

HERC2 Is an E3 Ligase That Targets BRCA1 for Degradation

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Abstract

The breast cancer suppressor BRCA1 forms a stable heterodimeric E3 ubiquitin ligase with BARD1. Each protein controls the abundance and stability of the other, and loss of the interaction leads to BRCA1 degradation. Here, we show that HERC2, a protein recently implicated in DNA damage repair, targets BARD1-uncoupled BRCA1 for degradation. HERC2 shuttles between the nucleus and the cytoplasm. Its COOH-terminal HECT-containing domain interacts with an NH₂-terminal degron domain in BRCA1. HERC2 ubiquitinates BRCA1; this reaction depends on Cys⁴⁷⁶² of HERC2, the catalytic ubiquitin binding site, and the degron of BRCA1. The HERC2-BRCA1 interaction is maximal during the S phase of the cell cycle and rapidly diminishes as cells enter G₂-M, inversely correlated with the steady-state level of BRCA1. Significantly, HERC2 depletion antagonizes the effects of BARD1 depletion by restoring BRCA1 expression and G₂-M checkpoint activity. Conversely, BARD1 protects BRCA1 from HERC2-mediated ubiquitination. Collectively, our findings identify a function for HERC2 in regulating BRCA1 stability in opposition to BARD1. The HERC2 expression in breast epithelial cells and breast carcinomas suggests that this mechanism may play a role in breast carcinogenesis.

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Introduction

The BRCA1 tumor suppressor is a key player in breast and ovarian carcinogenesis. In addition to its role in hereditary cancer (1), accumulated evidence indicates that BRCA1 dysfunction also results in sporadic basal-like breast cancer, an aggressive subtype of breast cancer with a poor prognosis (2). BRCA1 maintains genomic stability by functioning in DNA damage repair, cell cycle checkpoint activity, and apoptosis (3). One significant property of BRCA1 is that it is a cell cycle-regulated protein. Its expression level and phosphorylation status oscillate during the cell cycle (4–8). The expression level is regulated by both transcription and protein stability. Although it has been shown that BRCA1 turnover increases in S phase by ubiquitin-dependent proteolysis (7), features that regulate BRCA1 protein stability are largely unknown. One significant factor that regulates the stability of BRCA1 is the protein BARD1. BARD1 associates with BRCA1

to form a RING heterodimer that is essential for its stability, nuclear localization, and E3 ligase activity (9–14). Each protein controls the abundance and stability of the other in a heterodimerization-dependent manner, and loss of the interaction leads to BRCA1 degradation (9, 12). However, how BARD1-uncoupled BRCA1 is degraded remains unclear.

HERC2 is a highly mutable gene found at a deletion breakpoint hotspot on human chromosome 15q11-q13 (15–19). *HERC2* duplications in this region are thought to play a role in a 4-Mb deletion that causes Prader-Willi/Angelman syndrome. Whereas mutation of *HERC2* likely does not underlie Prader-Willi/Angelman syndrome in humans, homozygous mutation of this gene causes diverse phenotypes in mice (15) known as *rjs* (runty jerky sterile; ref. 20) and *jdj2* (juvenile development and fertility; ref. 21) that resemble symptoms of Prader-Willi syndrome. The *HERC2* gene encodes a giant 4834-amino acid protein of 528 kDa that is composed of 93 exons (15, 16). It is highly conserved in evolution and possesses multiple candidate functional domains including three RCC1-like domains, which have a role in Ran-dependent nuclear membrane trafficking, and a COOH-terminal HECT domain, a motif responsible for E3 ubiquitin ligase activity. The protein function of *HERC2* had long been undetermined. However, it was recently shown that *HERC2* is implicated in DNA damage repair functions. Bekker-Jensen et al. (22) showed that *HERC2* functions as an assembly factor for the complex of an E3 ubiquitin ligase RNF8 and an E2 ubiquitin-conjugating enzyme Ubc13 to promote Lys⁶³-linked polyubiquitin chains at sites of DNA damage in homologous recombination repair pathway of DNA double-strand breaks (DSB). Kang et al. (23) showed that *HERC2* is an E3 ligase that targets XPA (xeroderma pigmentosum A) for degradation in circadian control of nucleotide excision repair. Here, we

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report another function of HERC2 in relation to cell cycle and DNA damage response. HERC2 ubiquitinates BARD1-uncoupled BRCA1 and targets it for degradation. Furthermore, the regulation of BRCA1 stability by HERC2 is functionally relevant as evidenced by its effect on BRCA1-mediated G₂-M checkpoint activation.

Materials and Methods

Plasmids and RNA interference

Total RNA isolated from MCF10A cells was converted to single-strand cDNA using the PrimeScript RTase (TaKaRa). The cDNAs corresponding to protein fragments of human HERC2 (F1: 1–1295, F2: 1224–2329, F3: 2292–2923, F4: 2893–3591, F5: 3559–4834, F5-CT: 4252–4834 amino acids) were generated by PCR using PrimeSTAR Max DNA polymerase (TaKaRa) with the following pairs of primers: F1 (generated by connecting smaller fragments F1-1 and F1-2): F1-1, 5'-TAAGCTAGCGAAAAGATGCCCTCTGAATCTTTC-3' and 5'-TAATCTAGACCTAAACCAAGGAATTCCGG-3'; F1-2, 5'-TGCTAGCTTTGCTTGGTCATCATGTTC-3' and 5'-ATCTAGAATACTATTTCTGTAAACG-3'; F2, 5'-TTAGCTAGCTTCTGGACTGTGATTGACGGGAAG-3' and 5'-TAATCTAGACCTGCTTTTTCAGGATGTATAGC-3'; F3, 5'-TAAGCTAGCAAGTTAGAAAAGCACAAAATAAAG-3' and 5'-TAATCTAGAACTGCAGCCAAATCTTCTCTTC-3'; F4, 5'-TAAGCTAGCAAGCAGTGCAGGAGCTCAGGAATC-3' and 5'-TAATCTAGACAGAGCTCCAACAGCATATCTGC-3'; F5 (generated by connecting smaller fragments F5-1 and F5-2): F5-1, 5'-TAAGCTAGCCGGGCGGGGACAGGGGACAGG-3' and 5'-TAATCTAGATAATCGCCTTTGCCCCAGGTATAAAC-3'; F5-2, 5'-TAAGCTAGCGATGGCTGTAAAGTGCCTATG-3' and 5'-TCTCTAGACACAGCAGCCTACAG-3'; F5-CT, 5'-TAGGATCCCCTTACCAAATCTGGAGC-3' and 5'-TAGCTCATCTCTCGAGGACGTTTC-3'. Restriction sites used for subcloning are underlined. The HERC2 fragments were subcloned into pcDNA3 vectors in-frame with the NH₂-terminal Myc- or FLAG-tag. Mammalian expression plasmids for BRCA1, BARD1, and HA-ubiquitin were previously described (12, 24). Transfections were performed using the standard calcium phosphate precipitation method. Small interfering RNA (siRNA) oligonucleotides (Dharmacon) were synthesized to the following sequences (sense strand): HERC2#1 (5'-GGAAAGCACUGGAUUCGUU-3') and HERC2#2 (5'-GAAGGUGGUGUUCACUCA-3'). SMARTpool HERC2 siRNA mix, BARD1 siRNA mix, and control siRNA mix were purchased (Dharmacon). RNA duplexes (final concentration, 100 nmol/L) were transfected into the cells with Oligofectamine (Invitrogen). Cells were analyzed 72 hours after transfection.

Cell culture

All human cells were cultured in DMEM supplemented with 10% FCS and 1% antibiotic-antimycotic agent (Life Technologies). Where indicated, 0.1 μmol/L of proteasome inhibitor epoxomicin was added to cells 12 hours before harvesting. Cell cycle synchronization with a double-thymidine block was described (25). To examine the half-life of proteins *in vivo*, cells were incubated with 10 μg/mL cycloheximide

(Wako), a protein synthesis inhibitor, for the indicated time periods. To inhibit Crm1-dependent nuclear export of protein, cells were incubated with 15 ng/mL leptomycin B (Biomol) for 12 hours.

Immunoprecipitation, immunoblotting, and immunocytochemistry

Antibodies used were rabbit polyclonal antibodies to BRCA1 (C20, Santa Cruz Biotechnology), BARD1 (BL518, Bethyl Laboratories), and cyclin E1 (a gift from Dr. Yue Xiong, University of North Carolina at Chapel Hill) and mouse monoclonal antibodies to HERC2 (17, BD Biosciences), BRCA1 (MS110, Calbiochem; for immunohistochemical analysis), conjugated ubiquitin (FK2, Affiniti), phospho-histone H3 (Ser¹⁰; 3H10, Upstate), α- and β-tubulin (DMIA+BMIB, NeoMarkers), HA (12CA5, Boehringer), Myc (9E10, BabCo), and FLAG (M2, Sigma). BRCA1 antigen peptide (C20, Santa Cruz Biotechnology) was used as competing peptide in immunoprecipitation. Immunoprecipitation and immunoblotting methods, including the detection of *in vivo* ubiquitinated substrates, were previously described (24, 26). The relative amounts of protein detected by immunoblotting were determined by the lumino-image analyzer LAS3000 (Fujifilm). Indirect immunocytochemistry was performed as described (26) with 1 μg/mL of anti-BRCA1 and/or anti-HERC2 antibodies.

In vitro ubiquitin ligation assay

FLAG-HERC2-F5 E3 ligases were purified from HEK-293T cells with anti-FLAG immunoprecipitation followed by FLAG peptide elution and subjected to *in vitro* reaction with mammalian ubiquitin, E1, and His-UbcH5c as described (24, 26, 27). For substrate ubiquitination, 0.3 μg of GST-BRCA1(1–342) or GST (glutathione *S*-transferase) was added to the reaction.

Ionizing radiation-induced G₂-phase checkpoint

HCT116 cells were exposed to 10 Gy of ionizing radiation (IR). Immediately after IR, 100 ng/mL nocodazole was added to trap cells in mitosis, and samples were harvested by trypsinization 12 hours later. Cells were fixed by 70% ethanol, washed twice with PBS, and incubated in 0.25% Triton X-100 in PBS on ice for 15 minutes. After centrifugation, cell pellets were washed with PBS and incubated in 100 μL PBS containing 1% bovine serum albumin (BSA) and 0.75 μL of anti-phospho-histone H3 antibody for 3 hours. Cells were rinsed with PBS containing 1% BSA, incubated with FITC-conjugated goat anti-mouse IgG antibody (1:25 dilution), washed again, and incubated with PBS containing 50 μg/mL propidium iodide (PI) and 30 μg/mL RNase A for 1 hour. Cells were then analyzed by flow cytometry using FACSCalibur (Becton Dickinson).

Results

HERC2 is a BRCA1 degron-binding protein

We searched for proteins that mediate BRCA1 degradation by mass spectrometry analysis of BRCA1-associated proteins

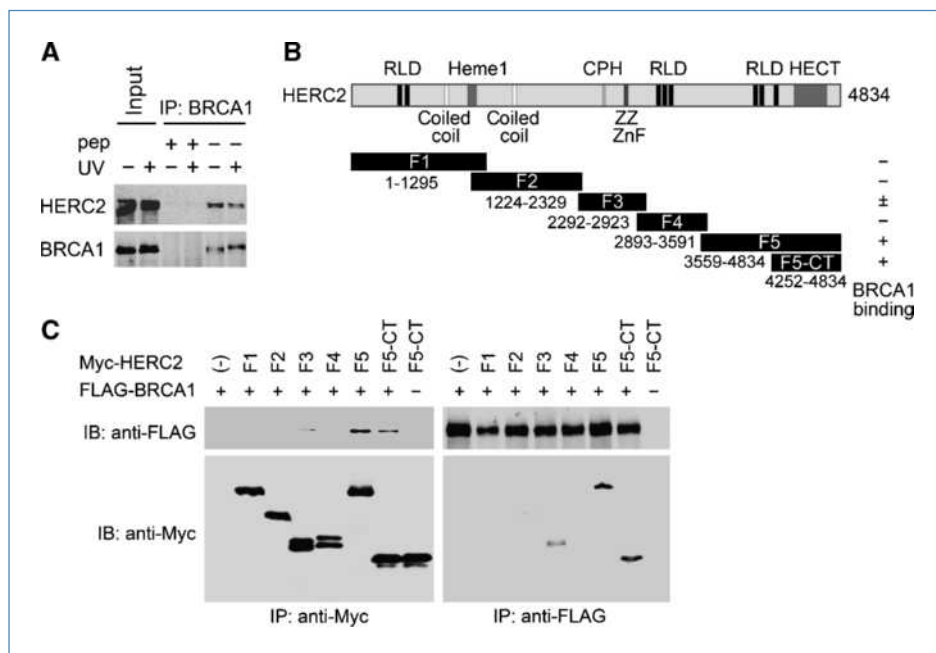


Figure 1. BRCA1 interacts with the COOH terminus of HERC2.

A, lysates prepared from HeLa cells before (-) or after (+) UV irradiation were immunoprecipitated with anti-BRCA1 antibody with (+) or without (-) competing antigen peptide and immunoblotted with the indicated antibodies. Inputs (3%) were also loaded. B, the indicated HERC2 fragments were generated with an NH₂-terminal Myc-tag and used to examine BRCA1 binding. The summary of the BRCA1 binding from C is shown at right. C, HEK-293T cells were cotransfected with full-length FLAG-BRCA1 and Myc-HERC2 fragments. Interaction between BRCA1 and HERC2 was assessed by immunoprecipitation (IP) followed by immunoblotting (IB) with indicated antibodies.

from MG132-treated cells and found HERC2 as one such candidate (Supplementary Fig. S1). Because HERC2 possesses a COOH-terminal HECT domain, a motif defining E3 ligases, we further analyzed HERC2 for its functional role in the BRCA1 complex. We first confirmed the interaction between endogenous BRCA1 and HERC2. HERC2 was readily detected in BRCA1 immunocomplexes and was specifically blocked by the competing BRCA1 antigen peptide (Fig. 1A). BRCA1 phosphorylation triggered by UV radiation did not affect its interaction with HERC2. To verify the specific interaction between HERC2 and BRCA1, we mapped the regions required for the interaction. A series of HERC2 fragments were cloned and fused in-frame with a Myc epitope-tag (Fig. 1B). Each fragment was tested for its ability to interact with FLAG-tagged full-length BRCA1 by coexpression in HEK-293T cells followed by coimmunoprecipitation. In the reciprocal combinations of immunoprecipitations and immunoblots, BRCA1 mainly interacts with fragment 5 (F5: 3559–4834) and F5-CT (4252–4834) of HERC2 (Fig. 1B and C). These are COOH-terminal fragments that contain the HECT domain. F3 also interacted weakly (Fig. 1B and C). We next mapped the region of BRCA1 required for its interaction with HERC2. A series of Myc-tagged BRCA1 deletions from the COOH terminus were tested for their binding to FLAG-HERC2-F5 (Fig. 2A). Because NH₂-terminal fragments of BRCA1 without BARD1 are unstable in cells (12), we cotransfected BARD1. HERC2-F5 was capable of binding to BRCA1(1–222) but not to BRCA1(1–135), suggesting that the binding site is located somewhere between amino acid residues 135 and 222 of BRCA1 (Fig. 2A and B). Because this region contained a degron domain (1–167) of BRCA1 that is responsible for the ubiquitination and instability of BRCA1 in cells (28), we further investigated this region. We con-

structed mutants lacking the RING domain (Δ RING: 65–772, 65–338) or degron region (Δ deg: 169–772, 169–338) in the context of BRCA1 NH₂-terminal fragments (Fig. 2A). BRCA1 lacking RING domain is incapable of binding to BARD1 (Fig. 2A; Supplementary Fig. S2A). Consistent with a previous report (28), Δ deg mutants were stable in cells, whereas Δ RING mutants were unstable (Supplementary Fig. S2B). Importantly, Δ degs were incapable of binding to HERC2-F5, whereas Δ RINGS were capable (Fig. 2A and C). Hence, the HECT-containing domain of HERC2 interacts with the degron domain of BRCA1 in a BARD1-independent manner.

HERC2 ubiquitinates BRCA1

To confirm that HERC2 indeed possesses ligase activity, FLAG-HERC2-F5 was immunoprecipitated from HEK-293T cells and subjected to an *in vitro* ubiquitin ligation assay. Polyubiquitin chain formation was detected with anti-conjugated ubiquitin antibody (FK2) in a manner dependent on E1, E2, ubiquitin, and ATP (Fig. 3A). The activity was inhibited by substitution of Cys⁴⁷⁶² in HERC2, a site conserved with the catalytic ubiquitin binding site of HECT domains, with serine (C4762S; Fig. 3B). This suggests that the reaction is carried out using the same mechanism as HECT E3 ligases. We next tested if BRCA1 could be ubiquitinated by HERC2 *in vitro* (Fig. 3C). GST-BRCA1(1–342) was purified and subjected to the *in vitro* ubiquitin ligation assay. GST-BRCA1(1–342) does not possess E3 activity *in vitro* unless BARD1 exists. The FLAG-HERC2-F5 immunocomplex was capable of ubiquitinating GST-BRCA1(1–342). BRCA1 ubiquitination was inhibited by the C4762S substitution, suggesting that the ubiquitination was not carried out by other E3(s) coprecipitating with HERC2.

We further tested *in vivo* BRCA1 ubiquitination mediated by HERC2. Myc-BRCA1 (Δ RING, 65–772) was transfected as a substrate. The RING deletion eliminates the possibility of BRCA1 autoubiquitination. When coexpressed with HERC2-

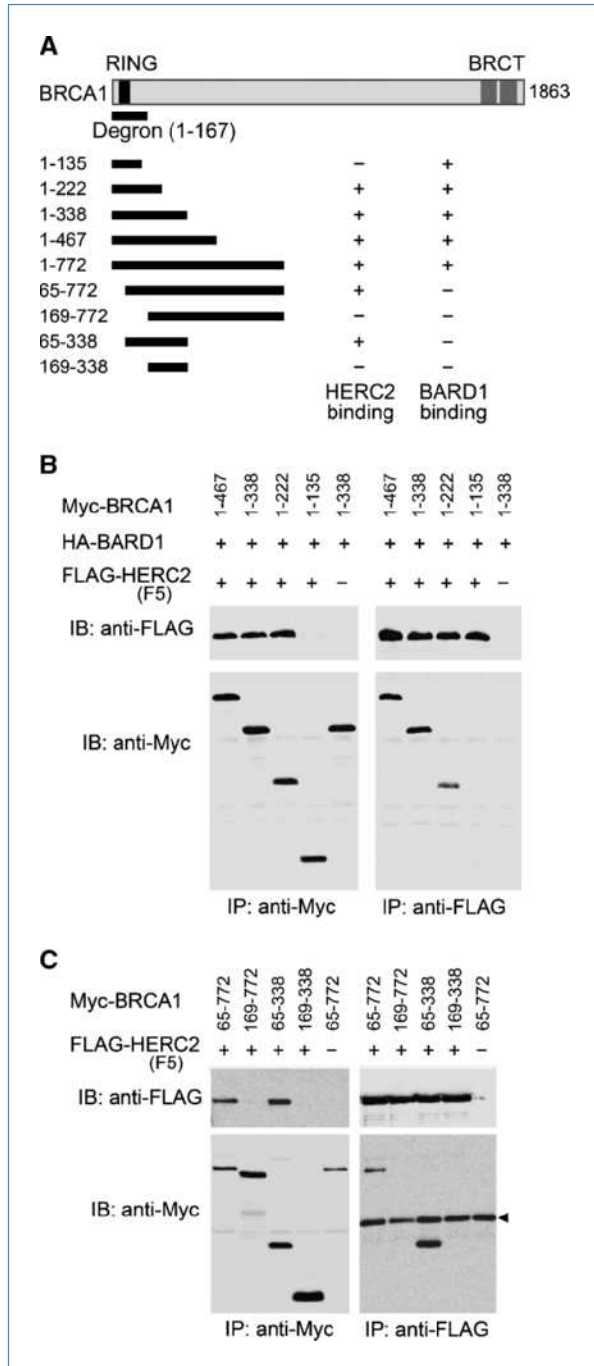


Figure 2. The HERC2 COOH terminus interacts with a BRCA1 degnon. A, the indicated BRCA1 fragments were generated with an NH₂-terminal Myc-tag and used to examine HERC2 binding. The summaries of the HERC2 binding from B and C and BARD1 binding are shown. B and C, interactions between FLAG-HERC2-F5 and Myc-BRCA1 fragments were analyzed as in Fig. 1C using the indicated plasmids and antibodies. Arrowhead, IgG.

F5, the ubiquitinated form of Myc-BRCA1(65–772) was readily detected (Fig. 4A). The ubiquitination was abolished by the C4762S mutation of HERC2 (Fig. 4A), although C4762S and wild-type HERC2 interacted with BRCA1 equally well (Fig. 4B). Consistent with the binding experiments, BRCA1 ubiquitination was not observed on deletion of the BRCA1 degnon domain (Δ deg, 169–772; Fig. 4C), indicating that ubiquitination is dependent on the degnon domain. Although ubiquitination does not always signal proteolysis, the degnon-dependent ubiquitination suggests that the BRCA1 ubiquitination mediated by HERC2 is likely a signal for proteolysis.

HERC2 interacts with BRCA1 in S phase

To determine the physiologic role for HERC2-BRCA1 interaction, we analyzed the interaction during the cell cycle by coupled immunoprecipitation and immunoblot because the BRCA1 protein expression level oscillates during the cell cycle. When cycling HeLa cells were synchronized by a double-thymidine block, the steady-state level of BRCA1 remained at a low level during S phase and increased as cells entered G₂-M phase (Fig. 5, 6 and 8 hours). BRCA1 was immunoprecipitated and HERC2 in the immunocomplex was detected by immunoblot. Importantly, the small amount of BRCA1 protein in fractions from S-phase cells coprecipitated more HERC2 than that from G₂-M- and G₁-phase cells (Fig. 5B, bottom). There was an inverse correlation between the amount of HERC2 in the BRCA1 complex and the BRCA1 steady-state level, supporting the idea that HERC2 is an E3 ligase that targets BRCA1 for degradation.

We next analyzed the cellular localization of HERC2 by immunofluorescent studies using a HERC2 antibody to examine whether HERC2 colocalizes with BRCA1 in cells. HERC2 mainly localized to the cytoplasm with the antibody used (Supplementary Fig. S3A). The specificity of the antibody was verified by siRNA depletion of HERC2. However, using different antibody, it has been shown that in addition to the cytoplasm, HERC2 localizes to the nucleus where it interacts with XPA (23). BRCA1 also mainly localizes to the nucleus. Therefore, we searched cellular condition where nuclear HERC2 expression is more evident. Because HERC2 interacts with BRCA1 in S phase (Fig. 5), we analyzed cellular localization of HERC2 during the cell cycle. HeLa cells were synchronized by a double-thymidine block, and HERC2 and BRCA1 were stained as cells progressed through the cell cycle. Nuclear HERC2 expression was more evident in S phase than that in G₁ phase and costained with BRCA1 (Supplementary Fig. S3B). The localization of HERC2 to both the cytoplasm and the nucleus prompted us to test whether it is a shuttle protein. Treatment of cells with leptomycin B, a Crm1 inhibitor, caused modest accumulation of nuclear HERC2 (Supplementary Fig. S3C), suggesting that HERC2 is a possible nuclear-cytoplasmic shuttle protein. A nuclear localization signal may exist in the fragment F3 because this particular fragment localized to the nucleus, whereas others remained cytoplasmic (Supplementary Fig. S3D).

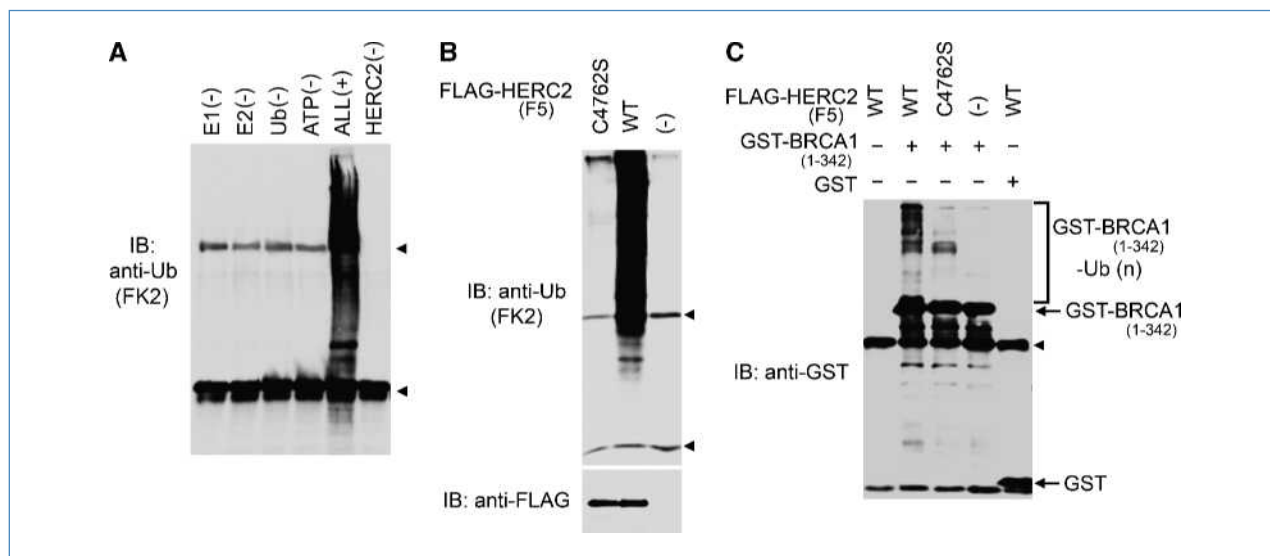


Figure 3. HERC2 ubiquitinates BRCA1 *in vitro* in a manner dependent on C4762S of HERC2. A, FLAG-HERC2-F5 was purified from HEK-293T cells and incubated with E1, E2, ubiquitin (Ub), and ATP or in the absence of the indicated reagents. Ubiquitinated products were detected by immunoblot with anti-conjugated ubiquitin antibody. B, wild-type or catalytically inactive C4762S mutant of FLAG-HERC2-F5 was subjected to the ubiquitin ligation assay as in A. Bottom, a portion of each FLAG-HERC2-F5 was immunoblotted with anti-FLAG antibody. C, wild-type or C4762S mutant of FLAG-HERC2-F5 was subjected to the ubiquitin ligation assay as in A in the presence of GST-BRCA1(1–342) or GST. Ubiquitinated BRCA1 was detected by immunoblot with anti-GST antibody. Arrowhead, IgG.

HERC2 targets BARD1-uncoupled BRCA1 for degradation

If HERC2 is an E3 ligase that targets BRCA1 for degradation, knockdown of HERC2 by siRNA could increase BRCA1 stability. We detected slight increase of BRCA1 amount by simple HERC2 knockdown when soluble fractions of S-phase cells were analyzed (Supplementary Fig. S4). We further searched for conditions under which HERC2 measurably degrades BRCA1. Most cellular BRCA1 forms a RING heterodimer with BARD1, an interaction necessary for BRCA1 protein stability (9, 12). The interaction with BARD1 masks the nuclear export signal of BRCA1, causing the BRCA1-BARD1 complex to be nuclear (10, 11). At the same time, BARD1 protects BRCA1 from ubiquitination (7). Therefore, we speculated that the nuclear BARD1 interaction protected BRCA1 from HERC2-mediated proteolysis. To test this possibility, we cotransfected HeLa cells with siRNA for HERC2 in combination with that for BARD1, and the half-life of BRCA1 protein *in vivo* was analyzed using cycloheximide. Inhibition of BARD1 expression markedly reduced BRCA1 stability as expected (Fig. 6A). Importantly, additional HERC2 inhibition significantly restored BRCA1 stability (Fig. 6A). Although BARD1 stability was also slightly restored by HERC2 inhibition, it was not due to less efficiency of siRNA knockdown because BARD1 mRNA expression in cells with BARD1/HERC2 siRNAs was inhibited at the same level as that in cells with BARD1/control siRNAs as measured by real-time quantitative reverse transcription-PCR analyses (Supplementary Fig. S5). We suspect that the restoration of BARD1 protein expression is caused by increased stability of BRCA1 because each protein controls the abundance and

stability of the other (9, 12). Reciprocally, we next tested whether addition of BARD1 protected BRCA1 from HERC2-mediated ubiquitination. FLAG-BRCA1 (I26A, 1–772), which is not capable of binding E2 and is enzymatically inactive (13), was transfected as a substrate to eliminate the possibility of BRCA1 autoubiquitination. When coexpressed with HERC2-F5, the ubiquitinated form of BRCA1 was readily detected (Fig. 6B). Significantly, cotransfection of BARD1 inhibited the ubiquitinated form of BRCA1 accompanied by the appearance of its unmodified form. Taking this result into consideration, it is interesting that the binding partner for BRCA1 was switched from HERC2 to BARD1 at the end of S phase and mitosis accompanied by an increase in the BRCA1 steady-state level (Fig. 5, 4–8 hours). Together, these results suggest that HERC2 is an E3 ligase that targets BARD1-uncoupled BRCA1 for degradation during S phase. Finally, ectopic expression of C4762S mutant of FLAG-HERC2-F5 significantly increased BRCA1 half-life, suggesting a dominant-negative effect of this catalytically inactive mutant and further supporting that HERC2 is an E3 ligase that targets BRCA1 for degradation (Supplementary S6).

Regulation of the stability of BRCA1 by HERC2 is functionally relevant

BRCA1 mediates G₂-M checkpoint activation by facilitating phosphorylation and activation of checkpoint kinases. Because HERC2 inhibition increased BRCA1 stability, we next tested if it affected G₂-M checkpoint arrest after IR. HCT116 cells transfected with each siRNA were exposed to IR, and nocodazole was added to trap the cells that passed

through the G₂-M transition in mitosis. Mitotic cells were identified by costaining with PI and anti-phospho-histone H3 antibody. IR treatment significantly reduced the number of cells trapped in mitosis by G₂-M checkpoint function (Supplementary Fig. S7). Depletion of BRCA1 or BARD1 significantly increased the number of mitotic cells when compared with control siRNA, illustrating the G₂-M checkpoint defect (Fig. 6C and D). Importantly, simultaneous depletion of HERC2 and BARD1 decreased the mitotic cell population to the level of control cells, suggesting restoration of the checkpoint function (Fig. 6C and D). Thus, the results suggest that HERC2 is able to affect BRCA1 function by regulating its stability in opposition to BARD1. We also tested single HERC2 depletion. It did not produce G₂-M checkpoint defect. It decreased, rather than increased, the number of mitotic cells to approximately a half of that with control siRNA (Fig. 6D). The observed effects of HERC2 depletion on BRCA1 stability and G₂-M checkpoint function were reproduced us-

ing two different siRNAs targeting independent sequences in HERC2, arguing against off-target effects.

HERC2 expression in breast cancer

The interaction between HERC2 and BRCA1 prompted us to investigate if HERC2 was expressed in breast cancer tissues. The breast cancer cells and noncancerous breast gland in biopsy specimens displayed HERC2 expression, whereas the surrounding mesenchymal cells did not (Supplementary Fig. S8A). Thirty-three of 60 cases of primary breast cancer expressed HERC2, and interestingly, HERC2 expression inversely correlated with BRCA1 expression in some cases, although it was not statistically significant (Supplementary Fig. S8B).

Discussion

In this report, we show that HERC2 is an E3 ligase that targets BRCA1 for degradation during the S phase of cell

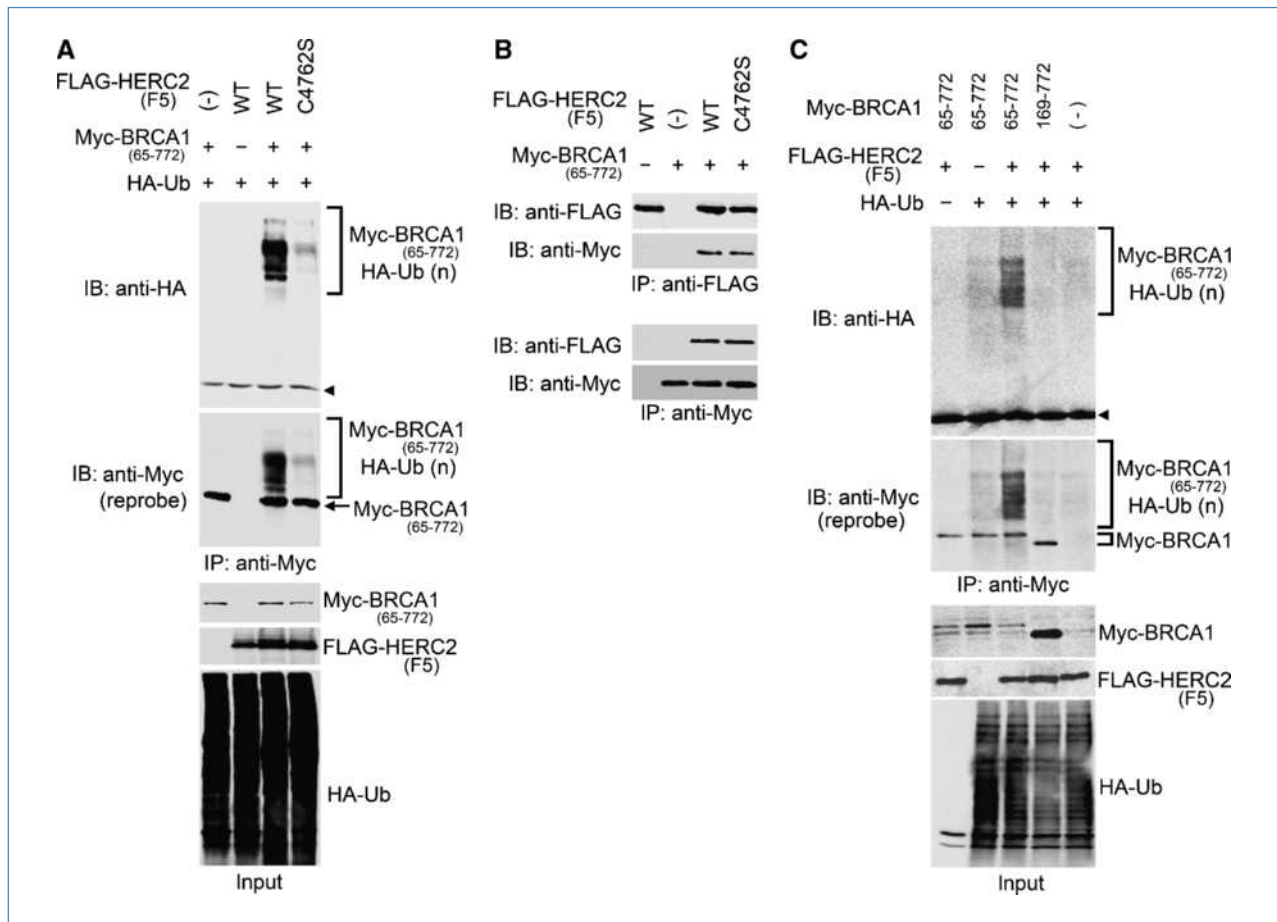


Figure 4. HERC2 ubiquitinates BRCA1 in a manner dependent on a degron of BRCA1. A, HEK-293T cells were transfected with the indicated plasmids and incubated with epoxomicin. Cells were harvested and boiled in 1% SDS lysis buffer, diluted, and immunoprecipitated with anti-Myc antibody. Ubiquitinated or unmodified Myc-BRCA1(65–772) was detected by immunoblot with anti-HA antibody or by reprobing with anti-Myc antibody, respectively. Inputs (3%) were also immunoblotted with the indicated antibodies (bottom three panels). B, HEK-293T cells were transfected with the indicated plasmids. Interaction between wild-type or C4762S mutant of FLAG-HERC2-F5 and Myc-BRCA1(65–772) was examined by immunoprecipitation followed by immunoblot using the indicated antibodies. C, HEK-293T cells were transfected with Myc-BRCA1 either with (65–772) or without (169–772) the degron plus the indicated plasmids. Ubiquitinated Myc-BRCA1(65–772) was detected as in A. Arrowhead, IgG.

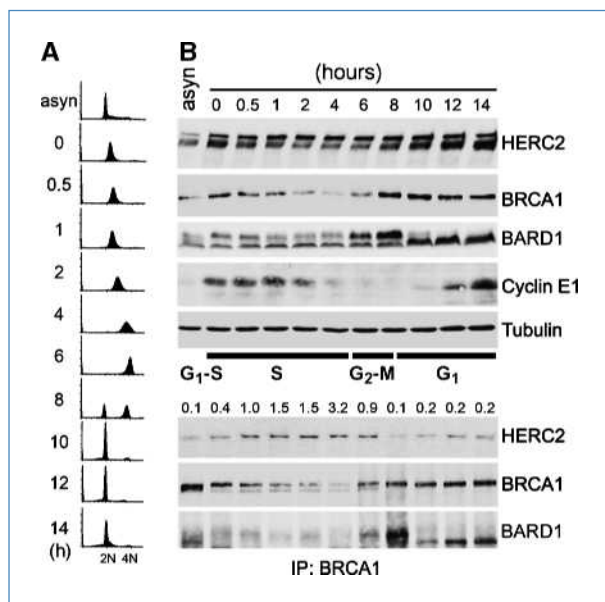


Figure 5. Cell cycle-dependent interaction of HERC2 with BRCA1. A, HeLa cells were synchronized by a double-thymidine block, and the cell cycle progression was monitored by fluorescence-activated cell sorting analysis. B, cell lysates were prepared with 0.5% NP40-based buffer at the indicated times after the release and analyzed by straight immunoblotting (top five panels) or by immunoprecipitation with anti-BRCA1 antibody followed by immunoblot using the indicated antibodies (bottom three panels). The ratios of HERC2 to BRCA1 protein in the BRCA1 immunoprecipitates determined by lumino-image analyzer are indicated based on normalization, where a ratio of HERC2 to BRCA1 in lane 3 was assigned the value of 1.0. Tubulin, loading control; cyclin E1, cell cycle control; asyn, asynchronous cells.

cycle. It has been reported that BRCA1 protein expression becomes maximal at late S and G₂-M phases (4–8). Because expression of BRCA1 mRNA is maximal in late G₁ and early S phases and decreases thereafter (7), the increase in the BRCA1 protein expression level during late S to G₂-M phase is mainly controlled by protein stability. BRCA1 is ubiquitinated and degraded during S phase, and decreased ubiquitination is accompanied by BRCA1 stabilization in G₂-M phase (7). Our results are consistent with the previous report and suggest that HERC2 is responsible for the ubiquitination and degradation of BRCA1. One of the regulators that affect HERC2-mediated proteolysis of BRCA1 seems to be BARD1 because the effects of HERC2 depletion on BRCA1 stability were particularly marked in the absence of BARD1, and because BARD1 overexpression protected BRCA1 from ubiquitination by HERC2. In addition, the binding partner for BRCA1 was switched from HERC2 to BARD1 during the period from late S phase to mitosis, accompanied by an increase in the BRCA1 steady-state level.

Because BRCA1 plays a critical role in DNA damage response, it is intriguing that HERC2 also has directly participated in DNA damage repair pathway. In response to IR, HERC2 is phosphorylated at Thr⁴⁸²⁷, which in turn binds to the forkhead-associated domain of RNF8, and is recruited to sites of DSBs where RNF8-oligomer complex bridges MDC1

and HERC2 (22). HERC2 facilitates assembly of the RNF8-Ubc13 complex, maintains the level of another E3 ligase RNF168, and is thereby required for ubiquitin-dependent retention of repair factors including 53BP1, RAP80, and BRCA1. Significantly, RNF8-Ubc13 fusion protein restored 53BP1 IR-induced foci formation in HERC2-depleted cells, suggesting that the major role of HERC2 in the pathway is to assemble the RNF8-Ubc13 complex. One question was whether E3 ligase activity of HERC2 has any role in the pathway. Expression of a catalytically inactive HECT domain of HERC2 rescued RNF8-Ubc13 interaction in HERC2-depleted cells, suggesting that the E3 ligase activity is not absolutely required for the complex assembly (22). If HERC2- and RNF8-dependent recruitment of BRCA1 to the site of DNA damage is required for the activation of G₂-M checkpoint function of BRCA1, depletion of HERC2 should produce G₂-M checkpoint failure. However, we showed that HERC2 depletion did not produce G₂-M checkpoint defect. This is an unusual feature for the proteins recruited to sites of DSB in relatively upstream events and indicates that HERC2 may have an additional role toward G₂-M checkpoint function. Because depletion of HERC2 complemented BRCA1 instability caused by depletion of BARD1 and restored G₂-M checkpoint function, we propose that HERC2 inhibits G₂-M checkpoint function by destabilizing BRCA1 or BRCA1-BARD1 complex. One possible scenario could be that HERC2 interacts with BRCA1 and inhibits its G₂-M checkpoint function during normal S phase or during recovery from the checkpoint. IR-induced phosphorylation of HERC2 may promote the retention of HERC2-BRCA1 on damaged chromosomes and inhibits the E3 ligase activity of HERC2 toward BRCA1. BARD1 may have some role in the inhibition. The ubiquitination mediated by RNF8 and RNF168 further facilitates the retention of BRCA1 through RAP80 (29–31) and promotes DNA repair and G₂-M checkpoint function. In siRNA knockdown experiments, excess amount of BRCA1 caused by HERC2 depletion may bypass the pathway and rescue the G₂-M checkpoint function.

Ubiquitin modification of target substrates signals many cellular processes other than proteasome-dependent proteolysis. HECT domain of HERC2 may catalyze different types of ubiquitin chains from that signals proteolysis. In this regard, it should be noted that ubiquitination of XPA by HERC2 also signals degradation and promotes circadian oscillation of XPA (23). Significantly, depletion of HERC2 improves repair efficiency of cisplatin-DNA damage by stabilizing XPA. HERC2 may generally regulate multiple DNA damage responses by ubiquitinating and degrading key players in the reactions.

In summary, we have identified a function for HERC2 in regulating BRCA1. Our findings suggest that BRCA1 protein stability is tightly regulated by at least two proteins, BARD1 and HERC2, and that this regulation is important for progression through the G₂-M cell cycle checkpoint. Interestingly, we found that approximately half of the breast cancer cases expressed detectable levels of HERC2 protein, and that HERC2 expression was inversely related to that of BRCA1 in some breast cancers, suggesting a mechanism that may lead to the emergence of BRCA1-negative breast cancers.

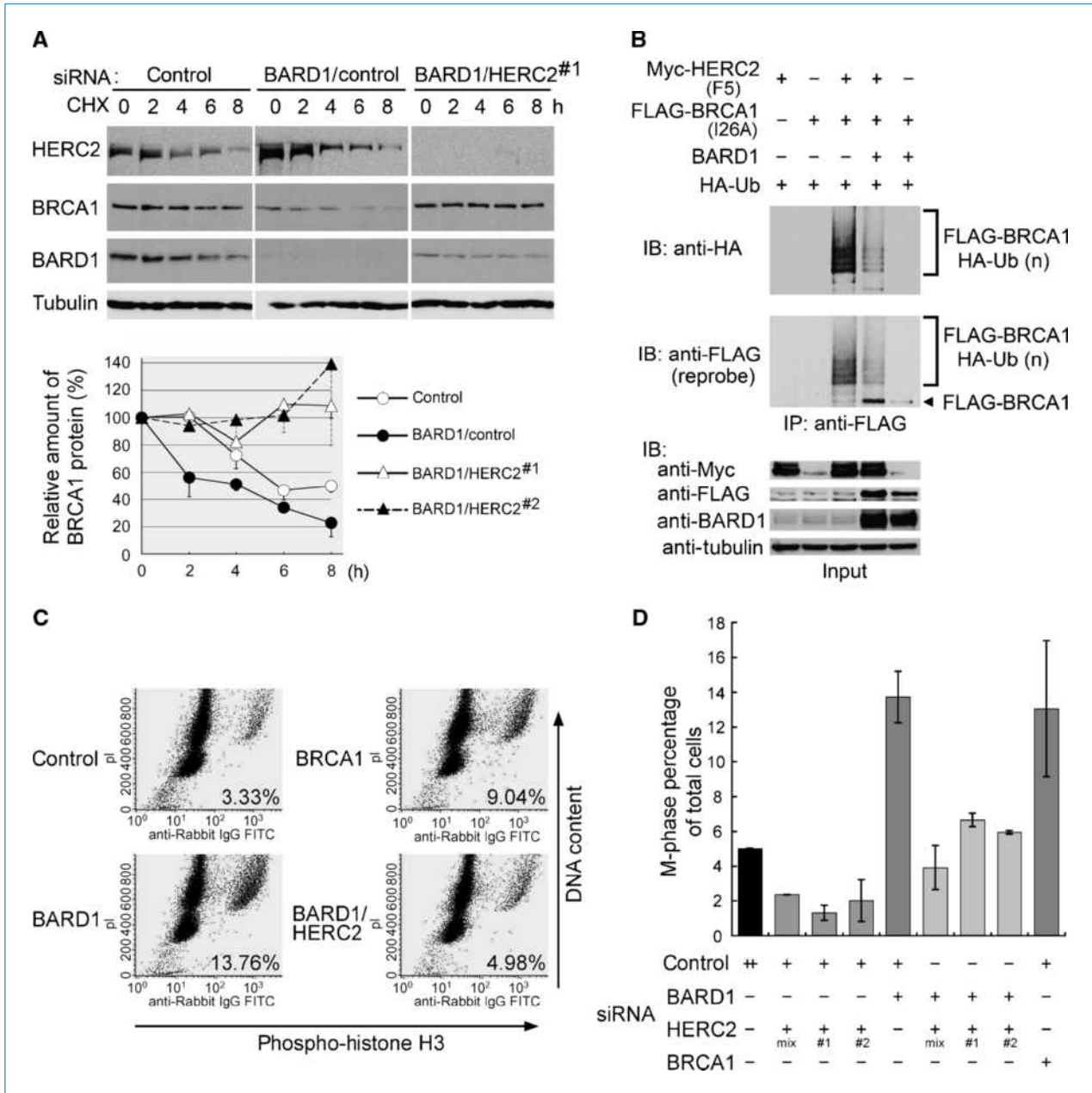


Figure 6. HERC2 targets BARD1-uncoupled BRCA1 for degradation. A, HeLa cells transfected with the indicated siRNA were incubated with cycloheximide (CHX) and chased for the indicated lengths of time. Top, whole-cell lysates were then immunoblotted with the indicated antibodies. Bottom, the amount of BRCA1 protein at each time point was quantitated, normalized relative to the amount of protein present in cells following the 0-h chase, and plotted against the chase time. Mean percentages from three individual experiments. Bars, SD. B, HEK-293T cells were transfected with the indicated plasmids. Ubiquitination of the catalytically inactive I26A mutant of FLAG-BRCA1(1-772) was detected as in Fig. 4A (top two panels). Inputs (3%) were also immunoblotted with the indicated antibodies (bottom four panels). C, HCT116 cells transfected with the indicated siRNAs were irradiated (10 Gy), followed by incubation with nocodazole for 12 h. Cells were costained for DNA content and histone H3 phosphorylation and analyzed by flow cytometry. Percentages of mitotic cells are shown. D, mean percentages of mitotic cells from a series of individual experiments performed as in C with the indicated siRNA. Bars, SD. Control, 5.06 ± 0.15 (n = 6); HERC2 (mix), 2.35 (n = 1); HERC2#1, 1.31 ± 0.43 (n = 2); HERC2#2, 2.01 ± 1.20 (n = 2); BARD1/control, 13.14 ± 1.95 (n = 6); BARD1/HERC2 (mix), 3.92 ± 1.29 (n = 6); BARD1/HERC2#1, 6.66 ± 0.38 (n = 3); BARD1/HERC2#2, 5.95 ± 0.10 (n = 3); BRCA1/control, 12.68 ± 3.62 (n = 6). mix, SMARTpool HERC2 siRNA mix.

Taken together with our demonstration that HERC2 functionally regulates BRCA1, this makes the potential role of HERC2 in breast cancer pathogenesis a critical issue for further study.

Disclosure of Potential Conflicts of Interest

Followings are the patents that may relate to the manuscript: (a) Ohta T. Carcinostatic method using BRCA1-BARD1 pathway WO-2005073379

(08/11/2005); (b) Ohta T and Sato K. Method for prevention of tumor WO-2007069795 (06/21/2007).

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