GABA_B1e promotes the malignancy of human cancer cells by targeting the tyrosine phosphatase PTPN12

Highlights
- GABA_B1e promotes the malignancy of breast cancer cells both in vitro and in vivo
- Specific phosphorylation of GABA_B1e is critical for its association with PTPN12
- GABA_B1e disrupts EGFR interacting with PTPN12 and induces EGFR-PI3K/Akt signaling
GABAB<sub>1e</sub> promotes the malignancy of human cancer cells by targeting the tyrosine phosphatase PTPN12

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SUMMARY

Neurotransmitter receptors are involved in cancer progression. Among them, the heterodimeric GABA<sub>B</sub> receptor, activated by the main inhibitory neurotransmitter GABA, is composed of the transmembrane GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits. The oncogenic role of the isoform GABA<sub>B1e</sub> (GB1<sub>e</sub>) containing only the extracellular domain of GABA<sub>B1</sub> remains unclear. We revealed that GB1<sub>e</sub> is largely expressed in human breast cancer (BrCa) cell lines as well as in BrCa tissues where it is upregulated. Moreover, GB1<sub>e</sub> promoted the malignancy of BrCa cells both in vitro and in vivo. We propose that GB1<sub>e</sub> favors EGFR signaling by interacting with PTPN12 to disrupt the interaction between EGFR and PTPN12, and phosphorylation of Y230 and Y404 on GB1<sub>e</sub> is required in this process. Our data highlight that the GABBR1 gene through the expression of the GB1<sub>e</sub> isoform might play an important oncogenic role in BrCa and that GB1<sub>e</sub> is of interest for the treatment of some cancers.

INTRODUCTION

Breast cancer (BrCa) is the most commonly diagnosed cancer and the leading cause of cancer death among women worldwide, with an estimation of nearly 2.3 million new cases and 685,000 deaths in 2020 (Sung et al., 2021). Although significant improvements have been made in BrCa diagnosis and treatment, it remains the leading cause of cancer death in women (Neophytou et al., 2018). It is therefore critical to identify new potential targets for the treatment of BrCa.

Neurotransmitter receptors are involved in cancer progression due to the modulatory effects of neurotransmitter on cancer cells (Schuller, 2008). The metabotropic receptor for the main inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Gassmann and Bettler, 2012), i.e. GABA<sub>B</sub> receptor, plays a controversial role in cancer cell growth, migration, invasion, and tumorigenesis. GABA<sub>B</sub> receptor agonists significantly reduced the incidence and number of gastric cancers in rats (Tatsuta et al., 1990), decreased the migration and metastasis of colon cancer in mice (Joseph et al., 2002; Thaker et al., 2005), inhibited the growth of human hepatocellular carcinoma cells both in vitro and in vivo (Wang et al., 2008), and reduced the growth of cholangiocarcinoma cells (Fava et al., 2005; Huang et al., 2013). In contrast, other studies revealed that GABA<sub>B</sub> receptor activation promoted the invasive ability of prostate and renal cancer cells (Azuma et al., 2003; Inamoto et al., 2007) and the metastasis of mouse breast 4T1 cancer cells (Zhang et al., 2014). We have also recently reported that GABA<sub>B</sub> receptor induced epidermal growth factor (EGFR) transactivation and promoted the invasion of human prostate cancer cells (Xia et al., 2017).

GABA<sub>B</sub> receptor is a G-protein-coupled receptor (GPCR) and is composed of GABA<sub>B1</sub> (GB<sub>1</sub>) and GABA<sub>B2</sub> (GB<sub>2</sub>) subunits (Pin et al., 2004), encoded by the GABBR1 and GABBR2 genes, respectively. GB<sub>1</sub> has at least 14 isoforms (GB<sub>1</sub>-a-l), among which GB<sub>1a</sub> and GB<sub>1b</sub> are the most abundant isoforms predominantly expressed in central nervous system (Jiang et al., 2012). GB<sub>1ab</sub> contains a large N-terminal extracellular domain (ECD) responsible for GABA, agonist and antagonist binding, a seven-transmembrane domain (TMD), and a C-terminal intracellular region. But the activation of the G protein is mediated by the TMD of GB<sub>2</sub> (Bettler et al., 2004; Pin and Bettler, 2016). Of interest, GB<sub>1e</sub> is a truncated GB<sub>1</sub> isoform resulting from the alternative splicing of GABBR1 gene, and it contains only the GB1<sub>ECD</sub> ended by nine extra C-terminal residues. GB1<sub>e</sub> is predominantly transcribed in human and rat peripheral tissues (Mizuta et al., 2008; Schwarz et al., 2000), but its function remains unknown.
In this study, we demonstrate that GB1e promotes the malignancy of human BrCa cells both in vitro and in vivo. GB1e is the predominant GB1 isoform, and it is upregulated in various human BrCa cell lines and tissues. Its expression level correlates with the tumorigenic potential of BrCa cell lines. Interestingly, GB1e is retrotranslocated from the ER lumen to the cytosol, where it undergoes proteasomal degradation or tyrosine phosphorylation. Phosphorylation of Y230 and Y404 on GB1e is critical for GB1e to hijack the protein tyrosine phosphatase non-receptor type 12 (PTPN12) from EGFR, which in turn promotes cancer cells growth and invasion. Our finding provides a new view for the treatment of BrCa by targeting the interactions between GB1e, PTPN12 and EGFR.

RESULTS

GB1e is abundant in human BrCa cell lines and tissues

GB1e, containing only the ECD of GB1a (Figures 1A and S1A), has been reported to be the predominant GB1 transcript in human and rat peripheral tissues (Schwarz et al., 2000). Our reverse-transcriptase PCR (RT-PCR) results showed that GB1e transcript (420 bps) was transcribed at different levels in the normal breast MCF10A cells and eight human BrCa cell lines representing major breast cancer subtypes, i.e. estrogen receptor (ER)+/progesterone receptor (PR)+ (MCF7 and T-47D), Her2+ (BT474, AU565, and SK-BR-3), and ER+/PR+/Her2− or triple negative (MDA-MB-453, MDA-MB-231, and BT-549) cell lines. As a negative control, GB1a/b transcript (570 bps) was only detected in cerebellar granule neurons (CGNs) (Figures 1B and S1B). Quantitative RT-PCR using GB1e-specific primers showed that highly tumorigenic MDA-MB-231 and BT-549 cells had higher levels of GB1e mRNA compared with the poorly tumorigenic MCF7 and T-47D cells (Figures S1B and S2).

The expression of GB1e protein in BrCa cell lines was assessed by immunoblotting with a monoclonal antibody recognizing the N-terminal sequence of GB1. GB1e protein (~78 kDa) was detected in all of the eight BrCa cell lines as well as in MCF-10A cells. Interestingly, higher level of GB1e protein was detected in BrCa cell lines with higher tumorigenic potential (Figures 1C and 1D). As a negative control, GB1a protein (~130 kDa) was only detected in CGNs.

We next examined the expression of GB1e mRNA and protein in 36 BrCa specimens and the matched para-cancerous tissues. Although GB1e transcript was the primary product in both BrCa and para-cancerous tissues, the average levels of GB1e transcript and protein were significantly higher in cancer specimens than those in para-cancerous tissues (Figures 1E, 1F, and S3). Moreover, cancer specimen was intensively immunostained with GB1 antibody, whereas the para-cancerous tissue was moderately stained (Figure 1G).

Collectively, different expression level of GB1e was detected in both human BrCa cell lines and tissues, and this GB1e level correlates with the tumorigenic potential of BrCa cell lines.

GB1e is secreted and retrotranslocated to the cytosol

We investigated the cellular distribution and secretion of GB1e. In MCF7 cells transfected with the Flag-tagged GB1e (MCF7-GB1e), GB1e was detected at the cell surface and in the cytoplasm, before and after cell membrane permeabilization, respectively (Figure S4A). The majority of GB1e was distributed in the cytoplasm as shown by the lower Flag signal under nonpermeabilized conditions (Figure 2A). GB1e was also secreted to the medium as revealed by the immunoblotting analysis of conditioned medium (CM) with an anti-GB1 antibody (Figure 2B). Of note, endogenous GB1e was also detected in the cells transfected with the empty plasmid. Two bands corresponding to ~100 and ~78 kDa were detected only in the CM but not in the total cell lysates (TCLs) where only the ~78 kDa was detected. We proposed that this 78 kDa band corresponds to glycosylated GB1e that could be secreted but also be present in the cytosol. In contrast, the secreted 100 kDa protein corresponds to a full glycosylated GB1e, with posttranslational modifications that occur in both ER and Golgi apparatus. This hypothesis is validated by the treatment of the cells with tunicamycin, an inhibitor of protein N-glycosylation, that resulted in one band of GB1e at ~65 kDa in the TCLs, whereas no band was detected in the CM (Figure 2C). We also treated the cells with brefeldin A, a compound disrupting the structure and function of the Golgi apparatus, and the results showed that the intensities of both ~100 and ~78 kDa bands in the CM were strongly reduced, whereas that of ~78 kDa in the TCLs was slightly increased (Figure 2D). These observations suggest that GB1e is modified by N-glycosylation, which occurs in both ER and Golgi apparatus.

Endoplasmic reticulum (ER) is a major site of protein synthesis, and increased protein synthesis may induce ER stress and then the retrotranslocation of proteins from the ER lumen to the cytosol, where proteins...
Figure 1. GB1e is abundant in human BrCa cell lines and tissues

(A) The schematic structure of GB1e, GB1a, and GB1b. SD, sushi domain; ECD, extracellular domain; TMD, transmembrane domain.

(B) mRNAs were isolated from the indicated human BrCa cell lines and subjected to RT-PCR analysis. Amplicons representing GB1a/b and GB1e were amplified by using the same primer pairs. Cerebellar granule neurons (CGNs) were used as the positive control for GB1a/b expression.

(C) Immunoblot analysis of GB1e in the indicated BrCa cell lines. MCF7 cells overexpressing GB1e (MCF7-GB1e) and CGNs were used as positive controls for GB1e and GB1a, respectively. β-actin was used as a loading control.

(D) Quantitative histograms of GB1e expression shown in panel (C). Fold changes of GB1e expression was normalized to β-actin. The data are presented as mean ± SEM (n = 3).

(E) Immunoblot analysis of GB1e in human representative BrCa and tumor adjacent tissues. β-actin was used as a loading control.

(F) Quantitative plot of GB1e expression normalized to β-actin. The data are presented as mean ± SEM (n = 3) and analyzed by unpaired t test. ***p < 0.001.

(G) Immunohistochemical analysis of paraffin sections of human BrCa and tumor adjacent tissues with an anti-GB1 antibody. Scale bar, 50 μm.

See also Figures S1, S2, S3 and S11.
undergo proteasomal degradation (Qu et al., 2021). It has been reported that ER-derived microsomes, containing the retrotranslocon channel that pulls proteins from the ER lumen at the expense of ATP, could be used to study protein export as a cell-free system in vitro (Schmitz et al., 2000; Wahlman et al., 2007). To investigate whether GB1e is retrotranslocated to the cytosol in BrCa cells with high GB1e expression, microsomes were isolated from MCF7 cells transfected with GB1e or from MDA-MB-231 cells with high level of endogenous GB1e. The purity and integrity of the isolated microsomes were indicated by the enrichment of the ER marker calnexin (Figure S4B). Trypsin treatment resulted in no degradation of microsomal GB1e in the absence of Triton X-100, but its complete degradation was detected in the presence of Triton X-100, indicating that GB1e is located within the microsomal lumen and no GB1e is attached to the outer surface of the microsomal membranes (Figure S4C). In the protein export assay, microsomes were resuspended in export buffer with or without ATP. The results showed that GB1e was detected in the microsome pellet but barely detectable in the supernatant in the absence of ATP. However, a time-dependent increase of GB1e
Figure 3. GB1e promotes the malignancy of human BrCa cells in vitro

(A–D) MCF7 and T-47D cells were stably transfected with GB1e or vector. (A) Immunoblot analysis assessing the amounts of GB1e with β-actin used as a loading control. (B) Cell proliferation was measured by adding CCK-8 solution to each well 24, 48, 72, or 96 h after cell plating in a 24-well plate (2 × 10^4 cells/well). (C) Clonogenic assay in which 200 cells were seeded into 24-well plates and cultured for 10–14 days with medium replacement for every 3 days. Colonies visible to the naked eyes were fixed and stained. Representative images were captured (left panel), and the numbers of colonies were counted (right panel). (D) Spheroid formation assay in which 2 × 10^4 cells were seeded into ultra-low attachment 24-well plates and cultured for 10–14 days.
was detected in the supernatant in the presence of ATP. Resuspension of microsomes in 50% reticulocyte lysate plus ATP also resulted in a time-dependent appearance of GB1e in the supernatant (Figure 2E). These data demonstrate the export of GB1e in this cell-free system. Finally, we showed that the treatment of MDA-MB-231 and MCF7-GB1e cells with MG132, a proteasomal inhibitor, increased GB1e levels in the TCLs (Figure 2F). The effect was observed at 6 hours posttreatment with a maximum amount of GB1e after 18 h, whereas GB1e levels in the CMs were not influenced by MG132. Altogether, our results demonstrate that GB1e is retrotranslocated from the ER lumen to the cytosol, where it undergoes proteasomal degradation.

GB1e promotes the malignancy of human BrCa cells in vitro

To investigate the function of GB1e in BrCa cells, GB1e was overexpressed in the poorly tumorigenic MCF7 and T-47D cells. Conversely, endogenous GB1e was silenced in the highly tumorigenic MDA-MB-231 and BT-549 cells by using two different shRNAs (sh-GB1 #1 and sh-GB1 #2) targeting two different regions of the GB1 mRNA (Figure S1B).

GB1e overexpression accelerated the proliferation of MCF7 and T-47D cells (Figures 3A and 3B), whereas GB1e silence remarkably slowed down the proliferation of MDA-MB-231 and BT-549 cells (Figures 3E and 3F). Moreover, GB1e overexpression markedly increased the numbers of colonies and spheroids formed by MCF7 and T-47D cells (Figures 3C and 3D), but GB1e silence significantly inhibited the clonogenicity and spheroid formation of MDA-MB-231 and BT-549 cells (Figures 3G and 3H). Furthermore, the numbers of migrated and invaded MDA-MB-231 and BT-549 cells were dramatically decreased by silenced GB1e (Figures 3I and 3J). These results demonstrate that GB1e has a tumorigenic role in BrCa cells.

GB1e silence suppresses the growth and metastasis of BrCa cells in nude mice

GB1e silenced or control MDA-MB-231 cells were subcutaneously injected into the dorsal flanks of athymic BALB/c mice. The tumors induced by GB1e silenced cells showed longer latency and smaller mean volume than those induced by control cells (Figures 4A and 4B). Correspondingly, the average tumor weights were dramatically reduced by GB1e silence (Figure 4C). In tumor xenografts induced by GB1e silenced cells, GB1e expression was barely detectable, and the percentage of Ki-67 positive cells was significantly decreased compared with that in the control tumors (Figure 4D). It suggested that GB1e promotes the growth of BrCa cells in vivo.

When GB1e silenced MDA-MB-231 cells were injected into the tail vein of athymic BALB/c mice, the lung metastasis was suppressed compared with the control MDA-MB-231 cells (Figures 4E and 4F). Large tumor cell mass was detected in the lungs of mice injected with control cells, but GB1e silence markedly inhibited the colonization of MDA-MB-231 cells in the lungs (Figure 4G). These results suggest that GB1e promotes the metastasis induced by MDA-MB-231 cells.

GB1e displaces EGFR-bound PTPN12 and enhances EGFR activity

The in vivo data indicate that GB1e is oncogenic. To investigate the oncogenic mechanism of GB1e, GB1e interacting proteins in MCF7-GB1e cells were analyzed by tandem LC/MS/MS, and PTPN12 appeared to be one of the candidate proteins (Table S1). Co-immunoprecipitation (co-IP) analysis revealed that Flag-GB1e and PTPN12 are in the same complex in MCF7 and T-47D cells transfected with either Flag-GB1e or the
empty vector (Figure 5A). Similar results were obtained in the reciprocal co-IP experiment (Figure 5B). These results suggest that both endogenous and overexpressing GB1e interact with PTPN12. Of note, a pair of sushi domains (SDs) is located at the N-terminus of GB1e (Figure S5A). SDs have been reported to mediate protein-protein interactions (Lehtinen et al., 2004; Terunuma, 2018). Interestingly, similar co-IP results were obtained with GB1e truncated with SD sequence (Figures S5B and S5C), indicating that the interaction between GB1e and PTPN12 is independent of GB1e SDs.

It was demonstrated that PTPN12 can dephosphorylate EGFR (Li et al., 2015; Nair et al., 2018; Sun et al., 2011). Co-IP experiments showed that the endogenous PTPN12 and EGFR are in the same complexes in MCF7 and T-47D cells stably transfected with GB1e as well as in GB1e silenced MDA-MB-231 and BT-549 cells (Figures 5C and 5D). Interestingly, the association between PTPN12 and EGFR was reduced in GB1e transfected cells (Figure 5C) but enhanced in GB1e silenced cells (Figure 5D). These results are consistent with a model that GB1e is a competitor for the interaction between PTPN2 and EGFR and these three
proteins cannot be in the same complex, which is in agreement with the absence of co-IP between GB1e and EGFR (Figure 5C).

To examine the effect of GB1e on EGFR activity, co-IP was performed using the anti-phosphotyrosine antibody 4G10 and immunoblotted with an anti-EGFR antibody. The results revealed that overexpression of GB1e dramatically increased EGFR phosphorylation in MCF7 and T-47D cells (Figure 5C), but GB1e silence significantly reduced EGFR phosphorylation in MDA-MB-231 and BT-549 cells (Figure 5D). These data suggested that GB1e limits the negative effect of PTPN12 on EGFR activity.

Figure 5. GB1e displaces EGFR-bound PTPN12 and enhances EGFR activity  
(A–C) TCLs from MCF7 and T-47D cells stably transfected with GB1e or vector were subjected to co-IP and/or IP analysis. (A–B) (Upper panels) The TCLs were immunoprecipitated with anti-Flag (A) or anti-PTPN12 (B) antibody, and the immunoprecipitates were probed with anti-PTPN12 and anti-GB1 antibodies. Mouse IgG was used as a negative control. (Lower panels) Immunoblot analysis assessing the levels of PTPN12, GB1e, and β-actin in the input. (C) TCLs were immunoprecipitated with 4G10 or anti-EGFR antibody; the precipitates were probed with anti-EGFR, anti-PTPN12, or anti-GB1 antibody. The input levels of EGFR and PTPN12 were evaluated by immunoblotting. The intensity of each immunoblot band was quantitated by ImageJ, and the data were presented as the fold changes (mean ± SEM; n = 3).

(D) TCLs from MDA-MB-231 and BT-549 cells transfected with sh-GB1 #1, #2, or sh-ctrl (control shRNA) were immunoprecipitated with 4G10 or anti-EGFR antibody, and the precipitates were probed with anti-EGFR and anti-PTPN12 antibodies. The input levels of EGFR and PTPN12 were analyzed by immunoblotting. The intensity of each immunoblot band was quantitated by ImageJ, and the data were presented as the fold changes (mean ± SEM; n = 3).

(E) Immunoblot analysis assessing the levels of pAkt (S473), total Akt, pERK1/2, and total ERK1/2 in the indicated cells. β-actin was used as a loading control. The intensity of each immunoblot band was quantitated by ImageJ, and the data were presented as the fold changes (mean ± SEM; n = 3).

See also Figures S5, S6, S7, S8 and Table S1.
Figure 6. Phosphorylation of Y230 and Y404 on GB1e is critical for GB1e to hijack PTPN12 from EGFR

(A and B) TCLs (A) and CM (B) from MCF7-GB1e cells were immunoprecipitated with 4G10 (A) or anti-Flag (A and B) antibody, and the precipitates were probed with anti-GB1 or 4G10 antibody. The levels of GB1e and β-actin in the input (A) were evaluated by immunoblotting.

(C) TCLs from MCF7-GB1e cells transfected with wild-type (WT) PTPN12, catalytic dead PTPN12 (C231S), or PTPN12 siRNA were immunoprecipitation with 4G10 antibody, and the precipitates were probed with anti-GB1 antibody. The input levels of GB1e, PTPN12, and β-actin were evaluated by immunoblotting.

The intensity of each immunoblot band was quantitated by ImageJ, and the data were expressed as the fold changes (mean ± SEM; n = 3).

(D) TCLs from MCF7 cells transfected with the indicated constructs were immunoprecipitated with 4G10 or anti-PTPN12 antibody, and the precipitates were subjected to immunoblotting with anti-GB1 antibody. The input levels of GB1e and PTPN12 were evaluated by immunoblot assay. The intensity of each immunoblot band was quantitated by ImageJ, and the data were expressed as the fold changes (mean ± SEM; n = 3).
We then analyzed the effects of GB1e on EGFR downstream signaling. GB1e overexpression significantly increased the level of Akt phosphorylation in MCF7 and T-47D cells, whereas GB1e silencing in MDA-MB-231 and BT-549 cells reduced Akt phosphorylation (Figure 5E). It was also the case in tumor xenografts induced by GB1e silenced MDA-MB-231 cells where Akt phosphorylation was markedly reduced (Figure S6A). This effect was specific for Akt signaling because neither overexpression nor silence of GB1e altered the activities of ERK1/2 and STAT3 (Figures 5E and S6B). Akt phosphorylation was abrogated by Tarceva (erlotinib), a small molecule inhibitor of EGFR (Sharma et al., 2007), suggesting that Akt activation was downstream of EGFR (Figure S6C).

We also brought results to show that the effects of GB1 shRNA on BrCa cells resulted from GB1e silence. GB1e silencing was rescued by transfecting GB1e silenced MDA-MB-231 cells with GB1e cDNA (Figure S7A) that significantly decreased the interaction between EGFR and PTPN12 (Figure S7B) but increased the phosphorylation of EGFR and Akt (Figures S7B and S7C). Moreover, GB1e rescue markedly increased the clonogenicity and migration capacity of GB1e silenced cells (Figures S7D and S7E). Altogether, these results suggested that GB1e induced a biased EGFR signaling by activating Akt specifically.

Finally, we showed that oncogenic effects of GB1e are not influenced by GABAB receptor agonist and antagonist. Indeed, although GB1e contains the same ECD as GB1a, neither baclofen nor the competitive antagonist CGP54626 had effects on cell proliferation, Akt phosphorylation, and colony formation (Figure S8).

Phosphorylation of Y230 and Y404 on GB1e is critical for GB1e to hijack PTPN12 from EGFR

We provided evidence that the phosphorylation state of GB1e is modulated by PTPN12, which has been reported to recognize specific phosphotyrosine residues on its substrates (Li et al., 2016). Tyrosine phosphorylated GB1e was detected in the TCLs from MCF7-GB1e cells but not in the CM (Figures 6A and 6B). The phosphorylation level of GB1e was dramatically reduced by transfected WT PTPN12, but increased by PTPN12 siRNA, while not significantly altered by the PTPN12 C231S mutant that had a dead catalytic site (Figure 6C).

To identify the phosphorylation sites on GB1e, we first performed a bioinformatics analysis. The potential phosphorylation sites, Y228, Y230, Y234, and Y404, were mutated to phenylalanine. The Y230F and Y404F mutations significantly reduced GB1e phosphorylation in MCF7 cells, whereas the expression level of each GB1e mutant was comparable with that of WT GB1e (Figure 6D). When both Y230 and Y404 were mutated, GB1e phosphorylation was further reduced (Figures 6D and S9A). Y230 and Y404 are therefore the main phosphorylation sites on GB1e.

We also showed that phosphorylation of Y230 and Y404 on GB1e is essential for GB1e to interact with PTPN12, to enhance EGFR-dependent Akt signaling, and for its oncogenic effects. Compared with WT GB1e, the mutants Y230F and Y404F and the double mutant dramatically decreased the co-IP between PTPN12 and GB1e (Figures 6D and S9B) but enhanced the co-IP between PTPN12 and EGFR (Figures 6E and S9C). These mutants also significantly reduced the phosphorylation levels of EGFR (Figures 6E and S9D) and Akt (Figures 6F and S9E). In contrast, the mutants Y228F and Y234F had a similar effect as the WT GB1e on these co-IP experiments (Figure 6D) and on the phosphorylation of EGFR (Figure 6E) and Akt (Figure 6F). Finally, the mutants Y230F and Y404F and the double mutant markedly decreased the colony numbers formed by MCF7 cells, but the Y228F and Y234F mutations on GB1e did not (Figure 6G). Altogether, these results suggested that the oncogenic effects of GB1e are mainly dependent on the phosphorylation of Y230 and Y404.

DISCUSSION

In this study, we made an observation that GB1e is the predominant GB1 isoform at both mRNA and protein levels in various human BrCa cell lines and tissues. Moreover, the expression level of GB1e correlates with...
the tumorigenic potential of BrCa cell lines and is upregulated in BrCa tissues. Interestingly, ER+/PR+, Her2+, and triple negative cell lines displayed low, medium, and high expression levels of GB1e, respectively, suggesting that GB1e expression correlates with the expression of ER, PR, and Her2 in breast cancer cells. GB1e not only enhances the proliferation, migration, and invasion of human BrCa cells in vitro but also promotes the growth and metastasis of BrCa cells in vivo. Furthermore, GB1e enhances EGFR signaling by displacing EGFR-bound PTPN12. Our results suggest an original mode of action of GB1e involved in the development and progression of human BrCa.

Although GB1e has a N-terminal signal peptide and it can be secreted as the other GB1a isoforms, our results showed an original mechanism of GB1e action. It is different from what have been reported previously that GABA<sub>a</sub> receptor induced EGFR transactivation in a G<sub>ia</sub>/G<sub>0</sub> protein dependent way (Xia et al., 2017). It also differs from the previous study that has shown the membrane-associated GB1<sub>a</sub> can form heterodimer with GB2 and disrupt the normal association between GB1<sub>a</sub> and GB2 but have no effect on GABA<sub>a</sub> receptor activation (Schwarz et al., 2000). Our data suggested that GB1e is retrotranslocated from the ER lumen to the cytosol. ER is the major site of protein synthesis and processing, and misfolded/unfolded proteins are identified and removed by a process called ER-associated degradation (ERAD), in which the proteins are transported to the cytosol and degraded by proteasomes (Oikonomou and Hendershot, 2020). However, the ERAD system is not always coupled to protein degradation. Clusterin, a secreted glycoprotein, can interact with conformationally altered Bax and inhibit the apoptosis of cancer cells once being retrotranslocated to the cytosol under stressed conditions (Nizard et al., 2007; Zhang et al., 2005). Our results indicated that GB1e might be recognized as a misfolded protein by ERAD components and then being retrotranslocated to the cytosol. It is in agreement with the observation that the isolated GB1 ECD appears unfolded in the absence of ligand (Geng et al., 2012). It is also consistent that neither baclofen nor CGP54626 influences the proliferation and clonogenicity of MCF7-GB1e cells, in agreement with that GB1e ectopically expressed in HEK293 did not bind CGP54626 (Schwarz et al., 2000).

Our results suggest that GB1e interacts with PTPN12 in the cytosol and then prevents this phosphatase to dephosphorylate EGFR and enhances Akt signaling, a pathway that could be responsible for the oncogenic role of GB1e. More than 20 substrates and binding partners of PTPN12 have been identified (Li et al., 2015; Nair et al., 2018). PTPN12 functions as a tumor suppressor in BrCa cells by dephosphorylating and inactivating EGFR, Her2, and other RTKs (Nair et al., 2018; Sun et al., 2011). Consistent with the published data, we detect an association between PTPN12 and EGFR in human BrCa cells. Our data suggest that GB1e and EGFR compete for PTPN12 interaction. Of note, GB1e SDs are not directly involved in this process, in contrast to the N-terminal SDs of GB1a that have been shown to interact with amyloid-β precursor protein, adherence-junction associated protein 1, PILRα-associated neural protein, and extracellular matrix protein fibulin-2 for axonal trafficking and synaptic transmission (Blein et al., 2004; Cinamarca et al., 2019; Rice et al., 2019).

Interestingly, the association between GB1e and PTPN12 is dependent on tyrosine phosphorylation of GB1e. Phosphorylation plays a key role in regulating protein function, and an estimated 30% of cellular proteins undergo phosphorylation (Qiu et al., 2020). Although the activity of GABA<sub>a</sub> receptor is not correlated with its overall phosphorylation state, the surface stability, endocytosis, and desensitization of GABA<sub>a</sub> receptor are regulated by phosphorylation of several serine residues in the intracellular regions of GB1 (S867, S917, and S923) and GB2 (Guett et al., 2010; Kuramoto et al., 2007; Pontier et al., 2006; Rose and Wickman, 2020). Phosphorylation of the GABA<sub>a</sub> ECD and tyrosine phosphorylation of GABA<sub>a</sub> receptor has not been reported yet. We find that phosphorylation of GB1e at multiple tyrosine sites, especially Y230 and Y404, plays a key role in disrupting EGFR-PTPN12 interaction and upregulating EGFR-Akt signaling cascade. Of note, Y230 and Y404 are located on two different lobes of the GB1 ECD (Figure S10A), but they are on two close faces that are nearby the interface between GB1a and GB2 ECD in the GABA<sub>a</sub> receptor. In addition, although Y230 is highly conserved during evolution, Y404 is conserved only in mammals (Figure S10B). Finally, the kinases that phosphorylate GB1e need to be further identified.

In addition to human breast cancer cells, GB1e expression was also detected in human prostate cancer cell lines (LNCaP, PC-3, and DU145) and tissues; moreover, four out of five human prostate cancer specimens showed increased protein expression of GB1e compared with matched tumor adjacent tissues (Figures S11A–S11C). Therefore, dysregulated GB1e expression may occur in various human cancers and is not specific to breast cancer. Moreover, the other human cancer cell lines including glioma (U251 and U87), hepatocarcinoma (BEL7402, HEPG2, HCCLM9), and colon cancer (Caco2, SW480) cell lines also showed GB1e
expression with variable levels (Figures S11A and S11B). Interestingly, GB1e protein level in the two glioma cell lines is lower than those in the other cancer cell lines. Because heterodimeric GABA<sub>B</sub> receptors are predominantly expressed in the central nervous system, whereas GB1e is the main GB1 isoform in the peripheral tissues, we assume that heterodimeric GABA<sub>B</sub> receptors and GB1e may play essential roles in the development and progress of brain tumor and nonneural-tissue-derived tumors, respectively.

Taken together, this study demonstrates that GB1e is a tumorigenic protein in BrCa cells and promotes the malignancy of BrCa cells both in vitro and in vivo. Cytosolic GB1e may undergo tyrosine phosphorylation or proteasomal degradation. Phosphorylation of Y230 and Y404 on GB1e is critical for GB1e to displace EGFR-bound PTPN12 and activate EGFR-Akt signaling pathway. This study, therefore, provides a new strategy for the treatment of BrCa by targeting the interactions between GB1e, PTPN12, and EGFR.

Limitations of the study
Although our in vitro and in vivo study clearly demonstrates that GB1e has a tumorigenic function in breast cancer cells and always is upregulated in breast cancer tissues, its exploitation in clinical value needed to be further studied. In addition, we did not analyze the function of the secreted GB1e. It is meaningful to further investigate whether secreted GB1e can promote breast cancer progression by regulating tumor microenvironment. In addition, it is also valuable to evaluate whether secreted GB1e is a potential marker for clinical diagnosis.

STAR METHODS
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  - Immunoprecipitation
  - Immunoblotting
  - Immunofluorescence
  - LC-MS/MS analysis
  - Immunohistochemistry
  - Microsome isolation
  - Protease protection assays
  - Protein export assay
  - Treat cells with GABA<sub>B</sub> receptor agonist
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103311.

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AUTHOR CONTRIBUTIONS
Conceptualization, J.X. and J.L.; Investigation, X.J., J.L., B.W., Y.Z., S.W., X.W., S.X., and Z.Z.; Methodology, X.J. and B.W.; Software, S.W.; Resources, P.Y. and Y.H.; Writing—Original Draft, X.J., B.W., J.L., P.R., and P.P.; Writing—Review & Editing, X.J., B.W., and P.R.; Funding Acquisition, J.L., P.R., and P.P.; Supervision, X.J. and J.L. All authors have revised the read and agreed version of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES
Azuma, H., Inamoto, T., Sakamoto, T., Kyama, S., Ubai, T., Shinohara, Y., Maemura, K., Tsuji, M., Segawa, N., Masuda, H., et al. (2003). Gamma-aminobutyric acid as a promoting factor of cancer metastasis; induction of matrix metalloproteinase production is potentially its underlying mechanism. Cancer Res. 63, 8090–8096.

Bettler, B., Kaumann, K., Mosbacher, J., and Gassmann, M. (2004). Molecular structure and physiological functions of GABAB receptors. Physiol. Rev. 84, 835–867.

Blein, S., Ginham, R., Uhrin, D., Smith, B.O., Soares, D.C., Veitel, S., McIlhinney, R.A., White, J.H., and Barlow, P.N. (2004). Structural analysis of the complement control protein (CCP) modules of GABAB receptor 1a: only one of the two CCP modules is compactly folded. J. Biol. Chem. 279, 48292–48306.

Dinamarca, M.C., Ravelli, A., Schneider, A., Fritzius, T., Früh, S., Rem, P.D., Stawarski, M., Lalanne, T., Turecek, R., Choo, M., et al. (2019). Complex formation of APP with GABAB receptors links axonal trafficking to amyloidogenic processing. Nat. Commun. 10, 1331.

Fava, G., Marucci, L., Glaser, S., Francis, H., De Morro, S., Benedetti, A., Alvaro, D., Venter, J., Meininger, C., Patel, T., et al. (2005). Gamma-Aminobutyric acid inhibits cholangiocarcinoma growth by cyclic AMP-dependent regulation of the protein kinase A’extracellular signal-regulated kinase 1/2’ pathway. Cancer Res. 65, 11437–11446.

Gassmann, M., and Bettler, B. (2012). Regulation of neuronal GABAB receptor functions by subunit composition. Nat. Rev. Neurosci. 13, 380–394.

Geng, Y., Xiong, D., Mosyak, L., Malito, D.L., Kniazeff, J., Chen, Y., Burmaka, S., Quick, M., Bush, M., Javitch, J.A., et al. (2012). Structure and functional interaction of the extracellular domain of human GABAB receptor GB2. Nat. Neurosci. 15, 970–978.

Guertg, N., Abdul Aziz, S., Holbro, N., Turecek, R., Rose, T., Seddik, R., Gassmann, M., Moes, S., Jenoe, P., Oertner, T.G., et al. (2010). NMDA receptor-dependent GABAB receptor internalization via CaMKII phosphorylation of serine 867 in GABAB2. Proc. Natl. Acad. Sci. U S A 107, 13924–13929.

Huang, Q., Zhu, C.L., Liu, C.H., Xie, F., Zhu, K., and Hu, S.Y. (2013). Gamma-aminobutyric acid binds to GABAB receptor to inhibit cholangiocarcinoma cells growth via the JAK/STAT3 pathway. Dig. Sci. 85, 734–743.

Inamoto, T., Azuma, H., Sakamoto, T., Kyama, S., Ubai, T., Kotake, Y., Watanabe, M., and Katsuoka, Y. (2007). Invasive ability of human renal cell carcinoma cell line Caki-2 is accelerated by gamma-aminobutyric acid, via sustained activation of ERK1/2 inducible matrix metalloproteinases. Cancer Invest. 25, 574–583.

Jiang, X., Xu, L., Zhang, Q., He, C., Zhang, Z., Yi, P., and Liu, J. (2012). GABAB receptor complex as a potential target for tumor therapy. J. Histochem. Cytochem. 60, 269–279.

Joseph, J., Nygård, H., Zander, K.S., and Enskeln, F. (2002). The neurotransmitter gamma-amino- butyric acid is an inhibitory regulator for the migration of SW 480 colon carcinoma cells. Cancer Res. 62, 6467–6469.

Kuramoto, N., Wilkins, M.E., Fairfax, B.P., Revilla-Sanchez, R., Terunuma, M., Tamaki, K., Iemata, M., Warren, N., Couve, A., Calver, A., et al. (2007). Phospho-dependent functional modulation of GABAB receptors by the metabolic sensor AMP-dependent protein kinase. Neurokin. 53, 233–247.

Lehtinen, M.J., Meri, S., and Jokiranta, T.S. (2004). Activation mechanism of neuronal GABAB receptors by the metabolic sensor AMP-dependent protein kinase. Neuron 50, 1345–1358.

Li, J., Davidson, D., Martins Souza, C., Zhong, M.C., Wu, N., Park, M., Muller, W.J., and Veillette, A. (2015). Loss of PTPN12 stimulates progression of ErbB2-dependent breast cancer by enhancing cell survival, migration, and epithelial-to-mesenchymal transition. Mol. Cell. Biol. 35, 4069–4082.

Mizuta, K., Osawa, Y., Mizuta, F., Xu, D., and Emala, C.W. (2008). Functional expression of GABAB receptors in airway epithelium. Am. J. Respir. Cell Mol. Biol. 39, 296–304.

Nair, A., Chung, H.C., Sun, T., Tysz, S., Dobrolecki, L.E., Dominguez-Vidana, R., Kury, S.J., Orellana, M., Renwick, A., Henke, D.M., et al. (2018). Combinatorial inhibition of PTPN12-regulated receptors leads to a broadly effective therapeutic strategy in triple-negative breast cancer. Nat. Med. 24, 505–511.

Neophytou, C., Boutsikos, P., and Papageorgis, P. (2018). Molecular mechanisms and emerging therapeutic targets of triple-negative breast cancer metastasis. Front. Oncol. 8, 31.

Nizard, P., Tetley, S., Le Dréan, Y., Waterin, T., Le Goff, P., Wilson, M.R., and Michel, D. (2007). Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol. Traffic 8, 554–565.

Okonomou, C., and Hendershot, L.M. (2020). Disposing of misfolded ER proteins: a troubled substrate’s way out of the ER. Mol. Cell Endocrinol. 500, 110630.

Pin, J.P., and Bettler, B. (2016). Organization and functions of mGlu and GABAB receptor complexes. Nature 540, 60–68.

Pin, J.P., Kniazeff, J., Binet, V., Liu, J., Maurel, D., Galvez, T., Duthey, B., Havlickova, M., Blahos, J., Prezeau, L., et al. (2004). Activation mechanism of
the heterodimeric GABA<sub>B</sub> receptor. Biochem. Pharmacol. 68, 1565–1572.

Pontier, S.M., Lahaie, N., Gingham, R., St-Gelais, F., Bonin, H., Bell, D.J., Flynn, H., Trudeau, L.E., McIlhinney, J., White, J.H., et al. (2006). Coordinated action of NSF and PKC regulates GABA<sub>B</sub> receptor signaling efficacy. EMBO J. 25, 2698–2709.

Qiu, W., Evans, C.A., Landels, A., Pham, T.K., and Wright, P.C. (2020). Phosphopeptide enrichment for phosphoproteomic analysis - a tutorial and review of novel materials. Anal. Chim. Acta 1129, 158–180.

Qu, J., Zou, T., and Lin, Z. (2021). The roles of the ubiquitin-proteasome system in the endoplasmic reticulum stress pathway. Int. J. Mol. Sci. 22, 1526.

Rice, H.C., de Malmazet, D., Schreurs, A., Frere, S., Van Molle, I., Volkov, A.N., Creemers, E., Vertkin, I., Nys, J., Ranavirous, F.M., et al. (2019). Secreted amyloid-β precursor protein functions as a GABA<sub>B1</sub>R<sub>1</sub> ligand to modulate synaptic transmission. Science 363, eaas4827.

Rose, T.R., and Wickman, K. (2020). Mechanisms and regulation of neuronal GABA<sub>B</sub> receptor-dependent signaling. Curr. Top Behav. Neurosc. Published online in advance. https://doi.org/10.1007/7854_2020_129.

Schmitz, A., Herrgen, H., Winkeler, A., and Herzog, V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. J. Cell Biol. 148, 1203–1212.

Schuller, H.M. (2008). Neurotransmission and cancer: implications for prevention and therapy. Anti-Cancer Drugs 19, 655–671.

Schwarz, D.A., Barry, G., Eliasof, S.D., Petrofski, R.E., Conlon, P.J., and Maki, R.A. (2000). Characterization of gamma-aminobutyric acid receptor GABA<sub>B1</sub>(<sup>Δ</sup>)<sub>261</sub> a GABA<sub>B1</sub>(<sup>Δ</sup>)<sub>261</sub> splice variant encoding a truncated receptor. J. Biol. Chem. 275, 32174–32181.

Sharma, S.V., Bell, D.W., Settleman, J., and Haber, D.A. (2007). Epidermal growth factor receptor mutations in lung cancer. Nat. Rev. Cancer 7, 169–181.

Sun, T., Aceto, N., Meerbrey, K.L., Kessler, J.D., Zhou, C., Migliaccio, I., Nguyen, D.X., Pavlova, N.N., Botero, M., Huang, J., et al. (2011). Activation of multiple proto-oncogenic tyrosine kinases in breast cancer via loss of the PTPN12 phosphatase. Cell 144, 703–718.

Sung, H., Ferlay, J., Siegel, R.L., Soerjomataram, I., Jemal, A., and Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. 71, 209–249.

Tatsuta, M., Ishi, H., Baba, M., Nakaizumi, A., Ichii, M., and Taniguchi, H. (1990). Inhibition by gamma-amino-n-butyric acid and baclofen of gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats. Cancer Res. 50, 4931–4934.

Terunuma, M. (2018). Diversity of structure and function of GABA<sub>B</sub> receptors: a complexity of GABA<sub>B</sub>-mediated signaling. Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci. 94, 390–411.

Thaker, P.H., Yokoi, K., Jennings, N.B., Li, Y., Rebhun, R.B., Rousseau, D.L., Jr., Fan, D., and Sood, A.K. (2005). Inhibition of experimental colon cancer metastasis by the GABA receptor agonist nembutal. Cancer Biol. Ther. 4, 753–758.

Wahlman, J., DeMartino, G.N., Skach, W.R., Bulleid, N.J., Brodsky, J.L., and Johnson, A.E. (2007). Real-time fluorescence detection of ERAD substrate retrotranslocation in a mammalian in vitro system. Cell 129, 943–955.

Wang, T., Huang, W., and Chen, F. (2008). Baclofen, a GABA<sub>B</sub> receptor agonist, inhibits human hepatocellular carcinoma cell growth in vitro and in vivo. Life Sci. 82, 536–541.

Xia, S., He, C., Zhu, Y., Wang, S., Li, H., Zhang, Z., Jiang, X., and Liu, J. (2017). GABA<sub>B</sub>R<sub>1</sub>-induced EGFR transactivation promotes migration of human prostate cancer cells. Mol. Pharmacol. 92, 265–277.

Zhang, D., Li, X., Yao, Z., Wei, C., Ning, N., and Li, J. (2014). GABAergic signaling facilitates breast cancer metastasis by promoting ERK1/2-dependent phosphorylation. Cancer Lett. 348, 100–108.

Zhang, H., Kim, J.K., Edwards, C.A., Xu, Z., Taichman, R., and Wang, C.Y. (2005). Clusterin inhibits apoptosis by interacting with activated Bax. Nat. Cell Biol. 7, 909–915.
# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-GB1            | Abcam  | Cat# ab55051; RRID: AB_941703 |
| anti-Flag           | Santa Cruz Biotechnology | Cat# sc-51590; RRID: AB_677316 |
| anti-PTPN12         | Santa Cruz Biotechnology | Cat# sc-65229; RRID: AB_632280 |
| anti-Ki-67          | Santa Cruz Biotechnology | Cat# sc-23900; RRID: AB_627859 |
| anti-EGFR           | Cell Signaling Technology | Cat# 2085; RRID: AB_1903953 |
| anti-pAkt(Ser473)   | Cell Signaling Technology | Cat# 4060; RRID: AB_2315049 |
| anti-total Ak       | Cell Signaling Technology | Cat# 9272; RRID: AB_329827 |
| anti-pERK1/2(T202/Y204) | Cell Signaling Technology | Cat# 4695; RRID: AB_390779 |
| anti-total ERK1/2   | Cell Signaling Technology | Cat# 9145; RRID: AB_2491009 |
| anti-total STAT3    | Cell Signaling Technology | Cat# 4904; RRID: AB_331269 |
| anti-Calnexin       | Cell Signaling Technology | Cat# 2679; RRID: AB_2223172 |
| HRP-conjugated anti-Flag antibody | Cell Signaling Technology | Cat# 86861; RRID: AB_2800094 |
| HRP-conjugated anti-rabbit IgG | Cell Signaling Technology | Cat# 7074; RRID: AB_2099233 |
| HRP-conjugated anti-mouse IgG | Cell Signaling Technology | Cat# 7076; RRID: AB_330924 |
| Alexa Fluor 488-conjugated anti-mouse IgG | Cell Signaling Technology | Cat# 4408; RRID: AB_10694704 |
| anti-β-actin        | Cell Signaling Technology | Cat# 4970; RRID: AB_2223172 |
| Phosphotyrosine antibody, clone 4G10 | Sigma-Aldrich | Cat# 05-321; RRID: AB_2891016 |
| **Biological samples** |        |            |
| Human breast cancer tissues | Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Tunicamycin         | YEASEN, Shanghai | Cat# 60251ES03 |
| Brefedlin A         | Beyotime, Shanghai | Cat# 51536 |
| Baclofen            | R&D Systems | Cat# 0796 |
| CGP54626            | R&D Systems | Cat# 1088 |
| Tarceva             | Sigma-Aldrich | Cat# 183319-69-9 |
| B-27 supplement (50x) serum free | ThermoFisher Scientific | Cat# 17504044 |
| Human EGF Recombinant Protein | ThermoFisher Scientific | Cat# PHG0311 |
| Human bFGF Recombinant Protein | ThermoFisher Scientific | Cat# PHG0261 |
| BD matrigel matrix growth factor reduced | BD Biosciences | Cat# 365234 |
| **Critical commercial assays** |        |            |
| PrimeScript RT reagent kit | Takara | Cat# RR047A |
| SYBR Green PCR master mix | Applied Biosystems | Cat# 430155 |
| QuikChange mutagenesis kit | Agilent Technologies | Cat# 200521 |
| Microsome isolation kit | Biovision | Cat# K249-50 |
| Lipofectamine™ 2000 Transfection Reagent | Invitrogen | Cat# 11668019 |
| PepMute™ siRNA Transfection Reagent | SigmaGen Laboratories | Cat# SL100566 |

(Continued on next page)
### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jianfeng Liu (jfliu@mail.hust.edu.cn).

#### Materials availability

Unique reagents generated in this study are available from the lead contact with a completed Material Transfer Agreement.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Experimental models: cell lines** | | |
| MCF7 | American Type Culture Collection (ATCC) | Cat# HTB-22; RRID: CVCL_0031 |
| T-47D | American Type Culture Collection (ATCC) | Cat# HTB-133; RRID: CVCL_0553 |
| BT-474 | American Type Culture Collection (ATCC) | Cat# HTB-20; RRID: CVCL_0179 |
| AU-565 | American Type Culture Collection (ATCC) | Cat# CRL-2351; RRID: CVCL_1074 |
| SK-BR-3 | American Type Culture Collection (ATCC) | Cat# HTB-30; RRID: CVCL_0033 |
| MDA-MB-453 | American Type Culture Collection (ATCC) | Cat# HTB-131; RRID: CVCL_0418 |
| MDA-MB-231 | American Type Culture Collection (ATCC) | Cat# HTB-26; RRID: CVCL_0062 |
| BT-549 | American Type Culture Collection (ATCC) | Cat# HTB-122; RRID: CVCL_1092 |
| MCF10A | American Type Culture Collection (ATCC) | Cat# CRL-10317; RRID: CVCL_0598 |
| HEK293T | American Type Culture Collection (ATCC) | Cat# CRL-11268; RRID: CVCL_1926 |
| Cerebellar granule neurons | This paper | N/A |
| **Experimental models: organisms/strains** | | |
| Balb/c nude mice | Beijing Vital River Laboratory | N/A |
| **Oligonucleotides** | | |
| See Table S2 for shRNA sequences of GB1 | This paper | N/A |
| See Table S3 for primers used for site-directed mutagenesis of GB1a and PTPN12 | This paper | N/A |
| See Table S4 for siRNA sequences of GB1 and PTPN12 | This paper | N/A |
| See Table S5 for RT-PCR primers of GB1a/1b and β-actin | This paper | N/A |
| See Table S6 for qRT-PCR primers of GB1a and β-actin | This paper | N/A |
| **Recombinant DNA** | | |
| pEGFP-N1-GB1a | This paper | N/A |
| pCDNA5/FRT-PTPN12 | This paper | N/A |
| **Software and algorithms** | | |
| Graphpad Prism 8 | GraphPad Software, Inc | N/A |
| ImageJ | ImageJ, NIH | N/A |
| **Other** | | |
| 4G10-conjugated agarose beads | Sigma-Aldrich | Cat# 16-101 |
| EGFR antibody-conjugated sepharose beads | Cell Signaling Technology | Cat# 8083; RRID: AB_10994874 |
| protein A-Sepharose beads | Millipore | Cat# 16-125 |
| protein G-Sepharose beads | Millipore | Cat# 16-266 |
Data and code availability
Data reported in this paper will be shared by the lead contact upon request.
This paper does not report original code.
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Cell lines
Human BrCa cell lines MCF7, T-47D, BT-474, AU-565, SK-BR-3, MDA-MB-453, MDA-MB-231, BT-549, and normal human breast epithelia cell line MCF10A were obtained from American Type Culture Collection. MCF7, T-47D, AU-565 and SK-BR-3 cells were maintained in complete DMEM (supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) with high glucose. BT-474 and BT-549 cells were maintained in complete RPMI-1640 medium. MDA-MB-453 and MDA-MB-231 were cultured in complete L-15 medium. MCF10A cells were grown in special medium purchased from Procell Life Science & Technology (Wuhan, China). Cerebellar granule neurons (CGNs) were dissected from the cerebella of one-week-old female Kunming mice. All cell lines were screened regularly for mycoplasma.

Tumor xenografts and experimental metastasis
Three to four-week-old female BALB/c nude mice were randomly divided into three groups (n = 5). 1 × 10⁶ of control or GB1e silenced MDA-MB-231 cells were injected subcutaneously into the dorsal flanks of mice. Starting three days post-injection, tumor size was measured every two to three days, and tumor volume was calculated according to the formula: length × width² ÷ 0.5. The mice were sacrificed 21 days post-injection. Tumors were harvested, weighed, and subjected to hematoxylin and eosin (H&E) staining or immunohistochemistry analysis.

For experimental metastasis experiment, 2 × 10⁵ control or GB1e silenced MDA-MB-231 cells were injected into the tail veins of female BALB/c nude mice (n = 5). Two weeks later, the mice were sacrificed and the lungs were excised. The metastatic nodules were fixed with Bouin solution (Sigma) and evaluated with H&E staining.

The protocols for animal study were approved by the Institutional Animal Care and Use Committee, TMC/HUST (approval No. 2019-S2336) in accordance with the Guide for the Care and Use of Laboratory Animals. The mice were maintained in grouped cages at a temperature of 20°C–24°C, humidity of 50%–60%, pathogen-free facility on a 12 hr light/dark cycle and fed ad libitum food and water.

METHOD DETAILS
Human BrCa specimens
Human BrCa and matched para-cancerous tissues were collected from Union Hospital, Tongji Medical College (TMC), Huazhong University of Science and Technology (HUST) in accordance with the protocol approved by the Ethics Committee of TMC/HUST (approval No. 2019-S1048). Surgically removed samples were snap-frozen in liquid nitrogen and stored at −80°C until use.

Plasmid construction
Human GB1e DNA (GenBank: AF301005.1) was synthesized by Sangon Biotech (Shanghai, China) and subcloned into plasmid pEGFP-N1 with Flag tag. Human PTPN12 cDNA (GenBank: NM_002835.4) was from Addgene (Watertown, MA, USA) and subcloned into pcDNA5/FRT plasmid with HA tag. GB1 shRNAs #1 or #2 was inserted into plasmid pLVX-shRNA2-Puro. GB1e, mutations (Y228F, Y230F, Y234F, Y404F, and Y230F/Y404F) and PTPN12 mutation (C231S) were made by using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The shRNA sequences and primer sequences for mutagenesis were listed in Tables S2 and S3.

GB1e with the deletion of sushi domain sequence (GB1eΔSDs) were amplified by using pEGFP-N1-Flag-GB1e as the template, and the forward and reverse primers were 5’-gatgcatactggaacgccacactca-3’ and
S’-gatggatcccaggttggcct-3’ respectively. For PCR amplification, 35 cycles of 95°C for 30 sec, 56°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min, were performed. The PCR product was cloned into plasmid pEGFP-N1 with Flag tag and verified by DNA sequencing.

Cell transfection
Plasmids encoding wild type (WT) GB1e, GB1e mutants (Y228F, Y230F, Y234F, Y404F, or Y230F/Y404F), WT PTPN12, or PTPN12 mutant (C231S) were transfected into MCF7 cells and/or T-47D cells by lipofectamine™ 2000 transfection reagent (Invitrogen), empty vectors were used as controls. MCF7 and T-47D cells transfected with WT GB1e were selected with G418 and to get stable cell lines.

GB1e or control siRNAs were transfected into MDA-MB-231 and BT-549 cells by using pepMute™ siRNA transfection reagent (SignaGen Laboratories, Rockville, MD, USA). shRNAs were synthesized based on siRNA sequences that efficiently silenced GB1e expression. Lentiviral particles containing sh-GB1e or sh-control were produced in HEK293T cells and used to infect MDA-MB-231 or BT-549 cells. Puromycin selection was performed to get stable GB1e silenced cell lines. PTPN12 siRNAs were transfected into MCF7 cells by using pepMute™ siRNA transfection reagent. The siRNA sequences were listed in Tables S4.

Reverse transcriptase and real time PCR
In reverse transcriptase (RT)-PCR, Trizol reagent (Invitrogen) was used to isolate total RNA from tissue specimens or cell lines. cDNA was made by using PrimeScript RT reagent kit (Takara Inc., Dalian, P.R. China) and subjected to 40 cycles of PCR. The products were separated by 2% agarose gels and analyzed by Bio-Rad Gel Doc XR+ System. Real time (qRT)-PCR was performed by using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The transcripts were analyzed by using the comparative Ct method. β-actin was used as an internal control. The primer sequences were listed in Tables S5 and S6.

Proliferation, colony and spheroid formation
For cell proliferation measurement, cells were seeded into 24-well plates (2×10^4/well). Cell Counting Kit-8 solution was added 24, 48, 72, or 96 h after cell plating, and OD_{450} was measured by a plate reader. In the clonogenicity assay, cells were seeded into 24-well plates (200/well) and cultured for 10–14 days with medium replacement for every three days. When the colonies reached the size visible to the naked eyes, they were fixed and stained with 1% crystal violet. Images were captured and the number of colonies was counted. In the spheroid formation assay, cells were seeded into Corning ultra-low attachment 24-well plates (2×10^4/well) and cultured in DMEM/F12 (1:1) medium supplemented with 2% B27, bFGF (20 ng/mL), and EGF (20 ng/mL) for 10–14 days. The number of spheroids was counted under microscope and the images were captured.

Transwell assay
Cell migration and invasion assays were assessed by modified Boyden chamber (Corning Costar, Rochester, NY, USA) containing a polycarbonate membrane filter with a pore size of 8.0 μm. For invasion assay, the upper chamber was pre-coated with Matrigel (Corning). 800 μL of medium containing 10% FBS was added to the lower chamber, 200 μL of cells (3×10^4 cells for migration and 5×10^4 cells for invasion) suspended in serum-free medium were added to the upper chamber and incubated at 37°C in 5% CO_{2} for 24 h. Cells remained on the top side of the membrane were gently removed, and those on the bottom surface were fixed and stained with 1% crystal violet. Images were captured by CellSens software under microscope at the magnification of 200×. The number of cells in each image was calculated by ImageJ software.

Enzyme linked immunosorbent assay (ELISA)
GB1e or empty vector transfected MCF7 cells seeded into 96-well plates were fixed with 4% formaldehyde, permeabilized or not with 0.2% Triton X-100/PBS, blocked with 3% BSA/PBS, and sequentially incubated with primary antibody against Flag epitope and HRP-conjugated secondary antibody at room temperature (RT) for 30 min. The plates were washed followed by addition of enhanced chemiluminescence (ECL) reagents. The fluorescence signals were measured by FlexStation 3 (Molecular Devices, USA).

Immunoprecipitation
Cells were lysed with pre-cold IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 1% protease inhibitor cocktails) on ice for 20 min. 500 μg of total cell lysates (TCLs) were mixed with 20 μL of 4G10-conjugated agarose beads or EGFR antibody-conjugated sepharose beads
in a final volume of 500 μL and incubated overnight at 4°C with continuous agitation. In another set of IP experiments, 500 μg of TCLs were mixed with 2 μg of anti-PTPN12 or -Flag antibodies in a final volume of 500 μL and incubated overnight at 4°C with continuous agitation. 20 μL protein A/G-Sepharose beads were then added to each sample and incubated at 4°C for another 4 h. The beads were then washed, resuspended in 2× electrophoresis loading buffer, and boiled for 10 min. The supernatants were subjected to immunoblotting or liquid chromatography-tandem MS (LC-MS/MS) analysis.

Immunoblotting
Immunoprecipitates, TCLs, condition medium, or tissue homogenates were resolved by SDS–PAGE followed by membrane transfer. Membranes were blocked, incubated with primary antibodies overnight at 4°C, and then probed with specific HRP-conjugated secondary antibodies at RT for 2 h. After extensive washing, the membranes were developed by ECL reagents.

Immunofluorescence
Cells seeded on coverslips (pre-coated with poly-lysine) placed in 12-well plates were cultured overnight and fixed with 4% formaldehyde at room temperature (RT) for 15 min. Afterwards, cells were rinsed, incubated with PBS in the presence or absence of 0.2% Triton X-100 for 5 min, and blocked with 3% BSA/PBS at RT for 30 min followed by incubating with primary antibody against GB1 overnight at 4°C. Subsequently, cells were washed, incubated with appropriate Alexa Fluor 488-conjugated secondary antibody against mouse IgG at RT for 1 h, washed, stained with DAPI for 10 min, and washed again. The coverslips were mounted on glass slides and the images were acquired by FV10-ASW 3.1 Viewer under Olympus FV1000 microscope (Tokyo, Japan).

LC-MS/MS analysis
The TCLs from GB1e or empty vector transfected MCF7 cells were immunoprecipitated with anti-Flag antibody, and the immunocomplex was resolved by SDS-PAGE followed by Coomassie Blue staining. Each lane was cut into four fragments and subjected to LC-MS/MS analysis.

Immunohistochemistry
Paraffin sections were incubated with 3% H2O2 to quench endogenous peroxidase activity, and then boiled in sodium citrate solution (10 mM, pH 6.0) to retrieve antigen. The slides were washed, blocked, and incubated with primary antibodies against GB1, Ki-67, or pAkt overnight at 4°C followed by incubation with HRP-conjugated secondary antibodies at RT for 1 h. The slides were washed and stained with DAB detection kit (ZSGB-BIO, Beijing, China). The intensity of the immunostaining was quantified by using Image-Pro Plus 6.0 software.

Microsome isolation
Microsomes were isolated from MDA-MB-231 and MCF7-GB1e cells by using a microsome isolation kit (Biovision, USA) according to manufacturer’s instructions. Briefly, cells were washed once with 1 mL cold PBS and centrifuged. Then, the cell pellets were resuspended in cold homogenization buffer and transferred to pre-chilled Dounce homogenizer. The cell pellets were gently homogenized on ice followed by adding additional homogenization buffer to fully suspend homogenate. Afterwards, the homogenates were transferred to microcentrifuge tube, vortexed for 30 sec, incubated on ice for 1 min, and then centrifuged at 10,000 g for 15 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and centrifuged at 20,000 g for 20 min at 4°C. The microsomal pellets were resuspended in cold PBS for further analysis. The purity and integrity of the microsomes were verified by the ratio of OD260/OD280 (>1.8) and immunoblotting with ER marker calnexin.

Protease protection assays
The microsomes isolated from MDA-MB-231 and MCF-GB1e cells were resuspended in PBS and treated or not with trypsin (100 μg/mL) and/or 1% Triton X-100 for 30 min on ice. The activity of trypsin was stopped by the addition of phenylmethylsulfonyl fluoride to 1 mM (Schmitz et al., 2000).

Protein export assay
Twofold export buffer plus ATP (4 mM MgCl2, 2 mM ATP, 20 mM creatine phosphate, 400 μg/mL creatine kinase in PBS, pH 7.5) or twofold export buffer minus ATP (4 mM MgCl2, 50 mM glucose, 60 U/mL...
hexokinase in PBS, pH 7.5) were added to microsomes resuspended in cold PBS. To analyze protein export in the presence of cytosol, microsomes were resuspended in 50% reticulocyte lysate plus ATP. All of the mixtures were incubated at 30°C for 20, 40, or 60 min followed by centrifugation (Schmitz et al., 2000).

**Treat cells with GABA<sub>B</sub> receptor agonist**

For cell proliferation assay, a total of 1×10⁴ MCF7-V or MCF-GB<sub>1α</sub> cells were seeded into 24-well plates and grown in complete DMEM for two days at 37°C with 5% CO₂. Cells were then incubated or not with GABA<sub>B</sub> receptor agonist baclofen (100 and 200 μM) or antagonist CGP54626 (5 and 10 μM) at 37°C for four consecutive days. The media were replaced every two days, and cell proliferation was examined every 24 h by using CCK-8 solution.

To examine the level of pAkt (S473), 4×10⁵ of MCF7-V or MCF-GB<sub>1α</sub> cells were seeded into 3.5-cm plates, maintained in complete DMEM for 2 days at 37°C with 5% CO₂ followed by serum starvation for 24 h. Cells were then incubated or not with baclofen (100 and 200 μM) or CGP54626 (5 and 10 μM) for 24 h at 37°C, and the TCLs were analyzed by immuno blot assay with antibodies against pAkt (S473), Akt, GB<sub>1</sub> and β-actin.

For clonogenic assay, 200 of MCF7-V or MCF-GB<sub>1α</sub> cells were seeded into 24-well plates and treated or not with baclofen (100 and 200 μM) or CGP54626 (5 and 10 μM) for 10–14 days. The media were replaced every 3 days. When colonies were visible to the naked eyes, the plates were washed, fixed, and stained with 1% crystal violet. Images were captured and the number of colonies was counted.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Immunoblot results were quantitated by using ImageJ software, and the optical density of each band was normalized to β-actin. The data were presented as mean ± SEM. Statistical analyses were performed by using GraphPad Prism 8 software with one-way ANOVA with post hoc test (Tukey), paired or unpaired t test. P value less than 0.05 was considered statistically significant and the levels are indicated as: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.