53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers

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Germ-line mutations in breast cancer 1, early onset (BRCA1) result in predisposition to breast and ovarian cancer. BRCA1-mutated tumors show genomic instability, mainly as a consequence of impaired recombinatorial DNA repair. Here we identify p53-binding protein 1 (53BP1) as an essential factor for sustaining the growth arrest induced by Brca1 deletion. Depletion of 53BP1 abrogates the ATM-dependent checkpoint response and G2 cell-cycle arrest triggered by the accumulation of DNA breaks in Brca1-deleted cells. This effect of 53BP1 is specific to BRCA1 function, as 53BP1 deletion did not alleviate proliferation arrest or checkpoint responses in Brca2-deleted cells. Notably, loss of 53BP1 partially restores the homologous-recombination defect of Brca1-deleted cells and reverts their hypersensitivity to DNA-damaging agents. We find reduced 53BP1 expression in subsets of sporadic triple-negative and BRCA-associated breast cancers, indicating the potential clinical implications of our findings.

BRCA1 and BRCA2 are large phosphoproteins involved in DNA-damage repair through homologous recombination (HR). Although BRCA1 and BRCA2 share many interacting proteins, they show little homology and are thought to have different roles in HR and other processes. BRCA1 is thought to be mainly a scaffold protein enabling interactions between different components of the HR machinery, whereas BRCA2 is directly involved in loading RAD51 to sites of damage or stalled replication forks.

Heterozygous BRCA1 and BRCA2 inactivation mutations are associated with an increased risk to develop breast and ovarian cancers. These tumors often show loss of heterozygosity of the wild-type allele and mutation of the p53 tumor suppressor4,5. Breast cancers that arise in BRCA1 mutation carriers are mostly high-grade tumors with the so-called triple-negative phenotype (that is, lacking expression of estrogen receptor and progesterone receptor and without amplification of human epidermal growth factor receptor (ERBB2/HER2))6. BRCA1-associated tumors also express basal epithelial cell markers, such as cytokeratin 5/6 (ref. 7), and cluster with basal-like breast cancers by gene expression profiling8. There is increasing evidence that a subset of sporadic tumors with a basal-like/triple-negative phenotype may have alterations in BRCA1-related pathways9. In contrast, BRCA2 mutation carriers develop mostly ER-positive breast cancers.

Whereas BRCA1 and BRCA2 function as tumor suppressors in breast and ovarian epithelium, homozygous deletion of BRCA1 or BRCA2 appears not to be tolerated during human or mouse development and in cultured primary cells such as mouse embryonic fibroblasts (MEFs) or stem cells10. Although concomitant deletion of p53 partially alleviates these phenotypes11, the incomplete rescue suggests the involvement of other factors in BRCA1/2 associated cancers. In search for such factors, using a candidate gene approach, knockout of 53BP1 was shown in a recent study12 to rescue Brca1 hypomorphic MEFs and mice from premature senescence. 53BP1, a DNA-damage response (DDR) factor involved in both HR and nonhomologous end joining (NHEJ), is known to be an activator of p53 (ref. 13). However, 53BP1 also has p53 independent function, and deletion of both 53BP1 and p53 has a synergistic effect on tumor development14,15.

The observations in the Brca1 hypomorphic mutants raise some intriguing questions. First, will 53BP1 ablation also rescue cells completely deficient for BRCA1, a situation that is common in BRCA1-associated tumors? In contrast to Brca1-null mice, the Brca1Δ11/Δ11 hypomorphic mice still express the natural BRCA1-Δ11 splice variant, which contains the conserved RING and BRCT domains13. The Brca1Δ11 allele is functionally active, as evidenced by the fact that homozygous Brca1Δ11/Δ11 mutants are viable on a p53 heterozygous background16. Other questions concern the mechanism by which deletion of 53BP1 rescues BRCA1-deficient cells and the potential relevance of 53BP1 status for BRCA1-associated cancers.

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In this work, we set out to explore these questions. We performed an unbiased transposon mutagenesis screen for factors that could restore normal growth of Brca1-null cells. Similar to the observations with Brca1Δ1/Δ1 hypomorphic mutants, clonal outgrowth of Brca1-null cells was rescued by a loss of function mutation of 53BP1. We show that cells lacking both Brca1 and 53BP1 have a partially restored HR pathway. The clinical relevance of these findings is indicated by our data showing that 53BP1 expression is reduced in a subset of basal-like/triple-negative breast cancers and in Brca1/2-associated breast cancers, suggesting positive selection for loss of 53BP1 function in these tumors.

RESULTS

53BP1 loss rescues proliferation defects of Brca1-null cells

Brca1 deletion in p53-proficient normal cells leads to a severe proliferation defect17. Cre/loxP-based conditional Brca1 knockout models would not be useful to screen for factors that enhance growth of Brca1-deficient cells, as Brca1-deleted cells are rapidly eliminated and the culture is rapidly overtaken by Brca1-proficient cells. To overcome this problem, we generated R26CreERT2 Brca1Sc o/E S cells with two different shRNAs that could be used to abrogate the growth defects of Brca1-null cells (Fig. 1a and Supplementary Fig. 1a). Furthermore, these cells contain the CreERT2 gene targeted to the Rosa26 locus, leading to expression of a tamoxifen-inducible CreERT2 recombinase fusion protein18. Incubation of these cells with 4-hydroxytamoxifen (4OHT) resulted in nearly complete switching of the Brca1Sc o allele and consequent loss of Brca1 protein expression (Supplementary Fig. 1b,c). Nonswitched R26CreERT2 Brca1Sc o/E S cells were effectively removed by puromycin selection (Supplementary Fig. 1e).

We used the piggyBac transposon system to perform an insertional mutagenesis screen for factors that rescue the proliferation defect of Brca1-deleted cells (Supplementary Data). We transfected R26CreERT2 Brca1Sc o/E S cells with plasmids containing an engineered piggyBac transposon and mouse codon-optimized piggyBac transposase. After induction of CreERT2-mediated deletion of the Brca1Sc o allele with 4OHT, we assayed for clonal survival of Brca1-deficient ES cells under puromycin selection (Supplementary Fig. 2a). The majority (294 out of 296) of surviving colonies analyzed contained both a switched and a nonswitched Brca1Sc o allele, indicating strong selection for allele duplication events (data not shown). Two clones that were completely Brca1 deleted showed identical patterns of piggyBac transposon integrations (Supplementary Fig. 2b), one of which mapped to intron 16 of the 53bp1 gene (Supplementary Fig. 2c) and correlated with abrogation of 53BP1 expression (Supplementary Fig. 2d), consistent with the reported ability of 53BP1 deletion to abrogate senescence and cell death in Brca1Δ1/Δ1 hypomorphic cells12. To validate the loss of 53BP1 expression as a survival factor in Brca1-null cells, we tested the effects of shRNA-mediated deletion of 53BP1 in R26CreERT2 Brca1Sc o/E S cells with two different shRNAs that efficiently suppressed 53BP1 expression, as determined by western blot analysis (Fig. 1b). The robust clonal growth arrest of R26CreERT2 Brca1Sc o/E S cells induced by 4OHT treatment was abolished when 53BP1 was depleted with either shRNA (Fig. 1c).

53BP1 loss rescues drug hypersensitivity of Brca1-null cells

A hallmark of BRCA1-deficient tumors is their cisplatin sensitivity21. Consistent with this, we observed enhanced cytotoxicity of cisplatin in our Brca1-deleted ES cells (Fig. 1d). shRNA-mediated loss of 53BP1 fully abolished the cisplatin sensitivity induced by Brca1 inactivation (Fig. 1d). We observed a similar reversal of drug sensitivity by 53BP1 shRNA, but not by p53 shRNA, treatment (Fig. 1e). Although shRNA-mediated inhibition of p53 suppressed the growth defects of Brca1-deleted cells (data not shown), it did not suppress cisplatin sensitivity, suggesting that p53 and 53BP1 provide distinct pathways for sustaining growth arrest in Brca1-deleted cells.

53BP1 loss blocks DNA-damage responses in Brca1-null cells

To investigate how suppression of 53BP1 or p53 alleviates the impaired proliferation of Brca1-knockout ES cells, we analyzed cell cycle profiles of 4OHT-treated R26CreERT2 Brca1Sc o/E S cells treated with control shRNA or shRNA causing depletion of 53BP1 or p53.
monitored by western blotting (Fig. 2a). In the absence of BRCA1, ES cells accumulate in G2 (Fig. 2b), which could reflect a checkpoint response induced by accumulation of unrepaired DNA damage. This G2 arrest is abrogated in 53BP1-deleted cells but not in p53-deleted cells, suggestive of an ataxia-telangiectasia mutated (ATM)-dependent checkpoint activation for which 53BP1 is essential. Consistent with this, we observed an increase in 53BP1 expression levels in Brca1-deleted cells (Fig. 2a). The less pronounced effect of p53 deletion on the G2 arrest is mirrored by the lower induction of p53 expression in response to Brca1 deletion, as detected by western blotting. p53 has a major role in the surveillance of chromosome integrity at the G1/S transition, although evidence for a relatively weaker p53-dependent checkpoint at the G2/M transition has also been reported22,23.

To address the possibility that the loss of 53BP1 affects the DNA damage response (DDR) induced by Brca1 deletion, we used MEFs that were established from mice carrying a Brca1<sup>5−13</sup> allele and a Brca2<sup>F−/−</sup>-null but not Brca1<sup>F−/−</sup>-null MEFs. The knockdown of 53BP1 in these cells led to a decrease in the occurrence of DNA breaks, reflected in diminished checkpoint responses.

In contrast to Brca1-knockout MEFs, proliferation of Brca2-knockout MEFs<sup>25</sup> was not rescued by shRNA-mediated depletion of 53BP1 (Fig. 3c). In contrast, p53 inhibition efficiently restored the proliferative capacity of Brca2-deleted cells. Consistent with the role of 53BP1 in mediating checkpoint responses specifically in cells lacking Brca1, 53BP1 abrogation did not affect CHK2 phosphorylation in BRCA1-deficient MEFs (Fig. 3b). This observation reflects the fundamentally distinct roles played by the BRCA1 and BRCA2 tumor-suppressor proteins in the DDR: whereas BRCA1 is required for the initial steps of the DDR response and signal amplification, BRCA2 functions downstream of the checkpoint signaling, promoting the HR pathway of DNA repair. Because 53BP1 acts during the early chromatin-remodeling events at the break, it is more likely to

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**Figure 2** 53BP1 depletion rescues cell-cycle defects of Brca1-null ES cells. (a) Western blot analysis of 53BP1 and p53 expression in R26<sup>CreERT2</sup> Brca1<sup>ScCo</sup> ES cells stably transduced with lentiviral shRNA vectors against 53bp1 or p53. Samples were taken before or at 4 and 9 d after 4OHT-induced deletion of Brca1. (b) Flow cytometry profiles of R26<sup>CreERT2</sup> Brca1<sup>ScCo</sup> ES cells stably transduced with nontargeting shRNA lentiviruses or shRNA vectors against 53bp1 and p53. Shown are percentages of cells in G1 and G2 before or 9 d after 4OHT-induced Brca1 deletion by CreERT2. Mean ± s.d. is shown from two experiments.
affect BRCA1-dependent signaling. In contrast, BRCA2 activation requires the damage signal generated at the break to be transduced downstream through at least two parallel pathways involving several response factors\(^\text{39}\). Thus, the loss of 53BP1 can be compensated by other regulatory mechanisms acting between the early events at the chromatin surrounding the break and the initiation of repair reactions. Although shRNA-mediated depletion of p53 rescues the proliferation defect of BRCA2-deficient cells, DDR activation shown by CHK2 phosphorylation persists. This suggests that CHK2-mediated p53 activation contributes to the senescence response induced by the loss of BRCA2. Consistent with the role of BRCA2 as the loader of RAD51 onto double-strand breaks (DSBs), the initiating step of recombinational DNA repair, we did not detect any rescue of RAD51 focus formation in BRCA2-deficient cells when either p53 or 53BP1 were depleted (data not shown).

Together, these results suggest that 53BP1 is required for efficient ATM-dependent checkpoint signaling to arrest cell-cycle progression in response to DNA damage accumulation in Brca1-deleted cells. Alternatively, 53BP1 loss might lead to more efficient DNA DSB repair and thereby reduce DDR activation.

### 53BP1 loss partially restores HR in Brca1-null cells

The observed abrogation of G2 accumulation, sensitivity to DNA crosslinkers and DDR may be due to a stimulation of BRCA1-independent DNA repair. Alternatively, checkpoint release might prevent the formation of DNA breaks by collapsing replication forks or allow the cells to continue cycling without actual repair of the damage. To investigate the effects of 53BP1 depletion on DNA repair, we analyzed RAD51 focus formation in Brca1\(^{-/-}\) and Brca1\(^{+/+}\) ES cells following treatment with ionizing irradiation (Fig. 4). Analysis of these ionizing radiation–induced RAD51 foci revealed a diminished response upon deletion of Brca1 (Fig. 4a,c), consistent with previous observations\(^\text{27}\), shRNA-mediated depletion of 53BP1 enhanced RAD51 focus formation in the absence of BRCA1, suggesting upregulation of HR in a BRCA1-independent manner. This process is not specific for ES cells, as we observed the same phenomenon in MEFs (Fig. 4b,d).

To determine whether 53BP1 depletion rescues homology-directed repair (HDR) in BRCA1-deficient cells, we measured gene-targeting efficiencies in R26\(^{CreERT2}\) Brca1\(^{SCo/\Delta}\) ES cells with or without shRNA-mediated depletion of 53BP1 or p53 using an isogenic Rb-targeting construct with a PGK-neo selection marker\(^\text{28}\). Whereas CreERT2-mediated depletion of Brca1 by addition of 4OHT abolished Rb gene targeting in R26\(^{CreERT2}\) Brca1\(^{SCo/\Delta}\) ES cells, this effect could not be reversed by shRNA-mediated depletion of p53 (Table 1). In contrast, we observed correct integration of the targeting vector at the Rb locus in 2 out of 160 53BP1-depleted R26\(^{CreERT2}\) Brca1\(^{SCo/\Delta}\) ES cells compared to 8 out of 78 R26\(^{CreERT2}\) Brca1\(^{SCo/\Delta}\) colonies, suggesting that 53BP1 loss leads to partial restoration of HDR in Brca1-null cells. This notion was supported by gene-targeting experiments with a promoterless Pim1-neo targeting construct\(^\text{29}\), showing correct integration in 4 out of 47 53BP1-depleted R26\(^{CreERT2}\) Brca1\(^{SCo/\Delta}\) ES cells compared to 9 out of 56 R26\(^{CreERT2}\) Brca1\(^{SCo/\Delta}\) colonies (Table 1). We conclude that depletion of 53BP1 restores HR activity in Brca1-null cells to 10–50% of that of BRCA1-proficient cells.

#### Basal-like breast cancers have low levels of 53BP1

The majority of BRCA1-associated tumors carry p53 mutations\(^\text{30,31}\). It is known that p53 loss can at least partially rescue BRCA1-deficient cells\(^\text{11,12}\). However, there may still be additional selection for aberrant expression of 53BP1, as suggested by our in vitro results and by the synergism in tumorigenesis observed in 53bp1\(^{-/-}\) p53\(^{-/-}\) knockout mice\(^\text{14,15}\). Therefore, we analyzed levels of 53BP1 mRNA in a publicly available dataset of 200 breast cancers, finding significantly lower expression of 53BP1 in basal-like breast cancers than in nonbasal-like breast cancers (Fig. 5a). Consistently, basal-like breast cancers were associated with significantly reduced gene-targeting efficiencies in 53BP1-depleted MEFs (Fig. 5b). These data support a role of 53BP1 in tumorigenesis in BRCA1-associated breast cancers and provide a mechanistic explanation for the incomplete efficacy of 53BP1 depletion in regulating HR.

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**Table 1. Gene-targeting frequencies in R26\(^{CreERT2}\) Brca1\(^{SCo/\Delta}\) ES cells**

| 4OHT\(^\text{a}\) | shRNA | Total analyzed | Nontargeted (%) | Targeted (%) |
|----------------|-------|----------------|-----------------|--------------|
| Rb             | NT    | 78             | 70 (90)         | 8 (10)       |
|                | + NT  | 78             | 78 (100)        | 0 (0)        |
|                | + 53BP| 160            | 158 (99)        | 2 (1)        |
|                | + p53 | 124            | 124 (100)       | 0 (0)        |
| Pim1           | NT    | 56             | 47 (84)         | 9 (16)       |
|                | + NT  | 11             | 11 (100)        | 0 (0)        |
|                | + 53BP| 47             | 43 (91.5)       | 4 (8.5)      |

\(^{\text{a}}\)4-hydroxytamoxifen (4OHT) was added to induce deletion of Brca1\(^{SCo}\).
available database of gene expression array data from 286 breast cancer specimens, all early stage (lymph-node negative) and treated with surgery and radiation therapy alone. We used previously described unsupervised clustering methods to identify basal-like breast cancers (BLCs), HER2-positive breast cancers and luminal A and luminal B subclasses of breast cancer. BLCs are characterized clinically as high-grade, invasive breast cancers that lack expression of estrogen receptor, progesterone receptor and HER2 (triple-negative phenotype) and are associated with a younger age of onset. Luminal A tumors are characterized by expression of the estrogen receptor (ER+), with luminal A tumors being mostly low-grade ER+ tumors and luminal B tumors being mostly high-grade ER+ tumors. We next calculated mean levels of 53BP1 expression for each breast cancer subtype (Fig. 5a). We found the lowest 53BP1 expression levels in the BLC subclass.

We have previously shown that a robust consensus clustering approach of breast cancer gene-expression data reveals two subclasses of BLC, which we labeled BA1 and BA2 (ref. 34). This approach also identifies two subclasses of HER2-positive cancers and three subclasses of luminal B tumors. We examined 53BP1 mRNA expression in these different subclasses in a combined dataset that includes the published datasets of two previous reports. In this combined dataset, we found that 53BP1 expression was clearly lowest in the BA2 subtype of BLC (P < 0.0001 versus normal, P < 0.0002 versus BA1) (Fig. 5b). These data suggest that a biologically relevant subset of BLC has low 53BP1 expression. To validate this finding at the protein level, we assayed a set of 504 breast cancer specimens from the Yale cohort by immunohistochemistry on tissue microarrays. The data are normalized, with mean expression of the combined data being set to 0. Expression of 53BP1 is significantly lower in the BA2 subtype (P < 0.0001 versus normal, P < 0.0002 versus BA1). (c, d) Distant relapse–free survival stratified by 53BP1 protein expression in all breast cancers (c) and in triple-negative (TN) breast cancers (d). Kaplan-Meyer survival curves are shown for breast cancers that scored positive for 53BP1 staining (black lines) and those that scored negative for 53BP1 staining (red lines).

53BP1 loss is associated with BRCA1/2 mutation status

To validate and extend the data from the Yale cohort, including assessment of 53BP1 protein expression in BRCA1/2-mutated familial breast carcinomas, we performed an independent larger-tissue microarray analysis on the Helsinki cohort of 1,187 patients (Table 3). We obtained these data using different 53BP1 antibodies, and the data were analyzed independently by separate pathologists (see Supplementary Fig. 4 for examples of 53BP1 staining patterns in the Helsinki cohort). Confirming the results obtained from the Yale cohort, lack of 53BP1 staining in the Helsinki cohort correlated independently with lack of estrogen receptor expression (P = 0.000004), lack of progesterone receptor expression (P = 0.003) and the triple-negative phenotype

Table 2 Low 53BP1 expression correlates with triple-negative status (Yale cohort)

| Features                  | 53BP1 expression |
|---------------------------|------------------|
|                           | Total (%) | Positive (%) | Negative (%) | P*    |
| Estrogen receptor         |           |             |              |       |
| Positive                  | 243 (55)   | 239 (63)    | 4 (6)        | <0.0001 |
| Negative                  | 200 (45)   | 140 (37)    | 60 (94)      |       |
| Progesterone receptor     |           |             |              |       |
| Positive                  | 226 (51)   | 222 (59)    | 4 (6)        | <0.0001 |
| Negative                  | 217 (49)   | 157 (41)    | 60 (94)      |       |
| ERBB2/HER2                |           |             |              |       |
| Positive                  | 78 (18)    | 77 (20)     | 1 (2)        | <0.0001 |
| Negative                  | 366 (82)   | 303 (80)    | 63 (98)      |       |
| Triple-negative (TN)      |           |             |              |       |
| Not TN                    | 311 (70)   | 305 (80)    | 6 (10)       | <0.0001 |
| TN                        | 132 (30)   | 75 (20)     | 57 (90)      |       |

*Fisher’s exact test.
Table 3 Loss or reduction of 53BP1 expression correlates with triple-negative status and BRCA1/2 mutation status (Helsinki cohort)

| Features                     | Total (%) | Normal (%) | Aberrant (%) | P  
|------------------------------|-----------|------------|--------------|------
| Estrogen receptor            | 1,053     | 834 (79.2) | 219 (20.8)   | 0.000004  
| Positive                     |           | 823 (80.3) | 11 (39.3)    |      
| Negative                     | 219 (20.8)| 202 (19.7) | 17 (60.7)    |      
| Progesterone receptor        | 1,050     | 703 (67.0) | 347 (33.0)   | 0.003  
| Positive                     |           | 692 (67.7) | 330 (32.3)   |      
| Negative                     | 347 (33.0)| 330 (32.3) | 17 (59.7)    |      
| ERBB2/HER2                   | 1,075     | 145 (13.5) | 142 (13.6)   | 0.103  
| Positive                     |           | 142 (13.6) | 142 (13.6)   |      
| Negative                     | 143 (13.5)| 142 (13.6) | 11 (39.3)    |      
| Triple-negative (TN)         | 1,018     | 930 (86.5)| 904 (86.4)   | 0.8   
| Not TN                       |           | 904 (86.4)| 904 (86.4)   |      
| TN                           | 143 (14.0)| 129 (13.0)| 129 (13.0)   |      
| BRCA1/2 mutation             | 1,187     | 1,108      | 1,079 (94.0)| 0.000004  
| Non-BRCA1/2                  |           | 1,079 (94.0)| 29 (7.4)    |      
| Sporadic                     | 374       | 371        | 3            | 0.001  
| Familial non-BRCA1/2         | 734       | 708        | 26           |      
| BRCA1/2                      | 79 (6.6)  | 69 (6.0)   | 10 (25.6)    | 0.0001  
| BRCA1                        | 35        | 30         | 5            | 0.003  
| BRCA2                        | 44        | 39         | 5            | 0.008  

*Fishers’s exact test, **BRCA1/2 versus sporadic, †BRCA1/2 versus familial non-BRCA1/2 tumors, ‡BRCA1/2 vs. all non-BRCA1/2 tumors (sporadic + familial non-BRCA1/2). §BRCA1 versus all non-BRCA1/2 tumors. ¶BRCA2 versus all non-BRCA1/2 tumors.

(\(P = 0.000004\)). In addition, the familial tumors from BRCA1/2 mutation carriers (79 tumors) showed the highest degree of aberrant 53BP1 reduction or loss compared to sporadic tumors (\(n = 374, P = 0.000003\)), familial carcinomas not attributable to BRCA1/2 mutations (\(n = 734, P = 0.001\)) or all non-BRCA1/2 tumors (\(n = 1,108, P = 0.0001\)). Both BRCA1- and BRCA2-associated tumors showed a significantly increased incidence of reduced 53BP1 staining compared to non-BRCA1/2 tumors (\(P = 0.003\) for BRCA1 and \(P = 0.008\) for BRCA2). Overall, these results show that loss of 53BP1 is more frequent among the most aggressive and difficult-to-treat triple-negative tumors as well as in tumors with BRCA1/2 mutations.

53BP1 expression and distant metastasis-free survival

We analyzed the survival data from the Yale cohort. There was significant association between 53BP1 status and distant metastasis-free survival, with 53BP1-negative tumors having significantly lower metastasis-free survival (\(P = 0.001\)). As most tumors that lack 53BP1 have a triple-negative phenotype, we analyzed distant metastasis-free survival in triple-negative breast cancers stratified by 53BP1 status. Amongst the triple-negative tumors, those that lack normal 53BP1 staining have decreased metastasis-free survival (\(P = 0.039\)) (Fig. 5d). As the cancers in this dataset were mostly early-stage, lymph node-negative cancers that did not receive any adjuvant treatment, these data suggest that early-stage triple-negative tumors with reduced 53BP1 may have a greater likelihood of metastasis in the absence of systemic treatment compared to triple-negative tumors with intact 53BP1.

DISCUSSION

BRCA1 is a large ubiquitously expressed protein that has a major role in DDR by HR. Its activity is extensively regulated by phosphorylation, sumoylation and interactions with many other proteins. Perhaps not surprisingly, BRCA1 is not only involved in HR but also in many other processes like cell-cycle control and transcriptional regulation. Despite its widespread expression and non–cell type–specific functions, mutations in BRCA1 are mainly associated with increased risk of breast and ovarian tumorigenesis. Loss of BRCA1 leads to severe proliferation defects in normal, non-cancerous cells—for instance, leading to lethality during embryonic development. Therefore, it seems likely that there are survival factors that allow BRCA1-deficient tumor cells to expand. In an unbiased genetic screen, we found that loss of 53BP1 rescues clonal outgrowth of Brca1-null ES cells. This result confirms the recently described rescue of Brca1Δ11/Δ11 hypomorphic mice by 53BP1 knockout. In addition, it shows that expression of the Brca1Δ11 splice variant is not required for rescue of BRCA1 deficiency by 53BP1 loss. This is important, as many human BRCA1-associated cancers are characterized by complete loss of BRCA1 expression. Further characterization indicated that BRCA1 and 53BP1 double-deficient cells are no longer hypersensitive to DNA cross-linking agents and do not spontaneously form DSBs or activate DDR. Suppression of ionizing radiation–induced CHK2 phosphorylation has been previously observed upon DNA interference–mediated 53BP1 depletion in U2OS cells and in 53bp1−/− MEFs. Here we show that 53BP1 status specifically affects the spontaneous induction of CHK2 phosphorylation when BRCA1 is lost. Our results are consistent with recently reported data on 53BP1-mediated suppression of DNA resection at DSBs and accumulation of single-stranded DNA ends in BRCA1 and 53BP1 double-deficient cells. In cell-free extracts, DNA damage–induced ATM activation and CHK2 phosphorylation are inhibited by 3′ single-stranded DNA overhangs generated during DNA-break processing in S/G2 (ref. 43). The decreased CHK2 phosphorylation that we observed in 53BP1-depleted Brca1-null cells could therefore result from decreased ATM signaling due to increased DNA resection or from increased DSB repair. The latter possibility is supported by the restoration of the ionizing radiation–induced RAD51 focus formation and the partial restoration of HR in 53BP1-depleted Brca1-null cells.

Although our data suggest that a certain level of HR repair can take place in the absence of both BRCA1 and 53BP1, 53BP1-depleted Brca1-null cells remain HR defective, as gene-targeting frequencies in these cells are reduced by a factor of 2–10 when compared to wild-type cells. Consistent with this, BRCA1-deficient tumors show excellent responses to therapies exploiting a HR defect, such as platinum drugs or PARP inhibitors. Also, Brca1-mutated mouse mammary tumors are highly sensitive to treatment with PARP inhibitors or platinum drugs. Whereas human BRCA1-mutated tumors can develop resistance to carboplatin by genetic reversion of the BRCA1 mutation—stressing the importance of BRCA1 function for HR—mouse mammary tumor models with large deletions in Brca1 cannot employ this mechanism and remain sensitive to cisplatin or carboplatin, even after multiple rounds of treatment. In contrast, Brca1Δ11/Δ11 mouse mammary tumors, which only express the Brca1Δ11 isoform, readily become resistant to cisplatin despite the fact that Brca1 exon 11 sequences are irreversibly deleted. This feature might be indicative of residual activity of the Brca1Δ11 isoform in HR.

At present, it is not clear whether 53BP1 loss contributes to development, therapy response and/or acquired resistance of BRCA1-deficient tumors. To explore this, we examined 53BP1 expression in independent cohorts of breast cancer patients from the US and Finland. These two tumor sets were analyzed by different antibodies and reviewed by independent pathologists using separate criteria, yet both cohorts showed a striking correlation of low 53BP1 expression levels with triple-negative...
tumors. Since most BRCA1-mutated breast cancers cluster in this subgroup, it was not unexpected to find that these tumors also lacked 53BP1 expression more often than other subsets of breast tumors. However, the often hormone receptor–positive BRCA2-associated tumors were also significantly enriched for 53BP1 aberrations. This may be indicative of a common selection for 53BP1 ablation in both types of HR-deficient breast cancers. This might occur via different routes, given the persistence of DDR activation and growth impairment in 53BP1-depleted BRCA2-deficient cells.

In conclusion, we have shown that 53BP1 loss alleviates the proliferation defect and DNA-damage hypersensitivity of Brca1-null cells and leads to partial restoration of HR in these cells. Furthermore, aberrant expression of 53BP1 is more common in BRCA1/2-associated breast cancers, which may hint at a role for 53BP1 loss in these tumors. 53BP1 is also lost in a subset of sporadic triple-negative breast cancers, suggesting a broader role for abnormalities in this pathway in breast tumorigenesis. Our results suggest that loss of 53BP1 may promote survival of BRCA1-deficient tumor cells after DNA damage induced by chemotherapy or irradiation. It is possible that 53BP1 loss may have different effects in BRCA1-deficient tumors versus sporadic triple-negative breast cancers. Regardless, 53BP1 might represent a candidate biomarker for predicting the response of HR-defective tumors to PARP inhibitors or platinum drugs.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

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Author Contributions

P.B., A.A., J. Bartek, M.T., S.G. and J.J. designed research; P.B., A.A., J.M.E., M.P. J. Bartkova, H.v.d.G., S.H., M.T., A.K., Q.Y., B.G.H., J.T., C.B., D.J.A. and H.N. performed research; R.D. contributed new reagents/analytical tools; P.B., A.A., J.M.E., M.P. J. Bartek, H.N., M.T., S.G. and J.J. analyzed data; P.B., A.A., J. Bartek, M.T., S.G. and J.J. wrote the paper.

Competing Financial Interests

The authors declare no competing financial interests.

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1. Venkitaraman, A.R. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell 108, 171–182 (2002).
2. Gudmundsdottir, K. & Ashworth, A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. Oncogene 25, 5864–5874 (2006).
3. Boulton, S.J. Cellular functions of the BRCA2 tumour-suppressor proteins. Biochem. Soc. Trans. 34, 633–645 (2006).
4. Collins, N. et al. Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12–13. Oncogene 10, 1675–1679 (1995).
5. Smith, S.A., Easton, D.F., Evans, D.G. & Ponder, B.A. Allele losses in the region 17q12–17q21 in familial breast and ovarian cancer involve the wild-type chromosome. Nat. Genet. 2, 128–131 (1992).
6. Haken, M. et al. The tumour biological features of BRCA1-induced breast and ovarian cancer. Eur. J. Cancer 33, 362–371 (1997).
7. Fouliks, W.D. et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J. Natl. Cancer Inst. 95, 1482–1485 (2003).
8. Jonkers, J. et al. Repeated observation of breast tumour subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. USA 100, 8418–8423 (2003).
9. Turner, N., Tutt, A. & Ashworth, A. Hallmarks of ‘BRCAness’ in sporadic cancers. Nat. Rev. Cancer 4, 814–819 (2004).
10. Evers, B. & Jonkers, J. Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. Oncogene 25, 5885–5897 (2006).
11. Haken, R. et al. The tumor suppressor gene BRCA1 is required for embryonic cell proliferation in the mouse. Cell 85, 1009–1023 (1996).
12. Liu, X. et al. Somatic loss of BRCA1 and p53 in mouse mammary tumors with features of human BRCA1-mutated basal-like breast cancer. Proc. Natl. Acad. Sci. USA 104, 12111–12116 (2007).
13. Wang, B., Matsuoka, S., Carpenter, P.B. & Elledge, S.J. 53BP1, a mediator of the DNA damage checkpoint. Science 298, 1435–1438 (2002).
14. Morales, J.C. et al. 53BP1 and p53 synergize to suppress genomic instability and lymphomagenesis. Proc. Natl. Acad. Sci. USA 103, 3310–3315 (2006).
15. Ward, I.M. et al. 53BP1 cooperates with p53 and functions as a haploinsufficient tumor suppressor in mice. Mol. Cell. Biol. 25, 10079–10086 (2005).
16. Xu, X. et al. Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. Nat. Genet. 28, 266–271 (2000).
17. Haken, M. et al. The tumor suppressor gene BRCA1 is required for embryonic cell proliferation in the mouse. Cell 85, 1009–1023 (1996).
18. Liu, X. et al. Somatic loss of BRCA1 and p53 in mouse mammary tumors with features of human BRCA1-mutated basal-like breast cancer. Proc. Natl. Acad. Sci. USA 104, 12111–12116 (2007).
19. 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).
20. Wang, W. et al. Chromosomal transposition of PiggyBac in mouse embryonic stem cells. Proc. Natl. Acad. Sci. USA 105, 9290–9295 (2008).
21. Bartz, S.R. et al. Small interfering RNA screens reveal enriched cisplatin cytotoxicity in tumors having both BRCA network and TP53 disruptions. Mol. Cell. Biol. 26, 9377–9386 (2006).
22. Kastan, M.B. & Bartek, J. Cell-cycle checkpoints and cancer. Nature 432, 316–323 (2004).
23. Taylor, W.R. & Stark, G.R. Regulation of the G2/M transition by p53. Oncogene 20, 1803–1815 (2001).
24. Silver, D.P. & Livingston, D.M. Self-excision retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol. Cell. Biol. 21, 233–243 (2001).
25. D’Souza, J. et al. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat. Genet. 29, 418–425 (2001).
26. Jazayeri, A. et al. ATM- and cell-cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat. Cell Biol. 8, 37–45 (2006).
27. Bhattacharyya, A., Ear, U.S., Koller, B.H., Weichselbaum, R.R. & Bishop, D.K. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. J. Biol. Chem. 275, 23899–23903 (2000).
28. te Riele, H., Robanus-Maandag, E. & Berns, A. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc. Natl. Acad. Sci. USA 89, 5128–5132 (1992).
29. te Riele, H., Robanus-Maandag, E., Clarke, A., Hooper, M. & Berns, A. Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. Nature 348, 649–651 (1990).
30. Holstege, H. et al. High incidence of protein-truncating TP53 mutations in BRCA1-related breast cancer. Cancer Res. 69, 3625–3633 (2009).
31. Manie, E. et al. High frequency of TP53 mutation in BRCA1 and sporadic basal-like carcinomas but not in BRCA1 luminal breast tumors. Cancer Res. 69, 663–671 (2009).
32. Ludwig, T., Chapman, D.L., Papaioannou, V.E. & Efstratiadis, A. Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. *Genes Dev.* 11, 1226–1241 (1997).

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

34. 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).

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

36. Scully, R. *et al.* Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* 90, 425–435 (1997).

37. Bartek, J. & Hodny, Z. SUMO boosts the DNA damage response barrier against cancer. *Cancer Cell* 17, 9–11 (2010).

38. Galanty, Y. *et al.* Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature* 462, 935–939 (2009).

39. Morris, J.R. *et al.* The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature* 462, 886–890 (2009).

40. Deng, C.X. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Res.* 34, 1416–1426 (2006).

41. Ward, I.M., Minn, K., van Deursen, J. & Chen, J. p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. *Mol. Cell. Biol.* 23, 2566–2563 (2003).

42. Bunting, S.F. *et al.* 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141, 243–254 (2010).

43. Shirotani, B. & Zou, L. Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Mol. Cell* 33, 547–558 (2009).

44. Silver, D.P. *et al.* Efficacy of neoadjuvant Cisplatin in triple-negative breast cancer. *J. Clin. Oncol.* 28, 1145–1153 (2010).

45. Fong, P.C. *et al.* Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N. Engl. J. Med.* 361, 123–134 (2009).

46. Rottenberg, S. *et al.* High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc. Natl. Acad. Sci. USA* 105, 17079–17084 (2008).

47. Rottenberg, S. *et al.* Selective induction of chemotherapy resistance of mammary tumors in a conditional mouse model for hereditary breast cancer. *Proc. Natl. Acad. Sci. USA* 104, 12117–12122 (2007).

48. Swisher, E.M. *et al.* Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance. *Cancer Res.* 68, 2581–2586 (2008).

49. Shafee, N. *et al.* Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. *Cancer Res.* 68, 3243–3250 (2008).
ONLINE METHODS

R26CreERT2 Brca1ScoΔ ES cells and mutagenesis screen. Details on the generation of R26CreERT2 Brca1ScoΔ ES cells and the piggyBac transposon mutagenesis screen are described in Supplementary Methods.

MEF isolation and immortalization. Heads and organs of E13.5 embryos were removed, and the remaining tissue was minced, rinsed in PBS and incubated for 30 min in 0.5 ml 0.05% (w/v) trypsin-EDTA (Gibco) at 37 °C. Cell aggregates were dissociated in DMEM supplemented with 10% (v/v) FBS (Gibco) and penicillin-streptomycin (Gibco). Cells were plated on 10-cm dishes and cryopreserved after 2 d as passage 1 MEFs. MEFs were immortalized by infection with retrovirus.50

Retroviral transductions. MEFs were transduced with pRetroSuper retroviruses encoding shRNAs targeting GFP51, 53BP1 (sh1, 5′-GCTATGGAGGATTGTGTG-3′; sh2, 5′-GGTGAAAGATATTTCCATA-3′) or p53 (ref. 52) as described previously (Supplementary Table 2). Briefly, HEK 293T packaging cells were transfected with pL-Clo helper vector together with either pRetroSuper alone or pRetroSuper plus retroviral vector encoding H&ECre recombinase.14 The culture medium was changed 24 h after transfection. Recipient MEFs were plated and then were infected 24 h later with retroviral supernatants. Additional infections were performed after 24 and 32 h. Twenty-four hours after the last infection, cells were washed and grown in medium containing 2–3 mg ml−1 puromycin.

Lentiviral transductions. R26CreERT2 Brca1ScoΔ ES cells were transduced with plK0-puro shRNA lentiviruses obtained from Mission Library clones (Sigma). In addition to the 53BP1 shRNAs mentioned above, we used shRNAs targeting p53 (sh1, 5′-CTACAAGAAGTCACGACAT-3′; sh2, 5′-AGAGATTTTACCTCCTAAGT-3′) and a nontargeting shRNA (SHC202, 5′-CAACAAGGATGAGGCACAA-3′) (Supplementary Table 2). After selection with 1.8 μg ml−1 puromycin, cells were switched by overnight incubation with 0.5 μM 4OHT. Four days after switching, cells were seeded at 1,000 cells cm−2 and assayed for clonal growth. Surviving colonies were fixed in formalin and stained with crystal violet.

Cell-cycle analysis. Cells were incubated with BrdU for 1 h, fixed with ethanol and incubated with mouse anti-BrdU (clone BU20A, Dako) and goat anti–mouse FITC conjugated secondary antibodies (DAKO). Cell-cycle distribution was measured by flow cytometry and analyzed using FlowJo software (FlowJo).

Cytotoxicity assays. R26CreERT2 Brca1ScoΔ ES cells were seeded in triplicate at 1,000 or 3,000 (in the case of switched cells with nontargeting shRNA) cells per well in 96-well plates. One day after seeding, cells were fed with medium containing either cisplatin or mitomycin C. Five days later, cell viability was assayed in an Envision plate reader (Perkin Elmer) using resazurin (cell titer blue, Promega).

MEF proliferation assays. Primary MEFs were seeded at 5,000 cells per well in 96-well plates. Cell number was determined after 24, 48, 72 and 96 h by incubating cells with 10 μg ml−1 G418, colonies were picked, expanded and lysed in direct lysis reagent (Viagen) containing 100 μg ml−1 proteinase K. Following heat inactivation of proteinase K, genomic DNA was used for PCR analysis or digested with appropriate restriction enzymes and analyzed by Southern blotting as described24,29.

Analysis of 53BP1 expression in human breast cancers. Details on analysis of 53BP1 expression in human breast cancers using tissue microarrays from the Yale and Helsinki cohorts or gene expression array datasets from previous reports35,35 are described in Supplementary Methods.

Antibodies. Antibodies used for immunoblotting of ES cells were mouse monoclonal anti–mouse BRCA1 (GH118), affinity-purified rabbit polyclonal anti–mouse BRCA1 against peptide 452–469, 53BP1 (ab21083, Abcam) and p53 (IMX25, Monosan). Immunocytochemical staining of ES cells was performed using human RAD51 (ref. 54) and goat anti–rabbit Alexa 588 secondary antibody (Molecular Probes).

Gene-targeting assays. R26CreERT2 Brca1ScoΔ ES cells were electroporated with linearized Rb-neo29 or Pim1-neo29 targeting constructs. After drug selection with 200 μg ml−1 G418, colonies were picked, expanded and lysed in direct lysis reagent (Viagen) containing 100 μg ml−1 proteinase K. Following heat inactivation of proteinase K, genomic DNA was used for PCR analysis or digested with appropriate restriction enzymes and analyzed by Southern blotting as described24,29.

NuPAGE-Novex 10% (w/v) bis-Tris gels or Tris-acetate 3–8% (w/v) (Invitrogen) were run according to the manufacturer’s instructions.

Immunofluorescence. ES cells were grown on coverslips, γ-irradiated with 10 Gy and fixed 6 h later using 2% (w/v) paraformaldehyde in PBS. Fixed cells were incubated with antibody in PBS containing 0.5% (w/v) BSA and 0.15% (w/v) glycine, mounted using Vectashield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and imaged with a Hamamatsu ORCA AG CCD camera on a Zeiss Axio Observer Z1 system. For quantification of RAD51 foci, images were converted into 8-bit gray-scale pictures using Image J software (ImageJ, US National Institutes of Health), and the fluorescence-intensity threshold was set based on a black-and-white intensity scale from 140 to 255. For each sample, the number of foci was counted in at least 20 individual nuclei.

MEFs were grown on coverslips and γ-irradiated with 10 Gy. Cells were allowed to recover for the indicated times, washed in PBS and swollen in hypotonic solution (85.5 mM NaCl, 5 mM MgCl2, adjusted to pH 7.0) for 5 min. Cells were fixed with 4% paraformaldehyde (10-min at room temperature), permeabilized by adding 0.03% (w/v) SDS to the fixative and immunostained as described44. Dried coverslips were mounted on microscope slides using the ProLong Antifade kit (Invitrogen) supplemented with 1 mg ml−1 DAPI, and viewed with a Leica DMi6000B fluorescence microscope. Images were acquired with a Leica DFC350 FX R2 digital camera using LAS-AF software (Leica). Image brightness and contrast were adjusted using Photoshop CS3 (Adobe). To quantify RAD51 foci, we determined the frequency of nuclei with more than 10 RAD51 foci at least 50 nuclei were analyzed for each sample.

Analysis of 53BP1 expression in human breast cancers. Details on analysis of 53BP1 expression in human breast cancers using tissue microarrays from the Yale and Helsinki cohorts or gene expression array datasets from previous reports35,35 are described in Supplementary Methods.

50. Jacobs, J.J. et al. Senescence bypass screen identifies TBX2, which represses Cdkn2a (p16ARF) and is amplified in a subset of human breast cancers. Nat. Genet. 26, 291–299 (2000).
51. Tarsounas, M. et al. Telomere maintenance requires the RAD51D recombination/repair protein. Cell 117, 337–347 (2004).
52. Dirac, A.M. & Bernards, R. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. J. Biol. Chem. 278, 11731–11734 (2003).
53. Palmero, I. & Serrano, M. Induction of senescence by oncogenic Ras. Methods Enzymol. 333, 247–256 (2001).
54. Tarsounas, M., Davies, D. & West, S.C. BRCA2-dependent and independent formation of RAD51 nuclear foci. Oncogene 22, 1115–1123 (2003).