




# HBXIP is a novel regulator of the unfolded protein response that sustains tamoxifen resistance in ER+ breast cancer

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Endocrine-therapy-resistant estrogen receptor-positive (ER+) breast cancer cells often exhibit an augmented capacity to maintain endoplasmic reticulum (EnR) homeostasis under adverse conditions. Oncoprotein hepatitis B X-interacting protein (HBXIP) is a known transcriptional coactivator that promotes cancer development. However, it is unclear whether HBXIP participates in maintaining EnR homeostasis and promoting drug resistance in ER+ breast cancer. Here, we report that tamoxifen-resistant (TmaR) breast cancer cells exhibit increased expression of HBXIP, which acts as an inactivator of the unfolded protein response (UPR) to diminish tamoxifen-induced EnR stress. We show that HBXIP deficiency promotes EnR-associated degradation, enhances UPR-element reporter activity and cellular oxidative stress, and ultimately attenuates the growth of TmaR cells *in vitro* and *in vivo*. Mechanistically, we demonstrate that HBXIP acts as a chaperone of UPR transducer inositol-requiring enzyme 1 $\alpha$  and diminishes production of reactive oxygen species (ROS) in TamR breast cancer cells. Upon loss of HBXIP expression, tamoxifen treatment hyperactivates IRE1 $\alpha$  and its downstream proapoptotic pathways and simultaneously induces accumulation of intracellular ROS. This elevated ROS programmatically activates the other two branches of the UPR, mediated by PKR-like ER kinase and activating transcription factor 6 $\alpha$ . Clinical investigations and Kaplan–Meier plotter analysis revealed that HBXIP is highly expressed in TamR breast cancer tissues. Furthermore, reinforced HBXIP expression is associated with a high recurrence and poor relapse-free survival rates in tamoxifen monotherapy ER+ breast cancer patients. These findings indicate that HBXIP is a regulator of EnR homeostasis and a potential target for TamR breast cancer therapy.

cancer. The endoplasmic reticulum (EnR) stress-activated unfolded protein response (UPR), a collective set of signaling pathways, has been demonstrated to be one of the most vital endocrine-therapy-resistant mechanisms (1). However, the detailed mechanisms by which the UPR pathways integrate their cytoprotective and proapoptotic outputs under EnR stress, such as TAM treatment, are still unknown.

EnR plays a crucial role in regulating protein homeostasis. When undergoing insatiable protein folding requirements, the cells will initiate EnR stress and cause UPR. The UPR is a prime candidate for one survival mechanism that, if successfully activated, could allow cells to survive the stress of endocrine therapies and confer a resistance phenotype (2). Three EnR protein sensors, protein kinase RNA-like endoplasmic reticulum kinase (PERK), activation transcription factor 6 $\alpha$  (ATF6), and inositol requiring enzymes 1 $\alpha$  (IRE1 $\alpha$ ), primarily mediate the UPR (3). The complex signaling network mediated by these proteins coordinates the response of cells to EnR stress. Chronic activation of the UPR signaling pathway is a key factor in cancer progression and has been widely accepted by the scientific and medical communities. The UPR is often abnormally activated when a tumor suppressor is absent or an oncogene is activated. Cancer cells can survive high protein synthesis and metabolic stress to promote tumor progression (4). However, activation of the UPR pathway is a double-edged sword, as persistent or irreplaceable EnR stress can activate apoptosis (5). At present, the combined use of chemotherapy and targeted drugs that inhibit oncogenic driver gene mutations to induce persistent EnR stress has become a promising cancer treatment strategy (6, 7). Although the UPR represents an ideal target for cancer treatment, it is also vital for cancer cells to develop chemotherapeutic drug resistance. Recent studies have shown that cancer cells with constitutive or acquired resistance to chemotherapy are resistant to EnR-stress-triggered cell death (8). Drug-resistant tumor cells often have a set of coordinated regulatory mechanisms to maintain appropriate EnR stress, promote survival, and prevent excessive stress-induced apoptosis (9). However, how is the

Breast cancer is the most prevalent type of cancer in women, and nearly 70% of cases are estrogen receptor-positive (ER+). Tamoxifen (TAM) has been universally used for the treatment of ER+ breast cancer. However, the inevitable emergence of resistance to TAM obstructs the successful treatment of this

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## HBXIP sustains tamoxifen resistance in ER+ breast cancer

UPR regulated in tamoxifen-resistant (TamR) ER+ breast cancer is not fully understood.

Hepatitis B virus X protein-binding protein (HBXIP), which is highly expressed in a variety of cancers, plays a vital role in tumor progression. As a vital oncogenic transcriptional coactivator, HBXIP modulates many crucial transcription factors such as c-Myc, p53, FAS, Sp1, E2F1, STAT4, and c-Myb (10), to regulate multiple signaling pathways, such as the PI3K/Akt, Hippo/YAP, MDM2/p53, and ERK1/2/NF- $\kappa$ B, promoting malignant tumor phenotype progression (11–13). Recently, our group found that HBXIP competes with NF-E2-related factor-2 (Nrf2) to bind with Kelch-like ECH associated protein 1 (Keap1), activate the Nrf2-ARE pathway, and enhance breast cancer cell antioxidant stress coping ability (14). However, it is unclear whether HBXIP plays a role in TamR ER+ breast cancer cells.

Here, we found that expression of HBXIP was significantly elevated in TamR breast cancer cells. HBXIP deficiency resensitizes TamR cells to tamoxifen (TAM) and attenuates the growth of TamR breast cancer *in vitro* and *in vivo*. Mechanistically, HBXIP inactivates the UPR to maintain EnR homeostasis in two ways: 1) HBXIP binds to the cytoplasmic region linker domain (LD) of IRE1 $\alpha$  to stabilize the IRE1 $\alpha$ -Bip complex and inactivate the IRE1 $\alpha$  pathway. 2) HBXIP reduces cellular ROS levels by activating the Nrf2-ARE pathway to inhibit the PERK and ATF6 pathways. Moreover, clinical analysis and Kaplan–Meier plotter investigations found that high expression of HBXIP was associated with high recurrence and poor survival rates in tamoxifen monotherapy ER+ breast cancer patients. Our findings provide evidence of the molecular mechanism that establishes HBXIP as a valuable regulatory element of TamR breast cancer treatment.

## Results

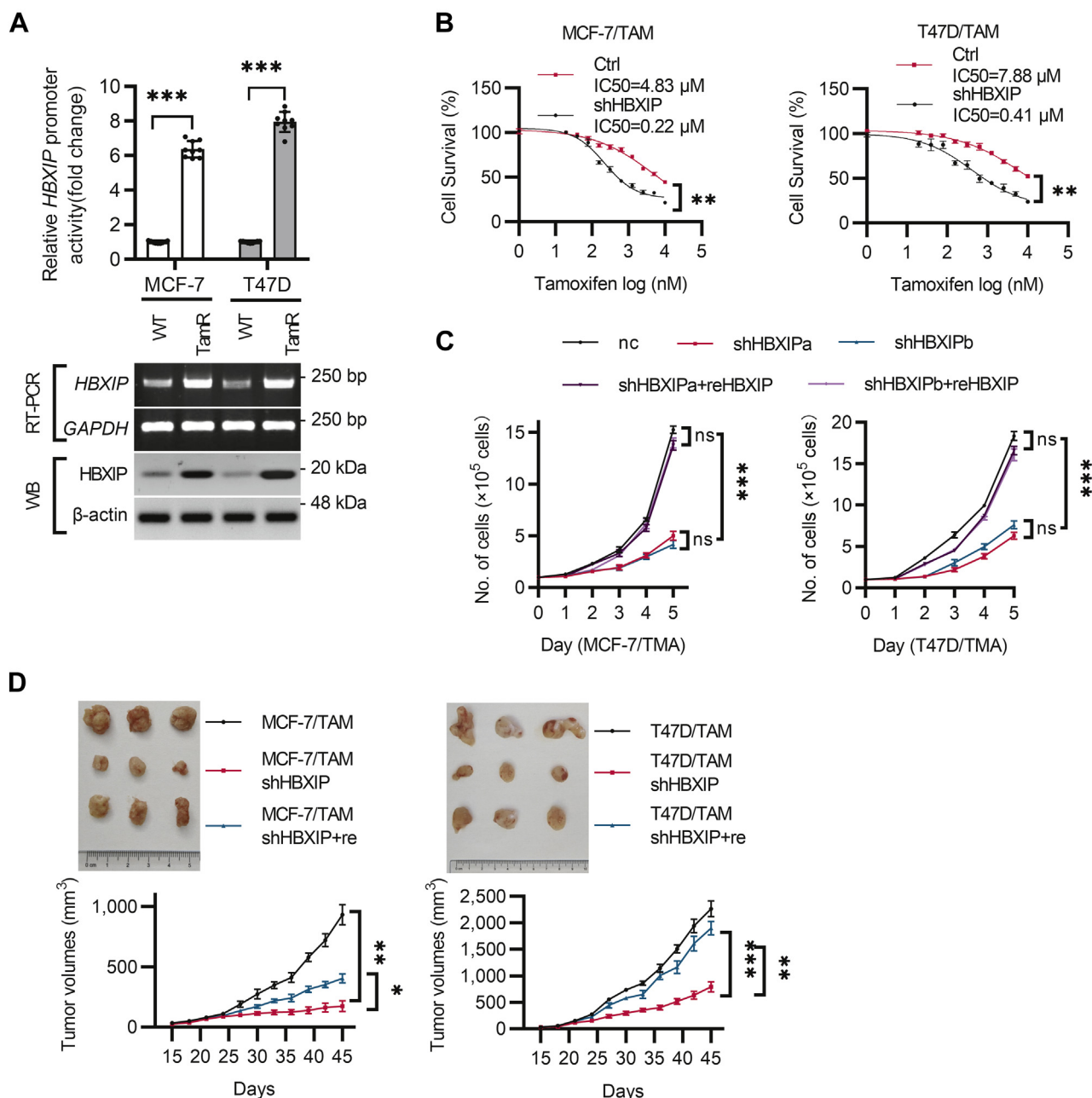
### HBXIP induces TAM resistance in ER+ breast cancer cells

TAM is a first-line drug for endocrine therapy in ER+ breast cancer. The emergence of TAM resistance and tumor recurrence is the foremost challenge in clinical treatment. To evaluate whether HBXIP is linked to TAM resistance in breast cancer, we established the TamR breast cancer cell lines MCF-7/TAM and T47D/TAM by screening with increasing doses of TAM for 8 months. Compared with the parent cells, the TAM IC<sub>50</sub> values of MCF-7/TAM and T47D/TAM cells were increased by 23.44- and 23.97-fold, respectively (Figs. S1–S6). HBXIP promoter activity and mRNA and protein expression levels were substantially increased in TamR cells compared with their parent cells (Fig. 1A). HBXIP deficiency markedly decreased TAM IC<sub>50</sub> values (Fig. 1B) and inhibited the growth of TamR cells (Fig. 1C). Reconstituting HBXIP expression in HBXIP-deficient TamR cells restored drug resistance and growth ability (Fig. 1, B and C). In addition, an *in vitro* soft agar colony formation assay revealed that the colony formation abilities of HBXIP-deficient TamR cancer cells were significantly inhibited by 1  $\mu$ M TAM. Reconstitution of HBXIP expression reinstated the colony formation abilities of HBXIP-deficient TamR cells in TMA soft agar (Fig. S2). These results

are consistent with previous studies showing that overexpression of HBXIP induced TAM resistance and enhanced the malignant growth ability of native MCF-7 and T47D cells in the presence of TAM *in vitro* and *in vivo* (15). Moreover, a xenograft tumor model was established to verify the roles of HBXIP in TamR breast cancer cells *in vivo*. The growth curve of xenograft tumors showed that weekly TAM administration (2 mg/kg, oral gavage) significantly inhibited the growth of HBXIP-deficient MCF-7/TAM and T47D/TAM xenograft tumors compared with the MCF-7/TAM and T47D/TAM groups (Fig. 1D). These results suggest that the reduction in HBXIP expression resensitized the xenografts to TAM treatment. Reconstituting HBXIP expression restored TAM resistance and xenograft growth abilities of HBXIP-deficient TamR cancer cells *in vivo* in response to TAM treatment (Fig. 1D). These findings demonstrate that enhanced HBXIP expression is involved in maintaining TAM resistance in ER+ breast cancer.

### HBXIP deficiency induces ER dilation and activation of IRE1 $\alpha$ -Xbp1 pathways

To explore the mechanisms involved in HBXIP-mediated drug resistance of TamR breast cancer cells, we examined the ultrastructural variations between HBXIP-deficient TamR cells and control cells using transmission electron microscopy. After treatment with 1  $\mu$ M TAM or 5  $\mu$ g/ml EnR stressor tunicamycin (Tm) for 48 h, apparent dilation of EnR was observed in stable HBXIP knockdown MCF-7/TAM and T47D/TAM cells (Fig. 2A). Dilation of the EnR is a hallmark feature of EnR stress. Studies have reported that EnR stress plays a critical role in generating drug resistance (16) by inducing the UPR as an adaptive response for maintaining EnR homeostasis (17, 18). Additionally, endoplasmic-reticulum-associated degradation (ERAD) is an integral part of the UPR because the expression of many ERAD genes is controlled by the UPR (19). To evaluate the roles of HBXIP in UPR pathway activation, we first evaluated whether HBXIP contributes to ERAD by evaluating the protein stability of the ERAD reporter CD3 $\delta$ -YFP (20), which was transiently expressed in stable HBXIP-deficient TamR cells and control cells. After 48 h of TAM treatment, there was increased CD3 $\delta$ -YFP accumulation in HBXIP-deficient cells than in control TamR cells (Fig. 2B). Reconstitution of HBXIP expression led to decreased CD3 $\delta$ -YFP levels in HBXIP-deficient TamR cells (Fig. 2B). These results indicate that HBXIP deficiency induces UPR activation and enhances ERAD in response to TAM treatment. Next, the UPR element (UPRE) reporter assay was conducted in TamR breast cancer cells and MEFs by employing the UPRE-Luc system (21). Knockdown of HBXIP expression in TamR cancer cells resulted in a significant increase in UPRE reporter activity during TAM treatment. Reconstitution of HBXIP expression markedly attenuated UPRE reporter activity (Fig. 2C). Enhancing the expression of HBXIP in native MCF-7 and T47D breast cancer cells also resulted in a significant decrease in UPRE reporter activity during TAM treatment (Fig. S3). Similar results were noted in HBXIP knockout MEFs.

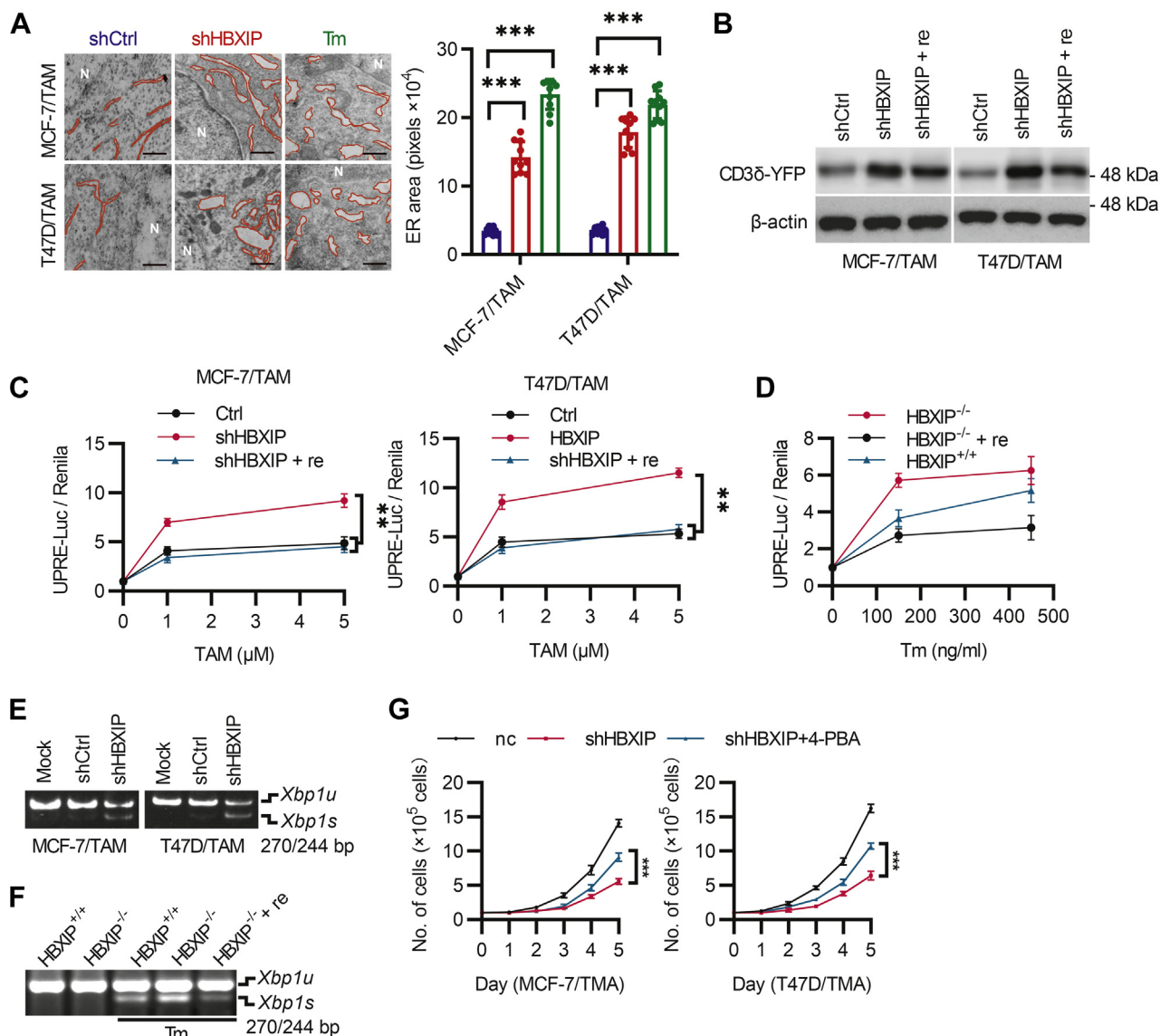


**Figure 1. HBXIP induces TAM resistance of ER+ breast cancer cells.** *A*, promoter activity assay, RT-PCR, and immunoblotting analysis of HBXIP transcription activity (upper panel); mRNA expression and protein expression (lower panel) in TamR cells and their parent cells. *B*, the repressive properties of increasing dose of TAM on TamR breast cancer cells and HBXIP deficient cells. The IC50 values of TAM were calculated. *C*, the growth capabilities of stable HBXIP knockdown (by two shRNAs of shHBXIPa and shHBXIPb) TamR cells, stable reconstituted HBXIP TamR cells, and control cells. *D*, photographs of dissected tumors from nude mice are shown (upper panel). The average volumes of tumors from nude mice transplanted with the indicated stable HBXIP knockdown and reconstituted TamR cells are shown (lower panel). The error bars indicate the  $\pm$ SD values as assessed by Student's *t* test. All experiments were performed at least three times. HBXIP, hepatitis B X-interacting protein; TAM, tamoxifen; TamR, tamoxifen-resistant.

UPRE reporter activity was considerably enhanced in HBXIP knockout MEFs, but weakened in HBXIP rescued MEFs after the indicated Tm treatment for 6 h (Fig. 2D). Moreover, the ERAD reporter and UPRE-Luc reporter assays are heavily dependent on the activation of IRE1 $\alpha$ -Xbp1 (21) which is the most conserved branch among UPR pathways. Upon activation, IRE1 $\alpha$  cleaves the *Xbp1u* mRNA into a spliced form *Xbp1s*(22). We confirmed these results using RT-PCR to examine *Xbp1* mRNA in TamR breast cancer cells and MEFs

under TAM or Tm treatment. *Xbp1s* mRNA levels were unevenly elevated in HBXIP knockdown TamR cells and HBXIP knockout MEFs, demonstrating that IRE1 $\alpha$ -Xbp1 signaling was activated (Fig. 2, E and F). Rescuing HBXIP expression in HBXIP knockout MEFs decreased *Xbp1s* mRNA level (Fig. 2F). Next, the UPR inhibitor 4-phenylbutyric acid (4-PBA) was employed to explore the UPR inhibitory role of HBXIP on TAM resistance in breast cancer cells. As shown in Figure 2G and Fig. S4, 4-PBA treatment reversed HBXIP-deficient cell

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**Figure 2. HBXIP deficiency induces ER dilation and activation of IRE1 $\alpha$ -Xbp1 pathways.** *A*, transmission electron microscopy in stable HBXIP knock-down TamR cells and control cells after treatment with 1  $\mu$ M TAM or 5  $\mu$ g/ml Tm. The dilated ER is marked by red lines. N, nucleus; scale bars, 2  $\mu$ m. The area (pixels/ER) was quantified by software NIH ImageJ. *B*, transiently expressed CD3 $\delta$ -YFP in TamR breast cancer cells that were stably knockdown and/or reconstitution of HBXIP. Immunoblot analyzed the protein levels of CD3 $\delta$ -YFP at 48 h post transfection under 1  $\mu$ M TAM treatment. *C*, the stable HBXIP knockdown TamR cells and control cells were cotransfected with the UPR-luciferase reporter system and/or pCMV-HBXIP vector as indicated. After 2 days of transfection, cells were treated with 1 and 5  $\mu$ M TAM for 48 h, and the luciferase activity was determined. *D*, the wild-type MEFs and HBXIP knockout MEFs were cotransfected with a UPR-luciferase reporter system and/or pCMV-HBXIP vector as indicated. After 48 h of transfection, the indicated MEFs were treated with 150 and 450 ng/ml Tm for 6 h, and the luciferase activity was measured. *E*, the RT-PCR analysis expression of *Xbp1u* and *Xbp1s* mRNA in stable HBXIP knockdown TamR cells and control cells and HBXIP expression reconstituted cells after treatment with 1  $\mu$ M TAM. *F*, expression of *Xbp1u* mRNA and *Xbp1s* mRNA in MEFs, HBXIP knockout and HBXIP expression reconstituted MEFs after treatment with 150 ng/ml Tm. *G*, the growth capabilities of native TamR cells and stable HBXIP knockdown TamR cells treated with 1 mM 4-PBA (or vehicle). The error bars indicate the  $\pm$ SD values as assessed by Student's *t* test. All experiments were performed at least three times. HBXIP, hepatitis B X-interacting protein; IRE1 $\alpha$ , inositol-requiring enzyme 1 $\alpha$ .

growth and colony formation abilities in the presence of TAM. Collectively, these results suggested that HBXIP is a negative UPR regulator and that HBXIP deficiency in TamR cancer cells, in combination with TAM treatment, promotes IRE1 $\alpha$ -Xbp1 pathway activation.

### HBXIP deficiency stimulates three arms of the UPR in cancer cells

The other two arms of the UPR, the ATF6 $\alpha$  and PERK pathways, also play critical roles in regulating EnR stress.

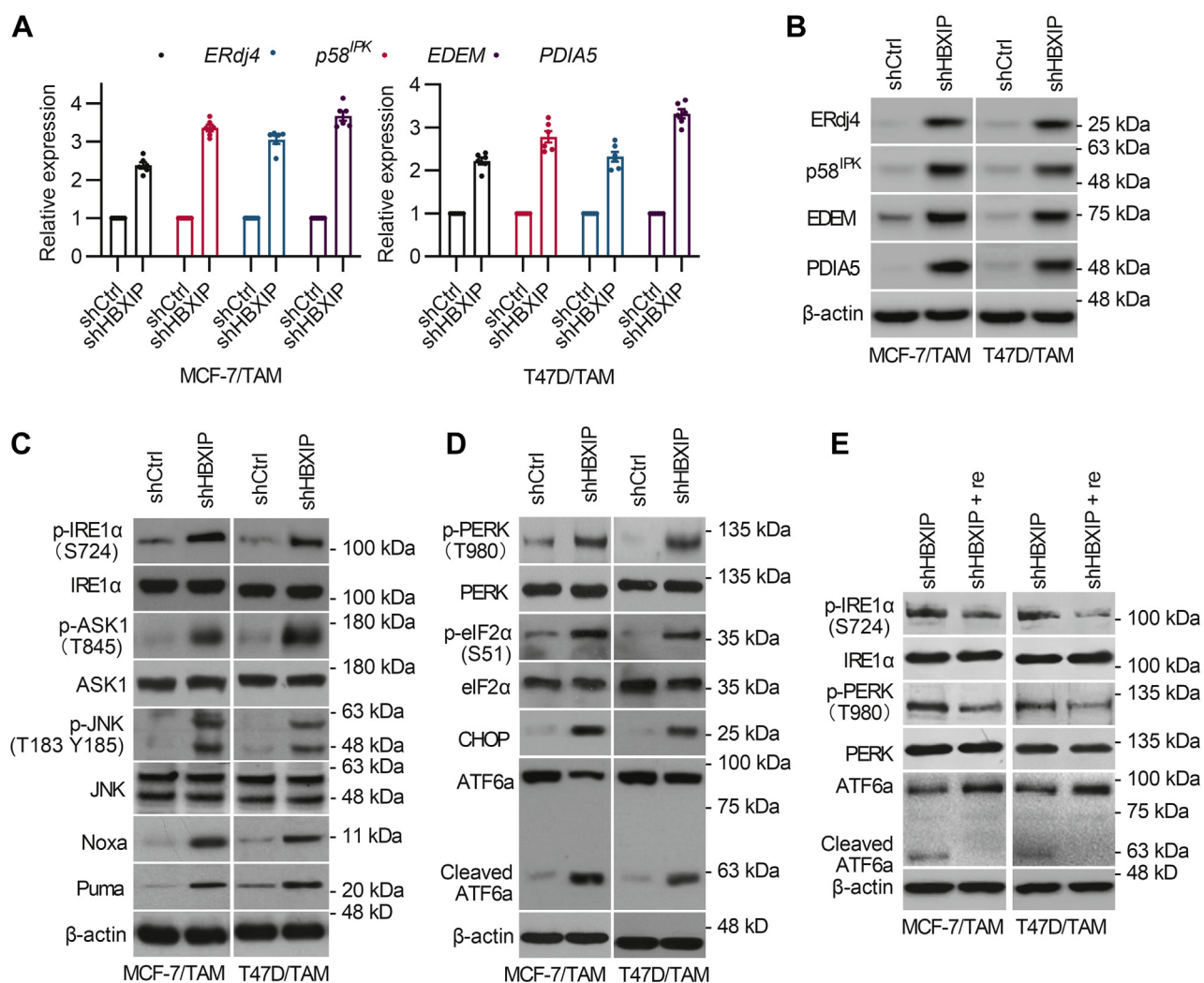
During EnR stress, ATF6 $\alpha$  converts an active transcription factor (TF) after cleavage by S1P/S2P proteases (22). PERK directly phosphorylates the TF eIF2 $\alpha$  and induces expression of the TF ATF4 and several UPR factors such as CHOP (22). We further examined whether downstream effectors of the IRE1 $\alpha$ -Xbp1 pathway were activated in HBXIP-deficient TamR breast cancer cells in response to TAM treatment. qPCR and Western blot analysis revealed that mRNA and protein expression levels of the Xbp1s target factors ERdj4, p58IPK, EDEM, and PDIA6 (23) were significantly upregulated in HBXIP-deficient TamR breast cancer cells after treatment

with TAM (Fig. 3, A and B). Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase (MAPK) family, which activates c-Jun N-terminal kinase and p38 in response to EnR stress (24). We found that silencing HBXIP in TamR cells triggered the phosphorylation of IRE1 $\alpha$ , ASK1, and JNK, which in turn induced enhanced expression of the proapoptotic factors Puma and Noxa in response to TAM treatment (Fig. 3C). Furthermore, HBXIP deficiency also induced the elevation of PERK and eIF2 $\alpha$  phosphorylation and CHOP and cleaved ATF6 $\alpha$  expression (Fig. 3D). These results indicate that HBXIP deficiency activates the PERK and ATF6 $\alpha$  pathways in TamR breast cancer cells treated with TAM. In HBXIP expression rescue assays, activation of the UPR pathways IRE1 $\alpha$ , PERK, and ATF6 $\alpha$  was attenuated by the reconstitution of HBXIP expression in HBXIP-deficient TamR breast cancer cells under TAM treatment conditions (Fig. 3E).

Collectively, the results suggest that HBXIP upregulation in TamR cancer cells mitigates the TAM-induced UPR.

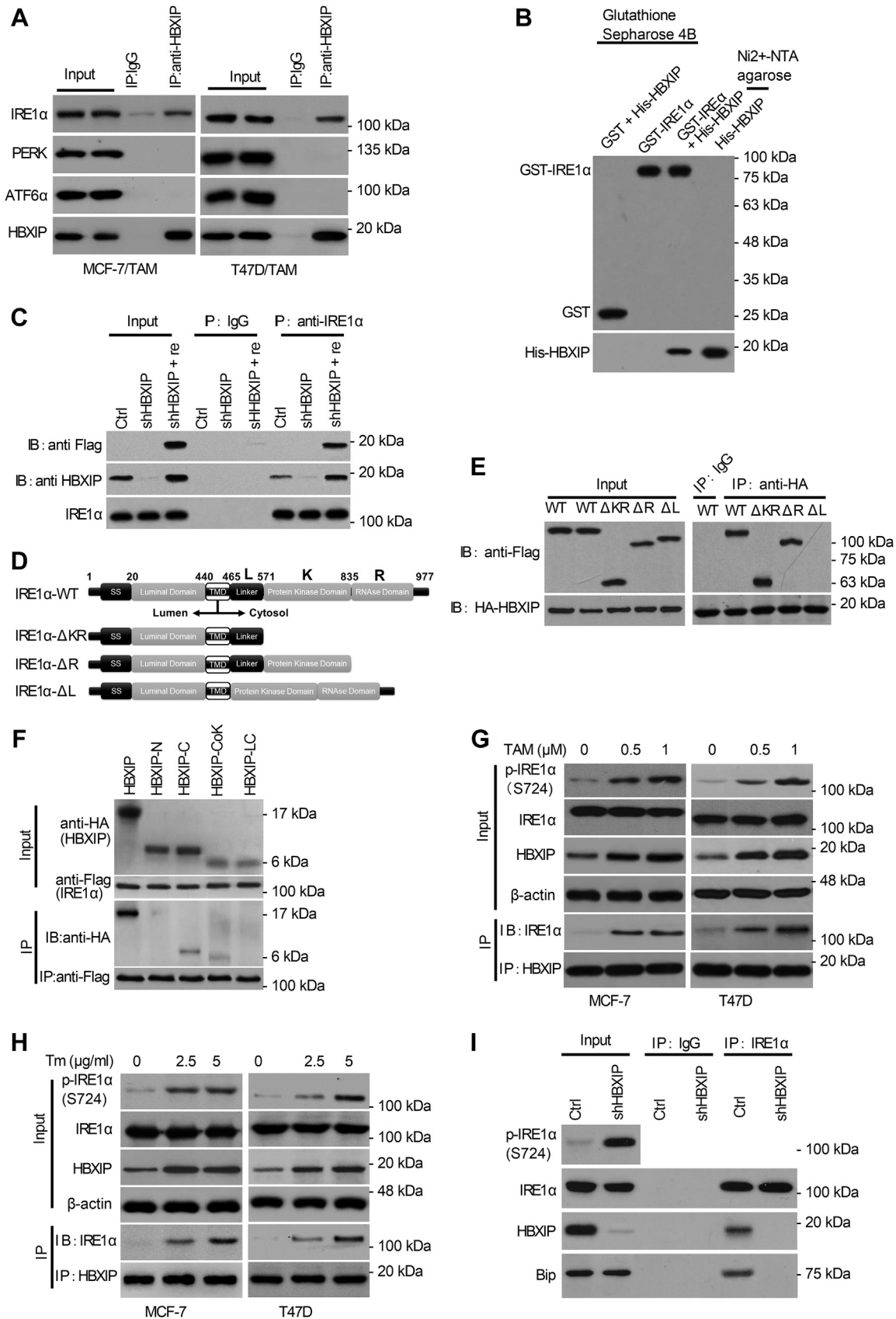
**HBXIP inactivates IRE1 $\alpha$  by binding to the cytosolic linker domain of IRE1 $\alpha$**

To clarify the intrinsic mechanism by which HBXIP inactivates the UPR in TamR breast cancer cells, we analyzed the interactions of HBXIP with IRE1 $\alpha$ , PERK, or ATF6 $\alpha$  proteins in TamR breast cancer cells by coimmunoprecipitation. An HBXIP-specific antibody was used to immunoprecipitate HBXIP in the indicated whole-cell extracts, and immunoblotting experiments detected coprecipitated IRE1 $\alpha$  but not PERK or ATF6 $\alpha$  (Fig. 4A). Because HBXIP primarily exists in cytoplasmic breast cancer cells (14), the binding site is likely located in the cytoplasmic region of IRE1 $\alpha$ . Therefore, the



**Figure 3. HBXIP deficiency stimulates three arms of the UPR in cancer cells.** A, qPCR quantified the expression of mRNAs of UPR-associated genes (*ERdj4*, *p58<sup>IPK</sup>*, *EDEM*, and *PDIA6*) in stable HBXIP knockdown TamR breast cancer cells and control cells treated with 1  $\mu$ M TAM for 48 h. B, immunoblot examined the expression of ERdj4, p58<sup>IPK</sup>, EDEM, and PDIA6 in stable HBXIP knockdown TamR breast cancer cells and control cells treated with 1  $\mu$ M TAM for 48 h. C, the activation of IRE1 $\alpha$  pathways in stable HBXIP knockdown and control TamR cancer cells post 1  $\mu$ M TAM treatment for 48 h. D, the activation of PERK pathway and ATF6 $\alpha$  in stable HBXIP knockdown and control TamR cancer cells at 48 h after 1  $\mu$ M TAM treatment. E, the activation states of IRE1 $\alpha$ , PERK, and ATF6 $\alpha$  in stable HBXIP knockdown and HBXIP reconstituted TamR breast cancer cells after 1  $\mu$ M TAM treatment for 48 h. The error bars indicate the  $\pm$ SD values as assessed by Student's *t* test. All experiments were performed at least three times. HBXIP, hepatitis B X-interacting protein; TAM, tamoxifen; TamR, tamoxifen-resistant.

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**Figure 4. HBXIP inactivates IRE1 $\alpha$  by binding with the linker domain of IRE1 $\alpha$ .** *A*, immunoprecipitation of endogenous HBXIP in TamR breast cancer cells and the coprecipitate was analyzed by immunoblotting against IRE1 $\alpha$ , PERK, and ATF6 $\alpha$ . *B*, the interaction between HBXIP and IRE1 $\alpha$  was determined by GST pull-down assays using GST-IRE1 $\alpha$  cyto (465–977 AA) and His-HBXIP fusion protein. *C*, immunoprecipitation of endogenous IRE1 $\alpha$  in HBXIP-deficient and reconstitution TamR breast cancer cells and the coprecipitates were analyzed by anti-Flag and anti-HBXIP Western blotting. *D*, schematic of the IRE1 $\alpha$

cDNA fragment coding for cytoplasmic region 465 to 977 AA of IRE1 $\alpha$  was cloned in the pGEX-4T1 vector. The subsequent GST-pulldown experiment verified that recombinant GST-IRE1 $\alpha$ cyto (cytoplasmic region) directly binds to His-HBXIP *in vitro* (Fig. 4B). Moreover, IRE1 $\alpha$  was immunoprecipitated in control MCF-7/TAM cells, HBXIP-deficient MCF-7/TAM cells with HBXIP expression reconstituted, and coprecipitated HBXIP was detected in control and HBXIP reconstituted cells using an anti-HBXIP antibody. Furthermore, the flag-tagged HBXIP was detected in the coprecipitate of the HBXIP-reconstituted group, indicating the specificity of HBXIP-IRE1 $\alpha$  binding (Fig. 4C). Next, we determined the domain of the IRE1 $\alpha$  protein responsible for binding to HBXIP. Based on known domains of the IRE1 $\alpha$  protein (25), we constructed a series of specific domain deletion mutants with flag tags (Fig. 4D). Coimmunoprecipitation assays were performed in HEK293 cells transiently coexpressing HA-HBXIP and flag-tagged IRE1 $\alpha$  mutants. In addition to the IRE1 $\alpha$  fragment lacking the linker domain (LD), IRE1 $\alpha$  mutants lacking the ribonuclease (RNase) domain (RD) or kinase and RNase domain (KRD) interacted with HBXIP (Fig. 4E). These results indicate that the LD domain of IRE1 $\alpha$  is responsible for the interaction between IRE1 $\alpha$  and HBXIP. The 91 C-terminal amino acids (83–173) are well conserved among species (Table S3) and contain several conserved functional regions, such as the CoRNR-like domain, KBS motif, and Leu zipper domain (14). We co-overexpressed IRE1 $\alpha$  and various functional domains containing HBXIP truncated mutations that were previously constructed (14) in HEK293 cells. Coimmunoprecipitation assays revealed that HBXIP-C and HBXIP-CoK, containing the CoRNR and KBS motifs, could bind to the IRE1 $\alpha$  protein (Fig. 4F). The tertiary structure of HBXIP (26) has an  $\alpha$  helix (86–95) and a  $\beta$  sheet formed by CoRNR-like box IVGVL (100–104) that form a hydrophobic pocket. We speculated that this hydrophobic pocket is responsible for binding to the nonpolar amino-acid-rich linker domain of IRE1 $\alpha$ . Accordingly, we designed an HBXIP<sup>mut</sup> mutant in which the "IVGVL" (100–104) motif was replaced with "GAGAG." Coimmunoprecipitation determined that HBXIP<sup>mut</sup> could not bind IRE1 $\alpha$  in cells (Fig. S5), suggesting that the "IVGVL (100–104)" motif of HBXIP was responsible for interacting with the linker domain of IER1 $\alpha$ .

Treating MCF-7 and T47D breast cancer cells with 0.5 and 1  $\mu$ M TAM for 12 h induced dose-dependent elevation of the phosphorylation level of IRE1 $\alpha$  (Fig. 4G). Coimmunoprecipitation determined that levels of the HBXIP-IRE1 $\alpha$  complex were elevated in a dose-dependent manner in response to with the dose of TAM (Fig. 4G). Similarly, treatment of MCF-7 and

T47D cells with increasing doses of Tm as indicated for 12 h activated the IRE1 $\alpha$  pathway in a dose-dependent manner (Fig. 4H). Coimmunoprecipitation assays revealed that levels of the intracellular HBXIP-IRE1 $\alpha$  complex were elevated as the Tm dose increased in TamR breast cancer cells (Fig. 4H). TAM or Tm, which can induce EnR stress, promotes formation of the IRE1 $\alpha$ -HBXIP complex. Therefore, we speculated that the interaction between HBXIP and IRE1 $\alpha$  helps ER+ breast cancer cells inhibit excessive EnR stress and promotes cell survival and drug resistance.

IRE1 $\alpha$  activity is primarily regulated by the EnR-resident chaperone-binding immunoglobulin protein (BiP), which regulates IRE1 $\alpha$  activity through combination with or separation from IRE1 $\alpha$  (27). We examined whether HBXIP influences the interaction between IRE1 $\alpha$  and BiP in MCF-7/TAM cells. Coimmunoprecipitation assays showed that HBXIP forms a heterologous trimer with IRE1 $\alpha$  and BiP in MCF-7/TAM cells. HBXIP deficiency resulted in the release of BiP protein from IRE1 $\alpha$  and elevation of the phosphorylation level of IRE1 $\alpha$  in response to TAM treatment (Fig. 4I). These results suggest that HBXIP stabilizes the IRE1 $\alpha$ -BiP complex by forming a heterologous trimer that regulates the inactivation of IRE1 $\alpha$  in TamR breast cancer cells during TAM incubation.

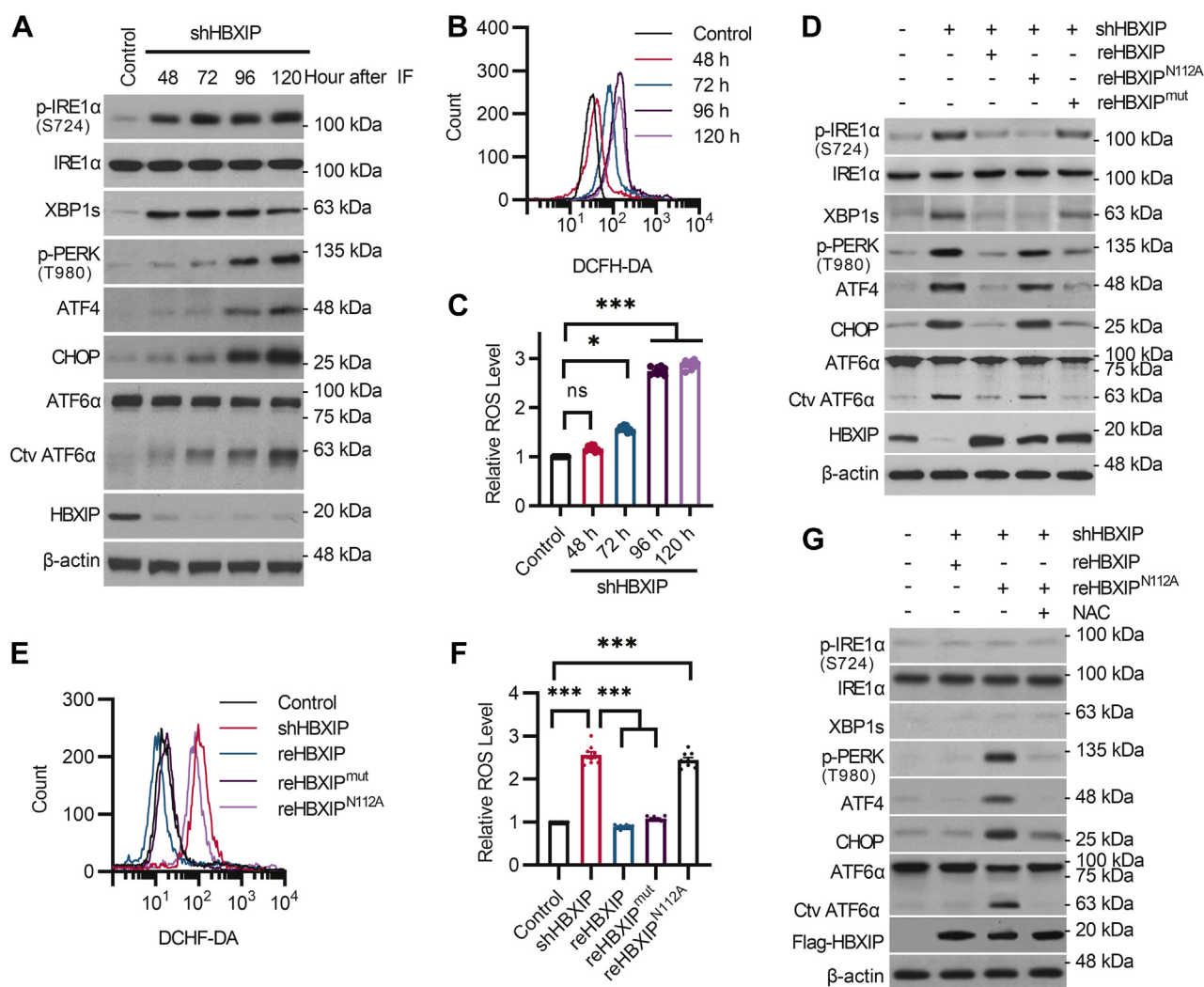
### HBXIP regulates activation of the PERK and ATF6 $\alpha$ pathways by modulating intracellular ROS

Next, the activation time course of the three arms of the UPR was investigated by transiently knocking down the expression of HBXIP using specific shRNA expressed by lentivirus pLKO-HBXIP (14). Under TAM treatment, phosphorylation levels of IRE1 $\alpha$  and protein levels of Xbp1s were significantly increased 48 to 120 h after lentivirus infection. Phosphorylation levels of PERK and protein levels of ATF4 and CHOP did not significantly increase until 96 h after infection, and cleaved ATF6 $\alpha$  levels significantly increased 72 h postinfection (Fig. 5A). HBXIP deficiency has a programmatic regulatory effect on activating the three UPR pathways in TamR breast cancer cells.

Studies have confirmed that high cellular ROS levels induce UPR activation (28–30), and our previous studies found that HBXIP diminishes cellular ROS by competitively binding to the oxidative stress sensor KEAP1 to activate the Nrf2-ARE pathway (14). Therefore, intracellular ROS levels were analyzed after lentivirus pLKO-HBXIP infection, and the results of DCFH-DA flow cytometry demonstrated that intracellular ROS level was markedly increased 72 h after lentivirus

constructs. E, various IRE1 $\alpha$  mutants constructs shown in D and HA-HBXIP were transiently expressed in HEK293 cells, proteins were immunoprecipitated with anti-HA agarose, and the precipitates were analyzed by anti-Flag Western blotting. F, various HA-tagged HBXIP mutants and Flag-IRE1 $\alpha$  were transiently expressed in HEK293 cells, proteins were immunoprecipitated with anti-Flag agarose, and the precipitates were analyzed by anti-HA Western blotting. G, MCF-7 and T47D cells were treated with dose increasing TAM as indicated for 12 h and subjected to immunoblotting to determine the activation of IRE1 $\alpha$  and HBXIP expression (upper panel). Aliquots of cellular protein extracts were subjected to immunoprecipitation with an anti-HBXIP antibody. The precipitates were analyzed by immunoblotting against IRE1 $\alpha$ . Western blotting for HBXIP was performed as a loading control (lower panel). H, MCF-7 and T47D cells were treated with dose increasing Tm as indicated for 12 h and subjected to immunoblotting to determine the activation of IRE1 $\alpha$  and HBXIP expression (upper panel). Aliquots of cellular protein extracts were subjected to immunoprecipitation with an anti-HBXIP antibody. The precipitates were analyzed by immunoblotting against IRE1 $\alpha$ . Western blotting for HBXIP was performed as a loading control (lower panel). I, immunoprecipitation of endogenous HBXIP in stable HBXIP knockdown MCF-7/TAM cells and control cells and the precipitates were analyzed by immunoblotting against HBXIP and BiP. All experiments were performed at least three times. HBXIP, hepatitis B X-interacting protein; TAM, tamoxifen; TamR, tamoxifen-resistant.

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**Figure 5. HBXIP regulates the activation of PERK and ATF6 $\alpha$  pathways by modulating intracellular ROS.** A, immunoblot analysis of the activation status of the three arms of the UPR in stable HBXIP knockdown MCF-7/TAM cells after 1  $\mu$ M TAM treatment for increasing time as indicated. B and C, a flow cytometry assay using DCFH-DA showed the intracellular ROS levels in TAM treatment cells as shown in A. D, Western blot analysis of the activation status of the three branches of the UPR in HBXIP deficient MCF-7/TAM cells reconstituted with HBXIP N112A mutant after treatment with 1  $\mu$ M TAM. E and F, a flow cytometry assay using DCFH-DA showed the intracellular ROS levels in TAM treatment cells as shown in D. G, immunoblot analysis of the inactivation effect of NAC treatment on the three arms of UPR in HBXIP N112A mutants reconstituted MCF-7/TAM HBXIP deficient cells. The error bars indicate the  $\pm$ SD values as assessed by Student's *t* test. All experiments were performed at least three times. HBXIP, hepatitis B X-interacting protein; TAM, tamoxifen.

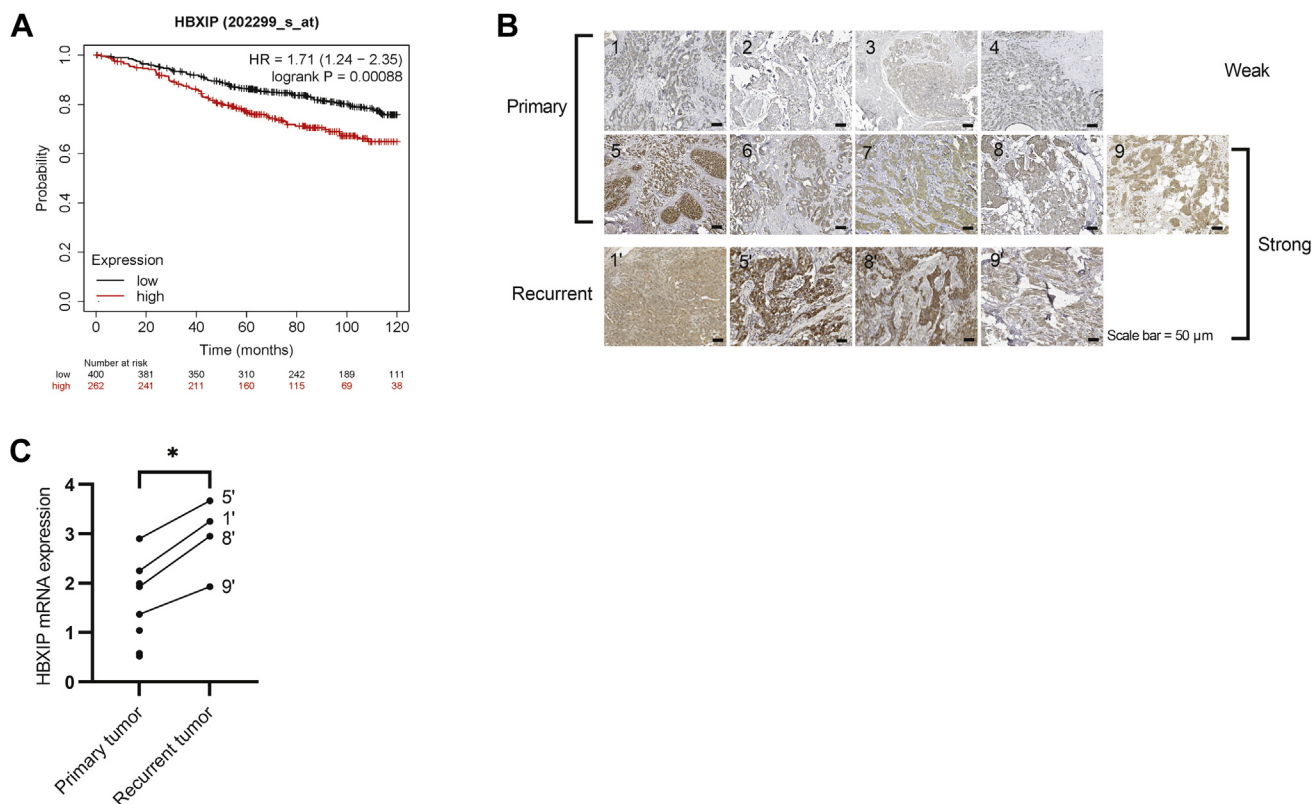
infection and dramatically elevated 96 h postinfection (Fig. 5, B and C). These changes in ROS levels are consistent with the time course of activation of the UPR branch pathways PERK and ATF6 $\alpha$ . To further confirm these results, we reconstituted wild-type HBXIP, HBXIP<sup>N112A</sup>, and HBXIP<sup>mut</sup> mutants in HBXIP-deficient MCF-7/TAM cells. The HBXIP<sup>N112A</sup> mutant could not bind Keap1 and lost its anti-ROS function (14), but it did bind to the LD domain of IRE1 $\alpha$  (Fig. S6). Compared with the wild-type HBXIP re-expression group, reintroducing the expression of HBXIP<sup>N112A</sup> inactivated IRE1 $\alpha$  and reduced levels of Xbp1s protein, expect in the PERK and ATF6 $\alpha$  pathways. In addition, re-expressing HBXIP<sup>mut</sup> (100–104, GAGAG), which loses IRE1 $\alpha$ -binding ability, inactivated the PERK and ATF6 $\alpha$  pathways but had little effect on the IRE1 $\alpha$ -Xbp1 pathway (Fig. 5D). Assessing the variation of intracellular ROS found that the reconstruction of HBXIP<sup>N112A</sup> did not attenuate the intracellular ROS compared with the wild-type

HBXIP and HBXIP<sup>mut</sup> reconstitution groups (Fig. 5, E and F). Moreover, diminishing intracellular ROS levels in HBXIP<sup>N112A</sup>-rescued cells by the antioxidant N-acetyl cysteine (NAC) inactivated the PERK and ATF6 $\alpha$  signaling pathways (Fig. 5G). Collectively, these results indicate that enhanced HBXIP maintains EnR homeostasis in TamR breast cancer cells during TAM treatment in two ways: (1) inactivating IRE1 $\alpha$  by binding to the LD domain (Figs. 2 and 4) inhibiting PERK and ATF6 $\alpha$  pathway activation by diminishing intracellular ROS through the Keap1-Nrf2 pathway (Fig. 5).

### High expression of HBXIP is a promoting and prognostic factor in TMA monotherapy breast cancer patients with respect to recurrence

To determine whether enhanced expression of HBXIP is related to TAM resistance in breast cancer, we first assessed





**Figure 6. High expression of HBXIP was a promoting factor for TMA monotherapy breast cancer patients to develop recurrence.** *A*, relapse-free survival analysis of TAM only treated ER+ breast cancer patients by the Kaplan–Meier plotter online resource. The plot was generated according to the HBXIP expression level (logrank  $p = 0.00088$ ). *B*, representative immunohistochemistry (IHC) images of HBXIP low expression (*Weak*) and high expression (*Strong*) in nine cases of TAM monotherapy ER+ breast cancer primary and recurrent tissues. *C*, the HBXIP mRNA expression level of primary and recurrent tissues as indicated in *B* was detected by qPCR. HBXIP, hepatitis B X-interacting protein; TAM, tamoxifen.

HBXIP expression in ER+ breast cancer patients ( $n = 662$ ) who were treated with TAM but not any other anticancer therapeutics using Kaplan–Meier meta-analyses (31). A prominent association between higher HBXIP expression and poorer 10-year relapse-free survival (RFS) was observed in TAM-treated patients than in the lower HBXIP expression group (Fig. 6A). Subsequently, the clinical relevance between HBXIP expression and tumor recurrence of ER+ breast cancer was investigated. We collected nine luminal-type tissues from patients treated with TMA monotherapy in 2015 (Table S2) and examined expression of HBXIP in primary breast cancer tissues collected at the original diagnosis by IHC staining. We found that HBXIP was highly expressed in five cases and expressed at low levels in four cases of primary breast cancer tissues (Fig. 6B). All patients were followed up for 60 months. During this period, four patients developed tumor recurrence, including three patients in the HBXIP strong staining group and one patient in the HBXIP weak staining group (Fig. 6B). IHC staining also demonstrated that protein levels of HBXIP in recurrent tissues were higher than that in primary tissues (Fig. 6B). Moreover, the qPCR results were consistent with the IHC staining and showed that mRNA levels of HBXIP in the recurrent tumor were significantly higher than that in the primary carcinoma (Fig. 6C). These clinical results are consistent with those of cell experiments *in vitro* and xenograft nude mouse model assays *in vivo*, which indicate a positive

correlation between the expression of HBXIP and TAM resistance in breast cancer. In conclusion, high expression of HBXIP is one of the important factors inducing TAM resistance and ER+ breast cancer recurrence.

## Discussion

HBXIP is a newly discovered oncoprotein that acts as a transcriptional coactivator to promote cancer development (10). In the present study, HBXIP was found to be substantially upregulated in TamR ER $\alpha$ + breast cancer cells, and its deficiency increased the sensitivity of tumor cells to TAM accompanied by activation of the UPR pathway. Cellular and biochemical analyses indicated that HBXIP physically interacts with the linker domain to inactivate IRE1 $\alpha$  by inhibiting BiP dissociation from the complex. HBXIP deficiency ameliorates ERAD, activates IRE1 $\alpha$  signaling, and elevates cellular ROS levels. The increased of ROS led to phosphorylation of PERK and cleavage of ATF6 $\alpha$  in TamR cancer cells. Clinical studies demonstrated that high expression of HBXIP was a promoting and prognostic factor for breast cancer TMA monotherapy patients to develop recurrence. Our findings highlight a strategy of targeting HBXIP to eradicate TamR in breast cancer.

Many conditions interfere with oxidative protein folding processes in the endoplasmic reticulum (EnR) lumen, leading

## HBXIP sustains tamoxifen resistance in ER+ breast cancer

to a cellular condition referred to as “EnR stress” (32). Tamoxifen has been reported to elevate endoplasmic reticulum stress in breast cancer (33). Adaptation to EnR stress is mediated by engagement of the unfolded protein response (UPR). The UPR is triggered by activating three molecular sensors, IRE1 $\alpha$ , PERK, and ATF6 $\alpha$ , which transmit information regarding the protein folding status in the ER lumen to the cytosol and nucleus to increase protein-folding capacity (5). The UPR is an adaptive cellular response that evolved to regulate protein-folding homeostasis, and if the UPR fails to resolve the misfolding condition, then cells undergo apoptosis (2). Hence, the UPR is becoming an attractive target for cancer therapy. In TamR breast cancer, it has been observed that activated IRE1 $\alpha$ , PERK, and eIF2 $\alpha$  levels are significantly reduced compared with their parental counterparts (34). Activation of the UPR/EnR stress pathway confers TAM sensitivity to TamR breast cancer cells by activating p38/JNK-induced apoptosis (34). As evident from the literature, acetylsalicylic acid (aspirin) is a tamoxifen sensitizer and was shown to reverse resistance elevating UPR/EnR stress (34). The avocado-derived toxin persin also augments the proapoptotic response of drug-resistant breast cancer cells to Tam by enhancing EnR stress signaling (35). Inhibition of PDE4D using either siRNAs or pharmacologic inhibitors restored tamoxifen sensitivity by activating EnR stress. Our results are consistent with these reports. Specifically, the IRE1-JNK signaling was found to be responsive to TRIM25 during EnR stress, suggesting that the IRE1-JNK pathway is the downstream effector of HBXIP. Inhibiting expression of HBXIP, as a negative regulator of EnR stress responses, conveyed TamR breast cancer cells Tam sensitivity by enhancing the UPR/EnR stress pathway. These results demonstrate that differential EnR stress status provides an opportunity for therapeutic intervention as it may sensitize cancer cells to induce apoptosis. HBXIP inhibitors in combination with tamoxifen may offer new strategies for overcoming resistance in TamR ER+ breast cancer patients.

As a core molecule in the UPR, activation of IRE1 $\alpha$  signaling affects cell fate during the endocrine treatment of ER+ breast cancer. Its activation and inactivation must be duly regulated. IRE1 $\alpha$  operates by forming a complex signaling platform at the ER membrane through the binding of adaptor proteins, controlling activation of the c-Jun N-terminal kinase (JNK), ERK, and NF- $\kappa$ B pathways (36). IRE1 $\alpha$  activity is specifically regulated by its associated factors, including the phosphatase PTP-1B (37), ASK1-interacting protein-1 (AIP1) (38), BAX inhibitor-1 (BI-1) (39) and some members of the BCL-2 protein family (40). In addition, other studies have identified the disulfide isomerase PDIA6 as an additional EnR foldase that binds to luminal IRE1 $\alpha$  and adjusts its signaling behavior (41). Previous studies have provided important insights into how IRE1 $\alpha$  activity can be regulated. Collectively, IRE1 $\alpha$ , with its partner's complex, is termed the UPRosome. In this complex, some of the partners involved enhance functions and stability, while others reduce them. The nature of the interaction between

IRE1 $\alpha$  and its partner in the complex is dynamically regulated based on tissue specificity or the type of insults (42). IRE1 $\alpha$  activation is regulated and fine-tuned by its regulatory partner from the EnR lumen and the cytoplasmic side. At present, nearly 30 proteins have been found to bind to IRE1 $\alpha$  and regulate its activity. For instance, a cytoskeleton myosin protein, nonmuscle myosin IIB (NMIIB), interacts with IRE1 $\alpha$  and regulates its oligomerization and activation (43). The AIP1-IRE1 association acts as an apoptotic signaling transducer and enhances IRE1 dimerization and downstream JNK/XBP1 activation (38). PDIA6, an ER-resident protein disulfide isomerase, negatively regulates IRE1 by binding to its luminal domain at cysteine 148; if oxidized, IRE1 $\alpha$  is activated. PDIA6-deficient cells cause hyperresponse to EnR stress with sustained autophosphorylation of IRE1 $\alpha$  and increased XBP1s and pJNK (41). Hsp47 directly binds directly to the IRE1 EnR luminal domain with high affinity, eliminating BiP from the complex to allow IRE1 oligomerization for optimal signaling (44). Mutant Jun activation domain-binding protein-1 (JAB1) downregulates the UPR signaling pathway through tight binding with the linker domain of IRE1 $\alpha$  and selects the UPR or cell death by association and dissociation with IRE1 $\alpha$ . Fortilin interacts with the cytoplasmic domain of IRE1, inhibits both kinase and RNase activities, and protects cells from apoptotic cell death (45). The Sec61 translocon forms a heterooligomeric complex with IRE1 $\alpha$  and regulates its oligomerization, activation, and inactivation. Loss of the IRE1 $\alpha$ -Sec61 translocon interaction and severe EnR stress conditions cause continuous IRE1 $\alpha$  activation and extended cleavage of XBP1u mRNA (46). In the present study, we found that enhanced levels of HBXIP bind to the linker domain of IRE1 $\alpha$  and form a heterotrimer with Bip, which inactivates IRE1 $\alpha$  in TamR breast cancer cells in response to Tam treatment. As a sensor of endoplasmic reticulum stress, the enhancement and attenuation of IRE1 $\alpha$  activity have important effects on the survival or apoptosis of tumor cells. It is unclear whether there is cross talk between the IRE1 $\alpha$ -JNK pathway and Keap1/Nrf2 pathway signaling, warranting further investigation in future work. Therefore, an in-depth study of the regulatory mechanism of IRE1 is of great value for the generation and treatment of drug resistance in ER $\alpha$ + breast cancer.

ROS play a crucial role in tumor progression and drug resistance. Various forms of ROS, primarily produced by mitochondria and oxidative protein folding (OPF), disturb EnR protein folding and induce EnR stress (47), which stimulates the UPR to cause apoptosis (28). Redox regulates EnR signaling transducers, such as BiP, IRE1 $\alpha$ , ATF6 $\alpha$ , and PERK, which are involved in UPR activation (28, 48). In instances where EnR stress levels surpass the UPRs folding ability, the pathway will trigger apoptosis. PERK induces apoptosis by sustaining levels of CHOP under ROS-mediated EnR stress (49). ATF6 regulates cell survival and tumor growth *via* downregulation of adaptive pathways such as mTOR (50). In addition, activation of IRE1 $\alpha$  recruits TNF receptor-associated factor 2 (TRAF2) to form a

heterocomplex and activates the downstream kinases ASK1 and JNK to induce apoptosis (51). IRE1 $\alpha$  induces apoptosis by directly interacting with the proapoptotic regulators BAX and BAK of the BCL-2 family (40). To maintain redox homeostasis of the EnR, ROS are consumed by oxidoreductases to ensure that OPF is maintained. The antioxidant response plays a pivotal role in ultimately persistent UPR-mediated cell death or survival determination (18). Cancer cells employ many factors to regulate the Nrf2-Keap1 pathway, which is a crucial pathway for protecting cells from oxidative stress and maintaining ER homeostasis. Our previous studies found that HBXIP can effectively compete with Nrf2 for binding to the KEAP1 protein and activate Nrf2-ARE signaling, thereby reducing cellular ROS levels (14). In this study, HBXIP deficiency potentially increased cellular ROS, activated PERK and ATF6 $\alpha$  signaling, and resensitized breast cancer cells to TMA treatment (Figs. 1 and 5). Coincident with our study, p62 (also known as sequestosome 1 (SQSTM1)), an adaptor protein involved in the autophagic process, can bind to KEAP1 at the Nrf2-binding site, activating Nrf2 and protecting cells against Tm-induced EnR-stress-mediated apoptosis (52). Tripartite motif-containing 25 (TRIM25), a crucial regulator of EnR stress, induces Nrf2 activation by directly targeting Keap1 to promote its ubiquitination and degradation, controls the UPR signaling pathway and ERAD, and reduces ROS levels and EnR-stress-induced hepatocarcinoma cell apoptosis (20). A better understanding of the intricate relationships between the UPR and oxidative stress may lead to the development of general pharmacologic agents for conquering drug resistance in cancer treatment.

In summary, this study reveals that HBXIP, an Nrf2 activator and ROS exterminator, binds to the with EnR stress sensor IRE1 $\alpha$  to restore cellular redox homeostasis and inactivate UPR signaling, which endows ER+ breast cancer cells with tamoxifen resistance. Therefore, targeted disruption of HBXIP might become a powerful strategy for breast cancer treatment.

## Experimental procedures

### Establishment of TamR breast cancer cells

MCF-7/TAM and T47D/TAM breast cancer cells resistant to tamoxifen were established according to the reported method (53). In brief, MCF-7 and T47D were seeded and cultured for 24 h and then changed media to 5% charcoal-stripped fetal calf serum (Sigma-Aldrich) in phenol red-free DMEM and RPMI1640 medium, respectively, and culture for 24 h. Then 0.1  $\mu$ M 4-OH tamoxifen (TAM) (Sigma-Aldrich) was added to the medium. The cells were continuously treated with gradually increasing doses of TAM for 8 months. MCF-7/TAM and T47D/TAM TamR breast cancer cells were finally obtained when cells can tolerate 1  $\mu$ M TAM.

### Xenografts

Take 6 to 8-week-old female BALB/c athymic nude mice and implant 17 $\beta$ -estrogen pellets (0.72 mg, 60 days release, innovative research in the United States) into the mice subcutaneously nape of the neck 3 days before injection before

cell inoculation. 1  $\times$  10<sup>7</sup> cells/0.2 ml of TamR, HBXIP-deficient or reconstituted cells resuspended respectively in 1:1 PBS/Matrigel (BD Biosciences) solution were injected into the mammary fat pad (MFP) of mice. Two weeks after inoculation, citric acid TAM (China Yangzijiang Company) 2 mg/kg in corn oil was administered *via* oral gavage every day. Tumor growth was regularly monitored, and size measurements were performed two times per week. After the experiment, the mice were euthanized. Tumor volume (V) is calculated by the following formula: Tumor volume (mm<sup>3</sup>) = length  $\times$  width<sup>2</sup>  $\times$  0.5. All animal studies were complied with relevant ethical regulations for animal testing and research, and all experiments conducted in the study were approved by the Institutional Animal Care and Use Committee of the Hebei University of Science and Technology. The details of other methods and key resources are described in the supporting information and Table S1.

### Statistical analysis

All experiments in this study were carried out in an individually triplicate. All data are shown as means  $\pm$  SD. Differences between groups were tested for statistical significance using the Student's *t* test or ANOVA. Statistical significance was determined at \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 by GraphPad Prism software (GraphPad Software).

### Data availability

The data supporting the findings in this study are available in the article, supporting information, or from the corresponding author upon reasonable request.

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*Supporting information*—This article contains supporting information (14, 23, 53–57).

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*Conflict of interest*—The authors declare that they have no conflicts of interest with the contents of this article.

*Abbreviations*—The abbreviations used are: ARE, antioxidant response element; ATF6 $\alpha$ , activation transcription factor 6 $\alpha$ ; HBXIP, hepatitis B X-interacting protein; Keap1, Kelch-like ECH-associated protein 1; IRE1 $\alpha$ , inositol requires enzymes 1 $\alpha$ ; NAC, N-acetyl-L-cysteine; NQO1, NAD(P)H quinone dehydrogenase 1;

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Nrf2, nuclear factor E2-related factor 2; PERK, protein kinase RNA-like endoplasmic reticulum kinase; Prdx1, peroxiredoxin 1; ROS, reactive oxygen species; Tm, tunicamycin.

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