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Supplementary Materials for

GREB1: An evolutionarily conserved protein with a glycosyltransferase domain links ERα glycosylation and stability to cancer

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Supplementary Figure Titles and Legends

Fig. S1: *GREB1* KO in ER^{+ve} breast cancer cell lines.

(**A**) Relative mRNA expression of *GREB1* was analyzed by qPCR in a panel of ERα^{+ve} or ERα^{-ve} cell lines. n = 2.

(**B**) gRNAs were designed to target the locus about 100bp downstream of ATG site in *GREB1*'s Exon 2 (upper panel). Genomic DNA of *GREB1*-KO clones was amplified by PCR and sequenced (lower panel). Unless specified, *GREB1*-KO clone 441 was used in subsequent experiments shown in the paper.

(**C**) GREB1 levels in MCF7-WT and *GREB1*-KO derivative cell lines with or without E2 stimulation.

(**D**) The growth of MCF7-WT and *GREB1*-KO cell lines was assessed by fluorimetric viability assay. n = 6; * $p < 0.05$ by two-way ANOVA with Holm-Sidak's multiple comparisons test.

(**E**) Growth of MCF7-WT and *GREB1*-KO cell lines was assessed by colony-formation assay using crystal violet staining. Photo credit: Sultan Abda Neja, Institute of Molecular and Cell Biology, A*STAR.

(**F**-**G**) MCF7-WT or *GREB1*-KO cells were subcutaneously injected into the flanks of the same NOD/SCID mice, each cell line on one side. The resulting tumors in these mice were harvested (**F**) and weighted (**G**). n = 8; **** $p < 0.0001$ by unpaired t-test. Photo credit: Anandhkumar Raju, Institute of Molecular and Cell Biology, A*STAR

(**H**) BT474-WT and BT474-*GREB1*-KO cell lines were treated with or without E2 stimulation. Levels of indicated proteins were analyzed by western blot.

(**I**) T47D-WT and T47D-*GREB1*-KO cell lines were treated with or without E2 stimulation. Levels of indicated proteins were analyzed by western blot.

(**J**) Growth of BT474-WT, BT474-*GREB1*-KO, T47D-WT and T47D-*GREB1*-KO cells was analyzed by colony formation assay with crystal violet staining. Photo credit: Eun Myoung Shin, Institute of Molecular and Cell Biology, A*STAR

Fig. S2: GREB1 modulates ERα signaling with direct interaction.

(A-E) MCF7-WT and *GREB1*-KO cells were transduced with Vec or *GREB1* expression construct.

(**A**) Protein levels were analyzed by western blot.

(**B**) mRNA expression levels were analyzed by qPCR. n = 2; ns = not significant, ** p < 0.01 by one-way ANOVA with Holm-Sidak's multiple comparisons test.

(**C**) Growth of transduced MCF7-WT and *GREB1*-KO cell lines was analyzed by colony formation assay with crystal violet staining. Photo credit: Sultan Abda Neja, Institute of Cell and Molecular Biology, A*STAR.

(**D**-**E**) ChIP of ERα was performed in transduced MCF7-WT and *GREB1*-KO cells. ERα recruitment at indicated promoters *GREB1* (**D**) and *XBP1* (**E**) were analyzed by qPCR. n = 2; ns = not significant, * p < 0.05, ** p < 0.01 by one-way ANOVA with Holm-Sidak's multiple comparisons test.

(**F**) Cytoplasmic (C) and nuclear (N) fractions of indicated cell lines were biochemically separated and blotted for the indicated proteins.

(**G**) *ESR1* constructs with corresponding regions were generated as depicted in this schematic.

(**H**) FLAG pulldown was performed and analyzed for indicated proteins by western blot in MCF7-WT cells expressing the ESR1 constructs. ns, non-specific band.

Fig. S3: GREB1 GT domain is important for GREB1 mediated cell proliferation.

(**A**) Multiple sequence alignment of the TAGT family of GT domains including GREB1 and its orthologs, J-base glycosyltransferases and phage TAGTs. Proteins are denoted by their accession numbers and species name. Red-filled circles indicate residues mutated in this study to create the GT-Mut construct.

(**B**) GREB1 was expressed in *S. cerevisiae.* Using the same lysates, O-GalNAcylation levels (left panel) and O-GlcNAcylation (right panel) were analyzed by western blot.

(**C**) HEK293T cells were transfected with OGT, FLAG-ERα together with either Vec or GREB1 expression constructs. FLAG-pulldown was performed; protein levels were analyzed by western blot.

Fig. S4: GREB1 O-GlcNAcylates ERα at T553 and S554 to regulate its stability.

(**A**) Percentage of glycosylated peptides at indicated sites.

(**B**) Densitometry analysis for ERα bands from western blot in Fig. 4C. n=4, **p<0.01, by two-way ANOVA.

(**C**) Densitometry analysis for ERα bands from western blot in Fig. 4D. n=4, non-significant (ns), by two-way ANOVA.

(**D-E**) MCF7-WT cells transduced with ERα-WT or ERα-2M were transfected with control siRNA (Cnt) or OGT siRNA for 48 hours and subsequently subjected to qRT-PCR; n=3; * p <0.05; *** p <0.001 by Student's t-test (**D**) and western blot (**E**).

(**F**) MCF7-WT ectopically expressing ERα-WT cells were transfected with siOGT. 48 hours post-transfection, cells were subjected to FLAG-pulldown, and O-GlcNAcylation of ERα were examined by western blot analysis.

(**G**) MCF7-WT cells transduced with ERα-WT or ERα-2M were transfected with siRNA Control (Cnt) or siOGT for 48 hours and subsequently subjected to cycloheximide

(CHX) treatment. The amount of remaining ERα after CHX treatment was analyzed by western blot.

(**H**) Western blots of CHX chase assays of cells with and without OGT knockdown by siRNA were analyzed by densitometry. Relative remaining protein was computed as the densitometry ratio between CHX treated and un-treated conditions, n=2.

(**I-J**) MCF7-WT cells (**I**) and GREB1-KO cells (**J**) were transduced with either shControl (shCnt) or shXBP1 and subsequently analyzed by qRT-PCR of indicated target genes in HBP pathway. n = 2; * $p < 0.05$, by unpaired t-tests.

(**K**-**M**) MCF7-WT cells transduced with ERα-WT were transfected with either control siRNA (siCnt) or siXBP1 were subjected to E2 treatment. mRNA levels of XBP1 were analyzed by qRT-PCR. * $p < 0.05$; ** $p < 0.01$ by Student's t-test (**K**). O-GlcNAcylation of ERα was examined by FLAG M2 pulldown followed by western analysis (**L**) and densitometry analysis which compute the ratio of O-GlcNAc band-1 and Flag- ERα band-2 (**M**).

Fig. S5: *GREB1* and breast cancer prognosis

(**A-B**) Kaplan Meier plots in a cohort of breast cancer patients regardless of treatments, plotting tumor's (**A**) *ESR1* and (**B**) *GREB1* mRNA as a function of overall patient survival.

(**C**-**D**) Kaplan Meier plots in a cohort of breast cancer patients treated with endocrine therapies, plotting tumor's (**C**) *ESR1* and (**D**) *GREB1* mRNA as a function of overall patient survival.

(**E**) Kaplan Meier plots in a cohort of $ERα^{-ve}$ breast cancer patients, plotting *GREB1* expression as a function of overall patient survival.

Supplementary Table Legends:

Table S1: ERα's interactors identified by mass spectrometry from MCF7-WT cells

FLAG-pulldown was performed in MCF7-WT cells expressing ERα-WT, and eluates were analyzed by mass spectrometry in the same experiment described in Fig. 7. Identified proteins are listed alphabetically, and highly similar proteins are grouped together.

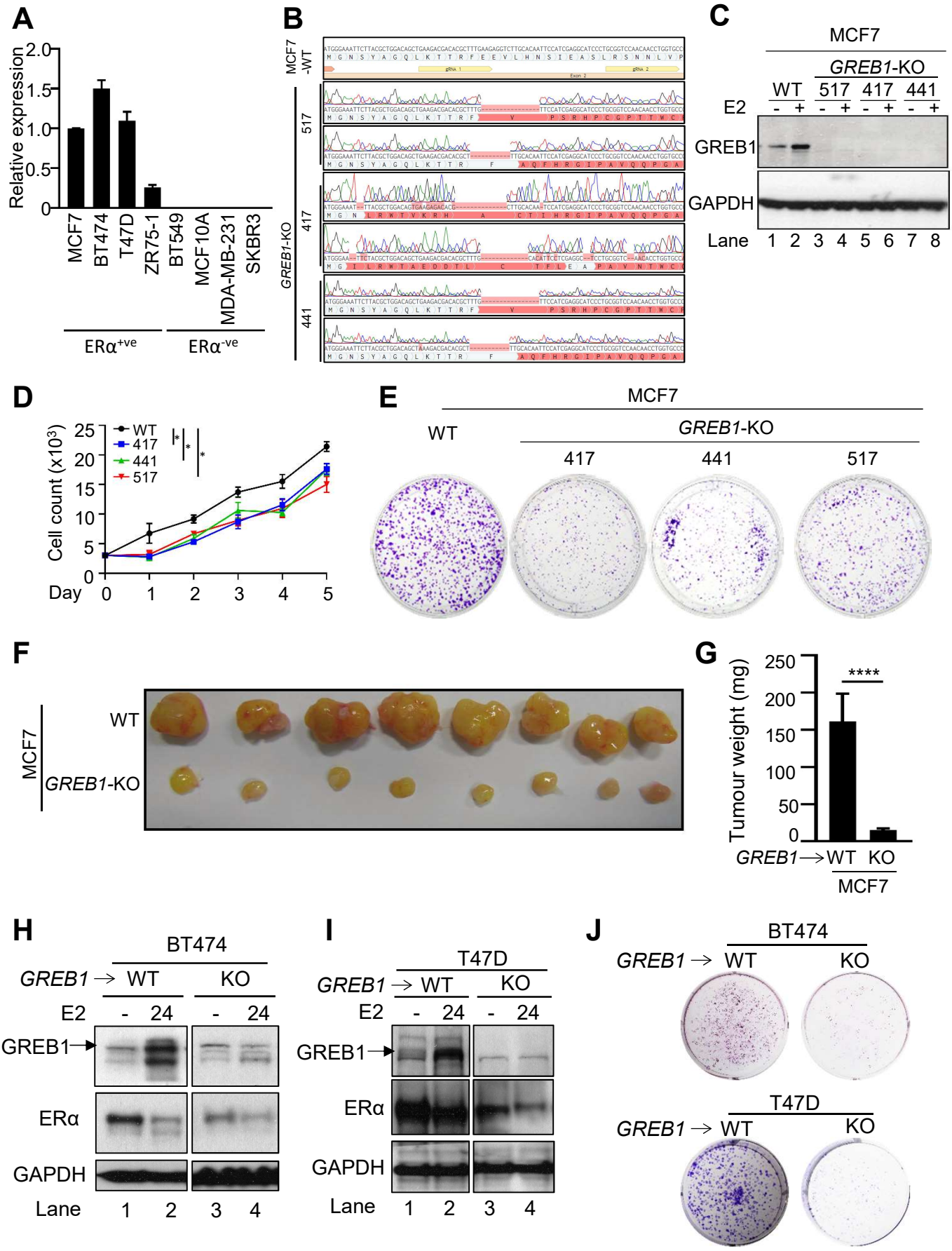
A
Relative expression
2.0
1.5
1.0
0.5
0
MCF7
BT474
T47D
ZR75-1
BT549
MCF10A
MDA-MB-231
SKBR3
ERα+ve
ERα-ve
B
MCF7 -WT
GREB1-KO
517
417
441
C
MCF7
GREB1-KO
WT
517
417
441
E2
- + - + - + - +
GREB1
GAPDH
Lane 1 2 3 4 5 6 7 8
D
Cell count (x10³)
25
20
15
10
5
0
WT
417
441
517
*
*
*
Day 0 1 2 3 4 5
E
MCF7
WT
GREB1-KO
417
441
517
F
MCF7
WT
GREB1-KO
G
Tumour weight (mg)
250
200
150
100
50
0
****
GREB1 → WT KO
MCF7
H
BT474
GREB1 → WT KO
E2 - 24 - 24
GREB1
ERα
GAPDH
Lane 1 2 3 4
I
T47D
GREB1 → WT KO
E2 - 24 - 24
GREB1
ERα
GAPDH
Lane 1 2 3 4
J
BT474
GREB1 → WT KO
T47D
GREB1 → WT KO

Fig. S1

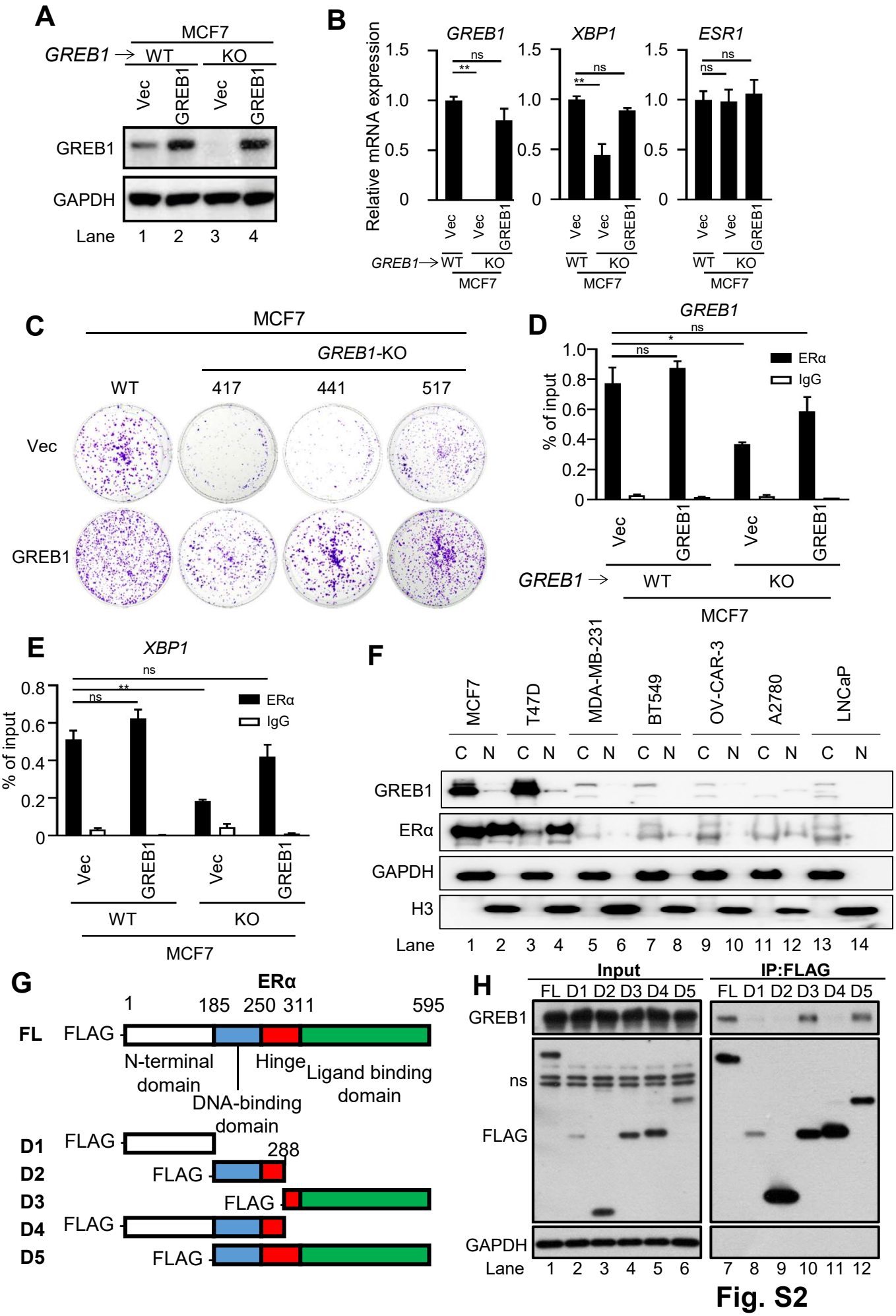
A
MCF7
GREB1 → WT KO
Vec GREB1 Vec GREB1
GREB1
GAPDH
Lane 1 2 3 4
B
GREB1
XBP1
ESR1
Relative mRNA expression
ns
**
GREB1→ WT KO
MCF7
C
MCF7
GREB1-KO
WT 417 441 517
Vec
GREB1
D
GREB1
ns
*
ERα
IgG
% of input
GREB1 → WT KO
MCF7
E
XBP1
F
MCF7 T47D MDA-MB-231 BT549 OV-CAR-3 A2780 LNCaP
C N
GREB1
ERα
GAPDH
H3
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
G
ERα
1 185 250 311 595
FL FLAG
N-terminal domain
Hinge
Ligand binding domain
DNA-binding domain
D1 FLAG 288
D2 FLAG
D3 FLAG
D4 FLAG
D5 FLAG
H
Input
IP:FLAG
FL D1 D2 D3 D4 D5
GREB1
ns
FLAG
GAPDH
Lane 1 2 3 4 5 6 7 8 9 10 11 12

Fig. S2

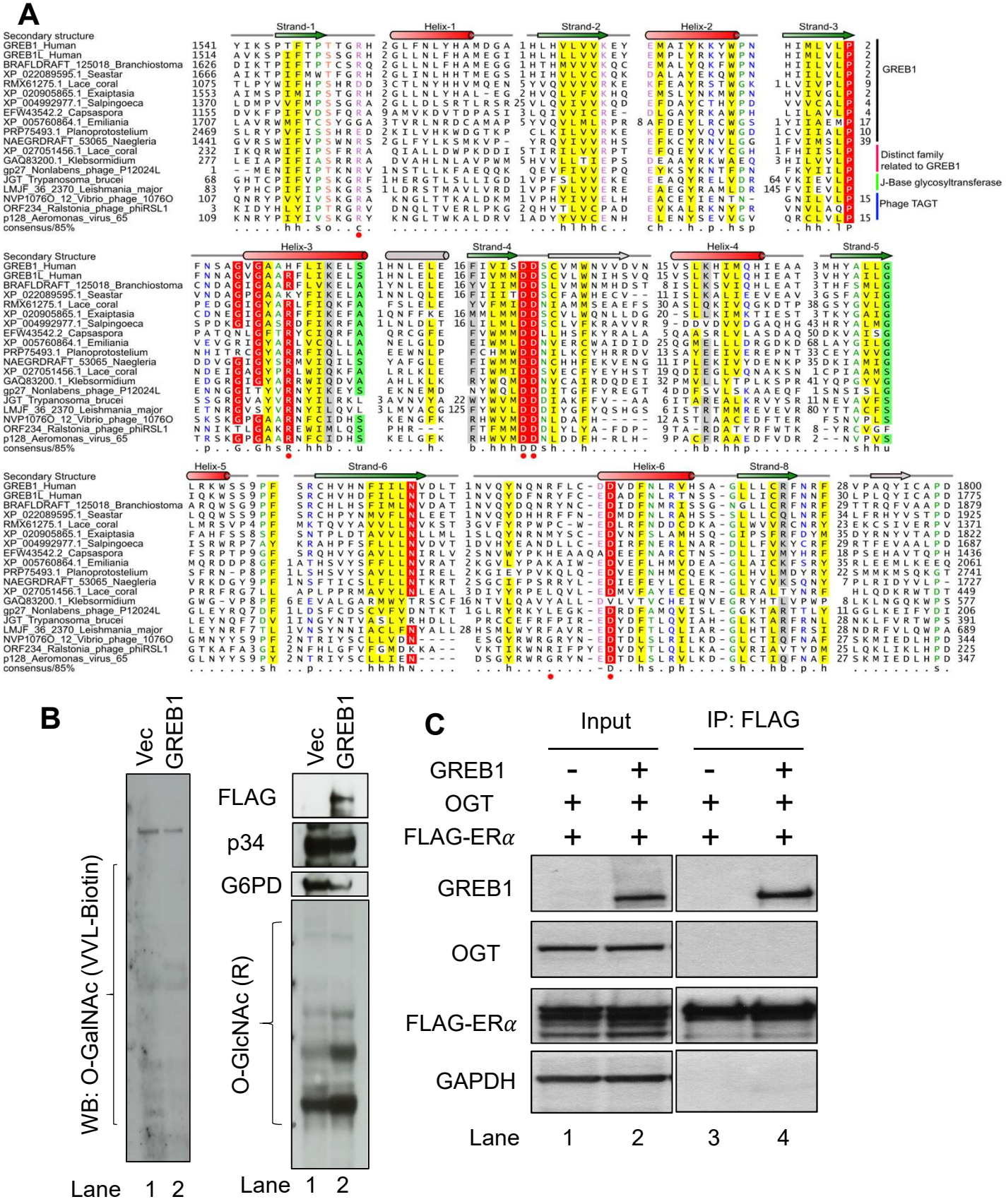
A
Secondary structure
Strand-1
Helix-1
Strand-2
Helix-2
Strand-3
GREB1_Human
GREB1L_Human
BRAFLDRAFT_125018_Branchiostoma
XP_022089595.1_Seastar
RMX61275.1_Lace_coral
XP_020905865.1_Exaiptasia
XP_004992977.1_Salpingoeca
EFW43542.2_Capsaspora
XP_005760864.1_Emiliania
PRP75493.1_Planoprotostelium
NAEGRDRAFT_53065_Naegleria
XP_027051456.1_Lace_coral
GAQ83200.1_Klebsormidium
gp27_Nonlabens_phage_P12024L
JGT_Trypanosoma_brucei
LMJF_36_2370_Leishmania_major
NVP1076O_12_Vibrio_phage_1076O
ORF234_Ralstonia_phage_phiRSL1
p128_Aeromonas_virus_65
consensus/85%
GREB1
Distinct family related to GREB1
J-Base glycosyltransferase
Phage TAGT
Helix-3
Strand-4
Helix-4
Strand-5
Helix-5
Strand-6
Helix-6
Strand-8
B
Vec
GREB1
WB: O-GalNAc (VVL-Biotin)
Lane 1 2
FLAG
p34
G6PD
O-GlcNAc (R)
Lane 1 2
C
Input
IP: FLAG
GREB1 - + - +
OGT + + + +
FLAG-ERα + + + +
GREB1
OGT
FLAG-ERα
GAPDH
Lane 1 2 3 4

Fig. S3

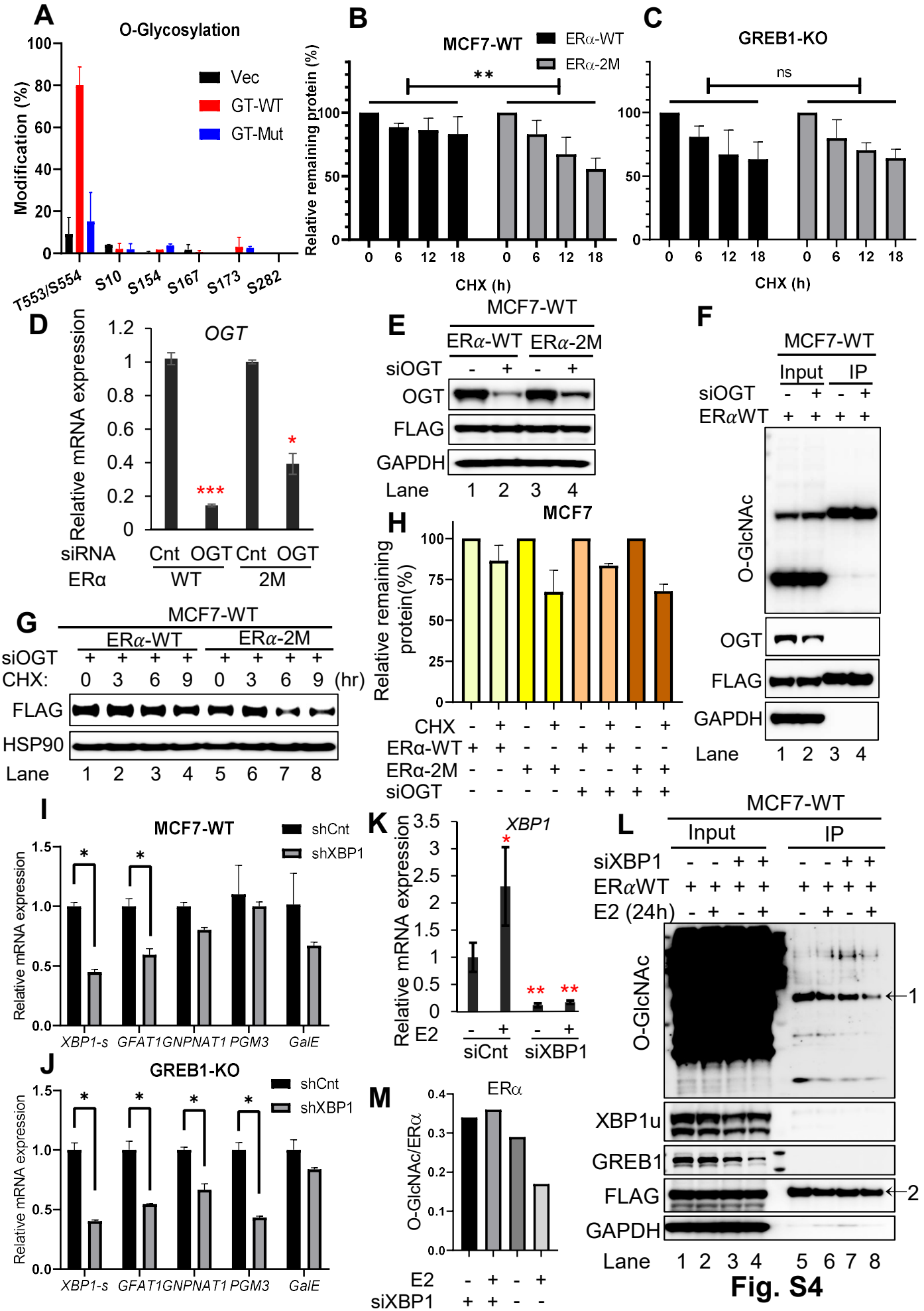
A
O-Glycosylation
Modification (%)
Vec
GT-WT
GT-Mut
T553/S554
S10
S154
S167
S173
S282
B
MCF7-WT
ERα-WT
ERα-2M
Relative remaining protein (%)
**
CHX (h)
C
GREB1-KO
ns
CHX (h)
D
OGT
Relative mRNA expression
siRNA
ERα
Cnt
OGT
WT
2M
E
MCF7-WT
ERα-WT
ERα-2M
siOGT
OGT
FLAG
GAPDH
Lane
F
MCF7-WT
Input
IP
siOGT
ERαWT
O-GlcNAc
OGT
FLAG
GAPDH
Lane
G
MCF7-WT
ERα-WT
ERα-2M
siOGT
CHX:
(hr)
FLAG
HSP90
Lane
H
MCF7
Relative remaining protein(%)
CHX
ERα-WT
ERα-2M
siOGT
I
MCF7-WT
shCnt
shXBP1
Relative mRNA expression
XBP1-s
GFAT1
GNPNAT1
PGM3
GalE
J
GREB1-KO
shCnt
shXBP1
Relative mRNA expression
XBP1-s
GFAT1
GNPNAT1
PGM3
GalE
K
XBP1
Relative mRNA expression
E2
siCnt
siXBP1
L
MCF7-WT
Input
IP
siXBP1
ERαWT
E2 (24h)
O-GlcNAc
XBP1u
GREB1
FLAG
GAPDH
Lane
M
ERα
O-GlcNAc/ERα
E2
siXBP1

Fig. S4

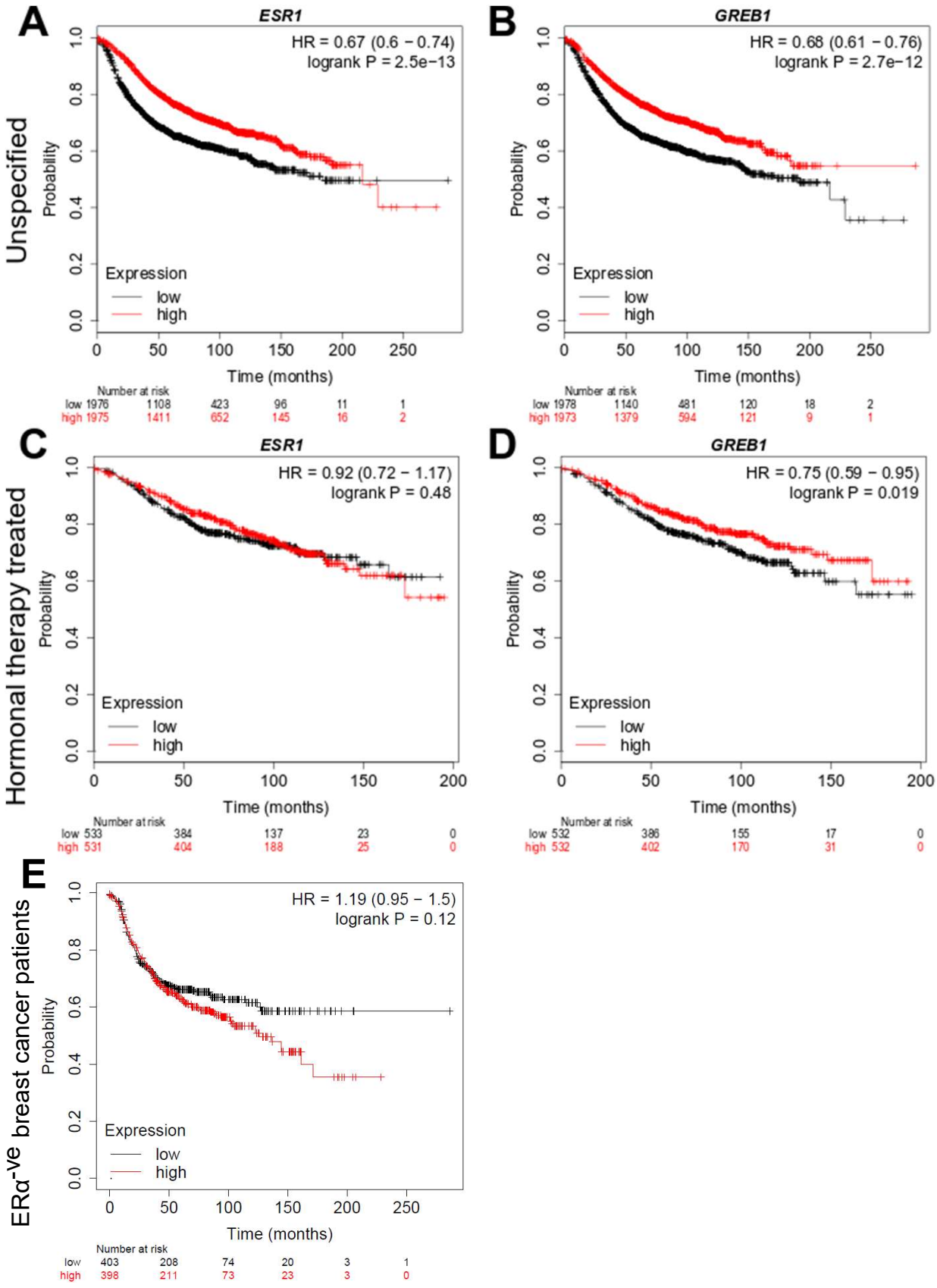
A
ESR1
HR = 0.67 (0.6 − 0.74)
logrank P = 2.5e−13
Unspecified
Probability
Expression
low
high
Time (months)
Number at risk
low 1976 1108 423 96 11 1
high 1975 1411 652 145 16 2
B
GREB1
HR = 0.68 (0.61 − 0.76)
logrank P = 2.7e−12
Probability
Expression
low
high
Time (months)
Number at risk
low 1978 1140 481 120 18 2
high 1973 1379 594 121 9 1
C
ESR1
HR = 0.92 (0.72 − 1.17)
logrank P = 0.48
Hormonal therapy treated
Probability
Expression
low
high
Time (months)
Number at risk
low 533 384 137 23 0
high 531 404 188 25 0
D
GREB1
HR = 0.75 (0.59 − 0.95)
logrank P = 0.019
Probability
Expression
low
high
Time (months)
Number at risk
low 532 386 155 17 0
high 532 402 170 31 0
E
HR = 1.19 (0.95 − 1.5)
logrank P = 0.12
ERα-ve breast cancer patients
Probability
Expression
low
high
Time (months)
Number at risk
low 403 208 74 20 3 1
high 398 211 73 23 3 0

Fig. S5

Table S1: ERα's interactors identified by mass spectrometry from MCF7-WT cells

26S proteasome proteins and subunits	CDC5L	HADHA	NONO	SLAIN2
Actin proteins Coatomer subunits	CHERP	HDAC1	NOP2	SLC25A3/5/6/11/13
ATP synthase subunits	CKAP4	HIST1H1C	NPM1	SMARC A5/D2
Elongation factors	CLNS1A	HIST1H2B	NRIP1	SMCHD1
Eukaryotic translation initiation factors	CLTC	HIST1HB1	NT5C2	SNW1
Exosome complex proteins	CNBP	HP1BP3	OTUD4	SPRR2A
Heat-sock proteins	CORO1C	HRNR	PABPC 1/4	SPTBN1
Heterogeneous nuclear ribonucleoproteins	CT45A10	HSD17B10	PCBP 1/2	SRP14
Keratin proteins	CTTN	ILF 2/3	PDIA6	SRPK1/2
Myosin proteins	DDB1	IMMT	PFKFB 2/3	SRPRB
Ribosomal proteins	DDX helicases	IVBS1ABP	PFM	SSB
Small nuclear ribonucleo proteins	DHX 9/15/30/36/57	JAK1	PKP3	SSBP1
Splicing factors	DIMT1	KCTD 5/17/2	PLRG1	STAU1
Tubulin proteins	DNAJA 2/3	KDELR2	POMGNT2	STK38
ACTR 2/3	DNAJC 9/13/21	KHDRBS1	PPM1B	STRAP
AIFM1	DRG1	KIF11	PPP2 CB/R1A/R2A	SVIL
AKAP8	DSG1	KPNA2	PRKRA	TAB1
ALB	DSP	L1RE1	PRMT5	TAF4
ALYREF	DSTN	LARP1	PRPF 3/4/19/31	TBL2
ANXA2	ELAVL1	LCE 1C/2B	PRRC2A	THRAP3
AP2 A1/B1	ELMSAN1	LCE2B	PTBP 1/3	TMED10
ARCN1	EMD	LIMA1	PTS	TMOD3
ARMCX3	EPB41L5	LMNA	PURA	TMPO
ASPH	EPRS	LMNB1	PURB	TOP1
ATAD3A	ERH	LRPPRC	PYM1	TRA2A
ATXN2L	**ESR1**	LRRC47	QPRT	TRIM28
BCLAF1	EWSR1	LUC7L2	RBBP4	TRMT1L
BMS1	FLG2	LYAR	RBM 10/39	TSPYL1
BRI3BP	FMR1	MAGED2	RBM4	TTN
C1QBP	FTSJ3	MAP 4/7	RFC1	TXNDC12
C7orf50	FUS	MATR3	RIO1	U2SOP2
CAD	FXR1	MCM 3/5	RPN1	UPF1/2
CALM1	G3BP 1/2	MOB2	RRBP1	USP10
CAMSAP3	G6PD	MOGS	RS27A	WDR5
CAPNS1	GAPDH	MOV10	RSBN1	WDR77
CAPRIN1	GLYR1	MSI2	SART3	XP32
CAPZB	**GREB1**	MTDH	SCYL2	XRCC 5/6
CASP14	GREB1L	MYBBP1A	SEMA3B	XRN2
CBX3	GTPBP4	NAP1L1	SERBP1	YBX 1/3
CCAR2	H1FX	NAT10	SGPL1	YME1L1
CCT 5/6A/7	H2AFZ	NCL	SIRT1	YWHA Q/Z