

Mutation of a single allele of the cancer susceptibility gene *BRCA1* leads to genomic instability in human breast epithelial cells

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Edited* by Bert Vogelstein, The Johns Hopkins University, Baltimore, MD, and approved September 13, 2011 (received for review July 12, 2011)

Biallelic inactivation of cancer susceptibility gene *BRCA1* leads to breast and ovarian carcinogenesis. Paradoxically, *BRCA1* deficiency in mice results in early embryonic lethality, and similarly, lack of *BRCA1* in human cells is thought to result in cellular lethality in view of *BRCA1*'s essential function. To survive homozygous *BRCA1* inactivation during tumorigenesis, precancerous cells must accumulate additional genetic alterations, such as *p53* mutations, but this requirement for an extra genetic "hit" contradicts the two-hit theory for the accelerated carcinogenesis associated with familial cancer syndromes. Here, we show that heterozygous *BRCA1* inactivation results in genomic instability in nontumorigenic human breast epithelial cells in vitro and in vivo. Using somatic cell gene targeting, we demonstrated that a heterozygous *BRCA1* 185delAG mutation confers impaired homology-mediated DNA repair and hypersensitivity to genotoxic stress. Heterozygous mutant *BRCA1* cell clones also showed a higher degree of gene copy number loss and loss of heterozygosity in SNP array analyses. In *BRCA1* heterozygous clones and nontumorigenic breast epithelial tissues from *BRCA* mutation carriers, FISH revealed elevated genomic instability when compared with their respective controls. Thus, *BRCA1* haploinsufficiency may accelerate hereditary breast carcinogenesis by facilitating additional genetic alterations.

breast cancer 1, early onset | human cell gene targeting | somatic cell knock-in

Women carrying heterozygous mutations in the cancer susceptibility gene *BRCA1* (1) are estimated to have a lifetime breast cancer risk of up to 85% (2). Wild-type *BRCA1* alleles are lost in the majority of these tumors, further underscoring *BRCA1*'s crucial tumor suppressor function (3). Paradoxically, biallelic disruption of *BRCA1* confers early embryonic lethality in mouse models (4–6), and similarly, lack of functional *BRCA1* in mature cells is thought to result in proliferation defects or cell death in view of *BRCA1*'s essential function in maintaining genomic integrity (7–9).

Although it remains unclear how *BRCA1* tumor cells survive biallelic *BRCA1* inactivation during tumorigenesis, evidence suggests a role for accumulated genetic alterations, such as *p53* mutations, in overcoming cell lethality (6, 10, 11). Because *BRCA1* tumors presumably follow the two-hit theory that explains the accelerated carcinogenesis in familial cancer syndromes (12), this requirement for an extra genetic "hit" in *BRCA1*-mediated tumorigenesis appears contradictory. A proposed mechanism that could facilitate such genetic alterations is genetic instability resulting from the inactivation of a single *BRCA1* allele (i.e., *BRCA1* haploinsufficiency) (7, 13, 14). *BRCA1* haploinsufficiency has also been suggested by several assays using breast epithelial cells from *BRCA1* carriers (15–21). However, to date there has been no

rigorous, defined experimental system to address *BRCA1* haploinsufficiency in human breast epithelial cells. Genetically engineered mouse models have been extensively studied to elucidate *BRCA1*'s function. However, in contrast to human *BRCA1* carriers, most animals bearing heterozygous *BRCA1* mutations do not demonstrate increased spontaneous breast or ovarian tumor formation (22–24), with the exception of a heterozygous mouse strain that is susceptible to other types of neoplasms (25). Thus, in the current study, we created a genetically defined experimental system derived from human breast epithelial cells and used it to address *BRCA1* haploinsufficiency in humans.

Results

We initially carried out gene targeting in human cell lines and generated isogenic cell models harboring heterozygous mutant *BRCA1* (Fig. 1). Two noncancerous breast epithelial cell lines were used for this study: the spontaneously immortalized MCF-10A cell line and a cell line immortalized by *hTERT* introduction (*hTERT*-IMEC) (26). These cell lines exhibit a basal-like phenotype (26, 27) (i.e., a gene expression signature similar to mammary basal epithelial cells from which *BRCA1* breast tumors are thought to originate). In addition, relative to cancer cells, these cell lines are genetically stable with wild-type *p53*. One of the most common pathogenic *BRCA1* mutations, 185delAG, which results in a 2-bp deletion at the coding region close to the N terminus, was heterozygously introduced in these cell lines via gene targeting (Fig. 1A).

We established two independently derived knock-in clones for both MCF-10A and *hTERT*-IMEC cell lines (hereafter termed *BRCA1*-het #1 and #2). Correct targeting was demonstrated by PCR amplification of the targeted genomic locus (Fig. 1B). Proper transcription from targeted alleles was confirmed by capillary electrophoresis and direct sequencing of RT-PCR products

Author contributions: H.K. and B.H.P. designed research; H.K., M.M., A.T., J.P. Garay, S.C., S.K., A.O., H.Y.W., Y.K., K.T., A.M.A., J.P. Gustin, J.C., G.M.W., R.C., S.A., L.H., A.L.G., C.M.H., Y.H., and K.E.B. performed research; H.K., Y.K., B.K., and C.M.H. contributed new reagents/analytic tools; H.K., M.M., A.T., J.P. Garay, S.C., S.K., A.O., H.Y.W., Y.K., B.K., K.T., A.M.A., J.P. Gustin, J.C., G.M.W., D.C., R.C., D.J., M.J.H., S.A., L.H., J.L., A.L.G., C.M.H., Y.H., E.G., A.K.M., K.V., P.A., K.E.B., and B.H.P. analyzed data; and H.K., M.M., J.L., E.G., A.K.M., K.V., P.A., and B.H.P. wrote the paper.

Conflict of interest statement: B.H.P. is a paid consultant for GlaxoSmithKline and is a paid member for the scientific advisory board of Horizon Discovery, Ltd.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1110969108/-DCSupplemental.

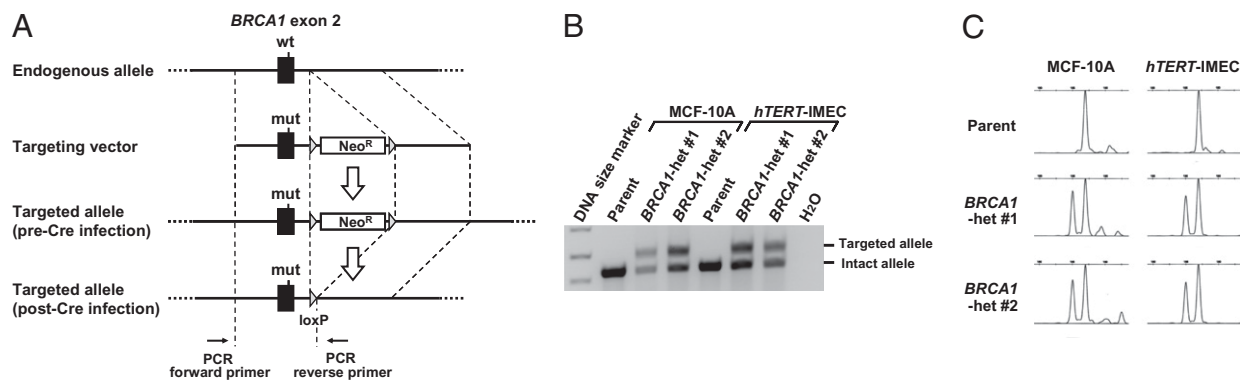


Fig. 1. Knock-in of *BRCA1* 185delAG mutation in MCF-10A and *hTERT-IMEC*. (A) A scheme for the knock-in process. Primers shown at the bottom were used for the PCR amplification in B. (B) PCR encompassing the targeted genomic locus. Targeted and intact alleles yield PCR products of 517 bp and 429 bp in size, respectively. Cell clones post Cre-loxP recombination were hereafter used in this study. Primers used for PCR are denoted in A. (C) Capillary electrophoresis of RT-PCR products encompassing the 2-bp deletion site in exon 2. Two neighboring peaks in *BRCA1* heterozygous clones represent PCR products of 186 bp and 188 bp in size, respectively.

(Fig. 1C and Fig. S1). In addition, we also isolated cell clones that underwent random integration of the targeting vectors within their genomes (hereafter termed “control”). These clones were used as control clones in all subsequent experiments. We also attempted to create MCF-10A and *hTERT-IMEC* cell clones carrying homozygous *BRCA1* 185delAG mutations by targeting the remaining wild-type alleles in *BRCA1*-het clones. However, despite several repeated attempts, we only obtained cell clones undergoing random integration of the targeting vector to the genome. This finding is consistent with the proposed model of cellular lethality because of biallelic *BRCA1* inactivation (7–9), although the possibility remains that unknown technical reasons may have accounted for the inability to obtain null clones.

To address *BRCA1* haploinsufficiency in humans, we employed several assays using our gene-targeted cell lines. Because it has been well established that *BRCA1* plays a pivotal role in homologous recombination (HR) repair of DNA double-strand breaks (also termed “gene conversion”) (28), we initially evaluated HR effi-

ciency in our MCF-10A-derived clones by stably transducing an EGFP reporter construct detecting HR (BABE-HR) (Fig. 2A). Through subsequent introduction of the restriction enzyme I-SceI, the SceGFP fragment present in BABE-HR is cleaved and then repaired by HR using an iGFP fragment as a template. This DNA recombination results in the reconstitution of a functional EGFP, which can be detected by fluorescence flow cytometry. Because of the technical difficulties of targeting the EGFP reporter construct to the same genomic position in all MCF-10A-derived isogenic clones, we isolated multiple single-cell subclones from each MCF-10A clone after BABE-HR transduction, transfected these subclones with the I-SceI-expressing plasmid, and then statistically compared their percentage of GFP positivities. This analysis revealed a significant decrease in HR repair in *BRCA1* heterozygous clones compared with their wild-type counterparts (Fig. 2B and C).

We also evaluated homology-mediated repair capacities in both MCF-10A and *hTERT-IMEC* isogenic clones using another EGFP reporter construct, which detects both HR and single-strand

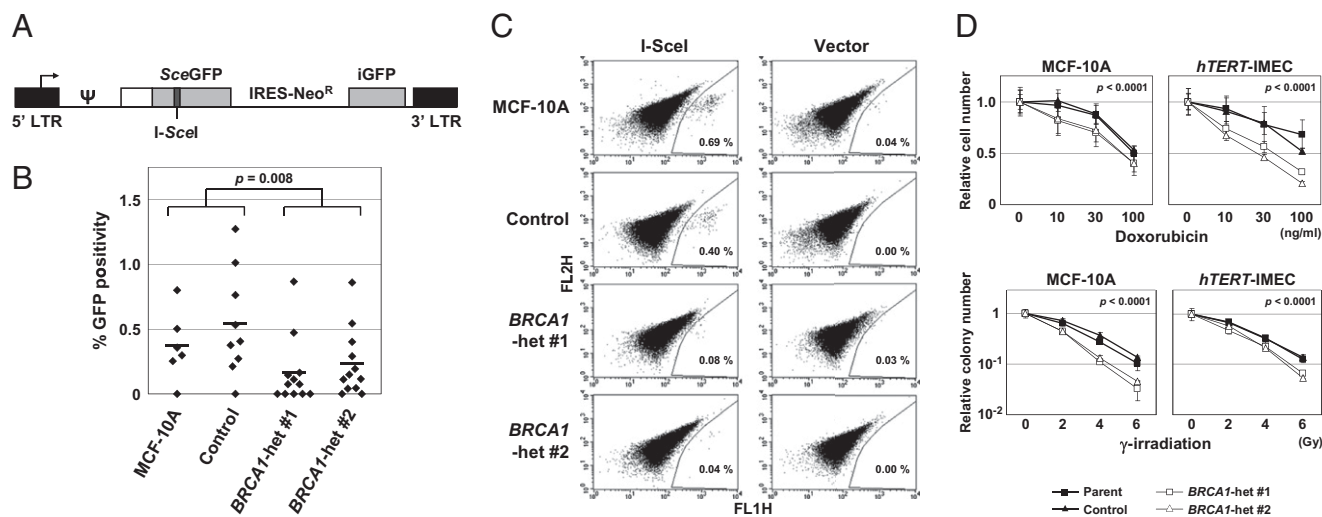


Fig. 2. DNA homologous recombination repair is suppressed in *BRCA1* heterozygous clones. (A) Retroviral vector-based GFP reporter construct BABE-HR measuring homologous recombination efficiency. SceGFP, inactive EGFP in which an 18-bp sequence is substituted with an I-SceI recognition site; iGFP, an internal portion of EGFP; arrows, transcription start sites; ψ , a packaging sequence. (B and C) HR repair assay. Multiple single-cell clones established after BABE-HR infection into MCF-10A-derived isogenic clones were transfected with an I-SceI-expressing plasmid, and GFP-positive ratios were determined by flow cytometry. Shown are a graphic representation of the entire results (B) and dot plots and percentage of GFP positivities in representative single-cell clones (C). Horizontal bars in B are averages. (D) Sensitivity of isogenic cell clones to Doxorubicin (Upper, $n = 5$) and γ -irradiation (Lower, $n = 3$). Data are shown relative to those of nontreated cells (mean \pm SD).

annealing repair (pEGFPx2-C1) (Fig. S2A). Of note, *BRCA1* is shown to be involved in both of these homology-mediated repair mechanisms (29). Assays were conducted based on transient transfection of I-SceI-cleaved pEGFPx2-C1, precluding the concern that the genomic positions of the integrated reporter constructs affect the results. Fluorescence flow cytometric analyses revealed a significant decrease in homology-mediated repair in both MCF-10A and *hTERT*-IMEC *BRCA1* heterozygous cell lines compared with wild-type counterparts (Fig. S2B).

In accord with reduced capacity for homology-mediated repair, cell survival assays demonstrated that *BRCA1* heterozygous clones have increased sensitivity to genotoxic stress conferred by either the chemotherapeutic drug Doxorubicin or γ -irradiation, both of which are known to cause DNA damage that is primarily repaired by HR (Fig. 2D). In contrast, we failed to detect a difference in the cytotoxicity of the poly(ADP-ribose) polymerase (PARP) inhibitor NU1025 between *BRCA1* heterozygous and wild-type clones (Fig. S3). Similarly, the PARP inhibitor ABT-888 currently being evaluated in clinical trials also had no difference in sensitivity in our isogenic clones. Notably, PARP inhibitors are thought to exert cell killing selectively in S-phase, unlike γ -irradiation and Doxorubicin, which exert cytotoxic effects in all phases of the cell cycle, including resting cells (30, 31). Thus, it may be that any elevated sensitivity to PARP inhibitors in *BRCA1* heterozygous clones is counterbalanced by G0/G1-dominant cell-cycle profiles and reduced proliferation capacities in these clones (see below). Overall, the data from DNA repair and cell survival assays demonstrated modest but significant impairment of DNA repair in *BRCA1* heterozygous cells.

We next explored other cellular properties previously described in *BRCA1*-deficient mouse embryos, such as deregulated expression of p21 protein and impaired proliferation (4). When *BRCA1* heterozygous human breast epithelial cells were propagated in exponential growth conditions and compared with their wild-type counterparts, p21 protein was up-regulated in immunoblot analyses similar to *BRCA1*-deficient mouse embryos (Fig. 3A), and cells were slightly but significantly more accumulated in the G0/G1 phase of the cell cycle (Fig. 3B and C). Slower cell growth in *BRCA1* heterozygous clones in comparison with their wild-type counterparts was also shown by proliferation assays

(Fig. 3D). In contrast, although G2 checkpoint deficiency upon γ -irradiation is a notable property of *BRCA1*-deficient cells (32), there was no attenuation in either induction or maintenance of G2 checkpoint responses after γ -irradiation in *BRCA1* heterozygous cell clones (Fig. S4). We also found no transformed phenotypes in *BRCA1* heterozygous clones by an EGF-free cell culture, as well as an anchorage independent growth assay. Collectively, these results demonstrated that cells with a single mutant *BRCA1* allele exhibit similar yet muted properties, as has been described for *BRCA1*-null cells.

To study how impaired DNA repair function caused by *BRCA1* haploinsufficiency impacts genomic integrity in human breast epithelial cells, we analyzed copy number (CN) changes, defined as the gain or loss of a given gene, in 389 cancer-related genes with the aid of a focused SNP array. *BRCA1* heterozygous MCF-10A clones and their wild-type counterparts were exposed to 6 Gy or mock γ -irradiation. A total of 30 single-cell subclones were then isolated from each γ -irradiated cell clone, as denoted in Tables S1 and S2, maintained for 4 wk, and analyzed with SNP arrays. Signal-intensity data were obtained for 1,223 of 1,421 markers in the Cancer SNP Panel, and the CNs of 389 of 408 cancer-related genes in the panel were determined. Of the SNP markers, 399 were informative for genotyping and enabled loss of heterozygosity (LOH) assessment in 186 genes.

The vast majority of the 389 genes have CN scores within 1.5 to 2.5, and no amplification or high CN gain was found in this dataset. Nonetheless, *BRCA1* heterozygous MCF-10A clones with or without 6 Gy γ -irradiation showed a significantly higher degree of CN loss compared with their wild-type counterparts (Fig. 4A and Table S1). Because this was significant in both nonirradiated ($P = 0.001$) and 6-Gy irradiated ($P = 0.006$) conditions upon individual analyses, the compromised genomic integrity is at least partially inherent to *BRCA1* heterozygous cells rather than a function of increased sensitivity to γ -irradiation, presumably reflecting a critical role for *BRCA1* against genomic DNA replication stress (33). Intriguingly, no difference in CN gain was detectable between *BRCA1* heterozygous and wild-type clones. In line with observed preferential CN loss in *BRCA1* heterozygous clones, the increase of LOH regions was also found in both 6-Gy and mock-irradiated *BRCA1* heterozygous clones compared with

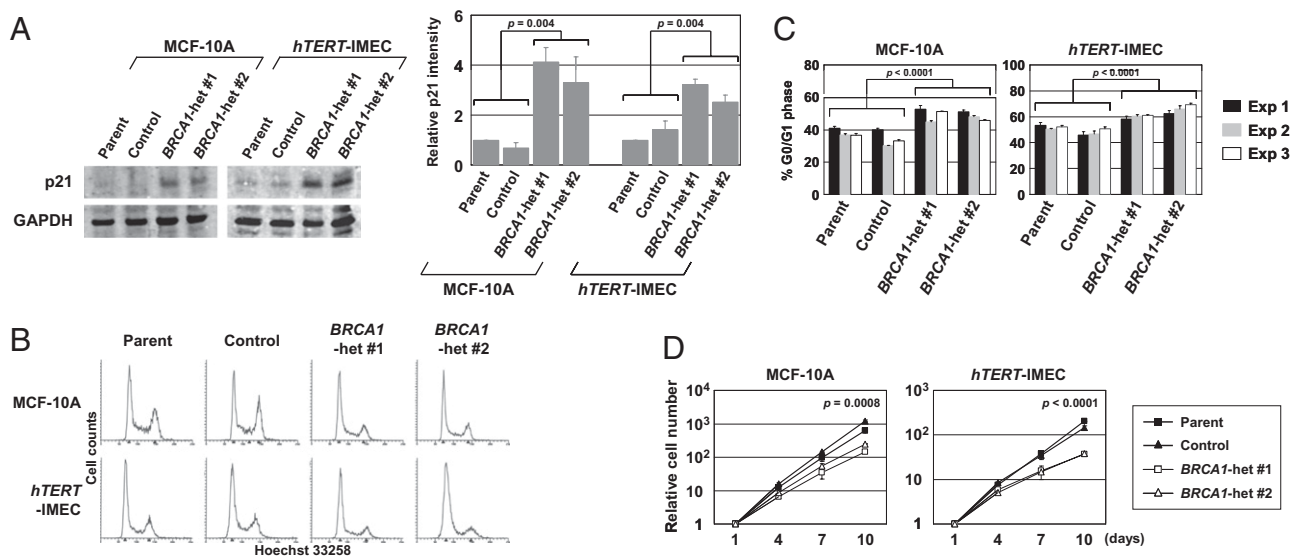


Fig. 3. *BRCA1* heterozygous clones have similar yet muted properties with *BRCA1*-null cells. (A) Western blot analysis of p21 in exponentially growing cells. Shown are a representative blot (Left) and densitometric analyses of three independent experiments (Right). Data were normalized to GAPDH and shown relative to each parental cell line (mean \pm SD). (B and C) Cell-cycle profiling by flow cytometry. Representative results (B) and G0/G1 populations (mean \pm SD; $n = 3$) in three independent experiments (C). (D) Cell proliferation assay. Cell numbers are indicated relative to those at day 1 (mean \pm SD; $n = 3$).

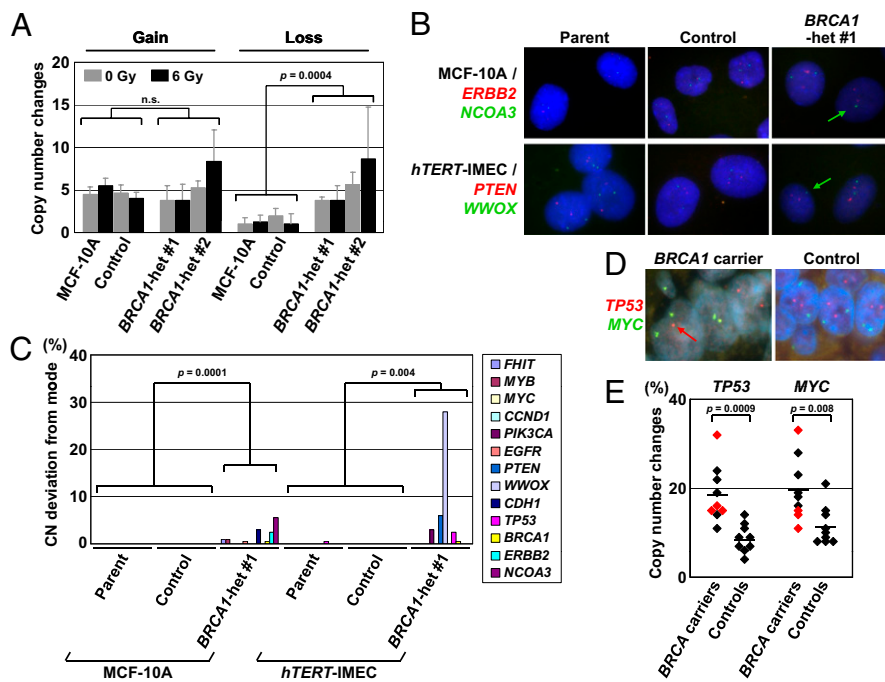


Fig. 4. Increased genomic instability in *BRCA1* heterozygous cells in vitro and in vivo. (A) MCF-10A–derived isogenic clones were exposed to 6 Gy or mock γ -irradiation and subjected to single-cell cloning. Single-cell subclones were analyzed with SNP arrays after 4 wk of culture, and genes exhibiting CN changes (< 1.5 or > 2.5) were counted (mean \pm SD). (B and C) FISH analyses of 13 genomic loci in isogenic cell clones. (B) Representative FISH images. Red and green spots represent *ERBB2* and *NCOA3* gene loci in MCF-10A clones and *PTEN* and *WWOX* loci in *hTERT*-IMEC clones, respectively. Green arrows indicate cells carrying single-copy genes labeled with green fluorescence. (C) Percentages of cells with allelic counts deviated from the modes. (D and E) FISH analyses of *TP53* and *MYC* gene loci in noncancerous breast epithelial tissues from *BRCA* mutation carriers and controls undergoing reduction mammoplasty. (D) Representative FISH images for a *BRCA1* mutation carrier and a control. A red arrow indicates a single *TP53* gene copy. (E) Percentage of cells undergoing CN changes. Red dots denote *BRCA2* mutation carriers. Horizontal bars represent averages.

their respective wild-type counterparts, although the difference was significant only in 6-Gy irradiated samples (Table S2).

To examine genomic alterations in a more stringent fashion—that is, at a single-cell resolution—we subsequently analyzed the isogenic cell clones with FISH. FISH visualizes individual alleles in each cell, and thereby enables the detection of subtle genomic CN changes in a small subset of cell populations that may be otherwise overlooked. Because of its high resolution, FISH can delineate genomic instability (measurable rate of genomic changes) in addition to aneuploidy or allelic imbalance (state of genomic changes) (34). For this analysis, we used probes to assess 13 genomic loci that are known to undergo frequent CN changes in sporadic or *BRCA1* mutant breast cancer (Table S3). As expected, deviation of an allele count from the mode was found in only 1 of 10,388 ($< 0.01\%$) cells in wild-type controls. In stark contrast, a total of 108 of 5,200 (2.08%) cells in *BRCA1* heterozygous clones derived from MCF-10A and *hTERT*-IMEC exhibited allele counts distinct from the modal populations, affecting 11 of 13 genomic loci (Fig. 4 B and C and Table S4). In *BRCA1* heterozygous clones, CN losses (88 cells) were dominant to CN gains (20 cells), concordant with our SNP array data (Fig. 4A and Table S1). In this FISH analysis, the *BRCA1* gene locus at chromosome 17q21.31 was retained in a total of 1,200 cells, with the exception of one cell each in *BRCA1* heterozygous MCF-10A and *hTERT*-IMEC clones (Table S4). Similarly, in our SNP array analysis, the *BRCA1* gene locus was retained and exhibited no CN changes in all of the examined single-cell subclones (Table S2). The retention of the *BRCA1* gene in *BRCA1* heterozygous single-cell subclones was further confirmed by a PCR amplification of the targeted gene locus followed by analytical electrophoreses. These results strongly suggest that the observed genomic instability phenotype in *BRCA1* heterozygous

clones was indeed attributable to *BRCA1* heterozygosity and not from a fraction of *BRCA1*-deficient cells emerging within *BRCA1* heterozygous populations.

Finally, we carried out a FISH analysis against nontumorigenic breast tissue specimens to determine if genomic instability in *BRCA1* heterozygous mutant cells is also detectable in vivo. Because of a limited number and amount of available *BRCA1* specimens, this analysis used five and four noncancerous breast tissues from known *BRCA1* and *BRCA2* carriers, respectively, and two genomic loci among the most frequently altered in *BRCA1* tumors—the *TP53* and *MYC* gene loci (11, 35)—were then analyzed. For controls, we used nine breast specimens from women with no personal or family history of breast cancers who had undergone reduction mammoplasties. As seen in Fig. 4 D and E, the *TP53* and *MYC* gene loci showed significantly more CN changes in breast epithelia from *BRCA* carriers compared with control samples. Virtually all of the CN changes detected in this analysis were losses of a single allele, consistent with our SNP array and FISH data for isogenic cell clones. It should be noted that control specimens also displayed a low level of CN changes and, specifically, CN loss. This result is because of the limitations of FISH using tissue samples. In this assay, some nuclei are cut into multiple sections, and thus a given section does not always contain all of the targets for a given FISH probe in a cell. The resultant false CN loss likely constitutes the background of the system. Chromosomal aberrations in breast tissues from *BRCA1* carriers have been previously suggested by LOH and array-based comparative genomic hybridization analyses that used pools of cells as samples (17, 21). Our current FISH data provide unambiguous evidence for this by demonstrating a low degree, but significant elevation of genomic instability in nontumorigenic breast epithelia from *BRCA* carriers.

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