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ORIGINAL ARTICLE

DBC2/RhoBTB2 functions as a tumor suppressor protein via Musashi-2 ubiquitination in breast cancer

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The gene encoding 'deleted in breast cancer 2' (*DBC2*), also referred to as *RHOBTB2* (Rho-related BTB domain-containing protein 2), is classified as a tumor suppressor gene. DBC2 is a substrate-specific adaptor protein for a novel class of Cullin-3 (CUL3)-based E3 ubiquitin ligases; however, it is unclear if the substrate adaptor function of DBC2 is required for its tumor suppressor activity. Furthermore, the key substrates of DBC2-mediated ubiquitination have yet to be identified. In the present study, we established a genome-wide human cDNA library-based *in vitro* ubiquitination target screening assay and identified Musashi-2 (MSI2) as a novel ubiquitination target protein of DBC2. MSI2 directly interacted with DBC2, and this interaction promoted MSI2 polyubiquitination and proteasomal degradation in breast cancer cells. Overexpression and knockdown experiments demonstrated that DBC2 suppressed MSI2-associated oncogenic functions and induced apoptosis. Immunohistochemistry analysis of a breast cancer tissue microarray revealed that DBC2 and MSI2 protein levels are inversely correlated in both normal breast tissues and breast cancer tissues. Taken together, these findings provide evidence that DBC2 suppresses tumorigenesis in breast cancer by ubiquitinating MSI2.

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INTRODUCTION

Ubiquitin-mediated post-translational modifications are essential for nearly all biological processes, including cell growth, differentiation and apoptosis.[1] Dysregulation of this phenomenon leads to irreversible changes in protein stability and can directly or indirectly promote a variety of pathological conditions, including cancer.[1] The process of ubiquitination involves multiple steps mediated by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and substrate-specific E3 ubiquitin ligases.[2] The most predominant class of E3 ligases is the family of really interesting new gene (RING)-finger domain-containing proteins. These proteins are subdivided into two groups: the monomeric RING-type E3 ligases and the multimeric RING-type E3 ligases, such as the Cullin-3 (CUL3)-based E3 ligases.[2] The substrate specificity of CUL E3 ligases is primarily mediated by distinct adaptor proteins that contain an F-box, SOCS-box, or a broad complex, tramtrack and bric-a-brac (BTB) domain.[2,3]

BTB domain-containing proteins comprise a new class of substrate-specific adaptors of the CUL3-based E3 ubiquitin ligase complex.[3,4] Previous studies have demonstrated that BTB protein-dependent CUL3-based E3 ubiquitin ligases are present in both mammals and non-mammalian species, including *Drosophila melanogaster*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans* and *Arabidopsis thaliana*.[5–9] BTB–CUL3 ubiquitin ligase complexes have essential roles in numerous biological processes, including embryonic development, apoptosis, cytokinesis, cell movement, Hedgehog signaling and responses to oxidative stress.[9–14]

Deleted in breast cancer 2 (DBC2), also referred to as Rho-related BTB domain-containing protein 2 (RhoBTB2), is a putative tumor suppressor protein that forms a complex with CUL3[15] and has a direct role in tumorigenesis.[16–18] A downregulation in *DBC2* expression resulting from homozygous deletion or promoter methylation has been observed in breast cancer,[16,19,20] and a previous study has reported that *DBC2* expression is lost in 60% of cases of breast cancers.[21] The antitumorigenic effects of DBC2 are mediated by the inhibition of cancer cell growth, proliferation, migration and invasion.[16,22] Furthermore, reduced *DBC2* expression is associated with distinct clinicopathological features of breast cancer, including human epidermal growth factor receptor 2 (HER2) status and p53 mutations.[19] In addition, downregulated *DBC2* expression has been observed in lung, gastric, bone and bladder carcinomas.[23–26] These findings indicate that DBC2 functions as both a tumor suppressor and a putative substrate-recruiting adaptor protein of the CUL3-based ubiquitin ligase complex; however, the relationship between these two distinct functions remains unclear. A better understanding of the multifunctional nature of DBC2 and identification of DBC2 ubiquitination substrates might inform the development of promising anticancer therapies.

RESULTS

Identification of MSI2 as a novel substrate for DBC2-dependent E3 ubiquitin ligases

In contrast to monomeric RING-finger E3 ubiquitin ligases, CUL3-based multimeric E3 ubiquitin ligases require BTB domain-containing proteins to provide substrate specificity.[6] Although DBC2 has been shown to interact with CUL3,[15] the substrates targeted by DBC2/CUL3-E3 ubiquitin ligase activity, including the target protein that potentially mediates the tumor suppressor

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function of DBC2, have yet to be identified. Therefore, we sought to identify candidate substrates of DBC2-dependent E3 ubiquitin ligase complexes. To this end, we adapted our previously reported *in vitro* genome-wide screening system of a human cDNA library.[27–29] To isolate selectively E3 ligase-specific substrates, we incorporated recombinant E1 proteins, E2 proteins, GST-DBC2, a CUL3-ROC1 protein complex and His-ubiquitin into this system (Supplementary Figure 1a and Supplementary Materials and Methods). The *in vitro* ubiquitination assay was conducted as described previously,[15,30] and the ubiquitin E3 ligase activity of the DBC2-CUL3 ligase complex was confirmed using western blot with an antibody against ubiquitin (data not shown). Using this approach, we isolated several highly ubiquitinated cDNA clones, one of which represented a novel ubiquitination target protein (Supplementary Figure 1b). The clones of interest were sequenced, and we conducted an extensive literature review of the relevant genes. The cDNA clone encoding Musashi-2 (MSI2), a protein with two RNA recognition motif domains, was selected for further analysis as its role in the oncogenesis of multiple cancers was the most well-studied compared with the other putative target proteins.[31–34]

We evaluated the potential role of DBC2 in MSI2 ubiquitination using *in vitro* binding assays with recombinant GST-MSI2 and [^{35}S] methionine-labeled DBC2. We observed a direct interaction between DBC2 and MSI2 (Figure 1a) but not between CUL3 and MSI2 (Supplementary Figure S2), suggesting that MSI2 ubiquitination is dependent on its interaction with DBC2. These findings were confirmed using co-immunoprecipitation and western blot assays with MDA-MB-231 breast cancer cells co-transfected with plasmids expressing DBC2 and MSI2 (Figure 1b). Immunofluorescence staining and confocal microscopy analysis revealed that DBC2 colocalized with MSI2 in the cytosol (Figure 1c). In addition, endogenous DBC2 and endogenous MSI2 co-precipitated with one another (Figure 1d).

DBC2 contains a GTPase domain and two BTB domains in its N- and C-terminal regions, respectively.[15] A previous study reported that the Y284D mutation in the first BTB domain of DBC2 disrupts its interaction with CUL3.[15] Co-immunoprecipitation and western blot assays revealed that both the C-terminal region of DBC2 (DBC2-C) and the DBC2 mutant protein with the Y284D mutation (DBC2-MT) interacted with full-length MSI2 (Figures 1e and f), indicating that the Y284 residue of DBC2 is required for its interaction with CUL3 but not for its interaction with its ubiquitination substrate MSI2. Taken together, these results demonstrate that DBC2 and MSI2 directly interact.

DBC2 promotes polyubiquitination-mediated proteasomal degradation of MSI2

Next, we evaluated the potential role of DBC2 in MSI2 ubiquitination. MSI2 was ubiquitinated in DBC2-overexpressing cells (Figure 2a, lane 2 versus lane 4), and this effect was enhanced in cells co-transfected with CUL3 and ROC1 (Figure 2a, lane 4 versus lane 8). In contrast, MSI2 was ubiquitinated at relatively lower levels in cells co-transfected with plasmids expressing CUL3 and ROC1 only (Figure 2a, lane 2 versus lane 6). This result was likely mediated by endogenous DBC2 (Figure 2a and Supplementary Figure S3), as MSI2 did not interact with CUL3 *in vitro* (Supplementary Figure S2). DBC2 also promoted K48-linked polyubiquitination of MSI2 (Supplementary Figure S4). As K48-linked ubiquitination is associated with proteasomal degradation of the target protein,[35] we investigated if DBC2 downregulated MSI2 protein levels via proteasomal degradation. We found that DBC2 decreased endogenous MSI2 protein levels in a concentration-dependent manner (Figure 2b), whereas DBC2 did not influence *MSI2* mRNA levels (Supplementary Figure S5). In addition, ROC1 and CUL3 overexpression enhanced DBC2-mediated MSI2 degradation (Figure 2c, lane 2 versus lane 4). In contrast, DBC2-MT did not significantly affect endogenous MSI2 levels (Figures 2b and c). Furthermore, MG132 treatment abolished the effect of DBC2 on MSI2 degradation (Figure 2d). Cellular ubiquitination assays demonstrated that polyubiquitination of endogenous MSI2 increased in DBC2-overexpressing cells compared with control cells, and CUL3-binding-deficient DBC2-MT did not promote endogenous MSI2 ubiquitination (Figure 2e). Moreover, the half-life of MSI2 decreased in cells transiently overexpressing DBC2 compared with control cells treated with the protein biosynthesis inhibitor cycloheximide (Figure 2f). Taken together, these results indicate that MSI2 is ubiquitinated by DBC2/CUL3 E3 ligase complexes and that this modification targets MSI2 for proteasomal degradation.

A previous study demonstrated that heat-shock protein 90 disrupts the intramolecular interaction between the GTPase and BTB domains of DBC2, thereby promoting the formation of the DBC2/CUL3 E3 ligase complex.[36,37] Consistent with these findings, MSI2 degradation was suppressed in MDA-MB-231 cells treated with the heat-shock protein 90-specific inhibitor geldanamycin (Supplementary Figure S6a). In addition, MSI2 inhibited the intramolecular interaction between the GTPase and BTB domains of DBC2 (Supplementary Figure S6b), indicating that the interaction between MSI2 and the BTB domains of DBC2 might relive the autoinhibitory conformation of DBC2. A previous study demonstrated that DBC2 is ubiquitinated by the CUL3 E3 ligase[15] and this effect was also observed in the present study. Interestingly, DBC2 ubiquitination and degradation was markedly suppressed in MSI2-overexpressing cells (Supplementary Figures S6d and S6e). In addition, DBC2 protein levels, but not *DBC2* mRNA levels, increased in MSI2-overexpressing cells (Supplementary Figure S6c), indicating that DBC2 primarily functions as a substrate-specific adaptor for the CUL3 ligase complex when the interaction between DBC2 and MSI2 is enhanced.

DBC2 knockdown enhances MSI2 protein stability

The results of the ubiquitination assays prompted us to further evaluate the role of endogenous DBC2 in the regulation of MSI2 protein levels. We infected MCF7 and MDA-MB-231 cells with lentiviral vectors expressing two different short hairpin RNA targeting *DBC2* (shDBC2). As *DBC2* mRNA levels were lower in shDBC2-expressing MCF7 cells compared with that in MDA-MB-231 cells (data not shown), we generated two independent stable shDBC2-expressing cell lines for further analysis. MSI2 protein levels significantly increased in DBC2-depleted cells, whereas *MSI2* mRNA levels were unaffected (Figures 3a and b). These results suggested that endogenous DBC2 specifically targets the MSI2 protein for degradation. Consistent with these findings, a cellular ubiquitination assay demonstrated that MSI2 ubiquitination was abolished and the half-life of MSI2 increased in DBC2-depleted cells (Figures 3c and d). Taken together, these data suggest that MSI2 ubiquitination in breast cancer cells is primarily mediated by DBC2.

DBC2 induces cell cycle arrest and apoptosis through MSI2 degradation

Recent studies reported that the oncogenic functions of MSI2 are mediated by p21, BAX, c-Myc and BCL-2.[32,38,39] Therefore, we investigated if DBC2 negatively regulates these proteins in breast cancer cells. DBC2 inhibited the MSI2-mediated downregulation of p21 but did not influence *p21* mRNA levels (Figure 4a and Supplementary Figure S7). In addition, DBC2, but not DBC2-MT, inhibited cell growth and proliferation in both control and MSI2-overexpressing cells ($P < 0.05$; Figures 4b and c). Furthermore, the proportion of cells in the sub-G1 and G0/G1 phases of the cell cycle increased in cells transiently expressing DBC2 and in shMSI2-expressing cells (Figure 4d). Interestingly after 48 h post-transfection, only the proportion of cells in the sub-G1 phase

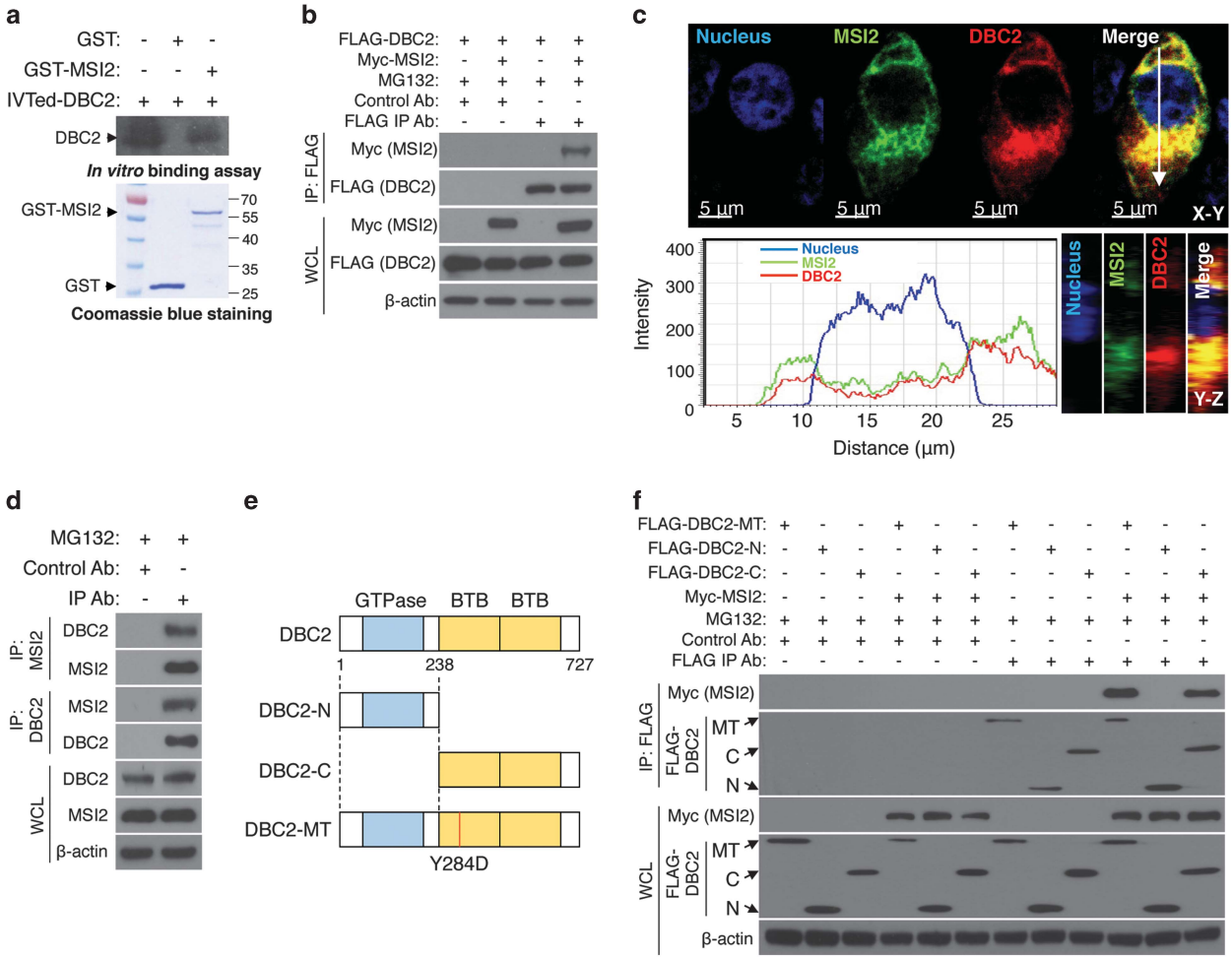


Figure 1. The interaction between DBC2 and MSI2. (**a**) DBC2 directly binds MSI2 *in vitro*. GST, GST-MSI2 and ^{35}S-methionine-labeled DBC2 were coincubated, and the samples were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresi (SDS–PAGE). (**b**) DBC2 complexes in MDA-MB-231 cells. The cells were transfected with FLAG-DBC2 and Myc-MSI2, and the protein complexes were immunoprecipitated (IP) using a control antibody and anti-FLAG. Western blot assays were conducted using the indicated antibodies. (**c**) DBC2 colocalizes with MSI2 in MDA-MB-231 cells. Cells cotransfected with dsRed-DBC2 (red fluorescence) and EGFP-MSI2 (green fluorescence) were treated with the proteasome inhibitor MG132 and analyzed using confocal microscopy. The nuclei were labeled with DAPI (4', 6-diamidino-2-phenylindole). The histogram presents the fluorescence intensity along the white arrow in the merged panel. (**d**) The interaction between endogenous DBC2 and MSI2. Protein complexes were immunoprecipitated from MDA-MB-231 cell lysates using control antibodies, anti-DBC2 and anti-MSI2. The protein interaction was confirmed using immunoblotting assays with the indicated antibodies. (**e**) Constructs of DBC2 functional domains and DBC2 mutant proteins. The Y284D DBC2 mutant protein (red line) is defective for CUL3 binding. The numbers indicate the amino-acid positions. (**f**) MDA-MB-231 cells were co-transfected with the indicated plasmids, and the interactions between DBC2 mutants and MSI2 were evaluated using immunoprecipitation and western blot assays with the indicated antibodies. The DBC2 C terminus and DBC2 Y284D mutant interact with MSI2. IVT, *in vitro* transcribed; WCL, whole-cell lysates.

increased in cells expressing DBC2 and shMSI2 (Supplementary Figure S8), suggesting that the inhibition of cell proliferation was due to DBC2-mediated cell death. To evaluate this hypothesis, apoptotic cells were labeled with Annexin V-fluorescein isothiocyanate and propidium iodide and analyzed using flow cytometry. The proportion of apoptotic cells increased by 11% in DBC2-overexpressing cells compared with control cells (Figure 4e), and a similar result was observed in cells expressing shMSI2 (Figure 4e). Previous studies demonstrated that MSI2 knockdown enhanced apoptosis, affected extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase phosphorylation, and altered the expression of the proapoptotic protein BAX and the antiapoptotic protein BCL-2.[39] To investigate the relationship between DBC2-mediated apoptosis and MSI2-associated signaling, we evaluated these effects in DBC2-overexpressing cells and shMSI2-knockdown cells using western blot assays. The levels of phosphorylated p38, mitogen-activated protein kinase, phosphorylated extracellular signal-regulated kinase and BCL-2 decreased in DBC2-overexpressing cells, and p21 and BAX levels increased. Similar results were observed in shMSI2-knockdown cells (Figure 4f), indicating that the effects observed in DBC2-overexpressing cells were not off-target effects. Taken together, these findings suggest that DBC2-induced cell cycle arrest and apoptosis are mediated by DBC2-induced MSI2 degradation.

Tumor-suppressive function of DBC2 is MSI2-dependent

To evaluate the hypothesis that MSI2 degradation is required for the tumor suppressor activity of DBC in breast cancer, we evaluated clonogenic growth and cell migration. Clonogenic growth was markedly suppressed in DBC2-overexpressing MDA-MB-231 cells but not in DBC2-MT-overexpressing MDA-MB--231 cells (Supplementary Figure S9). Similarly, both MSI2-

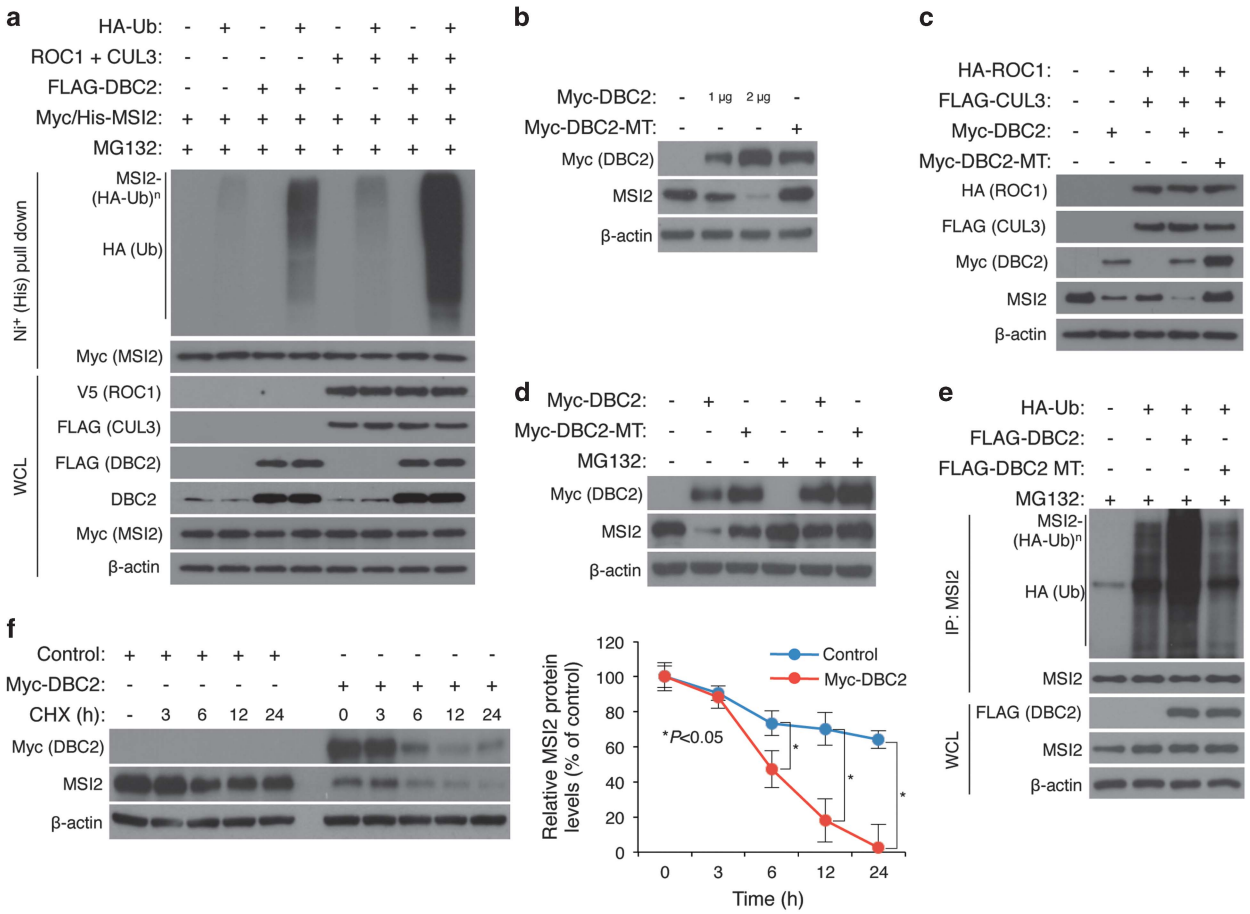


Figure 2. DBC2 induces MSI2 ubiquitination and degradation in breast cancer cells. (**a**) DBC2 induces MSI2 ubiquitination. MDA-MB-231 cells were co-transfected with the indicated plasmids and treated with the proteasome inhibitor MG132. MSI2 pull-down experiments were conducted using Ni-NTA beads, and the samples were analyzed using western blot assays with the indicated antibodies. Ub, ubiquitin. (**b**) DBC2 inhibits MSI2 protein expression. MDA-MB-231 cells were transfected with Myc-tagged DBC2 and DBC2-MT (Y284D mutant). MSI2 protein levels in cell lysates were detected using western blot assays with the indicated antibody. (**c**) DBC2-mediated downregulation of MSI2 is enhanced by ROC1 and CUL3 overexpression. MDA-MB-231 cells were co-transfected with the indicated plasmids, and the cell lysates were analyzed using western blot assays. (**d**) DBC2-mediated downregulation of MSI2 is inhibited by MG132. Cells transfected with plasmids expressing DBC2 and DBC2-MT were treated with 10 μM MG132 for 4 h and analyzed using western blot assays. (**e**) DBC2-mediated ubiquitination of endogenous MSI2 is dependent on CUL3-binding. MDA-MB-231 cells co-transfected with plasmids expressing DBC2, DBC2-MT and ubiquitin were treated with MG132. Protein complexes were immunoprecipitated from cell lysates using anti-MSI2. MSI2 ubiquitination was evaluated using western blot assays with an antibody against ubiquitin. (**f**) DBC2 inhibits MSI2 protein stability. MDA-MB-231 cells transfected with control and Myc-DBC2-expressing plasmids were treated with 40 μg/ml cycloheximide (CHX) for 3, 6, 12 or 24 h. DBC2 and MSI2 protein levels were evaluated using western blot assays. The graph shows the relative normalized intensities of the MSI2 bands. The data are presented as the mean ± s.d. of triplicate samples from at least two independent experiments. Student's *t*-test was used to determine statistical significance (*$P < 0.05$).

induced clonogenic growth and cell migration were suppressed by DBC2 but not by DBC2-MT (Figures 5a and b). To determine if these effects were independent of MSI2 degradation, we evaluated clonogenic growth, cell migration and proliferation in cells expressing shDBC2, shMSI2 and the shScramble control, either alone or in combination. Cell proliferation and clonogenic growth increased in DBC2 knockdown cells compared with control cells (Supplementary Figure S10 and figure 5c). In contrast, cell proliferation and clonogenic growth decreased in MSI2 knockdown cells compared with control cells. Interestingly, the effect of shDBC2 knockdown on cell proliferation and clonogenic growth was not observed in MSI2 knockdown cells. We observed a statistically significant difference ($P < 0.05$) between control and shDBC2 knockdown cells, but not between shMSI2 knockdown and shMSI2/shDBC2 double-knockdown cells (Supplementary Figure S10 and figure 5c). Similarly, cell migration was significantly enhanced in shDBC2-knockdown cells compared with control cells ($P < 0.01$), but this effect was not observed in shMSI2-knockdown cells (Figure 5d). These results indicate that the tumor suppressor functions of DBC2 in breast cancer cells are dependent on MSI2.

DBC2 protein level is inversely correlated with MSI2 protein level in breast cancer tissues

Next, we investigated the clinical relevance of DBC2 and MSI2 in breast cancer. The inverse correlation between DBC2 and MSI2 levels observed in shDBC2-knockdown cells was also observed in MDA-MB-361, SKBR3, T47D, ZR-75-1 and Hs578T breast cancer cell lines (Figure 6a).

To determine if DBC2 and MSI2 levels were also inversely correlated in clinical samples, we analyzed tissue microarrays of normal breast tissue ($n = 46$) and breast cancer tissue ($n = 80$) using immunohistochemistry (IHC) with specific antibodies against

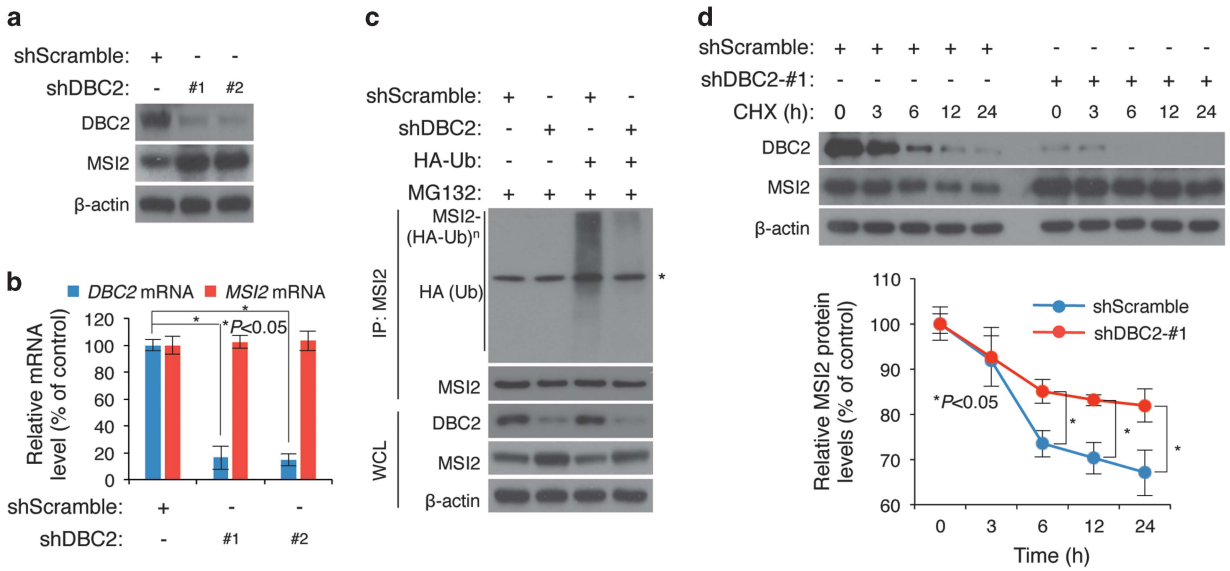


Figure 3. Endogenous DBC2 regulates MSI2 ubiquitination and stability in breast cancer cells. (**a** and **b**) MSI2 protein levels increased in DBC2-knockdown cells in the absence of changes in *MSI2* mRNA levels. MCF7 cells were stably infected with lentiviral vectors expressing two different shRNA constructs targeting DBC2 (nos. 1 and 2). DBC2 and MSI2 protein (**a**) and mRNA levels (**b**) were analyzed using western blot and quantitative reverse transcriptase–PCR (qRT–PCR), respectively. (**c**) DBC2 knockdown inhibits MSI2 ubiquitination. Control (shScramble) and shDBC2-knockdown cells transfected with a plasmid expressing HA-ubiquitin were treated with MG132. Endogenous MSI2 ubiquitination was evaluated using immunoprecipitation and western blot. The asterisk indicates a nonspecific band. (**d**) DBC2 knockdown enhances MSI2 protein stability. Control (shScramble) and shDBC2-knockdown cells were treated with cycloheximide (CHX) for the indicated period of time, and DBC2 and MSI2 protein levels were evaluated using western blot. The normalized intensity of each MSI2 band is expressed as the mean ± s.d. of triplicate samples from at least two independent experiments. Student's *t*-test was used to determine statistical significance (*$P < 0.05$).

DBC2 and MSI2. Consistent with what was observed in cell lines, the staining intensity of DBC2 and MSI2 in normal breast tissues and tumor tissues were inversely correlated. Compared with normal breast tissue, DBC2 protein levels decreased and MSI2 protein levels increased in the tumor tissues (Figure 6b). Staining intensity was scored on a scale of 0–3 (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). The quantitative analysis confirmed that DBC2 levels significantly decreased in tumor tissues compared with normal breast tissues ($P < 0.005$; Figure 6c). Lower staining scores (0 and 1) were observed in 25 of the 34 patients (73.53%) with stage I/II cancer and 39 of the 46 patients (84.78%) with stage III/IV cancer (Figure 6d). In addition, MSI2 levels significantly increased in tumor tissues compared with normal breast tissues ($P < 0.005$; Figure 6c), with higher staining scores (2 and 3) observed in 21 of 34 patients (61.76%) with stage I/II cancer and 33 of 46 patients (71.74%) with stage III/IV cancer (Figure 6d). The inverse correlation between DBC2 and MSI2 levels in breast cancer tissues was statistically significant ($n = 80$, $P = 0.018$, $r = -0.263$; Figure 6e). These findings prompted us to investigate the relationship between DBC2 and MSI2 protein levels and the clinicopathological parameters of the 80 breast cancer cases (Table 1). MSI2 levels were significantly greater in HER2-negative cancers ($P = 0.01$), whereas DBC2 levels were significantly lower in HER2-positive cancers ($P = 0.006$). Interestingly, there were no significant differences in Musashi homolog 1 (MSI1) proteins levels between normal tissues and tumor tissues (Supplementary Figure S11). Taken together, these results demonstrate that DBC2 and MSI2 protein levels are inversely correlated in breast cancer, and they are consistent with our findings that DBC2 interacts with MSI2 *in vitro* and DBC2 promotes MSI2 polyubiquitination/degradation in breast cancer cells.

DISCUSSION

DBC2, a protein identified 23 years ago, is a BTB domain-containing substrate adaptor for the CUL3 ubiquitin ligase complex and a multifunctional tumor suppressor protein.[15,16,18] However, the ubiquitination substrates that mediate its tumor suppressor activity have remained unknown. In the current study, we used a full-length human cDNA library[27–29] to establish an *in vitro* genome-wide ubiquitination substrate screening assay and identified MSI2 as a novel target of DBC2-dependent E3 ligases. We provide evidence for a direct interaction between DBC2 and MSI2 *in vitro* and in cell cultures and demonstrated that this interaction promotes the K48-linked polyubiquitination and proteasomal degradation of MSI2. In DBC2-knockdown cells, MSI2 levels and stability increased and MSI2 ubiquitination was abolished, whereas MSI2 protein stability increased in DBC2-overexpressing cells. Therefore, we conclude that DBC2 is indispensable to MSI2 ubiquitination and its subsequent proteasomal degradation.

A previous study found that MSI2 was upregulated in breast cancer tumors,[40] and MSI2 expression has been implicated with disease progression in several other cancers.[31,41–43] A chromosomal translocation resulting in a fusion gene of *MSI2* and the gene encoding homeobox A9 has been implicated in chronic myeloid leukemia progression,[41] and MSI2 is a prognostic marker in acute myeloid leukemia and a putative therapeutic target.[32,42,44] MSI2 is also a therapeutic target of treatment for myelodysplastic syndrome as it has a pivotal role in maintaining activated myelodysplastic syndrome stem cells.[45] MSI2 is a SRY-box 2-associated protein that has an essential role in tumor growth in glioma.[46] Elevated MSI2 protein levels are associated with cell invasion and a poor prognosis in hepatocellular carcinoma, and MSI2 promotes the progression of hepatitis B-associated hepatocellular carcinoma via the Wnt signaling pathway.[34,43] These results further support the hypothesis that MSI2 is an oncogenic

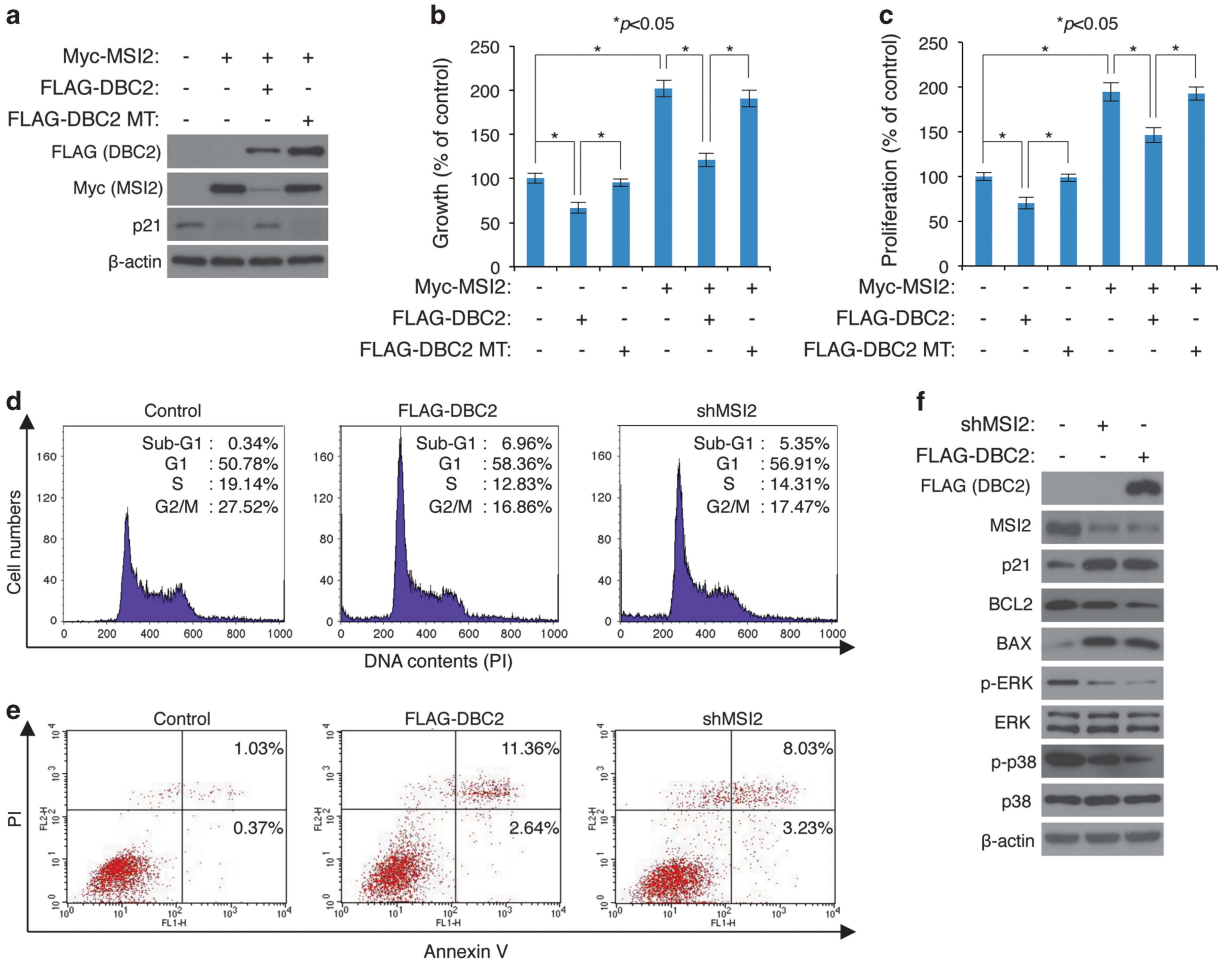


Figure 4. DBC2 regulates the effect of MSI2 on breast cancer cell survival. (**a**) DBC2 inhibits MSI2 function. Cells were co-transfected with plasmids expressing DBC2, DBC2-MT and MSI2. DBC2, MSI2 and p21 protein levels were analyzed using western blot. (**b** and **c**) DBC2 regulates MSI2-mediated cell growth and proliferation. MDA-MB-231 cells were co-transfected with plasmids expressing DBC2, DBC2-MT (Y284D mutant) and MSI2. Cell viability and proliferation were evaluated using the WST-1 assay (**b**) and BrdU (5-bromo-2'-deoxyuridine) incorporation assay (**c**), respectively. The results are represented as the mean ± s.d. of triplicate samples from at least two independent experiments. Student's *t*-test was used to determine statistical significance (*$P < 0.05$). (**d** and **e**) DBC2 overexpression and shRNA-mediated MSI2 knockdown induced cell cycle arrest and apoptosis. Cell cycle progression in control, shMSI2 knockdown and DBC2-overexpressing cells stained with propidium iodide (PI) was evaluated using flow cytometry. Apoptosis was analyzed using Annexin V and PI labeling and flow cytometry. (**f**) DBC2 inhibits apoptosis in MSI2-depleted cells. MSI2-knockdown cells and DBC2-overexpressing cells were analyzed using western blot with the indicated antibodies.

protein and a promising therapeutic target. Our findings suggest that a therapeutic approach taking advantage of the antitumorigenic effects of DBC2 might be a promising strategy for treating MSI2-associated diseases such as breast cancer.

Although we identified MSI2 as a ubiquitination substrate of DBC2, we could not exclude the possibility that other substrates mediated the antitumorigenic effects of CUL3/DBC2. Therefore, we investigated if MSI2 ubiquitination and degradation are required for DBC2-mediated suppression of breast tumorigenesis. Specifically, we investigated if (1) DBC2 inhibits MSI2 function, (2) DBC2-mediated inhibition of cell proliferation is mediated by cell cycle arrest and apoptosis, (3) the antitumorigenic effects of DBC2 are dependent on MSI2 and (4) if DBC2 and MSI2 protein levels are inversely correlated in breast tissue. Consistent with the results of *in vitro* experiments, DBC2 suppressed multiple MSI2 functions, including the inhibition of *p21* mRNA translation and the promotion of cell growth, in breast cancer cells. Although previous studies have demonstrated that MSI2 knockdown enhances p21 protein and mRNA levels, the MSI2-mediated downregulation of p21 protein levels is not entirely mediated by decreased mRNA stability.[32,47,48] A previous study reported that cell cycle arrest and apoptosis are induced in MSI2 knockdown cells.[39] Consistent with these findings, apoptosis was inhibited in MSI2-knockdown cells and DBC2-overexpressing cells. In addition, colony formation and cell migration were inhibited in MSI2 knockdown cells compared with DBC2-knockdown cells. These results suggested that the relationship between DBC2 and MSI2 might be disrupted in breast cancer. Previous reports have indicated that the downregulation of DBC2 in breast cancer results from a homozygous deletion or promoter methylation,[16,19,20] and *DBC2* expression is absent in 60% of breast cancers cases.[21] The loss of DBC2 in this context might result in excessive MSI2 protein levels. Consistent with this hypothesis, a statistically significant inverse correlation between DBC2 and MSI2 protein levels was observed in both normal breast tissue and breast cancer tissue. Furthermore, DBC2 levels significantly decreased and MSI2 levels significantly increased in

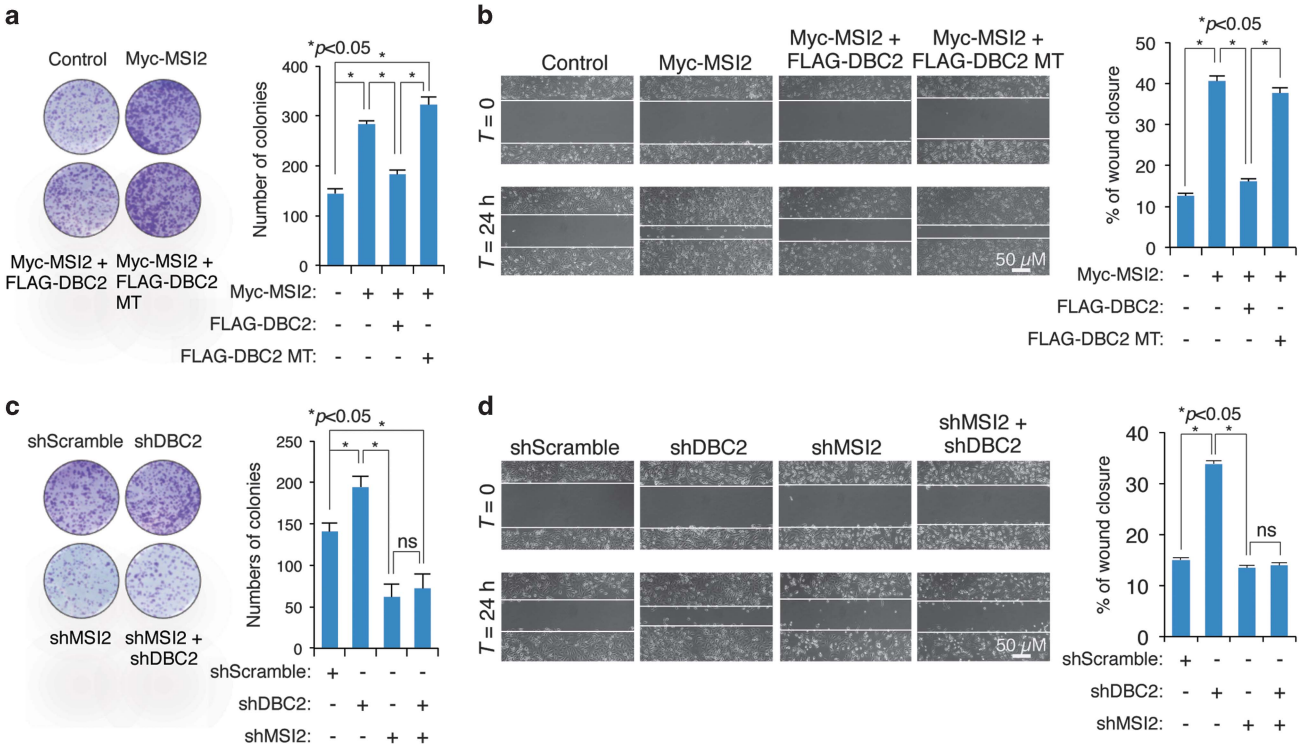


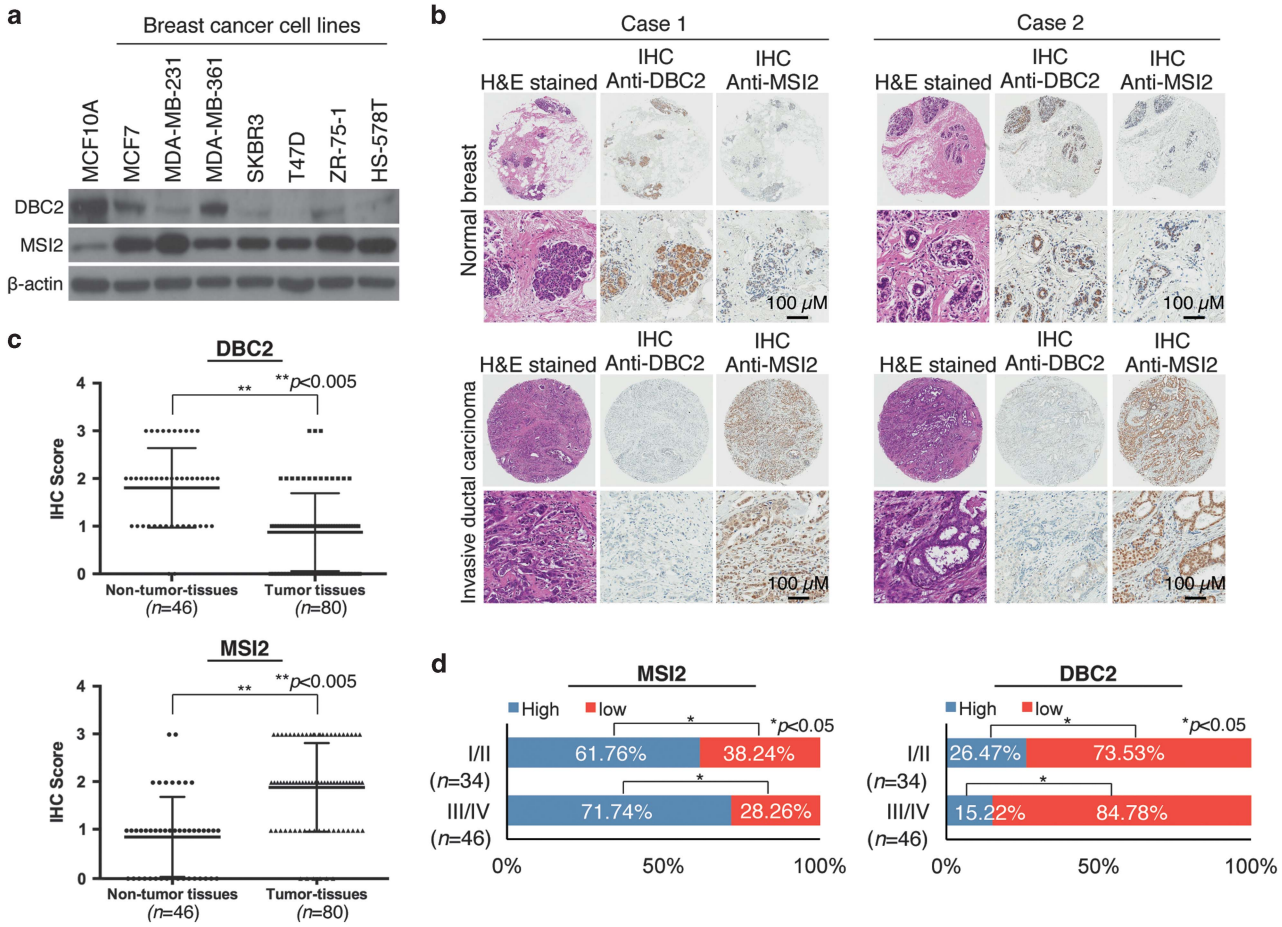
Figure 5. DBC2 inhibits MSI2-dependent clonogenic growth and cell migration. (**a**) MSI2-induced clonogenic growth is inhibited by DBC2 in a CUL3 binding-dependent manner. MDA-MB-231 cells were transfected with plasmids expressing MSI2, DBC2 and DBC2-MT. Cells were treated with G418 (500 μg/ml) for 2 weeks, stained with crystal violet and visualized. The number of colonies is expressed as the mean ± s.d. of triplicate experiments. (**b**) DBC2 inhibits cell migration via MSI2 degradation. MDA-MB-231 cells transfected with plasmids expressing DBC2, DBC2-MT and MSI2 were grown to 90% confluence. The cells were then wounded with a scratch, and wound closure was visualized and imaged. Wound closure (%) is expressed as the mean ± s.d. of triplicate experiments. (**c**) DBC2 knockdown enhances the clonogenic growth of breast cancer cells in an MSI2 dose-dependent manner. MDA-MB-231 cells were transduced with plasmids expressing shScramble (control), shDBC2 and shMSI2, and clonogenic growth was subsequently evaluated. (**d**) DBC2 knockdown-associated increase in cell migration is MSI2-dependent. The cells were transduced with plasmids expressing shScramble (control), shDBC2 and shMSI2, and wound closure was visualized and imaged. Wound closure (%) is expressed as the mean ± s.d. of triplicate experiments. *$P < 0.05$, Student's *t*-test. NS, nonsignificant.

breast cancer tissue compared with normal breast tissue. Therefore, we conclude that DBC2 exerts its antitumorigenic effects in an MSI2 ubiquitination/degradation-dependent manner.

The two MSI homologs (MSI1 and MSI2) share a common RNA recognition motif, but they differ in the length of their 3' termini.[49] We found that MSI2 is a specific substrate for the DBC2-dependent E3 ligase; however, an inverse correlation between MSI1 and DBC2 protein levels was not observed, indicating that the antitumorigenic effects of DBC2 in breast cancer are specifically dependent on MSI2. Katz *et al.*[40] analyzed RNA sequencing data from the Cancer Genome Atlas and, interestingly, found that *MSI2* was upregulated in at least 50% of breast cancer cases, whereas *MSI1* expression exhibited a roughly bimodal distribution. Although we made the interesting clinicopathological observation that MSI2 staining intensity was associated with HER2-negative status in breast cancer, Katz *et al.*[40] found that MSI1 and MSI2 expression were associated with HER2-positive breast cancer cells. Another group reported that methylation-mediated downregulation of MSI1 was associated with HER2-positive status.[50] These data indicate that, although MSI2 expression increased in breast cancer cells compared with the normal control tissues, the relationship between *MSI2* mRNA and protein expression in breast cancer remains unclear and requires further investigation.

The observation that DBC2 protein levels are downregulated in cancer cells compared with normal tissues is consistent with its role as a tumor suppressor.[16,18,20,26] A previous study reported that DBC2 promoter methylation is associated with HER2-positive status in breast cancer tissues.[19] However, we found that low DBC2 levels correlated with HER2-negative status, suggesting that promoter methylation does not have an important role in DBC2 protein levels in breast cancer cells. Rather, the post-translational ubiquitination of DBC2 by the CUL3 ubiquitin ligase might have a more important role, as DBC2 protein levels increased in cells treated with a proteasome inhibitor. Similarly, a previous study demonstrated that DNA methylation was not associated with the levels of proteins encoded by genes associated with cancer invasion and metastasis (for example ‚*ADAM23* (ADAM metallopeptidase domain 23), *ESR1* (estrogen receptor 1), *PGR B* (progesterone receptor isoform B), *CDH1* (cadherin 1 type 1 E-cadherin), *RASSF1A* (Ras association domain family member 1 isoform A), *SYK* (spleen tyrosine kinase), *TIMP3* (TIMP metallopeptidase inhibitor 3), *BRMS1* (breast cancer metastasis suppressor 1) and *SOCS1* (suppressor of cytokine signaling 1)) in breast cancer[51] or the DNA repair gene *MGMT* (O6-methylguanine-DNA methyltransferase) in ovarian cancer.[52]

Our study provides important insights into the tumor suppressor role of DBC2. We demonstrated that a DBC2-E3 ubiquitin ligase complex mediates MSI2 ubiquitination and degradation. In addition, our finding that DBC2 and MSI2 protein levels are inversely correlated in breast cancer cells and tissues might have clinicopathological implications. Further investigation into the relationship between DBC2 and MSI2 and the role of these proteins in the pathophysiological features of cancer, such as



e

Co-expression of DBC2 and MSI2 in breast cancer tissues

MSI2 expression	*n*	DBC2 expression			*p*-value	Spearman (*r*)
		-	+	++		
Breast cancer	80				0.018	-0.263
-		1	4	5		
+		6	9	1		
++		30	14	10		

Figure 6. Inverse correlation between DBC2 and MSI2 protein levels in breast cancer cell lines, normal breast tissues and breast cancer tissues. (**a**) DBC2 and MSI2 protein levels in breast cancer cell lines were evaluated using western blot assays. (**b**) DBC2 and MSI2 protein levels in normal breast tissue and breast cancer tissue. Immunohistochemistry (IHC) analysis of a breast cancer tissue microarray was conducted using antibodies against and DBC2 and MSI2. H&E, hematoxylin and eosin staining. (**c**) Inverse correlation between the staining intensity of DBC2 and MSI2 in normal breast tissue and breast cancer tissue. The staining intensity was assigned a score from 0 to 3 (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). The data are represented as the mean immunoreactive score ± s.d. $^{**}P < 0.005$ compared with normal breast tissue, using two-tailed Student's *t*-test. (**d**) The IHC staining intensity of DBC2 and MSI2 according to cancer stage; 0 and 1 were defined as low score, and 2 and 3 were defined as high scores. $^{*}P < 0.05$ compared with high staining intensity, usingtwo-tailed Student's *t*-test. (**e**) DBC2 protein levels are inversely correlated with MSI2 protein levels in breast cancer tissues, IHC score 0, (+) IHC score 1, (++) IHC scores 2 and 3. Spearman's ρ correlation coefficient between MSI2 and DBC2 ($P = 0.018$, $r = -0.263$).

metastasis and drug resistance, will advance our understanding of breast cancer development and recurrence.

MATERIALS AND METHODS

Plasmids, cell culture and reagents

Human *DBC2* and *MSI2* cDNAs were cloned into various expression plasmids (pcDNA3.1-Myc/His, pCMV-FLAG, pGEX, pEGFPC and pDsRed). We generated plasmids expressing DBC2 with the Y284D mutation (DBC2-MT) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA) according to the manufacturer's instructions. The human influenza hemagglutinin-tagged ubiquitin expression vector pMT123 was kindly provided by Dr Dirk Bohmann (University of Rochester, Rochester, NY, USA). Hemagglutinin-tagged ubiquitin mutant constructs (HA-Ub-K48R and HA-Ub-K63R) were gifts from Dr Zhijian Chen (University of Texas Southwestern Medical Center, Dallas, TX USA). The CUL3 and ROC1 expression vectors (V5-ROC1 and FLAG-CUL3) were kindly provided by Dr Xiong (University of North Carolina Chapel Hill, Chapel Hill, NC, USA). MDA-MB-231 and MCF7 cells (American Type Culture Collection, Manassas,

Table 1. Clinicopathological features of TMA stratified according to DBC2 and MSI2 expression

	No of cases	*Immunohistochemical staining,* n *(%)*					
		MSI2			*DBC2*		
		High	*Low*	**P-value*	*High*	*Low*	**P-value*
Age (years)				0.339			0.152
< 50	49	31 (63.2)	18 (36.7)		7 (14.2)	42 (85.7)	
> 50	31	23 (74.1)	8 (25.8)		9 (29)	22 (70.9)	
Tumor size (cm)				1.000			0.102
2–5	60	40 (66.6)	20 (33.3)		9 (15)	51 (85)	
> 5	20	14 (70.0)	6 (30.0)		7 (35.0)	13 (65)	
Tumor grade				0.469			0.263
I/II	34	21 (61.7)	13 (38.2)		9 (26.4)	25 (73.5)	
III/IV	46	33 (71.7)	13 (28.2)		7 (15.2)	39 (84.7)	
Endocrine response (ER/PR)				0.472			0.409
Positive	33	24 (72.7)	9 (27.2)		5 (15.1)	28 (84.8)	
Negative	47	30 (63.8)	17 (36.1)		11 (23.4)	36 (76.5)	
HER2				0.033*			0.012*
Positive	23	11 (47.8)	12 (52.1)		9 (39.1)	14 (60.8)	
Negative	57	43 (75.4)	14 (24.5)		7 (12.2)	50 (87.7)	

Abberviations: DBC2, deleted in breast cancer 2; ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; IHC, immunohistochemistry; MSI2, Musashi-2; PR, progesterone receptor; TMA, tissue microarray. Statistical analysis were performed by two-tailed Fisher's exact test, $*P < 0.05$ was considered significant. Low indicated IHC score 0, 1+. High indicated IHC score 2, 3+.

VA, USA) were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum. MG132 and cycloheximide were obtained from Sigma-Aldrich (St Louis, MO, USA).

Lentiviral-mediated RNA interference

MDA-MB-231 and MCF7 cells were transfected with plasmids using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The lentiviral vectors expressing shRNA *DBC2* and *MSI2* were obtained from Sigma-Aldrich. MSI2-knockdown cells were generated by transfecting cells with the shMSI2 plasmid. To generate shDBC2 lentiviral particles, 293 T cells were co-transfected with the vesicular stomatitis virus-G and pCMV-dR 8.2 plasmids in the presence of 8 μg/ml polybrene, and the viral particles were harvested 72 h later. The infected cells were selected using 1 μg/ml puromycin.

Western blotting and confocal fluorescence microscopy

The expression levels and cellular localizations of proteins after following transfection were determined by western blotting and confocal fluorescence microscopy, as described previously.[27] Detailed procedures are described in Supplementary Materials and Methods.

In vitro binding and immunoprecipitation assays

The *in vitro* binding and immunoprecipitation assays were conducted as described previously.[27] Detailed procedures are described in Supplementary Materials and Methods.

Cellular ubiquitination assay

The cellular ubiquitination assays were conducted as described previously.[27] Briefly, the immunoprecipitates were obtained by incubating cell lysates with anti-MSI2 and A/G Plus Agarose beads. The ubiquitinated His-tagged MSI2 was immunoprecipitated using Ni-NTA beads (Qiagen, Valencia, CA, USA) and visualized using immunoblotting assays with anti-HA.

Cell growth, proliferation, wound-healing and clonogenic assays

Cellular effects of DBC2 were analyzed by cell growth, proliferation, migration and clonogenic assays, as described previously. Detailed procedures are described in Supplementary Materials and methods.

Cell cycle and apoptosis assays

To analyze cell cycle dynamics, the cells were treated with 70% methanol and fixed overnight at 4 °C. The cell pellet was incubated with propidium iodide (50 μg/ml) supplemented with RNase A (100 μg/ml). To evaluate apoptosis, the cells were suspended in 100 μl of binding buffer and incubated with fluorescein isothiocyanate-conjugated Annexin V (Sigma-Aldrich). Cell cycle dynamics and apoptosis were evaluated using a BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA), and the data were analyzed using the Cell Quest program (BD Biosciences).

Tissue immunohistochemistry

The human breast cancer tissue array (110 cases; BC08111c), breast cancer normal tissue array (60 cases; CBB3) and breast cancer-metastasis-normal tissue array (55 cases; CBA4) were purchased from US Biomax Inc. (Rockville, MD, USA) and Super BioChips (Seoul, South Korea). DBC2, MSI1 and MSI2 protein expression in breast cancer tissues and matched normal breast tissues was evaluated using a standard immunohistochemistry protocol. The slides were incubated with antibodies against DBC2 (DB084; Delta Biolabs, Gilroy, CA, USA), MSI1 (ab52865; Abcam, Cambridge, UK) and MSI2 (ab156770, Abcam) overnight at 4 °C. Then, the cells were washed and subsequently incubated with a universal secondary antibody for 1 h at room temperature. DBC2- and MSI2-positive cells were quantified by a pathologist. The staining intensity was scored as follows: 0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong staining.

Statistical analysis

All data are expressed as mean ± s.d. of three independent experiments. The statistical analyses were conducted using the SPSS 10.0.5 software for Windows (SPSS Inc., Chicago, IL, USA). The relationship between MSI2 and DBC2 levels was analyzed using a two-tailed Fisher's exact test. Spearman's rank correlation coefficient analysis was used to examine the association

between MSI2 and DBC2 levels. Normally distributed data were evaluated using a two-tailed Student's *t*-test as indicated in the figure legends. Statistical significance is considered $P < 0.05$. There is an estimate of variation within each group, and the variance between groups is similar. All of the results from the study were included for statistical analysis with no randomization or blinding involved. No data points were excluded.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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