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Original Article

HORMAD1 inhibits senescence and promotes proliferation of triple negative breast cancer by facilitating ubiquitination-mediated degradation of p27

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Abstract: Objective: To investigate the role of the HORMA domain containing protein (HORMAD1) in senescence of triple negative breast cancer (TNBC) cells. Methods: We measured the levels of HORMAD1 and p27 mRNA levels and protein levels in TNBC cell lines using quantitative real -time polymerase chain reaction (PCR) and western blot respectively. We also used the cell counting kit-8 (CCK-8), flow cytometry assays to assess cell viability, cell cycle, apoptosis, immunofluorescence, and co-Immunoprecipitation, respectively. Results: We report for the first time that the depletion of HORMAD1 induces cellular senescence through the accumulation of p27 in -TNBC. The increased expression of p27 in HORMAD1-depleted cells was attributed to an impairment of the ubiquitin-mediated degradation of p27. In addition, ectopic expression of HORMAD1 blocked senescence caused by p27 accumulation, which was paralleled by an increase in the growth of TNBC cells. These findings indicate that the impairment of TNBC cell growth is caused by HORMAD1 depletion-induced senescence. Conclusion: Our findings suggest that downregulating HORMAD1 expression could serve as a strategy for restricting TNBC progression by inducing senescence by the p27 pathway.

Keywords: TNBC, HORMAD1, senescence, p27

Introduction

Emerging evidence has shown that the induction of senescence, an irreversible cell growth arrest, may function as a tumor-suppressive mechanism to restrict tumor expansion [1, 2]. However, the mechanisms underlying senescence in tumor cells remain unclear. The HORMA domain-containing protein (HORMAD1) is an important molecule that sustains the oncogenic characteristics of tumor cells [3-5]. Our previous study found that HORMAD1 enhanced triple-negative breast cancer (TNBC) cell tolerance to DNA damage [6]. HORMAD1 may be linked to the TNBC cell senescence phenotype, especially due to its close relationship to the p27 protein. Although the antiapoptotic function of HORMAD1 appears to largely contribute to tumor resistance to chemotherapy, its role in inhibiting senescence and promoting TNBC progression has not yet been elucidated. Given the relationship between DNA damage tolerance and cellular senescence, we conducted an in-depth study on the role of HORMAD1 in the regulation of cellular senescence. Considering the limited understanding behind the regulatory mechanisms of p27-mediated senescence induction, our results describe a novel function of HORMAD1, extending from anti-DNA damage response (DDR) roles to negatively regulating p27-mediated senescence. Therefore, targeting HORMAD1 may be a thera-

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peutic strategy to induce senescence and stop TNBC progression.

Materials and methods

Cell lines and cell culture

The human cancer cell lines MDA-MB-468 and BT549 were cultured as previously described [6]. TNBC cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Scientific) containing 10% fetal calf serum (Gibco) in a humidified incubator with 5% CO_2 at 37°C. The medium was refreshed every 1-2 days.

Knockdown and overexpression of HORMAD1

siRNA knockdown or PCDNA3.1 overexpression of HORMAD1 was carried out as previously described [6]. A lentiviral vector (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin) containing the same siRNA sequence was purchased from Genechem Co., Ltd. For some experiments, the knockout efficiency was maintained over a long period of time. Experiments were performed 24 to 48 hours post-transfection, as indicated.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Polymerase chain reaction (PCR) analysis was carried out as previously described [7]. The following primers were used for real-time PCR (RT-PCR): *HORMAD1* gene-specific primers (forward: 5'-GCC CAG TTG CAG AGG ACT C-3' and reverse: 5'-TCT TGT TCC ATA AGC GCA TTC T-3') and p27 *(CDKN1B)* gene-specific primers (forward: 5'-TGC AGA GAC ATG GAA GAG G-3' and reverse: 5'-TAG AAC TCG GGC AAG CTG-3'). These primers were purchased from Tsingke Biotechnology Co., Ltd.

Cell proliferation, cell cycle, and apoptosis detection

Cell proliferation was carried out using the cell counting kit-8 (CCK-8) assay, while cell cycle and apoptosis detection were analyzed by flow cytometry, as previously described [6].

Western blot

Western blot analysis was performed as previously described [6]. The following antibodies were used for western blotting: HORMAD1 (Sigma, HPA037850), HORMAD1 (Proteintech, 67091-1-Ig), p Retinoblastoma Protein (pRB) (Ser807/811) (Proteintech, 30376-1-AP), BAX (Proteintech, 50599-2-Ig), p16 (Proteintech, 10883-1-AP), caspase3 (Proteintech, 19677-1-AP), cyclinA (Selleck.cn, A5190), cyclinD1 (Proteintech, 60186-1-Ig), p27 (Proteintech, 25614-1-AP), ubiquitin (Proteintech, 10201-2-AP), γ-H2A Histone Family Member X (γH2AX) (ABclonal, AP0099), GAPDH (Proteintech, 10494-1-AP), and β-actin (Proteintech, 20536-1-AP). GAPDH or β-Actin was used as the internal control for each blot. Densitometry-based quantification was performed using ImageJ software.

Detection of senescence-associated β-galactosidase

A cell aging β-galactosidase staining kit (C-0602, Beyotime Institute of Biotechnology, China) was used to detect senescence-associated β-galactosidase (SA-β-Gal), following the manufacturer's instructions. TNBC cells were prepared as previously described and manipulated according to the above transfected/infected cancer cell lines with HORMAD1 knockdown or overexpression. Cells were seeded in duplicate in 24-well plates. After fixing cells with a fixative (Paraformaldehyde), an overnight incubation with the senescence detection kit was conducted. SA-β-Gal activity was visualized and quantified (three areas per well, more than 200 cells per condition). The number of senescent cells in each area was normalized to the number of total cells counted per area. Images were captured using the NIS-Elements Viewer® cell imaging station.

Immunofluorescence detection

Cancer cells exhibiting robust growth were selected and manipulated according to the above transfected/infected cancer cell lines with either HORMAD1 knockdown or overexpression. Cells were seeded in 24-well plates with a sterile glass slipper at the bottom. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 30 min. Subsequently, cells were washed three times with PBS for 5 min each. Then, cells were incubated with PBS containing 0.1% Triton X-100 for 30 min, followed by blocking with 5% BSA to

reduce binding of nonspecific proteins. A single target protein or primary antibody of two different genera of target protein (1:50-1:100) was incubated in a wet box at 4°C overnight. The next day, the cells were washed with PBS three times for 5 min each. Cells were incubated with fluorescent secondary antibodies (1:500) with two different excitation wavelengths for 1 h, and care was taken to avoid light during the incubation. After washing with PBS, staining was performed with DAPI for 10 min. The cell slide was inverted onto the slide for laser confocal observation.

Immunoprecipitation test

After 24-36 h of knockdown or overexpression of HORMAD1, cancer cells were treated with MG132 (5 M) for 12 h. Subsequently, cells were washed twice with precooled phosphate buffer saline (PBS), and lysed using a specialized non-denatured RIPA buffer containing protease inhibitors on ice for 15 mins. Cell lysates were scraped off and centrifuged at 12,000 rpm for 15 min at 4°C. The protein concentration was measured using the Bicinchoninic Acid method. According to the protein concentration, 5% of the total protein content for immunoprecipitation (IP) was aliquoted as the input group sample. For IP, the protein antibody (3-5 μg) and blank control IgG were added to the protein lysates and incubated overnight with gentle shaking at 4°C. The next day, the protein liquid containing IP antibody was added to the magnetic beads, washed with PBS, and incubated overnight again with gentle shaking at 4°C. The magnetic beads were separated, and 1× loading buffer was added at 95°C for 5 min. The following procedure was the same as that for the western blot analysis for protein expression.

Statistical analysis

All experiments were performed independently at least three times. The mean ± standard deviation (SD) was determined for each group. Statistical analyses were performed using the Student's *t*-test for individual comparisons in GraphPad Prism 8. Categorical variables are presented as numbers and percentages and were compared using chi-square and Fisher's tests. Data analyzed by parametric tests are represented by the mean ± standard error of the mean (SEM) of pooled experiments unless otherwise stated. $P < 0.05$ was considered significant.

Results

HORMAD1 silencing prevents the growth of TNBC cells in vitro

The identification of HORMAD1 as a novel target in TNBC [5, 6] prompted us to investigate the molecular consequences of its inhibition in an established cell culture. Our preliminary results show that HORMAD1 knockdown can promote docetaxel-induced apoptosis [6]. However, in subsequent studies, we found that stable HORMAD1-knockdown TNBC cell lines could not be screened by puromycin using the lentivirus shRNA-HORMAD1 vector hU6-MCS-Ubiquitin-EGFP-IRES-puromycin due to the slow growth and high mortality of cells (**Figure 1A**). The CCK-8 assay demonstrated that cell proliferation of the HORMAD1 knockdown group was significantly inhibited in TNBC cell lines (MDA-MB-468 and BT549) cultured in complete medium containing puromycin (0.5 μg/ml) (**Figure 1B**, **1C**). Flow cytometry results showed a significant increase in the G1 phase of the cell cycle in the two TNBC cell lines (**Figure 1D**, **1E**). The expression of cycle-related markers detected by western blot showed that there were no differences in cyclin D1 expression between the two groups of MDA-MB-468 cells. However, significant differences were found in p27 and cyclin A expression in both TNBC cell lines compared to the control group (**Figure 1F**).

To explore whether lentiviral knockdown of HORMAD1 affects cell apoptosis, flow cytometry was used to detect differences in apoptosis rates between the two groups of MDA-MB-468 cells. The results showed no significant difference in apoptosis rates between the two groups (**Figure 1G**, **1H**). Interestingly, while analyzing apoptosis using flow cytometry, a subset of nonspecific cells, named P2, showed a difference in proportion between the two groups (**Figure 1G**, **1I**). Additionally, the markers of apoptosis, cleaved caspase 3, and Bax, remained unchanged between the groups (**Figure 1J**). These results suggest that the cell cycle of TNBC cells can be altered with continuous knockdown of HORMAD1, without affecting apoptosis rates.

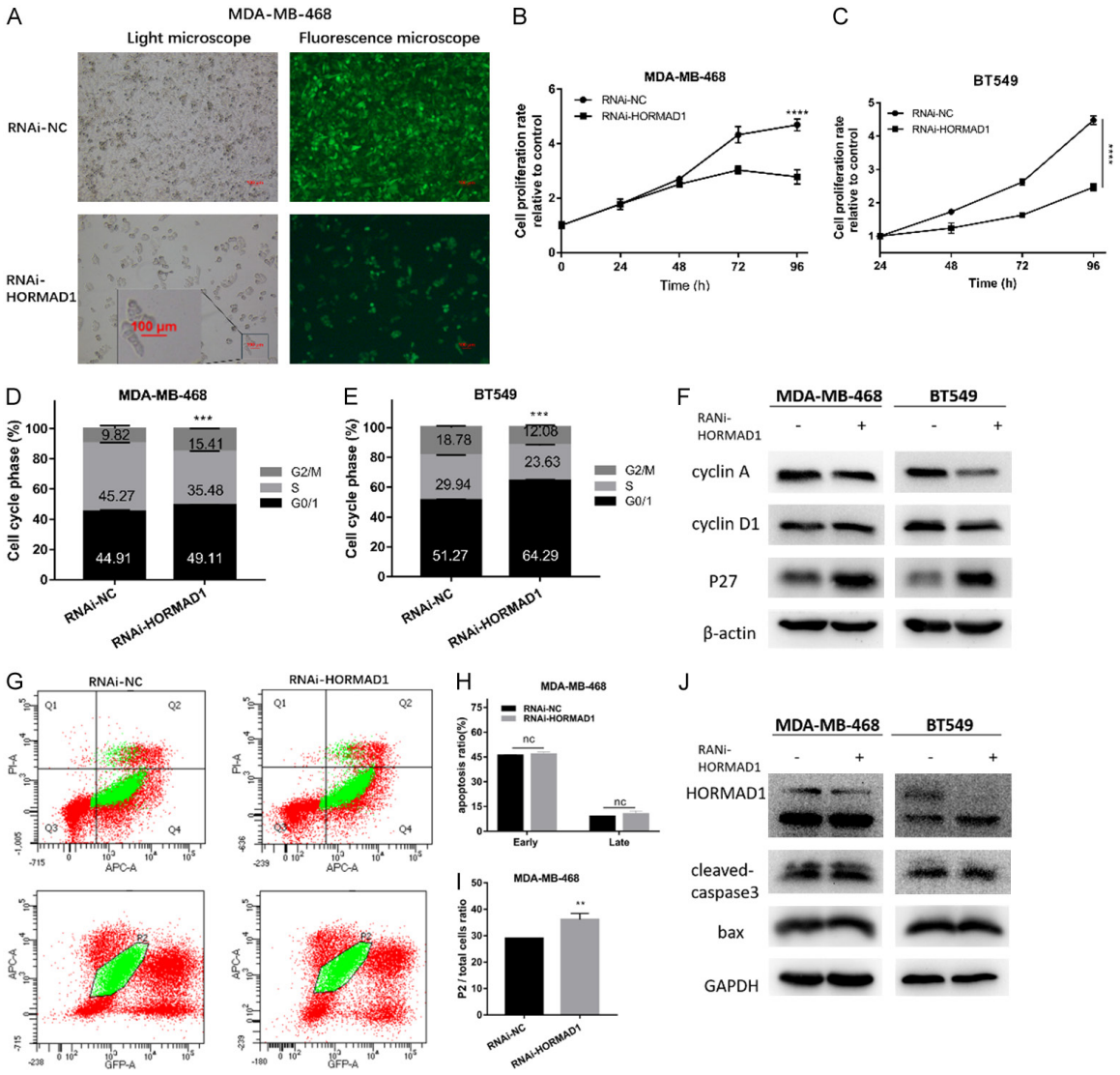

Figure 1. HORMAD1 knockdown inhibited the growth of triple negative breast cancer cells. A. Cell morphology and fluorescence (X 100) expression in MDA-MB-468 cells infected with RNAi-HORMAD1 were observed under fluorescence microscopy after 1 μg/ml puromycin was added for 15 days. Scale bar is 100 μm. B, C. The cell counting kit-8 assay was used to detect the proliferation curve of MDA-MB-468 and BT549 cells infected with RNAi-HORMAD1. D, E. Cell cycle changes in MDA-MB-468 and BT549 cells after RNAi-HORMAD1 treatment were detected by flow cytometry. F. Western blotting was used to detect the changes in the expression of cyclin-related proteins in different treatment groups. G. Flow cytometry was used to detect changes in apoptosis in MDA-MB-468 cells infected with RNAi-HORMAD1. A nonspecific cell population P2 (green cell population) was found. H. The apoptosis rate of the two groups was statistically analyzed after P2 cells were removed. I. The proportion of the P2 cell population in the HORMAD1 knockdown group was significantly higher than that of the control group. J. The expression of apoptosis-related proteins (Bax, cleaved-caspase3) was detected by western blot. HORMAD1, HORMA domain-containing protein1; RNAi, RNA inhibition; NC, negative control; ns, no significance; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

HORMAD1 silencing induces senescence in TNBC cells

Cellular senescence is a permanent state of cell cycle arrest characterized by a loss in the ability to replicate, as well as significant changes in cell morphology and gene expression. Telomere DNA sequences gradually shorten or even disappear, causing a series of DDRs. This process decreases chromosome stability and leads to cell cycle arrest, which may cause irreversible growth arrest [8]. Considering the role of HORMAD1 in DDR [4, 6, 9], it is important to note that many studies have shown a close

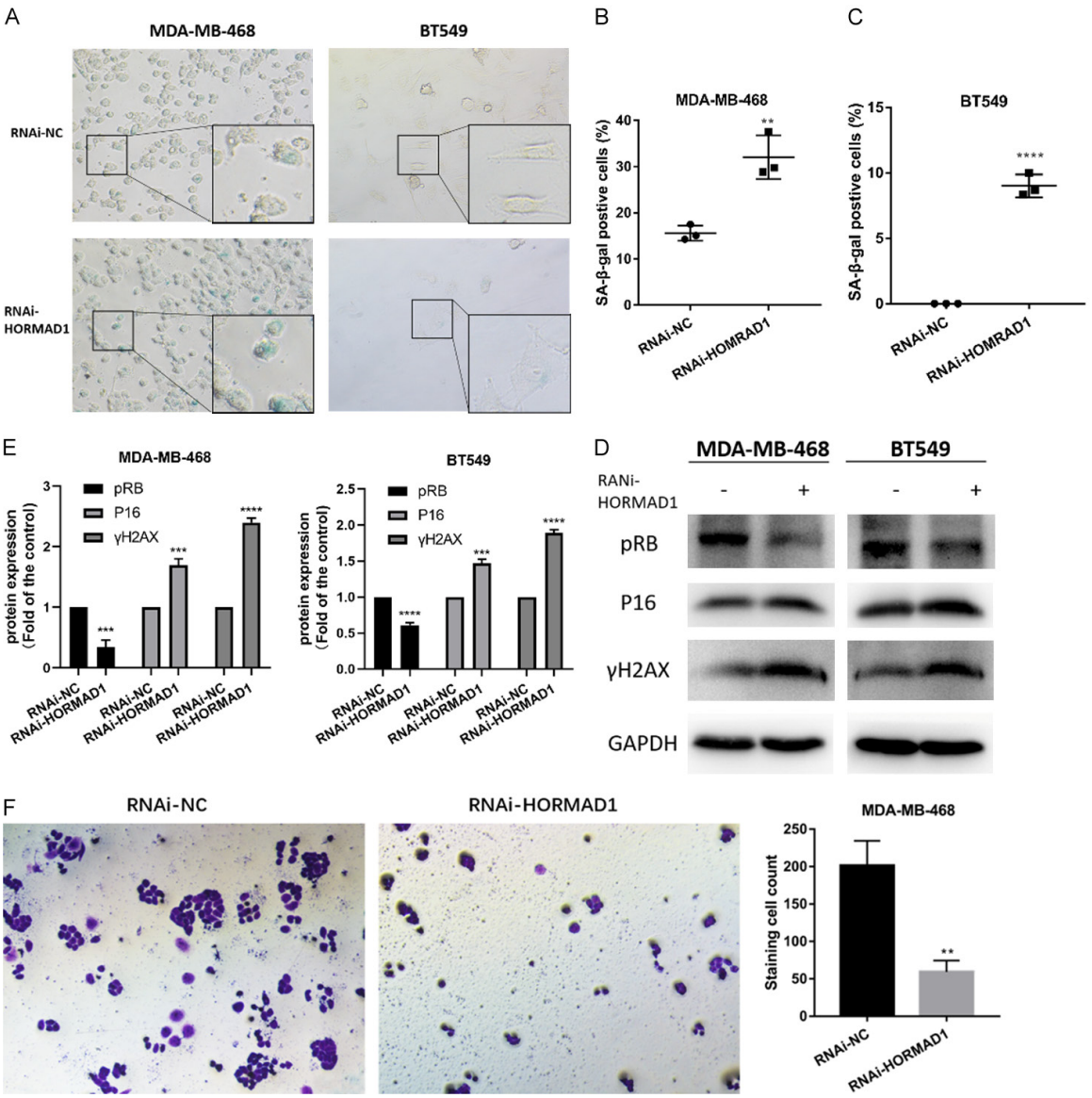

Figure 2. HORMAD1 inhibition can promote the senescence phenotype of triple negative breast cancer cells. A. Detection of senescence-related β-galactosidase (SA-β-gal) (X 200) expression in MDA-MB-468 and BT549 cells with different treatments. B, C. Statistical results of SA-β-gal for MDA-MB-468 and BT549 cells. D, E. Western blotting was used to detect the changes in pRB, p16, and γH2AX in different treatment groups of MDA-MB-468 and BT549 cells. F. Different treatment groups with crystal violet staining (X 200) in MDA-MB-468 cells and statistical analysis. HORMAD1, HORMA domain-containing protein1; RNAi, RNA inhibition; NC, negative control; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

relationship between p27 and cell senescence [10, 11]. We hypothesize that HORMAD1 is responsible for cancer cell senescence and that HORMAD1 knockdown promotes a senescent phenotype. To test this hypothesis, the expression of SA-β-Gal was assessed to measure cell senescence between different treatment groups. We found that the number of senescent cells was significantly increased in the HORMAD1 knockdown group compared to the RNAi-negative control (RNAi-NC) group in both MDA-MB-468 and BT549 cells (**Figure 2A-C**). Western blotting was used to detect the expression of senescence-related markers. The results showed that pRB, a senescence-related marker, was significantly reduced, and p16 and γH2AX expression was significantly increased, in cells with continuous knockdown of HORMAD1 compared to the control group (**Figure 2D**, **2E**).

These results prompted us to investigate whether siRNA knockdown of HORMAD1 could also induce an increase in senescent cells. SA-β-Gal staining results showed that the proportion of senescent MDA-MB-468 cells was significantly increased by siRNA knockdown of HORMAD1 (data not shown). However, in our previous experiments, we did not observe any evidence that siRNA knockdown of HORMAD1 expression affected the proliferation of TNBC cells [6]. It is speculated that siRNA-HORMAD1 may only transiently reduce the expression of HORMAD1, allowing the suppressed protein expression to be restored as the cells divide and proliferate. We further tested this hypothesis by using RNAi to knock down HORMAD1 continuously to explore its correlation with cell senescence. Crystal violet staining visually showed that after 3 days of puromycin screening, the number of cells in the HORMAD1 knockdown group was significantly reduced, with few living cells remaining (**Figure 2F**). These results suggest that transient and sustained knockdown of HORMAD1 can inhibit cell growth by inducing cellular senescence.

HORMAD1 negatively regulates p27 protein expression

In the previous results, we found that the expression level of p27 protein significantly increased after HORMAD1 knockdown (**Figure 1F**). Next, we examined the mutual regulatory relationship between HORMAD1 and p27 protein in tumor cells through positive and negative validation. The expression of the p27 gene after knockdown or overexpression of HORMAD1 was detected by RT-PCR. The results showed that, compared to the control group, there was no change in the expression level of the p27 gene in MDA-MB-468 cells, regardless of whether HORMAD1 was knocked down or overexpressed (**Figure 3A**). However, at the protein expression level, western blot results showed that p27 protein expression was increased when HORMAD1 expression was knocked down, whereas p27 protein levels were significantly decreased following HORMAD1 overexpression (**Figure 3B**). Using the same method in BT549 cells, we found similar trends (**Figure 3C**, **3D**).

To investigate further whether the increased expression of p27 protein caused by HORMAD1 knockdown can be reversed by overexpression of HORMAD1, we performed experiments in MDA-MB-436 cells. Western blot analysis showed that compared to the control group, the expression of p27 protein was significantly increased after HORMAD1 knockdown (**Figure 3E**; lane 1 vs. lane 2). In addition, compared to the HORMAD1 knockdown group alone, when HORMAD1 knockdown was accompanied by HORMAD1 overexpression, the increased p27 protein expression was attenuated (**Figure 3E**; lane 2 *vs.* lanes 4 and 5). To verify the specificity of this negative regulation, different siRNA-p27 knockdown sequences were constructed and tested in MDA-MB-436 cells. Western blot results showed that siRNA-p27#1 effectively knocked down the protein expression level of p27 without affecting the protein expression of HORMAD1 (**Figure 3F**).

To explore the spatial localization of these two proteins, an immunofluorescence assay was used to detect their expression. The results showed that the increased expression of p27 protein could be detected in different TNBC cells after siRNA knockdown of HORMAD1 (**Figure 3G**). Taking into account the knockdown efficiency of the siRNA, it was evident that p27 protein expression is lower in cells with high HORMAD1 expression (green arrow). Conversely, in cells with HORMAD1 knockdown, p27 protein expression was increased (red arrow), showing a typical negative correlation. Both proteins were mainly located in the cytoplasm. Moreover, HORMAD1 was negatively correlated with the expression of p27 (gene name: CDKN1B) on the data retrieval page of the GEPIA platform (http://gepia.cancer-pku.cn/) (**Figure 3H**).

To verify the hypothesis proposed above, BT549 cell lines were used as the cell model because of their rapid growth and division. Cells were collected at different times post-transfection with siRNA targeting HORMAD1. The western blot results showed that the expression of HORMAD1 was the lowest after 72 h of siRNA treatment. However, with the increase in transfection time, the expression of HORMAD1 tended to increase (**Figure 3I**). Intriguingly, p27 expression was negatively correlated with HORMAD1, consistent with the results in **Figure 1F**. The expression of p27 reached its peak at 72 h after siRNA treatment.

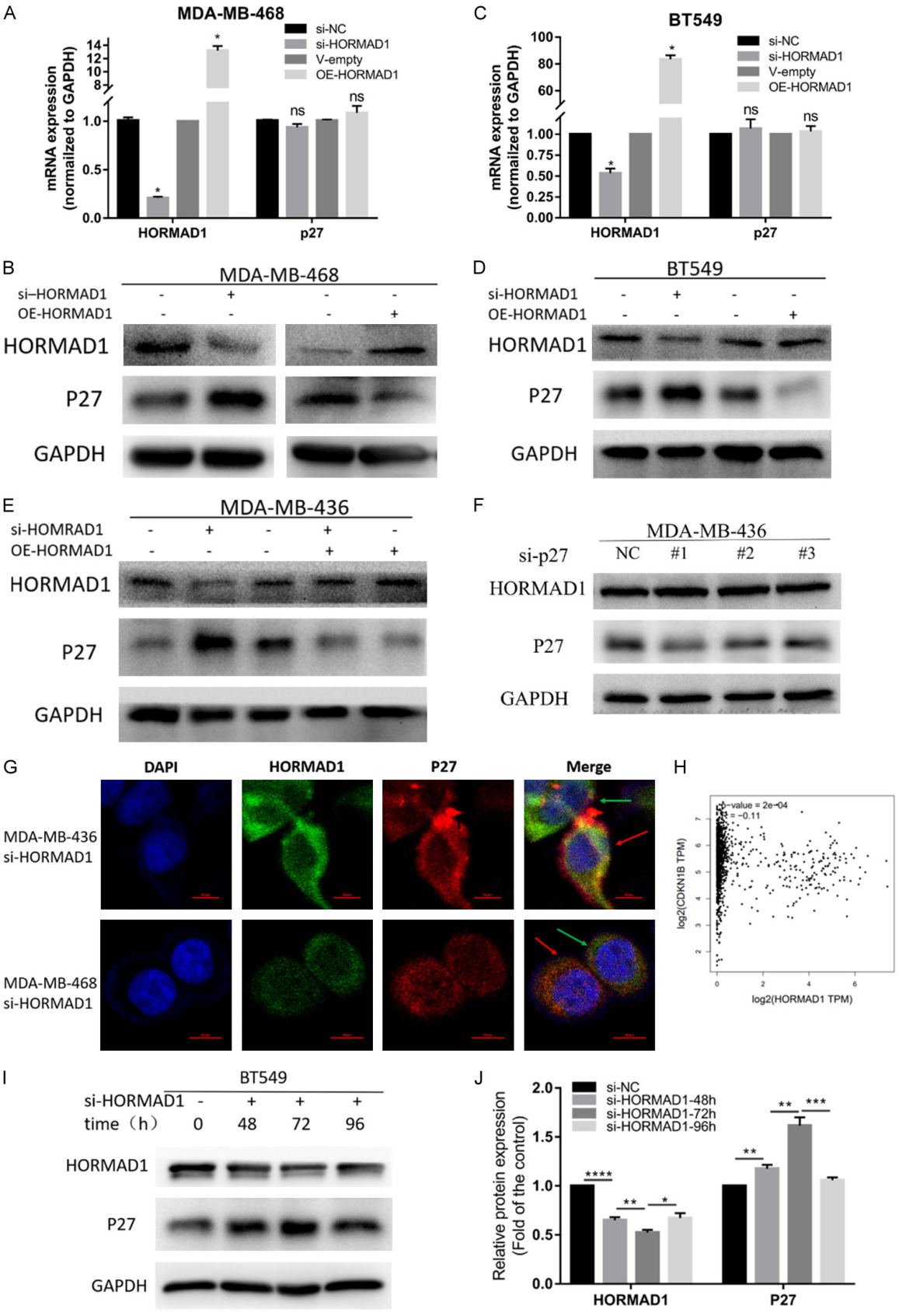
A
MDA-MB-468
si-NC
si-HORMAD1
V-empty
OE-HORMAD1
mRNA expression (normalized to GAPDH)
HORMAD1
p27
C
BT549
B
MDA-MB-468
si–HORMAD1
OE-HORMAD1
HORMAD1
P27
GAPDH
D
BT549
E
MDA-MB-436
si-HOMRAD1
F
si-p27
NC
#1
#2
#3
G
DAPI
HORMAD1
P27
Merge
MDA-MB-436 si-HORMAD1
MDA-MB-468 si-HORMAD1
H
log2(CDKN1B TPM)
log2(HORMAD1 TPM)
I
time（h）
0
48
72
96
J
si-HORMAD1-48h
si-HORMAD1-72h
si-HORMAD1-96h
Relative protein expression (Fold of the control)

Figure 3. HORMAD1 negatively regulates the expression of p27 protein in triple negative breast cancer cells. (A) Detection of p27 gene expression in MDA-MB-468 cells after HORMAD1 knockdown with siRNA or overexpression with vector-HORMAD1. (B) Detection of p27 protein expression in MDA-MB-468 cells after HORMAD1 knockdown with siRNA or overexpression. (C, D) The detection results of the same gene level and protein level in BT549 cells showed the same trend as (A) and (B). (E) Western blotting was used to detect the protein expression of p27 after HORMAD1 knockdown and overexpression in MDA-MB-436 cells. (F) Western blotting was used to detect the knockdown of the expression of p27 in MDA-MB-436 cells with different siRNA sequences. (G) Immunofluorescence detection of p27 protein expression and localization after HORMAD1 knockdown in TNBC cells. Scale bars is 10 μm. In both MDA-MB-436 and MDA-MB-468 cells, high expression of HORMAD1 corresponded to low expression of p27 protein (green arrow). Expression of p27 protein increased in HORMAD1 knockdown cells (red arrow). (H) Data retrieval within the GEPIA platform. (I, J) Western blotting was used to detect the expression of p27 protein after siRNA knockdown of HORMAD1 at different time points in BT549 cells. HORMAD1, HORMA domain-containing protein1; si-, siRNA-; OE, overexpression; V, vector; ns, no significance; NC, negative control group; *, $P < 0.05$;**, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

As transfection time increased, the expression of HORMAD1 tended to decrease (**Figure 3J**). Overall, these results suggest that HORMAD1 can indeed negatively regulate the expression of the p27 protein. This regulatory mechanism may be at the posttranscriptional modification level.

HORMAD1 induces a cell anti-senescence phenotype by inhibiting p27 expression

p27 is a typical marker of cellular senescence [12]. To verify that the increase in cell senescence after HORMAD1 knockdown is due to an increase in p27, we performed response experiments in TNBC cell lines. The results of SA-β-gal staining showed that HORMAD1 knockdown increased the expression of SA-β-gal in MDA-MB-468 and BT549 cells compared to the control group (**Figure 4A**, **4B**). Western blot analysis of senescence-related protein markers in different treatment groups of the two TNBC cell lines also found that the knockdown of HORMAD1 and p27 could restore inhibited pRB levels and reduce the increase in γH2AX, p16, and p27 proteins (**Figure 4C**).

Additionally, the CCK-8 assay was used in BT549 cells to detect cell proliferation changes in different treatment groups. When HORMAD1 was knocked down simultaneously with p27, the inhibited cell proliferation was restored (**Figure 4D**). Based on the phenotype phenomenon that HORMAD1 regulates p27 protein expression, western blotting was used to detect whether overexpression of HORMAD1 could reverse the protein expression changes observed with p27 knockout. The results showed that the expression levels of p27 and p16 proteins were reduced when HORMAD1 was knocked down or overexpressed simultaneously compared to the HORMAD1 group alone (**Figure 4E**). These results suggest that an increase in p27 caused by HORMAD1 knockdown may be the main cause of cell senescence.

HORMAD1 overexpression promotes ubiquitination-mediated degradation of p27

To explore the mechanism by which HORMAD1 negatively regulates p27 protein expression, we hypothesized that HORMAD1 might impact p27 expression at the posttranscriptional level, particularly by modifying its degradation pathway mediated by the E3 link-enzyme S-Phase Kinase-Associated Protein 2 (SKP2) [13]. To verify this hypothesis, we first treated two TNBC cell lines with cyclohexane (CHX) for western blot detection at different time points. The results showed that in BT549 cells overexpressing HORMAD1, p27 protein levels were significantly decreased compared to cells treated with CHX for 3 h (**Figure 5A**). Conversely, when HORMAD1 was knocked down in BT549 cells, p27 protein degradation slowed down compared to the control (**Figure 5B**). A consistent pattern was also observed in MDA-MB-468 cells (**Figure 5C**, **5D**), indicating that HORMAD1 promotes the degradation of p27. To further validate our hypothesis, immunoprecipitation was used to investigate whether overexpression of HORMAD1 increases the ubiquitination-mediated degradation of p27. The results showed that binding of the ubiquitin protein to the p27 protein was detected in both MDA-MB-468 and BT549 cells. In addition, treatment with MG132 effectively inhibited the degradation of p27 protein in the HORMAD1 overexpression group (**Figure 5E**, **5F**). These results indicate that HORMAD1 negatively regulates p27 expression by promoting the ubiquitination-mediated degradation of p27.

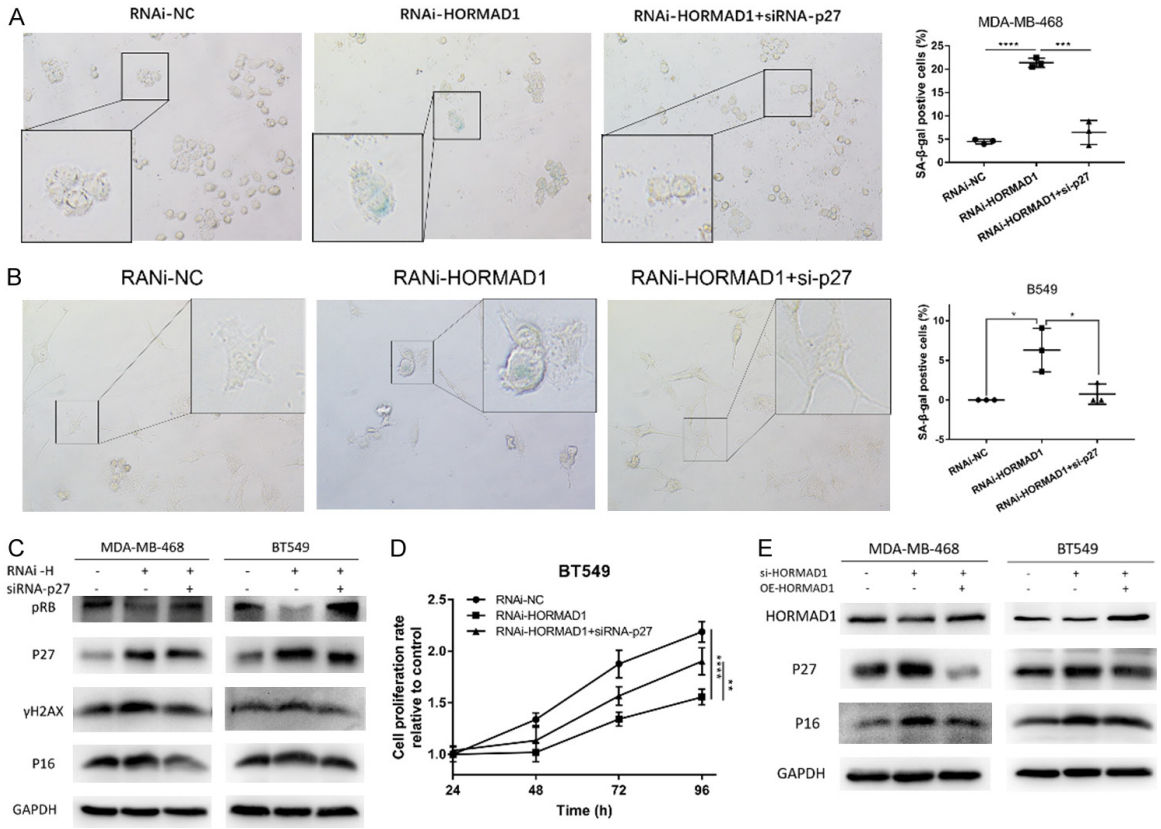

Figure 4. HORMAD1 plays an anti-senescence role by inhibiting the expression of p27 protein. A, B. Detection of senescence-related β-galactosidase (SA-β-gal) (X 200) after simultaneous HORMAD1 and p27 knockdown in MDA-MB-468 and BT549 cells. C. Western blotting was used to detect senescence-related markers in MDA-MB-468 and BT549 cells with simultaneous HORMAD1 and p27 knockdown. D. A cell counting kit-8 assay was used to detect the proliferation of BT549 cells in different treatment groups. E. Overexpression of HORMAD1 with knockdown of HORMAD1 can restore p16 and p27 suppression. HORMAD1, HORMA domain-containing protein1; γH2AX, γ-H2A Histone Family Member X; RNAi, RNA inhibition; si-, siRNA-; NC, negative control; OE, overexpression; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

Discussion

We have shown for the first time that HORMAD1 depletion results in the premature senescence of various types of TNBC cells through a p27-dependent pathway. The stabilization of p27 at the protein level was driven by the loss of function of HORMAD1. *In vivo* experiments confirmed the feasibility of using viral vector-mediated knockdown of target genes to inhibit tumor growth. Our findings provide therapeutic implications for senescence in the repression of tumor growth in various cancers in which HORMAD1 is highly expressed.

Cell senescence is a common feature of aging and cancer [14, 15], but the role of aging in the occurrence and development of cancer may be bidirectional or ambiguous [16, 17]. However, tumor cells typically possess anti-senescence properties, and promoting tumor cell senescence can be implemented as an effective anticancer strategy [18, 19].

Senescence is a prolonged state of growth arrest that can be caused by a variety of stimuli, including oncogene activation, oxidative and metabolic stress, and abnormal mitosis [7, 8, 20]. This physiologic process is implicated in age-related diseases [21, 22] and is an important anticancer mechanism for precancerous tumors [23]. Some of these preneoplastic lesions, including benign nevi, lung adenomas, and prostatic intraepithelial neoplasia, eventually replace this protective mechanism through the loss of key regulatory proteins like p53, RB, and p16, leading to the development of malignant tumors [18, 24, 25]. Moreover, tumor cells

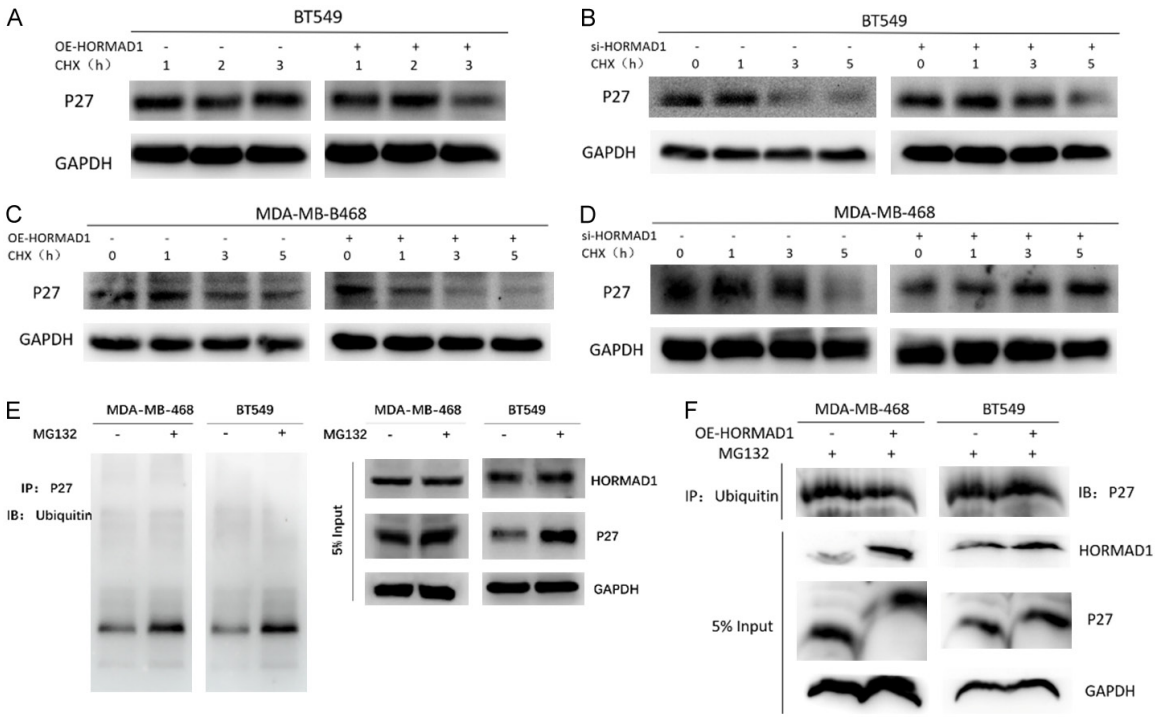

Figure 5. HORMAD1 promotes ubiquitin-mediated degradation of p27 in triple negative breast cancer cells. A, C. Overexpression of HORMAD1 in BT549 cells and MDA-MB-468 cells accelerated the degradation rate of the p27 protein. B, D. HORMAD1 knockdown in BT549 cells and MDA-MB-468 cells slowed the degradation rate of the p27 protein. E, F. The expression of p27 in MDA-MB-468 and BT549 cell lines was detected by immunoprecipitation assay. HORMAD1, HORMA domain-containing protein1; si-, siRNA-; OE, overexpression; CHX, cycloheximide.

that escape aging often exhibit genomic instability, high resistance to therapies [26-28], and more aggressive behavior [29]. However, the mechanisms of aging, especially in cancer cells that have inactivated RB and p53 signaling pathways, remain unclear.

Research has shown that the H3K4 methyltransferase SET Domain Containing 1A (SETD1A), which is abnormally overexpressed in tumors, inhibits senescence by transcriptionally activating SKP2 to inhibit p27. SETD1A is necessary for the maintenance of tumor cell mitosis and proliferation [30]. It has also been reported that CDKN1B (p27) is involved in the activation of the aging response in the absence of p21 and/or p16 activity [11, 24, 31]. Our results demonstrated for the first time that HORMAD1 can inhibit cellular senescence by inhibiting p27. This suggests that HORMAD1 may function as an oncogene in the process of tumor cell mitosis. At the same time, other researchers have used the combination of immunofluorescence and telomere fluorescence in situ hybridization to show that the aging of human cells is not entirely determined by telomere DNA damage, thus proving that both telomere and non-telomere DNA damage are determining factors of mammalian cell senescence [32]. Based on the role of HORMAD1 in DNA damage repair [6], we hypothesize that DNA damage repair defects caused by HORMAD1 knockdown also promote senescence of tumor cells with high HORMAD1 expression. This hypothesis will be one of the directions of our follow-up research. Activating the anti-aging program of tumor cells is a potential strategy for tumor treatment [33], and our results provide some support for the implementation of this approach.

Physiologically, HORMAD1 is only expressed in germ line tissues, such as the testicles, during the meiosis phase of cells [34]. However, in a pathologic state, HORMAD1 is highly expressed in specific subtypes of various malignant tumors, including TNBC [3, 35]. Interestingly, some researchers have found that HORMAD1

can be induced by hypoxia-inducible factor 1 in fetal nonalcoholic fatty liver and liver cancer tissues induced by a maternal high-fat diet and is widely expressed in the cytoplasm and nucleus of liver cancer cells [36]. Our immunofluorescence experiments also confirmed that HORMAD1 is expressed not only in the nucleus but also in the tumor cytoplasm (**Figure 3G**), indicating that HORMAD1 plays a role in tumor development that differs from its basic physiologic function.

Research indicates that targeting the Bcl-2-interacting cell death suppressor induces cellular senescence through regulation of the 14-3-3 zeta/Signal Transducer and Activator of Transcription 3 (STAT3)/SKP2/p27 pathway in glioblastoma cells [37]. In addition, the tumor suppressor gene Fbxo4, a direct substrate of Skp1-Cul1-F-box E3 ligase, can mediate the ubiquitination and degradation of FXR1, relieve its inhibition of p27 protein expression, promote the senescence phenotype of head and neck cancer cells, and inhibit tumor progression [38]. These results suggest that the p27 protein plays an important role in driving cells into a senescent state. Furthermore, SKP2 may be a crucial molecule in the upstream regulation of this process [39].

We also found that HORMAD1 negatively regulates the p27 protein and thus affects the aging properties of cells. This may be one of the mechanisms that HORMAD1 exerts in the cytoplasm to promote TNBC. In our studies, HORMAD1 depletion itself could constitute an aggregation-prone environment for p27, resulting in cellular senescence and alterations in cell fate. Our results therefore highlight the important role of HORMAD1 in protecting cells from entering the senescence program. The molecular mechanism by which HORMAD1 and p27 proteins participate in TNBC cells warrants further extensive study.

In conclusion, we have shown that HORMAD1 depletion induces senescence through regulation of p27 protein expression in various types of TNBC cells. Given the limited understanding of the regulatory mechanisms behind p27-mediated senescence induction, our findings reveal a novel function of HORMAD1, extending from anti-DDR roles to the negative regulator of p27-mediated senescence. Therefore, targeting HORMAD1 may serve as a therapeutic strategy for inducing senescence and preventing tumor progression, particularly in highly aggressive tumor cells.

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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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