

**Investigating perturbations in energy metabolism to identify targetable metabolic
vulnerabilities in cancer**

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ABSTRACT

Although metabolic differences between tumors and normal tissues were first observed over a century ago, the field of oncometabolism remained dormant in the subsequent years. It has only been until recently that dysregulation of cellular energetics was recognized as a hallmark of cancer. The advent of high throughput metabolomics technologies, computational systems biology and biochemical tools have aided in elucidating the various metabolic pathways exploited by cancer cells. Neoplastic cells strategically alter their metabolism to meet their bioenergetic and biosynthetic demands, which is particularly challenging in unfavorable microenvironments. However, emerging data indicate that cancer cells develop metabolic plasticity that allows them to rapidly remodel their metabolomes to survive and thrive in harsh tumor environments with limited nutrients and/or oxygen. The master regulators of cell growth and metabolism, AKT/mTOR (mechanistic/mammalian target of rapamycin) signaling axis plays a major role in governing metabolic processes essential to cancer cell survival and proliferation. This thesis highlights the crosstalk between the AKT/mTOR signaling and cancer metabolism. Specifically, my work elucidates plausible adaptive mechanisms that cancer cells turn on to survive energetic stress due to glutamine deprivation or inhibition of mitochondrial complexes I or IV. Specifically, the work in the thesis describes the changes in the glycolysis and citric acid cycle (CAC) resulting from the abrogation of the mitochondrial complex IV assembly protein, SCO2. These alterations in the metabolome were accompanied by rewiring of the AKT/mTOR signaling. The thesis also focuses on comparing energy production and utilization in colorectal cancer (CRC) cells and describes metabolic adaptations of CRC cells. To this end, we catalogued different energetic stressors and associated metabolic response networks that underpin adaptations of CRC cells to

energy stress thus revealing potential targetable metabolic vulnerabilities. Altogether, this work provides previously unrecognized insights into metabolic adaptation and plasticity of cancer cells.

ABRÉGÉ

Bien que des différences métaboliques entre les tumeurs et les tissus normaux aient été observées pour la première fois il y a plus d'un siècle, le domaine de l'oncométabolisme est resté relativement méconnu jusqu'à récemment. L'avènement des technologies de métabolomique à haut débit et de la biologie intégrative a contribué à élucider les différentes voies métaboliques exploitées par les cellules cancéreuses pour répondre à leurs demandes bioénergétiques et biosynthétiques. Répondre à de telles demandes est particulièrement difficile pour les cellules tumorales dans des microenvironnements défavorables. Cependant, de nouvelles données indiquent que les cellules cancéreuses développent une plasticité métabolique qui leur permet de remodeler rapidement leurs métabolomes pour survivre et proliférer dans des environnements tumoraux difficiles avec des nutriments et/ou de l'oxygène limités. Le régulateur principal de la croissance et du métabolisme cellulaire, AKT/mTOR (Mechanistic/Mammalian Target of Rapamycin), joue un rôle majeur dans la régulation des processus métaboliques essentiels à la survie et à la prolifération des cellules cancéreuses. Cette thèse met en évidence les interactions entre la signalisation AKT/mTOR et le métabolisme du cancer. En particulier, mon travail élucide les mécanismes adaptatifs que les cellules cancéreuses activent pour survivre au stress énergétique dû à la privation de glutamine ou à l'inhibition des complexes mitochondriaux I ou IV. Les travaux de cette thèse décrivent les changements dans le cycle de la glycolyse et de l'acide citrique (CAC) résultant de l'abrogation de la protéine du complexe mitochondrial IV, SCO2. Ces altérations du métabolome sont accompagnées d'une adaptation de la voie de signalisation AKT/mTOR. Cette thèse se concentre également sur la comparaison de la production et de l'utilisation d'énergie dans les cellules du cancer colorectal (CCR) et décrit les adaptations métaboliques des cellules du CCR. À cette fin, nous avons répertorié différents facteurs de stress énergétique et les réseaux de réponse

métabolique associés qui sous-tendent les adaptations des cellules CCR, révélant ainsi des vulnérabilités métaboliques potentielles ciblables. Dans l'ensemble, ces travaux fournissent des nouvelles connaissances sur l'adaptation métabolique et la plasticité des cellules cancéreuses.

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First and foremost, I would like to thank my parents and siblings for always encouraging me during my studies. You provided me with support in every form required. I am especially grateful to you, dad for advising me to pursue scientific research up to the graduate level. In some ways, I feel this degree is partly yours because I know you sacrificed your own goals of attaining an advanced degree to raise the family.

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PUBLICATIONS

Chapter 2 was published as an original research article:

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The following were published during my doctoral studies, but were not part of my principal projects:

Lo L, **Uchenunu O**, Botelho RJ, Antonescu CN, Karshafian R. (2022). AMP-activated protein kinase (AMPK) is required for recovery from metabolic stress induced by ultrasound microbubble treatment. *bioRxiv* 2022.03.02.482704 doi: <https://doi.org/10.1101/2022.03.02.482704>.

Igelmann S, Lessard F*, **Uchenunu O***, Bouchard J, Fernandez-Ruiz A, Rowell MC, Lopes-Paciencia S, Papadopoli D, Fouillen A, Ponce KJ, Huot G, Mignacca L, Benfdil M, Kalegari P, Wahba HM, Pencik J, Vuong N, Quenneville J, Guillon J, Bourdeau V, Hulea L, Gagnon E, Kenner L, Moriggl R, Nanci A, Pollak MN, Omichinski JG, Topisirovic I, Ferbeyre G. (2021). A hydride transfer complex reprograms NAD metabolism and bypasses senescence. *Molecular Cell*. 81(18):3848-3865. *co-second authors.

Papadopoli D, **Uchenunu O**, Palia R, Chekkal N, Hulea L, Topisirovic I, Pollak M, St-Pierre J. (2021). Perturbations of cancer cell metabolism by the antidiabetic drug canagliflozin. *Neoplasia*. 23(4):391-399.

Mzoughi S, Fong JY*, Papadopoli D*, Koh CM*, Hulea L*, Pigni P, Di Tullio F, Andreacchio G, Hoppe MM, Wollmann H, Low D, Caldez MJ, Peng Y, Torre D, Zhao JN, **Uchenunu O**, Varano G, Motofeanu CM, Lakshmanan M, Teo SX, Wun CM, Perini G, Tan SY, Ong CB, Al-

Haddawi M, Rajarethinam R, Hue SS, Lim ST, Ong CK, Huang D, Ng SB, Bernstein E, Hasson D, Wee KB, Kaldis P, Jeyasekharan A, Dominguez-Sola D, Topisirovic I, Guccione E. (2020). PRDM15 is a key regulator of metabolism critical to sustain B-cell lymphomagenesis. *Nature Communications*. 11(1):3520. *co-second authors.

Uchenunu O, Pollak M, Topisirovic I & Hulea L. (2019). Oncogenic kinases and perturbations in protein synthesis machinery and energetics in neoplasia. *Journal of Molecular Endocrinology*. 62(2):R83-R103.

Hulea L*, Gravel SP*, Morita M*, Cargnello M, **Uchenunu O**, Im YK, Lehhuédé C, Ma EH, Leibovitch M, McLaughlan S, Blouin MJ, Parisotto M, Papavasiliou V, Lavoie C, Larsson O, Ohh M, Ferreira T, Greenwood C, Bridon G, Avizonis D, Ferbeyre G, Siegel P, Jones RG, Muller W, Ursini-Siegel J, St-Pierre J, Pollak M, Topisirovic I. (2018). Translational and HIF-1 α -Dependent Metabolic Reprogramming Underpin Metabolic Plasticity and Responses to Kinase Inhibitors and Biguanides. *Cell Metabolism*. 28(6):817-832. *co-first authors

CONTRIBUTION TO ORIGINAL KNOWLEDGE

- 1) I deciphered the mechanisms underpinning metabolic adaptations of cancer cells to energy stress caused by dysfunction of mitochondrial complex IV. Specifically, I showed that the loss of SCO2 leads to a decrease in citric acid cycle (CAC) intermediates and compensatory increase in lactate production and other NAD⁺ regenerating reactions. Consistent with mitochondrial dysfunction due SCO2 deletion, I observed increase in pyruvate carboxylation and accumulation of lipid depots. In addition, the abrogation of SCO2 decreases the intracellular levels of most amino acids. Of note, SCO2 is thought to be a pivotal mediator of metabolic effects of tumor suppressor p53 (please see below). These results are therefore likely to be broadly applicable across the spectrum of neoplasia that are characterized by p53 dysfunction.
- 2) I provide evidence that SCO2 deletion leads to compensatory activation of AMPK and the IGF1R/AKT signaling axis, with paradoxical downregulation of mTORC1 signaling. These data implies that AMPK activation following the loss of SCO2 is essential to cell survival. We demonstrate similar rewiring of signaling in cells treated with mitochondrial complex III inhibitors. Furthermore, I demonstrated that SCO2 loss-induced signaling rewiring increases sensitivity of cells to IGF1R and AKT inhibitors, while decreasing the anti-proliferative effects of mTOR inhibitors. In long-term, these findings may inform strategies aiming to target cancer metabolism.
- 3) My data suggest that in addition to rendering HCT116 cells resilient to hypoxia, abrogation of SCO2 and accompanying signaling and metabolic reprogramming may increase the metastatic potential of colorectal cancer cells.

- 4) I showed that genetically distinct transformed colon cell lines are more sensitive to the drug- (i.e., phenformin) or nutrient depletion-induced (i.e., glutamine depletion) energy stress compared to normal colon epithelial cells.
- 5) I catalogued a compendium of similarities and differences in the metabolic profiles of colorectal cancer and normal colon epithelial cells under drug-induced (e.g., phenformin) or nutrient-deprivation (glutamine)-induced energy stress. These data imply that α -ketoglutarate carboxylation may be a potentially targetable common metabolic vulnerability across genetically distinct colorectal cancer cell lines.

These contributions, in combination with those that I made in collaboration (please see the appendix) provide hitherto unappreciated insights into metabolic rewiring of cancer cells thus contributing to better understanding of metabolic reprogramming of neoplasia. I hope that in long-term, my findings in concert with other similar efforts will help establish molecular bases for exploiting metabolic perturbations of cancer cells in the clinic.

CONTRIBUTIONS OF AUTHORS

The thesis titled: “Investigating perturbations in energy metabolism to identify targetable metabolic vulnerabilities in cancer” is based on five chapters.

The contributions of the authors who participated in this project are as follows:

Chapter 1: I wrote the manuscript including the creation of the figures. The chapter was reviewed and edited by Dr. Michael Pollak and Dr. Ivan Topisirovic.

Chapter 2: The study was conceived by Dr. Alexandre V. Zhdanov, Dr. Michael Pollak, Dr. Ivan Topisirovic, Dr. Dmitri Papkovsy and me. Dr. Alexandre V. Zhdanov and I designed and conducted experiments on gene expression, cell functioning, migration, signaling and metabolism. Dr. Dmitry Andreev and Dr. Pavel Baranov carried out RNA-sequence analysis. Dr. Laura Hulea Dr. Daina Avizonis and Dr. David Papadopoli assisted with metabolic experiments. Predrag Jovanovic carried out the transwell migration assay and analysis. Ye Wang and Phillipe Hutton carried out ELISA and Western blotting experiments. Dr. Alexandre V. Zhdanov and I wrote the initial draft of the manuscript. Dr. Michael Pollak, Dr. Ivan Topisirovic, Dr. Dmitri Papkovsy provided funding. All authors contributed to the interpretation of the data and editing the final version of the manuscript.

Chapter 3: Dr. Michael Pollak, Dr. Ivan Topisirovic and I conceived the study. I designed and conducted all the experiments. I wrote the initial draft of the manuscript. Dr. Michael Pollak and Dr. Ivan Topisirovic provided the necessary funding. Dr. Ivan Topisirovic edited the final version of the manuscript.

Chapter 4: I wrote the text. Reviews and edits were done by Dr. Ivan Topisirovic.

Appendix: The chapter highlights the different publications I was involved in, which delineated the mechanisms of metabolic rewiring in both normal and tumor cells. I provided critical

experimental and intellectual inputs in these studies. Specifically, with Igelmann et al., I was involved in conceptualizing and designing metabolic experiments. I also analyzed and interpreted the data which led to Fig. 4.1.1A-J. In the publication with Papadopoli et al., I designed and performed cell proliferation assay (Fig. 4.1.2A). I was also partly responsible for acquiring and analyzing the metabolic data for Mzoughi et al. This was done for Fig. 4.1.3A, Fig. 4.1.3C and Fig. 4.1.3D. Additionally, I designed, experimented, analyzed and created figures on cell proliferation assay for Hulea et al. This is illustrated as Fig. 4.1.4A. I also analyzed and interpreted the metabolic data used to produce Fig. 4.1.4C. Dr. Michael Pollak, Dr. Ivan Topisirovic and I conceived the structure of the chapter. Dr. Ivan Topisirovic edited the final version of the chapter.

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LIST OF ABBREVIATIONS

1,3-BPG: 1,3-biphosphoglycerate

1C: one-carbon unit

2C: two-carbon unit

2-DG: 2-deoxyglucose

2-HG: 2-hydroxyglutarate

2-PG: 2-phosphoglycerate

3-PG: 3-phosphoglycerate

3-PP: 3-phosphopyruvate

3-PS: 3-phosphoserine

4E-BP: 4E-binding protein

5,10-methylene-THF: 5,10-methylenetetrahydrofolate

5hmC: 5-hydroxymethylcytosine

6PGDH: 6-phosphogluconate dehydrogenase

6PGL: 6-phosphogluconase

α -KG: α -ketoglutarate

AABA: 2-amino-4-bis(aryloxybenzyl)aminobutanoic acid

ABAT: γ -aminobutyric acid transaminase

ACC: acetyl-CoA carboxylase

ACLY: ATP-citrate lyase

ACO: aconitase

ACS: acyl-CoA synthetase

ACSS2: acetyl-CoA synthetase 2

ADSL: adenylosuccinate lyase

ADSS: adenylosuccinate synthase

AGC: aspartate-glutamate carrier

ALDO: aldolase

ALKBH: AlkB homolog

ALL: acute lymphoblastic leukemia

ALT: alanine transaminase

AML: acute myeloid leukemia

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ARF: ADP ribosylation factor

ASNS: asparagine synthetase

AST: aspartate transaminase

ATP5O: ATP synthase subunit O

AU: arbitrary units

BCAA: branched chain amino acid

BPTES: bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide

CAC: citric acid cycle

CAD: carbamoyl phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase

CaMKK2: calcium/calmodulin-dependent protein kinase kinase β

CBS: cystathionine β -synthase

ChIP: chromatin immunoprecipitation

CLL: chronic lymphocytic leukemia

CML: chronic myeloid leukemia

COX: cytochrome c oxidase

CPT: carnitine palmitoyltransferase

CRC: colorectal cancer

CREB2: cAMP-responsive element binding 2

CRTC2: cyclic-AMP-regulated transcriptional co-activator 2

CS: citrate synthase

CSE: cystathionine γ -ligase

D-2-HGA: D-2-hydroxyglutaric aciduria

D2HGDH: D-2-hydroxyglutarate dehydrogenase

DHAP: dihydroxyacetone phosphate

DHFR: dihydrofolate reductase

DIC: differential interference contrast

DLBCL: diffuse large B-cell lymphoma

DMEM: Dulbecco's Modified Eagle's Medium

DMOG: dimethyloxallyl glycine

dNTP: deoxyribonucleotide

e3bp: e3 binding protein

EAA: essential amino acid

eEF2K: eukaryotic elongation factor 2 kinase

EGCG: epigallocatechin-3-gallate

EGF: epidermal growth factor

eIF2: eukaryotic translation initiation factor 2

eIF4E: eukaryotic translation initiation factor 4E

EMT: epithelial-mesenchymal transition

ETC: electron transport chain

ETF: electron-transferring flavoprotein

F-1,6-BP: fructose-1,6-biphosphate

F6P: fructose-6-phosphate

FA: fatty acid

FABP: fatty acid binding protein

FASN: fatty acid synthase

FATP: fatty acid transport protein

FBS: fetal bovine serum

FH: fumarate hydratase

FIH: factor inhibiting HIF

FLIM: fluorescence lifetime imaging

FNIP1/2: folliculin-folliculin interacting protein 1/2

FOXK1: forkhead/winged helix family k1

FTO: fat mass and obesity-associated

G6P: glucose-6-phosphate

G6PDH: glucose-6-phosphate dehydrogenase

GAAC: general amino acid control

GAB1: GRB2-associated binder 1

GABA: γ -aminobutyric acid

GAD: glutamate decarboxylase

GAP: glyceraldehyde-3-phosphate

GAP: GTPase-activating protein

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GCS: glycine cleavage system

GEF: guanine nucleotide exchange factor

GGT: γ -glutamyltransferase

GHB: γ -hydroxybutyrate

GLDC: glycine decarboxylase

GLUD: glutamate dehydrogenase

GLUL: glutamine synthetase

GLUT: glucose transporter

GMP: guanosine monophosphate

GMPS: guanosine monophosphate synthetase

GPD: glycerol-3-phosphate dehydrogenase

GPDH: glycerol-3-phosphate dehydrogenase

GPNA: γ -l-glutamyl-p-nitroanilide

GPx: glutathione peroxidase

GRB10: growth factor receptor-bound protein 10

GYS: glycogen synthase

HAT: histone acetyltransferase

HBV: hepatitis B virus

HCC: hepatocellular cancer

HCV: hepatitis C virus

HDAC: histone deacetylase

HIF: hypoxia inducible factor

HK: hexokinase

HLRCC: hereditary leiomyomatosis and renal cell cancer

HMT: histone methyl transferase

HOT: hydroxyacid-oxoacid transhydrogenase

HPV: human papillomavirus

HTC: hydride ion transfer complex

IDH: isocitrate dehydrogenase

IGF: insulin-like growth factors

IGF1R: insulin-like growth factor 1 receptor

IMP: inosine monophosphate

IMPDH: inosine monophosphate dehydrogenase

IR: insulin receptor

IRS-1: insulin receptor substrate-1

JmjC: Jumonji catalytic domain

KI: kinase inhibitor

L-2-HGA: L-2-hydroxyglutaric aciduria

L2HGDH: L-2-hydroxyglutarate dehydrogenase

LD: lipid droplet

LDH: lactate dehydrogenase

LDL: low-density lipoprotein

LHON: Leber hereditary optic neuropathy

LKB1: liver kinase B1

MAS: malate-aspartate shuttle

MAT: methionine adenosyl transferase

MCT: monocarboxylate transporter

MDH: malate dehydrogenase

ME: malic enzyme

MEF: mouse embryonic fibroblast

MPC1: mitochondrial pyruvate carrier complex 1

MPC2: mitochondrial pyruvate carrier complex 2

MS: methionine synthase

MTBSTFA: N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide

MTHFD: methylenetetrahydrofolate dehydrogenase

MTHFR: methylenetetrahydrofolate reductase

mTORC1: mechanistic target of rapamycin complex 1

NBCL: non-Hodgkin's B-cell lymphoma

NDPK: nucleoside diphosphate kinase

NEAA: non-essential amino acid

NHL: non-Hodgkin's lymphoma

NMuMG: Normal Murine Mammary Gland

NSCLC: non-small-cell lung cancer

OGDD: 2-oxoglutarate/iron (II)-dependent dioxygenases

OGDH: 2-oxoglutarate dehydrogenase

OXPHOS: oxidative phosphorylation

P5CS: pyrroline-5-carboxylate synthase

PARP: poly (ADP-ribose) polymerase

PC: pyruvate carboxylase

PCa: prostate cancer

PCK: phosphoenolpyruvate carboxykinase

PDAC: pancreatic ductal adenocarcinoma

PDC: pyruvate dehydrogenase complex

PDH: pyruvate dehydrogenase

PDK: pyruvate dehydrogenase kinase

PDK1: 3-phosphoinositide-dependent protein kinase 1

PDP: pyruvate dehydrogenase phosphatase

PEP: phosphoenolpyruvate

PFK: phosphofructokinase

PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase

PGI: phosphoglucose isomerase

PGK: phosphoglycerate kinase

PGM: phosphoglycerate mutase

PH: pleckstrin homology

PHD: prolyl hydroxylase

PHGDH: 3-phosphoglycerate dehydrogenase

PHGDH: phosphoglycerate dehydrogenase

PI: propidium iodide

PIP2: phosphatidyl inositol-4,5-biphosphate

PIP3: phosphatidyl inositol-3,4,5-triphosphate

PK: pyruvate kinase

PPAT: phosphoribosyl pyrophosphate amidotransferase

PPP: pentose phosphate pathway

PRA: 5-phosphoribosyl-1-amine

PRAS40: proline rich AKT1 substrate 40

PRDM15: PR/SET Domain 15

PRPP: phosphoribosyl pyrophosphate

PRPS: 5-phosphoribosylsynthetase

PRx: peroxiredoxin

PSAT1: phosphoserine aminotransferase 1

PSPH: phosphoserine phosphatase

PYCR: pyrroline-5-carboxylate reductase

R5P: ribose-5-phosphate

REDD1: regulated in development and DNA damage response 1

RHEB: ras homolog enriched in brain

RNR: ribonucleotide reductase

ROS: reactive oxygen species

RPI: ribose 5-phosphate isomerase

rpS6: ribosomal protein S6

RSK: p90 ribosomal protein S6 kinase

RTK: receptor tyrosine kinase

Ru5P: ribulose-5-phosphate

S6K: S6 kinase

SAH: S-adenosyl homocysteine

SAHH: S-adenosyl homocysteine hydrolase

SAICAR: succinylaminoimidazolecarboxamide ribose-5'-phosphate

SAM: S-adenosyl methionine

SCO2: synthesis of cytochrome c oxidase 2

SCS: succinyl-CoA synthetase

SDH: succinate dehydrogenase

SGLT: sodium-dependent glucose co-transporter

SGLT2: sodium-glucose cotransporter-2

SHMT: serine hydroxymethyltransferase

SITA: stable isotope tracer analysis

SITA: stable isotope tracer analysis

SLC25A: solute carrier family 25A

SLC2A: solute carrier 2A

SLC5A: solute carrier 5A

SOD2: superoxide dismutase 2

SSA: succinic semialdehyde

SSADH: succinic semialdehyde-dehydrogenase

SSP: serine synthesis pathway

STAT3: signal transducer and activation of transcription 3

STRAD: STE20-related kinase

TAG: triacylglycerol

TALDO: transaldolase

TBC1D7: TBC1 domain family member 7

TCSPC: time-correlated single photon counting

TET: ten-eleven translocation

TFAM: transcription factor a, mitochondrial

THF: tetrahydrofolate

TIGAR: TP53-induced glycolysis and apoptosis regulator

TISU: translation initiator of short 5' UTR

TKT: transketolase

TME: tumor microenvironment

TNBC: triple negative breast cancer

TPI: triose phosphate isomerase

TRx: thioredoxin

TSC: tuberous sclerosis complex

TSC2: tuberous sclerosis complex 2

UMP: uridine monophosphate

UMPS: uridine monophosphate synthetase

VHL: von-Hippel-Lindau

WT: wildtype

YY1: yin yang 1

CHAPTER 1: INTRODUCTION

1.1 Cancer burden and biology

Cancer imposes a significant burden on the Canadian healthcare system yearly as the population size grows and ages. Approximately 50 percent of Canadians are expected to be diagnosed with cancer in their lifetime (Canadian Cancer Statistics Advisory Committee, 2019) (Fig.1.1). In Canada, it was estimated that the number of new cancer cases will increase from 220,400 (1) to 225,800 cases (Canadian Cancer Statistics Advisory Committee, 2019) (Fig.1.1). This correlates with upsurge in cancer-related deaths (1) (Canadian Cancer Statistics Advisory Committee, 2019)



(Fig.1.1).

Fig. 1.1. Cancer-related statistics in Canada, 2019. (Canadian Cancer Statistics Advisory Committee, 2019).

Lung cancer was projected to be the leading cause of cancer-related deaths followed by colorectal, pancreatic and breast cancers (Canadian Cancer Statistics Advisory Committee, 2019). The steady rise in amount of cancer-associated mortality over the years is predominantly a reflection of poorly

understood biological underpinnings of the disease. Studying cancer etiology is particularly important in preventing and treating cancer, and thus reducing the cancer-related deaths. Cancer risk factors can be broadly classified into intrinsic and non-intrinsic risk factors. Unmodifiable intrinsic risks are spontaneous mutations that occur due to random errors in DNA replication (2). On the other hand, non-intrinsic risk factors include exogenous factors (carcinogens, viruses, physical activity, diet, smoking, UV radiation) and endogenous factors (metabolism, hormone levels, immunity, DNA damage response, genetic susceptibility) (2). Several studies link non-intrinsic factors to different types of cancers such as: smoking to lung cancer (3), *Helicobacter pylori* (*H. pylori*) to gastric cancer (Helicobacter & Cancer Collaborative (4), human papillomavirus (HPV) to cervical cancer (5) and head and neck cancer (6), hepatitis B virus (HBV), hepatitis C virus (HCV) to hepatocellular cancer (HCC) (7, 8) and UV radiation to melanoma (9). Overall, whether such risk factors are intrinsic or non-intrinsic, they may result in irreparable changes in the genome. Such lesions are frequently found in genes that regulate cellular processes including cell growth, proliferation, and cellular energetics (10, 11). This eventually leads to neoplasia.

There are over 200 different types of cancers (12). The diversity amongst the types of cancers makes them particularly challenging to diagnose and treat. Mutations in proto-oncogenes that generally regulate cell division, result in oncogenes that drive tumorigenesis (13). In addition, alterations in genes that act as tumor suppressors can trigger aberrant cell division (14). Molecular changes that are thought to underlie neoplasia are generally recognized as “hallmarks of cancer” that were originally published in 2000 (15). The hallmarks include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (15). In 2011, “hallmarks of cancer”

were revised to include cancer cell abilities to reprogram energy metabolism, evade immune destruction, generate genome instability and tumor-promoting inflammation (10). Tumorigenesis is reliant on metabolic reprogramming which is often a direct and indirect consequence of oncogenic mutations. Inactivated tumor suppressor genes can also dysregulate cellular metabolism (16). Cancer cells rewire their metabolism to support the energetic demands of unchecked proliferation, fuel biosynthetic pathways and alleviate potentially deleterious effects of reactive oxygen species (ROS) (17).

1.2 Cancer metabolism

1.2.1 Warburg effect

Almost a century ago, Otto Warburg observed that tumors uptake more glucose compared to surrounding healthy tissues (18). He also observed that the cancer cells even in the presence of physiological oxygen tension metabolize glucose into lactate (18). This is termed as the “Warburg effect” or “aerobic glycolysis”(19). Warburg went on further to propose that this phenomenon is specific to cancer cells and is caused by dysfunctional mitochondria (20). To this end, Warburg postulated that proliferating cells adapt to abrogation of mitochondrial functions by upregulating glycolysis to meet ATP demands (20).

Subsequent studies have contradicted Warburg’s hypotheses as aerobic glycolysis has been observed in normal proliferating thymocytes and lymphocytes (21, 22). Additionally, it was later found that defective mitochondria are not the cause of aerobic glycolysis in most cancer cells which is supported by data illustrating that the tumorigenic potential of cancer cells is markedly reduced by depleting mitochondrial DNA (23-25). In fact, most cancer cells not only retain their ability to carry out oxidative phosphorylation through fully functional mitochondria, but also reprogram their mitochondrial metabolism to circumvent the metabolic challenges associated with

their rapid proliferation (24, 26-29). Some cancer cells (e.g., HeLa, chronic lymphocytic leukemia (CLL) lymphocytes and melanoma) depend on oxidative phosphorylation (OXPHOS) and glycolysis to synthesize ATP (30-33). In particular, HeLa cells adapt their metabolism towards readily available substrates such as glucose and glutamine (which are catabolized in the mitochondria) (34). Exogenous lactate and acetate can also be oxidized by glioblastoma and breast cancer respectively to fuel neoplastic growth (35, 36). These and similar results illustrate the plasticity in cancer metabolism which is often needed to adapt to frequent changes in tumor microenvironment (TME). Other examples whereby cancer cells reprogram their cellular metabolism include increased glucose uptake (37), upregulated glycolysis (38), increased glutaminolysis (39), enhanced mitochondrial biogenesis (40) and perturbations in amino acid and lipid metabolism (41, 42).

Although the reduction of glucose into lactate produces fewer ATP molecules per glucose molecule when compared to its complete breakdown to produce carbon dioxide and water, ATP is synthesized faster and at comparable amounts via glycolysis (43-45). Equally important, glycolysis regenerates NAD^+ and provides proliferating cells with the necessary intermediates for synthesizing amino acids, lipids and nucleotides (46). Aerobic glycolysis is advantageous for cancer cells as it enhances NADPH production through the oxidative branch of the closely associated pentose phosphate pathway (PPP) to detoxify ROS while meeting the bioenergetic and biosynthetic requirements of proliferation (47)

1.2.2 Glucose metabolism

Glucose is an essential macronutrient for mammalian cells (48). It is either obtained directly through the hydrolysis of ingested polysaccharides and disaccharides (48) or biosynthesized in the liver through gluconeogenesis (49). Glucose serves as a major source for

ATP which is used to drive cellular processes (48). In addition, glucose is a predominant carbon source used to biosynthesize building blocks such as amino acids, fatty acids and nucleotides (50).

1.2.2.1 Glucose uptake

Due to its hydrophilicity, glucose cannot move freely across the cell membrane (48). Glucose uptake into cells is facilitated by a family of membrane proteins called hexose or glucose transporters (GLUTs) (51) (Fig. 1.2.). Glucose is also transported by a family of sodium-dependent glucose co-transporters (SGLTs) which are structurally and functionally different from the GLUTs (52). SGLTs are encoded by solute carrier 5A (*SLC5A*) genes (52) while the GLUTs are expressed by a family of genes referred to as solute carrier 2A (*SLC2A*) (51).

To date, 14 GLUTs were described and they are divided into 3 classes (51) (53). The class I GLUTs include GLUT1-4, and represent the best characterized class of GLUTs (51). Their expression varies between different tissues and cell types (51). Furthermore, GLUTs 1-4 have different affinities for glucose (51). GLUT1 is primarily found in erythrocytes, brain and skeletal muscles (54). GLUT2 is expressed mostly in the liver, pancreas, intestines, and kidneys but absent in skeletal muscles (55, 56). GLUT3 and GLUT4 are both found predominantly in the neurons, whereby GLUT4 is also highly expressed in the heart and skeletal muscles and is sensitive to insulin (57-59). Importantly, certain cancers (e.g., breast and colorectal cancer) engage multiple glucose transporters such as GLUT2 and GLUT3 to support neoplastic growth (60).

In normal cells, glucose uptake is a tightly regulated process that responds to changes in the cellular energy demands (61, 62). External regulation of glucose transport involves response to hormones (insulin and glucagon) and growth factors [epidermal growth factor (EGF) and insulin-like growth factors (IGF)] (63). To fuel high energy demanding cellular processes (e.g., mRNA translation) and biosynthesize the macromolecules necessary for proliferation, cancer cells

often upregulate glucose uptake (64). Tumors are typically poorly vascularized which limits their access to nutrients and oxygen (65-67). Hence, neoplastic cells must adapt their metabolism (e.g., by enhancing the expression of glucose transporters) to survive conditions wherein nutrients and oxygen are limited (68). To this end, the expression of *SLC2A1* is elevated in various types of cancers (69-71) including colorectal adenocarcinomas (69). In hepatocellular carcinoma, high levels of *SLC2A2* were reported to correlate with poor overall survival (72). *SLC2A3* and *SLC2A4* are also overexpressed in bladder cancer (73) and alveolar rhabdomyosarcoma (74) respectively. Similar to GLUTs, SGLTs (SGLT1 and SGLT2) were suggested to enhance glucose uptake in tumors (75). It was demonstrated that in metastatic lung cancer, *SLC5A2* expression (*SLC5A2* encodes SGLT2) was higher in metastatic tumors relative to primary tumors (76) suggesting that glucose metabolism can be modulated to promote tumor progression. Overall, cancer cells appear to utilize different strategies to upregulate glucose uptake thus fueling neoplastic growth.

1.2.2.2 Glycolysis

Upon transport into the cell, glucose is partly catabolized in the cytosol and subsequently oxidized in the mitochondria (48). Glycolysis is catabolic process whereby glucose through a series of reactions is broken down into pyruvate in the cytosol (48) (Fig. 1.2). This process produces 2 moles of pyruvate, ATP and NADH per mole of glucose (48) (Fig. 1.2.). In the absence of oxygen, pyruvate gets reduced by lactate dehydrogenase (LDH) to produce NAD⁺ and lactate (48). As Warburg observed, neoplastic cells can convert pyruvate into lactate even in the presence of sufficient oxygen (18). In contrast, in most normal tissues, pyruvate is typically oxidized in mitochondria under normal oxygen tension (48).

Hexokinases (HKs) catalyze the first committed step in glycolysis by phosphorylating glucose to produce glucose-6-phosphate (G6P) using an ATP molecule (48) (Fig. 1.2.). This traps

the glucose molecules by preventing their efflux back to the extracellular space (48). In turn, G6P inhibits HKs as a part of a negative feed-back loop (77). There are four main isoforms of HK found in mammals which vary in their localization and kinetics with respect to different substrates [i.e., glucose and ATP; reviewed in (78)]. G6P is then converted into fructose-6-phosphate (F6P) by phosphoglucose isomerase (PGI) and subsequently phosphorylated to produce fructose-1,6-biphosphate (F-1,6-BP) which is catalyzed by phosphofructokinase 1 (PFK1) (48) (Fig. 1.2.). The phosphorylation of fructose-6-phosphate is another rate-limiting step of glycolysis which consumes one ATP (48). Moreover, PFK1 is allosterically inhibited by ATP and activated by AMP (79). This suggests that the activity of PFK1 is dependent on the bioenergetic state of the cell.

F-1,6-BP is split by aldolase (ALDO) into two 3-carbon glycolytic intermediates that are isomers of each other: dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) (48) (Fig. 1.2). Since only GAP is further metabolized in glycolytic pathway, all DHAP molecules are isomerized into GAP by triose phosphate isomerase (TPI) (48) (Fig. 1.2). This signifies the end of the energy investment phase of glycolysis where two molecules of ATP are consumed to catabolize a molecule of glucose into three-carbon phosphate intermediates. The payoff phase of glycolysis begins when glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oxidizes and phosphorylates GAP to produce 1,3-biphosphoglycerate (1,3-BPG) by utilizing one NAD⁺ molecule per molecule of GAP in the process (48) (Fig. 1.2). 1,3-BPG is then converted to 3-phosphoglycerate (3-PG) by phosphoglycerate kinase (PGK) (48) (Fig. 1.2). This reaction also yields one ATP molecule for each converted 1,3-BPG molecule (48) (Fig. 1.2). Afterwards, phosphoglycerate mutase (PGM) isomerizes 3-PG to 2-phosphoglycerate (2-PG) and subsequently enolase dehydrates 2-PG to produce phosphoenolpyruvate (PEP) (48) (Fig. 1.2). Pyruvate kinase

(PK) catalyzes the last reaction of the payoff phase whereby PEP is irreversibly converted to pyruvate while simultaneously producing ATP (48) (Fig. 1.2).

Glycolytic enzymes are essential for promoting the survival and proliferation of malignant cells (80). Elevated HK2 expression has been observed in many cancers (81) including brain metastasis of breast cancer which is significantly associated with poor patient survival post-craniotomy (82). In addition, HK2 appears to be essential for tumorigenesis in *in vivo* models of KRAS-driven lung cancer and HER2+ breast cancer (83). PGI overexpression is involved in tumor metastasis and epithelial-mesenchymal transition (EMT) in breast cancer (84). Elevated levels of PFK mRNA have been observed in lung and colon carcinomas (85, 86). Notwithstanding that *GAPDH* is widely regarded as a housekeeping gene, its overexpression was observed in colorectal carcinoma (87) and pancreatic cancer (88, 89). Accordingly, it has been suggested that *GAPDH* may exert antiapoptotic roles in drug-resistant chronic myeloid leukemia (CML) cancer (90). Likewise, some cancer types (e.g., lung, gastric and pancreatic cancers) upregulate PGK or ALDO expression which parallels with increased cell motility, angiogenesis and metastasis (91-93). Overall, these findings demonstrate that dysregulated glycolytic gene expression is likely to play a central role in cancer initiation and progression.

In mammals, there are 2 genes (*PKLR* and *PKM*) which encode 4 tissue-specific isoforms (PKL, PKR, PKM1 and PKM2) of PK (94, 95). Intriguingly, although PKL is expressed in the liver, kidneys, pancreas and intestine while PKR is expressed in erythrocytes, they are both encoded by the *PKLR* gene (95). Alternative splicing of the *PKM* gives rise to two proteoforms: PKM1 (expressed in the brain, skeletal and cardiac muscles) and PKM2 (expressed in embryonic tissue, leucocytes, intestine, and thymus) (95, 96). Despite having the same catalytic function, PKM1 is constitutively active and more efficient at converting PEP into pyruvate relative

to PKM2 (95). Serine can bind and activate PKM2, whereby PKM2 activity is diminished when serine is unavailable (97). This reduction in PKM2 activity turns cells to a fuel-efficient state whereby more pyruvate is redirected to the mitochondria (97). Prior to this discovery, PKM2 was observed to interact directly with phosphotyrosine peptides resulting in the release of its allosteric activator, F-1,6-BP (98). Abrogating the enzymatic activity of PKM2 diverts glycolytic intermediates from energy production to anabolic processes (e.g. lipid synthesis) when cancer cells are stimulated by growth factors such as IGF (98). Consequently, growth factors which initiate the phosphorylation of tyrosine residues on signaling proteins inhibit PKM2 and this is thought to allow transformed cells to transition between bioenergetic and biosynthetic modes (98). Similar to serine and F-1,6-BP, succinylaminoimidazolecarboxamide ribose-5'-phosphate (SAICAR), which is a metabolic intermediate of *de novo* purine nucleotide biosynthesis pathway was also shown to allosterically activate PKM2 and promote cancer cell survival in glucose-deprived conditions (99). Accordingly, high PKM2 levels have been associated with many cancers including lung, cervical, prostate and brain malignancies (100-102). When malignant cells were engineered to express PKM1 instead of PKM2, the cells switched from aerobic glycolysis to OXPHOS which correlated with reduced tumor formation following xenotransplantation (102). As such, the regulation of PKM2 can be commandeered by neoplasia to increase metabolic flexibility. However, there are conflicting reports demonstrating that the abrogation or inhibition of PKM2 promotes tumor growth (103-105). Interestingly, in one of these reports, low PKM2 activity which was observed in lung cancer cells subjected to hypoxia led to the activation of PPP to generate sufficient NADPH for ROS detoxification and thus survival of cancer cells (105). Collectively, these studies indicate that the role of PKM2 in tumorigenesis is likely to be context-dependent.

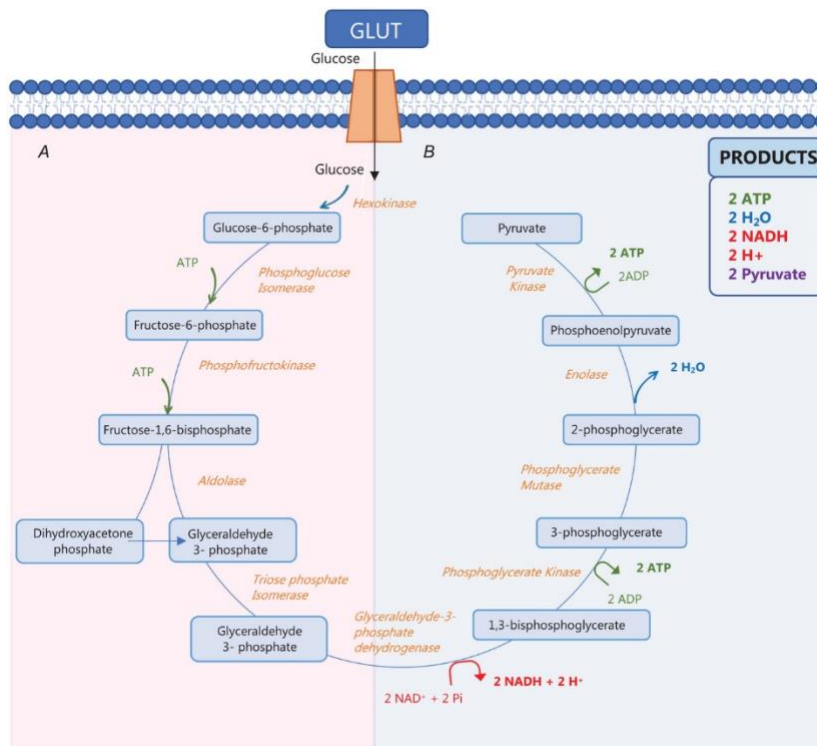


Fig. 1.2. Glucose uptake and glycolysis (106). Glucose is taken into the cell via specific glucose transporters (GLUTs). It is subsequently metabolized through a series of ten enzymatic reactions (orange) to produce pyruvate. Glycolysis is divided into an energy investment phase (A; pink box) which consumes ATP (green) and a payoff phase (B: blue box) which produces NADH, ATP and H₂O.

1.2.3 Pyruvate metabolism: Crossroads between glycolysis and citric acid cycle

1.2.3.1 Cytosolic reduction of pyruvate

Under anaerobic conditions, LDH catalyzes the reversible conversion of pyruvate to lactate with the concomitant oxidation of NADH in the cytosol of normal cells (48). Following its production, lactate is excreted into the extracellular space via monocarboxylate transporters (MCTs) (107). As mentioned above, it was observed that tumor cells metabolize glucose into lactate even in the presence of ample oxygen (20). Furthermore, the NAD⁺ pool generated by LDH is used to support successive rounds of glycolysis and increase the levels of anabolic intermediates

used by cancer cells (100, 108). Despite their high overall structural similarity, LDH exists as two distinct isoforms that exhibit significant differences in the charged residues surrounding the active sites. LDHA favors the conversion of pyruvate to lactate, while LDHB preferentially oxidizes lactate to pyruvate (109-111). Numerous studies demonstrate aberrant activation and abnormally high expression of LDHA in multiple cancers including colorectal cancer (CRC), liver cancer, non-small-cell lung cancer, pancreatic cancer and esophageal cancer (112-118). In addition to altering the metabolism to confer neoplastic growth advantage, the expression of LDHA promotes angiogenesis, cell invasion and metastasis (115, 119). While the upregulation of LDHB was shown to be essential to the proliferation of KRAS-driven lung cancer and triple negative breast cancer (120, 121), its low expression in hepatocellular carcinoma relative to noncancerous tissues correlated with unfavorable survival (122). This suggests that the role of LDHB in tumorigenesis seems to be context-dependent. Contrary to Warburg's findings, a recent hypothesis (i.e., "Reverse Warburg Effect") that supports oxidative mitochondrial metabolism in neoplasia proposes that stromal or transformed cells undergo aerobic glycolysis to produce lactate (123). Lactate is then released in the TME via MCT4 and is subsequently taken up through MCT1 transporter of surrounding cells as an energy source (123, 124). According to this model, LDHB catalyzes the oxidation of lactate to pyruvate which then enters the citric acid cycle (CAC). Metabolic cooperation of cancer cells within the TME is further substantiated in pancreatic and breast cancer models which revealed that lactate secreted by glycolytic hypoxic malignant cells can be taken up as fuel for aerobic tumor cells (36, 125). Moreover, secretion of lactate and subsequent acidification of TME is thought to induce angiogenesis while impairing tumor immunosurveillance (126). Of note, under physiological conditions, lactate released by brain, skeletal and muscle cells can be reconverted into glucose in the liver via the Cori cycle (127). Tumor cells extrude copious

amounts of lactate into the TME to maintain a high glycolytic flux which is thought to increase Cori cycle activity in the liver (128, 129). Overall, pyruvate-lactate metabolism impacts cancer fate in a cell-autonomous and systemic level.

1.2.3.2 Mitochondrial oxidative decarboxylation of pyruvate into acetyl-CoA

Under normoxia, pyruvate is chiefly imported into the mitochondrial matrix via the mitochondrial pyruvate carrier complex 1/2 (MPC1 and MPC2) to be irreversibly oxidatively decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH) complex (PDC) which is accompanied by NAD⁺ reduction (48, 130, 131). Essentially, this reaction links glycolysis, fatty acid metabolism and CAC (132). PDC is assembled from an e3 binding protein (e3BP) and multiple copies of three enzymes: E1 (pyruvate dehydrogenase), E2 (dihydrolipoyl transacetylase), and E3 (dihydrolipoyl dehydrogenase) (126, 133). E1 is the rate-limiting enzyme of the complex (126). At high concentrations, the products from the reaction (i.e., acetyl-CoA and NADH) inhibit the activity of PDC (134). Instantaneous post-translational regulation is achieved via kinases and phosphatases (134). Tissue-specific pyruvate dehydrogenase kinase isozymes (PDK1-4) which differ in their enzymatic activities, inactivate PDC by phosphorylating specific serine residues located on the α subunit of the E1 enzyme in the complex (135, 136). In turn, pyruvate dehydrogenase phosphatases 1/2 (PDP1/2) restore PDC enzyme activity via dephosphorylating key regulatory residues (126, 134). Thus, PDKs and PDPs help in maintaining the mitochondrial acetyl-CoA pool to support CAC activity. Acetyl-CoA is a precursor used by histone acetyltransferases (HATs) to catalyze the addition of acetyl groups to the histone N-terminal tails (137). Based on this, HATs link epigenetic regulation to the metabolic status of the cells as they are dependent on glucose availability, lipid metabolism and mitochondrial function (138-140).

This makes acetyl-CoA a versatile and critical metabolic intermediate to fuel anabolic reactions and epigenetic remodeling processes.

Reduced expression levels of *MPC1* or *MPC2* in cancer have been shown to correlate with poor patient survival (141, 142). Increased expression of MPCs in CRC cells was found to not only increase mitochondrial pyruvate oxidation while suppressing aerobic glycolysis, but also to impair anchorage-independent growth (141). In addition, aberrant regulations of PDKs and PDPs which control the activity of PDC have been associated with different cancers (143-145). For instance, the enhanced PDK1 activity has been linked to increased invasiveness and tumor growth in head and neck squamous cancer cells (146) whereas the upregulation of PDK4 was reported to correlate with increased migration and invasion in CRC (147). Moreover, the activation of PDP1 was observed to increase OXPHOS, decrease cell proliferation and reduce tumor growth in a lung cancer cell line xenograft model (143). Collectively, these studies suggest that the PD(K/P):PDC axis acts as metabolic switch in cancer cells which increases metabolic flexibility by allowing adaptation to fluctuations in nutrient and oxygen availability as well as bioenergetic requirements.

1.2.4 Citric acid cycle (CAC)

Acetyl-CoA produced by PDC can enter the citric acid cycle (CAC; also referred to as Krebs cycle or tricarboxylic acid cycle), where the carbon atoms are converted to CO₂ while simultaneously producing reducing equivalents (i.e. NADH and FADH₂) (131) (Fig. 1.3). CAC starts with the irreversible condensation of acetyl-CoA, derived from oxidative decarboxylation of pyruvate, β -oxidation of fatty acids or catabolism of amino acids (e.g., leucine, isoleucine and tryptophan), with oxaloacetate to produce the six-carbon citrate which is catalyzed by citrate synthase (CS) in the mitochondrial matrix (148). Through feedback inhibition, citrate suppresses the activity of CS (149). NADH, ATP and succinyl-CoA are also inhibitors of CS (148, 150).

Importantly, citrate downregulates glycolysis by allosterically inactivating glycolytic enzymes PFK1 and PFK2 (148, 151). Furthermore, high levels of citrate suppress PDC and succinate dehydrogenase (SDH), thus repressing the CAC (152, 153). The role of CS in neoplasia remains poorly understood considering that the abrogation of its activity appears to result in contrasting phenotypes. While reduced CS expression led to marked increases in glycolysis and migration of cervical cancer cells, silencing CS expression inhibited invasion and migration of ovarian carcinoma cells (154, 155). In healthy prostate epithelial cells, citrate exits the CAC via the dicarboxylate antiporter solute carrier family 25 (SLC25A1) to be eventually secreted into the prostatic fluid to facilitate the maturation and motility of spermatozoa (156). A defining metabolic phenotype of normal prostate epithelial cells, which is elevated citrate production and secretion, is reversed during tumorigenesis (156). Thus, low concentrations of citrate serve as a useful biomarker of prostate cancer, wherein the citrate levels inversely correlate with the stage of prostate cancer (PCa) (157).

In most of the non-transformed cells, citrate is either transported to the cytosol through SLC25A1, to serve as a precursor molecule for fatty acid and cholesterol biosynthesis, or it is retained in the mitochondrial matrix where it is reversibly isomerized to isocitrate by aconitase (ACO) (148) (Fig. 1.3). ACO dehydrates citrate into cis-aconitate, an intermediate product, which is then rehydrated into isocitrate (158) (Fig. 1.3). Zinc acts as a competitive inhibitor of mitochondrial aconitase (ACO2), which prevents citrate oxidation in the CAC (159). This is particularly important for the physiological functions of prostate epithelial cells (159). Similar to citrate, intracellular zinc acts as a biomarker for PCa, whereby its levels are inversely correlated to the progression of the disease (159, 160). Indeed, previous studies have suggested decreased levels of zinc as a biomarker for the early diagnosis of PCa (161, 162). Consistent with this, the

downregulation of an important zinc transporter, ZIP1, is associated with reduced zinc levels in prostate cancer (160, 163). Thus, the metabolic reprogramming that underlies the transformation of prostate cells comprises a switch from physiological citrate-producing epithelial cells to neoplastic citrate-oxidizing cells. Collectively, these findings suggest that the intracellular zinc levels not only dictate the metabolic state of prostate cells but could be associated with the development of PCa.

In normal conditions, isocitrate is oxidatively decarboxylated into the five-carbon α -ketoglutarate (α -KG) in a rate-limiting reaction catalyzed by NAD⁺-dependent isocitrate dehydrogenase (IDH) which concurrently produces the first NADH molecule of the CAC (148, 164) (Fig. 1.3). IDH is expressed in three isoforms: IDH1, IDH2, and IDH3 (151, 165). IDH1 is localized in the cytosol while IDH2 and IDH3 are found in the mitochondria (151, 165). Unlike mitochondrial IDH3 which depends on NAD(H), IDH1 and IDH2 prefer the use of NADP(H) as a cofactor (166). IDH1 and IDH2 have a high degree of sequence, structural and functional similarity between them (167, 168). Both isomers function as homodimers catalyzing the oxidative decarboxylation of isocitrate into α -KG while simultaneously producing NADPH necessary for protecting the cell against oxidative damage and fatty acid synthesis (169-172). Conversely, the reductive carboxylation of α -KG to isocitrate which is subsequently converted to citrate is important for regulating glycolysis and lipogenesis (173). The conversion of isocitrate to α -KG catalyzed by IDH3 is irreversible in contrast to the reversible processes of IDH1- and IDH2-catalyzed reactions (174, 175). IDH3 is regulated through the concerted activities of ions and molecules as citrate, ADP and calcium ions are allosteric activators while ATP, α -KG, NADPH and NADH suppress IDH3 activity (176-178). Importantly, in tumors where biosynthetic processes are upregulated and often accompanied with reduced mitochondrial respiration either

due to hypoxia or defective ETC complexes, the elevated intramitochondrial NADH/NAD⁺ ratio impedes isocitrate oxidative decarboxylation in the CAC (179, 180). The high NADH/NAD⁺ ratio is often associated with the mitochondrial export of citrate depots for synthesizing fatty acids, phosphoglycerides and cholesterol (179, 180). IDH1 is also a major producer of NADPH which is required for lipid synthesis and ROS detoxification (173, 181-184). Overall, this suggests that the activities and the direction of reactions catalyzed via IDH isoenzymes are governed by the NAD(P)H/NAD(P)⁺ ratios which in turn affects fatty acid and energy metabolism in both normal and malignant cells. This metabolic flexibility allows cancer cells to sustain essential anabolic processes and NADH generation for OXPHOS.

It is worth mentioning that although the tumorigenic effects of IDH1 and IDH2 mutants have been well characterized (discussed in detail below), the role of IDH3 in neoplasia has not been well studied. Elevated levels of IDH3 α , the catalytic subunit of IDH3, have been observed in GBM tumor samples as compared to normal brain tissue whereby it was found that elevated IDH3 α promotes tumor progression in glioma mouse models (185). Accordingly, overexpression of IDH3 α reduced α -KG levels thus preventing hypoxia inducible factor (HIF) 1 α degradation and enhancing tumor growth in cervical cancer models (186). However, other studies provided contradictory data regarding the association between IDH3 α expression and intracellular α -KG concentrations (187), thus suggesting that the mechanism by which IDH3 drives oncogenesis may be context-dependent.

α -KG, also known as 2-oxoglutarate, is subjected to irreversible oxidative decarboxylation by 2-oxoglutarate dehydrogenase (OGDH) enzymatic complex in the mitochondrial matrix to generate the four-carbon succinyl-CoA and the second NADH molecule of the CAC [(148) Fig. 1.3]. The OGDH complex is not only functionally analogous to the PDC, but also structurally

similar as the OGDH complex also consists of multiple copies of a thiamine pyrophosphate-dependent dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) (188). The OGDH complex requires thiamine pyrophosphate as a cofactor and is activated by calcium ions and suppressed by NADH and succinyl-CoA; two of the end products of the reaction it catalyzes (188-190). α -KG is an important cofactor for 2-oxoglutarate/iron (II)-dependent dioxygenases (OGDDs) including Jumonji catalytic (JmjC) domain containing N-methyl-lysine and N-methyl-arginine histone demethylases, which are known to epigenetically control gene expression (191). Aberrant regulation of these enzymes is implicated in many different types of cancers including breast and prostate cancer (191, 192). Hence, it is crucial for normal cells to maintain optimal intracellular concentrations of α -KG to preserve their epigenetic landscape. On the contrary, breast cancer cells appear to deplete their α -KG pool by upregulating OGDH levels and reduce α -KG into succinyl-CoA (193). This is congruent with reports demonstrating that the inhibition of OGDH results in the accumulation of α -KG which in turn increases levels of the α -KG-dependent chromatin modification 5-hydroxymethylcytosine (5hmC) (194, 195). This is mirrored by attenuated malignant progression and metastasis in p53^{-/-} pancreatic ductal adenocarcinoma and breast cancer (194, 195). Overall, this illustrates that alterations in α -KG levels result in epigenetic perturbations that play a major role in determining the fate of malignant cells.

Next, succinyl-CoA is hydrolyzed into succinate via succinyl-CoA synthetase (SCS) by substrate-level phosphorylation producing GTP in the process which is converted into ATP by mitochondrial nucleoside diphosphate kinase (NDPK) (148, 196, 197) Fig. 1.3. Succinyl-CoA also serves as a precursor in important biosynthetic pathways. For example, the 5-aminolevulinic acid, which is a condensation product of succinyl-CoA with glycine, is exported to the cytosol

which marks the first step of heme biosynthesis in the liver and erythroid cells (198). In addition to succinyl-CoA, succinate can be produced via the γ -aminobutyric acid (GABA) shunt, whereby GABA which is synthesized from glutamate by glutamate decarboxylase (GAD), is subsequently transaminated into succinic semialdehyde (SSA) through GABA transaminase (ABAT) that finally gets oxidized into succinate via SSA-dehydrogenase (SSADH) (199, 200). Although, there are limited studies on the GABA shunt in tissues other than neurons, recent findings showed that decreased expression of GAD impeded branched chain amino acid (BCAA) uptake in non-small-cell lung cancer (NSCLC) (201, 202). Furthermore, low expression of GAD reduced the proliferation of both NSCLC and castration-resistant prostate cancer cells (201, 202). These observations further highlight metabolic pathways that appear to be hijacked by transformed cells to drive tumorigenesis.

SDH, a component of complex II of the electron transport chain (ETC) located on the inner mitochondrial membrane, oxidizes succinate into fumarate [(148) Fig. 1.3]. Unlike the other CAC dehydrogenases that reduce NAD^+ , SDH produces FADH_2 (148) Fig. 1.3. The SDH complex consists of 6 subunits (SDHA, SDHB, SDHC, SDHD, SDHAF1 and SDHAF2) (203, 204). Increased levels of oxaloacetate inhibit SDH complex thereby downregulating the CAC (202). Fumarase, also referred to as fumarate hydratase (FH), catalyzes reversible hydration of fumarate into malate, while consuming water in the process [(148, 205) Fig. 1.3]. Loss-of-function mutations in the SDH complex and FH in the accumulation of succinate and fumarate, respectively (206). The accumulation of either succinate or fumarate has been associated with different cancers such as renal cell carcinoma, thus suggesting that SDH and FH exhibit tumor-suppressive properties (206).

Malate dehydrogenase (MDH) catalyzes the final enzymatic reaction which completes one turn of the CAC (148, 207). In this reaction, oxaloacetate is regenerated from malate with the concomitant reduction of NAD^+ to NADH (148, 207) Fig. 1.3. The resulting oxaloacetate can then be used for the next round of CAC (148, 207). MDH exists as two isoforms (MDH1 and MDH2) that differ in their subcellular localization and preference for cofactors (208, 209). MDH1 is found in the cytosol while MDH2 is localized in the mitochondrial matrix (208, 209). Both MDH isoforms are part of the malate-aspartate shuttle (MAS) in which MDH1 reduces cytosolic oxaloacetate into malate with the concurrent oxidation of NADH into NAD^+ while MDH2 predominantly catalyzes the reverse reaction, thus converting the mitochondrial malate pool into oxaloacetate and NADH (209). Hence, the mitochondrial malate reserves are composed of cytosolic-derived malate (imported to the mitochondria via the SLC25A11 transporter) and mitochondrial-based malate (produced by FH) (209). As such, MDH1 supports aerobic glycolysis by maintaining a high cytosolic NAD^+/NADH ratio while indirectly reducing mitochondrial NAD^+/NADH ratio. The MDH2 isoform is allosterically activated by elevated malate levels and inhibited by high concentrations of oxaloacetate (210). Elevated levels of MDH2 expression in prostate cancer have been associated with markedly shorter period of relapse-free survival post-chemotherapy (Liu et al., 2013). This suggests that aberrant MDH2 activity may be implicated in the metabolic reprogramming required for cancer cell progression (211).

Considering that the CAC enzymes evolved before the presence of oxygen on earth, it was proposed that the fundamental role of the CAC is the biosynthesis of metabolites (e.g. hemes, amino acids, nucleic acids, fatty acids) (148). However, CAC has evolved to also play a bioenergetic role by producing ATP, NADH and FADH_2 (148) Fig. 1.3. The NADH and FADH_2 subsequently feed the ETC via complex I and II respectively to ultimately generate ATP via

OXPHOS (148). Hence, under physiological conditions the CAC is coupled to OXPHOS due to the oxidation of NADH and FADH₂ (148).

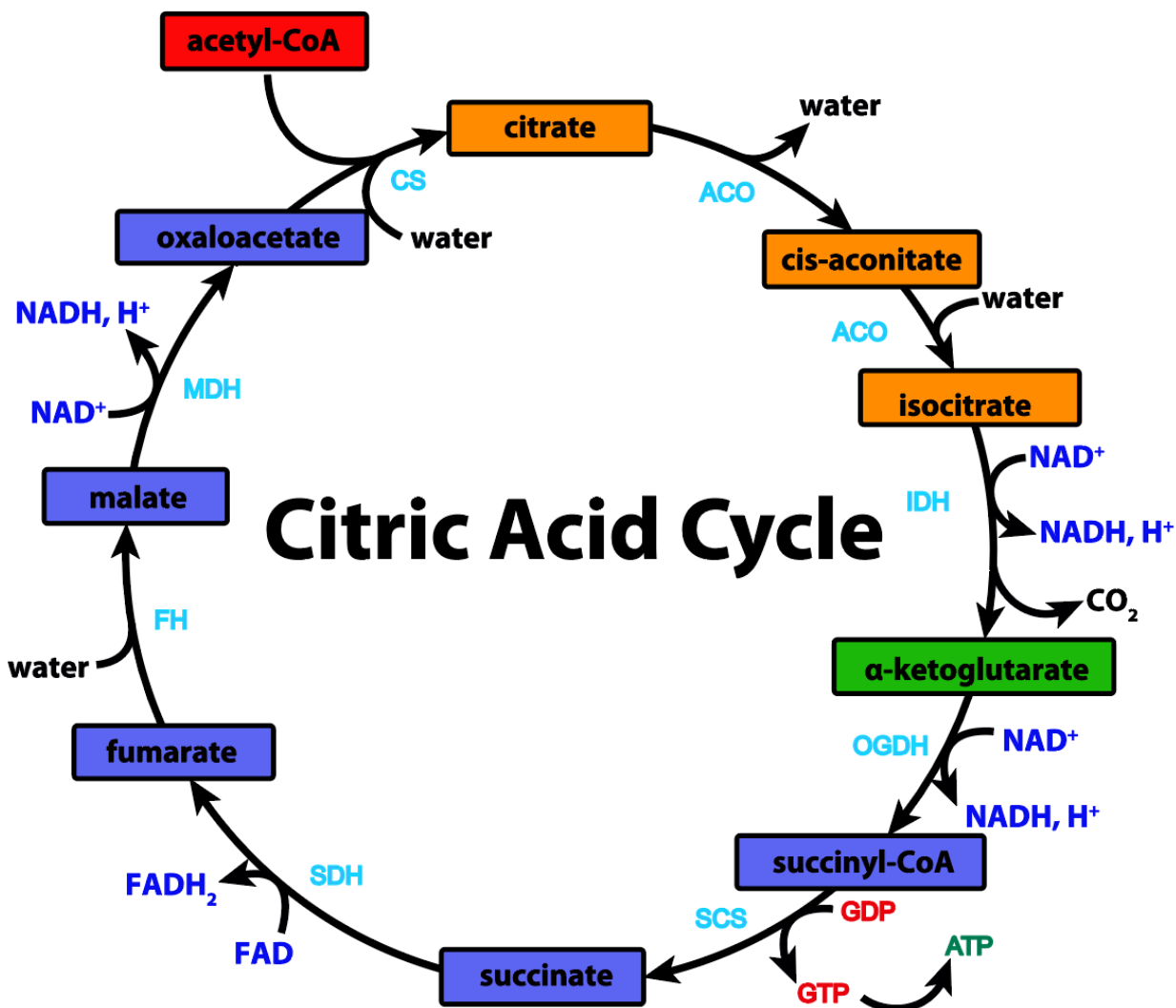


Fig. 1.3. Schematic diagram of the citric acid cycle (CAC). 2-carbon containing acetyl-CoA (shown in a red box) enters the CAC by condensing with 4-carbon oxaloacetate to produce 6-carbon citrate. Citrate is oxidatively decarboxylated through a series of enzymes to release CO₂ in the process while simultaneously producing 1 ATP molecule and reducing equivalents NADH (3) and FADH₂ (1). 6-carbon containing CAC intermediates are denoted in orange boxes while 5-carbon and 4-carbon containing CAC intermediates are represented with green and indigo boxes. The enzymes catalyzing CAC reactions are in cyan (adapted figure: George-stock.adobe.com).

1.2.4.1 Aberrant activity of CAC enzymes produces “oncometabolites”

1.2.4.1.1 2-Hydroxyglutarate (2-HG)

The intracellular levels of α -KG directly affect the activities of OGDDs including ten-eleven translocation (TET) family of DNA methylases, JmJc family of histone demethylases, prolyl hydroxylases (PHDs), AlkB homolog (ALKBH) DNA repair enzymes and fat mass and obesity-associated protein (FTO) which demethylates mRNA (212-215). OGDDs couple the oxidation of substrate to the oxidative decarboxylation of α -KG to produce succinate and CO₂ (214). Heterozygous somatic mutations observed in *IDH1* and *IDH2* have been known to indirectly modulate the activities of OGDDs (212, 216, 217). It was shown that missense mutations in *IDH1/2* genes (mostly affecting active site amino acid residues: R132 in *IDH1*, R140 and R172 in *IDH2*) result in the loss of normal enzymatic activity while attaining the ability to reduce α -KG into the D-enantiomer of 2-hydroxyglutarate (D-2-HG also known as R-2-HG) (212, 218-220). D-2-HG and L-2-HG (the L-enantiomer also referred to as S-2-HG) bind to the active sites of OGDDs as competitive inhibitors due to their structural similarities to α -KG (212, 216). Thus, these neomorphic mutations in *IDH1/2* lead to accumulation of D-2-HG which suppresses OGDDs thus leading to epigenetic alterations (via histone and DNA methylation). Considering that OGDDs play a role in regulating normal cellular differentiation, it is not surprising that *IDH1* and *IDH2* are frequently mutated genes in cancers such as glioma, acute myeloid leukemia (AML) and chondrosarcoma whereby *IDH1* and *IDH2* mutations are often associated with gene silencing and dedifferentiation of cells (221-223). This is consistent with findings that demonstrate either exogenously expressing mutant *IDH1/2* or treating non-transformed cells with cell-permeable D-2-HG impedes cell differentiation and inhibits H3K9 demethylases (224, 225).

Interestingly, D-2-HG and L-2-HG can accumulate in cells with wildtype *IDH1/2* genes (179, 226, 227). Studies have revealed that α -KG can be converted to D-2-HG by hydroxyacid-oxoacid transhydrogenase (HOT) while simultaneously producing SSA from γ -hydroxybutyrate (GHB) in normal cells (228, 229). Furthermore, overexpression of 3-phosphoglycerate dehydrogenase (PHGDH) in breast cancer, which catalyzes the first reaction of serine biosynthesis to produce 3-phosphohydroxypyruvate by NAD⁺-coupled oxidation of 3-PG has been suggested to reduce α -KG to D-2-HG (230). Although both D-2-HG and L-2-HG can be produced from α -KG, L-2-HG can be formed by LDHA and MDH1/2 in acidic pH and/or when oxygen is limited (231-234). Taken together, the studies suggest that cancer cells utilize L-2-HG and D-2-HG to reprogram epigenome and facilitate tumor progression (227, 235).

Under physiological conditions, the harmful effects of D-2-HG and L-2-HG are prevented as they are converted to α -KG by D-2-hydroxyglutarate dehydrogenase (D2HGDH) and L-2-hydroxyglutarate dehydrogenase (L2HGDH) respectively (236-238). Although intracellular levels of 2-HGs are kept at micromolar range by 2HGDHs, the millimolar levels of D-2-HG observed in patients diagnosed with type II D-2-hydroxyglutaric aciduria (D-2-HGA), AML and glioblastoma due to mutations in *IDH1/2* genes exceeds the catalytic ability of D2HGDH which leads to accumulation of D-2-HG in the plasma, urine and cerebrospinal fluids (216, 236, 239-242). In addition, loss-of-function mutations in *D2HGDH* and *L2HGDH* are responsible for autosomal recessive neurometabolic disorders, type I D-2-HGA and L-2-hydroxyglutaric aciduria (L-2-HGA) respectively (237, 242). Intriguingly, patients diagnosed with L-2-HGA are predisposed to developing brain tumors while patients diagnosed with D-2-HGA appear not to exhibit increased cancer incidence (241, 243). Of note, type II D-2-HGA patients which are characterized by mutations in *IDH1/2* typically present with more D-2-HG built-up in their plasma, urine and

cerebrospinal fluid as compared to type I D-2-HGA patients having inactivated D2HGDH (241, 242). Nonetheless, the mortality of patients with severe D-2-HGA is exceptionally high during infancy (241, 242). This significantly confounds potential comparisons of cancer susceptibility between different types of D-2-HGA (241, 242). Furthermore, considering that L-2-HG is five to ten times more potent than D-2-HG in suppressing OGDDs, this may potentially explain the discrepancy between L-2-HGA and D-2-HGA in cancer predisposition. Moreover, even in the absence of IDH1/2 mutations, elevated levels of D-2-HG and L-2-HG have been observed in different cancer types such as colorectal cancer, breast cancer, renal cell carcinoma and diffuse large B-cell lymphoma (235). Notwithstanding that the molecular underpinnings of 2-HG enantiomer function(s) in neoplasia remain incompletely understood, both enantiomers are likely to play a prominent role in tumorigenesis and tumor progression.

1.2.4.1.2 Succinate

Other than 2-HGs, OGDDs are susceptible to competitive inhibition by succinate due to its structural similarity to α -KG (191, 244). Inactivating mutations found in some of SDH complex subunits (SDHB, SDHC, SDHD and SDHAF2) lead to a buildup of succinate which is commonly observed in familial and sporadic paraganglioma and pheochromocytomas (245-248). Similarly to 2-HG, succinate acts as an oncometabolite by attenuating OGDD functions which includes abrogating the epigenetic remodeling by JmjC domain-containing histone demethylases and the TET family of DNA hydroxylases (244). Also, OGDDs like PHDs (i.e., PHD1-3) and factor inhibiting HIF (FIH) regulate HIF- α levels in response to alterations in intracellular oxygen tension. Namely, in normoxia specific proline (P402 and P564 in HIF-1 α ; P405 and P531 in HIF-2 α) and asparagine (N803 in HIF-1 α and N851 in HIF-2 α) residues in the HIF- α are hydroxylated by PHDs and FIH respectively to facilitate polyubiquitinylation and proteasomal degradation

mediated by the tumor suppressor von-Hippel-Lindau (VHL)-E3 ubiquitin ligase (249-254). As discussed in a greater detail below, HIF-1/2 regulates genes that are involved in both systemic responses to hypoxia, such as angiogenesis and erythropoiesis, and in cellular responses, such as alterations in glucose metabolism (255).

Mutations in *SDHD* and *SDHB* genes (which are commonly observed in hereditary paraganglioma and pheochromocytoma, respectively), activate hypoxic and angiogenic pathways, which is mediated by elevated succinate levels (255-257). These loss-of-function mutations in SDH subunits abolish the activity of the SDH complex leading to the accumulation of succinate in the mitochondrial matrix that consequently leaks into the cytosol to inhibit PHDs, ultimately stabilizing HIF-1/2 α (256, 258). Moreover, reduced expression of *SDHD* has been observed in gastric and colorectal carcinoma while patients with mutations in *SDHB* and *SDHC* are susceptible to developing early-onset renal cell or papillary thyroid carcinoma (259-262). Thus, defects in the SDH complex which are often associated with succinate buildup, lead to a “pseudohypoxic” state wherein HIF is activated even under normoxia. Collectively, mutations in SDH subunits result in accumulation of succinate that favors tumorigenesis and tumor progression via epigenetic dysregulation, and aberrant activation of HIF.

1.2.4.1.3 Fumarate

Inactivating mutations in the homotetrametric enzyme FH leads to the accumulation of fumarate which is commonly observed in patients diagnosed with fumaric aciduria, an autosomal recessive metabolic disorder typically associated with severe encephalopathy, as well as hereditary leiomyomatosis and renal cell cancer (HLRCC) (263, 264). Similar to the *SDH* genes, *FH* mutations are often germline, and are associated with the loss of the wild-type allele and decreased FH activity in the tumors (263, 265). FH is therefore regarded as a tumor

suppressor because decrease in its activity causes the intracellular buildup of the oncometabolite, fumarate, which akin to 2-HG and succinate competitively inhibits OGDDs including PHDs, histone demethylases and TET proteins (244, 266). HLRCC tumors in patients with germline FH mutations have been reported to increase not only HIF-1 α and HIF-2 α but also their gene targets such as *VEGFA* and *SLC2A1* which suggests that fumarate-mediated inhibition of HIF-1/2 α PHDs confers a survival advantage to HLRCC tumors (267). Additionally, recent findings show that the loss of FH and the subsequent accumulation of fumarate promotes EMT by inhibiting TET-mediated demethylation of the antimetastatic miRNA cluster miR-200, which results in the expression of EMT-related transcription factors (e.g., ZEB2) and markers (e.g., vimentin) (268). Overall, these studies demonstrate that accumulation of fumarate caused by mutations in *FH* promotes tumor growth and progression in the context of neoplasia of the kidney and potentially other malignancies.

1.2.4.2 CAC anaplerosis and cataplerosis

CAC intermediates serve as precursors for producing macromolecules such as succinyl-CoA for heme biosynthesis, citrate for fatty acid synthesis and oxaloacetate for aspartate and nucleic acid synthesis (269). The consumption of CAC intermediates is referred to as cataplerosis. In order to maintain CAC, which is necessary for generating the reducing equivalents (NADH and FADH₂) and energy required to sustain cell growth and proliferation, intermediates utilized in cataplerotic processes must be replenished (269). This process of replacing depleted pools of CAC intermediates is termed anaplerosis. Under normal physiological conditions, cataplerotic and anaplerotic reactions are coordinated to maintain the levels of CAC intermediates for biosynthetic and bioenergetic pathways in the cell (269, 270) .

The anaplerotic and cataplerotic metabolic pathways are organ-specific and are dependent on the organismal metabolic state (270). GTP-dependent phosphoenolpyruvate carboxykinase (PCK) isozymes (PCK1 localized in the cytosol and PCK2 localized in the mitochondrial matrix) catalyze the decarboxylation of oxaloacetate to PEP used for *de novo* glucose synthesis (gluconeogenesis) in the liver and kidneys (269). In skeletal muscles, PEP can be converted back to pyruvate that subsequently enters the CAC via acetyl-CoA (270). During fasting, characterized by low levels of insulin and elevated levels of glucagon and glucocorticoids, *PCK1* mRNA is upregulated to promote hepatic gluconeogenesis (271, 272). In addition, PCK1 activity is detected in nongluconeogenic tissues such as white and brown adipose tissues (273). It has been proposed that during glucose starvation, increased cAMP levels stimulate PCK1 transcription which indirectly mediates the synthesis of 3-glycerol phosphate (glyceroneogenesis) to form oxaloacetate needed for the re-esterification of free fatty acids in adipose and hepatic tissues released as very low-density lipoproteins (274, 275). An anaplerotic counterpart of PCK exists to prevent the extensive efflux of oxaloacetate from the CAC. Namely, the tetrametric ATP-dependent pyruvate carboxylase (PC) carboxylates pyruvate to form oxaloacetate which represents a key anaplerotic pathway whereby alanine and lactate are metabolized into pyruvate to replenish depleted oxaloacetate pools for gluconeogenesis, the urea cycle and lipogenesis (269, 276). PC is predominantly localized in the mitochondrial matrix and is highly catalytically active in many organs including liver, skeletal muscles, heart and pancreas (269). Considering that PC is allosterically activated by acetyl-CoA which is the key metabolite in fatty acid synthesis and β -oxidation, it suggests that fatty acid metabolism is linked to the regulation of anaplerotic flux via oxaloacetate and energy availability for gluconeogenesis (269, 277). Overexpression and/or elevated PC activity have been demonstrated to contribute to metabolic reprogramming and tumor

progression in several cancer models including NSCLC, breast and papillary thyroid cancer (278-280). Curiously, PCK2, the cataplerotic partner of PC, is also overexpressed in lung and breast cancer (281, 282). Glucose deprivation was shown to bolster the expression of PCK2 and increase PC activity in lung cancer models while being associated with the increased conversion of lactate to pyruvate and subsequently into PEP via oxaloacetate (281, 283). Also, amino acid starvation augments PCK2 expression required for the survival breast cancer cells in the context of amino acid withdrawal (282). These findings suggest that cancer cells may activate gluconeogenic and/or glyceroneogenic pathways previously assumed to be restricted to specialized tissues (adipose, liver and kidney) in order to survive nutrient scarcity. Although, these pathways eventually result in the production of glucose (via gluconeogenesis) and glycerol (via glyceroneogenesis), it is worth underscoring the importance of metabolic intermediates along these pathways which serve as precursors for building biomass in cancer cells. Finally, these findings illustrate high metabolic flexibility of cancer cells that allows them to adapt to fluctuations in TME.

1.2.4.2.1 CAC anaplerosis and glutamine metabolism

After glucose, glutamine is the second most consumed nutrient and most abundant amino acid in the blood stream making up over 20-25% of the free amino acid pool found at concentrations of 0.5-0.8 mM (284-286). Although digested food can be absorbed through the small intestine and metabolized to produce glutamine, most of the glutamine is catabolized in the small intestine and liver (286). Since skeletal muscles, adipose tissues and lungs are capable of synthesizing glutamine, glutamine is considered a non-essential amino acid (NEAA) (286). In skeletal muscles, the cytosolic concentration of glutamine ranges from 10 mM to 30 mM and serves as an important glutamine reserve that is readily released into the plasma in response to stress (287). Under certain conditions including injury or sepsis, glutamine uptake by kidneys,

gastrointestinal tract and immune cells is markedly elevated which renders glutamine a conditionally essential amino acid (288). Intestinal cells are particularly dependent on glutamine as they quickly become necrotic when deprived of glutamine (288). Unlike other NEAA, glutamine has a broad range of functions which include donating the amide nitrogen needed for *de novo* synthesis of nitrogen-containing metabolites (e.g. asparagine, purines and pyrimidines), fueling OXPHOS through the oxidative decarboxylation of glutamine-derived α -KG via the CAC, and serving as a substrate for ureagenesis and ammoniagenesis in liver and kidney, respectively (286). In addition, glutamine is utilized for ROS detoxification, acid-base buffering and gluconeogenesis (286, 289, 290). Essentially, under physiological conditions, glutamine is a source of carbon and nitrogen atoms to support a variety of metabolic pathways while indirectly maintaining systemic acid-base pH homeostasis.

Glutamine is imported into cells through several amino acid transporters including SLC1A5 (ASCT2) as well as SLC38A1 (SNAT1) and SLC38A2 (SNAT2) (291, 292). Glutamine can then be transported across the mitochondrial membranes by lesser-known and inadequately characterized mitochondrial glutamine transporters into the matrix to be deamidated by glutaminase (which is encoded by 2 genes: kidney-type *GLS* and liver-type *GLS2*) into glutamate and ammonia (293-295). Subsequently, mitochondrial glutamate is either further deamidated and oxidized to α -KG by glutamate dehydrogenase (GLUD) or transaminated into α -KG via amino acid transaminases such as alanine transaminases (ALT2 or GPT2) and aspartate transaminases (AST2 or GOT2) (296, 297). Alternatively, mitochondrial glutamate can be exported through SLC25A18 and SLC25A22 transporters to the cytosol where it participates in several metabolic activities that include serving as an exchange factor for extracellular cystine import via SLC7A11 (xCT), and being a precursor for *de novo* synthesis of other amino acids [e.g., alanine, aspartate,

serine and proline catalyzed by cytosolic ALT1, AST1, phosphoserine aminotransferase 1 (PSAT1) and pyrroline-5-carboxylate synthase (P5CS), respectively] (289, 298, 299). Moreover, glutamine serves as a substrate for biosynthesis of glutathione, a tripeptide ROS scavenger composed of glutamate, cysteine and glycine (289, 298, 299). In addition, glutamine-derived α -KG is implicated in a variety of the bioenergetic and biosynthetic activities of the CAC (289). α -KG also serves as a cofactor for OGDDs and drives the malate-aspartate shuttle via its role as an exchange factor (289). Overall, glutamine regulates a wide range of cellular activities as a direct or indirect substrate (via the functions of glutamate and α -KG) of key metabolic pathways and/or by acting as a cofactor in pivotal enzymatic reactions.

Similar to glucose, glutamine is thought to be frequently exploited by cancer cells to support neoplastic growth (300). The excessive demand for glutamine by cancer cells was first described by Harry Eagle when he demonstrated that for optimal growth, HeLa cells require 10-100-fold molar excess of glutamine relative to other amino acids in culture medium (301). Some cancer cell lines including those derived from pancreatic cancer, acute myelogenous leukemia, and small cell lung cancer have been shown to be “addicted” to glutamine inasmuch as they are strongly dependent on exogenous glutamine despite the fact that they can synthesize it from glucose (302). Glutamine metabolism is also upregulated in glioblastoma but also in non-transformed activated proliferating T-lymphocytes (39, 303). Interestingly, glutamine was found to activate glucose metabolism in cancer cells in which CAC intermediates are used as precursors for synthesizing other amino acids, nucleotides and fatty acids (39). In the absence of glucose, glutamine metabolism can be elevated in association with increased malic enzyme 1 (ME1) and PCK2 activities in an attempt to compensate for the loss of glucose as a bioenergetic and biosynthetic source in B-cell lymphoma and lung cancer cell lines (283, 304). It was also observed

that α -KG produced from glutamine was oxidatively decarboxylated in the CAC to produce either oxaloacetate that is subsequently converted to PEP by PCK2 or malate by MDH2 (39, 283, 305). PEP and malate are then exported to the cytosol for pyruvate synthesis (39, 283, 305). Cytosolic malate is oxidatively decarboxylated by ME1 to yield NADPH needed for fatty acid synthesis and pyruvate which is reduced to lactate via LDHA (39). In addition, it was reported that in pancreatic ductal adenocarcinoma (PDAC) cells glutamine-derived aspartate is transported into the cytosol to be converted into oxaloacetate by AST1 that is then reduced into malate by MDH1 and finally decarboxylated into pyruvate via NADPH-producing ME1 (306). The production of NADPH through this metabolic route is used to reduce glutathione for detoxifying ROS, hence highlighting the role of glutamine metabolism in maintaining cellular redox homeostasis (306).

Inside the mitochondria, the metabolic fate of glutamine-derived α -KG is closely associated with CAC enzymes and ETC complex activities or lack thereof (180, 307-309). Brown adipocytes reductively carboxylate glutamine-derived α -KG into isocitrate which is then converted into citrate and exported to the cytosol for *de novo* lipid synthesis (310). This cataplerotic route accounted for 90% of the total flux of glutamine into lipids in brown adipocytes (310). In the context of mitochondrial dysfunctions (including mutations in either FH or mitochondrial DNA, genetic lesions or pharmacological inhibition of complexes I or III of the ETC), α -KG produced from glutamine has been shown to be reductively carboxylated to allow lipogenesis and aspartate synthesis in rapidly proliferating malignant cells (180, 307, 311, 312). Furthermore, hypoxia has been demonstrated to promote the reductive carboxylation of glutamine-generated α -KG into isocitrate via IDH1 (313) or IDH2 in melanoma (314), with the concomitant elevated synthesis of 2-HG in several cancer cell lines with wildtype IDH1 and IDH2 (179). Furthermore, glutamine is the primary source of α -KG catalyzed into 2-HG by mutant IDH1 in glioma cells (308). This

preferential use of glutamine-derived α -KG is thought to constitute a targetable metabolic vulnerability in mutant IDH1-expressing cells as they are more sensitive to GLS inhibition as compared to WT IDH1-expressing cells (308).

Although many tumor cell types profoundly depend on glutamine metabolism to refill their diminished pools of CAC intermediates, it has been reported that the inhibition of glutaminolysis in some glioblastoma cell lines can induce a compensatory CAC anaplerotic mechanisms in which PC carboxylates glucose-derived pyruvate into oxaloacetate (315). Hence, glucose-dependent CAC anaplerosis via PC allows cells to become “independent” of glutamine. Conceivably, this could be, at least in part, an underlying mechanism of resistance against drugs targeting glutaminolysis (315). Additionally, *de novo* glutamine synthesis via glutamine synthetase (GLUL) activity in which glutamine is produced from the co-substrates, glutamate and ammonia, may render cells glutamine independent. GLUL expression has been associated with enhanced metastatic potential of HCC, while induction of GLUL expression by glutamine starvation promotes nucleotide synthesis and amino acid transport thereby maintaining cell survival and proliferation of glioma and breast cancer cell lines (316-318). Overall, these findings imply that metabolic rewiring caused by glutamine deprivation may decrease sensitivity of certain cancer subtypes to the therapies targeting glutamine metabolism.

1.2.5 ETC and OXPHOS

NADH and FADH₂ reducing equivalents that are largely generated via CAC, are chiefly oxidized in the ETC through a series of redox reactions that create an electrochemical gradient which is harnessed to produce ATP by OXPHOS (48, 319, 320). The ETC comprises of free electron carriers (ubiquinone and cytochrome C) and 4 bound protein complexes (I-IV) that are embedded within the inner mitochondrial membrane in close proximity to the mitochondrial

matrix where the most of CAC reactions are catalyzed (48). The reaction sequence begins with NADH and FADH₂ donating electrons to complex I (NADH-ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) respectively (48, 320). Electrons are subsequently transferred to ubiquinone (CoQ) followed by complex III and ultimately to complex IV via cytochrome C (48, 319, 320). Complex IV reduces oxygen (the final electron acceptor) to produce water in the process (48, 320). The movement of electrons along the ETC is coupled to protons being pumped into the intermembrane space by complexes I, III and IV (319, 320). The translocation of protons from the mitochondrial matrix to the intermembrane space creates a proton motive force, a proton gradient, used to drive the synthesis of ATP from ADP by complex V, ATP synthase (319, 321). Thus, the OXPHOS system includes the 4 complexes of the ETC which generates the proton motive force in addition to complex V which produces ATP.

1.2.5.1 Mammalian Complex I

Mammalian complex I, which is also referred to as NADH dehydrogenase, is the first and largest multicomponent enzyme complex of the ETC composed of 45 different subunits with a combined molecular mass of approximately 1 MDa (319, 322). As the name implies, complex I oxidizes NADH to NAD⁺, which involves the transfer of electrons from NADH to ubiquinone (CoQ) (323). Specifically, two electrons are transferred from NADH through a chain of cofactors including flavin mononucleotide and a series of eight iron-sulfur clusters arranged from low to high electron potentials, to CoQ consequently producing ubiquinol (QH₂) in the inner mitochondrial membrane (322, 323). This translocation of electrons, one at a time from NADH to CoQ, induces a conformational change in the complex that facilitates the pumping of four protons from the mitochondrial matrix to the intermembrane space (322, 324). It is thought that complex I generates approximately 40% of the proton flux (323). Defects in the complex I have been

associated with neurodegenerative diseases including Leigh's and Parkinson's disease (325-327). The role of complex I in tumorigenesis has been controversial whereby it was suggested that inactivation of complex I has both tumor-suppressive and tumor-promoting effects (328-330). Notably, these findings were obtained using models bearing different mutations in the various subunits of complex I. Thus, it is plausible that the discrepancy between the aforementioned studies stems from diverse genetic alterations that although impacting the function of complex I may not result in a common phenotype.

1.2.5.2 Mammalian Complex II

In parallel to complex I, electrons are fed into the ETC via complex II (SDH) which is also a component of the CAC (331). Structurally, SDHC and SDHD contain the CoQ binding site and are embedded in the inner mitochondrial membrane to anchor the entire complex (332, 333). The succinate binding pocket along with the iron-sulfur clusters and a flavoprotein are covalently attached to a FAD cofactor and are both found in SDHA and SDHB subunits which extend into the mitochondrial matrix (319, 334). Complex II converts FADH₂ to FAD while simultaneously reducing CoQ into QH₂ (331). Similar to complex I, electrons are transferred sequentially from FADH₂ to CoQ via iron-sulfur clusters (335). Unlike complex I, electron transport from FADH₂ to CoQ is not accompanied by proton translocation from the matrix to the intermembrane space (335). As a result of bypassing complex I, electrons transferred from complex II to CoQ forego the opportunity to be coupled to proton ejection by complex I.

Except for complex I and II, other lesser-known enzymes donate electrons to the CoQ pool (336). They include the electron-transferring flavoprotein (ETF)-ubiquinone oxidoreductase, the mitochondrial glycerol-3-phosphate dehydrogenase and the dihydroorotate dehydrogenase (DHODH) enzyme (337). The ETF-ubiquinone oxidoreductase enzyme is located on the

mitochondrial matrix-facing side of the inner mitochondrial membrane (338). ETF transfers electrons generated during the oxidation of fatty acids, branched chain amino acids, lysine, tryptophan and choline (338, 339). The mitochondrial glycerol-3-phosphate dehydrogenase is a component of glycerophosphate shuttle with a prominent role in linking glycolysis, OXPHOS and fatty acid metabolism. Moreover, the DHODH catalyzes a rate-limiting of the *de novo* pyrimidine synthesis pathway whereby dihydroorotate is converted to orotate (336, 340-342).

1.2.5.3 Mammalian Complex III

The electrons from QH₂ are donated to the dimeric complex III (referred to as cytochrome bc₁ complex or CoQ-cytochrome c reductase) of the ETC which is localized in the inner membrane of the mitochondria (319, 320). The catalytic subunits of the complex are cytochrome b (b_L and b_H), cytochrome c₁ and a high electron potential iron-sulfur cluster in the Rieske center (319, 320). Cytochrome b has two CoQ binding sites: (i) a QH₂ oxidation site (Q₀) located on the cytosolic side which is associated with the low potential cytochrome b_L and (ii) a Q[•] reduction site (Q_i) found on the mitochondrial matrix side and linked to the high potential cytochrome b_H (343). The oxidation of QH₂ into ubisemiquinone (QH[•]) after transferring an electron to the Rieske center is coupled with the release of two protons into the mitochondrial intermembrane space (344). The electron is subsequently transferred to cytochrome c₁, which then provides the electron to the mobile electron carrier, cytochrome c (344). Newly formed QH[•] found at the Q₀ site donates the second electron to cytochrome b_H at the Q_i site via cytochrome b_L (319). Next, the electron is lost to CoQ of the Q_i site from the reduced cytochrome b_H to regenerate QH[•] (319). A second QH₂ molecule is oxidized at the Q₀ site which leads to the ejection of two more protons into the intermembrane space (319). This is accompanied by one additional electron being transferred to the iron-sulfur cluster while the second electron is transferred by cytochrome b_H to QH[•] of the Q_i

site to yield QH₂ (319). Complex III deficiency underpins neurological disorders including Leigh syndrome and Leber hereditary optic neuropathy (LHON), whereby aberrant complex III function has also been observed in breast, colorectal cancer and lung cancer (345-348). This suggests that complex III may play a prominent role in neoplasia

1.2.5.4 Mammalian Complex IV

Functionally similar to CoQ, cytochrome c is loosely attached to the outer surface of the inner mitochondrial membrane by electrostatic interactions and shuttles electrons from complex III to complex IV (cytochrome c oxidase) where molecular oxygen is reduced to water (319-321). Complex IV is composed of 13 subunits with the 3 core components (*COX1*, *COX2* and *COX3*) encoded by the mitochondrial DNA (347). Considering the complexity of Complex IV and the fact that its subunits are assembled on both sides of the membrane, the enzyme complex requires 18 assembly factors, many of which have not been well-characterized (347, 349). The complex IV also contains cofactors (heme and copper) embedded into the core proteins, *COX1* and *COX2* (349). *COX1* has 2 heme molecules and a copper ion in the Cu_B site, while *COX2* has a Cu_A site formed by 2 copper ions (349). After cytochrome c is reduced by complex III, it moves along the surface of the membrane to interact with the *COX2* subunit of complex IV via electrostatic interaction while simultaneously transferring the electrons to the Cu_A site of the *COX2* subunit (319). The electrons are subsequently transported from Fe_a to the active binuclear center (Fe_{a3} and Cu_B) of *COX1* where oxygen is bound and converted to water (319). *COX3* is located on both sides of *COX1* along with *COX2*, stabilizes both *COX1* and *COX2* and is primarily involved in pumping protons into the intermembrane space (319, 335). Analogous to complex I and III, the donation of four electrons at a time to oxygen is coupled to the translocation of eight protons from the mitochondrial matrix (350). The four protons are used to form two water molecules while the

remaining protons enter the intermembrane space (350). As suspected, defects in mitochondrial complex IV resulting in decreased enzyme complex activity have been linked to neurodegenerative diseases such as LHON, Leigh-like syndrome and infantile-onset encephalopathy (347). Furthermore, mutations or aberrant expression of complex IV subunits, which either elevates or diminish the activity of the complex have been associated with shorter progression-free survival and increased invasiveness in different types of cancers including colorectal cancer, glioma, breast and esophageal cancer (351). The disruption of complex IV function was also demonstrated to induce Warburg Effect and enhance the metastatic potential of non-transformed skeletal myoblasts (351). This suggests that complex IV dysfunction may be crucial to metabolic reprogramming and tumor progression in a number of cancer subtypes.

1.2.5.5 Mammalian Complex V (F_1F_0 ATP synthase)

Altogether, complexes I, III and IV pump a total of ten protons into the intermembrane space generating an electrochemical proton gradient known as the mitochondrial membrane potential (335). The mitochondrial membrane potential together with the proton concentration creates a proton-motive force that is essential for energy production during OXPHOS because it couples the ETC complexes to the catalytic activity of complex V (335). Complex V comprises two functionally distinct multisubunit domains: an extra-membranous F_1 domain situated in the mitochondrial matrix and a transmembrane F_0 domain located in the inner mitochondrial membrane (319, 335). Protons are translocated into the mitochondrial matrix from the intermembrane space via the F_0 to F_1 domains (335). The movement of protons and the transfer of energy are coupled to a conformational change in the complex leading to the phosphorylation of ADP with inorganic phosphate to form ATP at the F_1 domain (335). Alternatively, complex V can hydrolyze ATP to drive the efflux of protons from the mitochondrial matrix into the

intermembrane space in order to sustain the proton-motive force (347, 352). This reverse-mode of complex V is typically observed in mitochondria with impaired respiration or leaky inner membranes (347, 352). Importantly, the persistent reverse-mode operation of complex V could deplete cellular ATP reserves, inevitably driving the cell into an energy crisis which could be exacerbated if there is an increased cellular demand for ATP (352, 353). Mutations in mtDNA genes encoding two of the F_0 subunits, ATP6 and ATP8 have been observed in patients with LHON, breast cancer and osteosarcoma (347, 354, 355). Surprisingly, the exogenous expression of mutant ATP6 T8993G in prostate cancer PC3 cell line through cybrid transfer was demonstrated to significantly impair mitochondrial ATP synthesis and result in bigger tumors in mice compared to wildtype ATP6 cybrids (356). Moreover, the decreased expression of the α subunit of complex V in colon cancer cells was associated with decreased sensitivity to chemotherapy (357). Altogether, these findings suggest that impaired OXPHOS due to the abrogation of complex V may play an important role in tumorigenesis and chemoresistance.

1.2.5.6 Reactive oxygen species (ROS)

Under physiological conditions, 0.2-2% of the electrons leak out from ETC complexes and interact with oxygen to produce ROS (319). Thus, low levels of ROS are inevitably generated as byproducts of cellular respiration (335). The main sites for ROS production in the ETC are complexes I and III where electrons leak to partially reduce oxygen to superoxide anion (335, 358). The rate at which superoxide anion is generated is dependent on the concentration of the one-electron donors and the rate at which these donors react with oxygen (335). When the iron-sulfur centers and flavin mononucleotide site of complex I accept electrons from NADH, they can react with oxygen to produce superoxide anion (335). Hence, the production of ROS is closely associated with the $NAD^+/NADH$ ratio, considering that a buildup of NADH which is usually

observed when the ETC is disrupted results in elevated levels of superoxide at complex I (335). The QH_2 oxidation site of complex III is another prominent location of superoxide anion production that creates an unstable $\text{QH}^\cdot$ intermediate that can react with oxygen to produce superoxide anion in both the mitochondrial intermembrane space and matrix (319, 335). Accordingly, inhibitors of complex I (e.g., rotenone and piericidin) and III (e.g., antimycin A) which block electron transfer in the ETC increase ROS production (319, 359).

ROS act as second-messengers in signaling pathways mediating various intracellular processes including autophagy and apoptosis (360, 361). Elevated ROS levels, however, are toxic to cells (319, 347). ROS damage DNA, lipids and proteins (323, 347). To counteract potentially deleterious effects of ROS, the cell utilizes antioxidant systems (335). To this end, the highly reactive and unstable super oxide anion is quickly converted to the more stable hydrogen peroxide by manganese SOD (Mn-SOD) localized in the mitochondrial matrix or copper/zinc SOD (Cu,Zn-SOD) located in the intermembrane space (335). The importance of Mn-SOD is underscored by the findings associating the loss of this enzyme with perinatal lethality (362, 363). The amount of hydrogen peroxide diffused out (or exported via aquaporins) of the mitochondria is dependent on the activities of catalase (commonly found in the mitochondria of heart and liver tissues), glutathione peroxidase/reductase (GPx) system and thioredoxin peroxidase/reductase system located in the mitochondrial matrix (335, 358). GPx uses hydrogen peroxide to oxidize glutathione (GSH) to glutathione disulfide (GSSG) (335, 364). Hydrogen peroxide also oxidizes the cysteine residue in the catalytic site of peroxiredoxin (PRx) that is subsequently reduced by thioredoxin (TRx) (335). Notably, both GPx and TRx antioxidant systems require NADPH for their reductive activities and thus, indirectly dependent on the various metabolic pathways that generate NADPH (335). Increased levels of ROS have been observed in the context of mitochondrial dysfunction

and suggested to drive bladder and prostate cancer progression (356, 365). Although heightened ROS levels promote DNA damage and genomic instability, the precise underlying mechanism of the role of ROS in tumorigenesis remains largely elusive.

1.2.6 Amino acids metabolism in cancer

In addition to glutamine, the levels of other NEAAs including serine, glycine, glutamate, alanine, aspartate and asparagine have been shown to have a major impact on cancer cell fate (298, 366). Although there are many well-characterized metabolic pathways to biosynthesize NEAAs, highly proliferative malignant cells occasionally require exogenous reserves of NEAAs to meet their high bioenergetic and biosynthetic demands. Consistent with this, emerging data indicate that production and availability of NEAAs play a central role in tumorigenesis, tumor progression and therapeutic responses across a broad range of cancers.

1.2.6.1 Aspartate

The CAC plays a prominent role in amino acid metabolism by providing metabolic precursors such as oxaloacetate and α -KG for the biosynthesis of various NEAAs (366). Aspartate is generated from oxaloacetate and glutamate-derived nitrogen by cytosolic (AST1) and mitochondrial (AST2) isoforms of glutamate oxaloacetate transaminase (366). Aspartate is involved in transferring electrons from the cytosol to the mitochondria via the malate-aspartate shuttle (MAS) which involves the exchange of mitochondrial aspartate for cytosolic glutamate and malate through the aspartate-glutamate carrier 1/2 (AGC1/2 or SLC25A12/SLC25A13 respectively) and malate- α -KG antiporters (OGC/SLC25A11) respectively (366, 367). Since the plasma concentration of aspartate is low and it is inefficiently imported into most cancer cells, *de novo* synthesis via ASTs is considered to be the primary source of aspartate in cancer cells (366). Recent reports have demonstrated that one of the major functions of mitochondrial respiration in

cancer cells is to support aspartate biosynthesis (311, 312). As aspartate is essential for nucleotide synthesis, it plays a key role in cellular proliferation (298). Therefore, aspartate availability is positively correlated with *in vivo* tumor growth whereas the inhibition of aspartate biosynthesis suppresses neoplastic growth in both breast and pancreatic cancers (306, 311, 312, 368, 369). In response to ETC inhibition, some cancer cell lines (e.g., ovarian cancer COLO-704, breast cancer MDA-MB-157 and acute monocytic leukemia NOMO-1) are able to maintain their intracellular aspartate levels by upregulating the expression of aspartate importers, SLC1A3 or SLC1A2 (370). Altogether, these findings indicate that aspartate biosynthesis plays a major role in metabolic reprogramming of cancer, whereby increase in aspartate levels provides malignant cells with flexibility to adapt to the defects in mitochondrial functions.

1.2.6.2 Asparagine

Asparagine is another NEAA that is thought to be indispensable for malignant cell proliferation under certain conditions including glutamine deprivation (366). In cancer cells, asparagine was found to enhance the expression of GLUL, the rate limiting enzyme for *de novo* glutamine synthesis (371). Intriguingly, albeit asparagine supplementation did not restore the levels of other NEAAs or CAC intermediates, it increased glutamine synthesis and breakdown leading to the recovery of global protein synthesis (371). Through its role as an amino acid exchange factor, asparagine also mediates amino acid uptake wherein intracellular asparagine is exchanged for extracellular amino acids (serine, histidine and arginine) (372). Ubiquitously expressed ATP-dependent asparagine synthetase (ASNS) irreversibly converts aspartate into asparagine by utilizing the amide nitrogen from glutamine (373). ASNS expression is induced in response to metabolic stressors including glutamine and glucose deprivation and ER stress which is mediated by the GCN2-EIF2 α -ATF4 and PERK-EIF2 α -ATF4 axis (298, 373, 374). Acute

lymphoblastic leukemia (ALL) cells frequently do not express functional ASNS and are thus reliant on exogenous asparagine (375, 376). Based on this, L-asparaginase that degrades and thus, limits availability of the asparagine is used as a first line therapy in ALL (377-379). In addition, high expression of ASNS is observed in castration-resistant prostate cancer, glioma and neuroblastoma wherein it correlates rapid tumor progression and poor prognosis (380, 381).

Notably, human cells do not express asparaginase and are thus incapable of breaking down asparagine (382, 383). Although the exogenous expression of ASNS enhances glutamine and nucleotide synthesis while inducing CAC anaplerosis, it suppresses proliferation and survival of cancer cells in the absence of exogenous glutamine (371). This suggests that the lack of asparaginase activity in humans may be an evolutionary adaptive mechanism to survive abnormal fluctuations in plasma glutamine levels (371). Recent reports indicate that low intracellular asparagine level impedes breast cancer metastasis which was associated with decreased expression of asparagine-rich EMT-related proteins (384). In summary, these findings indicate that asparagine metabolism, particularly in the absence of glutamine, regulates amino acid homeostasis, protein and nucleotide synthesis and thereby play a critical role in tumor cell proliferation and metastatic progression.

1.2.6.3 Serine and glycine

Serine can be exogenously taken up by the cells via amino acid transporters SLC6A14, SLC38A2, SLC1A5 and SLC38A5 (291, 298, 385, 386). In addition, serine can be biosynthesized from glycolytic intermediate, 3-PG (298). The rate-limiting step in the serine synthesis pathway (SSP) involves the oxidation of 3-PG to 3-phosphopyruvate (3-PP) by phosphoglycerate dehydrogenase (PHGDH), which is then transaminated to 3-phosphoserine (3-PS) by PSAT1 (387, 388). In the third and final step of the pathway, 3-PS is dephosphorylated into serine by

phosphoserine phosphatase (PSPH) (387). Some of the transporters (i.e., SLC38A2, SLC38A5 and SLC6A14) that facilitate the import of serine also mediate the uptake of other amino acids including glycine (291, 298, 385, 386). In addition to utilizing identical amino acid transporters, both serine and glycine are biosynthetically linked as serine can be converted to glycine in the cytosol and mitochondria by serine hydroxymethyltransferase (SHMT1) and (SHMT2) respectively (387, 389). Taking into account that the carbon and nitrogen atoms in serine and glycine are derived from 3-PG and glutamate, respectively during *de novo* biosynthesis, serine and glycine metabolisms are closely associated with glucose and glutamine metabolism (390). Recent findings show that many cancer cell types are dependent on exogenous supply of serine whereby serine starvation markedly inhibits the growth of some neoplastic cells in cell culture and *in vivo* (391). In turn, breast cancer and melanoma cells upregulate serine biosynthesis by diverting glycolytic intermediates to the SSP or increasing the expression of SSP enzymes including PHGDH (391-393). Accordingly, suppressing PHGDH levels in breast cancer cell lines was associated with decreased proliferation while the overexpression of PSAT1 in colon carcinoma stimulated cell proliferation and facilitated chemoresistance (392, 394). Expression of SHMT2 was shown to be induced by MYC or hypoxia (395). Importantly, increased SHMT2 expression appeared to prevent hypoxia-induced cell death by maintaining NADPH production and thus, bolstering ROS protection capacity (395). In the context of cancer, serine is regarded as the more conditionally essential amino acid relative to glycine because the proliferation rates of CRC and lung cancer cell lines were found to be unaltered by the absence glycine in the media, whereas the removal of only serine decreased proliferation to the same extent as the deprivation of both amino acids (396). The role of glycine metabolism in tumor cells has been controversial as it was reported that rapidly, but not slowly proliferating cancer cells depend on exogenous glycine (397).

Furthermore, it was proposed that glycine is only imported and metabolized by cells when extracellular serine is depleted (398). These findings suggest that transformed cells promote survival and proliferation by rewiring serine and glycine metabolism as a possible adaptive response to nutrient deprivation and oxidative stress (298).

1.2.6.4 Folate and methionine cycle

The SHMT catalytic activity requires a folate carrier, tetrahydrofolate (THF), as a cofactor which accepts one-carbon (1C) unit from serine to produce 5,10-methylenetetrahydrofolate (5,10-methylene-THF) in parallel to two-carbon (2C) unit glycine (366). The 5,10-methylene-THF subsequently undergoes a series of redox transformations by folate cycle enzymes to produce various 1C-THF species, including the regeneration of THF (399-401). The folate cycle begins with the reduction of folate (commonly known as vitamin B9) by dihydrofolate reductase (DHFR) into the biologically active THF. Folate cycle requires both mitochondrial and cytosolic isoforms of folate enzymes including the multifunctional NADP-dependent methylenetetrahydrofolate dehydrogenase (MTHFD1/2/1L) (399-401). Glycine contributes to the folate cycle by donating 1C unit to THF via the activity of glycine decarboxylase (GLDC) of the glycine cleavage system (GCS) to generate 5,10-methylene-THF (402). The folate cycle produces THF-containing intermediates that serve as precursors for thymidine synthesis and contribute to the production of purines, ATP, and NADPH (366, 391, 398). It is thought that serine is the more significant 1C unit contributor of the folate cycle relative to glycine (402). The preferential consumption of serine over glycine implies that a demand for 1C units might be driving serine-to-glycine conversion because this reaction donates 1C unit to the folate cycle while the reverse reaction consumes 1C unit from the folate cycle (398). This is consistent with existing data showing that glycine consumption inhibits angiogenesis and tumor growth in CRC, melanoma and liver cancer (403-

405). The conversion of glycine to serine depletes the 1C pool needed for nucleotide synthesis and cell proliferation (396). The folate cycle plays a prominent role in cancer cell metabolism. For instance, elevated MTHFD2 expression in breast cancer correlates with poor survival of patients (406). In addition, GLDC was found to be highly expressed in tumor-initiating cells of NSCLC wherein it induces glycolysis, intracellular serine level, and pyrimidine metabolism (394, 406). Although the GCS system supports tumorigenesis by generating 1C units for nucleotide synthesis, it is suggested that its primary role may be the detoxification/degradation of intracellular glycine (407, 408).

One of the 1C-THF species, 5-methyl-tetrahydrofolate (methyl-THF), which is generated by the folate cycle is used to drive the methionine cycle (366). In the methionine cycle, homocysteine is re-methylated to form methionine by methionine synthase (MS) using methyl-THF (366). Methyl-THF can be derived from the breakdown of 5,10-methylene-THF via cytosolic NADPH-dependent methylenetetrahydrofolate reductase (MTHFR) (366). Hence, the synthesis of methionine couples the methionine cycle to the folate cycle. Methionine can further be converted into S-adenosyl methionine (SAM) via the ATP-dependent methionine adenosyl transferase (MAT) (409). SAM acts as the principal donor of methyl groups for histone, DNA and RNA methylation reactions that underpin epigenetic and epitranscriptomic programs, (400). SAM is then demethylated by histone methyl transferase (HMT) to yield S-adenosyl homocysteine (SAH) which is ultimately converted to homocysteine via SAH hydrolase (SAHH) (366). This completes one turn of the methionine cycle. The homocysteine intermediate can leave the methionine cycle to enter a trans-sulfuration pathway consisting of the condensation of homocysteine with serine to produce cystathionine via cystathionine β -synthase (CBS), and the cleavage of cystathionine to cysteine by cystathionine γ -ligase (CSE) (410). Cysteine further

participates in glutathione synthesis and thus, antioxidant protection against ROS (411). 1C metabolic cycles are closely connected to other metabolic pathways (400, 412). Thus, it is not surprising that aberrant regulation of 1C metabolism is observed across a broad range of cancer types including CRC and HCC (413, 414).

Although malignant cells can synthesize methionine, most cancer cells rely on extracellular supply of methionine (415, 416). This phenomenon is only observed in transformed cells and is referred to as the Hoffman effect or methionine stress sensitivity (415, 416). It describes the underlying inhibitory mechanism of tumor cell proliferation when exogenous methionine is substituted for its metabolic precursor, homocysteine, which reflects the increased demand for metabolites derived from methionine (417, 418). The Hoffman effect is supported by recent data reporting that defects in the methionine cycle of lung tumor initiating cells result in loss of tumorigenic potential (419). This was attributed to alterations in cellular methylation due to reduction in SAM (419). In addition, breast cancer cell lines were demonstrated to be oxidatively stressed when methionine was replaced with homocysteine (418). The cells responded by redirecting homocysteine out of the methionine cycle for glutathione synthesis thus reducing production of methionine and SAM (418). Therefore, certain cancer cell types such as breast cancer reprogram their methionine metabolism in order to meet the demands for tumorigenesis and tumor survival.

1.2.7 Lipid metabolism

Lipids are a complex group of macromolecules with varying structures and functions (420). The large vacuoles in adipocytes and lipid droplets are the primary storage sites for lipids (421, 422) . They contain triglycerides that serve as important energy sources because they can be metabolized to produce ATP when nutrients are limited (420, 423). Although phospholipids are

the major component of biological membranes, other membrane lipids such as cholesterol, glycolipids and phosphatidylinositols facilitate membrane fluidity, cell recognition and function as secondary messengers, respectively (420, 424). Considering their versatile roles, lipids represent essential biomolecules in both normal and cancer cells (424).

1.2.7.1 *De novo* lipogenesis

The primary precursor of several lipid species are fatty acids (FAs), which are either derived from exogenous sources including diet or generated from lipids synthesized by lipogenic tissues (liver, breast and adipose tissues) (420, 423). The production of triglycerides and phospholipids involves the esterification of FAs with glycerol, which is generated from glycerol-3-phosphate (425). Glycerol synthesis is linked to glycolysis whereby glycerol-3-phosphate is produced from DHAP via glycerol-3-phosphate dehydrogenase (GPDH) (426). In turn, the main substrate for FA synthesis is cytosolic acetyl-CoA which is derived from acetate or citrate (423). The CAC produces mitochondrial citrate from either the reductive carboxylation of glutamine-derived α -KG or through the oxidative decarboxylation of glucose-generated pyruvate, that is exported to the cytosol (423). In the cytosol, citrate is cleaved to produce acetyl-CoA by ATP-citrate lyase (ACLY). Acetyl-CoA is then used as the substrate for the rate-limiting acetyl-CoA carboxylases (ACCs) to irreversibly generate the two-carbon donor malonyl-CoA (423). Eventually, the serial condensation of malonyl-CoA molecules and one priming acetyl-CoA by the NADPH-dependent fatty acid synthase (FASN) yields palmitate, the initial product of FA synthesis (423). Palmitate can be desaturated and/or elongated to generate other FA species (423). Considering that most normal tissues prefer the use of circulating lipids instead of *de novo* biosynthesis as the primary source of FAs, the role of FASN in non-transformed cells is of minor importance in well-nourished individuals (42). Nonetheless, a wide variety of tumors and pre-

malignant lesions upregulate FA synthesis through the coordinated expression of several lipogenic enzymes (42). The three main enzymes of FA biosynthesis- ACLY, ACC and FASN- are upregulated in several cancer types such as breast cancer and NSCLC (427-430). While it was demonstrated that silencing either ACC or FASN was associated with the induction of apoptosis and formation of ROS in breast and ovarian cancers, the inhibition of ACLY in human lung adenocarcinoma cell lines resulted in growth arrest in cell culture and *in vivo* (427, 429). When cancer cells are subjected to hypoxia or deprived of lipids, they increase acetate uptake via MCTs and by upregulating the expression of ATP-dependent acetyl-CoA synthetase 2 (ACSS2) which catalyzes the conversion of acetate to acetyl-CoA (35, 431, 432). Also, glioblastomas were shown to accumulate acetyl-CoA from glucose and acetate catabolism which was accompanied by elevated ACSS2 expression (35). Overall, the increase in lipogenesis even in the presence of exogenous lipid sources, allows tumor cells to meet their high metabolic demands and to adapt to exogenous lipid scarcity (424).

1.2.7.2 Fatty acid uptake

In addition to upregulating *de novo* FA synthesis, cancers have developed means to efficiently obtain lipids and FAs from the tumor microenvironment (433). Although lipids can be taken up by the cells through the endocytosis of low-density lipoprotein (LDL) via LDL receptor, free FAs are imported into the cells either via CD36 fatty acid translocase or the FA transport proteins (FATPs/SLC27A1-6) (434, 435). FA uptake can also be facilitated by FA binding proteins (FABPs) which are intracellular lipid chaperones (436). These different metabolic routes of obtaining FAs are often upregulated to meet the high metabolic needs of tumor cells (437-439). Consistently, the uptake of palmitate from the extracellular milieu has been demonstrated to promote migration and metastasis in squamous cell carcinoma (440). Thus, tumor cells develop

strategies to access FAs essential to neoplastic growth, whereby these mechanisms play a prominent role under conditions wherein *de novo* FA synthesis is suppressed (e.g., hypoxia) (441, 442).

1.2.7.3 Fatty acid oxidation

When nutrients are limiting, lipolysis disintegrates lipid droplets by cleaving acyl chains off triacylglycerides (423). The liberated FAs are coupled to CoA by acyl-CoA synthetase (ACS) (423). Subsequently, the FAs are converted to acylcarnitines by carnitine palmitoyltransferase 1 (CPT1) that are shuttled to the mitochondrial matrix via the carnitine-acylcarnitine translocase (SLC25A20) (423). Here, the FAs are transferred back to CoA by CPT2 (423). β -oxidation generates energy through the step-wise shortening of acyl-CoA molecules yielding one molecule of FADH_2 and NADH for every 2-carbon unit of acetyl-CoA removed from the acyl chain (423). The acetyl-CoA molecules produced can be oxidatively decarboxylated via the CAC to produce more NADH and FADH_2 (423). The reducing equivalents from both β -oxidation and CAC are finally oxidized via OXPHOS to produce ATP (423). Complete FA oxidation of palmitate produces approximately twice the amount of energy compared to the catabolism of glucose into carbon dioxide and water (423). Importantly, FA synthesis and oxidation are mutually exclusive and are regulated by negative feedback wherein CPT1 activity is inhibited by high levels of malonyl-CoA generated by ACC during FA synthesis (423). Considering that oxygen is required for FA oxidation, the catabolism of FAs is thought not to be a significant contributor to energy production in hypoxic tumor cells (443). In keeping with this, hypoxia increased the accumulation of lipid droplets, and suppressed β -oxidation by inhibiting the expression of acyl-CoA dehydrogenases (444). Paradoxically, it was reported that some neoplastic cells require β -oxidation, especially when subjected to metabolic stress such as hypoxia or glucose deprivation

(445). For example, the brain isoform of CPT1 (i.e., CPT1C), is induced by hypoxia or glucose depletion in tumor cells to support ATP production (445). These contradictory findings illustrate that understanding of the role of FA oxidation in neoplasia is incomplete. Nonetheless, these findings suggest that targeting FA catabolism may limit the metabolic plasticity of tumor cells.

1.2.8 Pentose Phosphate Pathway (PPP)

The pentose phosphate pathway (PPP), which is also referred to as the hexose monophosphate shunt, branches from the glycolytic pathway (446). In PPP, after G6P is formed by HK, it is irreversibly oxidized by the rate-limiting NADP-dependent G6P dehydrogenase (G6PDH) to produce 6-phosphoglucono- δ -lactone in the oxidative branch of the PPP (447). 6-phosphoglucono- δ -lactone is then hydrolyzed via 6-phosphogluconase (6PGL) to yield 6-phosphogluconate (446, 447). 6-phosphogluconate is oxidatively decarboxylated via NADP-dependent 6-phosphogluconate dehydrogenase (6PGDH) to generate ribulose-5-phosphate (Ru5P) (446, 447). Consequentially, the oxidative branch of the PPP produces 2 molecules of NADPH per molecule of G6P. The non-oxidative branch of the PPP involves the conversion of Ru5P by ribose 5-phosphate isomerase (RPI) to form ribose-5-phosphate (R5P) (447), which is followed by a series of reversible reactions catalyzed by transketolase (TKT) and transaldolase (TALDO). Both enzymes utilize glycolytic/gluconeogenic intermediates, such as F6P and G3P, to produce PPP intermediates and *vice versa* they can generate glycolytic/gluconeogenic intermediates from PPP intermediates (446). Hence, the PPP is coordinated with glycolysis and gluconeogenesis to control the production of NADPH and R5P (447, 448). Taken together, the PPP supports cell survival and proliferation by directly or indirectly providing NADPH needed to drive lipid biosynthesis and ROS scavenging, while simultaneously generating R5P molecules required for nucleotide synthesis (447).

Studies have suggested that cancer cells may reprogram their metabolism to redirect carbon units derived from glucose into both arms of the PPP (447). PPP reprogramming in tumor cells is facilitated due to the reversible nature of most of the reactions in the nonoxidative arm (446). PPP has also been thought to play a major role in metabolic plasticity of cancer cells (446). For example, when cells are under oxidative stress, the PPP is utilized to enhance NADPH production via the oxidative branch, and to simultaneously direct the nonoxidative branch towards re-synthesizing more G6P molecules needed to support the oxidative branch (446). Since G6PDH is negatively regulated by NADPH, it is expected that the activity of this enzyme is elevated in cancer cells with high NADPH consumption (449). In line with this, increased activity of G6PDH was reported in cervical cancer (450). In addition, it was shown that pancreatic cancer cells primarily engage the nonoxidative branch of PPP to produce ribonucleotides for the synthesizing nucleic acids (451, 452). This is occasionally supported by elevated TKT expression (451, 452). In summary, the aberrant expression, and activities of the PPP enzymes in cancer cells allow them to adapt to oxidative stress while supporting their rapid proliferative rates.

1.2.9 Nucleotide metabolism

Nucleotides play several diverse roles in a cell including providing energy to coenzymes in the form of ATP, acting as cofactors (e.g., FAD, NAD and NADP) and regulators (e.g., AMP and ADP) of various metabolic enzymes, along with providing the essential building blocks for nucleic acid synthesis (453). The synthesis of DNA and RNA is enhanced to support cell growth and proliferation especially in rapidly proliferating lymphocytes and cancer cells (454). Activated lymphocytes and tumor cells greatly induce *de novo* nucleotide synthesis pathways plausibly due to the limiting concentrations of nucleosides and nitrogenous bases available in blood (455).

1.2.9.1 Purine biosynthesis

R5P molecules, which are predominantly derived from the PPP, are used to produce phosphoribosyl pyrophosphate (PRPP) via 5-phosphoribosylsynthetase (PRPS) (454). The purine biosynthetic pathway takes place in the cytosol and commences with the conversion of PRPP into 5-phosphoribosyl-1-amine (PRA) by the rate-limiting PRPP amidotransferase (PPAT) (453, 456). PRA undergoes a series of reactions to build the purine rings which involves the use of glutamine and aspartate as the primary nitrogen atom providers while THF, bicarbonate, and glycine donate the carbon atoms needed to generate inosine monophosphate (IMP) (453, 454). The resulting IMP is used to synthesize both guanosine monophosphate (GMP) [via inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS)] and adenosine monophosphate (AMP) [through the activities of adenylosuccinate synthase (ADSS) and adenylosuccinate lyase (ADSL)] (456). *De novo* purine synthesis is regulated by product inhibition as AMP and GMP repress PRPS and PRA through a negative feedback loop (453). Enzymes involved in the nucleotide biosynthesis pathway such as IMPDH and PRPS2 have been shown to be important for MYC-driven oncogenesis because their abrogation correlates with decreased proliferation of B-cell lymphoma and small cell lung cancer (457, 458).

1.2.9.2 Pyrimidine biosynthesis

Unlike purine biosynthesis which begins with PRPP as the main precursor metabolite, pyrimidines are synthesized as free rings to which R5P molecules are subsequently attached (453). The first three reactions of *de novo* pyrimidine synthesis occur in the cytosol and are catalyzed by a trifunctional enzyme called carbamoyl phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase (CAD) (456). Specifically, CAD uses bicarbonate to convert glutamine into carbamoyl phosphate which represents the rate-limiting step of the pathway (456). Carbamoyl

phosphate co-reacts with aspartate to produce N-carbamoyl-L-aspartate that is ultimately dehydrated into dihydroorotate (453, 456). CAD catalyzes the assembly of the dihydroorotate ring by incorporating nitrogen atoms from glutamine and aspartate, while the carbon atoms of the ring are derived from aspartate and bicarbonate (454). As previously mentioned, dihydroorotate is oxidized into orotate by DHODH (453). The R5P molecules are linked to the orotate ring and subsequently decarboxylated into uridine monophosphate (UMP) via the catalytic activities of orotate phosphoribosyltransferase and orotidylic acid decarboxylase, respectively (453, 456, 459). Orotate phosphoribosyltransferase and orotidylic acid decarboxylase are components of the bifunctional uridine monophosphate synthetase (UMPS) (453, 456, 459). UMP acts as a negative regulator of pyrimidine biosynthesis through feedback inhibition of carbamoyl phosphate synthetase 2 domain of CAD (453). UMP is phosphorylated using 2 ATP molecules in sequential reactions catalyzed by UMP/CMP kinase and nucleoside-diphosphate kinase to produce UTP (459). UTP and glutamine serve as substrates of the ATP-dependent CTP synthase to generate CTP (459). Deoxyribonucleotides (dNTPs) such as dTTP are produced using dUMP as a precursor that can be methylated with 5,10-methylene-THF by thymidylate synthase to generate dTMP, which in turn is further phosphorylated to generate dTTP (460). dUMP is itself formed through the reduction and subsequent dephosphorylation of UTP by ribonucleotide reductase (RNR) or via the deamidation of dCMP derived from the hydrolysis of DNA (460, 461). *De novo* pyrimidine biosynthesis is upregulated in transformed breast cancer cells (462). The induction of *de novo* pyrimidine biosynthesis was accompanied by higher CAD expression and activity compared to non-transformed breast cells (462).

1.3 Dysregulation of metabolism in cancer

Perturbation in cellular energy metabolism and metabolic flexibility in cancer cells play a major role in tumor initiation, progression and therapeutic responses. Several transcription factors and signaling pathways that are frequently dysregulated in neoplasia are thought to orchestrate metabolic rewiring in cancer cells and underpin metabolic plasticity of neoplasia.

1.3.1 Transcriptional regulation of cancer metabolism

1.3.1.1 Hypoxia Inducible Factor (HIF)

It is proposed that one of the predominant mechanisms underlying the Warburg effect is the activation of the HIF complex, which functions as a transcription factor that is physiologically activated in response to hypoxia (463). Given that solid tumors rapidly outstrip their vascular supply because of their neoplastic growth, most cells in the tumor bed are thought to be exposed to low oxygen levels ranging between 1% - 2% (464, 465). The HIF complex is thought to upregulate the expression of genes to sustain cancer cell proliferation and survival under hypoxia (463). In some cancer subtypes, HIF is aberrantly activated even under normoxic conditions which is usually caused by genetic lesions such as the amplification of HER2 oncogene in breast cancer (466). HIF gene family comprises alpha and beta subunits that assemble into heterodimers to regulate transcription (467). Humans express 3 paralogues of HIF- α subunit (HIF-1 α , HIF-2 α /EPAS, HIF-3 α) as well as 2 HIF- β paralogues (HIF-1 β /ARNT, HIF-2 β /ARNT2) (467). HIFs appears to function in non-redundant manner whereby HIF-1 upregulates glycolytic genes while HIF-2 stimulates expression of genes associated with lipoprotein metabolism (468). Notably, genetic inactivation of the *VHL* tumor suppressor gene, which is common in clear cell renal carcinoma, leads to constitutive stabilization of HIF-1 α and HIF-2 α (254). Intriguingly, during the progression of clear cell renal carcinoma, the expression of HIF-1 α is occasionally abolished and

HIF-2 (a heterodimer of HIF-2 α and HIF-1 β) takes over the transcriptional control of genes previously regulated by HIF-1 (469). Considering that HIF regulates the transcription of genes encoding 13 different glucose transporters (e.g., GLUT1 and GLUT3) and glycolytic enzymes (e.g., HK1, HK2, LDHA, ENO1, PKM, PGK1, GAPDH and TPI), it is thought that it plays a major role in regulation of aerobic glycolysis (470). Furthermore, as an adaptive response, HIF promotes the transcription of genes that induce mitochondrial breakdown via autophagy such as BNIP and BNIP3L, when cells are subjected to chronic hypoxia (471, 472). HIF also attenuates oxidative decarboxylation in the CAC and induces reductive metabolism of glutamine which at least in part is achieved via suppression of OGDH (41, 473, 474). Taken together, HIF plays a prominent role in orchestrating metabolic reprogramming of neoplasia.

1.3.1.2 MYC

Similar to HIF, the MYC family of oncoproteins (CMYC, MYCL and MYCN) are transcription factors that are approximated to regulate the transcription of 15% of the whole human genome (472, 475). Although MYC expression is tightly regulated in non-transformed cells, MYC has been found to be overexpressed or aberrantly regulated in up to 70% of all types of cancers through diverse mechanisms, such as genetic copy-number gain (chromosome amplification or translocation), super-enhancer activation, and altered protein stability (476). For instance, aberrant expression of CMYC is observed in up to 70–80% of CRC (477). There is evidence demonstrating that enhanced MYC activation is a major promoter of malignant transformation, and that MYC-driven tumors and cancers driven by other oncogenes (e.g., *KRAS*) constitutively depend on elevated MYC levels for tumor progression (478, 479). Notably, aberrant MYC expression is postulated to play a major role in modulating global metabolic reprogramming, to enable the production of bioenergetic substrates and biomass needed to support neoplastic growth (480).

CMYC was reported to regulate the global metabolic rewiring of CRC by regulating 215 metabolic reactions (481). Similar to HIF, MYC acts as a promoter of glucose metabolism given that MYC directly upregulates the transcription of several glycolytic genes (e.g., *SLC2A1*, *HK2*, *ENO1*, *LDHA*) (37, 482). Interestingly, MYC cooperates with HIF-1 to upregulate the expression of key glycolytic genes in response to hypoxia, such as *HK2* and *PDK1* in MYC-driven Burkitt's lymphoma cells (38). This suggests that crosstalk between MYC and HIF-1 plays a prominent role in governing the glucose metabolism in lymphoma and potentially other cancer types (38).

In addition to regulating glucose metabolism, MYC also modulates amino acid metabolism (483, 484). MYC was reported to elevate the mRNA levels of large neutral essential amino acid transporters (*SLC7A5* and *SLC43A1*) (485). MYC also upregulates glutamine metabolism by activating glutamine transporters (*SLC1A5* and *SLC38A5*) and inducing the expression of glutamine-utilizing enzymes (*GLS1*, *PRPS2* and *CAD*) which collectively results in increased glutamine uptake (483, 486-488). Interestingly, a recent study showed that glutamine deprivation in cancer cells altered CMYC expression, wherein the major CMYC isoform is downregulated while the CMYC 1 isoform was upregulated (316, 489). The alterations in CMYC and CMYC 1 expression levels were mirrored by increased expression of *GLUL* which catalyzes *de novo* glutamine synthesis (316, 489). Consistent with its central role in glutamine metabolism, overexpression of MYC was shown to induce “glutamine addiction” and increase sensitivity of colon cancer cells and MYC-transformed rat fibroblasts to mitochondrial inhibitors including metformin (490). Furthermore, ATP production and oxygen consumption were elevated when glutamine rather than glucose was the carbon source in MYC-overexpressing rat fibroblast (490). Altogether, these findings illustrate that MYC-driven metabolic perturbations play a prominent role in tumorigenesis and tumor progression across diverse types of neoplasia.

1.3.1.3 p53

It is generally thought that the tumor suppressor gene, *TP53* is the most commonly mutated gene in cancer; it is mutated in over 50% of all cancer types (491). For example, mutations in *TP53* gene occur in approximately 40%-50% of sporadic CRC (492). p53, the protein product of the *TP53* gene, is widely recognized as a transcription factor that plays a central role in responses to a variety of cellular stressors (DNA damage, hypoxia, nutrient fluctuation and mitogenic oncogenes) by modulating cell proliferation, senescence, DNA repair, and apoptosis (493). However, there is an accumulating evidence that suggests that the tumor suppressor function of p53 is also mediated by its metabolic regulatory activities (491).

p53 has been shown to regulate glucose metabolism through the downregulation of glucose transporters (i.e., SLC2A1, SLC2A3 and SLC2A4) (494, 495). Furthermore, activated p53 inhibits the expression of several glycolytic enzymes such as HK2 and PGM (496-498). Conversely, p53 induces TIGAR (TP53-induced glycolysis and apoptosis regulator) which depletes intracellular fructose-2,6-bisphosphate levels leading to the inhibition of glycolysis (496-498). Although p53 suppress glucose uptake and glycolysis, reports indicate that it stimulates mitochondrial functions and OXPHOS (499). For example, p53 induces expression of SCO2 (Synthesis of Cytochrome c Oxidase 2) in CRC cells whereby the abrogation of the *SCO2* gene in cells with wildtype p53 protein recapitulated the metabolic switch from OXPHOS to aerobic glycolysis that occurs in p53-deficient cells (499). Collectively, these studies suggest that p53 suppresses glucose metabolism while stimulating mitochondrial respiration. Moreover, these results provide mechanistic evidence that links loss of p53 function with Warburg effect in cancer cells.

1.3.2 Metabolic regulation via cellular signaling pathways

Activating mutations in oncogenes and loss-of-function mutations in tumor suppressor genes result in the dysregulation of several intracellular signaling pathways. Although aberrant signaling pathways are well established as facilitators of uncontrolled proliferation and enhanced survival of tumor cells, abnormal function of transduction pathways also underpin critical metabolic processes needed to drive tumorigenesis (500).

1.3.2.1 RAS-RAF-MEK-ERK signaling pathway

The RAS-RAF-MEK-ERK signaling cascade, one of the most extensively studied signaling pathways, couples stimuli from the cell surface receptors to transcription factors that regulate gene expression. This pathway is frequently hyperactivated by genetic alterations in upstream signaling molecules such as receptor tyrosine kinases (RTKs) or activating mutations in downstream transducers (i.e., RAS and RAF) (501). The aberrant activation of the RAS-RAF-MEK-ERK prevents apoptosis while stimulating cell cycle progression (502). Moreover, there is growing body of evidence that implicates the constitutive activation of the RAS-RAF-MEK-ERK signaling pathway in inducing metabolic perturbations in cancer cells which includes promoting aerobic glycolysis (503).

1.3.2.1.1 RAS

Collectively, the three *RAS* genes (*HRAS*, *NRAS* and *KRAS*) are one of the most frequently mutated oncogenes in cancer with *KRAS* being the most prevalent (86%) (504). RAS proteins are GTPases that transduce extracellular signals from cell surface RTKs in response to growth factors and other extracellular stimuli to downstream intracellular effector pathways (e.g. ERK1/2 signaling pathway) (505). Mutations in RAS proteins have been demonstrated to profoundly affect metabolism in a variety of malignancies (506). For instance, oncogenic RAS has been implicated

in several nodes of glucose metabolism. Transcriptomic and stable isotope tracer analysis indicated that KRAS-transformed mouse embryonic fibroblasts, PDAC and CRC cells have elevated glucose uptake and glycolysis (507-509). The abrogation of mutant KRAS has been associated the downregulation of glucose transporter (*SLC2A1*) and several glycolytic genes (*HK1*, *HK2*, *PFK1* and *LDHA*) in PDAC (508). KRAS mutants also promote the nonoxidative arm of the PPP as illustrated by the increased carbon flux into glycolytic intermediates (G3P, F6P) that are then fed into the PPP and non-oxidative PPP intermediate (sedohepulose-7-phosphate) (508). Moreover, recent reports suggest that KRAS and HK1 interact in GTP-dependent manner, whereby this interaction is required to stimulate the hexokinase (509). In spite of indications of a direct GTP-dependent interaction between KRAS and HK1, KRAS predominantly regulates most metabolic pathways through MAPK-dependent signaling cascades to ultimately provide biomass for neoplastic growth (509, 510).

1.3.2.1.2 RAF

The serine/threonine RAF family of kinases which include ARAF, BRAF and CRAF become activated by GTP-bound RAS proteins (511). In addition, activating RAF mutations result in the constitutive activation of ERK1/2 signaling pathway (512). Among the three isoforms of RAF, BRAF has the highest mutation rate, occurring in 30–70% of melanomas, 30–50% of thyroid cancers, and 5–20% of colorectal cancers (511, 513). The majority of these tumors harbor BRAF V600E point mutation which favors the active structural conformation of BRAF causing the constitutive activation of ERK1/2 pathway (514). This ultimately promotes Warburg Effect and cell proliferation (515). Accordingly, BRAF-mutated CRC cell lines upregulate the glucose transporter *SLC2A1*, to increase glucose uptake (516). In addition, the BRAF V600E point mutation sustains melanoma cells by upregulating glycolytic enzymes including GAPDH,

PGAM1 and LDHA (517). Interestingly, it was shown that BRAF-mutated melanomas have reduced levels of the mitochondrial master regulator PGC1 α , and decreased OXPHOS (518). Collectively these findings provide insights in the role of RAF mutations in metabolic rewiring of neoplasia.

1.3.2.2 AMPK: Regulator of intracellular energy homeostasis

It is critical for cells to maintain energy homeostasis by coordinating energy consumption and production. Dysregulation of energy homeostasis is implicated in promoting various diseases such as type 2 diabetes, obesity, and cancer. AMP-activated protein kinase (AMPK) is considered to represent a major intracellular energy sensor, that is activated in response to alterations in energetic status of the cell caused by various stressors including nutrient deficiency, oxidative stress and hypoxia (519, 520). AMPK is a heterotrimeric serine/threonine kinase complex consisting of a catalytic α subunit (α 1 and α 2) and two regulatory β (β 1 and β 2) and γ (γ 1, γ 2 and γ 3) subunits (520-522). The expression of the different AMPK subunit isoforms is tissue-specific (523). Notably, AMPK α 1, β 1, and γ 1 subunits are ubiquitously expressed while the other AMPK subunit isoforms exhibit a more restricted expression pattern (523). For example, α 2 is highly expressed in skeletal and cardiac muscle, and it is the predominant α -subunit in these tissues (523). Cellular energy depletion typically results in the increase of AMP/ATP or ADP/ATP ratios, whereby AMP or ADP bind directly to the γ subunit of AMPK (523). This induces a conformational change in the α subunit and prevents dephosphorylation of the α subunit activation loop threonine residue (T172 in humans) by phosphatases (524-527). Threonine residue (T172 in humans) found in the activation loop of the α subunit is phosphorylated by upstream kinases including as the serine/threonine kinase liver kinase B1 (LKB1), and calcium/calmodulin-

dependent protein kinase kinase β (CAMKK2) (528, 529). The phosphorylation of AMPK at T172 increases AMPK activity by up to 100-fold in cell-free assays (526).

LKB1 functions as a constitutive heterotrimer found in complex with the kinase-dead STE20-related kinase (STRAD) and the STE20 family scaffolding protein (MO25) (530, 531). LKB1 is primarily responsible for the activation of AMPK under energy stress in most mammalian tissues (532). Nonetheless, AMPK can also be phosphorylated and activated independently of LKB1, in response to increased intracellular calcium levels by CAMKK2 (533). In this way, CAMKK2 couples calcium signaling to AMPK-dependent metabolic programs (534). Of note, CAMKK2 has been demonstrated to also activate AMPK following metabolic stressors, such as amino acid deprivation and hypoxia (535-537). Taken together, the role of AMPK as a sensor of metabolic stress is facilitated by the catalytic activities of both LKB1 and CAMKK2.

Importantly, it has been reported that the lysosomal membrane surface facilitates the phosphorylation of AMPK by LKB1 (538, 539). These studies provide mechanistic insights into AMPK activation under conditions of glucose deprivation (538, 539). In glucose-starved cells, LKB1 is tethered to the scaffolding protein, AXIN (538, 539). The LKB1-AXIN-AMPK complex was shown to be localized to the late endosome/lysosomes by binding to the lysosomal protein, LAMTOR1 (also known as Ragulator1), a component of v-ATPase-Ragulator complex (538).

There is accumulating evidence indicating that AMPK plays a major role in detecting oxidative stress. For example, ROS modulates AMPK activity by direct posttranslational modifications of the α -subunit (540, 541). However, the resulting effects of such modifications seem to be context-dependent. Exposure of HEK293 cells to H_2O_2 induced the oxidation and S-glutathionylation of C299 and C304 in the α -subunit, which result in activation of AMPK (541). Conversely, treating cardiomyocytes with H_2O_2 causes the oxidation of the C130 and C174 in the

α -subunit that coincides with the inhibition of AMPK (540). Although further work is warranted to define the role of AMPK in sensing ROS levels, these studies suggest that AMPK acts as a critical node linking energy and redox status in the cell.

AMPK not only senses the cellular energy status but also ATP levels by suppressing ATP-consuming biosynthetic pathways (e.g., protein synthesis and fatty acid synthesis), while concurrently promoting pathways that regenerate ATP through the breakdown of macromolecules and organelles (e.g., glycolysis and fatty acid oxidation). Specifically, energy stress activates AMPK which can promote glucose uptake by phosphorylating and degrading TXNIP, a negative regulator of glucose metabolism, resulting in the upregulation and surface translocation of SLC2A1 glucose transporter (542). Once glucose is catabolized to restore ATP to ADP/AMP ratios, AMPK is subsequently dephosphorylated which ultimately induces TXNIP to suppress glucose uptake as energy homeostasis is re-established (542). Conversely, AMPK prevents the storage of glycogen by inhibitory phosphorylation of glycogen synthases (GYS1 and GYS2) (543). It also downregulates gluconeogenesis through the phosphorylation and nuclear exclusion of cyclic-AMP-regulated transcriptional co-activator 2 (CRTC2) and class II histone deacetylases (HDACs), which are key cofactors for the transcription of gluconeogenic genes (544). Additionally, AMPK suppresses lipid synthesis through the direct inhibitory phosphorylation of acetyl-CoA carboxylase 1/2 (ACC1/2) which catalyzes the rate-limiting step of fatty acid synthesis (523, 545). In parallel to inhibiting fatty acid synthesis, activated AMPK stimulates fatty acid oxidation by reducing the levels of ACC2-generated malonyl-CoA (523). This alleviates the repression of CPT1 thus promoting fatty acid oxidation (523). In summary, AMPK-dependent inhibition of gluconeogenesis and lipid synthesis limits ATP consumption while promoting ATP-generating metabolic processes under energy stress.

Considering that protein synthesis is one of the most energetically demanding cellular process, AMPK inhibits this process when nutrients are limited. AMPK-dependent downregulation of protein synthesis is mostly mediated through the direct inhibition of mechanistic target of rapamycin complex 1 (mTORC1 complex) through two independent mechanisms: the activating phosphorylation of tuberous sclerosis complex 2 (TSC2), a negative mTORC1 regulator, and the inhibitory phosphorylation of RAPTOR (546, 547). AMPK also suppresses protein synthesis by phosphorylating eukaryotic elongation factor 2 kinase (eEF2K) which is a negative regulator of mRNA translation elongation (548). Notably, eEF2K can also be directly phosphorylated by mTOR (549), thus illustrating crosstalk between AMPK and mTOR signaling pathways.

Tumor-suppressive functions of AMPK have been described in prostate cancer wherein AMPK-mediated inhibition of lipogenesis was associated with tumor growth arrest (550). While the inhibition of AMPK due to a hypomorphic mutation decreases LKB1 expression and markedly accelerates tumor development in PTEN(+/-) mice, activating AMPK by administering well recognized mitochondrial inhibitors (such as metformin, phenformin or A-769662) to PTEN(+/-) mice significantly delayed tumor onset (551). Finally, it has been demonstrated that AMPK counteracts Warburg effect thus attenuating tumor growth in mouse models (552). Nevertheless, under certain contexts including nutrient and oxygen deprivation, AMPK activation engenders tumor cells with growth advantage by increasing metabolic plasticity and allowing cells to adapt to energy stress (553). This is for example illustrated by observations that under energy stress activated AMPK inhibits ACC1/2 in order to maintain NADPH levels by reducing NADPH-consuming fatty-acid synthesis while elevating NADPH-generating fatty-acid oxidation (554). This facilitated anchorage-independent growth and solid tumor formation in vivo mouse models

(554). These and other findings suggest that whereas AMPK plays a tumor suppressive role during tumor initiation, it attains a tumor promoting role once tumors outstrip their vasculature and are exposed to metabolic stress (555).

1.3.2.3 PI3K-AKT-mTOR signaling

The PI3K-AKT-mTOR signaling pathway underpins many essential cellular processes such as cell growth, protein synthesis, proliferation and energy metabolism (556). Similar to the RAS-RAF-MEK-ERK signaling cascade, the PI3K signaling pathway is activated when RTKs bind to growth factors, hormones or mitogens (557) (Fig. 1.4). This triggers the intracellular autophosphorylation of tyrosine residues on the RTKs which act as cell membrane anchors for PI3K (558-560). Subsequently, PI3K phosphorylates phosphatidyl inositol-4,5-bisphosphate (PIP₂) to yield phosphatidyl inositol-3,4,5-trisphosphate (PIP₃) (Fig. 1.4). PIP₃ translocates the serine/threonine kinase, AKT, to the cell membrane where it is phosphorylated within its activation loop (at T308) and activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (561) (Fig. 1.4). A major negative regulator of the PI3K signaling pathway, is the tumor suppressor PTEN which dephosphorylates PIP₃ into PIP₂ (562) (Fig. 1.4). Although the PI3K/AKT pathway has been reported to promote glucose uptake and glycolysis through the translocation of glucose transporter, SLC2A4 and activation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) respectively, the signaling pathway predominantly regulates various metabolic processes via its downstream effector mTOR (563-565).

mTOR is a serine/threonine kinase which acts downstream of the PI3K signaling pathway (Fig. 1.4). mTOR is the catalytic subunit of two functionally and structurally distinct protein complexes. The mTORC1 complex is composed of mTOR, mLST8, proline rich AKT1 substrate 40 (PRAS40), DEPTOR and RAPTOR while mTORC2 is composed mTOR, mLST8, mSIN1,

DEPTOR, PROTOR 1/2 and RICTOR (566-570). Between the two complexes, the functions of mTORC1 complex are better defined whereby it has been shown that it promotes glycolysis, lipid synthesis, cell growth and protein synthesis while the activities of mTORC2 are mostly obscure (570, 571). Nonetheless, mTORC2 has been demonstrated to regulate proliferation, cell survival and cytoskeletal organization primarily via the phosphorylation of AGC kinases including AKT, SGK1 and PKC (572, 573). While mTORC2 modulates metabolism mainly through the activation of AKT, the complex also regulates cytoskeletal remodeling and cell migration through the phosphorylation of PKC α , PKC δ , PKC ζ , PKC γ and PKC ϵ (570, 574-578). Also, mTORC2-mediated phosphorylation and activation of SGK1 promotes epithelial sodium ion transport and cell survival (579, 580).

1.3.2.3.1 Upstream activators of mTOR signaling

AKT phosphorylate and inhibits TSC2 which together with TSC1 and TBC1 domain family member 7 (TBC1D7) comprises tuberous sclerosis complex (TSC) (581) (Fig. 1.4). The TSC complex is GTPase-activating protein (GAP) that inhibits the activator of mTORC1, ras homolog enriched in brain (RHEB) GTPase (582, 583) (Fig. 1.4). Thus, the inhibition of the TSC complex by AKT results in the activation of mTORC1. DNA damage and hypoxia induce regulated in development and DNA damage response 1 (REDD1) protein which facilitates the dissociation of inhibitory 14-3-3 protein from TSC2 leading to mTORC1 inhibition (584-586) (Fig. 1.4).

Interestingly, ERK1/2 from the RAS-RAF-MEK-ERK signaling pathway can phosphorylate TSC2 (at S540 and S664) and RAPTOR (at S8, S696 and S863) directly which activate mTORC1 (587-590). In addition, ERK1/2 indirectly leads to the phosphorylation of TSC2 (at S1798) and RAPTOR (at S719, S721 and S722) via p90 ribosomal protein S6 kinase (RSK),

thus promoting mTORC1 signaling (591-594) (Fig. 1.4). The interaction between the RAS-RAF-MEK-ERK and PI3K-AKT-mTORC1 pathways results in negative regulation of each other's activity via cross-inhibition (595). For instance, the activation of ERK induces the phosphorylation of GRB2-associated binder 1 (GAB1) at six serine/threonine residues (T312, S381, S454, T476, S581, S597) which hinders GAB1-mediated recruitment of PI3K to the EGF receptor and thus decrease the phosphorylation of AKT (596). The ERK phosphorylation sites on GAB1 recruit the protein tyrosine phosphatase, SHP2, which dephosphorylates and regulate RASGAP and PI3K (595, 597). Conversely, AKT inhibits the MAPK signaling pathway via the phosphorylation of RAF (598). This is demonstrated in a study which show that high concentrations of IGF1 markedly activate AKT resulting in the phosphorylation and repression of RAF activity in breast cancer and melanoma cell lines (598-600). Intriguingly, it was shown that although activating BRAF mutations initiate nevi development, AKT activation is required to bypass MAPK-mediated cell cycle arrest or senescence (600). AKT reduces RAS-RAF-MEK-ERK signaling to levels it can cooperate with to transform nevi into melanomas (600). Collectively, these illustrate crosstalk between the RAS-RAF-MEK-ERK and PI3K-AKT-mTORC1 signaling pathways and thus, indicate cooperation between the two pathways in regulating metabolic processes and tumor development.

Amino acids, glucose, energy stress and oxygen and growth factors activate mTORC1 (570). Specifically, mTORC1 indirectly responds to high AMP/ATP ratio, glucose deprivation and hypoxia partly through the activation of AMPK which in turn inhibits mTORC1 as previously discussed (547, 601, 602) (Fig. 1.4). However, glucose starvation also suppresses mTORC1 complex independent of AMPK (603, 604). Glucose depletion inhibits Rag GTPases (RagA/RagB) which prevents the localization and activation of mTORC1 at the lysosome (603,

604) (Fig. 1.4). Additionally, mTORC1 senses amino acid levels through Rag GTPases (605, 606) (Fig. 1.4). Rag GTPases function as heterodimeric complexes of RagA or RagB with RagC or RagD and are attached to the lysosome through their association with the Ragulator/LAMTOR complex (that is composed of MP1, p14, p18, HBXIP and c7ORF59) (607, 608). The Ragulator/LAMTOR complex which associates with a v-ATPase at the lysosomal surface, not only acts as a scaffold for Rag GTPases but also has a guanine nucleotide exchange factor (GEF) activity for RagA/B (608). The presence of amino acids stimulates the GEF activity of the Ragulator in a v-ATPase-dependent manner resulting in RagA/B-GTP loading and the subsequent recruitment of mTORC1 to the lysosome where it also interacts with RHEB (608) (Fig. 1.4). Interestingly, a few additional components involved in amino acid sensing by mTORC1 have been identified such as the folliculin-folliculin interacting protein 1/2 (FNIP1/2) complex which acts as a GAP for RagC/D GTPases (609, 610). However, glutamine and asparagine have been demonstrated to stimulate mTORC1 independently of Rag GTPases but through ADP ribosylation factor (ARF) family GTPases (611-613). Of note, specific amino acids (e.g., leucine and arginine) are amongst the most potent activators of mTORC1. Leucine and arginine activate mTORC1 via GATOR1 and GATOR2 complexes (614). GATOR1 complex (composed of DEPDC5, Nprl2 and Nprl3) is inhibited by the GATOR2 complex (consisting of Mios, WDR24, WDR59, Seh1L and Sec13)(614). GATOR1 which suppresses mTORC1 activity by acting as a GAP for RagA/B, is anchored to the lysosome by another complex, KICSTOR (comprised of KAPTIN, ITFG2, c12orf66, and SZT2) (614, 615). Cytosolic leucine and arginine activate mTORC1 by binding and inactivating Sestrin2 and CASTOR1 respectively which are inhibitors of GATOR2 (616-620). In addition to the extensive studies on the effects of glucose and amino acids on mTORC1 signaling,

recent findings indicate that mTORC1 senses intracellular levels of nucleotides and lipids (621, 622).

Compared to mTORC1, there are fewer known activators of the mTORC2 complex. mTORC2 appears to be predominantly activated by insulin and other growth factors via PI3K (623). PI3K-dependent regulation of mTORC2 is facilitated by the mSIN1 subunit of the complex which contains a phosphoinositide-binding pleckstrin homology (PH) domain that inhibits mTORC2 in the absence of insulin but activates mTORC2 when insulin is present by binding to PIP₃ generated at the plasma membrane (624) (Fig. 1.4). Interestingly, PI3K promotes the association of mTORC2 with ribosomes which results in mTORC2 activation (625). When AKT is partially activated due to phosphorylation of T308, it phosphorylates mSIN1 (at T86) which induces mTORC2 to phosphorylate (at S473) and fully activate AKT (626). Although the partial activation of AKT is sufficient to phosphorylate and thus activate mTORC1, fully activated AKT phosphorylates a more diverse set of effectors containing an RXXS/T motif (568). As such, mTORC2 promotes mTORC1 signaling via AKT-dependent phosphorylation of TSC2 and other effectors. However, mTORC2 is regulated by mTORC1 as a result of a negative feedback loop between mTORC1 and PI3K (627, 628). Specifically, elevated mTORC1 increases the activity of one of its downstream effectors, S6 kinase (S6K), resulting in the phosphorylation and degradation of insulin receptor substrate-1 (IRS-1) which in turn recruits PI3K (627-629). Furthermore, mTORC1 phosphorylates and activates growth factor receptor-bound protein 10 (GRB10), which negatively regulates insulin/IGF-1 receptor signaling upstream of AKT and mTORC2 (630, 631). More recently, AMPK has been revealed as a positive regulator of mTORC2 during periods of glucose deprivation (632, 633). AMPK activates mTORC2 by phosphorylating mTOR and RICTOR when PI3K signaling is downregulated (632). While the existence of positive and

negative feedback loops between mTORC1 and mTORC2 signaling elucidate potential drug targets, the interconnectivity between the two complexes is indicative of cooperation between the mTOR complexes in mediating metabolic processes.

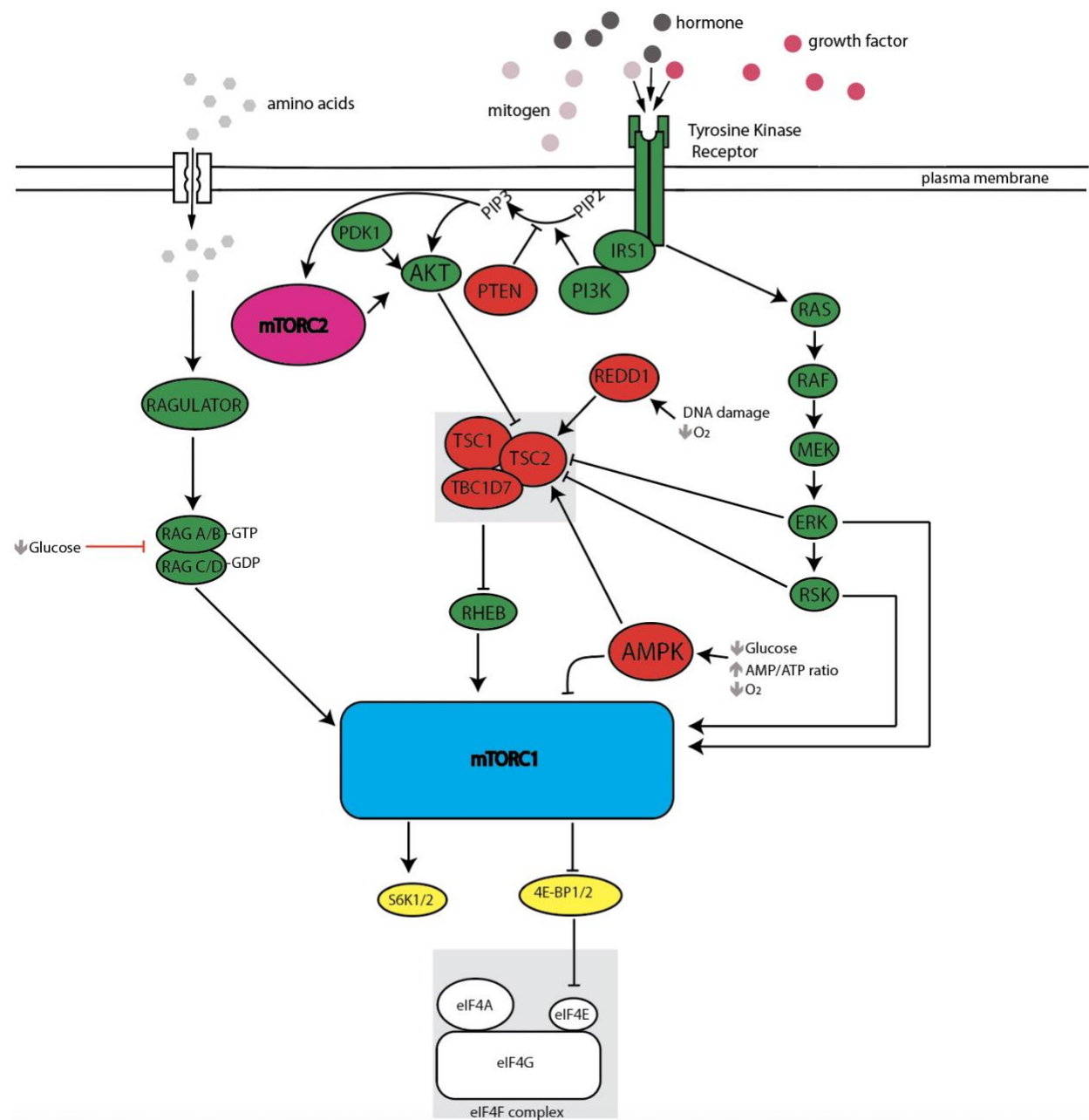


Fig. 1.4. mTOR signaling pathway. mTOR exists in two distinct complexes: mTORC1 (blue) and mTORC2 (magenta). mTOR is activated by hormones, growth factors via receptor tyrosine kinases as well as amino acids imported from the extracellular space. mTORC1 is also modulated by intracellular cues such as AMP/ATP ratio and levels of oxygen. Furthermore, mTORC1 signaling pathway interacts with the MAPK/RSK pathway. Upstream regulators of mTORC1 are shown in green (positive regulators) and red (negative regulators) while the downstream effectors are shown in yellow.

1.3.2.3.2 Systemic effects of mTOR signaling

mTOR signaling coordinates anabolic and catabolic processes to maintain energy homeostasis. In well-fed states, nutrient levels, and growth factors in the blood are increased which trigger anabolic processes while during starvation, catabolic processes are induced due to depleted growth factors and energy sources (570). The disruption of cellular energy homeostasis due to the upregulation or downregulation of mTOR result in tissue-specific abnormalities that are frequently lethal.

Under physiological conditions, when plasma glucose concentration is reduced, adaptive processes involving mTOR signaling such as gluconeogenesis are induced (570). Moreover, constitutive activation of mTORC1 signaling due to the abrogation of TSC1 in the liver diminishes the production of ketone bodies as compensatory energy sources during fasting (634). The importance of diminishing mTORC1 signaling when the levels of nutrient (such as amino acids and glucose) are low is further underscored by the findings that the whole-body knock-in mice expressing a constitutively active form of RagA die due to their inability to induce autophagy in the liver during the perinatal fasting period (603). In pancreatic β -cells, mTORC1 has a biphasic effect on their function. The hyperactivation of mTORC1 through the loss of TSC2 in β -cells

initially leads to increased pancreatic β -cell mass, elevated insulin production, and higher glucose tolerance in young mice which eventually leads to decreased β -cell mass, reduced insulin levels and hyperglycemia (635, 636). This is also consistent with prolonged mTORC1 hyperactivation induced by nutrient overload as commonly observed in type 2 diabetes and obesity (637, 638). Type 2 diabetes is often associated with diminished insulin production due to β -cell exhaustion caused by chronic exposure to excess of nutrients (i.e., glucose) (639). Furthermore, increased mTORC1 activity in insulin-responsive tissues results in insulin resistance because of enhanced negative feedback inhibition of insulin/PI3K/AKT signaling by S6K and via IRS-1 (638, 640). In addition, since mTORC2 fully activates AKT downstream of insulin-driven PI3K signaling, prolonged pharmacological or genetic inhibition of mTORC2 in the liver, muscle or adipocytes impedes response to insulin which is associated with glucose intolerance (641-645). In liver-specific RICTOR-knockout mice, AKT is inhibited which reduces glucokinase activity and glycolysis in the liver (641). Taken together, these findings provide evidence that both mTORC1 and mTORC2 regulate organismal glucose homeostasis.

In the presence of nutrients and insulin, mTORC1 promotes adipogenesis and lipogenesis (646). However, the inhibition of mTORC1 induces lipolysis and fatty acid oxidation (647). The hyperactivation of mTORC1 through the constitutive expression of activated RHEB in adipocytes upregulates lipogenesis while inhibiting lipolysis (648). Consistently, the abrogation of RAPTOR in the liver hinders mTORC1-driven *de novo* lipogenesis in the liver due to reduced expression of lipogenic genes including *Srebp1c* and *Fas* (649). Although the deletion of RAPTOR in adipocytes leads to hepatic steatosis, lipodystrophy and insulin resistance in mice, it also protects mice against diet-induced obesity and hypercholesterolemia because adipogenesis is downregulated (650, 651). Similar to mTORC1, mTORC2 regulates lipid homeostasis in the liver

and adipose tissue (646). The inhibition of mTORC2 through the loss of RICTOR in adipocytes markedly increases the overall lean mass of mice due to growth in the size of non-adipose organs such as heart, kidney, spleen, and bone (645, 652). Furthermore, the inactivation of RICTOR in adipocytes was associated with hyperinsulinemia, increased lipolysis in adipose tissue and insulin resistance in skeletal muscles and liver of mice (645, 652). Although hepatic insulin resistance is frequently linked to fatty liver due to the impaired ability of insulin to repress lipolysis, liver-specific deletion of RICTOR actually protects mice against high-fat diet-induced fatty liver (641, 642). Additionally, suppressing mTORC2 in the liver impairs insulin-driven hepatic lipogenesis which is accompanied by decreases in the expression of transcription factors (i.e., SREBP1c and PPAR γ) and lipogenic enzymes (e.g., ACC and FASN) (641). However, the expression of genes regulating fatty acid oxidation (e.g., *CPT1* and *PPAR α*) are increased (641). In summary, both mTORC1 and mTORC2 play pivotal tissue-specific roles in regulating organismal lipid metabolism.

mTORC1 signaling promotes and maintains muscle mass. For instance, mTORC1 induces muscle hypertrophy and prevents muscle atrophy resulting from disuse (653). Conversely, when mTORC1 signaling is downregulated, hypertrophy is impeded (653). It is worth mentioning that these effects on muscle growth are only observed during acute activation of mTORC1 signaling. Chronic activation of mTORC1 due to abrogation of TSC1 leads to muscle atrophy and low body mass because the muscles lack the ability to trigger autophagy which is a critical cellular process for muscle growth (654). Collectively, this suggests that periods of mTORC1 activation and inhibition is required for optimal muscle growth and function.

The PI3K-AKT-mTORC1 axis is frequently hyperactivated in cancer (655, 656). Hyperactivation of this axis stem from activating mutations in positive regulators including

PIK3CA and *AKT* genes, and/or inactivation of tumor suppressors that suppress this signaling axis including *PTEN* and *TSC* genes (655-657). These mutations occur in diverse cancer types including breast cancer, CRC and glioblastoma (658). More recently, activating mutations in mTOR have also been described in endometrial carcinoma, CRC, melanoma, renal cell cancer and bladder cancer (655, 656, 659). This has garnered interest as targeting the PI3K-AKT-mTORC1 pathway presents a potential therapeutic opportunity in cancer (658). Notwithstanding the modest success of applying allosteric mTOR and PI3K inhibitors in the clinic, the outcomes of these approaches were lesser than initially expected (655). This was largely attributed to incomplete understanding of the complexity of the mechanisms underpinning pro-oncogenic activities of the PI3K-AKT-mTORC1 pathway, and in particular the precise role of downstream effectors of mTOR in oncogenesis and tumor progression (655).

Considering that the effectors of mTORC1 and their impacts on cancer-related processes have been extensively covered in previous reviews (556, 565, 570, 647, 660), herein I will focus on findings highlighting the impacts of mTOR effectors on cellular metabolism pertinent to this thesis.

To date, the best studied downstream effectors of mTORC1 signaling are S6Ks (S6K1 and S6K2 in humans), and eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BP1-3 in humans) (661-663) (Fig. 1.4). Specifically, mTORC1 phosphorylates 4E-BPs (at T37/T46, followed by T70 and finally S65 in human 4E-BP1) (661-663). When unphosphorylated, 4E-BPs sequester eIF4E (mRNA cap-binding subunit of the eIF4F complex) and impedes its association with the scaffolding protein eIF4G1 (664). When mTORC1 phosphorylates 4E-BPs, they dissociate from eIF4E, and thus allow the eIF4F complex assembly (665) (Fig. 1.4). eIF4F complex recruits mRNA to the ribosome during translation initiation and is composed of

eIF4E, eIF4G1 and eIF4A (DEAD box RNA helicase) (666-669) (Fig. 1.4). mTORC1 regulates mRNA translation initiation via the phosphorylation of 4E-BPs and S6Ks (664, 670). In addition, mTORC1 stimulates translation elongation by directly or indirectly (via S6Ks) phosphorylating and inactivating eEF2K, whereby eEF2K negatively regulates ribosome translocation by phosphorylating and inhibiting eEF2 (670, 671). Importantly, protein synthesis is one of the most energy consuming processes in the cell (672). By altering the rates of global protein synthesis, mTORC1 therefore modulates cellular energy consumption (673).

Although mTORC1 stimulates global protein synthesis, translation of a subset of mRNAs is particularly sensitive to changes in mTORC1 activity (41, 674-678) (676-678). For example, the mTORC1-4E-BP-eIF4E axis regulates mitochondrial activity and biogenesis by selectively promoting the translation of nucleus-encoded mitochondria-related mRNAs [(such as components of complex I and V, mitochondrial ribosomal proteins and transcription factor a, mitochondrial (TFAM)] (679). Importantly, the mRNA that encodes these proteins typically harbor 5'UTR/TISU elements (Translation Initiator of Short 5' UTR; SAASATGGCGGC, in which S is C or G) and their translation is eIF4E sensitive, but unaffected by eIF4A inhibition (594, 679-681). Consequently, by inducing the translation of nucleus-encoded mitochondria-related mRNAs, mTORC1 upregulates ATP generating capacity to support the protein synthesis and other energy demanding cellular processes. In addition, the mTORC1-4E-BP axis regulates translation of mRNAs encoding enzymes involved in NEAA synthesis including PC, PHGDH, PSAT1 and ASNS (41). To this end, mTORC1-driven upregulation in translation of the aforementioned mRNAs has been shown to underpin metabolic plasticity of breast cancer, CRC and melanoma cells and determine their responses to the drug combinations of oncogenic kinase inhibitors and

biguanides (41). Collectively, these findings demonstrate that mTORC1-dependent translational control plays a major role in regulating metabolic rewiring of neoplasia.

mTOR can modulate glutamine metabolism via post-transcriptional and post-translational regulation of glutaminolysis-related genes (647, 660). Specifically, mTORC1 promotes glutaminolysis by increasing GLS levels through the S6K1-dependent regulation of CMYC (682) and activating GLUD through the abrogation of mitochondrial-localized sirtuin (SIRT4) which inhibits GLUD activity through ADP-ribosylation (683). While it is proposed that CMYC downregulates the expression of post-transcriptional repressors of GLS (i.e., miR-23a/b), mTORC1 promotes the proteasome-mediated degradation of a transcription factor, cAMP-responsive element binding 2 (CREB2), which contains a recognition motif in the promoter region of *SIRT4* (483). Thus, mTORC1 activates glutaminolysis via negative regulation of miR-23a/b and CREB2 which promotes GLS and GLUD activities respectively. mTORC2 may also regulate glutamine metabolism post-transcriptionally considering that it has been demonstrated to upregulate CMYC expression and a knockdown of RICTOR reduces the intracellular levels of α -KG that are probably derived from glutaminolysis (679, 684). Furthermore, mTORC2 may promote glutaminolysis, by inhibiting expression of GLUL via AKT-dependent cytoplasmic localization of GLUL transcription factors (FOXO3 and FOXO4) (685). Collectively, these reports firmly establish mTOR as a regulator of glutamine metabolism.

At the cellular level, mTOR regulates glucose metabolism chiefly through HIF-1 α and CMYC which increase the expression glucose transporters and glycolytic enzymes as previously discussed (647, 660). mTORC1 indirectly upregulates the transcription of HIF-1 α via the phosphorylation of signal transducer and activation of transcription 3 (STAT3) during hypoxia (686). Also, a recent study demonstrated that the transcription factor forkhead/winged helix family

k1 (FOXK1) is another mediator of mTORC1-regulated expression of HIF-1 α (687). mTORC1 also promotes the translation of HIF-1 α through the phosphorylation of its two downstream substrates: 4E-BP1 and S6K1 (686). This is consistent with reports illustrating that the deletion of TSC2, and thus activation of mTORC1 results in the accumulation of HIF-1 α while the pharmacological inhibition of mTORC1 with rapamycin decreases the expression of HIF-1 α (688, 689). Likewise, mTORC1 enhances the translation of *CMYC* mRNA in a 4E-BP1 and S6K1-dependent manner (682, 690). Given that glucose metabolic reprogramming is frequently observed in many cancers including breast, lung, pancreatic and colorectal cancers, it is not surprising that these cancers are also associated with the hyperactivation of mTOR, amplification of *CMYC* and increased aerobic glycolysis facilitated by elevated expression of glucose transporters such as SLC2A1 (656, 691, 692). mTORC2 regulates glucose metabolism through AKT which phosphorylates HK2 to promote its association with mitochondria and directly couple intramitochondrial ATP synthesis to glucose metabolism (693, 694). AKT also promotes glycolysis through phosphorylation and activation of PFK2 (563) and upregulating the expression of SLC2A1 (62). In fact, oncogenic activation of AKT has been shown to mediate aerobic glycolysis for the sustained growth and survival of cancer cells (695). mTORC2 also modulates glucose metabolism independent of AKT. In glioblastoma, mTORC2 inactivates class IIa histone deacetylases, which leads to the acetylation of FOXO1 and FOXO3 and thus activates *CMYC* to upregulate glycolytic gene expression (684). Taken altogether, the findings support that both mTORC1 and mTORC2 complexing are implicated in the regulation of glucose metabolism and metabolic reprogramming in cancer cells.

As previously stated, mTORC1 regulates mitochondrial activity through a 4E-BP-dependent selective translational regulation of mitochondrial-related mRNAs (679). Other studies

have further corroborated the role of mTOR signaling in mitochondrial metabolism. For example, the inhibition of mTORC1 with rapamycin reduces the mitochondrial membrane potential, oxygen consumption, and ATP synthesis while altering the mitochondrial phosphoproteome (696). The suppression of mTORC1 via silencing of RAPTOR downregulates genes encoding proteins involved in OXPHOS and mitochondrial biogenesis, thus impairing mitochondrial respiration (679, 697, 698). mTORC1 modulates mitochondrial respiration through transcriptional regulators such as PGC-1 α and yin yang 1 (YY1) (698, 699). In fact, mTOR and RAPTOR interact with YY1, and inhibition of mTOR disrupts the interaction and coactivation of YY1 by PGC-1 α (699). Contrary to mTORC1, the role of mTORC2 remains poorly understood. The inhibition of mTORC2 through silencing RICTOR was demonstrated to have no effect on the expression of ETC complexes I-IV proteins or mitochondrial respiration (700). Although the knockdown of RICTOR does not alter the levels of mitochondrial proteins such as ATP5O (ATP synthase subunit O, mitochondrial) and TFAM, it decreases α -KG levels which is a key CAC intermediate (679). Also, genes implicated in mitochondrial transport and function were among a set of genes that was proposed to be translationally downregulated by mTOR catalytic inhibitors but not by rapamycin, would suggest that mTORC2 is involved in the translational regulation of these genes (675, 680). However, this can also be explained by the inability of rapamycin to inhibit the phosphorylation of 4E-BP as previously reported (701, 702). Furthermore, subsequent studies confirmed that the mTORC1/4E-BP1 rather than mTORC2 regulates the translation of nuclear-encoded mRNAs with mitochondrial functions such as ATP5O (679, 680). Nonetheless, when stimulated by growth factors, mTORC2 localizes to the mitochondria-associated endoplasmic reticulum membranes where it phosphorylates and activates AKT (693). In turn, AKT phosphorylates HK2 which stimulates glycolysis, decreases the mitochondrial membrane potential and thus, affects

mitochondrial physiology and metabolism (693). Specifically, mTORC2 deficiency via the loss of RICTOR disrupts mitochondria-associated endoplasmic reticulum membranes leading to increases in mitochondrial membrane potential, ATP production and calcium uptake (693). Overall, although extensive studies have provided evidence elucidating the functions of mTORC1 in mitochondrial metabolism, further research is warranted to fill the gaps in knowledge regarding the role of mTORC2 in mitochondrial metabolism.

In addition to the metabolic processes discussed above, mTOR signaling is implicated in the biosynthesis and transport of amino acids (e.g., serine, methionine and cysteine), fatty acid synthesis and lipid metabolism, pentose phosphate pathway and nucleotide synthesis at various levels of regulation ranging from transcriptional to post-translational mechanisms (647, 660). This underscores the pertinent role of mTOR in mediating metabolic reprogramming of cancer cells. It is worth noting that although mTOR regulates several biosynthetic processes, mTOR activity is often regulated by the products of such processes. For example, mTOR regulates glucose and glutamine metabolism while being simultaneously regulated by glucose and glutamine (570, 647). As such mTOR is required to sense the energetic state of cells which promotes the anabolism or catabolism of nutrients. Even though there is an abundance of research elucidating the regulatory activities of mTORC1 in cancer metabolism, much is still left to uncover with regards to mTORC2. This thesis aims to further study the extensive crosstalk between mTOR signaling and metabolic reprogramming in the context of various energetic stressors in order to highlight vulnerabilities that can be targeted in cancer.

1.4 CENTRAL HYPOTHESIS AND OVERARCHING GOALS

Tumor cells must overcome energy stress as they outgrow their vascular supply. To this end, neoplastic cells must constantly adjust their metabolism to fluctuations in nutrients and oxygen in their microenvironment. Based on this, it is hypothesized that impeding the mechanism that allow cancer cells to adapt to energy stress will result in their death. Therefore, the overarching objective of this thesis is to dissect the mechanisms that underpin the alterations in the metabolome of cancer cells subjected to energy stress with a goal to identify potentially targetable and clinically exploitable vulnerabilities. Specifically, we set out to address the mechanisms that allow cancer cells to adapt to dysfunction of mitochondrial complex IV (Chapter 2), pharmacological inhibition of mitochondrial complex I, and nutrient deprivation (Chapter 3).

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CHAPTER 2: Mitochondrial complex IV defects induce metabolic and signaling perturbations that expose potential vulnerabilities in HCT116 cells

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2.1 ABSTRACT

Mutations in genes encoding cytochrome c oxidase (COX; mitochondrial complex IV) subunits and assembly factors (e.g., SCO1, SCO2, COA6) are linked to severe metabolic syndromes. Notwithstanding that *SCO2* is under transcriptional control of tumor suppressor p53, the role of mitochondrial complex IV dysfunction in cancer metabolism remains obscure. Herein, we demonstrate that the loss of *SCO2* in HCT116 colorectal cancer cells leads to significant metabolic and signaling perturbations. Specifically, abrogation of *SCO2* increased NAD⁺ regenerating reactions and decreased glucose oxidation through citric acid cycle while enhancing pyruvate carboxylation. This was accompanied by a reduction in amino acid levels and the accumulation of lipid droplets. In addition, *SCO2* loss resulted in hyperactivation of the IGF1R/AKT axis with paradoxical downregulation of mTOR signaling which was accompanied by increased AMPK activity. Accordingly, abrogation of *SCO2* expression appears to increase the sensitivity of cells to IGF1R and AKT, but not mTOR inhibitors. Finally, the loss of *SCO2* was associated with reduced proliferation and enhanced migration of HCT116 cells. Collectively, herein we describe potential adaptive signaling and metabolic perturbations triggered by mitochondrial complex IV dysfunction.

2.2 INTRODUCTION

Driven by genetic, epigenetic, environmental and other factors, malfunctions of citric acid cycle (CAC) enzymes, OXPHOS supercomplexes and other mitochondrial proteins have been

implicated in neoplastic transformation, tumor progression and/or therapeutic responses (1, 2). This is exemplified by inactivation of fumarate hydratase (FH), succinate dehydrogenase (SDH) or neomorphic mutations in isocitrate dehydrogenase (IDH) that result in accumulation of fumarate, succinate and 2-hydroxyglutarate, respectively (3-7). These metabolites are thought to contribute to tumorigenesis and tumor progression by interfering with several classes of α -ketoglutarate-dependent enzymes that govern key cellular functions (8). Cytochrome c oxidase (COX) assembly protein 2 (SCO2) is essential to the functioning of COX (mitochondrial complex IV). The loss of function or deletion of *SCO2* leads to abrogation of COX assembly and activity (9).

Though non-lethal diseases associated with deficiencies of COX are rare (10, 11), cell models bearing the relevant mutations are useful for understanding metabolic re-arrangements caused by mitochondrial complex IV deficiencies. To this end, studies on colon cancer HCT116 cell line devoid of *SCO2* (HCT116 *SCO2*^{-/-} cells) showed that COX deficiency is associated with decrease in O₂ consumption and slower proliferation. In addition, *SCO2*-deficient cells exhibit an increase in NADH, reactive oxygen species (ROS) and glycolysis, which are accompanied by reversed activity of F₁F₀ ATP-synthase (12-14). These perturbations are paralleled by dramatic changes in gene expression including induction of the factors implicated in epithelial-to-mesenchymal transition (EMT) (12-15). Importantly, HCT116 *SCO2*^{-/-} cells are more resilient to hypoxia than their *SCO2*-proficient counterparts (13), which in conjunction with induction of EMT phenotypes, suggests that dysfunction of mitochondrial complex IV may play a central role in metastatic progression. *SCO2* expression is regulated by p53 (12). Metabolic perturbations caused by the loss of *TP53* function are thus thought to be at least in part mediated by the downregulation of *SCO2* (12). The loss of p53 function was reported to result in the reduction of oxidative

phosphorylation (OXPHOS) and a compensatory increase in glycolysis (12) which is thought to be mediated by increase in hexokinase 2 (HK2) (16) and phosphoglycerate mutase (PGM) (17) as well as the reduction in TIGAR levels (18). Deletion of the *SCO2* gene via homologous recombination in wild type *TP53* HCT116 cells recapitulates the metabolic rewiring towards glycolysis that is observed in p53-deficient cells (12). However, it was also reported that wild type *TP53* glioma and colon cancer cells adapt to hypoxia by at least in part inducing *SCO2* expression (19). Moreover, the correlation between *SCO2* levels and cancer prognosis remains unclear. For instance, low expression of *SCO2* was associated with poor prognosis in breast and ovarian cancer patients (20-22). In contrast, TCGA analysis revealed amplification and subsequent increase in *SCO2* levels in metastatic cancers harboring *TP53* mutations, wherein high *SCO2* levels correlated with poor prognosis (23). Collectively these findings demonstrate that notwithstanding that *SCO2* is likely to play a major role in p53-dependent metabolic reprogramming and thus determine the fate of cancer cells, the underpinning mechanisms and associated clinical correlates remain incompletely understood. In the attempt to address these gaps in knowledge, we systematically examined metabolic perturbations induced by *SCO2* loss in HCT116 cells. As expected, dysfunction of mitochondrial complex IV in *SCO2*^{-/-} HCT116 cells resulted in dramatic metabolic reprogramming including elevated dependence on glucose and altered pyruvate and amino acid metabolism. These metabolic perturbations were accompanied by increased migration and sustained proliferation under extremely low (<0.1%) oxygen levels. Accordingly, *SCO2* deletion in HCT116 cells increases survival under hypoxia compared to the wildtype (WT) cells (13). Moreover, loss of *SCO2* resulted in activation of IGF1R/AKT axis and elevated sensitivity to the IGF1R and AKT inhibitors.

2.3 RESULTS

2.3.1 *SCO2* loss results in metabolic rewiring of HCT116 cells that is characterized by increased NAD⁺ regeneration

Mitochondrial complex IV deficiency caused by *SCO2* loss leads to impairment of the electron transport chain, decreased pyruvate oxidation and increased lactate production (13, 14). To identify metabolic pathways that are altered by abrogation of *SCO2*, we first quantified intracellular and extracellular steady-state levels of key metabolites. As expected, the loss or depletion of *SCO2* in HCT116 or A549 cells, respectively increased glucose uptake (Fig. 2.1A, Fig. S2.1A), extracellular lactate levels (Fig. 2.1B, Fig. S2.1B) and glycolytic intermediates including dihydroxyacetone phosphate (DHAP), 3-phosphoglycerol (3-PG) and 2-phosphoglycerol (2-PG) (Fig. 2.1C). This is in agreement with previous observations that *SCO2* deletion or OXPHOS inhibition using biguanides increases levels of 3-PG and lactate production in order to regenerate NAD⁺ necessary for maintaining glycolysis (13, 24, 25). The re-introduction of exogenous *SCO2* into *SCO2*^{-/-} HCT116 cells attenuated increased glucose uptake and lactate secretion thus confirming that *SCO2* attenuates glycolysis (Fig. S2.1C-D). Of note, notwithstanding repeated attempts, we could not achieve the rescue of *SCO2* expression to the levels of the endogenous protein (Fig. S2.1E). Taken together, loss of *SCO2* in HCT116 cells increases their dependence on glucose metabolism.

In contrast to DHAP, pyruvate levels were found to be lower in *SCO2*^{-/-} relative to WT HCT116 cells (Fig. 2.1C). This is likely due to the diversion of carbon units from the glycolytic pathway to glycerol biosynthesis via 3-phosphoglycerol (3-PG) as 3-PG was found to be higher in *SCO2*^{-/-} than in WT HCT116 cells (Fig. 2.1C). This is indicative of enhanced cytosolic glycerol-3-phosphate dehydrogenase (GPD1) activity which not only produces glycerol but also NAD⁺ (26). We hypothesize that this reaction in addition to the conversion of pyruvate to lactate is

necessary for maintaining glycolysis in the HCT116 *SCO2*^{-/-} cells. Indeed, the intracellular lactate/pyruvate ratio, which is traditionally used as a proxy for cytosolic NADH/NAD⁺ ratio and glycolytic activity (27) was elevated in the *SCO2*^{-/-} as compared to WT HCT116 cells (Fig. 2.1D). Since pyruvate oxidation is initiated by the pyruvate dehydrogenase (PDH), we investigated whether the *SCO2* status affects the levels and/or phosphorylation of PDH subunits. Pyruvate dehydrogenase kinases (PDK1-4) when stimulated by ATP, acetyl-CoA or NADH phosphorylate and inactivate PDH thus deflecting pyruvate from CAC under conditions of mitochondrial dysfunction and hypoxia (28). Surprisingly, we noted that both PDK1 levels and the phosphorylation of PDH subunit PDHE1- α 1 (S293) are decreased in *SCO2*^{-/-} cells relative to WT HCT116 cells in both normoxia and hypoxia (Fig. 2.1E). This was despite the fact that NADH levels are higher in *SCO2*^{-/-} than in WT HCT116 cells (13). Considering that PDK1 expression is stimulated by hypoxia inducible factor (HIF)-1 and 2 α , we next determined HIF-2 α levels. HIF-2 α induction under severe hypoxia (<0.1% O₂) was comparable between the cell lines (Fig. 2.1E). PDK1 levels and PDHE1- α 1 phosphorylation were however more strongly induced in WT as compared to HCT116 *SCO2*^{-/-} cells (Fig. 2.1E). Collectively, these findings show that although the loss of *SCO2* increased NADH levels (13), PDK1 activity is reduced in *SCO2*^{-/-} cells. Intriguingly, the decreased phosphorylation level of the PDHE1- α 1 subunit in HCT116 *SCO2*^{-/-} cells suggests that pyruvate oxidation via PDH is elevated relative to WT cells. Collectively, these findings show that despite the loss of *SCO2* which increased NADH levels (13), PDK1 activity is reduced in *SCO2*^{-/-} cells. Hence, although pyruvate oxidation may be elevated in HCT116 *SCO2*^{-/-} cells, this cannot be explained by NADH-mediated regulation of PDK1 activity. Recent studies demonstrated that one of the major functions of mitochondrial respiration is to produce sufficient amount of aspartate required to drive proliferation of cancer cells (29, 30). To this end, it is thought

that the most of cellular aspartate is derived from oxaloacetate produced in oxidative CAC (31). Consistent with the disruption of the oxidative CAC in *SCO2*^{-/-} HCT116 cells, steady-state aspartate levels were dramatically reduced in *SCO2*^{-/-} as compared to WT HCT116 cells (Fig. 2.2A). However, stable isotope tracer analysis (SITA) using ¹³C-labelled glucose show that in *SCO2*^{-/-} HCT116 cells, pyruvate may primarily undergo carboxylation followed by transamination to produce aspartate as illustrated by increase in m+3 fractions of malate, fumarate and aspartate isotopomers (Fig. 2.2B-C). In turn, the levels of the m+2 citrate, α -KG, fumarate, and malate isotopomers were markedly reduced in *SCO2*^{-/-} compared to WT HCT116 (Fig. 2.2C). These findings suggest that increased carboxylation of pyruvate may represent a compensatory mechanism for a decrease in CAC-derived aspartate in *SCO2*^{-/-} HCT116 cells. This, in addition to increased lactate production and contribution of 3-PG to glycerol synthesis may explain reduction in pyruvate levels in *SCO2*-deficient HCT116 cells.

2.3.2 Abrogation of *SCO2* in HCT116 cells leads to alterations in acetyl-CoA and lipid metabolism

Pyruvate is oxidized through PDH to produce acetyl-coenzyme A (acetyl-CoA), a 2-carbon unit that enters the CAC and yields NADH (32). Notwithstanding observed reduction in PDK1 activity in HCT116 cells devoid of *SCO2* (Fig. 2.1E), PDH is sensitive to product inhibition by NADH (33). Considering that NADH levels are elevated in *SCO2*^{-/-} vs. WT HCT116 cells (13) while *SCO2* loss decreased pyruvate oxidation through the CAC (Fig. 2.2B-C), we hypothesized that *SCO2* deletion is paralleled by a decrease in acetyl-CoA levels. Despite our inability to measure acetyl-CoA directly, we analyzed the effects of *SCO2* loss on acetyl-CoA-dependent pathways. Herein, we observed decreased levels of histone and α -tubulin lysine N-acetylation in *SCO2*^{-/-} relative to WT HCT116 cells in both normoxia and moderate hypoxia (3% O₂) (Fig. 2.3A).

Thus, although the phosphorylation of S293 residue of PDHE1- α 1 subunit often negatively correlates with PDH activity this is not the case for HCT116 *SCO2*^{-/-} cells. Moreover, PDH can be inhibited when phosphorylated at either S232 or S300 residues of PDHE1- α 1 subunit by PDK1-4 (34-36). Considering that acetyl-CoA carboxylation which generates malonyl-CoA is the rate-limiting step in fatty acid (FA) synthesis (37), we next monitored acetyl-CoA carboxylase (ACC) activity. Relative to WT, *SCO2*^{-/-} HCT116 cells exhibited increased activity of AMP-activated protein kinase (AMPK) that phosphorylates (S79) and inactivates ACC (Fig. 2.3B). Moreover, the levels of citrate, which allosterically activates ACC, were markedly decreased in *SCO2*^{-/-} as compared to WT HCT116 cells (Fig. 2.3C). Reduction of FA synthesis in *SCO2*^{-/-} HCT116 as compared to WT cells also aligns with the increase in both the steady-state and m+3 levels of 2- and 3-PG, which are triacylglycerol (TAG) precursors (Fig. 2.1C, Fig. 2.2C). Collectively, these data suggest that FA synthesis is reduced in *SCO2*-deficient vs. proficient cells. However, in contrast to WT cells, numerous Nile Red positive lipid droplets (LD), which are depots of TAGs, were observed in *SCO2*^{-/-} but not in WT HCT116 cells (Fig. 2.3D). *SCO2*-deficient mice have increased fat mass associated with reduced β -oxidation (38). Altogether, these findings suggest that the *SCO2* loss-induced decrease in mitochondrial β -oxidation is incompletely compensated by reduction in FA synthesis via suppression of ACC. Of note, we excluded the possibility that LDs contain FA that were imported from growth media by culturing cells in the presence of normal or FA-free FBS for 20 days, which did not affect the size or number of LDs (Fig. S2.2A). Importantly, HCT116 WT cells phenocopied the accumulation of LDs observed in *SCO2*-deficient cells when grown continuously under severe hypoxia (<0.1% O₂) (Fig. S2.2B) or in the presence of complex III inhibitors antimycin A (Ant A) or myxothiazol (Myx) (Fig. S2.2C). Chronically

hypoxic HCT116 *SCO2*^{-/-} cells also increased LD deposition, suggesting that in the presence of O₂ some FA may be metabolized likely through the peroxisomal β -oxidation (39).

2.3.3 Complex IV dysfunction in HCT116 cells results in increased succinate and 2-hydroxyglutarate levels

Steady-state metabolite analysis also revealed dramatic accumulation of succinate and 2-hydroxyglutarate (2-HG) in *SCO2*^{-/-} vs. WT HCT116 cells (Fig. 2.4A). As succinate is known to accumulate upon complex II inhibition (40-43) as well as following treatment with the complex III inhibitor Ant A (44), we reasoned that the increased succinate observed in cells devoid of *SCO2* was due to the impairment of the electron transport chain (ETC) and subsequent decrease in the activity of mitochondrial complex II (SDH). Indeed, steady state fumarate and malate levels were decreased, thus suggesting reduced utilization of succinate through CAC in *SCO2*^{-/-} as compared to WT HCT116 cells (Fig. 2.4A). In addition, 2-HG accumulated in HCT116 cells devoid of *SCO2*. While we could not distinguish the enantiomer of 2-HG that was elevated in HCT116 *SCO2*^{-/-} cells, sequencing analysis of *IDH1* and *IDH2* mRNAs in WT and HCT116 *SCO2*^{-/-} did not reveal neomorphic mutations (arginine residues R100, R109, R132 for *IDH1*, and R140, R149, R172 for *IDH2*) known to give rise to D-2-HG (Fig. S2.3A) (45). Low pH, reduced oxidation of α -ketoglutarate (α -KG) and increased NADH levels have been shown to enhance L-2-HG synthesis chiefly via lactate (LDH) and malate dehydrogenases (MDH) (46, 47). Although we did not detect differences in cellular pH between WT and *SCO2*^{-/-} HCT116 cells (Fig. S2.3B), the overall reductive state caused by ETC dysfunction and the lack of *IDH1* or *IDH2* mutations, suggest that it is likely that L-2-HG accumulates in HCT116 *SCO2*^{-/-} cells (48).

2.3.4 *SCO2* loss in HCT116 cells affects amino-acid metabolism

Amino acid metabolism is partly dependent on mitochondrial function. The CAC intermediates are used to biosynthesize amino acids including aspartate, asparagine, proline and glutamate (49). Moreover, as noted above, the essential role of mitochondrial respiration in producing aspartate to fuel neoplastic growth has been described (29, 30, 50). To this end, it was shown that the inhibition of mitochondrial complex I with metformin not only reduced the NAD⁺/NADH ratio but also suppressed aspartate biosynthesis (50). Consistently, we observed a decrease in proline, glutamate and asparagine levels in *SCO2*^{-/-} vs. WT HCT116 cells (Fig. 2.4B). In turn, levels of cysteine were higher in *SCO2*-deficient vs. proficient HCT116 cells (Fig. 2.4B). Considering that cysteine is a rate-limiting substrate in glutathione synthesis (51) and that the loss of *SCO2* results in increased ROS production (13), the observed increase in cysteine levels in *SCO2*^{-/-} HCT116 cells may be indicative of potential compensatory mechanisms that are triggered to protect cells from ROS-induced damage. In line with this, increased cysteine levels may be required to support the turnover of mitochondrial cysteine-rich proteins that also contribute to the antioxidant defense machinery (52). Accordingly, the levels of mitochondrial ROS scavenger superoxide dismutase 2 (SOD2) were elevated in *SCO2*^{-/-} compared to WT HCT116 cells (Fig. S2.4A).

Cancer cells use glutamine as an anaplerotic source for the CAC (53, 54). The loss of *SCO2* in HCT116 cells reduced intracellular glutamine levels compared to WT cells. Inhibition of OXPHOS results in reductive glutamine metabolism and consequent reductive carboxylation of α -KG to produce aspartate which is necessary for cancer cell proliferation (29, 30). A high α -KG/citrate ratio, commonly observed in cancer cells with mitochondrial dysfunction, is an indirect indication of reductive glutamine metabolism (55). Notwithstanding that the glutamine levels were lower in *SCO2*^{-/-} as compared to WT HCT116 cells, the α -KG/citrate ratio was dramatically

elevated in SCO2-deficient cells (Fig. 2.4B-C). Hence, although the deletion of *SCO2* decreased intracellular glutamine level in HCT116 cells, these data suggest that most of the glutamine is likely to be reductively metabolized in HCT116 *SCO2*^{-/-} cells (Fig. 2.4B). Altogether, these data suggest that the loss of *SCO2* causes major perturbations in amino acid metabolism.

2.3.5 Loss of mitochondrial complex IV function leads to induction of IGF1R/AKT axis with paradoxical reduction in mTOR signaling

To better understand the mechanism of metabolic adaptations to the disruption of mitochondrial complex IV function, we next investigated the effects of *SCO2* abrogation on mTOR signaling, which acts a major conductor of metabolic programs in the cell (56). Notably, phosphorylation of AKT catalytic site (T308) and hydrophobic motif (S473) were increased in *SCO2*^{-/-} as compared to WT HCT116 cells (Fig. 2.5A). Accordingly, *SCO2*^{-/-} HCT116 cells exhibited higher AKT-dependent phosphorylation (S9) of GSK3 β (Fig. 2.5A), thus indicating that AKT activity is higher in *SCO2*-deficient vs. proficient cells. Importantly, HCT116 WT cells treated with complex III inhibitors AntA and Myx showed similar increase in phosphorylation of AKT (S473) (Fig. 2.5B). Collectively, this indicates that disruption of terminal ETC complexes (III and IV) may result in compensatory AKT activation. Notably, the increase in AKT activity coincided with upregulation of insulin-like growth factor 1 receptor (*IGF1R*) mRNA and IGF1R β protein in *SCO2*^{-/-} vs. WT HCT116 cells (Fig. 2.5A and 2.5C), implying that the loss of mitochondrial complex IV function may lead to increased signaling through the IGF1R/AKT axis. Similar induction of *IGF1R* mRNA levels was observed in WT HCT116 cells maintained at 0.1% O₂ (Fig. 2.5C). Upregulated signaling via the IGF1R/AKT axis in *SCO2*^{-/-} HCT116 cells was also paralleled by an increase in the amount of IGF1 bound to IGF1R (Fig. 2.5D).

Surprisingly, despite increased AKT activity, *SCO2* loss in HCT116 cells led to a decrease in mTORC1 activity, as illustrated by reduction in phosphorylation of its downstream substrate 4E-binding protein 1 (4E-BP1; S65), and ribosomal protein S6 (rpS6; S240/244) which is a substrate of mTOR-dependent S6 kinases (S6Ks) (Fig. 2.5A). In turn, AMPK phosphorylation was increased in *SCO2*^{-/-} relative to HCT116 WT cells (Fig. 2.3B). Given that AMPK is a negative regulator of mTORC1 (57, 58) this may explain the reduction of mTORC1 signaling upon *SCO2* loss, despite increase in AKT activity. Altogether, these findings show that the impairment of mitochondrial complex IV function leads to increase in AKT and AMPK activity, which is accompanied by a decrease in mTORC1 signaling.

mTORC1 stimulates cell proliferation (59). *SCO2* loss-induced reduction in mTORC1 activity was therefore consistent with decreased proliferation of *SCO2*-deficient vs. proficient cells (Fig. 2.5E). Consistent with previous findings (13), the proliferation of *SCO2*^{-/-} HCT116 cells was less affected by exposure to 0.1% O₂ in comparison to WT HCT116 cells (Fig. 2.5E). These observed phenotypes appeared not to be limited to HCT116 cells. *SCO2* depletion in A549, HeLa and HT29 cells resulted in increased AKT phosphorylation (S473) and decreased phosphorylation of downstream mTORC1 substrates (Fig. S2.5A-C). Consistent with the findings in HCT116 cells, *SCO2* depletion also reduced proliferation of A549, HeLa and HT29 cells (Fig. S2.5D-F). Next, we re-expressed *SCO2* in *SCO2*^{-/-} HCT116 cells to exclude potential inadvertent effects caused by cellular adaptation to *SCO2* loss. Notwithstanding that as indicated above exogenous *SCO2* protein levels were significantly lower than the levels of endogenous *SCO2* protein (Fig. S2.1E, S2.5G), re-expression of *SCO2* in *SCO2*^{-/-} HCT116 cells resulted in partial rescue of cellular proliferation as compared to vector infected *SCO2*^{-/-} HCT116 cells (Fig. S2.5H). Re-expression of *SCO2* also partially rescued mTORC1 signaling as illustrated by increase in 4E-BP1 phosphorylation in

SCO2^{-/-} HCT116 cells expressing *SCO2* relative to control, vector infected *SCO2*^{-/-} HCT116 cells (Fig. S2.5G).

2.3.6 *SCO2* loss leads to increased cell migration and altered expression of EMT markers in HCT116 cells.

Mitochondrial dysfunction resulting in increased ROS and/or accumulation of oncometabolites such as 2-HG has been linked to elevated expression of EMT markers, increased cell migration and higher metastatic potential of cancer cells (60, 61). Accordingly, previous studies have shown higher levels of ROS and the mesenchymal marker, vimentin, in *SCO2*^{-/-} compared to HCT116 WT cells (13, 14). To this end, we next monitored the effects of *SCO2* loss on migration and expression of epithelial (E-cadherin) and mesenchymal (vimentin) markers using HCT116 cell model. These studies revealed that the loss of *SCO2* enhances migration of HCT116 cells (Fig. 2.5F). In addition to increased vimentin levels, *SCO2*-deficient HCT116 cells exhibited a decrease in E-cadherin levels as compared to WT HCT116 cells (Fig. 2.5G). Also the loss of *SCO2* in HCT116 correlated with elevated *ZEB1* mRNA levels (Fig. 2.5H), which encodes a key regulator of EMT (62). Moreover, re-expression of *SCO2* in *SCO2*^{-/-} HCT116 cells attenuated migration, reduced vimentin levels while increasing E-cadherin abundance (Fig. S2.5I-J). We observed that *SCO2*-deficient HCT116 cells exhibit attenuated mTORC1 signaling as compared to their *SCO2*-proficient counterparts (Fig. 2.5A). Inhibition of mTOR was shown to coincide with elevated phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2) and increased cell migration (63, 64). However, the phosphorylation of eIF2 α (S51) was not affected by the *SCO2* status in the HCT116 cells (Fig. S2.5K). Hence, although *SCO2* deletion in HCT116 cells reduced proliferation, it increased their migratory potential whereby this effect was independent of eIF2 α phosphorylation.

2.3.7 SCO2-deficient HCT116 cells exhibit increased susceptibility to IGF1R and AKT inhibitors.

Considering that the disruption of mitochondrial complex IV activity is paralleled by the increase in IGF1R β levels and AKT activity (Fig. 2.5A), we next investigated the effects of IGF1R and AKT inhibitors on the fate of transformed cells as a function of their SCO2 status. As compared to control (vehicle) treatment, allosteric pan-AKT inhibitor MK2206 reduced proliferation (Fig. 2.6A), attenuated progression from G1 to S phase of cell cycle (Fig. 2.6B) and decreased survival (Fig. 2.6C) of *SCO2*^{-/-} HCT116 cells more pronouncedly than in WT cells. Of note, depletion of SCO2 also potentiated anti-proliferative effects of MK2206 in A549 and HT29 cells as compared to the control, scrambled shRNA infected cells (Fig. S2.6A-B) albeit to a lesser extent than the complete SCO2 knock-out in HCT116 cells (Fig. 2.6A). Moreover, MK2206 reduced levels of glycolytic intermediates including DHAP and 3-PG in HCT116 *SCO2*^{-/-} cells to a greater extent than in control cells (Fig. S2.7A-B). Notably, comparable reduction of phosphorylated AKT levels was observed between WT and *SCO2*^{-/-} HCT116 cells thus excluding potential differences in AKT inhibition by MK2206 between the cell lines (Fig. 2.6D). The effect of MK2206 on mTORC1, as monitored by rpS6 (S240/244) and 4E-BP1 (S65) phosphorylation levels, were greater in SCO2-proficient vs. deficient HCT116 cells which is consistent with higher basal mTORC1 activity in the former cell line (Fig. 2.6D). In turn, although the allosteric (rapamycin) and active-site (torin 1) mTOR inhibitors abolished mTORC1 signaling in both cell lines as evidenced by the reduction in rpS6 (S240/244) and 4E-BP1 (S65) phosphorylation, their anti-proliferative effects were lesser in *SCO2*^{-/-} as compared to WT HCT116 cells (Fig. 2.6D-F). SCO2 depletion also attenuated anti-proliferative effects of mTOR inhibitors in A549 and HT29 cells (Fig. S2.8A-D). Similar to AKT inhibition, IGF1R/insulin receptor inhibitor, OSI-906 (linsitinib), reduced 4E-BP1 (S65)

phosphorylation and proliferation of HCT116 *SCO2*^{-/-} cells to a greater extent compared to WT cells (Fig. 2.6G-H). These findings suggest that the loss of *SCO2* may render HCT116 cells “addicted” to the IGF1R/AKT axis. In turn, HCT116 *SCO2*^{-/-} cells exhibit reduced mTORC1 signaling, which may explain their decreased sensitivity to mTOR inhibitors. Collectively, these results suggest that uncoupling of IGF1R/AKT and mTORC1 signaling may be required for adaptation of HCT116 cells to *SCO2* loss. mTOR is major stimulator of anabolic processes such as lipid and protein synthesis (65, 66). This alludes to a model whereby activation of IGF1R/AKT axis promotes survival of cancer cells with complex IV dysfunction by driving glycolysis, while concomitant suppression of mTOR is required to decrease energy consumption and thus compensate for disrupted mitochondrial ATP production.

2.4 FIGURES

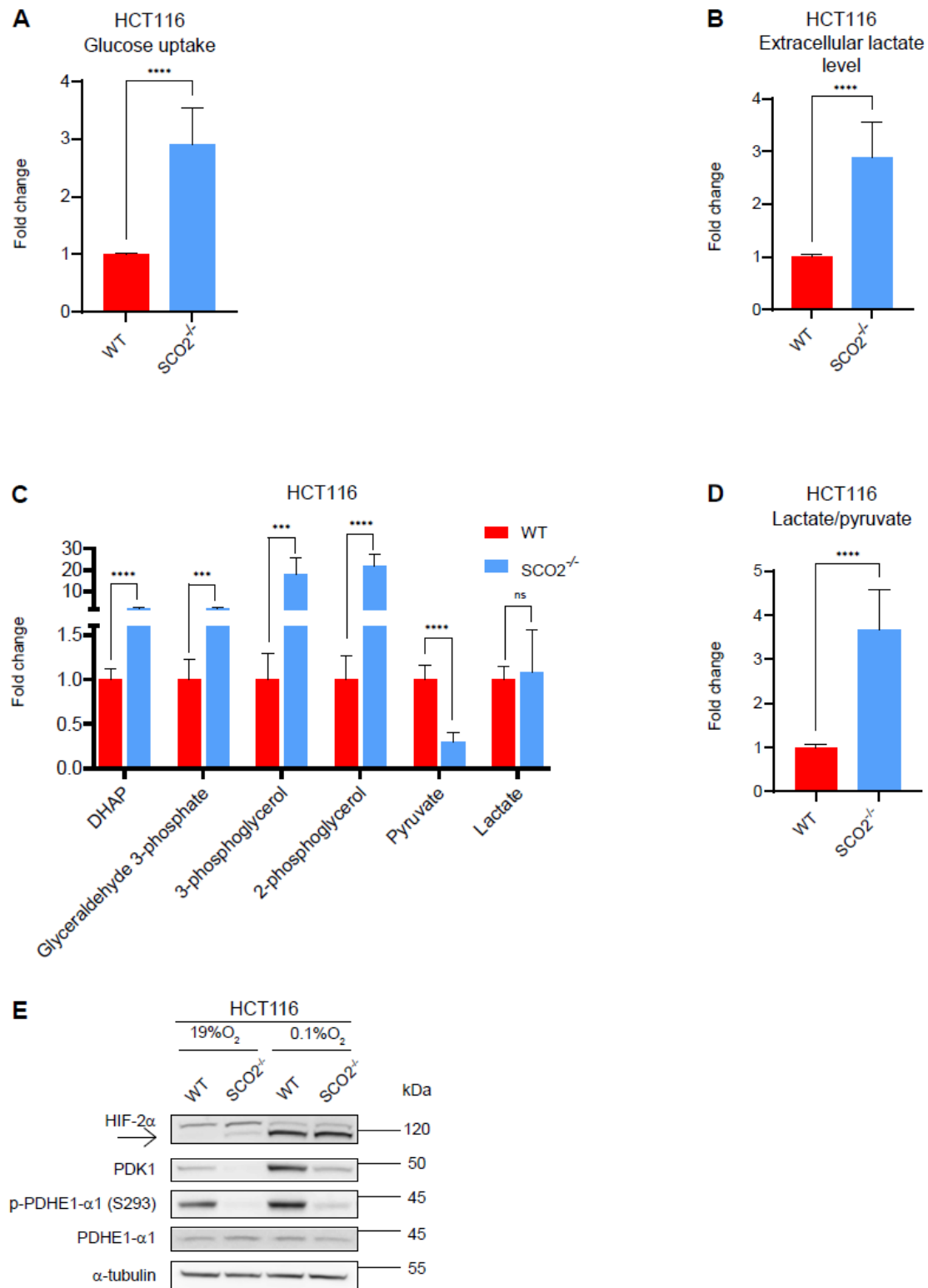


Fig. 2.1. *SCO2* loss in HCT116 cells rewires glucose metabolism while decreasing PDHE1- α 1 phosphorylation. **A-B,** Glucose uptake (**A**) and extracellular lactate levels (**B**) were determined using BioProfiler analyzer. Data are represented as mean fold change relative to the control, WT HCT116 cells (set to 1) +/- standard deviation (SD). **** $p < 0.0001$ (Unpaired 2-tailed t-test; $n=5$ independent experiments with 3 technical replicates in each). **C.** Intracellular levels of indicated metabolites in WT or HCT116 *SCO2*^{-/-} cells. Metabolite levels were monitored by GC-MS. Data are presented as mean fold change relative to HCT116 WT cells (set to 1) +/- SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Unpaired 2-tailed t-test; $n=3$ independent experiments with 3 technical replicates in each). **D.** Intracellular lactate/pyruvate ratio in WT or HCT116 *SCO2*^{-/-} cells. Intracellular lactate and pyruvate levels were determined by GC-MS. Obtained lactate/pyruvate ratio in WT HCT116 cells were set to 1. Data are presented as mean fold change of $n=3$ independent experiments with 3 technical replicates in each. **E.** Levels and phosphorylation status of indicated proteins in WT or *SCO2*^{-/-} HCT116 cells were monitored by Western blotting. α -tubulin served as a loading control. As indicated, cells were grown for 10 days under 19% and 0.1% O₂. Shown are representative Western blots from 3 independent experiments. Quantifications of Western Blots are shown in Fig. S2.9.

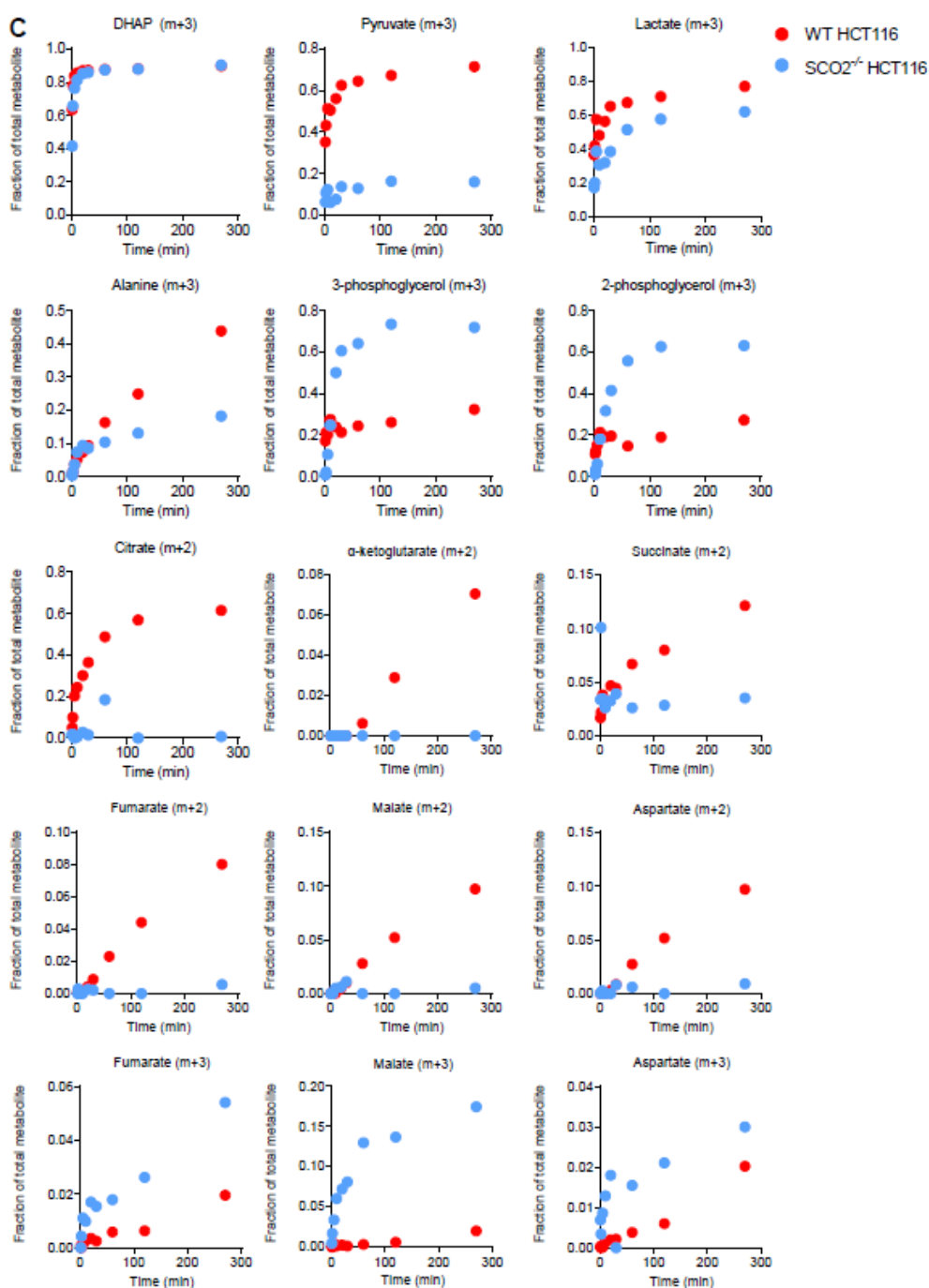
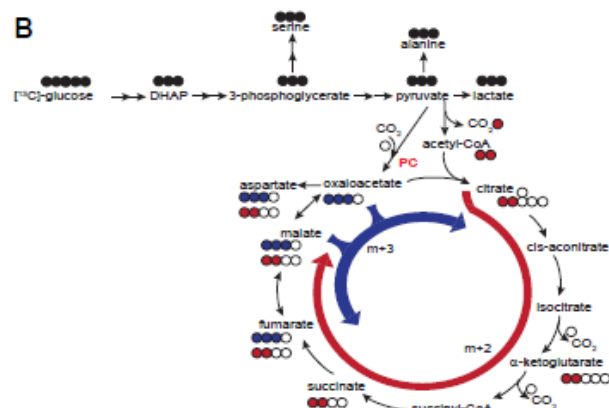
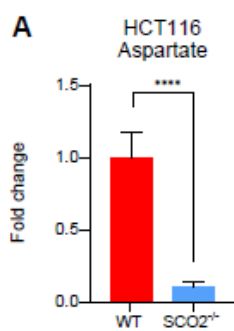


Fig. 2.2. *SCO2* loss in HCT116 cells reduces pyruvate oxidation while increasing pyruvate reduction and carboxylation. **A.** Intracellular levels of aspartate in WT and *SCO2*^{-/-} HCT116 cells. Aspartate levels were determined by GC-MS. Results are represented as mean fold change +/- SD in which the values calculated for WT HCT116 cells were set to 1. ****p<0.0001 (Unpaired 2-tailed t-test; n=3 independent experiments with 3 technical replicates in each). **B.** Schematic diagram of ¹³C incorporation into metabolites after incubation with ¹³C₆-glucose. Red arrow represents the incorporation of two ¹³C units via the oxidative carboxylation of CAC. Blue arrow represents the carboxylation of pyruvate via pyruvate carboxylase (PC) whereby resulting metabolites have three ¹³C units. **C.** Mass spectrometry analysis of glucose-derived metabolites (glucose flux) in *SCO2*^{-/-} and WT HCT116 cells. Cells were grown in the presence of ¹³C₆-glucose for 1, 2, 5, 10, 20, 30, 60, 120 and 270 min. Representative plots of 3 independent experiments are shown.

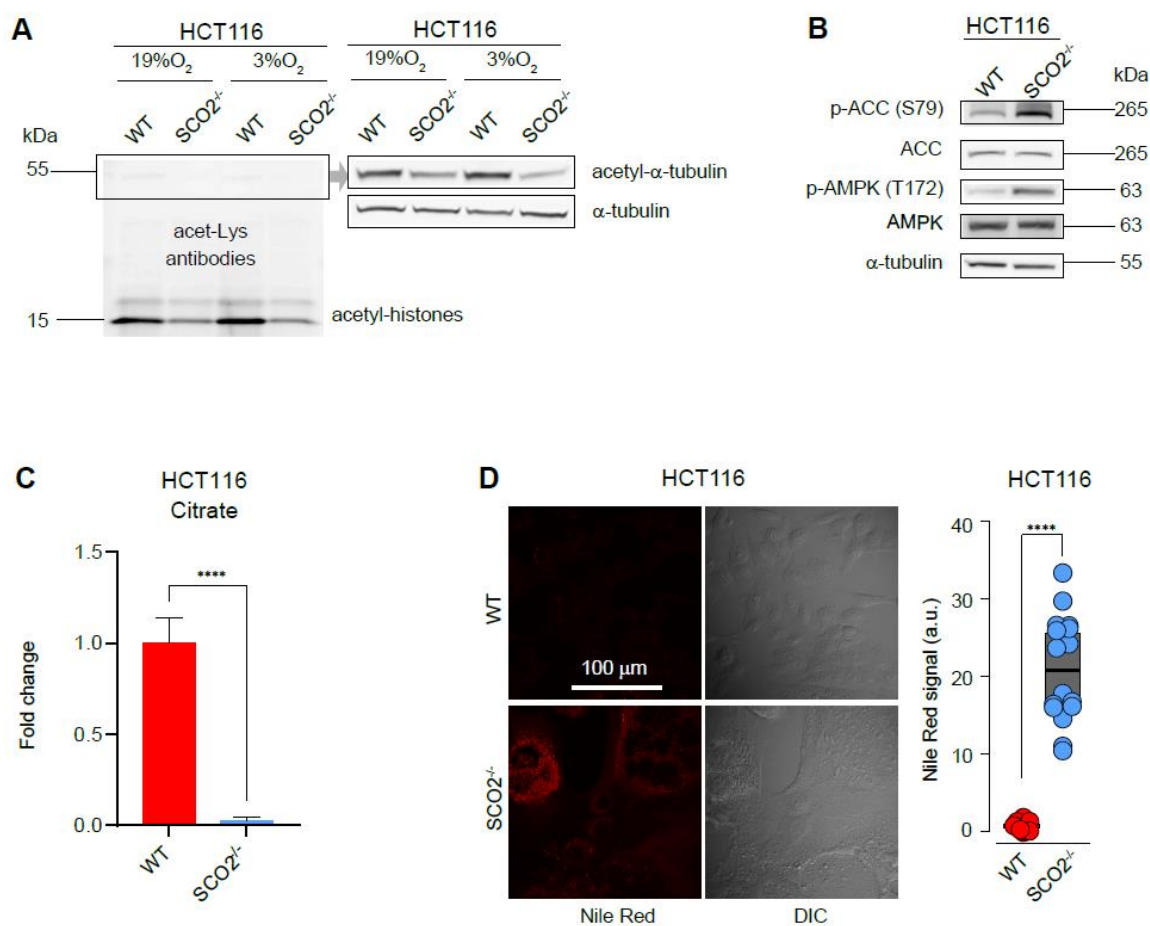


Fig. 2.3. Complex IV deficiency alters fatty acid metabolism in HCT116 cells. **A.** Western blot analysis of α -tubulin (Lys 40) and total histone acetylation levels in WT and SCO2^{-/-} HCT116 cells. Representative blots of 2 independent replicates are shown **B.** Phosphorylation status and levels of indicated proteins were assessed in WT and SCO2^{-/-} HCT116 by Western blotting. α -tubulin served as a loading control. Shown are representative blots of 3 independent experiments. **C.** Intracellular levels of citrate in WT and SCO2^{-/-} HCT116 cells. Citrate levels were determined by GC-MS. Results are represented as mean fold change $\pm$ SD whereby values obtained for WT HCT116 cells were set to 1. ****p<0.0001 (Unpaired 2-tailed t-test; n=3)

independent experiments with 3 technical replicates in each). **D.** Confocal microscopy of lipid droplets in WT and *SCO2*^{-/-} HCT116 cells using Nile Red staining. Fluorescence images are stacks of 5 focal planes taken with a 0.5 μ m step, with single-plane DIC images on the right (n=3 independent experiments). Results of a representative experiment (N = 16 cells for each cell line) are normalized to the mean fluorescence intensity in WT HCT116 cells and shown as individual data points in arbitrary units (a.u.); mean value in WT HCT116 cells was set to 1 a.u. ****p<0.0001 (Unpaired 2-tailed t-test). Quantifications of Western Blots are shown in Fig. S2.9.

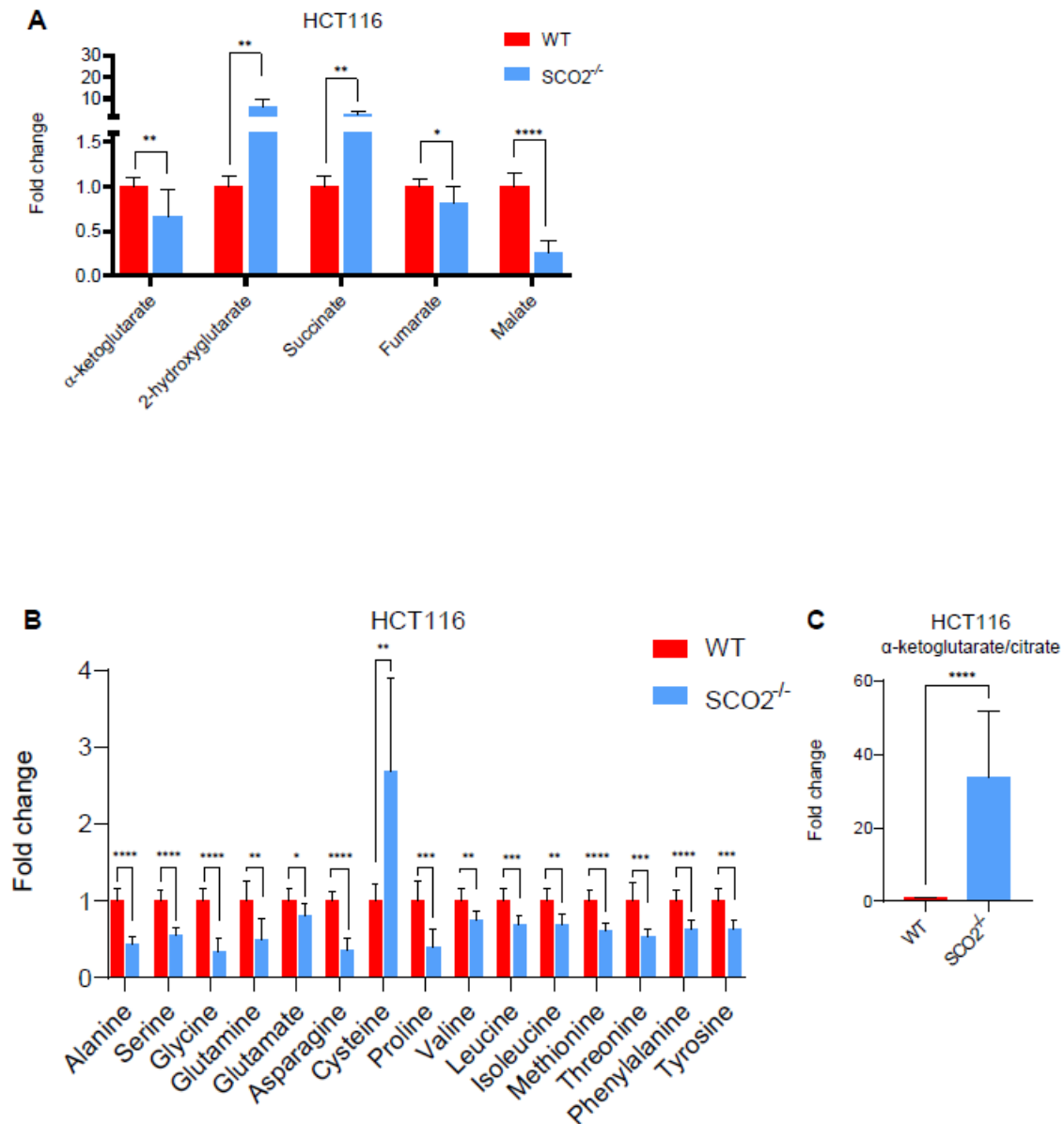


Fig. 2.4. *SCO2* abrogation in HCT116 cells results in asymmetry in CAC intermediates and decrease in the levels of the most amino acids. A-B, Intracellular levels of metabolites in WT and $SCO2^{-/-}$ HCT116 cells were monitored by GC-MS showing decreased levels of malate, fumarate, α -ketoglutarate while succinate level increased in $SCO2^{-/-}$ HCT116 cells. Data are

represented as mean fold change $\pm$ SD relative to WT HCT116 cells wherein values for each metabolite are set to 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Unpaired 2-tailed t-test; $n=3$ independent experiments with 3 technical replicates in each). C. Intracellular α -ketoglutarate/citrate ratio in WT and *SCO2*^{-/-} HCT116 cells was determined from the levels of respective metabolites detected by GC-MS. The values obtained for WT HCT116 cells were set at 1 and the results were represented as means of the ratios from 3 independent experiments with 3 technical replicates each $\pm$ SD. **** $p < 0.0001$ (Unpaired 2-tailed t-test).

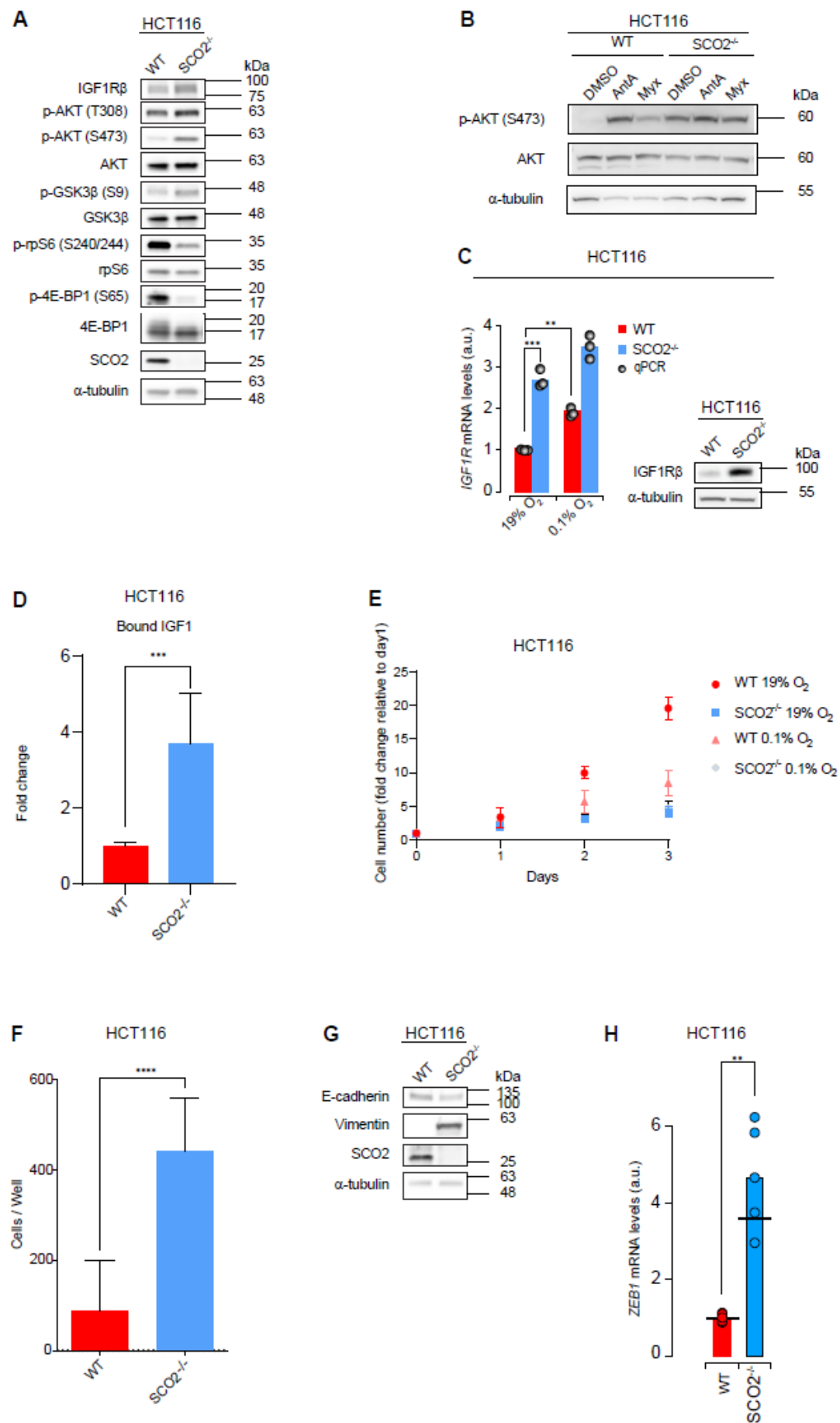


Fig. 2.5. *SCO2* loss in HCT116 cells leads to activation of AKT and AMPK, suppression of mTORC1, reduction in cell proliferation and increased cell migration.

A. Levels and phosphorylation status of indicated proteins were monitored by Western blotting in WT and *SCO2*^{-/-} HCT116 cells. α -tubulin served as a loading control. Shown are representative Western blots from 3 independent replicates.

B. WT HCT116 cells were treated with antimycin A (Ant A, 5 μ M) or myxothiazol (Myx, 2 μ M) treated for 8 days. Western blot analysis was employed to assess the levels and phosphorylation status of indicated proteins. α -tubulin served as a loading control. Representative blots from 3 independent experiments are shown.

C. *IGF1R* mRNA and IGF1R β protein levels were determined by RT-qPCR and Western blotting respectively. As indicated WT and *SCO2*^{-/-} HCT116 cells were maintained under 19% or 0.1% O₂ for 8 days. RT-qPCR data are presented as mean values of 3 independent experiments (columns) and individual data points; mean values obtained for HCT116 WT maintained under 19% oxygen was set to 1 a.u. **p<0.01, ***p<0.001 (Unpaired 2-tailed t-test). Representative Western blots from 3 independent experiments are shown. α -tubulin was used as a loading control.

D. Levels of bound IGF1 were calculated by monitoring free IGF1 levels in extracellular media of WT and *SCO2*^{-/-} HCT116 cells using ELISA. Data is represented as a mean fold change $\pm$ SD relative to WT HCT116 cells (set at 1). ****p<0.0001 (Unpaired 2-tailed t-test; n=3 independent replicates with 3 technical replicates each).

E. Numbers of viable WT (red) and *SCO2*^{-/-} (blue) HCT116 cells grown 0.1% (triangles) or 19% O₂ (squares) for indicated times was monitored by trypan blue exclusion using automated cell counter. Results are presented as the mean value $\pm$ SD relative to WT or *SCO2*^{-/-} HCT116 cell number grown in normoxia at day 1 which was set to 1 (n=4 independent experiments with 3 technical replicates each).

F. Transwell migration assay of WT and *SCO2*^{-/-} HCT116 cells. The columns represent the mean summarized cell counts of four fields from a single transwell

migration chamber. Data are presented as the mean value $\pm$ SD **** $p < 0.0001$ (Unpaired 2-tailed t-test; $n=3$ independent experiments with 2 technical replicates each). **G.** Levels of indicated proteins were monitored by Western blotting. α -tubulin was used as a loading control. Representative blots from 3 independent experiments are shown. **H.** *ZEB1* mRNA levels in WT and *SCO2*^{-/-} HCT116 cells were analyzed by RT-qPCR. Results are presented as mean values of 3 independent experiments (columns) and individual data points. Mean values obtained for WT HCT116 cells was set to 1 a.u. ** $p < 0.01$, *** $p < 0.001$ (Unpaired 2-tailed t-test). Quantifications of Western Blots are shown in Fig. S2.9.

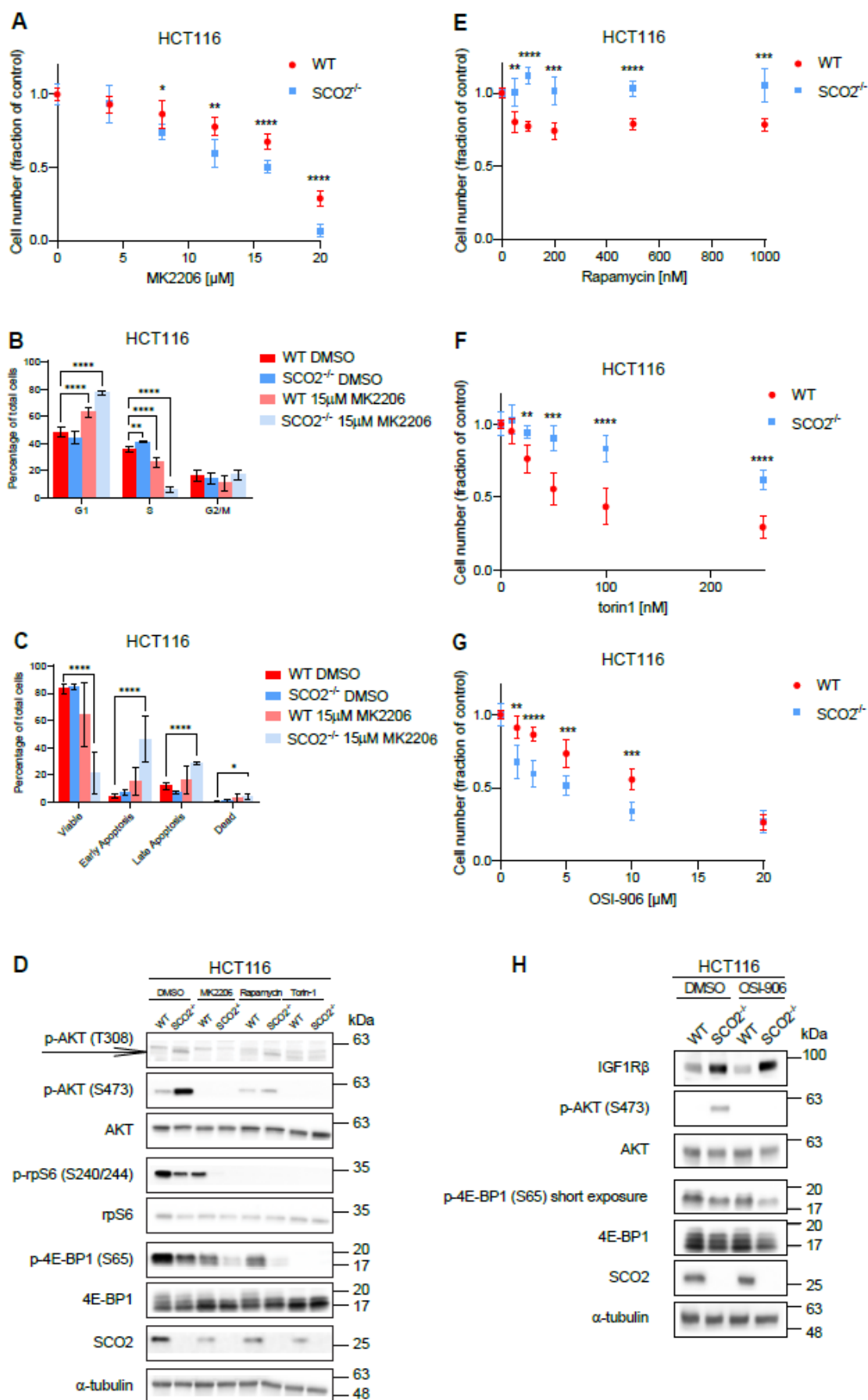


Fig. 2.6. Complex IV deficiency increases sensitivity of HCT116 cells to IGF1R and AKT, but not mTOR inhibitors. **A.** WT and *SCO2*^{-/-} HCT116 cells were treated with indicated concentrations of pan-AKT inhibitor MK2206 or a vehicle (DMSO) for 3 days. The number of viable cells at each time point was obtained using trypan blue exclusion and automated cell counter. Data are represented as the mean fraction of cells relative to the corresponding DMSO-treated controls which were set to 1. Experiments were carried out in 3 independent replicates (2 technical replicates each time). Bars represent +/- SD values. **B.** Cell cycle distribution of WT and HCT116 *SCO2*^{-/-} cells treated with MK2206 (15 μ M) or a vehicle (DMSO) for 24 h was determined by flow cytometry. The data are presented as mean percent values of the total cell population +/- the SD. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 (One-way ANOVA; Dunnette's posthoc test with WT HCT116 DMSO-treated cells as control; n=2 independent experiments with 3 technical replicates in each). **C.** WT and *SCO2*^{-/-} HCT116 cells were treated with MK2206 (15 μ M) for 48 h, stained with AnnexinV-FITC and PI subsequently monitored by flow cytometry. Percent of live, early apoptotic, late apoptotic and dead cells are shown relative to the total cell population. The data are presented as mean values +/- the SD. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 (One-way ANOVA; Dunnette's posthoc test with WT HCT116 DMSO-treated cells as control; n=2 independent experiments with 3 technical replicates each). **D.** Western blot analysis of indicated proteins isolated from WT and HCT116 *SCO2*^{-/-} cells treated with MK2206 (15 μ M), rapamycin (50 nM), torin 1 (250 nM), or a vehicle (DMSO) for 24 h. Representative blots from 2 independent experiments are shown. α -tubulin served as a loading control. **E-G,** WT and *SCO2*^{-/-} HCT116 cells were treated with indicated concentrations of mTOR [rapamycin (**E**) or torin 1 (**F**)], INSR/IGF1R inhibitors [OSI-906 (**G**)] or a vehicle (DMSO) for 3 days. Numbers of viable cells in each condition were determined by trypan blue exclusion using

automated cell counter. Data are represented as the mean fraction of cells relative to DMSO treated controls $\pm$ SD. Experiments were carried out in independent triplicate with 2 technical replicates each. **H.** WT and *SCO2*^{-/-} HCT116 cells were treated with OS1-906 (10 μ M) or a vehicle (DMSO) for 24 h. Levels and phosphorylation status of indicated proteins was determined by Western blotting. α -tubulin served as a loading control. Representative blots from 3 independent experiments are shown. Quantifications of Western Blots are shown in Fig. S2.9.

2.5 SUPPLEMENTAL FIGURES

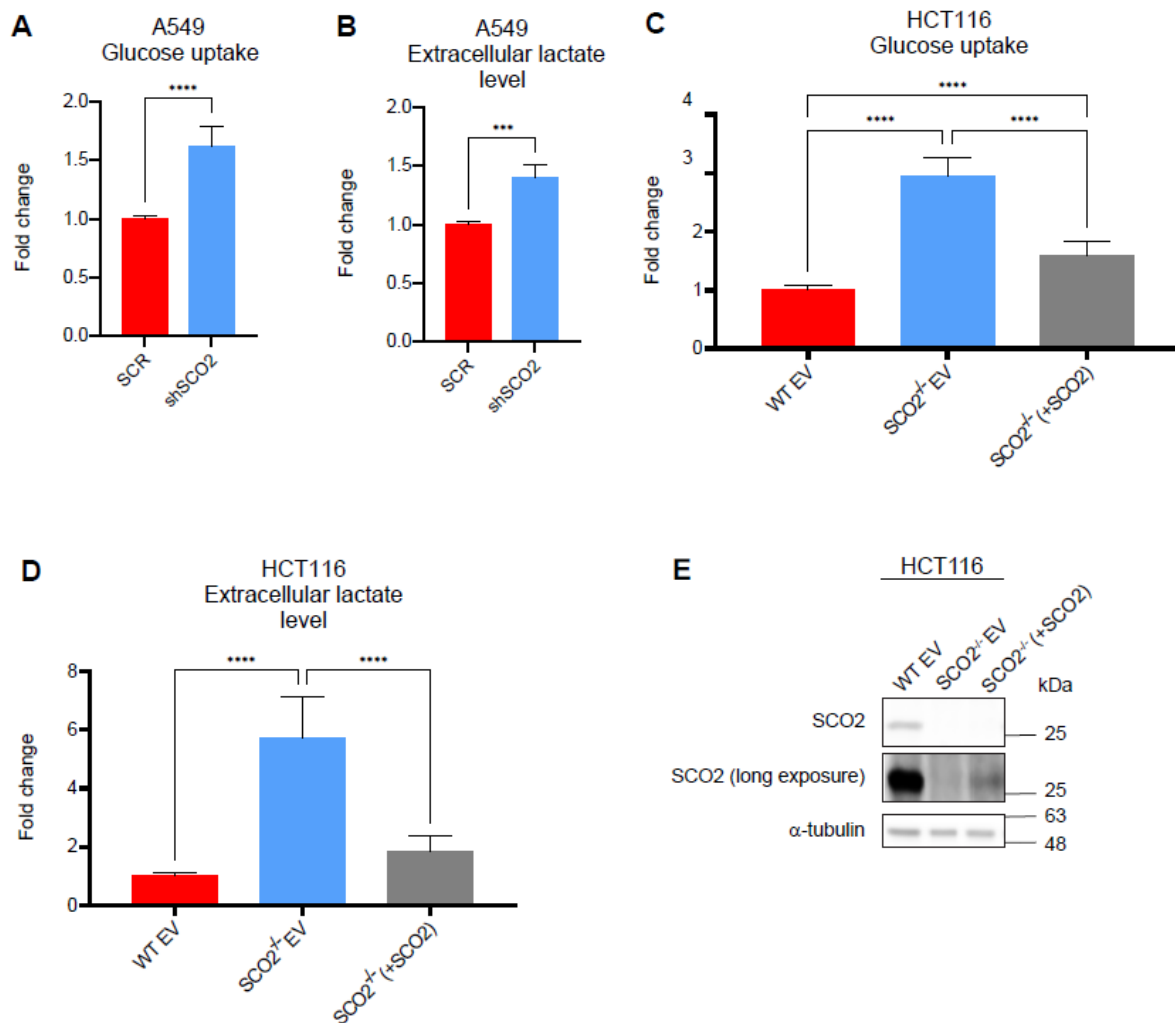


Fig. S2.1. SCO2 regulates glucose metabolism. A-D, Glucose uptake (**A** and **C**) and extracellular lactate levels (**B** and **D**) were monitored using BioProfiler analyzer in the indicated cell lines. Data are represented as mean fold change relative to the scrambled shRNA (SCR) control in A549 cells (**A-B**) or empty vector (EV) control in HCT116 cells (**C-D**) [SCR A549 and WT EV HCT116; set to 1 +/- standard deviation (SD)]. **** $p < 0.0001$, *** $p < 0.001$ ((**A-B**) Unpaired 2-tailed t-test; $n=2$ independent experiments with 3 technical replicates each. (**C-D**) One-way ANOVA; Tukey's multiple comparison post-hoc test; $n=4$ independent experiments with 1-3 technical replicates in each). **E.** Level of SCO2 in WT and *SCO2*^{-/-} HCT116 cells infected with an empty vector (EV) or *SCO2*^{-/-} HCT116 cells where *SCO2* was re-expressed (+SCO2) was monitored by Western blotting ($N=3$ independent experiments). α -tubulin served as a loading control. Quantifications of the Western blots are provided in Fig. S2.9.

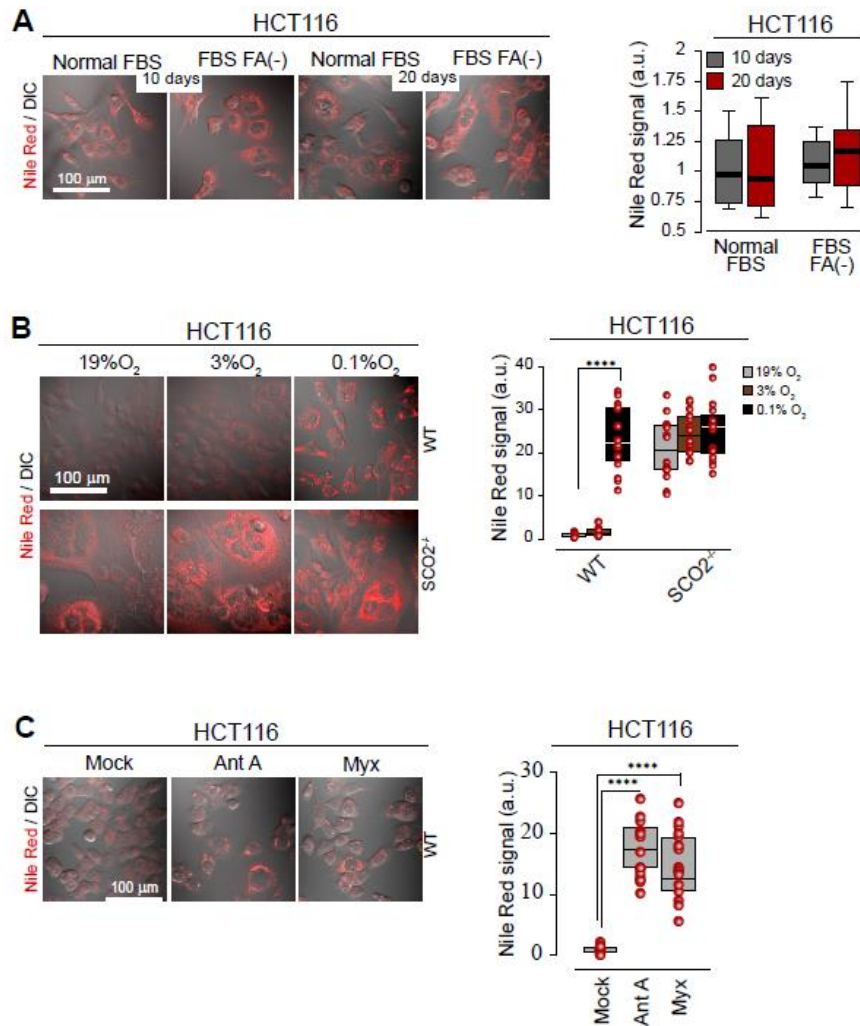


Fig. S2.2. Inhibition of complex III or IV of the electron transport chain results in lipid droplet accumulation in HCT116 cells. A-C, Confocal microscopy of lipid droplets using Nile Red staining with the fluorescent images composed of stacks of 5 focal planes taken with 0.5 μ m step subsequently superimposed with single-plane DIC images (N= 20 cells for each condition). **A.** SCO2^{-/-} HCT116 cells were grown for 10 and 20 days in the presence of regular fatty acid (FA+) and FA-deficient FBS (FA-). **B.** SCO2^{-/-} and WT HCT116 cells were maintained for 10 days at 19%, 3% or 0.1% O₂. **C.** WT HCT116 cells were treated with complex III inhibitors antimycin A (Ant A, 5 mM) or myxothiazol (Myx, 2 mM) for 10 days. All results are presented as median and

interquartile range (boxes) (A-C) along with individual data points (B-C). Mean value obtained for WT HCT116 cells was set to 1 a.u. ****p<0.0001 (Unpaired 2-tailed t-test).

A

IDH1

Annotated protein sequence with R100, R109, R132 highlighted

MSKKISGGSVVEMQGDGMTRIIWELIKEKLIFPYVELDLHSYDLGHNRDATNDQVTKDAABAIKKNVGVKCATITPDEKRVVEFKLQMWKSPNGTIRNLLGGTVFSEAIICKNIPRLVSGWVKPIHGRHAYGDOYRATDFVVPGPVKVEITYTPSDGTQKVTVLVHNFEEGGGVAMGMYNQDKSIEDFAHSSSQMALSEKGWPLYLSTKNITLKKYDGRFKDIFQEIYDKQYKQFEAQKIWYEHRLIDDMVAQAMKSEGGFIWACKNYDGDVQSDSVAQGYGSLGMMTSVLVCPDGKTVEAEAAHGTVTRHYRMYQKGGQETSTNPISIFAWTRGLAHRAKLDNNKELAFFANALEEVSIEETIEAGFMTKDLAACKIKGLPNVQSRSDYLNTEFMDKLGLENLKIKLAQAKL

	R100		R109				R132
WT	...ATACGGAATATTCTGGGTGGCACGGTCTTCAGAGAAGCCATTATCTGCAAAAATATCCCCCGCTTGTGAGTGGATGGGTAACCTATCATCATAGGTGGTCAT...						
SCO2 ^{-/-}	...ATACGGAATATTCTGGGTGGCACGGTCTTCAGAGAAGCCATTATCTGCAAAAATATCCCCCGCTTGTGAGTGGATGGGTAACCTATCATCATAGGTGGTCAT...						

IDH2

Annotated protein sequence with R140, R149, R172 highlighted

MAGYLRVVRSLCRASGSRPAWAPAALTAPTSQEQRHHYADKRIKVAKPVVEMDGGDEMTRIHWQFIKEKLILPHVDIQLKYFDLGLPNRDQTDQVTDSDALATQKYSVAVKCATITPDEARVVEFKLKKMWKSPNGTIRNLLGGTVFREPIICKNIPRLVPGWTKPIITIGRHAHGDQYKATDFVADRAAGTFKMFVTPKDGSGVKEWEVYNFPAGGVGMGMVNTDESISGFSAHSCFYAIQKKWPLYMSTKNTILKAYDGRFKDIFQEIFDKHYKTDFDKNKIWIYEHRLIDDMVAQVLKSSGGFVWACKNYDGDVQSDILAQGFGSLGMMTSVLVCPDGKTVEAEAAHGTVTRHYREHQKGRPTSTNPISIFAWTRGLEHHRGLDGNQDLIRFAQMLEKVCVETVESGAMTKDLGACIHLGNVNLKNEHFLNTTDFLDITKSNLDRALG

	R140		R149				R172
WT	...ACTATCCGGGAACATCTCTGGGGGGGACTGTCTTCGGGAGCCCATCATCTGCAAAAACATCCACGCGCTAGTCCTGGCTGGACCAAGCCCATCACCATTGGCAGGCACGCC...						
SCO2 ^{-/-}	...ACTATCCGGGAACATCTCTGGGGGGGACTGTCTTCGGGAGCCCATCATCTGCAAAAACATCCACGCGCTAGTCCTGGCTGGACCAAGCCCATCACCATTGGCAGGCACGCC...						

B

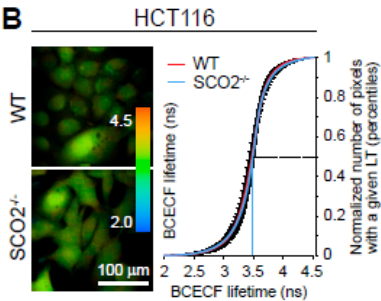


Fig. S2.3. HCT116 cells are devoid of mutations in *IDH1* and *IDH2* genes while the loss of *SCO2* does not affect intracellular pH. A. Sequence analysis of *IDH1* and *IDH2* genes in WT and *SCO2*^{-/-} HCT116 cells. Complete amino acid sequences of *IDH1* and *IDH2* proteins and DNA fragments containing the known mutations (R140, R149 and R172) that underlie D-2-HG production by *IDH* is provided. The absence of mutations was confirmed by RNAseq analysis (n=

2 independent experiments). **B.** The effects of *SCO2* on intracellular pH were assessed via confocal Fluorescence Lifetime Imaging (FLIM) of 2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) staining. Images are stacks of three focal planes taken with a 1 μm step. Right panel shows cumulative distributions of BCECF lifetime values in cells localized in the entire field of view (256 $\times$ 256 pixels); dotted lines demonstrate lifetime values that correspond to the 50th percentile (median) of the lifetime distribution (n=3 independent experiments).

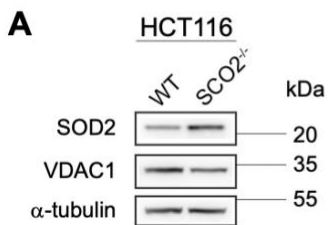


Fig. S2.4. *SCO2* deletion in HCT116 cells increases expression of SOD2. **A.** Levels of indicated proteins were monitored by Western blotting. VDAC1 and α -tubulin served as loading controls. The experiments are representative of 3 independent replicates. Quantifications of the Western blots are provided in Fig. S2.9.

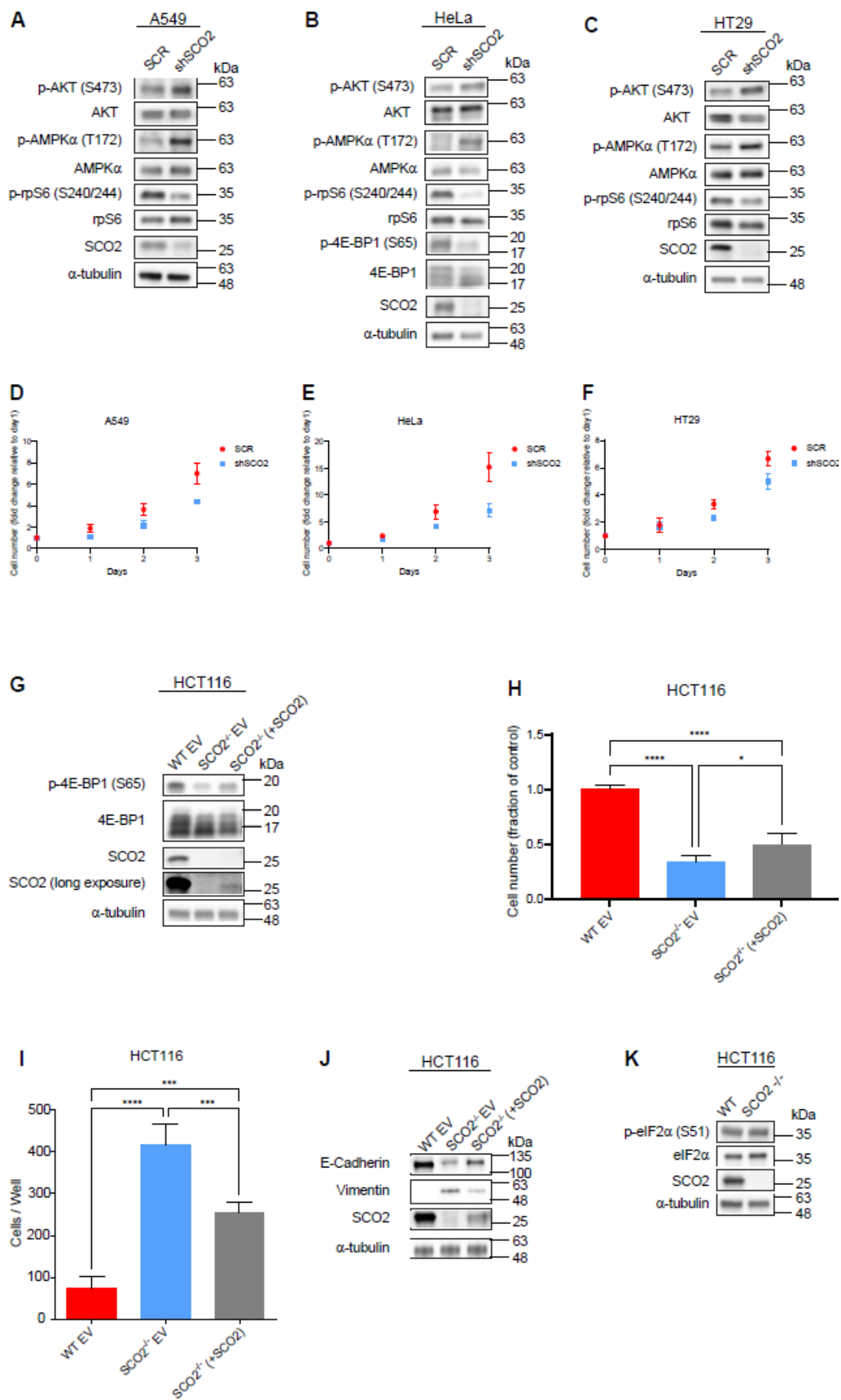


Fig. S2.5. *SCO2* loss rewires signaling, reduces proliferation and bolsters migration A-C,

Levels and phosphorylation status of indicated proteins were monitored by Western blotting after lentiviral knockdown of *SCO2* (sh*SCO2*) in A549 (**A**), HeLa (**B**) and HT29 (**C**) cells. In parallel, A549 (**A**), HeLa (**B**) and HT29 (**C**) cells were infected with scrambled shRNA (SCR). Shown are the representative Western blots from 3 (A549 and HeLa) or 2 (HT29) independent experiments. α -tubulin was used as a loading control. **D-F.** Numbers of viable SCR (red) and sh*SCO2* (blue) infected A549 (**D**), HeLa (**E**) and HT29 (**F**) cells grown for indicated times were determined by trypan blue exclusion using automated cell counter. Results are presented as the mean value $\pm$ SD whereby the number of SCR cells at day 1 was set to 1 (n=2 independent experiments with 3 technical replicates each for A549, n=4 independent experiments with 3 technical replicates each for HeLa, n=3 independent experiments with 3 technical replicates each for HT29). **G.** Levels and phosphorylation status of indicated proteins in WT EV, *SCO2*^{-/-} EV and *SCO2*^{-/-}(+SCO2) HCT116 cells were monitored by Western blotting. α -tubulin served as a loading control. Shown are representative Western blots from 3 independent experiments. **H.** Numbers of WT and *SCO2*^{-/-} HCT116 cells infected with empty vector (EV) and *SCO2*^{-/-} HCT116 cells re-expressing *SCO2* (+SCO2) grown for 3 days were assessed using trypan blue exclusion using automated cell counter. Results are presented as the mean value $\pm$ SD relative to WT EV HCT116 cell number which was set to 1 (n=2 independent experiments with 2-3 technical replicates each). **I.** Transwell migration assay of WT EV, *SCO2*^{-/-}EV and *SCO2*^{-/-}(+SCO2) HCT116 cells. Presented are the mean summarized cell counts of four fields from a single transwell migration chamber $\pm$ SD (****p<0.0001; ***p<0.001 One-way ANOVA; Tukey's multiple comparison post-hoc test; n=2 independent experiments with 2 technical replicates in each). **J.** Levels and phosphorylation status of indicated proteins were monitored by Western blotting in WT EV, *SCO2*^{-/-} EV and *SCO2*^{-/-}

(+SCO2) HCT116 cells. α -tubulin served as a loading control. Shown are representative Western blots from 3 independent experiments. **K.** Levels and phosphorylation status of indicated proteins were monitored by Western blotting in WT and *SCO2*^{-/-} HCT116 cells. α -tubulin served as a loading control. Shown are representative Western blots from 4 independent experiments. Quantifications of the Western blots are provided in Fig. S2.9.

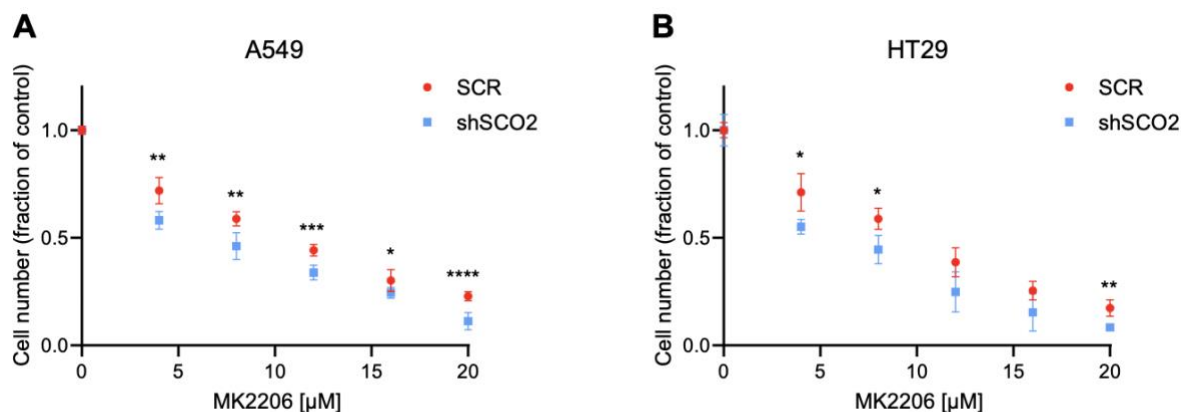


Fig. S2.6. Reduced complex IV activity increases sensitivity cancer cells to AKT inhibitor. A-B. A549 (**A**) and HT29 (**B**) cells were infected with scrambled shRNA (SCR) or SCO2 shRNA (shSCO2). The cells were treated with indicated concentrations of pan-AKT inhibitor MK2206 or a vehicle (DMSO) for 3 days. The number of viable cells were determined using trypan blue exclusion and automated cell counter. Data are presented as the mean fraction of cells relative to the corresponding DMSO-treated controls which were set to 1. Experiments were carried out in at

least 2 independent replicates (with 2 technical replicates each). Bars represent SD values. Data are represented as mean fold change $\pm$ SD relative to vehicle (DMSO) treated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Unpaired 2-tailed t-tests were carried out at each drug concentration tested).

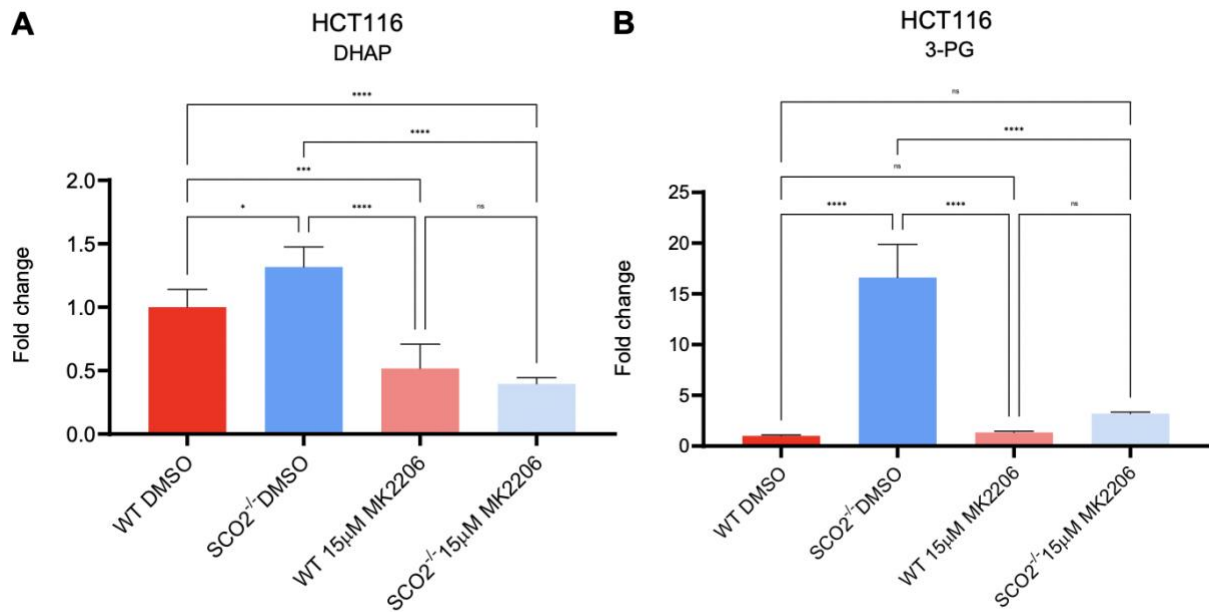


Fig. S2.7. Inhibition of AKT dampens the compensatory increase in glycolysis caused by the deletion of *SCO2* in HCT116 cells. **A-B**, Intracellular levels of DHAP (**A**) and 3-PG (**B**) in WT or *SCO2*^{-/-} HCT116 cells. Cells were seeded and treated with MK2206 (15 µM) for 24 h. Metabolite levels were monitored by GC-MS. Data are represented as mean fold change +/- SD relative to vehicle (DMSO) treated WT HCT116 cells. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.

**** $p < 0.0001$ (One-way ANOVA; Tukey's multiple comparison post-hoc test; $n = 2$ independent experiments with 3 technical replicates in each).

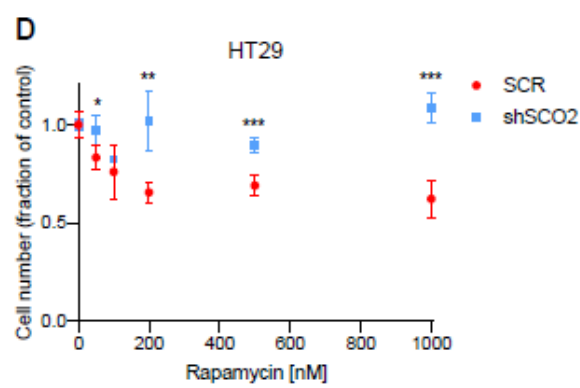
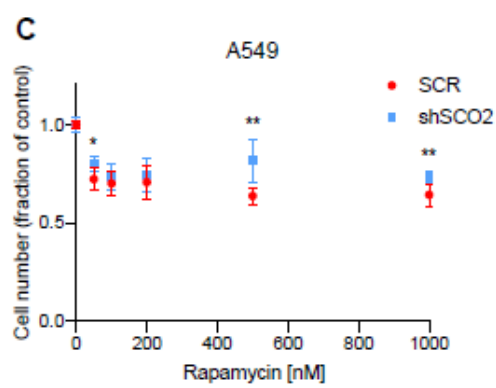
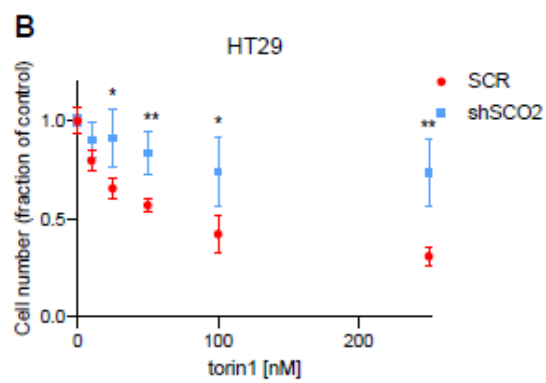
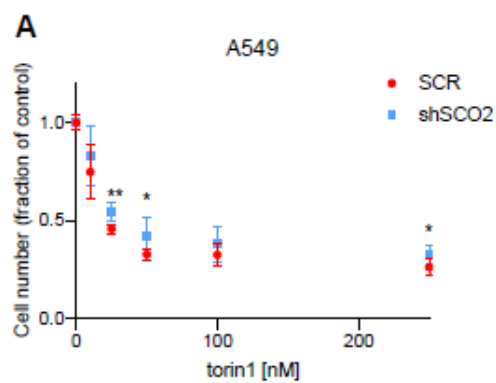
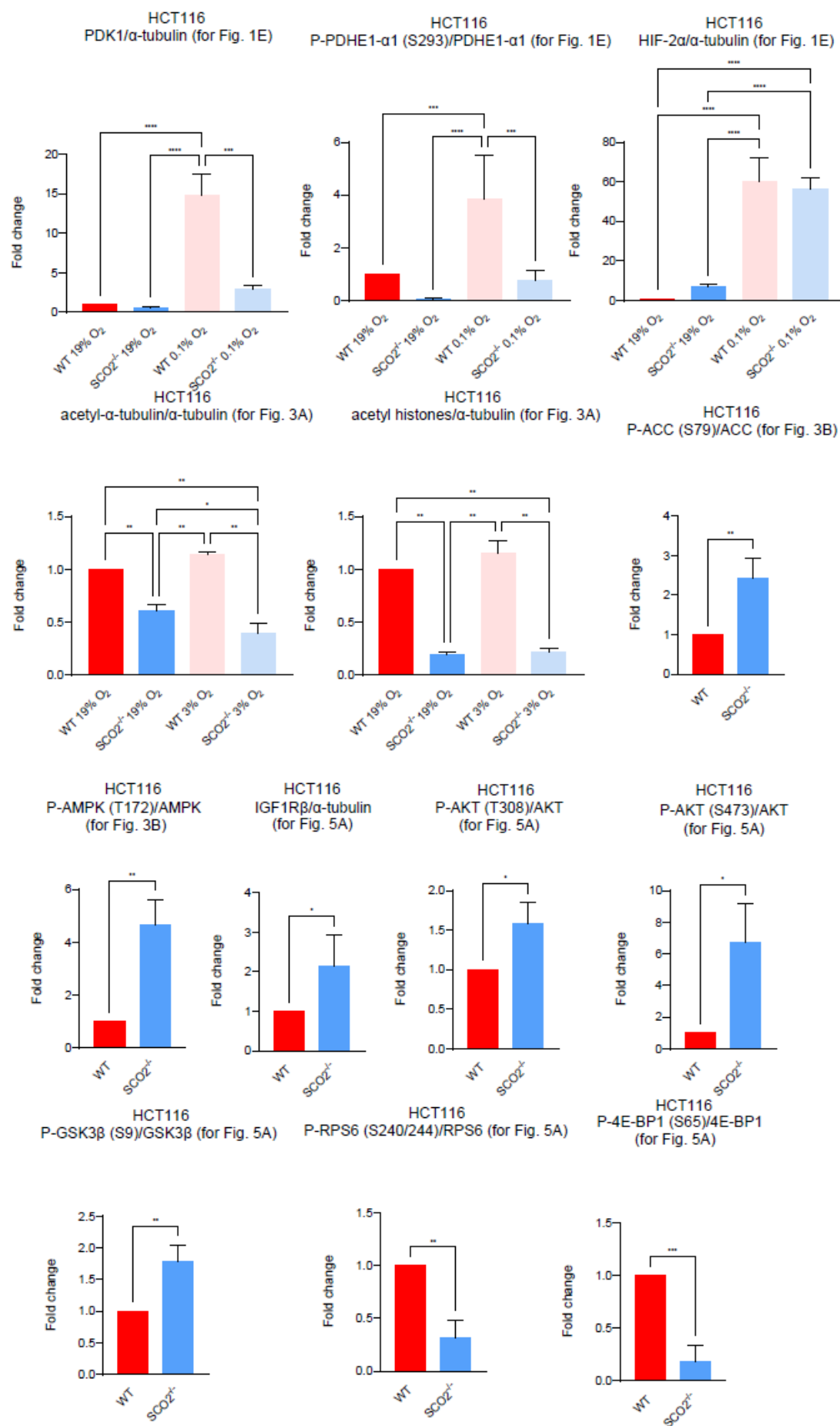
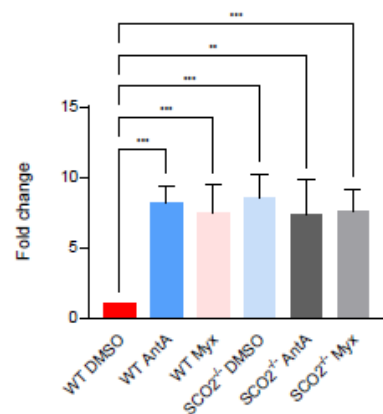


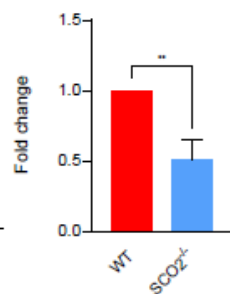
Fig. S2.8. Depletion of mitochondrial complex IV activity reduces sensitivity of cancer cells to mTOR inhibitors. A549 (**A-B**) and HT29 (**C-D**) cells were infected with scrambled shRNA (SCR) or SCO2 shRNA (shSCO2). The cells were treated with indicated concentrations of rapamycin (**A, C**) or torin 1 (**B, D**) or a vehicle (DMSO) for 3 days. Numbers of viable cells in each condition were determined by trypan blue exclusion using automated cell counter. Data are represented as the mean fraction of cells relative to DMSO treated controls +/- SD. Experiments were carried out in at least two independent times with 2 technical replicates each. Error bars represent SD values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Unpaired 2-tailed t-tests were carried out at each drug concentration tested).



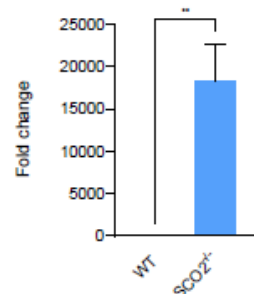
HCT116
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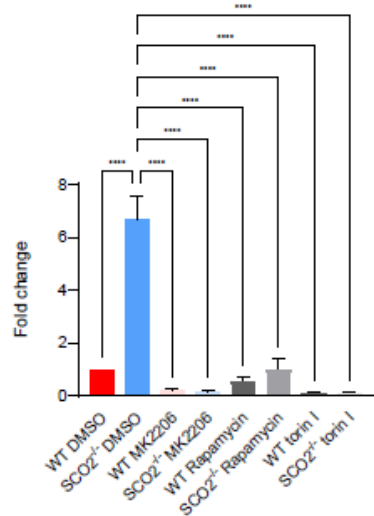
HCT116
E-Cadherin/ α -tubulin
(for Fig. 5G)



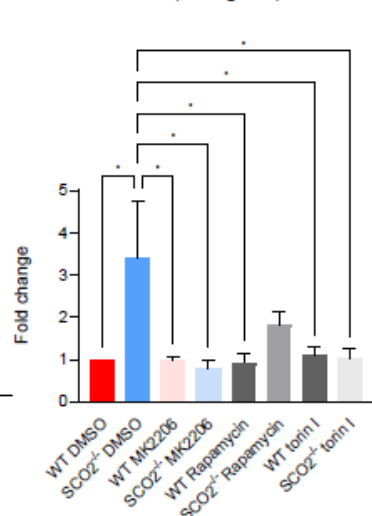
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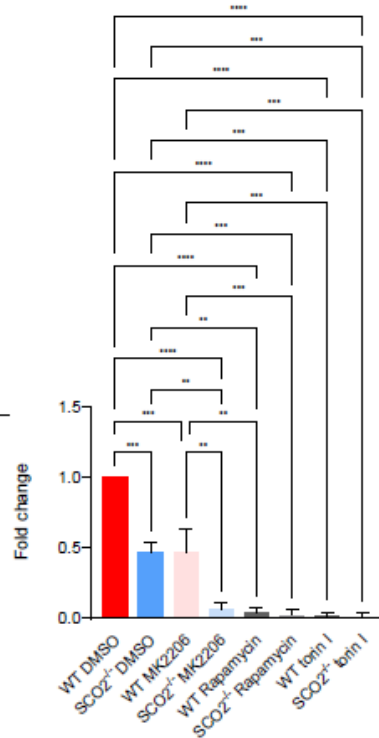
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P-AKT (S473)/AKT
(for Fig. 6D)



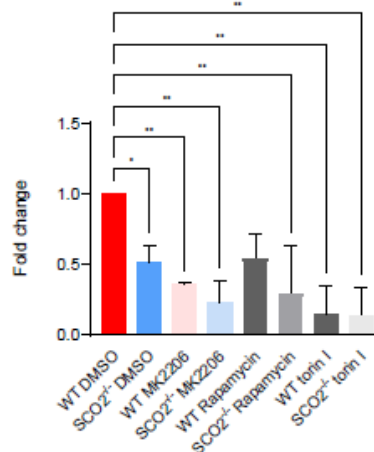
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(for Fig. 6D)



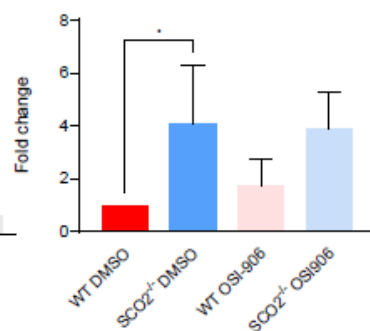
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(for Fig. 6D)



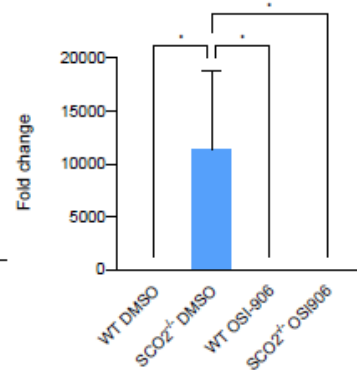
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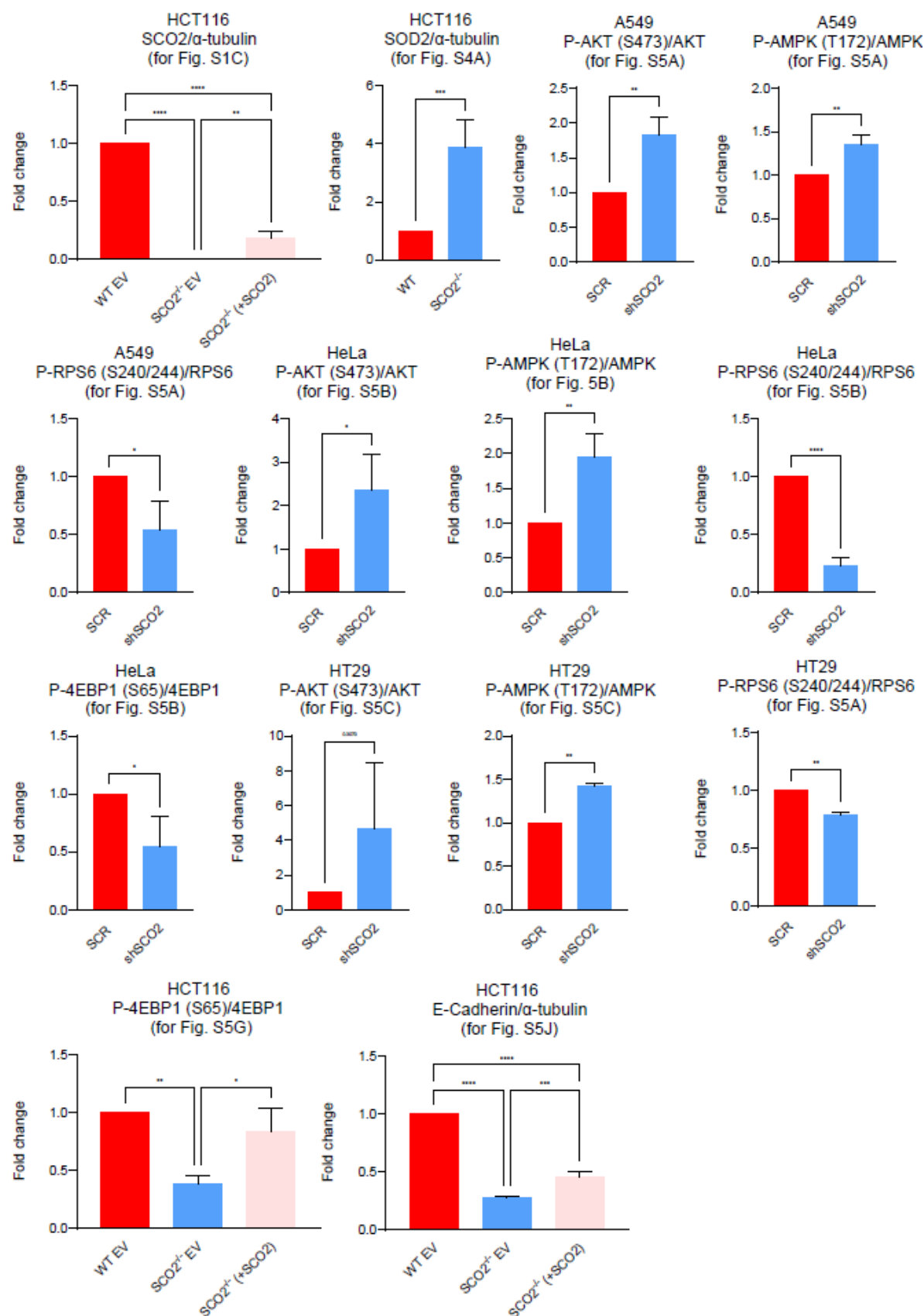


HCT116
IGF1R β / α -tubulin
(for Fig. 6H)



HCT116
P-AKT (S473)/AKT
(for Fig. 6H)





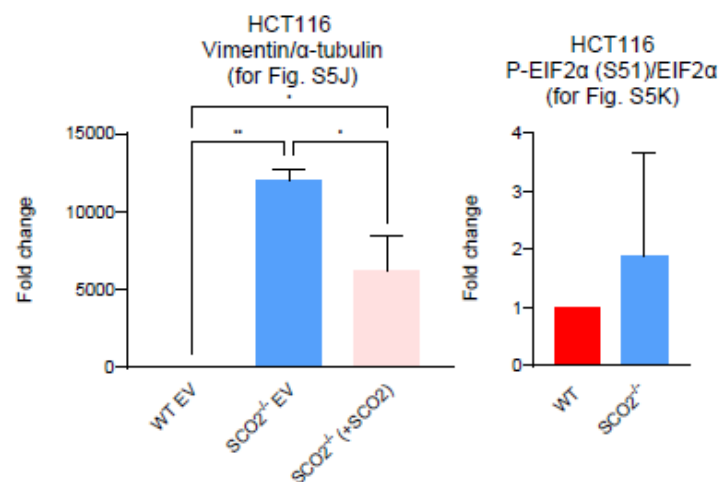
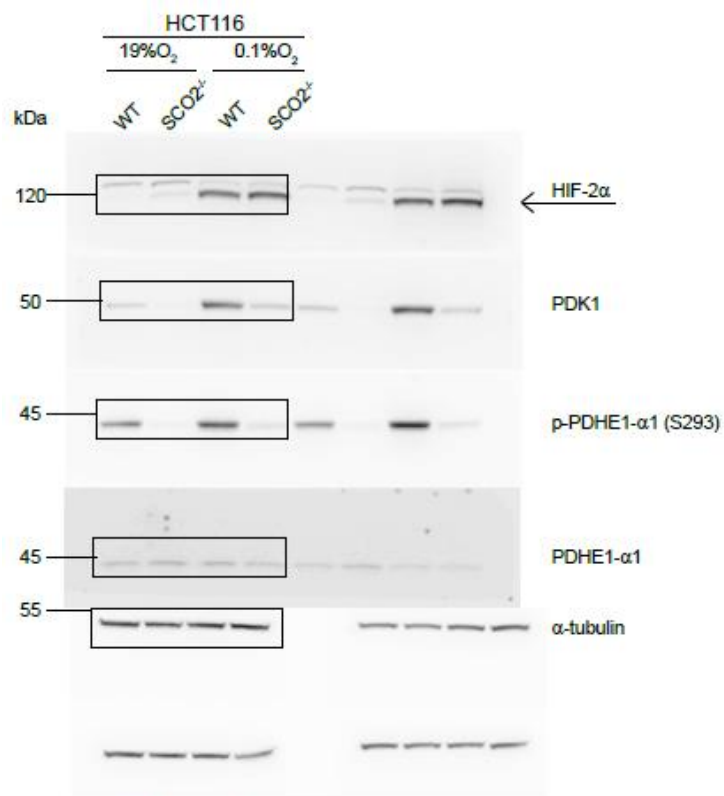
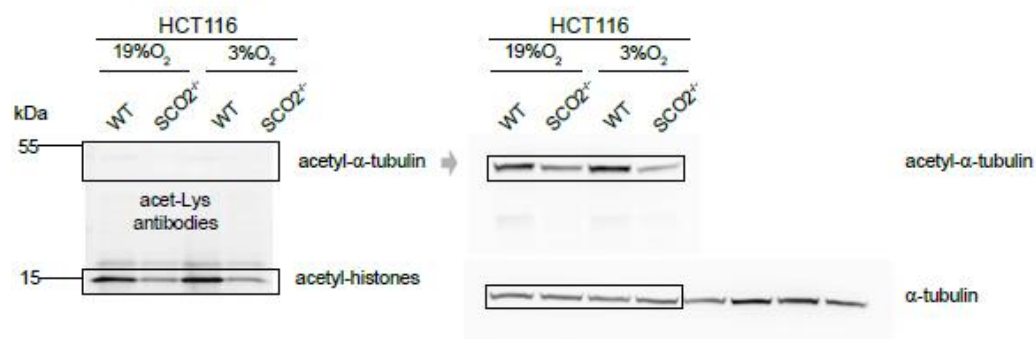


Fig. S2.9. Densitometry analysis of Western blots. Results represent mean fold changes in densitometry signals relative to the indicated controls. Signals for phospho-proteins and proteins of interest were normalized over corresponding total proteins or α -tubulin, respectively as

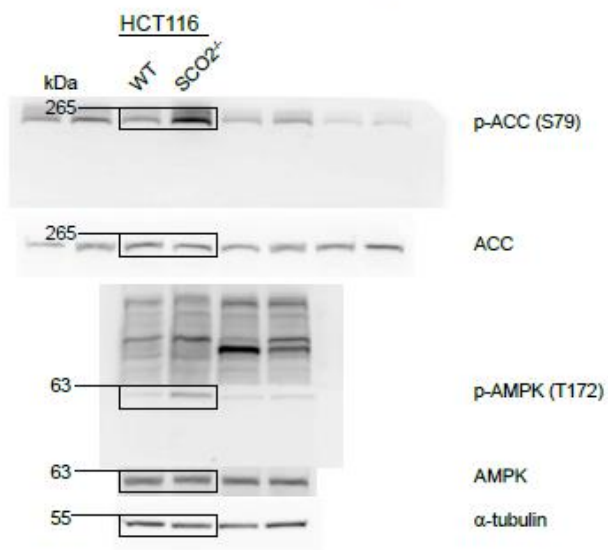
indicated. The experiments were repeated at least in independent duplicates; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Unpaired 2-tailed t-test, 1-way ANOVAs, and 2-way ANOVAs were done when two, three or more than three variables were investigated, respectively).



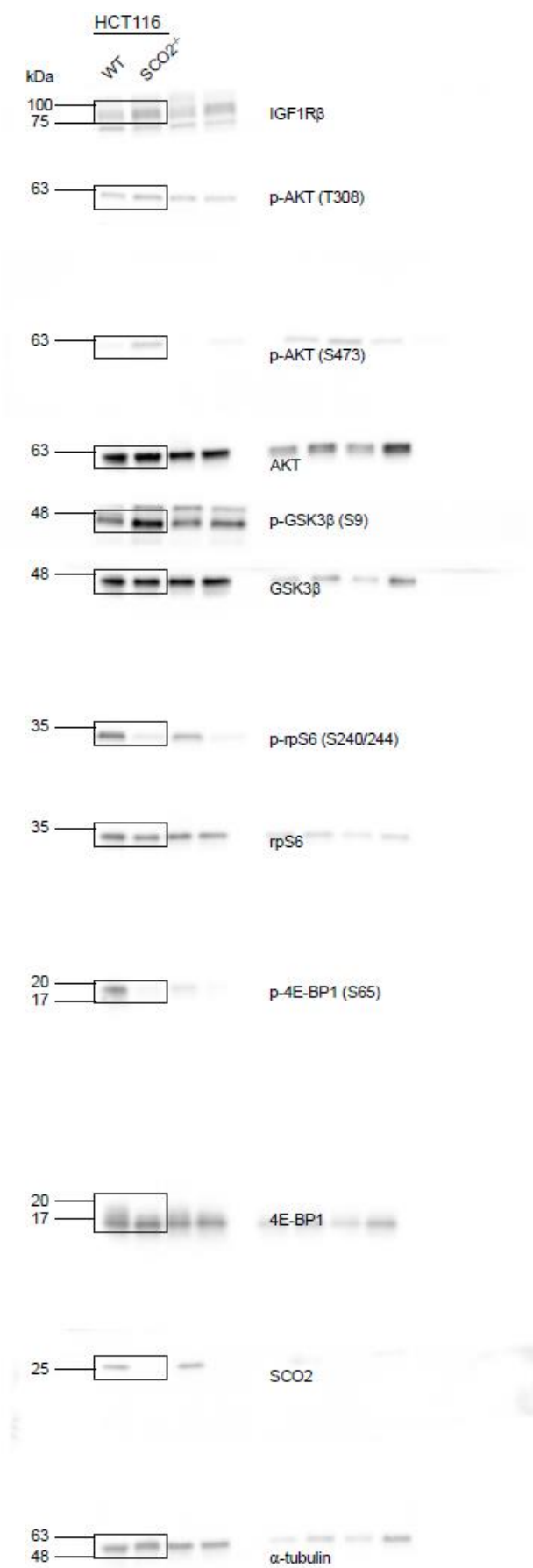
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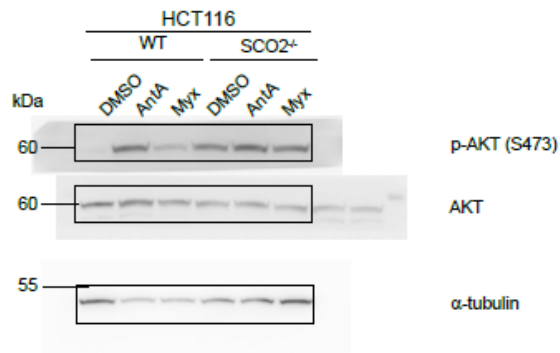
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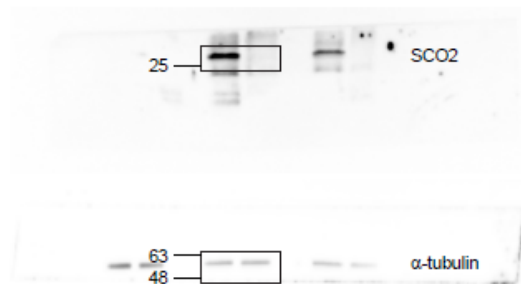
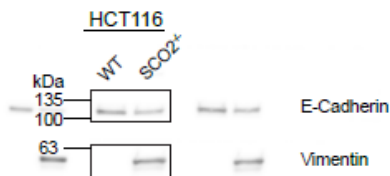
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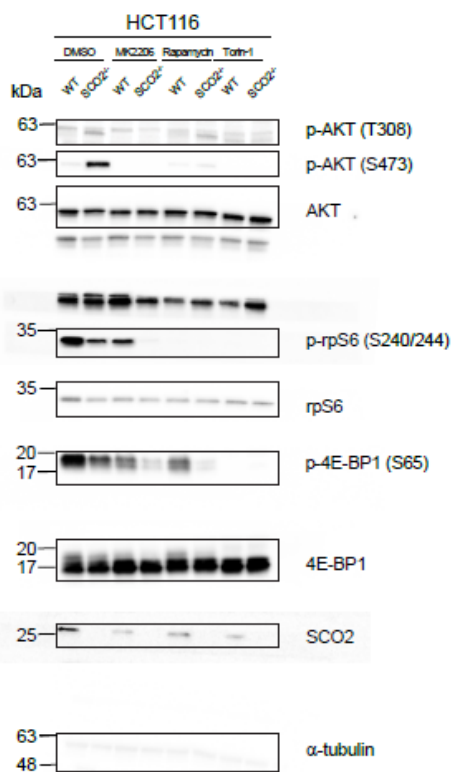
(for Fig. 5A)



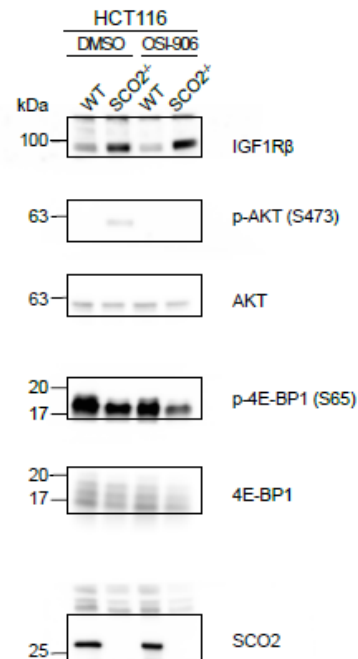
(for Fig. 5B)



(for Fig. 5G)

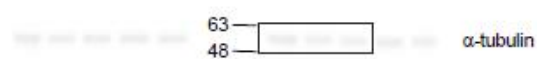
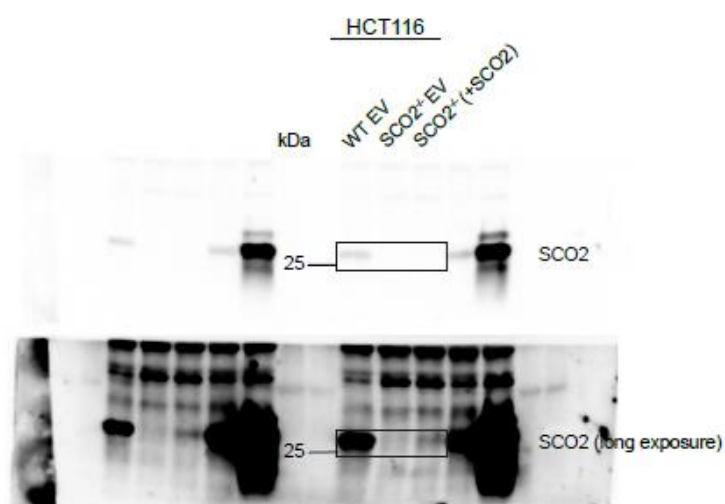


(for Fig. 6D)

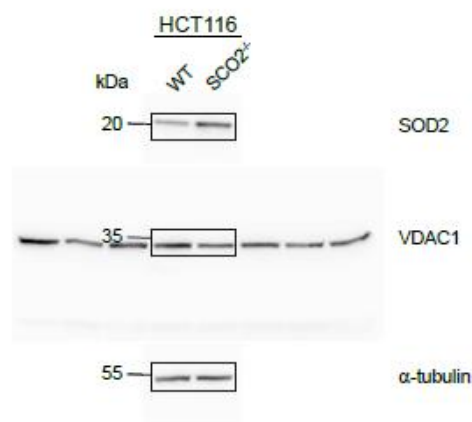


(for Fig. 6H)





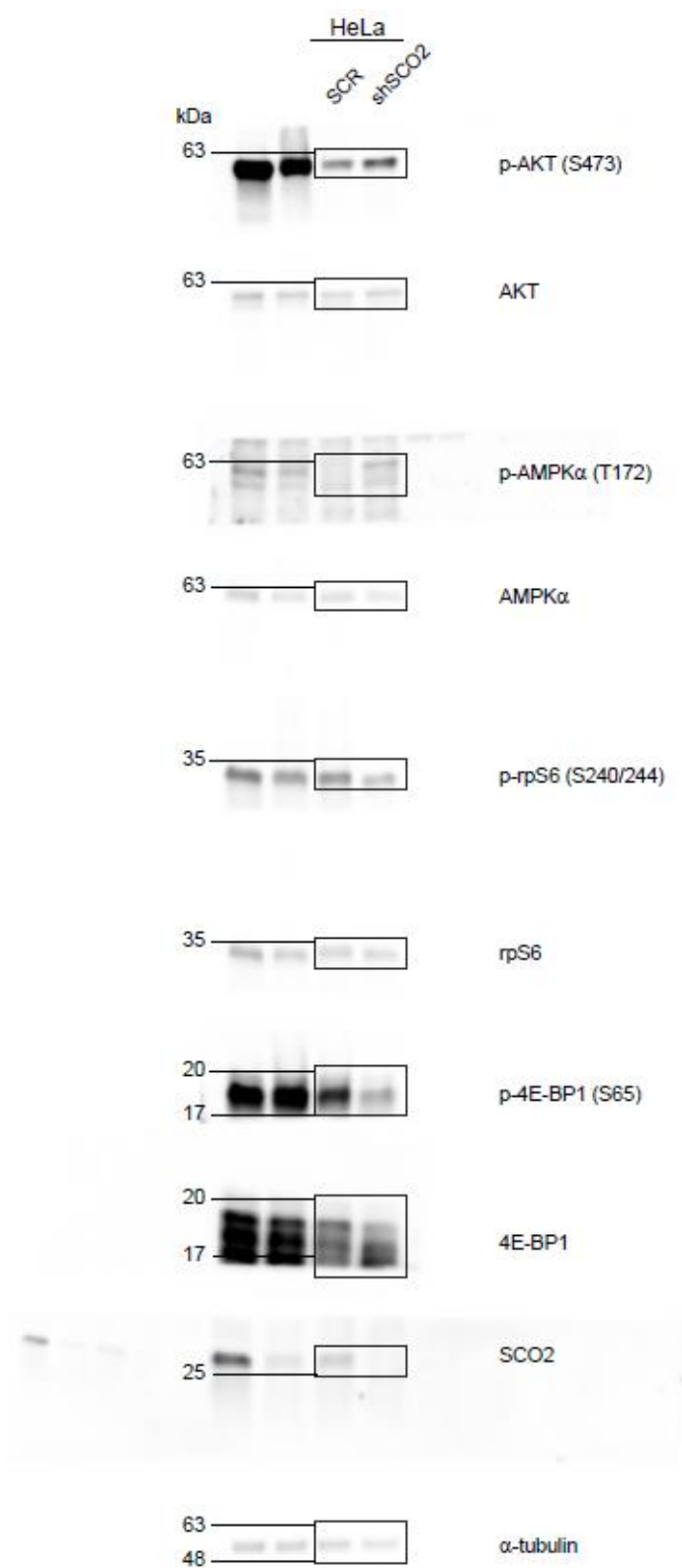
(for Fig. S1E)



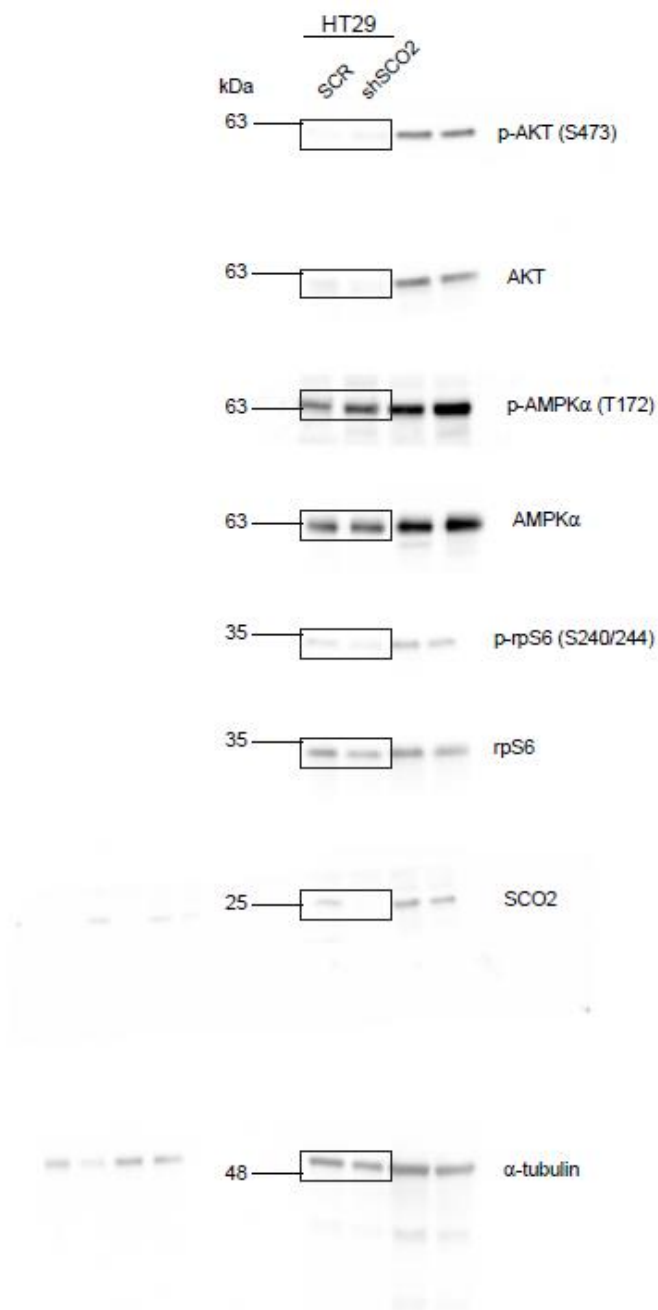
(for Fig. S4A)



(for Fig. S5A)



(for Fig. S5B)



(for Fig. S5C)

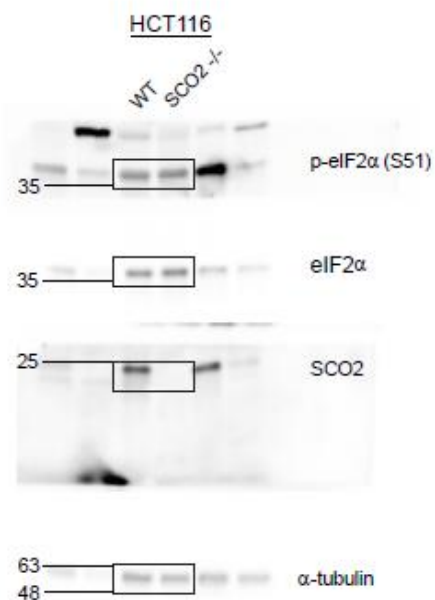
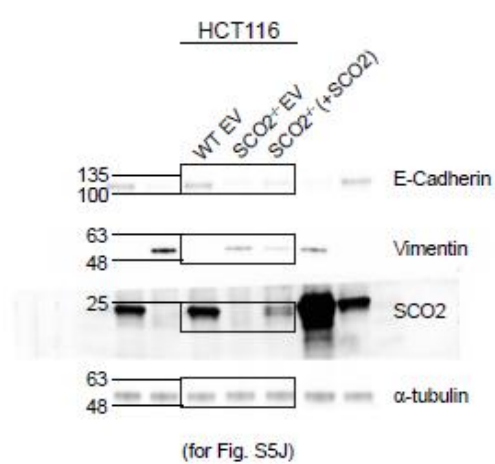
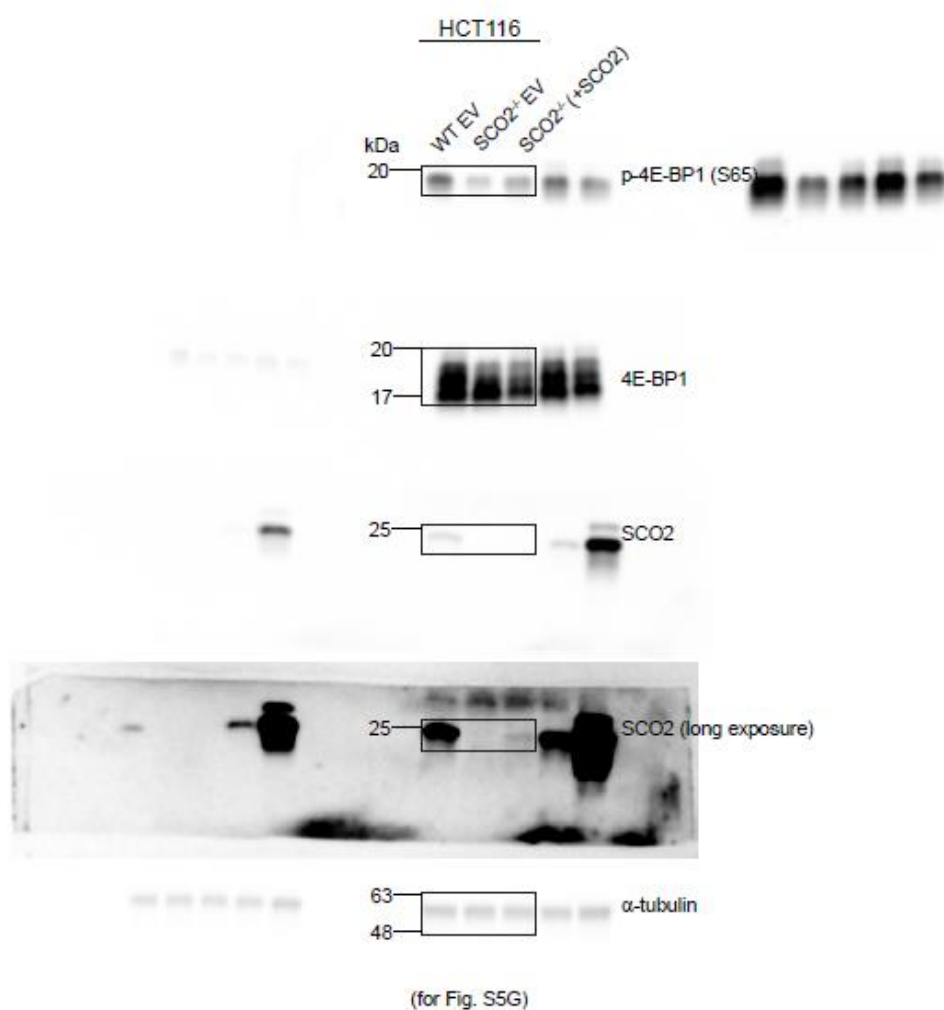


Fig. S2.10. Images and scans of Western blot films that are incorporated in the manuscript.

The boxes around the indicated protein bands show the approximate area of the x-ray films and nitrocellulose membrane images included in the figures.

2.6 DISCUSSION

Metabolic reprogramming of cancer cells allows them to adapt to changes in nutrient and oxygen availability and therapeutic insults (67, 68). To this end, it is considered that metabolic rewiring not only fulfils the energetic requirements of cancer cells, but also provides building blocks and maintains redox buffering capacity required to fuel growth and survival of cancer cells (67, 68). Herein, we report that disruption of mitochondrial complex IV function by *SCO2* loss is compensated by dramatic perturbations in metabolome and signaling. Most notably, *SCO2* loss in HCT116 cells leads to increase in glycolysis (12). This not only enables HCT116 cells with dysfunctional complex IV to meet the biosynthetic demands by providing carbon atoms but also allows for the regeneration of NAD⁺ which is necessary to maintain glycolysis and redox homeostasis. Indeed, although *SCO2* loss leads to overall increase in NADH levels in HCT116 cells (13), *SCO2*^{-/-} HCT116 cells upregulate NAD⁺ regenerating reactions. This is consistent with previous findings suggesting that conversion of pyruvate to lactate and the simultaneous production of NAD⁺ from NADH by lactate dehydrogenase are essential in the context of mitochondrial dysfunction (69). Moreover, *SCO2*^{-/-} HCT116 cells appear to exhibit increased activity of GPD1, as illustrated by the alterations in steady-state metabolite levels and increased glucose flux to 3-PG. Notably, although mitochondrial glycerophosphate dehydrogenase (GPD2) has been implicated in the reoxidation of cytosolic NADH via the ETC in prostate cancer (70), due to compromised mitochondrial functions this mechanisms is unlikely to be at play in *SCO2*-

deficient HCT116 cells. Hence, GPD1 is most likely involved in maintaining NAD⁺/NADH ratio in HCT116 *SCO2*^{-/-} cells. In addition, we observed large accumulation of lipids in HCT116 *SCO2*^{-/-} cells as compared to their WT counterparts. Lipid accumulation in *SCO2*-deficient cells was not caused by increased lipogenesis or FA uptake, thus indicating that the observed phenotype likely stems from impediments in β -oxidation of FA in the mitochondria. Finally, *SCO2* loss resulted in a reduction of most of the CAC intermediates and amino acids, which was paralleled by an increase in succinate and 2-HG. Although we did not formally test whether accumulation of these metabolites is sufficient to disrupt function of α -ketoglutarate-dependent enzymes (e.g. HIF- α prolyl hydroxylase, histone, DNA and RNA demethylases) (41, 67), these findings suggest that *SCO2* loss may result in epigenetic, transcriptional and epitranscriptional reprogramming.

Notably, the observed alterations in metabolic programs in *SCO2*-deficient HCT116 cells were paralleled by significant rewiring characterized by increased IGF1R/AKT activity that was accompanied by a reduction in mTORC1 signaling. This, at least in part, may be explained by elevated AMPK activity in *SCO2*-deficient HCT116 cells. Accordingly, *SCO2*^{-/-} HCT116 cells showed heightened sensitivity to IGF1R and AKT inhibitors, while they were less sensitive to mTOR inhibitors relative to *SCO2*-proficient cells. Previous studies demonstrated that AKT upregulates glycolysis (71). Since the loss of *SCO2* renders HCT116 cells heavily dependent on glycolysis to produce ATP and regenerate NAD⁺, it is plausible that these cells are more sensitive to IGF1R and AKT inhibitors because of their inhibitory effects on glycolysis. Strikingly, similar induction in AKT phosphorylation was observed in WT HCT116 cells treated with mitochondrial complex III inhibitors or subjected to hypoxia. Comparable to HCT116 *SCO2*^{-/-} cells, the decreased expression of *SCO2* in HeLa and A549 increased AKT activity while downregulating mTORC1. Although the precise mechanism(s) of induction of IGF1R/AKT axis in the context of

mitochondrial complex III or IV disruption remains elusive, these findings suggest that mitochondrial dysfunction may lead to critical reliance of neoplastic cells on AKT.

AMPK acts as a negative regulator of mTORC1, and it has recently been described that it may stimulate mTORC2 (72) and be activated by ROS (73). This suggests that AMPK upregulation in HCT116 *SCO2*^{-/-} cells may dampen mTORC1 signaling while sustaining mTORC2 and AKT activity. Indeed, we observed that disruption of *SCO2* results in marked increase in S473 AKT phosphorylation which is indicative of increased mTORC2 activity (74). In addition, mTORC2 may also exert AKT-independent effects of glucose metabolism as reported in glioblastoma (75). Of note, HCT116 *SCO2*^{-/-} cells produce ATP mainly in cytosol and have the ATP/protein ratio similar to that in WT cells (14), thus suggesting that AMP/ATP or ADP/ATP ratio may not play a major role in AMPK activation in this context. In turn, cytosolic Ca²⁺ levels are elevated in *SCO2*^{-/-} as compared to HCT116 WT cells (14) thereby suggesting that Ca²⁺/calmodulin dependent protein kinase kinase (CAMKK) may activate AMPK in cells exhibiting complex IV dysfunction. Also, AMPK has been found to be hyperactivated by CAMKK in mouse embryonic fibroblasts (MEFs) devoid of LKB1 kinase when subjected to oxidative stress (76). Considering that we observed similar phenotypes between cells with functional (HCT116) or dysfunctional LKB1 (A549, HeLa), it is likely that mechanisms other than LKB1 play a major role in AMPK activation in the context of mitochondrial complex IV dysfunction. Intriguingly, despite several attempts, we failed to knock down AMPK α 1 and/or α 2 subunits in HCT116 *SCO2*^{-/-} cells or deplete *SCO2* in AMPK α 1/ α 2^{-/-} mouse embryonic fibroblasts (MEFs) due to massive cell death. Notwithstanding that future investigations are required to establish the precise mechanism(s) of adaptation of cancer cells to the abrogated complex IV activity, these observations may suggest an important role for AMPK in this process.

Recent studies show that ROS plays pivotal roles in facilitating EMT and cell motility (77). HCT116 *SCO2*^{-/-} cells were previously shown to have elevated ROS levels (13). A prior study showed that ZEB1 represses E-cadherin and induces vimentin (62). Furthermore, elevated levels of 2-HG have been reported to increase the expression of *ZEB1* by inhibiting histone demethylase, which in turn decreased the expression of E-cadherin while concurrently increasing the expression of vimentin in HCT116 cells (78). Similarly, elevated levels of 2-HG were observed to stimulate migration, invasion of HCT116 cells and associated with distant metastasis (78). Collectively these findings suggest that metabolic and signaling adaptation to mitochondrial dysfunction caused by *SCO2* loss result in increased migratory potential of HCT116 cells.

Since *SCO2* represents one of the major metabolic targets of p53, together with the previous work, our study suggests that complex IV dysfunction plays a major role in metabolic perturbations of malignancies with defective p53 function. Importantly, we demonstrate that despite reducing proliferation in cell culture models, *SCO2* loss results in alterations in expression of EMT markers and increased migration of HCT116 cells. Considering the positive correlation of these phenomena and metastatic spread of cancer (79, 80), these findings suggest that alterations in *SCO2* and dysfunction of mitochondrial complex IV may increase the metastatic potential of cancer cells. Nonetheless, we acknowledge that our study has significant limitations as it was carried out on a very limited number of cell lines, while the precise mechanisms of signaling and metabolic perturbations remain elusive. Expanding these studies across various cancer cell lines and *in vivo* is therefore warranted to establish the general role of *SCO2* and disruption of complex IV function in metabolic reprogramming and accompanied signaling rewiring in neoplasia and its impact on cancer cell phenotypes.

2.7 METHODS

2.7.1 Cell lines and culture conditions

SCO2^{-/-} and WT HCT116 cells were provided by Dr. Paul M. Hwang (15). A549, HT29 and HeLa cells were obtained and authenticated by ATCC. All cells were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, 10 mM HEPES and 2 mM L-Glutamine, (provided by either MilliporeSigma or Wisent). Cells were maintained at 37°C, 5% CO₂ and 19% O₂, unless otherwise indicated.

2.7.2 Lentiviral infection of *SCO2* shRNA and expression constructs

Stable knockdown of *SCO2* in A549, HT29 and HeLa cells was generated using pLKO.1 lentiviral sh*SCO2*-containing vector (TRCN236560) obtained from Mission TRC genome-wide shRNA collections (MilliporeSigma). Non-mammalian shRNA control plasmid DNA was used as a negative control (MilliporeSigma: SHC002).

For *SCO2* cDNA generation, RNA was extracted from HCT116 cells using Trizol (ThermoFisher Scientific) according to manufacturer's instructions. Subsequently, cDNA was synthesized by SuperScript III Reverse Transcriptase (Invitrogen) and PCR amplified using Phusion High-Fidelity DNA polymerase (New England BioLabs) and the following primers:

Forward primer: 5' TATGCAACCGGTATGTTTGGTGGAGGTGGAGTTCTGAGC 3'

Reverse Primer: 5' TTGCAAGAATTCTCAAGACAGGACACTGCGGAAAGCCGC 3'

PCR cycling conditions: Initial denaturation was done at 98 °C for 3 min, 35 cycles of 98 °C for 30 secs, 60 °C for 30 s and 72 °C for 40 s and the final extension was done at 72 °C for 10 min.

Human *SCO2* cDNA was cloned into pLKO.1-puro empty vector control plasmid DNA (SHC001) using EcoRI and AgeI restriction enzymes (New England BioLabs). pLKO.1-puro empty vector (SHC001) was used as a negative control for the exogenous expression of *SCO2*.

Lentiviruses were produced as follows: HEK293T cells were co-transfected with 12 µg of shSCO2-containing plasmid (to knockdown *SCO2* in HeLa, HT29 and A549 cells) or *SCO2*-containing plasmid (to re-express *SCO2* in HCT116 *SCO2*^{-/-} cells), 8 µg of psPAX2 packaging plasmid and 4 µg of pMD2.G plasmid using jetPRIME transfection agent according to the manufacturer's protocol (Polyplus transfection). The media was changed 24h later and collected 48h post-transfection. The media was filtered through 0.45 µm filter (Fisher Scientific). The virus-containing media was mixed with fresh media at 1:1 ratio and added to already seeded A549, HT29 and HeLa cells. 4 µg/mL polybrene (MilliporeSigma) was added to the cells. Cells were re-infected the following day using freshly collected and filtered virus-containing media. 48h later, selection was done using 2 µg/mL of puromycin (Bio Basic).

2.7.3 Flow cytometry analysis of cell cycle

HCT116 *SCO2*^{-/-} and WT cells were seeded on 6-well plates and treated for 24 h MK2206 (15 µM) or vehicle (DMSO). The cells were then trypsinized, washed in PBS (Wisent) and then stained with 50 µg/mL propidium iodide (PI) in cold hypotonic buffer (0.1% sodium citrate and 0.1% Triton X-100 in water). Approximately 2.5×10^5 cells per condition were stained with 0.5 mL of the PI stain and analyzed on a LSR Fortessa cytometer (Becton Dickinson). Fluorescence was detected by excitation at 561nm and acquisition on the 610/20-A channel in linear scale. The distribution of cell cycle population was analyzed using the ModFit LT software.

2.7.4 Flow cytometry analysis of apoptosis

HCT116 *SCO2*^{-/-} and WT cells were seeded on 6-well plates and treated for 48h with MK2206 (15 µM) or vehicle (DMSO). Cells were trypsinized, counted and 1×10^5 cells were stained using Annexin V-FITC and PI for 20 min in the dark as per the manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit; Becton Dickinson). Samples were then analyzed with a LSR

Fortessa cytometer (Becton Dickinson). Fluorescence was detected by excitation at 488 nm and acquisition on the 530/30-A channel for FITC-Annexin V and by excitation at 561 nm and acquisition on the 610/20-A channel for PI. Cell populations were separated as follows: viable cells – Annexin V-/PI-; early apoptosis - Annexin V+/PI-; late apoptosis Annexin V+/PI+; dead Annexin V-/PI+; and expressed as % of total single cells.

2.7.5 GC-MS for Stable Isotope Tracer Analysis (SITA)

3×10^5 *SCO2*^{-/-} and WT HCT116 cells were seeded on 6-well plates to obtain 80% confluency 24 h later. For stable isotope tracer analysis (SITA), the supplemented McCoy's 5A medium was aspirated and replaced with fresh Dulbecco's Modified Eagle's Medium (DMEM) without glucose, glutamine and pyruvate, supplemented with 10% heat-inactivated FBS, 1% P/S, 2mM L-glutamine and 16.65 mM glucose to equilibrate the cells for 2 h. The equilibration media was replaced with DMEM containing 16.65 mM [U-¹³C]-glucose (Cambridge Isotope laboratories). Cells were incubated in this 'labelling media' for 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h and 4.5 h. The 6-well plates were taken out of the incubator and placed immediately on ice. The labelling media was aspirated, and cells were washed 3X with pre-chilled isotonic saline solution (on ice) and quenched on dry ice by adding 600 μ L of 80% methanol pre-chilled to -20°C. Cells were scraped from the plates and transferred to microcentrifuge tubes pre-chilled to -20 °C. Cell suspensions were lysed using a sonicator at 4 °C (10 minutes, 30 sec on, 30 sec off, high power setting with a Diagenode Bioruptor). This was repeated to ensure complete recovery of metabolites. Cell debris were discarded after centrifugation (16000 g, 4°C), supernatants were transferred to pre-chilled tubes and dried in a CentriVap cold trap (Labconco) overnight at 4°C. Dried pellets were dissolved in 30 μ L of pyridine containing methoxyamine-HCl (10 mg/mL) (MilliporeSigma) using a sonicator and vortex. Samples were incubated for 30 min at 70°C and

then transferred to injection vials containing 70 μ L of *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). Sample mixtures were further incubated at 70 °C for 1 h. 1 μ L of each sample was injected for GC–MS analysis. GC–MS instrumentation and software were all from Agilent. GC–MS methods and mass isotopomer distribution analyses were conducted as described (81). Data analyses were performed using the Agilent ChemStation and MassHunter software (Agilent).

2.7.6 GC-MS for Steady State metabolite analysis

3×10^5 HCT116 *SCO2*^{-/-} and WT cells were seeded on 6-well plates to obtain cells at 80% confluency 24 h later. The plates were quickly placed on ice after incubation period was over. Media were aspirated and cells were washed 3X with chilled isotonic saline solution. Subsequently, 300 μ L of 80% methanol pre-chilled to -20 °C was added to the cells. Cells were scraped from the wells and transferred to microcentrifuge tubes pre-chilled to -20 °C. 300 μ L more of the 80% methanol was added to the leftover cells in the wells, scraped, collected and pooled with the previously collected 300 μ L fraction. Cells were lysed, centrifuged and then the supernatants transferred into prechilled microcentrifuge tubes as previously described for SITA. The collected supernatants were spiked with 750 ng of myristic acid-D₂₇ (Millipore Sigma) to serve as an internal standard. Supernatants were dried overnight and derivatized as previously described for SITA. 1 μ L of the derivatized samples was injected for GC–MS analysis. The instrumentation and software used were identical to those used for SITA. Each metabolite was normalized to the peak intensity of myristic acid-D₂₇ and the average protein content derived from cells seeded in parallel and identical conditions to those collected for GC-MS steady state analysis. Data were expressed as fold change relative to WT HCT116 cells.

2.7.7 Glucose and lactate release assays

5×10^5 cells were seeded in triplicates in 6-well plates in 2 mL of media. The media were collected 24 h later. Cells were trypsinized and counted using an automated cell counter (Invitrogen). Media were spun down at 16000 g for 10 min and the supernatants transferred into microcentrifuge tubes. Glucose and lactate concentrations in the media were measured using a BioProfile 400 analyzer (Nova Biomedical). Total uptake and release were calculated by subtracting the concentrations from baseline glucose and lactate concentrations measured in samples of media incubated under identical conditions in 6-well plates without cells. Molar concentrations of the metabolites were normalized per cell and presented relative to the control.

2.7.8 Cell proliferation assays

1×10^5 cells were seeded in 6-well plates and incubated for 24 h. The media were replaced with treatment media containing MK2206, OSI-906, rapamycin, torin 1 or DMSO as a negative control and incubated for 72h. Treatment media were aspirated, and the cells were trypsinized. Complete media were added to stop the trypsinization. Samples were collected, stained with trypan blue to exclude dead cells and counted using an automated cell counter (Invitrogen). For hypoxic treatments, 1×10^5 cells were seeded in 6-well plates and placed in hypoxic chamber set to 0.1% O₂. Cells were counted daily over a span of 3 days in parallel to cells seeded and placed at 19% O₂.

2.7.9 Western blotting and antibodies

Cells were washed with ice-cold PBS and lysed for 20 min on ice in buffer A (Pierce RIPA) or B [50 mM Tris/HCL (pH 7.4), 5 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 250 mM mannitol, 1% (v/v) triton X-100, 1 mM DTT], both supplemented with 1× complete protease inhibitors and 1× PhosSTOP. The lysates were clarified at 4°C (10 min at 16,000 g), and

protein concentrations in the supernatants were determined using BCA™ kit. Samples were boiled in 1× Laemmli buffer at 95°C for 5 min, proteins were separated by SDS–PAGE (10–40 µg per lane) and transferred using wet mini-transfer system Hoefer™ TE 22 (Hoefer, CA) either onto 0.45 µm Immobilon™-P PVDF membranes (buffer A) or 0.45 µm nitrocellulose membranes (buffer B). In most cases, membranes were blocked in 5% BSA w/v in TBST buffer (0.1% Tween 20 in 1× TBS) for 1h, and then incubated with primary antibodies, which were prepared at 1:1000 dilution in 5% BSA in TBST (16 h at 4 °C). Antibodies against α -tubulin and HIF-2 α (1:1000 dilution), and the corresponding blocking solution were prepared using 5% (w/v) non-fat dry milk in TBST. Membranes were washed with TBST (3-5×5-10 min) and incubated for 1h with HRP-conjugated secondary antibodies, which were prepared at 1:5000 or 1:2500 in 5% milk/TBST. Primary antibodies against 4E-BP1#9644, p-4E-BP1 (S65) #9456, ACC #3662, anti p-ACC (S79) #3661, eIF2 α #2103S, p-eIF2 α (S51) #9721S, α -tubulin #2125, acetyl- α -tubulin (Lys40) #5335, AKT #4691, p-AKT (S473) #4060, p-AKT (T308) #13038, #4056 and #9275 , AMPK α #5832 and 2532, p-AMPK α (T172) #50081 and # 2535, E-cadherin #3195, IGF1R β #9750, mTOR #2972, p-mTOR (Ser2448) #2971, rpS6 #2217, p-rpS6 (S240/244) #2215, Snail #3879, Vimentin #5741 were all from Cell Signaling Technologies (Danvers, MA); VDAC1 #sc-8828 and rpS6 #sc-74459 from Santa Cruz Biotechnologies (Dallas, TX); SCO2 #PA5-76209 from Thermo Fisher Scientific, HIF-2 α # AF2886 from R&D Systems (Minneapolis, MN), SOD2 #ab16956, PDH E1- α # Ab110330 and p-PDH E1- α (Ser293) #Ab92696 from Abcam (Cambridge, UK), acetyl-lysine #ST1027 and α -tubulin #T5168 from MilliporeSigma. Secondary HRP-conjugated mouse anti-goat/sheep IgG #A9452, mouse anti-rabbit IgG #A1949, goat anti-mouse IgG #A0168 were from MilliporeSigma. After washing the membranes with TBST (3-5×5-10 min), specific protein bands were revealed by chemiluminescence using ECL™ Prime reagent on the LAS-3000 imager

(Fujifilm, Japan). As requested by reviewers, we performed densitometric analysis using ImageJ software. Herein, for each replicate intensities obtained for the bands of the proteins or phosphoproteins of interest were normalized against corresponding loading controls and total proteins, respectively. Resulting quantification (mean values across replicates +/- standard deviation) are provided in Fig. S9 while x-ray film scans and images of the immunoblots are included in Fig. S10.

2.7.10 Transwell migration assay

Cells were serum starved overnight (16 h) and plated onto 12-well-transwell migration inserts of 8 μm pore diameter (VWR) at 2.5×10^5 cells per well. 10% FBS-supplemented media was added to the lower chamber as the chemo-attractant and incubated for 24 h. Cells were fixed with 10% buffered formalin and stained with crystal-violet. Migrated cells were imaged under 200X light microscope. Four separate images (fields) were taken of each well. Each data point represents the summarized cell count of four fields from a single transwell migration chamber.

2.7.11 Generation of mRNA-seq libraries and *IDH1* and 2 mRNA sequencing analysis

The libraries for mRNA sequencing analysis were generated as in (82). Libraries were sequenced on an Illumina HiSeq 2000 system at the Beijing Genomics Institute (China).

2.7.12 Isolation of RNA and RT-PCR Analysis

Isolation of total RNA and reverse transcription reaction were performed using RNeasy® plus universal mini kit (Qiagen), high-capacity cDNA reverse transcription kit (Applied Biosystems). qPCR was conducted using SensiFAST™ SYBR® Lo-ROX kit (Bioline) on the AB7300 machine and analyzed using the 7300 System SDS Software (Applied Biosystems); reaction was controlled for the absence of genomic DNA amplification. Each experiment was carried out in independent triplicate. Primers were designed using Primer-BLAST program

(<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) for human genes encoding β -actin (NM_001101.5, forward: 5'-CGGCTACAGCTTCACCACCACG and reverse: 5'-AGGCTGGAAGAGTGCCTCAGGG) and IGF1R (NM_000875.5, forward: 5'-CGGGGAGAGAGCCTCCTGTGA and reverse: 5'-GCTGTTGGAGCCGCAGGCAT) and ZEB1 (NM_001128128.2, forward: 5'-GAAGACAAACTGCATATTGTGGAAG and reverse: 5'-CATCCTGCTTCATCTGCCTGA).

2.7.13 Live cell staining and confocal microscopy

Loading of the cells with fluorescent indicators was performed for 30 min using 1 μ M BCECF (whole cell pH probe) or 2 μ g/ml Nile Red (NR, lipid droplet stain) prepared in OptiMEM I medium. Live cell imaging was performed as follows: Fluorescence lifetime imaging (FLIM) of BCECF was performed at 37°C on an upright laser scanning Axio Examiner Z1 (Carl Zeiss) microscope equipped with 20x/1.0 W-Plan Apochromat dipping objective. Fluorescence decays were collected using a picosecond 488 nm laser (with emission collected at 512-536 nm), DCS-120 confocal (TCSPC) scanner, photon counting detector and SPCM software (Becker & Hickl GmbH). Data were analyzed with SPCImage (B&H) and Excel software. Fluorescence lifetime (LT) of BCECF was calculated using monoexponential fitting. The LT distribution histograms were obtained for 3 individual focal planes within each field of view (256×256-pixel matrixes). Cumulative LT distribution histograms were produced according to the algorithm developed in (83).

The NR staining was analyzed on an Olympus FV1000 confocal laser-scanning microscope with controlled CO₂, humidity and temperature. Fluorescence signals were collected with a UPLSAPO 60X/1.35 oil immersion Super Apochromat objective using 543 nm excitation and 550-650 nm emission wavelengths. The resulting z-stacked fluorescence and single plane differential

interference contrast (DIC) images were processed using FV1000 Viewer software (Olympus) and Adobe Photoshop.

2.7.14 IGF1 ELISA

1×10^6 *SCO2*^{-/-} and WT HCT116 cells were seeded in duplicates in 2 mL of media in 6-well plates and incubated for 24h. The media were collected 24h later and the cells were trypsinized and counted using an automated cell counter (Invitrogen). Media samples were spun down at 16000 g for 10 min and the supernatants were transferred into microcentrifuge tubes. ELISA was used to measure the concentrations of free IGF1 present in the media samples (Ansh Labs) according to the manufacturer's instructions. Briefly, media samples were added to IGF1 antibody coated microtiter wells. The wells were then washed and then incubated with horseradish peroxidase labelled antibody conjugate. At the end of the incubation period, a substrate solution was added to the wells until the color was adequately developed. After incubation with the substrate solution, an acidic stopping solution was added. Dual wavelength absorbance measurements were taken at 450 nm and at 630 nm. IGF1 concentrations were determined using a calibration curve. IGF1 bound to the cells were calculated by subtracting the concentrations of free IGF1 concentrations from baseline concentrations measured in samples of media incubated under identical conditions in 6-well plates without cells. Molar concentrations of the metabolites were normalized per cell and presented relative to the control.

2.7.15 Statistical analysis

Statistical analysis was performed using the results of 2-5 independent experiments. To ensure the accuracy and fidelity of the data, the experiments, when possible, were performed in several technical replicates.

The differences between *SCO2*^{-/-} and WT HCT116 cells (at different treatment conditions) in NR fluorescence, protein and mRNA levels, and other measured parameters were evaluated using unpaired 2-tailed t-test and non-parametrical Mann-Whitney U-test. Unpaired 2-tailed t-tests, two-way ANOVA (Dunnett's) multiple comparison posthoc test) and one-way ANOVA (Tukey's and Dunnett's multiple comparison posthoc tests) analyses were done on Prism (GraphPad).

2.7.16 Data Accessibility

Raw data are deposited at Mendeley (<https://data.mendeley.com/datasets/pyr8t5ng6r/draft?a=754177d8-d6f3-4de6-85b9-645dd4136d2f>).

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2.9 AUTHOR CONTRIBUTIONS

Author contributions: O.U., A.V.Z, D.B.P., M.N.P. and I.T. conceived the study. O.U., A.V.Z., designed and conducted experiments on gene expression, cell functioning, migration, signaling and metabolism. D.E.A and P.V.B. carried out RNA-sequence analysis. L.H. D.A., D.J.P assisted with metabolic experiments. P.J. carried out the transwell migration assay and analysis. Y.W. and P.H. carried out ELISA and Western blotting experiments. A.V.Z and O.U. wrote the initial draft of the manuscript. D.B.P., M.N.P. and I.T. provided funding. All authors contributed to the interpretation of the data and editing the final version of the manuscript.

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2.11 BRIDGING TEXT

This chapter investigates metabolic adaptations caused by mitochondrial dysfunction due to the inactivation of SCO2 in cancer cells. Loss of SCO2 induces energy stress which cancer cells adapt to by activating compensatory metabolic pathways (e.g., NAD⁺ regenerating pathways and pyruvate carboxylation). Metabolic reprogramming mediated by SCO2 deletion is accompanied by rewiring of the IGF1R/AKT axis and paradoxical decrease in mTORC1 signaling. This is paralleled by increased sensitivity of colorectal cancer cells to IGF1 and AKT inhibitors. This study therefore suggests that metabolic adaptations following energy stress may create rewiring of signaling pathways that may result in targetable vulnerabilities of cancer cells.

Considering these findings, and recent recognition of perturbations in energy metabolism as a hallmark of cancer cells, in chapter 3, we sought to define potential metabolic vulnerabilities of CRC cells. Herein, we induced energy stress by treating cells with mitochondrial complex I inhibitor (i.e., phenformin) or nutrient depletion (i.e., glutamine deprivation). Different stressors were used to discern the metabolic pathways activated by phenformin or glutamine starvation in genetically heterogeneous CRC cell lines but not in normal colon epithelial cells. These studies build on those carried out in the chapter 2 and have the overarching objective to identify potential commonalities in metabolic perturbations of genetically distinct CRC cells to various energetic stressors.

CHAPTER 3: Energy stress induces metabolic reprogramming in colorectal cancer

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3.1 ABSTRACT

Intratumor heterogeneity is commonly observed in colorectal cancers (CRCs) as evidenced by different genetic makeup of cancer cells in the same tumor bed. Intratumor heterogeneity represents a major challenge for therapeutic approaches to target neoplasia. This is because most targeted anti-cancer therapies, although effective in eradicating cancer cells that harbor specific mutations, are ineffective against selected subclonal populations that are inherently resistant and/or acquire resistance by losing their dependence on targeted mutation. Regardless of their genetic makeup, cancer cells must undergo metabolic reprogramming to survive energy stress. To this end, identifying mechanisms of metabolic adaptations of cancer cells that are independent of their genetic makeup holds a promise to overcome issues related to therapeutic challenges imposed by intratumor genetic heterogeneity. To address this, we metabolically profiled colorectal cancer (CRC) cell lines in the context of glutamine deprivation or phenformin treatment to comprehensively map the metabolic pathways that underlie adaptation to energy stress. We show that CRC cells are more sensitive to the antiproliferative effects of phenformin, or glutamine

deprivation compared to normal colon epithelial cells. Phenformin treatment and glutamine starvation downregulate mTORC1 signaling which concurrently suppress protein synthesis in transformed colon cells. Although phenformin and glutamine depletion lead to distinct metabolic profiles in HCT116 and HT29 cells, α -ketoglutarate carboxylation appears to be a common and potentially targetable metabolic vulnerability that may be exploited by combining biguanides with inhibitors of glutamine metabolism. Altogether, our studies provide initial evidence for potential commonalities in metabolic adaptations of CRC cell lines and lay foundation for further studies on a broader panel of CRC cell lines and *in vivo* models of CRC.

3.2 INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths and third most prevalent cancer amongst men and women globally (1). CRC death rates decreased in 2016 compared to 1970 in highly developed countries because of the rapid development of diagnosing technologies and improved therapeutics over the years (2). However, it is projected that by 2035, the incidence of CRC would rise to 2.5 million while the mortality rates of colon and rectal cancers will increase by 71.5% and 60% respectively worldwide (3). Genetic, lifestyle and environmental factors have been closely associated with the incidence and death rates of CRC, but the mechanisms underpinning the progression of CRC as well as potential molecular targets to treat this disease are still incompletely understood.

Despite the recent developments, nearly 70% of CRC patients develop liver metastasis which is the primary cause of mortality (4-6). The treatment protocol for CRC typically involves a combination of approaches including surgery, radiotherapy, systemic chemotherapy, targeted therapy and/or immunotherapy (2). However, there are limitations to the different approaches in managing CRC such as systemic toxicity, comorbidities, poor efficacy and acquired resistance (7,

8). Clinical management of CRC has been improved by the advent of targeted therapies (8). To this end, the aberrant expression of EGFR and VEGFR, activating mutations in PI3KCA, KRAS and BRAF, mutations in p53 are regarded as biomarkers which dictate the advancement of CRC and predict the responsiveness to targeted therapeutics (7, 8). While targeted therapy has been effective in hindering the progression of certain cancers such as the use of imatinib to treat BCR-ABL chronic myeloid leukemia (9), targeted approaches have yet to meet their expectations in CRC (7, 10-13).

Although cetuximab, a monoclonal antibody specific for EGFR is occasionally used to treat chemorefractory metastatic CRC, resistance to this drug develops via a number of mechanisms including constitutive activation of KRAS (14, 15). Malignant cell populations with activating gene mutations downstream of EGFR may be present at frequencies too low to be detected by insensitive techniques during genetic profiling of tumors (16). Consequently, the analyses of tumor biopsies may fail to capture the extensive molecular complexities of genetic heterogeneous tumors thus leading to ineffective treatment strategies (11). Intratumor and/or intermetastatic tumor genetic heterogeneity are commonly observed in solid tumors including CRC (11, 17-24). Genetic heterogeneity of tumors, which evolves as subclonal tumor cell populations change spatially and temporally during the development and progression of the disease, are often associated with shorter overall survival and adverse treatment outcomes of CRC (25-27). In addition, targeted therapies are thought to exert selection pressure that may allow better adapted malignant clonal cell populations to expand (28). This drawback in current targeted approaches needs to be addressed to effectively treat CRC.

During neoplastic growth, tumors rapidly outstrip their vasculature which results in hypoxic and nutrient-deficient tumor microenvironment (29-32). Therefore, in advanced solid

tumors, neoplastic cells are exposed to metabolic stress that they must overcome to meet their bioenergetic and/or biosynthetic demands (33). To achieve this, neoplastic cells rewire their energy metabolism to sustain proliferation and survival (34). Since the discovery of the Warburg effect, numerous studies have illustrated metabolic reprogramming in cancer under contexts of various oncogenic mutations and tumor microenvironment conditions (34). To this end, metabolic reprogramming is now regarded as a hallmark of cancer (34). Glucose and glutamine are the two most predominantly used nutrients for energy production and biosynthesis of macromolecules in cells (35, 36). Tumor cells consume more glucose and glutamine than the cells populating nonproliferating normal tissues (37). While CRC cells have been shown to survive glucose-deprived conditions, glutamine has been demonstrated to be required for their survival (38). Nonetheless, this apparent preference of glutamine over glucose as a biomass and bioenergetic source for CRC remains incompletely understood.

Metformin, a biguanide used as a first-line therapy for type 2 diabetes, exhibits antineoplastic effects across numerous cancer cell lines and models (39, 40). In addition, clinical studies revealed that metformin may be associated with reduced risk of colorectal adenoma incidence and CRC-specific mortality in diabetic patients with CRC (41, 42). In addition to its systemic effects that include decrease in insulin levels, metformin is thought to exert cell autonomous antitumoral effects by inhibiting mitochondrial complex I of the electron transport chain which leads to decreased ATP synthesis and an increase in the cellular AMP:ATP ratio (43). The elevated AMP:ATP ratio activates the cellular energy sensor, AMP-activated protein kinase (AMPK) which stimulates ATP generating catabolic processes such as fatty acid β -oxidation while suppressing energy consuming processes including protein synthesis in order to restore energy homeostasis (44, 45). Cells with impaired ability to activate AMPK due to the loss of LKB1 are

unable to restore ATP levels in response to biguanide-induced energetic stress (46, 47). Thus, cancer cells with defective LKB1-AMPK signaling are more sensitive to metformin compared to tumor cells that can activate AMPK (46). Taken together, AMPK activation seems to represent an adaptive response to biguanides that allows cells to bypass energy crisis. We previously showed that biguanides induce compensatory metabolic adaptations such as glucose addiction (48, 49). These adaptations represent potential drug targetable “metabolic vulnerabilities” that may be exploitable for treating CRC.

As all tumor cells require energy to proliferate, we hypothesize that the systematic mapping of the metabolic pathways that enable genetically distinct CRC cells adapt to glutamine deprivation and phenformin (a biguanide that is more potent than metformin) will highlight potentially clinically targetable metabolic vulnerabilities. Given that biguanides and nutrient depletion induce energy stress (50-52), we sought to investigate their effects as independent stressors of CRC cells. Specifically, we set out to identify potential common adaptive metabolic alterations in genetically diverse CRC cell lines subjected to energy stress caused by glutamine depletion or phenformin. We observed that CRC cells were more sensitive to the antiproliferative effects of phenformin and glutamine deprivation than normal colon epithelial cells. As expected, phenformin and glutamine starvation inhibited mTORC1 signaling which was associated with reduced global protein synthesis. Although phenformin led to distinct metabolic profiles in HCT116 and HT29 cells, glutamine deprivation resulted in relatively similar metabolic perturbations characterized by increased levels of intracellular amino acids in both cell lines. Moreover, phenformin and glutamine deprivation of CRC cells reveal that reductive α -ketoglutarate carboxylation may be a common mechanism underpinning adaptation of CRC cells to metabolic stress.

3.3 RESULTS

3.3.1 Metabolic stress reduces proliferation of transformed colorectal cell lines more strongly than non-transformed colon epithelial cells

Well-defined cancer cell lines that are frequently used as cell culture models have been shown to exhibit intra-cell line genetic variability (53, 54). Therefore, focusing on a single CRC cell line of defined genetic background may be insufficient to comprehensively recapitulate the intratumor genetic heterogeneity observed in patients and *in vivo* CRC models (55, 56). In order to simulate intratumor heterogeneity of CRC, and to mitigate cell line associated biases, we employed 2 genetically different human colon adenocarcinoma cell lines: HCT116 and HT29. One of the primary goals behind the study was to elucidate energy-stress response networks shared between different CRC cell lines (Fig. 3.1A). Both HCT116 (Fig. 3.1B) and HT29 (Fig. 3.1C) cells were treated with increasing concentrations of phenformin. Although the antiproliferative effects of biguanides across various cancer cell lines have been previously documented (49, 57-59), it was important to determine the phenformin concentration that inhibited proliferation by 50% of the control cells (i.e., IC_{50}). To facilitate direct comparisons of phenformin-induced metabolic perturbations between the cell lines we treated HCT116 and HT29 cells with the IC_{50} of phenformin (determined to be 0.6mM for both cells lines) (Fig. 3.1B-C). In addition to HCT116 and HT29 cells, SV40-transformed colorectal cell line (CCD841CoTr) derived from the same tissue as the normal epithelial colon cell line (CCD841CoN) were also treated with phenformin (Fig. 3.1D). At most of the tested concentrations, phenformin minimally reduced proliferation of CCD841CoN compared to CCD841CoTr cells (Fig. 3.1D). This confirms previous findings that phenformin has only a marginal effect on proliferation and survival of non-malignant cells (49).

We next assessed the dependence of CRC cell lines on glucose. Herein, we cultured HCT116 and HT29 cells in DMEM supplemented with FBS and different concentrations of glucose (Fig. 3.1E-F). Of note, this concentration range encompasses the normal blood glucose level (5 mM) and recommended cell culture (25 mM) levels. While glucose concentration below 2 mM dramatically reduced proliferation of both CRC cell lines, when cultured in 5mM of glucose, proliferation of HCT116 and HT29 was reduced by ~ 40% (Fig 3.1E) or was largely unaffected (Fig 3.1F) as compared to 25 mM glucose, respectively. Previous studies demonstrated that glutamine anaplerosis into citric acid cycle (CAC) may play an important role in normal colon but also in CRC (38, 60, 61). The core regions of solid tumors however often display glutamine deficiency (62). Therefore, we investigated glutamine dependencies in CRC cell lines. As expected, the proliferation of HCT116 (Fig 3.1G) and HT29 (Fig 3.1H) cells positively correlated with increasing glutamine concentration whereby the IC_{50} were determined to be 0.4mM for both cell lines. Although CCD841CoN cells exhibited no proliferative advantage over CCD841CoTr cells at any of the tested glutamine concentrations (Fig 3.1I), the proliferation of CCD841CoN cells was reduced by ~50% in the absence of glutamine in the media as compared to ~90% and 80% reduction in proliferation of HCT116 (Fig 3.1G) and HT29 (Fig 3.1H) cells, respectively. Collectively, these data suggest that CRC cells exhibit heightened sensitivity to the inhibitors of mitochondrial functions as compared to non-transformed colon epithelial cells, and that, at least in some contexts, CRC cells exhibit increased dependency on exogenous glutamine.

3.3.2 Phenformin and glutamine depletion downregulate mTORC1 activity and inhibit protein synthesis in CRC cell lines.

Since AMPK and mTORC1 act as sensors for nutrient availability cellular energy status, we next monitored AMPK/mTORC1 signaling at early (2 h) and late (16 h) time points after inducing

energy stress. Consistent with previous findings (63, 64), phenformin increased AMPK activity as illustrated by the phosphorylation of its α subunit (at T172) in both HCT116 (Fig 3.2A) and HT29 (Fig 3.2B) cells. Phenformin induced AMPK activity as early as 2 h in HCT116 cells which was augmented after 16 h of treatment (Fig 3.2A). In turn, induction of AMPK by phenformin was evident only after 16 h of treatment in HT29 cells (Fig 3.2B). Consistent with the role of AMPK in suppressing mTORC1 (65), increase in AMPK activity correlated with reduced mTORC1 signaling in both cell lines (Fig 3.2A-B). This was illustrated by the reduced phosphorylation of mTORC1 substrate, 4E-binding protein 1 (4E-BP1; S65) (Fig 3.2A), and mTORC1-dependent S6 kinases (S6Ks) substrate ribosomal protein S6 (RPS6; S240/244) (Fig. 3.2A-B). In contrast to phenformin treatment, glutamine deprivation did not result in appreciable effects on AMPK phosphorylation levels in both HCT116 (Fig 3.2B) and HT29 (Fig 3.2D) cells. Nonetheless, long term (16 h) deprivation of glutamine suppressed mTORC1 signaling as illustrated by the diminished phosphorylation of 4E-BP1 (S65) and RPS6 (S240/244) (Fig 3.2C-D). This suggests that glutamine deprivation represses mTORC1 activity independently of AMPK. However, glutamine-derived α -ketoglutarate level may be depleted which prevents GTP loading of RAGB and subsequent activation of mTORC1 via prolyl hydroxylases (PHDs) (66). Alternatively, glutamine may activate mTORC1 independently of RAGs via adenosine diphosphate ribosylation factor-1 (ARF1) GTPase (67). Notwithstanding that future investigations are required to determine the temporal changes of mTORC1 signaling in response to glutamine starvation, the data imply that the time taken to deplete intracellular glutamine pools cause a time lag between the observed mTORC1 inhibition and the depletion of exogenous glutamine levels.

Considering that mRNA translation is one of the most energetically demanding processes in the cell which is also regulated by AMPK and mTORC1 (68, 69), we assessed whether global

protein synthesis was affected by phenformin and glutamine deprivation. Phenformin inhibited global mRNA translation as illustrated by reduced polysome formation that is reflected by decreased polysome/80S monosome ratios in HCT116 (Fig 3.2E) and HT29 cells (Fig 3.2F). Similar to phenformin treatment, 16 h of glutamine depletion suppressed global mRNA translation in both HCT116 (Fig 3.2G) and HT29 cells (Fig 3.2H). Overall, these findings are consistent with previous observations suggesting that downregulation of mTORC1 activity and global protein synthesis plays a central role in adaptation to metabolic stress (49).

3.3.3 Phenformin induces metabolic perturbations in CRC cells.

Aberrant AMPK and mTOR signaling has been implicated in the metabolic reprogramming of cancer cells (70). Furthermore, mTOR inhibitors have been shown to modulate cell metabolism by suppressing the translation of mRNAs encoding metabolic enzymes and mitochondrial factors (49, 71-73). Thus, we determined the impact of phenformin on steady-state intracellular metabolites in order to identify metabolic pathways that are perturbed in CRC cells in response to metabolic stress. As expected (74), phenformin reduced the intracellular levels of CAC intermediates including citrate, succinate, fumarate and malate in HCT116 cells as compared to the control, vehicle treated cells (Fig 2.3A). Also consistent with prior findings (48), in HCT116 cells phenformin increased the levels of α -ketoglutarate and α -ketoglutarate/citrate ratio which is associated with the induction of reductive glutamine metabolism (75) (Fig 3.3A). Furthermore, the steady-state level of 2-hydroxyglutarate (2-HG) was elevated while γ -amino-butyric acid (GABA) was depleted in HCT116 cells treated with phenformin relative to the vehicle treated control (Fig 3.3A). Considering that 2-HG is synthesized from α -ketoglutarate while the GABA shunt represents anaplerotic CAC route via succinate (76, 77), these findings are consistent with observed phenformin-induced increase in α -ketoglutarate and decrease in succinate, respectively.

Since the synthesis of amino acids such as aspartate and alanine are dependent on mitochondrial activity (78-80), we expected that phenformin treatment will lead to reduction in intracellular steady-state levels of these amino acids. Indeed, a marked reduction in alanine and aspartate levels was observed in phenformin-treated HCT116 cells (Fig 3.3A). Although phenformin induced comparable perturbations in some steady-state metabolite levels in HT29 and HCT116 cells (e.g., citrate, succinate, α -ketoglutarate, 2-HG, GABA, aspartate and alanine), several differences in the phenformin-induced alterations of metabolite levels between these cell lines were noted (Fig 3.3B-C). For instance, fumarate and malate which were significantly elevated after 2 h exposure to phenformin were reduced to control levels after 16 h in HT29 (Fig 3.3B), but not HCT116 cells (Fig 3.3A). These findings suggest potential temporal differences in phenformin-induced metabolomes of HCT116 and HT29 cells (Fig 3.3C). Although the underpinning mechanisms remain to be established, these metabolic distinctions in the effects of phenformin on the metabolome of HCT116 and HT29 cells may be a reflection of genomic differences between the cell lines.

3.3.4 Glutamine deprivation depletes CAC intermediates while simultaneously increasing the levels of most amino acids in transformed colorectal cell lines.

CRC cells were reported to critically depend on glutamine as an anaplerotic substrate for CAC (61, 81). Moreover, biguanides induce glutaminolysis to support lipogenesis and aspartate synthesis (48, 49, 78, 82). Thus, we sought to investigate time-dependent metabolic perturbations of glutamine-deprived HCT116 and HT29 cells. In accordance with its role in supporting the CAC, glutamine depletion reduced the levels of CAC intermediates (i.e., citrate, malate, succinate, fumarate and malate) in HCT116 cells (Fig 3.4A). In contrast to energy stress induced by phenformin, long term glutamine deprivation of HCT116 cells decreased the α -

ketoglutarate/citrate ratio which is indicative of decreased reductive carboxylation of α -ketoglutarate (75). Intriguingly, 2 h of glutamine deprivation increased levels of both essential (e.g., valine, leucine, isoleucine, methionine, threonine, phenylalanine, tyrosine and histidine) and non-essential (e.g., cysteine, proline, serine and glycine) amino acids in HCT116 cells (Fig 3.4A). On the contrary, the levels a few amino acids such as alanine, glutamate, aspartate and asparagine were greatly depleted due to glutamine starvation (Fig 3.4A). Most of these observations were not limited to HCT116 cells. HT29 cells deprived of glutamine exhibited similar alterations in the levels of amino acids with concurrent decreases in CAC intermediates and α -ketoglutarate/citrate ratio (Fig 3.4B). Nonetheless, the effects of glutamine starvation on steady-state proline levels were remarkably different between HCT116 and HT29 cells (Fig 3.4A-B). Namely, while glutamine starvation led to an increase in proline levels in HCT116 cells, it was associated with a decrease in proline abundance in HT29 cells (Fig 3.4B-C). Although the cause of this apparent discrepancy in the effects of glutamine deprivation on proline levels between HCT116 and HT29 cells remains unclear, it is plausible that this and other observed discrepancies may stem from differences in the mechanisms that drive metabolic rewiring in these cell lines. For example, MYC has been demonstrated to promote proline biosynthesis from glutamine via glutamate by increasing the expression of enzymes in the pathway such as P5CS and P5C reductase (PYCR) (83). Considering that MYC expression is elevated in 50% of CRC (84), it is conceivable that differential expression of MYC (85) in HCT116 and HT29 glutamine-deprived cells may explain differences in proline levels.

3.4 FIGURES

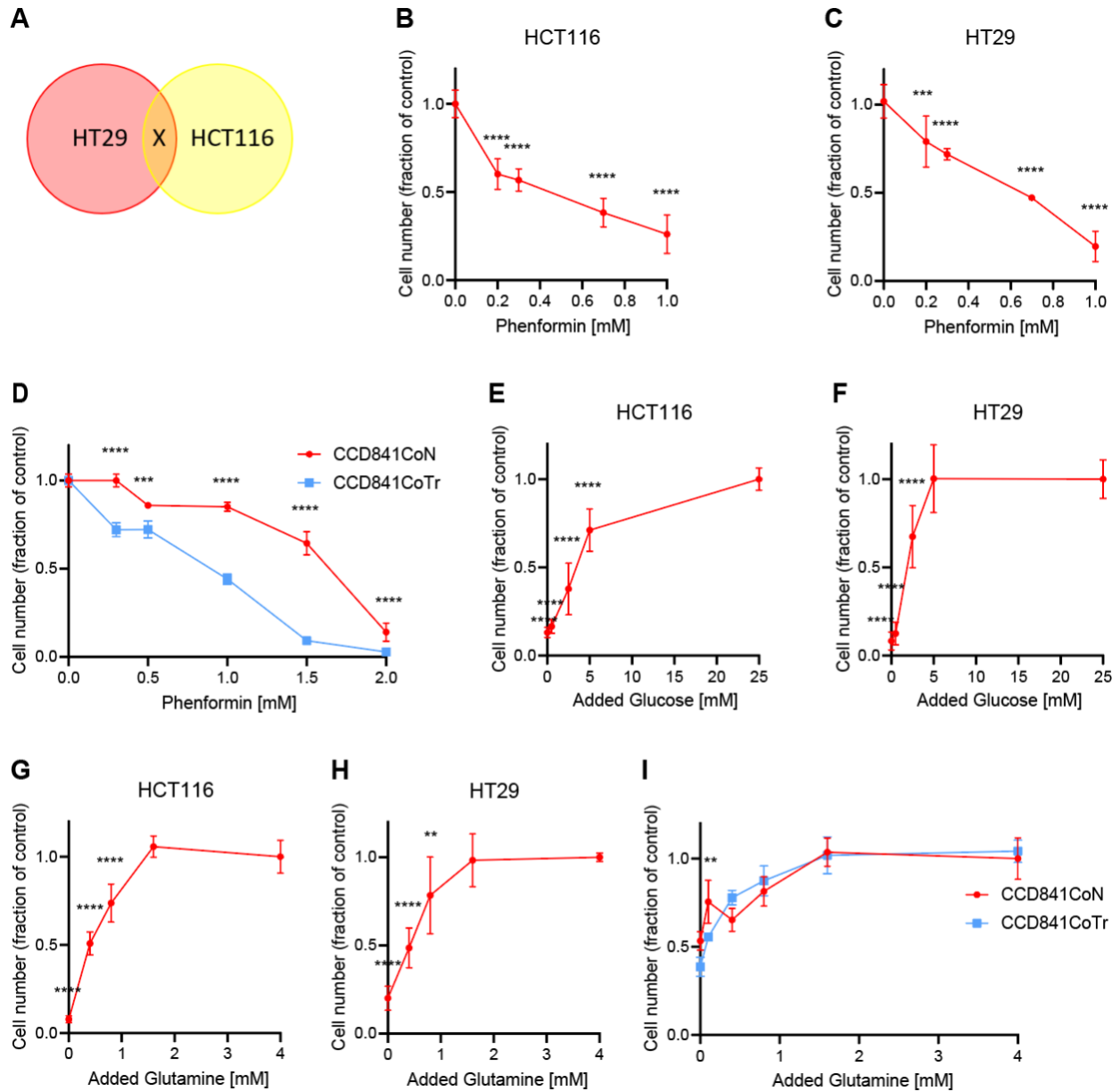


Fig. 3.1 Antiproliferative effects of nutrient deprivation and phenformin on epithelial colon cells. **A.** Venn diagram representing metabolic vulnerabilities after inducing energy stress in HCT116 and HT29. “X” denotes overlapping metabolic changes induced by energy stress in both HCT116 and HT29. **B-D.** HCT116 (**B**), HT29 (**C**), CCD841CoN and CCD841CoTr (**D**) cells were treated with indicated concentrations of phenformin or a vehicle (water) for 3 days. The number

of viable cells was determined using trypan blue exclusion and automated cell counter. Data are shown as the mean fraction of cells relative to the corresponding water-treated controls which were set at 1. The experiments were carried out in independent triplicates (with at least 2 technical replicates each time). Bars represent +/- SD values. **E-F**. HCT116 (**E**) and HT29 (**F**) cells were grown in media containing the indicated concentrations of glucose or vehicle (water) for 3 days. The number of live cells was determined using trypan blue exclusion and automated cell counter. Data are represented as the mean fraction of cells relative to the corresponding water-treated controls which were set at 1. The experiments were carried out in independent triplicates (with at least 2 technical replicates each time). Bars represent +/- SD values. **G-I**. HCT116 (**G**), HT29 (**H**), CCD841CoN and CCD841CoTr (**I**) cells were grown in media containing the indicated concentrations of glutamine or vehicle (water) for 3 days. The number of viable cells was determined using trypan blue exclusion and automated cell counter. Data are shown as the mean fraction of cells relative to the corresponding water-treated controls which were set at 1. The experiments were carried out in at least independent duplicates (with 2 technical replicates each time). Bars represent +/- SD values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (**B-C** and **E-H**, One-way ANOVA; Dunnett's posthoc test with water-treated cells as control; **D** and **I**, Two-way ANOVA; Tukey's posthoc test with water-treated cells as control).

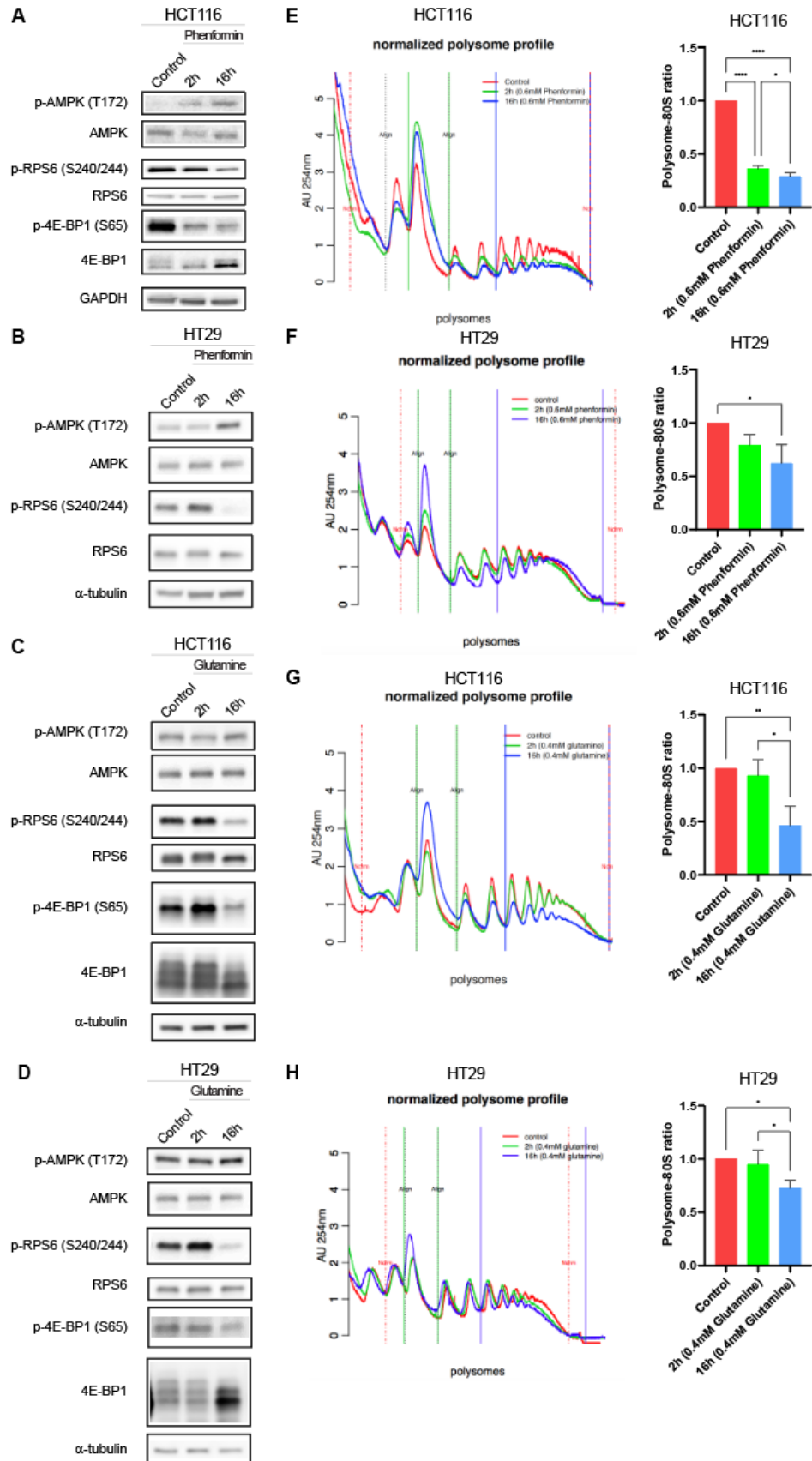


Fig. 3.2 Suppression of global mRNA translation is associated with the time course inhibition of mTORC1 signaling by phenformin and glutamine deprivation in CRC

A-B. Western blot analysis of indicated proteins isolated from HCT116 (**A**) and HT29 (**B**) cells treated with phenformin (0.6 mM) or a vehicle (water) for 2 h or 16 h. Representative blots from 3 independent experiments are shown. GAPDH and α -tubulin served as a loading control. **C-D.** HCT116 (**C**) and HT29 (**D**) cells were grown in media supplemented with FBS and 0.4 mM glutamine or 4 mM glutamine (water-treated control) for 2 h or 16 h. Levels and phosphorylation status of indicated proteins was determined by Western blotting. α -tubulin served as a loading control. The blots are representative of 3 independent experiments. **E-H.** HCT116 (**E**) and HT29 (**F**) cells were treated with phenformin (0.6 mM) or vehicle (water) for 2 h or 16 h. **G-H.** HCT116 (**G**) and HT29 (**H**) cells were in media supplemented with 0.4 mM glutamine or control (4 mM glutamine) for 2 h or 16 h. Polysome fractions were obtained by ultracentrifugation using 5-50% sucrose gradients. Representative images of the polysome fraction absorbance profiles (254 nm) are shown. Polysome to 80S monosome ratios were calculated by comparing the area of the 80S peak to the cumulative area under the polysome peaks. The ratios of the areas were made relative to the corresponding control cells which were set at 1 as displayed on the right of the corresponding polysome profiles. The experiments were carried out in independent triplicates. Bars represent $\pm$ SD values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (**E-H.** One-way ANOVA; Dunnette's posthoc test with water-treated cells as control).

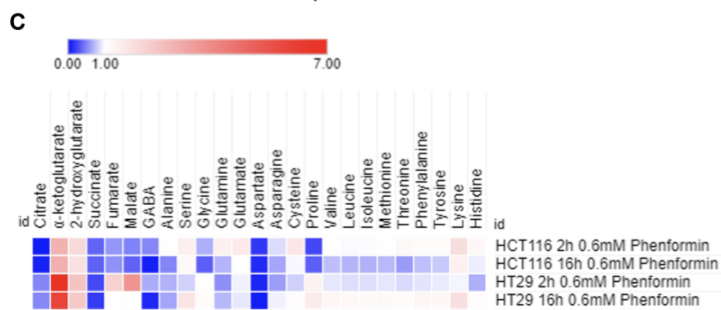
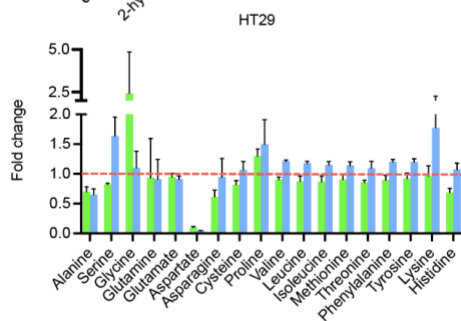
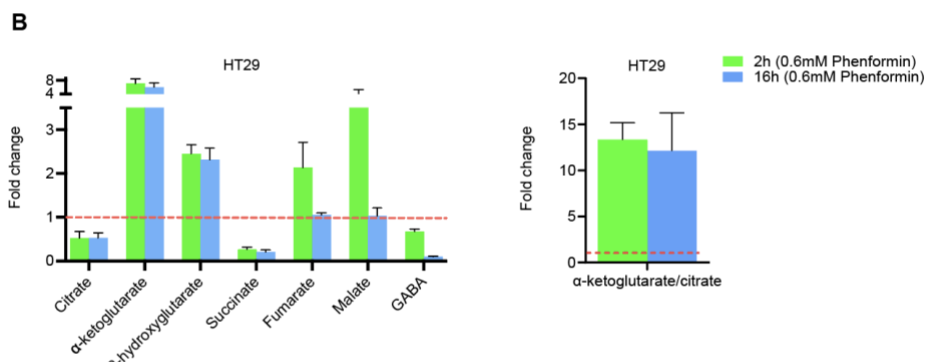
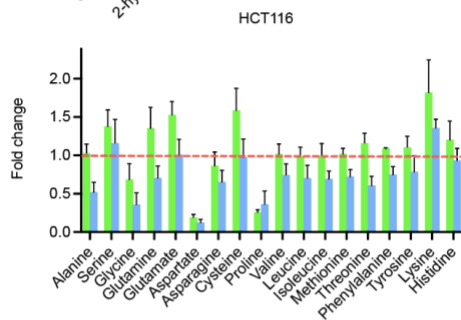
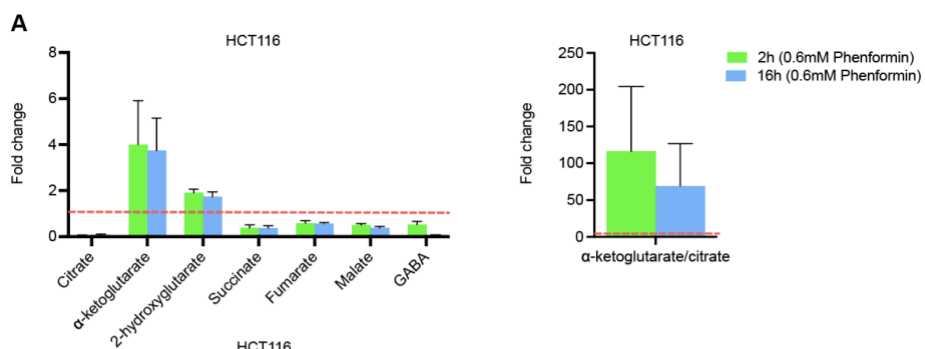


Fig. 3.3 Phenformin alters the metabolome of genetically heterogenous CRC cell lines. A-B. Intracellular levels of CAC-related metabolites and amino acids in HCT116 (**A**) and HT29 (**B**) cells were measured by GC-MS. The cells were treated as indicated and data are represented as mean fold change $\pm$ SD relative to control cells wherein values for each metabolite are set to 1 and displayed as the red-dashed line (N=3 independent experiments with technical triplicates each time). Intracellular α -ketoglutarate/citrate ratios in HCT116 (**A**) and HT29 (**B**) cells were determined from the steady state levels of α -ketoglutarate and citrate. The ratios calculated for the water-treated control cells were set at 1 (red-dashed line) and the results were represented as means of the ratios $\pm$ SD for the phenformin-treated cells of 3 independent experiments with 3 technical replicates each. **C.** Heatmap of the intracellular metabolites shown in Fig. **A** and **B**. The colors vary from blue (for metabolites whose levels were decreased in treatment conditions vs. control) to red (for metabolites whose levels were increased in treatment conditions vs. control).

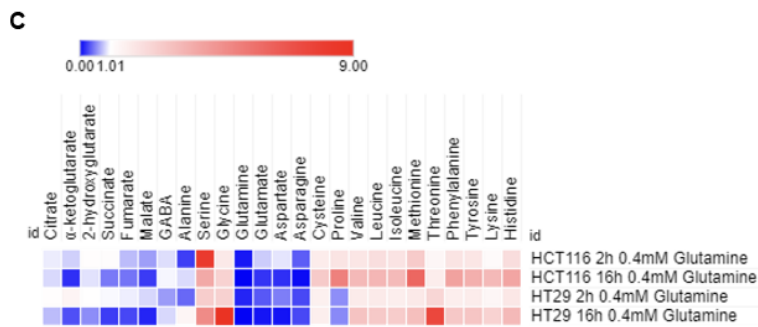
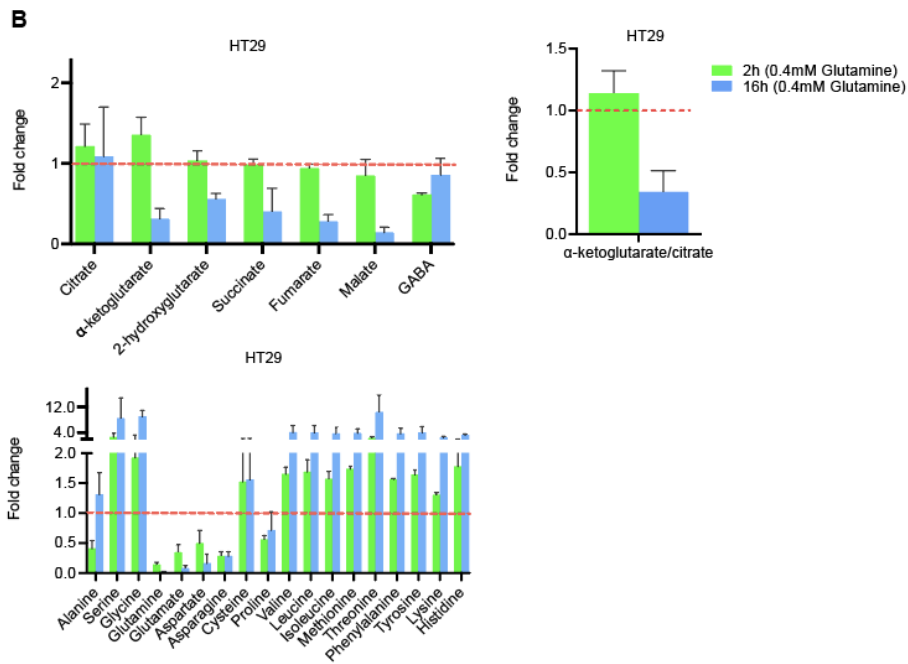
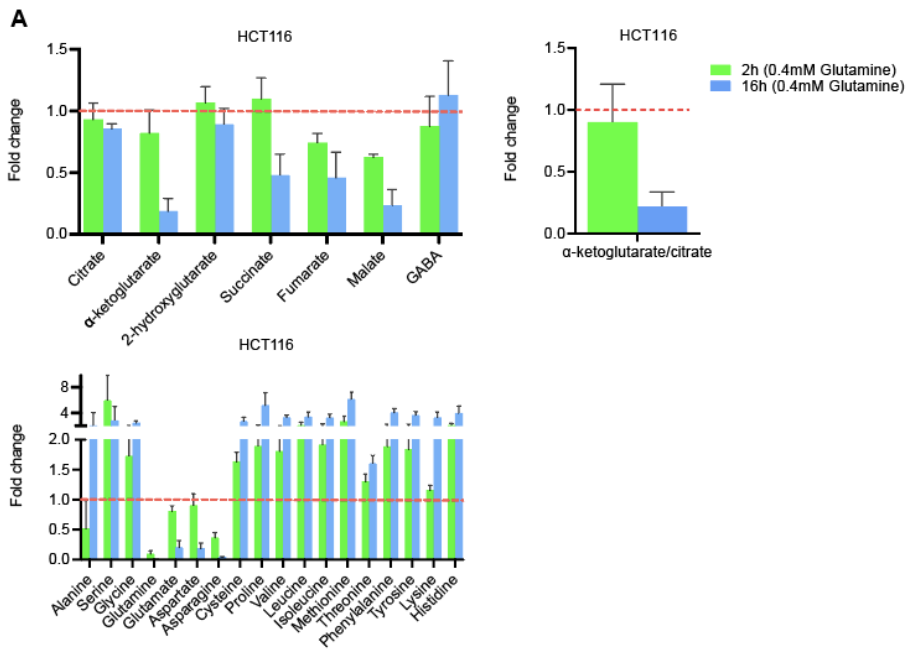


Fig. 3.4 Glutamine deprivation reduces the levels of CAC intermediates but increases the levels of most amino acids in genetically distinct CRC cell lines. A-B. Intracellular levels of metabolites in HCT116 (**A**) and HT29 (**B**) cells were detected by GC-MS. The cells were treated as indicated and data are shown as mean fold change $\pm$ SD relative to water-treated cells wherein values for each metabolite are set to 1 and displayed as the red-dashed line (N=3 independent experiments with technical triplicates each time). Intracellular α -ketoglutarate/citrate ratios in HCT116 (**A**) and HT29 (**B**) cells were calculated from the steady state levels of α -ketoglutarate and citrate. The ratios determined for the water-treated control cells were set at 1 (red-dashed line) and the results are presented as means of the ratios $\pm$ SD for the treated cells from 3 independent experiments with 3 technical replicates each. **C.** Heatmap of the intracellular metabolites shown in Fig. **A** and **B**. The colors vary from blue (for metabolites whose levels were decreased in treatment conditions vs. control) to red (for metabolites whose levels were increased in treatment conditions vs. control).

3.5 DISCUSSION

Oxygen and nutrient availability vary during tumor development (86), whereby both factors become limiting in poorly vascularized solid tumors (86). In response to the energy stress, cancer cells undergo metabolic reprogramming in order to meet their bioenergetic and biosynthetic demands (86). Previously published studies demonstrated that energy stress caused by phenformin has only a minimal impact on proliferation of non-transformed breast epithelial NMuMG cells as compared to NMuMG cells transformed with oncogenic Neu/ErbB2 (V664E) (referred to as NT2197) (49). Consistent with this finding, HCT116 and HT29 cells exhibited greater sensitivity to energy stress caused by phenformin relative to normal epithelial colon cells (CCD841CoN). This is evident as phenformin IC_{50} is lower in transformed colorectal cells (i.e., 0.9mM for

CCD841CoTr and 0.6 mM for both HCT116 and HT29) than in normal colon cells (i.e., 1.7 mM in CCD841CoN). Conversely, the IC₅₀ value for glutamine is higher in transformed colorectal cells (i.e., 0.4 mM in both HCT116 and HT29) than in normal colon cells (i.e., 0 mM in CCD841CoN).

Several driver mutations underlie tumor evolution and response to therapeutic insults in CRC. These mutations (APC; present in 80% of CRC), KRAS (35%–45%), p53 (50%), PIK3CA (20%–30%), and BRAF (10%) result in aberrant activation of the mTOR pathway (87-89). HCT116 and HT29 cells have missense mutations (P449T for HT29 and H1047R for HCT116) in PIK3CA (90) which lead to the constitutively active PI3K-AKT-MTOR signaling (91, 92). However, HCT116 and HT29 cells differ in the status of other driver mutations implicated in metabolic reprogramming. For instance, KRAS (G13D) is mutated only in HCT116 cells (93). In addition, HT29 cells have mutations in BRAF (V600E and T119S) and p53 (R273H) which are absent in HCT116 cells (90, 94-98). Despite BRAF and KRAS mutations promoting similar physiological phenotypes, they have been reported to induce different metabolic dependencies in CRC (96). Corroborating prior works on the effects of biguanides across several cancer cell lines (49, 73, 99, 100), we establish that regardless of the genetic differences between HCT116 and HT29 cells, the downregulation of mTORC1 activity and consequently, protein synthesis by phenformin may at least be partly mediated by AMPK. However, it is worth mentioning that in addition to AMPK-dependent mechanisms, inhibition of mTORC1 by biguanides may be mediated via RAG GTPases and/or REDD1 (100-102). Thus, future genetic approaches whereby expression of REDD1, RAG GTPases or AMPK subunits is modulated is required to discern the precise mechanism by which phenformin inhibits mTORC1 signaling in HCT116 and HT29 cells. Our data seem to validate reports that glutamine deprivation inhibits mTORC1 independent of AMPK activity (103). The inhibitory effects of glutamine deprivation

on mTORC1 activity seem to be mediated via RAGB (66). In turn, glutamine appears to stimulate mTORC1 via ADP-ribosylation factor 1 (ARF1) (67, 104). Based on this, further investigation is required to highlight the mechanisms by which glutamine starvation inhibits mTORC1 activity in the context of CRC. In addition to reducing global protein synthesis, biguanides and nutrient deprivation have also been shown to preferentially affect translation of specific subsets of mRNAs while only marginally affecting others (49, 73, 105). Future applications of ribosome and/or polysome profiling methods coupled with RNAseq are therefore warranted to catalogue mRNAs whose translation is perturbed in response to metabolic stress in the context of CRC.

Biguanides inhibit mitochondrial functions which leads to compensatory increase in glycolysis (48, 49, 106). Although the biguanide-induced increase in glycolysis is well-defined, the effect of biguanides on the levels of CAC intermediates appear to be context dependent (48, 49, 59, 74, 107, 108). To this end, although phenformin induced comparable changes in the levels of α -ketoglutarate, citrate and succinate in HCT116 and HT29 cells, the effects of phenformin on fumarate and malate appeared to be diminished with time in HT29 but not HCT116 cells. However, to fully establish potential discrepancies in the effects of phenformin on the metabolome of HT29 and HCT116 cells it is necessary to perform future metabolic flux analysis using stable isotope tracer labeling with ^{13}C -labelled substrates (such as glucose, glutamine or pyruvate). This and previously published studies (48, 49), suggest that phenformin induces reductive carboxylation of α -ketoglutarate. Given that glutamine is a major anaplerotic source (109, 110), CAC intermediate levels are expected to decrease once the intracellular glutamine levels become limiting. Importantly, glutamine deprivation appears to differentially affect CRC cell lines, whereby the effects of glutamine removal from the cell culture media led to dramatic inhibition of proliferation of HCT116 and HT29 but not CCD841CoTr cells. Notably, cells overexpressing

the MYC proto-oncogene (e.g., HT29) or oncogenic KRAS (e.g., HCT116) or PI3KCA mutations (e.g., HCT116 and HT29) have been shown to be highly dependent on glutamine (61, 111-114). It is therefore plausible that the context of cancer-driving mutation(s) dictate(s) glutamine “addiction” of cancer cells, whereby SV40-transformed CCD841CoTr cells show lesser dependence on glutamine. Collectively, these data are in good keeping with previous findings (61, 111-114) suggesting that the driver mutations of CRC may direct metabolic adaptations to metabolic stress caused by glutamine starvation or drugs interfering with mitochondrial functions.

Amino acid starvation leads to elevated levels of deacetylated tRNAs due to the failure of tRNA synthetase to aminoacylate tRNA when intracellular amino acid pools are insufficient (115, 116). Subsequently, the deacetylated tRNA molecules bind and activate GCN2 kinase (116). Once activated, GCN2 phosphorylates eIF2 α which is paralleled by reduction in global mRNA translation with concomitant increase in translation of a small subset of mRNAs enriched in those encoding stress-responsive transcription factors including ATF4 (116). We observed similar alterations in the intracellular levels of most essential amino acids (EAAs) and nonessential amino acids (NEAAs) in glutamine-deprived HCT116 and HT29 cells. This is consistent with recent findings demonstrating that cancer cells cultured in glutamine-deficient media significantly elevate the levels of amino acids via increased uptake of exogenous amino acids mediated by ATF4 transcriptional reprogramming (109, 110, 117, 118). Specifically, it was shown that glutamine deprivation triggers the general amino acid control (GAAC) pathway wherein ATF4 expression is upregulated (118, 119). In turn, ATF4 stimulates expression of genes encoding amino acid transporters (e.g., SLC7A5) which is associated with a time-dependent reactivation of mTORC1 and inhibition of autophagy (117, 118). Although glutamine-deprived cells also upregulate amino acid biosynthesis enzymes (e.g., asparagine synthetase) via GCN2/eIF2 α /ATF4 axis, they seem to

be unable to generate sufficient asparagine from the intracellular glutamine pools (109, 120). However, in contrast with the prior studies demonstrating that mTORC1 is reactivated in glutamine-starved cells (118), we observed no restoration of mTORC1 activity during 16 h of glutamine deprivation. Intriguingly, no observable changes to the levels of amino acids levels were detected when cells were grown in glutamine-deficient media that was supplemented with asparagine (109). Consistent with this, α -ketoglutarate or asparagine supplementation in glutamine-deficient media also rescued cell proliferation, restored mTORC1 activity and protein synthesis in glioblastoma and breast cancer cell lines (109, 110). Taken together, glutamine and/or asparagine appear to be required to sustain the mTORC1 activity in cancer cell lines, including CRC cells. In addition to enhanced amino acid uptake, it is plausible that the increase in the intracellular levels of EAA and NEAA in phenformin-treated or glutamine-deprived cells is caused by the induction of autophagy (121, 122). To this end, in the future we will set out to determine the role of autophagy in adaptation of CRC cells to energy stress induced by glutamine deprivation or biguanides.

CAC anaplerosis via α -ketoglutarate derived from glutamine is more pronounced in the tumors than in adjacent normal colon tissues (61). Thus, phenformin and drugs inhibiting glutaminolysis may have an optimal therapeutic index for treating CRC. Phenformin increases the reductive carboxylation of α -ketoglutarate, while glutamine deprivation impedes it. This suggests that combining biguanides and inhibitors of glutaminolysis may result in synergistic anti-neoplastic effects. Indeed, our findings have potential implications for therapeutic strategies targeting glutamine metabolism in CRC, as the efficacy of biguanides as antineoplastic drugs could be bolstered by combining it with inhibitors of glutamine metabolism.

Accordingly, CRC stem cells show greater sensitivity to metformin in glutamine-depleted media but not in glucose-deprived media (123). Furthermore, glutaminase C inhibitor (compound 968) enhanced the antineoplastic effects of metformin (123). In addition, CB-839 (a noncompetitive allosteric GLS1 inhibitor) exhibits marked antiproliferative effects across several cancer models (124-126) and prevents metabolic adaptation to MLN128 (an mTOR inhibitor) (127). Although our study provides pioneering insights into metabolic profiles of CRC in the context of metabolic stress. Future efforts to integrate energy stress-induced changes in gene expression, signaling and metabolome are warranted to establish the networks that underpin adaptive mechanisms of CRC cells and unravel potentially targetable metabolic vulnerabilities.

3.6 METHODS

3.6.1 Cell lines and culture conditions

HCT116, HT29, CCD841CoN and CCD841CoTr were obtained from ATCC. All cells were cultured in complete DMEM (i.e., DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin) (DMEM, FBS and penicillin/streptomycin were provided by Wisent). HCT116, HT29, CCD841CoN were maintained at 37 °C, 5% CO₂ and 19% O₂ while CCD841CoTr was incubated at 33 °C, 5% CO₂ and 19% O₂.

3.6.2 Glutamine deprivation

For western blotting, polysome profiling and GC-MS analysis, cells were seeded in complete DMEM 24 h before glutamine deprivation treatments. The cells were washed twice using DMEM without glutamine. After discarding the wash media, the cells were treated with DMEM containing 0.4mM glutamine supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. 1.8mL, 10.8mL and 27mL of treatment media were used for cells seeded in 6-well, 100mm and 150mm plates respectively.

3.6.3 Phenformin treatment

Cells seeded for western blotting, polysome profiling and GC-MS analysis were cultured in complete DMEM media 24 h prior to treatment. The cells were washed twice with DMEM without glucose. After discarding the wash media, cells were treated with DMEM containing 10mM glucose supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin and 0.6mM phenformin hydrochloride (Sigma). 1.8mL, 10.8mL and 27mL of the treatment media were used for cells seeded in 6-well, 100mm and 150mm plates respectively.

3.6.4 Metabolomics analysis

4.5×10^5 HCT116 and 5.7×10^5 HT29 cells were cultured on 6-well plates in technical triplicates to obtain cells approximately 80% confluency 48 h later. Cells were then treated with phenformin or deprived of glutamine for 2 h and 16 h. The plates were quickly placed on ice as soon as the treatment period was over. Media were discarded with an aspirator and the cells were washed with prechilled isotonic saline solution three times. Afterwards, the cells while on the plates were quenched on dry ice. 300 μ L of 80% methanol pre-chilled to -20 °C was added to the cells which were subsequently scraped from the wells and transferred into pre-chilled microcentrifuge tubes. This process was repeated with an additional 300 μ L of 80% methanol and pooled with the previously collected 300 μ L fraction. The cells were lysed at 4 °C (10 minutes, 30 sec on, 30 sec off, high power setting twice with a Diagenode Bioruptor). Cell debris were precipitated and discarded after centrifugation (16000 g, 4°C) while the supernatants were transferred into pre-chilled tubes, spiked with 750 ng of myristic acid-D₂₇ (Millipore Sigma) and dried in a CentriVap cold trap (Labconco) overnight at 4°C. Subsequently, the dried pellets were solubilized in 30 μ L of pyridine containing methoxyamine-HCl (10 mg/mL) (MilliporeSigma) using a sonicator and vortex. The samples were heated to 70°C for 30 min and then moved into GC-MS injection vials

containing 70 μL of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA). After homogenizing, the sample samples were reheated at 70 $^{\circ}\text{C}$ for 1 h. 1 μL of the derivatized samples was injected for GC–MS analysis. All GC–MS instrumentation and software (ChemStation and MassHunter) were from Agilent. Each metabolite was normalized to the peak intensity of myristic acid-D₂₇ and the average cell number derived from the cells seeded in parallel and identical conditions to those collected for GC-MS steady state analysis. Data were expressed as fold change relative to the control cells.

3.6.5 Cell proliferation assays

1×10^5 cells were seeded in technical duplicates in 6-well plates and incubated for 24 h. The seeding media were aspirated and substituted with treatment media for 72 h. Different concentrations of phenformin tested were prepared using DMEM containing 10 mM glucose supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. After the treatment period was over, the media were aspirated, and trypsin was added to the cells. Trypsinization was stopped by adding complete media to the wells after observing the cells were dislodged from the surface of the plates. The cell sample mixtures were collected and stained with trypan blue. Live cells were counted using an automated cell counter (Invitrogen).

3.6.6 Western blotting and antibodies

2.7×10^6 HCT116 and 3.4×10^6 HT29 cells were seeded in complete DMEM on 100mm plates. The seeding media were discarded 24 h later. Cells were then treated as previously described for glutamine deprivation and phenformin treatment. At the end of treatment period, the treatment media were aspirated, and the cells washed twice with ice-cold PBS while on ice. The cells were scrapped from the plates, collected into pre-chilled tubes and centrifuged (600 g at 4 $^{\circ}\text{C}$). The supernatants were carefully removed, the cell pellets were resuspended in lysis buffer [50 mM

Tris/HCL (pH 7.4), 5 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 250 mM mannitol, 1% (v/v) triton X-100, 1 mM DTT, 1× complete protease inhibitors and 1× PhosSTOP] and placed on ice for 20 min. The cell lysates were centrifuged (10 min at 16,000 g and 4°C), and the supernatant subsequently transferred into clean microfuge tubes. After calculating protein concentrations by using BCATM kit, the samples were boiled in 1× Laemmli buffer at 95°C for 5 min to denature and reduce the proteins. The proteins were separated by SDS–PAGE and transferred using wet mini-transfer system onto 0.45 µm nitrocellulose membranes. Membranes were placed in blocking buffer solution (5% BSA w/v in TBST buffer (0.1% Tween 20 in 1× TBS)) for 1 h at room temperature and then incubated with the appropriate primary antibodies which were diluted at 1:1000 in 5% BSA in TBST (overnight at 4 °C). The membranes were washed with TBST (3x 10 min at room temperature) and incubated for 1 h with HRP-conjugated secondary antibodies diluted at 1:5000 or 1:2500 in 5% skimmed milk w/v in TBST. Primary antibodies against 4E-BP1#9644, p-4E-BP1 (S65) #9456, α-tubulin #2125, GAPDH #2118, AMPKα #5832 and 2532, p-AMPKα (T172) #50081 and # 2535, p-rpS6 (S240/244) #2215 were all from Cell Signaling Technologies (Danvers, MA); rpS6 #sc-74459 from Santa Cruz Biotechnologies (Dallas, TX). Secondary HRP-conjugated mouse anti-rabbit IgG #A1949, goat anti-mouse IgG #A0168 were from MilliporeSigma. The membranes were washed (3X for 10 min at room temperature), ECL (Bio-Rad) mixture was added on the membranes and protein signals were revealed as chemiluminescent bands using Azure C300 Chemiluminescent Western Blot Imaging System (Azure Biosystems).

3.6.7 Polysome Profiling

6.8×10^6 HCT116 and 8.5×10^6 HT29 cells were seeded in complete DMEM on three 150mm plates per treatment condition. The cells were treated as previously described for glutamine deprivation

and phenformin treatment. As soon as incubation period was over, the cells were harvested as described (128). A linear 5% to 50% sucrose gradient was prepared and used to isolate the polysomes described in (128). Trizol (Thermo Fisher Scientific) was added to each fraction according to manufacturer's instructions and stored at -80 °C. Experiments were carried out in independent triplicates.

3.6.8 Statistical analysis

Statistical analysis was performed using data from 2-3 independent experiments. To ensure the precision, accuracy and fidelity of the data, the experiments, when possible, were done with at least duplicates. Two-way ANOVA (Dunnett's) multiple comparison posthoc test) and one-way ANOVA (Tukey's and Dunnett's multiple comparison posthoc tests) analyses were done on Prism (GraphPad).

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CHAPTER 4: DISCUSSION

4.1 Rewiring of signaling and metabolic networks underpins metabolic plasticity and adaptation of cancer cells to energy stress.

Although Otto Warburg first described metabolic reprogramming in cancer cells over a century ago, the recent resurgence of interest in studying cancer metabolism that were spurred by the latest technological developments established dysregulated cellular energetics as a hallmark of cancer (1, 2). Metabolic reprogramming is often coupled with the other hallmarks of cancers such as sustained proliferation, resistance toward apoptosis, and epigenetic remodeling (3). For instance, this is evident as the sustained proliferation of tumor cells is a bioenergetically and biosynthetically demanding process, whereby neoplastic cells must adapt their metabolomes to survive and thrive in nutrient and oxygen depleted environment (2). In essence, cancer cells must be metabolically flexible and plastic to balance energy-producing and energy-consuming processes while withstanding adverse stressors emanating from tumor microenvironment. Commonly altered metabolic processes that support tumor growth and progression include glycolysis, lipid metabolism, glutamine metabolism, mitochondrial metabolism and redox homeostasis. Initial research in cancer metabolism predominantly focused on processes that incorporate nutrients into the biomass (e.g., nucleotides, amino acids, and lipids) needed for proliferation (4). This established glucose and glutamine as the major sources for central carbon metabolism needed for cancer cell bioenergetic and biosynthetic processes (3, 4). In terms of bioenergetics, cancer cells often compensate for deficiency of either glucose or glutamine by augmenting the use of the available nutrient considering that the metabolism of both nutrients through the citric acid cycle (CAC) generates reducing equivalents for ATP production via oxidative phosphorylation (OXPHOS) (3-5). Cancer cells predominantly use reduced carbon to

biosynthesize a vast array of macromolecules including fatty acids, nucleotides and non-essential amino acids through the transformation of nutrients into distinct intermediates that serve as substrates for these biomolecules (3). Aerobic glycolysis produces less ATP albeit rapidly from substrate-level phosphorylation than OXPHOS (4). However, it is proposed that aerobic glycolysis maintains redox balance and facilitates anabolic pathways by providing carbon units to support cancer cell survival (3, 4, 6). Most biosynthetic reactions such as fatty acid synthesis are fueled by the reducing equivalent, NADPH (3). While NADPH is mainly produced through the oxidation of glucose via the pentose phosphate pathway, NADH is predominantly generated from glycolysis and CAC to drive OXPHOS (3). Cancer cell plasticity is necessary to allocate portions of carbon atoms derived from nutrients such as glucose and/or glutamine to various bioenergetic and biosynthetic processes (3). Overall, this illustrates how metabolic flexibility allows cancer cells to adapt to energetic stress, while fueling neoplastic growth and disease progression.

Compared to aerobic glycolysis which has been extensively studied in the context of tumorigenesis and disease dissemination, the importance of OXPHOS in tumor metabolism has been mostly underappreciated despite studies demonstrating that OXPHOS is a pivotal source for ATP production in various types of cancers such as lymphoma, melanoma and glioblastoma (7). Mitochondrial metabolism is not only necessary for sustaining the biosynthetic and bioenergetic activities of the CAC but also maintaining mitochondrial membrane potential which is required for proliferation (8). Although neoplastic cells often display distinct metabolic features defined by their tissue of origin, metabolic plasticity enables cancer cells to adjust their dependence between aerobic glycolysis and OXPHOS during cancer progression, in response to therapeutic insults or alterations in tumor microenvironment (7, 9-11). For example, when primary melanoma lesions (which are characterized as “oxidative tumors”) intravasate into circulation OXPHOS is

suppressed (9, 12). However, OXPHOS is upregulated after colonization of the metastatic site by the tumor cells (i.e., melanoma lung metastases) (9, 12).

CAC anaplerosis fueled by glucose and glutamine provides intermediates and reducing equivalents needed for *de novo* biosynthetic processes (3). However, not all biosynthetic reactions dependent on glutamine can be supported by glucose (3). This is because in addition to being a carbon source, glutamine supplies reduced nitrogen which is required for biosynthesizing non-essential amino acids and nucleotides (3). However, when glutamine is scarce cancer cells have been demonstrated to use branched-chain amino acids (BCAAs) as a nitrogen source (13). Overall, metabolic flexibility and plasticity allow cancer cells to meet their bioenergetic and biosynthetic needs which are vital to tumor growth, proliferation and survival. Recent discoveries indicate that cancer cell metabolism is dependent on various factors including (i) the availability of metabolic fuels, (ii) their metabolic plasticity and flexibility, and (iii) the tumor microenvironmental conditions [e.g., hypoxia, acidosis, etc. (14)].

Activation of oncogenes (e.g., *KRAS* and *PI3KCA*) or inactivation of tumor suppressors such as *PTEN* and *TP53* impact on key metabolic processes including glutamine metabolism, glycolysis and OXPHOS (15-19). As such, oncogenic mutations inadvertently result in metabolic dependencies while concurrently exposing metabolic vulnerabilities that could be targeted by therapeutic interventions. The precise mechanisms of how oncogenic lesions influence cellular metabolomes and how metabolic perturbations are orchestrated with the alterations in gene expression and signaling programs are still largely elusive. Major nutrient-sensing mTOR pathway is frequently dysregulated across a broad spectrum of neoplasia (20, 21). Aberrant mTOR signaling is emerging as a major factor implicated in metabolic rewiring of neoplasia [reviewed in (20-23)]. For example, we showed that mTORC1/4E-BP signaling axis coordinate reprogramming of

mRNA translation, mitochondrial functions and metabolic rewiring via controlling biosynthesis of the components of mitochondrial complexes and NEAAs including serine, asparagine and aspartate, respectively (24, 25). Similar interactions between mRNA translation machinery and metabolic programs are thought to be mediated by the GCN2/eIF2 α /ATF4 signaling axis (26, 27). The GCN2/eIF2 α /ATF4 axis mediates an adaptive stress response to amino acid deprivation by upregulating expression of genes involved in amino acid synthesis and transport (26, 28). Recent studies highlight that the crosstalk between these pathways and AMPK may play a major role in the coordination of metabolic and post-transcriptional gene expression programs in cancer cells thus endowing cancer cells with sufficient flexibility to adapt to a variety of stressor (29-31). These and similar post-transcriptional mechanisms are anticipated to collaborate with transcriptional programs directed by tumor promoting (e.g., c-MYC, HIF-1 α) or tumor suppressive transcription factors (e.g., p53) in shaping malignant metabolomes (32-36) (36). In turn, it is becoming apparent that metabolites including succinate, fumarate and 2-hydroxyglutarate affect gene expression by abrogating epigenetic and epitranscriptomic programs via interfering with histone, DNA and RNA demethylases (37). Collectively, these findings provide initial insights into the complexity of cellular networks that orchestrate signaling, gene expression and metabolic rewiring of neoplasia and indicate that future studies are warranted to dissect precise mechanisms that govern these processes.

Through my primary projects, I sought to capture metabolic perturbations induced by energetic stress in attempt to reveal potential targetable metabolic vulnerabilities in cancer. To this end, we show that glutamine deprivation or impaired mitochondrial complex I or IV activities result in energetic stress which is accompanied by a downregulation of mTORC1 signaling. However, while energetic stress due to complex I or IV dysfunction is associated with

compensatory increase in glycolysis and activation of AMPK, glutamine starvation had no apparent effect on AMPK. Based on this and previous literature (38), it appears that glutamine depletion inhibits mTORC1 activity independently of AMPK. Furthermore, we show that the inactivation of complex IV results in a paradoxical hyperactivation of AKT which we assume promotes survival of cancer cells by upregulating ATP producing processes (i.e., glycolysis), while minimally consuming energy via concomitant suppression of mTORC1 signaling. Consistently, HCT116 with impaired complex IV function exhibited heightened sensitivity to AKT inhibitors, while they were less sensitive to mTOR inhibitors, as compared to control cells with undisrupted complex IV function. Although the mechanism of activation of AKT accompanied by the paradoxical suppression of mTORC1 remains elusive, it is plausible that associated activation of AMPK dampens the AKT-dependent activation of mTORC1. Indeed, repeated attempts to abrogate expression or deplete AMPK subunits in HCT116 cells with defective complex IV resulted in massive cell death. This suggests that AMPK activity is essential for rewiring signaling pathways and survival of cells with impaired complex IV function.

In addition to rewiring of signaling pathways, we also observed that different types of stressors lead to distinct metabolic rewiring. For example, our data in conjunction with previous studies (39-45) revealed that rewiring of glutamine metabolism plays a major role in adaptation of cancer cells to aberrant ETC function. Consistent with findings showing that reductive glutamine metabolism is a function of the intracellular levels of α -ketoglutarate to citrate (46), we observed that impeding mitochondrial complex I or IV activity increases reductive glutamine metabolism. Although stable isotope tracer analysis with ^{13}C -glutamine is required to delineate how intracellular carbon flux is altered in response to the different energetic stressors, these findings

suggest that reductive glutamine metabolism may play a major role in adaptation of cancer cells to energy crisis caused by aberrant mitochondrial function.

4.2 Pharmacological strategies to target metabolic vulnerabilities in cancer

Metabolic reprogramming is thought to play a central role in tumorigenesis and tumor progression, whereby specific metabolic programs are thought to distinguish malignant from normal cells (11). Hence, targeting cancer-specific metabolic vulnerabilities should provide a sufficient therapeutic window to selectively eradicate neoplastic cells while exerting minimal toxicity in normal cells and tissues.

In addition to being the most abundant amino acid in the plasma, glutamine is metabolized by malignant cells at rates that appear to be higher than any other amino acid (10-100 times more than other amino acids) (47, 48). Although glutamine is often regarded as a nonessential amino acid, many types of cancers are dependent on extracellular glutamine, such as non-small cell lung cancer (NSCLC) and glioblastoma (49, 50). Furthermore, there is ample evidence suggesting that during stress, the need for glutamine seemingly exceeds the biosynthetic capacity of cells to provide sufficient amounts of *de novo* synthesized glutamine (51). Hence, glutamine is more accurately classified as a “conditionally essential amino acid” (51). Accordingly, the results of this thesis demonstrate the critical dependence of CRC cell lines on glutamine. For example, glutamine deprivation leads to reduction in mTORC1 activity and global protein synthesis which is paralleled with dramatically reduced proliferation of CRC cells. Although transformed colon cells showed no proliferative disadvantage compared to their parental non-transformed cells at any of the tested glutamine concentrations, future studies are required to elucidate the differences in glutamine metabolism between normal colon cells and CRC cells. In addition, our results in combination with studies from other groups (39-45) suggest that reductive glutamine metabolism may

constitute a targetable metabolic vulnerability in cancer cells that exhibit mitochondrial dysfunction. Based on this, I will discuss current and/or potential strategies to target glutamine metabolism in the context of my primary studies.

4.2.1 Pharmacological inhibition of glutamine uptake

The glutamine analog, γ -l-glutamyl-p-nitroanilide (GPNA), has been shown to have antitumor effects in glutamine-dependent NSCLC and triple-negative basal-like breast cancer (TNBC) cell lines (52, 53). Pharmacological treatment of tumor cells with GPNA not only reduced glutamine uptake, but also inhibited mTOR signaling and cell proliferation (52, 53). The effects of GPNA were proposed to be mediated through the inhibition of the glutamine transporter, SLC1A5 (52, 53). Moreover, a more potent SLC1A5 inhibitor 2-amino-4-bis(aryloxybenzyl)aminobutanoic acid (AABA; also referred to as V-9302) was demonstrated to reduce the viability of several cancer cell lines including breast, lung and CRC and attenuated the growth of corresponding xenografts tumor in mice (54). Consistent with the findings on the effects of glutamine deprivation on CRC cell lines reported in this thesis, V-9302 exposure decrease mTOR activity and diminish cancer cell proliferation (54).

Interestingly, subsequent studies showed that GPNA and V-9302 may exert their antineoplastic effects independently of SLC1A5 and that they may be reliant on different glutamine (SLC38A2) and leucine transporters (SLC7A5) (55-57). Given that GPNA is a substrate of a key enzyme in glutathione metabolism, γ -glutamyltransferase (GGT), additional reports suggested that the antitumor properties of GPNA in lung cancer were mediated by GGT (58). Specifically, it was proposed that GGT-catalyzes hydrolysis of GPNA into cytotoxic p-nitroaniline thus inducing apoptosis without exerting a major effect on glutamine uptake in lung cancer cells (58). Taken together, these data suggest that the anticancer activities of GPNA may

be context-dependent and more pleiotropic than previously thought. To this end, the antineoplastic effects of GPNA may be mediated by a combination of reduction in glutamine uptake and induction of oxidative stress. Deciphering of the molecular mechanisms of GPNA and V-9302 is further complicated by the intricate metabolic network involved in amino acid homeostasis. This is due to the existence of multiple amino acid transporters that import and export one or more amino acids (59). It was initially suggested that SLC7A5 is functionally coupled with SLC1A5 wherein glutamine imported by SLC1A5 is subsequently exchanged for essential amino acids (EAAs) (e.g., leucine) by SLC7A5 (60, 61). According to this model, leucine and other branched amino acids were thought to mediate the effects of glutamine on mTOR activation (60, 61). However, the pharmacological inhibition of SLC7A5 and SLC38A2 with V-9032, and abrogation of SLC1A5 led to a recently revised model of amino acid homeostasis (62). Herein it is thought that glutamine along with other NEAAs are predominantly accumulated in the cytosol by SLC38A1 which are then rapidly exchanged for other extracellular neutral NEAAs via SLC7A5 and SLC1A5 (62). The inhibition of SLC7A5 results in a rescue response thereby upregulating SLC38A2 and SLC1A4 (62, 63). This therefore suggests that SLC38A2 and SLC1A4 are functionally redundant (63). This model is corroborated by the findings showing that the knockout of SLC1A5 in liver, colon and lung adenocarcinoma cell lines failed to repress mTORC1 signaling or proliferation because of amino acid transporter redundancies (64, 65). However, a recent study using human head and neck squamous cell carcinoma cell cultures and xenografts showed that abrogating SLC1A5 alone was sufficient to increase oxidative stress, suppress mTORC1 signaling and attenuate tumor growth (64). Collectively, with new emerging findings demonstrating redundancies in the various amino acid transporters, the mechanisms of antineoplastic actions of glutamine transporter inhibitors remain obscure.

4.2.2 Pharmacological inhibition of glutaminolysis

Intracellular glutamine is converted to glutamate by glutaminases (GLS). Glutamate is an important precursor of glutathione which protects cells from oxidative stress (66). Glutamate can then be deamidated either through (GLUD1) or transaminases such as (GOT or GPT) to produce α -ketoglutarate which is incorporated into the citric acid cycle (CAC) (67, 68). Other than its incorporation into the CAC for the subsequent production of ATP, glutamine-derived α -ketoglutarate can be used for *de novo* fatty acid synthesis (indirectly through citrate), aspartate and nucleotide synthesis (ultimately through oxaloacetate) (67, 68).

Over the last decade, small-molecule allosteric inhibitors of GLS have been developed as anticancer therapies. These include compound 968, BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide), and CB-839 (69). GLS inhibitors differ in enzyme specificity and potency. While CB-839 and BPTES have the same molecular scaffold and are specific for GLS1, compound 968 is a pan-glutaminase inhibitor with moderate selectivity for GLS2 (69-72). Notably, compound 968 was demonstrated to suppress the proliferation of BPTES/CB-839-resistant luminal breast cancer cell lines (72). The increased sensitivity of luminal A breast cancer cell lines to compound 968 was reported to be dependent on the upregulation and preference of GLS2 over GLS1 for glutaminolysis (72). The antiproliferative effect of CB-839 in TNBCs with upregulated GLS1 was associated with elevated glutamine uptake, decreased glutamine catabolism and diminished OXPHOS (73). Consistent with this, GLS inhibition of TNBC suppressed mTORC1 activity and induced ATF4 expression (74). Importantly, although the inhibition of glutaminolysis increases intracellular levels of glutamine while glutamine deprivation or treatment with glutamine transporter inhibitors deplete glutamine levels, abrogating any of the two reduces mTORC1 signaling and decreases cell proliferation (54, 74). Unlike GLS1, the function of GLS2

remains unclear and appears to be context-dependent. For instance, while *GLS2* is transcriptionally upregulated by p53 (75) and has been considered a tumor suppressor in liver cancer and glioblastoma, it was also shown to be regulated by N-MYC in neuroblastoma (76, 77). These apparently conflicting roles of *GLS2* have hampered efforts in developing selective inhibitors against it.

In pre-clinical studies, BPTES is the most frequently used highly selective glutaminase inhibitor (78). However, it is less potent than its analog, CB-839, and its poor aqueous solubility restricts its application in *in vivo* models (78). To this end, CB-839 has been the only one tested in numerous clinical trials for many types of cancers including non-Hodgkin's lymphoma (NCT0207188), acute myeloid leukemia (NCT02071927), clear cell renal carcinoma (ccRCC) (NCT02771626), CRC (NCT02861300), TNBC (NCT03057600) and lung cancer (NCT02771626, NCT03831932, NCT03831932, NCT04250545). Although CB-839 used as a single-agent anticancer therapy profoundly inhibits glutaminolysis and arrest tumor cell growth in preclinical models, metabolic plasticity of cancer cells appears to significantly limit their efficacy (79) (80, 81). To this end, instead of relying solely on glutamine and specific glutamine transporters/glutaminolytic enzymes, cancer cells may switch to other metabolic routes that can be used as surrogate pathways to overcome therapeutic insults targeting glutamine metabolism (7). This in combination with a relatively modest success of therapeutic approaches targeting glutamine metabolism suggest that CB-839 and/or glutamine transporter inhibitors are unlikely to be effective as monotherapies.

4.2.3 Drug combinations with glutamine metabolism inhibitors

The mTOR signaling pathway regulates metabolism while integrating extracellular and intracellular signals emerging from the environment, nutrient availability and energetic status (82).

Importantly, mTOR inhibitors decrease energy consumption due to inhibition of global mRNA translation that is associated with reduced energy production (25). In this manner, mTOR inhibitors cause metabolic dormancy in cancer cells which explains their cytostatic effects observed in the clinic (25).

Strikingly, it was observed that CB-839 synergizes with the mTOR inhibitors AZD8055 and PP242 to reduce the proliferation of TNBC and glioblastoma cells (74, 83). Accordingly, the resistance to mTOR inhibitors in glioblastoma has been attributed to compensatory glutamine metabolism wherein GLS expression was upregulated by PP242 and led to concomitant increases in the intracellular levels of glutamate and α -ketoglutarate (83). Although these studies illustrated synergy between glutaminase inhibitors and active site mTOR inhibitors, they fail to address whether the synergistic effects are predicated on inhibiting mTORC1, mTORC2 or both. More recently it was demonstrated that glutamine deprivation, GLS knockdown, or exposure to CB-839 increased ROS levels, suppressed nucleotide synthesis and induced replication stress in a subset of ovarian cancer cells (84). The cells were more dependent on poly (ADP-ribose) polymerase (PARP) DNA repair and thus, sensitive to the PARP inhibitor, Olaparib (84). Moreover, DNA damage stimulated after treatment with certain cytotoxic drugs leads to abnormally high levels of PARP-mediated ribosylation, which is an energy costly process (85, 86). Taken together, these findings suggest that targeting multiple cellular processes increases the efficacy of drugs targeting glutamine metabolism which is likely due to the ability of these drug combinations to limit metabolic plasticity of neoplasia (84). Notwithstanding these promising preclinical results, future clinical trials are required to establish the efficacies of drug combinations targeting glutamine metabolism with other therapeutic modalities in cancer patients.

4.2.4 Therapeutics targeting OXPHOS in cancer

It has been shown that tumor cells devoid of mitochondrial DNA (mtDNA) lack tumorigenic potential, and form tumors only after acquiring healthy mtDNA from the host stromal cells (87). This was paralleled by restoration of respiratory activities in the tumor cells (87). This and similar studies illustrate the pivotal role of mitochondria in tumorigenesis and expose mitochondria as potential targets for anticancer drugs. The work in this thesis demonstrated that the inactivation of complex I or IV led to increased glucose uptake, glycolysis and reductive glutamine metabolism to compensate for the metabolic defects caused by mitochondrial (16, 24, 42, 88). This suggests that impairment in mitochondrial functions and consequent activation of compensatory metabolic pathways may constitute druggable metabolic vulnerabilities.

The anticancer activities of biguanides like metformin have been reported in numerous studies [reviewed in (89)]. Notably, results presented in this thesis confirmed previous findings that the antiproliferative effects of biguanides are more pronounced in malignant cells as compared to non-transformed cells (24), which together with proven safety of biguanides in the treatment of type 2 diabetes suggests sufficient therapeutic window (90). The mechanisms of the anti-neoplastic action of metformin and other biguanides is still unclear. For instance, it remains unclear whether biguanides act directly on the mitochondria by inhibiting complex I of the ETC because cancer cells deficient in mitochondrial DNA (rho0 cells) are sensitive to metformin (91). Furthermore, tumor cells with complex I mutations are more susceptible to metformin compared to cancer cells without the mutations (92). These reports oppose conclusions of previous studies showing that metformin causes time-dependent inhibition of complex I in isolated mitochondria (93) and that its anticancer effects are attenuated by the expression of *Saccharomyces cerevisiae* NADH dehydrogenase (NDI1) that is biguanide-insensitive (94). In addition, biguanides have been

proposed to exert their antiproliferative effects at clinically relevant concentrations by inducing oxidative stress through the inhibition of mitochondrial glycerol phosphate dehydrogenase (GPD2) (95, 96). At the therapeutically relevant drug concentrations (1-5 μ M) the reduced proliferation was not accompanied by suppression of mTOR signaling or diminished mitochondrial complex I activity (95, 96). These studies indicate that further research is required to determine the precise mechanism of the anti-neoplastic effects of biguanides.

Hyperinsulinemia is a risk factor for several cancer types and is associated with adverse prognosis in breast, colon and pancreatic cancer patients (97-99). This is consistent with epidemiological studies demonstrating that type-2 diabetes mellitus and obesity are linked with an increased risk for developing breast, colon, liver, pancreas and kidney cancer (100, 101). Insulin can promote tumorigenesis through its action on insulin/insulin-like growth factor receptors (IR/IGF1R) which activate downstream signaling pathways (commonly implicated in neoplasia) such as PI3K/AKT/mTOR and RAS/RAF/MEK/ERK (102). Hyperinsulinemia can indirectly exert mitogenic effects by elevating the levels of insulin-like growth factors (IGFs), sex hormones (i.e., androgens and estrogens) and increasing the expression of IGF1R (102-104). Mechanistically, metformin acts as an antidiabetic agent by reducing liver gluconeogenesis and increasing glucose uptake in skeletal muscles, thus decreasing blood glucose levels, improving insulin sensitivity and lowering insulin levels in diabetic patients (105). Consequently, the anticancer properties of metformin may be due to its systemic effects on glucose and insulin levels. As such, it is thought that the systemic effects of metformin may underly its anticancer properties especially in cancer patients with hyperinsulinemia. Nonetheless, the results of a large phase III randomized, placebo-controlled, double-blind clinical trial examining the effects of metformin versus placebo in over 3500 non-diabetic women with early-stage breast cancer position

biguanides as potential anticancer agents (106). Specifically, the administration of metformin led to weight loss, reduced circulating glucose and insulin levels, increased apoptosis and decreased proliferation of tumor cells (106, 107).

Following the promising results from preclinical studies, there are close to 100 ongoing clinical trials evaluating the anticancer effects of biguanides either as a single agent or in combination with other interventions (108). However, the results of the completed trials have been mostly disappointing due to the incomplete understanding of the molecular mechanism of biguanides. Another plausible reason for the failures in clinical studies evaluating the efficacy of metformin as an antitumor agent may be because many of these studies assume that the commonly prescribed dose of metformin for type-2 diabetes mellitus is sufficient to elicit its anticancer effects. Even though phenformin is more potent and bioavailable due to its hydrophobicity and permeability, metformin is the preferred choice of biguanide in over 90% of the clinical trials (109, 110). The primary reason for the lopsided application in clinical trials is due to phenformin-related toxicities such as lactic acidosis (111). This is in contrast with metformin which is well tolerated and largely nontoxic (112). Despite findings from retrospective epidemiological studies demonstrating that type-2 diabetes mellitus patients treated with metformin had reduced incidence rates of hepatocellular carcinoma, pancreatic cancer, breast cancer or colorectal adenoma, the bioavailability of metformin in treating cancer patients is not robust (113-118). In essence, preventing cancer does not equate to treating cancer. A predominant reason why the anticancer properties of metformin observed in *in vitro* experimental models are not apparent in clinical trials is because of the difference in the effective concentration of biguanides tested (95). In clinical trials, oral intake of biguanides results in plasma levels which are about 10-100 folds less than those used in preclinical studies (95). Preclinical studies employ metformin in the supra-

pharmacological levels (i.e., millimolar range) (95). Nonetheless, consistent efforts are being made to improve the potency of biguanides (119).

Developing small molecule inhibitors selectively targeting OXPHOS in cancer cells has remained a challenge because most conventional inhibitors of cellular respiration (e.g., rotenone) are also toxic to normal cells, and thus offer do not provide a suitable therapeutic window (120) (115). Novel potent OXPHOS inhibitors such as BAY 87-2243 and its analog IACS-010759 have suitable pharmacokinetic properties (121, 122). They have also been evaluated in clinical studies for acute myeloid leukemia, lymphoma and advanced solid tumors. While the clinical trials administering BAY 87-2243 was discontinued because of toxicity, IACS-010759 was demonstrated to be well-tolerated (123).

4.2.5 Drug combinations with biguanides

Given that cancer cells often undergo metabolic rewiring in response to biguanide-induced energy stress, targeting the compensatory metabolic adaptations through biguanide drug combination treatments is a potential pharmaceutical strategy that has been studied in various preclinical models (24, 124). Moreover, drug combinations that exhibit synergistic antineoplastic effects could mitigate the suboptimal pharmacokinetic issues associated with metformin by reducing its effective concentration.

One of the earliest proposed combinatorial biguanide treatments involved the use of glycolytic inhibitors such as 2-deoxyglucose (2-DG) to block compensatory increase in glycolytic ATP production and NADH oxidation (125, 126). Biguanides have demonstrated synergy with 2-DG to reduce cell viability and proliferation in preclinical models of prostate cancer, breast cancer and chemoresistant lung cancer stem cells (125, 127, 128). However, many clinical trials with 2-DG have been terminated due to lack of clinical efficacy and serious side effects (126).

Kinase inhibitors (KIs) such as erlotinib (EGFR inhibitor), gefitinib (EGFR inhibitor), trastuzumab (HER2 inhibitor), lapatinib (EGFR/HER2 dual inhibitor) and imatinib (BCR-ABL inhibitor) suppress glucose metabolism across various tumor cell models harboring mutations in the corresponding kinases (129-132). However, metabolic flexibility occasionally limits the therapeutic efficacy of these drugs. An example of this is vemurafenib, a BRAF inhibitor commonly used to treat melanoma, which also represses glucose metabolism (24, 133, 134). BRAF inhibitors provide only short-term benefits followed by the development of drug resistance which is accompanied with increased dependence on OXPHOS and glutamine metabolism (133, 135). In one of the studies highlighted in the thesis, we demonstrated that KIs targeting different oncogenic mutations synergize with phenformin across broad spectrum of cancer cell lines (24). Specifically, KIs antagonized the metabolic adaptive pathways activated by phenformin treatments (24). KIs diminished phenformin-induced increases in glycolysis and reductive glutamine metabolism (24). Furthermore, this indicates that the drug combination may be effective against genetic and metabolic heterogeneous tumors. In fact, phenformin is currently being tested in a phase I clinical trial to determine the optimal dosage for combination treatments with KIs (Dabrafenib and Trametinib) in patients with metastatic BRAF mutated melanoma (NCT03026517). Nonetheless, we demonstrated that mTORC1- and/or HIF1 α -dependent alterations in NEAA biosynthesis and glutamine metabolism, respectively provide cancer cells with metabolic flexibility that allows them to overcome KI and biguanide combinations (24). These data suggest that the applicability of KI/biguanide combinations in the clinic may be limited.

Curiously, preclinical studies have shown that BPTES bolsters the antiproliferative effects of phenformin in LKB1-deficient NSCLC (136). The loss of LKB1 impeded AMPK activation and sensitized tumor cells to the energy stress induced by the phenformin/BPTES drug

combination treatment (136). Abrogation of LKB1 impaired the metabolic flexibility of NSCLC cells and thus, rendered them susceptible to energetic crisis (136). Conceivably, the drug combination treatments impinge on essential metabolic and cellular processes regulated by mTOR signaling. Taken together, there seem to be promise in OXPHOS inhibitors/glutaminase inhibitors drug combination interventions particularly for tumors with limited metabolic flexibility and greatly dependent on glutamine metabolism and OXPHOS. Therefore, further research is required to identify the mechanisms that predict efficacy of combinatorial treatments incorporating small molecule inhibitors of OXPHOS. These studies should provide molecular basis to establish surrogate biomarkers that will facilitate stratification of the patients to identify potential responders to combinatorial approaches using OXPHOS inhibitors and other therapeutics.

4.3 CONCLUSION

Metabolic plasticity is an emerging feature of cancer cells that plays a prominent role in tumor dissemination and therapeutic responses. This thesis highlights previously unappreciated mechanisms underpinning metabolic flexibility of cancer cells in the context of energy stress caused by mitochondrial dysfunction due to abrogation of mitochondrial complex I or IV and/or nutrient deprivation. These findings provide initial insights into cellular networks underlying the metabolic adaptation of cancer cells to energy stress while exposing potential metabolic vulnerabilities. To this end, results reported in this thesis in concert with similar efforts in our laboratory and across the globe are likely to critically improve understanding of the molecular underpinnings of metabolic flexibility of cancer cells. In long-term, these efforts are anticipated to provide molecular bases for more effective approaches to identify and target metabolic vulnerabilities of neoplasia in the clinic.

4.4 LIMITATIONS AND FUTURE PERSPECTIVES

Notably, studies in chapters 2 and 3 were carried out in cell culture models that may not reflect complexity of organismal physiology and its impact on metabolic rewiring of neoplasia. Importantly, cell culture models are thought to insufficiently mimic rapid changes in nutrients, oxygen and hormonal milieu of cancer cells that occur *in vivo*. Tumor microenvironment affects metabolism of cancer cells. Reciprocally, cancer cells alter the metabolic composition of the extracellular milieu around them which often facilitate their progression (3). Additionally, metabolites produced by the host microbiota may promote or impede tumor initiation and progression (137). Taken together, studying the metabolic alterations of cancer cells warrants the use of *in vivo* models to fully capture the complexity of cancer metabolism. To this end, future studies using appropriate animal models are required to establish whether phenomena observed in chapters 2 and 3 also occur *in vivo*. Moreover, in chapter 3, we only monitored steady-state metabolite levels which are not indicative of flux through key metabolic pathways. This limitation will be addressed in future by performing stable isotope tracing analysis, which will allow us to monitor flux through key metabolic pathways upon abrogation of mitochondrial function and/or glutamine deprivation in CRC vs. non-transformed colon epithelial cells. Furthermore, the incorporation of systems biology approaches is required to elucidate the changes to gene expression and signaling pathways which underlie the metabolic adaptability of cancer cells to energy stress. These approaches will help us establish deeper insights into the mechanism underlying metabolic plasticity of cancer cells, which was an overarching objective of this thesis.

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APPENDIX - OTHER WORKS

A.1 Contributions to understanding of the molecular mechanisms of metabolic dysregulation in cancer

In addition to contributing to improving the knowledge of the molecular underpinnings of cancer metabolism through my own projects (Chapters 2 and 3), I was extensively involved in collaborative work aiming to dissect the mechanisms of metabolic reprogramming in both normal and cancer cells. To this end, I provided critical experimental and intellectual inputs in studies wherein we set out to unravel the mechanisms of metabolic plasticity of cancer cells (1-3). Moreover, we exploited potential avenues to target metabolic vulnerabilities of cancer cells and define mechanisms underlying the action of drugs targeting metabolic rewiring in neoplasia (3, 4). Collectively, these studies provide previously unappreciated insights into the mechanisms of metabolic plasticity of neoplasia. As mentioned in the introduction section, metabolic plasticity is a phenomenon whereby cancer cells instead of being addicted to one or few metabolic pathways can utilize a plethora of metabolites and/or nutrients via a variety of metabolic routes, while also being able to direct different metabolites and/or nutrients through the same metabolic pathway (5, 6). Metabolic plasticity thus allows cancer cells to rapidly alternate between metabolic pathways in order to adapt to a variety of stressors including therapeutic insults (5). In addition, our studies provided hitherto unappreciated mechanistic insights into anti-neoplastic action of the combinations of inhibitors of oncogenic kinases and biguanides as well as the sodium-glucose cotransporter-2 (SGLT2) inhibitors (3, 4). In large part, these results were summarized in our review article (7), while the major findings of each of these studies are outlined below.

A.1.1 Identification of hydride ion transfer complex that allows cells to overcome senescence and drives tumorigenesis.

Mitochondrial dysfunction, typically accompanied by decreased ATP levels and NAD⁺/NADH ratio, and increased reactive oxygen species (ROS) production is thought to play a major role in senescence (8, 9). Senescence constitutes a major tumor suppressive mechanism (10). In addition to its well established function as a transcription factor (11), signal transducer and activator of transcription 3 (STAT3) has also been reported to function in the mitochondria (12). Indeed, it has been shown that the abrogation of STAT3 function results in elevated ROS production and dysfunctional mitochondria in hematopoietic stem cells and hematopoietic progenitor cells (13). This leads to premature aging of blood cells (13). Moreover, it was suggested that oncogenic RAS-induced malignant transformation of primary cells requires STAT3-dependent regulation of mitochondrial functions (14). Altogether, these prior findings suggest a potential role of STAT3 in supporting mitochondrial activity in order to bypass senescence and drive oncogenesis.

My work with Igelmann et al. employed STAT3-deficiency and other models of cellular senescence to highlight the mechanisms that allow cells to reprogram their metabolism and adapt to mitochondrial dysfunction-associated senescence (1). Specifically, mitochondrial dysfunction-associated senescence was induced by depleting STAT3 in normal human fibroblasts (IMR90) (1). As expected, STAT3 depletion resulted in a reduction in cell proliferation and increased senescence-associated- β -galactosidase (SA- β -Gal) activity (1). This was accompanied by dramatic changes in the appearance of mitochondria including the disruption of cristae (1). STAT3 depletion not only disrupted the structural properties of the mitochondria but also suppressed their function (1). This was illustrated by elevated ROS levels, decreased NAD⁺/NADH ratio and reduced

oxygen consumption relative to control scrambled shRNA infected cells (1). We next observed that abrogating *TP53* or *RB1* tumor suppressors, which enables IMR90 cells to overcome STAT3 depletion-induced senescence resulted in normalization of NAD⁺/NADH ratio (1). This led us to hypothesize that a decrease in NAD⁺/NADH ratio may play a major role in metabolic perturbations that are related to induction of senescence. To test this tenet, we restored the NAD⁺/NADH ratio in STAT3-depleted cells by applying exogenous electron acceptor duroquinone or by expressing *Lactobacillus brevis* (LbNOX) or yeast (NDI1) NADH-oxidases. Strikingly, these interventions allowed STAT3 depleted cells to circumvent senescence (1). Collectively, these findings indicate that decrease in NAD⁺/NADH ratio plays a major role in the induction of senescence.

Notably, reduced expression of STAT3 increases p53 levels thereby inducing senescence (1, 15). As noted above, we showed that decreasing the expression of p53 in cells depleted of STAT3 circumvented senescence and restored the NAD⁺/NADH ratio (1). These findings suggest that loss of p53 facilitates senescence bypass by reprogramming redox metabolism to elevate NAD⁺/NADH ratios in STAT3-depleted cells. To further investigate metabolic perturbations in this model (Fig. A.1.1A), I carried out stable isotope tracer analysis (SITA) using fully labelled ¹³C-glucose in control or *TP53*-deficient STAT3-depleted IMR90 cells [Fig. A.1.1B-D; from Fig. 1J-O in (1)]. This revealed that p53 loss-induced bypass of the senescence in STAT3-depleted IMR90 cells is associated with increased pyruvate carboxylase (PC), malic enzyme 1 (ME1) and malate dehydrogenase 1 (MDH1) activity which was evidenced by the increase in ¹³C-pyruvate, ¹³C-malate and ¹³C-aspartate levels. We next sought to establish the mechanism of increased PC, ME1 and MDH1 activity in response to abrogation of p53 function. To this end, our bioinformatics analysis revealed that *PC*, *ME1* and *MDH1* are transcriptionally repressed by p53 while these

enzymes were downregulated in senescent cells (1). Moreover, we made a surprising observation whereby in cells that bypassed senescence, a proportion of PC was localized in the cytoplasm, which was in stark contrast with almost exclusively mitochondrial localization of this enzyme in senescent or control cells (1). Furthermore, a series of biophysical approaches in conjunction with immunofluorescence, immunoprecipitation and proximity ligation assays revealed that cytoplasmic PC, forms a complex with MDH1 and ME1 (1). Of note, MDH1 and ME1 are cytoplasmic enzymes (16, 17). Thus far, we concluded that the bypass of STAT3-depletion induced senescence is underpinned by formation of PC/ME1/MDH1 complexes. I next employed SITA analysis to further characterize metabolic activity of the latter complex, which revealed that PC supplies MDH1 with oxaloacetate in order to generate malate and oxidize NADH [Fig. A.1.1E-H; from Fig. 4J-K and Fig. S6I-L in (1)]. Malate is then subsequently converted back to pyruvate by ME1 which results in generation of NADPH (Fig. A.1.1A). Based on its ability to transfer hydride ions from NADH to NADP⁺ we named PC/ME1/MDH1 complexes a hydride ion transfer complex (HTC). Importantly, HTC recycles NAD⁺ which is a major cofactor in metabolic pathways known to fuel cancer growth [e.g., glycolysis and aspartate synthesis (18, 19)], while producing NADPH that drives lipid synthesis and provides ROS protection via stimulating regeneration of reduced glutathione (1). Indeed, overexpression of HTC enzymes in STAT3-depleted IMR90 cells averted senescence while restoring NAD⁺/NADH ratio, increasing NADPH levels and decreasing ROS (1). Based on this, we predicted that HTC may increase metabolic plasticity and thus play a major role in tumorigenesis. To test this, we overexpressed HTC enzymes in combination with oncogenic RAS and showed that this is sufficient to suppress senescence in mouse embryonic fibroblasts (MEF), drive tumorigenesis and support tumor growth in mice (1).

These experiments revealed that HTC is not only sufficient to overcome oncogene-induced senescence, but that it can also induce malignant transformation and drive neoplastic growth.

The importance of each enzyme within the HTC is highlighted by the findings that depletion of any of the HTC components leads to senescence. Namely, reducing the expression of PC, MDH1 or ME1 was accompanied by decreased NAD⁺/NADH in both IMR90 STAT3/p53 depleted fibroblasts or prostate cancer PC-3 cells (1). I performed SITA analysis of PC/STAT3/p53 depleted IMR90 cells supplemented with 3,4-¹³C-glucose (Fig. A.1.1I-J; from Fig. 1P-Q in (1)). As expected, PC depletion reduced the carbon flux into the HTC cycle as illustrated by the decrease in ¹³C-malate and ¹³C-aspartate levels (Fig. A.1.1J). It is also worthwhile to note that in addition to STAT3-depletion and oncogene-induced senescence, overexpression of HTC enzymes in normal human fibroblasts delayed replicative senescence while increasing the NAD⁺/NADH ratio (1). Altogether, these findings demonstrate that HTC plays a central role in overcoming senescence and driving tumorigenesis and tumor growth.

Importantly, the role of HTC in overcoming mitochondrial dysfunction appears not to be limited only to the senescence. For instance, overexpression of HTC enzymes diminished antiproliferative effects of mitochondrial complex I inhibitor piericidin A in human fibroblast (1). Furthermore, HTC assembly is induced by hypoxia (1). This is consistent with previous observations that hypoxia averts senescence in fibroblasts expressing oncogenic RAS (1, 20). Overall, these findings suggest that HTC plays a major role in adaptation to repression of mitochondrial functions.

In conclusion, these findings highlight the significance of HTC in maintaining cellular redox homeostasis under conditions of mitochondrial dysfunction. Specifically, we identified a cytosolic HTC complex that is formed by PC, ME1 and MDH1 and plays a paramount role in the

rewiring of NAD⁺ metabolism which allows cells to overcome senescence and become cancerous.

(1). Considering that HTC assembly is repressed by the tumor suppressors including *TP53*, *RB1* and *PTEN* (1), our findings further support the growing body of work that metabolic rewiring plays a major role in tumorigenesis caused by the loss of tumor suppressors (21-26). Accordingly, we observed increased expression and colocalization of HTC enzymes in prostate cancer mouse models and patient specimens. Importantly, by increasing the levels of electron acceptors (i.e., NAD⁺) while providing reducing equivalents (i.e., NADPH), HTC increases metabolic flexibility of cancer cells. The central role of HTC in metabolic plasticity is further illustrated by its ability to induce rewiring of metabolome thus allowing the cells to adapt to complex I inhibition and hypoxia. Significantly, HTC catalyzes the metabolic cycle with no net-carbon loss. This is important inasmuch as it is thus likely that HTC does not affect cellular pools of pyruvate, oxaloacetate and malate which are important precursors of a number of biosynthetic processes that are crucial for proliferation and survival of cancer cells (27-29). Although this study highlights cytosolic PC, MDH1 and ME1 as key components of the HTC complex, it is plausible that HTC comprises additional interacting partners. Future studies are presently conducted to further confirm the role of HTC in establishing metabolic plasticity of cancer cells in particular in the context of metastatic spread of the disease and drug resistance.

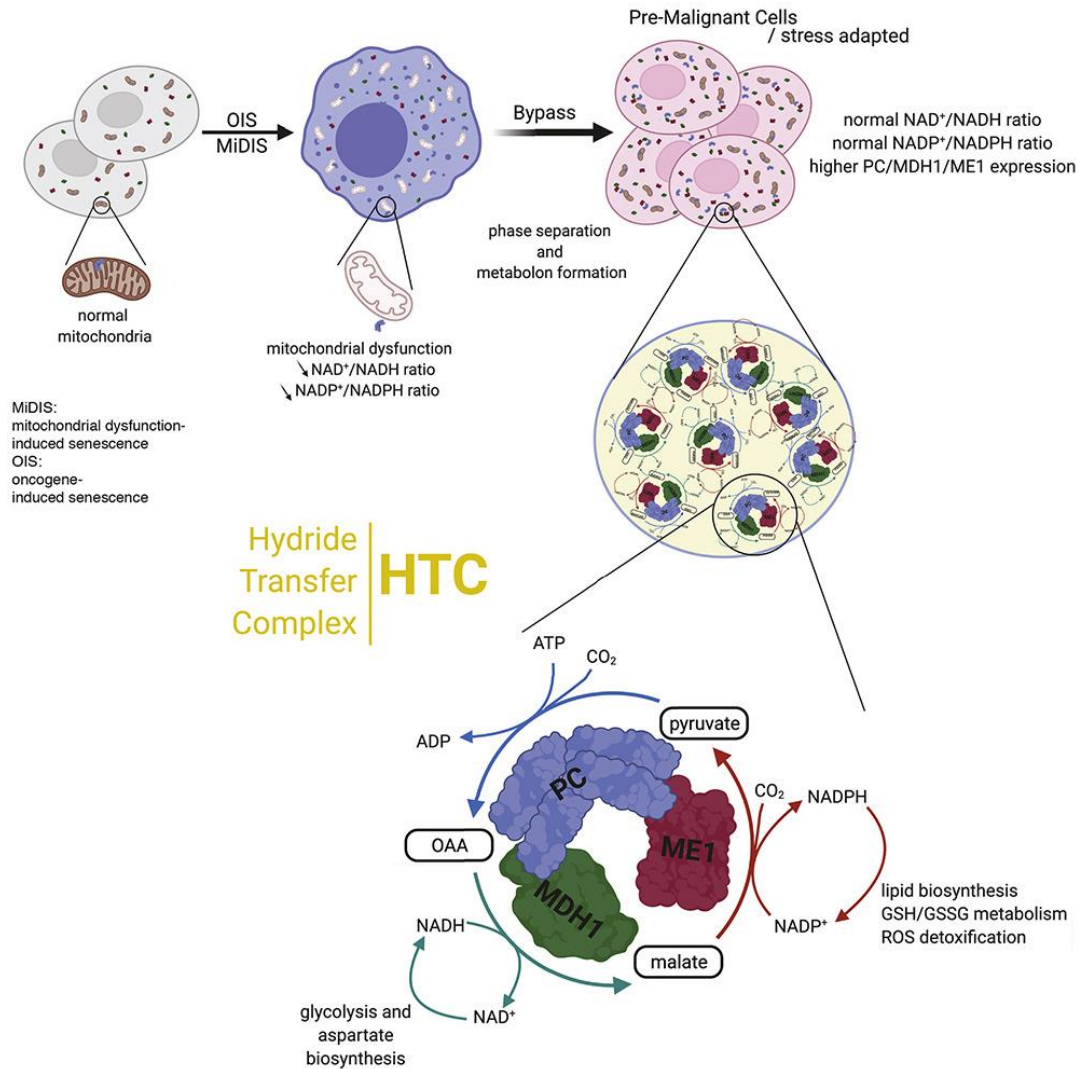


Fig. A.1.1A Schematic of the multi-enzymatic complex (hydride transfer complex) underlying metabolic reprogramming and senescence bypass. To bypass oncogene-induced senescence or mitochondrial dysfunction-induced senescence, the HTC which comprises of PC, MDH1 and ME1, catalyzes a metabolic cycle resulting in the regeneration of NAD^+ and $NADPH$. NAD^+ and $NADPH$ are key cofactors for essential metabolic processes including glycolysis and ROS detoxification. Figure is from (1).

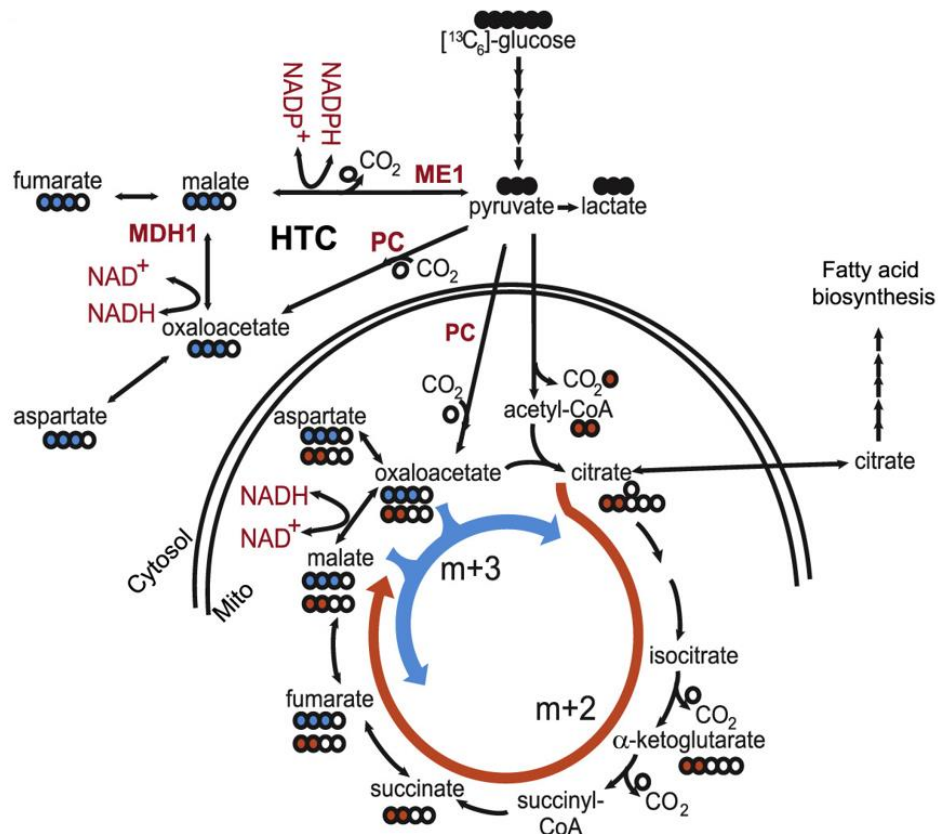


Fig. A.1.1B Model of NAD⁺ and NADPH regeneration by MDH1, ME1 and PC and schematic of ¹³C metabolite labeling patterns after incubating cells with ¹³C₆-glucose. ¹³C are denoted by filled circles. Red cycle is the forward direction of the citric acid cycle giving (m+2) intermediates (Red-filled circles). Blue cycle begins with pyruvate carboxylase (PC) and is characterized by (m+3) intermediates (Blue-filled circles). HTC, hydride transfer complex; mito, mitochondria. Figure is from (1).

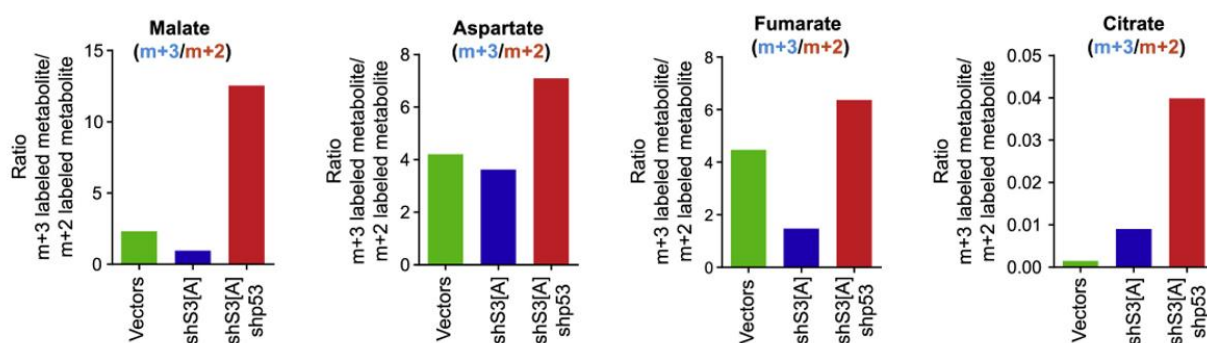


Fig. A.1.1C Reduced expression of p53 reprograms metabolism in STAT3-depleted IMR90 cells. Ratio of (m+3)/(m+2) isotopomers for the aforementioned metabolites in IMR90 cells expressing control vectors, a shRNA against STAT3 (shS3[A]) alone or in combination with an shRNA against p53 (shp53). The ratios were calculated using SITA data after 10 min of $^{13}\text{C}_6$ -glucose flux. Figure is from (1).

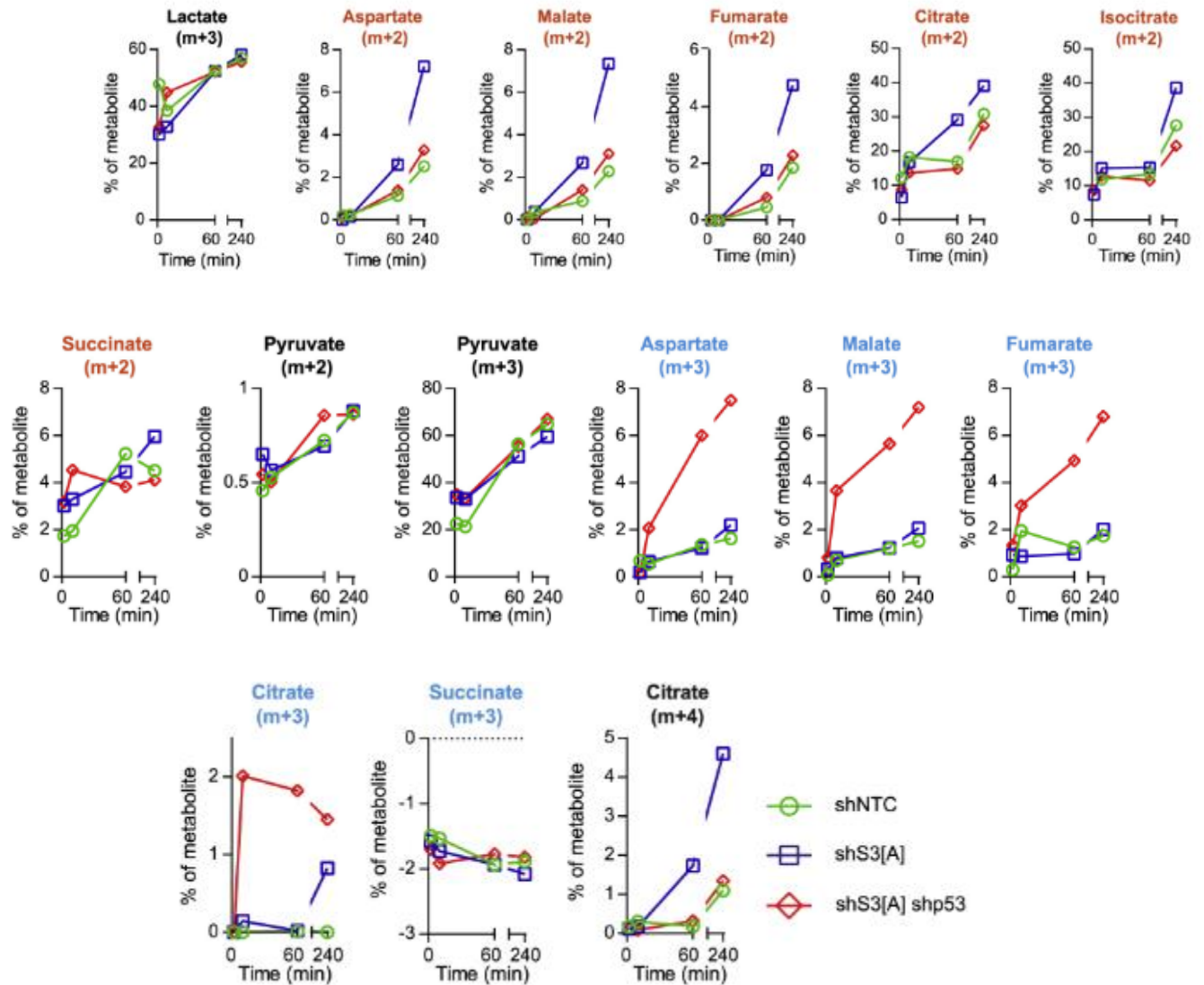


Fig. A.1.1D IMR90 cells depleted of STAT3 and p53 increases CAC anaplerosis via PC while decreasing entry into the CAC through pyruvate dehydrogenase. Mass spectrometry analysis of glucose-derived metabolites ($^{13}\text{C}_6$ -glucose flux) over time in IMR90 cells expressing a control shRNA (shNTC), a shRNA against STAT3 (shS3[A]) alone or in combination with an shRNA against p53 (shp53). Representative plots for the experiments are shown. Figure is from (1).

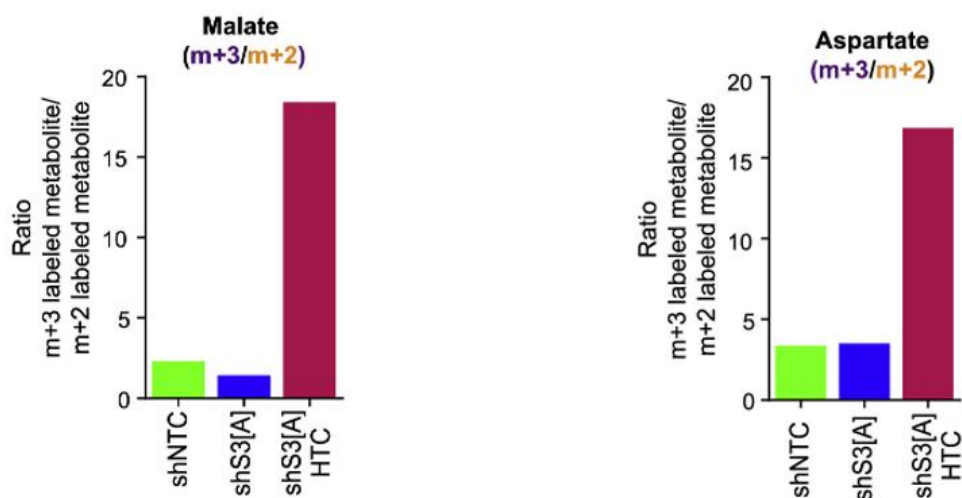


Fig. A.1.1E Expression of the hydride transfer complex enzymes reprograms metabolism in STAT3-depleted IMR90 cells. Ratio of (m+3)/(m+2) isotopomers for the indicated metabolites in IMR90 cells expressing control vectors, a shRNA against STAT3 with either control vectors (shS3[A]) or with vectors expressing HTC enzymes (shS3[A]HTC). The ratios were calculated using SITA data after 10 min of $^{13}\text{C}_6$ -glucose flux. Figure is from (1).

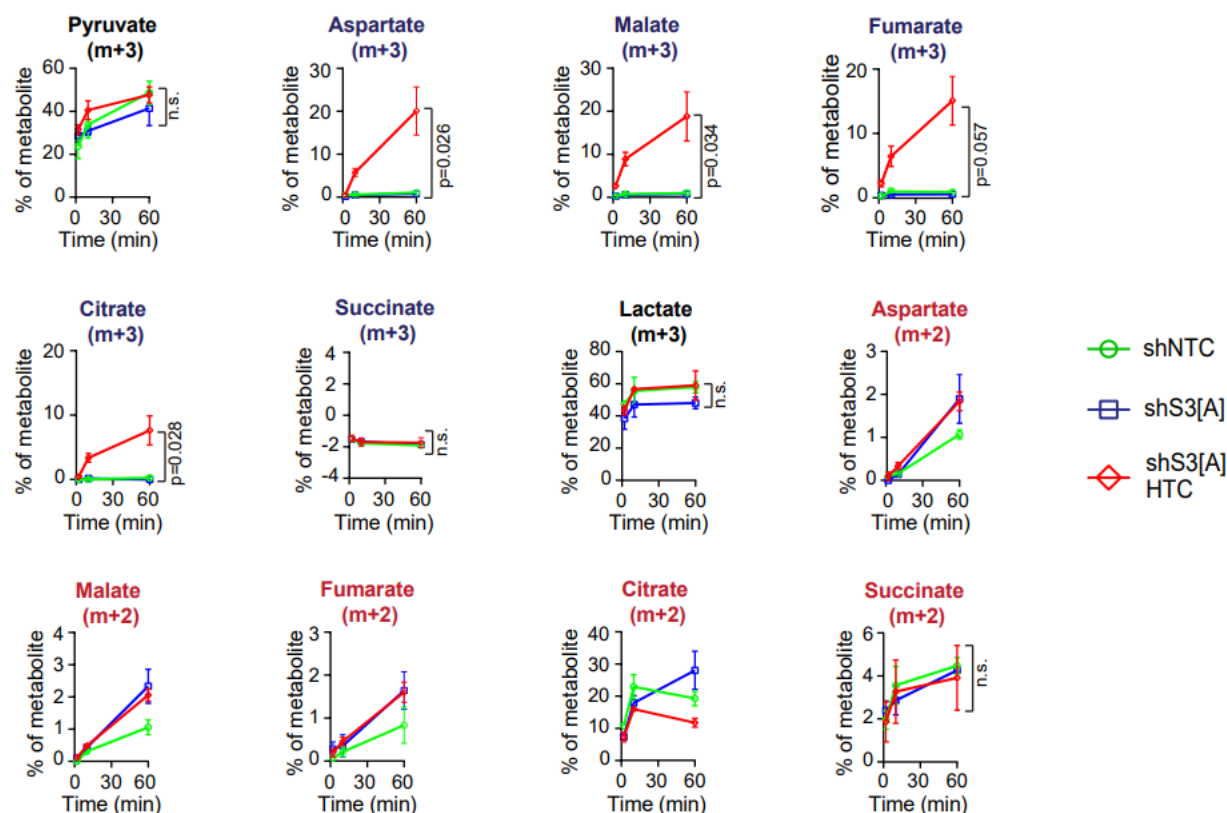


Fig. A.1.1F Expression of the hydride transfer complex enzymes increases CAC anaplerosis via PC in STAT3-depleted IMR90 cells. Mass spectrometry analysis of glucose-derived metabolites ($^{13}\text{C}_6$ -glucose flux) over time in IMR90 cells expressing a control shRNA (shNTC), a shRNA against STAT3 with either control vectors (shS3[A]) or with vectors expressing HTC enzymes (shS3[A]HTC). Mean percent of total labeled metabolites are shown. Figure is from (1).

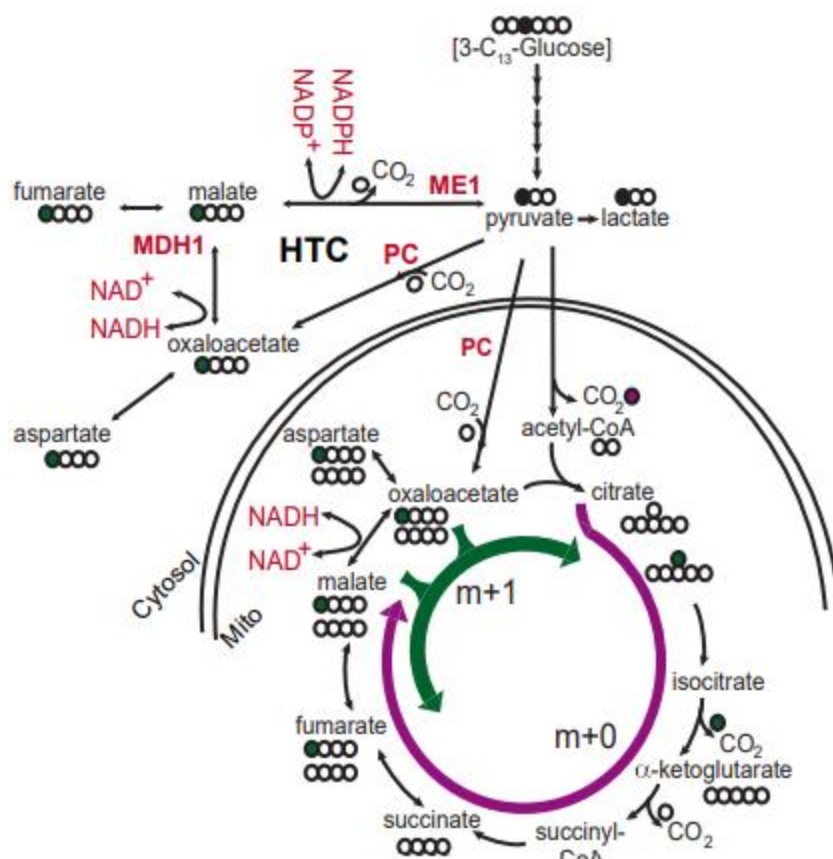


Fig. A.1.1G Schematic model of pyruvate carboxylase (PC) activity labeling pattern after 3-¹³C-glucose flux. 3-¹³C-glucose is metabolized into 3-¹³C-pyruvate. During the decarboxylation of 3-¹³C-pyruvate into acetyl-CoA, the labeled carbon is lost as heavy-labeled carbon dioxide and the subsequent citric acid cycle intermediates generated through oxidative decarboxylation are not labeled (magenta cycle). However, carboxylation of 3-¹³C-pyruvate into oxaloacetate by PC retains the labeled carbon and thus, each intermediate further derived from 3-¹³C-oxaloacetate is also labeled [(i.e., m+1 (green cycle))]. The filled circles show labelled carbon. Figure is from (1).

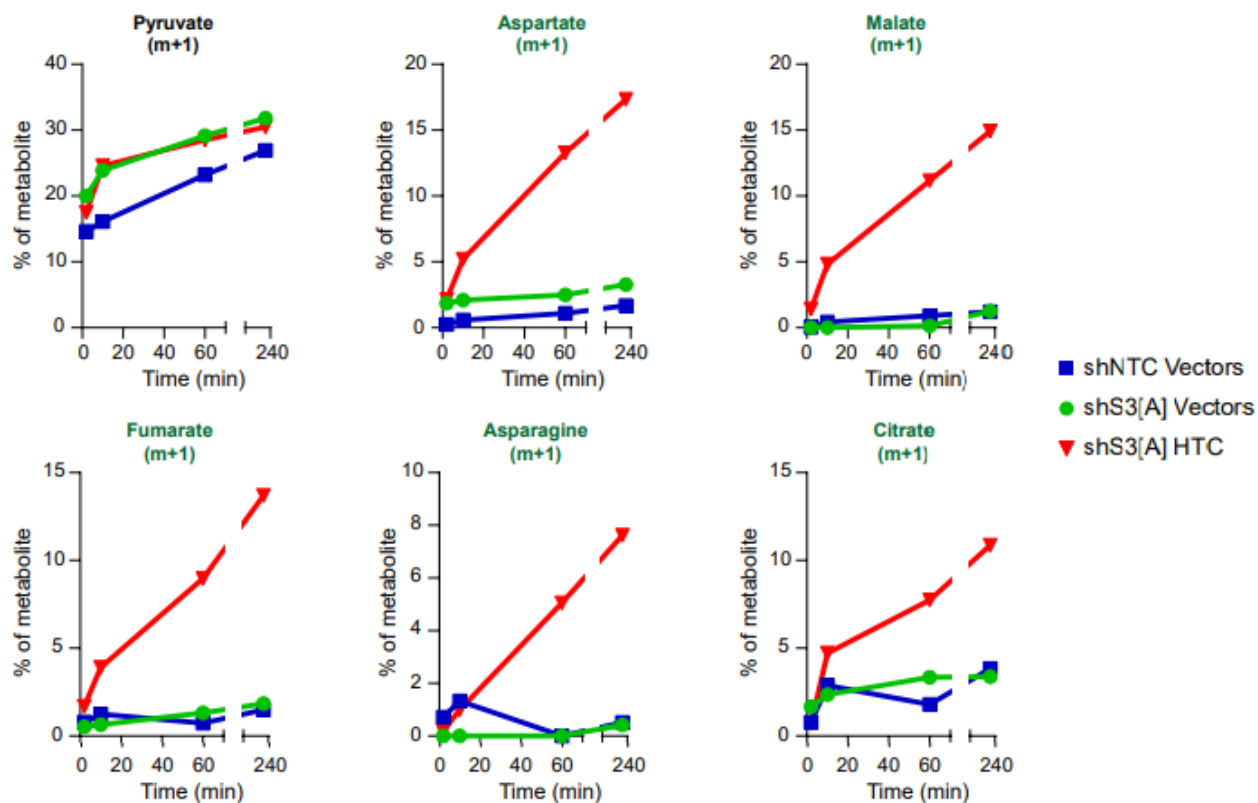


Fig. A.1.1H Expression of the hydride transfer complex enzymes increases CAC anaplerosis via PC in STAT3-depleted IMR90 cells. Mass spectrometry analysis of glucose-derived metabolites ($3\text{-}^{13}\text{C}$ -glucose flux) over time in IMR90 cells expressing a control shRNA (shNTC), a shRNA against STAT3 with either control vectors (shS3[A]) or with vectors expressing HTC enzymes (shS3[A]HTC). Figure is from (1).

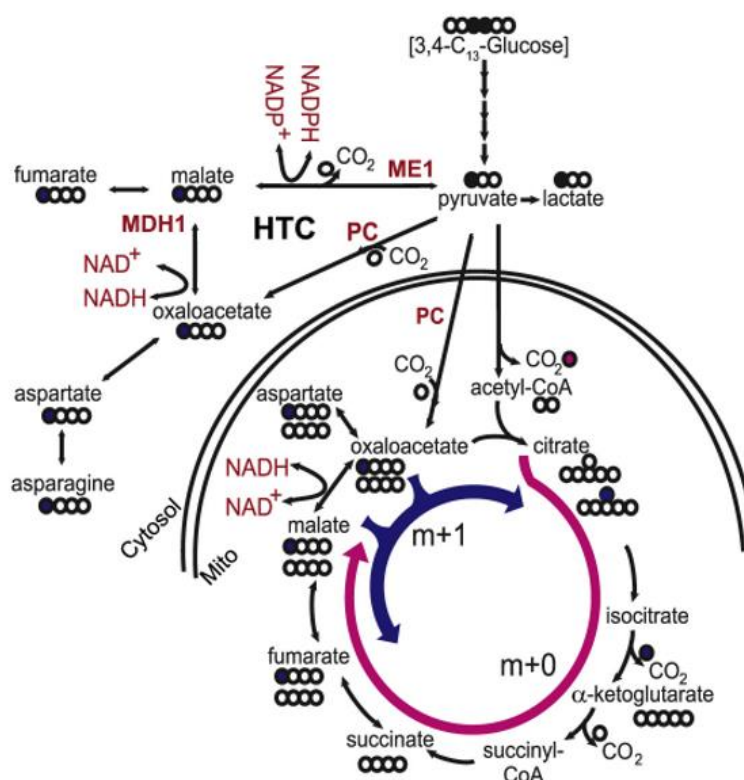


Fig. A.1.1I Schematic model of pyruvate carboxylase (PC) activity labeling pattern after 3,4- ^{13}C -glucose flux. 3,4- ^{13}C -glucose is metabolized into 3- ^{13}C -pyruvate. During the decarboxylation of 3- ^{13}C -pyruvate into acetyl-CoA, the labeled carbon is lost as heavy-labeled carbon dioxide and the subsequent citric acid cycle intermediates generated through oxidative decarboxylation are not labeled (magenta cycle). However, carboxylation of 3- ^{13}C -pyruvate into oxaloacetate by PC retains the labeled carbon and thus, each intermediate further derived from 3- ^{13}C -oxaloacetate is also labeled [(i.e., m+1 (blue cycle))]. The filled circles show labelled carbon. HTC, hydride transfer complex; mito, mitochondria. Figure is from (1).

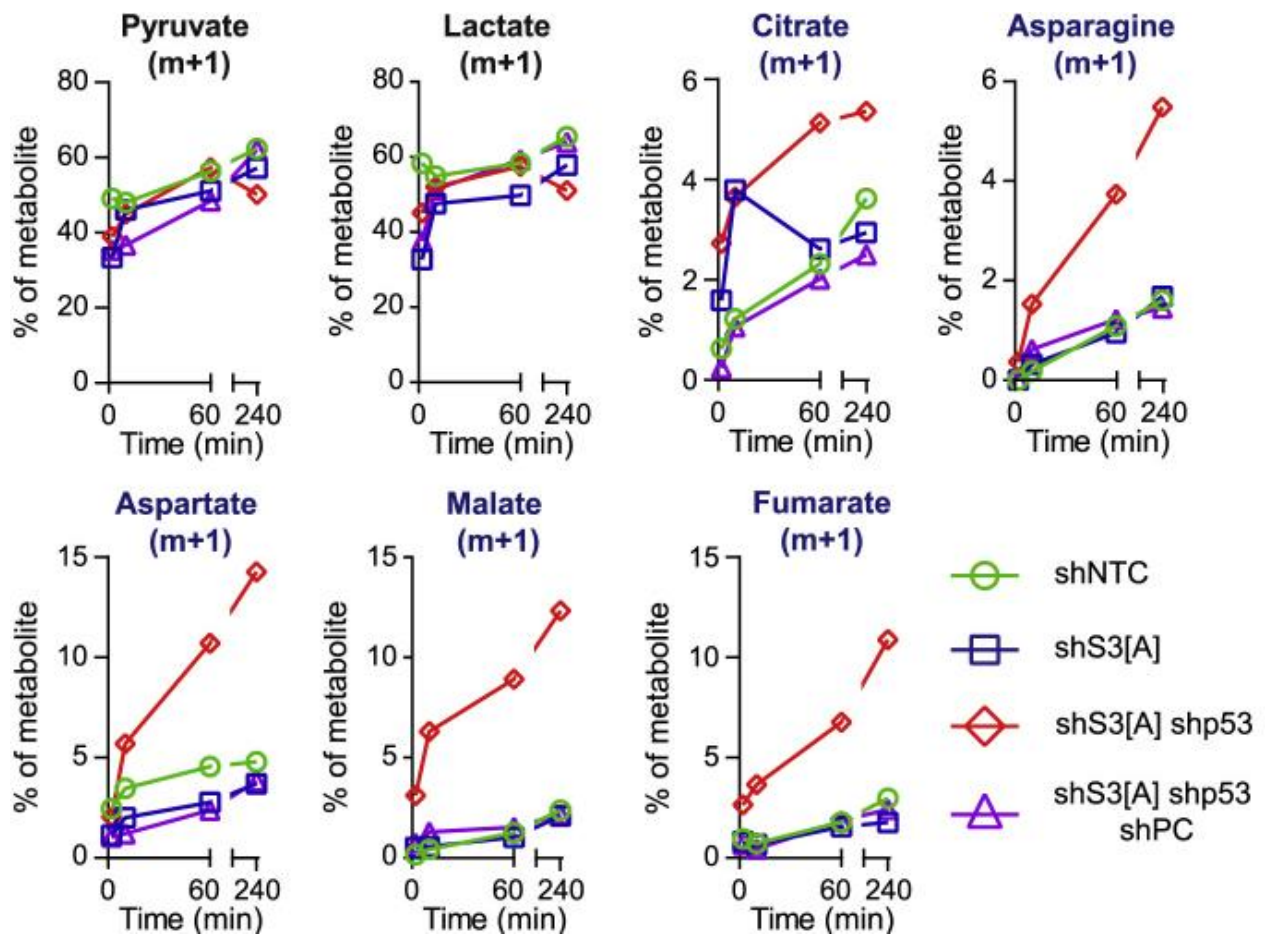


Fig. A.1.1J PC mediates metabolic reprogramming in IMR90 cells depleted of p53 and STAT3. Mass spectrometry analysis of glucose-derived metabolites ($3\text{-}^{13}\text{C}$ -glucose flux) over time in IMR90 cells expressing a control shRNA (shNTC), a shRNA against STAT3 alone (shS3[A]) or in combination with an shRNA against p53 (shp53), and/or a shRNA against PC (shPC). Figure is from (1).

A.1.2 Elucidating the mechanisms of the anti-neoplastic action of the sodium-glucose cotransporter-2 (SGLT2) inhibitors.

Increase in aerobic glycolysis is arguably one of the most recognized metabolic perturbations commonly observed in proliferating cells and neoplasia (30, 31). Over the last few decades, a panoply of studies have identified genes that are important regulators of glucose metabolism (32). Based on this, a number of lead compounds and/or drugs were developed and/or repurposed to target glucose metabolism in cancer (32). However, most of these efforts did not result in expected outcomes, thus suggesting that further investigation in the role of glucose metabolism in cancer is warranted. To this end, we investigated the mechanisms of antineoplastic action of canagliflozin, which is a sodium glucose cotransporter 2 (SGLT2) inhibitor that is commonly used to treat type 2 diabetes (4). SGLT2 transporters, expressed in the proximal convoluted tubule of the kidneys, are targeted when treating diabetes because they are responsible for roughly 90% of filtered glucose reabsorption (33). Thus, antihyperglycemic agents like canagliflozin inhibit the reabsorption of filtered glucose in the kidney which leads to increased glucose excretion and reduced blood glucose levels (33). The antiproliferative effects of canagliflozin have previously been shown in pancreatic and lung cancer (34, 35). Moreover, SGLT2 expression, which is generally thought to be limited to kidney cells, has been observed in prostate, glioblastoma, and pancreatic cancer (35, 36). However, the mechanism of anti-cancer action of canagliflozin remain obscure.

Supporting previously published studies (37-39), we showed that canagliflozin exhibits the antiproliferative effects in HER2+ (i.e., SKBR3 and BT-474) and luminal A (i.e., MCF7) breast cancer cell lines (4). I generated some of the cell proliferation data which compared the proliferative rates of control murine NT2197 breast cancer cells vs. those treated with

canagliflozin, other antidiabetic drugs like biguanide metformin or the additional SGLT2 inhibitor dapagliflozin [Fig A.1.2A; from Fig. 1C in (4)]. Surprisingly, the anti-proliferative effects of canagliflozin were not dependent on the availability of glucose (4). This suggested that the anti-proliferative effects of canagliflozin are independent of inhibition of glucose transport. In addition, the depletion of SGLT2 transporter neither reduced cell division nor rescued breast cancer cells from the antineoplastic effects of canagliflozin (4). Overall, these findings suggested a potential mechanism of anti-cancer action of canagliflozin that is independent of SGLT2.

Canagliflozin was previously shown to reduce cellular proliferation and decrease survival of prostate and lung cancer cells by inhibiting mitochondrial complex I (39). Also, HER2+ breast cancers have been shown to exhibit increased glutamine anaplerosis, to support neoplastic growth (40). Taking these findings into consideration, we sought to explore the effects of canagliflozin on mitochondrial metabolism in HER2+ breast cancer cells. Herein, we observed that relative to vehicle treated control, canagliflozin reduces oxygen consumption along with decreasing total ATP production in HER2-amplified SKBR3 breast cancer cells (4). In addition, canagliflozin reduced citrate, alpha-ketoglutarate and succinate levels while increasing glutamine levels as compared to vehicle treated control (4). SITA using labelled $^{13}\text{C}_5$ -glutamine showed reduced carbon flux into the citric acid cycle (CAC) through the sequential enzymatic activities of glutaminase 1 (GLS1) and glutamate dehydrogenase (GLUD) to produce α -ketoglutarate (4). In conclusion, these experiments demonstrated that canagliflozin alters mitochondrial metabolism of HER2+ breast cancer cells at least in part by inhibiting glutamine anaplerosis with a subsequent decrease in the levels of CAC metabolites.

Considering the observed inhibitory effects of canagliflozin on glutamine metabolism, we set out to further dissect the underlying mechanisms. We first demonstrated that the presence of

glutamine is required for the antineoplastic effects of canagliflozin as oxygen consumption and proliferation were reduced only in the presence of glutamine (4). Moreover, canagliflozin induced glutamine uptake while increasing glutamate excretion which suggests reduced GLUD activity in converting glutamate to alpha-ketoglutarate (4). This was supported by subsequent findings that canagliflozin greatly diminished GLUD activity (4). Altogether, the data suggests that canagliflozin inhibits proliferation of HER2-amplified breast cancer cell lines by interfering with glutamine metabolism via disruption of GLUD function.

Our findings support accruing evidence that glutamine metabolism may represent a valid therapeutic target in cancer. Indeed, recently several inhibitors of glutamine metabolism have been developed (41, 42). To this end, glutamine transporters, such SLC1A5, can be effectively targeted by drugs such as V-9302 (43). Moreover, several inhibitors of GLS1 including CB-839, BPTES and 968 have shown promising results in pre-clinical models (44). Importantly, CB-839 proceeded into clinical trials as a monotherapeutic agent and in combination with chemotherapy or immunotherapy for triple negative breast cancer (TNBC), non-small cell lung cancer (NSCLC) and non-Hodgkin's lymphoma (NHL) patients (ClinicalTrials.gov identifiers: NCT02771626, NCT02071862, NCT02071888). In contrast, fewer clinical trials have been carried out to date investigating the effects of GLUD inhibitors. Nevertheless, epigallocatechin-3-gallate (EGCG) is currently being assessed as an anticancer agent for colorectal cancer patients (NCT:02891538). The use of EGCG as an antineoplastic compound against colorectal cancer provides basis for exploring the effects of canagliflozin beyond HER2+ breast cancer. Collectively, our study provided pioneering evidence that the effects of canagliflozin in HER2-amplified breast cancer are unlikely to be mediated by SGLT2 and are more likely to be the consequence of abrogation of glutamine metabolism.

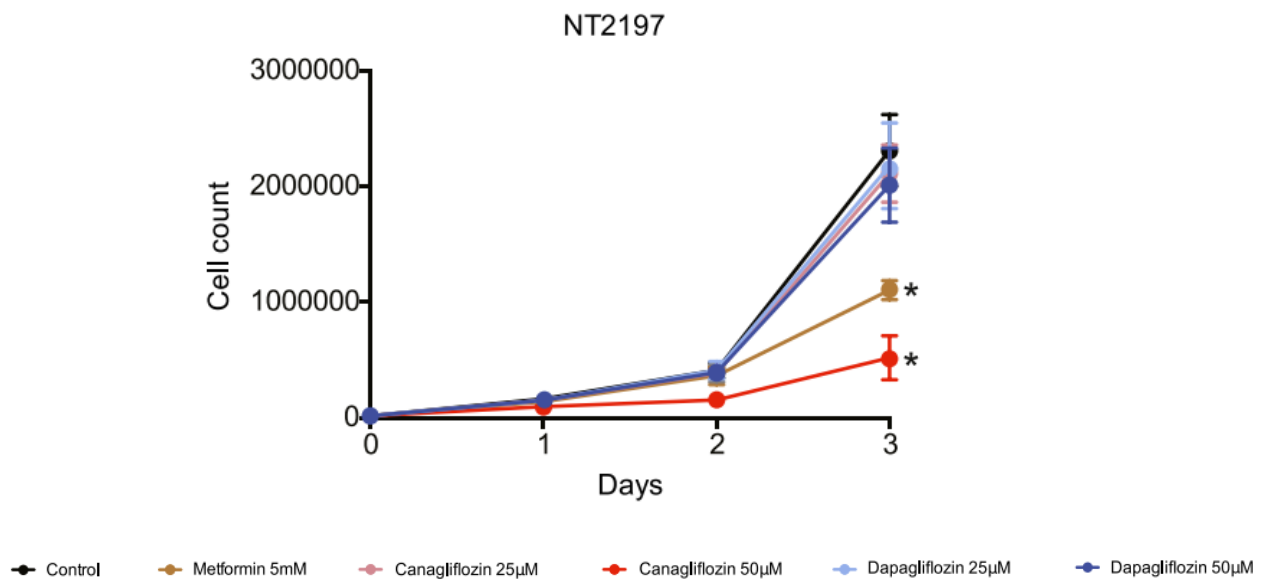


Fig. A.1.2A Canagliflozin reduces the proliferation of NT2197 cells. Number of viable NT2197 cells grown for indicated days was monitored by trypan blue exclusion using automated cell counter. Figure is from (4)

A.1.3 Establishing the role of PR/SET Domain 15 (PRDM15) in metabolic rewiring of B-cell lymphomas.

Transcription factors including c-MYC and HIF1 α , act as master regulators of cancer metabolism [summarized in the introduction section and (7)]. Nonetheless, the understanding of how transcriptional programs are integrated with metabolic rewiring in cancer is still incomplete. In collaboration with Mzoughi et al., we identified PRDM15 as transcription factor that is essential for metabolic reprogramming required to support survival and proliferation of B-cell lymphoma cells (2). PRDM15 is generally known to be involved in determination of cell identity during early development (45), but its function in adult tissues was largely unknown. Notwithstanding that we and others observed that PRDM15 is of the most highly overexpressed genes across the spectrum

of different non-Hodgkin's B-cell lymphomas (NBCL) such as follicular lymphoma, diffuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma (2, 46), its role in the etiopathogenesis and progression of these diseases remained elusive. This motivated us to investigate the role of PRDM15 in NBCL.

We first demonstrated that the abrogation of *PRDM15* using antisense oligonucleotides (AONs) reduces the survival of primary DLBCL cells and various B-cell lymphoma cell lines including P493-6, MC116, OCILY3, Karpas231, PR1, and HT (DLBCL), thus suggesting that PRDM15 plays a major role in B-cell lymphoma biology (2). Interestingly, except for having a slightly longer lifespan, tamoxifen-inducible PRDM15 adult whole-body KO mice (*Prdm15^{Δ/Δ};CreER* or *Δ/Δ* for short) had no conspicuous phenotypic differences compared to the controls (2). This indicates that PRDM15 is mostly expendable for normal adult murine homeostasis and adult stem cell renewal even though it has been previously described to be essential in embryonic stem cells and during development (2, 47, 48). However, considering that PRDM15 is upregulated in bone marrow B cells obtained from Eμ-Myc mice, we demonstrated that tamoxifen-inducible PRDM15 adult whole-body KO Eμ-Myc (*Prdm15^{Δ/Δ};CreER;Eμ-Myc*) mice delayed disease onset whereby *Prdm15^{Δ/Δ};CreER;Eμ-Myc* mice had a median disease-free survival of 332 days, compared to 107 days in control Eμ-Myc mice (2). Furthermore, the disruption of PRDM15 in primary tumor B-cells derived from tamoxifen-inducible PRDM15 KO Eμ-Myc mouse model decreased proliferation and increased apoptosis (2). In order to illustrate the cell-autonomous role of PRDM15 in tumor maintenance, primary tumors were extracted from *PRDM15^{F/F};CreER;Eμ-Myc* mice and transplanted into syngeneic recipient mice which were subsequently injected with tamoxifen to deplete PRDM15 in the tumor cells (2). While none of the tamoxifen-injected mice had palpable tumors at the end point of the experiment, all control

mice had tumors and greater disease burden. Collectively, these findings demonstrate that PRDM15 may act as a central factor driving B-cell lymphomagenesis and maintenance of B-cell lymphoma.

To gain further insights in the molecular mechanism(s) that underpin the role of PRDM15 in driving B-cell lymphomagenesis and disease progression, we carried out chromatin immunoprecipitation (ChIP)-sequencing and RNA sequencing experiments in PRDM15-proficient and deficient B-cells isolated from E μ -Myc mice to identify promoters that are occupied by PRDM15 in parallel with the changes in mRNA level. These analyses revealed that transcriptional targets of PRDM15 are enriched in metabolic genes including pyruvate kinase (*Pkm*), insulin receptor (*Insr*) and insulin like growth factor 1 receptor (*Igf1r*) (2). Moreover, gene ontology analysis of affected genes highlighted glycolysis and PI3K-AKT-mTOR signaling as two of the most highly affected functions upon *Prdm15* loss (2). We confirmed these findings by showing that the loss of *Prdm15* inhibited the activity of PI3K-AKT-mTOR signaling pathway (2). Intriguingly, suppression of mTOR signaling caused by *Prdm15* loss appeared not to be accompanied by the disruption of negative feedbacks that lead to activation of other tumorigenic signaling pathways including MAPK-ERK (2). It is worthwhile to note that the compensatory activation of the latter pathways has been shown to limit the efficacy of clinically used mTOR inhibitors (e.g., everolimus) in oncological indications (49, 50). Considering that mTOR plays a major role in regulation of cellular metabolism, while putative PRDM15 transcriptional targets were also enriched in genes known to regulate glucose metabolism [e.g., glucose transporter 1 (*SLC2A1*), pyruvate kinase (*PKM*), hexokinase 3 (*HK3*) and enolase (*ENO*)] (2), we next sought to document the effects of PRDM15 loss on the metabolome. I participated in these studies.

Indeed, loss of *Prdm15* in B-cell lymphomas derived from mice (i.e., *Prdm15^{F/F};CreER;Eμ-Myc* tumors treated with tamoxifen to deplete the expression of *Prdm15*) was accompanied by decrease in intracellular levels of glucose and glycolytic intermediates including fructose-6-phosphate, glucose-6-phosphate and glucose-1-phosphate (Fig. A.1.3A; from Fig. 6A in (2)). Likewise, the intracellular levels of a pentose phosphate pathway (PPP) intermediate sedoheptulose-7P were reduced when *Prdm15* was abrogated as compared to the control (Fig. A.1.3A; from Fig. 6A in (2)). Accordingly, in addition to the downregulation of glycolytic genes, the loss of *Prdm15* was also associated with the reduced expression of PPP enzymes (e.g., glucose-6-phosphate isomerase) (2). Nucleotides, CAC intermediates (e.g., malate, succinate, fumarate and α -ketoglutarate) and amino acids (asparagine, glutamine, glutamate and proline) were also reduced in B-cell lymphoma cells lacking *Prdm15* relative to *Prdm15*-proficient controls (Fig. A.1.3A; from Fig. 6A in (2)). Furthermore, OXPHOS was greatly diminished by deletion of *Prdm15* as compared to control B-cell lymphoma cells (2). Since c-MYC plays a prominent role in regulating glutamine metabolism (51) and our data suggests that c-MYC stimulates *PRDM15* transcription (2), I next performed SITA analysis whereby *Prdm15*-proficient (i.e., *Prdm15^{F/F};CreER;Eμ-Myc*) and tamoxifen-induced *Prdm15*-deficient B-cell (i.e. *Prdm15^{Δ/Δ};CreER;Eμ-Myc*) tumor lymphoma cells were labelled with $^{13}\text{C}_5$ -glutamine. This analysis revealed that although the total ^{13}C -labeled CAC intermediates levels were reduced in association with the loss of *PRDM15*, the fraction of ^{13}C -labeled CAC intermediates is mostly unaffected by *PRDM15* status (Fig. A.1.3B-D; from Supplementary Fig. 6D, G-H in (2)). Hence, CAC enzyme activities were unaffected by *PRDM15* status in the cell, and that the decrease in the total ^{13}C -labeled CAC intermediates levels observed in B-cell lymphomas devoid of *PRDM15* is at least partly due to decreased glutamine uptake and/or utilization (2). Taken altogether, these

findings established PRDM15 as a major regulator of metabolic rewiring in B-cell lymphoma, while suggesting that the loss of PRDM15 leads to metabolic catastrophe and subsequent death of B-cell lymphoma cells. These findings thus position PRDM15 as a major factor that underpins metabolic plasticity of B-cell lymphoma cells, whereby PRDM15 appears to be essential for survival and proliferation of B-cell lymphoma cells.

In conclusion, our study pointed out that although PRDM15 is essential for supporting metabolic programs of B-cell lymphoma, but not normal B-cells or other hematopoietic cells. This suggest that PRDM15 may constitute an appealing target for therapeutic intervention. Importantly, in addition to exhibiting low expression in adult tissues, the loss of *Prdm15* in mice did not result in any obvious phenotypic differences in the major examined organs (e.g., spleen, intestines and testes), thereby suggesting a wide therapeutic window for the potential PRDM15-targeted therapies for adults. Although the crystal structure of PRDM15 is yet to be determined and potential inhibitors of PRDM15 may still be in development, antisense oligonucleotides targeting PRDM15 seem to be a plausible approach (2, 52).

LC-MS Data		Tumor			
Metabolite	A	B	C		
Citrate	1.23	0.96	0.96	TCA Cycle	
α -ketoglutarate	0.47	0.47	0.53		
Succinate	0.49	0.55	0.37		
Fumarate	0.46	0.28	0.30		
Malate	0.61	0.54	0.62		
F6P/G6P/G1P	0.80	0.83	0.32	Glycolysis/PPP	
Sedoheptulose-7P	0.22	0.18	0.22		
Lactate	0.27	0.54	0.43		
AMP	0.63	1.05	1.20	Nucleotides	
ADP	1.02	0.59	0.56		
ATP	0.69	0.47	0.48		
GMP	0.49	0.68	0.84		
GDP	0.67	0.38	0.34		
GTP	0.49	0.38	0.40		
IMP	0.51	0.42	0.34		
CMP	0.31	0.55	1.13		
CDP	0.64	0.44	0.54		
CTP	0.46	0.38	0.55		
TTP	0.40	0.29	0.44		
UMP	0.33	0.70	1.15		
UDP	0.48	0.36	0.47		
UTP	0.36	0.31	0.42		
NAD	0.51	0.43	0.57		
NADP	0.38	0.19	0.16		
Proline	0.69	0.41	0.46	Amino acids	
Serine	1.21	0.70	0.79		
Alanine/Sarcosine	0.84	0.52	0.54		
Asparagine	0.88	0.50	0.46		
Glutamate	0.84	0.55	0.61		
Pyroglutamate	0.80	0.52	0.60		
Glutathione red	0.23	0.01	0.48		
Glutathione ox	1.22	0.49	1.19		
Betaine	0.73	0.70	0.56		
Hypotaurine	0.77	0.54	0.71		
Citruline	1.33	1.64	0.73	Other	
SAH	0.94	1.07	0.76		
SAM	1.16	0.68	0.69		
Taurine	0.85	0.57	0.66		
Ornithine	0.83	1.04	0.53		
Creatinine	0.61	0.56	0.65		
2-hydroxyglutarate	0.40	0.35	0.56		
UDP-N-Acetyl					
Glucosamine	0.44	0.43	0.53		
GC-MS Data					
Metabolite	D	E			
Pyruvate	0.562	0.896			
Glutamine	0.696	0.644			



Fig. A.1.3A PRDM15 transcriptionally reprograms metabolism in E μ -Myc tumor cells. Metabolomics analysis in Prdm15^{F/F};CreER;E μ -Myc vs. Prdm15 ^{Δ/Δ} ;CreER;E μ -Myc cells. The values in the upper panel represent the average intracellular level of each indicated metabolite in Prdm15 ^{Δ/Δ} ;CreER;E μ -Myc cells relative to the Prdm15^{F/F};CreER;E μ -Myc control cells

determined by LC-MS. The data were obtained from three independent tumors (i.e., A, B and C) with each tumor consisting of three technical replicates. The values in the bottom panel are average intracellular levels of pyruvate and glutamate measured in two independent tumors determined by GC-MS. Figure is from (2)

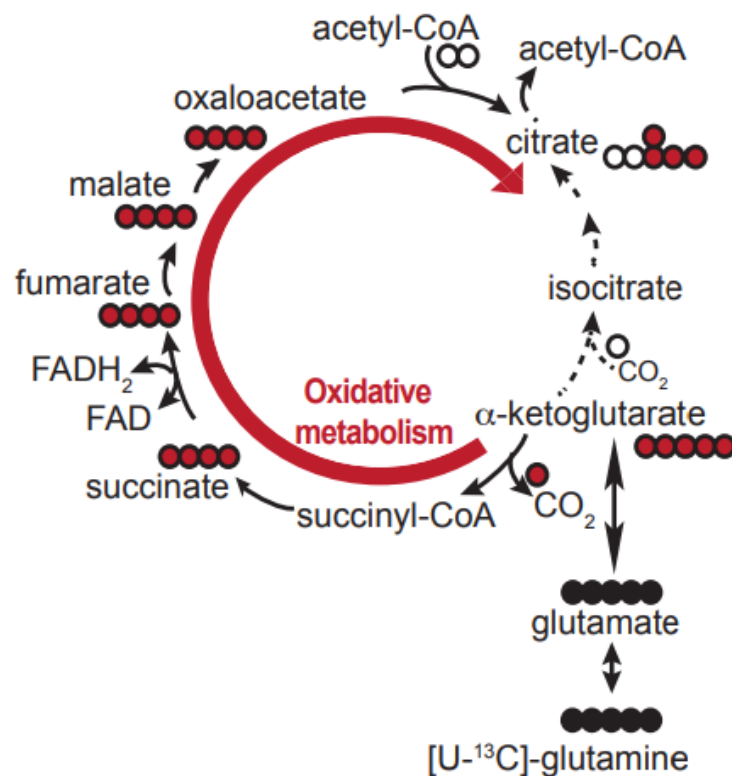


Fig. A.1.3B Schematic model of ^{13}C incorporation into CAC intermediates after incubating cells in media containing ^{13}C -glutamine. ^{13}C -glutamine is metabolized into ^{13}C - α -ketoglutarate which undergoes oxidative decarboxylation (red cycle) in the CAC to produce intermediates of the cycle. The filled circles show labelled carbon. Figure is from (2)

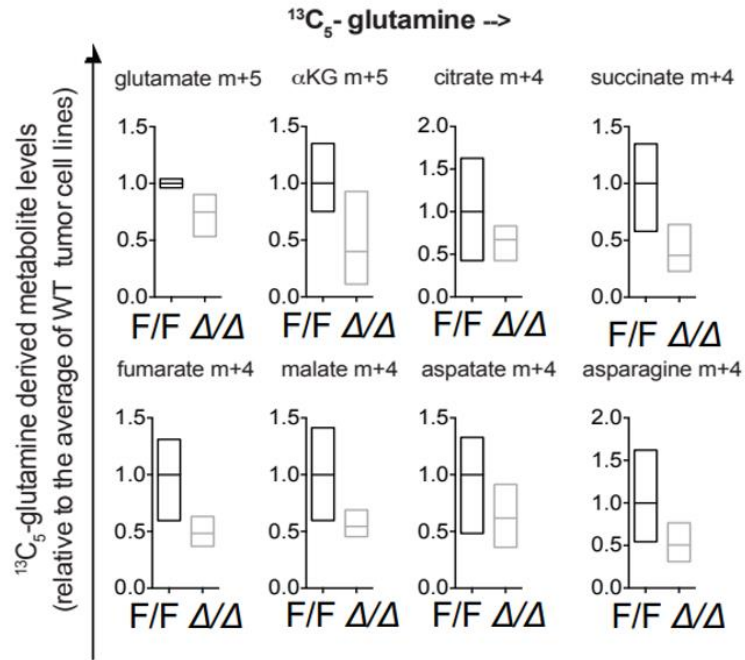


Fig. A.1.3C Loss of PRDM15 in Eμ-Myc tumor cells decreases the intracellular levels of CAC intermediates derived from the oxidative decarboxylation of glutamine-derived α -ketoglutarate. Prdm15^{F/F};CreER;Eμ-Myc and Prdm15^{Δ/Δ};CreER;Eμ-Myc cells were incubated with ¹³C₅-glutamine for 180 min. SITA were performed and the levels of indicated isotopomer ion amounts are shown as absolute amount and relative to the average of the Prdm15^{F/F};CreER;Eμ-Myc control cells. Figure is from (2).

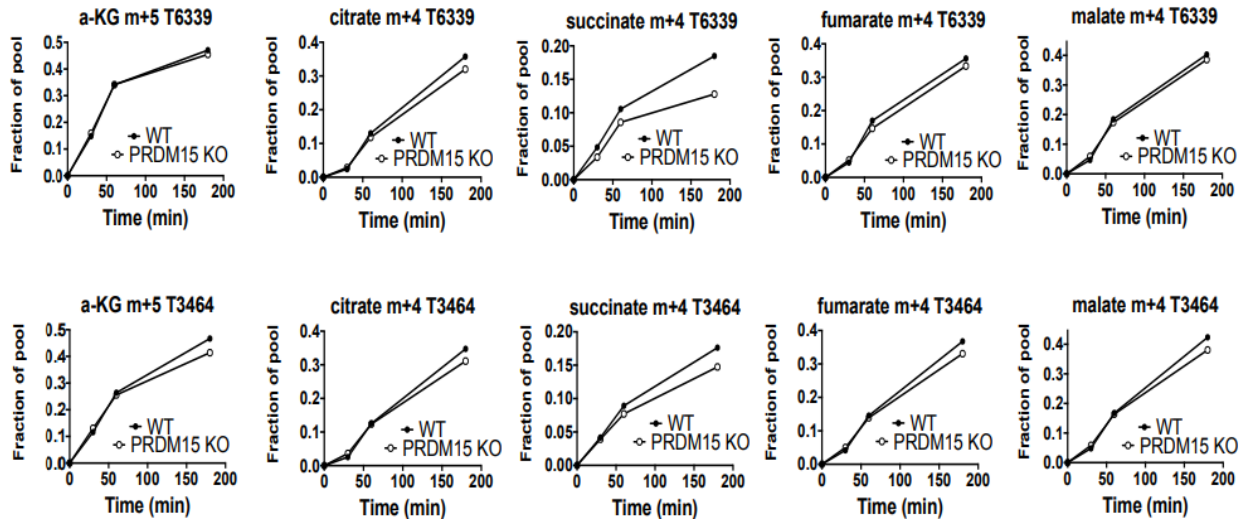


Fig. A.1.3D PRDM15 status has no effect on the activity of CAC enzymes. SITA of $\text{Prdm15}^{\text{F/F}};\text{CreER};\text{E}\mu\text{-Myc}$ (WT) and $\text{Prdm15}^{\Delta/\Delta};\text{CreER};\text{E}\mu\text{-Myc}$ (PRDM15) cells incubated with $^{13}\text{C}_5\text{-glutamine}$. The fraction of ^{13}C -CAC intermediates relative to the total pool labelled over time is shown. Data from two independent tumors (i.e., T63339 and T3464) is shown. Figure is from (2).

A.1.4 The role of mTORC1/4E-BP/eIF4E axis and HIF1 α in metabolic plasticity of cancer cells and responses to the combinations of inhibitors of oncogenic kinases and biguanides.

To date, attempts to target metabolic vulnerabilities of cancer cells have resulted only in a modest success (53). This is thought to be a consequence of incomplete understanding of the molecular underpinnings of metabolic rewiring in neoplasia (54). More recent evidence points out that when one of the major metabolic pathways is suppressed, cancer cells can compensate by activating alternative metabolic routes (54). For instance, anti-diabetic biguanides (e.g., metformin) exhibit anti-neoplastic effects by suppressing OXPHOS which at least in part is achieved via the inhibition of mitochondrial complex I (55-59). This decrease in OXPHOS is however compensated by increased uptake and utilization of glucose including its reduction to

lactate (59-62). The latter is thought to be required to regenerate NAD⁺ under conditions wherein the NADH dehydrogenase activity of complex I is reduced (60, 61). In turn, the vast majority of the inhibitors of oncogenic kinases (KIs) including HER2/EGFR inhibitor lapatinib, BRAF inhibitor vemurafenib, or BCR/ABL inhibitor imatinib suppress glucose uptake and glycolysis (63). Therefore, we and others rationalized that combining biguanides and KIs should result in synthetic lethality whereby compensatory increase in glycolysis required for adaptation to energy stress caused by biguanides should be disrupted by KIs (3, 64, 65). To test this hypothesis, we investigated the effects of combination of biguanides and KIs across the range of cancer cell lines whose neoplastic growth is driven by different oncogenic kinases (3). We opted to use phenformin in our experiments motivated by the findings that metformin is a less potent complex I inhibitor compared to phenformin (66), while phenformin has a more favorable pharmacokinetic and pharmacodynamic profile than metformin (67). It is important to note that although phenformin is not in clinical use for the treatment of type 2 diabetes due to relatively higher frequency of lactic acidosis as compared to metformin, its repurposing for oncological indications is well justified considering severity of the side-effects of anti-cancer therapeutics (68).

Using cell line models, we demonstrated that combining phenformin with KIs results in synergistic anti-proliferative effects and induction of apoptosis across a variety of cancer cell lines (3). More specifically, HER2 inhibitor lapatinib, BCR/ABL inhibitor imatinib and BRAF inhibitor vemurafenib synergized with phenformin in HER2⁺ breast cancer NT2197, BCR-ABL positive K562 myelogenous leukemia and BRAF-mutated A375 melanoma cell lines, respectively (3). Proliferation of HCT116 colon cancer cells is dependent on EGFR activity and is thus sensitive to lapatinib which acts as a dual HER2/EGFR kinase inhibitor (69). Of note, one of my contributions to this study was to carry out experiments showing that lapatinib and phenformin exert synergistic

anti-proliferative effects in HCT116 cells (Fig. A.1.4A; from Fig. 1B and Supplementary Fig. 2F-G of (3)). NT2197 cells were generated by expressing oncogenic Neu/ErbB2 (V664E) (rat orthologue of HER2) in Normal Murine Mammary Gland (NMuMG) cells (70). Importantly, NMuMG cells showed minimal sensitivity to phenformin, lapatinib or combination thereof as compared to NT2197 cells, thus suggesting that this drug combination does not induce major toxicity in normal cells (3). Finally, using xenograft NT2197 model, we showed that *in vivo* effects of a combination of phenformin and lapatinib are significantly stronger than using each drug alone (3).

Thus far, our results showed that phenformin and KIs exert synergistic effects across a variety of cell lines and “driver” oncogenic kinases. Considering the opposing effects of KIs and phenformin on glycolysis (71-75) (60), we next probed the effects of phenformin/KI combinations on metabolome. I actively participated in these studies. As expected, vemurafenib (referred to as PLX4032) and lapatinib inhibited phenformin-induced increase in glucose uptake and lactate production (Fig. A.1.4B; from Fig. 2B and Fig. 2F of (3)). In addition, it was previously shown that biguanides induce reductive glutamine metabolism (62). As described in chapter 2, reductive glutamine metabolism is characterized by the reductive carboxylation of α -ketoglutarate which allows sufficient production of citrate to fuel lipid synthesis under conditions where mitochondrial functions and/or CAC are blocked (76). We observed that vemurafenib and lapatinib largely antagonized phenformin-induced increase in the reductive glutamine metabolism (Fig. A.1.4C; from Fig. 2D and Fig. 2H of (3)). Findings obtained in cell culture were further confirmed *in vivo* using the NT2197 xenograft model (3). Altogether, these findings indicate that KIs attenuate adaptive metabolic rewiring induced in response to energy stress caused by phenformin treatment. However, the synergy between lapatinib and phenformin could not be explained solely by their

opposing effects on glucose metabolism inasmuch as combining phenformin with inhibitors of glycolysis (e.g., phosphofructokinase inhibitor 3PO), resulted only in weak additive effect (3).

Phenformin and KIs impinge on protein synthesis machinery (77) which is one of the most highly energetically demanding processes in the cells that is upregulated in cancer and linked to metabolic reprogramming in neoplasia (78, 79). We therefore investigated whether the effects of KIs and phenformin on protein synthesis may contribute to the observed synergistic effects. Herein, we first focused on two major signaling mechanisms known to regulate protein synthesis: the AMPK-mTORC1 axis and the MAPK-ERK pathway. As expected, phenformin activated AMPK while KIs such as lapatinib, vemurafenib and imatinib inhibited the MAPK-ERK pathway. However, at concentrations used, combining KIs and phenformin resulted in downregulation of mTORC1 signaling to a significantly higher extent than each drug alone (3). Intriguingly, although the active-site mTOR inhibitor, torin1, repressed mTORC1 and global protein synthesis to a higher extent than phenformin/lapatinib combination, the effects of torin1 on apoptosis were dramatically less pronounced than those observed when phenformin and lapatinib were combined (3). This can be explained by the findings that active-site mTOR inhibitors induce metabolic dormancy whereby the reduction in ATP synthesis is offset by the inhibition of energy demanding processes like protein synthesis (79). Hence, it is plausible that residual mTOR signaling in phenformin/lapatinib-treated cells is required to prevent metabolic dormancy and lead to energetic crisis which may explain higher induction of apoptosis in NT2197 cells treated with the latter combination as compared to torin1 treatment.

In addition to regulating global protein synthesis, alterations in mTORC1 signaling have been linked to selective perturbations in the pools of mRNAs that are being translated (80-84). We observed that phenformin/lapatinib combination not only inhibited global protein synthesis, but

also strongly reduced the translation of a number of “eIF4E-sensitive” mRNAs (e.g., *MCL1*, *BCL2* and *MYC*) (77) to the higher extent than either drug alone (3). We next confirmed that these effects on protein synthesis are mediated via the mTORC1-4E-BP-eIF4E axis by showing that is sufficient to rescue translation of “eIF4E-sensitive” mRNAs in cells treated with phenformin/KI combination to the levels comparable to those observed in control, vehicle treated cells (3). In addition to “classical” “eIF4E-sensitive mRNAs”, we observed that the loss of 4E-BPs alleviated the inhibitory effects of phenformin/lapatinib combination on translation of pyruvate carboxylase (*PC*), phosphoglycerate dehydrogenase (*PHGDH*), phosphoserine aminotransferase 1 (*PSAT1*) and asparagine synthetase (*ASNS*) mRNAs (3). Notably, proteins encoded by the aforementioned mRNAs are implicated in biosynthesis of non-essential amino acid (NEAA) aspartate (*PC*), asparagine (*ASNS*) and serine (*PHGDH* AND *PSAT1*) (85, 86). It was previously demonstrated that the mTORC1-4E-BP-eIF4E axis regulates translation of mRNAs involved in mitochondrial biogenesis (e.g., *TFAM*) and functions (e.g., *ATP5O*) (79), which was also confirmed in the experiments using phenformin/lapatinib combination (3). Collectively, these results suggested that synergistic effects of biguanides and KIs may at least in part be mediated by translational reprogramming of NEAA metabolism and bioenergetics via the mTORC1-4E-BP-eIF4E axis.

To test this, I carried out SITA with $^{13}\text{C}_6$ -glucose and 3- ^{13}C -glucose, which revealed that according to alterations in translations, NT2197 cells devoid of 4E-BP1/2 synthesize more serine and aspartate compared to 4E-BP1/2-proficient cells, whereby the flux via these biosynthetic pathways remained significantly elevated after treatment with phenformin and lapatinib combination in 4E-BP1/2 KO cells [Fig. A.1.4D-F; from Fig. 6B and Supplementary Fig. 6B of (3)]. The significance of aspartate and asparagine in determining the sensitivity of cells to phenformin/lapatinib combination was further underscored by the findings that supplementation

of the media with either aspartate or asparagine significantly attenuated the antiproliferative effects of phenformin/lapatinib combination even in 4E-BP1/2-proficient NT2197 cells (3). Conversely, inhibition of serine synthesis by depleting PHGDH potentiated the antiproliferative effect of the drug combination (3). Collectively, these findings show that translational reprogramming of NEAA biosynthesis via the mTORC1-4E-BP-eIF4E axis is a major determinant of the efficacy of combinations of phenformin with KIs.

Notwithstanding that abrogation of 4E-BP1/2 expression attenuated the effects of biguanide/KI combinations in cancer cell lines, it did not rescue proliferation rates to the levels observed in control vehicle-treated cells (3). This suggested that the factors other than the mTORC1-4E-BP-eIF4E axis are implicated in response to combinatorial biguanide/KI treatment. We observed that the HIF-1 α levels were more strongly reduced in NT2197 cells treated with phenformin/lapatinib drug combination compared to cells treated with either drug alone (3). Opposite to previous reports showing that in some contexts HIF-1 α levels are regulated at the level of translation in a 4E-BP-dependent manner (87), we determined that the changes in HIF-1 α levels in response to phenformin/lapatinib combination treatment of NT2197 cells were independent of the 4E-BP status in the cells (3). ¹³C₅-glutamine tracing experiments under conditions where HIF-1 α levels were induced by dimethyloxallyl glycine (DMOG), or in renal cancer RCC4 cells devoid or competent for Von Hippel Lindau (VHL) E3 ubiquitin ligase that marks HIF-1 α for degradation (88-90) revealed that HIF-1 α plays a major role in inducing reductive glutamine metabolism (3). Consistent with previous reports (91), we found that HIF-1 α negatively regulates α -ketoglutarate dehydrogenase (α KGDH) subunit E1 (OGDH) levels which limits oxidative, while bolstering reductive glutamine metabolism (3). Notably, phenformin/lapatinib combination treatment which had no major effect on the levels of HIF-1 α and OGDH in VHL-deficient renal cancer RCC4 cells,

reduced the levels of both proteins in their VHL-proficient counterparts (3). As mentioned above, increase in reductive glutamine metabolism limits anti-neoplastic efficacy of biguanides (62). Consistent with this VHL-deficient RCC4 cells exhibited reduced sensitivity to phenformin/lapatinib combination as compared to VHL-proficient RCC4 cells (3). This was accompanied with the inability of the drugs to suppress HIF-1 α levels and reductive glutamine metabolism in the absence of VHL (3).

Altogether, these findings unraveled the previously unappreciated mechanisms that mediate the effects of biguanide and KI combinations. More importantly, these results showed that cancer cells engage alternative metabolic pathways governed by the mTORC1-4E-BP-eIF4E and HIF-1 α dependent mechanisms to adapt to biguanide and KI combinations (Fig. A.1.4G; from (3)). These findings therefore suggest that instead of being “addicted” to one or few metabolic pathways, cancer cells exhibit metabolic plasticity whereby they can rewire metabolism using alternative mechanisms and pathways to avoid therapeutic insults and adapt to other types of stress. Considering that the combination of biguanides and KIs are being considered or are already in clinical trials (NCT03026517), these findings suggest that consideration of metabolic plasticity should play a major role in design and interpretation of these trials. Moreover, these and other emerging mechanisms that enhance metabolic flexibility of cancer cells [e.g., PGC1 α -dependent metabolic perturbations (92) and PDK1-dependent metabolic reprogramming (93)] are likely to limit the efficacy of targeting metabolic vulnerabilities of neoplasia in the clinic and dictate metastatic spread of the disease (6, 54). Collectively, these findings suggest that future research is warranted to improve understanding of the molecular underpinnings of metabolic reprogramming in cancer.

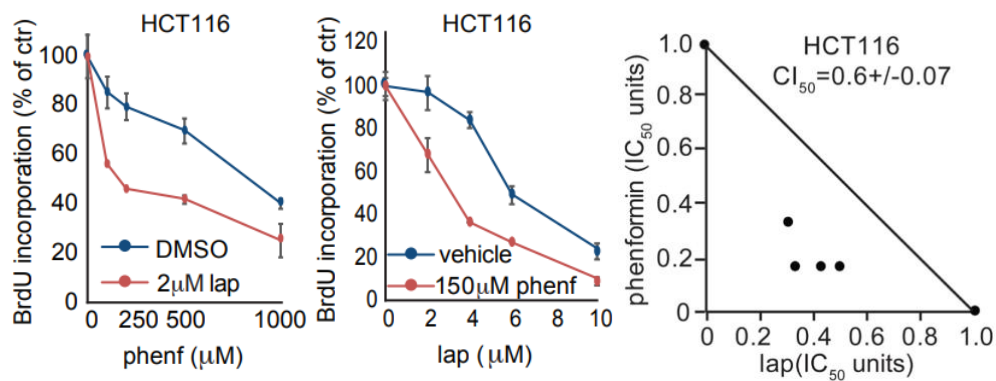


Fig. A.1.4A Lapatinib and phenformin work synergistically to inhibit the proliferation of HCT116 cells. HCT116 cells were treated with phenformin and/or lapatinib, at the indicated concentrations, for three days. Cell proliferation was monitored by BrdU incorporation and expressed as mean values +/- the SD of phenformin untreated (left panel) or lapatinib-untreated cells (middle panel). Combination Index 50 (CI₅₀) for lapatinib and phenformin (right panel) was calculated by isobologram method using the cell proliferation curves (left and middle panels) for HCT116. Data shown are representative of 2 independent experiments, each with 3 technical replicates. Figure is from (3).

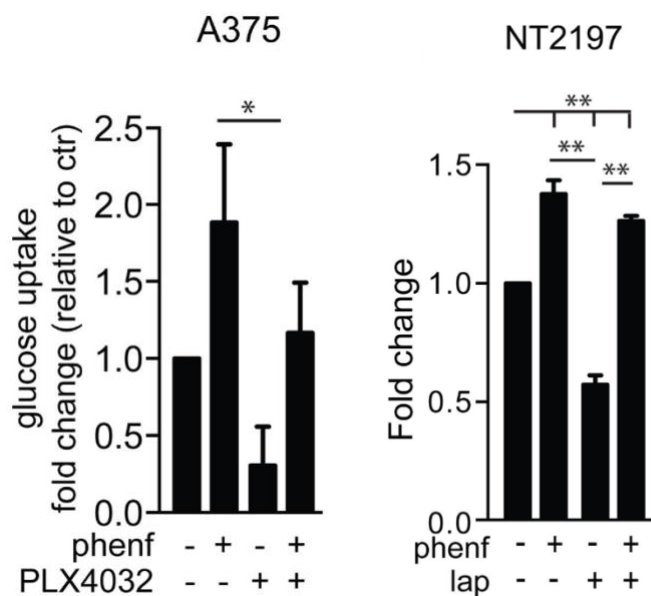


Fig. A.1.4B Kinase inhibitors counters phenformin-induced changes in glucose metabolism. A375 and NT2197 cells were treated with phenformin and/or kinase inhibitors (i.e., A375 cells were treated with PLX4032 while NT2197 cells were treated with lapatinib) for a day. Glucose uptake in drug-treated cells is presented relative to DMSO-treated control cells. Results are presented as mean values +/- the SD. Figure is from (3).

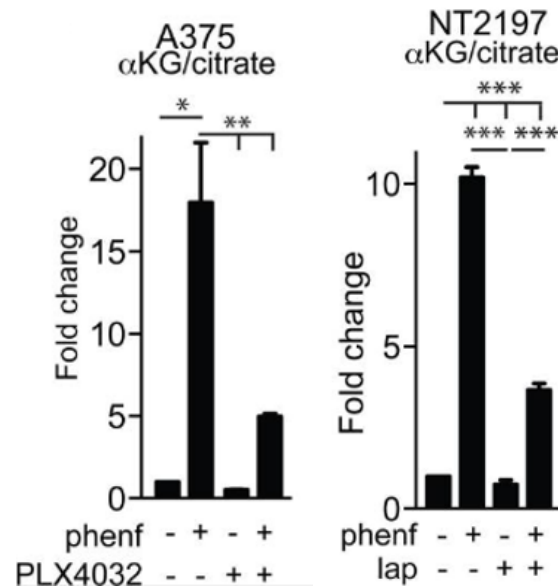


Fig. A.1.4C Kinase inhibitors opposes phenformin-induced changes in reductive glutamine metabolism. A375 and NT2197 cells were treated with phenformin and/or kinase inhibitors (i.e., A375 cells were treated with PLX4032 while NT2197 cells were treated with lapatinib) for a day. Intracellular α -KG/citrate ratios were determined by GC-MS and presented relative to DMSO-treated control cells. Results are presented as mean values +/- the SD. Figure is from (3).

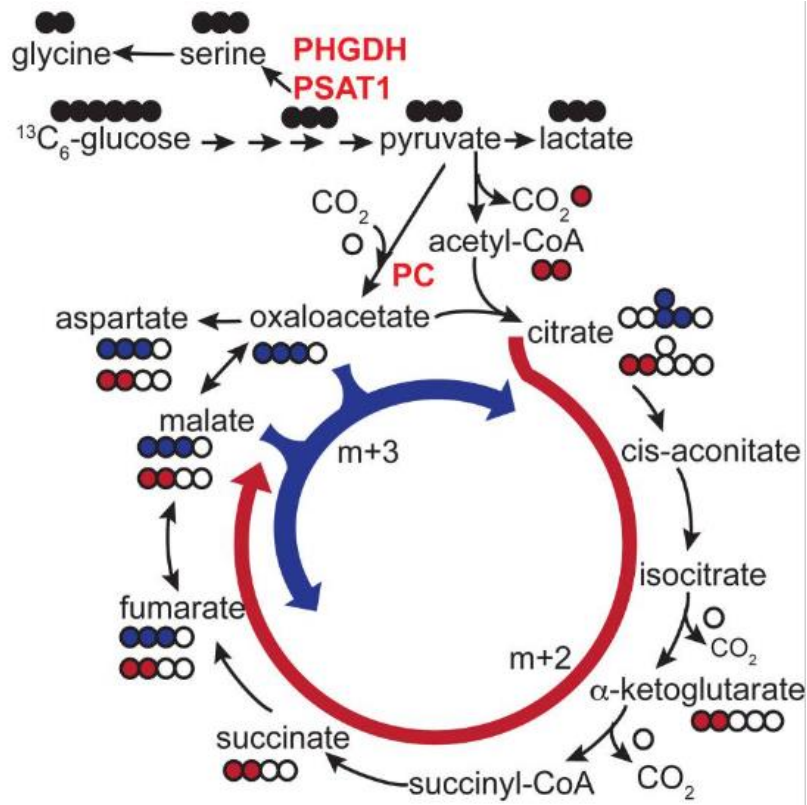


Fig. A.1.4D Schematic of ^{13}C metabolite labeling patterns after incubating cells with $^{13}\text{C}_6$ -glucose. ^{13}C are denoted by filled circles. Red cycle is the forward direction of the CAC giving (m+2) intermediates (Red-filled circles). Blue cycle begins with pyruvate carboxylase (PC) and is characterized by (m+3) intermediates (Blue-filled circles). Figure is from (3).

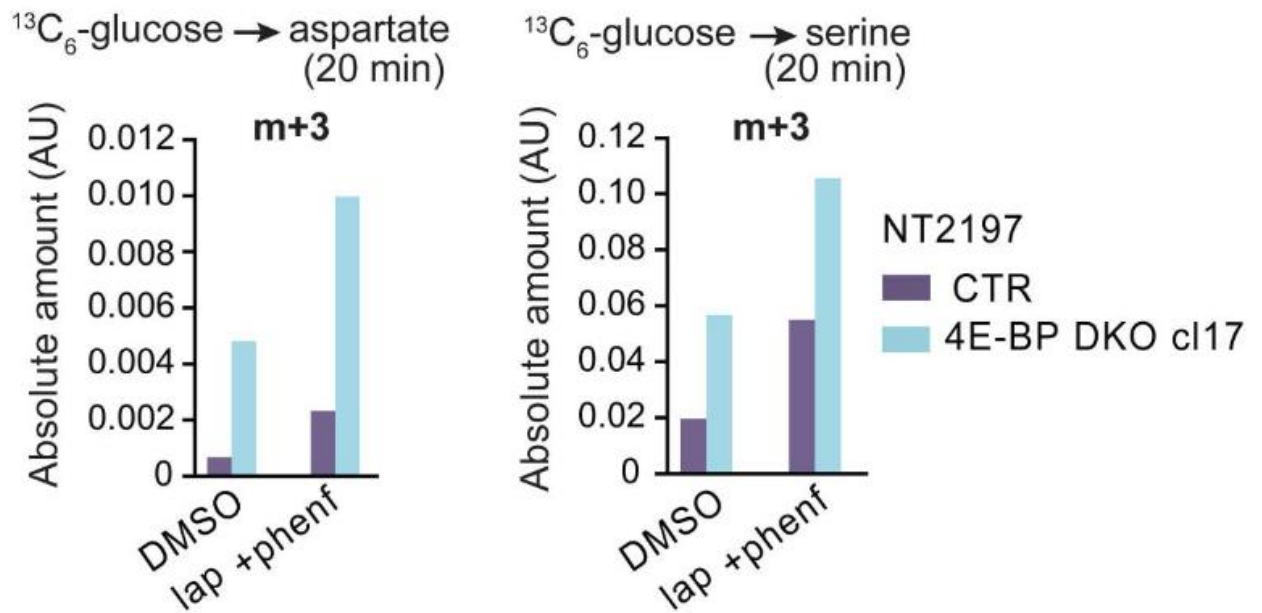


Fig. A.1.4E Deletion of 4E-BP in NT2197 cooperates with lapatinib and phenformin drug-induced increases in the levels of aspartate and serine. Abrogation of 4E-BP NT2197 WT and 4E-BP KO cells were incubated with $^{13}\text{C}_6$ -glucose for 20 min. SITA were performed and the absolute levels of aspartate (m+3) and serine (m+3) ions are shown. Data are representative of 2 independent experiments. AU: arbitrary units. Figure is from (3).

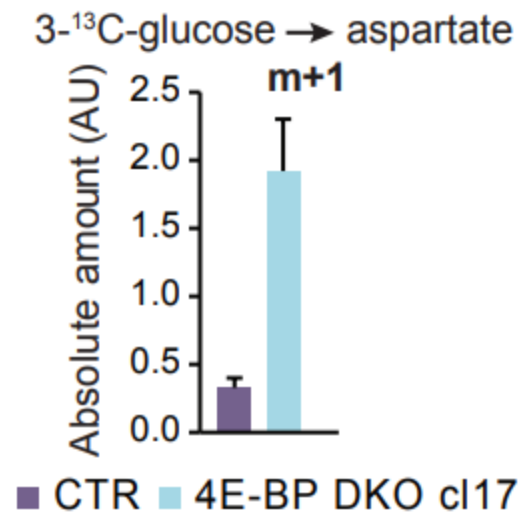


Fig. A.1.4F Loss of 4E-BP increases the activity of PC in NT2197 cells. NT2197 WT and 4E-BP KO cells were incubated with 3-¹³C-glucose for 10 min. SITA were performed and the absolute levels of (m+1) ions of aspartate are shown. Data represents the average of 3 independent experiments +/- SD. AU: arbitrary units. Figure is from (3).

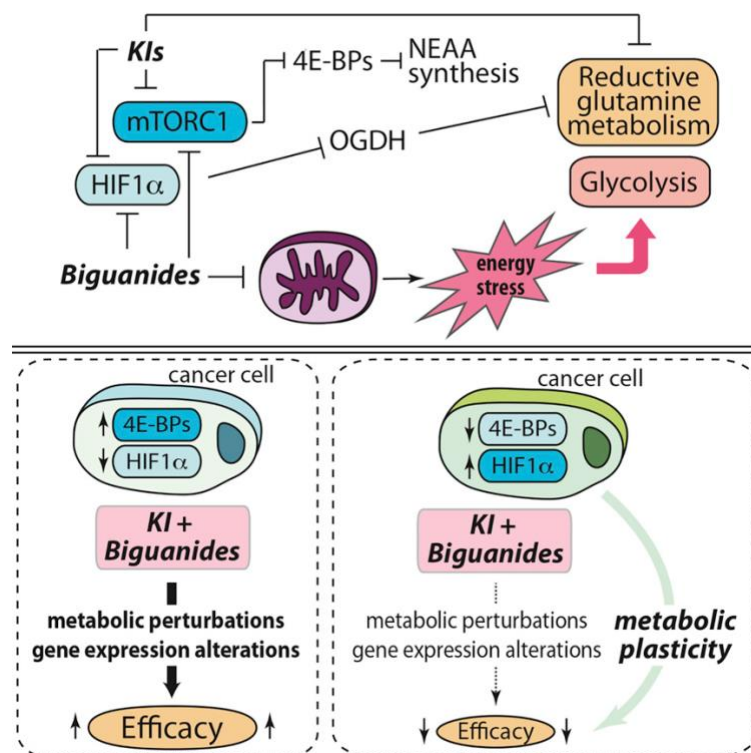


Fig. A.1.4G Schematic of the molecular mechanisms underlying the synergic efficacy of kinase inhibitors (KI) and biguanides in treating cancer cells. Cancer cells exhibit metabolic plasticity mediated by mTORC1/4E-BP1 and HIF-1 α . Drug combination treatments with KIs and biguanides can limit such metabolic plasticity. However, cancer cells can activate non-redundant adaptive mechanisms which allows them to survive the drug combinations. Figure is from (3).

A.2 CONCLUSION:

In this chapter, I described a number of collaborative studies in which I participated both experimentally and conceptually. Altogether, these studies provided hitherto unappreciated insights into mechanisms of metabolic reprogramming required to fuel neoplastic growth that also provide cancer cells with flexibility to adapt to fluctuations in nutrient and oxygen availability as well as to overcome therapeutic insults. Moreover, these findings highlighted previously unknown mechanisms of action of drugs targeting cancer metabolism and associated signaling pathways. Finally, these results in conjunction with a bevy of other recent studies highlighted the central role of metabolic plasticity in cancer maintenance, progression and therapeutic responses (54). Notwithstanding these advances in the field, there is still much to learn about the mechanisms governing metabolomes of cancer cells. Future research efforts addressing gaps in knowledge pertinent to the metabolic plasticity of neoplasia are thus required to provide the molecular basis for more efficient therapeutic strategies that take advantage of metabolic differences between normal and malignant cells.

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