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The *BRCA1* Pseudogene Negatively Regulates Anti-Tumor Responses through Inhibition of Innate Immune Defense Mechanisms

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Abstract

Innate immune defense mechanisms play a pivotal role in anti-tumor responses. Recent evidence suggests that antiviral innate immunity is regulated not only by exogenous non-self-RNA but also by host-derived pseudogene RNAs. A growing body of evidence also indicates a biological role for pseudogenes as gene expression regulators or immune modulators. Here we report an important role for *BRCA1P1*, the pseudogene of the *BRCA1* tumor suppressor gene, in regulating

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innate immune defense mechanisms in breast cancer cells. *BRCA1P1* expresses a long-noncoding RNA (lncRNA) in breast cancer cells through divergent transcription. Expression of lncRNA *BRCA1P1* is increased in breast tumors compared to normal breast tissues. Depletion of *BRCA1P1* induces an antiviral defense-like program including the expression of antiviral genes in breast cancer cells. Furthermore, *BRCA1P1*-deficient cancer cells mimic virus-infected cells by stimulating cytokines and inducing cell apoptosis. Accordingly, depletion of *BRCA1P1* increases host innate immune responses and restricts virus replication. In converse, overexpression of *BRCA1P1* reduces cytokine expression in breast cancer cells. Mechanistically, lncRNA *BRCA1P1* is localized in the nucleus, binds to the NF- κ B subunit RelA, and negatively regulates antiviral gene expression. Lastly, in a xenograft mouse model of breast cancer, depletion of *BRCA1P1* stimulates cytokine expression and local immunity, and suppresses tumor growth. Our results suggest an important role for *BRCA1P1* in innate immune defense mechanisms and anti-tumor responses. This mechanism of antiviral immunity regulated by a host-derived pseudogene RNA may guide the development of novel therapies targeting immune responses in breast cancer.

Keywords

Long non-coding RNA; Pseudogene; Breast Cancer; *BRCA1*; *BRCA1P1*; NF- κ -B; IFN- λ ; TNF- α ; Antiviral Response; Innate Immunity; Poly(I:C); Cytokines; Chemosensitivity; Tumor Growth; Apoptosis

Introduction

Innate antiviral immunity is a significant mechanism in cancer (1). It mediates intrinsic anti-tumor responses through several mechanisms, which include triggering of apoptosis of cancer cells, stimulating spontaneous DNA damage, and increasing antitumor efficacy of radiation and chemotherapy. Both cancer cells and virus-infected cells threaten the host by expressing neo-antigens and by evading control mechanisms of host immune surveillance. Cancer cells can actually mimic a viral infection process by activating RNA-sensing pattern recognition receptors (PRRs) and by stimulating cytokine production and activating cytotoxic immune cells, the latter killing infected cells via externally induced cell lysis and apoptosis. RIG-I and MDA5 of the RIG-I-like receptor (RLR) family are important PRRs involved in the detection of RNA viruses (2,3). RLRs initiate host antiviral responses that induce type I interferons (IFN) and other cytokines, leading to the transcription of hundreds of IFN-stimulated genes (ISGs) (4). Activation of RLRs by dsRNA ligands not only triggers host immune responses but can also directly induce apoptosis of cancer cells, in an IFN-dependent or independent manner (5). Consequently, cancer cells are highly susceptible to RLR-induced cell death via intrinsic and extrinsic apoptosis and immune activation, indicating that the signaling pathway driven by RLRs is a promising molecular pathway to target in cancer immunotherapy.

Interestingly, recent evidence suggests that antiviral innate immunity is regulated not only by exogenous non-self-RNA but also by host-derived pseudogene RNAs. Pseudogenes have been considered non-functional artifacts of evolutionary processes due to degenerative features such as the accumulation of disruptive mutations or their lack of regulatory

elements (6,7). However, a growing body of evidence indicates biological roles for pseudogenes as gene expression regulators or immune modulators. 5S ribosomal RNA pseudogene transcripts, in particular *RNA5SPI4I*, were shown to bind to RIG-I and induce the expression of antiviral cytokines during infection with herpes simplex virus type 1 or the related herpesvirus Epstein-Barr virus (8). Lethe, a pseudogene lncRNA, is selectively induced by pro-inflammatory cytokines or glucocorticoid receptor agonists, and serves as a functional regulator of inflammatory signaling (9). These data suggest that pseudogenes may play a role in regulating antiviral defense and inflammatory signaling pathways.

The chromosome 17q21 region containing *BRCA1* has a partially duplicated pseudogene, *BRCA1P1* (Gene ID: 394269, HUGO ID: 28470), which contains only three of the 24 exons of *BRCA1* (10–12). It also includes an insertion of the acidic ribosomal phosphoprotein P1 pseudogene (*RPLP1P4*) in exon 1a, displaying unique features of a chimeric pseudogene derived from the two parent genes, *BRCA1* and *RPLP1*. The presence of *BRCA1P1* on the same chromosome close to *BRCA1* appears to create a hotspot for homologous recombination (13), leading to genomic rearrangements between *BRCA1P1* and *BRCA1* in families with a high risk of breast and ovarian cancers. *BRCA1* deficiency is frequently observed in breast, ovarian, and other cancers (14–16). While the roles of *BRCA1* in regulating homologous recombination and DNA damage repair have been extensively studied, the biological relevance of *BRCA1P1* pseudogene in breast cancer has not been elucidated.

In this study, we discovered an important role for *BRCA1P1* in regulating antiviral program-like responses in breast cancer cells. In contrast to *BRCA1*'s involvement in homologous recombination repair, *BRCA1P1*-depleted cancer cells resemble virus-infected cells, and express high amounts of ISGs and cytokines, which ultimately makes them more susceptible to cell death. In a breast cancer xenograft mouse model, *BRCA1P1* depletion suppresses tumor growth and stimulates pro-inflammatory cytokines and local immunity. To our knowledge, this is the first study that demonstrates an important role of *BRCA1P1* pseudogene in anti-tumor responses through regulation of antiviral innate immunity and tumor growth.

Materials and Methods

Primary Breast Cancer Tissue Samples and RNA Extraction.

We obtained written informed consent from the patients for anonymous use of banked tumor tissues for research. The studies were conducted in accordance with recognized ethical guidelines of the Declaration of Helsinki, and were approved by an institutional review board at the University of Chicago hospitals. Methods for case selection, tumor RNA extraction, microarray and RNA sequencing were described previously (17). In brief, we selected female patient cases with invasive ductal carcinoma from our Breast Program Biospecimen Bank. Patients who had received neoadjuvant chemotherapy were excluded, as we were interested in pre-treatment gene expression. Areas of malignant tissue were isolated from frozen tissues and homogenized by the TissueLyzer LT (Qiagen, Valencia, CA). RNA was extracted using the Qiagen AllPrep DNA/RNA/Protein mini kit protocol (Qiagen, Valencia, CA). The integrity of RNAs was validated using a 2100 Bioanalyzer (Agilent,

Santa Clara, CA) at the University of Chicago Genomics Facility. RNAs with a minimum RNA integrity number of 8 were used for cDNA synthesis and qRT-PCR experiments. Molecular subtypes of the breast tumors were determined by mRNA expression of the PAM50 intrinsic classifier as previously described (17).

Animal Study and Handling.

All animals were humanely handled and monitored for health conditions according to the Institutional Animal Care and Use Committee (IACUC) approved protocols. Eight week old female *Foxn1^{nu/nu}* (Harlan) mice were anesthetized via inhalation with 2% vaporized isoflurane and were unilaterally injected with 4×10^6 MDA-MB-231 WT or *BRCA1*^{P1}-KO cells (100 μ l, 50% Matrigel, 8–9 animals per model) into the fourth inguinal mammary gland at the base of the nipple. Tumor measurements were performed weekly using calipers to calculate tumor volume using the formula: $1/2 (\text{Length} \times \text{Width}^2)$. The assessment was blind to the animal model and was performed by the same person throughout the study. Animal weight was monitored twice weekly. Spleens were collected from animals in each model at the end of the study for downstream experiments.

Cell Culture.

Primary human mammary epithelial cells (HMEC) were purchased from Lonza (Allendale, NJ). Breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were authenticated for species and unique DNA profile using short tandem repeat (STR) analysis by the provider (ATCC). Cells were cultured in media recommended by ATCC, and tested negative for mycoplasma contamination using the MycoAlert Kit (Lonza, Allendale, NJ).

Cell Transfection and Drug Sensitivity Assay.

Cells were seeded at $3\text{--}5 \times 10^5$ cells/well on 6-well plate and transfected with 20–50 nM of *BRCA1*^{P1}-ASO (LNA GapmeRs, Exiqon, Denmark) or 2 μ g of poly (I:C) (InvivoGen, San Diego, CA) using DharmaFECT 1 or 4 (Dharmacon, Lafayette, Co), or Lipofectamin 3000 transfection reagent (Invitrogen, Carlsbad, CA). One day after transfection, cells were transferred to 96-well cell culture plates at $1\text{--}1.5 \times 10^4$ cells/well. Chemotherapy drugs (5 μ M Doxorubicin or 4 μ M Camptothecin) (Sigma-Aldrich, St Louis, MO) were added to cells one day after transfer, and incubated for 24 hours. The Caspase-Glo 3/7 Assay (Promega, Madison, WI) was used to screen apoptotic cells by measuring luminescent signals using a Synergy H1 Plate Reader (BioTek, Winooski, VT).

CRISPR-Cas9 Knockout and Activation.

Guide RNAs (gRNA) were designed using the chopchop tools (<http://chopchop.cbu.uib.no/>), IDT Alt-R® CRISPR-Cas9 guide RNA (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM) or sgRNA designer (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgRNA-design>) and synthesized by Integrated DNA Technologies (Coralville, IA). Two pairs of gRNAs were designed to delete 1,147 bp (gRNAs 1 and 4) or 1,469 bp (gRNAs 2 and 3) of the *BRCA1*^{P1} sequence. For CRISPR activation (CRISPRa), three gRNAs were designed to target the *BRCA1*^{P1} promoter. DNA sequences of gRNAs will be

provided upon request. The knockout experiment was performed as previously described in the literature(18). Briefly, gRNAs were cloned into pSpCas9(BB)-2A-GFP (Addgene #48138) and pSpCas9(BB)-2A-Puro (Addgene #62988) using BbsI restriction enzyme site. For CRISPRa, we utilized SP-dCas9-VPR (Addgene #63798). MDA-MB-231 cells were co-transfected with gRNAs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) and incubated for two days. For the isolation of *BRCA1PI*-KO clones, cells were treated with puromycin (1 µg/ml) for one week, diluted to one cell per 100 µl media and plated into each well of a 96 well plate. Single colony cells were expanded and subjected to PCR validation and DNA sequencing.

Total RNA Extraction and Microarray Analysis.

T47D and MDA-MB-231 cells were transfected with control or *BRCA1PI*-ASOs, incubated for 24 hours, and processed for RNA purification. MDA-MB-231 cells with *BRCA1PI*-knockout or wild type genotype were also subjected to RNA purification. Total RNAs were isolated with the RNeasy kit (Qiagen, Valencia, CA) following the protocol provided by the manufacturer. To avoid any possibility of DNA contamination, total RNAs were treated with RNase-free DNase I (Qiagen, Valencia, CA). Quality control was performed with the Agilent 4200 TapeStation system (Agilent Technologies, Santa Clara, CA). RNAs with RNA integrity numbers greater than eight were selected and subjected to the Illumina Human HT12 microarray platform. The microarray data were analyzed by Significance Analysis of Microarrays (SAM) between control or *BRCA1PI*-ASO-treated cells or between *BRCA1PI*-knockout and wild type cells. The significant gene expression in ASO-treated cells in microarray experiments was identified and subjected to DAVID Functional Annotation Bioinformatics Microarray Analysis. The microarray datasets are available through GEO (GSE112572 & GSE112573).

Viral Infection Assays.

Sendai virus (SeV, Cantell strain) was purchased from Charles River Laboratories. Cells were infected with SeV at 5 HAU/mL and lysed at 8, 16, and 24-hours post-infection for quantitative real-time PCR (qRT-PCR). VSV-eGFP was kindly provided by Sean Whelan (Harvard). Cells were infected with VSV-eGFP at MOI of 0.005 and analyzed for eGFP expression using flow cytometry (LSR Fortessa flow cytometer, BD Biosciences) after 8, 16, and 24 hours later. For flow cytometry, cells were harvested and then pelleted by centrifugation at 300 x g for 5 min. Cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde in PBS at room temperature for 30 min, followed by washing cells twice with PBS. The main cell population was gated in the FCS/SSC blot and then analyzed in the FITC channel. Positive cells were above the cutoff value, which is set to 98% of measurable events of the negative control.

IncuCyte Live-Cell Proliferation and Apoptosis Analysis.

Cells were plated at 5,000 cells/well in 96-well plates and let to adhere for 24h. IncuCyte Caspase-3/7 Green reagent was added to the media at a 1:1000 dilution (Essen Bioscience No. 4440, Ann Arbor, MI). Images of live cells were acquired using a 10x objective every 2 hours (four images/well) with the IncuCyte® S3 Live-Cell Analysis System. Data was analyzed using IncuCyte analysis software. Cell density was quantified as a measure of cell

proliferation. Apoptosis was quantified as the Total Integrated Intensity of green fluorescent signal from cells with activated Caspase-3/7 (apoptotic cells) normalized to cell density calculated from phase-contrast images. The experiment was performed twice with 7–10 technical replicates.

Statistical Analysis.

Each experiment was repeated at least three times and the mean $\pm$ standard deviation (SD) or mean $\pm$ standard error (SE) is shown with T-TEST (unpaired, two-tailed) values. Statistical significance was set at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. We performed one-way ANOVA to compare the expression level across the breast tumor subtype groups. Statistical analysis was carried out using Microsoft Excel and Graphpad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Plots were generated using Graphpad Prism 6.0.

Availability of data and materials

The microarray datasets are available through GEO (GSE112572 & GSE112573). There are no restrictions on availability of data. The data that support the findings of this study are available from the corresponding author upon request.

More detailed materials and methods are included in Supplementary Information. The oligonucleotides and antibodies used in the experiments are listed in Supplementary Tables S1 and S2, respectively.

Results

The Genomic Structure and Degenerative Mutations in the *BRCA1P1* Pseudogene.

The chromosome 17q21 region comprising the *BRCA1P1* pseudogene shows parallel elements of genomic structure, as *BRCA1* is located head-to-head with *NBR2*, and *BRCA1P1* is located head-to-head with *NBR1* (Fig. 1A). Cross-species comparative analysis among chicken, mouse, and human revealed that the *BRCA1P1* pseudogene is present only in the human genome. A comparison of DNA sequences between *BRCA1* and *BRCA1P1* showed that the pseudogene contains only three exons (exons 1a, 1b and 2), with three major insertions that include a 343 bp insertion of the *RPLP1* pseudogene (*RPLP1P4*) in exon 1a (10) and two ALU element insertions in exon 1b and intron 1b of *BRCA1P1* (Fig. 1B). Notably, exon 2 contains a point mutation that alters the translation initiation codon (ATG) of *BRCA1P1* to ATA. Based on the degenerative mutations occurring in the pseudogene (insertions and a start codon mutation), we predicted no protein coding potential for *BRCA1P1* transcripts.

A Bi-directional Promoter Shapes the Transcription of the *BRCA1P1* Pseudogene.

To determine if the *BRCA1P1* pseudogene is expressed through the bi-directional promoter between *BRCA1P1* and *NBR1*, we cloned the 1,243 bp region of the *BRCA1P1* promoter into a luciferase reporter gene construct and assessed the promoter activity in T47D cells (Fig. 1C). Sequence homology analysis between the *BRCA1* and *BRCA1P1* promoters showed that 85.7% of the promoter regions were identical. The *BRCA1P1* promoter exhibits

a 9.6 ± 1.0 -fold increase in luciferase activity compared to a pGL3B empty vector. Using this active promoter, the *BRCA1P1* pseudogene is transcribed from exon 1a to exon 1b (including introns) with a resulting RNA of at least 1,629 nucleotides [details in Supplementary Methods (Characterization of *BRCA1P1* Transcripts) and Supplementary Fig. S1].

Expression of *BRCA1P1* is Increased in Breast Cancer Cells and Breast Tumors.

We surveyed *BRCA1P1* expression in breast cell lines and breast tumors of different molecular subtypes and genetic backgrounds (Fig. 1D-F). Three pairs of primers were designed to quantify the pseudogene transcripts from exon 1a, exon 1b, and intron 1a to exon 1b (unspliced form), respectively (Supplementary Fig. S2A). Because a spliced form of the *BRCA1P1* transcript was previously reported in HeLa cells (12), we first compared the expression between the spliced and unspliced (intron 1a to exon 1b) forms. This analysis showed that the majority of the *BRCA1P1* transcripts (98%) are not spliced in T47D and MDA-MB-231 cells (Supplementary Fig. S2B).

Using primers detecting unspliced transcripts of *BRCA1P1*, we assessed *BRCA1P1* expression in several breast cancer cell lines. A comparison of *BRCA1P1* expression between non-malignant and malignant breast cells showed an average 2.8-fold increased expression in breast cancer cell lines compared to primary mammary epithelial cells (HMEC) (Fig. 1D). Expression of *BRCA1P1* in MDA-MB-231 and T47D cells were 2.86 ± 0.96 and 3.63 ± 1.10 fold higher than that in HMEC, respectively. Next, we examined *BRCA1P1* expression in 36 frozen primary breast tissues of different molecular subtypes (Fig. 1E and F). Molecular subtypes of the breast tumors were determined by mRNA expression of the PAM50 intrinsic classifier (19). Expression of *BRCA1P1* increased by 2.1-fold in breast tumors compared to normal breast tissues, with no subtype-specific pattern of expression. Together, these data indicate that breast cancer cells and tumors exhibit elevated *BRCA1P1* expression compared to normal breast epithelial cells or normal breast tissues.

Depletion of *BRCA1P1* Induces Antiviral Immune Gene Expression.

To determine the biological role of the pseudogene in breast cancer, we inhibited expression of *BRCA1P1* in breast cancer cells using two different approaches. First, antisense oligonucleotides (ASO, LNA GapmeRs) were custom designed to target exon 1a of *BRCA1P1*. The efficiency of knockdown by ASO was validated at the RNA expression level using qRT-PCR (Supplementary Fig. S2C). The expression of *BRCA1P1* was significantly dampened by *BRCA1P1*-specific ASO, with no silencing effect on the expression of the parent genes (*BRCA1* and *RPLP1*) or adjacent genes (*NBR1*) in T47D and MDA-MB-231 cells. Secondly, we deleted the *BRCA1P1* pseudogene from the genome of MDA-MB-231 cells using CRISPR-Cas9 genome-editing tools (Supplementary Fig. S3A). Co-transfection of two guide RNAs (gRNA1 and 4) resulted in a partial deletion (1,147 bp) of the *BRCA1P1* pseudogene (Supplementary Fig. S3B), with no inhibitory effects on the parent gene (*BRCA1*) expression (Supplementary Fig. S3C).

To identify genes and pathways influenced by *BRCA1P1* loss or depletion, we performed genome-wide gene expression profiling (Fig. 2A). This analysis identified 3,981 upregulated

genes in *BRCA1P1*-ASO treated groups compared to control-ASO groups both in T47D and MDA-MB-231 cells, as well as 723 upregulated genes in *BRCA1P1*-KO cells compared to the WT cells. One hundred and thirteen genes were up-regulated in both *BRCA1P1*-KO and knockdown (ASO-treated) cells. Gene Ontology Enrichment analysis of the 113 overlapping genes revealed the antiviral defense response as one of the most upregulated pathways.

Validation of microarray data by qRT-PCR confirmed increased expression of antiviral ISGs [*IFIT3*, MDA5 (*IFIH1*), RIG-I (*DDX58*), *HERC6*, and *APOBEC3B*] in two clones of *BRCA1P1* KO compared to wild type control cells (Fig. 2B). Protein expression of IFIT3, MDA5, and RIG-I was also significantly increased in the KO clones compared to control cells (Fig. 2C). The data suggested that *BRCA1P1* deficiency triggered an innate immune defense program characterized by transcriptional upregulation of many antiviral genes.

Loss of *BRCA1P1* Stimulates Cytokine Expression.

Because the replication and spread of viral pathogens are restricted by innate immune mechanisms and most prominently cytokine signaling, we next examined whether loss of *BRCA1P1* stimulated cytokine expression. We used qRT-PCR to analyze cytokine expression because basal cytokine expression levels in breast cancer cells are extremely low, and thus often undetectable by microarrays. We found a dramatic increase in Tumor Necrosis Factor (*TNF*) expression in *BRCA1P1*-depleted cells compared to control cells (Fig. 2D). Expression of *TNF* was 10.7 ± 1.1 and 11.0 ± 0.8 fold greater in two *BRCA1P1*-KO clones, respectively, compared to the WT cells (left panel, Fig. 2D). *TNF* expression also gradually increased in T47D cells treated with increasing concentrations of *BRCA1P1*-ASOs (right panel, Fig. 2D). Of note, we did not observe any expression of type I IFNs (*IFNA1* and *IFNB1*) in MDA-MB-231 cells using qRT-PCR (Supplementary Fig. S4A and B). Copy number alteration (CNA) data in the Cancer Cell Line Encyclopedia (CCLE) revealed a frequent deletion of type I IFN genes in cancer cell lines (Supplementary Fig. S4D). In accord, MDA-MB-231 cells carry homozygous (deep) deletions of 17 subtypes of IFN- α and IFN- β genes. In contrast, the expression of type III IFN (*IFNL1*) was markedly increased in MDA-MB-231 cells, compared to nonmalignant HMEC cells (Supplementary Fig. S4C). Furthermore, loss of *BRCA1P1* led to a significant increase in *IFNL1* expression in MDA-MB-231 cells compared to the WT cells (left panel, Fig. 2E). Knockdown of *BRCA1P1* using ASO also increased *IFNL1* expression, compared to control-ASO treated cells (right panel, Fig. 2E). Collectively, our data indicate that loss of *BRCA1P1* stimulates the expression of cytokines (TNF- α and IFN- λ) and a range of antiviral ISGs.

Antiviral-like Program Induced by *BRCA1P1*-Depletion.

We next determined the effect of *BRCA1P1* loss on virus-induced antiviral gene expression and virus replication in MDA-MB-231 cells (Fig. 3A-C). Sendai virus (SeV, *Paramyxoviridae*) and eGFP-expressing vesicular stomatitis virus (VSV-eGFP, *Rhabdoviridae*) are well known to trigger innate immune responses via activation of the RLR signaling pathway. The expression of cytokine/chemokine (*TNF* and *CCL5*) and ISGs genes (*IFIT1* and *RSAD2*) during SeV infection was significantly higher in *BRCA1P1*-KO cells than in infected WT cells (Fig. 3A). In particular, the expression of *TNF* was greatly increased in *BRCA1P1*-KO cells compared to the WT cells at 24 hours post-SeV infection

(a $2,568.1 \pm 431$ -fold vs 361.7 ± 16.1 -fold increase). The expression of *IFNL1* and *IFNL2/3* was also greater in the KO cells at 8-hours post-infection (a $14,527 \pm 1,520$ -fold vs $5,754 \pm 320$ -fold increase) (Fig. 3B). In accord, cells depleted of *BRCA1PI* suppressed the replication of VSV-eGFP more effectively than did the WT cells [2.36 vs. 38.3 eGFP-positive cells (relative units)] (Fig. 3C). Collectively, these findings indicated that loss of *BRCA1PI* triggered a cytokine-mediated antiviral program.

Poly(I:C)-driven Antiviral Signaling in Breast Cancer Cells.

Previous reports showed that poly(I:C), a synthetic analogue of double-stranded RNAs, binds to cytosolic RIG-I and MDA5 receptors (20,21) and induces innate immune signaling that leads to the cytosol-to-nuclear translocation of several key transcription factors [e.g. NF- κ B, IFN-regulatory factors (IRFs), and AP-1] (22). To determine if *BRCA1PI* regulates cytokine expression through activation of transcription factors, we first examined poly(I:C)-driven nuclear translocation of NF- κ B in breast cancer cells (Supplementary Fig. S5A). In MDA-MB-231 cells, as in fibroblast cells, nuclear localization of the NF- κ B subunit RelA increased upon poly(I:C) transfection, starting from 4–8 hours after transfection. Next, as MDA-MB-231 cells express type III IFNs (Supplementary Fig. S4C), we examined nuclear transport of IRF-1, which plays a major role in type III IFN induction. We observed an increase in IRF-1 nuclear localization at 4–8 hours after poly(I:C) transfection. A rapid increase in *TNF* and *IFNL* expressions was also observed starting at 4 to 8-hours post-treatment, followed by increases in expression for ISGs (*IFIT3*, *IFIH1*, and *DDX58*), interleukins (*IL8*, *IL6*, *IL1A*, and *IL1B*), and the transcription factors (*IRF1*, *STAT1*, and *STAT2*) (Supplementary Fig. S5B and S5C).

We then compared poly(I:C)-induced gene expression between *BRCA1PI*-WT and KO cells (Fig. 3D). This analysis showed significant increases in the expression of *TNF* (a $4,660 \pm 136$ -fold vs. 575 ± 15 -fold increase), *IFNL1* (a $244,948 \pm 8,898$ -fold vs. $111,222 \pm 1,845$ -fold increase) and *IFNL2/3* (a $219,251 \pm 6,429$ -fold vs. $33,140 \pm 1,105$ -fold increase) in *BRCA1PI*-KO cells compared to the WT cells at 8-hours post-treatment. It is of note that we were able to measure gene expression only up to 8-hours post-treatment because poly(I:C)-transfected cells were severely apoptotic and not able to survive for longer times. The expression of interleukins (*IL1A*, *IL1B*, and *IL6*) and antiviral genes [*IFIH1* (MDA5) and *STAT1*] was also significantly higher in *BRCA1PI*-KO cells than in WT cells (Supplementary Fig. S6). These data suggested that depletion of *BRCA1PI* sensitized breast cancer cells to apoptosis by stimulating expression of cytokines.

We also investigated the effect of *BRCA1PI* over-expression on *TNF* and *IFNL* expression in breast cancer cells (Fig. 3E). To stimulate *BRCA1PI* expression using its own promoter and to express *BRCA1PI*-lncRNA in the proper subcellular location (nucleus), we used the CRISPR activation system (CRISPRa), which led to a 4.5-fold increase in *BRCA1PI* expression in MDA-MB-231 cells (left panel). Overexpression of *BRCA1PI* significantly decreased expression of *TNF* and *IFNL1* compared to the control vector-transfected cells (middle and right panels). The overexpression data together with the depletion data indicate that *BRCA1PI* regulates *TNF* and *IFNL* expression in breast cancer cells.

Association of *BRCA1P1*-lncRNA with the NF- κ B Subunit RelA.

To understand the mechanism whereby *BRCA1P1* regulates cytokine expression, we first determined the subcellular localization of *BRCA1P1*-lncRNA using two different methods (Fig. 4A). Fractionation of cellular compartments showed a strong enrichment of *BRCA1P1*-lncRNA in the nuclei of T47D, CAMA-1 and HCC-1937. Single molecule RNA-FISH (smRNA-FISH) revealed that the majority of *BRCA1P1* signals in cancer cells were restricted to the nucleus, whereas most *GAPDH* signals were detected in the cytoplasm (Fig. 4A). Together, these data showed nuclear localization of *BRCA1P1* transcripts.

We thus tested whether *BRCA1P1* interacts with transcription factors important for innate immune signaling in the nucleus of MDA-MB-231 cells. Specifically, we focused on NF- κ B, IRF-1 and STAT-1 because these transcription factors are known to regulate expression of *TNF*, *IFNL* and/or ISGs in response to viral infection or dsRNA stimulation. RNA immunoprecipitation (RIP) data revealed an association of *BRCA1P1*-lncRNA with the NF- κ B subunit RelA, but not with IRF1 or STAT-1 (Fig. 4B). *RNA18S5* (18S rRNA), which served as a negative control, exhibited no interaction with any of them. These data suggested a specific interaction of *BRCA1P1*-lncRNA with RelA in the nucleus.

Inhibition of RelA Activity by *BRCA1P1*.

To determine if the interaction of *BRCA1P1*-lncRNA with RelA influences the enrichment of RelA at the target promoters, we conducted chromatin immunoprecipitation (ChIP) of RelA proteins on the promoters of *TNF*, *IFNL1*, and *IFIH1* genes (Fig. 4C). We observed greater abundance of the RelA transcription factor at the promoters in *BRCA1P1*-KO cells compared to WT cells. Inversely, overexpression of *BRCA1P1* decreased RelA enrichment at the promoters of *TNF*, *IFNL1*, and *IFIH1* (Fig. 4D).

We also performed an electrophoretic mobility shift assay (EMSA) to determine whether *BRCA1P1*-lncRNA inhibits the association of RelA with the target promoters (Fig. 4E). As expected, we observed a strong association of recombinant RelA proteins (p65) with a NF- κ B cis-acting regulatory element at the TNF promoter, indicated by the retardation in electrophoretic mobility of the probe on polyacrylamide gel electrophoresis (PAGE) (lane 2, Fig. 4E). In contrast, the strength of this interaction decreased with increasing amounts (1 to 10 fmols) of *BRCA1P1*-lncRNA in a dose-dependent manner (lanes 3–5, Fig. 4E). The specificity of RelA's interaction with the motif was confirmed by further retardation in mobility (super-shift) resulting from interaction with anti-RelA antibodies (lane 6). These data further strengthen the deduction that *BRCA1P1*-lncRNA interferes with RelA's binding to target promoters.

Next, we generated truncated mutants of *BRCA1P1*-lncRNA and conducted RNA pull-down assays with recombinant RelA proteins (Fig. 4F and 4G). Whereas there was a strong association of *BRCA1P1*-lncRNA (1–1107 nt) with RelA (lane 1, Fig. 4G), deletion of exon 1a in *BRCA1P1*-lncRNA strongly decreased its interaction with RelA (lanes 3 and 4). In contrast, deletion of exon 1b and intron 1a had only a minor effect on the interaction (lane 2). These results suggest that exon 1a is important for the interaction of *BRCA1P1*-lncRNA with RelA. An RNA structure prediction showed that exon 1a forms a long stretch of small

hairpin loops, which might play a role in its interaction with RelA (Supplementary Fig. S7A), although this remains to be determined. Collectively, these data showed that *BRCA1P1*-lncRNA physically interacts with RelA in part through exon 1a, inhibits NF- κ B binding to promoters, and negatively regulates target gene expression.

Lastly, we determined whether depletion of NF- κ B abrogates *BRCA1P1* loss-driven *TNF* expression (Fig. 4H). Depletion of *BRCA1P1* increased *TNF* expression (a 57.8 ± 13.9 -fold increase) in control siRNA treated cells, which was reduced to a 23.2 ± 8.9 -fold increase in RelA siRNA treated cells. The expression of *IFNL1* was also reduced by RelA silencing (a 43.9 ± 9.2 vs 62.6 ± 6.2 -fold increase). Collectively, the data reinforce the observation that *BRCA1P1* regulates expression of *TNF* and *IFNL1* partly through NF- κ B.

Depletion of *BRCA1P1* Increases Apoptosis of Breast Cancer Cells.

A radical defense mechanism to restrict the spread of a viral infection is apoptosis of the infected cell. We found that *BRCA1P1*-deficient cells are apoptotic, with an increased frequency of caspase 3/7-positive cells in *BRCA1P1*-KO and *BRCA1P1*-ASO-treated cells compared to the WT cells and control-ASO-treated cells, respectively (left panels, Fig. 5A and 5B). The increases in apoptosis appeared to restrict the proliferation of these cells, demonstrated by clear decreases in cell density of *BRCA1P1*-KO cells and *BRCA1P1*-ASO-treated cells, compared to the respective control cells (right panels, Fig. 5A and 5B). To determine if the observed phenotypes (apoptosis and proliferation) are driven by *BRCA1P1* specifically, we re-expressed *BRCA1P1* in *BRCA1P1*-ASO-treated cells using CRISPRa (Fig. 5B). Re-expression of *BRCA1P1* reduced apoptosis and restored proliferation of *BRCA1P1*-ASO-treated cells to the same levels as those observed in control-ASO-treated cells, demonstrating the specificity of *BRCA1P1*-driven regulation of apoptosis and proliferation in breast cancer cells. Interestingly, we did not observe any change in cell proliferation or apoptosis driven by *BRCA1P1* depletion in HMEC cells (Fig. 5C), indicating that *BRCA1P1* regulates apoptosis in cancer cells but not in normal breast cells.

TNF- α Induced Apoptosis in *BRCA1P1*-depleted Cells.

We next tested whether cytokines induced apoptosis in *BRCA1P1*-KO cells. This is based on our data showing increased cytokine expression in *BRCA1P1*-KO cells (Fig. 2D and 2E), as well as previous literature reporting the pivotal role of TNF- α in the regulation of apoptosis (23,24). We treated *BRCA1P1*-KO and WT cells with poly(I:C) or TNF- α , and examined the effects on apoptosis and cytokine expression. Treatment of cells with poly(I:C) induced higher *TNF* expression (Fig. 3D) and more severe apoptosis in *BRCA1P1*-KO cells than it did in control cells (Fig. 5D). Treatment with TNF- α clearly increased apoptosis of *BRCA1P1*-KO cells with an average 3.9-fold increase compared to WT cells at 52-hours post-treatment (Fig. 5E). The increase in apoptosis was accompanied by strongly elevated expression of *TNF* and *IFNL1* in *BRCA1P1*-KO cells (Fig. 5F).

Since previous reports showed that TNF- α induces apoptosis through reactive oxygen species (ROS) and DNA damage (23–27), we determined whether apoptosis driven by *BRCA1P1* depletion is partly due to accumulation of ROS and DNA damage. In line with this, we observed significant increases in ROS and H2AX phosphorylation (γ -H2AX), a

well-known DNA damage marker, in *BRCA1PI*-KO cells compared to the WT cells (Fig. 5G and 5H). Our data indicate that TNF- α induces apoptosis of *BRCA1PI*-KO cells, partly through accumulation of ROS and DNA damage. Furthermore, we observed that *BRCA1PI*-depleted cells were more sensitive to genotoxic drugs, with increased apoptosis after doxorubicin and camptothecin treatment in *BRCA1PI*-KO cells (Fig. 5I), compared to the WT cells. Together, our data suggest that *BRCA1PI* depletion increases apoptosis and sensitizes breast cancer cells to genotoxic drug treatments.

Depletion of *BRCA1PI* Inhibits Tumor Growth *In Vivo*.

To determine the physiological role of *BRCA1PI* in regulating tumor growth and anti-tumor immunity *in vivo*, we injected MDA-MB-231 cells of either the *BRCA1PI* WT or KO genotype into the mammary glands of female athymic nude mice [(CrI:NU(NCr)-Foxn1nu]. In control mice that were injected with *BRCA1PI*-WT cells (n=8), tumor volumes increased over time, up to a volume of $494.9 \pm 241.4 \text{ mm}^3$ at day fifty-four post injection. In contrast, tumor volumes in mice with *BRCA1PI*-KO cells (n=9) remained below 50 mm^3 for all mice throughout the study. Tumors for this group of mice were below measurable limits after day thirty, except for one mouse with a tumor that reached 42 mm^3 by day fifty-four (Fig. 6A). Since our *in vitro* studies showed increased cytokine expression in *BRCA1PI*-depleted cells compared to WT cells (Fig. 2D and 2E), we also assessed tumor immunity regulated by *BRCA1PI* in our xenograft mouse model. Since athymic nude mice lack T-cells, we measured cytokine expression in spleen tissues harvested from mice with *BRCA1PI*-WT and *BRCA1PI*-KO tumors. The transcript amounts of the cytokines TNF- α , IL2, IFN- γ and IL12a in the spleen was significantly higher in mice with *BRCA1PI*-KO tumors compared to control mice with WT tumors (Fig. 6B). Collectively, these data suggest that *BRCA1PI* depletion suppresses tumor growth partly through stimulation of local immunity, ultimately leading to reduced proliferation and enhanced apoptosis of cancer cells.

Discussion

Innate immune defense pathways are critical for antitumor responses and the induction of apoptosis of cancer cells (1). Although the importance of antiviral mechanism is well known in cancer, the roles of host RNAs in antiviral defense mechanism have just begun to be elucidated. In this study, we discovered an immunogenic role for *BRCA1PI*-lncRNA in regulating antiviral defense mechanisms in breast cancer cells. Spread of a viral infection is restricted by three major defense mechanisms (22). The first involves the upregulation and activation of a set of antiviral proteins, which allow for virus sensing and attenuation of virus replication. The second, more drastic mechanism is the apoptosis of infected cells. The third is based on paracrine IFN signaling, which alerts non-infected cells. *BRCA1PI* is potentially involved in all three mechanisms. *BRCA1PI*-deficient cells were prone to apoptosis and highly expressed antiviral immune genes including IFNs, which are typical markers of virus-infected cells. Depletion of *BRCA1PI* suppressed tumor growth and stimulated local immunity in a breast cancer xenograft mouse model. Our findings thus reveal a novel mechanism of regulation of antiviral responses by a host pseudogene RNA in breast cancer cells.

Mechanistically, *BRCA1PI*-lncRNA binds the NF- κ B subunit RelA, inhibits the activity of RelA at its target promoters, and thereby negatively regulate transcription of antiviral genes. It is of note that *BRCA1PI*-lncRNA appears to interact with RelA irrespective of whether RelA dissociates (Fig. 4E-4G) or associates with chromatin (Fig. 4B and Supplementary Fig. S7B). It has been reported that the NF κ B complex binds chromatin and recruits a chromatin modifying complex (28–30). Specifically, the RelA subunit of NF κ B was reported to interact with histone deacetylases on chromatin (30). Thus, when *BRCA1PI*-lncRNA associates with RelA, it is likely to interact with histones indirectly through chromatin-bound RelA. In support of this, the level of *BRCA1PI* enrichment on H2B (0.59 ± 0.02 , relative unit) or H3 (0.47 ± 0.03) is similar to that of RelA (0.54 ± 0.08) (Fig. 4B and Supplementary Fig. S7B). Considering the vast numbers of nucleosome-associated histones on every chromosome, the data indicate that only a very small portion of histones associates with *BRCA1PI*-lncRNA, likely through RelA bound on chromatin. While further investigation will be required to determine the detailed mechanisms of *BRCA1PI*-regulated RelA activity, our data revealed a strong association of *BRCA1PI*-lncRNA with RelA. Interestingly, recent discoveries showed that cellular non-coding transcripts bind to RIG-I and/or its activator, the E3 ligase TRIM25, and promote RIG-I-mediated antiviral innate immune responses (8,31,32). Therefore, it is conceivable that there may be additional mechanisms through which *BRCA1PI* activates antiviral signaling, such as a direct effect of *BRCA1PI*-lncRNA on immunostimulatory dsRNAs or their sensors, or by regulating the activity of dsRNA-activated kinases, which warrants future investigation.

Our data also showed that *BRCA1PI* depletion stimulates the induction of pro-inflammatory cytokines in the spleen of our mouse model, suggesting an important role for *BRCA1PI* in regulating local immunity. However, since athymic nude mice lack T-cells, we were unable to evaluate the effects of *BRCA1PI*-deficiency on T-cells or other immune cells. Therefore, future studies using humanized mice will be needed to fully understand the role of *BRCA1PI*-lncRNA in modulating local immunity and the tumor microenvironment. Along these lines, as NF- κ B plays multiple roles in innate immunity and pro-inflammatory signaling, further investigation will be required to examine how NF- κ B regulates *BRCA1PI* depletion-driven antiviral immunity in a context-dependent manner. It also remains to be determined whether the interaction of *BRCA1PI*-lncRNA with RelA influences the RelA-mediated transcription of apoptosis genes, which may contribute to increased apoptosis in *BRCA1PI*-KO cells. Additionally, future studies on the detailed mechanisms of how *BRCA1PI* expression is regulated by upstream signals may increase our understanding of the regulatory circuits in *BRCA1PI*-mediated responses.

Despite some limitations, our study revealed a novel mechanism of innate immunity that is regulated by a pseudogene lncRNA. Regulation of innate immunity by the *BRCA1PI* pseudogene might open up new possibilities for multilayered immunotherapy for cancer control (33). Tumor immunity and immunotherapy have become increasingly important in treatment strategies for a variety of tumors. They also point to etiology and progression pathways that could be targeted for prevention. Clinical trials evaluating the safety and efficacy of intratumoral injection of STING agonists, TLR agonists, or poly(I:C) derivatives with innate immune modulators are currently completed or are ongoing (34–37). Oncolytic viruses have also emerged as important agents in cancer treatment as they offer the attractive

therapeutic combination of tumor-specific cell lysis together with immune stimulation (33,38). Considering the importance of innate immunity and antiviral sensing in tumor immunity, our findings on the regulation of innate immunity by *BRCA1P1* may have clinical relevance in the development of immunotherapies and should be further explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Minn AJ. Interferons and the Immunogenic Effects of Cancer Therapy. *Trends Immunol* 2015;36:725–37 [PubMed: 26604042]
2. Wies E, Wang MK, Maharaj NP, Chen K, Zhou S, Finberg RW, et al. Dephosphorylation of the RNA sensors RIG-I and MDA5 by the phosphatase PP1 is essential for innate immune signaling. *Immunity* 2013;38:437–49 [PubMed: 23499489]
3. Rehwinkel J, Gack MU. RIG-I-like receptors: their regulation and roles in RNA sensing. *Nature reviews Immunology* 2020;20:537–51
4. Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* 2011;1:519–25 [PubMed: 22328912]
5. Sistigu A, Yamazaki T, Vacchelli E, Chaba K, Enot DP, Adam J, et al. Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nat Med* 2014;20:1301–9 [PubMed: 25344738]
6. Balakirev ES, Ayala FJ. Pseudogenes: are they “junk” or functional DNA? *Annu Rev Genet* 2003;37:123–51 [PubMed: 14616058]
7. Zhang Z, Harrison PM, Liu Y, Gerstein M. Millions of years of evolution preserved: a comprehensive catalog of the processed pseudogenes in the human genome. *Genome Res* 2003;13:2541–58 [PubMed: 14656962]
8. Chiang JJ, Sparrer KMJ, van Gent M, Lassig C, Huang T, Osterrieder N, et al. Viral unmasking of cellular 5S rRNA pseudogene transcripts induces RIG-I-mediated immunity. *Nat Immunol* 2018;19:53–62 [PubMed: 29180807]
9. Raponi NA, Qu K, Zhang J, Mikhail M, Laberge RM, Chang HY. A mammalian pseudogene lncRNA at the interface of inflammation and anti-inflammatory therapeutics. *Elife* 2013;2:e00762 [PubMed: 23898399]
10. Barker DF, Liu X, Almeida ER. The BRCA1 and 1A1.3B promoters are parallel elements of a genomic duplication at 17q21. *Genomics* 1996;38:215–22 [PubMed: 8954804]
11. Brown MA, Xu CF, Nicolai H, Griffiths B, Chambers JA, Black D, et al. The 5' end of the BRCA1 gene lies within a duplicated region of human chromosome 17q21. *Oncogene* 1996;12:2507–13 [PubMed: 8700509]
12. Pettigrew CA, French JD, Saunus JM, Edwards SL, Sauer AV, Smart CE, et al. Identification and functional analysis of novel BRCA1 transcripts, including mouse Brca1-Iris and human pseudo-BRCA1. *Breast Cancer Res Treat* 2010;119:239–47 [PubMed: 19067158]

13. Puget N, Gad S, Perrin-Vidoz L, Sinilnikova OM, Stoppa-Lyonnet D, Lenoir GM, et al. Distinct BRCA1 rearrangements involving the BRCA1 pseudogene suggest the existence of a recombination hot spot. *Am J Hum Genet* 2002;70:858–65 [PubMed: 11880951]
14. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet* 2001;27:247–54 [PubMed: 11242102]
15. Scully R, Ganesan S, Vlasakova K, Chen J, Socolovsky M, Livingston DM. Genetic analysis of BRCA1 function in a defined tumor cell line. *Molecular cell* 1999;4:1093–9 [PubMed: 10635334]
16. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66–71 [PubMed: 7545954]
17. Han YJ, Boatman SM, Zhang J, Du XC, Yeh AC, Zheng Y, et al. LncRNA BLAT1 is Upregulated in Basal-like Breast Cancer through Epigenetic Modifications. *Scientific Reports* 2018;8:15572 [PubMed: 30349062]
18. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 2013;8:2281–308 [PubMed: 24157548]
19. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2009;27:1160–7 [PubMed: 19204204]
20. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, et al. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 2005;23:19–28 [PubMed: 16039576]
21. Reikine S, Nguyen JB, Modis Y. Pattern Recognition and Signaling Mechanisms of RIG-I and MDA5. *Front Immunol* 2014;5:342 [PubMed: 25101084]
22. Czerkies M, Korwek Z, Prus W, Kochanczyk M, Jaruszewicz-Blonska J, Tudelska K, et al. Cell fate in antiviral response arises in the crosstalk of IRF, NF-kappaB and JAK/STAT pathways. *Nature communications* 2018;9:493
23. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003;10:45–65 [PubMed: 12655295]
24. Voltan R, Secchiero P, Casciano F, Milani D, Zauli G, Tisato V. Redox signaling and oxidative stress: Cross talk with TNF-related apoptosis inducing ligand activity. *Int J Biochem Cell Biol* 2016;81:364–74 [PubMed: 27686849]
25. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 2005;120:649–61 [PubMed: 15766528]
26. Wheelhouse NM, Chan YS, Gillies SE, Caldwell H, Ross JA, Harrison DJ, et al. TNF-alpha induced DNA damage in primary murine hepatocytes. *Int J Mol Med* 2003;12:889–94 [PubMed: 14612962]
27. Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, et al. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. *Cancer Res* 2006;66:11565–70 [PubMed: 17178846]
28. Bhatt D, Ghosh S. Regulation of the NF-kappaB-Mediated Transcription of Inflammatory Genes. *Front Immunol* 2014;5:71 [PubMed: 24611065]
29. Lone IN, Shukla MS, Charles Richard JL, Peshev ZY, Dimitrov S, Angelov D. Binding of NF-kappaB to nucleosomes: effect of translational positioning, nucleosome remodeling and linker histone H1. *PLoS Genet* 2013;9:e1003830 [PubMed: 24086160]
30. Ashburner BP, Westerheide SD, Baldwin AS Jr. The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Molecular and cellular biology* 2001;21:7065–77 [PubMed: 11564889]
31. Lin H, Jiang M, Liu L, Yang Z, Ma Z, Liu S, et al. The long noncoding RNA Lnczc3h7a promotes a TRIM25-mediated RIG-I antiviral innate immune response. *Nature immunology* 2019;20:812–23 [PubMed: 31036902]
32. Jiang M, Zhang S, Yang Z, Lin H, Zhu J, Liu L, et al. Self-Recognition of an Inducible Host lncRNA by RIG-I Feedback Restricts Innate Immune Response. *Cell* 2018;173:906–19 e13 [PubMed: 29706547]

33. Demaria O, Cornen S, Daeron M, Morel Y, Medzhitov R, Vivier E. Harnessing innate immunity in cancer therapy. *Nature* 2019;574:45–56 [PubMed: 31578484]
34. Brody JD, Ai WZ, Czerwinski DK, Torchia JA, Levy M, Advani RH, et al. In situ vaccination with a TLR9 agonist induces systemic lymphoma regression: a phase I/II study. *J Clin Oncol* 2010;28:4324–32 [PubMed: 20697067]
35. Demaria O, De Gassart A, Coso S, Gesterhann N, Di Domizio J, Flatz L, et al. STING activation of tumor endothelial cells initiates spontaneous and therapeutic antitumor immunity. *Proc Natl Acad Sci U S A* 2015;112:15408–13 [PubMed: 26607445]
36. Dowling DJ, Tan Z, Prokopowicz ZM, Palmer CD, Matthews MA, Dietsch GN, et al. The ultra-potent and selective TLR8 agonist VTX-294 activates human newborn and adult leukocytes. *PLoS One* 2013;8:e58164 [PubMed: 23483986]
37. Caskey M, Lefebvre F, Filali-Mouhim A, Cameron MJ, Goulet JP, Haddad EK, et al. Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. *J Exp Med* 2011;208:2357–66 [PubMed: 22065672]
38. Gujar S, Pol JG, Kim Y, Lee PW, Kroemer G. Antitumor Benefits of Antiviral Immunity: An Underappreciated Aspect of Oncolytic Virotherapies. *Trends Immunol* 2018;39:209–21 [PubMed: 29275092]

Significance

This study identifies a novel mechanism of innate immunity driven by a host pseudogene RNA, that inhibits innate immune defense mechanisms and anti-tumor responses through regulation of antiviral gene expression.

Fig. 1. Genomic Structure, Promoter Activity and Expression of the *BRCA1P1* Pseudogene in Breast Cells and Tissues.

A, Schematic representation of the genomic organization of chromosome 17q21 (GRCh37/hg19), as shown in the NCBI genomic context (top). The UCSC genome browser view of chromosome 17q21 (NCBI36/hg18) shows the presence of the *NBR2* gene and *BRCA1P1* pseudogene in humans but not in mice or chickens (bottom). **B**, Depicted are the exonic and intronic structures of *BRCA1P1*. *BRCA1P1* retains only 3 exons (exon 1a, 1b and 2) out of 25 exons of the parent *BRCA1* gene. The numbers represent nucleotide positions starting with the transcription start site. Insertions of a pseudogene of acidic ribosomal phosphoprotein P1 (*RPLP1P4*) and two ALU elements are presented. **C**, Bidirectional promoters are located between *BRCA1* and *NBR2*, and between *BRCA1P1* and *NBR1*. Promoter activity was assessed in T47D cells using dual luciferase activity assays, which showed greater *BRCA1P1* promoter activity compared to a pGL3B empty vector. Error bars represent the standard deviation of three biological replicates (***) $p < 0.001$. **D**, qRT-PCR was performed on human primary mammary epithelial cells (HMEC), and 14 human breast cancer cell lines using the primers detecting the unspliced transcripts of *BRCA1P1*. Results were normalized to *RNA18S* (18S ribosomal RNAs) and fold induction is shown relative to HMEC cells. Data represent mean and SD of four to six independent experiments. **E** and **F**, *BRCA1P1* expression was analyzed in 36 frozen primary breast tissues (relative to expression in normal breast tissue) and grouped by molecular subtypes: normal breast

tissues (n=7), luminal (n=11), Her2-positive (n=5), basal-like breast tumors (n=13) (**E**). Expression of *BRCA1PI* showed a significant increase in breast tumors (n=29) compared to normal breast tissues (n=7) ($p=0.022$) (**F**).

Fig. 2. Upregulation of Antiviral Genes and Cytokines in *BRCA1P1*-Depleted Cells.

A, Experimental schema of gene expression microarray analysis in *BRCA1P1* knock-down (KD) and knock-out (KO) cells. T47D and MDA-MB-231 cells treated with antisense oligonucleotides (ASO) (left panel). MDA-MB-231 cells with *BRCA1P1*-wild type (WT) or KO genotype (right panel) were also subjected to RNA purification and microarray analysis. The analysis identified 3,981 and 723 upregulated genes in *BRCA1P1*-ASO treated and KO cells compared to controls, respectively, with 113 genes overlapping between KD and KO cells. **B**, qRT-PCR analysis of antiviral genes [*IFIT3*, *IFIH1* (MDA5), *DDX58* (RIG-I), *HERC6*, and *APOBEC3B*] in *BRCA1P1*-KO clones compared to the WT. Results were normalized to *RNA18S* and fold induction is shown relative to control WT cells. **C**, Western blot analysis showed increased expression of IFIT3, MDA5, and RIG-I in *BRCA1P1*-KO clones compared to the WT. β -Actin was used as an endogenous control. **D** and **E**, Expression of *TNF* or *IFNL1* mRNA was analyzed using qRT-PCR in *BRCA1P1*-KO cells (left panel) or *BRCA1P1*-ASO treated cells (right panel). Increasing concentration of *BRCA1P1*-ASO gradually increases expression *TNF* mRNA in T47D cells. All results were normalized to *RNA18S* and fold induction is shown relative to WT control cells (left panel) or control-ASO treated cells (right panel). Data represent mean and SD of n = 3 to n = 6 biological replicates, and are representative of at least two independent experiments. (**p<0.01, ***p<0.001, and ****p<0.0001).

Fig. 3. Antiviral Responses After Viral Infection, Poly(I:C) Transfection, or *BRCA1P1* Overexpression.

A and B, qRT-PCR analysis of *TNF*, *IFIT1*, *RSAD2*, *CCL5*, *IFNL1*, and *IFNL2/3* mRNA in *BRCA1P1*-KO and WT cells that were infected with Sendai virus (SeV, 5 HAU/mL) for the indicated times. Results were normalized to *GAPDH* and fold induction is shown relative to mock-infected control cells. Data represent mean and SD of $n = 3$ biological replicates, and are representative of at least two independent experiments. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) **C**, MDA-MB-231 WT and *BRCA1P1*-KO cells were infected with eGFP-expressing vesicular stomatitis virus (VSV-eGFP) at MOI of 0.005. The percentage of eGFP-positive cells was determined by flow cytometry for the indicated times. Error bars represent SD of three biological replicates. (** $P < 0.01$; **** $P < 0.0001$). **D**, qRT-PCR analysis of *TNF*, *IFNL1*, *IFNL2/3*, and *IL1B* mRNA in *BRCA1P1*-KO and WT cells transfected with poly(I:C) at 4 and 8 hours post-transfection (** $P < 0.01$; *** $p < 0.001$ vs poly I:C treated WT). **E**, *BRCA1P1* expression was stimulated in MDA-MB-231 cells using the CRISPR activation system (left panel), which led to a decrease in *TNF* and *IFNL1* expression (middle and left panels). Fold induction is shown relative to

control cells (no sgRNA treatment). Data represent mean and SD of $n = 3$ biological replicates, and are representative of at least two independent experiments (** $p < 0.01$; *** $p < 0.0001$).

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Fig. 4. *BRCA1P1*-lncRNA Interacts with Nuclear RelA and Regulates Its Binding to Promoters.

A, The intracellular expression level of *BRCA1P1* was quantified by qRT-PCR using cytoplasmic or nuclear fractions of breast cancer cells (T47D, CAMA1 and HCC-1937) (top). Representative images show single molecule RNA FISH results performed in MDA-MB-231 and HEK293 cells using twenty-six oligonucleotide probes tiling *BRCA1P1* (bottom). Fluorescent signals from *BRCA1P1*-lncRNA (red) were observed in nuclei, whereas *GAPDH* mRNA signals (control) were found in cytoplasm. **B**, RNA immunoprecipitation (RNA-IP) was conducted in MDA-MB-231 cells using antibodies against transcription factors (RelA, IRF1 and STAT1). Enrichment of *BRCA1P1* lncRNA is shown relative to input RNA (% input). 18S rRNA, *JPX* lncRNA and *BRCA1* mRNA were used as negative controls. Data represent mean and SD of n=3 to n=4 biological replicates, and are representative of at least two independent experiments (**p<0.01; ****p<0.0001 vs negative controls). **C** and **D**, Chromatin immunoprecipitation (ChIP) was performed in *BRCA1P1*-KO (**C**) or *BRCA1P1*-overexpression cells (**D**) using antibodies against RelA. Enrichment of RelA at the promoters of *TNF*, *IFNL1* and *IFIH1* is shown relative to input DNA (% input). Error bars represent SD of three biological replicates (*p<0.05; **p<0.01; ***p<0.001). **E**, Electrophoretic mobility shift assays (EMSA) were performed using biotin-labeled oligonucleotides containing the cis-acting regulatory NF- κ B element of the *TNF* promoter. Recombinant RelA proteins (200ng), *BRCA1P1*-lncRNA (1, 5 or 10 fmols) or anti-RelA antibodies (200 ng) were incubated with oligonucleotides (2 fmols) and subjected to 6% polyacrylamide gel electrophoresis (PAGE). The arrow indicates the mobility of free oligonucleotides (probe), oligonucleotides associated with RelA (shift) or oligonucleotides

associated both with RelA and anti-RelA antibodies (super-shift). **F**, Depicted are the truncated mutants of *BRCA1P1* with exon 1a to exon 1b (1–1107nt), exon 1a only (1–517nt), intron 1a to exon 1b (518–1107nt) or exon 1b only (676–1107nt). **G**, RNA pull-down assays were conducted using the truncated mutants of *BRCA1P1*-lncRNA, which were transcribed *in vitro*, labeled with biotin at their 3'ends, bound to streptavidin beads, and incubated with recombinant RelA (p65) proteins (left). Western blot analysis followed by RNA pull-down assays showed RelA proteins associated with the truncated mutants of *BRCA1P1* (right). **H**, qRT-PCR analysis of *TNF* and *IFNL1* mRNA in *BRCA1P1*-KO and WT cells that were transfected with RelA siRNA for 24 hours. Results were normalized to *RNA18S* and fold induction is shown relative to control siRNA treated cells. Data represent mean and SD of n=6 biological replicates, and are representative of at least two independent experiments (**p<0.01).

Fig. 5. Increased Apoptosis and Drug Sensitivity of *BRCA1P1*-Depleted Cells.

A, Apoptosis and proliferation of *BRCA1P1*-WT and KO cells were analyzed using the IncuCyte Live-Cell Imaging System. Apoptosis was quantified using green fluorescent signals from Caspase-3/7 positive apoptotic cells normalized to cell density (left), whereas cell density values were derived from phase-contrast images of cells to evaluate cell proliferation (right). Data represent mean and SD of $n = 4$ to 10 biological replicates, and are representative of at least two independent experiments. **B**, MDA-MB-231 cells were treated with control-ASO, *BRCA1P1*-ASO, or *BRCA1P1*-ASO followed by CRISPR activation of *BRCA1P1* (*BRCA1P1*-Over). Apoptosis and proliferation of the cells were analyzed using the IncuCyte system. **C**, HMEC cells were treated with control-ASO or *BRCA1P1*-ASO, and subjected to IncuCyte imaging. **D** and **E**, *BRCA1P1*-WT and KO cells were treated with poly(I:C) or TNF- α (25–30 ng/ml) for indicated times. Caspase-3/7 positive apoptotic cells were assessed using the IncuCyte Caspase-3/7 Green Assay. **F**, Expression of *TNF* or *IFNL1* mRNA was analyzed using qRT-PCR in *BRCA1P1*-KO and WT cells treated with TNF- α for 40 hours. All results were normalized to *RNA18S* and fold induction is shown relative to WT control cells (untreated). Data represent the mean and SD of $n = 3$ biological replicates, and are representative of at least two independent experiments (** $p < 0.001$). **G**, Reactive oxygen species (ROS) were measured in *BRCA1P1*-KO and WT cells using 2',7'-dichlorofluorescein diacetate (DCFDA). The Y axis represents the geometric mean of DCFDA fluorescence intensity. Data represent the mean and SD of $n = 4$ biological replicates, and are representative of at least two independent experiments (** $p < 0.01$). **H**, Western blot analysis of H2AX phosphorylation (γ -H2AX) in *BRCA1P1*-WT and KO cells. Histone3 (H3) was used as a loading control for nuclear proteins. **I**, Apoptosis was measured

in response to doxorubicin (Doxo) and camptothecin (Camp) in *BRCA1PI*-KO clones, using caspase 3/7 activity assays. Data represent the means of n=4 biological replicates, and are representative of at least two independent experiments (***) p -value <0.001 vs. wild type cells or control-ASO treated).

Fig. 6. Tumor Volume and Cytokine Expression in a *BRCA1/1*-KO Xenograft Mouse Model.

A, Tumor measurements were performed weekly starting at day 15 after cell injection. Each line represents the average weekly tumor volume in an animal during the study. **B**, Expression of pro-inflammatory cytokines (*TNF*, *IL2*, *IFNG* and *IL12A* mRNAs) were measured by qRT-PCR in spleen tissues collected at the end of the study. The data showed that the transcript amounts of the proinflammatory cytokines in the spleen was significantly higher in mice with *BRCA1/1*-KO tumors compared to control mice with WT tumors ($p < 0.05$). Fold change is shown relative to control mice with WT tumors. Two independent qRT-PCR experiments were performed and each dot represents the mean of $n=4$ biological replicates.