

## Original Article

# Long noncoding RNA HOXC-AS3 indicates a poor prognosis and regulates tumorigenesis by binding to YBX1 in breast cancer

Jun Su<sup>1\*</sup>, Bo Yu<sup>2\*</sup>, Chongguo Zhang<sup>3</sup>, Peiqiang Yi<sup>1</sup>, Huan Li<sup>1</sup>, Cheng Xu<sup>1</sup>, Lu Cao<sup>1</sup>, Peizhan Chen<sup>4</sup>, Min Li<sup>1</sup>, Kunwei Shen<sup>5</sup>, Jiayi Chen<sup>1</sup>

<sup>1</sup>Department of Radiation Oncology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, People's Republic of China; <sup>2</sup>State Key Laboratory of Oncogenes and Related Genes, Renji-Med X Clinical Stem Cell Research Center, Ren Ji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, People's Republic of China; <sup>3</sup>Department of Oncology, Second Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, People's Republic of China; <sup>4</sup>Clinical Research Center, Ruijin Hospital North, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China; <sup>5</sup>Comprehensive Breast Health Center, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, People's Republic of China. \*Equal contributors.

Received August 18, 2020; Accepted September 29, 2020; Epub October 15, 2020; Published October 30, 2020

**Abstract:** Multiple studies have highlighted the importance of long noncoding RNAs in tumorigenesis. However, the molecular mechanisms underlying the role of lncRNAs in breast cancer are not well understood. Recently, the lncRNA HOXC-AS3 has drawn significant attention due to its regulatory effects on the tumorigenesis of human cancers. However, the potential molecular mechanisms whereby it mediates breast cancer progression remain unknown. Based on public breast cancer expression data and using bioinformatics methods, we discovered significantly upregulated expression levels of HOXC-AS3 in diseased tissues. We verified this result in breast cancer samples and found that the expression of HOXC-AS3 was well correlated with the prognosis of breast cancer. *In vitro* and *in vivo* experimental evidence suggests that HOXC-AS3 has the potential to regulate tumorigenesis. Further, mechanistic studies demonstrated the potential of HOXC-AS3 in the transcriptional activation of TK1 via its binding to YBX1. Furthermore, the silencing of TK1 reversed HOXC-AS3-mediated increase in breast cancer cell growth and migration. In conclusion, these results indicated the potential value of HOXC-AS as a prognostic biological marker for breast cancer, and possibly, as a therapeutic target.

**Keywords:** Long noncoding RNAs, HOXC-AS3, tumorigenesis, YBX1, TK1

## Introduction

Breast cancer is associated with the highest morbidity and mortality among all cancers in women, accounting for 22.9% of all malignant tumors in women and 11.6% of cancer-related deaths [1, 2]. For decades, researchers have investigated the potential mechanisms underlying the progression and development of breast cancer. Thus, the diagnosis accuracy and treatment efficacy can be improved by identifying novel therapeutic targets.

To date, research on the underlying mechanisms in cancer has focused on the dysregulation of 2% of the genome that encodes proteins [3, 4]. Long noncoding RNAs (lncRNAs) are a

newly discovered type of RNA that were initially reported as spurious transcriptional noise, but have subsequently been revealed to have regulatory effects on carcinomas [5]. Since the initial study that found that the lncRNA Xist is of vital importance to the inactivation of the X chromosome [6], growing evidence has shown that the potential effects of lncRNAs on tumorigenesis may be more extensive. The therapeutic potential of lncRNAs has been demonstrated in multiple preclinical and clinical studies [7-9]. For instance, our group has previously suggested that the levels of lncRNA AFAP1-AS1 were associated with a poor prognosis of lung cancer, whose mechanism was attributed to the regulation of p21 expression and, thus, to cell proliferation [10]. In addition, activated

lncRNA H19 has been found to promote cell proliferation in breast cancer [11]. Moreover, in September 2015, the double-stranded DNA plasmid BC-819 (DTA-H19) was approved for two fast-track Phase III clinical trials, confirming its potential therapeutic effects on H19-overexpressing cancer cells, which was achieved by using the gene segment of diphtheria toxin-A with an H19 control sequence [12]. Despite this evidence, the biological functions of lncRNAs in breast cancer require further investigation in order to understand the molecular mechanisms underlying lncRNA-mediated protection in breast cancer.

To investigate whether the expression of lncRNAs is altered in breast cancer, we first used The Cancer Genome Atlas (TCGA) to check their expression level. We observed that the expression level of HOXC-AS3 was substantially increased in breast cancer tissues compared to adjacent non-tumor tissues. In addition, we found that HOXC-AS3 expression was associated with a poor prognosis of breast cancer. We further investigated how HOXC-AS3 affects carcinogenesis in the breasts. Overall, our findings suggest that HOXC-AS3 may serve as a prognostic marker and a potential therapeutic in breast cancer.

### Materials and methods

#### *Collection of tissues and ethical statement*

Our study included 60 patients undergoing surgical resection of primary invasive breast cancer at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, and the Second Affiliated Hospital of Nanjing Medical University since 2012. The following inclusion criteria were considered: (1) female patients diagnosed with unilateral disease, (2) invasive breast carcinoma confirmed via histological examination, (3) patients without diagnostic signs of metastasis, and (4) patients without presurgical treatment. Patients who did not meet the inclusion criteria were excluded from the study. Patients with inflammatory breast cancer or breast carcinoma *in situ* were also excluded. The follow-up monitoring for these patients was completed on September 10, 2019. The median follow-up time was 48.6 months. This study was approved by the Medical Ethical Committee of Ruijin Hospital (Shanghai, PR China) and Second Affiliated Hospital of Nanjing

Medical University (Nanjing, Jiangsu, PR China) and was conducted in accordance with the Helsinki Declaration. All patients were informed and consent forms for publication were signed. Pathological staging was defined using the AJCC 7<sup>th</sup> edition. The histopathologic information of the 60 patients was reconfirmed by senior pathologists, and the clinicopathological characteristics are summarized in **Table 1**.

#### *Acquisition of RNA expression data for breast cancer*

TCGA database was used to generate RNA sequencing data for breast cancer (<https://gdc-portal.nci.nih.gov/>). The lncRNA probes were obtained from the Ensembl database (<http://www.ensembl.org/index.html>), and the data were analyzed using R scripts.

#### *Cell culture*

The collection of breast cancer cell lines used in this study (MDA-MB-231, BCAP37, MCF-7, BT549, ZR7530, and T47D) was obtained from the Chinese Academy of Sciences, Institute of Biochemistry and Cell Biology (Shanghai, China). The cell lines were cultured and maintained in DMEM medium (GIBCO-BRL, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum at 37°C and 5% CO<sub>2</sub> conditions.

#### *Real-time qRT-PCR analysis*

TRIzol reagent (Invitrogen) was used to extract RNA, which was converted to cDNA using the PrimeScript RT reagent Kit, according to the manufacturer's instructions. To determine the expression levels of TK1, YBX1, and HOXC-AS3, qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China) and the results were normalized to the levels of  $\beta$ -actin using the  $2^{-\Delta\Delta Ct}$  method. **Table S1** shows the primers used in this study.

#### *siRNA knockdown*

Negative control RNA (si-NC) and individual small interfering RNA (siRNA) with target sequences were provided by Invitrogen (**Table S1**). The target sequences in YBX1 and TK1 have been described previously [13, 14]. MDA-MB-231 and T47D cells were transfected with the siRNAs and si-NC using Lipofectamine 2000 (Invitrogen) for 48 h before further experiments. The plasmid transfection of breast

## Roles of HOXC-AS3 in breast cancer

**Table 1.** The clinic-pathological factors of 60 breast cancer patients

Characteristics	(% of patients)	Expression of HOXC-AS3		P-value
		low	high	
Age				0.754
≤ 45	13 (21.67%)	7	6	
> 45	47 (78.33%)	23	24	
Histological grade (middle or low, high)				0.559
middle or low	44 (73.33%)	23	21	
high	16 (26.67%)	7	9	
ER				0.774
negative	17	9	8	
positive	43	21	22	
PR				0.432
negative	25	14	11	
positive	35	16	19	
HER2				0.117
negative	47	26	21	
positive	13	4	9	
TNM stage				< 0.01**
I and II	43 (71.67%)	28	15	
III and IV	17 (28.33%)	2	15	
Lymph node metastasis				< 0.01**
negative	24 (40%)	20	4	
positive	36 (60%)	10	26	
Tumor size				0.405
≤ 2 cm	19 (31.67%)	11	8	
> 2 cm	41 (68.33%)	19	22	

\*\*P < 0.01.

cancer cell line was performed using XtremeGENE™ HP DNA Transfection Reagent (Roche, CA, USA).

### Cell proliferation assays

The rate of cellular proliferation was determined using the Cell Proliferation Reagent Kit I (MTT) (Roche Applied Science) and Cell-Light EdU Apollo567 *In Vitro* Kit (EdU) (RiboBio, Guangzhou, China). MTT analysis was performed for the MDA-MB-231 and T47D cells in 96-well plates (2,000 cells/well) every 24 h. Forty-eight hours later, the EdU assay was performed in 24-well plates (2,000 cells/well) using fluorescence microscopy. All steps were conducted in quadruplicates. Eight hundred cells were inoculated into a 6-well plate to perform the colony-formation assay. After 14 days, methanol and crystal violet (0.1%) (Sigma Aldrich) were used to fix and stain the cells. The experiment was repeated three times for each group.

### Flow cytometry analysis of apoptosis

The MDA-MB-231 cells were trypsinized and collected 48 h after siRNA knockdown. Propidium iodide and FITC-Annexin V were used for staining, and flow cytometry (FACScan; BD Biosciences) was used to analyze the samples. The cells at different stages were discriminated, and the frequency of cells undergoing early apoptosis was compared with that of the control group. Data were collected from three technical repeats.

### Western blot analysis

RIPA buffer (Beyotime, Beijing, China) containing a protease inhibitor cocktail and phenylmethylsulfonyl fluoride (Roche, CA, USA) were used to lyse and collect cells. Total protein levels were determined using a protein assay kit from Bio-Rad (Epizyme, Shanghai, China). Proteins (50 µg) were subjected to a 10% SDS-polyacrylamide gel electrophoresis (SDS-

PAGE). Following the transfer of proteins onto a nitrocellulose membrane (Sigma), this was incubated with the indicated antibodies. ECL was used to visualize the protein bands, and band intensity was calculated using densitometry (Quantity One software; Bio-Rad, CA, USA) and  $\beta$ -actin was used as a control. The antibodies for toy-box binding protein 1 (YBX1) and thymidine kinase 1 (TK1) (1:1,000) were obtained from Abcam.

### *Tumor-formation assay using nude mouse*

Athymic BALB/c mice (at five weeks of age) were housed under specific pathogen-free conditions following the approval from the Shanghai Medical Experimental Animal Care Commission. MDA-MB-231 cells were transfected using shRNA and empty vectors, before being xenografted into female BALB/c nude mice. For the visible tumors, their volume was calculated as  $\text{length} \times \text{width}^2 \times 0.5$ , every 2 or 3 days. The same steps were performed for the mice that received shHOXC-AS3 and for those of the control group. After 29 days, the mice were sacrificed, and the tumors were collected for further measurements.

### *Chromatin immunoprecipitation (ChIP) assay*

The EZ-ChIP kit (Millipore, USA) was used for the ChIP assay, as per instructions provided by the manufacturer. YBX1 antibodies were acquired from Abcam. Cell lysis was performed according to the manufacturer's instructions, and then, cells were sonicated on wet ice. The samples were added to the magnetic beads and to the immunoprecipitating antibody. Finally, the DNA was freed from the protein/DNA complexes. The SYBR Green Master Mix (Takara) was used for qPCR to quantify the immunoprecipitated DNA. The percentage of the target DNA in relation to the input DNA was calculated based on equation 2 ( $[\text{Input Ct} - \text{Target Ct}] \times 0.1 \times 100$ ) and the data are presented as ChIP data.

### *RNA immunoprecipitation (RIP) assay*

The RIP assay was conducted using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) using the YBX1 antibodies obtained from Abcam. The nuclear lysate was prepared and stored at  $-80^{\circ}\text{C}$ , and then, the samples were added to the prepared magnetic

beads/antibody mixture, and the mixture was incubated overnight. Finally, the RNA was extracted using the kit for further quantitative RT-PCR detection.

### *Statistical analysis*

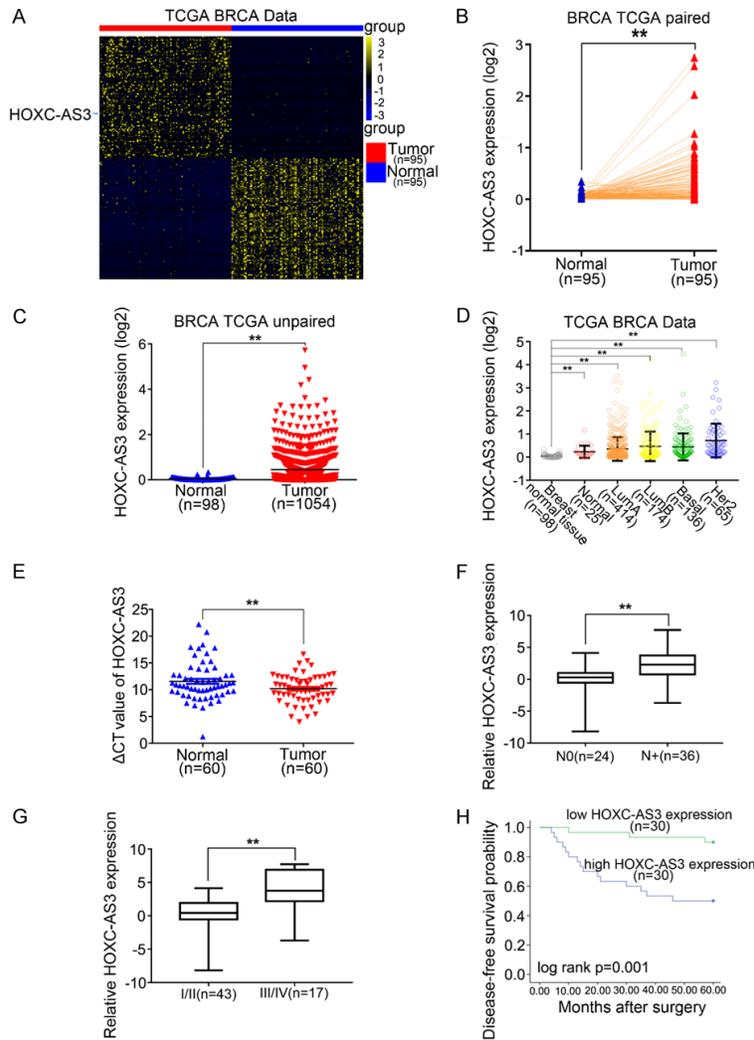
GraphPad Prism 7 (GraphPad Software, USA) and SPSS software package (version 17.0, SPSS, Inc.) were used for plotting and statistical analysis. Chi-square analysis and Student's t-test were used to explore the correlation between the HOXC-AS3 level and the clinical characteristics of our samples. The correlation between the clinical outcomes and HOXC-AS3 levels was evaluated using Kaplan-Meier analysis and log-rank test was used to correlate HOXC-AS3 expression with the survival outcomes and prognosis of breast cancer patients. A Cox proportional hazards analysis was used to evaluate the correlation between HOXC-AS3 level and DFS by computing the 95% confidence interval (CI) and the hazard ratio (HR). Multivariate Cox regression was used for the other covariates. All experiments were performed in triplicates. The experimental data were expressed as the means  $\pm$  deviations (SDs). Paired or unpaired T test was selected according to the sample conditions. *P*-values  $< 0.05$  were considered statistically significant.

## Results

### *Identification of HOXC-AS3 in breast cancer patients using TCGA database*

Raw RNA-Seq data of female breast cancer patients were obtained from the TCGA database. After removing the duplicate and paraffin specimens, we obtained the data of 95 paired specimens. To identify the differentially expressed lncRNAs, we removed the coding RNA. From 95 paired datasets, the top 100 dysregulated lncRNAs are shown in **Figure 1A**. Among these dysregulated lncRNAs, some lncRNAs have been reported previously. For example, previous studies found that LINC01614 acts as an unfavorable prognostic marker by regulating TGF- $\beta$  and FAK signaling in breast cancer [15]. Moreover, the expression level of LINC00052 could be used as a biomarker in breast cancer patients receiving HER3-targeting therapy [16]. Due to the regulatory effects of lncRNA AFAP1-AS1 on the apoptosis of cancer cells and their uncontrolled prolifera-

## Roles of HOXC-AS3 in breast cancer



**Figure 1.** Identification of HOXC-AS3 using bioinformatics analysis and HOXC-AS3 expression in breast cancer tissues and its clinical parameters. Raw microarray data were downloaded from TCGA, duplicate specimens and paraffin specimens were removed. Then, the raw data were analyzed using R scripts. A. The top 100 dysregulated lncRNAs present in TCGA breast cancer datasets. B. HOXC-AS3 expression in breast cancer tissues (n = 95) compared with normal paired breast tissues (n = 95) examined using the TCGA database. C. HOXC-AS3 expression in breast cancer tissues (n = 1,054) compared with normal unpaired breast tissues (n = 98) examined using the TCGA database. D. HOXC-AS3 expression in five subgroups of breast cancer and breast normal tissues from TCGA breast cancer dataset. E. HOXC-AS3 was tested in 60 pairs of breast cancer tissues using qRT-PCR. The expression levels of HOXC-AS3 in breast cancer tissues are significantly higher than in normal paired breast tissues. The  $\Delta$ Ct value was determined by subtracting the  $\beta$ -actin Ct value from the HOXC-AS3 Ct value. A smaller  $\Delta$ Ct value indicates a higher expression. F and G. HOXC-AS3 expression was significantly higher in patients with lymph node metastasis and an advanced TNM stage. H. Patients with high expression levels of HOXC-AS3 had reduced survival times compared with patients with low expression levels of HOXC-AS3 ( $P < 0.01$ , log-rank test). \* $P < 0.05$ , and \*\* $P < 0.01$ .

tion and subsequent metastasis, the increased level of the lncRNA AFAP1-AS1 is correlated with a reduced prognosis among breast cancer

patients [17]. Although some of these lncRNAs have been studied in breast cancer, the functions of many others are unclear. Among them, lncRNA HOXC-AS3 drew our attention because we previously found that it could mediate tumorigenesis in gastric cancer [13]. However, the role and molecular mechanism of HOXC-AS3 in breast cancer have not yet been studied.

In this study, we compared the expression level of HOXC-AS3 in breast cancer tissues with that of healthy tissues from the TCGA breast cancer database. The results indicated significantly increased expression levels of HOXC-AS3 in the tissues of breast cancer, both in paired and unpaired samples of breast cancer, as shown in **Figure 1B, 1C**. We then analyzed HOXC-AS3 expression in the TCGA dataset in five subgroups of breast cancer tissues (Basal, Her2, Luminal B, Luminal A, and Normal) and of normal breast tissues. We observed that HOXC-AS3 exhibited significantly higher levels in the five subgroups compared with the normal tissues, as shown in **Figure 1D**.

### *Upregulated HOXC-AS3 expression is significantly correlated with a poor prognosis in breast cancer*

The qRT-PCR analysis of the expression of HOXC-AS3 was performed in 60 paired tissues from breast cancer and adjacent healthy areas (**Figure 1E**). Approximately 70% (42 of 60) of the diseased tissues showed an increased expression of HOXC-AS3 ( $P < 0.01$ ).

Higher HOXC-AS3 levels were found in breast cancer patients with lymph node metastasis and in those with advanced TNM stages (III/IV)

## Roles of HOXC-AS3 in breast cancer

**Table 2.** Univariate and multivariate analysis of clinic pathologic factors for disease-free survival in 60 patients with breast cancer

Risk factors	Univariate analysis			Multivariate analysis		
	HR*	p value	95% CI	HR	p value	95% CI
HOXC-AS3 expression	6.724	< 0.01**	1.941~23.295	4.112	0.039*	1.071~15.797
TNM stage (I/II, III/IV)	0.179	< 0.01**	0.069~0.466	0.317	0.03*	0.112~0.895
Lymph node metastasis (negative, positive)	0.370	0.08	0.122~1.126			
Tumor size (≤ 2 cm, > 2 cm)	1.452	0.441	0.563~3.747			
Histological grade (middle or low, high)	1.912	0.305	0.554~6.607			
ER (positive, negative)	1.325	0.574	0.497~3.530			
PR (positive, negative)	0.874	0.781	0.339~2.256			
HER2 (positive, negative)	1.387	0.605	0.401~4.792			
Age (≤ 45, > 45)	0.650	0.496	0.118~2.247			

\*HR; hazard ratio. \*P < 0.05; \*\*P < 0.01.

(**Figure 1F, 1G**). Moreover, according to the Chi-square analysis, significant differences were found in the level of metastasis in the lymph nodes and the TNM stages ( $P < 0.01$ ) between the group with a high HOXC-AS3 expression ( $n = 30$ ) and that with reduced HOXC-AS3 levels (below the mean,  $n = 30$ ), whereas no differences were observed in other clinicopathological factors, including age at diagnosis ( $\leq 45, > 45$ ), histological grade, tumor size ( $\leq 2$  cm,  $> 2$  cm), and biomarkers (ER, PR, and Her-2) of the primary tumors (**Table 1**).

The correlation between the clinical outcomes and HOXC-AS3 levels was evaluated using Kaplan-Meier analysis, and the log-rank test was used to correlate HOXC-AS3 expression with the survival outcomes and prognosis of breast cancer patients. Higher expression levels of HOXC-AS3 were associated with a poor prognosis among patients diagnosed with breast cancer with a median disease-free survival (DFS) time of  $57.267 \pm 10.382$  months in the group with low expression levels and  $39.100 \pm 23.118$  months in the high expression group (**Figure 1H**) ( $P < 0.01$ ).

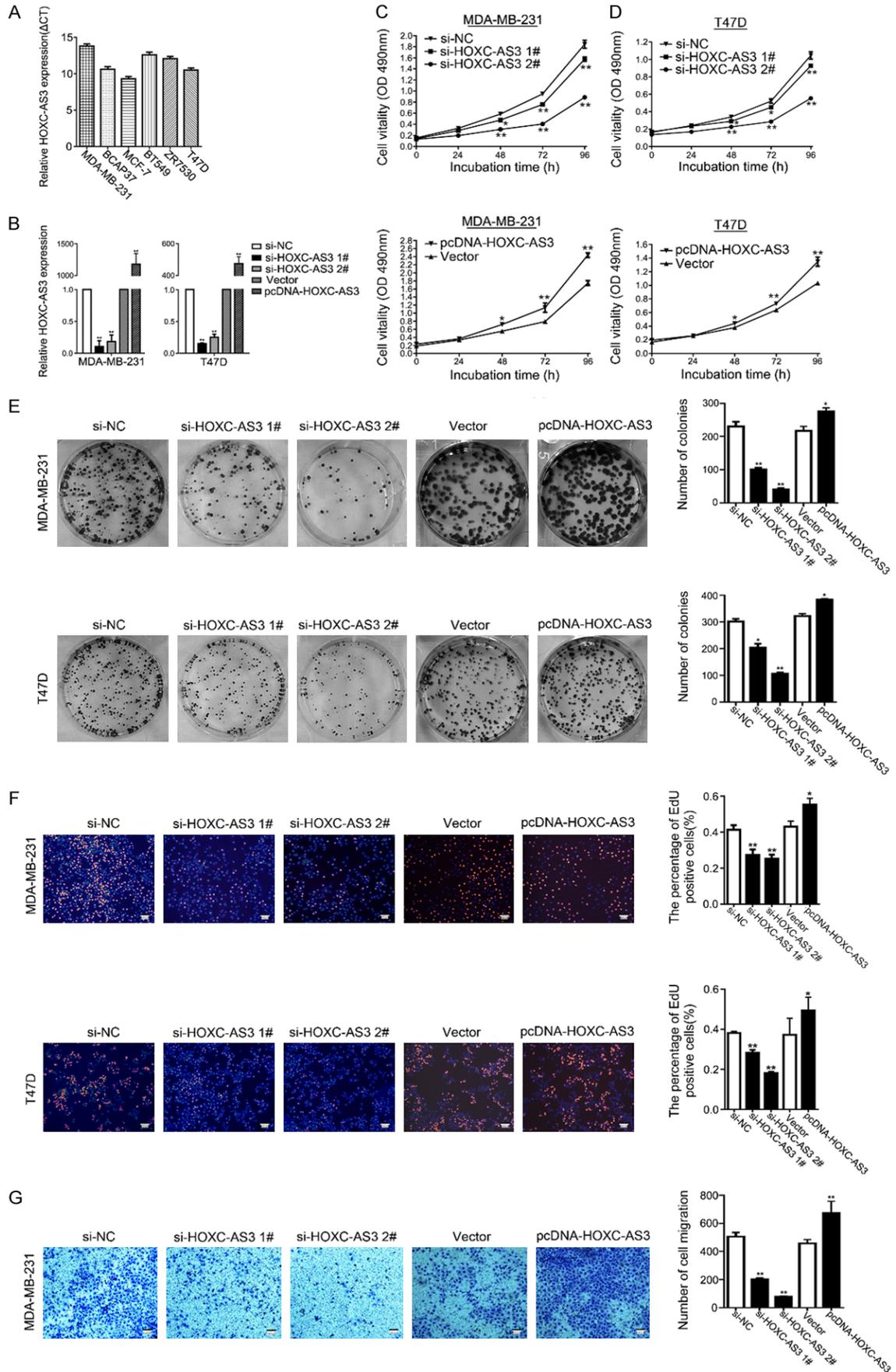
To confirm the prognostic value of HOXC-AS3 in determining the prognosis of breast cancer patients, a Cox proportional hazards regression model was used for the univariate and multivariate survival analyses. The results suggested that HOXC-AS3 was able to independently predict the disease-free survival of patients with breast cancer ( $P < 0.05$ ) (**Table 2**), as shown in **Figure 1H**. In conclusion, HOXC-AS3 could be of great importance to the progression and development of breast cancer.

### *HOXC-AS3 regulates breast cancer cell proliferation and migration in vitro*

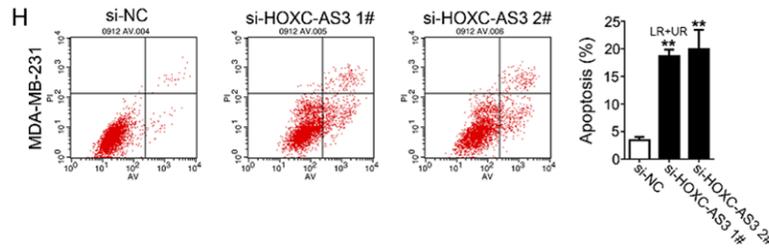
The MDA-MB-231 cell line with high metastatic potential, T47D cell line and low metastatic potential were used to mimic the subtypes of breast cancer. The expression levels of HOXC-AS3 were externally manipulated using plasmid-mediated overexpression and siRNA-mediated knockdown to investigate the corresponding function. The level of HOXC-AS3 expression was evaluated using qRT-PCR analysis, 48 h after transfection (**Figure 2A**). The results suggested remarkably reduced expression levels of HOXC-AS3 in si-HOXC-AS3-transfected breast cancer cells. In contrast, a remarkably upregulated HOXC-AS3 expression was observed in pc-DNA-HOXC-AS3-transfected breast cancer cells, as shown in **Figure 2B**.

The MTT analysis indicated that HOXC-AS3 knockdown inhibited cell proliferation. In contrast, the overexpression of HOXC-AS3 promoted the proliferation of the two cell lines (**Figure 2C, 2D**). Significantly decreased HOXC-AS3 levels and colony formation were observed among the two cell lines, according to the colony-formation analysis. Furthermore, HOXC-AS3 overexpression increased the number of clones, as shown in **Figure 2E**. A remarkable regulatory effect of HOXC-AS3 on cell proliferation was revealed using EdU assay (**Figure 2F**). Transwell assays indicated that cell migration was inhibited in the MDA-MB-231 cells whose HOXC-AS3 was knocked down, whereas it was increased in cells that overexpressed HOXC-AS3, compared to that in cells from the

# Roles of HOXC-AS3 in breast cancer



## Roles of HOXC-AS3 in breast cancer



**Figure 2.** HOXC-AS3 regulates breast cancer cell proliferation and migration *in vitro*. A. The expression of HOXC-AS3 was analyzed in breast cancer cells (MDA-MB-231, BCAP37, MCF-7, BT549, ZR7530, and T47D) using qRT-PCR. B. The relative expression levels of HOXC-AS3 in MDA-MB-231 and T47D cells transfected with an overexpression plasmid and siRNA of HOXC-AS3 (si-HOXC-AS3 1# and si-HOXC-AS3 2#) were measured using qPCR. C and D. MTT assays were performed after transfection to determine the cell viability of the transfected MDA-MB-231 and T47D cells. E. Colony-forming assays were conducted after transfection to determine the colony-forming capacity of the transfected MDA-MB-231 and T47D cells. The colonies were counted and captured. F. Edu assays were used to detect the cell proliferation after transfection. G. Transwell assays were used to investigate the changes in migratory abilities of MDA-MB-231 cells after transfection. H. At 48 h after transfection, MDA-MB-231 cells were stained and analyzed using flow cytometry. LR, early apoptotic cells. UR, terminal apoptotic cells. \* $P < 0.05$ , and \*\* $P < 0.01$ .

control group (Figure 2G). Flow cytometry results suggested that a reduced expression of HOXC-AS3 could remarkably induce MDA-MB-231 cell apoptosis (Figure 2H), which further confirmed the above evidence from another perspective.

### HOXC-AS3 regulates tumorigenesis *in vivo*

To study the role of HOXC-AS3 in cellular proliferation *in vivo*, we subcutaneously injected nude mice with MDA-MB-231 cells modified with either shHOXC-AS3 or control vectors. We observed a substantially reduced tumor growth in the shHOXC-AS3 group, compared with that of the control group (Figure 3A, 3B), which was consistent with the *in vitro* analysis. The mean tumor weight was remarkably reduced in the shHOXC-AS3 group compared with that in the control group at 29 days after administration (Figure 3C). IHC analysis, which was performed on the tumor tissues, suggested that the shCtrl group had higher expression levels of Ki-67 compared to shHOXC-AS3 group (Figure 3D). These results indicate that HOXC-AS3 can influence tumor growth *in vivo*.

### HOXC-AS3 interacts with YBX1

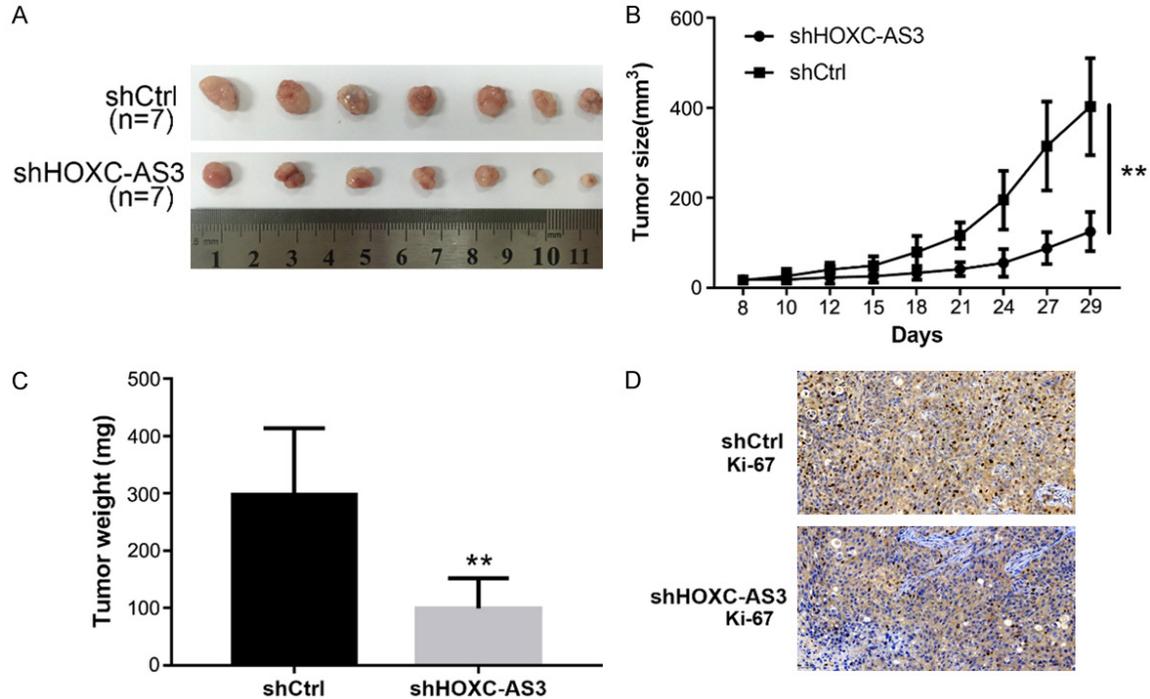
In our previous study, we observed that HOXC-AS3 regulates transcriptional gene expression by binding to YBX1 [13]. YBX1 is able to bind with various partners and regulate a variety of principal cellular pathways. For instance, the genetic inactivation function of YBX1 results in embryonic lethality [18]. More importantly, it

has been found that YBX1 overexpression is correlated with tumorigenic phenotypes [19-21], including breast cancer, and that multifunctional YBX1 is a powerful prognostic factor in breast cancer [22]. RNA immunoprecipitation (RIP) assay suggested that endogenous HOXC-AS3 expression was increased in the anti-YBX1 RIP fraction, compared with the IgG fraction in MDA-MB-231 cells (Figure 4A). The above results suggest that HOXC-AS3 plays a role in the tumorigenicity of breast cancer and may regulate the transcription of other genes by binding to YBX1.

### HOXC-AS3 can directly transcriptionally activate TK1 by binding to YBX1

Regarding the HOXC-AS3-related molecular mechanism of breast cancer, we refer to the results of our previous RNA transcriptome sequencing study, which involved inhibiting HOXC-AS3 and YBX1 in gastric cancer cell lines [13]. Among the common downstream regulatory genes of HOXC-AS3 and YBX1, thymidine kinase 1 (TK1) drew our attention due to its involvement in tumorigenesis and the changes induced by the knockdown of HOXC-AS3. As an enzyme in the nucleotide salvage pathway, TK1 can participate in cell proliferation, which makes it a potential biomarker for the proliferation of tumor tissues, including breast cancer [14, 23]. Moreover, the randomized phase III trial of Faslodex versus Exemestane in metastatic breast cancer indicated the potential of serum TK1 as a probable prognostic biomarker of breast cancer among patients receiving

## Roles of HOXC-AS3 in breast cancer



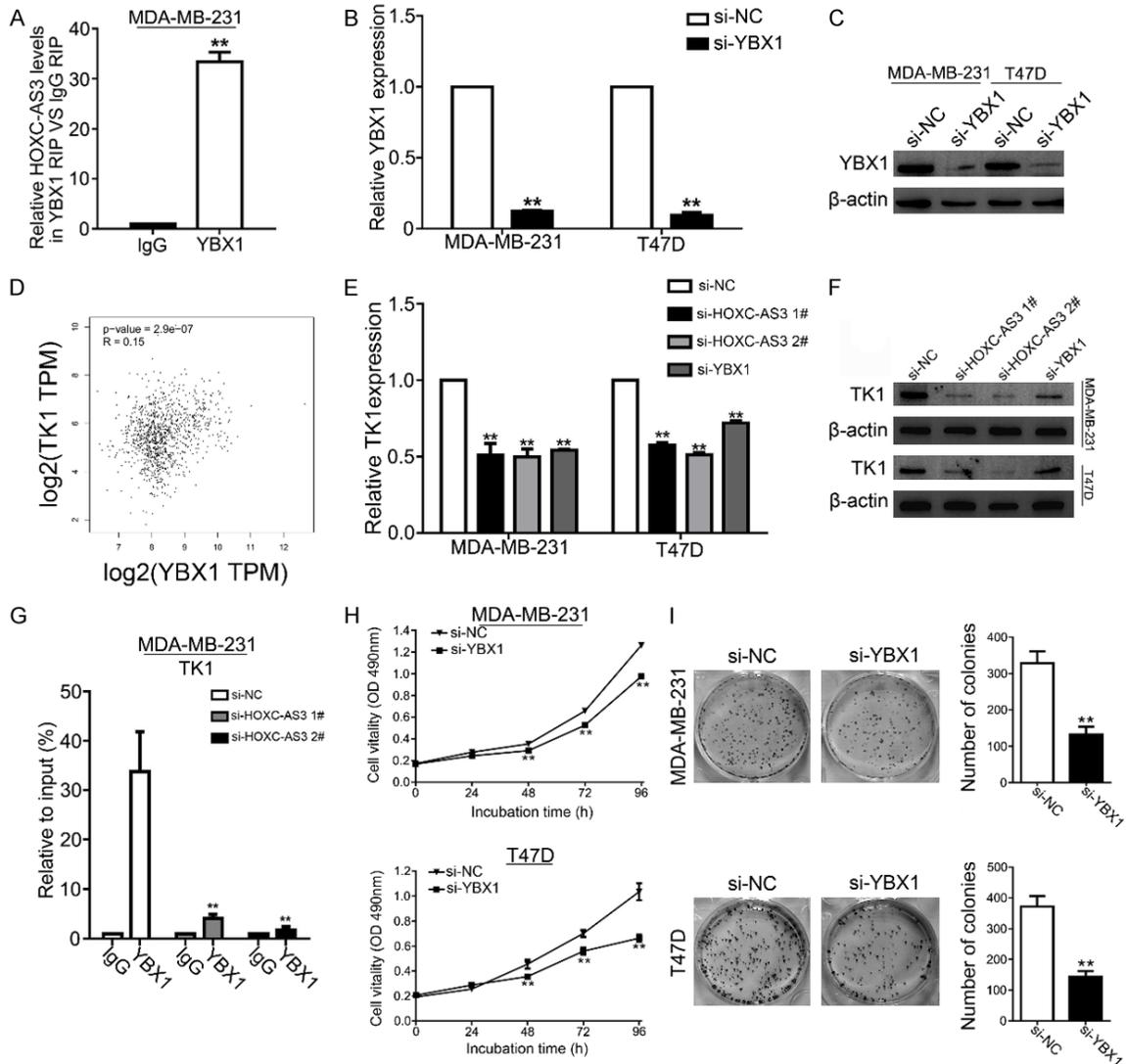
**Figure 3.** HOXC-AS3 regulates breast cancer cell growth *in vivo*. A. Scramble or shHOXC-AS3 was stably transfected into MDA-MB-231 cells, which was injected into nude mice (n = 7) (shHOXC-AS3 on the right, control on the left). B. Tumor volumes were calculated on the eighth day after injection and recorded every 2 or 3 days, thereafter. C. Tumor weights are represented as the mean  $\pm$  standard deviation (S.D.). D. Immunohistochemistry analysis revealed that the tumors developed from shHOXC-AS3-transfected cells displayed lower Ki-67 staining intensity than the control group. \*P < 0.05, \*\*P < 0.01.

endocrine therapy [24]. To confirm the function of YBX1 in breast cancer, we first transfected siRNAs into MDA-MB-231 and T47D cells to silence YBX1 expression, as shown in **Figure 4B, 4C**. We then analyzed the expression levels of YBX1 and TK1 and their correlation using data from the TCGA breast cancer database. These results demonstrate that the expression of YBX1 is correlated with the relative levels of TK1 (**Figure 4D**). We then evaluated the expression levels of TK1 mRNA and protein after the knockdown of HOXC-AS3 and YBX1 using qRT-PCR and Western blotting, respectively (**Figure 4E, 4F**). The chromatin immunoprecipitation (ChIP) assay suggested that the binding between the YBX1 protein and the TK1 promoter regions was positive under mediation by HOXC-AS3, which can be inhibited by silencing HOXC-AS3 (**Figure 4G**). In addition, colony formation and MTT assays suggested that YBX1 knockdown had inhibitory effects on cell proliferation and colony-forming ability in both cell lines, as shown in **Figure 4H, 4I**. Therefore, YBX1 function was further confirmed. These

results indicated that the knockdown of HOXC-AS3 and YBX1 coregulates TK1 expression.

Based on our analysis using the TCGA breast cancer dataset, in breast cancer tissues, the level of TK1 was noticeably increased, compared to that in their normal counterparts (**Figure 5A**). TK1 was knocked down in cell lines using siRNA transfection. After 48 h, TK1 expression at a gene and protein level was assessed using western blot and qRT-PCR. TK1 expression was significantly inhibited in si-TK1-transfected breast cancer cells (**Figure 5B, 5C**). MTT assays suggested that the knockdown of TK1 reduced the proliferation of the two cell lines (**Figure 5D**). Colony-formation assays also showed that silencing TK1 remarkably decreased the colony-forming ability of MDA-MB-231 and T47D cells (**Figure 5E**). Our results highlight the important role of TK1 in the tumorigenesis of breast cancer. Furthermore, the promotion of migration and cell growth mediated by HOXC-AS3 could be reversed by silencing TK1, as shown in **Figure**

## Roles of HOXC-AS3 in breast cancer



**Figure 4.** HOXC-AS3 Interacts with YBX1 and coregulates TK1. A. RIP experiments for YBX1 were performed, and the coprecipitated RNA was subjected to qRT-PCR for HOXC-AS3. In the RIP assay, the fold enrichment of HOXC-AS3 is relative to that of its matching IgG control. B and C. The qPCR and western blot assays detected the expression of YBX1 after the knockdown of YBX1. D. YBX1 level in breast cancer tissues showed a significant positive correlation with the relative level of TK1 expression in TCGA data. E and F. qRT-PCR and western blot assays detected the expression of TK1 after the knockdown of HOXC-AS3 and YBX1 in MDA-MB-231 and T47D cells. G. ChIP-qPCR of YBX1 of the promoter region of TK1 loci after the knockdown of HOXC-AS3 in MDA-MB-231 cells. Antibody enrichment was quantified relative to the amount of input DNA. Antibody directed against IgG was used as a negative control. H. MTT assays were used to determine the cell proliferation of MDA-MB-231 and T47D cells after YBX1 knockdown. I. Colony formation assays of MDA-MB-231 and T47D cells after YBX1 knockdown. The colonies were counted and captured. \* $P < 0.05$ , \*\* $P < 0.01$ .

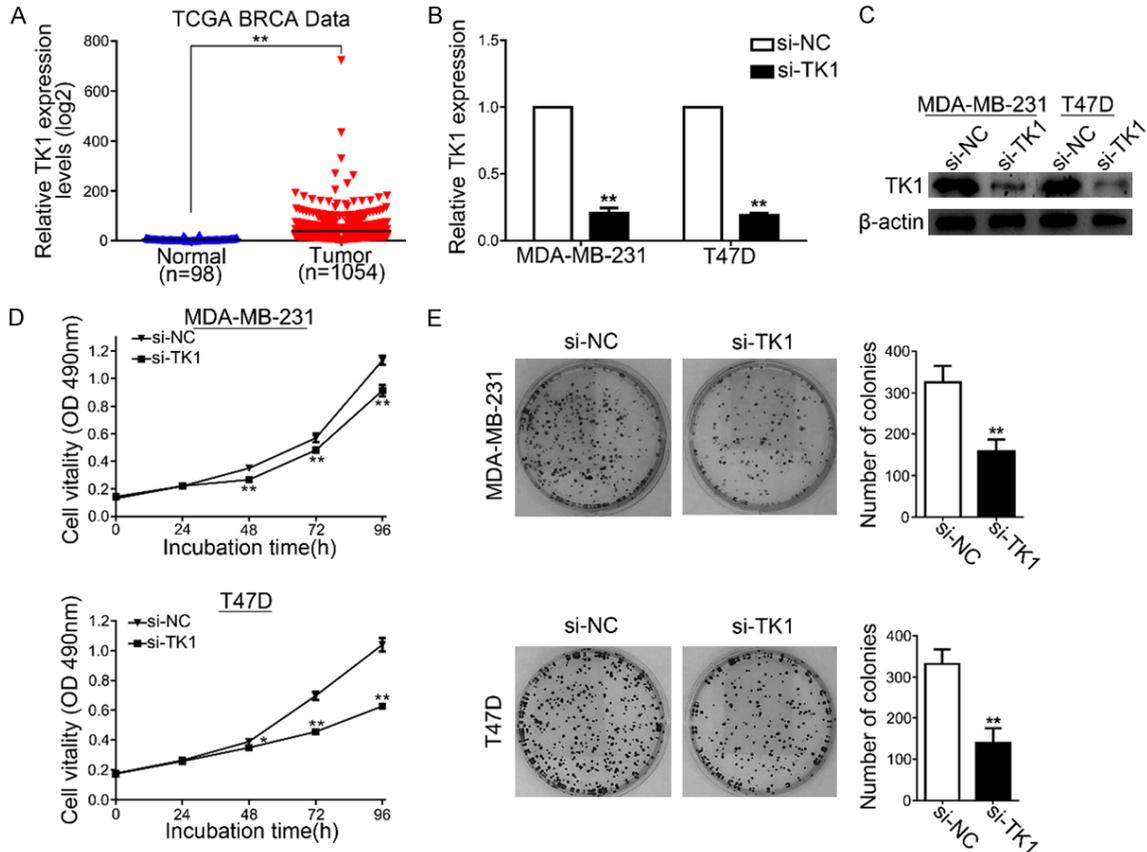
**6A-C.** This evidence suggests that HOXC-AS3 promotes the growth of breast cancer cells, which can be achieved, at least partially, by directly activating TK1 via the binding of YBX1.

### Discussion

The latest human gene sequencing data suggested that approximately 97% of the human

genome encodes noncoding RNAs (ncRNAs), from which lncRNAs are a newly discovered class [25, 26]. In recent years, increasing evidence has shown an association between the levels of lncRNAs and cancer pathogenesis [17, 27], indicating that lncRNAs might serve as diagnostic biomarkers during treatment and could also serve as a therapeutic target. Nonetheless, the potential molecular mecha-

## Roles of HOXC-AS3 in breast cancer



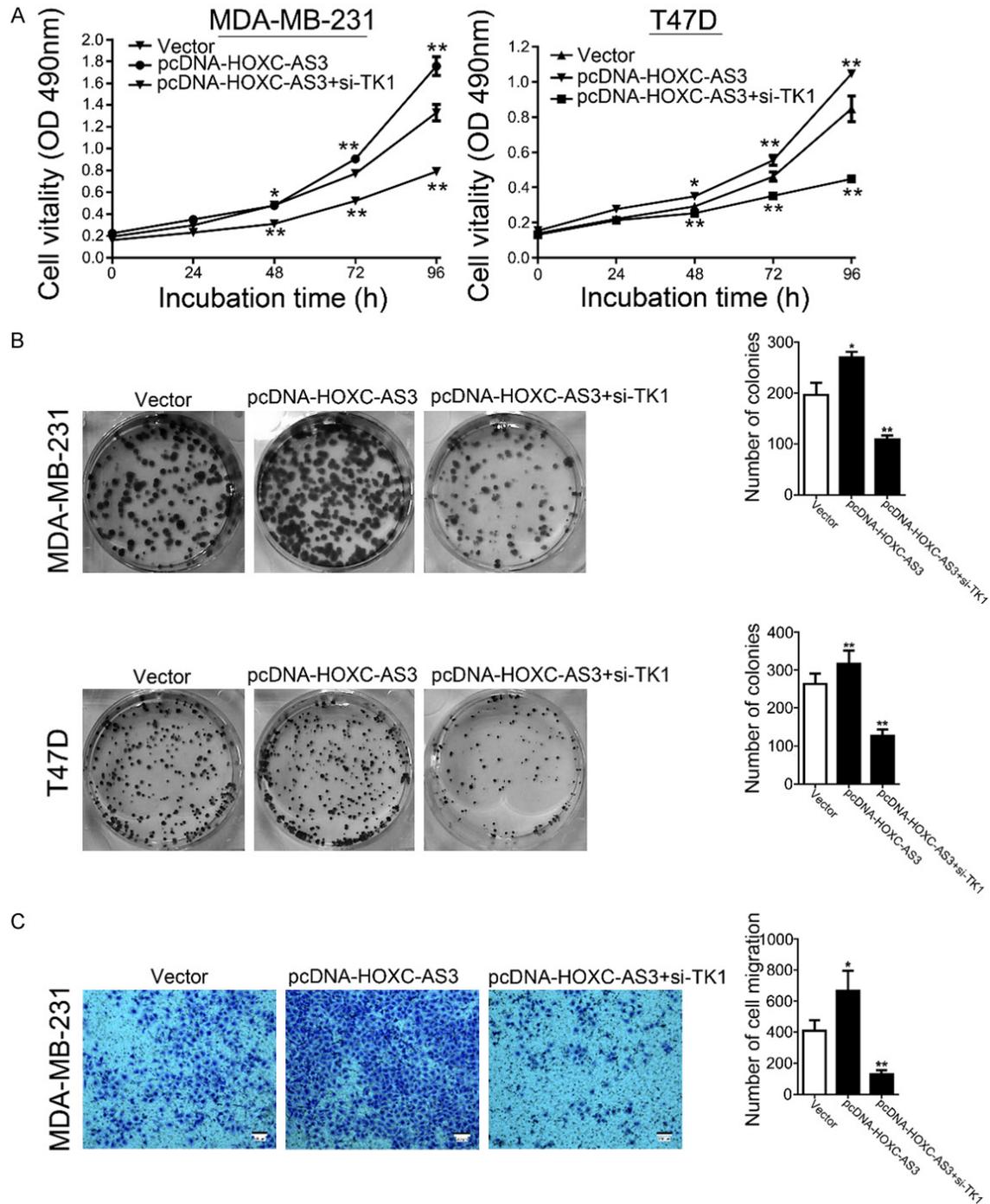
**Figure 5.** HOXC-AS3 directly transcriptionally activates TK1. A. TK1 expression in breast cancer tissues (n = 1,054) compared with normal unpaired breast tissues (n = 98) examined using the TCGA database (data from TANRIC). B and C. The qPCR and Western blot assays were used to analyze the expression of TK1 after TK1 knockdown. D. MTT assays were used to determine the cell proliferation of MDA-MB-231 and T47D cells after TK1 knockdown. E. Colony formation assays of MDA-MB-231 and T47D cells after TK1 knockdown. The colonies were counted and captured. \*P < 0.05, \*\*P < 0.01.

nisms underlying lncRNA actions remain largely unknown.

The lncRNA HOXC-AS3 is encoded on chromosome 12q13.13 and is an antisense transcript of HOXC10 [28, 29]. In our previous research, we found that lncRNA HOXC-AS3 is of great importance in the carcinogenesis of gastric cancer [13]. Previous studies have indicated that HOXC10 can interact with its natural antisense transcript lncRNA HOXC-AS3, thus processing the regulatory effects on mesenchymal stromal cell osteogenesis [30]. Recent research has suggested the promoting effects of HOXB13 on the invasion, migration, and proliferation of glioblastoma, due to the upregulation of the lncRNA HOXC-AS3 [31]. Very recently, research has shown that HOXC-AS3 may serve as a sponge for miR-3922-5p, which is able to increase the metastatic potential of

breast cancer [32]. Overall, HOXC-AS3 is of great significance to a number of biological tumor processes, but the corresponding biological functions have not been well identified. In particular, the molecular mechanisms of HOXC-AS3 during tumorigenesis of breast cancer remain unclear. To study the functional role of HOXC-AS3 in breast cancer, we assessed the publicly available TCGA breast cancer database and found that the expression level of HOXC-AS3 was significantly upregulated in breast cancer tissues, compared to normal breast tissues. We verified these results in tissue samples from breast cancer patients and found that increased HOXC-AS3 expression was associated with a poor prognosis, indicating that HOXC-AS3 might be an independent prognostic factor in breast cancer. The above results suggest the clinical value of HOXC-AS3 in the progression of breast cancer. Comparatively,

## Roles of HOXC-AS3 in breast cancer



**Figure 6.** TK1 reverses HOXC-AS3-mediated growth and migration promotion. A. MTT assays were used to detect cell proliferation after TK1 knockdown in MDA-MB-231 and T47D cells transfected with an overexpression plasmid of HOXC-AS3. B. Colony formation assays were used to detect the colony-forming capacity of MDA-MB-231 and T47D cells transfected with an overexpression plasmid of HOXC-AS3, and concurrently, with TK1 knocked down. The colonies were counted and captured. The above results showed that TK1 knockdown could reverse HOXC-AS3-mediated cell growth promotion. C. Transwell assays were used to investigate the changes in the migratory abilities of MDA-MB-231 cells transfected with an overexpression plasmid of HOXC-AS3 and si-TK1. \*P < 0.05, \*\*P < 0.01.

HOXC-AS3 expression was very low in normal breast tissues. HOXC-AS3 may also serve as a potential therapeutic target.

Generally, lncRNAs act through coactions with various RNA-binding proteins that lead to regulatory modulations of gene expression via his-

tone protein modification, RNA decay, DNA methylation, and chromosome reprogramming [33-36]. Evidence from our previous study revealed the potential contribution of HOXC-AS3 to tumorigenesis in gastric cancer cases by modulating gene transcription via the binding of toy-box binding protein 1 (YBX1) *in trans* [13]. The nucleic acid-binding domain in YBX1 protein can bind to the CCAAT-box, which is located on the gene promoter and is highly conserved in eukaryotes [37]. YBX1 can bind to the Y-box sequence located in the promoter region of genes and activate gene expression [38, 39]. YBX1 is of great significance for tumorigenesis, the upregulation of which was found to be correlated with a poor prognosis in a number of cancer types, such as breast cancer [40, 41]. Numerous studies have shown that lncRNAs can mediate carcinoma tumorigenesis by binding to YBX1 [42, 43]. Our findings suggest that HOXC-AS3 could directly interact with YBX1 in breast cancer cells.

Furthermore, we analyzed previous RNA-Seq results that involved the simultaneous silencing of both HOXC-AS3 and YBX1 in gastric cancer and detected these candidate genes in breast cancer. Compared with gastric cancer, we found slightly different results in breast cancer. Among the genes coregulated by HOXC-AS3 and YBX1, the expression of TK1 was significantly reduced after the knockdown of HOXC-AS3. TK1 is a nucleotide salvage pathway repair enzyme that can regulate cell cycle and proliferation [44, 45]. TK1 has an oncogenic role in tumorigenesis [46]. Moreover, previous studies have confirmed that YBX1 can bind to the promoter area of TK1 and affect TK1 transcription [39, 47], but the role of lncRNA in the regulation of TK1 by YBX1 has not been reported. Our ChIP assays showed that HOXC-AS3 knockdown inhibited the binding of YBX1 to the TK1 promoter region, and that TK1 was an important target of HOXC-AS3/YBX1-regulated genes. Our findings revealed that HOXC-AS3 partly restricts tumorigenesis of breast cancer through the direct transcriptional activation of TK1, by binding to YBX1. This indicated the important role of HOXC-AS3 in the biology of breast cancer.

### Acknowledgements

This work was supported in part by the National Natural Science Foundation of China (grants

81673102, 81602791, 81803164 and 81-972963) and National Key Research and Development Program of China (grant 2016-YFC0105409); Doctoral Innovation Fund of School of Medicine, Shanghai Jiaotong University (BXJ201918); Youth Foundation of Shanghai Health and Family Planning Commission (20164Y0066); Shanghai Municipal Education Commission-Gaofeng Clinical Medicine Grant Support (20171904); and Special Construction of Integrated Chinese and Western Medicine (ZHYY-ZXYJHZ X-2-2017-04).

### Disclosure of conflict of interest

None.

**Address correspondence to:** Jiayi Chen and Min Li, Department of Radiation Oncology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, China. Tel: +86-21-64370045; Fax: +86-21-64156886; E-mail: chenjiayi0188@aliyun.com (JYC); lm11866@rjh.com.cn (ML); Kunwei Shen, Comprehensive Breast Health Center, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, China. Tel: +86-21-64370045; Fax: +86-21-64156886; E-mail: kwshen@medmail.com.cn

### References

- [1] DeSantis CE, Ma J, Goding Sauer A, Newman LA and Jemal A. Breast cancer statistics, 2017, racial disparity in mortality by state. *CA Cancer J Clin* 2017; 67: 439-448.
- [2] Lee J, Blackmon BJ, Lee JY, Cochran DM Jr and Rehner TA. An exploration of posttraumatic growth, loneliness, depression, resilience, and social capital among survivors of Hurricane Katrina and the deepwater horizon oil spill. *J Community Psychol* 2019; 47: 356-370.
- [3] Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, Cabili MN, Jaenisch R, Mikkelsen TS, Jacks T, Hacohen N, Bernstein BE, Kellis M, Regev A, Rinn JL and Lander ES. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* 2009; 458: 223-227.
- [4] Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A and Bozzoni I. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* 2011; 147: 358-369.
- [5] Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A,

## Roles of HOXC-AS3 in breast cancer

- Bernstein BE, van Oudenaarden A, Regev A, Lander ES and Rinn JL. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci U S A* 2009; 106: 11667-11672.
- [6] Penny GD, Kay GF, Sheardown SA, Rastan S and Brockdorff N. Requirement for Xist in X chromosome inactivation. *Nature* 1996; 379: 131-137.
- [7] Yao N, Fu Y, Chen L, Liu Z, He J, Zhu Y, Xia T and Wang S. Long non-coding RNA NONHS-AT101069 promotes epirubicin resistance, migration, and invasion of breast cancer cells through NONHSAT101069/miR-129-5p/Twist1 axis. *Oncogene* 2019; 38: 7216-7233.
- [8] Gofrit ON, Benjamin S, Halachmi S, Leibovitch I, Dotan Z, Lamm DL, Ehrlich N, Yutkin V, Ben-Am M and Hochberg A. DNA based therapy with diphtheria toxin-A BC-819: a phase 2b marker lesion trial in patients with intermediate risk nonmuscle invasive bladder cancer. *J Urol* 2014; 191: 1697-1702.
- [9] Sidi AA, Ohana P, Benjamin S, Shalev M, Ransom JH, Lamm D, Hochberg A and Leibovitch I. Phase I/II marker lesion study of intravesical BC-819 DNA plasmid in H19 over expressing superficial bladder cancer refractory to bacillus Calmette-Guerin. *J Urol* 2008; 180: 2379-2383.
- [10] Yin D, Lu X, Su J, He X, De W, Yang J, Li W, Han L and Zhang E. Long noncoding RNA AFAP1-AS1 predicts a poor prognosis and regulates non-small cell lung cancer cell proliferation by epigenetically repressing p21 expression. *Mol Cancer* 2018; 17: 92.
- [11] Berteaux N, Lottin S, Monte D, Pinte S, Quatannens B, Coll J, Hondermarck H, Curgy JJ, Dugimont T and Adriaenssens E. H19 mRNA-like noncoding RNA promotes breast cancer cell proliferation through positive control by E2F1. *J Biol Chem* 2005; 280: 29625-29636.
- [12] Mizrahi A, Czerniak A, Levy T, Amiur S, Gallula J, Matouk I, Abu-lail R, Sorin V, Birman T, de Groot N, Hochberg A and Ohana P. Development of targeted therapy for ovarian cancer mediated by a plasmid expressing diphtheria toxin under the control of H19 regulatory sequences. *J Transl Med* 2009; 7: 69.
- [13] Zhang E, He X, Zhang C, Su J, Lu X, Si X, Chen J, Yin D, Han L and De W. A novel long noncoding RNA HOXC-AS3 mediates tumorigenesis of gastric cancer by binding to YBX1. *Genome Biol* 2018; 19: 154.
- [14] Zhu X, Shi C, Peng Y, Yin L, Tu M, Chen Q, Hou C, Li Q and Miao Y. Thymidine kinase 1 silencing retards proliferative activity of pancreatic cancer cell via E2F1-TK1-P21 axis. *Cell Prolif* 2018; 51: e12428.
- [15] Vishnubalaji R, Shaath H, Elkord E and Alajez NM. Long non-coding RNA (lncRNA) transcriptional landscape in breast cancer identifies LINC01614 as non-favorable prognostic biomarker regulated by TGFbeta and focal adhesion kinase (FAK) signaling. *Cell Death Discov* 2019; 5: 109.
- [16] Salameh A, Fan X, Choi BK, Zhang S, Zhang N and An Z. HER3 and LINC00052 interplay promotes tumor growth in breast cancer. *Oncotarget* 2017; 8: 6526-6539.
- [17] Ma D, Chen C, Wu J, Wang H and Wu D. Up-regulated lncRNA AFAP1-AS1 indicates a poor prognosis and promotes carcinogenesis of breast cancer. *Breast Cancer* 2019; 26: 74-83.
- [18] Uchiumi T, Fotovati A, Sasaguri T, Shibahara K, Shimada T, Fukuda T, Nakamura T, Izumi H, Tsuzuki T, Kuwano M and Kohno K. YB-1 is important for an early stage embryonic development: neural tube formation and cell proliferation. *J Biol Chem* 2006; 281: 40440-40449.
- [19] Dey A, Robitaille M, Remke M, Maier C, Malhotra A, Gregorieff A, Wrana JL, Taylor MD, Angers S and Kenney AM. YB-1 is elevated in medulloblastoma and drives proliferation in Sonic hedgehog-dependent cerebellar granule neuron progenitor cells and medulloblastoma cells. *Oncogene* 2016; 35: 4256-4268.
- [20] Matsumoto K and Bay BH. Significance of the Y-box proteins in human cancers. *J Mol Genet Med* 2005; 1: 11-17.
- [21] Huang J, Tan PH, Li KB, Matsumoto K, Tsujimoto M and Bay BH. Y-box binding protein, YB-1, as a marker of tumor aggressiveness and response to adjuvant chemotherapy in breast cancer. *Int J Oncol* 2005; 26: 607-613.
- [22] Maciejczyk A, Szelachowska J, Ekiert M, Matkowski R, Halon A, Lage H and Surowiak P. Elevated nuclear YB1 expression is associated with poor survival of patients with early breast cancer. *Anticancer Res* 2012; 32: 3177-3184.
- [23] Weigel EG, Burrup W, Kovtun R, Velazquez EJ, Felsted AM, Townsend MH, Ence ZE, Suh E, Piccolo SR, Weber KS, Robison RA and O'Neill KL. Membrane expression of thymidine kinase 1 and potential clinical relevance in lung, breast, and colorectal malignancies. *Cancer Cell Int* 2018; 18: 135.
- [24] McCartney A, Biagioni C, Schiavon G, Bergqvist M, Mattsson K, Migliaccio I, Benelli M, Romagnoli D, Bonechi M, Boccalini G, Pestrin M, Galardi F, De Luca F, Biganzoli L, Piccart M, Gradishar WJ, Chia S, Di Leo A and Malorni L. Prognostic role of serum thymidine kinase 1 activity in patients with hormone receptor-positive metastatic breast cancer: analysis of the

## Roles of HOXC-AS3 in breast cancer

- randomised phase III Evaluation of Faslodex versus Exemestane Clinical Trial (EFFECT). *Eur J Cancer* 2019; 114: 55-66.
- [25] Ponting CP, Oliver PL and Reik W. Evolution and functions of long noncoding RNAs. *Cell* 2009; 136: 629-641.
- [26] Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet* 2011; 12: 861-874.
- [27] Su J, Zhang E, Han L, Yin D, Liu Z, He X, Zhang Y, Lin F, Lin Q, Mao P, Mao W and Shen D. Long noncoding RNA BLACAT1 indicates a poor prognosis of colorectal cancer and affects cell proliferation by epigenetically silencing of p15. *Cell Death Dis* 2017; 8: e2665.
- [28] Pearson JC, Lemons D and McGinnis W. Modulating Hox gene functions during animal body patterning. *Nat Rev Genet* 2005; 6: 893-904.
- [29] Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E and Chang HY. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 2007; 129: 1311-1323.
- [30] Li B, Han H, Song S, Fan G, Xu H, Zhou W, Qiu Y, Qian C, Wang Y, Yuan Z, Gao Y, Zhang Y and Zhuang W. HOXC10 regulates osteogenesis of mesenchymal stromal cells through interaction with its natural antisense transcript lncHOXC-AS3. *Stem Cells* 2019; 37: 247-256.
- [31] Wang X, Sun Y, Xu T, Qian K, Huang B, Zhang K, Song Z, Qian T, Shi J and Li L. HOXB13 promotes proliferation, migration, and invasion of glioblastoma through transcriptional upregulation of lncRNA HOXC-AS3. *J Cell Biochem* 2019; 120: 15527-15537.
- [32] Shi SH, Jiang J, Zhang W, Sun L, Li XJ, Li C, Ge QD and Zhuang ZG. A novel lncRNA HOXC-AS3 acts as a miR-3922-5p sponge to promote breast cancer metastasis. *Cancer Invest* 2020; 38: 1-12.
- [33] Munschauer M, Nguyen CT, Sirokman K, Hartigan CR, Hogstrom L, Engreitz JM, Ulirsch JC, Fulco CP, Subramanian V, Chen J, Schenone M, Guttman M, Carr SA and Lander ES. The NORAD lncRNA assembles a topoisomerase complex critical for genome stability. *Nature* 2018; 561: 132-136.
- [34] Sanjana NE, Wright J, Zheng K, Shalem O, Fontanillas P, Joung J, Cheng C, Regev A and Zhang F. High-resolution interrogation of functional elements in the noncoding genome. *Science* 2016; 353: 1545-1549.
- [35] Hua JT, Ahmed M, Guo H, Zhang Y, Chen S, Soares F, Lu J, Zhou S, Wang M, Li H, Larson NB, McDonnell SK, Patel PS, Liang Y, Yao CQ, van der Kwast T, Lupien M, Feng FY, Zoubeidi A, Tsao MS, Thibodeau SN, Boutros PC and He HH. Risk SNP-mediated promoter-enhancer switching drives prostate cancer through lncRNA PCAT19. *Cell* 2018; 174: 564-575, e518.
- [36] Cho SW, Xu J, Sun R, Mumbach MR, Carter AC, Chen YG, Yost KE, Kim J, He J, Nevins SA, Chin SF, Caldas C, Liu SJ, Horlbeck MA, Lim DA, Weissman JS, Curtis C and Chang HY. Promoter of lncRNA gene PVT1 is a tumor-suppressor DNA boundary element. *Cell* 2018; 173: 1398-1412, e1322.
- [37] Dong J, Akcakanat A, Stivers DN, Zhang J, Kim D and Meric-Bernstam F. RNA-binding specificity of Y-box protein 1. *RNA Biol* 2009; 6: 59-64.
- [38] Kim ER, Selyutina AA, Buldakov IA, Evdokimova V, Ovchinnikov LP and Sorokin AV. The proteolytic YB-1 fragment interacts with DNA repair machinery and enhances survival during DNA damaging stress. *Cell Cycle* 2013; 12: 3791-3803.
- [39] Gai XX, Lipson KE and Prystowsky MB. Unusual DNA binding characteristics of an in vitro translation product of the CCAAT binding protein mYB-1. *Nucleic Acids Res* 1992; 20: 601-606.
- [40] Zhou LL, Ni J, Feng WT, Yao R, Yue S, Zhu YN, Tang HY, Lv LY, Feng JF and Zhu WG. High YBX1 expression indicates poor prognosis and promotes cell migration and invasion in nasopharyngeal carcinoma. *Exp Cell Res* 2017; 361: 126-134.
- [41] Lovett DH, Cheng S, Cape L, Pollock AS and Mertens PR. YB-1 alters MT1-MMP trafficking and stimulates MCF-7 breast tumor invasion and metastasis. *Biochem Biophys Res Commun* 2010; 398: 482-488.
- [42] Peng Z, Wang J, Shan B, Li B, Peng W, Dong Y, Shi W, Zhao W, He D, Duan M, Cheng Y, Zhang C and Duan C. The long noncoding RNA LINC00312 induces lung adenocarcinoma migration and vasculogenic mimicry through directly binding YBX1. *Mol Cancer* 2018; 17: 167.
- [43] Su W, Feng S, Chen X, Yang X, Mao R, Guo C, Wang Z, Thomas DG, Lin J, Reddy RM, Orringer MB, Chang AC, Yang Z, Beer DG and Chen G. Silencing of long noncoding RNA MIR22HG triggers cell survival/death signaling via oncogenes YBX1, MET, and p21 in lung cancer. *Cancer Res* 2018; 78: 3207-3219.
- [44] Hengstschlager M, Pfeilstocker M and Wawra E. Thymidine kinase expression. A marker for malignant cells. *Adv Exp Med Biol* 1998; 431: 455-460.
- [45] Mikulits W, Hengstschlager M, Sauer T, Wintersberger E and Mullner EW. Overexpression of thymidine kinase mRNA eliminates

## Roles of HOXC-AS3 in breast cancer

- cell cycle regulation of thymidine kinase enzyme activity. *J Biol Chem* 1996; 271: 853-860.
- [46] Alegre MM, Robison RA and O'Neill KL. Thymidine kinase 1 upregulation is an early event in breast tumor formation. *J Oncol* 2012; 2012: 575647.
- [47] Lipson KE, Chen ST, Konecki J, Ku DH and Baserga R. S-phase-specific regulation by deletion mutants of the human thymidine kinase promoter. *Proc Natl Acad Sci U S A* 1989; 86: 6848-6852.

## Roles of HOXC-AS3 in breast cancer

**Table S1.** The list of primers and the sequence of siRNAs

GENE	Forward primer	Reverse primer
human qRT-PCR		
HOXC-AS3	GTGGAGTAACAGCGCCATCT	CGGGTTTTGTTGCGTCTTGT
YBX1	GGACAAGAAGGTCATCGCAAC	TCTCCATCTCCTACTGCGA
TK1	GTTCTCAGGAAAAAGCACAGAG	GTCTTTGGCATACTTGATCACC
$\beta$ -actin	TGGCACCCAGCACAAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
ChIP-qPCR primers		
TK1	CACCCGGCCTGTATGTTTCT	TATGTGGCACAGGCTGAAGG
Sequences for siRNAs		
si-HOXC-AS3 1#	CAGAGUGGAGUACAGCGCCAUCUA	UAGAUGGCGCUGUUACUCCACUCUG
si-HOXC-AS3 2#	CGGUCAGUUUGGAGGAGUCACGUAU	AUACGUGACUCCUCAAACUGACCG
si-YBX1	CCACGCAATTACCAGCAAA	TTTGCTGGTAATTGCGTGG
si-TK1	GGCCGGACAACAAGAGAATT	UUCUCUUUGUUGUCCGCCTT