J Pathol. Author manuscript; available in PMC 2016 May 26.

Published in final edited form as:
J Pathol. 2014 November ; 234(3): 386–397. doi:10.1002/path.4404.

Up-regulation of the interferon-related genes in *BRCA2* knockout epithelial cells

Hong Xu[1,*], **Jian Xian**[2,*], **Emmanuelle Vire**[3,*], **Steven McKinney**[1], **Jason Wong**[1], **Vivien Wei**[4], **Rebecca Tong**[4], **Tony Kouzarides**[3], **Carlos Caldas**[2], and **Samuel Aparicio**[1,5]

[1]Department of Molecular Oncology, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, BC, V5Z 1L3, Canada

[2]Department of Oncology, University of Cambridge and Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Cambridge, CB2 0RE, UK

[3]Wellcome Trust/Cancer Research UK Gurdon Institute, The Henry Wellcome Building of Cancer and Developmental Biology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK

[4]Department of Microbiology and Immunology, University of British Columbia, 1365 - 2350 Health Sciences Mall, Vancouver, British Columbia, Canada, V6T 1Z3

[5]Department of Pathology and Laboratory Medicine, University of British Columbia, Wesbrook Mall, Vancouver, BC, V6T 2B5 Vancouver, BC

Abstract

BRCA2 mutations are significantly associated with early onset breast cancer, and the tumour suppressing function of BRCA2 has been attributed to its involvement in homologous recombination [1]-mediated DNA repair. In order to identify additional functions of BRCA2, we generated *BRCA2*-knockout HCT116 human colorectal carcinoma cells. Using genome-wide microarray analyses, we have discovered a link between the loss of BRCA2 and the up-regulation of a subset of interferon (IFN)-related genes, including *APOBEC3F and APOBEC3G*. The over-expression of IFN-related genes was confirmed in different human *BRCA2*$^{-/-}$ and mouse *Brca2*$^{-/-}$ tumour cell lines, and was independent of either senescence or apoptosis. In isogenic wild type *BRCA2* cells, we observed over-expression of IFN-related genes after treatment with DNA-damaging agents, and following ionizing radiation. Cells with endogenous DNA damage because of defective BRCA1 or RAD51 also exhibited over-expression of IFN-related genes. Transcriptional activity of the IFN-stimulated response element (ISRE) was increased in *BRCA2*

Correspondence: Dr. Samuel Aparicio, Department of Molecular Oncology, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, BC, V5Z 1L3, Canada, Phone: +1 604 675 8200, Fax: 604 675 8218, or Dr. Carlos Caldas, Cambridge Research Institute, Li Ka Shing Centre, Cambridge, CB2 0RE, UK, Phone: 01223 769 650, saparicio@bccrc.ca; Carlos.Caldas@cruk.cam.ac.uk.
*denotes equal contribution

No conflicts of interest were declared.

The GEO number for microarray analysis is GSE54830.

Author contributions

HX designed the study, carried out most experiments and wrote the manuscript. JX made the BRCA2 knockout cell lines in HCT116, performed DNA damage repair experiments on BRCA2 knockout cells and wrote part of the manuscript. EV performed the CHIP experiment and was involved in manuscript writing. SJ analyzed the data. JW carried out microarray analysis. RT and VW carried out experiments. TK, CC and SA were involved in study design.

knockout cells, and the expression of BRCA2 greatly decreased IFN-α stimulated ISRE reporter activity, suggesting that BRCA2 directly represses the expression of IFN-related genes through the ISRE. Finally, the colony forming capacity of *BRCA2* knockout cells was significantly reduced in the presence of either IFN-β or IFN-γ, suggesting that IFNs may have potential as therapeutic agents in cancer cells with *BRCA2* mutations.

Keywords

BRCA2; interferon; DNA damage

Introduction

Inherited *BRCA2* mutations predispose carriers to early onset breast, ovarian, and other cancers [2,3]. The primary role of BRCA2 is in HR-mediated DNA damage repair [4]. In *BRCA2* mutant cells, the formation of DNA damage foci by RAD51 filaments is reduced and HR repair efficiency is greatly compromised, leading to an increased error-prone DNA repair and ultimately, genomic instability [5,6]. In addition, a number of evidence supports a role for BRCA2 in transcriptional regulation. BRCA2 forms a complex with Smad3 and synergizes in regulating the transcription of Smad3-dependent luciferase reporters [7]. In *Arabidopsis*, BRCA2 is a major regulator of immune gene transcription [8]. In human cells, the expression of two innate immunity genes (*UCRP and UBE2L6*) were down-regulated after BRCA2 knock down [9]. Furthermore, BRCA2's interacting partner EMSY binds to the promoter of IFN-related genes and represses transcription in a BRCA2-dependent manner [10], supporting a link between the regulation of IFN-related genes and BRCA2/EMSY complex.

IFNs are produced not only by the immune system cells, but virtually by all human cells infected with pathogens[11,12]. There are three major functions for IFNs: innate immune response, activation of adaptive immune response, and anti-tumour activity [11]. IFNs directly induce apoptosis in many cancer cell lines, and boost the body's immune system to fight cancers. Indeed, a number of reports have demonstrated IFNs are effective in combination anti-cancer therapies for pancreatic cancers, lymphomas, melanomas, etc [13–18].

Although IFN responses have been studied for many years, new discoveries continue to be made and the complexity of the IFN-related network is increasing. Recently, a link between DNA damage and the IFN response was discovered. Following treatment with the topisomerase inhibitor etoposide, NF-κB was activated resulting in the induction of the IFN response [19]. STAT1, an upstream mediator of IFN signalling, plays an important role in DNA damage repair [20]. In addition, BRCA1, a major regulator of HR-based DNA repair, is required for the enhanced expression of type I IFN-related genes after IFN-γ stimulation [21–25]. Furthermore, an IFN-related gene signature was associated with resistance to chemotherapy and radiation therapy in breast cancer [26]. In this study, we reveal a link between BRCA2, another major contributor to HR, and IFN-related genes.

We identified an enrichment of up-regulated IFN-related genes in *BRCA2*-knockout human and mouse tumor cell lines. The expression of IFN-related genes is induced by exogenous DNA damage and also endogenous DNA damage. *BRCA2*$^{-/-}$ cells showed higher expression of promoter activity on ISRE and the expression of BRCA2 decreased IFN-α stimulated ISRE reporter activity. Our experimental results suggest that there are two pathways regulating IFN-related genes in *BRCA2*$^{-/-}$ cells, one is the endogenous DNA damage in *BRCA2*$^{-/-}$ cells, and the other is the direct transcriptional repression by BRCA2. Finally, IFN-β and IFN-γ reduced the colony forming capacity of *BRCA2* knockout cells, suggesting that a therapeutic window may be found to selectively kill cancers with BRCA2 deficiency.

Materials and Methods

Cell lines

HCT116 *BRCA2*$^{+/+}$ cells were from ATCC (CCL-247), and the *BRCA2*$^{-/-}$ cells were created in this study. Mouse mammary tumour *BRCA2* knockout cells (K14-Cre;Brca2$^{F11/F11;}$ p53$^{F2\text{-}10/F2\text{-}10}$) and control mouse mammary tumour *BRCA2* proficient cells (K14-Cre;Brca2 $^{wt/wt;}$ p53$^{F2\text{-}10/F2\text{-}10}$) were from Dr. Jos Jonkers' lab and were cultured as described [27]. HCC1937 and HCC1937/WT-BRCA1 were from Junjie Chen's lab [28] and are cultured in RPMI 1640 with 10% FBS. Two PEO1 cells maintained by different people (PEO1-CH and PEO1-SL) are from Dr. James Brenton's lab [29]. C4-2 cells are from Toshiyasu Taniguchi's lab [30]. PEO1 and C4-2 cells are cultured in RPMI 1640 with 10% FBS.

Targeted disruption of the human BRCA2 locus in HCT116

The gene targeting construct was generated by using a recombinant adeno-associated virus (rAAV) system and has been described by others [31]. More details can be found in supplementary methods and supplementary Figure S1.

RAD51 knockdown by shRNA interference

pGIPZ shRNAs for Rad51(RHS4430-98818235, RHS4430-99151947 and RHS4430-99157804) were bought from Open Biosystem. HCT116 cells were infect with lentivirus particle packaged with pooled shRNA for RAD51 or scramble control in MOI=5. 48hrs after infection, cells were split onto 10 cm dishes and fed with fresh medium supplemented with 0.5ug/ml puromycin for three days.

Microarray expression analysis

Total RNA was extracted using QIAzol™ lysis reagent (Qiagen, Maryland, USA), then hybridized to Affymetrix HuEx 1.0 exon chips. The microarray data were analyzed using the oneChannelGUI package of the R statistical programming language (R version 2.11.1, R Development Core Team, 2010). Raw intensity calls were normalized using quantile normalization [32] and probeset summarization (core plus extended) undertaken with RMA [33].

Drug treatment, antibodies and X-irradiation

Aphidicolin, Phleomycin and Camptothecin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and the Parp1 inhibitor from Kudos (Ku 0059436), Cambridge, UK. Paclitaxel was from Sigma. Irradiation was performed with a 250 kV *X*-ray unit at a dose rate of 4.7 Gy/minute.

Primary antibodies against ACTIN and γ-H2AX (phosphor S139) were purchased from Abcam (Cambridge, MA, USA). BRCA2 antibody was from Calbiochem (Ab-1). RAD51 antibody was from Abcam (ab213).

Apoptosis and senescence assay

Apoptosis was assayed via Annexin V-FITC and propidium iodide (PI) staining according to the manufacturer's protocol (Invitrogen Carlsbad, CA, USA). Senescence was measured by staining for senescence-associated β-Galactosidase (SA-β-gal) using a kit from Chemicon (Billerica, MA, USA).

Quantitative real-time reverse transcription PCR

Total RNA was isolated by miRNAeasy kit (Qiagen, Maryland, USA), and was treated by on-column DNase digestion. The RNA was quantified by Nanodrop and quality was then assessed with Agilent's 2100 Bioanalyzer. First strand cDNA was synthesized by using M-MLV (Invitrogen). Quantitative PCR was performed on an ABI 7900HT system using primers and probes listed in Supplementary Table 2. Relative cDNA amounts were estimated with the ΔΔCt method normalizing to three reference genes: 18S rRNA, ACTIN B, and LAMIN A (human) or *Actin B, Pgk1*, and *Hprt* (mouse).

Clonogenic assay

Cells were incubated in 6 cm plates. The medium with IFN-β (EMD calbiochem, Darmstadt, Germany) or IFN-γ (EMD calbiochem, Darmstadt, Germany) was changed every 3 – 4 days with fresh IFN added. After 10 days, colonies were fixed and stained with 4 mg/ml methylene blue dissolved in methanol.

Luciferase reporter assay

Firefly luciferase reporter construct with tandem ISRE promoter elements and constitutively expressing Renilla luciferase construct are from Qiagen. Luciferase reporter activity was measured using dual-luciferase reporter assay system from Promega.

Chromatin Immunoprecipitation (ChIP) Analysis

Chromatin was prepared from HCT116 cells and immunoprecipitations were performed as described previously [34]. Primer sequences for ISG15 promoter are: TCCCTGTCTTTCGGTCATTC and TTGGCTTCAGTTTCGGTTTC

Statistical analysis

Details of statistical analysis are in supplementary methods.

Results

Generation of a BRCA2 knockout human cell line

To identify the molecular mechanisms underlying the observed link between BRCA2 and DNA repair and discover additional functions of BRCA2, we generated *BRCA2* knockout HCT116 cells (Figure S1). These homozygous knockout cells did not produce BRCA2 protein (Figure S1e). Two homozygous *BRCA2* knockout cell clones (B18 and B46), as well as the parental HCT116 cells, were used for further analysis. The homozygous *BRCA2* knockout cells exhibited phenotypes consistent with those in previously published reports [35,36], including loss of Rad51 foci in the presence of double strand breaks (DSB) (Figure S2a), chromosomal rearrangements (Figure S2b), and elevated sensitivity to the DNA damaging agents Phleomycin and Parp1 inhibitors (Figure S2c, 2d).

In the absence of exogenous DNA damage, $BRCA2^{-/-}$ cells accumulate endogenous DNA damage, manifested by significantly increased numbers of γ-H2AX and 53BP1 foci observed by immunofluorescence (Figure 1a–d). Western blot analyses revealed expression of γ-H2AX in $BRCA2^{-/-}$ cells even in the absence of irradiation, whereas $BRCA2^{+/+}$ cells only expressed γ-H2AX following irradiation (Figure 1e). These results indicate that DNA damage occurs in *BRCA2* knockout HCT116 cells even in the absence of exogenous genotoxic stress.

Expression of IFN-related genes is up-regulated in *BRCA2* knockout HCT116 cells

We performed genome-wide microarray analyses to identify differentially expressed genes in wild type and *BRCA2*-deficient HCT116 cells. Genes that showed more than a two-fold change (in $BRCA2^{-/-}$ cells relative to wild type control cells) are listed in Supplementary Table 1. *BRCA2* was identified in the list of genes down-regulated in knockout cells, demonstrating that this assay is able to successfully identify differentially regulated genes. Several genes in the IFN response pathway -- including *APOBEC3G, APOBEC3F, IFI44* and *APOBEC3D* -- were up-regulated in *BRCA2*-deficient cells.

In order to identify functional enrichments in BRCA2-deficient cells, we entered the top 200 differentially expressed gene loci into GeneMANIA (a web-based version of algorithms used in the Cytoscape network visualization tool [37,38]). No pathway was enriched at a statistically significant level when only canonical nodes were considered. However a powerful feature of network visualization is the ability to extend the list of query genes by adding functionally similar genes. After adding 100 associated genes to the top 200 differentially expressed genes, functional networks were identified with type I IFN-related pathways at the top of the enrichment list with very low false discovery rate values (Table 1), followed by cytokine signalling and angiogenesis pathways.

To confirm the link between BRCA2 and type I IFN-related genes discovered from GeneMANIA, we performed quantitative RT-PCR analysis on genes in the type I IFN response pathway. Figure 2 shows that a number of IFN-related genes were expressed at higher levels in two independently derived HCT116 $BRCA2^{-/-}$ clones (#18 and #46), compared to isogenic $BRCA2^{+/+}$ cells. The most highly over-expressed genes included *APOBEC3G, OAS1, IFIT2, IRF3, ISG15*, *IFI44* and *IRF1* (all adjusted $p < 0.05$ for both

mutant clones) plus *APOBEC3F* and *MX1* (all adjusted $p < 0.05$ for clone #46). We also measured the mRNA levels of three types of IFNs. For type I IFN, there was no difference in the mRNA levels of IFN-α, measured by pan-specific primers for different IFN-α subtypes between *BRCA2* proficient and deficient cells. For IFN-β, both BRCA2 knockout clones showed decreased expression relative to *BRCA2* proficient cells. The transcription of the type II IFN, IFN-γ, was not detectable in both HCT116 wild type cells and *BRCA2* knockout cells (data not shown). For type III IFN, IFN-λ1 expression was significantly higher in two *BRCA2* knockout clones (clone46 and clone18) than wild type cells.

The observed over-expression of IFN-related genes in $BRCA2^{-/-}$ cells could potentially be explained by increased apoptosis. The percentage of apoptotic cells was slightly higher in the $BRCA2^{-/-}$ (mean 7.3%, n = 3 replicates) than the $BRCA2^{+/+}$ cells by flow cytometric analysis with Annexin V and PI staining (mean 2.6%, n = 4 replicates, Dunnett's test $p = 0.03$; Figure S3a, Figure S5b). To rule out an apoptotic/dead cell influence on our findings, we isolated the Annexin V-negative fraction (i.e. the non-apoptotic, live cells) by fluorescence activated cell sorting (Figure S3b), and performed quantitative RT-PCR on the Annexin V-negative $BRCA2^{+/+}$ and $BRCA2^{-/-}$ HCT116 cells. Some IFN response genes were still expressed at higher levels in the non-apoptotic $BRCA2^{-/-}$ cells than in wild type cells (*APOBEC3G, IFIT2, IRF1, MX1, PKR,* all adjusted $p < 0.05$, Figure S3c). Taken together, this suggests that the over-expression of IFN-related genes observed in $BRCA2^{-/-}$ cells is not restricted to apoptotic cells.

Some reports attribute the expression of IFN-related genes to cellular senescence [39,40]. To investigate this possibility, we measured the activity of β-galactosidase, a senescence marker, in $BRCA2^{-/-}$ and $BRCA2^{+/+}$ cells. Although the growth rate of $BRCA2^{-/-}$ cells was slower than that of $BRCA2^{+/+}$ controls, the percentage of senescent $BRCA2^{-/-}$ cells was similar to that of wild type cells (Figure S3d, Figure S6), indicating that the enhanced expression of IFN-related genes in $BRCA2^{-/-}$ cells is not a result of senescence. Collectively, these findings suggest that neither apoptosis nor senescence precedes the induction of IFN-related genes.

Up-regulation of IFN-related genes in BRCA2 deficient ovarian cancer cells and mouse tumor cells

To further understand the role of *BRCA2* and its relationship with IFN-related genes, the expression of IFN-related genes was evaluated in a *BRCA2* mutant ovarian cancer cell line, PEO1. Because of a point mutation in *BRCA2*, PEO1 cells are sensitive to cisplatin. The function of *BRCA2* was restored by a secondary mutation in *BRCA2* and the cell (C4-2) acquired cisplatin resistance [30]. The mRNA level of IFN-related genes was compared between two PEO1 cells (PEO1-CH and PEO1-SL) and C4-2 cells. Consistent with the result in HCT116, up-regulation of a number of IFN-related genes was observed in PEO1 cells relative to C4-2 cells (Figure 3a).

Furthermore, quantitative RT-PCR was used to measure IFN-related gene expression levels in mouse mammary tumor *Brca2* knockout and isogenic/wild type cells [27]. Since *OAS1* and *APOBEC3G* have the highest levels of expression among IFN-related genes in human HCT116 $BRCA2^{-/-}$ cells (Figure 3b), we focused our analysis in murine cells on these two

gene families. There are eight *OAS1* orthologs in the mouse genome, of which five were significantly up-regulated in *Brca2*$^{-/-}$ mouse cells (Figure 3b: *Oas1 B, C, D, E, F* all adjusted $p < 0.05$, estimated fold changes ranging from 1.9–8.5). The mouse genome does not contain orthologs of *APOBEC3G* or *APOBEC3F*, but does contain four genes in the cytidine deaminase family: *Apobec1, Apobec2, Apobec3* and *Aicda*. We analyzed the expression levels of *Apobec1* (the dominant deaminase in mouse [41]) and *Aicda* by RT-PCR. Transcript levels of *Apobec1* and two isoforms of *Aicda* were increased in *Brca2*$^{-/-}$ cells compared to wild type controls (Figure 3b: all adjusted $p < 0.05$). These results demonstrate that the over-expression of certain IFN-related genes in *BRCA*-deficient cells is a common feature of both human cells and mouse tumor cells.

IFN-related genes were induced by endogenous and exogenous DNA damage

The up-regulation of IFN-related genes following DNA damage has been reported in several human cancer cell lines [19,40]. *BRCA2* knockout cells accumulate endogenous DNA damage (Figure 1), and this may explain the increased expression of IFN-related genes. However, for HCT116 cells, the cells we used in this study, the relationship between the IFN response and DNA damage has not been analyzed yet. Therefore, we tested whether the transcription of IFN-related genes could be induced following DNA damage in wild type HCT116 cells.

DNA damage was induced with either ionizing radiation or drugs causing ssDNA and dsDNA breaks (Phleomycin, Camptothecin, or Aphidicolin). IFN-related genes were differentially up-regulated by the different DNA damaging agents (Figure S4). The set of IFN-related genes induced by DNA damage in wild type HCT116 cells was similar with that constitutively over-expressed in *BRCA2*$^{-/-}$ cells. In addition, the expression of IFN-related genes following DNA damage preceded apoptosis and senescence (Figure S5, S6). Furthermore, the up-regulation of IFN-related genes is not a general drug effect because a taxane (paclitaxel), a chemotherapy drug without direct DNA damaging activity, did not stimulate the expression of IFN-related genes (Figure S7).

As IFN-related genes are induced by DNA damaging agents, it is reasonable to hypothesize that they may also be induced in cells with endogenous DNA damage. To examine this hypothesis, we measured the expression of IFN-related genes in cells with defects in two important DNA damage repair genes, BRCA1 and RAD51. HCC1937 is a breast tumor cell line expressing only truncated BRCA1 and is hypersensitive to DNA damaging agents [28]. RT-PCR results demonstrated that IFN-related genes were over-expressed in HCC1937 cells comparing with HCC1937/WT-BRCA1 cells (Figure 4a). In addition, we knocked down RAD51, an important HR pathway component, in HCT116 cells. Comparing with scrambled control, IFN-related genes were up-regulated in RAD51 knockdown cells (Figure 4b). Taken together, the data suggest that increased expression of IFN-related genes can be caused by endogenous DNA damage.

BRCA2 modulates the expression of IFN-related genes through ISRE

Most IFN regulated genes have one or more ISRE elements (GAAANNGAAAG/CTC) in their promoters that function as enhancers for transcriptional activation by IFN-α and IFN-β

[42]. In order to evaluate whether the repression of the IFN response by BRCA2 was mediated through ISRE, we analyzed the activity of a luciferase reporter containing tandem ISRE in the promoter of a firefly luciferase gene. Relative luciferase activity was calculated by normalizing firefly luciferase activity to renilla luciferase activity (serving as a transfection control). The relative luciferase activity level was significantly higher in two *BRCA2* knockout clones than in wild type cells (Figure 5a), indicating that the BRCA2 is able to regulate the expression of IFN-related genes through promoter ISRE.

To further investigate the role of BRCA2 in transcriptional regulation of IFN-related genes, we measured the stimulation of ISRE reporter activity by IFN-α after expressing full-length BRCA2. The relative luciferase activity of ISRE was significantly increased following IFN-α treatment (p=0.00022) (Figure 5b). With the transfection of pRK5-BRCA2 construct, IFN-α stimulation on ISRE reporter activity was eliminated (p=0.18), suggesting that BRCA2 represses the transcription of IFN-related genes through the ISRE element. On the contrary, when RAD51construct was transfected into the cell, the IFN-α induced activation of ISRE activity was not affected (p=9e-05), consistent with the notion that the modulation of ISRE activity by BRCA2 is specific. The expression of BRCA2 in pRK5-BRCA2 transfected cells and RAD51 in RAD51-V5 transfected cells were confirmed by western blot (Figure S8).

A recent report shows that EMSY, which interacts with BRCA2, directly binds to the promoter of IFN-related genes and represses their expression [10]. In addition, the repressive effect of EMSY on IFN-related genes is dependent on BRCA2 [10]. These results suggest the importance of EMSY in the regulation of IFN-related genes. In order to further understand the relationship between BRCA2 and EMSY, we tested whether BRCA2 affected the chromatin-binding capacity of EMSY. We performed a CHIP assay for EMSY and analyzed its association with the promoter of one IFN-related gene, *ISG15* [10]. Consistent with Ezell et al., we observed an association between EMSY and the ISG15 promoter in wild type cells. Comparable results were observed in *BRCA2* knockout cells, wherein EMSY also bound to the ISG15 promoter (Figure 5c, p=0.96). Thus, BRCA2 doesn't affect the binding capacity of EMSY to the ISG15 promoter.

Exposure to IFNs inhibits *BRCA2*$^{-/-}$ cell growth

As IFNs repress cell growth and promote apoptosis, the over-expression of IFN-related genes in *BRCA2*$^{-/-}$ cells may affect cell viability. Indeed, we observed slower growth phenotype on *BRCA2*$^{-/-}$ cells comparing with *BRCA2*$^{+/+}$ cells. We assessed whether IFN treatment could further decrease the cell viability of *BRCA2*$^{-/-}$ cells and induce selective cell death. If so, there would be a possible therapeutic application of IFN treatment on BRCA2 deficient tumours. There are many type I IFNs, which bind to common receptors, and induce similar biological reactions [43]. As IFN signalling is known to involve crosstalk [44], we have tested the response of *BRCA2*$^{-/-}$ cells to both type I (IFN-β) and type II (IFN-γ) IFNs. Clonogenic assays were performed with wild type and *BRCA2*$^{-/-}$ HCT116 cells subjected to IFN-β or IFN-γ treatment. In the presence of IFN-β or IFN-γ, *BRCA2*$^{-/-}$ cells exhibited significantly reduced survival relative to wild type cells (p = 0.038 for Figure 6a, and p= 6.14e–10 for Figure 6b). The number of colonies formed and the size of the colonies

were both greatly reduced in the *BRCA2*$^{-/-}$ cells (Figure 6c and 6d). These results indicate that the growth of *BRCA2* knockout cells is repressed by both type I and type II IFNs.

Discussion

We have evaluated the role of *BRCA2* in transcriptional regulation at a genome-wide level by performing microarray analyses on *BRCA2* knockout and isogenic wild type cells. IFN-related genes are up-regulated in *BRCA2* knockout HCT116 cells and are in the most enriched functional category among all the over-expressed transcripts. These results were confirmed in both human and mouse cell lines, indicating the link between loss of *BRCA2* and the over-expression of IFN-related genes is evolutionarily conserved. In addition, we found that deficiency in other components of HR pathway, such as BRCA1 and RAD51, also results in the induction of IFN-related genes, suggesting that up-regulation of IFN-related genes is associated with endogenous DNA damage.

Over-expression of genes in the IFN signalling pathway has been observed in many types of tumours. Ovarian cancer with a *BRCA1* or *BRCA2* mutation exhibited high expression of IFN-related genes [45]. In breast cancer, an immune response gene expressing subgroup has been identified, and is associated with improved prognosis in triple negative breast cancers [46,47]. Recently, activated immune response has been associated with the loss of Fanconi anemia/*BRCA (FA/BRCA*) pathway in breast cancer patients, and has been validated as a biomarker of increased sensitivity to DNA damaging chemotherapy [48]. Our cell line model and knockdown experimental results are consistent with these clinical data, supporting that aberrations in *BRCA2* or other components in *BRCA* DNA damage repair pathway result in the direct activation of immune response.

By using a luciferase reporter assay, we found increased transcriptional responses (mediated by ISRE) in *BRCA2*$^{-/-}$ cells suggesting that, similar to transcription factors in the JAK/STAT pathway, BRCA2 also regulates IFN-related genes through the ISRE. The expression of BRCA2 reduced IFN-α stimulated ISRE reporter activity, further supporting a role for the BRCA2 complex in the direct regulation of IFN-related genes. Our CHIP assay showed that EMSY still binds to the promoter of IFN-stimulated genes in the absence of BRCA2, suggesting that the regulation of IFN-related genes by BRCA2 complex is independent of EMSY's chromatin binding capacity. CHIP result in *Arabidopsis* demonstrated that another BRCA2 interaction protein, RAD51, was recruited to the promoters of defence genes during plant immune response [8]. However, in our study, when RAD51 was over-expressed, it did not modulate IFN-α induced ISRE activity (in contrast with BRCA2 expression) (Figure 5b). The detailed mechanism of how BRCA2 and its interacting partners, EMSY and RAD51, regulate transcription of IFN-stimulated genes needs further investigation. But taken together, our experimental results suggest that IFN-related genes may be up-regulated in *BRCA2*$^{-/-}$ cells through two pathways – direct regulation by BRCA2 and indirectly by endogenous DNA damage in *BRCA2*$^{-/-}$ cells.

A number of IFN response genes have been linked to growth inhibition, senescence and apoptosis [49–52]. Our results suggest that under normal conditions, IFN-related genes are mostly repressed by the BRCA2 complex, and this repression is released in *BRCA2*

knockout cells. In the presence of IFN, the induction of IFN-related genes further increases in *BRCA2* knockout cells, and results in higher sensitivity of *BRCA2* knockout cells (than wild type cells) to both IFN-β and IFN-γ. Taken together, our results indicate that IFNs may have therapeutic potential in reducing the growth of cancer cells with *BRCA2* mutations. A better understanding of the effect of IFNs on *BRCA2* mutant cells under different genetic backgrounds and microenvironments would be necessary for this purpose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We greatly appreciate Dr. Jos Jonkers' lab for generously distributing mouse mammary tumour *BRCA2* knockout cells (K14-Cre; Brca2F11$^{/F11}$; p53$^{F2\text{-}10/F2\text{-}10}$) and control mouse mammary tumour *BRCA2* proficient cells (K14-Cre; Brca2 $^{wt/wt}$; p53$^{F2\text{-}10/F2\text{-}10}$), Junjie Chen's lab for distributing HCC1937 and HCC1937/WT-BRCA1 cells, Dr. James Brenton's lab for sharing PEO1 cells and Toshiyasu Taniguchi's lab for sharing C4-2 cells. We thank Sarah Mullaly, Damian Yap and members of the Aparicio and Caldas labs for critical reading of the manuscript.

References

1. Zhu J, Huang X, Yang Y. Innate immune response to adenoviral vectors is mediated by both Toll-like receptor-dependent and -independent pathways. Journal of virology. 2007; 81:3170–3180. [PubMed: 17229689]
2. Wooster R, Neuhausen SL, Mangion J, et al. Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12–13. Science. 1994; 265:2088–2090. [PubMed: 8091231]
3. Foster KA, Harrington P, Kerr J, et al. Somatic and germline mutations of the BRCA2 gene in sporadic ovarian cancer. Cancer Res. 1996; 56:3622–3625. [PubMed: 8705994]
4. Powell SN, Willers H, Xia F. BRCA2 keeps Rad51 in line. High-fidelity homologous recombination prevents breast and ovarian cancer? Mol Cell. 2002; 10:1262–1263. [PubMed: 12504001]
5. Thompson LH, Schild D. Homologous recombinational repair of DNA ensures mammalian chromosome stability. Mutat Res. 2001; 477:131–153. [PubMed: 11376695]
6. Yuan SS, Lee SY, Chen G, et al. BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. Cancer research. 1999; 59:3547–3551. [PubMed: 10446958]
7. Preobrazhenska O, Yakymovych M, Kanamoto T, et al. BRCA2 and Smad3 synergize in regulation of gene transcription. Oncogene. 2002; 21:5660–5664. [PubMed: 12165866]
8. Wang S, Durrant WE, Song J, et al. Arabidopsis BRCA2 and RAD51 proteins are specifically involved in defense gene transcription during plant immune responses. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:22716–22721. [PubMed: 21149701]
9. Tripathi MK, Chaudhuri G. Down-regulation of UCRP and UBE2L6 in BRCA2 knocked-down human breast cells. Biochemical and biophysical research communications. 2005; 328:43–48. [PubMed: 15670748]
10. Ezell SA, Polytarchou C, Hatziapostolou M, et al. The protein kinase Akt1 regulates the interferon response through phosphorylation of the transcriptional repressor EMSY. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:E613–621. [PubMed: 22315412]
11. Kim KS, Kang KW, Seu YB, et al. Interferon-gamma induces cellular senescence through p53-dependent DNA damage signaling in human endothelial cells. Mech Ageing Dev. 2009; 130:179–188. [PubMed: 19071156]
12. Le Page C, Genin P, Baines MG, et al. Interferon activation and innate immunity. Reviews in immunogenetics. 2000; 2:374–386. [PubMed: 11256746]

13. Lee J, Wang A, Hu Q, et al. Adenovirus-mediated interferon-beta gene transfer inhibits angiogenesis in and progression of orthotopic tumors of human prostate cancer cells in nude mice. Int J Oncol. 2006; 29:1405–1412. [PubMed: 17088978]
14. Hu G, Barnes BJ. Interferon regulatory factor-5-regulated pathways as a target for colorectal cancer therapeutics. Expert Rev Anticancer Ther. 2006; 6:775–784. [PubMed: 16759167]
15. Bouker KB, Skaar TC, Riggins RB, et al. Interferon regulatory factor-1 (IRF-1) exhibits tumor suppressor activities in breast cancer associated with caspase activation and induction of apoptosis. Carcinogenesis. 2005; 26:1527–1535. [PubMed: 15878912]
16. Jost E, Roos WP, Kaina B, et al. Response of pancreatic cancer cells treated with interferon-alpha or beta and co-exposed to ionising radiation. Int J Radiat Biol. 2010; 86:732–741. [PubMed: 20586542]
17. Salles G, Mounier N, de Guibert S, et al. Rituximab combined with chemotherapy and interferon in follicular lymphoma patients: results of the GELA-GOELAMS FL2000 study. Blood. 2008; 112:4824–4831. [PubMed: 18799723]
18. Payne MJ, Argyropoulou K, Lorigan P, et al. Phase II pilot study of intravenous high-dose interferon with or without maintenance treatment in melanoma at high risk of recurrence. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014; 32:185–190. [PubMed: 24344211]
19. Brzostek-Racine S, Gordon C, Van Scoy S, et al. The DNA damage response induces IFN. J Immunol. 2011; 187:5336–5345. [PubMed: 22013119]
20. Townsend PA, Cragg MS, Davidson SM, et al. STAT-1 facilitates the ATM activated checkpoint pathway following DNA damage. J Cell Sci. 2005; 118:1629–1639. [PubMed: 15784679]
21. Buckley NE, Hosey AM, Gorski JJ, et al. BRCA1 regulates IFN-gamma signaling through a mechanism involving the type I IFNs. Mol Cancer Res. 2007; 5:261–270. [PubMed: 17374731]
22. DelloRusso C, Welcsh PL, Wang W, et al. Functional characterization of a novel BRCA1-null ovarian cancer cell line in response to ionizing radiation. Mol Cancer Res. 2007; 5:35–45. [PubMed: 17259345]
23. Mullan PB, Hosey AM, Buckley NE, et al. The 2,5 oligoadenylate synthetase/RNaseL pathway is a novel effector of BRCA1- and interferon-gamma-mediated apoptosis. Oncogene. 2005; 24:5492–5501. [PubMed: 15940267]
24. Andrews HN, Mullan PB, McWilliams S, et al. BRCA1 regulates the interferon gamma-mediated apoptotic response. The Journal of biological chemistry. 2002; 277:26225–26232. [PubMed: 12011077]
25. Ouchi T, Lee SW, Ouchi M, et al. Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-gamma target genes. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:5208–5213. [PubMed: 10792030]
26. Weichselbaum RR, Ishwaran H, Yoon T, et al. An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105:18490–18495. [PubMed: 19001271]
27. Evers B, Drost R, Schut E, et al. Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008; 14:3916–3925. [PubMed: 18559613]
28. Scully R, Ganesan S, Vlasakova K, et al. Genetic analysis of BRCA1 function in a defined tumor cell line. Molecular cell. 1999; 4:1093–1099. [PubMed: 10635334]
29. Langdon SP, Lawrie SS, Hay FG, et al. Characterization and properties of nine human ovarian adenocarcinoma cell lines. Cancer research. 1988; 48:6166–6172. [PubMed: 3167863]
30. Sakai W, Swisher EM, Jacquemont C, et al. Functional restoration of BRCA2 protein by secondary BRCA2 mutations in BRCA2-mutated ovarian carcinoma. Cancer research. 2009; 69:6381–6386. [PubMed: 19654294]
31. Kohli M, Rago C, Lengauer C, et al. Facile methods for generating human somatic cell gene knockouts using recombinant adeno-associated viruses. Nucleic acids research. 2004; 32:e3. [PubMed: 14704360]

32. Bolstad BM, Irizarry RA, Astrand M, et al. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19:185–193. [PubMed: 12538238]
33. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003; 4:249–264. [PubMed: 12925520]
34. Dawson MA, Bannister AJ, Gottgens B, et al. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature. 2009; 461:819–822. [PubMed: 19783980]
35. Hakem R, de la Pompa JL, Mak TW. Developmental studies of Brca1 and Brca2 knock-out mice. Journal of mammary gland biology and neoplasia. 1998; 3:431–445. [PubMed: 10819537]
36. Jasin M. Homologous repair of DNA damage and tumorigenesis: the BRCA connection. Oncogene. 2002; 21:8981–8993. [PubMed: 12483514]
37. Warde-Farley D, Donaldson SL, Comes O, et al. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic Acids Res. 2010; 38:W214–220. [PubMed: 20576703]
38. Mostafavi S, Ray D, Warde-Farley D, et al. GeneMANIA: a real-time multiple association network integration algorithm for predicting gene function. Genome Biol. 2008; 9(Suppl 1):S4.
39. Coppe JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol. 2008; 6:2853–2868. [PubMed: 19053174]
40. Novakova Z, Hubackova S, Kosar M, et al. Cytokine expression and signaling in drug-induced cellular senescence. Oncogene. 2010; 29:273–284. [PubMed: 19802007]
41. Renard M, Henry M, Guetard D, et al. APOBEC1 and APOBEC3 cytidine deaminases as restriction factors for hepadnaviral genomes in non-humans in vivo. Journal of molecular biology. 2010; 400:323–334. [PubMed: 20546753]
42. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nature reviews Immunology. 2006; 6:644–658.
43. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol. 2005; 5:375–386. [PubMed: 15864272]
44. Der SD, Zhou A, Williams BR, et al. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:15623–15628. [PubMed: 9861020]
45. Jazaeri AA, Yee CJ, Sotiriou C, et al. Gene expression profiles of BRCA1-linked, BRCA2-linked, and sporadic ovarian cancers. Journal of the National Cancer Institute. 2002; 94:990–1000. [PubMed: 12096084]
46. Teschendorff AE, Miremadi A, Pinder SE, et al. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. Genome Biol. 2007; 8:R157. [PubMed: 17683518]
47. Petit V, Guetard D, Renard M, et al. Murine APOBEC1 is a powerful mutator of retroviral and cellular RNA in vitro and in vivo. Journal of molecular biology. 2009; 385:65–78. [PubMed: 18983852]
48. Mulligan JM, Hill LA, Deharo S, et al. Identification and validation of an anthracycline/cyclophosphamide-based chemotherapy response assay in breast cancer. Journal of the National Cancer Institute. 2014; 106:djt335. [PubMed: 24402422]
49. Stawowczyk M, Van Scoy S, Kumar KP, et al. The interferon stimulated gene 54 promotes apoptosis. The Journal of biological chemistry. 2011; 286:7257–7266. [PubMed: 21190939]
50. Andersen JB, Li XL, Judge CS, et al. Role of 2–5A-dependent RNase-L in senescence and longevity. Oncogene. 2007; 26:3081–3088. [PubMed: 17130839]
51. Couzinet A, Tamura K, Chen HM, et al. A cell-type-specific requirement for IFN regulatory factor 5 (IRF5) in Fas-induced apoptosis. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105:2556–2561. [PubMed: 18268344]
52. Lee SB, Rodriguez D, Rodriguez JR, et al. The apoptosis pathway triggered by the interferon-induced protein kinase PKR requires the third basic domain, initiates upstream of Bcl-2, and involves ICE-like proteases. Virology. 1997; 231:81–88. [PubMed: 9143305]

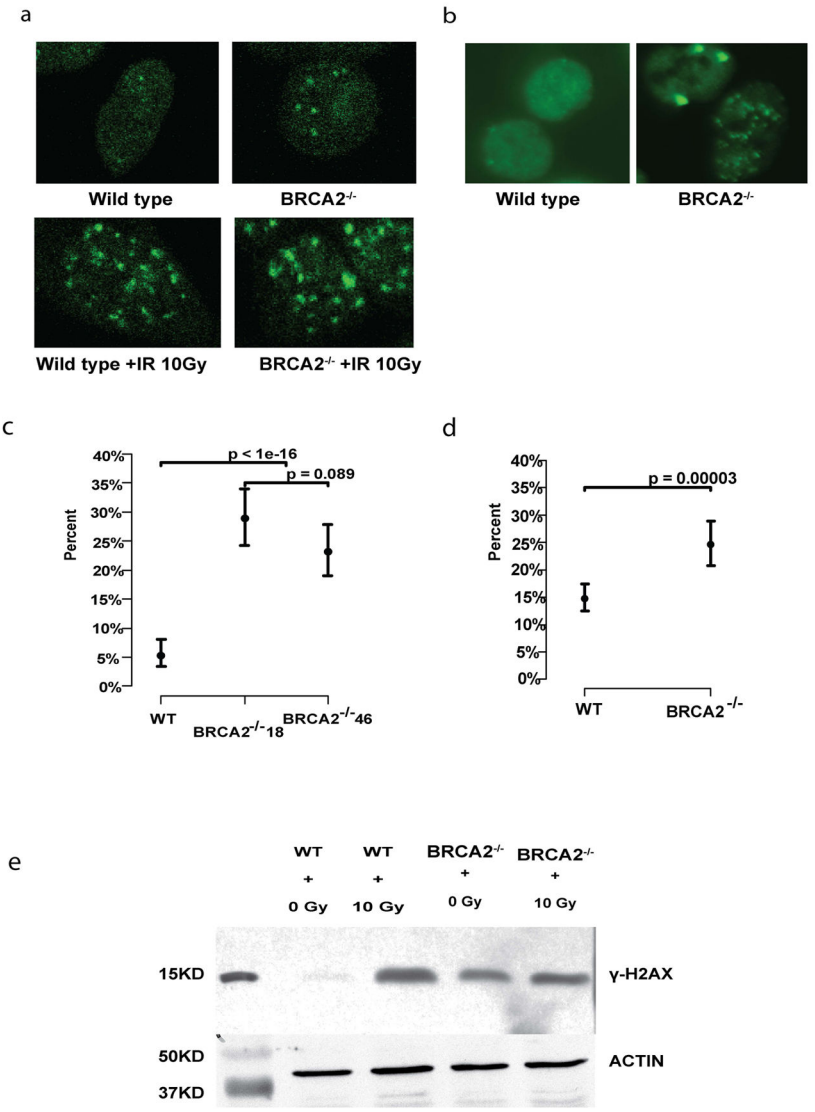

Figure 1. *BRCA2*$^{-/-}$ cells contain high levels of endogenous DNA damage

(a) Representative pictures of γ-H2AX foci in *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ HCT116 cells before and after irradiation. **(b)** Representative pictures of 53BP1 foci in *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ HCT116 cells. **(c)** Percentage of cells with >5 γ-H2AX foci in *BRCA2*$^{+/+}$ HCT116 cells and two HCT116 *BRCA2*$^{-/-}$ clones (clone #46 and clone #18). **(d)** Percentage of cells with >4 53BP1 foci in *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ HCT116 cells (clone #18). Data were obtained from n=3 independent experiments for each cell line. Error bars represent point-wise 95% confidence intervals. **(e)** Western blot analysis of γ-H2AX levels in *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ HCT116 cells before and after 10 Gray irradiation. ACTIN was used as a loading control.

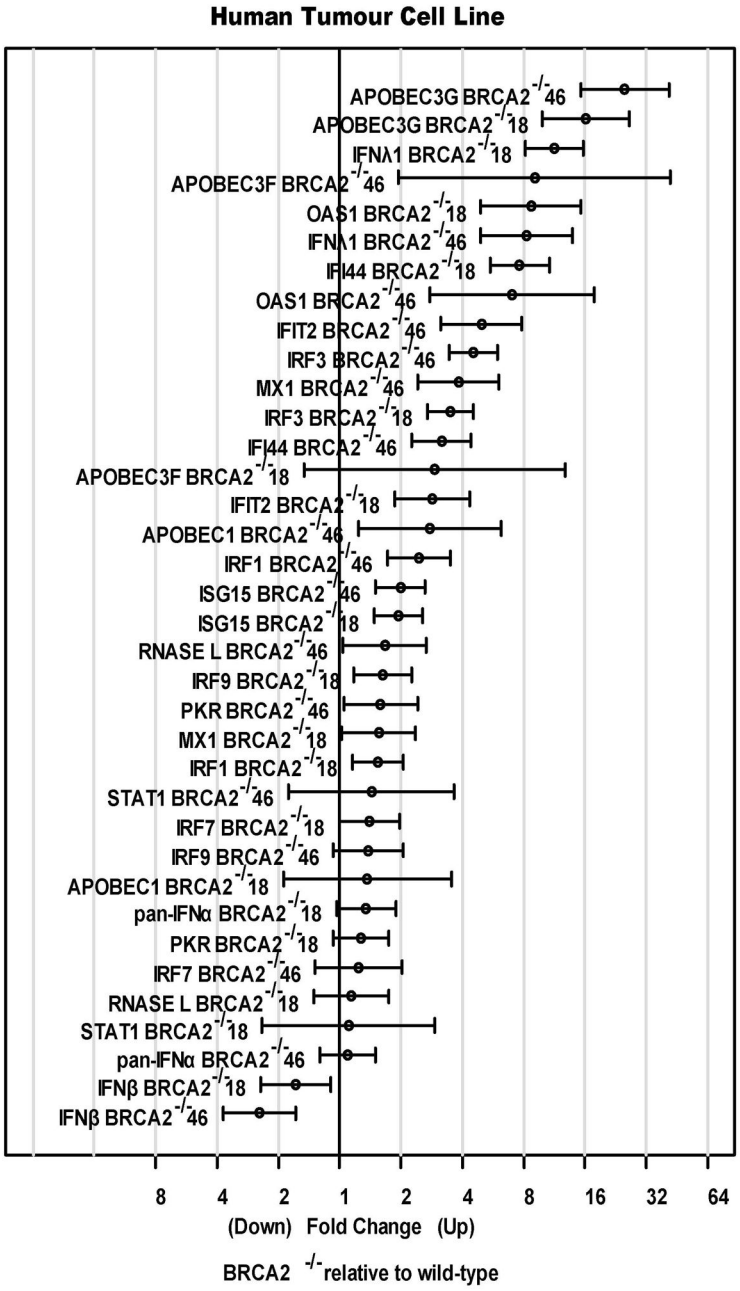

Figure 2. The expression of a number of IFN-related genes is up-regulated in *BRCA2*$^{-/-}$ HCT116 cells

The levels of several IFN-related gene transcripts were assessed by quantitative reverse transcription PCR in two *BRCA2*$^{-/-}$ clones and in isogenic *BRCA2*$^{+/+}$ HCT116 cells. Each gene was tested in 1–7 (average of 4) independent biological experiments, with each biological experiment containing three technical replicates. 18S rRNA, ACTIN B and LAMIN A were used as loading controls and the fold induction relative to wild type control was averaged across all biological and technical replicates via the ANOVA linear model. Error bars represent 95% confidence intervals.

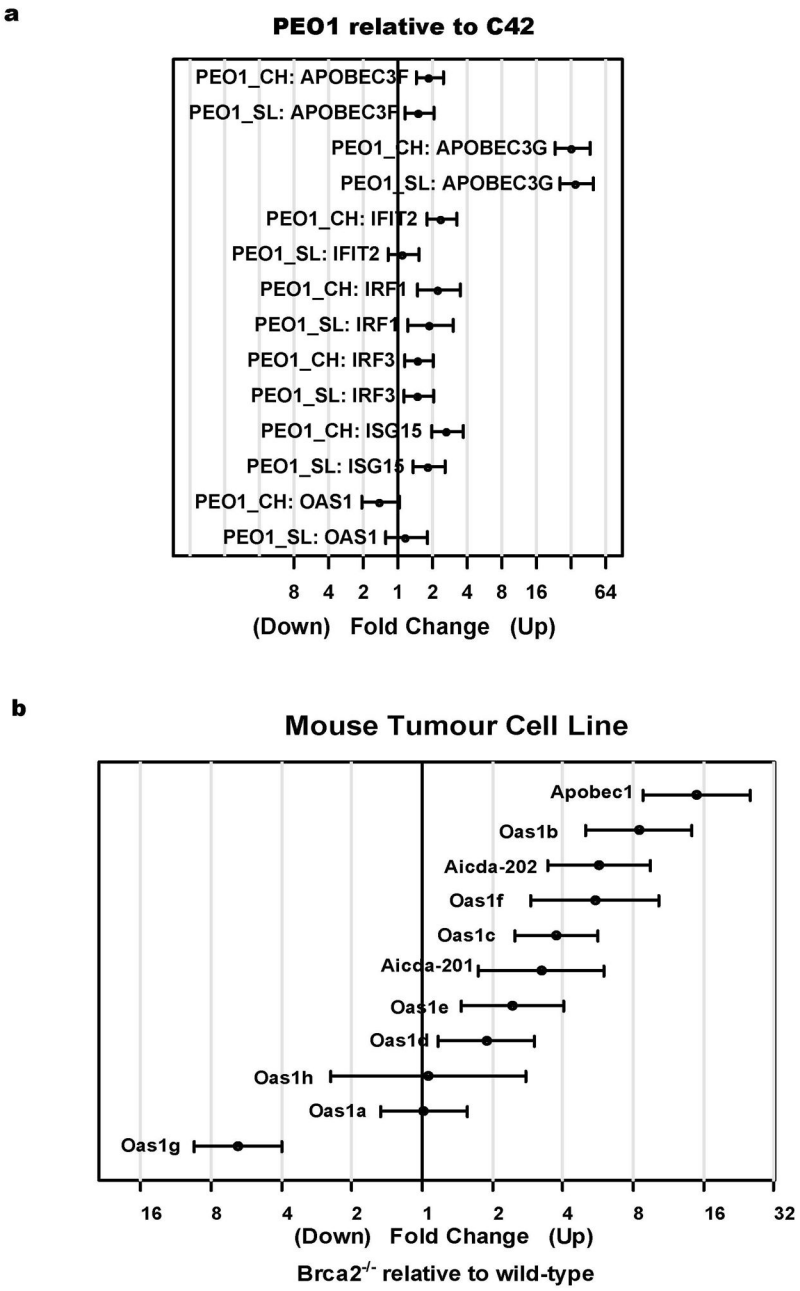

Figure 3. The induction of IFN-related genes in *BRCA2* deficient ovarian cancer cells and mouse tumor cells

(a) The up-regulation of IFN-related genes in two PEO1 clones (PEO1-CH and PEO1-SL), relative to C4-2 cells. **(b)** The expression of *OAS1* and *APOBEC3G* orthologs is up-regulated in *Brca2*$^{-/-}$ mouse tumour cells. Fold change estimates relative to wild type controls are shown. *Pgk1, Hprt* and *Actin B* were used as loading controls. Error bars represent 95% confidence intervals.

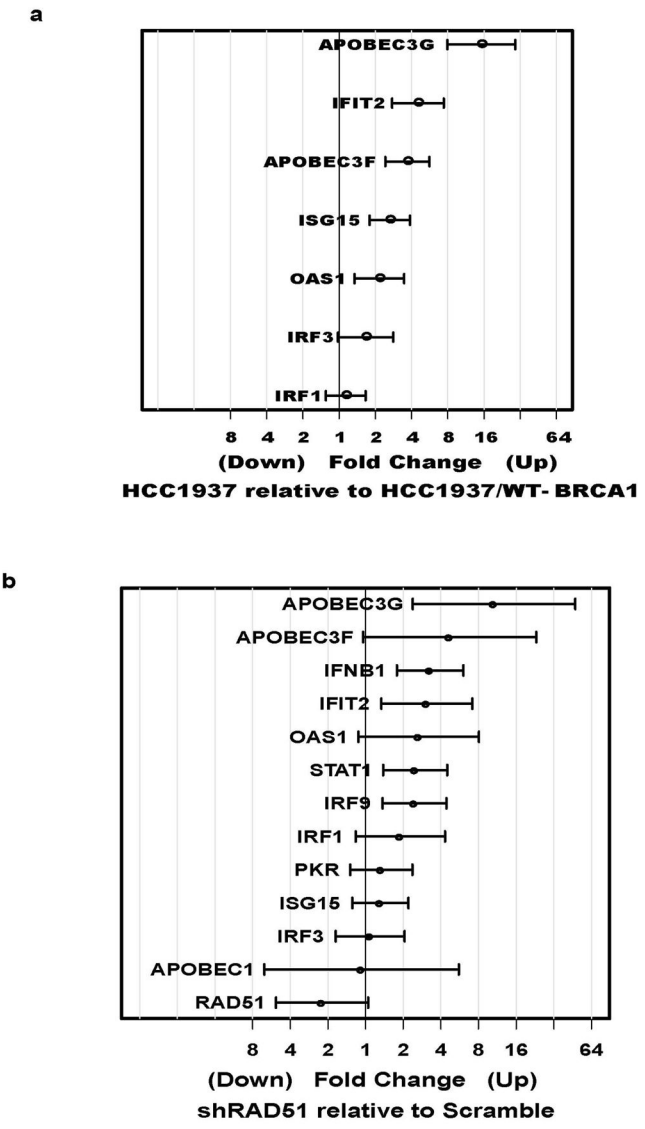

Figure 4. IFN-related genes are induced in cells with endogenous DNA damage

The expression of IFN-related genes was compared between HCC1937 cells relative to HCC1937/WT-BRCA1 cells **(a)** and RAD51 knocked down cells relative to cells infected with scramble shRNA in HCT116 **(b)**.

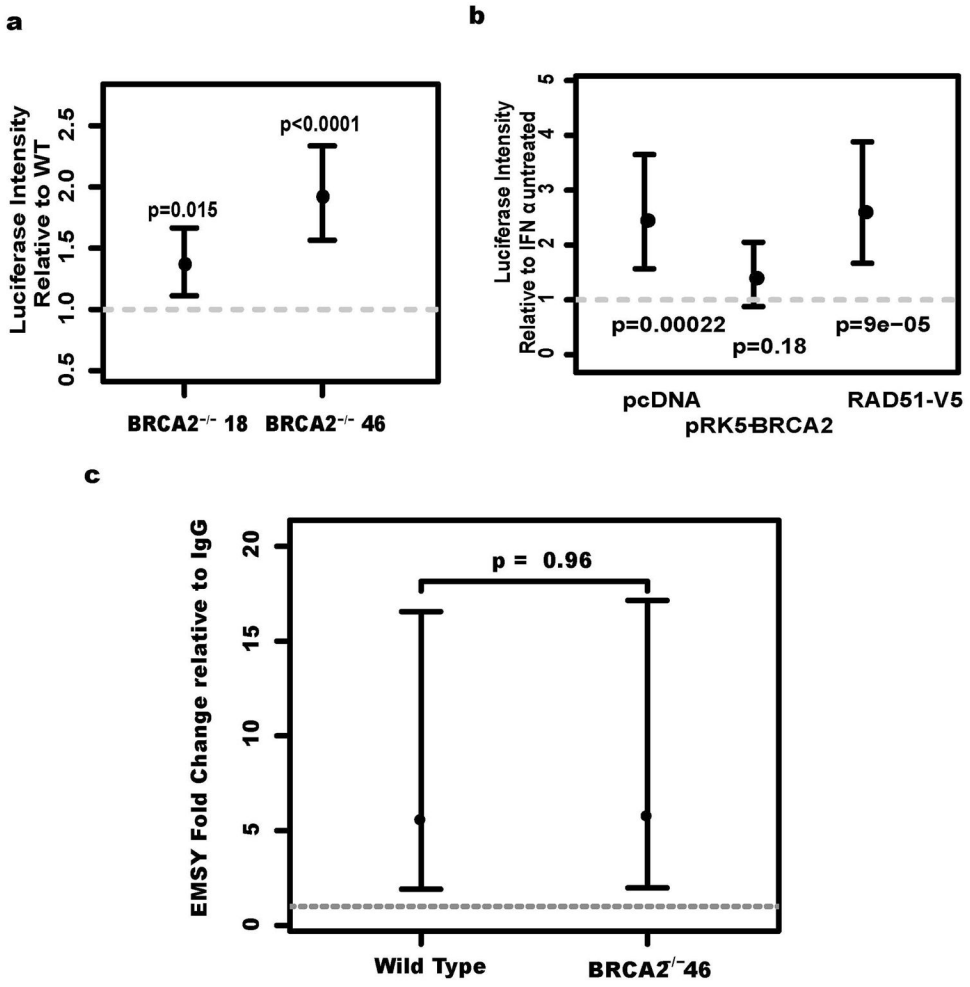

Figure 5. BRCA2 regulates the expression of IFN-related genes through ISRE

(**a**) Relative luciferase (Firefly) activity of a reporter construct with ISRE elements was compared between *BRCA2*$^{-/-}$ mutant clones and the WT clone. Error bars represent a 95% confidence interval for the relative intensity. **(b)** The effect of IFN-α treatment on ISRE promoter activity with the transfection of different constructs. Means and 95% confidence intervals are shown. **(c)** EMSY was CHIP from wt and *BRCA2*$^{-/-}$ cells, and the amount of DNA immunoprecipitated at ISG15 promoter was quantified by RT-PCR relative to IgG control.

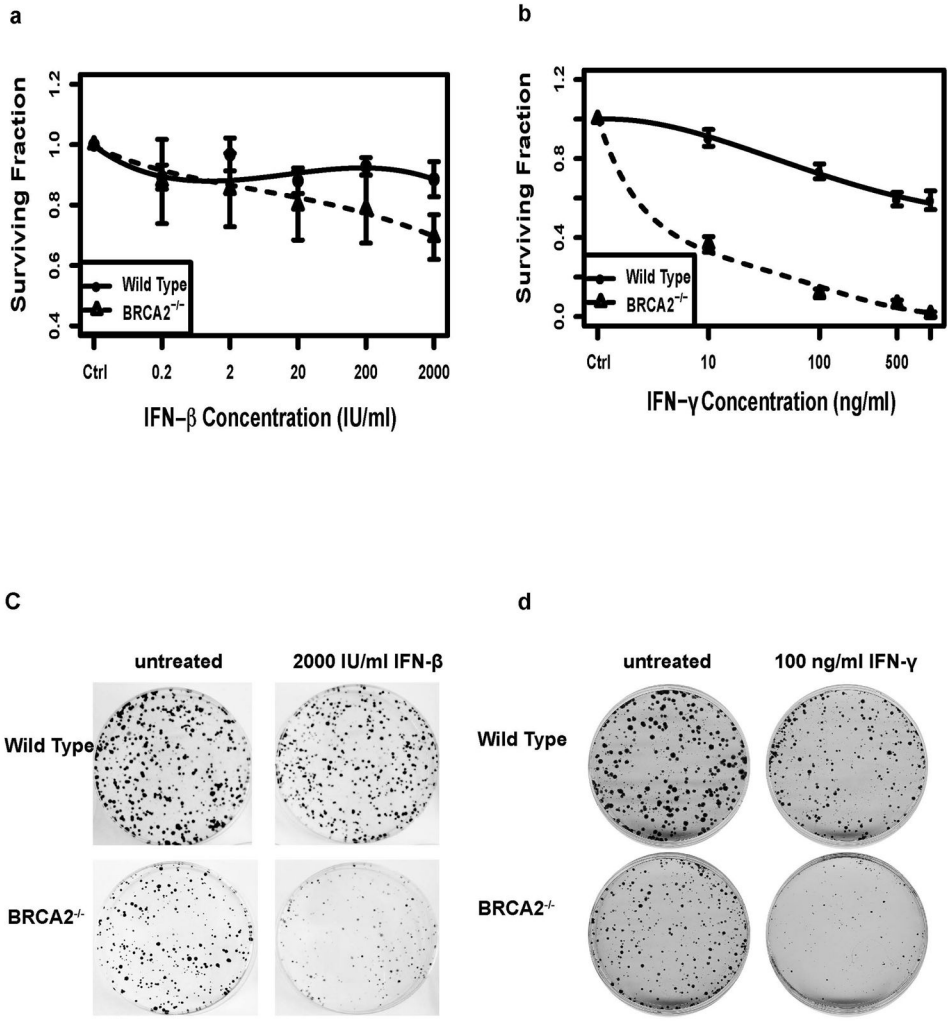

Figure 6. The colony formation capacity of *BRCA2*$^{-/-}$ cells is reduced by treatment with IFN-γ or IFN-β

Colony survival fraction of *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ HCT116 cells treated with different concentrations of IFN-β **(a)** or IFN-γ **(b)** for 10 days. Colonies were counted and values expressed as the percentage of viable colonies in treated versus untreated cells. Bootstrap standard error bars and cubic model fits are shown. Representative colony formation pictures are shown of *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells grown in the presence or absence of 2000 I.U./ml IFN-β **(c)** or 100 ng/ml IFN-γ **(d)**.

Table 1

Functional enrichment obtained by GeneMANIA analysis of the top 200 differently expressed genes in *BRCA2*$^{-/-}$ cells plus 100 associated genes

The top 200 differentially expressed genes were entered into GeneMANIA, allowing 100 genes to be associated with the query genes. The top 50 enriched functional GO annotations are listed.

GO annotation of query genes	FDR	Coverage
type I interferon-mediated signaling pathway	1.14E-14	18 / 66
response to type I interferon	1.14E-14	18 / 67
cellular response to type I interferon	1.14E-14	18 / 66
cytokine-mediated signaling pathway	2.05E-11	22 / 170
cellular response to cytokine stimulus	3.86E-11	22 / 177
response to cytokine stimulus	1.52E-10	23 / 212
blood vessel development	1.34E-07	18 / 171
vasculature development	4.35E-07	18 / 185
blood vessel morphogenesis	1.87E-06	16 / 157
angiogenesis	1.87E-06	15 / 135
cardiovascular system development	4.87E-06	20 / 275
circulatory system development	4.87E-06	20 / 275
anatomical structure formation involved in morphogenesis	1.34E-05	19 / 264
positive regulation of locomotion	1.10E-04	12 / 113
regulation of cellular component movement	1.63E-04	15 / 193
regulation of locomotion	1.64E-04	15 / 194
endothelial cell migration	1.99E-04	9/60
regulation of cell migration	2.91E-04	14 / 177
regulation of cell motility	3.39E-04	14 / 180
regulation of anatomical structure morphogenesis	6.63E-04	14 / 191
cytokine activity	1.35E-03	8/57
leukocyte chemotaxis	1.47E-03	8/58
extracellular matrix	1.51E-03	13 / 178
response to other organism	1.74E-03	14 / 211
MAPKKK cascade	1.74E-03	16 / 273
regulation of endothelial cell migration	2.12E-03	7/44
transforming growth factor beta receptor signaling pathway	2.32E-03	9/84
positive regulation of cell motility	2.33E-03	10 / 108
positive regulation of cell migration	2.33E-03	10 / 108
transmembrane receptor protein serine/threonine kinase	2.76E-03	11 / 136
positive regulation of cellular component movement	3.56E-03	10 / 114
sprouting angiogenesis	3.58E-03	5/19
cell chemotaxis	4.14E-03	8/70
regulation of cytokine production	4.14E-03	13 / 202
leukocyte activation	4.17E-03	15 / 267
MAP kinase tyrosine/serine/threonine phosphatase activity	4.52E-03	4/10

GO annotation of query genes	FDR	Coverage
negative regulation of immune system process	6.72E-03	7/55
MAP kinase phosphatase activity	6.72E-03	4/11
regulation of angiogenesis	6.87E-03	8/77
cytokine production	6.87E-03	13 / 215
epidermis development	6.87E-03	10 / 126
positive regulation of angiogenesis	7.26E-03	6/38
regulation of cell adhesion	7.26E-03	9 / 102
inflammatory response	7.44E-03	12 / 187
cellular response to interferon-gamma	8.87E-03	7/59