

SET binding protein 1 (SETBP1) suppresses cell proliferation in estrogen receptor-positive breast cancer

安東, 由貴

<https://hdl.handle.net/2324/7396175>

出版情報 : Kyushu University, 2025, 博士 (医学), 課程博士
バージョン :

権利関係 : This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature' s AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: <https://doi.org/10.1007/s12282-025-01667-w>.



SET binding protein 1 (SETBP1) suppresses cell proliferation in estrogen receptor-positive breast cancer.

Yuki Ando^{a,d}, Takaaki Masuda^{a,b}, Naoki Hayashi^a, Taro Tobo^c, Keisuke Kosai^a, Shohei Shibuta^a, Yuya Ono^a, Hajime Otsu^a, Yuichi Hisamatsu^{a,d}, Tomoharu Yoshizumi^d and Koshi Mimori^a

^aDepartment of Surgery, Kyushu University, Beppu Hospital, Oita, Japan

^bDepartment of Breast and Endocrine Surgery, Kochi Medical School, Kochi University, Kochi, Japan

^cDepartment of Clinical Laboratory Medicine, Kyushu University, Beppu Hospital, Oita, Japan

^dDepartment of Surgery and Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan

Short Title: SETBP1 expression in ER-positive BC

Corresponding Author: Koshi Mimori

Department: Surgery

Institute/University/Hospital: Kyushu University, Beppu Hospital

Street Name & Number: 4546, Tsurumihara

City, State, Postal code, Country: Beppu Oita, 874-0838, Japan

Tel: +81-977-27-1650

E-mail: kmimori@beppu.kyushu-u.ac.jp

Number of Tables: 2

Number of Figures: 5

Word count: Abstract: 248/250; body of text: 3719/4000

Abstract

Background: The single nucleotide polymorphism rs6507583 at the promoter of SET binding protein 1 (*SETBP1*) was implicated in estrogen receptor (ER)-positive breast carcinogenesis. Here, we evaluated the clinical and biological relevance of *SETBP1* expression in ER-positive breast cancer (BC).

Methods: The associations between SETBP1 expression and clinical outcomes in BC patients were analyzed in independent cohorts. The localizations of *SETBP1* expression in BC tissues were observed by immunohistochemical staining. Pathway analyses were conducted using TCGA dataset. An in vitro proliferation assay, protein phosphatase 2A (PP2A) activity assay, and gene expression analysis were performed in *SETBP1*-knockdown ER-positive BC cells. We investigated the factors influencing *SETBP1* mRNA expression using TCGA dataset. rs6507583 presence and *SETBP1* mRNA expression in 11 mammary cell lines and 56 BC tissue samples were examined by target sequencing and RT-qPCR, respectively.

Results: SETBP1 was downregulated in BC cells compared with normal ductal epithelial cells. Low *SETBP1* mRNA expression was an independent prognostic factor for poor recurrence-free survival. Pathway analyses revealed an inverse relationship between decreased *SETBP1* expression and the expression of E2F, MYC, and G2M checkpoint target genes in BC tissues. *SETBP1* knockdown promoted proliferation, inhibition of PP2A activity, and phosphorylation of MAPK in ER-positive BC. Low *SETBP1* expression was influenced by high *SETBP1* promoter methylation and DNA copy number *SETBP1* deletion. *SETBP1* expression with rs6507583 was lower than without rs6507583 in BC.

Conclusions: We demonstrated that low *SETBP1* expression could be a poor prognostic biomarker that promotes ER-positive BC proliferation, possibly via phosphorylation of MAPK.

Key words

28 SETBP1 • ER positive • breast cancer • proliferation • SNP

Background

Breast cancer (BC) is the most common cancer and the leading cause of cancer-related deaths among women worldwide including Japan [1–3], with approximately 70% of cases being estrogen receptor (ER) positive [4]. BC readily metastasizes, which limits the efficacy of further treatment [5]. A lack of understanding of the mechanism of ER-positive BC progression has hampered the development of efficient strategies to control disease progression and improve clinical outcomes. Thus, identifying driver genes contributing to the progression of ER-positive BC is of immense importance.

Single nucleotide polymorphism (SNP)(rs6507583) at the promoter of SET binding protein 1 (*SETBP1*) was implicated in breast carcinogenesis by BC genome-wide association analysis, and the association was stronger in ER-positive BC [6]. Importantly, it was reported that SNPs regulate the expression of target genes that affect tumorigenesis and the development of various cancers [7,8]. Altered *SETBP1* expression by SNPs may contribute to the progression of ER-positive BC.

SETBP1, discovered in 2001 [9], is located at chromosome 18q21.1. *SETBP1* encodes a large protein of 1542 amino acids localized in the cell nucleus and cytoplasm [10,11]. SETBP1 was initially found to interact with SE translation (SET) and with a small protein inhibitor of the tumor suppressor protein phosphatase 2A (PP2A) [12].

SETBP1 expression has been investigated in various diseases, including malignancies such as non-small cell lung cancer [11], hematologic malignancies [13], colorectal cancers [14], ovarian cancers [15], triple-negative BC [16], and gastric cancers [17,18], suggesting that SETBP1 functions either as an oncogene or a tumor suppressor depending on the cancer. However, the clinical and biological significance of SETBP1 in the development of ER-positive BC remains unknown.

Here, we analyzed the expression of SETBP1 in human ER-positive BC and determined the relationship between SETBP1 expression and survival in ER-positive

56 BC patients. Furthermore, we sought to determine the biological role of SETBP1 in the
57 progression of ER-positive BC.

Materials and Methods

Public datasets

Data from The Cancer Genome Atlas database (TCGA) via Broad Institute's Firehose (<http://gdac.broadinstitute.org/>), Cancer Cell Line Encyclopedia (CCLE) via the Cancer Dependency Portal (DepMap) (<https://depmap.org/portal/ccle/>), the Molecular Taxonomy of Breast Cancer International Consortium dataset (METABRIC) via cBioportal (https://www.cbioportal.org/study/summary?id=brca_metabric), and GSE19615 via PrognoScan analysis (<http://dna00.bio.kyutech.ac.jp/PrognoScan/>) were analyzed in this study. We obtained mRNA expression data from the TCGA dataset (1093 tumor tissues, 112 non-cancerous tissues), METABRIC dataset (1959 tumor tissue), GSE19615 dataset (115 tumor tissue), and CCLE dataset (47 cell lines). DNA copy number and methylation data were obtained from 313 tumor tissues and mutation data from 945 tumor tissues in the TCGA dataset. There were TCGA sequencing data were normalized with quantile normalization[19].

The prognostic value of *SET* mRNA expression in BC was assessed according to relapse-free survival using the Kaplan–Meier plotter (<http://kmplot.com/analysis/>), an online database that includes gene expression and clinical data.

Our patient cohort and sample collection

As the first dataset, 56 BC patients who underwent breast resection at the Kyushu University Beppu Hospital and its affiliated hospitals between 1990 and 1996 were enrolled in this study. As the second dataset, 55 BC patients who underwent breast resection at Kyushu University Beppu Hospital and its affiliated hospitals between 2016 and 2022 were enrolled in this study. Tissue samples from resected tumors for RNA extraction were immediately stored in RNAlater (Ambion, Austin, TX, USA), frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Tissue samples from resected tumors for DNA extraction were immediately frozen in liquid nitrogen and stored at

–80°C until DNA extraction. Corresponding non-cancerous breast tissues were also collected. Registration of clinicopathological characteristics and a prognostic follow-up were conducted after surgery. Written informed consent was obtained from each patient. The study design was approved by the institutional review boards and the ethics committee of the Kyushu University Institutional Review Board (approval number: 24121-00).

RNA extraction

Total RNA was extracted from frozen tissue samples and cell lines using Isogen (Nippon Gene, Tokyo, Japan), as described previously [20].

Reverse-transcription quantitative PCR (RT-qPCR)

RT was performed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) as described previously [21]. Quantitative polymerase chain reaction (qPCR) was performed using the LightCycler 480 SYBR Green I Master Mix (Roche, Basel, Switzerland) as described previously [22]. The primers used for *SETBP1* were 5'-GCTTACAACCTGCCCTGACCT-3' (sense) and 5'-AAGTTTCCTCCTTCCAGGGC-3' (antisense), and those used for *18s* were 5'-AGCCACATCGCTCAGACAC-3' (sense) and 5'-GCC CAATACG ACCAAATCC-3' (antisense). The expression level of *SETBP1* mRNA was normalized to that of *18s*.

Immunohistochemical analysis

Immunohistochemical analysis of *SETBP1* expression was performed using formalin-fixed paraffin-embedded surgical sections obtained from patients with ER-positive BC as described previously [23]. The primary rabbit polyclonal antibody against SETBP1 (bs-4944R; Bioss, Woburn, MA, USA) was used at a dilution of 1:1000. SET (ab1183; Abcam, Cambridge, UK) at a dilution of 1:500. The primary

mouse monoclonal antibody against Ki-67 (M7240; Dako, Glostrup, Denmark) was
used at a dilution of 1:100. Tumor histology was independently reviewed by two
researchers, including an experienced pathologist (T.T.)

The Ki-67 labeling index was expressed as the percentage of positively nuclear
staining cells among at least 500 invasive cells in the area scored. We performed the
analysis on our cohort of 55 patients.

Cell lines and cell culture

The human BC cell lines MDA-MB-468, HCC-1954, MCF10A, SKBR-3 (all
from ATCC), MDA-MB-361, ZR-75-1, MDA-MB-231 (all from KAC), MCF7, and
MRK-nu1 (both from Japanese Collection of Research Bioresources Cell Bank), and the
non-cancer HuMEC cells (Kurabo Industries Ltd.) were used in this study. The MDA-
MB-468, HCC-1954, ZR-75-1, MDA-MB-231, and SKBR-3 cell lines were cultured in
RPMI1640; the MCF7 and MDA-MB-361 cell lines were cultured in D-MEM, and
MRK-nu1 and HuMEC cells were cultured in DM160, with all media supplemented
with 10% fetal bovine serum (FBS). HuMEC cells were cultured using the
MammaryLife Comp kit (Kurabo Industries Ltd., Osaka, Japan). MCF10A cells were
cultured in MEGM (Lonza, Basel, Switzerland) supplemented with 100 ng/ml cholera
toxin. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

DNA extraction

DNA was extracted from tumor and normal tissue samples and from cell lines
using the AllPrep DNA/RNA Mini Kit (Qiagen, Hidden, Germany) according to the
manufacturer's instructions.

DNA sequencing

First, we amplified the extracted DNA, targeting a specific region of interest, using PCR. For amplification, we utilized the primers 5'-GTAAGATCCGAAGTGGTGGCA-3' (sense) and 5'-TTACACGCAGTCCCAGTTCA-3' (antisense). The PCR conditions were as follows: an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 98°C for 10 seconds, and extension at 68°C for 1 minute. After amplification, the PCR products were checked on an agarose gel, and the band corresponding to the target was excised and purified from the gel. Direct sequencing of these purified PCR products was conducted by FASMAC, using the Sanger sequencing method, as described previously [24,25].

Gene set enrichment analysis (GSEA)

The associations between *SETBP1* expression and previously defined gene sets were analyzed by GSEA using BC expression profiles from the appropriate TCGA dataset. The biologically defined gene sets were obtained from the Molecular Signatures Database v5.2 (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>).

Gene Ontology (GO) analysis

To determine the biological implications of low *SETBP1* expression, GO analysis was conducted using DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/tools.jsp>). This tool provided a comprehensive set of functional annotations for the evaluated genes, allowing us to categorize them into corresponding biological processes, cellular components, and molecular functions. The significance of the enriched GO terms was determined using the modified Fisher's exact test, with $P < 0.05$ considered significant.

siRNA-mediated knockdown experiments

Human *SETBP1*-specific and negative control siRNAs were purchased from Thermo Fisher and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. The BC cell lines were transfected with siRNA oligonucleotides using Lipofectamine RNAiMAX (Thermo Fisher) according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed as described previously [20]. The following antigen-specific antibodies were used: primary mouse polyclonal antibody against SETBP1 (bs-4944R; Bioss) at a dilution of 1:1000, primary rabbit polyclonal antibody against SET (ab1183; Abcam) at a dilution of 1:2000, a mixture of three specific rabbit monoclonal primary antibodies against phospho-cdk2 Tyr15, phospho-Histone H3 Ser10, and β -actin (ab136810; Abcam) at a dilution of 1:250, primary rabbit polyclonal antibody against p44/42 MAPK (k1/2) (#9102S; Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000, primary rabbit polyclonal antibody against phospho-p44/42 MAPK (k1/2) (#9101S; Cell Signaling Technology) at a dilution of 1:1000, primary mouse polyclonal antibody against PP2A C subunit (05-421; Millipore, Billerica, MA, USA) at a dilution of 1:1000, and primary mouse polyclonal antibody against β -actin (Santa Cruz Biotechnology) at a dilution of 1:10,000.

Colony formation assay

The colony formation assay was performed as described previously [26]. For siRNA-mediated knockdown of *SETBP1*, cells were plated at a density of 500/well (MCF7) or 2×10^4 /well (MDA-MB-361) in 6-well plates and incubated at 37°C under 5% CO₂. After 14 days, the colonies were stained using the Differential Quick Stain Kit (Sysmex) according to the manufacturer's instructions. Visible colonies were photographed using the FUSION SOLO S. Colony counts were determined using ImageJ software (version 1.80, NIH, Bethesda, MD, USA).

PP2A phosphatase activity assay

The protein phosphatase activity in each cell lysate was determined by measuring the generation of free phosphate from threonine phosphopeptide using the malachite green–phosphate complex assay, according to the instructions of the manufacturer (Upstate Biotechnology, Lake Placid, NY, USA). In brief, PP2A-specific reaction buffer (Millipore) containing 750 mM phosphopeptide substrate was added to cell lysates prepared in a low-detergent lysis buffer. After incubation for 10 min at 30°C, the malachite dye was added, and free phosphate was measured by optical density at 655 nm.

Statistical analysis

Associations between variables were analyzed using Student's t-test, the Mann–Whitney U test, or Fisher's exact test. The degree of linearity was assessed using Pearson's correlation coefficient. Recurrence-free survival (RFS) was estimated using the Kaplan–Meier method, and survival curves were compared using the log-rank test. Data analyses were conducted using JMP Pro version 17 software (SAS Institute) and R software version 4.3.1 (The R Foundation). Two-sided *P* values <0.05 were deemed statistically significant.

Results

Downregulation of *SETBP1* in BC

SETBP1 mRNA expression was significantly lower in BC tissues compared with normal breast tissues in the TCGA dataset ($P<0.05$) (Figure 1A). In 71.4% of cases, *SETBP1* mRNA expression was lower in BC tissues than in normal breast tissues, according to RT-qPCR, in our BC cohort (Figure 1B). Regarding ER status, *SETBP1* mRNA expression was lower in tumor tissues than in normal breast tissues in 61.3% of ER-positive cases and 94.4% of ER-negative cases (Figure 1C). ER-positive tumor tissues had significantly higher *SETBP1* mRNA expression compared with ER-negative tumor tissues in the TCGA dataset (Figure 1D). Immunohistochemical analysis showed that SETBP1 was highly expressed in the normal epithelial cells of ER-positive BC (Figure 1E).

These results indicate that *SETBP1* is downregulated in BC tumor cells.

RFS curves of BC patients according to *SETBP1* mRNA expression

We assessed the prognostic and clinical significance of *SETBP1* mRNA expression in BC. First, we evaluated the RFS rates according to *SETBP1* mRNA expression in patients with BC. In the univariate analysis, tumor size (≥ 2 cm) ($P<0.05$), menopause ($P<0.05$), and low *SETBP1* mRNA expression ($P<0.05$) were significantly associated with a lower RFS rate (Table 1). The multivariate analysis indicated that tumor size (≥ 2 cm) ($P<0.05$), menopause ($P<0.05$), and low *SETBP1* mRNA expression ($P<0.05$) were independent predictors of a poor prognosis in BC (Table 1). The observed trend toward poorer prognosis for lymph node metastasis in the METABRIC dataset, despite not reaching statistical significance, may have been influenced by differences in treatment strategies and surgical techniques for lymph node-positive cases across institutions. The RFS rate was significantly lower in the low than high *SETBP1* mRNA expression group in the METABRIC and GSE19615 datasets ($P<0.01$

and $P < 0.05$, respectively; Figure 2A). Furthermore, the RFS rate was lower in the low than high *SETBP1* mRNA expression group in ER-positive BC cases in the METABRIC and GSE1965 datasets ($P < 0.05$ and $P = 0.076$, respectively; Figure 2B). In the ER-negative BC cases, there was no significant difference in the RFS rate between the high and low *SETBP1* mRNA expression groups in the METABRIC and GSE1965 datasets ($P = 0.46$ and $P = 0.14$, respectively; Figure 2B).

These results suggest that low *SETBP1* expression indicates a poor prognosis in patients with ER-positive BC.

Clinicopathologic significance of *SETBP1* mRNA expression in BC

We analyzed the associations between *SETBP1* mRNA expression and clinicopathologic factors in BC patients at our hospital (Table 2A). We divided the patients into two groups: the T<N group (*SETBP1* mRNA expression was lower in BC than normal breast tissues) and T>N group (*SETBP1* mRNA expression was higher in BC than normal breast tissues). We found higher frequencies of ER-negative and PgR-negative statuses, but a lower frequency of lymphatic invasion, in the T<N than T>N group. In the METABRIC dataset, low *SETBP1* mRNA expression was associated with an older age, large tumor size, ER-negative, PgR-negative, and HER2-negative statuses, high histological grade, menopause, lymph node metastasis, and TNM stage (Table 2B).

These results suggest that low *SETBP1* mRNA expression is positively associated with malignant phenotypes.

Pathway analysis of *SETBP1* in ER-positive BC

To explore oncogenic pathways associated with *SETBP1* expression in ER-positive BC, we performed GSEA using TCGA dataset. *SETBP1* expression was negatively correlated with the expression of E2F, MYC, and G2M checkpoint target geneset in ER-positive BC (Figure 3A). E2F, MYC, and G2M checkpoint-related genes

play important roles in various biological processes, including cell proliferation and cell cycle [27] [28] [29]. Next, we performed GO analysis and observed downregulation of many genes involved in cell cycle-related and MAPK-related pathways (Figure 3B).

The results of the GSEA and GO analyses motivated us to investigate whether SETBP1 regulates tumor proliferation, cell cycle progression, and activation of the MAPK pathway in ER-positive BC.

Increased proliferation of ER-positive BC cells in vitro by *SETBP1* knockdown

We performed a colony formation assay using a knockdown system in two ER-positive BC cell lines, MCF7 and MDA-MB-361, to clarify the relationship between *SETBP1* and proliferative capacity. We found that *SETBP1* knockdown significantly reduced the proliferation of MCF7 and MDA-MB-361 cells (Figure 3C, 3D).

Ki-67 labeling index of *SETBP1* mRNA expression in ER-positive BC tissues

We investigated the relationship between Ki-67 labeling index and *SETBP1* expression in ER-positive BC tissues. To do this, the *SETBP1* mRNA level was divided into high and low groups based on the median value. As expected, the Ki-67 labeling index was significantly higher in the low *SETBP1* mRNA expression group compared to the high expression group ($P < 0.05$, Figure 3E), reinforcing the notion that low *SETBP1* expression promotes the proliferation of BC cells.

Activation of cell cycle progression by *SETBP1* knockdown

We evaluated the expression of histone H3 pSer10 (marker of cell cycle arrest at G2/M [30]) and CDK2 pTyr15 (marker of cell cycle arrest at G1/S [31]) in *SETBP1*-knockdown ER-positive BC cells (Figure 4A). The expression of both markers was suppressed, suggesting that *SETBP1* knockdown promotes cell cycle progression.

Suppressed PP2A activity and increased MAPK phosphorylation by *SETBP1* knockdown

SETBP1 has been reported to form a complex with SET and PP2A [32]. PP2A is considered a tumor suppressor protein since its inactivation is mediated primarily by increased expression of its endogenous inhibitors, such as SET and cellular inhibitor of phosphatase 2A [33]. However, it is unknown if the expression of SETBP1, which binds to and functions with SET, affects the activation of PP2A. Thus, we performed a PP2A activity assay using *SETBP1*-knockdown cells. PP2A activity was significantly suppressed in *SETBP1*-knockdown ER-positive BC cells compared with control siRNA ER-positive BC cells (Figure 4B). Activation of PP2A has been reported to inhibit phosphorylation of MAPK [34]. Therefore, we hypothesized that low *SETBP1* expression may activate the MAPK signaling pathway by inactivating PP2A activity in ER-positive BC cells. As expected, the ER-positive BC cell lines MCF7 and MDA-MB-361 with *SETBP1* knockdown showed increased phosphorylation levels of MAPK compared with control cells(Figure 4A).

These results suggest that *SETBP1* knockdown facilitates cell proliferation possibly via inactivation of PP2A, followed by activation of MAPK signaling in ER-positive BC cells.

Potential factors responsible for the downregulated *SETBP1* expression

Since DNA methylation, DNA copy number alterations, SNPs, and gene mutations are well-known factors that regulate gene expression [7,35,36], we investigated the relationship between each of these factors and *SETBP1* expression in BC. To do this, the *SETBP1* mRNA level was divided into high and low groups based on the median value.

First, we evaluated the relationship between the *SETBP1* promoter methylation rate and mRNA expression. The promoter methylation rate was significantly higher in

the low than high *SETBP1* mRNA expression groups (68.1% vs. 33.3%, $P < 0.05$, Figure 5A). The *SETBP1* promoter methylation rate was negatively correlated with *SETBP1* mRNA expression in the TCGA dataset ($R = -0.50$, $P < 0.01$, Figure 5A). When comparing ER-positive and ER-negative tumors from the TCGA dataset, the *SETBP1* promoter methylation rate was significantly lower in ER-positive BC (Supplementary Figure 1A).

Second, the relationship between the *SETBP1* DNA copy number status and gene expression level was assessed. The rate of *SETBP1* deletion was significantly higher in the low than high *SETBP1* mRNA expression groups (41.4% vs. 11.9%, $P < 0.05$, Figure 5B). Compared with the diploid cases, cases with *SETBP1* deletion had lower *SETBP1* mRNA expression ($P < 0.05$). Regarding ER status, the ER-positive BC cases had a lower rate of *SETBP1* deletion (Supplementary Figure 1B).

Next, we examined the relationship between the *SETBP1* rs6507583 SNP and *SETBP1* mRNA expression in 55 ER-positive BC cases from our BC cohort. rs6507583 was detected in only one case, and this case also showed the lowest *SETBP1* expression level (Figure 5C). Next, we investigated the rs6507583 SNP in human mammary cell lines and found that 2 of the 10 cell lines possessed rs6507583. The *SETBP1* expression level was lower in the two cell lines with rs6507583 (MRK-nu1 and SKBR-3 cells) than in the cell lines without this SNP (Figure 5D). Interestingly, SKBR-3 cells, possessing rs6507583, had low *SETBP1* mRNA expression with a low promoter methylation rate, whereas MDA-MB-231 cells, which lack rs6507583, had low *SETBP1* mRNA expression with a high promoter methylation rate. This suggests that the rs6507583 SNP and promoter methylation rate strongly contribute to the low *SETBP1* expression (Figure 5E).

Finally, the mutation rate in *SETBP1* was examined. The frequency of mutations in *SETBP1* was only 1.1% in the TCGA dataset in all BC cases (Figure 5F). The ER-positive and ER-negative cases had a comparable rate of mutations

347 (Supplementary Figure 1C).

348 These results suggest that the low expression of *SETBP1* may be induced by
349 the high promoter methylation rate, genomic deletions, and the rs6507583 SNP in BC.

350

Discussion

In this study, we found that low *SETBP1* expression contributed to the proliferation of ER-positive BC cells possibly by inducing MAPK phosphorylation. Furthermore, low *SETBP1* expression predicted a poor prognosis in ER-positive BC patients. To the best of our knowledge, this is the first study to provide evidence that *SETBP1* has a tumor suppressive role in ER-positive BC.

Our hospital cohort and the METABRIC dataset demonstrated that *SETBP1* expression was negatively associated with several malignant phenotypes, including tumor size, lymph node metastasis, histological grade, and TNM stage. However, in the high *SETBP1* mRNA expression group, lymphatic invasion is frequent in our hospital cohort. Lymphovascular invasion was evaluated only in our dataset, where the information was available. Given the small number of cases, the opposite result may have been observed. Further investigation is required. Additionally, low *SETBP1* expression was associated with poor prognosis in ER-positive BC. Our biological analysis indicated that low *SETBP1* expression accelerated cell proliferation, partly via cell cycle progression, in ER-positive BC. Furthermore, decreased *SETBP1* mRNA expression was attributed to DNA copy number deletion, increased promoter methylation, and the SNP rs6507583. These findings provide clinical and biological evidence that *SETBP1* plays a tumor-suppressive role in ER-positive BC.

Interestingly, low *SETBP1* expression in triple-negative BC has been reported to be associated with good prognosis [16]. Our study also showed that low *SETBP1* expression was not associated with a poor prognosis in ER-negative BC. These findings suggest *SETBP1* may have different functions in BC depending on ER status.

SETBP1 mRNA expression was significantly higher in ER-positive BC than in ER-negative BC. We found that *SETBP1* mRNA expression was decreased due to DNA copy number deletion, increased promoter methylation rate, and the rs6507583 SNP. ER-negative BC exhibited multiple factors contributing to the lower *SETBP1*

expression compared with ER-positive BC (Supplementary Figure 1A, 1B).

Our in vitro analysis revealed that *SETBP1* knockdown inhibited PP2A activity and increased MAPK phosphorylation, thereby enhancing cell cycle progression and proliferation in ER-positive BC cells. However, previous studies reported that SETBP1 overexpression leads to formation of the SETBP1/SET/PP2A complex, resulting in PP2A inhibition and subsequent cell proliferation in colorectal cancer, ovarian cancer, and acute myeloid leukemia [13,14,37]. These contradictory findings suggest that the role of SETBP1 in tumor progression varies depending on tissue type. Further research is needed to elucidate the tissue-dependent mechanisms of SETBP1 function.

ER-positive BC is treated effectively with endocrine therapies (ET) [37]. Low expression of *SETBP1* may lead to activation of the MAPK pathway, which is downstream of the ER [38], potentially diminishing the efficacy of ET. ER-positive BC cases with low *SETBP1* expression may benefit from combining ET and MAPK inhibitors [39]. *SETBP1* mRNA expression has the potential to serve as a crucial biomarker and therapeutic target for risk stratification and the design of personalized treatments in combination with MAPK inhibitors in ER-positive BC.

In summary, we demonstrated that that *SETBP1* inhibits tumor progression and is a prognostic biomarker in ER-positive BC.

Acknowledgments

The authors would like to thank M. Kasagi, S. Sakuma, T. Fukuda, N. Mishima, T. Kawano, and M. Utou for their technical assistance. This work was supported in part by the following grants and foundations: Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Science Research (grant numbers: 19K09176, 19H03715, 20H05039, 20K08930, 20K17556, 21K07179, 22K02903, 22K09006, 23K06765, 23K08074, 24K11766, 24K10384, and 24K02523); OITA Cancer Research Foundation; AMED (grant numbers: 23ck0106825h001, 23ck0106800h001,

22ama221501h0001, 21ck0106690s0201, 20cm0106475h0001, 20ck0106547h0001, and 20ck0106541h0001); Takeda Science Foundation; and The Princess Takamatsu Cancer Research Fund.

Disclosure Statement

One of the co-authors of this manuscript, Takaaki Masuda, serves as the editor of *Breast Cancer*. To maintain a fair and unbiased review process, the editorial office has ensured that Takaaki Masuda was not involved in the peer review or decision-making process for this manuscript.

Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*. 2021;71:209–49.
2. National Cancer Center Japan. [cited 2024 Apr 29]. Available from: https://ganjoho.jp/reg_stat/statistics/stat/cancer/14_breast.html
3. Kajiwarara Y, Takahashi A, Ueno H, Kakeji Y, Hasegawa H, Eguchi S, et al. Annual report on National Clinical Database 2020 for gastroenterological surgery in Japan. *Ann Gastroenterol Surg*. 2023;7:367–406.
4. Tong CWS, Wu M, Cho WCS, To KKW. Recent Advances in the Treatment of Breast Cancer. *Front Oncol*. 2018;8:227.
5. Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. Overview of resistance to systemic therapy in patients with breast cancer. *Adv Exp Med Biol*. 2007;608:1–22.
6. Michailidou K, Beesley J, Lindstrom S, Canisius S, Dennis J, Lush MJ, et al. Genome-wide association analysis of more than 120,000 individuals identifies 15 new susceptibility loci for breast cancer. *Nat Genet*. 2015;47:373–80.

- 437 7. Yang W, Zhang T, Song X, Dong G, Xu L, Jiang F. SNP-Target Genes Interaction
438 Perturbing the Cancer Risk in the Post-GWAS. *Cancers* . 2022;14:1–17.
- 439 8. Iguchi T, Nambara S, Masuda T, Komatsu H, Ueda M, Kidogami S, et al. miR-146a
440 Polymorphism (rs2910164) Predicts Colorectal Cancer Patients' Susceptibility to Liver
441 Metastasis. *PLoS One*. 2016;11:e0165912.
- 442 9. Minakuchi M, Kakazu N, Gorrin-Rivas MJ, Abe T, Copeland TD, Ueda K, et al.
443 Identification and characterization of SEB, a novel protein that binds to the acute
444 undifferentiated leukemia-associated protein SET. *Eur J Biochem*. 2001;268:1340–51.
- 445 10. Oakley K, Han Y, Vishwakarma BA, Chu S, Bhatia R, Gudmundsson KO, et al.
446 Setbp1 promotes the self-renewal of murine myeloid progenitors via activation of
447 Hoxa9 and Hoxa10. *Blood*. 2012;119:6099–108.
- 448 11. Li HR, Gao J, Jin C, Jiang JH, Ding JY. Downregulation of SETBP1 promoted non-
449 small cell lung cancer progression by inducing cellular EMT and disordered immune
450 status. *Am J Transl Res*. 2020;12:447–62.
- 451 12. Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a
452 potent inhibitor of protein phosphatase 2A. *J Biol Chem*. 1996;271:11059–62.
- 453 13. Acuna-Hidalgo R, Deriziotis P, Steehouwer M, Gilissen C, Graham SA, van Dam S,
454 et al. Overlapping SETBP1 gain-of-function mutations in Schinzel-Giedion syndrome
455 and hematologic malignancies. *PLoS Genet*. 2017;13:e1006683.
- 456 14. Torrejón B, Cristóbal I, Caramés C, Prieto-Potín I, Chamizo C, Santos A, et al.
457 Analysis of Potential Alterations Affecting SETBP1 as a Novel Contributing
458 Mechanism to Inhibit PP2A in Colorectal Cancer Patients. *World J Surg*. 2018;42:3771–
459 8.
- 460 15. Qiao H-Y, Zhang Q, Wang J-M, Jiang J-Y, Huan L-Y, Yan J, et al. TRIM29
461 regulates the SETBP1/SET/PP2A axis via transcription factor VEZF1 to promote
462 progression of ovarian cancer. *Cancer Lett*. 2022;529:85–99.
- 463 16. Chen LL, Zhang ZJ, Yi ZB, Li JJ. MicroRNA-211-5p suppresses tumour cell
464 proliferation, invasion, migration and metastasis in triple-negative breast cancer by
465 directly targeting SETBP1. *Br J Cancer*. 2017;117:78–88.
- 466 17. Fang F, Liu C, Li Q, Xu R, Zhang T, Shen X. The Role of SETBP1 in Gastric
467 Cancer: Friend or Foe. *Front Oncol*. 2022;12:1–10.
- 468 18. Li Q, Wu R, Wu F, Chen Q. KMT2D promotes proliferation of gastric cancer cells:
469 evidence from ctDNA sequencing. *J Clin Lab Anal*. 2021;35:e23721.
- 470 19. Garmire LX, Subramaniam S. Evaluation of normalization methods in mammalian
471 microRNA-Seq data. *RNA*. 2012;18:1279–88.
- 472 20. Masuda T, Xu X, Dimitriadis EK, Lahusen T, Deng C-X. “DNA Binding Region” of
473 BRCA1 Affects Genetic Stability through modulating the Intra-S-Phase Checkpoint. *Int*
474 *J Biol Sci*. 2016;12:133–43.
- 475 21. Hu Q, Masuda T, Sato K, Tobo T, Nambara S, Kidogami S, et al. Identification of
476 ARL4C as a Peritoneal Dissemination-Associated Gene and Its Clinical Significance in

477 Gastric Cancer. *Ann Surg Oncol*. 2018;25:745–53.

478 22. Nambara S, Masuda T, Nishio M, Kuramitsu S, Tobo T, Ogawa Y, et al. Antitumor
479 effects of the antiparasitic agent ivermectin via inhibition of Yes-associated protein 1
480 expression in gastric cancer. *Oncotarget*. 2017;8:107666–77.

481 23. Shimizu D, Masuda T, Sato K, Tsuruda Y, Otsu H, Kuroda Y, et al. CRMP5-
482 associated GTPase (CRAG) Is a candidate driver gene for colorectal cancer
483 carcinogenesis. *Anticancer Res*. 2019;39:99–106.

484 24. Kobayashi-Ishihara M, Terahara K, Martinez JP, Yamagishi M, Iwabuchi R, Brander
485 C, et al. HIV LTR-Driven Antisense RNA by Itself Has Regulatory Function and May
486 Curtail Virus Reactivation From Latency. *Front Microbiol*. 2018;9:1066.

487 25. Hashimoto M, Masuda T, Nakano Y, Tobo T, Saito H, Koike K, et al. Tumor
488 suppressive role of the epigenetic master regulator BRD3 in colorectal cancer. *Cancer*
489 *Sci [Internet]*. 2024; Available from: <http://dx.doi.org/10.1111/cas.16129>

490 26. Koike K, Masuda T, Sato K, Fujii A, Wakiyama H, Tobo T, et al. GET4 is a novel
491 driver gene in colorectal cancer that regulates the localization of BAG6, a
492 nucleocytoplasmic shuttling protein. *Cancer Sci*. 2022;113:156–69.

493 27. Johnson J, Thijssen B, McDermott U, Garnett M, Wessels LFA, Bernards R.
494 Targeting the RB-E2F pathway in breast cancer. *Oncogene*. 2016;35:4829–35.

495 28. Prall OW, Rogan EM, Musgrove EA, Watts CK, Sutherland RL. c-Myc or cyclin D1
496 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry. *Mol Cell*
497 *Biol*. 1998;18:4499–508.

498 29. Stark GR, Taylor WR. Analyzing the G2/M checkpoint. *Methods Mol Biol*.
499 2004;280:51–82.

500 30. Hans F, Dimitrov S. Histone H3 phosphorylation and cell division. *Oncogene*.
501 2001;20:3021–7.

502 31. Fagundes R, Teixeira LK. Cyclin E/CDK2: DNA Replication, Replication Stress
503 and Genomic Instability. *Front Cell Dev Biol*. 2021;9:774845.

504 32. Oaks J, Ogretmen B. Regulation of PP2A by Sphingolipid Metabolism and
505 Signaling. *Front Oncol*. 2014;4:388.

506 33. Kauko O, Westermarck J. Non-genomic mechanisms of protein phosphatase 2A
507 (PP2A) regulation in cancer. *Int J Biochem Cell Biol*. 2018;96:157–64.

508 34. Zheng H-Y, Shen F-J, Tong Y-Q, Li Y. PP2A Inhibits Cervical Cancer Cell
509 Migration by Dephosphorylation of p-JNK, p-p38 and the p-ERK/MAPK Signaling
510 Pathway. *Curr Med Sci*. 2018;38:115–23.

511 35. Ried T, Meijer GA, Harrison DJ, Grech G, Franch-Expósito S, Briffa R, et al. The
512 landscape of genomic copy number alterations in colorectal cancer and their
513 consequences on gene expression levels and disease outcome. *Mol Aspects Med*.
514 2019;69:48–61.

515 36. Jovanovic J, Rønneberg JA, Tost J, Kristensen V. The epigenetics of breast cancer.

Mol Oncol. 2010;4:242–54.

37. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E, ESMO Guidelines Working Group. Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2012;23 Suppl 7:vii11-9.

38. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev. 2008;29:217–33.

39. Hotokezaka H, Sakai E, Kanaoka K, Saito K, Matsuo K-I, Kitaura H, et al. U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. J Biol Chem. 2002;277:47366–72.

Figure Legends

Figure 1. *SETBP1* expression in breast cancer tissues. A. *SETBP1* mRNA expression in tumor tissues (T) and non-cancerous tissues (N) from a TCGA dataset. B. Waterfall plot showing the ratio of T to N in all BC cases. C. Waterfall plot showing the ratio of T to N according to ER status. Left: ER-positive BC. Right: ER-negative BC. D. *SETBP1* mRNA expression in T and N in our BC cohort and in tumor tissues according to ER status in the TCGA data. E. Immunohistochemical staining of *SETBP1* in T and N. N, normal tissue; T, tumor tissue.

Figure 2. Kaplan–Meier analysis of RFS in BC patients from the METABRIC and GSE19615 datasets. A. All BC cases in the METABRIC (left) and GSE19615 (right) datasets. B. ER-positive (top left) and -negative (top right) BC cases in METABRIC and ER-positive (bottom left) and -negative (bottom right) BC cases in GSE19615.

Figure 3. Association of low expression of *SETBP1* with cell growth in ER-positive breast cancer. A. Gene set enrichment analysis using TCGA dataset. B. GO analysis using the TCGA dataset. C. *SETBP1* mRNA expression using RT-qPCR and *SETBP1* protein expression using western blot analysis in *SETBP1*-knockdown and control cells. D. Colony formation assays using *SETBP1*-knockdown MCF7 and MDA-MB-361

cells. E. Ki-67 labeling index of *SETBP1* mRNA expression in ER-positive BC tissues.

* $P < 0.05$

Figure 4. Increased phosphorylation of MAPK and PP2A activity by *SETBP1*

knockdown in ER-positive BC cells. A. Expression levels of SET, PP2A, MAPK, phosphorylated MAPK, H3pSer10, and CDK2pTyr15 in *SETBP1*-knockdown and control cells (negative control). B. PP2A activity of *SETBP1*-knockdown and negative control cells.

Figure 5. Potential factors responsible for the downregulation of *SETBP1* expression. A.

Left: relationship between *SETBP1* mRNA expression and *SETBP1* promoter methylation rate in the TCGA dataset. Right: correlation between *SETBP1* mRNA expression and *SETBP1* promoter methylation rate in the TCGA dataset. R is the Pearson correlation coefficient. B. Left: relationship between *SETBP1* mRNA expression and *SETBP1* DNA copy number in the TCGA dataset. Right: relationship between *SETBP1* mRNA expression and *SETBP1* DNA copy number in the TCGA dataset. C. Relationship between the rs6507583 SNP and *SETBP1* mRNA expression in our BC cohort. D. Relationship between the rs6507583 SNP and *SETBP1* mRNA expression in BC cell lines. E. Relationship between the *SETBP1* promoter methylation rate and *SETBP1* mRNA expression in BC cell lines. F. Frequency of *SETBP1* mutations among all BC cases in the TCGA dataset.

Supplementary Figure 1. Comparison of factors affecting low *SETBP1* expression

according to ER status. A. Comparison of *SETBP1* promoter methylation rates in the TCGA dataset according to ER status. B. Comparison of *SETBP1* DNA copy number abnormalities in the TCGA dataset according to ER status. C. Frequency of *SETBP1* mutations among BC cases according to ER status in the TCGA dataset.

Table 1: Univariate and multivariate analyses of the clinicopathological factors affecting disease-free survival in the METABRIC dataset

Variable	HR	Univariate (95% CI)	<i>P</i> -value	HR	Multivariate (95% CI)	<i>P</i> -value
Menopause (Post/Pre)	1.35	1.17–1.56	<0.05	1.37	1.17–1.58	<0.05
ER status (+/-)	1.01	0.88–1.16	0.88			
PgR status (+/-)	0.96	0.85–1.07	0.45			
HER2 status (+/-)	1.07	0.88–1.03	0.48			
Tumor size (≥ 2 / < 2 cm)	1.19	1.06–1.33	<0.05	1.17	1.04–1.31	<0.05
Lymph node metastasis (+/-)	1.10	0.97–1.23	0.18			
<i>SETBP1</i> expression (low/high)	1.25	1.11–1.40	<0.05	1.26	1.10–1.45	<0.05

ER, Estrogen Receptor; PgR, Progesterone Receptor; CI, confidential interval; HR, hazard ratio.

Table 2: Correlations between *SETBP1* mRNA expression in tumor tissues and clinicopathological factors in breast cancer

(A) Our cohort

Variable	T<N (n =41) Number (%)	T>N (n =15) Number (%)	P-value
Age (y)			
Mean ± SD	56.5 ± 1.5	55.3 ± 2.7	0.66
Menopause			
Pre	5 (14.3)	5 (41.7)	0.06
Post	30 (85.7)	7 (58.3)	
Tumor size (cm)			
<2	24 (64.9)	7 (58.3)	0.47
≥2	13 (35.1)	5 (41.7)	
ER status			
+	20 (51.3)	12 (92.3)	<0.01
-	19 (48.7)	1 (7.7)	
PgR status			
+	16 (41.0)	12 (92.3)	<0.01
-	23 (59.0)	1 (7.7)	
Lymphatic invasion			
0	24 (64.9)	4 (30.8)	<0.05
1–3	13 (35.1)	9 (69.2)	
Vascular invasion			
0	33 (89.2)	11 (84.6)	0.96
1–3	4 (10.8)	2 (13.4)	
Lymph node metastasis			
+	21 (51.2)	9 (60.0)	0.33
-	20 (48.8)	6 (40.0)	
Distant metastasis			
+	2 (4.9)	0 (0)	0.53
-	39 (95.1)	15 (100)	

(B) METABRIC dataset

Variable	Low expression (n = 980)	High expression (n = 979)	P-value
Age (y)			
Mean ± SD	60.0 ± 0.4	62.1 ± 0.4	<0.05
Tumor size (cm)			
<2	385 (39.3)	456 (46.6)	<0.01
≥2	586 (59.8)	509 (52.0)	
ER status			
+	621 (63.4)	871 (89.0)	<0.01
-	359 (36.6)	108 (11.0)	
PgR status			
+	386 (39.4)	642 (65.6)	<0.01
-	594 (60.6)	337 (34.4)	
HER2 status			
+	180 (18.4)	67 (6.8)	<0.01
-	800 (81.6)	912 (93.2)	

Histological grade			
1, 2	322 (32.9)	608 (62.1)	<0.01
3	632 (64.5)	316 (32.2)	
Menopause			
Pre	233 (23.8)	190 (19.4)	<0.05
Post	747 (76.2)	789 (80.6)	
Lymph node metastasis			
+	505 (51.5)	398 (40.7)	<0.01
-	442 (45.1)	541 (55.3)	
Stage			
1	336 (34.3)	407 (41.6)	<0.01
2	228 (23.3)	200 (20.4)	
3	133 (13.6)	79 (8.1)	

ER, Estrogen Receptor; PgR, Progesterone Receptor; SD, standard deviation

Fig. 1

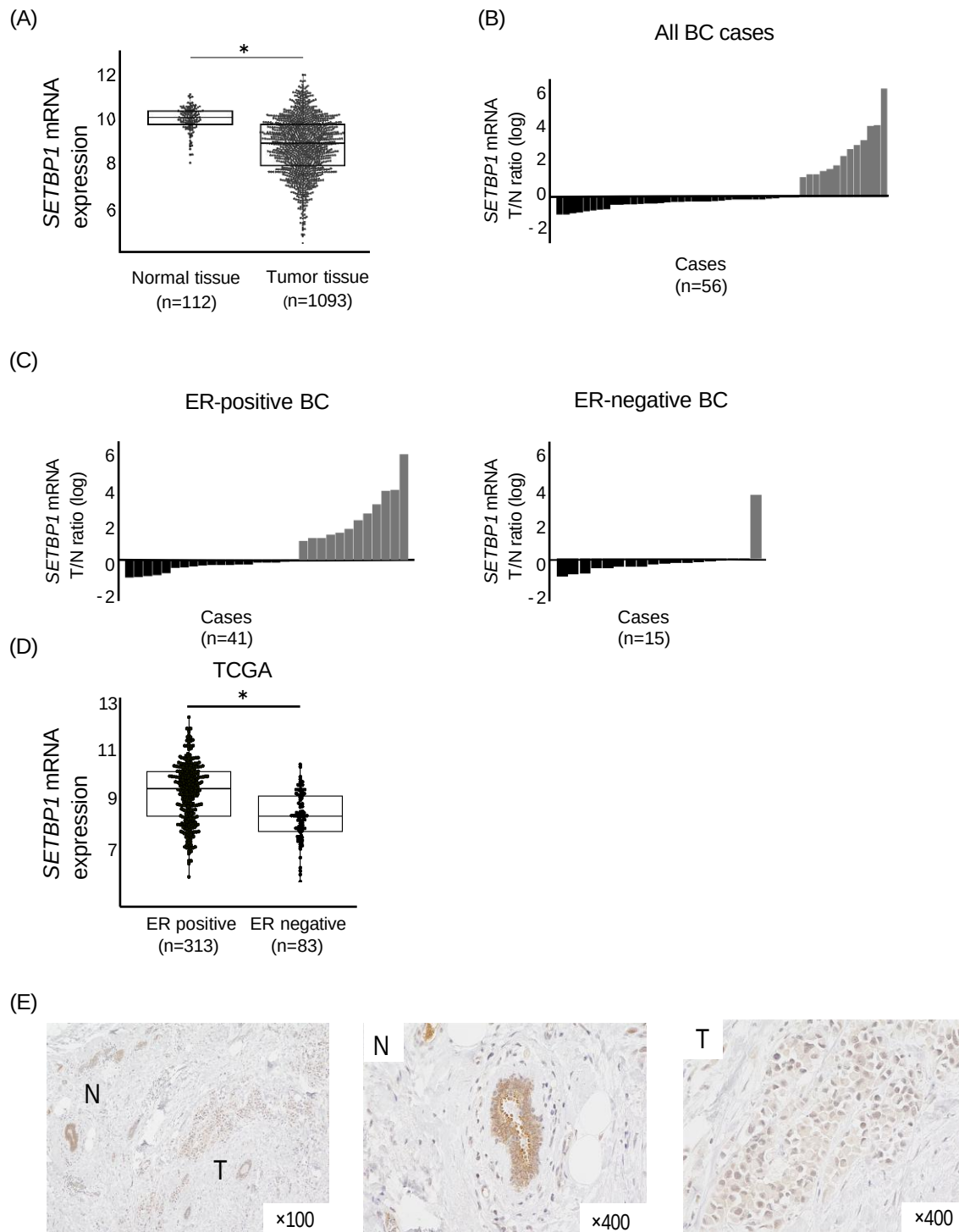
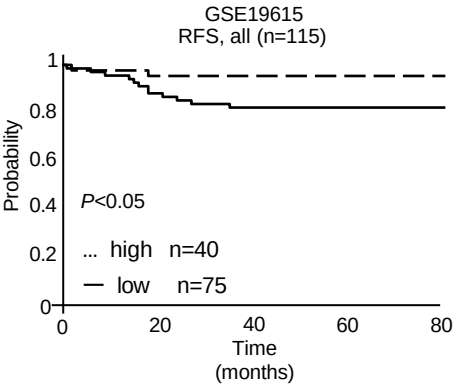
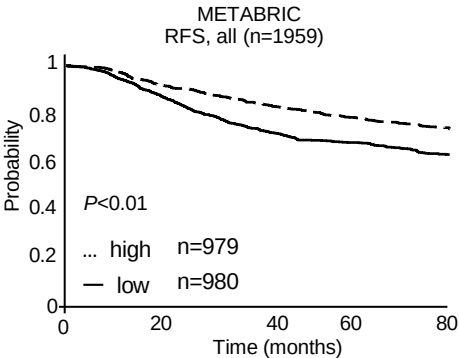


Fig. 2

(A)



(B)

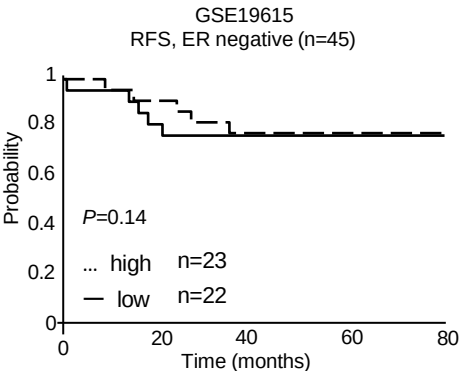
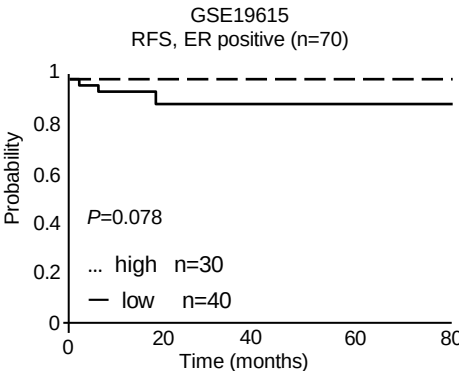
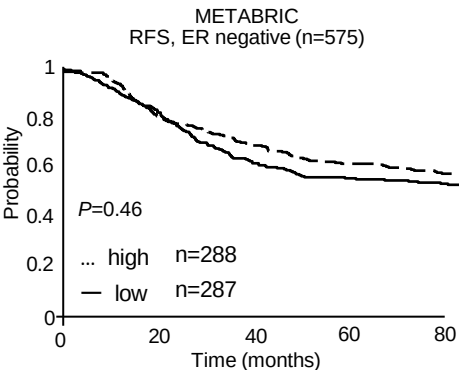
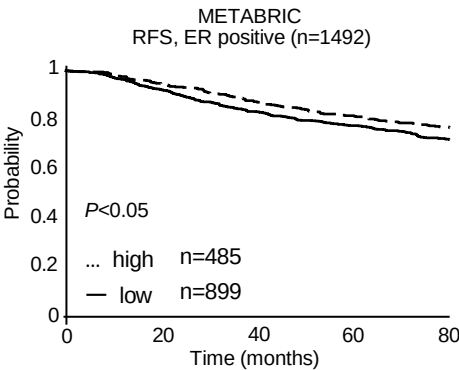


Fig. 3

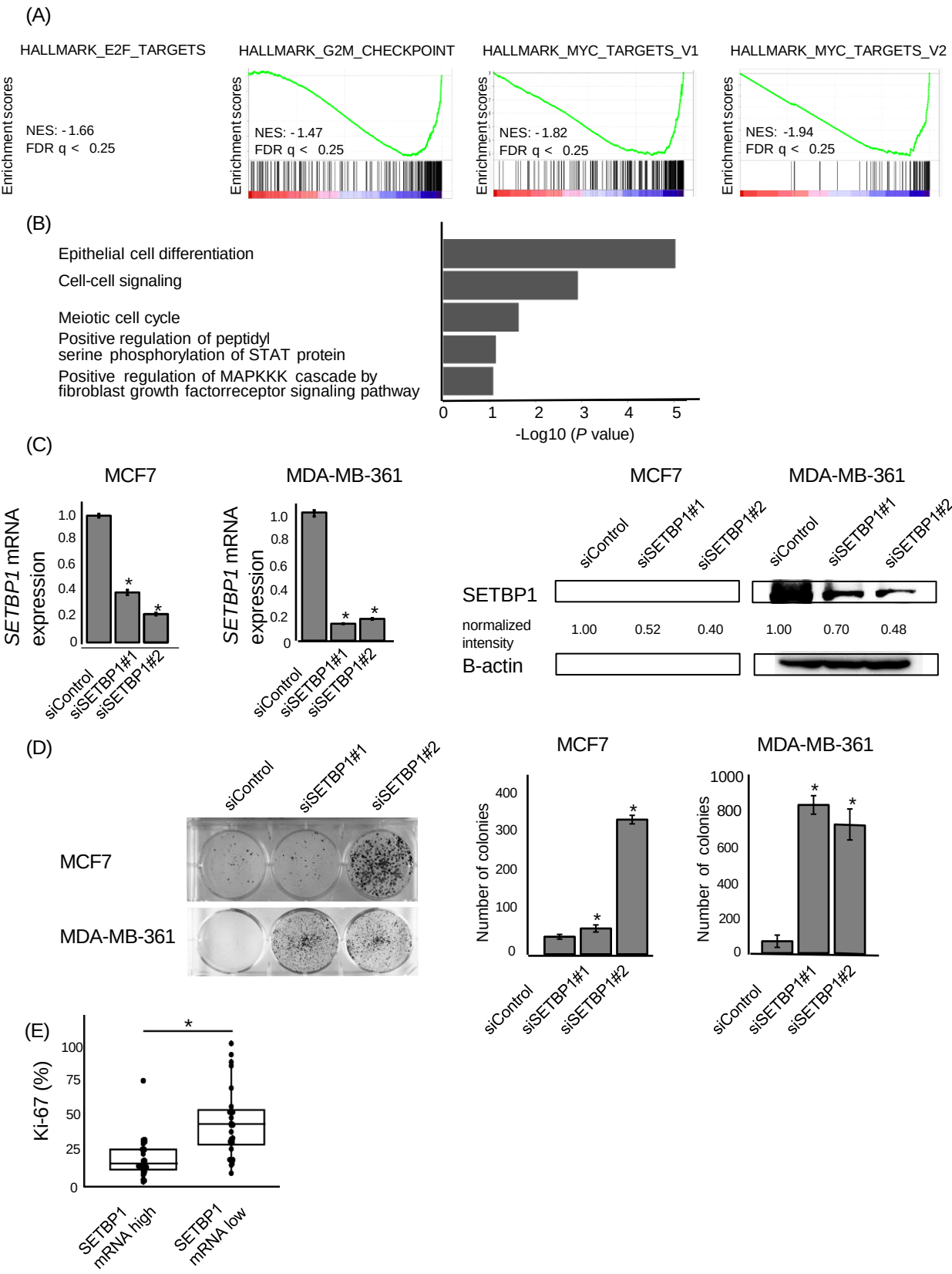


Fig. 4

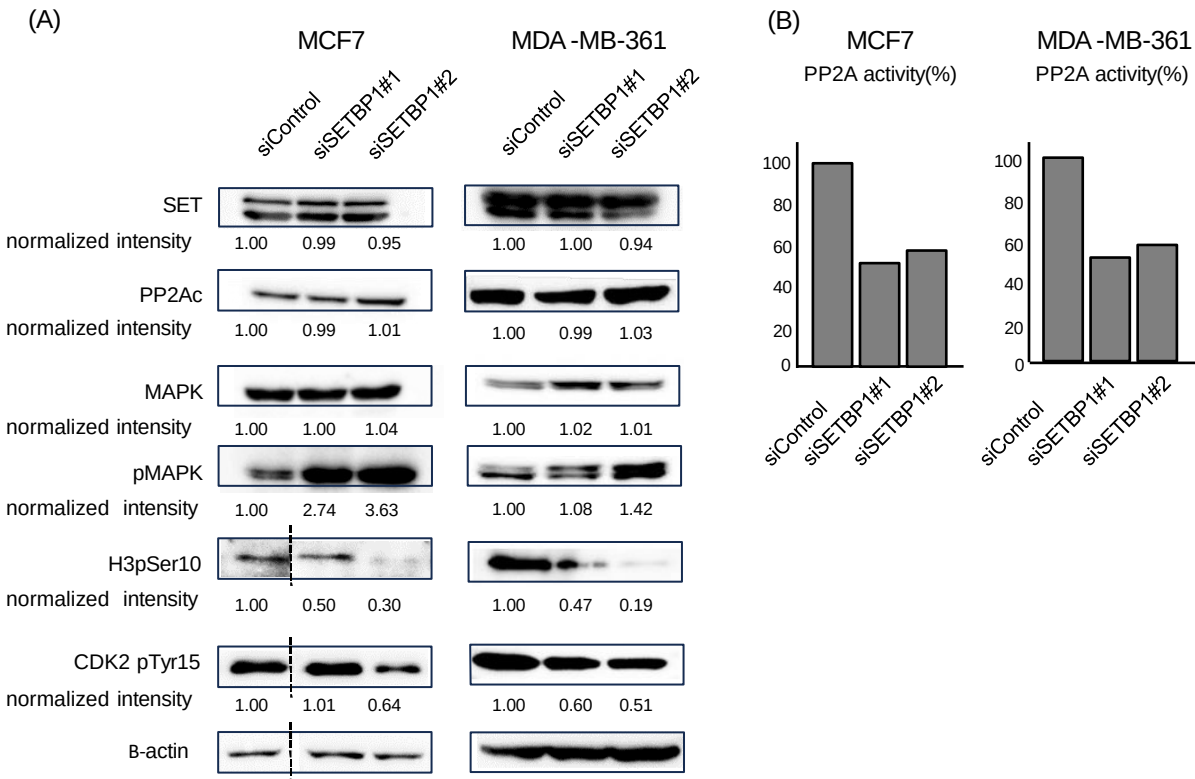
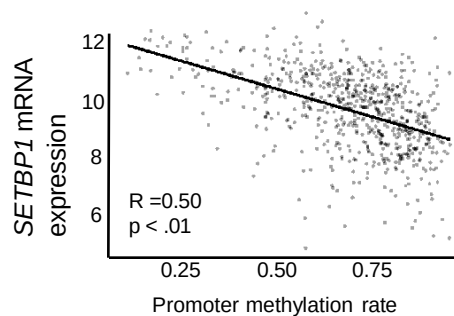
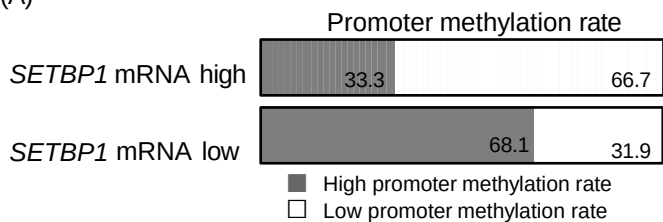
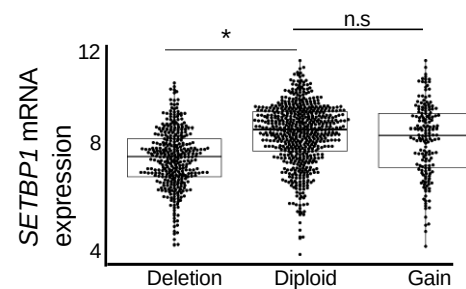
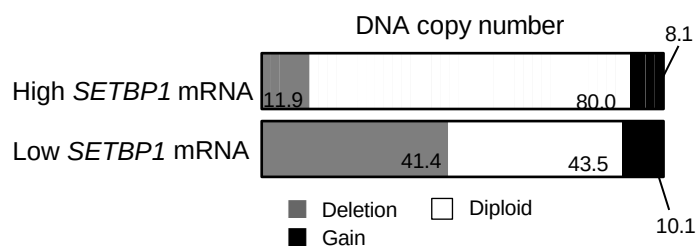


Fig. 5

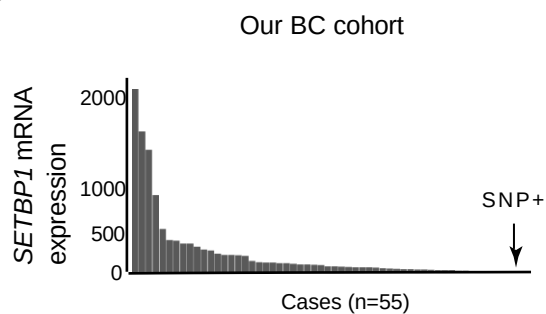
(A)



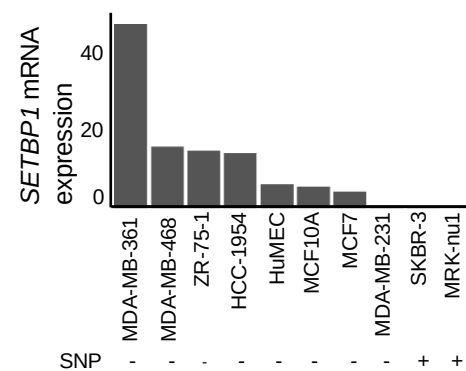
(B)



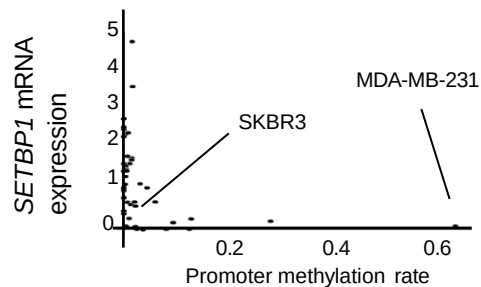
(C)



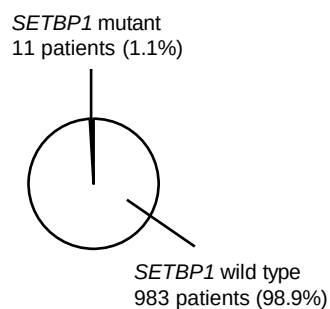
(D)



(E)

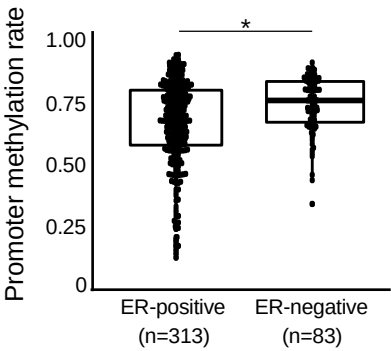


(F)

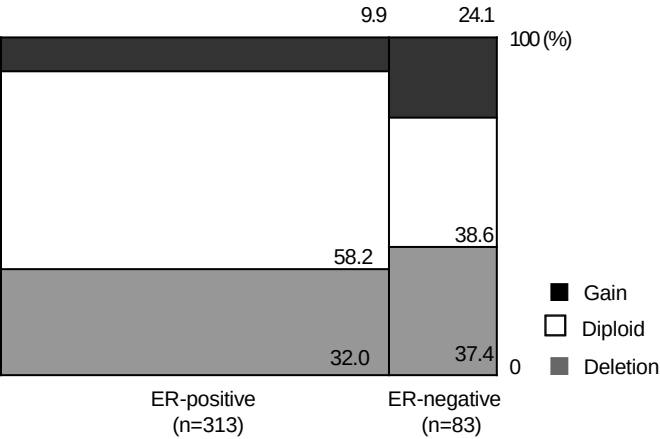


Supplementary Fig. 1

(A)



(B)



(C)

