Targeting Phosphorylation of Y-Box–Binding Protein YBX1 by TAS0612 and Everolimus in Overcoming Antiestrogen Resistance

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ABSTRACT

Nuclear expression of Y-box–binding protein (YBX1) is closely correlated with clinical poor outcomes and drug resistance in breast cancer. Nuclear translocation of YBX1 is facilitated by YBX1 phosphorylation at serine 102 by AKT, p70S6K, and p90RSK, and the phosphorylated YBX1 (pYBX1) promotes expression of genes related to drug resistance and cell growth. A forthcoming problem to be addressed is whether targeting the phosphorylation of YBX1 overcomes antiestrogen resistance by progressive breast cancer. Here, we found that increased expression of pYBX1 was accompanied by acquired resistance to antiestrogens, fulvestrant and tamoxifen. Forced expression of YBX1/S102E, a constitutive phosphorylated form, resulted in acquired resistance to fulvestrant. Inversely, YBX1 silencing specifically overcame antiestrogen resistance. Furthermore, treatment with everolimus, an mTORC1 inhibitor, or TAS0612, a novel multikinase inhibitor of AKT, p70S6K, and p90RSK, suppressed YBX1 phosphorylation and overcame antiestrogen resistance *in vitro* and *in vivo*. IHC analysis revealed that expression of pYBX1 and YBX1 was augmented in patients who experienced recurrence during treatment with adjuvant endocrine therapies. Furthermore, pYBX1 was highly expressed in patients with triple-negative breast cancer compared with other subtypes. TAS0612 also demonstrated antitumor effect against triple-negative breast cancer *in vivo*. Taken together, our findings suggest that pYBX1 represents a potential therapeutic target for treatment of antiestrogen-resistant and progressive breast cancer.

Introduction

Nearly 70% of breast cancers express ERα and are sensitive to endocrine therapy with a selective ER modulator, selective ER downregulator, and aromatase inhibitor (1). As an adjuvant therapy, these endocrine therapies provide marked benefits to patients with ERα-positive primary breast cancer and metastatic breast cancer (2, 3). However, the occurrence of resistant tumors to endocrine therapy through transformation from ERα-dependent into ERα-independent tumor growth results in severe loss of antitumor efficacy in the clinic (4). Thus, refractory tumor emergence is a major complication in breast cancer patients treated with adjuvant endocrine therapy (5, 6). Furthermore, approximately 20% of breast cancers depend on the human epidermal growth factor receptor 2 (HER2)/ERBB2 without expression of ERα (7, 8). Triple-negative breast cancer (TNBC), corresponding to 10% to 15% of breast cancer cases, defines by the loss of both ERα and HER2 (9).

Y-box–binding protein-1 (YBX1), a member of the cold-shock domain protein superfamily, plays a major role in enhanced expression of various drug resistance-related genes including ABCB1, MVP/LRP, TOP2A, CD44, CD49f, BCL2, and MYC, and other cell growth- and cycle-related genes in cancer cells (10–12). The increased expression of YBX1 is predictive of drug resistance and poor outcomes in patients with more than twenty different types of tumors (12). Of various human malignancies, the clinical and preclinical importance of YBX1 has been most extensively studied in breast cancer (12–15). Overexpression of YBX1 promoted tumorigenesis and malignant progression, implicating YBX1 as an oncoprotein for mammary tumors (16–19). Chen and colleagues recently reported that YBX1 sustains stemness properties and also that YBX1 is associated with breast cancer risk and outcome by single-cell landscape analysis, in mammary epithelium (20). Moreover, silencing of YBX1 induced marked downregulation of cell proliferation- and cell cycle-related genes (i.e., EGFR, HER2, FGFR2, and CDC6) and upregulation of ERα in human breast cancer cell lines (13, 21–25). YBX1 also stimulates the translation of mRNAs encoding transcriptional regulators of epithelial-to-mesenchymal transition (EMT) such as Snail and Twist, accelerating EMT in breast cancer cells (26, 27). Together YBX1 thus plays its oncogenic roles through pleiotropic regulatory mechanisms in breast cancer (12).

With regard to the reduced responsiveness to endocrine therapies, overexpression of YBX1-induced downregulation of ERα expression in breast cancer cells and the development of acquired resistance to tamoxifen in *in vivo* experimental models (22). Furthermore, expression of YBX1 was negatively correlated with ERα expression in tumor

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cells of patients with breast cancer (12, 13, 22). The top 500 genes that are positively correlated with YBX1 expression were identified, and among those genes, more than 60% genes are common to the top 500 genes that are negatively correlated with *ESR1* (23), implying that YBX1 may promote ERα-independent growth in breast cancer. On the other hand, it has been demonstrated that the nuclear and total cellular expression of YBX1 predicts malignant progression and poor outcomes in patients with breast cancer (13, 22, 23). Nuclear translocation of YBX1 is facilitated by its specific phosphorylation at Ser102 (pYBX1 Ser102), and pYBX1 Ser102 is suppressed by inhibitors of PI3K/AKT, mTOR, MEK, and p90RSK (16, 18, 28, 29). This suppression was accompanied by reduced nuclear translocation of YBX1 in cancer cells, indicating that both the AKT/mTOR/70S ribosome S6 kinase (p70S6K) and RAS/RAF/MEK/90S ribosome S6 kinase (p90RSK) signaling pathways play key roles in the activation of YBX1. Furthermore, the p70S6K family and the p90RSK family (p90RSK1-4), that phosphorylate YBX1 (S102), facilitate cell proliferation and survival (16, 29), and these kinases are highly activated in progressive breast cancer and numerous other human malignancies (30, 31).

Several reports have previously showed preclinical anticancer therapeutic approaches targeting YBX1, including a molecular decoy cell permeable peptide composed of nine amino acids that flank the YBX1 S102 site (32), Dectin-1 targeting vehicle delivering YBX1 antisense DNA (33), and long noncoding RNA that silences the expression of YBX1 (34). These experimental therapeutic trials exhibited a cytotoxic effect against cancer cells *in vitro*. However, further *in vivo* experiments and clinical trials by targeting YBX1 have not yet been reported. In the current study, we initially investigated whether pYBX1 promotes the development of resistance to antiestrogens in breast cancer cell. Moreover, clinical significance of pYBX1 was evaluated in recurrent tumors and TNBC, and we also newly presented our novel finding that everolimus and a new orally bioavailable multikinase inhibitor TAS0612 overcome antiestrogen resistance by breast cancer.

Materials and Methods

Cell lines

As previously described (23), the human breast cancer cell lines MCF-7, T-47D, SKBr-3, HCC1954, MDA-MB453, ZR-75-1, MDA-MB231, BT549, Hs578T, and HCC1143 were purchased from the ATCC. SUM149PT and SUM159PT cells were purchased from Asterand. KPL-1 was purchased from Health Science Research Resources Bank. Cell culture conditions were described previously (23). All cell lines were passaged for $\leq$6 months and were not further tested or authenticated by the investigators. Two fulvestrant-resistant cell lines, designated as T-47D/FR-1 and T-47D/FR-2, were established from T-47D cells through continuous exposure to stepwise increasing concentrations of fulvestrant (up to 1 μmol/L) for approximately 6 months (22). T-47D/FR-1 and T-47D/FR-2 were established in different flasks and were not cloned.

Chemicals

TAS0612 was prepared at Taiho Pharmaceutical Co., Ltd as described compound #34 in PCT Patent Application WO2017/200087 (35). We generated an antibody against YBX1, designated as (st1968), by immunizing New Zealand white rabbits with a synthetic peptide representing the YBX1 C-terminal amino acid residues 299–313 (36). This antibody detects cytoplasmic and nuclear YBX1 in IHC analysis. An antibody against YBX1 (EP2708Y, #ab76149; Abcam) was used for the Western blotting analysis. Other antibodies were shown in Supplementary Materials and Methods. Fulvestrant (#14409-25MG) and dextran-coated charcoal (#C6241-20G) were purchased from Sigma-Aldrich. Hygromycin B (#085-06153), tamoxifen (#209–14361), and 17β-estradiol (#052–04041) were purchased from Wako Pure Chemical Industries. Everolimus (#S1120) and AZD8055 (#S1555; ref. 37) were purchased from Selleck Chemicals. U0126 (#22428101; ref. 38) was purchased from Promega. LY294002 (#440202; ref. 39) was purchased from Calbiochem.

Immunofluorescence

Cells were plated on glass coverslips in 24-well plates and allowed to attach overnight. Then, cells were rinsed with PBS and then fixed in 4% paraformaldehyde/PBS for 30 minutes. Cells were rinsed twice with PBS and then permeabilized with 0.5 mL of solution containing 5% BSA, 0.2% Triton X-100 in PBS for 90 minutes. After 1 hour of blocking with 2% BSA, the cells were incubated overnight with primary anti-YBX1 antibody (1:100, ab76149 (EP2708Y); Abcam) at 4°C in 1% BSA in PBS. Cells were then rinsed three times with PBS and incubated with CF488 conjugated anti-goat IgG antibody (1:500, 20225; Biotium) in 1% BSA in PBS for 60 minutes. Coverslips were mounted on slide glasses using gel mount and we performed fluorescence imaging with KEYENCE BZ-8000.

Mice

The Animal Ethics Committee of Kyushu University (Fukuoka, Japan) reviewed and approved all the animal experimental procedures in this study (Approval number: A30-070-0), which were conducted according to the recommendations of the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, NIH, Department of Health and Human Services, Bethesda, MD). Female BALB/c nu/nu athymic nude mice (aged 6–7 weeks) were purchased from CLEA and housed in microisolator cages under a 12-hour light/dark cycle. Water and food were supplied *ad libitum*. Animals were observed for tumor growth, activity, feeding, and pain according to the guidelines of the Harvard Medical Area Standing Committee on Animals.

Xenograft studies

Approximately 5.0×10^6 T-47D/FR-2 cells or 5.0×10^6 SUM159PT cells in 200 μL of 50% Matrigel were orthotopically implanted into the fourth mammary fat pad on both sides. T-47D/FR-2 cells showed estradiol-independent growth *in vitro* and *in vivo*. Thus, 17β-estradiol pellets were not implanted prior to inoculation. Tumor sizes were measured, and tumor volumes (mm^3) were calculated as follows: length $\times$ width^2 $\times$ 0.5. When tumors reached 50 mm^3, mice were randomly allocated into groups ($n = 8$/group). The mice was treated with everolimus [2 mg/kg/mouse in 0.5% carboxymethyl cellulose sodium salt (Wako Pure Chemical Industries, Ltd.), daily], TAS0612 [50 mg/kg/mouse in 0.5% hydroxypropyl methylcellulose (Shin-Etsu Chemical Co., Ltd) with diluted McIlvaine buffer solution (pH 3.0; Nacalai Tesque), daily], or tamoxifen citrate (500 μg/mouse in peanut oil, daily, subcutaneously). The tumors were harvested after 2 weeks, stored at −80°C, or immediately fixed in 10% paraformaldehyde overnight at 4°C.

Patient selection

We selected 61 patients with breast cancer who were treated in the Japan Community Health Care Organization, Kurume General Hospital (Kurume, Japan) from 2005 to 2013. The study was approved by the Institutional Review Board of the hospital (Approval number: 292).

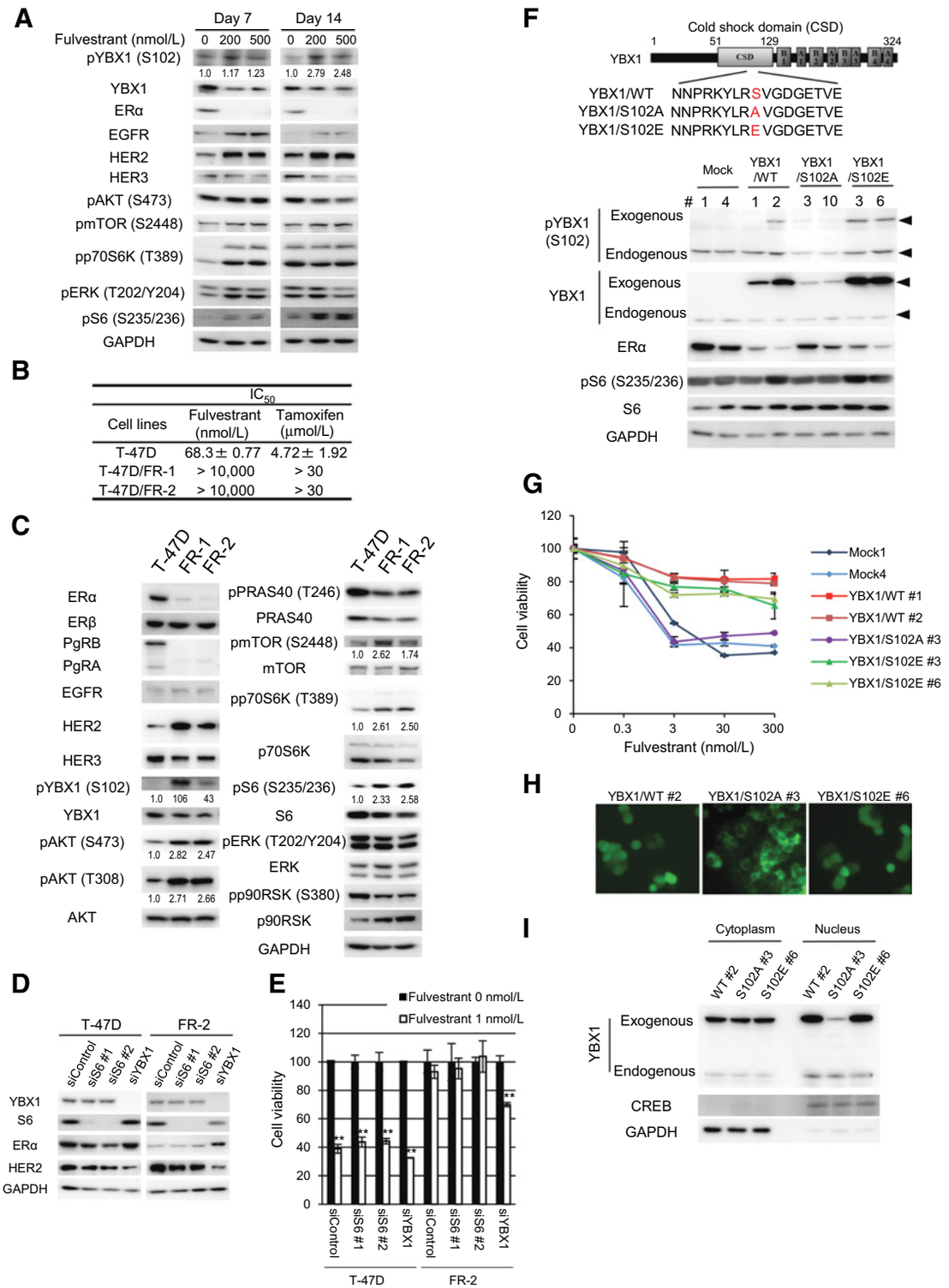


Figure 1.
The constitutive phosphorylation of YBX1 and acquired resistance to fulvestrant in breast cancer cells. **A,** The effect of fulvestrant on expression of pYBX1, ERα, EGFR family protein, and ERK/p90RSK and AKT/mTOR signaling molecules in T-47D. Cells were treated with fulvestrant at the indicated concentrations for 7 or 14 days. **B,** Comparison of cellular sensitivity to fulvestrant and tamoxifen between fulvestrant-resistant cell lines and their parental-sensitive cell line. IC_{50} values are average of triplicate dishes for each cell line. (*Continued on the following page.*)

Written informed consent was obtained from all patients. Primary and secondary tumor tissue specimens ($n = 12$) were selected in this study. These cases were defined as hormonal therapy–resistant, and subsequent analysis was performed using these tissue sections. An overview of the clinical information of recurrent patients (i.e., age of cases, sex, pathological diagnosis of primary and secondary tumor, status of menopause, duration of hormonal therapy, and content of the treatments) is presented in Supplementary Table S1. The dataset of YBX1 and pYBX1 (S102) expression of 397 patients with invasive breast cancer was obtained from TCGA [Breast Invasive Carcinoma (TCGA, Provisional) dataset of the cBioPortal for Cancer Genomics; http://www.cbioportal.org; Supplementary Table S2].

IHC analysis

Biopsy sample tissues of breast were immediately fixed in 10% neutral-buffered formalin. Paraffin-embedded tissue samples were cut (4 μm), examined on a coated glass slide, and IHC stained for YBX1 (st1968; 1:2,000; produced in our laboratory), pYBX1 (1:100; Cell Signaling Technology), and pS6 (1: 100; Cell Signaling Technology). For YBX1 and pYBX1, the IHC analysis was performed using the Ventana BenchMark XT automated immunostainer (Ventana Automated Systems, Inc.). Immunostaining with pS6 was performed using the Bond-Max system (Leica Microsystems). Positive controls, for each antibody (breast cancer), and negative controls were run in parallel.

Image analysis

An automated determination of total YBX1 (cytoplasmic and nuclear YBX1), pYBX1, and pS6 staining areas was performed as previously described using the "WinROOF" software (version 5.7; Mitani Corporation). Digital images of cancer cells were selected for clarity from five high-power fields (×400) for each IHC specimen, using a charge-coupled device digital camera (DXM1200; Nikon). The staining area of cancer cells was measured in 800 to 1,000 tumor cells and averaged.

Statistical analysis

Experimental results were expressed as mean ± SD (**Figs. 1B** and **E**, **2A** and **D**, **3B**, and **4D**). Statistical differences between groups were assessed by two-tailed Student *t* test. A *P* value of less than 0.05 was considered significant. In comparing multiple groups, Tukey–Kramer multiplicity adjustment was applied for all pairwise comparisons.

In vitro assays

WST assay, cell proliferation assay, preparation of charcoal-stripped serum, cell transfection, and Western blotting analysis are described in Supplementary Materials and Methods.

Results

The phosphorylation status of YBX1 is closely associated with resistance to antiestrogen

Of various phosphorylation sites of YBX1 protein, the phosphorylation at YBX1 Ser102 has been known to be closely associated with its biological functions (16, 29). We first assessed whether the phosphorylation of YBX1 Ser102 was closely correlated with acquired resistance to antiestrogens in breast cancer cells. We initially examined the effect of long-term treatment with fulvestrant on YBX1 phosphorylation status in T-47D cells. Treatment with fulvestrant for 7 and 14 days increased the expression of EGFR, HER2, pmTOR, and pS6 accompanied with markedly reduced expression of ERα (**Fig. 1A**). It was noted that the expression of pYBX1 was upregulated although YBX1 itself was similarly expressed (**Fig. 1A**).

We subsequently compared YBX1 phosphorylation status in two fulvestrant-resistant cell lines (FR-1 and FR-2), which we previously established (22). Both FR-1 and FR-2 showed not only resistance to fulvestrant but also to tamoxifen, another representative therapeutic antiestrogen (**Fig. 1B**). Both resistant cell lines showed increased expression of pYBX1, and also markedly reduced expression of ERα and progesterone receptor (PgR) accompanied by enhanced expression of pmTOR, pAKT, pp70S6K, and pS6, as compared with their drug sensitive counterpart (**Fig. 1C**). As AKT/mTOR/p70S6K/S6 signaling pathway and YBX1 are activated in resistant cell lines, we examined whether YBX1 or S6 could be specifically associated with antiestrogen resistance. Treatment with YBX1-siRNA enhanced ERα expression and suppressed HER2 expression in T-47D/FR-2 cells, consistent with our previous study (22). In contrast, treatment with S6-siRNA did not affect expression of ERα and HER2 (**Fig. 1D**). As shown in **Fig. 1E**, YBX1 silencing augmented cellular sensitivity to fulvestrant in T-47D/FR-2 cells but S6 silencing did not, indicating YBX1 specifically induced antiestrogen resistance in T-47D/FR-2 cells.

We next assessed whether YBX1 Ser102 phosphorylation *per se* affects altered sensitivity to antiestrogens in breast cancer cells. We established stable cell lines expressing the YBX1 constitutively active mutant (YBX1/S102E), inactive mutant (YBX1/S102A) and YBX1 wild type (YBX1/WT), to confirm whether constitutive phosphorylation of YBX1 can selectively contribute to antiestrogen resistance (**Fig. 1F**). As shown in **Fig. 1F**, ERα expression was markedly decreased in both YBX1/WT and YBX1/S102E cells versus mock cells, but not in YBX1/S102A cells. YBX1/WT and YBX1/S102E cells showed higher resistance to fulvestrant than mock while YBX1/S102A cells showed similar sensitivity to fulvestrant as mock (**Fig. 1G**). YBX1/WT and YBX1/S102E showed more abundant expression of exogenous YBX1 in the nucleus than YBX1/S102A cells, when the exogenous cytoplasmic YBX1 was similarly expressed in the cytoplasm of YBX1/WT, YBX1/S102A and YBX1/S102E cells (**Fig. 1H** and **I**). This finding was consistent with previous studies (16, 25), indicating that nuclear translocation of YBX1 was promoted through its phosphorylation at S102. Enhanced expression of YBX1 and/or

(*Continued.*) Relative drug sensitivity for each cell line is normalized by IC_{50} value for T-47D as shown in parentheses. **C,** Western blots showing expression of ERα, PgR, EGFR family protein, pYBX1, and ERK/p90RSK and AKT/mTOR signaling molecules in T-47D, FR-1, and FR-2 cells. **D,** Western blots showing YBX1, S6, ERα, and HER2 expression after treatment with YBX1 siRNA (10 nmol/L) or S6 siRNA (10 nmol/L) for 24 hours in T-47D and T-47D/FR-2 cells. **E,** T-47D and T-47D/FR-2 cells were treated with YBX1 siRNA (10 nmol/L) or S6 siRNA (10 nmol/L) for 24 hours, exposed to fulvestrant for 72 hours, and subjected to cell proliferation assays. Data represent the mean ± SD of triplicate dishes. **, $P < 0.01$, two-sided Student *t* test. Values are expressed as the percentage of the value in the absence of drugs. **F,** Top, Schema for YBX1 S102 point mutation. Bottom, Western blots showing pYBX1, YBX1, ERα, and pS6 expression in T-47D cells stably transfected with the YBX1/WT, YBX1/S102A, and YBX1/S102E mutant expression vector. **G,** The sensitivity to fulvestrant was assessed using WST assays for 3 days. Data shown represent mean ± SD of triplicate measurements (percentage cell viability relative to DMSO-treated control). **H,** The GFPs, GFP-YBX1/WT, GFP-YBX1/S102A, and GFP-YBX1/S102E, were expressed in YBX1/WT #2, YBX1/S102A #3, and YBX1/S102E #6 cells. **I,** Expression of endogenous and exogenous YBX1 in cytoplasm and nucleus in YBX1/WT #2, YBX1/S102A #3, and YBX1/S102E #6 cells. CREB is specific marker for nuclear protein, and GAPDH is specific marker for cytoplasmic protein. pYBX1, pAKT (S473), pAKT (T308), pmTOR (S2448), pp70S6K (T389), and pS6 (S235/236) levels were quantified, and the results were indicated as fold change relative to control or parental cells.

pYBX1 may thus play a crucial role in acquired resistance to antiestrogens.

Everolimus suppresses YBX1 phosphorylation in antiestrogen-resistant cell lines

YBX1 phosphorylation is mediated through AKT/mTOR/p70S6K and RAF/MEK/p90RSK signaling pathways (12, 16, 18, 28, 29). We examined whether everolimus, an inhibitor of mTORC1, can suppress YBX1 phosphorylation and overcome antiestrogen resistance. Everolimus significantly inhibited cell proliferation of FR-1 and FR-2 than T-47D cells (**Fig. 2A**). Everolimus also suppressed phosphorylation of p70S6K, S6, and YBX1, but not p90RSK, in antiestrogen-resistant cells (**Fig. 2B**). Furthermore, LY294002 (PI3K/AKT inhibitor) suppressed the phosphorylation of YBX1, AKT, mTOR, and S6 in resistant cells, whereas U0126 (MEK inhibitor) did not (**Fig. 2C**). Collectively, both proliferation and enhanced phosphorylation of YBX1 by acquired antiestrogen resistance in FR cells are mediated through the AKT/mTOR/p70S6K pathway rather than the RAF/MEK/p90RSK pathway.

We examined whether pYBX1 plays an independent survival factor as downstream molecules of AKT/mTOR/p70S6K pathway in breast cancer cells using constitutively active and phosphomimetic mutant YBX1/S102E. The constitutively active mutant YBX1/S102E cells showed approximately 100-fold higher resistance to everolimus than mock and YBX1/WT (**Fig. 2D**). Phosphorylation of endogenous YBX1 and S6 was suppressed by everolimus at similar levels in both YBX1/WT and YBX1/S102E (**Fig. 2E**). By contrast, expression of exogenous YBX1/S102E was not affected by everolimus (**Fig. 2E**), indicating that cell proliferation signals by YBX1/S102E is not under regulation of mTORC1, and also that YBX1/S102E potentially functions as an independent proliferation and survival factor.

We next compared cellular sensitivity to everolimus, AZD8055 (mTORC1/C2 inhibitor), U0126 and fulvestrant in six human breast cancer cell lines including three ERα(+)/HER2(−) cell lines and three ERα(−)/HER2(+) cell lines. Although ERα-positive cell lines showed higher sensitivity to fulvestrant than ERα-negative cell lines, all six cell lines showed similar high sensitivity to everolimus and AZD8055 (Supplementary Table S3). Basal expression levels of YBX1, pYBX1 and other signaling molecules in these six cell lines were compared by western blot analysis in Supplementary Fig. S1. As shown in **Fig. 2F**, everolimus inhibited pmTOR, pp70S6K and pS6 in a dose-dependent manner in all cell lines. Furthermore, everolimus inhibited pYBX1 in all cell lines, indicating that cell proliferation and pYBX1 are regulated by mTOR/p70S6K pathway in the ERα(+)/HER2(−) and ERα(−)/HER2(+) cell lines tested.

TAS0612, a multikinase inhibitor of AKT, p70S6K, and p90RSK, suppresses the phosphorylation and nuclear translocation of YBX1

TAS0612 (**Fig. 3A**) is a new orally bioavailable multikinase inhibitor, which suppresses the binding of AKT/p90RSK/p70S6K to their substrates (35, 40). On the basis of the IC_{50} values obtained after treatment with TAS0612, eight breast cancer cell lines were classified into two groups: sensitive (T-47D, FR-1, and FR-2) and moderately sensitive (KPL-1, MCF-7, SKBr-3, HCC1954, and MDA-MB453; **Fig. 3B**). TAS0612 inhibited pYBX1, pPRAS40 and pS6 in ERα(+)/HER2(−) and ERα(−)/HER2(+) cell lines (**Fig. 3C**). In contrast, the expression levels of pAKT, pp90RSK, and pp70S6K were increased following treatment with relatively higher doses of TAS0612 (**Fig. 3C**). Because TAS0612 inhibits the binding of p90RSK, AKT, or p70S6K to their substrates (35, 40), the accumulated phosphorylation forms of p90RSK, AKT, and p70S6K are totally inactive.

Subsequently, we examined the effect of TAS0612 on fulvestrant resistant cell lines. TAS0612 effectively suppressed phosphorylation of YBX1, PRAS40, mTOR, and S6 in resistant cells (**Fig. 3D**). Assessment by immunocytochemical analysis and cell fractionation revealed that everolimus and TAS0612 suppressed the nuclear translocation of YBX1 in a fulvestrant-resistant cell line (**Fig. 3E** and **F**). Furthermore, we confirmed that TAS0612 and everolimus suppressed phosphorylation of YBX1 and S6 under the same conditions (**Fig. 3F**). It was previously reported that expression of cell proliferation- and cell cycle–related genes was under control of YBX1 (12). TAS0612 suppressed the expression of such proliferation- and cell cycle–related genes, EGFR, HER2, cyclin B1/D1/E and CDC6 (**Fig. 3G**), accompanied by induction of apoptosis (**Fig. 3H**). Thus, TAS0612 markedly inhibits phosphorylation of YBX1 and S6, and reduces the expression of YBX1-targeted cell proliferation–related genes.

TAS0612 inhibits cell proliferation and suppresses the phosphorylation of YBX1 in TNBC cell lines

Approximately 10% to 15% of all patients with breast cancer are TNBC, a type that is intrinsically resistant to endocrine and chemotherapeutic drugs (9). We evaluated YBX1 and pYBX1 (S102) expression of 397 patients with invasive breast cancer [Breast Invasive Carcinoma (TCGA, Provisional)] obtained from The Cancer Genome Atlas (https://cancergenome.nih.gov/; Supplementary Table S2; refs. 41, 42). YBX1 were expressed at similar levels in four subtypes of breast cancer, but pYBX1 expression was found to be significantly higher in tumors of patients with TNBC as compared with other subtypes (**Fig. 4A** and **B**). On the basis of expression levels of pYBX1 protein in breast cancer cell lines tested, TNBC cell lines also showed relatively higher expression levels of pYBX1 as compared with other ER(+) or HER2(+) cell lines (**Fig. 4C**).

Of the six TNBC cell lines that are intrinsically resistant to fulvestrant because of ERα loss, three (i.e., MDA-MB231, SUM149PT, and HCC1143) are resistant to everolimus and AZD8055. The other three cell lines (i.e., SUM159PT, BT549, and Hs578T) are sensitive to these drugs *in vitro* (**Fig. 4D**). Four cell lines, SUM149PT, SUM159PT, BT549, and Hs578T, showed cellular sensitivity to TAS0612 (**Fig. 4D**). The basal expression levels of pYBX1 and its upstream regulatory signaling molecules varied among the six TNBC cell lines (Supplementary Fig. S2A).

Everolimus inhibited expression of pYBX1 in the aforementioned three everolimus sensitive cell lines (SUM159PT, BT549, and Hs578T) and one resistant (HCC1143) cell line, but not in two resistant cell lines, MDA-MB231 and SUM149PT (Supplementary Fig. S2B). In contrast, as shown in **Fig. 4E**, TAS0612 effectively suppressed expression of pYBX1 in all TNBC cell lines, including two everolimus-resistant cell lines (MDA-MB231 and SUM149PT). Furthermore, TAS0612 markedly suppressed YBX1-targeted genes and induced apoptosis accompanied by reduction of pYBX1 expression (**Fig. 4F**). On the basis of these findings, it is suggested that mTOR/p70S6K is the main pathway involved in phosphorylation of YBX1 and proliferation in SUM159PT, BT549, and Hs578T cells.

Treatment with TAS0612 or everolimus shows antitumor effects against antiestrogen-resistant cancer cells and TNBC cells

In an antiestrogen-resistant T-47D/FR-2 orthotopic xenograft mice model, tamoxifen did not suppress tumor growth (**Fig. 5A** and **B**). In contrast, everolimus or TAS0612 markedly suppressed tumor growth (tumor volume and weight) in a FR-2 xenograft model (**Fig. 5A** and **B**). There was no marked weight loss in everolimus or TAS0612-treated

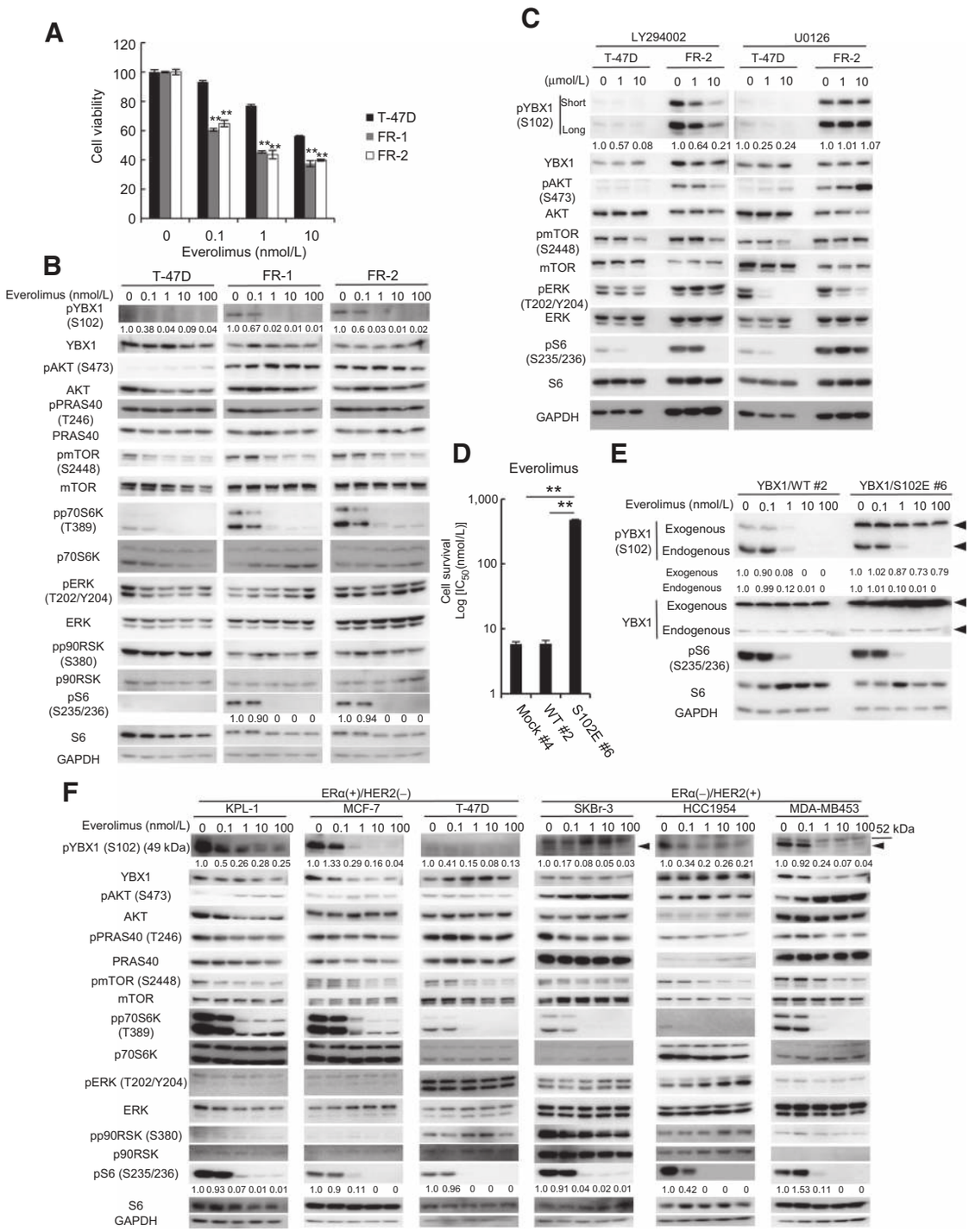


Figure 2.

Everolimus suppresses YBX1 phosphorylation in antiestrogen-resistant cells. **A,** The sensitivity to everolimus was assessed using WST assays for 3 days. Data shown represent mean ± SD of triplicate measurements (percentage cell viability relative to DMSO-treated control). **, $P < 0.01$, two-sided Student *t* test. **B,** Effect of everolimus on expression and phosphorylation of ERK/p90RSK and AKT/mTOR signaling molecules. Cells were exposed to everolimus for 6 hours. **C,** Effect of LY294002 and U0126 on expression and phosphorylation of ERK/p90RSK and AKT/mTOR signaling molecules. Cells were treated with LY294002 and U0126 for 6 hours. **D,** The sensitivity to everolimus was assessed using WST assays for 3 days. Data represent the IC_{50} mean ± SD of triplicate dishes for each cell line. **, $P < 0.01$, two-sided Student *t* test. **E,** Inhibitory effect of everolimus on expression of pYBX1 and pS6. Cells were treated with everolimus at the indicated concentrations for 6 hours. **F,** Effect of everolimus on expression and phosphorylation of ERK/p90RSK and AKT/mTOR signaling molecules. Cells were treated with everolimus at the indicated concentrations for 6 hours. pYBX1 and pS6 levels were quantified and the results were indicated as fold change relative to control cells.

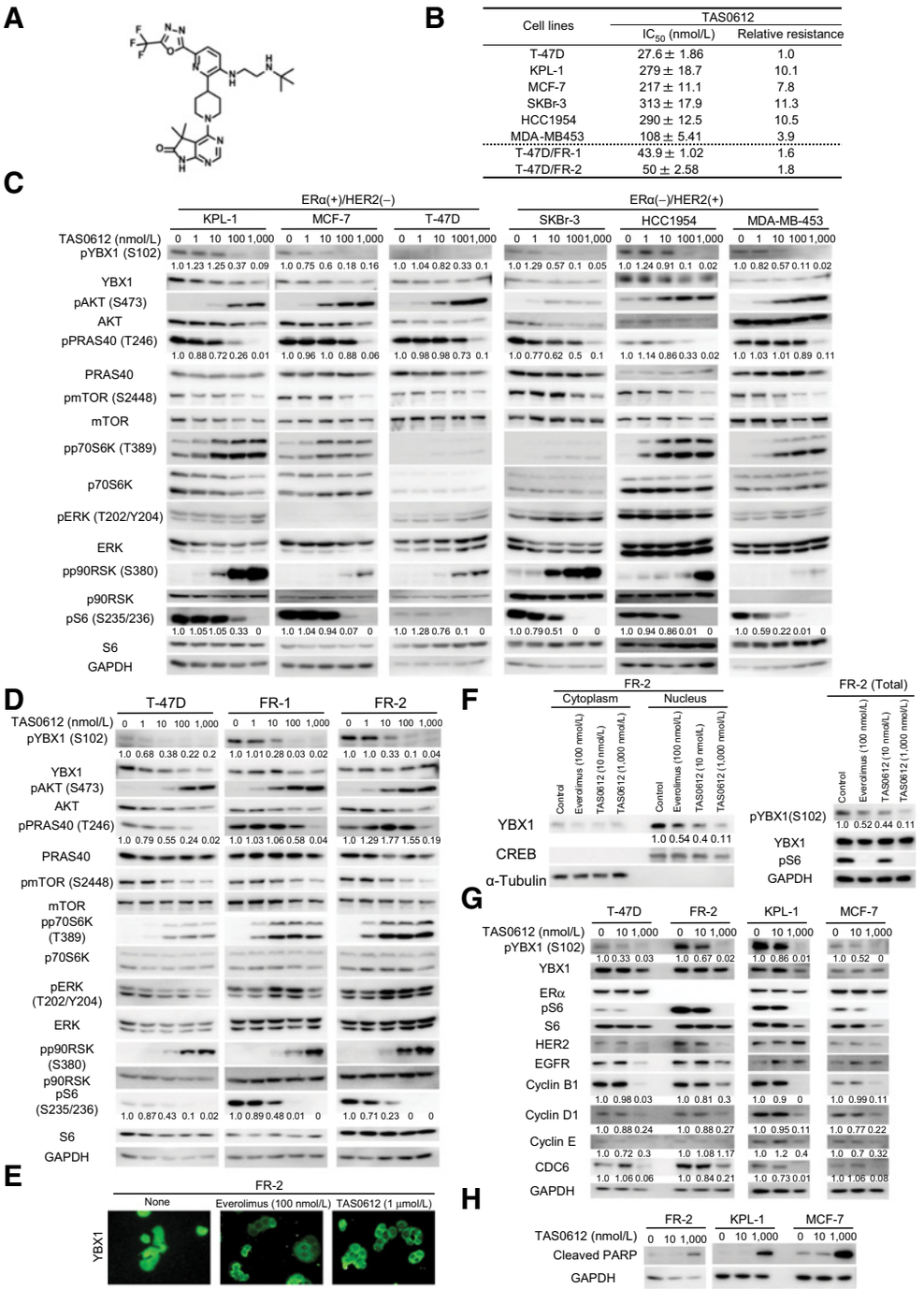
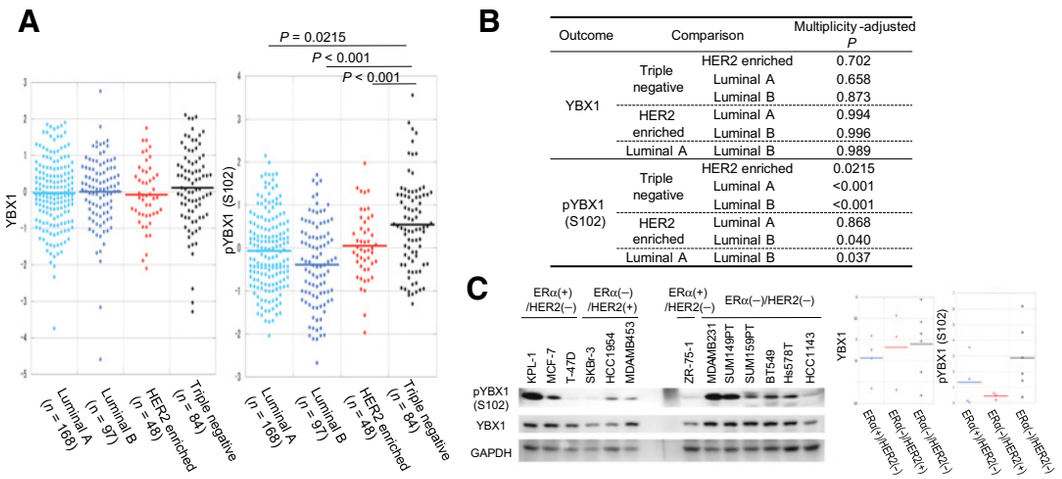


Figure 3.

A novel multikinase inhibitor TAS0612 suppresses YBX1 phosphorylation in fulvestrant-resistant FR cells. **A,** Structure of TAS0612. **B,** WST assay for 3 days by 8 breast cancer cell lines. Data represent the IC_{50} mean ± SD of triplicate dishes for each cell line. Relative drug sensitivity for each cell line is normalized by IC_{50} value for T-47D as shown in relative resistance ratio. **C** and **D,** Effect of TAS0612 on expression and phosphorylation of ERK/p90RSK and AKT/mTOR signaling molecules in 6 breast cancer cell lines (**C**) or T-47D, FR-1, and FR-2 cells (**D**). Cells were treated with TAS0612 at the indicated concentrations for 6 hours. **E,** Immunofluorescent staining for YBX1. FR-2 cells were treated with everolimus or TAS0612 for 24 hours and then stained with YBX1. **F,** Effect of everolimus or TAS0612 on the nuclear translocation of YBX1. Cytoplasmic and nuclear extracts were prepared at 24 hours after treatment with everolimus and TAS0612. CREB and α-tubulin are shown as a loading control for nuclear extract and cytoplasmic extract, respectively. **G,** Effect of TAS0612 on expression of YBX1-targeted genes, including HER2, EGFR, cyclin B1/D1/E, and CDC6, in four breast cancer cell lines. Cells were treated with TAS0612 at the indicated concentrations for 72 hours. **H,** The effect of TAS0612 on cell apoptosis. Cells were treated with TAS0612 at the indicated concentrations for 72 hours. pYBX1, pPRAS40, pS6, YBX1, cyclin B1/D1/E, and CDC6 levels were quantified, and the results were indicated as fold change relative to control cells.



B

Outcome	Comparison		Multiplicity-adjusted *P*
YBX1	Triple negative	HER2 enriched	0.702
		Luminal A	0.658
		Luminal B	0.873
	HER2 enriched	Luminal A	0.994
		Luminal B	0.996
	Luminal A	Luminal B	0.989
pYBX1 (S102)	Triple negative	HER2 enriched	0.0215
		Luminal A	<0.001
		Luminal B	<0.001
	HER2 enriched	Luminal A	0.868
		Luminal B	0.040
	Luminal A	Luminal B	0.037

D

Cell lines	ERα	HER2	PIK3CA	PTEN	BRAF	KRAS	HRAS	Fulvestrant (nmol/L)	TAS0612 (nmol/L)	Everolimus (nmol/L)	AZD8055 (nmol/L)	U0126 (μmol/L)
T-47D	+	-	H1047R	WT	WT	WT	WT	68.3 ± 0.77 (1.00)	27.6 ± 1.86 (1.00)	0.74 ± 0.22 (1.00)	33.1 ± 0.99 (1.00)	> 30
ZR-75-1	+	-	WT	-	WT	WT	WT	243 ± 5.27 (3.56)	27.1 ± 2.52 (0.98)	5.85 ± 0.24 (7.90)	35.9 ± 1.60 (1.08)	27.7 ± 5.30
MDA-MB231	-	-	WT	WT	G464V	G13D	WT	> 10,000	6,150 ± 620 (222.2)	> 1,000	957 ± 27.2 (29.0)	22.8 ± 1.62
SUM149PT	-	-	WT	WT	WT	WT	WT	> 10,000	135 ± 3.05 (4.89)	> 1,000	490 ± 15.5 (14.8)	17.7 ± 0.03
SUM159PT	-	-	H1047L	WT	WT	WT	G12D	> 10,000	187 ± 5.24 (6.79)	6.46 ± 0.77 (8.73)	163 ± 23.4 (4.92)	11.7 ± 1.02
BT549	-	-	WT	-	WT	WT	WT	> 10,000	333 ± 24.4 (12.1)	5.60 ± 0.70 (7.57)	60.2 ± 5.62 (1.82)	> 30
Hs578T	-	-	WT	WT	WT	WT	G12D	> 10,000	227 ± 14.9 (8.23)	8.03 ± 0.23 (10.9)	62.3 ± 4.45 (1.88)	> 30
HCC1143	-	-	WT	WT	WT	WT	WT	> 10,000	2,744 ± 134.1 (99.4)	> 1,000	558 ± 11.2 (16.9)	26.5 ± 3.02

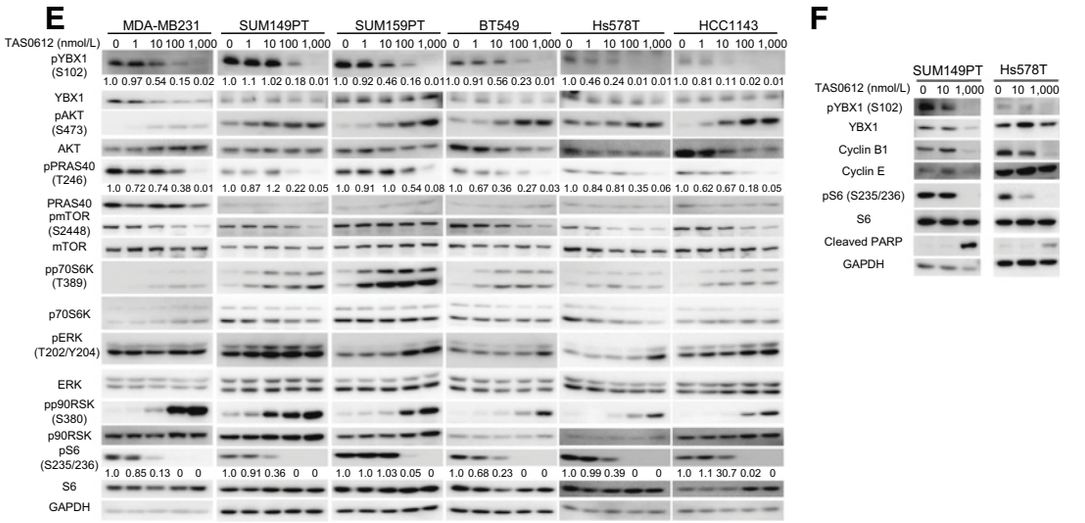


Figure 4.

TAS0612 suppresses YBX1 phosphorylation in TNBC cells. **A,** Dot plot analyses of YBX1 and pYBX1 (S102) expression among Luminal A ($n = 168$), Luminal B ($n = 97$), HER2-enriched ($n = 48$), and triple-negative ($n = 84$) subtypes. Data obtained from TCGA [Breast Invasive Carcinoma (TCGA, Provisional)] dataset using cBioPortal. **B,** Statistics calculated by two-sided Student *t* test with Tukey–Kramer multiplicity adjustment for all pairwise comparisons. **C,** Expression of YBX1 and pYBX1 (S102) in 13 breast cancer cell lines (left). Dot plot analyses of YBX1 and pYBX1 (S102) expression (normalized to GAPDH) among 13 breast cancer cell lines (right). **D,** IC_{50} values of fulvestrant, TAS0612, everolimus, AZD8055, and U0126 in TNBC, ER(−)/HER2(−), human breast cancer cell lines. IC_{50} values are average of triplicate dishes for each cell lines. Relative drug sensitivity for each cell line is normalized by IC_{50} value for T-47D as shown in parentheses. IC_{50} values of T-47D are presented in Supplementary Table S3. IC_{50} values of another ER(+) cell line, ZR-75-1, are also presented as controls. **E,** The effect of TAS0612 on expression and phosphorylation of ERK/p90RSK and AKT/mTOR signaling molecules in six TNBC cell lines. Cells were treated with TAS0612 at the indicated concentrations for 6 hours. pYBX1, pPRAS40, and pS6 levels were quantified, and the results were indicated as fold change relative to control cells. **F,** Effect of TAS0612 on the expression of YBX1-targeted genes in two TNBC cell lines. Cells were treated with TAS0612 at the indicated concentrations for 72 hours.

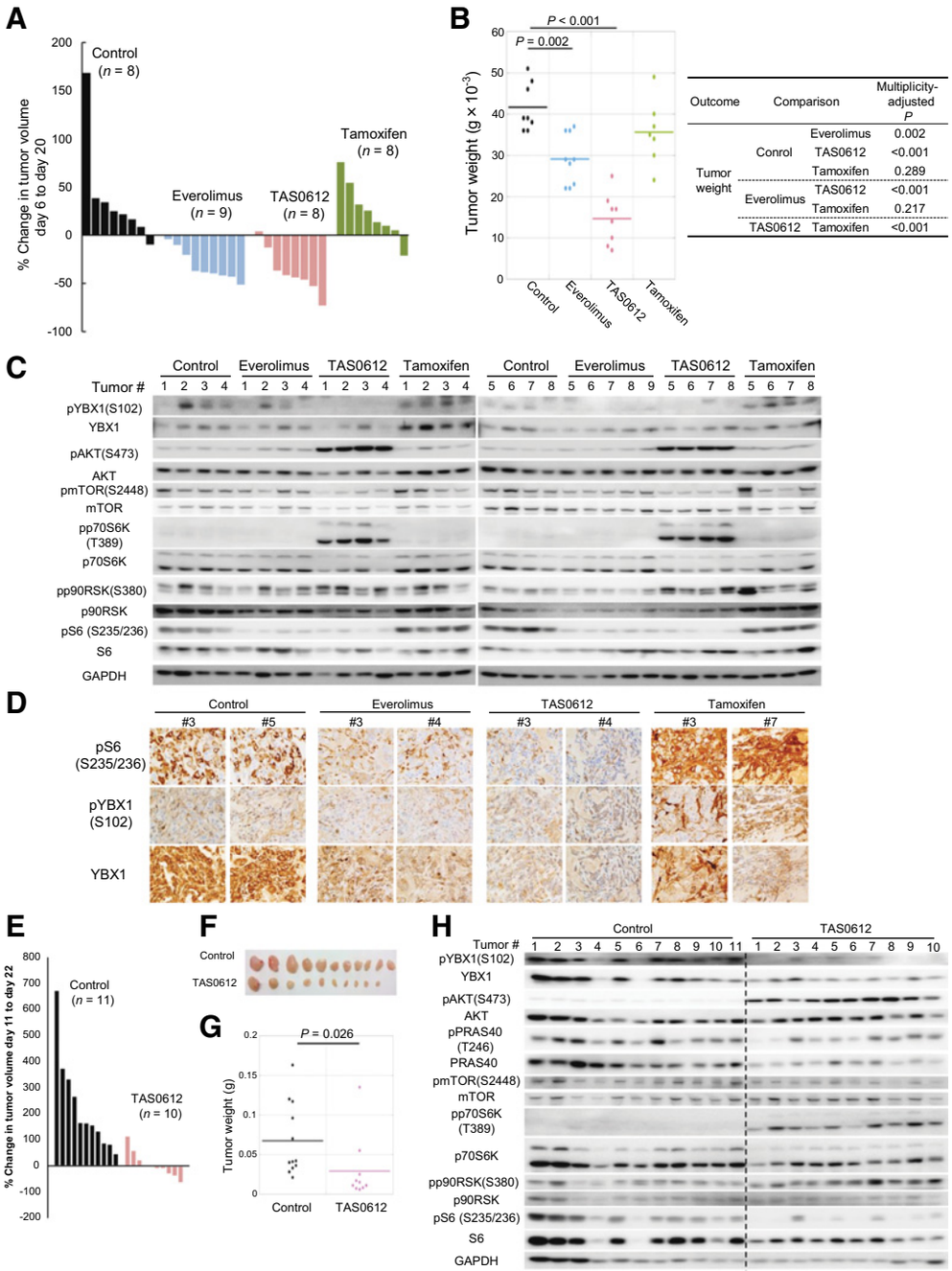


Figure 5.

TAS0612 or everolimus shows antitumor effect against antiestrogen-resistant cells and TNBC cells *in vivo*. **A,** T-47D/FR-2 cells were orthotopically implanted into the fourth mammary fat pad on both sides. After tumor established, mice were sorted into 4 groups and treated with everolimus (2 mg/kg/mouse, daily, orally), TAS0612 (50 mg/kg/mouse, daily, orally), or tamoxifen citrate (500 μg/mouse, daily, s.c.). Waterfall plot depicting change in tumor volume after 14 days of treatment with agents as indicated. Each of the four experimental groups of mice is treated as part of the same experiment. Each bar represents an individual tumor (control, $n = 8$; everolimus, $n = 9$; TAS0612, $n = 8$; tamoxifen, $n = 8$). **B,** Comparison of tumor weights of four experimental groups on day 20 after inoculation. Statistics calculated by two-sided Student *t* test with Tukey–Kramer multiplicity adjustment for all pairwise comparisons. **C,** Western blot analysis of tumors treated with everolimus, TAS0612, or tamoxifen for 14 days. **D,** IHC analysis of YBX1, pYBX1, and pS6 expression in FR-2 tumors after 14 days of treatment with everolimus, TAS0612, or tamoxifen. Representative tumor samples of each group are shown. **E,** SUM159PT cells were orthotopically implanted into the fourth mammary fat pad on both sides. After tumor established, mice were treated with TAS0612 (50 mg/kg/mouse, daily, orally). Waterfall plot depicting change in tumor volume after 11 days of treatment with agent as indicated. Each of the two experimental groups of mice is treated as part of the same experiment. Each bar represents an individual tumor (control, $n = 11$; TAS0612, $n = 10$). The image (**F**) and weight (**G**) of SUM159PT tumors are shown. Statistics calculated by two-sided Student *t* test. **H,** Western blot analysis of tumors treated with TAS0612 for 11 days.

mice (Supplementary Fig. S3). The expression of pYBX1 and pS6 was decreased in almost all everolimus- or TAS0612-treated tumors, compared with control or tamoxifen-treated tumors (**Fig. 5C**). In addition, IHC analyses also revealed that treatment with everolimus or TAS0612 decreased the expression of pYBX1 and pS6 (**Fig. 5D**). Collectively, TAS0612 or everolimus overcomes acquired resistance to antiestrogen.

We also examined whether TAS0612 showed any antitumor effect on TNBC by an orthotopic xenograft mouse model of SUM159PT. Treatment with TAS0612 significantly suppressed tumor growth in terms of both volume and weight (**Fig. 5E–G**). This effect was accompanied by reduced expression of pYBX1 and pS6 in almost all TAS0612-treated tumors (**Fig. 5H**). In addition, there was increased accumulation of inactive pAKT, pp70S6K, and pp90RSK in treated tumors (**Fig. 5H**). Collectively, TAS0612 is effective in the treatment of TNBC.

The expression of YBX1 and pYBX1 is enriched in tumors of patients refractory to endocrine therapy

We finally examined whether the increased expression of pYBX1 and YBX1 plays a clinically important role in breast cancer resistance to endocrine therapy. We followed up 61 ERα-positive patients during treatment with tamoxifen, exemestane, anastrozole, and a luteinizing hormone-releasing hormone agonist, and found that 12 patients relapsed after receiving endocrine therapy (**Fig. 6A**; Supplementary Table S1). All nonrecurrent patients ($n = 49$) showed low expression of YBX1 in pretreatment tumors, but recurrent patients ($n = 12$) already showed relatively higher expression of YBX1 in pretreatment tumors ($P = 0.05$) when assessed by IHC analysis (**Fig. 6A**). These recurrent patients showed variable levels of YBX1 in pretreatment tumors of recurrent patients (**Fig. 6A** and **B**). Especially, pretreatment tumors of pt. #2, #3, and #4 showed relatively higher expression of YBX1 than other tumors.

Of the 12 patients who were refractory to endocrine therapy, seven premenopausal women were treated with tamoxifen or a luteinizing hormone-releasing hormone agonist for 2 to 6 years, whereas five postmenopausal women were treated with aromatase inhibitors for 5 to 11 years, as adjuvant therapy, followed by tumor recurrence (Supplementary Table S1). **Figure 6B** shows representative IHC images of YBX1, pYBX1, and pS6 in all 12 clinical samples before (pre) and after (post) the administration of endocrine therapy. YBX1 is expressed in both the cytoplasm and nucleus, whereas pYBX1 is mainly expressed in the nucleus of cancer cells in most recurrent samples (**Fig. 6B**). The expression levels of YBX1, pYBX1, and pS6 were quantitated by analyzing the expression levels of five independent areas per tumor, and heat map data of pre- and post-endocrine therapy are presented (**Fig. 6C**). The posttreatment tumors (pt. #5–12) with recurrent periods >3 years showed more abundant expression of YBX1, pYBX1, and pS6 versus the pretreatment tumors. Of note, four patients (pt. #1–4) showed recurrence within 3 years, and three of those (pt. #2–4) demonstrated increased expression of both YBX1 and pYBX1 in pretreatment tumors versus posttreatment tumors (**Fig. 6B** and **C**). The expression of pYBX1 and YBX1 thus closely correlates with the acquirement of drug resistance in ERα-positive breast cancer following treatment with endocrine therapeutic drugs.

Discussion

Our previous study demonstrated that overexpression of YBX1 induces marked reduction of ERα, accompanied by acquired resistance to antiestrogen (22). In this study, we newly found that the acquired antiestrogen resistance was specifically characterized by a marked increase in the expression of pYBX1 with a marked decrease in ERα expression. Exogenous expression of the constitutive active YBX1 (YBX1/S102E) specifically induced a marked decrease in ERα expression and resistance to fulvestrant in breast cancer cells. Conversely, YBX1 silencing specifically induced increased ERα expression and sensitivity to fulvestrant, resulting in overcoming acquired antiestrogen resistance. The YBX1 phosphorylation status *per se* thus limits the responsiveness to antiestrogens in breast cancers through its regulatory role in the expression of ERα and its targeted genes. We also found that enhanced expression of pYBX1 is accompanied by concomitant activation of AKT/mTOR/p70S6K and/or RAF/MEK/p90RSK signaling pathways (**Fig. 6D**). Treatment with inhibitors of PI3K/AKT, mTOR, and MEK suppresses phosphorylation of YBX1 and also overcome antiestrogen resistance, by breast cancer cells (**Fig. 6D**).

In our therapeutic experimental models, everolimus overcame acquired resistance to antiestrogen (**Fig. 5**). Fulvestrant resistant cells showed a 10-fold higher collateral sensitivity to everolimus versus their sensitive counterpart, together with increased expression of pYBX1 (**Figs. 1C** and **2A**). The increased expression of pYBX1 in FR cells was suppressed by everolimus and a PI3K inhibitor. However, this effect was not induced by a MEK inhibitor (**Fig. 2C**). The expression of pYBX1 and cell proliferation in these resistant cells mainly depend on the AKT/mTOR/p70S6K pathway. Collectively, everolimus is effective for overcoming antiestrogen resistance in breast cancer with activated AKT/mTOR/p70S6K pathway (**Fig. 6D**).

On the other hand, Ichikawa and colleagues recently reported that TAS0612 exhibits strong antitumor effect in preclinical tumor models with deregulated RAS and PI3K pathway activities, and evaluations are currently ongoing to support clinical development of TAS0612 (40). TAS0612 was also found to overcome antiestrogen resistance through suppression of pYBX1 expression and the nuclear translocation of YBX1 (**Fig. 3** and **5**). Furthermore, although TAS0612 suppressed the expression of pYBX1 in all tested six TNBC cell lines, MDA-MB231 and HCC1143 did not show cellular sensitivity to TAS0612, and these two cell lines are only sensitive to U0126 (**Fig. 4D**). Concerning the MEK/ERK signaling pathway, phosphorylated ERK (pERK) forms homodimers and translocates to the nucleus (43). The pERK directly activates various transcription factors including Elk-1 and Ets-2, resulting in upregulation of c-Fos, c-Jun, c-Myc and cyclin D1 in cancer cells including breast cancer (43). It is generally accepted that the RAF/MEK/ERK pathway not through p90RSK directly leads to enhanced cell proliferation. Thus, cell proliferation in MDA-MB231 and HCC1143 may be directly induced by MEK/ERK.

Furthermore, TAS0612 showed potent antitumor effects and marked inhibition of pYBX1 and pS6 in the experimental therapeutic model of TNBC (**Fig. 5E–G**). The RAF/MEK/p90RSK pathway is often activated in numerous human malignancies, and selective targeting of the p90RSK family has been expected to modulate chemoresistance (31). The MEK/ERK cascade is also a major target for the development of potent therapeutics against progressive breast cancer (44). **Figure 4A** and **B** demonstrates significantly enhanced expression of pYBX1 in TNBC as compared with other subtypes of breast cancer, suggesting pYBX1 contributes to progression of some of TNBC. It is expected that TAS0612 targeting both the AKT/mTOR/p70S6K and RAF/MEK/p90RSK signaling pathways may improve the therapy for progressive breast cancer including TNBC.

Phosphorylation of YBX1 and S6 is facilitated by the AKT/mTOR/p70S6K and RAF/MEK/p90RSK signaling pathways and was

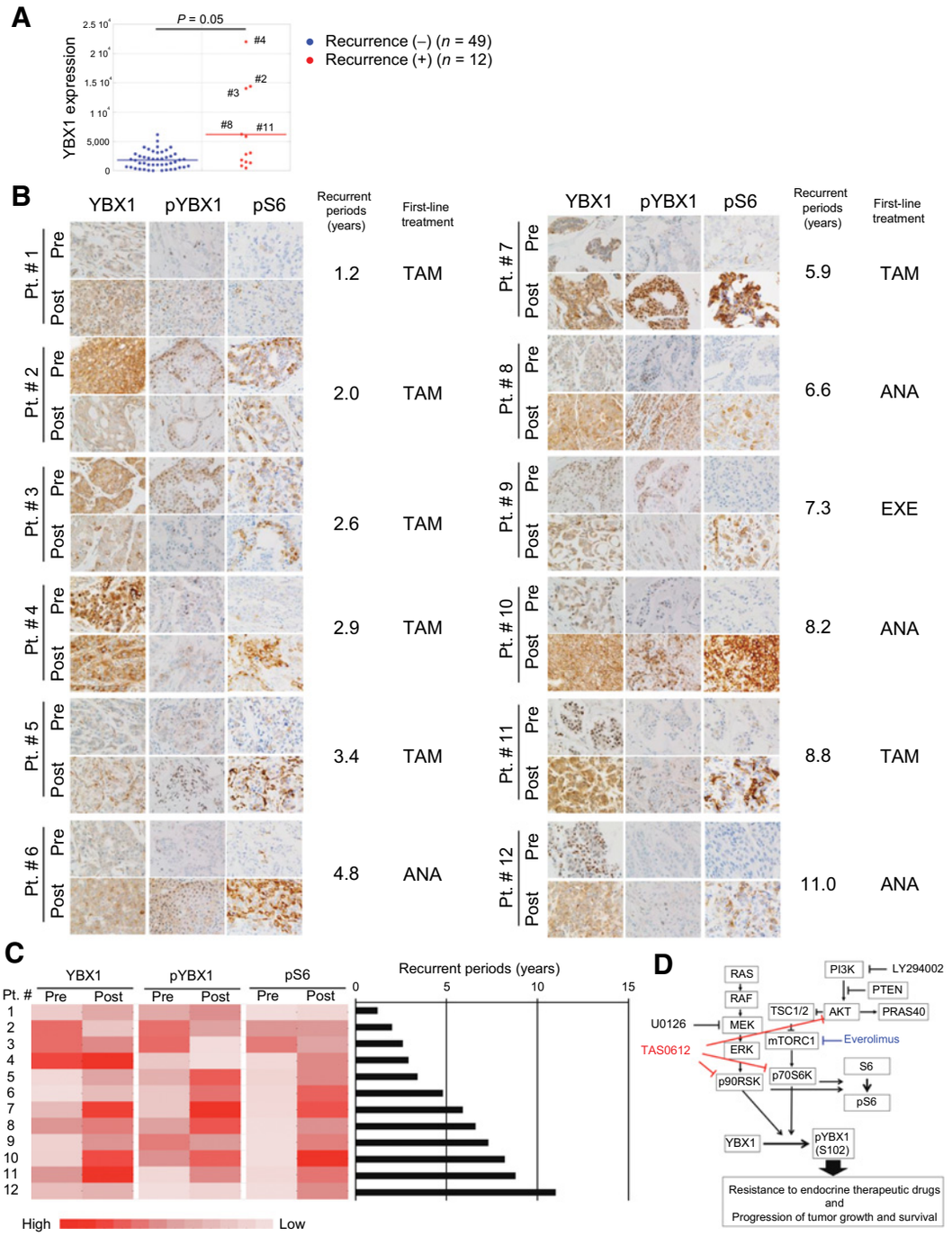


Figure 6.

Enhanced expression of YBX1 and pYBX1 in recurrent breast cancer after adjuvant endocrine therapies. **A,** Dot plots showing the expression levels of YBX1 quantified by IHC analysis in the primary tumors of patients who did not relapse ($n = 49$) after the initial surgical operation and in the primary tumors of patients who relapsed ($n = 12$). The recurrent tumors with higher YBX1 expression are indicated by patient number. Statistics calculated by two-sided Student *t* test. An overview of the clinical information of each recurrent patient is presented in Supplementary Table S1. **B,** IHC analysis of YBX1, pYBX1, and pS6 expression in breast cancer specimens (×400 original magnification) in paired preendocrine and postendocrine therapeutic samples of cancer patients. **C,** Differential expression of YBX1, pYBX1, and pS6. The heatmap depicts the expression of YBX1, pYBX1, and pS6 in paired preendocrine and postendocrine therapeutic samples of patients with cancer; red and white rectangles indicate high and low protein expression, respectively (left). Recurrent periods of each patient (right). **D,** Mechanistic model depicting how drugs targeting YBX1 phosphorylation pathway overcomes progression and resistance to endocrine therapeutics in breast cancer. YBX1 is phosphorylated by AKT/mTOR/p70S6K and/or MEK/ERK/p90RSK signaling pathway in breast cancer cells. YBX1 phosphorylation *per se* thus plays a crucial role in cell proliferation, survival, and drug resistance. TAS0612 or everolimus is expected to overcome progression and drug resistance. ANA, anastrozole; EXE, exemestane; TAM, tamoxifen.

markedly inhibited by TAS0612 or everolimus in fulvestrant resistant cells, resulting in overcoming acquired antiestrogen resistance (**Fig. 6D**). YBX1 silencing also augmented cellular sensitivity to fulvestrant in T-47D/FR-2 cells, but S6 silencing did not affect cellular sensitivity to fulvestrant. Furthermore, forced expression of YBX1 phosphomimic mutant (YBX1/S102E) induced fulvestrant resistance, but expression levels of pS6 and S6 were not affected by the phosphomimic mutant. These data indicate that pYBX1 as a downstream molecule of AKT/mTOR/p70S6K and RAF/MEK/p90RSK signaling pathways specifically induced antiestrogen resistance. However, pS6 was not associated with sensitivity to antiestrogen and cell growth in fulvestrant resistant cells. Therefore, inhibition of pYBX1 is required to inhibit cell growth and survival in fulvestrant-resistant cells. We conclude that pYBX1 functions as an oncogenic driver of drug resistance in breast cancer. It is also stressed that expression of pYBX1 and YBX1 is often upregulated in recurrent posttreatment tumors after adjuvant therapy (**Fig. 6B** and **C**) as well as TNBC (**Fig. 4A** and **B**). Determination of YBX1 and its phosphorylated form may also contribute to the prediction of responses to anticancer drugs and the progression status of breast cancer.

Disclosure of Potential Conflicts of Interest

S. Hattori is a statistical consultant at Chugai Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Hattori, J. Akiba, M. Tanaka
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