AKT-Phosphorylated FOXO1 Suppresses ERK Activation and Chemoresistance by Disrupting IQGAP1-MAKP Interaction

Pan et al.

Appendix

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Appendix Methods

Tandem affinity purification of proteins

293T cells were transfected with SFB backbone vector or SFB-tagged FOXO1. 24 h after transfection, cells were lysed by NETN buffer (20 mM Tris-HCl, pH8.0, 100 mM NaCl, 1mM EDTA, 0.5% Nonidet P-40) with 50 mM β-glycerophosphate, 10 mM NaF, and 1μg/mL pepstatin-A at 4°C for 3 h. The supernatant were incubated with streptavidin sepharose beads (GE) at 4°C overnight. The beads were washed with NETN buffer for three times and then eluted by 2 mM biotin (Sigma) for 1 h at 4°C twice. The elution products were incubated with S-protein agarose beads (Novagen) overnight at 4°C and after three times wash the products bound to S-protein agarose beads were subjected to SDS-PAGE and visualized by silver staining or Code-blue staining.

Protein sequence analysis by LC-MS/MS

The identities of eluted proteins from tandem affinity purification were revealed by mass spectrometry performed by the Taplin Biological Mass Spectrometry Facility at Harvard. Briefly, excised gel bands were cut into approximately 1 mm^3 pieces. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure (Shevchenko et al, 1996). Gel pieces were washed and dehydrated with acetonitrile for 10 min. followed by removal of acetonitrile. Pieces were then completely dried in a speed-vac. Rehydration of the gel pieces was with 50 mM ammonium bicarbonate solution containing 12.5 ng/μl modified sequencing-grade trypsin (Promega) at 4ºC. After 45 min., the excess trypsin solution was removed and replaced with 50 mM ammonium bicarbonate solution to just cover the gel pieces. Samples were then placed in a 37ºC room overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The

extracts were then dried in a speed-vac (~1 h). The samples were then stored at 4ºC until analysis.

On the day of analysis the samples were reconstituted in 5 - 10 µl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 5 µm C18 spherical silica beads into a fused silica capillary (125 µm inner diameter x ~20 cm length) with a flame-drawn tip (Peng & Gygi, 2001). After equilibrating the column each sample was loaded via a Famos auto sampler (LC Packings) onto the column. A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid).

As peptides eluted they were subjected to electrospray ionization and then entered into an LTQ Velos ion-trap mass spectrometer (Thermo Fisher Scientific). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program, Sequest (Thermo Fisher Scientific) (Eng et al, 1994). Spectral matches were manually examined and multiple identified peptides per protein were required.

Appendix References

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Huang H, Regan KM, Lou Z, Chen J, Tindall DJ (2006) CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. *Science* **314:** 294-297

Peng J, Gygi SP (2001) Proteomics: the move to mixtures. *J Mass Spectrom* **36:** 1083-1091

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68:** 850-858

Appendix Table S1. FOXO1-associated proteins identified by tandem affinity purification and mass spectrometry*

Name of Protein Identified	ID	Number of Peptides Identified
PRKDC	IPI:IPI00296337.2	61
XRCC6	IPI:IPI00644712.4	38
FOXO1	IPI:IPI00289866.6	36
XRCC5	IPI:IPI00220834.8	35
YWHAE	IPI:IPI00000816.1	26
MYH9	IPI:IPI00019502.3	24
MYH10	IPI:IPI00397526.3	22
HSPA8	IPI:IPI00003865.1	16
DDX21	IPI:IPI00015953.3	16
RPS3_IPI:IPI00011253.3	IPI:IPI00011253.3	16
TUBB2C	IPI:IPI00007752.1	12
HSPA9	IPI:IPI00007765.5	12
HNRNPU	IPI:IPI00479217.1	11
RPA1	IPI:IPI00020127.1	10
PARP1	IPI:IPI00449049.5	10
YWHAQ	IPI:IPI00018146.1	9
ACTB	IPI:IPI00021439.1	8
YWHAZ	IPI:IPI00021263.3	8
HSPA5	IPI:IPI00003362.2	8
RPL4	IPI:IPI00003918.6	8
ACTA2	IPI:IPI00008603.1	8
SLC25A5	IPI:IPI00007188.5	8
ACACA	IPI:IPI00011569.2	8
MYBBP1A	IPI:IPI00005024.3	8
NPM1	IPI:IPI00220740.1	7
YWHAH	IPI:IPI00216319.3	7
YWHAB	IPI:IPI00216318.5	7
DDX5	IPI:IPI00017617.1	7
RPS3A	IPI:IPI00419880.6	7
YWHAG	IPI:IPI00220642.7	6
RPL3	IPI:IPI00550021.4	6
IQGAP1	IPI:IPI00009342.1	6
RPL7	IPI:IPI00030179.3	6
EPRS	IPI:IPI00013452.9	6
RPL6	IPI:IPI00329389.8	6
HNRNPM	IPI:IPI00171903.2	6
C1QBP	IPI:IPI00014230.1	5
CKAP4	IPI:IPI00141318.2	5
TUBA4A	IPI:IPI00007750.1	5
RPS2	IPI:IPI00013485.3	5
RPS6	IPI:IPI00021840.1	5
RPS4X	IPI:IPI00217030.1	5
CYFIP1	IPI:IPI00644231.3	5
EEF1A2	IPI:IPI00014424.1	5
ACTBL2	IPI:IPI00003269.1	5
DHX9	IPI:IPI00844578.1	5
TUBB	IPI:IPI00011654.2	4
SSBP1	IPI:IPI00029744.1	4
HSPA1A	IPI:IPI00304925.5	4

RPS8	IPI:IPI00216587.9	4
SPTAN1	IPI:IPI00744706.2	4
TUBA1C	IPI:IPI00166768.3	4
RSL1D1	IPI:IPI00008708.5	4
RPL18	IPI:IPI00215719.6	4
HIST1H4J	IPI:IPI00453473.6	4
RPL13P12	IPI:IPI00397611.2	4
VIM	IPI:IPI00418471.6	4
RPL8	IPI:IPI00012772.8	4
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RPS14	IPI:IPI00026271.5	3
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GNL3	IPI:IPI00003886.3	3
KPNA2	IPI:IPI00002214.1	3
SYNCRIP	IPI:IPI00018140.3	3
DDX3X	IPI:IPI00215637.5	3
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FLNA	IPI:IPI00302592.2	3
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PTCD3	IPI:IPI00783302.1	3
RPS13	IPI:IPI00221089.5	3
SFN	IPI:IPI00013890.2	3
RPA2	IPI:IPI00013939.3	3
SERBP1	IPI:IPI00410693.3	2
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SETD7	IPI:IPI00028366.2	2
DHX30	IPI:IPI00411733.4	2
DNAJA1	IPI:IPI00012535.1	2
MRPS9	IPI:IPI00641924.2	2
PTBP1	IPI:IPI00179964.5	2
SLC25A13	IPI:IPI00007084.3	2
PTPLAD1	IPI:IPI00008998.3	2
ILF2	IPI:IPI00005198.2	2
DBT	IPI:IPI00003944.1	2
USP7	IPI:IPI00003965.5	2
MATR3	IPI:IPI00017297.1	2
RAD50	IPI:IPI00107531.1	2
EP300	IPI:IPI00020985.4	2
HNRNPA3	IPI:IPI00419373.1	2
RPL21P19	IPI:IPI00247583.5	2
HSPA7	IPI:IPI00011134.2	2
RPL11	IPI:IPI00376798.3	2
NAP1L1	IPI:IPI00023860.1	2
TUBB1	IPI:IPI00006510.1	2
RPL27	IPI:IPI00219155.5	2
GTPBP4	IPI:IPI00385042.4	2
HSPA1L	IPI:IPI00301277.1	2
SPTBN1	IPI:IPI00005614.6	2
NCL	IPI:IPI00444262.3	2
LRPPRC	IPI:IPI00783271.1	2

PC	IPI:IPI00299402.1	2
RPL13A	IPI:IPI00304612.9	2
RPS18	IPI:IPI00013296.3	2
ABCF2	IPI:IPI00005045.1	2
KCTD12	IPI:IPI00060715.1	2

* Proteins with two or more peptides detected are considered confidently identified

Appendix Table S2. Sequences for shRNAs

Gene	Sequence
sh IQGAP1-1	5′-CCGGGCCCACATTGTGCCTTTATTTCTCGAGAAATAAAGGCACAATGTGGGCTTTTTG-3′
sh IQGAP1-2	5′-CCGGCCTCAGATTCAAGACCTATATCTCGAGATATAGGTCTTGAATCTGAGGTTTTTG-3′
sh FOXO1-1	5′-CCGGGCCGGAGTTTAGCCAGTCCAACTCGAGTTGGACTGGCTAAACTCCGGCTTTTTG-3′
sh FOXO1-2	5′-CCGGATCTACGAGTGGATGGTCAACTCGAGTTGACCATCCACTCGTAGATCTTTTTG-3′
sh FOXO3-1	5′-CCGGCAGACCCTCAAACTGACACAACTCGAGTTGTGTCAGTTTGAGGGTCTGTTTTTG-3′
sh FOXO3-4	5′-CCGGGTCACTGCATAGTCGATTCATCTCGAGATGAATCGACTATGCAGTGACTTTTTG-3′

Appendix Figure Legends

Figure S1. Diverse functions of FOXO1 binding proteins, PTEN expression in PCa cell lines and S319 phosphorylation-enhanced interaction of FOXO1 and IQGAP1.

(**A**) Gene ontology analysis reveals the categories ($p \leq 0.05$) of diverse functions of FOXO1 binding proteins identified by tandem affinity purification-coupled mass spectrometry.

(**B**) Western blot analysis of PTEN protein expression in DU145 (PTEN-positive) and LNCaP (PTEN-negative) PCa cell lines. ERK2 was used as loading control.

(**C**) Two replicates of experiments shown in Fig 1F. In vitro protein binding assay. GST and GST-FOXO1-3 (amino acids 211-419) purified from bacteria were subjected to AKT kinase assay with IgG or HA-AKT-CA immunoprecipitated from HA-AKT-CA-transfected C4-2 cells before incubating with in vitro translated Flag-IQGAP1 for protein binding assay.

(**D**) Western blot bands of IQGAP1 proteins in GST pull down and input samples as shown in Fig 1F were quantified first. The quantitative value of IQGAP1 in GST pull down samples was then normalized by the value of IQGAP1 in input samples, and normalized value in each group was further normalized to that in the GST-FOXO1-3 plus IgG group. Quantification and normalization were performed in a similar manner for the western blot bands in the two repeated experiments shown in (**C**). The data from three replicates (n=3) were used to generate the figure shown in this panel.

Figure S2. Quantitative data of the western blots shown in Fig 2.

(**A**) FOXO1 proteins co-IP by IQGAP1 from cell lysates mock treated or treated with λ protein phosphatase as shown in Fig 2A and the repeated experiment (replication 2) were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further

normalized to the value in mock treated group. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**B**) FOXO1 proteins co-IP by IQGAP1 from cells transfected with control vector pcDNA3.1, constitutively active HA-AKT-CA and kinase dead HA-AKT-DN as shown in Fig 2B and the repeated experiment (replication 2) were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further normalized to the value in cells transfected with the control vector pcDNA3.1. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**C**) FOXO1 proteins co-IP by IQGAP1 from cells transfected with control siRNA (siControl) and PTEN-specific siRNA (siPTEN) as shown in Fig 2C and the repeated experiment (replication 2) were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further normalized to the value in cells transfected with siControl. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**D**) FOXO1 proteins co-IP by IQGAP1 from cells mock treated (DMSO) or treated with the PI3K inhibitor LY294002 as shown in Fig 2D and the repeated experiment (replication 2) were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further normalized to the value in cells treated with DMSO. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**E**) IQGAP1 proteins co-IP by Flag-tagged FOXO1 proteins from cells transfected with Flag-FOXO1-WT, T24A, S256A, S319A and T24A/S256A/S319A (or A3) as shown in Fig 2e and the repeated experiment (replication 2) were quantified and normalized to the quantified value of IP-ed Flag-FOXO1 proteins. The normalized values were further normalized to the value in cells transfected with FOXO1-WT. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**F**) IQGAP1 proteins co-IP by Flag-FOXO1 proteins from cells transfected with Flag-tagged, transcription-deficient Flag-FOXO1-537 (can be phosphorylated by AKT) and Flag-FOXO1-537-A3 (resistant to AKT phosphorylation) as shown in Fig 2F and the repeated experiment (replication 2) were quantified and normalized to the quantified value of IP-ed Flag-FOXO1 proteins. The normalized values were further normalized to the value in cells transfected with FOXO1-537. The data from two replicates (n=2) were used to generate the figure shown in this panel.

Figure S3. Quantitative data of the western blots shown in Fig 3.

(**A**) MAPK pathway protein (c-Raf, MEK2 or ERK2) co-IP by IQGAP1 from cells infected with lentivirus expressing control shRNA (shControl) and FOXO1-specific shRNA (shFOXO1) as shown in Fig 3C and the repeated experiment (replication 2) were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further normalized to the value in cells infected with shControl. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**B**) MAPK pathway protein (c-Raf, MEK2 or ERK2) co-IP by IQGAP1 proteins, from cells transfected with control vector (pcDNA3.1), Flag-tagged transcription-deficient Flag-FOXO1-537 (can be phosphorylated by AKT) and Flag-FOXO1-537-A3 (resistant to AKT phosphorylation) as shown in Fig 3D and the repeated experiment (replication 2), were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further normalized to the value in cells transfected with pcDNA3.1. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**C**) MAPK pathway protein (c-Raf, MEK2 or ERK2) co-IP by IQGAP1 proteins, from cells transfected with control vector (pcDNA3.1), Flag-FOXO1-WT, Flag-FOXO1-NESm (primarily in the nucleus) and Flag-FOXO1-NLSm (cytoplasmic form) as shown in Fig 3E and the repeated

experiment (replication 2), were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further normalized to the value in cells transfected with pcDNA3.1. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**D**) Two replicates of experiments shown in Fig 3F. Western blot analysis of whole cell lysate and co-IP samples in LNCaP cells 24 h transfected with indicated plasmids. E.V., empty vector.

(**E**) MAPK pathway protein (c-Raf, MEK2 or ERK2) co-IP by IQGAP1 proteins, from cells transfected with empty vector (EV, pcDNA3.1), HA-tagged FOXO1 peptide FOXO1-S319, phospho-mimicking peptide HA-FOXO1-IQBP-S319E and non-phosphorylatable peptide HA-FOXO1-S319A as shown in Fig 3F, were quantified and normalized to the quantified value of IP-ed IQGAP1. The normalized values were further normalized to the value in cells transfected with EV. Quantification and normalization were performed in a similar manner for the western blot bands in the two repeated experiments shown in (**D**). The data from three replicates (n=3) were used to generate the figure shown in this panel.

Figure S4. Quantitative data of the western blots shown in Fig 4.

(**A**) Western blot bands of p-ERK1/2, in cells infected with lentivirus expressing control shRNA (shControl) or two independent FOXO1-specific shRNAs (#1 and #2) as shown in Fig 4A and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total ERK2. The normalized values were further normalized to the value in cells infected with shFOXO1#1. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**B**) Western blot bands of p-ERK1/2, in cells infected with lentivirus expressing control shRNA (shControl) or two independent FOXO1-specific shRNAs (#1 and #2) with or without shRNA-resistant Flag-tagged FOXO1 (S1R or S2R) as shown in Fig 4B and the repeated experiment

(replication 2), were quantified and normalized to the quantified value of total ERK2. The normalized values were further normalized to the value in cells infected with shFOXO1#1. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**C**) Western blot bands of p-ERK1/2, in cells infected with lentivirus expressing control shRNA (shControl), FOXO1-specific shRNA, and/or IQGAP1-specific shRNA as shown in Fig 4C and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total ERK2. The normalized values were further normalized to the value in cells infected with shFOXO1. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**D**) Western blot bands of p-ERK1/2, in cells transfected with Myc-IQGAP1 with or without Flag-tagged transcription-deficient Flag-FOXO1-537 (can be phosphorylated by AKT) or with or without Flag-FOXO1-537-A3 (resistant to AKT phosphorylation) as shown in Fig 4D and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total ERK2. The normalized values were further normalized to the value in cells transfected with Myc-IQGAP1. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**E**) Western blot bands of p-ERK1/2, in cells transfected with empty vector (EV, pcDNA3.1), Flag-tagged transcription-deficient Flag-FOXO1-537 (can be phosphorylated by AKT) or Flag-FOXO1-537-A3 (resistant to AKT phosphorylation) and with or without EGF treatment as shown in Fig 4E and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total ERK2. The normalized values were further normalized to the value in cells transfected with EV and treated with EGF. The data from two replicates (n=2) were used to generate the figure shown in this panel.

Figure S5. Quantitative data of the western blots shown in Fig 5.

(**A**) Western blot bands of p-AKT-S473 (p-FOXO1-S319 or p-ERK1/2), in LNCaP cells (left panel), C4-2 cells (middle panel) or C4-2B cells (right panel) treated with vehicle (DMSO), the AKT inhibitor MK2206 or the PI3K/mTOR dual inhibitor NVP-BEZ235 as shown in Fig 5A and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total AKT (total FOXO1 or total ERK2). The normalized values were further normalized to the value in cells treated with DMSO. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**B**) Western blot bands of p-AKT-S473 (p-FOXO1-S319 or p-ERK1/2), in LNCaP cells transfected with empty vector (EV) or expression vector for small FOXO1 phospho-mimicking peptide HA-FOXO1-IQBP(SE) and treated with or without the AKT inhibitor MK2206 and/or the new protein synthesis inhibitor cycloheximide (CHX) as shown in Fig 5B and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total AKT (total FOXO1 or total ERK2). The normalized values were further normalized to the value in cells transfected with EV and treated with vehicle. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**C**) Western blot bands of p-AKT-S473 (p-FOXO1-S319 or p-ERK1/2), in LNCaP cells transfected with control vector pcDNA3.1, Flag-FOXO1-NESm or Flag-FOXO1-NLSm and treated with vehicle (DMSO) or the AKT inhibitor MK2206 as shown in Fig 5 C and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total AKT (total FOXO1 or total ERK2). The normalized values were further normalized to the value in cells transfected with pcDNA3.1 and treated with vehicle. The data from two replicates (n=2) were used to generate the figure shown in this panel.

(**D**) Western blot bands of p-AKT-S473 (p-FOXO1-S319 or p-ERK1/2), in LNCaP cells transfected with control shRNA (shControl) or two independent IQGAP1-specific shRNA shIQGAP1-1 and shIQGAP1-2 and treated with or without the AKT inhibitor MK2206 and/or

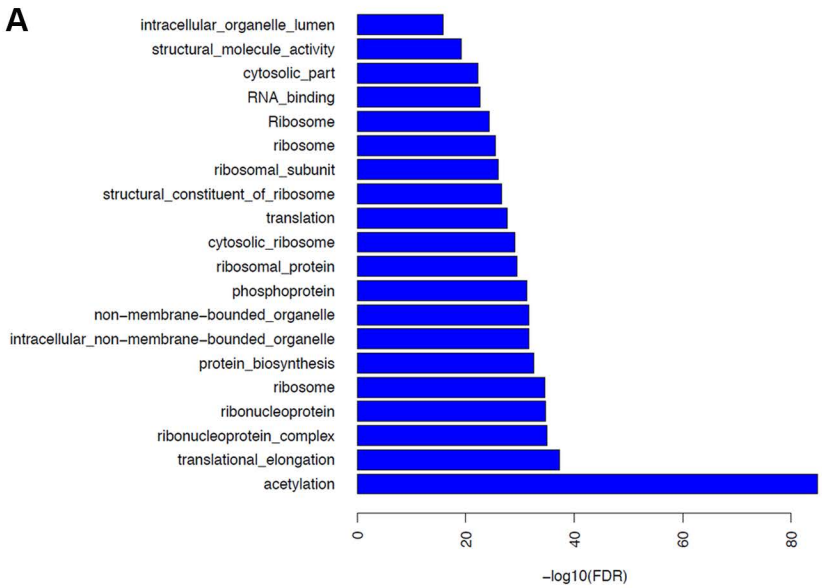
the new protein synthesis inhibitor cycloheximide (CHX) as shown in Fig 5E and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total AKT (total FOXO1 or total ERK2). The normalized values were further normalized to the value in cells transfected with shControl and treated with vehicle. The data from two replicates (n=2) were used to generate the figure shown in this panel.

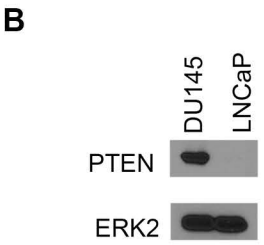
Figure S6. Quantitative data of the western blots shown in Fig 6.

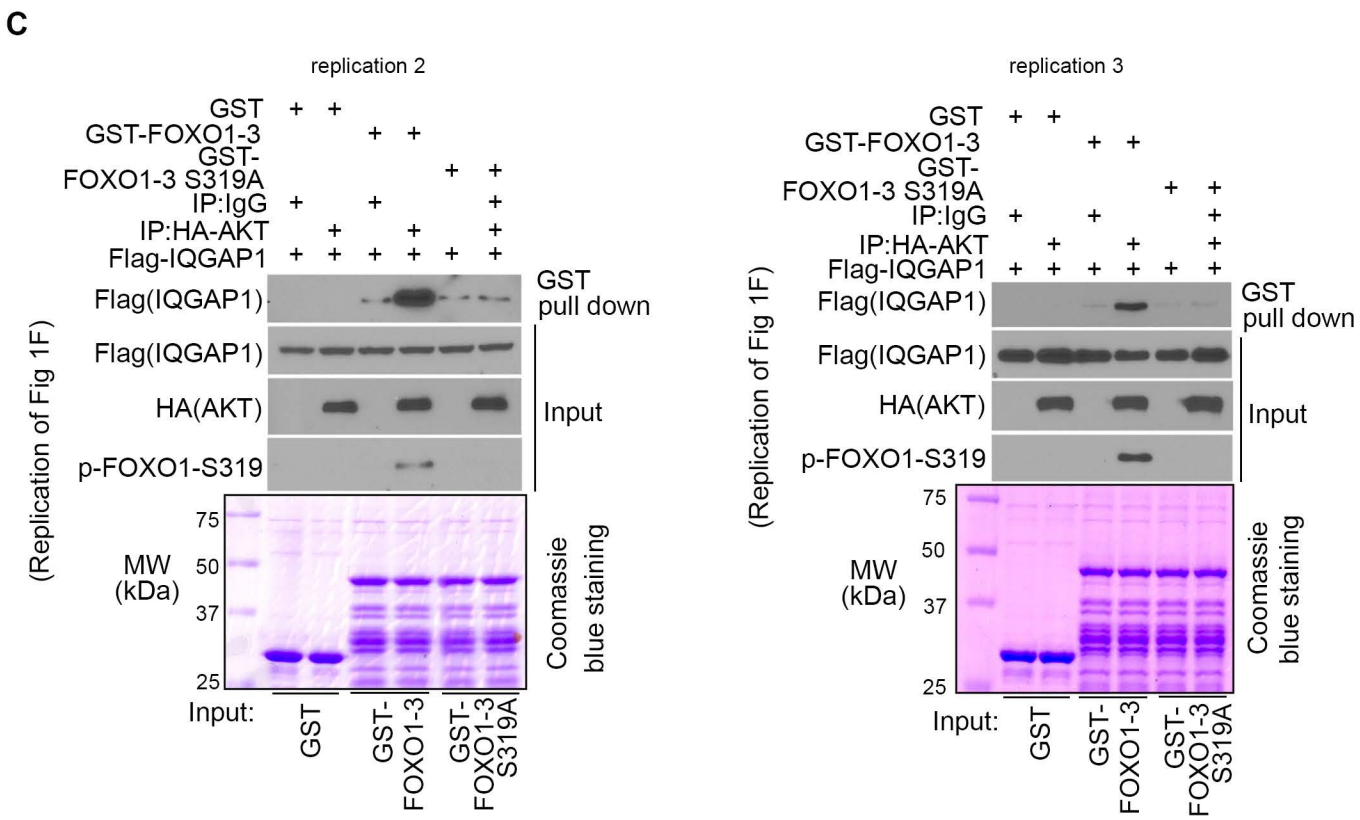
(**A**) Western blot bands of p-AKT-S473 (or p-ERK1/2), in LNCaP (left panel) and BT474 (right panel) cells infected with lentivirus expressing control shRNA (shControl) or IQGAP1-specific shRNA shIQGAP1 and treated with vehicle (DMSO) or paclitaxel as shown in Fig 6A and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total AKT (or total ERK2). The normalized values were further normalized to the value in cells infected with shControl and treated with DMSO. The data from two replicates (n=2) were used to generate the figure shown in this panel.

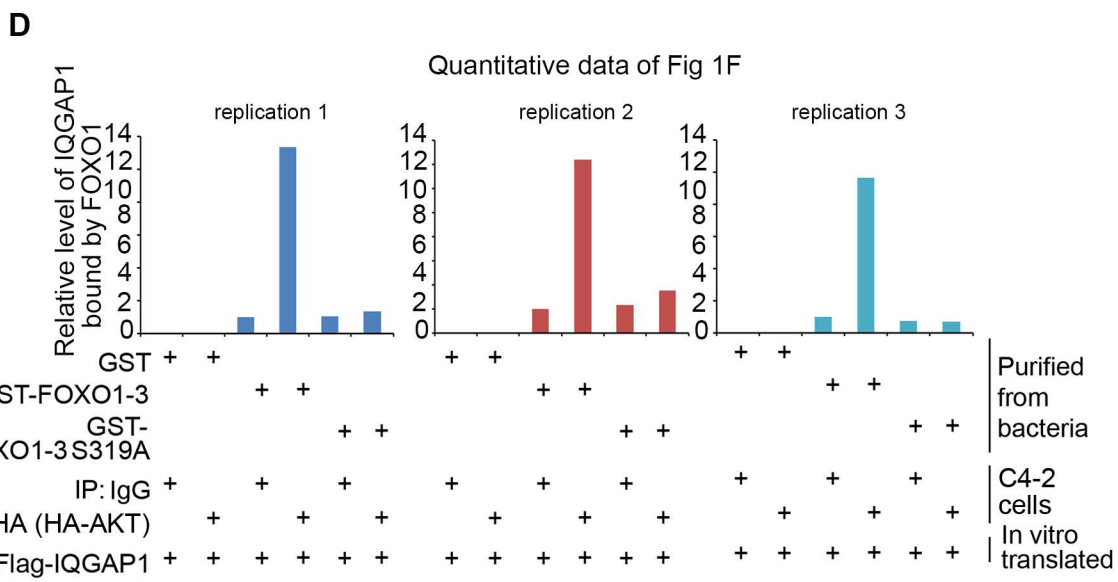
(**B**) Western blot bands of p-AKT-S473 (or p-ERK1/2), in LNCaP (left panel) and BT474 (right panel) cells transfected with empty vector (EV) or expression vector for small FOXO1 phospho-mimicking peptide HA-FOXO1-IQBP(SE) and treated with vehicle (DMSO) or paclitaxel as shown in Fig 6B and the repeated experiment (replication 2), were quantified and normalized to the quantified value of total AKT (or total ERK2). The normalized values were further normalized to the value in cells transfected with EV and treated with DMSO. The data from two replicates (n=2) were used to generate the figure shown in this panel.

Appendix Figure S1

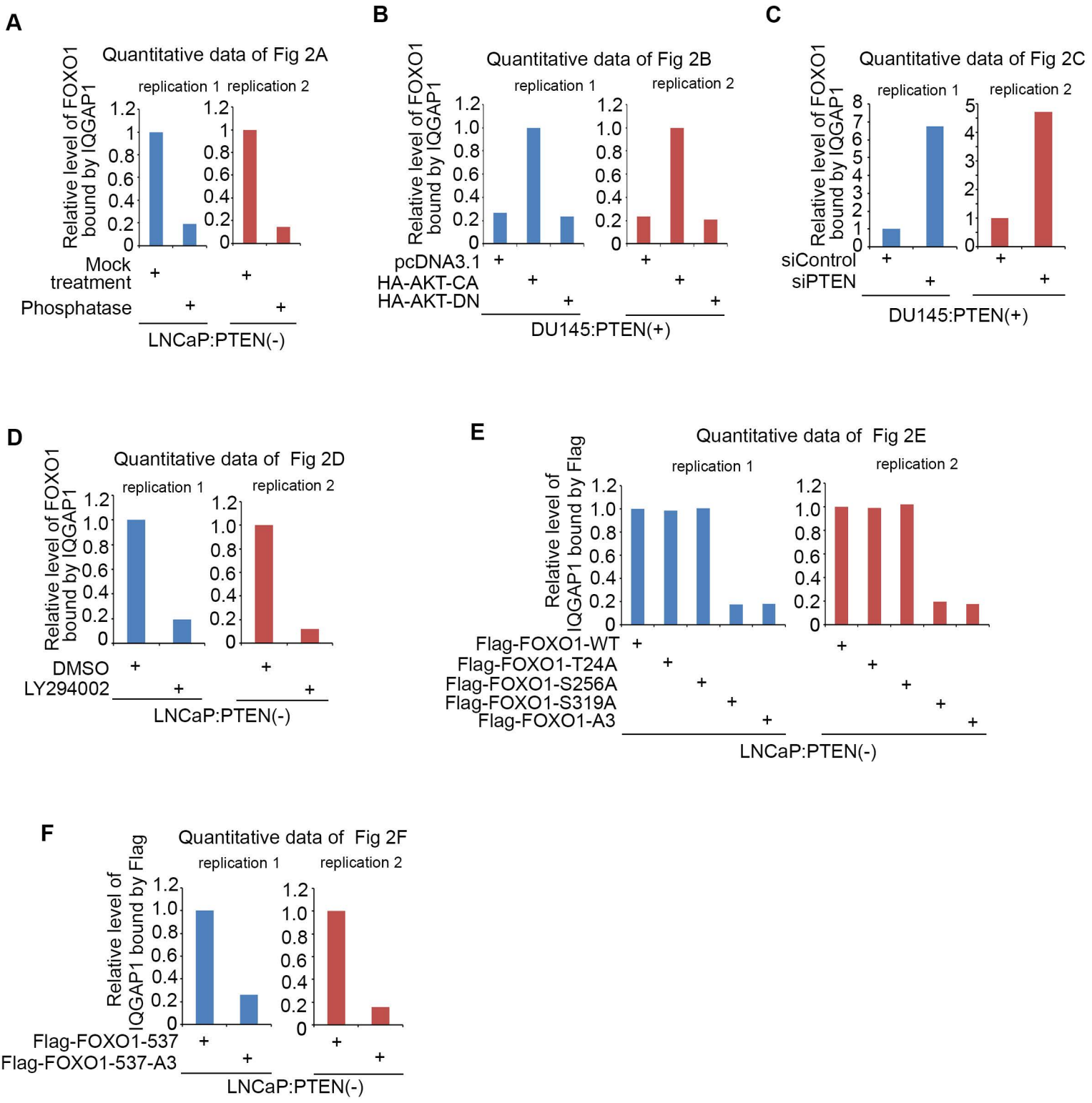








Appendix Figure S2



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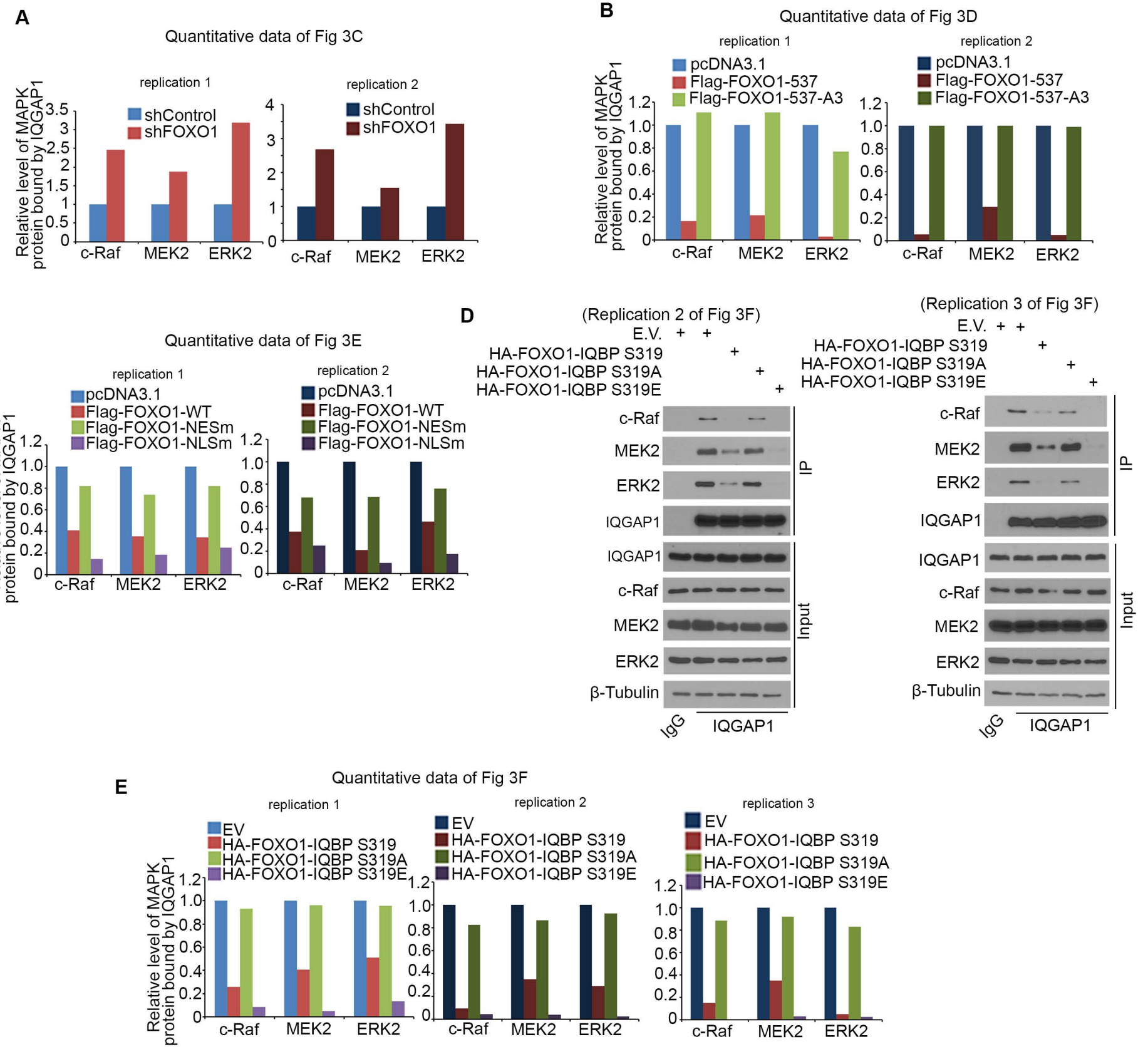

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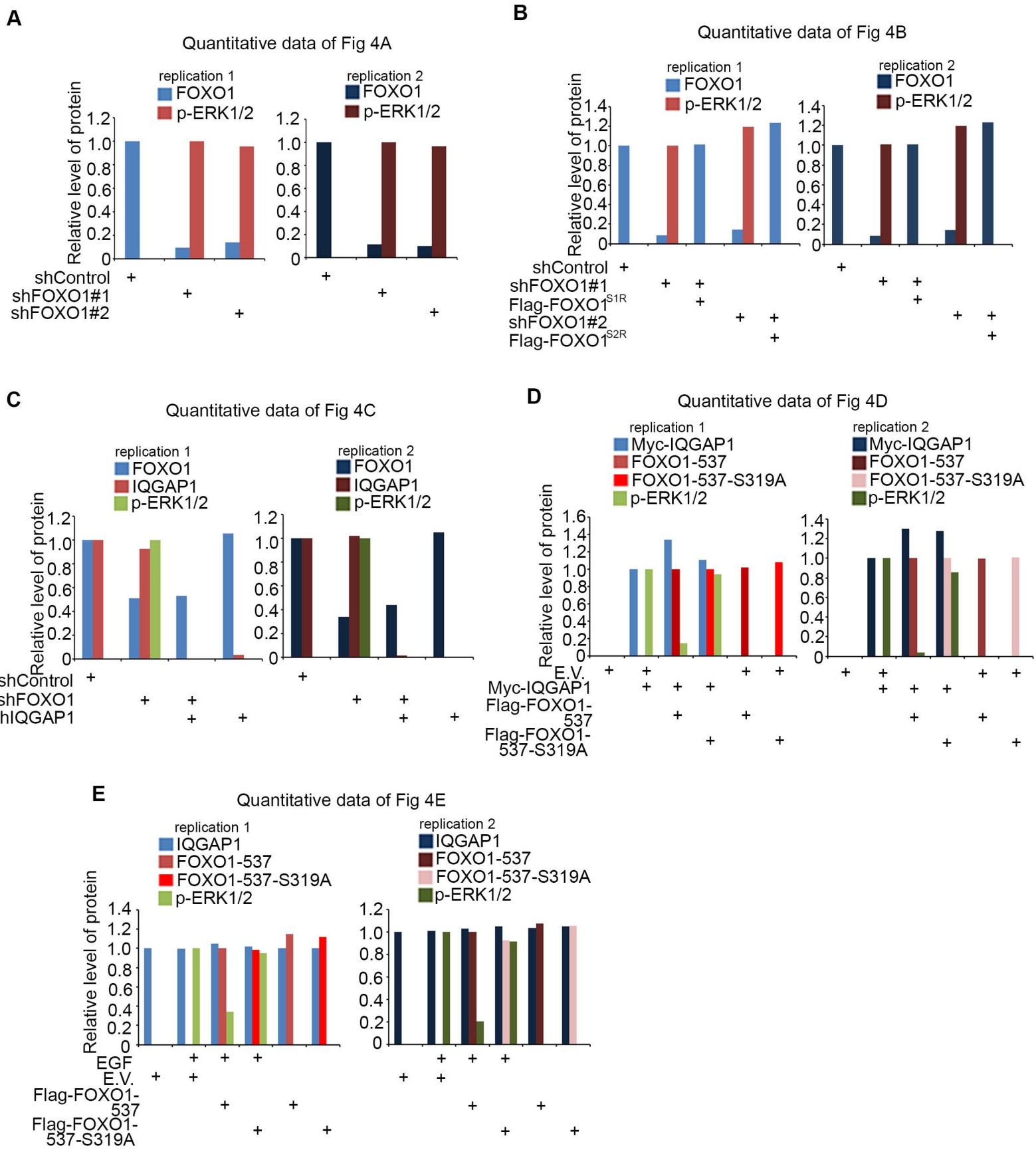
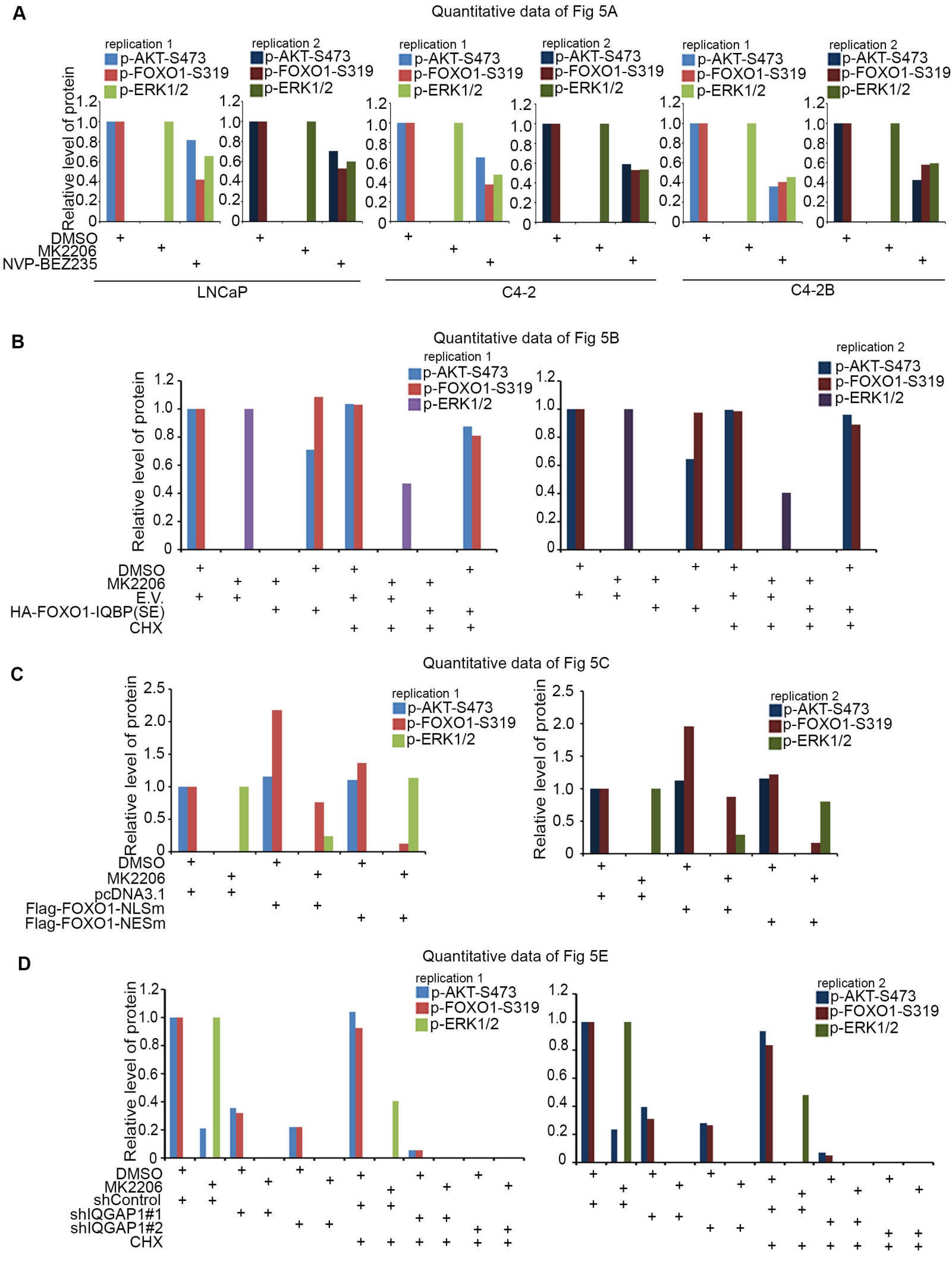


Appendix Figure S5



Appendix Figure S6