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Modification of PLAC8 by UFM1 affects tumorous proliferation and immune response by impacting PD-L1 levels in triple-negative breast cancer

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ABSTRACT

Background Triple-negative breast cancer is characterized by a poor prognosis and lack of targeted treatments, and thus, new targeting markers and therapeutic strategies are urgently needed. We previously indicated that PLAC8 promotes tumorigenesis and exerts multidrug resistance in breast cancer. Therefore, we aimed to characterize the PLAC8-regulated network in triple-negative breast cancer.

Methods We measured the levels of PLAC8 in breast cancer cell lines and found that PLAC8 is post-translationally modified by ubiquitin-fold modifier 1 (UFM1). Then, we revealed a new regulatory system of PD-L1 by PLAC8 in triple-negative breast cancer. We also tested the molecular functions of PLAC8 in triple-negative breast cancer cell lines and measured the expression of PLAC8 and PD-L1 in breast cancer tissues.

Results PLAC8 was generally highly expressed in triple-negative breast cancer and could be modified by UFM1, which maintains PLAC8 protein stability. Moreover, PLAC8 could promote cancer cell proliferation and affect the immune response by regulating the level of PD-L1 ubiquitination. Additionally, among patients with breast cancer, the expression of PLAC8 was higher in triple-negative breast cancer than in non-triple-negative breast cancer and positively correlated with the level of PD-L1.

Conclusions Our current study discoveries a new PLAC8-regulated network in triple-negative breast cancer and provides corresponding guidance for the clinical diagnosis and immunotherapy of triple-negative breast cancer.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Triple-negative breast cancer (TNBC) does not well respond to endocrine therapy, targeted therapy and immunotherapy and PLAC8 plays an important role in breast cancer progression. Therefore, exploration of its accurate functions in TNBC might be perspective.

WHAT THIS STUDY ADDS

⇒ We identify post-translational modification of PLAC8 by ubiquitin-fold modifier 1 and PD-L1 by PLAC8 and PLAC8/PD-L1 activation regulates TNBC proliferation and immune responses.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study might provide new targets to potentiate the efficacy of immunotherapy in TNBC.

INTRODUCTION

Breast cancer is still the most common malignant disease in women.[1 2] Nearly 20% of all breast cancers are classified as triple-negative breast cancer (TNBC), which lacks or exhibits minimal expression of progesterone, estrogen and HER2 receptors.[3] Although TNBC occupies a small proportion of all patients with breast cancer, it has a worse prognosis than other types of breast cancer. Thus, many current studies focusing on TNBC are aimed at providing new tools for diagnosis, treatment, and related treatment targets.[4–6] For example, PARP is an enzyme involved in single-strand DNA break repair, known as base excision repair, and its inhibition effectively constrains BRCA-associated TNBC. PARP inhibitors are routinely used in clinical trials for TNBC.[3] Despite objective responses to PARP inhibitors and improvements in progression-free survival compared with standard chemotherapy in BRCA-associated triple-negative patients with breast cancer, the benefits are limited and transitory.[3 7] Here, we intended to identify better indicators of TNBC treatment and diagnosis. Our previous research suggested that PLAC8 can promote breast cancer cell growth and participate in the process of tamoxifen and adriamycin resistance.[8–10] As we mentioned in our review,[11] PLAC8 also plays an important role in various cancers and is involved in tumor progression, including in programmed cell death,

cancer stemness and cancer immunity. This finding indicates that PLAC8 has important clinical significance in breast cancer. Therefore, we investigated the underlying mechanisms of PLAC8 in TNBC. In this study, we found that PLAC8 expression is generally higher in TNBC than in non-TNBC. When we knocked down the expression of PLAC8 in TNBC cells, we found that cell proliferation is inhibited. Additionally, PLAC8 could be modified and maintained by ubiquitin-fold modifier 1 (UFM1), which belongs to a small subclass of ubiquitin proteins[12] in TNBC cells. Similar to the process of ubiquitination, UFM1 conjugates to target proteins and has been demonstrated to modulate several cellular activities. Post-translational modification by UFM1 plays an important role in breast cancer.[13 14] In addition to cell proliferation, PLAC8 can also affect the TNBC immune response. PD-1/PD-L1 blockade therapy has emerged as a promising new treatment in breast cancer, and studies have reported that PD-L1 is more highly expressed in TNBC and is involved in the tumor immune processes.[15] Our results demonstrated that PLAC8 interacts with PD-L1 and affects its expression in TNBC.

Our study proposes for the first time that UFM1 can modify PLAC8 to maintain its protein stability in TNBC. Moreover, the PLAC8-regulated axis controls cell progression and affects the immune response in TNBC. Together, fully understanding the potential function of the PLAC8 is helpful to provide a theoretical basis for the clinical diagnosis and effective targeting of TNBC.

MATERIALS AND METHODS

Cell culture and plasmid construction

The human breast cancer cell lines MCF-7, T47D and HS578T were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 0.01 mg/mL bovine insulin. ZR-75–1, HCC-1937, BCAP-37, and BT549 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% glutamine. SK-BR-3 was maintained in McCoy's 5A Medium supplemented with 10% fetal bovine serum. MDA-MB-231 was maintained in Leibovitz's L-15 Medium supplemented with 10% fetal bovine serum and incubated cultures at 37°C without CO_2. The whole cells except MDA-MB-231grew in a humid atmosphere containing 5% CO_2 at 37°C.

The shRNA sequences of PLAC8 were described as earlier.[9] The corresponding siUFM1 sequence were as follows:

siUFM1#1: GUUGGAAGUUGUUAAUAUC.
siUFM1#2: GAACUGCGGAUUAUUCCUA.
siUFM1#3: GCGGAUUAUUCCUAGAGAU.

pcDNA3.1–3*FLAG-PLAC8 and pcDNA3.1–3*FLAG-PLAC8^{mut} were designed and purchased from Generay. pcDNA3.1–3*FLAG-PD-L1 was purchased from NovoPro. And pcDNA3.1-HA-UB was purchased from Addgene.

Reagents

Cycloheximide (#HY-12320), Chloroquine (#HY-17589A) and MG-132 (#HY-13259) were purchased form MedChemExpress (MCE).

Western blot analysis

Western blots analysis was performed as previously described.[8] The primary antibodies PLAC8 (#13885, 1:500) and PD-L1(#13684, 1:1000) were purchased from Cell Signaling Technology. UFM1 (#109305, 1:1000) was purchased from Abcam. FLAG tag antibody (#AP1013A-eV, 1:1000) was purchased from Abcepta. These antibodies GAPDH (#sc-47724, 1:1000) and HA (#sc-7392, 1:1000) were purchased from Santa Cruz Biotechnology. Densitometric analysis was performed by ImageJ. The data were corrected by background subtraction and normalized against GAPDH as an internal control.

RNA isolation and quantitative real-time PCR

These were performed as described previously.[9] In general, total RNA was extracted from cells and tissues using TRIzol (Invitrogen, Carlsbad, California, USA) reagent. All samples were amplified thrice by real time, and the expression was normalized to GAPDH. The following primer sequences were used:

GAPDH forward 5′-TGACTTCAACAGCGACACCCA-3′
GAPDH reverse 5′-CACCCTGTTGCTGTAGCCAAA-3′
PLAC8 forward 5′-GGAACAAGCGTCGCAATGAG-3′
PLAC8 reverse 5′-AAAGTACGCATGGCTCTCCTT-3′
UFM1 forward 5′-AGCTCACCTAATTGTTCACATGG-3′
UFM1 reverse 5′-CACTGTAGGGTGTTGTCTTTCTT-3′
PD-L1 forward 5′-TGGCATTTGCTGAACGCATTT-3′
PD-L1 reverse 5′-TGCAGCCAGGTCTAATTGTTTT-3′.

Cell proliferation assay

For CCK-8 assay, cells (0.5×10^4 were seeded onto a 96-well culture plate for 24, 48, or 72 hours). The absorbance was measured at 450 nm by using a BioTek ELx800 absorbance microplate reader. For colony formation assay, 500 cells were seeded in a 6-well plate and cultured for 14 days in complete medium. Then colonies were fixed and dyed with 0.1% crystal violet.

Coimmunoprecipitation assay

Cell lysates (the remaining 10% of sample was used to prepare inputs) were precleared with IgG and subsequently incubated with primary antibody, while rabbit IgG was used as the negative control. Protein A/G-agarose (#sc-2003; Santa Cruz, California, USA; 20 µL) was then added and incubated overnight. The precipitates were washed five times with PBS and analyzed by Western blotting.

UFMylation modification detection

Cells were lysed with strong denatured lysate (150M Tris, pH 8.0, 5%SDS, 30% glycerol), and then boiled at for 10 mins. The lysates then were centrifuged at high speed and supernatant was immunoprecipitated with the antibodies. The UFMylation of PLAC8 was detected by western blot.

Ubiquitination assay

Cells were transfected with the indicated plasmids and treated with 10 µM Chloroquine for 12 hours or 10 µM MG-132 for 6 hours before harvesting. Then cells were lysed and centrifuged at high speed while the remaining 10% of sample was used to prepare inputs. The lysates were subjected to immunoprecipitation with IgG or PD-L1 antibody overnight. The ubiquitination of endogenous PD-L1 was detected by western blot.

Flow cytometry analysis of membrane PD-L1

Cells were collected and then incubated with PBS (0.5% bovine serum albumin) for 30 mins at room temperature. The cells were probed with PD-L1 antibody (#14-5982-82, eBioscience) at room temperature for 60 mins. Cell suspensions were washed twice in PBS and stained with indicated fluorescent-labeled antibodies for 30 mins. After washing three times with PBS, the cells were analyzed using flow cytometry and data were analyzed using FlowJo X software.

Immunofluorescence and immunohistochemical staining

Immunohistochemical staining was performed as previously described.[8] Briefly, all sections were incubated 40 mins at room temperature with the primary antibody. Then, three PBS washes were performed, after which the slides were exposed to the secondary antibody for 30 mins at room temperature. We washed it three times with PBS, 5 mins each time, then with DAB for 5 mins, tap water for 10 mins, hematoxylin counterstain for 2 mins, tap water again for 10 mins, and then routine dehydration, transparency, mounting, and microscopy. And for each staining, a positive control was included (human breast cancer tissues), as well as a negative control, without the primary antibody or with rabbit/mouse IgG. The slices were stained with PLAC8 (1:200), PD-L1 (1:200), Ki-67 (1:500), CD3 (1:1000), and CD8 (1:1000).

Jurkat co-culture and IL-2 ELISA assay

MDA-MB-231 and HCC-1937 cells were treated with IFN-γ for 24 hours and Jurkat cells were stimulated by 50 ng/L PMA and 1 µg/mL Ionomycin for 24 hours. Subsequently, MDA-MB-231 cells or HCC-1937 cells were seeded in 96-well plates. After the cells adhered, the supernatants were discarded, and Jurkat cells were added to MDA-MB-231 or HCC-1937 cells at a ratio of 4:1 in 150 µL media. The supernatants were collected after 48 hours and examined by Human IL-2 ELISA kit (#70-EK102-96, Multi-Science) according to the manufacturer's instruction.

Tissue specimens

A total of 118 samples of breast cancer tissues were obtained from the Sir Run Run Shaw Hospital, which is affiliated with Zhejiang University, from patients who had been histopathologically and clinically diagnosed from 2002 to 2012. Protein expression was evaluated by two pathologists (double blinded) and was divided into four scores: strong, +3; moderate, +2; weak, +1; and negative, 0. Specimens with scores of +3 or +2 were defined as having high expression, and those with scores of+1 or 0 were defined as having low expression.

Tumor xenografts

Female nude mice (4 weeks old) and female BALB/c mice (5–6 weeks) were purchased from Shanghai Laboratory Animal Center and housed in a specific pathogen-free environment according to the Ethics Committee for Animal Studies of Zhejiang University (SRRSH202107012, Hangzhou, China). Stable transfected MDA-MB-231 cells ($1\times10^{*6}$ per mouse) or 4T1 cells ($0.5\times10^{*6}$ per mouse) were injected into the right subaxillary region.

Mice were monitored and measured twice per week using a slide caliper including tumor length (L), and width (W). Tumor volume (mm^3) = $\pi/6\times$length × width.[2] Mice were then sacrificed and some tumor tissues were fixed with 10% paraformaldehyde for immunohistochemical analysis and western analysis. Animal care and experiments involved in this study were performed in accordance with Accreditation of Laboratory Animal Care International guidelines.

Statistical analysis

Data were analyzed in Excel and graphed in GraphPad Prism V.7.0 software (San Diego, California, USA). The comparisons between multiple groups were performed using multiple comparisons by one-way analysis of variance. Comparisons between groups were performed using Student's t-test and an unpaired two-tailed Student's t-test was used to compare mean data. Data were independently obtained from at least three experiments. The results are presented with statistical significance or p value (*p<0.05; **p<0.01; NS, indicated not statistically significant).

RESULTS

Oncogenic function of PLAC8 in TNBC cell proliferation

We assessed PLAC8 mRNA expression in the Oncomine online database and found that its level is higher in TNBC according to the Bittner breast database (figure 1A). Similar results were obtained from the GEO database (GSE45827) (figure 1B). We then further tested the expression of PLAC8 in TNBC cells and found that PLAC8 is generally highly expressed in these cells (figure 1C). These results provided the first hint that PLAC8 might have important functions in TNBC development. We used MDA-MB-231 and HCC-1937 cell lines to knock down PLAC8 with sh-RNA (online supplemental figure S1A,B). PLAC8 deficiency inhibited MDA-MB-231 and HCC-1937 cell proliferation (figure 1D,E). PLAC8 knockdown also suppressed colony formation relative to control cells (figure 1F,G). We also determined the roles of PLAC8 in vivo and found that depletion of PLAC8 decreased tumor volume (figure 2A–D). Knockdown of PLAC8 did not impact mouse weight (figure 2B). IHC images further indicated that PLAC8 promotes cell proliferation (figure 2E). These assays together indicated

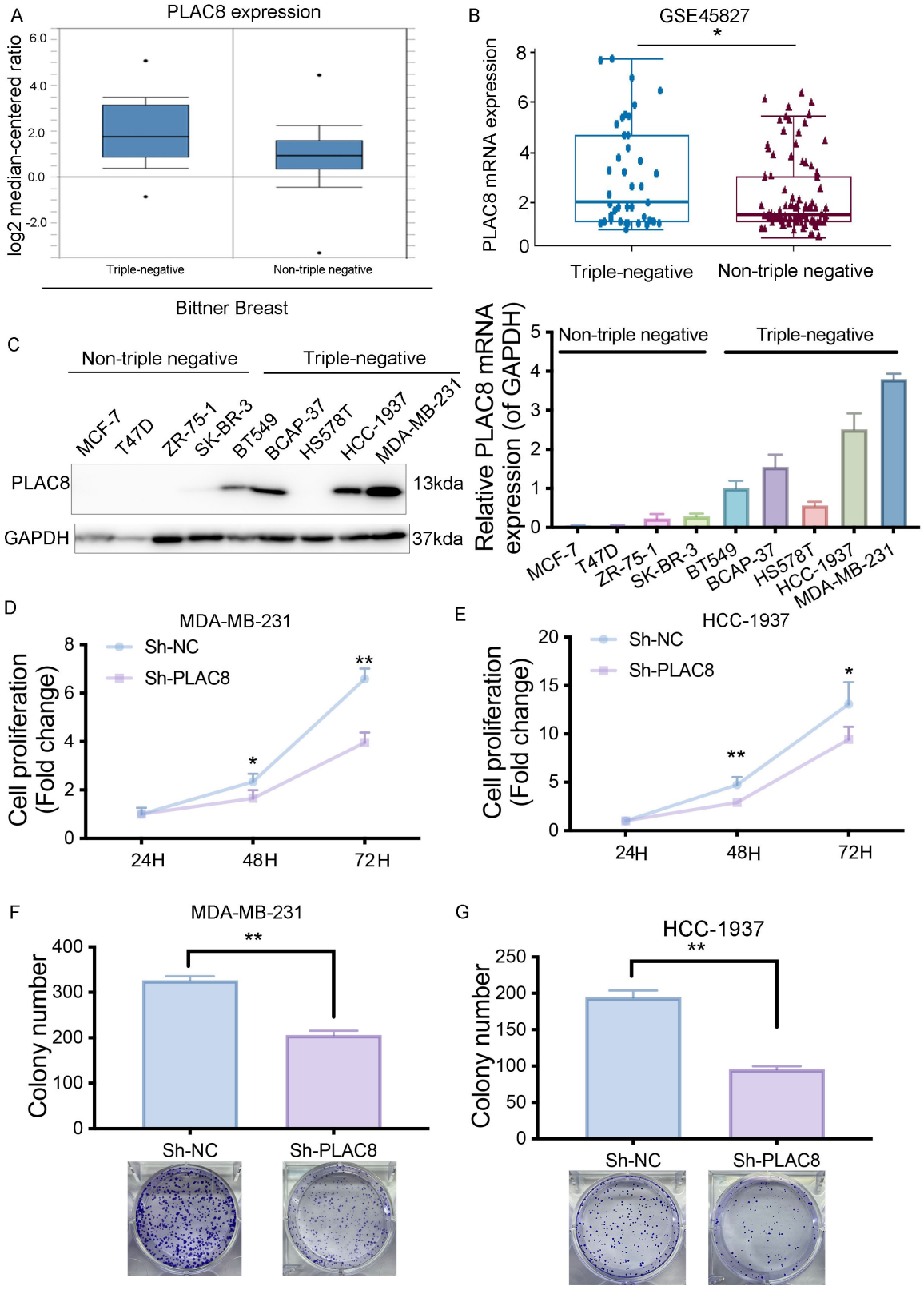


Figure 1 High PLAC8 expression is related to triple-negative breast cancer (TNBC) progression. (A) PLAC8 mRNA expression in TNBC and non-TNBC samples in the Bittner breast database of the Oncomine database. (B) The level of PLAC8 expression among triple-negative breast cancer and non-TNBC samples in the Gene Expression Omnibus database (GSE45827). (C) Western blot and RT-PCR analysis of PLAC8 levels in breast cancer cell lines (MCF-7, T47D, ZR-75–1, SK-BR-3, BT549, BCAP-37, HS578T, HCC-1937 and MDA-MB-231). (D, E) MDA-MB-231 and HCC-1937 cells stably expressing control or PLAC8 shRNAs. Cell proliferation was then determined by CCK-8 assay. Clonogenic survival assays were assayed in MDA-MB-231 cells (F) or HCC-1937 cells (G) that stably expressed control or PLAC8 shRNAs. Data are presented as the means±SD of three independent experiments. *p<0.05; **p<0.01.

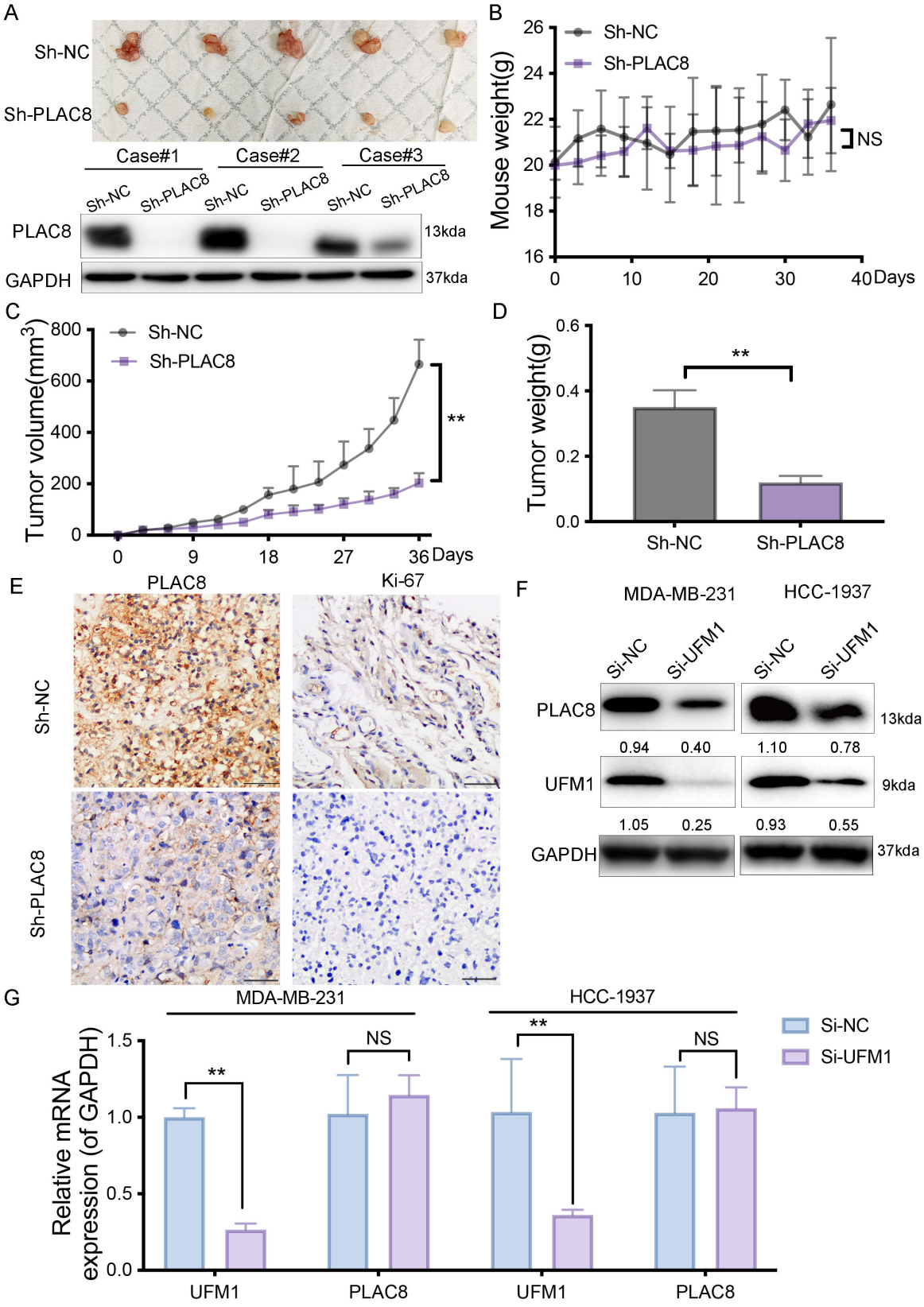


Figure 2 PLAC8 expression impacts triple-negative breast cancer tumorous progression. (A) Images of tumors from different mouse groups. Western blot analysis of PLAC8 expression in tumor tissues. GAPDH served as a loading control. (B) Mouse weight in different groups. (C) Tumor volume and (D) tumor weight in mice. (E) IHC staining of PLAC8 and Ki-67 in tumor tissues. Scale bar represents 50μm. (F) Western blot and (G) RT-PCR analyses of UFM1 expression in MDA-MB-231 and HCC-1937 cells with PLAC8 depletion 72 hours and 48 hours after transfection. The expression was normalized to GAPDH. Data are presented as the means±SD of three independent experiments.**p<0.01; NS, not significant; IHC, immunohistochemical; UFM1, ubiquitin-fold modifier 1.

that PLAC8 contributes to cell growth in TNBC, which is consistent with our previous results.

UFMylation of PLAC8 maintains its stability

To better understand PLAC8-associated mechanisms in TNBC, we explored whether the UFMylation pathway has a role in the regulation of PLAC8 expression. We knocked down UFM1 in MDA-MB-231 and HCC-1937 cells (online supplemental figure S1C,D) and found that the level of PLAC8 protein decreases (figure 2F). However, the level of PLAC8 mRNA did not change (figure 2G). Thus, we examined the effect of UFMylation on PLAC8 stability in MDA-MB-231 and HCC-1937 cells treated with cycloheximide (CHX), an inhibitor of protein synthesis, and found that PLAC8 stability is reduced after UFM1 depletion (figure 3A). Furthermore, we confirmed that the UFMylation modification of endogenous PLAC8 can be detected in both MDA-MB-231 and HCC-1937 cells (figure 3B). To identify the UFMylation site in the PLAC8 protein, we generated lysine-to-arginine (Lys-to-Arg) mutation in PLAC8 (figure 3C). Of the generated mutation, the mutation at K103 blocked UFMylation of PLAC8, indicating that the PLAC8 UFMylation site resides at the Lys 103 residue (figure 3C). Consequently, the expression of FLAG-PLAC8, but not FLAG-PLAC8^{mut}, impaired cell viability, which was reduced when PLAC8 was knocked down (figure 3D,E). PLAC8 knockdown suppressed colony formation relative to control cells, and this suppression was rescued by the overexpression of FLAG-PLAC8, but not FLAG-PLAC8^{mut} (online supplemental figure S2A,B). Taken together, these data revealed that PLAC8 is UFMylated at K103 and that PLAC8 UFMylation promotes cell growth in TNBC cells.

PLAC8 interacts with glycosylated and ubiquitinated PD-L1

PLAC8 is reported to be involved in the cancer immune response,[11] so we further tested whether there is a connection between PLAC8 and the immune response in TNBC. As shown in figure 4A,B, knockdown of PLAC8 induced the secretion of interleukin-2 (IL-2) by Jurkat cells. Additionally, it has been reported in the literature that PD-L1 is highly expressed in TNBC.[15] We measured the expression of PD-L1 in breast cancer cell lines and found that it is mainly glycosylated in these cells (figure 4C). The Oncomine online database showed that PD-L1 expression is relatively higher in TNBC (online supplemental figure S2C,D). The GEO database (GE65194) also showed that the level of PD-L1 is higher in TNBC (online supplemental figure S2E). These results indicated that there may be a positive connection between PLAC8 and PD-L1 and PLAC8 may be involved in TNBC immunity through PD-L1. Immunofluorescence was used to determine the location of PLAC8 and PD-L1 (figure 4D). We then knocked down the expression of PLAC8 in MDA-MB-231 and HCC-1937 cells and found that PD-L1 protein expression also decreases (figure 4E and figure 5A), but that the mRNA level did not change (online supplemental figure S2F). Considering that interferon gamma (IFN-γ) has been reported to induce PD-L1 expression, we investigated whether this is also the case in PLAC8 knockdown TNBC cell lines. As shown in online supplemental figure S3G, knockdown of PLAC8 also decreased PD-L1 expression in the presence of IFN-γ, suggesting that PLAC8 is a dependent regulatory factor of PD-L1 expression in TNBC cells. Furthermore, we used CHX to explore whether PLAC8 affects PD-L1 stability. As shown in figure 4F,G, PLAC8 depletion decreased the steady-state levels of PD-L1 stability. This effect was blocked by treatment with the lysosome inhibitor chloroquine but not the proteasome inhibitor MG132 (figure 5B). In addition, we examined the endogenous interaction between PLAC8 and PD-L1 in MDA-MB-231 and HCC-1937 cells and confirmed that PLAC8 interacts with PD-L1 (figure 5C). Next, we examined whether PLAC8 regulates the ubiquitination of PD-L1. As shown in figure 5D, we found increased polyubiquitination of PD-L1 in PLAC8 knockdown cells. Overall, these data indicated that PLAC8 regulates PD-L1 ubiquitination in TNBC.

PLAC8 regulates the immune response in TNBC by impacting PD-L1 levels

Flow cytometry demonstrated that knockdown of PLAC8 causes decreased expression of PD-L1 on the surface of MDA-MB-231 and HCC-1937 cells (figure 6A). We next aimed to identify the function of PLAC8 in immune regulation, therefore, we cocultured MDA-MB-231 and HCC-1937 cells with Jurkat cells. ELISA results showed that knockdown of PLAC8 in TNBC cells promotes the secretion of IL-2 by Jurkat cells (figure 6B,C). Altogether, our experiments indicated that high PLAC8 expression in TNBC cells promotes cell proliferation and inhibits T lymphocyte activity by upregulating PD- L1 expression. To confirm the function of the PLAC8/PD-L1 axis in tumor growth in vivo, we implanted cells into the mammary fat pads of immunocompetent BALB/c mice, and the tumor volume was measured twice a week. Our results showed that PLAC8 knockdown significantly decreases the tumor size (figure 6D,E). IHC staining of the tumor tissues demonstrated that the expression of PD-L1 is decreased and $CD3^+CD8^+$ T cell infiltration is induced when PLAC8 was knocked down (figure 6F), supporting that PLAC8 modulates the immune response in a mouse model by regulating PD-L1 expression.

PLAC8 expression positively correlates with PD-L1 levels in TNBC

We further assessed the clinical significance of the PLAC8-mediated PD-L1 pathway in TNBC patients. While examining PLAC8 and PD-L1 protein expression in human non-triple-negative breast and TNBC tissues, we observed that a higher percentage of triple-negative patients with breast cancer have elevated PLAC8 and PD-L1 expression (figure 7A–C). We then evaluated the potential association between PLAC8 and PD-L1 protein levels in TNBC samples by IHC. We also observed a significant correlation between the expression levels of PLAC8 and PD-L1

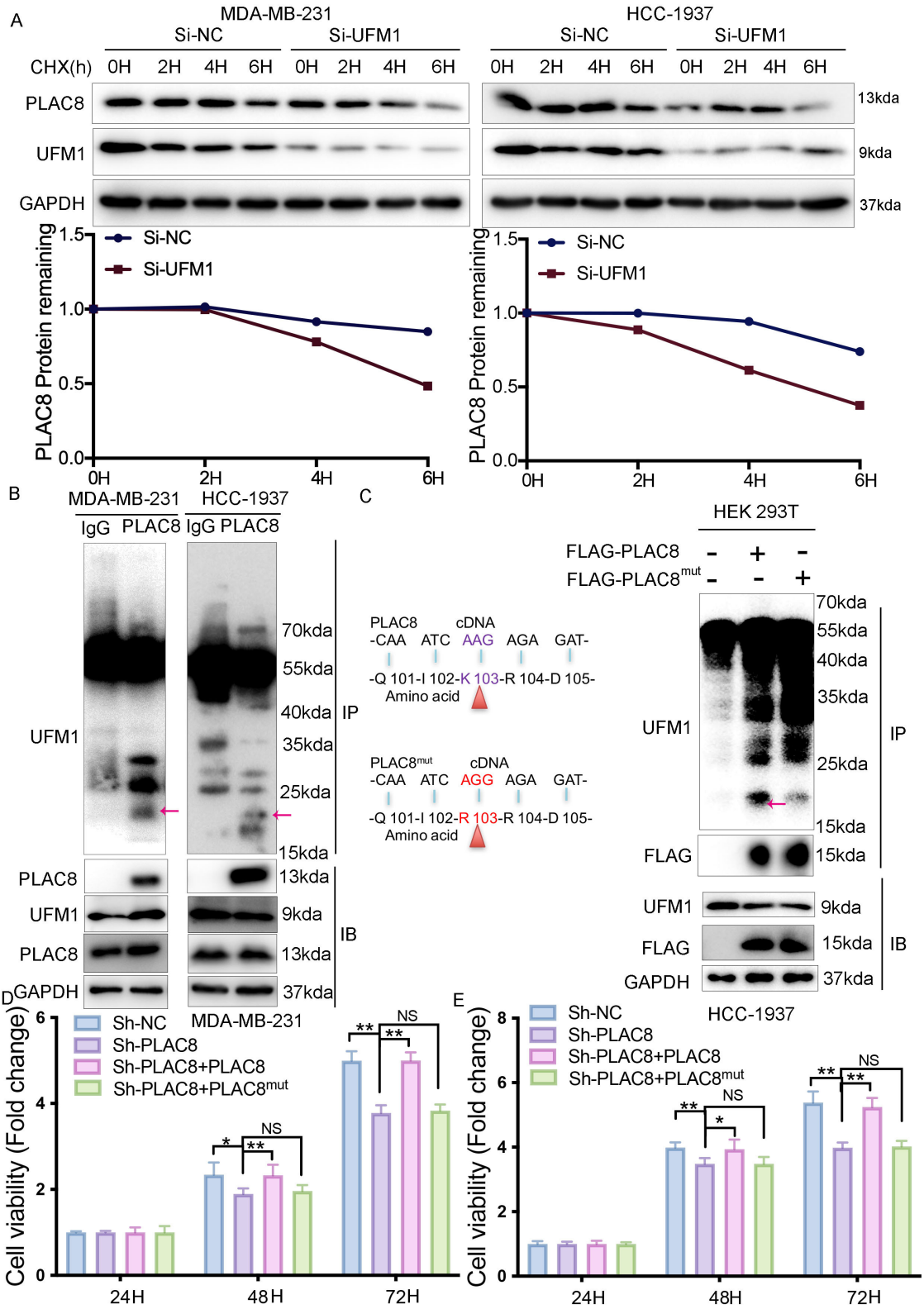


Figure 3 UFMylation stabilizes the PLAC8 protein. (A) PLAC8 protein expression was examined by western blot in MDA-MB-231 and HCC-1937 cells with UFM1 depletion 72 hours after transfection. The cells were treated with 10 µg/mL cycloheximide (CHX) for the indicated times. PLAC8 protein levels were quantified based on GAPDH. (B) Western blot analysis of PLAC8 UFMylation in MDA-MB-231 and HCC-1937 cells. (C) Schematic diagram of the WT PLAC8 protein and the mutation at L103. HEK 293T cells were transfected with Flag-PLAC8 WT or Flag-PLAC8 (K103R) mutant expression vectors, and PLAC8 UFMylation was determined by western blot. (D) and (E) MDA-MB-231 and HCC-1937 cells stably expressing shPLAC8 and transfected with the Flag empty vector, Flag-PLAC8 or Flag-PLAC8 (K103R) were seeded into plates. Cell proliferation was then determined by CCK-8 assay at 24 hours, 48 hours and 72 hours. Data are presented as the means±SD of three independent experiments. *p<0.05, **p<0.01; NS, not significant; UFM1, ubiquitin-fold modifier 1.

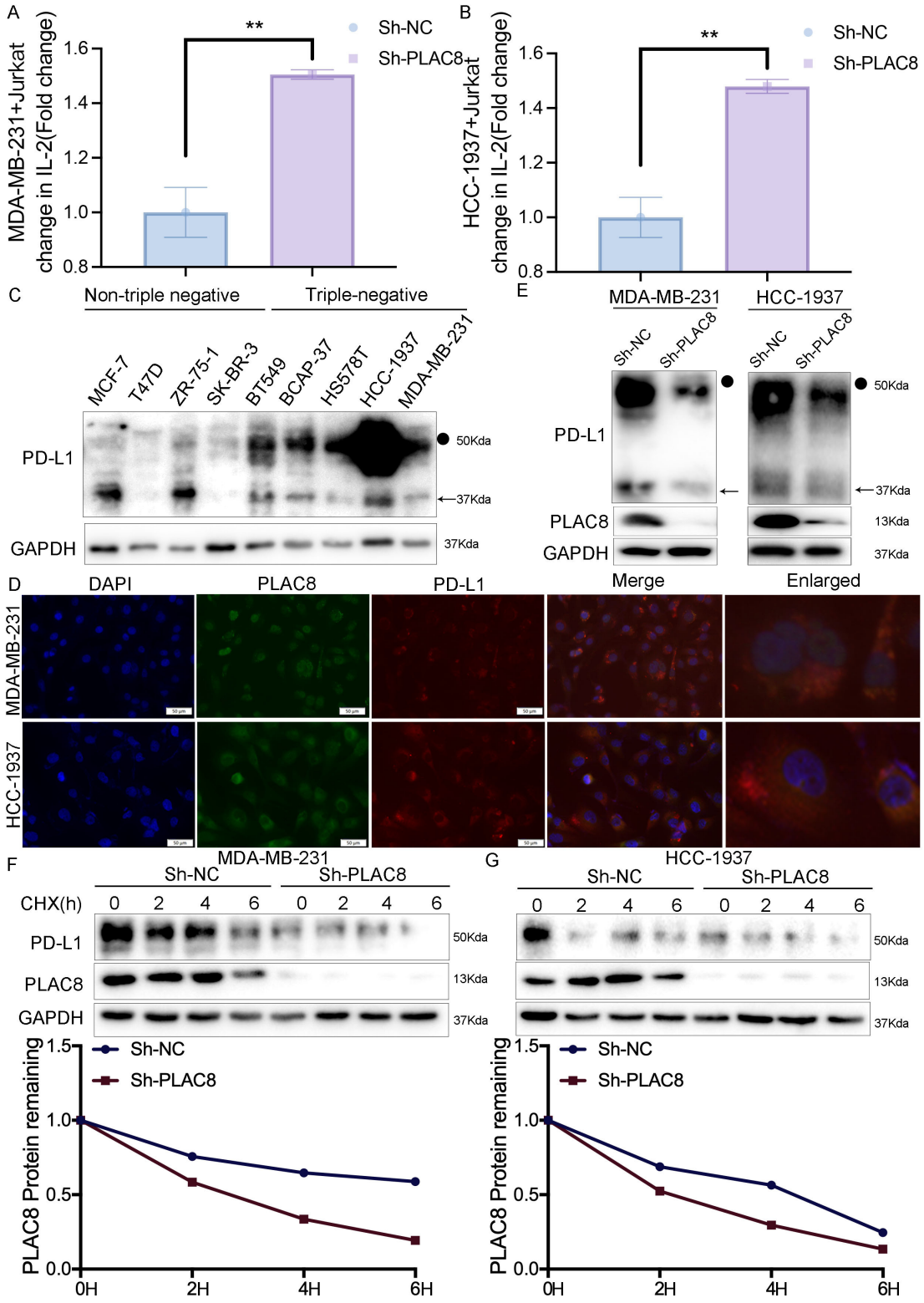


Figure 4 PLAC8 binds to the PD-L1 protein and impacts its stability. (A) and (B) Jurkat cells were cocultured with MDA-MB-231 or HCC-1937 cells with or without PLAC8 depletion for 48 hours, and IL-2 secretion was detected by ELISA. (C) Western blot analysis of PD-L1 expression in breast cancer cell lines (MCF-7, T47D, ZR-75–1, SK-BR-3, BT549, BCAP-37, HS578T, HCC-1937 and MDA-MB-231). (D) Intracellular localization analysis of PLAC8 (green) and PD-L1 (red) in MDA-MB-231 and HCC-1937 cells by immunofluorescence assay. Nuclei (blue) were stained with DAPI. Scale bar represents 50μm. (E) Western blot analysis of PLAC8 and PD-L1 in MDA-MB-231 and HCC-1937 cells with PLAC8 depletion 72 hours after transfection. (F) and (G) PD-L1 protein stability was examined by western blot in MDA-MB-231 and HCC-1937 cells with PLAC8 depletion 72 hours after transfection. The cells were treated with 10 μg/mL cycloheximide (CHX) for the indicated times. PD-L1 protein levels were quantified based on GAPDH. Data are presented as the means±SD of three independent experiments. **p<0.01.

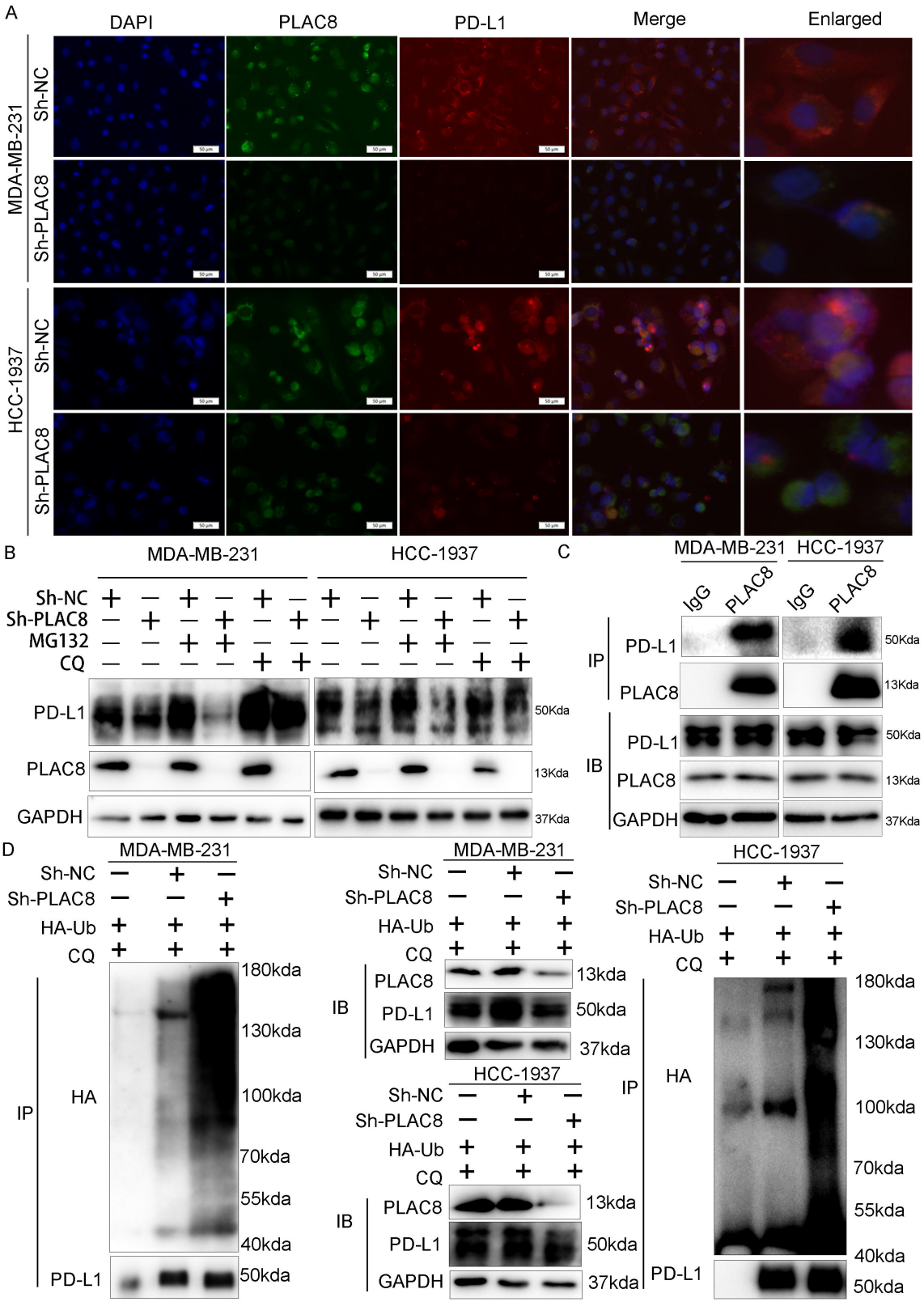


Figure 5 PLAC8 regulates PD-L1 ubiquitination. (A) Intracellular localization analysis of PLAC8 (green) and PD-L1 (red) in MDA-MB-231 and HCC-1937 cells with PLAC8 depletion by immunofluorescence assay. Nuclei (blue) were stained with DAPI. Scale bar represents 50μm. (B) Western blot analysis of PD-L1 expression in MDA-MB-231 and HCC-1937 cells with or without PLAC8 depletion in the presence of the proteasome inhibitor MG132 (10 μM, 6 hours) or the lysosome inhibitor chloroquine (10 μM, 24 hours). (C) Endogenous PD-L1 was analyzed by immunoprecipitation with a PLAC8 antibody in MDA-MB-231 and HCC-1937 cells. (D) MDA-MB-231 and HCC-1937 cells were transfected with HA-Ub plasmid for 48 hours and treated with chloroquine (10 μM, 24 hours). Ubiquitination of endogenous PD-L1 was analyzed by immunoprecipitation with a PLAC8 antibody and followed by western blot analysis in MDA-MB-231 and HCC-1937 cells with or without PLAC8 depletion.

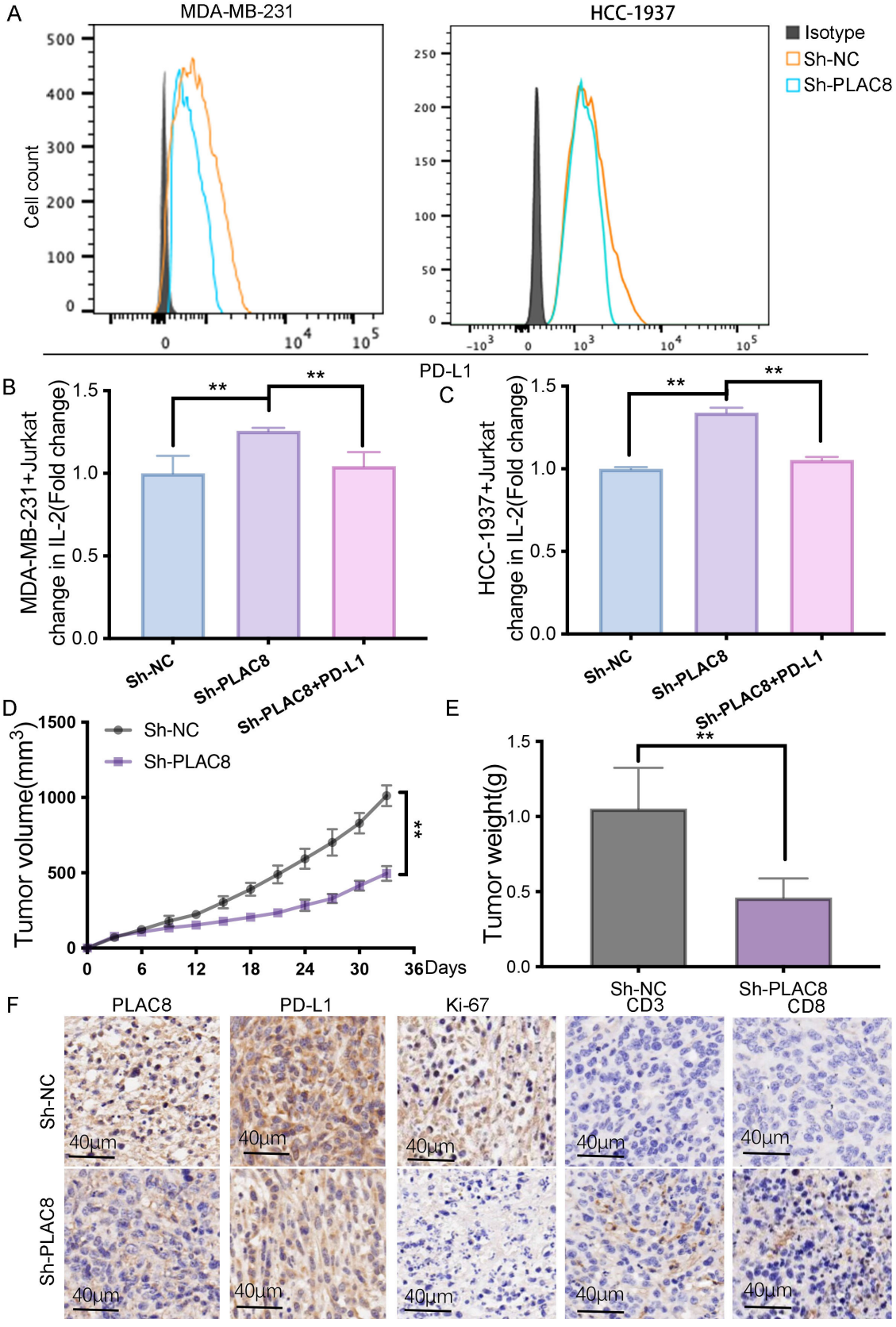
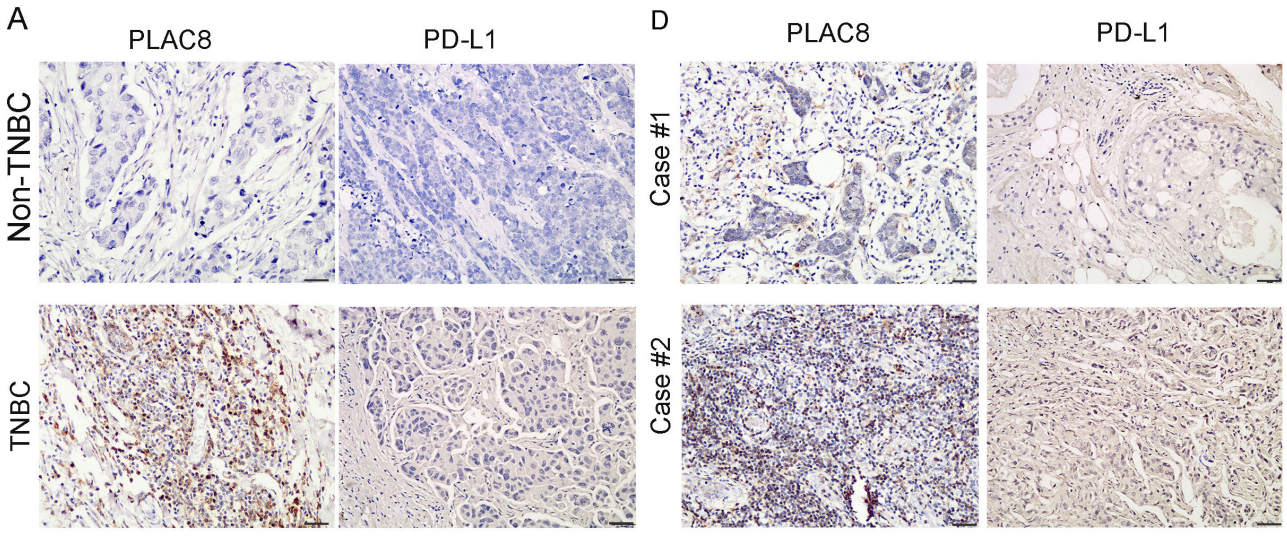


Figure 6 Knockdown of PLAC8 affects the cancer immune response by suppressing PD-L1 expression in triple-negative breast cancer. (A) Flow cytometry plots measuring PD-L1 expression on the cell membrane of MDA-MB-231 and HCC-1937 cells with or without PLAC8 depletion. (B) and (C) Jurkat cells were cocultured with MDA-MB-231 or HCC-1937 cells with or without PLAC8 depletion for 48 hours, and IL-2 secretion was detected by ELISA. (D) Tumor volume and (E) tumor weight in mice. (F) IHC staining of PLAC8, PD-L1, Ki-67, CD3 and CD8 in tumor tissues. Scale bar represents 40μm. Data are presented as the means±SD of three independent experiments. **p<0.01; IHC, immunohistochemical.



B

Subtype	PLAC8 expression		Total	
	Low	High		
Non-TNBC	19	11	30	P=0.044
TNBC	35	53	88	
Total	54	64	118	

C

Subtype	PD-L1 expression		Total	
	Negative	Positive		
Non-TNBC	21	9	30	P<0.001
TNBC	30	58	88	
Total	51	67	118	

E

PD-L1 expression	PLAC8 expression		Total	
	Low	High		
Negative	18	12	30	P=0.005
Positive	17	41	58	
Total	35	53	88	

Figure 7 Correlations between PLAC8 and PD-L1 expression in triple-negative breast cancer (TNBC). (A) IHC staining of PLAC8 and PD-L1 proteins in human non-triple-negative breast cancer and TNBC samples. (B) and (C) Correlation analysis between PLAC8 and PD-L1 expression in human non-triple-negative breast cancer and triple-negative breast cancer status. (D) and (E) Correlation analysis between PLAC8 expression and PD-L1 expression in triple-negative breast cancer. Scale bar represents 50μm.

(figure 7D,E). Altogether, these results suggested that the PLAC8-mediated expression of PD-L1 can be physiologically significant and may be clinically relevant in TNBC patients.

DISCUSSION

This study provides a plausible rationale for developing therapeutic interventions involving the PLAC8 pathway for managing TNBC progression, and experimental and clinical evidence supports a potentially novel and interesting mechanism in TNBC. Here, we have shown that UFMylation of PLAC8 can potentially influence tumorous promotion and immune response in TNBC cells by reducing PD-L1 ubiquitination. To our knowledge, this is the first study examining the molecular function of PLAC8 protein regulation by UFMylation in TNBC. The PLAC8 protein was responsible for the glycosylation-dependent stabilization of PD-L1.

PLAC8 is a cysteine-rich protein that has been found to play an important role in a variety of tumors.[16 17] However, the precise role of PLAC8 in cancer cells is still complicated, due in large part to the fact that current studies have demonstrated that PLAC8 displays multifaceted functions in different cancers and that specific targets of PLAC8 are less well understood in cancer. Our previous study found that PLAC8 can inhibit breast cancer cell apoptosis,[8] and PLAC8 is involved in multidrug resistance in breast cancer.[9] Taken together, these data demonstrate that PLAC8 is an important regulator of breast cancer progression. It remains largely unknown whether and how PLAC8 contributes to TNBC progression. In this study, we first analyzed two different breast cancer databases, including the Oncomine and GEO databases, and found that PLAC8 is more highly expressed in TNBC than in other types of breast cancer. In addition, we measured PLAC8 protein and mRNA levels in breast cancer cell lines by western blot and RT–PCR, respectively, and in breast cancer tissues by immunohistochemical staining, which showed that PLAC8 expression is generally higher in triple-negative patients with breast cancer compared with all types of patients with breast cancer. Further experiments confirmed that PLAC8 can promote TNBC cell growth in vitro and in vivo.

We further discovered the underlying PLAC8 regulatory mechanisms in TNBC. The regulation of protein stability, such as through the ubiquitin–proteasome pathway,[18–20] is a critical issue central to the comprehension of the molecular basis of carcinogenesis. Previously, we identified that PLAC8 can be ubiquitinated by curcumin.[9] To date, the post-translational regulation of PLAC8 is not fully understood, which indicates that an in-depth study of PLAC8 post-translational modifications can help us to understand the related molecular mechanisms of this protein in breast cancer. UFMylation is a recently identified ubiquitin-like modification with essential biological functions.[21] It was reported that UFMylation of the MRE11 protein promotes ATM activation and serves as a therapeutic target.[22] UFM1 conjugates to target proteins via the E1 and E2-like enzymes UBA5 and UFC1, respectively, and the E3 ligase UFL1.[23 24] UFM1 also plays an important role in breast cancer.[13] For example, the ASC1 protein is modified by UFM1 to participate in the transcriptional activation of ERalpha and regulates the development of breast cancer.[23] Our previous study identified SLC7A11 as a new UFMylation substrate in breast cancer.[14] All of these studies support that UFMylation might be a key regulatory factor in breast cancer. In this study, we first determined that the PLAC8 protein expression decreases when UFM1 is knocked down. Then, we confirmed that UFM1 contributes to PLAC8 protein stability. To identify the UFMylation site in PLAC8, we generated a lysine-to-arginine (Lys-to-Arg) mutation at lysine 103 and confirmed that PLAC8 is UFMylated at K103. These results clarified a novel mechanism of PLAC8 post-translational regulation in TNBC.

As most triple-negative patients with breast cancer are still refractory or nonresponsive to immune checkpoint therapies, identifying immunotherapies and combination strategies is a major priority.[25] Over the past decade, researchers have reported that PLAC8 might be an immune-related gene in cancer.[26 27] PLAC8 overexpression inhibits the activation of T cells, which suggests that PLAC8 might be an immunosuppressive factor regulating the immune response in TNBC. PD-L1 has been identified as a promising checkpoint in cancer, and several studies have also implied that the PD-L1 protein is highly expressed in TNBC.[15 28 29] PD-L1 is constitutively expressed on the surface of cancer cells and interacts with PD-1, which is expressed on immune cells.[30 31] The IMPASSION130 clinical trial showed OS improvement with atezolizumab (an anti-PD-L1 antibody) plus nab-paclitaxel versus placebo plus nab-paclitaxel in the PD-L1 immune cell-positive TNBC population, with a median increase of 7.5 months.[32] However, another clinical trial, IMPASSION131, did not show a significant benefit from combining atezolizumab with paclitaxel, which was in contrast with the findings from the IMPASSION130 trial of this first-line immune checkpoint blockade for metastatic TNBC.[33] The reasons for this difference remain undefined and indicate that PD-L1 might be a promising predictive biomarker in TNBC. Therefore, exploring the underlying molecular mechanism of PD-L1 expression may provide more clinical evidence for immunotherapies in TNBC. In our study, PD-L1 expression was relatively higher in TNBC cells and tissues. This led us to speculate that there might be a correlation between PLAC8 and PD-L1 expression in TNBC and that PLAC8 may regulate PD-L1 expression. Breast cancer cells and tissues showed that the level of PLAC8 positively correlates with the expression of PD-L1. We next determined the precise regulatory mechanism between PLAC8 and PD-L1. Although the 33 kDa non-glycosylated form of PD-L1 can be detected in cells, the majority of PD-L1 is glycosylated in TNBC, with a heterogeneous molecular weight ranging from 45 to 55 kDa.[34] Additionally, glycosylation of PD-L1

stabilizes the PD-L1 protein in cancer cells and regulates the PD-L1/PD-1 interaction.[34] Further experiments in our study confirmed that the glycosylated PD-L1 protein, which was detected at approximately 50 kDa, is the major form found in TNBC cells and that knockdown of PLAC8 mainly reduced the level of glycosylated PD-L1. After knocking down PLAC8, PD-L1 protein levels decreased, but the level of PD-L1 mRNA did not change. Coimmunoprecipitation experiments suggested that PLAC8 binds to PD-L1. Post-translational modifications have emerged as an important aspect of PD-L1 biology in different cancers.[35] The PD-L1 protein can be deubiquitinated and ubiquitinated by various proteins, such as USP22, CSN5 and ARIH1.[29 36 37] Ubiquitin ligases regulate the stability of proteins that are marked by ubiquitination and trafficked to the proteasome or lysosome for degradation.[38] Therefore, we assessed whether PLAC8 can impact the ubiquitination of PD-L1. We treated MAD-MB-231 and HCC-1937 cells with MG-132, which can inhibit proteasome activity, and chloroquine, which can inhibit lysosome activity. Unexpectedly, the reduction in PD-L1 protein induced by PLAC8 knockdown was rescued by chloroquine. Together these data demonstrate that PLAC8 stabilizes the PD-L1 protein via regulating its ubiquitination.

We also began to characterize the molecular functions of PLAC8 in TNBC. On the one hand, PLAC8 could promote TNBC cell growth. On the other hand, our functional assays showed that knockdown of PLAC8 reduces the cell surface expression of PD-L1 and drastically reduces the immunosuppression of T cells, confirming that PLAC8 plays an immunosuppressive role through upregulating PD-L1 protein expression in TNBC.

CONCLUSIONS

In summary, we first illuminate that PLAC8 regulates PD-L1 ubiquitination, which promotes PD-L1 expression in TNBC. Additionally, PLAC8 itself can be modified by UFM1, and this modification maintains its stability. The PLAC8-regulated network can affect tumorous proliferation and immune response in TNBC. Our research conclusions can provide a basis for novel clinical diagnosis and immune treatment options for TNBC, although further investigations are needed to validate our findings.

Contributors LBW is responsible for the overall content as the guarantor. MSM, YXC and LBW have designed the study. MSM, YXC and JJY have performed the experiments and acquired data. MSM and LBW have written the manuscript. JJY, YFC, LX, FYJ, JCZ, SWJ, ZQL, CC, JHZ and XZ edited the manuscript. All authors approved the final version of the manuscript.

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Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s).

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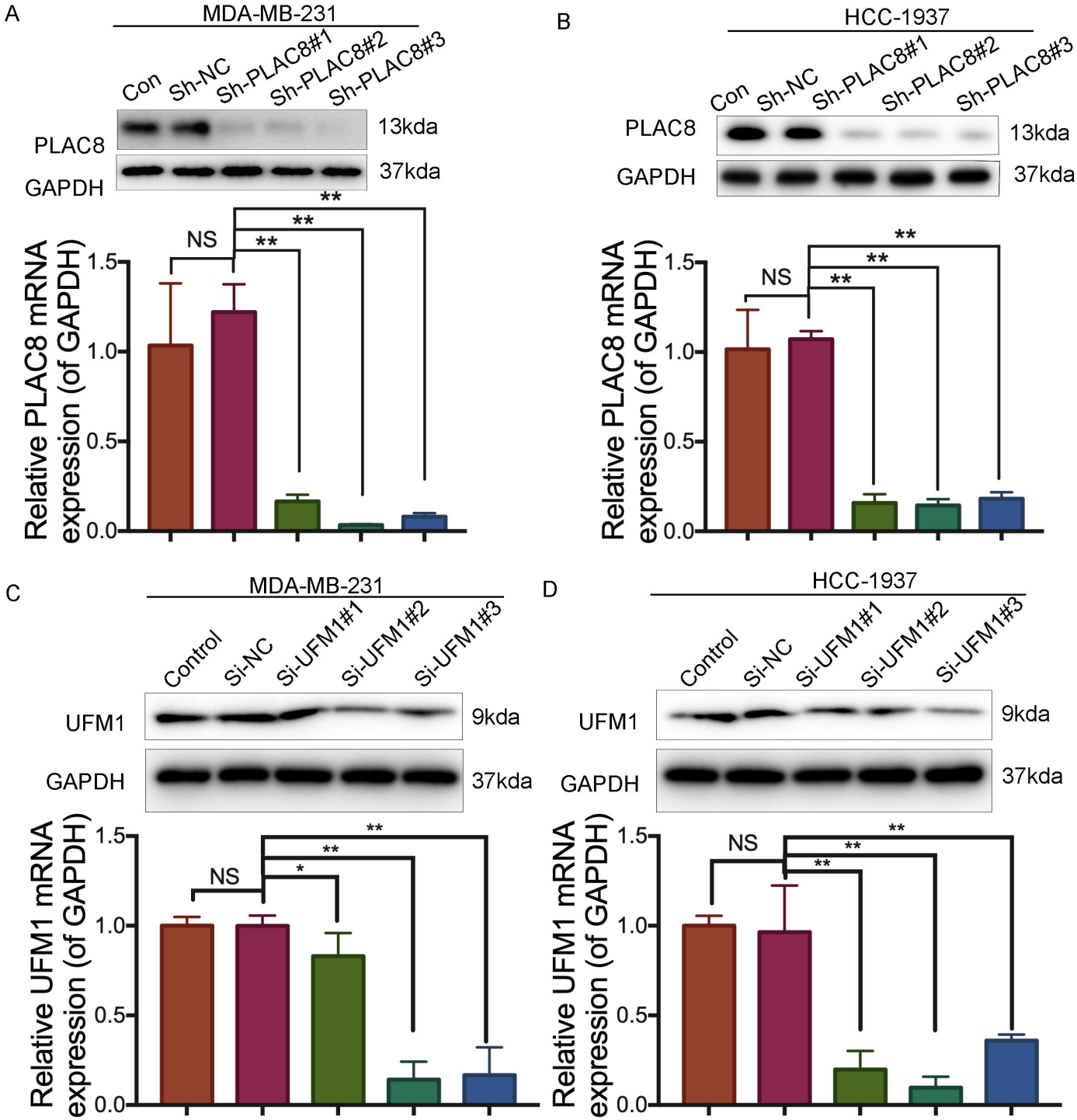
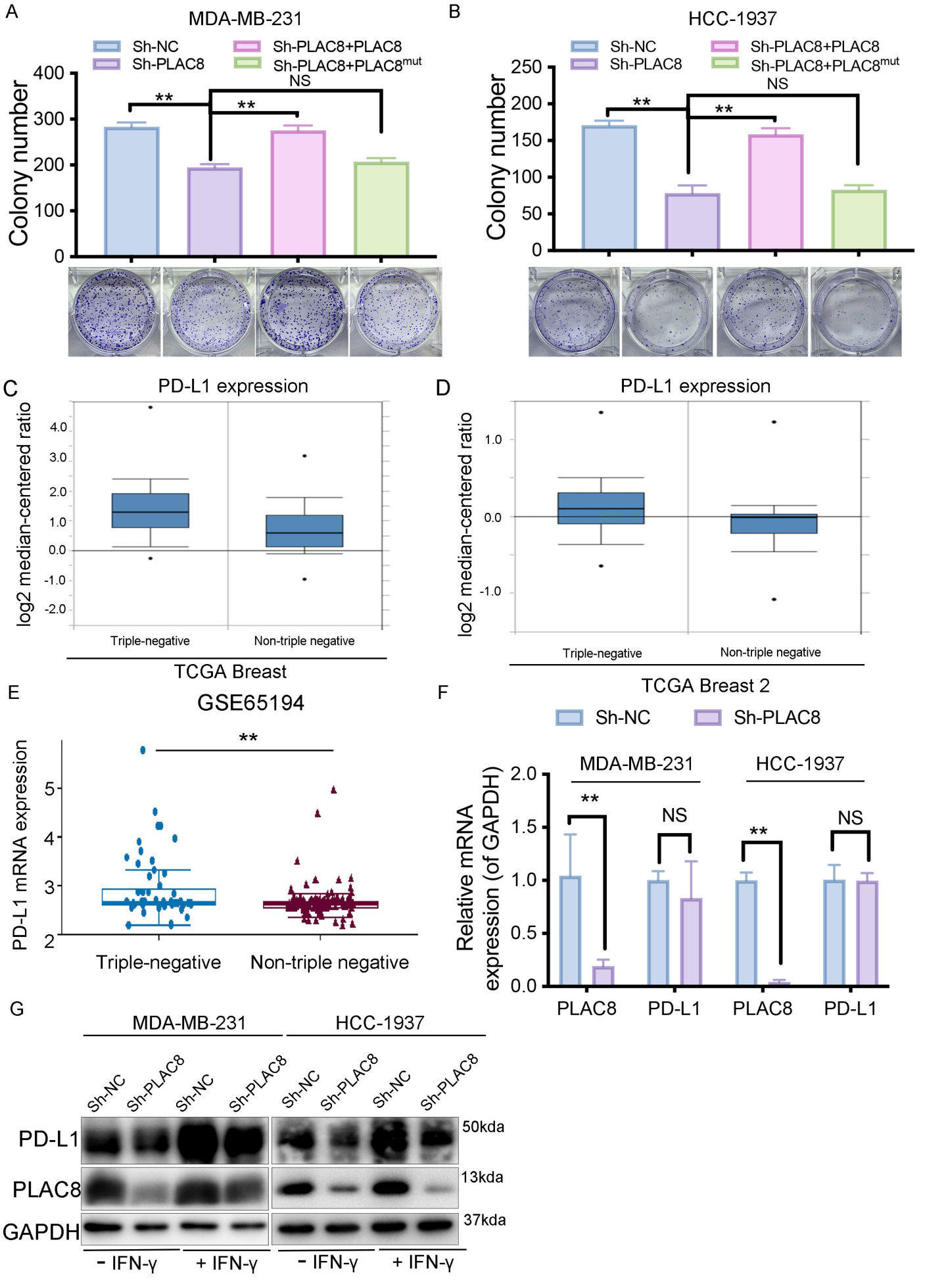

A
MDA-MB-231
Con
Sh-NC
Sh-PLAC8#1
Sh-PLAC8#2
Sh-PLAC8#3
PLAC8
13kda
GAPDH
37kda
Relative PLAC8 mRNA expression (of GAPDH)
NS
**
B
HCC-1937
C
MDA-MB-231
Control
Si-NC
Si-UFM1#1
Si-UFM1#2
Si-UFM1#3
UFM1
9kda
Relative UFM1 mRNA expression (of GAPDH)
*
D
HCC-1937


A
MDA-MB-231
Sh-NC
Sh-PLAC8
Sh-PLAC8+PLAC8
Sh-PLAC8+PLAC8mut
Colony number
400
300
200
100
0
**
**
NS
B
HCC-1937
Sh-NC
Sh-PLAC8
Sh-PLAC8+PLAC8
Sh-PLAC8+PLAC8mut
Colony number
250
200
150
100
50
0
**
**
NS
C
PD-L1 expression
log2 median-centered ratio
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
Triple-negative
Non-triple negative
TCGA Breast
D
PD-L1 expression
log2 median-centered ratio
1.0
0.0
-1.0
Triple-negative
Non-triple negative
TCGA Breast 2
E
GSE65194
PD-L1 mRNA expression
5
4
3
2
**
Triple-negative
Non-triple negative
F
Sh-NC
Sh-PLAC8
MDA-MB-231
HCC-1937
Relative mRNA expression (of GAPDH)
2.0
1.5
1.0
0.5
0.0
**
NS
**
NS
PLAC8
PD-L1
PLAC8
PD-L1
G
MDA-MB-231
HCC-1937
Sh-NC
Sh-PLAC8
Sh-NC
Sh-PLAC8
Sh-NC
Sh-PLAC8
Sh-NC
Sh-PLAC8
PD-L1
PLAC8
GAPDH
50kda
13kda
37kda
- IFN-γ
+ IFN-γ
- IFN-γ
+ IFN-γ

Supplementary Figure 1. The expression of PLAC8 in (A) MAD-MB-231 and (B) HCC-1937 cells transfected with or without PLAC8 shRNAs was explored by western blot analysis 72 h after transfection and RT–PCR analysis 48 h after transfection. The expression of UFM1 in (C) MAD-MB-231 and (D) HCC-1937 cells transfected with or without UFM1 siRNAs was explored by western blot analysis 72 h after transfection and RT–PCR analysis 48 h after transfection. Data are presented as the means ± SD of three independent experiments. *p < 0.05; **p < 0.01; ns, not significant.

Supplementary Figure 2. (A) and (B) Clonogenic survival assays were performed. (C) and (D) PD-L1 mRNA expression in TNBC and non-TNBC samples in the TCGA breast and TCGA breast 2 databases. (E) The relative levels of PD-L1 mRNA in the GSE65194 database. (F) RT–PCR analysis of PLAC8 and PD-L1 mRNA in MDA-MB-231 and HCC-1937 cells with PLAC8 depletion 48 h after transfection. (G) PLAC8 and PD-L1 protein expression in stable PLAC8 knockdown cells treated with or without IFN-γ was detected by western blot. Data are presented as the means ± SD of three independent experiments. *p < 0.05; **p < 0.01; ns, not significant.