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The Multifaceted Contributions of Mitochondria to Cellular Metabolism

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Abstract

Although classically appreciated for their role as the powerhouse of the cell, the metabolic functions of mitochondria reach far beyond bioenergetics. Mitochondria catabolize nutrients for energy, generate biosynthetic precursors for macromolecules, compartmentalize metabolites for the maintenance of redox homeostasis, and function as hubs for metabolic waste management. We discuss the importance of these roles in both normal physiology and in disease.

Introduction

The transition to a highly oxidizing atmosphere in early earth development created a selective pressure that favored organisms with respiratory capacity[1,2] including heterotrophic anaerobes, which consumed aerobic prokaryotic microbes (protomitochondrion).[3] Following endosymbiosis, mitochondrial signals have been synchronized with the eukaryotic cell[4]. This integral relationship is demonstrated by the compartmentalized nature of cellular metabolism, in which mitochondrial reactions are required components of metabolic pathways.

Mitochondria coordinate cellular adaptation to stressors such as nutrient deprivation, oxidative stress, DNA damage and ER stress.[5] Although long known to be critical for bioenergetics, emerging research shows that mitochondrial metabolism is multifaceted, mirroring their diverse functions. In addition to ATP, mitochondria produce metabolic precursors for macromolecules such as lipids, proteins, DNA and RNA. Mitochondria also generate metabolic by-products, such as reactive oxygen species (ROS) and ammonia, and possess mechanisms to clear or utilize waste products.

In this review, we discuss the metabolic functions of mitochondria as bioenergetics powerhouses, biosynthetic centres, balancers of reducing equivalents, and waste management hubs. Metabolic compartmentalization is instrumental for mitochondria to perform these functions. We highlight how mitochondrial metabolism supports their diverse functions in cell biology and how metabolism is compartmentalized in normal physiology and disease. A deeper understanding of mitochondrial contributions to metabolism will

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further elucidate their roles in disease and may reveal co-dependent pathways to target in therapies.

Mitochondria are the powerhouses of the cell

Cells consume fuels such as sugars, amino acids and fatty acids to generate energy in the form of ATP and GTP.[6] Nutrients are metabolized and shuttled into the tricarboxylic acid (TCA) cycle, and through iterative oxidations, electrons are stored in the reducing equivalents NADH and $FADH_2$.[6] These carriers deposit electrons into the electron transport chain (ETC) in the Inner Mitochondrial Membrane (IMM), and use electron flow to pump protons into the intermembrane space.[7,] Protons flow down their electrochemical gradient through F_1F_0-ATP synthase to generate ATP.[8] Whereas oxidative phosphorylation is the largest source of cellular ATP, the potential energy generated by the ETC is also harnessed for biosynthetic purposes. Many diseases arise when the ETC is perturbed.[9,10] We discuss how mitochondria integrate fuel metabolism to generate energy for the cell, encompassing both classical and unconventional fuel sources (Figure 1).

Pyruvate

Pyruvate is generated by a number of sources, depending on nutrient availability and tissue, including glucose catabolism (thought to be a major source), and lactate.[11–13] Pyruvate utilization in the cytosol versus mitochondria is one of the clearest examples of how compartmentalization is a major determinant of cellular bioenergetics. In healthy tissue, the fate of pyruvate is dependent on oxygen availability and mitochondrial respiratory capacity.[14] In normoxia, pyruvate is generated via glycolysis and transported across the IMM through the Mitochondrial Pyruvate Carrier (MPC).[15,16] Pyruvate is further catabolized inside mitochondria through the TCA cycle. During hypoxia, mitochondrial respiration is repressed, causing cells to adaptively sink electrons onto pyruvate through lactate dehydrogenase (LDH), generating lactate in the cytosol.[17] This pathway is engaged in muscle during exercise, the intestines, and the renal medulla of the kidneys.[18–20] Otto Warburg observed that cancer cells rewire glucose metabolism for lactate synthesis even in normoxia, known as the Warburg Effect.[14,21] Additional studies must be performed to determine the net catalytic activity of LDH in tumors, given that metabolic tracing studies in lung cancer patients have demonstrated that lactate is a major source of TCA cycle intermediates.[13] The extent of LDH-mediated pyruvate production may depend on *in vitro* versus *in vivo* models of tumor metabolism, emphasizing the need to test metabolic flux *in vivo*.

The critical role of pyruvate compartmentalization in bioenergetics and metabolism is highlighted by recent elegant studies of the MPC.[15,16] Pharmacological inhibition of MPC represses mitochondrial pyruvate uptake, shifting reliance to glycolysis for ATP production. This shift is evident in cancer cells, which repress MPC1 to promote the Warburg Effect, and in myocytes of diabetic mice, which elevate glucose consumption in response to MPC inhibition[22,23] Suppression of MPC accelerates proliferation in intestinal stem cells,[24] suggesting that the role of MPC is context-dependent and sensitive to mitochondrial respiratory capacity and/or nutrient availability.

Within mitochondria, pyruvate may enter the TCA cycle via the activity of two distinct enzymes: Pyruvate Dehydrogenase Complex (PDC), which generates acetyl CoA, and Pyruvate Carboxylase (PC), which generates oxaloacetate.[25] Although PDC and PC both catalyze the flux of pyruvate into the TCA cycle, their enzymatic activities can be distinguished by stable isotope tracing[26,27], and their metabolic roles do not appear to be interchangeable. PDC deficiency is sufficient to rewire energy metabolism towards aerobic glycolysis despite the potential adaptive node for TCA cycle anaplerosis (a process to replenish TCA cycle intermediates), mediated by PC.[28] Many cancers favor PC-mediated anaplerosis, although the factors that dictate the choice for pyruvate flux between PC and PDC are little studied.[27,29,30] Therefore, these enzymes may have important functions beyond TCA cycle flux for bioenergetics.

Glutamine and Branched Chain Amino Acids (BCAAs)

The catabolism of glutamine, the most abundant amino acid, often starts in the mitochondria and its carbon and nitrogen atoms are distributed into macromolecules throughout the cell, including TCA cycle intermediates (important in bioenergetics), amino acids, nucleotides, glutathione, and lipids.[31]

In mitochondria, glutaminase (GLS) converts glutamine into glutamate and ammonia. Either transaminase or glutamate dehydrogenase (GDH) converts glutamate into α-ketoglutarate.[32,33] Glutamine anaplerosis sustains TCA cycle intermediates in conditions of limiting glucose and MPC inhibition, demonstrating the potential flexibility of these metabolic nodes.[34,35] Glutamine anaplerosis is critical for meeting the energetic requirements of proliferative cells, such as T cells during the transition from quiescent naïve T cells to effector cells, and in cancers, particularly with MYC elevation.[32,36,37] GLS inhibition suppresses proliferation, and GLS inhibitors are being evaluated in clinical studies for a number of cancers.[31,38,39] However, sensitivity to GLS inhibition *in vitro* is not always consistent *in vivo,* and is dependent on extracellular cystine levels.[40] This emphasizes the need for investigators to study the effect of the microenvironment on metabolic dependencies and to validate experiments *in vivo*.

Although glutamine transporters at the plasma membrane have been identified,[41] the mitochondrial glutamine transporter has not been fully characterized.[42,43] This critical area of research is challenging to address because there are likely multiple mechanisms for glutamine import.

The BCAAs leucine, isoleucine, and valine are a major source of cellular energy via acetyl CoA and succinyl CoA generation.[44] The tissue of origin dictates dependency on BCAA catabolism in normal physiology and in cancer.[45] In normal physiology, myocytes and adipocytes activate mitochondrial BCAA catabolic enzymes to support ATP production during exercise or fasting and differentiation, respectively.[46,47] BCAA catabolism is repressed in maple syrup urine disease, which is caused by mutations to branched-chain keto acid dehydrogenase (BCKDH) and causes dysfunction of immune cells, skeletal muscle and the central nervous system.[48] Although mitochondrial BCAA catabolism is critical in these pathologies, it is unknown how BCAAs are imported into the mitochondria. Identifying their

transport mechanisms will be critical to our understanding of mitochondrial BCAA catabolism in cellular homeostasis.

Fatty Acid Oxidation

Palmitate, a 16-carbon fatty acid (FA), stores 39KJ/g of energy compared to 16KJ/g stored in glucose.[49] Therefore, FAs are a major source of cellular energy, particularly under conditions of nutrient stress. Mitochondrial FA import is a rate-determining step for fatty acid oxidation (FAO) and demonstrates how metabolic compartmentalization adapts to cellular state. As long chain FAs are unable to cross mitochondrial membranes, mitochondria have evolved an intricate set of reactions and transporter activities to allow fat to access mitochondrial β-oxidation machinery. The outer mitochondrial membrane (OMM) enzyme carnitine palmitoyl transferase 1 (CPT1) forms acylcarnitines from fatty acyl CoAs.[50] Acylcarnitines are shuttled into mitochondria through the carnitine–acylcarnitine translocase (SLC25A20) in the IMM. CPT2 liberates FA from carnitine, initiating FAO.[51] Acetyl CoA from FAO is used for the TCA cycle as well as for aspartate and nucleotide synthesis.[52]

CPT1 activity is tightly controlled by a network of metabolites, linking it to cellular nutrient status. Malonyl CoA, generated by the enzyme acetyl CoA carboxylase (ACC), represses CPT1 to inhibit acylcarnitine import.[53] Malonyl CoA is the initiating metabolite for FAS, and its levels dictate the balance of fat synthesis or oxidation within a cell. In low energy conditions, AMP-activated protein kinase (AMPK) phosphorylates and inhibits ACC, decreasing malonyl CoA and increasing CPT1 activity.[54] ACC2 is also hydroxylated by the dioxygenase prolyl hydroxylase 3 (PHD3).[55] Hydroxylation promotes ACC2 activity in nutrient abundance. These enzymes are altered in some cancers and human diseases as the mechanism that dictates fat utilization. PHD3 is suppressed in cancers that rely on FAO, such as AML and prostate cancer, and elevated in cancers that rely on FAS such as breast and non-small-lung-cell cancer.[55–57] Reciprocally, AMPK is linked to fat utilization in diseases and cancers.[58,59]

The dynamic regulation of FAO is key to cellular physiology. FAO is fundamental for the survival and function of memory $CD8^+$ T cells, unlike effector cells that rely on glycolysis and glutaminolysis for energy.[60,61] Likewise, FAO is activated in insulin resistance, in which free fatty acids provide a compensatory fuel for repressed glucose uptake.[62,63]

Mitochondria are biosynthetic hubs

Mitochondria participate in the biosynthesis of nucleotides, FAs, cholesterol, amino acids, glucose, and heme (Figure 2).[64] These biosynthetic pathways are engaged in stress responses, and are often mis-regulated in disease.[5] Rather than being dysfunctional, highly proliferative cells such as cancer cells and activated T cells rely on mitochondrial metabolites to form biomass.[5,65] Below we review the mitochondrial compartmentalization of anabolic pathways and its role in cell stress responses and disease.

Nucleotides

The 1C metabolic pathway involves a set of reactions that generate and transfer activated one carbon (1C) units for *de novo* nucleotide synthesis, compartmentalize amino acids, and contribute to redox homeostasis. The co-factor tetrahydrofolate (THF) is the carrier that mediates 1C transfer reactions for *de novo* nucleotide synthesis.[66,67] Activated THF molecules are generated through an oxidative/reductive cycle that catabolizes serine (to generate glycine) in the mitochondria and synthesizes serine in the cytosol.

The carrier SLC25A32 imports THF into the mitochondria, where it is converted by serine hydroxymethyltransferase (SHMT2) into 5,10 methylene-THF and glycine. Like many enzymes in 1C metabolism, SHMT2 is bi-directional. SHMT2 favors production of glycine and 5,10 methylene-THF, and cells deficient in mitochondrial 1C metabolism are glycine auxotrophs.[68] In the absence of SHMT2, cytosolic SHMT1 reverses flux to compensate[69] demonstrating how metabolic flexibility among subcellular compartments is critical to stress adaptation.

Mitochondrial methylenetetrahydrofolate dehydrogenase (MTHFD2) converts 5,10 methylene-THF to 10-formyl-THF. MTHFD2 expression is regulated by mTORC1, and is critical for growth and proliferation.[70] MTHFD2 is overexpressed in many human cancers[71] and mitochondrial biogenesis and SHMT2/MTHFD2 expression are promoted during T-cell activation to support proliferation.[72] 10-formyl-THF has multiple fates: conversion into THF by 10-formyl-THF dehydrogenase, production of formyl-methionine for mitochondrial translation, or hydrolyzation to formate by MTHFD1L. Mitochondrial contributions to this pathway are critical, as mitochondrial formate is the main carbon source for cytosolic 1C metabolism [66]

The IMM enzyme dihydroorotate dehydrogenase (DHODH), which oxidizes dihydroorotate to orotate, is required for *de novo* pyrimidine synthesis.[73] Consistent with their reliance on 1C metabolism, T cells require DHODH for clonal expansion and differentiation into effector cells.[74] DHODH is targeted in autoimmune disorders and inhibition suppresses myeloid differentiation of AML cells.[75] DHODH activity is also elevated in response to DNA damage and upon genotoxic chemotherapy treatment to increase nucleotide synthesis for DNA repair.[76,77]

Citrate

In addition to generating electron carriers for the ETC, TCA cycle intermediates such as citrate regulate anabolic reactions. Mitochondrial citrate controls anabolic reactions by directly acting as the carbon source for FAs, cholesterol and ketone bodies through ATP citrate lyase (ACLY),[78] and by allosteric modulation. Citrate is generated by citrate synthase (CS) or through the reduction of α-ketoglutarate by isocitrate dehydrogenase (IDH).[79–81] Mitochondrial citrate is exported by the malate-citrate antiporter SLC25A1.[82] In the cytosol, citrate is converted to acetyl CoA via ACLY, which can access many pathways, including conversion to malonyl CoA by the activity of ACC (as described above). Cytosolic citrate is a potent allosteric regulator of ACC by increasing its polymerization and activity.[83]

Regulation of citrate export may provide a physiological node for the cell to communicate lipid homeostasis to the mitochondria. SLC25A1 is sensitive to membrane rigidity, and high levels of cholesterol or acidic phospholipids in the IMM repress mitochondrial citrate export.[84] Moreover, fasting causes a 40% reduction in mitochondrial citrate export.[85] Although these studies indicate that citrate export is affected by lipid abundance, it is unknown if repression of SLC25A1-mediated citrate export affects ACC2 polymerization and FAS initiation.

Acetyl CoA is required for epigenetic modifications such as histone acetylation.[86–88] Thus, fat metabolism may be intimately linked with the epigenetic state, although it is unknown whether the connection is direct. The emerging role of mitochondrial metabolism in epigenetic reprogramming may extend beyond acetyl CoA to include other mitochondrial metabolites such as succinate, fumarate, and ROS, which directly affect the activity of Fe (II)/α-KG-dependent dioxygenases, including hydroxylases, DNA demethylases and histone demethylases.[89]

Amino Acids

The mitochondria is a hub for amino acid synthesis, including glutamine, glutamate, alanine, proline, and aspartate. Glutamine synthetase (GS) condenses glutamate and ammonia to make glutamine.[90] GS has been reported to have activity in cytosol and mitochondria, and its biological role may differ depending on its subcellular localization. GS has a "weak" mitochondrial localization sequence and is imported into the mitochondria in the liver, whereas GS is cytoplasmic in astrocytes.[91] In glioblastoma, GS generates a source of glutamine for *de novo* purine synthesis.[92] However, in breast cancer cells, GS-derived glutamine is not used for *de novo* nucleotide synthesis.[93] One possible explanation for this difference is the subcellular localization of GS in these systems.

Glutamate is generated by and utilized as a nitrogen source for numerous reactions.[94] Glutamate metabolism stratifies in proliferating and quiescent cells; proliferating cells elevate the expression of glutamate-dependent transaminases, whereas quiescent cells suppress them.[95] Many of the glutamate-dependent transaminases, such as glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) have two (cytosolic and mitochondrial) isoforms.[95] It will be key for future studies to elucidate the role of subcellular compartmentalization of glutamate metabolism in proliferation.

Proline and ornithine metabolism are centrally mitochondrial. The mitochondrial enzyme Pyrroline-5-carboxylate synthase (P5CS) generates pyrroline-5-carboxylate (P5C), which can be used for proline and ornithine production.[96] Ornithine is made by ornithine amino transferase (OAT) and proline is produced through reduction of P5C by Pyrroline-5-carboxylate reductase (PYCR). The mechanisms underlying compartmentalization of proteinogenic amino acids, such as proline and glutamate are little studied.[97]

Gluconeogenesis

Gluconeogenesis is predominantly a cytosolic process, although the initiating step by PC occurs inside the mitochondria.[98] PC-derived oxaloacetate is converted to malate and exported from the mitochondria for the remaining steps of gluconeogenesis.[99] This export

can occur through SLC25A1 (citrate-malate antiporter), SLC25A11 (α-ketoglutarate-malate antiporter) or SLC25A10 (dicarboxylate-phosphate antiporter).[100] The dominant mechanism for malate export in gluconeogenesis is unknown. Furthermore, it is unclear if metabolic stressors such as nutrient deprivation or hypoxia dictate this mechanism. In the cytosol, phosphoenolpyruvate carboxykinase (PCK) converts oxaloacetate into phosphoenol pyruvate (PEP) for gluconeogenesis.[101] The mitochondrial isoform of this enzyme, PCK2, has no known connections to gluconeogenesis.[101]

Heme

Heme metabolism illustrates an extraordinary example of metabolic compartmentalization. The committed step of the pathway is catalyzed by mitochondrial aminolevulinate synthase (ALAS), which generates ALA from glycine and succinyl CoA.[102] ALA is exported via SLC25A38 and, through four cytosolic reactions, is converted into coproporphyrinogen III (CPGIII). Next, CPGIII enters the intermembrane mitochondrial space through the ATP-dependent transporter ABCB6 for further catalysis by coproporphyrinogen oxidase (CPOX).[103] The intermembrane space is a region in which few metabolic reactions occur. The terminal step of heme synthesis is in the mitochondrial matrix, in which ferrochelatase (FECH) catalyzes the insertion of ferrous iron into the macrocycle.[104] As heme biosynthesis generates H_2O_2 in the intermembrane region, we speculate that there may be direct links between heme metabolism and ROS-sensitive signaling pathways.

Mitochondria balance redox equivalents

The mitochondria and cytosol have distinct requirements for NAD^+, and proper compartmentalization of redox equivalents is crucial for maintenance of cellular homeostasis and survival in response to environmental stressors.[105–107] The cytosol is a more oxidizing environment in which the NAD^+/NADH ratio ranges between 60-700.[108] Conversely, mitochondria employ more reductive metabolic reactions, and the NAD^+/NADH ratio is approximately 7-8.[108] To sustain the imbalanced distribution of NAD, mammalian cells engage indirect pathways (Figure 3) because there is no known mammalian transporter for NAD^+ or NADH, contrary to yeast which facilitate NAD transport through NDT1.[109]

Malate-Aspartate Shuttle

The malate-aspartate shuttle is ubiquitously engaged to generate cytosolic NAD^+ and mitochondrial NADH.[110] This cycle involves an oxidation or reduction catalyzed by malate dehydrogenase (MDH1: cytosolic, MDH2: mitochondrial), a transamination catalyzed by glutamate-oxaloacetate transaminase (GOT1:cytosolic, GOT2: mitochondrial), and two antiporters localized to the IMM (aspartate-glutamate antiporter AGC and malate α-ketoglutarate antiporter MαA).[111] Compartmentalization of reducing equivalents through the malate-aspartate shuttle is key for survival in stress conditions such as exercise, in which cytosolic NAD^+ is required to promote glucose catabolism and mitochondrial NADH for ATP production.[112] Moreover, in PDAC cancers with oncogenic KRAS, glutamine is fluxed through the malate-aspartate shuttle to raise the NADPH/$NADP^+$ ratio for glutathione synthesis.[113] When oxidative phosphorylation is repressed, cells utilize the reverse flux of

GOT1 to generate aspartate[114,115]. In addition to its regulation of redox balance, the malate-aspartate shuttle may also contribute to cellular amino acid compartmentalization.

Citrate-Malate Shuttle

In contrast to the malate-aspartate shuttle, the citrate-malate shuttle functions equally (with respect to reducing equivalents), but is less studied in the context of disease. Similar to malate-aspartate shuttle, the citrate-malate shuttle utilizes both isoforms of MDH. However, MDH activity is paired with CS, ACLY, and the malate-citrate antiporter (CIC).[116] Rather than elevating cytosolic aspartate, the citrate-malate shuttle increases cytosolic citrate levels. Therefore, flux through the citrate-malate shuttle promotes FAS through citrate compartmentalization.[117] Thus, although both the malate-aspartate and citrate-malate shuttles balance reducing equivalents through MDH activity, these shuttles are not interchangeable. The implications of cytosolic citrate accumulation in the malate-citrate shuttle are yet to be defined beyond FAS. For example, flux through the citrate-malate shuttle may also affect epigenetics through ACLY activity and acetyl CoA production.[88]

α-glycerophosphate Shuttle

The α-glycerophosphate shuttle is a unique redox balancing pathway, which intersects the mitochondria but does not directly affect mitochondrial NAD/NADH.[118] The α-glycerophosphate shuttle is composed of cytosolic and mitochondrial α-glycerophosphate dehydrogenase (cGPDH and mGPDH). In this cycle, cGPDH utilizes NADH to reduce dihydroxyacetone phosphate (DHAP) to glycerophosphate (GAP) and generate cytosolic NAD^+. GAP is subsequently oxidized to DHAP by the flavin-dependent mGPDH, which directly deposits electrons into the ETC. The α-glycerophosphate shuttle is tightly linked to glycolysis and is highly active in brown adipose tissue (BAT) to regenerate cytosolic NAD^+ while simultaneously sinking electrons into the ETC for thermogenesis.[118] As this pathway is engaged in highly glycolytic cells, it would be interesting for future studies to investigate the potential role of this redox shuttle in cancer.

One Carbon Metabolism

MTHFD is among the largest contributors to cellular NADPH, in addition to the pentose phosphate pathway and malic enzyme (ME).[119] MTHFD isozymes are bi-directional, however, stable isotope tracing of NADPH revealed that the mitochondrial MTHFD favors NADPH production, and the cytosolic isoform favors $NADP^+$ production.[120] The 1C metabolic pathway is an adaptive mechanism to survive oxidative stress. Upon ETC inhibition, flux through the mitochondrial arm of 1C metabolism is activated for NADPH/$NADP^+$ balance.[121] NADPH is required for reduction of glutathione for clearance of ROS. In cancer cells, flux through the mitochondrial 1C pathway generates cytosolic NADPH for FAS.[122]

Mitochondria orchestrate waste management

The by-products of metabolic reactions are often depicted as waste. However, emerging studies have revealed a functional role for metabolic by-products such as lactate, ammonia, ROS and hydrogen sulfide (H_2S).[12,13,93,123,124] The study of metabolic by-products is a

growing area of research, especially in cancer, in which metabolic by-products accumulate in the tumor microenvironment (TME)[125] (Figure 4A). Mitochondria are indispensable in cellular waste management (Figure 4B–D). Below, we review the pathways that mitochondria utilize to re-purpose cellular waste.

Ammonia

Ammonia is generated in mammalian cells by amino acid lyases and nucleotide deaminases, however, the largest contributor to ammonia in mammals is the microbiome.[126] Ammonia is a neurotoxin that is sustained below 50 μM in plasma of healthy adults, and can induce seizure when plasma levels become elevated.[122] Moreover, high ammonia may induce autophagy in some cultured cells.[127,128] To evade toxicity, mammalian cells possess three ammonia-assimilating enzymes: carbamoyl phosphate synthetase 1 (CPS1), GS, and GDH.

The urea cycle is a sink for ammonia, ultimately generating urea, which cannot be metabolized by mammalian enzymes. CPS1 is the rate-limiting step of the urea cycle, generating carbamoyl phosphate (CP).[129] N-acetyl glutamate (NAG) is an essential activator of CPS1, and congenital NAGS mutations cause hyperammonemia.[130] CP is condensed with ornithine by ornithine carbamoyltransferase (OTC) to generate citrulline, which is exported through ORNT1, the citrulline-ornithine antiporter for the remaining steps of the cycle. Interestingly, in KRAS/LKB1 mutant cancer, CP from CPS1 is diverted into *de novo* pyrimidine synthesis.[131] The mechanism of CP export from the mitochondria is unknown and may be a potential therapeutic target.

Although urea is a metabolic waste product for mammalian cells, urease-positive bacteria in the microbiome re-catabolize 15-30% of urea to regenerate ammonia.[132] Consequently, similar to congenital mutations in urea cycle enzymes, the microbiome can contribute to hyperammonemia.[126,133] Beyond ammonia metabolism, many microbial metabolites intersect host biology and their roles remain an active area of research.[134]

GDH and GS assimilate ammonia, generating glutamate and glutamine. Glutamate contributes to the urea cycle through conversion to aspartate by GOT2 and mitochondrial export via AGC1/2. GDH is a bidirectional enzyme, and high ammonia levels reverse the direction of GDH, favoring the reductive activity.[135] This bi-directionality is particularly relevant in breast cancers, as ammonia accumulates in the TME, driving GDH towards glutamate synthesis.[93] Beyond the TME, physiological niches with high ammonia levels (the microbiome, liver, and kidneys) may promote the reductive activity of GDH. Additionally, GDH-mediated ammonia assimilation requires NAD(P)H and therefore may contribute to redox balance.

ROS

Mitochondria generate, sequester and interconvert ROS in response to stressors such as hypoxia, nutrient availability, cytokine stimulation and changes in mitochondrial membrane potential.[136] ROS are generated from the reduction of oxygen (O_2) to superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). Mitochondrial ROS are generated in reactions such as NADPH oxidase (NOX4) and the Fenton Reaction, and through electron

leak from ETC complexes[123], although NOX4 is not strictly localized to mitochondria.[137] ROS are highly reactive and inflict oxidative damage to macromolecules.[138]

Mitochondria rely on ROS clearance to protect the concentrated iron-sulfur clusters in the ETC and iron-dependent enzymes such as aconitase. Superoxide dismutase (SOD2) converts superoxide into a less reactive molecule, H_2O_2.[123] Cellular H_2O_2 can be degraded to water by catalase, glutathione peroxidase (GPx), and peroxiredoxin (Prx), however mitochondria do not have catalase and only a single splice variant of GPx4 has been demonstrated to be localized to mitochondria.[139,140] Mitochondria rely on the combined activities of peroxiredoxins (Prx3 and Prx5), thioredoxins (Trx2), and thioredoxin reductase 2 (TRXR2) to decompose the locally generated H_2O_2.[141]

Beyond toxicity, ROS are potent mitogen signaling agents that foster proliferation, differentiation, and migration.[123,142] Specifically, ROS oxidize cysteine residues, linking mitochondria to signaling cascades. ROS inactivates the catalytic cysteine of phosphatase 1B (PTP1B), enabling receptor tyrosine phosphorylation required for growth-factor signaling.[143] ROS inactivate PTEN, which represses the PI-3 Kinase/AKT signaling cascade and PHDs to repress HIF hydroxylation.[144,145] In breast cancer, low levels of the mitochondrial sirtuin 3 promote HIF stabilization through ROS, stimulating the Warburg Effect.[146] In macrophages, mROS promote the antibacterial innate immune response, and mice harboring mROS-deficient macrophages are susceptible to infection.[147] Similarly, mitochondria provide ROS for B-cell and T-cell activation.[148,149] ROS are thus critical to proliferating systems.

Hydrogen Sulfide

H_2S is produced in the microbiome by sulfur-reducing bacteria and by mammalian cells through cystathionine β synthase (CBS), cystathionine γ lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST).[150] H_2S-producing enzymes are localized to the cytosol and mitochondria, depending on the tissue type.[124]

High levels of H_2S are toxic and repress respiration through complex IV inhibition.[137] To dampen H_2S toxicity, mitochondria sequentially oxidize H_2S generating thiosulfate, sulfite, and ultimately sulfate.[151] The first and last reactions catalyzed by flavin-dependent sulfide quinone reductase (SQR) and sulfite oxidase (SO) directly deposit electrons onto Coenzyme Q (CoQ) of the ETC.[136] In CoQ deficiency, H_2S oxidation is significantly repressed.[152] The intermediate oxidation step of H_2S is catalyzed by thiosulfate reductase (TR) and requires oxidized glutathione as an electron sink. Because the enzymes for glutathione synthesis are cytosolic, mitochondria must import glutathione for this process. Glutathione can utilize the dicarboxylate carrier SLC2510 and the α-kg carrier SLC25A11 for import, although a selective mechanism of transport remains unknown and may be pivotal for H_2S clearance.[153]

H_2S metabolism is directly linked with oxidative phosphorylation.[154] Hypoxia represses H_2S detoxification through respiratory chain inhibition.[124] Interestingly, the microbiome, which has the highest H_2S levels, is hypoxic in some regions.[155] The mechanism for H_2S tolerance in the microbiome remains unknown. H_2S production and clearance may be critical in diseases such as cancer and diabetes, which are associated with altered respiration.

Future directions of mitochondrial metabolism in cellular homeostasis and disease

Here we discuss the multifaceted contributions of mitochondria to cell metabolism as bioenergetic powerhouses, biosynthetic centres, balancers of reducing equivalents and waste management hubs. Although mitochondrial pathways are well defined, the mechanisms by which metabolites are compartmentalized remain elusive. Identifying the transporters that coordinate metabolic flux for key pathways such as amino acid and glutathione import will be important directions for future research[156]. Given that mitochondrial metabolism is critical to many diseases, transporters that enable metabolic compartmentalization may be promising therapeutic targets[5,65,157–159]. It will also be key to consider mitochondrial metabolite concentrations, which differ from whole cell concentrations[160], to better inform the kinetics of mitochondrial enzymes under different cellular stress conditions and in disease. Mitochondrial concentrations are critical when studying bi-directional enzymes such as transaminases and enzymes in 1C metabolism.

It will be important for future studies to probe the physiological contributions of mitochondria to cell biology. Metabolism is not always comparable when studying *in vitro* and *in vivo* models. These differences may dictate the efficacy of therapies, such as the glutaminase inhibitor in cancer.[40] The extent to which a physiological niche alters mitochondrial contributions to metabolism and cell/tissue function has not been well explored. For example, metabolic by-products accumulate in the TME, increasing the necessity for cancer cells to engage waste management pathways[13,93,125]. Disparities between model systems may be avoided by performing *in vitro* studies in media with physiological metabolite concentrations, using model systems that represent the 3-dimensional architecture of the tissue being studied, and performing experiments *in vivo*[161–163].Future studies in this exciting and growing field will continue to reveal the roles of mitochondrial metabolism in cellular homeostasis and disease.

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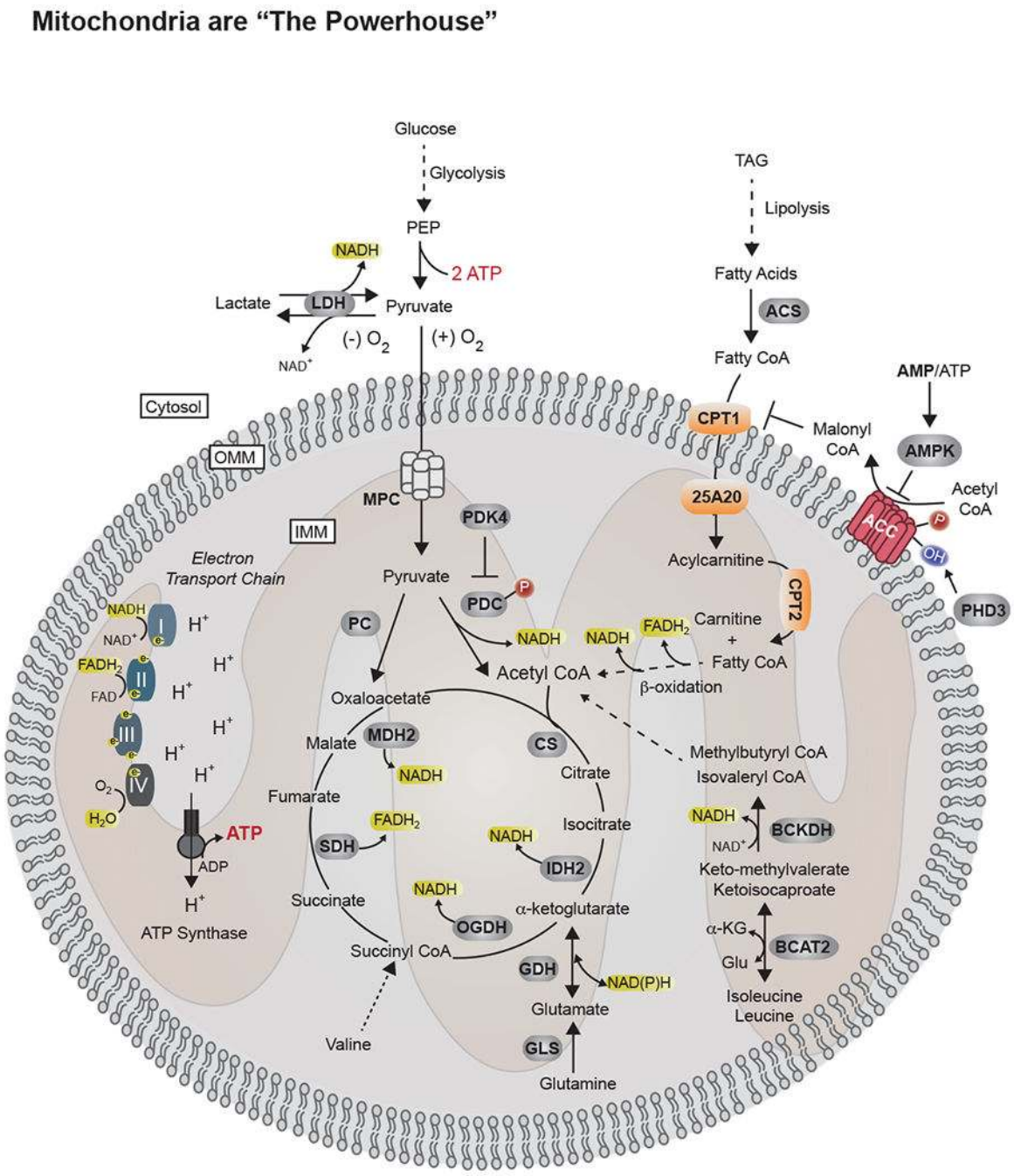


Figure 1: Mitochondria are the powerhouse of the cell.

Mitochondria integrate fuel metabolism to generate energy in the form of ATP. Mitochondria oxidize pyruvate (derived from glucose or lactate), fatty acids, and amino acids to harness electrons onto the carriers NADH and $FADH_2$. NADH and $FADH_2$ transport these electrons to the electron transport chain, in which an electrochemical gradient is formed to facilitate ATP production through oxidative phosphorylation. Enzymes have the following abbreviations: LDH: lactate dehydrogenase, VDAC: Voltage-dependent anion channel, MPC: mitochondrial pyruvate carrier, PDC: pyruvate dehydrogenase complex, PC: pyruvate

carboxylase, CS: citrate synthase, IDH2: isocitrate dehydrogenase 2, OGDH: α-ketoglutarate dehydrogenase, SDH: succinate dehydrogenase, MDH2: malate dehydrogenase 2, GLS: glutaminase, GDH: glutamate dehydrogenase, BCAT2: branched chain amino transferase 2, BCKDH: branched chain ketoacid dehydrogenase, PHD3: prolyl hydroxylase 3, AMPK: adenosine monophosphate kinase, ACC: Acetyl CoA Carboxylase, , ACS: acyl CoA synthetase, CPT1/2: carnitine palmitoyltransferase 1/2. Electrons and reducing equivalents are shown in yellow.

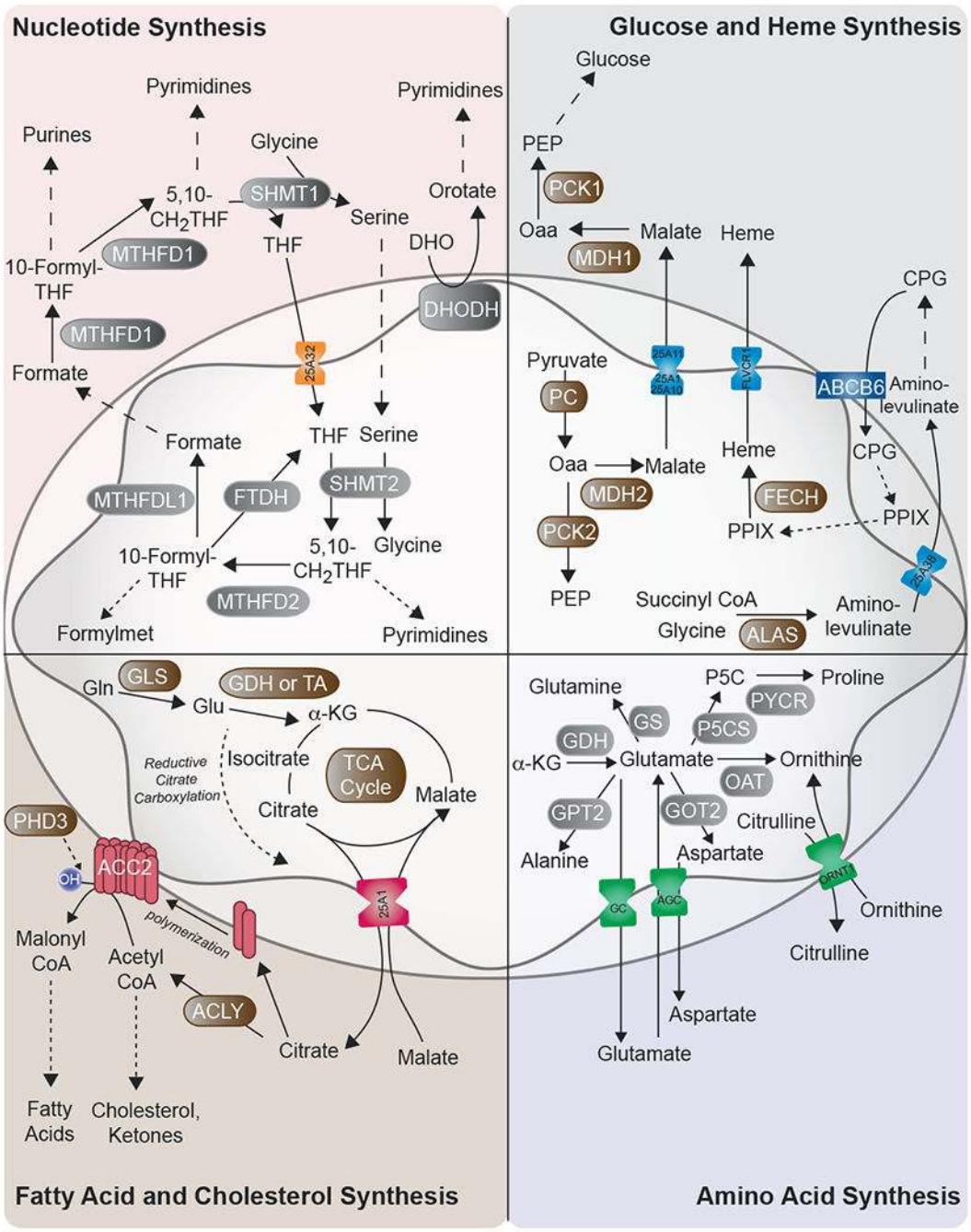


Figure 2. Mitochondria are biosynthetic hubs.

The mitochondria are a critical source of building blocks for biosynthetic pathways including nucleotide synthesis, fatty acid and cholesterol synthesis, amino acid synthesis, and glucose and heme synthesis. Compartmentalization is a key feature of biosynthetic pathways. While many of the enzymes listed are bi-directional, arrows are drawn to highlight the biosynthetic functions. Enzymes are circled in grey and brown with the following abbreviations: *Nucleotide Synthesis:* MTHFD1/2: methylenetetrahydrofolate dehydrogenase, SHMT1/2: serine hydroxymethyltransferase, DHODH: dihydroorotate

dehydrogenase, FTDH: formate dehydrogenase. *Fatty Acid and Cholesterol Synthesis:* GLS: glutaminase, GDH: glutamate dehydrogenase, TA: transaminase, ACLY: ATP citrate lyase, ACC2: acetyl CoA carboxylase, PHD3: prolyl hydroxylase 3, MPC: mitochondrial pyruvate carrier. *Amino Acid Synthesis:* GDH: glutamate dehydrogenase, GS: glutamine synthetase, P5CS: Pyrroline-5-carboxylate synthase, PYCR1: Pyrroline-5-carboxylate reductase 1, OAT: ornithine aminotransferase, GOT2: glutamate oxaloacetate transaminase 2, GPT2: glutamate pyruvate transaminase 2, GC: glutamate carrier, AGC: aspartate-glutamate carrier, ORNT1: ornithine translocator. *Glucose and Heme Synthesis:* PCK1/2: phosphoenolpyruvate carboxykinase, MDH1/2: malate dehydrogenase, PC: pyruvate carboxylase, ALAS: aminolevulinate synthase, FECH: ferrochetolase, ABCB6: ATP binding cassette subfamily B member 6, FLVCR: feline leukemia virus subgroup C receptor 1.

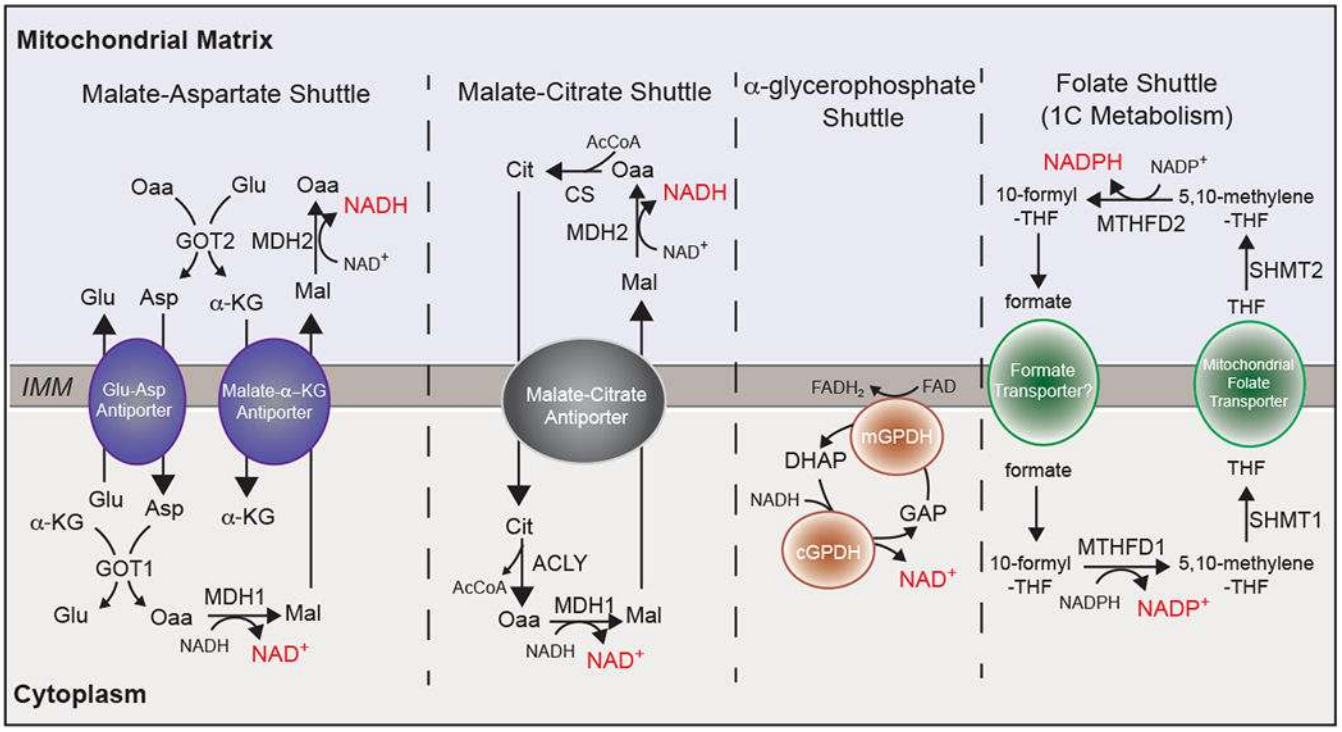
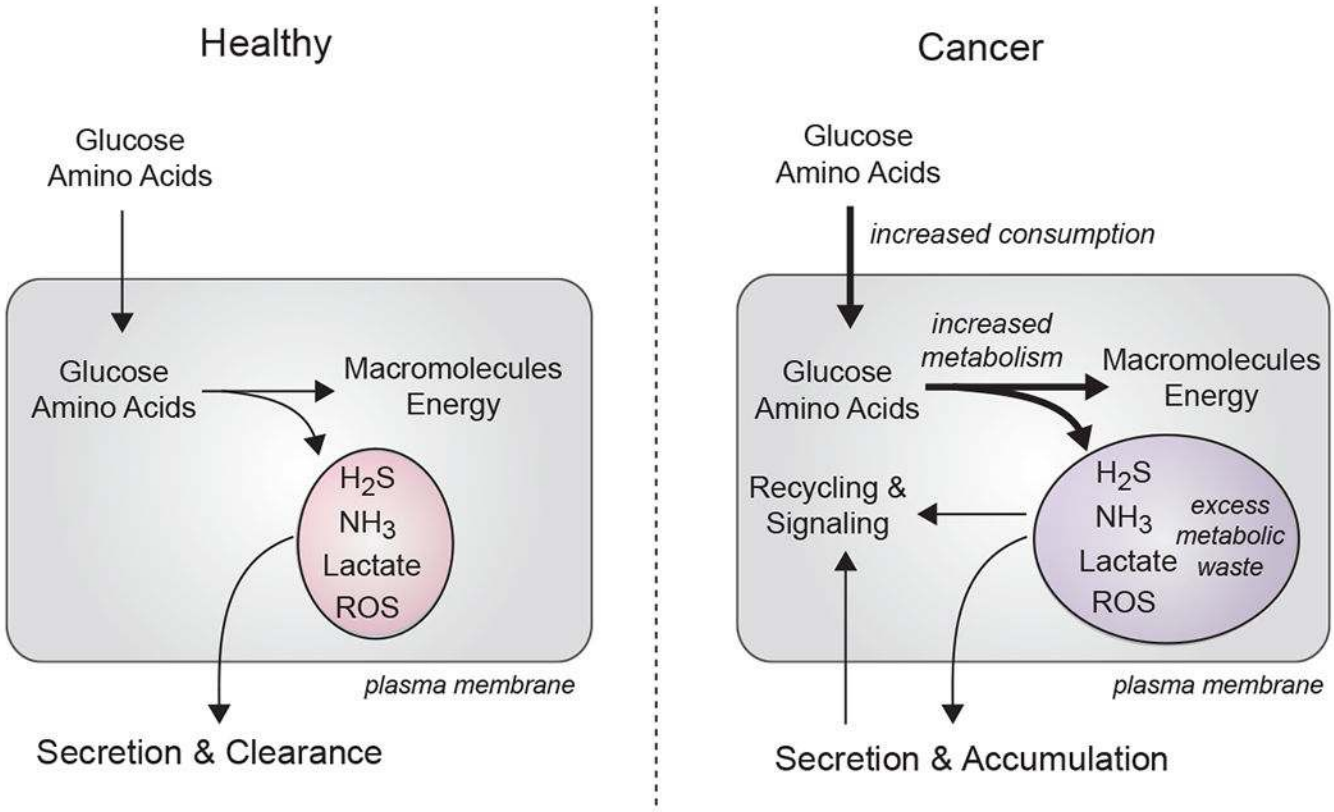


Figure 3. Mitochondria balance redox equivalents.

In the absence of a direct mode for NAD transport, cells rely on compartmentalized flux of metabolites to support balance of reducing equivalents NAD/NADH, and NADP/NADPH. Generally, redox shuttles favor cytosolic NAD^+ synthesis and mitochondrial NADH synthesis. Enzymes and transporters have with the following abbreviations: *Malate-aspartate shuttle*. GOT1/2: Glutamate oxaloacetate transaminase, MDH1/2: malate dehydrogenase, ME1: malic enzyme 1, Glu-Asp antiporter: glutamate-aspartate antiporter, Malate-α-KG Antiporter: malate-α-ketoglutarate antiporter. *Malate-citrate shuttle*. ACLY: ATP citrate lyase, MDH1/2: malate dehydrogenase, CS: citrate synthase. *α-glycerophosphate shuttle*. (m/c)GPDH: mitochondrial/cytosolic glycerol-3-phosphate dehydrogenase. *Folate Shuttle (1C Metabolism)*: MTHFD1/2: methylenetetrahydrofolate dehydrogenase, SHMT1/2: serine hydroxymethyltransferase.

a

Waste accumulates in the tumour microenvironment



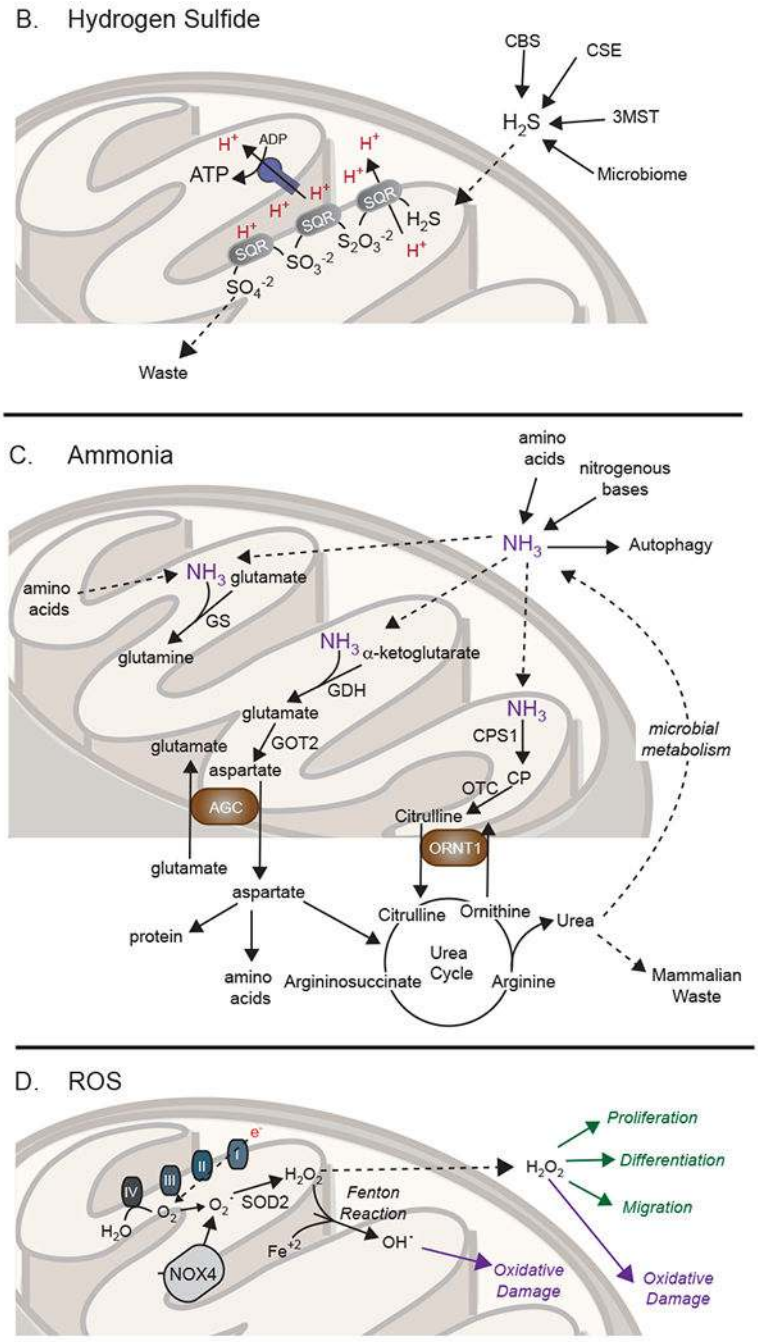


Figure 4. Mitochondria orchestrate waste management.

(A). Tumor cells increase nutrient consumption and metabolic fitness relative to healthy tissue, leading to accumulation of waste products in the tumor microenvironment. To manage metabolic waste, cancer cells engage recycling pathways for these metabolic by-products. (B). *Ammonia*. Production of and metabolic clearance of ammonia (NH_3) in cell metabolism. NH_3 is generated by amino acid and nucleotide catabolism. NH_3 is assimilated in the mitochondria through GS (glutamine synthetase), GDH (glutamate dehydrogenase), and CPS1 (carbamoyl phosphate synthase 1). CPS1 initiates the urea cycle for production of

the metabolic waste product urea. Urea can be re-catabolized by urease positive bacteria in the microbiome to regenerate NH_3. AGC: aspartate-glutamate carrier, ORNT1: ornithine translocator (C). *Hydrogen Sulfide*. Production of and metabolic clearance of hydrogen sulfide (H_2S) in cell metabolism. H_2S is generated by the mammalian enzymes CBS (cystathionine β synthase), CSE (cystathionine γ lyase), 3-MST (3-mercaptopyruvate sulfurtransferase) and from the metabolic reactions in the microbiome. H_2S is cleared by iterative oxidation catalyzed by sulfide quinone reductase (SQR), thiosulfate reductase (TR), and sulfite oxidase (SO). TR utilizes oxidized glutathione (GS^-) as a sink for electrons. Oxidations catalyzed by SQR and SO are linked to mitochondrial ETC and oxidative phosphorylation. (D). *Reactive Oxygen Species*. Reactions that generate and sequester ROS (reactive oxygen species). ROS are generated in the mitochondria through the ETC and NOX4 (NADPH oxidase). SOD2 (superoxide dismutase 2) converts superoxide into a the less reactive molecule hydrogen peroxide (H_2O_2). In the mitochondria, H_2O_2 is turned over by combined functions of periodxins (Prx) and thioredoxins (Trx). H_2O_2 also reacts with Fe^{+2} (the Fenton Reaction) to generated $OH\cdot$ in the mitochondria. ROS inflict oxidative damage to proteins in the mitochondria and cytosol, and also function as potent mitogen signaling agents.