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**Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR).**

Juan M. García-Martínez<sup>1</sup>, Jennifer Moran<sup>2</sup>, Rosemary G. Clarke<sup>3</sup>, Alex Gray<sup>4</sup> Sabina C Cosulich<sup>5</sup>, Christine M. Chresta<sup>5</sup> and Dario R. Alessi<sup>1</sup>

1. MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland.
2. Division of Signal Transduction Therapy, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland.
3. Division of Cell Biology and Immunology Division of Cell Biology and Immunology, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland.
4. Division of Molecular Physiology, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland.
5. Cancer Bioscience, AstraZeneca, Alderley Park, Cheshire, SK10 4TG, England.

Correspondence to DRA (d.r.alessi@dundee.ac.uk)  
Telephone 44-1382, 344 241 Fax 44-1382, 223 778

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Running title: Novel small molecule mTOR inhibitor.

**Abstract.**

mTOR stimulates cell growth by phosphorylating and promoting activation of AGC family kinases such as Akt, S6K and SGK. mTOR complex-1 (mTORC1) phosphorylates the hydrophobic motif of S6K, whereas mTORC2 phosphorylates the hydrophobic motif of Akt and SGK. Here we describe the small molecule Ku-0063794, which inhibits both mTORC1 and mTORC2 with an  $IC_{50}$  of ~10 nM, but does not suppress the activity of 76 other protein kinases or 7 lipid kinases including Class 1 PI 3-kinases at 1000-fold higher concentrations. Ku-0063794 is cell permeant, suppresses activation and hydrophobic motif phosphorylation of Akt, S6K and SGK, but not RSK, an AGC kinase not regulated by mTOR. Ku-0063794 also inhibited phosphorylation of the T-loop Thr308 residue of Akt phosphorylated by PDK1. We interpret this as implying phosphorylation of Ser473 promotes phosphorylation of Thr308 and/or induces a conformational change that protects Thr308 from dephosphorylation. In contrast, Ku-0063794 does not affect Thr308 phosphorylation in fibroblasts lacking essential mTORC2 subunits, suggesting that signalling processes have adapted to enable Thr308 phosphorylation to occur in the absence of Ser473 phosphorylation. We found that Ku-0063794 induced a much greater dephosphorylation of the mTORC1 substrate 4E-BP1 than rapamycin, even in mTORC2 deficient cells, suggesting a form of mTOR distinct from mTORC1 or mTORC2 phosphorylates 4E-BP1. Ku-0063794 also suppressed cell growth and induced a G1-cell cycle arrest. Our results indicate that Ku-0063794 will be useful in delineating the physiological roles of mTOR and may have utility in treatment of cancers in which this pathway is inappropriately activated.

**Introduction**

The mammalian Target Of Rapamycin (mTOR) protein kinase lies at the nexus of signalling pathways that regulate cell growth and proliferation. Many cancer driving mutations in genes such as receptor tyrosine kinases, Ras, PI 3-kinase and PTEN stimulate proliferation, growth and survival by activating the mTOR kinase. Two mTOR complexes have been characterised, termed mTOR Complex-1 (mTORC1) and mTOR Complex-2 (mTORC2) (reviewed in [1, 2]). mTORC1 consists of mTOR, Raptor and mLST8 and is acutely inhibited by rapamycin [3-5]. mTORC1 is activated by insulin and growth factors via a PI 3-kinase controlled pathway, involving Akt mediated phosphorylation of PRAS40 and tube

### Materials and methods.

**Materials.** Protein G-Sepharose, glutathione-Sepharose, were purchased from Amersham Bioscience.  $^{32}\text{P}$ -ATP was from Perkin-Elmer; IGF1 was from Invitrogen. Tween-20, DMSO, Phorbol-12-Myristate-13-Acetate (TPA) and dimethyl pimelimidate were from Sigma, and CHAPS and Rapamycin were from Calbiochem. Akti-1/2, PI-103, PD0325901-CL was synthesised by Dr Natalia Shpiro at the University of Dundee. Ku-0063794 was synthesised at AstraZeneca. The wild type control mLST8<sup>+/+</sup> and mLST8<sup>-/-</sup> knockout mouse embryonic fibroblast cells (MEFs) were described previously [17] and kindly provided by David Sabatini (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts). The wild type control Rictor<sup>+/+</sup> and Rictor<sup>-/-</sup> knockout MEFs were described previously [29] and kindly provided by Mark Magnuson (Vanderbilt University School of Medicine, Nashville, Tennessee). The wild type control Sin1<sup>+/+</sup> and Sin1<sup>-/-</sup> knockout MEFs were described previously [16] and kindly provided by Bing Su (Yale University School of Medicine, New Haven).

**Antibodies.** The following antibodies were raised in sheep and affinity purified on the appropriate antigen: anti-mLST8 (S837B, 3<sup>rd</sup> bleed) was raised against the human full length mLST8 protein expressed in *E.coli* (used for immunoblotting), anti-Raptor (S682B, 3<sup>rd</sup> bleed; residues 1-20 of human Raptor MESEMLQSPLLGLGEEDEAD, used for immunoblotting and immunoprecipitation), anti-Rictor (S654B, 3<sup>rd</sup> bleed; residues 6-20 of human Rictor RGRSLKNLRVGRND, used for immunoblotting in 293 cells and immunoprecipitation), anti-Rictor (S274C, 1<sup>st</sup> bleed; residues 6-20 of mouse Rictor RGRSLKNLRIRGRND, used for immunoblotting), anti-Sin1 (S8C, 1<sup>st</sup> bleed) was raised against the human full length Sin1 protein expressed in *E.coli* (used for immunoblotting). Anti-NDRG1 (S276B, 2<sup>nd</sup> bleed) was made in sheep using recombinant GST-fusion of full-length NDRG1 (used for immunoblotting). An antibody that recognizes NDRG1 phosphorylated at Thr346, Thr356 and Thr366 (S911B, 2<sup>nd</sup> bleed; termed pNDRG1 3xThr-P) was raised against the nonapeptide RSRSHpTSEG, whose sequence is common to all three SGK1 phosphorylation sites on NDGR1 (used for immunoblotting). Anti-Akt1 (S695B, 3<sup>rd</sup> bleed; residues 466 - 480 of human Akt1 RPHFPQFSYSASGTA, used for immunoblotting and immunoprecipitation), anti-S6K (S417B, 2<sup>nd</sup> bleed; residues 25 - 44 of human S6K AGVFDIDLDPEDAGSEDEL, used for immunoblotting and immunoprecipitation). Anti-GST (S902A, 1<sup>st</sup> bleed) was raised against the GST tag expressed from pGex4T (used for immunoblotting). Anti-PRAS40 (S115B, 1<sup>st</sup> bleed; residues 238 - 256 of human PRAS40 DLPRPRLNTSDFQKLKRY, used for immunoblotting). An antibody that recognizes PRAS40 phosphorylated at Thr246 (S114B, 2<sup>nd</sup> bleed) was raised against the residues 240 - 251 of human PRAS40 CRPRLNTpSDFQK (used for immunoblotting). Anti-RSK2 (S382B, 1<sup>st</sup> bleed; residues 712 - 734 of human RNQSPVLEPVGRSTLAQRRGIKK, used for immunoblotting and immunoprecipitation); Anti-FoxO1 (S504A, 1<sup>st</sup> bleed) was made in sheep using recombinant GST-fusion FoxO1 (2-655, used for immunoblotting). Anti- $\beta$  Tubulin (H-235, #sc-9104), phospho-RSK Thr227 (#sc-12445-R) and the total mTOR antibody (#sc-1549) were purchased from SantaCruz Biotechnology. For phospho-immunoblotting of the hydrophobic motif of SGK1(Ser422) we employed the Thr389 S6K antibody (# 9205) from Cell Signalling Technology which we previously demonstrated cross-reacted with the phosphorylated Ser422 of SGK1 [30]. The phospho-Akt Ser473 (#9271), Thr308 (#4056), Thr450 (#9267), phospho-S6K Thr389 (#9234), phospho S6 ribosomal protein Ser235 (#4856), total S6 ribosomal protein (#2217), 4E-BP1 total (#9452), phospho 4E-BP1 Thr37/Thr46 (#9459), phospho 4E-BP1 Ser65 (#9451), phospho-ERK Thr202/Tyr204 (#9101), total ERK (#9102), phospho-RSK Thr573 (#9346), phospho-RSK Thr380 (#9341), phospho-FoxO1/O3 Thr24/32 (#9464), phospho-GSK3 $\alpha/\beta$  Ser21/9 (#9331), the phospho-mTOR Ser2481 (#2974) and the phospho-mTOR Ser2448 (#2971) were purchased from Cell Signaling Technology. For phospho-immunoblotting of the phosphorylated T-loop of S6K we employed the pan-PDK1 site antibody from Cell Signaling Technology (#9379) as previously described [31]. The GSK3 $\alpha/\beta$  antibody (44-610)

was purchased from Biosource. The Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Thermo Scientific.

**General methods.** Tissue culture, immunoblotting, restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols. DNA constructs used for transfection were purified from *E. coli* DH5 $\alpha$  using Qiagen plasmid Mega or Maxi kit according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, UK, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. For transfection studies, typically ten 10-cm-diameter dishes of HEK-293 cells were cultured and each dish was transfected with 5-10  $\mu$ g of the indicated plasmids using the polyethylenimine method [32]. Cellular levels of phosphatidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) were measured as described previously [33]. MEFs were cultured with additional non-essential amino acids and 1% sodium pyruvate solution.

**Buffers.** The following buffers were used: Tris lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 0.3 % (w/v) CHAPS, 1 mM sodium orthovanadate, 10 mM sodium- $\beta$ -glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.15 M NaCl, 0.1 % (v/v) 2-mercaptoethanol, 1 mM benzamidine and 0.1 mM phenylmethylsulphonylfluoride], Buffer A [50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA and 0.1 % (v/v) 2-mercaptoethanol], Hepes lysis buffer [40 mM Hepes (pH 7.5), 120 mM NaCl, 1 mM EDTA, 0.3 % (w/v) CHAPS, 10 mM sodium pyrophosphate, 10 mM sodium- $\beta$ -glycerophosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1 mM benzamidine and 0.1 mM phenylmethylsulphonylfluoride], Hepes kinase buffer [25 mM Hepes (pH 7.5), 50 mM KCl], TBS-Tween Buffer [50 mM Tris/HCl pH 7.5, 0.15 M NaCl and 0.1% (v/v) Tween-20] and Sample Buffer [50 mM Tris/HCl pH 6.8, 6.5% (v/v) Glycerol, 1% (w/v) SDS, and 1 % (v/v) 2-mercaptoethanol].

**Cell lysis.** HEK293, HeLa or MEF cells were cultured and treated as described in the figure legends. Following treatment, cells were rapidly rinsed twice with ice cold PBS and then lysed using Tris lysis buffer. Whole-cell lysates were centrifuged (18,000  $\times$  g at 4 °C for 20 min), supernatants were removed and stored in aliquots at -80 °C until required.

**Specificity kinase panel.** All assays were performed at The National Centre for Protein Kinase Profiling (<http://www.kinase-screen.mrc.ac.uk/>) as previously described [34]. Briefly, all assays were carried out robotically at room temperature (21 °C) and were linear with respect to time and enzyme concentration under the conditions used. Assays were performed for 30 min using Multidrop Micro reagent dispensers (Thermo Electron Corporation, Waltham, MA, U.S.A.) in a 96-well format. The abbreviations for each kinase are defined in legend to Table 1. The concentration of magnesium acetate in the assays was 10 mM and [ $\gamma$ -<sup>33</sup>P]ATP (~800 cpm/pmol) was used at 5  $\mu$ M for CK2 $\alpha$ , DYRK3, EF2K, ERK1, ERK8, GSK3 $\beta$ , HIPK2, IGF1R, IRR, MARK3, MKK1, p38 $\gamma$  MAPK, p38 $\delta$  MAPK, PAK4, PIM2, Akt1, PLK1, PKC $\zeta$  and PRK2; 20  $\mu$ M for CaMKK $\beta$ , CDK2/cyclin A, CHK1, CHK2, CK1 $\delta$ , CSK, EPH-B3, FGF-R1, IR, JNK1 $\alpha$ 1, JNK2 $\alpha$ 2, MAPKAP-K2, MSK1, MST2, MST4, p38 $\beta$  MAPK, PKA, PAK5, PAK6, PDK1, PIM1, PIM3, PKC $\alpha$ , ROCKII, PRAK, S6K1, SGK1, SYK, VEGFR and YES1; or 50  $\mu$ M for AMPK, BRSK2, BTK, CaMK1, DYRK1a, DYRK2, EPH-A2, ERK2, IKK $\epsilon$ , LCK, MELK, NEK2A, NEK6, p38 $\alpha$ , PhK $\gamma$ 1, Akt2, PKD1, RSK1, RSK2, SRPK1 and TBK1, in order to be at or below the K<sub>m</sub> for ATP for each enzyme [34].

#### **Lipid kinase panel.**

Sphingosine Kinase 1 (SPHK1) was assayed as follows: SPHK1 (diluted in 50mM Tris/HCl pH 7.5, 150mM NaCl, 5mM MgCl<sub>2</sub>, 1mM EGTA, 1mM DTT) was assayed against Sphingosine in a final volume of 50  $\mu$ l containing 50mM Tris/HCl pH 7.5, 150mM NaCl, 5mM MgCl<sub>2</sub>, 1mM EGTA, 10 $\mu$ M Sphingosine, 10 $\mu$ M ATP, 1mM DTT and incubated for 30 min at room temperature.

Sphingosine Kinase 2 (SPHK2) was assayed as follows: SPHK2 (diluted in 50mM Tris/HCl pH 7.5, 200mM KCl, 5mM MgCl<sub>2</sub>, 1mM EGTA, 1mM DTT) was assayed

against Sphingosine in a final volume of 50  $\mu$ l containing 50mM Tris/HCl pH 7.5, 200mM KCl, 5mM MgCl<sub>2</sub>, 1mM EGTA, 10 $\mu$ M Sphingosine, 1 $\mu$ M ATP, 1mM DTT and incubated for 30 min at room temperature.

Choline Kinase was assayed as follows: Choline Kinase (diluted in 25mM glycine-NaOH pH 8.5, 67mM KCl, 5mM MgCl<sub>2</sub>) was assayed against Choline in a final volume of 50 $\mu$ l containing 25mM glycine-NaOH pH 8.5, 67mM KCl, 5mM MgCl<sub>2</sub>, 1mM Choline, 1 $\mu$ M ATP, 1mM DTT and incubated for 30 min at room temperature. These three assays were stopped by addition of 50 $\mu$ l Kinase Glo Plus Reagent, incubated for 10 min at room temperature and read for 1 sec/well.

Class 1 PI3K $\alpha$  was assayed as follows: PI3K $\alpha$  (diluted in 50mM Hepes pH 7.5, 150mM NaCl, 0.02% Na Cholate, 1mM DTT) was assayed against PtdIns(4,5)P<sub>2</sub> diC8 in a final volume of 50 $\mu$ l containing 37mM Hepes pH 7.5, 111mM NaCl, 0.02% Na Cholate, 5mM DTT, 5mM MgCl<sub>2</sub>, 1mM ATP, 2 $\mu$ M PtdIns(4,5)P<sub>2</sub> and incubated for 70 min at room temperature. Assays were stopped by addition of a 5.5 $\mu$ l solution of 50mM EDTA and 0.02% (w/v) sodium Cholate. 25 $\mu$ l of the resultant mixture was transferred to Lumitrac 200 plate. Detection mix (41mM Hepes pH 7.5, 123mM NaCl, 1.7 $\mu$ g GST-GRP1, 0.16 $\mu$ M PtdIns(3,4,5)P<sub>3</sub> Biotin, 1.6 $\mu$ g Streptavidin Allophycocyanin, 0.96 $\mu$ g/ml Eu Chelate labelled antibody) was added to give a final volume of 50 $\mu$ l and incubated for 20 min at room temperature before reading.

Class 1 PI3K $\beta$  was assayed as follows: PI3K $\beta$  (diluted in 50mM Hepes pH 7.5, 150mM NaCl, 0.02% Na Cholate, 1mM DTT) was assayed against PtdIns(4,5)P<sub>2</sub> diC8 in a final volume of 50 $\mu$ l containing 37mM Hepes pH 7.5, 111mM NaCl, 0.02% Na Cholate, 5mM DTT, 5mM MgCl<sub>2</sub>, 1mM ATP, 2 $\mu$ M PtdIns(4,5)P<sub>2</sub> and incubated for 70 min at room temperature. Assays were stopped by addition of 5.5 $\mu$ l 50mM EDTA and 25 $\mu$ l transferred to Lumitrac 200 plate. Detection mix (41mM Hepes pH 7.5, 123mM NaCl, 1.7 $\mu$ g GST-GRP1, 0.16 $\mu$ M PtdIns(3,4,5)P<sub>3</sub> Biotin, 1.6 $\mu$ g Streptavidin Allophycocyanin, 0.96 $\mu$ g/ml Eu Chelate labelled antibody) was added to give a final volume of 50 $\mu$ l and incubated for 20 min at room temperature before reading.

Class 2 PI3K $\gamma$  was assayed as follows: PI3K $\gamma$  (diluted in 20mM Tris/HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM DTT, 0.5mM EGTA, 0.02% CHAPS) was assayed against phosphoinositide substrate in a final volume of 50 $\mu$ l containing 19mM Tris/HCl pH 7.5, 143mM NaCl, 0.96mM EDTA, 0.96mM DTT, 0.48mM EGTA, 0.02% CHAPS, 20 $\mu$ M phosphatidylinositol, 0.2mM ATP, 2mM MgCl<sub>2</sub> and incubated for 30 min at room temperature. Assays were stopped by addition of 5.5 $\mu$ l 50mM EDTA, 2% CHAPS and 25 $\mu$ l transferred to Lumitrac 200 plate. Detection mix (18.6mM Tris/HCl pH 7.5, 140mM NaCl, 0.9mM DTT, 0.02% CHAPS, 1.4 $\mu$ g SGK PX, 0.06 $\mu$ M PtdIns(3)P Biotin, 1.6 $\mu$ g Streptavidin Allophycocyanin, 0.64 $\mu$ g/ml Eu Chelate labelled antibody) was added to give a final volume of 50ml and incubated for 30 min at room temperature before reading.

Class 3 VPS34 was assayed as follows: VPS34 (diluted in 20mM Tris/HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM DTT, 0.5mM EGTA, 0.02% CHAPS) was assayed against phosphoinositide substrate in a final volume of 50 $\mu$ l containing 19mM Tris/HCl pH 7.5, 143mM NaCl, 0.96mM EDTA, 0.96mM DTT, 0.48mM EGTA, 0.02% CHAPS, 20 $\mu$ M phosphatidylinositol, 0.2mM ATP, 2mM MnCl<sub>2</sub> and incubated for 60 min at room temperature. Assays were stopped by addition of 5.5 $\mu$ l 50mM EDTA, 2% CHAPS and 25 $\mu$ l transferred to Lumitrac 200 plate. Detection mix (18.6mM Tris/HCl pH 7.5, 140mM NaCl, 0.9mM DTT, 0.02% CHAPS, 1.4 $\mu$ g SGK PX, 0.06 $\mu$ M PtdIns(3)P Biotin, 1.6 $\mu$ g Streptavidin Allophycocyanin, 0.64 $\mu$ g/ml Eu Chelate labelled antibody) was added to give a final volume of 50ml and incubated for 30 min at room temperature before reading.

**mTOR complexes kinase assays.** HEK293 cells were freshly lysed in Hepes lysis buffer. 1-4 mg of lysate was pre-cleared by incubating with 5-20  $\mu$ l of Protein G-Sepharose conjugated to pre-immune IgG. The lysates extracts were then incubated with 5-20  $\mu$ l of Protein G-Sepharose conjugated to 5-20 $\mu$ g of either the Rictor, the Raptor antibody, or pre-immune IgG. All antibodies were covalently conjugated to Protein G-Sepharose. Immunoprecipitations were carried out for 1 hour at 4°C on a vibrating platform. The immunoprecipitates were washed four times with Hepes lysis

buffer, followed by two washes with Hepes kinase buffer. For Raptor immunoprecipitates used for phosphorylating S6K1, the initial two wash steps the buffer included 0.5M NaCl to ensure optimal kinase activity [7]. GST-Akt1 was isolated from serum deprived 293 cells incubated with PI-103 (1 $\mu$ M for 1 h) [24]. GST-S6K1 was purified from serum deprived 293 cells incubated with rapamycin (0.1 $\mu$ M for 1 h) [25]. mTOR reactions were initiated by adding 0.1 mM ATP, 10 mM Mg in the presence or absence of Ku-0063794 and GST-Akt1 (0.5  $\mu$ g) or GST-S6K1 (0.5  $\mu$ g). Reactions were carried out for 30min at 30°C on a vibrating platform and stopped by addition of SDS sample buffer. Reactions were then filtered through a 0.22  $\mu$ m Spin-X filter and samples subjected to electrophoresis and immunoblot analysis.

**Kinase assays.** HEK293 were lysed in Tris lysis buffer. In order to perform Akt and S6K assays, 500  $\mu$ g of lysate was incubated with 5  $\mu$ g of the corresponding antibody conjugated to Protein G-Sepharose. To perform SGK1 activity assays, 50  $\mu$ g of transfected lysate was incubated with 5  $\mu$ g of glutathione-Sepharose. All the incubations were performed for 1 hour at 4 °C on a vibrating platform. Kinase activity was assayed exactly as described previously [35] using the Crosstide peptide (GRPRTSSFAEG) at 30  $\mu$ M. Incorporation of [<sup>32</sup>P]-phosphate into the peptide substrate was determined by applying the reaction mixture on to P81 phosphocellulose paper and scintillation counting after washing the papers in phosphoric acid. One unit of activity was defined as that which catalysed the incorporation of 1 nmol of [<sup>32</sup>P]-phosphate into the substrate.

**GST-pull down of transfected SGK1 for immunoblotting analysis.** 36 hours post-transfection, HEK293 cells were lysed in Tris lysis buffer. 0.5 mg of lysate was affinity-purified on glutathione-Sepharose. Incubations were carried out for 1 hour at 4°C on a vibrating platform. The resultant precipitates were then washed two times with Tris lysis buffer containing 0.5 M NaCl, followed by two washes with Buffer A. The immunoprecipitates were resuspended in 20  $\mu$ l of sample buffer, filtered through a 0.22  $\mu$ m Spin-X filter, and samples subjected to electrophoresis and immunoblot analysis as described below.

**Immunoblotting.** Total cell lysate (5-20  $\mu$ g) or immunoprecipitated samples were heated at 95°C for 5 min in sample buffer, and subjected to polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membrane. Membranes were blocked for 1 hour in TBS-Tween buffer containing 10% (w/v) skimmed milk. The membranes were probed with the indicated antibodies in TBS-Tween containing 5% (w/v) skimmed milk or 5% (w/v) BSA for 16 hours at 4°C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent.

**Cell growth.** MEFs were seeded in 24-well plates (20000 cells per 1.91 cm<sup>2</sup> well) and grown overnight in the presence of 10% foetal bovine serum. Cells were then treated in the presence or absence of rapamycin or Ku-0063794 and the medium was changed every 24 h with freshly dissolved inhibitors. Every 24h of treatment, cells were washed once with Phosphate Buffered Saline (PBS), and fixed in 4% (v/v) paraformaldehyde in PBS for 15 min. After washing once with water, the cells were stained with 0.1% Crystal Violet in 10% ethanol for 20 min and washed three times with water. Crystal Violet was extracted from cells with 0.5 ml of 10% (v/v) ethanoic (acetic) acid for 20 min. The eluate was then diluted 1:10 in water, and absorbance at 590 nm was quantified.

**Flow Cytometric Analysis of Cell Cycle Distribution.** Adherent MEFs were harvested by trypsinisation, washed once in PBS, and re-suspended in ice-cold 70% (v/v) ethanol in water. Cells were washed twice in PBS plus 1% (w/v) bovine serum albumin and stained for 20 min in PBS plus 0.1% (v/v) Triton X-100 containing 50 g/ml propidium iodide and 50 g/ml RNase A. The DNA content of cells was determined using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software. Red fluorescence (585 $\pm$ 42 nm) was acquired on a linear scale, and pulse width analysis was used to exclude doublets. Cell cycle distribution was determined using FlowJo software (Tree Star Inc.).



## Results.

**Ku-0063794 is a specific mTORC1 and mTORC2 inhibitor.** The structure of Ku-0063794 is shown in Figure 1A. It inhibited the activity of endogenous immunoprecipitated mTORC1, assayed employing S6K1 as substrate (Fig 1B) and mTORC2, assayed using Akt as substrate (Fig 1C) with an  $IC_{50}$  of  $\sim 10$  nM. To evaluate the specificity of Ku-0063794, we studied the effect of Ku-0063794 at ATP concentrations, which approximate the  $K_m$  constant for ATP, towards a panel of 76 protein kinases. At 1  $\mu$ M Ku-0063794, which completely suppressed mTORC1 and mTORC2 activity (Fig 1B & 1C), none of the kinases on the specificity panel were significantly inhibited (Table 1). Even at 10  $\mu$ M the only enzyme on the panel that was inhibited more than 2-fold was MAP-kinase-kinase-1, which was decreased  $\sim 55\%$  (Table 1). Ku-0063794 even at concentrations of 10  $\mu$ M, did not significantly inhibit seven lipid kinases tested (Class I PI 3-kinase $\alpha$  and PI 3-kinase $\beta$ , Class II PI 3-kinase-B, Class III VPS34 as well as Spingosine kinase-1, Spingosine kinase-2, and Choline kinase) (Table 2).

**Ku-0063794 inhibits mTORC1 activity in HEK-293 cells.** We next investigated the effect of adding increasing amounts of Ku-0063794 on the activity and phosphorylation of S6K1 in 293 cells cultured in the presence of serum (Fig 2A). Under these conditions, concentration of Ku-0063794 as low as 30 nM, almost ablated S6K1 activity and phosphorylation of the hydrophobic motif (Thr389). Consistent with phosphorylation of the T-loop of S6K1 being dependent upon prior phosphorylation of the hydrophobic motif, Ku-0063794 inhibited phosphorylation of the T-loop residue (Thr229). Ku-0063794 also inhibited phosphorylation of the ribosomal S6 protein, an S6K substrate (Fig 2A). The ability of Ku-0063794 to suppress S6K1 activity and phosphorylation was rapid, with maximal inhibition seen within 10 min and sustained for at least 4 h, the longest time point examined (Fig 2B). Ku-0063794 also suppressed S6K1 activity and phosphorylation induced by IGF1 stimulation of serum starved HEK293 cells, although higher concentrations were required to fully inhibit S6K1, compared to cells cultured in serum (compare Fig 2A with Fig 2C). For example, in the presence of serum, 30 nM Ku-0063794 suppressed S6K1 activity by  $\sim 90\%$ , whereas following IGF1 stimulation, 30 nM Ku-0063794 suppressed S6K1 activity by  $\sim 50\%$ . In the presence of IGF1, a concentration of 300 nM Ku-0063794 was necessary to reduce S6K1 activity  $\sim 90\%$ . mTORC1, and hence S6K1, is also activated by nutrients such as amino acids. We observed that Ku-0063794 induced a dose-dependent inhibition of the phosphorylation of S6K1 and S6 protein induced following the replenishment of amino acids to 293 cells incubated in medium lacking these nutrients (Fig 2D). Concentrations of 100-300 nM Ku-0063794 were required to fully suppress amino acid induced phosphorylation of S6K1 and S6 protein (Fig 2D).

Recent work has shown that mTORC1 is phosphorylated at Ser2448 [36], a reaction probably mediated by S6K1 [37, 38]. In contrast, mTORC2 autophosphorylates at Ser2481 [36]. We observed that Ku-0063794 suppressed phosphorylation of both Ser2448 and Ser2481 in a dose-dependent and time dependent manner similar to S6K1 (lower panels of Fig 2A to 2C).

**Ku-0063794 inhibits mTORC2 activity in HEK-293 cells.** To investigate whether Ku-0063794 inhibited mTORC2, we studied how it affected Akt activity and hydrophobic motif (Ser473) phosphorylation in HEK-293 cells. In the presence of serum (Fig 3A) or following IGF1 stimulation (Fig 3B), Ku-0063794 led to a dose-dependent suppression of Akt activity, accompanied by inhibition Ser473 phosphorylation. As observed with S6K1, the activity and phosphorylation of Akt was inhibited by lower concentrations of Ku-0063794 when cells were cultured in serum compared to stimulation with IGF1 (compare Fig 3A and 3B). IGF1 induces a  $\sim 6$ -fold greater activation of Akt than serum (compare Fig 3A and 3B), which is likely to account for the higher concentrations of Ku-0063794 required to inhibit Akt and S6K in IGF1-stimulated cells compared with serum. The effects of Ku-0063794 on Akt activity and phosphorylation were observed within 10 min and sustained for up to 48 h (Fig 3C & 3D). Phosphorylation of the Akt substrates PRAS40 at Thr246 [39],

GSK3 $\alpha$ /GSK3 $\beta$  at Ser21/Ser9 [40] and Foxo-1/3a at Thr24/Thr32 [41] was also inhibited to a similar extent by Ku-0063794 as Akt activity and phosphorylation in cells cultured in serum (Fig 3A). In cells stimulated with IGF1, which induced a greater activation of Akt than observed with Serum, even concentrations of 1  $\mu$ M Ku-0063794 did not inhibit Akt phosphorylation and activation completely (Fig 3B). At 1  $\mu$ M Ku-0063794 in IGF-1 stimulated cells, Akt1 activity was reduced to  $\sim$ 0.5 U/mg which is  $\sim$ 50% of Akt activity observed with cells cultured in serum (Fig 3A). This may explain why phosphorylation of GSK3 and Foxo are not completely suppressed with 1  $\mu$ M Ku00637954 in IGF1 stimulated compared to serum treated cells (Compare Fig 3A and Fig 3B).

We also studied the effect that Ku-0063794 had on the Turn-Motif Thr450 residue of Akt1, whose phosphorylation is regulated by mTORC2 [26, 27]. Even after 48 h treatment with 1  $\mu$ M Ku-0063794, phosphorylation of Akt at Thr450 was only moderately reduced (Fig 3D). This is consistent with phosphorylation of Thr450 being introduced in Akt shortly after its synthesis and turning over very slowly [26, 27].

**Ku-0063794 inhibits phosphorylation of Akt at Thr308 in 293 cells.** We observed that in addition to inhibiting AktSer473 phosphorylation, Ku-0063794 suppressed phosphorylation of Akt at Thr308 (Fig 3A to 3D). This was unexpected, as some previous work suggested that the phosphorylation of Thr308 by PDK1 is not directly influenced by mTORC2. Ku-0063794 was not suppressing Thr308 phosphorylation by inhibiting PI 3-kinase or PtdIns(3,4,5)P<sub>3</sub> production, as IGF1 induced similar enhancement of PtdIns(3,4,5)P<sub>3</sub> levels in the absence or presence of 1  $\mu$ M Ku-0063794, under conditions which the PI 3-kinase inhibitor PI-103, ablated IGF1 induced PtdIns(3,4,5)P<sub>3</sub> production (Fig 4A). To further evaluate the effect that Ku-0063794 had on Thr308 phosphorylation, we overexpressed in HEK293 cells a mutant form of GST-Akt1 in which Ser473 was changed to Asp in an attempt to mimic the Akt phosphorylated form of mTORC2 and tested how Ku-0063794 affected phosphorylation at Thr308. We found that IGF1 induced phosphorylation of GST-Akt1[Ser473D] at Thr308 was not suppressed by Ku-0063794 to the same extent as endogenous Akt in these cells, suggesting that Ku-0063794 is not directly inhibiting PDK1-mediated phosphorylation of Thr308 (Fig 4B). In parallel studies Ku-0063794 also suppressed Thr308 phosphorylation of wild type GST-Akt1 to a greater extent than GST-Akt1[Ser473D] (Fig 4B). The dual PI 3-kinase and mTOR inhibitor PI-103, inhibited phosphorylation of Thr308 on both GST-Akt1[Ser473D] and wild type-Akt1 (Fig 4B). We also examined the effect that Ku-0063794 had on the phosphorylation of Akt at Thr308 in mouse embryonic fibroblast cells (MEFs) lacking the critical mTORC2 subunits Rictor [29] (Fig 4C), mLST8 [17] (Fig 4D) and Sin1 [16] (Fig 4E). Consistent with previous work, IGF1 induced phosphorylation of Thr308, but not Ser473 in mTORC2 deficient cells (Fig 4C to 4E). However, Ku-0063794 did not inhibit phosphorylation of Thr308 in any of the mTORC2 deficient cells, under conditions which it suppressed Thr308 phosphorylation in control wild type cells (Fig 4C to 4E). Treatment of mTORC2 deficient cells with PI-103 abolished phosphorylation of Thr308.

**Ku-0063794 inhibits SGK1 but not RSK.** We have shown that SGK1 activity and phosphorylation of its hydrophobic motif (Ser422) is regulated by mTORC2 but not mTORC1 [21]. This finding has recently been supported by elegant genetic studies in *C.elegans* [42, 43]. SGK1 activity and hydrophobic motif phosphorylation should therefore be inhibited by Ku-0063794, but not rapamycin. As it is not possible to assay phosphorylation and activity of endogenous SGK1 [21], we investigated how Ku-0063794 affected activity and phosphorylation of overexpressed full length SGK1 in 293 cells cultured in the presence of serum (Fig 5A). We observed that Ku-0063794 inhibited SGK1 activity and Ser422 phosphorylation in a dose dependent manner, to the same extent as Akt and S6K1 phosphorylation (Fig 5A). We also examined the phosphorylation of the N-myc downstream-regulated gene-1 (NDRG1), a well-characterised substrate for SGK1, which is not phosphorylated in SGK1 knockout mouse tissues [44]. Employing an antibody recognising the SGK1-phosphorylated form of NDRG1, we found that Ku-0063794 inhibited NDRG1 phosphorylation to the same extent as it suppressed SGK1 activity. Phosphorylation

of endogenous NDRG1 was also inhibited by Ku-0063794 in HeLa (Fig 5B) cells as well as wild type MEFs (Fig 5C). The dual PI 3-kinase/mTOR inhibitor PI-103, but not rapamycin or the Akt inhibitor Akti-1/2 [45], reduced phosphorylation of NDRG1 in both HeLa cells and wild type MEFs.

To ensure Ku-0063794 was not inhibiting the phosphorylation and activity of all AGC kinases, we studied the effect that Ku-0063794 had on the activation of the RSK, that is activated by ERK1/ERK2 pathway and not regulated by mTOR. HEK293 cells were stimulated with phorbol ester, which markedly enhanced ERK as well as RSK phosphorylation and activity (Fig 5D). Ku-0063794 did not inhibit phorbol ester induced ERK or RSK phosphorylation and RSK activation under conditions in which the MAP kinase kinase inhibitor PD 0325901-CL [46], ablated ERK phosphorylation and RSK activation (Fig 5D).

**Ku-0063794 induces complete dephosphorylation of 4E-BP1.** Consistent with mTOR regulating 4E-BP1 phosphorylation, we found that treatment of wild type MEFs (Fig 6A) or HEK-293 (Fig 6B) with Ku-0063794, induced a marked increase in the electrophoretic mobility of 4E-BP1, which was accompanied by complete dephosphorylation of Thr37, Thr46 and Ser65. We observed that rapamycin, reduced phosphorylation of 4E-BP1 and enhanced electrophoretic mobility to a significantly lower extent than Ku-0063794, even in mTORC2 deficient Sin1 knockout MEFs (Fig 6A and 6B).

**Ku-0063794 suppresses cell growth and induces G1-cell cycle arrest.** We compared the effect that Ku-0063794 and rapamycin had on growth of wild type and mLST8 deficient MEFs. We selected these cells, as despite lacking mTORC2, they proliferate at similar rates in serum as wild type control fibroblasts. Moreover, mLST8-fibroblast cell lines proliferate more rapidly than Rictor or Sin1 deficient MEFs (data not shown). We observed that Ku-0063794 suppressed growth of both wild type (Fig 7A) and mLST8 deficient (Fig 7C) MEFs more markedly than rapamycin. Flow cytometric analysis indicated that Ku-0063794 increased the proportion of wild type (Fig 7B) and mLST8 knockout MEFs (Fig 7D) in the G1 cell cycle state about 2-fold, compared to rapamycin which increased the proportion of cells in G1 by 1.5-fold.

## Discussion.

Our results establish that Ku-0063794 is a potent and highly specific inhibitor of mTOR as it does not significantly inhibit 76 other protein kinases tested as well as 7 lipid kinases including class 1a PI-3K $\alpha$  and PI 3-K $\beta$ . mTOR belongs to the PI 3-kinase related kinases (PIKK) subgroup as it possesses significant similarity to PI 3-kinases [47]. Many widely used PI 3-kinase inhibitors including wortmannin [48], LY294002 [48], PI-103 [49] and NVP-BEZ235 [50], inhibit mTOR with similar potency as Class 1 PI 3-kinase. Recently specific PI 3-kinase inhibitors have been described that do not inhibit mTOR, such as s GDC-0941 [51]. Our data demonstrate that Ku-0063794 does not inhibit PI 3-kinase *in vivo*, as Ku-0063794 does not suppress increases in cellular levels of PtdIns(3,4,5)P<sub>3</sub> following IGF-1 stimulation of HEK-293 cells (Fig 4A). Moreover, Ku-0063794 does not inhibit Akt Thr308 phosphorylation in three different mTORC2 deficient MEF cell lines (Fig 4C to 4E) under conditions which PI-103 inhibited PtdIns(3,4,5)P<sub>3</sub> production and Thr308 phosphorylation.

Two other mTOR specific inhibitors that do not inhibit PI 3-kinase have recently been described namely, PP242 [52] and Torin [53]. Although the chemical structure of Torin has thus far not been disclosed, the chemical structures of PP242 and Ku-0063794 are quite distinct. We have synthesised PP242 and compared its specificity to Ku-0063794 and observed that it is less selective. At 1  $\mu$ M PP242, 22 of the 76 kinases tested were inhibited by >50%, whereas at 10  $\mu$ M PP242, 46 out of the 76 kinases were inhibited (data available upon request). In contrast, 10  $\mu$ M Ku-0063794 only suppressed 1 out of 76 kinases over 2-fold (Table 1). Torin was reported to be highly selective [53]. The availability of chemically unrelated mTOR inhibitors will be useful in confirming specificity of physiological responses observed when using these reagents. Our findings using Ku-0063794 are similar to those reported for the PP242 [52] and Torin [53], as all 3 inhibitors reduced hydrophobic motif as well as T-loop phosphorylation of endogenous S6K as well as Akt. We also demonstrated Ku-0063794, but not rapamycin or Akti-1/2, inhibited phosphorylation and activation of SGK1 as well as its physiological substrate NDGR1. This is consistent with our findings that SGK1 is regulated by mTORC2 and not the mTORC1 inhibitor rapamycin (reviewed in [54]). We would expect Torin and PP242 to also inhibit SGK1 activation and phosphorylation.

The finding that Ku-0063794 as well as Torin [53] and PP242 [52] inhibited Akt Thr308 phosphorylation is arguably unexpected, as previous work suggested that Thr308 and Ser473 phosphorylation can occur independently from each other. The initial evidence for this was that an Akt-Thr308A mutant overexpressed in IGF1 stimulated 293 cells, was still phosphorylated at Ser473 and vice versa [55]. Moreover, in cells lacking mTORC2 activity, Akt is still phosphorylated at Thr308 despite not being phosphorylated at Ser473 [16, 17, 29]. In PDK1 deficient cells [56] and in mouse tissues lacking PDK1 [57, 58], Akt is still phosphorylated at Ser473, but not at Thr308. One explanation for the effect that mTOR inhibitors such as Ku-0063794 have on Akt Thr308 phosphorylation, would be if Akt phosphorylation of Ser473 generated a docking site for PDK1 to interact and phosphorylate Thr308, in the same way in which phosphorylation of the hydrophobic motifs of S6K/SGK by mTOR promotes T-loop phosphorylation of these enzymes by PDK1. The evidence against this stems from previous studies undertaken in knockin cells [59, 60] and in mouse tissues [61] that express a PIF-pocket mutant form of PDK1 that is unable to interact with the hydrophobic motifs of S6K and SGK1. In these knockin-studies it was demonstrated that Akt was normally phosphorylated at Thr308, under conditions which S6K and SGK are not phosphorylated at their T-loop residue, suggesting that Akt Thr308 phosphorylation was not dependent on PDK1 interacting with Akt via its PIF-pocket. How can the effects on Thr308 phosphorylation in mTOR inhibitor and knockin/knockout studies be reconciled? One possibility is that in wild type cells, phosphorylation of Akt at Ser473 does indeed promote PDK1 interaction and contribute towards maximal phosphorylation of Thr308 by PDK1. However, in knockout cells permanently lacking mTORC2 activity or in PDK1 PIF-pocket mutant knockin cells, Akt activation may be so essential for survival, that signalling networks

adapt so that Akt can be phosphorylated by PDK1 at Thr308 independently of Ser473 phosphorylation. Evidence to support the idea that PDK1 might interact with Ser473 after its phosphorylation by mTORC2 might stem from recent observations made in PDK1-PH domain knockin mice, that express a form of PDK1 that can not interact with PtdIns(3,4,5)P<sub>3</sub>. In tissues or cells derived from these animals, Akt is still phosphorylated at Thr308, albeit to ~3-fold lower level than in wild type tissues or cells [62]. This observation was unexpected, as previous evidence indicated that PDK1 needed to interact with phosphoinositides at the plasma membrane in order to phosphorylate Akt at Thr308 [19]. However, if phosphorylation at Ser473 by mTORC2 does stimulate interaction of PDK1 with Akt this may bypass the need for PDK1 to interact with PtdIns(3,4,5)P<sub>3</sub> and enhance phosphorylation of Thr308 without the need of PDK1 binding to phosphoinositides. In future work it would be important to investigate the effect on Akt Thr308 phosphorylation of allosteric PIF-pocket PDK1 inhibitors being elaborated [63, 64] or mTORC2 specific inhibitors (if such compounds could be developed).

Another possibility to account for the effect of mTOR inhibitors on Akt Thr308 phosphorylation, is if the lack of Akt Ser473 phosphorylation leads to destabilisation of Akt, promoting dephosphorylation of Thr308 by protein phosphatase(s). Indeed this idea is supported by structural analysis of Akt2, where it has been demonstrated that hydrophobic motif phosphorylation induces a marked disorder to order transition of the kinase domain, resulting in stabilisation of alphaC helix with concomitant restructuring of the T-loop and reconfiguration of the structure of the catalytic domain [65]. Treatment of cells with Ku-0063794 will inhibit Akt Ser473 phosphorylation and may lead to less stable Akt conformation, with Thr308 being more exposed and accessible to becoming dephosphorylated by protein phosphatase(s). It would be interesting to investigate whether in mTORC2 deficient knockout cells, in which Thr308 phosphorylation is unaffected, the activity of the protein phosphatase(s) that act on Thr308 is reduced, or if Akt is stabilised in some way that enables it to function without Ser473 phosphorylation.

Ku-0063794 (Fig 6), as well as Torin [53] and PP242 [52], induce a much greater dephosphorylation of 4E-BP1 than rapamycin, suggesting that these drugs will more effectively suppress protein synthesis resulting from over activation of the PI 3-kinase/Akt pathways. Both mTORC1 and mTORC2 activity would be inhibited in mTORC2 deficient cells treated with rapamycin. However, we still observed significant phosphorylation of 4E-BP1 at Thr37/Thr46 and Ser65 in Sin-1 knockout MEFs treated with rapamycin, under conditions, which the hydrophobic motif of both S6K and Akt are not phosphorylated (in agreement with lack of mTORC1 and mTORC2 activity). In contrast, treatment of Sin-1 knockout MEFs with Ku-0063794, led to a much greater dephosphorylation of 4E-BP1 than observed with rapamycin. Similar results were also found with the Torin [53] and PP242 [52] inhibitors. Taken together these results indicate that rapamycin may not inhibit the phosphorylation of 4E-BP1 by mTORC1 and/or there may be a rapamycin resistant form of mTOR, distinct from mTORC2. The existence of such a complex might also explain why Ku-0063794 inhibits proliferation of mTORC2 deficient mLST8 knockout cells more effectively than rapamycin (Fig 7C to 7D). It would be interesting to see whether it was possible to isolate a form of mTOR capable of phosphorylating 4E-BP1, which was inhibited by Ku-0063794, but not rapamycin.

There is great expectation that inhibitors of the PI 3-kinase-PDK1-Akt-mTOR signalling axis will have utility as anti-cancer drugs, as this signalling network is inappropriately activated in the majority of human cancers. Rapamycin and its analogues, are being extensively deployed for the treatment of various cancers [66]. It will be interesting to evaluate whether inhibitor such as Ku-0063794, that targets both mTOR complexes is a more effective anti-cancer agent than rapamycin. This might be expected, as mTOR inhibitors such as Ku-0063794, unlike rapamycin derivatives, will inhibit Akt and SGK isoforms that are likely to play vital roles in driving the proliferation of many cancers. The finding that mTOR inhibitors such as Ku-0063794 induce more marked dephosphorylation of 4E-BP1 than rapamycin, also holds promise that these drugs will be more effective at suppressing protein synthesis

required for growth and proliferation of cancer cells than rapamycin derivatives. Most importantly, Ku-0063794 and other mTOR inhibitors will also be very useful reagents to dissect and validate the molecular roles that mTOR plays and hence extend our repertoire of knowledge of how this master regulator of cell growth operates.

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**Table 1. Effect of Ku-0063794 on 76 protein kinases.** Results are presented as percentage of kinase activity compared to control incubations in which Ku-0063794 was omitted. Protein kinases were assayed as described at the material and methods section. The results are an average of a triplicate determination  $\pm$  standard deviation. Asterisks indicates inhibition of  $> 2$ -fold. Abbreviations not defined in main text: AMPK, AMP-activated protein kinase; BRSK, brain-specific kinase; BTK, Bruton agammaglobulinemia tyrosine kinase; CaMK1, calmodulin-dependent kinase; CaMKK, CaMK kinase; CDK, cyclin dependent kinase; CHK, checkpoint kinase; CK, casein kinase; CSK, C-terminal Src kinase; DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; EF2K, elongation-factor-2-kinase; EPH, ephrin; FGF-R, fibroblast-growth-factor receptor; GSK3, glycogen synthase kinase 3; HIPK, homeodomain-interacting protein kinase; IGF1-R, insulin-like growth factor 1 receptor; IKK, inhibitory  $\kappa$ B kinase; IR, insulin receptor; IRR, insulin related receptor; JNK, c-Jun N-terminal kinase; Lck, lymphocyte cell-specific protein tyrosine kinase; MAPKAP-K, MAPK-activated protein kinase; MARK, microtubule-affinity-regulating kinase; MELK, maternal embryonic leucine-zipper kinase; MKK1, MAPK Kinase-1; MLCK, smooth-muscle myosin light-chain kinase; MNK, MAPK-integrating protein kinase; MSK, mitogen- and stress-activated protein kinase; MST, mammalian homologue Ste20-like kinase; NEK, NIMA (never in mitosis in *Aspergillus nidulans*)-related kinase; PAK, p21-activated protein kinase; PHK, phosphorylase kinase; PIM, provirus integration site for Moloney murine leukaemia virus; PKA, cAMP-dependent protein kinase; PKD, protein kinase D; PLK, polo-like kinase; PRAK, p38-regulated activated kinase; PRK, protein kinase C-related kinase; ROCK, Rho-dependent protein kinase; SRPK, serine-arginine protein kinase; SYK, spleen tyrosine kinase; TBK1, TANK-binding kinase 1; VEGFR, vascular endothelial growth factor receptor; YES1, Yamaguchi sarcoma viral oncogene homologue 1, n.d. not determined.

**Table 2. Effect of Ku-0063794 on 7 lipid kinases.** Results are presented as percentage of lipid kinase activity compared to control incubations in which Ku-0063794 was omitted. Lipid kinases were assayed as described in the Materials and Methods, in the absence or presence of the indicated concentration of Ku-0063794. The results are an average of a triplicate determination  $\pm$  standard deviation.

**Figure 1. Ku-0063794 inhibits both mTORC1 and mTORC2 complexes *in vitro*.** (A) Structure of Ku-0063794. (B & C) HEK-293 cell extracts were subjected to immunoprecipitation with pre-immune (IgG), anti-Raptor (B) or anti-Rictor antibody (C). The immunoprecipitates were incubated with dephosphorylated GST-S6K1 (B) or GST-Akt1 (C) in the presence of the indicated concentrations of Ku-0063794 or vehicle. Kinases assays were performed for 30 min in the presence of MgATP and then subjected to immunoblot analysis with the indicated antibodies. Similar results were obtained in three independent experiments.

**Figure 2. Ku-0063794 inhibits mTORC1 activity *in vivo*.** (A & B) HEK-293 cells cultured in the presence of 10% foetal bovine serum, were treated for 30 min in the absence or presence of the indicated concentrations of Ku-0063794 (A) or with 1  $\mu$ M Ku-0063794 for the indicated time (B). Cells were lysed and S6K1 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity  $\pm$  standard error from three different samples, with each sample assayed in duplicate. Cell lysates were also analysed by immunoblotting with the indicated antibodies. (C) As in (A) except that cells were deprived of serum for 16 hours prior to stimulation with 50 ng/ml of IGF-1 for 20 min. (D) As in (C) except that after 16 hours of serum deprivation, cells were incubated for 1 hour in amino acid free EBSS medium containing 10% dialysed serum. Cells were then incubated in the absence or presence of the indicated amount of Ku-0063794 for 30 min, prior to re-addition of physiological levels of amino acids for an additional 30 min. Immunoblots are representative of three different experiments.

**Figure 3. Ku-0063794 ablates mTORC2 *in vivo*.** HEK-293 cells were cultured in serum, stimulated with IGF-1 in the absence or presence of Ku-0063794 as described in the legend to Figure 2. Akt1 was immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity  $\pm$  standard error from three different samples, with each sample assayed in duplicate. Cell lysates were analysed by immunoblotting with the indicated antibodies. Immunoblots are representative of three different experiments. In (D) medium containing freshly dissolved Ku-0063794 was replaced every 12 h.

**Figure 4. Further investigation of the effect of Ku-0063794 on Akt Thr308 phosphorylation.** (A) HEK-293 cells were deprived of serum for 16 hours, treated for 30 min in the absence or presence of the indicated concentrations of Ku-0063794 or PI-103 then stimulated with 50 ng/ml of IGF-1 for 10 min. Cells are lysed with 0.5 M trichloroacetic acid and amounts of PtdIns (3,4,5)P<sub>3</sub> determined. Each bar represents the mean  $\pm$  standard error from three independent samples. (B) HEK-293 were transfected with a DNA construct encoding wild type or mutant GST-Akt1. 24h post-transfection, cells were deprived of serum for 16 hours and stimulated IGF-1 for 20 min, in the presence or absence of inhibitors as described in (A). Cell lysates were analysed by immunoblotting with the indicated antibodies and Immunoblots shown are representative of 3 separate experiments. (C to E) The indicated wild type (wt) or knockout (ko) MEFs were deprived and stimulated with IGF-1 for 20 min, in the presence or absence of inhibitors as described in (A). Cell lysates were analysed by immunoblotting with the indicated antibodies. Similar results were obtained in three independent experiments.

**Figure 5. Ku-0063794 suppresses hydrophobic motif phosphorylation and activation of SGK1 but not RSK.** (A) HEK-293 cells were transfected with a DNA construct encoding GST-SGK1 (full-length enzyme). Cells were cultured in the presence of 10% foetal bovine serum in order to maintain PI 3-kinase pathway activity. 36h post-transfection, cells were treated for 30 min in the absence or presence of the indicated concentrations of Ku-0063794 or PI-103. Cells were lysed, SGK1 was affinity-purified on glutathione-Sepharose and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity  $\pm$  standard error from three different samples, with each sample assayed in duplicate. Affinity purified SGK1 was also subjected to immunoblotting with an anti-GST antibody (SGK1-Total) as well as an anti-Ser422 phosphospecific antibody. Cell lysates were also analysed by immunoblotting with the indicated non-SGK antibodies. (B & C) HeLa cells or the indicated wild type (wt) or knockout (ko) MEFs, were cultured in the presence of 10% serum, were treated for 30 min in the absence or presence of the indicated concentrations of inhibitors. Cells were lysed and extracts analysed by immunoblotting with the indicated antibodies. Immunoblots are representative of three different experiments. (D) HEK-293 cells were deprived of serum for 16 hours, treated for 30 min in the absence or presence of 1  $\mu$ M Ku-0063794 or 0.2  $\mu$ M PD 0325901 then stimulated with 400 ng/ml of Phorbol-12-Myristate-13-Acetate (TPA) for 15 min. RSK was immunoprecipitated with an antibody recognising all isoforms and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity  $\pm$  standard error from two different samples, with each sample assayed in duplicate. Cell lysates were also analysed by immunoblotting with the indicated antibodies.

**Figure 6. Ku-0063794 induces a marked dephosphorylation of 4E-BP1.** (A & B) The indicated wild type (wt) or knockout (ko) MEFs (A) or HEK-293 cells (B) were cultured in the presence of 10% serum, and treated for 30 min in the absence or presence of the indicated concentrations of inhibitors. Cells were lysed and extracts analysed by immunoblotting with the indicated antibodies. Immunoblots are representative of three different experiments.

**Figure 7. Ku-0063794 inhibits cell growth and induces a G1 cell cycle arrest.** The indicated wild type (wt) or knockout (ko) MEFs were split at low density (20000 cells per 1.91 cm<sup>2</sup> dish) and cells cultured for 1, 2 or 3 days in the absence (DMSO) or presence of 3  $\mu$ M Ku-0063794 or 100 nM rapamycin. (A & C) Cell numbers were measured in quadruplicate using a Crystal Violet staining as described at the Material and Methods section. The data is presented as average  $\pm$  standard error percentage of cells relative to the DMSO control. Flow cytometric analysis was also undertaken as described in the Materials and Methods. (B & D) DNA was stained with propidium iodide, and cellular content was analysed. The percentage of cells in G1, S, or G2 phases were determined from triplicate dishes for each condition using CellQuest software. Two way ANOVA and Bonferroni posttests were performed, \* indicates  $p < 0.001$  compared to DMSO Control. # indicates  $p < 0.001$  compared to Rapamycin.

Kinase	Percentage activity remaining		Kinase	Percentage activity remaining	
	1 $\mu$ M	10 $\mu$ M		1 $\mu$ M	10 $\mu$ M
	Ku-0063794	Ku-0063794		Ku-0063794	Ku-0063794
<i>Akt1</i>	98 $\pm$ 8	90 $\pm$ 1	<i>MLCK</i>	82 $\pm$ 4	104 $\pm$ 3
<i>Akt2</i>	97 $\pm$ 8	107 $\pm$ 9	<i>MNK1</i>	111 $\pm$ 0	75 $\pm$ 12
<i>AMPK</i>	97 $\pm$ 1	106 $\pm$ 3	<i>MNK2<math>\alpha</math></i>	87 $\pm$ 5	92 $\pm$ 1
<i>Aurora B</i>	n.d.	86 $\pm$ 6	<i>MSK1</i>	101 $\pm$ 6	117 $\pm$ 7
<i>BRSK2</i>	90 $\pm$ 1	72 $\pm$ 7	<i>MST2</i>	81 $\pm$ 10	102 $\pm$ 3
<i>BTK</i>	85 $\pm$ 7	81 $\pm$ 21	<i>MST4</i>	87 $\pm$ 5	86 $\pm$ 8
<i>CAMK1</i>	96 $\pm$ 1	101 $\pm$ 6	<i>NEK2A</i>	92 $\pm$ 10	86 $\pm$ 2
<i>CAMKK<math>\beta</math></i>	108 $\pm$ 5	94 $\pm$ 0	<i>NEK6</i>	74 $\pm$ 4	83 $\pm$ 10
<i>CDK2/Cyclin A</i>	89 $\pm$ 5	90 $\pm$ 6	<i>P38<math>\alpha</math> MAPK</i>	101 $\pm$ 6	96 $\pm$ 5
<i>CHK1</i>	87 $\pm$ 8	86 $\pm$ 7	<i>P38<math>\beta</math> MAPK</i>	106 $\pm$ 7	83 $\pm$ 5
<i>CHK2</i>	97 $\pm$ 2	96 $\pm$ 9	<i>P38<math>\delta</math> MAPK</i>	72 $\pm$ 1	87 $\pm$ 4
<i>CK1<math>\delta</math></i>	112 $\pm$ 15	109 $\pm$ 12	<i>P38<math>\gamma</math> MAPK</i>	89 $\pm$ 8	91 $\pm$ 1
<i>CK2<math>\alpha</math></i>	104 $\pm$ 0	85 $\pm$ 12	<i>PAK4</i>	110 $\pm$ 1	75 $\pm$ 4
<i>CSK</i>	90 $\pm$ 1	99 $\pm$ 3	<i>PAK5</i>	101 $\pm$ 1	81 $\pm$ 3
<i>DYRK1a</i>	94 $\pm$ 10	80 $\pm$ 7	<i>PAK6</i>	75 $\pm$ 3	100 $\pm$ 24
<i>DYRK2</i>	92 $\pm$ 1	96 $\pm$ 12	<i>PDK1</i>	89 $\pm$ 5	91 $\pm$ 2
<i>DYRK3</i>	88 $\pm$ 9	84 $\pm$ 1	<i>PhK<math>\gamma</math>1</i>	89 $\pm$ 2	116 $\pm$ 3
<i>EF2K</i>	88 $\pm$ 7	108 $\pm$ 0	<i>PIM1</i>	89 $\pm$ 4	98 $\pm$ 15
<i>EPH-A2</i>	108 $\pm$ 8	96 $\pm$ 2	<i>PIM2</i>	86 $\pm$ 11	78 $\pm$ 0
<i>EPH-B3</i>	100 $\pm$ 8	78 $\pm$ 4	<i>PIM3</i>	114 $\pm$ 12	75 $\pm$ 7
<i>ERK1</i>	102 $\pm$ 0	87 $\pm$ 1	<i>PKA</i>	115 $\pm$ 7	85 $\pm$ 12
<i>ERK2</i>	102 $\pm$ 8	92 $\pm$ 9	<i>PKC<math>\alpha</math></i>	106 $\pm$ 13	86 $\pm$ 18
<i>ERK8</i>	88 $\pm$ 1	92 $\pm$ 5	<i>PKC<math>\zeta</math></i>	87 $\pm$ 9	84 $\pm$ 0
<i>FGF-R1</i>	102 $\pm$ 8	87 $\pm$ 10	<i>PKD1</i>	80 $\pm$ 4	101 $\pm$ 3
<i>GSK3<math>\beta</math></i>	95 $\pm$ 11	91 $\pm$ 5	<i>PLK1</i>	76 $\pm$ 2	94 $\pm$ 2
<i>HIPK2</i>	89 $\pm$ 5	89 $\pm$ 1	<i>PRAK</i>	67 $\pm$ 4	87 $\pm$ 8
<i>IGF1R</i>	117 $\pm$ 0	89 $\pm$ 1	<i>PRK2</i>	95 $\pm$ 5	105 $\pm$ 12
<i>IKK<math>\beta</math></i>	104 $\pm$ 3	89 $\pm$ 3	<i>ROCKII</i>	75 $\pm$ 4	86 $\pm$ 5
<i>IKK<math>\epsilon</math></i>	99 $\pm$ 1	100 $\pm$ 5	<i>RSK1</i>	100 $\pm$ 4	86 $\pm$ 2
<i>IR</i>	81 $\pm$ 1	67 $\pm$ 5	<i>RSK2</i>	103 $\pm$ 7	104 $\pm$ 1
<i>IRR</i>	105 $\pm$ 7	71 $\pm$ 4	<i>S6K1</i>	104 $\pm$ 11	98 $\pm$ 4
<i>JNK1<math>\alpha</math>1</i>	80 $\pm$ 5	93 $\pm$ 4	<i>SGK1</i>	76 $\pm$ 6	64 $\pm$ 6
<i>JNK2<math>\alpha</math>2</i>	101 $\pm$ 4	103 $\pm$ 13	<i>Src</i>	n.d.	86 $\pm$ 5
<i>LCK</i>	69 $\pm$ 5	104 $\pm$ 9	<i>SRPK1</i>	101 $\pm$ 1	94 $\pm$ 2
<i>MAPKAP-K2</i>	98 $\pm$ 3	81 $\pm$ 10	<i>SYK</i>	88 $\pm$ 3	83 $\pm$ 5
<i>MARK3</i>	93 $\pm$ 6	93 $\pm$ 15	<i>TBK1</i>	97 $\pm$ 8	89 $\pm$ 4
<i>MELK</i>	91 $\pm$ 6	72 $\pm$ 4	<i>VEGFR</i>	92 $\pm$ 8	92 $\pm$ 8
<i>MKK1</i>	96 $\pm$ 4	43 $\pm$ 7*	<i>YES1</i>	94 $\pm$ 10	90 $\pm$ 1

Table 1



Kinase	Percentage activity remaining	
	Ku-0063794 (1 $\mu$ M)	Ku-0063794 (10 $\mu$ M)
<i>PI3 Kinases</i>		
<i>CLASS I</i>		
<b>PI3K<math>\alpha</math></b>	97 $\pm$ 2	95 $\pm$ 3
<b>PI3K<math>\beta</math></b>	95 $\pm$ 9	84 $\pm$ 22
<i>CLASS II</i>		
<b>PI3KC2<math>\beta</math></b>	126 $\pm$ 13	82 $\pm$ 12
<i>CLASS III</i>		
<b>VPS34</b>	101 $\pm$ 4	93 $\pm$ 1
<i>Other Lipid Kinases</i>		
<b>SPHK1</b>	108 $\pm$ 3	99 $\pm$ 7
<b>SPHK2</b>	119 $\pm$ 2	116 $\pm$ 3
<b>Choline Kinase</b>	102 $\pm$ 1	102 $\pm$ 1

Table 2

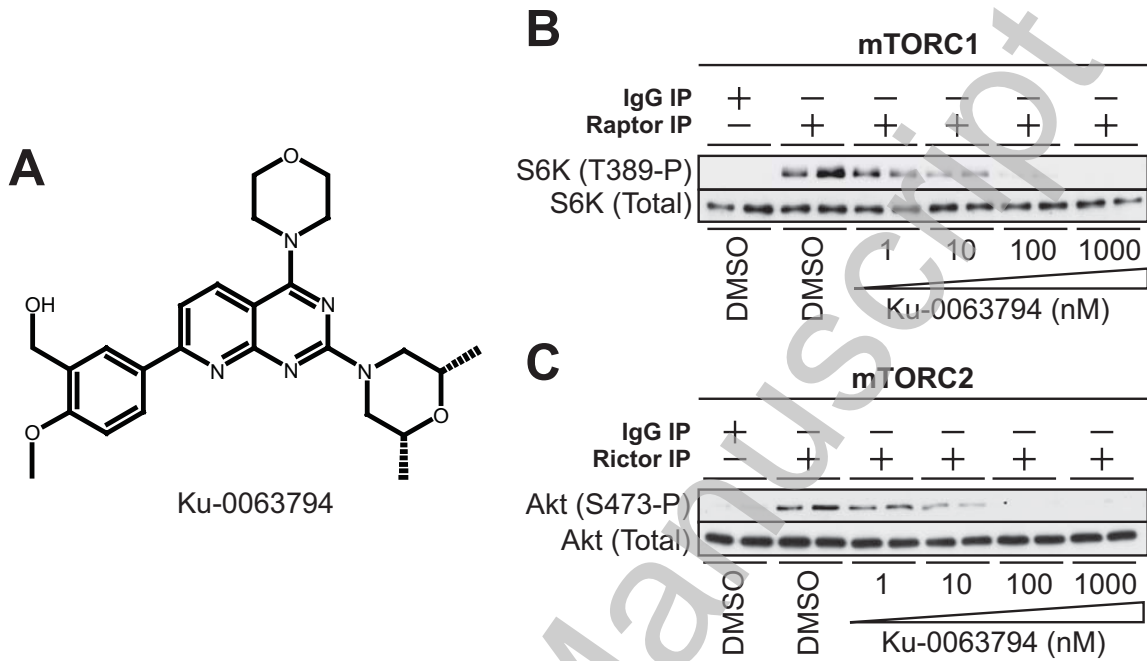


Fig. 1

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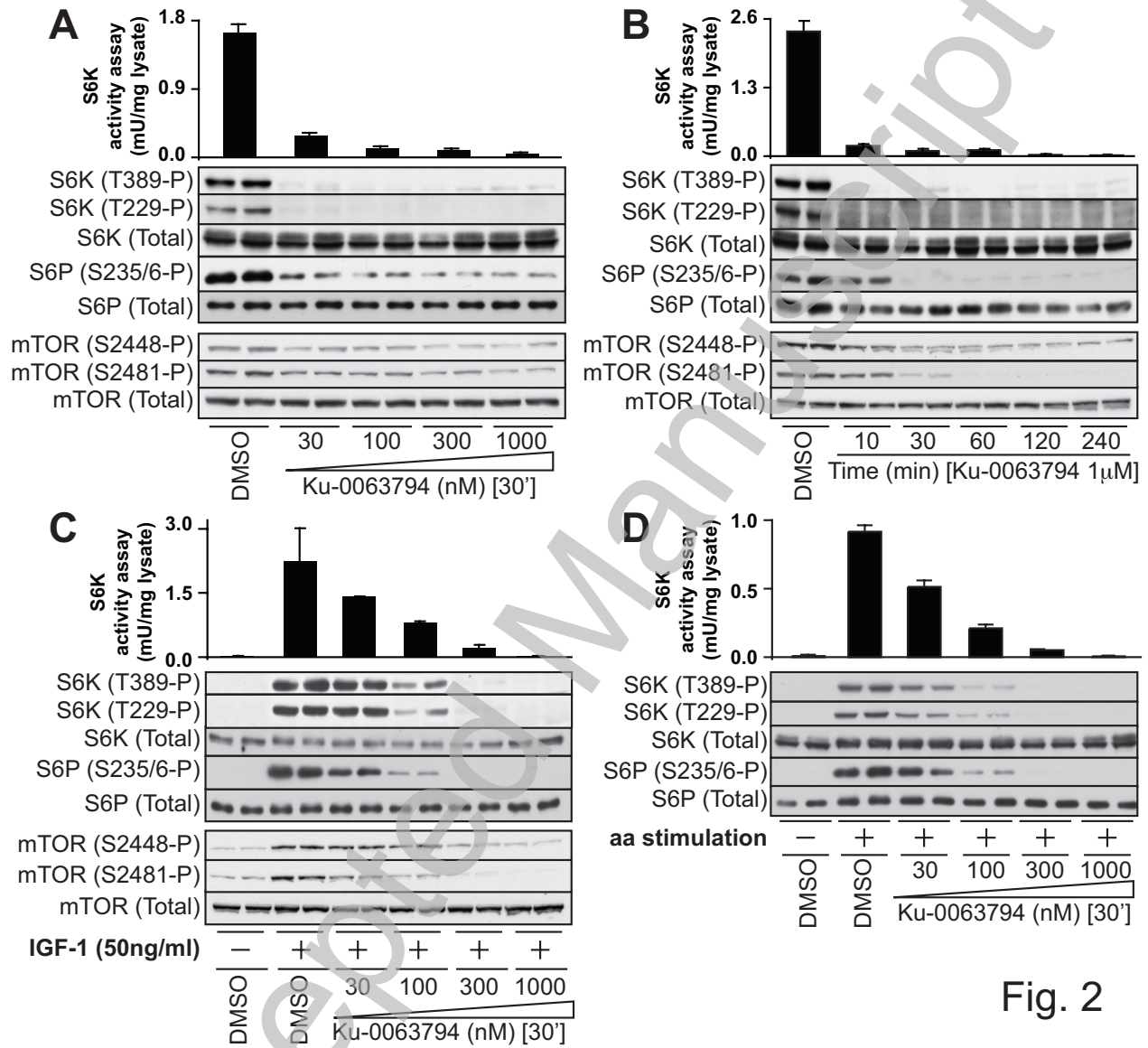
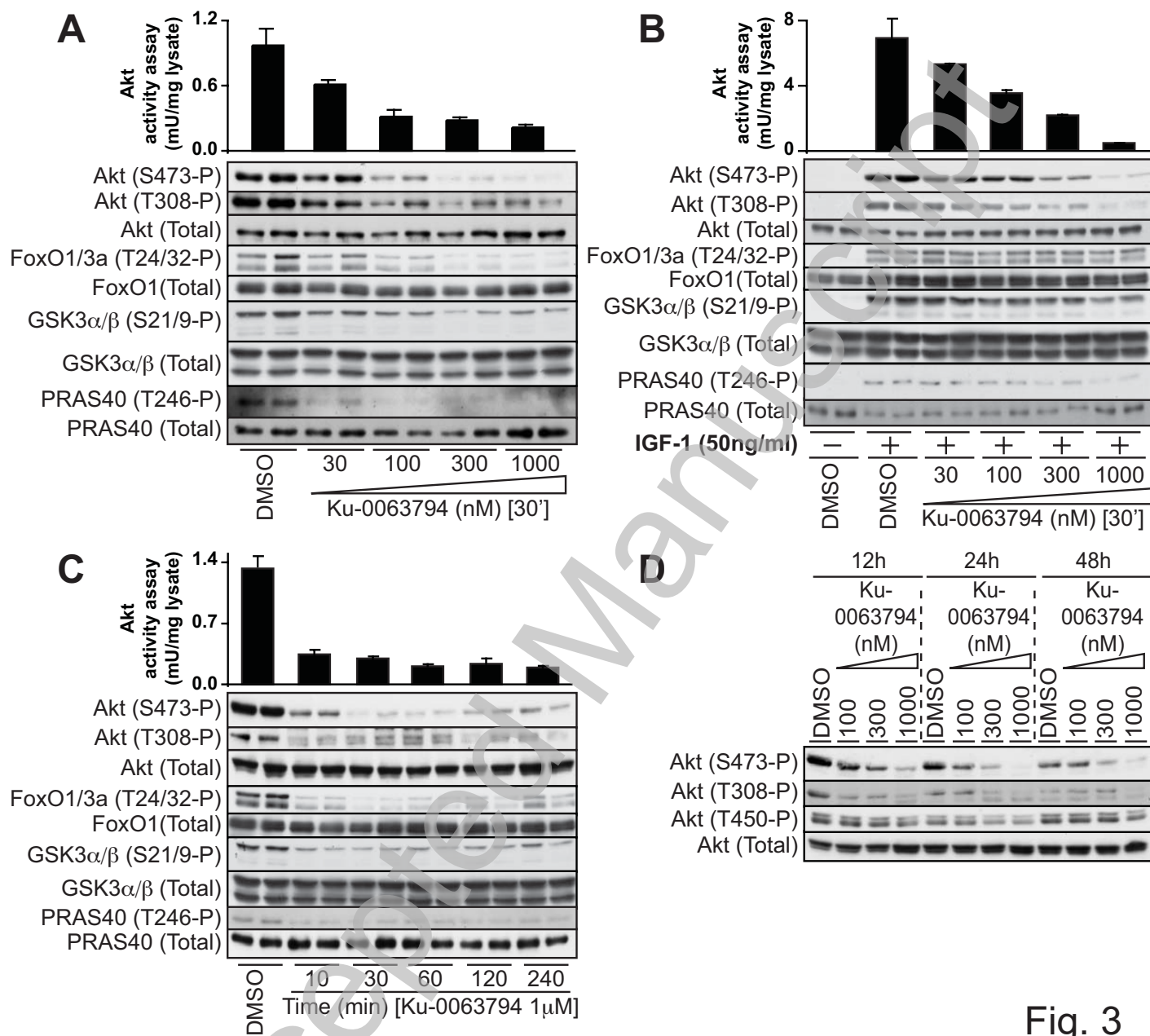


Fig. 2

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**Fig. 3**

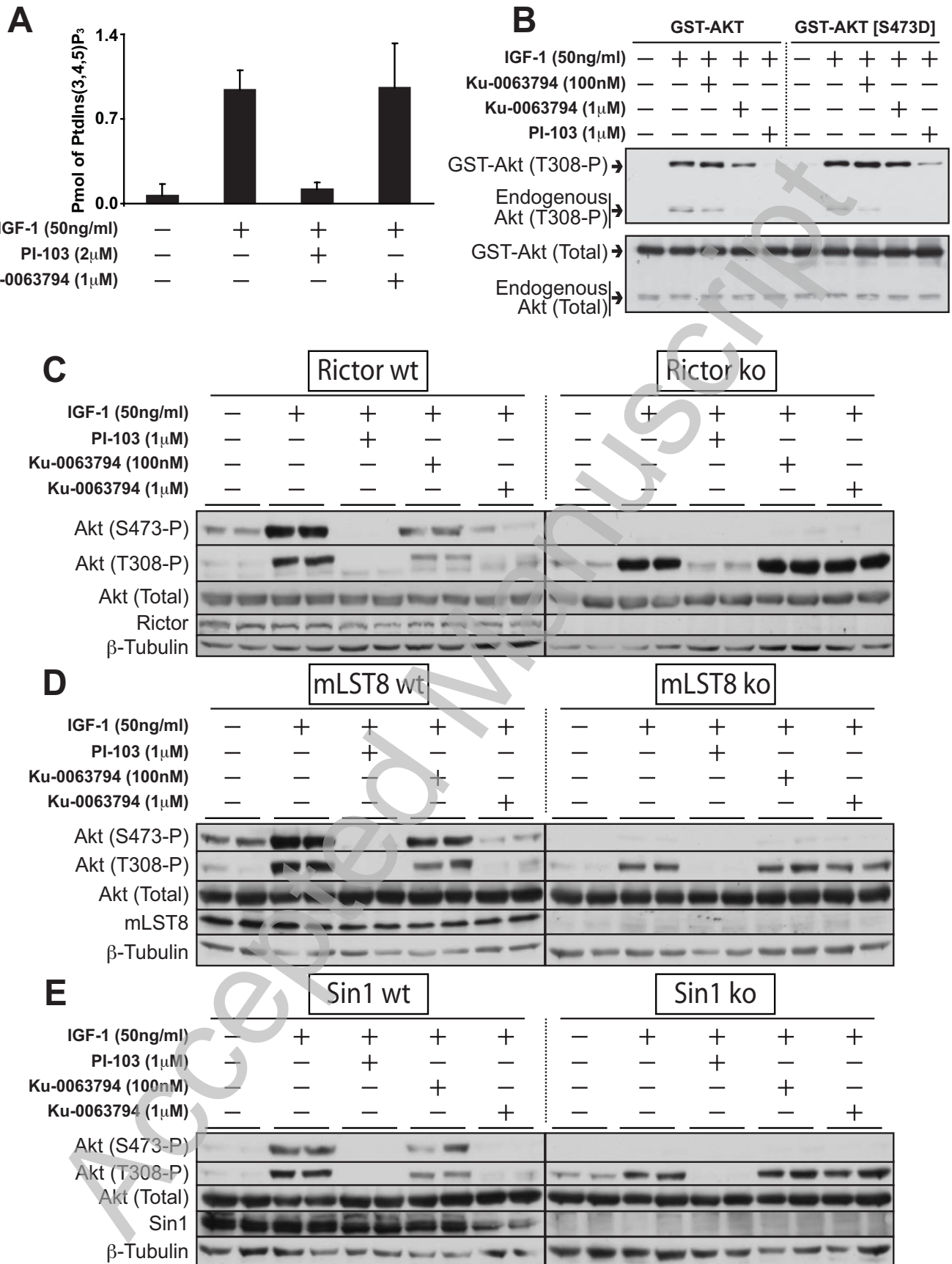


Fig. 4

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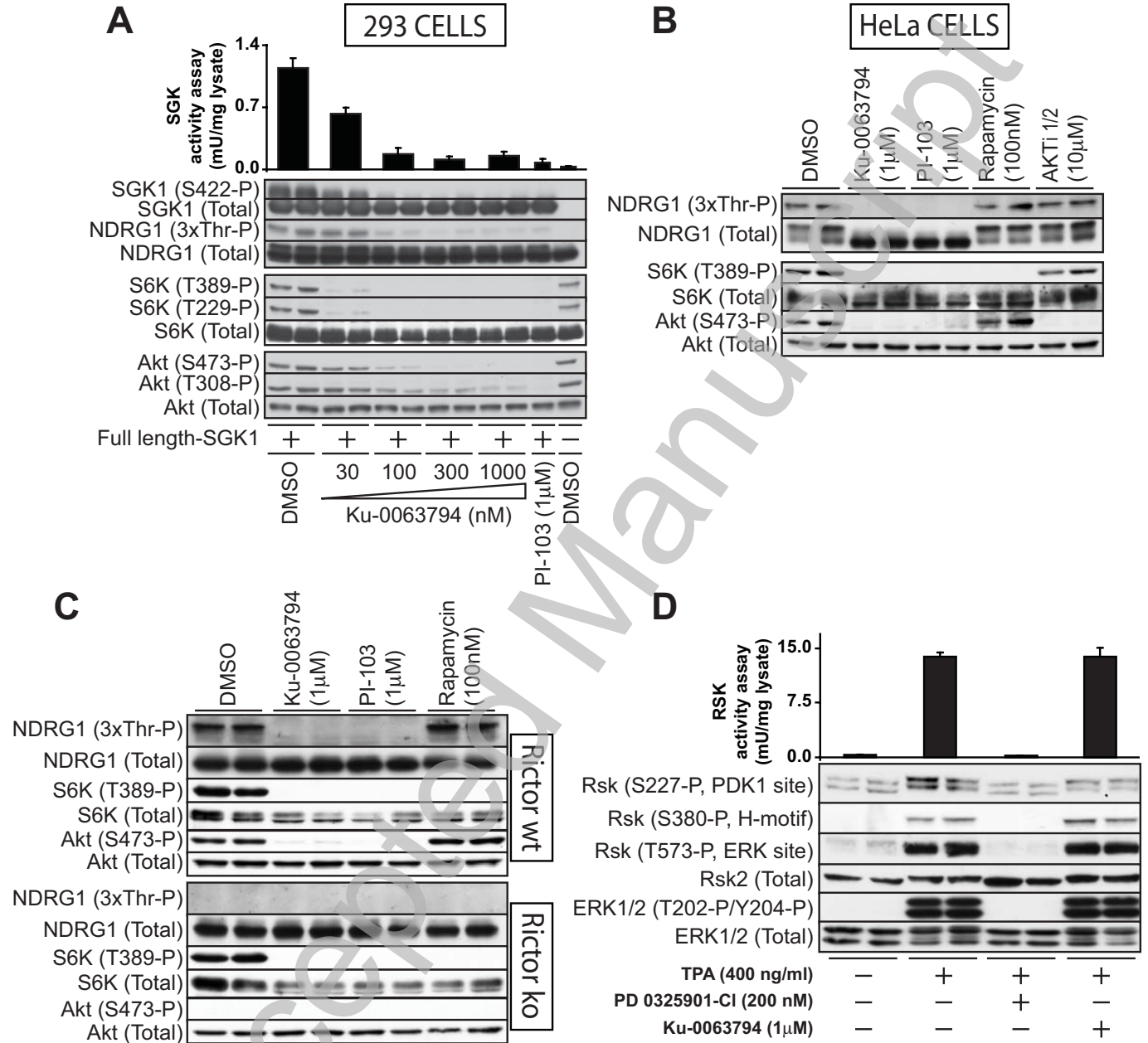


Fig. 5

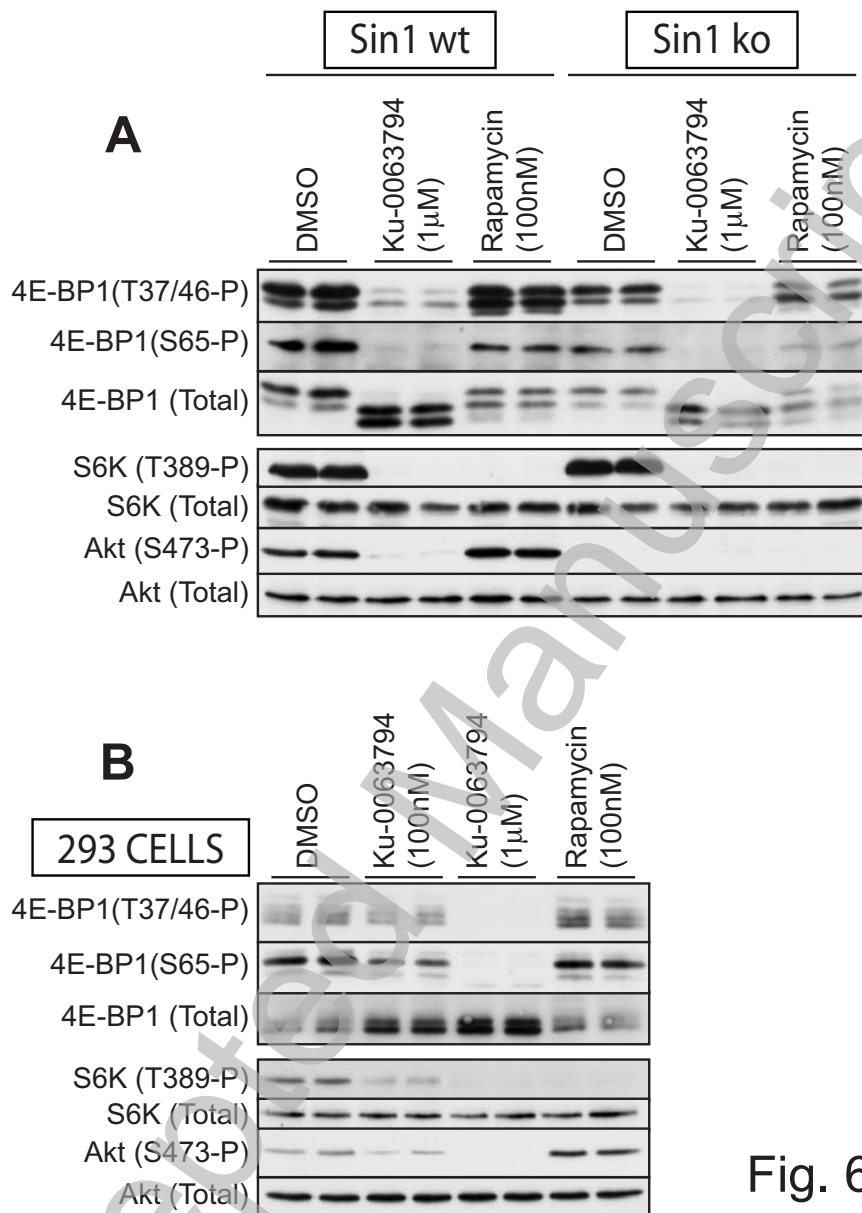


Fig. 6

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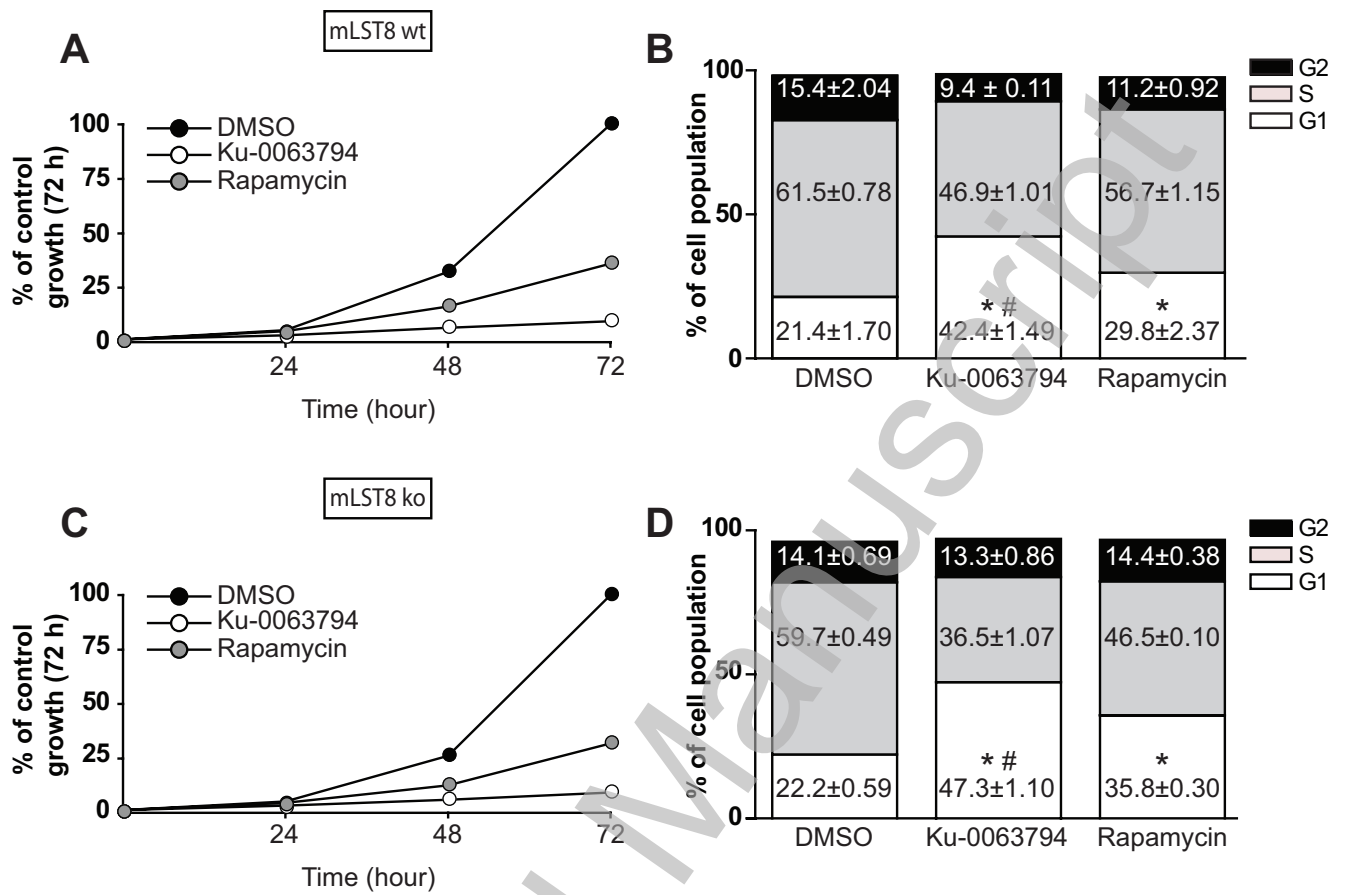


Fig. 7

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