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Original Article

MUS81 inhibits cell proliferation and migration in breast cancer by promoting the expression of CDKN2A(p16^{INK4a})

Rong Xie[1*], Deyu Kong[2*], Xinyu Su[1], Lixi Zhang[1], Jinnan Wan[1], Yanhong Yan[3]

[1]Department of Thyroid and Breast Surgery, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, Hubei, China; [2]Department of Medical Oncology, The First Affiliated Hospital of Kunming Medical University, Kunming 650000, Yunnan, China; [3]Department of Physical Examination, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, Hubei, China. [*]Equal contributors.

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Abstract: MUS81 has been recognized as a significant tumor suppressor - essential for DNA damage repair and maintaining chromosomal stability. However, its biological function and expression profile in breast cancer (BC) remain unclear. In this investigation, we examined the relationship between MUS81 expression and the proliferative and migratory capacities of BC cells. MUS81 mRNA and protein levels were markedly lower in breast cancer tissues and cell lines than in adjacent normal tissues and non-tumorigenic MCF-10A cells. Functional assays revealed that MUS81 overexpression suppressed, while MUS81 silencing enhanced, BC cell proliferation and motility, as demonstrated by CCK-8, colony formation, wound-healing, and Transwell experiments. In vivo, MUS81 overexpression markedly reduced Ki-67 expression in xenograft tumors. Although MUS81 did not alter CDKN2A mRNA expression, immunohistochemistry and Western blot analyses showed that p16^{INK4a} protein levels increased following MUS81 overexpression. Furthermore, the modulation of p16^{INK4a} expression by MUS81 was abolished by pretreatment with cycloheximide or MG132, suggesting that MUS81 stabilizes p16^{INK4a} by preventing proteasome-mediated degradation. Collectively, these findings indicate that MUS81 works as a tumor suppressor in BC by inhibiting proliferation and migration through post-translational stabilization of p16^{INK4a}.

Keywords: MUS81, breast cancer, proliferation, migration, CDKN2A(p16^{INK4a})

Introduction

Globally, breast cancer is the most widespread cancer in women and is the top cause of cancer-related deaths in the female population [1, 2]. Patients with early-stage, non-metastatic breast cancer have a 70%-80% chance of being cured, but advanced BC with distant metastases remains incurable [3, 4]. BC is a molecularly heterogeneous disease, categorized into four subtypes based on molecular features: luminal A and luminal B (expressing the Estrogen receptor (ER) and/or progesterone receptor (PR)), HER2-enriched, and triple-negative BC (lacking ER, PR, and HER2 expression) [5, 6]. Prior investigations have discovered genes that frequently undergo mutations and/or amplifications in tumor cells, including TP53 (41% of tumors), MYC (20%), CCND1 (16%), FGFR1 (11%) and GATA3 (10%), associated with BC [7-9]. Further research is necessary to discover gene changes as novel indicators for assessing cancer progression and enhancing the therapeutic efficacy of BC.

The MUS81 gene encodes a structure-specific DNA endonuclease that partners with EME1/MMs4 to resolve Holliday junctions (HJs), crucial for DNA double-strand break (DSB) repair and maintaining chromosomal integrity [10-12]. In 2004, McPherson et al. found that 73% of MUS81$^{-/-}$ mice and 50% of MUS81$^{+/-}$ mice died from various spontaneous tumors, including lymphoma, breast cancer, and prostate cancer, indicating MUS81 as a potential tumor suppressor gene in mice [13]. In a recent in vitro

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study, it was found that MUS81 haploinsufficiency in the human colon cancer cell line HCT116 can activate intra-S-phase and G2/M checkpoints, promoting cellular replication and indicating a role for MUS81 in colorectal cancer progression [14, 15]. Additionally, Wu et al. observed a marked reduction in the expression of MUS81 mRNA and protein in hepatocellular carcinoma tissues, with low MUS81 expression correlating with poor differentiation, metastasis, and prognosis in liver cancer patients [16]. Several studies propose Mus81 inhibition as a novel strategy to enhance the sensitivity of colon and breast cancer cells to chemotherapeutic agents [17, 18].

However, the significance and expression of MUS81 in human BC are largely unexplored. Therefore, we conducted this study to investigate the biological effects of MUS81 knockdown and overexpression. The proliferation and migration of human BC cell lines were examined in a series of experiments. We also investigated the molecular mechanisms by which MUS81 inhibits BC progression.

Materials and methods

Cell lines

Breast cancer cell lines MDA-MB-231, MDA-MB-468, BT549, MCF-7, BT474, T47D, and the non-tumorigenic MCF-10A were obtained from the American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in L15 medium, MDA-MB-468, BT549, and T47D cells in RPMI-1640 medium, and MCF-7 and BT474 cells in DMEM medium, all supplemented with 10% fetal bovine serum (FBS). MCF-10A cells were maintained in DME/F12 medium with 10% FBS, 10 μg/mL insulin, 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, and 0.5 μg/mL hydrocortisone. MDA-MB-231 cells were incubated at 37°C without CO_2, whereas the other cells were kept at 37°C in a humidified incubator with 5% CO_2.

Patients and samples

Breast cancer samples and nearby normal tissues were collected from 16 patients who underwent surgery at the Breast and Thyroid Surgery Center, Central Hospital of Wuhan, from 2022 to 2023. No patients received neoadjuvant therapy, including chemotherapy, targeted therapy, or endocrine therapy, prior to surgery. All samples were gathered right after surgery, frozen in liquid nitrogen, and then stored at -80°C until analysis could be performed. Approval for this research was granted by the Medical Research Ethics Committee of the Central Hospital of Wuhan (IRB: WHZXKYL-2022-033). Informed consent was secured from each patient.

In vivo experiments

The Experimental Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, approved all animal studies. Ten 3- to 4-week-old BALB/c-nu nude mice were obtained from Molebao (Wuhan) Biotechnology Co., Ltd. and randomly divided into two groups. In the xenograft experiment, a 100 μL cell suspension of stably transduced MDA-MB-231 (1×10^7) cells was injected subcutaneously into the left axilla of mice. Following 4 to 5 weeks of feeding, the xenograft tumors were excised, fixed in 4% formaldehyde, and readied for immunohistochemical analysis (Biossci).

RNA extraction and quantitative real-time PCR (QRT-PCR)

Using the Trizol reagent from Invitrogen, USA, total RNA was extracted from cells and tissues and subsequently reverse transcribed into cDNA with the PrimeScript® RT reagent Kit from Takara, Japan. Quantitative real-time PCR (qRT-PCR) was conducted with the TB Green Premix Ex Taq II kit (Takara Bio Inc., Japan). The PCR primers for MUS81 were: forward 5'-TGTGTGGACATTGGCGAGAC-3' and reverse 5'-CTGCAAAGGTCATCCAGTCG-3'. The PCR primers for CDKN2A were: forward 5'-GATCCAGGTGGGTAGAAGGTC-3' and reverse 5'-CCCCTGCAAACTTCGTCCT-3'. GAPDH expression was quantified and served as an internal reference. MUS81 transcripts were standardized against GAPDH and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Gene overexpression and knockdown

Lentivirus overexpressing MUS81 and lentiviruses carrying RNAi targeting MUS81 were acquired from Jikai Gene Chemical Technology Co., Ltd., Shanghai, China. The breast cancer cells were transfected with lentivirus in a cell culture medium containing HitransGP (25×

magnification). Stably transduced cells were established by screening with puromycin (Sigma-Aldrich). The overexpression and knockdown efficiencies of the lentivirus were measured by PCR and western blotting.

Western blotting

Cells and tissues were lysed with 1× cell lysis buffer from Biosharp. The total proteins were then separated using SDS-PAGE and transferred to a PVDF membrane from Merck Millipore. The membranes were probed with primary antibodies that target MUS81 (ab14387, Abcam, 1:1,000 dilution), $p16^{INK4a}$ (ab108349, Abcam, 1:1,000 dilution), and GAPDH (ab8245, Abcam, 1:3,000 dilution), followed by incubation with the corresponding secondary antibodies. GAPDH served as the loading control.

Cell proliferative assays

Cells were placed in a 96-well plate, with five wells designated per group, each containing 3,000 cells. Following 1, 2, 3, 4, and 5 days of incubation, 10 μL of CCK-8 (cell counting kit-8, Dojindo, Japan) reagent was introduced to each well. The measurement of absorbance at 450 nm was conducted with a microplate reader (Thermo Fisher Scientific). For the colony formation experiment, the cells (1×10^3 cells per well, six wells per group) were cultured in 6-well plates. Following a 14-day incubation period for clone visibility, they were fixed with 4% paraformaldehyde for 20 minutes and stained with crystal violet dye (Aspen) for 30 minutes. Colony counts were performed using a light microscope. Each experiment was conducted three times.

Cell migration experiments

The upper chamber of a Transwell insert with 8.0-μm pores (Corning, New York, USA) received a 200 mL tumor cell suspension containing 0.1% serum (2 × 10^4 cells per well). Subsequently, 600 μL of culture medium with 20% serum was introduced into the upper chamber. Following an incubation period of 24-48 h, invaded cells were stained with 0.1% crystal violet for 10 minutes and counted under a microscope. The images were taken at ×400 magnification. In the scratch experiment, cells were grown in 6-well plates, ensuring sufficient coverage of the plate bottom after wall adhesion. A straight line was drawn on each cell group using a 10 μL pipette suction head, PBS was employed to wash away the detached cells. Subsequently, 2 mL of serum-free culture medium was added to each cell group, and the scratch width was recorded under an inverted microscope at the beginning and after 48 hours.

Immunohistochemical staining

Immunohistochemistry (IHC) was utilized to identify MUS81 and Ki67 expression in transplanted tumors. In brief, paraffin sections of these tumors underwent dewaxing, rehydration and were subjected to microwave heat-induced antigen retrieval using citrate buffer (pH 6.0). Afterward, the sections were exposed to 3% H_2O_2 to inhibit endogenous peroxidase activity. The sections were blocked with 10% goat serum after being rinsed with TBS and then incubated overnight at 4°C with the primary antibody. Sections were incubated with the matching HRP-conjugated secondary antibody for an hour at room temperature the following day. Visualization was achieved using diaminobenzidine, followed by hematoxylin counterstaining. Subsequently, the sections underwent dehydration and drying through an alcohol gradient. Ultimately, the sections were rendered transparent using xylene, sealed with neutral gum, and then examined and photographed under a microscope. The images were taken at ×200 magnification.

Fluorescence immunocytochemical staining

The tumor cells were placed on coverslips, fixed in 4% paraformaldehyde for 20 minutes, permeabilized using 0.3% Triton X-100, and blocked with goat serum for an hour. Next, the cells were exposed to primary antibodies specific for MUS81 (11018-1-AP, Proteintech Group; 1:50 dilution) or CDKN2A (10883-1-AP, Proteintech Group; 1:400 dilution) and kept at 4°C overnight. Following this, the cells were treated with HRP mouse anti-rabbit immunoglobulin (IgG; 1:2,000 dilution), stained with DAPI (300 nmol/L) for nuclear visualization, and imaged using a fluorescence microscope. The images were taken at ×400 magnification.

Statistical methods

The Mann-Whitney U-test was employed to evaluate the expression level of MUS81 in breast cancer tissue, while the Student's t-test

was utilized to compare group differences. The statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0, and a *p*-value below 0.05 was deemed statistically significant.

Result

Down-regulation of MUS81 in BC

Our investigation into the expression pattern of MUS81 in BC started with an analysis of mRNA sequencing data of 1085 BC samples from the TCGA database and 291 normal breast samples from the GTEx database using the GEPIA2 platform (http://gepia2.cancer-pku.cn/). The mRNA expression of MUS81 was found to be lower in BC tissues (**Figure 1A**). Subsequently, these findings were confirmed at both the cellular and tissue levels using western blotting and qRT-PCR techniques. The relative expression of MUS81 in BC cells was notably reduced compared to that in MCF-10A cells (**Figure 1B**, **1D**). Furthermore, western blotting and PCR showed lower MUS81 expression in BC tissues than in the adjacent paracancerous tissues (**Figure 1C**, **1E**). These findings indicated that MUS81 is downregulated in BC.

MUS81 knockdown and BC progression

Owing to the down-regulation of MUS81 in BC, we investigated the functional consequences of overexpressing or knocking down MUS81 in BC cell lines exhibiting low or high expression levels.

The reduction of MUS81 in MCF-10A and T47D cells was achieved through stable transfection with lentiviruses carrying RNAi (MUS81 RNAi). Western blotting and PCR confirmed the transfection efficiency (**Figure 2A**, **2B**). Cell proliferation assays using CCK-8 and colony formation demonstrated increased proliferation in MCF-10A and T47D cells with stable MUS81 suppression compared to cells transfected with the negative control (RNAi NC; **Figure 2C**, **2D**). Furthermore, scratch and Transwell assays indicated enhanced migration ability of BC cells upon stable transfection with MUS81 RNAi (**Figure 2E**, **2F**).

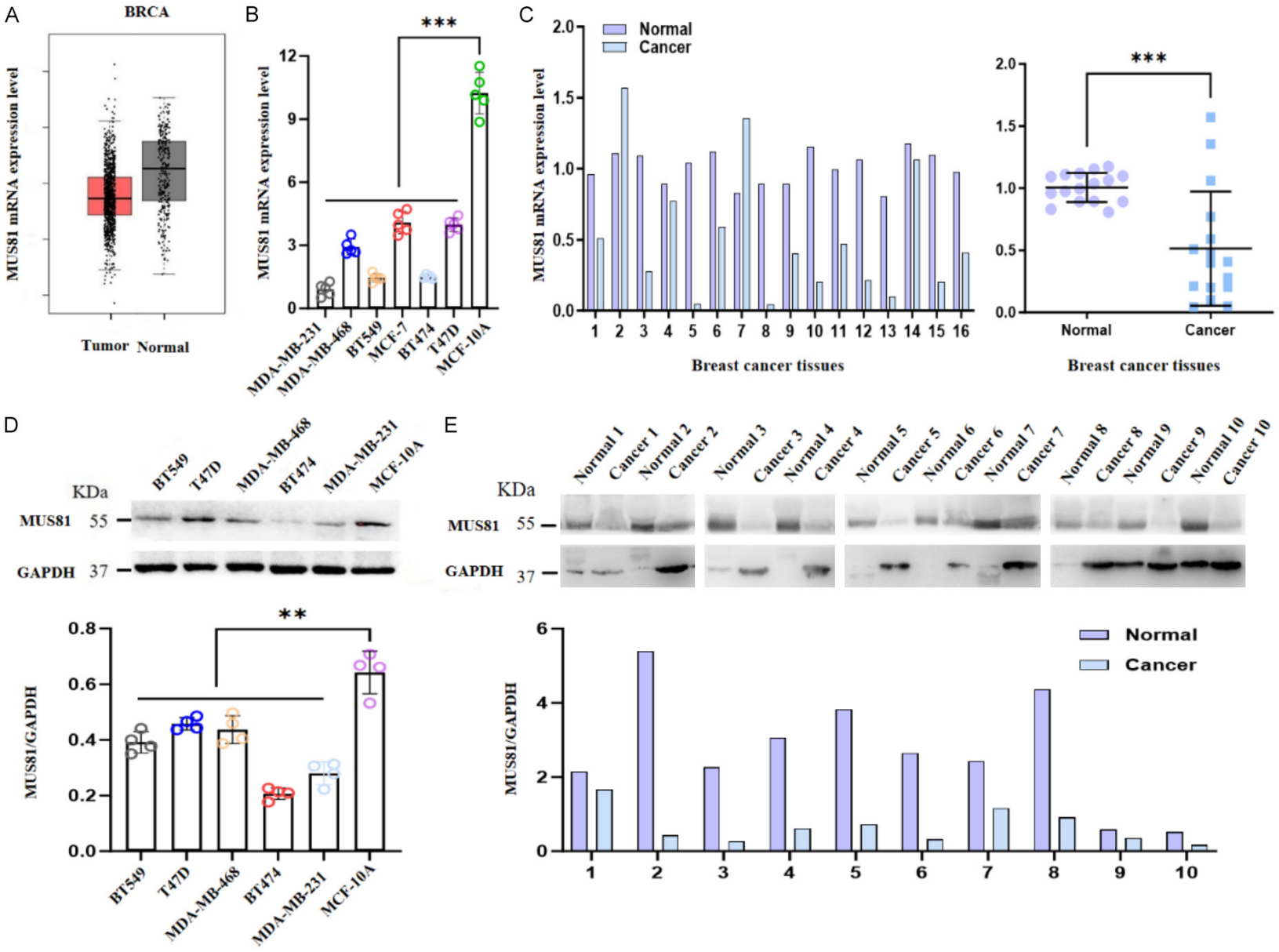
MUS81 overexpression and BC suppression

The overexpression of MUS81 in MDA-MB-231 and BT549 cells was achieved through stable transfection with a lentivirus overexpressing MUS81. Western blotting and PCR confirmed the transfection efficiency (**Figure 3A**, **3B**). CCK-8 and colony formation assays demonstrated that MUS81 overexpression reduced the growth of BC cells compared to cells transfected with the empty vector (Mock; **Figure 3C**, **3D**). The migratory capacity of BC cells transfected with MUS81 was notably lower than that of the control group (**Figure 3E**, **3F**).

Moreover, the subcutaneous xenografts in mice were established through stable overexpression of MUS81. Immunohistochemistry was then conducted to assess the expression of MUS81 and Ki-67 in the xenografts. The findings revealed a notable decrease in Ki-67 expression after MUS81 overexpression (**Figure 3G**). Overall, these findings indicate that MUS81 suppresses the development and aggressiveness of BC cells.

MUS81 promoting the stability and expression of the CDKN2A protein

Transfection with the interference lentivirus (MUS81 RNAi) and lentivirus overexpressing MUS81 (MUS81) resulted in low and high expression levels in T47D and MDA-MB-231 cells, respectively. QRT-PCR results indicated that after the upregulation or downregulation of MUS81, the expression level of CDKN2A mRNA did not change significantly (**Figure 4A**). A significant reduction in the growth of BC cells and tumor weight of their formed subcutaneous xenografts in nude mice was noted upon overexpression of MUS81 (Supplementary Figure 1). Immunohistochemistry results suggested that the expression of $p16^{INK4a}$ significantly increased following the overexpression of MUS81 in subcutaneously implanted tumors (**Figure 4B**). Immunofluorescence analysis was conducted to detect the localization and expression of $p16^{INK4a}$. The findings indicated a significant increase in nuclear CDKN2A expression following MUS81 overexpression (**Figure 4C**). Western blotting showed that with the overexpression or depletion of MUS81, CDKN2A protein ($p16^{INK4a}$) levels increased or decreased accordingly (**Figure 4D**). Western blotting was performed after treatment with actinomycin X (CHX, a protein synthesis inhibitor) or MG-132 (a proteasome inhibitor) in the control group and MUS81 low-expression or overexpression cells. The results showed that the levels of $p16^{INK4a}$ were significantly altered (**Figure 4E**,


A
BRCA
MUS81 mRNA expression level
Tumor
Normal
B
***
MUS81 mRNA expression level
MDA-MB-231
MDA-MB-468
BT549
MCF-7
BT474
T47D
MCF-10A
C
Normal
Cancer
MUS81 mRNA expression level
Breast cancer tissues
***
Normal
Cancer
Breast cancer tissues
D
KDa
BT549
T47D
MDA-MB-468
BT474
MDA-MB-231
MCF-10A
MUS81 55
GAPDH 37
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MUS81/GAPDH
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Normal 1
Cancer 1
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Normal 6
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Normal 7
Cancer 7
Normal 8
Cancer 8
Normal 9
Cancer 9
Normal 10
Cancer 10
MUS81 55
GAPDH 37
Normal
Cancer
MUS81/GAPDH

Figure 1. MUS81 is downregulated in BC. A. GEPIA2 platform predicting the expression of MUS81 in BC and normal controls. B, C. QRT-PCR indicating the expression level of MUS81 mRNA in BC cells, MCF-10A, BC tissues and corresponding paracancerous tissues (n=16). Significance levels are denoted as ***$P < 0.001$. D, E. Western blotting showing the expression level of MUS81 protein in BC cells, MCF-10A, BC tissues and corresponding paracancerous tissues (n=10). Left panel: Representative Western blotting images. Right panel: A bar graph showing the mean data of all subjects analyzed in each group. Significance levels are denoted as **$P < 0.01$.

4F). Images of original western blots were showed in Supplementary Figure 2. These data suggested that MUS81 promotes the stability and expression of $p16^{INK4a}$ by inhibiting proteasome-mediated degradation.

Discussion

MUS81 is a structure-specific DNA endonuclease initially identified by Interthal et al. in 2000 [19]. It has the capability to reinitiate stalled replication forks and resolve HJs through the formation of heterodimers with EME1 or EME2. This enzyme is essential for the repair of DSBs and the preservation of chromosome integrity [20-22].

Although numerous investigations have suggested the potential role of MUS81 in human malignancies [14, 15, 23], its specific function and expression pattern in human BC are largely unexplored. This research aimed to predict the expression pattern of MUS81 in BC using bioinformatics and reveal the low expression level of MUS81 in BC. The reduced expression of MUS81 in BC tissues and cell lines was then confirmed through western blotting and PCR. Thus, we hypothesized that MUS81 exerts an anti-tumor effect in BC. Further data confirmed that MUS81 inhibited the proliferation and migration of BC cells. These findings offer new insights into the anti-tumor effects of MUS81 in BC and suggest the potential for developing therapies to treat this disease. However, the precise mechanism by which MUS81 influences BC progression remains unclear.

Despite evidence indicating a positive role of MUS81 in DNA replication, there is limited understanding of its regulation during S phase. Most known information pertains to negative regulatory mechanisms that prevent the untimely action of MUS81 nucleases on replication intermediates [21]. Accumulating evidence supports the essential role of MUS81 in cell proliferation, with its activation leading to DNA damage [24, 25]. Furthermore, MUS81-dependent DNA damage is modulated by cyclin-dependent kinase 1/2 (CDK1/2), which phosphorylates both MUS81 and its scaffold protein, SLX4. This phosphorylation promotes the formation of the active MUS81-SLX4 complex on chromatin during S phase, suggesting an anti-tumor effect of MUS81 [26, 27]. Consistent with our findings, MUS81 overexpression suppressed the growth and migration of BC cells, an effect that was reversed by MUS81 RNAi. To obtain deeper insight into the mechanisms by which MUS81 inhibits the proliferation of BC cells, cyclin-dependent kinase-related proteins were examined. Our study showed that low expression or overexpression of MUS81 markedly inhibited or promoted the expression of the CDKN2A protein ($p16^{INK4a}$), suggesting a new antitumor mechanism involving the cell cycle.

The $p16^{INK4a}$ protein is one of the tumor suppressor proteins, encoded by the cell cycle dependent kinase inhibitor 2A (CDKN2A) gene [28]. Mitogen-stimulated progression through the G1 phase of the cell cycle and initiation of DNA replication in S phase are coordinated by various cyclin proteins and cyclin-dependent kinases (CDKs), the activities of which are regulated by CDK inhibitors [29]. The $p16^{INK4a}$ protein blocks the cell cycle by inhibiting CDK4/CDK6, which maintains RB in its active, antiproliferative state [30, 31]. The inactivation of $p16^{INK4a}$ by promoter methylation or genetic change is increasingly frequent with advancing stages of various tumors, suggesting that $p16^{INK4a}$ inactivation may promote the progression of cancers, such as breast, lung, colorectal cancers and multiple myeloma [32, 33]. This study showed that with the overexpression or depletion of MUS81, the level of $p16^{INK4a}$ protein increases or decreases accordingly, but there is no significant change in its mRNA level. The result suggested MUS81 could promote the expression of $p16^{INK4a}$ protein by inhibiting its degradation during the post-translational modification of CDKN2A. We speculate that MUS81 may affect the progression of BC by regulating the expression of $p16^{INK4a}$ protein.

Conclusions

In conclusion, our research indicates that MUS81 is down-regulated in human BC, and its decreased expression correlates with the

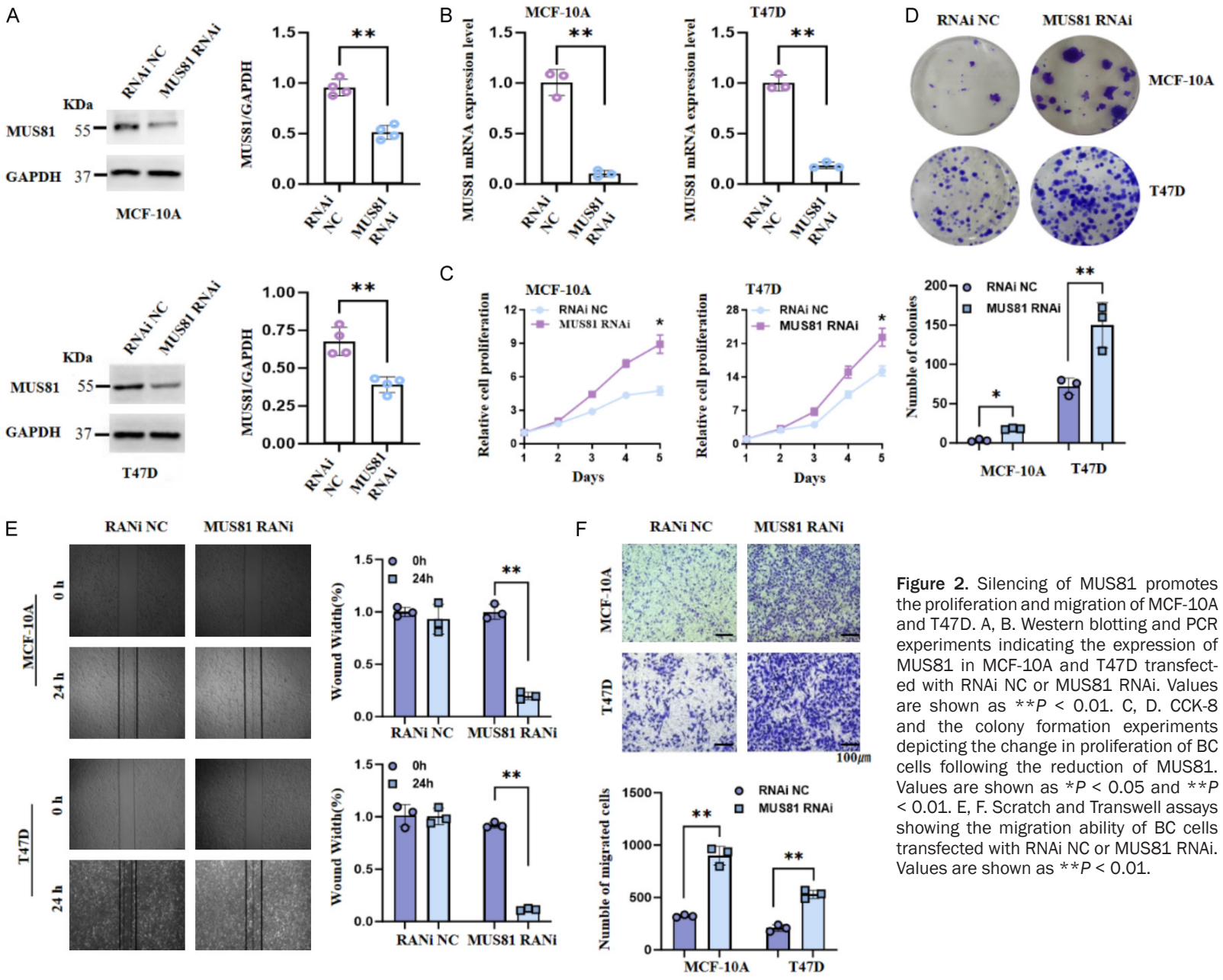

Figure 2. Silencing of MUS81 promotes the proliferation and migration of MCF-10A and T47D. A, B. Western blotting and PCR experiments indicating the expression of MUS81 in MCF-10A and T47D transfected with RNAi NC or MUS81 RNAi. Values are shown as $^{**}P < 0.01$. C, D. CCK-8 and the colony formation experiments depicting the change in proliferation of BC cells following the reduction of MUS81. Values are shown as $^{*}P < 0.05$ and $^{**}P < 0.01$. E, F. Scratch and Transwell assays showing the migration ability of BC cells transfected with RNAi NC or MUS81 RNAi. Values are shown as $^{**}P < 0.01$.

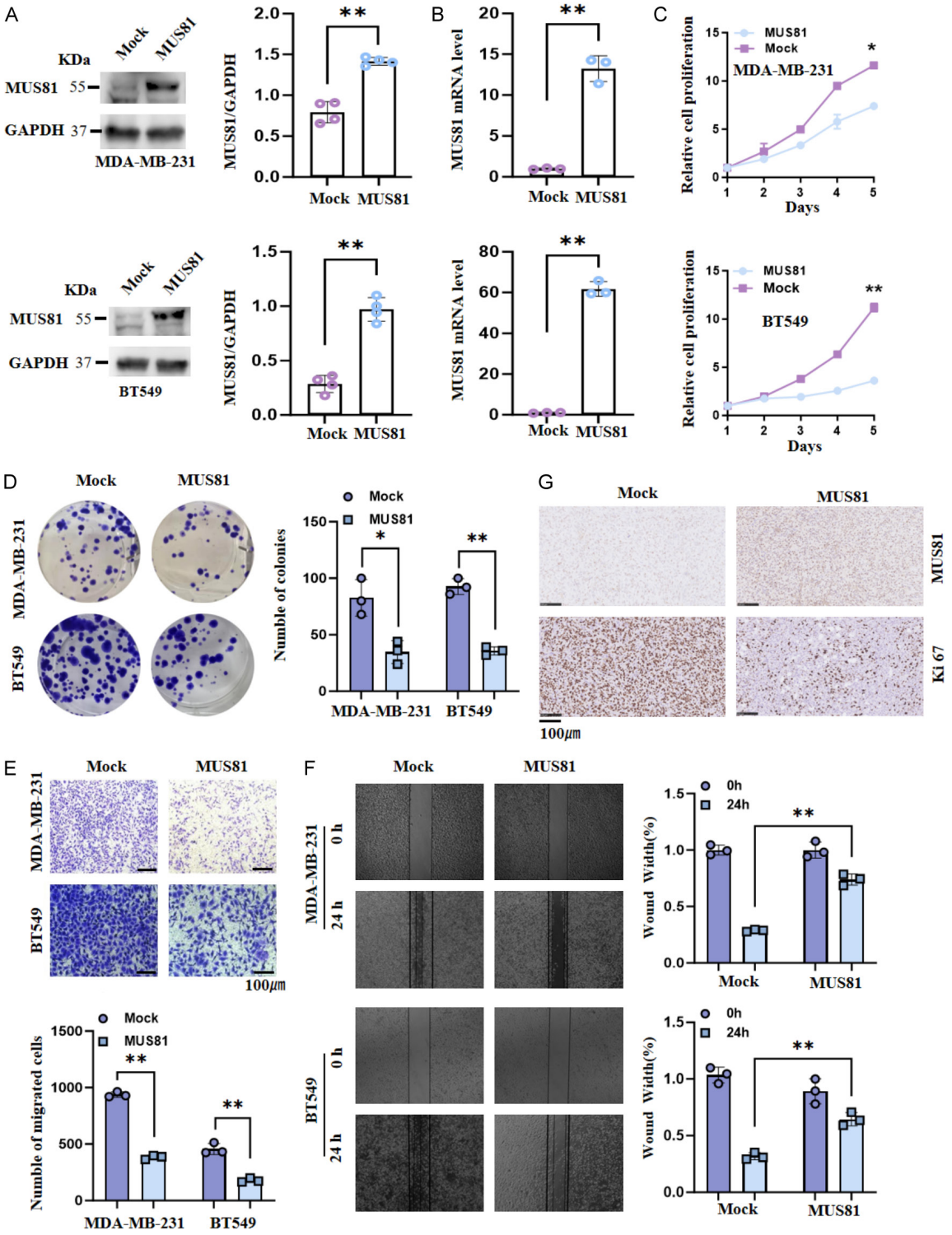

Figure 3. Overexpressing MUS81 represses the proliferation and migration of MDA-MB-231 and BT549. A, B. Western blotting and PCR experiments indicating the expression of MUS81 in MDA-MB-231 and BT549 transfected with Mock or MUS81. Significance levels are denoted as $^{**}P < 0.01$. C, D. CCK-8 and the colony formation experiments depicting the change in proliferation of BC cells following the overexpression of MUS81. Significance levels are denoted as $^{*}P < 0.05$ and $^{**}P < 0.01$. E, F. Scratch and Transwell experiments showing the migration ability of BC cells transfected with Mock or MUS81. Significance levels are denoted as $^{**}P < 0.01$. G. Immunohistochemistry staining of subcutaneous xenografts showing the expression of MUS81 and Ki 67.

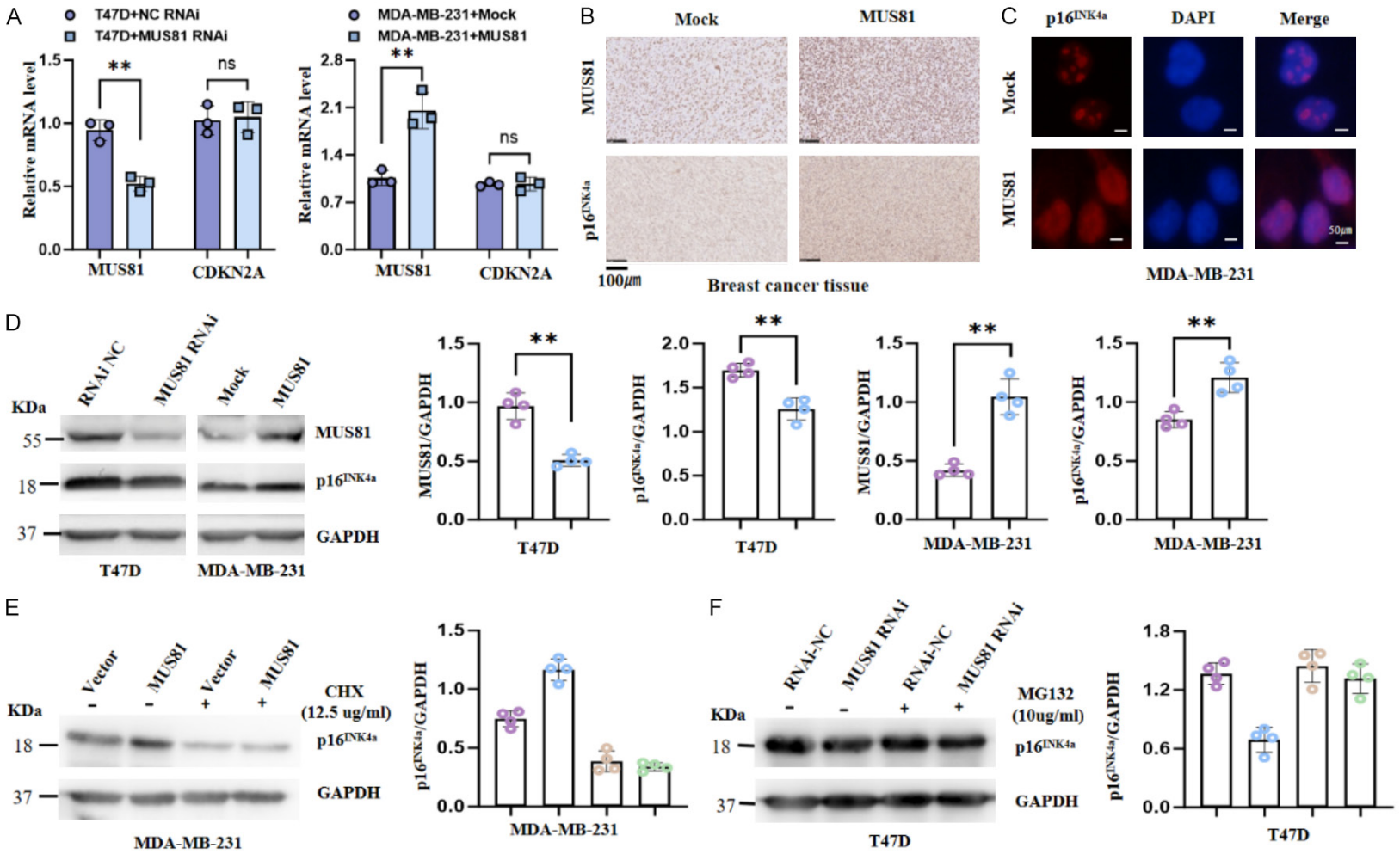

Figure 4. Low-expression or overexpression MUS81 inhibits or promotes the expression of CDKN2A protein ($p16^{INK4a}$). A. QRT-PCR indicating the expression of MUS81 and CDKN2A in T47D and MDA-MB-231 transfected with negative control or MUS81 RNAi or MUS81. Data are expressed as n.s.: no significance, $^{**}P < 0.01$. B. Immunohistochemical staining of xenograft tumor showing the expression of MUS81 and $p16^{INK4a}$. C. Immunofluorescent cytochemistry indicating the expression of $p16^{INK4a}$ in MDA-MB-231 transfected with mock or MUS81. D. Western blotting displaying the expression of MUS81 and $p16^{INK4a}$ in T47D and MDA-MB-231 transfected with negative control or MUS81 RNAi or MUS81. Data are expressed as n.s.: no significance, $^{**}P < 0.01$. E, F. Western Blotting showing the protein level of CDKN2A after CHX (12.5 ug/ml) or MG-132 (10 ug/ml) treatment in the control group and MUS81 low-expression or overexpression cells.

development and aggressiveness of BC. It is still uncertain if MUS81 is linked to the prognosis of BC patients, and the molecular mechanism behind its anti-tumor effect in BC has not been identified. Although we demonstrated that MUS81 protects $p16^{INK4a}$ from proteasome-mediated degradation, we did not clearly elucidate the molecular pathway or interaction through which this regulation occurs.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Yanhong Yan, Department of Physical Examination, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, Hubei, China. Tel: +86-15072401566; E-mail: 79325172@qq.com

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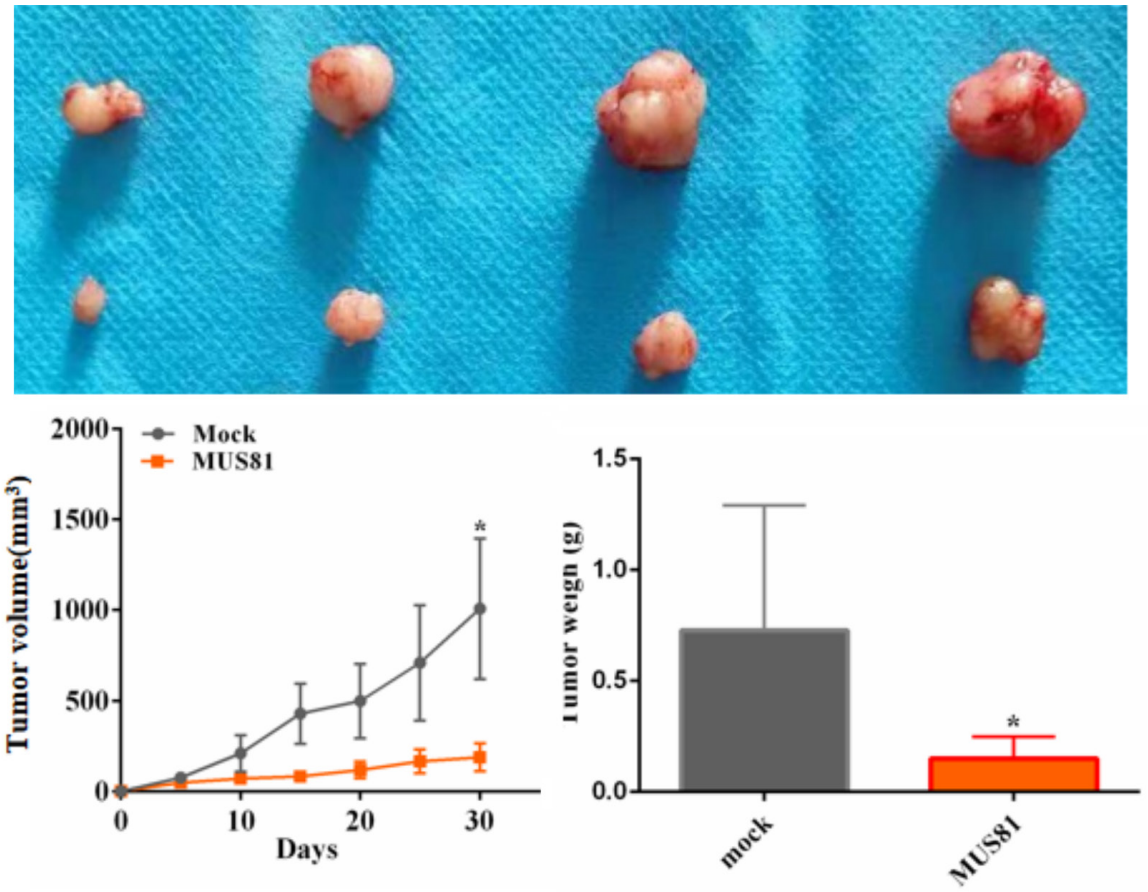
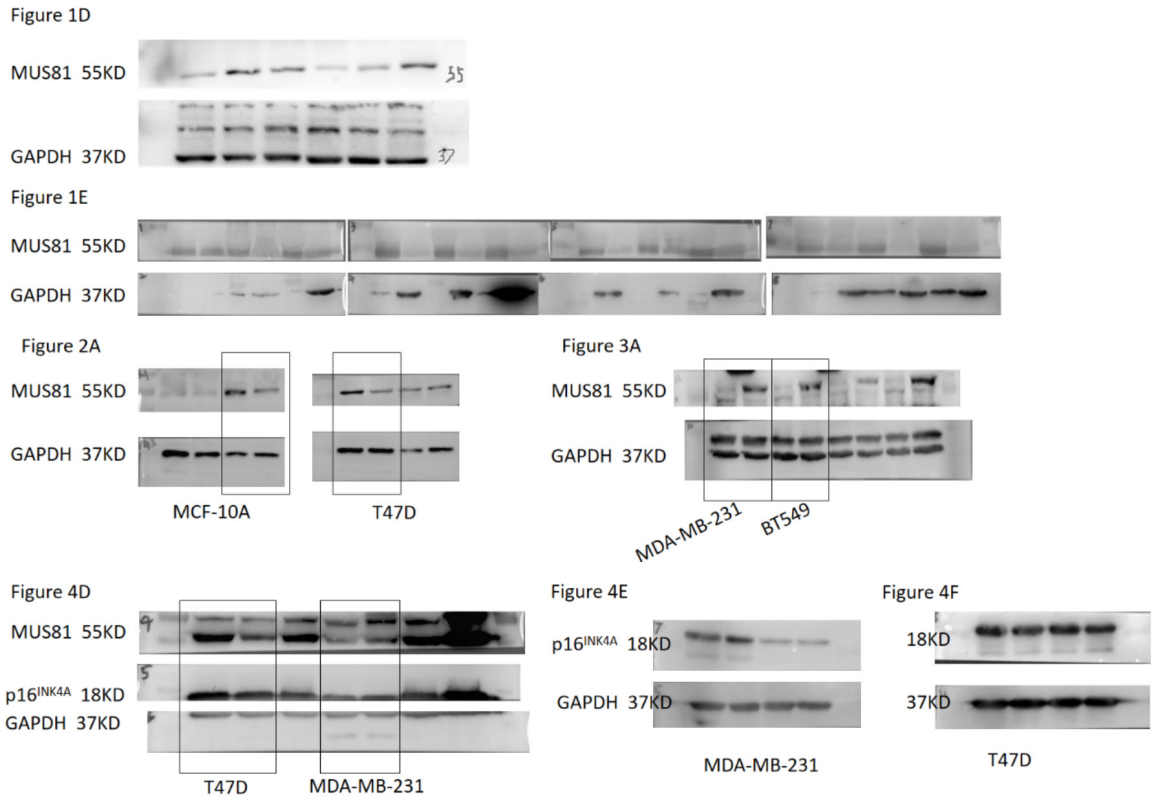


Supplementary Figure 1. Representative images (upper panel), in vivo growth curves (lower left panel), and weight at the end points (lower right panel) of xenografts formed by subcutaneous injection of MDA-MB-231 cells stably transfected with mock, MUS81 into the left axilla of mice.



Supplementary Figure 2. Images of the original western blots.