Supplementary Materials for

SFXN1 is a mitochondrial serine transporter required for one-carbon metabolism

Nora Kory, Gregory A. Wyant, Gyan Prakash, Jelmi uit de Bos, Francesca Bottanelli, Michael E. Pacold, Sze Ham Chan, Caroline A. Lewis, Tim Wang, Heather R. Keys, Yang Eric Guo, David M. Sabatini

correspondence to: sabatini@wi.mit.edu

This PDF file includes:

Materials and Methods
Figs. S1 to S6
References

Other Supplementary Materials for this manuscript includes the following:

Tables S1 and S2

Materials and Methods

Reagents

Reagents were obtained from the following sources: the antibodies that recognize SFXN1 (HPA019543), SFXN2 (HPA026834), SFXN3 (HPA048105), SFXN1, 2, and 3 (HPA008028) and SHMT2 (HPA020549) from Atlas Antibodies; AKT (4691), CALR (12238), Catalase (12980), Citrate Synthase (14309), Cytochrome c oxidase subunit 4 isoform 1 (COX4; 4850), GOLGA1 (13192), RPSS6KB1 (2708), SHMT1 (12612), VDAC (4661), the myc (2278) and HA epitopes (3724) and HRP-coupled anti-rabbit secondary antibody as well as Normal Donkey Serum from Cell Signaling Technology (CST); the FLAG epitope from CST (2368) and Sigma (F1804); LAMP2 (sc-18822), LMNA (sc-20680), MTHFD1 (sc-271412), MTHFD2 (sc-390708), TOM20 (sc-11415) and HRP-labeled anti-mouse secondary from Santa Cruz Biotechnology (SCBT); SFXN4 (CSB-PA744046LA01HU) and SFXN5 (CSB-PA819464LA01HU) from Cusabio; MTHFD1L (ab116615) from Abcam; Cytochrome c oxidase subunit 1 (COX1; 459600) from Invitrogen; and anti-conjugated pyridoxal antibody from Advanced Targeting Systems (AB-T149). The 2,3,3-$^{2}H_3$-serine was obtained from Cambridge Isotope Laboratories; [3H] serine and alanine from American Radiolabeled Chemicals, Inc.; amino acids and galactose from Sigma Aldrich; X-tremeGENE 9 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies and from Invitrogen; anti-HA magnetic beads from ThermoFisher Scientific; glucose from Westnet Inc. (# BM-675); ANTI-FLAG M2 Agarose Affinity Gel and sodium formate from Sigma; egg phosphatidylcholine, *E. coli* total lipids, and the lipid extruder from Avanti Polar Lipids; Bio-Beads SM-2 Adsorbents from Biorad Laboratories; filter membranes for extrusion and supports from Whatman; Cell-Tak from Corning.

Cell lines and plasmids

The pMXs-IRES-Bsd vector was from Cell Biolabs. K562 and HeLa cells were purchased from ATCC. The identities of the K562, Jurkat, and HeLa cells used in this study were authenticated by STR profiling. Sequences of human SFXN1, *S. cerevisiae* FSF1 and *Drosophila melanogaster* Sfxn1-3 (CG11739) and Sfxn2 (CG6812) were synonymously mutated to remove the proto-spacer adjacent motif (PAM) sequence and/or codon-optimized for expression in human cells.

Plasmid name	Addgene ID	Reference
pMXs_FLAG-SFXN1	110634	This study
pMXs_SFXN1	110635	This study
pMXs_FLAG-SFXN2	110636	This study
pMXs_FLAG-SFXN3	110637	This study
pMXs_FLAG-SFXN4	110638	This study
pMXs_FLAG-SFXN5	110639	This study
pMXs_SFXN5	110640	This study
pMXs_FLAG-scFSF1	110641	This study
pMXs_FLAG-dmSfxn1-3 (CG11739)	110642	This study
pMXs_FLAG-dmSfxn2 (CG6812)	110643	This study
pLentiCRISPR Metabolism sgRNA library	110066	(*15*)

Cell culture

Unless otherwise indicated, Jurkat, K562, and HEL cells were cultured in RPMI (Life Technologies) supplemented with 10% Inactivated Fetal Calf Serum (IFS, Sigma and Gemini), 2 mM glutamine, and penicillin/streptomycin. HeLa and HEK-293T cells were cultured in DMEM (Life Technologies) supplemented with 10% IFS and penicillin/streptomycin. HEK-293T cells used for virus production were cultured in IMDM (Life Technologies) supplemented with 20% IFS, and penicillin/streptomycin. To prepare media lacking serine or glycine, RPMI without amino acids and glucose (US Biological) was supplemented with amino acids except serine and glycine and 11 mM glucose according to the RPMI 1640 formulation (Life Technologies) with the addition of 130 μM alanine (except in CRISPR screens) as well as dialyzed IFS and penicillin/streptomycin. Serine or glycine, or both amino acids (for control media) were added to the media in experiments as indicated. Dropout media for other amino acids was prepared accordingly. To compare proliferation of cells in glucose to proliferation in galactose, RPMI without amino acid and glucose was supplemented with amino acids, dialyzed IFS, and either 11 mM glucose or galactose.

Virus production

HEK-293T cells were co-transfected with the pLentiCRISPR sgRNA library, the VSV-G envelope plasmid and the ΔVPR lentiviral packaging plasmid, or with pMXS plasmids and retroviral packaging plasmids Gag-Pol and VSV-G, using X-TremeGene 9 Transfection Reagent. The culture medium was exchanged 24 hours after transfection with the same medium instead supplemented with 30% IFS. The virus-containing supernatant was collected 48 hours after transfection and spun for 5 min at 400 x g to eliminate cells.

Transduction of cell lines

Cells were seeded at a density of 1 x10^6 cells/mL in RPMI containing 8 μg/mL polybrene (EMD Millipore), and then transduced with lentivirus by centrifugation at 2,200 RPM for 45 min at 37°C. After an 18-hour incubation, cells were pelleted to remove virus, washed twice in PBS and then re-seeded into fresh culture medium containing puromycin or blasticidin, and selected for 72 hours.

CRISPR/Cas9-mediated generation of knockout cell lines

Human SFXN1, SFXN2, SHMT1, SHMT2, MTHFD2 were depleted using the lentiviral pLentiCRISPRv1 system. The following sense (S) and antisense (AS) oligo-nucleotides were cloned into pLentiCRISPRv1:

sgSFXN1_3 (S): caccgGATAGGAAGAATGTCAGCCC
sgSFXN1_3 (AS): aaacGGGCTGACATTCTTCCTATCc
sgSFXN1_5 (S): caccgGTTCATAATGAATGGAGGGA
sgSFXN1_5 (AS): aaacTCCCTCCATTCATTATGAACc
sgSFXN2_1 (S): caccgGGACTGGGCCAAGGTGATGG
sgSFXN2_1 (AS): aaacCCATCACCTTGGCCCAGTCCc
sgSHMT1_2 (S): caccgGAAGGAGAGTAACCGGCAGA
sgSHMT1_2 (AS): aaacTCTGCCGGTTACTCTCCTTCc
sgSHMT2_1 (S): caccgGAGAAGGACAGGCAGTGTCG
sgSHMT2_1 (AS): aaacCGACACTGCCTGTCCTTCTCc

sgMTHFD2_2 (S): caccgGGTGGAAAGGGCGAAGGCGA
sgMTHFD2_2 (AS): aaacTCGCCTTCGCCCTTTCCACCc

SFXN3 was depleted using pX330 with the following guide:
sgSFXN3_10 (S): caccgGAGTGCCACCACTGGAGCTG
sgSFXN3_10 (AS): aaacCAGCTCCAGTGGTGGCACTCc

After selection of guide-transfected or transduced cells, cells were single-cell FACS-sorted into 96-well plates. Cell clones with the desired knockouts were identified by western blotting. Control cells were generated by targeting the AAVS1 locus as described before (*14*). Double knockout cells were generated by sequential rounds of single cell cloning.

CRISPR/Cas9 negative selection genetic screens

Cells were infected with a sgRNA library targeting 3000 metabolic genes and transporters and containing 499 control sgRNAs (*15*). 48 hours after infection cells, were selected with puromycin for 72 hours. After 48 hours of recovery post-selection, cells were seeded into RPMI with or without serine or glycine as indicated. Cells were passaged every other day, with seeding densities of 250,000 cells/ml for Jurkat cells and 175,000 cells/ml for K562 cells until reaching ~14-15 population doublings (PDs) in full media and glycine-deficient media or ~9 population doublings in serine-deficient media (because cells proliferated more slowly in the absence of serine compared to in full RPMI media). Wild-type and *SFXN1*-null cells in the *SFXN1* synthetic lethality screen grown in glycine-deficient media were resuspended in fresh media lacking glycine every day from PD ~8 to the end of the screen to prevent accumulation of secreted glycine. DNA was extracted from 30-50 x10^6 cells using the QIAamp DNA Blood Maxi Kit (QIAGEN). sgRNA inserts were PCR amplified using Ex Taq DNA Polymerase (Takara). The resultant PCR products were purified and sequenced on a HiSeq 2500 (Illumina) (primer sequences provided below) to monitor the change in the abundance of each sgRNA between the initial and final cell populations.

Primer sequences for sgRNA quantification
Forward:
AATGATACGGCGACCACCGAGATCTACACGAATACTGCCATTTGTCTCAAGATCT
A
Reverse:
CAAGCAGAAGACGGCATACGAGATCnnnnnnTTTCTTGGGTAGTTTGCAGTTTT
(nnnnnn denotes the sample barcode).

Illumina sequencing primer
CGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCT
AGCTCTAAAAC
Illumina indexing primer
TTTCAAGTTACGGTAAGCATATGATAGTCCATTTTAAAACATAATTTTAAAACTGC
AAACTACCCAAGAAA

Sequencing reads were aligned to the sgRNA library and the abundance of each sgRNA was calculated. sgRNAs with less than 30 counts in the initial cell pool were removed from

downstream analyses. The log_2 fold-change in abundance of each sgRNA was calculated for each treatment condition after adding a pseudocount of one. For the *SFXN1* synthetic lethality screen sgRNAs with less than 24 counts in the initial cell pool were removed from downstream analyses. A pseudocount of one was added to all sgRNAs and counts were normalized by number of reads in each sample multiplied by one million. Gene scores were defined as the average log_2 fold-change in the abundance of all sgRNAs targeting a given gene between the initial and final cell populations and calculated for all treatment conditions. The differential gene score was calculated as the difference in gene scores between treatment conditions.

<u>Cell proliferation assays</u>

10,000 cells per well were seeded into 96-well plates in triplicate. Cell titer glo reagent (Promega) was added to one plate immediately after seeding and luminescence was measured, while a second plate was read-out 4 days after seeding. Number of doublings in 4 days was determined by calculating the log_2 fold-change in signal between day 0 and 4. For formate rescue experiments, 1 mM formate was added to media at the time of seeding (Jurkat cells) or cells were cultured in formate-containing media for 1 week prior to the experiment (K562 cells) unless indicated otherwise.

<u>MS-based metabolomics and quantification of metabolite abundances</u>

Metabolite abundance using LC/MS-based metabolomics was measured and quantified as previously described (*15*). Briefly, Jurkat or K562 cells were seeded at densities of 0.6 $x10^6$ per ml and 0.33 $x10^6$ per ml, respectively. 24 hours later, 1.5-2 $x10^6$ Jurkat or 1 $x10^6$ K562 cells were harvested, washed once in ice-cold 0.9% saline prepared with LC-MS-grade water, and extracted with 80% methanol containing 500 nM isotope-labeled amino acids as internal standards (Cambridge Isotope Laboratories). The samples were vortexed for 10 min at 4°C and centrifuged at 17,000 x g. The supernatant was dried by vacuum centrifugation at 4°C. Samples were stored at -80°C until analyzed. On the day of analysis, samples were resuspended in 50-100 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 (*15, 42*). Amino acids were normalized to their respective internal standards, purine synthesis intermediates were normalized to the glutamate internal standard. Folate measurements were performed as previously described (*43, 44*). For GC-MS analysis of amino acids, samples were prepared as for LC-MS analysis, but after drying the samples were derivatized using N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma-Aldrich) as previously described (*45*).

<u>Serine tracing experiments</u>

Cells were cultured for 12 hours in serine deficient RPMI containing 285 µM 2,3,3-$^2H_3$-serine (Cambridge Isotope Laboratories), supplemented with 10% dialyzed serum. Cells were extracted for LC-MS metabolite analysis as described above. For LC-MS analysis of labeled nucleotides, dried samples were resuspended in 40 µL LC-MS-grade water and 10 µL were injected. Mass isotopomer distributions were corrected for natural abundance using in-house algorithms as previously described (*45*).

In parallel, culture supernatant was collected and after removal of cells by centrifugation, 20 µL were extracted for GC-MS analysis with 300 µL acetone containing 1 µg norvaline. Samples were vortexed briefly and spun 10 min at 4°C. 200 µl of the supernatant were dried down using a nitrogen dryer and derivatized and analyzed as described above. Mass isotopomer

distributions were determined by integrating metabolite ion fragments and corrected for natural abundance using in-house algorithms as previously described (*45*). Glycine levels were normalized to norvaline.

Immunofluorescence assays and STED imaging

For immunofluorescence assays 50,000 HeLa cells were plated in a 24-well glass bottom imaging plate (Cellvis, Mountain View, CA) and transfected with 500 ng of the cDNAs for FLAG-Sideroflexin constructs 16 hours later. 48 hours after transfection, cells were rinsed twice with PBS and fixed with 3% paraformaldehyde with 0.1% glutaraldehyde in PBS for 10 minutes. The fixation and all subsequent steps were performed at room temperature. Cells were rinsed three times with PBS and permeabilized with 0.3% NP40, 0.05% Triton X-100, 0.1% BSA in PBS for 3 minutes. After rinsing three times with wash buffer (0.05% NP40, 0.05% Triton-X 100, 0.2% BSA in PBS) samples were blocked for 1 hour in blocking buffer (0.05% NP40, 0.05% Triton-X 100, 5% Normal Donkey Serum). The samples were incubated with primary antibody in blocking buffer for 1 hour, washed three times with wash buffer, and incubated with secondary antibodies produced in donkey (diluted 1:500 in blocking buffer) for 30 minutes in the dark, washed three times with wash buffer, and rinsed three times with PBS. The primary antibodies used were directed against COX4 (CST; 1:250 dilution), the FLAG epitope (Sigma, 1:1000 dilution) and TOM20 (SCBT, 1:1000). Secondaries antibodies conjugated with Alexa 488 and 568 were used for confocal microscopy and antibodies conjugated with ATTO594 and ATTO647N were used for STED imaging.

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 488 nm and 647 nm. Images were processed with FIJI (*46*). STED imaging was carried out on a commercial Leica TCS SP8 STED 3X setup with a white light excitation laser. Wavelengths of 594 and 650 nm were used to excite ATTO594 and ATTO647N respectively. For detection HyD 1 and HyD 2 were used and set to 604-644 and 665-705 nm windows. The 775-nm depletion laser was used for both dyes. The two colors were imaged sequentially line by line. STED images were deconvolved to reduce noise using the Richardson-Lucy algorithm implemented in the python-microscopy package (python-microscopy.org). Line profiles were generated from the raw data using FIJI.

Mitochondrial isolations for immunoblot analyses

30 $x10^6$ Jurkat or K562 cells expressing the HA-mito tag or a control tag were washed 1x in PBS, 1x in KPBS according to (*42*). 5 µl of the cell suspension in 1 ml KPBS was lysed in 50 µl of 1% Triton lysis buffer to obtain whole cell protein levels. The rest was lysed using 8 or 5 strokes for Jurkat and K562 cells, respectively, with a 30 ½ G needle. Lysates were spun for 1 min at 1000 x g to pellet unbroken cells, and subsequently incubated with 100 µl HA-magnetic beads for 4 min. Beads were washed 3x in KPBS, and mitochondria lysed in 50 µl lysis buffer for 10 min. Beads were removed using the magnet, and samples were spun 10 min at 17,000 x g to remove residual beads and insoluble material. SDS-PAGE loading dye was added to each sample, and for Jurkat cells 6 µl of whole cell lysate and 9 µl of the mitochondrial fraction were analyzed by SDS-PAGE, for K562 cells 12 µl each of the whole cell lysate and

mitochondrial fraction. For analysis of pyridoxal-conjugated proteins, isolated mitochondria were processed as described previously (*40*).

In vitro transport assays

In vitro transport assays were performed as described previously (*47, 48*) with the following specifications. FLAG-SFXN1 was purified from HEK-293T cells stably expressing the protein using FLAG-affinity purification and eluted off the beads with the FLAG peptide.

1% Phosphatidylcholine was added to total *E. coli* lipids in chloroform, evaporated under nitrogen and desiccated overnight. Dried lipids were hydrated in inside buffer (20 mM MES pH 7, 50 mM NaCl, 20 mM KCl) by freeze-thawing in liquid nitrogen 20x times. Lipids were extruded 6x through a 0.4 µm filter and 15x through a 0.1 µm filter. 15 µg of purified SFXN1 protein were reconstituted into 1.2 mg liposomes using a detergent:lipid ratio of 5:1 with the addition of 1 mM DTT in inside buffer rotating at 4°C for 1 hour. The proteoliposomes were incubated for 3 sequential rounds with Bio-Beads to remove detergent. A liposome-only control was prepared by replacing the protein with inside buffer in the reconstitution reaction. Incorporation of protein into liposomes was assessed using a glycerol gradient centrifugation assay. [3H] Serine at 1 µM and alanine at 500 nM were used as substrates and a buffer containing 20 mM Tris-HCL pH 7.4 and 100 mM NaCl was used as outside buffer in the transport reactions. Different lots of total *E. coli* lipid extracts were tested and ones that optimally preserved SFXN1 activity were used in experiments.

Amino acid uptake into isolated mitochondria

Mitochondria from wild-type or *SFXN1*-null Jurkat cells were prepared according to (*49*). Briefly, cells (~1 g starting material) were resuspended in low tonicity buffer (100 mM sucrose, 10 mM MOPS pH 7.2, 1 mM EGTA, 0.1% BSA) and disrupted with 20 strokes in a homogenizer containing a pure PTFE head (VWR International) and two strokes with a dounce tissue grinder with tight-fitting pestle (DWK Life Sciences Kimbl Kontes). After adjusting tonicity, lysates were spun twice at 950 x g to remove unbroken cells and nuclei, and mitochondria were pelleted by centrifuging at 10,000 x g for 10 min. The supernatant as well as the top, white part of the pellet containing other membranes was aspirated and the brown/yellow mitochondrial pellet was washed in isolation buffer (210 mM mannitol, 70 mM sucrose, 10 mM MOPS pH 7.2, 1 mM EGTA, 0.1% BSA) before resuspending in mitochondria incubation media (125 mM KCl, 10 mM MOPS pH 7.2, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 10 mM NaCl, 1 mM EGTA). Mitochondrial preparations were adjusted for protein content. 200 µl volume uptake reactions with 0.75 µM [3H] serine, 0.175 mM pyridoxal phosphate, 1 µM NAD+, and 2 µM tetrahydrofolate or 3 µM [3H] glutamate (*50*) were initiated by adding ~100 µg of mitochondria to incubation media containing substrates at 30°C. 50 µl samples were collected immediately after start of the reaction and at 1, 2, 3 mins and added to mixed cellulose-ester filters (Fisher Scientific, 0.22 µm pore size) connected to a vacuum line (adapted from (*51*)). Filters were washed with 3 mL of ice cold TBS and the radioactivity remaining on the filters was measured using a scintillation counter. Blank reaction samples, in which mitochondria were replaced with incubation media, were used to subtract the background. The uptake rate was calculated from the first two minutes of the reaction.

Mitochondrial characterization

For quantification of mitochondrial mass and morphology, Jurkat cells were stained with MitoTracker DeepRed FM (Life Technologies, M22426) at 25 nM for 1h before analysis by flow cytometry or fluorescence microscopy. For microscopy, nuclei were stained with Hoechst 33342 fluorescent stain (Molecular Probes) at 2 µg/ml and z-stacks with 250 nm step size were taken at 100x magnification. FIJI was used to generate max intensity z-projections and measure mitochondrial length (*46*). To measure mitochondrial membrane potential cells were stained with 200 nM tetramethylrhodamine, methyl ester, perchlorate (TMRM; Life Technologies, T668) in RPMI for 20 min at 37°C, washed once with PBS, and resuspended in fresh PBS for flow cytometry analysis of live cells. Where indicated, cells were incubated with 10 µM FCCP for 10 min prior to adding TMRM dye.

Analysis of mtDNA copy number was performed as previously described (*52*). Briefly, genomic and mitochondrial DNA were extracted from cells using the QIAamp DNA mini kit according to the manufacturer's instructions (Qiagen). The following primers targeting the mitochondrial gene ND1 and the nuclear gene RUNX2 were used to assess mtDNA copy number by qPCR by normalizing Ct values of ND1 to those of RUNX2.

ND1_F: CCC TAA AAC CCG CCA CAT CT
ND1_R: GAG CGA TGG TGA GAG CTA AGG T
RUNX2_F: CGC ATT CCT CAT CCC AGT ATG
RUNX2_R: AAA GGA CTT GGT GCA GAG TTC AG.

Oxygen consumption rates (OCR) of intact cells were measured using an XF24 Extracellular Flux Analyzer (Agilent). 200,000 cells were seeded on Seahorse XF24 cell culture plates coated with Cell-tak and assayed after incubation at 37°C for 1 h. Three basal OCR measurements were taken, followed by sequential injections of 1 µM oligomycin, 3 µM FCCP, and 1 µM antimycin A, taking three measurements following each treatment. Cellular respiration was calculated by subtracting the OCR after Antimycin A treatment from the basal or FCCP-stimulated OCR.

Bioinformatics analyses

SFXN1 topology was predicted using Protter (*20*). For construction of the phylogenetic tree, Sideroflexin homologue protein sequences (SFXN1_HUMAN Q9H9B4, SFXN2_HUMAN Q96NB2, SFXN3_HUMAN Q9BWM7, SFXN4_HUMAN Q6P4A7, SFXN5_HUMAN Q8TD22, FSF1_YEAST Q12029, Q9VN13_DROME, Q9VVW3_DROME) were aligned using MUSCLE (*53*). The PHYLIP proml module (*54*) was used to construct the phylogenetic tree and FigTree software v.1.4.3 to visualize it. Percent sequence similarities were calculated with the NCBI blastp tool. Graphpad Prism 7 software was used to generate the heat map of Sideroflexin RNA expression based on data from the Cancer Cell Line Encyclopedia (broadinstitute.org/ccle, (*55*)).

Statistical analyses

Two-tailed t tests were used for comparison between two groups. All comparisons were two-sided, and P values of less than 0.05 were considered to indicate statistical significance. All error bars denote standard deviations between biological replicates unless indicated otherwise.

Fig. S1.

(A) Serine levels in cells decrease when cells are cultured in the absence of serine. Serine levels were measured by LC-MS in extracts from wild-type Jurkat cells (mean ± SD; $n = 3$; $^{**}P < 0.01$).
(B) Full list of genes with differential gene scores of <-1.5 from K562 cell screen. 1C – one-carbon; PLP – pyridoxal phosphate.
(C) Individual sgRNA scores for *SFXN1* in Jurkat and K562 cells.
(D) Immunoblot showing deletion of one-carbon gene products in single cell clones. Lysates prepared from indicated knockout cells were equalized for total protein amount and analyzed by immunoblotting for the levels of the indicated proteins.

Fig. S2.

(A) Protein levels of the 5 human Sideroflexins in different tissues (data from Human Proteome Map (*56*))
(B) mRNA levels of SFXN1 in normal human tissues. TPM (Transcripts Per Kilobase Million) levels were extracted from GTEx Portal V7 (mean ± SD).
(C) mRNA levels of SFXN1 in human cancers. Leukemias and lymphomas are amongst the cancers with the highest mRNA levels of SFXN1 (indicated in red). RPKM (Reads Per Kilobase Million) levels were extracted from the Cancer Cell Line Encyclopedia ((*55*); mean ± SD).

Fig. S3.

(A) Serine levels are increased and glycine levels are reduced in *SFXN1*-null K562 cells and the cellular serine/glycine ratio is increased as in cells with deletion of known components of the mitochondrial one-carbon pathway. Serine and glycine levels were measured by LC-MS in extracts from wild-type K562 cells or single-cell-derived control and knockout clones (mean ± SD; $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).
(B) SFXN1 deletion does not result in a reduction of folate or THF. The THF to folate ratio of *SFXN1*-null Jurkat cells is restored by expression of the sgRNA-resistant *SFXN1* cDNA (mean ± SD; $n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$). THF – tetrahydrofolate.
(C) Purine intermediates accumulate in *SFXN1*-null K562 cells. Purine intermediates were measured by LC-MS in extracts from wild-type K562 cells or single-cell-derived control and knockout clones (mean ± SD; $n = 3$).
(D) Serine depletion causes accumulation of purine synthesis intermediates. Purine intermediates were measured by LC-MS in extracts from wild-type Jurkat cells, or single-cell-derived *SFXN1*-null cells incubated for 24 hours in the indicated media (mean ± SD; $n = 3$).
(E) One-carbon pathway map indicating genes that scored in the *SFXN1* synthetic lethality screen. Two-tailed t tests were used for comparisons between groups.

Fig. S4.

(A) SDS-PAGE and Coomassie blue staining was used to analyze recombinant FLAG-SFXN1 purified from HEK293T cells.
(B) Steady-state kinetic analysis of SFXN1 transport reveals a K_m of 170 μM for serine. Transport over time of [3H] serine (1 μM, indicated by asterisk) in the presence of increasing concentrations of unlabeled serine.

(C) *SFXN1*-null cells have a proliferation advantage compared to wild-type cells in media with low concentrations of cystine. Proliferation of wild-type Jurkat or single cell-derived knockout cells was assayed in full RPMI media (not containing alanine and containing 235 μM cystine) or RPMI containing 130 μM alanine or 10 μM cystine as indicated (mean ± SD; $n = 3$; ****$P < 0.0001$).

Fig. S5.
(A) Loss of known components of the mitochondrial one-carbon pathway but not SFXN1 results in glycine auxotrophy. Proliferation of wild-type Jurkat or single cell-derived knockout cells was assayed in full or glycine-deficient RPMI media as indicated.
(B) Indicated endogenous Sideroflexin proteins are present in purified mitochondria from Jurkat and K562 cells. Mitochondria were affinity-purified using the HA-Mito-tag (*42*). HA-immunoprecipitates and cell lysates prepared from wild-type cells expressing the HA-mito tag or a control mito-tag and were analyzed by immunoblotting for the levels of the indicated proteins. CS – citrate synthase, mitochondrial matrix marker; VDAC1 – voltage-dependent anion channel, mitochondrial outer membrane marker; CALR – calreticulin, ER marker; GOLGA1 – Golgin subfamily A member, Golgi marker; LAMP2 – lysosome-associated membrane glycoprotein, lysosomal marker; CAT – catalase, peroxisomal marker; RPS6KB1 – Ribosomal protein S6 kinase beta-1, cytosolic marker; LMNA – Lamin A, nuclear marker.
(C) Immunoblot showing loss of Sideroflexins in Jurkat single cell-derived clones. Lysates prepared from indicated knockout cells were equalized for total protein amount and analyzed by immunoblotting for the levels of the indicated proteins. Exp. – exposure.
(D) Immunoblot showing levels of SFXN1 and SFXN3 in Jurkat cells expressing the *SFXN1* or *SFXN3* cDNA. Lysates prepared from indicated knockout cells were equalized for total protein amount and analyzed by immunoblotting for the levels of the indicated proteins. # indicates that the overexpressed SFXN3 protein is recognized by the SFXN1 antibody.
(E) Expression of SFXN1 or SFXN3 rescues growth and glycine auxotrophy of cells lacking SFXN1 and SFXN3 (mean ± SD; $n = 3$; **$P < 0.01$, ***$P < 0.001$).
(F) Immunoblot showing Sideroflexin homologue protein levels in wild-type cells and in cells lacking SFXN1 and SFXN3 expressing an empty vector (EV) or indicated Sideroflexin cDNAs. Lysates prepared from indicated cells were equalized for total protein amounts and analyzed by immunoblotting for the levels of the indicated proteins. # indicates that the SFXN1 antibody recognizes overexpressed SFXN3. ° indicates that the SFXN1 antibody recognizes overexpressed SFXN5. The SFXN1-3 antibody recognizes SFXN1, 2, and 3 and their orthologues. The asterisk denotes constructs containing an N-terminal FLAG-tag. Two-tailed t tests were used for comparisons between groups.

Fig. S6.
(A) mtDNA content of wild-type, *SFXN1*-null and *SFXN1&3* double knockout Jurkat cells (mean ± SD; $n = 3$; ***$P < 0.001$; N.S. – not significant).
(B) Mitochondrial mass per cell as determined by flow cytometry analysis of wild-type, *SFXN1*-null and *SFXN1&3* double knockout Jurkat cells stained with MitoTracker DeepRed FM. The histograms were normalized and smoothened.
(C) Max intensity z-projections of confocal images of mitochondria visualized by MitoTracker (magenta in merged images) were used to measure mitochondrial length of wild-type, *SFXN1*-null and *SFXN1&3* double knockout Jurkat cells. Nuclei were stained with

Hoechst DNA stain (blue) (mean ± SD; *n* > 220; ****$P < 0.0001$; N.S. – not significant). Scale bar is 5 µm.
(D) Oxygen consumption rate (OCR) and respiration of wild-type, *SFXN1*-null and *SFXN1&3* double knockout Jurkat cells determined by Seahorse Extracellular Flux Analysis (mean ± SD; $n \geq 5$ technical replicates; ***$P < 0.001$, ****$P < 0.0001$).
(E) Proliferation of wild-type, *SFXN1*-null and *SFXN1&3* double knockout Jurkat cells was assayed in RPMI containing glucose or galactose as the carbon source as indicated (mean ± SD; $n = 3$; ***$P < 0.001$, ****$P < 0.0001$).
(F) Relative mitochondrial membrane potential as assessed by flow cytometry analysis of wild-type, *SFXN1*-null and *SFXN1&3* double knockout Jurkat cells stained with tetramethylrhodamine, methyl ester, and perchlorate (TMRM). Indicated cells were treated with 10 µM FCCP. The histograms were normalized and smoothened.
(G) Expression of the mitochondrially translated Cytochrome c oxidase (COX; complex IV) subunit 1 (COX1) is reduced in *SFXN1&3* double knockout cells. Lysates prepared from indicated cells were equalized for total protein amounts and analyzed by immunoblotting for mitochondrially (COX1) and nuclear encoded mitochondrial proteins (COX4, CS, SHMT2). CS – citrate synthase.
(H) Pyridoxal-conjugation of proteins is not decreased in mitochondria of *SFXN1*-null and *SFXN1&3* double knockout cells. Mitochondria were isolated from wild-type, *SFXN1*-null, and *SFXN1&3* double knockout Jurkat cells expressing the HA-mito tag or a control tag. To stably conjugate pyridoxal to its cognate binding proteins samples were reduced using sodium cyano borohydride ($NaBH_3CN$). VDAC1 – voltage-dependent anion channel; CS – Citrate Synthase; Exp – Exposure.
Type or paste caption here. Maintain formatting or style as "SM caption." Create a page break and paste in the figure above the caption.

References

42. W. W. Chen, E. Freinkman, T. Wang, K. Birsoy, D. M. Sabatini, Absolute Quantification of Matrix Metabolites Reveals the Dynamics of Mitochondrial Metabolism. *Cell* **166**, 1324-1337 e1311 (2016).
43. L. Chen, G. S. Ducker, W. Lu, X. Teng, J. D. Rabinowitz, An LC-MS chemical derivatization method for the measurement of five different one-carbon states of cellular tetrahydrofolate. *Anal Bioanal Chem* **409**, 5955-5964 (2017).
44. N. Kanarek *et al.*, Histidine catabolism is a major determinant of methotrexate sensitivity. *Nature* **559**, 632-636 (2018).
45. C. A. Lewis *et al.*, Tracing compartmentalized NADPH metabolism in the cytosol and mitochondria of mammalian cells. *Molecular cell* **55**, 253-263 (2014).
46. J. Schindelin *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012).
47. G. A. Wyant *et al.*, mTORC1 Activator SLC38A9 Is Required to Efflux Essential Amino Acids from Lysosomes and Use Protein as a Nutrient. *Cell* **171**, 642-+ (2017).
48. S. Wang *et al.*, Metabolism. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science* **347**, 188-194 (2015).
49. A. Panov, Z. Orynbayeva, Bioenergetic and Antiapoptotic Properties of Mitochondria from Cultured Human Prostate Cancer Cell Lines PC-3, DU145 and LNCaP. *PlosOne* **8**, (2013).
50. A. Minn, J. Gayet, Kinetic study of glutamate transport in rat brain mitochondria. *Journal of Neurochemistry* **29**, 873-881 (1977).
51. D. K. Bricker *et al.*, A Mitochondrial Pyruvate Carrier Required for Pyruvate Uptake in Yeast, Drosophila, and Humans. *Science* **337**, 96-100 (2012).
52. W. W. Chen *et al.*, Inhibition of ATPIF1 ameliorates severe mitochondrial respiratory chain dysfunction in mammalian cells. *Cell Rep* **7**, 27-34 (2014).
53. R. C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792-1797 (2004).
54. J. Felsenstein, PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* **5**, 164-166 (1989).
55. J. Barretina *et al.*, The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603-607 (2012).
56. M. S. Kim *et al.*, A draft map of the human proteome. *Nature* **509**, 575-581 (2014).