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Estrogen causes ATBF1 protein degradation through the estrogen-responsive E3 ubiquitin ligase EFP

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Abstract

We previously revealed that tumor suppressor ATBF1 formed an autoregulatory feedback loop with estrogen-ER α signaling to regulate estrogen-dependent cell proliferation in breast cancer cells. In this loop, ATBF1 inhibits the function of estrogen-ER α signaling while ATBF1 protein levels are fine-tuned by estrogen-induced transcriptional upregulation as well as ubiquitin proteasome pathway (UPP)-mediated protein degradation. Here we show that the estrogen-responsive finger protein (EFP) is an E3 ubiquitin ligase mediating estrogen-induced ATBF1 protein degradation. Knockdown increases but overexpression of EFP decreases ATBF1 protein levels. EFP interacts with and ubiquitinates ATBF1 protein. Furthermore, we show that EFP is an important factor in estrogen-induced ATBF1 protein degradation in which some other factors are also involved. In human primary breast tumors, due to both as directly-upregulated ER α target gene products, the levels of ATBF1 protein are positively correlated with the levels of EFP protein. However, the ratio of ATBF1 protein to EFP protein is negatively correlated with EFP protein levels. Functionally, ATBF1 antagonizes EFP-mediated cell proliferation. These findings not only establish EFP as the E3 ubiquitin ligase for estrogen-induced ATBF1 protein degradation, but further support the autoregulatory feedback loop between ATBF1 and estrogen-ER α signaling and thus implicate ATBF1 in estrogen-dependent breast development and carcinogenesis.

Keywords

Estrogen; ATBF1; Protein degradation; E3 ubiquitin ligase; EFP; Breast cancer

Introduction

ATBF1, whose gene was first isolated from human hepatoma cells based on the ability of its product to bind an AT-rich enhancer element of the human α -fetoprotein (AFP) gene [1], is a transcription factor composed of 3,703 amino acid residues containing 1 ATPase A-motif, 2 DEAH box-like sequences, 4 homeodomains and 23 zinc finger motifs involved in transcriptional regulations and protein-protein interactions of cancer-related genes. A number of studies suggest that ATBF1 is a potential tumor suppressor gene. For example, it suppresses the expression of AFP oncoprotein by binding to the AT motif competitively with hepatocyte nuclear factor 1 (HNF1), and cooperates with p53 to activate the CDKN1A (p21) tumor suppressor resulting in cell cycle arrest [2]. In addition, ATBF1 regulates

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myoblastic differentiation and interacts with Myb to repress the transcriptional activity of this oncoprotein [3]. It also interacts with PIAS3 in suppressing oncogenic STAT3 signaling [3]. In prostate cancer, frequent somatic mutations were identified, and a germline mutation is significantly associated with an increased prostate cancer risk [4].

In breast cancer, although few mutations were detected, ATBF1 undergoes frequent genomic deletion and transcriptional downregulation [5–7], and ATBF1 mRNA expression is significantly associated with better patient survival [8]. There also appears to be an association between ATBF1 mRNA expression and ER α positivity in human breast tumors [8]. In characterizing the relationship between ATBF1 and estrogen-ER α signaling, we have identified an autoregulatory feedback loop between ATBF1 and estrogen-ER α signaling, which may play an important role in estrogen-dependent breast development and tumorigenesis [9, 10]. In this loop, while it inhibits the function of estrogen-ER α signaling by at least in part selectively competing for ER α binding with the steroid receptor coactivator and potent oncoprotein AIB1 [10–12], ATBF1 protein levels are fine-tuned by estrogen-induced transcriptional upregulation and protein degradation [9]. We demonstrated that estrogen upregulated the transcription of ATBF1 by the direct binding of ER α onto its promoter and that the estrogen-responsive ubiquitin proteasome pathway (UPP) is involved in estrogen-induced ATBF1 protein degradation [9]. The deep mechanism for estrogen-induced ATBF1 protein degradation involving the UPP remains to be identified.

The UPP has emerged as a major player in fine-tuning protein levels in a variety of cellular processes. Protein degradation mediated by the UPP involves protein ubiquitination and subsequent proteasomal destruction [13]. Ubiquitination is operated by three critical enzymes, including the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme (UBC) and E3 ubiquitin ligase [14, 15]. The UPP has been well implicated in estrogen-ER α signaling, primarily through regulating the expression of multiple E3 ubiquitin ligases [16]. The estrogen-responsive finger protein EFP is one such E3 ubiquitin ligase. It is a member of the RING finger family of E3 ubiquitin ligases identified by the binding of ER α onto its genomic sequence, the existence of a typical estrogen-responsive element (ERE), and its estrogen-dependent expression pattern in tissues [17, 18]. EFP is essential for estrogen-dependent cell proliferation [19], and its protein expression positively associates with lymph node metastasis, ER α status, and poor prognosis of breast cancer patients [20]. Here, we show that EFP is an E3 ubiquitin ligase mediating estrogen-induced ATBF1 protein degradation, casting further light on the importance of ATBF1 in estrogen-dependent breast development and carcinogenesis.

Experimental Materials and Methods

Plasmids, reagents and cell lines

The plasmids of pCMV-ER α , FLAG-ATBF1, HA-ATBF1, HA-KLF5, HA-Ub and HA-tagged ATBF1 protein deletion mutants were described previously [10, 21]. The plasmid of EFP in the vector pFLAG-CMV-2 was a gift from Dr. Dong-Er Zhang at the Scripps Research Institute [22]. The coding region of EFP was subcloned into the pcDNA3.1 vector to express EFP used in the ubiquitination analysis. Plasmids of FLAG-ATBF1D and FLAG-ATBF1F were obtained by subcloning ATBF1D and ATBF1F into the FLAG-pcDNA3 vector from HA-ATBF1D and HA-ATBF1F plasmids, respectively [10]. Plasmids for EFP deletion mutants were created by cloning PCR products into the FLAG-pcDNA3 vector with the EFP plasmid as the template. Primer sequences for creating EFP deletion mutants are listed in Table S1.

The 17 β -estradiol (E2), MG132, polyclonal anti-FLAG, anti-HA and anti- β -actin antibodies, monoclonal anti-FLAG M2 antibody, anti-HA affinity gel, and protease inhibitor mixture

cocktail were purchased from Sigma. Monoclonal anti-ER α antibody was obtained from Upstate. Monoclonal anti-EFP antibody was obtained from BD Biosciences. Goat anti-rabbit second antibody was obtained from Cell Signaling Technology. Polyclonal anti-ATBF1 antibodies were described previously [10].

Breast cancer cell lines MCF7 and T-47D and prostate cancer cell line 22Rv1 were purchased from American Type Culture Collection (Manassas, VA) and propagated following standard protocols from ATCC. For hormone-free experiments, cells were grown in phenol-free media supplemented with 5% dextran-charcoal-stripped fetal bovine serum (FBS, Hyclone) for 72 hours before seeding for experiments.

Gene expression analysis

Gene expression was quantified by real time PCR as described in our previous studies [23, 24]. Primer sequences for all the genes are listed in Table S2.

RNA interference (RNAi) and plasmid transfection assays

RNAi against ATBF1 and EFP genes and ATBF1-specific siRNAs and the negative control siRNA against the luciferase gene were described previously [10]. A siRNA pool containing three EFP-specific siRNAs from the Santa Cruz Biotechnology company was used for RNAi against the EFP gene. Plasmid transfection assays were performed with Lipofectamine 2000 (Invitrogen) following the protocol recommended by the manufacturer.

Cycloheximide (CHX) chase assays

Cells were seeded into 12-well plates at a density of 2×10^5 cells per well 24 hours before transfection with different combinations of ATBF1, EFP and control plasmids in triplicate. Forty hours post-transfection, CHX chase assays were performed as described previously [9].

Immunoprecipitation (IP) and western blot assays

IP and western blot assays were performed as described previously [10].

In vivo ubiquitination assays

Cells grown in 100 mm dishes were transfected with plasmids of FLAG-ATBF1D or FLAG-ATBF1F, HA-Ub, pCMV-ER α or CMV-EFP or siRNA against EFP as indicated in figures. Forty hours post-transfection, cells were treated with MG132 at the final concentration of 20 μ M for four hours. At the end of treatments, cells were washed twice with ice-cold PBS and then lysed in modified RIPA buffer [10]. Cell lysates were centrifuged to collect supernatants, which were then incubated with anti-FLAG M2 antibody overnight with rotation at 4°C. After washing three times with modified RIPA buffer, immunoprecipitates, together with cell lysates as input, were boiled for 10 minutes in the SDS-PAGE loading buffer, resolved in 10% SDS-PAGE gels, and then blotted with anti-HA-Ub, anti-FLAG, anti-ER α , anti-EFP and anti- β -actin antibodies, respectively, following standard western blot procedures.

Immunohistochemistry analysis of human primary breast tumors

Immunohistochemistry was performed on multiple tumor arrays prepared from 140 formalin-fixed, paraffin-embedded surgical specimens of breast cancer with anti-ATBF1 (1:400 dilution) and anti-EFP (1:400 dilution) antibodies, respectively. All tumor tissues dotted on the arrays were pathologically confirmed. The staining procedures followed the protocols recommended by the manufacturers and the preimmune sera were used as the negative control. The staining levels were scored with the modified McCarty's H-scoring

system based on the percentage of positive cells and the staining intensity [25]. The staining was grouped as negative (–, H-score <50), weakly positive (+, H-score 50–100), moderately positive (++, H-score 100–200), and strongly positive (+++, H-score > 200).

Cell proliferation assays

Cell proliferation was analyzed as described previously [10]. Briefly, MCF7 and T-47D were seeded in 12-well plates at a density of 1×10^5 cells per well with (2- ^{14}C)-thymidine added in media as an internal control. On the following day, after washed three times with media to remove unincorporated thymidine, MCF7 cells were transfected with siRNAs against ATBF1, EFP and luciferase genes, and T-47D cells with FLAG-ATBF1, FLAG-EFP and control plasmids as indicated in Figure 6. Forty-eight hours after transfection, media were replaced with media containing (methyl- ^3H)-thymidine. After being incubated for another four hours for thymidine incorporation, cells were washed three times with PBS and transferred onto glass microfiber filters (VWR Scientific) to measure ^3H and ^{14}C radioactivity in ScintiSafe Econo 1 solution (Fisher Scientific) using an LS 6500 multi-purpose scintillation counter (Beckman Coulter). Rates of DNA synthesis were indicated by the ratio between ^3H and ^{14}C readings in each sample. The expression of ATBF1 and EFP proteins was monitored by western blot analysis.

Statistical Analysis

Statistical analysis was performed using SAS and SPSS software packages to compare differences among different groups as indicated in figure legends, and $P < 0.05$ was considered statistically significant.

RESULTS

The estrogen-responsive E3 ubiquitin ligase EFP mediates ATBF1 protein degradation

We demonstrated that ATBF1 protein was degraded by estrogen-induced UPP [9]. Estrogens function mainly by regulating the expression of a variety of ER α target genes [26]. We hypothesized that ATBF1 protein degradation by estrogen-induced UPP should be mediated by certain estrogen-responsive E3 ubiquitin ligases that can specifically recognize, ubiquitinate and degrade ATBF1 protein. While ATBF1 is implicated in suppressing estrogen-dependent cell proliferation [9, 10], the estrogen-responsive E3 ubiquitin ligase EFP is an essential factor for estrogen-dependent cell proliferation [19]. Therefore, we tested whether EFP could mediate the degradation of ATBF1 protein. We first applied RNAi to knock down EFP expression and found that knockdown of EFP expression significantly increased ATBF1 protein levels in MCF7 cells (Figure 1A). We then coexpressed EFP and ATBF1 and analyzed their protein expression. Coexpressed EFP dramatically reduced ATBF1 protein levels (Figure 1B).

As shown in our previous results, reconstituted estrogen-ER α signaling in 22Rv1 cells dramatically caused ATBF1 protein degradation [9], suggesting that the reconstituted estrogen-ER α signaling induces the expression of certain E3 ubiquitin ligases for ATBF1 degradation. We examined whether EFP could be induced by the reconstituted estrogen-ER α signaling in 22Rv1 cells and found that both EFP protein and mRNA could be significantly induced by the reconstituted estrogen-ER α signaling (Figure S1A and S1B). We also tested the role of some other estrogen-responsive E3 ubiquitin ligases, including SKP2, Cul-4A, E6-AP, EFP, BCA2, MDM2, and RNF11, in the estrogen-induced ATBF1 protein degradation and none of them were responsible for estrogen-induced ATBF1 protein degradation (data not shown).

To further determine whether EFP reduces ATBF1 protein levels through protein degradation, we performed CHX chase assays. Even when protein synthesis was inhibited, EFP still significantly decreased ATBF1 protein levels (Figure 1C and 1D). These results suggest that the estrogen-responsive E3 ubiquitin ligase EFP mediates estrogen-induced ATBF1 protein degradation, although we cannot rule out the involvement of other E3 ligases.

EFP interacts with ATBF1 protein

Protein interaction between an E3 ubiquitin ligase and its substrate is required for protein degradation in the UPP [15]. To further investigate whether EFP is an E3 ubiquitin ligase for ATBF1 protein degradation, we examined whether EFP and ATBF1 co-existed in the same protein complex. 22Rv1 cells were cotransfected with expression constructs for HA-ATBF1 and FLAG-EFP, and subjected to IP with anti-HA affinity gel and western blotting with anti-FLAG antibody. EFP protein was efficiently precipitated in ATBF1 and EFP cotransfected cells, but not in control cells transfected with an empty vector and EFP plasmid (Figure 2A). These results indicate that EFP and ATBF1 proteins exist in the same protein complex in transfected cells. We then performed IP with anti-ATBF1 antibody in MCF7 cells, which also express higher levels of ATBF1 protein, and western blotting with anti-EFP antibody. Endogenous EFP was clearly detected in the protein complexes precipitated with anti-ATBF1 antibody (Figure 2B). KLF5 is a bona fide EFP-interacting protein [27], and its interaction with EFP was used as the positive control in the IP experiments (Figure 2A and 2B). Taken together, these results further show a protein interaction between EFP and ATBF1.

To map the domains mediating the interaction between EFP and ATBF1, we performed co-IP in 22Rv1 cells transfected with FLAG-EFP and HA-tagged ATBF1 protein deletion mutants or with HA-ATBF1 and FLAG-tagged EFP protein deletion mutants. Among six overlapping deletion mutants that span the entire ATBF1 protein, including ATBF1A to ATBF1F, ATBF1D and ATBF1F interacted with EFP but ATBF1A, ATBF1B, ATBF1C or ATBF1E did not (Figure 2C and S2A; Table 1). To further narrow the interaction regions in ATBF1D and ATBF1F, we then examined the interaction of smaller deletion mutants with EFP. After two rounds of deletion mapping, three independent small deletion mutants still showed interactions with EFP (Figure 2C and S2B–D; Table 1), including ATBF1D3 (residues 1800 to 1950), ATBF1D6 (residues 2101 to 2300) and ATBF1F1 (residues 3001 to 3450). Further deletion of these mutants abolished their interactions with EFP. To map minimal EFP domains mediating the interaction with ATBF1, we first divided the whole EFP protein into two deletion mutants: EFPN containing the RING finger domain and EFPC containing the B-box-coiled-coil domain and a SPRY domain considering that the B-box-coiled-coil domain in RING proteins acting as E3 ubiquitin ligases is often involved in the interaction with their substrates [19]. EFPC strongly interacted with ATBF1, but not EFPN (Figure 2D and S3A). Next, we divided EFPC into three deletion mutants: EFP1 containing the B1 and B2 boxes, EFP2 containing the B2 box and the coiled-coil domain and EFP3 containing the SPRY domain. EFP1 and EFP2 bound to ATBF1, but not EFP3 (Figure 2D and S3B). When EFP1 was further divided into EFP4, EFP5 and EFP6, its interaction with ATBF1 was abolished (Figure 2D and S3C). EFP4 and EFP5 contain the B1 box and EFP6 contains the B2 box. Further deletion of EFP2 showed that EFP7 and EFP10 containing the intact coiled-coil domain interacted with ATBF1, but not EFP8 and EFP9, which disrupts the coiled-coil domain (Figure 2D and S3C–D). Taken together, EFP1 containing the B1 and B2 boxes and EFP10 containing the intact coiled-coil domain are minimal interacting domains of EFP with ATBF1, indicating that the B-box-coiled-coil domain is sufficient for mediating the interaction of EFP with ATBF1. The B-box-coiled-coil domain also mediates

the interaction of EFP with other substrates [19]. These data further confirm the interaction and identify the domains mediating the interaction between EFP and ATBF1.

EFP promotes the ubiquitination of ATBF1 protein

Ubiquitination is a key step in protein degradation by the UPP, which is completed by the E3 ubiquitin ligase [15]. To further characterize the role of EFP in the degradation of ATBF1 protein, we investigated whether EFP could promote the ubiquitination of ATBF1 protein. Considering the huge size of the ATBF1 protein (3,703aa, ~400 kDa), which makes it challenging to analyze any ATBF1 modifications with the full-length protein in SDS-PAGE gels, we used ATBF1D and ATBF1F, which mediate the interaction of ATBF1 with EFP, for the ubiquitination analysis. Plasmids of FLAG-ATBF1D (or FLAG-ATBF1F), CMV-EFP and HA-tagged ubiquitin (HA-Ub) were cotransfected into 22Rv1 cells. After treatment with MG132 at a final concentration of 20 μ M for four hours, cells were subjected to IP with anti-FLAG antibody overnight, and ubiquitinated ATBF1D or ATBF1F proteins were detected by immunoblotting with anti-HA antibody. As shown in Figure 3(A) and 3(B), a significantly higher level of smear of HA-Ub-modified ATBF1D and ATBF1F proteins was detected in EFP-expressing cells compared to cells without EFP expression. Without EFP expression, cotransfected ubiquitin could cause weaker ubiquitination of both ATBF1D and ATBF1F (Figure 3A and 3B), suggesting that EFP is not the only factor for ATBF1 ubiquitination. These results suggest that EFP can ubiquitinate ATBF1 protein, supporting the role of EFP in ATBF1 protein degradation.

Since we showed that ATBF1 protein was degraded by estrogen-induced UPP [9], based on which EFP was identified, we next investigated whether estrogen could induce the ubiquitination of ATBF1 protein and the role of estrogen-induced EFP expression in the ubiquitination. 22Rv1 cells grown in normal media were cotransfected with siRNAs and plasmids of FLAG-ATBF1D (or FLAG-ATBF1F), pCMV-ER α and HA-Ub, and subjected to IP with anti-FLAG antibody and western blotting with anti-HA antibody. We found that estrogen significantly induced the ubiquitination of ATBF1 protein (Figure 3C and 3D). When the estrogen-induced EFP expression was simultaneously knocked down, however, the estrogen-induced ATBF1 protein ubiquitination was significantly reduced (Figure 3C and 3D), suggesting the role of EFP in estrogen-induced ATBF1 protein degradation.

ATBF1D and ATBF1F deletion mutants were identified as the domains that interacted with EFP to mediate estrogen-induced ATBF1 protein degradation

We demonstrated that both ATBF1 protein degradation by estrogen and protection by MG132 had a dose- and time-dependent manner [9]. To further confirm that EFP mediates estrogen-induced ATBF1 protein degradation, we then investigated whether ATBF1D and ATBF1F were degraded by estrogen and protected by MG132 as ATBF1. ATBF1D or ATBF1F was cotransfected with ER α into 22Rv1 cells grown in hormone-free media. Forty hours post-transfection, cells were treated with different concentrations or different times of the same concentration of E2. Dose-dependent protein degradation by estrogen was detected for both ATBF1D and ATBF1F (Figure 4A). Treatment of E2 at 1 μ M for 0–24 hours showed time-dependent protein degradation of ATBF1D and ATBF1F in the same cell system (Figure 4B). While ATBF1D and ATBF1F were degraded by estrogen in a dose- and time-dependent manner, the expression of EFP protein was simultaneously induced by estrogen in a dose- and time-dependent manner (Figure 4A and 4B), consistent with the role of EFP in estrogen-induced ATBF1 protein degradation. In 22Rv1 cells cotransfected with ER α and ATBF1D or ATBF1F and treated with 1 μ M of E2, we also added MG132 at 0–40 μ M for four hours or at 20 μ M for 0–8 hours, and dose- and time-dependent protein protection from estrogen-induced degradation was detected again for both ATBF1D and ATBF1F (Figure 4C and 4D). These results reveal that EFP-interacting domains in ATBF1

could be degraded by estrogen and estrogen-induced EFP and protected by MG132 as ATBF1, and further support the role of EFP in estrogen-induced ATBF1 protein degradation.

EFP is an important factor mediating estrogen-induced ATBF1 protein degradation

To weigh the importance of EFP in estrogen-induced ATBF1 protein degradation, we compared the degradation of ATBF1 protein between estrogen and EFP. Using 22Rv1 cells grown in hormone-free media and transfected with ER α and each of 19 ATBF1 deletion mutants (Figure 2C) following treatments with E2, we found that estrogen degraded nine ATBF1 deletion mutants, including ATBF1D, ATBF1F, ATBF1D1, ATBF1F1, ATBF1D3, ATBF1D5, ATBF1D6, ATBF1D7 and ATBF1F6 (Table 1; Figure S4A). Using 22Rv1 cells transfected with EFP and each of the 19 ATBF1 deletion mutants, we found that EFP degraded six ATBF1 deletion mutants, including ATBF1D, ATBF1F, ATBF1D1, ATBF1F1, ATBF1D3 and ATBF1D6 (Table 1; Figure S4B). The bigger ATBF1 deletion mutants that could be degraded by estrogen were also degraded by EFP, including ATBF1D, ATBF1F, ATBF1D1 and ATBF1F1 (Table 1; Figure S4). Neither estrogen nor EFP degraded the other bigger ATBF1 deletion mutants, including ATBF1A, ATBF1B, ATBF1C, ATBF1E, ATBF1D2 and ATBF1F2 (Table 1; Figure S4). The bigger ATBF1 deletion mutants which were degraded by both estrogen and EFP also interacted with EFP (Figure 2D and S2; Table 1). For the nine smaller ATBF1 deletion mutants, including ATBF1D3, ATBF1D4, ATBF1D5, ATBF1D6, ATBF1D7, ATBF1F3, ATBF1F4, ATBF1F5 and ATBF1F6, estrogen could degrade ATBF1D3, ATBF1D5, ATBF1D6, ATBF1D7 and ATBF1F6, but EFP only interacted with and degraded ATBF1D3 and ATBF1D6 (Table 1; Figure S4). Although they were degraded by estrogen, ATBF1D5, ATBF1D7 or ATBF1F6 did not interact with and were not degraded by EFP (Table 1; Figure S4). There was no ATBF1 deletion mutant interacting with and degraded by EFP but not degraded by estrogen. Taken together, these data suggest that EFP is an important factor in estrogen-induced ATBF1 protein degradation, and there are some other factors also responsible for estrogen-induced ATBF1 protein degradation, consistent with ubiquitination data (Figure 3).

Correlation between ATBF1 and EFP protein levels in human breast tumors

As direct target gene products of estrogen/ER α , the expression of EFP and ATBF1 proteins is demonstrable in normal mammary glands and breast cancer [8, 20]. EFP was a significant prognostic factor for breast cancer and patients with higher levels of EFP protein had a worse prognosis [20]. ATBF1 was an independent prognostic factor for disease-free survival and patients expressing high levels of ATBF1 had a better prognosis [8]. To explore whether ATBF1 protein is degraded by EFP, we concomitantly analyzed their expression in 140 breast tumors from different patients by immunohistochemistry. The expression of EFP and ATBF1 proteins is shown in Figure 5(A) and summarized in Table 2. ATBF1 protein expression appeared not to negatively correlate with EFP protein expression (Table 2). Instead, most tumors expressing higher levels of EFP protein also expressed higher levels of ATBF1 protein (Table 2). Both EFP and ATBF1 proteins were positively associated with ER α positivity, while the association of EFP protein with ER α positivity was more significant than ATBF1 protein (Figure 5B). Statistical analysis with the SAS software found a positive correlation between the expression of ATBF1 and EFP proteins with a Spearman correlation coefficient of 0.31495 ($P < 0.05$, Table 2; Figure 5C). However, the ratio between ATBF1 protein to EFP protein was negatively associated with the EFP protein expression levels with a Spearman rank correlation coefficient of -0.52493 ($P < 0.0001$, Table 2; Figure 5D), indicating that more EFP protein, greater ATBF1 protein degradation. These results not only support previous results that both EFP and ATBF1 are direct

upregulated target gene products of estrogen/ER α [8, 20], but also indicate the role of EFP in estrogen-induced ATBF1 protein degradation.

ATBF1 antagonizes EFP-mediated breast cancer cell proliferation

As direct target gene products of estrogen-ER α signaling, EFP is essential for estrogen-dependent breast cancer cell proliferation but ATBF1 inhibits estrogen-dependent breast cancer cell proliferation [9, 10, 18, 19]. In this study, we revealed that EFP mediated estrogen-induced ATBF1 protein degradation. It is therefore possible that ATBF1 can antagonize EFP-mediated breast cancer cell proliferation. To that end, we knocked down the expression of EFP and ATBF1 in MCF7 cells, which express a substantial level of both ATBF1 and EFP proteins (Figure 6C, [10, 19]), and analyzed cell proliferation. Knockdown of EFP expression significantly reduced cell proliferation by 50% (Figure 6A), consistent with the results of a previous study [19]. When ATBF1 was co-knocked down, however, the reduction of cell proliferation by EFP knockdown was significantly attenuated by 56% (Figure 6A). We also reconstituted the expression of EFP and ATBF1 in T-47D cells, which express detectable EFP but not ATBF1 protein (Figure 6C), and analyzed cell proliferation. Overexpression of EFP significantly promoted cell proliferation by 80% (Figure 6B). When ATBF1 was co-reconstituted, however, the promotion of cell proliferation by EFP overexpression was significantly attenuated by 28% (Figure 6B). Knockdown by RNAi and overexpression by plasmids of ATBF1 and EFP were monitored by western blot analysis (Figure 6D and 6E). These results not only show that ATBF1 antagonizes EFP-mediated breast cancer cell proliferation, but provide another mechanism for how ATBF1 inhibits estrogen-dependent breast cancer cell proliferation [10]. These results also functionally argue the degradation of ATBF1 protein by EFP.

Discussion

In our previous studies, we showed that ATBF1 formed an autoregulatory feedback loop with estrogen-ER α signaling to regulate estrogen-dependent cell proliferation [9, 10]. ATBF1 inhibits estrogen-ER α signaling while estrogen upregulates ATBF1 transcription but degrades its protein through estrogen-induced UPP. In this study, we examined different estrogen-responsive E3 ubiquitin ligases for their role in estrogen-induced ATBF1 protein degradation and demonstrate that EFP, an ER α target gene product [17, 28], is responsible for estrogen-induced ATBF1 protein degradation.

EFP is a well-established downstream target gene of estrogen-ER α signaling [18, 29]. The expression of EFP mRNA and protein can be efficiently induced in response to estrogen treatment in human breast cancer cells and positively associated with ER α expression status in human primary breast cancer tumors [17, 20]. EFP protein belongs to the RING-finger-B box-coiled coil (RBCC) motif family and many members of the family function as E3 ubiquitin ligases in UPP-mediated protein degradation. EFP was identified as an E3 ubiquitin ligase by the findings that it could ubiquitinate and degrade substrates such as 14-3-3 sigma and DDX58 [19, 30]. We found that the reconstitution of estrogen-ER α signaling in ER α -negative cells significantly induced EFP expression accompanying ATBF1 protein degradation (Figure S1 and 4). Coexpression of EFP significantly decreased ATBF1 protein levels but knockdown of EFP increased ATBF1 protein levels (Figure 1). *In vitro* and *in vivo* experiments demonstrated that EFP formed a complex and directly interacted with ATBF1 protein (Figure 2). On the other hand, EFP significantly promoted the ubiquitination of ATBF1 protein as estrogen-ER α signaling (Figure 3). The domains in ATBF1 protein mediating its interaction with EFP were also degraded by both EFP and estrogen-ER α signaling and protected from degradation by the UPP inhibitor (Figure 4, 5 and S4; Table 1). These findings show that EFP is an E3 ubiquitin ligase responsible for estrogen-induced ATBF1 protein degradation.

More evidence for EFP as an E3 ubiquitin ligase for estrogen-induced ATBF1 protein degradation came from the expression analysis between EFP and ATBF1 proteins in human primary breast tumors (Figure 5). Both EFP and ATBF1 are target genes of estrogen-ER α signaling [9, 18]. When estrogen-ER α signaling is activated, the expression of EFP and ATBF1 can be upregulated at the same time [9]. In agreement with this observation, a positive correlation between the expression of ATBF1 and EFP proteins was identified. The greater degradation of ATBF1 protein by high levels of EFP (Figure 1–5, [9]) is a potential mechanism for the negative association between the ratio of ATBF1 to EFP protein and the EFP protein levels.

EFP plays an essential role in promoting estrogen-dependent breast development and tumorigenesis [19, 28]. Loss-of-function of EFP attenuates responses to estrogen and significantly decreases estrogen-dependent cell growth and proliferation in epithelial cells [28]. In MCF7 cells, knockdown reduces but overexpression of EFP enhances tumor xenograft formation in athymic mice [19]. Overexpression of EFP also makes MCF7 cells estrogen-independent, as EFP-overexpressing MCF7 cells form tumors in the absence of estrogen [19]. In human breast cancer, EFP is not only overexpressed, but its expression is positively associated with lymph node metastasis and poor prognosis of breast cancer patients [17, 20, 31]. Conversely, ATBF1 is frequently inactivated in human breast cancer and can significantly inhibit breast cancer cell growth and proliferation [7, 8, 10]. ATBF1 mRNA expression was an independent prognostic factor for disease-free survival and patients expressing high levels of ATBF1 mRNA had a better prognosis [8]. Moreover, knockout of ATBF1 in mice significantly increases cell proliferation of mammary epithelial cells and enhances mammary gland duct branching [10]. These observations in breast development and tumorigenesis are consistent with our findings that EFP mediates the degradation of ATBF1 protein. Our functional analysis in breast cancer cells further showed that ATBF1 and EFP antagonized each other in the control of cell proliferation (Figure 6).

There appears to be an autoregulatory feedback loop involving ATBF1, estrogen-ER α signaling and EFP (Figure 7). In this loop, estrogen-ER α signaling fine-tunes the ATBF1 protein level by upregulating its transcription but degrading its protein through the simultaneous upregulation of EFP. Meanwhile, ATBF1 imposes constraints preventing estrogen-ER α signaling from overactivation. Considering the respective critical roles of ATBF1, estrogen-ER α signaling and EFP in breast development and tumorigenesis, we suggest that this autoregulatory feedback loop is essential in estrogen-dependent breast development and tumorigenesis. In normal breast epithelial cells, the autoregulatory feedback loop can normally run to regulate breast development since estrogen concentration, ER α status and the expression of ATBF1 and EFP are all under proper control in multiple cascades of levels [32–37]. In the initiation and progression of breast cancer, however, the autoregulatory feedback loop becomes a causal factor of breast cancer through overactivation of estrogen-ER α signaling, overexpression of EFP and inactivation of ATBF1 [6–8, 19, 20, 31, 38–40]. The overactivation of estrogen-ER α signaling, overexpression of EFP and inactivation of ATBF1 turn the autoregulatory feedback loop into a vicious circle, consequently contributing to the initiation and progression of breast cancer. We are currently testing the role of the autoregulatory feedback loop in estrogen-dependent breast development and tumorigenesis by using ATBF1 knockout mice combined with ER α knockout mice in different estrogen environments.

In summary, our findings in this study establish EFP as the first E3 ubiquitin ligase of ATBF1 and suggest the importance of ATBF1 in estrogen-dependent breast development and tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Morinaga T, Yasuda H, Hashimoto T, Higashio K, Tamaoki T. A human alpha-fetoprotein enhancer-binding protein, ATBF1, contains four homeodomains and seventeen zinc fingers. *Mol Cell Biol.* 1991; 11:6041–6049. [PubMed: 1719379]
2. Mori Y, Kataoka H, Miura Y, Kawaguchi M, Kubota E, Ogasawara N, Oshima T, Tanida S, Sasaki M, Ohara H, Mizoshita T, Tatematsu M, Asai K, Joh T. Subcellular localization of ATBF1 regulates MUC5AC transcription in gastric cancer. *Int J Cancer.* 2007; 121:241–247. [PubMed: 17330845]
3. Kaspar P, Dvorakova M, Kralova J, Pajer P, Kozmik Z, Dvorak M. Myb-interacting protein, ATBF1, represses transcriptional activity of Myb oncoprotein. *J Biol Chem.* 1999; 274:14422–14428. [PubMed: 10318867]
4. Xu J, Sauvageot J, Ewing CM, Sun J, Liu W, Isaacs SD, Wiley KE, Diaz L, Zheng SL, Walsh PC, Isaacs WB. Germline ATBF1 mutations and prostate cancer risk. *Prostate.* 2006; 66:1082–1085. [PubMed: 16637072]
5. Cleton-Jansen AM, van Eijk R, Lombaerts M, Schmidt MK, Van't Veer LJ, Philippo K, Zimmermann RM, Peterse JL, Smit VT, van Wezel T, Cornelisse CJ. ATBF1 and NQO1 as candidate targets for allelic loss at chromosome arm 16q in breast cancer: absence of somatic ATBF1 mutations and no role for the C609T NQO1 polymorphism. *BMC Cancer.* 2008; 8:105. [PubMed: 18416817]
6. Kai K, Zhang Z, Yamashita H, Yamamoto Y, Miura Y, Iwase H. Loss of heterozygosity at the ATBF1-A locus located in the 16q22 minimal region in breast cancer. *BMC Cancer.* 2008; 8:262. [PubMed: 18796146]
7. Sun X, Zhou Y, Otto KB, Wang M, Chen C, Zhou W, Subramanian K, Vertino PM, Dong JT. Infrequent mutation of ATBF1 in human breast cancer. *J Cancer Res Clin Oncol.* 2007; 133:103–105. [PubMed: 16932943]
8. Zhang Z, Yamashita H, Toyama T, Sugiura H, Ando Y, Mita K, Hamaguchi M, Kawaguchi M, Miura Y, Iwase H. ATBF1-a messenger RNA expression is correlated with better prognosis in breast cancer. *Clin Cancer Res.* 2005; 11:193–198. [PubMed: 15671546]
9. Dong XY, Guo P, Sun X, Li Q, Dong JT. Estrogen up-regulates ATBF1 transcription but causes its protein degradation in estrogen receptor-alpha-positive breast cancer cells. *J Biol Chem.* 2011; 286:13879–13890. [PubMed: 21367855]
10. Dong XY, Sun X, Guo P, Li Q, Sasahara M, Ishii Y, Dong JT. ATBF1 inhibits estrogen receptor (ER) function by selectively competing with AIB1 for binding to the ER in ER-positive breast cancer cells. *J Biol Chem.* 2010; 285:32801–32809. [PubMed: 20720010]
11. Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M, Sellers WR, Brown M. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer cell.* 2004; 6:263–274. [PubMed: 15380517]
12. Zhang H, Yi X, Sun X, Yin N, Shi B, Wu H, Wang D, Wu G, Shang Y. Differential gene regulation by the SRC family of coactivators. *Gene Dev.* 2004; 18:1753–1765. [PubMed: 15256502]
13. Zhang H, Sun L, Liang J, Yu W, Zhang Y, Wang Y, Chen Y, Li R, Sun X, Shang Y. The catalytic subunit of the proteasome is engaged in the entire process of estrogen receptor-regulated transcription. *EMBO J.* 2006; 25:4223–4233. [PubMed: 16957778]
14. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998; 67:425–479. [PubMed: 9759494]

15. Pickart CM. Mechanisms underlying ubiquitination. *Annu Rev Biochem.* 2001; 70:503–533. [PubMed: 11395416]
16. Zhang J, Guenther MG, Carthew RW, Lazar MA. Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Gene Dev.* 1998; 12:1775–1780. [PubMed: 9637679]
17. Ikeda K, Orimo A, Higashi Y, Muramatsu M, Inoue S. Efp as a primary estrogen-responsive gene in human breast cancer. *FEBS Lett.* 2000; 472:9–13. [PubMed: 10781795]
18. Inoue S, Orimo A, Hosoi T, Kondo S, Toyoshima H, Kondo T, Ikegami A, Ouchi Y, Orimo H, Muramatsu M. Genomic binding-site cloning reveals an estrogen-responsive gene that encodes a RING finger protein. *Proc Natl Acad Sci USA.* 1993; 90:11117–11121. [PubMed: 8248217]
19. Urano T, Saito T, Tsukui T, Fujita M, Hosoi T, Muramatsu M, Ouchi Y, Inoue S. Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth. *Nature.* 2002; 417:871–875. [PubMed: 12075357]
20. Suzuki T, Urano T, Tsukui T, Horie-Inoue K, Moriya T, Ishida T, Muramatsu M, Ouchi Y, Sasano H, Inoue S. Estrogen-responsive finger protein as a new potential biomarker for breast cancer. *Clin Cancer Res.* 2005; 11:6148–6154. [PubMed: 16144914]
21. Chen C, Sun X, Guo P, Dong XY, Sethi P, Cheng X, Zhou J, Ling J, Simons JW, Lingrel JB, Dong JT. Human Kruppel-like factor 5 is a target of the E3 ubiquitin ligase WWP1 for proteolysis in epithelial cells. *J Biol Chem.* 2005; 280:41553–41561. [PubMed: 16223724]
22. Zou W, Zhang DE. The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase. *J Biol Chem.* 2006; 281:3989–3994. [PubMed: 16352599]
23. Dong XY, Chen C, Sun X, Guo P, Vessella RL, Wang RX, Chung LW, Zhou W, Dong JT. FOXO1A is a candidate for the 13q14 tumor suppressor gene inhibiting androgen receptor signaling in prostate cancer. *Cancer Res.* 2006; 66:6998–7006. [PubMed: 16849544]
24. Dong XY, Rodriguez C, Guo P, Sun X, Talbot JT, Zhou W, Petros J, Li Q, Vessella RL, Kibel AS, Stevens VL, Calle EE, Dong JT. SnoRNA U50 is a candidate tumor-suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer. *Hum Mol Genet.* 2008; 17:1031–1042. [PubMed: 18202102]
25. McCarty KS Jr, Miller LS, Cox EB, Konrath J, McCarty KS Sr. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch Pathol Lab Med.* 1985; 109:716–721. [PubMed: 3893381]
26. Cottone E, Orso F, Biglia N, Sismondi P, De Bortoli M. Role of coactivators and corepressors in steroid and nuclear receptor signaling: potential markers of tumor growth and drug sensitivity. *Int J Biol Marker.* 2001; 16:151–166.
27. Zhao KW, Sikriwal D, Dong X, Guo P, Sun X, Dong JT. Oestrogen causes degradation of KLF5 by inducing the E3 ubiquitin ligase EFP in ER-positive breast cancer cells. *Biochem J.* 2011; 437:323–333. [PubMed: 21542805]
28. Orimo A, Inoue S, Minowa O, Tominaga N, Tomioka Y, Sato M, Kuno J, Hiroi H, Shimizu Y, Suzuki M, Noda T, Muramatsu M. Underdeveloped uterus and reduced estrogen responsiveness in mice with disruption of the estrogen-responsive finger protein gene, which is a direct target of estrogen receptor alpha. *Proc Natl Acad Sci USA.* 1999; 96:12027–12032. [PubMed: 10518570]
29. Ueyama K, Ikeda K, Sato W, Nakasato N, Horie-Inoue K, Takeda S, Inoue S. Knockdown of Efp by DNA-modified small interfering RNA inhibits breast cancer cell proliferation and in vivo tumor growth. *Cancer Gene Ther.* 2010; 17:624–632. [PubMed: 20467453]
30. Gack MU, Shin YC, Joo CH, Urano T, Liang C, Sun L, Takeuchi O, Akira S, Chen Z, Inoue S, Jung JU. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature.* 2007; 446:916–920. [PubMed: 17392790]
31. Thomson SD, Ali S, Pickles L, Taylor J, Pace PE, Lymboura M, Shousha S, Coombes RC. Analysis of estrogen-responsive finger protein expression in benign and malignant human breast. *Int J Cancer.* 2001; 91:152–158. [PubMed: 11146438]
32. Briskin C, O'Malley B. Hormone action in the mammary gland. *Cold Spring Harb Perspect Biol.* 2010; 2:a003178. [PubMed: 20739412]
33. Ido A, Miura Y, Tamaoki T. Activation of ATBF1, a multiple-homeodomain zinc-finger gene, during neuronal differentiation of murine embryonal carcinoma cells. *Dev Biol.* 1994; 163:184–187. [PubMed: 8174773]

34. Ikeda K, Inoue S, Orimo A, Sano M, Watanabe T, Tsutsumi K, Muramatsu M. Multiple regulatory elements and binding proteins of the 5'-flanking region of the human estrogen-responsive finger protein (efp) gene. *Biochem Bioph Res Co.* 1997; 236:765–771.
35. Nakajima A, Maruyama S, Bohgaki M, Miyajima N, Tsukiyama T, Sakuragi N, Hatakeyama S. Ligand-dependent transcription of estrogen receptor alpha is mediated by the ubiquitin ligase EFP. *Biochem Bioph Res Co.* 2007; 357:245–251.
36. Shimada N, Suzuki T, Inoue S, Kato K, Imatani A, Sekine H, Ohara S, Shimosegawa T, Sasano H. Systemic distribution of estrogen-responsive finger protein (Efp) in human tissues. *Mol Cell Endocrinol.* 2004; 218:147–153. [PubMed: 15130519]
37. Watanabe M, Miura Y, Ido A, Sakai M, Nishi S, Inoue Y, Hashimoto T, Tamaoki T. Developmental changes in expression of the ATBF1 transcription factor gene. *Brain Res.* 1996; 42:344–349.
38. Horie K, Urano T, Ikeda K, Inoue S. Estrogen-responsive RING finger protein controls breast cancer growth. *J Steroid Biochem Mol Biol.* 2003; 85:101–104. [PubMed: 12943693]
39. Sakuma M, Akahira J, Suzuki T, Inoue S, Ito K, Moriya T, Sasano H, Okamura K, Yaegashi N. Expression of estrogen-responsive finger protein (Efp) is associated with advanced disease in human epithelial ovarian cancer. *Gynecol Oncol.* 2005; 99:664–670. [PubMed: 16140366]
40. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *The New Engl J Med.* 2006; 354:270–282.

Figure 1. Identification of the estrogen-responsive EFP as an E3 ubiquitin ligase that mediates estrogen-induced ATBF1 protein degradation

(A) Knockdown of endogenous EFP in MCF7 cells increased endogenous ATBF1 protein levels. (B) EFP overexpression reduced ATBF1 protein levels in cotransfected cells. (C, D) Overexpression of EFP shortened the half-life of ATBF1 protein in 22Rv1 cells as determined by CHX chase assay. Western blotting was applied to detect protein expression (C), and band intensities for ATBF1 protein in both EFP and control (Con) groups were determined by the Image J program and plotted over times of treatment, with the readings at the “0” time point defined as 100% and others normalized accordingly (D). * $P < 0.01$; ** $P < 0.005$ when compared to their control counterparts.

Figure 2. Evaluation of protein interaction between ATBF1 and EFP by immunoprecipitation (IP) combined with western blotting

(A) 22Rv1 cells were cotransfected with FLAG-EFP and HA-ATBF1 or HA-KLF5 plasmids, and cell lysates were precipitated with anti-HA affinity gel and blotted with anti-FLAG antibody. (B) Cell lysates from MCF7 cells were precipitated with anti-ATBF1 (aATBF1) or anti-KLF5 (aKLF5) antibody and blotted with anti-EFP antibody (aEFP). (C) Summary of mapping results for the interactions of different deletion mutants of ATBF1 protein with EFP protein as determined by co-IP and western blotting. Full-length ATBF1 protein (residues 1-3703) is shown at the top, with zinc finger motifs marked by blank lozenges, DEAH box- and DEAD box-like sequences by blank rectangles, homeodomains by solid rectangles, and ATP-binding site by a solid circle. (D) Summary of mapping results

for the interaction of different EFP deletion mutants with ATBF1 protein. Full-length EFP protein is shown at the top with different domains indicated. For panels (C) and (D) black and white bars indicate positive and negative interactions between ATBF1 and EFP, respectively, with the name of the mutant and residues spanned shown to the right of each bar. Results of co-IP and western blotting for (C) and (D) are shown in Figure S2 and S3, respectively.

Figure 3. Ubiquitination of ATBF1D (A, C) and ATBF1F (B, D) induced by EFP and estrogen-ER α signaling

Plasmids for HA-Ub, FLAG-ATBF1D or FLAG-ATBF1F, EFP or ER α and siRNAs against EFP or the luciferase gene were cotransfected into 22Rv1 cells as indicated on the top of each panel, and MG132 treatment was applied for four hours. Cell lysates were subjected to co-IP combined with western blotting (image at the top of each panel) or western blotting alone (lower 5 images in each panel) with the indicated antibodies.

Figure 4. Estrogen induces the degradation of ATBF1D and ATBF1F and the expression of EFP protein (A, B), while the proteasome inhibitor MG132 prevents such degradation (C, D). Plasmid for ATBF1D or ATBF1F was transfected into 22Rv1 cells, which were then treated with different concentrations of E_2 for 24 hours (A), or different times of 1 μ M of E_2 (B), and with 1 μ M of E_2 for 24 hours followed by different concentrations of MG132 for 4 hours (C) or with 20 μ M of MG132 for different times (D). Western blotting was performed to examine protein expression, and β -actin served as the loading control for each gel.

Figure 5. Immunohistochemical and statistical analysis of EFP and ATBF1 proteins in human primary breast tumors
(A) Immunohistochemistry of EFP and ATBF1 proteins in four representative breast tumors (RBT). RBT1 expressed higher levels of EFP protein but lower levels of ATBF1 protein, RBT2 higher levels of both EFP and ATBF1 proteins, RBT3 lower levels of EFP protein but higher levels of ATBF1 protein, and RBT4 was negative for both EFP and ATBF1 proteins. (B) The association of EFP and ATBF1 protein expression levels with ER α positivity in breast tumors. (C) Positive correlation between the expression of EFP protein and the expression of ATBF1 protein. The expression levels of ATBF1 protein were plotted against the expression levels of EFP protein. (D) Negative correlation between the expression ratio of ATBF/EFP proteins and the expression of EFP protein. The expression ratio between ATBF1 and EFP proteins was plotted against the expression levels of EFP protein. The data for statistical analysis are listed at the bottom of the charts in (C) and (D).

Figure 6. ATBF1 antagonizes EFP-mediated cell proliferation in ERα-positive breast cancer cells as measured by DNA synthesis rates

(A) In MCF7 cells, knockdown of EFP reduces cell proliferation, but the reduction is partially rescued by simultaneous knockdown of ATBF1. SiEFP, siATBF1 and siLuc are siRNAs against EFP, ATBF1 and the luciferase gene, respectively. (B) In T-47D cells, overexpression of EFP enhances cell proliferation, but the enhancement is attenuated by simultaneous expression of ATBF1. * $P < 0.001$; ** $P < 0.005$; *** $P < 0.05$. Expression of ATBF1, EFP and β-actin was monitored by western blotting in parental MCF7 and T-47D cells (C), MCF-7 cells transfected with siRNAs (D), and T-47D cells transfected with ATBF1 and EFP (E).

Figure 7. Proposed model of the autoregulatory feedback loop between ATBF1 and estrogen-ER α signaling in ER α -positive breast cancer cells
While estrogen-ER α signaling induces the transcription of both EFP and ATBF1, ATBF1 suppresses the function of estrogen-ER α signaling, and EFP degrades ATBF1 protein.

Table 1Degradation of ATBF1 deletion mutants by estrogen-ER α signaling and EFP

ATBF1 deletion mutants	Degradation by Estrogen-ER α	Interaction with EFP	Degradation by EFP
ATBF1	+	+	+
ATBF1A	-	-	-
ATBF1B	-	-	-
ATBF1C	-	-	-
ATBF1D	+	+	+
ATBF1E	-	-	-
ATBF1F	+	+	+
ATBF1D1	+	+	+
ATBF1D2	-	-	-
ATBF1F1	+	+	+
ATBF1F2	-	-	-
ATBF1D3	+	+	+
ATBF1D4	-	-	-
ATBF1D5	+	-	-
ATBF1D6	+	+	+
ATBF1D7	+	-	-
ATBF1F3	-	-	-
ATBF1F4	-	-	-
ATBF1F5	-	-	-
ATBF1F6	+	-	-

Table 2

Expression summary of EFP and ATBF1 proteins in 140 breast tumors

EFP ATBF1	+++ n=18 248.6±34.5	++ n=48 148.9±23.6	+ n=40 65.4±10.4	- n=34 20.0±12.3
+++ (n)	11	26	11	3
++ (n)	3	11	12	9
+ (n)	2	5	11	5
-(n)	2	6	6	17
Staining levels	224.2±121.1	209.8±123.4	144.4±105.7	79.1±79.3
ATBF1/EFP	0.93±0.51	1.40±0.80	2.17±1.42	4.11±4.11