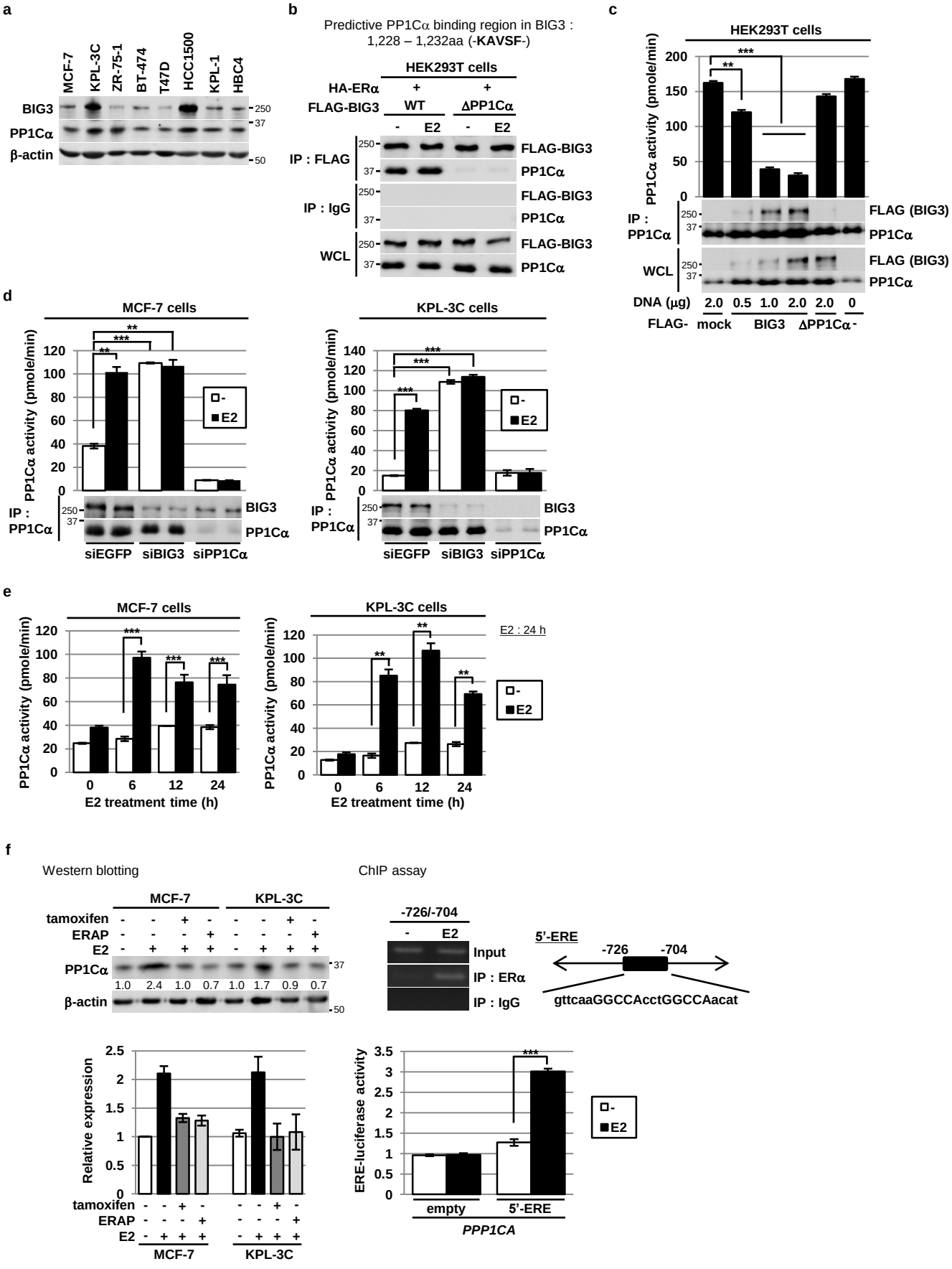
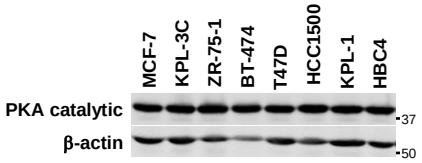

a
MCF-7
KPL-3C
ZR-75-1
BT-474
T47D
HCC1500
KPL-1
HBC4
BIG3
PP1Cα
β-actin
250
37
50
b
Predictive PP1Cα binding region in BIG3 :
1,228 – 1,232aa (-KAVSF-)
HEK293T cells
HA-ERα + +
FLAG-BIG3 WT ΔPP1Cα
- E2 - E2
IP : FLAG
IP : IgG
WCL
FLAG-BIG3
PP1Cα
c
HEK293T cells
PP1Cα activity (pmole/min)
IP : PP1Cα
WCL
FLAG (BIG3)
PP1Cα
DNA (μg) 2.0 0.5 1.0 2.0 2.0 0
FLAG- mock BIG3 ΔPP1Cα -
d
MCF-7 cells
KPL-3C cells
PP1Cα activity (pmole/min)
-
E2
IP : PP1Cα
BIG3
PP1Cα
siEGFP siBIG3 siPP1Cα
e
MCF-7 cells
KPL-3C cells
PP1Cα activity (pmole/min)
E2 treatment time (h)
E2 : 24 h
f
Western blotting
ChIP assay
MCF-7
KPL-3C
tamoxifen
ERAP
E2
PP1Cα
β-actin
1.0 2.4 1.0 0.7 1.0 1.7 0.9 0.7
-726/-704
Input
IP : ERα
IP : IgG
5'-ERE
-726
-704
gttcaaGGCCAcctGGCCAacat
Relative expression
ERE-luciferase activity
empty
5'-ERE
PPP1CA

g

Position	Sequence	PSSM Score
228 - 248	QLLYLECILSVLSSSSSSMHL	43.4
318 - 338	**VARTIYYIAAELVRLVGSVDS**	**24.2**
439 - 459	ELSQGKGLSEGQVQLLLLRLE	74.4
496 - 516	GNERSLDISISVTTDTGQTTL	26.2
1106 - 1126	SGSSAAKVVLTLSTQADRLFE	42.4
1225 - 1245	**VSQKAVSFIHDILTEVLTDWN**	**20.6**
1449 - 1469	GLIEVWIILLEQLTAAVSNCP	83.0

h



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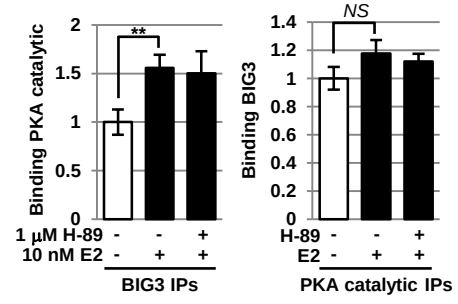

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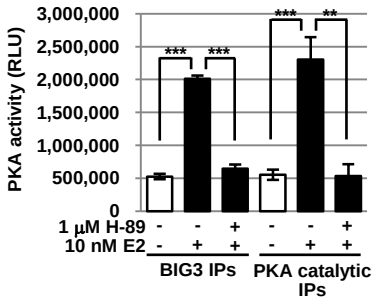
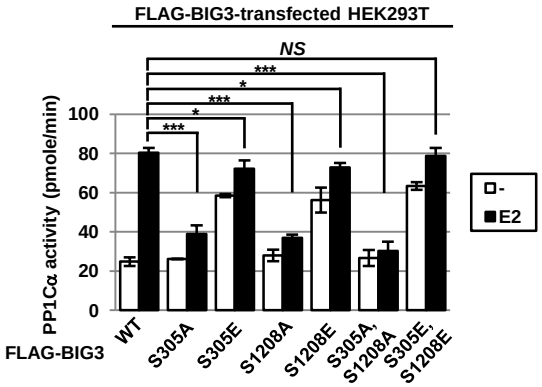


k

Target site	Score
T162	0.79
S305	0.79
S689	0.81
S925	0.83
S1208	0.78
S1763	0.80

(*Analysis by NetPhos 3.1*)

l



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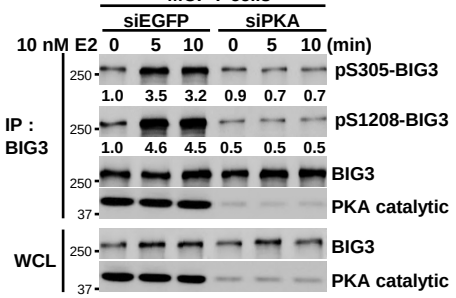


n

S305-Peptide (DHGRG**S**GCSCTAPALSGPVAR**)**
+ recombinant PKA for 30 min at 30˚C

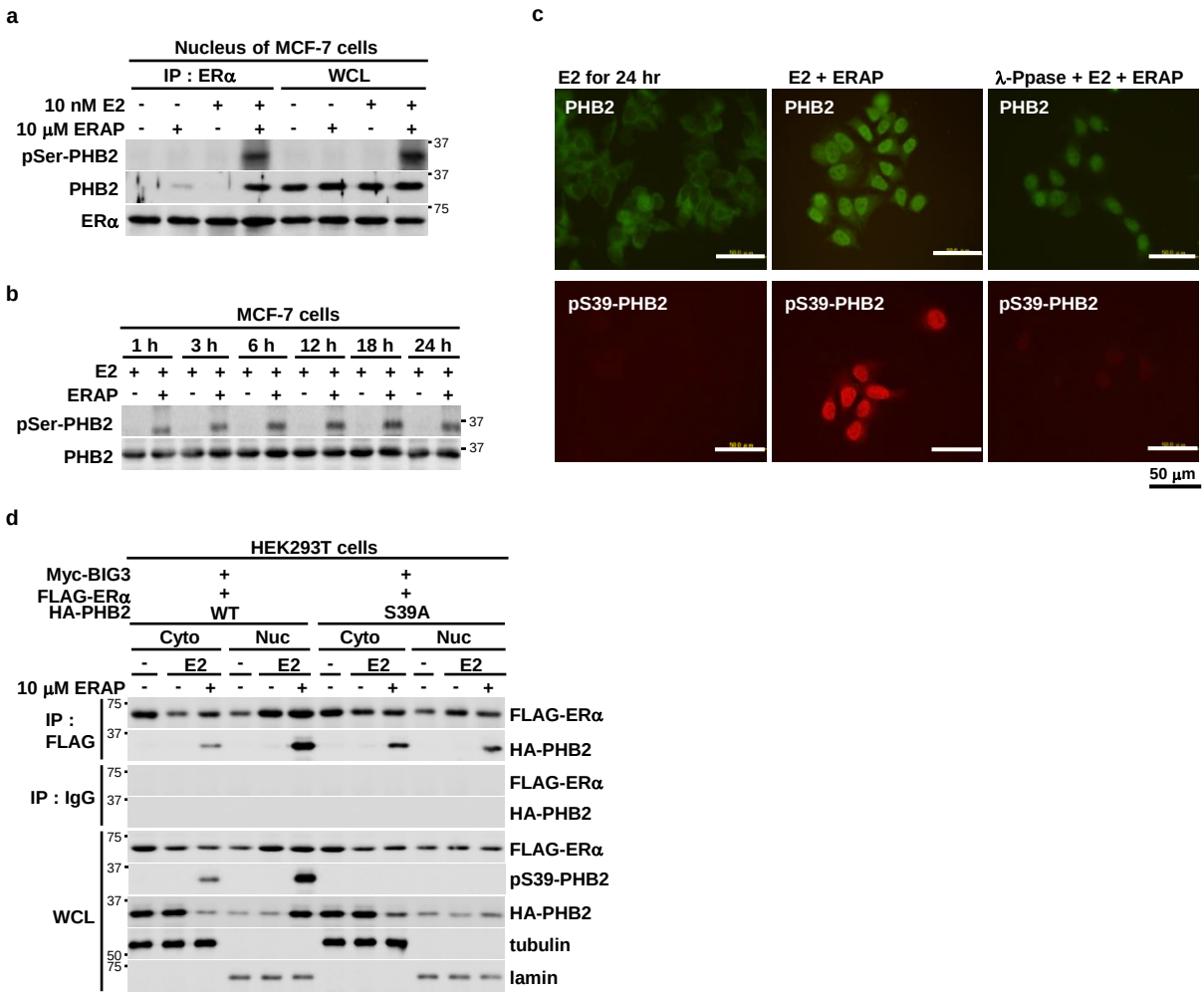
Score	Sequence	Modification
83.14	GSGCSCTAPALSGPVAR	-
57.63	G**S**GCSCTAPALSGPVAR	2S: Phospho 4C: Carboxymethyl 6C: Propionamide

S1208-Peptide (RCW**S**LVAPH**)**
+ recombinant PKA for 30 min at 30˚C

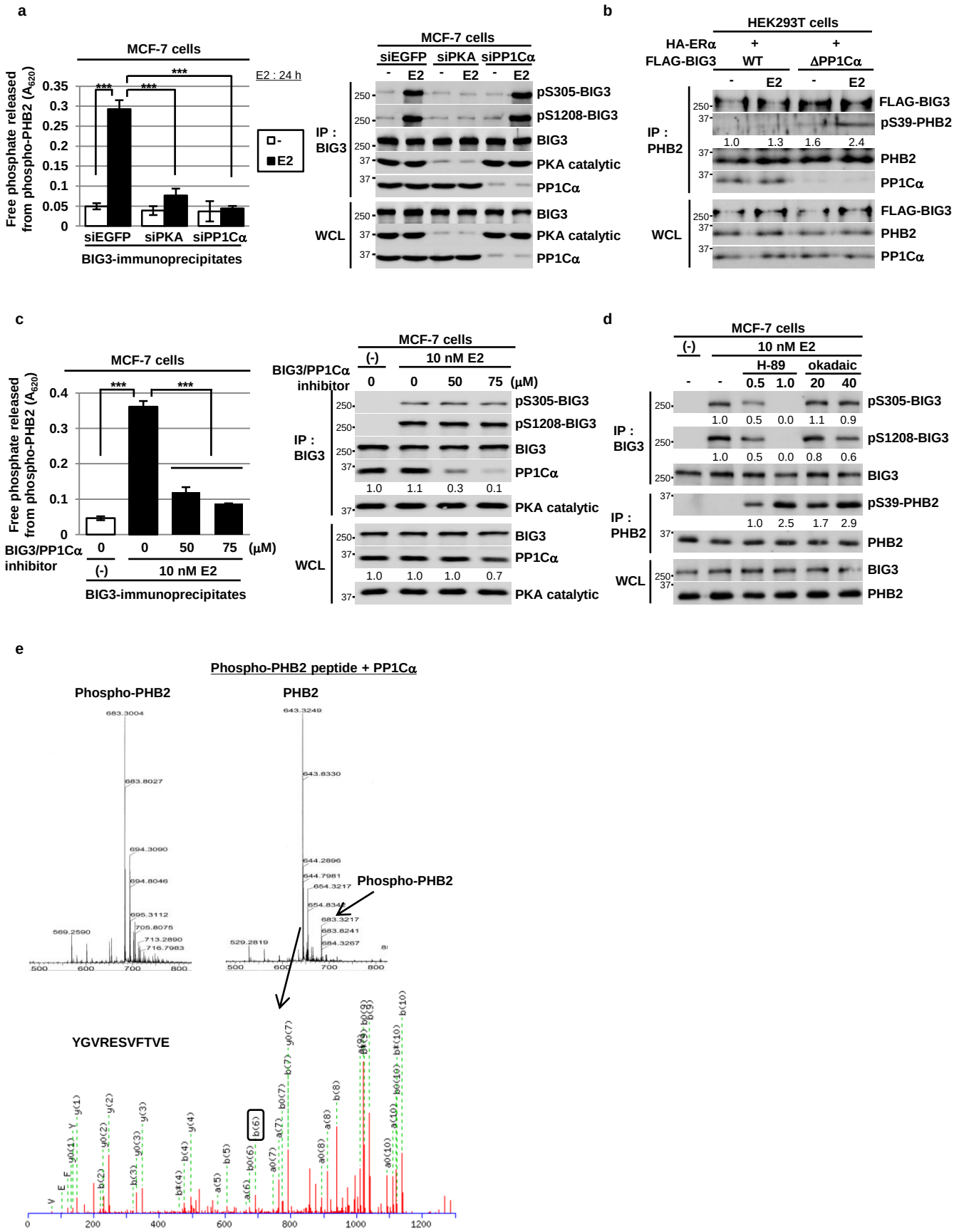
Score	Sequence	Modification
72.15	CWSLVAPH	-
56.34	CW**S**LVAPH	1C: Propionamide 3S: Phospho

Supplementary Figure 1 | BIG3 regulated PP1Cα activity through its phosphorylation by PKA. (**a**) Expression patterns of BIG3 and PP1Cα in breast cancer cell lines. β-actin served as a quantitative internal control. (**b**) Identification of the predictive PP1Cα binding region in BIG3. The indicated FLAG-tagged BIG3 constructs (WT; full-length BIG3, ΔPP1Cα; BIG3 deleting PP1Cα-binding region, 1,228-KAVSF-1,232) and HA-tagged ERα construct transfected-HEK293T cells were immunoprecipitated using an anti-FLAG antibody. (**c**) The inhibitory effects of BIG3 overexpression on phosphatase activity of PP1Cα-immunoprecipitates in FLAG-tagged BIG3 or ΔPP1Cα construct-transfected HEK293T cells using *p*NPP as a substrate. These data represent the means ± s.e.m. of three independent experiments. (**d**) Effects of siPP1Cα and siBIG3 on phosphatase activity of PP1Cα immunoprecipitates using *p*NPP as a substrate in MCF-7 and KPL3C cells. These data represent the means ± s.e.m. of three independent experiments. (**e**) Phosphatase activity of PP1Cα after E2 stimulation in MCF-7 (left) and KPL-3C (right) cells. These data represent the means ± s.e.m. of three independent experiments. (**f**) Positive

feedback regulation of *PPP1CA* transactivation. Left, Effects of tamoxifen on *PPP1CA* expression. For immunoblot analysis (upper), β-actin served as a loading control. For real-time PCR analysis (lower), the data are expressed as the fold-increase over untreated cells (set at 1.0). These data represent the mean ± s.e.m. of three independent experiments. Right, ChIP assays of the transactivation of *PPP1CA* through an ERE motif in 5' upstream (upper), and luciferase assays of the transactivation of *PPP1CA* using a luciferase reporter containing an ERE motif conserved within 5' upstream of the *PPP1CA* gene (lower). The data represent the mean ± s.e.m. of three independent experiments. (**g**) The predicted PKA binding regions in BIG3, as determined using Hou *et al.*[24] and the PSIVER software. The bold letters indicate the potential PKA binding regions in BIG3. (**h**) Expression patterns of PKA protein in breast cancer cell lines. (**i**) Statistical analysis of PKA and BIG3 binding to BIG3 (right) and PKA (left) immunoprecipitates, respectively. These data are expressed as the fold-increase over untreated cells (set at 1.0), and represent the mean ± s.e.m. of three independent experiments. (**j**) Statistical analysis of *in vitro* PKA activity of BIG3 and PKA immunoprecipitates. These data represent the mean ± s.e.m. of three independent experiments. (**k**) The predicted phosphorylation sites of BIG3 by PKA, as determined using NetPhos 3.1 software. (**l**) Phosphatase activity of PP1Cα in a pseudo-phosphorylation mutant of BIG3 (S305E and S1208E) and alanine mutant of BIG3 (S305A and S1208A). (**m**) The inhibitory effects of siPKA on BIG3 phosphorylation. Representative results are shown from one of two experiments. (**n**) 2DICAL analysis of engineered peptides representing S305 and S1208 on BIG3 with recombinant PKA (recPKA). ***P*<0.01, ****P*<0.001 (two-sided Student's *t*-test)

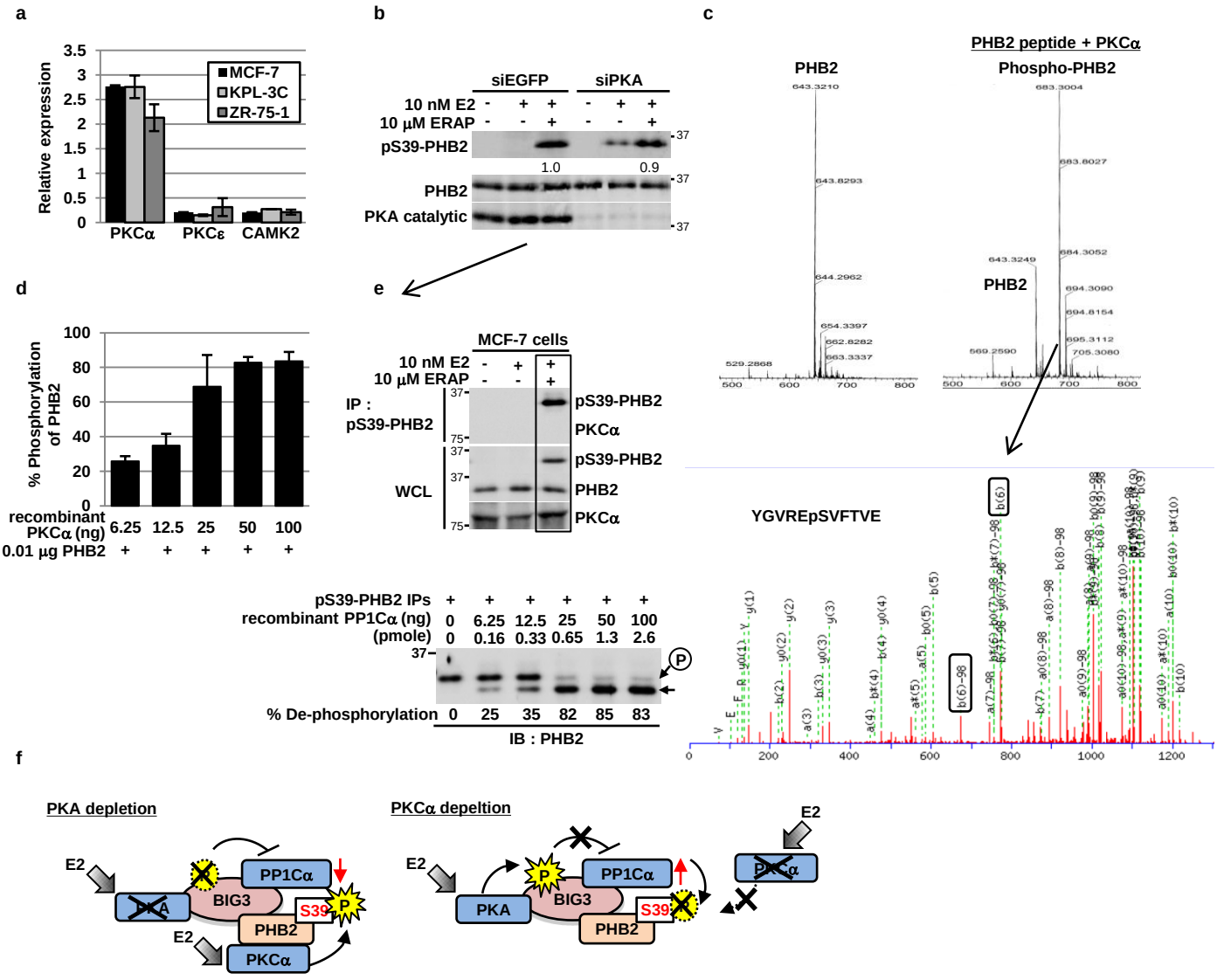


Supplementary Figure 2 | PHB2 is phosphorylated at S39 via PKCα. (**a**) Serine phosphorylation of PHB2 in ERα immunoprecipitates of the nuclear fraction of MCF-7 cells after E2 ± ERAP treatment for 24 h. (**b**) Time course of serine phosphorylation of PHB2 in MCF-7 cells after E2 ± ERAP treatment for the indicated time. (**c**) Representative immunofluorescence images of PHB2 phosphorylation at S39; PHB2 (green), phosphorylated S39 PHB2 (red). (**d**) The subcellular localization of PHB2 phosphorylation at S39 in the presence of E2 in the cytoplasmic (Cyto) and nuclear (Nuc) fractions of the indicated PHB2 (WT, S39A) and ERα construct-transfected HEK293T cells. a/b-tubulin (tublin) and laminin B (lamin) were used as loading controls for the cytoplasmic and nuclear fractions, respectively.

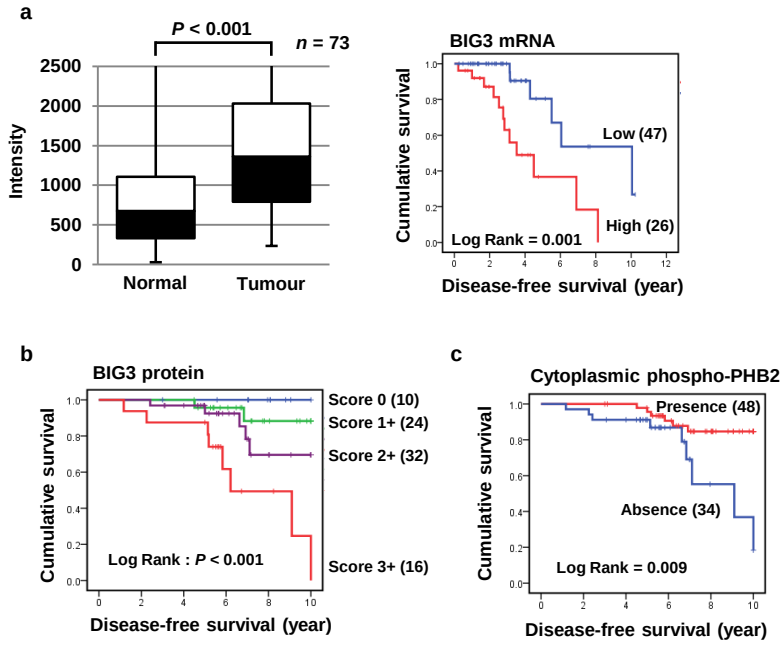


Supplementary Figure 3 | Phosphatase activity of BIG3-PP1Cα regulates PHB2 phosphorylation. (**a**) Phosphatase activity (left) and Western blot analyses (right) of BIG3 immunoprecipitates of PP1Cα- and PKA-depleted MCF-7 cells against phospho-S39 PHB2 peptide. Phosphatase activity was measured using malachite green. These data represent the means ± s.e.m. of three independent experiments. (**b**) The inhibitory effect of PP1Cα on PHB2 S39 phosphorylation. The indicated

FLAG-tagged BIG3 (WT, ΔPP1Cα) and HA-tagged ERα-transfected HEK293T cells, followed by immunoprecipitation with an anti-PHB2 antibody. (**c**) The inhibitory effect of BIG3-PP1Cα binding inhibitor on PP1Cα phosphatase activity. MCF-7 cells were treated with BIG3-PP1Cα binding inhibitor for 24 h in the presence of E2 and were immunoprecipitated using BIG3 antibody. (**d**) The effects of PKA inhibitor H-89 and PP1Cα inhibitor okadaic acid on BIG3 phosphorylation (S305 and S1208) and PHB2 phosphorylation (S39). MCF-7 cells were treated with H-89 or okadaic acid for 24 h in the presence of E2 and were immunoprecipitated using BIG3 or PHB2 antibody. (**e**) Q-TOF spectra indicating dephosphorylation of phospho-PHB2-peptide by PP1Cα in positive ion mode. The dephosphorylation of peptide is exemplarily shown for *m*/*z* 643.3. Upper, the left spectrum is an untreated control, and the right spectrum was acquired after dephosphorylation with PP1Cα. Lower, product ion spectrum of the PHB2 peptide YGVRESVFTVE, with a precursor mass of *m*/*z* 643.3. *** $P<0.001$ (two-sided Student's *t*-test).



Supplementary Figure 4 | PKCα is confirmed to be responsible kinase for PHB2 S39 phosphorylation. (**a**) Expression patterns of *PKCα, PKCε*, and *CAMK2* in ERα-positive breast cancer cell lines (MCF-7, KPL-3C and ZR-75-1) using real-time PCR. (**b**) The inhibitory effects of siPKA on PHB2 phosphorylation at S39 in MCF-7 cells after E2 ± ERAP treatment for 24 h. (**c**) Q-TOF spectra of direct PHB2 peptide phosphorylation by PKCα in positive ion mode. The phosphorylation of peptide is exemplarily shown for *m*/*z* 683.3. Upper, the left spectrum is an untreated control, and the right spectrum was acquired after phosphorylation with PKCα. Lower, product ion spectrum of the phosphor-S39 PHB2 peptide YGVRE(pS)VFTVE, with a precursor mass of *m*/*z* 683.3. (**d**) Statistical analysis of the ratio of phosphorylation at S39 in full-length PHB2 by PKCα. These data are expressed as the percentage of phosphorylated band in total PHB2 band and represent the mean ± s.e.m. of four independent experiments. (**e**) Dephosphorylation of PHB2 phosphorylation at S39 immunoprecipitated by phospho-specific PHB2 (S39) antibody from MCF-7 cells treated with E2/ERAP for 24 h. (**f**) Schematic illustration of BIG3-PKA-PP1Cα tri-complex under the depletion of PKA (left) and PKCα (right).



Supplementary Figure 5 | BIG3 overexpression was predictive of worse outcomes in ERα-positive breast cancer. (**a**) mRNA expression of *BIG3* in patients with ERα-positive breast cancer (left) and Kaplan-Meier curves of overall survival as a function of *BIG3* expression (right) based on the TCGA data set. (**b**, **c**) Kaplan-Meier analysis of survival associated with BIG3 protein (**b**) and cytoplasmic PHB2 phosphorylation at S39 (**c**) in representative ERα-positive breast cancer specimens.

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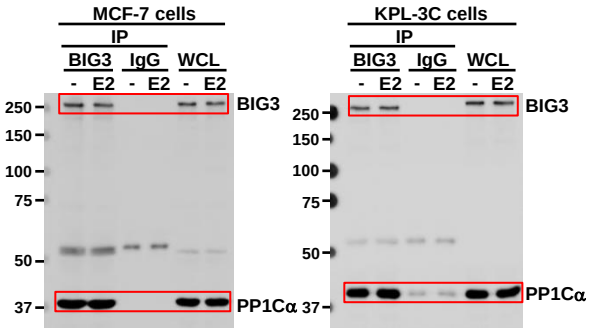

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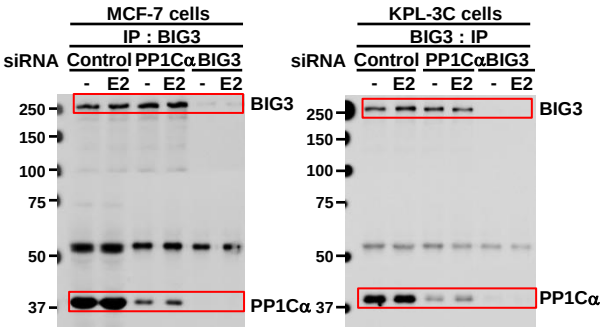

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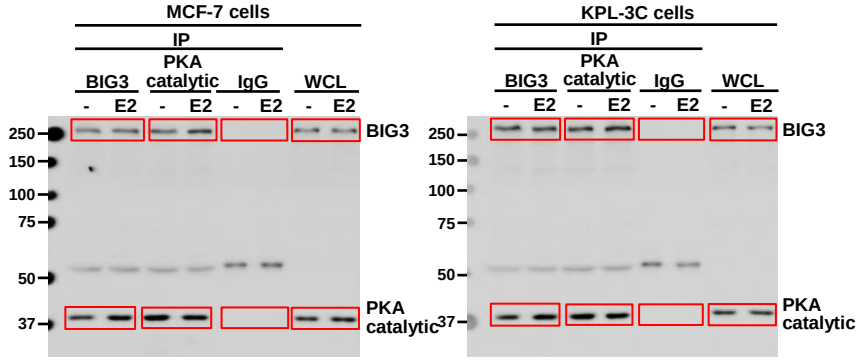

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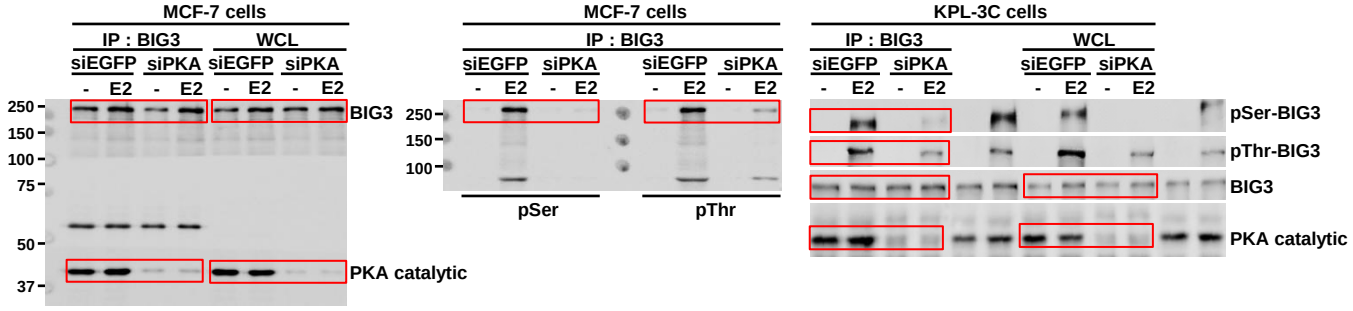

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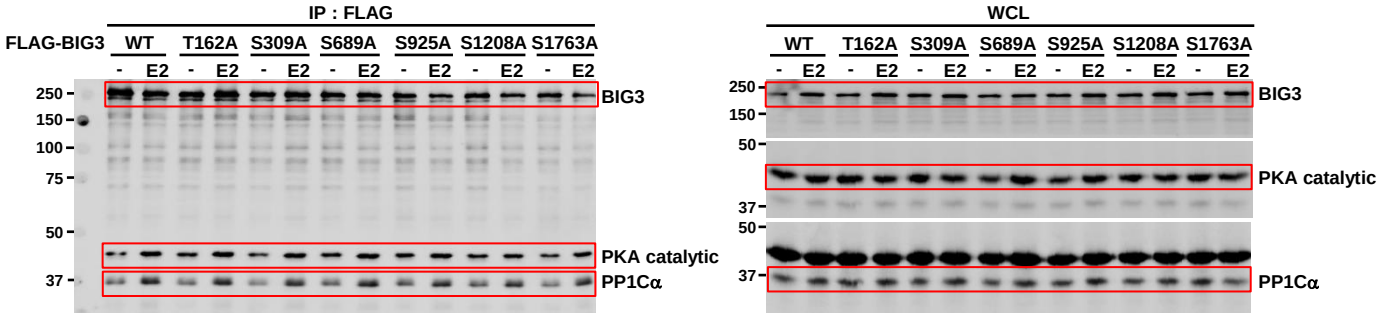

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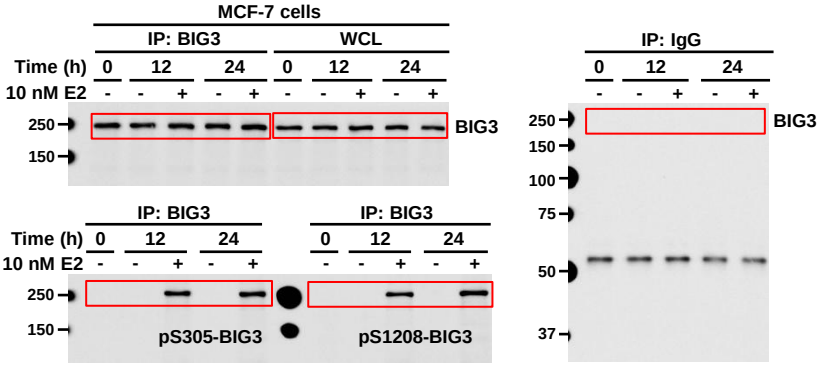

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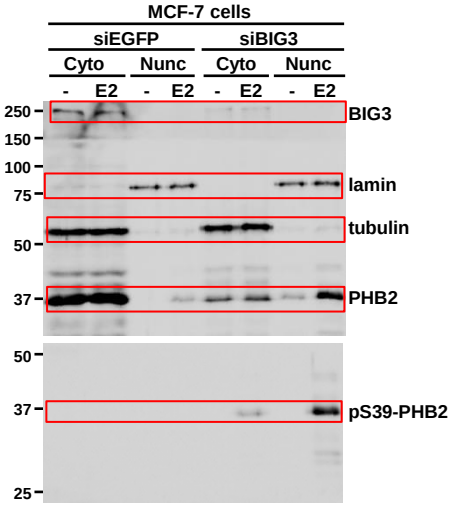

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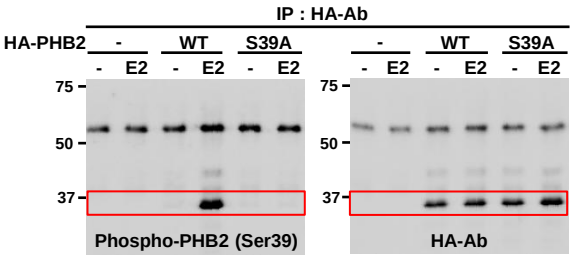

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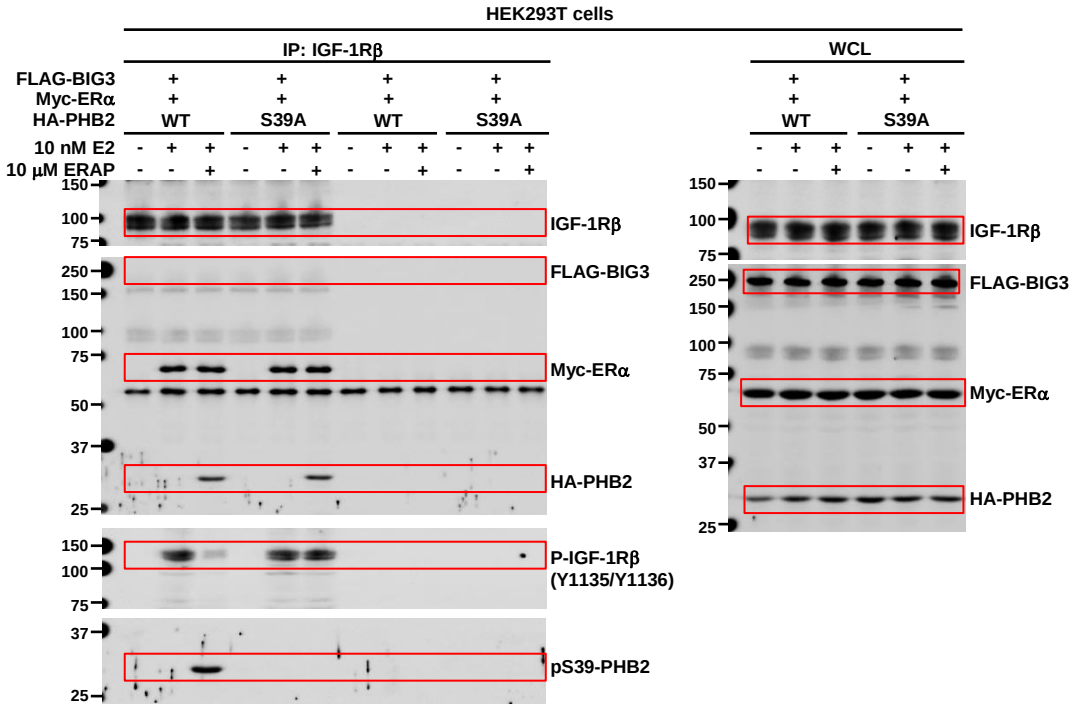

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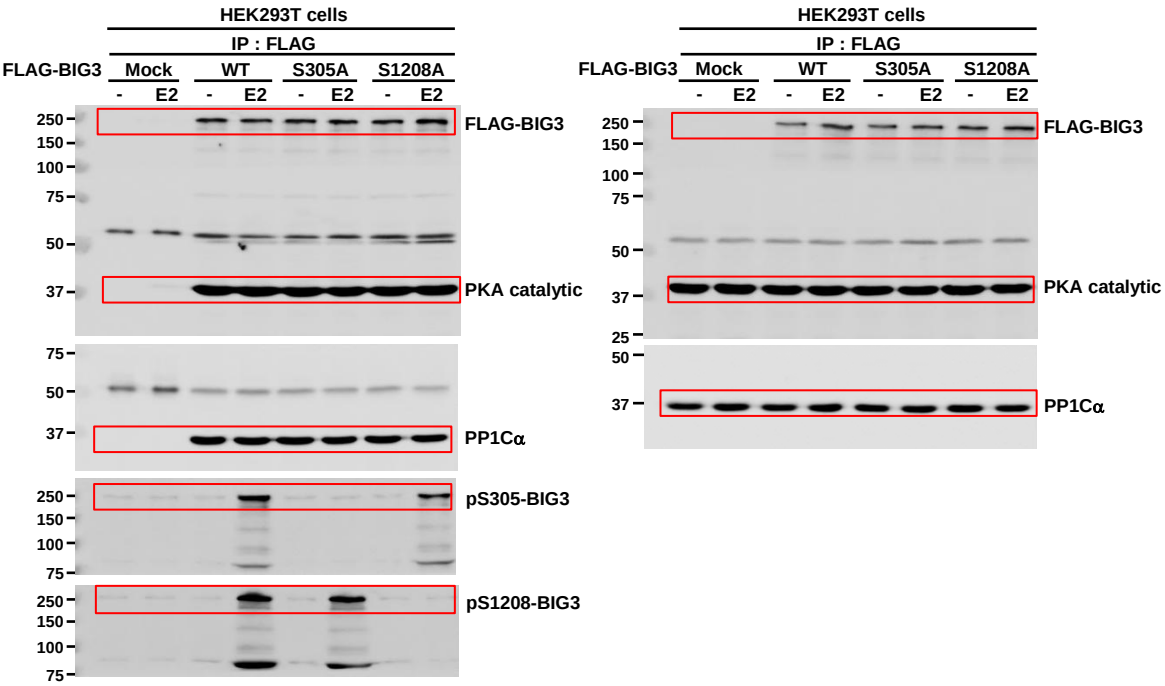

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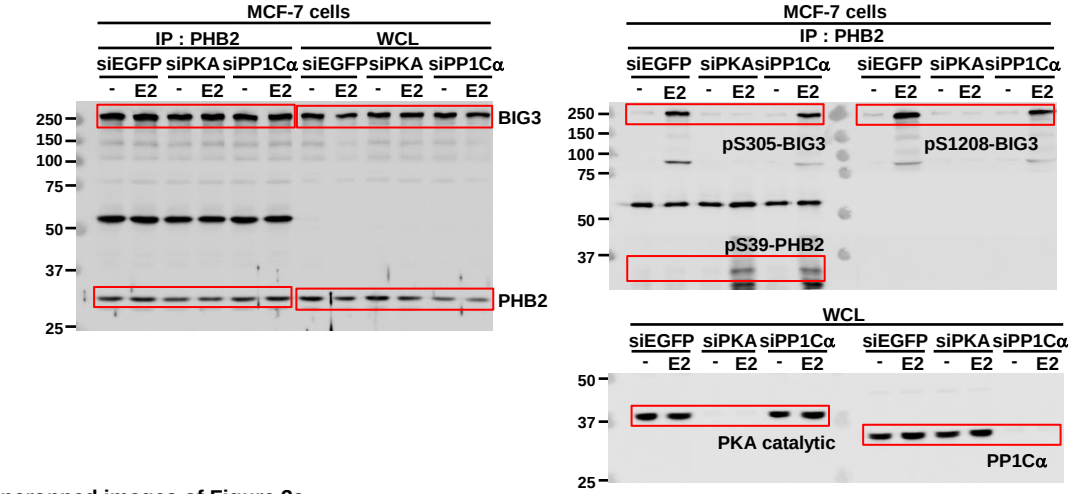

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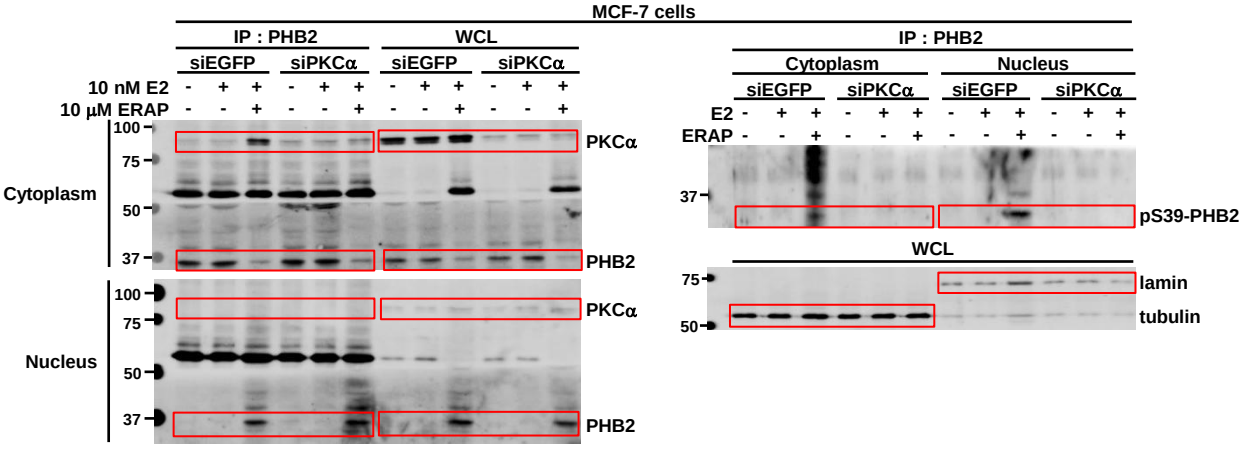

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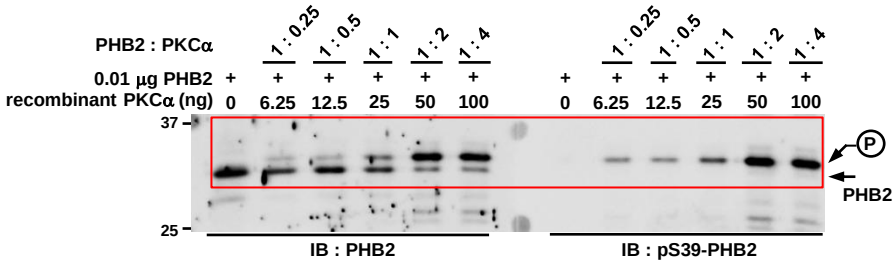

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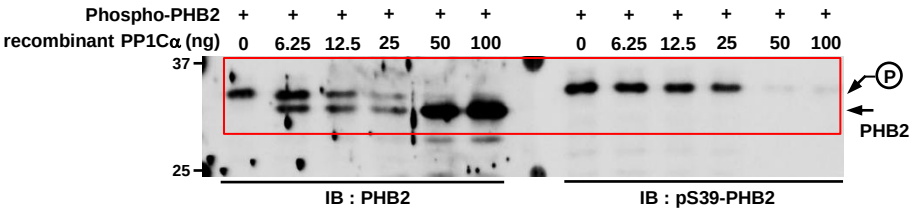

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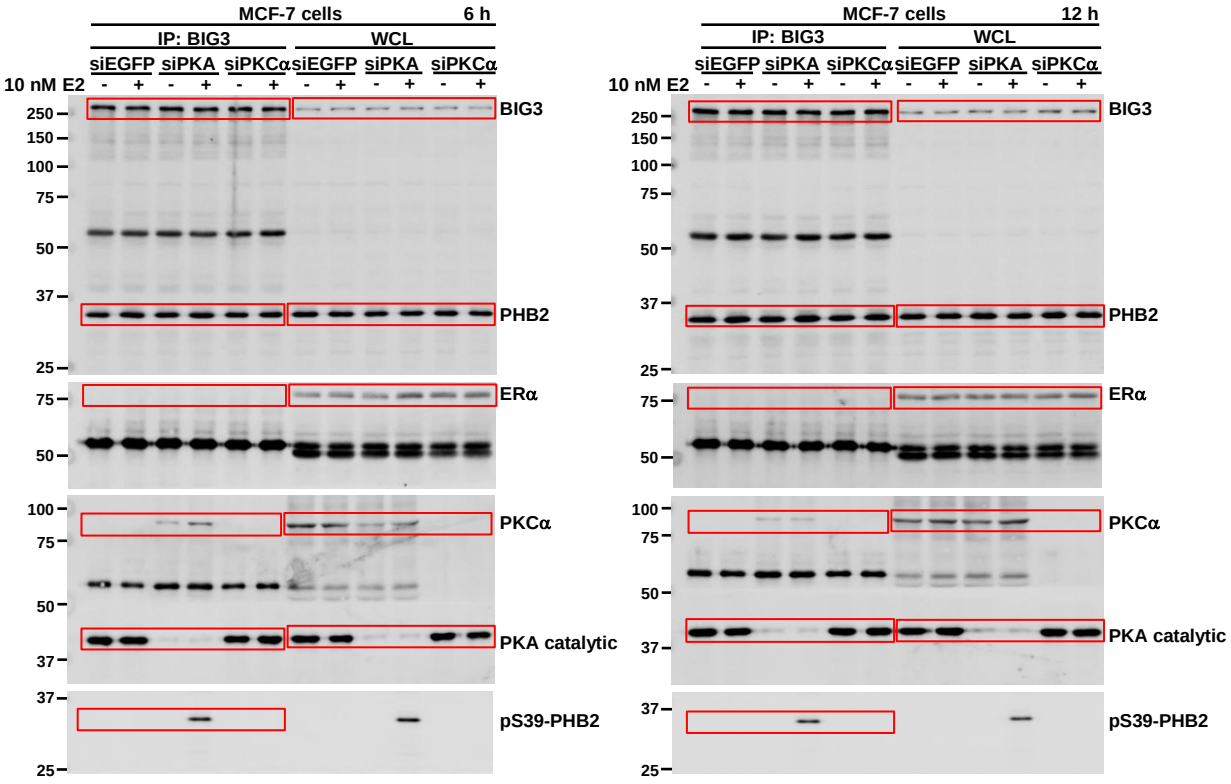

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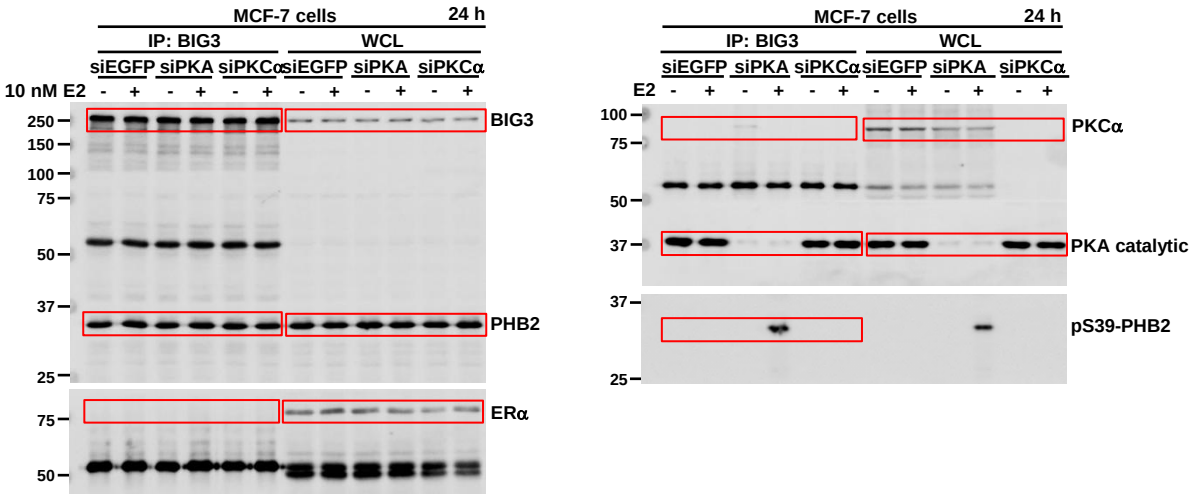

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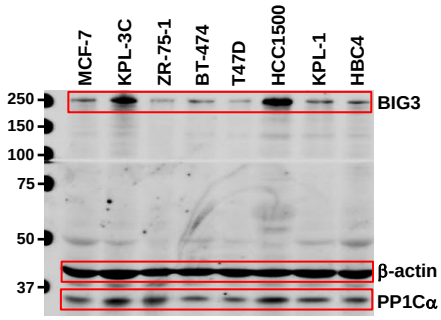

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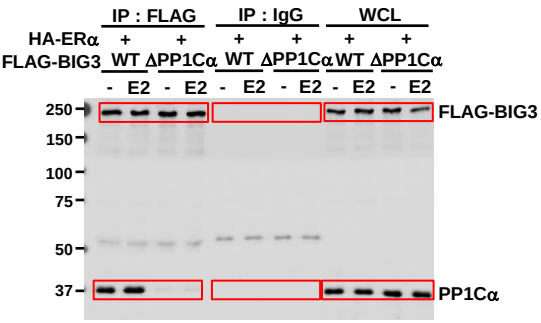

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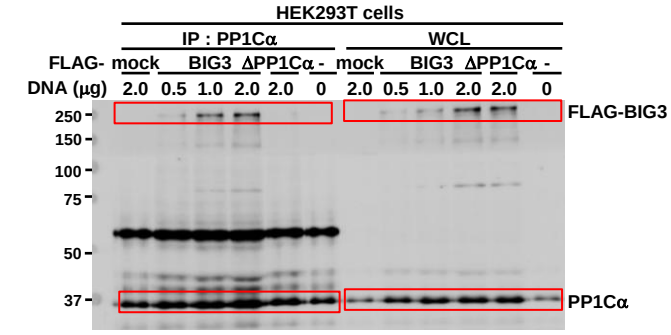

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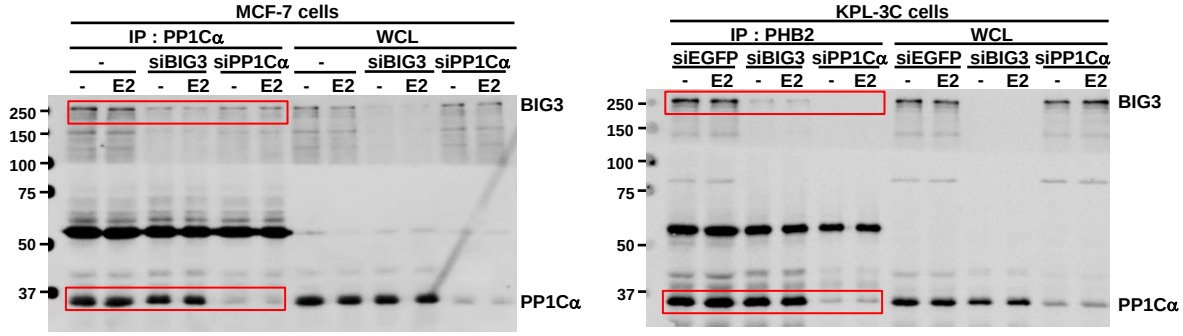

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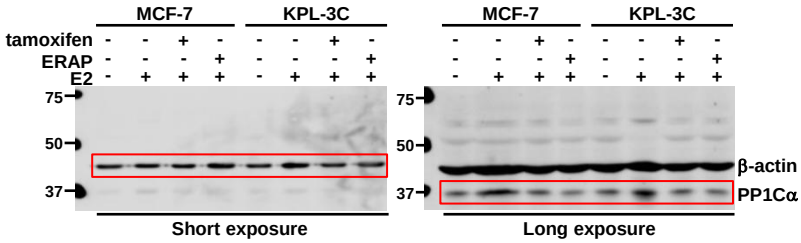

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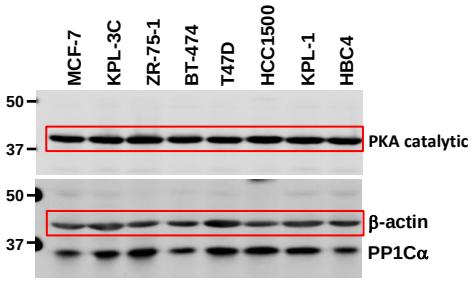

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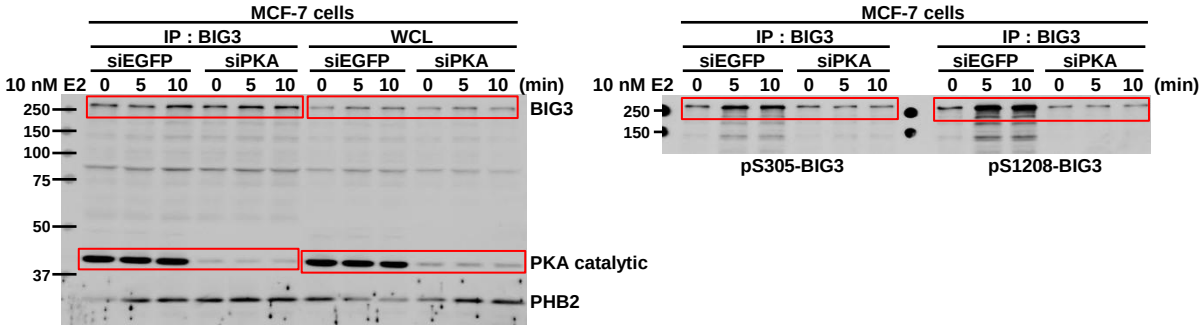

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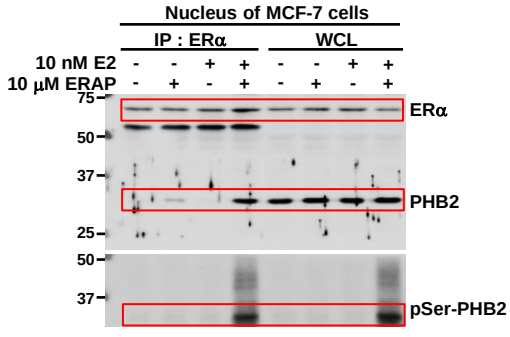

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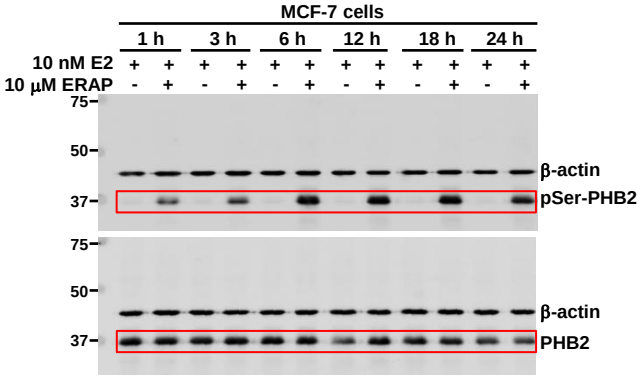

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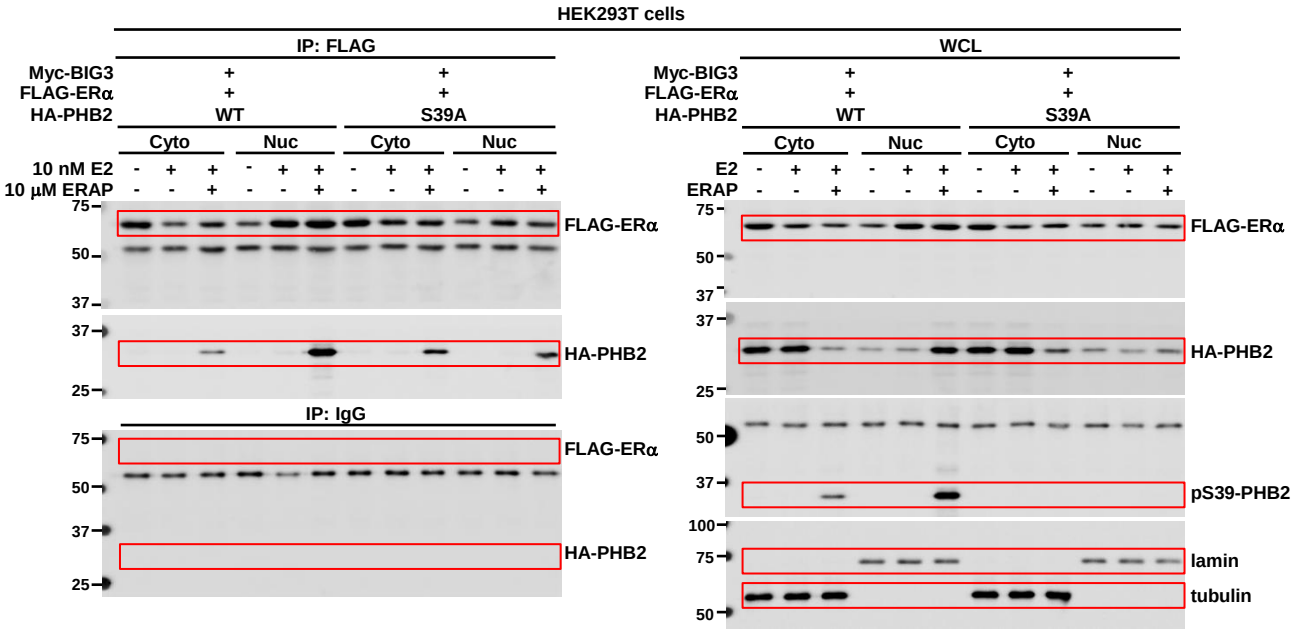

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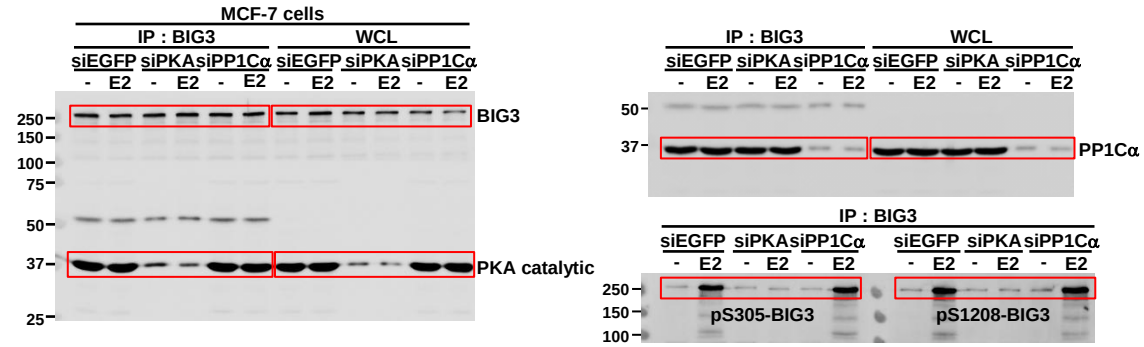
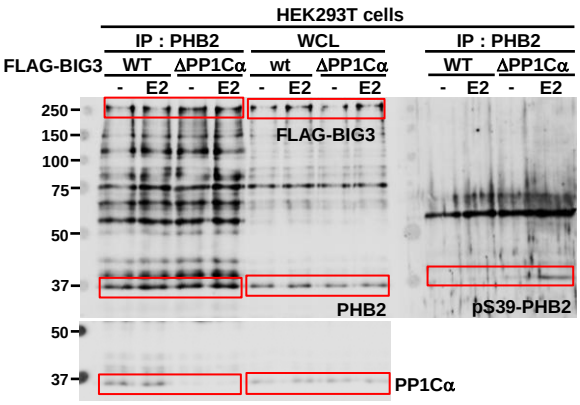
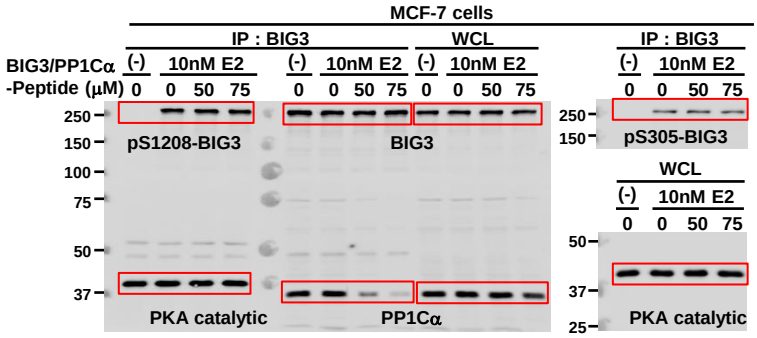


Uncropped images of Supplementary Figure 3b



Uncropped images of Supplementary Figure 3c



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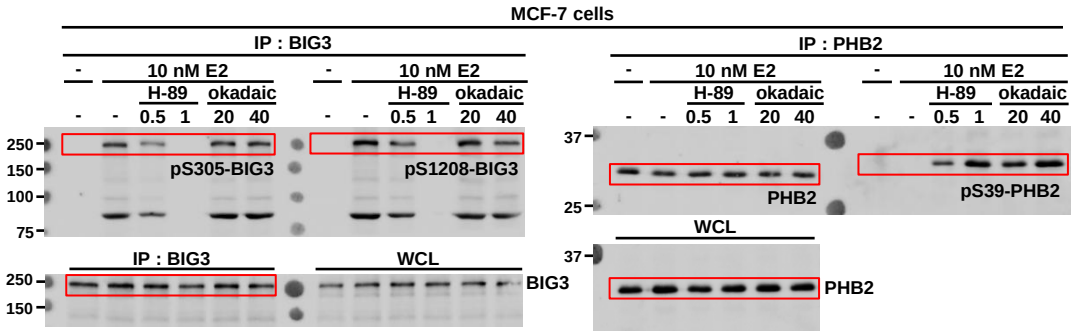
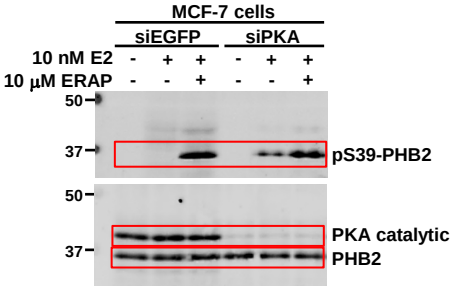
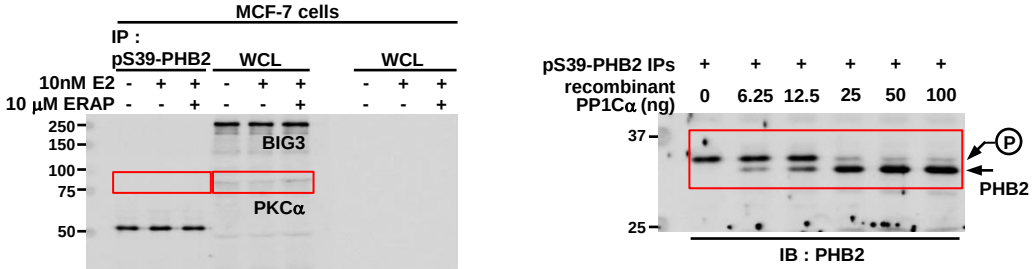
Uncropped images of Supplementary Figure 3d



Uncropped images of Supplementary Figure 4b



Uncropped images of Supplementary Figure 4d



Supplementary Figure 6 | Full-length images of immunoblots. Uncropped images of scanned immunoblots in Figures and Supplementary figures with size marker indications (kDa)

Supplementary Table 1. Mascot Search Results MS/MS Fragmentation of GSGCSCTAPALSGPVAR (upper) and CWSLVAPH (lower) found in BIG3_HUMAN in Sprot

Score	Mr(calc)	Delta	Sequence	Site Analysis
57.6	1741.7165	-0.0212	GSGCSCTAPALSGPVAR	Propionamide C6, Carboxymethyl C4, Phospho S2; 45.13%
57.6	1741.7165	-0.0212	GSGCSCTAPALSGPVAR	Propionamide C4, Carboxymethyl C6, Phospho S2; 45.13%
47.6	1741.7165	-0.0212	GSGCSCTAPALSGPVAR	Propionamide C6, Carboxymethyl C4, Phospho S5; 4.45%
47.6	1741.7165	-0.0212	GSGCSCTAPALSGPVAR	Propionamide C4, Carboxymethyl C6, Phospho S5; 4.45%
37.1	1741.7165	-0.0212	GSGCSCTAPALSGPVAR	Propionamide C6, Carboxymethyl C4, Phospho T7; 0.40%
37.1	1741.7165	-0.0212	GSGCSCTAPALSGPVAR	Propionamide C4, Carboxymethyl C6, Phospho T7; 0.40%
20.6	1741.7052	-0.0100	SEQPGSILGPECASCK	
18.3	1741.7178	-0.0225	PNGTFSLHYFPYYG	
18.0	1741.7520	-0.0567	QNKQSSESAVSSTVNP	
18.0	1741.7520	-0.0567	QNKQSSESAVSSTVNP	

Score	Mr(calc)	Delta	Sequence
56.3	1062.4358	0.0069	CWSLVAPH
27.3	1062.4383	0.0043	GPGTQLPEGQ
21.5	1062.4747	-0.0321	GTATVLSTPH
21.5	1062.4747	-0.0321	GTATVLSTPH
20.4	1062.4035	0.0391	LSAVLSQEG
20.3	1062.4821	-0.0394	VSCPLPPVT
19.1	1062.4284	0.0142	GPDRESAPH
19.0	1062.4747	-0.0321	AGPGTGLPGGLS
18.6	1062.3916	0.0511	YVCIPGCT
17.2	1062.4747	-0.0321	HDVVLVGGST

Supplementary Table 2. Clinical characteristic and results of immunohistochemistry of ERα-positive breast cancer specimens.

Case	BIG3 intensity (0-3)	PHB2 intensity (0-3)		DFI (year)	Recurrence	Age	Menopause	Tumour size	Lymph node metastasis	Stage
		Nucleus	Cytoplasm							
1	2	2	1	5.00	+	44	Pre	< 2 cm	n0	I
2	0	2	1	8.09		48	Post	2.1 - 5 cm	n0	I
7	1	2	2	6.48		44	Pre	< 2 cm	n0	IIA
9	2	3	1	10.00		69	Post	2.1 - 5 cm	n1	IIB
10	2	2	1	6.92	+	58	Post	2.1 - 5 cm	n1	II
12	2	0	1	7.17		63	Post	2.1 - 5 cm	n0	IIA
20	1	3	2	10.00		48	Pre	2.1 - 5 cm	n0	I
22	1	0	1	8.81		71	Post	< 2 cm	n0	IIA
29	0	0	0	7.02		82	Post	< 2 cm	n0	IIA
30	3	3	1	5.83	+	47	Pre	2.1 - 5 cm	n0	IIA
32	1	0	0	6.84	+	70	Post	< 2 cm	n1	IIB
35	1	2	1	4.51	+	55	Post	< 2 cm	n0	II
36	1	3	1	7.22		69	Post	2.1 - 5 cm	n0	IIA
37	1	3	1	8.05		58	Post	< 2 cm	n0	I
39	3	0	0	6.73		42	Pre	2.1 - 5 cm	n0	I
41	1	3	1	9.48		45	Pre	2.1 - 5 cm	n0	I
43	3	0	1	8.24		51	Post	< 2 cm	n0	I
44	3	1	2	5.75		58	Post	2.1 - 5 cm	n0	I
45	2	3	2	8.01		48	Pre	2.1 - 5 cm	n1	I
46	2	0	0	5.62		46	Pre	2.1 - 5 cm	n0	I
47	2	0	0	5.54		40	Pre	2.1 - 5 cm	n0	I
48	3	1	0	1.17	+	51	Post	2.1 - 5 cm	n0	IIA
49	1	3	1	10.00		49	Pre	< 2 cm	n0	I
50	2	2	1	7.05		55	Post	< 2 cm	n0	I
51	2	0	1	6.38		44	Pre	< 2 cm	n0	I
55	2	0	1	6.13		55	Post	2.1 - 5 cm	n0	I
57	0	1	1	7.06		71	Post	2.1 - 5 cm	n1	IIA
58	2	0	0	5.83		46	Pre	< 2 cm	n0	I
61	2	3	1	9.06		67	Post	< 2 cm	n0	IIA
62	2	3	2	9.98		48	Pre	< 2 cm	n1	I
70	2	3	1	10.00		51	Post	2.1 - 5 cm	n0	I
73	2	3	2	10.00		59	Post	< 2 cm	n0	I
74	3	0	0	7.11	+	55	Post	< 2 cm	n1	I
76	2	0	1	5.62		55	Post	< 2 cm	n0	I
77	3	0	1	6.21	+	63	Post	< 2 cm	n0	I
78	1	0	0	6.08		72	Post	< 2 cm	n0	I
79	0	3	2	5.57		54	Post	2.1 - 5 cm	n0	II
82	3	0	0	9.10	+	45	Pre	2.1 - 5 cm	n0	II
85	1	3	1	7.17		50	Pre	2.1 - 5 cm	n1	II
86	2	3	0	6.99		50	Pre	< 2 cm	n0	I
88	1	3	2	9.81		46	Pre	< 2 cm	n0	I
89	0	2	1	3.00		52	Pre	2.1 - 5 cm	n0	II
92	3	0	0	10.00	+	40	Pre	2.1 - 5 cm	n0	II
93	3	0	1	5.18	+	49	Pre	< 2 cm	n0	I
95	2	0	0	4.99		58	Post	< 2 cm	n0	I
96	2	0	0	4.00		78	Post	2.1 - 5 cm	n0	II
100	3	0	0	5.14	+	46	Pre	2.1 - 5 cm	n0	II
101	2	0	0	3.08		52	Pre	< 2 cm	n0	I
102	3	0	0	5.66		44	Pre	< 2 cm	n0	I
103	2	0	1	4.98		58	Post	< 2 cm	n0	I
117	1	0	0	4.49		48	Pre	< 2 cm	n0	I
118	1	1	0	4.68		64	Post	< 2 cm	n1	II
120	2	0	0	6.63	+	57	Post	< 2 cm	n0	II
122	2	1	1	3.12		53	Post	2.1 - 5 cm	n0	I
124	0	3	0	10.00		39	Pre	2.1 - 5 cm	n0	I
125	2	0	0	5.20		70	Post	< 2 cm	n0	II
129	2	0	0	2.42	+	68	Post	2.1 - 5 cm	n0	II
135	2	0	1	4.67		70	Post	< 2 cm	n0	I
136	1	0	0	5.38		47	Post	< 2 cm	n0	I
138	1	0	0	6.75		51	Pre	< 2 cm	n0	IIA
145	2	0	0	4.80		56	Post	< 2 cm	n0	I

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146	1	3	2	9.99		58	Post	< 2 cm	n0	I
155	2	0	0	4.66		68	Post	2.1 - 5 cm	n0	II
156	0	2	1	9.05		46	Pre	< 2 cm	n0	I
159	3	0	0	2.25	+	40	Pre	2.1 - 5 cm	n0	II
161	1	1	1	5.43		48	Pre	2.1 - 5 cm	n0	I
163	1	3	1	9.34		49	Pre	< 2 cm	n0	I
168	1	3	1	7.81		51	Post	2.1 - 5 cm	n0	II
170	0	2	2	8.08		63	Post	2.1 - 5 cm	n1	II
174	1	0	1	5.72		43	Pre	2.1 - 5 cm	n0	II
176	3	3	2	5.51		80	Post	2.1 - 5 cm	n0	II
180	3	0	0	5.00		78	Post	2.1 - 5 cm	n0	II
181	2	0	1	5.66		70	Post	< 2 cm	n0	I
182	3	0	0	5.60		68	Post	< 2 cm	n0	I
187	1	3	2	7.73		58	Post	< 2 cm	n0	I
192	2	3	1	8.04		71	Post	2.1 - 5 cm	n0	II
193	3	0	0	5.37		46	Pre	2.1 - 5 cm	n0	II
195	0	3	0	7.96		49	Post	< 2 cm	n0	I
198	2	0	0	4.93		54	Post	< 2 cm	n0	I
203	1	0	1	6.68		50	Pre	< 2 cm	n0	I
205	1	1	1	5.26		44	Pre	2.1 - 5 cm	n0	II
208	1	3	2	10.00		47	Pre	< 2 cm	n1	I

Supplementary Table 3. The sequences of each primer set.

Genes	Sequence	Purpose
BIG3	5'-CGGAATTCATGGAAGAAATCCTGAGGA AGC-3′ (forward) 5′-ATAGTTTAGCGGCCGCACAATGATGTCATAGACACGG-3′ (reverse)	Cloning
BIG3 mutant (T162A)	5'-GTGCGGGCAGCCCTCAGTCAA-3′ (forward) 5′-TTGACTGAGGGCTGCCCGCAC-3′ (reverse)	Cloning
BIG3 mutant (S305A)	5'-TCAGGCTGCGCCTGCACTGCG-3′ (forward) 5′-CGCAGTGCAGGCGCAGCCTGA-3′ (reverse)	Cloning
BIG3 mutant (S305E)	5'-GGCCGAGGAGAAGGCTGCTCC-3′ (forward) 5'-GGAGCAGCCTTCTCCTCGGCC-3′ (reverse)	Cloning
BIG3 mutant (S689A)	5'-CGGCTCCTGGCCCTCTCCAAT-3′ (forward) 5′-ATTGGAGAGGGCCAGGAGCCG-3′ (reverse)	Cloning
BIG3 mutant (S925A)	5'-GCACGGCTGGCCTGCGCTCTA-3′ (forward) 5′-TAGAGCGCAGGCCAGCCGTGC-3′ (reverse)	Cloning
BIG3 mutant (S1208A)	5'-CGCTGCTGGGCCCTTGTGGCC-3′ (forward) 5′-GGCCACAAGGGCCCAGCAGCG-3′ (reverse)	Cloning
BIG3 mutant (S1208E)	5'-CGCTGCTGGGAACTTGTGGCC-3′ (forward) 5'-GGCCACAAGTTCCCAGCAGCG-3′ (reverse)	Cloning
BIG3 mutant (S1763A)	5'-AGATACATCGCCATGCAGAAC-3′ (forward) 5′-GTTCTGCATGGCGATGTATCT-3′ (reverse)	Cloning
PHB2	5′-CGGAATTCCAGACCGTGCATCATGGCCCAGAACTTGAAGGA-3′ (forward) 5′-CCGCTCGAGTTTCTTACCCTTGATGAGGCTGT-3′ (reverse)	Cloning
PHB2 (S39A)	5′-GTGCGCGAAGCCGTGTTCACC-3′ (forward) 5′-GGTGAACACGGCTTCGCGCAC-3′ (reverse)	Cloning
PKCα	5'-CCCAAGAATGAAAGCAAGCA-3' (forward) 5'-CCGAAACTCCAAAGGAAAGG-3' (reverse)	Real-time PCR
PKCε	5'-TCAAGCAGCACCCATTCTTC-3' (forward) 5'-TCAGGGCATCAGGTCTTCAC-3' (reverse)	Real-time PCR
CAMK2	5'-TCAGGGCATCAGGTCTTCAC-3' (forward) 5'-GGGGAGAGAGGCAATGAAGA-3' (reverse)	Real-time PCR
β2-microglobulin	5'-AACTTAGAGGTGGGGAGCAG-3' (forward) 5'-CACAACCATGCCTTACTTTATC-3' (reverse)	Real-time PCR

The underlines indicate the recognition sites of restriction enzymes.
Double-underlines indicate the mutation sites.