

**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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**Abbreviations:** BIG3, brefeldin A-inhibited guanine nucleotide-exchange protein 3; ER, estrogen receptor; PHB2/REA, prohibitin 2/repressor of estrogen receptor activity;

**Running Title;** BIG3 as a molecular-target for breast cancer

**Abstract**

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 co-immunoprecipitation 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 ER $\alpha$ , and resulted in suppression of the ER $\alpha$  transcriptional activity. On the other hand, when BIG3 was present, BIG3 trapped PHB2/REA in cytoplasm and inhibited its nuclear translocation, and caused enhancement of ER $\alpha$  transcriptional activity. Our results imply that BIG3 overexpression is one of the important mechanisms causing the activation of the estrogen/ER $\alpha$  signaling pathway in the hormone-related growth of breast cancer cells.

## Introduction

Breast cancer is the most common cancer among women worldwide; 1.15-million new cases and 410,000 deaths caused by breast cancer in 2002 (Parkin *et al.*, 2005). Incidence of breast cancer is increasing in most countries and the increasing rate is much higher in countries where its incidence was previously low (Parkin *et al.*, 2005). 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 (Berry *et al.*, 2005; Yager, *et al.*, 2006). 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 a better management to 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 (Bange *et al.*, 2001; Petricoin *et al.*, 2002). 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 (Park *et al.*, 2006; Shimo *et al.*, 2007; Lin *et al.*, 2007; Shimo *et al.*, 2008; Ueki *et al.*, 2008), bladder cancer (Kanehira *et al.*, 2007a; Kanehira *et al.*, 2007b), synovial sarcomas (Nagayama *et al.*, 2004; Nagayama *et al.*, 2005), testicular seminoma (Okada *et al.*, 2004) and renal cell carcinoma (Togashi *et al.*, 2005; Hirota *et al.*, 2006; Dobashi *et al.*, 2009). 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 anti-cancer 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 (Nishidate *et al.*, 2004; Saito-Hisaminato *et al.*, 2002) and identified dozens of molecules that were over-expressed in a great majority of breast cancers and 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 ER $\alpha$  transcriptional activity. Our findings imply BIG3 to be a promising target for development of novel anti-cancer drugs for breast cancer.

## 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 (Nishidate *et al.*, 2004). Among the transactivated genes, we in this study focused on Brefeldin A-inhibited guanine nucleotide-exchange protein 3 (*BIG3*). We confirmed its upregulation in 9 of 12 clinical breast cancer specimens, compared with normal breast ductal cells or with whole mammary gland by semiquantitative RT-PCR (Figure 1a). Subsequent northern-blot analysis confirmed overexpression of its 15-kb transcript in breast cancer cell lines (Supplementary Figure 1). On the other hand, BIG3 expression was hardly detectable in any of 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; 3348bp) in the 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 2,177 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 (Chardin *et al.*, 1996; Jackson *et al.*, 2000; Cox *et al.*, 2004; Casanova *et al.*, 2007).

To investigate the biological function of the *BIG3* protein, we first generated an anti-*BIG3* polyclonal antibody and found it to recognize the endogenous *BIG3* protein of approximately 250 kDa in a breast cancer cell line, SK-BR-3, as well as exogenously-expressed *BIG3* in COS7 cells without any cross-reactivity to other proteins (Supplementary Figure. 2a). 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) (Figure 1b). Subsequent immunohistochemical analysis using anti-*BIG3* antibody showed its strong staining in the cytoplasm of two different histological subtypes of breast cancer, the papillotubular carcinoma and the solid-tubular carcinoma (Figure 1c), while any staining was detected neither in the normal breast tissue (Figure. 1d) nor in heart, lung and liver (Supplementary Figure 2b) in concordance with the results of northern-blot analysis.

#### *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 BT-474, which expressed a high-level of BIG3, using a mammalian vector-based RNA interference technique. 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 (Figure 2a, b; left panels). In concordance with the knockdown effect, MTT (Figure 2a, b; middle panels) and colony formation assays (Figure. 2a, b; right panels) revealed significant growth-suppressive effects by si#2 and si#3, but not significant by si#1 (mean absorbance of MTT assays in SK-BR-3 cells transfected with siBIG3 (si#1, si#2 and si#3) versus si-mock: si#1 versus si-mock, mean = 1.90 versus 2.39, difference = -0.49, 95% CI = -0.98 to -0.009,  $P = 0.053$ ; si#2 versus si-mock, mean = 0.48 versus 2.39, difference = -1.90, 95% CI = -2.44 to -1.37,  $P = 0.003$ ; si#3 versus si-mock, mean = 0.94 versus 2.39, difference = -1.44, 95% CI = -1.95 to -0.93,  $P = 0.005$ ; mean absorbance of MTT assays in BT-474 cells transfected with siBIG3 (si#1, si#2 and si#3) versus si-mock: si#1 versus si-mock, mean = 2.23 versus 2.41, difference = -0.18, 95% CI = -0.72 to -0.36,  $P = 0.30$ ; si#2 versus si-mock, mean = 0.34 versus 2.41, difference = -2.07, 95% CI = -2.63 to -1.51,  $P = 0.004$ ; si#3 versus si-mock, mean = 0.43 versus 2.41, difference = -1.98, 95% CI = -2.54 to -1.42,  $P = 0.004$ ). We also generated siRNA that contained a 3-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 (Supplementary Figure 3) or BT-474 cells (mean absorbance of MTT assays in BT-474 cells transfected with mismatch-siBIG3 (mismatch si#3) versus si-mock: mismatch si#3 versus si-mock, mean = 2.32 versus 2.41, difference = -0.09, 95% CI = -0.65 to 0.47,  $P = 0.57$ ) (Figure 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 prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) (Montano, *et al.*, 1999) 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) and HA-tagged PHB2/REA (PHB2/REA-HA) (see Materials and methods). These plasmids were co-transfected 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 co-precipitated with PHB2/REA-HA (Figure 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 co-precipitated with BIG3-Flag (Figure 3a; right panel). We additionally confirmed that BIG3-Flag was co-precipitated with endogenous PHB2/REA in SK-BR-3 cells (Figure 3b).

We subsequently performed immunocytochemical staining analysis with anti-PHB2/REA polyclonal antibody using breast cancer cell line, SK-BR-3, (see Materials and methods) and observed staining of endogenous PHB2/REA mainly as speckled pattern in cytoplasm in most of the breast cancer cells (Figure 3c, middle panel). In a small subset of the cells, its staining was observed in both cytoplasm and nucleus (Figure 3c, right panel; arrows). Since PHB2/REA was reported to selectively repress the transcriptional activity of ER $\alpha$  through its interaction with ER $\alpha$  in the nucleus (Montano, *et al.*, 1999; Delage-Mourroux *et al.*, 2000; Kasashima *et al.*, 2006), we investigated a possibility of a direct interaction between BIG3 and ER $\alpha$ , 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 cytoplasm and be translocated to the nucleus in ER $\alpha$ -positive cells (Montano, *et al.*, 1999; Delage-Mourroux *et al.*, 2000; Kasashima *et al.*, 2006), we hypothesized that BIG3 might interact with PHB2/REA and interfere its nuclear translocation. Therefore, we performed immunocytochemical staining to examine the subcellular distribution of PHB2/REA protein in a presence or an absence of BIG3. Figure 4a showed that endogenous PHB2/REA was also localized in 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 (Figure 4b, left panels), 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 (Figure 4b, right panels). These findings suggest that BIG3 interacted with PHB2/REA and interfered its nuclear translocation.

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 (Figure 4c). 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 (Figure 4d). 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 (Supplementary Figure 4). These data further support that BIG3 trapped PHB2/REA in the cytoplasm, and resulted in inhibition of its nuclear translocation and the interaction with ER $\alpha$ .

#### *BIG3 up-regulates ER $\alpha$ transcriptional activity though its interaction with PHB2/REA*

To examine whether BIG3 protein could enhance the ER $\alpha$  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 reporter assay (see Materials and methods). We confirmed the knockdown of BIG3 expression by western-blot analysis (Figure 5a), and found that the depletion of BIG3 expression caused the significant decrease of ER $\alpha$  transcriptional activity in MCF-7 cells (mean relative ER $\alpha$  transcriptional activity in MCF-7 cells transfected si-BIG3 after E2 stimulation versus MCF-7 cells transfected with si-EGFP after E2 stimulation; 0.65 versus 0.99, difference = -0.35. 95% CI = -0.38 to -0.31,  $P$  = 0.0006; mean relative ER $\alpha$  transcriptional activity in MCF-7 cells transfected with si-BIG3 without E2 stimulation versus MCF-7 cells transfected si-EGFP without E2 stimulation; 0.93 versus 0.94, difference = -0.02. 95% CI = -0.27 to 0.23,  $P$  = 0.57) (Figure 5b). These findings suggest that the presence of BIG3 protein is likely to enhance the ER $\alpha$  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 last two decades. However, the proportion of patients having benefit by presently available treatments is still limited (Berry *et al.*, 2005; Yager, *et al.*, 2006). Hence, it is urgent to further develop new anticancer agents that are highly specific to malignant cells, with minimal or no adverse reactions. 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 anti-cancer 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 (Jackson *et al.*, 2000; Cox *et al.*, 2004; Casanova *et al.*, 2007). 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 through the exocytic pathway and class-III is implicated in endocytosis and in actin dynamics at the plasma membrane. Little is unknown about the roles of class-II Arf (Jackson *et al.*, 2000; Cox *et al.*, 2004; Casanova *et al.*, 2007). All of Arf-GEFs identified to date are characterized by a central catalytic domain of approximately 200 amino acids referred to as the Sec7 domain that is sufficient for GEF activity (Jones *et al.*, 1999; Jackson *et al.*, 2000; Cox *et al.*, 2004; Casanova *et al.*, 2007). Brefeldin A-inhibited guanine nucleotide-exchange proteins 1 and 2 (BIG1 and BIG2) contain highly conserved sec7 domains that catalyze replacement of ARF-bound GDP by GTP to initiate membrane vesicle formation (Cox *et al.*, 2004; Casanova *et al.*, 2007). 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 class-I Arfs (Cox *et al.*, 2004; Yamaji *et al.*, 2000). 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 estrogen receptor  $\alpha$  (ER $\alpha$ )-selective coregulator (Montano, *et al.*, 1999; Delage-Mourroux *et al.*, 2000; Kasashima *et al.*, 2006), as a candidate. We demonstrated their *in vivo* interaction and co-localization at the cytoplasm of breast cancer cells. We further confirmed that endogenous PHB2/REA was localized in cytoplasm under the presence of BIG3 regardless with or without stimulation of E2, but it translocated to the nucleus, and repressed the transcriptional activity under the absence of BIG3 protein (Figure 4 and 5). Moreover, all of ER $\alpha$ -positive breast cancer cells we examined showed the overexpression of PHB2/REA (data not shown). 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 ER signaling pathway by overexpression of BIG3 in breast cancer *in vivo* (Figure 6). Additionally, these findings may explain why there is no apparent correlation between PHB2/REA expression and ER $\alpha$  inactivation in breast cancer cases. Furthermore, although further analysis of the BIG3 function by evaluation of activation of ER-downstream genes will be necessary, our data should contribute to 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 regardless 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 for breast cancer cells. Since inhibition of the interaction between BIG3 and PHB2/REA may lead to rescue 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. Our data should contribute to a better understanding of breast carcinogenesis, and imply that *BIG3* is a promising molecular target for breast cancer treatment.

## **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 and mouse fibroblast cell line, NIH3T3 were purchased from American Type Culture Collection (ATCC, Rockville, MD), and cultured under their respective depositors' recommendations. HBC4 and HBC5 were kind gifts from Dr. Takao Yamori of

Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. All cells were cultured in appropriate media; i.e. RPMI-1640 (Sigma-Aldrich, St. Louis, MO) for HBC4, HBC5, HCC1937, T47D, BT-549, HCC1395, ZR-75-1, BT-483, HCC1143, HCC1500 and HCC1599 (with 2mM L-glutamine); Dulbecco's modified Eagle's medium (Sigma-Aldrich) for BT-474, OCUB-F, COS-7 and NIH3T3; EMEM (Sigma-Aldrich) for MCF-7 and BT-20 (with 10µg/ml Insulin); McCoy (Sigma-Aldrich) for SK-BR-3 (with 1.5mM L-glutamine); L-15 (Roche, Basel, Switzerland) for MDA-MB-231, MDA-MB-157 and MDA-MB-453. Each medium was supplemented with 10% fetal bovine serum (FBS; Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). MDA-MB-231, MDA-MB-157 and MDA-MB-453 cell lines were maintained at 37°C in atmosphere of humidified air without CO<sub>2</sub>, and other cell lines were maintained at 37°C in atmosphere of humidified air with 5% CO<sub>2</sub>. Tissue samples from surgically-resected breast cancers and their corresponding clinical information were obtained from Department of Breast Surgery, Cancer Institute Hospital, Tokyo after obtaining written informed consent.

#### *Semiquantitative reverse transcription-PCR analysis*

Microdissection of breast cancer cells was performed as described previously (Nishidate *et al*; 2004). Total RNAs were extracted from each of microdissected breast cancer clinical samples, microdissected normal breast ductal cells and breast cancer cell lines using RNeasy Micro Kits (Qiagen, Valencia, CA), and purchased polyA (+) RNAs isolated from mammary gland, heart, lung, liver, kidney and bone marrow from Takara Clontech (Kyoto, Japan). Subsequently, T7-based amplification and reverse transcription were carried out as described previously (Nishidate *et al*; 2004). Three-microgram aliquots of each amplified RNA were reversely transcribed for single-stranded cDNAs using oligo (dT)<sub>12-18</sub> primer and Superscript II (Invitrogen, Carlsbad, CA) at 42°C for 60 min. We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR by monitoring *β2-microglobulin* (*β2-MG*) as a quantitative internal control. PCR amplification was performed using EX-taq polymerase (Takara) and the

cDNAs as templates with the following primers: 5'-CTTGACAAGGCCTTTGGAGT-3' and 5'-CAATATGCTTTTCCCGCTTT-3' for *BIG3*; 5'-AACTTAGAGGTGGG AGCAG-3' and 5'-CACAACCATGC CTTACTTTATC-3' for  $\beta$ 2-MG. PCRs were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification. The PCR conditions were 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec for 21 cycles for  $\beta$ 2-MG and 28 cycles for *BIG3*. The PCR products were resolved by electrophoresis on 2.0% ethidium bromide-stained agarose gels, and band intensity was quantified with the use of NIH Image analysis software (<http://rsb.info.nih.gov/nih-image/>).

#### *5' rapid amplification of cDNA ends (5' RACE)*

5' RACE experiments were carried out using 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'-GCCTCCTTCTGCAGCTTCCTCAGGATTT-3') and a 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). The PCR products were cloned using TA cloning kit (Invitrogen) and sequences were determined by DNA sequencing (ABI3700; PE Applied Biosystems, Foster, CA).

#### *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'-ATAGTTTAGCGGCCGCACAATGATGTCATAGACACGG-3' (underlines indicate recognition sites of restriction enzymes). The PCR product was inserted into the *EcoRI* and *NheI* 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 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), respectively. The two recombinant peptides were expressed in *Escherichia coli*, BL21 codon-plus strain (Stratagene, La Jolla, CA), respectively, and purified using Ni-NTA resin agarose (Qiagen) 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) according to supplier's instructions. We confirmed that this antibody could specifically recognize endogenous BIG3 protein in breast cancer cell line, SK-BR-3 cells. An affinity-purified anti-BIG3 antibody was used for western blot, immunocytochemical and immunohistochemical analyses as described below.

#### *Western blot analysis*

To examine the expression of endogenous BIG3 protein in breast cancer cell lines (MDA-MB-231, MDA-MB-453, SK-BR-3, T47D, ZR-75-1, BT-483, HCC1395, BT-20, BT-549, HCC1143, HCC1500, HCC1937 and MCF-7), cells were lysed with lysis buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% NP-40 and 0.5% CHAPS) including 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA). After homogenization, cell lysates were incubated on ice for 30 min and centrifuged at 14,000 rpm for 15 min to separate only supernatant from cell debris. The amount of total protein was measured by protein assay kit (Bio-Rad), and then the proteins were mixed with SDS-sample buffer and boiled for 5 min before loading at 7.5% SDS-PAGE gel. After electrophoresis, the proteins were blotted onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, United Kingdom). After blocking with 4% BlockAce blocking solution (Dainippon Pharmaceutical. Co., Ltd, Osaka, Japan) for 1 h,

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 (1:10000 dilution; GE Healthcare) for 1 h, and protein bands were visualized by ECL detection reagent (GE Healthcare).

#### *Immunocytochemical staining*

To examine the subcellular localization of endogenous BIG3 and PHB2/REA proteins in breast cancer cells, MCF-7 or T47D cells were seeded at  $1 \times 10^5$  cells per well (Lab-Tek II Chamber Slide System; Nalgen Nunc International, Naperville, IL) under estrogen-free conditions as describe below. Cells were then fixed with Phosphate Buffered Saline (PBS; Sigma-Aldrich) 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 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) diluted at 1:1000 for 1 h. Nuclei were counter-stained with 4', 6'-diamidine-2'-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under 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 4µm sections of paraffin-embedded breast cancer tissue (sample No. 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). Specimens were deparaffinized by the treatment with xylene and ethanol, then processed for antigen retrieval by autoclave at 108°C for 15 min in antigen retrieval solution, high pH (DAKO Cytomation, Glostrup, Denmark) and treated with peroxidase blocking reagent

(DAKO Cytomation) for 1 h. Tissue sections were incubated with anti-BIG3 polyclonal antibody diluted at 1:150 for 1 h and followed by horseradish peroxidase-conjugated secondary antibody (DAKO Cytomation) for 30 min. Specific immunostaining was visualized with peroxidase substrate (3, 3'-diaminobenzidine tetrahydrochloride) (DAKO liquid DAB+ chromogen; DAKO Cytomation). Finally, tissue specimens were stained with hematoxylin to discriminate nucleus from cytoplasm.

#### *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 (Shimokawa *et al*; 2003). The siRNA expression vectors against *BIG3* (psiU6BX3.0-BIG3) were prepared by cloning of double-stranded oligonucleotides into the *BbsI* site in the psiU6BX3.0 vector. The target sequences of synthetic oligonucleotides for siRNAs were as follows; 5'-AAGGTCCTATGGATCTAGGTA-3' for si-#1, 5'-AAGAAAGCA TCGCAGTCTCAG-3' for si-#2, 5'-AAGATGCGTTCT CTGCCACAC-3' for si-#3, 5'-AATATTCGATCTCTGCCACAC-3' for si-m#3 (bold letters indicate mismatched sequence in si-#3). All of the constructs were confirmed by DNA sequencing (ABI3700; PE Applied Biosystems). Human breast cancer cell lines, SK-BR-3 and BT-474, were plated onto 10 cm dishes (1 x 10<sup>6</sup> cells/dish) and transfected with 8µg 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 transfection reagent (Roche) according to the manufacturer's instructions. After 24 h of transfection, cells were re-seeded for colony formation assay (1 x 10<sup>6</sup> cells/10 cm dish), RT-PCR (1 x 10<sup>6</sup> cells/10 cm dish) and 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay (2 x 10<sup>5</sup> cells/well). We selected psiU6BX3.0-introduced SK-BR-3 or BT-474 cells with culture medium containing 0.2mg/ml or 1.0mg/ml of neomycin (geneticin; Invitrogen), respectively. We changed 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 semi-quantitative RT-PCR using specific primer sets;



5'-GCCCTTGAAGCCAATATTCC-3' and 5'-AGATGGTTTCAGTGGGCTTG-3' for *BIG3*; 5'-AACTTAGAGGTGGGAGCAG-3' and 5'-CACAACCATGCCTTACTTTATC-3' for  $\beta$ 2-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) to assess the colony number. To quantify cell viability, MTT assays were performed with cell counting kit-8 (Wako, Osaka, Japan) according to manufacturer's recommendations. Absorbance at 570 nm wavelengths was measured with a Microplate Reader 550 (Bio-Rad). These experiments were performed in triplicate.

Furthermore, we used siRNA oligonucleotides (Sigma Aldrich Japan KK, 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 EGFP (siEGFP) were as follows: si-*BIG3*; 5'-GAUGCGUUCUCUGCCACACTT-3', siEGFP (control); 5'-GCAGCACGACUUCUUAAG-3'. MCF-7 or ZR-75-1 (2.5 X10<sup>5</sup> cells in 10cm dish) cells were transfected with those siRNAs using Lipofectamin RNAiMAX (Invitrogen, Carlsbad, CA) in OptiMEM (Invitrogen) medium according to the instructions of manufacture. After 24 h of the transfection, the cells were treated with 1 $\mu$ M of E2 (17 $\beta$ -estradiol; Sigma-Aldrich), and then were applied for immunocytochemical and western blot analyses using anti-PHB2/REA polyclonal antibody (Abcam, Cambridge, UK), anti-ER $\alpha$  monoclonal antibody (LAB VISION, Fremount, CA) and anti-*BIG3* polyclonal antibody according to the method described in the western blot and immunocytochemical analyses sections. Fluorescent images were obtained under TCS SP2 AOBS microscope (Leica).

#### *Immunoprecipitation and mass spectrometry*

To identify an interacting protein(s) with *BIG3* protein, BT-549 cells were plated onto 15-cm dishes (1 x 10<sup>7</sup> cells/dish) and transfected with 20 $\mu$ g of pCAGGSnH3F-Mock (without insertion) or pCAGGSnH3F-*BIG3* using

FuGENE6 reagent (Roche) according to the manufacturer's instructions. After 48-h incubation, the cells were lysed with 0.1% NP-40 lysis buffer as described in western blot analysis section. The cell lysates were pre-cleaned with normal mouse IgG and rec-Protein G Sepharose 4B (Zymed, San Francisco, CA) 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, proteins on beads were eluted with SDS-sample buffer by boiling for 5 min. The eluted protein samples were separated by SDS-PAGE using NuPAGE 4-12% Bis-Tris gel (Invitrogen). Proteins in polyacrylamide gel were silver stained by SilverQuest Silver Staining Kit (Invitrogen) according to the manufacturer's instructions. Bands that were specifically observed in the BIG3-transfected lane were excised with a clean, sharp scalpel and the extracted proteins were applied for PMF (Peptide Mass Fingerprint) analysis using MALDI TOF-MS (Shimadzu Biotech, Tsukuba, Japan).

#### *Co-immunoprecipitation assay*

For co-immunoprecipitation assay, plasmids designed to express the entire coding sequence of PHB2/REA were constructed using the following primer set; 5'-CGGAATTCCAGACCGTGCATCATGGCCCAGAACTTGAAGGA-3' and 5'-CCGCTCGAGTTTCTTACCCTTGATGAGGCTGT-3' (underlines indicate the recognition sites of restriction enzymes), and inserted in frame into the *EcoRI* and *XhoI* 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 of pCAGGSn3FC-BIG3 and pCAGGSnHC-PHB2/REA. After 48 h of the transfection, the cells were lysed with 0.1% NP-40 lysis buffer as described in western blot analysis section. The cell lysates were pre-cleaned 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, co-precipitated 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*

For immunocytochemical staining, 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 AG, Goettingen, Germany). The cells were maintained at 37°C in atmosphere of humidified air with 5% CO<sub>2</sub>. Transfection with BIG3 (pCAGGSnH3F-BIG3) expression vector was performed using FuGENE6 transfection reagent (Roche) and phenol red-free Opti-MEM (Invitrogen), according to the manufacturer's instructions. After 24 h of the transfection, the media were exchanged with phenol red-free Opti-MEM containing 1µM 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 anti-rabbit secondary antibody (Molecular Probe, Eugene, OR) diluted at 1:1000.

*Estrogen responsive element (ERE) reporter gene assays*

An ERE reporter gene construct and fluorescent SEAP assay kit were purchased from Clontech (Takara, Kyoto, Japan). 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. After 24 h of 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, 1µM of E2 was treated for 48 h for SEAP assay and western blot analysis, respectively. The SEAP reporter assay was performed using SEAP assay kit (Takara, Clontech) according to the supplier's recommendations.

*Statistical analysis*

All experimental data are summarized as the mean value with 95% confidence intervals (CIs). All statistical analyses were performed using the two-sample *t* test with Welch's correction, and were two-side. Differences were considered to be statistically significant at a value of *P* less than 0.05.

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### Figure legends

**Figure 1** Expression profiles of 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.  $\beta$ 2-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 cytoplasm in all of cancer tissues investigated, including papillo-tubular (Sample nos. 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 x100.

**Figure 2** Effect of 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).  $\beta$ 2-MG served as the quantity control. MTT and colony formation assays showed a significant decrease in the number of colonies by knockdown of *BIG3* in SK-BR-3 cells (middle and right panels). The value shown in MTT assay are the means and upper 95% confidence intervals for three independent experiments performed in triplicate. Error bars are 95% CIs. \* $P=0.003$ ; \*\* $P=0.005$ . (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 (mis-match si#3) (left panels). MTT and colony formation assay showed a decrease in the number of colonies by knockdown of *BIG3* in BT-474 cells (middle and right panel). The value shown in MTT assay are the means and upper 95% confidence intervals for three independent experiments performed in triplicate. Error bars are 95% CIs. \* $P=0.004$ ; \*\* $P=0.004$ .

**Figure 3** Interaction of *BIG3* and 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 cytoplasm, but the nuclear localization was observed in some cells (arrows).

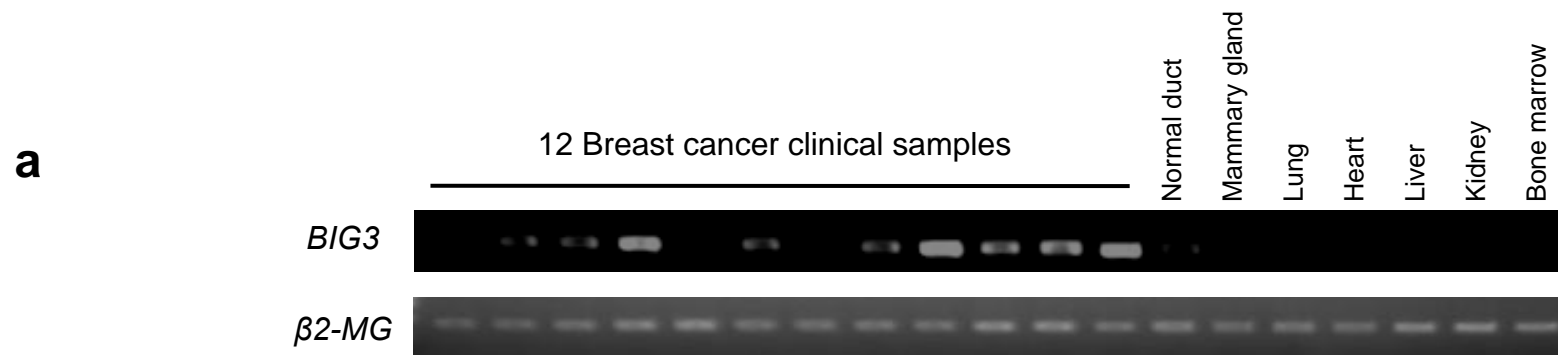
**Figure 4** Inhibition of the nuclear translocation of PHB2/REA by overexpression of BIG3. **(a)** Subcellular localization of PHB2/REA in MCF-7 cells, which overexpressed BIG3 protein. Endogenous PHB2/REA protein was located in cytoplasm of MCF-7 cells with or without treatment of E2. The arrows show 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 (left panels), when BIG3 protein was exogenously expressed. The arrows show endogenous PHB2/REA in nucleus of T47D cells. T47D cells were transfected with Flag-tagged BIG3 (BIG3-Flag; green) and treated with 1 $\mu$ M of E2 for 24 h (right panels). Endogenous PHB2/REA (red) remained in cytoplasm in the presence of BIG3 (the right panels; Merge). The arrows show co-localization of endogenous PHB2/REA and exogenous BIG3 in cytoplasm of T47D cells. **(c)** Expression of BIG3, ER $\alpha$  and PHB2/REA at the protein level in MCF-7 cells treated with si-BIG3 oligonucleotide. After 24 h of 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. **(d)** Subcellular localization of endogenous PHB2/REA in BIG3-depleted cells. MCF-7 cells were treated with si-BIG3 or si-EGFP. After 24 h of the siRNA treatment, the cells were treated with E2 for 48 h, and then were analyzed by immunocytochemical staining.

**Figure 5** Regulation of ER $\alpha$  transcriptional activity through its interaction between PHB2/REA and BIG3. **(a)** Knockdown of BIG3 expression at the protein level in the cells treated with si-BIG3. After 24 h of the transfection with si-BIG3, 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 served as a loading control for western blot analysis. The arrow indicates the BIG3 protein.

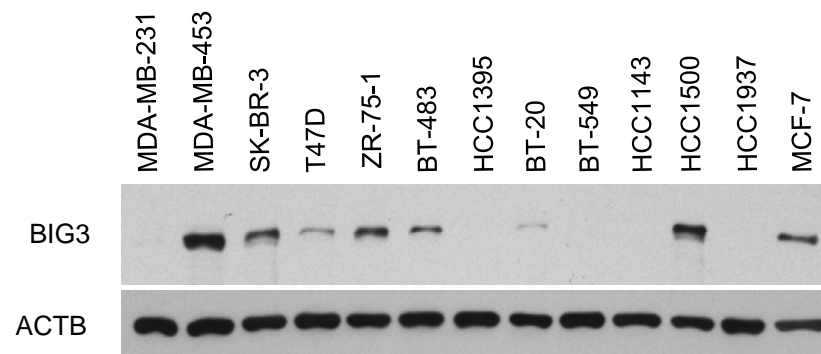
(b) SEAP assay to determine the transcriptional activity of ER $\alpha$  in BIG3-depleted cells. MCF-7 cells were transfected with siRNA-oligonucleotides of BIG3 (si-BIG3) or EGFP (si-EGFP; control). After 24 h of the transfection, the cells were washed with PBS (-) at three times. Subsequently, the 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 $\mu$ M of E2 for additional 48 h. The SEAP reporter activity was measured relative to the response to E2 in the mock-transfected cells, which was set at 1.0. The value shown in SEAP assay are the means and upper 95% confidence intervals for three independent experiments performed in duplicate. \* $P=0.0006$ .

**Figure 6** Schematic presentation of up-regulation of ER-signaling pathway by BIG3. In the absence of BIG3, PHB2/REA is translocated to the nucleus, binds to ER $\alpha$ , and represses the transcriptional activity of the estradiol (E2)-ligand ER $\alpha$  (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 regardless with or without E2 stimulation (right panel), resulting in the constitutive activation of the ER signaling.

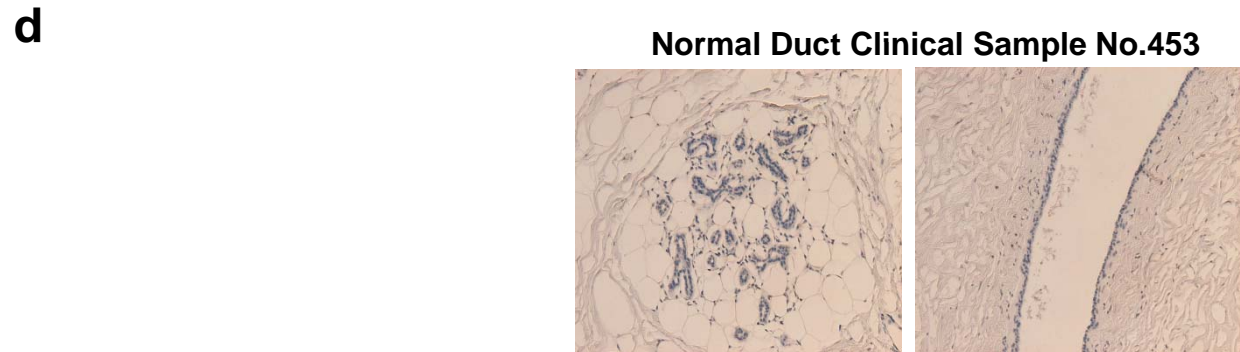
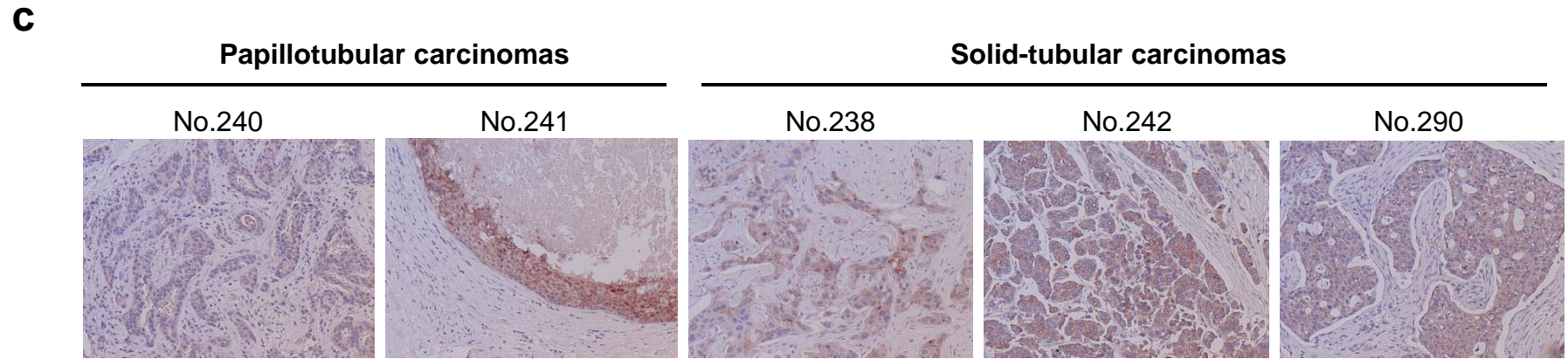
**Figure 1**



**b**

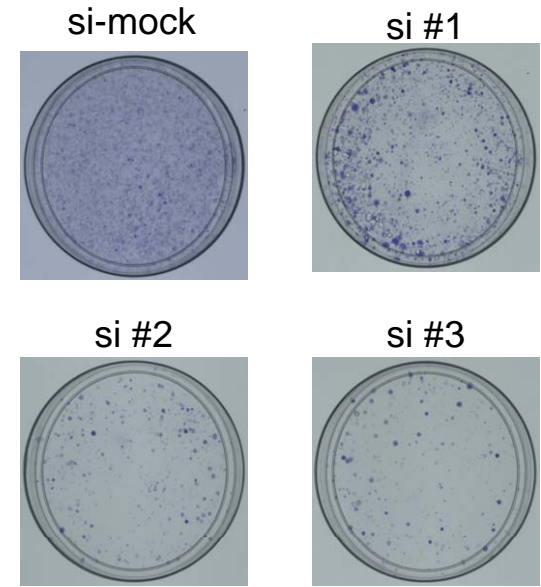
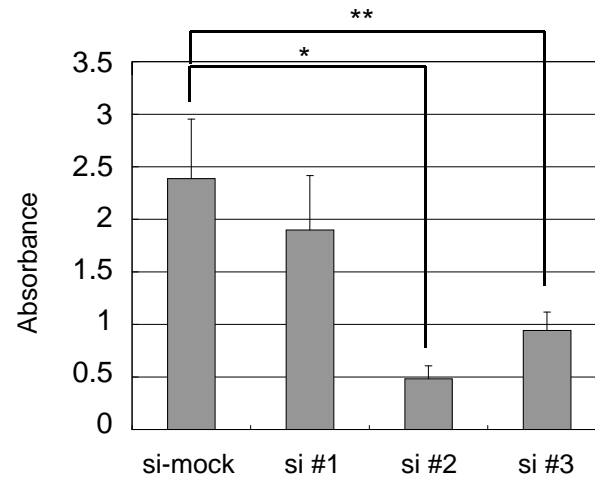
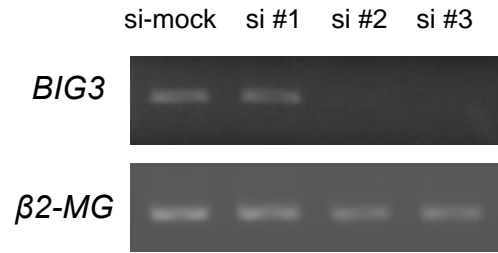


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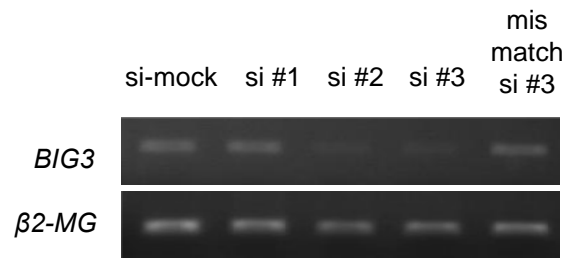


**Figure 2**

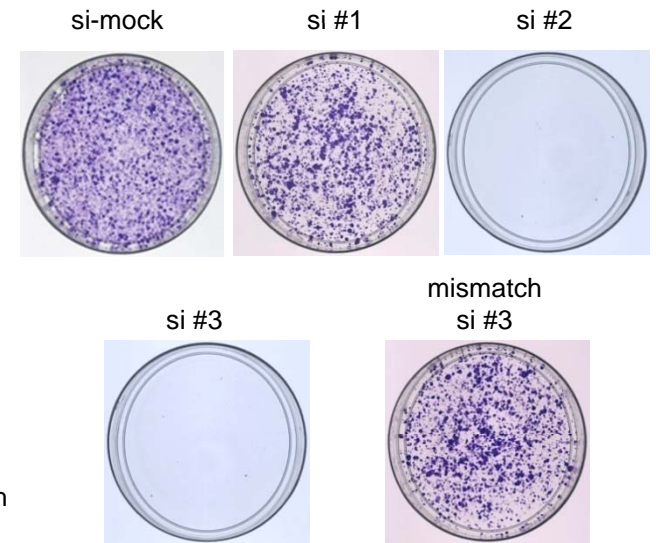
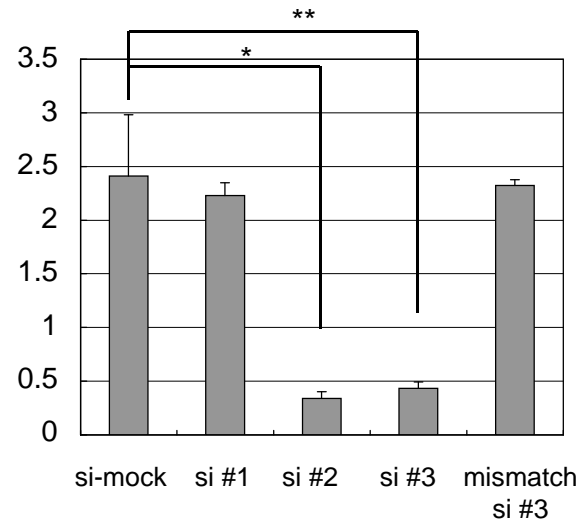
**A**



**B**

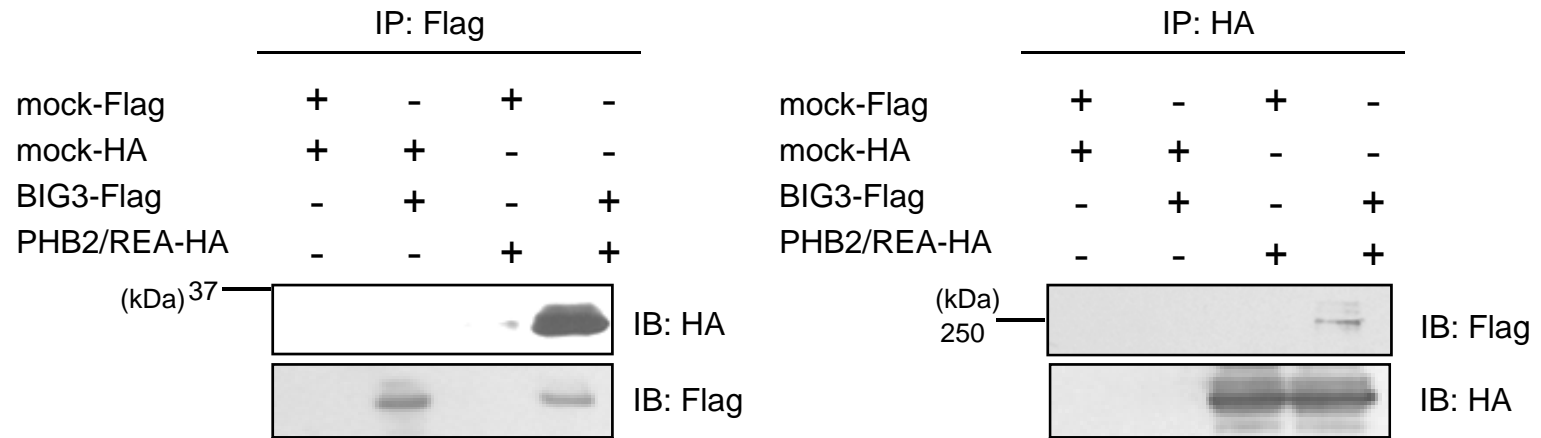


Absorbance

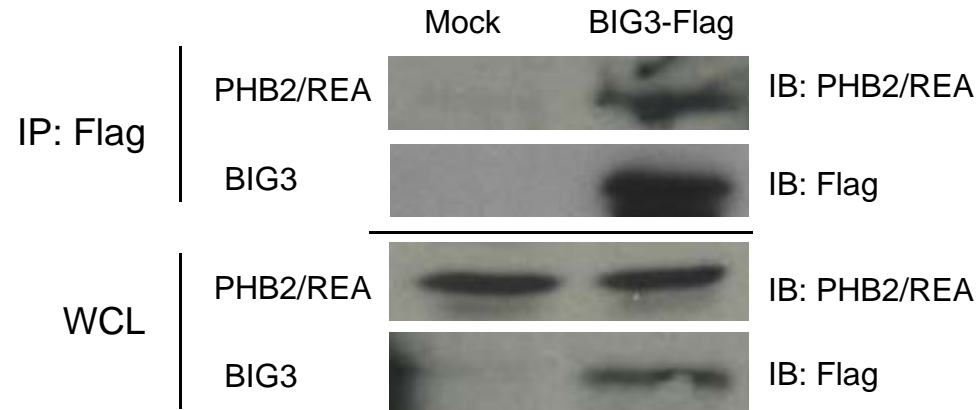


**Figure 3**

**a**

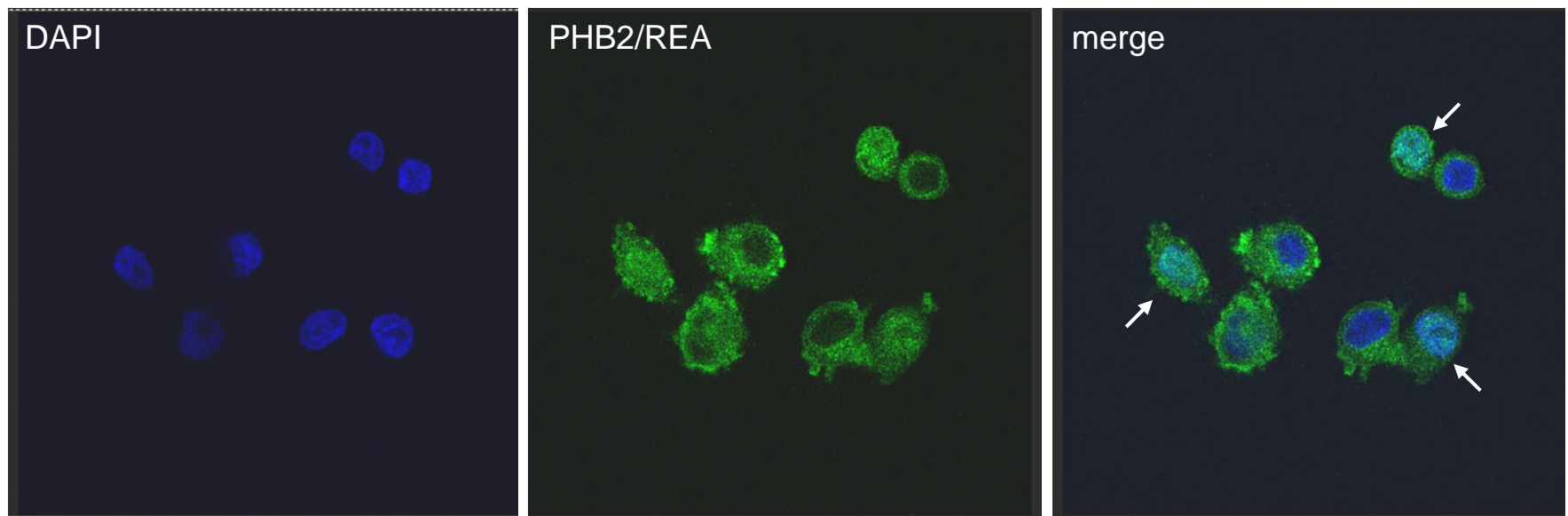


**b**



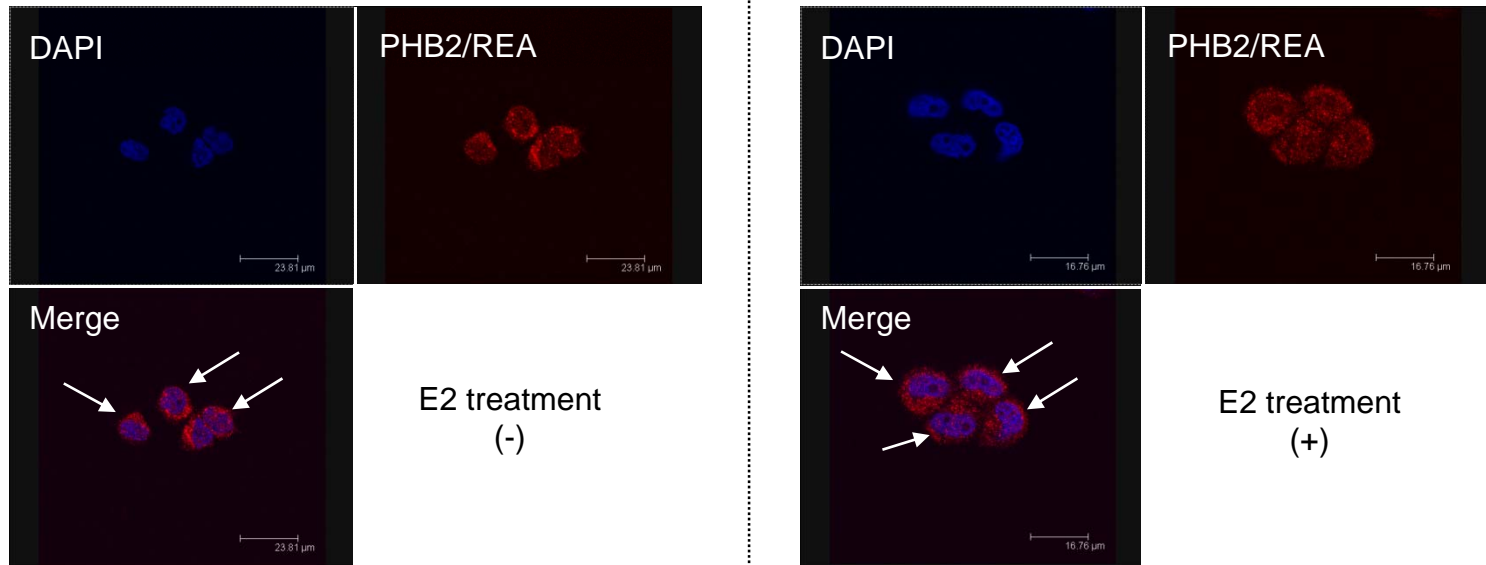
## Figure 3 continue

**c**

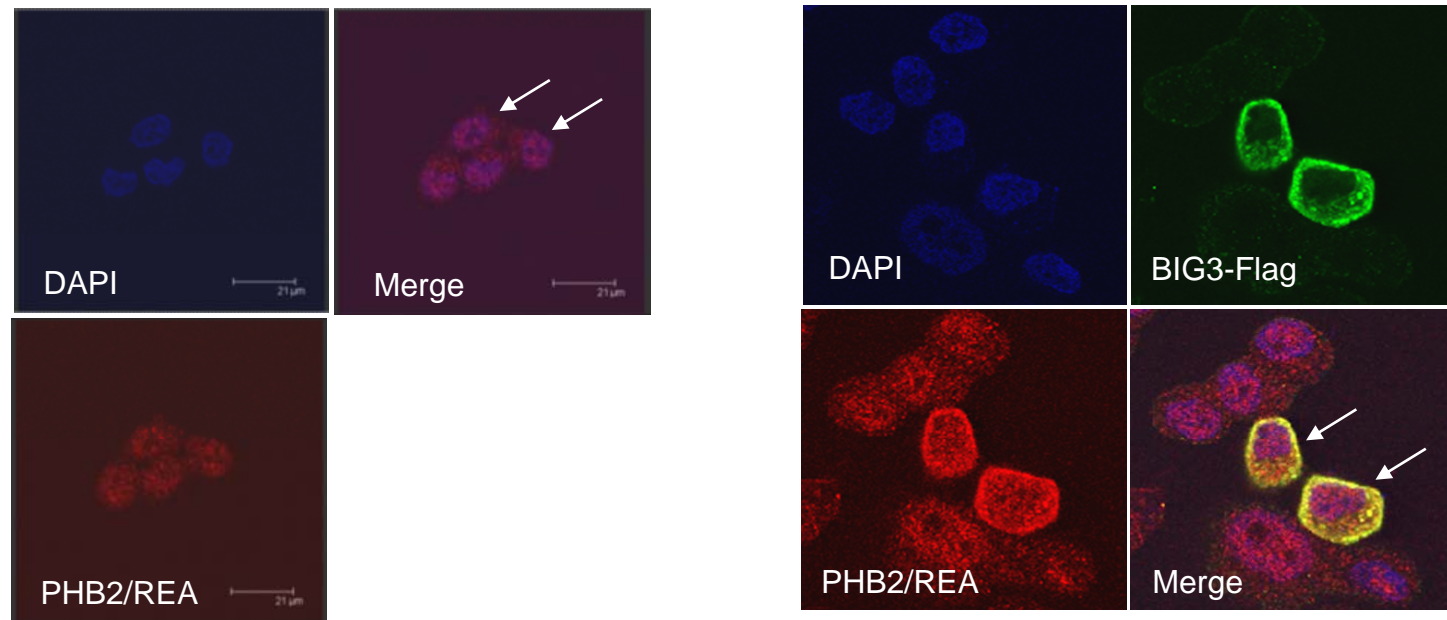


**Figure 4**

**a**



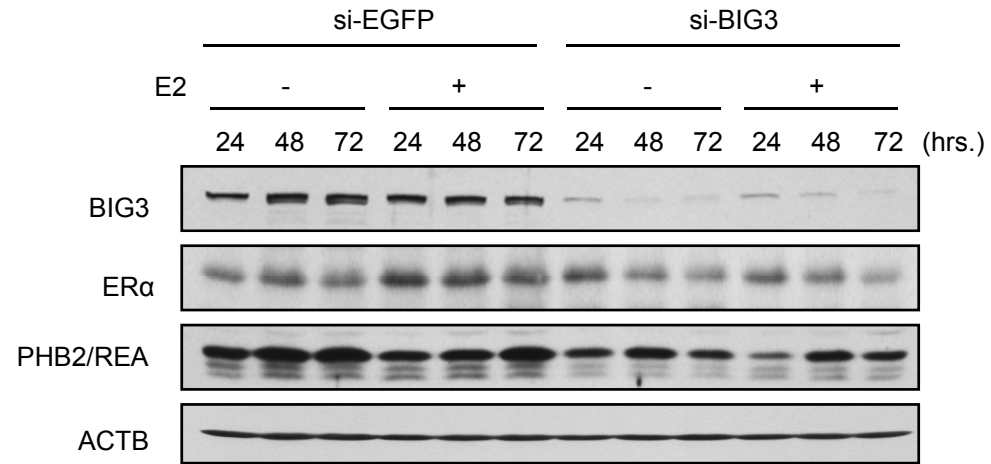
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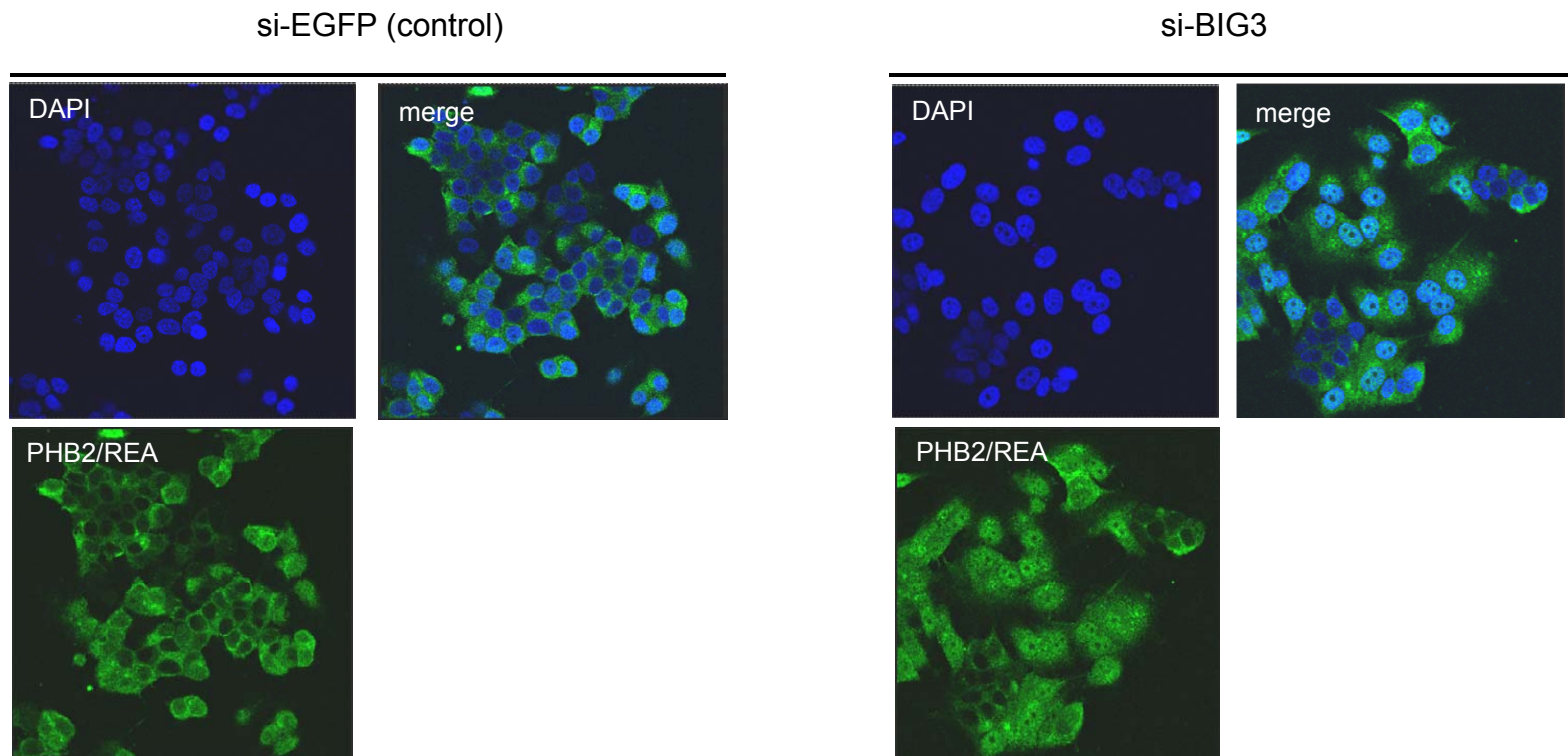


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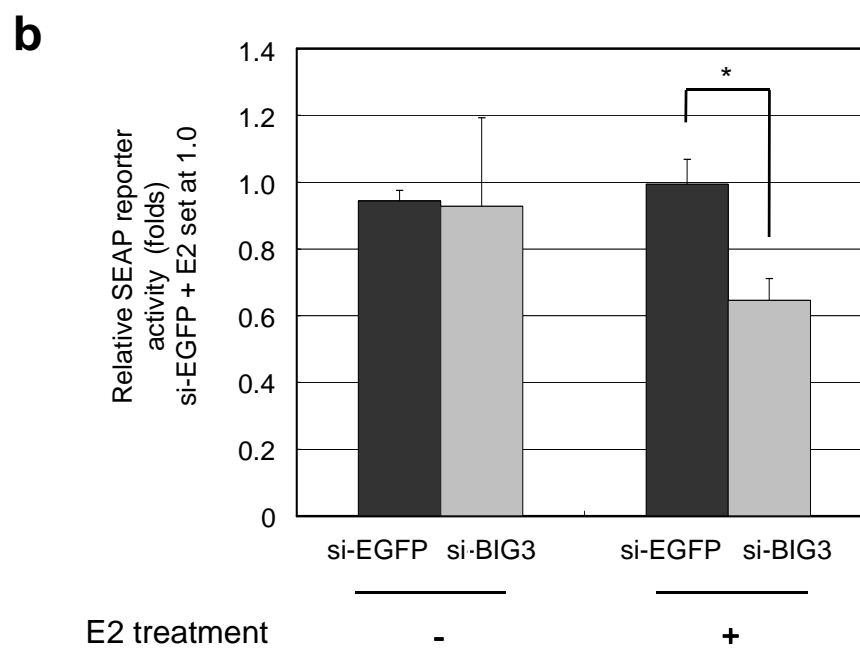
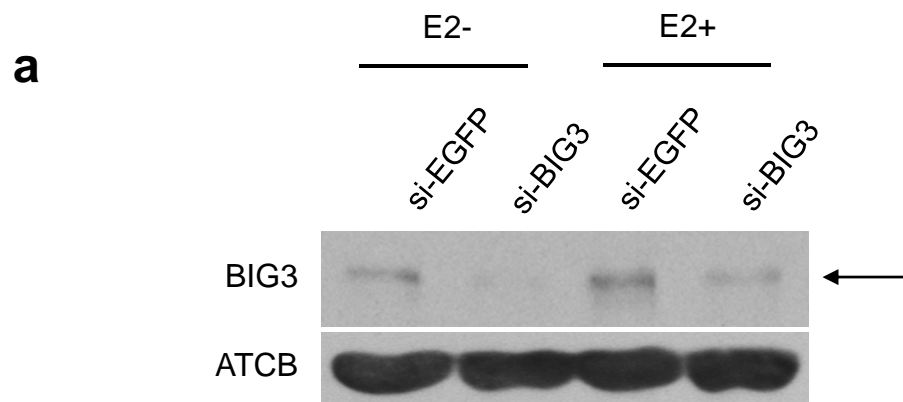
**c**



**d**

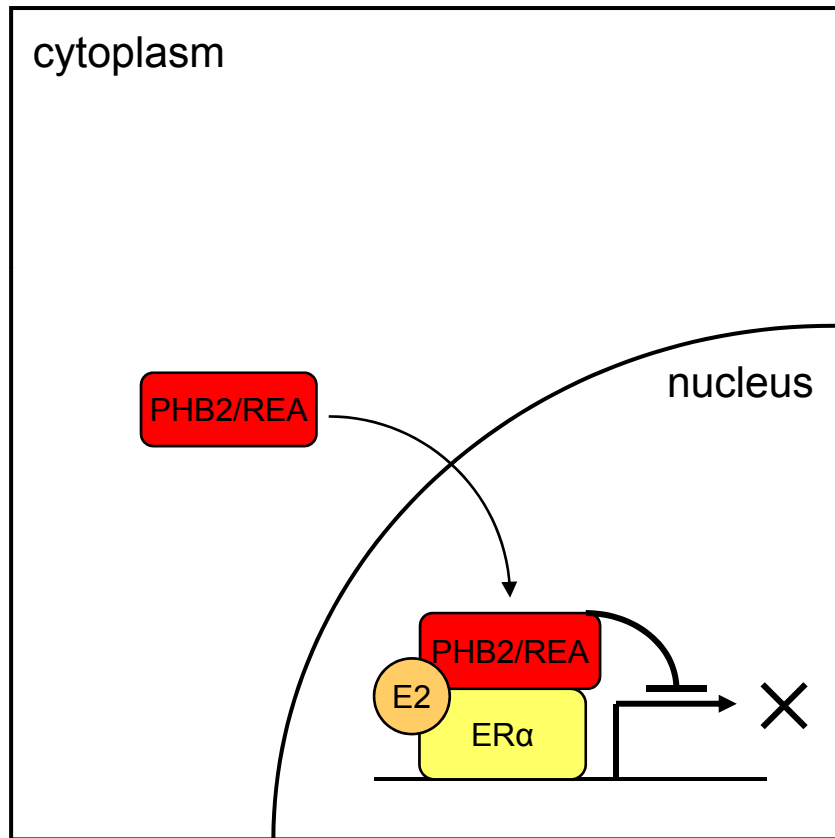


**Figure 5**



**Figure 6**

Absence of BIG3



Presence of BIG3

