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**Supplemental Information**

# Transmembrane Inhibitor of RICTOR/mTORC2 in Hematopoietic Progenitors

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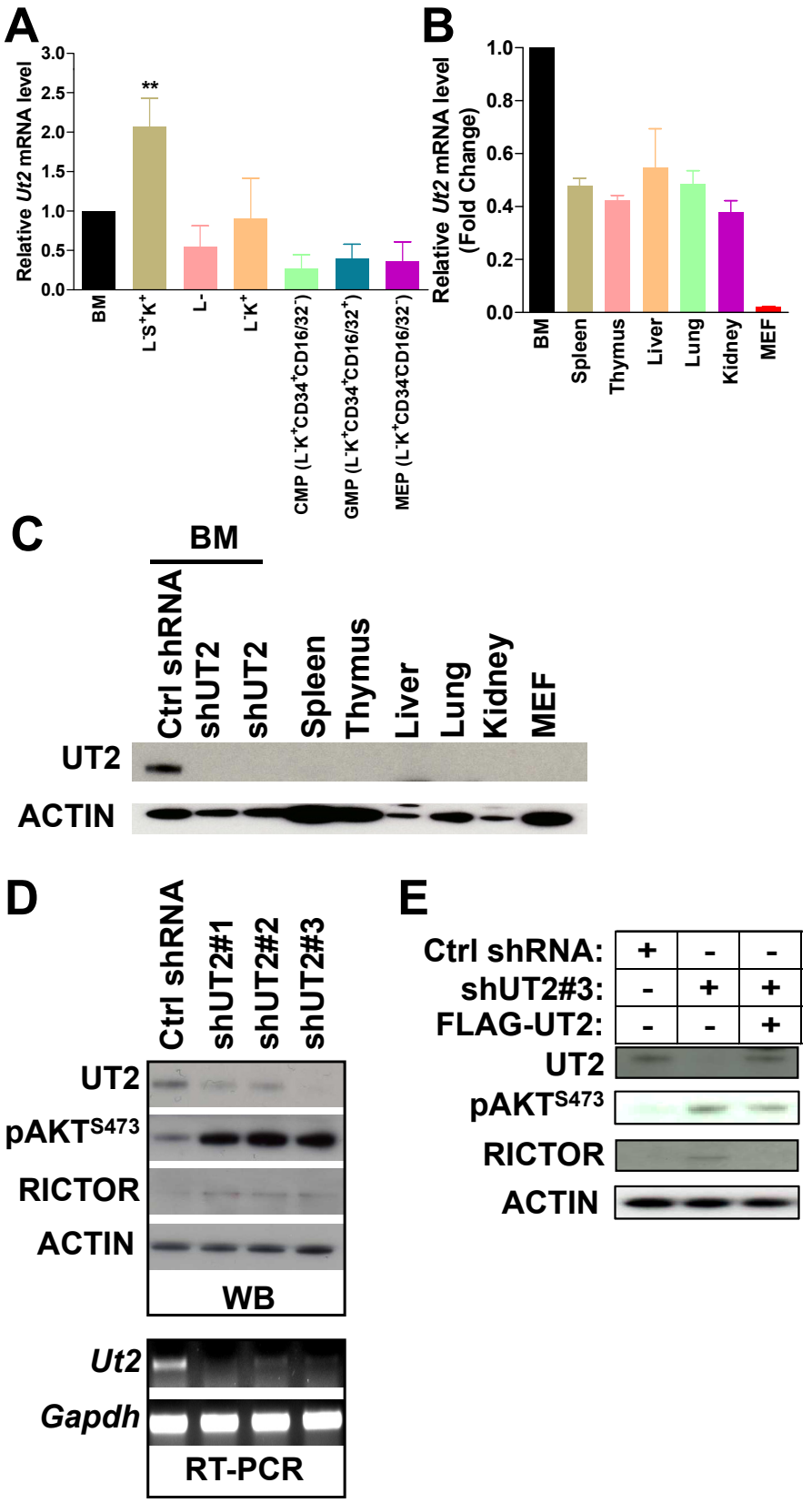
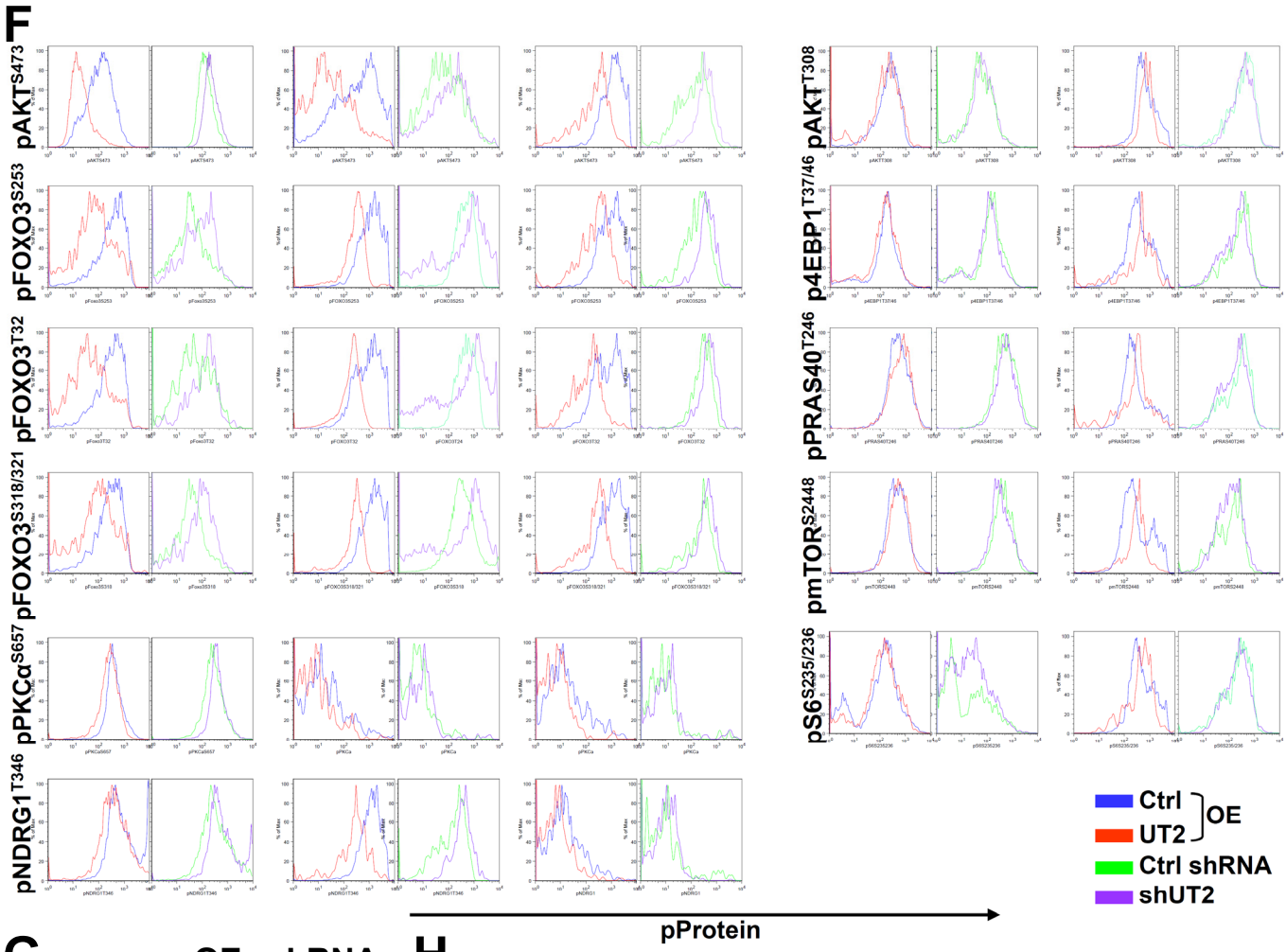
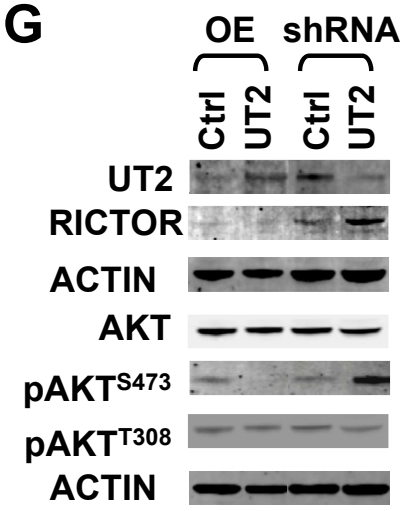
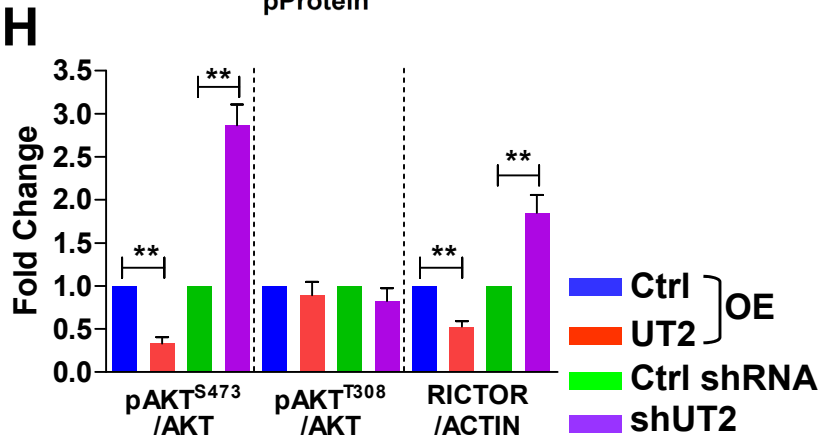
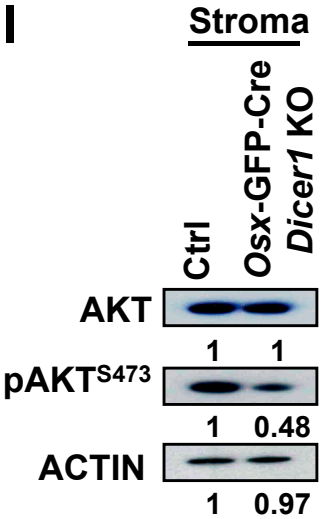



Lee et al., Figure S1.

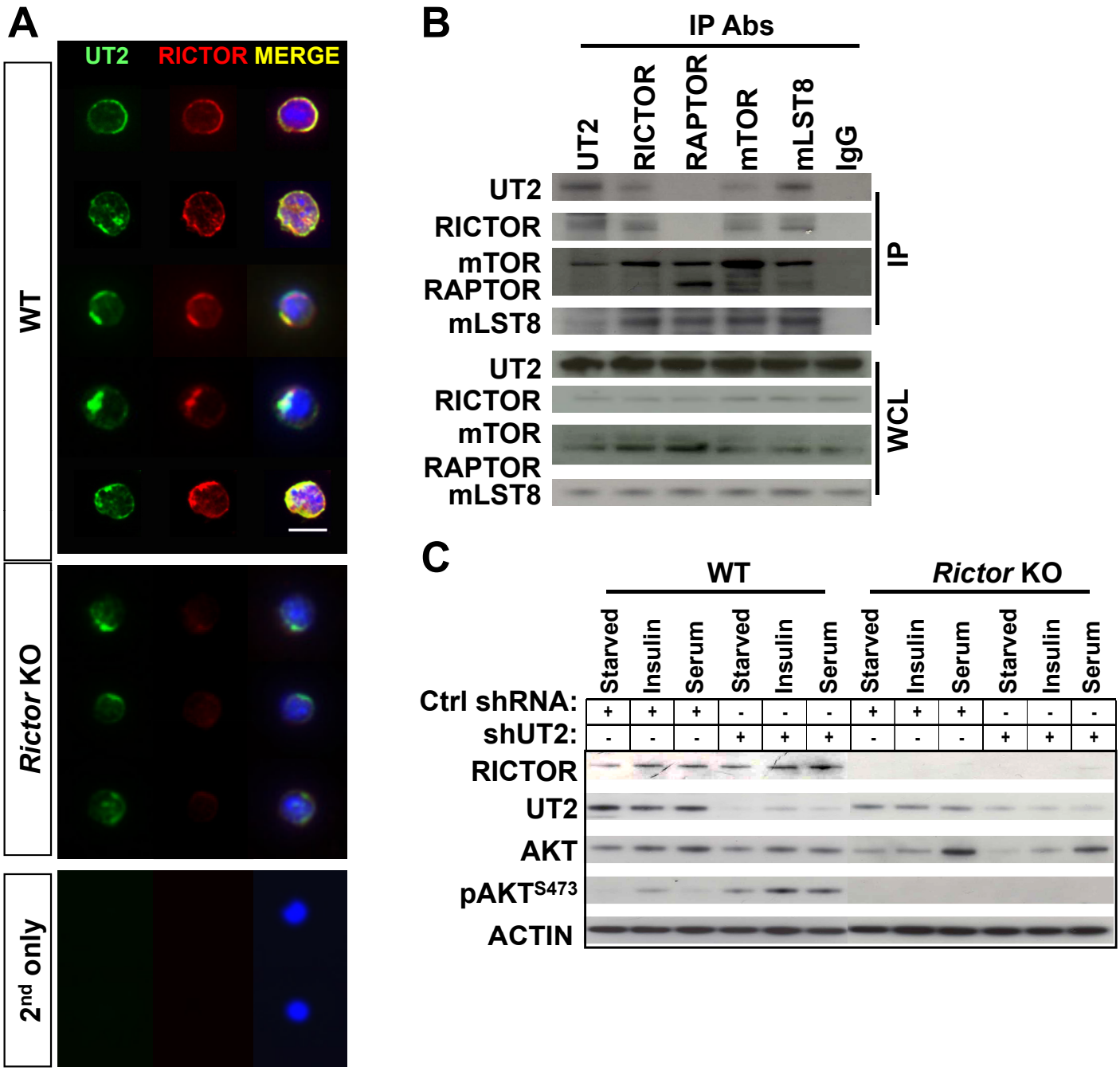



Lee et al., Figure S1 continued.

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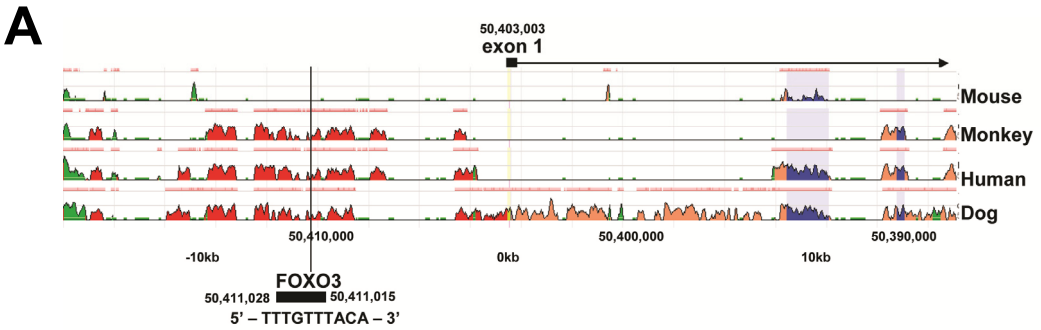
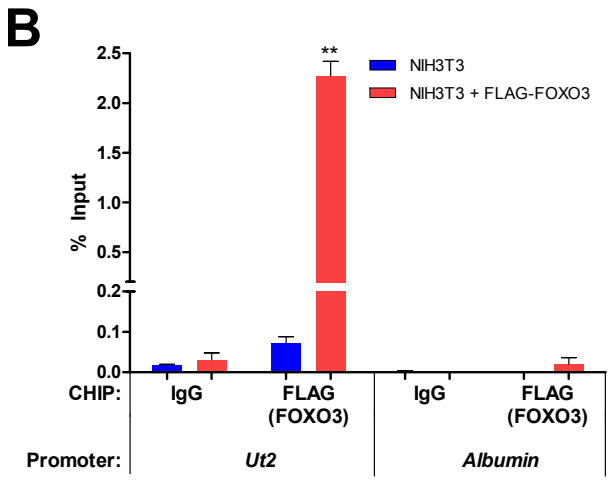
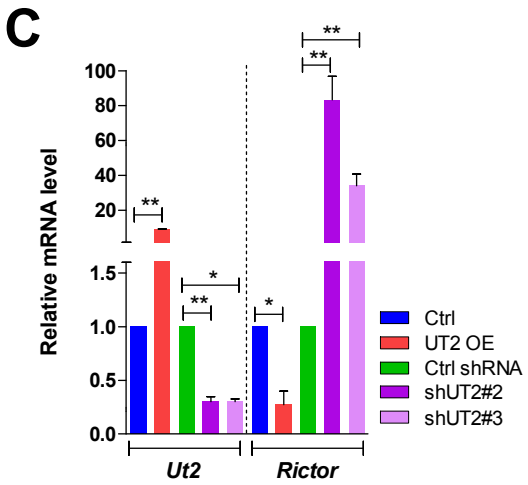
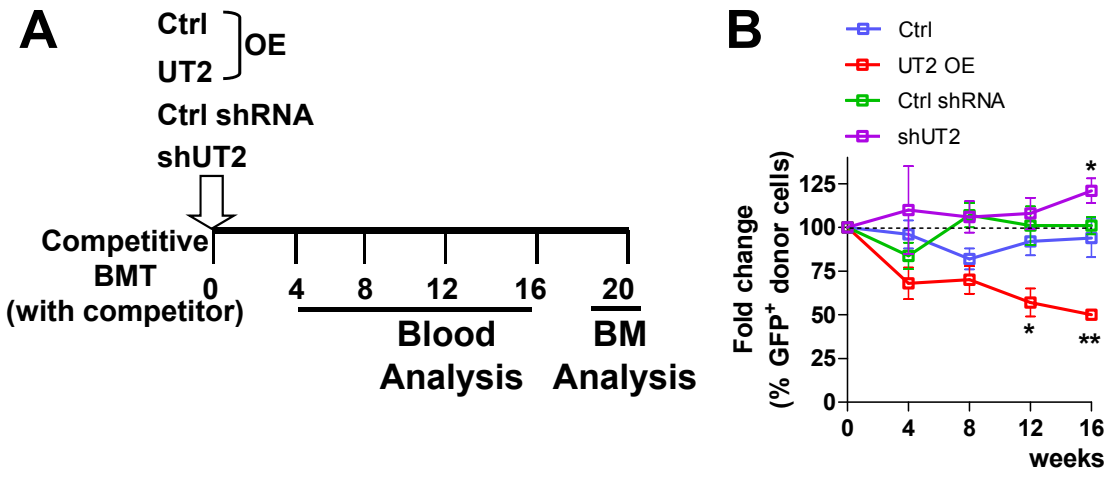



**Lee et al., Figure S3.**




**Lee et al., Figure S4.**

## SUPPLEMENTAL FIGURE LEGENDS

**Figure S1, related to Figure 1. Characterization and Expression profile of UT2 in primary hematopoietic stem and progenitor cells (HSPCs).**

**(A)** qRT-PCR analysis for *Ut2* mRNA from primary hematopoietic stem and progenitor cells. Data are means ± s.e.m. (n = 3 experiments; two-tailed, unpaired t-test). **p < 0.01.
**(B)** qRT-PCR analysis for *Ut2* mRNA from adult tissues (n = 3 experiments; two-tailed, unpaired t-test).
**(C)** Western blot analysis for UT2 protein from adult tissues. The shRNA lanes indicate the specificity of the antibody.
**(D)** Primary hematopoietic cells from depletion of UT2 were analyzed by western blotting using the indicated antibodies (top panels) and RT-PCR (bottom panels).
**(E)** Restoration of depletion of UT2 primary hematopoietic cells with UT2 expression vector.
**(F)** Flow cytometry was performed on sorted overexpressing or shRNA-depleted for UT2 $GFP^{+}$ cells from over-expression and depletion of UT2 primary hematopoietic cells, and these were then processed for flow cytometry (n = 2-3 experiments).
**(G)** Western blot from hematopoietic (BM) cells overexpressing (left panels) and depleted (right panels) of UT2 were analyzed by an infrared western-blotting method (Odyssey system) using the indicated antibodies. Beta-ACTIN (β-ACTIN) was used as a loading control.
**(H)** Graph shows the fold change of the indicated normalized protein ratios by infrared method (Odyssey system) (n = 4 experiments). Data are means ± s.e.m. **p < 0.01.
**(I)** *Osx*-GFP-Cre;*Dicer1* KO mice showed decreased $pAKT^{S473}$ expression *in vivo*. Stromal cells from control and *Dicer1* KO mice were isolated, and analyzed by western blotting using the indicated antibodies (n = 3 mice pool per each genotype).

**Figure S2, related to Figure 2. Interaction between UT2 and mTORC2/RICTOR.**

**(A)** Wild-type and *Rictor* KO primary hematopoietic cells immunostained for UT2 (green) and RICTOR (red). Scale bars, 10 μm**.**
**(B)** Immunoblot analyses for the presence of the indicated components of the mTORC2 in immunoprecipitates prepared from primary hematopoietic cell lysates with antibodies against UT2, RICTOR, RAPTOR, mTOR, or mLST8.
**(C)** Wild-type and *Rictor* KO BM cells expressing shRNA constructs against control or UT2 were starved for 3 hr, and then restimulated with insulin (1 μg/mL) or serum (10 % serum), respectively, for 30 min prior to lysis, and analyzed by western blotting (n = 6 mice pool per each genotype).

**Figure S3, related to Figure 3. FOXO3 binds to this conserved site within the *Ut2* promoter *in vivo.***

**(A)** Schematic representation of mouse *Ut2* promoter region, and FOXO3 binding site predicted by rVISTA 2.0 (http://rvista.dcode.org/).
**(B)** ChIP analysis for Flag-FOXO3 or mock expressing NIH3T3 cells that used the indicated antibodies, respectively. Data are means ± s.e.m. (n = 3 experiments; two-tailed, unpaired t-test). **p < 0.01.
**(C)** UT2 regulates *Rictor* expression in primary hematopoietic cells. qRT-PCR for *Rictor* expression from overexpressing or shRNA-depleted for UT2 primary hematopoietic cells.

Data are expressed as mean ± s.e.m. (n = 3 experiments; two-tailed, unpaired t-test). *p < 0.05. **p < 0.01.

**Figure S4, related to Figure 4. Role of UT2 in primary hematopoietic cells.**
**(A)** Experimental scheme for competitive BMT experiments.
**(B)** The normalized fold changes (% of donor $GFP^+$ cells) from recipients were determined by fluorescence-activated cell sorting (FACS) at the indicated times. Data are expressed as mean ± s.e.m. (n = 6-9 biological replicated experiments, two-tailed, unpaired t-test). *p < 0.05. **p < 0.01.
**(C)** The normalized $GFP^+$ hematopoietic stem and progenitor ($Lin^-c\text{-}KIT^+SCA1^+$) populations were determined by FACS at 20 weeks post-transplantation. Data are means ± s.e.m. (n = 6-7 biological replicated experiments, two-tailed, unpaired t-test). *p < 0.05.
**(D)** $GFP^+$ cells were sorted from primary hematopoietic cells of recipients, and were plated in cytokine-supplemented methylcellulose medium. Data are means ± s.e.m. (n = 6-7 biological replicated experiments, two-tailed, unpaired t-test). *p < 0.05. **p < 0.01.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Mice and Animal Procedures

All mice were kept in a specific pathogen-free facility at Massachusetts General Hospital. All mice studies and breeding were carried out under the approval of Institutional Animal Care and Use Committee of Massachusetts General Hospital. *Foxo1/3/4*$^{floxed}$;*Mx1*-Cre (Paik et al., 2007; Sykes et al., 2011; Tothova et al., 2007) and *Rictor*$^{floxed}$;*Mx1*-Cre (Shiota et al., 2006) mice were generated previously. To examine T-ALL model in mice, hematopoietic bone marrow precursors were transduced with a constitutively active form of NOTCH1 (ICN) and transplanted into recipient mice (Chiang et al., 2008; Lee et al., 2012; Piovan et al., 2013). For competitive bone marrow transplantation (BMT) assay, wild-type mice (CD45.2) were administered 150 mg/kg 5-flurouracil (5-FU) (Kalaitzidis et al., 2012; Lee et al., 2011; Sykes et al., 2011). Recovered bone-marrow (BM) cells was stimulated overnight in RPMI/10% FBS with murine IL-3, IL-6, and stem cell factor. BM cells were then transduced twice with UT2-expressing retroviruses or lentiviruses expressing shRNA against mouse UT2, respectively, and transplanted into lethally irradiated recipient mice (CD45.1) with competitive BM cells (CD45.1). Beginning four weeks after transplantation and continuing for 16 weeks, blood from the tail veins of recipient mice, was subjected to ammonium-chloride potassium (ACK) red cell lysis and quantities donor cell engraftment.

### Materials

The *Ut2* cDNA was cloned into the retrovirus and lentivirus expression vector. FLAG expression vectors for UT2 full length and ΔC mutant were generated by restriction enzyme digestion with BamHI-XhoI and PCR-based mutagenesis. The MIG-myr-*Foxo3* WT, AAA mutant, and *Akt* construct were kindly provided by Dr. S. Sykes. And MIG-*Notch1* (ICN) was kindly provided by Dr. D. Kalaitzidis. FLAG-*Foxo3* construct was a kind gift of Dr. DS Lim (Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Korea). Myc-*Rictor* vector was purchased from Addgene. The pLKO-shUT2 lentiviral vectors were purchased from Open Biosystems. For pLKO-GFP-shUT2, the GFP marker was replaced by a puromycin resistance cassette subcloned into the BamHI and KpnI sites of pLKO. For luciferase reporter constructs,

PCR-amplified murine *Ut2* promoter fragment from 129/Sv mouse tail genomic DNA was inserted into the XhoI and HindIII sites of pGL3-Basic (Promega). Antibodies to AKT, pAKT$^{S473}$, pAKT$^{T308}$, PTEN, FOXO3, pFOXO3$^{S253}$, pFOXO3$^{T32}$/pFOXO1$^{T24}$, pFOXO3$^{S318/321}$, mTOR, pmTOR$^{S2448}$, RICTOR, pRICTOR$^{T1135}$, S6, pS6$^{S235/236}$, 4EBP1, p4EBP1$^{T37/46}$, PRAS40, pPRAS40$^{T246}$, RAPTOR, mLST8, ERK1/2, PDK1, PP2A, PI3K p85, MYC and β-ACTIN were from Cell Signaling Technology. Antibodies to UT2 (C14ORF37), RICTOR, PKCα, and pPKCα$^{S657}$, pERK1/2$^{T202/204}$ were from Santa Cruz Biotechnology, and RICTOR, UT2 (C14ORF37) were from Abcam, and FLAG M2 antibody was from Sigma.

**Cell culture, Virus production, Transfection, and Stimulation**

The retrovirus and lentivirus expression constructs were transfected using Fugene 6 (Promega) into 293FT or 293TL cells, respectively. Media containing the recombinant retrovirus was collected for transduction at 48 hours post transfection, filtered through a 0.45 μm filter, and concentrated with PEG-it Virus Concentration Solution (System Biosciences) overnight at 4 °C (Sun et al., 2009). Viruses were precipitated next day and resuspended with PBS. For luciferase reporter assay, HEK293T cells ($3 \times 10^5$/well of a 6-well plate) were transfected with 1 μg *Foxo3* expression plasmid, 200 ng pGL3-Ut2 promoter constructs, and 25 ng pRL-CMV by employing polyethylenimine (Polysciences). Forty-eight hours later, cells were harvested, and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase values were divided by RENILLA luciferase values to calculate transfection efficiency. Primers used for the construct of the *Ut2* promoter were were as follow: *Foxo3_Ut2*-F, 5'- AAT TCT CGA GAG TCA AAG AGT AGC TGT TCA TGA A and *Foxo3_Ut2*-R, 5'- AAT TAA GCT TCT AAA AGC AAA ATG AGA GAG AGG A. Cell proliferation of GFP$^+$ BM cells expressing UT2 or shRNA-mediated depletion of UT2 cells were followed by cell counting of samples in triplicate using a cellometer (Nexcelom Bioscience). For serum and insulin stimulation experiments, BM cells expressing UT2 or shRNA-mediated depletion of UT2 BM cells were deprived of serum for 3 hr, then FBS or insulin was add back at 10% serum or 1μg/mL insulin concentration, respectively, for 30 min prior to lysis (Guertin et al., 2006; Jacinto et al., 2006).

**Protein Analyses**

For immunoblotting, cells were lysed in RIPA buffer (Cell Signaling Technology) supplemented with Halt protease and phosphatase inhibitor cocktails (Thermo Scientific). Western blot analysis was carried out according to standard methods (Sykes et al., 2011). Equal amounts of total protein from lysates were subjected to SDS-PAGE, transferred to PVDF membrane (invitrogen), and membranes were probed by overnight incubation with appropriate primary antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies and ECL detection reagent (GE Healthcare) and quantification of protein bands (Multigauge V3.0, Fujifilm). And Data were imaged and quantitated with the Odyssey Infrared Imaging System (LI-COR Biosciences). For standard immunoprecipitation experiments, all cells, with the exception of those used to isolate mTORC were lysed with Triton X-100 containing lysis buffer (Peterson et al., 2009) and all protein extracts were pre-cleared with Protein-A/G agarose (Santa Cruz Biotechnology) and incubated with Protein A/G agarose bound with antibodies at 4 °C. Immunoprecipitates were washed four times with lysis buffer and boiled for 10 minutes in 1X LDS sample buffer (Invitrogen). For immunoprecipitations of mTORC, cells were lysed in mTORC lysis buffer (ice-cold CHAPS-containing lysis buffer) (Guertin et al., 2006;

Huang et al., 2008; Kim et al., 2002; Peterson et al., 2009; Sarbassov et al., 2004; Sarbassov et al., 2005). Immunoprecipitates were washed washed four times with mTORC lysis buffer. Samples were resolved by SDS-PAGE and proteins transferred to PVDF and visualized by immunoblotting as described above. mTORC2 in vitro kinase assay was performed as described previously (Guertin et al., 2006; Huang et al., 2008; Jacinto et al., 2006; Kim et al., 2002; Peterson et al., 2009; Sarbassov et al., 2004; Sarbassov et al., 2005). For Chromatin Immunoprecipitation (ChIP) experiments, we performed using a Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore) in accordance with the manufacturer's instructions (Lee et al., 2008). The immunoprecipitated DNA fragments were recovered and subjected to qPCR using the primers. Primer sequences were as follow: *Foxo3*_ChIP-F, 5'- ACA CGA AGC AAT GTT TTG TTT TA and *Foxo3*_ChIP-R, 5'- AAG GAA GTC TCC CCT TCA CC; *Albumin*_ChIP-F, 5'- CTC CAG ATG GCA AAC ATA CG and *Albumin*_ChIP-R, 5'- TCT GTG TGC AGA AAG ACT CG.

**Quantitative (real-time) reverse-transcriptase PCR (qRT-PCR)**
Reverse transcription and quantitative PCR were performed as previously described (Lee et al., 2011; Lee et al., 2008). Briefly, one microgram of total RNA was extracted from BM cells using RNeasy Plus Mini Kit (Qiagen). cDNA was made with iScript cDNA Synthesis Kit (Invitrogen). Quantitative RT-PCR was performed with SYBR Green Mix (Applied Biosystem) and a StepOne Real-Time PCR System instrument (Applied Biosystem), and data were normalized by the abundance of *Gapdh* mRNA. The normalized Ct values were measured by using the $2^{(-\Delta Ct)}$ calculation method. The sequences of specific primers are available upon request.

**Immunofluorescence assay**
Immunofluorescence assays were performed as previously described (Sykes et al., 2011). BM cells from wild-type and *Rictor*-deficient animals were placed on coated slides via cytospin (4 min at 450 rpm), and fixed with 1% paraformaldehyde for 10 minutes. After washing, cells were permeabilized with methanol and blocked with 5 % BSA. Slides were stained with anti-RICTOR (Abcam) and anti-UT2 antibodies at 4 °C overnight. After washing, slides were incubated with secondary antibodies conjugated with AlexaFluor 488 or 594 (Invitrogen), respectively, together with DAPI.

**Flow Cytometry and Antibodies**
BM cells and leukocytes were harvested and subjected to red cell lysis (Lee et al., 2011; Sykes et al., 2011). For phosphoflow experiments, we performed as previously described (Kalaitzidis et al., 2012; Sykes et al., 2011). Briefly, Sorted $GFP^+$ cells were fixed with 1.6% paraformaldehyde (PFA) and permeablized with ice-cold 95% methanol. Stained cells were analyzed with an LSRII and FACSCalibur flow cytometer. Cell sorting was performed with a FACSAriaII instrument (Becton Dickinson). Data acquisition and analysis were performed with Cell Quest Pro or Diva software (BD Biosciences) and with FlowJo software (Tree Star), respectively.

**Colony Formation Assays**
For assessing hematopoietic progenitor cell activity, sorted $GFP^+$ BM cells were counted and plated in methylcellulose medium (M3434, STEMCELL Technologies). The colony number is counted 7 days after plating.

**Statistical Analysis**

Sample size required for the experiments was estimated based upon results of preliminary data. In vitro and in vivo data were analyzed with a two-tailed, unpaired Student's T Test (GraphPad Prism (GraphPad Software Inc.) and SigmaPlot 10.0 software (SPSS Inc.)). Values of $p < 0.05$ were considered statistically significant ($*p < 0.05$; $**p < 0.01$). The Kaplan-Meier log-rank test was used to analyze mouse survival data using GraphPad Prism (GraphPad Software Inc.). No blinding or randomization was performed for any of the experiments.

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