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The ErbB3 binding Protein EBP1 regulates ErbB2 protein levels and tamoxifen sensitivity in breast cancer cells

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Abstract

The ErbB2/3 heterodimer plays a critical role in breast cancer progression and in the development of endocrine resistance. EBP1, an ErbB3 binding protein, inhibits HRG-stimulated breast cancer growth, decreases ErbB2 protein levels and contributes to tamoxifen sensitivity. We report here that ectopic expression of *EBP1* in Estrogen Receptor (ER) positive breast cancers that express ErbB2 at both high and low levels decreased ErbB2 protein levels. ErbB2 protein expression was also increased in mammary glands of *Ebp1* knock out mice. To define the mechanism of ErbB2 down regulation, we examined the effects of EBP1 on ErbB2 mRNA levels, transcription of the ErbB2 gene and ErbB2 protein stability. We found that ectopic expression of *EBP1* decreased steady state levels of endogenous ErbB2 mRNA in all cell lines tested. *EBP1* overexpression decreased the activity of an ErbB2 promoter reporter in cells which overexpress ErbB2. However, reporter activity was unchanged or increased in cells which express low endogenous levels of ErbB2. We also found that ectopic expression of *EBP1* accelerated ErbB2 protein degradation and enhanced ErbB2 ubiquitination in cells which express both low and high levels of ErbB2. Treatment with proteasome inhibitors prevented this decrease in ErbB2 protein levels. Ablation of EBP1 expression led to tamoxifen resistance that was abrogated by inhibition of ErbB2 activity. These results suggest that EBP1 inhibits expression of ErbB2 protein levels by multiple mechanisms and that EBP1's effects on tamoxifen sensitivity are mediated in part by its ability to modulate ErbB2 levels.

Keywords

EBP1; ErbB2; tamoxifen; breast cancer

INTRODUCTION

A wealth of clinical data has demonstrated the aberrant expression of ErbB family members in breast cancer [1] [2]. The ErbB2 gene is amplified in 20-30% of breast carcinomas contributing to more aggressive disease [3]. The overexpression of ErbB2 has been successfully exploited therapeutically by use of the monoclonal antibody Trastuzumab and by tyrosine kinase inhibitors. ErbB3 is also overexpressed in many breast tumors [4] and coexpression of ErbB2 and ErbB3 is significantly associated with decreased survival [5]. Oncogenic signaling through ErbB3 can abrogate the effectiveness of ErbB directed

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therapies [6]. The ErbB2/ErbB3 receptor pair forms the most potent mitogenic receptor complex *in vitro* [7] and is key to the proliferation of human breast cancer cells that express these receptors [8].

Endocrine resistance is often associated with enhanced expression of members of the ErbB receptor family, especially ErbB2. Multiple clinical studies indicate that ErbB2 expression portends a poorer prognosis with tamoxifen therapy [9]. This is important as approximately half of breast cancers that overexpress ErbB2 also express hormone receptors [10]. It has been demonstrated in both cell culture and animal models for many years that the enhanced expression of ErbB2 leads to the ability of cells to bypass normal endocrine responsiveness [11] [12].

The biological activity and expression of ErbB2 and ErbB3 are regulated by a host of interacting proteins that may be potential targets for development of new therapies. Our laboratory has been interested in proteins that regulate ErbB3, as the ErbB3 receptor has impaired tyrosine kinase activity [13, 14], necessitating its interactions with other proteins to exert its biological effects. An ErbB3 binding protein (EBP1) was isolated in our laboratory during a yeast two-hybrid screen for ErbB3 interacting proteins [15]. Overexpression of *EBP1* inhibits growth of ErbB2/3 expressing cell lines. Ectopic expression of *EBP1* promotes G2/M cell cycle arrest and cellular differentiation [16]. Overexpression of *EBP1* inhibits the transcription of reporter genes controlled by Cyclin D1, Cyclin E and c-myc promoters and the transcription of endogenous E2F1 and c-myc genes via its binding to an E2F1 consensus element [17-19]. The ability of EBP1 to repress transcription requires its interaction with histone deacetylase 2 (HDAC2), Rb and Sin3A [18, 19] [20].

Our previous work has demonstrated that ectopic expression of *EBP1* inhibits growth of MCF-7 and AU565 breast cancer cell lines in response to HRG and interferes with HRG induced growth signals, such as the activation of AKT. In addition, ErbB2 protein levels are decreased in MCF-7 and AU565 cells transfected with *EBP1* [21]. In the current study, we were interested in determining the mechanism of ErbB2 downregulation in ER+ breast cancer cells. We found that EBP1 decreased steady state levels of *ErbB2* mRNA in all cell lines tested. However, *ErbB2* promoter activity was decreased only in cells which overexpress ErbB2. EBP1 decreased ErbB2 protein stability in cells which express both high and low levels of ErbB2 via a proteasome mediated pathway. We also found that tamoxifen resistance induced by ablation of EBP1 expression was abrogated by inhibition of ErbB2 activity.

MATERIALS AND METHODS

Cell Culture

MCF-7, T47D and BT474 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in a humidified atmosphere of 5% CO₂ in air in RPMI 1640 (Biofluids, Rockville, MD) and 10% FBS (Sigma, St. Louis, MO).

Reagents

Heregulin β 1 (HRG β 1) was obtained from R & D Systems Inc. (Minneapolis, MN), EGF and 4-hydroxy-tamoxifen (OHT) from Sigma, and Geneticin (G418) from Invitrogen (Carlsbad, CA). The proteasome inhibitor ALLnL was from Sigma and MG132 from Calbiochem (San Diego, CA).

Plasmids

A full-length *EBP1* cDNA (GenBank NM006191) was generated by PCR with specific reverse and forward primers containing *EcoRI* and *BamHI* restriction sites using a pcDNA-*EBP1* vector as a template [17]. This cDNA includes all 3 possible translation initiation sites of *EBP1* and encodes the largest form of the protein. The EGFP-*EBP1* plasmid was constructed by cloning full length *EBP1* into the *BamHI/ EcoRI* sites of the EGFP-C1 vector (Clontech, Palo Alto CA) and the *EcoRI/BamHI* sites of the CMV10 vector (Sigma) respectively. The pcDNA3 ErbB2 expression plasmid was a generous gift of Dr. Yossi Yarden.

Creation of stably transfected cell lines

To establish *EBP1* overexpressing stable transfectants, subconfluent cells in 100-mm tissue culture dishes were transfected with 10 µg of EGFP, or EGFP-*EBP1* expression plasmids using Fugene-6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Cells were selected in G418 (500 µg/ml) for 4 weeks and mass cultures obtained.

To generate *EBP1* silenced cell lines, cells were seeded into 96 well plates and transduced with lentiviral particles (MOI 25) with shRNA targeted to NT 302-322 of the *PA2G4* (*EBP1*) (Genbank NM_006191.1) gene (Mission shRNA, Sigma) according to the manufacturer's protocol. Cell lines were selected in 2 µg/ml of puromycin and surviving colonies of cells expanded as mass cultures. Four independent transductions of this lentiviral construct were performed and designated Clones 1-4. The control was an *EBP1* directed lentivirus (NT 776-793) that was previously demonstrated not to change *EBP1* expression [22].

Western blot Analysis

Total cell extracts were prepared by direct lysis of cells with lysis buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM NaCl, 1% Triton X-100, 0.5 mM DTT, and 1× Complete™ protease inhibitor]. Protein concentrations were measured using a detergent compatible kit (Bio-Rad, Richmond, CA). Proteins (30 µg per well) were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted as described [15]. The *EBP1* antibody was from Upstate (Lake Placid, NY), the anti-actin and GAPDH antibodies from Sigma, the ubiquitin antibodies were from Cell Signaling (Danvers, MA). Images were quantified using IMAGE-J software (NIH). Where indicated, blots were stripped in Restore Western blot Stripping Buffer™ (Pierce, Rockford, IL) as directed by the manufacturer and reprobed.

Immunohistochemical analysis

Mammary glands (#4) were excised and fixed in 10% buffered neutral formalin. Sections of formalin-fixed, paraffin-embedded tissues were cut to 5 µm. Slides were stained with rabbit ErbB2 and *EBP1* antibodies (both from Upstate) diluted 1:100 using the standard avidin-biotin method (Vecta-Stain Kit, Vector Labs, Burlingame, CA) with Harris hematoxylin as a counterstain [23].

Cell Growth Assays

For studies assessing the effect of tamoxifen on cell growth, cells (5×10^3) were plated in 96 well plates in complete media. After a 24-hour attachment period, the medium was replaced with complete medium containing hydroxy-tamoxifen (OHT) at the indicated concentrations. Relative live cell numbers were determined at Day 4 using a Promega Proliferation Reagent (Promega, Madison, WI) as per manufacturer's instructions with absorbance being read at 490nm using a Dynex plate reader.

RNA Isolation and PCR analysis

RNA was extracted from whole cell lysates, DNase treated and converted into cDNA using an AMV reverse-transcription system (Promega) in the presence of random hexamers (Invitrogen). The cDNA was used for conventional PCR or quantitative real-time PCR (RT-qPCR) with specific gene primers as follows: **ErB2** forward 5'- gggagaatggggtctcaaa 3' or agccgcgagcaccaagt and reverse 5'- ctctccctggggtgtcaagt or ttggggcaggtaggtgagtt **GAPDH** (NM_002046): forward 5'- ccaccatggcaaatcc -3' and Reverse 5'- tcgctcctggaagatggtg. An MYIQ real-time PCR detection system and SYBR green PCR mix (BioRad) were used to carry out the real-time PCR. The relative quantitation of targeted genes was determined by the comparative $\Delta\Delta C_t$ (threshold) method using GAPDH as an internal control [24]. All data were analyzed from three independent experiments and statistical significance was validated by Student's *t*-test.

Dual Luciferase Assay

5×10^4 cells/well were transfected with 0.5 μ g of GFP or GFP-*EBP1*, 0.5 μ g of *ErbB2*-luc (a firefly luciferase reporter gene under the control of the -500 to +1 region of the *ErbB2* promoter [25] and 5ng of the pRL-TK vector (a *Renilla* luciferase reporter gene under the control of the thymidine kinase promoter) using Fugene 6. Forty-eight hours after transfection, cells were lysed and luciferase activity determined using a dual-luciferase reporter assay (Promega). The activities of *Renilla* luciferase were used to normalize any variations in transfection efficiency.

Statistical Analysis

Data were analyzed using a two-tailed Students *t*-test and a $p < 0.05$ was deemed statistically significant.

RESULTS

Modulation of EBP1 expression affects ErbB2 protein levels

Our previous published results indicated that ectopic expression of *EBP1* decreased protein levels of ErbB2 in MCF-7 (ER+) and AU565 (ER-) cells [21]. Overexpression of ErbB2 is a major problem in development of endocrine resistance in ER+ cell lines [9] and devising new ways to decrease ErbB2 levels are important for future therapeutic development. We were therefore interested in assessing the effects of EBP1 in ER+ cell lines that express ErbB2 at both high and low levels. We overexpressed *EBP1* in ER+ ErbB2 high BT474 cells and ER+, ErbB2 low T47D cells. Ectopic expression of *EBP1* decreased ErbB2 levels three fold in BT474 cells and five- fold in T47D cells as determined by densitometric analysis (Fig. 1a,b). However, the levels of ErbB2 in BT474 *EBP1* transfectants were still much greater than those observed in control T47D cells. Elimination of EBP1 expression by shRNA in T47D cells enhanced ErbB2 protein levels four fold (Fig. 1c) as we previously described for MCF-7 cells [21].

To further determine the physiological effect of Ebp1 on endogenous ErbB2 levels, we examined expression of ErbB2 in adult mammary epithelium in a knock out mouse we recently developed [26]. We found that in mammary epithelial cells, ErbB2 expression was increased in mammary tissue of *Ebp1*^{-/-} 10 week old female mice (Fig. 1d).

Steady state mRNA levels are decreased after EBP1 transfection

To determine a mechanism for the decreased protein levels of ErbB2, we first examined steady state ErbB2 mRNA levels in *EBP1* transfected and control cell lines which express high (BT474, AU565) or low (MCF-7, T47D) levels of ErbB2. The expression of the EBP1-

GFP transgene is shown in Figure 1a,b for T47D and BT474 cells lines. The expression of the FLAG-tagged EBP1 in the AU565 and MCF-7 EBP1 transfectants has previously been published [21]. We found by RT-qPCR that ectopic expression of *EBP1* led to statistically significant decreases in steady state ErbB2 mRNA levels in all cell lines tested (Fig. 2). ErbB2 mRNA levels were decreased about 50% in BT474, AU565 and MCF-7 cells and 66% in T47D cells. However, the levels of ErbB2 mRNA found in BT474 and AU565 cells stably overexpressing *EBP1* were significantly higher than those found in control T47D and MCF-7 cells. Thus, while EBP1 overexpression lowers ErbB2 mRNA levels in cells expressing high levels of ErbB2, mRNA levels do not reach those observed in cells with normally low endogenous levels.

Ectopic expression of EBP1 affects the activity of the ErbB2 promoter

We next determined if EBP1 could affect *ErbB2* transcription using an *ErbB2* promoter – luciferase reporter that encodes the 500 bp proximal promoter upstream from the transcription start site. Transient transfection of *EBP1* into COS7, BT474, and AU565 cells led to significant decreases in reporter activity (Fig.3a). However, the level of reporter activity in BT474 *EBP1* transfectants was higher than that observed in T47D controls. In contrast, the activity of the reporter was not significantly changed in T47D and significantly increased in MCF-7 cells (Fig. 3a). In *EBP1* stable BT474 transfectants, a statistically significant 58% decrease in luciferase activity was observed as compared to vector controls (Fig. 3b). No changes in reporter activity were observed in T47D stable *EBP1* transfectants (Fig. 3b).

Effects of EBP1 on ErbB2 protein stability

As ErbB2 protein levels were decreased more than mRNA in both BT474 and T47D *EBP1* stable transfectants, we next examined ErbB2 protein stability. ErbB2 protein levels have been demonstrated to be regulated by proteasome mediated degradation [27]. We examined ErbB2 levels after inhibition of protein biosynthesis with cycloheximide (20 µg/ml). We found that in BT474 cells the expression of ErbB2 protein was significantly lower at 8 hours after cycloheximide treatment as compared to vector controls (Fig. 4a). In T47D cells, ErbB2 protein levels were also lower after 8 hours of cycloheximide treatment in *EBP1* transfected cells (Fig. 4b) as compared to vector controls.

We next determined if the increased ErbB2 degradation observed after ectopic expression of *EBP1* could be rescued by proteasome inhibition. We used the proteasome inhibitors MG132 and ALLnL to stabilize ubiquitinated ErbB2 in BT474 and T47D cells in which *EBP1* had been overexpressed. As expected, ErbB2 levels in the absence of the inhibitor were decreased in both BT474 and T47D *EBP1* transfectants as compared to controls (Figs. 5a,b). ErbB2 levels were increased when EBP1 was ablated in T47D cells (Fig. 5B). Addition of ALLnL was able to overcome the decrease in ErbB2 protein observed in BT474 *EBP1* transfectants. However, MG132 was not able to restore ErbB2 levels (data not shown). This is of interest as ALLnL inhibits the activity of both the proteasome and calpain I [27]. In T47D *EBP1* transfectants both MG132 (Fig. 5b) and ALLnL (data not shown) resulted in restoration of ErbB2 levels. In EBP1 silenced cells, ErbB2 levels were slightly decreased in the presence of the proteasome inhibitor (Fig. 5b). EBP1 itself has been demonstrated to be a target of ubiquitination. Two isoforms of EBP1 have been reported: a p42 pro-apoptotic form and a p48 anti-apoptotic form. The p42 isoform appears to be preferentially degraded in cancer cells [28]. However, endogenous EBP1 levels were not increased by proteasome inhibitors in either cell line at this time point. Only the 48kD form of EBP1 was observed (Fig. 5a, b). The GFP-EBP1 fusion protein continued to be overexpressed at about the same level (data not shown).

Since ErbB2 is degraded through a mechanism that involves its polyubiquitination [29], we tested if ectopic expression of *EBP1* could elevate ubiquitination of ErbB2. Examination of whole cell lysates revealed that treatment with proteasome inhibitors increased ubiquitination as expected (Fig. 5c,d left panels). ErbB2 was next immunoprecipitated from lysates of cells treated with proteasome inhibitors for 6 hours and proteins analyzed by western blotting with anti-ubiquitin and ErbB2 antibodies. Ubiquitination of ErbB2 was enhanced by treatment with proteasome inhibitors as expected [27] in BT474 Cells (Fig. 5c, right panel). In contrast, ubiquitinated ErbB2 was not observed in the presence of MG132 in T47D GFP controls. This suggests that there is a very low steady-state level of ubiquitination of ErbB2 in control T47D cells. ErbB2 ubiquitination was increased in *EBP1* transfected cells as compared to vector controls for both cell lines (Fig. 5, C,D right panels).

EBP1 affects the response to tamoxifen through an ErbB2-mediated pathway

We first tested if ectopic expression of *EBP1* could enhance tamoxifen sensitivity. We found that sensitivity to low doses of tamoxifen was significantly enhanced in T47D *EBP1* transfectants (Fig. 6a). To determine if this enhanced sensitivity was due to a decrease in ErbB2 levels, we transiently transfected an expression plasmid for *ErbB2*. This resulted in a reversal of the effects of *EBP1* on tamoxifen sensitivity and induction of tamoxifen resistance (Fig. 6a) as previously demonstrated.

We previously showed that elimination of *EBP1* induced tamoxifen resistance in MCF-7 cells and upregulated ErbB2 expression [21]. Similarly, Fig. 1C shows that ErbB2 expression was upregulated after elimination of *EBP1* expression in T47D cells. We therefore determined if silencing of *EBP1* could also induce tamoxifen resistance in T47D cells. *EBP1* silenced cells were treated with OHT and cellular sensitivity assessed. *EBP1* knock out cells (now ER+ and ErbB2 high) were no longer sensitive to OHT (Fig. 6b). To determine if the tamoxifen resistance that occurred after *EBP1* silencing was due primarily to the increase in ErbB2 levels, we treated *EBP1* knock out T47D cells with the anti-ErbB2 antibody Herceptin in addition to OHT. We found that Herceptin restored sensitivity to tamoxifen that had been lost due to *EBP1* silencing (Fig. 6c, left panel), suggesting *EBP1*'s primary mechanism of action may be via decreases of ErbB2 levels. In contrast, Herceptin treatment had no effect on the growth or the response to tamoxifen of T47D cells transduced with a control lentivirus (Fig. 6c, right panel).

DISCUSSION

Increasing data support the clinical importance of specific ErbB heterodimers and their interacting partners in breast cancer development [2]. We have previously shown that the ErbB3 binding protein *EBP1* inhibited the growth of ErbB2/ ErbB3 expressing breast cancer cell lines in response to HRG in part by downregulating protein levels of ErbB2 [21]. We now show here that the *EBP1*-induced inhibition of ErbB2 protein was due to changes at the level of both ErbB2 transcription and protein stability.

The ability of *EBP1* to regulate ErbB2 at multiple levels was somewhat surprising. However, *EBP1* has previously been demonstrated by both biological and structural analysis to be multifunctional [30]. The crystal structure of *EBP1* [30] reveals that it has domains that may interact with DNA, RNA and protein. Indeed, many independent groups have demonstrated that *EBP1* binds DNA [31, 32], RNA [33-36] and proteins [15, 37, 38] with varying biological consequences. What controls this differential activity of *EBP1* is unknown. *EBP1* is a phosphoprotein and it is possible that specific post translational modifications may result in activation of different functions. For example, we have shown that phosphorylation of *EBP1* at Ser363 is essential for its ability to bind DNA through interactions with Sin3A [39]. Extensive data from Ye's group indicate that phosphorylation

of EBP1 at Ser 360 affects its interaction with nuclear AKT [37], caspase 3[40] and Bre1 [28].

We first found that steady state mRNA levels of ErbB2 were decreased in *EBP1* transfected cells. Possible changes in transcriptional rate were suggested by studies indicating that EBP1 decreased the activity of a luciferase reporter construct containing the ErbB2 proximal promoter in cells that overexpress ErbB2. However, the activity of this promoter construct was not decreased by ectopic expression of *EBP1* in cells with normal levels of ErbB2 expression, although mRNA levels were decreased. It is possible that a different complement of transcriptional activators exist in cells that express low levels of ErbB2 that are differentially affected by EBP1. Currently, we do not know if EBP1 inhibits the ErbB2 promoter directly, or through interactions with other proteins. EBP1 is a member of the SF 00553 protein family of DNA binding proteins, and thus may be predicted to bind directly to the ErbB2 promoter alone or in a complex of nuclear proteins. We previously showed via ChIP and EMSA analysis that EBP1 can directly bind to promoters containing E2F1 [19] or Androgen Response Elements [20, 24]. In addition, it is possible that EBP1 may interact with other transcriptional activators or repressors to decrease ErbB2 transcription. The -500 bp region of the ErbB2 promoter contains an Ap2 γ site. Proteins such as Wwox have been shown to inhibit ErbB2 transcription by interfering with Ap2 γ recruitment [41]. Whether or not EBP1 interacts with Wwox and Ap2 γ remains to be determined.

The protein levels of ErbB2 in both BT474 and T47D *EBP1* transfectants cannot be wholly accounted for by changes in mRNA levels. Thus, some additional EBP1 regulated processes may govern final protein levels. We unexpectedly found that ErbB2 protein stability was changed in *EBP1* transfectants.. EBP1 has no consensus sites for ubiquitin ligase activity. In addition, EBP1 does not bind directly to human ErbB2 ectopically expressed in murine cells [15] or endogenous ErbB2 in BT474 cells (data not shown). However, it is possible that EBP1 enhances the expression, activity or recruitment of E3 ligases such as Chip [27] or LRIG [42] that are important in ErbB2 ubiquitination and degradation. Of interest, EBP1 itself is subjected to ubiquitin mediated degradation. EBP1 protein has two forms: a p42 form which is a tumor suppressor and a p48 form that is a tumor promoter [43]. E3 ubiquitin ligase hBRE1 can promote EBP1 ubiquitination and degradation in prostate cancer cell lines[28]. However, only the p42 tumor suppressor form is affected in human prostate cancer cells. In our studies, inhibition of proteasome activity did not induce the appearance of the p42 band, nor increase expression of the p48 form.

We have previously found that knock out of EBP1 in MCF-7 cells results in tamoxifen resistance. We similarly found that inhibition of EBP1 expression renders T47D cells tamoxifen resistant. To further explore the mechanism of this inhibition, we found that blocking ErbB2 activity with Herceptin reversed the EBP1 induced tamoxifen resistance. Thus, we postulate that EBP1 may regulate tamoxifen sensitivity in part by modulating levels of ErbB2. Similarly, the transcription factor PAX2 represses ErbB2 transcription by competing with SRC-3 for binding to Estrogen response elements within an intronic region of the *ErbB2* gene. The repression of ErbB2 transcription by PAX 2 results in tamoxifen sensitivity [44].

In summary, we have found that ectopic expression of *EBP1* suppresses ErbB2 protein levels in ER+ human breast cancer cell lines that express ErbB2 at both high and low levels. EBP1 affected both transcription of *ErbB2* mRNA and ErbB2 protein levels by altering ErbB2 stability and enhancing ubiquitination. Abrogation of EBP1 protein expression rendered T47D cells tamoxifen resistant and this resistance was mediated via the ErbB2 pathway. Thus, restoration of EBP1 or EBP1 signaling pathways may provide a new alternative in hormone refractory breast cancer.

Acknowledgments

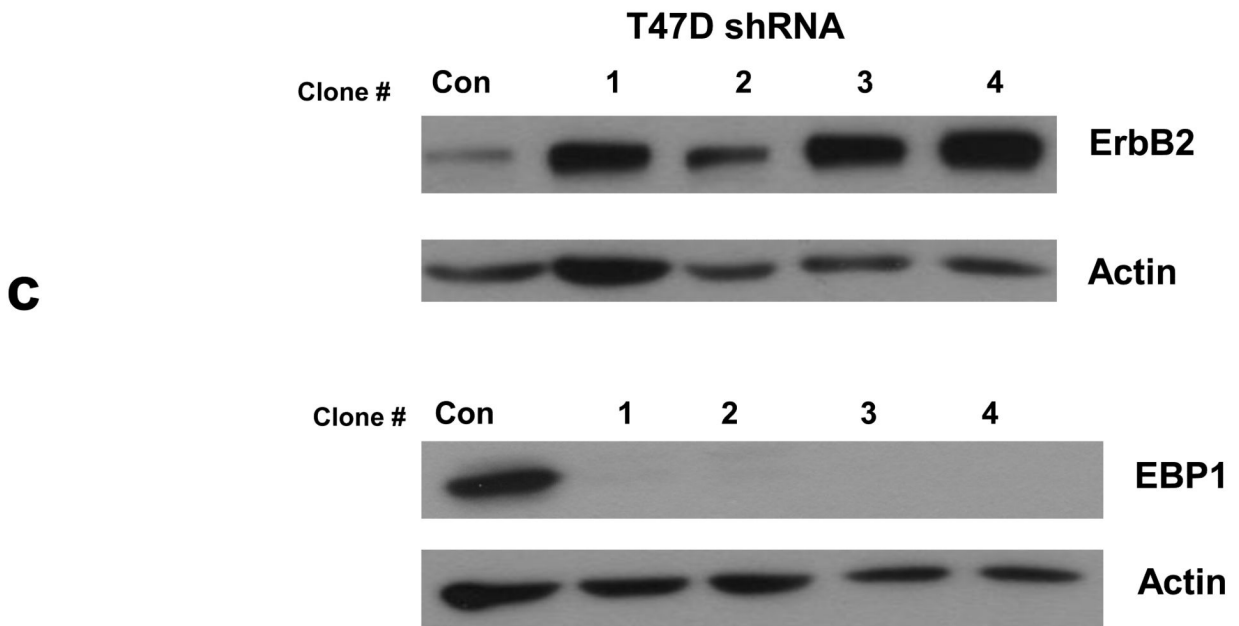
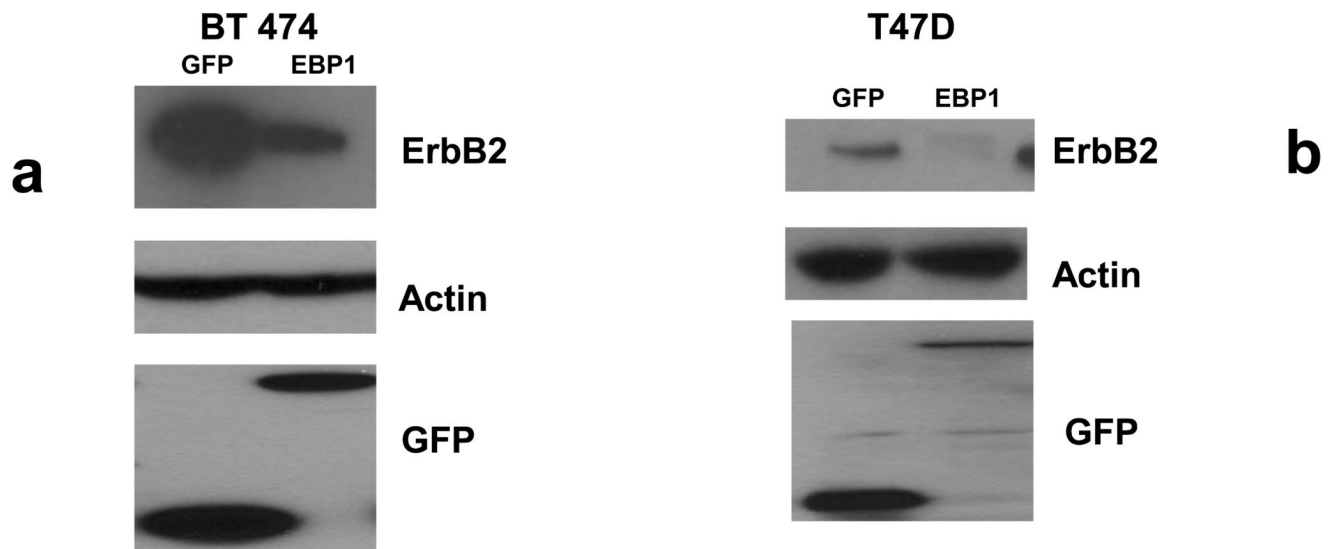
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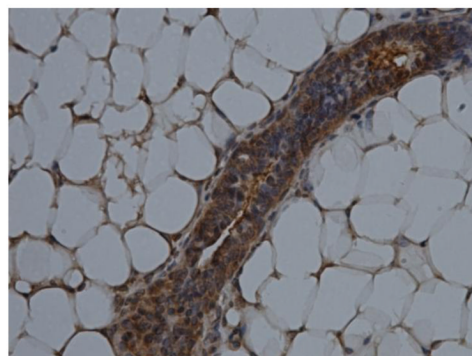
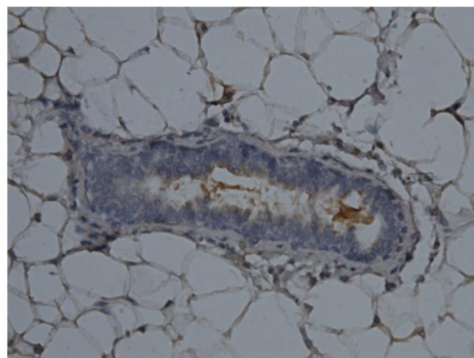
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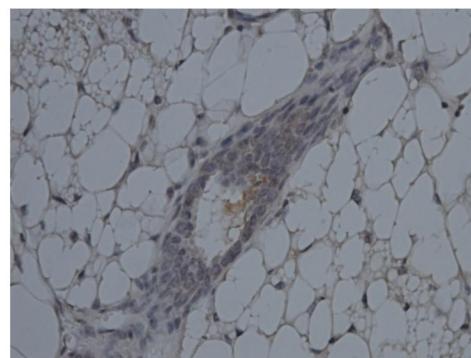
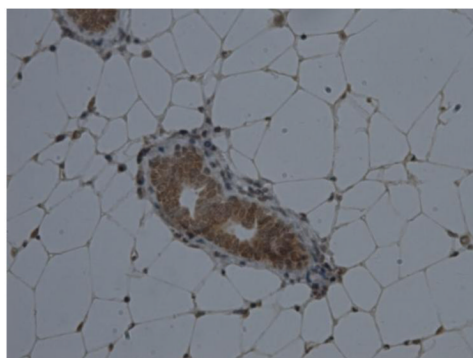
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d

ErbB2



EBP1

Wild type

Knock out

Fig. 1. Effect of modulation of EBP1 expression on ErbB2 protein levels

a,b) Lysates of logarithmically growing BT474 or T47D vector control cells (GFP) or *EBP1* transfected cells (EBP1) were analyzed by Western blotting with antibodies for ErbB2, Actin or GFP as indicated. Data are representative of 3 experiments. **c)** T47D cells were transduced with Control (Con) or EBP1 (1-4) directed lentiviruses and ErbB2, EBP1 or actin levels measured by Western blotting. Clones 1-4 are directed against the same EBP1 NT sequence, but represent independent experiments. **d.** Number 4 mammary glands were isolated from female 10 week old wild type (3) or Ebp1 knock out mice (3). Immunohistochemical analysis was performed for ErbB2 or EBP1 as indicated. A representative field is shown.

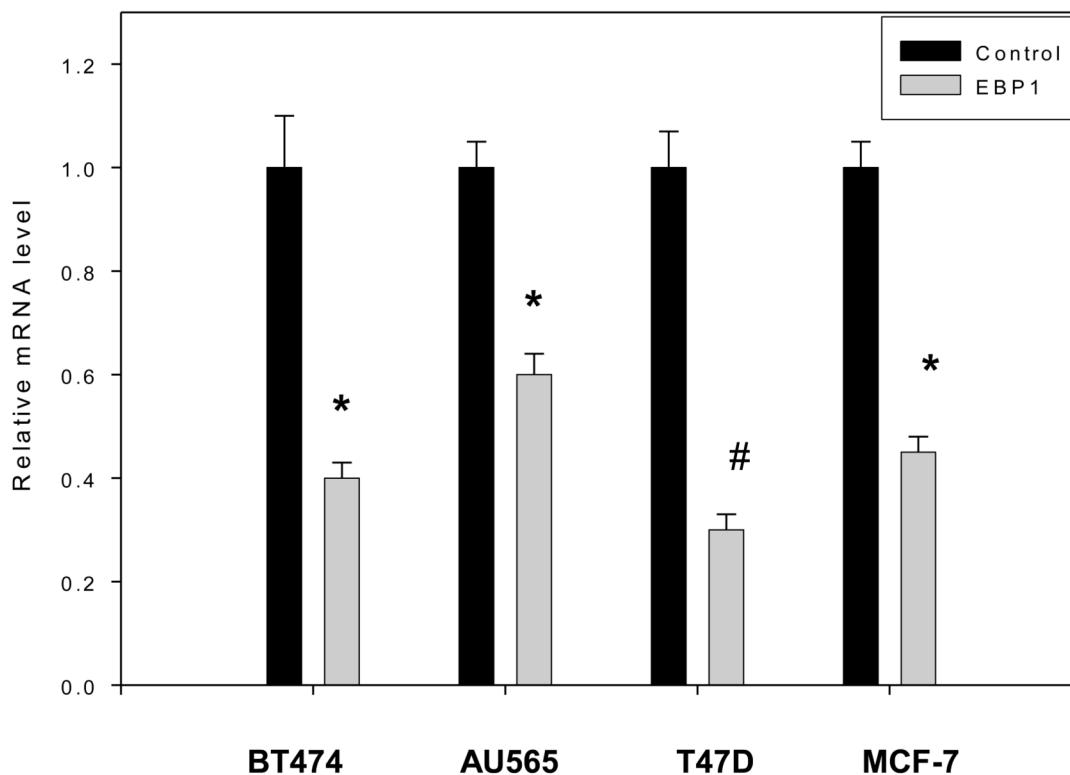


Fig. 2. Steady state levels of ErbB2 mRNA in vector control and EBP1 transfected cells
The cells lines indicated were stably transfected with EBP1 (see Fig. 1 and [21]). Total RNA (triplicate wells) was then extracted to detect the steady-state levels of ErbB2 and GAPDH mRNAs by quantitative RT-PCR. The mRNA level observed in control BT474 cells was set at 1. All values are presented in relation to this standard. Bars represent means \pm SEM. The graph is representative of one of three independent experiments. * $P < 0.05$ compared with control vector; # $P < .01$ compared with control vector.

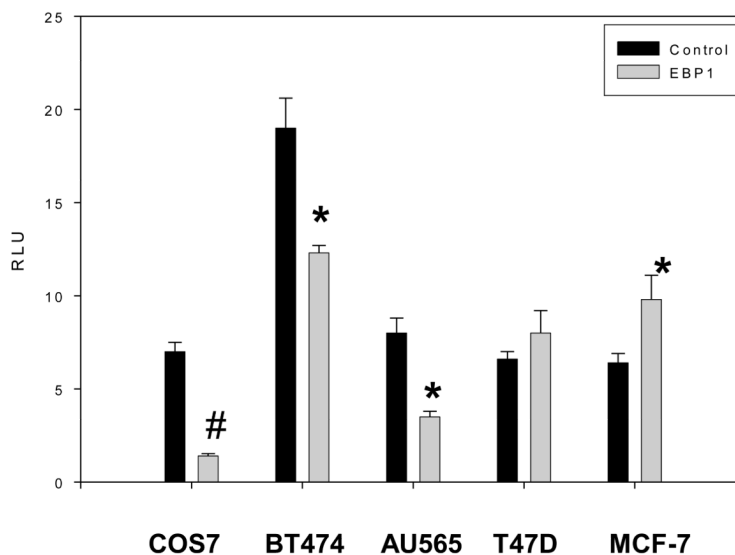
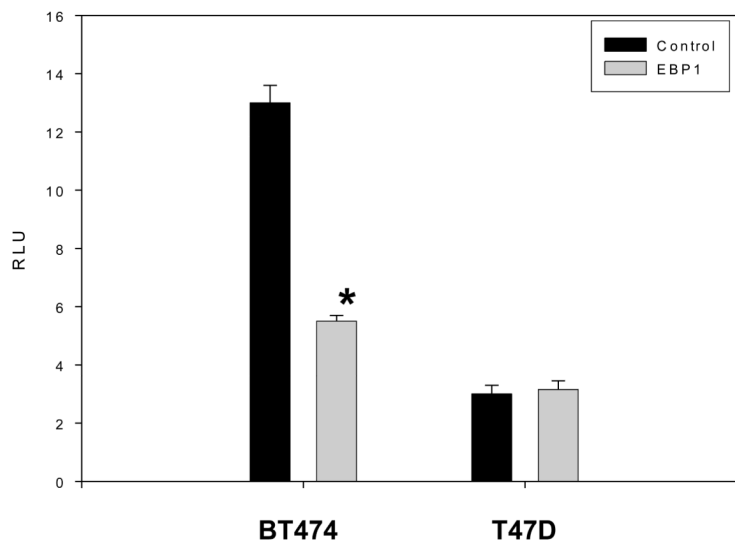
a**b**

Fig. 3. Effect of Ebp1 on ErbB2 promoter activity

a) The cell lines indicated were transiently co-transfected with ErbB2-luc, pRL-TK and GFP (Control) or GFP-EBP1. After 48hrs, cells were lysed and relative luciferase units were determined as described in the Material and Methods. The data are expressed as Relative Light Units (RLU) which is the ratio of ErbB2-luc RLU: pRL-TK RLU for each sample. Each bar represents the mean \pm S.D. of 8 wells. The figure is representative of 3 independent experiments. **b)** Cells lines stably transfected with EBP1 or GFP (control) were transiently transfected with ErbB2-luc and pRL-TK. Luciferase activity was determined as described in A. *P<0.05 compared with control vector; #P<.01 compared with control vector.

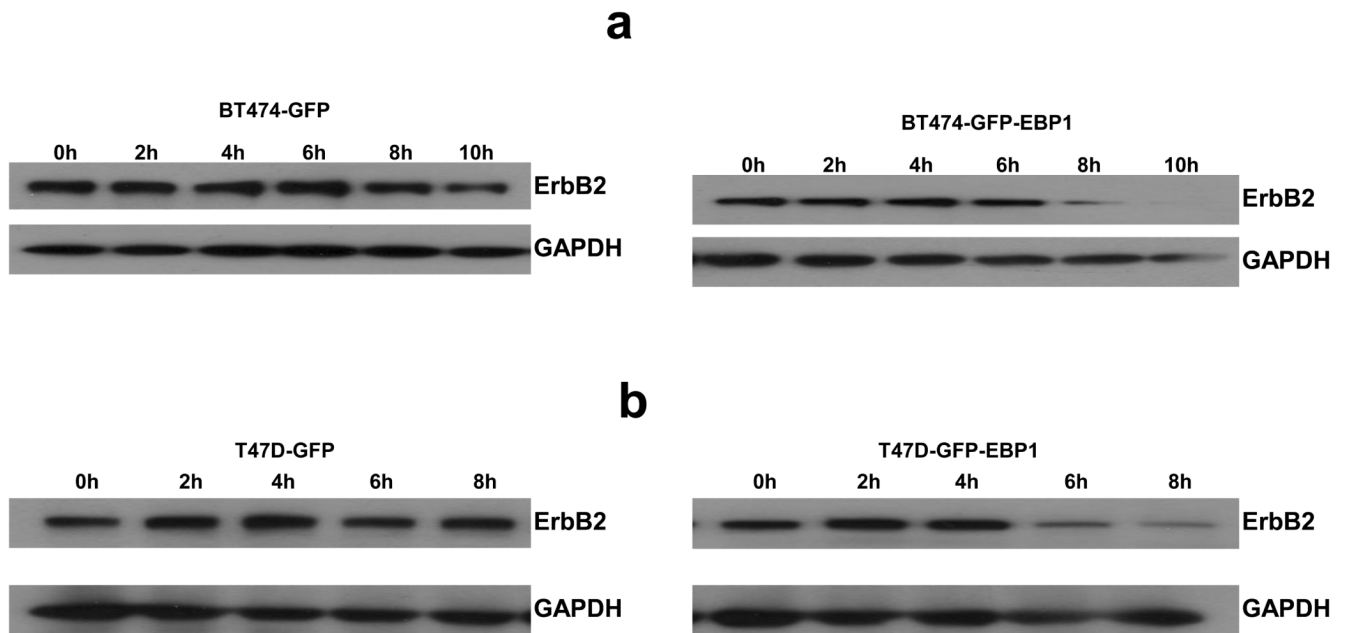
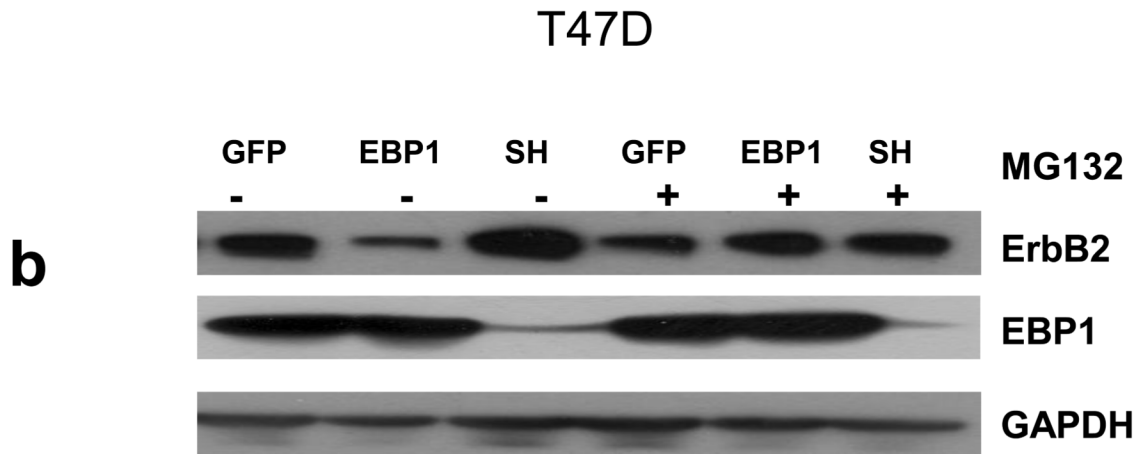
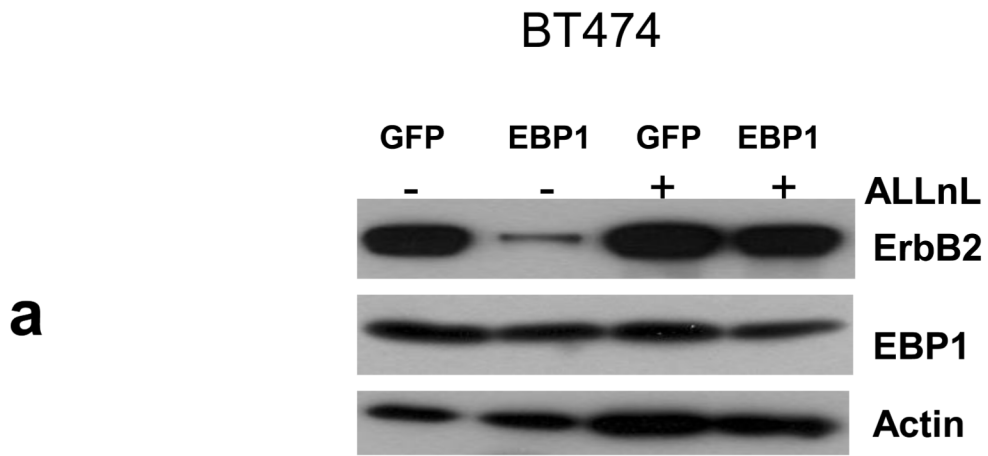


Fig. 4. Decreased stability of ErbB2 protein after ectopic expression of EBP1
Control GFP or EBP1 stably transfected BT474 (**a**) or T47D cells (**b**) were treated with cycloheximide. Cells lysates were obtained at the indicated time points and analyzed by Western blotting with ErbB2 or GAPDH antibodies as indicated.



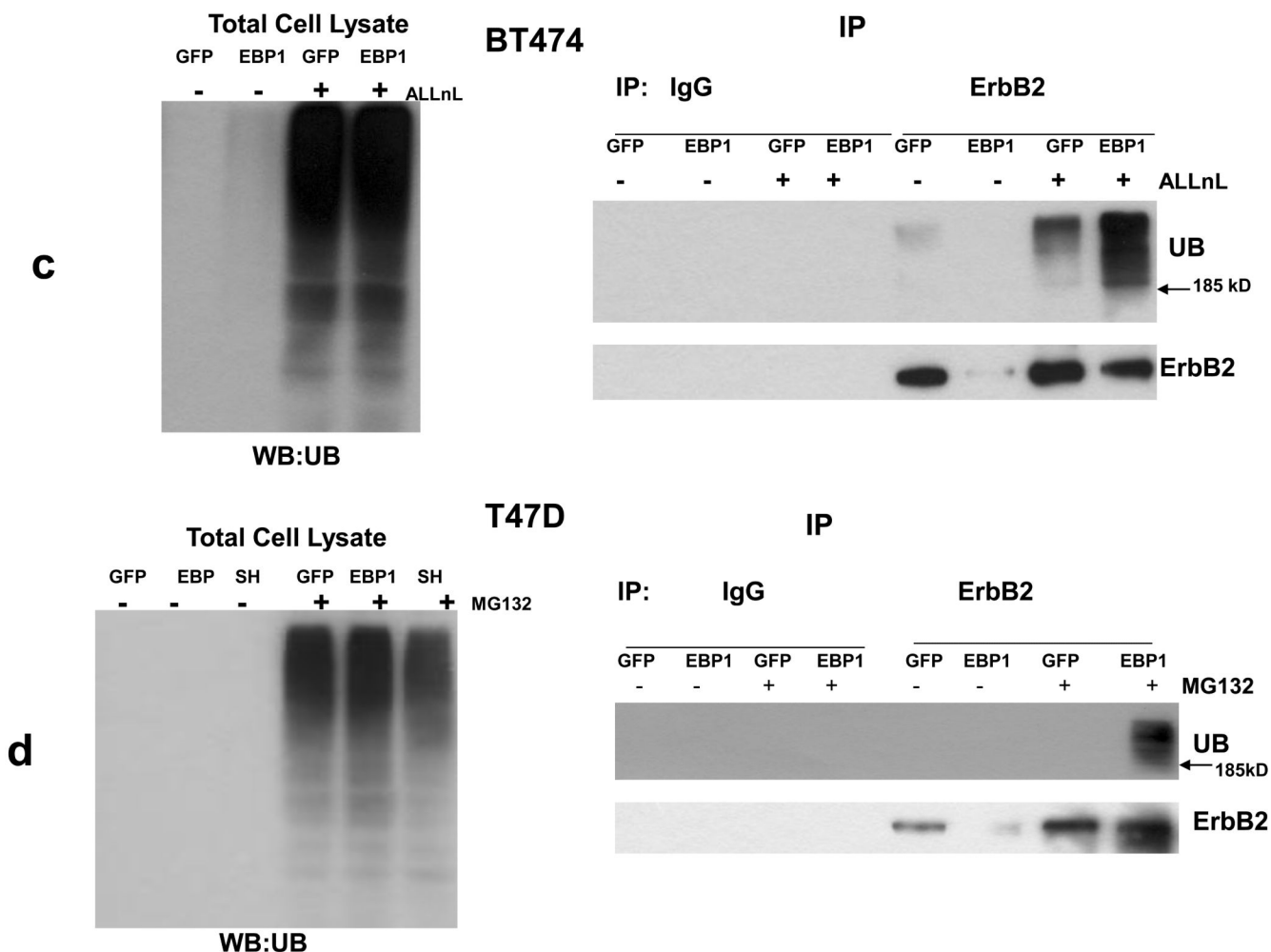


Fig. 5. EBP1 induced degradation of ErbB2 can be rescued by proteasome inhibition
a,b BT474 GFP control and *EBP1* transfected cells (**a**) and T4D control (GFP) cells and cells in which EBP1 was transfected (EBP1) or ablated (SH) (**b**) were treated with the indicated proteasome inhibitors for 6 hours prior to lysis. Cells lysates were analyzed by Western blotting with antibodies against ErbB2, EBP1 or actin or GAPDH as indicated. **c,d** BT474 GFP control and *EBP1* transfected cells and T4D control (GFP) cells and cells in which EBP1 was transfected (EBP1) or ablated (SH) (**d**) were treated with the indicated proteasome inhibitors for 6 hours prior to lysis. (**Left panels**) Cell lysates were resolved by SDS-PAGE and analyzed by Western blotting using an antiubiquitin antibody. (**Right Panels**) ErbB2 was immunoprecipitated from these cell lysates with IgG or anti-ErbB2 antibodies. Precipitated proteins were analyzed by Western blotting with antibodies to Ubiquitin or ErbB2 as indicated. Representative of three independent experiments.

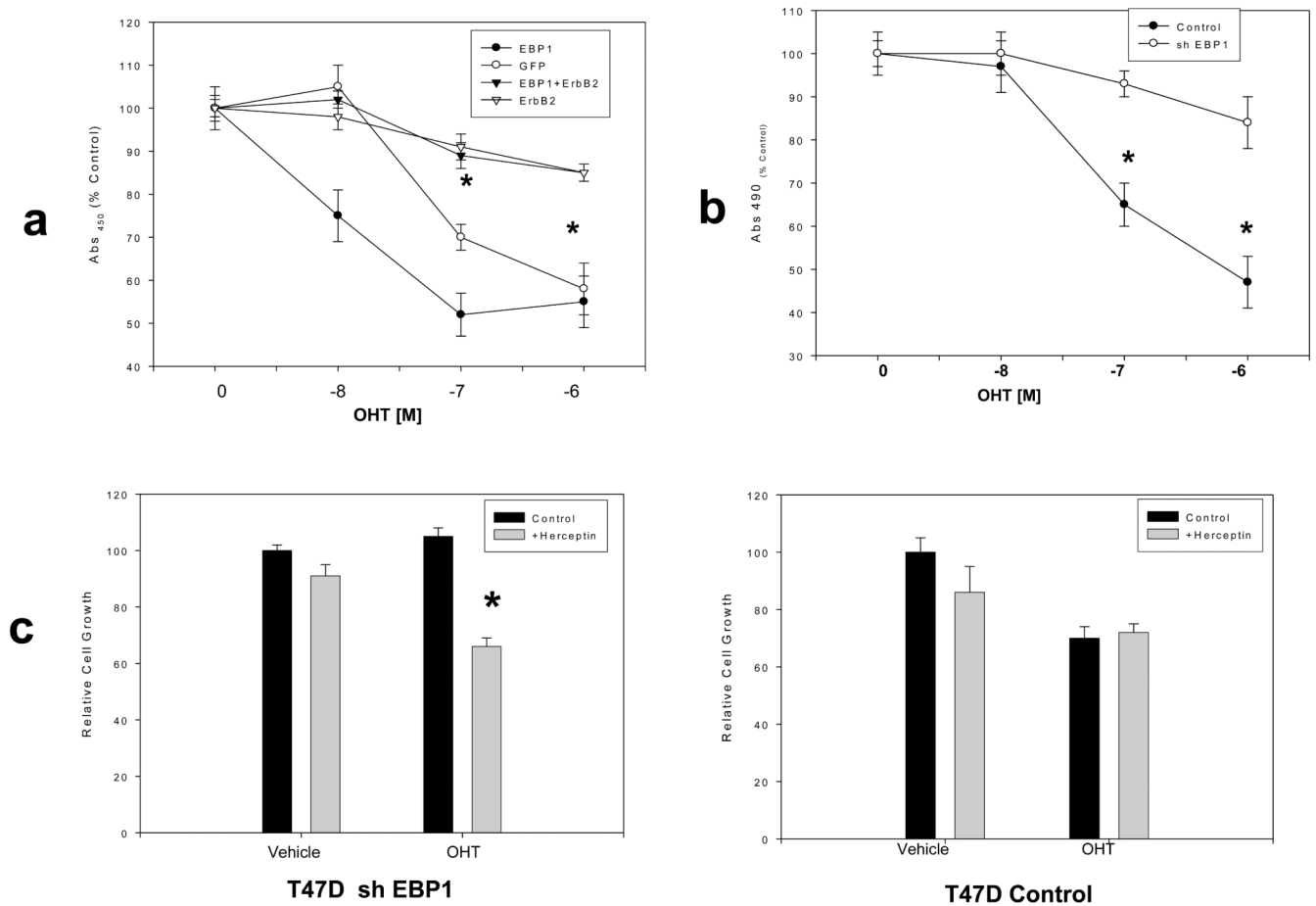


Fig. 6. Resistance of EBP1 knock out cells to tamoxifen is mediated by ErbB2

a) T47D vector control, *EBP1* transfected cells, or these cells transiently transfected with an *ErbB2* expression vector were treated with the indicated concentrations of OHT. Viable cells were quantified four days later using a Promega Cell Proliferation assay. Each data point represents the Mean \pm S.D. of 6 wells. Similar results were observed in two independent experiments. *—differences between control and ErbB2 transfected cells at indicated concentrations of OHT were significant at $P < 0.05$. **b)** T47D cells stably transduced with a control lentivirus (control) or an shRNA *EBP1* targeted lentivirus (clone 4, Fig.1C), were incubated for 4 days in the presence of OHT at the indicated concentrations or vehicle control. Viable cells were quantified using a Promega Cell Proliferation assay. Each data point represents the Mean \pm S.D. of 6 wells. Similar results were observed in two independent experiments. **c)** EBP1 ablated T47D cells (left panel) or T47D controls (right panel) were treated with OHT (10^{-7} M) and/or Herceptin ($250\mu\text{g/ml}$) as indicated. Relative cell growth was determined by MTT assays at Day 4. 100% growth=growth in the absence of either OHT or Herceptin. In b and c * $P < 0.05$ compared with control vector; # $P < .01$ compared with control vector.