TUSC4 Functions as a Tumor Suppressor by Regulating BRCA1 Stability

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Abstract

BRCA1 expression is lost frequently in breast cancers in which it promotes malignant development. In the present study, we performed a global expression analysis of breast cancer cells in which the tumor-suppressor candidate gene TUSC4 was silenced to gain insights into its function. TUSC4 silencing affected genes involved in cell cycle and cell death, which have broad reaching influence on cancer development. Most importantly, we found a cluster pattern of gene-expression profiles in TUSC4-silenced cells that defined a homologous recombination (HR) repair defect signature. Mechanistic investigations indicated that TUSC4 protein could physically interact with the E3 ligase Herc2, which prevents BRCA1 degradation through the ubiquitination pathway. TUSC4 silencing enhanced BRCA1 polyubiquitination, leading to its degradation and a marked reduction in HR repair efficiency. Notably, ectopic expression of TUSC4 suppressed the proliferation, invasion, and colony formation of breast cancer cells in vitro and tumorigenesis in vivo. Furthermore, TUSC4 silencing was sufficient to transform normal mammary epithelial cells and to enhance sensitivity to PARP inhibitors. Our results provide a set of genetic and biologic proofs that TUSC4 functions as a bona fide tumor suppressor by regulating the protein stability and function of BRCA1 in breast cancer. Cancer Res; 75(2); 378–86. ©2014 AACR.

Introduction

Loss of tumor-suppressor gene function leading to genomic instability is a well-known prerequisite toward cancer development (1, 2). Through genome-wide sequencing, many tumor suppressors were found frequently deleted or mutated in various cancers (3). Tumor-suppressor candidate 4 (TUSC4); also known as nitrogen permease regulator-like 2 (NPRL2), was first identified in a human lung cancer homozygous deletion region on chromosome 3p21.3. NPRL2/TUSC4 is highly conserved in species ranging from yeasts to humans (4). Sequence analysis demonstrated that NPRL2/TUSC4 possesses a protein-binding domain other than the nitrogen permease domain and that overexpression of NPRL2/TUSC4 reduces lung cancer proliferation both in vitro and in vivo (5). Homozygous deletions in chromosome 3p21.3 were also frequently detected in breast cancers (6). Indeed, we confirm through microarray and pathway analyses that loss of NPRL2/TUSC4 was associated with cancer development and multiple molecular functions such as cell cycle and cell death. Interestingly, we have identified that loss of NPRL2/TUSC4 expression resulted in a similar pattern to homologous recombination (HR) repair defects when clustered with our previously established HR defect gene signature profile (7).

BRCA1 expression is tightly regulated at both the transcriptional and protein level throughout the different stages of the cell cycle (17, 18), knowledge of how BRCA1 protein stability is regulated is still very limited. Our data suggest that one mechanism of regulation occurs through direct binding between NPRL2/TUSC4 and HERC2, the E3 ligase of BRCA1 (19), thus inhibiting HERC2-mediated BRCA1 degradation. Together, we have demonstrated that NPRL2/TUSC4 is a bona fide tumor-suppressor gene in breast cancer, and its function in suppressing genomic instability and cancer development may be at least in part through protecting the protein stability of BRCA1.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell culture and plasmids

The U2OS, MDA-MB-231, and MCF-10A cell lines were purchased from the ATCC. McCoy’s 5A medium (CellGro;10-050-CV) supplemented with 10% FBS was used to maintain U2OS cells, RPMI-1640 medium (Corning; 10-104-CV) supplemented with 10% FBS was used to culture MDA-MB-231 cells, and serum-free mammary epithelial growth medium (Clonetics; CC-3051) containing insulin, hydrocortisone, EGF, and bovine pituitary extract was used to maintain MCF-10A cells. All cells were incubated under humidified conditions in 5% CO2. The pCMV5-3/C2 plasmid was obtained from EMD Biosciences (133407-82-6), and cycloheximide was purchased from BD Biosciences (612366). MG132 was purchased from Sigma. Anti-HERC2 antibody was purchased from Santa Cruz Biotechnology (sc-6954), anti-Flag M2 (F3165), anti-FLAG (10157-1-AP), anti-BRCA1 antibody was purchased from Santa Antibodies and reagents.

DNA Sequencing and Microarray Facility confirmed the identities of all plasmids.

Antibodies and reagents

Anti-NPRL2/TUSC4 antibody was purchased from Proteintech (10157-1-AP), anti-BRCA1 antibody was purchased from Santa Cruz Biotechnology (sc-6954), anti-Flag M2 (F3165), anti-Flag M2 Affinity gel (Sigma; A2220), and anti-β-actin (A2066) antibodies were purchased from Sigma. Anti-HERC2 antibody was purchased from BD Biosciences (612366). MG132 was purchased from EMD Biosciences (133407-82-6), and cycloheximide was obtained from Sigma (C7698). G418 was purchased from Sigma (C7698). G418 was purchased from Sigma (A1720). Full-length NPRL2/TUSC4 was amplified using a TOPO TA cloning kit for subcloning (Invitrogen; 45064) with the sense primer 5'-AATGGCCAGCGGCTGCCGCA-3' and antisense primer 5'-TCACTTCAGGCTGAGATGA-3'.

RNA extraction and RT-PCR

RNA was extracted by TRIzol reagent (Life Technologies; 15596026) and reverse transcription was conducted using the SuperScript III Kit (Invitrogen), and BRCA1 was amplified using RT-PCR with the sense primer 5'-CAGCGATATTTCCAGAGC-3' and antisense primer 5'-CTGTTTCCCGACTGTGGTT-3'. CyclinH was used as internal control.

RNA interference

Stable knockdown of NPRL2/TUSC4 expression was established via RNAi using lentiviral vector shRNA (Sigma; MISSION; NM660545). NPRL2/TUSC4 was targeted with a lentiviral particle of MISSION shRNA as well as MISSION control shRNA for 24 hours. BRCA1-expressing plasmids were then transfected into NPRL2/TUSC4-knockdown cells to induce reexpression of BRCA1. After 48 to 72 hours, flow cytometry was performed to detect GFP-positive cells.

Immunoprecipitation and immunoblotting

For immunoprecipitation of FLAG-NPRL2/TUSC4, U2OS cells were first transfected with an empty vector or FLAG-NPRL2/TUSC4 plasmids. Seventy-two hours after transfection, G418 was added to the medium for selection purposes. After stable clones were isolated from the pool, whole cellular extracts were incubated with RIPA buffer as described previously (20), and the products were immunoprecipitated with anti-FLAG M2 affinity gel for 8 hours at 4°C. After washing, the complexes were eluted with 3×FLAG peptide and evaluated using SDS-PAGE. For immunoprecipitation of the binding between HERC2 and BRCA1, cell lysate was preclared with A/G Agarose beads (Santa Cruz Biotechnology; SC-2003) and incubated with 1 μg of antibody at 4°C overnight. Precipitate was then washed and eluted in 5× SDS buffer and subjected to SDS-PAGE and immunoblotting. For immunoblotting, after samples were separated using electrophoresis, membranes were blocked with 5% milk diluted in Tris buffer with 0.1% Tween 20 for 1 hour at room temperature. The primary antibody was diluted in 5% BSA in PBS with sodium azide (Sigma; S227), and then incubated with the membranes for 2 hours at room temperature. Subsequently, membranes were washed with PBS with 0.1% Tween 20 and incubated with secondary antibody. Finally, signals of the bound antibody were detected using enhanced chemiluminescence (GE Healthcare; RP2232).

In vitro proliferation and PARP inhibition assays

MTT (Sigma; M5655) was used to evaluate the proliferation of cells. Briefly, cells were counted and seeded in a 96-well flat-bottomed plate. After 96 hours, cells were incubated with MTT substrate (Sigma; 20 mg/mL) for 4 hours, and the cultures were removed and replaced with DMSO. The optical density was measured spectrophotometrically at 570 nm. The colony formation assay was performed by seeding 200 cells in 6-well plates. Olaparib and rucaparib were added to the culture medium, and the cells were compared with untreated control cells. Colonies were scored after 3 weeks. All experiments were repeated three times.

Microarray analysis

The mirVana RNA Isolation Kit (Ambion) was used to isolate total RNA. A 500 ng of total RNA was used with a Sentrix Human-6 Expression Bead Chip (Illumina) for labeling and hybridization. BeadArray Reader was used for chip scanning (Illumina). As described previously (7), the gene-expression profile was subjected to normalization and log transformation. The NextGENe software program was used to identify genes whose expression differed in two clusters, and a t test was used to separate genes with significantly different expression (P < 0.001). The Ingenuity Pathway Analysis system was used for gene enrichment analysis.

Homologous recombination repair and flow cytometry analyses

The plasmids DR-GFP, pcAGGS, and pcBAse were gifts from Dr. Maria Jasim (Memorial Sloan-Kettering Cancer Center, New York, NY). U2OS cells were first treated with NPRL2/TUSC4 and BRCA1 siRNA as well as control siRNA for 24 hours. BRCA1-expressing plasmids were then transfected into NPRL2/TUSC4-knockdown cells to induce reexpression of BRCA1. After 48 to 72 hours, flow cytometry was performed to detect GFP-positive cells.
using a FACSCalibur and the CellQuest software program (Becton Dickinson). Three independent experiments were performed to obtain mean values and their SDs. Cell-cycle analysis was performed at the MD Anderson Cancer Center Flow Cytometry and Cellular Imaging Facility.

Tumor growth in nude mice and immunohistochemistry
Six-week-old female athymic nu/nu mice (NCI, average weight 25 g) were used in this study. The MD Anderson Institutional Animal Care and Use Committee approved the animal protocol. A total of $5 \times 10^6$ MDA-MB-231 cells with and without NPRL2/TUSC4 overexpression or $1 \times 10^7$ MCF-10A cells with and without knockdown of NPRL2/TUSC4 expression were injected into the mammary fatpads of mice. Tumors were measured from 1 week after MDA-MB-231 cells injection and monitored weekly, whereas MCF-10A tumors were observed 1 month after cell injection. At least 5 nude mice were used for each group. Human breast tissue samples (Biomax) were embedded in Xylene and 100%, 95%, 70%, 50% ethanol, respectively, for deparaffinization, slides were then incubated with NPRL2/TUSC4 antibody at 4°C overnight followed by antigen retrieval. Then samples were processed and evaluated immunohistochemically under microscope after being dehydrated and stabilized.

Transfection and ubiquitination assay
U2OS cell transfection was conducted using Oligofectamine (Life Technologies; 12252-001). Plasmids encoding HA-tagged ubiquitin were transfected in U2OS cells with and without knockdown of NPRL2/TUSC4 expression. Forty-eight hours after transfection, cells were harvested and lysed with RIPA buffer. Precipitated BRCA1 protein was isolated using SDS-PAGE and detected using an antiubiquitin antibody (Cell Signaling Technology; 3933).

Statistical analysis
All statistical analyses were performed with a two-tailed Student t test.

Results
NPRL2/TUSC4 expression is reduced in breast cancer and correlates with breast cancer progression
To characterize whether NPRL2/TUSC4 expression is associated with breast cancer, we performed Western blotting to measure the expression of NPRL2/TUSC4 in nontransformed breast cell lines, including HMEC, MCF-10A, and MCF-12A, and breast cancer cell lines with both luminal and basal-like subtypes (Fig. 1A). We observed that NPRL2/TUSC4 expression was markedly higher in the nontransformed cell lines than that in breast cancer cell lines. We also evaluated NPRL2/TUSC4 expression in normal breast tissue and breast carcinomas using immunohistochemical staining, and found that NPRL2/TUSC4 expression was lower in tumor tissue than in the matched adjacent normal breast tissue (Fig. 1B). Furthermore, The Cancer Genome Atlas–based analysis of mRNA expression in invasive breast carcinomas demonstrated a significant difference between the survival rates in patients with unaltered NPRL2/TUSC4 expression and those with downregulated expression level.
NPR2/TUSC4 expression (P < 0.01; refs. 21, 22). Specifically, the survival rate of 100 months was 24.7% in 889 patients with downregulation of NPR2/TUSC4 expression (Z-score threshold, ±1), whereas the survival rate in these patients dropped sharply to 0% after 100 months. In comparison, the survival rate was 40% in patients with unaltered NPR2/TUSC4 expression after 200 months (P < 0.01; Fig. 1C). Comparison of patients with upregulated and unaltered NPR2/TUSC4 expression did not demonstrate any significant differences in survival rate (Supplementary Fig. S1). These results strongly suggest that low NPR2/TUSC4 expression is associated with the cancer phenotype, indicating that NPR2/TUSC4 may play an important role as a tumor suppressor in patients with breast cancer.

To systematically evaluate the tumor-suppressive function of NPR2/TUSC4, we first performed microarray analysis comparing NPR2/TUSC4-knockdown cell lines and control cell lines (Supplementary Fig. S2). Gene Expression Omnibus accession number: GSE60535). We then examined the differentially expressed genes in these cells using the Ingenuity Pathway Analysis system (QIAGEN). Comparison of the NPR2/TUSC4-knockdown and control cells ranked cancer as one of the top disease and disorder pathways, further suggesting that NPR2/TUSC4 functions as a tumor-suppressor gene (Supplementary Fig. S3A). In addition, a high proportion of genes in the NPR2/TUSC4-deficient gene signatures were involved in the canonical pathways of DNA damage response and breast cancer regulation, such as cell-cycle regulation and DNA damage responses (Supplementary Fig. S3B).

Owing to the low NPR2/TUSC4 expression in breast cancer and the association with poor breast cancer patient survival rates, we explored the role of NPR2/TUSC4 in the development of breast cancer and the possible functional pathways that NPR2/TUSC4 is involved in. It has been reported that multiple cancers have mutations in or epigenetically silenced HR-related genes, which indicated potential association between HRD and cancer development (15, 16). Because the NPR2/TUSC4 gene signature shows several genes involved in DNA damage response, we suspected that low expression of NPR2/TUSC4 contributes to the deficiency of HR repair, which also drives genomic instability in breast cancer development. We performed a cluster analysis of a NPR2/TUSC4-knockdown microarray signature with previously established HRD gene signatures (7). The heatmap demonstrated that NPR2/TUSC4-deficient cells formed a cluster with HRD gene signatures (Fig. 1D), whereas the control cells separated from NPR2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under NPRL2/TUSC4-knockdown samples.

NPRL2/TUSC4 deficiency impairs HR repair by downregulation of BRCA1 expression.

The major conserved pathway used in mammalian cells to maintain genetic integrity and DNA fidelity is HR repair (23, 24). Here, we have identified an association between the expression profile of NPRL2/TUSC4-knockdown and BRCA1-deficient HRD gene signature. We suspected that loss of TUSC4 will also affect the foci formation of BRCA1, so we carried out phenotypic examination to test whether TUSC4 is required for BRCA1 foci formation by immunostaining. We performed BRCA1 foci staining followed by IR and UV irradiation. TUSC4 knockdown significantly abolished the BRCA1 foci formation after irradiation, whereas control siRNA did not affect the formation of BRCA1 foci (Fig. 2A and B). We further evaluated HR repair efficiency by a standard HR repair analysis system (20, 25) with NPR2/TUSC4-deficient U2OS model cells. We found that NPR2/TUSC4-knockdown cells had a significant decrease in HR reporter activity compared with control cells, which suggested impaired HR repair efficiency (Fig. 2C). We used BRCA1-knockdown cells as a positive indicator of HR repair to confirm HR repair efficiency. NPR2/TUSC4-knockdown cells presented comparative reduction in HR repair efficiency with BRCA1-knockdown cells, which were around 40% to 50% lower than that in control cells. To confirm that the defective HR repair efficiency was not caused by transfection efficiency or inaccurate efficiency from I-Scel, we reintroduced BRCA1 expression into the NPR2/TUSC4-knockdown cells and observed a significant increase in HR repair efficiency.

Surprisingly, we found that knockdown of NPR2/TUSC4 expression reduced BRCA1 protein expression (Fig. 2D), indicating that the decrease in HR repair efficiency in the NPR2/TUSC4-knockdown cells may have resulted from abnormal BRCA1 protein expression. These results are consistent with our above findings that NPR2/TUSC4-knockdown cells have HRD gene expression patterns similar to those in BRCA1-deficient cells, which were used to generate our HRD gene signatures. These results revealed for the first time a novel function of NPR2/TUSC4 in that disruption of its expression decreases BRCA1 expression and functions.

NPRL2/TUSC4 regulates BRCA1 protein stability.

We next sought to determine how NPR2/TUSC4 affects BRCA1 protein expression. To that end, we first sought to determine whether reduced BRCA1 protein expression after NPR2/TUSC4 knockdown was caused by altered cell-cycle distribution because BRCA1 expression is known to be cell-cycle dependent. We carried out a cell-cycle analysis and did not observe a significant difference in G1, G2-M, or S-phase distribution between control and NPR2/TUSC4-knockdown cells (Fig. A3A, Supplementary Fig. S4), indicating that decreased BRCA1 expression after NPR2/TUSC4 knockdown was not due to shifts in the cell cycle. To determine whether such changes occur through transcriptional regulation, we performed quantitative reverse transcriptase PCR to measure BRCA1 mRNA expression in control cells and cells with NPR2/TUSC4 knockdown. We identified no significant BRCA1 mRNA differences after NPR2/TUSC4 knockdown, whereas there is a significant drop in NPR2/TUSC4 mRNA (Fig. 3B), thus ruling out the possibility that BRCA1 expression by NPR2/TUSC4 was regulated at the mRNA level. Next, we sought to determine whether NPR2/TUSC4 regulates BRCA1 protein stability. To answer this question, we conducted cycloheximide chase experiments to determine BRCA1 stability in control and NPR2/TUSC4-knockdown U2OS cells. As shown in Fig. 3C, NPR2/TUSC4 knockdown reduced the half-life of BRCA1 from about 20 hours to less than 6 hours, suggesting that NPR2/TUSC4 plays an essential role in stabilizing BRCA1 at the protein level (Fig. 3D and E). In addition, to determine whether BRCA1 protein stability is regulated by NPR2/TUSC4 via the proteasome pathway, we treated control and NPR2/TUSC4-knockdown U2OS cells with the proteasome inhibitor MG132. As shown in Fig. 3F, MG132 restored BRCA1 expression.
in cells with NPRL2/TUSC4 knockdown but only slightly increased the BRCA1 protein expression in control cells. This result suggests that NPRL2/TUSC4 regulates BRCA1 protein stability via the proteasome-dependent pathway.

**NPRL2/TUSC4 regulates BRCA1 protein stability via the ubiquitination pathway**

It has been previously reported that HERC2 is an E3 ligase that targets BRCA1 for degradation (18). To determine whether NPRL2/TUSC4 regulates BRCA1 stability via HERC2, we examined BRCA1 ubiquitination under normal versus NPRL2/TUSC4 knockdown conditions. Surprisingly, we observed no signs of ubiquitination regardless of the NPRL2/TUSC4 knockdown status. However, after treatment with MG132, Western blotting shows that BRCA1 is highly ubiquitinated upon NPRL2/TUSC4 knockdown, whereas control cells exhibited comparatively reduced BRCA1 ubiquitination (Fig. 4A), indicating that knockdown of NPRL2/TUSC4 expression caused a robust increase in BRCA1 protein polyubiquitination. We also confirmed previous findings that downregulation of HERC2 expression leads to increased expression of BRCA1 regardless of the presence of NPRL2/TUSC4 (Fig. 4B and C). Under both conditions, BRCA1 expression was markedly upregulated after depletion of HERC2.

The next question to be answered was whether NPRL2/TUSC4 participates in ubiquitination of BRCA1 via HERC2 indirectly or stabilizes BRCA1 directly by binding. We performed immunoprecipitation with established NPRL2/TUSC4-overexpressing U2OS cell lines to determine the relationships among NPRL2/TUSC4, BRCA1, and HERC2. We found that NPRL2/TUSC4 physically interacts with HERC2 but not with BRCA1 (Fig. 4D), which suggests that NPRL2/TUSC4 regulates BRCA1 stability via interaction with HERC2. Considering previous reports on NPRL2/TUSC4 interactions (26, 27), we suspected that NPRL2/TUSC4 may prevent physical interaction between BRCA1 and HERC2. The binding between these two proteins has been previously described in Hela cell lines (19), so we further performed immunoprecipitation to determine whether overexpression of NPRL2/TUSC4 weakens this binding. As shown in Fig. 4E, although endogenous HERC2 physically binds BRCA1, overexpression of NPRL2/TUSC4 interrupts the binding, indicating that NPRL2/TUSC4 may regulate BRCA1 stability by preventing physical interaction between BRCA1 and HERC2.

**NPRL2/TUSC4 suppresses the tumorigenicity of human breast cancer cells**

Our data so far suggest that NPRL2/TUSC4 functions as a breast tumor suppressor in part through positive regulation of BRCA1 stability. We postulated that overexpression of NPRL2/TUSC4 would, therefore, suppress breast tumor proliferation in vitro and in vivo. To test this, we compared the proliferation rate of the breast cancer cell line MDA-MB-231 with or without NPRL2/TUSC4 overexpression. Colony formation assays indicated markedly reduced proliferation of NPRL2/TUSC4-overexpressing cells.
Because NPRL2/TUSC4 effectively inhibits breast cancer cell growth in vitro, we further examined the effect of NPRL2/TUSC4 overexpression in a xenograft mouse model of breast cancer. We injected control or NPRL2/TUSC4-overexpressing MDA-MB-231 cells into the mammary fatpads of female nude mice. We then monitored and measured tumor growth weekly. Six weeks after injection, 5 of 10 mice injected with NPRL2/TUSC4-overexpressing cells remained tumor free, whereas all 5 mice injected with control cells had large tumors (Fig. 5A and B; Table 1).

NPRL2/TUSC4 deficiency transforms normal mammary epithelial cells

Previous reports indicate that HR repair defect sensitizes cancer cells to DNA-damaging drugs (14, 15) and the PARP inhibitors (10, 11). Thus, NPRL2/TUSC4-deficient cells with HR repair defects are likely to be more sensitive to the treatment of PARP inhibitors, which can effectively inhibit the repair of single-strand DNA breaks. To test this hypothesis, we examined PARP inhibitor sensitivity in cells with NPRL2/TUSC4 deficiency. We performed colony formation assay in U2OS cells with NPRL2/TUSC4 knockdown after PARP inhibitor olaparib and rucaparib treatment, as well as control cells. All cells were treated with 1 μmol/L of cycloheximide for 0 to 24 hours. D, curves of BRCA1 level normalized to 0 hours after cycloheximide treatment, whereas blue curves indicate control cells, and red curves indicate NPRL2/TUSC4-knockdown cells. E, bar graph of BRCA1 half-life. Control cells have BRCA1 half-life of about 20 hours, whereas NPRL2/TUSC4-knockdown cells have BRCA1 half-life of about 5 hours.

Discussion

BRCA1 plays a key role in DNA damage responses, including HR repair. BRCA1 executes cellular functions in multiple
complexes to safeguard genomic stability, and therefore loss of BRCA1 expression is associated with aggressive phenotypes of breast carcinoma (13, 28). However, very little is known about the mechanism of how BRCA1 expression is regulated during different stages of cancer development. Several previous studies demonstrated that BRCA1’s stability is regulated via ubiquitination pathways (19, 29), but the network underlying this regulation is not fully characterized. Several previous studies demonstrated that BRCA1 expression is associated with aggressive phenotypes of breast carcinoma (13, 28). However, very little is known about the mechanism of how BRCA1 expression is regulated during different stages of cancer development. Several previous studies demonstrated that BRCA1’s stability is regulated via ubiquitination pathways (19, 29), but the network underlying this regulation remains unknown. We previously established HR repair deficiency gene signatures and identified 230 genes that predict HRD across tumor types (7). We surprisingly identified similar HRD gene signature patterns in NPRL2/TUSC4- and BRCA1-deficient cells. This suggests an association between NPRL2/TUSC4 and BRCA1, and provides a rationale to explore the network surrounding these proteins. Furthermore, NPRL2/TUSC4 knockdown cells exhibited enhanced sensitivity to the PARP inhibitors olaparib and rucaparib, which supports potential clinical use of PARP inhibitors to treat NPRL2/TUSC4-deficient breast cancer.

Previously reported data indicated that BRCA1 degradation is proteasome dependent, with HERC2 being the critical E3 ligase in this process. In the present study, we demonstrate for the first time that expression of NPRL2/TUSC4 can positively regulate BRCA1 stability by preventing physical interaction between BRCA1 and HERC2, which in turn protects BRCA1 from ubiquitination and degradation. Because NPRL2/TUSC4 deficiency correlates with decreased BRCA1 protein expression, and considering the previously reported capacity of NPRL2/TUSC4 to inhibit binding between PDK1 and its coactivator Src (30), we suspected that NPRL2/TUSC4 plays a similar role in disrupting the interaction between BRCA1 and HERC2. Indeed, we found that NPRL2/TUSC4 physically interacts with BRCA1’s E3 ligase HERC2, but not with BRCA1 itself. Overexpression of NPRL2/TUSC4 markedly weakened BRCA1 interaction with HERC2 in immunoprecipitation experiments. This observation explains how NPRL2/TUSC4 overexpression can protect BRCA1 from degradation and significantly reduce the proliferation of breast cancer. Thus, the regulatory mechanisms of NPRL2/TUSC4 function in blocking the AKT pathway and stabilizing BRCA1 protein expression may be similar. NPRL2/TUSC4 does not have phosphorylation or E3 ligase activity according to functional domain analysis, so we speculate that its potential functions in the DNA damage response network and as a tumor suppressor are through mechanisms of physical interaction to affect the stability of target proteins.

In addition, our data demonstrate that depletion of NPRL2/TUSC4 leads to diminished BRCA1 DNA damage foci after irradiation. This may be attributed to decreased BRCA1 protein

Figure 4. NPRL2/TUSC4 regulates BRCA1 stability via the ubiquitination pathway by blocking the binding of HERC2 to BRCA1. A, NPRL2/TUSC4 knockdown increased ubiquitination level of BRCA1 compared with control cells (after MG132 enrichment for ubiquitination). B, HERC2 knockdown rescued BRCA1 expression level, with and without the presences of NPRL2/TUSC4. C, bar graph, a significant BRCA1 reduction after NPRL2/TUSC4 knockdown (P < 0.05). BRCA1 increases after HERC2 knockdown with NPRL2/TUSC4 presence (P < 0.05), or without NPRL2/TUSC4 (P < 0.001). All values were compared with control cells. D, NPRL2/TUSC4 physically interacts with HERC2 but not BRCA1. E, NPRL2/TUSC4 overexpression reduced the binding between BRCA1 and HERC2 in Hela cell lines. A 2% input was used to indicate the level of HERC2, IgG control antibody and BRCA1 antibody were used to perform immunoprecipitation (IP) in both control and NPRL2/TUSC4 overexpression cells. A total of 4 mg of protein were used to perform in each condition.

A 2% input was used to indicate the level of HERC2, IgG control antibody and BRCA1 antibody were used to perform immunoprecipitation (IP) in both control and NPRL2/TUSC4 overexpression cells. A total of 4 mg of protein were used to perform in each condition.
expression as well as reduced recruitment of BRCA1 to DNA damage foci. Furthermore, double knockdown of NPRL2/TUSC4 and BRCA1 in an HR repair assay reduced the HR repair efficiency more than knockdown of NPRL2/TUSC4 or BRCA1 expression alone, suggesting that in addition to positive regulation for BRCA1 protein stability, NPRL2/TUSC4 may regulate HR function through BRCA1-independent pathways (data not shown).

Table 1. Tumorigenicity of orthotopically implanted control and TUSC4 overexpression MDA-MB-231 cells

<table>
<thead>
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<th>Number of mice with tumors (%)</th>
<th>Week 1</th>
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<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
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<td>3/5 (60)</td>
<td>5/5 (100)</td>
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NOTE: A total of 5 × 10^5 cells from MDA-AB-231 control and two independent NPRL2/TUSC4-overexpression MDA-AB-231 cell lines (NPRL2/TUSC4 #7 and NPRL2/TUSC4 #13) were injected per mouse into mammary fat pads glands of 6-week-old female nude mice. Each cell line was injected in 5 different mice, and tumor sizes were analyzed.

Table 2. Tumorigenicity of orthotopically implanted control and TUSC4-knockdown MCF10A cells

<table>
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<th>Week 5</th>
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NOTE: A total of 1 × 10^6 cells from MCF-10A control and two independent NPRL2/TUSC4-knockdown MCF-10A cell lines (NPRL2/TUSC4 #1 and NPRL2/TUSC4 #4) were injected per mouse into mammary fat pads glands of 6-week-old female nude mice. Each cell line was injected in 5 different mice, and tumor sizes were analyzed.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Peng, G. Peng, S.-Y. Lin
Development of methodology: Y. Peng, E. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Peng, H. Dai, C.-C.-J. Lin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Peng, E. Wang, G. Peng, S.-Y. Lin
Writing, review, and/or revision of the manuscript: Y. Peng, C.-C.-J. Lin, W. Mo, S.-Y. Lin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Mo
Study supervision: S.-Y. Lin

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