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KBTBD7, a novel human BTB-kelch protein, activates transcriptional activities of SRE and AP-1

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In this study, a novel member of BTB-kelch proteins, named KBTBD7, was cloned from a human embryonic heart cDNA library. The cDNA of KBTBD7 is 3,008 bp long and encodes a protein product of 684 amino acids (77.2 kD). This protein is highly conserved in evolution across different species. Western blot analysis indicates that a 77 kD protein specific for KBTBD7 is wildly expressed in all embryonic tissues examined. In COS-7 cells, KBTBD7 proteins are localized to the cytoplasm. KBTBD7 is a transcription activator when fused to GAL4 DNA-binding domain. Deletion analysis indicates that the BTB domain and kelch repeat motif are main regions for transcriptional activation. Overexpression of KBTBD7 in MCF-7 cells activates the transcriptional activities of activator protein-1 (AP-1) and serum response element (SRE), which can be relieved by siRNA. These results suggest that KBTBD7 proteins may act as a new transcriptional activator in mitogen-activated protein kinase (MAPK) signaling. [BMB reports 2010; 43(1): 17-22]

INTRODUCTION

The BTB domain (also known as the POZ domain) was originally identified as a conserved motif present in the *Drosophila melanogaster* bric-á-brac, tramtrack and broad complex transcription regulators and in many pox virus zinc finger proteins (1). This evolutionarily conserved protein-protein interaction motif is often found at the N-terminus of developmentally regulated zinc-finger transcription factors, as well as in some actin associated proteins bearing the kelch motif. Approximately two-thirds of the full-length human BTB genes also encodes C2H2 zinc finger modules, whereas one half of the remaining entries also contains the kelch motif (2). In most of BTB proteins, the BTB domain acts as a protein-protein interaction module that is able to both self-associate and interact with non-BTB proteins (3). The kelch motif is an ancient and evolutionarily-widespread sequence motif of 44-56 amino acids in length. It occurs as groups of five to seven repeats and has been identified in proteins of otherwise distinct molecular architecture, termed the kelch-repeat superfamily (4). Recently, the BACK domain (BTB and C-terminal kelch) has been described in several BTB-kelch proteins, however, its function is unknown (5).

Thus far, the function of only a few of BTB-kelch proteins is known, including transcription regulation (6), cytoskeleton regulation (7), tetramerization and gating of ion channels (8), protein ubiquitination/degradation (9). Recent studies of BTB family genes suggest their extensive involvement in development and disease. For example, NAC-1 is related to tumor recurrence and is essential for tumor growth and survival (10). KLHL7 antibodies are associated with various cancers, and in some patients also with neurological disease (11). Gigaxonin is a novel and distinct cytoskeletal BTB protein that may represent a general pathological target for neurodegenerative disorders (12). However, the physiological and biochemical functions of novel BTB-kelch proteins remain largely uncharacterized.

With the aim of identifying genes with transcription regulatory activity that are involved in human development and diseases, a novel BTB-kelch gene KBTBD7 was cloned from a human embryonic cDNA heart library. KBTBD7 encodes a protein with a predicted length of 684 amino acids that contains a BTB domain at the N-terminus, five kelch repeats at the C-terminus, and a BACK domain between the BTB and Kelch domains. Western blot analysis shows that a 77 kD transcript product is expressed broadly in early human embryonic tissues. We show that the KBTBD7 protein has a transcription activation activity in MCF-7 cells. Overexpression of KBTBD7 in the cell activates the transcriptional activities of the serum response element (SRE) and activator protein-1 (AP-1), suggesting that KBTBD7 may act as a new transcriptional activator in the mitogen-activated protein kinases MAPK signaling pathway.

RESULTS

Molecular characterization and evolutionary conservation of the KBTBD7 gene

We cloned a novel gene that was named KBTBD7 (FJ150424)

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as approved by HUGO Nomenclature Committee. The full-length KBTBD7 gene (Supplement Fig. 1A) consists of an open reading frame of 2,055 bp extending from the first ATG code at nucleotide 310 to a TGA stop code at 2,362. AACATGG sequence consistent with the Kozak rule was found in the start region of ORF and potential polyadenylation signal (AATAAA) was found at the 3′ untranslated region (UTR). The deduced KBTBD7 protein had 684 amino acids with a calculated molecular mass of 77.2 kDa. Furthermore, SMART analysis results indicated there existed a BTB domain at the NH2-terminus and five kelch motifs in the COOH terminus (Supplement Fig. 1B). Comparison of the KBTBD7 sequence with the genomic sequence showed that the KBTBD7 gene maps to chromosome 13q14.11 and spans approximately 10 kb. The KBTBD7 gene consists of one exon and has no introns.

BLAST searches using the sequence of KBTBD7 identified closely related sequences in Homo sapiens, Pan troglodytes, Bos taurus, Mus musculus, Canis familiaris, Danio rerio and Rattus norvegicus (Supplement Fig. 2). Sequence comparison of BTB motif among its orthologues indicates that the degree of conservation in this domain is quite high among vertebrate members (Fig. 1A). Sequence comparisons of BACK and Kelch-repeats show that these two domains are also conserved during evolution (Fig. 1B, C). A phylogenetic tree analysis using DNAstar software suggests that KBTBD7 is an evolutionarily conserved gene (Fig. 1D).

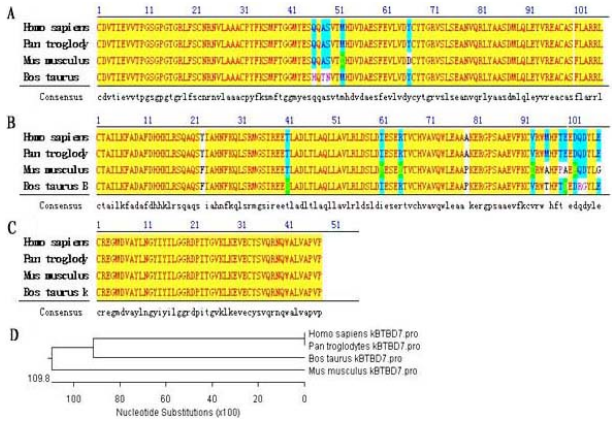

Fig. 1. KBTBD7 is conserved during evolution. (A, B) and (C) Amino acid sequence alignment of BTB domain (A), BACK domain (B) and Kelch repeats motif (C) in Homo sapiens, Pan troglodytes, Bos taurus, and Mus musculus indicates that BTB domain, BACK domain, and Kelch repeats motif are conserved during evolution. (D) A phylogenetic tree analysis of the KBTBD7 and other homologue proteins shown in (A, B) and (C) suggested that KBTBD7 is an evolutionarily conserved gene.

KBTBD7 is a cytoplasmic protein

To examine the subcellular location of KBTBD7, the pEGFP-C3-KBTBD7 was transfected into *COS-7* cells, and the cells were visualized with epifluorescence microscope after labeled with DAPI for nucleus. EGFP-KBTBD7 fusion protein distributes in cytoplasm (Fig. 2A) and DAPI binds to DNA (Fig. 2B). The combined image (Fig. 2C) shows that KBTBD7 protein exists in cytoplasm.

Expression of KBTBD7 protein at embryo stage

In an effort to understand the expression pattern of the KBTBD7 gene in early human embryogenesis, we generated antibody using the GST-KBTBD7 fusion protein (Supplement Fig. 3) and performed Western blot analysis on multiple embryonic. A 77 kDa protein product with slightly different expression levels was detected in all of the ten embryonic tis-

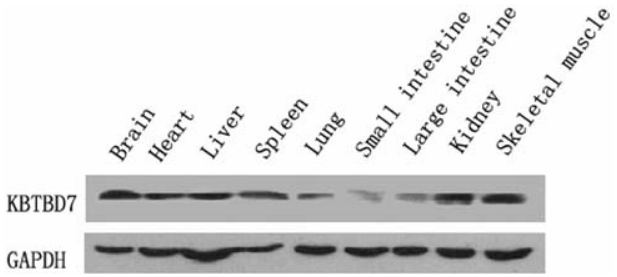

Fig. 3. Western blot analysis of KBTBD7 in human embryonic tissues Western blot analysis indicate that a 77kD protein recognized by antiKBTBD7 antobodies is detected at different levels of expression in brain, heart, liver, spleen, lung, kidney, skeletal muscle and intestine of embryo. GAPDH was used as a control for the equivalent amount of loaded protein (total protein) in each other. A band at 36 kD was also detected.

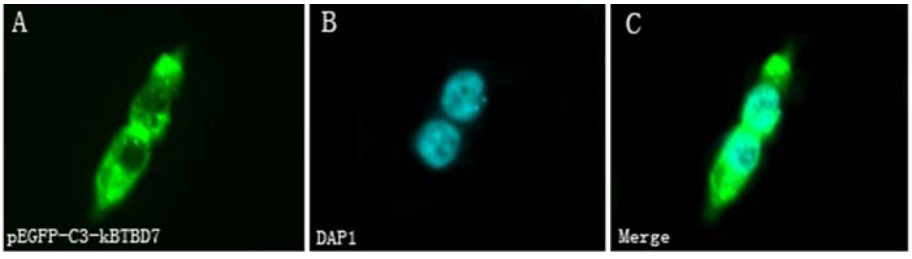

Fig. 2. Cellular localization of the KBTBD7 protein in COS-7 cells (A) EGFP- KBTBD7 is localized in the cytoplasm when transfected into the COS-7 cells. (B) The nucleus of the cells was stained with DAPI. (C) The combined image of (A) and (B), showing subcellular localization of KBTBD7 when overexpressed in COS-7 cells.

sues examined (Fig. 3). The result was consistent with the expression of KBTBD7 in multiple tissues examined by RT-PCR (Supplement Fig. 4), suggesting that KBTBD7 is a broadly expressed protein that may be involved in the human embryonic development.

KBTBD7 is a transcriptional activator

Previous studies have shown that BTB proteins family members are transcription factors; most of them are repressors and few of them are activators. Although KBTBD7 shares conserved regions with other BTB proteins, the potential role of KBTBD7 in transcription is unknown. We examined KBTBD7 for transcriptional activator and/or repressor activity; pCMV-BD-KBTBD7 was co-transfected with pL8G5-Luc reporter gene and pLexA-VP16 into COS-7 cells. As shown in Fig. 4A, when co-transfected with pL8G5-Luc plasmid, the GAL4-KBTBD7 fusion protein activated luciferase activity by 2.4 fold, suggesting that KBTBD7 functions as a transcriptional activator.

To further identify potential transcriptional regulatory domains in KBTBD7, we constructed five truncated GAL4-KBTBD7 fusion proteins: pCMV-BD-BTB (54-169 aa), pCMV-BD-BTB-BACK (54-280 aa), pCMV-BD-BACK (174-280 aa), pCMV-BD-BACK-Kelch (174-603 aa) and pCMV-BD-Kelch repeat (383-603 aa) (shown in Fig. 4A). pCMV-BD-BTB increased luciferase activity by 1.8 fold and the pCMV-BD-Kelch repeat increased activity by 1.7 fold. These results indicate that both the N-terminal BTB domain and the C-terminal Kelch repeat motif contribute to the transcriptional activation by KBTBD7. In contrast, the BACK domain seems to be a transcriptional repressor as it reduces activation by both the BTB domain and kelch repeat motifs, suggesting that the BACK domain may play a transcriptional repressive role in KBTBD7.

KBTBD7 activates SRE and AP-1-mediated transcriptional activation

MAPK signal transduction pathways are the most widespread mechanisms of eukaryotic cell regulation (13). In order to investigate the role of KBTBD7 in cell signal transduction, and specifically in MAPK-mediated transcriptional regulation, pathway-specific reporter gene assays were performed to measure the transcription of AP-1 and SRE by KBTBD7 in COS-7 cells, using pAP-1-Luc and SRE-luciferase reporters respectively. Expression of KBTBD7 significantly increased AP-1 transcriptional activity by approximately 4.0 fold (Fig. 4D, E) and SRE transcription by 2.5 fold (Fig. 4B, C).

To verify that the transcriptional activation we observed was due to the activity of the KBTBD7 protein, we used RNA interference to block the translation of KBTBD7. MCF-7 cells were transfected with pSUPER-RNAi-KBTBD7, together with pAP-1-LUC (or pSRE-LUC) and pCMV-Tag2C-KBTBD7. As shown in Fig. 4, pSUPER-RNAi-KBTBD7 blocked the activiation by KBTBD7. Taken together, our results suggest that KBTBD7 regulates components involved in the MAPK signaling pathway in cells.

DISCUSSION

Proteins containing a N-terminal BTB/POZ domain and four to six kelch motifs within the C-terminal region constituted a subgroup of the kelch superfamily. Thus far, the function of a few of BTB/kelch proteins is known. BTB/kelch protein Keap1 plays a role in gene expression by sequestering a transcription

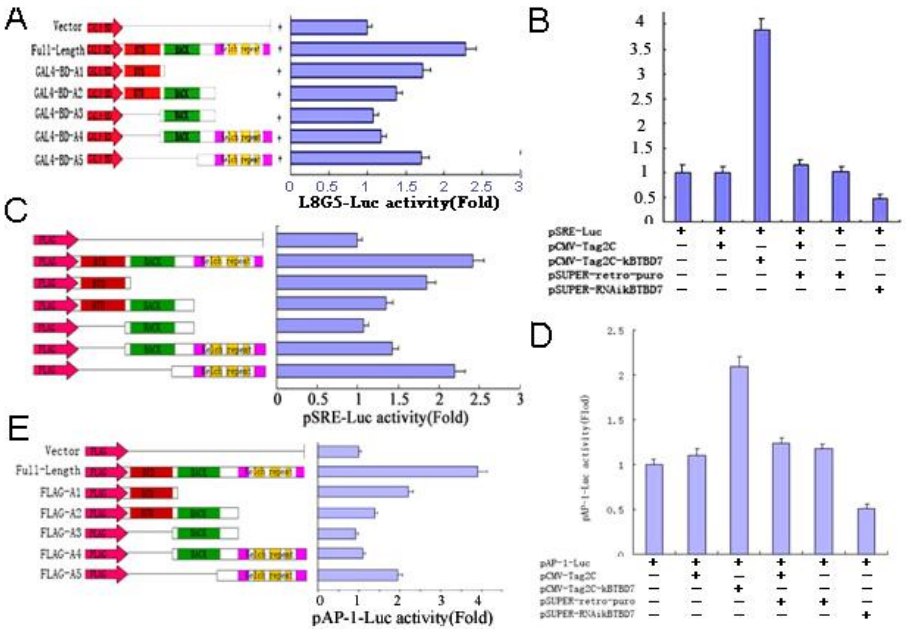

Fig. 4. Luciferase reporter assay of KBTBD7 (A) The transcriptional activity of full-length and truncated KBTBD7 fusion proteins (GAL4-BD-KBTBD7, GAL4-BD-A1 to GAL4-BD-A5) in MCF-7 cells. (B, D) Overexpressing KBTBD7 activates transcriptional activities of pSRE (B) and pAP-1(D) in MCF-7. (C, E) Overexpressing full-length and truncated KBTBD7 fusion proteins (FLAG-KBTBD7, FLAG-A1 to FLAG-A5) has a different impact on transcriptional activities of pSRE (C) and pAP-1(E) in MCF-7.

factor Nrf2 in the cytoplasm under normal conditions (14). A series of domain function analyses of Keap1 showed that the BTB domains are crucial for Transcription factor Nrf2 degradation (9). A recent study, however, indicates that Keap1 might also have a function in cell morphology and organization by binding to the SH3 domain of myosin- VIIa and associates with it in specialized adhesion junctions through the kelch-repeat domain (15). BTB/kelch protein Mayven was predominantly expressed in the human brain and was predicted to be important in the organization of the actin cytoskeleton by binding directly with actin through their kelch repeats (16). Diverse other cellular functions of the BTB/kelch proteins have been mentioned in our previous report (17). For example, The promyelocytic leukemia zinc finger (PLZF) protein containing an N-terminal BTB/POZ domain which is required for dimerization and transcriptional repression is a transcription factor disrupted in patients with t(11;17)(q23; q21)-associated acute promyelocytic leukemia (6). In this study, we isolated a novel member of the human BTB-kelch transcription factor family, KBTBD7, from a human embryonic cDNA heart library. This gene contains an N-terminal BTB domain and five C-terminal kelch motifs and like other BTB-kelch family proteins, KBTBD7 is highly conserved during evolution, suggesting that KBTBD7 might also play an important role in development and disease.

Previous studies have shown that BTB proteins family members are transcription factors; most of them are repressors and few of them are activators. To explore the effect of KBTBD7 on transcription regulation, a luciferase reporter assay was employed to explore the possibility that KBTBD7 activates transcription. Our results show that whole KBTBD7 exerts a modest (2.0-3.0 fold) activating effect on transcription of luciferase. In addition, the BTB domain and the Kelch domain were individually able to increase reporter gene activity, although the effect was somewhat weaker than that of whole KBTBD7. These results indicate that, like some BTB domain proteins, KBTBD7 is a transcriptional activator, in which the BTB domain and Kelch domain are required for full activation activity.

Using transient transfection and reporter assays, we have shown that KBTBD7 activates transcription via both SRE and Ap-1 components of MAPK signaling pathways. MAPK-mediated signaling pathways are involved in multiple cellular processes through phosphorylation of specific endpoint targets such as Elk-1 and SRE, which form a ternary complex together with SRF to induce expression of c-fos and other early response genes. Previous report shows that heart-specific deletion of SRF in the embryo by using a new beta MHC-Cre transgenic mouse line results in lethal cardiac defects (18). The absence of SRF in cardiomyocytes and smooth muscle cells (SMC) leads to ultra-structural defects in contractile/cytoskeletal assembly (19). SRE is one of the several cis-elements which mediate c-fos induction and it is recognized by a dimer of the serum response factor (SRF) that recruits the monomeric ternary complex factors (TCFs), whose members include Elk-1, SAP-1 or SAP-2 (20). The c-fos products heterodimerize with c-Jun proteins form AP-1 complexes. The DNA-binding activity of AP-1/c-Jun was found to be dramatically increased in failing hearts, an increase that was not observed in compensatory cardiac hypertrophy. In addition, AP-1/c-Jun activation in cardiomyocytes resulted in reduction of α-MHC mRNA. Activation of AP-1 involves the direct phosphorylation/dephosphorylation of AP-1 components as well as the phosphorylation and activation of transcription factors that induce elevated expression of c-Jun or c-fos (21). Our results suggested that KBTBD7 is a new BTB-kelch family protein that activates transcriptional activities mediated by MAPK signaling pathways in the cell and a role for KBTBD7 in processes involved in heart disease.

The myc-interacting zinc finger protein (MIZ-1) was located in the cell cytoplasm and regulated by association with microtubules and can activate LDLR transcription in response to changes in the cytoskeleton (22). Using transient transfection and subcellular location assays, we have shown that KBTBD7 protein exists in cytoplasm. One possible scenario is that KBTBD7 proteins act in synergy with other transcription factors to integrate information from multiple extracellular signals and, in turn, to induce the necessary cellular changes required for tissue specification and morphogenesis as an active transcriptional regulator in MAPK pathways.

In conclusion, KBTBD7 activates the transcriptional activities of AP-1 and SRE. These results suggest that KBTBD7 may act, directly or indirectly, as a positive transcriptional regulator in MAPK-mediated signaling pathways. It is likely that KBTBD7 proteins act in synergy with other transcription factors to integrate information from multiple extracellular signals to induce the necessary cellular changes required for tissue specification and morphogenesis and it may also play roles in the regulation of heart development and in heart disease.

MATERIALS AND METHODS

Construction of cDNA library of human embryonic heart

Embryos were obtained with the consent of the patients and according to the guidelines approved by Hunan Normal University of Ethics Committee, and with the approval of the Changsha Women and Children's Hospital, People's Republic of China. Construction of cDNA library of human embryonic heart was performed according to the methods described previously (23).

Blast searching and bioinformatics analysis

BLASTn program was used to search human EST database and identify the cytological locus of genes and to look for exons and introns. Analysis of KBTBD7 sequence was performed by DNAstar software (24). Amino acid sequence alignment was performed with the CLUSTAL W program (25).

Full-length KBTBD7 cDNA cloning and bioinformatics analysis

PCR was performed on a PCRSPRINT reactor (Thermo Hybaid) with one pair of degenerate oligonucleotide primers PS and PAS (Supplement Table 1) corresponding to open reading frame of KBTBD7 (Accession number: NM_032138). Amplification was carried out at 94°C, 4 min; 94°C, 30 s; 60°C, 30 s; and 72°C, 2 min for 35 cycles; then 72°C, 10 min. The amplification products were separated by agarose gel and the bands were cloned into pMD18-T vector (TaKaRa). The transformants were randomly chosen and sequenced with 3730 DNA Sequencer (ABI PRISM) according to manufacturer's procedures.

Expression of GST fusion proteins and preparation of KBTBD7 polyclonal antibody

For expressing GST fusion proteins, the PCR products of KBTBD7 (546-595 aa) with the primers P4S/P4AS (Supplement Table 1) were cloned into pGEX4T-1 vector by *EcoR* I and *Xho* I sites, and confirmed by DNA sequencing. The recombinant vector was transformed into *E.coli* BL21 (Invitrogen), and expression was then induced with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 26°C. The GST-KBTBD7 (546-595 aa) soluble fusion protein was purified on a glutathione-Sepharose column (Amersham Biosciences) according to the manufacturer's protocol and used to immunize rabbits for polyclonal antiserum production. The polyclonal antibody was purified and the specificity of antibody was confirmed using to methods described previously (17).

Protein extraction and Western blotting analysis

The following fetal tissues were used: brain, kidney, heart, liver, lung, muscle, stomach and small intestine. Western blotting analysis was performed with anti-KBTBD7 of or anti-GAPDH antibodies (Santa cruz) as the first antibody. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody, and detection was performed using the ECL.

Plasmid construction

The L8G5-Luc, pSRE-Luc and pAP-1-Luc constructs used were generated previously in the lab (24). To generate a fusion protein of KBTBD7 with enhanced green fluorescent protein (EGFP), the KBTBD7 ORF was amplified by PCR (primer P2S, P2A3) (Supplement Table 1) and then subcloned into the *Eco*RI and *Bam*H1 sites of the pEGFP-C3 vector. To generate a fusion protein of KBTBD7 with GAL4 or FLAG tag, the KBTBD7 ORF was amplified by PCR with primers P3S/P3AS or P2S/P2AS (Supplement Table 1) and then subcloned in-frame into the *Eco*RI and *Xba*I site of the pCMV-BD or *Eco*RI and *Bam*HI site of pCMV-Tag2C, respectively. To generate a fusion protein of BTB, BTB-BACK, BACK, BACK-Kelch and Kelch-repeat fragments with GAL4 or FLAG tag, the five fragments were amplified by PCR with primers P5S/P5AS, P6S/P6AS, P7S/P7AS, P8S/P8AS and P9S/P9AS (Supplement Table 1), respectively, and then subcloned to the *Bam*HI and *Eco*RI sites of the pCMV-BD and pCMV-Tag2C .

Cell culture, transient transfection, and subcellular localization analysis

COS-7 cells used in all studies were maintained and passaged according to standard methods described previously (24) and transfected with pEGFP-C3-KBTBD7 using lipofectamine (Invitrogen) according to the company's protocol. Subcellular localization of the EGFP-KBTBD7 fusion protein was detected 48 h following transfection by fluorescence microscopy (Nikon, E400).

Transient expression reporter gene assay and deletion analysis

COS-7 cells were transfected using lipofectamine as described above. pCMV-BD-KBTBD7 or other truncated GAL4-KBTBD7 fusion constructs, or pCMV-Tag2C-KBTBD7 were co-transfected into COS-7 cells along with the pL8G5-Luc reporter and pAP-1-Luc or pSRF-Luc. The luciferase activity assay was performed 48 h later according to the methods described previously (24).

RNAi analysis

A pSUPER.retro.puro vector-based system was used to deliver siRNA into MCF-7 cells. A pair of oligonucleotides was designed by the RNAi program (http://www.openbiosystems.com/RNAi). The sequences were s1: 5′GATCCCCGGATGTGGCATATCTCAATTTCTAGAGAATTGAGATATGCCACATCCTTTTTC3′ and anti-s1: 5′TCGAGAAAAAGGATGTGGCATATCTCAATTCTCTAGAAATTGAGATATGCCACATCCGGG3′. The oligos were annealed and cloned according to the instructions (OligoEngine). Luciferase activity assay was performed in MCF-7 cells transfected with pSRE-Luciferase (or pAP1-Luciferase), pCMV-Tag2C-kBTBD7, and pSUPER-RNAi-KBTBD7.

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