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Original Article

Genome-wide target interactome profiling reveals a novel *EEF1A1* epigenetic pathway for oncogenic lncRNA *MALAT1* in breast cancer

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Abstract: Breast cancer is the most common cancer in women worldwide, accounting for approximately 500,000 deaths each year. *MALAT1* is a highly conserved long noncoding RNA (lncRNA), and its increased expression is associated with relapse and metastatic progression in breast cancer. We performed RNA reverse transcription-associated trap sequencing (RAT-seq) to characterize the genome-wide target interaction network for *MALAT1* and showed that *MALAT1* interacted with multiple pathway target genes that are closely related to tumor progression and metastasis. Notably, *MALAT1* bound to the promoter regulatory element of the translation elongation factor 1-alpha 1 gene *EEF1A1*. Knockdown of *MALAT1* by shRNA caused significant downregulation of *EEF1A1* in breast cancer MDA-MB231 and SKRB3 cells. Using a luciferase reporter assay, we showed that knockdown of *MALAT1* reduced the promoter activity of *EEF1A1* in these two breast cancer cells. Chromatin immunoprecipitation (ChIP) assay indicated that *MALAT1* regulated *EEF1A1* by altering the histone 3 lysine 4 (H3K4) epigenotype in the gene promoter. *MALAT1* was overexpressed in breast cancer tissues and breast cancer cells. Knockdown of *MALAT1* reduced cell proliferation and invasion by arresting cells at the G0/G1 phase. Ectopic overexpression of *EEF1A1* reversed the altered tumor phenotypes induced by *MALAT1* shRNA treatment. These data suggest an epigenetic mechanism by which *MALAT1* lncRNA facilitates a pro-metastatic phenotype in breast cancer by *trans*-regulating *EEF1A1*.

Keywords: LncRNA, interactome, *MALAT1*, epigenetics, histone methylation, breast cancer, *EEF1A1*

Introduction

Breast cancer is the most common cancer among women throughout the world, with more than 2 million estimated new patients reported in 2018 [1, 2]. Despite extensive research, breast cancer is still the leading cause of cancer mortality in women. Thus, more studies are needed to investigate the underlying molecular mechanisms involved in mammary tumor progression and metastasis. These studies may help develop new biomarkers or targets for disease prognosis and treatment.

Emerging evidence has demonstrated that long non-coding RNAs (lncRNAs) may function as oncogenes or tumor suppressors, and therefore they could serve as biomarkers or therapeutic targets for cancer patients [3-6]. Transcriptome analyses reveal that up to 80% of the human genome is transcribed into RNAs, whereas only < 2% of the human genome DNA is composed of protein-coding genes [7, 8]. Thus, the majority of the human genome is transcribed into non-coding RNAs. LncRNAs are defined as transcripts longer than 200 nucleotides without protein-coding capacity. LncRNAs are actively involved in various biological processes and function as epigenetic regulators of gene expression at the transcriptional and post-transcriptional levels [9-13]. Consistent with their role in these regulatory processes, it is not surprising that lncRNAs are also aberrantly expressed in various cancers, including breast cancer [14-19].

The nuclear-retained Metastasis-Associated Lung Adenocarcinoma Transcript 1 (*MALAT1*),

also referred as Nuclear-Enriched Abundant Transcript 2 (NEAT2), is a highly conserved lncRNA among mammals [20]. The gene encodes a long non-coding RNA with a length of ~8000 nt. *MALAT1* was initially identified as a prognostic marker for metastasis and survival in early-stage non-small cell lung carcinoma [21]. Later studies have shown that it is also upregulated in multiple malignancies, including breast [22], liver [23], colon [24], stomach [25], and bladder [26]. Its upregulation is associated with tumorigenesis or disease progression [27-29]. However, contradictory effects of *MALAT1* have been reported on tumorigenesis and development in breast cancer. It has been observed that high expression of *MALAT1* is associated with poor relapse-free survival in breast cancer [30]. *MALAT1* promotes proliferation and invasion in breast cancer cells *in vitro* [31-33]. Moreover, genetic loss or systemic delivery of antisense oligonucleotides targeting *MALAT1* in mice with established mammary tumors resulted in slower tumor growth, significant differentiation into cystic tumors and decreased metastasis [34, 35]. In contrast, studies by Eastlack showed that *MALAT1* abundance correlates with inhibition of oncogenic cell function in breast cancer [36]. Similarly, a recent work also reported a tumor suppressive role of *MALAT1* in breast cancer metastasis [37].

Profiling the lncRNA-DNA interaction network associated with *MALAT1* would help define its physiologic functions. In this study, we employed a reverse transcription-associated trap sequencing (RAT-seq) assay to characterize the genome-wide interactome for *MALAT1* lncRNA. Using this approach, we have identified potential interacting genes and defined a *MALAT1* lncRNA-DNA interactome network in breast cancer. Notably, we identified the translation elongation factor *EEF1A1* as a novel *MALAT1*-interacting gene, and showed that *MALAT1* enhanced tumor progression in breast cancer cells by *trans*-targeting the *EEF1A1* promoter.

Materials and methods

Cell culture

Five human breast cancer cell lines (MDA-MB231, MDA-MB436, SKBR3, MCF7, and T47D) and viral packaging 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). A non-tumorigenic human breast cell line (MCF10A) was obtained as a gift from Dr. Markus Covert's lab, Department of Bioengineering, Stanford University. Human breast cancer cell lines were routinely maintained in DMEM medium (Sigma, MO) and MCF10A in MEGM Mammary Epithelial Cell Growth Medium BulletKit (Lonza), containing 10% (v/v) fetal bovine serum (Sigma, MO), 100 U/ml of penicillin sodium and 100 μg/ml of streptomycin sulfate (Invitrogen, CA), in a 37°C humidified incubator with 95% air, 5% CO_2.

Bioinformatic analysis

The Cancer Genome Atlas (TCGA) RNA-sequencing and corresponding clinical data were downloaded from the TCGA website and normalized with TCGA Assembler and R software as noted in the previous study [38]. Data from 32 mammary paracancer tissues and 32 mammary cancer tissues were used for gene expression clustering analysis. Heatmap and Volcano Plot were generated by R software. Gene ontology enrichment analysis for RAT sequences was performed with Cytoscape software and ClueGO plug-in following the previous study [38].

Construction of plasmids

To construct the *MALAT1* knockdown vector, four short hairpin RNAs (shRNAs) against *MALAT1* were cloned into a lentiviral vector (Addgene, plasmid #8453) to construct two separate lentiviral vectors (Table S1). In each vector, two shRNAs were cloned in tandem under the control of H1 and U6 promoters, respectively, as previously described [39]. To construct the *EEF1A1* overexpression vector, *EEF1A1* cDNA was amplified and cloned into the vector (Addgene, #73310). All of the recombinant vectors were confirmed by DNA sequencing. Primers for plasmid construction are listed in Table S1.

Cell transfection

The lentiviruses were prepared in 293T cells using Lipofectamine 3000 transfection reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. MDA-MB231 and SKBR3 cells were grown to 70% confluence before being transfected with viral supernatants containing 5 mg/ml polybrene (Sigma, MO). Stable

cell clones were screened by administration of puromycin or blasticidin (Invitrogen).

RNA isolation and real-time Q-PCR

Total RNA was extracted by TRIzol reagent (Sigma, MO) from cells and stored at -80°C. RT-PCR reaction was performed with an Eppendorf Thermal Cycler. Complementary DNA (cDNA) was synthesized with M-MLV reverse transcriptase (Thermo Fisher Scientific, CA) according to the manufacturer's protocol. The amplification of target sequences was performed with Kapa HiFi PCR Kit (Kapa Biosystem, USA). Quantitative real-time PCR was carried out on ABI Prism 7900HT (Applied Biosystems) using SYBR GREEN PCR Master (Applied Biosystems, USA) following the manufacturer's protocols. All the samples were normalized to β-ACTIN according to the 2-ΔΔCt method [40].

Western blot

Cells were lysed in RIPA buffer in the presence of the protease inhibitor cocktail. The protein concentration was measured with a BCA protein assay kit (Thermo Scientific). Equal amounts of protein were resolved by SDS-PAGE and subjected to Western blot analysis with detection by the Odyssey infrared imaging system. Primary antibodies to *EEF1A1* and GAPDH were obtained from Abcam (MA, USA). Secondary antibodies were obtained from LicorOdyssey (Nebraska, USA).

Cell proliferation assay

Cell proliferation was measured with the cell counting kit-8 (CCK8) assay (Sigma, MO) according to the manufacturer's protocol. Briefly, cells were seeded in 96-well plates in triplicate at a density of 1×10^3-2×10^4 per well. CCK-8 solution (10 μl) was added to each well at 0, 24, 48, 72 and 96 hrs, respectively, followed by incubation for 3 hrs at 37°C. The absorbance was measured at 490 nm using a microplate reader.

Cell invasion assays

Transwell invasion assays were performed using 6-well BD Biocoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) according to the manufacturer's recommendations. Homogeneous single cell suspensions (3×10^5 cells/well) were added to the upper matrigel-coated chambers in serum-free medium. The lower chamber of the Transwell was filled with culture media containing 10% FBS. After incubating at 37°C for 24 h, the non-invading cells on the top of the Transwell were scraped off with a cotton swab. Invading cells were stained with 0.1% crystal violet for 10 min at room temperature and counted under a light microscope [41].

Cell-cycle analysis by flow cytometry

The trypsinized cells were washed with PBS and fixed with 70% cold ethanol at 20°C overnight. After washing, the fixed cells were resuspended in 500 μl PBS with propidium iodide and RNase A, followed by incubation at 37°C for 30 min in the dark. The suspended cells were analyzed using the FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Data on cell cycle distribution were analyzed using ModFit LT 3.0 software.

Luciferase reporter assay

To construct luciferase reporter vectors, the *EEF1A1* promoter was amplified from genomic DNA by PCR and subcloned into pGL3-Basic vector (Addgene, #E1751) using KpnI/XhoI (Thermo Fisher Scientific, CA). Primers for plasmid construction are listed in Table S1. Non-treated cells (CTL), shNC cells and shMALAT1 cells were cultured in 48-well plates. Each cell line was respectively transfected with pGL3-Basic vector or luciferase reporter vector comprising *EEF1A1* promoter using Lipofectamie 3000 (Invitrogen, USA). Luciferase activity was measured with a luminometer using the Dual-Luciferase reporter assay system (Promega, Madison WI, USA) according to the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with a ChIP assay kit (Millipore, NY) following the protocol provided by the manufacturer with slight modifications as previously described [41]. In brief, shMALAT1 and normal cells were fixed with 1% formaldehyde and sonicated on ice. The sonicated chromatin was immunoprecipitated with anti-H3K4me3 antibody and anti-IgG antibody (Cell Signaling, MA). Precipitated DNA was sub-

jected to quantitative PCR analysis. For comparison, the ChIP data are presented as relative values by normalizing to PCR signals of input DNA.

Reverse transcription-associated trap (RAT) assay

A nuclear lncRNA RAT assay [38, 42] was modified to identify the genome-wide targets that interact with *MALAT1* lncRNA. In brief, cells were cross-linked with 2% formaldehyde and lysed with hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.4% NP-40, RNase inhibitor 100 U/ml, 1× protease inhibitors). Nuclei were resuspended in 1× reverse transcription buffer. The gene strand-specific reverse transcription was performed in situ in the nucleus in the presence of biotin-dCTP. After 30 min of reverse transcription of *MALAT1* with Maxima Reverse Transcriptase (Thermo Fisher Scientific, CA) at 65°C, the reaction was stopped by adding 4 μl 0.5 M EDTA. After the resuspended nuclei were lysed with 0.3% SDS, the chromatin complex was sonicated on ice for 15 min (10 s on and 10 s off) using a Branson sonicator with a 2-mm microtip. The biotinylated-*MALAT1*-cDNA/chromatin DNA complex was pulled down with biotin-streptavidin magic beads (Invitrogen, CA). After washing, the pulled-down sample was treated with 10 mg/mL proteinase K at 70°C for 1 hour to reverse the cross-links, followed by extraction. The extracted DNAs were further digested by Mbo1 into small fragments and ligated with the NEBNext adaptors (NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina) to construct the library. The library DNAs were subjected to Illumina sequencing (Shanghai Biotechnology, Shanghai). For RAT-Seq control, we performed a RAT assay by replacing *MALAT1* complimentary primers with random primers and constructed a control library for high-throughput sequencing [38, 40].

RAT-seq data analysis

After Next Generation Sequencing, the adapter sequences were removed from the raw data using Illumina annotated adapter sequences, and reads that became shorter than 20 nt were filtered out. The remaining reads were further filtered to remove very low quality reads using the fastq_quality_filter program of the FASTX package (version: 0.0.13, http://hannonlab.cshl.edu/fastx_toolkit/index.html). Following filtering of raw reads, the RAT-Seq clean reads were mapped to the human genome (hg19) using Bowtie (version: 0.12.8). MACS (version: 1.4.2) was used to identify the DNA regions that interacted with the *MALAT1* lncRNA by peaks detecting. The peaks were visualized using the Integrative Genomics Viewer (IGV) tools and UCSC genome browser. To reduce the background, the RAT-Seq data were normalized over the control RAT-Seq data in the RAT assay [43].

Statistical analysis

All experiments were performed in triplicate and the data are expressed as mean ± SD (Standard Deviation). The comparison between two groups was analyzed by the Student's t-test using the GraphPad Prism software version 4.0. The difference among multiple groups was determined by analysis of variance (ANOVA). Results were considered statistically significant at $P < 0.05$.

Results

Genome-wide target analysis of MALAT1 by RAT-seq

The role of *MALAT1* lncRNA in breast cancer remains to be characterized. We proposed to use a RAT-seq approach [38, 42] to identify the genome-wide targets that interact with *MALAT1* lncRNA (**Figure 1A**). Cells were cross-linked with formaldehyde to fix the structure of the lncRNA-chromatin conformations. Then, *MALAT1* was reverse transcribed at 60°C *in situ* using *MALAT1*-specific complementary primers in the presence of biotin-dCTP. The *MALAT1* biotin-cDNA chromatin complex was pulled down with streptavidin beads, and the *MALAT1*-interacting target DNAs were purified for library construction and DNA sequencing. A random oligonucleotide was used to construct the RAT control library for sequencing [43].

After adjusting over the control data, the *MALAT1* RAT-seq data were characterized by the assignment of gene ontology (GO) terms with Cytoscape software. GO enrichment analysis revealed that *MALAT1* lncRNA might be involved in many different biological processes (**Figure 1B**). Using RAT-Seq, we found that *MALAT1* lncRNA bound to many pathway gene

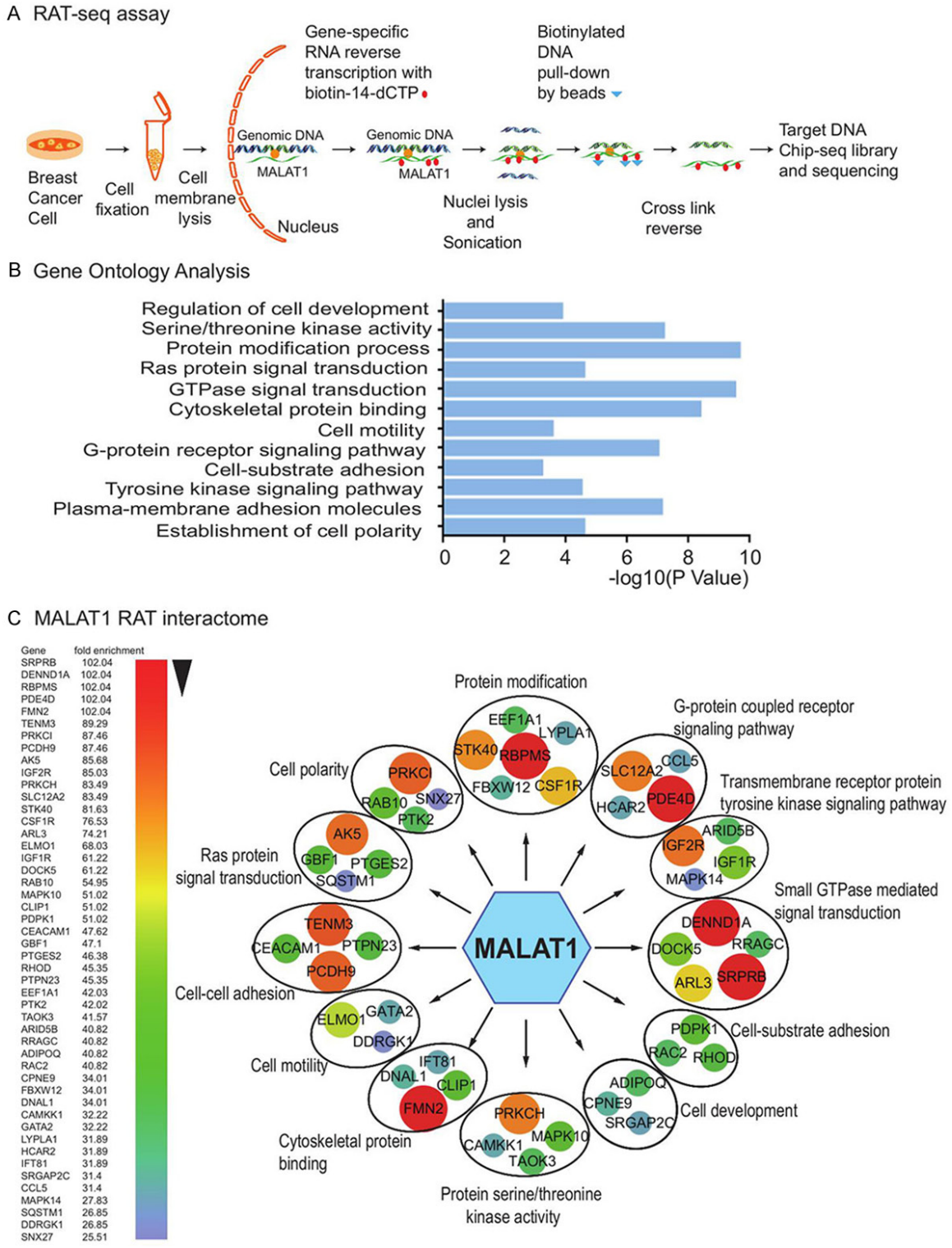

Figure 1. Interactome of *MALAT1* lncRNA by RAT-seq. A. RAT-seq assay. *MALAT1* lncRNA was *in situ* reverse transcribed using *MALAT1*-specific complementary primers at 60°C with biotin-dCTP. The biotin-*MALAT1* cDNA chromatin complex was isolated by streptavidin beads and cDNAs were isolated for Illumina library sequencing. RAT-seq will generate a genome-wide target interaction network for *MALAT1* lncRNA in breast cancer cells. B. Gene ontology enrichment pathway analysis of the *MALAT1* RAT-Seq data. GO enrichment was analyzed with Cytoscape software. C. The *MALAT1* RAT-seq interactome. The *MALAT1* interactome was drawn based on the enrichment fold of the top RAT-Seq pathway target genes.

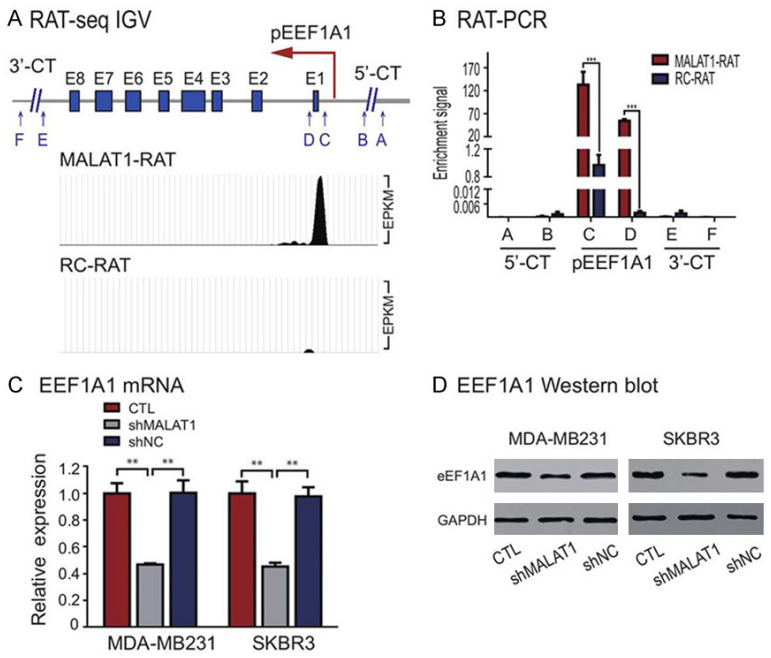

Figure 2. *MALAT1* binds to the *EEF1A1* promoter and epigenetically regulates its activity. A. The RAT-seq IGV binding of *MALAT1* lncRNA at the *EEF1A1* locus. *MALAT1*-RAT: the RAT-seq library created by the *MALAT1*-specific complementary primers; RC-RAT: the RAT-seq control library created by random oligonucleotide primers; p*EEF1A1*: *EEF1A1* promoter; 3'-CT, 5'-CT: the 3'- and 5'-control sites; E1-E8: *EEF1A1* exons. B. Quantitation of *EEF1A1* binding in the *MALAT1*-specific RAT-seq products and the negative control RAT-seq products. C. EEF1A1 expression levels by Q-PCR in MALAT1-knockdown cells. β-Actin was used as an internal control. $^{**}P < 0.01$ as compared with the control groups. D. Western blot of eEF1A1. Note the reduced expression of eEF1A1 in *MALAT1*-knockdown breast cancer cells. GAPDH was used as control.

targets that are closely related to tumor progression (**Figure 1C**).

MALAT1 directly binds to the EEF1A1 promoter and regulates its activity

We hypothesized that *MALAT1* would directly bind to the promoters of its target genes, where it could epigenetically regulate transcription. Thus, we focused on those target genes whose promoters interacted with *MALAT1*, and whose transcription was also affected by shMALAT1 knockdown. Among the RAT-seq targets, the translation elongation factor 1-alpha 1 gene *EEF1A1* met both of these criteria. *EEF1A1* abundance was the most affected by *MALAT1* shRNA knockdown among all tested gene targets (Figure S1).

EEF1A1 encodes an isoform of the alpha subunit of the elongation factor-1 complex, which mediates the delivery of aminoacyl tRNAs to the ribosome [44]. The RAT-seq data showed that *MALAT1* was primarily enriched within the *EEF1A1* promoter regulatory element region (**Figure 2A**). No enrichment of *MALAT1* lncRNA was observed in the random oligo RAT-seq control group.

We also collected the RAT-seq library samples and used Q-PCR to map the enrichment of *MALAT1* at the *EEF1A1* gene locus. In the *EEF1A1* promoter, we detected a significantly higher binding signal in the *MALAT1* RAT-seq group than that in the random RAT-seq group (**Figures 2B**, S2, sites C, D). There were only low background signals at the 5'-CT and the 3'-CT control sites in the same locus.

We then explored whether *MALAT1* knockdown affected the activity of its target gene. We collected shMALAT1-treated and random shNC-treated breast cancer cells. As compared with the shNC and untreated groups (CTL), shMALAT1 treatment significantly reduced the *EEF1A1* mRNA abundance both in MDA-MB231 and SKBR3 breast cancer cells (**Figure 2C**). Using Western blot, we also found that shMALAT1 treatment decreased EEF1A1 protein levels (**Figure 2D**).

MALAT1 epigenetically regulates the EEF1A1 promoter

To delineate the molecular mechanisms underlying the role of *MALAT1*, we first constructed the *EEF1A1* promoter-luciferase vector and performed a luciferase reporter assay in breast cancer cells. The *EEF1A1* promoter-pGL3 luciferase vector was transfected into the *MALAT1*-knockdown MDA-MB231 and SKBR3 breast cancer cells, and luciferase activity was quantitated and standardized over the pGL3 vector control. As seen in **Figure 3A**, the relative luciferase activity was significantly lower in shMALAT1 group than that in the shNC control group, suggesting that the activity of the *EEF1A1* promoter is regulated by *MALAT1* lncRNA.

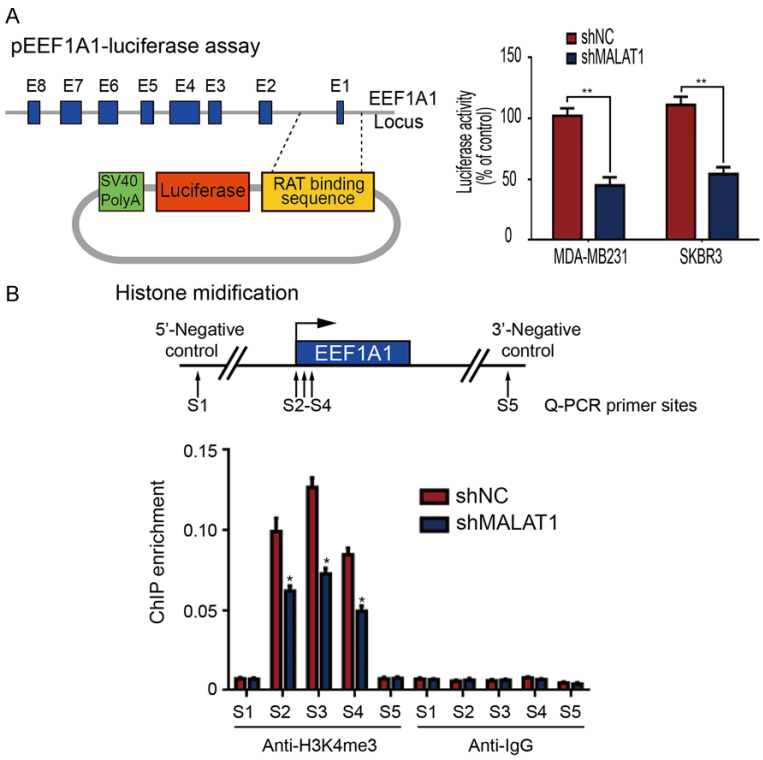

Figure 3. *MALAT1* epigenetically regulates EF1A1. A. p*EEF1A1*-luciferase assay. The *EEF1A1* promoter (p*EEF1A1*) sequence was cloned into the upstream of luciferase gene. Luciferase reporter assay was performed in CTL group, shNC group and shMALAT1 group by co-transfecting respectively with pGL3-Basic vector or luciferase reporter vector. Data were adjusted over the negative control (CTL) and were represented as means ± SD. $^{**}P < 0.01$ as compared with the control groups. B. Quantitation of Histone 3-K4 (H3K4) trimethylation. All data are presented as the relative values after normalization over the input DNA. $^{*}P < 0.05$ as compared with control.

We then performed a chromatin immunoprecipitation (ChIP) assay to examine the epigenotype in the *EEF1A1* promoter and asked whether *MALAT1* regulated the expression of *EEF1A1* through histone modification. Q-PCR was used to measure the status of histone methylation. As compared with the vector control group, breast cancer cells transfected with shMALAT1 showed a significant decrease in H3K4 methylation in the promoter (**Figure 3B**, sites S2-S4). There was no statistical difference detected at the 5'- and 3'-negative control sites (S1 and S5), as well as in the IgG control group. These data suggest that *MALAT1* regulates *EEF1A1* epigenetically by altering H3K4 in the gene promoter.

MALAT1 is aberrantly upregulated in breast cancers

To explore the role of *MALAT1* in breast cancer, we downloaded gene expression data of 64 mammary tissues from TCGA database, including 32 paracancer tissues and 32 tumor tissues. Unsupervised hierarchical clustering analysis showed that *MALAT1* was dysregulated in breast cancers (**Figure 4A**). Expression of *MALAT1* was significantly higher in the breast cancer tissues than in paracancer tissues (**Figure 4B**).

We also compared the expression of *MALAT1* in breast cancer cell lines, including MDA-MB231, MDA-MB436, SK-BR3, MCF-7, and T-47D, and one non-breast cancer cell line (MCF10A). *MALAT1* was overexpressed in breast cancer cell lines as compared to normal MCF10A cells (**Figure 4C**). Among them, MDA-MB-231 is a highly aggressive and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks the expression of ER (oestrogen receptor), PR (progesterone receptor), and HER2 (human epidermal growth factor receptor 2). In contrast, SK-BR3 overexpresses HER2 and EpCAM and is capable of forming poorly differentiated tumors in immunocompromised mice. These two breast cancer cells were thus used as the target cell lines for the following knockdown assay.

MALAT1 knockdown suppresses tumor phenotypes in breast cancer cells

To investigate the biological functions of this lncRNA, we used short hairpin RNA (shRNA) to knockdown *MALAT1* in two breast cancer cell lines MDA-MB231 and SKBR3. Four shRNA sequences were designed to target MALAT1 and were cloned in tandem in two separate lentiviral vectors (shMALAT1-1, -2). Using Q-PCR, we found that *MALAT1* expression was downregulated in shMALAT1-transfected cells compared with non-transfected control (CTL) and non-targeting (random) control (shNC)-transfected cells (**Figure 5A**).

Using the Cell Counting Kit-8 (CCK8) assay, we found that cell proliferation was decreased in

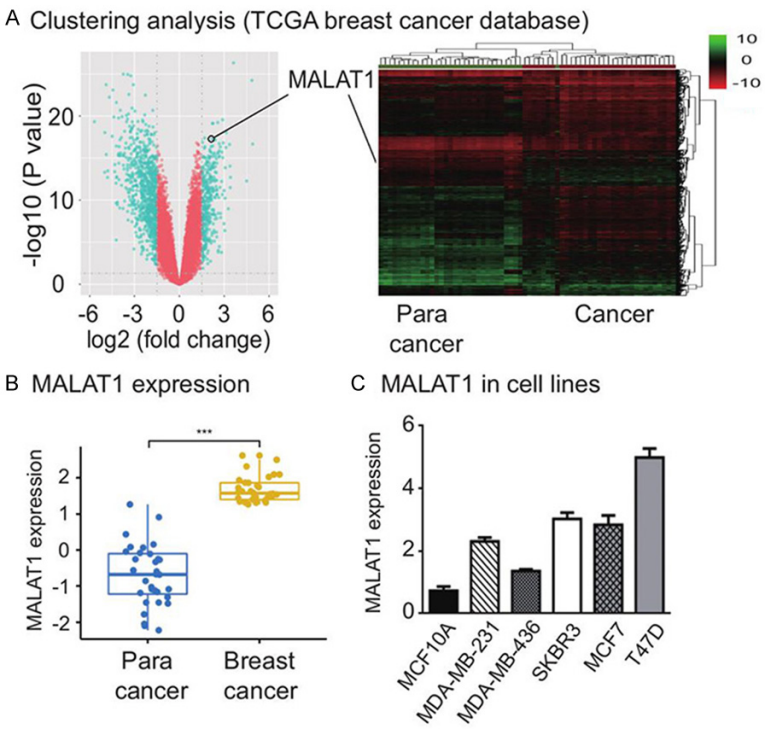

Figure 4. *MALAT1* is dysregulated in breast cancer. A. Unsupervised hierarchical clustering analysis of the significantly differentially expressed genes in paracancer tissues and tumor tissues. Data from 64 mammary tissues was downloaded from TCGA database. In the heatmap, the normalized expression values are represented in shades of green and red, indicating the expression being above and below the median expression value across the samples. B. The normalized expression level of *MALAT1* in 64 mammary tissues (32 paracancer tissues and 32 tumor tissues). ***P < 0.01 as compared with the paracancer group. C. Q-PCR quantitation of *MALAT1* expression in breast cancer cell lines.

the shMALAT1-treated group in comparison with the CTL and shNC groups (**Figure 5B**). Flow cytometry analysis showed that the silencing of *MALAT1* arrested cells at the G0/G1 phase (**Figure 5C**). Only the shMALAT1-1 (shMALAT1) knockdown cells were used for the following assays.

MALAT1 knockdown inhibits tumor invasion

We examined whether *MALAT1* was involved in cell invasion in MDA-MB231 and SKBR3 cells using a transwell assay. The number of invading cells in the shMALAT1 group was decreased compared with that in the CTL group and the shNC groups (**Figure 6**).

EEF1A1 overexpression reverses the inhibitory effect of MALAT1 knockdown

Given the fact that *MALAT1* directly binds to the *EEF1A1* promoter and controls its activity, we asked whether overexpression of *EEF1A1* was able to rescue the effect of *MALAT1* knockdown in breast cancer cells. We constructed an *EEF1A1* overexpression vector by cloning its cDNA sequence into the lentiviral plasmid vector containing the blasticidin + gene. *EEF1A1* was overexpressed in the shMALAT1 (puromycin+)-treated MDA-MB231 and SKBR3 breast cancer cells. The empty lentiviral vector was used as the negative control. After puromycin and blasticidin selection, stable clones were used for functional assays.

Using Q-PCR and Western blot assays, we confirmed that *EEF1A1* was highly expressed in the overexpression group (**Figure 7A**). CCK8 assay showed that overexpression of *EEF1A1* reversed the effect of *MALAT1* silencing on cell proliferation in both breast cancer cells (**Figure 7B**). Flow cytometry analyses also showed that the inhibition of cell cycle progression by *MALAT1* knockdown was rescued in the *EEF1A1*-overexpression group (Figure S3).

Using a transwell assay, we showed that ectopic expression of *EEF1A1* reversed the shMALAT1-induced inhibition of cell invasion in MDA-MB231 and SKBR3 breast cancer cells (**Figure 7C**). Collectively, these results revealed that *MALAT1* promoted cell proliferation and cell invasion, at least in part by activating the *EEF1A1* pathway.

Discussion

This study demonstrates a new epigenetic mechanism by which *MALAT1* promotes malignant phenotypes in breast cancer cells. Using RNA reverse transcription-associated trap sequencing, we characterized the genome-wide interaction network targets for *MALAT1* lncRNA, identifying the translation elongation factor 1-alpha 1 gene *EEF1A1* as a critical tar-

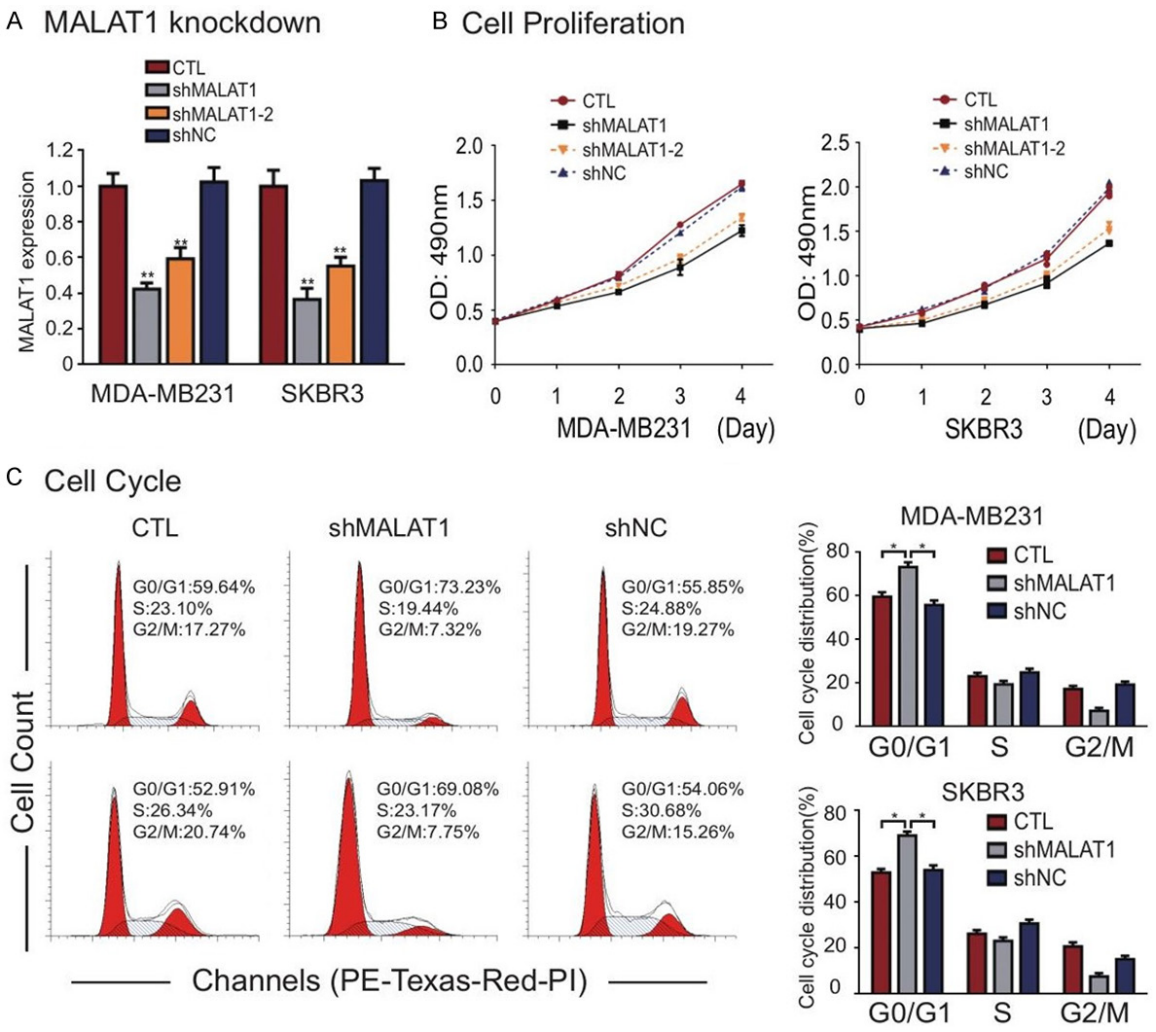

Figure 5. The role of *MALAT1* in cell proliferation and cell cycle. A. MALAT1 shRNA knockdown in two breast cancer cell lines. MALAT1 expression was examined by Q-PCR in CTL (non-transfected control), shNC (random shRNA non-targeting control), shMALAT1 (MALAT1 shRNA-1 transfected cells), and shMALAT1-2 (MALAT1 shRNA-2 transfected cells). β-Actin was used as an internal control. $^{**}P < 0.01$ as compared with CTL and shNC controls. B. Cell Proliferation. CCK-8 assay was used to determine cell growth viability at 0, 24, 48, 72 and 96 hour time points. C. Cell cycle. Flow cytometry was used to measure cell cycle profile with propidium iodide staining. Cell numbers were counted according to DNA content of G0/G1, S and G2/M phases. The statistical results are shown on the right panel. $^{*}P < 0.05$ as compared with the control groups.

get of *MALAT1*. *MALAT1* interacted with the promoter regulatory element of *EEF1A1* and affected the status of H3K4 methylation in the gene promoter. *EEF1A1* was downregulated in *MALAT1* knockdown breast cancer MDA-MB231 and SKRB3 cells. Knockdown of *MALAT1* reduced the promoter activity of *EEF1A1* in breast cancer cells, leading to decreased cell proliferation, cell invasion, and cell cycle arrest. In contrast, ectopic over-expression of *EEF1A1* reversed these altered tumor phenotypes. Thus, *MALAT1* plays an oncogenic role in breast cancer, at least in part by epigenetically regulating the *EEF1A1* pathway.

A number of studies have reported involvement of *MALAT1* in tumor progression and metastasis in various cancers. A recent study found that *MALAT1* acts as a proto-oncogene in hepatocellular carcinoma by upregulating the oncogenic splicing factor SRSF1, resulting in the activation of the Wnt and mTOR pathways [23]. In lung cancer, *MALAT1* has been found to promote lung cancer development by binding with the stemness transcription factor OCT4 at its enhancer regions [45]. High levels of OCT4/*MALAT1* correlated with the poor prognosis. Moreover, it has been reported that high expression of *MALAT1* is associated with poor

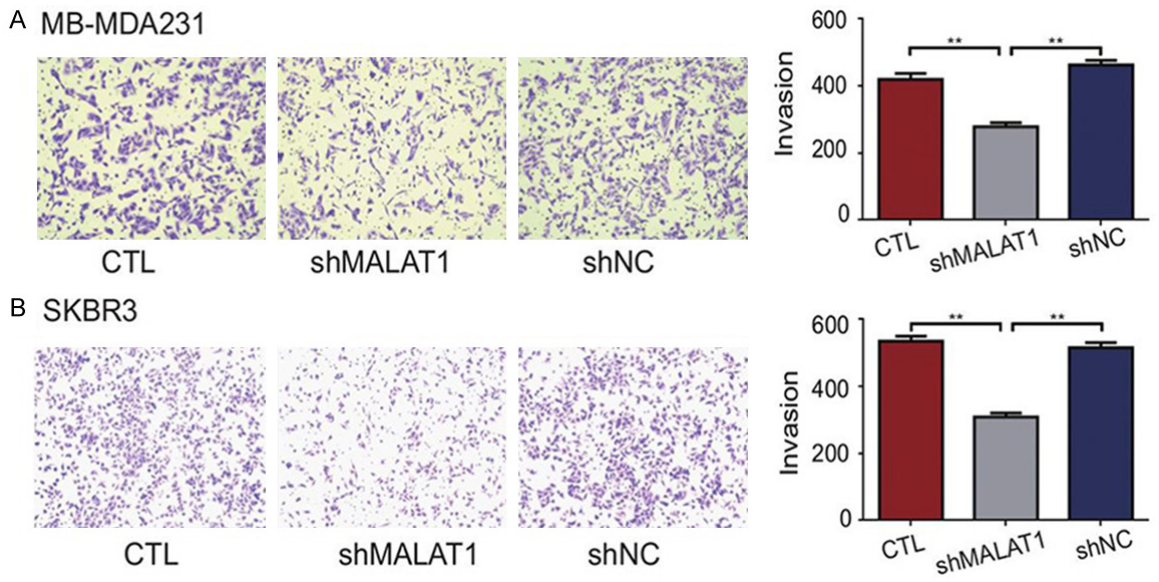

Figure 6. *MALAT1* knockdown inhibits cell invasion in breast cancer cells. Representative images of invading MDA-MB231 cells (A) and SKBR3 cells (B) are showed on the left panel. shNC: random shRNA control; shMALAT1: Cells that were transfected with MALAT1 shRNA-1. Quantitation of invaded cells is shown in the right panel, mean ± SD, $^{**}P < 0.01$ as compared with the control groups.

relapse-free survival in patients with breast cancer [30]. However, conflicting observations were also reported, which suggested that *MALAT1* functions as a tumor suppressor gene [36, 37]. These discrepancies may arise from the different models used in these studies. The role of *MALAT1* may vary depending on formation of a complex with proteins or other RNA species in a cell- or tissue type-specific manner.

Numerous proteins have been shown to interact with *MALAT1*. *MALAT1* has been implicated in affecting pre-mRNAs alternative splicing through interacting with SR proteins, such as SRSF1, SRSF2, and SRSF3 [46, 47]. *MALAT1* binds with human antigen R (HuR), thereby acting as regulatory complex in dedifferentiating breast cancer cells [48, 49]. In the present study, we identified *EEF1A1* as a critical gene target of *MALAT1* in breast cancer cells. *EEF1A1* plays a crucial role in regulating eukaryotic protein synthesis by triggering the initiation of translation elongation [44]. *EEF1A1* was also found to participate in various biological processes, such as heat shock response [50], signal transduction [51, 52] and cytoskeleton regulation [53]. Accumulating evidence demonstrates that *EEF1A1* is aberrantly overexpressed in many tumors and may be involved in tumor progression. For example, the eEF1A1 factor co-locates and combines with PAK4, promoting metastasis and progression of gastric cancer cells [54]. *EEF1A1* is upregulated in hepatocarcinoma, where it enhances tumor progression and correlates with poor prognosis [55]. The ubiquitin-like protein FAT10 promotes tumor proliferation by stabilizing *EEF1A1* expression [56].

It has been found that *EEF1A1* is a downstream target of the TGF-β signaling pathway. Type I TGF-β receptor (TβR-I), an essential component of TGF-β signaling, phosphorylates eEF1A1 at Ser300 in vitro and in vivo [57]. Decreased Ser300 phosphorylation in human breast cancer correlates with increased proliferation and tumor progression. In our study, we show that *MALAT1* binds to the *EEF1A1* promoter region and epigenetically regulates its activity. It is not surprising that *EEF1A1* overexpression indeed reverses the inhibitory effect of *MALAT1* knockdown on cell proliferation and invasion. Thus, *MALAT1* may promote cell proliferation and cell invasion in part by activating the *EEF1A1* pathway in breast cancer. It should be noted that *EFF1A1* is a major member of the eukaryotic elongation factor family that regulates protein synthesis. The eEF1 complex plays an indispensable function in eukaryotic protein peptide chain elongation in eukaryotic cells. Knockdown of this factor may affect cellular functions

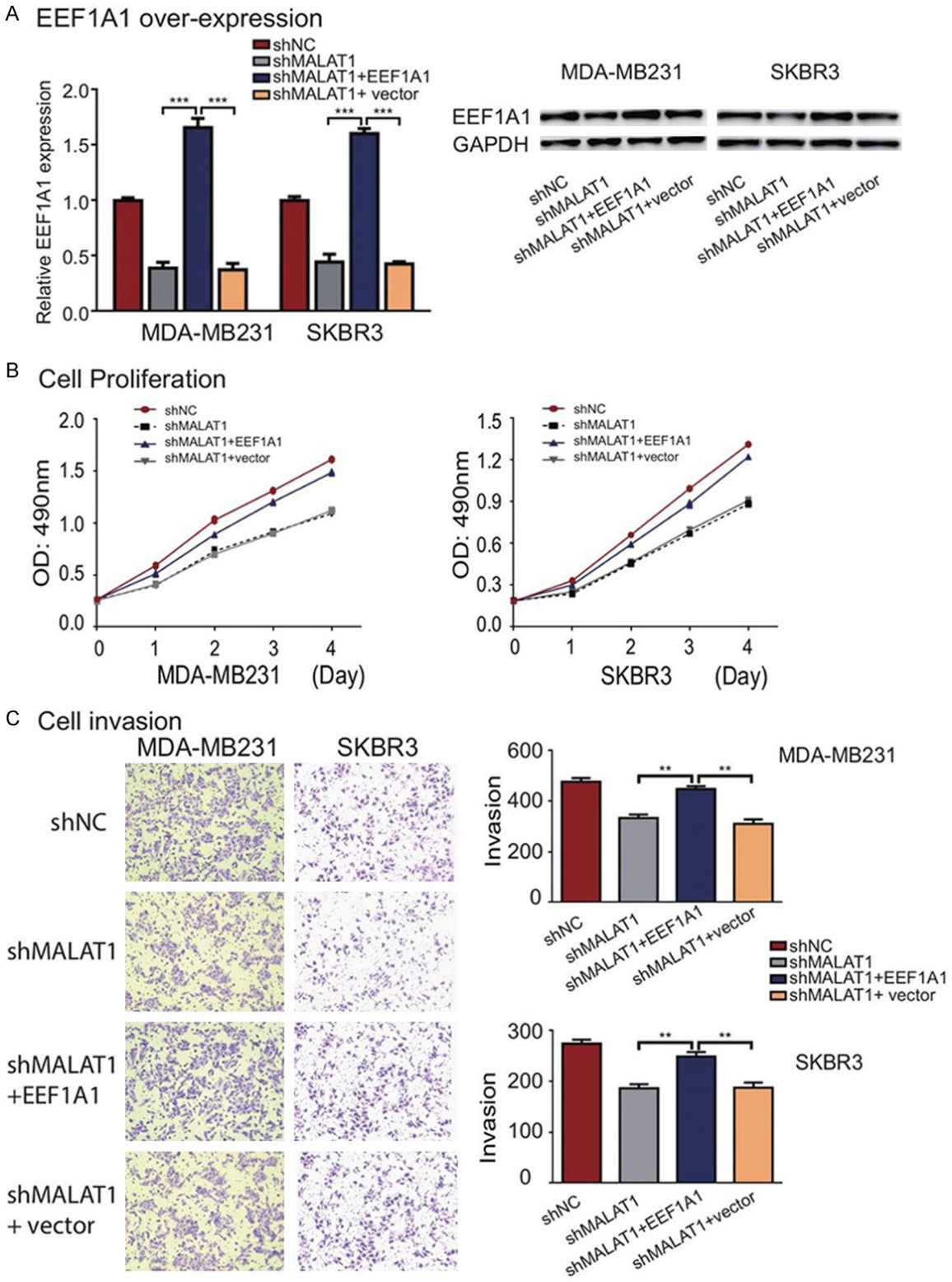

Figure 7. *EEF1A1* rescues the effect induced by *MALAT1* knockdown. A. Overexpression of *EEF1A1* in breast cancer cells. The expression of *EEF1A1* was quantitated by Q-PCR. shNC: random shRNA control; shMALAT1: Cells that were transfected with MALAT1 shRNA-1. β-Actin was used as an internal control. $^{***}P < 0.001$ as compared with the control groups. Overexpression of *eEF1A1* in breast cancer cells was measured by Western blot. B. Cell growth viability as measured by CCK-8 assay. C. Cell invasion as examined by Transwell assay. Quantitation of invaded cells was shown as mean ± SD, $^{**}P < 0.01$ as compared with the control groups.

through other "off-target" pathways. Considering the critical role of *EEF1A1*, we used the overexpressed *EEF1A1* to rescue the effect of *MALAT1* knockdown in breast cancer cells. Nevertheless, further study is needed to clarify whether the *MALAT1-EEF1A1* interaction has an effect on other downstream molecules in the TGF-β signaling pathway.

In conclusion, using a RAT-seq high-throughput approach, we have demonstrated that *MALAT1*, a highly conserved lncRNA, enhances proliferation and invasion by *trans*-targeting *EEF1A1* in breast cancer cells. By binding to the *EEF1A1* promoter element, it regulates H3K4 methylation to activate the promoter. Knockdown of *MALAT1* alters H3K4 methylation and reduces *EEF1A1* promoter activity. These data expand our current knowledge of the *MALAT1* interacting network and related epigenetic mechanisms, laying the foundation for developing new therapeutic strategies to treat breast cancer.

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Disclosure of conflict of interest

None.

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Table S1. Oligonucleotide primers used for PCR

ID	Oligo Name	Oligo sequence	Product size
RT-PCR			
MALAT1	JH5239	TGGGATGGTCTTAACAGGGA	141 bp
	JH5240	CCTGAAGGTGTTCGTGCCAA	
PARP2	JH5251	CTGTGGACCCAGAGTGTACA	160 bp
	JH5252	CTGAAGTTCCTCTGGGCATC	
SENP5	JH5253	CTGCCAGGAACGAGGGTAGC	134 bp
	JH5254	GCCTCCAAACCCAGTATTCCA	
PRKCH	JH5255	GAAGGCGAGTCCACCAGATC	147 bp
	JH5256	TATGGACGACACAGGTGCAC	
GNG2	JH5259	ACAACACCGCCAGCATAGCA	161 bp
	JH5260	TGAAGCCGGAACAGGGGTCA	
EEF1A1	JH5261	GATTCGGGCAAGTCCACCACT	164 bp
	JH5262	ATACCACGTTCACGCTCAGC	
CTNNA2	JH5263	GGCAGCTCGAGTCATACACAT	151 bp
	JH5264	ACGTTGGCACTCAGGGCTTC	
IGF1R	JH3461	ACTCATTGTTCTCGGTGCACGC	151 bp
	JH3464	GACCTGTGTCCAGGGACCATGG	
β-Actin	J880	CAGGTCATCACCATTGGCAATGAGC	135 bp
	J881	CGGATGTCCACGTCACACTTCATGA	
MALAT1 shRNAs			
1	JH5077	CACAGGGAAAGCGAGTGGTTGGTAA	
2	JH5078	GATCCATAATCGGTTTCAAGGTA	
3	JH5075	CTGGGCTTCTCTTAACATTTA	
4	JH5076	GGCCAAATGTTGAAGTTAAGTT	
Control (shNC)		GCAGCAACTGGACACGTGATCTTAA	
RAT primers			
MALAT1	JH4491	CAACTGCTTGCAGTCCTGCGAC	
	JH4492	CAAGGTCTTTTAATCACCTTCG	
	JH4493	CTGCCCTCAAAAGCTTCAGAC	
	JH4494	ATAGACCCCTGACTTTCTGGAA	
	JH4495	GTGAAGGGTCTGTGCTAGATC	
	JH4631	CATCTAGGCCATCATACTGC	
	JH4633	AAGCATTGCCCTTCTATTGGTA	
Negative control	JH5849	ATGGACTGATGATCTTATGC	
	JH5850	TACATAGTAGATCAGATACT	
RAT EEF1A1 binding			
P1	JH5563	CAGCACGCAAAGCTTCCCAG	167 bp
	JH5564	AGCAGTCAACTCCATAGCCT	
P2	JH5611	CTGGACATCTGGAGTACCAG	185 bp
	JH5612	TGGGGCGAAGGGTCGGGACT	
P3	JH5491	GCTCCGGTGCCCGTCAGTG	196 bp
	JH5492	ACGTTCACGGCGACTACTGC	
P4	JH5613	ATGCGGGCCAAGATCTGCAC	139 bp
	JH5614	TGAGACTACCCCCGTCCGAT	
P5	JH5565	TAGCAGGCCCATGGATCTCA	199 bp
	JH5566	CTGGTCTGAACTCCTGACCT	
P6	JH5497	AGAGGCTCAACAGGGAAGTC	179 bp
	JH5498	TTCTGGTGAGGGCATCTGCA	

Luciferase			
	JH5879	TCTATCGATAGGTACCGCCCGCTCTCGTCATCACTGAG	1284 bp
	JH5880	GATCGCAGATCTCGAGATGACGACAATGTTGATATGAGTC	
ChIP primers			
S1	JH5563	CAGCACGCAAAGCTTCCCAG	167 bp
	JH5564	AGCAGTCAACTCCATAGCCT	
S2	JH5493	AGTTCGAGGCCTTGCGCTTA	167 bp
	JH5494	AGCGTCGCAGCAGGTCATC	
S3	JH5613	ATGCGGGCCAAGATCTGCAC	139 bp
	JH5614	TGAGACTACCCCCGTCCGAT	
S4	JH5996	AATCGGACGGGGGTAGTCTC	125 bp
	JH5997	ATCTTTCCGCTCACGCAACT	
S5	JH5497	AGAGGCTCAACAGGGAAGTC	179 bp
	JH5498	TTCTGGTGAGGGCATCTGCA	
EEF1A1 overexpression			
	JH5926	CGTGACGCGGGATCCGCCACCATGGGAAAGGAAAAGACTCATATCAAC	1386 bp
	JH5927	CGCCGCTGCCGCTAGCTTTAGCCTTCTGAGCTTTCTGGGCA	

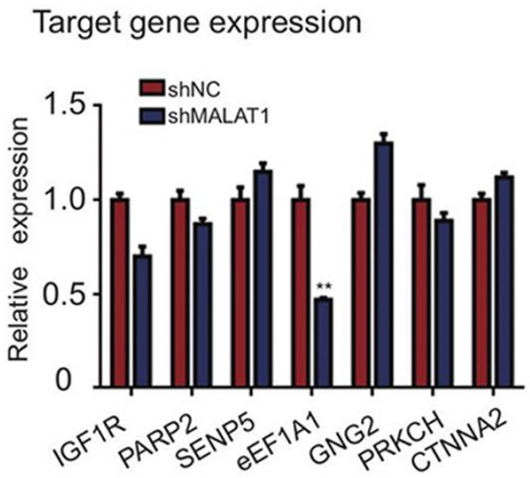

Figure S1. Expression of *MALAT1* target genes. Quantitation of *MALAT1* target genes as measured by qPCR. All data shown are mean ± SD. $^{**}P < 0.01$ as compared with normal control.

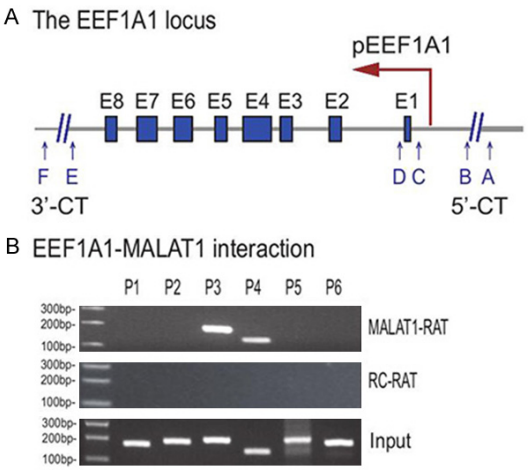

Figure S2. *MALAT1* binding in the eEF1A1 promoter. A. The *EEF1A1* gene locus. p*EEF1A1*: *EEF1A1* promoter; 3'-CT, 5'-CT: the 3'- and 5'-control sites; E1-E8: *EEF1A1* exons; p*EEF1A1*: *EEF1A1* promoter. B. Specific binding of *MALAT1* lncRNA to the *EEF1A1* promoter. *MALAT1*-RAT: the RAT-seq library created by the *MALAT1*-specific complementary primers; RC-RAT: the RAT-seq control library created by random oligonucleotide primers; input: sample aliquots collected before pulldown in the RAT-seq samples.

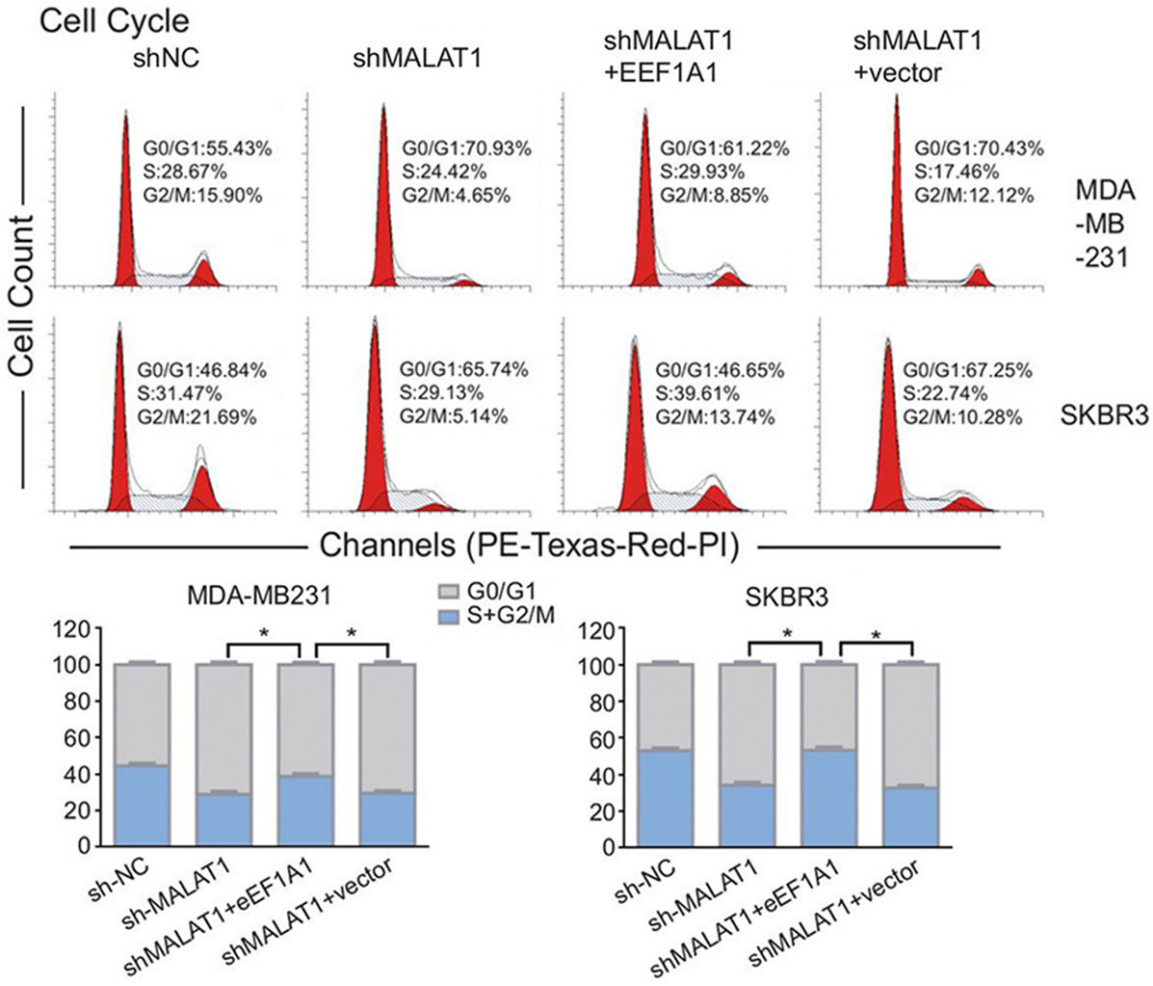

Figure S3. EF1A1 rescues the effect of *MALAT1* knockdown (cell cycle). Cell Cycle. shMALAT1 cells were transfected with *EEF1A1* overexpression vector or control vector. Cell cycle profile was examined by flow cytometry with propidium iodide staining, cell number were counted according to DNA content of G0/G1, S and G2/M phases.