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Deubiquitinating Enzyme MINDY1 Facilitates Immune Escape in Breast Cancer by Maintaining the Stability of Immune Checkpoint Protein PD-L1

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Featured with frequent recurrence and distant metastasis, the mortality of breast cancer (BC) increased year by year in recent decades. MINDY lysine 48 deubiquitinase 1 (MINDY1) played an oncogenic role in several tumors. The role of MINDY1 deserved further study in BC. A wide ranges of assays including qRT-PCR, Western blotting, CCK-8 flow cytometry analysis, co-immunoprecipitation assay, Pearson's coefficient tests, and tumor xenograft assay were designed and carried out to determine the role of MINDY1 in BC. Both MINDY1 and programmed death-ligand 1 (PD-L1) is upregulated in BC tissues and cells. In addition, knockdown of MINDY1 attenuated cell viability and promoted the activation of T cells. Mechanistically, MINDY1 stabilized PD-L1 protein by interacting with PD-L1 in BC cells, and MINDY1 is positively associated with PD-L1 in BC tissues. Moreover, rescue assay revealed that the effect of MINDY1 silencing on cell viability and T cell activation was reversed by PD-L1 overexpression. The *in vivo* study also demonstrated that the effect of MINDY1 knockdown on tumor growth induced by was counteracted by PD-L1 overexpression. In conclusion, we identified PD-L1 as a novel target of MINDY1 and established a significant association between MINDY1 and the cancer immune response. Importantly, our findings reveal that MINDY1 promoted BC progression via PD-L1-mediated immune evasion.

Keywords: breast cancer; immune escape; MINDY lysine 48 deubiquitinase 1; PD-L1
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Introduction

Breast cancer (BC) is a prevalent gynecological neoplasm characterized by the massive proliferation of mammary epithelial cells (Keenan and Tolaney 2020; Yin et al. 2020). In China, BC has the highest incidence among female malignant tumors and continues to increase annually with over 300,000 women being diagnosed each year, which emerged as a significant public health concern, exerting a profound impact on women's well-being, the national economy, and societal progress (Howard and Olopade 2021). Histologically, BC can be categorized into 4 subtypes: luminal A, luminal B, HER2-enriched, and triple-negative (Borri and Granaglia 2021; Derakhshan and Reis-Filho 2022). Among these subtypes, triple-negative breast cancer was regarded as the most aggressive form characterized by a higher propensity for recurrence and metastasis (Zou et al. 2022). To date, limited targeted therapies have demonstrated efficacy against BC (Leon-Ferre and Goetz 2023). Hence, it is imperative to investigate innovative and efficacious therapeutic targets in order to enhance the diversity of therapeutic approaches.

The programmed cell death-1 receptor (PD-1), located on the surface of immune effector cells, serves as an inhibitor of immune checkpoint (Jiang et al. 2019). In the context of cancer, upregulation of programmed death-ligand 1 (PD-L1) expression appears to be a prominent mechanism facilitating immune evasion (Dermani et al. 2019). Numerous

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studies have demonstrated the efficacy of blocking PD-L1 using specific antibodies such as pembrolizumab or atezolizumab (Jiang et al. 2019; Hu et al. 2021). However, in the early stages of BC, patients who received a combination of immune checkpoint inhibitors and neoadjuvant chemotherapy exhibited significantly complete pathological responses (Jalalvand et al. 2021). Therefore, studying the upstream regulatory mechanisms of PD-L1 expression in BC is of great significance for further understanding the function of this immunosuppressive molecule.

Ubiquitination, a crucial post-translational modification for maintaining cellular homeostasis, has been increasingly implicated in various processes including DNA repair, cell cycle progression, apoptosis, and antigen presentation (Li et al. 2016; Lim et al. 2016). Given its profound impact on cellular processes, the regulation of ubiquitylation is governed by specialized Ub proteases known as deubiquitinases (DUBs) (Zhu et al. 2021; Dong et al. 2023). MINDY lysine 48 deubiquitinase 1 (MINDY1), also known as FAM63A, is a member of the motif interacting with ubiquitin (MIU) containing novel DUB family, which is identified as a cysteine protease DUBs (Abdul Rehman et al. 2016; Kristariyanto et al. 2017). MINDY1 functions as an exo-DUB, displaying remarkable specificity in cleaving long K48-linked polyUb chains (Kristariyanto et al. 2017). Previous studies have reported the oncogenic role of MINDY1 in several malignancies (Luo et al. 2021; Tang et al. 2021). To be specific, MINDY1 promotes cell growth and cell cycle progression by stabilizing YAP in bladder cancer (Luo et al. 2021). Besides, MINDY1 facilitates tumor growth *in vitro* and *in vivo* by stabilizing estrogen receptor α (Tang et al. 2021). Nevertheless, the role of MINDY1 in BC requires further research.

In the current research, we proposed a hypothesis that MINDY1 facilitates immune escape by maintaining the stability of immune checkpoint protein PD-L1 in BC.

Materials and Methods

Clinical samples

The BC tissues (n = 35) and adjacent non-tumor tissues (n = 35) were obtained from patients undergoing surgery at the Yiling People's Hospital. Prior to the operation, none of the participants had undergone radiosurgery or chemosurgery. The clinical tissues were stored in liquid nitrogen at −80°C for qRT-PCR analysis. This study was authorized by the Ethics Committee of Yiling People's Hospital, and all participants provided written informed consent. The association of MINDY1 with clinical features of breast cancers patients were shown in Table 1.

Cells

Human breast epithelial cells (MCF-10A) and BC cells (MCF-7 and MDA-MB-231) were obtained from the Cell Resource Center (Shanghai, China) and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100-U/mL of penicillin, 100-μg/mL of streptomycin and 0.05-mg/mL of gentamicin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. T cells were isolated from blood samples of healthy controls. The heparin anticoagulant was added to remove serum. Then, the red blood cell lysis buffer was added to lyse haemocytes for 10 min at room tem-

Table 1. Association of MINDY1 with clinical features of breast cancers.

Characteristics	Cases (n=35)	MINDY1 level		*P* value
		High (n=18)	Low (n=17)	
Age, years				NS
< 65	15	7	8	
≥ 65	20	11	9	
Tumor size				NS
< 2 cm	17	8	9	
≥ 2 cm	18	10	8	
Tumor grade				NS
I + II	21	11	10	
III + IV	14	7	7	
Tumor stage				NS
T1 + T2	16	9	7	
T3 + T4	19	9	10	
Estrogen receptor status				*$p < 0.05$
Positive	24	13	11	
Negative	11	5	6	
Lymph node metastasis				NS
Present	18	9	9	
Absent	17	9	8	

NS means non-significant.

Table 2. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'→3')
Human PD-L1 forward	TGCCGACTACAAGCGAATTACTG
Human PD-L1 reverse	CTGCTTGTCCAGATGACTTCGG
Human MINDY1 forward	ACTCCAGTGACACCAACCTCGT
Human MINDY1 reverse	TCAGCTCACACAGTCCGTGGTA
Human GAPDH forward	GTCTCCTCTGACTTCAACAGCG
Human GAPDH reverse	ACCACCCTGTTGCTGTAGCCAA
Human beta-actin forward	CATTGCTGACAGGATGCAGAAGG
Human beta-actin reverse	TGCTGGAAGGTGGACAGTGAGG

perature. The supernatant was removed after centrifugation for 5 min. Cell pellets were then washed and resuspended in 5 mL of bioclean phosphate buffer saline solution, and cell number was counted. At last, CD3 positive cells were selected using flow cytometry.

Plasmids and transfection

Small interfering RNA (siRNA) targeting MINDY1 (siMINDY1#1 and siMINDY1#2) and negative control (si-NC) were purchased from Ruibo Biotechnology (Guangzhou, China). The full length of PD-L1 were inserted into pcDNA3.1 vector to overexpress PD-L1 with empty vector as control. Wild type (WT) and mutant MINDY1 plasmids were purchased from Hanbio Biotechnology (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for plasmid transfection.

qRT-PCR

Total RNA was extracted from BC tissues, adjacent tissues, and cells using TRIzol reagent (Life Technologies, USA). The extracted RNA was reversely transcribed into complementary DNA (cDNA) utilizing the ReverTra Ace qPCR RT kits (Toyobo, Japan). Then, in consonance with the manufacturer's directions, RT-qPCR was performed using the SYBR® Premix Ex TaqTM II reagent kit (RR820A, Takara) on an ABI7500 real-time qPCR system (7500, ABI Company, Oyster Bay, NY, USA). RNA expression was calculated by $2^{-\Delta\Delta Ct}$ method, and GAPDH and β-actin served as internal controls. Sequences of primers were presented in Table 2.

Western blotting

Protein was isolated from MCF-10A, MCF-7 and MDA-MB-231 cells using RIPA lysis buffer. The protein sample was transferred into PVDF membrane after separated by 10% SDS-polyacrylamide gel electrophoresis. Subsequently, the membranes were blocked with 5% defatted milk and incubated with the primary antibodies at 4°C overnight. The membranes were incubated with the secondary antibody at room temperature for 1 h. The protein bands were visualized by an enhanced chemiluminescence (ECL) kit (Boster, China). The gray value was determined by ImageJ software. The detailed information of specific primary antibodies was provided in Table 3.

Cell counting kit-8 (CCK-8) assay

After transfection, MCF-7 and MDA-MB-231 cells were seeded into the 96-well plate (1×10^4 cells/ml). After incubation for 24, 48 and 72 h, cells in each well were added with 10 μl CCK-8 solution for 2 h incubation at 37˚C. Hereafter, the optical density was detected using a microplate reader (BioTek Instruments, Inc.) at 450 nm wavelength.

Colony formation assay

Colony formation assays are performed under anchorage-independent conditions. After transfection with different plasmids, MCF-7 and MDA-MB-231 cells were seeded into 6-well plates (1,000 cells/well, low-adhesion plates) containing complete media, and the plate was swirled to ensure an even distribution of the cell. The culture medium was changed every 3 days. 14 days later, cells were washed twice with PBS after removing the media. Then, the colonies were immobilized and stained with crystal violet (Beyotime). The images of colonies were observed and photographed by using a digital camera, and the numbers of colonies were counted via Image J software.

In vitro cell co-culture

Total 1×10^5 CFSE (Solabio Life Sciences, Beijing, China)-labelled $CD3^+$ T cells were cocultured with MCF-7 and MDA-MB-231 (the ratio of $CD3^+$ T cells to BC cells is 5:1) in a 96-well plate containing anti-CD3 (2 μg/mL), anti-CD28 (1 μg/mL), and recombinant human Interleukin-2 (rhIL-2, 20 IU/mL). Subsequently, the percentage of proliferated T cells ($CD3^+CD28^+$ T cells) was determined by flow cytometry.

Table 3. Primary antibodies information.

Antibody	Dilution	Number	Source
PD-L1	1:1000	ab205921	Abcam
MINDY1	1:1000	PA5-55825	Invitrogen
Myc	1:1000	ab32072	Abcam
GAPDH	1:1000	ab8245	Abcam

Flow cytometry analysis

The single-cell suspension was resuspended in the staining buffer, Pacific blue-interferon-γ and perCP-CD3 and were added to medium. Following immobilization and permeabilization, cells were then intracellular treated with perCP-conjugated IFN-γ antibody (Catalog #: IC285C, BD Biosciences, Shanghai, China). The BD fluorescent-activated cell sorting canto II instrument was utilized for quantifying the cells, while flow Jo software was employed for data analysis.

Co-immunoprecipitation assay

Cells were treated with NP-40 lysis buffer containing a cocktail of protease inhibitors. The cell lysate was precleared with rabbit IgG for 2 h and subsequently subjected to immunoprecipitation using anti-IgG or anti-MINDY1 overnight at 4°C. Then, Protein A/G PLUS-Agarose beads (Santa Cruz) were added and incubated for another 2 h at 4°C. The immunocomplexes were washed with lysis buffer for three times and separated by western blotting.

Tumor xenograft model

Total 15 BALB/c (nu/nu) mice (female, 20-24 g, 4-week-old) were obtained from Vital River (Beijing, China). All mice were housed in a sterile SPF room (24±2°C, a 12 h light/dark cycle) with ad libitum access to food and water. After transfection with si-NC, si-MINDY1#1, and si-MINDY1#1+PD-L1 lentivirus, MCF-7 cells (1×10^7) were collected and then subcutaneously injected into the mice (5 mice in each group). Tumor volume was recorded from day 7 to day 24 by detecting the tumor length and width. The tumor volume was calculated as (length × width^2)/2. All mice were sacrificed on day 24, and the tumors were isolated, photographed and weighed.

Statistical analysis

The experimental data are expressed as the mean ± standard deviation (SD). All statistical analysis was performed using Graphpad 7.0 software. Student's t test was used for comparison between groups, differences between multiple groups were used one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

Result

MINDY1 is upregulated in BC tissues and cells

We attended to evaluate the role of MINDY1 in BC. According to Fig. 1A, MINDY1 level was increased in BC tissues compared to adjacent non-tumor tissues. Moreover, high MINDY1 level is positively associated with estrogen receptor (ER) status (Table 1). Additionally, the MINDY1 mRNA and protein levels were also higher in MDA-MD-231 and MCF-7 cells than that in MCF-10A cells (Fig. 1B). Taken together, MINDY1 presented high level in BC tissues and cells.

Knockdown of MINDY1 suppressed BC cell proliferation and promoted T cell activation

Considering the high level of MINDY1, we effectively knocked down MINDY1 level by transfection of si-MINDY1#1 or si-MINDY1#2 (Fig. 2A). Moreover, the proliferative ability of MCF-7 and MDA-MD-231 cells was also attenuated by knockdown of MINDY1 (Fig. 2B). Besides, colony formation assay displayed that knockdown of MINDY1 effectively suppressed the proliferation of MCF-7 and MDA-MD-231 cells (Supplementary Fig. S1A). Then, MCF-7 and MDA-MD-231 cells were co-treated with T cells, and flow cytometry analysis demonstrated that the ratio of proliferated T cells (CD3+CD28+T cells) and IFN-γ positive T cells were enhanced by knockdown of MINDY1 (Fig. 2C-F). To summarize, inhibition of MINDY1 attenuated BC cell proliferation and facilitated T cell activation.

MINDY1 maintains the stability of PD-L1

In mechanism, MINDY1 was widely reported to serve as a deubiquitinating enzyme. First, the MINDY1 protein level was significantly inhibited by transfection of si-MINDY1#1 or si-MINDY1#2 (Fig. 3A). Besides, PD-L1 protein level was reduced by silence of MINDY1 in MCF-7 and MDA-MD-231 cells, and this downregulation was reversed by adding MINDY1-WT instead of mutant MINDY1^{C137A} (Fig. 3B). The co-immunoprecipitation assay revealed that endogenous MINDY1 and PD-L1 were

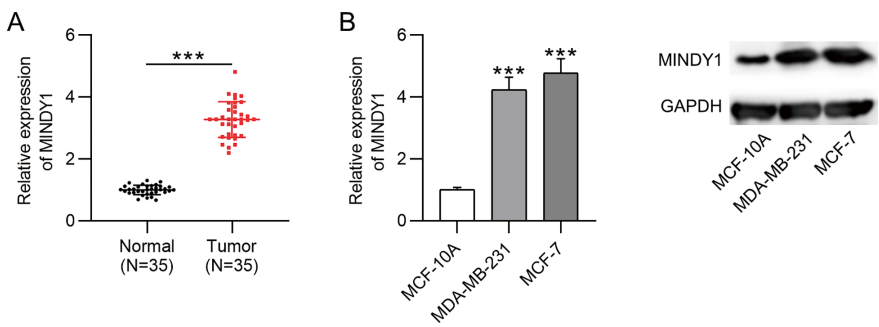

Fig. 1. MINDY1 is upregulated in BC tissues and cells.
(A) The measurement of MINDY1 mRNA level in BC tissues (n = 35) and adjacent tissues (n = 35). (B) The measurement of MINDY1 mRNA and protein levels in MCF-10A, MCF-7 and MDA-MB-231 cells. ***P < 0.001.

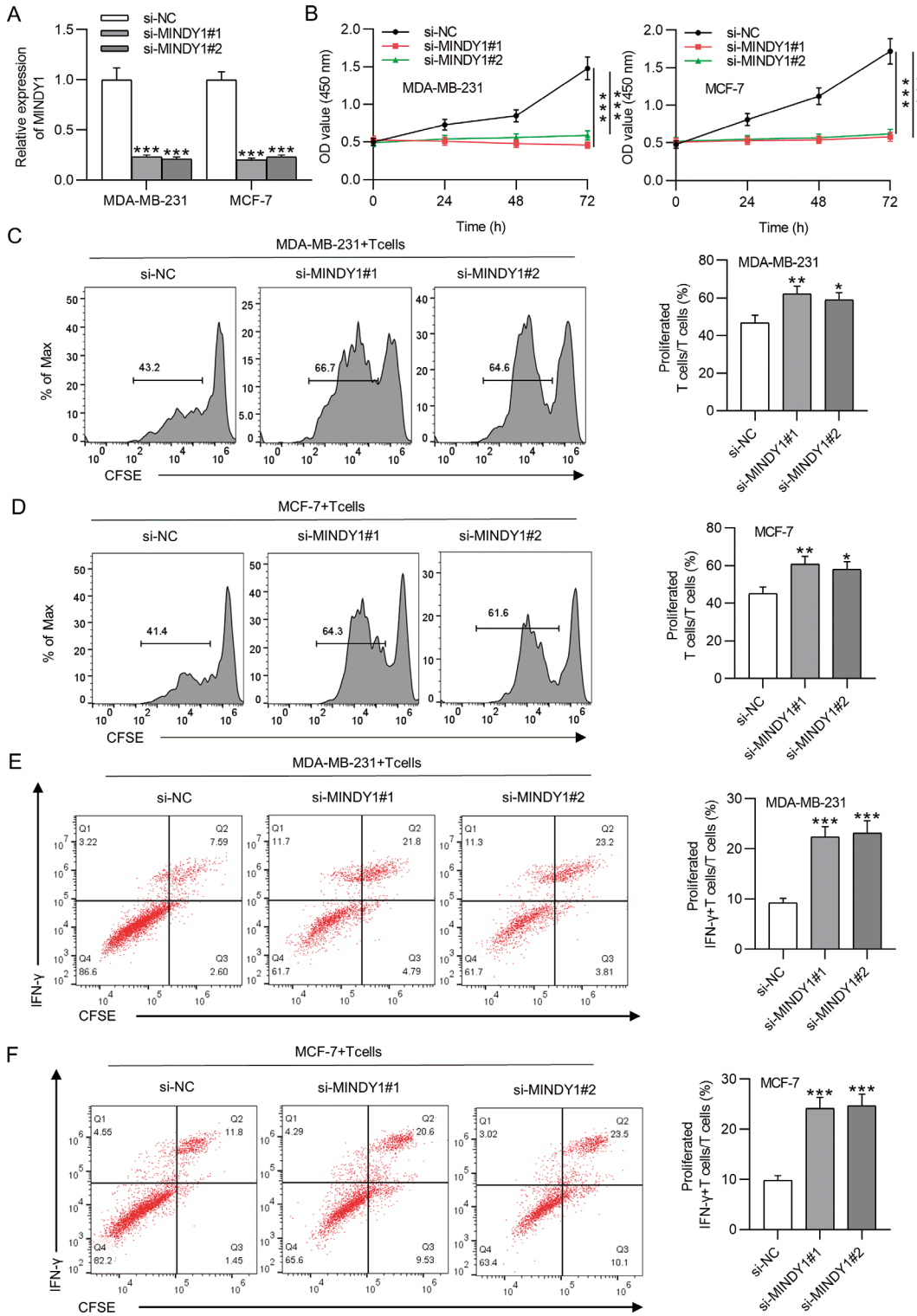

Fig. 2. Knockdown of MINDY1 suppressed BC cell proliferation and promoted T cell activation. (A) The knockdown efficiency of si-MINDY1#1/2 was analyzed by qRT-PCR. (B) Cell viability of BC cells was assessed by CCK-8 assay. (C-F) CD3+ T cells and IFN-γ+ T cells were detected by flow cytometry analysis. *P < 0.05, **P < 0.01, ***P < 0.001.

co-immunoprecipitated from the lysates of MDA-MD-231 and MCF-7 cells, indicating the combination between MINDY1 and PD-L1 (Fig. 3C). However, the qRT-PCR analysis uncovers that MINDY1 did not affect PD-L1 mRNA (Fig. 3D). We then evaluate the effect of MINDY1 on the stability of PD-L1 protein. MDA-MD-231 and MCF-7 cells are treated with cycloheximide and transfected with si-MINDY1#1/2, and we discover that the half-life of

PD-L1 is significantly shortened by knockdown of MINDY1 (Fig. 3E-H). Collectively, MINDY1 maintains the stability of PD-L1 by interacting PD-L1 protein in MDA-MD-231 and MCF-7 cells.

MINDY1 promotes BC cell proliferation and T cell activation by upregulating PD-L1

We then explore the expression and role of PD-L1 in BC. As shown in Fig. 4A, PD-L1 level in BC tissues is

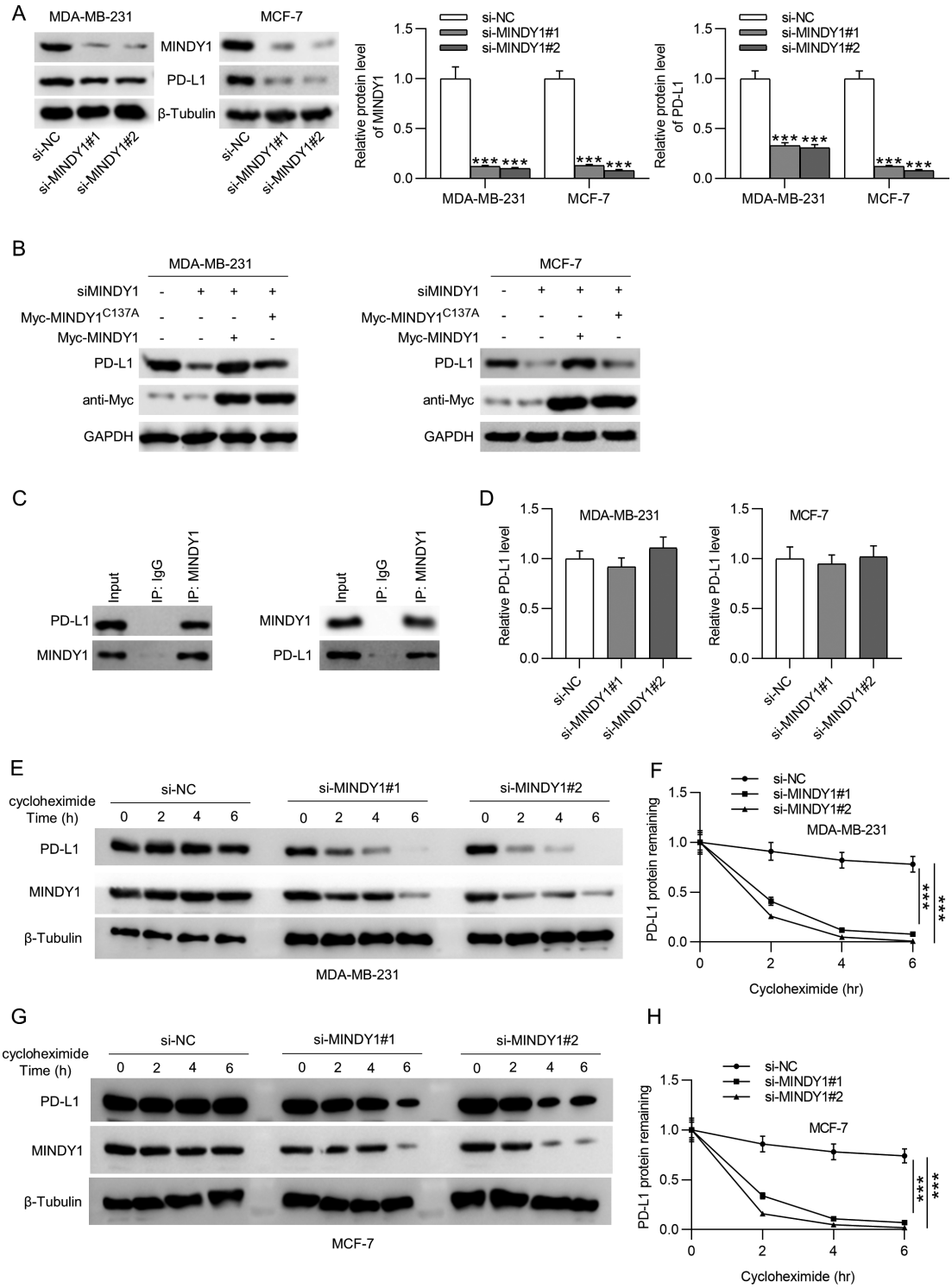

Fig. 3. MINDY1 maintains the stability of PD-L1.
(A-B) The MINDY1, PD-L1, Myc proteins were analyzed by western blot analysis in BC cells. (C) The interaction between MINDY1 and PD-L1 was evaluated by co-immunoprecipitation assay. (D) The measurement of mRNA level of PD-L1 in BC cells transfected with si-MINDY1#1/2. (E-H) The stability of PD-L1 in response of MINDY1 silencing was analyzed by Western blotting. ***P < 0.001.

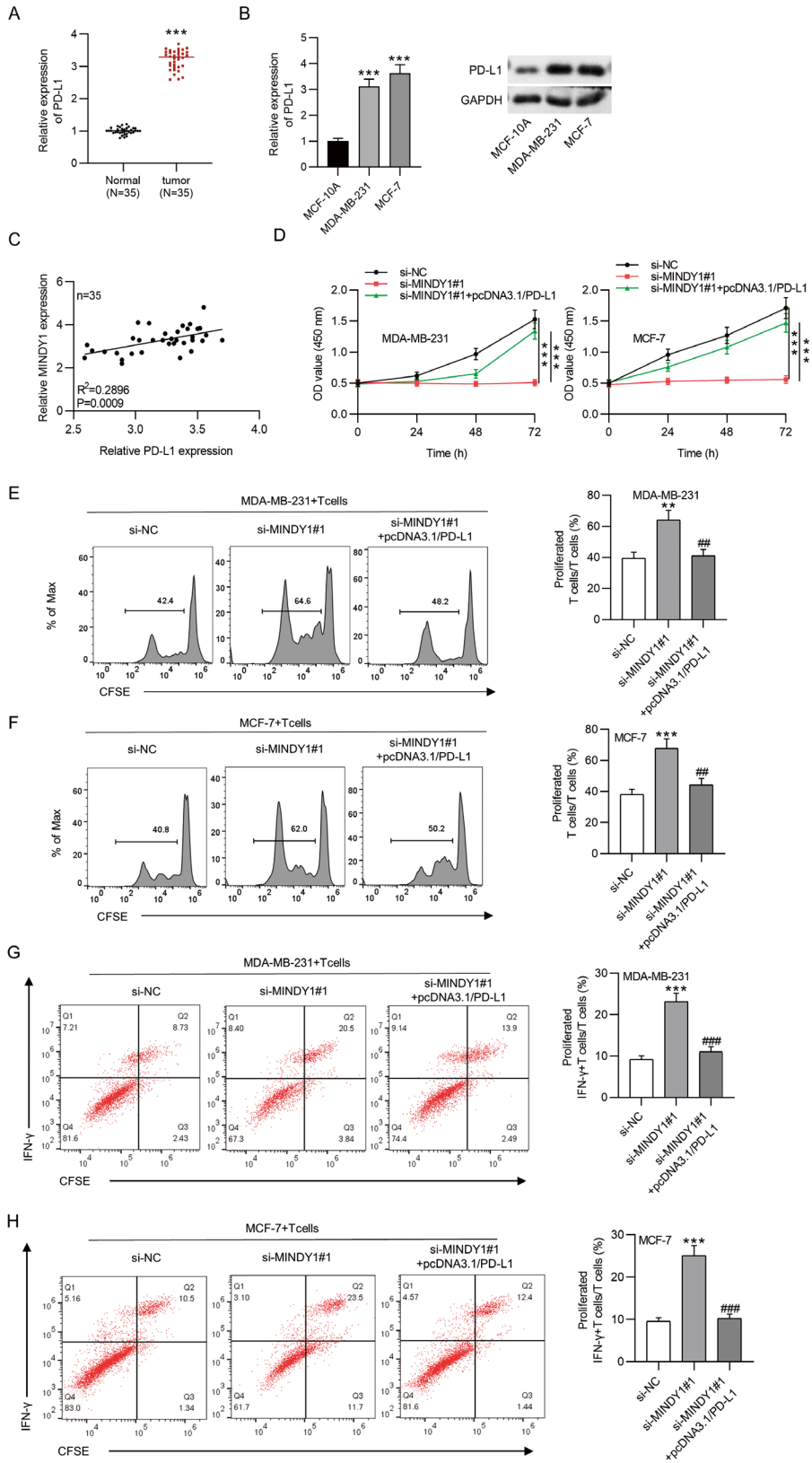

Fig. 4. MINDY1 promotes BC cell proliferation and T cell activation by upregulating PD-L1.
(A) The measurement of PD-L1 mRNA level in BC tissues (n = 35) and adjacent tissues (n = 35). (B) The measurement of PD-L1 mRNA and protein levels in BC cells and normal breast epithelial cells. (C) The relationship between MINDY1 and PD-L1 in BC tissues was analyzed by Pearson's coefficient tests. (D) Cell viability of BC cells was assessed by CCK-8 assay. (E-H) $CD3^+$ T cells and IFN-γ^+ T cells were detected by flow cytometry analysis. **P < 0.01, ***P < 0.001 vs. si-NC group. $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs. si-MINDY#1 group.

higher than that in non-tumor tissues. Similarly, PD-L1 mRNA and protein levels are upregulated in MDA-MD-231 and MCF-7 cells compared to MCF-10A cells (Fig. 4B). Moreover, PD-L1 level is positively associated with MINDY1 level in BC tissues (Fig. 4C). The rescue assay demonstrates that the inhibitive effect of MINDY1 knockdown on cell proliferation is reversed by overexpression of PD-L1 (Fig. 4D). Additionally, the increased ratio of proliferated $CD3^+$ T cells/T cells and IFN-γ^+ T cells/T cells induced by MINDY1 inhibition is also neutralized by PD-L1 overexpression (Fig. 4E-H). Altogether, MINDY1 promotes the immune escape of BC cells by upregulating PD-L1.

MINDY1 depletion inhibits tumor growth by regulating PD-L1 in vivo

At last, the role of MINDY1 and PD-L1 *in vivo* is explored. Tumor size, volume and weight are significantly suppressed by knockdown of MINDY1, while overexpression of PD-L1 reverses this trend (Fig. 5A-C). To conclude, MINDY1 depletion inhibits tumor growth by regulating PD-L1 *in vivo*.

Discussion

Featured with frequent recurrence and distant metastasis, the mortality of BC increased year by year in recent decades (Farshbafnadi et al. 2021). Despite the application of advanced technologies including chemotherapy, radiotherapy, and immunotherapy, BC patients still presented poor prognosis (Criscitiello et al. 2021; Cejuela et al. 2022). Thus, further exploration of the underlying mechanisms in BC is of great importance. In our study, we focused on the role of MINDY1 in BC. We discovered that MINDY1 was upregulated in BC cells and tissues. Moreover, knockdown of MINDY1 inhibited BC cell proliferation and facilitated T cell activation. In mechanism, MINDY1 promoted the stabilization of PD-L1 by interacting with PD-L1, and MINDY1 level was positively related to PD-L1 level in BC tissues. Importantly, the rescue assay indicated that overexpression of PD-L1 reversed the inhibitive effect of MINDY1 silencing on cell viability and immune escape of BC cells.

MINDY1, a novel deubiquitinating enzyme, exhibits a preference for cleaving lengthy polyUb chains (Abdul Rehman et al. 2021). As an emerging family of DUBs, MINDY1 potentially plays specialized roles in maintaining proteostasis (Li et al. 2023). The oncogenic role of MINDY1 has been identified in bladder cancer (Luo et al. 2021). Particularly, MINDY1 was upregulated and facilitated cell growth and cell cycle progression by interacting ERα in BC (Tang et al. 2021). Similarly, we also discovered the upregulation of MINDY1 in BC. Moreover, MINDY1 promoted the proliferative activity of BC cells.

As the immune checkpoint protein, the accumulation of stabilized PD-L1 proteins on the tumor cell membrane played a critical role in immune evasion of various tumor cells (Zak et al. 2015). In detail, these proteins interacted with the receptor PD-1 on T cell membranes to allow tumor cells to evade detection by the immune system (Wu et al. 2022). This interaction within the PD-L1/PD-1 axis pro-

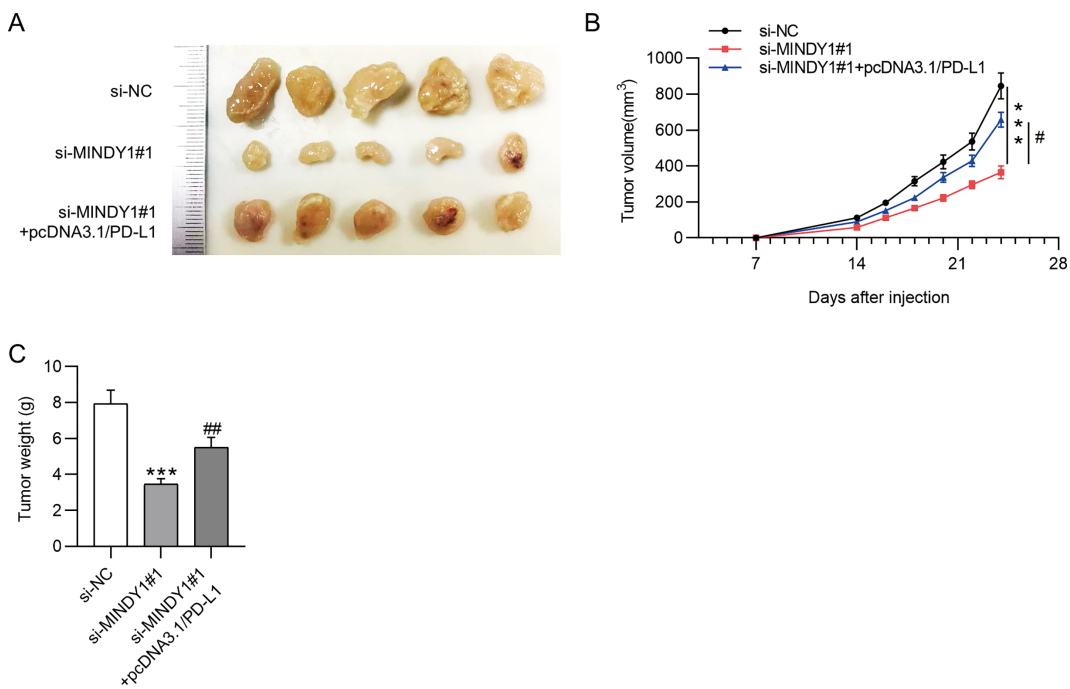

Fig. 5. MINDY1 depletion inhibits tumor growth by regulating PD-L1 *in vivo*.
(A) The represented images of xenograft tumor in mice. (B) the tumor volume was recorded on day 7, 14, 16, 18, 20, 22, and 24. (C) The xenograft tumor was isolated on day 24 and weighed. ***$P < 0.001$ vs. si-NC group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. si-MINDY#1 group.

vides inhibitory signals that suppress T cell activity and thereby contribute to immune evasion of cancer cells (Lim et al. 2023). In our study, we also demonstrated that knockdown of MINDY1 significantly facilitated the activation of T cells, implying the inhibitive effect of MINDY1 knockdown on immune evasion.

Recent reports have elucidated various post-translational mechanisms for controlling the abundance of PD-L1 (Cha et al. 2019; Yamaguchi et al. 2022). For instance, LINC00184 (a lncRNA) promoted cell proliferation and immune escape by sponging miR-105-5p and targeting PD-L1 in prostate cancer (Zhang et al. 2022). Moreover, KCNQ1OT1 (a lncRNA) from tumor cell-derived exosomes ubiquitinated PD-L1 to mediate immune escape by targeting miR-30a-5p/USP22 axis in colorectal cancer (Xian et al. 2021). However, further investigation is warranted to explore the relationship between PD-L1 stabilization and ubiquitin proteasome system (Li et al. 2016; Hsu et al. 2018). Interestingly, MINDY1 has been reported to deubiquitylate ERα in BC and YAP in bladder cancer (Luo et al. 2021; Tang et al. 2021). In this study, we discovered that MINDY1 promoted the stabilization of PD-L1 and subsequently impaired cancer cell immunity. Specifically, MINDY1 exhibited specific interaction with PD-L1 and effectively prevents its proteasomal degradation, and then induced immune escape by reducing not only proliferated $CD3^+$ T cells but also IFN-γ^+ T cells. Importantly, the rescue assay validated that overexpression of PD-L1 counteracted the suppressive effect of MINDY1 silencing on BC cell viability and immune escape.

In conclusion, we identified PD-L1 as a novel target of MINDY1 and established a significant association between MINDY1 and the cancer immune response. Importantly, our findings reveal that MINDY1 promoted BC progression via PD-L1-mediated immune evasion. Consequently, targeting the MINDY1 holds great potential for improving patient outcomes in cancer immunotherapy.

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Author Contributions

Liang Ren and Li Wang was the main designer of this study. Liang Ren, Li Wang, Zhewei Cao, Xuelin Yi, Yiran Chen, Yang Yang and Ya Liu performed the experiments and analyzed the data. Yunfei Jiang, Liang Ren, Li Wang and Ya Liu drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
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