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Loss of SETD2 induces a metabolic switch in renal cell carcinoma cell lines toward enhanced oxidative phosphorylation

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Abstract

SETD2, a histone H3 lysine trimethyltransferase, is frequently inactivated and associated with recurrence of clear cell renal cell carcinoma (ccRCC). However, the impact of SETD2 loss on metabolic alterations in ccRCC is still unclear. In this study, SETD2 null isogenic 38E/38F clones derived from 786-O cells were generated by zinc finger nucleases, and subsequent metabolic, genomic, and cellular phenotypic changes were analyzed by targeted metabolomics, RNA-sequencing, and biological methods, respectively. Our results showed that, compared to parental 786-O cells, 38E/38F cells had elevated levels of MTT/Alamar blue levels, ATP, glycolytic/mitochondrial respiratory capacity, citrate synthase (CS) activity, and TCA metabolites such as aspartate, malate, succinate, fumarate, and α -ketoglutarate. The 38E/38F cells also utilized alternative sources beyond pyruvate to generate acetyl-CoA for the TCA cycle. Moreover, 38E/38F cells showed disturbed gene networks mainly related to mitochondrial metabolism and the oxidation of fatty acids and glucose, which was associated with increased PGC1 α , mitochondrial mass, and cellular size/complexity. Our results indicate that SETD2 deficiency

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

induces a metabolic switch toward enhanced oxidative phosphorylation in ccRCC, which can be related to PGC1 α -mediated metabolic networks. Therefore, this current study lays the foundation for the further development of a global metabolic analysis of cancer cells in individual patients, which ultimately will have significant potential for the discovery of novel therapeutics and precision medicine in SETD2 inactivated ccRCC.

Graphical Abstract

Keywords

clear cell renal cell carcinoma (ccRCC); SETD2; mitochondria; oxidative phosphorylation; PGC1 α

Introduction

Kidney and renal pelvis cancer is the 10th most common cancer in the United States, with an estimated 63,340 new cases and 14,970 associated deaths in 2018.¹ Renal cell carcinoma (RCC) is the predominant type, accounting for up to 85% of all kidney cancers. Of those RCC patients, 70–75% are sub-typed as clear cell RCC (ccRCC).² Although surgery remains the standard of care for patients with early-stage ccRCC (stages I–III), roughly 30% of these patients will progress to distant metastases following resection of localized tumors. Despite recent advances in systemic therapy, median survival drops to ~2 years after development of metastatic disease.³

A common molecular feature of ccRCC is the inactivation of the von Hippel-Lindau (VHL) tumor suppressor protein through a variety of mechanisms.² VHL, an E3 ubiquitin ligase, regulates the cellular response to hypoxia through its interaction with hypoxia inducible factor-1 (HIF-1). Under normoxic conditions, VHL mediates ubiquitylation and proteasomal degradation of HIF-1 via interaction with the oxygen-dependent degradation domain core of HIF-1.⁴ The inactivation of VHL in ccRCC cell leads to elevated activity of HIF-1 and a number of downstream genes essential for tumor metabolism and angiogenesis, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and GLUT1.^{2, 4} However, the loss of VHL has been shown to be insufficient for tumorigenesis in mice,⁵ suggesting that other factors are also involved in ccRCC onset and progression.

In addition to functional alterations of VHL, several genes regulating chromatin remodeling in close genomic proximity to VHL are frequently mutated in RCC, including those of the SWI/SNF chromatin remodeling complex (PBRM1 and BAP-1) and histone lysine 36 trimethylase SETD2.^{6, 7} SETD2, the histone methyltransferase responsible for the trimethylation of H3K36, is inactivated in approximately 10–32% of ccRCC cases.^{6, 8} SETD2 promotes transcriptional elongation and plays important roles in double-stranded break repair, DNA methylation, and RNA splicing.^{9, 10} In ccRCC, the homozygous loss of SETD2 activity leads to a global decline of H3K36me3 histone markers and is associated with reduced survival and increased risk of recurrence.¹⁰ Moreover, metastatic ccRCC exhibits markedly reduced H3K36me3 compared to matched primary ccRCCs.¹¹ These reports strongly suggest that SETD2 mutations drive the ccRCC progression, but the specific mechanism remains unclear.

Increasingly, kidney cancer is being recognized as a metabolic disease, and many mutated genes in RCC, such as VHL, fumarate hydratase (FH) and succinate dehydrogenase (SDH), are involved in cellular respiration and energy metabolism.¹² HIF-1-induced genetic reprogramming promotes a classic Warburg phenotype in RCC through VHL or mechanisms dependent on metabolic enzymes.^{12–14} For instance, loss of VHL in RCC leads to a HIF-1-dependent reprogramming of energy metabolism that includes elevated glucose uptake, glycolysis, and lactate production accompanied by a reciprocal decrease in respiration under aerobic conditions.¹³ Inactivation of FH, a tricarboxylic acid (TCA) cycle enzyme, in RCC results in glucose-mediated generation of cellular reactive oxygen species (ROS) and ROS-dependent HIF-1 stabilization, which consequently enhances aerobic glycolysis and reduces reliance on mitochondrial respiration in RCC.¹⁴ However, the impact of SETD2 inactivation on the metabolic phenotype of RCC is rarely reported and remains unknown.

In this study, we explored metabolic alterations in SETD2 deficient cell lines. Two independent SETD2 null isogenic clones were generated from 786-O cells by zinc finger nucleases, and ensuing changes in metabolism, genes and cellular phenotypes were analyzed using targeted metabolomics, RNA-seq and molecular biological methods, respectively.

Methods and Materials

Cell culture

The 786-O ccRCC cell line (ATCC, Manassas, VA, USA) was cultured in RPMI 1640 medium (Corning, Corning, NY, USA) supplemented with 10% FBS (HyClone, South Logan, UT, USA), 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA), and 2 mM L-glutamine (Gibco, Grand Island, NY, USA). SETD2 null isogenic cell lines including 38E and 38F were generated by the zinc finger nuclease method as previously described,¹⁰ and were cultured under identical conditions to those of 786-O cells.

Cell proliferation assay

The proliferation rates of each cell were determined by MTT (Sigma, St. Louis, MO, USA) and Alamar Blue (Invitrogen, Carlsbad, CA, USA). For the MTT test, 100 μ L of MTT (1 mg/ml) solution was added into 96-well plates and incubated at 37 °C for 2 h. After

removing media, 100 μ L of isopropanol/1N HCl (v:v = 24:1) was added into each well to dissolve formazan crystals, and the absorbance at 570 nm was measured by a microplate reader (BioTek Epoch, Winooski, VT, USA). For the Alamar Blue assay, 100 μ L of Alamar Blue (10% in medium) was added into 96-well plates and incubated at 37 °C for 2 h. Then, the absorbance of Alamar Blue solution was measured by a microplate reader (BioTek Epoch) at 600 nm.

Measurement of cellular ATP

Cellular ATP levels were measured using the CellTiter-Glo ATP assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, 25 μ L of CellTiter-Glo reagent was added into each well of a 384-well plate. After mixing contents for 2 min on a shaker to induce cell lysis, the plate was then incubated at room temperature for 10 min. The luminescence was measured by a microplate luminometer (BioTek, VT, USA).

Seahorse assay

Mitochondrial and glycolytic rates were measured by a Seahorse XF96 extracellular flux analyzer (Seahorse Biosciences, Billerica, MA, USA) with Mitochondrial Stress Test (MST) and Glycolysis Stress Test (GST) assay kits. Cells (1.8×10^4 per well) were plated in quadruplicate, using XF96 extracellular flux assay plates in 200 μ L XF base medium equilibrated to a pH of 7.4. After adherence for 2 hours at 37 °C, media were changed to GST or MST buffer. For MST, compounds were added in the following order: oligomycin (1 μ M), FCCP (1 μ M), and rotenone/antimycin A (0.5 μ M). For GST, compounds were added in the following order: glucose (10 mM), oligomycin (1 μ M), and 2-DG (50 mM). Values for each measurement were averaged across quadruplicate wells and displayed as oxygen consumption rate (OCR, pmol O₂/min) for MST and extracellular acidification rate (ECAR, mpH/min) for GST.

Metabolic enzymatic activity assay

The activity of citrate synthase (CS) was measured by commercial kits (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. In brief, the absorbance of cell samples was recorded at 412 nm and then normalized to cell mass following the addition of acetyl-coA and oxaloacetate reagents for 10 min.

Targeted metabolomics analysis with GC-MS

Intracellular metabolites were extracted by methanol/H₂O (v:v = 80:20) and analyzed using GC-MS. Briefly, extracted samples were incubated with 30 μ L O-methylhydroxylamine hydrochloride solution in pyridine (Sigma-Aldrich, St. Louis, MO, USA) at 60 °C for 90 min. Next, 70 μ L of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA, Sigma, St. Louis, MO, USA) was added and placed at 60 °C for 30 min. GC-MS spectral acquisition was performed on an Agilent 7890 GC-5975C MSD system (Agilent Technologies, Inc., Santa Clara, CA, USA). For analysis, 1 μ L derivatized sample was injected using the splitless mode, and helium (purity > 99.999%) was used as the carrier gas. The flow rate of carrier gas was 0.5 mL/min. The temperature of injection port and transfer line was 250°C and 290°C, respectively. Chromatographic separation was performed on a

Zorbax DB5-MS + 10 m Duragard Capillary Column (30 m x 250 μ m x 0.25 μ m). The column temperature was maintained at 60 °C for 1 min, then increased at a rate of 10 °C/min to 325 °C and held at this temperature for 10 min. The solvent delay was 9.69 min. Mass spectral signals were recorded in full scan mode using electron ionization (EI, 70 eV), ion source temperature was 230°C, EM voltage was 1225 V, and mass range was 50–600 m/z with a scan frequency of 2.7 scans/second.

The external calibration curves were used for quantification, and different concentrations of external standard solutions (encompassing 3–5 orders of magnitude) were prepared and run together with study samples. While the GC-MS data were collected in the scanning mode, the raw GC-MS data were extracted in a targeted way to monitor 11 compounds of our strong interest (mainly in the TCA cycle), using Agilent MassHunter software (Version B. 09.00). The parameters used to extract the data were all developed and optimized using pure standards, and the targeted m/z values were listed in Table S1. The metabolite levels were then further normalized to the protein concentration of each sample.

Measurement of oxygen consumption rates

The oxygen consumption rates of cells were detected by an oxygen electrode assay with 10 mM glucose. The basal oxygen consumption rate, the non-glycolytic rate after addition of sodium fluoride (NaF, pyruvate dehydrogenase phosphatase inhibitor, Sigma), and the ability of cells to oxidize lactate to form pyruvate for mitochondrial respiration were determined.

RNA-Seq analysis

The RNA-Seq analysis was performed as previously described.¹⁰ Briefly, the genomic RNA was extracted from cells using the RNease Mini Kit (Qiagen, Hilden, Germany), and residual DNA was removed by DNase I. DNA libraries were prepared with Illumina's TruSeq RNA sample preparation V2 kit, and final amplification was performed using TruSeq bar-coded primers. Amplified DNAs (DNA libraries) were sequenced on the Illumina Genome Analyzer II (Illumina, San Diego, CA, USA). We performed analysis of RNA sequencing data sets using cufflinks⁴¹ and ChIPRNAseq-PRO⁴² software packages to generate prediction models of transcript splice isoforms. RNA abundance was predicted by calculating the fragments per kilobase of exon per million fragments mapped (FPKM) of each transcript prediction. Changes in FPKM were converted to log₂ values and were visualized using a heatmap. The gene ontology (GO) analysis was performed using STRING software.

Western Blotting

Cells were harvested and lysed in ice-cold radio immunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail and phosphatase inhibitors (Sigma, MO, USA). Protein concentrations were determined by BCA kit (Bio-Rad, Hercules, CA, USA). Briefly, 10 μ g of protein were loaded on 12% tris-glycine polyacrylamide gel prior to being transferred to a PVDF membrane on ice. After transfer, membranes were blocked with 5% milk in tris-buffered saline with 0.1% Tween-20 (TBST) for 30 min. Membranes were then incubated with primary antibodies: anti-H3K36me3 (1:1000, Active Motif, Carlsbad, CA), anti-CS (1:1000, Cell Signaling, Danvers, MS), and anti-PGC1 α (1:1000, Cell Signaling) at

4 °C overnight. After washing with TBST, membranes were incubated with goat anti-rabbit/mouse HRP (1:10,000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. After washing with TBST, membranes were observed by chemiluminescent kit (Thermo Scientific, Carlsbad, CA, USA).

MitoTracker Red staining

Mitochondria were labeled with MitoTracker Red (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were stained with 100 nM MitoTracker Red at 37 °C for 30 min. After washing with PBS, stained cells were observed by confocal microscopy (LSM 510, Zeiss, Germany).

Statistical analysis

All data, expressed as mean $\pm$ SEM, were analyzed by one-way ANOVA with Tukey's post hoc analysis to control for multiple comparisons using SPSS software (Version 22.0, IBM Corporation, USA); the significance threshold (α) was set to 0.05. Principal component analysis (PCA) of metabolites data was performed using SIMCA-P 11.5 software (Umetrics, Umea, Sweden) as previously described.¹⁵

Results

Loss of SETD2 increases overall metabolic activity of ccRCC cells

We first performed proliferation assays to measure the growth rates of SETD2 deficient cells. MTT luminescence was significantly higher in 38E and 38F cells at 3 hours after plating (Figure 1A, S1), and this finding was also corroborated by comparable results obtained from the Alamar Blue assay (Figure 1B), suggesting a higher intracellular oxidase activity in SETD2 deficient ccRCC cells. Moreover, we observed higher ATP levels in 38E and 38F cells than those of 786-O cells (Figure 1C). In addition, loss of SETD2 led to a global decline of H3K36me3 (Figure 1D–E), and increased cell size and intercellular complexity as indicated by forward scatter (FSC) and side scatter (SSC) histograms from 38E and 38F cells (Figure S2).

Loss of SETD2 enhances mitochondrial and glycolytic metabolism in ccRCC cells

Seahorse results showed that SETD2 deficient 38E/38F cells had both higher mitochondrial (Figure 2A) and glycolytic (Figure 2B) metabolic rates than those of 786-O cells. For MST (Figure 2C), compared to 786-O cells, 38E and 38F cells had 153.9% and 121.0% of the basal mitochondrial OCR, 251.3% and 147.1% of the proton leak-derived OCR, and 104.8% and 107.9% of the ATP synthesis-derived OCR, respectively. The OCR associated with maximal respiration, spare respiratory capacity, and non-mitochondrial oxygen consumption in 38E and 38F cells were 601.5% and 1322.4%, 1516.6% and 3438.9%, and 320.2% and 167.9% higher than those of 786-O cells, respectively. For GST (Figure 2D), 38E and 38F cells had 167.8% and 133.1% of ECAR, and 79.5% and 102.5% of glycolytic capacity compared to 786-O cells, respectively. Glycolytic reserves for 38E and 38F were 2.6- and 2.4-fold higher than 786-O cells, respectively. Non-glycolytic acidification in 38E and 38F cells was more than 2-fold higher than that of 786-O cells. Additionally, 786-O cells consumed oxygen at a rate of 50.7 pmol O₂/min with an acidification rate of 58.2 mpH /min,

while 38E and 38F cells had OCRs 2.98- and 2.33-folds higher, as well as ECARs 1.81- and 2.01-folds higher than those of 786-O cells, respectively (Figure 2E).

Loss of SETD2 enhances TCA cycle metabolic enzyme activity in ccRCC cells

We further analyzed the activity of CS, the first rate-limiting enzyme in the TCA cycle, in SETD2 deficient 38E/38F cells. Our data showed significantly elevated CS activity in 38E and 38F cells compared to 786-O cells (Figure 3A), but no significant difference in CS protein was observed between 786-O and 38F cells (Figure 3B), suggesting the elevated CS activity to be independent of CS protein level.

Loss of SETD2 increases TCA cycle metabolite production in ccRCC cells

To further validate observed metabolic changes of the TCA cycle, GC-MS-based targeted metabolomics was performed. The PCA score plot showed clear separation between 786-O and 38E/38F groups, and TCA metabolites had similar patterns of alterations in 38E/38F cells (Figure 4A–C). Further analysis showed 38E/38F cells had significantly higher levels of aspartate, malate, succinate, fumarate, and α -ketoglutarate, and lower levels of lactate than 786-O cells (Figure 4C). Furthermore, levels of citrate, isocitrate, cis-aconitic acid, and glutamate in 38E/38F cells were slightly increased, but no significant difference between 786-O and 38E/38F groups was observed (Figure S3).

SETD2-deficient ccRCC cells utilize alternative sources of acetyl-CoA beyond pyruvate for maintenance of TCA cycle activity

Base oxygen consumption of isogenic SETD2-deficient 38F cells was 41.2% higher than 786-O cells (Figure S4). After NaF addition, the oxygen consumption of 786-O cells was reduced by 30.2%, but remained unchanged for 38F cells, suggesting that SETD2-deficient ccRCC cells could utilize additional sources of acetyl-CoA beyond pyruvate for maintaining TCA cycle activity. The addition of lactate increased oxygen consumption in both 38F and 786-O cells, indicating the oxidation of lactate to form pyruvate. Furthermore, results from the TCGA-KIRC dataset showed SETD2 homozygous deficient ccRCC tumors to have lower levels of *PDPI* and higher levels of *SHMT2* (both involved in pyruvate metabolism) compared to WT tumors (Figure S5).

Loss of SETD2 disturbs gene networks of mitochondrial and fatty acid metabolism

RNAseq data showed SETD2 deficiency to cause dysregulation of many genes in ccRCC cells, and gene enrichment analysis showed the main biological processes affected by SETD2 to be mitochondrial, lipid (fatty acids), glucose, coenzyme, and purine metabolism (Figure 5A–B). In addition, PGC1 α and its related gene networks were altered by SETD2 mutations (Figure 5C). For instance, gene networks related to AMPK signaling such as *AMPK α 2*, glucose metabolism such as *SLC2A4* (GLUT4) and glycogen synthase 2 (*GYS2*), fatty acid metabolism such as Acetyl-CoA carboxylase 2 (*ACC2*) and carnitine palmitoyltransferase 1 (*CPT1*), mitochondrial function such as *PPARGC1A* (PGC1 α) and ETC subunit composition genes (complex I to V) were significantly enhanced in isogenic SETD2-deficient 38F cells (Figure 6A–B). Moreover, genes involved in fatty acid metabolism such as hepatic lipase (*LIPC*), *CPT1B*, acyl-CoA synthases 2 and 5 (*ACSL2* /

ACSL5), and acyl-CoA dehydrogenase (*ACADL*) were also up-regulated in SETD2-deficient 38F cells (Figure S6).

Loss of SETD2 increases PGC1 α and mitochondrial mass in ccRCC cells

Since PGC1 α is a central regulator of mitochondrial oxidative phosphorylation and fatty acid metabolism,¹⁶ we hypothesized that the elevated metabolic activity in SETD2-deficient ccRCC cells is associated with increased PGC1 α expression. Indeed, we found that isogenic SETD2-deficient 38F cells had a significant increase of the PGC1 α protein (2.5-folds) compared to 786-O cells (Figure 7A). In addition, staining of the living cells with a mitochondrial stain revealed higher mitochondrial mass in isogenic SETD2-deficient 38E/38F cells than that in 786-O cells (Figure 7B–C). Although PGC1 α protein expression is not available in public genomic datasets such as The Cancer Genome Atlas, we examined the mRNA expression of PPARGC1A, the gene that encodes PGC1 α , in SETD2 wild-type and mutant tumors. In our analysis, we found that the SETD2 mutant group produced a slightly higher expression although the difference was not significant (Figure S7, $p > 0.05$).

Discussion

Although the connection between RCC and dysregulated metabolism has been previously reported,¹² the effects of SETD2 inactivation on ccRCC metabolic phenotype and its contribution to tumor metastasis are still unknown. In this study, we observed higher luminescence of MTT and Alamar Blue in SETD2 deficient 38E/38F cells than 786-O cells. MTT is reduced by a variety of cytosolic and mitochondrial oxidases such as NAD(P)H, FADH, FMNH, and NADH.¹⁷ Alamar Blue is reduced by the same cohort of enzymes as that for MTT as well as cytochromes involved in the respiratory chain.¹⁸ Thus, these results suggest that loss of SETD2 leads to increased overall metabolic activity in ccRCC cells. Moreover, the elevated ATP levels also suggest increased mitochondrial respiration in SETD2-deficient cells. Further, Seahorse results showed that SETD2 inactivation induced a significant increase in the glycolytic and mitochondrial respiration rates in RCC cells. SETD2-deficient cells had higher basal glycolytic rates and a shut-down of ATP synthesis in the electron transport chain (ETC) by oligomycin led to increased ECAR. There was very little increase in ECAR of 786-O cells after oligomycin addition, suggesting that 786-O cells were operating at the near maximal glycolytic rate under basal conditions and thus had a limited ability to adapt to increased energy demand. Moreover, SETD2-inactivated cells showed more pronounced effects on mitochondrial respiration compared to 786-O cells. The addition of FCCP (an ETC uncoupler) revealed that SETD2-deficient cells have an enormous capacity to meet the energetic demand. In contrast, the OCRs in response to FCCP in 786-O cells were lower than their initial rates, creating an overall negative spare respiratory capacity. Although many cancers exhibit the classic Warburg effect and rely less on mitochondrial respiration for their energetic demand, a similar metabolic switch observed in this study has been reported in prostate cancer and was associated with poor overall survival.¹⁹ Thus, our results indicate that SETD2 inactivation can enhance both glycolytic and mitochondrial respiration with greater capacity for mitochondrial oxidative metabolism in ccRCC cells.

The Seahorse results further supported the elevated activities of TCA enzymes such as CS in SETD2-deficient cells. The activity of CS, responsible for the conversion of citrate from acetyl-CoA and oxaloacetate, was significantly increased in SETD2-deficient cells, although there was no apparent increase in CS protein. In addition, we observed increased levels of TCA cycle metabolites such as aspartate, malate, succinate, fumarate, and α -ketoglutarate in SETD2-deficient 38E/38F cells. Since pyruvate to acetyl-CoAs conversion is essential for maintenance of the TCA cycle, and since pyruvate dehydrogenase (PDH) is usually inactivated in tumor cells,²⁰ our results suggest a potential link between SETD2 deficiency and pyruvate metabolism in ccRCC cells. Interestingly, we found several key genes that regulate pyruvate metabolism, such as *SHMT2* and *PDP1*, to be altered in homozygous SETD2-mutated RCC from the TCGA-KIRC dataset.^{21, 22} *SHMT2* is involved in glycine-serine metabolism as an alternative route to generate pyruvate²¹ and, thus, its up-regulation might divert pyruvate for the production of acetyl-CoA in SETD2-deficient RCCs. *PDP1*, an activator of PDH to generate acetyl-CoA,²² was reduced in SETD2-deficient tumors, suggesting a decreased reliance on pyruvate as a source of acetyl-CoA for the TCA cycle in SETD2-deficient RCCs. This hypothesis is partly supported by the reduced oxygen consumption in 786-O cells but not SETD2-deficient 38F cells after treatment with NaF (a PDP1 inhibitor).²² These results suggest that pyruvate is a major source of acetyl-CoA in 786-O cells, but SETD2-deficient cells may use other fuel sources to generate acetyl-CoA for the TCA cycle.

To further explore the underlying molecular mechanisms, RNAseq and gene network analysis was performed in 786-O and 38E/38F cells. We found that loss of SETD2 disturbed networks related to glucose, mitochondrial, and fatty acid metabolism. For instance, *GLUT4*, a principal transporter for cellular glucose uptake,²³ was significantly increased in SETD2-deficient cells. PGC1 α , a master regulator for cellular energy homeostasis,¹⁶ was also elevated by SETD2 inactivation. Additionally, both mitochondrial uncoupling genes (*UCP2*, *UCP3*, and *SLC25A27*) and ETC subunit composition genes (*NDUSF4*, *SDHA*, *COX5b*, and *ATP5A1*) were significantly up-regulated in SETD2-deficient cells. Indeed, the up-regulation of mitochondrial biogenesis has been reported in cancers, and some cancers have mutations in mitochondrial TCA cycle enzymes.²⁴ PGC1 α has been recognized as a key stimulator for mitochondrial biogenesis, which can induce oxidative phosphorylation and TCA cycle gene expression.^{16,25} Moreover, PGC1 α has also been shown to promote colon and liver carcinogenesis growth via enhancing mitochondrial and fatty acid metabolism.²⁶ Thus, our results indicate that enhanced mitochondrial oxidative metabolism in SETD2-deficient ccRCC cells may be influenced by PGC1 α expression. This hypothesis is supported by the observed elevation in PGC1 α protein, and its associated increase in mitochondrial mass, cell size, and intercellular complexity in SETD2-deficient cells. As previously reported, PGC1 α mRNA has multiple isoforms,²⁷ and its mRNA levels may not correlate with PGC1 α protein expression. Moreover, there are likely other factors in tissue that could influence PGC1 expression such as VHL and HIF1- α ,²⁸ while our SETD2 isogenic cells have the same VHL/HIF phenotype. Thus, comparison of PGC1 isoforms and protein expression in SETD2 WT vs mutation tumor of ccRCC patients is needed in future studies.

Although we examined independently generated SETD2 deficient cell lines, our results in cultured cells may not accurately reflect the in vivo tumor microenvironment.²⁹ First, cultured cells are limited to selection of rapidly proliferating clones under nonphysiological conditions and our results should be confirmed in murine models in vivo to better recapitulate the tissue microenvironment. Second, although we detected increased levels of TCA metabolites, we did not see a correlation with RNA sequencing or Western blot of key enzymes. Similarly, a prior study did not see a correlation in human RCC tumors between RNA sequencing and direct measurement of metabolites.³⁰ Third, there are likely other factors involved in mitochondrial biogenesis beyond PGC1 α ,²⁴ thus future studies should examine the influence of the tumor microenvironment on biogenesis.

Conclusion

Taken together, our study observed that loss of SETD2 is associated with a metabolic switch in ccRCC cell lines toward enhanced oxidative phosphorylation and lipogenesis, and its mechanism can be potentially related to PGC1 α -mediated metabolic networks (Figure 8). Moreover, our results suggest a need for a comprehensive metabolomics analysis of cancer cells with SETD2 inactivation in vivo to specifically identify pathways involved in this metabolic switch, which provides a number of opportunities to identify novel therapeutic targets in kidney cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Loss of SETD2 increases the metabolic rate in ccRCC cells. The cell metabolic rate was measured by (A) MTT (n=3) and (B) Alamar Blue assays (n=3) at 3 hours after cell plating. (C) Cellular ATP production was measured by CellTiter-Glo reagent (n=3). (D-E) Western blot and quantitative analysis for H3K36me3 histone protein (*p<0.05).

Figure 2.

Loss of SETD2 enhances glycolytic and mitochondrial respiration in ccRCC cells. (A) Mitochondrial stress test (MST) and (B) glycolysis stress test (GST) were performed on Seahorse (n=3). ECAR and OCR were measured by addition of glucose fuel source and different metabolic inhibitors. (C) Mitochondrial activity and (D) glycolytic activity metabolic parameters were calculated from MST and GST, respectively. (E) OCR vs. ECAR of SETD2 isogenic cell lines under conditions of basal respiration (*p<0.05).

Figure 3. Loss of SETD2 enhances the TCA cycle metabolic enzyme in ccRCC cells. (A) Evaluation of citrate synthase (CS) activity in 786-O and isogenic SETD2-deficient 38E/38F cells (n=3, *p<0.05). (B) Western blot for CS protein expression in ccRCC cells.

Figure 4. Targeted GC-MS analysis of 786-O and isogenic SETD2-deficient 38E/38F cells. (A) PCA score plot and (B) scatter plot based on TCA cycle metabolites from 786-O, 38E, and 38F cells. (C) Quantitative analysis of aspartate, malate, succinate, fumarate, α -ketoglutarate, and lactate concentration in each cell type ($n=3$, $*p<0.05$).

Figure 5. RNA-seq analysis of 786-O and isogenic SETD2-deficient 38E and 38F cells. (A) Visualization of the gene alterations between 786-O and 38E/38F cells (red indicates up-regulated genes and green indicates down-regulated genes compared to 786-O cells). (B) Gene enrichment analysis revealed the cellular biological processes affected by SETD2 inactivation. (C) Identification of disturbed gene regulation networks in SETD2-deficient 38E/38F cells.

Figure 6.

Gene network analysis of isogenic SETD2-deficient ccRCC cell lines. (A) Activation of pathways that promote glucose uptake, mitochondrial biogenesis, and fatty acid oxidation in SETD2-deficient cells. (B) Genes involved in ETC subunit composition were increased in 38F cells (red indicates up-regulated genes and blue indicates down-regulated genes compared to 786-O cells).

Figure 7.

Loss of SETD2 increases PGC1 α and mitochondrial mass in ccRCC cells. (A) Western blot for PGC1 α protein expression in 786-O and isogenic SETD2-deficient 38F cells (** $p<0.01$). (B) MitoTracker Red staining for 786-O, 38E, and 38F cells (400x magnification). (C) Relative mitochondrial mass in each cell type as determined by MitoTracker Red staining ($n=3$, * $p<0.05$).

Figure 8. Schematic diagram of PGC1α overexpression and enhanced mitochondrial oxidative metabolism induced by SETD2 inactivation in ccRCC cells. Elevated TCA metabolites (green) in SETD2-deficient cells may be shunted toward increased fatty acid synthesis, leading to cancer metastasis.