53BP1 loss abrogates DNA damage responses in *Brca1* null cells and is associated with triple-negative and BRCA-mutated breast cancers

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Transposon mutagenesis screen

Recently it was demonstrated that piggyBac transposons can be efficiently used to introduce genome wide modifications in ES cells[1]. Therefore we decided to make use of a piggyBac transposon containing the MSCV5'LTR for our genetic screens. We transfected *R26*^{CreERT2}*;Brca1*$^{SCo/\Delta}$ ES cells with MSCV5'LTR transposon and mouse codon optimized piggyBac transposase, switched the *Brca1*^{SCo} allele and assayed for clonal survival under puromycin selection (**Supplementary Fig. 2a**). From a total of in 296 picked colonies all but 2 clones appeared to contain both a switched and a non-switched *Brca1*^{SCo} allele, suggesting selection of allele duplication events (data not shown). This phenomenon has also been observed by others[2]. Southern blot analysis of the 2 clones that only contained the switched *Brca1*$^{\Delta 5\text{-}6}$ and the *Brca1*$^{\Delta 5\text{-}13}$ alleles revealed identical patterns of transposon insertions (**Supplementary Fig. 2b**). Since both clones were derived from the same subpool of transfected cells it is likely that they are identical subclones. The locations of the transposon insertions were sequenced using a splinkerette PCR approach. One of the insertions appeared to be within the *53bp1* gene (**Supplementary Fig. 2c**). Western blot analysis revealed that 53BP1 expression is abrogated in the surviving BRCA1 deficient clones (**Supplementary Fig. 2d**).

Supplementary Methods

Generation and culturing of *Brca1*^{SCo} ES cells

Brca1$^{wt/\Delta 5\text{-}13}$ ES cells[3] were electroporated with a *Brca1*^{SCo} targeting vector and selected for integration of the construct using a flirted Neo-TK selection marker (**Supplementary Fig. 1**). Correctly targeted clones were identified by Southern blot analysis with a *Brca1* exon 11 probe on *BamHI* digested genomic DNA. Targeting was confirmed by long range PCR over the 5' homology arm. The Neo-TK selection marker was removed by transient expression of pFlpe (a kind gift from Dr. Francis Stewart) and subsequently cells were targeted with a *Rosa26-CreERT2* construct[4] with an EF1α-Bsd resistance marker (**Supplementary Fig. 1**). ES cells were cultured on gelatin coated plates in 60% buffalo red liver (BRL) cell

conditioned medium supplied with 10% fetal calf serum, 0.1 mM β-mercaptoethanol (Merck) and 10^3 U/ml ESGRO LIF (Millipore) as described[5].

Genotyping

PCR primers used for genotyping are 5'-GCTGTTCTCCTCTTCCTCATC-3' in combination with 5'-AGGTCTGCCTGCCTCTACTTC-3' (158 bp product) for the *Brca1*^{SCo} allele and 5'-GTGGGCTTGTACTCGGTCAT-3' (125 bp product) for the switched *Brca1*$^{\Delta SCo}$ allele. Presence of the *Brca1*$^{\Delta 5\text{-}13}$ and absence of *Brca1* wt alleles was confirmed by PCR using primers 5'-TATCACCACTGAATCTCTACCG-3' +5'-TCCATAGCATCTCCTTCTAAAC-3' (431 bp product) and 5'-CACCTGCTCTGGCTGATG-3' + 5'-AGGTCTGCCTGCCTCTACTTC-3' (158 bp product) respectively. For Southern blots to detect targeted integration of the *Brca1*^{SCo} construct we used a probe in *Brca1* exon 11 generated using primers 5'-TGTGGAGCCATGTGGCAC-3' and 5'-GGTTCAGGTGAGGGCGGC-3'. Long range PCR to confirm targeting was performed using primers 5'-GGCTAGCCTGCAACTGTTGAC-3' and 5'-ACGTCGAGTGCCCGAAGG-3' (3 kb product). Targeting of the *Rosa26* locus with *CreERT2-Bsd* was verified using a *Rosa26* 5' probe generated with T3 and T7 primers from plasmid pH607[6]. Switching of the *Brca1*^{SCo} allele was analyzed by Southern blot using a *Brca1* intron 7 probe on EcoRV digested genomic DNA. The following primers were used to generate the probe: 5'-GGAGCAGAGCAAGTCATTGT-3' and 5'-TCAGAAAGTCAGGGGATCTG-3'.

PiggyBac transposon mutagenesis screen

One day before transfection 2.5 x 10^6 *R26*^{CreERT2}*;Brca1*$^{SCo/\Delta}$ ES cells were seeded on a 10cm dish. The next day cells were transfected with 18 μg piggyBac MSCV5' LTR transposon (kindly provided by Wei Wang and Pentao Liu, Wellcome Trust Sanger Institute, Hinxton, UK), 2 μg mPB transposase[1] and 500 ng CMV-eGFP (Clontech) plasmids using Lipofectamine 2000 (Invitrogen). Mock transfected and non-transfected controls were included. Upon overnight incubation, culture medium was refreshed and successful transfection was confirmed by analysis of GFP expression. 24 hr after transfection, cells were passaged to 3 x 10cm dishes and switched by overnight incubation with 0.5 μM 4OHT. Two days after switching, cells from each plate were passaged to 6 x 10 cm dishes and grown in the presence of 1.8 μg/ml puromycin. Pools of surviving cells and 296 puromycin resistant

colonies were harvested and analyzed by PCR and Southern blotting for switchings status of the *Brca1*^{SCo} allele. Subsequently, transposon insertion sites of confirmed *Brca1*$^{\Delta/\Delta}$ samples were determined using splinkerette PCR in combination with shotgun cloning and sequencing or direct high throughput 454 sequencing. Sequence data were analyzed and insertions were mapped on the mouse genome using iMapper[7] or the BLAT algorithm[8].

Splinkerette PCR on randomly sheared DNA fragments

Genomic DNA was isolated using the Gentra Puregene DNA isolation kit (QIAGEN) and processed for 454 sequencing of insertion sites using the shear-splink method (Koudijs et al, in preparation). In brief, DNA was sheared using a Covaris DNA shearer and end-repaired using the End-it repair kit (Epicenter Technologies). Subsequently, splinkerette adapaters containing 454 recognition sequences were ligated to the blunt-ended fragments and ligation products were amplified by PCR1. Upon size fractionation by agarose gel electrophoresis, a nested splinkerette PCR2 was performed with primers adapted for 454 sequencing and PCR products were purified using the QIAEX II gel extraction kit (Qiagen). The following primers were used for PCR1: 5' CGAAGAGTAACCGTTGCTAGGAGAGACC 3' and 5'-TAAATAAACCTCGATATACAGACCGATAAA-3'. Primers for PCR2 were: 5'-GCCTTGCCAGCCCGCTCAGATGAGACTGGTGTCGACACTAGTG-3' and 5'-GCCTCCCTCGCGCCATCAGTTTTACGCATGATTATCTTTAACGTACGTC-3'.

Gene expression array analysis

Gene expression array data from 286 cases of early stage breast cancer measured on Affymetrix U133A chips, published by Wang and colleagues was analyzed[9]. Robust consensus clustering techniques were used to classify the breast cancer cases into basal, HER2-positive, Luminal A and Luminal B subtypes[10]. The mean expression of probes corresponding to 53BP1 for the samples in each subtype was calculated.

Combined dataset analysis: The dataset of Richardson et al[11] represents 40 breast cancer tumors (out of which 18 are basal-like tumors) and 7 normal samples measured on Affymetrix U133 2 Plus chips and it is available as raw CEL files at GEO (GSE3744).

Data integration: The dataset of Richardson et al was first made compatible with the Wang et al data by restricting it to the probe sets of the U133A chip and by processing it with the mas5 software available in the Affymetrix package at http://www.bioconductor.org. Systematic source and batch bias adjustment in the two datasets was performed by the

distance-weighted discrimination (DWD) method which has been shown to be suited for the correction of the systematic biases associated with micro array data sets[9, 12]. The DWD calculations were carried out using a DWD JAVA implementation publicly available at https://genome.unc.edu/pubsup/dwd. The settings used for the input variables in the DWD software were non-standardized DWD, centered at the second mean.

Tissue microarray and patient information

Yale cohort*:* A tissue microarray comprising duplicate cores of tumors from 504 patients with a median follow-up of 6.7 years was used for this study. All patients in this study were treated surgically with breast conserving surgery with or without axillary lymph node dissection between 1975 and 2003. Following breast conserving surgery, patients were treated with standard daily whole breast irradiation, at the radiation oncology facilities of the Department of Therapeutic Radiology, Yale University School of Medicine. Adjuvant systemic chemotherapy and/or adjuvant hormone therapy was administered as clinically indicated in accordance with standard practices during this time interval. The specimens were collected under a Institutional Review Board approved protocol.

Helsinki cohort*:* The tissue microarrays from the Helsinki cohort have been described in detail previously[13, 14]. The numbers of tumors and family history are included in **Table 1B**. Permissions for this study were obtained from the Ethics Committee (E8) of the Helsinki University Central Hospital and from the Ministry of Social Affairs and Health in Finland. Patient samples were used with written informed consent.

Immunohistochemical staining for 53BP1

Yale cohort*:* Immunohistochemical analysis of the tissue microarrays from the Yale cohort was performed on 5-µm-thick tissue sections prepared from formalin-fixed, paraffin-embedded archival tissue from the tissue block constructed. Tissue sections were deparaffinized, staining was processed by the Ventana system using Ventana HX system BenchMark (Ventana Medical Systems). Anti-53BP1 rabbit polyclonal antibody (Bethyl Labs) was used at a dilution of 1:500. All procedures were performed automatically in the Ventana system. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide for 4 minutes. Antigen retrieval was performed using CC1 (Cell

Conditioning Solution, Ventana Medical Systems, Cat # 950-124) with extended time of 72 minutes. The tissue sections were incubated overnight with primary antibody at room temperature. Ventana's Universal Secondary Antibody (Cat # 760-4205) was applied for 12 minutes followed by chromogenic detection kit DABMap (Ventana Medical Systems, Cat # 760-124). Slides were counterstained with Hematoxylin and dehydrated and cleared before coverslipping from Xylene. DAB (3,3-diaminobenzidine tetrahydrochloride dehydrate) was then applied as a chromogenic substrate. For immunohistochemical evaluation of 53BP1, nuclear labeling of tumor cells was classified as either negative (if no staining or positive staining was present in <10% of tumor cells) or positive (if $\geq$10% of tumor cells stained positively).

Helsinki cohort*:* The protocol used to stain and analyze the tissue microarrays from the Helsinki cohort was as described[13, 14]. Briefly, tumor sections from the Helsinki cohort were analyzed by a very sensitive method allowing for detection of very low levels of 53BP1. A tumor was scored as having reduction or loss of 53BP1 when more than 60% of cancer cells in the lesion were 53BP1 negative (while the remaining minor fraction of cancer cells showed detectable and variable expression), or when the overall staining intensity was grossly reduced in the tumor cells as compared to normal cells present on the same section. Both of these patterns clearly contrasted with strong 53BP1 staining of normal tissues and stromal cells surrounding the cancer cell nests. Binding of the primary mouse monoclonal antibody against 53BP1 (a kind gift from Thanos Halazonetis, University of Geneva) was detected by the sensitive Vectastain Elite kit (Vector Laboratories), followed by DAB chromogen reaction with the Nickel sulphate enhancement step. Nuclear counterstaining was omitted to detect even the weak nuclear staining signal.

Statistical analysis

Yale cohort*:* Descriptive statistics comparing 53BP1 expression with conventional markers of tumor aggressiveness in the Yale cohort were analyzed by standard χ^2 tests, or, when appropriate, Fisher's exact test. Estimates of survival were calculated by the Kaplan-Meier product-limit method, and the differences were assessed by the log-rank test. Probabilities of survival were calculated from the date of breast carcinoma diagnosis to the study endpoints. The study endpoints were local relapse, distant metastasis-free survival, and overall survival,

including all deaths. Loco-regional recurrence was defined as clinically and biopsy-proven relapse in the ipsilateral breast. Distant metastases were defined as clinical evidence of distant disease based on clinical and/or radiographic evidence. Multivariate survival analysis using Cox's proportional hazard regression model was carried out to assess the independent contribution of each variable to survival. All *P* values were two-tailed, and the 0.05 level was considered statistically significant. A computer program package SAS (Version 9.1, SAS Institute) was used for all statistical testing and management of the database.

Helsinki cohort*:* Statistical analysis of the data obtained from the Helsinki cohort was conducted with the SPSS version 12.0.1 for Windows (SPSS Inc). The differences in dichotomous variables were tested by the chi- square test or Fisher's exact test. All p-values are two-sided and due to multiple testing, p-value <0.01 was considered significant.

Supplementary References

1. Wang,W. *et al.* Chromosomal transposition of PiggyBac in mouse embryonic stem cells. *Proc Natl Acad Sci U S A* **105**, 9290-9295 (2008).

2. Chang,S., Biswas,K., Martin,B.K., Stauffer,S., & Sharan,S.K. Expression of human BRCA1 variants in mouse ES cells allows functional analysis of BRCA1 mutations. *J Clin Invest* **119**, 3160-3171 (2009).

3. Liu,X. *et al.* Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proc Natl Acad Sci U S A* **104**, 12111-12116 (2007).

4. Hameyer,D. *et al.* Toxicity of ligand-dependent Cre recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues. *Physiol Genomics* **31**, 32-41 (2007).

5. Wielders,E., Dekker,M., & Te Riele,H. Generation of double-knockout embryonic stem cells. *Methods Mol Biol* **530**, 205-218 (2009).

6. Vooijs,M., Jonkers,J., & Berns,A. A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. *EMBO Rep* **2**, 292-297 (2001).

7. Kong,J., Zhu,F., Stalker,J., & Adams,D.J. iMapper: a web application for the automated analysis and mapping of insertional mutagenesis sequence data against Ensembl genomes. *Bioinformatics* **24**, 2923-2925 (2008).

8. Kent,W.J. BLAT--the BLAST-like alignment tool. *Genome Res* **12**, 656-664 (2002).

9. Wang,Y. *et al.* Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* **365**, 671-679 (2005).

10. Alexe,G. *et al.* High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates. *Cancer Res* **67**, 10669-10676 (2007).

11. Richardson,A.L. *et al.* X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell* **9**, 121-132 (2006).

12. Benito,M. *et al.* Adjustment of systematic microarray data biases. *Bioinformatics* **20**, 105-114 (2004).

13. Bartkova,J. *et al.* Aberrations of the MRE11-RAD50-NBS1 DNA damage sensor complex in human breast cancer: MRE11 as a candidate familial cancer-predisposing gene. *Mol Oncol* **2**, 296-316 (2008).

14. Tommiska,J. *et al.* The DNA damage signalling kinase ATM is aberrantly reduced or lost in BRCA1/BRCA2-deficient and ER/PR/ERBB2-triple-negative breast cancer. *Oncogene* **27**, 2501-2506 (2008).

15. Dirac,A.M. & Bernards,R. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J Biol Chem* **278**, 11731-11734 (2003).

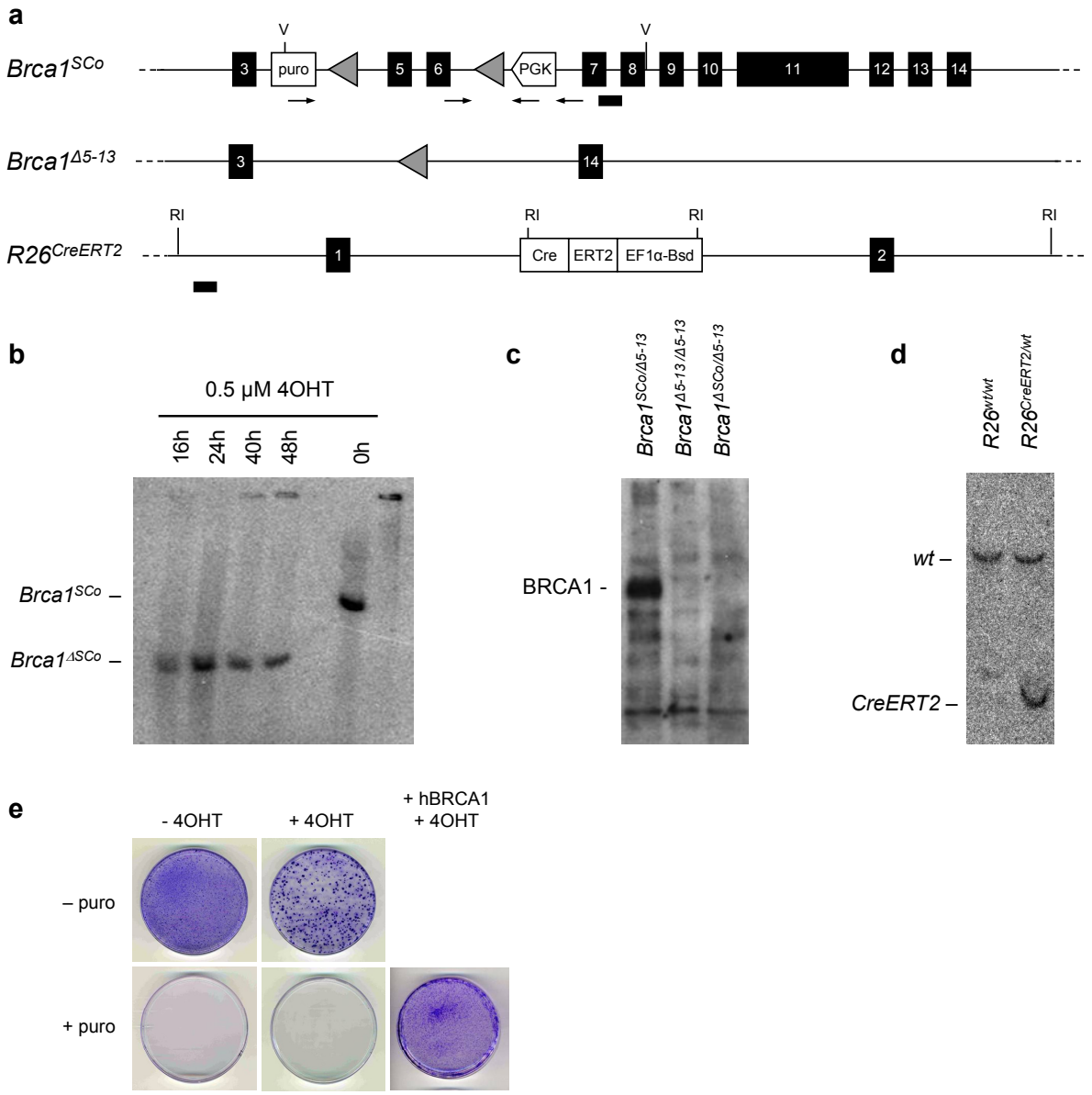
16. Tarsounas,M. *et al.* Telomere maintenance requires the RAD51D recombination/repair protein. *Cell* **117**, 337-347 (2004).

Supplementary Table 1 Overview of shRNA sequences

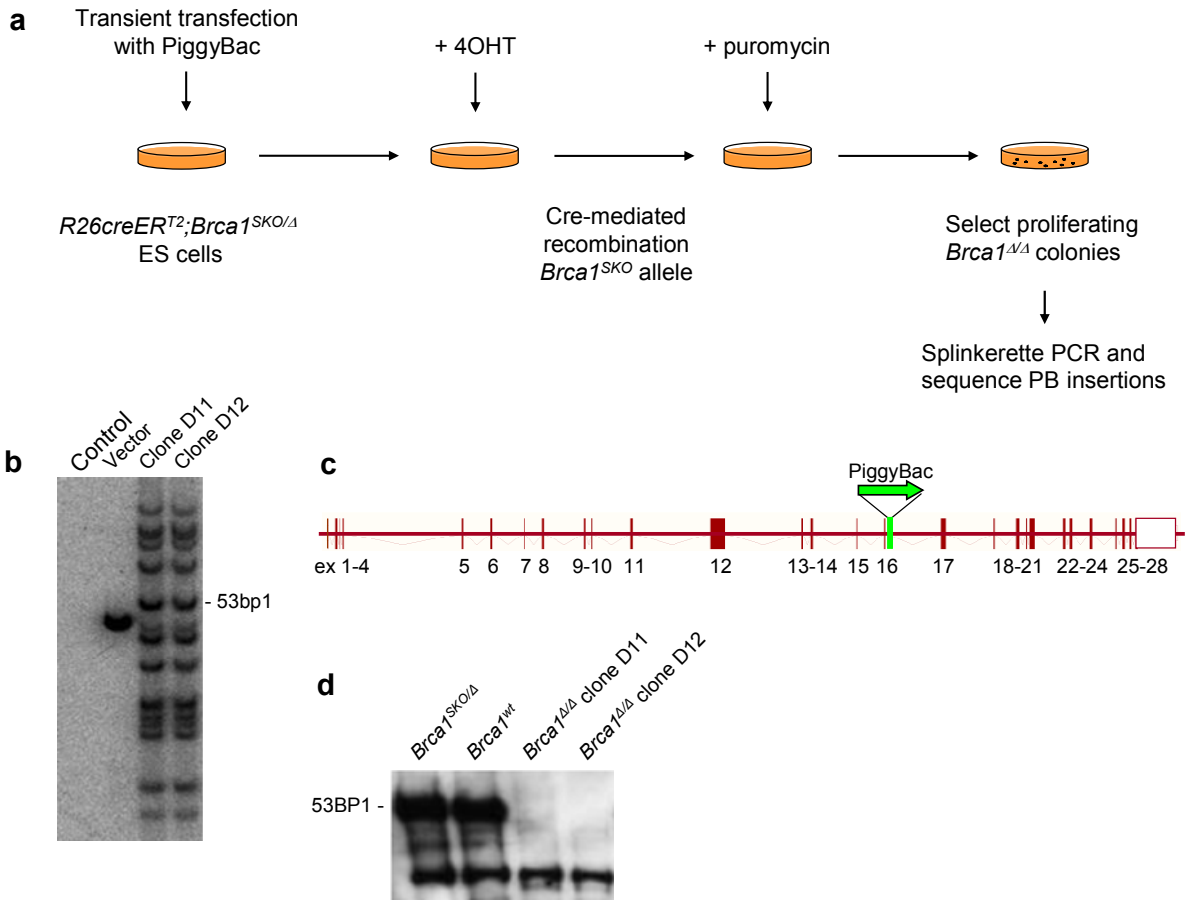
Name	Sequence (5'-3')	Reference
53BP1sh1	GCTATTGTGGAGATTGTGTTT	
53BP1sh2	GCGTAGAAGATATTTCACCTA	
P53sh1	CTACAAGAAGTCACAGCACAT	
P53sh2	AGAGTATTTCACCCTCAAGAT	
P53sh used in MEF studies	GTACATGTGTAATAGCTCC	[15]
NTsh	CAACAAGATGAAGAGCACCAA	
GFPsh	GCTGACCCTGAAGTTCATC	[16]

Supplementary Table 2 Correlation between 53BP1 expression and other characteristics (Yale cohort)

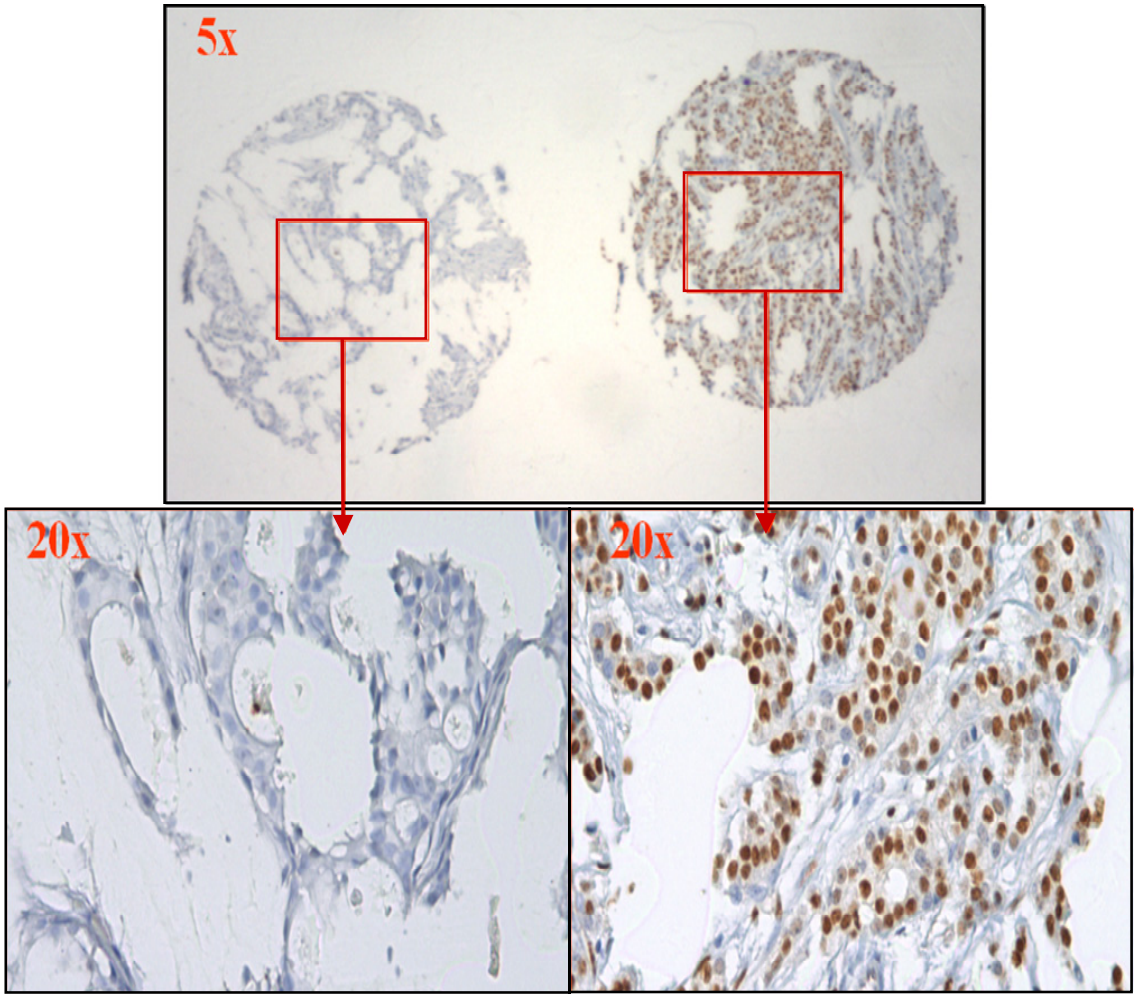
		53BP1 Expression		
Features	Number	Positive (%)	Negative (%)	*p*
Age				**<0.0001**
≤ 50	189	145(38%)	44 (68%)	
> 50	255	234(62%)	21(32%)	
Race				0.7658
White	375	319(84%)	56(86%)	
Black	56	48(13%)	8(12%)	
Others	13	12(3%)	1(2%)	
Histology				0.0498
Ductal	371	331(88%)	50(77%)	
Lobular	33	27(7%)	7(11%)	
Others	28	20(5%)	8(12%)	
Tumor Size				0.3956
T_1	314	267(77%)	47(82%)	
T_2	90	80(23%)	10(13%)	
Nodal Status				0.1434
Negative	210	172(45%)	38(58%)	
Positive	72	63(17%)	9(14%)	
Unknown	162	144(38%)	18(28%)	



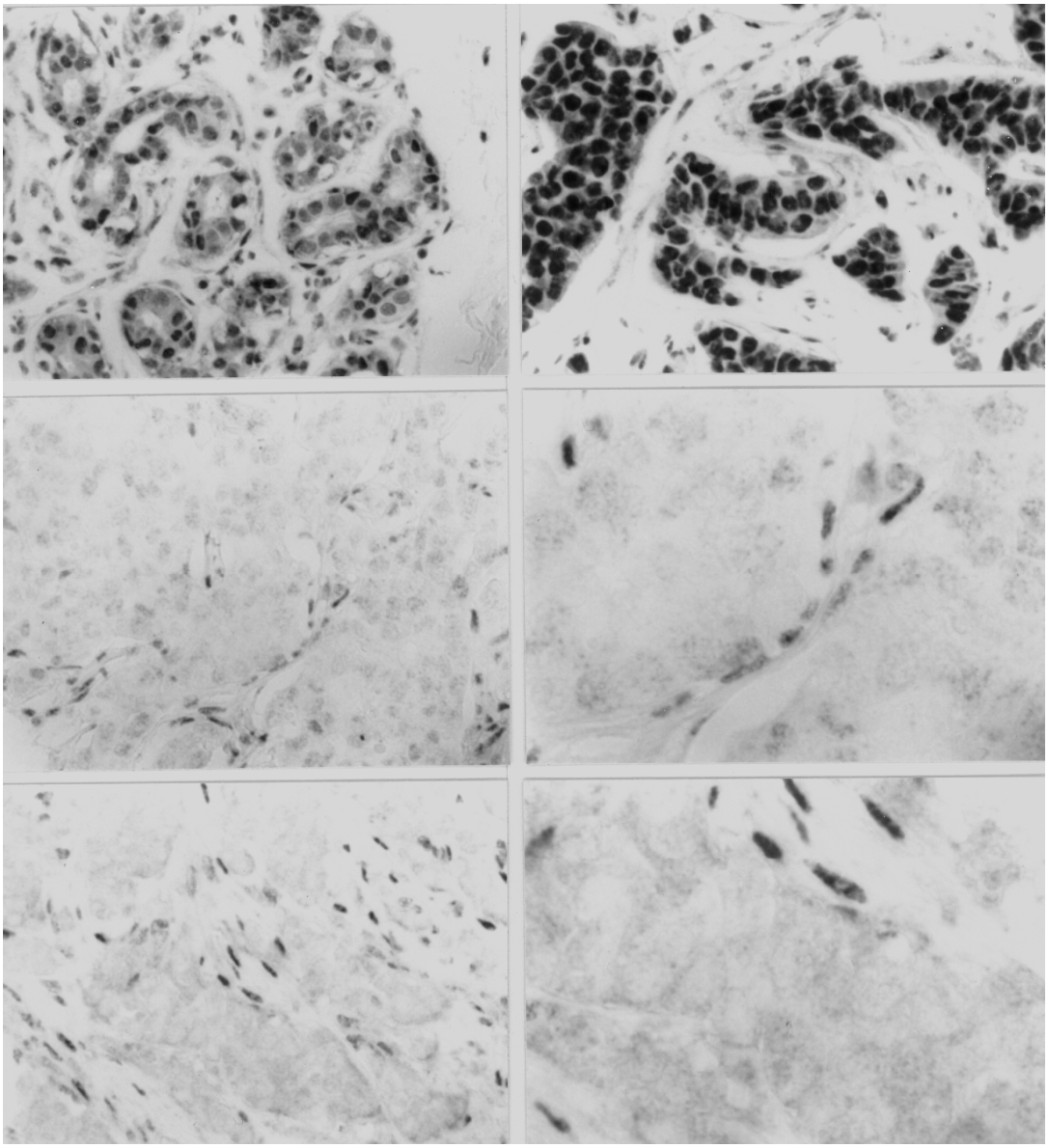
Supplementary Figure 1 Generation and validation of *Brca1*$^{SCo5\text{-}6}$, *Brca1*$^{\Delta 5\text{-}13}$ and *R26*$^{CreERT2\text{-}Bsd}$ alleles in mouse ES cells. (**a**) Schematic overview of mutant alleles in *Brca1*^{SCo} ES cells. Positions of primers for genotyping of the *Brca1*^{SCo} allele are indicated. (**b**) Southern blot showing efficient switching of *Brca1*^{SCo} ES cells by overnight incubation with 0.5μM 4-hydroxytamoxifen (4OHT). EcoRV digested genomic DNA was hybridized with a *Brca1* intron 7 probe. (**c**) Western blot showing absence of BRCA1 protein 3 days after 4OHT induced switching of *Brca1*^{SCo} ES cells. Lysate of a *K14Cre;Brca1*$^{\Delta 5\text{-}13/\Delta 5\text{-}13}$*:p53*$^{\Delta 2\text{-}10/\ \Delta 2\text{-}10}$ mammary tumor[3] was used as a negative control. (**d**) Southern blot showing targeting of *Cre*^{ERT2} to the *Rosa26* locus of *Brca1*$^{SCo/\Delta 5\text{-}13}$ ES cells. EcoRI digested genomic DNA was hybridized with the *Rosa26* 5'probe (pHA607). (**e**) Crystal violet staining of untransfected *R26*^{CreERT2}*;Brca1*$^{SCo/\Delta}$ ES cells vs. cells complemented with a human *BRCA1* BAC clone (hBRCA1). CreERT2-mediated deletion of the *Brca1*^{SCo} allele was achieved by addition of 4OHT.



Supplementary Figure 2 A piggyBack transposon mutagenesis screen in *R26*^{CreERT2};*Brca1*$^{SCo/\Delta}$ ES cells identifies *53bp1* insertions in surviving Brca1-deficient colonies. (**a**) Outline of the transposon mutagenesis screen. PiggyBac (PB) transposon containing the MSCV5'LTR promoter/enhancer and PB transposase are cotransfected in *R26*^{CreERT2};*Brca1*$^{SCo/\Delta}$ ES cells. Upon Cre-mediated recombination, BRCA1-deficient ES colonies are selected using puromycin and analyzed for proliferation rescuing PB transposon insertions. (**b**) Southern blot analysis of surviving BRCA1-deficient ES cell colonies D11 and D12. Genomic DNA from control ES cells, control ES cells with 5 ng MSCV5'LTR transposon vector and clones D11 and D12 was digested with BamHI and hybridized with a transposon specific probe. The band corresponding to the insertion in *53bp1* is indicated. (**c**) Schematic drawing of the PB transposon insertion in *53bp1* in *R26*^{CreERT2};*Brca1*$^{\Delta/\Delta}$ ES cell clones D11 and D12. (**d**) Western blot showing loss of 53BP1 expression in *R26*^{CreERT2};*Brca1*$^{\Delta/\Delta}$ ES cell clones D11 and D12, which carry inactivating piggyBac transposon insertions in intron 16 of *53bp1*.



Supplementary Figure 3 Example of 53BP1 protein expression in human breast cancers (Yale cohort). Tissue microarray slides were processed for immunohistochemistry using an antibody specific for 53BP1 protein. Shown are representative specimens that stained negative (left panel) or positive (right panel) for 53BP1.



Supplementary Figure 4 Examples of aberrantly reduced 53BP1 protein in subsets of human breast carcinomas (Helsinki cohort)**.** Immunohistochemical detection of 53BP1 in human breast carcinomas shows moderate to high nuclear expression in normal human breast tissue (top left) and the majority of invasive breast tumors (top right), in contrast to aberrant reduction or loss of 53BP1 in subsets of BRCA-deficient tumors (middle panels) and ER-PR-HER2 triple-negative carcinomas (bottom panels). The middle and bottom panels show paired images of the same tumor at lower magnification (left) and high magnification (right), respectively. Note the very low or absent staining for 53BP1 seen selectively in tumor nests, in contrast to preserved normal level of 53BP1 in the stromal cells of the tumors shown in the middle and bottom panels.