

## A Mammary-Specific, Long-range Deletion on Mouse Chromosome 11 Accelerates *Brcal*-Associated Mammary Tumorigenesis<sup>1,2</sup>

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### Abstract

We engineered a mammary-specific knockout model for *Brcal* deficiency that also lacks the majority of one chromosome 11 to determine whether tumor susceptibility loci reside on this chromosome that cooperate with the loss of *Brcal* during mammary cancer formation. *Brcal*-deficient females that are haploinsufficient in 60 cM of chromosome 11 exhibited accelerated mammary tumorigenesis in comparison to *Brcal* conditional knockout mice. On the histopathologic level, these tumors were either adenocarcinomas or benign, inflammatory lesions. Like human BRCA1-associated breast cancers, mammary carcinomas in this new mouse model were ER $\alpha$ -negative and of basal epithelial origin. *Brcal* deficiency and haploinsufficiency in 60 cM of chromosome 11 caused widespread genome instability as determined by spectral karyotyping analysis. In addition to the verification of the long-range deletion event, the spectral karyotyping analysis revealed that the duplication of the genome and higher degree of aneuploidy occur rather late in tumor progression. Despite chromosomal rearrangements near the *Trp53* locus as determined by fluorescence *in situ* hybridization, the *Trp53* gene was transcriptionally active. The analysis of the coding sequence and expression pattern of p53 and p21 suggests that loss-of-heterozygosity of *Trp53* caused by somatic mutations contributes to accelerated mammary tumorigenesis in this model.

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### Introduction

Germ line mutations of the *breast cancer-associated gene-1* (*BRCA1*) are responsible for a 55% to 85% cumulative lifetime risk of breast cancer by age 70 [1–3]. BRCA1 is a multidomain protein that is suggested to play a role in a variety of cellular functions including maintenance of genomic stability, DNA double-strand break repair, transcriptional regulation, and cell cycle and spindle checkpoint control [4,5]. Although the precise mechanisms for BRCA1's ability to prevent breast cancer initiation are not clearly defined, targeted gene deletion models of *Brcal* provided important insights into the biological functions of this gene for embryonic development, tissue homeostasis, and cancer initiation [6]. Our previous studies on a conditional knockout model of *Brcal* demonstrated that the ablation of this tumor-suppressor gene from the mammary epithelium is sufficient to induce neoplastic transformation and mammary cancer after a long latency. Haploinsufficiency in p53 greatly

accelerates mammary carcinogenesis in this model for hereditary human breast cancer [7].

Epidemiological evidence in humans as well as genetic studies in *Brcal*-deficient murine cancer models suggest that subsequent somatic mutations, including p53 and possibly additional modifier loci, are responsible for variations in the latency of *Brcal*-associated mammary

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tumorigenesis. Although *BRCA1* gene mutations are rare in sporadic breast cancers, the expression of full-length *BRCA1* transcripts and the protein is reduced in a subset of sporadic malignancies [8,9], suggesting that genetic or epigenetic alterations in noncoding, regulatory regions near *BRCA1* play a role in sporadic breast cancer. On the basis of a meta-analysis for commonly deleted regions in sporadic breast cancers that also identified a modified chromosomal segment on 17q21 near *BRCA1* [10], Biggs et al. [11] suggested that there might be tumor-suppressor genes near *Brcal* that may play a role in cancer initiation. The latter research team used Cre/loxP-based chromosome engineering as a method to delete large portions (up to 5 Mb) of the mouse chromosome 11, which contains the *Brcal* and *p53* genes, to identify additional putative tumor susceptibility genes that cooperate with these more prominent tumor-suppressor loci. These large deletions, however, tend to cause lethality in homozygous mutants, and segmental haploidy in the germ line can cause abnormalities in other organs that may interfere with the analysis of tumor susceptibility loci in specific adult tissues such as the mammary gland.

To address whether putative tumor susceptibility loci on chromosome 11 are able to cooperate with the loss of *Brcal* during mammary carcinogenesis, we generated a mammary-specific knockout of *Brcal* that is also haploid-deficient in nearly the entire chromosome 11. Females deficient in *Brcal* that also lack approximately 60 cM of one chromosome 11 develop mammary cancer after a significantly shorter latency compared to females that carry only a conditional mutation of *Brcal*. Like familial BRCA1-associated breast cancers in humans, the adenocarcinomas that appeared in *Brcal*-deficient mice that are haploinsufficient in chromosome 11 are ER $\alpha$ -negative and of basal epithelial origin. Unlike previous reports, these lesions do not exhibit an up-regulation of ErbB2. Using spectral karyotyping (SKY) analysis, we demonstrate that these mammary cancers have a highly unstable genome that includes chromosomal rearrangements in the central region of the homologous chromosome 11, which did not undergo the long-range deletion event. Using fluorescence *in situ* hybridization (FISH), we determined that the chromosomal breaks occurred near the *Trp53* locus. However, the *Trp53* gene itself was not translocated to other chromosomes and was transcriptionally active. The sequence analysis of the coding region of *p53* suggests that, in a significant subset of cases, loss-of-heterozygosity of *Trp53* caused by somatic mutations is a contributing factor to accelerated tumorigenesis in this breast cancer model.

## Materials and Methods

### Mice

The generation of WAP-Cre and MMTV-Cre transgenic lines as well as the conventional Wap knockout mice and the *Brcal* conditional knockout model was described previously [7,12,13]. All animals used in this study were treated humanely and in accordance with institutional guidelines and federal regulations.

### Whole Mount Staining of Mammary Glands and Histologic Analysis of Mammary Tumors

Protocols for the preparation of mammary gland whole mounts and hematoxylin and eosin-stained sections of formalin-fixed tissues were described previously [14]. The entire hematoxylin and eosin-stained sections of representative mammary gland lesions were digitized at high resolution using a whole-slide scanning microscope (Zeiss, Jena, Germany) with image capture software from MicroBrightField, Inc.

Composite images were analyzed and annotated by Dr. Bob Cardiff using an Internet-based image database with integrated virtual microscopy software (Zoomify) at the Center for Comparative Medicine, University of California, Davis.

### Immunostaining

A basic protocol for immunohistochemistry and immunofluorescent staining of paraffin-embedded mammary gland specimens was described previously [15]. We used the following primary antibodies:  $\alpha$ -CK5 (1:500 dilution),  $\alpha$ -CK6 (1:500 dilution), and  $\alpha$ -CK14 (1:1000 dilution) from Covance (Berkeley, CA);  $\alpha$ -CK8 (TROMA-I; 1:250 dilution) from the Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA;  $\alpha$ -SMA (NCL-SMA; 1:50 dilution) from Novocastra Laboratories, Ltd (Newcastle upon Tyne, United Kingdom);  $\alpha$ -pStat3 (58E12, 1:50 dilution) from Cell Signaling Technology (Danvers, MA); and  $\alpha$ -ER $\alpha$  (MC-20, 1:500 dilution) and  $\alpha$ -p21 (C-19, 1:200 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA). The cytokeratins were visualized with mouse-specific or rabbit-specific Alexa Fluor 594 and 488-conjugated secondary antibodies (1:1000 dilution) from Invitrogen (Carlsbad, CA). The slides were mounted with Vectashield containing 1.5  $\mu$ g of 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA). For immunohistochemistry of ER $\alpha$ , pStat3, and p21, we used the corresponding biotinylated secondary antibodies (1:200 to 1:1000 dilution) and Vectastain Elite ABC kit (Vector). 3,3-Diaminobenzidine (DAB) was used as a chromogen, and slides were counterstained with Mayer's hematoxylin. Bright field and fluorescence images of histologic slides were taken on a Zeiss Axio Imager microscope equipped with a SPOT FLEX camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

### Spectral Karyotyping and FISH

The SKY and FISH analyses were performed on short-term cultured mammary cancer cells that were derived from primary tumors similar to a published protocol by Medina and Kittrell [16]. The annotation of the karyotypes was performed at the Cytogenetic Core facility of the VanAndel Institute, Grand Rapids, MI. The composite karyotypes for each tumor were assigned based on the individual analysis of more than 20 metaphases. The FISH analyses were carried out at the Genome Imaging Facility at Albert Einstein College of Medicine. The genomic probe for the *Trp53* locus was kindly provided by Thomas Ried (National Institutes of Health, Bethesda, MD). The methodology for the chromosomal labeling of *Trp53* and *Septin 9* (*Sept9*) were described previously [7,17].

### Reverse Transcription-Polymerase Chain Reaction and Sequencing of the *Trp53* Coding Region

To determine the transcriptional activation of the *Trp53* gene, we isolated total RNA from tumor cell pellets using standard guanidinium thiocyanate-phenol-chloroform extraction. A SuperScript II kit from Invitrogen with oligo dT primers was used to perform the first strand synthesis. The primer sequences and PCR conditions for the amplification of a 1166-bp region of the *Trp53* coding sequence were described previously [18]. Polymerase chain reaction products were gel-purified and cloned into the TOPO TA vector (Invitrogen). A minimum of three clones per tumor sample were sequenced from both directions using standard M13 forward and M13 reverse primers. The contigs were assembled and analyzed using the Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, MI).

### Western Blot Analysis

The preparation of whole-cell extracts of clarified cell lysates and the experimental procedures for Western blot analysis were described previously [19]. The following antibodies were used:  $\alpha$ -ActB (I-19; 1:2000 dilution) and  $\alpha$ -p53 (FL-393; 1:1000 dilution) from Santa Cruz Biotechnology;  $\alpha$ -p21 (sx118; 1:1000 dilution) from BD Biosciences (San Jose, CA);  $\alpha$ -ErbB2 (1:200 dilution) from Abcam (Cambridge, MA); and  $\alpha$ -p19/Arf (Ab-1; 1:1000 dilution) and  $\alpha$ -Mdm2 (Ab-2; 1:1000 dilution) from EMD Biosciences (San Diego, CA).

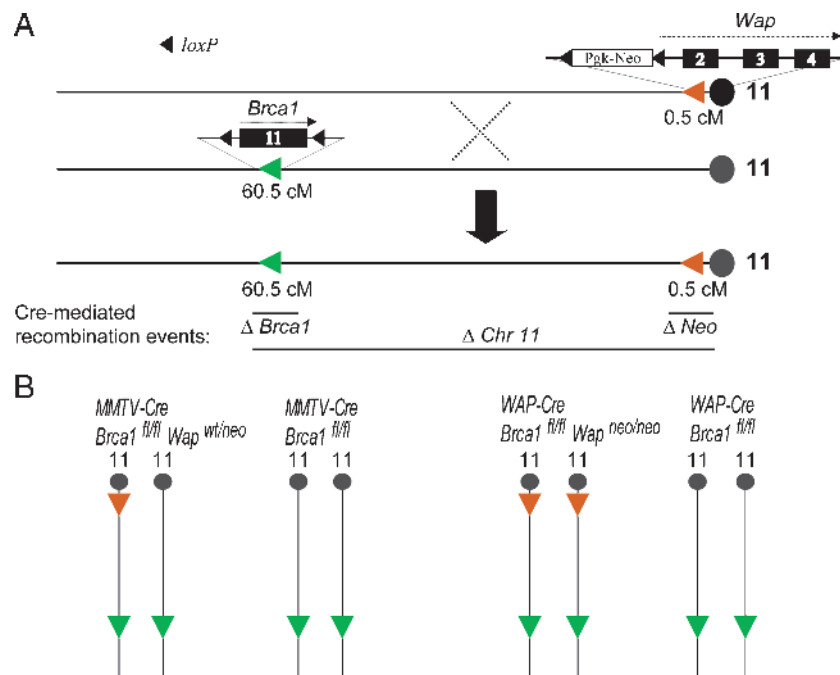
### Results

#### Accelerated Mammary Tumorigenesis in *Brca1*-Deficient females That Lack 60 cM of Chromosome 11

To determine whether tumor susceptibility loci reside on chromosome 11 that cooperate with the loss of *Brca1* during mammary cancer formation, we have engineered a mammary-specific knockout model for *Brca1* deficiency that also lacks nearly the entire chromosome 11. Figure 1A illustrates the generation of a mouse chromosome 11 that contains *loxP* sites that are approximately 60 cM apart. The targeted replacement of exon 1 of the *whcy acidic protein* (*Wap*) gene with a floxed neomycin selection marker was described previously [13]. The *Wap* gene is located in close proximity (approximately 0.5 cM) to the centromere of chromosome 11. Similar to the *Wap* locus, the *Brca1* gene resides on the antisense strand but at a distance of approximately 60.5 cM away from the centromere. We previously described the gen-

eration of a *Brca1* conditional knockout allele that contains *loxP* sites adjacent to exon 11 [7]. Homozygous *Brca1* floxed (*Brca1<sup>fl/fl</sup>*) male mice were bred with females containing the *Wap* knockout locus to generate female mice that carry both targeted chromosomes 11. These females were subsequently mated with homozygous *Wap* knockout (*Wap<sup>neo/neo</sup>*) males to generate *Wap<sup>neo/neo</sup>* offspring that also carry a *Brca1* floxed allele as a result of crossover events between both genetically modified chromosomes 11 during meiosis in the oocytes. The resulting *Brca1<sup>fl/wt</sup> Wap<sup>neo/neo</sup>* mice were bred to obtain the floxed chromosomes 11 in homozygosity (*Brca1<sup>fl/fl</sup> Wap<sup>neo/neo</sup>*). The presence of the floxed loci on both homologous chromosomes prevents the segregation of the targeted loci through subsequent crossover events that occur quite frequently within the 60-cM region between *Wap* and *Brca1*. Because pups thrive poorly on *Wap*-deficient dams [13], the maintenance of this new strain also requires a prolonged lactation period of at least 4 weeks.

To generate a mammary-specific knockout of *Brca1* and a long-range deletion on chromosome 11, *Brca1<sup>fl/fl</sup> Wap<sup>neo/neo</sup>* females were crossed with MMTV-Cre (line A) or WAP-Cre transgenic males that also possess two *Brca1* floxed alleles. This breeding strategy avoids the segregation of the mutant *Brca1* and *Wap* loci during the introduction of the Cre transgenes. MMTV-Cre and WAP-Cre *Brca1<sup>fl/fl</sup> Wap<sup>wt/neo</sup>* males were subsequently mated with *Brca1<sup>fl/fl</sup> Wap<sup>neo/neo</sup>* females in an attempt to create mice that carry the respective Cre transgenes in a homozygous chromosome 11 floxed background. This strategy worked for the WAP-Cre transgene in the expected mendelian fashion, but we only obtained seven viable MMTV-Cre *Brca1<sup>fl/fl</sup> Wap<sup>neo/neo</sup>* mice after



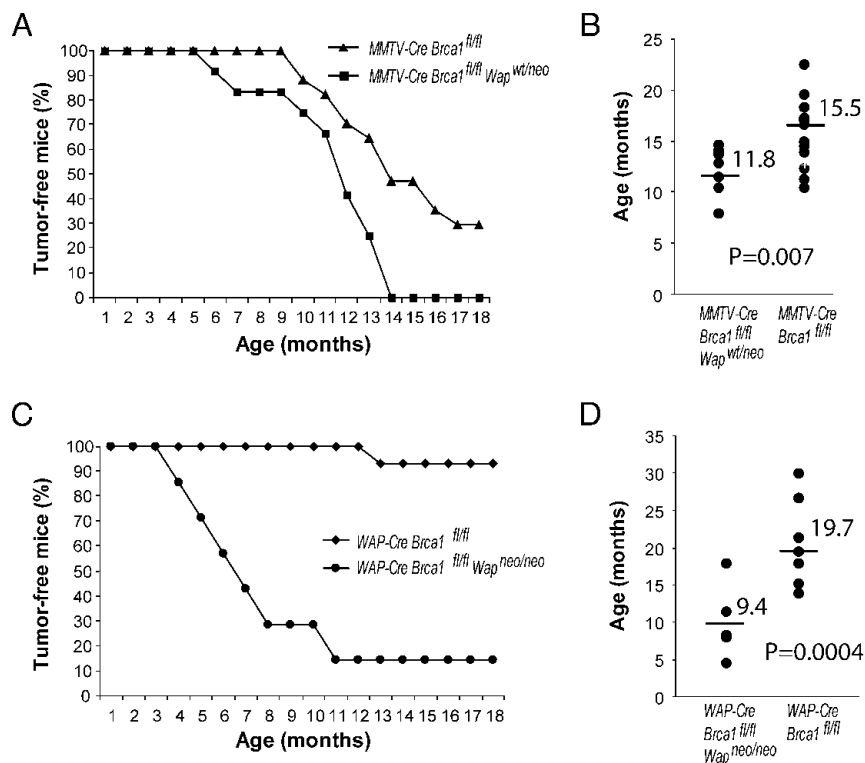
**Figure 1.** Generation of conditional knockout mice that are *Brca1*-deficient and that lack 60 cM of chromosome 11. (A) A crossover event between the two homologous chromosomes 11 that carry a targeted *Wap* locus and a conditional knockout allele of *Brca1*, respectively, produces a single chromosome with the two targeted loci. Because *Wap* and *Brca1* are directly oriented on the antisense strand of chromosome 11, the expression of Cre recombinase is able to catalyze two short-range excision events (i.e., exon 11 of *Brca1* and the PGK-*neo* selection marker of *Wap*) and a long-range deletion of approximately 60 cM between the two targeted loci. (B) A mammary-specific deletion of *Brca1* and a very large portion of chromosome 11 is achieved in experimental animals that carry the MMTV-Cre or WAP-Cre transgenes in a homozygous *Brca1* floxed background where one or both homologous chromosomes 11 contain a targeted *Wap* locus (*Wap<sup>wt/neo</sup>* and *Wap<sup>neo/neo</sup>*, respectively). MMTV-Cre and WAP-Cre-based *Brca1* conditional knockout females that carry two wild type *Wap* alleles served as controls.

genotyping more than 40 litters. Three of the seven MMTV-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>neo/neo</sup>* mice succumbed to T-cell proliferative disorders and exhibited a wasting syndrome between the ages of 4 and 7 months. One mouse developed a mammary cancer, one mouse died during a surgical procedure, and two mice are still alive to date. Because the MMTV-Cre transgene is not located on chromosome 11 and the MMTV-Cre-mediated deletion of *Brca1* does not cause embryonic lethality, this observation might suggest that the long-range deletion event might occur quite efficiently during embryogenesis in various cell types that efficiently express Cre under the MMTV-LTR [20]. Because of embryonic lethality, we maintained MMTV-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>wt/neo</sup>* along with WAP-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>neo/neo</sup>* females as the two experimental groups (Figure 1B) and compared their tumor latency to the two control cohorts of regular *Brca1* conditional knockouts (i.e., MMTV-Cre and WAP-Cre *Brca1<sup>fl/fl</sup>* mice). All females were multiparous and had delivered two to three litters. As illustrated in Figure 2, the MMTV-Cre-mediated long-range deletion event on just one of the two homologous chromosomes 11 was sufficient to accelerate *Brca1*-associated mammary tumorigenesis. All experimental animals had succumbed to mammary cancer by the age of 14 months (Figure 2A). The mean tumor latency was shortened by approximately 4 months (Figure 2B). The most noticeable difference was observed in the WAP-Cre-based experimental group. We previously reported that, in comparison to MMTV-Cre *Brca1<sup>fl/fl</sup>* females, WAP-Cre-based conditional *Brca1* knockout mice have a reduced incidence in mammary tumor formation and a prolonged latency [7]. This fact was confirmed in our current control cohort where only very few WAP-Cre *Brca1<sup>fl/fl</sup>* fe-

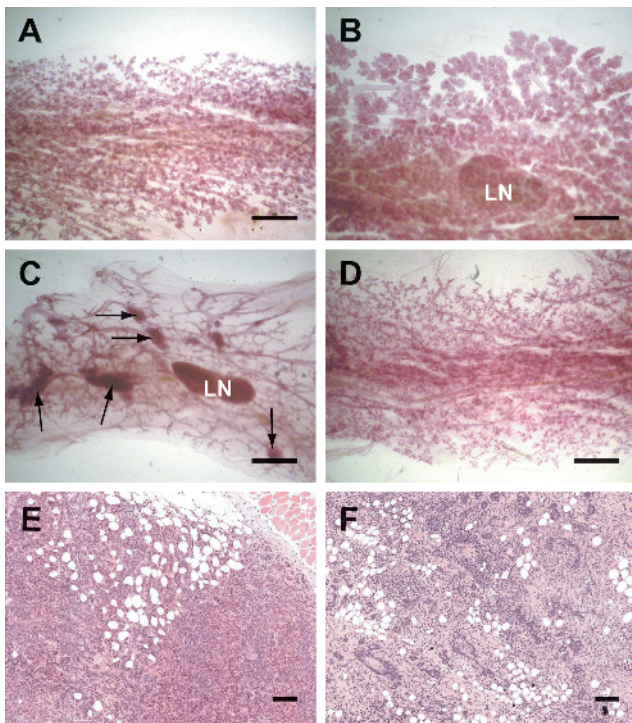
males developed mammary cancer by the age of 18 months (Figure 2C). The likelihood that a long-range deletion event occurs on one of the two homologous chromosomes 11 had a significant impact on the tumor-free survival of the experimental females, and their average life expectancy was shortened by approximately 10 months (Figure 2, C and D).

### Mammary Tumors That Arise in MMTV-Cre and WAP-Cre-Mediated Chromosome 11 Deletion Models Exhibit Differences in Their Histopathologic Features

The mammary-specific deletion of *Brca1* causes a selective growth inhibition of *Brca1*-deficient epithelial cells and impaired mammo-genesis [7]. The severity of the phenotypic abnormalities during ductal elongation and alveologenesis can vary between animals and depends greatly on the expression pattern of the Cre recombinase driven by the *MMTV-LTR* or the *Wap* gene promoter. None of the MMTV-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>wt/neo</sup>* females was able to lactate during the first and second gestation periods owing to impaired alveologenesis (Figure 3A), and some animals also exhibited a reduction in ductal branching (not shown). The developmental abnormalities in these experimental animals were probably a direct consequence of *Brca1* deficiency in basal and luminal epithelial cells throughout the developing ductal system [20]. In contrast, the expression of the WAP-Cre transgene occurs mainly during alveolar development. This transgene is also known to exhibit a mosaic expression pattern, which, depending on the deleted gene, can result in a negative selection of knockout cells. These knockout cells can be replaced in the developing alveolus by proliferating alveolar progenitors that do not express Cre [15].



**Figure 2.** Tumor-free survival of *Brca1*-deficient females that lack 60 cM of chromosome 11 (MMTV-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>wt/neo</sup>*,  $n = 12$ , and WAP-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>neo/neo</sup>*,  $n = 7$ ) in comparison to *Brca1* conditional knockout mice (MMTV-Cre *Brca1<sup>fl/fl</sup>*,  $n = 17$ , and WAP-Cre *Brca1<sup>fl/fl</sup>*,  $n = 15$ ). Mice were monitored twice weekly for a period of 18 months and killed when a tumor became palpable. (A, C) Kaplan-Meier curves. (B, D) Mean age of onset of palpable mammary tumors (horizontal bars). Each marker represents the age of onset of the first palpable tumor per mouse.



**Figure 3.** Differences in mammarygenesis and the histopathology of mammary tumors in MMTV-Cre-based (A, C, E) and WAP-Cre-based (B, D, F) *Brca1*-deficient females that also carry a 60 cM floxed region of chromosome 11. (A–D) Carmine red-stained mammary gland whole mounts in postpartum dams several hours after delivering the offspring (A, B) and in 1-year-old multiparous females (C, D). *LN* indicates lymph node. Bar, 1 mm. Multifocal lesions were only present in the MMTV-Cre-based deletion models (C, arrows). (E, F) Hematoxylin and eosin-stained sections of mammary tumors. Bar, 100  $\mu$ m.

Therefore, most WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females were able to lactate despite a reduction in the overall alveolar density (Figure 3B). The subsequent analysis of mammary glands from parous, tumor-bearing animals showed that many MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> females exhibited multifocal lesions throughout the ductal tree (Figure 3C), and a subset of these animals had several palpable mammary cancers. Because multifocal tumorigenesis was a rare event in the control cohort, this observation suggests that the MMTV-Cre-mediated long-range deletion event occurs in a significant subset of epithelial cells. Similar to the controls, WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females generally did not exhibit multiple macroscopic and microscopic lesions (Figure 3D). This was likely the result of a more restricted expression of the WAP-Cre transgene in luminal epithelial cells of developing alveoli during pregnancy and lactation. Also, the histopathologic analysis of large mammary cancers revealed important differences in both models. The mammary cancers that arose in the MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> females were predominantly adenocarcinomas that invaded into the surrounding stroma (Figure 3E). In contrast, a significant number of WAP-Cre-mediated mammary tumors were classified as benign lesions (Figure 3F). These palpable tumors were soft and contained large inflammatory regions and reactive hyperplasia. On the histopathologic level, these lesions exhibited features of severe acute and chronic mastitis with fibrosis. These large mammary tumors, which tend to appear in WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females within a month after weaning

of their last litter, had a significant negative impact on the general health of the animals that consequently needed to be euthanized. None of the WAP-Cre *Brca1*<sup>fl/fl</sup> control mice exhibited such distinct mammary lesions. We are currently unable to fully explain why only WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females with accelerated mammary tumorigenesis predominantly developed benign, inflammatory lesions. The *Stat3* gene, which is located in very close proximity to *Brca1*, is required for the initiation of apoptosis of differentiated alveolar cells after weaning of the young. Females that lack *Stat3* in secretory epithelial cells exhibit a delay in mammary gland remodeling and have a higher incidence in mastitis [21]. We therefore considered that a long-range deletion event on both homologous chromosomes 11 might result in *Stat3*-deficiency in differentiated epithelial cells. In support of this notion, active *Stat3* was not present in most epithelial cells within hyperplastic lesions from WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females. In contrast, nuclear *Stat3* was expressed in nearly all fibrous stromal cells adjacent to these benign lesions (Figure W1, A and B). However, we never observed impaired remodeling and prolonged survival of a significant subset of secretory epithelial cells throughout the mammary glands of WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females immediately after weaning of the offspring. This suggests that such long-range recombination events occur rarely and do not lead to widespread milk stasis and mastitis.

#### *Mammary Cancers That Are Deficient in Brca1 and Haploinsufficient in Chromosome 11 Express Basal Epithelial Cell Markers*

The histopathologic examination of large mammary tumors that arose in the WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females revealed that these tumors were mostly benign, inflammatory lesions. A subsequent analysis of expression patterns of cytokeratins (CKs) 5, 6, 8, and 14 as well as smooth muscle actin (SMA) showed that all different epithelial subtypes were present in these hyperplastic and fibrocystic lesions (not shown). We therefore focused our attention on the expression of these markers in the invasive mammary cancers that predominantly arose in the MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> mice. These cancer cells expressed low levels of CK5 compared to adjacent normal epithelial cells (Figure 4A), and they expressed moderate levels of CK6 (Figure 4B). The bulk of these tumors contained many cancer cells that expressed high levels of CK14 but not CK8 (Figure 4, C and D). In addition, we used mammary cancers from MMTV-*neu* mice as positive and negative controls for the examination of these cellular markers. In contrast, MMTV-*neu* tumors arise in the luminal epithelial compartment, and consequently, they express higher levels of CK8 and little CK14 compared to adjacent normal mammary ductal cells (Figure 4, E and F). Analogous to CK14, MMTV-*neu*-overexpressing mammary cancer cells exhibited very little expression of SMA (Figure W2B). The expression of this myoepithelial marker was significantly higher in *Brca1*-deficient mammary cancers, in particular, in cells that invaded into the stroma. Within the bulk of the tumor, the SMA staining was somewhat reduced and seemed to be weaker than the immunoreactivity in well-differentiated ductal cells that were engulfed by the invading cancer cells (Figure W2A). Collectively, the analysis of the cytokeratin markers and SMA expression pattern shows that mammary cancers that are deficient in *Brca1* and haploinsufficient in chromosome 11 are of basal epithelial origin, and they are distinctly different from *Her2/neu*-overexpressing mammary cancers and benign lesions that arose in the WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> females. Like *Her2/neu*-overexpressing tumors, mammary cancers from MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> females lack the expression of the estrogen receptor (Figure W2, C and D).

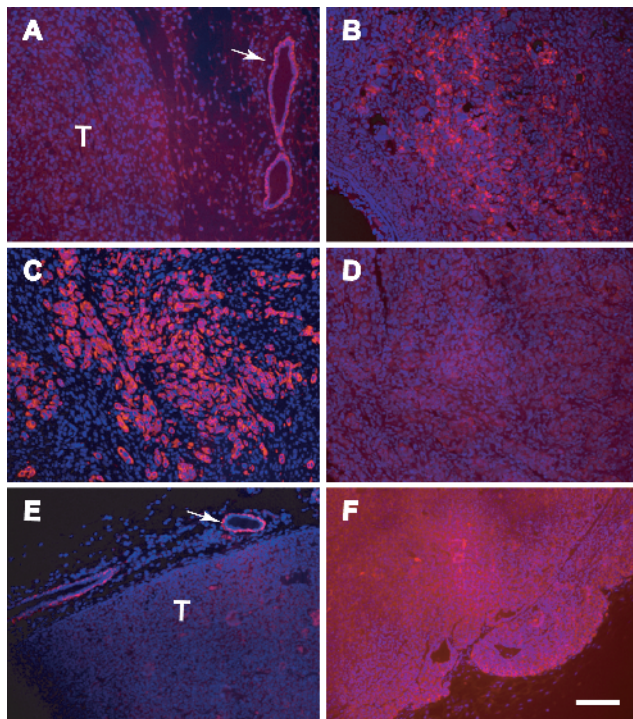
However, ER $\alpha$ -positive mammary epithelial cells were present in hyperplastic lesions in WAP-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>neo/neo</sup>* mice (Figure W2E).

### *Brca1* Deficiency and Haploinsufficiency in 60 cM of Chromosome 11 Cause Widespread Genome Instability

Previous studies demonstrated that *Brca1*-deficient mammary cancer cells were aneuploid, and chromosomal translocations as well as loss and gain of chromosomal regions were observed [22,23]. To assess the extent of genome instability in mammary cancers that arose in MMTV-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>wt/neo</sup>* females, we performed a SKY analysis on short-term cultured mammary cancer cells that were derived from two tumor-bearing animals (Figure 5). The SKY analysis confirmed that these cancers originated from mammary epithelial cells that were haploinsufficient in a very large portion of one chromosome 11, suggesting that such large segmental deletion events of more than 60 cM can be achieved through Cre-mediated recombination. According to information obtained from the Mouse Genome Informatics database, more than 1500 annotated genes and expressed sequences reside between *Wap* and *Brca1* on chromosome 11. The fact that neoplastic mammary epithelial cells are able to survive with a single copy of such a large number of genes is remarkable, and this finding clearly underlines the extraordinary plasticity that cancer cells possess. It might also suggest that mammary epithelial cells are capable of developing mechanisms that will allow them to compensate for the lack of important loci that are expressed in these cells. Normal

cells in other tissues and organs might not have that ability, and they express different genes located at chromosome 11 that are essential for their survival. For example, segmental deletions and the resulting haploidy of just 3 to 4 cM within the same region of chromosome 11 cause early embryonic lethality [24]. In addition, mutagenesis screens have identified 59 lethal mutations between the *Trp53* gene and the *Wnt3* locus, which resides approximately 2.5 cM distal of *Brca1* [25].

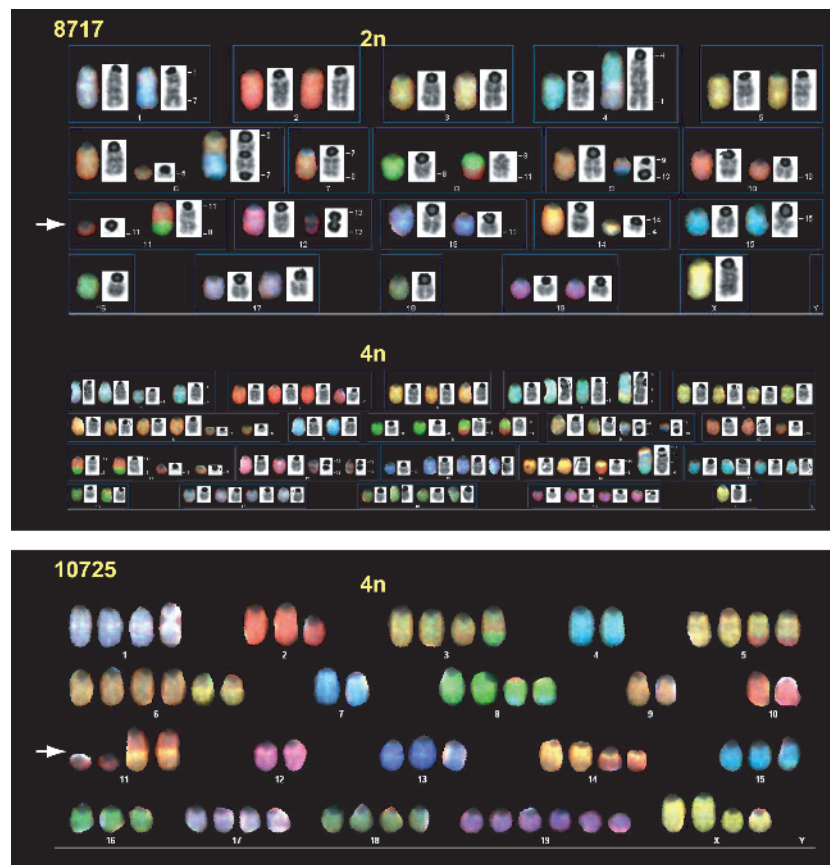
Besides confirming the long-range deletion event, the SKY analysis and examination of more than 20 metaphase spreads per tumor also demonstrates that *Brca1* deficiency and chromosome 11 haploinsufficiency cause widespread genome instability with numerous chromosomal aberrations that are also summarized in Table 1. A comparison of the specific aberrations between 2n and 4n cells [for example, the translocation event T(8C1;11C)] shows that the duplication of the genome and a higher degree of aneuploidy occur rather late in tumor progression. In addition to the long-range deletion event, both mammary cancers exhibited chromosomal breaks and translocations within the central region of the homologous chromosome 11, which is known to contain the *Trp53* tumor-suppressor gene. We used FISH analysis to determine whether the *Trp53* locus was affected or lost as a consequence of the chromosomal rearrangements (Figure 6). In addition to the *Trp53* probe and the chromosome 11 paint, we used a genomic probe encompassing the *Septin 9* (*Spet9*) locus to label the distal region of chromosome 11. This particular chromosomal segment, which is orthologous to human 17q25.3, has been reported to be amplified and overexpressed in a subset of murine adenocarcinomas and human breast cancer cell lines [17]. The results of this study show that the chromosomal breaks and translocations seemed to have occurred in both tumors in close proximity of the *Trp53* locus. In addition, the dissociation of *Trp53* from the *Sept9* locus indicates that, unlike the distal region of chromosome 11, the *p53* gene itself was not translocated. Because *Sept9* is located outside the long-range deletion event, it was still present on the two short chromosomes 11 in 4n metaphase spreads.



**Figure 4.** Mammary cancers that are deficient in *Brca1* and haploinsufficient in chromosome 11 are of basal epithelial origin. Immunostaining of cytokeratins 5 (A), 6 (B), 14 (C, E), and 8 (D, F) using Alexa Fluor 594-conjugated secondary antibodies (red) in mammary cancers from MMTV-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>wt/neo</sup>* females (A–D) and *Her2/neu*-overexpressing controls (E, F). The slides were counterstained with DAPI (blue nuclei). Arrows in panels A and E indicate the positive staining of cytokeratins 5 and 14 in normal mammary epithelial cells adjacent to the primary neoplasm. Bar, 100  $\mu$ m.

### *Somatic Mutations in p53 Contributed to Brca1-Associated Mammary Tumorigenesis*

The previous work on the mammary-specific *Brca1* deletion model shows that rearrangements in chromosome 11 and aberrant *p53* expression contribute to neoplastic transformation [7]. In accordance with this finding, Weaver et al. [23] reported that *Brca1*-deficient mammary cancers showed a loss of the proximal portion of chromosome 11, which may have included the *Trp53* gene. Because both tumors that were examined by SKY and FISH exhibited chromosomal breaks and translocations within the central region of chromosome 11, we assumed that these translocations might have affected the expression of *p53*. Surprisingly, the results of a reverse transcription-polymerase chain reaction assay show that this gene is transcriptionally active in these two and two additional cancers (Figure 7A). One of four tumors examined expresses a *p53* protein, which seems to be transcriptionally inactive based on the lack of *p21* expression (Figure 7B). The notion that this tumor contains a somatic mutation in the coding region of *Trp53* is supported by the fact that the *p53* protein is highly up-regulated. HC11 cells that possess a transcriptionally inactive mutant of *p53* and that lack *p21* expression were used as appropriate controls. After examining the expression of *p53*, we sequenced the entire coding region of the *p53* transcripts in the four different cancer cases (Table 2). Somatic mutations were detected in three mammary cancers, and two of these mutations lead to a premature termination of the coding



**Figure 5.** The SKY analysis of adenocarcinomas from two MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> females. Arrows indicate chromosomes 11 in both karyotypes that underwent Cre-mediated long-range deletion events. In addition, the SKY analysis revealed that both homologous chromosomes 11 carried translocations with chromosomes 8 and 14, respectively.

region. Tumor 8717, which expresses the nonfunctional p53 protein, exhibited a deletion of three nucleotides that caused the absence of a single amino acid (R246) from the DNA binding domain of p53. We were unable to identify a somatic mutation of *p53* in tumor 10725, and the underlying alteration of the function of the encoded p53 protein (as assessed by the lack of p21 expression) remains elusive. Neither the down-regulated expression of p19/Arf (*Cdkn2a*) nor the overexpression of Mdm2 contributed to the functional inhibition of p53 in all four cancers (Figure 7B). It should also be noted that the examination of p53 and p21 expression can be skewed by the presence of stromal cells within the primary cancer cell cultures. In one instance, this was evident in explanted tumor cells of from animal 11704, which exhibited a weak expression of p21 due to the presence of fibroblasts. P21, however, could not be detected by immunostaining in the nuclei of neoplastic epithelial cells of the corresponding primary lesion from this animal (Figure W3). In summary, our study shows that, despite chromosomal rearrangements in the central portion of the chromosome 11, which did not undergo Cre-mediated excision, the *Trp53* locus was still transcriptionally active. Somatic mutations in *p53* were responsible for the absence or functional inhibition of this tumor suppressor in a subset of *Brca1*-associated mammary cancers in our animal model.

Human *Brca1*-associated breast cancers more frequently exhibit a basal phenotype, and they lack expression of ER $\alpha$ , PR, and ErbB2 [26–28]. In contrast, Brodie et al. [22] reported that most *Brca1*-deficient mammary tumors in mice overexpressed ErbB2. In addition

to this apparent inconsistency between humans and mice, we also asked whether the expression of ErbB2 could have been altered by the long-range Cre-mediated deletion event or the observed translocations that involve the homologous chromosome 11. According to

**Table 1.** Composite Karyotypes of Two Mammary Cancers After Examination of More Than 20 Metaphases Per Tumor Using SKY Analysis.

Composite karyotype of tumor 8717

**2n cells:** 40,XX[cp8]/38,X,Der(4)T(1E;4D3)[6],Del(4D)[2],+Del(6B)[8],-7[8], Del(8C3)[8],T(8C1;11C)[8],Dic(9A.13A)[8],-10[3],Del(10A)[3],Del(10C)[2], **Del(11A2-D)[8]**,+Del(12C)[3],Der(12;12)T(12A2;12A1)Del(12A2)[8],Del(13C)[8], Der(14)T(4D3;14B)[8],-16[8][cp8]

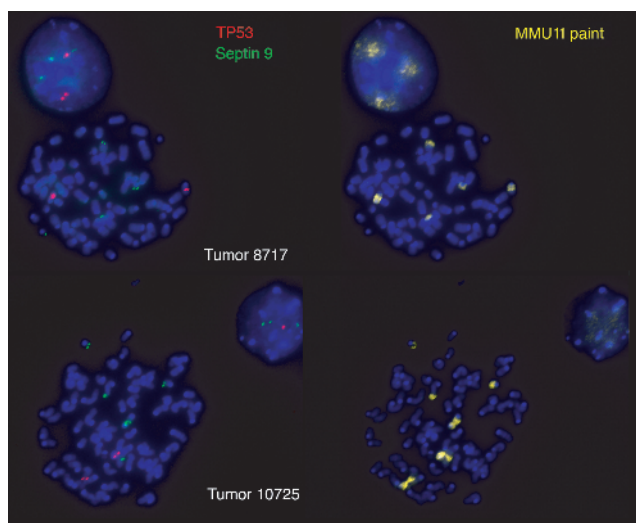
**4n cells:** 67-77<4n>,X or XX,-1[3],-2[4],-3[4],Der(4)T(1E;4D3)X1[1], Der(4)T(1E;4D3)X2[5],+Del(6B)X2[7],-7X1[1],-7X2[5],Del(8C3)X2[7], T(8C1;11C)X1[1],T(8C1;11C)X2[6], Dic(9A.13A)X1[1],Dic(9A.13A)X2[6],-10[2],Del(10A)X1[1], Del(10A)X2[2],Del(10C)[4], **Del(11A-D)X2[7]**,Der(12;12) T(12A2;12A1)Del(12A2)X1[2], Der(12;12)T(12A2;12A1)Del(12A2)X2[5],-13[3],Del(13C)X1[4],Del(13C)X2[3], Der(14)T(4D3;14B)X1[2],Der(14) T(4D3;14B)X2[4],-15[5],-16X1[2],-16X2[5],-17[3][cp7]

Composite karyotype of tumor 10725

**2n Cells:** 40,XX[cp4]

**4n Cells:** 67-83<4n>,XX,Del(XB)X1-2[17],-1[5],+1[2],Del(2F)X1-2[17],-3[5], Der(3)T(3D;9E)X1-2[16],Der(3)T(3D;9E;8C)[6],-4X2[16],Der(4)T(4D;15E)[4], T(4D;15E)X1-2[16],Der(5)T(5E;3;11)X2[17],Del(6B)[3],+Der(6)T(5E;6B)X2[17],-7X2[17], Del(8B)[3],Der(8)T(4;8C)X2-3[17],-9X2[17],10X2[17],T(10C;12C)X2[2], **Del(11A2-D)X2[17]**, Der(11)T(11C;14B)[2],T(11C;14B)X1-2[17],-12X2[17],-13X1-2[10],Der(14)T(11C;14B)[2],-15[4],-16[7],-18[4],+19[2][cp17]

The long-range deletion event in chromosome 11 is highlighted in bold font. Please note that in contrast to tumor 8717, tumorigenic cells with a 2n karyotype were not present in tumor 10725. It is likely that the normal karyotype of 2n cells in this tumor represents that of dividing fibroblasts that were present in the short-term culture.

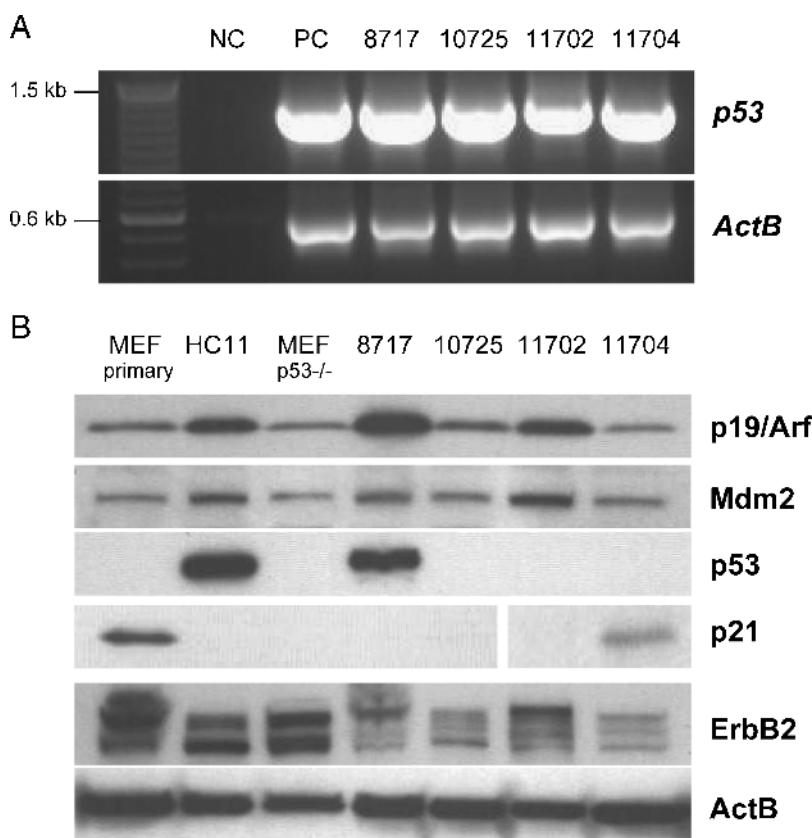


**Figure 6.** Fluorescence *in situ* hybridization to determine the localization of the *Trp53* gene (red) and the *Sept9* locus (green) in adenocarcinomas from two MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> females. A specific paint (MMU11) was used to counterstain individual fragments of chromosome 11.

the current Mouse Genome Informatics sequence information, the *ErbB2* gene resides proximal of *Brca1* on chromosome 11 (i.e., 57 cM from the centromere) and therefore within the long-range deletion segment. The Western blot analysis, however, shows that the ErbB2 protein expression was not significantly altered in *Brca1*-deficient cells that were haploinsufficient in a large portion of chromosome 11 (Figure 7B). The levels of ErbB2 expression in the mammary cancer cells were equal to or lower than the expression level observed in nontumorigenic, immortalized mammary epithelial cells (Figure 7B, HC11 cells in lane 2). Therefore, the previously reported up-regulation of ErbB2 may not be a common feature or necessity in *Brca1*-deficient murine mammary cancers.

## Discussion

In the *Brca1* conditional knockout model generated by Xu et al. [7], the Cre-mediated deletion of exon 11 of *Brca1* results in a shift from a predominantly full-length transcript to a shorter mRNA splice variant that encodes a *Brca1* protein of approximately 90 kDa [29]. This short *Brca1* isoform occurs, albeit at much lower levels, in the normal mammary gland. The sole expression of the *Brca1* Δexon 11 isoform in mammary epithelial cells is sufficient to initiate neoplastic transformation after a long latency [7]. It has been recently suggested



**Figure 7.** Somatic mutations caused p53 deficiency in mammary cancers that are deficient in *Brca1* and haploinsufficient in chromosome 11. (A) Reverse transcription–polymerase chain reaction analysis of the transcriptional activation of the *Trp53* gene in four mammary cancers from MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> mice; *NC* indicates negative control [total RNA from p53-deficient mouse embryonic fibroblasts (MEFs)]; *PC*, positive control (total RNA from primary wild type MEFs). (B) Western blot analysis to determine the expression of the p53 protein and its downstream target p21<sup>Cip1</sup> as well as the expression of upstream regulators of p53 (p19/Arf and Mdm2) and the ErbB2 receptor tyrosine kinase. Beta-actin (*ActB*) served as a loading control. Cell lysates from primary wild type and p53-deficient MEFs were used as positive and negative controls for p21 and p53 expression. Because wild type p53 is barely detectable in primary MEFs, we used HC11 cell extracts as a positive control for the expression of mutant p53, which is known to be expressed at much higher levels. HC11 cells also served as a control for the baseline expression of ErbB2 in untransformed mammary epithelial cells.



**Table 2.** Mutations in the Coding Region of the *Trp53* Gene in Four Independent Tumor Samples of Brca1-Deficient Mammary Cancers That Are Haploinsufficient in Chromosome 11.

Tumor No.	Mutation	Location	Location
8717	Del(735-737)	Del(R246)	DNA binding domain
10725	wild type	—	—
11702	T927G transversion	Y324Stop	Homo-oligomerization domain
11704	C421T transition	Q141Stop	DNA binding domain

that the estrogen receptor (ER $\alpha$ ) plays a role in premalignant transformation in this model [30]. Elevated levels of estrogen resulted in accelerated mammary cancer formation in Brca1-deficient mice that lacked one functional *Trp53* allele. Similar to this report, we also observed that ER $\alpha$  was predominantly expressed in the benign, inflammatory lesions that originated in WAP-Cre *Brca1<sup>fl/fl</sup> Wap<sup>neo/neo</sup>* females. Although these mammary tumors were palpable and relatively large, they were distinctly different from adenocarcinomas of similar size that originated in the MMTV-Cre-mediated Brca1-deficient mammary cancer model that was haploinsufficient in chromosome 11. The latter model seems to more closely resemble key features of human *Brca1*-associated breast cancers. They exhibit a basal phenotype as assessed by the expression profile of keratins, they are ER $\alpha$ -negative, and they do not exhibit an up-regulation of ErbB2. The differences between these two models, therefore, do not support the notion that Brca1-deficient mammary cancers originate from ER $\alpha$ -positive precursor lesions. Recently, published findings by Jones et al. [31] demonstrate that, although the overexpression of ER $\alpha$  accelerates Brca1-associated mammary tumorigenesis, exogenous levels of this steroid hormone receptor increases the incidence in preneoplastic lesions and mammary cancers that are ER $\alpha$ -negative. Because it has been shown that ER $\alpha$  can stimulate the proliferation of human and murine mammary epithelial cells in a juxtacrine or paracrine manner [32–34], it is possible that ER $\alpha$  promotes the neoplastic growth of adjacent Brca1-deficient mammary epithelial cells that lack ER $\alpha$ .

ER $\alpha$ -negative adenocarcinomas that are Brca1-deficient and haploinsufficient in chromosome 11 exhibited normal or reduced levels of ErbB2, and we therefore concluded that the previously reported up-regulation of ErbB2 is not a requirement for Brca1-associated mammary cancer in mice. Similarly, the conditional deletion of the 3-prime region of *Brca1* (i.e., exons 22–24) resulted in ErbB2-negative lesions as determined by immunostaining [35]. Whereas the long-range, Cre-mediated deletion event in our model will result in a complete ablation of exons 11 through 24 of *Brca1* (Figure 1A), the homologous chromosome 11, however, will retain the expression of the  $\Delta$ exon 11 isoform of *Brca1*. Hence, the sole expression of this splice form may not be responsible for any differences in ErbB2 expression in Brca1-deficient mammary cancers as suggested by McCarthy and colleagues [35].

Females deficient in Brca1 that also lack approximately 60 cM of chromosome 11 develop mammary cancer after a significantly shorter latency compared to females that carry only a conditional mutation of *Brca1*. Nonetheless, the increase in tumorigenicity and penetrance of the phenotype is quite similar to Brca1 conditional knockout mice that lack one copy of the *Trp53* gene [7]. This observation suggests that the loss of heterozygosity of *Trp53* is the predominant genetic event responsible for an accelerated onset of the disease. In agreement with this conclusion, somatic mutations in *Trp53* in the remaining homologous chromosome 11 were tightly linked to the occurrence of mammary cancers. Conversely, this finding might also suggest that this large portion of chromosome 11 does not contain additional tumor-

suppressor genes that play a role in the initiation of Brca1-deficient mammary cancer as previously hypothesized. Consequently, the search for tumor-suppressor loci that modify the disease onset has to include other regions of the genome.

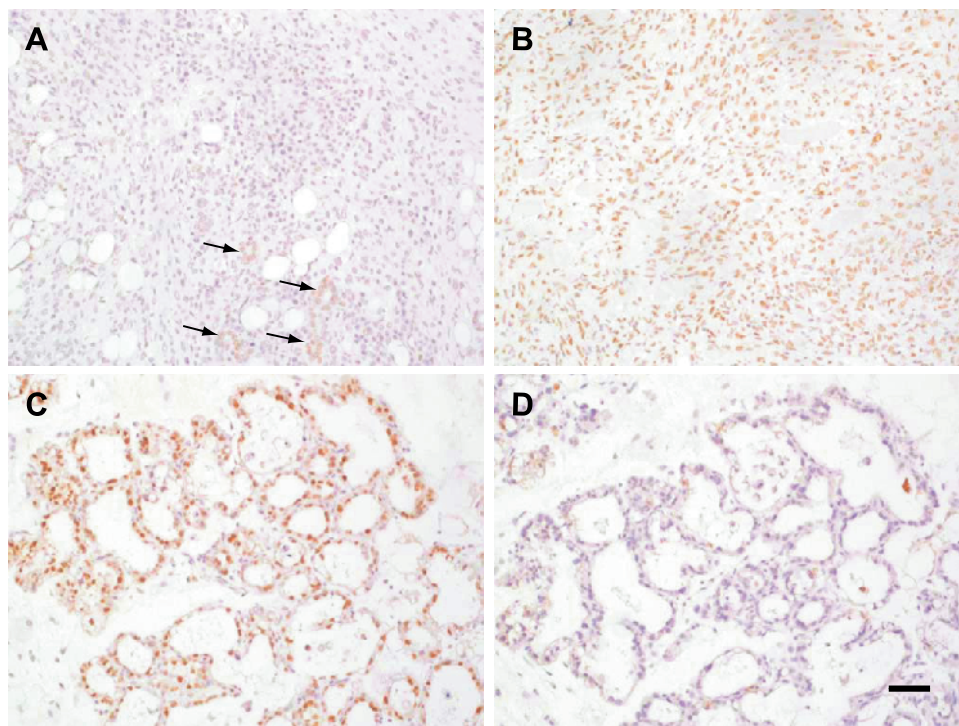
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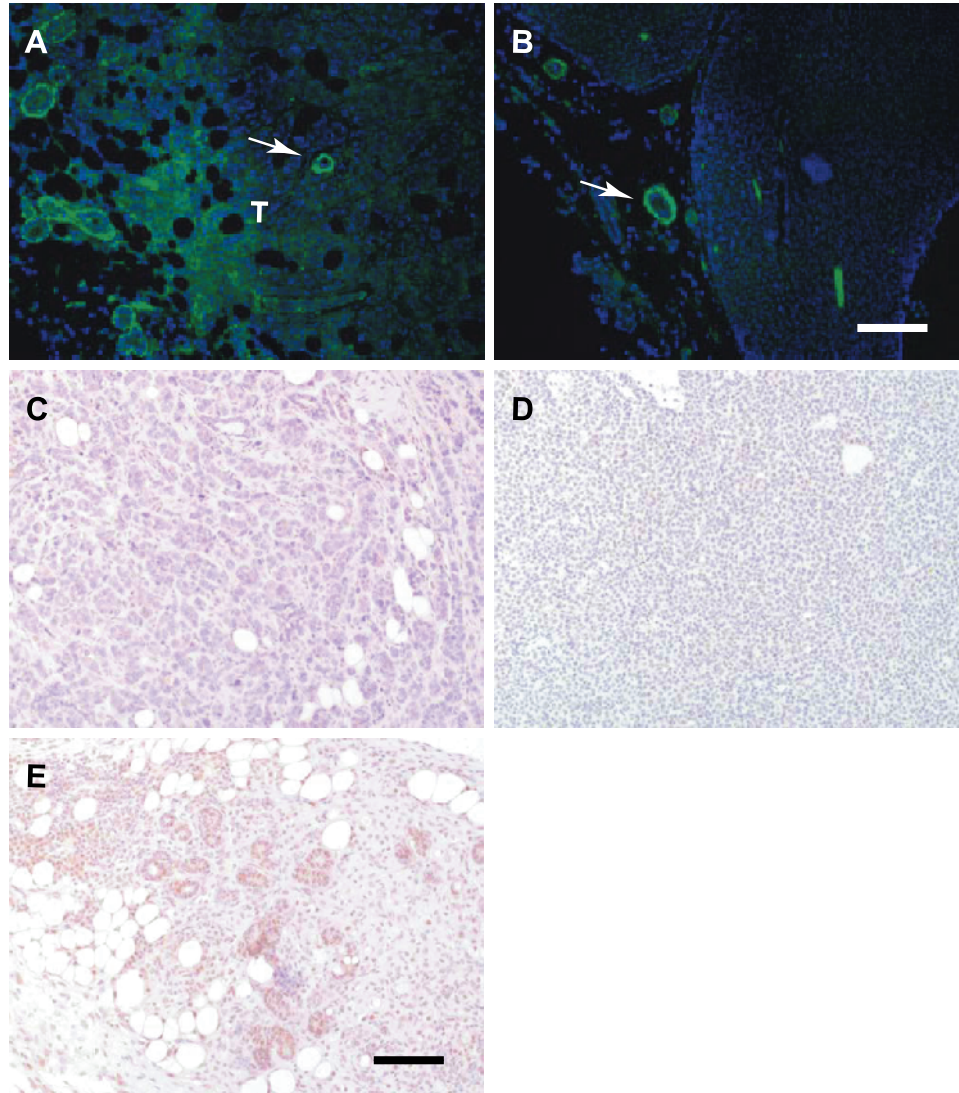
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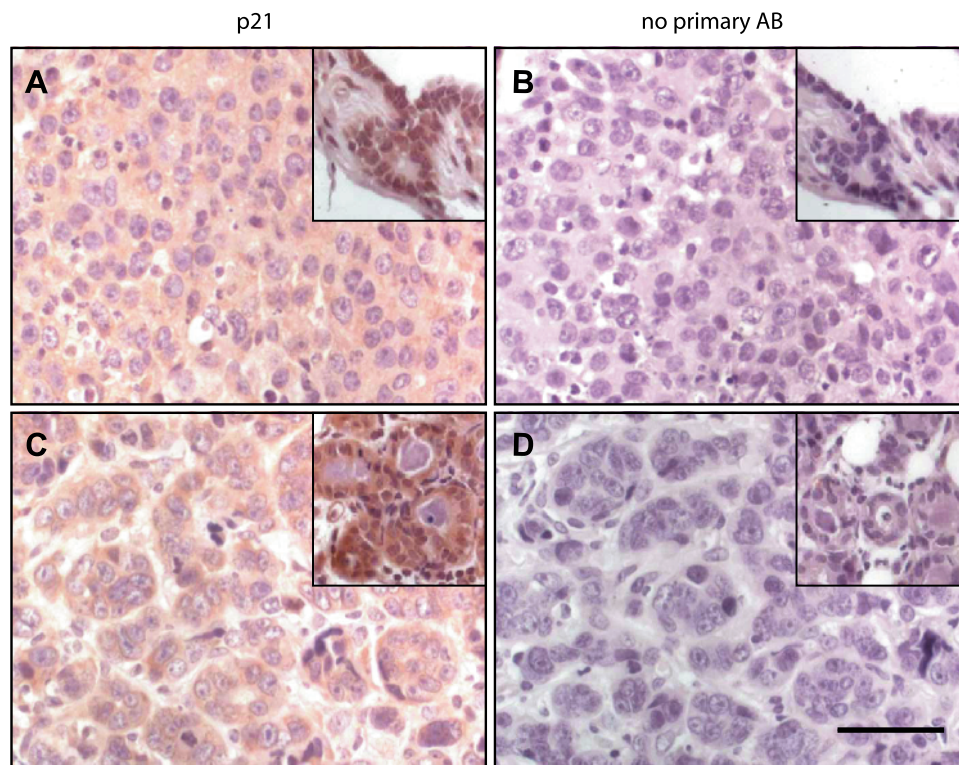
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**Figure W1.** Mammary epithelial cells in benign lesions of WAP-Cre *Brca1<sup>fl/fl</sup>* *Wap<sup>neo/neo</sup>* females lack expression of active Stat3. Immunostaining of tyrosine phosphorylated Stat3 in epithelial cells (A) and adjacent fibrous stromal cells (B) of the same hyperplastic lesion of a multiparous female. Arrows in panel A indicate few isolated epithelial cells with active Stat3. Mammary tissue from a female 48 hours after weaning the pups (C) served as a positive control. (D) Matching section of panel C, without the primary antibody against Stat3 (negative control). The slides were counterstained with hematoxylin. Bar, 50  $\mu$ m.



**Figure W2.** Mammary cancers that are deficient in *Brca1* and haploinsufficient in chromosome 11 are of basal epithelial origin and lack expression of estrogen receptor alpha ( $ER\alpha$ ). Immunofluorescence staining of SMA using an Alexa Fluor 488-conjugated secondary antibody (green) in a mammary cancer from an MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> female (A) and *Her2/neu*-overexpressing control (B). The slides were counterstained with DAPI (blue). Arrows in panels A and B indicate the positive staining of SMA in normal mammary epithelial cells adjacent to the primary neoplasm. Bar, 100  $\mu$ m. (C–E) Immunostaining of  $ER\alpha$  in mammary tumors from an MMTV-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>wt/neo</sup> female (C), a *Her2/neu*-overexpressing mouse as an  $ER\alpha$ -negative control (D), and a WAP-Cre *Brca1*<sup>fl/fl</sup> *Wap*<sup>neo/neo</sup> female (E). The slides were counterstained with hematoxylin. Bar, 100  $\mu$ m.



**Figure W3.** Brca1-deficient mammary cancers that are haploinsufficient in chromosome 11 lack nuclear expression of p21<sup>Cip</sup>. Immunostaining of p21 (A, C) as well as their corresponding controls without primary antibody (B, D) in neoplastic epithelial cells of tumor 11704 (A, B) and tumor 10725 (C, D). Insets show normal epithelial tissues adjacent to the primary tumor of the same histologic section. The slides were counterstained with hematoxylin. Bar, 50  $\mu$ m.