

ERK-Mediated Phosphorylation of EZH2 Regulates HER2 Expression on Long-term Genistein-induced Acquired Endocrine Resistance in Estrogen-Receptor-Positive Breast Cancer Cells

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Research

Keywords: genistein, breast cancer, endocrine resistance, ERK, phospho-EZH2, H3K27me3, HER2

Posted Date: November 19th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-108061/v1>

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Abstract

Background

Genistein, a soy isoflavone, is the most important phytoestrogen in typical oriental diet. Many studies have shown that genistein at lower concentrations promotes breast cancer cells growth through the estrogen receptor pathway. However, recent research has found that long-term consumption of low doses of genistein results in hormone-independent growth phenotypes of MCF-7 tumors, with increased expression of HER2. Overexpression of HER2 has been causally associated with endocrine therapy resistance in human breast cancer. The mechanism by which prolonged exposure to genistein leads to increased HER2 expression is unclear. Whether genistein-induced HER2 expression is the cause of endocrine resistance remains to be determined.

Methods

We selected the MCF-7 and T47D breast cancer cells model with higher ER α and lower HER2. It was investigated whether prolonged exposure to genistein induced TAM-sensitive breast cancer cells to TAM-refractory cells by increasing HER2 expression. Furthermore, it was explored whether HER2 expression and endocrine resistance were associated with EZH2.

Results

We found that genistein had estrogen-like effect and inhibited HER2 expression during short-term exposure. However, long-term exposure to genistein induced acquired endocrine resistance, because of increased expression of HER2. During long-term exposure to genistein, the continuous activation of ERK1/2 phosphorylated EZH2 at Ser21, resulting in a decrease of lysine 27 trimethylation. As H3K27me₃ level decreased, the expression of IL-6 and IL-8 increased, and HER2 level gradually increased, forming a feedback loop of ERK1/2 / EZH2/ IL-6 and IL-8 / HER2.

Conclusions

These findings indicated that high HER2 expression caused by EZH2 phosphorylation was an important mechanism of endocrine resistance. The study also provided a new insight for genistein-induced acquired endocrine resistance. For breast cancer patients, long-term use of soy supplements has potential health risk. Especially, monitoring dietary exposure to genistein is advisable when treated with tamoxifen.

Background

Approximately 70% of breast cancers express estrogen receptor α (ER α) and merit the use of endocrine therapies, such as the estrogen receptor (ER) antagonist tamoxifen (TAM) [1]. However, ER-positive breast cancer frequently acquires resistance to TAM after long-term treatment, which is a serious therapeutic problem [2, 3].

Multiple mechanisms are responsible for the development of endocrine resistance. Compelling evidence suggests that the human epidermal growth factor receptor (HER) family plays a critical role in mediating endocrine therapy resistance [4-8]. The amplification of the HER2 locus can overcome the growth inhibitory effects imposed by TAM in ER-positive breast cancers [9]. The mechanism by which HER2 overexpression mediates TAM resistance is the crosstalk between ER α and HER2 initiates intracellular kinase cascades, such as MAPK signaling, promoting growth and progression in breast cancer cells, negating the inhibitory effects of TAM [10].

Phytoestrogens are compounds derived from plants that have estrogenic properties and are abundant in the human diet, particularly Soy. Genistein (GE), a major phytoestrogen in soybeans found in processed foods, induces genomic ER signaling in the developing breast cancer [11, 12]. Although no studies in humans have been conducted, some studies have shown that genistein negates the inhibitory effects of TAM in breast cancer cell lines and animal models [13-15]. Previous study has demonstrated that long-term exposure to genistein leads to estrogen-independence growth in ER-positive breast tumors and results in increased expression of HER2 [16]. The exact cause of abnormal growth factor signaling caused by genistein is unknown. Genistein may not have estrogenic properties when acting on ER-positive breast cancer for a long time, may cause the development of acquired endocrine resistance in breast cancer, in which HER2 may play an important role.

Enhancer of zeste homolog 2 (EZH2) is frequently overexpressed in human bladder, breast, colon and prostate cancers [17]. Studies have shown that EZH2 specifically catalyzes trimethylation of histone H3 lysine 27 (H3K27me₃), resulting in transcriptional repression and chromatin compaction [18-20]. Several studies have reported that epigenetic alterations are associated with endocrine resistance in breast cancer [21, 22]. Although roles of EZH2 in driving cancer proliferation and invasion are extensively characterized [23, 24], few studies investigate the association of EZH2 with acquired endocrine resistance. Data from a study suggest that low H3K27me₃ levels were significantly associated with aromatase inhibitor resistance in breast cancer patients [25]. Notably, genistein induced PI3K/AKT non-genomic ER signaling to phosphorylate and repress the histone methyltransferase EZH2. As a result, this signaling reduces levels of H3K27me₃ in chromatin [26]. However, it is not clear whether inhibition of EZH2 histone methyltransferase activity is associated with increased HER2 expression and endocrine therapy resistance.

Therefore, we selected the MCF-7 and T47D breast cancer cells model with higher ER α and lower HER2. We have investigated whether prolonged exposure to genistein can induce TAM-sensitive breast cancer cells to TAM-refractory cells by increasing HER2 expression. During long-term exposure to Genistein, the phosphorylation of EZH2 and the levels of H3K27me₃ were observed, and their effects on TAM sensitivity were evaluated. Furthermore, it was explored whether HER2 was physically associated with EZH2. The data reported here reveal genistein regulates TAM resistance through EZH2/ H3K27me₃/HER2 axis in breast cancer.

Methods

Reagents

E₂ and TAM were purchased from Sigma Aldrich (St. Louis, MO, USA). GSK-J4 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Genistein and U0126 were obtained from Beyotime Biotechnology (Nanjing, Jiangsu, China). Recombinant Human IL-6 and IL-8 were purchased from PeproTech (Cranbury, NJ, USA).

Cell Culture

Human breast cancer cell lines MCF-7, T47D were maintained in DMEM/F-12 medium (Yuanpei, Shanghai, China) supplemented with 10% fetal bovine serum (Biological Industries, Israel), 100 units/mL penicillin (Beyotime, Nanjing, Jiangsu, China), 100 µg/mL streptomycin (Beyotime, Nanjing, Jiangsu, China), and 100 mM nonessential amino acids (Life Technologies, Grand Island, NY, USA) at 37 °C in 5% CO₂ atmosphere. To observe the short-term effects of E₂ or genistein on cells, cells were switched to MEM medium without phenol red (Life Technologies, Grand Island, NY, USA) plus 5% charcoal-stripped fetal bovine serum (Biological Industries, Israel), supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 100 mM nonessential amino acids for at least 3 days before the experiments.

RNA interference

The 21-nucleotide duplex siRNAs for EZH2, HER2 and one negative control siRNA were synthesized by Santa Cruz (Dallas, Texas, USA). Transfection was carried out using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Transfection efficiency was evaluated in every experiment by RT-qPCR 24 hours later to ensure that cells were transfected.

Quantitative Real-Time RT-PCR (qPCR)

Total RNA from the two cell lines was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA using the ExScript RT reagent (Abm, Zhenjiang, Jiangsu, China). Real-time RT-PCR was performed using StepOnePlus Real Time PCR System (Roche Group, Basel, Switzerland) with specific primers for EZH2, HER2, IL-6, IL-8 and GAPDH expression was used to normalize for variance. Real-time fluorescence monitoring of the PCR products was performed with SYBR Green I fluorescent dye (Abm, Zhenjiang, Jiangsu, China). The expression levels of specific genes are reported as ratios of expression of GAPDH in the same master reaction.

Western Blotting Analysis

Cells were lysed with whole cell lysis buffer, frozen at -80°C and thawed three times to rupture the cell membranes. Samples of the lysates were incubated for 30 min on ice to lyse the nuclei, and then centrifuged at 12,000 rpm for 20 min at 4°C. Equal amounts of protein (20 µg) were subjected to western blot analysis. Western blotting was performed by standard procedures.

Proliferation Assay

Breast cancer cells were seeded in 96-well plates (4000 cells/well). At the end of the cell treatment, cell proliferation was assayed using Cell Counting Kit-8(CCK-8) (Apexbio, Houston, TX, USA) as per the manufacturer's protocol.

Statistical Analysis

Experimental values are presented as mean \pm standard deviation (SD). At least three independent trials were performed for each experiment. Statistical analytical Methods and numbers of data points analyzed for each experiment are described in figure legends. Statistically significant analysis by two-way ANOVA with Sidak's multiple comparison test ($\alpha = 0.05$) was performed in experiments graphed in Figure 1a–c, 4d, 4f, 5e, 5f. One-way ANOVA with Dunnett's test ($\alpha = 0.05$) was performed in experiments graphed in Figures 1g and 5d. Independent two-samples t-test was performed in experiments graphed in Figures 1h, 1i, 4c, 4e, 5a-c. For all analyses, the p values < 0.05 were considered statistically significant.

Results

Elevated HER2 promotes to genistein - induced acquired endocrine therapy resistance

It has been shown that long-term consumption of low doses genistein promotes MCF-7 tumor growth and results in non-hormone dependent tumors with increased expression of HER2 [16]. Long-term TAM treatment also induces overexpression of HER2 as a resistance mechanism to hormonal therapy [9, 27]. The different plasma levels of genistein were consistently observed in Asian and Western populations. For example, the plasma levels were about 0.02 $\mu\text{mol/L}$ in English and 0.26 $\mu\text{mol/L}$ in Japanese and Korean [28, 29]. In this study, 1 μM was selected as the study dose of genistein. Using ER-positive breast cancer cells, we investigated whether genistein induces resistance to endocrine therapy. After long-term (3 weeks) genistein exposure followed by a 24h withdrawal, cells were treated with different concentrations of TAM for 48h. Compared with the parallel cells, the MCF-7 cells (Fig. 1a) and T47D cells (Fig. 1b) by prolong genistein stimulus and withdrawal showed resistance to TAM, while T47D cells treated with E_2 remained normal TAM sensitivity (Fig. 1c). Western blot analysis showed that HER2 levels decreased after genistein exposure for 24h (Fig. S1a and b), which was similar to E_2 (Fig. S1c). HER2 levels were significantly increased after 3 weeks of exposure to genistein in MCF-7 cells (Fig. 1d). During long-term genistein exposure, T47D cells were subjected to western blot analysis at 2 days, 1 week, 2 weeks, 3 weeks, respectively. HER2 expression decreased initially, then increased gradually. The levels were higher than that in the parental cells at 3 weeks (Fig. 1e and g). The HER2 levels increased substantially after TAM treatment for 24h (Fig. S1d) and further increased during prolonged TAM treatment (Fig. 1f and g). We investigated whether endocrine resistance is associated with up-regulation of the HER2. Under the action of HER2 siRNA (Fig. 1h), genistein-induced resistant cells recovered their sensitivity (Fig. 1i). All these results indicate that HER2 might play an important role in genistein-induced acquired endocrine resistance.

ERK1/2 signal activation caused by long-term exposure to genistein is associated with HER2 elevation

The results of preclinical studies have indicated that acquired TAM resistance is associated with increased activities of ERK [30]. Genistein induces phosphorylation of ERK1/2 within a certain amount of time, and ERK activity starts at 3h and peaks at 24 h [31]. In the present study, the 24h exposure of the T47D cells to genistein induced phosphorylation of ERK1/2 (Fig. S2a), as TAM did (Fig. S2b). During long-term genistein exposure, T47D cells were subjected to western blot analysis at 2 days, 1 week, 2 weeks, 3 weeks, respectively. Phosphorylation of ERK1/2 increased after 1 week (Fig. 2a).

Phosphorylation of ERK1/2 increased at 3 weeks in MCF-7 cells (Fig. 2b). The results of TAM treatment were the same as those of long-term genistein exposure (Fig. 2c). We observed a similar trend in the changes of HER2 protein levels and ERK1/2 phosphorylation levels after long-term genistein exposure. MAP kinase displayed markedly increased activity in cell lines overexpressing HER2 [32]. In this study, Knockout of HER2 gene decreased phosphorylation of ERK1/2 (Fig. S2c). When MEK inhibitor U0126 treated T47D cells, inhibition of ERK1/2 phosphorylation down-regulated the levels of HER2 (Fig. S2d), genistein-induced (Fig. 2d) and TAM-induced (Fig. 2e) HER2 expression. These results indicate that activation of ERK1/2 signal caused by long-term exposure to genistein interacts with the expression of HER2.

EZH2 phosphorylation at Ser21 increases and H3K27me3 levels reduce during long-term genistein exposure

EZH2 is an estradiol-regulated gene and its promoter contains functional estrogen-response elements [33]. After TAM treatment for 24h, the mRNA and protein levels of EZH2 were down-regulated, as well as H3K27me3 levels (Fig.S3a). During long-term TAM treatment, EZH2 and H3K27me3 levels were also decreased (Fig. 3a). TAM inhibition of EZH2 expression may be caused by its antagonism against ER.

As a phytoestrogen, genistein promoted the expression of ER α target gene after 24h exposure (Fig.S3b). It also increased the levels of EZH2 and H3K27me3 in MCF-7 cells and T47D cells (Fig.S3c). During long-term genistein exposure, EZH2 protein levels increased at 2 days and 1 weeks, and fell back to the same level as the control at 2 weeks and 3 weeks. Remarkably, the levels of H3K27me3 increased first, and decreased at 2 weeks and 3 weeks (Fig. 3b). The expression of EZH2 decreased slightly and the levels of H3K27me3 also decreased at 3 weeks in MCF-7 cells (Fig. 3c). Collectively, it seems highly likely that **decreased** trimethylation at H3K27 by long-term genistein exposure was not a result of decreased total levels of EZH2, because EZH2 levels remained unchanged or decreased slightly in response to long-term genistein.

Phosphorylation at Ser21 altered the affinity of EZH2 for its substrate, histone H3, which reduces EZH2 methyltransferase activity [34]. We hypothesized that genistein regulates trimethylation at H3K27 by influencing the phosphorylation of EZH2. To validate our hypothesis, we first examined and compared the phosphorylation levels of EZH2 at Ser21 between genistein and TAM. After long-term TAM treatment, the phosphorylation of EZH2 at Ser21 was gradually increased (Fig. 3c). During long-term genistein exposure, the phosphorylation of EZH2 at Ser21 decreased first, and increased at 2 weeks and 3 weeks in T47D

cells. Likewise, the phosphorylation of EZH2 also increased at 3 weeks in MCF-7 cells (Fig. 3b). These results suggest that phosphorylation of EZH2 at Ser21 may be responsible for the decrease of H3K27me3 during long-term genistein exposure. Unlike genistein, TAM had a stronger inhibitory effect on H3K27 trimethylation, due to enhancing phosphorylation of EZH2 and suppressing expression of EZH2 by antagonizing ER.

ERK decreases the H3K27 trimethylation through phosphorylation of EZH2, contributing to HER2 expression and endocrine resistance

We observed a similar trend in the changes of EZH2 phosphorylation and ERK1/2 phosphorylation after long-term genistein exposure. Accordingly, we evaluated whether phosphorylated ERK1/2 affects EZH2 phosphorylation, H3K27me3 levels. When MEK inhibitor U0126 treated T47D cells for 24h after 3 weeks with genistein, the results showed that inhibition of ERK1/2 phosphorylation downregulated genistein-induced EZH2 Ser21 phosphorylation, and up-regulated genistein-decreased H3K27me3 (Fig. 4a). When U0126 was combined with TAM, the results showed that inhibition of ERK1/2 phosphorylation also downregulated TAM-induced EZH2 phosphorylation at Ser21, up-regulated TAM-decreased H3K27me3 (Fig. 4b). Phosphorylation of EZH2 at Ser21 results in decreased EZH2 activity and H3K27me3 levels [34]. Other data suggest that low H3K27me3 levels are significantly associated with resistance to aromatase inhibitors in breast cancer patients [25]. To investigate whether H3K27me3 level was related to the expression of HER2 and the sensitivity to TAM, after exposure to 1 μ M genistein for 3 weeks, T47D cells were treated with EZH2 siRNA or GSK-J4, an H3K27 demethylase inhibitor. The results showed that GSK-J4 elevated the levels of H3K27me3, inhibited the expression of HER2 (Fig. 4c), and restored TAM sensitivity in T47D cells long exposed to genistein (Fig. 4d). However, EZH2 gene knockout lowered the levels of H3K27me3, promoted the expression of HER2 (Fig. 4e), and diminished the efficacy of TAM (Fig.4f). Increased phospho-EZH2 was not a result of increased total levels of EZH2, because phosphorylation of EZH2 remained unchanged in response to knocking down EZH2 expression (Fig. 4e). The above results suggested that activated ERK1/2 signaling phosphorylates EZH2 and reduces the trimethylation of H3K27. It has been reported that phosphorylation of EZH2 at Ser21 reduces the methylation for H3K27 [34]. Our findings revealed that reduction of H3K27me3 was involved in increasing expression of HER2 and reducing the sensitivity of the cells to TAM.

Increased IL-6 and IL-8 induced by genistein is involved in HER2 elevation and endocrine resistance

A decrease of H3K27me3 by EZH2 deficiency resulted in derepression of silenced IL-6 and IL-8 in MCF-7 or another ER-positive T47D cells [35]. IL-6 is involved in TAM resistance through the downstream activation of multiple signaling pathways [36-38]. Previous study suggests a close link between IL-8 and traditional chemotherapy drug resistance [39]. IL-6 and IL-8 may play critical roles in acquired endocrine resistance caused by genistein. Therefore, we analyzed expression of IL-6 and IL-8 in cells stimulated by prolong genistein. Both IL-6 mRNA and IL-8 mRNA significantly increased after 3 weeks of genistein exposure (Fig. 5a). After 24 h treatment, like E₂, genistein inhibited IL-6 mRNA and IL-8 mRNA in MCF-7 cells (Fig. S4a), while only inhibited the expression of IL-6 mRNA in T47D cells (Fig. S4b). TAM, like

prolong genistein, increased the expression of IL-6 and IL-8 mRNA (Fig. S4c and d). Our observations confirmed that knockdown of EZH2 gene elevated expression of IL-6 and IL-8 mRNA (Fig. 5b), and GSK-J4 as an H3K27 demethylase inhibitor decreased expression of IL-6 and IL-8 mRNA (Fig. 5c). We analyzed the involvement of IL-6 and IL-8 in HER2 expression and TAM resistance in breast cancer. Here we demonstrated that exogenous IL-6 or IL-8 (24h treatment with recombinant IL-6 or IL-8) elevated expression of HER2 in T47D cells (Fig. 5d). Results of further investigation indicated that IL-6 or IL-8 diminished the efficacy of to TAM (Fig. 5e and f). Together, these results indicate that the elevation of HER2 promoted by IL-6 or IL-8 may be the cause of TAM resistance induced by genistein and EZH2 inactivation and H3K27me3 level reduction were involved in the enhanced expression of IL-6 and IL-8.

Discussion

Despite endocrine therapy has dramatically improved survival in ER-positive breast cancer patients, resistance to treatment is common, resulting in metastatic relapse that cannot be cured [40, 41]. Much evidence has demonstrated that increased growth factor signaling, in particular the HER2 pathway contributes to endocrine therapy resistance [42]. HER2 gene expression can be down-regulated by E₂ in the MCF-7 breast cancer cell line [43] through direct transcriptional repression of the HER2 gene [44]. In the current study, genistein, like E₂, reduced the levels of HER2 and TAM increased the levels of HER2 after short-term treatment. Therefore, we believe that genistein may directly inhibit the transcription of HER2 gene through ER. After long-term exposure, genistein increased the expression of HER2, which is consistent with previous report in an athymic mice xenograft model [16]. Here we demonstrate that long-term exposure to genistein causes the development of acquired endocrine resistance in ER-positive breast cancer cells, in which HER2 may play an important role.

Many observations have confirmed the link between increased HER2 activity and phosphorylation of the downstream MAPK/ERK pathway [45]. TAM was shown to activate ERK in ER-positive MCF-7 and T47D cells but not in ER-negative MDA-MB-231 cells [46]. Unrestrained MAPK signaling phosphorylates Ser-118 in the ER [47, 48], alters the ER association with corepressors of transcription [49], leading to loss of the inhibitory effect of TAM, which is a viable mechanism for MAPK to cause TAM resistance. Our previous study has shown that genistein induces activation of ERK1/2, starting at 3h and peaking at 24 h [31]. In the present study, phosphorylation of ERK1/2 was high again from week 1 during long-term genistein exposure. Our study found that HER2 gene knockout resulted in decreased phosphorylation of ERK1/2. When inhibiting the phosphorylation of ERK1/2, the levels of HER2 protein were down-regulated. These data suggest that HER2 and ERK1/2 interact in a positive feedback regulation mode, which may be the reason for the unconstrained activation of ERK1/2. Meanwhile, HER2 expression is at a high level. Nonetheless, the data presented imply that MAPK may play a causal role in genistein-induced TAM resistance in ER-positive breast tumor cells.

Our further results suggest that the activated ERK1/2 signaling phosphorylated of EZH2 at Ser21 and down-regulated H3K27me3 level. EZH2 as a part of the polycomb repressive complex 2 (PRC2), possesses histone H3K27-specific methyltransferase activity [50]. Histone H3K27 methylation by EZH2 is

an important mechanism of gene silencing [51]. Previous publications report that EZH2 is an estrogen-regulated gene [33, 52, 53]. Here we found that genistein, as a phytoestrogen, up-regulated the levels of EZH2 and H3K27me3 in ER-positive breast cancer cells. However, after long-term genistein exposure, EZH2 protein levels have not changed significantly and the phosphorylation of EZH2 at Ser21 significantly increased. Phosphorylation of EZH2 at Ser21 dissociates EZH2 from chromatin resulting in decreased EZH2 activity and H3K27me3 levels [34], which is consistent with our results. We speculate that due to increased phosphorylation of EZH2, EZH2 protein levels did not increase as they did with short-term genistein exposure. Recent study has shown that EZH2 was evidently less enriched in TAMR cells [54]. Study has found that low H3K27me3 level were significantly associated with aromatase inhibitor resistance [25]. In the current study, we demonstrated that decrease of H3K27me3 caused by EZH2 deficiency was associated with increased HER2 expression and genistein-induced TAM resistance. On the contrary, increase of H3K27me3 inhibited the expression of HER2 and restored TAM sensitivity in T47D cells long exposed to genistein.

E_2 is effective in suppressing TNF α induction of the IL-6 and IL-8 genes in MCF-7 cells [55]. Our findings indicated that genistein was equally effective in suppressing the expression of IL-6 and IL-8 mRNA, possibly due to its estrogen-like activity. Long term anti-hormone therapy alters the function of ER α to create an inflammatory microenvironment in breast cancer [56]. However, we found that genistein up-regulated the expression of IL-6 and IL-8 after long-term exposure. Both IL-6 and IL-8 increased the expression of HER2 and diminished the efficacy of TAM. These data suggest that the up-regulation of inflammatory cytokine increased the levels of HER2, which may play a role in acquired endocrine resistance induced by long-term exposure of genistein. Unfortunately, the mechanism of IL-6 or IL-8 regulating HER2 expression has not been reported yet and further investigations are required to determine it.

In addition, we explored how inflammatory cytokines is elevated and which factors regulate them. Our data indicated that expression of IL-6 and IL-8 were significantly increased upon EZH2 silencing and significantly decreased upon H3K27 methylation enhancement. Our study disclosed EZH2 levels and activity were correlated negatively with inflammatory cytokines and that H3K27 methylation may account for the epigenetic repression, which is consistent with previous report [35]. For the first time, we have found that genistein induced TAM resistance via the EZH2/ H3K27me3/inflammatory cytokine /HER2 axis.

In summary, our work reveals a critical epigenetic program that determines HER2 expression as well as cell fate in response to TAM treatment (Fig. 6). In sensitive cells upon short-term exposure of genistein, the expression of HER2 is suppressed because of its estrogen-like function. However, long-term genistein exposure results in sustained activation of ERK1/2 and therefore induces a inactivation of epigenetic enzymes EZH2 by phosphorylation and decrease of H3K27me3 level, which causes high expression of inflammatory cytokines and HER2. Maintenance of HER2 protein at high level phosphorylates the downstream ERK1/2 and reprograms ER α -dependent transcriptional machinery, which renders acquired resistance phenotypes in breast cancer cells. TAM plays a similar role in this pathway. The difference is

that TAM directly increases HER2 expression and inhibits EZH2 expression by antagonizing ER, even in the short term.

Conclusions

Taken together, our findings provide a compelling foundation for elucidating the endocrine resistance mechanism induced by genistein in breast cancer. The timing of exposure to phytoestrogens may be a key component in determining its effects. This study provides a comprehensive understanding of the health risks of dietary exposure to phytoestrogens in breast cancer patients. Breast cancer patients, especially those on TAM should be cautioned against the long-term use of soy supplements and purified products in order to achieve more lasting results.

Abbreviations

| | |
|----------|--|
| ER | estrogen receptor |
| TAM | tamoxifen |
| HER | human epidermal growth factor receptor |
| GE | genistein |
| EZH2 | enhancer of zeste homolog 2 |
| H3K27me3 | trimethylation of histone H3 lysine 27 |
| PRC2 | polycomb repressive complex 2 |

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to the publication of the article.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by National Natural Science Foundation of China (81573183, 81673205) and the Center for Global Health, School of Public Health, Nanjing Medical University. The project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Authors' contributions

CH and QZ are responsible for design, specific operation and data analysis of the experiment; BY, WX, KJ, KY, MZ are responsible for operation of the experiment; ZL is responsible for the writing of the article, experimental design and financial support. All authors read and approve the final manuscript.

Acknowledgements

Not applicable.

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Figures

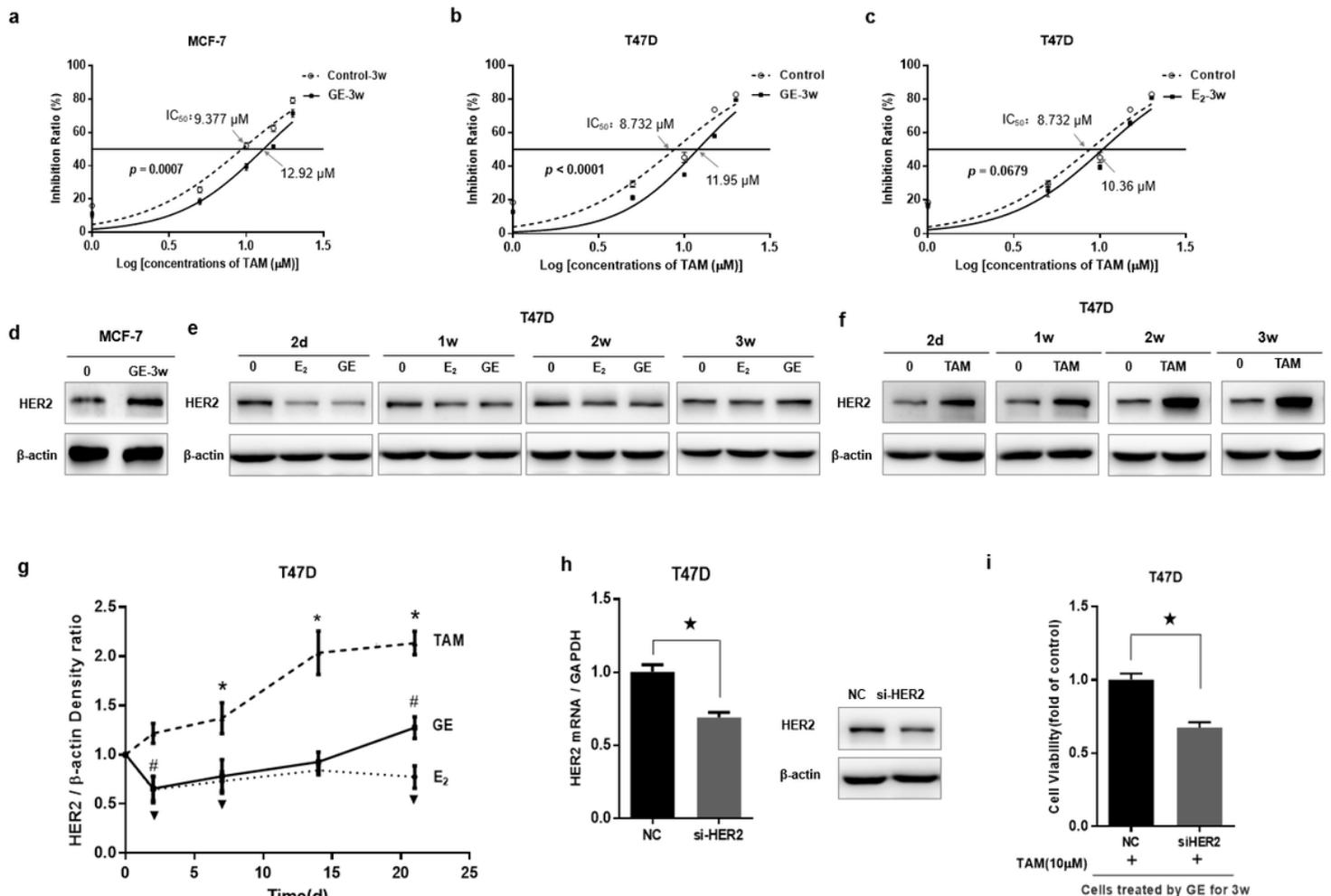


Figure 1

Elevated HER2 promotes to genistein - induced acquired endocrine therapy resistance (a) MCF-7 cells were treated by 1 μM genistein for 3 weeks. (b) T47D cells were treated by 1 μM genistein for 3 weeks. (c) T47D cells were treated by 1 nM E2 for 3 weeks. After 3 weeks, the drug was removed, and after 24h, cells were treated with TAM for 48h. The cell viability was determined in quadruplicate, and the IC50s were calculated. (d) Western blot analysis was used to determine HER2 levels at 3 weeks after exposure to 1 μM genistein in MCF-7 cells. (e) Western blot analysis was used to determine HER2 levels at 2 days, 1 week, 2 weeks and 3 weeks after exposure to 1 μM genistein or 1 nM E2 in T47D cells. (f) Western blot analysis was used to determine HER2 levels at 2 days, 1 week, 2 weeks and 3 weeks after treatment with 1 μM TAM in T47D cells. (g) Expression of HER2 was normalized to the β -actin control in each lane in triple. *# ∇ $p < 0.05$ vs. control cells. (h) The mRNA levels of HER2 were assessed by PCR and the protein levels of HER2 were measured by western blot analysis in T47D cells transfected with HER2 siRNA for 6h. (i) T47D cells were treated by 1 μM genistein for 3 weeks. After 3 weeks, genistein was removed, and cells were transfected with NC or HER2 siRNA for 6h respectively, and then treated with 10 μM TAM for 48h. Data of the mRNA are expressed as the mean \pm SD (n = 3). control group. Data of the cell viability are expressed as the mean \pm SD (n = 4), \boxtimes $p < 0.05$ vs. NC group.

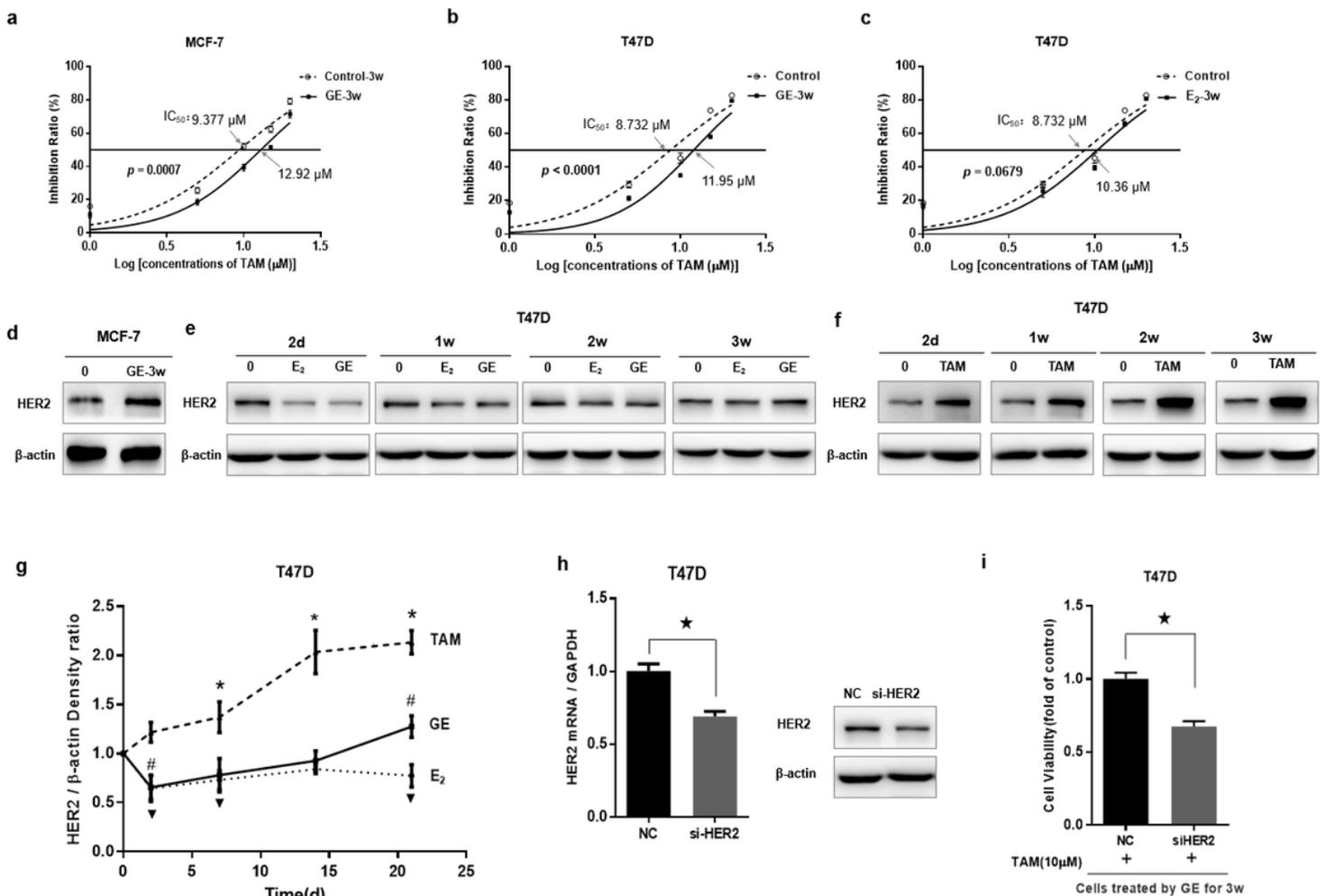


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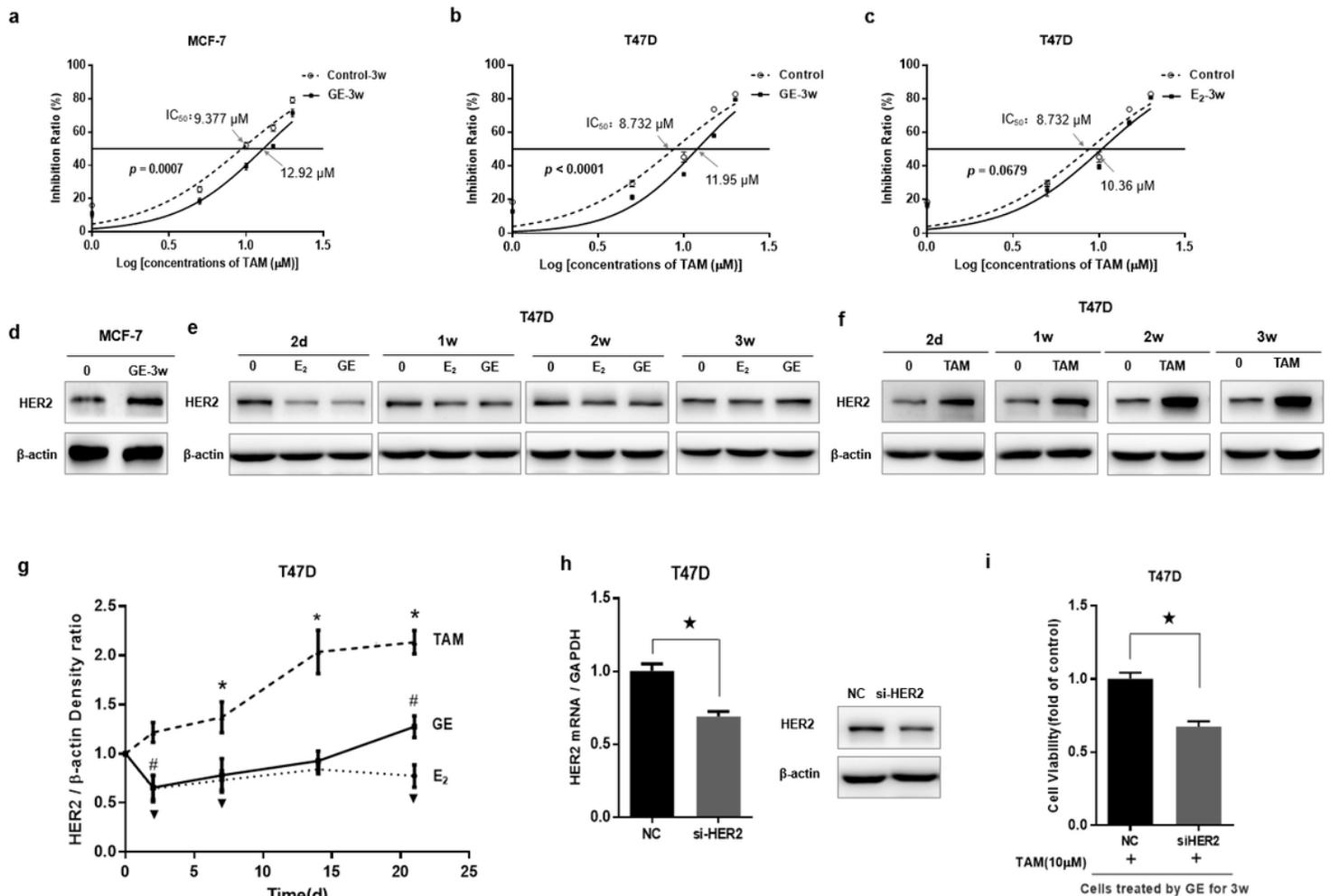


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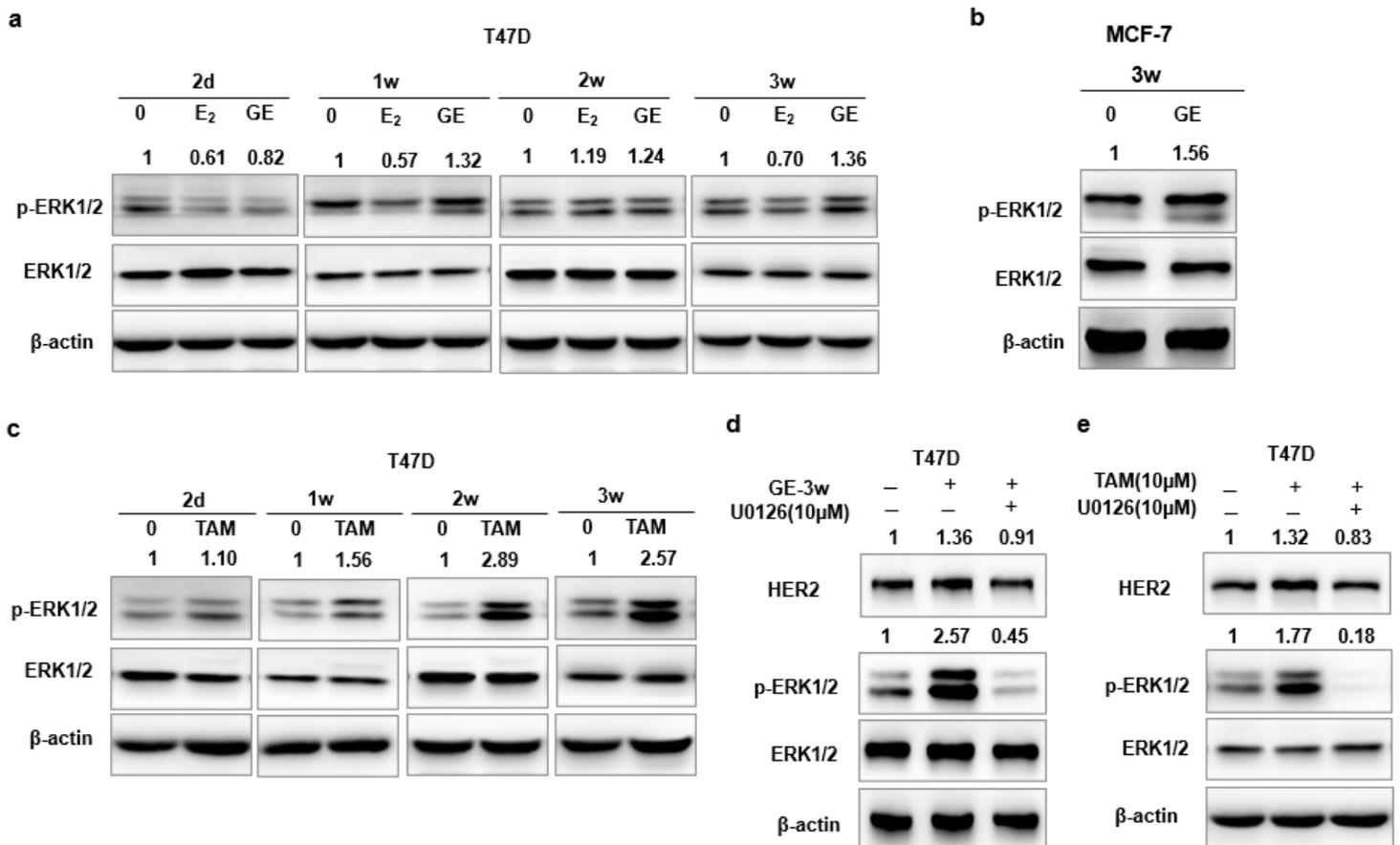


Figure 2

ERK1/2 signal activation caused by long-term exposure to genistein is associated with HER2 elevation (a) Western blot analysis was used to determine phospho-ERK1/2 and ERK1/2 levels at 2 days, 1 week, 2 weeks and 3 weeks after exposure to 1 μ M genistein or 1 nM E2 in T47D cells. (b) Western blot analysis

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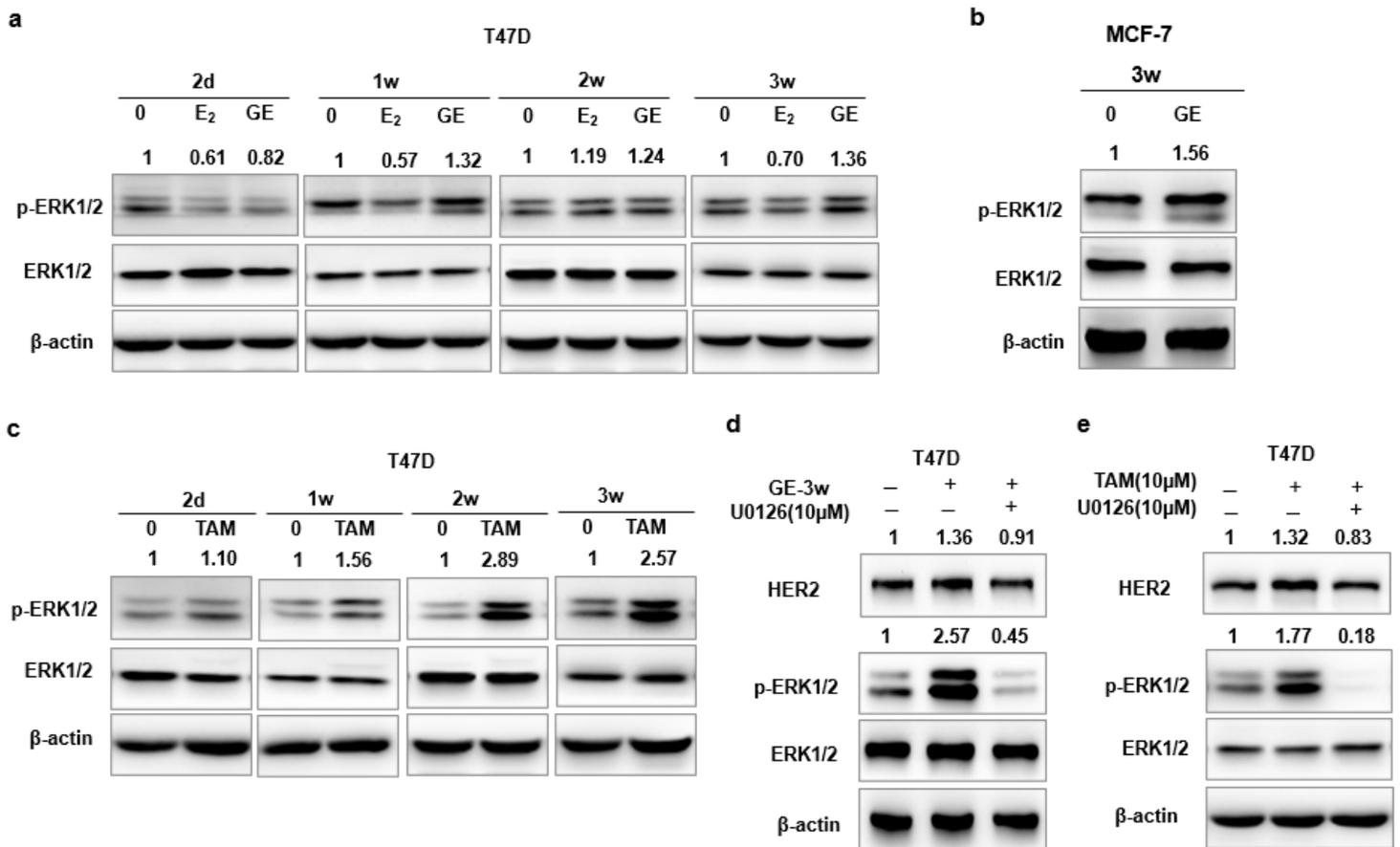


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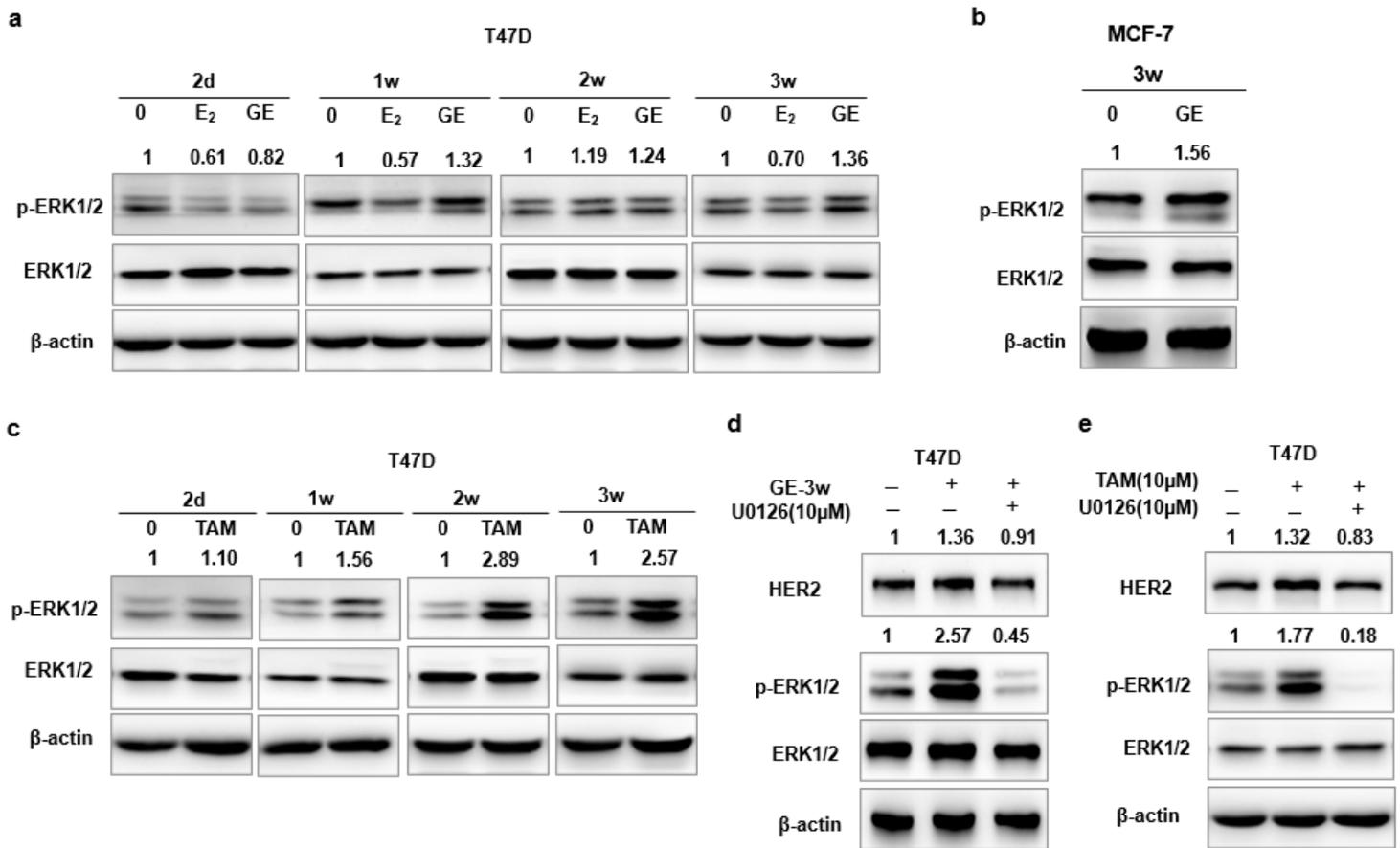


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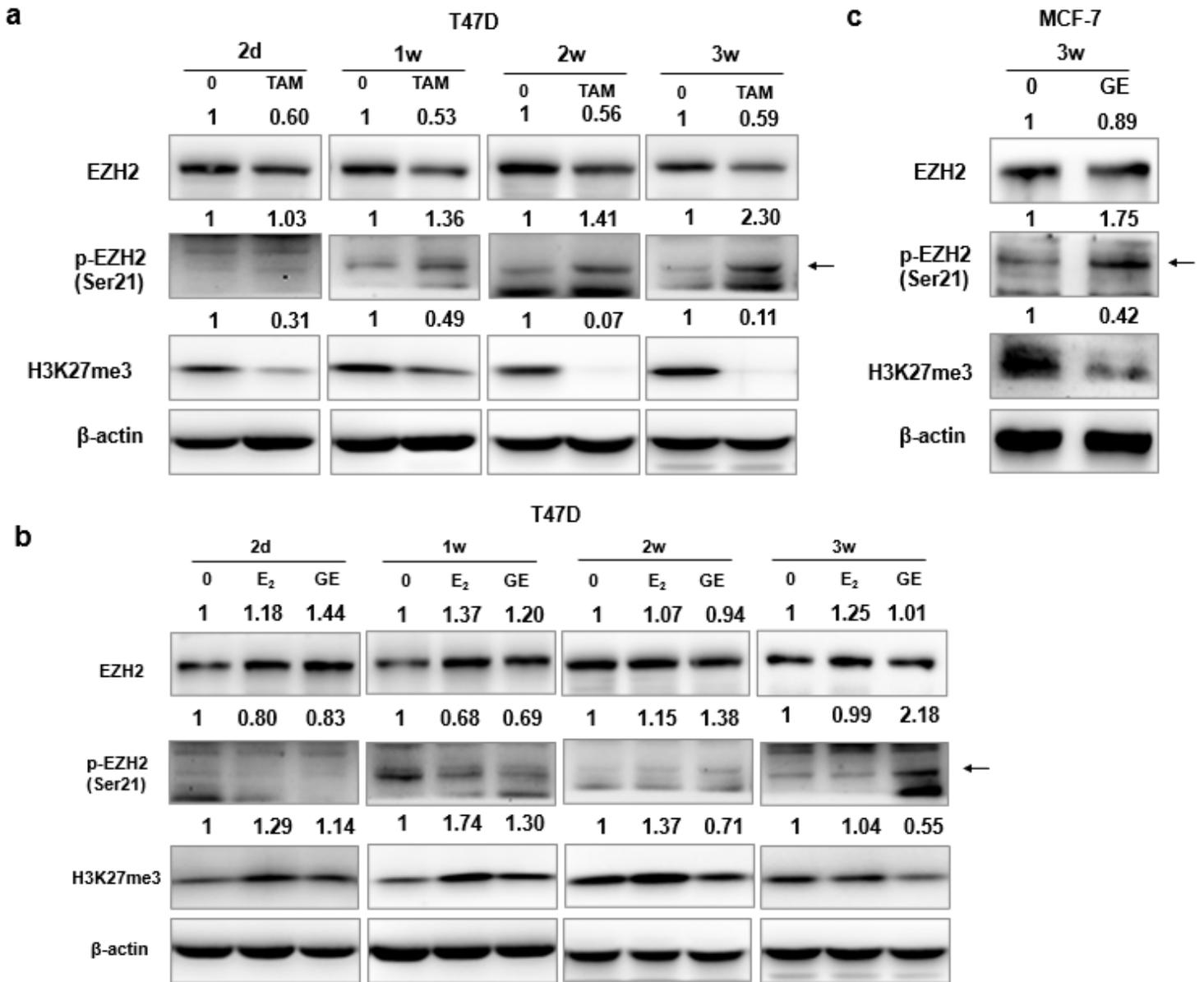


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EZH2 phosphorylation at Ser21 increases and H3K27me3 levels reduce during long-term genistein exposure (a) Western blot analysis was used to determine phospho-EZH2(Ser21), EZH2 and H3K27me3 levels at 2 days, 1 week, 2 weeks and 3 weeks after treatment with 1 μ M TAM in T47D cells. (b) Western blot analysis was used to determine phospho-EZH2(Ser21), EZH2 and H3K27me3 levels at 2 days, 1 week, 2 weeks and 3 weeks after exposure to 1 μ M genistein or 1 nM E₂ in T47D cells. (c) Western blot analysis was used to determine phospho-EZH2(Ser21), EZH2 and H3K27me3 levels at 3 weeks after exposure to 1 μ M genistein in MCF-7 cells.

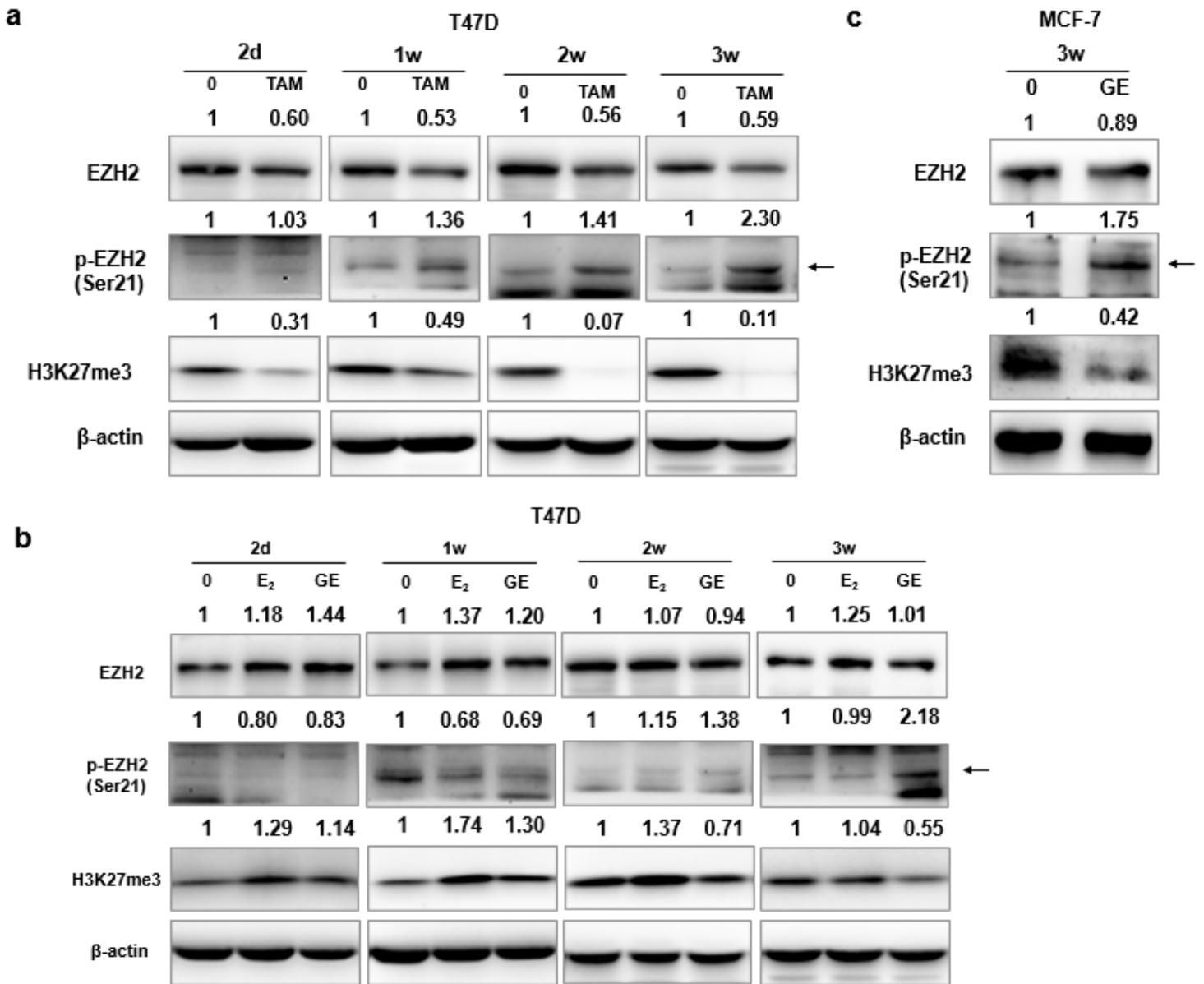


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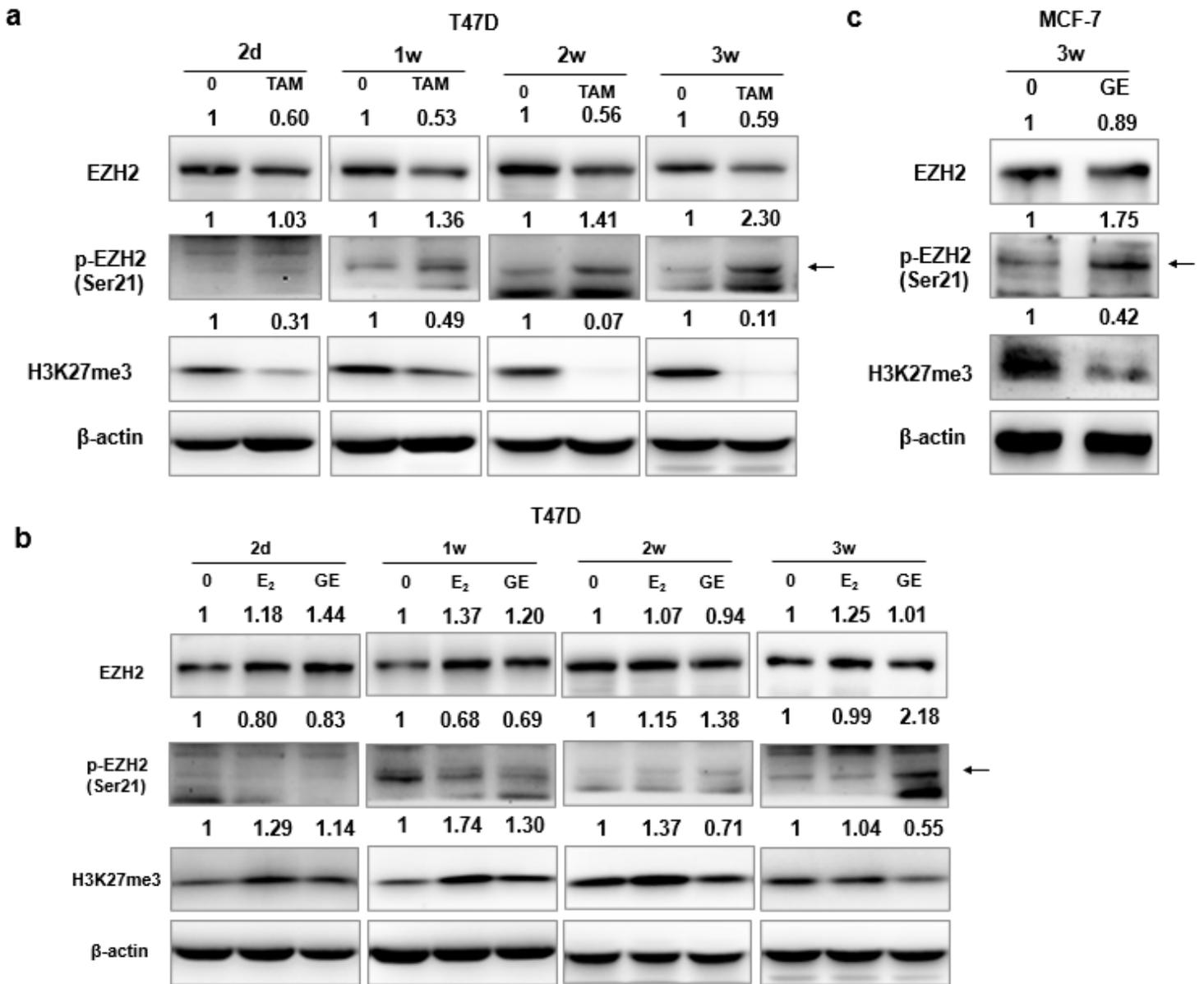


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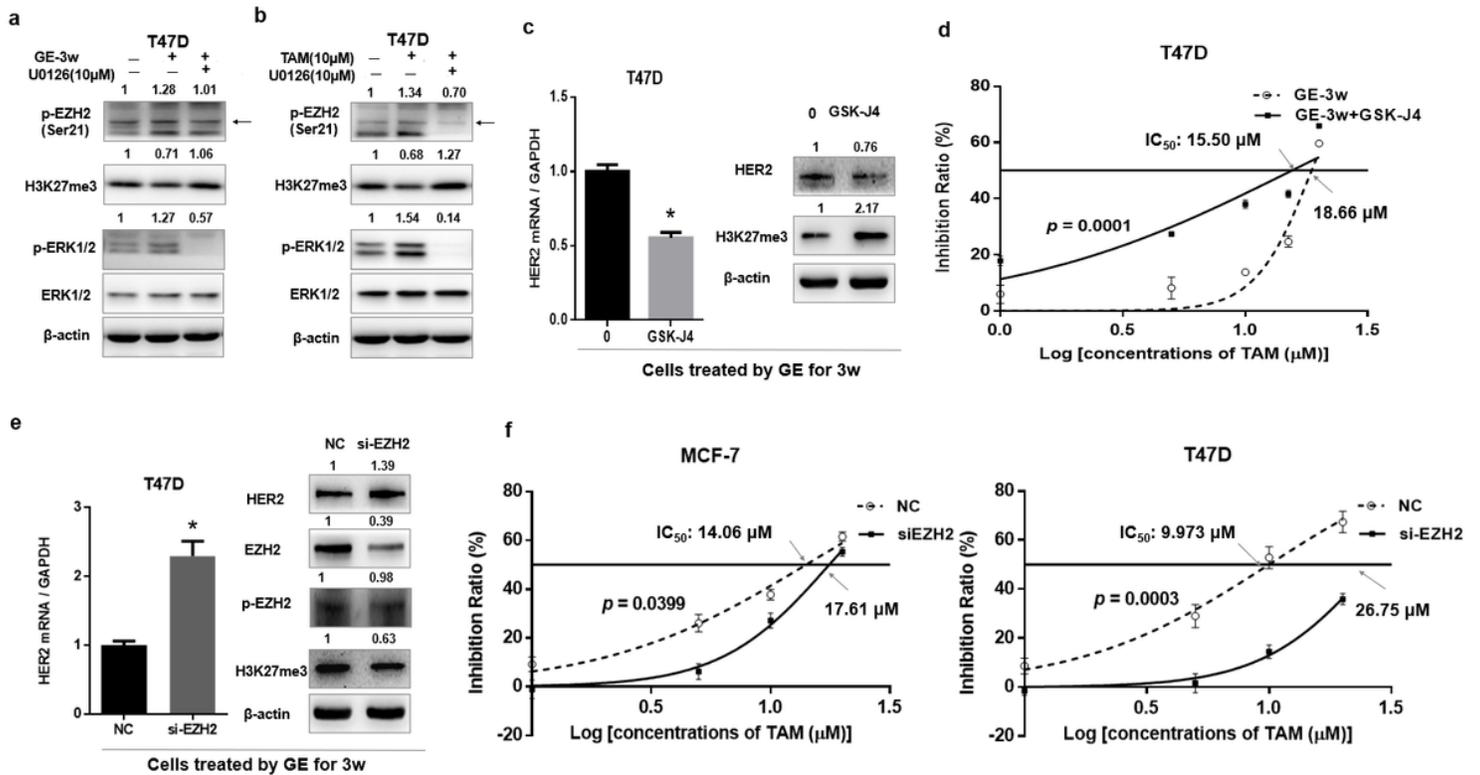


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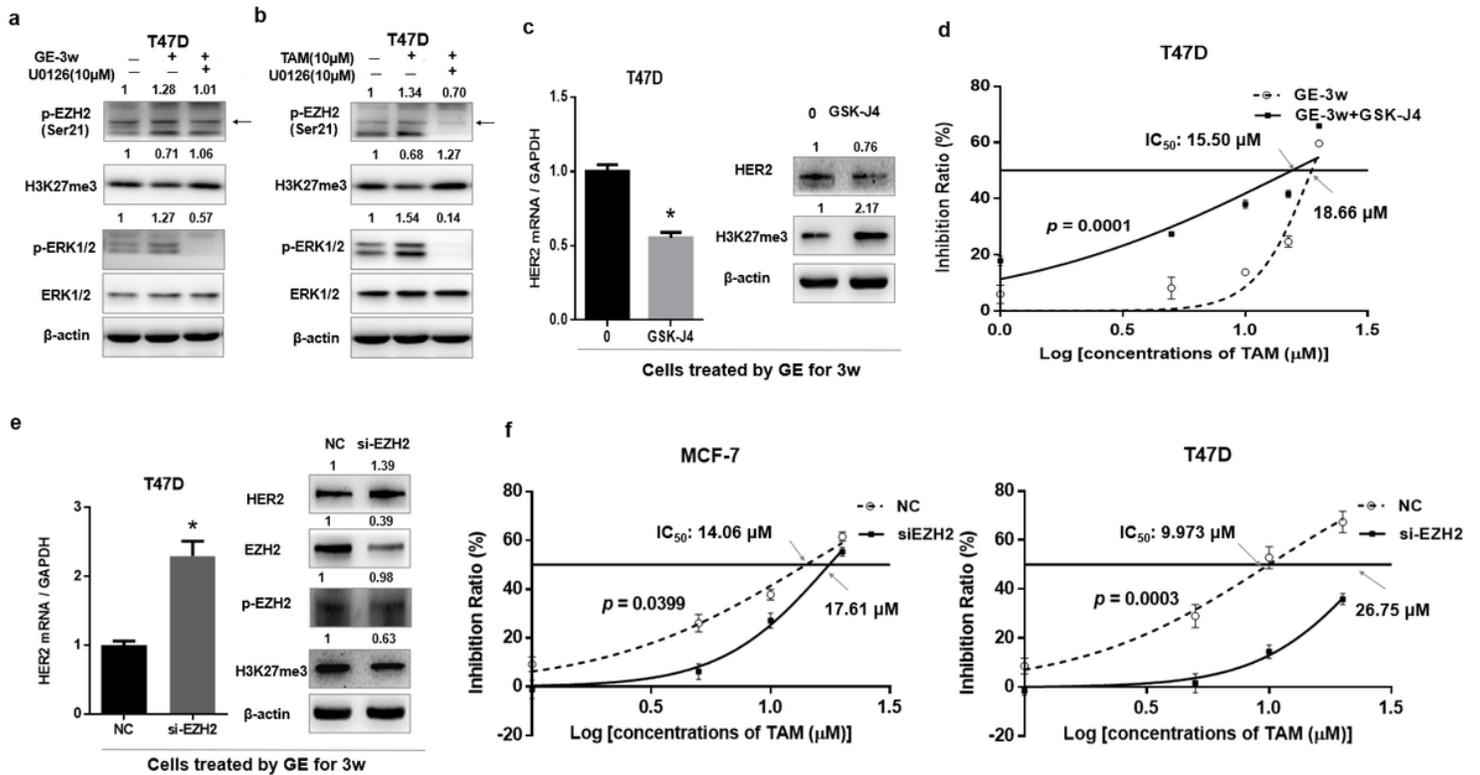


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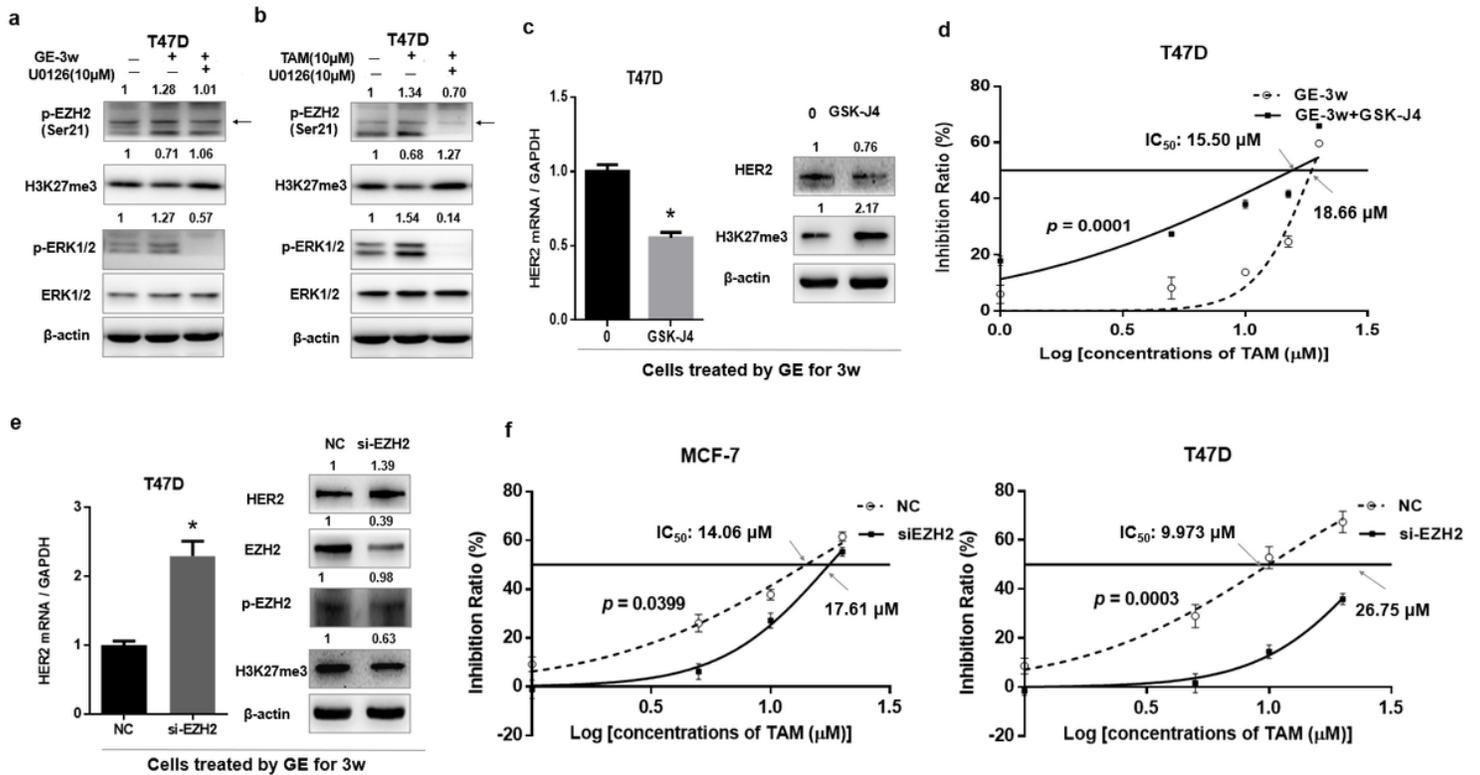


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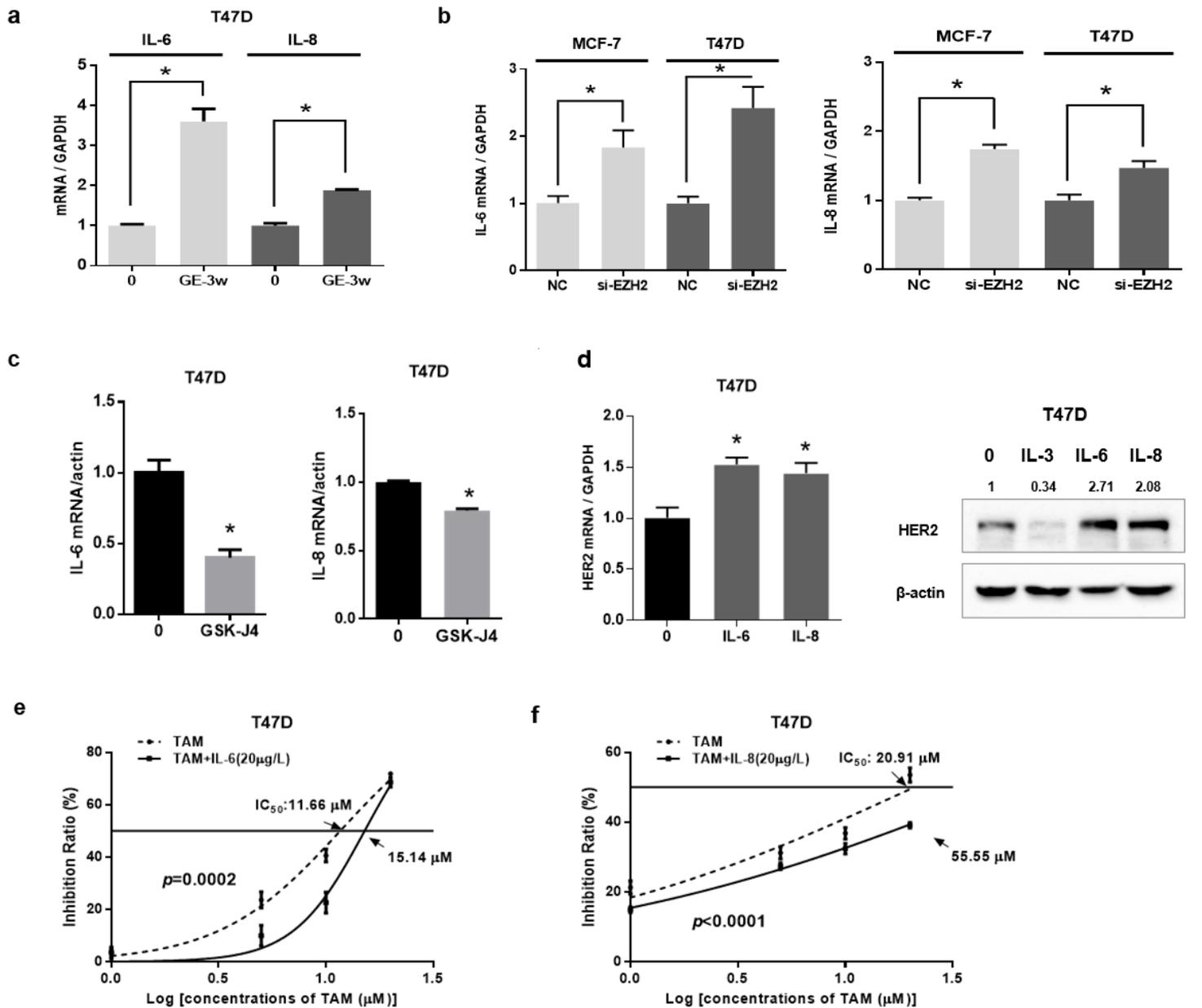


Figure 5

Increased IL-6 and IL-8 induced by genistein is involved in HER2 elevation and endocrine resistance (a) The mRNA levels of IL-6 and IL-8 were assessed by PCR in T47D cells treated with 1 μM genistein for 3 weeks. (b) The mRNA levels of IL-6 and IL-8 were assessed by PCR in MCF-7 cells and T47D cells transfected with EZH2 siRNA for 6h. (c) The mRNA levels of IL-6 and IL-8 were assessed by PCR in T47D cells treated with 5 μM GSK-J4 for 12h. Data of the mRNA are expressed as the mean \pm SD (n = 3), *p < 0.05 vs. control group. (d) The mRNA levels of HER2 were assessed by PCR and the protein levels of HER2 were measured by western blot analysis in T47D cells treated with IL-6 (20 $\mu\text{g/L}$) or IL-8 (20 $\mu\text{g/L}$) for 24 h. (e) T47D cells were treated with 20 $\mu\text{g/L}$ IL-6 for 24h, and then treated with TAM (1 μM -20 μM) for 48h. (f) T47D cells were treated with 20 $\mu\text{g/L}$ IL-8 for 24h, and then treated with TAM (1 μM -20 μM) for 48h. The cell viability was determined by CCK8 assay in quadruplicate, and the IC₅₀s were calculated. Data of the mRNA are expressed as the mean \pm SD (n = 3), *p < 0.05 vs. control group.

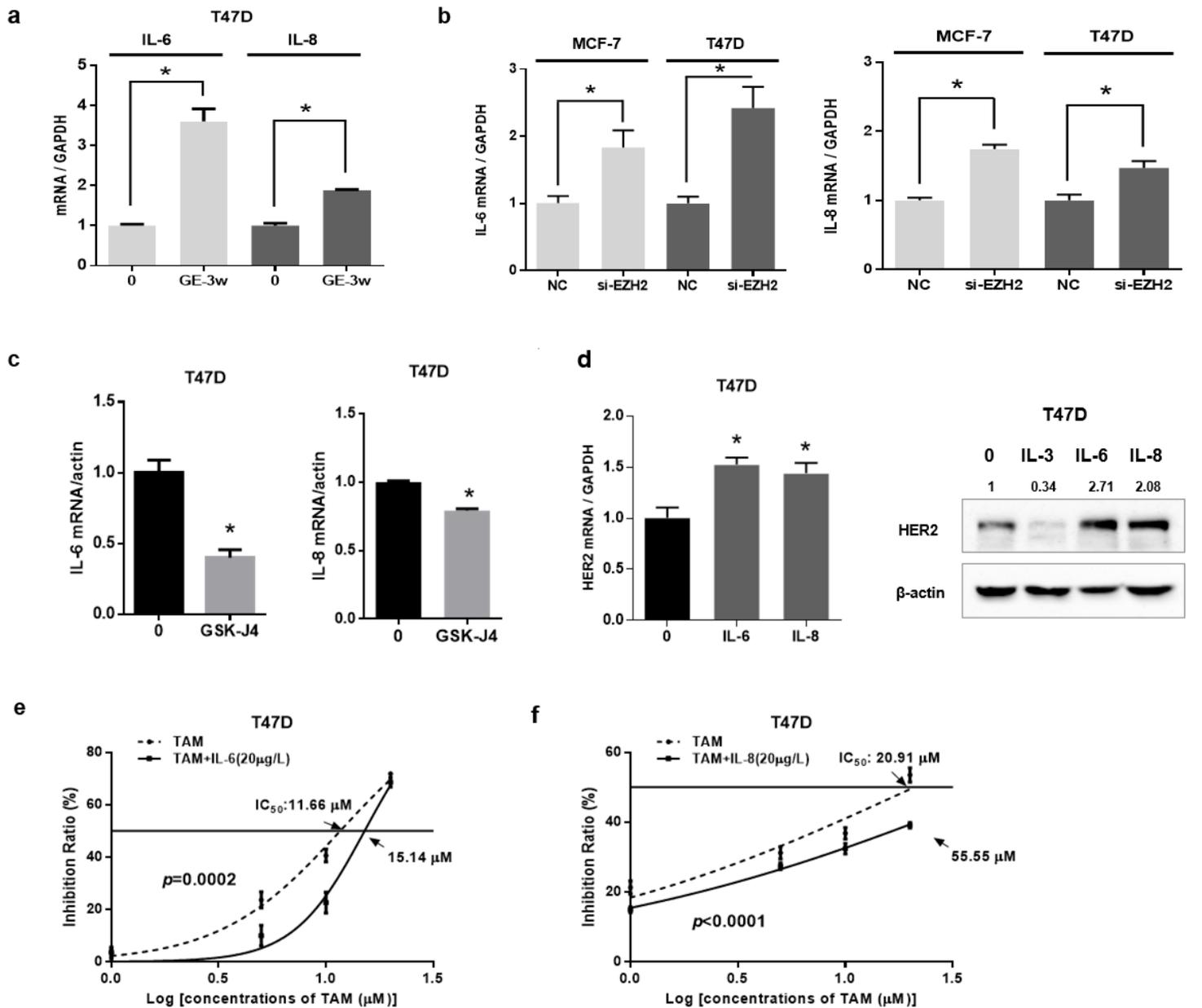


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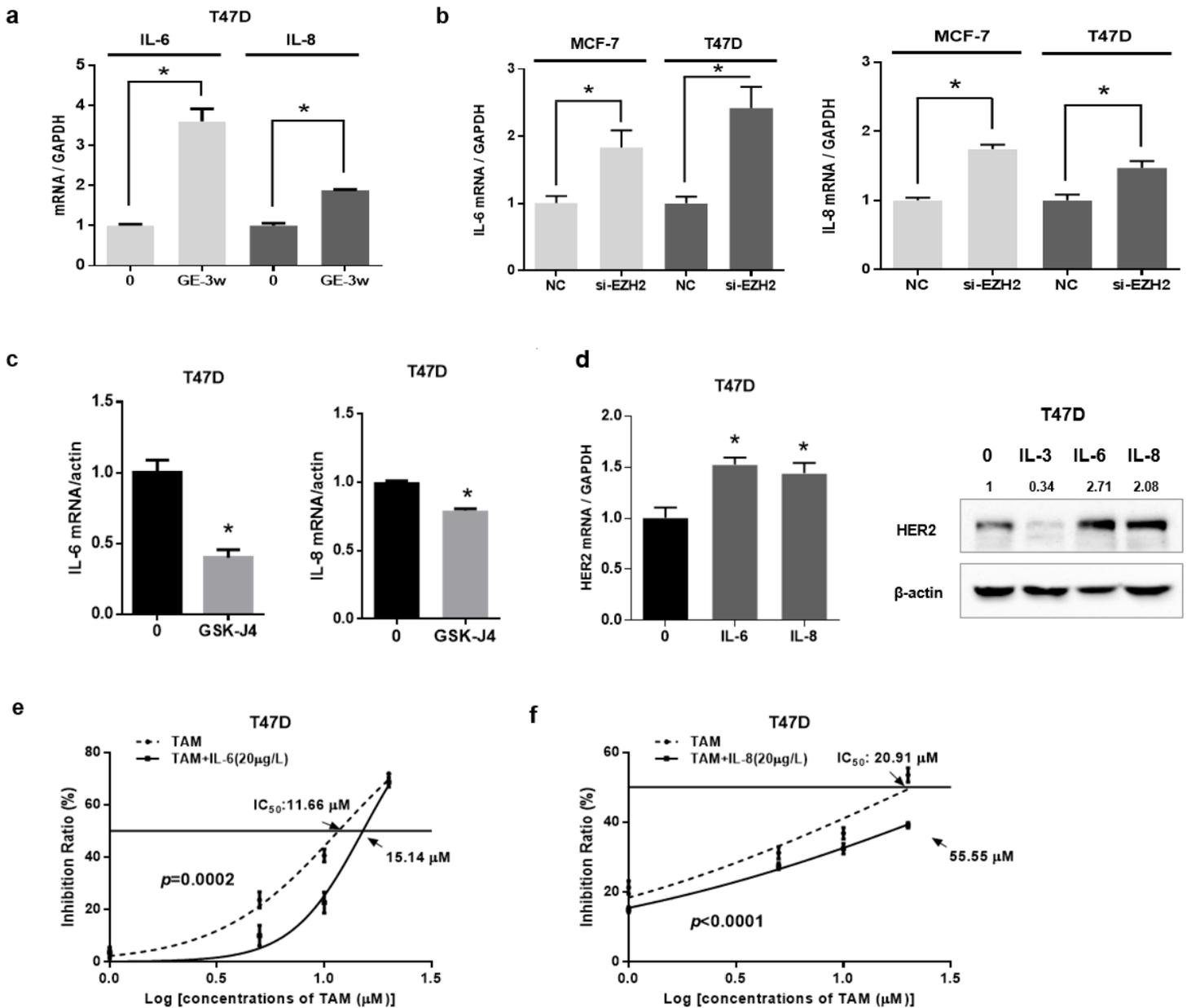


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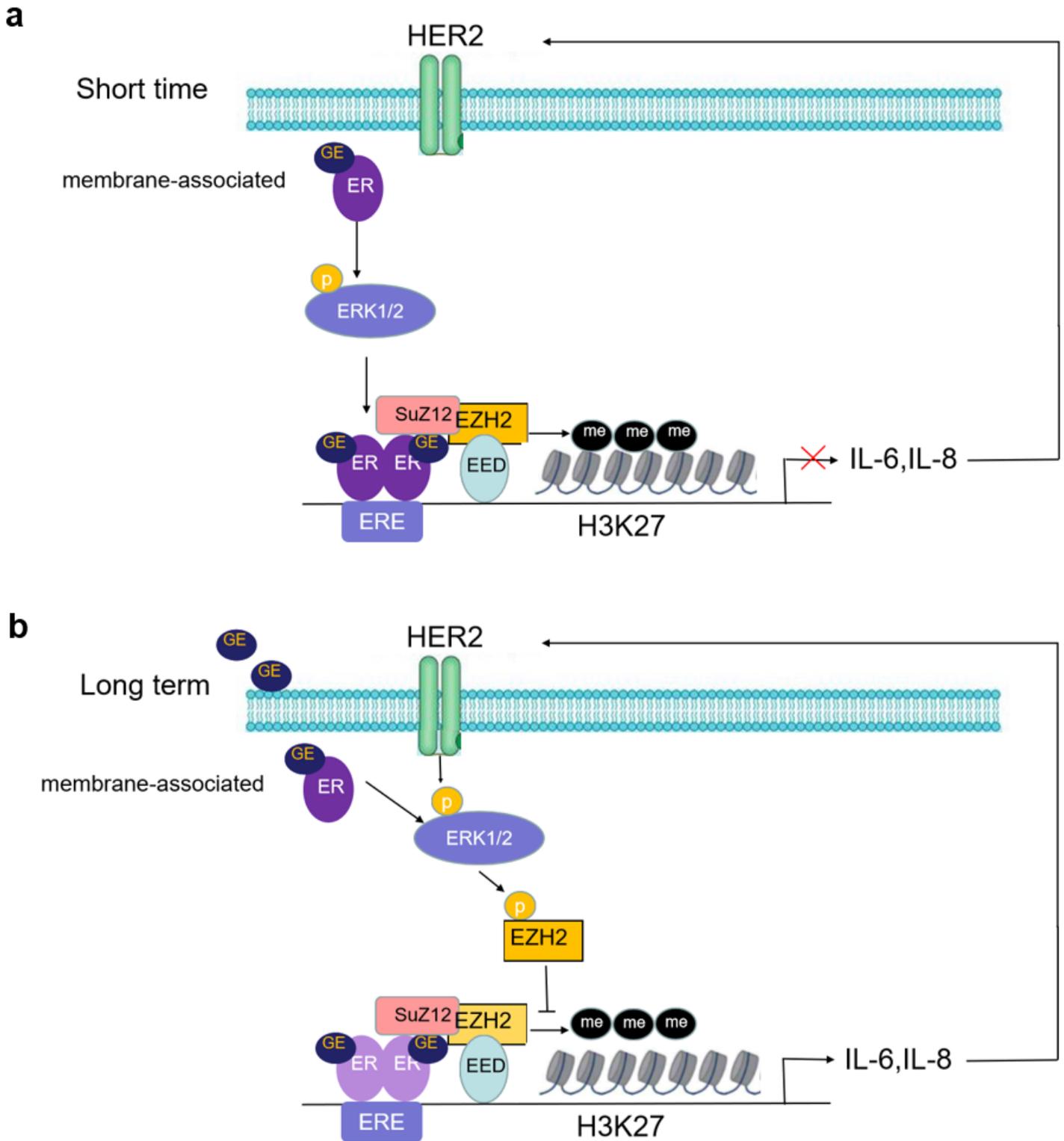


Figure 6

Role of EZH2 in expression of HER2 mediated by genistein in ER -positive breast cancer cells (a) Genistein plays an estrogen-like role in short-term exposure. In the cytoplasm, genistein binds to ER, rapidly activates ERK1/2 signaling and enhances the nuclear transcription function of ER, thereby promoting the expression of EZH2 and enhancing the histone methylation function. H3K27me3 inhibits the expression of IL-6 and IL-8, and thus inhibits the expression of HER2. (b) After long-term exposure

with genistein, the continuous activation of ERK1/2 signal leads to phosphorylation of EZH2 Ser21, resulting in decrease of H3K27me3 level, which alleviates its inhibitory effect on IL-6 and IL-8. IL-6 and IL-8 increase HER2 level, and HER2 further activates ERK1/2 signal, forming the feedback loop of ERK1/2 / EZH2/H3K27me3/ IL-6 and IL-8/ HER2.

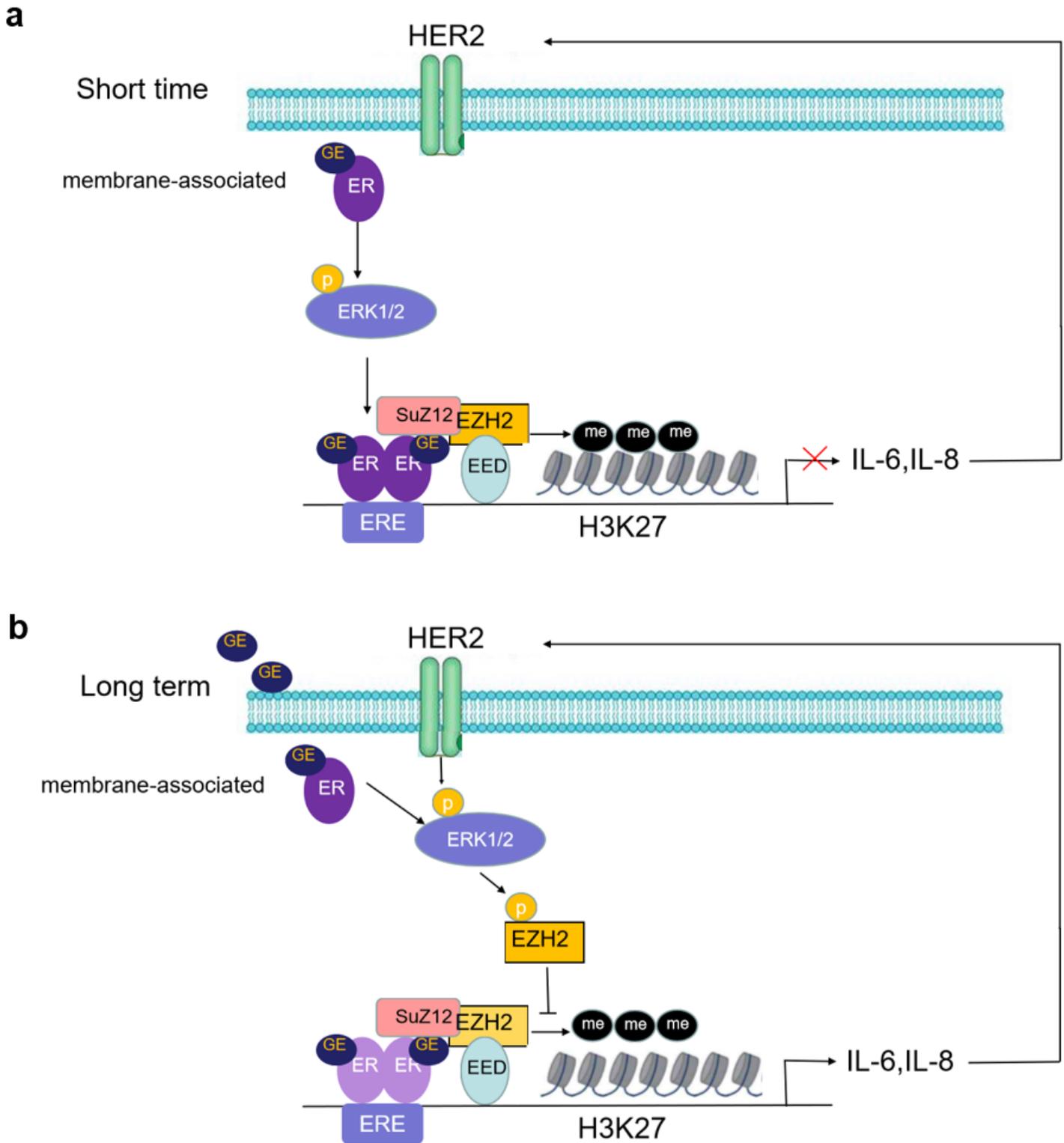


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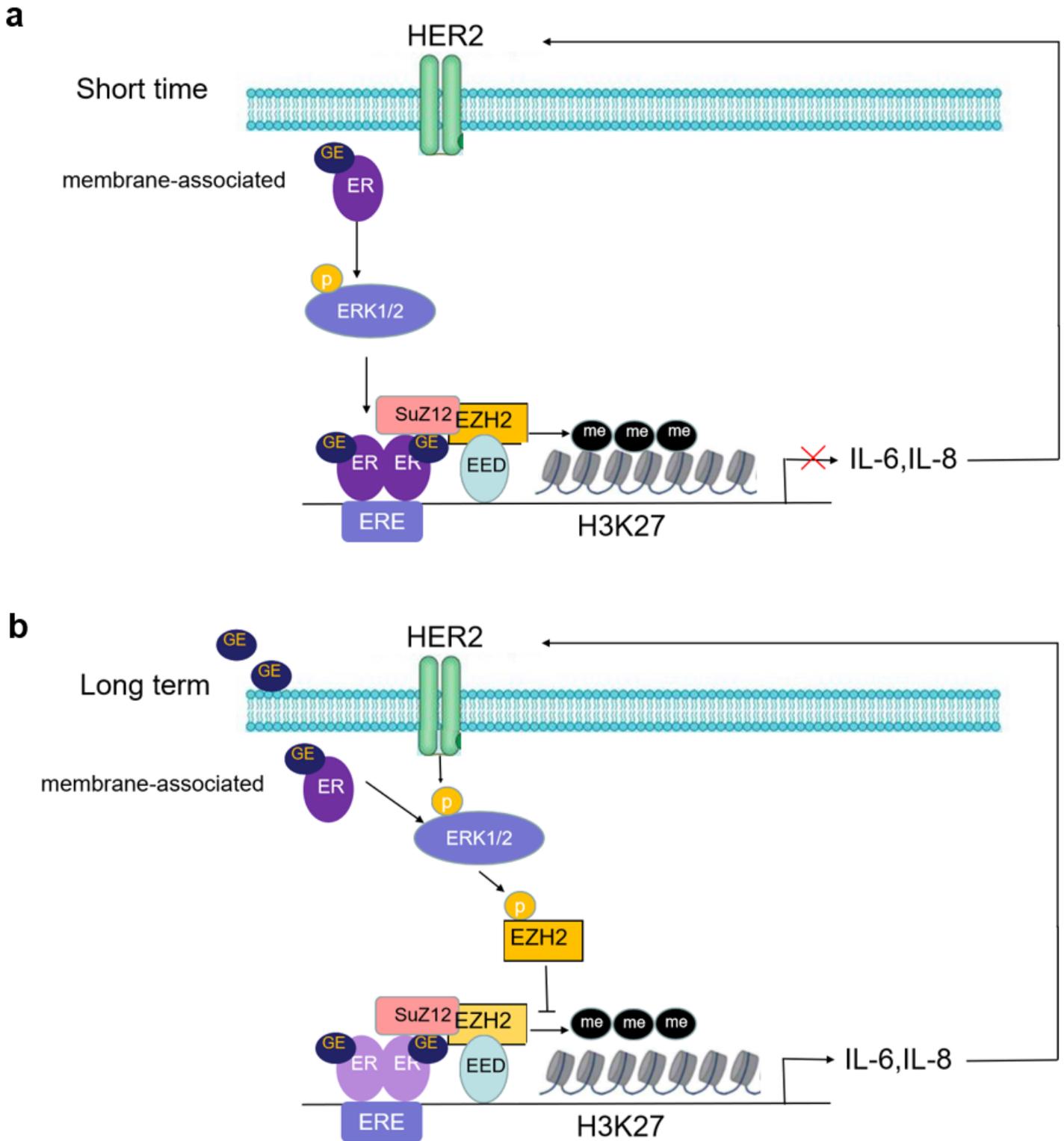


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