BRCA1 loss activates cathepsin L–mediated degradation of 53BP1 in breast cancer cells

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BRCA1 loss activates cathepsin L–mediated degradation of 53BP1 in breast cancer cells

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Introduction

BRCA1 is a well-established tumor suppressor, and women carrying germline mutations in BRCA1 have a high risk of developing breast and ovarian cancer (Neuhausen and Marshall, 1994; Wooster and Weber, 2003). Tumors that arise often lack expression of estrogen and progesterone receptors and Her2, being classified as triple-negative breast cancers (TNBC) and with resistance to genotoxic drugs. The mechanisms responsible for decreased 53BP1 transcript and protein levels in tumors remain unknown. Here, we demonstrate that BRCA1 loss activates cathepsin L (CTSL)–mediated degradation of 53BP1. Formation of this pathway rescued homologous recombination repair and allowed BRCA1-deficient cells to bypass growth arrest. Importantly, depletion or inhibition of CTSL with vitamin D or specific inhibitors stabilized 53BP1 and increased genomic instability in response to radiation and poly(adenosine diphosphate–ribose) polymerase inhibitors, compromising proliferation. Analysis of human breast tumors identified nuclear CTSL as a positive biomarker for TNBC, which correlated inversely with 53BP1. Importantly, nuclear levels of CTSL, vitamin D receptor, and 53BP1 emerged as a novel triple biomarker signature for stratification of patients with BRCA1–mutated tumors and TNBC, with potential predictive value for drug response. We identify here a novel pathway with prospective relevance for diagnosis and customization of breast cancer therapy.

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Introduction

BRCA1 is a well-established tumor suppressor, and women carrying germline mutations in BRCA1 have a high risk of developing breast and ovarian cancer (Neuhausen and Marshall, 1994; Wooster and Weber, 2003). Tumors that arise often lack expression of estrogen and progesterone receptors and Her2, being classified as triple-negative breast cancers (TNBC; Turner and Reis-Filho, 2006). BRCA1 participates in DNA double-strand break (DSB) repair, S and G2/M phase cell-cycle checkpoints after damage, control of centrosome numbers, maintenance of heterochromatin, and transcriptional regulation of several genes (Scully and Livingston, 2000; Mullan et al., 2006; Zhu et al., 2011). In addition, BRCA1 function is linked to epigenetic mechanisms such as DNA methylation and miRNA biogenesis (Shukla et al., 2010; Kawai and Amano, 2012; Tanic et al., 2012).

Recruitment of BRCA1 to DNA DSBs facilitates repair by homologous recombination (HR), and loss of BRCA1 results in genomic instability characterized by unrepaired DNA breaks and complex chromosomal rearrangements that compromise cell viability (Scully et al., 1997a; Moynahan et al., 1999; Snouwaert et al., 1999). As such, BRCA1 knockout mice and mice carrying a BRCA1 deletion mutant (BRCA111/111) are embryonic lethal (Xu et al., 2001; Evers and Jonkers, 2006). Although lethality...
Accumulation of 53BP1 in this context promotes indiscriminate NHEJ and chromosomal instability that ultimately causes proliferation arrest or cell death. Conversely, in cells double deficient in BRCA1 and 53BP1, end-resection is allowed, rescuing HR (Bunting et al., 2010). Consistent with this model, 53BP1 loss reduces the sensitivity of BRCA1-deficient cells to genotoxic agents such as cisplatin and mitomycin C (Bouwman et al., 2010) and to poly(ADP-ribose) polymerase inhibitors (PARPi; Farmer et al., 2005; Bunting et al., 2010), compounds at the forefront for breast cancer therapy (Gartner et al., 2010). Thus, BRCA1-deficient cells are thought to down-regulate 53BP1 as a means to ensure proliferation/viability.

Up-regulation of 53BP1 levels represents a promising strategy for treatment of breast tumors with the poorest prognosis and for improving their response to PARPi and other DNA-damaging strategies. However, we lack knowledge about how 53BP1 mRNA and protein levels are down-regulated in cancer cells. We previously identified a pathway regulating 53BP1 protein levels (Gonzalez-Suarez et al., 2011; Redwood et al., 2011a,b). Up-regulation of the cysteine protease cathepsin L (CTSL) leads to accumulation of the protease in the nucleus, degradation of 53BP1 protein, and defects in NHEJ. Importantly, inhibition of CTSL activity by treatment with vitamin D or specific inhibitors stabilizes 53BP1 protein levels and rescues NHEJ defects (Gonzalez-Suarez et al., 2011).
Here, we demonstrate that BRCA1-deficient cells activate CTSL-mediated degradation of 53BP1 as a means to overcome genomic instability and growth arrest. In addition, depletion or inhibition of CTSL in these cells increases genomic instability in response to ionizing radiation (IR) or PARPi. Lastly, we identify high levels of nuclear CTSL and low levels of 53BP1 and vitamin D receptor (VDR) as a novel signature in subsets of breast cancer patients. We envision that the status of nuclear CTSL, VDR, and 53BP1 could be used for customization of breast cancer therapy.

Results

**BRCA1-deficient cells activate CTSL-mediated degradation of 53BP1 to bypass growth arrest**

Previous studies demonstrated that loss of 53BP1 rescues the BRCA1-deficient phenotype (Cao et al., 2009; Bouwman et al., 2010; Bunting et al., 2010). We also showed that CTSL regulates the stability of 53BP1 (Gonzalez-Suarez et al., 2011; Redwood et al., 2011a,b). Here, we investigated whether breast tumor cells are able to down-regulate 53BP1 upon loss of BRCA1 to restore proliferation/viability and if CTSL is one of the factors responsible for the depletion of 53BP1. The human breast cancer cell line MCF7, which is BRCA1 and 53BP1 proficient, was depleted of BRCA1 via lentiviral transduction with shRNAs (Fig. 1 A and Fig. S1 A). As previously shown in human fibroblasts (Tu et al., 2011), depletion of BRCA1 in MCF7 cells induces growth arrest (Fig. 1 B). BRCA1-deficient cells did not show differences in the levels of 53BP1 and p107, a known target of CTSL degradation, were monitored by Western blot. Note how cathepsin inhibition stabilizes 53BP1. (F) MCF7 cells growing asynchronously or growth arrested for 48 h by serum deprivation were analyzed for cell cycle profile (left) and for levels of CTSL, 53BP1, and BRCA1 by Western blot (right). Representative experiment of three biological repeats. Either Lamin A/C or β-tubulin was used as loading control.
and the BRCA1-deficient breast cancer cell line HCC1937. Ectopic expression of BRCA1 resulted in stabilization of 53BP1 in both cell lines (Fig. 1 G), revealing a novel function of BRCA1 in the stabilization of 53BP1 protein.

To determine if 53BP1 loss is responsible for the bypass of growth arrest in BRCA1-deficient cells, we depleted 53BP1 before BRCA1 depletion. As shown in Fig. 2 A, we achieved a marked reduction of both 53BP1 and BRCA1 proteins. Importantly, previous depletion of 53BP1 prevented the characteristic growth arrest that follows BRCA1 depletion (Fig. 2 B). These data indicate that loss of 53BP1 allows BRCA1-deficient cells to bypass growth arrest. Interestingly, cells that were depleted of 53BP1 before depletion of BRCA1 also up-regulated CTSL (Fig. 2 A), which is consistent with normal 53BP1 levels. In contrast, BOGA cells were unable to form 53BP1 IRIF (Fig. 3 A), which is consistent with normal 53BP1 levels. Of BRCA1 were not affected by depletion of CTSL (Fig. S2 B). Intriguingly, we observed a slight increase in BRCA1 protein in BOGA cells depleted of CTSL, suggesting a possible feedback mechanism of CTSL on BRCA1 protein levels, a notion that remains to be tested.

We previously demonstrated that vitamin D inhibits CTSL activity and stabilizes 53BP1 protein in mouse embryonic fibroblasts (MEFs; Gonzalez-Suarez et al., 2011). Here, we show that treatment of BOGA cells with vitamin D (1α,25-dihydroxy-vitamin D3) stabilized the levels of 53BP1 (Fig. 2 D). Similarly, treatment with the cathepsin inhibitor E-64 led to increased levels of 53BP1 protein (Fig. 2 E). These data demonstrate that cells growth arrested after BRCA1 loss activate CTSL-mediated degradation of 53BP1 to bypass the growth arrest imposed by BRCA1 deficiency. In addition, depletion or inhibition of CTSL can increase 53BP1 levels in the context of BRCA1 deficiency with potential therapeutic effects. Interestingly, growth arrest induced by serum deprivation in MCF7 cells also led to decreased levels of BRCA1, up-regulation of CTSL, and down-regulation of 53BP1 (Fig. 2 F). These data suggest that BRCA1 might regulate CTSL-mediated degradation of 53BP1 during the cell cycle and that the growth arrest induced by BRCA1 depletion itself could contribute to the activation of CTSL-mediated degradation of 53BP1.

CTSL-mediated degradation of 53BP1 rescues HR defects in BRCA1-deficient cells

BRCA1 deficiency impairs DNA end-resection at DSBs and formation of RAD51-coated filaments that facilitate subsequent HR steps (Scully et al., 1997a,b; Moynahan et al., 1999; Snouwaert et al., 1999; Bhattacharyya et al., 2000; Sung et al., 2003; Schlegel et al., 2006). Interestingly, loss of 53BP1 in BRCA1-deficient cells partially rescues HR and accumulation of RAD51 at IR-induced foci (IRIF; Bunting et al., 2010). Here, we determined how CTSL-mediated degradation of 53BP1 impacts 53BP1 and RAD51 recruitment to DNA DSBs. We show that growth-arrested MCF7 cells immediately after BRCA1 depletion retained their ability to recruit 53BP1 protein to IRIF (Fig. 3 A), which is consistent with normal 53BP1 levels. In contrast, BOGA cells were unable to form 53BP1 IRIF (Fig. 3 B), which is consistent with their decreased 53BP1 levels. Next, we determined if the deficiency in 53BP1 foci formation could be rescued by inhibiting CTSL. BOGA cells treated with vehicle were defective in the formation of 53BP1 IRIF, whereas treatment with vitamin D rescued 53BP1 IRIF (Fig. 3, C and D; and Fig. S3 A).

In contrast to 53BP1, RAD51 recruitment to IRIF was inhibited shortly after BRCA1 depletion in growth-arrested cells (Fig. S3 B). This defect was rescued in BOGA cells at 1 h after IR (Fig. 4, A and B). The rescue was not because of an increase in BRCA1 levels, as BOGA cells were unable to form BRCA1-labeled IRIF (Fig. S3 C). We tested whether activation of CTSL-mediated degradation of 53BP1 is behind the rescued recruitment of RAD51 to DSBs in BOGA cells. First, we show that stabilization of 53BP1 by vitamin D treatment reduced the extent of RAD51 IRIF (Fig. 4, A and B), revealing
of 53BP1 hinders NHEJ in BRCA1-deficient cells. However, these cells are still able to repair DSBs although at a lower rate, suggesting that repair by HR or alternative NHEJ might remain relatively intact, which is consistent with RAD51 foci being able to form early after IR.

Cells deficient in HR become dependent on alternative pathways of DNA DSB repair, which often form complex chromosomal aberrations that trigger cell cycle arrest or death. Loss of 53BP1 is sufficient to reduce the extent of aberrant chromosome structures in BRCA1-deficient cells (Bunting et al., 2010). Here, we determined whether stabilization of 53BP1 in BRCA1-deficient cells exacerbates the extent of genomic instability after IR by analyzing chromosomal aberrations in metaphase spreads. We did not find a profound increase in chromosome aberrations after IR in BOGA cells (Fig. 5 B), in agreement with the deficiency in BRCA1 and 53BP1. However, stabilization of 53BP1 in this context by vitamin D treatment significantly increased the percentage of metaphases with aberrant chromosomes after IR. Thus, CTSL inhibition could represent a novel strategy to induce radiosensitivity in specific types of breast tumors.

To confirm that inhibition of CTSL-mediated degradation of 53BP1 is responsible for the increase in chromosomal aberrations after IR, we analyzed genomic instability in BOGA cells depleted of CTSL. These cells showed a marked increase in genomic instability after IR by analyzing chromosomal aberrations in metaphase spreads. We did not find a profound increase in chromosome aberrations after IR in BOGA cells (Fig. 5 B), in agreement with the deficiency in BRCA1 and 53BP1. However, stabilization of 53BP1 in this context by vitamin D treatment significantly increased the percentage of metaphases with aberrant chromosomes after IR. Similarly, stabilization of 53BP1 in BOGA cells by treatment with the cathepsin inhibitor E-64 markedly increased genomic instability after IR (Fig. 5 C). Consistent with the increase in genomic instability, treatment of BOGA cells with vitamin D or E-64 significantly reduced their recovery from IR (Fig. 5, D and E). Thus, CTSL inhibition could represent a novel strategy to modulate HR efficiency in BRCA1-deficient cells.

Consequences of CTSL-mediated degradation of 53BP1 for DNA repair and genomic stability

To determine the functional consequences of CTSL-mediated degradation of 53BP1 in BRCA1-deficient cells, we evaluated the kinetics of DNA DSB repair by performing comet assays under neutral, nondenaturing conditions (Olive et al., 1990). Fig. 5 A shows that BOGA cells exhibited defects in the fast phase of repair corresponding to classical NHEJ (Iliakis et al., 2004), which is consistent with our previous finding that up-regulation of CTSL in MEFs leads to defects in the fast phase of repair through degradation of 53BP1 (Gonzalez-Suarez et al., 2011). Furthermore, inhibition of CTSL with vitamin D rescued the kinetics of DNA DSB repair, mirroring control cells (Fig. 5 A). These results suggest that CTSL-mediated degradation of 53BP1 hinders NHEJ in BRCA1-deficient cells. However, these cells are still able to repair DSBs although at a lower rate, suggesting that repair by HR or alternative NHEJ might remain relatively intact, which is consistent with RAD51 foci being able to form early after IR.

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genomic instability, in agreement with the resistance of cells double deficient in 53BP1 and BRCA1 to this treatment. Interestingly, stabilization of 53BP1 by vitamin D increased the extent of chromosomal aberrations in response to PARPi, suggesting that inhibition of CTSL-mediated degradation of 53BP1 could induce sensitivity to PARPi.

Increased levels of nuclear CTSL in TNBC and tumors from patients with BRCA1 germline mutations

Although CTSL is one of the most abundant proteases in the endosomal/lysosomal compartment, it has also been identified in the nucleus (Goulet et al., 2004; Duncan et al., 2008). We previously showed that up-regulation of CTSL leads to accumulation of the protease in the nucleus and degradation of 53BP1 (Gonzalez-Suarez et al., 2011). Recent studies demonstrated that loss of 53BP1 is more frequent in TNBC and BRCA1-mutated human breast cancer (Bouwman et al., 2010). Here, we determined whether up-regulation of CTSL occurs in human breast cancers and if it correlates with decreased levels of chromosomal aberrations after IR, when compared with cells deficient in either BRCA1 or CTSL alone (Fig. 6 A). Furthermore, we determined if the effect of vitamin D increasing genomic instability after IR in BOGA cells is mediated by 53BP1 by monitoring chromosomal aberrations in cells doubly depleted of 53BP1 and BRCA1. In these cells, the combination of vitamin D and IR did not result in the profound increase in genomic instability (Fig. 6 B) that we observed in BOGA cells (Fig. 5 B). These results demonstrate that vitamin D exerts its effect in part by stabilizing 53BP1 levels and that the extent of CTSL-mediated degradation of 53BP1 is a key determinant of the ability of BRCA1-deficient cells to deal with the DNA damage generated by IR and putatively other genotoxic agents.

BRCA1-deficient cells are exquisitely sensitive to PARPi (Bryant et al., 2005; Drew et al., 2011). Importantly, loss of 53BP1 reduces the sensitivity of these cells to PARPi (Bunting et al., 2010; Aly and Ganesan, 2011). We assessed whether stabilization of 53BP1 in BOGA cells would increase the extent of genomic instability induced by PARPi. As shown in Fig. 6 C, treatment of BOGA cells with PARPi did not result in profound genomic instability, in agreement with the resistance of cells double deficient in 53BP1 and BRCA1 to this treatment. Interestingly, stabilization of 53BP1 by vitamin D increased the extent of chromosomal aberrations in response to PARPi, suggesting that inhibition of CTSL-mediated degradation of 53BP1 could induce sensitivity to PARPi.
In addition, given the inhibitory effect of vitamin D on this pathway, we monitored the levels of VDR, which mediates most of vitamin D’s cellular effects. We performed immunohistochemical analyses of multitumor tissue microarrays (TMAs) constructed with tissue from 249 patients with sporadic breast cancer (Fig. 7 and Table 1) classified into four molecular subtypes: luminal A, luminal B, Her2, and triple negative.

Immunohistochemical scores (Hscores; ranging from 0 to 300) for Ki67, ER, CTSL, 53BP1, and VDR provided a semi-quantitative measurement of their expression for each tumor subtype (Pallares et al., 2009). As shown in Fig. 7, staining of CTSL was both cytoplasmic and nuclear, whereas 53BP1 staining was only nuclear. Table 1 summarizes the immunohistochemistry results. Whereas cytoplasmic CTSL Hscores were similar in all tumor subtypes, nuclear CTSL Hscores were markedly enhanced in triple-negative tumors. In agreement with the in vitro findings, these high nuclear CTSL Hscores concur with lower 53BP1 Hscores in TNBC compared with all other tumor types. Furthermore, using the median nuclear Hscores for CTSL and 53BP1 of 0 and 150, respectively, as cut-off points with identical statistical power, we confirmed statistically significant differences in CTSL and 53BP1 expression among molecular tumor types, with TNBC emerging as a remarkably different tumor subtype. Table 2 shows that 60% of triple-negative tumors elicited Hscores for nuclear CTSL >0, a frequency more than twofold higher than for any other molecular type (P = 0.0013). Also, 75% of triple-negative tumors expressed 53BP1 Hscores below 150 compared with 39–49% of all other tumor types (P = 0.0049), clearly showing that high expression of nuclear CTSL and low expression of 53BP1 is significantly more associated with TNBC than any other molecular type. Thus, we identified nuclear CTSL as a novel biomarker for subsets of TNBC patients (Fig. S5). Importantly, this new signature (high nuclear CTSL and low 53BP1) could serve to stratify TNBC patients.

Next, we analyzed breast tumors from patients with germline mutations in BRCA1 (n = 18) or BRCA2 (n = 14) by immunohistochemistry (Tables 3 and 4). In comparison with sporadic TNBC, tumors from patients with BRCA1 germline mutations elicited the same high Hscores for nuclear CTSL (P = 0.95) and low Hscores for 53BP1 (P = 1). In contrast, tumors from patients with BRCA2 germline mutations had nuclear CTSL Hscores significantly lower than BRCA1-related tumors. Accordingly, 53BP1 Hscores were higher in tumors from patients with BRCA2 germline mutations than in BRCA1-related tumors or all molecular subtypes of sporadic tumors. These results support our in vitro data for a role of CTSL in the degradation of 53BP1 in BRCA1-deficient cells. Importantly, Fig. 8 A shows a statistically significant inverse linear correlation between Hscores for nuclear 53BP1 and nuclear CTSL in all tumor subtypes with positive nuclear CTSL expression. However, a coefficient of determination of only 6.6% indicates that 93.4% of the variability in 53BP1 Hscores cannot be accounted for by increases in nuclear CTSL. Thus, additional factors might contribute to CTSL-mediated degradation of 53BP1 in these tumors. Identifying these factors could help to discriminate subsets of patients in which this pathway is activated.

Previous studies in human colon cancer cells showed a correlation between expression of VDR and cystatin D, an inhibitor of several cathepsins including CTSL, and up-regulation of cystatin D by vitamin D (Alvarez-Díaz et al., 2009). In addition, our in vitro data show that vitamin D inhibits CTSL-mediated degradation of 53BP1. Vitamin D actions require a functional nuclear VDR (Dusso et al., 2005). Because VDR levels are reduced in several human cancers and BRCA1 loss causes defective VDR translocation to the nucleus (Deng et al., 2009), we hypothesized that there might be a threshold for nuclear VDR that inhibits CTSL-mediated degradation of 53BP1. High levels of nuclear VDR could explain the signature of tumors with high
patients with TNBC (in which BRCA1 is frequently somatically altered) and tumors from patients with BRCA1 germline mutations (Tables 2 and 4). Based on our in vitro data, these signatures could potentially be used as predictors of the response of specific tumors to radiation, cross-linking reagents, and PARPi.

**Discussion**

Breast cancers classified as triple negative or BRCA1 deficient are among the most aggressive and difficult to treat. These tumors harbor similar DNA repair deficiencies and gene expression profiles (Foulkes et al., 2010). Of particular relevance is the loss of BRCA1 function and decrease in 53BP1 levels, two factors with a decisive role in the choice of DNA DSB repair mechanisms: HR or NHEJ (Bouwman et al., 2010). Recent landmark studies demonstrated that loss of 53BP1 allows survival of BRCA1-deficient cells and induces their resistance to DNA-damaging therapeutic strategies (Cao et al., 2009; Bothmer et al., 2010; Bouwman et al., 2010; Bunting et al., 2010). Thus, stabilization of 53BP1 levels represents a promising new strategy for the treatment of these cancers. However, before this study, no information was available about how the levels of 53BP1 mRNA and/or protein are down-regulated in breast tumor cells.

This study demonstrates that up-regulation of CTSL is a mechanism responsible for lowering 53BP1 protein levels in BRCA1-deficient cells, allowing bypass of the characteristic

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**Figure 7.** A new signature for subsets of TNBC patients. Immunohistochemical analysis was performed in breast tumor TMAs from 249 patients, which included four molecular subtypes: luminal A, luminal B, Her2, and triple negative. Representative images of immunohistochemical labeling with Ki67, ERα, Her2, CTSL, and 53BP1 are shown. Note that although cytoplasmic CTSL is observed in all tumor subtypes, nuclear CTSL is markedly up-regulated in a subset of TNBC. In addition, TNBC tumors exhibit a marked decrease in 53BP1. Bars, 100 μm.
of BRCA1 function, which is anticipated to contribute to the pro-
duction of 53BP1 in a subset of TNBC and tumors from patients with
est median levels of nuclear CTSL concur with the lowest levels
between 53BP1 and nuclear CTSL in human tumors. The high-
treatment with PARPi.

depletion and that increased CTSL activity impacts mechanisms
that CTSL is up-regulated in breast cancer cells after BRCA1
activity could represent a strategy for cancer treatment (Lankelma
et al., 2010). Our previous studies in MEFs revealed novel roles
in cell cycle regulation (Rb family members) and DNA repair
(Gonzalez-Suarez et al., 2011). The present study shows
that CTSL can be activated in BRCA1-deficient cells to lower 53BP1
levels (Bouwman et al., 2010), indicating that different mecha-
isms can be activated in BRCA1-deficient cells to lower 53BP1
levels and ensure survival. Importantly, depletion of CTSL or
inhibition of its activity stabilizes 53BP1 protein levels and in-
duces genomic instability in BRCA1-deficient cells after IR or
with PARPi.

Furthermore, we show a significant negative correlation
between 53BP1 and nuclear CTSL in human tumors. The high-
est median levels of nuclear CTSL concur with the lowest levels
of 53BP1 in a subset of TNBC and tumors from patients with
BRCA1 germline mutations and with low nuclear VDR levels.
This study has revealed a new pathway, activated upon loss of
BRCA1 function, which is anticipated to contribute to the pro-
gression of breast cancers with the poorest prognoses. Inhibition
of this pathway by treatment with vitamin D or cathepsin inhibi-
tors could provide a new therapeutic strategy for breast cancer.
Importantly, the status of the pathway offers great potential
as a predictive biomarker for response to therapy.

CTSL up-regulation after growth arrest by
BRCA1 loss and serum deprivation

In a variety of cancers, up-regulation of CTSL has been asso-
ciated with increased invasiveness, metastasis, and overall de-
gree of malignancy (Jedeczko and Sloane, 2004; Skryzdelowa
et al., 2005; Goecheva and Joyce, 2007). Thus, inhibition of CTSL
activity could represent a strategy for cancer treatment (Lankelma
et al., 2010). Our previous studies in MEFs revealed novel roles
for CTSL in the degradation of nuclear factors with functions
in cell cycle regulation (Rb family members) and DNA repair
(Gonzalez-Suarez et al., 2011). The present study shows
that CTSL is up-regulated in breast cancer cells after BRCA1
depletion and that increased CTSL activity impacts mechanisms

Table 1. Immunohistochemical analysis of CTSL, 53BP1, and VDR expression in sporadic human breast cancer

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Molecular type</th>
<th>n (%)</th>
<th>Fisher exact test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear CTSL &gt;0</td>
<td>Luminal A</td>
<td>23 (23.2)</td>
<td>0.0013</td>
</tr>
<tr>
<td></td>
<td>Luminal B</td>
<td>22 (31.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ErbB2 (-1)</td>
<td>12 (27.3)</td>
<td></td>
</tr>
<tr>
<td>Triple negative (-1)</td>
<td>21 (60.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>53BP1 &lt;150</td>
<td>Luminal A</td>
<td>59 (59.6)</td>
<td>0.0049</td>
</tr>
<tr>
<td></td>
<td>Luminal B</td>
<td>35 (50.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ErbB2 (-4)</td>
<td>21 (51.2)</td>
<td></td>
</tr>
<tr>
<td>Triple negative (-4)</td>
<td>9 (25.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear VDR &lt;120</td>
<td>Luminal A</td>
<td>46 (48.4)</td>
<td>0.34</td>
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<tr>
<td></td>
<td>Luminal B</td>
<td>40 (61.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ErbB2 (-4)</td>
<td>25 (58.1)</td>
<td></td>
</tr>
<tr>
<td>Triple negative (-4)</td>
<td>16 (48.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values denote the absolute [n] and the relative (%) frequencies of tumors with Hscore values above (nuclear CTSL) or below (nuclear 53BP1 and VDR) the me-
dian Hscore values for each protein in the overall population of sporadic breast cancer. Bolded frequency values highlight the molecular subtype responsible
for the statistically significant difference identified with the Fisher exact test that compares all molecular types (bold p-value if significant difference).
of DNA DSB repair. Several lines of evidence implicate BRCA1 in transcriptional regulation, chromatin remodeling (Bochar et al., 2000; Mullan et al., 2006), and maintenance of heterochromatin silencing (Zhu et al., 2011). Epigenetic mechanisms such as DNA methylation and expression of miRNAs have been linked to BRCA1 function (Shukla et al., 2010; Kawai and Amano, 2012; Tanic et al., 2012). Although the mechanism by which BRCA1-deficient cells activate CTSL remains unknown, the latency in the activation of this pathway indicates that CTSL is not a direct transcriptional target of BRCA1. Rather, we speculate that the loss of BRCA1 might result in alterations in chromatin structure that either make the CTSL gene more permissive to transcriptional activation over time and/or alter the stability of CTSL mRNAs. Those BRCA1-deficient cells that are able to activate CTSL-mediated degradation of 53BP1 would be poised to continue proliferation.

Furthermore, we show that cells growth arrested in G0/G1 by serum deprivation also exhibit low BRCA1, high CTSL, and low 53BP1 levels, suggesting a functional relationship between these proteins during the cell cycle. We envision a model where the decrease in BRCA1 levels in G0/G1 phases contributes to CTSL-mediated degradation of 53BP1. Up-regulation of CTSL could also trigger a feedback mechanism that lowers BRCA1 protein levels. This is supported by studies showing that BRCA1 is a target for degradation by cysteine proteases of the cathepsin family, although the specific cathepsin was not identified.

Table 3. Immunohistochemical analysis of CTSL, 53BP1, and VDR expression in tumors from patients with BRCA1 or BRCA2 germline mutations

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Mutation type</th>
<th>H score</th>
<th>M-W test p-value (vs. sporadics)</th>
<th>M-W test p-value (BRCA1 vs. BRCA2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic CTSL</td>
<td>BRCA1 mutation [-4]</td>
<td>119 (34.2)</td>
<td>110 [100, 138]</td>
<td>60–190</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-1]</td>
<td>118 (46.2)</td>
<td>120 [100, 120]</td>
<td>100–150</td>
</tr>
<tr>
<td>Nuclear CTSL</td>
<td>BRCA1 mutation [-4]</td>
<td>38 (45.2)</td>
<td>30 [15, 45]</td>
<td>0–180</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-1]</td>
<td>15 (22.7)</td>
<td>4 [0, 15]</td>
<td>0–75</td>
</tr>
<tr>
<td>53BP1</td>
<td>BRCA1 mutation [-1]</td>
<td>111 (28.4)</td>
<td>115 [100, 125]</td>
<td>70–175</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-1]</td>
<td>198 (43.6)</td>
<td>210 [185, 220]</td>
<td>110–270</td>
</tr>
<tr>
<td>Cytoplasmic VDR</td>
<td>BRCA1 mutation [-3]</td>
<td>86 (38.5)</td>
<td>100 [50, 100]</td>
<td>0–150</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-3]</td>
<td>145 (36.7)</td>
<td>150 [115, 165]</td>
<td>100–200</td>
</tr>
<tr>
<td>Nuclear VDR</td>
<td>BRCA1 mutation [-1]</td>
<td>66 (52.9)</td>
<td>53 [27, 100]</td>
<td>0–180</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-3]</td>
<td>175 (57.8)</td>
<td>170 [135, 193]</td>
<td>110–300</td>
</tr>
</tbody>
</table>

Values are mean and median Hscores for nuclear and cytoplasmic CTSL, 53BP1, and VDR in BRCA1- or BRCA2-related tumors. Min–Max denotes minimal and maximal values within the tumor. Dispersion is assessed by SD and percentiles ([P25, P75]); (−X) represents X missing values. Bolded p-values highlight the statistical significance of differences measured by Mann-Whitney test (M-W) between each tumor mutation subtype versus the overall population of sporadic breast cancer or between tumors with BRCA1 versus BRCA2 germline mutations.

Table 4. Frequency of CTSL, 53BP1, and VDR expression within BRCA1- and BRCA2-related tumors

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Mutation type</th>
<th>n (%)</th>
<th>Fisher exact test p-value (vs. sporadics)</th>
<th>Fisher exact test p-value (BRCA1 vs. BRCA2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear CTSL &gt;0</td>
<td>BRCA1 mutation [-4]</td>
<td>12 (85.7)</td>
<td>0.0001</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-1]</td>
<td>7 (53.8)</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>53BP1 &lt;150</td>
<td>BRCA1 mutation [-1]</td>
<td>2 (11.8)</td>
<td>0.0019</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-1]</td>
<td>11 (84.6)</td>
<td>0.0210</td>
<td></td>
</tr>
<tr>
<td>Nuclear VDR &lt;120</td>
<td>BRCA1 mutation [-1]</td>
<td>3 (16.7)</td>
<td>0.0027</td>
<td>0.0051</td>
</tr>
<tr>
<td></td>
<td>BRCA2 mutation [-1]</td>
<td>8 (72.7)</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

Values denote the absolute (n) and the relative (%) frequencies of BRCA1- or BRCA2-related tumors with Hscore values above (nuclear CTSL) or below (nuclear 53BP1 and VDR) the median Hscore values for each protein in sporadic breast tumors. Bolded p-values highlight the statistically significant difference measured by Fisher exact test comparing a molecular subtype with either the overall population of sporadic breast cancer or between tumors with BRCA1 versus BRCA2 germline mutations.
a lesser effect in cells with normal CTSL expression. This is likely because up-regulation of CTSL leads to an increase in the levels of nuclear CTSL, which is low relative to other cellular compartments in normal cells (Gonzalez-Suarez et al., 2011). Although our attempts to localize nuclear CTSL in MCF7 BOGA cells by Western blot were unsuccessful, the immunohistochemical analysis of TMA clearly showed that a subset of TNBC and BRCA1-deficient tumors present with high levels of nuclear CTSL. Interestingly, these tumors are often also deficient in nuclear VDR and 53BP1.

The ability to impact the choice of DNA DSB repair pathway could have profound consequences for cancer therapy. In tumor cells that activate CTSL-mediated degradation of 53BP1 as a means to ensure survival, cathepsin inhibition could stabilize 53BP1, increase genomic instability, and induce growth (Blagosklonny et al., 1999). Future studies need to determine if CTSL functions as a regulator of 53BP1 and BRCA1 protein stability during the cell cycle.

Vitamin D and cathepsin inhibitors can modulate DNA DSB repair choice

Our previous study in MEFs (Gonzalez-Suarez et al., 2011) and the present study in human breast cancer cells reveal an unprecedented role for vitamin D and cathepsin inhibitors in the regulation of DNA DSB repair choice. By stabilizing 53BP1 protein levels in the context of BRCA1 deficiency, CTSL inhibitors facilitate repair of DSBs by NHEJ while inhibiting HR. Importantly, our data indicate that both vitamin D and cathepsin inhibitors impact 53BP1 stability, especially in cells that up-regulate CTSL, i.e., BRCA1-deficient cells, showing a lesser effect in cells with normal CTSL expression. This is likely because up-regulation of CTSL leads to an increase in the levels of nuclear CTSL, which is low relative to other cellular compartments in normal cells (Gonzalez-Suarez et al., 2011). Although our attempts to localize nuclear CTSL in MCF7 BOGA cells by Western blot were unsuccessful, the immunohistochemical analysis of TMA clearly showed that a subset of TNBC and BRCA1-deficient tumors present with high levels of nuclear CTSL. Interestingly, these tumors are often also deficient in nuclear VDR and 53BP1.

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showed a role for VDR in the up-regulation of cathepsin inhibitors (Alvarez-Díaz et al., 2009). Thus, it is tempting to speculate that vitamin D interventions could lead to VDR-induced expression of cystatins and the attenuation of CTSL-mediated degradation of 53BP1. Future studies testing this hypothesis might lead to new strategies of targeted therapy.

The combination of low nuclear VDR or high nuclear CTSL with low 53BP1 levels offers great potential for the stratification of BRCA1-deficient and TNBC patients into different subgroups and as a predictive biomarker for the response of these patients to current therapies. In particular, the use of PARPi as single agents or in combination with radiation or chemotherapy is a leading strategy for breast cancer management, especially for HR-deficient tumors (Farmer et al., 2005; Hellemey et al., 2005; Fong et al., 2009; Tutt et al., 2010; Drew et al., 2011). However, a significant fraction of these cancers acquire resistance to PARPi. A recent study in cell culture and mouse models demonstrated that loss of 53BP1 reduces the sensitivity of BRCA1-deficient cells to PARPi (Bunting et al., 2010).

Our study suggests that BRCA1-deficient and TNBC patients that exhibit low nuclear VDR, high nuclear CTSL, and low 53BP1 levels are likely to be proficient in HR and resistant to PARPi. Therefore, these patients will not benefit from this specific treatment unless levels of 53BP1 are stabilized. For these patients, treatment with vitamin D or CTSL inhibitors to stabilize 53BP1 levels in combination with PARPi might result in the most effective therapy.

Materials and methods

Cells maintained in DMEM, 10% FBS, and antibiotics/antimycotics were transduced with shRNAs and selected in media containing 0.5 mg/ml Genetecin G418 or 2 µg/ml puromycin. For exceptions, one shBRCA1 [5'-TCACTGTA-CAATTAATGTCG-(5')]; 1, 5'-GGAATTCCTAGAGATAGTGTTGG-3', 2, 5'-TATAGCTGTTGGGGACTAG-3'; 3, 5'-CCCATTAGTTTCTCTAAA-3'; 4, 5'-GCC-CCACCTATGTACTGAAAT-3'; 5, 5'-CCACAATCTAAGTTACGTTG-3']. Lentiviral transductions were performed as previously described (Gonzalez-Suarez et al., 2009). In brief, 293T cells were transfected with viral packaging (pHR 8-2.23) and envelope (pCMV-VSV-G) plasmids along with the vector containing the shRNA of interest. After 48 h, viral media was collected and used to infect target cells in one 4–6-h infection. Cells were allowed to recover for 48 h and treated with the appropriate selection drug. Viral packaging and envelope plasmids were gifts from S. Stewart (Washington University, St. Louis, MO). HA-BRCA1 (Addgene) transient transfections were performed using the X-tremeGENE HP transfection reagent (Roche). For growth arrest by serum deprivation, MCF7 cells were incubated in DMEM, 0.1% FBS, and antibiotics/antimycotics for 48 h. DNA content was monitored after ethanol fixation and propidium iodide labeling (1 mg/ml in water).

Cell treatments

Vitamin D. Cells were incubated with 10^{-7} M 1α,25-dihydroxyvitamin D3 (Sigma-Aldrich) for 24–48 h, as indicated. Aliquots of 1 nM 1α,25-dihydroxyvitamin D3 were resuspended in 1 ml BGS and diluted in DMEM to a final concentration of 10% BGS. BGS was the vehicle control.

Cell treatments

E-64. Cells were incubated with the broad-spectrum cathepsin inhibitor E-64 (Sigma-Aldrich) diluted in water at a concentration of 10 µM for 24 h.

PARPi. Cells were treated with the PARPi EB-47 (Sigma-Aldrich) diluted in water at a concentration of 1 µg/ml for 48 h.

IR. For determining the extent of genomic instability, cells were irradiated with 2 Gy and analysis of metaphase spreads was performed 24 h.
after IR. For assaying formation of IRIF, cells were irradiated with 8 Gy and fixed and processed for immunofluorescence 1, 3, or 6 h after IR as indicated. For comet assays, cells were irradiated with 8 Gy.

Western blotting
Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.5% SDS) supplemented with PMSF, protease inhibitors (Sigma-Aldrich), and 20 mM DTT. Protein detection was performed using the following antibodies: BRCA1 (Santa Cruz Biotechnology, Inc.), 53BP1 (Novus Biologicals), CTSL (Santa Cruz Biotechnology, Inc.), β-Tubulin (Sigma-Aldrich), and p107 (Santa Cruz Biotechnology, Inc.).

Immunofluorescence
For immunofluorescence of RAD51 and 53BP1, cells were plated on coverslips and fixed in 3.7% formaldehyde and 0.2% Triton X-100 in PBS for 20 min at room temperature. Coverslips were washed and blocked for 1 h at 37°C in 1% BSA and 0.1% Triton X-100 in PBS. Incubation with antibodies recognizing RAD51 (1:200, Santa Cruz Biotechnology, Inc.) or 53BP1 (1:1,000, Novus Biologicals) was performed for 1 h at 37°C, followed by washing in PBS and incubation with Alexa Fluor 488 goat anti–rabbit (1:1,000; Invitrogen) secondary antibody for 1 h at 37°C. After washing in PBS, coverslips were mounted using Vectashield with DAPI (Vector Laboratories).

Proliferation assay
To quantify growth upon depletion of BRCA1, cells were plated in triplicate at 150,000 cells/well and counted 96 h later. Treated cells were irradiated with 2 Gy and incubated for 3 days at 37°C, after which time 1 ml of fresh media was added and the cells were incubated for an additional 7 days. For each concentration of ethanol, and washed with PBS. Ki67, ERα, and Her2 were stained with hematoxylin. Appropriate positive and negative controls were used to determine molecular subtype. Comparative studies of CTSL, VDR, and 53BP1 expression were performed on sequential serial sections. Antibody retrieval for CTSL and ERα was achieved by heat treatment at 95°C for 20 min. For a high pH solution [Dako], heat-induced antigen retrieval for 53BP1 and Ki67 was performed in a low pH solution [Dako]. Before staining the sections, endogenous peroxidase was blocked. Primary antibodies and incubation times were as follows: CTSL (1:50; Santa Cruz Biotechnology, Inc.); incubation overnight at 4°C; 53BP1 (1:2,500; Novus Biologicals); incubation 20 min at room temperature; VDR (1:2,000; Abcam); incubation 20 min at room temperature; Ki67 (Ready-to-use; Dako); incubation 20 min at room temperature), and Her2 (Ready-to-use; Dako). The reaction was visualized with the Streptavidin-Biotin Complex (Dako) for CTSL and Envision Flex (Dako) for 53BP1, Ki67, and ERα. Sections were counterstained with hematoxylin. Appropriate positive and negative controls were also tested.

Hscores provide a semiquantitative measurement of protein expression per tumor by taking into consideration the percentage of positive

Novel pathway responsible for 53BP1 loss in cancer • Grotsky et al.
cells and the intensity of their staining. An H-score ranging from 0 (no immune reaction) to 300 (maximal immunoreactivity) was obtained with the formula H-score = 1× (% light staining) + 2× (% moderate staining) + 3× (% strong staining). The reliability of such scores for the interpretation of immunohistochemical staining in TMAs has been reported previously (Pallares et al., 2009).

H-score staining was evaluated according to a standard protocol (Hercep-Test; Dako) and scored as four intensities [i.e., negative = 0; weak = 1; moderate = 2×; and strong = 3×], considering negative Her2 expression for intensity values of 0, 1+, and 2+ when there was no amplification by FISH and positive for intensity values of 3+ and 2+, when 2+ was amplified by FISH. For each marker, there were a variable number of non-assessable cases caused by technical problems including no representative counter sample left in the cylinders, detachment, cylinders missed while constructing the array, necrosis, and absence of viable tumor cells in the TMA sections.

Statistical analysis

For the in vitro experiments, a two-tailed student’s t-test was used to calculate statistical significance of the observed differences. Excel 2010 (Microsoft) was used for the calculations. In all cases, differences were considered statistically significant when P < 0.05. For some figures the 95% confidence interval based on an exact binomial distribution was calculated to determine significant differences among samples. For the TMA studies, a Kruskal-Wallis test was used to test the statistical significance of the observed differences in CTSL, VDR, and 53BP1 Hscores between molecular breast tumor subtypes. Tumors were partitioned according to cut-off nuclear Hscores for CTSL, 53BP1, and VDR selected by their median values as >0, <150, and <120, respectively. Once cut-off points were applied, Fisher exact test was used to assess the statistical significance of the differences in the distribution of the two categories of CTSL, 53BP1, and VDR Hscores above or below cut-off points for all breast tumor types. In the analysis of BRCA1 and BRCA2 samples, we used the Mann-Whitney test to analyze Hscore differences between them as well as differences of each of them with the sample of sporadic tumors, and we used the Fisher exact test to assess differences in the distributions of grouped defined by the same cut-off points used for the sporadic tumors. The subsample of TNBC was also compared with the sample of germinial BRCA1 mutated cancers using the same statistical tests. The Pearson correlation coefficient together with linear regression models assessed the statistical significance of the relationship between nuclear CTSL and 53BP1 Hscores. R package was used to perform all TMA statistical tests. Differences were considered significant when P < 0.05.

Online supplemental material

Fig. S1 shows an entire BRCA1 Western blot as well as Western blots of 53BP1, CTSL, VDR, and 53BP1 Hscores in MDA-MB-231 cells and by different BRCA1 shRNAs. Fig. S2 shows qRT-PCR results for the sh53BP1/shBRCA1 and the shBRCA1/shCTSL doubly depleted cells. Fig. S3 shows results from immunofluorescence studies of CTSL, BRCA1, and RAD51 IFI in the generated cell lines and upon different treatments. Fig. S4 shows RAD51 foci formation data at 3 and 6 h after IR. Fig. S5 shows results from immunofluorescence studies of 53BP1, BRCA1, and CTSL, VDR, and 53BP1 Hscores between molecular breast tumor subtypes.

References


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