Supplementary Materials for

SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway

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Materials and Methods

Materials

Reagents

Reagents were obtained from the following sources: the antibody against SAMTOR (NBP1-94062) from Novus Biologicals; the antibody against Nprl3 (HPA011741) from Atlas Antibodies; antibodies against LAMP2 (sc-18822), MAT2A (sc-166452), ubiquitin (sc-8017), and HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; the antibody against raptor (2818718) from EMD Millipore; antibodies against phospho-T389 S6K1 (9234), S6K1 (2708), phospho-T398 dS6K, phospho-S65 4E-BP1 (9451), 4E-BP1 (9644), mTOR (2983), HRP-labeled anti-rabbit secondary antibody and the myc (2278) and FLAG (2368) epitopes from Cell Signaling Technology (CST); antibodies against the HA epitope from CST (3724) and Bethyl laboratories (A190208A); SAM (13956) from Cayman Chemical; [^{3}H] SAM from American Radiolabeled Chemicals, Inc. SAH (A9384), RPMI, anti-FLAG M2 affinity gel, and amino acids from Sigma Aldrich; DMEM from SAFC Biosciences; Effectene transfection reagent from Qiagen; XtremeGene9 and Complete Protease Cocktail from Roche; Alexa 488, 568 and 647-conjugated secondary antibodies, Schneider's media, and Inactivated Fetal Bovine Serum (IFS) from Invitrogen; amino acid-free RPMI, and amino acid-free Schneider's media from US Biologicals; and anti-HA magnetic beads, methionine-free RPMI from ThermoFisher Scientific. Antibodies against Wdr24 and SZT2 were generously provided by Jianxin Xie of Cell Signaling Technology, Inc. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University).

Plasmids

Plasmid name	Addgene ID	Reference
FLAG-SAMTOR in pRK5	100508	This study
FLAG-Kaptin in pRK5	87041	Wolfson et al. 2017
FLAG-metap2 in pRK5	32004	Peterson et al. 2011
FLAG-DEPDC5 in pRK5	46340	Bar-Peled et al. 2013
FLAG-S6K1 in pRK5	100509	Burnett et al. 1998
FLAG-metap2 in pLJM1	100510	This study
FLAG-Kaptin in pLJM1	100511	This study
HA-metap2 in pRK5	100512	This study
HA-SAMTOR in pRK5	100513	This study
HA-SAMTOR(G172A) in pRK5	100514	This study
HA-SAMTOR(D190A) in pRK5	100515	This study
HA-RagA in pRK5	99710	Sancak et al.
HA-RagC in pRK5	99718	Sancak et al.
HA-RagA(Q66L) in pRK5	99712	Sancak et al.
HA-RagC(S75N) in pRK5	99719	Sancak et al.
Myc-metap2 in pRK5	100516	This study

Myc-SAMTOR in pRK5	100517	This study
GFP-metap2 pLC242	100518	This study
GFP-Sestrin2 pLC242	100519	This study
GFP-SAMTOR pLC242	100520	This study
MAT2A (sg1_resistant) in pCW57.1	100521	This study
pLJC5-FLAG-SAMTOR	102420	This study

Methods

Cell lines and tissue culture

HeLa, HEK-293T, p53 -/- MEFs were cultured in DMEM with 10% IFS and supplemented with 2 mM glutamine. These cell lines were maintained at 37°C and 5% CO_2. Drosophila S2R+ cells were cultured in Schneider's media with 10% IFS at 25°C and 5% CO_2.

Transfections

For the transfection of cDNA expression constructs into HEK-293T cells, 1.5 – 2 million cells were seeded in 10 cm dishes. Using the polyethylenimine method (*39*), cells were transfected 24 hours after seeding with the indicated pRK5 based expression vectors. Experiments were done 36-48 hours after transfection. The total amount of DNA transfected was normalized to 5 µg with the empty pRK5 vector. The following amounts of cDNA were used in the indicated figures.

Fig1C: 250 ng FLAG-metap2, 250 ng FLAG-SAMTOR, 350 ng FLAG-DEPDC5, or 75 ng FLAG-KPTN.

Fig 2A: 2 ng FLAG-S6K1 and the amounts as indicated in the figure for the HA-tagged constructs.

Fig 2C: 2 ng FLAG-S6K1, 150 ng HA-RagA/C, or HA-RagA (Q66L) and HA-RagC (S75N).

Fig 2D: 2 ng FLAG-S6K1 and 0 ng, 25 ng, or 50 ng HA-SAMTOR.

Fig 3F: 150 ng HA-metap2, 150 ng HA-SAMTOR, 200 ng HA-SAMTOR (G172A), or 190 ng HA-SAMTOR (D190A).

Fig 3G: 2 ng FLAG-S6K and 0 ng, 25 ng, or 100 ng HA-tagged SAMTOR wild-type or mutant.

Fig 4B: 50 ng HA-metap2 or 25 ng HA-SAMTOR.

Fig 4F: 2 ng FLAG-S6K, 25 ng HA-metap2, 10 ng HA-SAMTOR, or 40 ng of HA-SAMTOR (G172A).

Fig S1A: 150ng FLAG-metap2, 250ng FLAG-Depdc5, 150ng HA-Nprl3, 150ng HA-Nprl2, 750ng HA-SZT2, 50ng HA-KPTN, 50ng HA-ITFG2, 50ng C12orf66, 150ng Myc-SAMTOR.

Lentiviral production and lentiviral infections

HEK-293T cells were seeded at a density of 750,000 cells per well of a 6-well plate in DMEM with 20% IFS. 24 hours after seeding, VSV-G envelope and CMV ΔVPR packaging plasmids were co-transfected with either pLJM1 containing cDNAs, pLentiCRISPRv2 with indicated guide sequences, or pCW57.1_tTA with the MAT2A (sg1_resistant) cDNA, using XTremeGene 9 transfection reagent (Roche). 12 hours after transfection, the media was changed to DMEM 20% IFS. 36 hours after the media change, the virus-containing supernatant was collected and passed through a 0.45 µm filter. Target cells were plated in 6-well plates with 8 µg/mL polybrene and incubated with virus containing media. Infections with pLentiCRISPRv2 were spinfected at 2200 rpm for 45 minutes at 37°C. 24-48 hours later, the media was changed to fresh media containing either puromycin for pLJM1 or pLentiCRISPR or blasticidin for pCW57.1_tTA.

Cell lysis and immunoprecipitations

Cells were rinsed with cold PBS and lysed in lysis buffer (1% Triton, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hepes pH 7.4, 2.5 mM MgCl2 and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer)). Cell lysates were cleared by centrifugation in microcentrifuge (15,000 rpm for 10 minutes at 4°C). Cell lysate samples were prepared by addition of 5X sample buffer (0.242 M Tris, 10% SDS, 25% glycerol, 0.5 M DTT, and bromophenol blue), resolved by 8%-16% SDS-PAGE, and analyzed by immunoblotting.

For anti-FLAG immunoprecipitations, anti-FLAG M2 Affinity Gel (SIGMA A2220) was washed with lysis buffer three times then resuspended to a ratio of 50:50 affinity gel to lysis buffer. 25 µL of a well-mixed slurry was added to cleared lysates and incubated at 4°C in a shaker for 90-120 minutes. For anti-HA immunoprecipitations, magnetic anti-HA beads (Pierce) were washed three times with lysis buffer. 30 µL of resuspended beads in lysis buffer was added to cleared lysates and incubated at 4°C in a shaker for 90-120 minutes.

Immunoprecipitates were then washed three times, once with lysis buffer and twice with lysis buffer with 500 mM NaCl. Immunoprecipitated proteins were denatured by addition of 50 µL of SDS-containing sample buffer (0.121 M Tris, 5% SDS, 12.5% glycerol, 0.25 M DTT, and bromophenol blue) and boiled for 5 minutes. Denatured samples were resolved by 8%-12% SDS-PAGE, and analyzed by immunoblotting.

RNAi in Drosophila S2R+ cells and qPCR

The dsRNA against dSesn was designed as described in (*7*). To minimize off-target effects, we used the DRSC tool at **http://flyrnai.org/RNAi_find_frag-free.html** and excluded regions of 19-mer-or-greater identity to any other Drosophila transcripts. The dsRNA targeting GFP was used as a negative control. The dsRNA against dSamtor was picked from searching CG3570 at DRSC/TRiP Functional Genomics Resources website: **http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl**. DRSC24231 was used in this work. Primer sequences used to amplify DNA templates for dsRNA synthesis for GFP, dSamtor and dSesn, including underlined 5' and 3' T7 promoter sequences, are as follows:

F-dsGFP primer:
GAATTAATACGACTCACTATAGGGAGAAGCTGACCCTGAAGTTCATCTG

R-dsGFP primer:
GAATTAATACGACTCACTATAGGGAGATATAGACGTTGTGGCTGTTGTAGTT
F-dsdSamtor primer:
GAATTAATACGACTCACTATAGGGAGATGGAATCCTACAGAGCCGAGGG
R-dsdSamtor primer:
GAATTAATACGACTCACTATAGGGAGACGTACCCGTAGCAGTCCAATCCTG
F-dsdSesn primer:
GAATTAATACGACTCACTATAGGGAGAGACTACGACTATGGCGAAGTGAA
R-dsdSesn primer:
GAATTAATACGACTCACTATAGGGAGATCAAGTCATATAGCGCATTATCTCG

On day one, 2 million S2R+ cells were plated in 6-well culture dishes in 1.5 ml of Schneider's media with 10% IFS. Cells were transfected with 2 µg of each dsRNA using Effectene transfection reagent (Qiagen) after 12-24 hours. On day three, a second round of dsRNA transfection was performed. On day five, 1.2 million cells were plated in 12-well culture dishes coated with fibronectin in advance. 3-4 hours later, cells were rinsed once with amino acid-free Schneider's media, and starved for either methionine or leucine by replacing the media with methionine or leucine-free media for 1 hour. To stimulate with methionine or leucine, the media was replaced with complete Schneider's media for 30 minutes. Cells were then rinsed with cold PBS once, lysed in lysis buffer, and subjected to immunoblotting for the levels of phospho-T398 dS6K and total dS6K.

To validate knockdown of dSamtor and dSesn, the following primer pairs were used in qPCR reactions due to the lack of available antibodies to these proteins. We used alpha-tubulin as internal standard control. The data were analyzed via the ΔΔCt method as described previously (*21*).

F-alpha-tubulin: CAACCAGATGGTCAAGTGCG
R-alpha-tubulin: ACGTCCTTGGGCACAACATC
F-dSamtor: GACCAACGATGGGAAGGTGG
R-dSamtor: GCTCTGTAGGATTCCAGGAGT
F-dSesn: TCCGCTGCCTAACGATTACAG
R-dSesn: TTCACCAGATACGGACACTGA

Sequence analyses of SAMTOR

We assessed the sequence conservation of SAMTOR with the PHMMER online tool (https://www.ebi.ac.uk/Tools/hmmer/search/phmmer) and performed secondary structure predictions using the HHPred online tool (https://toolkit.tuebingen.mpg.de/#/tools/hhpred). Note: the name for SAMTOR in the BioPlex dataset is C7orf60. When we searched C7orf60 on multiple websites, including Genecards and Uniprot, we found that C7orf60 is also associated with another name: Probable BMT2 (Base Methyltransferase Of 25S rRNA 2) homolog. BMT2 is a nuclear RNA methyltransferase in *Saccharomyces cerevisiae* (*40*). However, in our own extensive analyses we could find no similarity between human C7orf60 (SAMTOR) and yeast BMT2 at the protein sequence level. We suspect that the BMT2 name was erroneously assigned in an automated fashion to C7orf60 because both contain a predicted Class I Rossmann fold methyltransferase domain.

Generation of CRISPR/Cas9 genetically modified cells with loss of SAMTOR or MAT2A

To generate HEK-293T or HeLa cells with loss of SAMTOR, the following sense (S) and antisense (AS) oligonucleotides encoding the guide RNAs were cloned into pX330:

sgSAMTOR_guide1_S: caccgGAAATACTGCTCGTGCGCAG
sgSAMTOR_guide1_AS: aaacCTGCGCACGAGCAGTATTTCc

Control cells were generated by targeting the AAVS1 locus as described before (*10, 41*). On day one, 2 million HEK-293T cells were seeded in a 10-cm plate. Twenty-four hours after seeding, each well was transfected with 1μg shGFP pLKO, 1 μg of the pX330 guide construct and 3 μg of empty pRK5 using XtremeGene9. Two days after transfection, cells were moved to a new 10-cm plate into puromycin containing media. Forty-eight hours after selection, the media was switched to media not containing puromycin. Cells were allowed to recover for 1 week after selection prior to single-cell sorting with a flow cytometer into the wells of a 96-well plate containing 150 μl of DMEM supplemented with 30% IFS.

For HeLa cells, on day one, 1 million cells were plated in a 10-cm dish. 24 hours later, the cells were transfected with 1 μg shGFP pLKO and 1 μg of the pX330 guide construct using FuGENE. Selection with puromycin was started the following day to eliminate untransfected cells. 48 hours after selection, the medium was aspirated and replenished with fresh medium without puromycin and the cells were single-cell sorted as described above. Cells were grown for two weeks and the resultant colonies were trypsinized and expanded. Cell clones were validated via immunoblotting.

Human SAMTOR, mouse SAMTOR, and MAT2A were depleted using the lentiviral pLentiCRISPRv2 system. The following sense (S) and antisense (AS) oligonucleotides were cloned into pLentiCRISPRv2:

Human SAMTOR
sgSAMTOR_1 (S): caccgGAAATACTGCTCGTGCGCAG
sgSAMTOR_1 (AS): aaacCTGCGCACGAGCAGTATTTCc
sgSAMTOR_2 (S): caccgGATATGGAGCCAGGGGCCGG
sgSAMTOR_2 (AS): aaacCCGGCCCCTGGCTCCATATCc

Mouse Samtor
sg*Mm*Samtor_1 (S): caccgGCAGGAGAAGCTGTCCGGGG
sg*Mm*Samtor_1 (AS): aaacCGCCACTAAGACCACTCCAGc
sg*Mm*Samtor_2 (S): caccgCTCCGCAAGAAGTACCGCGA
sg*Mm*Samtor_2 (AS): aaacTCGCGGTACTTCTTGCGGAGc
sg*Mm*Samtor_3 (S): caccgATGAACGCTCTTCACCACCC
sg*Mm*Samtor_3 (AS): aaacGGGTGGTGAAGAGCGTTCATc

Human MAT2A
sgMAT2A_1 (S): caccgTTAAAGGAGGTCTGTGCCGG
sgMAT2A_1 (AS): aaacCCGGCACAGACCTCCTTTAAc

Lentivirus was produced and used to infect cells as described above. To give Cas9 time to cut the targeted locus, experiments were performed at least one week after transduction.

Generation of the MAT2A doxycycline-repressible system

The MAT2A cDNA was amplified from cDNA prepared from total cell HEK-293T RNA. The following synonymous mutations were introduced by overhang extension PCR into the MAT2A coding sequence: 144G>A to remove a BamHI restriction site and 939G>A to mutate the protospacer adjacent motif of the sgMAT2A_1 sgRNA sequence. Using NheI and BamHI restriction sites, the MAT2A_sg1 cDNA was cloned downstream of the tetO element in the lentiviral pCW57.1 vector, which encodes the tet/dox-repressible tTA trans-factor and a blasticidin resistance gene. Lentivirus was produced as described above and was used to transduce wild-type HEK-293T cells. After 24 hours, blasticidin was added to the cells to remove untransduced cells. After 48 hours of selection, cells were transduced with lentivirus produced from pLentiCRISPRv2 with the MAT2A_sg1 guide sequence. After puromycin selection, cells were single cell sorted using flow cytometry into 96-well plates containing DMEM 30% IFS. Resulting clones were expanded and screened by replica plating for sensitivity to 30 ng/mL doxycycline. Positive clones were then screened by immunoblotting for MAT2A protein in whole cell lysates.

Immunofluorescence assays

Immunofluorescence assays were performed as described previously (*10*). Briefly, for the experiment in Figure 2B, 2 million cells growing in a 10 cm dish and plated 24 hours before were transfected with 150 ng of the cDNAs for GFP-metap2, GFP-SAMTOR, or GPF-Sestrin2 in pIC242. After 24 hours, 400,000 cells were counted and plated on fibronectin-coated glass coverslips (TED PELLA, Inc.) in 6-well tissue culture plates. For the experiment in Figure 4E, 400,000 HEK-293T cells were plated on fibronectin-coated glass coverslips in 6-well tissue culture plates. After 24 hours, the slides were rinsed once with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. The slides were then rinsed three times with PBS and the cells permeabilized with 0.05% Triton X-100 in PBS for 5 minutes at room temperature. The slides were rinsed three times with PBS and then blocked for 1 hour in Odyssey blocking buffer at room temperature. The slides were incubated with primary antibody in Odyssey blocking buffer at 4°C overnight, rinsed three times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:1000 in Odyssey blocking buffer) for 50 minutes at room temperature in the dark, and washed three times with PBS. The primary antibodies used were directed against mTOR (CST; 1:100-1:300 dilution), LAMP2 (Santa Cruz Biotechnology; 1:300 dilution). Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) containing DAPI.

Images were acquired on a Zeiss AxioVert200M microscope with a 63X oil immersion objective and a Yokogawa CSU-22 spinning disk confocal head with a Borealis modification (Spectral Applied Research/Andor) and a Hamamatsu ORCA-ER CCD camera. The MetaMorph software package (Molecular Devices) was used to control the hardware and image acquisition. The excitation lasers used to capture the images

were 405 nm, 488 nm, 561 nm and 640 nm. DAPI channel is not shown in the main images, but it is in the insets as a blue signal.

In the experiment in Figure 2B, an Alexa568-conjugated secondary antibody was used for the mTOR staining and the excitation wavelength was 561 nm, while an Alexa647-conjugated secondary antibody was used for the LAMP2 staining and the excitation wavelength was 640 nm. The GFP signal was detected by excitation with the 488 nm laser without use of a primary or secondary antibody.

In the experiment in Figure 4E, an Alexa488-conjugated secondary antibody was used for mTOR staining and the excitation wavelength was 488 nm, while an Alexa568-conjugated secondary antibody was used for the LAMP2 staining and the excitation wavelength was 561 nm.

Purification of proteins expressed in human cells for SAM binding assays

For radiolabelled SAM binding assays using FLAG-tagged wild-type SAMTOR (figure 3B), suspension HEK-293F cells were seeded at 2.5 million cells/ml, and the pRK5-FLAG-SAMTOR cDNA was transfected using polyethylenimine. 72-96 hours after transfection, cells were rinsed one time in cold PBS and lysed in 1% Triton lysis buffer (1% Triton, 40 mM Hepes pH 7.4, 2.5 mM MgCl2 and 1 tablet of EDTA-free protease inhibitor [Roche] per 25 ml buffer). Following anti-FLAG immunoprecipitation, the beads were washed 4 times with lysis buffer containing 500 mM NaCl and the protein was eluted in FLAG Elution Buffer (40 mM Hepes pH 7.4, 150 mM NaCl, 2.5 mM MgCl2 and 0.5 mg/ml FLAG peptide) for 1 hour at 4°C. The eluted protein was further purified via size-exclusion chromatography on a Superdex S75 10/300 column (GE Healthcare) equilibrated in running buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM DTT) and concentrated to approximately 1 mg/ml. 1 µl of the protein was examined by SDS-PAGE followed by Coomassie blue staining for purity analysis. 5 µg of purified FLAG-SAMTOR protein was used in each sample in the experiment in Figure 3B.

For radiolabeled SAM binding assays using HA tagged SAMTOR (wild-type, D190A and G172A) (Figure 3E), 6 million HEK-293T cells were plated in a 15 cm plate. 24 hours after plating, the cells were transfected using polyethylenimine with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 12 µg HA-GST Rap2A, 15 µg HA-SAMTOR wild-type or mutants. The total amount of plasmid DNA in each plate was normalized to 20 µg total DNA with empty-pRK5. In figure 3E, each plasmid was transfected to five plates. 48 hours after transfection, cells were lysed as previously described and the lysates with same plasmid transfected was mixed and combined.

SAM binding assay

Anti-FLAG (Sigma) or anti-HA magnetic beads (Pierce) were blocked by rotating in 1 µg/µl bovine serum albumin (BSA) for 30 minutes at 4 °C, then washed three times in lysis buffer, and re-suspended in an equal volume of lysis buffer.

30 µl of a bead slurry was added to each of the purified proteins or clarified cell lysates and incubated for 90 minutes at 4°C. The beads were then washed as previously and incubated for one hour on ice in cytosolic buffer (0.1% Triton, 40 mM HEPES pH 7.4, 10 mM NaCl, 150 mM KCl, 2.5 mM $MgCl_2$) with the indicated amount of [^{3}H]-labeled SAM and unlabeled SAM or SAH. At the end of one hour, the beads were aspirated dry and rapidly washed four times with binding wash buffer (0.1% Triton, 40

mM HEPES pH 7.4, 300 mM NaCl, 2.5 mM MgCl2). The beads were aspirated dry again and resuspended in 80 µl of cytosolic buffer. Each sample was mixed well and then 15 µl aliquots were separately quantified using a TriCarb scintillation counter (Perkin Elmer). This process was repeated in pairs for each sample, to ensure similar incubation and wash times for all samples analyzed across different experiments.

For radiolabeled SAM binding assays using HA tagged SAMTOR (wild-type, D190A and G172A), an immunoprecipitation for each sample was performed in parallel. After washing three times as previously described, the proteins were eluted in lysis buffer with 500 mM NaCl and 1 mg/ml HA peptide for 1 hour at 30°C. The eluted proteins were denatured by the addition of sample buffer and boiled for 5 minutes at 95°C, resolved by 10 % SDS-PAGE, and analyzed with Coomassie blue staining.

K_d and K_i calculations

The affinities for SAM and SAH of human FLAG-SAMTOR were determined by first normalizing the bound [^{3}H]-labeled SAM concentrations across three separate binding assays performed with varying amounts of cold SAM or SAH. These values were plotted and fit to a hyperbolic equation (Cheng-Prusoff equation) to estimate the IC_{50} value. K_d or K_i values were derived from the IC50 value using the equation: K_d or K_i = IC_{50} / (1+([^{3}H]SAM)/K_d).

In vitro GATOR1-SAMTOR dissociation assay

HEK-293T cells stably expressing endogenous FLAG-tagged Depdc5 were lysed and subjected to anti-FLAG immunoprecipitations as described above. The GATOR1-SAMTOR complexes immobilized on the FLAG beads were washed twice in lysis buffer with 250 mM NaCl, and then incubated for 25 minutes in 0.3 ml of cytosolic buffer (0.1% Triton, 40 mM HEPES pH7.4, 10 mM NaCl, 150 mM KCl, 2.5 mM MgCl2) with the indicated concentrations of SAM or SAH in the cold. The amount of GATOR1, SAMTOR, GATOR2, and KICSTOR that remained bound was assayed by SDS-PAGE and immunoblotting as described previously.

LC/MS-based metabolomics and quantification of metabolite abundance

LC/MS-based metabolomics were performed and analyzed as previously described (*42, 43*), with 500 nM isotope-labeled internal standards were used. SAM standards were purchased from Cayman Chemical (13956), and SAH from SIGMA (A9384). Briefly, 80% methanol extraction buffer with 500 nM isotope-labeled internal standards was used for whole cell metabolite extraction. Samples were briefly vortexed and dried by vacuum centrifugation. Samples were stored at -80°C until analyzed. On the day of analysis, samples were resuspended in 100 µL of LC/MS grade water and the insoluble fraction was cleared by centrifugation at 15,000 rpm. The supernatant was then analyzed as previously described by LC/MS (*42, 43*).

Statistical analyses

Two-tailed t tests were used for comparison between two groups. All comparisons were two-sided, and P values of less than 0.001 were considered to indicate statistical significance.