

Supplementary Information for

The m⁶A RNA demethylase FTO is a HIF-independent synthetic lethal partner with the VHL tumor suppressor

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Datasets S1 to S5

SI Materials and Methods

Application of MiSL Algorithm for Identification of SL Partners of VHL-Deficient ccRCC

SL partners of VHL-Loss in ccRCC were identified using the MiSL algorithm. The basic steps of the MiSL algorithm were the same as described in Sinha et al (1). The steps include a Boolean implication-based analysis using mutation and copy number data followed by a ccRCC -specific differential expression analysis. The Boolean implication-based analysis was done using relevant pan-cancer TCGA data and TCGA ccRCC data. For the pan-cancer analysis, the first step is to select relevant tumor types for analysis. We identified tumor types where VHL mutations and/or deletions were frequent. VHL mutation and deletion was determined using TCGA Level 3 data. TCGA Level 3 mutation data directly identifies samples with mutation in VHL. A tumor sample was said to have VHL deletion if there existed a segment in the TCGA Level 3 data that overlapped with the genomic locus of the VHL gene and had a segment mean ≤ -0.3 in the tumor sample but not in the corresponding normal sample. VHL mutations were found to be recurrent in ccRCC whereas VHL deletions were present in multiple TCGA tumor types. In the case of VHL deletions, we further restricted to tumor types where VHL deletions were associated with concordant lowered expression of VHL (as per t-test, p-value < 0.05) to ensure that VHL deletion was not a passenger deletion in the tumor type of interest. The above filtering steps identified 26 tumor types where VHL deletions were recurrent and were thus included in the pan-cancer analysis.

Next, a VHL-Loss feature was defined using TCGA Level 3 mutation, deletion and gene expression data. For a tumor type, a tumor sample was said to have VHL-loss if it had: (1) VHL mutation or (2) VHL deletion combined with low VHL expression (expression in the 10th percentile compared to all samples in that tumor type). The rest of the samples were classified as wild-type, excluding samples that were hard to classify like samples that were did not have mutation or deletion in VHL but had low VHL expression (10th percentile) and VHL-deleted samples with medium VHL expression (between 10-50th percentile) were excluded from the analysis.

Preliminary candidate SL genes of VHL-Loss were identified by extracting HILO Boolean implications (which are mutual exclusion relationships) between VHL-Loss and SL gene deletion and/or HIHI Boolean implications (which are subset relationships) between VHL-Loss and SL gene amplification in pan-cancer data and within ccRCC data. Boolean implications, which are statistical IF-THEN relationships between pairs of variables, were detected using a statistical test consisting of two parts: first, the Fisher's exact test was used to test dependence, then sparseness of a specific quadrant was tested by using a maximum-likelihood estimate of the error rate for the points in the sparse quadrant. An implication was considered significant if the p-value from the Fisher test was less than a cutoff threshold (always less than 0.05) and the error rate was less than 0.14. For each candidate alteration, the Boolean implication analysis was only restricted to tumor types where the candidate alteration was likely to be a non-passenger alteration. A deletion in gene *A* was considered to be a non-passenger in a tumor type if *A* was differentially down-regulated (as per t-test, p-value < 0.05) in samples with deletions in *A* versus the remainder of the samples. Similarly, amplification in gene *A* was considered to be a non-passenger in a tumor type if *A* is differentially up-regulated in samples with amplification of *A*. Finally, the genes identified by pan-cancer analysis were further filtered down to only include genes that are differentially over-expressed in the presence of the VHL mutated/deleted samples versus the wildtype in ccRCC (as per t-test, p-value < 0.05, fold-change > 1.2).

FTO Transcriptomic and Proteomic Analysis

Tumor-vs-normal gene expression and protein data analysis for FTO was done in three different datasets. These include differential expression analysis for TCGA KIRC tumor versus normal, differential analysis of FTO in E-MTAB6692 gene expression data and differential analysis of FTO in CPTAC gene expression and protein data. E-MTAB6692 is a kidney cancer meta-dataset comprising of a total of 347 samples including both primary tumors and tumor-free renal tissues from six independent GEO datasets. This meta-dataset consisted of datasets generated from the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) which were further

preprocessed with RMA normalization, merged, and batch effect-corrected via Combat method. The CPTAC dataset, generated by the Clinical Proteomic Tumor Analysis Consortium, is a clear cell renal cell carcinoma (ccRCC) dataset with comprehensive genomic, epigenomic, transcriptomic, proteomic, and phosphoproteomic characterization of treatment-naive ccRCC and paired normal adjacent tissue samples. Differential analysis of FTO gene or protein expression in tumor versus normal samples was performed using Wilcoxon rank sum test.

Cell Lines and Cell Culture

The human clear cell renal carcinoma cell lines UMRC2-vec and UMRC2-VHL cells were a gift from Dr. Othon Iliopoulos (MGH, (2)). The 786-OM1A cell line was a generous gift from Dr. Joan Massague (MSKCC, (3)). The 786-OM1A-vec and 786-OM1A-VHL cells was generated by introducing control virus (Plasmid #1764, Addgene) and VHL expression virus (Plasmid #19234, Addgene). All cell lines were authenticated from the original source and were used within 6 months of receipt. Additionally, cells were tested upon receipt for viability, cell morphology and the presence of Mycoplasma and viruses (Charles River Laboratories). All cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37°C and 5% CO₂.

Patient Samples

All patients who participated in this study provided written informed consent for collection and research use of their materials and use of these samples was approved by the Stanford University Institutional Review Board (IRB #34175) in accordance with recognized ethical guidelines per the U.S. Common Rule. For more information about the patients, please see dataset S5.

CRISPR Knockdown, shRNA and siRNA

Oligos for FTO sgRNA are as following: 5'- CTCTCGTTCCTCGGCAGTCG -3' (sgRNA#1) and 5'- GAAGCGCACCCCGACTGCCG-3' (sgRNA#2). Plasmid backbone pX458 (CRISP-cas9-GFP) was kindly as a gift from Dr. Mike Bassik (Stanford, (4)). Cells were transiently transfected with corresponding CRISPR plasmid, followed by sorting for GFP positive cells into 96 well plate (1 cell/well). Cells were then grown for 4-6 weeks (passaged from 96-well plate to 24-well plate to

12-well plate to 6 well plate) and eventually tested for FTO knockout by western blot analysis. Oligos for control, FTO and ARNT shRNA were synthesized as previously described (5). The sequence are as follows: Oligos were cloned into shRNA lentiviral plasmid vector (p246, kindly as a gift from Bassik lab). The shRNA sequences are as follows: 5'-TCACCAAGGAGACTGCTATTT-3' (shFTO#2), 5'-CCCATTAGGTGCCCATATTTA-3' (shFTO#3), 5'-TCTCGCATCCTCATTGGTAAT-3' (shFTO#5), 5'-GCAGGTCTGAAGTTCAT-3' (shCtrl for shFTO#2, shFTO#3, and shFTO#5) and 5'-AAATAAACCATCTGACTTCTC-3' (shARNT), 5'-ACAAGATGAAGAGCACCAA-3' (shCtrl for shARNT). Stable cell lines were generated using previously published protocol (6). The ON-TARGET plus smart pool siRNAs for siCON (D-001810-10-20), siFTO(L-004159-01), siHIF1A (L-004018-00) and siHIF2A (L-004814-00) were purchased from Dharmacon. The knockdown was modified from the manufacturer's instructions. Briefly, cells (1000 cells/well for 96-well plate; 50,000 cells/well for 12-well plate; 100,000 cells/well for 6 well plate) were plated and cultured overnight. The cells were transfected with Lipofectamine RNAiMAX (13778, Thermo Fisher Scientific) mixed with siRNA(s). Forty-eight hours after transfection, transfected cells were trypsinized and washed and plated into the assays described below.

Cell Viability Assay

For cell viability, cells were seeded in 96-well plates at 1000 cells/well and cultured overnight. Cells were transfected with indicated siRNA(s) according to manufacturer's instruction. Viable cells were quantified with Cell titer Blue assay (Promega G8080) according to manufacturer's instructions at indicated timepoints. All experiments were performed in triplicate.

Cell Growth Assay (Crystal Violet)

Cells were seeded in 96-well plates at 1000 cells/well and cultured overnight. The cells were transfected with indicated siRNA(s) according to manufacturer's instruction. 72 hours post the transfection, cells were fixed with 5% Neutral formalin and stained with 1% crystal violet. All experiments were performed in triplicate.

2-D Colony Formation Assay

Cells (500) were seeded in 6-well plate and incubated for 14 days after which they were fixed and stained with 1% crystal violet prepared in 100% ethanol. For the colony assay with cells transfected with siRNA(s), 500,000 parental cells were seeded into a 12-well plate overnight. Cells were transfected with indicated siRNA(s) according to manufacturer's instruction. Forty-eight hours post transfection, cell were trypsinized and 500 cells were plated to 6-well plates and incubated for 14 days. Colonies were fixed and stained with 1% crystal violet prepared in 100% ethanol.

For the dimethyl α -ketoglutarate rescue experiments, 500 cells were transfected with indicated siRNA were plated in 6 well plates. Cells were cultured with DMEM supplemented with 3.5 mM dimethyl α -ketoglutarate (Sigma, 349631) for 14 days. For the meclofenamic acid experiments, 500 cells were plated into 6 well plates. After 24 hours, cells were treated with vehicle, 20 μ M or 100 μ M meclofenamic acid (Cayman Chemical, 70550) for 14 days (after 7 days new media with drug was replaced). All experiments were performed in triplicate.

3-D Soft Agar Assay

The soft agar assay was modified from previously reported (7). Briefly, 1.4 % noble agar (Sigma, St. Louis, MO) in PBS was melted in the microwave and cooled to 40-42°C in a water bath. A 0.7% agar/medium (1 mL/ 6 well) base layer was added to each well for anchorage independent growth. The plates were left at room temperature for 15 minutes to allow the agar to solidify. 1 ml of 0.35% agar/medium containing 5,000 cell was add on top of the solidified agar. Cells were cultured at 37°C in humidified incubator for 14 - 21 days. For the meclofenamic acid experiments, cells were treated 24 hours after plating with vehicle, 20 μ M or 100 μ M meclofenamic acid (Cayman Chemical, 70550) for 14 days (after 7 days new media with drug was replaced). All experiments were performed in triplicate. Colonies with sizes $\geq 50 \mu$ m were counted under a light microscope.

Apoptosis Assay

For assay of apoptotic cells, 0.1 million cells were seeded into 6-well plates and cultured overnight. Cells were transfected with indicated siRNA(s) according to manufacturer's instruction. Seventy-two hours post-transfection, cells were harvested and stained with Apoptosis Detection Kit (Cat#: 640930, Biolegend) according to the manufacturer's instructions. Apoptotic cells were analyzed with LSR FORTRESSA (BD Biosciences) and Flowjo (Becton, Dickinson & Company). All experiments were performed in triplicate.

Immunohistochemical Staining

For Immunohistochemical staining, tumor specimens were fixed in 10% (v/v) neutral buffered formalin and embedded in paraffin using routine methods. Tumor sections were subsequently deparaffinized with xylene, rehydrated in ethanol solutions and subjected to antigen retrieval using 10 mM citric acid buffer (pH 6.0) and microwaved for 10 min. Slides were probed with primary antibodies overnight at 4°C, followed by secondary detection using biotinylated anti-rabbit antibody (Vector Laboratories) and streptavidin-HRP conjugated antibody (EMD Millipore), both for 30 min at 37°C. Negative controls for all samples were tissue sections treated with secondary antibodies alone. Proteins were visualized with DAB Chromogen System (DAKO), counterstained with Hematoxylin (VWR) and mounted on slides with Fluoromont-G (Southern Biotech). The primary antibodies used for IHC staining were KI67 (1:100, ab16667, Abcam) and FTO (1:200, D2V11, Cell signaling). Slides were scanned with NanoZoomer (Hamamatsu). Slides were analyzed with equipped NDP view 2 software (Hamamatsu). For FTO IHC scoring, ten 200x magnified fields were scored semi-quantitatively by assessing the percentage of cells with positive staining and the overall staining intensity of these cells.

The kidney cancer tissue microarray was purchased from US Biomax (KD601) that contains 30 ccRCC cases with normal adjacent tissue. For scoring, the percentage of cells with positive FTO staining, the % of cells with the core were evaluated: no positive staining = 0, 1-25% = 1, 25-50% = 2, 50-100% = 3. The staining intensity of cells with positive FTO staining were evaluated: no

staining = 0, weak staining = 1, moderate staining = 2, strong intense staining = 3. The two scores were multiplied to result in an overall score ranging from 0-9. For KI67 quantification, positive cell per area (mm²) were counted.

TUNEL Staining

ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100, Sigma-Aldrich) was used for TUNEL staining according to manufacturer's instruction. For TUNEL quantification, positive cell per area (mm²) were counted.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA synthesis and qRT-PCR were set-up using BioRad iScript cDNA synthesis kit (Cat: 1708891) and iTaq SYBR MasterMix (Cat: 1725120). Real time quantitative PCR was performed (Applied Biosystems 7500) using the cycling conditions: as per manufacturer's instructions. All qRT-PCRs were set-up in triplicates using three biological replicates for each sample. The PCR primers used for qRT-PCR are as follows: FTO-forward (5'-TCACCAAGGAGACTGCTATTT-3'), FTO-reverse (5'-TCACCAAGGAGACTGCTATTT-3'), ARNT-forward (5'-CTGCCAACCCCGAAATGACAT-3'), ARNT-reverse (5'-CGCCGCTTAATAGCCCTCTG-3'), SLC1A5-forward (5'-CCGCCTTGGCAAGTACATTCT-3'), SLC1A5-reverse (5'-GGCAGGATGAAACGGCTGA-3'), housekeeping gene 18S-Forward: 5'-GCCCGAAGCGTTTACTTTGA-3'; 18S-Reverse: 5'-TCCATTATTCCTAGCTGCGGTATC-3' TBP-forward (5'-CCACTCACAGACTCTCACAAC-3'), and TBP-reverse (5'-CTGCGGTACAATCCCAGAACT-3'),

Western Blot and Antibodies

Whole cell lysates were isolated in RIPA buffer, followed by quantification of protein concentration by Pierce BCA protein assay Kit (Thermo Scientific). Protein samples were gel electrophoresed on 4-12% Bis-Tris gels (Thermo Scientific), followed by transfer to polyvinylidene difluoride membrane. Post blocking in 5% milk in PBST buffer, blots were incubated overnight in primary

antibodies at 4⁰C. Blots were developed using chemiluminescent kit (34096, Thermo Scientific) and visualized with ChemiDoc XRS+ imaging system equipped with Image Lab Software (Bio-Rad Laboratories). Antibodies used were: anti-FTO (1:1000, D2V1I, Cell signaling), anti-SLC1A5 (1:1000, V501, Cell signaling) anti-HIF1A (1:1000, NB100-449, Novus Biologicals), anti-HIF2A (1:1000, NB100-122, Novus Biologicals), and anti-Beta Actin (1:5000, 8H10D10, Cell signaling).

m⁶A ELISA

For RNA-seq assay, total RNA samples were extracted from cells with miRNeasy mini kit (217004, Qiagen). RNA concentration was measured by NanoDrop 2000 (Thermo Scientific). Global m⁶A was measured with m⁶A RNA Methylation Quantification Kit (Cat#: P-9005-96, EpiGentek) according to manufacture instruction. Briefly, 200 ng total RNA per sample was use for m⁶A measurement.

RNA-seq and m⁶A-seq Assays

For RNA-seq assay, total RNA was extracted from cells with miRNeasy mini kit (217004, Qiagen) Total RNA was delivered to Novogene for next generation sequencing. Libraries were constructed with PrepX mRNA library kit (WaferGen) and sequenced to 50 bp using TruSeq SBS kit on Illumina Hiseq system. The raw reads were mapped using STAR and transcript quantification was done using HTSeq. FPKM was used for transcript quantification. Differential analysis was performed using DESeq2. DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting p-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate(FDR). Genes with an adjusted p-value <0.05 found by DESeq2 were assigned as differentially expressed.

m⁶A-seq assays were performed according to the published protocol with minor modifications (8). Poly(A)+ RNA was enriched from total RNA with RNA fragmentation reagents (Ambion). Specific m⁶A antibody (202003, Synaptic Systems) was applied for immunoprecipitation (IP). The reads from the input and m⁶A IP sequencing libraries were aligned

to hg19/hg38 reference genome using STAR exomePeak with default setting was used to m⁶A peak calling (9). BedGraph files were generated using deeptool's bamCompare that compared the respective IP and input files after normalizing for sequencing depth. The bedGraph files were visualized in IGV.

Gene Set Enrichment Analysis (GSEA) was used to analyze the enriched signaling pathways (10). For GSEAPreranked, the genes were ranked using p-value based scores from differential analysis multiplied by +1 (hypermethylation or upregulation) and -1 (hypomethylation or downregulation). Gene set enrichment analysis (GSEA) was performed using GSEAPreranked from the GenePattern web-site. For GSEAPreranked, the number of permutations was set to 1000. The raw RNA sequencing data and m⁶A data has been uploaded to GEO (GSE139123).

Gene-Specific m⁶A qPCR

qRT-PCR was applied to assess the relative abundance of the selected mRNA in m⁶A antibody IP samples and input samples. cDNA synthesis and qRT-PCR were set-up using BioRad iScript cDNA synthesis kit (Cat: 1708891) and iTaq SYBR MasterMix (Cat: 1725120) according to manufacturer's instruction. The enrichment of m⁶A mRNA was calculated by the IP mRNA divided by the input mRNA (input %). The primer used for SLC1A5 validation are as follows: primer 1-forward (5'-GGGGCAGGACTCCTCCAAAA -3'), primer 1-reverse (5'-GGCTCTGTGCTTCTCGACTCC-3'), primer 2-forward (5'- TGAAGAGTGAGCTGCCCCCTG-3'), primer 2-reverse (5'- CCTTCCTCAGTGGGGACTGG-3').

Glutamine Consumption Assay by Liquid Chromatography-Mass Spectrometry

For glutamine consumption assay, 0.15 million of tumor cells were plated in 60 mm dish and cultured overnight. Cell were transfected with indicated siRNA. Post transfection (48 hours), the cells were washed with PBS. Media was replaced with glutamine free DMEM supplemented with 2mM of glutamine (Sigma, G7513). The media was collected at indicated timepoints and stored at -80°C for glutamine measurement. For mass spectrum analysis of glutamine in the media, 2 μL medium was mixed with 600 μL Acetonitrile:H₂O (80:20). Extract was vortexed and centrifuged

at 15,000 rpm for 15 min. 200 μ L supernatant was transferred to LC-MS vials. Quantitative LC-MS analysis was performed using an Agilent 1290 UHPLC system equipped with an Agilent 6545 Q-TOF mass spectrometer (Santa Clara, CA, US). A hydrophilic interaction chromatography method (HILIC) with a ZIC-pHILIC column (150 x 2.1 mm i.d., 5 μ m; Merck) was used for compound separation at 35 °C with a flow rate of 0.3ml/min. The mobile phase A consisted of 20 mM ammonium bicarbonate in water and mobile phase B was acetonitrile. The gradient elution was 0 – 1 min, 80 % B; 1 – 3 min, 80 % B \rightarrow 40 % B; 3 – 4 min, 40 % B; 4 – 4.5 min, 40% \rightarrow 80%B; 4.5 – 6 min, 80%B. The overall runtime was 6 min and the injection volume was 2 μ L. Agilent Q-TOF was operated in negative mode and the relevant parameters were as listed: ion spray voltage, 3500 V; nozzle voltage, 1000 V; fragmentor voltage, 125 V; drying gas flow, 11 L/min; capillary temperature, 300 °C, drying gas temperature, 320 °C; and nebulizer pressure, 40 psi. A full scan range was set at 50 to 1200 (m/z). The reference mass were 119.0363 and 980.0164. The acquisition rate was 2 spectra/s. Data processing was performed with Agilent Profinder B.08.00 (Agilent technologies). Glutamine peak was extracted with retention time (RT) = 3.9min and target mass = 146.0691. The mass tolerance was set to +/-15ppm and RT tolerance was +/-0.2 min.

Animal Studies

All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Stanford University in accordance with institutional and National Institutes of Health guidelines. To establish orthotopic ccRCC xenografts, Rag2^{-/-}IL2rg^{-/-} double knockout mice aged from 6 and 12 weeks were injected with a solidified collagen plug (BD Bioscience) (100 μ L containing 1×10^6 ccRCC cells) under the renal capsule as previously described (11). Briefly, cells were suspended in ice-cold neutralized collagen and 100 μ L of the cell/ collagen slurry was dropped into one well of a six-well cell-culture plate. After the plate was incubated at 37°C for 30 min to allow the collagen plug to solidify, the plug was inserted under the renal capsule of the

mouse using fine tip forceps. Sixty days post the implantation, tumors were harvested and weighed. Total tumor weight was calculated by using the weight of tumor bearing kidney minus the tumor free kidney.

Supplementary Figure 1

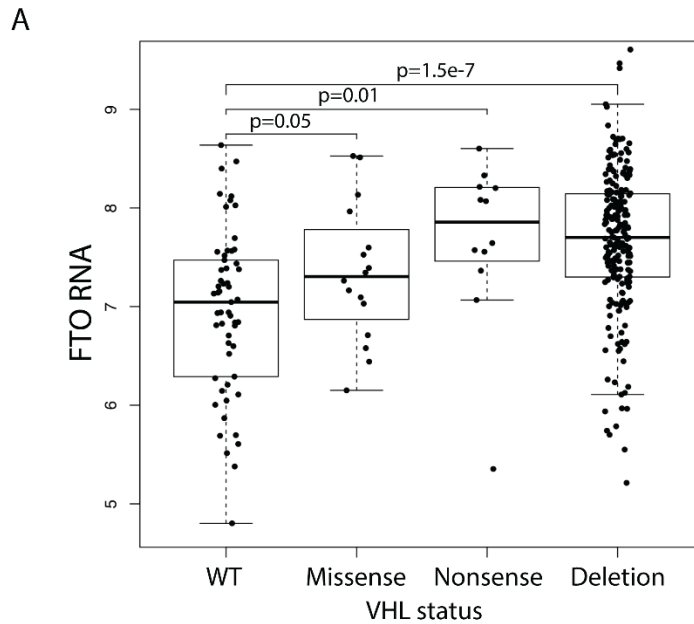


Fig. S1. FTO expression is increased in ccRCC tumors with VHL mutations or deletions compared to VHL wild type tumors.

A. FTO mRNA expression in samples from TCGA data. Samples are grouped based on VHL status (VHL wild type, WT; VHL missense mutation, Missense; VHL nonsense mutation, Nonsense; VHL deletions, Deletion).

Supplementary Figure 2

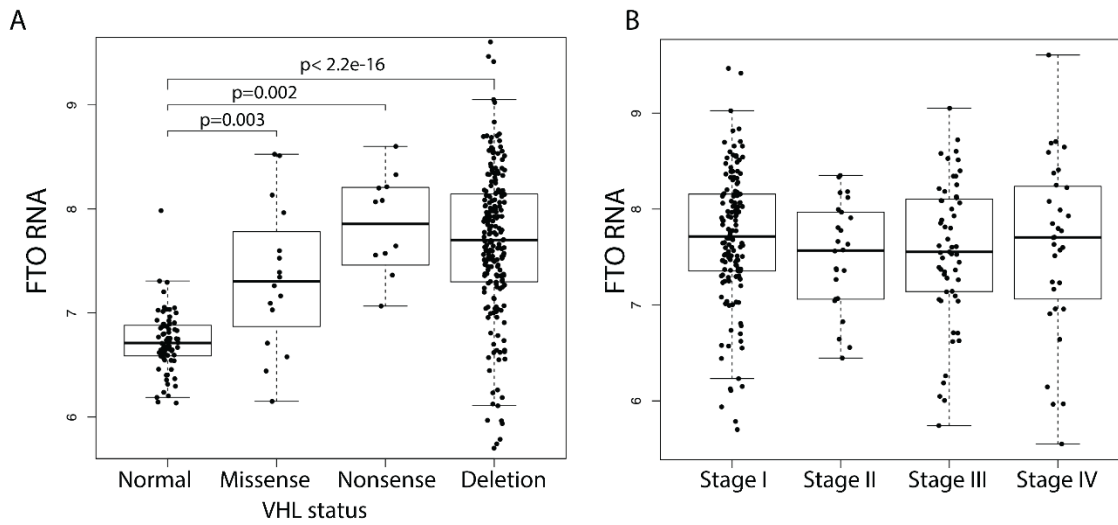


Fig. S2. FTO mRNA expression in normal tissue and ccRCC tumors.

A. FTO mRNA expression in samples from TCGA data comparing FTO expression in normal tissue to ccRCC tumor tissue grouped by VHL mutation or deletion status. **B.** FTO mRNA expression in ccRCC tumors (stage I-IV) from TCGA, $p = 0.28$.

Supplementary Figure 3

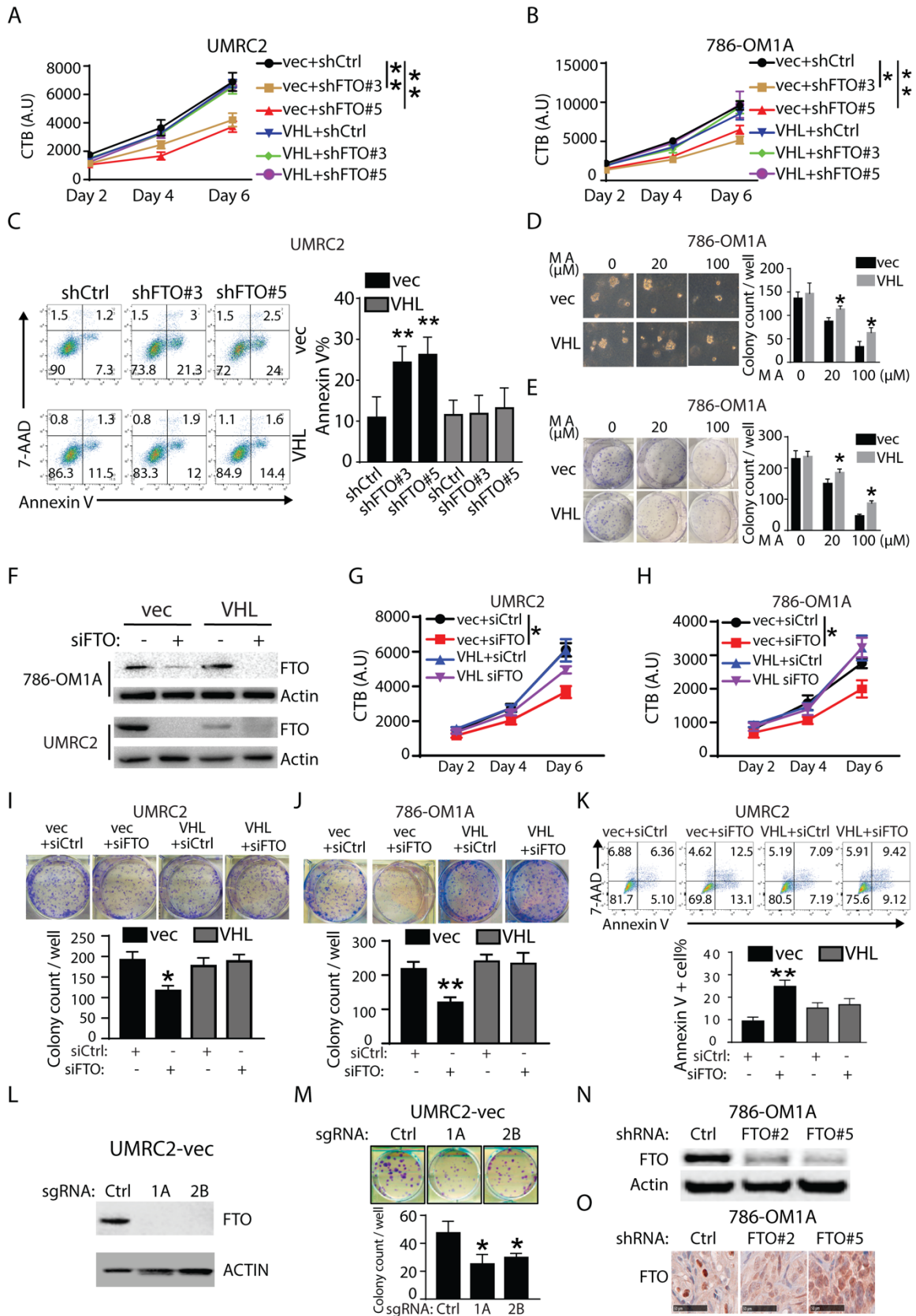


Fig. S3. FTO inhibition reduces VHL-deficient ccRCC growth and survival.

A-B. Cell viability of UMRC2-vec or UMRC2-VHL (**A**) and 786-OM1A-vec or 786-OM1A-VHL (**B**) cells transfected with shControl, shFTO#3 and shFTO#5. Data represent the average \pm SD. * $p < 0.05$ and ** $p < 0.01$ for indicated group versus shCtrl. **C.** Flow cytometry analysis of annexin V in UMRC2-vec, UMRC2-VHL, 786-OM1A-vec or 786-OM1A-VHL cells transfected with shControl, shFTO#3 and shFTO#5. **D-E.** Picture (left) and quantification (right) of 3-D soft agar colonies (**D**) and 2-D colonies (**E**) in 786-OM1A-vec or 786-OM1A-VHL cells treated with vehicle or meclufenamic acid (MA). Data represent the average \pm SD. * $p < 0.05$ and ** $p < 0.01$ for indicated group compared to 786-OM1A-vec under each treatment. **F.** Western blot analysis of FTO expression in UMRC2-vec, UMRC2-VHL, 786-OM1A-vec or 786-OM1A-VHL cells transfected with siControl, and siFTO. **G-H.** Cell viability of UMRC2-vec, UMRC2-VHL (**G**), 786-OM1A-vec or 786-OM1A-VHL (**H**) cells transfected with siControl, and siFTO. Data represent the average \pm SD. * $p < 0.05$ for indicated group versus siCtrl. **I-J.** Picture (top) and quantification (bottom) of 2-D colonies in UMRC2-vec, UMRC2-VHL (**I**), 786-OM1A-vec or 786-OM1A-VHL (**J**) cells transfected with siControl, and siFTO. Data represent the average \pm SD. * $p < 0.05$ for indicated group versus siCtrl. **K.** Annexin V analysis of UMRC2-vec and UMRC2-VHL cells transfected with shControl, shFTO#3 and shFTO#5. Data represent the average \pm SD. * $p < 0.05$ and ** $p < 0.01$ for indicated group versus siCtrl. **L.** Western blot analysis of FTO expression in UMRC2-vec cells transfected with CRISPR-Cas9 control sgRNA and two CRISPR-Cas9 sgRNAs targeting FTO. **M.** Macroscopic picture (top) and quantification of colonies (bottom) of UMRC2-vec cells transfected with CRISPR-Cas9 control sgRNA and two CRISPR-Cas9 sgRNAs targeting FTO. **N.** Western blot analysis of FTO expression in 786-OM1A cells transfected with shCtrl, shFTO#2 and shFTO#5. **O.** Immunohistochemical analysis of FTO in tumors taken from mice with orthotopic subrenal capsule injection of shCtrl or shFTO#2 786-OM1A cells.

Supplementary Figure 4

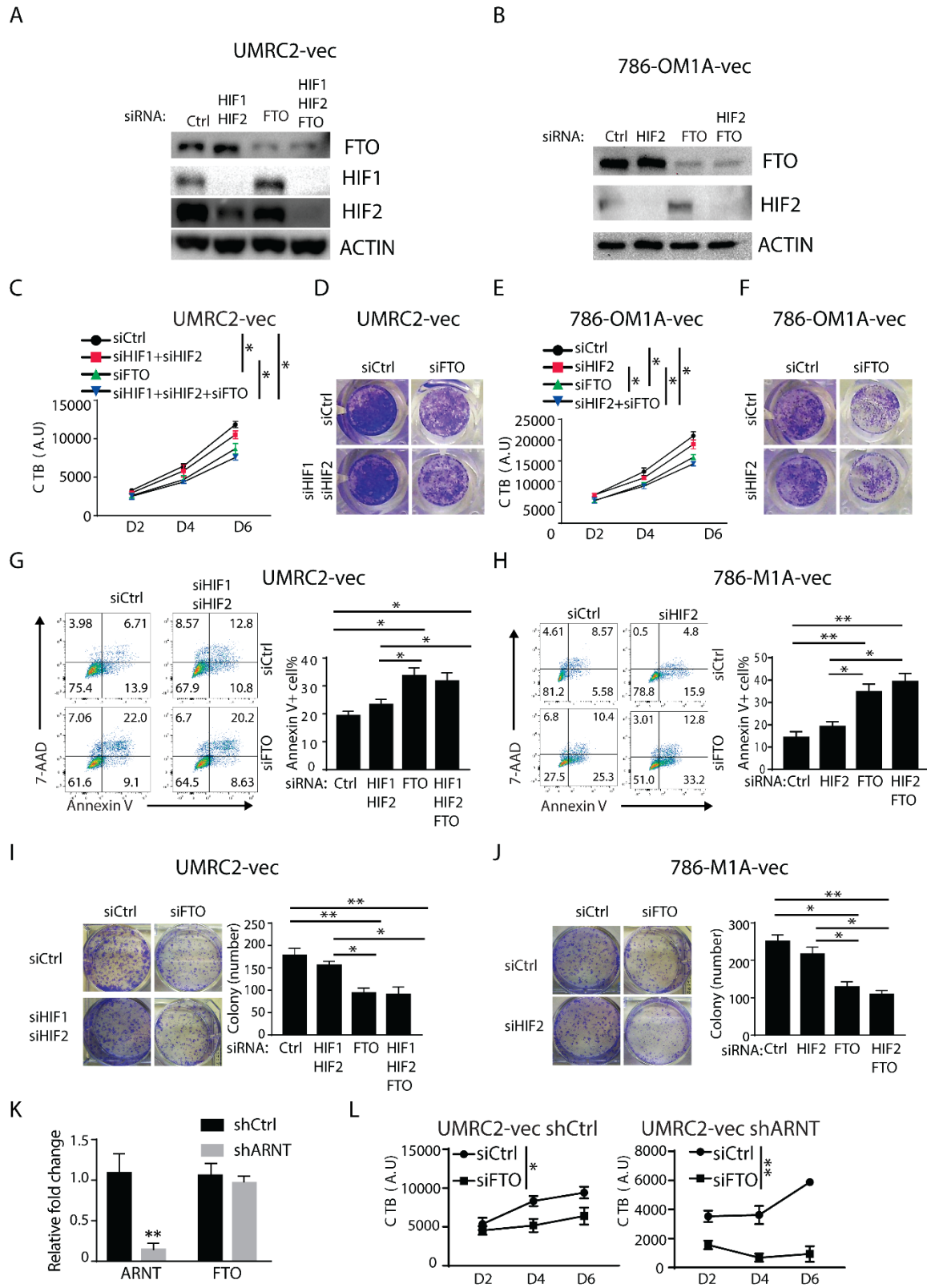


Fig. S4. FTO regulates VHL-deficient ccRCC cell growth and survival in HIF-independent manner.

A-B. Western blot analysis of FTO, HIF1A and HIF2A expression in UMRC2-vec (**A**) and 786-OM1A-vec (**B**) cells transfected with indicated siRNA. **C-J.** Cell viability (**C** and **E**), crystal violet staining (**D** and **F**), annexin V analysis (**G** and **H**) and 2-D colonies (**I** and **J**) of UMRC2-vec (**C**, **D**, **G** and **I**) and 786-OM1A-vec (**E**, **F**, **H** and **J**) cells transfected with indicated siRNA. Data represent the average \pm SD. * $p < 0.05$ and ** $p < 0.01$. **K.** Real time PCR analysis of shCtrl and shARNT UMRC2-vec cells transfected with siCtrl or siFTO. Data were normalized to TBP expression. Data represent the average \pm SD. * $p < 0.05$ and ** $p < 0.01$. **L.** Viability of shCtrl and shARNT UMRC2-vec cells transfected with siCtrl or siFTO at days 2, 4, and 6 following siRNA treatment. Data represent the average \pm SD. * $p < 0.05$ and ** $p < 0.01$.

Supplementary Figure 5

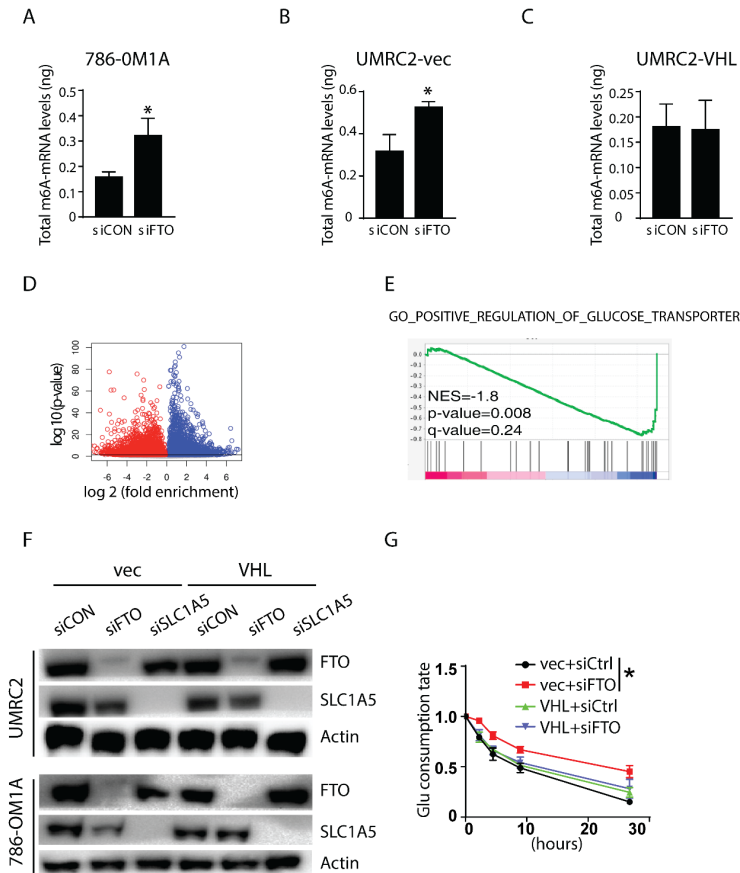


Fig. S5. FTO regulates SLC1A5 expression and glutamine uptake in VHL deficient ccRCC cells.

A-C. Global m⁶A levels in 786-OM1A (**A**), UMRC2-vec (**B**) and UMRC2-VHL (**C**) cells transfected with siControl (siCtrl) or siFTO. Data represent the average \pm SD. * $p < 0.05$. **D.** Volcano plot of differential m⁶A changes between shFTO and shControl UMRC2 cells. The x axis refers to the log₂ fold change in m⁶A levels and y axis refers to the log₁₀(p-value). **E.** Gene set enrichment analysis (GSEA) for the glucose transporter gene set from MSigDB. Genes were ranked based on the degree of differential expression between shFTO and shControl cells. A negative enrichment score indicates that genes belonging to these gene sets were significantly downregulated in shFTO compared to shControl cells. **F.** Western blot analysis of FTO and SLC1A5 expression in UMRC2-vec, UMRC2-VHL, 786-OM1A-vec and 786-OM1A-VHL cells transfected with indicated siRNAs. **G.** Glutamine consumption rate of UMRC2-vec and UMRC2-VHL cells transfected with siCtrl and siFTO at 0,2,4,8 and 24 hours after siRNA treatment. Data represent the average \pm SD. * $p < 0.05$ for indicated group versus siCtrl.

Captions for datasets S1 to S5

Dataset S1: Detailed MiSL pipeline scores for SL candidates from the MiSL analysis.

Dataset S2: List of differentially expressed genes based on RNAseq analysis of shFTO and shControl UMRC2 cells.

Dataset S3: List of differentially methylated genes based on m⁶A seq analysis of shFTO and shControl UMRC2 cells.

Dataset S4: Integrative Analysis of m⁶A seq and RNAseq data.

Dataset S5: ccRCC patient information.

SI References

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