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Original Article

IGF2BP2-modified UBE2D1 interacts with Smad2/3 to promote the progression of breast cancer

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Abstract: Recent studies have suggested that ubiquitin-conjugating enzyme E2D1 (UBE2D1) is involved in tumor progression. In this study, we found that UBE2D1 expression was upregulated in breast cancer (BC) and was related to the prognosis of BC patients. Through in vitro and in vivo experiments, we demonstrated the aberrant expression of UBE2D1 promoted the proliferation and migration of BC cells, and the IGF2BP2-mediated N6-methyladenosine (m6A) modification increased the stability of UBE2D1 mRNA. Mechanistically, UBE2D1 expression regulated the activity of TGF-β signaling through modulating the expression and the phosphorylation level of Smad2/3. Furthermore, UBE2D1 directly bound to Smad2/3 and affected the subsequent binding of Smad2 and Smad3, which is a necessary step for TGF-β signaling activation. Thus, our study reveals a pro-oncogenic role of UBE2D1 in the progression of BC and may provide novel strategies for BC treatment.

Keywords: UBE2D1, IGF2BP2, Smad2/3, breast cancer, tumor progression

Introduction

Breast cancer (BC) has been a life-threatening problem to women's health worldwide, accounting for nearly one-third of the new cancer cases among women in the United States in 2023 [1]. Although with the better understanding of BC, the early diagnosis of BC has been improved, and a variety of treatment options, including surgery, endocrine therapy, chemotherapy, radiotherapy, targeted therapy and immunotherapy have been developed [2], the prognosis remains unsatisfactory, especially for triple-negative breast cancers and metastatic breast cancers [3-5]. Due to the high complexity and heterogeneity of BC, more therapeutic targets need to be identified. Recent studies have shown that the ubiquitin-conjugating enzyme E2D (UBE2D) family, which consists of four highly conserved discrete genes, UBE2D1-4, plays an important role in tumorigenesis [6, 7]. Particularly, some reports have suggested that UBE2D1 is involved in several irreplaceable carcinogenic pathways in tumors and that UBE2D1 promotes tumor growth by regulating p53 ubiquitination and degradation [8-10]. In addition, it has been reported that silencing UBE2D1 reduces SMAD4 ubiquitination and inhibits gastric cancer cell migration through the TGF-β/SMAD4 pathway [11]. Another study shows that UBE2D1 is involved in Cisplatin resistance in gastric cancer [12]. Furthermore, the persistence of the RNF128 Iso1-UBE2D1 complex is conducive to the stability of mutant p53, promoting the survival of Barrett's esophagus cells [9]. In addition, the upregulation of UBE2D1 is associated with the poor prognosis in patients with lung adenocarcinoma or bladder cancer [13, 14]. However, the role of UBE2D1 in BC development is unclear, which prompted us to carry out the current study.

Transforming growth factor β (TGF-β) is a key regulator of BC progression and metastasis by

Table 1. shRNA sequences used in the experiments

Gene name	Sequence (5'-3')
sh1-IGF2BP2	TTCCCGCATCATCACTCTTAT
sh2-IGF2BP2	AGTGAAGCTGGAAGCGCATAT
sh1-mettl14	CCATGTACTTACAAGCCGATA
sh2-mettl14	GCTAATGTTGACATTGACTTA
sh-SMAD2	GCAGAACTATCTCCTACTACT
sh-SMAD3	GGCTGCTCTCCAATGTCAACA
sh1-UBE2D1	CTTCTTTCTCACTGTACATTT
sh2-UBE2D1	AGTACCAGATATTGCACAAAT
sh1-IGF2BP1	CTCCGCTTGTAAGATGATCTT
sh2-IGF2BP1	GCAGTGGTGAATGTCACCTAT
sh1-IGF2BP3	GCCTCCTCCTAAGACTTAGGA
sh2-IGF2BP3	CGGTGAATGAACTTCAGAATT

Table 2. qPCR primers used in the experiments

Gene name	Sequence (5'-3')
IGF2BP2-F	CGAGGGCACTTGTGAGAAGCG
IGF2BP2-R	TGTTCATGGTGCTGTCCACGTG
Mettl14-F	CTGAAAGTGCCGACAGCATTGG
Mettl14-R	CTCTCCTTCATCCAGATACTTACG
Smad2-F	GGGTTTTGAAGCCGTCTATCAGC
Smad2-F	CCAACCACTGTAGAGGTCCATTC
Smad3-F	TGAGGCTGTCTACCAGTTGACC
Smad3-R	GTGAGGACCTTGTCAAGCCACT
UBE2D1-F	ATCAAGGTGGAGTCTTCTTTCTC
UBE2D1-R	CCATTACTGTTTATGTTTGGATGGT
GAPDH-F	GGTGAAGGTCGGAGTCAACG
GAPDH-R	CCATGTAGTTGAGGTCAATGAAG

regulating a variety of cellular functions such as cell invasion, migration and epithelial-mesenchymal transition (EMT) [15, 16]. Notably, Smad2/3 is the central mediator of TGF-β signaling pathway. In canonical TGF-β signaling, activated TGF-β type I receptor (TGF-βR1) phosphorylates Smad2/3, which then binds to SMAD4 to form transcription complexes and translocate into the nucleus where they regulate the expression of target genes [17]. Studies have shown that the Nodal signaling-activated TGF-β/Smad2/3 pathway regulates the stem-like properties of BC cells [18]. Recent evidence also suggests that the TGF-β/Smad2/3 pathway controls the EMT, metastasis, and survival of BC cells [18, 19].

In this study, we revealed that UBE2D1 was significantly upregulated in BC tissues and promoted the proliferation and invasion of BC cells in vivo and in vitro. In addition, the stability of UBE2D1 mRNA was enhanced upon its binding to the N6-methyladenosine (m6A) reader IGF2BP2. Furthermore, UBE2D1 promoted the progression of BC through the TGF-β/Smad2/3 pathway by interacting with Smad2/3. Hence, our results suggest that UBE2D1 may be a potential therapeutic target for the treatment of patients with BC.

Materials and methods

Cell culture and stable cell line construction

MCF-10A, MCF-7, MDA-MB-231, and 293T cells were maintained in high-glucose DMEM containing 10% FBS (Wisent, Canada) and 1% penicillin and streptomycin (P/S). To generate stable cell lines, lentivirus expressing the indicated gene was produced in 293T cells, and the virus-containing medium was collected to infect the target cells. After infection for 6-8 h, cells were continuously cultured in fresh medium for 48 h culture before puromycin treatment for two weeks to select the cells that stably expressing the indicate gene, which was further confirmed by RNA and protein detection.

Plasmid construction

Construction of shRNA-expressing plasmids: The primers of specific shRNA were designed, synthesized, and cloned into plko.l-puro vector. All plasmids were confirmed by sequencing. The shRNA sequences were shown in **Table 1**.

Construction of overexpression plasmids: The cDNA of indicated gene was cloned into mammalian expression vector PLVX-3Flag gene overexpression experiments. All constructs were confirmed by sequencing.

RNA extraction and qPCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, America) following the manufacturer's instructions. After quantification, total RNA was reverse transcribed into cDNA (Vazyme, China) followed by qPCR reaction using SYBR GREEN (Vazyme, China) was prepared for use in a qPCR system. The primers used for qPCR were shown in **Table 2**.

CCK8 assay

Cell proliferation was measured by CCK-8 assay. Briefly, 3000 cells per well were seeded into 96-well plate and cultured for 24 h, 48 h, 72 h, and 96 h. CCK-8 reagent (Zomanbio, China) was then added to each well at the indicated time point. After 1-2 h incubation, the light absorbance value of the wells at 450 nm was measured.

Colony formation assay

Briefly, 1000 cells per well were seeded into six-well plate in triplicate and cultured for approximately 2 weeks. Then, the cells were fixed and stained with crystal violet solution. The number of cell clones was counted under microscope.

Wound healing assay

Briefly, cells at a density of 90-100% confluence in the six-well plate were scratched using a pipette tip. The closure of the scratch was observed under microscope and measured at 0 h, 24 h, and 48 h.

Transwell assay

Briefly, 10000 cells in serum free medium were added to the upper chamber of transwell, while high-glucose DMEM medium containing 20% FBS was added to the lower well. Cells were cultured for approximately 16 h, and the migrated cells to the lower side of the membrane were fixed, stained with crystal violet solution, and counted under microscope.

Xenograft tumor model in nude mice

BALB/c-nu nude female mice aged 4-6 weeks were used in our in vivo study. To generate xenograft tumor, 1-4 × 10^6 stable cells/100 ul PBS was inoculated subcutaneously into the flank area in the middle of the posterior axillary in the nude mice. The tumor growth was monitored for 1-2 weeks. At the end of the experiment, the tumors were dissected, weighed and measured, and then fixed with formalin for further analysis.

Nucleoplasm separation of protein assay

Briefly, cell pellet was lysed in 200 μl of cytoplasmic protein extraction reagent A with PMSF (PUMOKE, China) at 4°C. Then, Reagent B (Beyotime, China) was added to the lysate followed by vortex and centrifugation. The supernatant was immediately removed, and the pellet was resuspended with 50 μl of nuclear protein extraction reagent supplemented with PMSF followed by vortex and centrifugation. The supernatant was collected as the nuclear protein faction.

Western blot

Briefly, cells were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors. After protein quantification, the protein samples were separated by SDS, transferred onto PVDF membrane. Then the membrane was incubated with primary antibody against the target protein at 4°C overnight, followed by incubation with corresponding secondary antibody at room temperature. After extensive washing, the protein bands were visualized with ECL (Axygen, America). The primary antibodies used in this study were: anti-METTL14, IGF2BP2, Smad2, and Smad3 (Proteintech, China).

Co-immunoprecipitation (Co-IP)

Co-IP was performed to identify interacting proteins. Briefly, cell pellets were collected and lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors. A 30 μl aliquot of cell lysate was saved as input in western blotting, while the rest of the cell lysate was subjected to IP with indicated primary antibody, and the immune complex was precipitated with magnetic beads and analyzed by western blotting.

RNA-binding protein immunoprecipitation (RIP)-qPCR

RIP-qPCR is used to study RNA-binding to target proteins in cells. Briefly, the binding of IGF2BP2 (Proteintech, China) to myc mRNA was analyzed by using IGF2BP2-specific antibody to precipitate RNA-protein complex. The bound RNA was reverse transcribed and quantified qPCR (BersinBio, China).

Methylated RNA immunoprecipitation (meRIP)-qPCR

meRIP was applied to determine N6-methyladenosine (m6A) modification of UBE2D1 RNA.

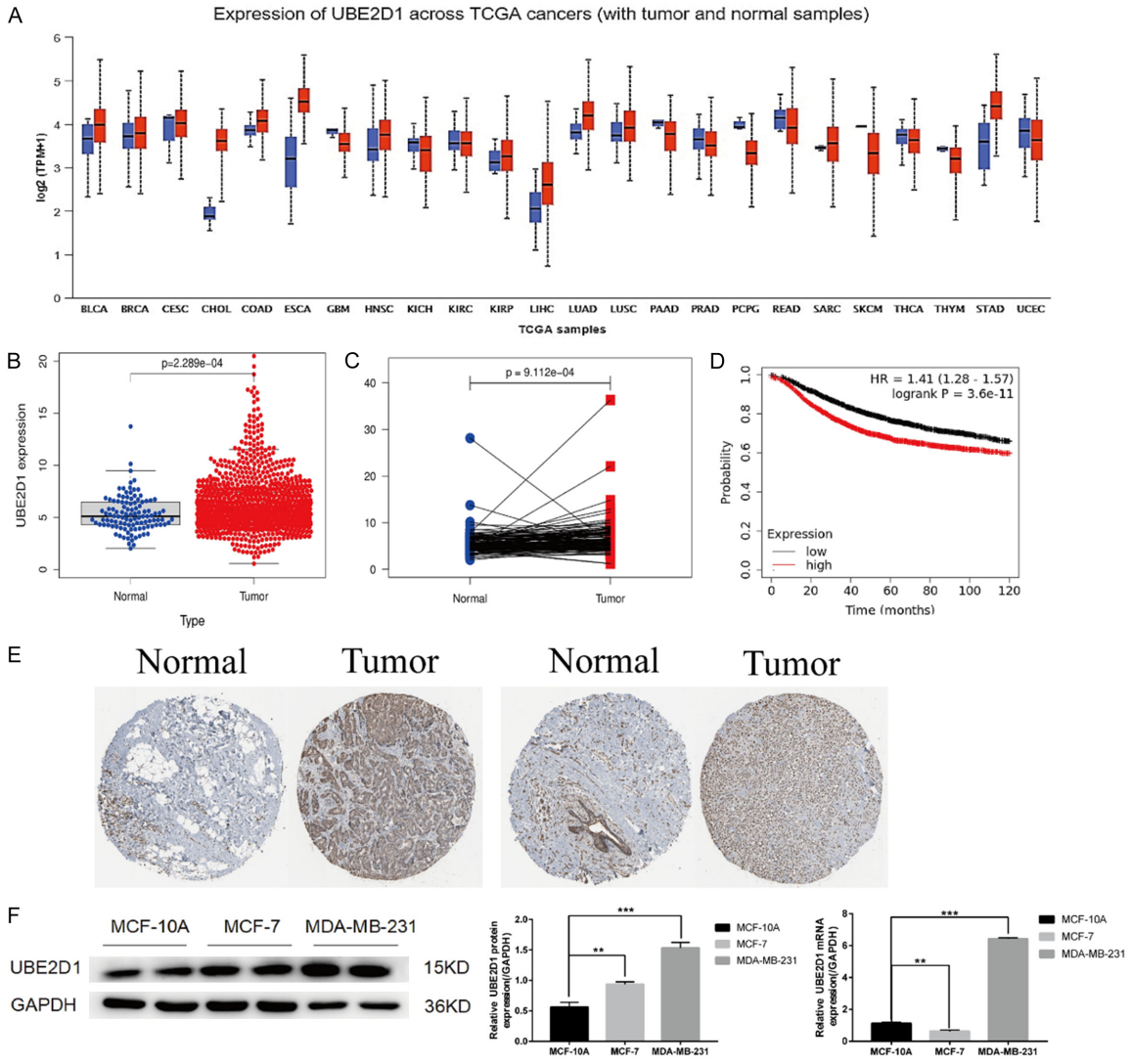


Figure 1. UBE2D1 is highly expressed in breast cancer cells and associated with poor prognosis. A. UBE2D1 expression in pancarcinoma; B, C. UBE2D1 expression in BC samples from the TCGA database; D. Prognostic model based on UBE2D1 expression in BC; E. UBE2D1 expression in BC tissues; F. UBE2D1 expression in BC cell lines as determined by western blot.

Briefly, Co-IP was used to incubate random RNA fragments with anti-methylated RNA (m6A) antibodies (ABclonal, America) to obtain RNA methylation-modified fragments. Then, qPCR is used to directly quantify the enriched RNA (BersinBio, China).

Statistical analysis

Data were analyzed with GraphPad Prism 7 software, and each experiment was repeated three times. Data were expressed as the mean ± standard deviation. Difference between two groups was compared using t-test, and a difference was considered statistically significant when $P < 0.05$.

Results

UBE2D1 expression was upregulated in BC cells and was associated with poor prognosis

To determine the role of UBE2D1 in BC, we analyzed UBE2D1 expression levels in a wide variety of tumors using TCGA data (UALCAN) and found a significant upregulation of UBE2D1 in various types of tumors, including BC (**Figure 1A**). In addition, we downloaded BC data from TCGA, and an R language analysis showed that UBE2D1 was highly expressed in BC tissues (**Figure 1B**, **1C**). Kaplan-Meier plotter demonstrated that patients with high UBE2D1 expression had a significantly shorter overall survival

(OS) than those with low UBE2D1 expression (**Figure 1D**). Furthermore, the immunohistochemistry (IHC) staining data on Human Protein Atlas also showed that UBE2D1 was highly expressed in BC tissues (**Figure 1E**). Importantly, we experimentally measured the protein level of UBE2D1 in BC cells by Western blotting and found that UBE2D1 level was significantly higher in BC cells than in normal MCF-10A BC cells (**Figure 1F**). Together, these results indicate that UBE2D1 expression is upregulated in BC and is negatively correlated with the prognosis of BC patients.

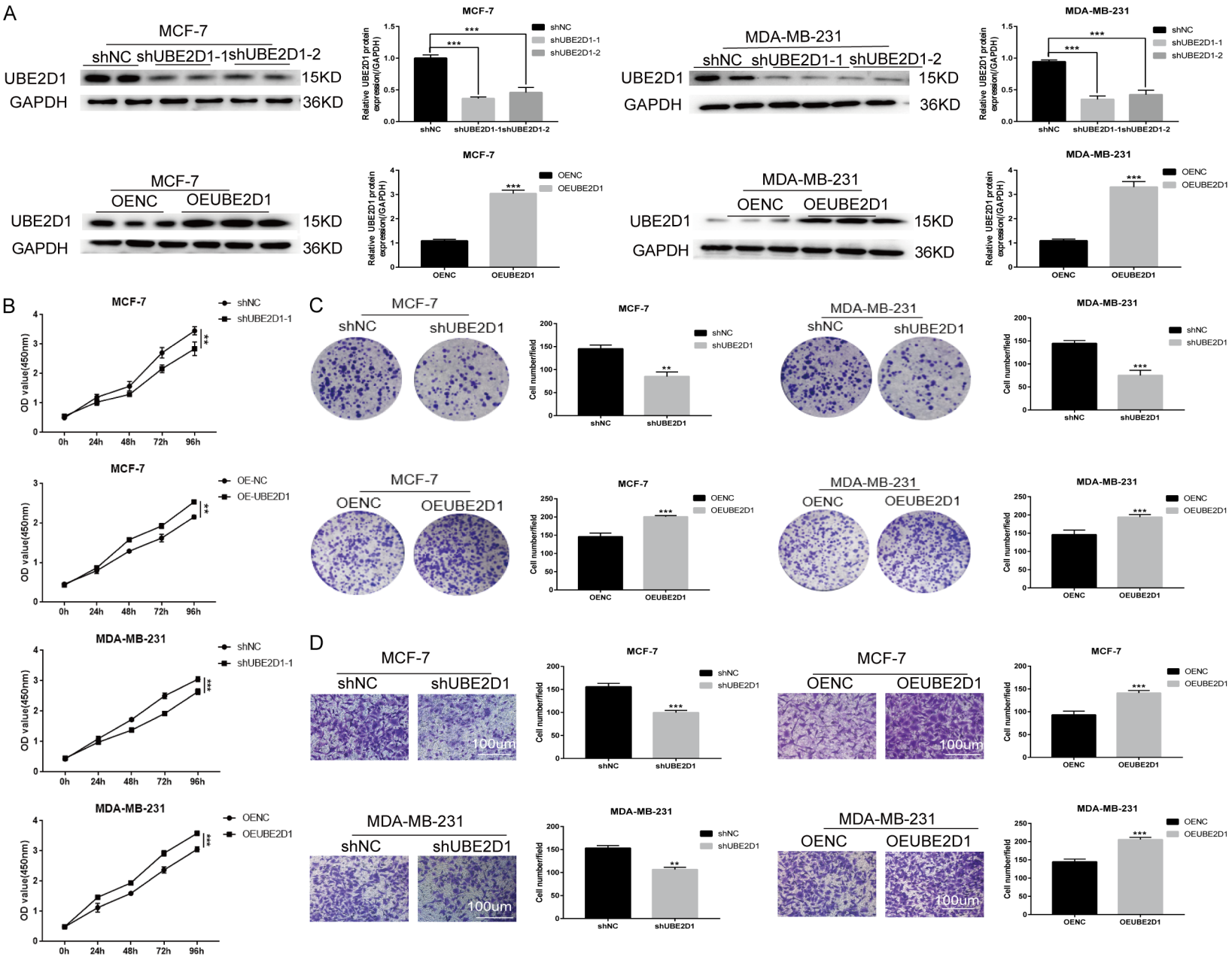
UBE2D1 promoted BC cell proliferation and invasion

To further explore the biological function of UBE2D1, vectors with UBE2D1 knocked down or overexpressed were constructed and we expressed UBE2D1 knockdown or UBE2D1 overexpression plasmid in MCF-7 and MDA-MB-231 cells, and the successful knockdown or overexpression was confirmed by western blotting (**Figure 2A**). CCK-8 assay showed that knocking down UBE2D1 inhibited, while UBE2D1 overexpression promoted cell proliferation (**Figure 2B**). Consistently, colony formation experiments showed that knocking down UBE2D1 suppressed, whereas UBE2D1 overexpression enhanced the colony formation of BC cells (**Figure 2C**). Furthermore, knocking down UBE2D1 inhibited, while overexpression of UBE2D1 promoted the migration of these cells, as determined by transwell assay (**Figure 2D**). We also used Edu staining (50 μM for 3 h) to confirm that UBE2D1 knockdown decreased the proliferation of MCF-7 and MDA-MB-231 cells (**Figure 2E**). Finally, we verified the effect of UBE2D1 on xenograft tumor growth in vivo. MDA-MB-231 cells stably expressing shUBE2D1 were subcutaneously injected into nude mice, and the results showed that knocking down UBE2D1 significantly inhibited tumor growth, as indicated by the smaller tumor size (**Figure 2F**). Collectively, these results suggest that UBE2D1 may act as an oncogene to promote the growth and migration of BC cells.

m6A reader protein IGF2BP2 bound to and stabilized UBE2D1 mRNA in BC cells

Given our data above that UBE2D1 was aberrantly upregulated in BC and promoted BC cell proliferation and migration, we first explored the mechanism underlying high UBE2D1 expression in BC. Posttranscriptional modification has been recognized as an important regulatory step in normal and pathological processes. Notably, the m6A moiety is the most abundant mRNA modification, and mRNA methylation is regulated mainly by m6A readers. As one of the m6A readers, IGF2BPs recognizes and binds to the conserved GG (m6A) C sequence in mRNA, which increases the stability of mRNA. Therefore, we searched UBE2D1 RNA-binding proteins using the CLIP-seq data of starBase (http://starbase.sysu.edu.cn/starbase2/index) and identified a significant positive correlation between IGF2BPs and UBE2D1, suggesting IGF2BPs as UBE2D1 mRNA-binding proteins (**Figures 3A**, S1A, S1B). Subsequently, we examined the mRNA and protein levels of UBE2D1 in cells with IGF2BP1, IGF2BP2, or IGF2BP3 knockdown by qPCR and Western blot and observed a significant change with IGF2BP2 knockdown (Figure S1C, S1D). Therefore, we focused our study on IGF2BP2.

First, we generated stable BC cells with either IGF2BP2 knockdown or overexpression and observed that UBE2D1 protein level was significantly reduced in IGF2BP2 knockdown cells but was significantly increased in IGF2BP2-overexpressing cells (**Figure 3B**, **3C**). In addition, we also generated stable BC cells with METTL3 and METTL14 knockdown or METTL3 and METTL14 overexpression and found that METTL3 and METTL14 knockdown decreased, while METTL3 and METTL14 overexpression increase UBE2D1 protein level (**Figures 3D**, **3E**, S1F). Notably, mutation at the catalytic domain of METTL3 lost this effect, suggesting that the m6A activity was required for METTL3 regulation of UBE2D1 (Figure S1E). Furthermore, we predicted the m6A binding site on UBE2D1 through the SRAMP website (**Figure 3F**), which was confirmed by MeRIP experiment showing that UBE2D1 could indeed bind to the catalytic active site of m6A (**Figure 3G**). To determine how IGF2BP2 regulated UBE2D1 level, we employed RIP experiments to explore the relationship between IGF2BP2 and m6A modification. The results demonstrated that IGF2BP2 bound to UBE2D1 mRNA (**Figure 3H**). In addition, using actinomyces D treatment, we found that the degradation of UBE2D1 mRNA was increased by IGF2BP2 knockdown but was decreased by IGF2BP2 overexpression (**Figure**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MCF-7
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UBE2D1
GAPDH
15KD
36KD
MCF-7
shNC
shUBE2D1-1
shUBE2D1-2
Relative UBE2D1 protein expression/(GAPDH)
MDA-MB-231
shNC shUBE2D1-1 shUBE2D1-2
MDA-MB-231
MCF-7
OENC OEUBE2D1
OENC
OEUBE2D1
MDA-MB-231
OENC OEUBE2D1
B
MCF-7
shNC
shUBE2D1-1
OD value(450nm)
0h 24h 48h 72h 96h
MCF-7
OE-NC
OE-UBE2D1
MDA-MB-231
shNC
shUBE2D1-1
MDA-MB-231
OENC
OEUBE2D1
C
MCF-7
shNC shUBE2D1
Cell number/field
MDA-MB-231
shNC shUBE2D1
MCF-7
OENC OEUBE2D1
MDA-MB-231
OENC OEUBE2D1
D
MCF-7
shNC shUBE2D1
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MDA-MB-231
OENC OEUBE2D1

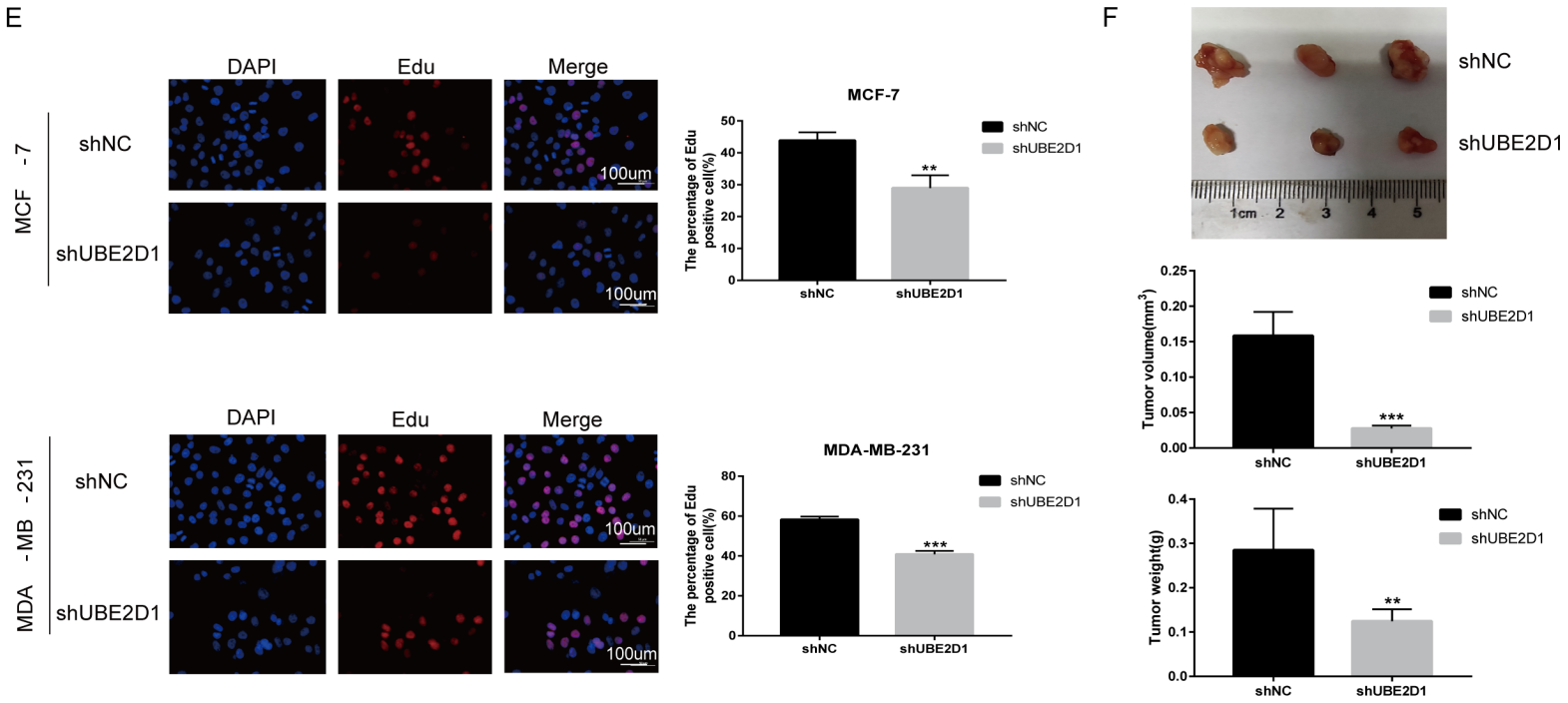
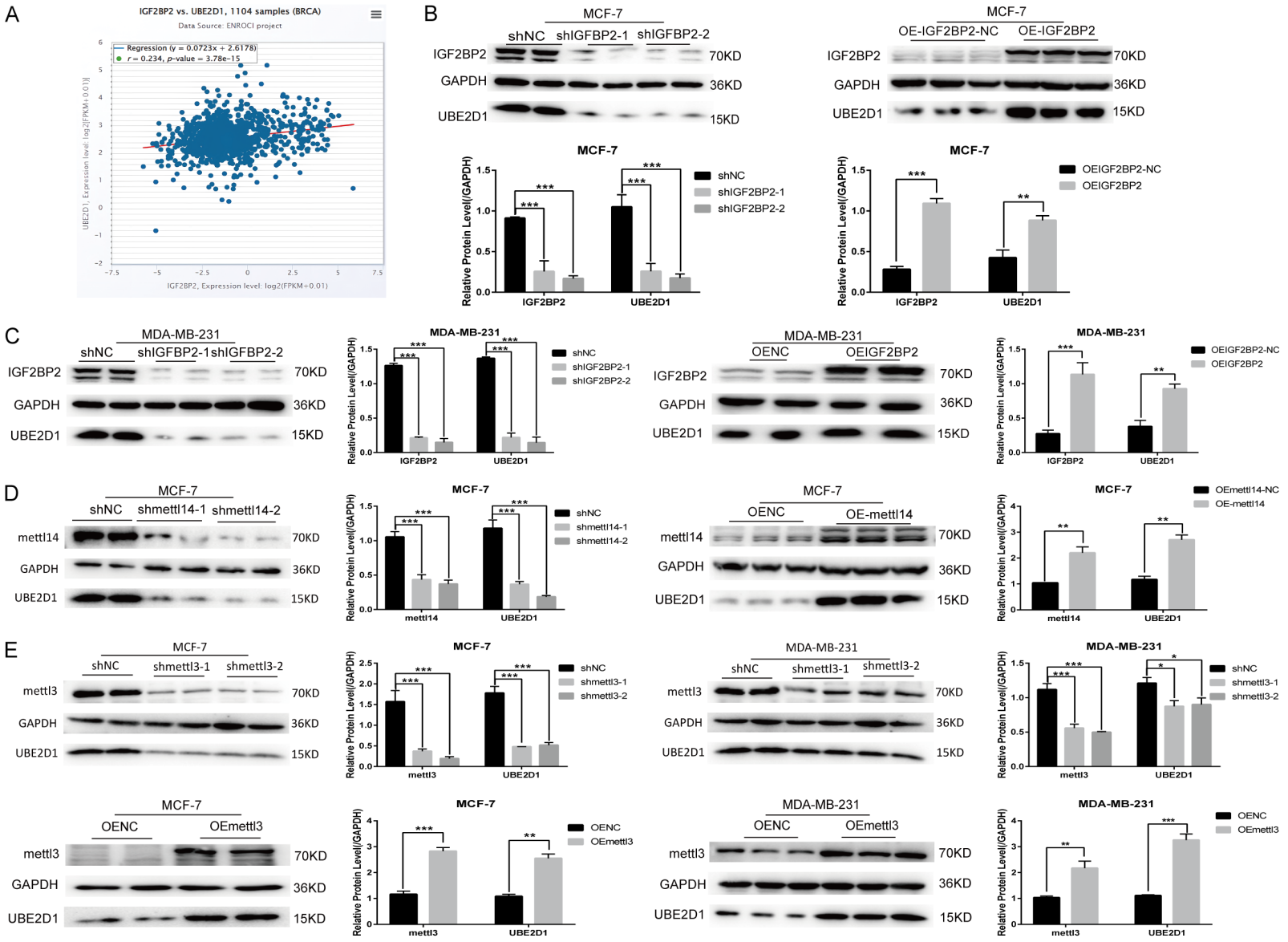


Figure 2. UBE2D1 promoted the proliferation and migration of BC cells. A. The successful knockdown or overexpression of UBE2D1 was determined by western blotting. B. The proliferation of UBE2D1-overexpressing cells was determined by CCK-8 assay. C. Colony formation assay of BC cells with UBE2D1 knockdown or overexpression. D. Transwell assays of BC cells with UBE2D1 knockdown or overexpression. E. The proliferation of cells with UBE2D1 knockdown or overexpression was measured by EdU assay. F. The growth of UBE2D1 knockdown-cell-derived tumor in nude mice.


A
IGF2BP2 vs. UBE2D1, 1104 samples (BRCA)
Data Source: ENROCI project
Regression (y = 0.0723x + 2.6178)
r = 0.234, p-value = 3.78e-15
UBE2D1, Expression level: log2(FPKM+0.01)
IGF2BP2, Expression level: log2(FPKM+0.01)
B
MCF-7
shNC shIGFBP2-1 shIGFBP2-2
IGF2BP2 70KD
GAPDH 36KD
UBE2D1 15KD
MCF-7
OE-IGF2BP2-NC OE-IGF2BP2
IGF2BP2 70KD
GAPDH 36KD
UBE2D1 15KD
MCF-7
Relative Protein Level/(GAPDH)
shNC
shIGF2BP2-1
shIGF2BP2-2
IGF2BP2
UBE2D1
MCF-7
OEIGF2BP2-NC
OEIGF2BP2
C
MDA-MB-231
shNC shIGFBP2-1 shIGFBP2-2
IGF2BP2 70KD
GAPDH 36KD
UBE2D1 15KD
MDA-MB-231
OENC OEIGF2BP2
IGF2BP2 70KD
GAPDH 36KD
UBE2D1 15KD
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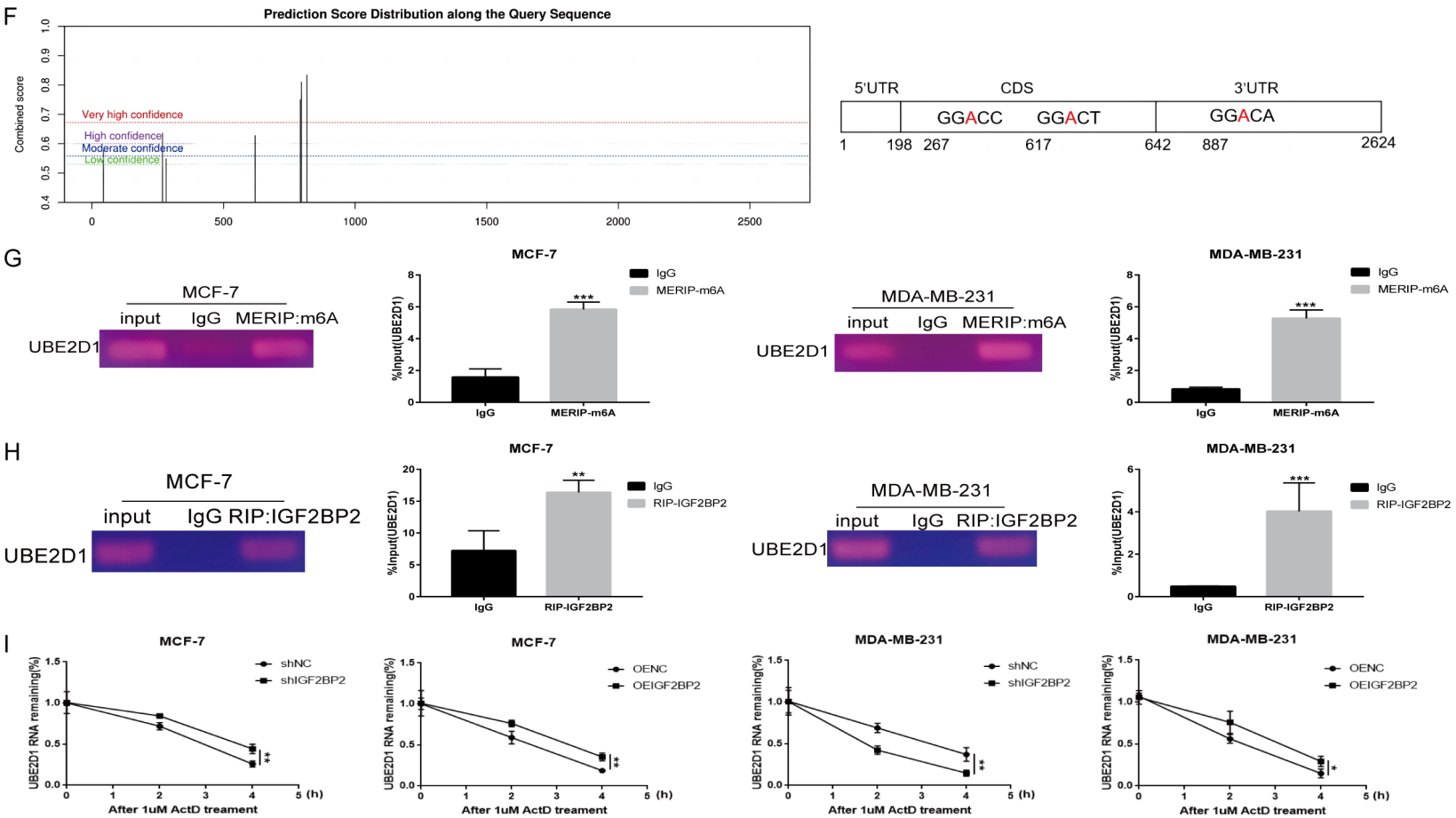


Figure 3. m6A reader protein IGF2BP2 bound to and stabilized UBE2D1 mRNA in BC cells. A. Correlation between UBE2D1 and IGF2BP2; B, C. After IGF2BP2 was knocked down and overexpressed, the expression of Western blot analysis of UBE2D1 in cells with IGF2BP2 knockdown or overexpression; D, E. Western blot analysis of UBE2D1 in cells with METTL3/4 knocked down or overexpression; F. The distribution of m6A modification sites on UBE2D1 and the sites with high confidence on UBE2D1 mRNA; G. MeRIP assay showed the m6A binding site in UBE2D1; H. IGF2BP2 binding to UBE2D1 as determined by RIP assay; I. The stability of UBE2D1 after actinomycin D treatment.

3I). Therefore, we concluded that the increased m6A modification on UBE2D1 mRNA in BC cells enhanced the stability of the mRNA through IGF2BP2 binding.

IGF2BP2 promoted BC cell proliferation and migration

To explore the biological function of IGF2BP2 in BC, CCK-8 assays were performed to evaluate the effect of IGF2BP2 on cell proliferation, and the results showed that knocking down IGF2BP2 inhibited, while IGF2BP2 overexpression promoted BC cell proliferation (**Figure 4A**). Colony formation assays further confirmed this effect, in which IGF2BP2 knockdown inhibited the colony formation of BC cells, while IGF2BP2 overexpression led to the opposite effect (**Figure 4B**). Moreover, the transwell assay results revealed that knocking down IGF2BP2 attenuated, whereas IGF2BP2 overexpression enhanced the migration of BC cells (**Figure 4C**), which was also verified by the wound healing assay (**Figure 4D**).

UBE2D1 enhanced the activation of TGF-β/Smad2/3 signaling pathway in BC

To further explore the mechanism underlying the oncogenic function of UBE2D1 in BC, we downloaded TCGA data, and a pathway enrichment analysis revealed that UBE2D1 was significantly enriched in the TGF-β/Smad signaling pathway. In addition, a protein interaction analysis showed a clear interaction between UBE2D1 and Smad (**Figure 5A-C**), suggesting that UBE2D1 may influence the progression of BC by affecting the TGF-β pathway.

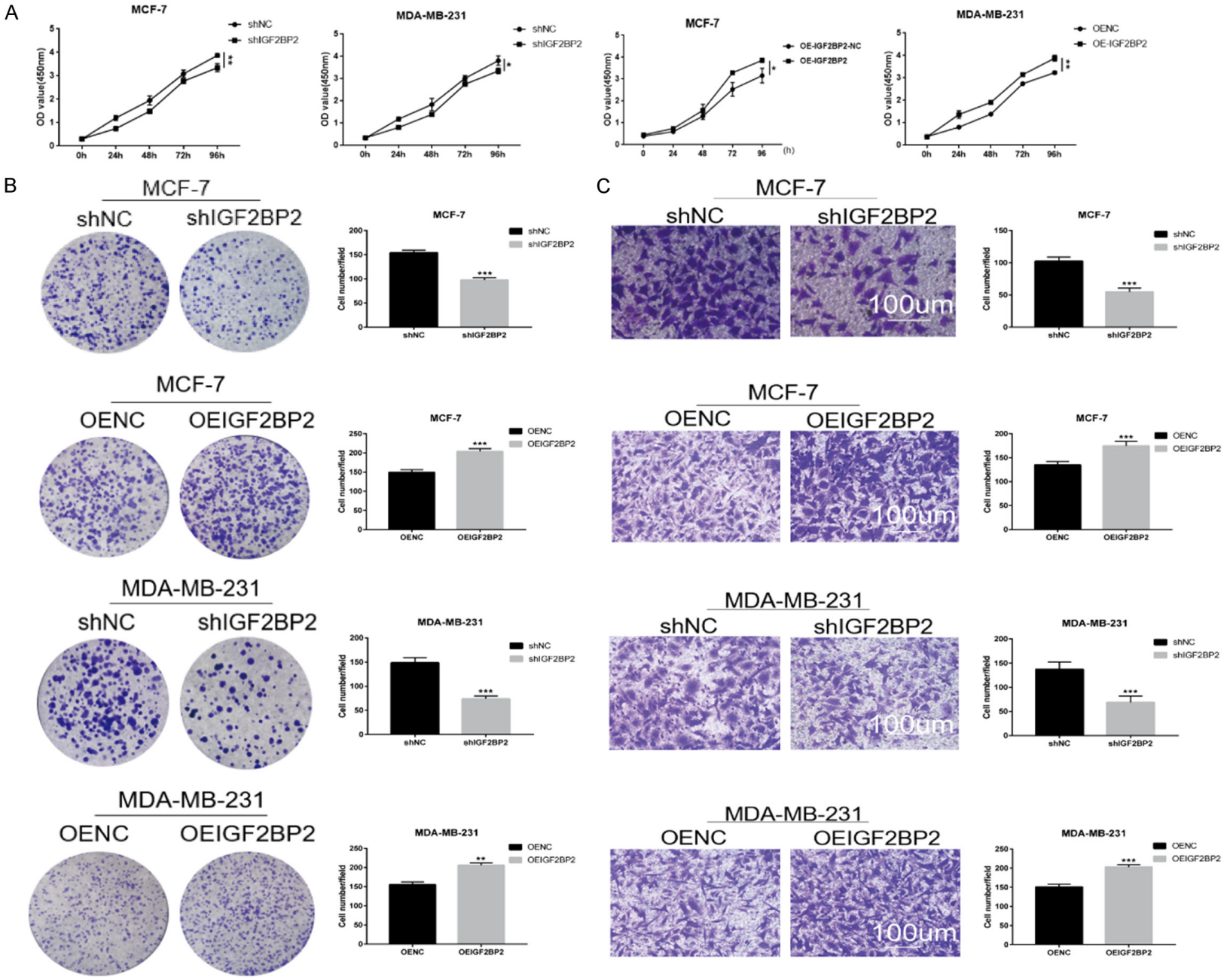
Indeed, we found that Smad2/3 and p-Smad2/3 levels were significantly reduced by UBE2D1 knockdown; while, their levels were significantly upregulated by UBE2D1 overexpression (**Figure 5D**), which was consistent with the qPCR results (**Figure 5E**). To determine if protein degradation was involved in this effect, we subsequently treated cells with CHX and MG132 and didn't observe significant changes in the expression levels of Smad2/3 and p-Smad2/3 (**Figure 5F**), suggesting that UBE2D1 might regulate the expression of Smad2/3 and p-Smad2/3 through a non-ubiquitin/proteosome degradation pathway. Furthermore, nucleoplasmic separation experiments in BC cells revealed that UBE2D1 and Smad2/3 were located in the cytoplasm, while p-Smad2/3 was located in the nucleus (**Figure 5G**). Moreover, Co-IP experiments demonstrated a binding of UBE2D1 to Smad2/3 (**Figure 5H**). Taken together, these results suggested that UBE2D1 not only inhibits its TGF-β pathway activation by directly inhibiting Smad2/3 and p-mad2/3 level, but also affects the occurrence and development of BC by binding to Smad2/3 in BC cells.

TGF-β/Smad2/3 signaling was an important oncogenic pathway in BC

To further clarify the important role of Smad2/3 in BC, we constructed MCF-7 cells stably expressing shSmad2 and shSmad3 for Smad2/3 knockdown. CCK-8 assay results showed that knocking down Smad2/3 inhibited the proliferation of BC cells, while Smad2/3 overexpression showed the opposite effect (**Figure 6A**). Similar effects were observed in colony formation assay in which knocking down Smad2/3 inhibited, while Smad2/3 overexpression promoted the colony formation of BC cells (**Figure 6B**). Consistently, transwell assay also showed that knocking down Smad2/3 inhibited, whereas overexpression of Smad2/3 enhanced the migration of BC cells (**Figure 6C**).

UBE2D1 participated in BC cell proliferation and migration by promoting the interaction between Smad2 and Smad3

This study has found that UBE2D1 not only regulates the expression of Smad2/3 but also binds to Smad2/3. However, it was not clear how UBE2D1 regulates Smad2/3 activity. Therefore, Co-IP was performed to explore whether UBE2D1 affects the binding between Smad2 and Smad3. The results showed that the binding between Smad2 and Smad3 was weakened by UBE2D1 knockdown (**Figure 7A**). In addition, we downloaded Smad2/3 and UBE2D1 target genes from the Cistrome Cancer web site, and an enrichment analysis indicated that the target genes downstream of Smad2 were enriched in TGF-β pathway, Smad3 and UBE2D1 as well as were related to the EGF/EGFR pathway (**Figure 7B-D**), another growth-promoting oncogenic pathway. These results suggest that UBE2D1 further regulates the expression of downstream genes by mediating the binding of Smad2 and Smad3 and thus participates in BC occurrence. Furthermore, among the 382 genes identified at the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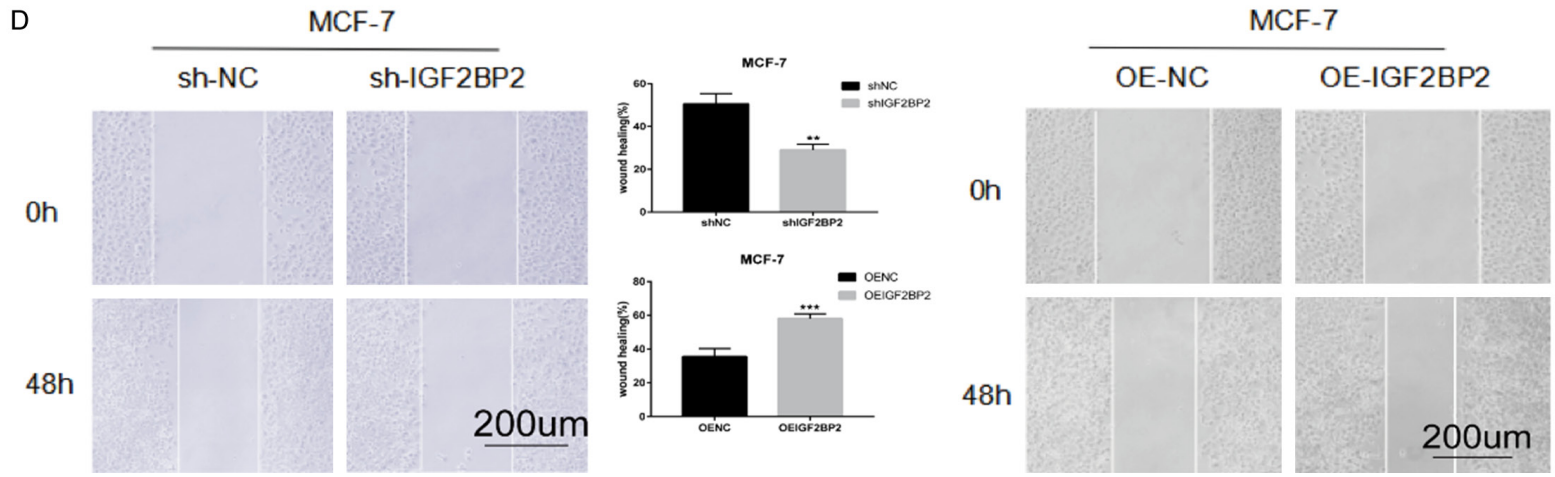
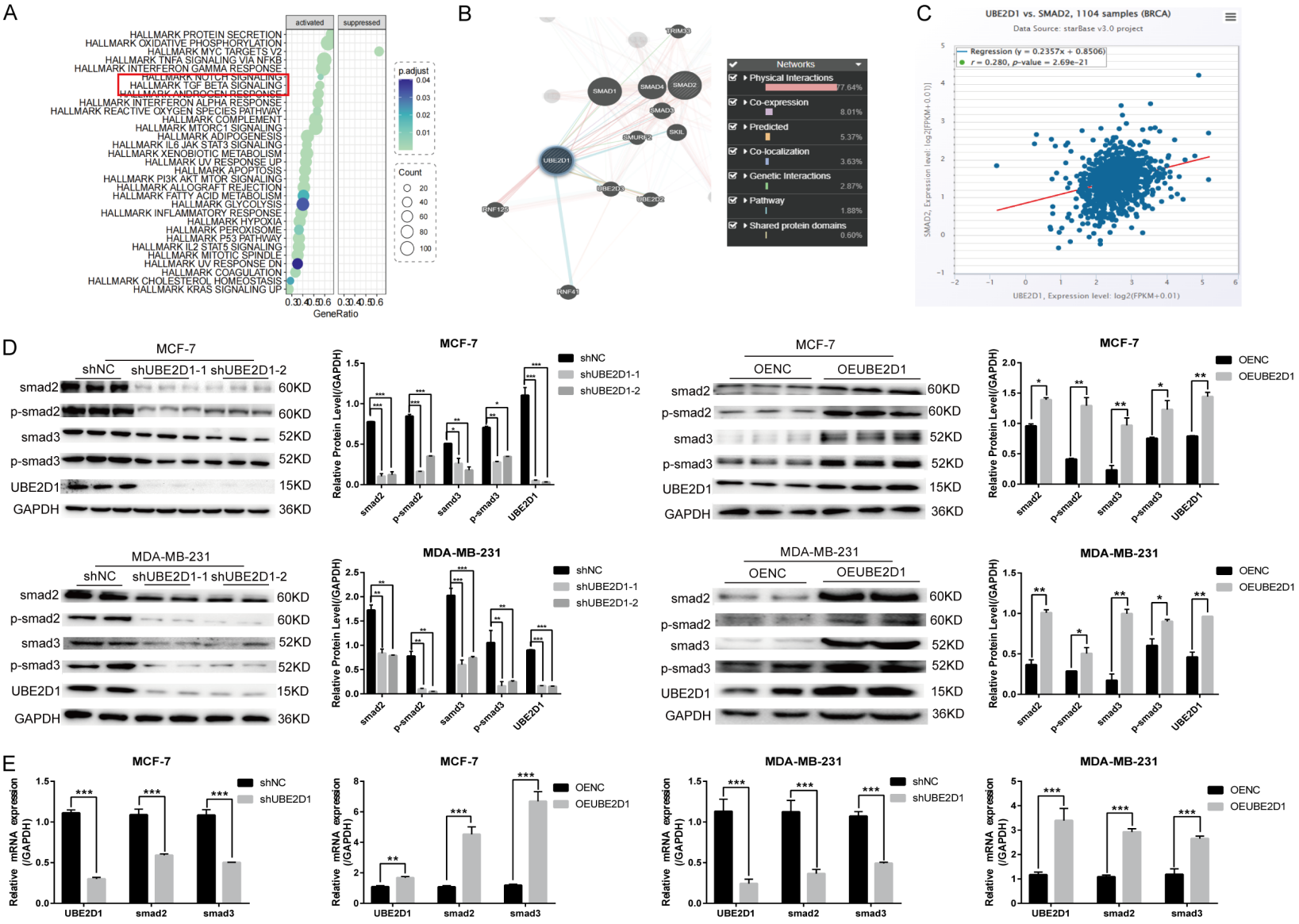


Figure 4. IGF2BP2 promoted BC cell proliferation and migration. A. The proliferation of IGF2BP2 was measured by CCK-8 assay. B. Colony formation assay of IGF2BP2. C. Transwell assays of BC cells with IGF2BP2 knockdown or overexpression. D. Wound healing assays of BC cells with IGF2BP2 knockdown or overexpression.


A
activated
suppressed
HALLMARK PROTEIN SECRETION
HALLMARK OXIDATIVE PHOSPHORYLATION
HALLMARK MYC TARGETS V2
HALLMARK TNFA SIGNALING VIA NFKB
HALLMARK INTERFERON GAMMA RESPONSE
HALLMARK NOTCH SIGNALING
HALLMARK TGF BETA SIGNALING
HALLMARK ANDROGEN RESPONSE
HALLMARK INTERFERON ALPHA RESPONSE
HALLMARK REACTIVE OXYGEN SPECIES PATHWAY
HALLMARK COMPLEMENT
HALLMARK MTORC1 SIGNALING
HALLMARK ADIPOGENESIS
HALLMARK IL6 JAK STAT3 SIGNALING
HALLMARK XENOBIOTIC METABOLISM
HALLMARK UV RESPONSE UP
HALLMARK APOPTOSIS
HALLMARK PI3K AKT MTOR SIGNALING
HALLMARK ALLOGRAFT REJECTION
HALLMARK FATTY ACID METABOLISM
HALLMARK GLYCOLYSIS
HALLMARK INFLAMMATORY RESPONSE
HALLMARK HYPOXIA
HALLMARK PEROXISOME
HALLMARK P53 PATHWAY
HALLMARK IL2 STAT5 SIGNALING
HALLMARK MITOTIC SPINDLE
HALLMARK UV RESPONSE DN
HALLMARK COAGULATION
HALLMARK CHOLESTEROL HOMEOSTASIS
HALLMARK KRAS SIGNALING UP
p.adjust
Count
GeneRatio
B
TRIM33
SMAD1
SMAD4
SMAD2
SMAD3
SMURF2
SKIL
UBE2D1
UBE2D3
UBE2D2
RNF126
RNF41
Networks
Physical Interactions 47.64%
Co-expression 8.01%
Predicted 5.37%
Co-localization 3.63%
Genetic Interactions 2.87%
Pathway 1.88%
Shared protein domains 0.60%
C
UBE2D1 vs. SMAD2, 1104 samples (BRCA)
Data Source: starBase v3.0 project
Regression (y = 0.2357x + 0.8506)
r = 0.280, p-value = 2.69e-21
SMAD2, Expression level: log2(FPKM+0.01)
UBE2D1, Expression level: log2(FPKM+0.01)
D
MCF-7
shNC
shUBE2D1-1
shUBE2D1-2
smad2
p-smad2
smad3
p-smad3
UBE2D1
GAPDH
60KD
52KD
15KD
36KD
Relative Protein Level/(GAPDH)
OENC
OEUBE2D1
MDA-MB-231
E
Relative mRNA expression /(GAPDH)
shUBE2D1

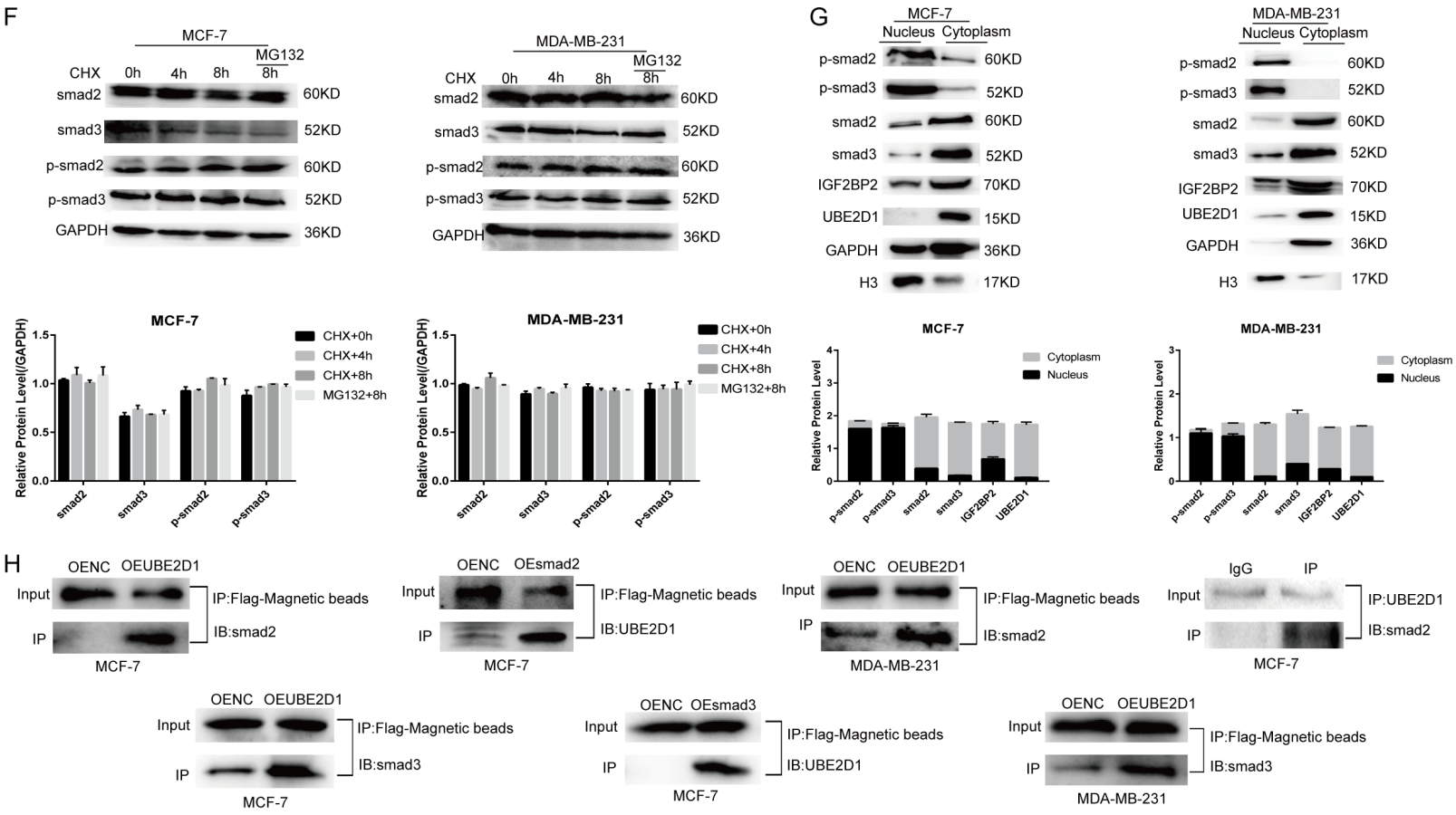
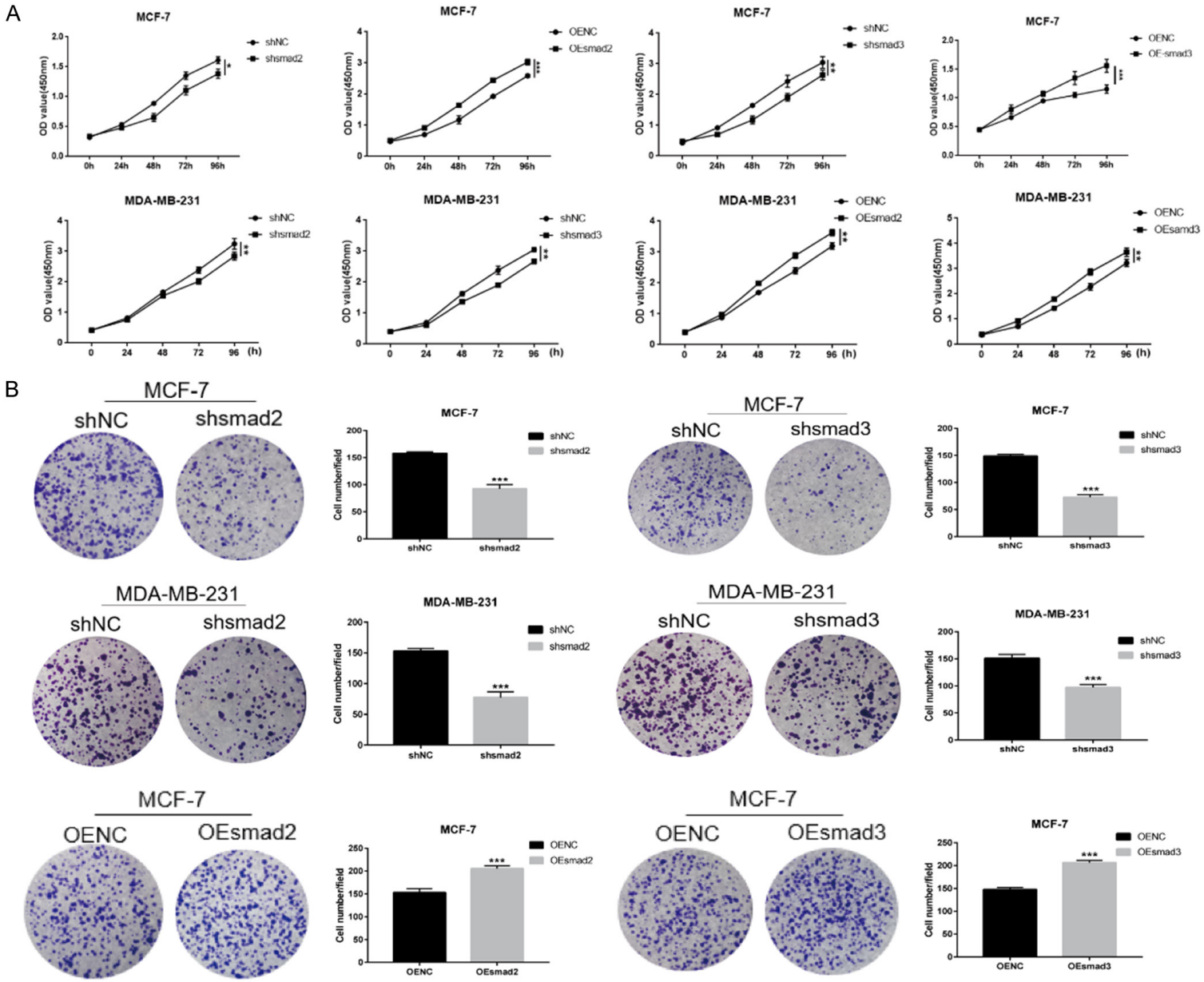


Figure 5. UBE2D1 promoted the activation of TGF-β/Smad2/3 signaling pathway in BC. A. Pathway enrichment analysis of UBE2D1 target genes. B, C. Correlation between UBE2D1 and Smad2/3 levels. D. Western blot analysis of Smad2/3 and p-Smad2/3. E. qPCR analysis of Smad2/3 and p-Smad2/3. F. Western blot assay of Smad2/3 and p-Smad2/3 in CHX and MG132-treated cells. G. Western blot analysis of the nucleoplasmic distribution of target genes. H. Co-IP indicated the interaction between UBE2D1 and Smad2/3.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MCF-7
shNC
shsmad2
OD value(450nm)
0h 24h 48h 72h 96h
MCF-7
OENC
OEsmad2
MCF-7
shNC
shsmad3
MCF-7
OENC
OE-smad3
MDA-MB-231
shNC
shsmad2
0 24 48 72 96 (h)
MDA-MB-231
shNC
shsmad3
MDA-MB-231
OENC
OEsmad2
MDA-MB-231
OENC
OEsamd3
B
MCF-7
shNC
shsmad2
Cell number/field
MCF-7
shNC
shsmad3
MDA-MB-231
shNC
shsmad2
MDA-MB-231
shNC
shsmad3
MCF-7
OENC
OEsmad2
MCF-7
OENC
OEsmad3

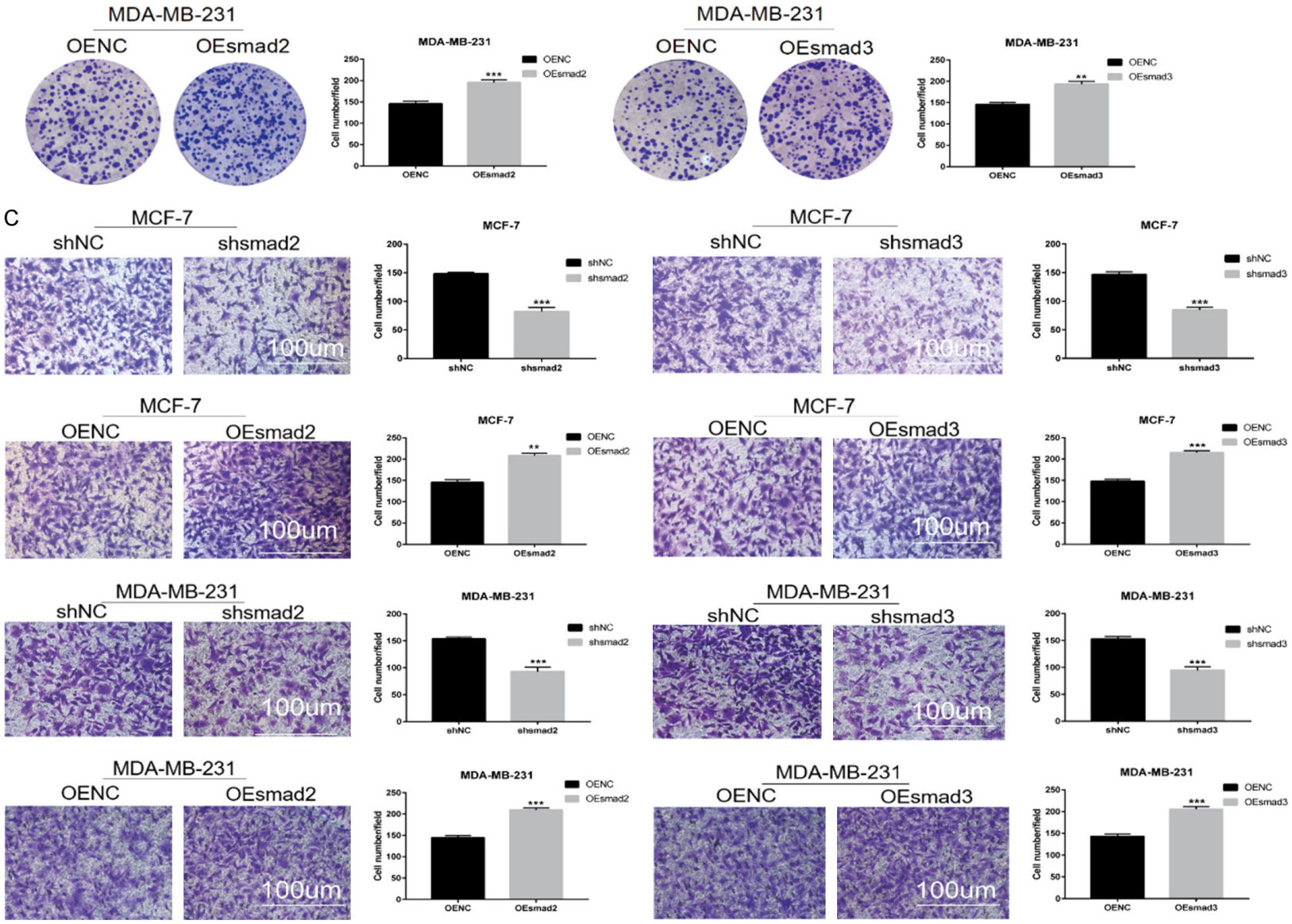
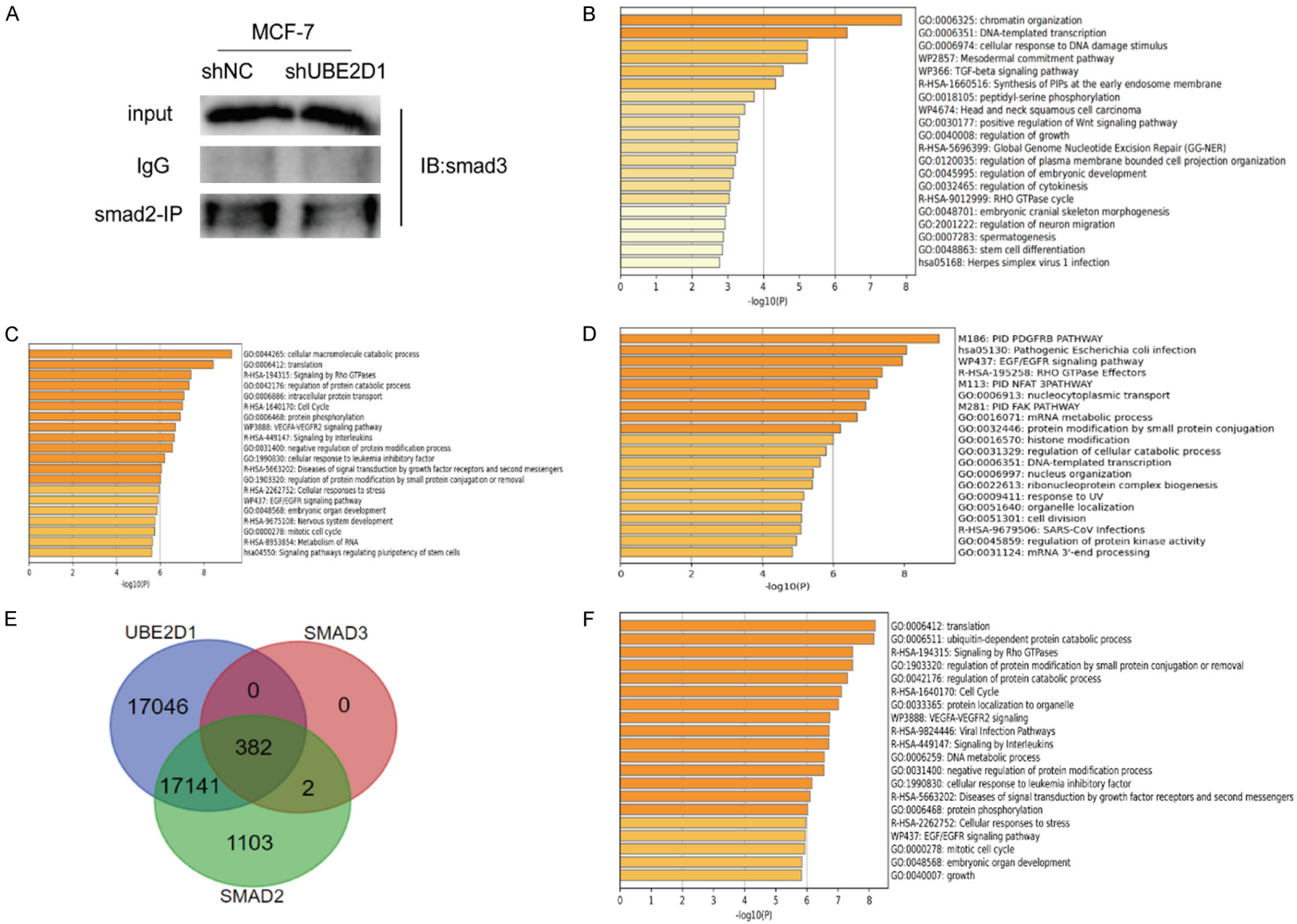


Figure 6. TGF-β/Smad2/3 was an important oncogenic pathway in BC. A. The effect of Smad2/3 on cell proliferation was measured by CCK-8 assay. B. The effect of Smad2/3 on colony formation. C. The effect of Smad2/3 on cell migration was determined by transwell assay.


A
MCF-7
shNC
shUBE2D1
input
IgG
smad2-IP
IB:smad3
B
GO:0006325: chromatin organization
GO:0006351: DNA-templated transcription
GO:0006974: cellular response to DNA damage stimulus
WP2857: Mesodermal commitment pathway
WP366: TGF-beta signaling pathway
R-HSA-1660516: Synthesis of PIPs at the early endosome membrane
GO:0018105: peptidyl-serine phosphorylation
WP4674: Head and neck squamous cell carcinoma
GO:0030177: positive regulation of Wnt signaling pathway
GO:0040008: regulation of growth
R-HSA-5696399: Global Genome Nucleotide Excision Repair (GG-NER)
GO:0120035: regulation of plasma membrane bounded cell projection organization
GO:0045995: regulation of embryonic development
GO:0032465: regulation of cytokinesis
R-HSA-9012999: RHO GTPase cycle
GO:0048701: embryonic cranial skeleton morphogenesis
GO:2001222: regulation of neuron migration
GO:0007283: spermatogenesis
GO:0048863: stem cell differentiation
hsa05168: Herpes simplex virus 1 infection
-log10(P)
C
GO:0044265: cellular macromolecule catabolic process
GO:0006412: translation
R-HSA-194315: Signaling by Rho GTPases
GO:0042176: regulation of protein catabolic process
GO:0006886: intracellular protein transport
R-HSA-1640170: Cell Cycle
GO:0006468: protein phosphorylation
WP3888: VEGFA-VEGFR2 signaling pathway
R-HSA-449147: Signaling by Interleukins
GO:0031400: negative regulation of protein modification process
GO:1990830: cellular response to leukemia inhibitory factor
R-HSA-5663202: Diseases of signal transduction by growth factor receptors and second messengers
GO:1903320: regulation of protein modification by small protein conjugation or removal
R-HSA-2262752: Cellular responses to stress
WP437: EGF/EGFR signaling pathway
GO:0048568: embryonic organ development
R-HSA-9675108: Nervous system development
GO:0000278: mitotic cell cycle
R-HSA-8953854: Metabolism of RNA
hsa04550: Signaling pathways regulating pluripotency of stem cells
-log10(P)
D
M186: PID PDGFRB PATHWAY
hsa05130: Pathogenic Escherichia coli infection
WP437: EGF/EGFR signaling pathway
R-HSA-195258: RHO GTPase Effectors
M113: PID NFAT 3PATHWAY
GO:0006913: nucleocytoplasmic transport
M281: PID FAK PATHWAY
GO:0016071: mRNA metabolic process
GO:0032446: protein modification by small protein conjugation
GO:0016570: histone modification
GO:0031329: regulation of cellular catabolic process
GO:0006351: DNA-templated transcription
GO:0006997: nucleus organization
GO:0022613: ribonucleoprotein complex biogenesis
GO:0009411: response to UV
GO:0051640: organelle localization
GO:0051301: cell division
R-HSA-9679506: SARS-CoV Infections
GO:0045859: regulation of protein kinase activity
GO:0031124: mRNA 3'-end processing
-log10(P)
E
UBE2D1
SMAD3
SMAD2
17046
0
0
382
17141
2
1103
F
GO:0006412: translation
GO:0006511: ubiquitin-dependent protein catabolic process
R-HSA-194315: Signaling by Rho GTPases
GO:1903320: regulation of protein modification by small protein conjugation or removal
GO:0042176: regulation of protein catabolic process
R-HSA-1640170: Cell Cycle
GO:0033365: protein localization to organelle
WP3888: VEGFA-VEGFR2 signaling
R-HSA-9824446: Viral Infection Pathways
R-HSA-449147: Signaling by Interleukins
GO:0006259: DNA metabolic process
GO:0031400: negative regulation of protein modification process
GO:1990830: cellular response to leukemia inhibitory factor
R-HSA-5663202: Diseases of signal transduction by growth factor receptors and second messengers
GO:0006468: protein phosphorylation
R-HSA-2262752: Cellular responses to stress
WP437: EGF/EGFR signaling pathway
GO:0000278: mitotic cell cycle
GO:0048568: embryonic organ development
GO:0040007: growth
-log10(P)

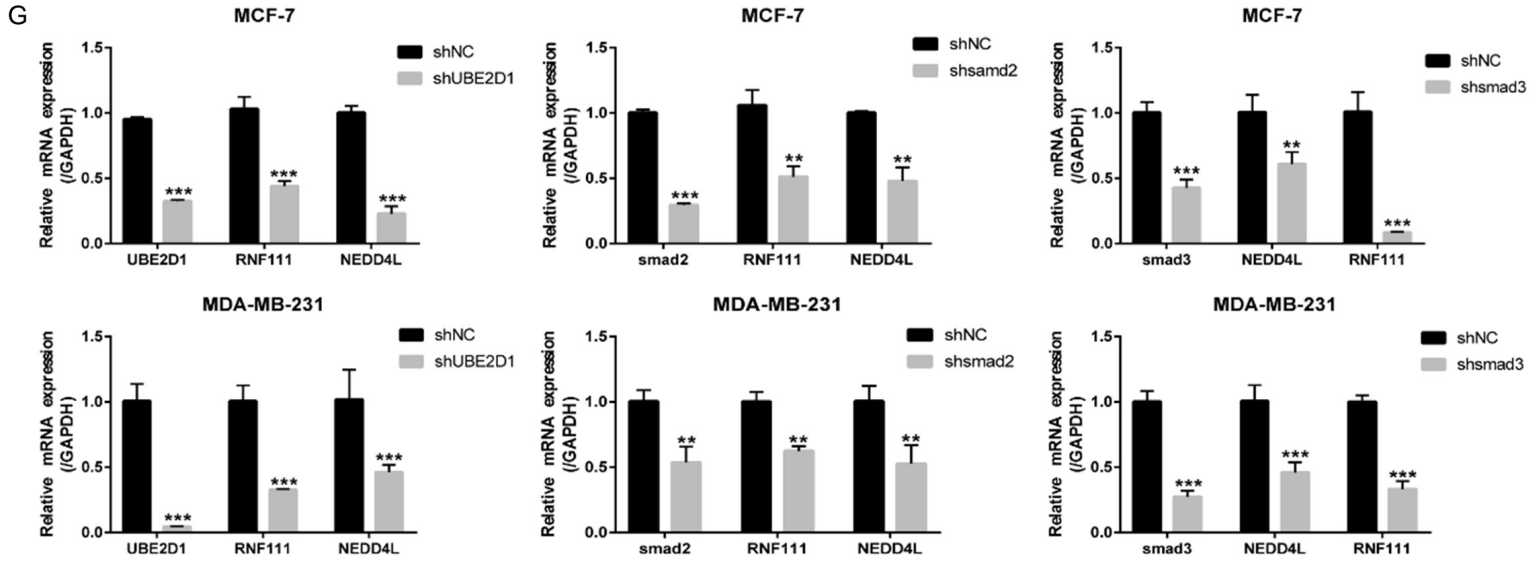


Figure 7. UBE2D1 participated in BC cell proliferation and migration by promoting the interaction of Smad2 and Smad3. A. Co-IP of Smad2 and Smad3 in cells with UBE2D1 knockdown. B. The enrichment of Smad2 target genes. C. The enrichment of Smad3 target genes. D. The enrichment of UBE2D1 target genes. E. The intersection genes of UBE2D1 and SMAD2/3. F. Enrichment of 382 cross-target genes. G. The changes in the mRNA expression levels of target genes determined by qPCR.

intersection of Smad2, Smad3 and UBE2D1, we chose RNF111 and NEDD4L, which are highly correlated to the EGF/EGFR pathway after enrichment, for subsequent experimental validation (**Figure 7E**, **7F**). A qPCR analysis showed that the expression levels of RNF111 and NEDD4L were regulated by UBE2D1 and Smad2/3 (**Figure 7G**), suggesting that UBE2D1 promotes the proliferation and migration of BC cells by affecting the binding of Smad2/3 in the TGF-β signaling pathway, thus affecting the expression of downstream target genes.

Discussion

With advances in basic and clinical research, the treatment for patients with BC has greatly improved [4]. However, there are still many challenges, such as the drug resistance in luminal breast cancer and HER-2-positive breast cancer as well as the lack of effective therapeutic targets for triple-negative breast cancer, that require the identification of novel molecular biomarkers for the diagnosis and prognosis of BC [2, 5, 20]. UBE2D1 is known to regulate the development of various cancers. In our study, we analyzed the data of 33 types of cancers in the TCGA database and found that the expression of UBE2D1 was upregulated in most tumors, including esophageal carcinoma, liver hepatocellular carcinoma, and stomach adenocarcinoma. Through a differential expression and prognosis analysis of the TCGA data, we found that UBE2D1 expression was upregulated in BC and was also negatively associated with OC of patients with BC. Therefore, we measured UBE2D1 expression in BC patient samples as well as BC cell lines and confirmed the findings from bioinformatics analysis. To further explore the role of UBE2D1 in BC, we conducted in vivo and in vitro experiments and found that overexpression of UBE2D1 promoted the proliferation and migration of BC cells.

Moreover, we explored the mechanism underlying the high UBE2D1 expression in BC. RNA modification is a common epigenetic modification mode, among which methylation at adenosine N6 (m6A) modification is catalyzed by the RNA methyltransferases (writers) METTL3, METTL4 and WTAP. The reversible demethylation of M6A-modified RNA sites is catalyzed by the demethylases (erasers) FTO and ALKBH5. The m6A-modified RNA sites can be recognized and bound by an m6A-binding protein (reader), which regulates RNA metabolism. These modifications regulate RNA translation, splicing, transposition, degradation, and processing [21]. In this study, we found that the expression level of UBE2D1 decreased significantly by METTL3 knockdown. To further elucidate the key molecular mechanism underlying m6A modification-induced increase of UBE2D1 mRNA level, we reviewed our previous analysis on UBE2D1 mRNA-binding proteins using CLIP-seq data (starBase) and found that UBE2D1 mRNA bound to a m6A reader protein: insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs) [22]. Importantly, our in vitro experimental results showed that the expression level of UBE2D1 was significantly reduced by IGF2BP2 knockdown but was significantly increased by IGF2BP2 overexpression, which was further verified by MeRIP-qPCR.

TGF-β signaling plays a key role in tumor progression. Most cancer cells benefit from increased TGF-β expression and TGF-β signaling, resulting in cancer invasion and spread, increased stem cell population, and acquired cancer therapy resistance [23]. To explore the specific mechanism by which UBE2D1 promotes the progression of BC and to confirm the involvement of TGF-β signaling in the cancer-promoting effect of UBE2D1, we conducted a pathway analysis with TCGA data and found UBE2D1 was significantly enriched in TGF-β signaling, P53 signaling, TNF-α/NF-kB signaling, etc. Further analysis of UBE2D1-interacting proteins suggested that the TGF-β/Smad pathway might be an essential contributor to the UBE2D1-promoting effects in cancer. Subsequent experiments confirmed the role of the TGF-β/Smad2/3 pathway in promoting the proliferation and migration of BC cells.

Smad2/3 is the central mediator of the TGF-β signaling pathway [24]. Undoubtedly, the regulation of Smad activity and expression depends on the interaction of Smad proteins with their partners in cells. Therefore, the identification and characterization of molecules that interact with Smads are key to understanding how the Smad pathway functions [25]. Notably, through protein interaction analysis, we found that both UBE2D1 and Smads interacted with each other. Through a series of biochemical and cell experiments, we determined that UBE2D1 regulated the protein levels of Smad2/3 and p-Smad2/3, hence the activity of TGF-β signaling. In addi-

tion, we found that UBE2D1 bound to Smad2/3 as UBE2D1 and Smad2/3 were located mainly in the cytoplasm, while p-Smad2/3 was located in the nucleus. Therefore, we speculate that UBE2D1 ultimately regulates the progression of BC by binding to Smad2/3 and is involved in the nuclear translocation of p-Smad2/3 and SMAD4 complex. Since the binding between Smad2 and Smad3 is a key event in the TGF-β signaling pathway [17], we further explored whether UBE2D1 affect the binding of Smad2 to Smad3. As expected, the Co-IP results showed that the binding between Smad2 and Smad3 was weakened by UBE2D1 knockdown.

In conclusion, our study revealed that IGF2BP2-mediated UBE2D1 mRNA methylation plays an important role in promoting the proliferation and migration of BC cells. We also identified that UBE2D1 not only interacted with Smad2/3 but also promoted the Smad2 and Smad3 interaction, thereby facilitating the progression of BC. Hence, UBE2D1 may serve as a novel therapeutic target for BC treatment.

Acknowledgements

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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cer, decreasing ubiquitination of SMAD4. Infect Agent Cancer 2021; 16: 63.
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A

RBP	GeneID	GeneName	GeneType	ClusterNum	ClipExpNum	ClipSiteNum	HepG2(log2FC)	K562(log2FC)	Pan-Cancer
IGF2BP2	ENSG00000072401	UBE2D1	protein_coding	25	7	41	NA	NA	22
IGF2BP1	ENSG00000072401	UBE2D1	protein_coding	15	5	24	0.687	NA	11
IGF2BP3	ENSG00000072401	UBE2D1	protein_coding	17	5	20	NA	NA	24

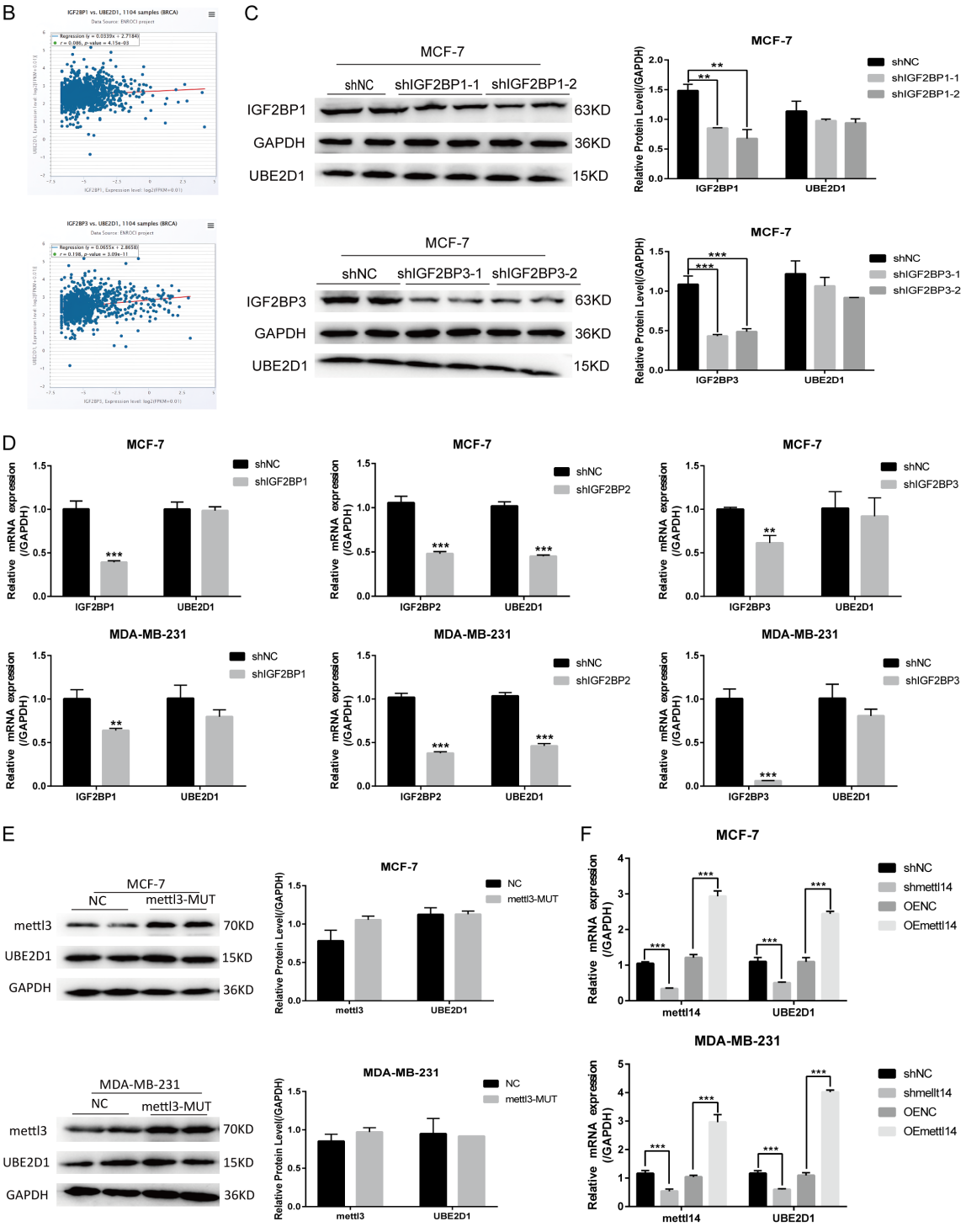

Figure S1. A, B. Correlation of UBE2D1 with IGF2BPs; C, D. Western blot and qPCR assays of UBE2D1 expression in IGF2BPs knockdown cells. E. Western blot analysis of UBE2D1 in cells with METTL3 mutation at the catalytic site; F. qPCR analysis of UBE2D1 mRNA expression in cells with METTL14 knockdown or METTL14 overexpression.