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ARTICLE

# Growth arrest and apoptosis induction in androgen receptor-positive human breast cancer cells by inhibition of USP14-mediated androgen receptor deubiquitination

Yuning Liao[1] · Xiaohong Xia[1] · Ningning Liu[2] · Jianyu Cai[1] · Zhiqiang Guo[1] · Yanling Li[1] · Lili Jiang[1] · Q. Ping Dou[1,3,4] · Daolin Tang[5,6] · Hongbiao Huang[1] · Jinbao Liu[1]

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## Abstract

It has been well known that androgen receptor (AR) is critical to prostate cancer development and progression. It has also been documented that AR is expressed in more than 60% of breast tumors, which promotes the growth of estrogen receptor-negative ($ER^-$)/AR-positive ($AR^+$) breast cancer cells. Thus, AR might be a potential therapeutic target for AR-positive/ER-negative breast cancer patients. Previously we reported that in prostate cancer cells proteasome-associated deubiquitinase ubiquitin-specific protease 14 (USP14) stabilized AR protein level by removing its ubiquitin chain. In the current study, we studied the USP14-AR protein interaction and cell proliferation status after USP14 reduction or inhibition in breast cancer cells, and our results support the conclusion that targeting USP14 is a novel strategy for treating AR-responsive breast cancer. We found that inhibition of USP14 accelerated the K48-ubiquitination and proteasome-mediated degradation of AR protein. Additionally, both genetic and pharmacological inhibition of USP14 significantly suppressed cell proliferation in AR-responsive breast cancer cells by blocking $G_0/G_1$ to S phase transition and inducing apoptosis. Moreover, AR overexpression inhibited USP14 inhibition-induced events, suggesting that AR deubiquitination by USP14 is critical for breast cancer growth and USP14 inhibition is a possible strategy to treat AR-positive breast cancer.

## Introduction

Breast cancer, an increasing threat to women in the world, is considered as a heterogeneous disease. Breast cancer can be divided into three major subtypes, based on the status of estrogen receptor (ER), progestogen receptor (PR), and HER2 status [1, 2]. Sex steroid hormones are critical to the growth and development of the uterus and breast/prostate in women/men [3]. Estrogen/ER is a highly attractive target for anti-breast cancer strategies; however, current endocrine therapies are ineffective for 25–30% of ER-negative ($ER^-$) breast cancers. Therefore, identifying new, novel therapeutic targets in advanced $ER^-$ breast cancer is critical and urgent.

Yuning Liao, Xiaohong Xia and Ningning Liu contributed equally to this work.

✉ Hongbiao Huang
hhb800616@126.com

✉ Jinbao Liu
jliu@gzhmu.edu.cn

[1] Affiliated Cancer Hospital of Guangzhou Medical University; Protein Modification and Degradation Lab, SKLRD, School of Basic Medical Sciences, Guangzhou Medical University, Guangzhou, Guangdong, China

[2] Guangzhou Institute of Cardiovascular Disease, the Second Affiliated Hospital, Guangzhou Medical University, Guangzhou, Guangdong, China

[3] The Molecular Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA

[4] Departments of Oncology, Pharmacology and Pathology, School of Medicine, Wayne State University, Detroit, MI, USA

[5] The Third Affiliated Hospital, Center for DAMP Biology, Key Laboratory for Major Obstetric Diseases of Guangdong Province, Key Laboratory of Reproduction and Genetics of Guangdong Higher Education Institutes, Protein Modification and Degradation Laboratory, Guangzhou Medical University, Guangzhou, Guangdong, China

[6] Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA

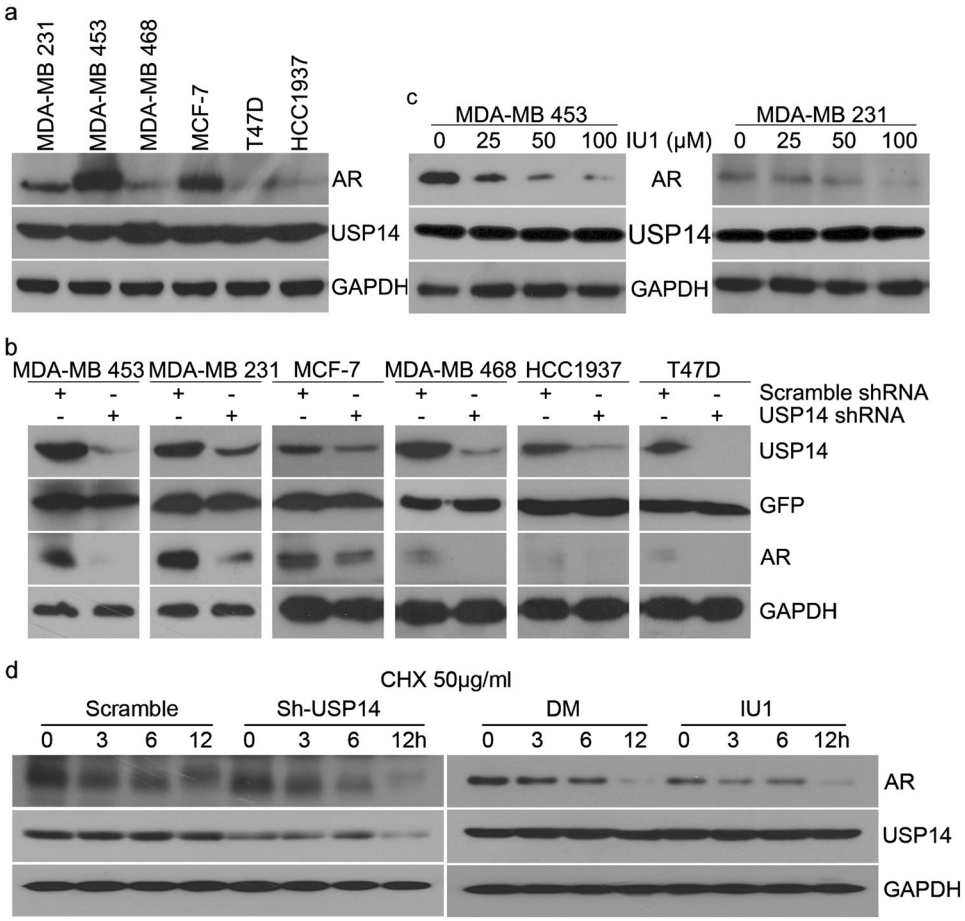
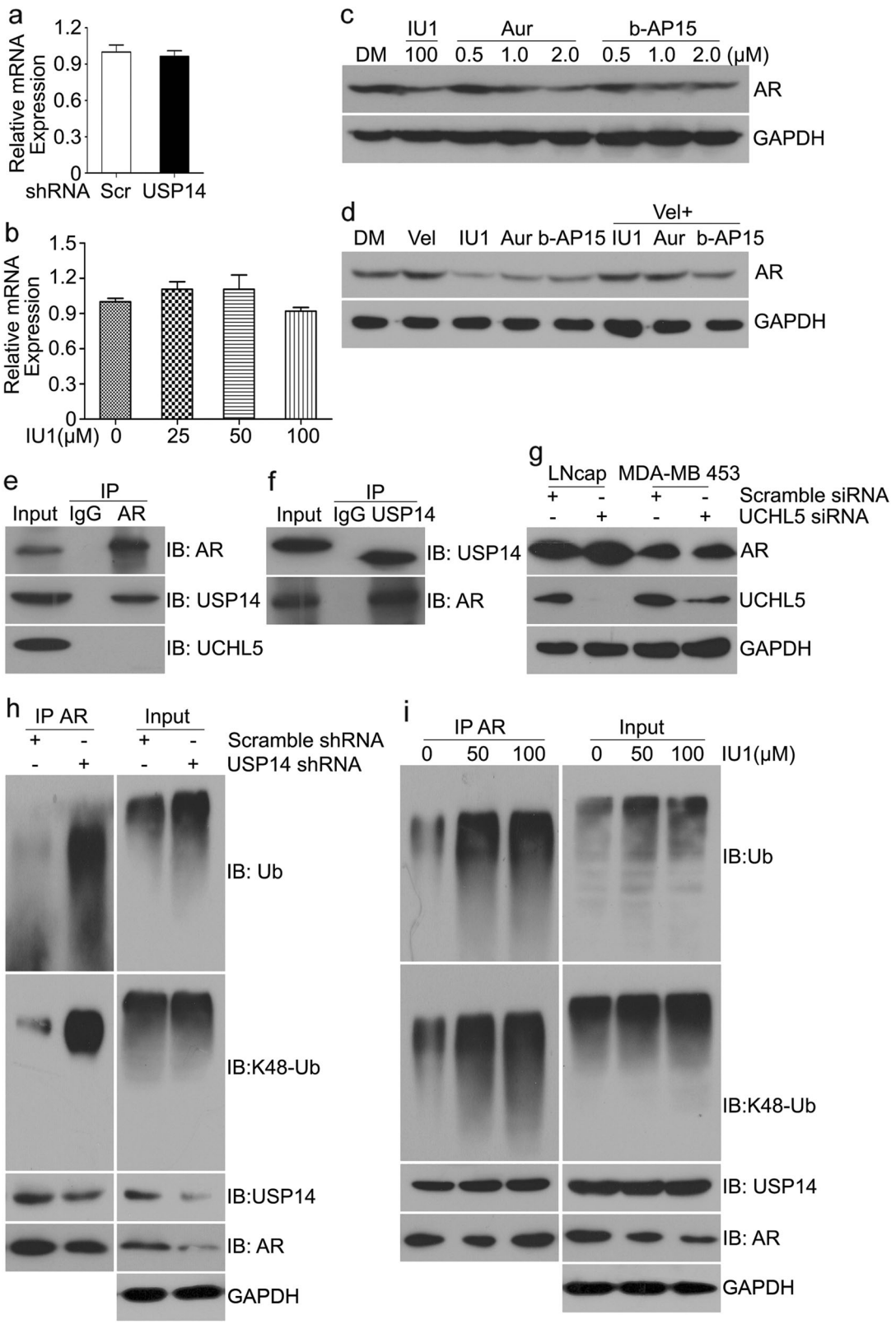



**Fig. 1** USP14 inhibition or silencing downregulates AR protein level in breast cancer cells. **a** Protein lysates were collected from the indicated breast cancer cells. Western blot assay was used to detect the expression of USP14 and AR proteins. GAPDH was used as an internal control. **b** Protein lysates were collected from the indicated breast cancer cells stably expressing USP14 shRNA or control shRNA. Western blot assay was used to detect the expression of USP14, GFP, and AR proteins. **c** Protein lysates were collected from the indicated breast cancer cells treated with the indicated concentrations of IU1 for 48 h. Western blot assay was used to detect the expression of USP14 and AR proteins. **d** MDA-MB-453 cells stably expressing USP14 or control shRNA or treated with IU1 or DMSO, and then treated with cycloheximide (CHX) for various lengths of time. Western blot assay was used to detect AR and USP14 protein level

Expression of AR is a phenomenon in most breast tumors, irrespective of ER status [4, 5]. Although AR has an anti-proliferative effect in $ER^+$ breast cancer by antagonizing ER [6], recent studies have also shown that AR facilitates the proliferation of $ER^-$ breast carcinomas [3, 7, 8]. Indeed, AR signaling inhibitors, small molecules that bind AR and inhibit its nuclear translocation, such as enzalutamide and bicalutamide, have shown some efficacy in treating advanced $ER^-/AR^+$ breast cancer, which established a potential strategy for treating anti-$ER^-/AR^+$ breast cancer by targeting AR [7, 9]. Recent study also shows that the level of AR expression may represent a valuable prognostic marker or tool for treatment selection in breast cancer [10]. These findings collectively suggest that androgens may promote breast carcinogenesis, and AR could be developed as a therapeutic target for breast cancers.

AR is highly regulated by the ubiquitin proteasome system. Ubiquitination of AR may affect or even change its function and location, or promote its degradation; AR ubiquitination can be reversed by deubiquitination mediated by deubiquitinases (DUBs) [11]. Increasing levels of E3 ubiquitin ligases, such as MDM2, CHIP, and SIAH2, have been shown to co-regulate AR and therefore control AR stability and activity [12–14]. The function of DUBs, key effectors of deubiquitination, is to remove mono-ubiquitin (Ub) or poly-Ub chains from target proteins, resulting in protein degradation or prevention of degradation, and by doing so, DUBS are involved in the regulation of multiple cellular processes. Indeed, several DUBs, including USP26, USP12, USP10, and USP7, have been reported to interact with AR protein and overcome the Ub-ligase effects of MDM2, CHIP, or SIAH2 [15–19].

There are three DUBs, USP14, UCHL5, and Rpn11 (POH1) present in mammalian 19 S proteasome complexes. Rpn11 is an intrinsic subunit of 19 S regulatory particle, whereas USP14 and UCHL5 reversibly associate with 19 S proteasome, indicative of attractive and versatile roles for these DUBs [20–22]. As a member of the ubiquitin-specific processing protease (USP) family, USP14 has been reported to be overexpressed in various cancers, including multiple myeloma, ovarian carcinoma, and colorectal cancer [22–24]. Different from numerous DUBs, the deubiquitinating activity of USP14 is activated by proteasome [25–27]. USP14 counteracts the function of proteasome by mediating


a
Relative mRNA Expression
1.2
0.9
0.6
0.3
0
shRNA Scr USP14
b
Relative mRNA Expression
1.5
1.2
0.9
0.6
0.3
0
IU1(μM) 0 25 50 100
c
IU1 Aur b-AP15
DM 100 0.5 1.0 2.0 0.5 1.0 2.0 (μM)
AR
GAPDH
d
Vel+
DM Vel IU1 Aur b-AP15 IU1 Aur b-AP15
AR
GAPDH
e
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Input IgG AR
IB: AR
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Input IgG USP14
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LNcap MDA-MB 453
+ - + - Scramble siRNA
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+ - + - Scramble shRNA
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IB: Ub
IB:K48-Ub
IB:USP14
IB: AR
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i
IP AR
Input
0 50 100 0 50 100 IU1(μM)
IB:Ub
IB:K48-Ub
IB: USP14
IB: AR
GAPDH

◀ **Fig. 2** Loss of USP14 expression/function accumulates the poly-K48-ubiquitination of AR and promotes AR degradation. **a** Total RNAs were collected from MDA-MB-453 cells stably expressing USP14 shRNA or control shRNA and subjected to $RT^2$-PCR analysis. Three independent experiments were performed. Mean ± S.D. ($n = 3$). **b** Total RNAs were extracted from MDA-MB-453 cells treated with IU1 for 24 h and subjected to $RT^2$-PCR analysis. Three independent experiments were performed. Mean ± S.D. ($n = 3$). **c** Protein lysates were collected from MDA-MB-453 cells treated with the indicated doses of IU1, auranofin (Aur), or b-AP15 for 48 h. Western blot assay was used to detect AR protein level. **d** Protein lysates were collected from MDA-MB-453 cells exposed to IU1 (100 μM), Aur (1 μM), and b-AP15 (1 μM) in the presence or absence of bortezomib/ Velcade (50 nM) for 24 h and western blot assay was used to detect AR protein level. **e**, **f** Protein lysates were collected from MDA-MB-453 cells. Co-immunoprecipitation assay was performed to detect AR, USP14, and UCHL5 interaction. **g** Protein lysates were collected from LNCaP and MDA-MB-453 cells treated with UCHL5 siRNA for 48 h. Western blot assay was used to detect AR and UCHL5 protein level. **h** Protein lysates were collected from MDA-MB-453 cells stably expressing USP14 shRNA or control shRNA. Co-immunoprecipitation assay was performed using AR antibody beads, and immunoblotted for ubiquitin (Ub), K48-Ub, USP14, and AR. Cells were exposed to MG132 (10 μM) for 6 h before harvest. **i** Protein lysates were collected from MDA-MB-453 cells treated with IU1 for 48 h. Co-immunoprecipitation assay was performed using AR antibody beads, and immunoblotted for ubiquitin (Ub), K48-Ub, USP14, and AR. Cells were exposed to MG132 (10 μM) for 6 h before harvest

rapid deubiquitination and reducing the anchoring time of ubiquitin conjugates, and thereby suppressing the degradation of the substrate proteins [27, 28]. Previously we found that the proteasome-associated DUB USP14 promoted the cell cycle in prostate carcinoma cells by deubiquitination and stabilization of AR [11]. The current study demonstrated that USP14 is crucial for the growth and survival of $AR^+/ER^-$ breast cancer, which was dependent on AR status.

## Results

### USP14 regulates AR protein level in breast cancer

In the current study, we first determined the expression levels of AR and USP14 proteins in six breast cancer cell lines using Western blot analysis. We observed high expression of USP14 protein in all six breast cancer cell lines (Fig. 1a). However, AR expression was higher in MDA-MB-453, MDA-MB-231, and MCF7 cell lines than in MDA-MB-468, HCC1937, and T47D lines (Fig. 1a). Therefore, profile of USP14 expression is not positively correlated with that of AR. This phenomenon is similar to our previous findings from prostate cancer cell lines [11], which might suggest a potential role of USP14 in the progression of breast carcinomas. We previously reported that USP14 regulates the expression or function of AR in prostate cancer cells [11]. We then applied USP14 short hairpin RNA (shRNA) in breast cancer cells to knockdown USP14 and studied the consequent effect on the AR expression; we found that inhibition of USP14 expression by its specific shRNA treatment caused great decrease in AR expression in $AR^+$ breast cancer cells (Fig. 1b). To verify whether this shRNA effect is dependent on inhibition of the deubiquitinating activity of USP14, we used IU1, a potent pharmacological inhibitor that selectively inhibits USP14 by preventing its docking on the proteasome [27], and tested the effect of USP14 inhibition by IU1 on the AR expression in breast cancer cells. We found that similar to USP14 shRNA (Fig. 1b), inhibition of USP14 by IU1 also significantly decreased AR protein level in breast cancer cells (Fig. 1c), supporting the conclusion that proteasomal DUB USP14 regulates the expression of AR in breast cancer cells. To determine whether USP14 regulates the stability of AR protein, we used cycloheximide (CHX). Treatment of control MDA-MB-453 (either scramble shRNA or parental) cells with CHX for up to 12 h caused decreased levels of AR (Fig. 1d), suggesting a contribution of AR protein synthesis to endogenous AR protein levels; however, co-treatment of CHX and USP14 shRNA or IU1 resulted more rapid decrease in levels of endogenous AR protein (Fig. 1d), strongly suggest that deubiquitination of AR protein by USP14 is essential for its protein stability.

### USP14 interacts with, and stabilizes AR protein

It has been shown that DUBs regulate their substrate proteins on multiple levels, including post-translational and protein–protein interaction. DUBs can directly bind to target proteins, and can also interact with transcription factors or histone-associated proteins, thereby regulating transcription [29]. Thus, we investigated whether USP14 regulates the transcription of AR. Data from RT-PCR assay demonstrated that the mRNA level of AR was not affected by either genetic or pharmacological inhibition of USP14 (Fig. 2a, b). We therefore hypothesized that inhibition or silencing of USP14 could induce AR downregulation through enhancing AR degradation. To test effects of other inhibitors that potently inhibit both USP14 and UCHL5, we used auranofin (Aur) [30] or b-AP15 [31] and determined if they could affect AR expression in breast cancer MDA-MB-453 cells. We found that both Aur and b-AP15 decreased the expression of AR in a dose-dependent fashion (Fig. 2c), and the reduction can be rescued by using bortezomib (Velcade/Vel), a selective 20 S proteasome inhibitor (Fig. 2d).

To determine if there is any interaction between AR protein and USP14 or UCHL5 protein, we performed co-immunoprecipitation (co-IP) for AR, USP14, and UCHL5. We found that USP14, but not UCHL5, directly binds AR protein (Fig. 2e, f). In addition, reduction of UCHL5 by its siRNA did not affect the expression of AR (Fig. 2g),

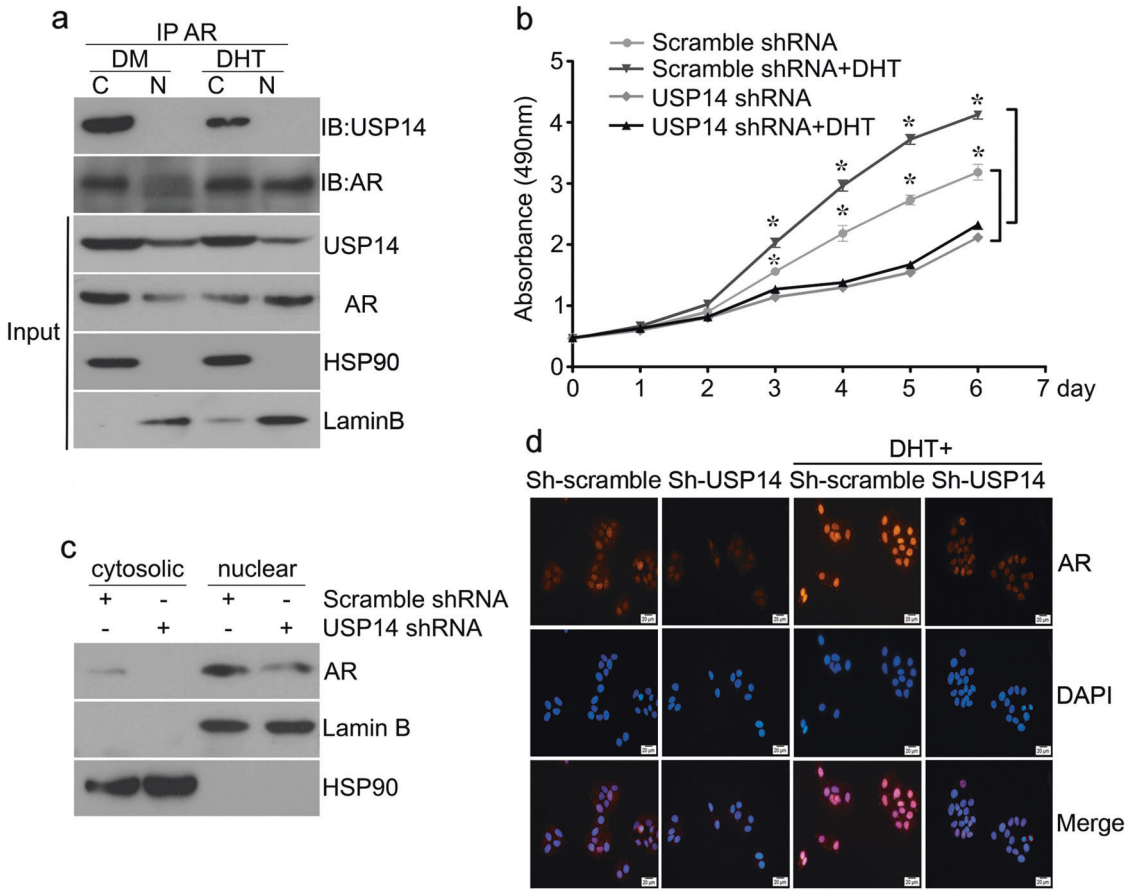



**Fig. 3** USP14 in the cytoplasm is critical to the nuclear import of AR. **a** MDA-MB-453 cells were treated with DHT (10 nM) for 24 h. Cytosolic and nuclear protein fractions were prepared, which were then immunoprecipitated with AR antibody beads, and immunoblotted for AR and USP14. HSP90 was used as a cytoplasmic control. Lamin B was used as a nuclear control. **b** MDA-MB-453 cells stably expressing USP14 shRNA or control shRNA were exposed to DHT (10 nM) for indicated days. Cell viability was detected using MTS assay. Error bars correspond to a 95% CI of three independent experiments. $*P < 0.05$. **c** Cytosolic and nuclear protein lysates were extracted, respectively, from MDA-MB-453 cells stably expressing USP14 or control shRNA exposed to DHT (10 nM) for 24 h. Western blot assay was used to detect AR expression. **d** MDA-MB-453 cells stably expressing USP14 shRNA or control shRNA were exposed to vehicle (Veh) or DHT (10 nM) for 24 h. Immunofluorescence microscopy shows endogenous AR (orange) and nucleus (blue). Scale bars represent 20 μm

suggesting that USP14 but not UCHL5 recruited on the19S proteasome plays a selective role in the deubiquitination of AR. To further confirm that USP14 is a DUB of AR, we determined the effect of IU1 or USP14 shRNA on the abundance of poly-ubiquitinated and K48-poly-ubiquitinated AR using co-IP. We found that IU1 and USP14 knockdown dramatically increased levels of ubiquitinated and K48-ubiquitinated AR (Fig. 2h, i), suggesting that USP14 is an AR DUB, capable of deubiquitinating and thereby stabilizing AR protein.

## USP14 is not required for AR translocation

DUBs could regulate the localization or function of target proteins. USP10 is a bona fide DUB that deubiquitinates p53 and AR in the cytoplasm and enhances their nuclear import and transcriptional activity [19, 32]. Whether USP14 could co-translocate with AR into the nucleus is unclear. To determine the interaction between AR and USP14 protein in the nucleus, we performed co-IP for AR and USP14 using both nuclear and cytosolic preparations of MDA-MB-453 cells treated with DHT or control solvent for 24 h. We found that USP14 does not interact with AR in the nucleus under DHT stimulation (Fig. 3a). In addition, western blot analysis and immunofluorescent staining assay indicated that USP14 silencing significantly downregulated the abundance of AR in both the nucleus and cytoplasm under androgen stimulation (Fig. 3c, d), suggesting that cytosolic USP14 is not required for AR translocation and that USP14 silencing-induced decrease of nuclear AR could be due to

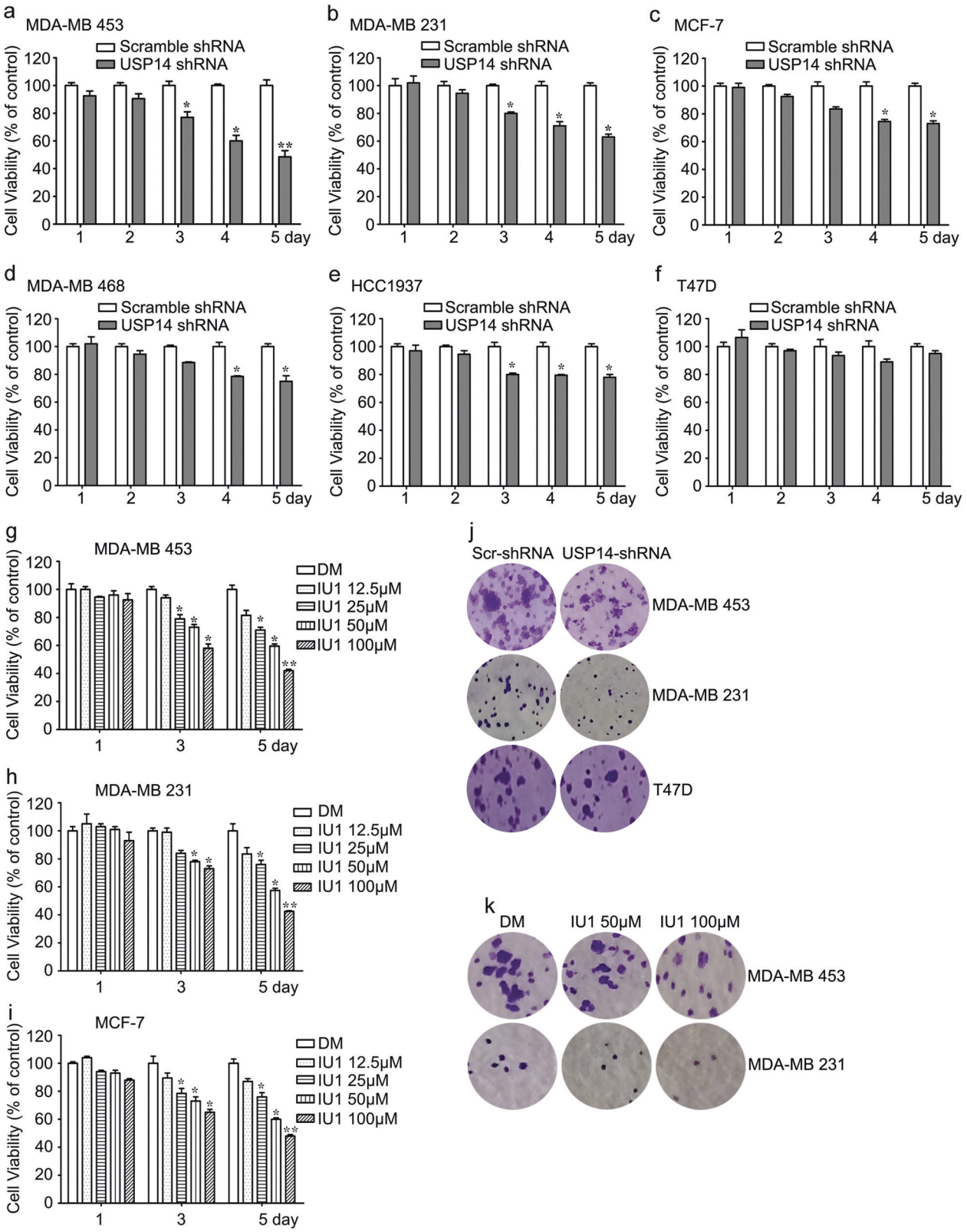
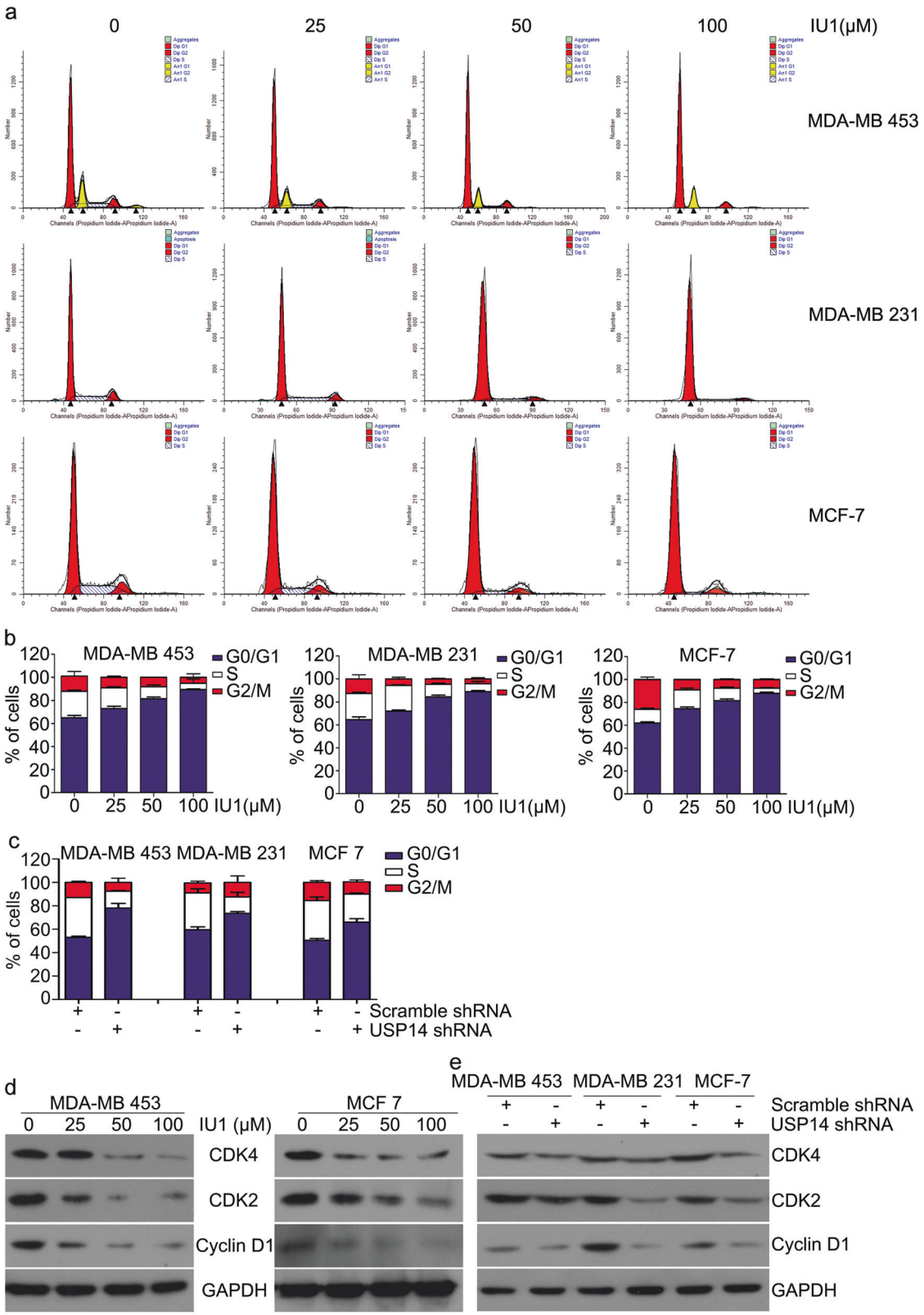



**Fig. 4** USP14 inhibition or silencing suppresses the proliferation of $AR^{+}$ breast tumor cells. **a–f** MDA-MB-453, MDA-MB-231, MCF7, MDA-MB-468, HCC1937, and T47D cells were exposed to USP14 shRNA daily for up to 5 days. **g–i** MDA-MB-453, MDA-MB-231, and MCF7 cells were exposed to IU1 for 1, 3, and 5 days. Cell viability was detected using MTS assay. Error bars correspond to 95% CI of three independent experiments. $*P < 0.05$, $**P < 0.01$ vs. each vehicle control. **j** Colony formation assay was performed in the indicated breast cancer cells stably expressing control shRNA or USP14 shRNA for 2 weeks; representative images are shown. **k** Colony formation assay was performed in the indicated breast cancer cells exposed to IU1 for 2 weeks; representative images are shown


a
0
25
50
100
IU1(μM)
MDA-MB 453
MDA-MB 231
MCF-7
b
MDA-MB 453
MDA-MB 231
MCF-7
G0/G1
S
G2/M
% of cells
0 25 50 100 IU1(μM)
c
MDA-MB 453 MDA-MB 231 MCF 7
G0/G1
S
G2/M
% of cells
Scramble shRNA
USP14 shRNA
d
MDA-MB 453
MCF 7
0 25 50 100 IU1 (μM)
CDK4
CDK2
Cyclin D1
GAPDH
e
MDA-MB 453 MDA-MB 231 MCF-7
Scramble shRNA
USP14 shRNA
CDK4
CDK2
Cyclin D1
GAPDH

◀ **Fig. 5** USP14 inhibition or silencing blocks $G_0/G_1$ to S phase transition in breast cancer cells. **a** Fluorescence-activated cell sorting analysis (FACS) was performed on the indicated breast cancer cells exposed to IU1 for 48 h. ($n = 3$). **b** The percentage of cells in each population was calculated. Mean ± S.D. ($n = 3$). **c** FACS analysis was performed on the indicated breast cancer cells that stably expressed USP14 shRNA or control shRNA for 48 h. The percentage of cells in each population was calculated. Mean ± S.D. ($n = 3$). **d** Protein lysates were collected from the indicated breast cancer cells treated with IU1 for 48 h. Western blot assay was used to detect CDK4, CDK2, and cyclin D1 protein expression. GAPDH was used as an internal control. **e** Protein lysates were collected from the indicated breast cancer cells stably expressing USP14 shRNA or control shRNA. Western blot assay was used to detect CDK4, CDK2, and cyclin D1 expression. GAPDH was used as an internal control

the decrease of total AR protein. To investigate the effect of USP14 knockdown on DHT-mediated events in MDA-MB-453 cells, we compared the proliferation rates of USP14 shRNA knockdown or control cells after treatment with DHT for up to 7 days using MTS assay. We found that DHT promoted the proliferation of the control group, but had little enhancing effect on the proliferation of the USP14 knockdown group (Fig. 3b). Collectively, the results show that USP14 regulates the total AR level but not AR translocation, and mediates the responsiveness of $AR^+/ER^-$breast cancer to androgen.

## Inhibition of USP14 suppresses the proliferation of breast cancer

To study the potential role of USP14 in the proliferation of breast tumor cells, we determined the effect of USP14 knockdown on breast cell viability. We found that USP14 inhibition by shRNA significantly suppressed the proliferation of MDA-MB-231, MDA-MB-453, MDA-MB-468, HCC1937, and MCF7 breast cancer cell lines (Fig. 4a–e); however USP14 knockdown did not affect the proliferation of T47D cells (Fig. 4f), which could be related to the fact that these cells express very high levels of ER and very low levels of AR (Fig. 1a). In addition, we also used the pharmacological USP14 inhibitor IU1 (12.5, 25, 50, 100 μM) and tested the effect on the proliferation of breast cancer cells. Inhibition of USP14 by IU1 caused a concentration-dependent, significant suppression on the proliferation of MDA-MB-453, MDA-MB-231, and MCF7 carcinoma cells (Fig. 4g–i). To test the long-term effect of USP14 inhibition or silencing on breast carcinoma cells, we performed breast cancer cell colony formation assay using either IU1 treatment or USP14 shRNA-generated stable clones. USP14 inhibition by either IU1 or specific shRNA dramatically decreased the colony formation of MDA-MB-453 and MDA-MB-231 cells after 2 weeks; however, the identical experimental conditions had little or no inhibitory effect on T47D colonies. (Fig. 4j, k).

## Inhibition of USP14 causes $G_0/G_1$ arrest and apoptosis in breast cancer cells

We then studied the underlying mechanism by which USP14 regulates the growth of $AR^+$ breast cancer cells. We monitored the cell cycle progression of each group of breast cancer cells exposed to various concentrations of IU1 (25, 50, 100 μM), and found that inhibition of USP14 activity dramatically induced $G_0/G_1$ cell cycle arrest after 48 h, associated with decreased population in $S/G_2/M$ phases (Fig. 5a, b). In addition, silencing USP14 expression with stable expression of shRNA also caused an arrest of cells at $G_0/G_1$ (Fig. 5c), indicating that USP14 regulates the $G_1$ to S transition in androgen-responsive breast cancer cells. To explore the molecular mechanism by which USP14 regulates the $G_1$–S transition, Western blot analysis was performed to detect levels of several $G_1$ and S key players. We found that the IU1 treatment cause a decrease in the protein level of cyclin D1, CDK4, and CDK2, all of which have been shown to be essential for $G_0/G_1$ to S phase transition (Fig. 5d). In USP14-stable knockdown MDA-MB-231, MDA-MB-453, and MCF7 cells protein levels of cyclin D1, CDK4, and CDK2 were also decreased (Fig. 5e). Collectively, these results indicate that USP14 regulates levels of key cell cycle regulators cyclin D1, CDK4, and CDK2 and consequently the $G_1$–S phase transition in breast cancer cells.

We then determined whether apoptosis induction is also involved in the growth inhibition by USP14 inhibition or knockdown in breast cancer cells. To do so, we performed several specific assays, including annexinV-fluorescein isothiocyanate (FITC)/propidium iodide (PI)-staining, PARP cleavage, and Bcl-2 protein expression using western blot analyses. Bcl-2 is an anti-apoptotic protein, while p89 cleavage fragment of PARP is a molecular marker of apoptosis. We found that USP14 inhibition significantly induced apoptosis (Fig. 6a–d), as evident by PARP cleavage, and downregulation of the anti-apoptotic protein Bcl-2, in $AR^+/ER^-$ breast cancer (Fig. 6e, f), and moderately induced apoptosis in $AR^+/ER^+$ MCF7 cells (Fig. 6a–f).

## AR overexpression suppresses USP14 inhibition-induced events

To study the functionality of AR deubiquitination by USP14, we first compared the effects of AR depletion in the $AR^+/ER^-$ MDA-MB-453 cells to T47D cells that express high ER and low AR. We found that AR depletion by siRNA led to $G_1$ arrest and apoptosis in MDA-MB-453, but not in T47D (Fig. 7a, b); consistently, AR depletion decreased levels of CDK4, CDK2, cyclin D1, and Bcl-2 in MDA-MB-453, but not T47D cells (Fig. 7c, d). Next, we determined if re-introduction of AR would rescue the

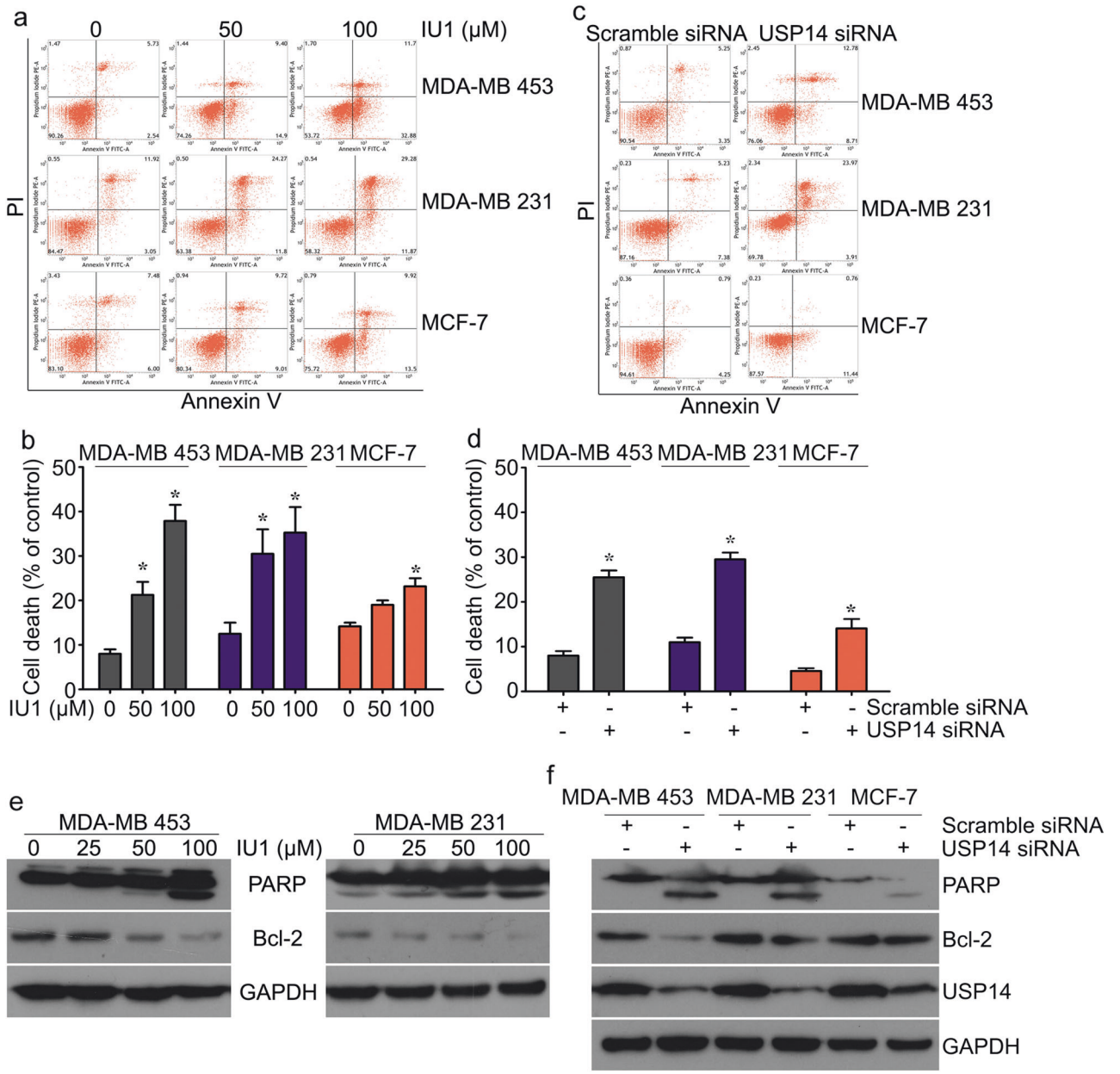
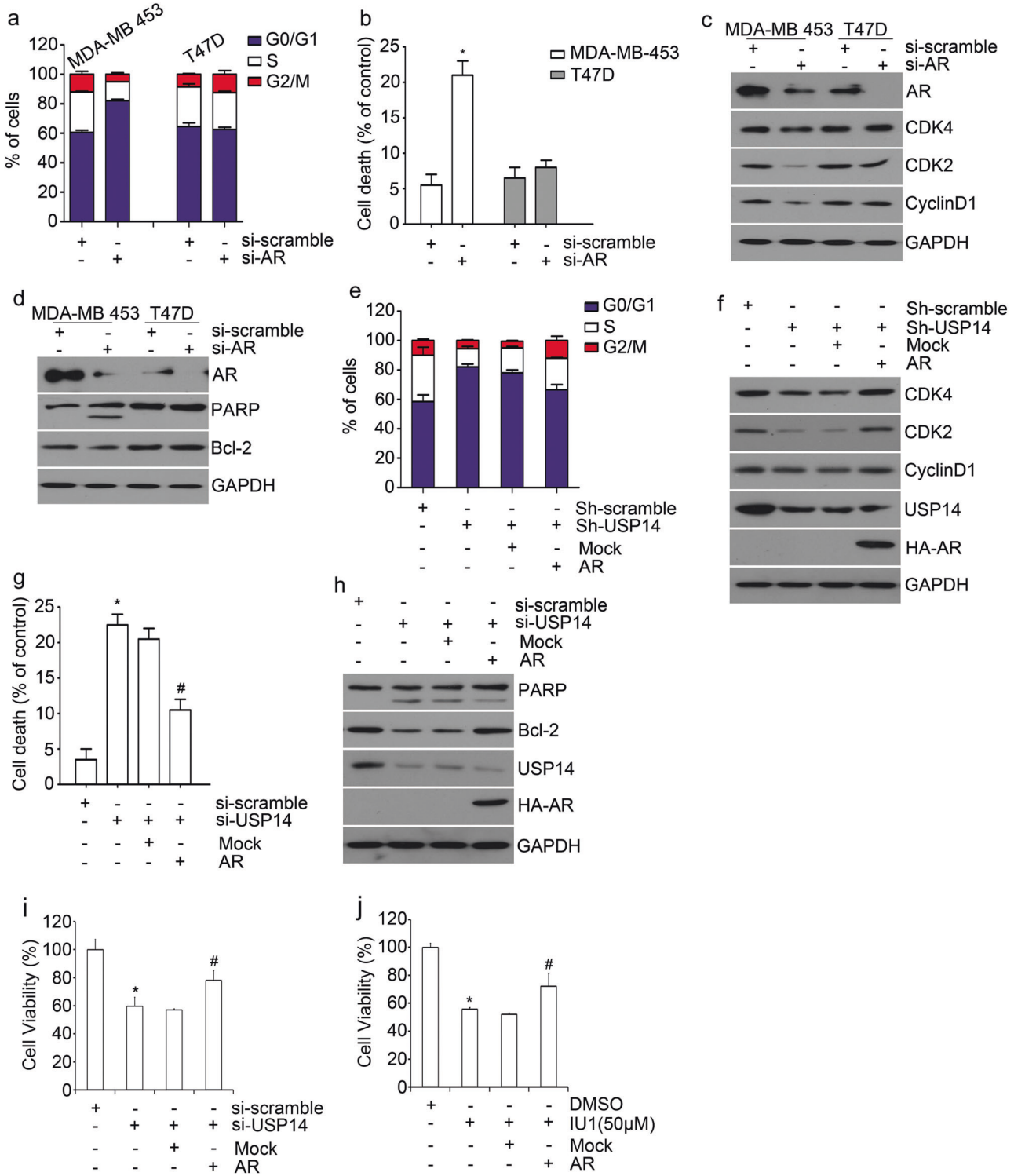



**Fig. 6** USP14 inhibition or silencing induces apoptosis in $AR^{+}/ER^{-}$ breast cancer cells. The indicated breast cancer cell lines treated with IU1 for 72 h were collected. Flow cytometry analysis with annexinV-FITC/PI staining was used to calculate the apoptotic cells. Representative images (**a**) and cell death population (**b**) from three independent replicates are shown. Mean ± S.D. ($n = 3$). *$P < 0.05$ vs. each vehicle control. The indicated breast cancer cells treated with USP14 siRNA or control siRNA for 72 h were collected. Flow cytometry analysis with annexinV-FITC/PI staining was used to calculate the apoptotic cells. Representative images (**c**) and cell death population (**d**) are shown. Mean ± S.D. ($n = 3$). *$P < 0.05$ vs. each vehicle control. **e** Protein lysates were collected from the indicated breast cancer cells treated with IU1 for 48 h. Western blot assay was used to detect PARP and Bcl-2 protein expression. **f** Protein lysates were collected from the indicated breast cancer cells treated with USP14 siRNA or control siRNA for 48 h. Western blot assay was used to detect PARP and Bcl-2 expression. GAPDH was used as an internal control

growth inhibition induced by USP14 knockdown. To do so, MDA-MB-453 cells stably expressing control shRNA or USP14 shRNA were transfected with a tagged AR vector (HA-AR) or a control vector (Mock), followed by measurement of cell cycle progression. Overexpression of AR was able to rescue cells from the USP14 silencing-induced $G_1$ arrest (Fig. 7e). Western blot analysis from the same experiment confirmed increased levels of CDK4, CDK2, and Cyclin D1 protein after overexpressing AR (Fig. 7f). Additionally, AR overexpression inhibited cell apoptosis




and growth suppression induced by USP14 silencing or IU1 treatment (Fig. 7g–j). Collectively, these findings demonstrate the functionality of AR deubiquitination by USP14 in promoting breast cancer cell proliferation.

## Discussion

Breast cancer is a common threat to women's health in the whole world. ER, PR, and HER2 play important roles in promoting the development and progression of most breast

◀ **Fig. 7** AR overexpression inhibits USP14 silencing-induced $G_1$–S arrest and apoptosis. **a** Fluorescence-activated cell sorting analysis (FACS) was performed on the indicated breast cancer cells treated with the AR siRNA or control siRNA for 48 h. Cells in each population were calculated from three independent replicates. Mean ± S.D. ($n = 3$). **b** The indicated breast cancer cells treated with AR siRNA or control siRNA for 72 h. Flow cytometry analysis with annexinV-FITC/PI staining was used to calculate the apoptotic cells. Apoptotic populations from three independent replicates are shown. Mean ± S.D. ($n = 3$). $^*P < 0.05$ vs. each vehicle control. **c** and **d** Protein lysates were collected from the indicated breast cancer cells exposed to AR siRNA or control siRNA for 48 h. Western blot assay was used to detect the expression of AR, CDK4, CDK2, cyclin D1, PARP, and Bcl-2. **e** MDA-MB-453 cells stably expressing USP14 or control shRNA were transduced with AR or control vector for 48 h and subjected to FACS analysis. Cells in each population were calculated from three independent replicates. Mean ± S.D. ($n = 3$). **f** Protein lysates were collected from MDA-MB-453 cells stably expressing USP14 or control shRNA transduced with AR or control vector for 48 h. Western blot assay was used to detect the expression of CDK4, CDK2, cyclin D1, USP14, and HA-tag. **g** MDA-MB-453 cells exposed to USP14 siRNA or control siRNA with or without AR or control vector for 72 h were collected. Flow cytometry analysis with annexinV-FITC/PI staining was used to calculate the apoptotic cells. Apoptotic populations from three independent replicates are shown. Mean ± S.D. ($n = 3$). $^*P < 0.05$ vs. vehicle control. $^{\#}P < 0.05$ vs. si-USP14. **h** Western blot assay was used to detect the expression of PARP, Bcl-2, USP14, and HA-tag. GAPDH was used as an internal control. **i** MDA-MB-453 cells were exposed to USP14 siRNA or control siRNA with or without AR or control vector for 72 h. **j** MDA-MB-453 cells were exposed to IU1 with or without AR or control vector for 72 h. MTS assay was used to detect cell viability. Error bars correspond to 95% CI of three independent replicates. $^*P < 0.05$ vs. each vehicle control. $^{\#}P < 0.05$ vs. si-USP14 or IU1 treatment

cancers. Blocking these receptors can lead to significant suppression of the development of many breast cancers. However, triple-negative breast cancer (TNBC), which is $ER^-$, $PR^-$, and $HER2^-$ and comprised 15–20% of breast cancers, has the worst prognosis and vital organ metastases due to the lack of effective therapeutic targets [2, 33, 34]. Recent studies have identified AR as a novel therapeutic target in breast cancer, unveiling a great treatment opportunity for TNBC patients. Our current study showed that USP14, one of the 19 S proteasome-associated DUBs, stabilizes AR protein level by deubiquitinating the K48-ubiquitin chain on AR. Loss of USP14 expression/function dramatically decreased AR level, blocked $G_0/G_1$ to S phase transition, and triggered cell apoptosis in $AR^+$ breast cancer cells, suggesting that targeting USP14/AR axis could be a potential strategy for TNBC therapy.

Proteasomes recruit UCHL5 and USP14 to regulate the degradation of proteins. Inhibition of both UCHL5 and USP14 dramatically accumulates total K48-poly-ubiquitinated proteins [30, 31, 35], suggesting that UCHL5 and USP14 are required for the proteasome-mediated degradation of most proteins. However, the function of UCHL5 and USP14 for a specific protein remains largely unknown. Previously we identified USP14 as an AR DUB on 19 S proteasome by cleaving its Ub-chain in LNCaP cells. The current study confirmed that USP14 removes the K48-ubiquitin chain on AR and subsequently inhibits proteasome-mediated degradation of AR, consistent with the finding that USP14 inhibited proteasome and mediated deubiquitination by reducing the anchoring duration of ubiquitin substrates on the proteasome [27, 28]. We wondered if UCHL5 exerts the same or opposite effects for AR. We found that dual inhibitors of USP14 and UCHL5 such as b-AP15 [31] and auranofin [30] reduced AR protein level in both prostate and breast cancer cells. We further explored the effect of UCHL5 knockdown on AR protein level and protein interaction between UCHL5 and AR. We found that UCHL5 silencing did not affect the expression of AR, and UCHL5 did not interact with AR via protein–protein binding under tested experimental condition, suggesting that USP14, but not UCHL5, is required to remove the ubiquitin chain on AR and stabilize AR protein on the 19 S particle.

DUBs have emerged as a class of novel therapeutic targets or biomarkers for anticancer strategies. Indeed, several DUBs have been reported to regulate AR expression or transcriptional activity. As an example, USP26 physically interacts with AR and influences AR ubiquitination and transcriptional activation [17]. Also, USP12 co-localizes with AR in the cytoplasm and promotes AR transcriptional activity by confronting the ubiquitin-dependent degradation of AR [15]. USP7 seems to be required to bind AR to chromatin, mediating its activity [16]. Moreover, USP10 not only binds AR, resulting in increased transcriptional activity, but also deubiquitinates the histone variant H2A.Z, both of which are required for $AR^-$mediated gene activation [18, 19]. Therefore, our current study investigated the function of USP14 for AR translocation. We found that USP14 could not translocate AR into the nucleus under DHT stimulation. Nevertheless, loss of USP14 reduced the volume of nuclear AR under DHT stimulation, which may result from the reduction of cellular AR after USP14 silencing.

We further investigated the molecular mechanisms by which USP14 contributes to breast cancer development and progression. USP14 inhibition or silencing significantly inhibits the growth of MDA-MB-453 and MDA-MB-231 breast cancer cells, and modestly suppresses the growth of breast cancer cells with either low AR expression (MDA-MB-468 and HCC1937) or high AR/ER expression (MCF7). In addition, USP14 inhibition or silencing-induced $G_0/G_1$ arrest in MDA-MB 231, MDA-MB 453, and MCF7 breast cancer cells, associated with significantly reduced levels of CDK4, CDK2, and cyclin D1 proteins. This finding was consistent with findings from our previous study on prostate cancer, suggesting that USP14 plays an

important role in cell cycle regulation. Moreover, inhibition or silencing of USP14 dramatically induced higher levels of cell death and PARP cleavage in MDA-MB 231 and MDA-MB 453 than in MCF7 breast cancer cells. Surprisingly, treatment of USP14 inhibition or knockdown had no or little effect on breast cancer T47D cells that express high level of ER and very low level of AR. USP14 silencing-induced cell cycle arrest and apoptosis could be rescued by over-expressing AR, indicating that cell death and growth arrest are direct results of inhibition of AR deubiquitination. These findings also suggest that USP14 is definitely required for the growth and survival of $AR^+/ER^-$ breast cancers.

In conclusion, this work has provided novel insights into the interaction between proteasome-associated DUB USP14 and AR, and the functional role of deubiquitination of AR by USP14 in AR-positive human breast cancer cells including TNBC. Furthermore, the current research has provided a mechanistic base for targeting USP14 as a therapeutic strategy for treating patients with $AR^+/ER^-$ breast cancer.

## Materials and methods

### Materials

IU1 (catalog no. sc-361215), USP14 (sc-76817), AR (sc-29204), and UCHL5 (c-76797) siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). b-AP15 (S4920), MG132 (S2619), and bortezomib (S1013) were purchased from Sellectchem (Houston, TX, USA). Aur (catalog no. BML-EI206-0100) was obtained from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). MTS (catalog no. G111) was from Promega Corporation (Madison, WI, USA). AnnexinV-FITC/PI apoptosis detection kits (KGA107) were purchased from Keygen Company (Nanjing, China). Co-IP assay kit (14311D) was obtained from Life Technologies (Carlsbad, CA). Antibodies were from the following corporations: anti-GAPDH (MB001), anti-CDK2 (BS1050), anti-GFP (BSAP0675M), anti-Bcl-2 (BS70205) (Bioworld Technology, Inc., Louis Park, MN, USA); anti-Ubiquitin (catalog no. 3936), anti-PARP (9532), anti-CDK4 (12790), anti-USP14 (11931), anti-Lamin B (13435), anti-HSP90 (4877), HA-tag (3724), anti-K48-ub (12805), anti-cyclin D1 (2922) (Cell Signaling Technology, Beverly, MA, USA); anti-AR (ab108341), anti-UCHL5 (ab124931) (Abcam, USA).

### Cell lines and cell cultures

The following cell lines are from American Type Culture Collection (Manassas, VA, USA): MDA-MB-453, MDA-MB-231, MCF7, MDA-MB 468, HCC1937, T47D, and LNCaP. HCC1937 and LNCaP were grown in RPMI 1640, 10% fetal bovine serum (FBS) and penicillin/streptomycin. MDA-MB 453, MDA-MB 231, MCF7, MDA-MB 468, and T47D cells were grown in HyClone DMEM, 10% FBS and penicillin/streptomycin.

### Cell viability assay

Cell viability was detected using MTS assay (CellTiter 96Aqueous One Solution reagent) as we previously reported [36]. Briefly, cultured MDA-MB-453, MDA-MB-231, MCF7, MDA-MB-468, HCC1937, or T47D cells were digested and suspended at $2 \times 10^4$ cells/ml medium, and then randomly seeded with 100 µl cell suspensions in 96-well plate. The cells were incubated for 24 h and then exposed to IU1 or USP14 shRNA for one to 5 days. MTS reagent was added to each well. After 3 h reaction at 37°, the absorbance of density of each well was read at a wavelength of 490 nm with a microplate reader (Sunrise, Tecan).

### Flow cytometry analysis of cell cycle and apoptosis

For cell cycle assay, the indicated breast cancer cells were exposed to IU1 or USP14/control shRNA for 48 h. Cells were collected and then washed three times with 4°C PBS, and then resuspended with 500 µl PBS plus 2 ml 70% ethanol at 4°C for 12 h. Cells were then centrifuged and washed with 4°C PBS again, followed by 50 µg/ml PI, 100 µg/ml RNaseA, 0.2% Triton-X-100 complex incubation for 30 min at 4°C. The flow cytometry was used to analyze the stained cells. Cell apoptosis analysis was performed as we previously described [37]. Briefly, the indicated breast cancer cells were exposed to IU1 or USP14/control siRNA for 72 h. Cells were collected and washed three times with 4°C PBS. Then cells were resuspended with 500 µl annexinV-FITC binding buffer, 5 µl annexinV-FITC, and 5 µl PI mixture in each group. After incubation for 30 min, flow cytometry was used to analyze the stained cells.

### Clonogenic assay

Clonogenic assay was performed as previously described [38]. The indicated breast cancer cells were exposed to IU1 or USP14/control shRNA for 48 h. Then the cells in each group were digested, resuspended and randomly seeded in 60 mm dishes supplemented with 10% FBS DMEM medium, cultured in an atmosphere of 5% $CO_2$ for 2 weeks. Cells were fixed with 4% paraformaldehyde for 15 min, then washed with PBS twice, followed by crystal violet solution incubation for 5 min. Colonies >60 µm were counted from three independent repeated experiments.

## siRNA transfection

SiRNA transfection was performed as previously described [39]. Briefly, the indicated breast cancer cells were randomly seeded in 60 mm dishes for 24 h. RPMI opti-MEM, lipofectamine RNAiMax (Invitrogen) reagent and siRNAs (Santa Cruz, CA) targeting human USP14/UCHL5/AR or siRNAs(Santa Cruz, CA) with non-specific sequences mixtures were prepared respectively, then the mixtures was added in each group and cultured for 72 h. Fresh medium was replaced appropriately after transfection for 6 h.

## Lentivirus USP14 shRNA transfection

Lentivirus (pLent-4in1shRNA-GFP) containing human USP14 (NM-005151) shRNA or control shRNA was purchased from VigeneBio (Shandong, China). Exponentially growing cells were randomly seeded in 60 mm dishes. When the cells were cultured overnight and reached 50% confluence, medium containing lentiviruses and polybrene (5 μg/ml; Santa Cruz, CA, USA) was added at a multiplicity of infection of 10 and mixed with the cells. After overnight incubation, supernatant in each well was replaced with DMEM containing 10% FBS and cultured for 48 h. For selection of stably-transfected cells, we proceeded with puromycin selection performed by aspirating the medium and replacing it with fresh medium containing puromycin (Santa Cruz, CA, USA) at the concentration of 2 μg/ml and then aspirating and replacing with freshly prepared selective medium and culturing the surviving cells approximately every 2 days.

## RNA extraction and PCR assay

RNAs were extracted from MDA-MB 453 cells treated with IU1 or USP14/control shRNA for 24 h with RNAiso plus (TaKaRa Biotechnology, Dalian, China) and performed according to the manufacturer's instructions. The Purity and concentration of RNAs in each sample were read with 260:280 nm. The First-strand cDNA was synthesized with 1 μg total RNA and the use of PrimeScript RT Master Mix kit (TaKaRa, Dalian, China). Real-time quantitative PCR was used to measure the mRNA expression levels of AR. GAPDH, a housekeeping gene, was here used as an internal control. SYBR Premix Ex Taq$^{TM}$ kit (TaKaRa, Dalian, China) was used according to the manufacturer's instructions. The primers in this study for PCR were as follow, AR: F: 5′-GGTGAGCAGAGTGCCCTATC-3′; R: 5′-GAAGAC CTTGCAGCTTCCAC-3′; GAPDH: F:5′-TCCCATCACCATCTTCCA-3′; R: 5′-CATCACGCCA-CAGTTTCC-3′.

## Protein expression and interaction analysis

Protein interaction (Co-IP) analysis was performed as described in a previous study [11]. In brief, antibodies and dynabeads (Invitrogen) mixtures were prepared overnight. Then the cell lysates extracted from MDA-MB 453 were added in the antibodies-beads mixtures. After incubation and rotation at 4°C for 1 h, the antibodies-prays mixtures were washed with PBS-T for three times. Then the mixtures were suspended with appropriate SDS loading buffer and separated from dynabeads. Western blot was used to analyze protein expression. This assay was performed as described previously [40].

## Immunofluorescence microscopy

Cells stably expressing USP14 or scramble shRNA were exposed to DHT 10 nM for 24 h. Then 4% paraformaldehyde was used to fixed cells for 15 min. 0.5% Triton-X was used to permeabilize cells for 5 min. 5% BSA (bovine serum albumin, Sigma) was used to block for 30 min. And then the cells were incubated with AR primary antibodies (Abcam) overnight at 4°C. Next the cells were incubated with anti-rabbit IgG-cy3 secondary antibodies (Bioworld) for 1 h. DAPI (4′,6-diamidino-2-phenylindole, Abcam) was used to indicate the nucleus. Images were acquired using an Olympus fluorescence microscope with ×400 magnification.

## Plasmids and transfection of cells

The plasmid HA-AR encoding a fusion protein of AR and HA-tag or control vector was purchased from Genechem (Shanghai, China). Exponentially growing MDA-MB-453 cells stably expressing USP14 or scramble shRNA were seeded in six-well plates for 24 h. The cells were transfected with plasmid HA-AR or control vector mixed with lipofectamine 2000 reagent (Life Technologies). Then the cells were incubated for 48 h for further analysis.

## Statistical methods

Data are presented as mean ± SD from three independent experiments where applicable. To determine statistical probabilities, Unpaired Student's *t* test or one way ANOVA is used where appropriate. Statistical analysis was performed by GraphPad Prism5.0 software (GraphPad Software) and SPSS 16.0. A $P$ value of $>0.05$ was considered statistically significant.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.



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