Title

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Journal

Chemical Research in Toxicology, 34(7)

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Publication Date

2021-07-19

DOI

10.1021/acs.chemrestox.1c00206

Peer reviewed

HHS Public Access

Author manuscript

Chem Res Toxicol. Author manuscript; available in PMC 2022 July 19.

Published in final edited form as:

Chem Res Toxicol. 2021 July 19; 34(7): 1814–1821. doi:10.1021/acs.chemrestox.1c00206.

ATF3 Modulates the Resistance of Breast Cancer Cells to Tamoxifen through an N^6–Methyladenosine-Based Epitranscriptomic Mechanism

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Abstract

Tamoxifen has been used for years for treating estrogen receptor-positive breast cancer; drug resistance, however, constitutes one of the main challenges for this therapy. We found that the

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.1c00206.

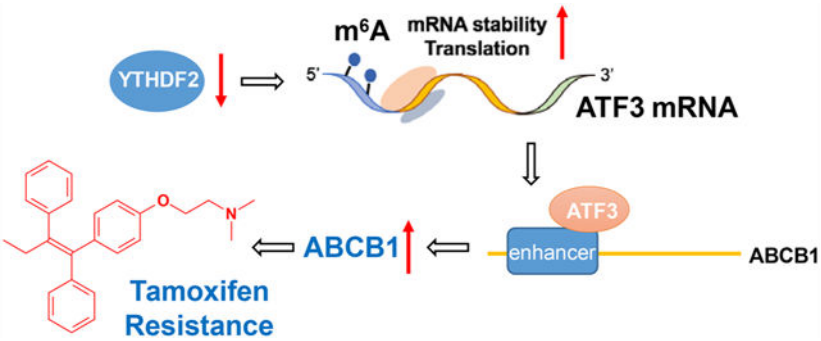
Tables of sequences for shRNA and overexpression plasmids primers, sequences of qPCR primers, primer sequences used for the SELECT assay, and primers sequences used for ChIP-qPCR and figures of Western blot images, cell viability, polysome traces, and ChIP-seq data (PDF)

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.chemrestox.1c00206

The authors declare no competing financial interest.

protein expression level of ATF3 is significantly higher in tamoxifen-resistant (TamR) MCF-7 cells than the corresponding parental cancer cells. In addition, ATF3 protein expression is positively correlated with the resistance of TamR MCF-7 cells to 4-hydroxytamoxifen (4-OHT). Mechanistically, elevated ATF3 protein expression in TamR MCF-7 cells results from a lower level of expression of YTHDF2, an m^{6}A reader protein, and the ensuing stabilization and increased translational efficiency of ATF3 mRNA. Additionally, TamR MCF-7 cells exhibited decreased methylation at A131, a consensus motif site for m^{6}A, in the 5′-untranslated region (5′-UTR) of ATF3 mRNA. Moreover, augmented ATF3 stimulates the expression of ABCB1, an efflux pump that confers drug resistance in breast cancer cells, and ATF3 itself is also positively regulated by adenylate kinase 4. Together, our results uncovered a novel molecular target for m^{6}A modification (i.e., ATF3 mRNA) and the epitranscriptomic regulator for this target (i.e., YTHDF2). We also illustrated the role of ATF3 in drug resistance, revealed its downstream target (i.e., ABCB1), and suggested ATF3 as a candidate therapeutic target for overcoming drug resistance in cancer cells.

Graphical Abstract



INTRODUCTION

Breast cancer is one of the common causes of cancer deaths.[1] Hormone therapy and chemotherapy have been developed for the clinical treatment of breast cancer.[2] Among them, tamoxifen, as a partial estrogen receptor (ER) antagonist,[3] has been used clinically for approximately 30 years as a chemotherapeutic agent for treating ER-positive breast cancer.[4] Nevertheless, therapeutic resistance is still a main hurdle in tamoxifen therapy.[5]

Multiple factors contribute to therapeutic resistance in cancer cells,[6,7] including augmented drug efflux, diminished drug uptake, activation of DNA repair mechanisms, evasion of drug-induced apoptosis, etc.[8] The CREB/ATF family of transcription factors are leucine zipper proteins that bind to the cAMP response element (CRE) and are able to homo- or heterodimerize to regulate gene expression.[9] Previous studies have demonstrated that this family of transcription factors are important for the transcriptional activation of genes encoding inflammatory mediators and pro-inflammatory cytokines.[9–11] ATF3, a member of this family of transcription factors, assumes important roles in regulating the expression of many genes associated with inflammation[12] and cancer progression.[13–15] ATF3 can be induced by many chemotherapeutic agents,[16] e.g., cisplatin,[17] doxorubicin,[18]

and paclitaxel;[19] nonetheless, it remains unclear if ATF3 also plays a role in therapeutic resistance to tamoxifen.

The ATP-binding cassette (ABC) transporter superfamily of membrane proteins transport various molecules across extra- and intracellular membranes.[20] Mutations in these genes result in neurological diseases, retinal degeneration, defective bile transport, and aberrant drug response.[20] The ABCB subfamily is unique because it contains both full transporters, which harbor two nucleotide-binding folds (NBFs) and two trans-membrane (TM) domains, and half transporters, which assemble into a functional transporter through homo- or heterodimerization.[21] Among them, *ABCB1* was the first human ABC transporter cloned[22] and manifested with an ability to confer multidrug resistance to cancer cells.[23] Additionally, ABCB1 was found to transport the active metabolite of tamoxifen, i.e., 4-hydroxytamoxifen (4-OHT).[24]

N^6-methyladenosine (m^6A) is the most prevalent internal modification in mRNA, and this methylation represents a crucial mechanism for modulating gene expression in many physiological processes.[25] In addition, the m^6A-mediated epitranscriptomic mechanism assumes important roles in cancer chemo- and radiotherapy. For example, METTL3, the catalytic subunit of the m^6A writer complex,[26] promotes the therapeutic resistance of pancreatic cancer cells.[27] Likewise, FTO—a demethylase for m^6A and m^6A_m[28,29] —regulates the therapeutic resistance of cervical squamous cell carcinoma by demethylating β-catenin mRNA.[30]

In this study, we uncovered that tamoxifen-resistant (TamR) MCF-7 breast cancer cells exhibit an elevated level of ATF3 protein. We also identified ATF3 as a potential driver for tamoxifen resistance, which involves its transcriptional regulation of ABCB1. Moreover, the expression level of ATF3 protein in the TamR MCF-7 cells is modulated by both YTHDF2, an m^6A reader protein, and adenylate kinase 4.

EXPERIMENTAL PROCEDURES

Materials.

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich.

Cell culture.

Parental and TamR MCF-7[31] human breast cancer cells were generous gifts from Dr. David Eastmond at UC Riverside and Dr. Guangdi Wang at Xavier University, respectively. The breast cancer cells were maintained in DMEM supplemented with 10% FBS (Invitrogen) and antibiotics. In addition, 1 μM 4-OHT was included in the culture medium of TamR MCF-7 to maintain the drug-resistant subline. The cells were cultured at 37 °C in an incubator containing 5% CO_2.

shRNA and Plasmids.

The sequences for shRNA and primers are listed in Table S1. Scrambled shRNA with a hairpin sequence of 5′-CCT AAG GTT AAG TCG CCC TCG CTC TAG CGA GGG CGA CTT AAC CTT AGG-3′ (Addgene, Cambridge, MA) was employed as a negative

control, as described previously.[32] All primers and oligodeoxyribonucleotides used for the construction of shRNA plasmids were obtained from Integrated DNA Technologies. All shRNAs were cloned into the Age I/EcoR I site of the pLKO.1 vector (Addgene, plasmid # 10878). The coding sequences of the ATF3 plasmid were cloned into the NheI and AgeI restriction sites of the pLJM1-EGFP vector (Addgene, plasmid # 19319). Plasmids for shAK4–1, shAK4–2, AK4, YTHDF2, and YTHDF2 W432A were previously described.[33,34] All constructs were confirmed by Sanger sequencing.

Lentivirus Production and Stable Cell Line Generation.

HEK293T cells were transfected with pLKO.1/puro-shRNAs and pLJM1-EGFP-ATF3 plasmids together with pLTR-G (plasmid # 17532) envelope plasmid and pCMV-dR8.2 dvpr (plasmid # 8455) package plasmid using PolyFect transfection reagent (Qiagen). Viral particles were collected 48 h later and filtered through a 0.45 μm sterile filter. Cells were infected for 48 h with a 5:1 mixture of viral particles and DMEM containing 1 μM 4-OHT. The cells were screened with 2 μg/mL puromycin for a week and cultured in complete DMEM supplemented with 1 μg/mL puromycin, together with 1 μM 4-OHT (for TamR MCF-7 cells).

Western Blot.

MCF-7 and TamR MCF-7 cells, which were cultured in a 6-well plate until their confluency levels reached 40–50%, were treated for 24 h with 4-OHT at the indicated concentrations. The cells were lysed with CelLytic M cell lysis reagent (Sigma-Aldrich), and the supernatant was collected for Western blot analysis. Primary antibodies employed for Western blot analysis included those recognizing human ATF3 (Santa Cruz Biotechnology, sc-81189), YTHDF2 (EMD Millipore, Q2490423), ABCB1 (ABclonal, A11747), and AK4 (Santa Cruz Biotechnology, sc-271161). Goat anti-rabbit IgG (whole molecule)-peroxidase antibody (Sigma-Aldrich, A0545) and m-IgGκ BP-HRP (Santa Cruz Biotechnology, sc-516102) were used as secondary antibodies. Membranes were also probed with anti-GAPDH antibody (Santa Cruz Biotechnology, sc-32233) to confirm equal protein loading.

Real-Time-Quantitative PCR (RT-qPCR).

Cells were seeded in 6-well plates at a 40% confluence level and treated with the indicated compounds. Total RNA was extracted from cells using a total RNA kit (VWR). Approximately 1.0 μg of RNA was reverse transcribed by employing M-MLV reverse transcriptase (Promega) and an oligo-$(dT)_{18}$ primer. After a 50 min incubation at 37 °C, the reverse transcriptase was deactivated by heating at 75 °C for 15 min. RT-qPCR experiments were performed using an iQ SYBR Green Supermix kit (Bio-Rad) on a Bio-Rad iCycler system, and the running conditions were at 95 °C for 3 min and 45 cycles at 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 45 s. The comparative cycle threshold (Ct) method ($\Delta\Delta C_t$) was used for the relative quantification of gene expression,[35] and the primers are listed in Table S2. The total mRNA level of each gene was normalized to that of *GAPDH*.

Proliferation Assay.

Cells were cultured in 96-well plates (6000 cells/well). Compounds at indicated concentrations were added into the plates after the cells were cultured for 12 h. Cell proliferation was determined after treatment with compounds for 72 h. Cell viability was determined with a Cell Counting Kit-8 (Dojindo Molecular Technologies) according to the vendor's instructions, and absorbance at 450 nm was recorded in a Synergy H1 multilabel reader (BioTek). The data were normalized to control groups (ethanol) and represented as mean ± SD of results from three independent measurements.

Single-Base Elongation- and Ligation-Based qPCR Amplification (SELECT) Assay.

SELECT assay was performed as previously described.[36] Briefly, total RNA and up and down primers were mixed and annealed. DNA polymerase, ATP, and SplintR were mixed and subsequently added to the mixture. The final reaction mixture was incubated at 40 °C for 20 min, denatured at 80 °C for 20 min, and subsequently cooled to 4 °C. RT-qPCR was subsequently performed using the same conditions as described above. The sequences for up and down primers and qPCR primers are listed in Table S3.

Polysome Profiling.

Sucrose solutions were prepared in a polysome buffer (10 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 100 μg/mL cycloheximide, and 1% Triton X-100). Sucrose density gradients (15–50%, w/v) were freshly prepared in SW 41 ultracentrifuge tubes (Backman) using a Gradient Master (BioComp Instruments). Cells were pretreated with 100 μg/mL cycloheximide at 37 °C for 5 min, followed by washing with cold PBS containing 100 μg/mL cycloheximide. The cells were then lysed in polysome lysis buffer for 30 min on ice. Cell debris was removed by centrifugation at 10 000 RCF for 10 min at 4 °C. The same amount of polysome lysate was loaded onto sucrose gradients followed by centrifugation at 35 000 rpm for 120 min at 4 °C in an SW 41 Ti rotor. The resulting sample was eluted at a flow rate of 1.5 mL/min through an automated density gradient fractionation system (Brandel Inc.) that continuously monitored OD_{254} values. Aliquots of polysome fraction were used for real-time PCR analysis.

ChIP-qPCR.

Cells were seeded in a T75 flask, and about 1×10^7 cells were cross-linked with 0.75% formaldehyde at room temperature for 8 min and quenched with 125 mM glycine for 5 min. After washing with 1× PBS three times, the cells were lysed for chromatin immunoprecipitation. Chromatin was sheared using a Qsonica sonicator with 42% amplitude for 50 s and then using Covaris S220 sonicator for 10 min with a peak incident power of 140, a duty cycle of 10%, and 200 cycles per burst at 4 °C. Chromatin immunoprecipitation was performed using ATF3 antibody (Cell Signaling Technology, 33593) and Protein A/G Plus-Agarose (Santa Cruz Biotechnology). Finally, the DNA was eluted and purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using the primers listed in Table S4.

Bioinformatic Analysis of Publicly Available Data Sets.

ChIP-seq data for ATF3 in human K562 cells was retrieved from ENCODE Consortium[37,38] using accession number ENCSR028UIU and was visualized by UCSC genome browser.[39] Two putative enhancer regions were chosen on the basis of signal intensity and ENCODE cCREs database.[40] The distribution of m^{6}A in ATF3 mRNA data was retrieved from the MeT-DB v2.0 web site.[41]

RESULTS AND DISCUSSION

TamR MCF-7 Cells Exhibit Elevated Expression of ATF3 Protein, Which Confers Tamoxifen Resistance.

ATF3 can be induced by various chemotherapeutic drugs, including cisplatin, doxorubicin, and paclitaxel.[17–19] In this study, we set out to examine whether ATF3 modulates tamoxifen resistance in breast cancer cells and to understand the underlying molecular mechanism. To this end, we first assessed the relative expression levels of ATF3 in MCF-7 breast cancer cells and the isogenic TamR cells. We found that the mRNA level of ATF3 exhibits a ~1.5-fold increase, which is accompanied by a much more pronounced elevation of ATF3 protein in TamR MCF-7 cells than parental MCF-7 cells (Figure 1A,B).

We next asked if an increased expression of ATF3 protein contributes to therapeutic resistance in these cancer cells. To this end, we stably overexpressed the *ATF3* gene in parental MCF-7 cells and knocked down the expression of this gene in TamR MCF-7 cells using two separate sequences of shRNAs (Figure S1). Proliferation assay results showed that ATF3 overexpression elicited increased the resistance of MCF-7 cells to 4-OHT, and reciprocally shRNA-mediated suppression of ATF3 resensitized TamR MCF-7 cells to 4-OHT (Figure 1C). These results demonstrate that elevated expression of ATF3 confers an increased resistance of MCF-7 cells to tamoxifen.

TamR MCF-7 Cells Exhibit Attenuated YTHDF2 Expression and Augmented Stabilization of ATF3 mRNA.

The above results revealed a much more pronounced increase of ATF3 protein relative to its mRNA in TamR MCF-7 cells over parental MCF-7 cells, suggesting the involvement of a post-transcriptional mechanism in ATF3 regulation. Previous studies revealed that m^{6}A in mammalian mRNAs modulates their stabilities and translation efficiencies, and the m^{6}A-based regulation of ATF3 contributes to axon regeneration in an adult mammalian nervous system.[42] Hence, we next asked if the increased expression of ATF3 protein in TamR MCF-7 cells arises from an m^{6}A-mediated epitranscriptomic mechanism.

Our recent study showed that the m^{6}A reader protein YTHDF2 exhibits a lower level of expression in TamR MCF-7 cells than MCF-7 cells,[34] and the binding with YTHDF2 to m^{6}A-bearing mRNAs is known to trigger their decay.[43] Thus, we investigated whether augmented expression of ATF3 protein in TamR MCF-7 cells emanates from diminished expression of YTHDF2. To test this, we overexpressed wild-type YTHDF2 and its W432A mutant, which is defective in m^{6}A binding,[33] in TamR MCF-7 cells. Our results showed that the overexpression of YTHDF2 led to a significantly attenuated level of ATF3 protein

in TamR MCF-7 cells; the overexpression of the W432A mutant, however, was unable to alter the level of ATF3 (Figure 2A,B). Reciprocally, the suppression of YTHDF2 in MCF-7 cells with two different sequences of shRNAs led to an elevated expression of ATF3 protein (Figure 2C,D and Figure S2A).

We also examined whether the decreased YTHDF2 level affects the half-life ($t_{1/2}$)[44] of ATF3 mRNA in TamR MCF-7 cells. Our results showed that the $t_{1/2}$ values of ATF3 mRNA are 2.12 and 1.35 h in TamR MCF-7 and parental MCF-7 cells, respectively (Figure 2E). This result supports that the attenuated expression of YTHDF2 led to a stabilization of ATF3 mRNA in TamR MCF-7 cells.

We next investigated whether YTHDF2 modulates the resistance of MCF-7 cells toward 4-OHT. Proliferation assay results showed that the knockdown of YTHDF2 elicits diminished the sensitivity of MCF-7 cells to 4-OHT (Figure 2F). On the contrary, the overexpression of YTHDF2 in TamR cells did not affect their proliferation, suggesting that the extent of decrease in ATF3 protein expression was not adequate to resensitize TamR cells to 4-OHT (Figure S2B). In this vein, it is worth noting that, while the overexpression of YTHDF2 could lead to a significant decrease in the level of ATF3 protein in TamR MCF-7 cells, the level is still approximately 13-fold higher than that observed in parental MCF-7 cells (Figure 2B).

We next analyzed the publicly available m^{6}A sequencing data by using the Met-DB v2.0 database.[41,45] The result showed that most m^{6}A peaks are located in the 5′- and 3′-untranslated regions (UTRs) of ATF3 mRNA (Figure 3A). Recent studies showed that the heat shock-elicited overexpression of HSP70 and DNAJB4 proteins occurs through a mechanism involving cap-independent translation enabled by m^{6}A in the 5′-UTRs of the mRNAs of the two genes.[46–49] Thus, we asked whether a similar mechanism contributes to the augmented expression of ATF3 in TamR MCF-7 cells. Our results from SELECT assay[36] showed that adenosine residue 131 in the 5′-UTR of ATF3 mRNA from TamR MCF-7 cells was with decreased m^{6}A levels compared with parental MCF-7 cells (Figure 3B,C and Figure S2C). Hence,etth the diminished N^6-methylation at adenosine 131, together with the decreased YTHDF2 level, results in the stabilization of ATF3 mRNA in TamR MCF-7 cells.

Next, we investigated the translation efficiency of ATF3 mRNA by conducting the polysome profiling assay. Our results showed that the translation efficiency of ATF3 mRNA is elevated in TamR MCF-7 cells relative to parental MCF-7 cells (Figure 3D and Figure S3). The overexpression of YTHDF2, but not its W432A mutant, in TamR MCF-7 cells led to a decreased level of the polysome-bound ATF3 mRNA (Figure 3E and Figure S3). Reciprocally, the knockdown of YTHDF2 in parental MCF-7 cells resulted in an increased loading of ATF3 mRNA to polysome fraction (Figure 3F and Figure S3). These results support that attenuated YTHDF2 expression in TamR MCF-7 cells gives rise to the stabilization of ATF3 mRNA and promotes its loading to ribosome to increase its translation.

ATF3 Transcriptionally Activates ABCB1 to Confer Resistance of MCF-7 Cells to 4-OHT.

ABCB1, an efflux pump on the apical membrane of endothelial cells, transports the primary active metabolite of tamoxifen, i.e. 4-OHT.[24] ATF4, another member of the ATF/CREB

family of transcription factors, can bind to a stress-responsive enhancer and stimulate the transcription of ABCB1, which leads to dynamic drug resistance in acute myeloid leukemia.[50] Thus, we assessed whether ATF3 could transcriptionally regulate the expression of ABCB1 and confer resistance to tamoxifen. Our results showed that the expression levels of ABCB1 mRNA and protein are significantly increased in TamR MCF-7 cells relative to parental MCF-7 cells (Figure 4A,C). Additionally, the shRNA-mediated knockdown of ATF3 attenuated both the protein and mRNA levels of ABCB1 in TamR MCF-7 cells (Figure 4A,C). Moreover, the overexpression of YTHDF2, but not the W432A mutant, resulted in a significantly diminished level of ABCB1 protein in TamR MCF-7 cells (Figure 4A,B). However, the overexpression of ATF3 in parental MCF-7 cells did not affect the ABCB1 level (Figure S4A), suggesting that the ATF3-mediated regulation of ABCB1 is restricted to the drug-resistant cellular environment.

We then asked whether the elevated expression of ABCB1 in TamR MCF-7 cells contributes to resistance to 4-OHT. Proliferation assay results showed that the genetic depletion of ABCB1 rendered TamR MCF-7 cells more sensitive to 4-OHT (Figure 4D and Figure S4B). These results suggest that ATF3 transcriptionally activates the expression of ABCB1, thereby conferring tamoxifen resistance.

We next investigated the mechanism through which ATF3 regulates ABCB1 expression. In this vein, the exposure of leukemia cells to daunorubicin was shown to activate an integrated stress response-like transcriptional program to induce ABCB1 through the remodeling and activation of an ATF4-bound, stress-responsive enhancer.[50] Therefore, we asked whether ATF3 could bind to these enhancers to promote the transcription of ABCB1. To this end, we first analyzed the ChIP-seq data retrieved from the publicly available data sets and found that two enhancer regions are potentially bound by ATF3 (Figure S4C). In addition, our ChIP-qPCR results confirmed that these two enhancers could be bound by ATF3 compared with a control region that is 5 kb upstream of the E1 enhancer, and the occupancy of ATF3 was significantly higher in TamR MCF-7 cells than parental MCF-7 cells (Figure 4E). These data suggest that ATF3 can bind to the enhancer regions of ABCB1 and thus promote its transcription in TamR MCF-7 cells.

ATF3 is Positively Regulated by AK4.

Our recent study showed that an increased expression of adenylate kinase 4 (AK4) modulates the resistance of breast cancer cells to tamoxifen through an m^6A-based epitranscriptomic mechanism.[34] Thus, we asked whether there exists a connection between ATF3 and AK4 in conferring resistance of MCF-7 cells to tamoxifen. To test this, we first assessed the relative expression level of ATF3 in MCF-7 cells with the overexpression of AK4 and in TamR MCF-7 cells upon the stable knockdown of AK4. Our results showed that the overexpression of AK4 in parental MCF-7 cells elicited an elevated expression level of ATF3, and the genetic depletion of AK4 led to a decreased level of ATF3 (Figure S5A,B). On the contrary, we found that neither the overexpression of ATF3 in MCF-7 cells nor the knockdown of ATF3 in TamR MCF-7 cells modulates the expression level of AK4 protein (Figure S5C,D). Together, these results suggest that ATF3 constitutes a downstream target of AK4.

DISCUSSION

Tamoxifen has been employed for many years in treating ER-positive breast cancer; nonetheless, drug resistance has been the most challenging issue for this therapy.[4,51] Understanding the molecular mechanism of resistance to this drug may offer new venues for overcoming therapeutic resistance. Here, we uncovered that an elevated level of ATF3 contributes to the resistance of MCF-7 breast cancer cells to tamoxifen, and this process entails an elevated expression of ABCB1. Our findings corroborated the previous observation that ATF3 can be induced by several chemotherapeutic agents[17–19] and provided new mechanistic insights by underscoring that the increased expression of ATF3 in tamoxifen-resistant cells arises from the decreased expression of the m^6A reader protein YTHDF2 and the ensuing elevated stability of ATF3 mRNA, which ultimately promotes the translation of ATF3.

The m^6A-mediated epitranscriptomic mechanism is known to modulate therapeutic resistance.[27,30] Additionally, we revealed recently that TamR MCF-7 cells exhibit an increased expression of METTL3, which leads to elevated levels of m^6A in the 5′-UTR of AK4 mRNA and augmented expression of AK4 protein, thereby conferring resistance to tamoxifen.[34] Here, our work unveils another novel molecular target for the m^6A-based epitranscriptomic mechanism (i.e., ATF3 mRNA) and uncovers the epitranscriptomic regulator for this target (i.e., YTHDF2). We also found that AK4 could positively regulate the expression of ATF3, suggesting ATF3 as a downstream target of AK4. It will be important to explore, in the future, how chronic exposure to tamoxifen elicits a decreased expression of YTHDF2 and how AK4 stimulates the expression of ATF3.

ABCB1 induction was shown to result in resistance to paclitaxel and olaparib in ovarian cancer cells.[52] Additionally, ATF4 was found to induce ABCB1 expression through binding to enhancer regions of ABCB1 in daunorubicin-resistant leukemia cells.[50] Here, we uncovered that another transcription factor of this family, i.e. ATF3, could bind to the enhancers of ABCB1 to activate its transcription, which ultimately gives rise to drug resistance.

Together, our results demonstrated a new role of ATF3 in drug resistance, revealed its downstream target (i.e., ABCB1), and suggested ATF3 as a candidate therapeutic target for mitigating drug resistance in cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R01 CA210072 and R01 ES032024). The authors would like to thank Prof. Guangdi Wang for providing the drug-resistant cell line employed in this study.

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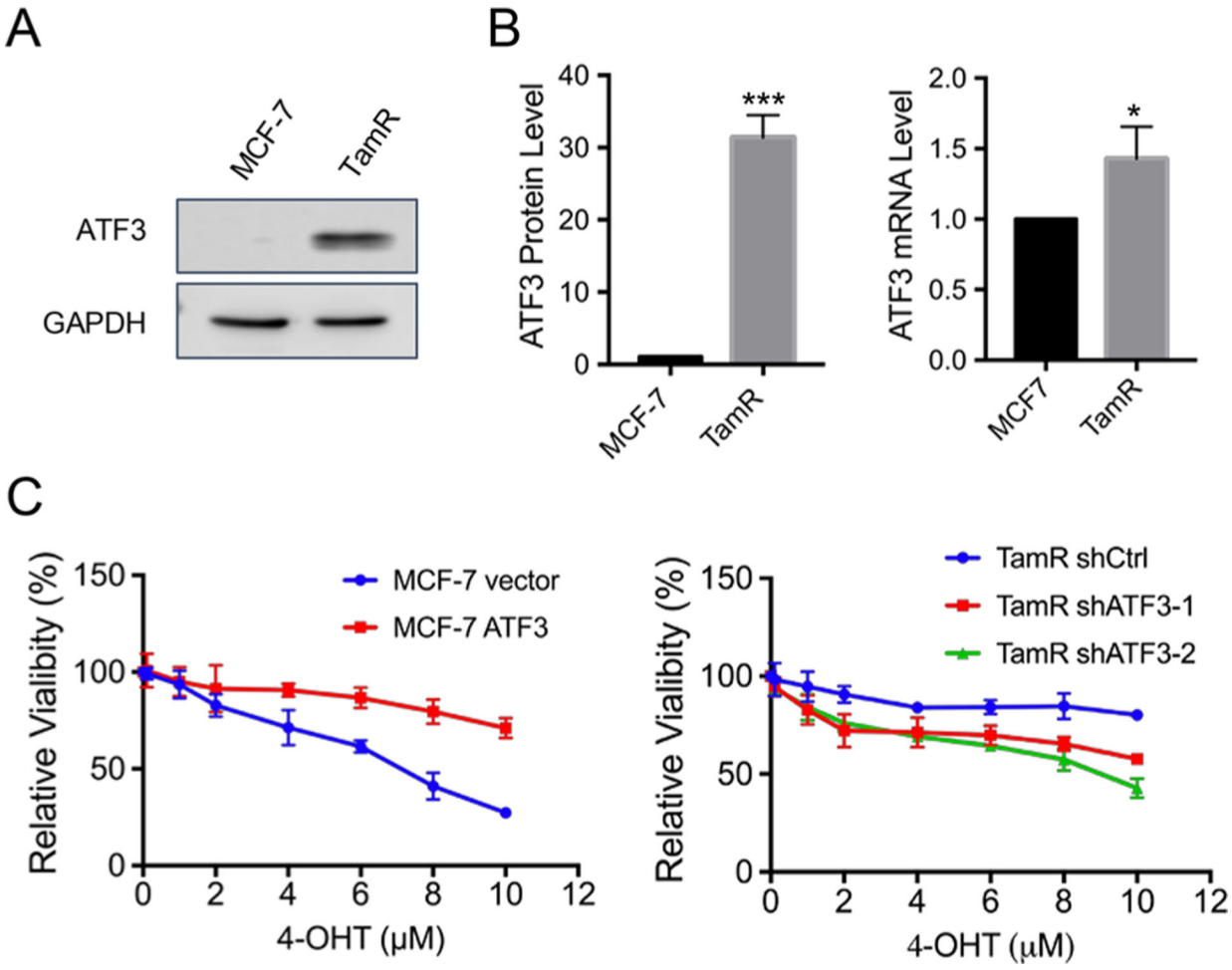

Figure 1.

ATF3 protein is expressed at higher level in TamR MCF-7 cells than parental MCF-7 cells, which confers resistance to 4-OHT. (A) Western blot for monitoring the relative levels of expression of ATF3 protein in TamR MCF-7 and parental MCF-7 cells. (B) Relative levels of ATF3 protein and mRNA levels in the drug-resistant/parental MCF-7 cells. The Western blot signal for ATF3 was measured on the basis of its band intensity using ImageJ, normalized against that of the loading control (i.e., GAPDH), and displayed relative to the level observed in parental MCF-7 cells. (C) Parental MCF-7 cells with a stable overexpression of ATF3 and TamR MCF-7 cells with a stable knockdown of ATF3 were incubated with different concentrations of 4-OHT for 72 h, and cell viability was monitored with CCK8. The data were normalized against the control groups (ethanol) and displayed as mean ± SD ($n = 3$). The p values were calculated on the basis of unpaired, two-tailed Student's t-test: *, $0.01 \leq p < 0.05$; ***, $p < 0.001$.

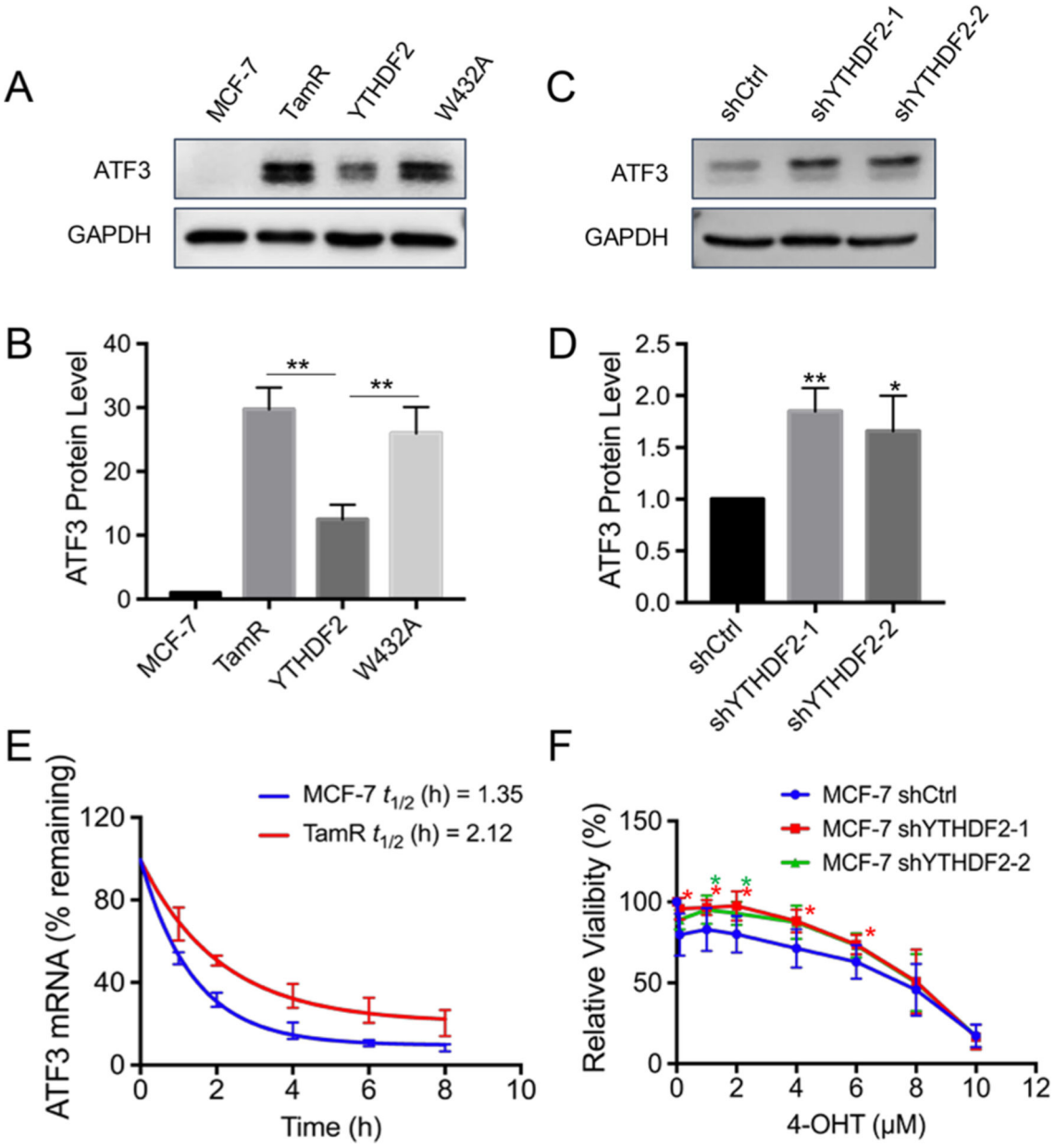

Figure 2.

TamR MCF-7 cells display decreased YTHDF2 expression, which stabilizes ATF3 mRNA. (A) Western blot images and (B) quantification results for examining alterations in the expression of ATF3 protein in TamR MCF-7 cells after the overexpression of YTHDF2 or its mutant defective in m^6A binding, i.e., YTHDF2 W432A. (C) Western blot images and (D) quantification results for monitoring the levels of ATF3 protein in MCF-7 cells after the knockdown of YTHDF2. The values in the bar graphs of (B) and (D) were calculated from the ratios in band intensities of ATF3 over those of GAPDH and further normalized to that of MCF-7 cells. (E) Time-dependent changes in the relative levels of ATF3 mRNA in MCF-7 and TamR MCF-7 cells after treatment with actinomycin D. (F) Parental MCF-7 cells with a stable knockdown of YTHDF2 were incubated with different concentrations of

4-OHT for 72 h, and cell viability was monitored using CCK8. The data were normalized against the control group (ethanol) and represented by mean ± SD ($n = 3$). The p-values were calculated on the basis of unpaired, two-tailed Student's t-test: *, $0.01 \leq p < 0.05$; **, $0.001 \leq p < 0.01$.

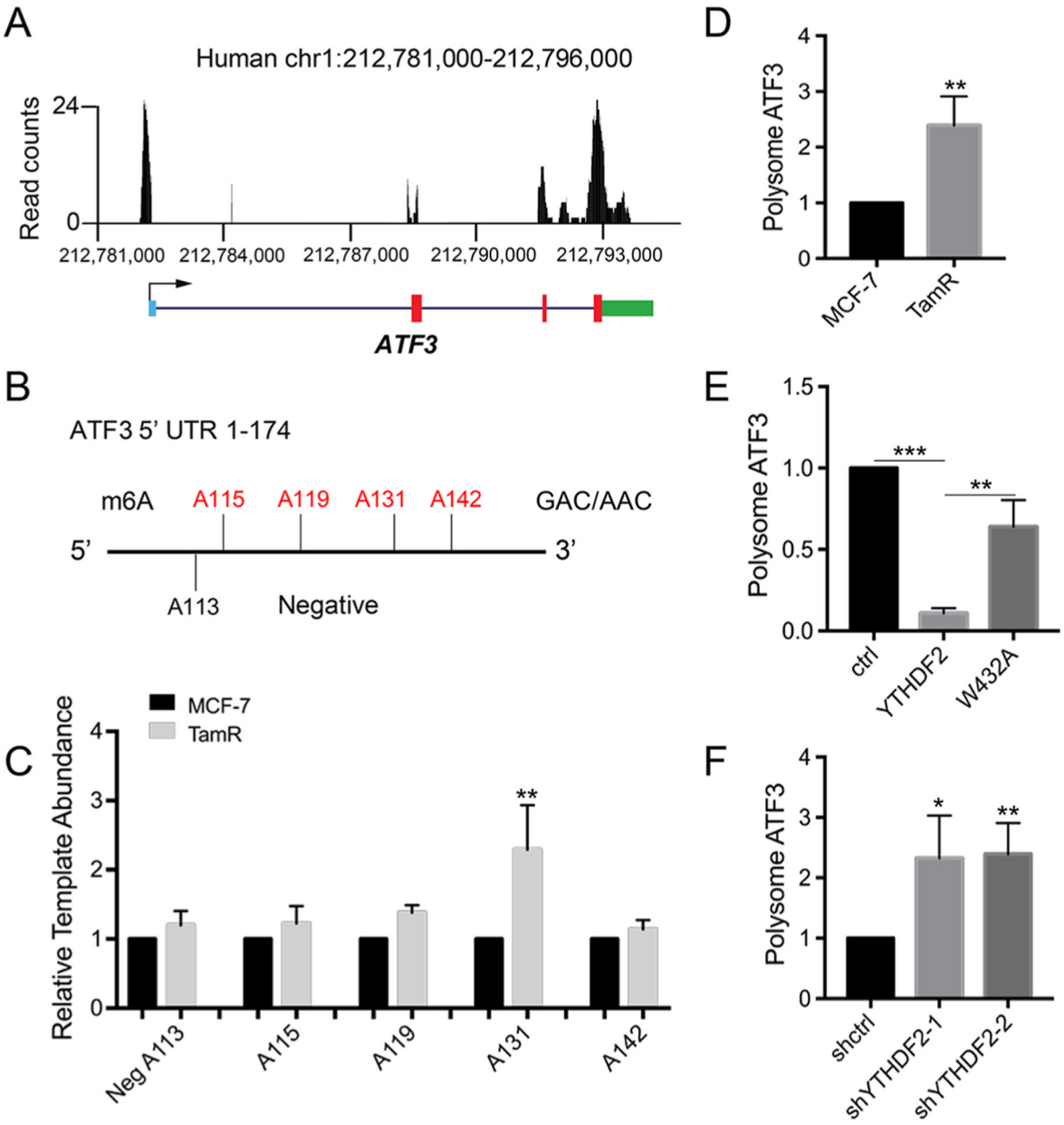

Figure 3.

m^{6}A level at adenosine 131 in the 5′-UTR of ATF3 mRNA was lower in TamR MCF-7 cells than parental MCF-7 cells, and decreased YTHDF2 promotes the translation of ATF3. (A) Integrated Genome Browser track showing the distribution of m^{6}A peaks in ATF3 mRNA. The data were obtained from MeT-DB v2.0 (http://180.208.58.19/metdb_v2/), where the 5′-UTR, CDS, and 3′-UTR are shown in blue, red, and green bars, respectively, and intron regions are designated with a thin yellow line. (B) Putative m^{6}A sites in thte 5′-UTR of ATF3 mRNA. (C) Relative template abundances of ATF3 5′-UTR measured by the SELECT method ($n \geq 3$). A total of five sites were monitored, four at m^{6}A motif site (GAC/AAC) and one at a CAG site (as a negative control, "Neg"). The *p*-values were calculated versus the negative control. (D) Relative levels of polysome-bound ATF3 mRNA in parental and TamR

MCF-7 cells. (E) Quantification of changes in levels of polysome-bound ATF3 mRNA in TamR MCF-7 cells after the overexpression of YTHDF2 or YTHDF2-W432A. (F) Bar chart displaying the levels of polysome-bound ATF3 mRNA in MCF-7 cells after a knockdown of YTHDF2. The data were normalized against the control groups and represented as mean ± SD ($n = 3$). The p-values were calculated on the basis of unpaired, two-tailed Student's t-test: *, $0.01 \leq p < 0.05$; **, $0.001 \leq p < 0.01$; ***, $p < 0.001$.

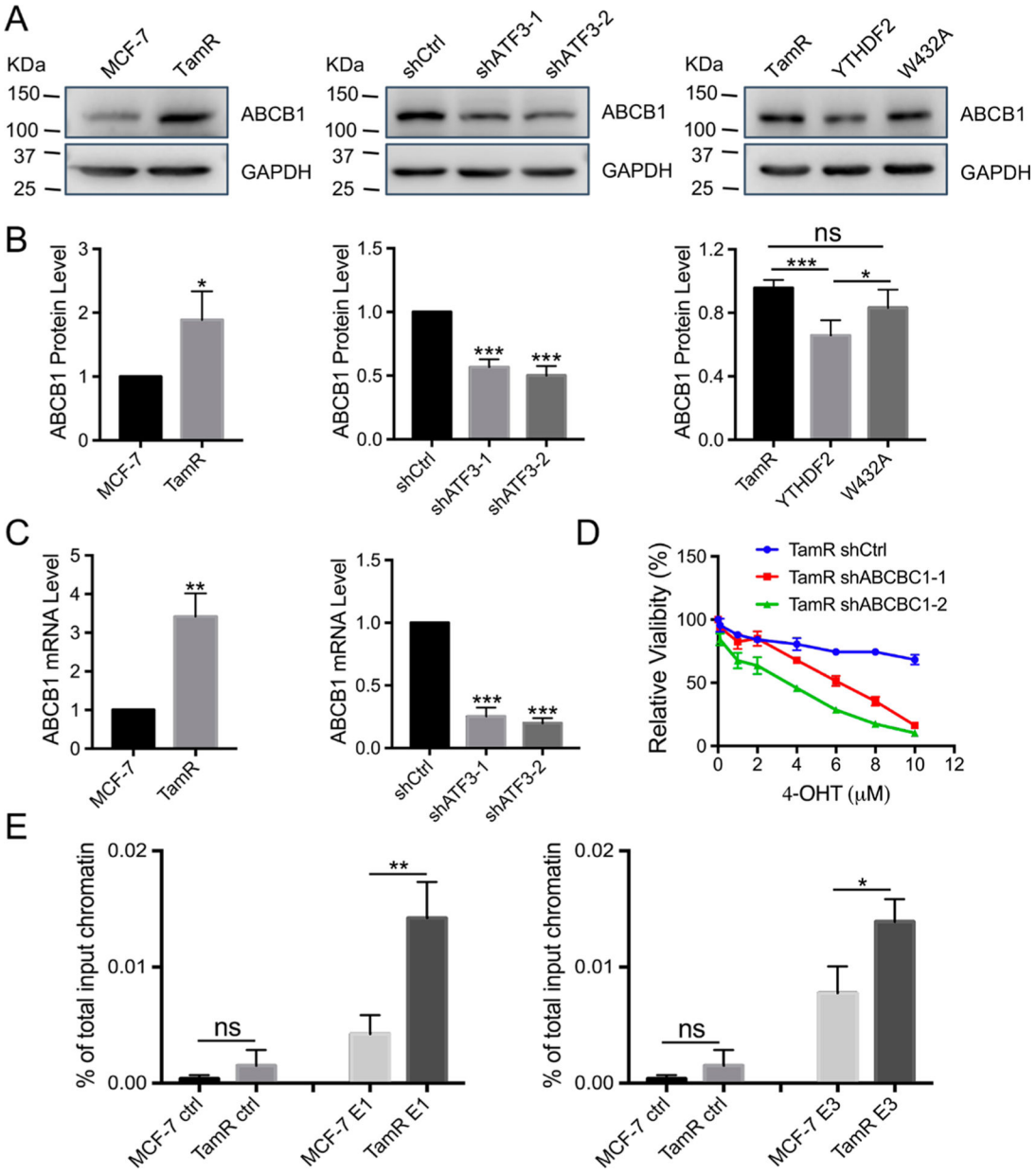

Figure 4.

ATF3 transcriptionally regulates ABCB1 in TamR MCF-7 cells. (A) Western blot images and (B) quantification results for examining the levels of ABCB1 protein in MCF-7 and TamR MCF-7 cells, TamR MCF-7 cells after knockdown of ATF3, or TamR MCF-7 cells upon the overexpression of YTHDF2 and its W432A mutant. The relative levels of ABCB1 protein were measured from band intensities using ImageJ and are depicted relative to the level detected for control cells. (C) Relative levels of ABCB1 mRNA in parental and TamR MCF-7 cells or in TamR MCF-7 cells after knockdown of ATF3. (D) TamR MCF-7 cells with a stable knockdown of ABCB1 were treated with different concentrations of 4-OHT for 72 h, and cell viability was measured using CCK8. The data were normalized against the control groups (ethanol) and represented as mean ± SD ($n = 3$). (E) ChIP-qPCR data showed

thw occupancy of ATF3 in the enhancer regions of *ABCB1* genes in TamR MCF-7 cells. The *p*-values were calculated on the basis of unpaired, two-tailed Student's *t*-test: *, $0.01 \leq p < 0.05$; **, $0.001 \leq p < 0.01$; ***, $p < 0.001$; ns, not significant (i.e., $p > 0.05$).