Breast Cancer Amplified Sequence 2, a Novel Negative Regulator of the p53 Tumor Suppressor

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Abstract
Breast cancer amplified sequence 2 (BCAS2) was reported previously as a transcriptional coactivator of estrogen receptor. Here, we report that BCAS2 directly interacts with p53 to reduce p53 transcriptional activity by mildly but consistently decreasing p53 protein in the absence of DNA damage. However, in the presence of DNA damage, BCAS2 prominently reduces p53 protein and provides protection against chemotherapeutic agent such as doxorubicin. Deprivation of BCAS2 induces apoptosis in p53 wild-type cells but causes G2-M arrest in p53-null or p53 mutant cells. There are at least two apoptosis mechanisms induced by silencing BCAS2 in wild-type p53-containing cells. Firstly, it increases p53 retention in nucleus that triggers the expression of apoptosis-related genes. Secondly, it increases p53 transcriptional activity by raising p53 phosphorylation at Ser46 and decreases p53 protein degradation by reducing p53 phosphorylation at Ser157. We show for the first time that BCAS2, a small nuclear protein (26 kDa), is a novel negative regulator of p53 and hence a potential molecular target for cancer therapy. [Cancer Res 2009;69(23):8877–85]

Introduction
The sequence of breast cancer amplified sequence 2 (BCAS2) is the same as DAM1 (Mas musculus DNA amplified in mammary carcinoma mRNA; GenBank accession no. AB020623) deposited at National Center for Biototechnology Information in July 2001 (1–3). The BCAS2 gene maps to chromosome 1p13.3-21 region and contains 678 bp encoding 225 amino acids, with a predicted molecular mass of 26 kDa (2). BCAS2 was recently characterized as a transcriptional cofactor that enhances estrogen receptor-mediated gene expression (4). In this article, we found that BCAS2 associates with the tumor suppressor p53 protein; we have also probed the cellular and molecular effects of this interaction.

Tumor suppressor protein p53 is a multifunctional protein that regulates cell growth and death by responding to various genotoxic and oncogenic stresses (5). Tight regulation of p53 activity is essential for maintaining normal cell growth and for preventing tumorigenesis. Mutations in p53 are a hallmark of at least 50% of all human cancers (6, 7). The p53 protein has been shown to interact with several cellular and viral proteins including SV40 large T, human papillomavirus E6 protein, adenovirus E1b, MDM2, E2F, p300/CREB, NUMB, and ASPP proteins (8, 9). These proteins are a mixture of general and apoptosis-specific regulators of p53 and regulate how p53 protein determines the fate of cells (10).

In this study, BCAS2 interaction partners were identified and characterized using glutathione S-transferase (GST) pull-down assays combined with mass spectrometry. One such protein shown to bind BCAS2 was p53; its interaction was confirmed by further GST pull-down in vitro and through reciprocal immunoprecipitation in vivo. Because BCAS2 is a nuclear protein, we further examined whether BCAS2 could regulate p53 transactivation and apoptosis. Here, we show for the first time that BCAS2 is a novel negative regulator of p53 that directly interacts with p53 protein, decreasing p53 transcription activity. Deprivation of BCAS2 induces cell apoptosis in p53 wild-type cells but causes G2-M arrest in p53-null or p53 mutant cells. It seems that, beyond p53, other cellular factors exist that interact with BCAS2 and are involved in the inhibition of cell growth.

Materials and Methods
In vitro GST pull-down analysis. The GST and GST fusion protein plasmids were transfected into Escherichia coli BL21 (DE3) cells. These fusion proteins were purified using glutathione-Sepharose 4B beads (GE Life Sciences) according to the manufacturer’s procedures. His-tagged p53 was synthesized by transformed pRSET-p53 plasmid into E. coli BL21 (DE3) cells and then purified by Ni-NTA agarose beads (Qiagen). The purified His-tagged p53 protein was incubated with the immobilized GST or GST-fused proteins beads and these bound proteins were subjected to Western blotting with anti-His or anti-GST antibodies.

Preparation of cytoplasmic and nuclear extracts. Nuclear and cytoplasmic fractions were prepared from MCF-7 cells treated with the appropriate anti-BCAS2 short hairpin RNAs (shRNA) for 48 h as described previously (11).

Fluorescence microscopy assay. MCF-7 cells were fixed and then doubly stained with anti-p53 and anti-BCAS2 antibodies followed by incubation with FITC-conjugated anti-rabbit (Abcam) and Alexa Fluor–conjugated anti-mouse antibodies (Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole. Fluorescent images were monitored using a Carl Zeiss confocal microscope (LSM 510 META).

Comunmunoprecipitation and Western blot analysis. To determine BCAS2-p53 binding, MCF-7 cells were transfected with full-length or deletion p53 and BCAS2 plasmids. Forty-eight hours after transfection, cell lysates were harvested and immunoprecipitated with the antibodies indicated in procedures described previously (11, 12).

Transient transfection and luciferase assays. Transient transfections were done by plasmid electroporation into MCF-7 or H1299 cells. Forty-eight hours after transfection, the cells were assayed using a Dual-Glo Luciferase...
Assay System (Promega) according to the manufacturer's instructions and detected using a Luminoskan Ascent luminometer (Thermo). The intensity of the firefly luciferase signals generated was normalized against an internal control (Renilla luciferase pRL-CMV).

Cell cycle analysis. Cells transfected with the indicated plasmids were collected and fixed and then stained with propidium iodide and analyzed by flow cytometry (BD FACSCalibur).

Cell proliferation assay. MCF-7, H1299, C33A, and LNCaP cells were transfected by electroporation. Twenty-four hours after transfection, MCF-7, C33A, and H1299 cells were replated at 1 × 10⁵ per well in 12-well culture plates. LNCaP cells were reseeded at 2 × 10⁴ per well in 24-well culture plates. The total cell numbers were counted at 24, 48, 72, and 96 h time points by trypan blue staining.

Real-time quantitative PCR. Total cellular RNA was extracted using Trizol reagents (Invitrogen) according to the manufacturer's instructions. Real-time PCR was done with a Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol.

Chromatin immunoprecipitation-quantitative PCR assay. Chromatin immunoprecipitation assays were done as described previously (11). For chromatin immunoprecipitation-quantitative PCR (qPCR), DNA was purified with a QIAquick PCR purification kit (Qiagen) and subjected to analysis by real-time qPCR. One percent of the preclear supernatant from the reaction lacking primary antibody was saved as total input of chromatin.

Statistical analysis. Statistical procedures were carried out using SPSS 16.0 software. ANOVA analysis was done. P values < 0.05 were considered significant.

Results

Protein-protein Interaction between BCAS2 and p53 tumor suppressor. To characterize BCAS2 function, we first examined which proteins could interact with BCAS2 by examining the nuclear extracts of MCF-7 cells by GST pull-down assays followed by mass spectrometry (Fig. 1A, top). Tumor suppressor protein p53 was just one of numerous potential BCAS2-interacting proteins identified (data not shown); immunoblotting confirmed that p53 was bound to GST-BCAS2 fusion protein (Fig. 1A, bottom).

We then asked whether BCAS2 could directly interact with p53. MDM2 protein is a well-studied p53 binding protein, and residues 1 to 110 are required for p53 binding (13). GST-MDM2 (120) covering MDM2 amino acid sequence 1 to 120 was constructed and regarded as a positive control for p53 binding. The His-p53 protein synthesized by E. coli was incubated with the various purified GST-fusion proteins in GST pull-down assay. The results showed that the GST-MDM2 (120) and GST-BCAS2 proteins could be pulled down with the purified His-p53 protein (Fig. 1B, top, lanes 2 and 4). These data indicate that BCAS2 directly interacted with p53.

When investigating the interaction between endogenous BCAS2 and p53 in MCF-7 cells containing wild-type p53, p53 protein could be detected by anti-BCAS2 antibody (Fig. 1B, bottom). The detection of BCAS2 (molecular weight, 26 kDa) by precipitated p53 antibody was hampered by the immunoglobulin light chain, which is

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Interaction between BCAS2 and p53 in vitro and in vivo. **A,** BCAS2 associates with p53 in vitro. GST-BCAS2 and GST proteins were incubated with MCF-7 nuclear extracts and analyzed by silver staining (top). GST-pull-down proteins were analyzed by Western blot (WB) with p53 antibody (bottom). **B,** top, BCAS2 directly interacts with p53 in vitro. The His-p53 protein incubated with the GST, GST-BCAS2, or GST-MDM2 (120). The bound proteins were analyzed by Western blot using an anti-His or anti-GST antibody. Bottom, association of endogenous BCAS2 and p53 in vivo. Cell lysates of MCF-7 were subjected to immunoprecipitation (IP) with antibody against BCAS2 and then immunoblotted with p53 antibody (bottom). **C,** nuclear colocalization of BCAS2 and p53 by confocal laser microscopy. Bar, 10 μm.
25 kDa (data not shown). Instead, we conducted the confocal microscopy assay that showed endogenous BCAS2 and p53 localized inside the MCF-7 nucleus (Fig. 1C). In summary, our data showed that BCAS2 can directly interact with p53 in vitro and in vivo.

**Overexpression of BCAS2 reduces p53 protein and transcriptional activity.** The tumor suppressor p53 is a well-known transcription factor that can activate downstream target genes (5, 10). Having identified a direct interaction between BCAS2 and p53, we investigated whether BCAS2 affected p53 transcriptional activity. Transcription of the p21 gene is reportedly enhanced by p53 binding to its promoter region, a stretch that contains two copies of the p53 response element (14). Doxorubicin, a DNA-damaging agent, is a potent inducer of p53 protein and p53-dependent transcriptional activity (15). Firstly, we used the p21 promoter (5 μg; kindly provided by Dr. Bert Vogelstein) along with p3XFLAG-BCAS2 (15 or 20 μg) and internal control pRL-CMV (0.1 μg) as an assay model to determine p53 transcriptional activity (16). The total amount of plasmid per dish was equalized by adding empty vectors. The results showed that the luciferase activity of the p21 promoter decreased (top), accompanied with increased BCAS2 protein expression and decreased levels of p53 and p21 (bottom) in MCF-7 cells, regardless of doxorubicin treatment (Fig. 2B) or no treatment (Fig. 2A). However, that the quantitation of immunoblot by increasing FLAG-BCAS2 plasmids was not detectable accordingly can be explained by the transfection efficiency having reached its limits. Likewise, H1299 cells (lung cancer with null p53 and weak endogenous p21) were cotransfected with a p53 expression plasmid in combination with a p21-Luc promoter plasmid. Overexpression of BCAS2 reduces p53 protein and transcriptional activity.

**Reciprocal binding domains between BCAS2 and p53.** To verify our hypothesis that BCAS2 is an inhibitor of p53, we mapped the domains within p53 and BCAS2 responsible for interacting with BCAS2 and p53, respectively. HA-tagged deletion mutants of p53 were constructed and named p53-Δ1, p53-Δ2, p53-Δ3, p53-Δ4, and p53-Δ5 (Fig. 3A, top). Figure 3A showed that p53-Δ2, p53-Δ3, and p53-Δ4 complexed with BCAS2...
protein (bottom), indicating that p53 residues 132 to 324 are responsible for binding BCAS2, the same region required for DNA binding and nuclear signaling (17). Likewise, BCAS2 has two coiled-coil domains (Fig. 3B, top) that interact with other proteins (18, 19); we generated wild-type or single- or double-domain deletion mutants named BCAS2-FL, BCAS2-dCC1, BCAS2-dCC2, and BCAS2-dCC1+2, respectively (Fig. 3B, top), to answer whether coiled-coil domains of BCAS2 were involved in the interaction with p53 protein. Figure 3B showed that those BCAS2 mutants lacking either one or both domains were unable to bind p53 (bottom). When deletion mutants of BCAS2 were introduced into MCF-7 cells, p53 and p21 protein expression was less disrupted than by full-length BCAS2 (Fig. 3C). This result indicated that the BCAS2 coiled-coil domains were responsible for binding to p53 and regulating its activity.

Depletion of BCAS2 affects p53-dependent transcription. To evaluate the functions of BCAS2 gene by RNA interference (11, 12, 20), four BCAS2 small interfering RNA sequences were designed and cloned into pSUPER plasmid and named according to the BCAS2 sequence to be disrupted (Supplementary Fig. S1). As shown in Fig. 4A, BCAS2 protein expression was significantly diminished by shBCAS2#434 and mildly diminished by shBCAS2#135 and shBCAS2#510 shRNAs in 293T cells (top). To further determine that the shBCAS2#434 construct could knock down BCAS2 gene expression is on-target effect, we generated the mutant plasmid FLAG-BCAS2 (mutant), in which the sequence corresponding to

Figure 3. Analysis of the mutual binding domains between BCAS2 and p53 proteins. A, schematic representation of the various p53 deletion mutants constructed (top). Specific domains are labeled as follows: TA, transactivation domain; DBD, specific DNA-binding domain; TET, tetramerization domain; NLS, nuclear localization signal; NES, nuclear export signal. Mapping of p53 domains used for binding with BCAS2. MCF-7 cells were transfected with HA-p53-FL or p53 deletion mutants. Cell extracts were immunoprecipitated with BCAS2 antibody. Bound proteins were subjected to Western blot analysis (bottom). B, map of BCAS2 domains. Two coiled-coil domains were identified and named CC1 (residues 139-173) and CC2 (residues 192-219), respectively (top). Mapping the BCAS2 domains required for interacting with p53. Cells were transfected with FLAG-BCAS2-FL or BCAS2 deletion mutants. Cell extracts were immunoprecipitated with p53 antibody. Bound proteins were subjected to Western blot analysis (bottom). C, effect of BCAS2 mutants on the expression of p53 and p21 protein in MCF-7 cells. MCF-7 cells were transfected with the indicated plasmids, and total cell lysates were subjected to Western blot analysis (top). The expression levels of p53 and p21 protein were measured as described in Fig. 2.
shBCAS2#434-targeted sequence (position 434-452) was mutated to 5′-AATCGAGCAAGCTCAAAAA-3′. After cotransfection of wild-type or mutant BCAS2 plasmid with shBCAS2#434 into 293T cells, Western blot analysis showed that shBCAS2#434 only inhibited wild-type protein expression (Fig. 4A, bottom, lane 4) but not mutant type expression (lane 6). In summary, the designed sequence of shBCAS2#434 targeted the BCAS2 gene and diminished RNA expression (data not shown).

We have shown that BCAS2 repressed the p53 transcriptional ability (Fig. 2). We therefore examined whether BCAS2 knockdown could increase the p53 transcriptional activity of other p53-targeted genes, such as p21, MDM2, PUMA, NOXA, and BAX (10, 21). qPCR assays showed significantly increased gene expression of p21, PUMA, NOXA, and MDM2 but not BAX in MCF-7 cells treated with shBCAS2#434 compared with the control (pSUPER) in both doxorubicin treatment and no treatment (Fig. 4B). Two other cell types, LNCaP (prostate cancer cells containing wild-type p53), presented similar patterns of p53-induced gene expression (p21, PUMA, MDM2, and NOXA) after BCAS2 depletion as MCF-7 (Supplementary Fig. S2A) and A549 (lung cancer cells with wild-type p53) showed the increased gene expression of PUMA, MDM2, and NOXA but not p21 (Supplementary Fig. S2B). It can be explained the different cell contexture influenced p53 binding efficiency to its targeted genes. To avoid off-target effects, we further treated MCF-7 cells with the other targeting sequences, shBCAS2#135, which could decrease BCAS2 protein expression, too (Fig. 4A, top). Similarly, qPCR of shBCAS2#135-treated cells showed the significantly increased gene expression of p21, PUMA, NOXA, and MDM2 but not BAX (Fig. 4B). To directly examine how BCAS2 depletion would affect p53-dependent transcription, RNA interference studies showed that shBCAS2#434 could increase p21 promoter activity in MCF-7 cells by ∼3.5-fold compared with

Figure 4. Depletion of BCAS2 expression increases the p53 transcriptional activity. A, top, identification of effective shRNA targets to BCAS2. Four BCAS2 shRNA clones were analyzed for inhibition of endogenous BCAS2 protein. Bottom, cells were transfected with shBCAS2#434 along with BCAS2 (wild-type) or mutant constructs and analyzed by Western blot. B, effect of BCAS2 knockdown on p53-targeted genes expression was analyzed by qPCR. C, silencing BCAS2 expression increases p21 promoter reporter activity. D, depletion of BCAS2 expression increased the protein level of p21 and PUMA by Western blot.
shBCAS2#331 and mock controls (Fig. 4C). Figure 4D shows that p21 and PUMA protein levels were higher in shBCAS2#434-treated MCF-7 cells compared with mock, pSUPER, and shBCAS2#331. Taken together, interfering with BCAS2 expression in wild-type p53 cancer cells induces p21 and PUMA RNA and protein expression, indicating decreasing p53 transcriptional activity.

Reducing BCAS2 expression affects p53 nuclear retention and post-translational modifications. The expression of BCAS2 mildly reduced p53 protein expression (Fig. 2). We therefore investigated whether the depletion of BCAS2 would increase the amount of p53 protein. As shown in Fig. 5A, the p53 protein levels were mildly increased in MCF-7 cells treated with shBCAS2#434 compared with mock, pSUPER, and shBCAS2#331 in normal condition but in the presence of doxorubicin; the significant increase of p53 protein was shown in shBCAS2#434 (Fig. 5A, lane 8). To further assess whether BCAS2 regulates p53 protein turnover, Fig. 5B shows that there was more p53 protein in shBCAS2#434-treated cells than in cells transfected with the control shRNAs. This indicates that the half-life of p53 protein in the shBCAS2#434-treated cells was longer than in cells treated with control shRNAs.

Besides being transcription-dependent, the p53-induced apoptotic program also includes a transcription-independent pathway (5, 22), probably mediated by cytosolic p53 protein (5, 10, 22–24). In our studies, most p53 protein in MCF-7 cells is located in the nucleus (Fig. 1C). Hence, we examined whether knockdown of BCAS2 would affect the relative distribution of p53 between the cytosol and the nucleus. Our results show that p53 protein was increasingly nuclear in shBCAS2#434-treated MCF-7 cells (Fig. 5C, lane 6) post-transfection, indicating that BCAS2 depletion increases nuclear p53 protein. Taken together, the knockdown of BCAS2 caused the induction of p53 transcriptional activity by nuclear p53 protein.

The transactivation function of p53 is believed to be regulated through protein accumulation and post-translational modifications such as phosphorylation (25, 26). We therefore examined changes in post-translational modifications to p53 on BCAS2 induction and deprivation of BCAS2 (Fig. 5D). The effect on the extent of p53 phosphorylation was analyzed after normalization to total p53 level. As shown in Fig. 5D, introduction BCAS2 in combination with doxorubicin treatment in MCF-7 cells caused a measurable decrease at Ser\(^{46}\) phosphorylation status and increase at Ser\(^{315}\) of p53 (left). In contrast, downregulation of BCAS2 by shRNA transfection increased the levels of Ser\(^{46}\) but reduced Ser\(^{315}\) phosphorylation (right). The protein p53 phosphorylation at Ser\(^{315}\) is reported to be required for its nuclear export and degradation by Hdm2 (27), and phosphorylation at Ser\(^{46}\) is involved in p53-dependent transcription activity (26). It may explain BCAS2-degrading p53 protein by increasing Ser\(^{315}\) phosphorylation and BCAS2-reducing p53 phosphorylation levels of Ser\(^{46}\).

Figure 5. Effect of BCAS2 on p53 protein stability, nuclear retention, and post-translational modifications. A, knockdown BCAS2 gene expression affects p53 protein expression in cells without and with doxorubicin treatment. B, effect of BCAS2 on p53 stability. As described in A, 48 h after transfection, the protein synthesis inhibitor cycloheximide (CHX) was added (100 μg/mL) to the cells at the different time points indicated. Band densities were quantitated using a UVP BioSpectrum-AC Imaging System. We set the p53 protein level in each control treatment as 100%, so the densitometer measured changes as a percentage of each control treatment. C, subcellular location of p53 by shBCAS2 treatment. Blots were probed for p53, BCAS2, poly(ADP-ribose) polymerase (PARP; for the nuclear fraction control), and tubulin (cytosolic fraction). D, left, BCAS2 suppresses the phosphorylation levels of p53 at Ser\(^{46}\) and induces phosphorylation at 315; right, deprivation of BCAS2 increases p53 phosphorylation at Ser\(^{46}\) but reduces at Ser\(^{315}\). The number presented the ratio of phosphorylated p53 to the corresponding total p53 protein in doxorubicin-treated experiments (control, set at 1).
Reducing the expression of BCAS2 increases p53-dependent apoptosis in cancer cells. Tumor suppressor p53 was reported to play an important role in apoptosis and cell cycle arrest (28). Cell proliferation assays were done to determine the biological effects of knocking down BCAS2 gene expression on p53 activity. MCF-7 and LNCaP cells containing wild-type p53 (29, 30) were transfected with the pSUPER, shBCAS2#434, and shBCAS2#331 vectors separately to determine the cell growth rates. H1299 cells containing null p53 and C33A cells harboring mutant p53 were also examined. The results showed that silencing BCAS2 expression by shBCAS2#434 caused dramatic decreases in the cell growth rates of MCF-7 and LNCaP cells compared with mock, pSUPER, and shBCAS2#331 control cells (Fig. 6A and Supplementary Fig. S3A, respectively). The dead cell number significantly increased with time at 72 and 96 h in MCF-7 cells treated with shBCAS2#434 (Supplementary Fig. S4A). The corresponding level of BCAS2 protein decreased in shBCAS2#434-treated cells at 48 h (Fig. 6A, bottom). The results from the trypan blue exclusion assay showed that knocking down BCAS2 expression in MCF-7 cells with shBCAS2#434 caused a dramatic increase (>40%) in cell death after 72 h (Supplementary Fig. S4A). Similar results were obtained by flow cytometry analysis, whereby the percentage of cells in sub-G1 phase increased at 72 h in MCF-7 cells treated with shBCAS2#434 compared with mock and pSUPER.
vector controls (Supplementary Fig. S5; Supplementary Table S1). Likewise, growth rates for H1299 and C33A cells treated with shBCAS2#434 were lower compared with the control groups (Fig. 6B and Supplementary Fig. S3B, respectively). The corresponding level of BCAS2 protein was reduced 48 h after treatment (Fig. 6B and Supplementary Fig. S3B, bottom, respectively). Conversely, H1299 cells did not experience the apoptotic phenomenon seen in the MCF-7 cells following shBCAS2#434 treatment (Supplementary Fig. S4B). The percentage of cells in sub-G1 phase did not change significantly in H1299 cells (Supplementary Table S2) and C33A cells (Supplementary Table S3) treated with pSUPER, shBCAS2#331, and shBCAS2#434, respectively. The appearance of sub-G1 means that cells are undergoing apoptosis (31), induced by shBCAS2#434 in wild-type p53-containing cells (MCF-7) but not in p53-null cells (H1299) or p53 mutant cells (C33A). Therefore, we can infer that apoptosis caused by silencing BCAS2 expression in MCF-7 (wild-type p53) cells can be mediated by p53. To further establish this, shp53 plasmid silencing BCAS2 expression in MCF-7 (wild-type p53) cells can be mediated by p53. To further establish this, shp53 plasmid was introduced to knock down p53 gene expression in MCF-7 cells. Cell cycle analysis after the indicated treatments showed that the sub-G1 cell populations of the shp53 and shBCAS2#434-treated cells were the same as control (Fig. 6C; Supplementary Table S4). The immunoblot presented p53 depletion in cells treated with shp53 (Fig. 6C, bottom, lane 2). BCAS2 depletion in cells with shBCAS2#434 (lanes 3-5), and ablation of both p53 and BCAS2 in the combined shp53- and shBCAS2-treated cells (lane 5). In summary, silencing BCAS2-induced apoptosis is p53-dependent. However, shBCAS2#434 caused cell cycle arrests at the G2-M phase in p53-null H1299 cells and p53 mutant C33A cells (Supplementary Tables S2 and S3, respectively). This suggests that, beyond p53, BCAS2 may interact with other cellular factors to cause p53-independent cell growth arrest (Supplementary Fig. S6).

**Ectopic overexpression of BCAS2 rescues p53-mediated growth inhibition.** Because silencing BCAS2 expression caused either cell apoptosis (wild-type p53) or growth arrest (p53-null and mutant), we investigated whether ectopic expression of BCAS2 could rescue cells from p53-induced growth inhibition. Previous reports showed that low doses (50 nmol/L) of doxorubicin caused cell growth arrest through the activation of the p53 pathway and p21 induction (15). Therefore, MCF-7 cells were transiently transfected with FLAG-BCAS2 or FLAG-vector plasmids and then treated with 50 nmol/L doxorubicin for 48 h. Using a cell proliferation assay, we showed that doxorubicin-induced growth arrest in MCF-7 cells could be rescued by BCAS2 overexpression as shown in Fig. 6D (left). Similar results were also observed by flow cytometry analysis (Fig. 6D, right; Supplementary Table S5). In response to doxorubicin, the percentage of the G1 population increased in MCF-7 control cells and in MCF-7 cells treated with FLAG-vector at the expense of the respective S-phase populations. By comparison, in doxorubicin-treated MCF-7 cells overexpressing BCAS2, the percentage of the G1 population decreased, whereas the S-phase population increased (Fig. 6D, right; Supplementary Table S5). The p53, p21, and BCAS2 protein expression levels derived from each treatment were compiled with the biological results (Fig. 6D, right and bottom). Taken together, introducing BCAS2 in MCF-7 cells treated with low doses of doxorubicin could decrease p21 protein expression, thereby accounting for the BCAS2-mediated protection from doxorubicin; this would imply that BCAS2 antagonizes p53-mediated growth inhibition.

**Discussion**

We discovered that BCAS2 is a novel p53 binding protein. Loss of p53 function is a characteristic of almost all human tumors (32). We show that the binding of BCAS2 reduces p53 function by BCAS2 mildly but consistently decreasing p53 protein in normal condition (Fig. 24-C). However, in the presence of DNA damage, BCAS2 prominently reduces p53 protein and provides protection against chemotherapeutic agent such as doxorubicin (Fig. 6D). How BCAS2 is involved in p53 protein degradation will become interesting. Because BCAS2 is a small-sized protein (26 kDa) and only contains two coiled-coil domains that are reportedly responsible for the other protein interaction, it is less likely to function as E3 ligase activity for protein degradation. On the other hand, MDM2 induces degradation and nuclear export of p53 by acting as a p53-specific E3 ubiquitin ligase (6, 13, 33–35). It is interesting to examine whether BCAS2 plays as a scaffold or adaptor molecule to mediate MDM2 or other E3 ligase-related proteins for p53 protein degradation. Various negative apoptotic regulators of p53 were recently identified that reduce p53 protein activity through different mechanisms (6, 8, 14, 15, 21, 36–42). For example, MDM2, PACT, aurora kinase A, and E6 function by causing p53 protein ubiquitination and degradation (6, 13, 33, 43). Transcriptional activation of p53 is also repressed by p202 via binding to the PXXP motifs of p53 (39) and by LSD1 through demethylated p53 protein (31). Notably, HSP27 and PFTm function as small-sized protein inhibitors of p53 by promoting the assembly of p53 into complexes (6, 15, 40). Here, we describe the novel functioning of BCAS2 as a small-sized p53 binding protein, not previously known to be a p53 inhibitor.

Silencing BCAS2 for 72 h without stress treatment causes ~40% cells apoptotic in p53-containing cells (MCF-7; Fig. 6D; Supplementary Fig. S4; Supplementary Table S1). There are several possible explanations for this. Firstly, the increases of p53 Ser46 phosphorylation (Fig. 5D) prominently induce p53 activity that trigger apoptosis. Secondly, in addition to p21, PUMA is also highly stimulated in both RNA and protein levels (Fig. 4B and D). PUMA is a key mediator of p53-dependent apoptosis and targets to p53 that can release p53 from inhibitory p53-BCL-XL complexes and then induce mitochondrial outer membrane permeabilization (6). Thirdly, deprivation of BCAS2 only without stress in p53-null cells results in growth arrest (Supplementary Tables S2 and S3), implying that BCAS2 may also have direct effect in cell growth. This and our unpublished finding of accumulation of DNA damage after BCAS2 knockdown suggest that BCAS2 may be involved in DNA damage repair and RNA splicing. Interestingly, BCAS2 is also known as sp27, a protein associated with the RNA spliceosome (3). sp27 can form a pre-mRNA splicing complex with Pso4/Prp19, Cdc5L, and Prlg1. The Pso4 complex was reported to be involved in DNA repair and RNA splicing (44–49). The function of BCAS2 in the cell cycle process remains unknown. It would be interesting to identify the BCAS2-interacting proteins that induce p53-independent cell cycle arrest at G2-M.

In sum, our hypothesis model shows that the binding of BCAS2 reduces p53 function, whereby silencing the BCAS2 gene causes p53 release to function as a transcription factor toward p21, NOXA, MDM2, and PUMA gene expression, resulting in an amplification

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5 Weng and Chen, unpublished results.
of apoptotic signals leading to cell death in p53 wild-type cells. In p53-null or p53 mutant cells, other cellular factors exist to interact with BCAS2 to inhibit cell growth in a p53-independent manner (Supplementary Fig. S6).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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