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Supplemental Information

Inhibition of PI3K/mTOR Leads to Adaptive Resistance in Matrix-Attached Cancer Cells

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Inventory of Supplemental Information

Supplemental Data

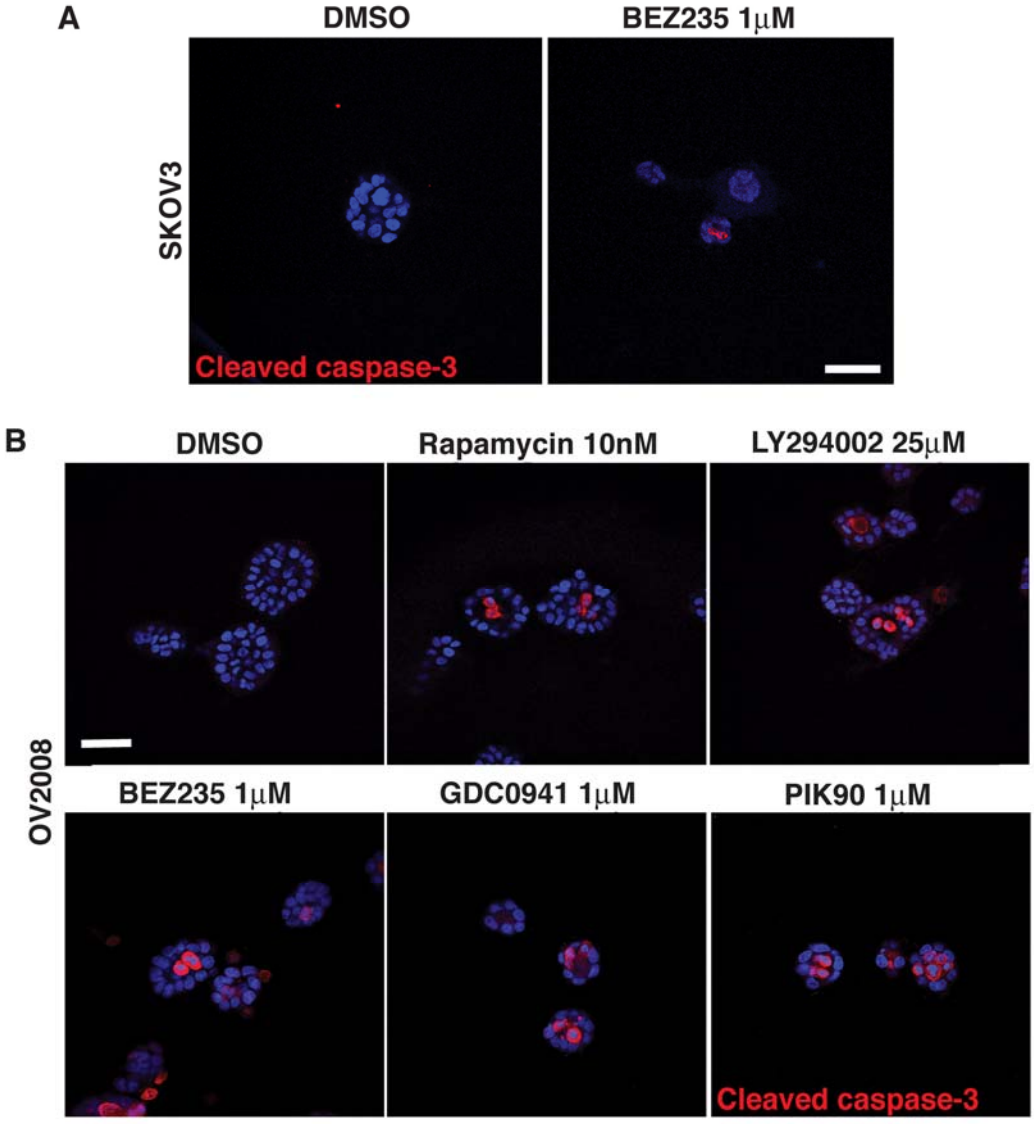
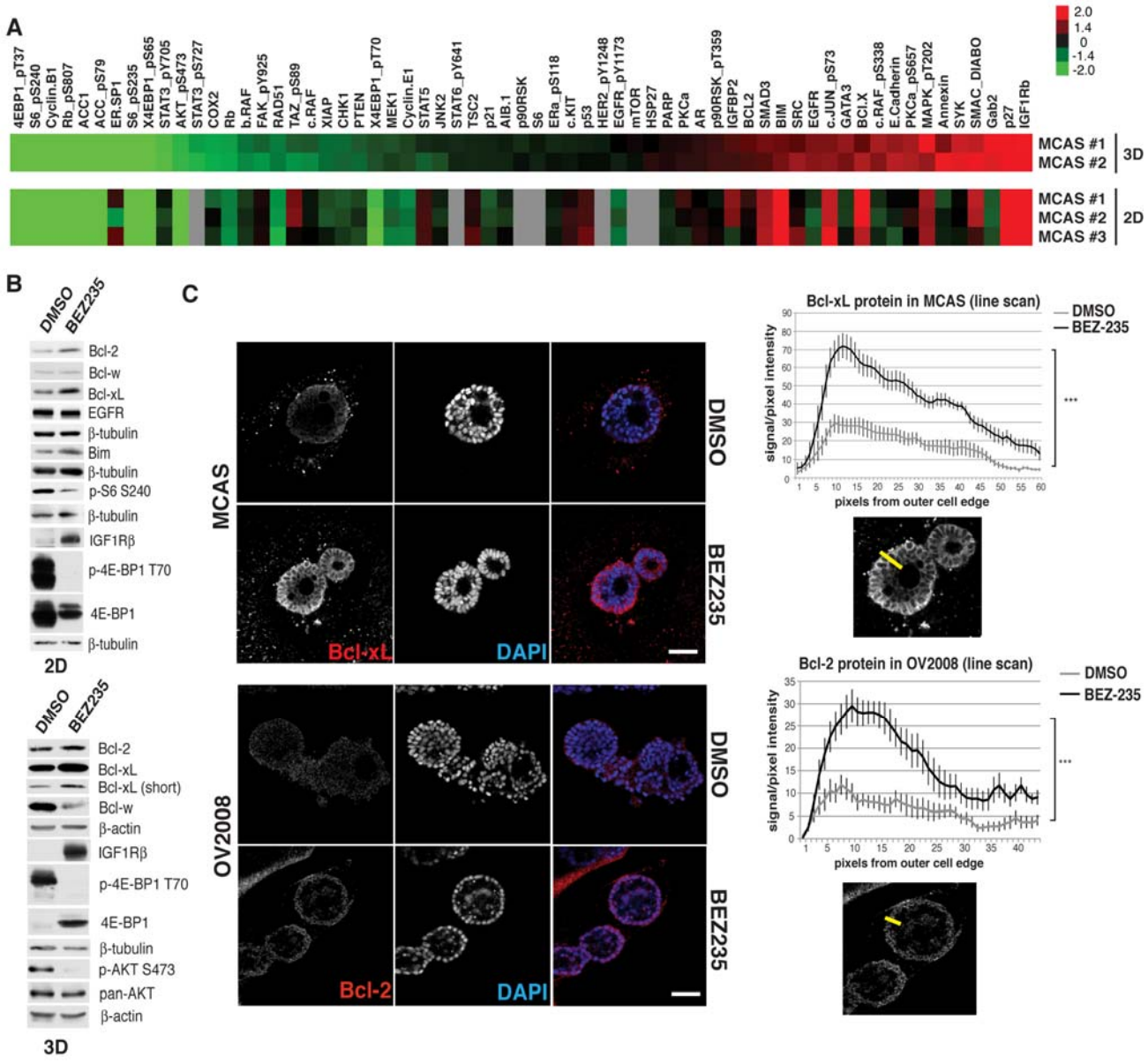


Figure S1, related to Figure 1.
Multiple PI3K and mTOR inhibitors induce death of the inner cells of tumor cell spheroids. **(A)** SKOV3 cells were grown for four days in reconstituted basement membrane and treated for an additional 48h with BEZ235. The cells were fixed and stained for cleaved caspase-3 (red) and DAPI (blue) and imaged by confocal microscopy. **(B)** OV2008 ovarian cancer cells were grown for four days in reconstituted basement membrane and treated for an additional 48h with the indicated drugs after which the cells were fixed and stained for cleaved caspase-3 (red) and DAPI (blue) and imaged by confocal microscopy. Scale bars 50μm.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ER.SP1
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X4EBP1_pS65
STAT3_pY705
AKT_pS473
STAT3_pS727
COX2
Rb
b.RAF
FAK_pY925
RAD51
TAZ_pS89
c.RAF
XIAP
CHK1
PTEN
X4EBP1_pT70
MEK1
Cyclin.E1
STAT5
JNK2
STAT6_pY641
TSC2
p21
AIB.1
p90RSK
S6
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HER2_pY1248
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BIM
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EGFR
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MAPK_pT202
Annexin
SYK
SMAC_DIABO
Gab2
p27
IGF1Rb
MCAS #1
MCAS #2
3D
MCAS #1
MCAS #2
MCAS #3
2D
B
DMSO
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Bcl-w
Bcl-xL
EGFR
β-tubulin
Bim
β-tubulin
p-S6 S240
β-tubulin
IGF1Rβ
p-4E-BP1 T70
4E-BP1
β-tubulin
2D
DMSO
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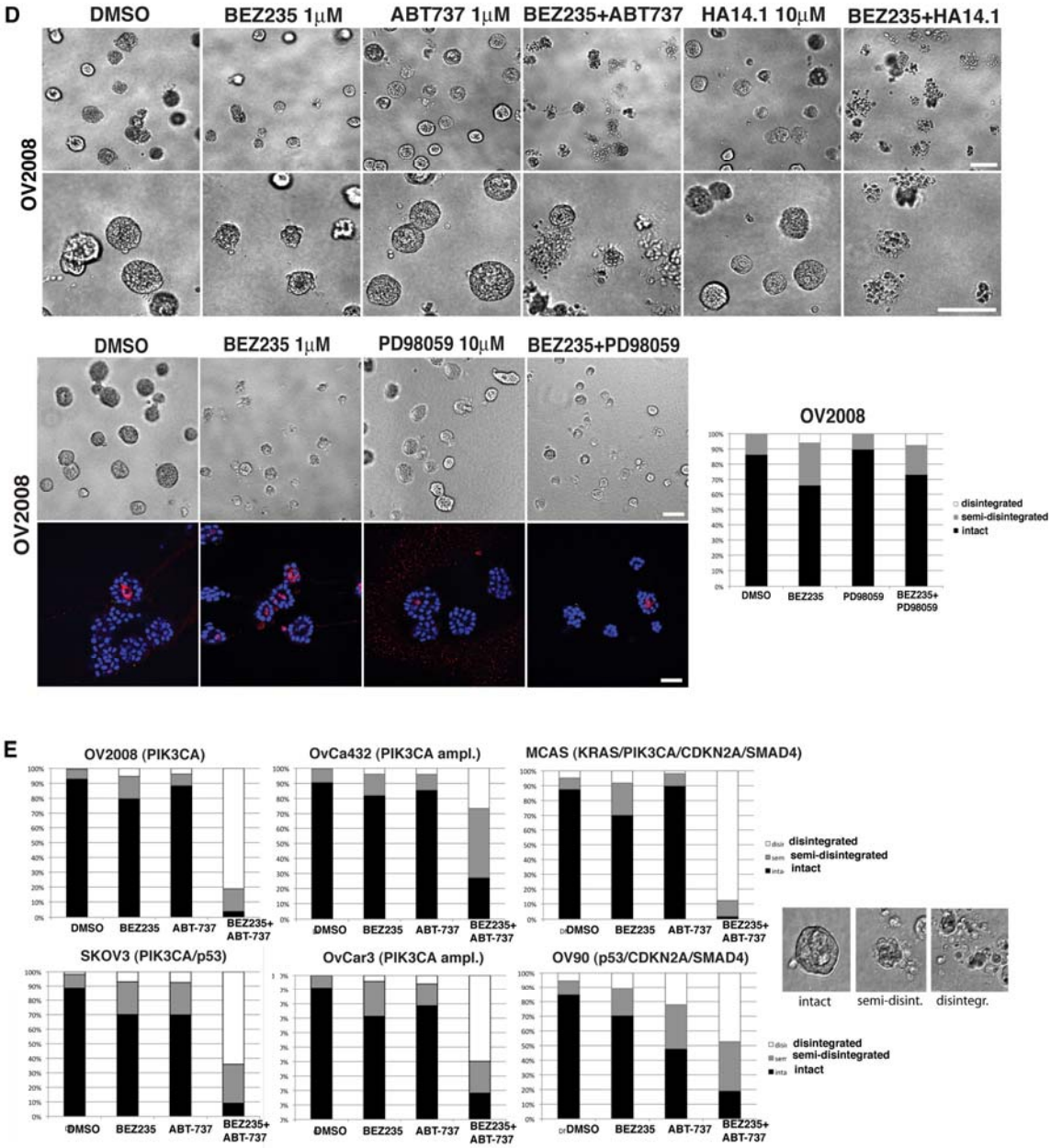
Bcl-xL
Bcl-xL (short)
Bcl-w
β-actin
IGF1Rβ
p-4E-BP1 T70
4E-BP1
β-tubulin
p-AKT S473
pan-AKT
β-actin
3D
C
MCAS
DMSO
BEZ235
Bcl-xL
DAPI
OV2008
DMSO
BEZ235
Bcl-2
DAPI
Bcl-xL protein in MCAS (line scan)
DMSO
BEZ-235
signal/pixel intensity
pixels from outer cell edge
***
Bcl-2 protein in OV2008 (line scan)
DMSO
BEZ-235
signal/pixel intensity
pixels from outer cell edge
***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BEZ235 1μM
ABT737 1μM
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HA14.1 10μM
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OV2008
DMSO
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PD98059 10μM
BEZ235+PD98059
OV2008
OV2008
DMSO
BEZ235
PD98059
BEZ235+ PD98059
disintegrated
semi-disintegrated
intact

E
OV2008 (PIK3CA)
OvCa432 (PIK3CA ampl.)
MCAS (KRAS/PIK3CA/CDKN2A/SMAD4)
SKOV3 (PIK3CA/p53)
OvCar3 (PIK3CA ampl.)
OV90 (p53/CDKN2A/SMAD4)
DMSO
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ABT-737
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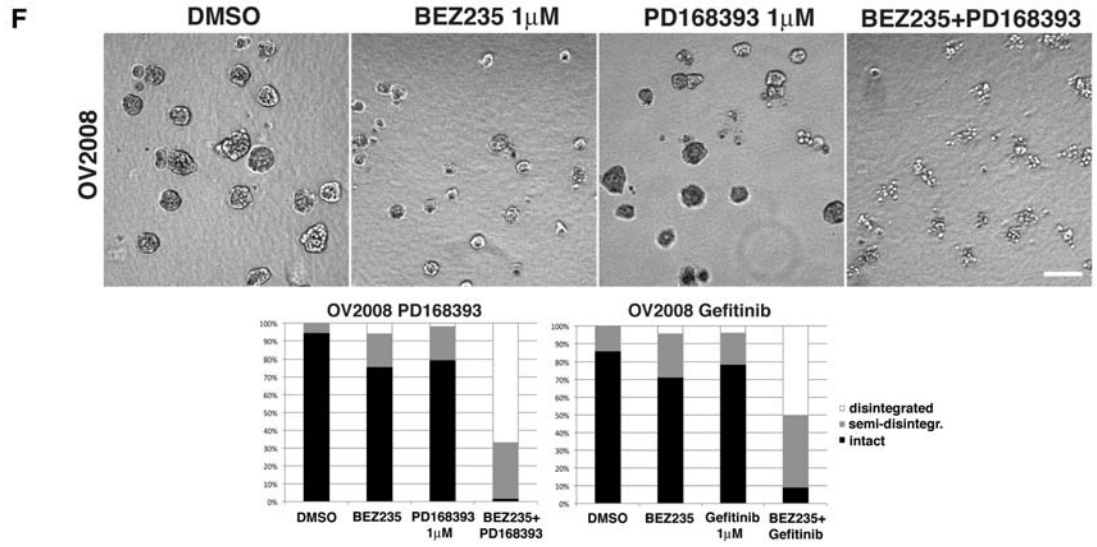
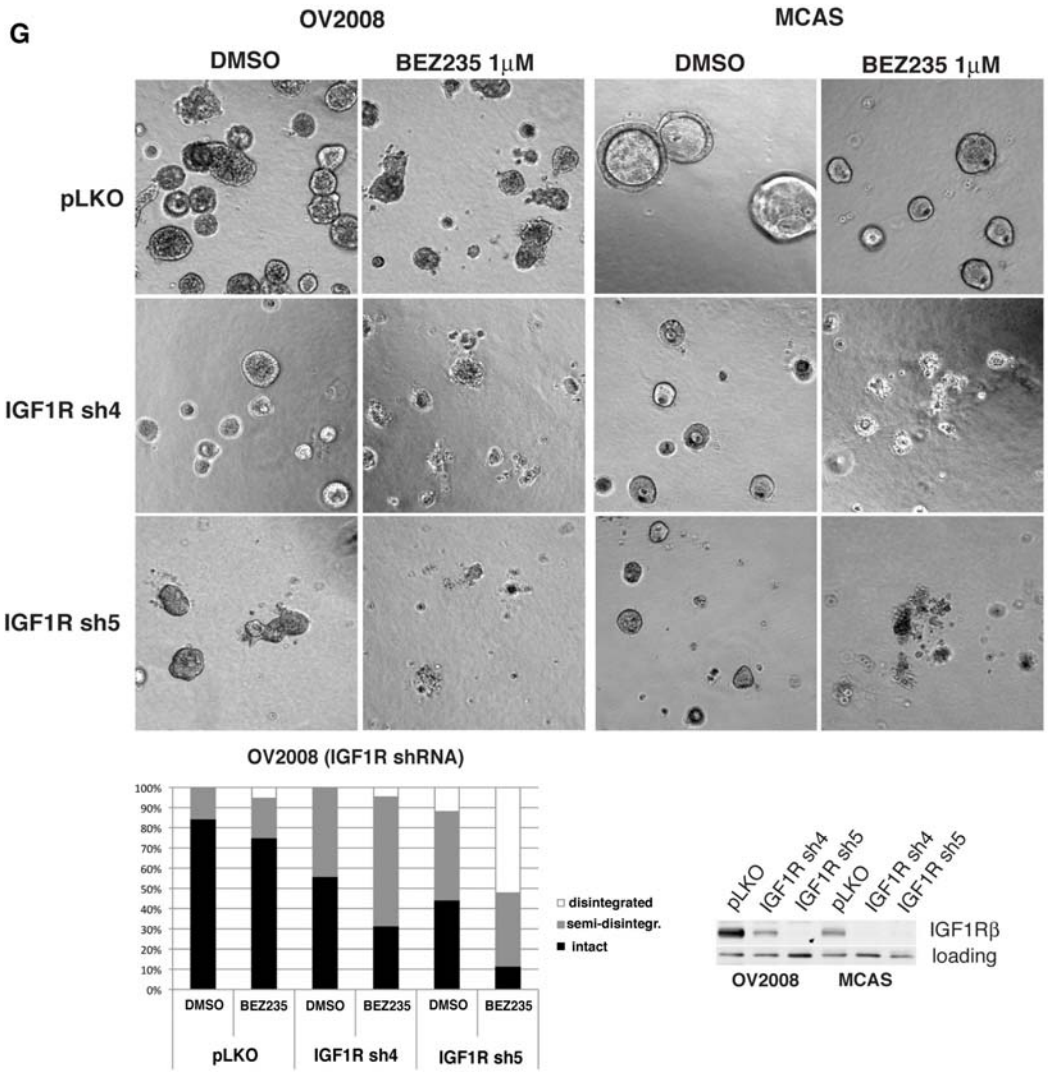
semi-disintegrated
intact
intact
semi-disint.
disintegr.


F
DMSO
BEZ235 1μM
PD168393 1μM
BEZ235+PD168393
OV2008
OV2008 PD168393
OV2008 Gefitinib
DMSO
BEZ235
PD168393 1μM
BEZ235+ PD168393
Gefitinib 1μM
BEZ235+ Gefitinib
disintegrated
semi-disintegr.
intact


G
OV2008
MCAS
DMSO
BEZ235 1μM
pLKO
IGF1R sh4
IGF1R sh5
OV2008 (IGF1R shRNA)
DMSO
BEZ235
pLKO
IGF1R sh4
IGF1R sh5
disintegrated
semi-disintegr.
intact
pLKO
IGF1R sh4
IGF1R sh5
IGF1Rβ
loading
OV2008
MCAS

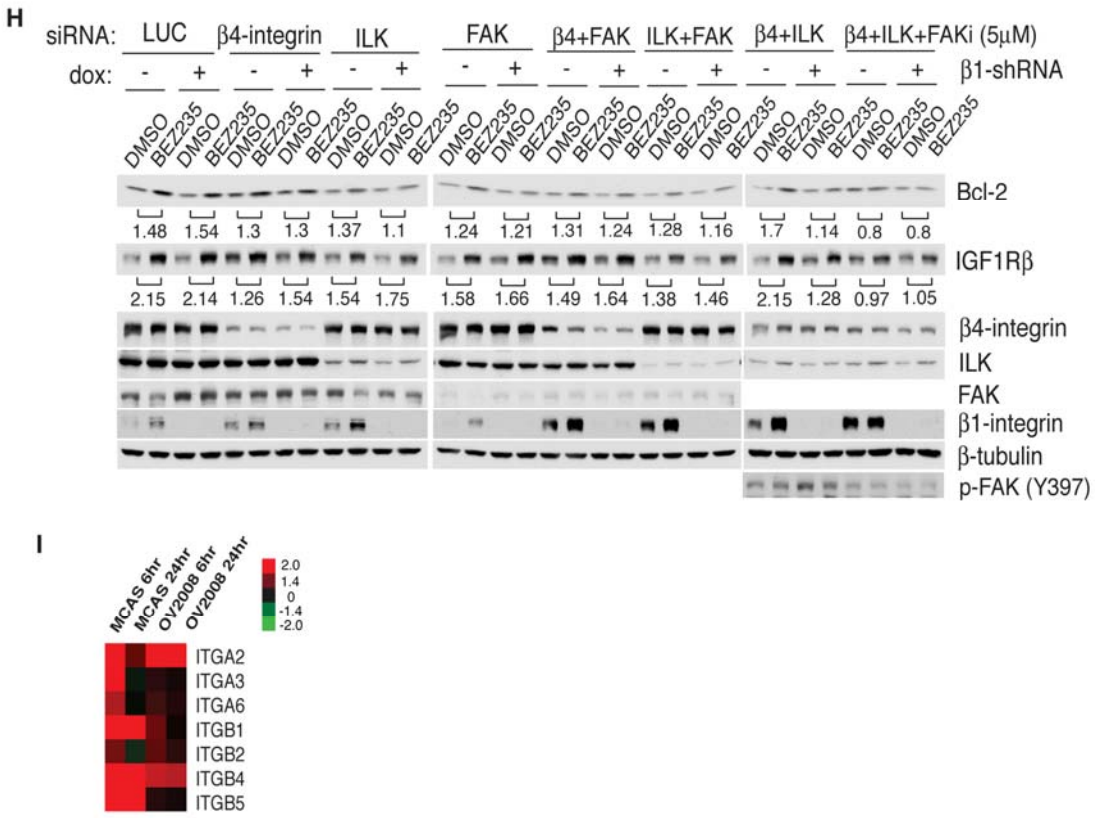
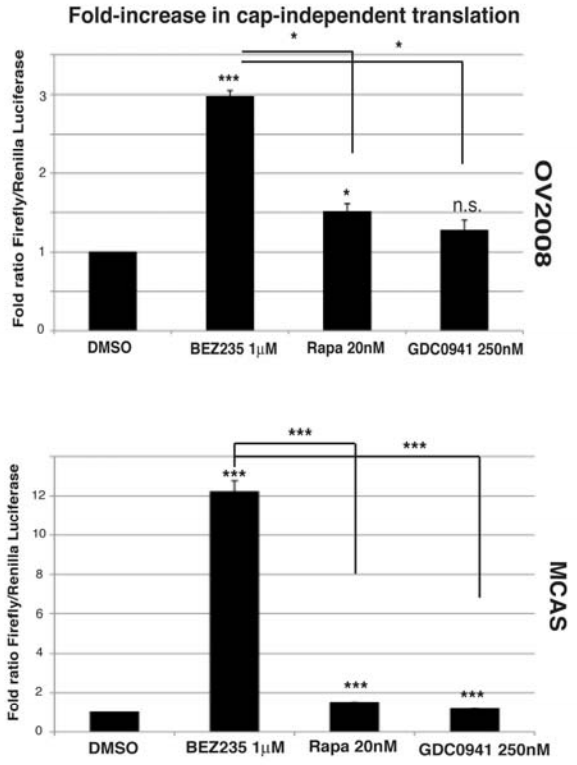


Figure S2, related to Figure 2.
(A) MCAS cells were cultured either in monolayers (2D) or in Matrigel (3D) and treated with BEZ235 or DMSO for 48h. Protein lysates were analyzed by RPPA (red indicates signals up-regulated upon BEZ235 treatment, green, those that were down-regulated). Samples were normalized to DMSO-treated controls. Proteins that were significantly affected in OV2008 RPPA analysis ($p<.05$, Student's T-Test) and up- or down-regulated greater than 1.3-fold in MCAS cells are shown. Grey boxes indicate antibodies that were not analyzed in the 2D analysis. (**B)** Some of the up- and down-regulated proteins from MCAS RPPA were selected for further validation by Western blot analysis. Known downstream targets of PI3K and mTOR pathway were down-regulated upon BEZ235 treatment (p-S6 S240, p-AKT S473 and p-4E-BP1 T70), whereas 4E-BP1, Bcl-2, Bcl-xL and IGF1Rβ were all up-regulated upon BEZ235 treatment. (**C)** Immunofluorescence analysis of Bcl-xL and Bcl-2 in BEZ235-treated tumor spheroids. OV2008 and MCAS cells were grown in Matrigel for 4d, treated with DMSO or BEZ235 for 48h, fixed and stained for DAPI (blue) and Bcl-2 (red) or Bcl-xL (red), and imaged by confocal microscopy. Scale bar 50μM. Bcl-2 and Bcl-xL were highly enriched in the outer spheroid cells. Fluorescence intensity was analyzed by line scans from the outer edge towards the mid-structure (as shown below the graphs in yellow scale bar, 60 pixels for MCAS and 43 pixels for OV2008). 16 structures were analyzed for each condition, and line scans were performed for each structure from three different angles at 10 pixel width. (**D)** OV2008 cells were treated with two Bcl-2 family inhibitors HA14.1 or ABT-737

alone or in combination with BEZ235. Both Bcl-2 family inhibitors in combination with BEZ235 were able to cause apoptosis of the outer cells. Inhibition of MEK pathway in combination with PI3K/mTOR pathway does not lead to death of the outer cells. OV2008 cells were treated with PD98059 alone or in combination with BEZ235 for 48h, cells were fixed, scored for structural integrity and stained for cleaved caspase-3 (red) and DAPI (blue) and imaged with DIC or confocal microscopy. Confocal scale bar 50μm, phase contrast scale bar 200μm. (**E)** Six ovarian cancer cell lines were treated with DMSO, BEZ235, ABT737 or in combination and scored for 3D structural integrity (examples of scored structures shown in side panel). Combination treatment causes disintegration/cell death in all six cell lines. (**F)** EGFR inhibition in combination with BEZ235 treatment also causes death of outer cells. OV2008 cells were treated with Gefitinib or PD168393 alone or in combination with BEZ235 for 48h in 3D and scored for structural integrity. Scale bar 200μm. (**G**) Depletion of IGF1R in combination with BEZ235 treatment results in death of outer cells. IGF1R was knocked-down in OV2008 and MCAS cells with shRNAs and treated with BEZ235 for 48h in 3D. The OV2008 structures were scored for structural integrity and the efficiency of the knock-down was validated by Western blot. (**H)** Integrin signaling contributes to adaptive response. OV2008 cells expressing doxycyclin inducible β1-integrin shRNA were grown in monolayer culture and transfected with siRNAs targeting β4-integrin, ILK, FAK or combinations of these in the absence or presence of doxycylin to induce β1-integrin knock-down or with 5μM FAK inhibitor PF573228. In addition the cells were treated with DMSO or BEZ235 for 24 hours. Bcl-2 and IGF1Rβ up-regulation was monitored by Western blots, quantitated and normalized to β-tubulin. The numbers indicate relative change in Bcl-2 or IGF1R protein levels compared to the DMSO control. (**I)** Integrin mRNAs that were significantly ($p<0.01$) upregulated (fold-change) in MCAS and OV2008 after BEZ235 treatment are shown. Data derived from microarray analysis described in Figure 3.

A



B

OV2008 LUC values, related to Figure 5A

Time	Condition	AVG Firefly	AVG Renilla
6h	DMSO	33677.78	178486.7
	BEZ235	31292.89	132035.6
	Torin1	26168.89	105680.4
24h	DMSO	44508.44	330529.8
	BEZ235	68380	129271.6
	Torin1	60926.22	131881.3
48h	DMSO	38638.67	305132
	BEZ235	44607.11	77427.6
	Torin1	28486.67	117104.4

MCAS LUC values, related to Figure 5A

Time	Condition	AVG Firefly	AVG Renilla
6h	DMSO	1794728	9044549.8
	BEZ235	1929414.2	4263674.7
	Torin1	1541306.2	3490588.4
24h	DMSO	966186.7	3840131.1
	BEZ235	186742.7	83590.7
	Torin1	335266.2	261413.8
48h	DMSO	207329.8	826673.8
	BEZ235	35089.3	22605.3
	Torin1	65126.2	40204.9

LUC values related to Figure S3A

Cell Line	Condition	AVG Firefly	AVG Renilla
OV2008	DMSO	3916.0	136805.3
	BEZ235	1905.3	22573.8
	Rapamycin	3746.2	87084.9
	GDC0941	1176.0	33013.8
MCAS	DMSO	304972.9	4596996.0
	BEZ235	60005.3	74054.7
	Rapamycin	191311.6	1931767.6
	GDC0941	125976.0	1633943.1

LUC and LUC mRNA (qPCR) values related to Figure 5B

5'UTR LUC dmso	5'UTR LUC BEZ	BCL2 IRES dmso	BCL2 IRES BEZ
94761947	44326981	278838	689663
5'UTR LUC mRNA dmso	5'UTR LUC mRNA BEZ	BCL2 IRES LUC mRNA dmso	BCL2 IRES LUC mRNA BEZ
2.384	3.793	1.191	2.199

Figure S3, related to Figure 5.

(A) A dual luciferase reporter, in which Renilla luciferase expression is mediated by cap-dependent translation and firefly luciferase expression by CrPV IRES cap-independent translation, was used to monitor cap-independent translation in OV2008 and MCAS cells in response to DMSO, BEZ235, 20nM Rapamycin or 250nM GDC0941 treatment. * $p<0.05$, *** $p<0.005$. (**B**) Raw LUC values for all the CrPV and BCL-2 reporter assays.

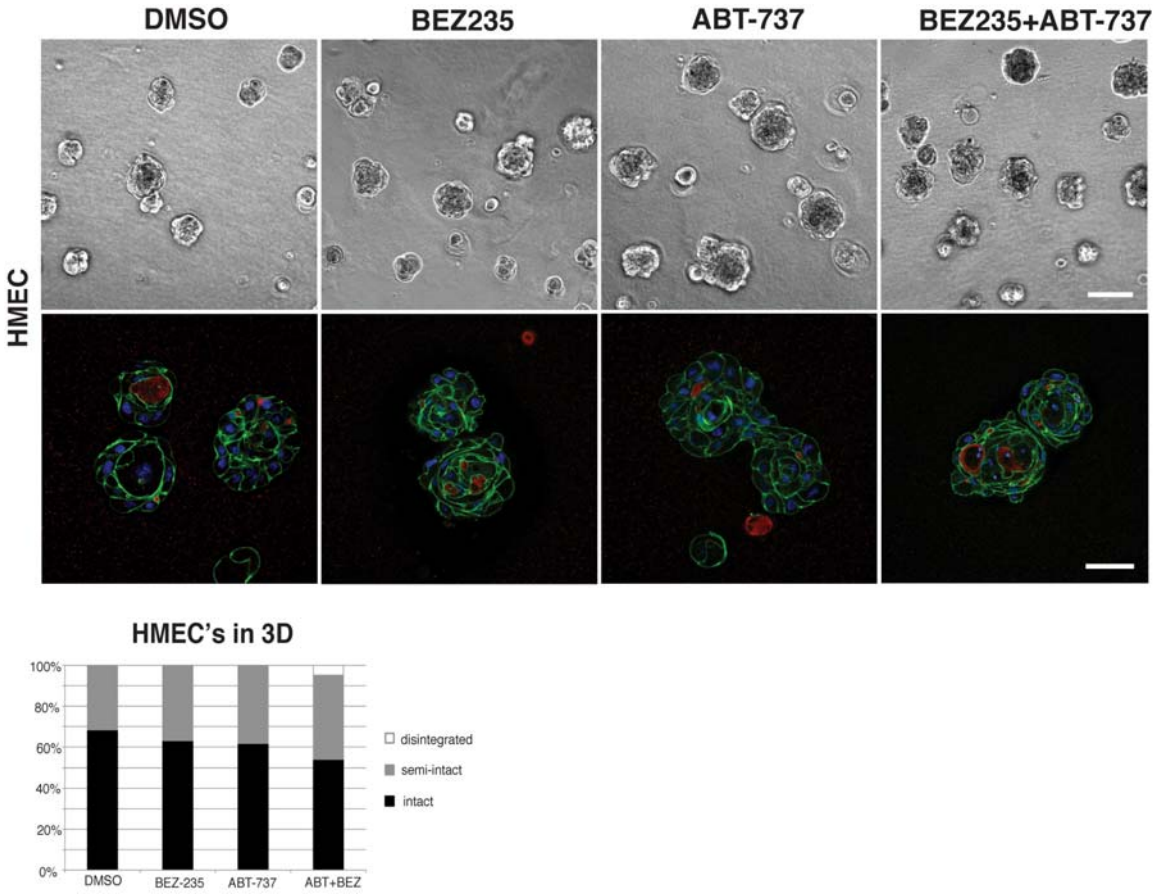


Figure S4 related to Figure 6.
Non-immortalized, non-transformed human mammary epithelial cells (HMEC's) do not show sensitivity to dual inhibition of PI3K/mTOR and Bcl-2. HMEC's were grown for 8d in Matrigel and treated for 48h with dmso, BEZ235, ABT-737 or a combination of both. The cells were fixed, stained for DAPI (blue), cleaved caspase-3 (red) and phalloidin (green) and imaged by confocal microscopy. Confocal scale bar 50µm, phase contrast 200µm. The structural integrity was scored as described in Figure S2.

Supplemental Experimental Procedures

Antibodies and Reagents

Drugs and inhibitors: Rapamycin (a kind gift from Dr. J. Blenis), LY294002 (Sigma), BEZ235 (Axon Medchem) (Maira et al., 2008), FAK-inhibitor PF573228 (Tocris #3239), GDC0941 (Axon Medchem), PIK-90 (Axon Medchem), ABT-737 and ABT-263 (Selleck Chemicals), HA14.1 (Selleck chemicals), PD98059 (Calbiochem), PD168393 (EMD Chemicals), Gefitinib (Axon Medchem) and Torin1 (a kind gift from Dr. N. Grey) were all dissolved in DMSO and used at indicated concentrations. Assays with BEZ235 when not mentioned were performed with 1μM concentration. For mouse experiments, GNE493 was kindly provided by Genentech and ABT-737 by Abbott Pharmaceuticals.

Antibodies: Bcl-2 (D55G8 #4223, Cell Signaling Technologies = CST, Epitomics #1017, DAKO #M0887), p90RSK pT359 (#9344 CST), RSK1/2/3 (32D7 #9355 CST), IGF1R pY1135 (#3918 CST), IGF1Rβ (Santa Cruz # sc-713 and CST #3018), p-ERK (Invitrogen #44680G), total MAPK (#9107 CST), EGFR pY1173 (53A5 #4407 CST), EGFR (#4267 CST), β-tubulin (Invitrogen), β-actin (Invitrogen), 4E-BP1 pT37/46 (#2855 CST), 4E-BP1 pT70 (#9455 CST), total 4E-BP1 (53H11 #9644 CST), AKT pS473 (D9E #4060 CST), pan-AKT (#2920 CST), Bcl-w (#2724 CST), c-Jun pS73 (#3270 CST), Bcl-xL (54H6 #2764 CST), Bim (Epitomics #1036), S6 pS240/244 (#5364 CST), S6 (#2317 CST), p-S6 S235/236 (#4856 CST), eIF2α pS51 (#3398 CST), eIF2α (#9723 CST), Ki67 (Invitrogen), Cleaved caspase-3 (#9661 CST), β1-integrin (BD Biosciences #610468), β4-integrin (CST #4707), FAK (Millipore #05-537), ILK (CST #3862), FAK pY397 (CST #8556), FOXO1 (CST #2880), FOXO3 (CST #2497), FOXO4 (CST #9472), BAD S112 (CST #5284).

mRNA microarray and data analysis

Total RNA was isolated according to manufacturer's protocol with TRIzol-reagent (Invitrogen) 6h and 24h after treatment with 1 μM BEZ235 or DMSO vehicle control from 3D grown structures. All samples were prepared in triplicate. cRNA was hybridized to the Illumina HumanHT-12 v4 Expression BeadChips and processed according to the manufacturer's protocol. Background correction and quantile normalization of data was performed in GenomeStudio software (Illumina, Inc.). Spots with detection p-value >0.01 in both conditions were eliminated. Differential expression between experimental groups was defined as $p<0.01$. Gene expression heatmaps were generated with Cluster 3.0 and Java TreeView 1.1.1.

FOXO transcriptional targets were identified from a published review (van der Vos and Coffer, 2011) and from MetaCore (*GeneGo* Inc). Targets shown to be positively regulated by FOXO were included. For enrichment analysis, genes were considered up-regulated using 1.3 fold change and $p<0.01$ thresholds. p-values were calculated using the Hypergeometric probability distribution distribution in Sisa (http://home.clara.net/sisa/). The data from the microarrays has been deposited in GEO database with accession number GSE28992.

Analysis and quantitation of primary samples

Primary tumor cells were grown in 3D reconstituted basement membranes for eights days after which the indicated cellular signaling inhibitors were added for 48h. To visualize DNA fragmentation and permeability of nuclear envelope - phenotypes associated with cellular death, 3D cultures were stained with ethidium bromide and DAPI (Invitrogen). Images of stained structures were obtained using Nikon Ti-E motorized inverted microscope equipped with A1R scan head with spectral detector and resonant scanner. The images were acquired using pinhole size 2.4 μm. To quantify the amount of ethidium bromide bound to fragmented DNA, the ratio of total red spectral signal (-EtD1-565nm exitation/578nm emission) and blue spectral signal (-DAPI-359nm excitation/ 461nm emission) was obtained on blinded samples using Nikon Elements software. JMP 8 statistical software was used to graph the distribution of total red/blue ratio per structure.

Plasmids, siRNAs and shRNAs

Inducibe β1-integrin-shRNA (pTRIPZ) was obtained from Open Biosystems (RHS4696-99404134), other shRNA's were obtained from RNAi Consortium: pLKO shIGF1R: sh4 (5'-CCGGCCTTGGACGTTCTTTCAGCATCTCGAGATGCTGAAAGAACGTCCAAGGTTTTT-3'), sh5 (5'-CCGGCCAAGCCTGAGCAAGATGATTCTCGAGAATCATCTTGCTCAGGCTTGGTTTTT-3'), shFOXO3 (5'-CCGGCAAACTGACACAAGACCTACA-CTCGAGTGTAGGTCTTGTGTCAGTTTGTTTTTG-3') were used in shRNA experiments and generated according to standard protocol in 293T cells. ON-TARGET SMARTpool siRNAs targeting LUC and human FOXO1, FOXO3, FAK, ILK and ITGBβ4 were purchased from Dharmacon. siRNA's were transfected according to standard protocol and experiments were performed within 48-72h of transfection.

Cell culture, cell lines and virus production

All the cell lines were a kind gift from Dr. D. Slamon (UCLA). OV2008, MCAS, SKOV3, OVCAR3, OvCa432 and OV90 cells were all passaged in 1:1 mixture of MCDB105 (Cell Applications Inc.) and 199 (Gibco) media supplemented with 10% inactivated calf serum (Gibco), 2mM L-glutamine (Gibco) and penicillin-streptomycin (Gibco). With 3D experiments, serum was reduced to 2%. Cells were routinely cultured in 37°C with 5% CO_2. Lentiviruses for shRNAs in pLKO- andd pTRIPZ plasmids were made in 293T cells according to standard protocol and selected with Puromycin for 3d. MCF10A cells were cultured as previously described (Debnath et al., 2003). HMEC's were obtained from Lonza and cultured in MEGM media (Lonza) according to providers instructions. T-47D, MDA-MB-468 and HCC-1569 breast cancer cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin. 3D structures for protein and microarrays were grown and harvested as follows: Cells were seeded on polyHEMA (Sigma #192066) coated (6mg/ml in 95% EtOH and allowed to dry completely) 24-well plates, 20000 cells/well, in 750µl of media supplemented with 2% serum and 2% Matrigel and allowed

to grow for 4 days. Cells were fed with 750µl of media supplemented with 2% serum and 2% Matrigel and drugs were also added at this timepoint. After two additional days six wells were pooled and cells were harvested for protein and mRNA analysis.

Immunofluorescence staining and Microscopy

3D structures were fixed and stained as previously described for MCF-10A cells (Debnath et al., 2003). EtBr from the LIVE/DEAD cell stain kit (Invitrogen) was used to stain for dying cells in 3D and 5 µM Hoechst 33342 (Invitrogen) to counterstain nuclei. Phase contrast microscopy was used to image 3D structures as previously described (Debnath et al., 2003).

For confocal imaging of 3D structures, we used Multispectral Multimode Laser Scanning Confocal Microscope: Nikon TE-200U inverted microscope with Nikon C1 point scanning confocal with spectral detection, wide range of Nikon 40x, 1.4 NA magnification/NA DIC and phase optics, Nikon halogen trans illuminator with 0.52 NA LWD and 0.85 NA Dry condenser, 405nm, 488nm & 561nm laser lines, Nikon EZ-C1 software, TMC vibration-isolation table. 3D images are shown as one section from mid-structure.

RPPA assay and Western blot

Sample preparation and probing with antibodies to the indicated proteins, normalization of data points, and analysis were performed according to the previously described method (Hennessy, 2011). 3D and 2D Western blot samples were harvested as described above and lysed in radio immunoprecipitation assay buffer (Tris-HCl 50mM pH 7.4, NaCL 150mM, NP-40 1%, Sodium deoxycholate 0.5%, SDS 0.1%) supplemented with protease and phosphatase inhibitors (Leupeptin, Aprotinin, 1mM PMSF, Pepstatin, 1mM sodium fluoride, 1mM sodium orthovanadate). Lysates were clarified by centrifugation at 13,000 ***g*** for 10 minutes. Clarified lysates were boiled in 1x sample buffer (0.04 M Tris-HCl pH 6.8, 1% SDS, 1% β-mercaptoethanol and 10% glycerol) for 10 minutes and resolved by 4-20% SDS-PAGE gradient gels (Invitrogen), transferred PVDF membranes (Whatman), blocked with 5% BSA in TBS (25mM Tris, 140 mM NaCl, 2.7 mM KCl, pH 7.4), 0.1%

Tween 20, for 1h at room temperature and probed by antibodies o/n. Membranes were subsequently probed with secondary antibodies linked to horseradish peroxidase (HRP; Santa Cruz). Western blot membranes were developed using enhanced chemiluminescent substrate for detection of HRP (PIERCE). Western blot results were prepared using Kodak film developer and scanner Epson 3000. IRES enrichment score was calculated by Fisher's exact test.

IRES luciferase reporter assays

The bi-cistronic dual luciferase reporter plasmid CrPV IRES was a kind gift from Dr. G. Wagner (Harvard Medical School). The plasmid was transiently transfected into OV2008 and MCAS cells using FuGENE (Roche). The next day cells were split for the assay 300,000 cells/well (6-well plate) for 24h hours, then treated with DMSO, 1μM BEZ235 or 0.5μM Torin1 for 6h, 24h and 48h and analyzed using the Dual-Luciferase Reporter Assay System (Promega). The Bcl-2 IRES and unstructured 5'UTR plasmids were a kind gift from Dr. R. Lloyd (Baylor College of Medicine) and were used as previously described (Suo et al., 2010). Briefly, OV2008 cells were transfected with one of the two constructs as above, treated with DMSO or 1μM BEZ235 for 24h, and luminescence values measured and normalized to total Luciferase mRNA levels as detected by qPCR. A paired two-tailed Student's t-test was performed on pre-normalized ratios to determine statistical significance. All experiments were performed in triplicate, and in three independent experiments.

Quantitative real-time PCR

Total mRNA was isolated with TRIzol (Invitrogen) according to manufacturers instructions. cDNA was generated with SuperScript First Strand synthesis kit (Invitrogen) according to manufacturers instructions, and 0.2-2mg of total RNA was used for the RT-PCR reaction. RT-PCR reactions were diluted to 200ml with H_2O and 2ml were used per q-PCR reaction. Power SYBR Green (Applied Biosystems) was used for q-PCR, primers used for q-PCR were:

Luciferase forward 5'-TCTGATTACACCCGAGGGGG-3'

Luciferase reverse 5’-CGGTAAGACCTTTCGGTACTTCGTC-3’,

RPLPO forward 5’-ACGGGTACAAACGAGTCCTG-3’,

RPLPO reverse 5’-CGACTCTTCCTTGGCTTCAA-3’.

Supplemental Reference

van der Vos, K. E., and Coffer, P. J. (2011). The extending network of FOXO transcriptional target genes. Antioxid Redox Signal *14*, 579-592.