Xanthohumol suppresses oestrogen-signalling in breast cancer through the inhibition of BIG3-PHB2 interactions

Tetsuro Yoshimaru1, Masato Komatsu1, Etsu Tashiro2, Masaya Imoto2, Hiroyuki Osada3, Yasuo Miyoshi4, Junko Honda5, Mitsunori Sasa6 & Toyomasa Katagiri1

1Division of Genome Medicine, Institute for Genome Research, The University of Tokushima, Tokushima, Japan, 2Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Kanagawa, Japan, 3Antibiotics Laboratory, RIKEN CSRS, Saitama, Japan, 4Department of Surgery, Division of Breast and Endocrine Surgery, Hyogo College of Medicine, Hyogo, Japan, 5Department of Surgery, National Hospital Organization Higashitokushima Medical Center, Tokushima, Japan, 6Department of Surgery, Tokushima Breast Care Clinic, Tokushima, Japan.

Xanthohumol (XN) is a natural anticancer compound that inhibits the proliferation of oestrogen receptor-α (ERα)-positive breast cancer cells. However, the precise mechanism of the antitumour effects of XN on oestrogen (E2)-dependent cell growth, and especially its direct target molecule(s), remain(s) largely unknown. Here, we focus on whether XN directly binds to the tumour suppressor protein prohibitin 2 (PHB2), forming a novel natural antitumour compound targeting the BIG3-PHB2 complex and acting as a pivotal modulator of E2/ERα signalling in breast cancer cells. XN treatment effectively prevented the BIG3-PHB2 interaction, thereby releasing PHB2 to directly bind to both nuclear- and cytoplasmic ERα. This event led to the complete suppression of the E2-signalling pathways and ERα-positive breast cancer cell growth both in vitro and in vivo, but did not suppress the growth of normal mammary epithelial cells. Our findings suggest that XN may be a promising natural compound to suppress the growth of luminal-type breast cancer.

Oestrogen-receptor-α (ERα) plays a pivotal role in the development and progression of breast cancer. The current endocrine therapies for breast cancer are mainly based on targeting ERα signalling using selective ERα modulators (e.g., tamoxifen and raloxifene), ERα downregulators (e.g., fulvestrant), and aromatase inhibitor (AI)1–3. Of these treatments, tamoxifen, which inhibits breast cancer cell growth through the competitive binding of ERα, is a standard treatment offered to patients with ERα-positive breast cancer. However, tamoxifen treatment often fails, and patients succumb to recurrent, endocrine-resistant tumours4,5. Moreover, AI, which blocks oestrogen (E2) synthesis, provides substantial clinical benefits, such as good efficacy, a significant increase in disease-free survival, and a prolonged time to disease recurrence in postmenopausal women with ERα-positive breast cancer, compared with tamoxifen treatment. Nevertheless, some patients who have undergone AI treatment still relapse6,7. The precise molecular events that determine alterations in the effectiveness of these endocrine therapies remain unknown.

We previously reported that the oncoprotein brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) and tumour suppressor prohibitin 2 (PHB2) complex play a pivotal role in E2 signalling modulation in ERα-positive breast cancer8,9. Namely, BIG3 binds PHB2, thereby inhibiting the E2-dependent suppressive ability of PHB2 and resulting in constitutive ERα activation. Considering these findings, strategies utilising the tumour suppressive activity of PHB2 upon its release from BIG3 by inhibitors of protein-protein interaction (PPI) may represent novel therapies for breast cancer, although PPI has been difficult to target with small molecules or synthetic peptide inhibitors. Indeed, we further demonstrated that a dominant-negative peptide, ERAP, which specifically disrupts the BIG3-PHB2 interaction, leads to the inhibition of multiple ERα-signalling pathways driving the growth of breast cancer by reactivating PHB2 tumour suppressive activity. However, because this peptide is difficult to use in clinical practice due to its limited stability, it is necessary to identify alternative long-term stable antagonistic compounds targeting the BIG3-PHB2 interaction.
**Results**

Xanthohumol inhibits the BIG3-PHB2 interaction and mediates the repression of multiple E2-induced activation events. Previous reports show that XN inhibits the proliferation of ERα-positive breast cancer cells and specifically binds to the prohibitin proteins PHB1 and PHB2. Moreover, we recently reported that BIG3-PHB2 complex formation plays a crucial role in ERα-positive breast cancer cell growth. Considering these findings, we attempted to investigate the possibility of XN as an inhibitor targeting the BIG3-PHB2 interaction. Co-immunoprecipitation experiments revealed that XN dose-dependently inhibited the complex formation of endogenous BIG3 and PHB2 in the ERα-positive breast cancer cell lines MCF-7 and KPL-3C, which highly express both BIG3 and PHB2 (Fig. 1a and Supplementary Fig. S1a). Similar results were obtained with ERAP treatment, a dominant-negative peptide inhibiting the BIG3-PHB2 interaction by its direct binding to PHB2 that we previously developed (Fig. 1a and Supplementary Fig. S1a). We also demonstrated that XN directly bound to recombinant PHB2 protein in vitro (Supplementary Fig. S1b). In addition, we found that XN inhibited the in vitro PHB2-ERAP interaction in a dose-dependent manner (Supplementary Fig. S1c), suggesting the possibility that XN caused the specific inhibition of BIG3-PHB2 complex formation by its direct binding to PHB2.

We next investigated the subcellular distribution of endogenous PHB2 in ERα-positive breast cancer cells following XN treatment. The results showed that in the presence of E2, treatment with XN led to a decrease in cytoplasmic PHB2, thereby substantially increasing the amount of nuclear PHB2 (Fig. 1b, WCL). Furthermore, co-immunoprecipitation experiments with an anti-ERα antibody indicated that the PHB2 released from BIG3 by XN treatment interacts with cytoplasmic and nuclear ERα in a dose-dependent manner (Fig. 1b, IP: ERα).

Next, we sought to elucidate the effect of XN on nuclear ERα function as a transcriptional factor. After validating E2-dependent PHB2 nuclear translocation by XN treatment using immunocytochemical approaches with MCF-7 and KPL-3C cells (Fig. 1c and Supplementary Fig. S1d), we analysed ERα transcriptional activity by luciferase reporter assays with oestrogen-responsive element (ERE) reporters. XN significantly inhibited E2-induced ERα transcriptional activity in a dose-dependent manner in MCF-7 and KPL-3C cells (minimum effective dose, 5 μM; Fig. 1d and Supplementary Fig. S1e).

We previously demonstrated that inhibiting the BIG3-PHB2 interaction interfered with E2-induced, non-genomic ERα activation pathways. We next examined the effects of XN on the phosphorylation status of Akt and MAPK, which are the downstream signalling molecules of the non-genomic pathways. As expected, Akt (S473) and p42/44 MAPK (T202/Y204) phosphorylation levels were increased after 24 h of E2 stimulation in MCF-7 cells, whereas 30 μM XN treatment clearly abrogated the E2-induced phosphorylation levels of both proteins as well as that induced by ERAP treatment (Fig. 1e). In addition, 30 μM XN treatment remarkably reduced the phosphorylation levels at all five sites (S104/S106, S118, S167, S305 and Y537) within ERα, which are involved in ERα transcriptional activity, DNA-binding, co-activator binding, protein stability and cell proliferation in ERα-positive breast cancer cells.

To examine the effect of XN on breast cancer cell proliferation, we measured the E2-dependent cell growth of ERα-positive breast cancer cell lines, MCF-7 or KPL-3C, with an MTT assay at 24 h after treatment with XN. The results showed that XN treatment significantly reduced the E2-stimulated cell growth in a dose-dependent manner (IC_{50} = 7.3 μM and 8.2 μM in the MCF-7 and KPL-3C cells, respectively; Fig. 2a). By contrast, 50 μM XN remarkably inhibited the cell proliferation of both MCF-7 and KPL-3C cells, indicating the inhibition of E2-dependent cell growth (Fig. 2a). In fact, similar results were obtained with MCF-7 cell growth in the absence of E2 (Supplementary Fig. S2a), suggesting that 50 μM XN inhibits cell growth by independent mechanisms targeting the BIG3-PHB2 interaction. By contrast, compared with MCF-7 or KPL-3C cells, 30 μM XN had little effect on the growth of normal mammary epithelial MCF-10A cells that do not express ERα (Fig. 2b and Supplementary Fig. S2b). Accordingly, it must be noted that 50 μM XN treatment induced a non-specific inhibitory effect on the growth of MCF-10A cells. Furthermore, we also demonstrated that 30 μM XN treatment had no inhibitory effects on E2-induced growth of siRNA-mediated PHB2-depleted cells (Supplementary Fig. S2c), indicating that XN suppresses E2-induced cell growth through its specific inhibition of BIG3-PHB2 interaction. These results indicate that 30 μM XN specifically and completely inhibited multiple aspects of the E2/ERα-signalling network via the tumour-suppressive ability of endogenous PHB2 released from BIG3, resulting in complete suppression of E2-dependent breast cancer cell growth.

We next investigated the duration of the inhibitory effect of XN by an MTT assay. In contrast with ERAP, the inhibitory effect of XN on the growth of the MCF-7 and KPL-3C breast cancer cells was maintained for 96 h after XN treatment without affecting cell morphology (Fig. 2c and Supplementary Figs. S2d–g). In addition, XN also significantly suppressed E2-induced expression of the ERα-target genes TFF1 and CCND1 for 96 h after XN treatment (Fig. 2d). Taken together, our findings strongly suggest that the inhibitory effect of XN on the responsiveness of ERα-positive breast cancer cells has much higher stability than that of ERAP.

Next, we examined the effects of XN on the cell cycle distribution of MCF-7 cells by flow cytometry and microscopy analyses. The population of cells in the G2/M phase increased after a 24 h E2 stimulation, whereas the population in the G1 phase, but not sub-G1 population, increased after 30 μM XN treatment, indicating that XN suppressed cell growth by inducing a G1 arrest and caused no cell phenotypic alterations, similar to ERAP (Fig. 3a,b; ref. 9). By con-s
contrast, high dose of XN treatment causes cell phenotypic alterations regardless of E2 stimulation (Supplementary Fig. S3). Similarly, XN has been reported to induce apoptosis in several cancer cell lines, including leukemia, prostate cancer, and hepatoma cell lines. We previously reported that XN acts as an autophagy modulator by directly binding to the N-domain of valosin-containing protein (VCP), which is essential for autophagosome-lysosome fusion and the formation of autolysosomes in cancer cells. Therefore, we wished to clarify the effect of XN on VCP function in ERα-positive breast cancer cells. We first examined VCP expression in various cell lines, and found high expression of VCP in breast cancer cell lines as well as in colon and lung cancer cell lines and a mammary epithelial cell line (Fig. 3c). Next, because XN was also reported to induce endoplasmic reticulum (ER) stress via inducing the loss of VCP-mediated ER-associated degradation (ERAD) activity, we examined the effect of XN on ERα transcriptional activity were evaluated using luciferase assays. The data represent the mean ± SE of three independent experiments (** P < 0.01, *** P < 0.001 in two-sided Student's t-test). (e) Immunoblot analyses were performed to evaluate the inhibitory effects of XN on E2-induced Akt (Ser473), MAPK (Thr202/Tyr204), and ERα (Ser104/Ser106, Ser118, Ser167, Ser305 and Tyr537) activities in MCF-7 cells. The blots were cropped, and the full-length blots were included in the supplementary information.
Although 50 μM XN treatment might cause apoptosis through its inhibition of VCP function (Fig. 3e). Taken together, our data clearly demonstrate that XN suppresses E2-induced cell growth via specific disruption of the BIG3-PHB2 interaction regardless of VCP function.

**XN inhibits ERα-positive breast cancer tumour growth in a xenograft mouse model.** To determine the antitumour activity of XN in vivo, KPL-3C orthotopic breast cancer xenografts were developed in nude mice. Once the tumours were fully established, various concentrations of XN (0.3 and 1.0 mg kg⁻¹), ERAP (14 mg kg⁻¹), or vehicle alone were administered daily by intraperitoneal injection for 36 days. The animals also received daily treatments of E2 (6 mg per day). Daily E2 treatment induced the time-dependent growth of KPL-3C tumours, whereas XN treatment caused a significant inhibition of E2-induced tumour growth comparable with that observed after ERAP treatment (Fig. 4a and Supplementary Fig. S4a). No toxicity or significant body weight changes were observed throughout these experiments (Supplementary Fig. S4b). To clarify the mechanisms of the in vivo antitumour effect of XN, we first examined its effect on the BIG3-PHB2 interaction. Co-immunoprecipitation experiments in each tumour revealed that 1.0 mg kg⁻¹ and 0.3 mg kg⁻¹ XN treatment effectively inhibited the formation of the endogenous BIG3-PHB2 complex, resulting in nuclear translocation of PHB2 in tumours (Fig. 4b). Substantially increased nuclear PHB2 staining was observed in tumours treated with XN in a dose-dependent manner by immunohistochemistry (Fig. 4c). We next examined the effects of XN on the activation of the non-genomic ERα signalling pathway. As expected, considerable suppression of Akt and MAPK phosphorylation was observed in tumours treated with XN (Fig. 4d), demonstrating that XN had potent in vivo antitumour activity. Furthermore, to evaluate the effect of XN treatment on the cell cycle in tumours, we investigated the expression of the proliferative markers, Ki67 and PCNA by immunohistochemistry. Ki67 was known to be expressed in all phases of the active cell cycle (G1, S, G2 and M phase), and PCNA in most abundant during the S phase. As shown in Fig. 4e, Ki67 was clearly detected in every tumour, whereas PCNA was not detected in tumours treated with 1.0 mg kg⁻¹ XN or 14 mg kg⁻¹ ERAP, indicating that XN treatment suppresses tumour growth by inducing G1-arrest. Collectively, these results suggest that XN acts as an effective therapeutic agent against ERα-positive breast cancer by inducing G1-arrest via targeting the BIG3-PHB2 interaction.

**Discussion**

Previous studies have shown that the BIG3-PHB2 complex plays a critical role in promoting breast cancer cell growth, and strategies capable of inhibiting this interaction may represent novel therapies.
Figure 3 | Xanthohumol suppresses E2-dependent cell growth independently of apoptosis induction and VCP function. (a) Flow cytometric analyses were performed to evaluate the effect of XN treatment on the cell cycle. MCF-7 cells were treated for 24 h with E2 and/or XN or ERAP. (b) Representative cell morphologies of MCF-7 or MCF-10A cell following XN treatment are shown. (c) Expression patterns of VCP, BIG3, and PHB2 in breast cancer and normal epithelial cell lines. Colorectal cancer cell lines (HCT116 and SW480) and an epidermoid carcinoma cell line (A431) were used as positive controls for VCP expression. β-Actin served as a quantitative internal control. The blots were cropped, and the full-length blots were included in the supplementary information. (d) The CHOP and GRP78 expression levels following XN treatment were evaluated using real-time PCR. The data represent the mean ± SE of three independent experiments (* P < 0.05, ** P < 0.01, *** P < 0.001 in two-sided Student’s t-test). (e) An MTT assay was performed to evaluate the inhibitory effect of XN on the E2-dependent growth of VCP-depleted cells. The data represent the mean ± SE of three independent experiments (** P < 0.01, *** P < 0.001 in two-sided Student’s t-test).
Figure 4 | Xanthohumol has in vivo anti-tumour efficacy in xenograft models of human ERα-positive breast cancer. (a) XN inhibits tumour growth in a human breast cancer KPL-3C xenograft mouse model. The tumour size represents the mean ± SE of each group (n = 5). (* P < 0.05, ** P < 0.01 in two-sided Student’s t-test). (b) The inhibitory effects of XN treatment on the BIG3–PHB2 interactions were evaluated in tumours. The blots were cropped, and the full-length blots were included in the supplementary information. (c) Statistical analyses of the intensity (upper) and representative immunohistochemical staining (lower) of nuclear PHB2 protein in tumours. The data represent the mean ± SE of five different points (* P < 0.05, ** P < 0.01 in two-sided Student’s t-test). (d) Immunoblot analyses were performed to evaluate the effects of XN on the phosphorylation levels of Akt and MAPK proteins in tumours. The blots were cropped, and the full-length blots were included in the supplementary information. (e) Representative immunohistochemical staining of nuclear Ki67 (upper) and PCNA (lower) in tumours (left) and statistical analyses of the nuclear PCNA intensity (right).
for breast cancer. In this study, we demonstrated that a natural anti-
compound, XN, which binds to endogenous PHB2, pre-
vented the BIG3-PHB2 interaction, thereby releasing PHB2 to
inhibit both nuclear- and membrane-associated ERz activation.
These data are in accordance with our previous observations that a
dominant-negative peptide, ERAP targeting the BIG3-PHB2 inter-
action suppressed the growth of ERz-positive breast cancer cells2.
Collectively, therapeutic strategies utilising the reactivation of PHB2
upon its PPI inhibitor-mediated release from BIG3 appeared to effec-
tively treat luminal-type breast cancer cells, especially those with
docrine resistance. However, because the inhibitory effect of
ERAP is maintained for only 24 h3, it is difficult to use in clinical
practice. Therefore, we tested the inhibitory effect of XN, which
directly binds to the PHB2 protein, as a selective antagonistic com-
 pound targeting the BIG3-PHB2 interaction and found that the
inhibitory effect of XN demonstrated long-term stability was main-
tained for up to 96 h (Figs. 2c and d). Moreover, no toxicity or
significant body weight loss by daily intraperitoneal injection with
effective doses for 36 days were observed. In addition, previous stud-
ies have shown that long-term treatment with XN causes no adverse
effects on female mouse reproduction5–7. From a therapeutic perspec-
tive, XN targeting of the BIG3-PHB2 interaction provides excellent
therapeutic indices with minimal off-target effects, thereby leading to
better quality-of-life for patients with breast cancer.

Another interesting finding of this study is that 30 μM XN spec-
ifically inhibited multiple E2-induced cell growth pathways in breast
cancer by targeting of the BIG3-PHB2 interaction despite the fact that
XN is known to exert a broad spectrum of antitumour actions, such as
apoptosis induction6,29–31, and NF-κB inhibition6,29,31. In particular, we
previously reported that XN bound directly to the N domain of VCP,
thereby inducing endoplasmic reticulum (ER) stress via the loss of
VCP-mediated ER-associated degradation (ERAD) activity2. Here, we
here examined the effect of XN on ER stress induction and demon-
strated that 50 μM XN, but not 30 μM XN, caused remarkable up-
regulation of the ER stress-response genes CHOP and GPR78 in breast
cancer cells regardless of E2 stimulation (Fig. 3d). These findings sug-
gest that the antitumour effect of 30 μM XN is dependent on
the inhibition of the BIG3-PHB2 interaction, but not on the loss of
VCP function. However, high-dose (50 μM) XN treatment might
cause loss of VCP function, thereby leading to the accumulation of
unfolded proteins and resulting in ER-stress in cancer cells36–37.
Furthermore, we observed that 30 μM XN resulted in complete sup-
pression of the E2-induced cell growth in VCP-depleted cells (Fig. 3e).
Taken together, XN (30 μM) treatment appears to be a natural anti-
tumour compound targeting BIG3-PHB2 complex formation in
breast cancer cells, although it will be necessary to clarify the effect
of XN treatment on other binding partners involved in NF-κB signal-
ling and apoptosis-related proteins.

Furthermore, we recently demonstrated that the combination of
tamoxifen and ERAP induced more potent anti-tumour activity in vivo
and in vitro compared with either treatment alone4. Thus, a combina-
tion of the current endocrine therapies and BIG3-PHB2 interaction
inhibitors, such as XN or ERAP, may lead to more effective combined
effects on the intrinsic and acquired endocrine resistance of breast
cancer through the different modes of actions of these drugs. In con-
clusion, our findings show that the selective BIG3-PHB2 interaction
inhibitor XN could completely suppress E2-dependent ERz activation
and had growth-suppressive effects on breast cancer cells both in vitro
and in vivo. These findings suggest that this BIG3-PHB2 interaction
inhibitor may have great potential in the treatment of luminal type
breast cancers, especially those with endocrine resistance.

Methods

Ethical statement. All experiments in this study were conducted according
to protocols reviewed and approved by the Committee for Safe Handling of Living
Modified Organisms (Permission number 26–40) and the Institutional Animal Care
and Use Committee (Permission number 13155) in the University of Tokushima.

Cell lines and culture conditions. Human breast cancer cell lines (MCF-7, ZR-75-1,
BT474, SK-BR-3, MDA-MB-453, MDA-MB-231), a mammary epithelial cell line
(MCF-10A), colorectal cancer cell line (HCT116, SW480), and epidermoid
carcinoma cell line (A431) were purchased from American Type Culture Collection
(ATCC, Manassas, VA, USA). The KPL-3C cells were established, characterized, and
kindly provided by Dr. Jun-ichi Kurebayashi (Kawasaki Medical School)43. All of the
cells were cultured under the respective depositor’s recommendations. The
MCF-
7 cells were seeded in 48-well plates (2 × 103 cells mL−1), 24-well plates (1 × 104 cells
mL−1), 6-well plates (3 × 105 cells mL−1), or 10-cm dishes (2 × 105 cells 10 mL−1) in
MEM (Life Technologies, Rockville, MD, USA) supplemented with 10% FBS
(Nichirei Biosciences, Tokyo, Japan), 1% antibiotic/antimycotic solution (Life
Technologies), 1 mM sodium pyruvate, and 10 μg mL−1 insulin (Sigma, St. Louis, MO, USA).
These cells were maintained at 37°C with 5% CO2. The next day, the medium was changed to phenol
red-free DMEM/F12 (Life Technologies), supplemented with FBS, antibiotic/
antimycotic solution, NEAA, sodium pyruvate, and insulin. After 24 h, the cells were treated
with 10 nM 17-estradiol (E2, Sigma) ± xanthohumol or peptides (e.g., ERAP).

Antibodies and immunoblot analyses. The anti-BIG3 monoclonal antibody
was generated by Sigma-Aldrich (Tokyo, Japan). Briefly, a rat was immunised with
purified His-tagged human BIG3 protein (459–572 aa). The iliac lymph nodes were
collected and fused with myeloma cells, resulting in the formation of a hybridoma.
Immunoblot analyses were performed as described previously4. After SDS-PAGE, the
membranes blotted with proteins were blocked with 4% BlockAce solution
(Dainippon Pharmaceutical, Osaka, Japan) for 1 h and then incubated with
antibodies against the following proteins: BIG3 (1:1,000); PHB2 (1:1,000, Abcam,
Cambridge, UK); ERs (SP-1, 1:500; Thermo Fisher Scientific, Fremont, CA, USA;
Akt, phospho-Akt (S473) (S85771, 1:1,000); p44/p42 MAPK, phospho-p44/p42 MAPK
(T202/Y204) (1:1,000); VCP (valosin-containing protein) (1:500), and α/β-
tubulin (1:1,000) (Cell Signaling Technology, Danvers, MA, USA); β-actin (AC-15; 1:5,000)
and LaminB1 (1:100, Sigma) using standard procedures. All of the experiments were
performed in triplicate at a minimum.

Immunoprecipitation. Immunoprecipitation analysis was performed as described
previously4. The cell lysates were pre-cleared with normal IgG and rec-Protein G
Sepharose 4B (Life Technologies) at 4°C for 3 h. Then, the supernatants were
incubated with antibodies against BIG3 (3 μg) and ERs (5 μg) at 4°C for 12 h. Next, the
 antibodies were precipitated with rec-Protein G Sepharose 4B at 4°C for 1 h. The
immunoprecipitated protein complexes were washed three times with lysis buffer.
Subsequent SDS-PAGE and immunoblot analyses were performed as described above.

Direct binding of XN with recombinant PHB2 protein. Direct binding of XN to
recombinant PHB2 protein was evaluated by XN-beads pull-down assay. The 3.6 μg
of 6×His-tagged recombinant PHB2 (His-PHB2) were incubated with 3 μL control-
beads or XN-beads together with 1 mg mL−1 BSA for 4 h. Then, the bound fractions
were immunoblotted with anti-6×His antibody (Takara, Shiga, Japan).

Ni-resin agarose pull-down assay. Binding inhibition of ERAP-ERz interaction
by XN was assayed by Ni-resin agarose pull-down. The binding assay was performed
by mixing 0.27 nmole 6×His-tagged recombinant PHB2, 0.27 nmole HA-ERAP, and
0.5 to 3 times moles of XN for 1 h at 4°C, followed by capturing Ni-NTA agarose
(Qiagen, Hilden, Germany) for 2 h at 4°C. The bound fractions were washed with
5 mM imidazole five times and further eluted by SDS-sample buffer for immunoblot
analyses.

Inhibition of the BIG3-PHB2 interaction by xanthohumol. The XN was isolated and
purified as described previously4,7. XN was dissolved in dimethylsulphoxide
(DMSO) at a concentration of 100 nmol mL−1, and then suspended in phenol red-free
DMEM/F12 at the concentrations indicated. Treatment with 0.03% DMSO
equivalent to 30 μM XN had no effect on E2-induced cell growth, ERz-target gene
expression, Akt (S473), MAPK (T202/Y204), and Erz (S104/S106, S118, S167, S305
and T337) activities, and BIG3-PHB2 interactions (Supplementary Fig. S5a–d).
A dominant-negative peptide (ERAP; 11R-GGG-QMQLDLTLQQR), which was
designed to specifically inhibit the BIG3-PHB2 interaction, was synthesised as
described previously4 and used as a positive control. To examine the effects of XN on
the inhibition of endogenous BIG3-PHB2 complex formation, MCF-7 cells were
reated with 10 nM E2 ± XN. The BIG3-PHB2 interactions were assessed 24 h later
using co-immunoprecipitation followed by immunoblot analysis, as described above.

Nuclear/cytoplasmic fractionation. Nuclear and cytoplasmic fractions of MCF-7
cells were prepared using the NE-PER nuclear and cytoplasmic extraction reagent
(Thermo Fisher Scientific) as described previously36. α/β-Tubulin and laminin B were
used as loading controls for the cytoplasmic and nuclear fractions, respectively.

Immunocytochemical staining measurement of the nuclear translocation
of PHB2. MCF-7 cells were seeded at 5 × 103 cells per well in 8-well chambers
(Laboratory-Tek II Chamber Slide System, Nalgene, Nunc International) for 48 h
and then treated with E2 ± XN or ERAP for 24 h. The staining procedures
were conducted as described previously.

www.nature.com/scientificreports
Luciferase reporter assay. Transfections of ERE-luciferase reporter into MCF-7 or KPL-3C cells were performed by ERE reporter assay kit (Qigene). The enclosed Renilla-luciferase reporter was monitored as an internal control. Briefly, 16 h post-transfection, the culture medium was changed to assay medium (Opti-MEM, 10% FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, and 10 μg/mL insulin). After 8 h, the cells were exposed to E2 ± XN for 24 h. Then, the cells were harvested and analysed for luciferase and Renilla-luciferase activities using the Promega dual luciferase reporter assay (Promega KK, Tokyo, Japan) as described previously.

Cell proliferation assay. The cell proliferation assays were performed using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) as described previously. The data represent the mean ± SE of three independent experiments.

Real-time PCR. The expression of the ERα target genes (TFF1 and CCND1) and endoplasmic reticulum-responsive genes (CHOP and GRP78) was evaluated by real-time RT-PCR as described previously. Each sample was normalised to the β2-MG mRNA content, and the results were expressed as the fold change over untreated cells at 0 h (set at 1.0). The data represent the mean ± SE of three independent experiments. The primers were as follows: TFF1 5′-GCGCTTCTTAGGCAAAAT-3′, 5′-CTCCCTCTGTGCAAAAGG-3′, CDND1 5′-CAGAAGTGTGGTGGAAGG-3′ and 5′-CAGTGCAGGACGCAGAAGA-3′, GRP78 5′-CCCAAAGTTTGAGAGG-3′ and 5′-CACAATCTGGCGAGCAG-3′, and β2-MG 5′-AACCTAGGAGGTGGCAGAAG-3′ and 5′-CAACACCATGGCTTCATTTACT-3′.

Cell cycle assay. The cell cycle assay was performed as described previously. The cells were fixed in ice cold 70% ethanol, incubated with 20 μg/mL propidium iodide (Sigma) and 1 mg/mL RNase A (Sigma), and analysed by flow cytometry using a FACScalibur with CellQuest software (Becton-Dickinson, San Jose, California, USA).

In vivo tumour growth inhibition. KPL-3C cell suspensions (1 × 10^5 cells per mouse) were mixed with an equal volume of Matrigel (BD) and injected (200 μL total) into the mammary fat pads of 6-week-old female BALB/c nude mice (Charles River Laboratories, Tokyo, Japan). The mice were housed in a pathogen-free isolation facility with a 12-h light/dark cycle and were fed rodent chow and ad libitum.

Immunohistochemical staining of xenografts. To examine the PHB2, Ki67 and PCNA protein expression in KPL-3C xenograft tumours, we stained 3-μm-thick sections of formalin-fixed and paraffin-embedded tumour tissue with antibodies, as previously described.

Statistical analyses. Student’s t-test was used to determine the significance of the differences among the experimental groups. Values of P < 0.05 were considered significant.
39. Shan, H., Takahashi, T., Bando, Y., Izumi, K. & Uehara, H. Inhibitory effect of soluble platelet-derived growth factor receptor \(\beta\) on intraosseous growth of breast cancer cells in nude mice. Cancer Sci. 102, 1904–1910 (2011).

Acknowledgments

The authors thank Dr. Jun-ichi Kurebayashi (Kawasaki Medical School) for the gifts of the KPL-3C breast cancer cell line. This work was supported by the Project Future of Relay For Life Japan, a grant/research support from Tokushima Breast Care Clinic, a Grant-in-Aid for Scientific Research on Innovative Areas (MEXT KAKENHI Grant Number 251347212), Grants-in-Aid for Scientific Research (B) (MEXT KAKENHI Grant Number 25293079) and (C) (MEXT KAKENHI Grant Number 26461948) and IMSUT Joint Research Project.

Author contributions

T.Y. performed all of the experiments and prepared the draft of the manuscript. M.K. performed the in vivo experiments. E.T., M.I. and H.O. performed isolation and purification of XN, and in vitro binding assay for the direct binding of XN to recombinant PHB2 protein. Y.M., J.H. and M.S. discussed the interpretation of antitumor effect of XN or ERAP on the ER\(\alpha\)-signalling pathway in breast cancer. T.K. was involved in the conception and design of all of the studies, the interpretation of data and preparing the draft and final version of the manuscript. All of the authors read and approved the final manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: Toyomasa Katagiri is a stock holder and an external board member of OncoTherapy Science, Inc. The other authors have declared that no conflicts of interest exist.

How to cite this article: Yoshimaru, T. et al. Xanthohumol suppresses oestrogen-signalling in breast cancer through the inhibition of BIG3-PHB2 interactions. Sci. Rep. 4, 7355; DOI:10.1038/srep07355 (2014).

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/4.0/