SUPPLEMENTARY FIGURES

Fig.S1. Skp2 Regulates ERα Levels and Stability in Breast Cancer Cells

A) Quantitative analysis of Skp2 protein in ERα–negative and positive cell-lines. B) Quantitative analysis of ERα protein in MCF-7 cells subjected to siRNA mediated Skp2 depletion. C) Quantitative analysis of ERprotein in MCF-7 cells subjected to adenovirus mediated Skp2 over-expression. D) Quantitative analysis of ERα protein in MCF-7 cells subjected to adenovirus mediated Skp2 over-expression followed by Cycloheximide treatment for indicated times.

Fig.S2. Cul-7 and Rbx-1 are Essential for Skp2 Mediated Proteasomal Degradation of ERα in Breast Cancer Cells

A) Western analysis of ERα protein in MCF-7 cells subjected to siRNA mediated knockdown of indicated Cullin proteins and Rbx-1, in the absence or presence of ligand. B) Quantitative analysis of ERα protein in MCF-7 cells subjected to siRNA mediated depletion of Cul4B, Cul-7 or Rbx-1. C) Quantitative analysis of ERα protein in MCF-7 cells subjected to siRNA mediated depletion of various Cullin protein isoforms or Rbx-1 followed by adenovirus mediated Skp2 over-expression.

Fig.S3. Phosphorylation of ERα at Serine-294 is Required for its Ubiquitination by Skp2 and Impacts Ability of Skp2 to Alter ERα Protein Levels and Gene Regulation

A) Quantitative analysis of ERα protein in Cos-1 cells subjected to dose-dependent overexpression of Skp2-WT or S294A phospho-mutant. B) Western analysis of extracts from MDA-MB-468 cells transfected with WT or S294A mutant ERα in the presence of control or Skp2 adenovirus and treated with cycloheximide (50μg/ml) for the indicated times. C) HEC-1 cells were transfected with 2ERE-pS2-Luciferase expression plasmid along with wild type or S294A ERα and β-galactosidase in the presence or absence of Skp2 adenovirus, treated for 24h with either Veh or E2 (10nM), and monitored for luciferase activity. D) Silver stained gel for mass-spectrometry analysis of ERα upon Skp2 or p38MAPK over-expression in MCF-7 breast cancer cells. E) Quantitative analysis of ERα protein in MCF-7 cells subjected to adenovirus mediated Skp2 over-expression in the presence or absence of p38MAPK inhibitor. F) Quantitative analysis of p38MAPK protein in ERα–negative and positive cell lines.

Fig.S4. ERα Knockdown Blocks the Transcriptional Regulation of ER-Target Genes by Skp2 or p38MAPK, Skp2 and phospho-p38MAPK are Recruited to Target Genes, and Flow Cytometry Reveals Enhancement of S-Phase Entry by Skp2 and p38MAPK

Real-time PCR to monitor expression of ERα target genes, A) TFF1 and B) GREB1 in MCF-7 cells subjected to siRNA against ERα for 24h followed by infection with control, Skp2, p38MAPK or ERα adenovirus also for 24h and treatment with Veh (0.1% EtOH), E2 (10nM) or TOT (1_M) for 4h before harvest. The Western blot shows the extent of ERα protein knockdown with siRNA treatment. C) and D) ChIP assays: MCF-7 cells treated for 45 min, 4h or 24h with Veh (0.1% EtOH), E2 (1nM) or TOT (1μM), and then washed with cold PBS (pH 7.2) were harvested for ChIP assays using anti-ERα, Skp2, phospho-p38MAPK or IgG negative control antibody. Data are represented as recruitment index (Specific antibody signal/IgG signal ratio) and genes analyzed are p21 and p27. E) and F) ChIP re-ChIP analysis: MCF-7 cells treated with Veh (0.1% EtOH) or E2 (10nM) for 4h, were subjected to ChIP assays as described in Materials and Methods, using anti-Skp2, p38MAPK or IgG negative control antibody. Immunoprecipitated material from ChIP pull-downs were recovered with 10mM dithiothreitol in IP buffer at 37°C for 30 min, diluted and subjected to a second round of immunoprecipitation using specific ERα antibody. Data are represented as recruitment index (Specific antibody signal/ IgG signal ratio) and genes analyzed are TFF1 and GREB1. G) MCF-7 cells were processed and subjected to Propidium Iodide staining followed by Flow-cytometry analysis using BD-FACS-Canto. The graphs show relative number of cells in each phase of the cell cycle which is directly proportional to the surface area of the respective peak of any cell cycle stage. H) MCF-7 cells were synchronized at different stages of the cell cycle as described in Materials and Methods. Propidium Iodide staining followed by Flow-cytometry analysis was performed using BD-FACS-Canto and the relative number of cells in each phase of the cell cycle is shown.

Fig. S5: Impact of loss of ERα in mouse mammary gland or MCF-7 cells on Skp2 and p38MAPK protein levels.

A) Western analysis in mammary tissue protein extracts from WT of ERα knockout (ERKO) mice. Each lane shows extract from a different mouse. B) Western analysis on whole cell extracts from MCF-7 cells transfected with siGL-3 or siRNA against ERα.

Fig.S6. Skp2-S64A Mutant Defective in ERα Interaction/Ubiquitination has No Impact on ERα Target Gene Expression or Cell Proliferation, whereas Constitutively Active Phosphomimic Skp2-S64E Enhances Gene Expression and Cell Proliferation

A) Cos-1 cells transfected with pCMV-Skp2-WT, pCMV-Skp2-S64A (phosphorylation defective mutant) or pCMV-Skp2-S64E (constitutively active phospho-mimic) along with ERα

and HA-Ubiquitin (HA-Ub) or alone and treated with MG-132 for 5h before harvest, 24h after transfection, were subjected to ubiquitination assay for ERα. B) Hec-1 cells transfected with pCMV-2ERE-pS2-Luciferase, ERα and Renilla Luciferase (internal control to assess transfection efficiency) along with pCMV-Skp2-WT, Skp2-S64A or Skp2-S64E were treated for 24h with either Veh (0.1% EtOH) or E2 (10nM), 6h post transfection and thereafter were harvested and monitored for luciferase activity. C) Proliferation of MCF-7 cells with over-expressed Skp2-WT, Skp2-S64A or Skp2-S64E mutant followed by treatment with Veh (0.1% EtOH) or 10nM E2 and analysis of cell density using WST reagent.

Fig. S7: Assessment of Changes in ERα (ESR1) mRNA after Knockdown of Skp2 or p38MAPK Inhibition in MDA-MB-468 and MDA-MB-453 Cells.

Real-time q-PCR analysis of ERα mRNA in A) MDA-MB-468 or B) MDA-MB-453 cells transfected with scramble (control) or Skp2 siRNA, or treated or not with p38MAPK inhibitor for 24h followed by control vehicle, estradiol (E2, 10nM) or tamoxifen (TOT, 1μM) treatment for 24h.

Fig. S8: Effect of knockdown of Skp2 and p38MAPK on expression of pS2 and GREB1 mRNA in MCF-7 cells.

A) Real-time q-PCR analysis of pS2/TFF1 mRNA or B) GREB1 mRNA in MCF-7 cells transfected with scramble (control), Skp2, or p38MAPK siRNA for 24h followed by 24h treatment with control vehicle or estradiol (E2, 10nM).

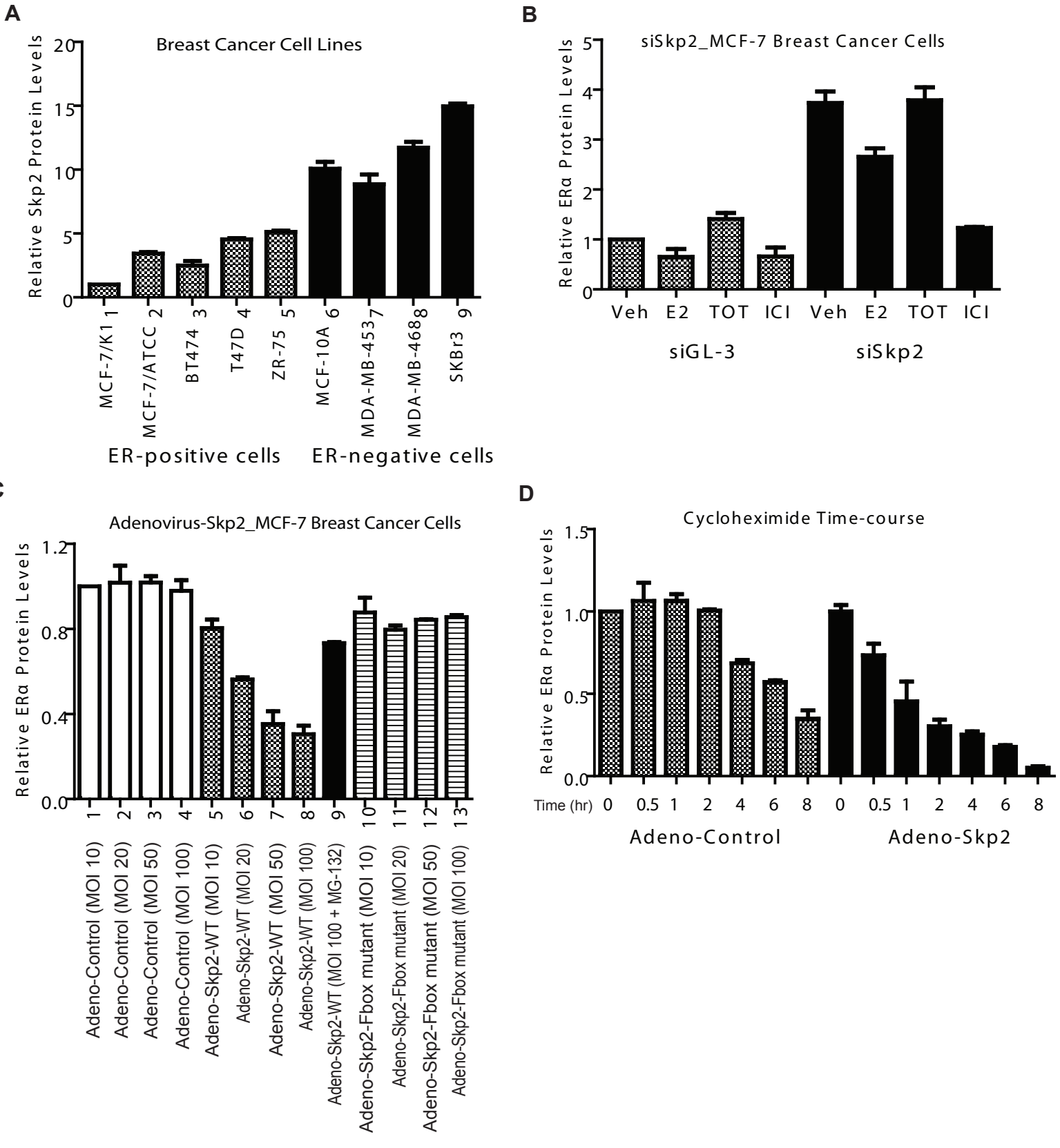

A
Breast Cancer Cell Lines
Relative Skp2 Protein Levels
20
15
10
5
0
MCF-7/K1 1
MCF-7/ATCC 2
BT474 3
T47D 4
ZR-75 5
MCF-10A 6
MDA-MB-453 7
MDA-MB-468 8
SKBr3 9
ER-positive cells
ER-negative cells
B
siSkp2_MCF-7 Breast Cancer Cells
Relative ERα Protein Levels
5
4
3
2
1
0
Veh E2 TOT ICI Veh E2 TOT ICI
siGL-3
siSkp2
C
Adenovirus-Skp2_MCF-7 Breast Cancer Cells
Relative ERα Protein Levels
1.2
0.8
0.4
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13
Adeno-Control (MOI 10)
Adeno-Control (MOI 20)
Adeno-Control (MOI 50)
Adeno-Control (MOI 100)
Adeno-Skp2-WT (MOI 10)
Adeno-Skp2-WT (MOI 20)
Adeno-Skp2-WT (MOI 50)
Adeno-Skp2-WT (MOI 100)
Adeno-Skp2-WT (MOI 100 + MG-132)
Adeno-Skp2-Fbox mutant (MOI 10)
Adeno-Skp2-Fbox mutant (MOI 20)
Adeno-Skp2-Fbox mutant (MOI 50)
Adeno-Skp2-Fbox mutant (MOI 100)
D
Cycloheximide Time-course
Relative ERα Protein Levels
1.5
1.0
0.5
0.0
Time (hr) 0 0.5 1 2 4 6 8 0 0.5 1 2 4 6 8
Adeno-Control
Adeno-Skp2

Fig.S1

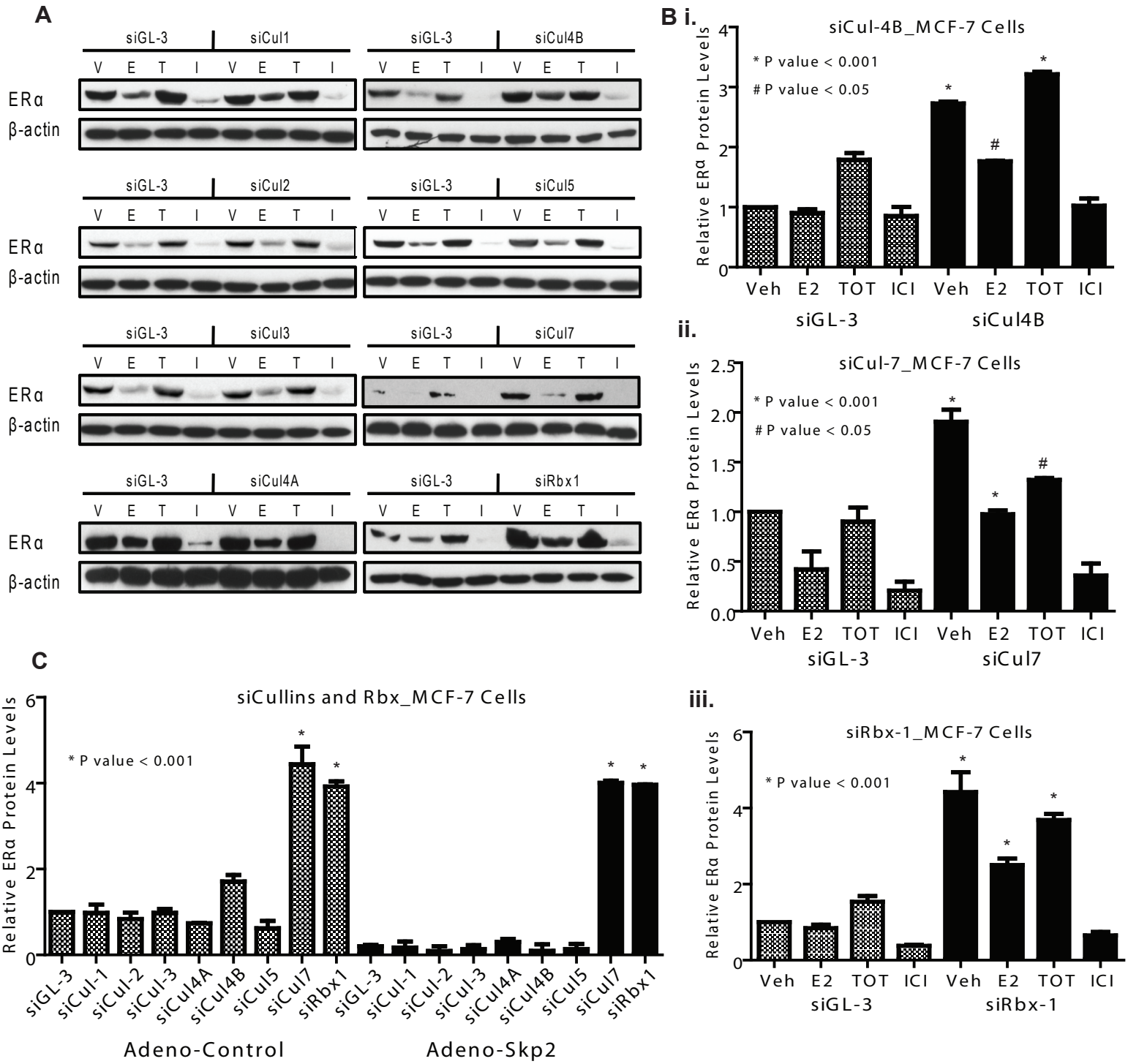
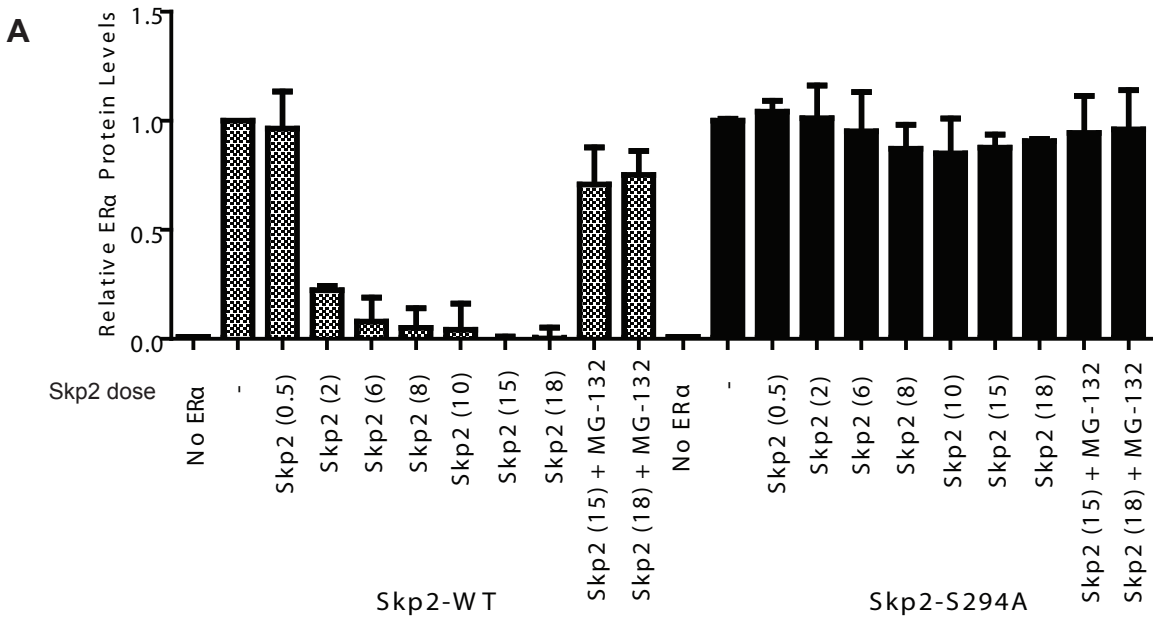
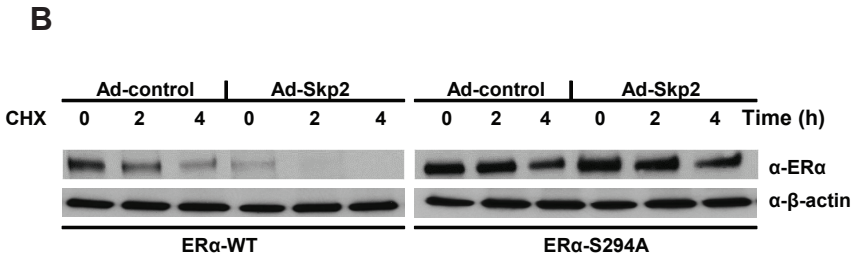
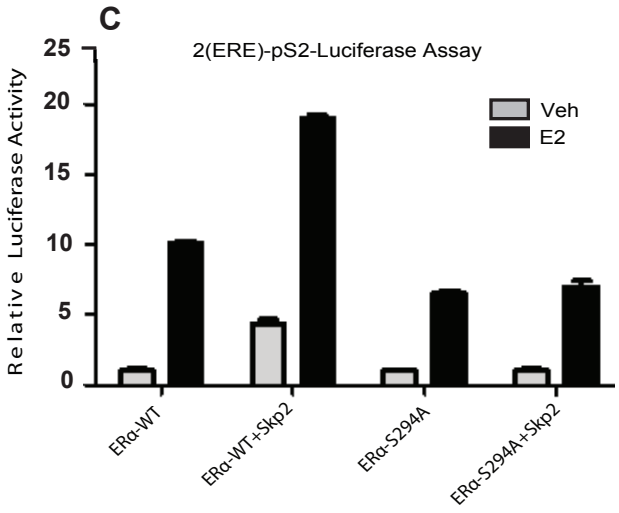
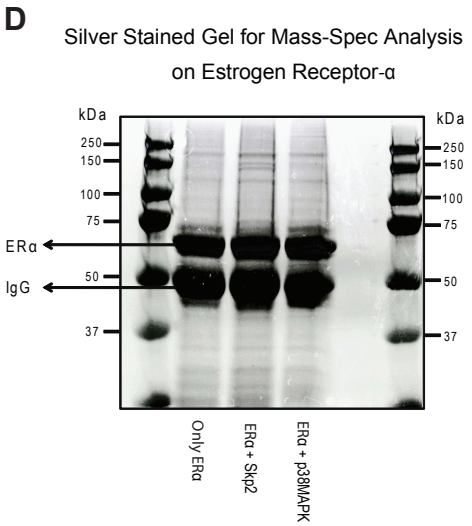

A
siGL-3
siCul1
siCul4B
siCul2
siCul5
siCul3
siCul7
siCul4A
siRbx1
V E T I
ERα
β-actin
B i.
siCul-4B_MCF-7 Cells
* P value < 0.001
# P value < 0.05
Relative ERα Protein Levels
Veh E2 TOT ICI Veh E2 TOT ICI
siGL-3
siCul4B
ii.
siCul-7_MCF-7 Cells
siGL-3
siCul7
C
siCullins and Rbx_MCF-7 Cells
siGL-3 siCul-1 siCul-2 siCul-3 siCul4A siCul4B siCul5 siCul7 siRbx1
Adeno-Control
Adeno-Skp2
iii.
siRbx-1_MCF-7 Cells
siRbx-1

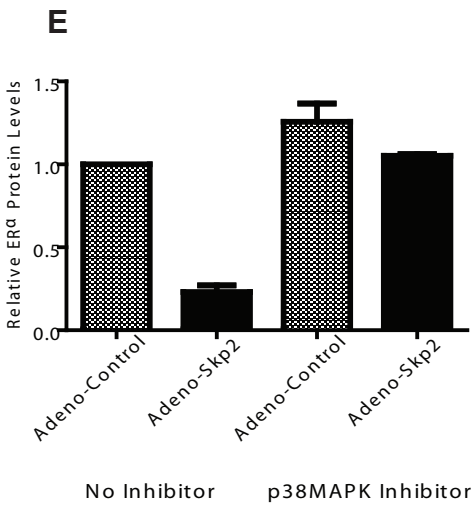
Fig.S2


A
Relative ERα Protein Levels
1.5
1.0
0.5
0.0
Skp2 dose
No ERα
-
Skp2 (0.5)
Skp2 (2)
Skp2 (6)
Skp2 (8)
Skp2 (10)
Skp2 (15)
Skp2 (18)
Skp2 (15) + MG-132
Skp2 (18) + MG-132
No ERα
-
Skp2 (0.5)
Skp2 (2)
Skp2 (6)
Skp2 (8)
Skp2 (10)
Skp2 (15)
Skp2 (18)
Skp2 (15) + MG-132
Skp2 (18) + MG-132
Skp2-WT
Skp2-S294A


B
Ad-control
Ad-Skp2
Ad-control
Ad-Skp2
CHX
0 2 4 0 2 4 0 2 4 0 2 4 Time (h)
α-ERα
α-β-actin
ERα-WT
ERα-S294A


C
2(ERE)-pS2-Luciferase Assay
Relative Luciferase Activity
25
20
15
10
5
0
Veh
E2
ERα-WT
ERα-WT+Skp2
ERα-S294A
ERα-S294A+Skp2


D
Silver Stained Gel for Mass-Spec Analysis
on Estrogen Receptor-α
kDa
250
150
100
75
50
37
ERα
IgG
Only ERα
ERα + Skp2
ERα + p38MAPK


E
Relative ERα Protein Levels
1.5
1.0
0.5
0.0
Adeno-Control
Adeno-Skp2
Adeno-Control
Adeno-Skp2
No Inhibitor
p38MAPK Inhibitor

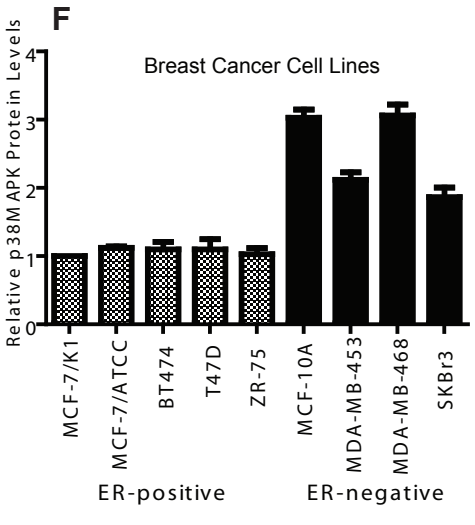

F
Breast Cancer Cell Lines
Relative p38MAPK Protein Levels
4
3
2
1
0
MCF-7/K1
MCF-7/ATCC
BT474
T47D
ZR-75
MCF-10A
MDA-MB-453
MDA-MB-468
SKBr3
ER-positive
ER-negative

Fig.S3

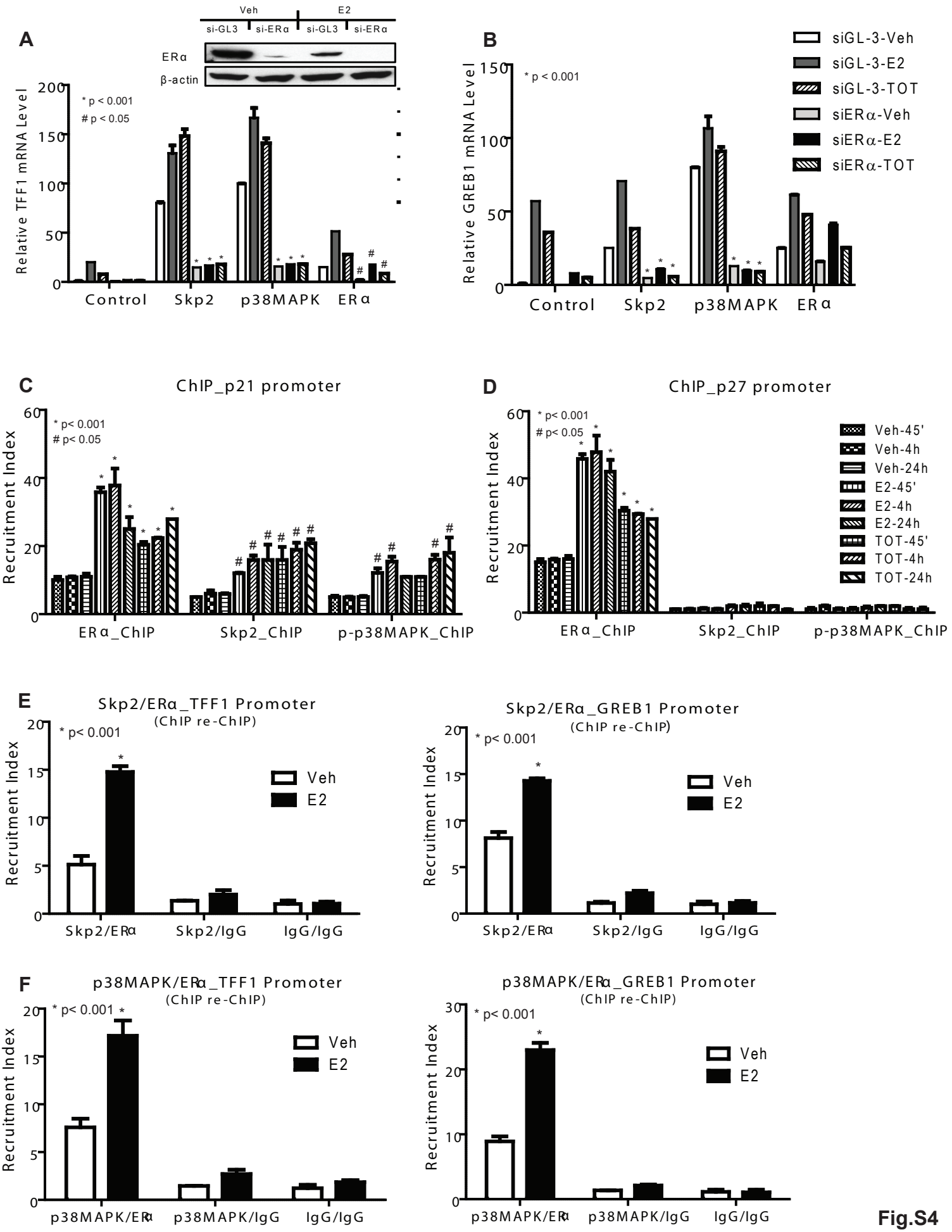
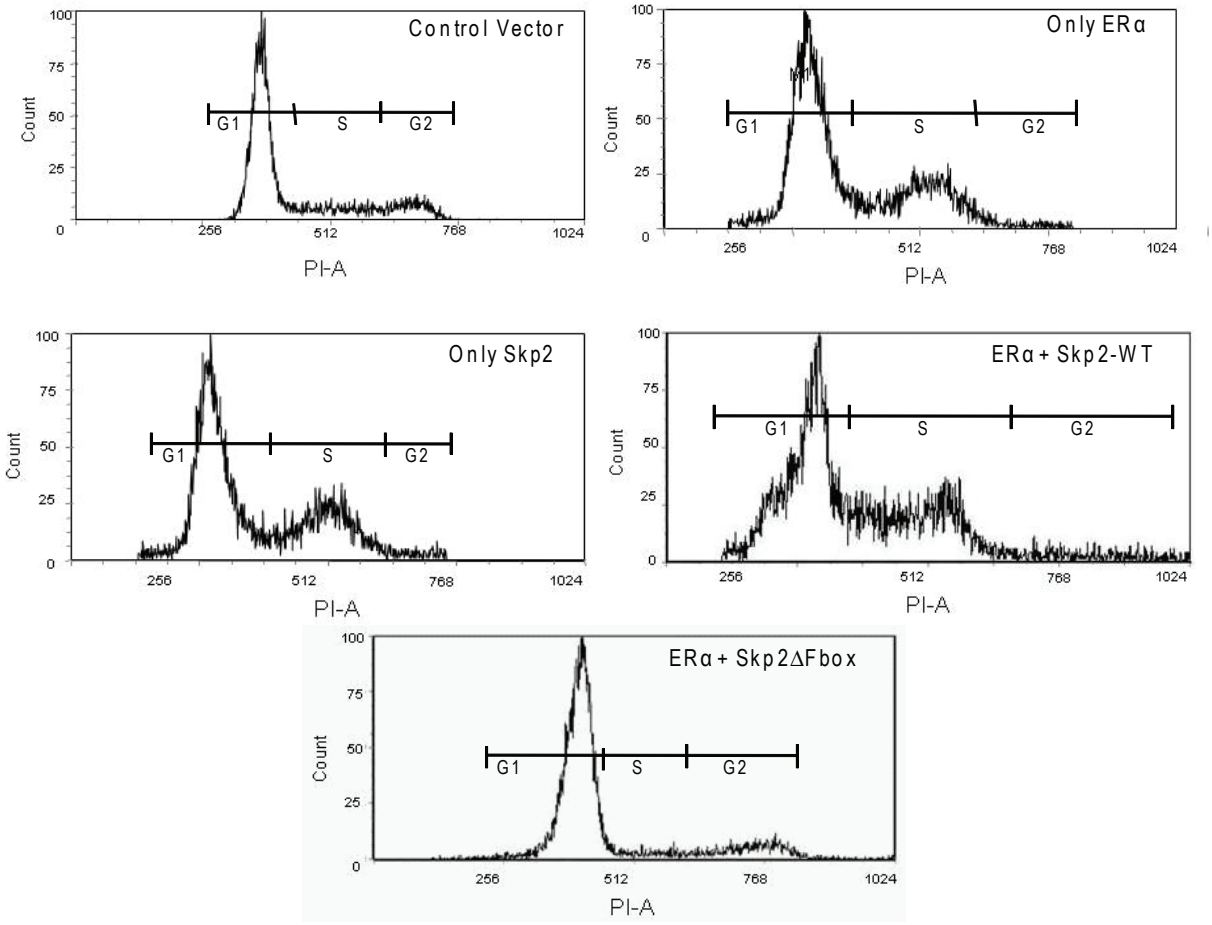

A
Veh
E2
si-GL3
si-ERα
si-GL3
si-ERα
ERα
β-actin
Relative TFF1 mRNA Level
* p < 0.001
# p < 0.05
Control
Skp2
p38MAPK
ERα
B
siGL-3-Veh
siGL-3-E2
siGL-3-TOT
siERα-Veh
siERα-E2
siERα-TOT
Relative GREB1 mRNA Level
* p < 0.001
Control
Skp2
p38MAPK
ERα
C
ChIP_p21 promoter
Recruitment Index
* p< 0.001
# p< 0.05
ERα_ChIP
Skp2_ChIP
p-p38MAPK_ChIP
D
ChIP_p27 promoter
Recruitment Index
* p< 0.001
# p< 0.05
Veh-45'
Veh-4h
Veh-24h
E2-45'
E2-4h
E2-24h
TOT-45'
TOT-4h
TOT-24h
ERα_ChIP
Skp2_ChIP
p-p38MAPK_ChIP
E
Skp2/ERα_TFF1 Promoter
(ChIP re-ChIP)
Recruitment Index
* p< 0.001
Veh
E2
Skp2/ERα
Skp2/IgG
IgG/IgG
Skp2/ERα_GREB1 Promoter
(ChIP re-ChIP)
Recruitment Index
* p< 0.001
Veh
E2
Skp2/ERα
Skp2/IgG
IgG/IgG
F
p38MAPK/ERα_TFF1 Promoter
(ChIP re-ChIP)
Recruitment Index
* p< 0.001
Veh
E2
p38MAPK/ERα
p38MAPK/IgG
IgG/IgG
p38MAPK/ERα_GREB1 Promoter
(ChIP re-ChIP)
Recruitment Index
* p< 0.001
Veh
E2
p38MAPK/ERα
p38MAPK/IgG
IgG/IgG

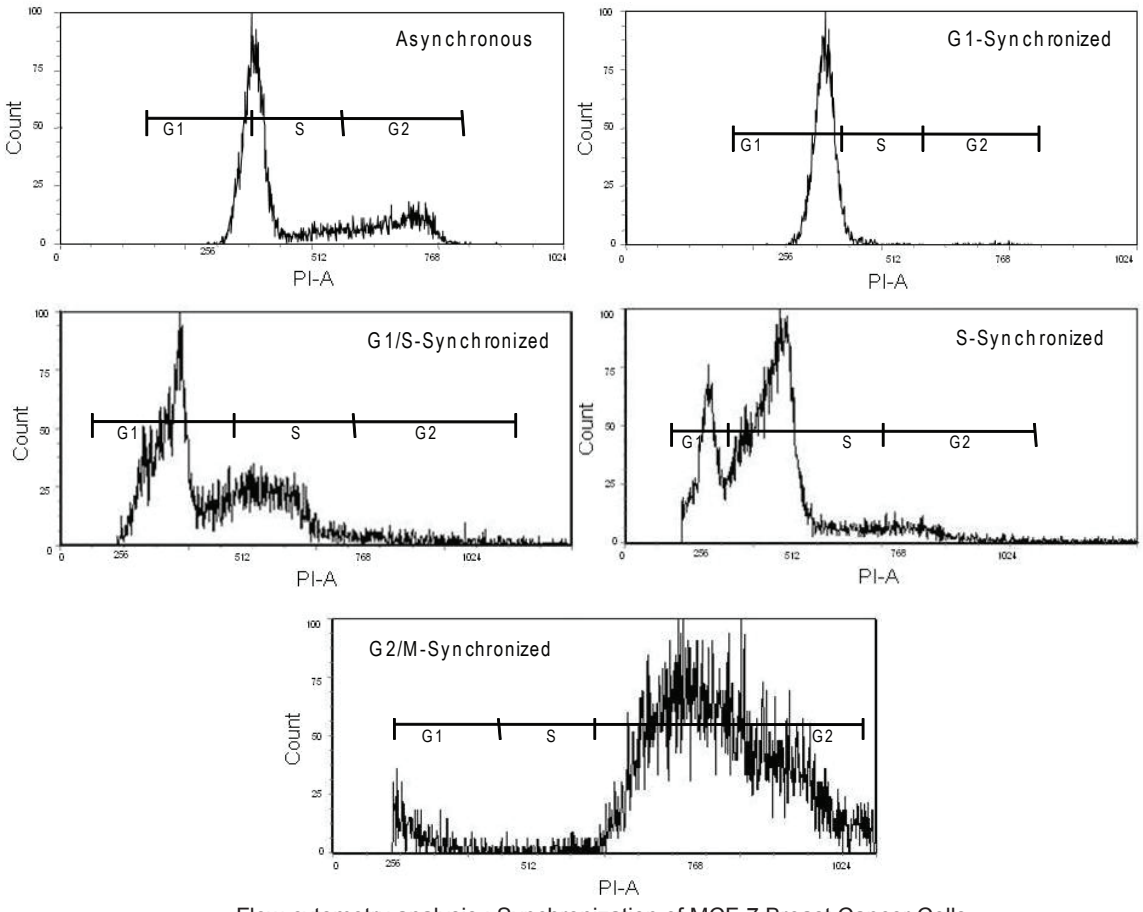
Fig.S4

G



Flow-cytometry analysis: S-phase Entry of MCF-7 Breast Cancer Cells

H



Flow-cytometry analysis : Synchronization of MCF-7 Breast Cancer Cells

Fig.S4

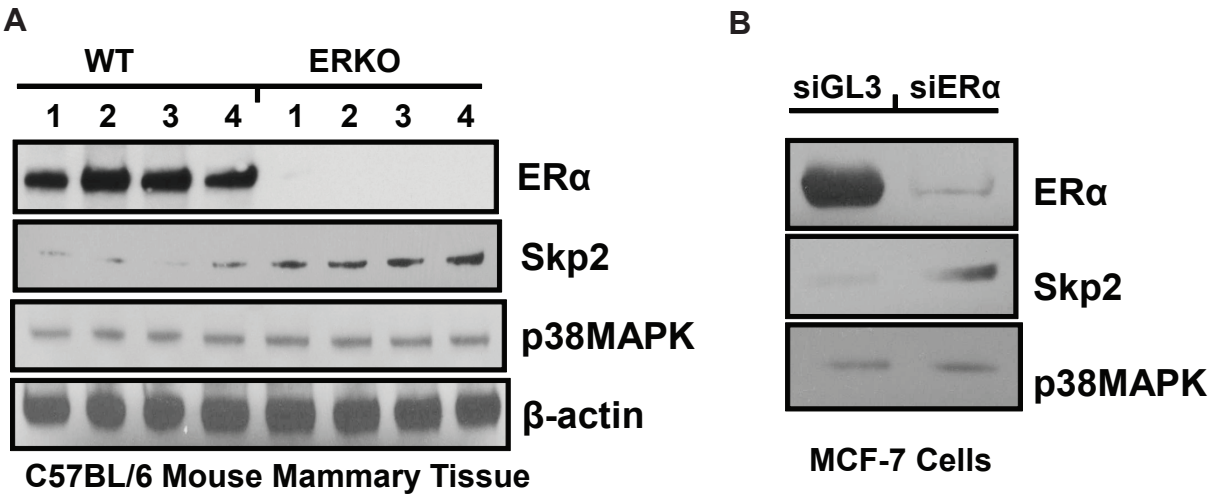
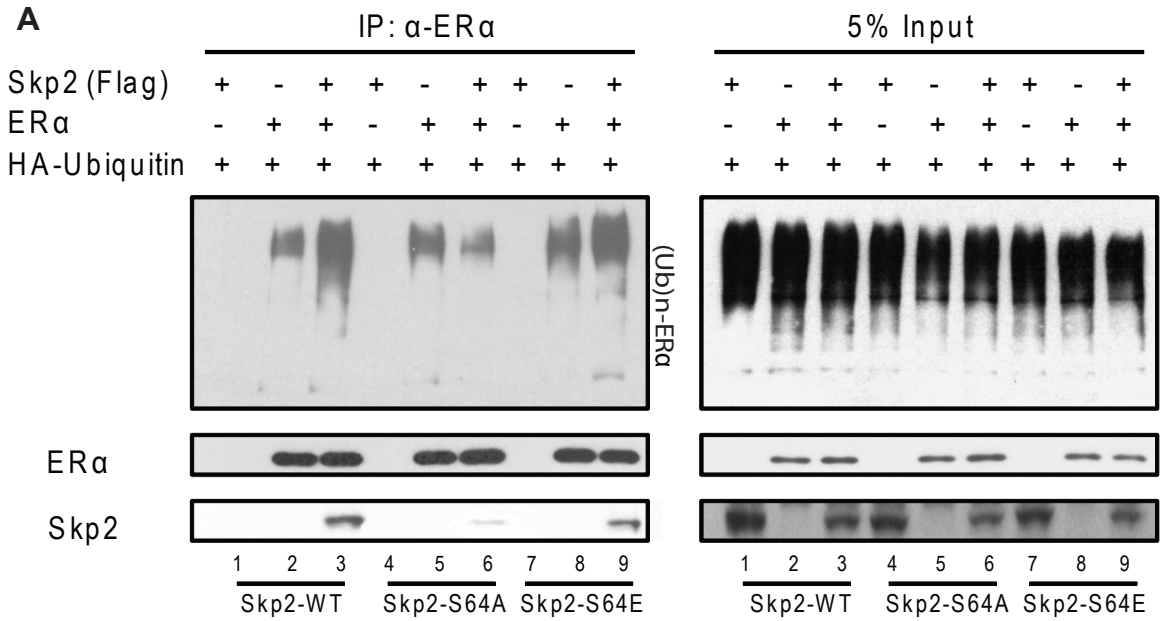
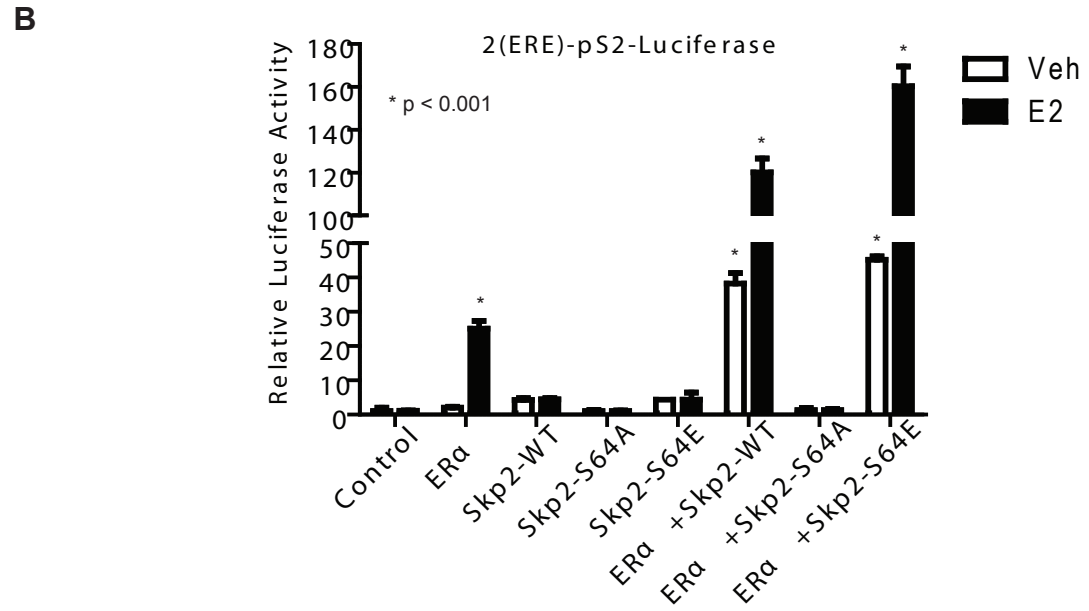

A
WT
ERKO
1 2 3 4 1 2 3 4
ERα
Skp2
p38MAPK
β-actin
C57BL/6 Mouse Mammary Tissue
B
siGL3
siERα
ERα
Skp2
p38MAPK
MCF-7 Cells

Fig.S5


A
IP: α-ERα
5% Input
Skp2 (Flag)
ERα
HA-Ubiquitin
(Ub)n-ERα
ERα
Skp2
1 2 3 4 5 6 7 8 9
Skp2-WT
Skp2-S64A
Skp2-S64E


B
2(ERE)-pS2-Luciferase
* p < 0.001
Relative Luciferase Activity
Veh
E2
Control
ERα
Skp2-WT
Skp2-S64A
Skp2-S64E
ERα +Skp2-WT
ERα +Skp2-S64A
ERα +Skp2-S64E

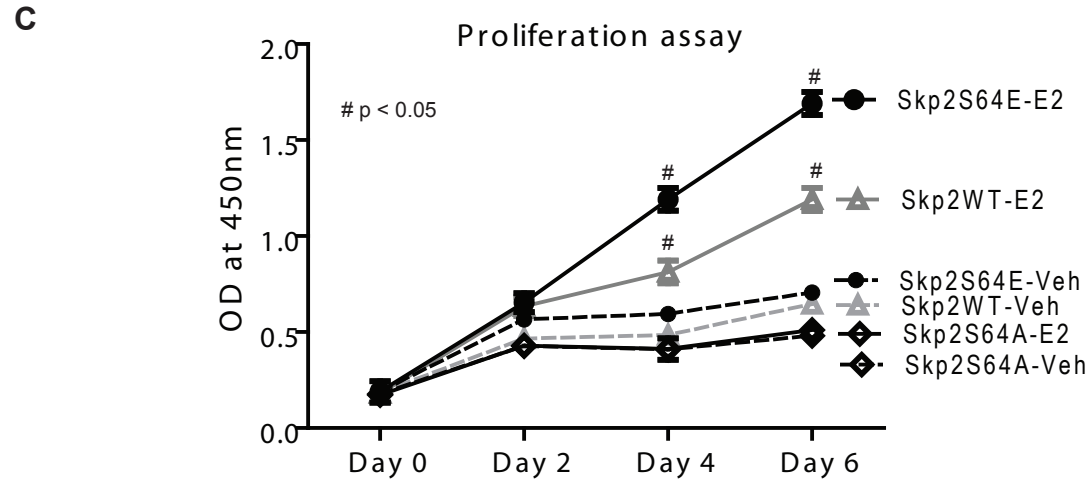
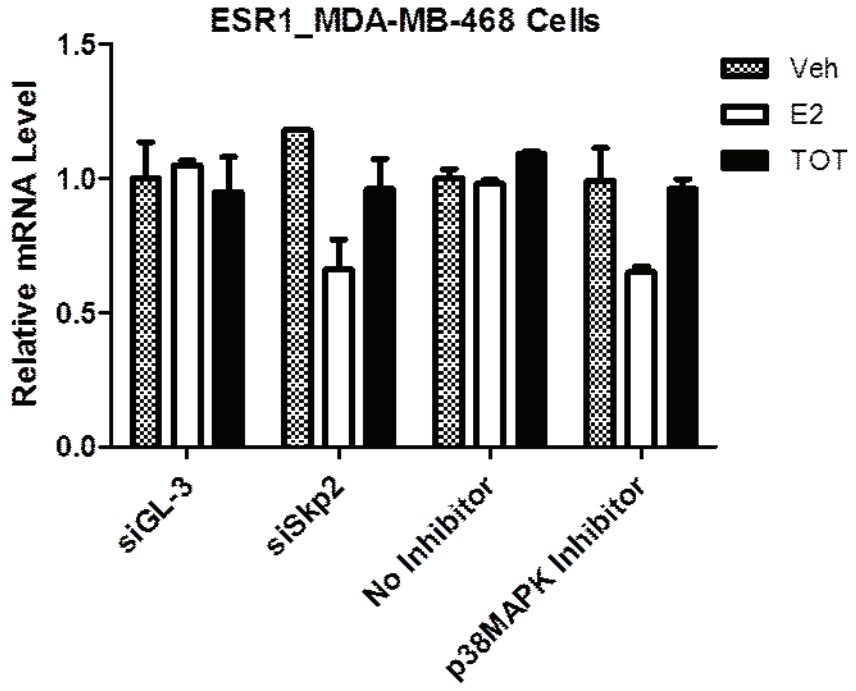

C
Proliferation assay
# p < 0.05
OD at 450nm
Day 0
Day 2
Day 4
Day 6
Skp2S64E-E2
Skp2WT-E2
Skp2S64E-Veh
Skp2WT-Veh
Skp2S64A-E2
Skp2S64A-Veh

Fig.S6

A


ESR1_MDA-MB-468 Cells
Relative mRNA Level
1.5
1.0
0.5
0.0
Veh
E2
TOT
siGL-3
siSkp2
No Inhibitor
p38MAPK Inhibitor

B

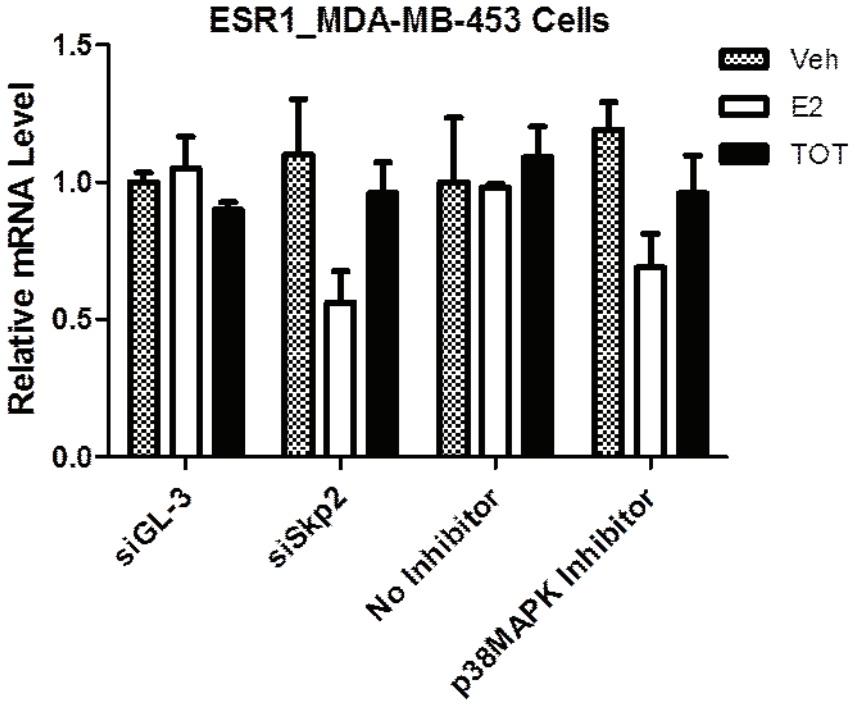
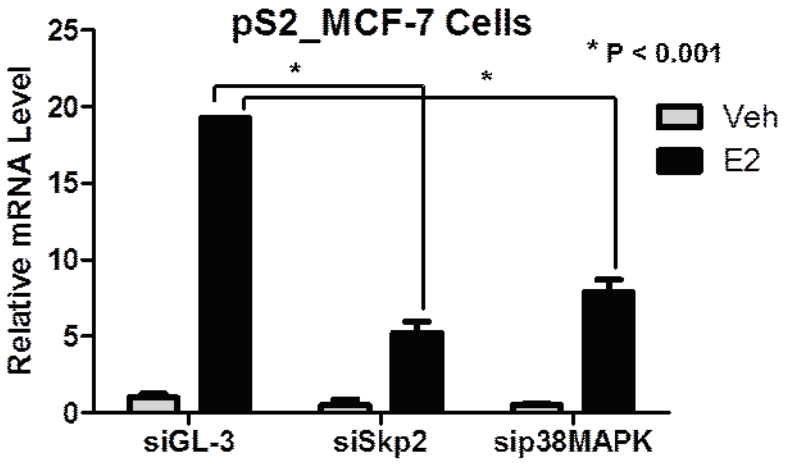

ESR1_MDA-MB-453 Cells
Relative mRNA Level
1.5
1.0
0.5
0.0
Veh
E2
TOT
siGL-3
siSkp2
No Inhibitor
p38MAPK Inhibitor

Fig.S7

A


pS2_MCF-7 Cells
* P < 0.001
Relative mRNA Level
25
20
15
10
5
0
*
*
Veh
E2
siGL-3
siSkp2
sip38MAPK

B

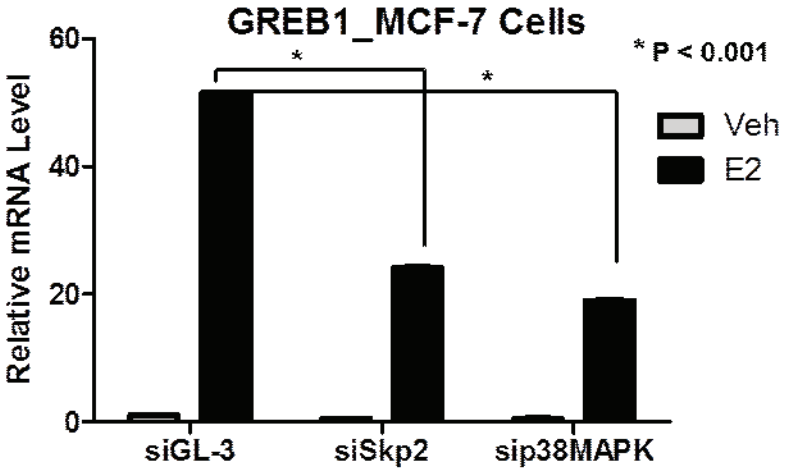

GREB1_MCF-7 Cells
* P < 0.001
Relative mRNA Level
60
40
20
0
*
*
Veh
E2
siGL-3
siSkp2
sip38MAPK

Fig.S8