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MTBP is Over-expressed in Triple Negative Breast Cancer and Contributes to its Growth and Survival

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Abstract

Triple negative breast cancer (TNBC) is a clinically aggressive subtype of breast cancer commonly resistant to therapeutics that have been successful in increasing survival in ER+ and HER2+ breast cancer patients. As such, identifying factors that contribute to poor patient outcomes and mediate the growth and survival of TNBC cells remain important areas of investigation. *MTBP* (MDM2 Binding Protein), a gene linked to cellular proliferation and a transcriptional target of the MYC oncogene, is over-expressed in human malignancies, yet its contribution to cancer remains unresolved. Evaluation of mRNA expression and copy number variation data from The Cancer Genome Atlas (TCGA) revealed *MTBP* is commonly over-expressed in breast cancer and 19% show amplification of *MTBP*. Increased transcript or gene amplification of *MTBP* significantly correlated with reduced breast cancer patient survival. Further analysis revealed that while *MTBP* mRNA is over-expressed in both ER+ and HER2+ breast cancers, its expression is highest in TNBC. MTBP mRNA and protein levels were also significantly elevated in a panel of human TNBC cell lines. Knockdown of *MTBP* in TNBC model systems induced apoptosis and significantly reduced TNBC cell growth and soft agar colony formation, which was rescued by expression of shRNA-resistant *Mtbp*. Notably, inducible knockdown of *MTBP* expression significantly impaired TNBC tumor growth, *in vivo*, including in established tumors. Thus, these data emphasize MTBP is important for the growth and survival of TNBC and warrants further investigation as a potential novel therapeutic target. Implications: MTBP significantly contributes to breast cancer survival and is a potential novel therapeutic target in TNBC.

Keywords

MTBP; apoptosis; proliferation; growth; breast cancer

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Introduction

The use of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) as biomarkers is standard practice in the clinical management of breast cancer. Their expression directs the use of targeted therapeutics such as tamoxifen and trastuzumab that have dramatically improved patient survival. Unfortunately, such improvements in clinical outcomes have not been realized in the management of triple negative breast cancer (TNBC), a subset of breast cancers lacking *HER2* amplification and expression of ER and PR (1). TNBC comprises 10–20% of breast cancer cases and is more commonly identified in younger women and those with African American or Hispanic heritage (2). It is clinically aggressive, correlating with an increased risk of distant recurrence within three years following treatment and a significant decrease in overall patient survival, compared to receptor positive cases (3, 4). While there has been some success in exploiting novel molecular targets, such as PARP inhibitors in BRCA1 mutant tumors with errors in DNA break repair (5, 6), these cases are isolated and applicable to only select TNBCs. Other targets such as mTOR, Src, and HER1 tested in phase II clinical trails have shown only minimal success (7–9). Thus, there is a need to identify and test the therapeutic efficacy of novel molecular targets in TNBC.

The Mdm2 (Two) Binding Protein (MTBP) was first identified as a potential tumor suppressor that binds Mdm2, a negative regulator of p53 (10). However, subsequent genetic studies indicated it functions independent of Mdm2, and instead, contributes to tumor development induced by the Myc oncogene (11–13). Recently, MTBP has been implicated in regulating proliferation and cell cycle progression (12, 14, 15). *MTBP* is a transcriptional target of MYC, and its protein expression increased in response to pro-proliferative signals and decreased upon growth factor withdrawal (12). In mouse models, *Mtbp* heterozygosity led to reduced levels of Mtbp protein and this inhibited Myc-induced B cell proliferation, resulting in a significant delay in lymphoma development (12). Furthermore, siRNA-mediated knockdown of *MTBP* was reported to delay cell cycle progression through the S and G2/M phases of the cell cycle (14, 15). Therefore, MTBP appears to contribute to the development and possibly the maintenance of tumors through regulation of proliferation, but further investigation is needed.

Here, we report *MTBP* is overexpressed and amplified in breast cancer, correlating with decreased patient survival. Notably, *MTBP* mRNA expression was highest in TNBC. shRNA-mediated knockdown of *MTBP* in human TNBC cell lines inhibited their expansion and induced apoptosis, *in vitro*, as well as significantly reduced tumor growth, *in vivo*. Our data reveal MTBP significantly contributes to breast cancer and is a potential novel therapeutic target in the treatment of TNBC.

Material and Methods

Patient Data

Patient survival and gene expression data for 844 breast cancers was accessed from The Cancer Genome Atlas public data portal (https://tcga-data.nci.nih.gov/tcga/) January–April 2013. For Kaplan-Meier survival curves, normalized RNA-Seq data (version 2, level 3) was

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used as gene expression values and the median was used to classify samples into high and low expression groups. Log-rank tests were used to compare survival between groups. Box and whisker plots (box represents first and third quartiles, thick band is median value, and bars extend to +/− 1.58 the interquartile range divided by the square root of the number of samples), were applied to describe *MTBP* gene expression values. Groups were compared using a Wilcoxon rank sum test. Gene copy number alteration (CNA) and survival data for 913 breast cancers was obtained from the cBioPortal for Cancer Genomics (http://www.cbiopor2tal.org/public-portal/) May 2013.

Cell Culture, vectors, transfection, and infection

The human cell lines MDA-MD-231, HCC1806, and HCC1937 were cultured as described by the American Type Culture Collection (Manassas, VA) and were provided by Dr. Jennifer Pietenpol. Cells were transfected with Effectene (Qiagen, Germantown, MD) or were infected with retroviruses, as previously described (16). *MTBP* shRNA 19mer sequences (shRNA1 GGAGAGTGTTCTAGCTATT or shRNA2 GAAACACAGTATTACCGAG) and non-targeting control (GACTTACGAGATCAGAAAG) were used in pSuper constitutive expression constructs (Oligoengine, Seattle, WA) and were adapted to the dox-inducible system (pInducer) generously provided by Dr. Thomas Westbrook (17) using the RNAi central shRNA retriever (http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA).

Proliferation, cell cycle, apoptosis and transformation assays

For measurement of proliferation, 1,000 to 5,000 cells were plated in triplicate and MTT assays were performed as per manufacture's protocol (Sigma, St. Louis, MO). Cell cycle (Dean-Jett-Fox analysis) and apoptosis (subG1 DNA content) were evaluated with FlowJo software (TreeStar Inc., Ashland, OR) following DNA staining with propidium iodide and flow cytometry. Apoptosis was also evaluated by flow cytometry analysis of Annexin V-APC binding (Life Technologies, Pittsburgh, PA) and Caspase 3 cleavage by Western blot (see below). Cell viability was assessed by Trypan Blue Dye exclusion. Soft agar assays were performed as previously described (18). For dox-inducible shRNA experiments, 0.5–1 μg/ml of dox was added to the cultures.

Mice

Female athymic nude mice (5–6 week old; Harlan, Indianapolis, IN) were injected subcutaneously in the flank with 3×10^6 HCC1806 cells. Mice were housed with drinking water supplemented with 5% sucrose with or without 2 g/L of dox (Research Products International Corp., Prospect, IL) that was changed every 48 hours beginning on the day of injection or 10 days later. Tumor volume was calculated from measurements with electronic calipers. At time of sacrifice, mice were photographed, and tumors were extracted, photographed and weighed. A piece of each tumor was frozen for Western blot analysis. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee and followed all federal and state rules and regulations.

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Western blotting and quantitative real-time PCR analysis

For Western blotting, cells or tumors that were infected or transiently transfected (see above) were harvested after 48 hours or at indicated times and were lysed as previously reported (16, 18). Equal amounts of protein were resolved by SDS-PAGE and Western blotted using antibodies specific for MTBP (B5, Santa Cruz Biotechnology, Santa Cruz, CA), cleaved Caspase 3 (D175, Cell Signaling Technology, Danvers, MA), MYC (C33, Santa Cruz Biotechnology) and β-ACTIN (AC15, Sigma, St. Louis, MO). To evaluate mRNA expression, total RNA was isolated, cDNA was generated, and qRT-PCR for *MTBP* and *β-ACTIN* levels was performed as previously described (12). mRNA data are relative to *β-ACTIN* levels.

Statistical evaluation

Wilcoxon rank sum test (Figs 1A and 2A), log rank tests (Figs 1B–C), student's t-test (Figs 2–6), and Cox regression analysis were used to compare data. Error bars represent standard deviation (Figs 2–5) or standard error of the mean (Fig 6).

Results

MTBP is overexpressed in human breast cancer and correlates with decreased patient survival and triple negative status

We previously detected MTBP/Mtbp overexpression in human lymphoma cell lines and primary murine lymphomas (12). *MTBP* was also reported amplified in colorectal carcinoma and multiple myeloma (19, 20), as well as several human cancer cell lines (21), suggesting its overexpression contributes to human cancers. To specifically evaluate *MTBP* expression in human breast cancer, mRNA expression and patient survival data for 844 breast cancers from The Cancer Genome Atlas (TCGA) were assessed. *MTBP* was significantly elevated in breast cancer samples compared to normal breast tissue ($p=2.2\times10^{-16}$; Fig 1A). When cancers were separated by their *MTBP* expression, those patients whose breast cancers had elevated *MTBP* expression exhibited reduced overall survival compared to patients whose breast cancers had lower levels of *MTBP* (p=0.0337; Fig 1B). A Cox regression analysis also showed that increased *MTBP* levels are significantly linked with worse patient survival (p=0.033). Moreover, *MTBP* was amplified in 19% of breast cancers, and this amplification decreased overall patient survival compared to tumors without amplified *MTBP* (p=0.01955; Fig 1C; 22, 23). These data indicate *MTBP* overexpression is common and thus, likely selected for during breast cancer formation and/or progression. The results also show that elevated levels of *MTBP* correlate with reduced breast cancer patient survival.

To assess MTBP levels in different subtypes of breast cancers, we separated the TCGA breast cancer patient samples into clinically relevant subgroups: estrogen-receptor positive (ER+), HER2 positive (HER2+), and triple negative (TN: ER−, PR−, HER2−) tumors. While *MTBP* mRNA was elevated significantly in all three subgroups compared to normal breast tissue, the triple negative breast cancers (TNBC) expressed significantly more *MTBP* than the ER+ or HER2+ subgroups (Fig 2A). This finding was supported by the observation that *MTBP* mRNA was also significantly elevated in a panel of human TNBC cell lines compared to normal human mammary epithelial cells (HMECs; Fig 2B). To determine

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whether the increased mRNA levels translated into increased protein, the levels of MTBP protein were assessed. MTBP protein levels were elevated in all of the TNBC cells (Fig 2C). In comparison, the oncogenic transcription factor MYC, which has previously been shown to positively regulate *MTBP* expression and to be elevated in aggressive breast cancers, was also elevated in these same cells (12, 24). Therefore, *MTBP* mRNA levels are the highest in patient samples of the clinically aggressive TNBC subtype, and TNBC cell lines have high levels of MTBP *mRNA* and protein.

Reducing MTBP levels inhibit TNBC cell proliferation

Considering Mtbp expression increases in response to pro-proliferative factors (12), and MTBP is highly overexpressed in TNBC, we questioned whether reducing levels of MTBP in human TNBC cells would alter their ability to proliferate. To begin to test this concept, we knocked down *MTBP* expression with two different *MTBP* shRNAs in the MDA-MB-231 and HCC1806 TNBC cell lines. While both cell lines overexpress MTBP (Fig 2B), they represent distinct subtypes of TNBC (mesenchymal-like and basal-like, respectively), and HCC1806 cells have an *MTBP* amplification (21, 25). In both cell lines, reduced MTBP expression resulted in a decrease in proliferation that correlated with the amount of MTBP protein present, where *MTBP* shRNA1 was more effective at reducing MTBP protein levels than *MTBP* shRNA2 (Fig 3A). Similarly, anchorage-independent growth in soft agar was significantly reduced for both the MDA-MB-231 and the HCC1806 cells when *MTBP* was knocked down (Fig 3B). To ensure the observed effects were due to reduced MTBP expression, shRNA resistant murine Mtbp was co-expressed with *MTBP* shRNA1. The murine Mtbp rescued the ability of MDA-MB-231 cells to form colonies in soft agar in the presence of *MTBP* shRNA1, while cells co-transfected with vector control and *MTBP* shRNA1 still showed decreased colony formation (Fig 3C). These results indicate knockdown of *MTBP* inhibits TNBC expansion and anchorage-independent growth.

To further evaluate the anti-proliferative effects of *MTBP* knockdown in TNBC cells, *MTBP* shRNA1 was adapted to a lentiviral doxycycline (dox)-inducible system (17). Using the three TNBC cell lines MDA-MB-231, HCC1806, and the basal-like HCC1937 cells that also over-express MTBP (Fig 2C), we observed that dox-induced *MTBP* shRNA1 resulted in reduced MTBP protein expression within 24 hours (Fig 4A) and significantly decreased (37–40% reduced) proliferation within 72 hours (Fig 4B) in all three lines. The steady-state levels of MYC remained unchanged (Fig 4A). Expression of the shRNA resistant murine Mtbp allowed *MTBP* shRNA1 expressing MDA-MB-231 cells to continue growing at rates analogous to that of cells treated with vehicle control (Fig 4C). Therefore, disruption of MTBP expression with constitutive or inducible shRNA caused a reduction in the ability of TNBC cells to form colonies in soft agar and to proliferate.

MTBP knockdown induces apoptosis in TNBC cells

To investigate the biological reason *MTBP* knockdown inhibited the expansion of TNBC cells, HCC1806 cells expressing the dox-inducible *MTBP* shRNA1 or the non-targeting shRNA control were cultured with or without dox for 72 hours. At this time, there were visibly fewer adherent and more floating dox-treated *MTBP* shRNA1 expressing cells compared to vehicle control treated cells and non-targeting shRNA expressing cells cultured

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with or without dox (Fig 5A). Evaluation of the cell cycle revealed no significant difference in G1, S or G2/M distribution of the cells where *MTBP* shRNA1 had been induced with dox compared to vehicle control (Fig 5B). In contrast, dox-treated *MTBP* shRNA1 expressing HCC1806 cells had an increase in the percentage of cells with sub-G1 DNA content (Fig 5B). There was also a significant decrease in viability (Fig. 5C) and an increase in Annexin V positive (Fig 5D) *MTBP* shRNA1 containing cells. Moreover, in HCC1806 cells with *MTBP* shRNA1, cleaved Caspase 3 was visible after the addition of dox compared to vehicle control or to those cells with the non-targeting shRNA control (Fig 5E). Thus, shRNA-mediated knockdown of *MTBP* in TNBC cells induced apoptosis, without detectable alterations in phases of the cell cycle.

MTBP loss inhibits TNBC growth, *in vivo*

To evaluate whether *MTBP* knockdown would alter TNBC growth *in vivo*, HCC1806 cells expressing dox-inducible *MTBP* shRNA1 were subcutaneously injected into the flanks of athymic nude mice. Their drinking water was supplemented with or without dox. By day 7, tumors in mice receiving dox to induce *MTBP* shRNA1 showed a statistically significant decrease in volume compared to tumors in control mice not receiving dox (24 mm^3 vs 43 mm^3; $p<0.0001$), and this difference continued to increase through the duration of the experiment (Fig 6A). At the time of sacrifice (day 21), tumors that expressed *MTBP* shRNA1 due to dox exposure were smaller in volume and weighed significantly less than the tumors from control mice (Fig 6A–C). There was a 70% reduction in tumor volume in the mice expressing *MTBP* shRNA1 compared to controls. These tumors showed reduced levels of MTBP protein, verifying *MTBP* shRNA1 expression persisted over the course of the experiment (Fig 6C). MYC protein levels were similar in all tumors (Fig. 6C).

We also tested whether established TNBC tumors would be affected by knockdown of *MTBP*. Specifically, HCC1806 cells expressing the dox-inducible *MTBP* shRNA1 were injected into the flanks of nude mice at the same time as cells for the experiment described above and were allowed to grow. After 10 days when the tumors averaged 100–150 mm^3, these mice were given dox to induce *MTBP* shRNA1 expression. Within 72 hours these tumors were significantly smaller than tumors that were not exposed to dox (188 mm^3 vs 350 mm^3; $p=0.0057$); yet, tumors did not completely disappear (Fig 6A). Instead, after the initial decrease in tumor size, a significant reduction in the rate of tumor growth was observed that was analogous to the rate of tumor growth for the mice that received dox on day one. At sacrifice (day 21), the tumors from the mice that received dox to induced *MTBP* shRNA1 after tumors had established were smaller and weighed significantly less than the tumors from mice that never received dox (Fig 6B–C). The tumors were similar in size and weight to the tumors from mice that been exposed to dox for the entire experiment. These data collectively indicate that targeting *MTBP*, reducing its expression, significantly limits the growth of TNBC cells *in vivo*, including established TNBC tumors.

Discussion

Studies have linked MTBP to cancer (11, 12, 19, 20, 26), but little was known about MTBP in established cancer cells, particularly how it influenced proliferation, cellular survival, and

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patient outcomes. Here, we show *MTBP* is overexpressed in human breast cancer, and this correlated with significantly decreased patient survival. Notably, among the different breast cancer subtypes, we determined *MTBP* expression was highest in the TNBC subtype, which lacks targeted therapies and is known for being clinically aggressive (1, 3, 4). Experiments also revealed that reducing *MTBP* expression in human TNBC cell lines with shRNA significantly inhibited cell expansion by inducing apoptosis. The growth inhibitory effects of *MTBP* knockdown in TNBC cells were also observed *in vivo*, in xenografts and importantly, in established TNBC tumors. Therefore, this study identifies MTBP as an important indicator of poor breast cancer patient prognosis and triple negative status as well as being critical for the growth and survival of TNBC cells. The results of this study support further investigation into MTBP as a novel therapeutic target in TNBC.

This study reveals that MTBP overexpression contributes in a significant way to human breast cancer and increases understanding of MTBP in cancer. Specifically, we previously, reported MTBP is overexpressed in human and murine B cell lymphomas (12). Others have shown the region of the genome encoding *MTBP* is amplified in colorectal cancer and multiple myeloma (19, 20). Similarly, evaluation of copy number variation data from The Cancer Genome Atlas (TCGA) indicate *MTBP* is amplified in many types of human cancer (13, 22, 23). For breast cancer, we determined *MTBP* amplification occurred in 19% of the tumors, and this significantly correlated with decreased patient survival. Since most breast cancer deaths are associated with metastasis, the current analysis suggests *MTBP* overexpression is a potential novel indicator of aggressive breast cancers with increased metastatic potential that are more likely to result in patient death. However, this concept conflicts with experimental data that indicate decreased MTBP expression increases cell migration, invasion, and metastasis (11, 26). It is possible that both are correct if, as has been shown for MYC (27), MTBP is temporarily downregulated when cancer cells move and is then upregulated after cancer cells seed metastatic sites and begin to proliferate again. However, there is also one report showing that decreased *MTBP* expression in a narrow subset of head and neck cancer correlated with reduced survival (28). Thus, although MTBP could have a tissue-specific or cancer cell mutation-specific function, much of the data suggest that in multiple hematopoietic and non-hematopoietic human cancers, including breast cancer, MTBP overexpression is selected for and contributes to cancer development and progression. The current study links *MTBP* overexpression in breast cancer to advanced disease and poor patient prognosis.

Data from multiple groups, including our own, suggest MTBP has a critical function in proliferation, and that this significantly contributes to tumor development (12, 13, 15). Specifically, we previously determined that an Mtbp haploinsufficiency suppressed proliferation mediated by the Myc oncogene, significantly inhibiting the ability of Myc to induce B cell lymphoma development (12). Moreover, *MTBP* mRNA and protein expression increased in response to oncogene expression (MYC and E2F1) or growth factor exposure, and *MTBP* was shown to be a transcriptional target of MYC (12). Others have indicated MTBP contributes to cell cycle progression by linking MTBP to DNA replication origins and mitotic progression (14, 15). Additionally, we recently determined that elevated levels of MTBP resulted in enhanced cellular proliferation and transformation, *in vitro* and *in vivo*

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(13). Here we show *MTBP* is overexpressed in breast cancers and its expression is the highest in the TNBC subtype. TNBCs are reported to have a higher proliferative index when compared to receptor positive high-grade invasive carcinomas (29). In addition, elevated MYC transcriptional activity, which is correlated with decreased breast cancer patient survival, is linked to increased proliferation in breast cancer, and the TNBC subtype has the highest MYC transcriptional activity (24). We recently determined patients with breast cancers that express high levels of both *MYC* and *MTBP* have a worse prognosis than those with just high *MYC* expression (13), suggesting cooperation between MYC and MTBP overexpression in breast cancer. Therefore, MTBP appears to be a pro-proliferative factor where its overexpression supports the increased proliferative capacity of cancer cells, which is associated with poor patient survival in many human cancers (29–35).

Deletion of *Mtbp* is embryonic lethal in mice, indicating it has an indispensable function in development (11). Here we show that MTBP also has an essential function in breast cancer cell survival. Knockdown of *MTBP* in human TNBC cell lines using constitutive or inducible *MTBP* shRNA severely limited TNBC growth, *in vitro* and *in vivo*, due to induction of apoptosis. This is similar to the oncogene addiction mediated cell death observed when an oncogene, such as MYC, is knocked down in cancer cells (36–38), suggesting TNBC cancer cells can become reliant on MTBP for their continued growth and survival. Support of this concept was reported when knockdown of *MTBP* with siRNA in HeLa cells delayed DNA replication or mitosis and also led to cell death (14, 15). Although we did not detect cell cycle changes with *MTBP* knockdown in TNBC cells, as was reported for HeLa cells (14, 15), we did observe significant apoptosis resulting in reduced TNBC cell survival. The apoptosis that occurred upon *MTBP* knockdown resulted in a reduction in the ability of the TNBC cells to grow in soft agar and *in vivo* in mice. Notably, utilizing inducible *MTBP* shRNA revealed that, *in vivo*, established breast cancers rely on MTBP for their continued growth. Therefore, our data identify MTBP as a protein TNBC cells need to survive and grow. Thus, MTBP is a potential novel therapeutic target in TNBC warranting further investigation. Moreover, additional studies are needed to examine whether MTBP has a similar essential function in other human malignancies, including receptor positive subtypes of breast cancers.

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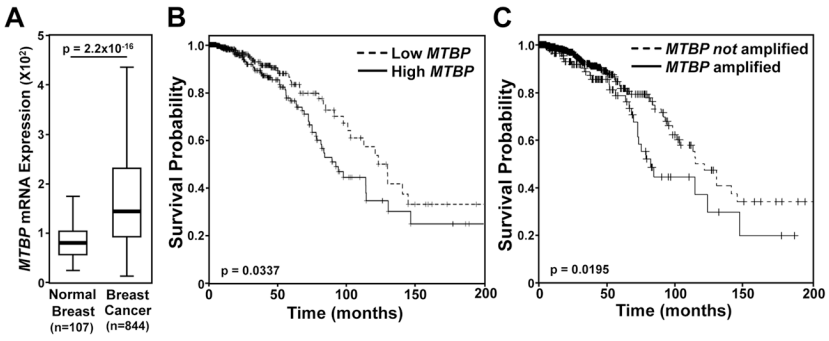

Figure 1. MTBP overexpression in breast cancer decreases survival

(A, B) RNA-Seq mRNA expression data for normal and breast cancer tissue from TCGA database. (A) Box and whisker plot of relative *MTBP* mRNA expression in normal and cancerous breast tissue with "n" indicating the number of samples. (B) Kaplan Meier survival curves for breast cancer patients divided by the median value into low and high *MTBP* mRNA expression (n=421/group). (C) Kaplan Meier survival curves of *MTBP* gene copy number in breast cancer samples with (n=171) and without (n=742) amplified *MTBP* from the cBio Portal for Cancer Genomics (22, 23). p-values calculated using a Wilcoxon rank sum test for A and a log-rank test for B–C.

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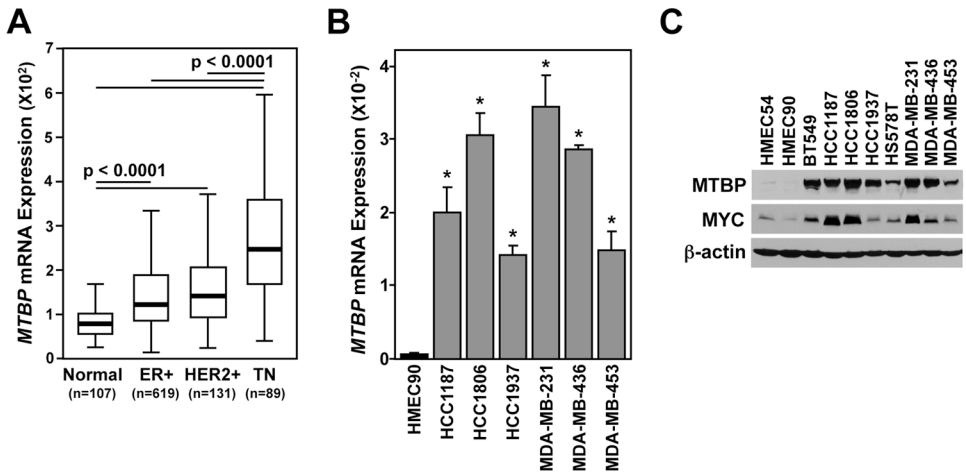

Figure 2. MTBP overexpressed in triple negative breast cancer

(A) Box and whisker plot of relative *MTBP* mRNA expression from TCGA RNA-Seq mRNA expression data, representing normal breast tissue and breast cancers divided into estrogen receptor positive (ER+), human epithelial growth factor receptor 2 positive (HER2+), or triple negative (TN: ER−, PR−, HER2−). The “n” indicates the number of samples. (B) Expression of *MTBP* mRNA measured by qRT-PCR in TNBC cell lines and normal human mammary epithelial cells (HMECs); *p≤0.0007. (C) Western blots of whole cell lysates of TNBC cell lines and HMECs for the proteins indicated.

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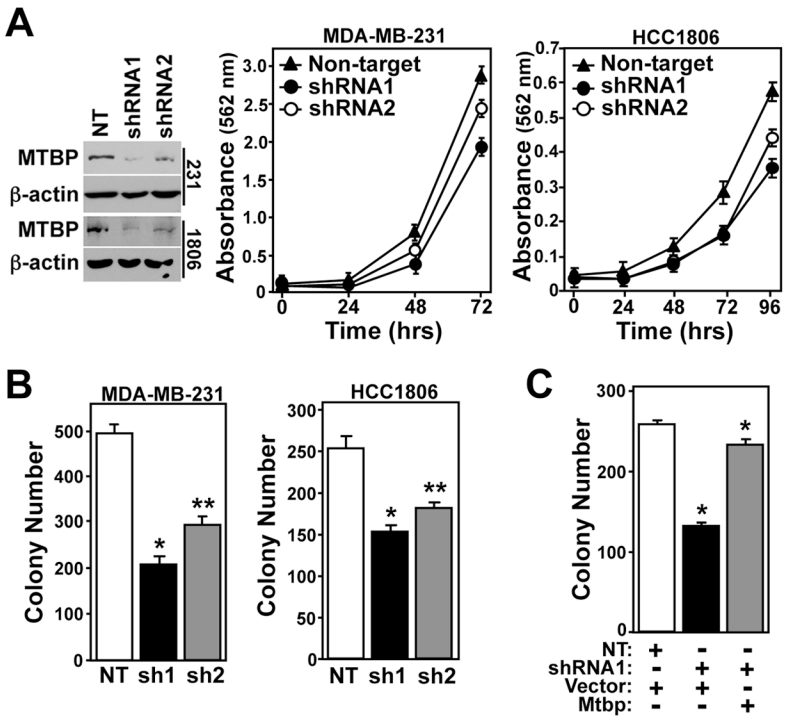

Figure 3. *MTBP* knockdown inhibits proliferation and colony growth

MDA-MB-231 or HCC1806 cells were transfected with constitutively expressing *MTBP* shRNA1, shRNA2, or non-targeting (NT) control shRNA vectors. (A) Whole cell lysates were Western blotted, and cells were subjected to MTT assays at 24 hour intervals (for MDA-MB-231 at 48–72 hrs $p<0.001$ for NT vs. shRNA1 or shRNA2 and $p<0.01$ for shRNA1 vs. shRNA 2; for HCC1806 $p<0.01$ NT vs. shRNA1 or shRNA2 at 24–96 hrs and $p=0.0013$ at 96 hrs). (B) Cells were subjected to soft agar colony formation assay (MDA-MB-231 *$p=0.0004$, **$p=0.0021$ and HCC1806 *$p=0.0036$, **$p=0.0136$ for NT vs. shRNA1 or shRNA2, respectively). (C) MDA-MB-231 cells transiently transfected with *MTBP* shRNA1 or non-targeting shRNA and shRNA1-resistant murine Mtbp or vector control were subjected to soft agar colony formation assay (*$p<0.0001$ for NT vs. shRNA1 and shRNA1 vs. shRNA1 + Mtbp).

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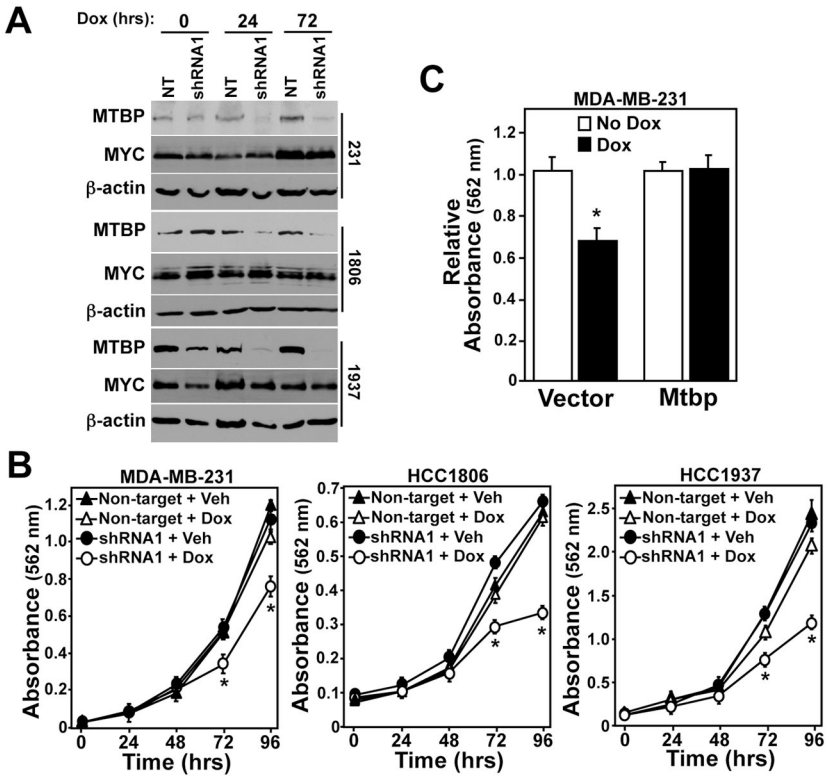

Figure 4. Inducible *MTBP* shRNA inhibits cell expansion

MDA-MB-231, HCC1806, and HCC1937 cells expressing doxycycline (dox) inducible *MTBP* shRNA1 or non-targeting (NT) shRNA control. (A) Western blots of whole cell lysates not exposed to dox or at intervals after the addition of dox. (B) Cells were cultured with dox or vehicle (veh) control and proliferation was monitored by MTT assay at 24 hr intervals (*$p<0.01$ shRNA1 + veh vs. shRNA1 + dox). (C) MTT assay of MDA-MB-231 cells expressing dox inducible *MTBP* shRNA1 and constitutively expressing shRNA1-resistant murine Mtbp or empty vector control after 72 hours in the presence of dox or vehicle control (no dox; *$p=0.0027$).

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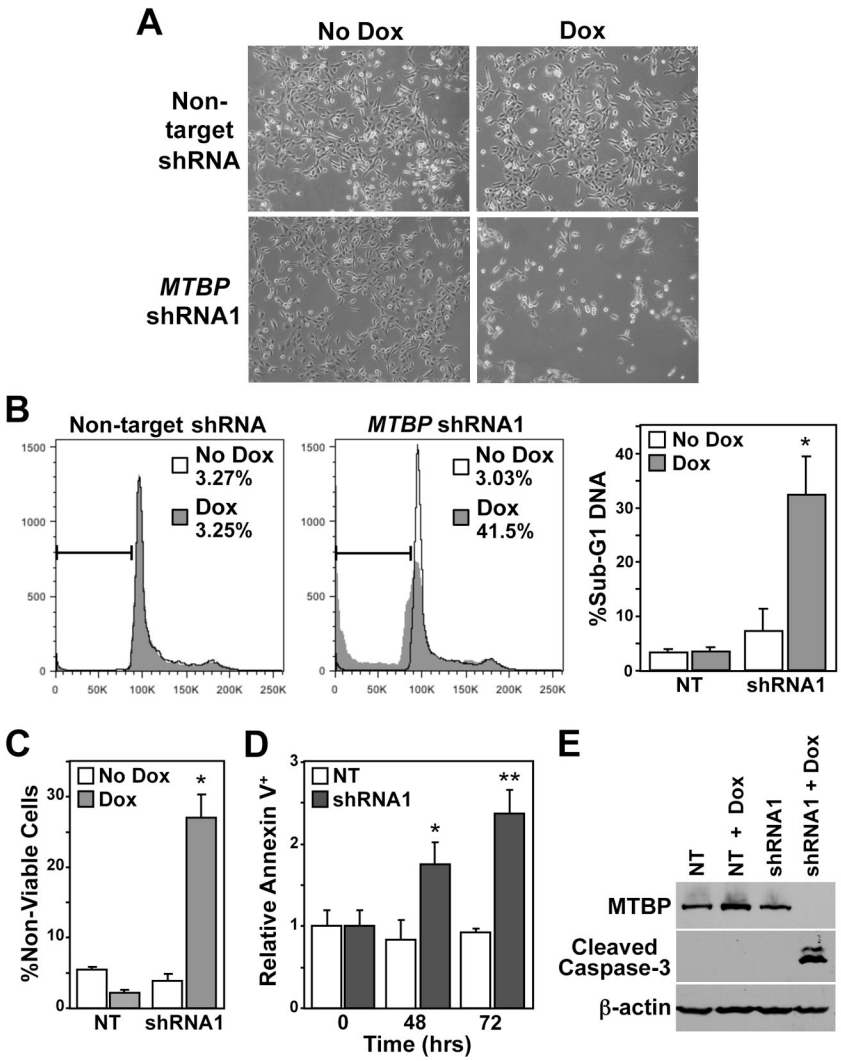

Figure 5. MTBP knockdown induces apoptosis

HCC1806 cells expressing doxycycline (dox) inducible *MTBP* shRNA1 or non-targeting (NT) shRNA control incubated with dox or vehicle control (no dox) for 72 hours. (A) Representative light microscopy images. (B) The percentage of sub-G1 DNA content, following staining of DNA with propidium iodide, was measured by flow cytometry. Representative histograms with the percentage of sub-G1 DNA indicated (left); the mean of the data obtained shown on the right (*p=0.0008). (C) Cell viability was determined by Trypan Blue Dye (*p≤0.0003 *MTBP* shRNA dox vs. *MTBP* shRNA no dox or NT dox). (D) The percentage of Annexin V positive cells relative to samples at time 0 was measured by flow cytometry (*p=0.034, **p=0.0069). (E) Western blots for the indicated proteins were performed on whole cell lysates.

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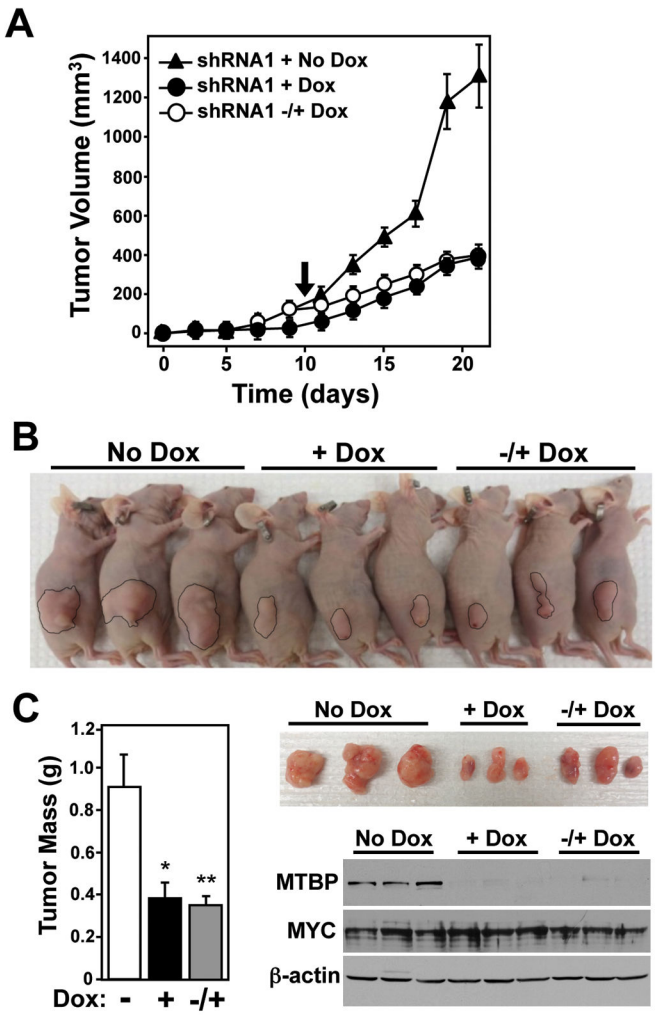

Figure 6. Knockdown of *MTBP* inhibits breast cancer growth *in vivo*.

(A) HCC1806 cells were injected subcutaneously into the flanks of nude mice (n=10/group) on day 0. Mice received drinking water with (+Dox) or without dox (No Dox) at day 0, or water with dox starting at day 10 (−/+ Dox; indicated by arrow). Tumor volume was measured at intervals ($p<0.001$ for shRNA1 + No Dox vs. shRNA1 + Dox for days 7–21 and for shRNA1 + No Dox vs. shRNA1 −/+ Dox days 13–21). (B) On day 21, mice were sacrificed and photographed. A representative photo of all three treatment groups is shown with the tumors outlined in black. (C) Tumors were extracted, photographed and weighed (*$p=0.0058$ and **$p=0.0024$ compared to no dox). A representative photograph is shown. Protein lysates from representative tumors were western blotted for the indicated proteins.