Biochemical and Biophysical Research Communications 444 (2014) 290–295

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

HUWE1 interacts with BRCA1 and promotes its degradation in the ubiquitin–proteasome pathway

Xiaozhen Wang [a,b], Guang Lu [a], Li Li [a], Juan Yi [a], Kaowen Yan [a], Yaqing Wang [a], Baili Zhu [a], Jingyu Kuang [a], Ming Lin [a], Sha Zhang [a], Genze Shao [a,b,*]

[a] Department of Cell Biology, Peking University Health Science Center, Beijing 100191, China
[b] Institute of Systems Biology, Peking University, Beijing 100191, China

ARTICLE INFO

Article history:
Available online 14 December 2013

Keywords:
HUWE1
BRCA1
Ubiquitination
Degradation

ABSTRACT

The cellular BRCA1 protein level is essential for its tumor suppression activity and is tightly regulated through multiple mechanisms including ubiquitn–proteasome system. E3 ligases are involved to promote BRCA1 for ubiquitination and degradation. Here, we identified HUWE1/Mule/ARF-BP1 as a novel BRCA1-interacting protein involved in the control of BRCA1 protein level. HUWE1binds BRCA1 through its N-terminus degron domain. Depletion of HUWE1 by siRNA-mediated interference significantly increases BRCA1 protein levels and prolongs the half-life of BRCA1. Moreover, exogenous expression of HUWE1 promotes BRCA1 degradation through the ubiquitin–proteasome pathway, which could explain an inverse correlation between HUWE1 and BRCA1 levels in MCF10F, MCF7 and MDA-MB-231 breast cancer cells. Consistent with a functional role for HUWE1 in regulating BRCA1-mediated cellular response to DNA damage, depletion of HUWE1 by siRNA confers increased resistance to ionizing radiation and mitomycin. These data indicate that HUWE1 is a critical negative regulator of BRCA1 and suggest a new molecular mechanism for breast cancer pathogenesis.

1. Introduction

BRCA1 is an important tumor suppressor associated with breast cancer. It plays critical roles in multiple cellular processes including DNA damage repair, the cell cycle, and checkpoint control [1–3]. Loss of BRCA1 leads to deficiency in the repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) and leads to genomic instability that is associated with transformation. Germline mutations of BRCA1 strongly predispose women to breast and ovarian cancer and accounts for a significant proportion of hereditary breast cancer [4]. However, while a BRCA1 mutation is rare among sporadic cancers, reduced BRCA1 protein levels is often detected in breast cancers without a BRCA1 mutation [5,6]. The underlying mechanisms for this phenomenon are not fully understood. Given the critical roles of BRCA1 in tumor suppression, it is assumed that reduced BRCA1 protein levels might result in an insufficient or loss of BRCA1 function, and could therefore contribute to tumorigenesis by accelerating genomic instability. Thus, identification of BRCA1 regulators is important for our understanding the mechanisms underlying breast cancer pathogenesis.

The cellular BRCA1 protein level is tightly controlled through multiple forms of regulation including both transcriptional and posttranscriptional mechanisms. First, the human *Brca1* gene is transcriptionally regulated [7], and this transcription can be inhibited by hypermethylation of its promoter, which is often observed in many types of breast cancer [8]. Second, BRCA1 is regulated by posttranscriptional modifications such as ubiquitination. For example, HERC2 and SCF^{FBXO44} have been recently identified as E3 ligases responsible for BRCA1 ubiquitination and proteolysis [9,10]. In addition, E2T, an ubiquitin-conjugating enzyme, is also involved in the regulation of BRCA1 [11]. These findings highlight an essential role of the ubiquitin–proteasome system in controlling BRCA1 levels.

The HECT-domain E3 ligase HUWE1 (HECT, UBA and WWE domain containing 1) is involved in the ubiquitination and degradation of multiple proteins, including p53 [12], Mcl-1 [13], N-Myc [14], Cdc6 [15], TopBP1 [16], Polβ [17], and MyoD [18]. HUWE1 plays diverse biological roles and is upregulated in multiple diseases, including breast cancer [19]. HUWE1 is highly expressed in 80% of breast cancer cell lines, while its expression in normal breast cells (MCF10A) is low [12]. Previously, we used mass spectrometry to determine that HUWE1 resided within the BRCA1-Merit40/RAP80 complex [20], indicating that BRCA1 might act as

Abbreviations: HUWE1, HECT, UBA and WWE domain containing 1; CHX, cycloheximide; HMECs, human mammary epithelial cells; IR, ionizing radiation; MMC, mitomycin; NLS, nuclear localization signal; siRNA, short interfering RNA.

* Corresponding author at: Department of Cell Biology, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100191, China. Fax: +86 10 82805119.
E-mail address: gzshao@bjmu.edu.cn (G. Shao).

0006-291X/$ - see front matter © 2014 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.12.053

a novel candidate substrate of HUWE1. To identify a role for HUWE1 in regulating BRCA1, in the present study we characterized the interaction between HUWE1 and BRCA1. Our results demonstrate that HUWE1 enhances BRCA1 ubiquitination and degradation and regulates BRCA1 activity in protecting against DNA damage. These findings elucidate new molecular mechanisms underlying breast cancer tumorigenesis.

2. Materials and methods

2.1. Plasmids and reagents

Plasmids pcDNA3.1-FH-HUWE1, pEYFP-HUWE1, and pcDNA4-BRCA1-myc-His/C61Gwere generated by a PCR-based subcloning strategy using pFastBac-Mule or pcDNA3-HA-BRCA1 plasmids as templates. pcDNA-ARF-BP1 was provided by Wei Gu (Columbia University), and pFastBac-Mule was provided by Xiaodong Wang (University of Texas Southwestern Medical Center). Additional details are provided in Supplementary Materials and methods.

2.2. Immunoprecipitation, immunostaining, CHX chase assay, and IR/MMC sensitivity assays

Experimental procedures and details of immunoprecipitation, immunostaining, Western blotting, the CHX chase assay, and IR/MMC sensitivity analyses are provided in Supplementary Materials and methods.

3. Results

3.1. HUWE1 interacts with BRCA1

To search for proteins that mediate BRCA1 ubiquitination and proteasomal degradation, we purified the BRCA1-MERIT40/Rap80complex as previously described [20], HUWE1 was identified resided in the complex by mass spectrometry analysis (Supplementary Table S1). To confirm the interaction between HUWE1 and BRCA1, HEK293T cells expressing Flag-HA-HUWE1 protein were subjected to co-immunoprecipitation assays. HUWE1 co-immunoprecipitated with anti-BRCA1 antibody, but not control rabbit IgG (Fig. 1A). The reciprocal co-immunoprecipitation verified this interaction (Fig. 1B). These results indicate that HUWE1 interacts with BRCA1 *in vivo*.

To further characterize the interaction between HUWE1 and BRCA1, we mapped the regions of HUWE1 that are required for interaction with BRCA1. Plasmids expressing a series of HA-tagged HUWE1 fragments (F1–5) were constructed (Fig. 1C), and their ability to associate with full-length BRCA1 was assessed in HEK293T cells by co-immunoprecipitation following transfection. BRCA1 interacted primarily with HUWE1 F3, but not F4, suggesting

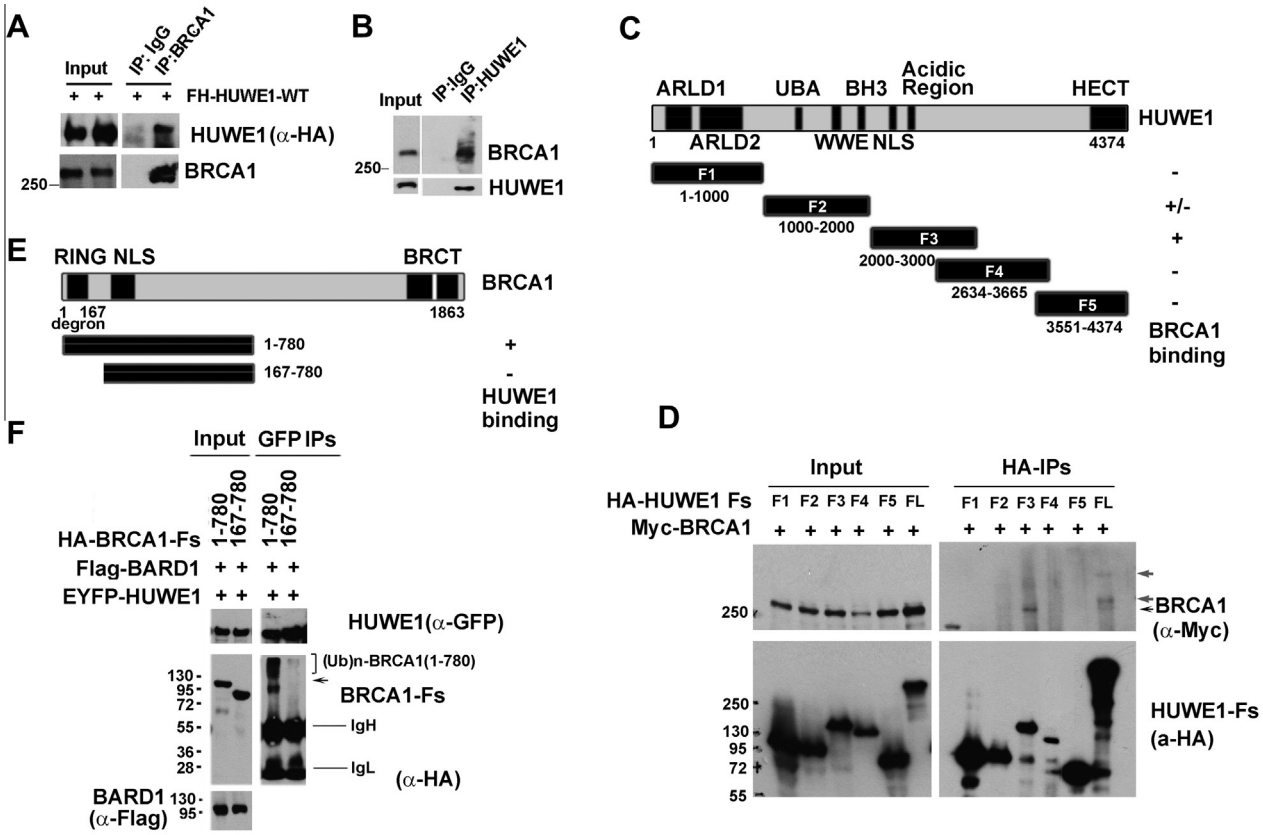

Fig. 1. HUWE1 interacts with BRCA1. (A) HEK293T cells were transfected with pcDNA3.1-Flag-HA-HUWE1, and cell lysates were then subjected to immunoprecipitation (IP) with control IgG or α-BRCA1antibody, followed by immunoblotting using α-HA antibody to target Flag-HA-HUWE1, or α-BRCA1 to target endogenous BRCA1. (B) The reciprocal immunoprecipitation/immunoblot was performed. HEK293T cell lysates were subjected to IP with control IgG or α-HUWE1 antibody, followed by immunoblotting using α-BRCA1 or α-HUWE1 antibodies to target endogenous proteins. (C) To map the region of HUWE1 required for association with BRCA1, a series of deletion plasmids were constructed. (D) HA-tagged HUWE1 fragments (F1–F5) or full length HUWE1 (FL) was co-expressed with Myc-tagged full-length BRCA1 in HEK293T cells (input). IP was performed using anti-HA-agarose beads (HA-IPs), followed by immunoblotting with α-Myc to detect BRCA1 or α-HA to detect HUWE1. The black arrow indicates the position of BRCA1 immunoprecipitated by F3; the gray arrows show a shifted migration of BRCA1 protein immunoprecipitated by full-length HUWE1. (E) To map the region of BRCA1 required for association with HUWE1, HA-tagged BRCA1 fragments were constructed. (F) HA-BRCA1 fragments were co-expressed with Flag-BARD1 and EYFP-HUWE1 in HEK293T cells (input), and then immunoprecipitated with α-GFP antibody (GFP IPs). Immunoblotting of BRCA1 and HUWE1 was performed using α-HA and α-GFP antibodies, respectively. The black arrows indicate the position of the 1–780 BRCA1 fragment immunoprecipitated with HUWE1, while the bracket shows a high molecular weight band shift. Results are representative of three independent experiments.

that the 2000–2634 aa region of HUWE1 is essential to mediate its interaction with BRCA1 (Fig. 1D, black arrow). This region contains a nuclear localization signal (NLS) region (2236–2255 aa), and a 52-amino acid, glutamic/aspartic acid-rich domain (2410–2461 aa) (Fig. 1C). Notably, the BRCA1 protein immunoprecipitated by full-length HUWE1 displayed a shifted migration (Fig. 1D, gray arrow), suggesting that BRCA1 might be modified by a mechanism that is preferentially enacted by full-length HUWE1.

We next mapped the region of BRCA1 required for its interaction with HUWE1. Two HA-tagged BRCA1 deletions (Fig. 1E) were tested for binding to GFP-tagged HUWE1 (EYFP-HUWE1). Because NH2-terminal fragments of BRCA1 without BARD1 are unstable in cells [9,21], BARD1 was cotransfected. HUWE1 immunoprecipitated BRCA1 (1–780) (Fig. 1F, black arrow) but not BRCA1 (167–780), suggesting that the HUWE1 binding site within BRCA1 is located between amino acid residues 1 and 167. Interestingly, this region of BRCA1 encompasses a degron domain that mediates the ubiquitination and instability of BRCA1 [9,22]. The immunoprecipitated BRCA1 1–780 fragment also showed a high molecular weight band shift (Fig. 1F, bracket), indicating that HUWE1 may contribute to a posttranslational modification of BRCA1. Based on the known function of HUWE1 as an E3 ubiquitin ligase [12–17,23], this shift is most likely attributed to ubiquitination. These results suggest that the NLS-containing, acidic amino-rich region of HUWE1 (2000–2634 aa) interacts with the degron domain of BRCA1 and may be associated with its ubiquitination.

3.2. HUWE1 decreases the stability of BRCA1

Ubiquitination by E3 ligases is often associated with degradation [24]. To determine whether HUWE1 regulates BRCA1 protein stability, HUWE1 was expressed exogenously in HEK293T cells, and levels of endogenous BRCA1 were assessed. BRCA1 levels were slightly reduced by HUWE1 in a dose-dependent manner (Fig. 2A). These results were verified in Hela cells by immunofluorescent microscopy (Fig. 2B). However, the level of ectopic BRCA1 was not significantly affected in cells co-expressing HUWE1 in 293T cells (Supplementary Fig. S1). This result may be due to the increased expression of BARD1 caused by BRCA1 over-expression, which may in turn, have inhibitory effects on HUWE1. Conversely, knockdown of HUWE1 in MCF-10F cells using two specific siRNAs resulted in elevated BRCA1 and BARD1 protein levels (Fig. 2C).

To evaluate whether HUWE1 regulates BRCA1 in a UPS-dependent manner, we determined the effects of HUWE1 on BRCA1 protein stability at defined intervals after treatment with cycloheximide (CHX) to block *de novo* protein synthesis. Overexpression of WT HUWE1, not the E3 ligase enzymatic-dead mutant C4341A [13], accelerated BRCA1 degradation (Fig. 2D).

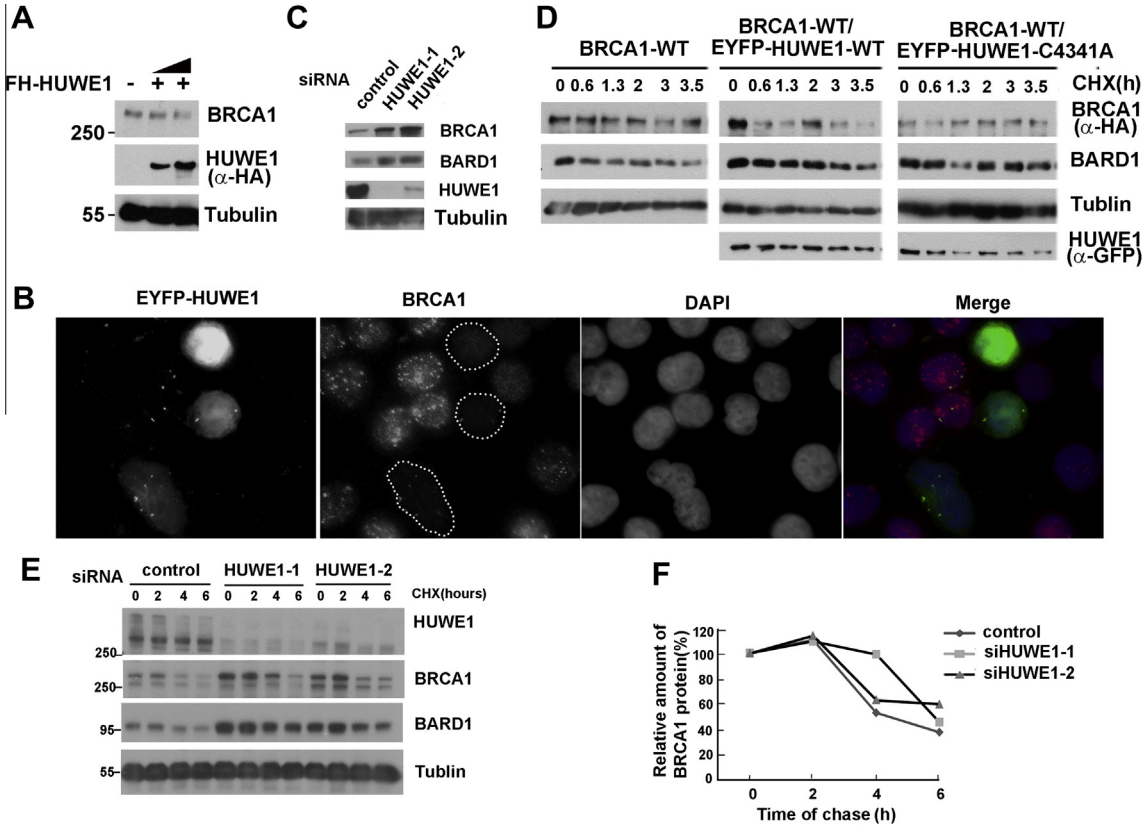

Fig. 2. HUWE1 decreases the stability of BRCA1. (A) Dose-dependent degradation of endogenous BRCA1 by HUWE1. HEK293T cells were transfected with 0, 0.8 or 2.0 μg of Flag-HA-HUWE1 plasmid, followed by immunoblotting with α-BRCA1 or α-HA antibody. Tubulin is shown as a loading control. (B) HUWE1 expression downregulates endogenous BRCA1. Forty-eight hours after transfection with EYFP-HUWE1, HeLa cells were fixed and immunostained with anti-BRCA1 antibody. Cells expressing EYFP-HUWE1 are circled with a white, dotted line, while DAPI stains all cells. A merged photo of all three stains is shown. (C) HUWE1 knockdown stabilizes endogenous BRCA1 protein. MCF10F cells were transfected with non-specific siRNA (control), two siRNAs targeting different sites within HUWE1 (siHUWE1-1 and siHUWE1-2). After 72 h, cell lysates were subjected to immunoblotting with the indicated antibodies. (D) Over-expression of HUWE1 accelerates the degradation of BRCA1. HEK293T cells were transiently transfected with HA-BRCA1 plasmid alone or together with EYFP-HUWE1 WT or C4341A mutant plasmid. Forty-eight hours post-transfection, cells were treated with cycloheximide (CHX), and lysates were collected for immunoblotting at the indicated times. (E) siRNA depletion of HUWE1 increases the half-life of BRCA1. MCF-10F cells were transfected with siRNA and then incubated with CHX for the indicated times. Levels of endogenous HUWE1, BRCA1 and BARD1 were determined by immunoblotting. Tubulin is shown as a loading control. (F) Levels of BRCA1 in panel E were quantified and normalized to 100% at 0 h CHX. All results are representative of three independent experiments.

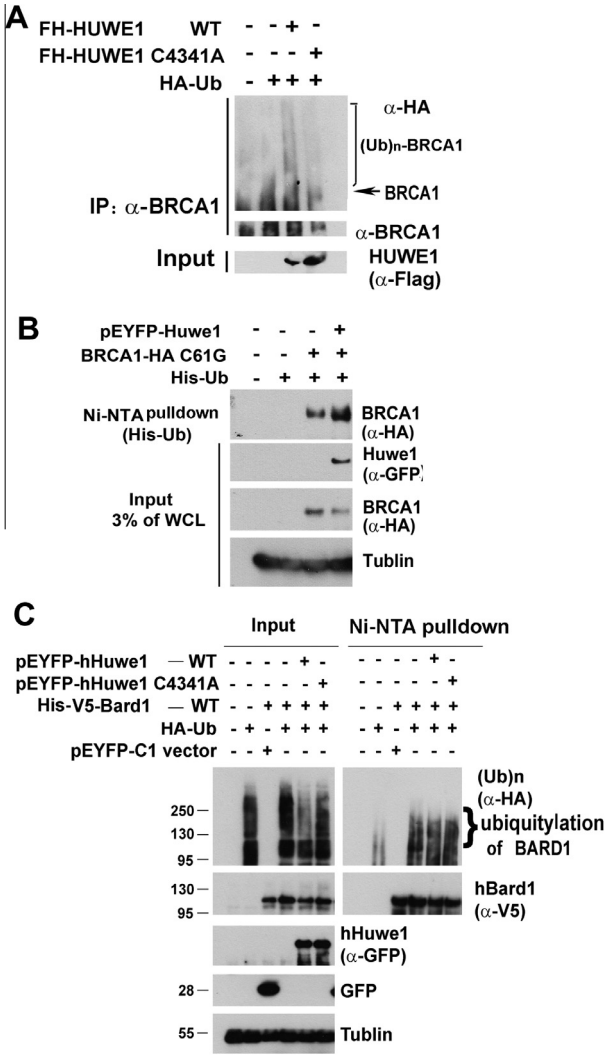

Fig. 3. HUWE1 promotes BRCA1 ubiquitination. (A) Wild-type, but not C4341A mutant HUWE1, promotes polyubiquitination of BRCA1. HA-Ub, Flag-HA-HUWE1 WT and C4341A plasmids were co-transfected into HEK293T cells. BRCA1 was immunoprecipitated under denaturing conditions using α-BRCA1 antibody, and polyubiquitinated BRCA1 (BRCA1-(Ub)*n*) and total BRCA1 were detected by Western blotting with α-HA and α-BRCA1antibodies, respectively. α-Flag is shown as a control for exogenous HUWE1 expression. (B) HUWE1 promotes polyubiquitination of the BRCA1 C61G mutant. His-Ub, EYFP-HUWE1 and BRCA1-HA C61G plasmids were co-transfected into HEK293T cells. Following Ni^{+2}-NTApulldown, ubiquitinated BRCA1 C61G was detected by immunoblotting with α-HA antibody. Expression of HUWE1 (α-GFP), BRCA1 (α-HA) and tubulin in the input are shown as control. (C) HUWE1 does not contribute to the ubiquitination of BARD1. HEK293T cells were transfected and His-V5 tagged BARD1was pulled down with Ni^{+2}-NTA resin, followed by immunoblotting as indicated. BARD1 (α-V5), HUWE1 (α-GFP) and tubulin in the input are shown as control. All results are representative of three independent experiments.

Conversely, BRCA1 was stabilized in MCF10F cells by transfection with siRNA against HUWE1 (Fig. 2E), leading to an extended half-life (Fig. 2F). These results demonstrate that HUWE1 promotes the degradation of BRCA1through UPS.

It is known that BRCA1 associates with BARD1 to form a RING heterodimer, which is essential for stability [25,26]. Over-expression of BRCA1 could result in an increase in BARD1 protein, which in turn could repress the ubiquitination of BRCA1 [7,9]. To avoid the antagonistic effect of BARD1, we utilized the C61G BRCA1 mutant that is unable to efficiently form a heterodimer with BARD1 to examine the effect of HUWE1 on BRCA1 stability [21,26,27]. Ectopic BRCA1 mutant C61G and EYFP-HUWE1Wt or C4341A were co-transfected into HEK-293T cells and BRCA1 C61G protein stability was accessed. While BRCA1 C61G was unstable in cells transfected HUWE1 Wt, but significantly stabilized in C4341A mutant transfected cells (Fig. S2). This result suggests HUWE1 preferentially promotes BARD1-uncoupled BRCA1 for degradation.

3.3. HUWE1 promotes BRCA1 ubiquitination

Previous studies demonstrate that HUWE1 causes the degradation of its substrates through the ubiquitin–proteasome pathway [12,15,18]. To determine whether HUWE1-dependent downregulation of BRCA1 is a consequence of BRCA1 ubiquitination, we employed HUWE1 C4341A mutant, which preserves the ability of HUWE1 to bind the substrate but prevents the transfer of an ubiquitin moiety [13]. Denaturing immunoprecipitation using an anti-BRCA1 antibody revealed that overexpression of WT HUWE1, but not the C4341A mutant, enhances the polyubiquitination of endogenous BRCA1 in HEK293T cells (Fig. 3A).

To verify these findings, we performed Ni+2-NTA pulldown assays of BRCA1 following transfection of His-Ub. BRCA1 associates with BARD1 to form a RING heterodimer, which represses BRCA1 ubiquitination [25]. To avoid the antagonistic effect of BARD1, we used a free-state BRCA1 C61G mutant that cannot couple with BARD1 [21]. Results show that HUWE1 co-expression enhances the levels of BRCA1 C61G recovered by Ni+2-NTA pulldown (Fig. 3B); further indicating that HUWE1 causes degradation of BRCA1 through the ubiquitin–proteasome pathway.

Like BRCA1, BARD1 levels are enhanced by HUWE1 siRNA (Fig. 2C and E). Therefore, we investigated whether HUWE1 can target BARD1 for polyubiquitination. However, BARD1 ubiquitination was not significantly affected by either WT or mutant HUWE1 (Fig. 3C), indicating that BARD1 is not a substrate for HUWE1 ubiquitination. Collectively, these data indicate that HUWE1 targets BRCA1, but not BARD1, for polyubiquitination and degradation.

3.4. Biological relevance of HUWE1-dependent regulation of BRCA1

Because HUWE1 promotes BRCA1 ubiquitination and degradation, we wondered whether it might contribute to the reduced BRCA1 protein levels in sporadic breast cancer. To determine whether the levels of these proteins are inversely correlated, we examined three breast cancer cell lines (MCF10F, MCF7 and MDA-MB-231) as compared to normal human mammary epithelial cells (HMECs). HUWE1 levels were high in MCF10F, MCF7 and MDA-MB-231 cells, but low in HMECs; whereas the inverse pattern was observed for BRCA1 (Fig. 4A). This finding is consistent with a physiological role for HUWE1 in negatively regulating BRCA1 in sporadic breast cancer.

Based on the function of BRCA1 in DNA damage repair, cell-cycle checkpoint and apoptosis, cells deficient in BRCA1 are highly sensitive to DNA damaging reagents [2,20]. To investigate the biological consequences of HUWE1-mediated BRCA1 degradation, we assessed the effects of HUWE1 on the DNA damage and repair response. MCF10F cells were depleted of HUWE1 by siRNA and treated with IR or MMC, and then cell survival was assessed. The results show that RNAi inhibition of HUWE1 confers increased resistance of MCF-10F cells to IR and MMC (Fig. 4B and C), which is consistent with a role for HUWE1 in repressing BRCA1-mediated DNA damage repair. In addition, HUWE1 depletion also induced an inhibitory effect on MCF-10F growth (Fig. S3). Thus, the results suggested that HUWE1-dependent regulation of BRCA1 function plays a critical role in various cellular processes.

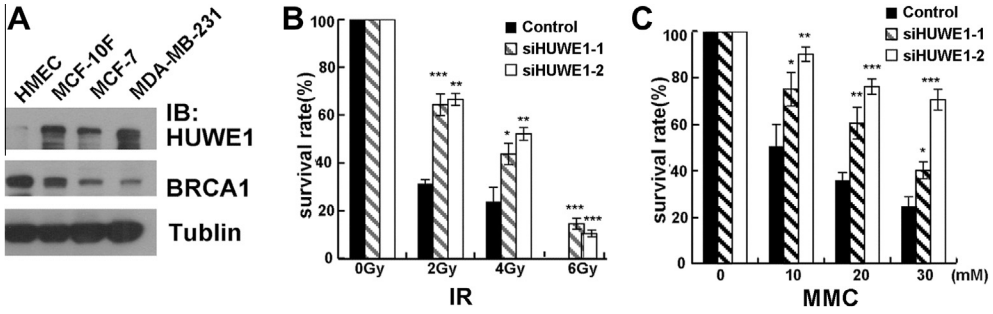

Fig. 4. Elevated HUWE1 expression in breast cancer cells inversely correlates with BRCA1 expression and HUWE1 siRNA confers resistance of MCF-10F cells to IR and MMC. (A) Inverse correlation of BRCA1 and HUWE1 expression in breast cancer cell lines and normal human mammary epithelial cells (HMECs) as determined by immunoblotting. MCF10F cells were transfected with control or HUWE1 siRNA and then treated with IR(B) or MMC(C). After 12 days, the cell survival rate (% of total population) was quantified. Values represent the average + SD of three independent experiments.

4. Discussion

BRCA1 is a multi-functional tumor suppressor and plays critical roles in the DNA damage repair, cell cycle and checkpoint control. Fine tune of BRCA1 levels is thus essential for the maintenance of genome integrity and suppression of malignancy. BARD1 couples with BRCA1 to form a RING heterodimer that is essential for BRCA1 stability, nuclear localization, and E3 ligase activity [25]. Recently, several proteins have been identified to control BRCA1 protein turnover, including HERC2 [9] and SCF^{FBXO44} [10]. These two E3 ligases display several differences in their shared role of BRCA1 degradation. HERC2 associates with BRCA1 in the S phase and targets BARD1-uncoupled BRCA1 for degradation [9]. However, FBXO44 coprecipitates with BRCA1 and BARD1 and affects the stability of both two proteins, suggesting an interaction with the BRCA1/BARD1 complex [10]. In this report, we identified HUWE1 as a new negative regulator of BRCA1, as demonstrated by both overexpression and silencing of HUWE1 in breast cancer cells. Our results suggest that HUWE1 regulates BRCA1both in the presence and absence of BARD1. In this sense, HUWE1 may have a role in regulating BRCA1 that overlaps or is complementary to both HERC2 and SCF^{FBXO44}. Consistent with our findings, BRCA1 protein was also found to be significantly accumulated in *Mule*-deficient MEF and B cells [28]. These data strongly suggest BRCA1 is a substrate for HUWE1 E3 ligase *in vivo*.

Similar to HERC2, HUWE1 is also a member of the HECT family of E3 ligases, and seemed to preferentially target BARD1-uncoupled BRCA1 for ubiquitination (Figs. 3B and S2) [9]. Unlike HERC2, HUWE1 possesses an ubiquitin-associated domain (UBA), which is an ubiquitin receptor that exists in many proteins involved in diverse cellular functions [29]. As such, HUWE1 might bind to ubiquitinated BRCA1 through the UBA domain. Indeed, BRCA1 that precipitated with HUWE1 displayed a shift in migration in the immunoblot (Fig. 1B, D and F), indicating the existence of modifications to BRCA1. Moreover, BRCA1 can be auto-ubiquitinated when dimerized with BARD1 through K6-linked polyubiquitin chains [30,31], a modification that was reported to be bound by the UBA domain of UBXN1 [32]. Importantly, this binding can inhibit the E3 ligase activity of BRCA1/BARD1. It remains to be investigated whether the UBA domain of HUWE1 has similar features as that of UBXN1. However, given the existence of a large quantity of cellular BRCA1/BARD1 heterodimers, this possibility would have important implications in the regulation of BRCA1 protein levels as well as its function. Based on the significant accumulation of BRCA1 and BARD1 upon HUWE1 depletion in mammary cell lines such as MCF-7 and MCF-10F (Fig. 2C and E), it is plausible that HUWE1 could be a major negative regulator of BRCA1.

Consistent with a previous report [13], we have also shown that cells exhibited increased resistance to IR and DNA damaging agents including MMC and cisplastin when HUWE1 was depleted. This result is consistent with a role of increased BRCA1 levels and function in the DNA damage repair. The resultant increased BRCA1 would favor DNA damage repair, especially DSBs, by HR, and thus improve the survival rate after genotoxic stress. These data suggest a crucial role of HUWE1 in the suppression of HR and thus favor NHEJ repair [33]. Indeed, Hao et al observed impaired IgH CSR in *Mule*-deficient B cells [28]. These findings strongly suggest that the HUWE1-dependent regulation of BRCA1 has profound impacts on the choice of DNA repair mechanisms. In addition, HUWE1 has also been demonstrated to be involved in DNA replication, base-excision repair and ATM-dependent DNA damage response [15–17,28]. Consistently, we also found that HUWE1 is promptly downregulated and translocated to the nucleus in response to IR (Supplementary Fig. S4). These findings strongly support a role of HUWE1 in DNA damage repair [34].

In addition to its role in the control of BRCA1, HUWE1 was also demonstrated to regulate p53and Mcl-1, two essential proteins involved in the cell cycle control and apoptosis [12,13]. Therefore, dysregulation of HUWE1 would result in deficit in BRCA1, p53 and Mcl-mediated cellular functions such as DNA damage repair, cell cycle, and apoptosis, leading to genomic instability and transformation, which is an important mechanism for tumorigenesis. Consistent with this, HUWE1 was found to be frequently over-expressed in many types of cancers including breast cancer [19,23]. These data suggest that HUWE1 may have oncogenic function, playing roles in the initiation, development, and progression of breast cancer as well as other types of cancers.

Author contributions

G.S. designed the research; X.W., G.L., L.L., J.Y., K.Y., Y.W., B.Z., Y.W., and J.K. performed the research; M.L. and S.Z. contributed to protein purification; X.W. and G.L. analyzed the data; X.W. and G.S. wrote the paper.

Acknowledgments

We thank Dr. Xiaodong Wang at University of Texas Southwestern Medical Center, Dr. Wei Gu at Columbia University, for generously providing the pFastBac-Mule and pcDNA3-ARF-BP1 plasmid, respectively. This study was supported by the National Natural Science Foundation of China (Grant 81272915); Beijing Natural Science Foundation (Grant 7122099); and Grant (20110001110013) from the Doctoral Program of Higher Education; The Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (JWSL44-8); 985 Program, Ministry of Education of China.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.053.

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