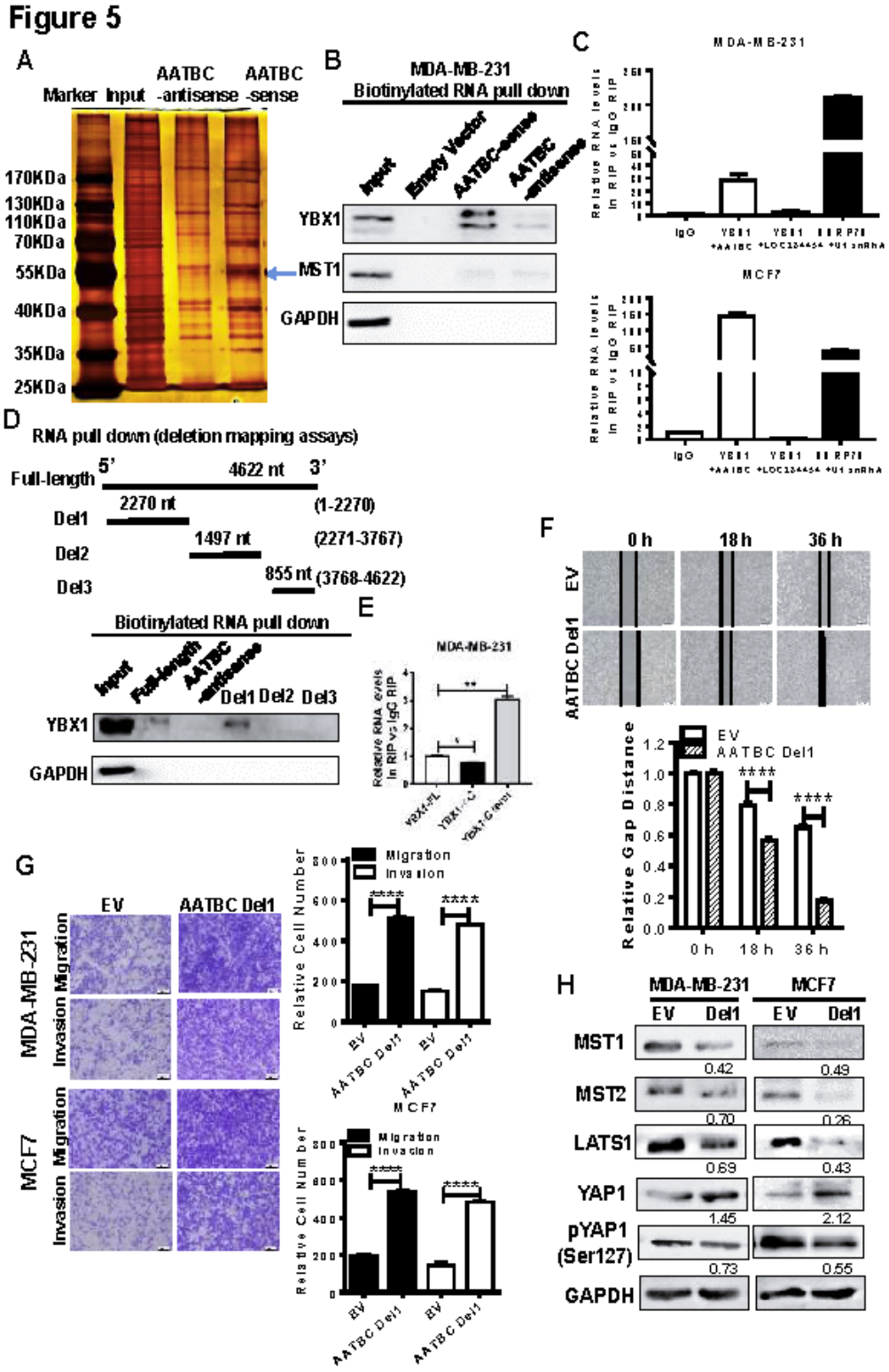

Figure 5

AATBC binds with YBX1 through 1-2270 bp region of AATBC. a RNA pulldown experiments were performed using biotinylated AATBC in MDA-MB-231 lysates. Interacting proteins were resolved, visualized by silver staining, and identified by LC-MS/MS. The arrow indicates the approximate position of YBX1. b The interaction between AATBC and YBX1 was detected by western blotting after RNA pull-down assays of the sense and antisense AATBC in MDA-MB-231 cell lysates. c RIP experiments of AATBC in MDA-MB-231 and MCF7 cells using anti-YBX1 and IgG antibodies. The fold enrichment of lncRNAs are shown relative to the matched control IgG RIP experiments. The lncRNA LOC284454 and SNRNP70 were used as negative and positive controls, respectively. RIP, RNA immunoprecipitation. d RNA pull-down assays were performed using the AATBC deletion mapping constructs in MDA-MB-231 cell lysates. e RIP experiment was performed. Transfect empty vector, YBX1 full length or YBX1 deletion mutants (C-terminal and △C) into MDA-MB-231 cells respectively. 48h later, collect cells for RIP experiment. qPCR detects the specific structural region of AATBC binding YBX1. (FL: full length; △C: C-terminus deletion; C-term: C-terminus.) f AATBC Del1 (1-2270 bp) promoted migration of MDA-MB-231 by wound healing assay. g AATBC Del1 (1-2270 bp) promoted migration and invasion of MDA-MB-231 and MCF7 cells by transwell assay with or without Matrigel. h Western blotting showed that the Hippo signaling pathway was regulated by AATBC Del1 (1-2270 bp).EV: the empty vector; NC: negative control siRNA; AATBC Del1: the AATBC Del1 (1-2270 bp) overexpression vector. Scale bars = 100 μm. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 6

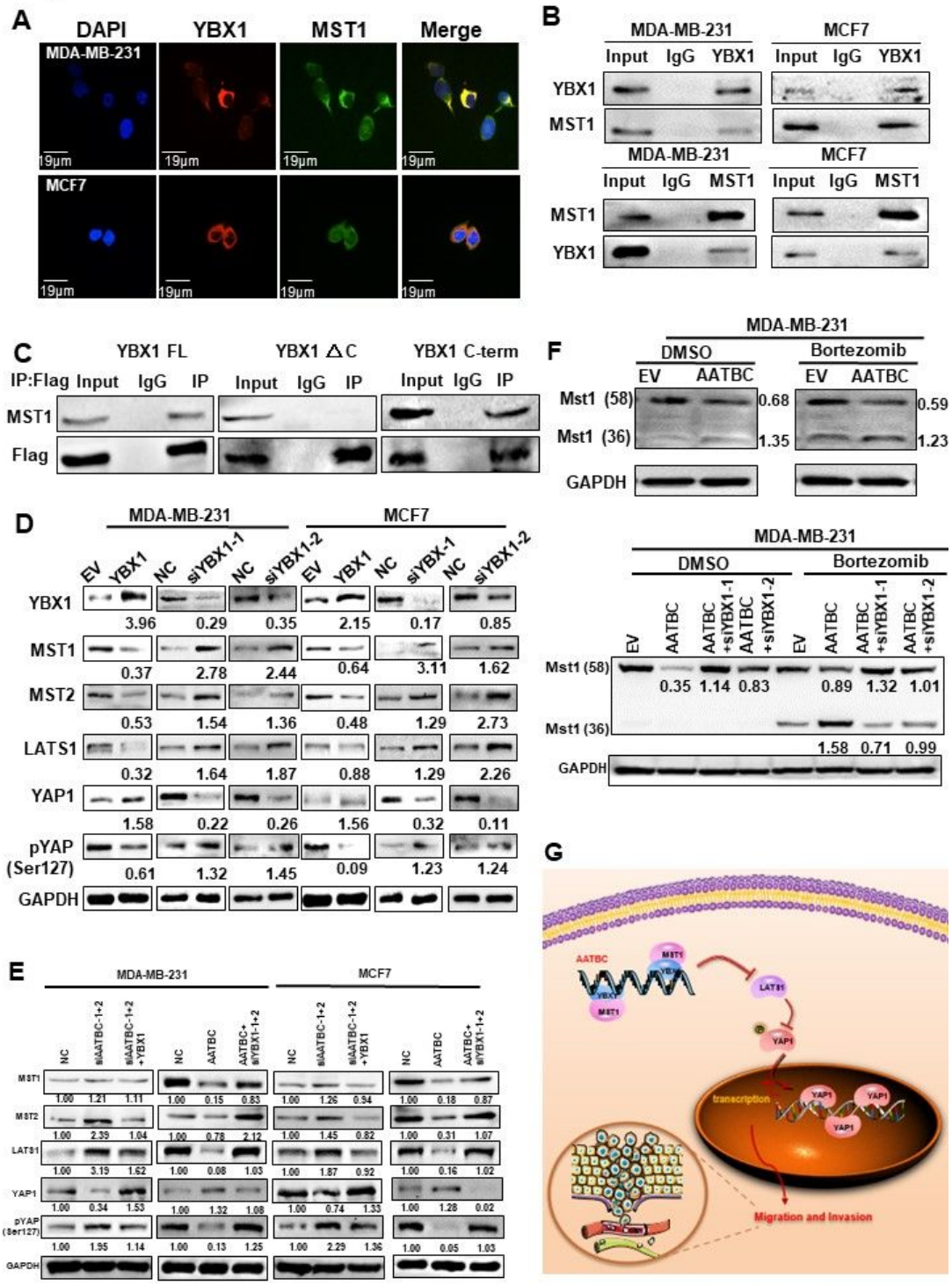

Figure 6

YBX1 participates in the AATBC-mediated regulation of the YAP1/Hippo pathway by binding to MST1. a Immunofluorescence was performed using anti-YBX1 and anti-MST1 antibodies in MDA-MB-231 cells. DNA was stained with DAPI. Scale bars = 19 μm. b Immunoprecipitation was performed in MDA-MB-231 and MCF7 cell lysates using anti-YAP1 and anti-MST1 antibodies. c Co-immunoprecipitation test to detect the interaction between Mst1 and YBX1 truncation. d Western blot analysis of the YAP1/Hippo

pathway components after YBX1 overexpression or knockdown in MDA-MB-231 and MCF7. e Western blot analysis was used to determine if YBX1 participates in the regulation of AATBC with respect to activation of the YAP1/Hippo pathway in MDA-MB-231 and MCF7 through overexpresion or knockdown of AATBC or YBX1. f MDA-MB-231 cells were transfected with the empty vector and scrambled control siRNA or the AATBC overexpression vector or co-transfected with YBX1 siRNA and the AATBC overexpression vector for 48 h, which was followed by treatment with DMSO or 25 nM bortezomib for 24 h. MST1 was detected by western blotting. g AATBC may act as a YBX1 scaffold, regulating YAP1/Hippo signaling through an AATBC/YBX1/MST1 axis that inhibits YAP1 phosphorylation and promotes its translocation and transcriptional activity. AATBC: the ATTBC overexpression vector; DAPI: 4',6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide.

Supplementary Files

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