RNA BIOLOGY
2024, VOL. 21, NO. 1, 1–15
https://doi.org/10.1080/15476286.2023.2286101

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Post-transcriptional regulation of BIRC5/survivin expression and induction of apoptosis in breast cancer cells by tristetraprolin

Suhad Al-Yahya[a], Maher Al-Saif[a], Maha Al-Ghamdi[b], Walid Moghrabi[a], Khalid S.A. Khabar[a], and Norah Al-Souhibani [a]

[a]Molecular Biomedicine Department, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; [b]Biomedical Physics Department, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia

ABSTRACT

Inhibition of apoptosis is one of the hallmarks of cancer and is a target of various therapeutic interventions. BIRC5 is an inhibitor of apoptosis that is aberrantly expressed in cancer leading to sustained growth of tumours. Post-transcriptional control mechanisms involving RNA-binding proteins and AU-rich elements (AREs) are fundamental to many cellular processes and changes in the expression or function of these proteins can promote an aberrant and pathological phenotype. BIRC5 mRNA has an ARE in its 3′ UTR making it a candidate for regulation by the RNA binding proteins tristetraprolin (TTP) and HuR (ELAVL1). In this study, we investigated the binding of TTP and HuR by RNA-immunoprecipitation assays and found that these proteins were associated with BIRC5 mRNA to varying extents. Consequently, BIRC5 expression decreased when TTP was overexpressed and apoptosis was induced. In the absence of TTP, BIRC5 mRNA was stabilized, protein expression increased and the number of apoptotic cells declined. As an ARE-mRNA stabilizing protein, recombinant HuR led to upregulation of BIRC5 expression, whereas HuR silencing was concomitant with downregulation of BIRC5 mRNA and protein and increased cell death. Survival analyses demonstrated that increased TTP and low BIRC5 expression predicted an overall better prognosis compared to dysregulated TTP and high BIRC5. Thus, the results present a novel target of ARE-mediated post-transcriptional regulation.

ARTICLE HISTORY
Revised 10 November 2023
Accepted 14 November 2023

KEYWORDS
Apoptosis; BIRC5; post-transcriptional regulation; RNA-binding protein; tristetraprolin

Introduction

Breast cancer has been reported to be the highest in estimated new incidence among females in the United States, comprising 30% of all new cancer cases. However, breast cancer has been estimated to rank fourth in deaths among females in the US, an improvement from previous years when it came in second place as the leading cause of death from cancer [1]. One of the hallmarks of cancer is the evasion of programmed cell death (apoptosis) [2]. Apoptosis is an important physiological process for removal of unhealthy and damaged cells. On the other hand, disproportionate cell death, such as that occurs with certain chemotherapeutic agents, can destroy vital non-renewable cells. Therefore, maintaining a proper balance is crucial for sustaining cellular and tissue homoeostasis. Aberrant apoptosis mechanisms are increasingly being recognized as major targets of therapy, in conjunction with efforts to decelerate proliferation and abnormal growth of tumour masses by means of targeting the cell cycle. Thus, selective induction of apoptosis has become the focus of recent clinical approaches [3–5]. However, in order to be selective, in that only tumour cells are targeted and not normal cells, the approach should focus on normalizing overexpression or under expression of key factors involved in apoptosis [6].

BIRC5 (also called survivin) is a member of the Inhibitors of Apoptosis Protein (IAP) family that inhibits apoptosis by interfering with the caspase cascade. BIRC5 is overexpressed in many cancers including breast, colon, cervical, and prostate cancers, and is associated with a poor prognosis [7–11]. In addition to the inhibition of cell death, BIRC5 is involved in other aspects of mitosis and cell division, such as regulation of chromosome-microtubule attachment and cytokinesis [12].

Transcriptional regulation of BIRC5 occurs via p53 and PTEN [13, 14] and several signalling pathways such as the PI3/Akt and Stat3 pathways [15, 16]. BIRC5 is post-translationally modified by acetylation [17], ubiquitylation [18], and phosphorylation, the latter being a requirement for its function in apoptosis inhibition [19, [20].

Post-transcriptionally, BIRC5 has been reported to be targeted by a number of microRNAs in bladder, colorectal, and breast cancers [21–24]. AU-rich elements (AREs) are well-characterized cis-acting elements present in the 3′ UTR of many mRNAs. AREs are recognized by various RNA binding proteins (RBPs) that bind to the ARE and elicit several regulatory functions, such as mRNA decay, stabilization, and translational control. Tristetraprolin (ZFP36, TTP) is a well-known ARE-binding protein that induces the mRNA decay of inflammatory cytokines such as TNF, IL-8, GM-CSF, and IL-10 [25], [26] and cancer genes involved in mitosis, invasion and metastasis such as NEK2, TOP2A, PLAU, MMP1, PLAUR, VEGF, and CXCR4 [27–30]. TTP is the product of an early response gene that

CONTACT Norah Al-Souhibani noalsuhaibani@kfshrc.edu.sa; Khalid S.A. Khabar khabar@kfshrc.edu.sa Molecular Biomedicine Department, Molecular Biomedicine Department, (JCIA-Accredited Academic Medical Center), Takhasusi Road, Riyadh P3354, MBC-03, Saudi Arabia

Supplemental data for this article can be accessed online at https://doi.org/10.1080/15476286.2023.2286101

is downregulated in cancer [28,31]. On the other hand, the RBP Human antigen R (ELAVL1, HuR) is an ARE mRNA stabilization factor that is upregulated in cancer [32,33].

BIRC5 mRNA contains an ARE at its 3′ UTR that can subject it to regulation by the RBPs TTP and HuR. In this study, we present a detailed characterization of ARE-mediated regulation of BIRC5 expression. We demonstrate that TTP and HuR interact with BIRC5 mRNA and regulate its stability and protein expression. We also show that modulation of TTP and HuR expression resulted in downstream effects on induction or inhibition of apoptosis via regulation of BIRC5 expression.

Materials and methods

Cell culture

The breast cancer tumorigenic cell lines MDA-MB-231, MCF-7, and T47D, the normal-like mammary cell line MCF10A, and the kidney cancer cell line HEK293 were obtained from ATCC (Rockville, MD). MDA-MB-231, MCF-7, T47D, HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific) supplemented with 10% Foetal Bovine Serum (FBS, Invitrogen) and 1% penicillin–streptomycin (100 units/mL penicillin and 100 µg/mL streptomycin) antibiotics and 2 mM L-glutamine. MCF10A cells were cultured in a 1:1 mixture of Ham's F12 and DMEM fortified with HuMEC supplement (Life Technologies), 10% FBS and antibiotics. TTP/zfp36$^{+/+}$ and TTP/zfp36$^{-/-}$ mouse embryonic fibroblasts (MEFs) were obtained as previously described [28] and were grown in DMEM supplemented with 10% FBS and antibiotics. HEK293 Tet-On Advanced cells (Clontech, Mountain View, CA) were used in Tetracycline-induced expression experiments and were cultured in DMEM supplemented with 10% Tet System-Approved FBS (Clontech), 100ug/ml G418 (Sigma), and 5% Penicillin–Streptomycin (Invitrogen, Carlsbad, CA). Cells are routinely tested for mycoplasma contamination.

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA was extracted using Trizol (ThermoFisher) as per manufacturer's instructions. A DNAse treatment step is included during RNA preparation to avoid contamination with genomic DNA. The reverse transcription (RT) reaction was performed using M-MLV reverse transcriptase (Invitrogen) and 5 µg RNA. Quantitative PCR (qPCR) was performed as multiplex reactions in a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA) using FAM-labelled gene-specific TaqMan probes (ThermoFisher Scientific) and VIC-labelled endogenous control probes (ThermoFisher Scientific). Samples were amplified in triplicate, and quantification of relative expression was performed using the $\Delta\Delta C_t$ method. For the reporter mRNA stability assay, Fluor® Universal qPCR Master Mix (New England Biolabs, MA) was used to detect amplification of reporter products.

Plasmids and ARE-reporter constructs

The pCR3.1-TTP and pcDNA3.1-HuR plasmids used are as described previously [34]. BIRC5 Human-tagged ORF clone (NM_001168) was purchased from OriGene. RPS30 promoter-linked reporter expression constructs containing BIRC5 3' UTR, BIRC5 ARE, BIRC5 mutated ARE (NM_001168.3) and TNF 3' UTR sequences were synthesized by ligating two annealed synthetic complimentary oligonucleotides with BamHI and XbaI overhangs as previously described [35]. Annealed oligos were cloned into the 3' UTR of a RPS30-NanoLuciferase vector. Tet-ON TTP inducible constructs were described previously [36]. A list of primers and plasmid constructs used is available in Supplemental Material.

RNA interference

RNA interference was performed using siRNA duplexes against TTP (NM_ 003407, TTP siRNA-1 sense 5′-GACGGAACUCUGUCACAAG–3′ and antisense 5′–CUUGUGACAGAGUCCGUC–3′ and TTP siRNA-2 sense 5′–GUUGUGGAUGAAGUGGCAG-3′ and anti-sense 5′–CUGCCACUUCAUCCACAAC-3′) and HuR (NM_001419) (HuR siRNA-1, sense, 5′-GCCUGUUCAGCAGCAUUGG-3′ and antisense, 5′-CCAAUGCUGCUGAACAGGC-3′ and HuR siRNA-2, sense 5′-UGUGAAAGUGAUCCGCGAC-3′ and antisense, 5′-GUCGCGGAUCACUUUCACA-3′). TTP and HuR siRNAs, including non-specific controls, were custom-made by Metabion (Germany). BIRC5 siRNA was purchased from Santa Cruz Biotechnology (sc-29499, Santa Cruz, CA, USA). The efficiency of siRNA silencing was determined by immunoblotting. After transfection of siRNA, cells were harvested for flow cytometry, or for measurement of BIRC5 protein in addition to full-length and cleaved caspase 3 and caspase 7 protein levels.

Transfection and reporter activity assessment

Transfections were performed using Lipofectamine 2000 or Lipofectamine LTX transfection reagent (ThermoFisher Scientific) to transfect HEK293 cells or MDA-MB-231 cells, respectively, in reduced serum media. For TTP overexpression, MDA-MB-231 cells were transfected with pCR3.1-TTP plasmid or empty vector control. HuR and BIRC5 overexpression was carried out in MCF10A cells. For silencing experiments, MCF-7 cells were transfected with 100 nM of TTP siRNA. Similarly, 50 nM of HuR siRNA or BIRC5 siRNA were transfected into MDA-MB-231 cells. For reporter experiments, HEK293 cells or HEK293 Tet-On Advanced cells were seeded in 96-well plates and transfected with 25 ng of reporter alone (HEK293 cells) or co-transfected with 10 ng Tet-ON TTP inducible constructs (HEK293 Tet-ON). Induction of TTP expression was achieved with doxycycline (0.25 µM), luciferase activity was assessed 24 h after transfection using Nano-Glo® Dual-Luciferase® Reporter Assay (Promega), and detection was performed using the Varioskan Flash multimode plate reader (Thermo Fisher Scientific). For the reporter mRNA stability assay, HEK293 cells were transfected with RPS30M1-Nluc-DU reporter for 24 h then treated with

ActD (5ug/ml) for 1 h, 2, 4, and 7 h followed by RNA extraction with Trizol for RT-qPCR as indicated earlier.

Immunoprecipitation of RNP complexes

For TTP-IP, MDA-MB-231 cells were transfected with TTP, C124R, or PCR3.1 empty vector plasmid for 24 h. The immunoprecipitation procedure was carried out as described previously [28]. TTP-associated mRNA was subjected to RT-qPCR, as described above, using FAM-labelled probes for BIRC5 and normalized to a VIC-labelled probe for GAPDH (Applied Biosystems).

In an alternative method, HEK-293 cells (10 cc plates) were transfected with pcDNA3.1 vectors expressing HA-tagged TTP or myc-tagged HuR along with 100 ng of RPS30-SGFP-BIRC5-ARE (details on the reporter constructs were previously described in [35]). To immunoprecipitate HA-tagged TTP, Protein G Sepharose 4-Fast Flow (GE Healthcare, Cat#17-0618-01), beads coupled to either monoclonal anti-HA or anti-myc antibodies as the negative control were used. Anti-myc antibody was used to immunoprecipitate HuR protein, and anti-HA antibody was used as a negative control. In a third experiment, HEK293 cells were transfected with myc-tagged HuR alone or co-transfected with TTP plasmid as a competing RNA-binding protein. Anti-myc antibody was used to precipitate HuR protein in HuR- and TTP-transfected samples and IgG was used as a control. RT-qPCR for the transfected reporter gene was performed using a FAM-labelled probe for RPS30-SGFP-BIRC5-ARE and normalized to VIC- labelled RPLPO.

mRNA stability

The mRNA stability assay was done by seeding TTP/zfp36$^{+/+}$ and TTP/zfp36$^{-/-}$ MEFs in 6-well plates in regular serum conditions. The following day, media was replaced with 0.5% serum for 18 h then re-stimulated with 10% serum for 2 h followed by the addition of Actinomycin D (ActD, 5 µg/ml) to the cells. Total RNA was extracted at 0, 2, 4, 8 and 24 h for RT-qPCR using TaqMan expression assays for mouse Birc5 and β-actin as the endogenous control. Similarly, mRNA stability assays were performed in MDA-MB-231 cells transfected with 0.1 ug TTP plasmid or MCF10A cells transfected with 1 ug of HuR plasmid. ActD (5 ug/ml) was added the next day for 0, 2,4, 8, and 24 h and total RNA was extracted for RTqPCR. The reporter mRNA stability assay has been described above.

Immunoblotting

Immunoblotting was performed as described previously [28]. Primary antibodies used were as follows: rabbit anti-BIRC5 (# 2803, Cell Signaling Technology, Danvers, MN, USA), goat anti-TTP (sc-8458, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat-anti HuR (sc-5483, Santa Cruz Biotechnology), rabbit anti caspase 3 (#9665, Cell Signalling Technology), rabbit anti-cleaved caspase 3 (# 9664, Cell Signaling Technology), rabbit anti-caspase 7 (#12817, Cell Signalling), rabbit anti-cleaved caspase 7 (# 8438, Cell Signaling Technology), rat anti-HA (Roche, USA), rabbit anti-tubulin (Cell Signaling # 2125) and rabbit anti β-actin (Cell Signaling # 4970), and rabbit anti-GAPDH (Cell Signaling #2118).

Flow cytometry

Apoptosis was assessed by flow cytometry using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & Propidium Iodide (PI) (Life Technologies). Cells were harvested and washed in cold PBS, centrifuged and re-suspended in 100 µL of 1X annexin-binding buffer at a density of $1 \times 10[6]$ cells/mL. Alexa Fluor® 488 annexin V (5 µL) and PI working solution (1uL 100 µg/mL) were added to the 100 µL suspension and incubated at room temperature for 15 min. Four hundred-microliter 1X annexin-binding buffer was added to the cell suspension, mixed and analysed by flow cytometry at 530 nm and 575 (or equivalent) using 488 nm excitation. Cells were analysed on an LSR II flow cytometer (Becton Dickinson, Mountain View, CA, USA), and data was acquired using BD FACSDiva software.

Apoptosis-associated caspase 3 and caspase 7 levels were assessed by flow cytometry. Harvested cells were centrifuged, fixed with 400 µL 4% formaldehyde, permeabilized with permeabilization buffer (BD Biosciences), incubated with anti-cleaved caspase 3 or caspase 7 antibody (Asp175), (Asp198), Cell Signaling, dilution 1:100) on ice for 1 h, then washed twice with FACS buffer (2% FBS in distilled H_2O). Cells were then incubated with FITC-conjugated goat anti-rabbit IgG (ab6717, Abcam, 1:400) for 30 min in the dark, washed twice and resuspended in FACS buffer. Fluorescence was analysed on an LSR II flow cytometer as indicated above.

Cancer patient data

BIRC5, TTP and HuR mRNA expression levels for 389 Invasive Ductal Breast Cancer (IDBC) patients and 61 corresponding normal-matched samples were downloaded from The Cancer Genome Atlas (TCGA) database through the Oncomine web-based platform. Log2 median-centred intensity ratios were used, and an expression of 1.7-fold of normal was the determining criterion for upregulation.

Kaplan–Meier survival analysis was performed using the Kaplan-Meier Plotter portal (kmplot.com) for breast cancer. Overall survival (OS), relapse-free survival (RFS), and distant metastasis-free survival (DMFS) data were used and split into high and low BIRC5 mRNA expression groups based on the best cut-off option. The BIRC5 probe selected was 202095_s_at and all available data sets were used for the survival analysis. Similarly, OS, RFS, and DMFS survival curves were generated for TTP with inverted BIRC5 mRNA expression in addition to HuR and non-inverted BIRC5 mRNA expression. Survival curves were generated with their associated log rank p-values and hazard ratios (HR) from the portal. A p-value of < 0.05 was considered statistically significant.

Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA, USA). Data were presented as mean ±

SEM. Statistical analyses were performed using two-tailed unpaired Student's t-test when comparing two columns, while one-way ANOVA was used to analyse three or more data columns with Dunnett's post-hoc multiple comparison test when comparing breast cancer cells to MCF10A as the control. P-values < 0.05 were considered statistically significant, and experiments were repeated at least three times unless stated otherwise in the figure legends. Quantification of immunoblots was performed using Image J software, and normalization was performed using the internal control.

Results

BIRC5 overexpression in invasive breast cancer patients and prognostic significance

We examined BIRC5 mRNA expression in a study cohort consisting of 389 invasive ductal breast cancer (IDBC) patient samples and 61 corresponding normal-matched samples from TCGA database. Based on a differential expression fold of 1.7 or greater, IDBC samples demonstrated significantly higher expression levels of BIRC5 mRNA (~2.6-fold, $P < 0.0001$) compared to normal patient samples (Figure 1A).

Similarly, BIRC5 mRNA and protein expression were elevated in several human breast cancer cell lines, triple negative (ER, PR, and Her2 receptor-negative) MDA-MB-231 cells, ER (+) MCF-7 and T47D cells compared to the normal-like ER (-) mammary cell line, MCF10A (Figures 1 B,C). BIRC5 mRNA was upregulated at least 15-fold in MCF-7 cells ($P < 0.0001$) and greater than 20-fold in T47D ($P < 0001$) and MDA-MB-231 cells ($P < 005$) compared to normal-like MCF10A cells. BIRC5 mRNA demonstrated slightly increased expression in T47D cells compared to MDA-MB-231 cells, whereas an almost twofold increase was evident compared to MCF-7 cells. BIRC5 protein was clearly upregulated in all three breast-cancer cell lines compared to MCF10A with a somewhat greater band in MDA-MB -231 cells (Figure 1C). For comparison, TTP and HuR protein levels were examined as well. MCF10A cells demonstrated

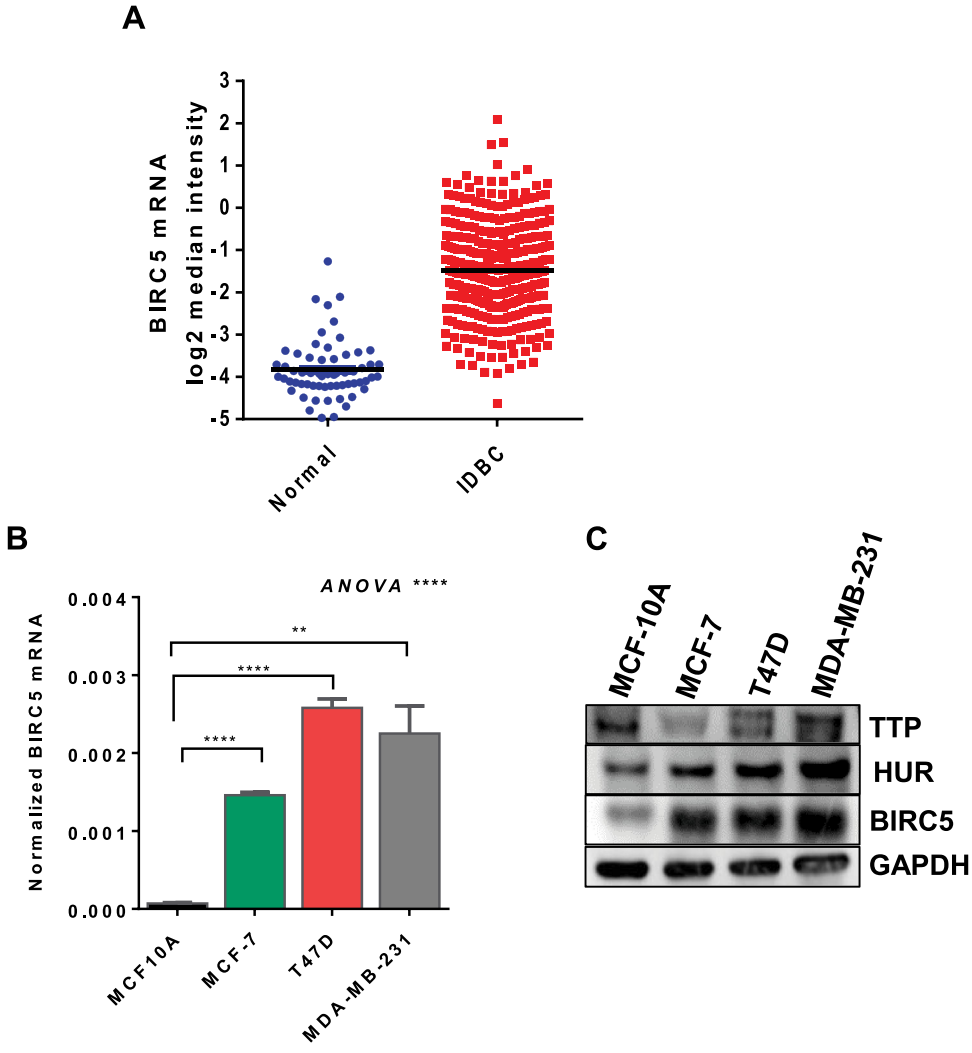

Figure 1. BIRC5 expression in breast cancer. (A) BIRC5 mRNA expression in invasive ductal breast cancer patients compared with normal patients. (B and C) BIRC5 mRNA and protein expression in the breast cancer cell lines MDA-MB-231, MCF-7, and T47D and the normal mammary cell line MCF10A. TTP and HuR protein levels were also examined in these cells. Cells were seeded in 6-well plates overnight. RNA and protein were extracted the following day for RT-qPCR and immunoblotting. BIRC5 mRNA was quantified using TaqMan specific primers and normalized to endogenous RPLPO. Protein levels were detected using anti-BIRC5, anti-TTP, and anti-HuR antibodies and normalized to GAPDH as the endogenous control. Results are from one experiment representative of three independent experiments. Statistical significance was achieved by one-way ANOVA with Dunnett's post-hoc multiple comparison test using MCF10A as the control, ****$P < 0.0001$.

a clear single lower band demonstrating un-phosphorylated active TTP protein. MCF-7 cells displayed a lighter band, indicating less overall TTP protein, but at the same time, one that is gradually being phosphorylated as evident by the spread-out band. T47D and MDA-MB-231 cells displayed two bands for TTP protein, a lower un-phosphorylated one, which is obviously less dense than that in MCF10A, and a higher molecular weight band representing phosphorylated and inactive TTP protein. HuR displayed a similar pattern of expression to BIRC5, which, as an RBP known to stabilize BIRC5, explains higher BIRC5 protein expression.

In order to investigate the prognostic significance of BIRC5 expression in breast cancer patients, we examined the effect of high and low BIRC5 mRNA expression on overall survival (OS), relapse-free survival (RFS), and distant metastasis-free survival (DMFS) in breast cancer patients using the KM plotter portal. As shown in Figures 2 A-C, the results indicate that high BIRC5 expression correlated poorly with OS (HR = 1.8, logrank P = 1e-06), RFS (HR = 1.92, log rank P < 1e-16) and DMFS (HR = 1.88, logrank P = 2.7e-15) compared to low expression. This demonstrates that the upregulation of BIRC5 in breast cancer patients is associated with decreased survival and overall poor prognosis.

Post-transcriptional regulation of BIRC5

In order to understand the mechanisms behind BIRC5 overexpression in breast cancer, we decided to focus on ARE-mediated post-transcriptional regulation. A characteristic feature of AREs in the 3′ UTR is that their presence confers an instability phenotype to the mRNA. Examination of human BIRC5 3' UTR sequence showed two putative TTP binding sites in the region 1975–1993 (NM_001168). Therefore, in order to test the functionality of BIRC5 ARE, we transfected HEK293 cells with RPS30 promoter-driven nano-luciferase reporter constructs containing either BIRC5 ARE, BIRC5 3′ UTR, BIRC5 mutant ARE, TNF 3′ UTR, or a non-ARE-containing control 3′ UTR (BGH) (Figure 2D). A statistically

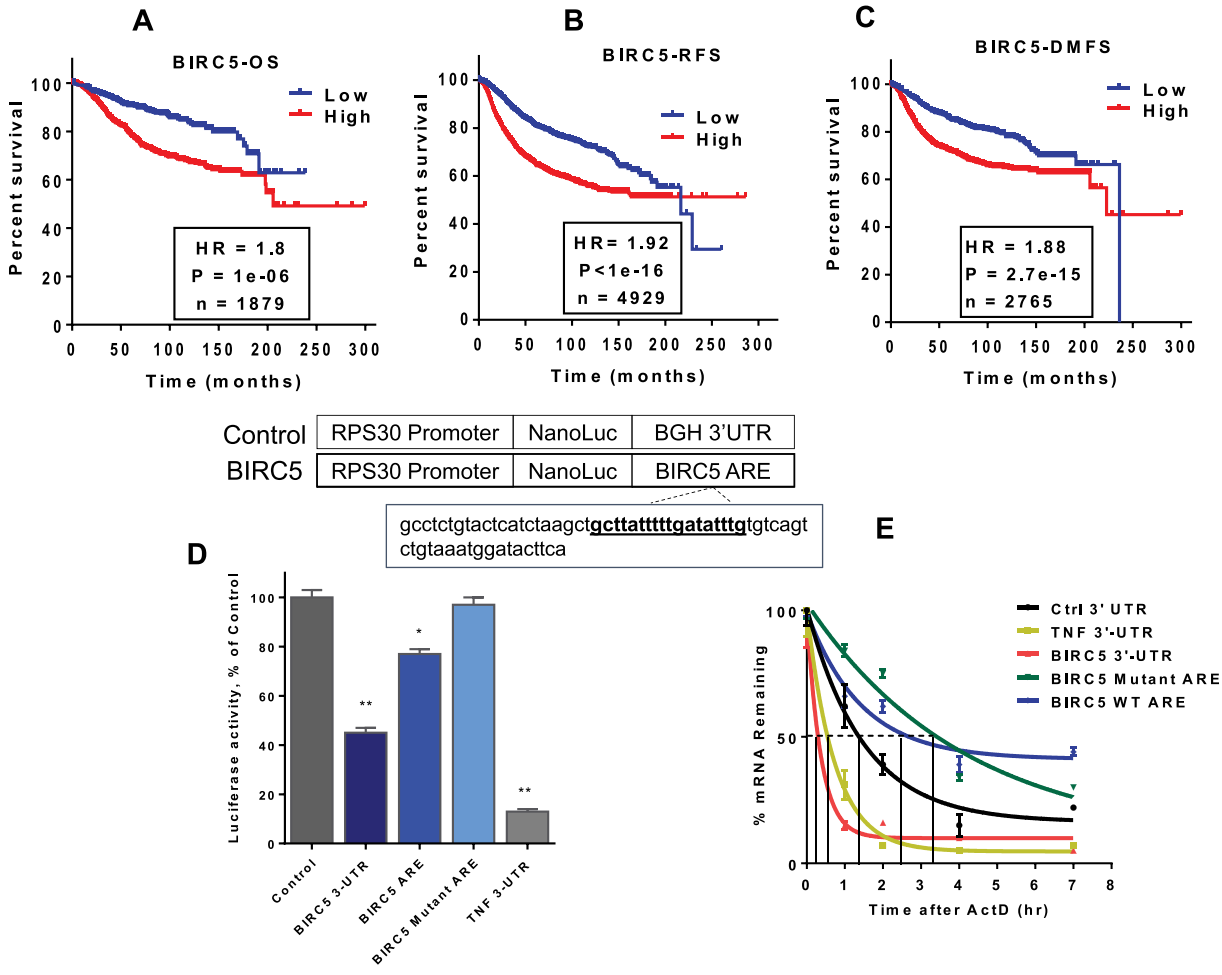

Figure 2. Breast cancer patient survival curves for high and low BIRC5 mRNA expression. (A-C) Kaplan–Meier curves for overall survival (OS), relapse-free survival (RFS), and distant metastasis-free survival (DMFS) were obtained from the KM-plotter portal. (D) assessment of BIRC5 are reporter activity. HEK293 cells were transfected with 25 ng of RPS-30 promoter-linked Nano-luciferase reporters containing BIRC5 ARE, BIRC5 3' UTR, BIRC5 mutated ARE, or TNF 3' UTR as a positive control. BGH 3' UTR was used as the negative control. Luciferase activity was measured after 24 h of transfection and presented as percent of control. Results are from one experiment representative of three independent experiments, *** P < 0.001, **** P < 0.0001 (Student's t-test). (E) assessment of reporter mRNA stability. Cells were transfected with RPS30M1-nluc-DU reporters containing BIRC5 ARE, BIRC5 3' UTR, BIRC5 mutated ARE, or TNF 3' UTR as a positive control. BGH 3' UTR was used as the negative control. Cells were treated with ActD (5 ug/ml) for the times indicated and RNA was extracted for RT-qPCR. Results are from one representative experiment of two independent experiments.

significant reduction (25%) in expression of BIRC5 ARE was detected, whereas the entire 3′ UTR resulted in a stronger (~50%) reduction in luciferase reporter activity compared to the non-ARE control 3′ UTR. No significant destabilizing effect was detected with BIRC5 mutant ARE reporter as well. This indicates that, while BIRC5 ARE is functional as a destabilizing factor, the entire length of the 3′ UTR or other regions present in the complete 3′ UTR are required for enhanced mRNA destabilizing activity. We also examined the mRNA stability of the reporters by means of Actinomycin D chase experiment. Cells were transfected with the reporter constructs as indicated for Figure 2D and then treated with ActD for the indicated times. RNA was then extracted for RT-qPCR. The mRNA stability of the reporters shows that both TNF 3′ UTR and BIRC5 3′ UTR were less stable than the control 3′ UTR (0.5 and 0.25 compared to greater than 1 h, respectively). BIRC5 WT ARE mRNA stability was greater than the control (~2.5 h) and BIRC5 mutant ARE demonstrated the highest reporter mRNA stability of all.

This indicates the importance of BIRC5's entire 3' UTR for destabilization to occur.

Correlation of BIRC5 expression with the RNA-binding proteins TTP and HuR

Since AU-rich mRNAs (ARE-mRNAs) are regulated by the RNA binding proteins, tristetraprolin (TTP, ZFP36) and HuR, which promote mRNA decay and stability, respectively, we examined their interrelationships with BIRC5 expression. We used the fit model to examine the correlation of mRNA expression of BIRC5 with that of TTP and HuR for the TCGA IDBC patient data. A significant negative correlation was found with TTP (Figure 3A, $P < 0.0001$, $r = -0.53$) indicating that BIRC5 mRNA increases as TTP is downregulated. However, HuR correlated positively with BIRC5 (Figure 3B, $P < 0.0001$, $r = 0.49$), demonstrating a parallel pattern of expression for HuR and BIRC5.

Association of BIRC5 mRNA with TTP and HuR

The presence of an active ARE in BIRC5 3' UTR and the results of the correlation studies prompted a close examination of the association of TTP and HuR proteins with BIRC5 mRNA. Therefore, we performed RNA-IP experiments to determine if the RNA binding proteins were bound to BIRC5 mRNA. In one experiment MDA-MB-231 cells were used due to the abundant expression of BIRC5. However, due to low TTP expression, the cells were transfected with wild-type or non-binding mutant TTP plasmid constructs. Overexpressed TTP protein was immunoprecipitated with anti-TTP antibody or normal IgG as a negative control. Associated BIRC5 mRNA was then assessed by RT-qPCR. As demonstrated in Figure 3C, BIRC5 mRNA was clearly associated with wild-type TTP compared to the control and the non-binding mutant (~3.5-fold increase in associated BIRC5 mRNA, $P < 0.01$).

In an alternative method, HEK293 cells were transfected with HA-tagged TTP plasmid and co-transfected with an RPS30 promoter-driven SGFP reporter construct containing BIRC5 ARE 3' UTR. TTP protein was immunoprecipitated with anti-HA or anti-myc as a negative control. In agreement with the previous IP experiment, SGFP reporter mRNA containing BIRC5 ARE 3′ UTR was enriched nearly threefold (Figure 3D, $P < 0.0001$) in the sample precipitated with anti-HA compared to that with anti-myc antibodies.

Similarly, we investigated HuR binding to RPS30-SGFP-BIRC5 -ARE by immunoprecipitation of overexpressed myc-tagged HuR protein in HEK293 cells using anti-myc antibody with anti-HA as a control. BIRC5 ARE mRNA was enriched nearly 20-fold in the anti-myc sample compared to the control, clearly demonstrating that HuR indeed binds to BIRC5 mRNA (Figure 3E). This prompted us to examine the extent of binding competition between HuR and TTP, so we transfected HEK293 cells with HuR plasmid alone or with TTP plasmid. HuR protein was then precipitated with anti-myc or IgG as a control antibody. BIRC5 ARE mRNA was enriched more than 30-fold in the HuR sample and 15-fold when TTP was co-transfected compared to the control (Figure 3F). Enrichment of BIRC5 ARE mRNA was reduced by 50% when TTP was co-transfected compared to the samples transfected with HuR alone ($P < 0.0001$). These findings indicate that TTP and HuR proteins compete to physically associate with BIRC5 mRNA with HuR showing greater affinity than TTP for BIRC5 mRNA.

TTP overexpression triggers apoptosis by downregulating BIRC5

We were interested in the functional effect of TTP binding to BIRC5 mRNA on the regulation of expression and the downstream consequences on apoptosis. Therefore, we overexpressed TTP protein by transfecting MDA-MB-231 cells with increasing amounts of HA-tagged TTP plasmid. Then, we looked at endogenous BIRC5 and caspase 3 protein levels as a result of TTP overexpression. The dose-dependent increase in recombinant TTP is demonstrated in Figure 4A as well as endogenous TTP protein levels. BIRC5 protein expression decreased as a result of TTP overexpression in a dose-dependent manner (Figure 4A). Full-length caspase 3 protein level decreased in the same manner as BIRC5, while that of cleaved caspase 3 increased in parallel with increasing amounts of TTP recombinant protein. Indeed, this effect was present with the lowest amount of plasmid transfected (0.1 μg). Examination of apoptosis by Annexin V also showed an increase in the number of apoptotic cells with increased TTP expression (Figure 4B). Caspase 3 staining by flow cytometry confirmed immunoblotting findings, demonstrating a dose-dependent increase as well (Figure 4C). Additional confirmation of TTP regulation of BIRC5 mRNA was achieved by performing reporter experiments using a tetracycline-inducible system for TTP expression. TTP induction led to a significant decrease in BIRC5 3' UTR (>25%, $P < 0.0001$) and BIRC5 ARE (~50%, $P < 0.0001$) compared to BIRC5 mutant ARE and the non-ARE control (Figure 4D). TNF 3' UTR was used as the positive control and showed more than 90% reduction in luciferase activity when doxycycline was added. BIRC5 mRNA stability as a result of TTP overexpression using the lowest amount of TTP (0.1 ug) was also performed (Figure S4-A) showing a reduction in BIRC5 mRNA half-life when TTP was overexpressed compared to the control. The results show that TTP does indeed regulate BIRC5 mRNA

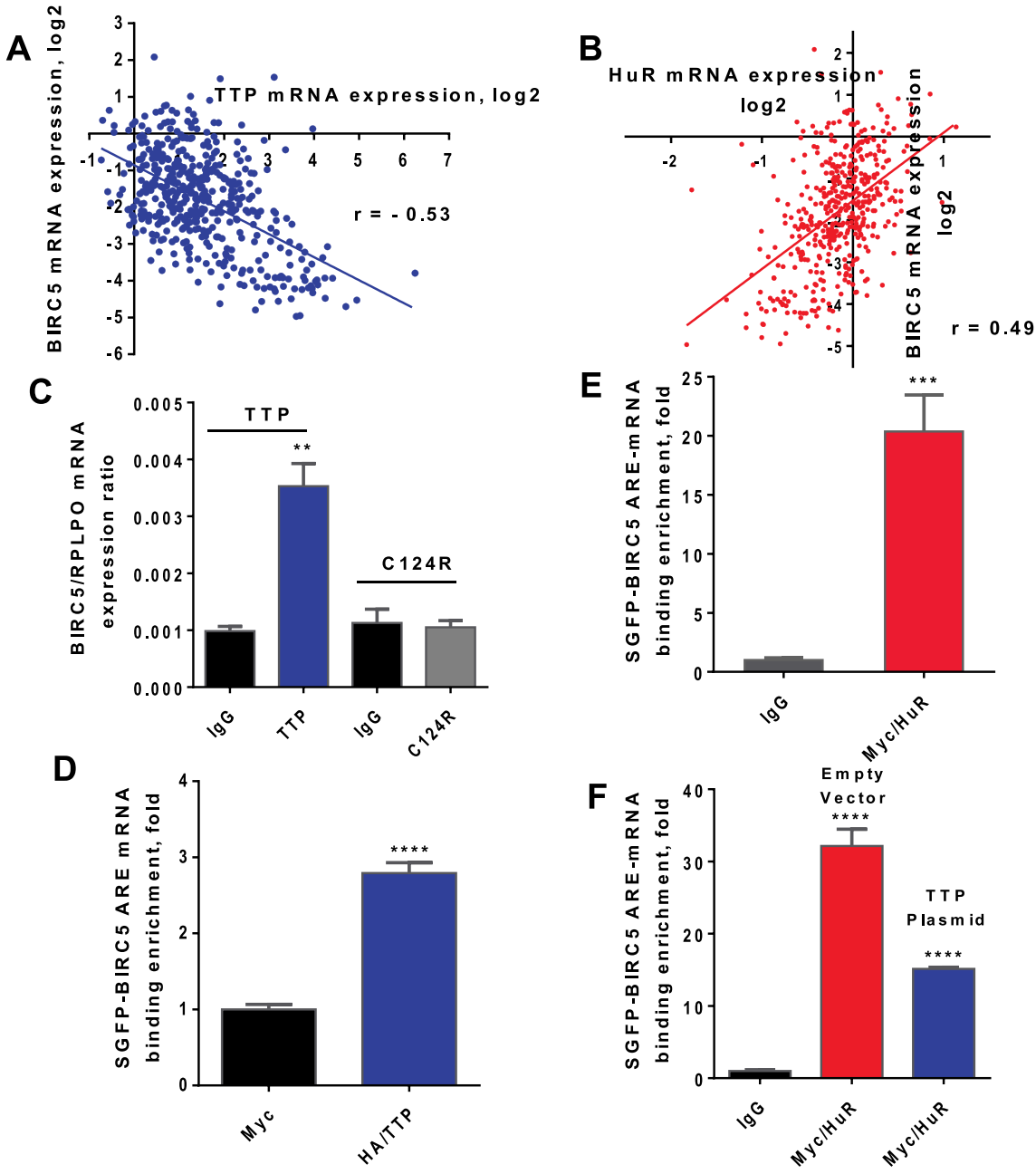

Figure 3. BIRC5 correlation with TTP and HuR. (A and B) Pearson correlation plots of BIRC5 and TTP mRNA expression levels or BIRC5 and HuR for invasive ductal breast cancer patients obtained from the oncomine portal. Linear regression and *R* values were generated and are shown. Immunoprecipitation of TTP-associated BIRC5 mRNA, in MDA-MB-231 cells. (C) Cells were seeded and transfected the following day with TTP or mutant TTP (C124R) plasmid (2 ug/100 cm plate). TTP protein was immunoprecipitated using anti-TTP or IgG as a negative control and associated BIRC5 mRNA quantification was done by RT-qPCR and normalized to RPLPO. Data are from two independent experiments. $^{**}P < 0.005$, $^{****}P < 0.0001$ (Student's *t*-test) (D) HEK293 cells were transfected with HA-tagged TTP plasmid (2 ug/100 cm plate) and TTP protein was immunoprecipitated using anti-HA-coupled beads or anti-myc-coupled beads as a negative IP control. BIRC5 mRNA binding enrichment was assessed by RT-qPCR using a FAM-labelled probe for BIRC5 and normalized to VIC-labelled RPLPO. Data are from two independent experiments. $^{****}P < 0.0001$ (Student's *t*-test). Immunoprecipitation of HuR-associated BIRC5 mRNA, (E) HEK293 cells were transfected with myc-tagged HuR plasmid (2 ug/100 cm plate) and HuR protein was immunoprecipitated using anti-myc-coupled beads or anti-HA-coupled beads as a negative IP control. BIRC5 mRNA binding enrichment was assessed by qPCR and normalized to RPLPO. Data are from two independent experiments. $^{***}P < 0.005$ (Student's *t*-test). (F) competitive binding immunoprecipitation assay. HEK293 cells were transfected with myc-tagged HuR plasmid alone or co-transfected with HA-tagged TTP plasmid. Anti-myc antibody was used to precipitate HuR protein in HuR and TTP transfected samples and IgG was used as the control. BIRC5 mRNA binding enrichment was assessed by RT-qPCR using a FAM-labeled probe for BIRC5 and normalized to VIC-labeled RPLPO. Data are from two independent experiments. $^{****}P < 0.0001$ (Student's *t*-test).

and that the presence of an ARE in its 3' UTR is sufficient for destabilization.

In parallel, we knocked down BIRC5 expression in MDA-MB -231 cells using small interfering RNA (siRNA) against BIRC5 and analysed cleaved caspase 7 levels and apoptotic activity. Immunoblotting confirmed knockdown of BIRC5 protein and showed a corresponding increase in cleaved caspase 7 level (Figure 4E). Consequently, the number of apoptotic cells has nearly doubled (Figures 4F,G, $P < 0.05$) upon silencing of BIRC5. The findings demonstrate that upregulation of TTP expression

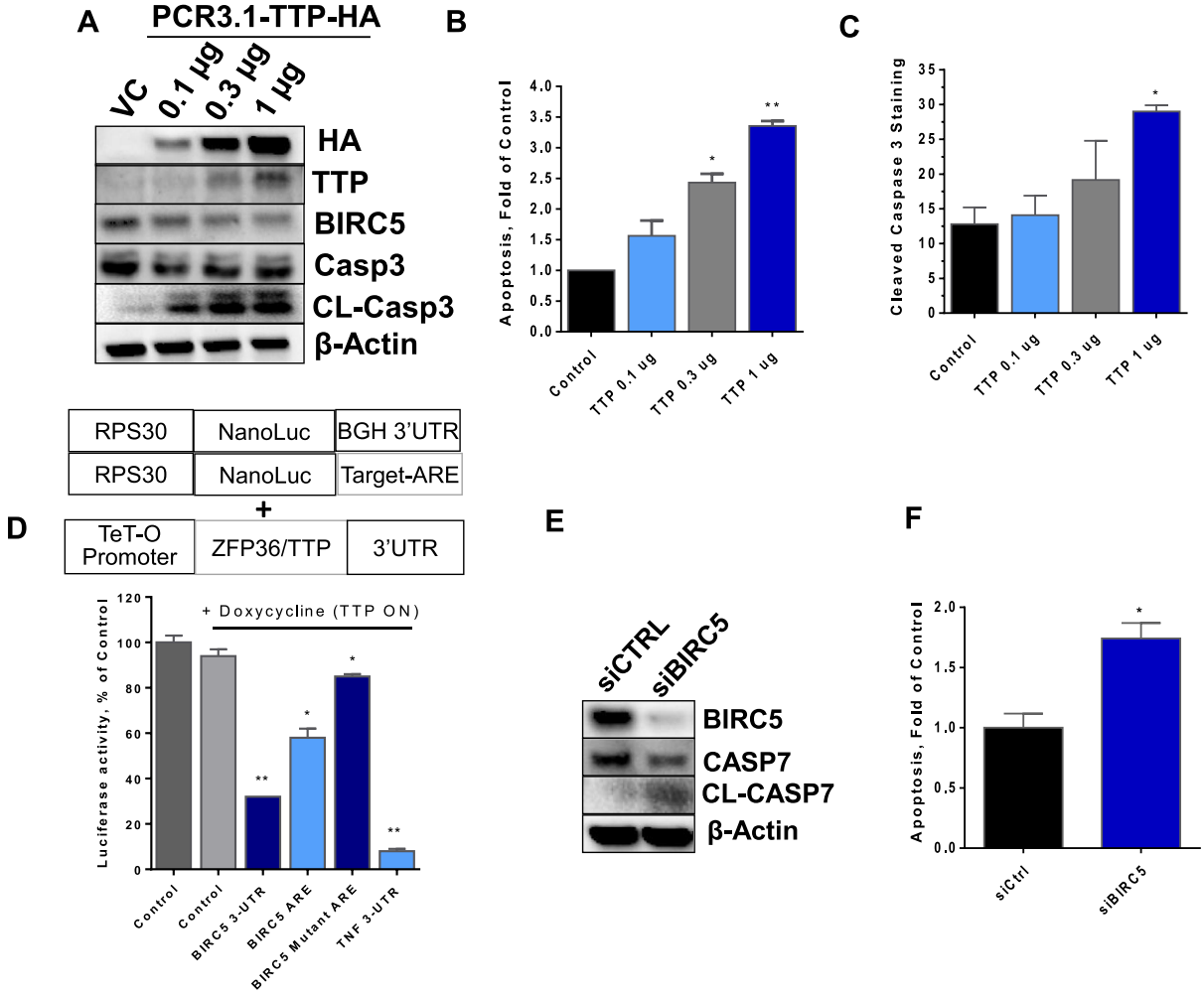

Figure 4. TTP regulation of BIRC5 expression and apoptosis. (A) MDA-MB-231 cells were transfected with increasing amounts of HA-tagged TTP plasmid. Total protein was extracted after 24 h for immunoblotting using anti-HA, anti-BIRC5, anti-full-length and cleaved caspase 3, and anti-β-actin as the loading control. (B and C) MDA-MB-231 cells were transfected as in (A) and after 24 h, cells were harvested and fixed for Annexin V or cleaved-caspase 3 staining, $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). Results are from one experiment representative of three independent experiments. Reporter assessment of TTP induction. (D) HEK293 Tet-ON cells were transfected with 25 ng of RPS-30 promoter-linked Nano-luciferase reporters containing BIRC5 ARE, BIRC5 3' UTR, BIRC5 mutated ARE, or TNF 3' UTR as a positive control and co-transfected with a Tet-O-inducible TTP expression plasmid (10 ng) as described in materials and methods. BGH 3' UTR was used as the negative control. To induce TTP, doxycycline (0.25 µM) was added and luciferase activity was measured after 24 h, $^{*}P < 0.05$, $^{****}P < 0.0001$ (Student's *t*-test). Effect of BIRC5 knockdown on apoptosis. (E & F) MDA-MB-231 cells were transfected with siBIRC5 or control siRNA for 24 h. Cells were harvested for immunoblotting or fixed for Annexin V assay, $^{*}P < 0.05$ (Student's *t*-test). Immunoblotting was performed using anti-BIRC5, anti-full-length and cleaved caspase 7, or anti-β-actin as the loading control.

triggers apoptosis in breast cancer cells as a result of BIRC5 downregulation in a manner similar to silencing of BIRC5 expression.

Prognostic relevance of TTP and BIRC5 expression

To evaluate the prognostic significance of TTP and BIRC5 expression in breast cancer patients, we analysed survival curves in patients with high TTP and low BIRC5 versus low TTP and high BIRC5 expression using the Kaplan Meier plotter database [37]. To accomplish this, we looked at high and low TTP mRNA expression levels and inverted BIRC5 in order to reflect accurately the relative expression patterns of TTP and BIRC5. Patients with high TTP and low BIRC5 had a better prognosis as they were associated with better RFS (HR = 0.63, $P < 1e\text{-}16$), OS (HR = 0.66, $P = 8.9e\text{-}05$), and DMFS (HR = 0.67, $P = 5.5e\text{-}07$) than low TTP and high BIRC5 expression levels, which is similar to what is seen in cancer (Figure 5A-C). We also examined RFS and DMFS in ER/PR positive patients who had high TTP and low BIRC5 (Figures 5 D,E). The plots indicated an even better prognosis (HR = 0.56, 0.44, respectively). However, the number of patients was much less, resulting in lower, but statistically significant, P values ($P < 0.0001$, < 0.005, respectively). In addition, RFS, OS, and DMFS survival curves were performed using the expression of TTP with inverted BIRC5 and HuR and mutant p53 (Figure S3 A-C, Supplementary Material). The findings show that high TTP and low BIRC5 mRNA expression predicts an overall good prognosis in breast cancer patients.

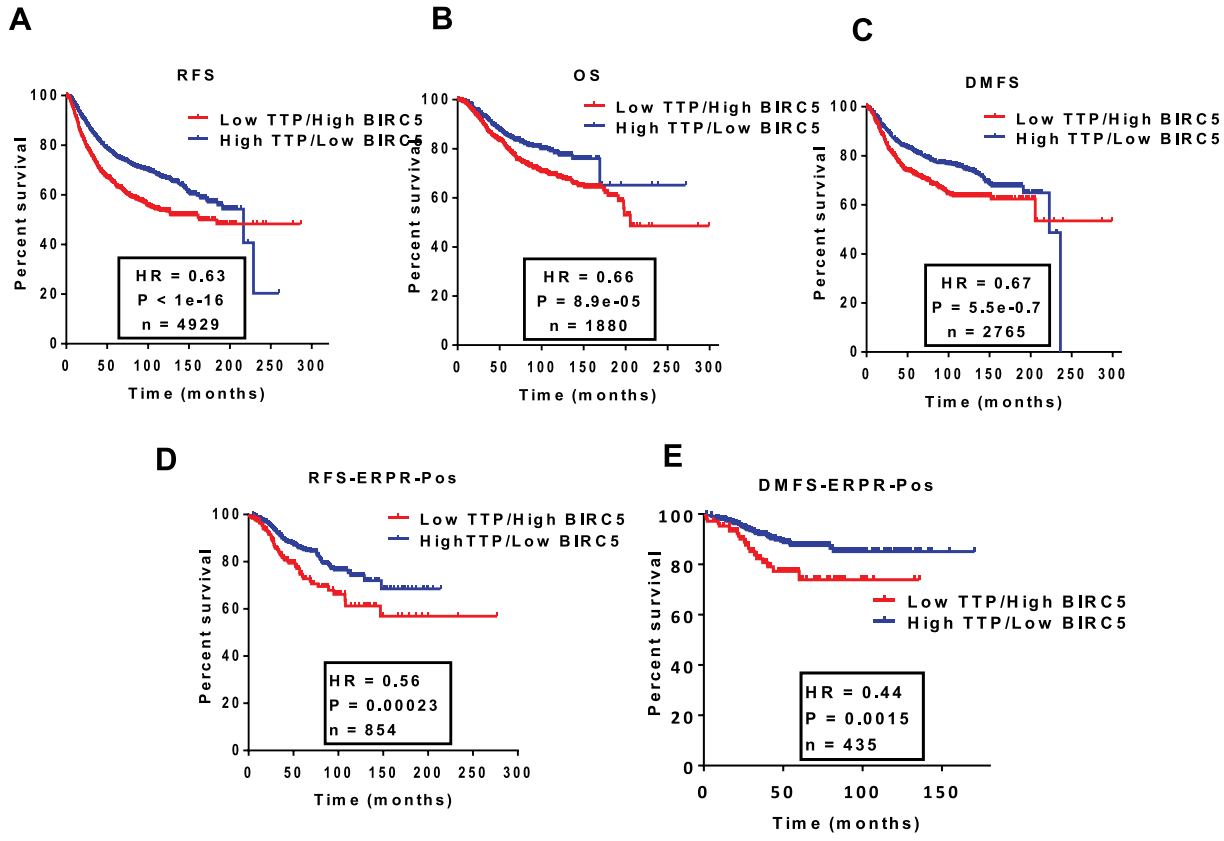

Figure 5. Survival analysis of TTP and BIRC5 co-expression. (A-C) Kaplan-Meier survival curves were generated using the *KMplot* portal. OS, RFS, and DMFS survival curves were generated in which expression levels were split into high and low TTP, based on the best cut-off option, with inverted BIRC5 mRNA expression (e.g. high TTP, low BIRC5). (D and E) RFS and DMFS survival curves were generated as in (A-C) using only ER/PR positive data.

Loss of TTP expression increases BIRC5 mRNA stability and expression

We then examined BIRC5 mRNA and protein expression in TTP knockout mouse embryonic fibroblasts (MEFs, zfp36 $^{+/+}$ and zfp36 $^{-/-}$). Birc5 mRNA expression increased almost threefold in cells lacking TTP (Figure 6A, $P < 0.0001$). A definite increase in Birc5 protein expression was observed as well in TTP knockouts compared to the wild-type MEFs (Figure 6B). Furthermore, we examined Birc5 mRNA half-life in the presence and absence of TTP by performing mRNA stability experiments. Actinomycin D (5 µg/ml) was added to TTP wild-type and knockout MEFs for 2, 4, 8, and 24 h after which total RNA was extracted for RT-qPCR. Birc5 mRNA was definitely more stable in the absence of TTP (half-life in TTP WT = 2.67 h ±1.15 SD and in TTP KO = 9.3 h ±2 SD, Figure 6C) demonstrating an almost 3.5-fold increase in mRNA half-life compared to TTP expressing cells.

We wanted to find out if a similar increase in BIRC5 expression would be achieved by siRNA knockdown of TTP in breast cancer cells that express the RNA-binding protein. Therefore, we chose the ER+ MCF-7 cell line in which TTP is fairly abundant [28] (Figure 1C). MCF-7 cells were transfected with two types of siTTP or a control siRNA for 24 and 48 h. Total protein was extracted for immunoblotting to confirm TTP silencing and to examine BIRC5 expression. Figure (6D) shows a reduction in TTP protein at 24 h, while BIRC5 protein showed an obvious increase after 48 h of transfection with both siRNAs. siTTP 1 caused a somewhat greater reduction in TTP protein at 24 h than siTTP 2, whereas BIRC5 expression was clearly affected and a definite increase was demonstrated with both siRNAs. Apoptosis decreased significantly with TTP silencing, demonstrating a 40% to 50% reduction (Figure 6E) as did cleaved caspase 7 staining showing a similar pattern of reduction (Figure 6F). The reduction in apoptosis and cleaved caspase 7 staining appeared slightly greater with siTTP 2 than siTTP 1. These reductions are in accord with BIRC5 upregulation and inhibition of apoptosis.

To validate these findings, we attempted to rescue cells from apoptosis by forced expression of BIRC5 and compare that to TTP silencing. To that end, MCF10A cells were transfected with increasing amounts of BIRC5 plasmid for 24 h and the cells were then harvested for immunoblotting or apoptosis analysis. All transfected amounts of BIRC5 plasmid led to dramatic reductions in the number of apoptotic cells (Figure 6G) and BIRC5 protein overexpression was confirmed by immunoblotting (Figure 6H). Although not as dramatic as BIRC5 overexpression, the findings clearly suggest that TTP siRNA knockdown leads to an upregulation of BIRC5 expression and a corresponding decrease in apoptosis.

HuR upregulates BIRC5 and downregulates cleaved caspase 3

We demonstrated earlier elevated levels of BIRC5 mRNA bound to immunoprecipitated HuR protein, indicating that the RNA-binding protein is associated with BIRC5 mRNA to a large extent (Figure 3E). We decided to investigate the effect of

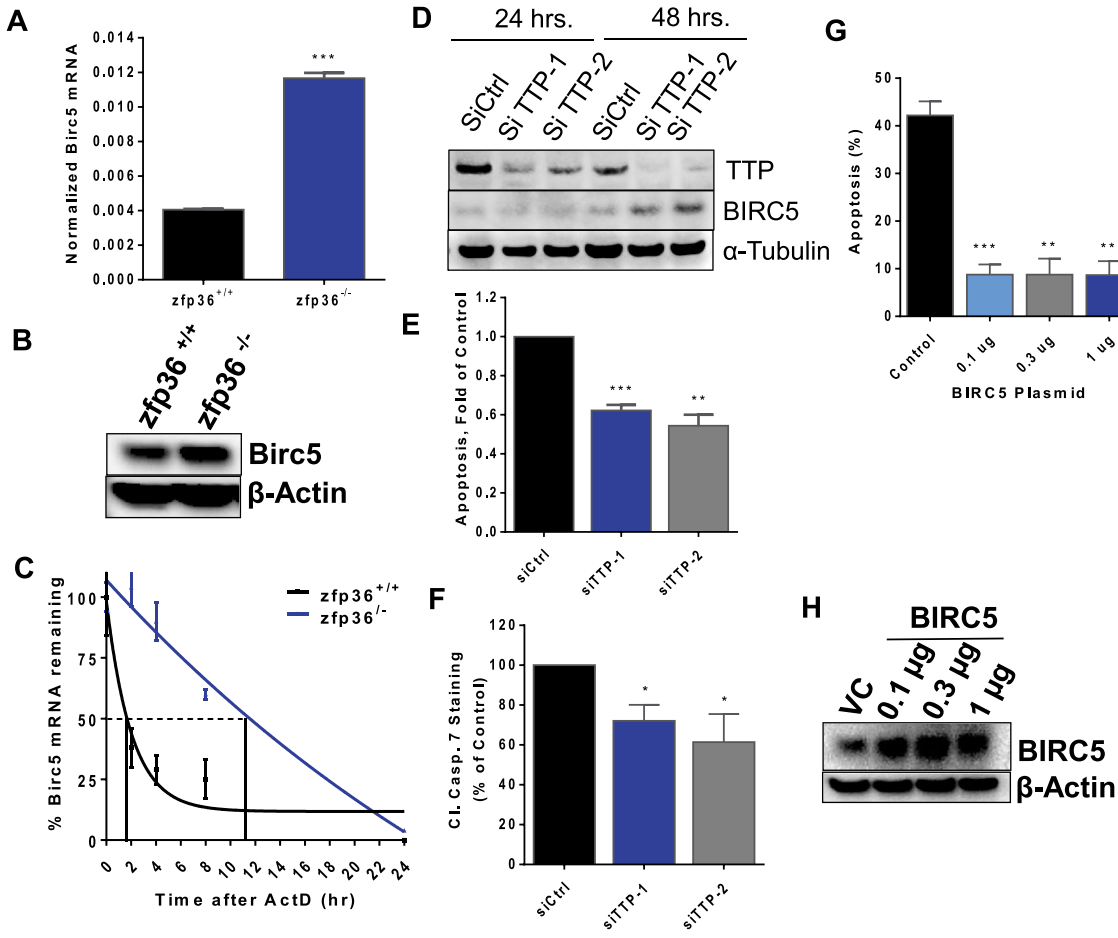

Figure 6. Birc5 mRNA expression in the absence of TTP. (A and B) zfp36$^{+/+}$ and zfp36$^{-/-}$ MEFs were seeded at 70% confluency overnight then total protein or RNA were extracted for immunoblotting or RT-qPCR. Birc5 mRNA expression was measured with TaqMan specific primers for mouse Birc5 and normalized to mouse β -actin. Data are from one experiment representative of three independent experiments, *** $P < 0.0001$ (Student's *t*-test). Birc5 protein expression was assessed by probing with anti-mouse Birc5 antibody and anti-mouse β-actin as the loading control. Data are from one experiment representative of two independent experiments. (C) BIRC5 mRNA stability in TTP wild-type and knockout MEFs. Cells were seeded in 6-well plates in incubated in regular serum overnight. Cells were serum-starved the next day by replacing media with low-serum media (0.5% FBS) for 18 h then re-stimulated with 10% serum for 2 h followed by addition of Actinomycin D (ActD, 5 µg/ml) to the cells. Total RNA was extracted at 0, 2, 4, 8, and 24 h for RT-qPCR using TaqMan expression assays for mouse Birc5 and β-actin as the endogenous control. The one-phase decay model was used to estimate BIRC5 mRNA half-life. The data are from one experiment representative of two independent experiments.

TTP silencing upregulates BIRC5 and inhibits apoptosis. (D) MCF-7 cells were transfected with two siRNAs against TTP or a control siRNA and incubated for 24 and 48 h. Total protein was extracted for immunoblotting and probed with anti-TTP, anti-BIRC5, and anti-Tubulin antibodies as the endogenous control. Image is from one experiment representative of three independent experiments. (E and F) MCF-7 cells were transfected with two siRNAs against TTP or a control siRNA for 24 h after which the cells were harvested and fixed for Annexin V assay or cleaved caspase 7 staining. Data represent the mean of three independent experiments, *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ (Student's *t*-test). BIRC5 overexpression inhibits apoptosis. MCF10A cells were transfected with increasing amounts of BIRC5 plasmid and incubated for 24 h. (G) Cells were harvested and fixed for Annexin V assay. Data represent the mean of three independent experiments, **$P < 0.005$, ***$P < 0.001$ (Student's *t*-test). (H) immunoblotting confirmation of BIRC5 overexpression.

recombinant HuR expression in MCF10A cells on BIRC5 upregulation. BIRC5 mRNA showed a modest yet statistically significant upregulation (Figure 7A). BIRC5 protein levels demonstrated an almost twofold increase compared to the vector control 48 h post-HuR plasmid transfection during which HuR protein increased as well (Figure 7B). A possible explanation for the modest upregulation in BIRC5 levels is that MCF10A cells express wild-type p53 and, according to a previous study in oesophageal cells [38], overexpression of HuR stabilizes p53 mRNA, which in turn leads to a downregulation of BIRC5. Full-length Caspase 3 increased slightly at 24 h, whereas cleaved Caspase 3 protein levels declined steadily upon HuR overexpression, suggesting inhibition of apoptosis (Figure 7B). Furthermore, BIRC5 mRNA stability increased with HuR overexpression (Figure S4-B). Conversely, silencing HuR in MDA-MB-231 cells with two different siRNAs led to reductions in BIRC5 mRNA (Figure 7C) and protein (Figure 7D) and to a greater extent with siRNA 2 ($P < 0.05$). Full-length Caspase 3 decreased with HuR silencing and a corresponding increase in cleaved caspase 3 with siHuR 2. The number of apoptotic cells also increased with siHuR 2 (Figures 7D,E). These results point to a stabilizing effect of HuR on BIRC5 mRNA and subsequent inhibition of apoptosis.

HuR and BIRC5 expression correlate poorly with patient survival

As indicated in the survival analyses presented earlier, BIRC5 upregulation by itself is a negative predictor of overall patient

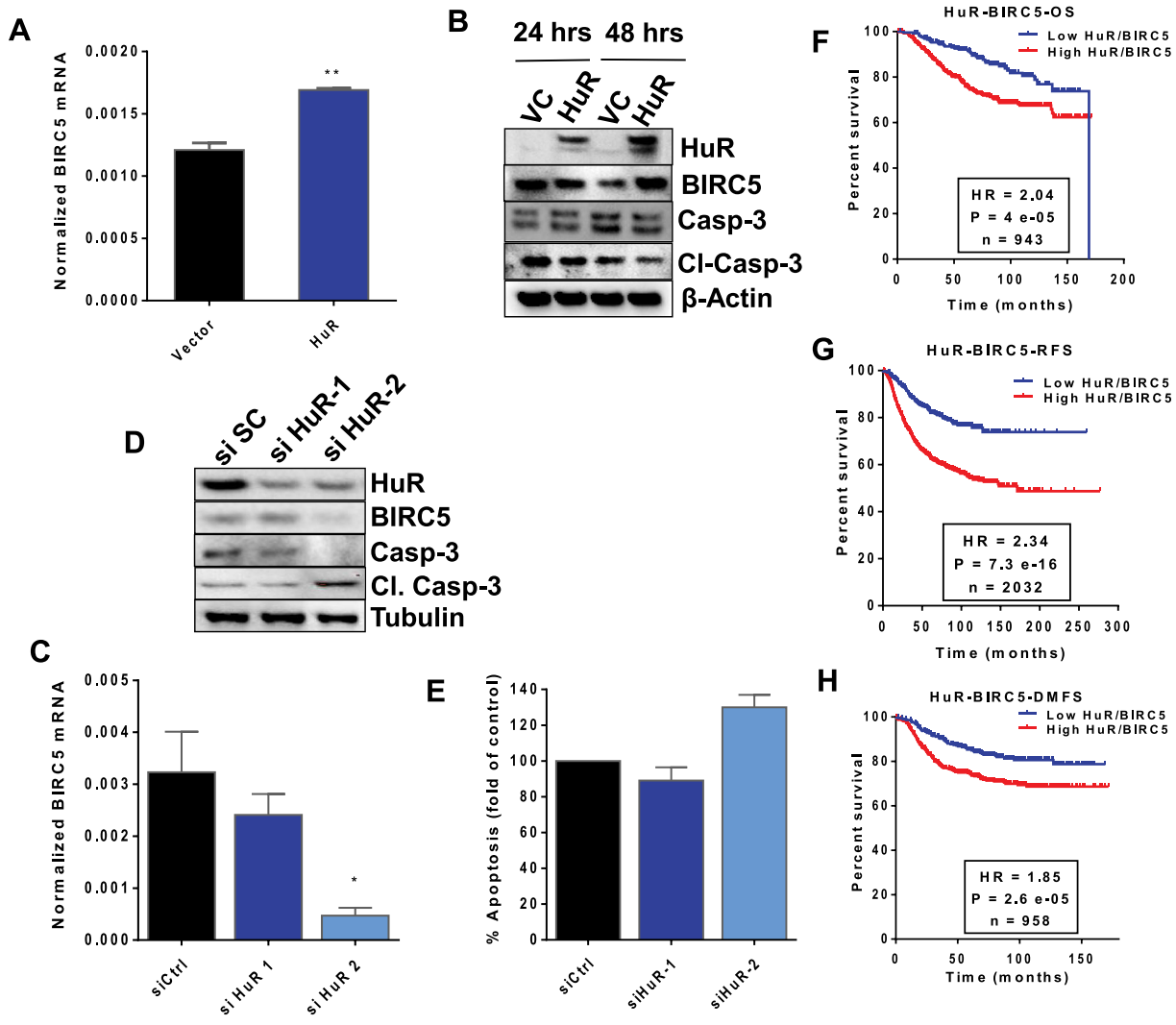

Figure 7. HuR regulation of BIRC5 expression. (A) MCF10A cells were transfected with HuR plasmid or empty vector for 24 h. Total RNA was then extracted for RT-qPCR using BIRC5 TaqMan expression assay and normalized to human RPLPO. The data are from one experiment representative of three independent experiments, $^{**}P < 0.005$ (Student's *t*-test). (B) MCF10A cells were transfected as in (A) for 24 and 48 h. Total protein was extracted for immunoblotting with anti-HuR, anti-BIRC5, anti- full-length and cleaved caspase 3 and anti- β-actin as the loading control. MDA-MB-231 cells were transfected with two siRNAs against HuR or a control siRNA for 48 h and either; (C) total RNA was extracted for RT-qPCR using a BIRC5 TaqMan probe and normalized to human RPLPO, $^{**}P < 0.005$ (Student's *t*-test), or (D) total protein was extracted for immunoblotting and probed with anti-HuR, anti-BIRC5, anti-full-length and cleaved caspase 3, and anti-tubulin as the loading control, or (E) cells were fixed for Annexin V assay. Survival analysis of HuR and BIRC5 co-expression. (F-H) Kaplan-Meier survival curves were generated using the *KMplot* portal. OS, RFS, and DMFS survival curves were generated wherein expression levels were split into high and low HuR and BIRC5 (co-expression, non-inverted) based on the best cut-off option, (e.g. high HuR, high BIRC5).

survival. Given the fact that HuR is an mRNA-stabilizing protein that we have demonstrated to bind to BIRC5 mRNA and upregulate expression, we predicted that increased expression of both HuR and BIRC5 would also indicate an even worse prognosis than that with BIRC5 alone. Indeed, survival analysis using the KM plotter portal showed poor OS, RFS, and DMFS when both HuR and BIRC5 are upregulated compared to low expression (Figure 5 F-H). The hazard ratios for OS and RFS were 2 or greater compared to 1.9 or less for BIRC5 alone. RFS, in particular, demonstrated a poorer prognosis with an HR = 2.34 (P = 7.3e-16, n = 2032) compared to HR = 1.92 (P < 1 e-16, n = 4929) for BIRC5 alone. This indicates that the upregulation of both HuR and BIRC5 correlate poorly with patient survival and suggest a poor prognosis for breast cancer patients.

Discussion

Post-transcriptional control of gene expression is increasingly becoming the focus of many studies, particularly due to its aberrant nature in many types of cancer as opposed to normal cells where it is tightly maintained and controlled. ARE-mediated mechanisms of post-transcriptional regulation represent important means for controlling the mRNA stability of many genes that are active in inflammation and cancer. TTP and HuR are among the most characterized ARE-interacting RNA binding proteins (RBPs), which have been estimated to be over 1000 in number [39]. TTP binds to AREs located in the 3' UTR by means of two tandem zinc finger domains and subsequently recruits a deadenylase complex of proteins that ultimately degrade the ARE-mRNA. HuR, on

the other hand, contains three RNA recognition motifs (RRMs) whereby two of them interact with AREs [40] and the third RRM binds to the poly(A) tail [41]. This interaction eventually leads to the stabilization of ARE-mRNAs by HuR [42].

Many targets of TTP have been identified, especially in inflammation wherein it was first identified as a protein that destabilizes TNF-α [25] and other cytokines as well [43, 44]. TTP role as a tumour suppressor was later demonstrated with the identification of target cancer genes that play active roles in invasion, metastasis and angiogenesis. Our previous findings [27, 28, 30] and those of others [45–47] have uncovered targets of post-transcriptional control, specifically TTP regulation, that have various roles in the progression of cancer. In this study, we have identified a novel target of TTP that inhibits apoptosis and is upregulated in cancer. We have substantiated, by multiple levels of evidence, that TTP directly targets BIRC5 mRNA and that HuR competes for binding to BIRC5 3' UTR. We demonstrated that TTP downregulates BIRC5 endogenous expression in breast cancer cells. As a result, proliferation is controlled by means of induction of apoptosis. Furthermore, RNA-IP experiments demonstrated the association of TTP with BIRC5 mRNA and TTP wild-type and knockout MEFs showed BIRC5 upregulation and increased mRNA stability in the absence of TTP.

It has been reported that both HuR and TTP share similar binding preferences in the 3' UTR and that TTP has equal inclination towards binding to AU-rich and U-rich regions [48]. While BIRC5 ARE demonstrates overlapping ARE sequences within a G-rich region typical of class I AREs, it does not conform entirely to this classification, whereas, within the same 15 nt region, a typical HuR binding site is present upstream of the TTP binding site. This might explain the much greater enrichment of BIRC5 mRNA with HuR IP compared to that with TTP.

The association of TTP with BIRC5 mRNA has functional significance in cancer, which is illustrated by the downregulation of BIRC5 expression and the subsequent induction of apoptosis. In fact, the least amount of TTP transfected into MDA-MB-231 cells (0.1 μg) induced apoptosis to a degree similar to the silencing of BIRC5 (Figure 4), despite the fact that BIRC5 protein abundance appeared greater with TTP overexpression. As a result, a reduced level of apoptosis would be assumed when recombinant TTP was used than that with BIRC5 silencing. A possible explanation would be that TTP induces apoptosis by targeting other ARE-mRNAs involved in induction of cell death and not only by regulation of BIRC5 expression. Johnson et al. [49] reported TTP induction of apoptosis by sensitizing cells to TNF-α at specific TTP expression levels. They also alluded to the possibility of TTP/TIS11 interacting with mRNAs involved in the apoptotic pathway. They demonstrated later that multiple sequences in the TTP protein are required for this function to take place [50]. To our knowledge, this is the first report of TTP's association with BIRC5 mRNA leading to its ARE-mediated destabilization and subsequent induction of apoptosis.

TTP expression has been demonstrated to be higher in normal cells than in tumours where it is suppressed [28, 31]. We have shown here that BIRC5 expression displays an opposing pattern of expression where it is downregulated in normal breast cells compared to cancer cells. We attempted to mimic the relationship between TTP and BIRC5 expression in normal conditions in our KMplot survival analysis by inverting BIRC5 expression. This showed a good prognosis for breast cancer patients and supports our findings as well.

In contrast, HuR upregulated BIRC5 expression and, subsequently, cleaved caspase 3 protein level was reduced. Donahue et al. [38] had previously reported an HuR-mediated increase in BIRC5 expression by binding to a region in the 3' UTR, but the stabilization only occurred in the absence of p53 in oesophageal epithelial cells. Here we show by RNA-IP in HEK293 cells, which express wild-type p53, that HuR is highly associated with BIRC5 mRNA, and to a much greater extent than that with TTP. Both RNA-binding proteins appear to compete with each other for the ARE region. Furthermore, HuR is upregulated in cancer, which reflects favourably on BIRC5 expression by promoting upregulation of BIRC5 mRNA. Considering the fact that both HuR and BIRC5 are upregulated in cancer and TTP is not,

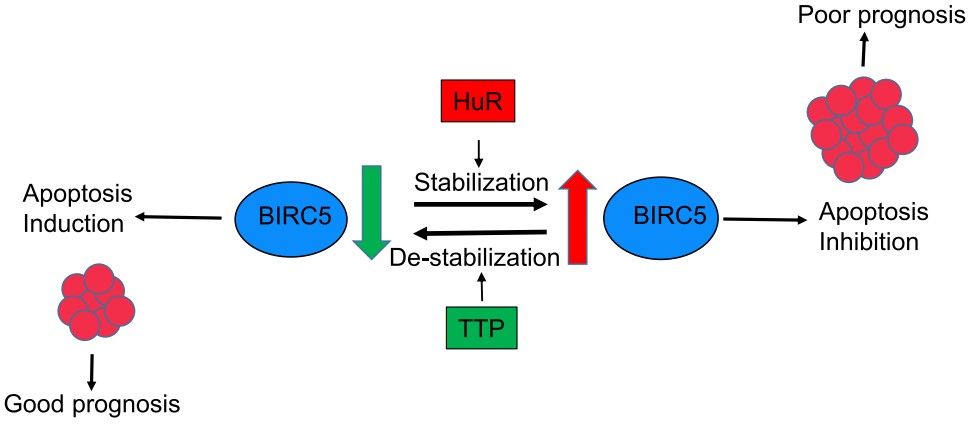

Figure 8. Schematic illustration of TTP and HuR regulation of BIRC5 and apoptosis. The RNA binding proteins TTP and HuR both bind to BIRC5 mRNA. TTP binding leads to ARE-mediated destabilization of BIRC5 mRNA and reduced expression. This eventually induces apoptosis in cells resulting in less proliferation and, thus, predicts a good prognosis. On the other hand, HuR stabilizes BIRC5 mRNA, increasing its expression leading to the inhibition of apoptosis and supporting cell proliferation. Upregulation of both HuR and BIRC5 results in a worse prognosis than BIRC5 alone.

this tips the balance in favour of inhibition of apoptosis in cancer cells and stimulating their proliferation. This positive correlation and reciprocal expression are also reflected in the patient survival data analysed here in which high expression of both HuR and BIRC5 predicted a poorer prognosis than that with BIRC5 alone.

Chemoresistance to cancer drugs is a major problem in the treatment of cancer leading to its recurrence and poor patient prognosis. Increased expression of BIRC5 has been implicated in the chemoresistance of several cancers, including breast cancer [51–54] subsequently affecting patient survival prognosis as alluded to earlier. Numerous therapeutic approaches have been attempted to downregulate BIRC5 and enhance sensitivity to cytotoxic medications [51, 55, 56]. On the other hand, TTP dysregulation in cancer has been attributed to a variety of mechanisms. One is its phosphorylation by several kinases such as ERK2 [57], p38 MAPK [58], JNK [59], MK2 [60] and PLK1 [61] leading to increased stability and, at the same time, reduced destabilizing activity [62, [63]. Kinase activity is upregulated in tumours making kinases important targets for cancer treatment [64, [65]. Indeed, inhibition of PLK1 using volasertib resulted in decreased TTP phosphorylation, decreased stability of the protein, and subsequently reduced tumour growth in mice [61]. Further elucidation of the role of PLK1 inhibition on BIRC5 expression and apoptosis in cancer cell lines may provide useful information on enhancing TTP regulation of BIRC5 and other ARE-cancer gene targets.

To conclude, we have demonstrated regulation of BIRC5 expression post-transcriptionally via the RNA-binding proteins TTP and HuR through control of mRNA stability. Figure 8 illustrates the relationship between TTP, HuR, and BIRC5 and the effect it has on apoptosis and cancer cell survival. On the one hand, TTP induces apoptosis by binding to BIRC5 3' UTR, promoting mRNA decay and downregulating the expression of BIRC5. The end result is reduced proliferation of cancer cells and better patient survival. On the other hand, HuR binding to BIRC5 3' UTR stabilizes its mRNA and upregulates its expression leading to inhibition of cell death and poor patient prognosis.

Acknowledgments

The authors would like to thank Mr Pulicat Manogaran and Mr Amer Almazrou for their assistance with flow cytometry.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

Intramural funding from King Faisal Specialist Hospital and Research Center (RAC # 2150004) supported this work.

Author contributions

Khalid S.A. Khabar: conceptualization, reviewing and editing final draft. Norah Al-Souhibani: conceptualization, methodology, validation, original draft writing and editing, formal analysis, supervision and project administration. Suhad Al-Yahya, Walid Moghrabi, Maher Al-Saif and Maha Al-Ghamdi: investigation, validation, formal analysis, resources, editing and reviewing final draft.

Data availability statement

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Norah Al-Souhibani http://orcid.org/0000-0003-2614-7396

References

[1] Siegel RL, Miller KD, Fuchs HE, et al. Cancer statistics, 2021. *Ca A Cancer J Clinicians*. 2021;71(1):7–33. doi: 10.3322/caac.21654
[2] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57–70. doi: 10.1016/S0092-8674(00)81683-9
[3] Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin. 2005;55(3):178–194. doi: 10.3322/canjclin.55.3.178
[4] Li GY, Jung KH, Lee H, et al. A novel imidazopyridine derivative, HS-106, induces apoptosis of breast cancer cells and represses angiogenesis by targeting the PI3K/mTOR pathway. Cancer Lett. 2013;329(1):59–67. doi: 10.1016/j.canlet.2012.10.013
[5] Khan KH, Blanco-Codesido M, Molife LR. Cancer therapeutics: targeting the apoptotic pathway. Crit Rev Oncol Hematol. 2014;90(3):200–219. doi: 10.1016/j.critrevonc.2013.12.012
[6] Kasibhatla S, Tseng B. Why target apoptosis in cancer treatment? Mol Cancer Ther. 2003;2(6):573–580.
[7] Sohn DM, Kim SY, Baek MJ, et al. Expression of survivin and clinical correlation in patients with breast cancer. Biomed Pharmacother. 2006;60(6):289–292. doi: 10.1016/j.biopha.2006.06.008
[8] Xu C, Yamamoto-Ibusuki M, Yamamoto Y, et al. High survivin mRNA expression is a predictor of poor prognosis in breast cancer: a comparative study at the mRNA and protein level. Breast Cancer. 2014;21(4):482–490. doi: 10.1007/s12282-012-0403-9
[9] Hernandez JM, Farma JM, Coppola D, et al. Expression of the antiapoptotic protein survivin in colon cancer. Clin Colorectal Cancer. 2011;10(3):188–193. doi: 10.1016/j.clcc.2011.03.014
[10] Zhou XL, Wang M. Expression levels of survivin, bcl-2, and KAI1 proteins in cervical cancer and their correlation with metastasis. *Genet Mol Res*. 2015;14(4):17059–17067. doi: 10.4238/2015.December.16.6
[11] Zhang M, Ho A, Hammond EH, et al. Prognostic value of survivin in locally Advanced prostate cancer: study based on RTOG 8610. Int J Radiat Oncol Biol Phys. 2009;73(4):1033–1042. doi: 10.1016/j.ijrobp.2008.06.1489
[12] Altieri DC, Altieri DC. Survivin – The inconvenient IAP. Survivin – the inconvenient IAP. Seminars In Cell & Developmental Biology. 2015;39:91–96. doi: 10.1016/j.semcdb.2014.12.007
[13] Mirza A, McGuirk M, Hockenberry TN, et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene. 2002;21(17):2613–2622. doi: 10.1038/sj.onc.1205353
[14] Guha M, Plescia J, Leav I, et al. Endogenous tumor suppression mediated by PTEN involves survivin gene silencing. Cancer Res. 2009;69(12):4954–4958. doi: 10.1158/0008-5472.CAN-09-0584
[15] Asanuma H, Torigoe T, Kamiguchi K, et al. Survivin expression is regulated by coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor via phosphatidylinositol 3-Kinase/AKT Signaling pathway in breast cancer cells. Cancer Res. 2005;65(23):11018–11025. doi: 10.1158/0008-5472.CAN-05-0491
[16] Gritsko T, Williams A, Turkson J, et al. Persistent activation of Stat3 Signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. Clin Cancer Res. 2006;12(1):11–19. doi: 10.1158/1078-0432.CCR-04-1752

[17] Wang H, Holloway MP, Ma L, et al. Acetylation directs survivin nuclear localization to repress STAT3 oncogenic activity. J Biol Chem. 2010;285(46):36129–36137. doi: 10.1074/jbc.M110.152777
[18] Vong QP, Cao K, Li HY, et al. Chromosome alignment and segregation regulated by ubiquitination of survivin. Science. 2005;310(5753):1499. doi: 10.1126/science.1120160
[19] O'Connor DS, Grossman D, Plescia J, et al. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. Proc Natl Acad Sci U S A. 2000;97(24):13103–13107. doi: 10.1073/pnas.240390697
[20] Barrett RMA, Osborne TP, Wheatley SP. Phosphorylation of survivin at threonine 34 inhibits its mitotic function and enhances its cytoprotective activity. Cell Cycle. 2009;8 (2):278–283. doi: 10.4161/cc.8.2.7587
[21] Yang R, Liu M, Liang H, et al. miR-138-5p contributes to cell proliferation and invasion by targeting survivin in bladder cancer cells. Mol Cancer. 2016;15(1):82–82. doi: 10.1186/s12943-016-0569-4
[22] Li P-L, Zhang X, Wang L-L, et al. MicroRNA-218 is a prognostic indicator in colorectal cancer and enhances 5-fluorouracil-induced apoptosis by targeting BIRC5. Carcinogenesis. 2015;36 (12):1484–1493. doi: 10.1093/carcin/bgv145
[23] Hu Y, Xu K, Yagüe E. miR-218 targets survivin and regulates resistance to chemotherapeutics in breast cancer. Breast Cancer Res Treat. 2015;151(2):269–280. doi: 10.1007/s10549-015-3372-9
[24] Wang C, Zheng X, Shen C, et al. MicroRNA-203 suppresses cell proliferation and migration by targeting BIRC5 and LASP1 in human triple-negative breast cancer cells. J Exp Clin Cancer Res. 2012;31(1):58–58. doi: 10.1186/1756-9966-31-58
[25] Lai WS, Carballo E, Strum JR, et al. Evidence that tristetraprolin binds to AU-Rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol. 1999;19(6):4311–4323. doi: 10.1128/MCB.19.6.4311
[26] Stoecklin G, Tenenbaum SA, Mayo T, et al. Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. J Biol Chem. 2008;283(17):11689–11699. doi: 10.1074/jbc.M709657200
[27] Hitti E, Bakheet T, Al-Souhibani N, et al. Systematic analysis of AU-Rich element expression in cancer reveals common functional clusters regulated by key RNA-Binding proteins. Cancer Res. 2016;76(14):4068–4080. doi: 10.1158/0008-5472.CAN-15-3110
[28] Al-Souhibani N, Al-Ahmadi W, Hesketh JE, et al. The RNA-binding zinc-finger protein tristetraprolin regulates AU-rich mRNas involved in breast cancer-related processes. Oncogene. 2010;29(29):4205–4215. doi: 10.1038/onc.2010.168
[29] Lee HH, Son YJ, Lee WH, et al. Tristetraprolin regulates expression of VEGF and tumorigenesis in human colon cancer. Int J Cancer. 2010;126(8):1817–1827. doi: 10.1002/ijc.24847
[30] Al-Souhibani N, Al-Ghamdi M, Al-Ahmadi W, et al. Posttranscriptional control of the chemokine receptor CXCR4 expression in cancer cells. Carcinogenesis. 2014;35(9):1983–1992. doi: 10.1093/carcin/bgu080
[31] Brennan CM, Kuwano Y, Alkharouf N, et al. The mRNA-destabilizing protein tristetraprolin is suppressed in many cancers, altering tumorigenic phenotypes and patient prognosis. Cancer Res. 2009;69(12):5168–5176. doi: 10.1158/0008-5472.CAN-08-4238
[32] de Silanes I L, Fan J, Yang X, et al. Role of the RNA-binding protein HuR in colon carcinogenesis. Oncogene. 2003;22 (46):7146–7154. doi: 10.1038/sj.onc.1206862
[33] Denkert C, Weichert W, Pest S, et al. Overexpression of the embryonic-lethal abnormal vision-like protein HuR in ovarian carcinoma is a prognostic factor and is associated with increased cyclooxygenase 2 expression. Cancer Res. 2004;64(1):189–195. doi: 10.1158/0008-5472.CAN-03-1987
[34] Al-Ahmadi W, Al-Ghamdi M, Al-Haj L, et al. Alternative polyadenylation variants of the RNA binding protein, HuR: abundance, role of AU-rich elements and auto-regulation. Nucl Acids Res. 2009;37(11):3612–3624. doi: 10.1093/nar/gkp223
[35] Hitti E, Al-Yahya S, Al-Saif M, et al. A versatile ribosomal protein promoter-based reporter system for selective assessment of RNA stability and post-transcriptional control. RNA. 2010;16 (6):1245–1255. doi: 10.1261/rna.2026310
[36] Al-Haj L, Al-Ahmadi W, Al-Saif M, et al. Cloning-free regulated monitoring of reporter and gene expression. BMC Mol Biol. 2009;10(1):20. doi: 10.1186/1471-2199-10-20
[37] Lánczky A, Győrffy B. Web-based survival analysis tool tailored for medical Research (KMplot): development and implementation. *J Med Internet Res*. 2021;23(7):e27633. doi: 10.2196/27633
[38] Donahue James M, Chang Elizabeth T, Xiao L, et al. The RNA-binding protein HuR stabilizes survivin mRNA in human oesophageal epithelial cells. Biochem J. 2011;437(1):89–96. doi: 10.1042/BJ20110028
[39] Gerstberger S, Hafner M, Tuschl T. A census of human RNA-binding proteins. Nat Rev Genet. 2014;15(12):829–845. doi: 10.1038/nrg3813
[40] Ma W-J, Cheng S, Campbell C, et al. Cloning and characterization of HuR, a ubiquitously expressed elav-like protein. J Biol Chem. 1996;271(14):8144–8151. doi: 10.1074/jbc.271.14.8144
[41] Ma WJ, Chung S, Furneaux H. The elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. Nucl Acids Res. 1997;25(18):3564–3569. doi: 10.1093/nar/25.18.3564
[42] Fan XC, Steitz JA. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRnas. EMBO J. 1998;17(12):3448–3460. doi: 10.1093/emboj/17.12.3448
[43] Panganiban RP, Vonakis BM, Ishmael FT, et al. Coordinated post-transcriptional regulation of the chemokine system: messages from CCL2. J Interferon Cytokine Res. 2014;34(4):255–266. doi: 10.1089/jir.2013.0149
[44] Khabar KSA. Post-transcriptional control of cytokine gene expression in health and disease. J Interferon Cytokine Res. 2014;34 (4):215–219. doi: 10.1089/jir.2013.0151
[45] Young LE, Dixon DA. Posttranscriptional regulation of cyclooxygenase 2 expression in colorectal cancer. Curr Colorectal Cancer Rep. 2010;6(2):60–67. doi: 10.1007/s11888-010-0044-3
[46] Sanduja S, Blanco FF, Young LE, et al. The role of tristetraprolin in cancer and inflammation. Front Biosci. 2012;17(1):174–188. doi: 10.2741/3920
[47] Essafi-Benkhadir K, Onesto C, Stebe E, et al. Tristetraprolin inhibits ras-dependent tumor vascularization by inducing vascular endothelial growth factor mRNA degradation. ?Mol Biol Cell. 2007;18(11):4648–4658. doi: 10.1091/mbc.e07-06-0570
[48] Bhandare S, Goldberg DS, Dowell R, et al. Discriminating between HuR and TTP binding sites using the k-spectrum kernel method. PLoS One. 2017;12(3):e0174052. doi: 10.1371/journal.pone.0174052
[49] Johnson BA, Geha M, Blackwell TK. Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival. Oncogene. 2000;19(13):1657–1664. doi: 10.1038/sj.onc.1203474
[50] Johnson BB, Blackwell TK. Multiple tristetraprolin sequence domains required to induce apoptosis and modulate responses to TNFα through distinct pathways. Oncogene. 2002;21 (27):4237–4246. doi: 10.1038/sj.onc.1205526
[51] Virrey JJ, Guan S, Li W, et al. Increased survivin expression confers chemoresistance to tumor-associated endothelial cells. Am J Pathol. 2008;173(2):575–585. doi: 10.2353/ajpath.2008.071079
[52] Moriai R, Tsuji N, Moriai M, et al. Survivin plays as a resistant factor against tamoxifen-induced apoptosis in human breast cancer cells. Breast Cancer Res Treat. 2009;117(2):261–271. doi: 10.1007/s10549-008-0164-5
[53] Wang S, Huang X, Lee CK, et al. Elevated expression of erbB3 confers paclitaxel resistance in erbB2-overexpressing breast cancer cells via upregulation of survivin. Oncogene. 2010;29 (29):4225–4236. doi: 10.1038/onc.2010.180
[54] Nestal de Moraes G, Vasconcelos FC, Delbue D, et al. Doxorubicin induces cell death in breast cancer cells regardless of survivin and XIAP expression levels. Eur J Cell Biol. 2013;92(8–9):247–256. doi: 10.1016/j.ejcb.2013.08.001
[55] Olie RA, Simões-Wüst AP, Baumann B, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res. 2000;60(11):2805.

[56] Faversani A, Vaira V, Moro GP, et al. Survivin family proteins as novel molecular determinants of doxorubicin resistance in organotypic human breast tumors. Breast Cancer Res. 2014;16(3):R55–R55. doi: 10.1186/bcr3666

[57] Taylor GA, Thompson MJ, Lai WS, et al. Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by Mitogen-activated protein Kinase in vitro. J Biol Chem. 1995;270(22):13341–13347. doi: 10.1074/jbc.270.22.13341

[58] Mahtani KR, Brook M, Dean JLE, et al. Mitogen-activated protein Kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. Mol Cell Biol. 2001;21(19):6461–6469. doi: 10.1128/MCB.21.9.6461-6469.2001

[59] Cao H, Dzineku F, Blackshear PJ. Expression and purification of recombinant tristetraprolin that can bind to tumor necrosis factor-[alpha] mRNA and serve as a substrate for mitogen-activated protein kinases. Arch Biochem Biophys. 2003;412(1):106–120. doi: 10.1016/S0003-9861(03)00012-2

[60] Stoecklin G, Stubbs T, Kedersha N, et al. MK2-Induced tristetraprolin: 14-3-3 complexes prevent stress granule association and ARE-mRNA decay. EMBO J. 2004;23(6):1313–1324. doi: 10.1038/sj.emboj.7600163

[61] Al-Qahtani QH, Moghrabi WN, Al-Yahya S, et al. Kinome inhibition reveals a role for polo-like kinase 1 in targeting post-transcriptional control in cancer. Mol Oncol. 2021;15(8):2120–2139. doi: 10.1002/1878-0261.12897

[62] Hitti E, Iakovleva T, Brook M, et al. Mitogen-activated protein Kinase-activated protein Kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/Uridine-rich element. Mol Cell Biol. 2006;26(6):2399–2407. doi: 10.1128/MCB.26.6.2399-2407.2006

[63] Clement SL, Scheckel C, Stoecklin G, et al. Phosphorylation of tristetraprolin by MK2 impairs AU-rich element mRNA decay by preventing deadenylase recruitment. Mol Cell Biol. 2011;31(2):256–266. doi: 10.1128/MCB.00717-10

[64] Fabbro D, Cowan-Jacob SW, Möbitz H, et al. Targeting cancer with small-molecular-weight Kinase inhibitors. In: Kuster B, editor Kinase inhibitors: methods and protocols. Totowa, NJ: Humana Press; 2012. pp. 1–34.

[65] Marmiroli S, Fabbro D, Miyata Y, et al. Phosphorylation, Signaling, and cancer: targets and targeting. *Biomed Res Int*. 2015; 601543–601543.