S100A2 is a BRCA1/p63 coregulated tumour suppressor gene with roles in the regulation of mutant p53 stability

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Here, we show for the first time that the familial breast/ovarian cancer susceptibility gene, BRCA1, along with interacting ΔNp63 proteins, transcriptionally upregulate the putative tumour suppressor protein, S100A2. Both BRCA1 and ΔNp63 proteins are required for S100A2 expression. BRCA1 requires ΔNp63 proteins for recruitment to the S100A2 proximal promoter region, while exogenous expression of individual ΔNp63 proteins cannot activate S100A2 transcription in the absence of a functional BRCA1. Consequently, mutation of the ΔNp63/p53 response element within the S100A2 promoter completely abrogates the ability of BRCA1 to upregulate S100A2. S100A2 shows growth control features in a range of cell models. Transient or stable exogenous S100A2 expression inhibits the growth of BRCA1 mutant and basal-like breast cancer cell lines, while short interfering RNA (siRNA) knockdown of S100A2 in non-tumorigenic cells results in enhanced proliferation. S100A2 modulates binding of mutant p53 to HSP90, which is required for efficient folding of mutant p53 proteins, by competing for binding to HSP70/HSP90 organising protein (HOP). HOP is a cochaperone that is required for the efficient transfer of proteins from HSP70 to HSP90. Loss of S100A2 leads to an HSP90-dependent stabilisation of mutant p53 with a concomitant loss of p63. Accordingly, S100A2-deficient cells are more sensitive to the HSP-90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin, potentially representing a novel therapeutic strategy for S100A2- and BRCA1-deficient cancers. Taken together, these data demonstrate the importance of S100A2 downstream of the BRCA1/ΔNp63 signalling axis in modulating transcriptional responses and enforcing growth control mechanisms through destabilisation of mutant p53.

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BRCA1 was initially cloned in 1994 as one of the genes predisposing to early-onset breast and ovarian cancer. 1 BRCA1 germline mutations confer a cumulative lifetime risk of 50–85 and 12–60% of developing breast and ovarian cancer, respectively. 2 Somatic BRCA1 mutations are rare in sporadic breast cancer, but BRCA1 expression is downregulated in ~30% of sporadic cases. 3 BRCA1 is known to have multiple roles including DNA damage repair, cell cycle checkpoint control and transcriptional regulation. Although BRCA1 does not bind to DNA in a sequence-specific manner, it facilitates transcriptional control at a number of different levels (e.g. interacts with transcription factors, the RNA polymerase II holoenzyme complex and proteins involved in chromatin remodelling; for a review see Mullan et al. 4). Through these multiple interactions, BRCA1 can coactivate or corepress a large number of target genes involved in its downstream functions.

Our group has recently identified the ΔNp63 family of proteins as novel BRCA1 interactors with important roles in BRCA1-mediated tumour suppression. 5 Our data suggest that the transcriptional control of ΔNp63 expression by BRCA1 is important for the maintenance of genomic stability and for the normal differentiation of mammary tissue. The tumour suppressor role of p53 in breast tissue was also demonstrated by the finding that mutant p53 proteins may drive tumourigenesis through the formation of p53–SMAD complexes, which interacts with and opposes p63 function, leading to enhanced metastasis. 6 The family of S100 proteins is a highly conserved group of more than 20 members of small (9–13 kDa) acidic calcium-binding proteins. 7 Numerous S100 proteins are known to be overexpressed in cancers, while S100A2 is thought to primarily act in a tumour-suppressive role. 8–10 S100A2 expression is downregulated in several tumour types including breast cancer, 11 with promoter methylation being one mechanism responsible for this. 12 A number of S100 proteins have been shown to interact with the p53 tumour suppressor, interactions that may be influenced through p53 phosphorylation and acetylation events, potentially activating as well as inhibiting p53. 13 Specifically, S100A2 is known to interact with wild-type p53 and other p53 family members to enhance their transcriptional activity. 13 S100A2 has been shown previously
to be a direct transcriptional target of both p63 and p73 proteins. The function of S100A2 (also called S100L) is still unknown but it is localised to the basal layer of normal human epidermis and hair follicles, possibly reflecting its p63-dependent activation.

In this study, we show that S100A2 is a coregulated target of both BRCA1 and ΔNp63, requiring both proteins to be expressed and fully functional for optimal S100A2 expression. We observe consistent growth inhibitory effects in multiple breast cancer cell lines and non-tumourigenic breast cell lines, consistent with its role as a tumour suppressor in breast tissue. S100A2 knockdown results in an increase in mutant p53 and p63 with a concomitant loss of p63. We demonstrate that the observed increase in p53 is owing to HSP90-dependent stabilisation and S100A2-depleted cells are therefore more sensitive to the HSP90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG). Taken together, these data suggest a role for a BRCA1–ΔNp63-S100A2 signalling pathway important for the growth control of breast tissue through the regulation of HSP90–client protein interaction.

Results

We have consistently observed S100A2 as a positively regulated BRCA1 target gene following several microarray experiments (data not shown). To validate this, we first stably reconstituted wild-type BRCA1 into the BRCA1 mutant HCC1937 and basal-like (BRCA1 low-expressing) MDA-MB-468 cells. Figure 1a shows western blot analyses of HCC1937-BR cells showing marked upregulation of S100A2 protein following BRCA1 reconstitution relative to the empty vector (EV) control cell line, HCC1937-EV. Figure 1aii confirmed that this effect was transcriptional with an approximate fivefold upregulation of S100A2 mRNA. Similar effects were observed with MDA-MB-468 cells relative to EV controls (Figures 1bi and ii). In contrast, siRNA knockdown of BRCA1 in non-tumourigenic HME-1 (Figures 1ci and ii) or luminal MCF7 cells (Figures 1di and ii) resulted in respective downregulation of S100A2 proteins and mRNAs. S100A2 was also shown to be downregulated in BRCA1-associated tumours using publically available data sets (Supplementary Figure 1). Clearly, S100A2 is regulated by BRCA1 at a protein and mRNA level in multiple breast cell lines and in primary breast cancers.

As detailed in the Introduction, we recently identified the ΔNp63 family of proteins as BRCA1 transcriptional targets. BRCA1 transcriptionally regulates these proteins through specific interaction with ΔNp63; to drive a positive ΔNp63 regulatory loop, S100A2 has already been described as a p63 target gene, so we decided to investigate the mechanism underpinning S100A2 upregulation. Both HCC1937- and MDA-MB-468 BRCA1-reconstituted cell lines were treated (alongside EV controls) with ΔNp63siRNA. As Figures 2a and b show, we observed strong induction of ΔNp63 in BRCA1-reconstituted cells (relative to EV controls) accompanied by pronounced upregulation of S100A2. However, ΔNp63 siRNA totally abrogated S100A2 protein and mRNA in BRCA1-reconstituted cells, showing that the BRCA1–ΔNp63 complex is a crucial regulator of S100A2. To demonstrate the ΔNp63 specificity, we performed ΔNp63-, TAp63- and p53-specific siRNA knockdowns in MCF7 cells (Supplementary Figure 2A) and immunoblotted for S100A2. As Figure 2ci shows, only ΔNp63 knockdown reduced S100A2 levels and this was consistent for γ- or α/β-specific siRNAs (Figure 2cii, knockdowns shown in Supplementary Figure 2B). Similar results are also seen with knockdown of mutant p53 in the MDA-BR
p63 often transcriptionally activates basal target genes in collaboration with the AP2 family of proteins.\textsuperscript{18} We were able to show that AP2\textsubscript{a} was also required for S100A2 activation (Figure 2cii, knockdowns shown in Supplementary Figures 2Bii and iii). Finally, we decided to investigate if exogenous expression of p63 could bypass the requirement for BRCA1 expression and activate S100A2 independently. BRCA1 mutant HCC1937 cells were transfected with either an EV construct or different \( \Delta N\) or TA-p63 isoforms. As Figure 2d shows, only BRCA1 reconstitution restored S100A2 expression, suggesting that for S100A2 promoter activation, BRCA1 was still required regardless of p63 levels. These data together show that both BRCA1 and \( \Delta N\)p63 proteins (in collaboration with AP2\textsubscript{a}) are required for optimal activation of S100A2 transcription.

Next, we wanted to define exactly how BRCA1 modulated S100A2 promoter activity. We PCR cloned the proximal S100A2 promoter and transfected into a variety of cell lines with modulated BRCA1. For both HCC1937- and MDA-MB-468 BRCA1-reconstituted cells, we observed significant

![Figure 2](https://example.com/figure2.png)

**Figure 2.** (a) Western blot analysis of BRCA1 mutant HCC 1937 cells stably transfected with either EV or wild-type BRCA1 (BR) and transiently transfected with siRNA targeting \( \Delta N\)p63 (DNp63si) or scrambled control (Scr). Blots were probed with p63, S100A2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. (ii) Real-time polymerase chain reaction (PCR) analysis of the same samples as (i) with specific primers for S100A2 and \( \beta\)-tubulin as a housekeeper. (b) Western blot analysis of BRCA1 low MDA468 cells stably transfected with either EV or BR and transiently transfected with siRNA targeting \( \Delta N\)p63 (DNp63si) or Scr. Blots were probed with p63, S100A2 and GAPDH as a loading control. (i) Real-time PCR analysis of the same samples as (i) with specific primers for S100A2 and \( \beta\)-tubulin as a housekeeper. (c) Western blot analysis of MCF-7 cells transiently transfected with siRNA targeting \( \Delta N\)p63 (DNp63si), TAp63 (TAp63si), p53 (p53si) or Scr. Blots were probed with S100A2 and GAPDH as a loading control. (ii) Western blot analysis of MCF-7 cells transiently transfected with siRNA targeting p63\textsubscript{a}, p63\textsubscript{a} and \( \beta\) (p63\textsubscript{a}+/\-si), all p63 isoforms (pan-p63), AP2\textsubscript{a} (AP2\textsubscript{a}si), AP2\textsubscript{b} (AP2\textsubscript{b}si) and Scr. Blots were probed with S100A2 and GAPDH as a loading control. (d) Western blot analysis of HCC BR cells or HCC 1937 cells transiently transfected with EV, \( \Delta N\)p63\textsubscript{a}, \( \Delta N\)p63\textsubscript{y}, TAp63\textsubscript{a} or TAp63\textsubscript{y}. Blots were probed for S100A2, p63 and GAPDH as a loading control.
luciferase activities that were markedly reduced with siRNA against ΔNp63 (Figures 3ai and ii). Similarly, BRCA1 and ΔNp63 siRNA knockdowns in MCF7 cells reduced S100A2 luciferase, while p53 knockdown had minimal effect (Figure 3aiii). In addition to the previously characterised p53 binding site located ~2200 bp upstream of the untranslated exon 1 of the S100A2 gene, Kirschner et al. described a second p63-specific binding site located just upstream of the first exon. While deletion of the p53-responsive site had no effect on the observed luciferase activity, we could demonstrate that the predicted ΔNp63 binding site was responsible for this activation since site-directed mutagenesis of this site completely abrogated the BRCA1-dependent luciferase activation in both HCC1937 and MDA-MB-468 cell models (Figures 3bi and ii, respectively). We also PCR cloned two regions within intron 2 of S100A2, which have been shown to be p63 responsive. Although these regions were p63 responsive, they did not appear to be consistently regulated by BRCA1 (Supplementary Figure 3); therefore, these promoter regions were not studied further. Chromatin immunoprecipitation (ChIP) assays showed localisation of both BRCA1 and ΔNp63 to the S100A2 proximal promoter region, while an upstream (~2 kb) region showed no obvious recruitment of either protein (Figure 3ci). Finally, we could demonstrate the requirement for ΔNp63 for BRCA1 recruitment to the S100A2 promoter using ΔNp63 siRNA knockdown followed by ChIP assay, showing total loss of BRCA1 recruitment following ΔNp63 siRNA (Figure 3cii). Taken

Figure 3  (a) Luciferase reporter assay of (i) HCC EV and wild-type BRCA1 (BR) cells and (ii) MDA EV and BR cells transiently transfected with siRNA targeting ΔNp63 (p63si) or scrambled control (Scr). Cells were then transfected with either S100A2 promoter construct (S100A2pro) or the EV control (pGL3). Renilla was used to normalise for transfection efficiency. Values are expressed as relative luciferase units (RLUs) normalised to pGL3 and Renilla. (iii) Luciferase reporter assay of MCF7 cells transiently transfected with siRNA targeting BRCA1 (BRsi), ΔNp63 (ΔNsi), p53 (p53si) or Scr. Cells were then transfected with either S100A2 promoter construct (S100A2pro) or the EV control (pGL3). Renilla was used to normalise for transfection efficiency. Values are expressed as RLUs normalised to pGL3 and Renilla. (b) Luciferase reporter assay of (i) HCC EV and BR cells and (ii) MDA EV and BR cells transfected with either an S100A2 promoter construct (S100A2pro), an S100A2 promoter construct with one p53/p63 binding site deleted (Del), an S100A2 promoter construct with one p53/p63 binding site deleted and the second mutated (SDM) or the EV control (pGL3). Renilla was used to normalise for transfection efficiency. Values are expressed as RLUs normalised to pGL3 and Renilla. (c) ChIP assay of MDA468 BR cells showing localisation of p63 and BRCA1 on the S100A2 promoter. One per cent of sonicated lysate before immunoprecipitation was used as a positive control (input) and IgG pulldown was used as a negative control (IgG1 and IgG2a). In addition, polymerase chain reaction (PCR) primers for a region upstream of the S100A2 promoter show specificity for the promoter. (ii) ChIP assay of MDA468 BR cells transiently transfected with siRNA targeting ΔNp63 (ΔNp63si) or Scr showing recruitment of BRCA1 and p63 to the S100A2 promoter. One per cent of sonicated lysate before immunoprecipitation was used as a positive control (input) and IgG pulldown was used as a negative control (IgG1 and IgG2a). In addition, PCR primers for a region upstream of the S100A2 promoter show specificity for the promoter.
together, these data demonstrate that DNP63-dependent recruitment to a specific site in the S100A2 promoter is required for its transcriptional activation.

S100A2 has previously been reported to act as a tumour suppressor gene in breast tissue; thus, we wanted to address the functional consequences of S100A2 loss and how important this protein was for BRCA1-mediated tumour suppression. Stable exogenous S100A2 expression was performed in the BRCA1-defective HCC1937, MDA-MB-468 and transient S100A2 expression in the SUM149 cells (Figures 4ai–ii and Supplementary Figure 4Aii) resulted in a reproducible inhibition of cell growth, as assessed by 5-day growth curves (Figures 4bi–ii and Supplementary Figure Ai). Again knockdowns of S100A2 in BRCA1-proficient, non-

Figure 4  (a) Western blot analysis of (i) MDA468 or (ii) HCC1937 cells stably transfected with either EV or S100A2 or (iii) HME-1 cells transiently transfected with siRNA targeting S100A2 or scrambled control (Scr) probed for S100A2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. (b) Five-day growth curve of the same samples as (a). (c) Western blot analysis of (i) MDA BR or (ii) HCC BR cells transiently transfected with siRNA targeting S100A2 or Scr. Blots were probed for S100A2, p53, p63 and β-tubulin as a loading control. Western blot analysis of (iii) MDA 468 and (iv) HCC 1937 cells stably transfected with either EV or S100A2. Blots were probed for S100A2, p53, p63 and β-tubulin as a loading control. (d) Real-time polymerase chain reaction (PCR) analysis of (i) MDA BR or (ii) HCC BR cells transiently transfected with two independent siRNAs targeting S100A2 (S100A2 no. 2 and S100A2 no. 3) and Scr using primers specific for S100A2, p53, p63 (pan), CyclinG2, Sharp1 and Caveolin1 (Cav1). Real-time PCR analysis of (iii) MDA 468 and (iv) HCC 1937 cells stably transfected with either EV or S100A2 using primers specific for S100A2, p53, p63 (pan), CyclinG2, Sharp1 and Caveolin1 (Cav1)
tumourigenic HME1 and MCF10-A cells (Figure 4aiii and Supplementary Figure 4Bii) resulted in enhanced proliferation (Figure 4bii and Supplementary Figure 4B). Consistent with previous findings, the effect of S100A2 on cell numbers appears to be through the regulation of transition from the S to G2/M phase of the cell cycle and not through the regulation of cell viability.20 Although PARP cleavage (indicative of apoptosis) is not observed when S100A2 is modulated (Supplementary Figure 4C), there is an increase in the number of cells in the S phase (as measured by bromodeoxyuridine (BrdU) incorporation) when S100A2 is stably overexpressed in HCC1937 and the opposite seen when S100A2 is depleted by siRNA (Supplementary Figure 4D). An interesting observation was made that while BRCA1 and ΔNp63 knockdowns resulted in S100A2 downregulation, S100A2 siRNA also resulted in an increase in mutant p53 protein levels with a concomitant reduction in ΔNp63 protein levels in both HCC1937-BR and MDA-MB-468-BR cell lines (Figures 4c–ii). Similar effects were also observed with BRCA1 and ΔNp63siRNA (Supplementary Figure 5), as well as in the BRCA1 low expression MDA468-EV cells (Supplementary Figure 2C). Using the converse approach of stable overexpression of S100A2 in the BRCA1-deficient MDA-MB-468 and HCC1937 cells, we observed a decrease in mutant p53 protein levels and an increase in ΔNp63 protein levels (although ΔNp63 levels were considerably lower than those observed in BRCA1-proficient cell lines) (Figures 4c–ii and iv). Although the effect on p53 levels was not transcriptional, the effect on ΔNp63 was clearly evident at an mRNA level (Supplementary Figure 5B). Previous studies have demonstrated that mutant p53, particularly ‘gain-of-function’ mutant p53 proteins are able to interfere with the tumour-suppressive role of p63 to drive metastasis.8 Accordingly, the expression of p63 target genes, CyclinG2, Sharp1 and Caveolin1, were all modulated in an S100A2-dependent manner (Figure 4d). Studies are ongoing to determine the exact mechanism of how S100A2 regulates p63; however, our data suggests that S100A2 modulates p63 through a mechanism independent of p63 binding to tumour-suppressive complexes.6

As S100A2 levels appeared to antagonise mutant p53 expression, we hypothesised that S100A2 may somehow alter the stability of mutant p53 to suppress tumourigenesis. It has been documented that certain p53 missense mutants are dependent on chaperones such as HSP90 for their stability.22 Given the ability of S100A2 to interact with the cochaperone HSP70/HSP90 organising protein (HOP) and therefore modulate the transfer of client proteins from HSP70 to HSP90,23 we investigated the effect of S100A2 on the chaperone-dependent folding and thus the stability of mutant p53. Levels of mutant p53 in the denatured and native conformation were quantified by immunoprecipitation using specific antibodies (PAb-240 and PAb-1620, respectively) and compared with total levels as determined by immunoprecipitation with an antibody that detects total p53 levels (DO-1). Following knockdown of S100A2 in MDA468-BR cells, total p53 increased as shown previously (Figure 5ai and Supplementary Figure 6A). As the majority of the contact mutant (R280H) p53 in the MDA-MB-468 cell line is found in the native conformation,23 it was only possible to compare the overall levels of mutant p53. Knockdown of S100A2 resulted in an increase in the native form of mutant p53, normalised to total p53 protein (Figure 5ai and Supplementary Figure 6A). Conversely, stable overexpression of S100A2 in the HCC1937 cells resulted in the expected decrease in total p53 (Figure 5bi and Supplementary Figure 6Aii) with a decrease in the native form and an increase in the denatured form of mutant p53 (Figures 5bii and iii and Supplementary Figure 6Aii). To determine if S100A2 modulated the binding of mutant p53 to HSP90, we assessed the amount of denatured mutant p53 interacting with HSP90 by immunoprecipitation. In MDA468-BR cells, knockdown of S100A2 resulted in an increase in denatured p53 interacting with HSP90 (Figure 5ci). Conversely, overexpression of S100A2 in the HCC1937 cell led to a decrease (Figure 5cii). We confirmed that S100A2 interacted with HOP (but not HSP90) by immunoprecipitation (Figure 5di) and demonstrated that loss of S100A2 expression results in increased HOP–HSP90 interaction (Figure 5di) and therefore predicts for increased client protein transfer from HSP70 to HSP90. Next, we utilised the HSP-90 inhibitor, 17-AAG, to evaluate the dependency of mutant p53 for HSP90-mediated folding. Knockdown of S100A2 in MDA468-BR cells led to the expected increase in mutant p53, but this was abrogated by treatment with 17-AAG (Figure 5ei). These cells were also more sensitive to the inhibitor (relative to scrambled control siRNA-treated cells) (Figure 5fi and Supplementary Table 1). Similar results are also seen in the HCCBR and HME-1 cells following siRNA (Supplementary Figure 6B and Supplementary Table 1).

Figure 5  (a) Quantitation by densitometry of immunoprecipitation (IP) in MDA BR cells transiently transfected with two independent siRNAs targeting S100A2 (S100A2 no. 2 and S100A2 no. 3) and scrambled control (Scr). (i) Total p53 was immunoprecipitated using DO1 and (ii) folded/native conformation p53 was immunoprecipitated using pAB1620. (b) Quantitation by densitometry of IP in HCC1937 cells stably transfected with EV or S100A2. (i) Total p53 was immunoprecipitated using DO1. (ii) Folded/native conformation p53 was immunoprecipitated using pAB1620 and (iii) denatured p53 was immunoprecipitated using pAB240. (c) Co-immunoprecipitation of (i) MDA BR cells transiently transfected with siRNA targeting S100A2 (SA2si) and Scr and (ii) HCC1937 cells stably transfected with EV or S100A2 (SA2). Cell lysates were immunoprecipitated with pAB240, and then following western blot analysis, probed for HSP90. (d) IP-western blot analysis of MDA BR cells. Whole-cell lysate was immunoprecipitated with either S100A2 antibody or an IgG control followed by western blot analysis. Blots were probed with HSP90, HOP or S100A2. (ii) IP-western blot of MDA BR cells was transiently transfected with two independent siRNAs targeting S100A2 (S100A2 no. 2 and S100A2 no. 3) and Scr. Whole-cell lysates were immunoprecipitated with either S100A2 or IgG control followed by western blot analysis. Blots were probed for HSP90, HOP and S100A2. (e) Western blot analysis of MDA468 BR cells transiently transfected with two independent siRNAs targeting S100A2 (SA2si no. 2 and no. 3) or Scr. Cells were then treated with 4 μM 17-AAG or vehicle control for 16 h. Blots were probed with p53, S100A2 or β-tubulin as a loading control. (ii) Western blot analysis of HCC1937 cells stably transfected with EV control or S100A2 (SA2). Cells were treated with 2 μM 17-AAG or vehicle control for 16 h. Blots were probed with p53, S100A2 and β-tubulin as a loading control. (f) Dose–response curve of (i) MDA BR cells transiently transfected with two independent siRNAs targeting S100A2 (S100A2 no. 2 and no. 3) or Scr and (ii) HCC1937 cells stably transfected with EV or S100A2 (SA2). Cells were treated with the indicated range of concentration of 17-AAG for 72 h before cell viability was assessed by MITT. Cell survival was normalised to vehicle control (100%).
as well as in HCC1937 cells stably reconstituted with S100A2, with S100A2 conferring over 10-fold decrease in sensitivity to 17-AAG compared with EV control (Figures 5eii and fii and Supplementary Table1). Using cell line data from Lehmann et al., IC50 values for the 17-AAG analogue, 17-dimethylaminoethylamino-17-demethoxygeldanamycin.
(17-DMAG), were extrapolated and correlation with S100A2 expression levels (as determined by microarray analysis of the same publically available data sets utilised in the study) was assessed (Supplementary Figure 6C and Supplementary Table1). This showed highly significant correlation between S100A2 levels and sensitivity to HSP90 inhibition. Taken together, these data highlight the important role of S100A2 as a regulator of mutant p53 protein levels and sensitivity to HSP90 inhibition.

**Discussion**

In this study, we have identified S100A2 as an integral component of BRCA1 tumour suppression downstream of the BRCA1–ΔNp63 transcription complex. We show that S100A2 consistently represses cell proliferation in a range of breast cell lines and that S100A2 expression is doubly important for regulating both the optimal transcription of p63 and for helping to destabilise mutant p53 protein levels. We have shown that S100A2 interferes with the transfer of client proteins from HSP70 to HSP90, therefore inhibiting the folding and stabilisation of the mutant protein. Inhibition of HSP90 abrogates the increase in mutant p53 observed when S100A2 expression is lost and S100A2-deficient cells are acutely sensitive to the HSP90 inhibitor, 17-AAG.

The role of S100A2 in tumour suppression is still poorly described. S100A2 is the only member of the S100 family that consistently represses cell proliferation in a range of breast cancer cell lines and that S100A2 expression levels (as determined by microarray analysis of the same publically available data sets utilised in the study) was assessed (Supplementary Figure 6C and Supplementary Table1). This showed highly significant correlation between S100A2 levels and sensitivity to HSP90 inhibition. Taken together, these data highlight the important role of S100A2 as a regulator of mutant p53 protein levels and sensitivity to HSP90 inhibition.

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ionising radiation as well as PARP inhibitors. Our work further highlights the interplay between BRCA1 and HSP90 inhibition. We propose that BRCA1-deficient breast tumours, where abrogation of p53 function through mutation, appear to be a prerequisite for BRCA1-defective cancers to progress, would be extremely sensitive to 17-AAG treatment owing to the loss of S100A2 expression and thus increased HSP90-dependent stabilisation of mutant p53. In addition, 17-AAG may further downregulate BRCA1 levels, and thus S100A2, resulting in greater drug sensitivity.

HSP90 is an attractive drug target owing to the fact that most inhibitors display selectivity for tumour over normal cells. Currently, there are 17 different HSP90 inhibitors in clinical trials in a variety of different cancer types. However, no HSP90 inhibitor has been FDA approved to date. Although phase I clinical trials of geldanamycin derivatives showed promising results, monotherapy showed little activity in phase II. This may be due to the suboptimal doses of the drugs used to avoid toxicity. However, HSP90 inhibitors have shown promising results when used in combination with other agents such as herceptin for the treatment of Her2positive breast cancers. As with most modern targeted therapies, personalising treatments to match the patients’ genetic profile or particular tumour types may increase the effectiveness of HSP90 inhibition. S100A2 may therefore represent a novel biomarker of response to HSP90 inhibitors and BRCA1-deficient tumours may benefit from the combination of HSP90 inhibition with DNA-damaging agents such as PARP inhibitors. Alternatively, other agents, which have been shown to interfere with HSP90 binding to client proteins, such as histone deacetylase inhibitors, may also prove useful in treating BRCA1-deficient cancers.

Materials and Methods
Cell lines. All cell lines were purchased from ATCC (in partnership with LGC Standards, Middlesex, UK). Cell lines were characterised by isoenzyme/cytochrome c oxidase I assay and short tandem repeat analysis by the cell bank. Full details of the HCC-EV/BR, MDA468-EV/BR and MCF7 cell lines are provided in Mullan et al. and Tkocz et al. HCC1937 and MDA468-EV and -S100A2 cells were generated by stable retrovirus transfection of the cells with (i) pBabe EV or pBabe S100A2 constructs, respectively. Infected cells were selected in the presence of 1 μg/ml puromycin. All other cell lines were maintained as described previously.

Cloning of S100A2 and its promoter. Flag-tagged S100A2 was PCR amplified and cloned into pBabe-puro EV. Primers are listed in Supplementary Materials and Methods. S100A2 regulatory regions were cloned into pGL3 basic and used at a final concentration of 100 nM. siRNA sequences are S100A2 no. 2, and used are: S100A2 – F, 5’-GCTCTCCTCCTGGTCTG-3’ and R, 5’-TCTCCA GAGAATCTGACAT-3’; p53 – F, 5’-TCCAGGTGGAAGAAATTTGCC-3’ and R, 5’-GATGTTGTCAGTACATGAGGCC-3’; cloning full-length Flag-tagged S100A2 – F, 5’-GCCATGGACTACAAGGAGCGATGACAAAGATGTGACTTCCTGAG-3’ and R, 5’-GCTTCAGGTTCTGGTCTG-3’. All other primers have been described previously.

Luciferase assays. Cells were co-transfected with the relevant luciferase constructs and Renilla using Gene Juice (Novagen, Middlesex, UK) according to the manufacturer's instructions. After 24 h, cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, USA) and luciferase and Renilla activities were assessed by luminescence using 0-luciferin and coelenterazine as substrates, respectively. Site-directed mutagenesis was carried out using KOD polymerase (Novagen) according to the manufacturer's instructions.

ChIP assays. ChIP assays were performed as described previously. PCR was performed on extracted DNA using Taq DNA polymerase (Roche) according to the manufacturer’s instructions. Primers used are as in Kirschner et al.

HSP90 inhibition. For dose–response curves, cells treated with a range of concentrations of 17-AAG (log 10−12−log 10−6 M) for 72 h before cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Dorset, UK). For other studies, cells were incubated with 4 μM (MDA-MB-468) or 2 μM (HCC1937) 17-AAG for 16 h.

S100A2 expression in breast tumours. Publically available data sets, with BRCA1 mutation/methylation status were analysed using Oncomine (www.oncomine.org) for S100A2 expression.

Correlation of S100A2 expression and 17-DMAG IC50 values. 17-DMAG IC50 values for a range of cell lines were extrapolated from Lehmann et al. and used as described previously. The probe set for S100A2 was identified and the relevant expression levels were compared with BRCA1 mutation/methylation status by Oncomine for S100A2 expression.

BrdU incorporation. Cells were treated with 10 μM BrdU for 20 min minutes before fixing with 4% paraformaldehyde. Cells were then stained with the FITC mouse anti-BrdU set (BD Pharmingen, Oxford, UK) according to the manufacturer’s instructions. BrdU-positive cells were then counted and expressed as a percentage of total cells.

Conflict of Interest
The authors declare no conflict of interest.

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