Activation of an estrogen/estrogen receptor signaling by BIG3 through its inhibitory effect on nuclear transport of PHB2/REA in breast cancer

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Breast cancer is known to be a hormone-dependent disease, and estrogens through an interaction with estrogen receptor (ER) enhance the proliferative and metastatic activity of breast tumor cells. Here we show a critical role of transactivation of BIG3, brefeldin A-inhibited guanine nucleotide-exchange protein 3, in activation of the estrogen/ER signaling in breast cancer cells. Knocking-down of *BIG3* expression with small-interfering RNA (siRNA) drastically suppressed the growth of breast cancer cells. Subsequent coimmunoprecipitation and immunoblotting assays revealed an interaction of BIG3 with prohibitin 2/repressor of estrogen receptor activity (PHB2/REA). When BIG3 was absent, stimulation of estradiol caused the translocation of PHB2/REA to the nucleus, enhanced the interaction of PHB2/REA and ERα, and resulted in suppression of the ERα transcriptional activity. On the other hand, when BIG3 was present, BIG3 trapped PHB2/REA in the cytoplasm and inhibited its nuclear translocation, and caused enhancement of ERα transcriptional activity. Our results imply that BIG3 overexpression is one of the important mechanisms causing the activation of the estrogen/ERα signaling pathway in the hormone-related growth of breast cancer cells. (*Cancer Sci* 2009; 100: 1468–1478)

Breast cancer is the most common cancer among women worldwide; the breast cancer incidence and death rates for Japanese were 102.8 and 17.1 per 100 000 population, respectively, in the year 2006.[1,2] Incidence of breast cancer is increasing in most countries including the USA and Japan, and the increasing rate is much higher in countries where its incidence was previously low.[1,2] It has been known that breast cancer is a hormone-dependent disease, and estrogens through an interaction with estrogen receptor (ER) drastically enhance the proliferative and metastatic activity in breast tumor cells.[3,4] However, despite the clinical benefit of interruption of the ER function with synthetic anti-estrogen drugs such as tamoxifen, the precise mechanism of an estrogen/ER signaling pathway in breast cancer progression is not well understood. Therefore, further characterization of the pathophysiologic roles of this pathway and development of novel drugs targeting this pathway should be eagerly expected to provide better management for breast cancer patients.

Gene-expression profile analysis can generate a considerable amount of information for characterizing the nature of individual cancers; such information should be applied for extraction of potential molecular targets for improving clinical strategies to treat neoplastic diseases.[5,6] Through the genome-wide expression analysis of a large number of microdissected clinical cancer materials, we have identified dozens of genes that function as oncogenes in the process of development and/or progression of breast cancer,[7–11] bladder cancer,[12,13] synovial sarcomas,[14,15] testicular seminoma,[16] and renal cell carcinoma.[17,18] These molecules are considered to be good candidates for development of new therapeutic modalities. Since cytotoxic anti-cancer drugs often cause severe adverse reactions, it is obvious that careful selection of novel target molecules on the basis of well-characterized mechanisms of action should be very helpful to develop effective anticancer drugs with the minimum risk of adverse events. Toward such goals, we performed expression profile analysis of 81 breast tumors and 29 normal human tissues by means of a cDNA microarray representing 23 040 cDNAs or ESTs,[19,20] and identified dozens of molecules that were over-expressed in a great majority of breast cancers and were low or undetectably expressed in normal human organs.

Among many over-expressed genes in breast cancers, we report in this study identification and characterization of a novel gene, brefeldin A-inhibited guanine nucleotide-exchange protein 3 (*BIG3*), a novel member of the BIG1/Sec7p subfamily of ADP ribosylation factor-GTP exchange factors (ARF-GEFs), to be a key molecule regulating an estrogen/estrogen receptor (ER) signaling pathway in breast cancer. We also demonstrate an interaction of BIG3 with prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) protein, and that their interaction can enhance the ERα transcriptional activity. Our findings imply BIG3 to be a promising target for development of novel anti-cancer drugs for breast cancer.

Materials and Methods

Cell lines and clinical samples. Human breast cancer cell lines HCC1937, MCF-7, MDA-MB-231, SK-BR-3, T47D, BT-549, HCC1395, MDA-MB-157, BT-20, MDA-MB-453, ZR-75-1, BT-483, BT-474, HCC1143, HCC1500, HCC1599, and OCUB-F, as well as African green monkey SV40-transfected kidney fibroblast cell line, COS-7, were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and cultured under their respective depositors' recommendations. All cells were cultured according to previous reports.[7–11] Tissue samples from surgically resected breast cancers and their corresponding clinical information were obtained from the Department of Breast Surgery, Cancer Institute Hospital, Tokyo, and the Division of Breast and Endocrine Surgery, Department of Surgery, St Marianna University School of Medicine, after obtaining written informed consent. This study, as well as the use of all clinical materials described above, were approved by individual institutional Ethical Committees.

Semiquantitative reverse transcription-PCR analysis. Extraction of total RNA and subsequent cDNA synthesis were performed as

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described.[19] PolyA (+) RNAs isolated from the mammary gland, heart, lung, liver, kidney, and bone marrow were purchased from Takara Clontech (Kyoto, Japan). The PCR primer sequences were 5′-CTTGACAAGGCCTTTGGAGT-3′ and 5′-CAATATGCT TTTCCCGCTTT-3′ for *BIG3*; and 5′-AACTTAGAGGTGGGAG CAG-3′ and 5′-CACAACCATGCCTTACTTTATC-3′ for *β2-MG*.

5′ rapid amplification of cDNA ends (5′ RACE). 5′ RACE experiments were carried out using the SMART RACE cDNA amplification kit (Takara Clontech) according to the manufacturer's instructions. For the amplification of the 5′ part of *BIG3* cDNA, a gene-specific primer (5′-GCCTCCTTCTGCAGCTTCCTCAG GATTT-3′) and the universal primer mixture supplied in the kit were used. The cDNA template was synthesized from mRNA extracted and purified from MDA-MB-453 breast cancer cells, using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR products were cloned using the TA cloning kit (Invitrogen) and sequences were determined by DNA sequencing (ABI3700; PE Applied Biosystems, Foster, CA, USA).

Construction of *BIG3* expression vectors. To construct *BIG3* expression vector, the entire coding sequence was amplified by PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). Primer sets were 5′-CGGAATTCATGGAAGAAATCCTGAGGAAGC-3′ and 5′-ATAGTTTAGCGGCCGCACAATGATGTCATAGACA CGG-3′ (underlining indicates recognition sites of restriction enzymes). The PCR product was inserted into the *Eco*RI and *Not*I sites of pCAGGSnH3F expression vector in frame with a hemagglutinin (HA) tag at the N-terminus and a Flag-tag at the C-terminus. DNA sequences of the construct were confirmed by DNA sequencing (ABI3700; PE Applied Biosystems).

Generation of anti-BIG3 specific polyclonal antibody. Plasmids were designed to express two fragments of BIG3 (codons 459–572 and 799–1200) using pET21a (+) vector in frame with a T7 tag at the N-terminus and a histidine (His) tag at the C-terminus (Novagen, Madison, WI, USA), respectively. The two recombinant peptides were expressed in *Escherichia coli*, BL21 codon-plus strain (Stratagene, La Jolla, CA, USA) and purified using Ni-NTA resin agarose (Qiagen, Valencia, CA, USA) according to the supplier's protocols. The purified recombinant proteins were mixed together and then used for immunization of rabbits (Medical and Biological Laboratories, Nagoya, Japan). The immune sera were subsequently purified on antigen affinity columns using Affigel 15 gel (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's instructions.

Western blot analysis. Cells were lyzed with lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, and 0.5% CHAPS) including 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). The amount of total protein was estimated by a protein assay kit (Bio-Rad), and then proteins were mixed with SDS-sample buffer and boiled before loading onto 7.5% SDS-PAGE gel. After electrophoresis, the proteins were blotted onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK), and blocked with 4% BlockAce solution (Dainippon Pharmaceutical, Osaka, Japan) for 1 h. The membranes were incubated with purified anti-BIG3 polyclonal antibody for another 1 h to detect endogenous BIG3 protein. Finally, the membrane was incubated with HRP-conjugated secondary antibody (1:10 000 dilution; GE Healthcare) for 1 h, and protein bands were visualized by ECL detection reagent (GE Healthcare).

Immunocytochemical staining. MCF-7 or T47D cells were seeded at 1×10^5 cells per well (Laboratory-Tek II Chamber Slide System; Nalgen Nunc International, Naperville, IL, USA) under estrogen-free conditions as described below. Cells were then fixed with phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) containing 4% paraformaldehyde at 4°C for 30 min and rendered permeable with PBS containing 0.1% Triton X-100 at 4°C for 2 min. Subsequently, the cells were covered with 3% BSA in PBS for 1 h to block non-specific hybridization followed by incubation with anti-BIG3 polyclonal antibody or anti-PHB2/REA polyclonal antibody (Abcam, Cambridge, UK) diluted at 1:500 or 1:500 for another 1 h. After washing with PBS (–), cells were stained by Alexa 488-conjugated anti-rabbit secondary antibody (Molecular Probe, Eugene, OR, USA) diluted at 1:1000 for 1 h. Nuclei were counter-stained with 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under a TCS SP2 AOBS microscope (Leica, Tokyo, Japan).

Immunohistochemical staining. To examine the expression of BIG3 protein in breast cancer and normal tissues, we prepared slides of 4-μm sections of paraffin-embedded breast cancer tissue (sample no. 214, 240, 241, 238, 242, and 290), normal mammary tissue (sample no. 453) and other commercially available normal human tissues (lung, heart, and liver) (BioChain, Hayward, CA, USA). The staining using anti-BIG3 polyclonal antibody diluted at 1:150 was performed as described previously.[7]

Gene-silencing effect by small interfering RNA. We had established a vector-based RNAi (RNA interference) expression system using psiU6BX3.0 siRNA expression vector as described previously.[21] The target sequences of synthetic oligonucleotides for siRNAs were as follows: 5′-AAGGTCCTATGGATCTAGGTA-3′ for si-#1, 5′-AAGAAAGCATCGCAGTCTCAG-3′ for si-#2, 5′-AAGATGCGTTCTCTGCCACAC-3′ for si-#3, and 5′-AA**T**AT**T** CG**A**TCTCTGCCACAC-3′ for si-m#3 (bold letters indicate mismatched sequence in si-#3). All of the constructs were confirmed by DNA sequencing.

Human breast cancer cell lines SK-BR-3 and BT-474 were plated onto 10-cm dishes (1×10^6 cells/dish) and transfected with 8 μg each of psiU6BX3.0-Mock (without insertion) and psiU6BX3.0-BIG3 (si-#1, si-#2, si-#3, and si-m#3 including three-base substitutions in #3) using FuGENE6 reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were re-seeded for colony formation assay (1×10^6 cells/10 cm dish), RT-PCR (1×10^6 cells/10 cm dish), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (2×10^5 cells/well). We selected psiU6BX3.0-introduced SK-BR-3 or BT-474 cells with culture medium containing 0.2 mg/mL or 1.0 mg/mL of neomycin (geneticin; Invitrogen), respectively. We changed the culture medium twice a week. Total RNAs were extracted from the cells after 3-day incubation with neomycin, and then the knockdown effect of siRNAs was examined by semiquantitative RT-PCR using specific primer sets; 5′-GCCCTTGAAGCCAA TATTCC-3′ and 5′-AGATGGTTTCAGTGGGCTTG-3′ for *BIG3*; and 5′-AACTTAGAGGTGGGAGCAG-3′ and 5′-CACAACCAT GCCTTACTTTATC-3′ for *β2-MG* as an internal control. SK-BR-3 or BT-474 cells expressing siRNA were grown for 4 weeks in selective media containing 0.2 mg/mL or 1.0 mg/mL of neomycin, and then fixed with 4% paraformaldehyde at 4°C for 30 min before staining with Giemsa's solution (Merck, Whitehouse Station, NJ, USA) to assess the colony number. To quantify cell viability, MTT assays were performed with cell counting kit-8 (Wako, Osaka, Japan) according to the manufacturer's recommendations. Absorbance at 570 nm wavelengths was measured with a Microplate Reader 550 (Bio-Rad). These experiments were performed in triplicate.

We also used siRNA oligonucleotides (Sigma Aldrich Japan, Tokyo, Japan) due to its high transfection efficiency to further verify the subcellular localization of PHB2/REA protein in cells which *BIG3* gene was knocked-down by siRNA. The sequences targeting *BIG3* (si-BIG3) or Enhanced green fluorescent protein (*EGFP*) (si-EGFP) were as follows: si-BIG3, 5′-GAUGCGUUCU CUGCCACACTT-3′; si-EGFP (control); 5′-GCAGCACGACU UCUUCAAG-3′. MCF-7 or ZR-75-1 (2.5×10^5 cells in 10-cm dish) cells were transfected with those siRNAs using Lipofectamin RNAiMAX (Invitrogen) in OptiMEM (Invitrogen) medium according to the instructions from the manufacturer. Twenty-four hours later, the cells were treated with 1 μM of E2 (17β-estradiol;

Sigma-Aldrich), and then were subjected to immunocytochemical and western blot analyses using anti-PHB2/REA polyclonal antibody and anti-BIG3 polyclonal antibody, respectively. Fluorescent images were obtained under a TCS SP2 AOBS microscope (Leica). Nuclear-translocated PHB2/REA protein was observed in si-EGFP or si-BIG3-transfected cells by a phase contrast microscopy at 24 h after E2 treatment, and the number of cells with nuclear-translocated PHB2/REA per 100 cells was counted. These experiments were performed in duplicate.

Immunoprecipitation and mass spectrometry. BT-549 cells were plated onto 15-cm dishes (1×10^7 cells/dish) and transfected with 20 μg of pCAGGSnH3F-Mock (without insertion) or pCAGGSnH3F-BIG3 using FuGENE6 reagent (Roche). After 48 h incubation, the cells were lyzed with 0.1% NP-40 lysis buffer as described in 'Western blot analysis' section. The cell lysates were precleaned with normal mouse IgG and rec-Protein G Sepharose 4B (Zymed, San Francisco, CA, USA) at 4°C for 1 h. Subsequently, the lysates were incubated with anti-Flag M2 agarose (Sigma-Aldrich) at 4°C for overnight. After washing five times with lysis buffer, the protein samples were separated by SDS-PAGE (4–12% Bis-Tris gel) (Invitrogen). Proteins in polyacrylamide gel were silver stained with the SilverQuest Silver Staining Kit (Invitrogen). Bands that were specifically observed in the BIG3-transfected lane were excised, and the extracted proteins were subjected to PMF (peptide mass fingerprint) analysis using MALDI TOF-MS (Shimadzu Biotech, Tsukuba, Japan).

Co-immunoprecipitation assay. Plasmids designed to express the entire coding sequence of PHB2/REA were constructed using the following primer set: 5′-CGGAATTCCAGACCGTGCATC ATGGCCCAGAACTTGAAGGA-3′ and 5′-CCGCTCGAGTTTC TTACCCTTGATGAGGCTGT-3′ (underlining indicates the recognition sites of restriction enzymes), and inserted in frame into the *Eco*RI and *Xho*I sites of pCAGGSnHC expression vector with an HA tag at the C-terminus (pCAGGSnHC-PHB2/REA). BIG3 expression vector (pCAGGSnH3F-BIG3) was subcloned into pCAGGSn3FC expression vector in frame with a Flag-tag at the C-terminus (pCAGGSn3FC-BIG3). COS-7 cells were transiently transfected with either or both pCAGGSn3FC-BIG3 and pCAGGSnHC-PHB2/REA. Forty-eight hours after the transfection, the cells were lyzed with 0.1% NP-40 lysis buffer as described in 'Western blot analysis' section. The cell lysates were precleaned at 4°C for 1 h, and subsequently incubated with anti-Flag M2 agarose (Sigma-Aldrich) or monoclonal anti-HA agarose conjugate (Sigma-Aldrich) at 4°C for overnight. After washing with lysis buffer, coprecipitated proteins were separated by SDS-PAGE. Finally, we performed western blot analysis using anti-HA high affinity (3F10) rat monoclonal antibody (Roche) or anti-Flag M2 monoclonal antibody (Sigma-Aldrich) to detect the exogenously expressed PHB2/REA or BIG3 protein, respectively.

Cell culture, transfection under estrogen-free conditions. T47D or MCF-7 cells were cultured with the following media: phenol red-free RPMI-1640 (Invitrogen), supplemented with 10% FBS and 1% antibiotic/antimycotic solution (Sigma-Aldrich) filtered with minisart-plus (Sartorius, Goettingen, Germany). The cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2. Transfection with BIG3 (pCAGGSnH3F-BIG3) expression vector was performed using FuGENE6 transfection reagent (Roche) and phenol red-free Opti-MEM (Invitrogen), respectively. Twenty-four hours after the transfection, the media were exchanged with phenol red-free Opti-MEM containing 1 μM of E2 (17β-estradiol; Sigma-Aldrich) and then incubated for 24 h. Immunocytochemical staining was performed using anti-Flag rabbit polyclonal antibody (Sigma-Aldrich) diluted at 1:500, and Alexa 594-conjugated antirabbit secondary antibody (Molecular Probes, Eugene, OR, USA) diluted at 1:1000.

Estrogen responsive element (ERE) reporter gene assays. MCF-7 cells were transfected with siRNA-oligonucleotides of BIG3 (si-BIG3) or EGFP (si-EGFP; control) using Lipofectamin RNAiMAX (Invitrogen) in phenol red-free OptiMEM (Invitrogen) medium. Twenty-four hours after the transfection, the cells were washed with PBS (–) at three times, and were transfected with estrogen-responsive reporter gene (pERE-TA-SEAP) vector using phenol red-free OptiMEM for 12 h. Subsequently, 1 μM of E2 was treated for 48 h for SEAP assay and western blot analysis. The SEAP reporter assay was performed using an SEAP assay kit (Takara Clontech) according to the supplier's recommendations.

Statistical analysis. Statistical significance was calculated by Student's *t*-test, using Statview 5.0 software (SAS Institute, Cary, NC, USA). A difference of $P < 0.05$ was considered to be statistically significant.

Results

Overexpression of BIG3 in breast cancer cells. To elucidate the mechanism of breast carcinogenesis and identify molecules that could be applicable as targets for development of novel therapeutic drugs, we previously performed genome-wide gene-expression profile analysis of 81 breast cancers using cDNA microarray representing 23 040 cDNAs.[19] Among the transactivated genes, in this study we focused on *BIG3*. We confirmed its up-regulation in nine of 12 clinical breast cancer specimens, compared with normal breast ductal cells or with whole mammary gland by semiquantitative RT-PCR (Fig. 1a). Subsequent northern blot analysis confirmed overexpression of its 15-kb transcript in breast cancer cell lines (Supporting Fig. S1). On the other hand, BIG3 expression was hardly detectable in any normal human organs except the brain (data not shown) as concordant to the results of cDNA microarray analysis.

Since the assembled cDNA sequence of *BIG3* (LOC202451; 3348 bp) in the National Center for Biotechnology Information (NCBI) database was much smaller than the 15-kb transcript indicated by northern blot analysis, we performed the exon-connection and 5′ RACE experiments, and obtained the full-length cDNA sequences of *BIG3* consisting of 14 763 nucleotides (Genebank accession; AB252196) encoding a protein of 2177 amino acids. The *BIG3* gene consists of 34 exons, spanning an approximately 183-kb genomic region on the chromosomal band 6q23.3. The simple modular architecture research tool (SMART) program predicted a presence of a Sec7 domain (586–798 amino-acids) that might be required for the protein transport through the Golgi apparatus, in the BIG3 protein.[22–25]

To investigate the biological function of the BIG3 protein, we firstly generated an anti-BIG3 polyclonal antibody and found it to recognize the endogenous BIG3 protein of approximately 250 kDa in breast cancer cell line SK-BR-3, as well as exogenously expressed BIG3 in COS-7 cells without any cross-reactivity to other proteins (Supporting Fig. S2a). We further investigated endogenous expression of BIG3 protein in cell lysates from 13 breast cancer cell lines by western blot analysis and found that six of them (MDA-MB-453, SK-BR-3, ZR-75-1, BT-483, HCC1500, and MCF-7) expressed a high level of BIG3 protein, two (T47D and BT-20) expressed it at a low level, and no expression was observed in the remaining five cell lines (MDA-MB-231, HCC1395, BT-549, HCC1143, and HCC1937) (Fig. 1b). Subsequent immunohistochemical analysis using anti-BIG polyclonal antibody showed its strong staining in the cytoplasm of two different histological subtypes of breast cancer, the papillotubular carcinoma (214, 240, and 241) and solid-tubular carcinomas (238, 242, and 290) (Fig. 1c), while any staining was detected neither in the normal breast tissue (Fig. 1d) nor in heart, lung, and liver (Supporting Fig. S2b) in concordance with the results of northern blot analysis. These findings suggest that BIG3 was up-regulated in breast cancer cells at the protein level as well as at the transcriptional level.

Effect of BIG3 on cell growth. To ascertain a possible role of BIG3 in mammary carcinogenesis, we knocked down the expression of endogenous *BIG3* in breast cancer cell lines SK-BR-3 and

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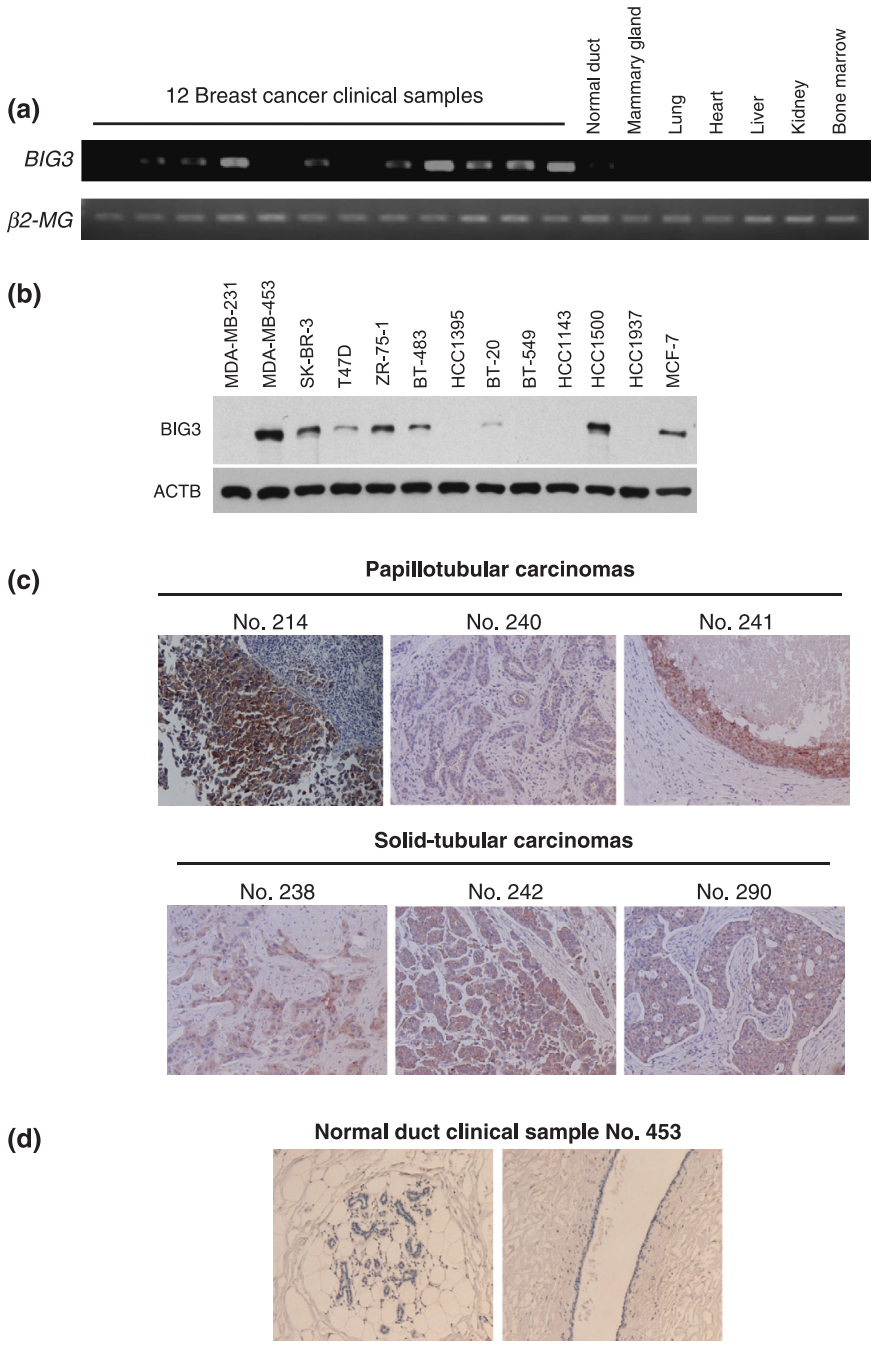

Fig. 1. Expression profiles of brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3). (a) Expression of *BIG3* in 12 clinical breast cancer samples (4T, 5T, 110T, 214T, 138T, 327T, 341T, 411T, 631T, 758T, 624T, and 869T) and normal human tissues (normal duct, microdissected normal mammary ductal cells, mammary gland, lung, heart, liver, kidney, and bone marrow) examined by semi-quantitative RT-PCR. *β2-MG* served as the quantity control. (b) Expression of endogenous BIG3 protein in 13 breast cancer cell lines examined by western blot analysis using an affinity-purified anti-BIG3 polyclonal antibody. ACTB (beta-actin) served as a loading control. (c,d) The results of immunohistochemistry of breast cancer and normal breast tissue sections. The endogenous BIG3 protein was stained with anti-BIG3 polyclonal antibody. The expression was hardly detectable in normal breast tissues (Sample no. 453) as shown in (d), but cancer cells were stained mainly at the cytoplasm in all of cancer tissues investigated, including papillotubular (Sample nos. 214, 240, and 241) and solid-tubular carcinomas (Sample nos. 238, 242, and 290) as shown in (c). Representative figures are from microscopic observation with original magnification of ×100.

BT-474, which expressed a high-level of *BIG3*, using a mammalian vector-based RNA interference technique (see 'Materials and Methods'). Introduction of either of two *BIG3*-specific siRNA constructs (si-#2 and si-#3) significantly suppressed the *BIG3* mRNA expression, compared with a control siRNA construct, si-mock or si-#1 (Fig. 2a,b; left panels). In concordance with the knockdown effect, MTT (Fig. 2a,b; middle panels) and colony formation assays (Fig. 2a,b; right panels) revealed significant growth-suppressive effects by si-#2 and si-#3 (MTT assays: SK-BR-3; *, $^{**}P < 0.001$, BT-474; *, $^{**}P < 0.001$; unpaired *t*-test). We also generated siRNA that contained a three-base substitution in si#3 sequence (mismatch si#3), and found no suppressive effect on the expression of *BIG3* or on cell growth of SK-BR-3 (Supporting Fig. S3) or BT-474 cells (Fig. 2b). These observations suggest that *BIG3* has a critical function in the growth of the breast cancer cells.

Identification of PHB2/REA as an interacting protein of BIG3. Since the biological functions of BIG3 are totally unknown, we searched for a protein(s) interacting with BIG3 by immunoprecipitation and mass spectrometry analyses. Lysates of BT-549 cells transfected with a pCAGGSnH3F-BIG3 vector or a pCAGGSnH3F-Mock (mock control) were extracted and immunoprecipitated with anti-Flag M2 monoclonal antibody (see 'Materials and Methods'). Protein complexes were silver-stained on SDS-PAGE gels. An approximately 30-kDa protein, which was seen in immunoprecipitates of cell lysates transfected with the Flag-tagged BIG3 plasmid but not in those with mock control plasmid, was extracted and its peptide sequences were determined by mass-spectrometry analysis (data not shown). This approach identified PHB2/REA[26] as a candidate interacting with BIG3. To investigate the biological significance of their interaction, we constructed plasmids designed to express Flag-tagged BIG3 (BIG3-Flag)

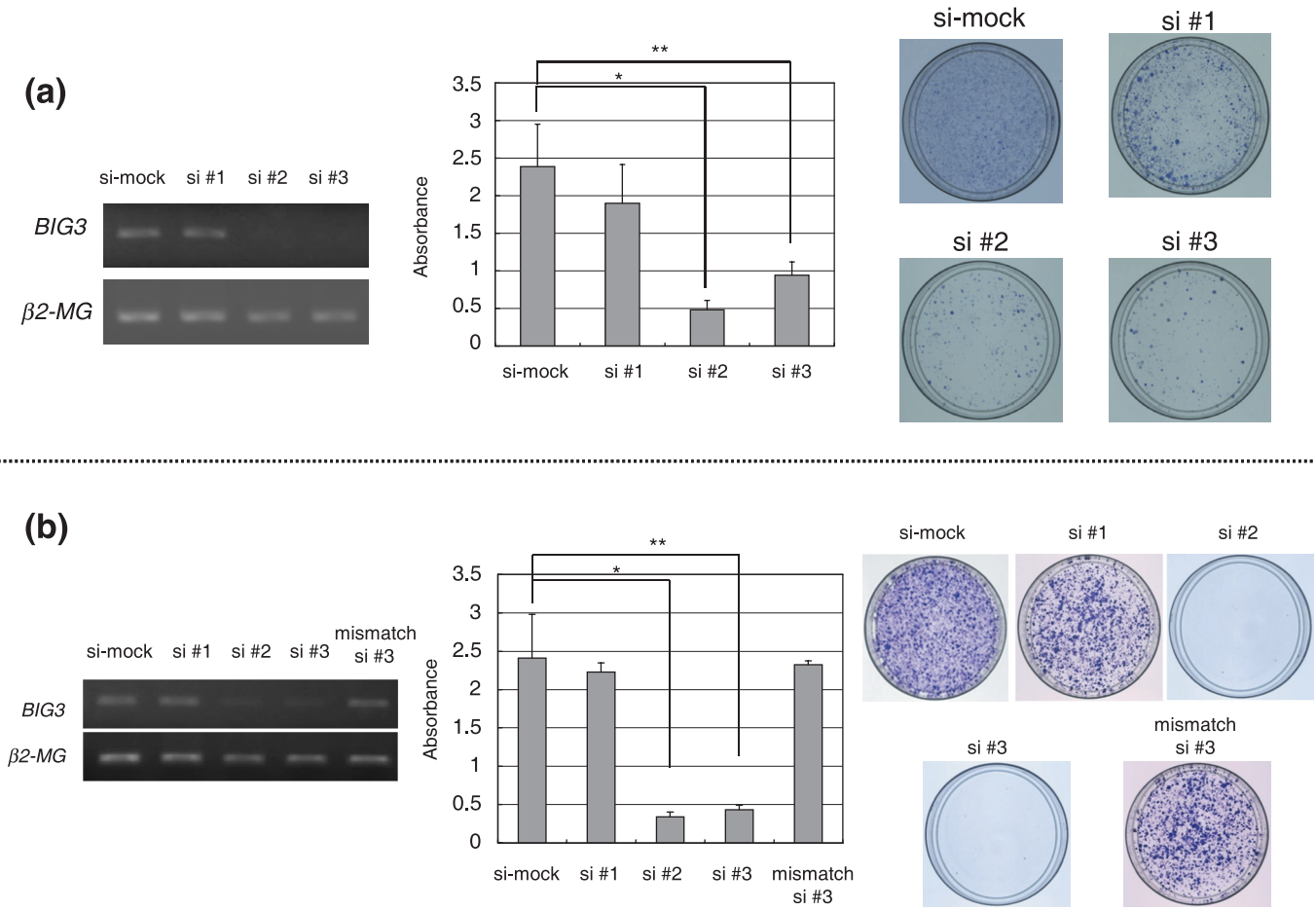

Fig. 2. Effect of brefeldin A-inhibited guanine nucleotide-exchange protein 3 (*BIG3*) on cell growth. (a) Semiquantitative RT-PCR showing the suppression of endogenous expression of *BIG3* in SK-BR-3 breast cancer cells (si-#2, si-#3) at 3 days after neomycin selection (left panels). *β2-MG* served as the quantity control. MTT and colony formation assay showed a significant decrease in the number of viable cells by knockdown of *BIG3* in SK-BR-3 cells after 4 days or 1 month of neomycin selection (middle and right panel) (MTT assay; *, **$P < 0.001$; unpaired *t*-test). (b) Semiquantitative RT-PCR demonstrates the suppression of endogenous expression of *BIG3* in BT-474 breast cancer cells by *BIG3*-specific siRNAs (si-#2, si-#3) (left panels), while knockdown effect of mismatch siRNA (mismatch #3) is not observed (mismatch si-#3) (left panels). MTT and colony formation assay showed a decrease in the number of viable cells by knockdown of *BIG3* in BT-474 cells (middle and right panel) (MTT assay; *, **$P < 0.001$; unpaired *t*-test).

and HA-tagged PHB2/REA (PHB2/REA-HA) (see 'Materials and Methods'). These plasmids were cotransfected into COS-7 cells, and then the proteins were immunoprecipitated with anti-Flag antibody. Immunoblot of the precipitates using anti-HA antibody indicated that BIG3-Flag was coprecipitated with PHB2/REA-HA (Fig. 3a; left panel). Conversely, we performed immunoprecipitation using anti-HA antibody and then immunoblot analysis of the precipitates using anti-Flag antibody. The results showed that PHB2/REA-HA was coprecipitated with BIG3-Flag (Fig. 3a; right panel). We additionally confirmed that BIG3-Flag was coprecipitated with endogenous PHB2/REA in SK-BR-3 cells (Fig. 3b).

We subsequently performed immunocytochemical staining analysis with anti-PHB2/REA polyclonal antibody using the breast cancer cell line SK-BR-3 (see 'Materials and Methods') and observed staining of endogenous PHB2/REA mainly as a diffused pattern in the cytoplasm in most of the breast cancer cells (Fig. 3c; middle panel). In a small subset of the cells, its staining was observed in both the cytoplasm and nucleus (Fig. 3c; right panel, arrows). Since PHB2/REA was reported to selectively repress the transcriptional activity of ERα through its interaction with ERα in the nucleus,[26–28] we investigated the possibility of a direct interaction between BIG3 and ERα, but failed to indicate their interaction (data not shown).

Inhibition of nuclear translocation of PHB2/REA by BIG3. Since PHB2/REA was shown to be localized mainly at the cytoplasm and to be translocated to the nucleus in ERα-positive cells,[26–28] we hypothesized that BIG3 might interact with PHB2/REA and interfere with its nuclear translocation. Therefore, we performed immunocytochemical staining to examine the subcellular distribution of PHB2/REA protein in the presence or absence of BIG3. Figure 4(a) showed that endogenous PHB2/REA was also localized in the cytoplasm of MCF-7 cells, in which BIG3 protein was overexpressed, with or without treatment of E2. Moreover, we confirmed that endogenous PHB2/REA was translocated into the nucleus of T47D cells, in which BIG3 was expressed at a very low level (Fig. 4b) after E2 treatment. On the other hand, PHB2/REA remained in the cytoplasm even with E2 treatment when BIG3 was exogenously introduced into T47D cells (Fig. 4c). Moreover, we examined whether intracellular-localization of PHB2/REA is dependent with BIG3 expression in breast cancer tissue by immunohistochemical staining analysis with anti-PHB2/REA and anti-BIG3 antibodies. The result showed that intracellular-localization of PHB/REA was mostly consistent with the cytoplasmic localization of BIG3 protein in breast cancer tissue (Sample no. 242) (Fig. 4d). These findings suggest that BIG3 interacted with PHB2/REA and interfered with its nuclear translocation in breast cancer cells.

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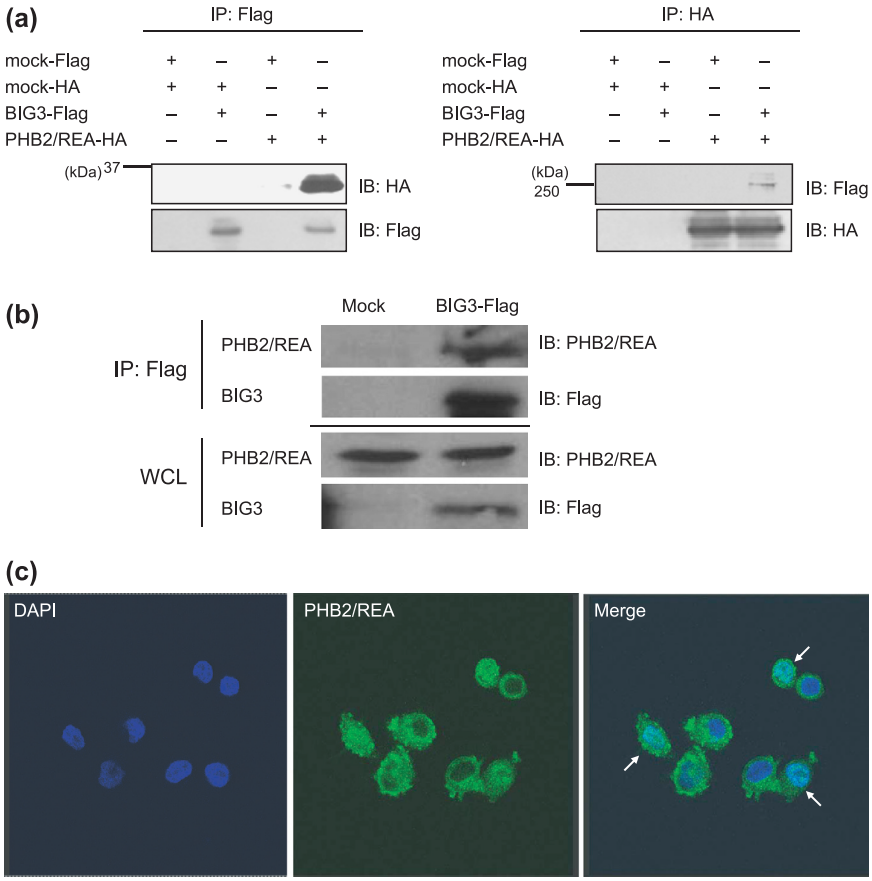

Fig. 3. Interaction of brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) and prohibitin 2/repressor of estrogen receptor activity (PHB2/REA). (a) Interaction of exogenous BIG3 and exogenous PHB2/REA protein. COS-7 cells were transfected with a combination of Flag-tagged mock (mock-Flag), Flag-tagged BIG3 (BIG3-Flag), HA-tagged mock (mock-HA), and HA-tagged PHB2/REA (PHB2/REA-HA); immunoprecipitated with anti-Flag M2 agarose or anti-HA high affinity (3F10) rat monoclonal antibody; and immunoblotted with anti-HA high affinity (3F10) rat monoclonal antibody (left panels) or anti-Flag M2 monoclonal antibody (right panels), respectively. (b) Confirmation of interaction between exogenous BIG3 and endogenous PHB2/REA protein. SK-BR-3 breast cancer cells were transfected with the Flag-tagged BIG3 (BIG3-Flag), immunoprecipitated with anti-Flag M2 agarose, and immunoblotted with anti-PHB2/REA polyclonal antibody. WCL indicates whole cell lysate. (c) Endogenous expression of PHB2/REA in breast cancer cells. Immunocytochemistry was performed in SK-BR-3 breast cancer cells using an anti-PHB2/REA polyclonal antibody (green) and DAPI (blue) to discriminate nucleus. Endogenous PHB2/REA was localized mainly in the cytoplasm, but the nuclear localization was observed in some cells (arrows).

Furthermore, we investigated the subcellular localization of endogenous PHB2/REA in MCF-7 cells in which endogenous BIG3 expression was knocked down using the siRNA oligonucleotide of BIG3 (si-BIG3). The significant knockdown of BIG3 expression was observed in si-BIG3-transfected MCF-7 cells, but not in si-EGFP-transfected cells as a control (Fig. 4e). PHB2/REA was localized in the nucleus at 48 h after the E2 treatment, but was present in the cytoplasm in those treated with si-EGFP (Fig. 4f). Moreover, the cell population of nuclear translocated-PHB2/REA was significantly increased in si-BIG3-transfected MCF-7 cells ($P = 0.0074$; unpaired t-test) with E2 treatment, compared with those in si-BIG3-transfected MCF-7 cells without E2 treatment (Fig. 4g). Additionally, we also confirmed that the knockdown of BIG3 expression also led to the nuclear-translocation of PHB2/REA in ZR-75-1 cells, which expressed a high-level of BIG3 (Supporting Fig. S4a,b). These data further support that BIG3 trapped PHB2/REA in the cytoplasm, and resulted in inhibition of its nuclear translocation and the interaction with ERα.

BIG3 up-regulates ERα transcriptional activity though its interaction with PHB2/REA. To examine whether BIG3 protein could enhance the ERα transcriptional activity in breast cancer cells, we transfected with estrogen-responsive reporter gene (pERE-TA-SEAP) vector followed by the transfection with either of the si-BIG3 or si-EGFP into MCF-7 cells, and then performed a reporter assay (see 'Materials and Methods'). We confirmed the knockdown of BIG3 expression by western blot analysis (Fig. 5a), and found that the depletion of BIG3 expression caused the significant decrease of ERα transcriptional activity in MCF-7 cells (mean relative ERα transcriptional activity in MCF-7 cells transfected with si-BIG3 after E2 stimulation *vs* MCF-7 cells transfected with si-EGFP after E2 stimulation; $P = 0.0005$; unpaired t-test) (Fig. 5b). These findings suggest that the presence of BIG3 protein is likely to enhance the ERα transcriptional activity though inhibition of nuclear translocation PHB2/REA in breast cancer cells.

Discussion

Identification and characterization of cancer-related genes and their products have contributed to the development of molecular-targeting drugs for cancer therapy in the past two decades. However, the proportion of patients benefiting from presently available treatments is still limited.[3,4] Hence, further developing new anticancer agents that are highly specific to malignant cells with minimal or no adverse reactions is urgent. Through the precise expression profile analysis of breast cancer, we identified *BIG3* to be significantly overexpressed in the great majority of breast cancer cases and breast cancer cell lines. The immunohistochemical analysis also supported the high level of endogenous BIG3 protein in breast cancer cells in concordance with the results of northern blot analysis. Knockdown of the endogenous *BIG3* expression resulted in remarkable growth suppression of breast cancer cells. These results implied that BIG3 could serve as a valuable target for development of anticancer agents for breast cancer.

ADP ribosylation factors (Arfs) are approximately 20-kDa GTPases that have key roles in the regulation of protein trafficking and guanine-nucleotide exchange in eukaryotic cells.[22–24] The Arfs require accessory proteins to facilitate nucleotide exchange (GTP exchange factors [GEFs]) and GTP hydrolysis (GTPase activating proteins [GAPs]). The mammalian Arfs are divided into three classes I–III: class-I Arf is involved in transport though the exocytic pathway and class-III is implicated in endocytosis and in actin dynamics at the plasma membrane. Little is known about the roles of class-II Arf.[21–23] All of Arf-GEFs identified to date are characterized by a central catalytic domain

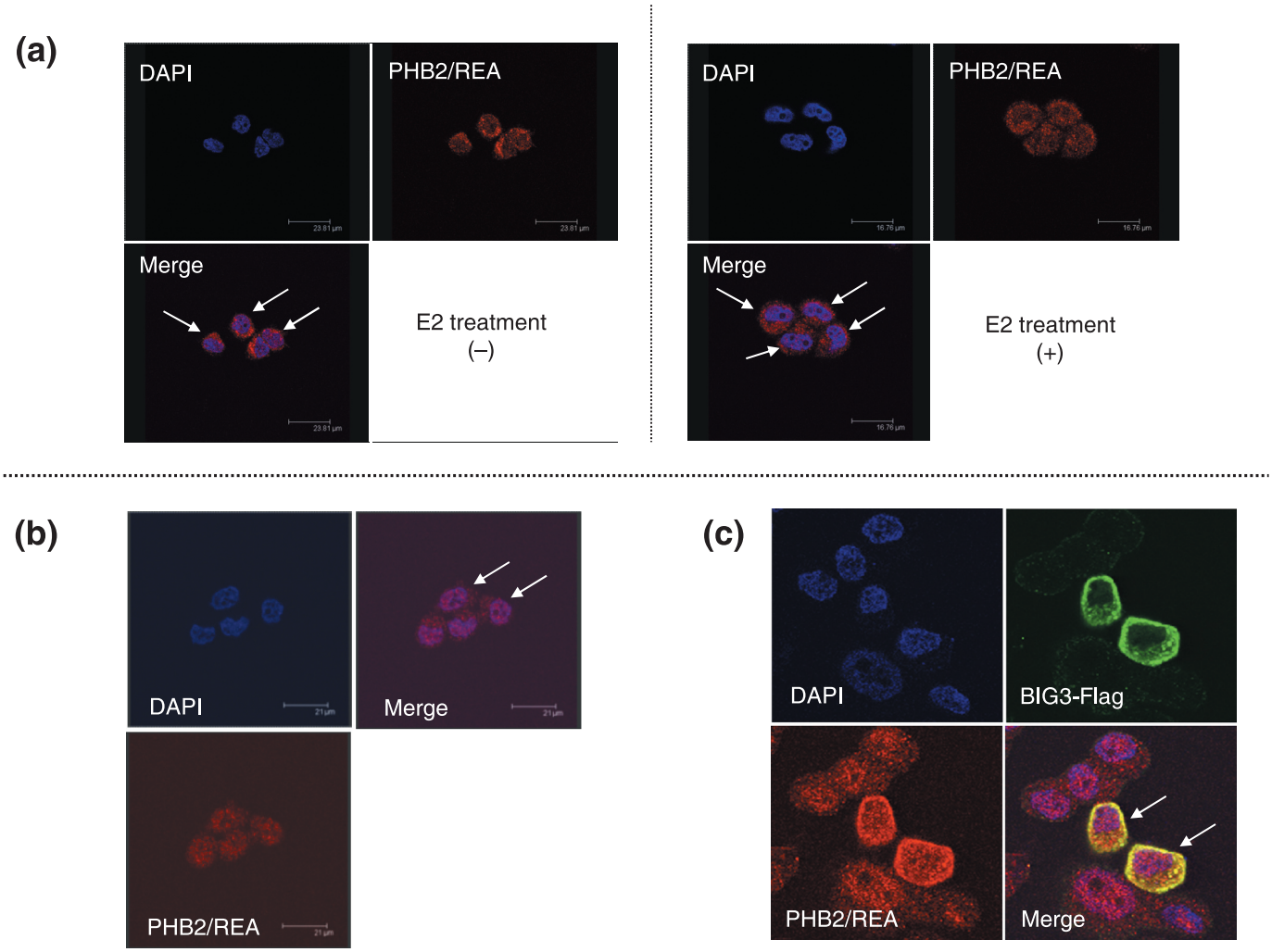

Fig. 4. Inhibition of the nuclear translocation of prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) by overexpression of brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3). (a) Subcellular localization of PHB2/REA in MCF-7 cells, which overexpressed BIG3 protein. Endogenous PHB2/REA protein was located in the cytoplasm of MCF-7 cells with (right panels) or without treatment of E2 (left panels). The arrows show the cytoplasmic PHB2/REA in MCF-7 cells. (b) Subcellular localization of PHB2/REA in T47D cells in which an expression level of BIG3 protein was very low. The arrows show endogenous PHB2/REA in nucleus of T47D cells. (c) Subcellular localization of PHB2/REA in the exogenously BIG3-transfected T47D cells. T47D cells were transfected with Flag-tagged BIG3 (BIG3-Flag; green) and treated with 1 μM of E2 for 24 h (upper right panel). Endogenous PHB2/REA (bottom left panel; red) remained in cytoplasm in the presence of BIG3 (bottom right panel; Merge). The arrows show colocalization of endogenous PHB2/REA and exogenous BIG3 in the cytoplasm of T47D cells. (d) Intracellular-localization of PHB2/REA and BIG3 proteins in breast cancer tissue. Cytoplasmic localization of PHB2/REA was mostly consistent with that of BIG3 protein in breast cancer tissue (Sample no. 242). The rectangular regions in each upper panel (100-fold) are enlarged (lower panels; 400-fold). (e) Expression of BIG3, estrogen receptor (ER)-α, and PHB2/REA at the protein level in MCF-7 cells treated with si-BIG3 oligonucleotide or a control si-EGFP oligonucleotide. Twenty-four hours after the transfection with each siRNA oligonucleotide, the cells were treated with E2 for 24, 48, or 72 h, and then were analyzed by western blot analysis. si-EGFP was used as a control siRNA. ACTB served as a loading control for western blot analysis. (f) Subcellular localization of endogenous PHB2/REA in BIG3-depleted cells. MCF-7 cells were treated with si-BIG3 or si-EGFP. Twenty-four hours after the siRNA treatment, the cells were treated with E2 for 48 h, and then were analyzed by immunocytochemical staining. (g) Nuclear-translocated PHB2/REA protein in si-BIG3-transfected MCF-7 cells after E2 treatment. Knockdown of BIG3 expression at the protein level in the cells treated with si-BIG3. Twenty-four hours after the transfection with si-BIG3 or si-EGFP, the cells were treated with E2 for 48 h, and then were analyzed by western blot analysis with anti-BIG3 antibody. The si-EGFP was used as a control siRNA. ACTB served as a loading control for western blot analysis (upper panels). Nuclear-translocated PHB2/REA protein was observed in si-BIG3 or si-EGFP-transfected cells by microscopy at 48 h after with or without E2 treatment. Then, the number of cells with nuclear-translocated PHB2/REA per 100 cells was graphed (lower panel) ($P = 0.0074$; unpaired t-test).

of approximately 200 amino acids referred to as the Sec7 domain that is sufficient for GEF activity.[22–24,29] Brefeldin A-inhibited guanine nucleotide-exchange proteins 1 and 2 (BIG1 and BIG2) proteins contain highly conserved sec7 domains that catalyze replacement of ARF-bound GDP by GTP to initiate membrane vesicle formation.[22,23] Although it was considered to belong to the sec7/Arfs protein family, BIG3 contains a single highly conserved Sec7 domain and shares partially the only 25% identity in amino acid sequences with BIG1 and BIG2 proteins, that were initially isolated from bovine brain cytosol on the basis of their brefeldin A-sensitive activation of calss-I Arfs.[22,30] Therefore, to elucidate the biological significance of BIG3 in breast cancer cells, we screened a protein(s) interacting with BIG3 and identified PHB2/REA, which is known to be an ERα-selective coregulator,[26–28] as a candidate. We demonstrated their *in vivo* interaction and colocalization at the cytoplasm of breast cancer cells. We further confirmed that endogenous PHB2/REA was localized in the cytoplasm under the presence of BIG3 with

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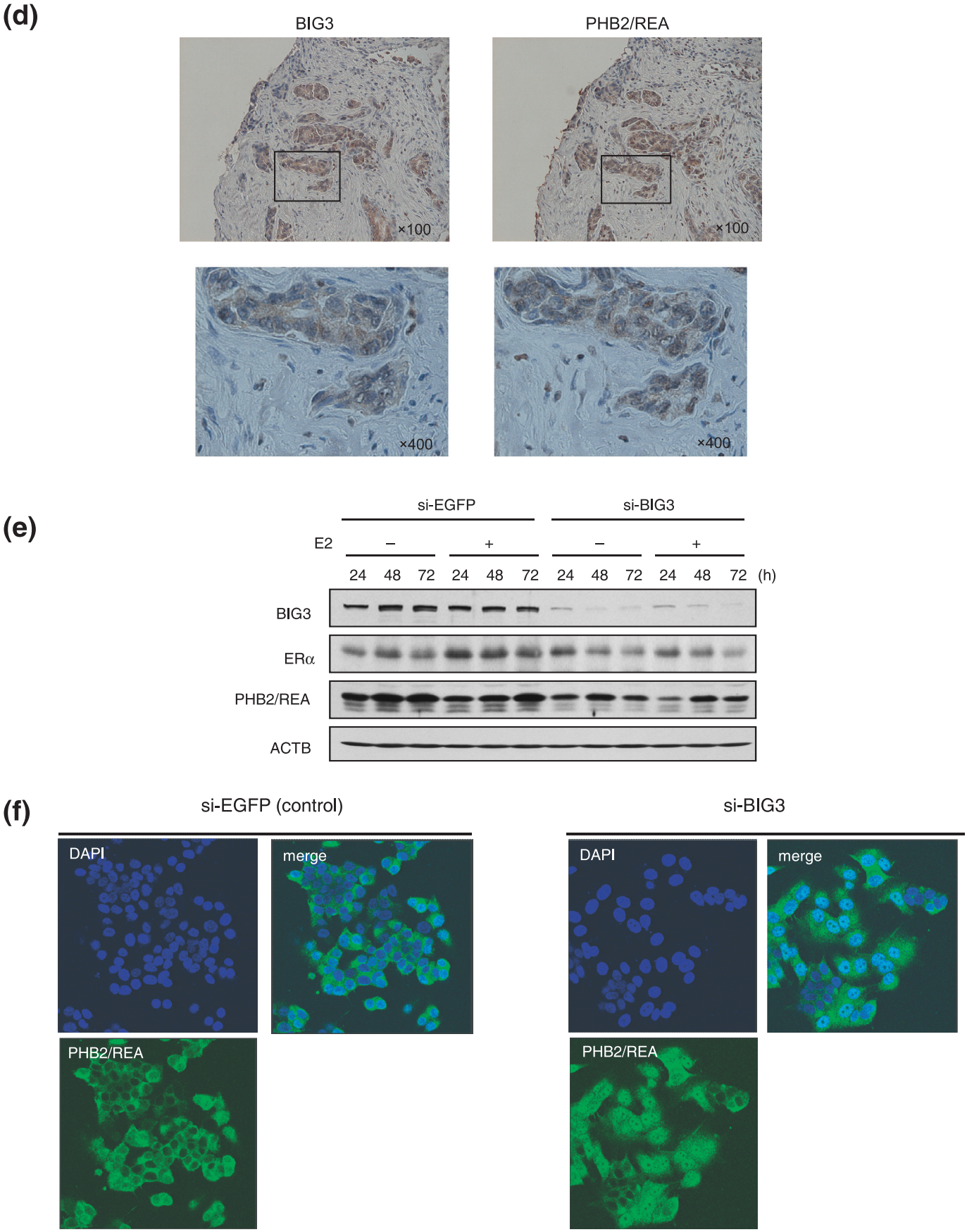

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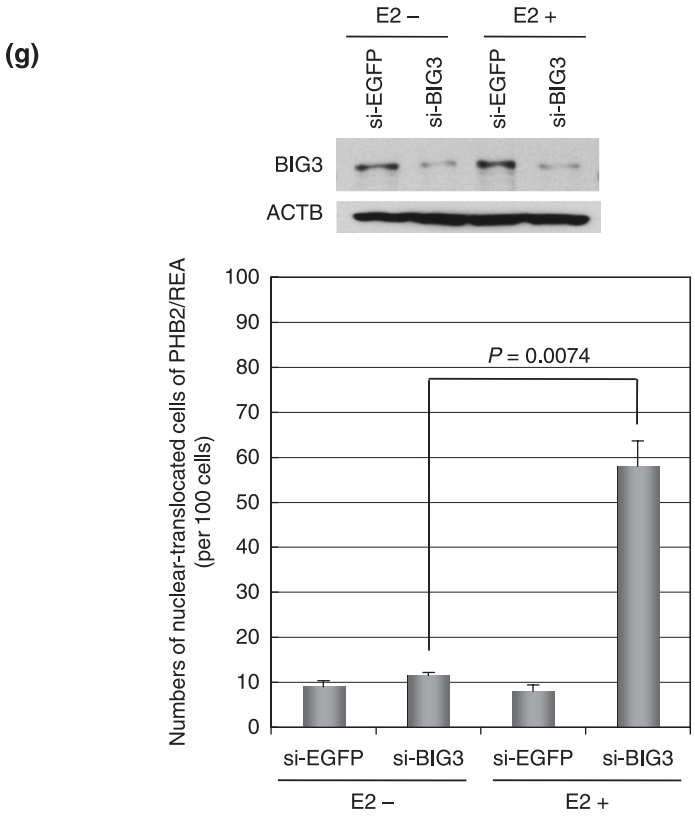

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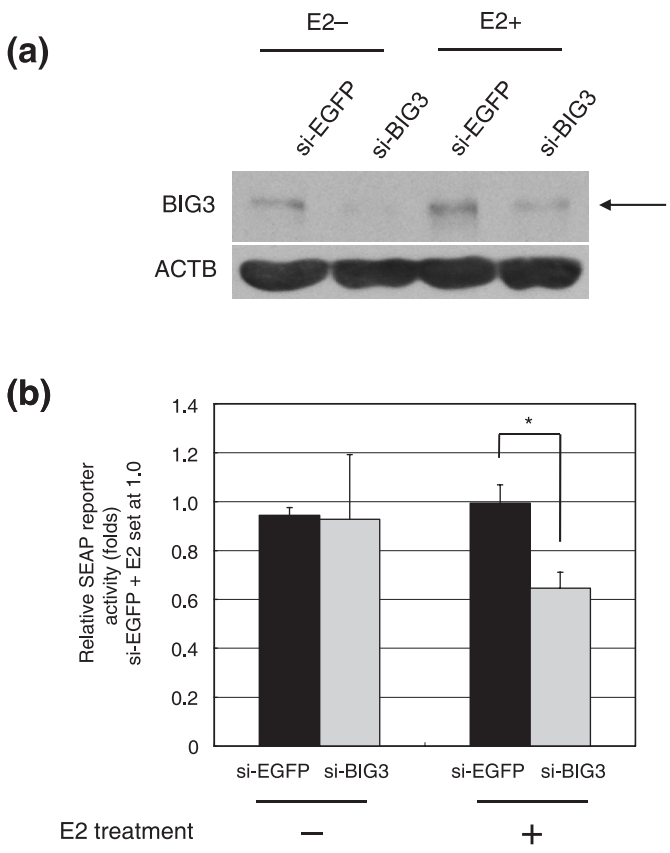

or without stimulation of E2, but it translocated to the nucleus, and repressed the transcriptional activity under the absence of BIG3 protein (Figs 4 and 5). Moreover, all ERα-positive breast cancer cells we examined showed overexpression of PHB2/REA (data not shown). Although expression levels of BIG3 were similar in MCF-7 (ER-positive) and SK-BR-3 (ER-negative) cells (Fig. 1), the different pattern of localization of PHB2/REA in those cell lines might be due to discordant ER status. Therefore, it suggests the possibility that PHB2/REA protein may function as the negative-feedback mechanism to suppress the activated ER signaling. Taken together, these results imply the constitutive activation of the ER signaling pathway by overexpression of BIG3 in breast cancer *in vivo* (Fig. 6). Additionally, these findings may explain why there is no apparent correlation between PHB2/REA expression and ERα inactivation in breast cancer cases. Furthermore, although further analysis of the BIG3 function by evaluation of activation of ER-downstream genes will be

Fig. 5. Regulation of estrogen receptor (ER)-α transcriptional activity though its interaction between prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) and brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3). (a) Knockdown of BIG3 expression at the protein level in the cells treated with si-BIG3. Twenty-four hours after the transfection with si-BIG3 or si-EGFP, the cells were treated with E2 for 48 h, and then were analyzed by western blot analysis with anti-BIG3 antibody. The si-EGFP was used as a control siRNA. ACTB served as a loading control for western blot analysis. The arrow indicates the BIG3 protein. (b) SEAP assay to determine the transcriptional activity of ERα in BIG3-depleted cells. MCF-7 cells were transfected with si-BIG3 or si-EGFP. Twenty-four hours after the transfection, the cells were washed with PBS (–)three times. Subsequently, the estrogen-responsive reporter gene (pERE-TA-SEAP) vector was transfected using phenol red-free OptiMEM in MCF-7 cells for 12 h and treated with 1 μM of E2 for additional 48 h. The secreted alkaine phosphatase (SEAP) reporter activity was measured relative to the response to E2 in the mock-transfected cells, which was set at 1.0 (*$P = 0.0005$; unpaired *t*-test). Three independent experiments performed in duplicate.

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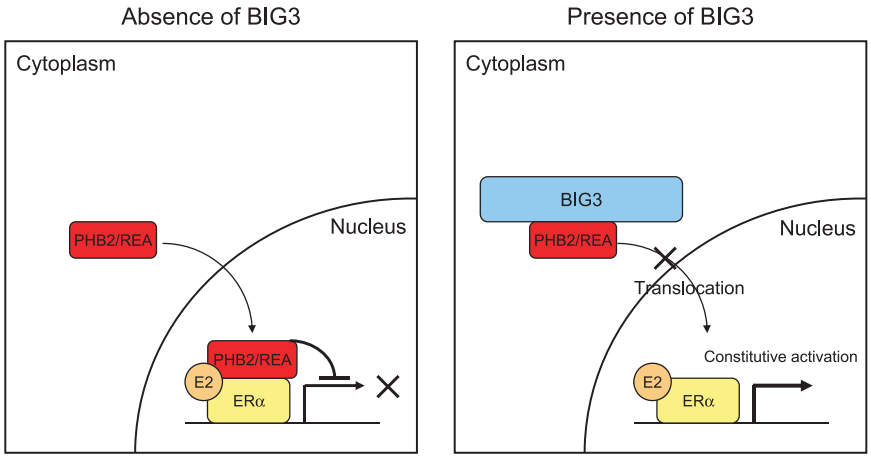

Fig. 6. Schematic presentation of up-regulation of estrogen receptor (ER)-signaling pathway by brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3). In the absence of BIG3, prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) is translocated to the nucleus after E2 stimulation, binds to ERα, and represses the transcriptional activity of the estradiol (E2)-ligand ERα (left panel). On the other hand, in the presence of BIG3, PHB2/REA binds to BIG3 in the cytoplasm and its nuclear translocation is inhibited with or without E2 stimulation (right panel), resulting in the constitutive activation of the ER signaling.

necessary, our data should contribute to a more profound understanding of the estrogen/ER signaling pathway in breast cancer carcinogenesis.

Our findings demonstrated for the first time that endogenous PHB2/REA was trapped in the cytoplasm under the presence of BIG3 with or without stimulation of E2, while it was translocated to the nucleus and repressed the transcriptional activity without a high level of BIG3 protein in the cytoplasm. The overexpression of BIG3 might play an important role in determining the sensitivity of estrogen-target drugs to breast cancer cells. Since inhibition of the interaction between BIG3 and PHB2/REA may lead to rescuing the nuclear-translocation of PHB2/REA protein in ER-dependent breast cancer cells, the inhibitor for their interaction would be a possible valuable target to develop agents against breast cancer. Furthermore, as shown in Figure 2, we found that knockdown of BIG3 expression by siRNA resulted in growth suppression of breast cancer cell regardless of treatment of E2. In addition, Fig. 5 showed that inhibitory effects on transactivation of ERα by knockdown of BIG3 were not observed in the absence of E2. These findings suggest the possibility that overexpression of BIG3 also might enhances tumorigenesis by independent mechanisms with the effects on ERα transactivation, although further analysis of the BIG3 functions on ER-independent pathways will be necessary. Our data should contribute to a better understanding of breast carcinogenesis, and imply that *BIG3* is a promising molecular target for breast cancer treatment.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Northern blot analysis of breast cancer cell lines (BT-549, HCC1935, MDA-MB-157, BT-20, MDA-MB-453, ZR-75-1, BT474, HCC1143, HCC1500, HCC1599, and OCUB-F) and normal human tissues (mammary gland, lung, heart, liver, kidney, and brain), showing the expression of the transcript of brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) approximately 15 kDa in breast cancer cell lines and weakly in the brain.

Fig. S2. (a) Evaluation of specificity of affinity-purified anti-brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) polyclonal antibody. Expression of endogenous BIG3 protein in SK-BR-3 breast cancer cells was detected as a single band by western blot analysis. The arrow indicates approximately 250 kDa of BIG3 protein. (b) Immunohistochemical staining of BIG3 in normal human tissue sections (heart, lung, and liver). The expression of BIG3 protein in heart, lung, and liver were hardly detectable.

Fig. S3. (a) No knockdown effect with mismatch siRNA in SK-BR-3 by semi-quantitative RT-PCR (mismatch si#3). (b) MTT and (c) colony formation assays show no reduction in the number of viable cells by the mismatch si#3 as well as si-mock.

Fig. S4. (a) Knockdown of brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) expression at the protein level in si-BIG3 transfected-ZR-75-1 cells. Twenty-four hours after the transfection of si-BIG3 or si-EGFP as a control, the cells were treated with E2 for 48 h, and then were analyzed by western blot analysis. The si-EGFP was used as a control siRNA. ACTB (beta-actin) served as a loading control for western blot analysis. The arrow indicates an approximately 250-kDa BIG3 protein. (b) Subcellular localization of endogenous prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) in BIG3-depleted ZR-75-1 cells. ZR-75-1 cells were treated with si-BIG3 or si-EGFP. Twenty-four hours after the treatment of each siRNA, the cells were treated with E2 for 48 h, and then were analyzed by immunocytochemical staining with anti-PHB2/REA antibody. The arrows indicate endogenous prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) protein in nucleus of BIG3-depleted ZR-75-1 cells (right panels). On the other hand, endogenous PHB2/REA protein was observed in the cytoplasm of si-EGFP-treated cells.

Supplementary Methods

Northern blot analysis. Northern blot membrane for breast cancer cell lines was prepared as described previously.[1] The breast cancer northern blots were hybridized with [a^{32}P]-dCTP-labeled PCR products of brefeldin A-inhibited guanine nucleotide-exchange protein 3 (*BIG3*) cDNA prepared by RT-PCR (see below). Pre-hybridization, hybridization, and washing were performed as described previously.[2] The blots were autoradiographed with intensifying screens at –80°C for 14 days. A specific probe for *BIG3* (459 bp) was prepared by RT-PCR using the following primer sets: 5′-CAAGCTT GCTTACAGAGACCTG-3′ and 5′-GGGCCAAACCTACCAAAGTT-3′, and was radioactively labeled with Megaprime DNA labeling system (GE Healthcare, Buckinghamshire, UK).

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