OS cell lines and mouse models

Primary mouse OS cell cultures were derived from tumors generated in either *Osx*-Cre *p53*$^{fl/fl}$*pRb*$^{fl/fl}$ mice (fibroblastic OS model) (1) or *Osx*-Cre TRE-p53.1224 *pRb*$^{fl/fl}$ model (osteoblastic OS model) (2) (all obtained directly from mouse models of OS). Primary human OS (OS17) was derived from a primary human OS xenograft generously provided by Dr Peter Houghton (Nationwide Children's Hospital, Ohio). The material was expanded in NOD scid gamma (NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice, the tumor isolated and mechanically dissociated and the resultant cell culture used up to passage 10. Primary human OS (SJOS001112_X1, SJOS010929_X1, SJOS001107_X1, SJOS001107_X2) were derived from primary human xenografts collected from patients with osteosarcoma at SJCRH and available through Dr Michael A Dyer (St Jude Children's Research Hospital) shared resource (https://hospital.stjude.org/dbstp/). Human OS cell lines MG-63, Saos-2, U2OS, G-292, 143B and SJSA-1 were purchased from ATCC (MG63 from ECACC cell line remainder from ATCC; no authentication performed by the authors). Normal human osteoblasts were derived from bone marrow aspirates from the posterior iliac crest of healthy human adult donors (17–35 years of age), with informed consent (IMVS/SA Pathology normal bone-marrow donor program RAH#940911a). The cells were outgrown from the bony spicules that were collected following filtration of the BM through a 70μm filter. Normal mouse osteoblastic cells were derived from crushed and collagenase I (3mg/mL) digested long bones of 6-8-wk old C57Bl/6 mice. Cell cultures were established by mechanically dissociating tumor material with a scalpel and plating in αMEM supplemented with 10% FCS (non-heat inactivated), 1% Penicillin/Streptomycin and 1% Glutamax supplement (media the same for all primary cell cultures).

High-throughput siRNA whole genome screen

OS cell line 494H (Cre:lox model derived cell line representative of fibroblastic OS passage 8 from derivation from primary tumor) were grown and transfected with SMARTpool siRNAs using DharmaFECT lipid 3 transfection reagents (Dharmacon GE). The mouse siRNA library (Dharmacon GE) consists of 16,874 individual protein coding genes consisting of the following gene families all designed to RefSeq52: Protein kinases (G-003505), GPCR (G-003605), Phosphatases (G-003705), Ion channel (G-003805), Drug targets (G-004655), Remaining genome (G005005). SMARTpools were obtained in 384-well plate format, hydrated and diluted in the Victorian Centre for Functional Genomics (VCFG) to 1 nmol using 1× siRNA buffer (Dharmacon GE). Each well in this library contained a SMARTpool of 4 distinct siRNA species targeting different sequences of the target transcript. Cells were reverse transfected (3) at 400 cells per well with 40nM of the siGENOME SMARTpool library complexed for 20 minutes with DharmaFECT 3 using the Sciclone ALH3000 (Caliper Life Sciences) and BioTek 406 (BioTek) liquid handling robotics. Cell lines were transfected in quadruplicate (day 0) and one set of duplicates treated as control (siRNA only) and a second set of duplicates treated after 48hrs (day 2) with 100nM doxorubicin. At 120hrs post

transfection (day 5) the cell viability for each well was quantified on the basis of the direct measurement of intracellular ATP using the CellTiter-Glo luminescent assay (Promega) at a 1:2 dilution. All luminescent measurements were taken on the Synergy H4 high-throughput multimode microplate reader (BioTek). Assay robustness was measured on each plate using the Z'factor metric, the dynamic range between the positive (siPLK1) and negative (Mock, lipid only transfection) control wells. A Z'factor of >0.3 was required for a plate to met quality control metrics and be included in the final analysis. To identify targets that significantly caused loss of viability, all target well values were normalized to the average of the mock transfected wells on each plate and then subjected to sample-based normalization using a robust z-score approach (4). Each well on duplicate plates was then averaged for a single value for each gene target (5). For the duplex deconvolution screen, transfection followed the protocol above, with the final siRNA concentration of 25nM. Given that all targets are theoretically hits at this point we could not use sample-based normalization. Viability values for individual siRNAs were normalized to the average of a large number (~30) negative controls per plate (all 4 siRNAs were on the same plate for internal reference) and averaged between the duplicate plates. The threshold to be considered a hit was derived from the relative fold change (less than 0.7 compared to control) associated with a robust z-score cut of +2 or -2. RNAi screen data is available from Pubchem with PubChem accession ID #1053208 (primary screen) and PubChem AID# 1053209 (validation screen).

The list of hits from the screen was compiled. Those that increased cell death were analyzed separately from those they caused increased survival. The lists of candidates were assessed using Ingenuity Pathway Analysis software (Qiagen) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 algorithm (http://david.abcc.ncifcrf.gov/) (6). Reported statistics are those derived form each program respectively.

Small molecule screen against primary mouse OS paired samples

Three independent sets of paired primary and metastatic OS cell lines (Cre:lox model derived cell line representative of fibroblastic OS, less than or equal to passage 8 from derivation (1)) were plated at 400 cell/well in a 384-well plate format. After 24hrs the cells were treated with the 131 different compounds in an 11-point dose response ranging from 0.01-10mM. All assays were performed in duplicate. At 48hr post treatment the cell viability for each well was quantified on the basis of the direct measurement of intracellular ATP using the CellTiter-Glo luminescent assay (Promega) at a 1:2 dilution. All luminescent measurements were taken on the EnSpire plate reader (PerkinElmer, Waltham, MA, USA). Data was plotted and the IC_{50} calculated using Prism 6 software.

Small molecule screen against primary human xenograft-derived OS

Excess, de-identified tumor material was collected from patients with osteosarcoma at SJCRH in agreement with local institutional ethical regulations and institutional review board approval. Tumor tissue was initially implanted in the flank location of NOD scid gamma (NSG,

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice. After sufficient tumor growth, orthotopic xenografts were created by processing the flank tumor tissue into a single-cell suspension by enzymatic and mechanical dissociation and injection into the femur of CD-1 nude mice homozygous for the *Foxn1*^{nu} spontaneous mutation. All xenograft lines were assayed for optimal growth conditions *in vitro* in 384-well plates, including plating density, DMSO-sensitivity, and positive control compounds before being validated and assayed for drug sensitivities. Disassociated cells were plated in 384-well plates, 24 hours after plating cells were treated with drug compounds in a 10-point 3-fold dose response dilution with top concentration ~10mM (effective assay range 30uM-40pM). At 72hr post treatment the cell viability for each well was quantified on the basis of the direct measurement of intracellular ATP using CellTiter-Glo luminescent assay (Promega) at a 1:2 dilution, taken with EnVision plate reader (Perkin Elmer, Waltham, MA, USA). EC50 values were calculated by first log10 transform the raw luminescence signal (RLUs). Then for single compound experiment the signal was normalized to give percent inhibition relative to the positive control (staurosporine) and negative control (DMSO) using the following equation: *% inhibition* = [100* (negative control mean - compound value)] / [(negative control mean - positive control mean).]

Small molecules and dose response calculations

For *in vitro* studies, compounds were dissolved in DMSO as 10mM solutions and subsequently diluted to working concentrations in DMSO to maintain an equivalent DMSO v/v % in all treatment groups (unless otherwise stated all chemicals/drugs were purchased from Selleckchem). Doxorubicin was obtained from St Vincent's Hospital Pharmacy (Melbourne, Australia) and diluted to a 2mg/ml solution in PBS. Spliceostatin A was generously provided by Prof Yoshida (Chemical Genetics Laboratory. RIKEN, Saitama, Japan) (7).

Drug concentration effects on cell viability were assessed by plating cells at 2500 per well in white 96 well plates with clear bottoms, 24 h prior to treatment. Drug treatments were applied in serially diluted concentrations ranging from 0.01nM to 10μM depending on the compound being assessed and were normalized to 0.1 or 0.2% v/v DMSO per well depending on the assay. Treatments were carried out over 48hr dependent on the compound being assessed. Viability was quantified using CellTitre-Glo (Promega) using an EnSpire plate reader (PerkinElmer). Half maximal inhibitory (IC_{50}) values were calculated using Prism 6.0e software nonlinear regression parameters and a log (inhibitor) vs response variable slope four parameter equation. After assessing every pairwise combination of two compounds, synergy was calculated as the percentage that an experimentally derived value deviated from a theoretical additive interaction value derived using the Bliss Additivity model (8). Bliss additivity values predict a combined response (C) of two agents with individual effects (A) and (B) by C= A+B-(AxB), where (A) and (B) represent fractional inhibition between 0 and 1. Experimental responses greater than 15% above the predicted combined response value were considered to indicate synergism.

Cell death assays

Cells were treated with the indicated drug or vehicle control for 48 h prior to harvesting. Cells were washed with PBS and 100,000 cells were stained in 1x Annexin Binding buffer (BD Biosciences) diluted 1:20 with Annexin V-APC (1mg/ml) (BD Pharmingen) and 7-Aminoactinomycin D (7AAD) (100μg/ml) (Life Technologies) for 15 min protected from light. Following the addition of 4 volumes of 1x Annexin Binding buffer, apoptotic cells were quantified using FACS (LSRFortessa, BD Biosciences). Live cells (Annexin V negative, 7AAD low) and cells in early and late stages of apoptosis (Annexin V positive, 7AAD low/high) were analyzed using FlowJo software (v8.8.7) (Ashland, OR, USA). Each cell line was assessed in duplicate in three separate biological experiments.

Western blots

Protein lysates were prepared in RIPA lysis buffer (50mM Tris-HCl pH7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl, 2mM EDTA, 50mM NaF). Protein (10-25μg) was electrophoresed on 10% Bis Tris or 4-12% Bis Tris gradient NuPAGE Novex protein gels (Life Technologies) and transferred to PVDF membrane (Merck Millipore). Membranes were blocked in 5% skim milk in TBST (20mM Tris, 150mM NaCl, 0.1% Tween-20) for 1 h before incubation with primary antibodies diluted in 5% skim milk in TBST overnight at 4°C, or for 1 h at room temperature in the case of pan-actin. Primary antibodies: cleaved caspase-3 (Asp175, clone 5A1E; Rabbit mAb; 1:500), phospho-mTOR (Ser2448; clone D9C2; Rabbit mAb; conc: 1:1000), mTOR (clone 7C10, Rabbit mAb, conc: 1:1000), phospho-p70 S6 kinase (Thr389, clone 108D2; Rabbit mAb; conc: 1:1000), phospho-p70 S6 kinase (Ser371; conc: 1:1000), phospho-4E-BP1 (Thr37/46, clone 236B4; Rabbit mAb; conc: 1:1000), pan actin (Ab-5, mouse monoclonal antibody, Thermo Scientific, 1:3000). Secondary antibodies: goat anti-rabbit IgG, HRP-linked and goat anti-mouse HRP-linked. Antibodies were from Cell Signaling Technologies unless otherwise noted.

siRNA knockdown (not high throughput)

Cells were transfected 24 hrs after seeding with ON-TARGETplus siRNA SMARTpools (Dharmacon GE) (20nM final concentration) complexed with DharmaFECT 3 in Opti-MEM reduced serum media (Life Technologies). Cells transfected with a non-targeting ON-TARGETplus control siRNA SMARTpool (cat # D-001810-02) or mock transfection (lipid only) served as controls in every assay. All assays were carried out in culture medium without antibiotics. In assays that combined drug treatments, drug treatment was commenced 24 h after siRNA transfection. Cell viability was assessed 72 h after transfection by trypsinisation and counting using a BioRad TC10 automated counter (Hercules, CA, USA).

Gene expression analysis

Expression of transcripts in mouse OS samples was assessed using RNA-seq data derived from both the *Osx*-Cre $p53^{fl/fl}pRb^{fl/fl}$ mice (fibroblastic OS model) and *Osx*-Cre TRE-p53.1224

pRb$^{fl/fl}$ model (osteoblastic OS model). Three independent cell cultures from anatomically similar sites from the fibroblastic OS (1) and the osteoblastic OS (2) models were analyzed. RNA-Seq libraries were constructed from 3-15μg of total RNA at the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Australia). RNA-Seq libraries were sequenced on the Illumina HiSeq 2000 with 100bp paired-end reads at the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Australia). RNA-Seq reads were aligned to the mouse genome build mm9/NCBI37 using Casava 1.7 and Bowtie v0.12.2 mapping software. We used a differential expression analysis pipeline developed at the Victorian Life Sciences Computation Centre, University of Melbourne. This pipeline is based on the Tuxedo protocol (Tophat, Cufflinks) and EdgeR/Voom with HTSeq-count (Source code available at: https://bitbucket.org/jessicachung/rna_seq_pipeline). Aligned reads (see above) were summarised using HTSeq (9). Normalization factors were calculated using the edgeR method="TMM" corresponding to the weighted trimmed mean of M-values (to the reference) (10), where the weights are from the delta method on Binomial data. Normalized read counts (moderated log counts per million) and differential expression were generated using a protocol from Voom (11). The raw dataset was deposited in Gene Expression Omnibus (GEO) database with accession number GSE58916.

For gene expression the human OS lines MG63, Saos-2, U2OS, G-292, 143B, SJSA-1 and OS17 were used as well as 6 independent primary human osteoblasts. RNA was prepared using Trisure (Bioline). RNA was prepared from mouse primary tissues and cell lines using Trisure or RNA extraction kits (Bioline). All RNA was DNase treated prior to reverse transcription. cDNA was prepared as directed by the manufacturer (Agilent or Bioline) and quantitative real-time PCR (qPCR) performed as previously described (12). Oligonucleotide sequences are listed in Supplemental Table 1. Primer/probes for qPCR were from IDT (Ohio, USA) and sequences are previously described (2). The relative gene expression was normalized to the *Hprt* or *PGK1* housekeeping gene and calculated by the $2^{-\Delta CT}$ method.

***In vivo* treatment with GSK2126458**

Balb/c nu/nu mice were used as recipients for transplant of luciferase tagged OS cells. All experiments were approved by the AEC (St. Vincent's Hospital, Melbourne). For *in vivo* imaging cell lines were infected stably with pMMP-LucNeo virus (gift from A Kung, Dana-Farber Cancer Institute). 75,000 cells were implanted subcutaneously on the back flank of Balb/c nu/nu recipients. Cells were resuspended in extracellular matrix (Cultrex PathClear BME Reduced Growth Factor Basement Membrane Extract). The tumors were allowed to establish for 7 days prior to initial imaging (designated Day 0). For imaging *in vivo* mice were anesthetized (isoflurane), injected with d-luciferin at 15mg/ml i.p. (Perkin Elmer), and imaged with the IVIS 200 Imaging System (Xenogen) using an auto-exposure and a manual exposure of 1 s with F-stop 1 and Bin 4. To quantitate the bioluminescence from the tumors, identical circular regions of interest were drawn to encircle the tumor on each flank, and the integrated flux of photons (photons/second) within each region of interest was determined by using the

LIVING IMAGES software package (Xenogen/Perkin Elmer) as instructed by the manufacturer. GSK2126458 was dissolved in DMSO then dissolved in 0.5% hydroxypropylmethylcellulose (Sigma) and 0.2% Tween-80 (Sigma) in purified water (13, 14). Animals were treated with GSK2126458 (Selleckchem) by oral gavage at 1.5mg/kg/day 5 days per week for 4 weeks or vehicle control (15). Micro-computed tomography (μCT) analysis was performed performed at the endpoint only according to standard procedures using a Skyscan 1076 scanner (Skyscan/Bruker) (2, 16, 17). Mice were anesthetized with 100μl/10g of body weight with ketamine. Animals were scanned at a voxel size of 9μm, reconstructed using NRecon (Skyscan) with smoothing of 1, ring artifact reduction of 6 and beam-hardening correction of 35%. 3D modeling of the bones were done using CVol (Skyscan) applying surface rendering after using the marching cube tight model in CTAn (Skyscan), or CTvox (Skyscan) applying volume rendering or maximum intensity projection.

Statistical analysis (not including siRNA screen)

Each experiment is represented as the mean ± SEM (error bars) calculated from biological replicates unless otherwise stated. Unpaired parametric Student's *t*-test was used to compare results unless otherwise stated. Where indicated in legends, unpaired nonparametric Mann Whitney ranks test or 2-way analysis of variance (2-way ANOVA) were used to compare groups. Data was log transformed to normalize variances before 2-way ANOVA. In all analyses, statistical significance of $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$ are represented as *, **, ***, **** respectively. Prism 6.0e software was used for statistical analyses.

References:

1. Walkley CR, Qudsi R, Sankaran VG, Perry JA, Gostissa M, Roth SI, et al. Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Dev 2008; 22: 1662-76.

2. Mutsaers AJ, Ng AJ, Baker EK, Russell MR, Chalk AM, Wall M, et al. Modeling distinct osteosarcoma subtypes in vivo using Cre:lox and lineage-restricted transgenic shRNA. Bone 2013; 55: 166-78.

3. Ziauddin J, Sabatini DM. Microarrays of cells expressing defined cDNAs. Nature 2001; 411: 107-10.

4. Birmingham A, Selfors LM, Forster T, Wrobel D, Kennedy CJ, Shanks E, et al. Statistical methods for analysis of high-throughput RNA interference screens. Nat Methods 2009; 6: 569-75.

5. Falkenberg KJ, Gould CM, Johnstone RW, Simpson KJ. Genome-wide functional genomic and transcriptomic analyses for genes regulating sensitivity to vorinostat. Scientific Data 2014; 1: Article number: 140017.

6. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4: 44-57.

7. Kaida D, Motoyoshi H, Tashiro E, Nojima T, Hagiwara M, Ishigami K, et al. Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA. Nat Chem Biol 2007; 3: 576-83.

8. Fitzgerald JB, Schoeberl B, Nielsen UB, Sorger PK. Systems biology and combination therapy in the quest for clinical efficacy. Nat Chem Biol 2006; 2: 458-66.

9. Anders S, Pyl PT, Huber W. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics 2015; 31: 166-9 (originally obtained from bioRxiv http://dx.doi.org/10.1101/002824).

10. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol 2010; 11: R25.

11. Law CW, Chen Y, Shi W, Smyth GK. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol 2014; 15: R29.

12. Walkley CR, Shea JM, Sims NA, Purton LE, Orkin SH. Rb Regulates Interactions between Hematopoietic Stem Cells and Their Bone Marrow Microenvironment. Cell 2007; 129: 1081-95.

13. Posch C, Moslehi H, Feeney L, Green GA, Ebaee A, Feichtenschlager V, et al. Combined targeting of MEK and PI3K/mTOR effector pathways is necessary to effectively inhibit NRAS mutant melanoma in vitro and in vivo. Proc Natl Acad Sci U S A 2013; 110: 4015-20.

14. Villanueva J, Infante JR, Krepler C, Reyes-Uribe P, Samanta M, Chen HY, et al. Concurrent MEK2 mutation and BRAF amplification confer resistance to BRAF and MEK inhibitors in melanoma. Cell Rep 2013; 4: 1090-9.

15. Knight SD, Adams ND, Burgess JL, Chaudhari AM, Darcy MG, Donatelli CA, et al. Discovery of GSK2126458, a Highly Potent Inhibitor of PI3K and the Mammalian Target of Rapamycin. ACS Medicinal Chemistry Letters 2010; 1: 39-43.

16. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J Bone Miner Res 2010; 25: 1468-86.

17. Singbrant S, Russell MR, Jovic T, Liddicoat B, Izon DJ, Purton LE, et al. Erythropoietin couples erythropoiesis, B-lymphopoiesis, and bone homeostasis within the bone marrow microenvironment. Blood 2011; 117: 5631-42.

Supplemental Table 1.

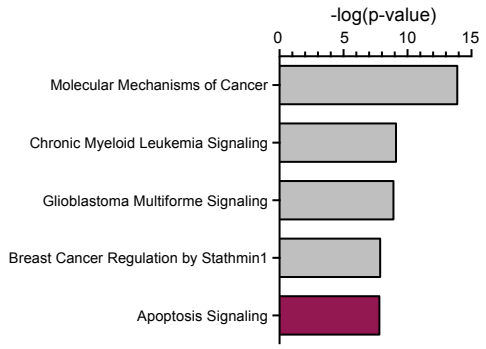
Primer Sequences used for qPCR

Gene	Forward	Reverse
Cse1L	5'-TCATCCCCTATATCCCCACTC-3'	5'-GGGTTAGCTTTGCAGGTTATTC-3'
Nxf1	5'-ACGAAGGGAAGTCATACAACG-3'	5'-CCTCAAATCGGGAAGACTGTAC-3'
Phf5a	5'-GTGTGGCTATCGGAAGACTG-3'	5'-GCCCTGGTAAGATCCATAGTTAC-3'
U2af2	5'-GAGTTGTATCCACGGTAGTCC-3'	5'-CCTGTGGCACTATCCTTAACC-3'

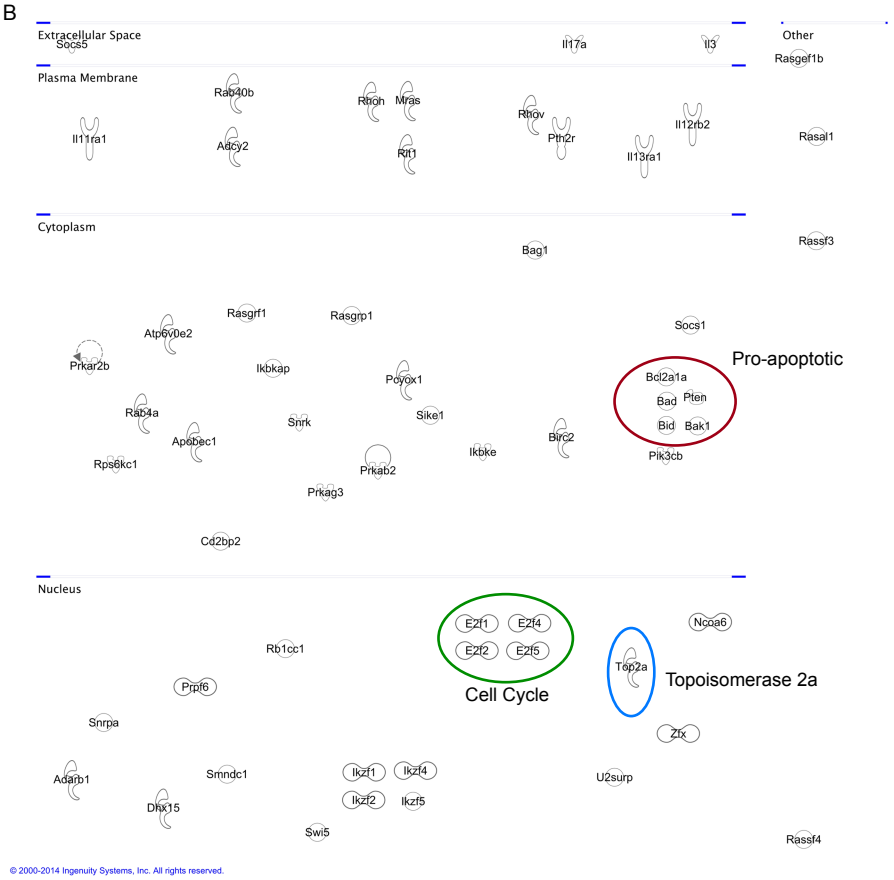
Gene/ Species/ Assay	Forward Primer 5'→3'	Reverse Primer 5'→3'	Reference
Pik3ca / Mouse / Sybr	AACATCTGGCAAAACCAAGGC	CCACACAGTCCCCAATGGAT	
Pik3cb / Mouse / Sybr	GGGGCAGTTCATCTTCTAAGC	GGGCGGAAGCACACTCATAA	
Mtor / Mouse / Sybr	CTGCAGAATCGTCAAGGGGT	ATCTCCCTGGCTGCTCCTTA	
Rptor / Mouse / Sybr	CCTATGGGAACATCCGAGCC	ACTGAGCTGCCCGATTCTTC	
Rictor / Mouse / Sybr	CGCTCGTGGGCAGGTATTAT	CGAGCAGACCTCGCCTTATT	
PIK3CA/ Human / Sybr	CCTGATCTTCCTCGTGCTGCTC	ATGCCAATGGACAGTGTTCCTCTT	Henken et al (2011) Molecular Cancer 10:71
PIK3CB / Human / Sybr	CTAATGTGTCAAGTCGAGGTGGAA	GGAAAATCTCTCGGCAGTCTTGT	Ghavampour et al (2013) PLOS One 8(9):e73981
PIK3CD / Human / Sybr	GAGAGAAGACAAACCCCGCT	GTTTCCCTTCGCAGATGTGT	
PIK3CG/ Human / Sybr	ACCACCGAATGTCTGTGTTTC	TGCAAAACTATTGCCGGAACG	

Supplemental Figure 1. Suppressors of doxorubicin induced cell death from siRNA screen

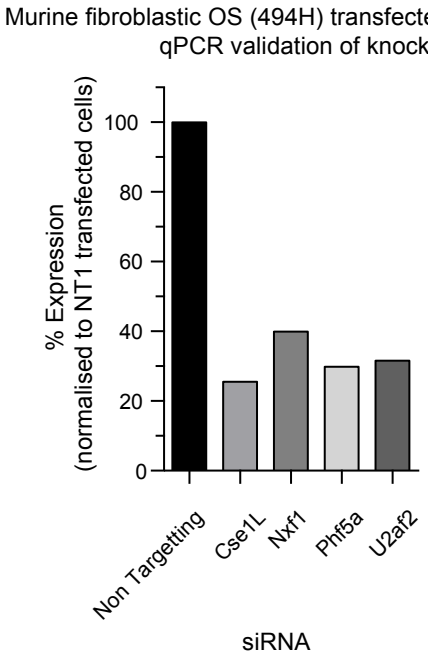
A



B

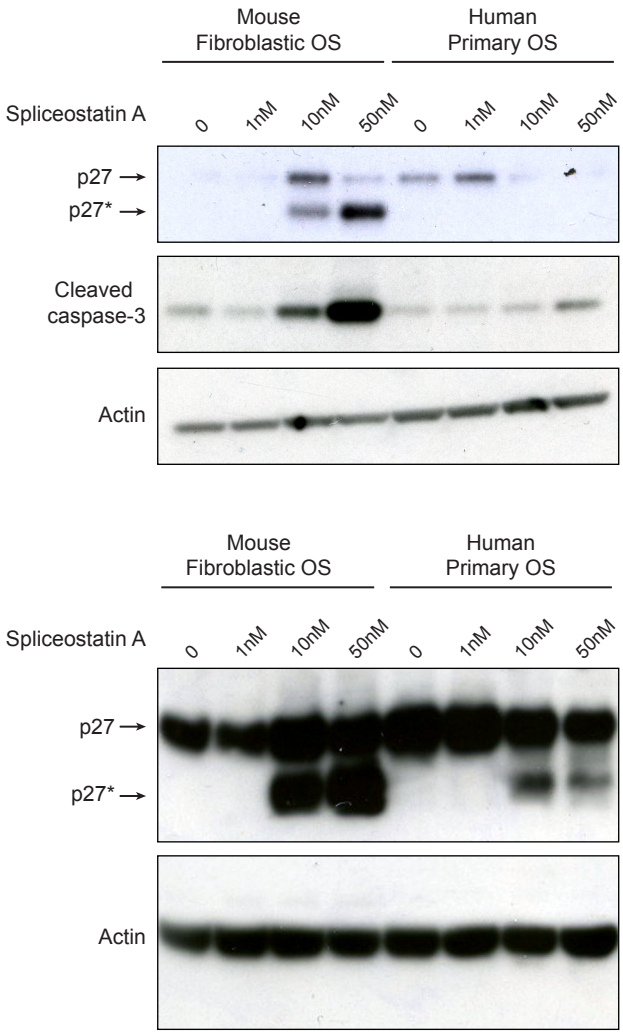


C

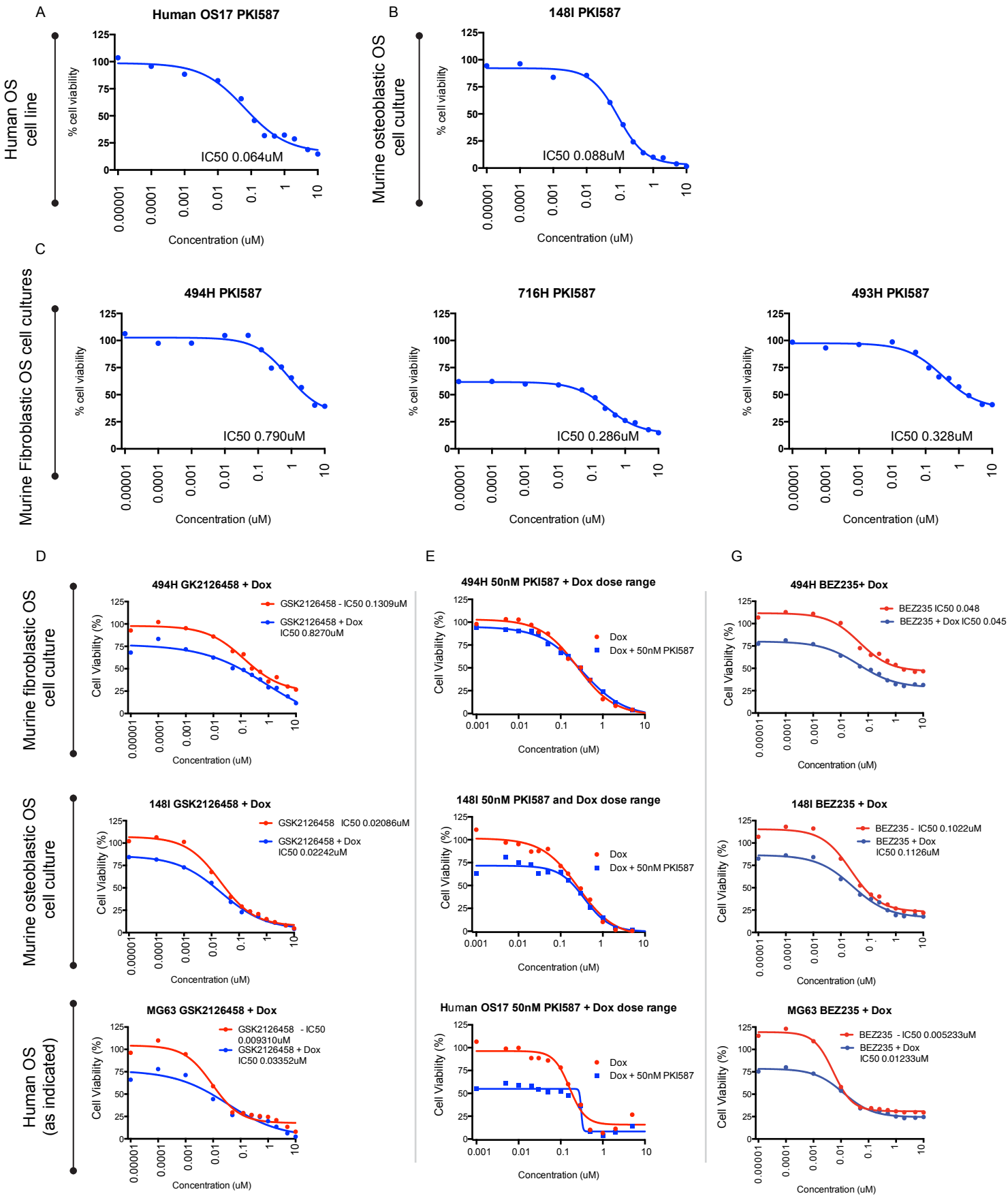


Supplemental Figure 1.
A. Ingenuity pathwy analysis of siRNA that decreased cell death in response to doxorubicin (suppressors). **B**. Visualisation of the siRNA fulfilling this category using Igenuity Pathway Analysis. **C**. qPCR of OS 494H transfected withnon targeting siRNA (NT) or the indicated siRNA to demonstrate efficient knockdown.

Supplemental Figure 2.

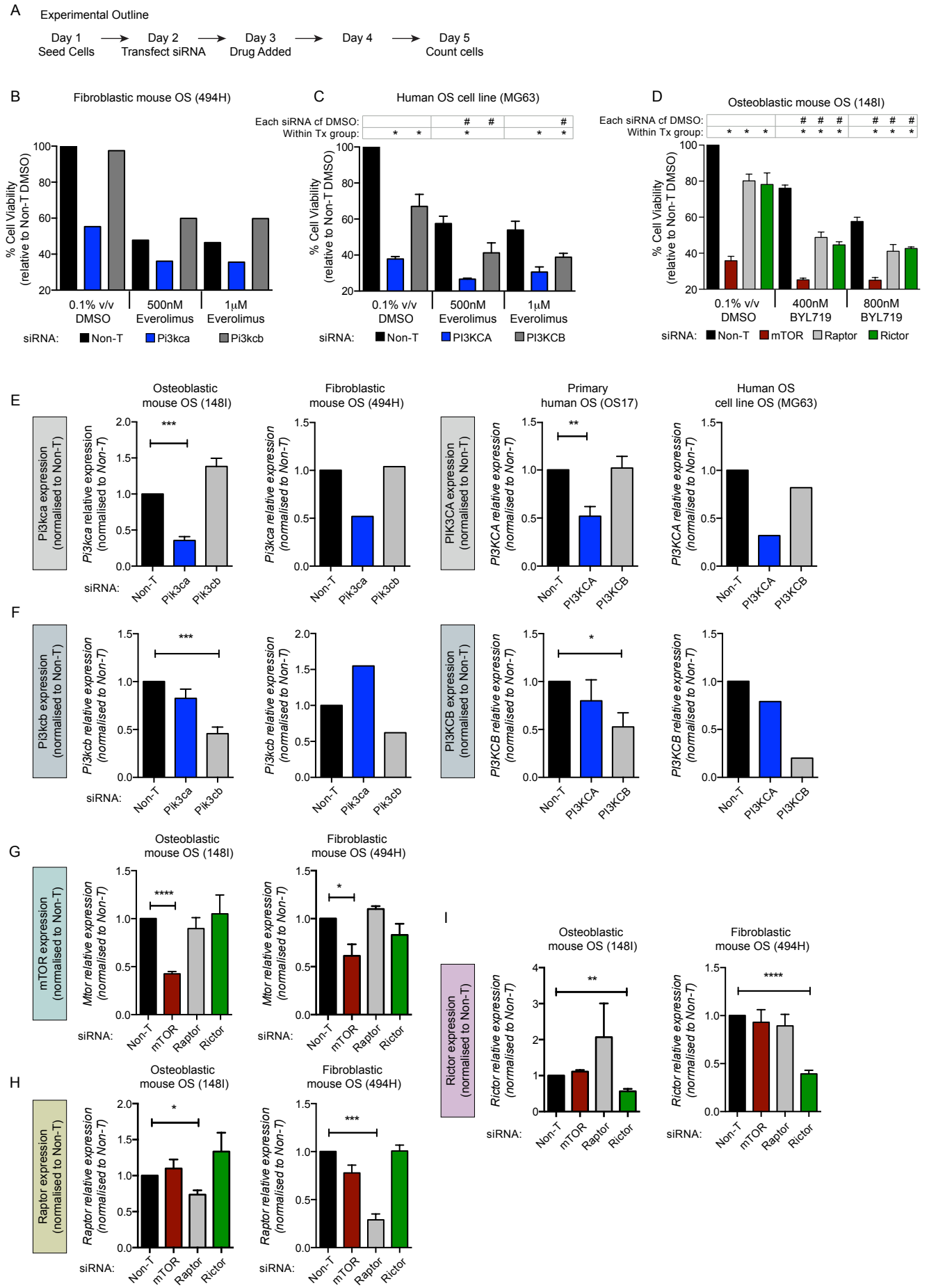


Supplemental Figure 2.
A. Dose response of murine fibroblastic OS (494H) and primary human OS cell culture (OS17) to spiceostatin A. Levels of p27 and the aberrantly spliced isoform p27* are indicated; cleaved caspase-3 and actin loading control. **B**. Overexposed blot from A.



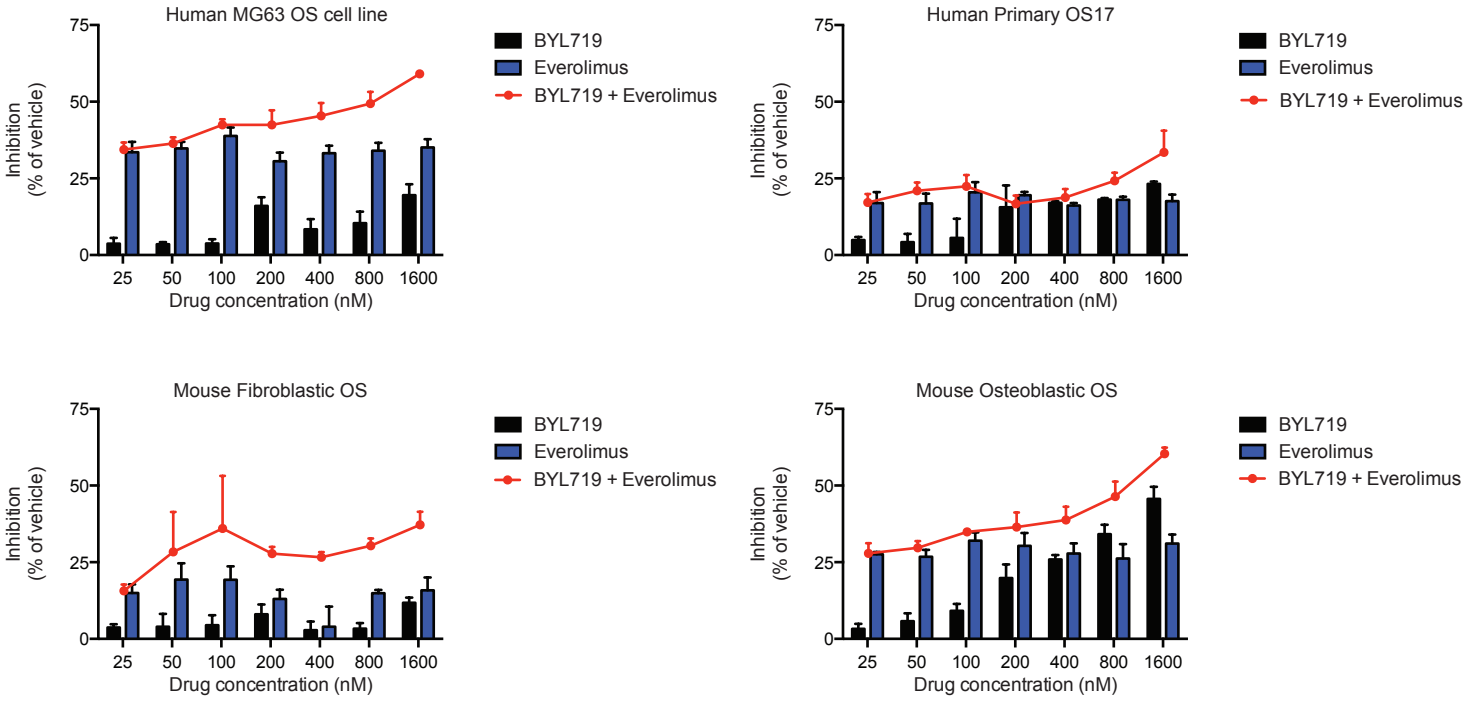
Supplemental Figure 3.
Dose response curves of human primary OS (OS17) (**A**), murine osteoblastic OS (148I)(**B**) and murine fibroblastic OS (494H, 716H, 493H)(**C**) to PKI587. Dose response curves of murine fibroblastic OS (494H), Murine osteoblastic OS (148I) and human OS cell line (MG63) or primary human OS derived cell culture (OS17) treated with doxorubicin and GSK2126458 (**D**), PKI587 (**E**) and BEZ235 (**F**).
(D) Fixed dose of doxorubicin with variable GSK2126458
(E) Fixed dose of PKI587 and variable dose of doxorubicin
(F) Fixed dose of doxorubicin with variable BEZ235

Supplemental Figure 4.

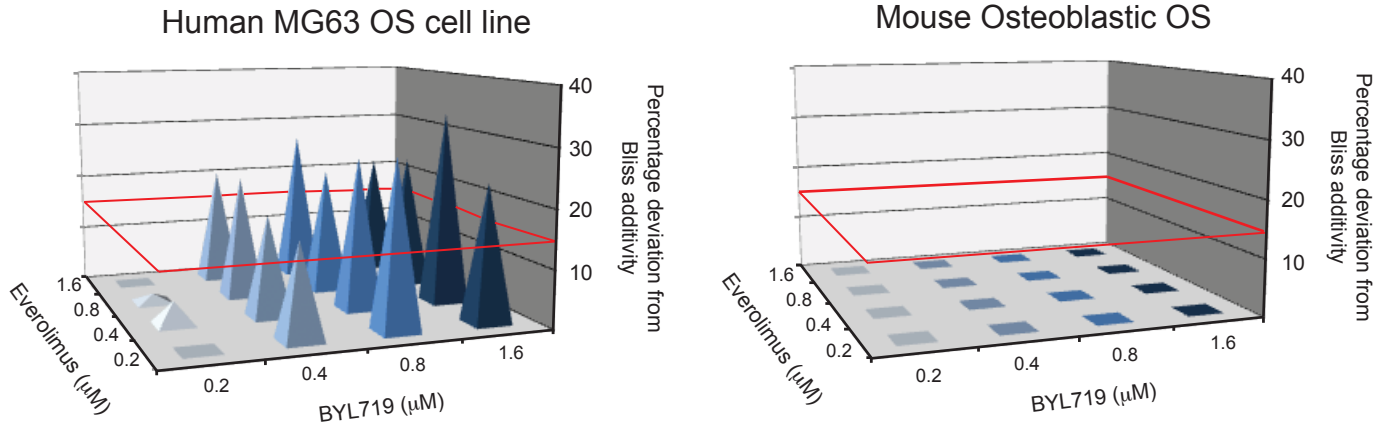


Supplemental Figure 4.
A. Experimental outline; **B**. SMARTpool siRNA for PI3K subunits in mouse OS and treatment with everolimus; **C**. SMARTpool siRNA for PI3K subunits in human OS and treatment with everolimus; **D**. SMARTpool siRNA for mTOR, Raptor, Rictor subunits in mouse OS and treatment with PI3KCA inhibitor BYL719 as indicated; qPCR for Pi3kca and PI3KCA (**E**) or Pi3kcb and PI3KCB (**F**) respectively in cells treated with the indicated siRNA in human and mouse OS cells as indicated; qPCR for mTOR (**G**), Raptor (**H**) and Rictor (**I**) in mouse OS cells.

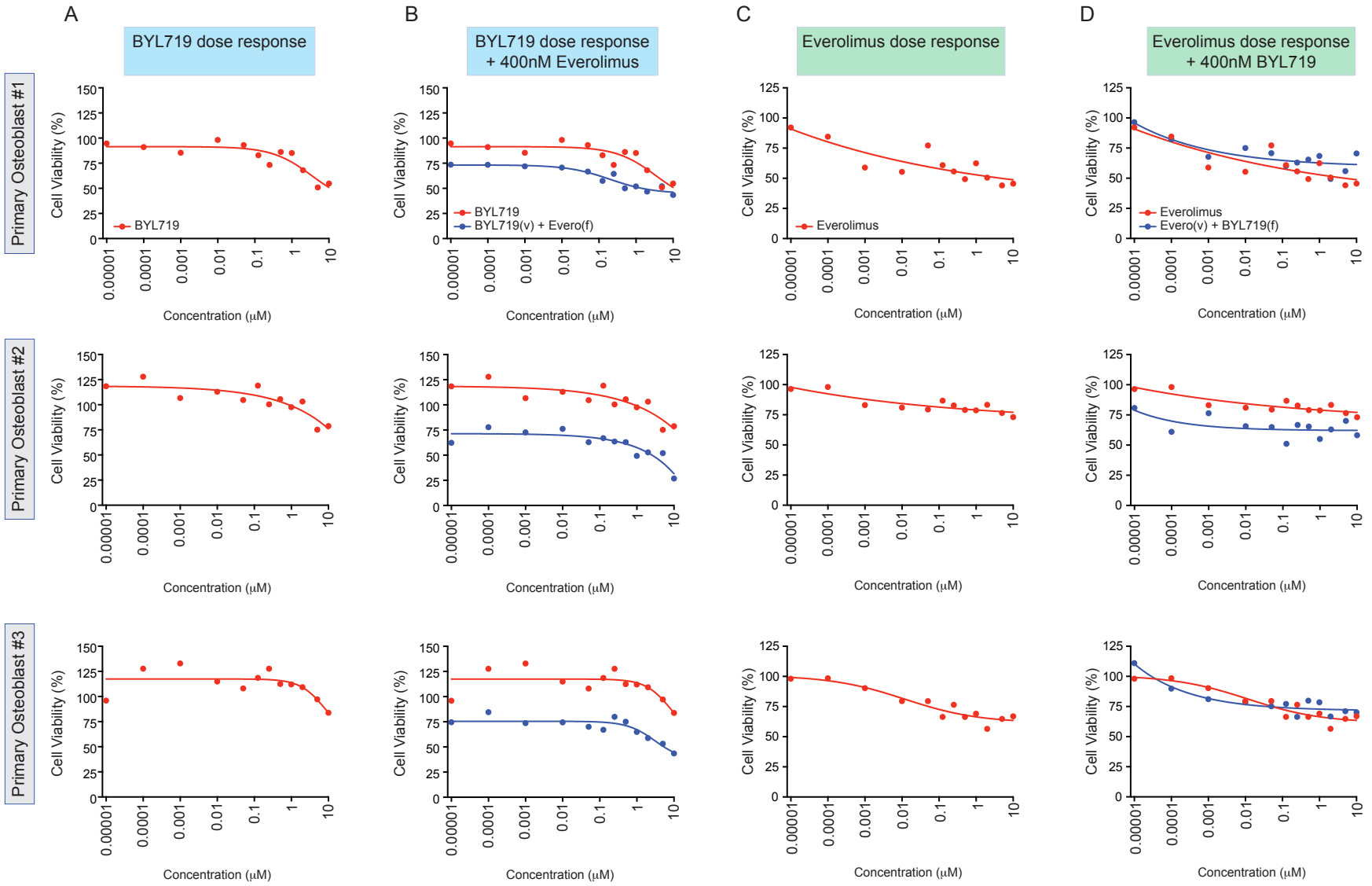
Supp Figure 5 : BYL719 and Everolimus Synergy Matrix - combined results (Mean+/-SEM)(n=3 except OS17 n=2)
Plotted as Inhibition, as a percentage of vehicle control.



Supp Figure 5: BYL719 and Everolimus Synergy Matrix - Bliss additivity calculation of Synergism
- combined results (Mean percentage deviation from Bliss additivity from n=3 expts, except OS17 n=2.
Plotted as Percentage deviation from Bliss additivity.
Synergism is currently defined as greater than 15% deviation from Bliss additivity score (red line).

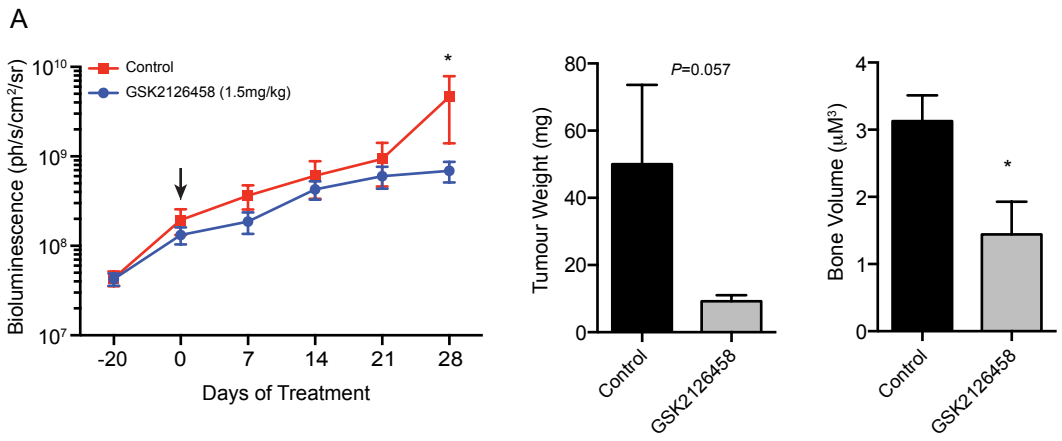


Supplemental Figure 6.



Supplemental Figure 6.
Dose response curves of primary mouse osteoblasts (three independently isolated cultures from wild-type C57Bl/6 mouse long bones, designated 1, 2, 3 respectively)
(**A**) Dose response of BYL719 (Pik3ca inhibitor) alone
(**B**) Dose response of BYL719 (Pik3ca inhibitor) alone (red line, data from A), Dose response of BYL719 (BYL719(v)) with a fixed dose of 400nM Everolimus (Evero(f); blue line).
(**C**) Dose response of Everolimus (mTOR inhibitor) alone
(**D**) Dose response of Everolimus (mTOR inhibitor) alone (red line, data from C), Dose response of Everolimus (Evero(v)) with a fixed dose of 400nM BYL719 (BYL719(f); blue line).
All data points are means of triplicate replicates per osteoblast culture; Dose response curve calculated using Prism 6 software.
Fixed doses of BYL719 and Everolimus derived from doses that elicited a synergistic interaction between the BLY719 and everolimus based on BLISS synergy scores (Figure 6D).

Supplemental Figure 7. GSK2126458 treatment reduces OS tumour proliferation *in vivo*.



Supplemental Figure 7.
A. GSK2126458 induces reduced OS proliferation and mineral deposition in vivo. Luciferase tagged murine fibroblastic OS (494H) were grafted subcutaneously and allowed to establish for 20 days. At day 20 (indicated as 0 on the graph) size was quantitated by luminescence and treatment with 1.5mg/kg/day GSK2126458 was performed 5 days per week for 4 weeks. Bioluminescence imaging to assay tumor burden in mice at day 0, 14, 21 and 28 post treatment. Total tumor bioluminescence and tumor bone volume (an indicator of tumor size) were significantly reduced following treatment with GSK2126458. Data are expressed as mean ±SEM (n=6 per cohort). * p <0.05 calculated using Mann Whitney ranks test.