Cysteine-linked dimerization of BST-2 confers anoikis resistance to breast cancer cells by negating proapoptotic activities to promote tumor cell survival and growth

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Almost all breast tumors express the antiviral protein BST-2 with 67%, 25% and 8.2% containing high, medium or low levels of BST-2, respectively. Breast tumor cells and tissues that contain elevated levels of BST-2 are highly aggressive. Suppression of BST-2 expression reprograms tumorigenic properties of cancer cells and diminishes cancer cell aggressiveness. Using structure/function studies, we report that dimerization of BST-2 through cysteine residues located in the BST-2 extracellular domain (ECD), leads to anoikis resistance and cell survival through proteasome-mediated degradation of BIM—a key proapoptotic factor. Importantly, BST-2 dimerization promotes tumor growth in preclinical breast cancer models in vitro and in vivo. Furthermore, we demonstrate that restoration of the ECD cysteine residues is sufficient to rescue cell survival and tumor growth via a previously unreported pathway—BST-2/GRB2/ERK/BIM/Cas3. These findings suggest that disruption of BST-2 dimerization offers a potential therapeutic approach for breast cancer.

Results

Comparative analysis of BST-2 protein in breast tumors. In agreement with previous meta-analysis of BST-2 mRNA,3,11 assessment of BST-2 protein levels using Protein Atlas (http://www.proteinatlas.org) confirms presence of BST-2 protein in breast tumors (Figure 1a). The data show that more breast tumors contain elevated BST-2 protein compared with the levels of estrogen receptor, progesterone receptor, HER2 or Myc. These data suggest that BST-2 may have a significant role in breast cancer and could be a valuable therapeutic target.

BST-2 mediates adhesion of breast cancer cells to components of the tumor microenvironment. To investigate the role of BST-2 on cellular interactions, we performed an adhesion assay using previously reported6 4T1 cells stably expressing scrambled shRNA (shCTL—BST-2 expressing) or BST-2-targeting shRNA (shBST-2—BST-2 suppressed) (Supplementary Figure 1). Results show that BST-2-expressing incoming cancer cells efficiently adhere to monolayers of shCTL cells compared with BST-2-suppressed cells. When the monolayer consists of BST-2-suppressed cells, adhesion of BST-2-suppressed cells was also reduced (Figure 1b, image and bar). Additional experiments using MCF-7 cells overexpressing (OE) empty vector or wild-type BST-2 show that BST-2 is required for cell to cell or cell to ECM interactions. Although MCF-7-vector cells adhere marginally to the monolayer, OE-BST-2 enhances MCF-7 adherence (Figure 1c). In addition, MCF-7-OE-BST-2 cells efficiently adhere to collagen (Figure 1d) and fibronectin (Figure 1e) pre-coated plates compared with MCF-7-vector cells. These data indicate that resident cancer cells anchored on supportive structure or incoming cancer cells in suspension require BST-2 for efficient adhesion.
Adhesive interaction between fibroblasts and breast cancer cells upregulate BST-2 expression. To further evaluate the significance of BST-2-mediated adhesive interactions, we show that adhesion between monolayers of Cal51 (Figure 1f) or MDA-MB-231 (Figure 1g) and several strains of fibroblasts significantly upregulates BST-2 expression in the cancer cells. The magnitude of BST-2 upregulation may depend on the basal level of cancer cell BST-2 level (Figure 1h) and the magnitude of fibroblast-mediated BST-2 upregulation (Figures 1f and g) are lower in
Cal51 compared with MDA-MB-231. In addition, fibroblasts contain varying levels of BST-2 (Figure 1i), but their BST-2-inducing capability is comparable (Figures 1f and g). These data indicate that interactions between cancer cells and fibroblasts may regulate cancer cell BST-2 and may promote cellular reprogramming.

BST-2 expression is required for efficient growth of breast cancer cells in suspension. To assess the biological relevance of BST-2-mediated adhesive interactions, we first examined the effects of BST-2 on anchorage independency and spheroid formation. MCF-7-OE-BST-2 cells produced larger spheroids compared with MCF-7-vector cells (Figure 1j). These data indicate that BST-2 promotes survival and growth of cancer cells in suspension; suggesting that breast tumor cells that are anchorage independent due to high levels of BST-2 may undergo anoikis in circulation in the absence of BST-2.

BST-2 dimers are present in breast cancer cells and dimerization is regulated by ECD cysteine residues. Owing to the effective role of BST-2 in cell adhesion, we evaluated the structural property of BST-2 that has a role in cell adhesion. We engineered MCF-7 cells (Figure 2a) stably expressing WT BST-2 that is predominantly dimer (OE-BST-2D) and dimerization-deficient BST-2 that is expressed as monomers (OE-BST-2M). PCR analysis shows efficient expression of BST-2D and BST-2M mRNA (Figure 2b), whereas western blots confirm presence of BST-2D or BST-2M in these cells (Figure 2b). Functionally, BST-2D and BST-2M increase viability, proliferation and invasion of MCF-7, albeit with subtle differences (Figure 2c).

BST-2 dimers mediate adhesion of breast cancer cells to components of the tumor microenvironment. Here, we assessed whether the variant of BST-2 in cancer cells is critical for BST-2-BST-2 interactions that mediate cell to cell and/or cell to matrix adhesion. As expected, OE-BST-2D but not OE-BST-2M significantly increases adhesion of cancer cells to collagen (Figure 2d) and fibronectin (Figure 2e), despite comparable expression (Figure 2b). OE-BST-2D cells have increased ability to bind low BST-2-expressing and high BST-2-expressing cells (Figure 2f). In contrast, OE-BST-2M cells have reduced ability to bind cells, irrespective of the level of BST-2 on the monolayer (Figure 2f). Further analyses show that adhesion of cancer cells expressing different levels and variants of BST-2 increases when shBST-2 monolayers are forced to express BST-2D but not BST-2M (Figure 2g).

The significance of BST-2 dimerization was further appreciated in experimental settings where adhesive interactions of cancer cells with other cells were examined. Compared with OE-BST-2D cells, OE-BST-2M cancer cells have reduced ability to bind endothelial cells—HUVECs (Figure 2h) and immune cells—macrophages (Figure 2i, white background) and monocytes (Figure 2j). Induction of BST-2 in macrophages with the BST-2 agonist, IFNα, results in increased adherence of cancer cells expressing the different variants of BST-2 to IFNα+ macrophages compared with IFNα- macrophages (Figure 2i, white and gray backgrounds). The increased adherence of OE-BST-2M cells to IFNα+ macrophages could be attributed to enhancement of endogenous BST-2D. Furthermore, monocytes, irrespective of their level of BST-2, adhere efficiently to monolayers of shCTL cells compared with shBST-2 cells (Figure 2j, pink and blue bars). The effect of BST-2 is dependent on the variant of BST-2, as monocytes expressing shCTL and shBST-2 adhere more efficiently to BST-2D-expressing monolayers (Figure 2j). These data indicate that the level and variant of BST-2 in cancer cells may determine the rate of immune cell adherence.

Next, we performed adhesion in the presence and absence of recombinant BST-2 (rBST-2). Results show that rBST-2 efficiently blocks adhesion of BST-2-expressing cancer cells but has no effect on adhesion of BST-2-suppressed cells (Figure 2k), indicating that BST-2 is responsible for the observed adhesion. Furthermore, rBST-2 specifically blocks adhesion of OE-BST-2D cells but has no effect on OE-BST-2M cells (Figure 2i), confirming that the variant of BST-2 (D or M) is crucial in cancer cell adhesion.

We confirmed the role of BST-2 dimerization on adhesion by seeding equivalent numbers of cells on rBST-2 pre-coated plates. Compared with shCTL cells, shBST-2 cells were unable to adhere efficiently to rBST-2-coated plates (Figure 2m). Importantly, OE-BST-2D increases cell adherence, whereas OE-BST-2M did not (Figure 2n). These data suggest that recombinant human BST-2 binds to both murine and human BST-2 in cancer cells and blocks cancer cell to cancer cell adhesion.

BST-2 dimerization regulates anchorage independency. As BST-2 dimerization is critical for cellular and matrix interactions, we showed that BST-2 dimerization is crucial for colony formation and anchorage-independent growth. As expected, 4T1 shCTL cells form significantly larger colonies compared with 4T1 shBST-2 cells (Figure 3a). OE-BST-2D but not OE-BST-2M efficiently rescues colony formation in shBST-2 cells (Figure 3a), indicating that BST-2 dimerization is required for growth of cancer cells independent of anchor. These findings were confirmed with MCF-7 cells (Figure 3b). The difference in the ability of OE-BST-2D and OE-BST-2M cells to grow in suspension is not because of the level of BST-2 (Figures 3c and d) but can be attributed to the variant of BST-2 (Figure 3d, red brackets—BST-2 shifts in non-reducing gels). These data indicate that BST-2 expressed as dimers may endow cancer cells the ability to cluster, survive and grow in suspension—a characteristic of aggressive epithelial-derived tumor cells.

BST-2 dimerization promotes adherent-independent survival of cancer cells by inhibiting anoikis. If BST-2 dimerization is involved in protection of cancer cells from anoikis, cells expressing BST-2D will survive under anoikis conditions. Indeed, following poly-HEMA-mediated induction of anoikis, shBST-2 cells have significant reduction in viability compared with shCTL cells (Figures 4a and b). OE-BST-2D but not OE-BST-2M rescues viability of shBST-2 cells (Figures 4a and b). The inability of BST-2M cells to survive under anoikis conditions is due to the variant of BST-2 because BST-2 mRNA is higher in BST-2M cells compared with shBST-2 cells (Figure 4c).
Next, we assessed the levels of molecules implicated in anoikis, including BIM and caspase-3 (Cas3). BIM mRNA was suppressed in shBST-2 and OE-BST-2M cells compared with shCTL and OE-BST-2D cells in normal conditions (Figure 4d). However, upon induction of anoikis, the level of BIM mRNA (Figure 4d), BIM protein and cleaved Cas3 (Figure 4e) increased. These observations were confirmed using MCF-7 cells where OE-BST-2M cells in suspension showed reduced survival, increased BIM mRNA, and increased BIM and cCas3 proteins (Supplementary Figures 2a-c), despite elevated...
BST-2 mRNA (Supplementary Figure 2d). These results suggest that BST-2 dimerization promotes cancer cell survival by inhibiting anoikis.

**BST-2 dimerization results in phosphorylation of BST-2 in cancer cells.** To explore the mechanism by which BST-2 promotes cellular interactions, we showed that dimerization of BST-2 molecules activates BST-2. Western blot analysis of input protein following exposure of cells to vehicle or rBST-2 shows that the levels of GAPDH, phospho-tyrosine (p-Tyr), and growth factor receptor-bound protein 2 (GRB2) are similar (Figure 5a). In contrast, immunoprecipitation with anti-BST-2 antibody reveals that BST-2 is tyrosine phosphorylated in cancer cells in a manner that is dependent on BST-2 dimerization (Figure 5b). These data suggest that BST-2D-expressing cells contain activated and more function-relevant BST-2.

To determine the role of activated BST-2 in cancer cells, we started by investigating the level of GRB2—a docking protein that binds to phospho-tyrosine residues of activated receptors and recruits ERK1/2 to the signaling complex. The level of GRB2 remained the same in quiescent cells (Figure 5b). But upon BST-2 activation, higher GRB2 and ERK1/2 were bound to phospho-BST-2 in shCTL and OE-BST-2D cells (Figure 5b). Remarkably, GRB2 and ERK1/2 bound to BST-2 in OE-BST-2M cells did not increase upon BST-2 activation (Figure 5b).

We confirmed that BST-2 is tyrosine phosphorylated using a mutant form of BST-2 that is able to form dimers but the cytoplasmic tail tyrosine residues at positions 6 and 8 had been substituted with alanine residues (OE-BST-2DΔTy). The level of anti-BST-2-precipitated p-Tyr, GRB2 and ERK1/2 did not change between vehicle and rBST-2-treated OE-BST-2DΔTy cells (Figure 5b), suggesting that phospho-Y6/Y8 recruits GRB2.

**BST-2 dimerization results in ERK-mediated BIM phosphorylation.** As BIM protein is degraded by the proteasome following its phosphorylation by kinases—ERK1/2 and JNK,13-16 we used 12-O-tetradecanoylphorbol-13-acetate (TPA) to impair proteasome activity. TPA treatment resulted in a significant increase in the levels of phosphorylated BIM (P-BIM) in OE-BST-2D cells, whereas OE-BST-2M cells did not show this increase (Figure 5c). These results suggest that BST-2 dimerization promotes anchorage-independent growth by inducing ERK-mediated BIM phosphorylation and, subsequently, by inhibiting apoptosis.

**Figure 3** Covalent dimerization of BST-2 is important for anchorage-independent growth of breast cancer cells. (a) Representative images and quantitation of growth of 4T1 cells expressing different levels and variants of BST-2 on soft agar for 30 days. (b) Representative images and quantitation of growth of Vector, OE-BST-2D or OE-BST-2M MCF-7 cells on soft agar for 30 days. Clones were stained with crystal violet and imaged at 10X. To calculate colony size, the diameters of colonies from five different fields were measured, averaged and a percent calculated relative to either shCTL for 4T1 cells or Vector for MCF-7 cells, which were set to 100%. (c) FACS analysis of levels of variants of BST-2 on the surface of 4T1 shBST-2 cells. Numbers in parenthesis correspond to mean fluorescence intensity of BST-2 expression presented as percent. (d) Western blot analysis of total BST2 protein under reducing and non-reducing conditions. Red brackets depict shift in BST-2D but not in BST-2M sizes under different conditions. Experiments were repeated at least three times with similar results. Error bars represent S.E.M. and significance was taken at *P<0.05, **P<0.001 and ***P<0.001. NS, not significant.
(TPA) to induce survival signal and phosphorylate BIM. TPA treatment phosphorylates ERK1/2 and BIM in OE-BST-2D cells but not in OE-BST-2M cells, although total ERK1/2, BIM and phosphorylated JNK were unchanged from vehicle-treated cells (Figure 5c). These data indicate that activation of ERK1/2 and phosphorylation of BIM are dependent on BST-2 dimerization.

To confirm that ERK1/2-mediated BIM phosphorylation is BST-2 dimerization dependent, we exposed OE-BST-2D and OE-BST-2M cells to FR180204, an ERK1/2 specific inhibitor. Comparisons with vehicle, FR180204 had no effect on protein levels in OE-BST-2D and OE-BST-2M cells (Figures 5c and d). However, inhibition of ERK1/2 activity in OE-BST-2D but not OE-BST-2M cells treated with TPA resulted in increased total BIM and reduced pERK1/2 and pBIM (Figures 5c and d). Importantly, total ERK1/2 and pJNK were not affected by FR180204 (Figure 5c).

BST-2 dimerization induces proteasomal degradation of BIM. As BST-2 downregulates BIM, we examined whether this downregulation occurs via proteasomal degradation. Although TPA activates/phosphorylates ERK1/2 and decreases BIM levels in OE-BST-2D cells, MG132 treatment results in accumulation of BIM in TPA-treated cells (Figures 5e and f). Importantly, TPA, MG132 or TPA/MG132 has no effect on the levels of pERK1/2 and BIM in OE-BST-2M cells (Figures 5e and f). The concentration of inhibitors used were non-toxic (Figure 5g) and not responsible for the observed BST-2-independent reduction of pERK1/2 in MG132-treated cells (Figures 5e and f). Together, results in Figure 5 suggest that BST-2 dimerization promotes ERK1/2 activation, BIM phosphorylation/degradation and inhibition of Cas3 activation that culminate in enhanced anoikis resistance – a phenotype required by cancer cells to survive in circulation.

CTC clusters of metastatic breast cancer patients are enriched in BST-2. The clinical significance of BST-2-mediated cell clustering and survival was evaluated using data from a publicly available dataset to compare the levels of BST-2 in circulating tumor cells (CTCs). Intrapatient comparison of BST-2 in CTC singlets versus CTC clusters shows that 8 out of 10 patients have CTC clusters that express higher BST-2 than their respective CTC singlets (Figure 6a). On the average, CTC clusters express higher (~2 fold) BST-2 compared to CTC singlets (Figure 6b). Further analysis shows that BIM RNA inversely correlates with BST-2 RNA in CTCs (Figure 6c), supporting the findings in Figures 4d and e and further suggest that BST-2 may facilitate cancer cell clustering, thus protecting cancer cells from hemodynamic shear stress in circulation.

BST-2 dimerization regulates the growth of triple-negative breast cancer cells in mice. Compared with shCTL, shBST-2 cells have a significant decrease in primary tumor growth (Figures 7a-c). Analysis of tumor volume (TV)
and final tumor mass show that OE-BST-2D but not OE-BST-2M rescues tumor growth potential of shBST-2 cells (Figures 7a-c). OE-BST-2D and shCTL but not shBST-2 and OE-BST-2M cells efficiently metastasize as evidenced by increased luciferase expression over time (Figures 7a and d). In addition, we observed increase spontaneous pulmonary metastases of shCTL and OE-BST-2D but not shBST-2 and OE-BST-2M tumors (Figures 7e and f). The decrease in lung metastasis in shBST-2 and OE-BST-2M tumor-bearing mice could be attributed to smaller primary tumors, although the effect of BST-2 on primary tumor is distinct from its effect on lung metastasis. Alternatively, dimerization-competent OE-BST-2D cancer cells could associate with BST-2-expressing lung-associated cells, and such association may protect cancer cells from apoptosis. Indeed, BIM levels in the lungs of shCTL and OE-BST-2D tumor-bearing mice were
significantly reduced compared with the level in shBST-2 and OE-BST-2M tumor-bearing mice (Figure 7g). These data indicate that BST-2 dimerization is required for tumor growth and that disruption of BST-2 dimerization may render metastatic cells susceptible to apoptotic insult in the lungs.

According to Kaplan–Meier’s survival analysis, growth of shCTL and OE-BST-2D tumors culminates in death with mean overall survival of 37.5 and 41.0 days for OE-BST-2D and shCTL tumor-bearing mice, respectively (Figure 7h). At variance, the mean overall survival for shBST-2 and OE-BST-2M tumor-bearing mice was undefined (Figure 7h). Together, these data suggest that disruption of BST-2 dimerization may serve to prevent tumor growth and metastatic colonization of the lungs, thus increasing overall survival of tumor-bearing hosts.

On the basis of these observations, we propose a new model for cancer cell survival and growth in which dimeric BST-2 orchestrates pro-adhesive and anti-anoikis stimuli (Figure 8). The principle of this new model is that dimeric BST-2 allows interaction between cancer cells and the tumor microenvironment that promotes the survival, growth and metastasis of tumor cells.

**Discussion**

Here we provide evidence for structural and molecular link between BST-2 and breast cancer by highlighting the following:

First, the BST-2 ECD cysteine residues mediate formation of BST-2 dimers in cancer cells. Previous studies demonstrated the effect of BST-2 dimers in protection against viral infection.18 However, these studies did not evaluate the involvement of BST-2 dimerization in altering cancer cell behavior. Our study identifies BST-2 dimerization as critical in the promotion of cancer cell adhesion. We found that cancer cells expressing dimeric BST-2 efficiently adhere to other
cancer cells, potential stromal cells, and ECM proteins. Thus, cancer cells expressing BST-2 dimers may serve as a target or docking sites for other cells and ECM proteins to bind to tumors. The interaction between BST-2-expressing primary tumor cells and other resident stromal cells may regulate the expression of other factors in secondary organs, thus conditioning metastatic sites for subsequent arrival of tumor cells, especially tumor cells that express dimeric BST-2. It remains to be determined how BST-2 mediates the adhesion of cancer cells to components of the ECM. Perhaps, the cysteine residues involved in BST-2 dimerization may associate with cysteine residues found on fibronectin type II domain.19

Second, similar to the role of BST-2 in adhesion, the ability of BST-2 to promote survival and growth of cancer cells in suspension is controlled by BST-2 dimerization. Cancer cell adhesion is intricately related to the ability of such cells to survive in suspension. We report that cells expressing BST-2 dimers and not monomers activate intracellular signaling that result in the degradation of BIM and blockade of Cas3 activation, culminating in anoikis resistance and cell survival. Therefore, BST-2:BST-2 dimerization may transmit survival signals or suppress proapoptotic factors in breast cancer cells, creating a microenvironment that allows cells to grow independent of anchor. In our studies, we identified BST-2/GRB2/ERK/BIM/Cas3 as an important pathway in anoikis evasion by breast cancer cells. This BST-2-directed cell reprogramming allows cancer cells to survive in circulation. Evidently, BST-2 is present in circulating breast cancer cells, and levels are elevated in cells that circulate as clusters. Whether BST-2 is directly linked to cancer cell clustering and survival in human blood is yet to be determined. Meta-analysis
of data from CTCs shows an inverse correlative association between BST-2 and BIM. This concept was experimentally validated with breast cancer cells in vitro and with lung tissues from tumor-bearing mice. Of note, the tumorigenic activity of BST-2 dimerization is operative across species (mouse and human) and is independent of the aggressive nature of the cells.

Third, our study extends our knowledge of the molecular mechanism of anoikis evasion and the positive impact of the proteasome on tumor growth. The loss of cell viability and growth arrest observed in vitro and in vivo following expression of monomeric BST-2 is dependent on blockade of GRB2 recruitment and ERK1/2 activation, proteasomal degradation of BIM and activation of Cas3. Whether or not other anti- or pro-apoptotic factors are involved is yet to be determined. Also unknown are the kinases that catalyze and co-ordinate this complex BST-2/GRB2/ERK/BIM/Cas3 pathway. Activation of ERK1/2 in cells expressing BST-2 dimers may be orchestrated by serine/threonine kinases, such as Src or Ras known to phosphorylate ERK.\[20,21\] In addition to accumulation of BIM protein, BIM mRNA was upregulated in cells expressing reduced levels of BST-2 or monomers of BST-2. It is unclear how BST-2 dimerization can lead to reduced BIM at the RNA level. In immune cells, BST-2 is negatively regulated by MYD88/PI3K.\[22\] Possibly in cancer cells, BST-2 may activate PI3K to phosphorylate FOXO3A—a transcription factor that induces BIM expression upon its dephosphorylation and nuclear translocation.\[25\] Although the identity of the BST-2 tyrosine residues that are phosphorylated is yet to be revealed, it is known that the cytoplasmic tail of BST-2 contains two tyrosine residues at positions 6 and 8 that become phosphorylated upon virus-mediated BST-2 activation.\[26\] Possibly, these or other tyrosines present in the different domains of BST-2 are phosphorylated. Indeed, in silico analysis using the PPSP software (http://ppsp.biocuckoo.org/)\[27\] revealed that BST-2 contains four phosphorylatable tyrosines at positions 6, 8, 153 and 154.\[28\] Aside from tyrosines, the cytoplasmic tail of BST-2 contains phosphorylatable serines and a threonine.

Fourth, we provide evidence that cells expressing monomeric BST-2 are unable to grow in the mammary gland. As monomeric BST-2 is deficient in adhesion and anchorage independency, it is possible that these cells were unable to make contact with each other or with mammary gland resident cells. The lack of increased metastatic tumor growth in the lungs persuades us to speculate that tumor cells expressing monomeric BST-2 may alter the tumor environment landscape by changing the type of immune cells that are recruited to the tumor microenvironment because of changes in the expression of signaling cytokines and chemokines.\[29–31\] It is also possible that cells expressing monomeric BST-2 may not survive in circulation thus limiting the number of cancer cells that may reach metastatic sites. Noteworthy, although expression of BST-2M in cells almost completely repressed

Figure 8 Hypothetical model of BST-2-mediated anoikis resistance. Wild-type dimer-forming BST-2 (BST-2D) in cancer cells is activated upon cell to cell or cell to ECM interaction. BST-2 activation results in phosphorylation of the cytoplasmic tail (CT), presumably at the tyrosines residues located at positions 6 and 8. Other phosphorylation events independent of these tyrosines are possible. Phosphorylated BST-2 recruits GRB2 (an adaptor protein that recognize p-Tyr), facilitating activation of yet to be identified kinase(s), such as Src or Ras, which in turn phosphorylates ERK. Phospho-ERK then phosphorylates BIM resulting in subsequent proteasomal degradation and removal of BIM. In the absence of BIM, mitochondrial membranes remain intact and pro-Cas3 is not cleaved and activated (c-Caspase-3). The end result is that cancer cells overcome anoikis, survive under harsh conditions and grow/metastasize.
tumor formation, shBST-2 cells (containing low levels of BST-2D) were able to form primary tumors, albeit small. Although the reason for BST-2M-mediated repression of tumor growth is yet to be determined, it is possible that functionally distinct signals may be elicited by BST-2M and shBST-2 cells and that BST-2M signals may have a negative growth effect on tumor cells (autocrine). It is also plausible that BST-2M signals may be transmitted to other distal cells to inhibit cell growth (paracrine).

In summary, we have demonstrated how BST-2 activity shapes the function of breast cancer cells. We identify BST-2/GRB2/ERK/BIM/Cas3 as the pathway regulating BST-2-mediated cancer cell adhesion, anoikis resistance, anchorage-independence, cell survival and growth. Our findings may motivate development of new targeted treatments based on disruption of BST-2 homodimerization in tumors.

Materials and Methods

Cell lines. The murine triple-negative breast cancer cell line-4T1 and the luminal A breast cancer cell line—MCF-7, respectively, are kind gifts from Drs. Lyse Norian and Wei Zhou Zhang of the University of Iowa, Iowa City, IA, USA. All cells were maintained according to ATCC guidelines (Manassas, VA, USA).

Animals. Five-week-old female BALB/cAnNCr mice purchased from Harlan (Indianapolis, IN, USA) were used. Tumor-bearing mice were killed when they became moribund. TV was calculated as: TV = 0.5(length × width)².32 Experiments involving mice were approved by the University of Iowa Animal Care and Use Committee (IACUC).

Mice injections and live animal imaging. Orthotopic mammary tumors were generated by implanting 300 000 cancer cells into the 10th mammary fat pad of 5-week-old female mice. Before imaging, mice were anesthetized, weighed and injected intraperitoneally with d-luciferin (Sigma-Aldrich, St. Louis, MO, USA). Mice of 5-week-old female mice. Before imaging, mice were anesthetized, weighed and injected intraperitoneally with d-luciferin (Sigma-Aldrich, St. Louis, MO, USA). Mice were imaged using the Xenogen IVIS three-dimensional optical imaging system (Caliper Life Sciences, Hopkinton, MA, USA). Luciferase expression was quantified with Living Image Software (Caliper Life Sciences). Primary tumors were weighted and photographed post-mortem. Pulmonary nodules were quantified by manual counting.

Generation of BST-2-overexpressing cancer cells. MCF-7 cells, which contain low levels of endogenous BST-2D or 4T1 shBST-2 cells in which endogenous mouse BST-2 was downregulated,3 were stably transfected with either empty pcDNA3.1 (Vector for MCF-7 cells or shBST-2 for 4T1 cells), pcDNA3.1 containing dimerization-competent wild-type human BST-2 (BST2D) or pcDNA3.1 containing dimerization mutant BST2D in which cysteine residues at positions 53, 63 and 91 were replaced with alanine residues (BST2M). These BST2 constructs are a kind gift from Dr. John Guatelli of UCSD (La Jolla, CA, USA) and Dr. Klaus Strebel of NIH (Bethesda, MD, USA).18,33 Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) was used for the transfections and the amounts used were adjusted according to the manufacturers’ instructions. Transfected cells were selected with G418 at 500 μg/ml and stable cells were used in all experiments. Note that shBST2 is a shRNA specific for mouse BST2 and does not affect the expression of human BST-2.

Assessment of BST-2 protein expression and phosphorylation. Western blots were performed as previously described.34–36 Briefly, protein extracts from MCF-7 or 4T1 cells expressing variants of BST2 (Vector, OE-BST-2D, OE-BST-2M, shCTL, shBST2, OE-BST-2D OE-BST-2M) were isolated and assayed under reducing (β-ME + heat) and non-reducing (heat only) conditions as previously described.38 For BST2 dimerization and activation/phosphorylation studies, equivalent numbers (300,000 cells) of shCTL, shBST2, OE-BST-2D, OE-BST-2M and OE-BST-2DΔTy cells were seeded in six-well plates. Four hours later, cells were treated with 200 ng per well of rBST2 or equivalent volume of vehicle for 1 h. Equivalent concentrations of total proteins from the cells were used to immunoprecipitate BST2 using anti-BST2 antibodies (AIDS reagents program, Germantown, MD, USA). Immunoprecipitates were separated and probed with anti-phospho-tyrosine, anti-GRB2, and anti-ERK1/2 antibodies (Cell Signaling Beverly, MA, USA). The species-appropriate IRDye secondary antibody was used followed by detection with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Evaluation of BST-2 surface protein. Approximately, 1 x 10⁶ 4T1 cells were treated with either APC-conjugated anti-human BST-2 antibody (BioLegend, San Diego, CA, USA) or equivalent volumes of vehicle for 1 h at 4 °C. Cells were washed and stained with 7-AAD viability dye (BioLegend) for 15 min. Using FACS Calibur flow cytometer (BD, San Jose, CA, USA), at least 10,000 events were collected per sample. FACS data were analyzed by Flowjo software (TreeStar, Ashland, OR, USA).

Assessment of RNA levels. Isolation of RNA was accomplished using a RNeasy mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions. For cDNA synthesis, equivalent amounts of RNA treated with DNase I (Qiagen) were reverse-transcribed with high capacity cDNA reverse transcription kit (ABI, Carlsbad, CA, USA). The cDNA was amplified with target-specific primers. Quantitative reverse transcription real-time qPCR (RT-qPCR) was carried out using ABI 7500 FAST thermal cycler. Primers used: GAPDH-forward: 5′-GGTGAAGCAAGGTCAACGTC-3′, reverse: 5′-CAGGAGGCTTTCAGATG-3′; β-actin-forward: 5′-TTTTCTTCCTGGGCTTC-3′; BIM-forward: 5′-AATGGAGGACGAGTCAAGCA-3′; human BST-2 RT-qPCR primer assays (SA-Biosciences, Frederick, MD, USA) as well as shBST2-forward: 5′-AGAAGGGCTTACCCAGATG-3′; reverse: 5′-CTTTGTCTCCTGGGCTTC-3′; PKH67Green-forward: 5′-TGCTCTTCCA CATACAGCG-3′; and Cas3-forward: 5′-CAAACCTCGTGGATCAAACA-3′; reverse: 5′-CCCCCTTCCAGGATA TCCATT-3′.

Cell to cell adhesion assay. Cells of interest were grown to confluence in a 96-well plate. In all, 20,000 cancer cells of interest were labeled with PKH67Green fluorescent cell linker (Sigma-Aldrich). Labeled cells were added to the appropriate monolayers and allowed to incubate for 4 h. Non-adhered cells were washed off with PBS and plates were read at 485 nm/535 nm (excitation/emission) wavelengths using a Tecan Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) to determine the rate of adhesion. Values are represented as relative fluorescence intensity (RFI) or as percentage.

Cell to ECM protein adhesion assay. Wells of a 96-well plate were coated with 50μl of 50 μg/ml collagen or fibronectin (Sigma-Aldrich) and incubated at 37 °C for 4 h. Nonspecific sites were blocked with 40 μl of 2 mg/ml bovine serum albumin in PBS and then wells were washed once with PBS. In all, 0.5 μl PKH67Green (Sigma-Aldrich) labeled cancer cells (MCF-7 Vector, BST-2D or BST-2M or 4T1 shCTL or shBST-2 cells) were added to pre-coated wells and allowed to adhere for 4 h. Non-adhered cells were washed off with PBS and plates were read using a Tecan plate reader as described in the previous paragraph.

Blockade of cell to cell adhesion. BST-2D-overexpressing MCF-7 cells or 4T1 shCTL cells were plated to confluence in a 96-well plate and blocked with water (Vehicle) or 200 ng/well of rBST2 (Sino Biological Inc., Beijing, China) for 4 h. Cell monolayers were washed twice with PBS. 25,000 PKH67Green-labeled MCF-7 cells expressing Vector, OE-BST-2D or OE-BST-2M were added to MCF-7 cell monolayers. On the other hand, 25,000 PKH67Green 4T1 cells expressing shCTL or shBST2 were added to 4T1 shCTL monolayers. Cells were allowed to adhere for 4 h at 37 °C. Plate was washed twice with PBS and fluorescence was read with a Tecan plate reader as described previously.

Assessment of cancer cell viability and determination of small molecule IC₅₀. A total of 10 MCF-7 cells expressing Vector, OE-BST-2D or OE-BST-2M or 4T1 cells expressing shCTL, shBST2, OE-BST-2D, OE-BST-2M or OE-BST-2DΔTy were seeded in 96-well plates for 24 h. Cells were then left untreated or treated with 0, 0.5, 1 or 2 μM of MG132, 0, 10, 20, 40 μM of FR180204; or 0, 10, 20, 40 μM of TPA. These cells were then incubated with 20 μl of 5 mg/ml MT T reagent for 3.5 h followed by removal of media, addition of 150 μl/well of MTT solution (0.1% NP-40 and 4 mM HCI in isopropanol) and rocking for 15 min to determine the effect of different BST2 constructs in cancer cell viability and to determine the IC₅₀ of the different small molecules used. Absorbance at 590 nm was read using a Tecan Infinite M200 Pro plate reader.
Induction of cell survival and analysis of the signal transduction pathway. Equivalent numbers (300,000 cells) of relevant cells were seeded on six-well plates and treated with DMSO (Vehicle), 20 nM of the survival signal TPA (Sigma-Aldrich), 1 μM of the proteasome inhibitor MG132 (Sigma-Aldrich), a combination of TPA and MG132 (TPA/MG132), 20 μM of the ERK1/2 kinase inhibitor FR180204 (Sigma-Aldrich), or a combination of TPA and FR180204 (TPA/FR180204) for 24 h following IgGμ determination. Equivalent concentrations of total proteins from the cells were separated on a RAGE-gel and probed with an anti-BST2 antibody (AIDS reagents program), anti-cleaved Cas9, anti-BIM, and anti-GAPDH antibodies (Santa Cruz Biotechnology, Dallas, TX, USA), as well as with anti-ERK1/2, anti-pERK1/2, anti-pJNK, anti-pAKT S473 and anti-pBIM antibodies (Cell Signaling). The species-appropriate IRDye secondary antibodies were used followed by detection with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Evaluation of cancer cell proliferation. A total of 10 000 relevant cells were seeded in 96-well plates for 24 h. Bromodeoxyuridine or 5-bromo-2′-deoxyuridine (BrDU) (Calbiochem, Billerica, MA, USA) assay was carried out according to the manufacturer’s instructions. Absorbance at 450 nm was read using a Tecan Infinite plate reader.

Colony formation assay. In all, 24-well plates were coated with 500 μl of 0.5% agar and allowed to solidify. Following, 4T1 shCTL, shBST-2, OE-BST-2D or OE-BST-2M and MCF-7 Vector, OE-BST-2D- or OE-BST-2M-expressing cells were seeded at 20 000 cells per well. Following, 4T1 shCTL, shBST-2, OE-BST-2D or OE-BST-2M were starved for 6 h, suspended in serum-free medium and were plated on top of the Matrigel layer. In all, 600 μl of culture medium containing 30% FBS and 5 μl of complete RPMI was added on top of the agar layer. Growth medium was replaced twice a week and cells were allowed to form colonies for 30 days. Colonies were stained with crystal violet and imaged using a Nikon Eclipse Ti microscope adjusted with a Nikon digital sight camera (Nikon, Tokyo, Japan). The diameters of colonies from five different fields were measured, averaged and a percent calculated relative to either shCTL for 4T1 cells or Vector for MCF-7 cells, which was set to 100%.

Invasion assay. The apical chamber of 24-well cell culture inserts (Merck Millipore, Billerica, MA, USA) were coated with 1.5 mg/ml of Matrigel (100 μl) (Sigma-Aldrich) and allowed to solidify for 3 h. A total of 250 000 MCF-7 cells expressing Vector, OE-BST-2D- or OE-BST-2M were starved for 6 h, suspended in serum-free medium and were plated on top of the Matrigel layer. In all, 600 μl of culture medium containing 30% FBS and 5 μl of culture medium (109 × read counts) was added to the basal chamber of the unit and cells were allowed to invade through the membranous barrier for 24 h at 37 °C. Noninvasive cells were washed off; invasive cells were fixed with 4% PFA, permeabilized with 1% methanol, labeled with Giemsa stain and imaged. Images were processed using ImageJ software (NIH, Bethesda, MD, USA). Cells from five different fields were blind counted and averaged.

Experimental induction and analysis of anoikis. U-bottom 96-well plates were coated with 50 μl of sterile 95% ethanol or 50 μl of 12 mg/ml poly-HEMA in 95% ethanol (Sigma-Aldrich) and allowed to dry for 72 h under the hood, as previously described. Poly-HEMA prevents cells from attaching to the plastic. Following, 4T1 shCTL, shBST-2, OE-BST-2D or OE-BST-2M; and, MCF-7 Vector, OE-BST-2D- or OE-BST-2M-expressing cells were seeded at 20 000 cells per well. Plates were centrifuged at 1200 x g for 10 min and then incubated at 37 °C for 48 h. Cells were collected to test cell viability using Trypan blue (Life Technologies) and a MTT assay (Life Technologies). The rest of the cells were pelleted and kept at −20 °C until used for RNA and protein isolation.

Meta-analysis. The publically available Gene Expression Omnibus ( GEO) data set GSE51827, which contains RNAseq data from CTCA tissues and clusters isolated from metastatic breast cancer patients was used to determine the levels of BST-2 mRNA. RPKM units were calculated using the formula: RPKM = (10^8 × read counts/(total mapped reads × exon length)). Intrapatient comparisons were performed by plotting CTC singlets BST-2 levels along with CTC clusters BST-2 levels of the same patient. Correlation analyses of BIM and BST-2 levels were performed with all samples excluding patient samples whose BIM or BST-2 levels were zero. Moreover, for analyses of BST-2 expression in human mammary cancer epithelial cells (Cal51 and MDA-MB-231) co-cultured with different strains of human fibroblasts (CCD1112SK, WI38, HFF1 and HFF2), the GEO dataset GSE41678 were used. All data points were included in the analyses.

Statistics. Statistical analysis of significant differences was performed with unpaired t-test assuming Gaussian distribution with Welch’s correction or using a non-parametric Kolmogorov–Smirnov test (GraphPad Prism software, San Diego, CA, USA). Error bars represent S.D. for transcript data and S.E.M. for other data. Kaplan–Meier survival plots were analyzed using the Gehan–Breslow–Wilcoxon test (GraphPad Prism software). A probability (P) value of 0.05 or lower was considered significant.

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
CMO conceptualized experiment, WDM executed experiments, CMO and WDM analyzed data and prepared the manuscript. All authors read and approved the final version of the manuscript.

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