A novel tumor suppressor ZBTB1 regulates tamoxifen resistance and aerobic glycolysis through suppressing HER2 expression in breast cancer

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Transcriptional repressor zinc finger and BTB domain containing 1 (ZBTB1) is required for DNA repair. Because DNA repair defects often underlie genome instability and tumorigenesis, we determined to study the role of ZBTB1 in cancer. In this study, we found that ZBTB1 is down-regulated in breast cancer and this down-regulation is associated with poor outcome of breast cancer patients. ZBTB1 suppresses breast cancer cell proliferation and tumor growth. The majority of breast cancers are estrogen receptor (ER) positive and selective estrogen receptor modulators such as tamoxifen have been widely used in the treatment of these patients. Unfortunately, many patients develop resistance to endocrine therapy. Tamoxifen-resistant cancer cells often exhibit higher HER2 expression and an increase of glycolysis. Our data revealed that ZBTB1 plays a critical role in tamoxifen resistance in vitro and in vivo. To see if ZBTB1 regulates HER2 expression, we tested the recruitments of ZBTB1 on HER2 regulatory sequences. We observed that over-expressed ZBTB1 occupies the estrogen receptor α (ERα)-binding site of the HER2 intron in tamoxifen-resistant cells, suppressing tamoxifen-induced transcription. In an effort to identify potential microRNAs (miRNAs) regulating ZBTB1, we found that miR-23b-3p directly targets ZBTB1. MiR-23b-3p regulates HER2 expression and tamoxifen resistance via targeting ZBTB1. Finally, we found that miR-23b-3p/ZBTB1 regulates aerobic glycolysis in tamoxifen-resistant cells. Together, our data demonstrate that ZBTB1 is a tumor suppressor in breast cancer cells and that targeting the miR-23b-3p/ZBTB1 may serve as a potential therapeutic approach for the treatment of tamoxifen resistant breast cancer.

Estrogen receptor (ER) α is the key mediator of estrogen functions in the breast and plays prominent roles in breast cancer (1–3). In common with other nuclear receptors, ERs regulates target genes by recruiting transcriptional co-regulators and components of the basal transcription machinery (4–6). The ligand-bound ERα, depending on the nature of the ligand, recruits and interacts with coregulatory proteins that can either enhance (co-activators) or repress (co-repressors) its transcriptional activity. Ligand-activated ERα binds to estrogen-response elements (ERE) of target genes such as trefoil factor 1 (TFF1) and c-Myc (7–9).

Approximately 70% of all breast cancers are ERα positive at the time of diagnosis, and anti-estrogen therapies, such as tamoxifen, are very important in premenopausal women breast cancer management (10, 11). The selective estrogen receptor modulator, tamoxifen, can bind to the ER and block the interaction between estrogen and the ER (12, 13). Tamoxifen is the most prolific therapeutic drug for the treatment of ER-positive breast cancer, showing effective tumor growth inhibition and prevention of disease recurrence (14, 15). Unfortunately, not all patients with ER-positive breast cancer respond to tamoxifen. Moreover, many patients that receive tamoxifen as adjuvant therapy eventually acquire tamoxifen resistance. Therefore, studies on the mechanism of tamoxifen resistance are very important to improve the prognosis of breast cancer patients (16–19).

It has been recognized that activation of the tyrosine kinase HER2 (human epidermal growth factor receptor-2) is one of the major mechanisms contributing to the tamoxifen resistance (20). HER2 is amplified and overexpressed in 20 to 30% of invasive breast cancers and has been implicated as a major player in tamoxifen resistance (12, 21–23). Some breast tumors that exhibit tamoxifen resistance are characterized by elevated HER2 levels, and acquired tamoxifen-resistant cell lines exhibit HER2 overexpression. Some ERα-positive tumors with the poor outcome tend to have high HER2 levels (24–26). Although HER2 overexpression is not the universal mechanism for all tamoxifen-resistant tumors, it is an important strategy to repress HER2 expression for overcoming tamoxifen resistance in some breast cancer patients. Paired box gene 2 has been shown to play a critical role in HER2 expression in tamoxifen-resistant cells and paired box gene 2 predicts clinical outcome of tamoxifen therapy in breast cancer patients (12, 27). The cross-talk between HER2 and ERα has been well-established. There is an ERα-binding site on the HER2 intron and ERα is responsible for HER2 regulation (27). In tamoxifen-sensitive cell line MCF-7, tamoxifen represses HER2 expression through co-repressor recruitments by ERα. However, tamoxifen does not repress HER2 expression in tamoxifen-resistant cells for ERα recruits...
co-activators instead of co-repressors onto HER2 intron (27). These findings suggest a new mechanism for HER2 regulation in ERα positive breast cancer (22, 27, 28).

Transcriptional repressor ZBTB1 is required for DNA repair and lymphoid development. However, the role of ZBTB1 in cancer remains to be investigated (29–31). Here, we found that ZBTB1 is down-regulated in breast cancer and the low expression of ZBTB1 indicates poor outcome in breast cancer treatments. ZBTB1 sensitized resistant cells to tamoxifen through HER2 regulation. Moreover, we found that miR-23b-3p directly target ZBTB1 and then regulate tamoxifen resistance and aerobic glycolysis.

Results

ZBTB1 is down-regulated in breast cancer

POK/ZBTB family was significantly down-regulated at mRNA level in breast tumors compared with normal tissues. Although each major subtype of breast cancer exhibits lower level of ZBTB1, a significant lower level of ZBTB1 was observed in HER2-positive and triple-negative than luminal breast tumors (Fig. 1A). To study the alteration of ZBTB1 protein expression, we performed immunohistochemistry (IHC) using patient breast tissues and found that ZBTB1 protein exhibited a lower level in breast tumors than normal tissues (Fig. 1, B and C). Breast cancer with low ZBTB1 expressions had shorter overall survival (Fig. 1D). These results indicated that ZBTB1 may be a novel suppressor for breast cancer.

ZBTB1 sensitizes breast cancer cells to tamoxifen

Because ZBTB1 is down-regulated in breast cancer, we decided to carry out a series of experiments to examine the effect of ZBTB1 on the growth of breast cancer cells. Cell proliferation assays revealed that overexpression of ZBTB1 reduced the proliferation of MCF-7 and T47D cells (Fig. 2, A and B), whereas ZBTB1 shRNA treatment enhanced the proliferation of...
of these two cell lines (Fig. 2, C and D). To exclude the off-target effect of ZBTB1 shRNA, we reintroduced shRNA-resistant ZBTB1 into knockdown MCF-7 cells and found that this construct of ZBTB1 restored cell proliferation (Fig. S1). Soft agar assay showed that ZBTB1 inhibited anchorage-independent MCF-7 and T47D cell growth (Fig. 2, E and F).

To examine the effect of ZBTB1 on tumor growth in vivo, ZBTB1 stably expressing cells were injected into nude mice. As shown in Fig. 2, G and H, the decreases in the sizes and weights of tumors excised from animals of the ZBTB1-overexpressing group were observed as compared with those of the control group. Taken together, these data indicate that ZBTB1 plays an
important role in the growth of breast cancer cells both in vitro and in vivo.

Zbtb1 regulates tamoxifen resistance in breast cancer cells

~70% of breast cancers are ER-positive and thus disruption of ER function using tamoxifen is the main therapeutic strategy employed in targeting the disease. However, tamoxifen resistance is a major challenge in breast cancer treatment (12, 22). To investigate the role of ZBTB1 in tamoxifen resistance in breast cancer, we generated the tamoxifen-resistant cell line MCF-7/TamR. We then measured ZBTB1 expression using Western blotting in cells. MCF-7/TamR cells were infected with lentivirus expressing ZBTB1 in MCF-7/TamR cells which stably express ZBTB1 and then treated with an indicated amount of TAM for 5 days. Cells were then harvested and assessed for cell proliferation by MTT assays. At least three independent experiments were performed. C, cells described in B were transfected along with plasmids expressing ERE-TK-LUC reporter and PRL-TK (internal control) followed by vehicle (Veh) or E2 treatment for 24 h. The relative luciferase values are expressed as mean ± S.E. At least three independent experiments were performed. D, MCF-7/TamR cells, which stably expressed ZBTB1 or control plasmids were injected into nude mice. After 2 weeks mice were injected with Veh or TAM and tumors were measured with Vernier calipers at the indicated times. Each group included 6 mice. E, tumor weights were calculated and shown as a scatting plot with median and whiskers from minimum to maximum. The scale bar indicates 1 cm, *, p < 0.05; **, p < 0.01.
ZBTB1 conferred tamoxifen resistance in MCF-7 cells (Fig. S3, A and B). An increasing number of studies have shown that tamoxifen plays an agonist role in ERα-mediated transcription in tamoxifen-resistant cells (12, 22). In this study, we found that re-expression of ZBTB1 could inhibit ERα transcriptional activity by tamoxifen treatment in MCF-7/TamR cells (Fig. 3C). However, knockdown of ZBTB1 induced ERα transcriptional activity by tamoxifen treatment in MCF-7 cells (Fig. S3C).

To determine the role of ZBTB1 on tamoxifen resistance in vivo, ZBTB1 stably expressing cells were injected into nude mice and then mice were treated with tamoxifen. As expected, tamoxifen treatment alone did not significantly alter tumor growth compared with the vehicle control group in mice injected with MCF-7/TamR cells. However, tamoxifen treatment could further potentiate tumor growth inhibition by ZBTB1 (Fig. 3, D and E). These results suggest that ZBTB1 sensitizes breast cancer cells to tamoxifen in vivo.

**ZBTB1 suppresses HER2 expression in MCF-7/TamR cells**

HER2 has been shown to play a critical role in tamoxifen resistance. Our data above showed that ZBTB1 exhibits a much lower level in HER2 overexpressing than luminal breast cancer as well normal tissues. This result indicates a reverse association between HER2 and ZBTB1 expressions. To investigate whether ZBTB1 could regulate HER2 expression in tamoxifen-resistant cells, we first compared HER2 expression in MCF-7/TamR with MCF-7 cells and found that HER2 was present at significantly higher levels in MCF-7/TamR cells (Fig. 4A).

To determine whether ZBTB1 could regulate HER2 expression in tamoxifen-resistant cells, we first compared HER2 expression in MCF-7/TamR and MCF-7 cells and found that HER2 was present at significantly higher levels in MCF-7/TamR cells (Fig. 4A). We then investigated the effect of ZBTB1 on HER2 expression and found that re-expression of ZBTB1 inhibited HER2 expression at both mRNA and protein levels in the presence of tamoxifen (Fig. 4, B and C, Fig. S4, A and B).

Our and other groups’ studies show that ERα and its co-activators, such as amplified in breast cancer 1 (AIB1) and mediator subunit 1 (MED1) bind HER2 intron to regulate HER2 expression in tamoxifen-resistant cells (22, 27). To study the molecular mechanisms under HER2 regulation by ZBTB1, we performed chromatin immunoprecipitation (ChIP) assays to detect the ERα, AIB1, and MED1 recruitments on HER2 intron in MCF-7/TamR cells (Fig. 4A). We then investigated the effect of ZBTB1 on HER2 expression and found that re-expression of ZBTB1 inhibited HER2 expression in both mRNA and protein levels in the presence of tamoxifen (Fig. 4, B and C, Fig. S4, A and B).

**Figure 4. ZBTB1 suppresses HER2 expression in MCF-7/TamR cells.** A, Western blotting analysis of HER2 levels in MCF-7 and MCF-7/TamR cells. B and C, MCF-7/TamR cells were infected with lentivirus expressing ZBTB1 in MCF-7/TamR cells, which stably express ZBTB1 and then treated with TAM for 24 h. Cells were then harvested and assessed for real-time PCR (B) and Western blotting assays (C). D and E, the cells described in B were treated with Veh or TAM for 1 h and ERα and its co-activators (D) and co-repressors (E) recruitments on HER2 intron were detected by ChIP assay. F, ZBTB1 interacts with ERα. MCF-7 cells were treated with and without 100 nM TAM for 1 h. Cell lysates were immunoprecipitated with either anti-ZBTB1 antibody or control IgG. The precipitates were analyzed by Western blotting using anti-ERα. G, ChIP assay for ZBTB1 occupancy on HER2 intron. MCF-7, MCF-7/TamR, and ZBTB1 expressing MCF-7/TamR cells were treated with 100 nM TAM for 1 h and ChIP assay using ZBTB1 antibody was followed. At least three independent experiments were performed. The relative recruitments versus vehicle are expressed as mean ± S.D. *, p < 0.05; **, p < 0.01.
that miR-23b-3p inhibition suppressed tamoxifen-induced HER2 expression at mRNA and protein levels via targeting ZBTB1 (Fig. 6, B and C, Fig. S8, B and C). We then performed ChIP assays to detect the effect of miR-23b-3p on ERα and its coregulator recruitments on the ERα-binding region of HER2 intron in MCF-7/TamR and BT474 cells. As a result, we found that miR-23b-3p knockdown diminished MED1 and AIB1 but not ERα recruitments induced by tamoxifen (Fig. 6D, Fig. S8D). However, miR-23b-3p knockdown restored co-repressors such as N-CoR and HDAC1 recruitments by ERα onto HER2 intron (Fig. 6E, Fig. S8E).

**ZBTB1 is a direct target of miR-23b-3p in MCF-7/TamR cells**

To further investigate the role of ZBTB1 in tamoxifen resistance, we used 2 target prediction programs, TargetScan and miRanda, to predict miRNAs that target ZBTB1. Our analysis predicted 6 potential ZBTB1-targeting miRNAs. First, we investigate microRNAs, which are dysregulated in tamoxifen-resistant cells and found only miR-23b-3p exhibited a higher level in MCF-7/TamR than MCF-7 cells (Fig. S6). Then, we performed Western blotting and found miR-23b-3p overexpression decreased ZBTB1 expression in MCF-7 and T47D cells, however, miR-23b-3p inhibition increased ZBTB1 expression in MCF-7/TamR and BT474 cells (Fig. 5, A–D, Fig. S7, A–D). Thus, we decided to study the role of miR-23b-3p in tamoxifen resistance. We generated WT and mutant ZBTB1 3'-UTR expression plasmids that were fused to a luciferase reporter according to the matched sequence between ZBTB1 3'UTR and miR-23b-3p (Fig. 5F). We found that miR-23b-3p overexpression significantly inhibited luciferase activity of WT but not mutant reporter genes in MCF-7 and T47D cells. However, miR-23b-3p silencing specifically enhanced the luciferase activity of WT but not mutant ZBTB1 3'-UTR in MCF-7/TamR and BT474 cells (Fig. 5, F and G, Fig. S7, E and F).

**MiR-23b-3p confers tamoxifen resistance via targeting ZBTB1 in MCF-7/TamR cells**

To investigate the role of miR-23b-3p in tamoxifen resistance, we transferred MCF-7/TamR and BT474 cells with anti-miR-23b-3p oligo and performed MTT (3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide) assay. Our results showed that miR-23b-3p inhibition significantly sensitized resistant cells to tamoxifen treatment. In addition, knockdown of ZBTB1 diminished the effect of miR-23b-3p inhibition on tamoxifen response in MCF-7/TamR and BT474 cells (Fig. 6A, Fig. S8A). Our data also indicated that miR-23b-3p inhibition suppressed tamoxifen-induced HER2 expression at mRNA and protein levels via targeting ZBTB1 (Fig. 6, B and C, Fig. S8, B and C). We then performed ChIP assays to detect the effect of miR-23b-3p on ERα and its coregulator recruitments on the ERα-binding region of HER2 intron in MCF-7/TamR and BT474 cells. As a result, we found that miR-23b-3p knockdown diminished MED1 and AIB1 but not ERα recruitments induced by tamoxifen (Fig. 6D, Fig. S8D). However, miR-23b-3p knockdown restored co-repressors such as N-CoR and HDAC1 recruitments by ERα onto HER2 intron (Fig. 6E, Fig. S8E).
MiR-23b-3p/ZBTB1 regulates aerobic glycolysis in tamoxifen-resistant cells

Recent studies established the association of aerobic glycolysis and tamoxifen resistance. Inhibition of aerobic glycolysis can reverse tamoxifen resistance (33, 34). To investigate the role of miR-23b-3p/ZBTB1 in aerobic glycolysis, we decided to carry out a series of experiments on tamoxifen-resistant cells. Lactate production and glucose uptake increases were observed in MCF-7/TamR cells compared with MCF-7 cells (Fig. S9). More importantly, miR-23b-3p inhibition or ZBTB1 re-expression significantly reduced lactate production and glucose uptake in MCF-7/TamR and BT474 cells in the presence of tamoxifen (Fig. 7A–D, Fig. S10, A–D). Western blotting indicated that miR-23b-3p inhibition or ZBTB1 re-expression suppressed the expressions of HKII (hexokinase II) and LDHA (lactate dehydrogenase A), which are critical for the glycolytic pathway (Fig. 7E, Fig. S10E). Moreover, knockdown of ZBTB1 diminished the inhibitory effect of anti-miR-23b-3p oligo on lactate production and glucose uptake (Fig. 7, F and G). Our Western blotting assays also indicated that miR-23b-3p regulates HKII and LDHA expressions via targeting ZBTB1 (Fig. 7H). These results suggested the role of miR-23b-3p/ZBTB1 in aerobic glycolysis of tamoxifen-resistant cells.

Discussion

Through this study, we have identified a key role of the ZBTB1 protein in tamoxifen resistance through HER2 modulation in breast cancer. First, we found that ZBTB1 is down-regulated in breast cancer and it suppresses breast cancer cell growth in vitro and in vivo. Second, we showed that ZBTB1 is down-regulated in tamoxifen-resistant cells and re-expression of ZBTB1 sensitizes breast cancer cells to tamoxifen. Third, ZBTB1 is recruited onto the intron of the HER2 gene in breast cancer cells and is required for HER2 expression repression in tamoxifen-resistant cells. Finally, we found that ZBTB1 is a direct target of miR-23b-3p and miR-23b-3p/ZBTB1 regulates tamoxifen resistance and aerobic glycolysis in tamoxifen-resistant cells.

Human ZBTB family proteins consist of 49 members who encode transcriptional factor that have critical roles in cell fate decision and lineage commitment and other development...
processes (35, 36). This family of proteins contains a POZ/BTB domain at the N terminus and multiple Krüppel-type zinc fingers at the C terminus. The POZ/BTB domain is involved in protein–protein interactions and the zinc finger region mediates sequence-specific binding to DNA elements (37). POK proteins, ZBTB1 included, have been shown to commonly act as transcriptional repressors by directly binding specific consensus sequences on DNA and interacting with co-repressors such as N-CoR, SMRT, and Sin3a via the POZ domain at the N terminus (29, 38). Multiple studies showed that ZBTB1 deficiency results in a severely impaired lymphoid development, and to a less extent, myeloid development in mice. In addition to immune cell developments, ZBTB1 is also required for DNA repair in human UV-irradiated Hela cells (29–31, 39). Although several ZBTB members have been shown to play critical roles in tumorigenesis, the role of ZBTB1 in cancer is unclear. Here, we used UALCAN to explore TCGA database and found that ZBTB1 was down-regulated at mRNA level in breast cancer (32, 40). Our IHC assay using specific ZBTB1 antibody confirmed ZBTB1 expression alteration at the protein level in breast cancer. In line with this observation, ZBTB1 suppressed breast cancer cell proliferation and tumor growth. These results suggest ZBTB1 function as a tumor repressor in breast cancer. Although there is no correlation between ZBTB1 mRNA level and overall survival in TCGA database, we observed the correlation between ZBTB1 protein level and total survival in breast cancer patients. One possible reason is that TCGA integrates ZBTB1 expression data at mRNA level using RNA-Seq, however, we measured ZBTB1 expression at protein level using IHC. Sometimes they can be very different for translation regulation by many molecules such as micro-RNAs (41). Another possible reason is that IHC analysis can differentiate ZBTB1 expression in cancer cells from other types of cells, such as lymphocytes of breast tumors, but RNA-seq of TCGA may include ZBTB1 expression in all types of cells. Thus, different expression data may lead to the discordant findings.

Tamoxifen resistance is one of the major challenges for the successful treatment of breast cancer and HER2 overexpression is associated with a poor outcome in tamoxifen-treated patients. Therefore, HER2 expression repression is required for the anti-proliferative effects of tamoxifen (12, 22). In this study, we found that ZBTB1 exhibited a very low level in tamoxifen-resistant cells and re-expression of ZBTB1 sensitized resistant cells to tamoxifen treatment. Importantly, ZBTB1 is required for HER2 repression by tamoxifen. Our and other groups’
studies have identified the critical role of cross-talk between ERα and HER2 in tamoxifen resistance. ERα is present on HER2 intron and either enhance or suppress HER2 expression by co-regulator recruitments in the presence of tamoxifen. In tamoxifen-sensitive cells, tamoxifen repress HER2 by ERα recruiting co-repressors such as N-CoR and HDAC1 (12, 22). In a sharp contrast, ERα recruits co-activators instead of co-repressors in tamoxifen-resistant cells. We found that ZBTB1 is present on the ERα-binding site of HER2 intron and is required for co-repressor recruitments in the presence of tamoxifen. MiR-23b-3p is found to be overexpressed and directly diminishes ZBTB1 expression in tamoxifen-resistant cells. As schematically illustrated in Fig. 8, this action diminished the inhibitory effect of ZBTB1 on HER2 expression. MiR-23b has been shown to play an important role in cytoskeletal remodeling and miR-23b expression correlates with the development of metastases in breast cancer patients (42). During preparation of this manuscript, a published study suggests that miR-23b-3p regulates amino acid metabolism and high expression of miR-23b-3p correlates with reduced survival in ER+ breast cancer patients (43). Here, our study suggests that miR-23b-3p is overexpressed in tamoxifen-resistance cells and play important roles in glycolysis and tamoxifen resistance. These studies indicate that miR-23b may play different roles in different process and different types in breast cancer. Further studies including knockout mouse model is required for the role of miR-23b in vivo.

Cancer cells exhibit aberrant metabolism characterized by high glycolysis even in the presence of abundant oxygen. This phenomenon, known as the Warburg effect or aerobic glycolysis, facilitates tumor growth with elevated glucose uptake and lactate production (33, 44, 45). The association of aerobic glycolysis and tamoxifen resistance has been well-established (33). In this study, we showed that miR-23b-3p confers tamoxifen resistance and aerobic glycolysis through directly target ZBTB1. HER2 has been shown to play a critical role in aerobic glycolysis through AKT (46, 47). It is interesting to investigate whether miR-23b-3p/ZBTB1 regulate aerobic glycolysis through AKT and its downstream molecules such as mTOR and p-STAT3. The research on the molecular mechanism of a recent study has shown that aerobic glycolysis of cancer cells is linked with macrophage M2 in mice. Macrophage M2 is contrast to classic macrophage (M1) and facilitates cancer progression (48, 49). This implies the association between tamoxifen resistance and tumor immunology. However, the linkage between tumor immunology and tamoxifen resistance remains to be established. Because ZBTB1 is essential for T, B, and NK cell development, we deduced that ZBTB1 may serve as a bridge to link the cancer cells and immune cells (30, 31). It is interesting to study the relationship between tamoxifen resistance, aerobic glycolysis, and immune escape using this model in breast cancer. Of note, it is worthy to pay efforts to screen ZBTB1 agonist for breast cancer treatments for one hand it suppresses cancer cell growth and the other hand it enhances immune cell development.

Materials and methods

Cell culture

The human breast cancer cell lines MCF-7, T47D, and BT474 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell lines were authenticated using STR profiling (supporting information). The expression status of ER was further confirmed by Western blotting before used in the experiments. All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS (HyClone, Thermo Fisher Scientific, Florence, KY) at 37 °C.
with 5% CO₂ in tissue culture incubators. 4-Hydroxytamoxifen (TAM) was purchased from Sigma. Tamoxifen-resistant cells (MCF-7/TamR) were developed by culturing tamoxifen-sensitive MCF-7 cells in the presence of TAM (gradually increases content from 0.1 to 1 μM) for more than 1 year as described previously (50). HER2-overexpressing cell line BT474 was also used for tamoxifen resistance according to the previous study (22). For experiments involving TAM treatments, cells were routinely cultured in phenol red-free DMEM plus 10% charcoal-stripped FBS for at least 3 days before the treatments.

**Plasmids and lentiviral vector preparation**

The plasmids pERE-TK-Luc and pRL-CMV were described previously (50). To generate a lentiviral construct, ZBTB1 was cloned into pCDH-CMV (System Biosciences, Mountain View, CA). High titer lentiviruses were generated by transient transfection in 293T cells as described previously (22). Anti-miR-23b-3p inhibitor, miR-23b-3p, and the control oligonucleotides were purchased from GeneCopoeia (Rockville, MD). The human-specific shRNAs targeting ZBTB1 were synthesized by Genetech Co., Ltd. (Shanghai, China) and inserted into hU6-MCS-CMV-puromycin for commercial lentivirus package. The plasmids pERE-TK-Luc and pRL-CMV were described previously (50). In brief, MCF-7 cells were treated with 100 nM TAM or vehicle for 1 h. Cells were harvested and lysed in 0.5 ml lysis buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 0.25% Nonidet P-40, 1 mM DTT and protease inhibitor tablets from Roche (Biolab, GA, USA). The immunoprecipitation was performed with anti-ZBTB1 antibodies (Thermo, Waltham, MA). Real-time PCR amplification was performed with DNA extracted from the immunoprecipitates. The primers for HER2 intron were described previously (27).

**Transient transfections and reporter gene assays**

For transfection, cells were plated in 24-well–plates containing phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped FBS, and the plasmids were transfected with Lipofectamine 2000 (Invitrogen). Following transfection, transient transfections in 293T cells as described previously (22). Anti-miR-23b-3p inhibitor, miR-23b-3p, and the control oligonucleotides were purchased from GeneCopoeia (Rockville, MD). The human-specific shRNAs targeting ZBTB1 were synthesized by Genetech Co., Ltd. (Shanghai, China) and inserted into hU6-MCS-CMV-puromycin for commercial lentivirus package. Sequences of shRNAs were as follows: shRNA-1: 5′-UGG-CAUGAAACUACCACGCUAUA-3′; shRNA-2: 5′-GAGCC-CAAGUUCAACGGUAATT-3′ (29). shRNA-resistant ZBTB1 plasmid was generated by mutating nucleotides in the target site: 5′-TGGCATAATATTTACATATTATA-3′ (shown in lower cases) according to a previous study (29).

**Real-time PCR**

Total RNA was isolated from cells with an Rnasy Mini kit (Qiagen, Hilden, Germany) or TRizol (Invitrogen) reagent according to the manufacturer’s instructions. Total RNA from each sample was reverse transcribed with oligo(dT)₂₀ using SuperScript® III Reverse Transcriptase (Invitrogen) followed by real-time PCR. Primers for miR-23b-3p were purchased from GeneCopoeia (Rockville, MD). Real-time PCR was performed with SYBR Green PCR Master Mix reagents using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

**ChIP**

ChIP analysis was performed as described previously (50). In brief, MCF-7 and MCF-7/TamR cells were treated for 1 h with vehicle (ethanol) or 100 nM TAM and immediately fixed by adding 37% formaldehyde to the medium to a final concentration of 1%. After PBS washing, cells were harvested and the nuclear lysates were sonicated to generate an average DNA size of 0.5–1 kb. The immunoprecipitation was performed with anti-ERα, MED1, AIB1, N-CoR, HDAC1 (Santa Cruz, CA), and ZBTB1 antibodies (Thermo, Waltham, MA). Real-time PCR amplification was performed with DNA extracted from the immunoprecipitates. The primers for HER2 intron were described previously (27).

**MTT assay**

MTT (Sigma-Aldrich) assay was performed as described previously (22). Cells were seeded into a 96-well–plate with 10,000 cells each well. MTT was added to the medium to a final concentration of 0.5 mg/ml and then the medium was removed and 0.2 ml of DMSO was added. After incubation for 30 min at room temperature, the absorbance of the converted dye was measured at 570 nm using a Synergy spectrophotometer (Biolab, GA, USA).

**Soft agar assay**

For anchorage-independent growth, 5 × 10³ cells were plated on 6-well–plates containing a bottom layer of 0.6% agarose in DMEM and a top layer of 0.3% low melting temperature-agarose in DMEM. Colonies were scored and photographed after 2 weeks of growth.

**Co-immunoprecipitation**

Co-immunoprecipitation assay was performed as described previously (50). In brief, MCF-7 cells were treated with 100 nM TAM or vehicle for 1 h. Cells were harvested and lysed in 0.5 ml of lysis buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 0.25% Nonidet P-40, 1 mM DTT and protease inhibitor tablets from Roche (Penzberg, Germany) and immunoprecipitation was performed with anti-ZBTB1 (Thermo, Waltham, MA). The beads were washed and resolved by SDS-PAGE, followed by immunoblotting using anti-ERα (Santa Cruz, CA).

**Measurements of lactate production and glucose uptake**

Lactate production and glucose uptake were measured using Glucose Uptake Colorimetric Assay Kit and Lactate Assay Kit II (Biovision, Milpitas, CA) according to the manufacturer’s protocols. For measurement of lactate production 5 × 10⁵ cells were seeded in a 6-well–plate and incubated overnight in DMEM containing 10% FBS. To measure the secretion of lactate, the cells were incubated in DMEM without FBS. After incubation for 6 h, the supernatant was collected and mixed with the reaction buffer for 30 min at room temperature in the dark. The lactate levels were measured at 450 nm in a microplate reader and normalized with cell number. For glucose uptake assay, 5,000 cells were seeded into a 96-well–plate, and incubated overnight. Cells were washed with PBS and then starved for glucose by preincubating with 100 ml of Krebs-Ringer-phosphate-HEPES (KRPH) buffer containing 2% BSA for 40 min. Ten microliters of 10 mM 2-deoxyglucose was added and incubated for 20 min. Reactions were conducted as the manufacturer’s protocol. The final supernatant was used for...
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determination of glucose uptake at 412 nm in a microplate reader. The results were normalized to cell number.

Immunohistochemistry staining
IHC staining of human breast cancer was carried out essentially as previously described (50). In brief, the slide was deparaffinized and subjected to heat-induced antigen retrieval using citrate buffer. The tissue sections were then incubated with primary antibodies against ZBTB1 (Thermo, Waltham, MA) overnight at 4°C. The slide was subsequently treated with anti-rabbit secondary antibody and then developed using avidin-conjugated horseradish peroxidase with diaminobenzidine as the substrate. Hematoxylin was used for counterstaining and the images were visualized and captured using axioplan imaging 2e microscope (Weimar, Germany). The IHC results were assigned a mean score considering both the intensity of staining and the proportion of cells with an unequivocal positive reaction. The brown or brownish-yellow particles were defined as positive cells; 1) staining intensity as the standard: unstained, 0 score; pale yellow, 1 score; brownish-yellow, 2 scores; brown, 3 scores; 2) percentage of stained cells in total cells as the standard: ≤5%, 0 score; 6–25%, 1 score; 26–50%, 2 scores; 51–75%, 3 scores; ≥76%, 4 scores. The score of each sample was calculated as the product of scores in 1 and 2. The score ≥8 is regarded as high expression and the score < 8 is low level expression.

Human tissue analysis
90 breast tumors including adjacent noncancerous tissues were obtained from the 1st and 2nd Affiliated Hospital of Yichun University. 75 breast tumors are ER-positive and these patients all received endocrine therapy (31 received chemotherapy). 19 breast tumors are HER2-positive and all received anti-HER2 therapy. 8 breast tumors are triple negative and all received chemotherapy. All cases were female with 28–81 years of age (mean age: 52.4 years) and the follow-up time was 4–76 months (mean time: 54.7 months). Tissue samples were used for ZBTB1 expressions using IHC. The study was conducted in accordance with the Declaration of Helsinki principles and approved by the Ethics Review Boards of the 1st and 2nd Affiliated Hospital of Yichun University. All the samples derived from the 1st and 2nd Affiliated Hospital of Yichun University were obtained with informed consent under the approved protocols.

Animal experiments
All the experimental procedures involving animals were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No.8023, revised 1978). The protocol has been approved ethically by the Administration Committee of Experimental Animals, Medicine School, Yichun University, Yichun, China, and the protocol number is IACUC-2016013. The procedures were described previously (50). In brief, 1 × 10⁷ MCF-7 or MCF-7/TamR cells that stably expressed ZBTB1 or control plasmids were injected into the abdominal mammary fat pad of 6-week-old female nude mice. After 2 weeks, we randomly allocated the mice to groups in which they received placebo or tamoxifen pellets (Innovative Research of America, Sarasota, Florida, USA). Tumor growth was monitored by caliper measurements. Excised tumors were weighed, and portions were frozen in liquid nitrogen.

Statistical analysis
All the experiments were repeated at least three times. Presented data are shown at mean ± S.D. of three experiments if no specific description. Student’s t test and one-way analysis of variance test for multiple group comparisons were performed using SPSS. p < 0.05 was considered statistically significant. Estimation of overall survival was performed using the Kaplan-Meier method, and differences between survival curves were determined with the log-rank test.

Data availability
All the data are within the manuscript and supporting information. All the data are to be shared upon request (Jiajun Cui, Yichun University, cui_jj@hotmail.com).

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Abbreviations—The abbreviations used are: ER, estrogen receptor; ERE, estrogen-response element; HER2, human epidermal growth factor receptor-2; IHC, immunohistochemistry; AIB1, amplified in breast cancer 1; MED1, mediator subunit 1; N-CoR, nuclear receptor co-repressor 1; HDAC1, histone deacetylase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; LDHA, lactate dehydrogenase A; HKII, hexokinase II; DMEM, Dulbecco’s modified Eagle’s medium; shRNA, short hairpin RNA; IP, immunoprecipitation; IB, immunoblot; FBS, fetal bovine serum; TAM, 4-hydroxytamoxifen.

References


ZBTB1 sensitizes breast cancer cells to tamoxifen


