Supplementary information

Xanthohumol suppresses oestrogen-signalling in breast cancer through specific inhibition BIG3-PHB2 interaction.

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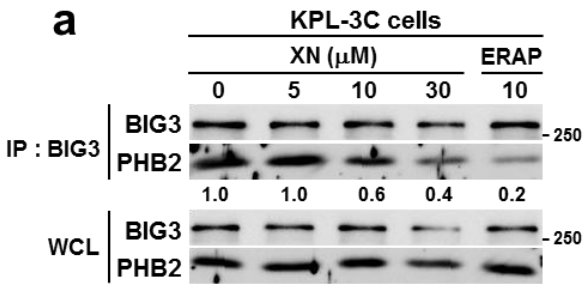
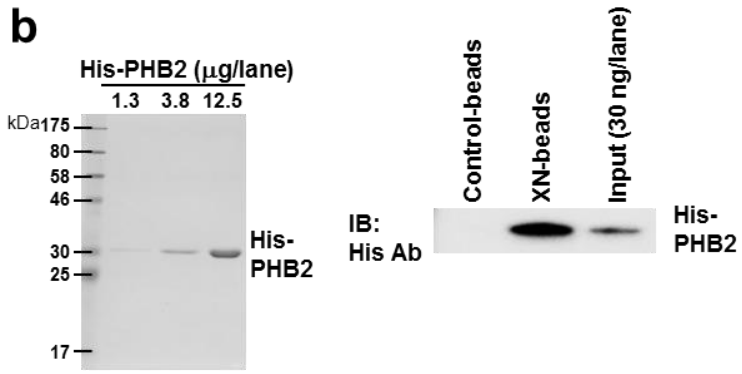
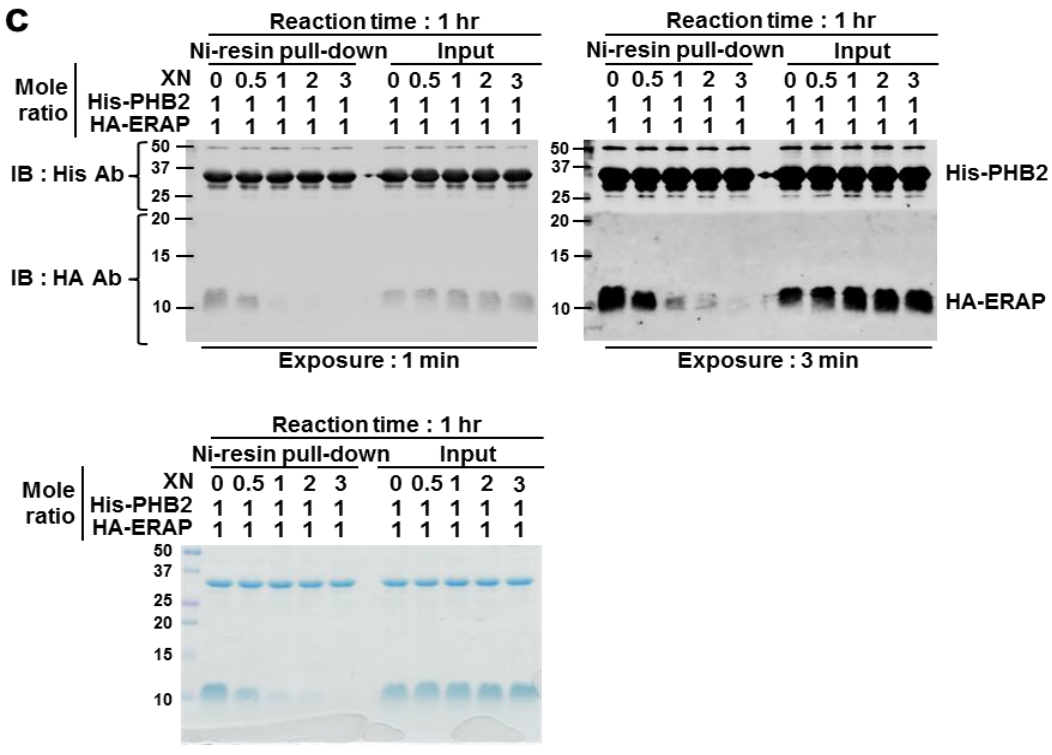
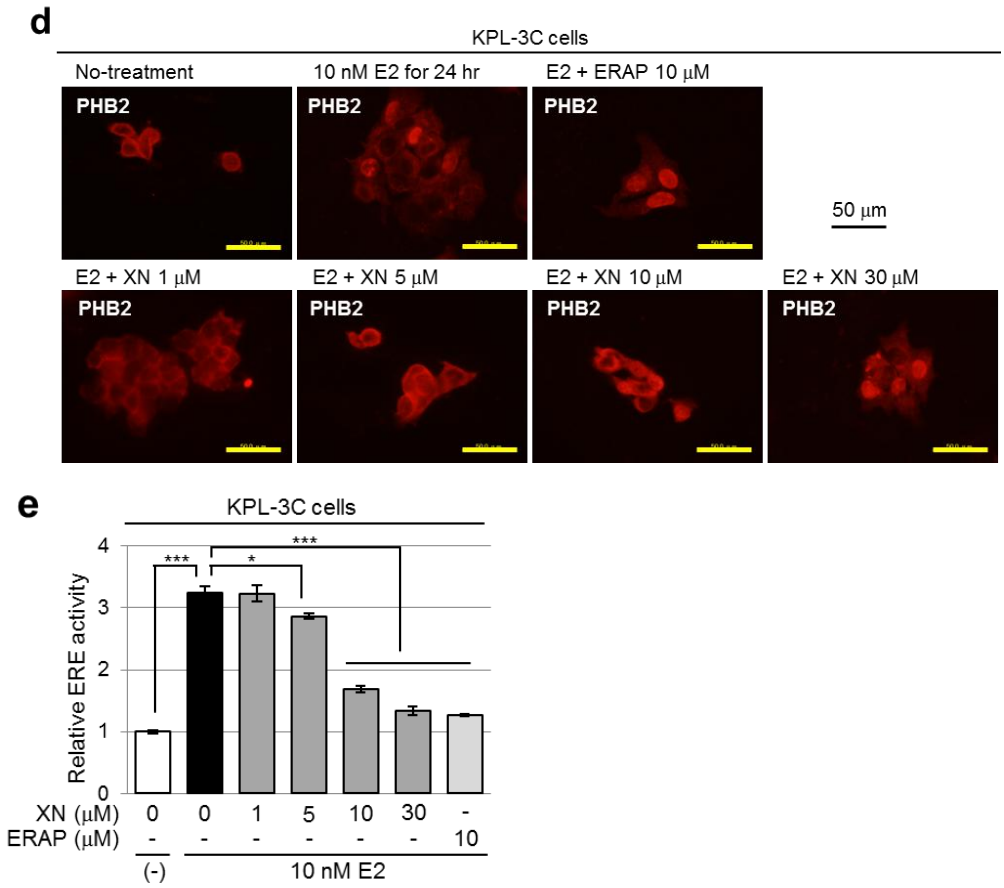
Supplementary Figures



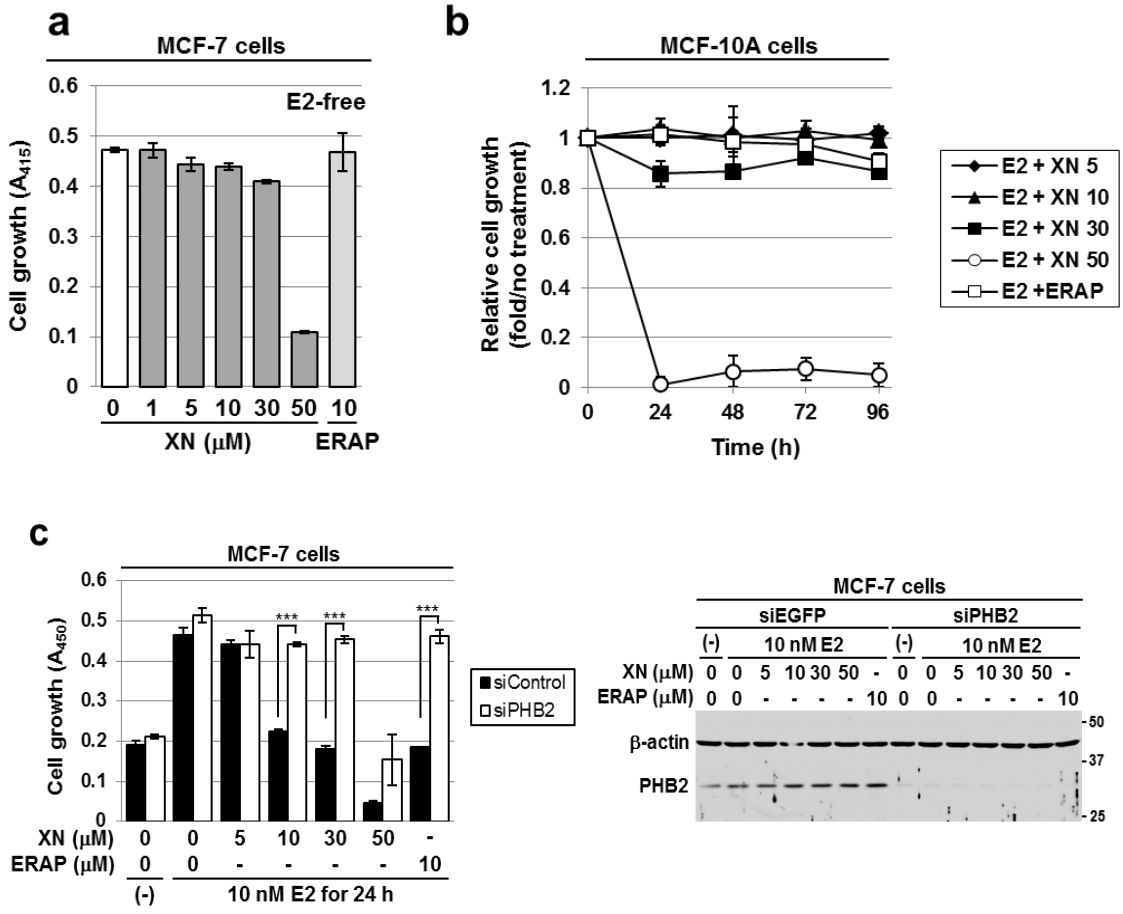


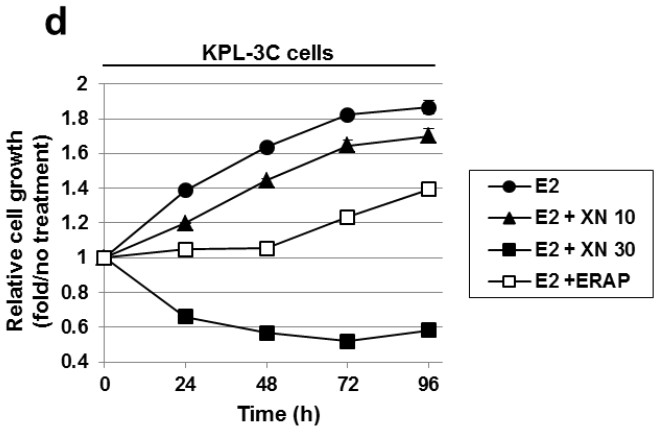


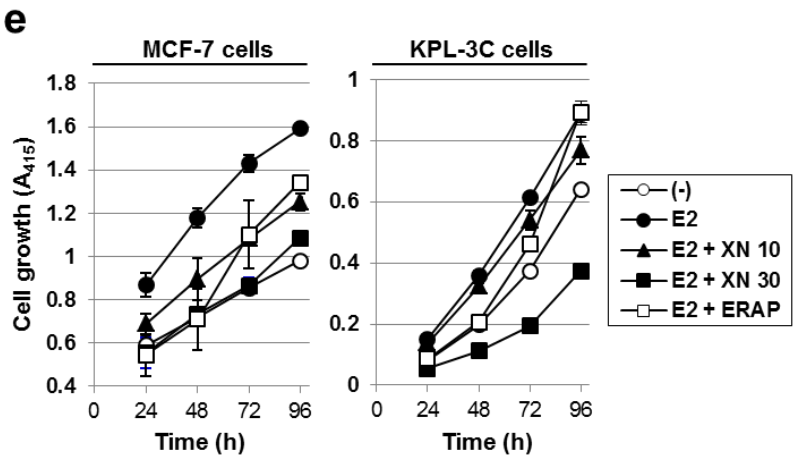


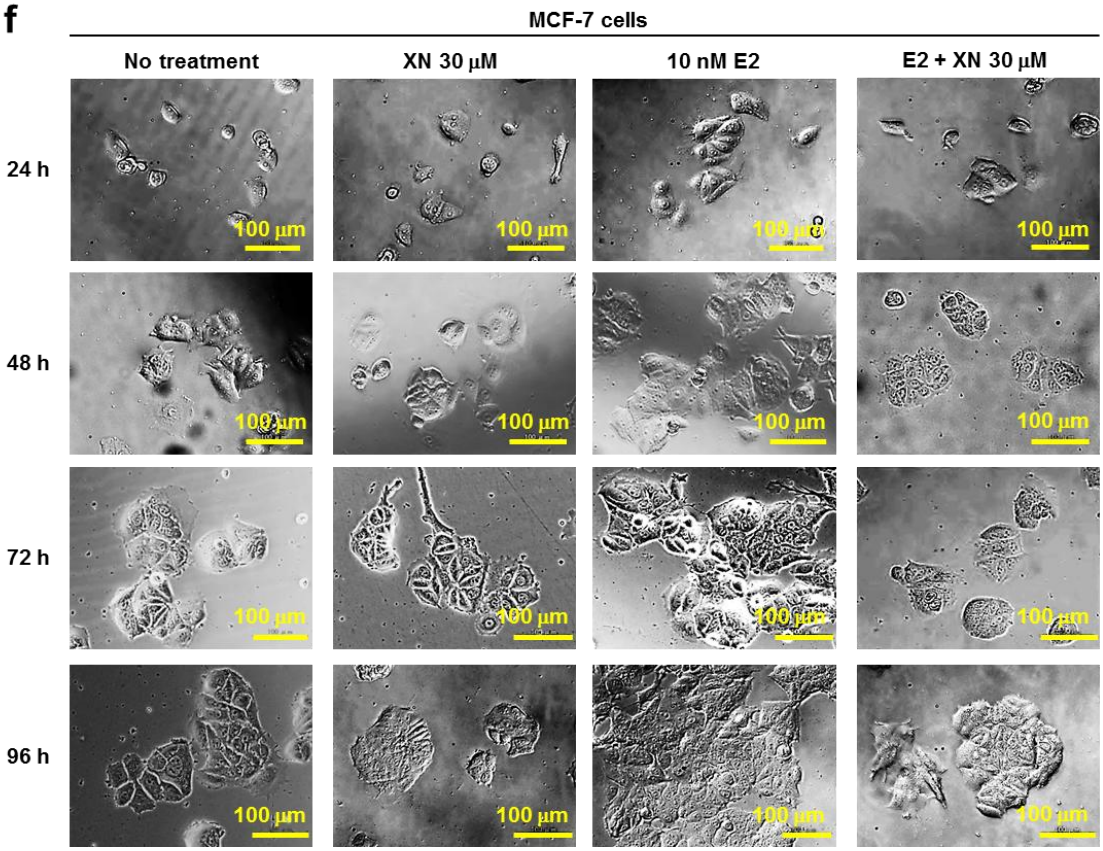
Supplementary Figure S1 | Xanthohumol inhibits BIG3-PHB2 interaction and promotes the nuclear translocation of PHB2 in KPL-3C cells. (a) The inhibitory effects of XN treatment on BIG3-PHB2 interactions were evaluated in KPL-3C. The blots were cropped, and the full-length blots were included in the supplementary information. (b) (left) The purification of recombinant PHB2. The indicated amount of 6 x His-tagged recombinant PHB2 (His-PHB2) were stained with CBB. (right) Direct binding of XN to PHB2 *in vitro*. The 3.6 μg of 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. The blots were cropped, and the full-length blots were included in the supplementary information. (c) Direct inhibition of PHB2-ERAP interaction by XN was evaluated. The mixture of 0.27 nmole 6 x His-tagged recombinant PHB2 (His-PHB2) and 0.27 nmole HA-ERAP were incubated with 0.5 to 3 times moles of XN for 1 h. His-PHB2 was then captured with Ni-NTA agarose, and the bound fractions were immunoblotted. (d) Representative immunofluorescence images of the subcellular localization of PHB2 are shown. (e) The inhibitory actions of XN on

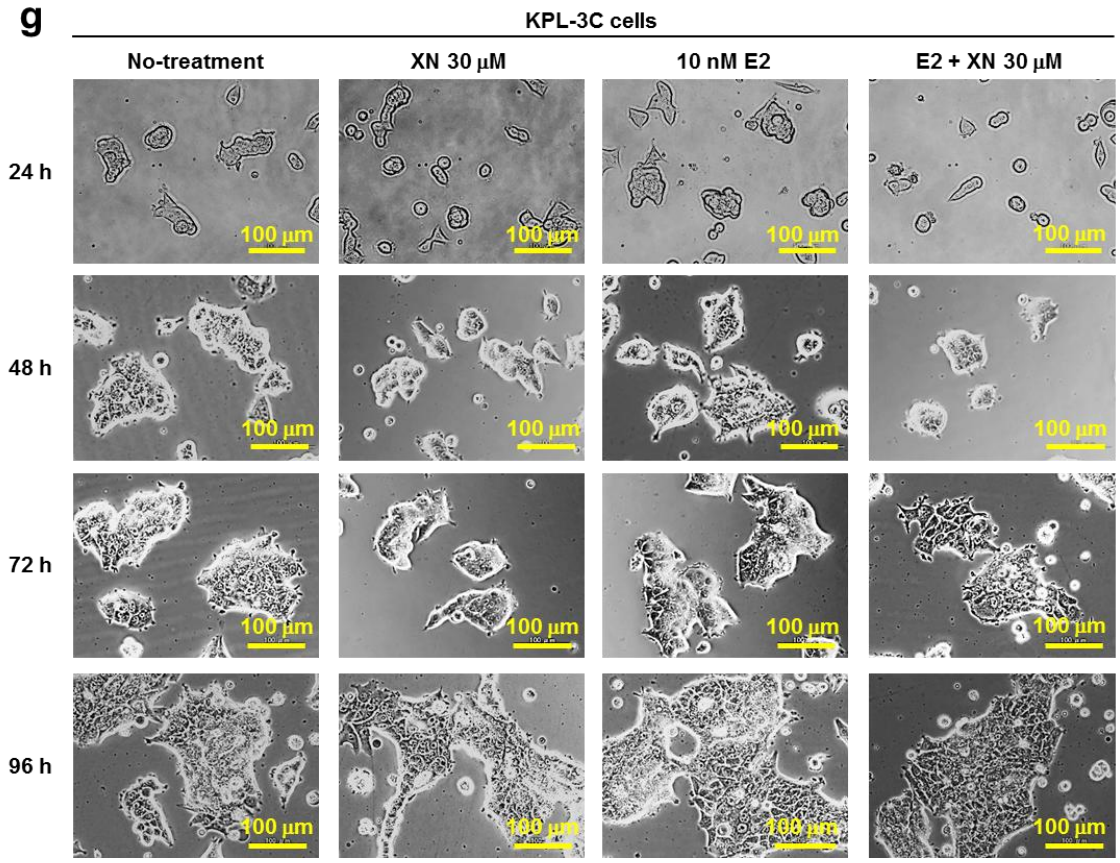
ERα transcriptional activity were evaluated using luciferase assays. The data represent the mean ± SE of three independent experiments (* P < 0.05, *** P < 0.001 in two-sided Student's *t*-test).



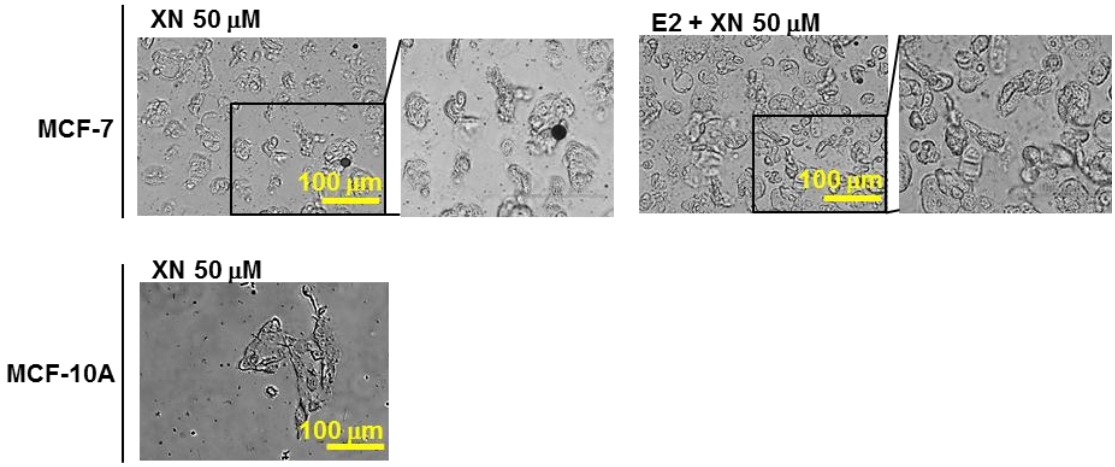

d
KPL-3C cells
Relative cell growth (fold/no treatment)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0
24
48
72
96
Time (h)
E2
E2 + XN 10
E2 + XN 30
E2 +ERAP


e
MCF-7 cells
KPL-3C cells
Cell growth (A_{415})
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
96
Time (h)
(-)
E2
E2 + XN 10
E2 + XN 30
E2 + ERAP

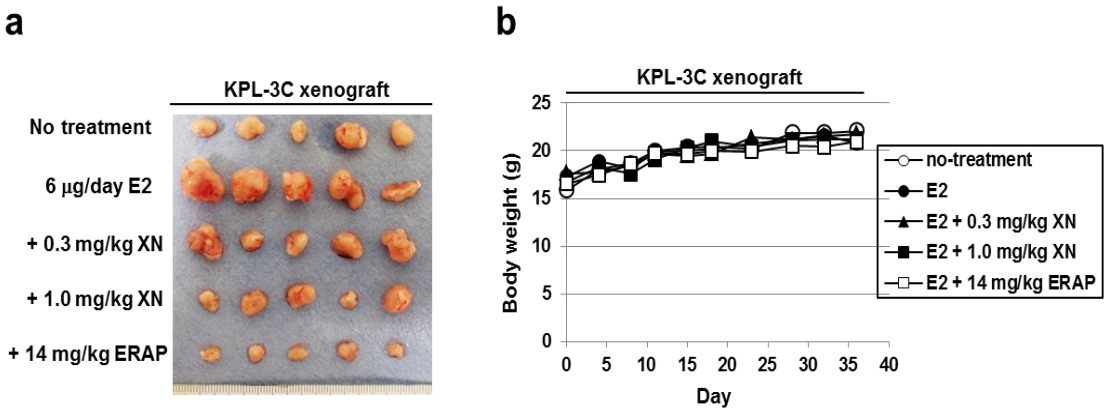

f
MCF-7 cells
No treatment
XN 30 μM
10 nM E2
E2 + XN 30 μM
24 h
48 h
72 h
96 h
100 μm



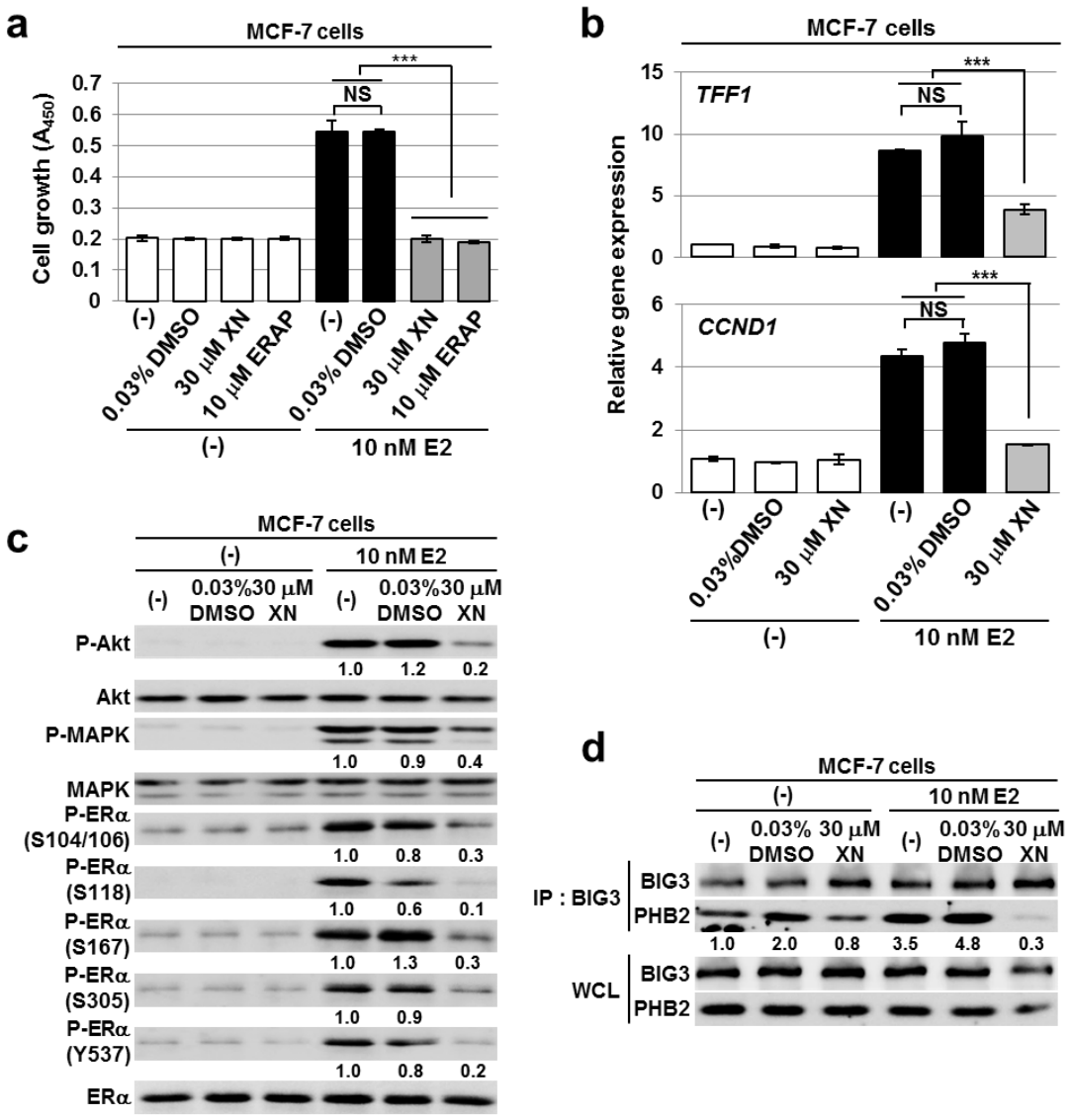
Supplementary Figure S2 | Xanthohumol suppresses E2-dependent cell growth for long-term stability. (a,b) MTT assay was performed to evaluate the inhibitory effect of XN on growth of MCF-7 cells under E2-free conditions for 24 h (a) and of the BIG3-negative mammary epithelial cell line MCF-10A for the times indicated (b). (c) (left) MTT assay was performed to evaluate the inhibitory effect of XN on the E2-dependent growth of PHB2-depleted cells. The data represent the mean ± SE of three independent experiments (*** $P < 0.001$ in two-sided Student's t-test). (right) Immunoblot analyses were performed to evaluate the PHB2 expression. (d,e) The duration of inhibitory effects of XN on growth of KPL-3C (d,e) and MCF-7 cells (d) were measured for the times indicated. The results were expressed at the fold increase over untreated cells at each time (set at 1.0, d), and measured at absorbance at 450 nm (e). The data represent the mean ± SE of three independent experiments. (f,g) Representative cell morphologies of MCF-7 (f) or KPL-3C (g) by XN treatment for the times indicated.



Supplementary Figure S3 | High dose of xanthohumol causes cell phenotypic alterations. Representative cell morphologies of MCF-7 or MCF-10A by 50 μM XN treatment are shown.



Supplementary Figure S4 | Xanthohumol has *in vivo* anti-tumor efficacy in xenograft models of human ERα-positive breast cancer. (a) KPL-3C xenograft tumors at day 36 after the indicated treatments. (b) The body weights of the KPL-3C xenograft mice are indicated after XN or ERAP treatment. The body weight represent the mean ± SD of each group (n=5).



Supplementary Figure S5 | Vehicle of Xanthohumol (0.03%DMSO) does not affect the multiple E2-induced activation events. The inhibitory effects of 0.03% DMSO on cell growth (a), ERα-target gene expression (b), E2-induced Akt (S473), MAPK (T202/Y204), and ERα (S104/S106, S118, S167, S305 and Y537) activities (c), and BIG3-PHB2 interactions (d) were measured in MCF-7 cells for 24 h. Gene expressions were expressed as the fold increase over untreated cells (set at 1.0). The data represent the mean ± SE of three independent experiments (** $P < 0.001$ in two-sided Student's *t*-test). ERAP was used as a positive control for the inhibition of the E2-dependent cell growth. The blots were cropped, and the full-length blots were included in the supplementary information.

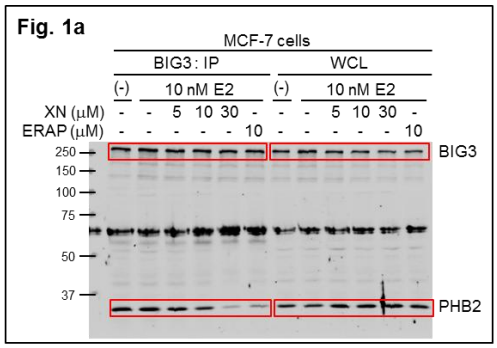


Fig. 1a
MCF-7 cells
BIG3 : IP
WCL
(-)
10 nM E2
XN (μM)
ERAP (μM)
BIG3
PHB2

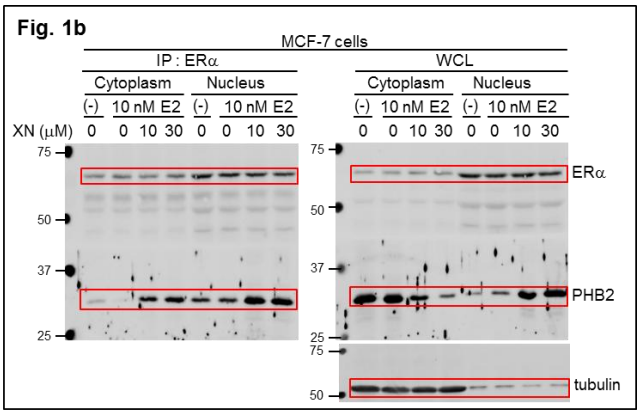


Fig. 1b
MCF-7 cells
IP : ERα
WCL
Cytoplasm
Nucleus
(-)
10 nM E2
XN (μM)
ERα
PHB2
tubulin

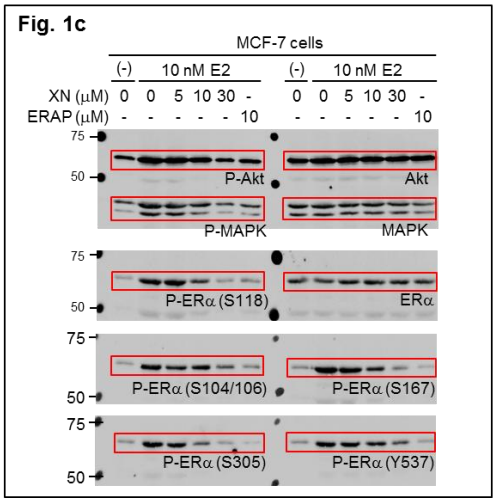


Fig. 1c
MCF-7 cells
(-)
10 nM E2
XN (μM)
ERAP (μM)
P-Akt
Akt
P-MAPK
MAPK
P-ERα (S118)
ERα
P-ERα (S104/106)
P-ERα (S167)
P-ERα (S305)
P-ERα (Y537)

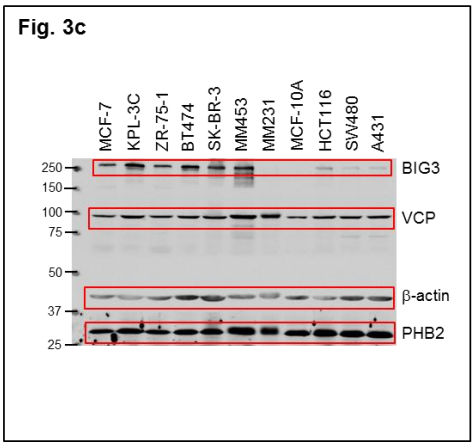


Fig. 3c
MCF-7
KPL-3C
ZR-75-1
BT474
SK-BR-3
MM453
MM231
MCF-10A
HCT116
SW480
A431
BIG3
VCP
β-actin
PHB2

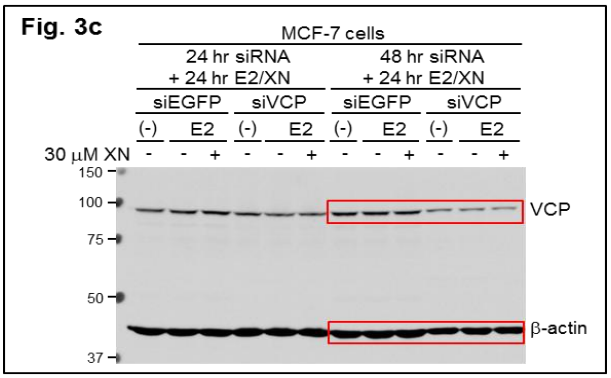


Fig. 3c
MCF-7 cells
24 hr siRNA
+ 24 hr E2/XN
48 hr siRNA
+ 24 hr E2/XN
siEGFP
siVCP
(-)
E2
30 μM XN
VCP
β-actin

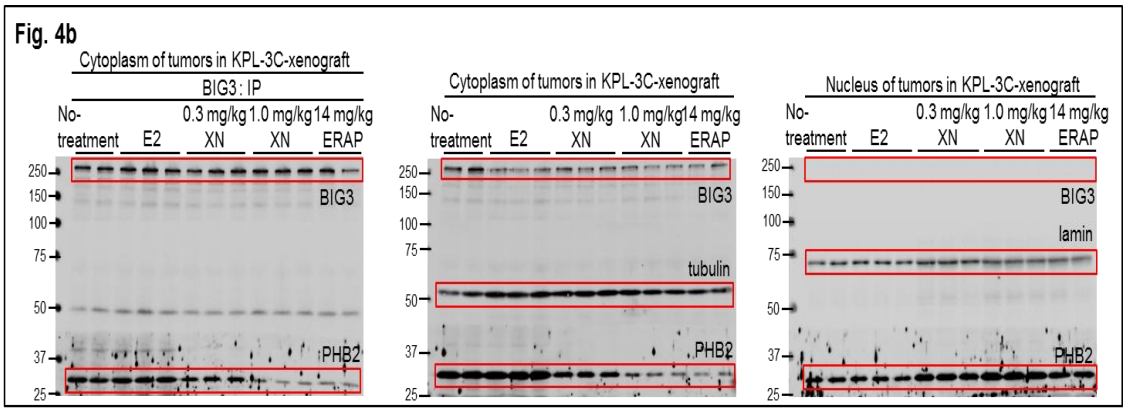
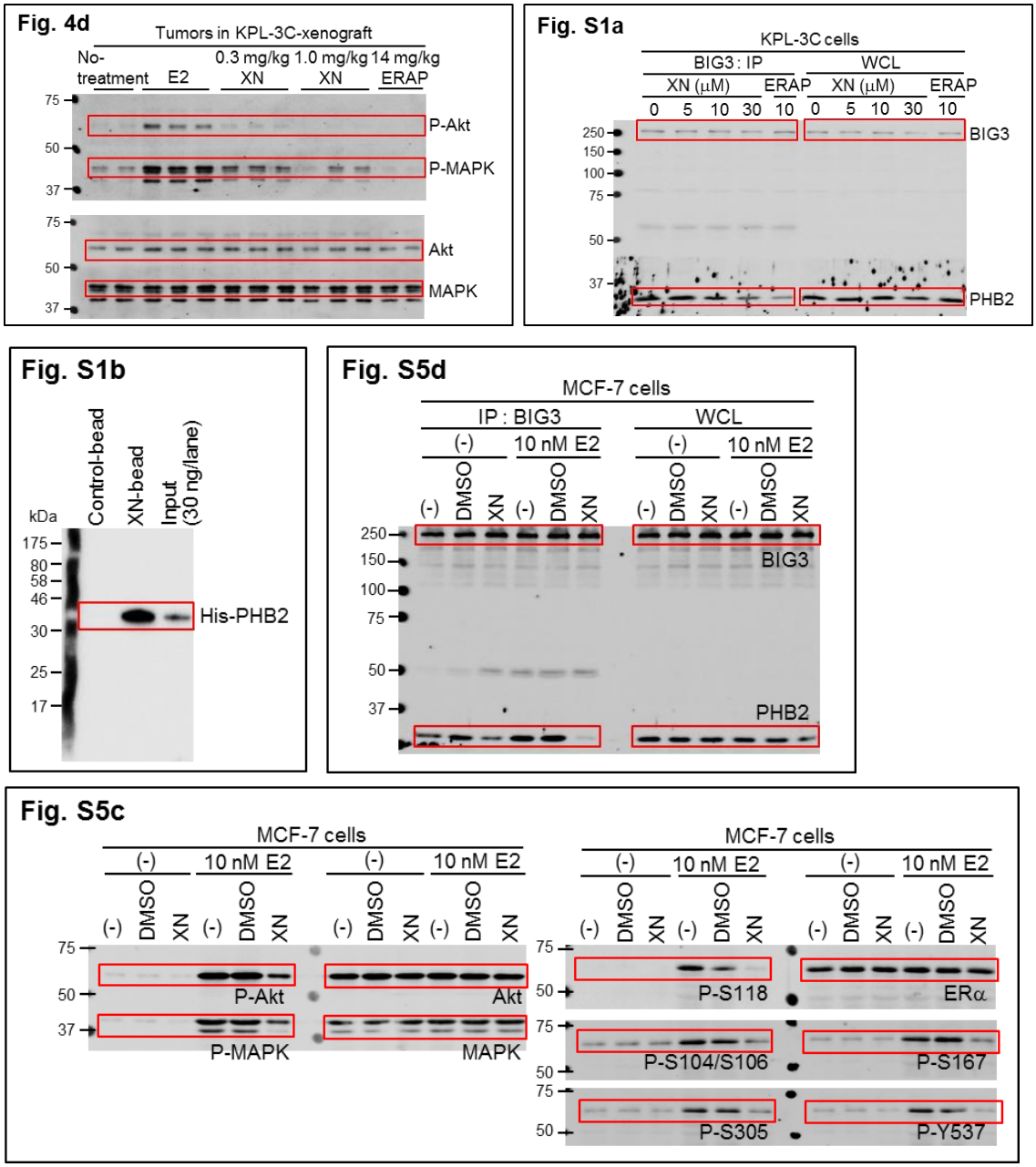


Fig. 4b
Cytoplasm of tumors in KPL-3C-xenograft
BIG3 : IP
No-treatment
E2
0.3 mg/kg XN
1.0 mg/kg XN
14 mg/kg ERAP
BIG3
PHB2
Cytoplasm of tumors in KPL-3C-xenograft
tubulin
Nucleus of tumors in KPL-3C-xenograft
lamin



Supplementary Figure S6 | Full-length of images of all limmunoblots in Figures and Supplementary Figures.