Supporting Information

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SI Materials and Methods

Plasmids and siRNA. The reporter constructs estrogen-responsive element–Luc, catD-Luc, and pS2-Luc and expression vectors for estrogen receptor α (ERα), Flag-tagged ERα, and HA-tagged ERα have been described previously (1). Expression vectors for siRNA-resistant TANK-binding kinase 1 (TBK1) containing a silent mutation in the 3′ nucleotide of a codon in the middle of the siRNA-binding site were generated by recombinant PCR. Myc-, HA-, or Flag-tagged ERα mutants were generated using a Quik-Change Mutagenesis Kit (Strategene). Primers for S→A mutations are as follows:

S305A1: 5′-CAGGGACAAGGCCAGGGCGTTCTTCTTAG-3′

S305A2: 5′-AACGCCCTGGCCTTGTCCCTGACGGCC-GAC-3′

S468A1: 5′-CCAGAGCCTTCAGGGTGCTGGACAG-3′

S468A2: 5′-CCTGAAGGCTCTGGAAGAGAAGGACC-3′

S578A1: 5′-GCAAGGCATGCGATGAAGTAGAG-3′

S578A2: 5′-CGCATGCCTTGCAAAAGTATTAC-3′

All plasmids were verified by restriction enzyme analysis and DNA sequencing.

Real-Time RT-PCR. First-strand cDNA was generated from total RNA using random priming and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time PCR was performed using QuantiTect SYBR Green PCR Master Mix (Qiagen) in triplicate and was analyzed on an ABI Prism 7700 analyzer (Applied Biosystems). All real-time values were normalized to GAPDH. The primer sequences are as follows:

pS2: 5′-GGAGAACAAGGTGATCTGCG-3′ (forward) and 5′-CACACTCCTCTTCTGGAGGG-3′ (reverse)

C3: 5′-GTGACAATGTACCATGCTAAGG-3′ (forward) and 5′-TAGTGTTCTTGGCATCCTGAG-3′ (reverse)

cyclin D1: 5′-CTGGCCATGAACTACCTGGA-3′ (forward) and 5′-GTCACACTTGATCACTCTGG-3′ (reverse)

cathepsin D: 5′-GACACAGGCACTTCCCTCAT-3′ (forward) and 5′-GTAGTAGCGGCCGATGAAGA-3′ (reverse)

c-fos: 5′-AAGGAGAATCCGAAGGGAAAGGAATAAGAT-GGCT-3′ (forward) and 5′-AGACGAAGGAAGACGTGTA-AGCAGTGCAGCT-3′ (reverse)

Bcl-xL: 5′-TTTGAATCCGCCACCATGTCTCAGAGCAAC-CGGGAGCTG-3′ (forward) and 5′-TTTCTCGAGCTTTCC-GACTGAAGAGTGAGCCCA-3′ (reverse)

GAPDH: 5′-CATGTTCGTCATGGGTGTGAACCA-3′ (forward) and 5′-AGTGATGGCATGGACTGTGGTCAT-3′ (reverse)

ERα: 5′-ATGACCATGACCCTCCAC (forward) and 5′- GT-CAGACCGTGGCAGGGAA (reverse)

TBK1: 5′-TGAAGAGGAGACAACAACAAG-3′ (forward) and 5′-GTGCCATACAGAGAAACAAAC-3′ (reverse)

Cell Growth Assay. Cells were seeded at a density of 5×10^4 per well in six-well plates. Triplicate wells were then harvested on days 0, 2, 4, and 6 and were counted using a Countess Automated Cell Counter (Invitrogen). Cell culture medium was replenished every other day.

Assessment of Colony Formation in Soft Agar. Aliquots of 10^4 MCF-7 cells were resuspended in 1 mL of 0.35% agar (wt/vol) in DMEM/FBS. The aliquots were poured into six-well dishes on top of a 1.5-mL layer of 0.5% agar (wt/vol) in DMEM/FBS, allowed to solidify, and incubated for 21 d at 37 °C in the presence of 5% (vol/vol) CO_2. The dishes were photographed, and the colony size was estimated, using the Image-Pro Plus 4.1 software package (Media Cybernetics), by determining the total area that the colonies occupied in the photographs.

Cell Migration Assays. Cells were seeded onto six-well dishes in 10% (vol/vol) FBS/RPMI medium 1640 and maintained at 37 °C with 5% (vol/vol) CO_2 until they reached 95% confluence. The monolayer cells were wounded by a sterile pipette tip to create a 1-mm cell-free path. Culture medium was removed, and the samples were washed with PBS, followed by culturing in 10% (vol/vol) FBS/RPMI medium 1640. Cells were fixed in 3.7% (vol/vol) paraformaldehyde at the indicated time intervals (every 12 h) and photographed under a low-magnification microscope. The distances between the wounding center and the front of the migrating cells (vertical axis) were measured for statistical analysis.

Animal Experiments. Two days after implantation of estrogen pellets (17β-estradiol, 0.36 mg per pellet, 60-d release; Innovative Research of America), 1×10^7 tumor cells were injected into the abdominal mammary fat pad of 6-wk-old female nude mice. When tumors reached a volume of ∼200 mm^3, we randomly allocated the mice to groups in which they received a placebo or the indicated drugs. BX795 (Santa Cruz Biotechnology) was administered at a dose of 1 μg per mouse and tamoxifen (Sigma) at a dose of 30 μg per mouse every 2 d unless indicated. Tumor growth was monitored by caliper measurements. Excised tumors were weighed, and portions were frozen in liquid nitrogen or fixed in 4% (vol/vol) paraformaldehyde for further study.

Cohort. There were a total of 473 formalin-fixed and paraffin-embedded tumor samples and 171 paired adjacent normal tissue samples in our study. All tumors were primary and untreated before surgery. The clinical pathological information for all tumors was obtained (Table S2). All subjects received a radical mastectomy or modified radical mastectomy. The axillary lymph nodes were routinely dissected, and lymph node metastasis was determined based on histological examination. Tumor size was defined as the maximum tumor diameter measured on the tumor samples at the time of surgery. Histological types of the total 473 samples were defined according to the World Health Organization (WHO) classification criteria (2007) into grade I (111 cases), grade II (293 cases), and grade III (69 cases). Clinical stage was also defined according to the WHO classification criteria (2007) into stage I (79 cases), stage II (308 cases), stage III (84 cases), and stage IV (2 cases). Among the samples, 119 tumor samples and paired adjacent tissue samples were obtained from a tissue bank maintained in the Department of Pathology at the General Hospital of Jinan Military Region, Jinan, Shandong, China. Two hundred tumor samples and 52 paired adjacent normal tissue samples were obtained from a tissue bank maintained in the Department of Pathology at the General Hospital of the Chinese People's Liberation Army, Beijing, China. The remaining 154 tumor samples were obtained from a tissue bank

maintained at the Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing, China. After surgery, the majority of these subjects received adjuvant chemotherapy alone (cyclophosphamide, methotrexate, and fluorouracil or an anthracycline-based regimen) or combined chemotherapy and endocrine therapy, with or without radiotherapy. Subjects with adjuvant endocrine therapy usually received tamoxifen treatment (20 mg/d) for a maximum of 5 y.

Immunohistochemistry. Immunohistochemistry staining for TBK1 was performed on the paraffin-embedded tissue microarray. H&E staining was performed to ensure the cancer tissue and normal epithelia were present. The tissue microarrays were performed on 4-μm thick sections. Briefly, tissue slides were deparaffinized and rehydrated through a graded alcohol series. The endogenous peroxidase activity was prohibited with 3% (vol/vol) hydrogen peroxide solution for 15 min. Antigen retrieval was performed by placing the slides in 10 mM sodium citrate buffer (pH 6.0) and maintained at a subboiling temperature for 10 min. To block nonspecific staining, the slides were immersed in 10% (vol/vol) normal goat serum in PBS for 30 min. Primary antibody was then used overnight at 4 °C in a humidified chamber. Slides were washed in PBS, followed by anti-mouse secondary antibody (Polink-2 HRP DAB Detection Kit; Dako) for 30 min at room temperature. Quality assessment was performed on each batch of slides by including a negative control consisting of eliminating the primary antibody to preclude nonspecific signal. Breast carcinomas known to expresses high levels of TBK1 protein were used as a positive control.

All staining was assessed by pathologists blinded to the origination of the samples using a semiquantitative method. Each specimen was assigned a score according to the intensity of the nucleic and cytoplasmic staining. Tissue was scored (H-score) (2) based on the total percentage of positive cells and the intensity of the staining (1+, 2+, or 3+), where H = (% "1+" × 1) + (% "2+" × 2) + (% "3+" × 3) (3). A minimum of 100 cells were evaluated in calculating the H-score. The final immunoreactive score was determined by multiplying the intensity score by the extent of the score of stained cells, ranging from 0 (the minimum score) to 3 (the maximum score). As for ERα, ERα S305, and cyclin D1, we defined a score of 0 as negative and a score of >0 as positive. As for TBK1, we defined the median value (score = 1.5) of TBK1 expression in tumor tissues as the cutoff value. Thus, high TBK1 expression was defined as 1.5 < TBK1 score ≤ 3, and low TBK1 expression was defined as 0 ≤ TBK1 score ≤ 1.5.

Ethics Statement. All animals were handled in strict accordance with the *Guide for the Care and Use of Laboratory Animals* (4) and the principles for the utilization and care of vertebrate animals (5), and all animal work was approved by the Institutional Animal Care Committee of Beijing Institute of Biotechnology. The study using clinical samples was approved by the Ethical Committees of the General Hospital of Jinan Military Region; General Hospital of the Chinese People's Liberation Army; and Southwest Hospital, Third Military Medical University. Informed consent was obtained from all subjects or their relatives.

1. He X, et al. (2010) c-Abl regulates estrogen receptor alpha transcription activity through its stabilization by phosphorylation. *Oncogene* 29(15):2238–2251.
2. McClelland RA, et al. (1990) Automated quantitation of immunocytochemically localized estrogen receptors in human breast cancer. *Cancer Res* 50(12):3545–3550.
3. Finn RS, et al. (2013) Quantitative ER and PgR assessment as predictors of benefit from lapatinib in postmenopausal women with hormone receptor-positive, HER-2 negative metastatic breast cancer. *Clin Cancer Res*, 10.1158/1078-0432.CCR-13-1260.
4. Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC).
5. US Office of Science and Technology Policy (1985) Laboratory animal welfare: U.S. government principles for the utilization and care of vertebrate animals used in testing, research, and training. *Fed Regist* 50(97):20864–20865.

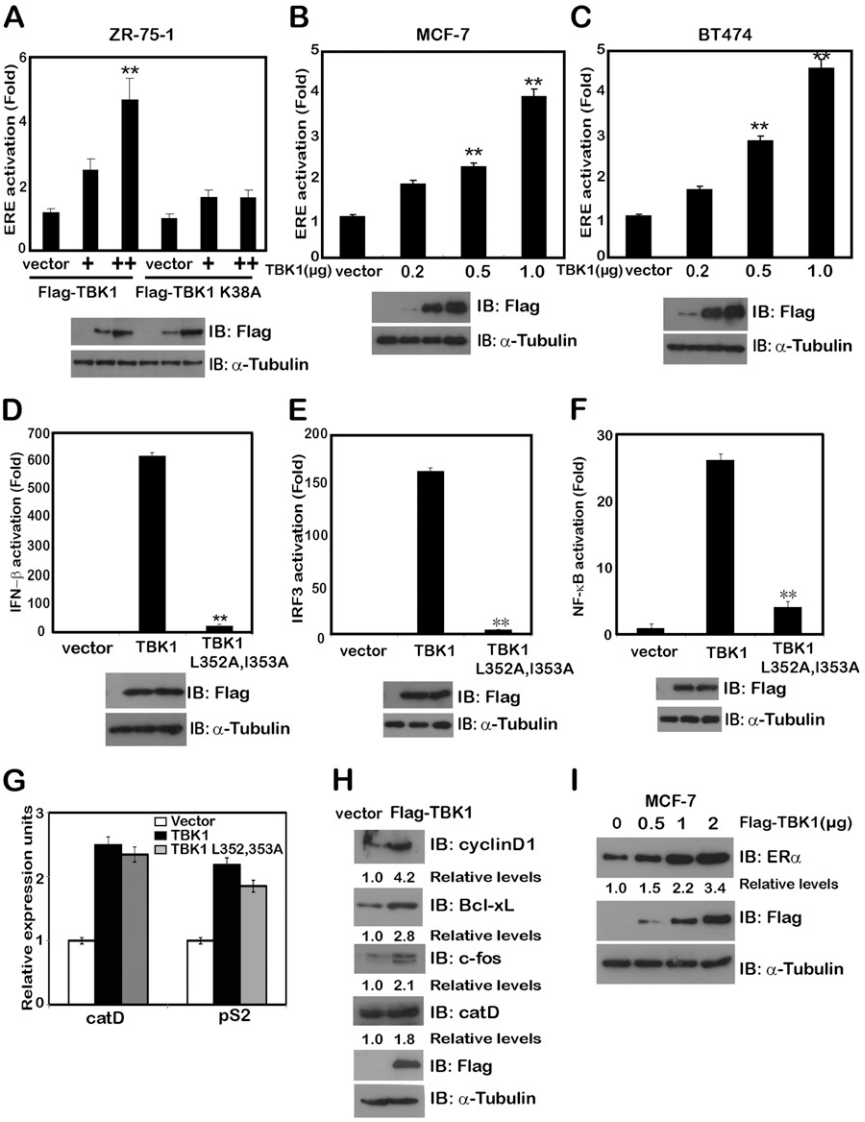

Fig. S1. TBK1 regulates the transcriptional activity of ERα independent of its role in the innate immune response (Fig. 1). (*A*) ZR-75-1 cells were cotransfected with estrogen-responsive element (ERE)-Luc together with the increased amounts of expression vector for TBK1 or TBK1 K38A. The Luc activity was measured 24 h later and normalized for transfection efficiency. IB, immunoblot. MCF-7 (*B*) or BT474 (*C*) cells were transfected with ERE-Luc together with the indicated amounts of expression vector for TBK1. The Luc activity was measured 24 h later and normalized for transfection efficiency. The 293T cells were transfected with interferons (IFN)-β–Luc (*D*), interferon regulatory transcription factor 3-Luc (*E*), or NF-κB–Luc (*F*) together with TBK1 or TBK1 L352, 353A. The Luc activity was measured 24 h later and normalized for transfection efficiency. (*G*) MCF-7 cells were transfected with TBK1 or TBK1 L352A, I353A, and real-time PCR was performed using the primers specific for pS2 and catD. (*H*) MCF-7 cells were transfected with Flag-TBK1 expression plasmid. Whole-cell lysates were analyzed by immunoblotting with anti-cyclin D1, anti–Bcl-xL, anti–c-fos, and anti-catD antibodies or anti-cyclin D1. α-Tubulin was used as an equal loading control. (*I*) MCF-7 cells were transfected with an increasing amount of Flag-TBK1. Whole-cell lysates were analyzed by immunoblotting with anti-TBK1 or anti-ERα antibody. α-Tubulin was used as an equal loading control. Cell-based studies were performed at least three independent times with comparable results. Data represent mean ± SEM. The Student *t* test was used for statistical analysis (**$P < 0.01$).

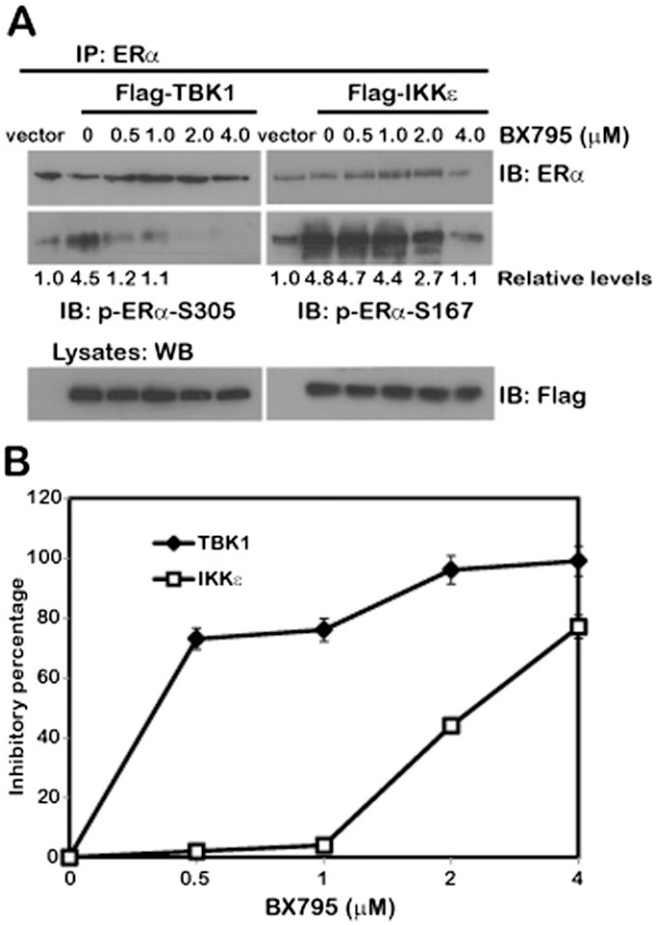

Fig. S2. BX795 (1 μM) is a specific inhibitor of TBK1 (Fig. 3). (*A*) MCF-7 cells transfected with TBK1 or IκB kinase ε (IKKε) were treated with different doses of BX795 for 2 h. Whole-cell lysates were analyzed by immunoblotting with anti–p-ERα–S305, ERα, anti–p-ERα–S167 antibody, or anti-Flag antibody. IP, immunoprecipitate. (*B*) Densities of ERα bands in Fig. 2*A* were quantified, and the BX795-mediated ERα inhibitory percentage was calculated as $I = 100 \times (C - T)/C$, where C = relative levels determined by software-based quantification in control and T = relative levels determined by software-based quantification in treatment (1).

1. Clark K, Plater L, Peggie M, Cohen P (2009) Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: A distinct upstream kinase mediates Ser-172 phosphorylation and activation. *J Biol Chem* 284(21):14136–14146.

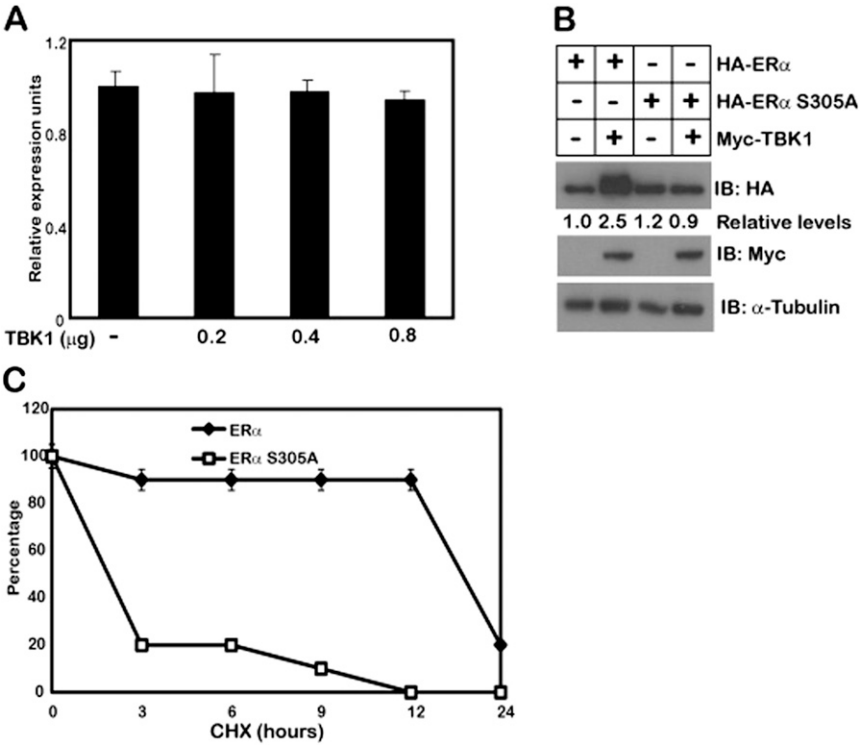

Fig. S3. TBK1 increases the stability of ERα primarily through phosphorylation of ERα at serine 305 (Fig. 4). (*A*) MCF-7 cells were transfected with different doses of TBK1 expression plasmids. RNA was extracted 24 h later, and the ERα mRNA level was analyzed by quantitative real-time RT-PCR. (*B*) MCF-7 cells were transfected with Myc-TBK1 together with HA-ERα or HA-ERα S305 mutant. Whole-cell lysates were analyzed by immunoblotting with anti-HA or anti-Myc antibody. (*C*) Densities of ERα bands in Fig. 4*D* were quantified and expressed as a percentage of each control band density. CHX, cycloheximide. The numbers below some Western blots indicate relative levels determined by software-based quantification of the representative experiment shown.

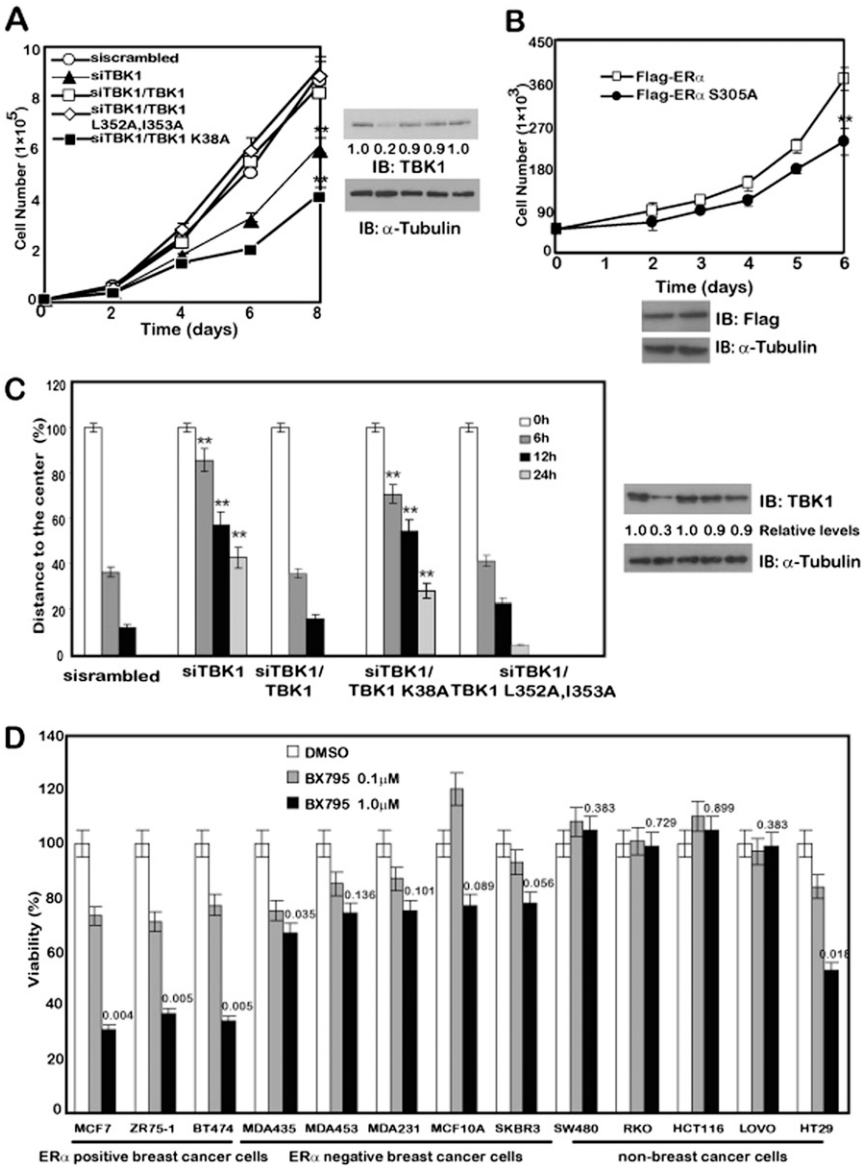

Fig. S4. TBK1 regulates breast cancer cell growth (Fig. 5). (*A*) Stable clones of ZR-75-1 cells transfected with scrambled siRNA (siscrambled); TBK1 siRNA oligos (siTBK1) together with Flag-vector; Flag-TBK1; Flag-TBK1 L352, 353A; or Flag-TBK1 K38A were seeded at a density of 5×10^4 per well in six-well plates. Cells were trypsinized at the indicated times and counted with a Coulter Counter. (*B*) Two stably transfected clones with WT ERα or ERα S305A were seeded at a density of 5×10^4 per well in six-well plates. Cells were trypsinized at indicated times and counted with a Coulter Counter. (*C*) Indicated stable clones were wounded, and healing was followed over the indicated times. (*D*) Indicated cell lines were treated with different dosages of BX795. Relative cell viability was assayed 6 d after BX795 treatment. The *P* value was shown as indicated. Cell-based studies were performed at least three independent times with comparable results. Data represent mean ± SEM. The Student *t* test was used for statistical analysis (**$P < 0.01$).

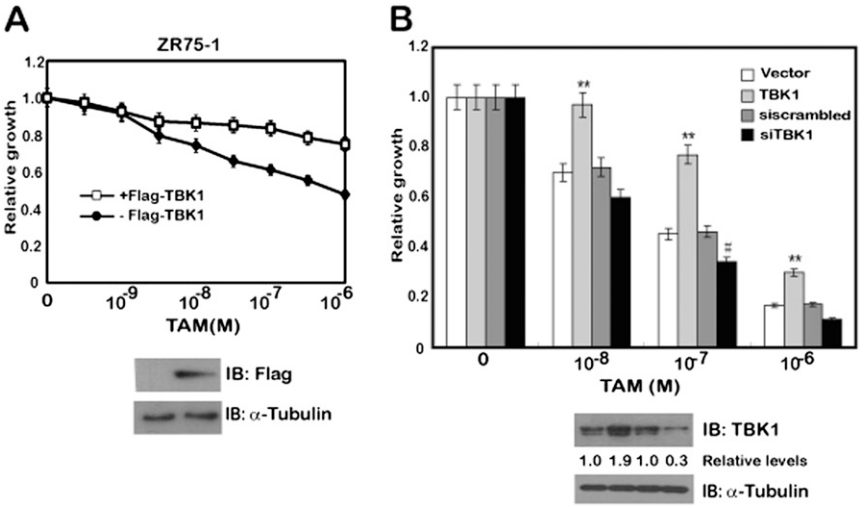

Fig. S5. TBK1 has a major role in the resistance of breast cancer cells to tamoxifen treatment (Fig. 5). (*A*) ZR-75-1 cells transfected with TBK1 were treated with a range of concentrations of tamoxifen. Cell viability was assessed 7 d after the tamoxifen treatment. (*B*) Stable clones of ZR-75-1 cells transfected with scrambled siRNA, siTBK1 together with Flag-vector, or Flag-TBK1 were treated with a range of concentrations of tamoxifen. Cell viability was assessed 7 d after the tamoxifen treatment. $^{**}P < 0.01$ and $^{\#}P < 0.01$ vs. control Flag-vector and scrambled siRNA, respectively. Cell-based studies were performed at least three independent times with comparable results. Data represent mean ± SEM. The Student *t* test was used for statistical analysis ($^{**}P < 0.01$).

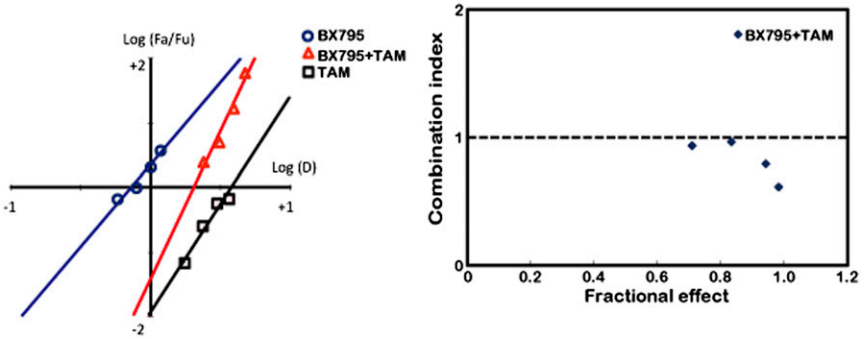

Fig. S6. Combination therapy of BX795 and tamoxifen against TBK1-overexpressed ZR-75-1 xenograft tumors in nude mice (Fig. 5). To investigate a potential synergistic effect of BX795 and tamoxifen in nude mice bearing TBK1-overexpressed ZR-75-1 xenograft exposed to BX795 and tamoxifen, we applied the median effect analysis described by Chou and Talalay (1). The results were plotted with the median-effect analysis, log (Fa/Fu) with respect to log (D). Fa and Fu are fractions of tumors that were affected (inhibited) or unaffected by drugs. D represents doses of drugs. All data fit well with linear regression analysis (all r^2 values were larger than 0.92), indicating the results are highly reliable. All combination indices were calculated to be well below 1, indicating a strong synergy from cotreatment.

1. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 22:27–55.

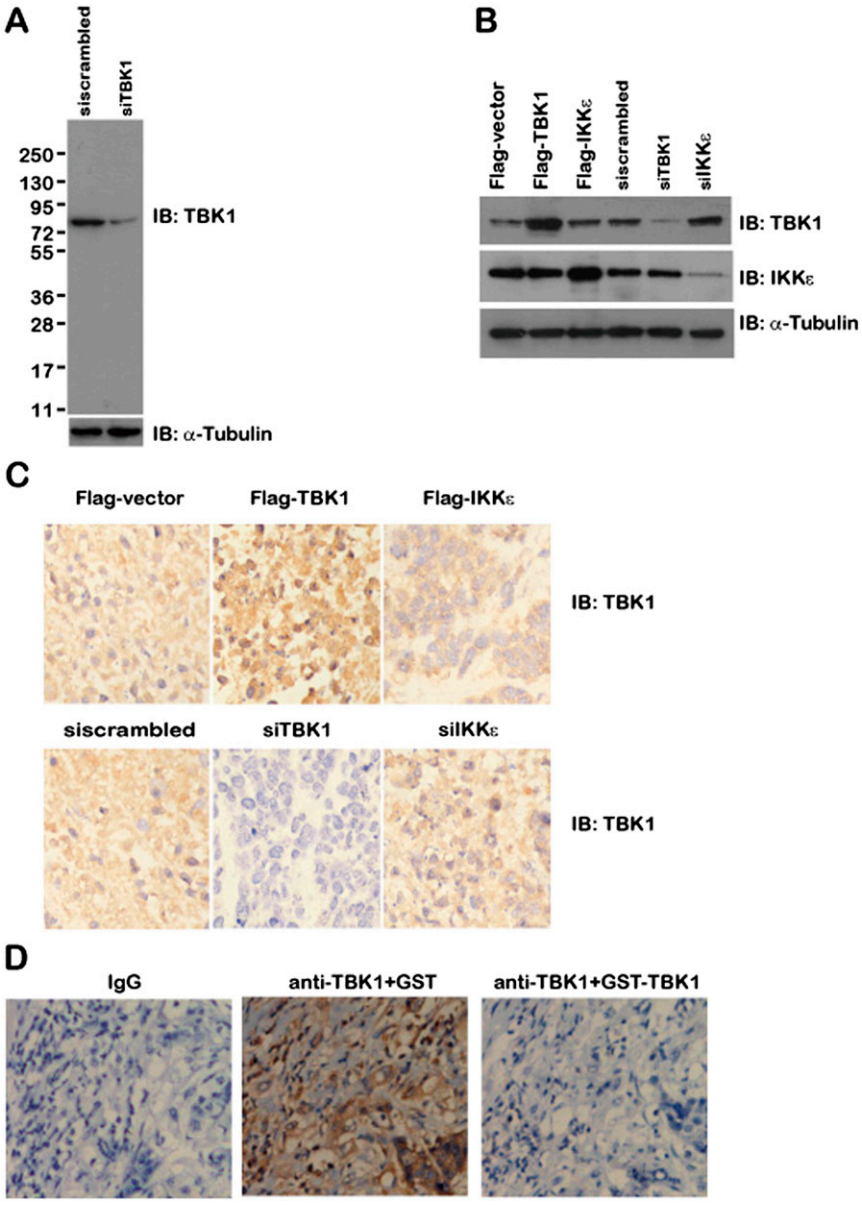

Fig. S7. Validation of antibody specificity to TBK1 (Fig. 6). (*A*) Immunoblot analysis of lysates from MCF-7 cells transfected with TBK1 siRNA using antibody specific for TBK1. (*B*) Immunoblot analysis of lysates from tumors derived from MCF-7 cells expressing TBK1, IKKε, TBK1 siRNA, or IKKε siRNA using antibodies specific for TBK1 or IKKε. (*C*) Immunofluorescence staining of TBK1 from tumors derived from MCF-7 cells expressing TBK1, IKKε, TBK1 siRNA, or IKKε siRNA using antibodies specific for TBK1. (Magnification: 400×.) (*D*) Immunohistochemical staining of breast cancer specimens incubated with normal IgG or anti-TBK1. To validate antibody specificity, anti-TBK1 antibody was preincubated with recombinant GST-TBK1 protein for 1 h before application to tissue. (Magnification: 400×.)

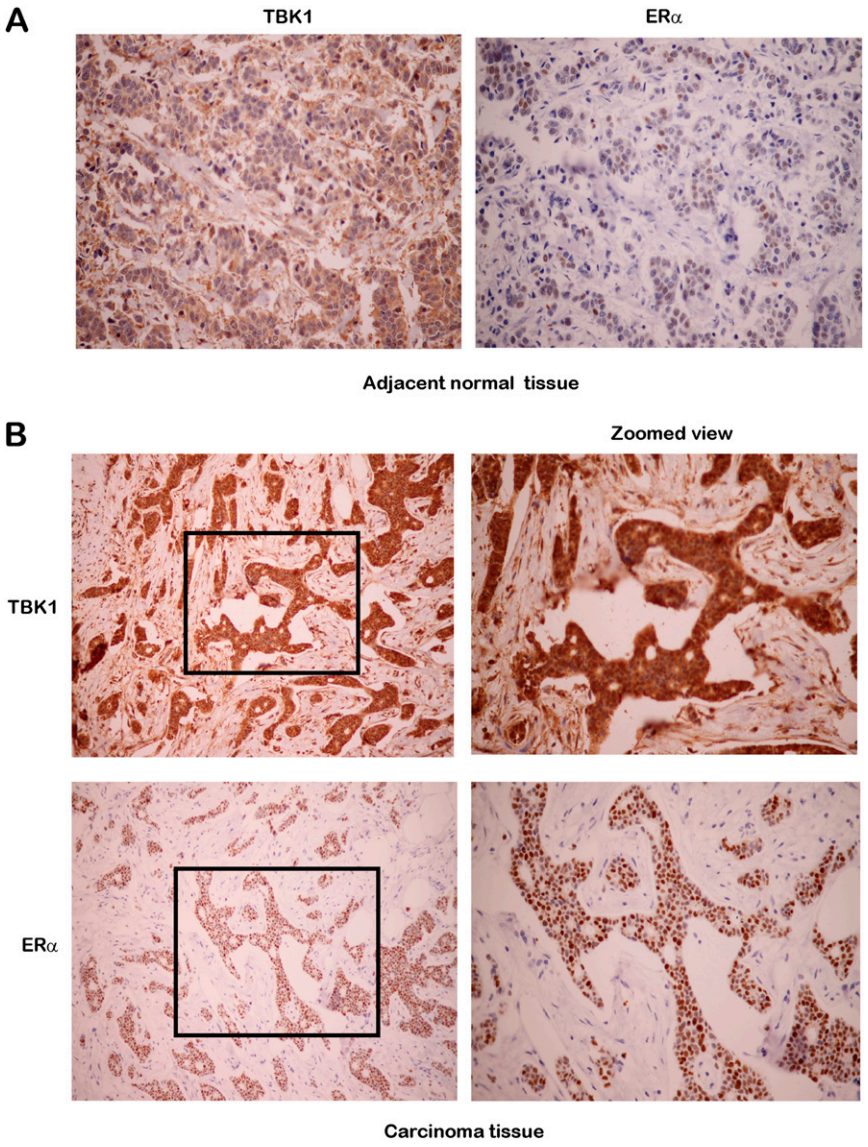

Fig. S8. TBK1 is positively correlated with ERα in patients with breast cancer (Fig. 7). (*A*) Expression of ERα and TBK1 in normal tissues adjacent to breast cancer. (Magnification: 400×.) (*B*) Expression of ERα and TBK1 in human breast cancer tissues. (Magnification: 200×.) The boxed areas (*Left*) are magnified (*Right*). (Magnification: 400×.)

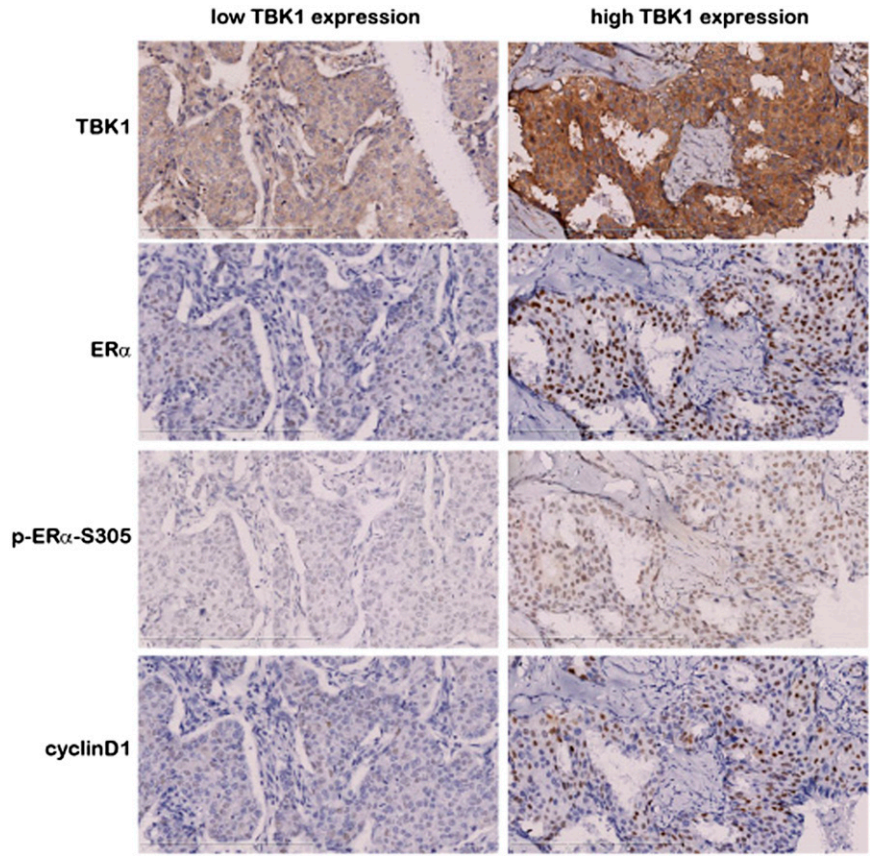

Fig. S9. TBK1 is positively correlated with ERα, ERα Ser-305, and cyclin D1 in patients with breast cancer (Fig. 7). Representative images from immunohistochemical staining of ERα, ERα Ser-305, and cyclin D1 in tumors from TBK1 high expression and low expression groups. (Magnification: 400×.)

Table S1. Combination therapy of BX795 and tamoxifen against TBK1-overexpressed ZR-75-1 xenograft tumors in nude mice

Therapy	Dose range, μg per mouse	Route	Schedule	Mice (five per group)
BX795	0.6, 0.8, 1.0, 1.2	i.v. injection	Every 2 d	20
TAM	36, 48, 60, 72	i.v. injection	Every 2 d	20
BX795 + TAM	(0.6 + 36), (0.8 + 48), (1.0 + 60), (1.2 + 72)	i.v. injection	Every 2 d	20
Control	Placebo	i.v. injection	Every 2 d	5

TAM, tamoxifen.

Table S2. Clinical characteristics of the patient cohort with breast cancer in this study

Clinical characteristics	Jinan*	Beijing†	Chongqing‡	Total
No. of patients	119	200	154	473
Tumor samples	119	200	154	473
Paired adjacent normal tissue samples	119	52	0	171
Follow-up	63	0	154	217
Age, y				
≤50	65	109	106	280
>50	54	91	48	193
Histological grade				
I	18	42	51	111
II	80	120	93	293
III	21	38	10	69
T stage				
T1	40	18	64	122
T2	59	144	79	282
T3	12	19	7	38
T4	8	19	4	31
N stage				
N0	58	137	78	273
N1	32	53	63	148
N2	19	7	10	36
N3	10	3	3	16
M stage				
M0	119	200	152	471
M1	0	0	2	2
Clinical stage				
I	26	15	38	79
II	60	153	95	308
III	33	32	19	84
IV	0	0	2	2

M, metastasis (distant); N, node (lymph); T, tumor (size).

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