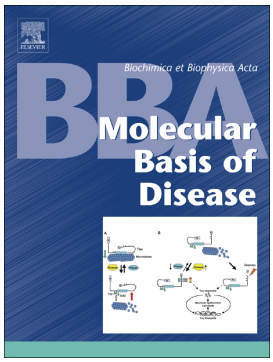

Intermediary metabolism: An intricate network at the crossroads of cell fate and function

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Intermediary metabolism: an intricate network at the crossroads of cell fate and function

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Abstract

Intermediary metabolism is traditionally viewed as the large, highly integrated network of reactions that provides cells with metabolic energy, reducing power and biosynthetic intermediates. The elucidation of its major pathways and molecular mechanisms of energy transduction occupied some of the brightest scientific minds for almost two centuries. When these goals were achieved, a sense that intermediary metabolism was mostly a solved problem pervaded the broader biochemical community, and the field lost its vitality. However, intermediary metabolism has recently been re-energized by several paradigm-shifting discoveries that challenged its perception as a self-contained system and re-positioned it at the crossroads of all aspects of cell function, from cell growth, proliferation and death to epigenetics and immunity. Emphasis is now increasingly placed on the involvement of metabolic dysfunction in human disease. In this review, we will navigate from the dawn of intermediary metabolism research to present day work on this ever-expanding field.

List of abbreviations[1]

Acetyl CoA	Acetyl coenzyme A
ACLY	ATP-citrate lyase
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAD	BCL-2 associated agonist of cell death
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2-like protein 4
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma-extra large
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide, oxidized form
$FADH_2$	Flavin adenine dinucleotide, reduced form
FAO	Fatty acid oxidation
$Fru1,6P_2$	Fructose 1,6-bisphosphate
FTO	Fat mass and obesity-associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT1	Glucose transporter 1
GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
2HG	2-Hydroxyglutarate
HIF-1α	Hypoxia inducible factor 1 α
HK	Hexokinase

IDH	Isocitrate dehydrogenase
IDO	Indoleamine oxygenase
IL-2	Interleukin-2
IMM	Inner mitochondrial membrane
ITAM	Immunoreceptor tyrosine-based activation motif
JHDM	Jumonji domain-containing histone demethylase
KEAP1	Kelch-like ECH-associated protein 1
αKG	α-Ketoglutarate
K_m	Michaelis constant
LCK	Lymphocyte-specific PTK
LKB1	Liver kinase B1
m^6A	Methylation at the N^6 position of adenine
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mtDNA	Mitochondrial DNA
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NOX	NADPH oxidase
NRF2	Nuclear factor erythroid-2-related factor 2
OAA	Oxaloacetate
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PI3K	Phosphoinositide-3-kinase
PPP	Pentose phosphate pathway
PRX	Peroxiredoxin
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTK	Protein tyrosine kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SAM	S-Adenosyl methionine
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
TCR	T cell receptor
Teff	Effector T
TET	Ten-eleven translocation
TNFα	Tumor necrosis factor α
TRX	Thioredoxin
TSC	Tuberous sclerosis complex
VDAC	Voltage-dependent anion channel

[1] This list contains abbreviations used at least twice in the main text.

1. Foreword

Intermediary, or intermediate, metabolism is the subfield of biochemistry traditionally concerned with the vast and highly integrated network of biochemical reactions that provides cells with forms of energy for immediate use (i.e., metabolic energy), reducing power and biosynthetic intermediates. Its establishment as a research field dates back to the first decades of the twentieth century, when it became a very active area of investigation; yet, its foundations were laid more than a century before, by the work of Antoine Lavoisier on the chemical nature of respiration [1]. In spite of intensive research efforts, progress in this emerging field throughout most of the nineteenth century was restricted by very limited conceptual knowledge and the inexistence of suitable analytical methods. Nonetheless, significant advances were made, paving the way for the groundbreaking discoveries of the first four decades of the twentieth century. The many Nobel Prizes awarded to researchers working in this field from the late 1910s to the 1950s attest to the enormous vitality of intermediary metabolism during this period, seen by many as its Golden Age. At the end of this period, intermediary metabolism was already contending with the growing influence of molecular biology, whose own Golden Age started with the publication of the double-helix structure of deoxyribonucleic acid (DNA), by James Watson and Francis Crick, in 1953 [2] – interestingly, the year Hans Krebs and Fritz Lipmann were awarded the Nobel in Physiology or Medicine for the discovery of the citric acid cycle and coenzyme A, respectively. Despite having receded into the background, the field progressed steadily and, by the 1970s, the classical pathways of intermediary metabolism were firmly established, as were the mechanistic principles behind the energy transducing processes. Between the 1960s and the 1990s, major advances were made in our understanding of intermediary metabolism regulation. Nonetheless, the field´s vitality gradually waned. Then, at the turn of the millennium, interest was rekindled by a succession of seminal discoveries which repositioned intermediary metabolism at the crossroad of all aspects of cellular function, ultimately touching on all facets of physiology and pathology. In particular, it is now clear that the study of intermediary metabolism is crucial for our understanding of many diseases, ranging from the classical metabolic diseases, such as type 2 diabetes and obesity, to cancer.

This review starts with an historical overview of the establishment of intermediary metabolism as a research field. This is followed by a brief presentation of the classical pathways of intermediary metabolism. The focus will then shift to the control of intermediary metabolism and to how metabolites, metabolic proteins and by-products of intermediary metabolism impinge on cell function and death, either directly or through their impact on signaling and on the epigenetic

state. Considering its vastness, it would be impossible to describe in a single review all the pathways of intermediary metabolism and their multiple connections to all other cellular processes. Thus, rather than comprehensive, this review is intended to be representative. We will focus on a few selected topics, referencing researchers whose work was of fundamental importance for the advancement of each subfield. Importantly, our discussion will be restricted to the cellular level. We apologize in advance to all other researchers, past and present, whose collective substantial contributions to the field we could not discuss.

2. Historical background

Antoine de Lavoisier, often described as the "father of chemistry", could also, with equal justice, be acknowledged as the "father of physiological chemistry" and, ultimately, the "father of intermediary metabolism". Indeed, it was Lavoisier who, at the end of the eighteenth century, first described a physiological process from a chemical perspective (Timeline). Specifically, he proposed that respiration is a slow combustion (oxidation) that burns foodstuffs, forming carbonic acid and water. He hypothesized that respiration took place in the lungs, producing the heat that maintains animal temperature and was somewhat connected to mechanical work [1]. It was also Lavoisier who first described chemically the global conversion of grape must into ethanol and carbon dioxide, i.e., alcoholic fermentation. However, the fact that this conversion is a metabolic process, taking place in a living cell, eluded him and his contemporaries [3]. That alcoholic fermentation requires the action of microorganisms was first acknowledged in the first half of the nineteenth century by Charles Cagniard-Latour, Friedrich Kützing and Theodor Schwann, but this was outright rejected and even ridiculed by three of the most influential scientists of the time, Jöns Berzelius, Justus von Liebig and Friedrich Wöhler. However, extensive work by Louis Pasteur in the 1850s established, once and for all, that fermentation involves the action of a microorganism. Pasteur's work, carried out with financial backing from the alcoholic fermentation industries, also showed that fermentation can generate other end-products, such as lactate and acetate, accounting for vast quantities of wine becoming spoilt [3]. This finding strengthened the view that had emerged in the early nineteenth century that fermentations are widespread chemical processes underlying many of the chemical changes occurring within plants and animals [3,4].

<Timeline>

Lavoisier's findings and proposals regarding respiration captivated the interest of chemists, biologists and physiologists in the intermediary reactions through which foodstuffs are broken down and thoroughly transformed into their simple end-products. Scientists also sought an explanation for the intriguing fact that the oxidation of foodstuffs can occur at the moderate temperatures of animals' bodies, while it requires much higher temperatures when taking place outside the body. Empirical and theoretical advances made during the nineteenth century led to the identification of carbohydrates, fats and proteins as the three main classes of foodstuffs and to the establishment of chemical equations for their global oxidation. However, in spite of an increased knowledge concerning the structural formulas and reactivity of organic compounds, namely the step-by-step oxidations that they can undergo in the test tube, the elucidation of the intermediate stages of the oxidation of foodstuffs was still a daunting task, in the face of the complexity of these very large molecules and the inherently low levels of intermediary metabolites, well below the sensitivity limits of the analytical methods available at the time. By the end of the nineteenth century, work by Emil Fischer and others showed that foodstuffs are composed of small units joined by chemical bonds that are hydrolysed during digestion. This finding was a major breakthrough, as researchers could now deal with much simpler molecules featuring much more tractable chemical reactivities [4]. Fischer's work also formed the basis for our understanding of enzyme specificity.

Another major breakthrough in intermediary metabolism research took place in 1897, when Eduard Buchner produced the first truly cell-free extracts from yeast cells and demonstrated that they could bring about fermentation upon addition of sugars. Buchner also identified the active constituent of the extract, which he proposed to be a protein, naming it "zymase" [5] (later identified as a collection of enzymes). The possibility to carry out controlled *in vitro* studies of fermentation dramatically accelerated the pace of research towards a complete understanding of its reactions. Equally important, the finding that fermentation does not require a living cell established a new conception of all metabolic processes, ultimately providing the framework for twentieth century biochemistry. It must be stressed that, although Pasteur had described fermentation as "respiration without air", his work even suggesting that it is prevented by the presence of oxygen, no direct links had been yet suggested between the two processes [3]. Nonetheless, biochemists hoped that, similar to what had been achieved with fermentation, cellular respiration might also be "extracted" from organized tissues, greatly simplifying the elucidation of all the reactions involved in the complete oxidation of foodstuffs [4].

The twentieth century got off to a good start: in 1904, the general mechanism of fatty acid β-oxidation was deducted by Franz Knoop, one of the most enthusiastic researchers in the field. When Knoop fed dogs phenyl derivatives of ordinary aliphatic fatty acids and analyzed the partial decomposition products present in the dogs' urine, he consistently found phenylacetate. Unfortunately, β-oxidation would remain, for almost three decades, the only reasonably known sequence of intermediary metabolism reactions – the discovery that the two-carbon piece split off from fatty acids was acetyl coenzyme A (acetyl CoA), a nodal metabolite at the crossroads between multiple metabolic pathways, would take even longer. As a consequence, how carbohydrates and proteins were converted to fat, as established from animal feeding experiments carried out already in the nineteenth century, would also remain elusive for several decades [4].

In 1905-1908, Arthur Harden and William Young made an important contribution to our understanding of alcoholic fermentation, showing that it produces a sugar phosphate ester and that it requires, besides "zymase", inorganic phosphate and a low molecular weight, heat-stable fraction. This fraction, which they named "cozymase", was later found to contain the oxidized form of the coenzyme nicotinamide adenine dinucleotide (NAD^+) [6–8]. Modest progress was also being achieved in the study of different stages of cellular respiration. This study was mostly initiated by Thorsten Thunberg, who established adequate experimental conditions for its systematic study in isolated tissues, by manometry. By the late 1900s, his research efforts, combined with those of Federico Battelli and Lisa Stern, led to the identification of a tricarboxylic acid (TCA) and three dicarboxylic acids (citrate and succinate, fumarate and malate, respectively) that were oxidized by isolated tissues [4,9]. Unfortunately, twenty years would have to elapse before these carboxylic acids, with no obvious structural relation to the molecules used by animals as fuels, could be incorporated into a coherent account of cellular respiration. The fact that it was commonly, but wrongly assumed that biological oxidations consisted of successive additions of oxygen directly to the substrate molecules might have contributed to this delay. In the early 1910s, Heinrich Wieland had already produced indirect experimental evidence for an alternative oxidation mechanism, consisting of successive removal of pairs of hydrogen atoms, which afterwards combined with oxygen to form water. He had also proposed that hydrogen removal was catalyzed by dehydrases (later termed dehydrogenases). Unfortunately, his proposal, which proved out to be correct, did not win general acceptance and was even fiercely rejected by the eminent biochemist Otto Warburg, who would go on to be awarded the Nobel in Physiology or Medicine, in 1931, for his "discovery of the nature and mode of action of the respiratory enzyme"

and who is now viewed as the “father of oncometabolism”, to acknowledge his seminal discovery of a tumor-specific metabolic phenotype, the so-called Warburg effect [4,10].

Anaerobic carbohydrate metabolism was also being studied in the muscle, which was known to store glycogen and accumulate lactate in the resting state, when deprived of oxygen. German physiologists had long associated the formation of lactate with muscular activity and, once it became accepted, although not unequivocally demonstrated, that lactate was derived from glucose (or from its storage form, glycogen), there was a general expectation that muscle glycolysis, as the anaerobic decomposition of carbohydrates that occurs in animal tissues was then known, shared important similarities with the lactic acid fermentation of microorganisms. There was also a strong conviction that these processes and yeast alcoholic fermentation were mostly similar, only branching off at some late step to generate their specific end-products. The discovery made by Otto Meyerhof, in 1918, that muscle glycolysis requires the same coenzyme as yeast alcoholic fermentation further supported the existence of an underlying metabolic unity in all life forms. Meyerhof’s initial goal was to understand the mechanisms by which the energy stored in foodstuffs is used to perform mechanical work and, more generally, how energy and chemical transformations are linked to cellular function. However, by the late 1920s, his efforts were also directed towards the establishment of the intermediate steps of the degradation of foodstuffs. Of note, it was Meyerhof who pioneered, in 1926, the *in vitro* study of muscle glycolysis, through the establishment of a protocol for the preparation of a fully glycolytic cell-free muscle extract and the successful utilization of the general method of inhibiting a step in an overall pathway to cause the accumulation of an intermediate. With this protocol, Meyerhof confirmed Gustav Embden’s earlier observation of the formation of sugar phosphate esters in muscle press juice [4,7]. As a result, the idea that these esters might be mere side-products of fermentation, rather than true intermediates, was finally dismissed and the chemistry of phosphorus in biological systems became a hot topic in biochemical research. In 1927, Cyrus Friske and Yellapragada SubbaRow and Grace and Philip Eggleton independently reported the discovery of phosphocreatine; two years later, the discovery of adenosine triphosphate (ATP) was reported by Karl Lohmann and by Friske and SubbaRow [11]. The role of ATP as the nearly universal energy donor in biological systems was proposed, in 1941, by Lipmann. Lipmann also proposed the term “energy-rich phosphate bond” and introduced the squiggle notation (~P) to represent it [12].

Throughout the first three decades of the twentieth century, progress towards the goal of establishing all individual steps that transform foodstuffs entering an organism into the final products that leave it was slow, in spite of numerous efforts. Nonetheless, conceptual knowledge

was steadily accumulating, enabling scientists to delineate critical features of both the individual steps and the whole chain of reactions. In particular, there was presumptive evidence that: (i) the different pathways of intermediary metabolism are, to a large extent, composed of a small number of basic reactions that are repeatedly used in similar situations; for instance, it was expected that, similar to what had been observed for pyruvate, other α-keto acids also underwent decarboxylation, giving off CO_2; (ii) at some point in their degradation, all foodstuffs converge to a reduced number of ubiquitous intermediates (e.g., pyruvate, acetate and certain dicarboxylic acids); (iii) each dehydrogenation reaction is carried out by its own individual dehydrogenase; (iv) some of the intermediary processes have to be cyclic; (v) the degradation and synthesis of foodstuffs are linked, likely through acetate, thus compensating for the continuous variations in the availability of these foodstuffs [4].

In 1933, the first coherent scheme of glycolysis that stood the test of time was finally proposed by Embden and, five years later, the glycolytic pathway was formally elucidated, with major contributions from Meyerhof and collaborators. Meanwhile, in 1935, a major progress was made towards the elucidation of the aerobic metabolism, when Albert Szent-Györgyi discovered that the highly respiratory pigeon breast muscle retained its oxidative capacity for a long time after its disintegration and suspension in aqueous media. With this new experimental setup, Szent-Györgyi confirmed the rapid oxidation of the dicarboxylic acids succinate, fumarate, malate and oxaloacetate (OAA), also concluding that these substances act as catalysts, rather than as traditional fuel molecules. A year later, Frederick Stare and Carla Baumann provided final proof of this catalytic effect, by showing that increases in respiration could be observed upon addition of very small quantities of the acids and that the amounts of these acids in the medium did not decrease during the process. In 1937, Krebs made two decisive contributions to the field. First, he observed that, similar to the above-mentioned carboxylic acids, citrate can act as a catalyst in cellular respiration. Shortly after, he found that this TCA could be synthesized from OAA and a substance which could be derived from carbohydrates, such as pyruvate or acetate. Krebs was now able to formulate a complete scheme for the final oxidation of carbohydrates: by a cyclic sequence of reactions involving several carboxylic acids as intermediates, one acetate equivalent is oxidized and the OAA consumed in the condensing reaction is regenerated. It was also Krebs who proposed the designation citric acid cycle [9]. The identification of acetyl CoA as the pyruvate derivative that condenses with OAA, by Severo Ochoa, Joseph Stern and Feodor Lynen had to wait until 1951.

In the 1940s, Albert Lehninger and Eugene Kennedy showed that, in eukaryotic cells, both the TCA cycle and β-oxidation occur exclusively in the mitochondria and Engelhardt proposed that the key function of aerobic metabolism is ATP generation [13]. Molecular details of what would be called oxidative phosphorylation (OXPHOS) also started to emerge. In particular, David Keilin, drawing on his work on the cytochrome system and on the work by Warburg, Wieland and others on the respiratory hydrogen carriers, came to the concept of the respiratory chain: a water-insoluble complex of redox carriers, operating sequentially between redox coenzymes and molecular oxygen (O_2). And yet, the mechanism by which electron transfer was coupled to ATP regeneration still eluded researchers. In 1961, after two decades of intensive work by a large number of outstanding scientists, the mystery was finally solved, when Peter Mitchell formulated his revolutionary "coupling hypothesis", later known as the "chemiosmotic hypothesis". After almost two decades of intensive scrutiny, Mitchell´s hypothesis was finally promoted to the status of theory [14]. The question of how intermediary metabolism is regulated was yet to be answered. Significant advances would be made between the 1960s and the 1990s, namely on the unique metabolism of skeletal muscle concerning the oxidation of glucose and fatty acids (the Randle cycle [15]. However, this critical aspect failed to attract the attention of the broader biochemical community, who regarded intermediary metabolism as self-contained and self-regulated.

The widespread perception of intermediary metabolism as a solved problem started to change in the late 1990s. One of the harbingers of this new dawn of intermediary metabolism was the discovery, made in 1996, by Xiaodong Wang and colleagues, that apoptosis can be initiated by the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol [16], thus linking metabolic energy generation to a plethora of physiological and pathological processes. Importantly, this discovery opened a new way to look at intermediary metabolism, showing that the energy generation machinery has a central role not only in sustaining cellular life, but also in regulating cell death, ultimately defining life as we know it. The finding that intermediary metabolism is controlled by signaling pathways was yet another major contributor to this perception, as was the discovery that reactive oxygen species (ROS), generated inside aerobic cells mostly as by-products of intermediary metabolism, influence many cellular processes, such as proliferation, differentiation, metabolic adaptation and immune functions. Indeed, immunometabolism is currently a hot topic in biological and medical research, as is the impact of intermediary metabolites in gene expression through epigenetics. Altogether, these and many other findings have triggered a second, very much disease-oriented Golden Age of intermediary

metabolism. After a long tenure in the background, intermediary metabolism is coming again to the forefront.

3. Classical pathways of intermediary metabolism: an overview

Despite their colossal diversity, all known organisms, from bacteria to the highest mammals, share the same three fundamental requirements: metabolic energy, reducing power and biosynthetic intermediates. Remarkably, there are also strong similarities in the ways they meet these requirements. This is nowhere more evident than in the fact that there are only two general processes of metabolic energy generation: fermentations and cellular respirations. Also, although different types of fermentation exist, originating a variety of end-products, they are all strikingly similar, one of the many manifestations of the general principle of economy and simplicity underlying intermediary metabolism across all species.

Both fermentations and cellular respirations involve the oxidation of fuel molecules, the distinction lying on the type of final electron acceptor they use: fermentations employ an endogenous acceptor, i.e., a metabolite that is formed during the fermentation process, whereas cellular respirations employ an exogenous acceptor. Of note, endogenous acceptors allow only for a very partial oxidation of the fuel molecules, whereas exogenous acceptors permit their complete oxidation. As an important corollary, the energy yields of cellular respirations are much higher than those of fermentations. On the other hand, fermentations do not depend on a supply of exogenous electrons acceptors and can proceed at much higher rates than cellular respirations. In this section, we will briefly describe the classical metabolic pathways for the generation of metabolic energy, reducing power and biosynthetic intermediates.

Heterotrophs rely on nutrients present in the diet to meet all their fundamental requirements. To be able to enter cells, most nutrients must first undergo digestion, an extracellular hydrolysis process that releases their smaller constituents, most notably monosaccharides, amino acids and fatty acids. In exceptional circumstances, such as under conditions of nutrient depletion, cells resort to autophagy to satisfy their needs. Through this mechanism, which involves the delivery of cytoplasmic materials to the lysosome for degradation, cells recycle soluble components and old organelles and use the resulting products, e.g., amino acids, for protein synthesis, energy production and replenishing energy storage. Inside the cells, monosaccharides, amino acids and fatty acids undergo multi-step degradation processes, organized in so-called metabolic pathways, to generate energy, reducing power and/or

biosynthetic intermediates. In the case of energy and reducing power generation, degradation is necessarily accompanied by oxidation.

Aerobic cells derive most of their energy from cellular respiration, using O_2 as the final electron acceptor (Figure 1). Although the initial steps of degradation are necessarily specific for each fuel molecule, they all converge to a cyclic metabolic pathway – the TCA cycle – to complete their oxidation. At the end of the TCA cycle, all fuel molecules have been completely oxidized to carbon dioxide. Oxidation occurs by successive dehydrogenations, i.e., by the successive removal of pairs of hydrogen atoms. These dehydrogenations are catalyzed by dehydrogenases, whose activities depend on redox coenzymes, acting as electron acceptors. The electrons sequentially extracted from fuel molecules are transferred to the oxidized forms of these coenzymes, NAD^+ and flavin adenine dinucleotide (FAD), converting them to their reduced forms, NADH and $FADH_2$. Due to the very low intracellular levels of these coenzymes, the accepted electrons must be quickly transferred to other species, so that the coenzymes can be converted back to their oxidized forms to receive electrons from additional fuel molecules. Thus, these two redox coenzymes can be viewed as electron carriers.

<Figure 1>

NADH and $FADH_2$ cannot transfer their electrons to O_2 directly. Instead, this transfer involves several redox molecules in tandem, located in the inner mitochondrial membrane (IMM) and collectively forming the electron transport chain (ETC). Besides four very large protein complexes (complexes I, II, III and IV), the ETC also contains two much smaller and highly mobile molecules: cytochrome *c* and ubiquinone (cytochrome *c* is loosely attached to the outer leaflet of the IMM). Most ATP is regenerated during the transfer of electrons from NADH and $FADH_2$ to O_2, through OXPHOS. From a thermodynamic point of view, OXPHOS couples a strongly spontaneous process to an otherwise unfavorable process, i.e., the global oxidation of NADH and $FADH_2$ by O_2 and the phosphorylation of adenosine diphosphate (ADP) by inorganic phosphate, respectively. Coupling is provided by an electrochemical gradient across the IMM, resulting from the shuttling of protons from the mitochondrial matrix to the intermembrane space by ETC complexes I, III and IV. Proton shuttling is energised by the redox reactions that these very large protein complexes, located in the inner mitochondrial membrane, undergo, as electrons flow from the reduced coenzymes to O_2. ATP synthesis is driven by an ATP synthase (also known as complex V), a protein complex also located in IMM, using the potential energy of the electrochemical gradient that is

released when protons flow back to the mitochondrial matrix through a pore formed by this complex.

Figure 1A illustrates aerobic cellular respiration with glucose – a key molecule in human intermediary metabolism – as the substrate. The initial degradation of glucose converts it into two molecules of pyruvate, through a sequence of 10 steps, all taking place in the cytosol and collectively named glycolysis. In the presence of an adequate supply of O_2, pyruvate is translocated to the mitochondrial matrix, where it is converted, by oxidative decarboxylation, to the acetyl moiety of acetyl CoA. This acetyl moiety then condenses with OAA to generate citrate, which, after a 9-step cyclic sequence, regenerates OAA. At the end of this sequence, known as the TCA cycle, all 6 carbon atoms of glucose have been fully oxidized and excreted as CO_2. A small amount of ATP (4 molecules) has also been generated by a process called substrate-level phosphorylation, which uses the energy released in the cleavage of high-energy bonds: the acylphosphate bond of 1,3-bisphosphoglycerate and the thioester bond of succinyl CoA, cleaved during glycolysis and the TCA cycle, respectively. However, most of the energy released during the oxidation of glucose is stored in the form of NADH (10 molecules) and $FADH_2$ (2 molecules). Altogether, the transfer of electrons from NADH and $FADH_2$ to O_2 will generate ca. 26 ATP molecules, via OXPHOS.

The sequence glycolysis $\rightarrow$ oxidative decarboxylation of pyruvate $\rightarrow$ TCA cycle $\rightarrow$ OXPHOS is often referred to as the central axis of intermediary metabolism. Monosaccharides other than glucose, as well as several amino acids and glycerol, are initially converted to glycolytic and/or TCA cycle intermediates (Figure 2), while activated fatty acids (i.e., in the form of acyl CoA) are initially converted to acetyl CoA via a sequence of four steps called β-oxidation, each cycle of β-oxidation removing a two-carbon fragment in the form of acetyl CoA (Figure 3). This pathway is particularly active in organs such as the liver, heart and skeletal muscle. Once within the central axis, all fuel molecules are metabolized the same way as glucose.

<Figure 2>

<Figure 3>

In the absence of an adequate O_2 supply, as is the case in muscle tissue during intense physical activity, pyruvate is reduced to lactate, with the concomitant regeneration of NAD^+ (Figure 1B). Fermentation of glucose has a very low energy yield, generating only 2 ATP molecules

per glucose molecule, both by substrate-level phosphorylation. Lactate can then be excreted to the blood stream to be converted back to glucose by the liver, in a process called gluconeogenesis. In some tissues, lactate can also act as a significant source of pyruvate for energy generation [17].

It is important to note that both glycolysis and the TCA cycle can be used for purposes other than ATP generation. Notably, these two metabolic pathways can also be used for the generation of biosynthetic intermediates. For instance, citrate, a TCA cycle intermediate, can pass across the mitochondrial membrane to the cytosol, where it is cleaved into OAA and acetyl CoA to support a variety of biosynthetic processes, including the synthesis of fatty acid and sterols. Many amino acids, as well as purines and pyrimidines, are derived from two other TCA cycle intermediates, α-ketoglutarate (αKG) and OAA, while succinyl coenzyme A (succinyl CoA) is a precursor of the porphyrins required for the synthesis of cytochromes and blood pigments. More recently, it has been found that some metabolic intermediates are also used as epigenetic modifications, as discussed in section 6. Of note, if TCA cycle intermediates are removed from the cycle, their pools must necessarily be replenished to permit the cycle's continued function. The process of intermediate replenishment is known as anaplerosis. Pyruvate and glutamine/glutamate are major anaplerotic substrates (see also section 6) [18].

Respiration and fermentation are not the only intracellular fates of fuel. For instance, glucose is also the substrate of choice for the generation of reducing power, in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH), the preferred electron donor for both biosynthesis and antioxidant defence. This reduced coenzyme can be generated by the pentose phosphate pathway (PPP; Figure 4A), a pathway that is also used for the synthesis of ribose 5-phosphate. It is, thus, especially active in rapidly dividing cells. Glucose also feeds the hexosamine biosynthesis pathway (HBP). This pathway is essential for the synthesis of UDP-N-acetylglucosamine, the primary sugar donor for protein glycosylation. Other molecules such as glutamine, fatty acids and amino acids can also act as substrates for the HBP, and this pathway is now commonly viewed as a nutrient signaling pathway [19]. Glucose not needed for immediate use is stored in the form of glycogen, a glucose homopolymer than can be very quickly mobilized, ensuring adequate glucose levels between meals and also during period of strenuous exercise (Figure 4B). Of note, all these pathways branch from glycolysis at the level of fructose 6-phosphate.

<Figure 4>

As can be appreciated, intermediary metabolism is a complex network comprising an enormous set of reactions, organized in metabolic pathways linked at many intersections. The large majority of these reactions is catalyzed, their spontaneous (i.e., non-catalyzed) rates being incompatible with life as we know it. This is seemingly a major burden to the cell, as it has to express a large number of metabolic enzymes. However, it provides a critical means to kinetically regulate metabolism. This can be achieved at different levels: through allosteric regulation of the enzymes involved, for an acute effect, or through covalent modification and/or regulation of their expression, for a more sustained response.

4. Adjusting intermediary metabolism to cell function, physiological state and environmental conditions

Although all cells share the same fundamental requirements, the relative importance of these requirements and the metabolic strategies adopted to meet them are both cell type- and context-specific, depending on the set of metabolic enzymes expressed by the cells, whether these cells are actively dividing or in a quiescent state and the availability of different nutrients, among other factors.

To adjust intermediary metabolism to cell function and to distinct physiological states and environmental conditions, cells divert the flow of metabolites to new desired directions, at the many intersections within the metabolic network. For many decades, intermediary metabolism was mostly perceived as a self-contained, self-regulated network of reactions, ensuring metabolic homeostasis via intrinsic regulators that match supply to demand, independently of signaling pathways or any other forms of control external to the network. This perception received a certain support from the discovery, in the 1960s, of three metabolic enzymes, the muscle isoforms of glycogen phosphorylase, phosphofructokinase and fructose-1,6-bisphosphatase, that are allosterically regulated by adenine nucleotides [20]. In line with this discovery, Daniel Atkinson introduced the concept of energy charge (EC) of the adenylate pool – EC = (ATP+ 1/2ADP)/[ATP + ADP + AMP] – proposing that this parameter, meant to provide a quantitative estimate of the cellular energy status, acts as an intrinsic regulator of intermediary metabolism [21]. However, the number of metabolic enzymes allosterically regulated by them turned out to be rather small [22]. Over the years, we have come to realize that the role of adenine nucleotides in the regulation of intermediary metabolism is mostly indirect and that metabolic homeostasis requires the action of additional players. In particular, commitment to energy-demanding processes, such as cell growth and division, requires the ability to detect and integrate signals extrinsic to the metabolic network,

namely those related to nutrient availability. This ability is also critical for the maintenance of whole-body energy balance, where hormones such as insulin play a critical role [23]. A description of the regulation of intermediary metabolism at the organismic level is, however, beyond the scope of the present review.

4.1 Balancing catabolism and anabolism

In most eukaryotic cells, cellular energy status is permanently sensed by a kinase cascade. The first component of this cascade to be identified was AMP-activated protein kinase (AMPK), an evolutionarily conserved serine/threonine protein kinase named after AMP, its allosteric regulator. AMPK is activated in response to low cellular energy status, signalled by low AMP:ATP or ADP:ATP ratios. AMPK not only monitors changes in these ratios, it is also actively engaged in restoring energy balance. Briefly, AMPK activation upregulates ATP generation through stimulation of nutrient uptake, autophagy, mitochondrial biogenesis and ATP-generating pathways (i.e., glycolysis and β-oxidation), among other processes, and it concomitantly inhibits ATP-consuming processes, namely biosynthetic pathways, cell cycle progression and apoptosis [22,24,25]. As can be appreciated, AMPK action extends well beyond intermediary metabolism and biosynthesis. In fact, with at least 60 downstream targets [26], AMPK is at the crossroads between intermediary metabolism and a panoply of cellular processes. Acute metabolic reprogramming is achieved through enzyme phosphorylation, whereas sustained reprogramming involves modulation of gene expression at both the transcript and protein levels [22].

AMPK only exhibits significant activity in its phosphorylated form. In most mammalian tissues, AMPK is mainly phosphorylated by liver kinase B1 (LKB1), a serine/threonine kinase with a well-defined tumor suppressor activity [27], but it can also be phosphorylated by other kinases [22]. Whether AMPK mediates the tumor suppressor functions of LKB1 is still not clear [22,28]. In fact, besides AMPK, LKB1 also activates, through phosphorylation, a family of 12 kinases related to AMPK with critical roles in cell growth, metabolism and polarity [29]. Tellingly, cells and tissues where the AMPK or LKB1 genes have been knocked out exhibit higher increases in AMP:ATP and ADP:ATP ratios than their normal counterparts in response to energy stresses such as muscle contraction, ischemia in cardiac muscle or treatment with metformin (a diabetes drug that inhibits mitochondrial complex I and promotes FAO) [22].

By allosterically binding to phospho-AMPK, AMP not only modulates its activity, it also almost completely abolishes its dephosphorylation by protein phosphatases. In addition, AMP binding to unphosphorylated AMPK makes it a better substrate for LKB1 [22]. These effects on

AMPK phosphorylation/dephosphorylation can also be produced by ADP, but at concentrations 10-fold higher than those required for AMP [24]. At high concentrations, ATP competitively binds to the allosteric sites of AMPK, antagonizing all three effects of AMP on AMPK. Altogether, this system provides an ultrasensitive response to small changes in intracellular AMP:ATP and AMP:ADP ratios [22]. Unsurprisingly, AMPK activation is most notable under ATP-depleting cellular stress conditions, such as nutrient and oxygen depletion and redox imbalance [30].

4.2. Linking cell proliferation to cellular energy status and environmental conditions

Cell division requires an ample supply of nutrients to meet its high demand for energy, reducing power and biosynthetic intermediates. In aerobic organisms, ample oxygen levels are also critical. Thus, it is paramount that cross-talk exists between growth factor signaling, nutrient-, oxygen- and energy-sensing pathways, energy generation, biosynthesis and cell cycle progression. This crosstalk is provided, in part, by the aforementioned LKB1/AMPK system via the mechanistic target of rapamycin (mTOR) pathway, a signaling pathway involved in the regulation of cell cycle progression in many species and known to be deregulated in most human cancers [31]. A key role is played by mTOR complex 1 (mTORC1), through its regulatory role in biosynthesis, intermediary metabolism and autophagy [32,33].

The mTOR protein kinase constitutes the catalytic subunit of mTORC1. Through phosphorylation of the mTOR substrates ribosomal protein S6 kinase beta-1 (S6K1) and eIF4E binding protein (4EBP), mTORC1 ultimately promotes the translation of numerous proteins, namely transcription factors and cell growth regulators. Examples include sterol regulatory element-binding protein (SREBP) transcription factors, which control the expression of metabolic genes involved in fatty acid and cholesterol biosynthesis, hypoxia inducible factor 1 α (HIF-1α), which controls the expression of several glycolytic genes, c-myc and cyclin D1 [27,32]. Furthermore, mTORC1 also promotes cell growth by suppressing protein degradation, most notably by preventing autophagy. Interestingly, the extent of autophagy induction is largely determined by the relative activities of mTORC1 and AMPK [32]. mTORC1 is a downstream mediator of several growth factor- and mitogen-dependent signaling pathways, such as the insulin/ insulin-like growth factor-1 (IGF-1), Wnt and inflammatory cytokine tumor necrosis factor α (TNFα) pathways. All of these pathways inhibit a major inhibitor of mTORC1 activity, the tuberous sclerosis complex (TSC) [27,32].

Under conditions of nutrient deprivation, hypoxia or other conditions that result in energy stress, the LKB1/AMPK system inhibits the mTOR pathway through activation of TSC, which in turn

arrests cell cycle progression in G1, prior to S phase [34]. The recently unveiled glucose-sensing role of AMPK likely contributes to this control. AMPK's ability to sense glucose availability in the absence of changes in adenine nucleotide ratios is mediated by the glycolytic enzyme aldolase, which catalyzes the conversion of fructose 1,6-bisphosphate ($Fru1,6P_2$) into glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) (Figure 1A). Upon glucose deprivation, $Fru1,6P_2$ levels rapidly decrease, triggering the formation of an AMPK activation complex and, ultimately, AMPK activation [24].

The ability of the LKB1/AMPK system to cause cell cycle arrest may involve other players. For instance, this system mediates signaling by the tumor suppressor protein p53 and the cyclin-dependent kinase inhibitor p21. In tumor cells, LKB1 stimulates the activities of p53 and p21 [35,36], while AMPK controls p53-dependent apoptosis [37]. Interestingly, AMPK is also activated downstream of p53 [38] by Sestrin 1 and Sestrin 2, known p53 target genes that inhibit mTORC1 signaling [39]. In addition, PRKAB1, another p53 target gene, encodes the AMPKβ1 regulatory subunit, which also downregulates mTOR activity [40]. In addition, the LKB1/AMPK axis also integrates cell growth and intermediary metabolism by phosphorylating several transcriptional coactivators, such as p300 histone acetyltransferase [41], histone deacetyltransferases (HDACs) [42,43] and the CRTC family of CREB coactivators [44].

5. Reactive oxygen species (ROS) as modulators of signaling pathways

While the realization that intermediary metabolism is controlled by signaling pathways was groundbreaking, the finding that metabolites and metabolic by-products are active players in signaling and in epigenetics was truly paradigm shifting, re-positioning intermediary metabolism at the crossroads of all aspects of cell function. In this review, we will briefly address these aspects, by discussing the involvement of ROS (section 5.2) in signaling and how certain metabolites regulate the epigenetic state (section 6).

5.1. ROS as inevitable by-products of aerobic metabolism

In spite of the undeniable advantages of aerobic cellular respiration over lactic acid fermentation, the presence of O_2 in the intracellular milieu constitutes a permanent threat to aerobic cells. Indeed, the electronic configuration of this molecule makes it especially susceptible to univalent electron transfer [45], which might well be considered as the most inescapable of all problems faced by aerobic cells, as the sequential reduction of O_2 generates a series of very unstable species. When the intracellular levels of these species, collectively referred to as ROS, are

not properly controlled, they react extensively and indiscriminately with biomolecules, generating additional reactive species, culminating in the propagation of radical chain reactions [46,47]. Damage inflicted by ROS in biomolecules and, ultimately, cell structures, may be so massive as to result in cell death. ROS accumulation has been linked to many diseases, including neurodegenerative disorders, cancer, diabetes and premature aging, although their precise role in these diseases remains mostly unknown [48,49]. Unsurprisingly, aerobic organisms have developed a plethora of cellular antioxidant defense mechanisms, comprising both enzymatic and non-enzymatic components, to maintain ROS at harmless levels [50–52]. Dietary non-enzymatic antioxidants include the hydrosoluble vitamin C (ascorbate) and the liposoluble vitamin E and carotenoids (e.g., β-carotene and lycopene), while endogenous non-enzymatic antioxidants include several low molecular weight thiols, most notably glutathione (GSH). GSH is the major soluble endogenous non-enzymatic antioxidant and is highly abundant in all cell compartments. Besides its critical role in detoxifying ROS, GSH also converts oxidized vitamins C and E back to their active forms, among other fundamental functions. Enzymatic antioxidants include superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPXs) and peroxiredoxins (PRXs). Cells protect themselves from oxidative damage by regulating the levels of these enzymatic antioxidants [53,54].

Within the cell, O_2 becomes partially reduced when it abstracts electrons from certain ubiquitous cofactors involved in univalent electron transfer, most notably the reduced metal centers, flavins and quinones present in ETC complexes [46,49,55–57]. This reduction results in the generation of the superoxide anion radical ($O_2^{\bullet-}$) (equation 1). Due to its charge, $O_2^{\bullet-}$ is unable to permeate lipid bilayers, but it can cross the outer mitochondrial membrane (OMM) through specific voltage-dependent anion channels (VDACs) [46,57].

$$O_2 + e^- \longrightarrow O_2^{\bullet-} \quad (1)$$

$O_2^{\bullet-}$ can quickly oxidize solvent-exposed Fe^{2+} present in the $[4Fe\text{-}4S]^{2+}$ clusters of certain dehydratases. The resulting $[4Fe\text{-}4S]^{3+}$ product is unstable and releases free iron into the intracellular milieu, leaving the iron-sulfur cluster in an inactive $[3Fe\text{-}4S]^{+}$ form. Besides inactivating the dehydratases, the oxidation of these clusters poses an additional threat to cells, as iron released from the oxidized clusters may participate in the Fenton reaction, generating the $OH^{\bullet}$ radical (discussed below) [45,58]. This radical is the most potent form of ROS, being able to oxidize most biomolecules. On the other hand, its extremely short half-life restricts its action to its

production site [47,59]. To minimize $OH^{\bullet}$ generation from $O_2^{\bullet-}$, SODs located in the cytosol and in the mitochondrial matrix dramatically accelerate the dismutation of $O_2^{\bullet-}$ into O_2 and hydrogen peroxide (H_2O_2) (equation 2).

$$2\ O_2^{\bullet-} + 2\ H^+ \longrightarrow\ H_2O_2 + O_2 \qquad (2)$$

As is the case with $O_2^{\bullet-}$, H_2O_2 toxicity is mostly indirect, resulting mainly from its conversion into the $OH^{\bullet}$ radical by reacting with free intracellular Fe^{2+} (Fenton reaction; equation 3) and other transition metals [58].

$$Fe^{2+} + H_2O_2 \longrightarrow (FeO)^{2+} + OH^- + H^+ \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \qquad (3)$$

H_2O_2 and $O_2^{\bullet-}$ can also generate the $OH^{\bullet}$ radical via the Haber-Weiss reaction (equation 4) [60].

$$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^- \qquad (4)$$

Cells rely on several enzymatic antioxidants to avoid excessive intracellular H_2O_2 levels. Through the action of catalase, present in the peroxisomes of most cells, H_2O_2 is very quickly converted to water (H_2O) and O_2 (equation 5). To prevent the inactivation of catalase by H_2O_2 as it is reduced to H_2O, NADPH binds to each of the four catalase's subunits and provides the necessary reducing equivalents [61].

$$2\ H_2O_2 \longrightarrow\ 2\ H_2O + O_2 \qquad (5)$$

Elimination of H_2O_2 can also be achieved through the action of GPXs and PRXs, which are both widely distributed across different cell compartments (cytosol, mitochondria and the endoplasmic reticulum (ER)) [57]. In the case of GPXs, conversion of H_2O_2 to H_2O is accompanied by reduction of GSH to glutathione disulfide (GSSG) (equation 6). GSSG can be reduced back to GSH by the action of GSH reductase, using NADPH as the electron donor, which is then converted to its oxidized form ($NADP^+$) (equation 7) [62].

$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O \qquad (6)$$

$$GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+ \quad (7)$$

In the case of PRXs, the cysteine residue present in the active site of these highly abundant thiol protein homodimers reacts with H_2O_2, forming the corresponding sulfenic acid (Cys-SOH), which then reacts with the cysteine residue on the adjacent monomer to form a disulfide bond (Cys-S-S-Cys). This bond is then reduced by thioredoxin (TRX) to complete the catalytic cycle. Afterwards, oxidized TRXs are reduced by thioredoxin reductase, using NADPH as the electron donor [63]. Of note, all these three enzyme families depend on NADPH to maintain their catalytic activity, making NADPH generation a centerpiece of effective antioxidant defense. NADPH also participates actively in ROS production in phagocytic cells [57], but this aspect is out of the scope of the present review.

5.2. ROS-mediated signaling

For a long time, ROS were viewed as purely harmful entities, lacking a physiological function. However, in the past two decades, it has become apparent that ROS play a critical role as modulators of a variety of signaling pathways. This unsuspected role was first hinted in the late 1990s, namely when it was shown that binding of epidermal growth factor (EGF) to its receptor (EGFR) produced an immediate burst of ROS, which was not toxic and was followed by increase in tyrosine phosphorylation [64]. Another study showed the involvement of ROS released by mitochondria in the physiological adaptation to hypoxia. Specifically, ROS were shown to induce the expression of the hypoxia inducible factor (HIF) target gene erythropoietin whose protein product stimulates red blood cell production in the bone marrow [65]. Along the same lines, it has now been shown that reducing endogenous ROS levels inhibits the proliferation of both vascular smooth muscle cells and tumor cells [66] and that H_2O_2 reversibly inhibits centrosome-bound protein phosphatase (Cdc14B), allowing activation of cyclin dependent kinase 1 (Cdk1) and, consequently, mitotic progression [67], whereas inhibition of endogenous ROS production causes cell-cycle arrest in the G1 phase [68], among other effects. The current perception is that the action of ROS on signaling extends to a wide variety of processes other than proliferation, such as metabolic adaptation and immune responses [49,55,56].

While the role of ROS as mediators of signaling pathways is now generally accepted, how this role is exerted is still incompletely understood, as are the mechanisms that ensure that ROS are not completely removed from the intracellular milieu by the robust antioxidant systems

present in all cells. It is already known that ROS activate the nuclear factor erythroid-2-related factor 2/Kelch-like ECH-associated protein 1 (NRF2/KEAP1) pathway, which serves as a master regulator of ROS levels. In particular, ROS promote specific modifications in KEAP1 cysteine residues, preventing NRF2 from being degraded by modified KEAP1, consequently leading to NRF2 translocation to the nucleus and the activation of specific cytoprotective genes [9].

Another unsolved question regards the intracellular concentration of H_2O_2 associated with signaling. It has been suggested that it is in the low nanomolar range, but the assessment of ROS levels remains challenging, especially *in vivo* [49,55,56]. Importantly, it has been shown that the levels of H_2O_2 required for signaling do not cause meaningful changes in the GSSG:GSH intracellular ratio [57]. Which ROS are involved in signaling is another open question, but the inability to cross biological membranes, poor stability and non-specific reactivity compromise the potential cell signaling capabilities of $O_2^{\bullet-}$ and $OH^{\bullet}$. H_2O_2, on the other hand, is relatively stable and able to cross biological membranes, allowing it to diffuse away from its place of origin. Thus, H_2O_2 is likely the only ROS mediating signaling [48,49].

The most well characterized, but by no means the only, mechanism of ROS-mediated signaling involves the oxidation of cysteine residues within redox-sensitive proteins to their sulfenic form (Cys-SOH). Target cysteine residues exhibit unusually low pK_a values (of 4 or lower; K_a is the dissociation constant), by virtue of their local environment. At physiological pH, these unique cysteine residues exist as thiolate anions (Cys-S^-), which are more susceptible to oxidation than their protonated counterparts (i.e., the corresponding cysteine thiol (Cys-SH)). The enhanced susceptibility to oxidation exhibited by the cysteine residues of these proteins may provide the degree of specificity required in signaling. At high H_2O_2 concentrations, the sulfenic form is further oxidized, giving rise to sulfinic (Cys-SO_2^-) and sulfonic (Cys-SO_3^-) forms. These oxidative modifications induce allosteric changes within the target protein that have functional consequences in the protein itself and, ultimately, in the signaling pathways in which it participates. Importantly, while Cys-SOH can be converted back to Cys-S^-, thus restoring protein function, the generation of Cys-SO_2H and Cys-SO_3H is irreversible [49,55,56,69].

Potential targets of ROS-mediated signaling include members of the protein tyrosine phosphatase (PTP) superfamily, whose activities are decreased by oxidation, as well as certain protein tyrosine kinases (PTKs), which are activated by oxidation [69]. One such kinase is AMPK. A cytoplasmic form of ataxia-telangiectasia mutated (ATM), the product of the gene mutated in human ataxia-telangiectasia, may also be involved in the activation of AMPK by ROS, as suggested by a lower effect observed in fibroblasts from ataxia-telangiectasia patients or mouse embryo

fibroblasts lacking ATM. Also, the fact that cells lacking LKB1 are less susceptible to ATM-dependent activation of AMPK by oxidative stress suggests the involvement of this upstream kinase, which has been shown to be phosphorylated by ATM. Whether this phosphorylation by ATM has any effect on LKB1 activity remains to be demonstrated [22].

The extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, which plays an important role in various cellular processes such as cell growth, differentiation, survival and cell death, and the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway [49,70] are also regulated by ROS. In the case of the PI3K/AKT pathway, oxidation by H_2O_2 of a cysteine residue in the active site of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) leads to the inactivation of its lipid phosphatase activity, causing an increase in the intracellular PIP3 levels, which, in turn, activates AKT and, ultimately, increases the expression of various cell survival-related genes, indicating a functional role for elevated intracellular ROS [71]. ROS also play a key role in a variety of other pathways. For instance, they act on proteins such as protein tyrosine phosphatase non-receptor type 1 (PTP1B) and MAPK, inactivating these phosphatases via inhibition of their dephosphorylation activity. In addition, the tyrosine-protein kinases LYN and SYK, whose phosphorylation is modulated by H_2O_2 in the mitochondrial intermembrane space, are necessary for signaling downstream of JNK and AKT [72].

In summary, ROS can activate or deactivate a variety of proteins involved in signal transduction. However, unlike most signaling pathways, ROS-mediated signaling also has the potential to be nonspecific, in the sense that the oxidation of critical cysteine residues of phosphatases and other signaling molecules can be achieved in a non-controlled fashion by stark variations in the redox environment of a cell. The realization that ROS are not only damaging molecules, but also key signaling molecules, opens doors to unveiling redox mechanisms with the potential to become therapeutic targets in many diseases.

6. Regulation of epigenetic state by intermediary metabolism

Epigenetic control of gene expression is another way through which intermediary metabolism contributes to tuning eukaryotic cell function to environmental stimuli. Covalent modifications in DNA and RNA, as well as in DNA-associated proteins called histones, comprise a portion of our understanding of the field of epigenetics. These modifications, which include methylation, acetylation, phosphorylation and ubiquitination, ultimately influence the expression of genes at the transcriptional and translational levels. For instance, acetylation of histones, particularly at gene promoter regions, is generally associated with activation of gene expression,

as the addition of an acetyl group neutralizes the interaction of their positively charged N-termini with the negatively charged backbone of DNA. In contrast, histone (particularly on lysines 9 and 27 of histone 3) and DNA methylation, by influencing the recruitment of transcription factors and chromatin remodeling enzymes, have been linked to transcriptional repression. Importantly, these modifications may be temporary, as they can be removed by enzymes, such as DNA demethylases. As such, epigenetic modifications are sometimes described as dynamic.

Intermediary metabolism and epigenetics are linked through the enzymes involved in the epigenetic modification. Specifically, epigenetic "writers" utilize metabolites as substrates, cofactors and allosteric regulators to make covalent modifications, whereas epigenetic "erasers" depend on cofactors and allosteric regulators to remove their substrates, i.e., the metabolites used in the covalent modifications. The correct deposition and removal of epigenetic modifications requires the careful coordination of all these players. In fact, aberrant epigenetic modifications can lead to a variety of pathologies. A detailed discussion of the biochemistry and crosstalk between intermediary metabolism and epigenetics across diverse cell types and disease pathologies is out of the scope of this review, and the interested reader is referred to a number of outstanding reviews that explore these aspects in great detail [73–79]. Our discussion focuses on the principles of control exerted by the products of intermediary metabolism on a subset of dynamic epigenetic modifications—the acetylation of histones and the methylation of histones, DNA and RNA. As an example of the crucial role metabolism plays in regulating epigenetic state impacting human pathology, this section also discusses how the coordination of metabolic and epigenetic reprogramming mediates cancer progression, as aberrations in epigenetics are now appreciated to be potent driving forces of tumorigenesis.

Histone acetylation and deacetylation

Histone lysine acetylation is catalyzed by a family of enzymes called histone acetyltransferases (HATs), which transfer the acetyl group from acetyl CoA onto histone lysine tails. Histone lysine deacetylation is catalyzed by two families of enzymes: NAD^+-dependent sirtuins and NAD^+-HDACs. Sirtuin-mediated deacetylation involves the cleavage of NAD^+ cofactor, generating the products nicotinamide (NAM), *O*-acetyl ADP-ribose and a deacetylated histone [80]. Given the central role of NAD^+ as an electron acceptor for oxidative reactions (section 3) [74], it is tempting to speculate that mitochondrial function and cellular energy status may be communicated through fluctuations in NAD^+ levels to mediate nuclear sirtuin deacetylation activities [81]. In contrast, the activity of the other class of histone deacetylases, the classical

HDACs, has been shown to be regulated by lactate and ketones bodies, such as β-hydroxybutyrate (βHB) (Figure 5B).

<Figure 5>

Histone/DNA/RNA methylation and demethylation

Methylation of chromatin occurs on either lysine and arginine residues of histones or directly on the DNA bases cytosine and adenine. Histones can be mono-, di- or tri-methylated, with the majority of these marks associated with transcriptional repression. While mammalian DNA has recently been reported to be methylated on adenine [82], much more is known about the function of cytosine methylation, which almost exclusively occurs in the context of CpG dinucleotide "islands" [83]. More recently, functional roles for the most prevalent modification on RNA – methylation at the N^6 position of adenine (m^6A) – have been described [84]. Histone, DNA and RNA methyltransferases (HMTs, DNMTs and METTLs, respectively) all require the methyl donor S-adenosyl methionine (SAM), generated from methionine and/or serine through their participation in the folate and methionine cycles, respectively (Figure 5A). Within the methionine cycle, SAM is generated from methionine ligation to ATP by methionine adenosyltransferase (MAT). Serine can support SAM synthesis through either *de novo* synthesis of ATP or by providing one-carbon units to regenerate methionine from homocysteine, although the significance of the latter mechanism under normal cell culture conditions is still controversial [85]. Cancer cells overexpress serine/methionine synthesis enzymes [86–92] and display increased transporter activity of these amino acids, as well as of glutamine [93–95].

Aside from the lysine-specific demethylase (LSD) family of histone demethylases that require FAD as a cofactor, the majority of enzymes that perform demethylation reactions – namely, Jumonji domain-containing histone demethylases (JHDMs), ten-eleven translocation (TET) and fat mass and obesity-associate protein (FTO) and alkylated DNA repair protein (AlkB) family enzymes – classify as iron/αKG-dependent dioxygenases, requiring the metabolic cofactor αKG (Figure 5B), as well as molecular oxygen and iron. In mammalian development, αKG is crucial for promoting histone and DNA demethylation to coordinate gene expression changes associated with early differentiation of primed pluripotent stem cells [96] and maintenance of naïve stem cell self-renewal [97]. In contrast, the TCA cycle metabolites succinate and fumarate act as negative regulators of iron/αKG-dependent dioxygenases (Figure 5B).

"Ink" sources

The major "ink" for acetylation, acetyl CoA, can be produced in the mitochondria from the oxidative decarboxylation of glucose-derived pyruvate, catalyzed by the pyruvate decarboxylase complex (PDC) (Figure 1A) [98]. Additional sources of mitochondrial acetyl CoA include fatty acid oxidation (FAO) and ketogenic amino acid catabolism, depending on cell type [76]. Because acetyl CoA cannot pass through the mitochondrial membrane, extramitochondrial acetyl CoA is generated from the efflux-able TCA cycle intermediate citrate through the activity of the enzyme ATP-citrate lyase (ACLY). In fact, it has been shown that ACLY knockdown impairs histone acetylation [98]. Citrate pools can be replenished by anaplerosis, using glutamine as the initial substrate, leading to increased glutamine consumption. Stress conditions, such as hypoxia, that perturb cellular redox balance can also induce reductive carboxylation of glutamine to maintain citrate levels for acetyl CoA generation.

As illustrated in Figure 5A, in addition to glucose, it is now appreciated that a number of amino acids can be utilized by cells to synthesize the major substrates for the "writers" of acetylation (acetyl CoA) and methylation (S-adenosyl methionine or SAM). This is especially true for cancer cells, due to the existence of hypoxic regions in the tumor microenvironment, among other stresses [99–101], or that overexpress serine/methionine synthesis enzymes [86–92] and display increased transporter activity of these amino acids [93–95]. The participation of these nutrient sources in intermediary metabolism also provides "erasers" with cofactors (αKG, NAD^+ and FAD) and allosteric regulators (lactate, succinate, fumarate and either the R or S enantiomer of 2-hydroxyglutarate (2HG)).

Although it was traditionally believed that the "ink" (substrates/cofactors) to perform such modifications was unlimited, emerging evidence suggests otherwise; namely, the activities of epigenetic enzymes can indeed be limited by metabolic activity, as it defines the intracellular levels of their substrates. For example, numerous functionally important post-translational modifications on histones have been reported, yet histone lysine acetylation and methylation appear to be uniquely constrained by dynamic changes in cell metabolism. This dynamic control stems from the fact that the cellular levels of the substrates acetyl CoA and SAM are in the 10^{-3} to 10^{-2} mM range, roughly matching the Michaelis constant (K_m) of HATs and histone/DNMTs using them (K_m in the 10^{-4} to 10^{-2} mM range) [78]. The same relation of matching concentrations with K_m holds true for the cofactors and allosteric regulators of histone deacetylases and histone/DNA demethylases. In contrast, epigenetic modifications such as phosphorylation and ubiquitination are considered recalcitrant towards metabolic control due largely to the fact that kinases and

ubiquitin ligases, the enzymes that perform these modifications, typically have K_m values in the 10^{-3} mM range and are, therefore, saturated by cellular ATP levels (in the mM range).

Dysregulated metabolism fuels epigenetic changes during cancer progression

Acetylation and methylation exert effects on gene expression programs that can directly impact malignant transformation. For example, tumor tissue often displays increased DNA methylation compared to normal tissue [83,102]. Hypermethylation of CpG islands in gene promoters has been reported to shut off the tumor suppressors retinoblastoma protein (Rb), Von-Hippel Lindau (VHL), adenomatous polyposis coli (APC), $p16^{INK4a}$, hMLH1 and BRCA1 [83,103]. It was recently reported that loss of the tumor suppressors LKB1 in pancreatic cancer cells [89] or PKC λ/ι in prostate cancer cells [92] fueled increased flux through serine, glycine and one-carbon metabolism to promote SAM biosynthesis, DNA methylation and subsequent gene expression changes favoring tumor development. Conversely, serine [85] and methionine [88] depletion results in reduced cellular SAM levels, thereby impacting downstream histone and DNA methylation. It remains to be determined whether dietary restriction of SAM precursors also augments the efficacy of conventional chemotherapies [104] in suppressing tumor growth by destabilizing chromatin methylation events.

Tumors have also been reported to increase acetate uptake [105,106]; in hypoxic cells, the acetyl CoA synthetases ACSS1 and ACSS2 generate acetyl CoA from acetate to promote global histone acetylation [107]. A recent report also demonstrated that blocking acetyl CoA generation by ACLY reduced histone acetylation and inhibited pancreatic carcinogenesis [108], highlighting the potential therapeutic value of targeting acetyl CoA production pathways to disrupt cancer cell plasticity and chromatin regulation.

Some of the strongest evidence suggesting that altered metabolism promotes cancer development beyond merely sustaining tumor anabolism is the discovery and functional characterization of mutations in isocitrate dehydrogenase (IDH) isoforms 1 and 2 in gliomas, acute myeloid leukemias and subsets of chondrosarcomas and lymphomas [109]. These neomorphic IDH mutant cells produce the R enantiomer of the oncometabolite 2HG (R-2HG) [110,111], which competes with the cofactor αKG in the enzyme active site to inhibit JHDM-mediated demethylation of histone lysine residues and TET-mediated hydroxylation and subsequent demethylation of 5-methylcytosine of DNA. Ectopic expression of mutant IDH drives a pathogenic accumulation of R-2HG that blocks cell differentiation [109,112], while disruption of mutant IDH activity inhibits the proliferation of leukemia and glioma cells and induces cell differentiation [113–

115], suggesting that mutant IDH-mediated production of 2HG may be both necessary and sufficient to initiate certain cancers characterized by a loss of endogenous differentiation mediated by chromatin remodeling. More recently, the S enantiomer of 2HG (S-2HG), produced under hypoxic conditions by promiscuous enzyme activity of malate dehydrogenase and lactate dehydrogenase, was reported to phenocopy some of the repressive chromatin signatures incurred by R-2HG accumulation [116]. Intriguingly, it was proposed that R-2HG may also possess tumor suppressor functions through a mechanism in which R-2HG-mediated inhibition of the RNA demethylase FTO enriches m^6A RNA levels and decreases the stability of messenger RNAs encoding crucial proteins for cancer cell proliferation and survival [117].

Although it is becoming increasingly clear that metabolic reprogramming in cancer helps maintain a cancer-associated – and, at least in some cases outlined above, cancer-driving – epigenetic state, it is still unclear at which stages of cancer progression might targeting metabolite-epigenetic interactions prove most useful. On one hand, while mutations in metabolic enzymes are relatively rare (with the exception of IDH isoforms 1 and 2, FDH and SDH mutations in certain subtypes of cancer), mutations in chromatin modifiers, as well as histone variants, are now known to occur frequently during cancer initiation and progression. Such mutations free cancer epigenetics from regulation by metabolic inputs and are likely positively selected in tumors. On the other hand, supplementation of metabolites such as cell-permeable αKG [118] and vitamin C [119] are sufficient to reverse carcinogenesis induced by tumor suppressor loss or oncometabolite accumulation in some instances. Therefore, metabolic interventions aimed at reverting a tumor epigenetic state towards a more normal cell-like state may provide benefit at multiple stages of cancer progression by distinct mechanisms. Further studies are needed to clarify the necessary and sufficient metabolic bottlenecks that can be therapeutically exploited in various cancer types.

7. Metabolic control of apoptosis

Apoptosis is an ancient, highly regulated cell suicide program that plays a fundamental role in embryonic development, tissue homeostasis and immunity. In mammalian cells, apoptosis can be executed by two well-characterized pathways: the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrial) pathway. In both pathways, cell death results from the activation of cysteine proteases of the caspase family, but the two pathways are triggered by different stimuli and fulfill different roles. The extrinsic pathway is initiated by ligand-induced activation of death receptors on the plasma membrane and ensures the elimination of unwanted cells during development. The intrinsic pathway is triggered by a variety of intracellular stress signals, such as

DNA damage and oxidative stress [120]. In this section, we will discuss how the intrinsic pathway is regulated by intermediary metabolism.

The main regulators of the intrinsic pathway belong to the B-cell lymphoma 2 (BCL-2) protein family. Members of this family can be classified into three subclasses: the pro-apoptotic BH3-only proteins, such as BCL-2 associated agonist of cell death (BAD), BCL-2-like protein 11 (BIM), BH3 interacting-domain death agonist (BID), BCL-2-modifying factor (BMF), p53 upregulated modulator of apoptosis (PUMA) and NOXA; the pro-survival BCL-2-like proteins, including BCL-2, B-cell lymphoma-extra large (BCL-xL) and induced myeloid leukemia cell differentiation (MCL-1); and the pore-forming or death effector proteins, namely BCL-2-like protein 4 (BAX) and BCL-2 homologous antagonist/killer (BAK) proteins. In response to an apoptotic signal, BH3-only proteins activate BAX and BAK, either by direct binding to their monomeric forms, inducing conformational changes, or by binding to pro-survival BCL-2-like proteins [121]. As a result, BAX and BAK monomers self-associate into oligomeric complexes that perforate the OMM. OMM permeabilization allows for the release into the cytosol of large mitochondrial intermembrane space proteins, including the so-called apoptogenic factors, chiefly cytochrome *c* [122–124]. Released cytochrome *c* then forms a complex with apoptotic protease activating factor 1 (APAF1), the apoptosome, which in turn activates caspase-9 [125]. This initiator caspase precipitates a proteolytic caspase cascade by activating executor caspases, such as caspase-3, -6 and -7, leading to widespread intracellular proteolytic degradation and, ultimately, cell death [126,127].

Cytochrome *c* was the first protein shown to participate in both intermediary metabolism and apoptosis [16]. Since then, several proteins with key roles in apoptosis were found to also play a role in intermediary metabolism, as exemplified by members of the BCL-2 family which relay information about the cytosolic metabolic status to mitochondria: BAD resides in a complex containing the glucokinase, an hexokinase (HK) isozyme that regulates glucose-driven mitochondrial respiration in liver and pancreatic beta cells [128]; NOXA activation is intimately associated with upregulation of the PPP during glucose starvation [129]; BAX was implicated in intermediary metabolism, ROS production and mitochondrial dynamics [130,131]. Interestingly, BAX-deficient cells are more dependent on glycolysis, exhibiting reduced mitochondrial respiration [132]. Other examples of molecules exhibiting this dual function include apoptosis-inducing factor (AIF), which has a role in the ETC (complexes I and III), modulating OXPHOS via the oxidoreductase CHCHD4/MIA40 [133]. But the molecule which is truly at the interface of intermediary metabolism and apoptosis is the protein forming the VDAC. Located on the OMM, VDAC is key in the crosstalk between mitochondria and cytosol. From one side, VDAC controls cellular energy production and

metabolism. On the other side, it regulates mitochondria-mediated apoptosis. VDAC interacts with many proteins that are involved in the activation of apoptosis, namely HK, BCL-2 and BCL-xL. Upon signaling, VDAC oligomerizes to form a large channel to facilitate the release of apoptotic proteins located in the mitochondrial intermembrane space, such as cytochrome *c* and AIF [134]. Furthermore, VDAC has a key role in Ca^{2+} homeostasis and oxidative stress [126].

Apoptosis can also be triggered by alterations in intermediary metabolism [135]. For instance, several studies have shown that disruptions in any of the ETC complexes are intimately associated with apoptosis. In animal models, pharmacological inhibition of complex I activity using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone mimics Parkinson's disease by promoting neuronal cell degeneration and apoptosis [136]. Complex II has been linked to cell death following the identification of mutations in the gene coding for one of its subunits, succinate dehydrogenase complex flavoprotein subunit A (SDHA), in Leigh syndrome, a neurodegenerative disease involving neuronal cell death [137,138]. Complex III inhibition results in inactivation of *de novo* pyrimidine biosynthesis and consequently activation of p53, which triggers apoptosis [139], whereas suppression of complex IV sensitizes cells to apoptosis [140]. Inhibition of complex V promotes a decline in mitochondrial respiration efficiency and consequently a decrease in ATP generation. Reduction in cellular ATP levels activates AMPK, in turn propelling a switch from anabolic to catabolic reactions, thus impairing cell growth and, ultimately, inducing cell death [48]. Due to this dual role in metabolism and cell death, the ETC must be tightly regulated. Of note, in a normal cell, a decrease in mitochondrial respiration reduces mitochondrial membrane potential, resulting in deficient Ca^{2+} uptake and protein trafficking and, finally, cell death [141,142]. Interestingly, human cells lacking mitochondrial DNA (mtDNA) (i.e., r0 cells) are resistant to apoptosis, while upregulation of mtDNA sensitizes cancer cells to apoptosis [143]. Such close relationship between mitochondria and cell death poses opportunities to develop new and effective drug-induced apoptosis targets for cancer therapy [144].

8. Metabolic control of the immune system

Another major breakthrough in the second Golden Age of intermediary metabolism was the discovery of an intricate interplay between metabolic pathways and immune cell differentiation and function, giving rise to the growing field of immunometabolism [145].

Upon infection by pathogens, an immune response must be mounted to eradicate them. T cells (or T lymphocytes) are critical players in this response. These immune cells belong to the adaptive arm of the immune system responsible for distinguishing self from nonself. They can be

divided into two main groups: $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. T cells that have not encountered their cognate antigen (peptide), i.e., naïve T cells, are actively maintained in a quiescent state, characterized by low metabolic activity, transcription and translation. Mounting a T cell response requires their activation by specialized antigen presenting cells (APC) and clonal expansion. Proliferating T cells then differentiate into specialized effector T (Teff) cell subtypes: Th1, Th2, Th17, etc [146,147]. They can also differentiate into regulatory T cells (Tregs). A rare subset of $CD4^+$ T cells dedicated to maintaining immune homeostasis and suppressing immune responses, $CD4^+$ $FOXP3^+$ Tregs can be generated in the thymus (thymic Tregs) or arise in the periphery from naïve T cells (peripheral Tregs). One of the broad mechanisms through which Tregs exert their suppressive function is by inhibiting Teff cell activation, proliferation and function [148,149]. In a matter of days, one single naïve T cell can divide and give rise to millions of Teff cells, leading to clearance of antigen. While most Teff cells die afterwards (contraction phase), a few long-lived memory T cells remain, which can be summoned in the event of a second encounter with the same antigen (recall response) [150].

Upon activation, naïve T cells re-enter the cell cycle and their metabolism is reprogrammed to support cell growth, proliferation and differentiation [151]. Of note, while Teff cells rely on aerobic glycolysis, fostering anabolism, memory T cells favor FAO. In fact, white adipose tissue is a known reservoir for memory T cells [152]. Strikingly, inducing mitochondrial fusion in Teff cells upregulates OXPHOS and FAO and is sufficient to confer them characteristics of memory T cells and enhance the anti-tumor activity of adoptively transferred T cells [153].

T cells require two signals to become fully activated [154]. Signal 1 is delivered upon recognition via the T cell receptor (TCR) of its cognate peptide epitope presented by a major histocompatibility complex (MHC) molecule on the surface of an APC. Of note, while the TCR, a heterodimer (TCRα and TCRβ), is responsible for ligand recognition, the signaling downstream of TCR engagement is transduced by the CD3 dimers associated with the TCR (CD3$\delta\varepsilon$, CD3$\gamma\varepsilon$ and CD3$\zeta\zeta$), which together form the TCR/CD3 complex. Upon TCR engagement with peptide-MHC, lymphocyte-specific protein tyrosine kinase (LCK) is recruited to the TCR/CD3 complex and phosphorylates signaling motifs present in the cytoplasmic portion of the CH3 dimers called immunoreceptor tyrosine-based activation motifs (ITAMs). Zeta chain of TCR-associated protein kinase 70 (ZAP70) then binds to the phosphorylated ITAMs and propagates the TCR signal in the form of an intracellular phosphorylation cascade, resulting in extracellular calcium influx and activation of the MAPK and PI3K pathways [155]. Signal 2, also known as co-stimulation, is delivered by the T cell surface receptor CD28. Upon binding to cell surface molecules CD80 or

CD86 on APCs, CD28 triggers signaling events critical for T cell survival, cytokine production, actin remodelling and long-term proliferation. Molecules downstream of CD28 include Vav1, guanine nucleotide exchange factor (GEF), which binds to proteins that anchor the actin cytoskeleton to the cell membrane, the pro-survival molecule BCL-xL, as well as LCK and PI3K [156]. Signal 1 or signal 2 are not sufficient to activate a T cell. In fact, exposure to signal 1 alone renders T cells anergic, i.e., trapped in a hyporesponsive state for long periods of time. They are no longer activated by signal 1 and signal 2 and fail to upregulate the T cell activation-associated metabolic machinery [157]. Optimal T cell activation is achieved by including, in addition to signal 1 and signal 2, what some call "signal 3" – interleukin-2 (IL-2). First identified as T cell growth factor (TCGF), IL-2 is essential for maximum T cell proliferation and generation of either Teff cells or memory T cells. Indeed, IL-2 deprivation is one of the mechanisms used to suppress Teff cell responses (see below). Of note, Teff cells secrete IL-2 themselves upon activation; IL-2 signaling is thus both paracrine and autocrine [158].

T cell activation is accompanied by an increase in nutrient uptake, cellular respiration, glutaminolysis, mitochondrial biogenesis, one carbon metabolism, PPP, fatty acid synthesis and mevalonate metabolism (for sterol synthesis) [146]. Indeed, while resting naïve T cells mainly rely on cellular respiration to generate metabolic energy, activated T cells upregulate lactic acid fermentation in the presence of ample oxygen, providing a fast source of energy while allowing the diversion of TCA cycle intermediates for anabolism to aid cell growth. Glucose transporter 1 (GLUT1) and glycolytic enzymes (e.g., HK) are upregulated upon T cell co-stimulation via CD28 signaling, driven chiefly by activity of the PI3K-AKT-mTOR pathway [159], as well as by the transcription factors c-Myc and HIF-1α [160,161]. TCR signaling upregulates pyruvate dehydrogenase kinase 1 (PDHK1), which in turn activates pyruvate dehydrogenase (PDH), redirecting pyruvate from entering the TCA cycle to being converted to lactate [162]. Blocking the conversion of pyruvate to lactate leads to a sharp decrease in the production of the pro-inflammatory antitumor cytokines interferon-γ (IFNγ) by T cells and TNFα by monocytes [163,164]. The mechanism behind these observations involves the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a moonlighting protein: besides being a glycolytic enzyme, it also suppresses translation by binding to the 3'-untranslated region (3'-UTR) of a variety of messenger RNA (mRNA) molecules, as first described in 1977 in fibroblasts [165]. Decreased glycolytic fluxes free more GAPDH inside the cell to suppress translation, resulting in less cytokine production [163,164].

Various studies have highlighted the importance of mitochondria to the metabolic reprogramming of T cells. Genetic ablation of the complex III of the ETC in Teff cells abrogates antigen-specific expansion and IL-2 production; the same intervention specifically in Tregs vanquishes their suppressive function [166,167].

In spite of tremendous progress in the past few years defining the metabolic requirements of T cells, the exact interplay between immunological clues, nutrients and intrinsic metabolic programs in determining T cell survival and function remains ill defined, especially for T cells residing in tissues. Nevertheless, one niche has offered valuable insight into such interactions: cancer.

Cancer metabolism: impact on the antitumor immune response

Metabolic reprogramming has emerged as a hallmark of carcinogenesis [168–170]. With the advent of cancer immunotherapy as a successful approach to treat previously incurable cancers [171–173], recent efforts have aimed to dissect the impact of deranged tumor metabolism in anticancer immune responses in the tumor microenvironment.

One mechanism by which tumors evade immune surveillance is Teff cell anergy induction [174]. In addition, the tumor microenvironment is impoverished in extracellular glucose, preventing naïve $CD4^+$ T cells from becoming pro-inflammatory Teff cells, instead differentiating into suppressive Tregs. Similarly, inhibiting glycolysis shifts naïve T cell polarization from a Th17 cell to a Treg fate [175].

Unlike Teff cells, Tregs rely mainly on cellular respiration [176,177]. This ability to function in low glucose, high lactate environments is in line with the fact that many solid tumors become enriched in Tregs, one of many mechanisms in place to dampen antitumor immune responses [178,179].

Amino acid metabolism also plays a key role both in immunity and in carcinogenesis. Upon activation, T cells express several amino acid transporters. Leucine uptake induces downstream mTORC1 signaling. Deletion of the leucine transporter SLC75A in T cells abrogates $CD8^+$ T cell clonal expansion and $CD4^+$ T cell differentiation into Teff cells (Th1, Th17), but not into peripheral Tregs [180]. Arginine is important for $CD8^+$ T cell survival and antitumor activity [181]. Indeed, myeloid cell production of arginase I decreases arginine availability in the tumor microenvironment and concomitantly T cell antitumor response [182]. Tryptophan is another amino acid required for optimal T cell function. Depletion of tryptophan by cells present in healthy (e.g., maternal-fetal interface) and tumor tissue via the enzyme indoleamine oxygenase (IDO)

arrests the cell cycle in T cells [183,184]. Similarly, cysteine depletion by myeloid-derived suppressor cells (MDSCs) in the tumor limits T cell activation [185]. Notably, of the many mechanisms described for Treg-mediated Teff cell inhibition, a significant proportion can be seen as metabolically-driven. Tissue Tregs constitutively express high levels of CD25 (high affinity IL-2 receptor subunit), GLUT1, CD98 (amino acid transporter) and CD71 (transferring receptor), directly depleting IL-2, glucose, leucine and Fe^{2+}, respectively, from the milieu, and induce IDO and arginase expression in APCs, diminishing the amount of tryptophan and arginine available, respectively [146,148,186–188]. Finally, glutamine is an important source of carbon and energy both for cancer cells and for T cells. Cancer cells display high fluxes of glutaminolysis, induced by the oncogenic transcription factor c-Myc [189]. TCR signaling also induces c-Myc activity and concomitantly glutaminolysis. Glutamine depletion leads to an increase in relative Treg numbers in detriment of Th1 cell numbers [190], while deleting the glutamine transporter SLC15A in naïve $CD4^+$ T cells impairs their differentiation into Th1 and Th17 cells, but not into Tregs [191].

Not surprisingly, a myriad of inhibitors targeting glycolysis, glutaminolysis, FAO and OXPHOS have been shown to modulate immune responses in autoimmunity and cancer [145]. Yet, virtually all cell types in our body use these pathways, leading one to expect treatments based on blocking them to be overtly toxic or simply ineffective. Nevertheless, the current understanding is that these can be selective, as they can preferentially target the cell type(s) with the greatest demand for the targeted pathway. Indeed, simultaneous blockade of glycolysis and glutaminolysis combined with metformin treatment has been shown to delay or even prevent immune rejection of allogeneic skin (i.e., from an individual with different MHC alleles) and heart transplants [192]. This outcome was likely due to the fact that furiously dividing immune cells recognizing the foreign transplant are more dependent on rapid energy generation than adult skin or heart cells. However, further starving the tumor microenvironment of glucose or amino acids would limit tumor growth, yet also curtail antitumor T cell responses, while providing nutrients would enhance Teff cell activation, proliferation and function, but also aid tumor cell growth. Still, some metabolic interventions at the tumor microenvironment level have yielded promising results in boosting tumor elimination when paired with immune checkpoint blockade to unleash T cell responses, including enhancing ROS production by administering mitochondrial uncouplers [193] and blocking IDO to increase tryptophan levels [194]. An alternative strategy is to manipulate immune cell metabolism in isolation. In cancer immunotherapy, a common approach is to expand tumor-specific T cells *ex vivo* and then infuse them into the patient. Interestingly, restricting glycolysis [195] or enhancing mitochondrial fusion and function [153,196] have been shown to

result in cell therapy products enriched in memory-like $CD8^+$ T cells significantly more effective in controlling tumor growth *in vivo*. Enhanced cancer therapy regimens are likely to result from the continued study of the metabolic interactions between cancer cells and immune cells.

9. Concluding remarks

Nowadays, the fundamental importance of intermediary metabolism is beyond dispute. In this review, we focused on some of the ways in which it impinges on all aspects of cellular function, ultimately touching many facets of physiology and pathology. Besides its intrinsic scientific value, a deep knowledge of intermediary metabolism has also undeniable practical value, as exemplified by the production of high-value products, such as fuels and drugs, through rational metabolic manipulation. However, this aspect, so called metabolic engineering, is beyond the scope of our review.

Further advancements in the field will undoubtedly depend on the combined use of relevant model organisms and clinical data, as well as on increasingly powerful analytical techniques and computational tools for data analysis. Yet, despite the renaissance in intermediary metabolism research of the last few decades, the long period in which it laid dormant took a heavy toll on the current number of scientists with a solid training in the field. Thus, to keep up this momentum, one of the most pressing challenges might be the development of effective strategies for teaching intermediary metabolism [197]. One must focus on explaining the chemical principles governing metabolic pathways, rather than presenting them as a very large assortment of intracellular reactions happening in the background. Such approach would demolish the idea that intermediary metabolism is tedious, highlighting the elegance of both its unifying aspects and interwoven nature. Ultimately, it would help shift intermediary metabolism from a topic that students memorize and put aside after their examinations to a vibrant field to which they will want to make a contribution, as the next generation of intermediary metabolism researchers.

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Timeline. Achievements in intermediary metabolism research.

Figure 1. Overview of the two main metabolic processes used by human cells to generate energy using glucose as the fuel molecule. A. Aerobic cellular respiration. When glucose is the substrate, this energy-transducing process starts with glycolysis, a 10-step sequence taking place in the cytosol that converts one molecule of glucose into two molecules of pyruvate, generating 2 molecules of adenine triphosphate (ATP) and 2 molecules of reduced nicotinamide adenine dinucleotide (NADH). Pyruvate in then transported into the mitochondria, where it is converted into acetyl coenzyme A (acetyl CoA) through oxidative decarboxylation. In the process, each pyruvate molecule releases one carbon atom in its fully oxidized state, as a carbon dioxide (CO_2) molecule. The reaction also generates one molecule of NADH. The acetyl moiety transported by acetyl CoA then enters the tricarboxylic acid (TCA) cycle through condensation with the TCA intermediate oxaloacetate, which generates citrate. After a 9-step sequence, oxaloacetate is regenerated and 2 carbon atoms leave the cycle in the form of CO_2. The process also generates 1 molecule of guanosine triphosphate (GTP), 3 molecules of NADH and 1 molecule of reduced flavin adenine dinucleotide ($FADH_2$). At the end of the TCA cycle, all 6 carbon atoms of glucose have been fully oxidized and excreted as CO_2. At this stage, most of the energy released during the oxidation of glucose is stored in the form of NADH (10 molecules) and $FADH_2$ (2 molecules). Altogether, the transfer of electrons from NADH and $FADH_2$ to O_2 will generate ca. 26 ATP molecules, by oxidative phosphorylation (OXPHOS). This transfer is mediated by the 6 components of the electron transport chain (ETC) depicted in the figure. **B.** Lactic acid fermentation. In the absence of an adequate O_2 supply, as is the case in muscle tissue during intense physical activity, pyruvate is reduced to lactic acid with the concomitant regeneration of NAD^+. Altogether, the conversion of pyruvate to lactate generates 2 molecules of ATP per molecule of glucose.

Abbreviations not defined in the figure legend: 1,3BPG, 1,3-bisphosphoglycerate; Cyt *c*, cytochrome *c*; CI, complex I or NADH-Q oxidoreductase; CII, complex II or succinate-Q reductase; CIII, complex III or Q-cytrochrome *c* oxidoreductase; CIV,Complex IV or cytochrome *c* oxidase; CV, complex V or ATP synthase; DHAP, dihydroxyacetone phosphate; $F1,6P_2$, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; FAD, oxidized flavin adenine dinucleotide; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GDP, guanosine diphosphate; IMM, Inner mitochondrial membrane; NAD^+, oxidized nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; Q, Coenzyme Q or ubiquinone; succinyl CoA, succinyl coenzyme A.

Enzyme codes: E1, hexokinase; E2, phosphoglucose isomerase; E3, 6-phosphofructo-1-kinase; E4, aldolase; E5, triose phosphate isomerase; E6, glyceraldehyde 3-phosphate dehydrogenase; E7, phosphoglycerate kinase; E8, phosphoglucomutase; E9, enolase; E10, pyruvate kinase; E11, pyruvate dehydrogenase; E12, citrate synthase; E13, aconitase; E14, isocitrate dehydrogenase; E15,

α-ketoglutarate dehydrogenase; E16, succinyl-CoA synthase; E17, succinate dehydrogenase; E18, fumarase; E19, malate dehydrogenase; E20, lactate dehydrogenase.

Figure 2. Overview of the complete oxidation of different amino acids and monosaccharides. The complete oxidation of these fuel molecules begins with their conversion into glycolytic and/or tricarboxylic (TCA) cycle intermediates or into acetyl coenzyme A (acetyl CoA). Once within the so-called central axis of intermediary metabolism (i.e., the sequence glycolysis → oxidative decarboxylation of pyruvate → TCA cycle → OXPHOS; see Figure 1), all fuel molecules are metabolized the same way as glucose. The arrows depicting the conversion of fuel molecules into glycolytic and TCA cycle intermediates or acetyl CoA represent the whole conversion, not necessarily a single step.

Figure 3. Overview of β-oxidation, the initial stage of fatty acid oxidation. Before entering the mitochondrial matrix to undergo oxidation, fatty acids are activated in the cytosol. Once in the mitochondria, the activated form (i.e., acyl coenzyme A (acyl CoA)) is converted to acetyl coenzyme A (acetyl CoA) by a recurring sequence of 4 steps. Acetyl CoA is then fully oxidized in the tricarboxylic acid (TCA) cycle (Figure 1A). The electrons removed to the fuel molecules in the two hydrogenation steps are transferred to the oxidized forms of nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD). The reduced forms of these coenzymes (NADH and $FADH_2$) then transfer their electrons to the electron transport chain (ETC), for the generation of energy via oxidative phosphorylation (OXPHOS) (Figure 1A).
Enzyme codes: E1, acyl-CoA dehydrogenase; E2, enoyl-CoA-hydratase; E3, 3-hydroxyacyl-CoA dehydrogenase; E4, β-ketothiolase.

Figure 4. Intracellular fates of glucose. **A.** The oxidative phase of the pentose phosphate pathway (PPP) generates ribose 5-phosphate (ribose5P) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). **B.** Prior to its addition to an existing glycogen chain, glucose is converted to an activated form – uridine diphosphate-glucose (UDP-glucose), a process that requires energy in the form of uridine triphosphate (UTP-glucose). **C.** Glycolysis. The dashed arrow represents the 9 remaining steps of glycolysis (Figure 1A).
Abbreviations not defined in the figure legend: G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; $(glucose)_n$, glycogen molecule with *n* glucose residues; $(glucose)_{n+1}$, glycogen molecule with (*n+1*) glucose residues; 6PG, 6-phosphogluconate; 6PGL, 6-phosphoglucono-δ-lactone; ribulose5P, ribulose 5-phosphate; UDP, uridine diphosphate.
Enzyme codes: E1, glucose-6-phosphate dehydrogenase; E2, gluconolactonase; E3, 6-phosphogluconate dehydrogenase; E4, phosphopentose isomerase; E5, phosphoglucomutase; E6, glucose-1-phosphate uridyltransferase; E7, glycogen synthase.

Figure 5. Crosstalk between intermediary metabolism and epigenetic regulation. A. A variety of nutrients is processed by the pathways of intermediary metabolism to generate substrates, cofactors and allosteric regulators of epigenetic modifiers. Nutrient catabolism of glucose-derived pyruvate, fatty acids and ketogenic amino acids (AAs) are the major sources of mitochondrial acetyl coenzyme A (acetyl CoA), the major substrate for histone acetylation. In order to support histone acetylation in the nucleus, acetyl CoA must first be converted to citrate in the mitochondria, transported across the mitochondrial membrane, and cleaved back into acetyl CoA in the cytosol or nucleus. Some cells also possess the ability to generate acetyl CoA from acetate as a nutrient source. Glutamine, one of the main anaplerotic substrates, generates α-ketoglutarate (αKG). αKG, succinate, fumarate and the oncometabolite 2-hydroxyglutarate (2HG; the R enantiomer produced by mutant isocitrate dehydrogenase (IDH) and the S enantiomer produced by various dehydrogenases under hypoxic conditions) can all regulate the function of histone and DNA demethylases. The mitochondrial electron transport chain (ETC) activity regulates the availability of oxidized nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) cofactors necessary for sirtuins (histone deacetylase) and lysine-specific histone demethylase (LSD) family demethylases. Methionine is the main precursor for S-adenosyl-methionine (SAM), an important product of the methionine cycle used as the substrate for histone/DNA/RNA methylation. Serine— either derived from glucose or transported into the cell—supports SAM levels either through nucleotide synthesis or donating its side chain carbon to the folate-mediated one-carbon (1C) cycle, which is coupled to the methionine cycle. **B.** Regulation of acetylation, deacetylation, methylation and demethylation by various metabolic inputs. Activators are denoted by pointed arrows, while inhibitors are denoted by blunted arrows. In the case of histone deacetylases and demethylases, the inhibitors for NAD^+-independent classical histone deacetyltransferase (HDAC) and LSD family enzymes are highlighted in yellow.

Abbreviations not defined in the figure legend: Ac, acetylation motif; ALKBH5, alkylated DNA repair protein homolog 5; CoA, coenzyme A; DNMT, DNA methyltransferase; $FAD/FADH_2$, reduced/oxidized forms of flavin adenine dinucleotide; FTO, fat mass and obesity-associated protein; HMT, histone methyltransferase; JHDM, Jumonji domain-containing histone demethylase; Me, methylation motif; METTL, RNA methyltransferase; NAD^+/NADH, reduced/oxidized forms of nicotinamide adenine dinucleotide; SAH, S-adenosylhomocysteine; SIRT, sirtuin family of histone deacetylases; TET, ten-eleven translocation.

Credit Author Statement

A.M.U. conceptualized the review, wrote sections 1, 2, 3 and 9, provided editorial input to all sections and reviewed and edited the whole manuscript. L.M.R.F. wrote section 8 and reviewed and edited the whole manuscript. A.M.L. wrote section 6 and prepared the corresponding figures. T.L.S. wrote section 7 and contributed to section 5. M.C.S. wrote section 5 and prepared timeline and all other figures. M.C.A. wrote section 4. All authors provided comments on and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Milestones in intermediary metabolism research span four centuries
- Metabolites and metabolic by-products are involved in cell signaling
- Metabolism regulates programmed cell death
- Several metabolites link intermediary metabolism to epigenetics
- Intermediary metabolism plays a key role in immune cell specification and function

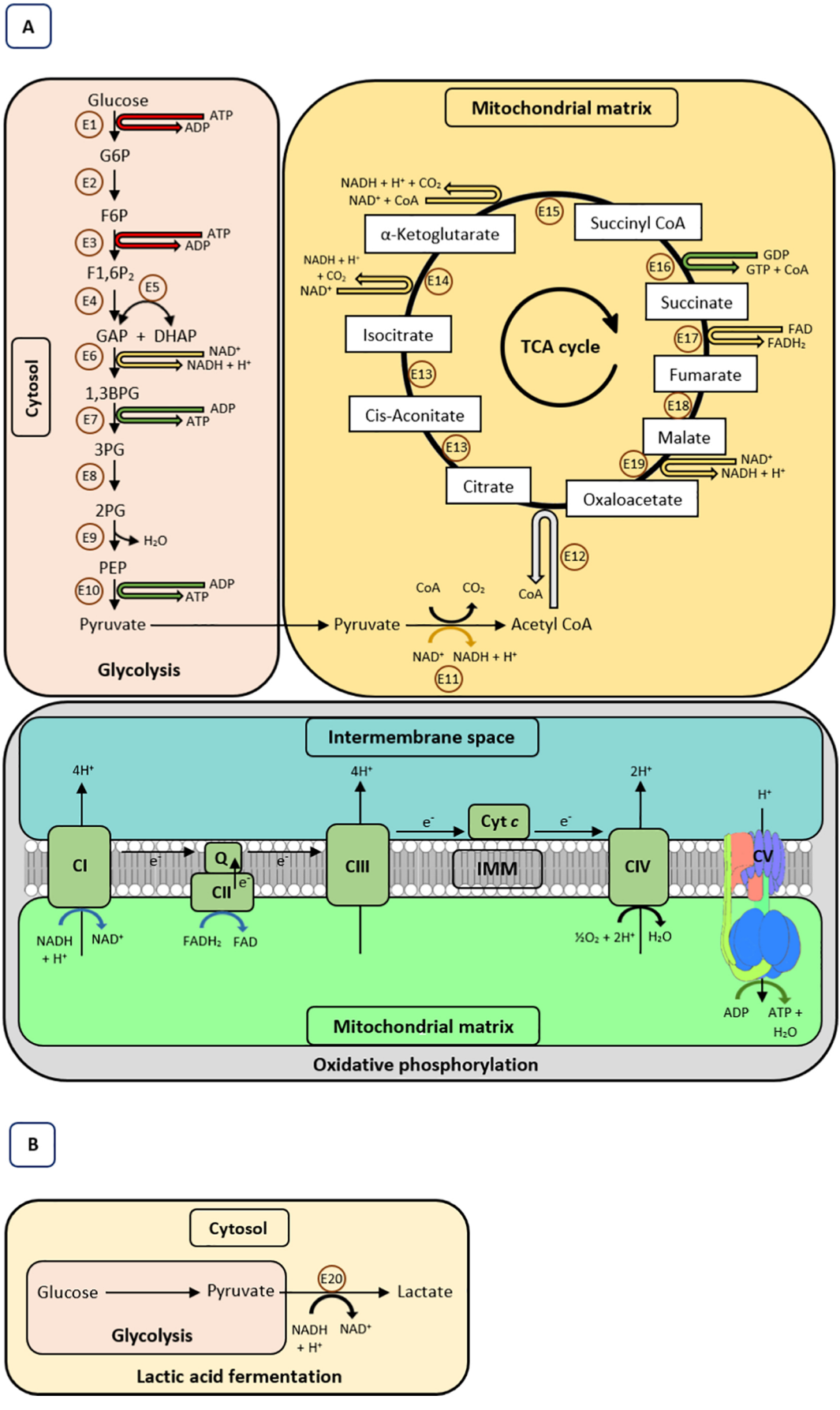
A
Glucose
E1
ATP
ADP
G6P
E2
F6P
E3
ATP
ADP
F1,6P2
E4
E5
GAP + DHAP
E6
NAD+
NADH + H+
1,3BPG
E7
ADP
ATP
3PG
E8
2PG
E9
H2O
PEP
E10
ADP
ATP
Pyruvate
Glycolysis
Cytosol
Mitochondrial matrix
NADH + H+ + CO2
NAD+ + CoA
E15
Succinyl CoA
α-Ketoglutarate
E16
GDP
GTP + CoA
NADH + H+
+ CO2
NAD+
E14
Succinate
Isocitrate
TCA cycle
E17
FAD
FADH2
E13
Fumarate
Cis-Aconitate
E18
E13
Malate
E19
NAD+
NADH + H+
Citrate
Oxaloacetate
E12
CoA
CO2
CoA
Pyruvate
Acetyl CoA
NAD+
NADH + H+
E11
Intermembrane space
4H+
4H+
2H+
H+
e-
Cyt c
e-
CI
e-
Q
e-
CII
CIII
IMM
CIV
CV
NADH
+ H+
NAD+
FADH2
FAD
½O2 + 2H+
H2O
ADP
ATP +
H2O
Mitochondrial matrix
Oxidative phosphorylation
B
Cytosol
Glucose
Pyruvate
E20
Lactate
Glycolysis
NADH
+ H+
NAD+
Lactic acid fermentation

Figure 1

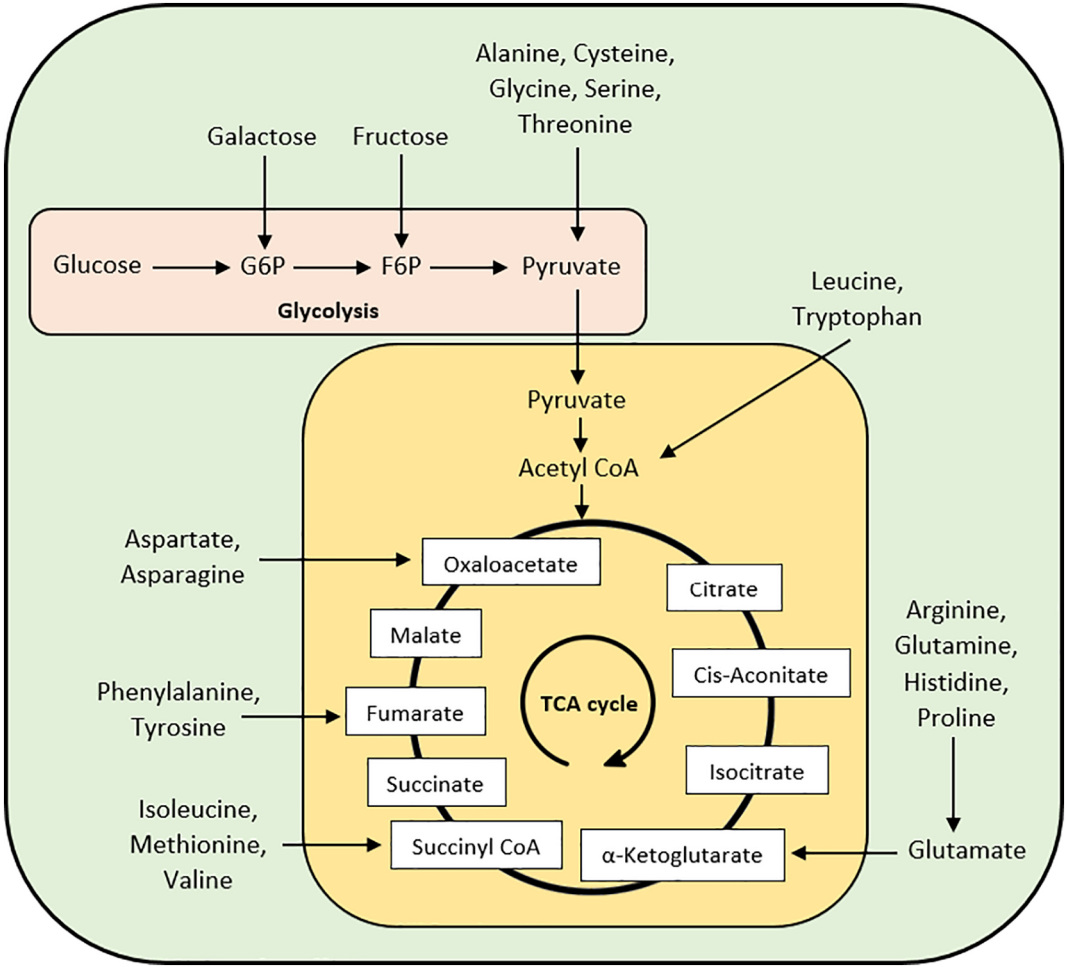
Alanine, Cysteine,
Glycine, Serine,
Threonine
Galactose
Fructose
Glucose
G6P
F6P
Pyruvate
Glycolysis
Leucine,
Tryptophan
Pyruvate
Acetyl CoA
Aspartate,
Asparagine
Oxaloacetate
Citrate
Malate
Cis-Aconitate
Arginine,
Glutamine,
Histidine,
Proline
Phenylalanine,
Tyrosine
Fumarate
TCA cycle
Isocitrate
Succinate
Isoleucine,
Methionine,
Valine
Succinyl CoA
α-Ketoglutarate
Glutamate

Figure 2

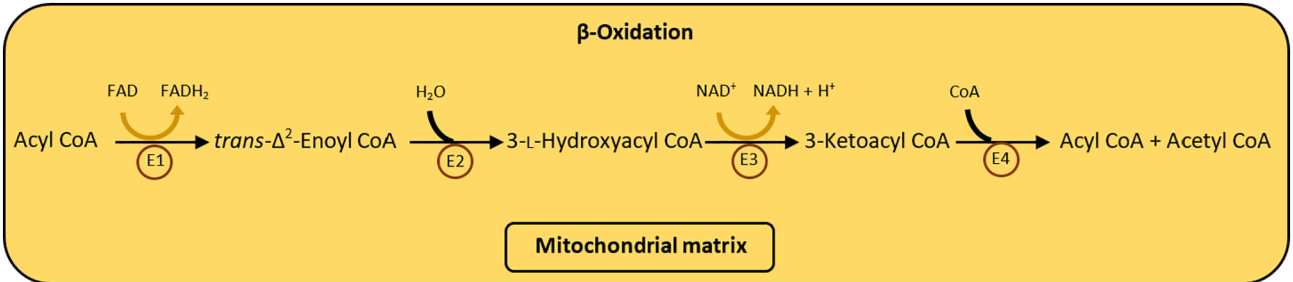
β-Oxidation
FAD
FADH2
H2O
NAD+
NADH + H+
CoA
Acyl CoA
E1
trans-Δ2-Enoyl CoA
E2
3-L-Hydroxyacyl CoA
E3
3-Ketoacyl CoA
E4
Acyl CoA + Acetyl CoA
Mitochondrial matrix

Figure 3

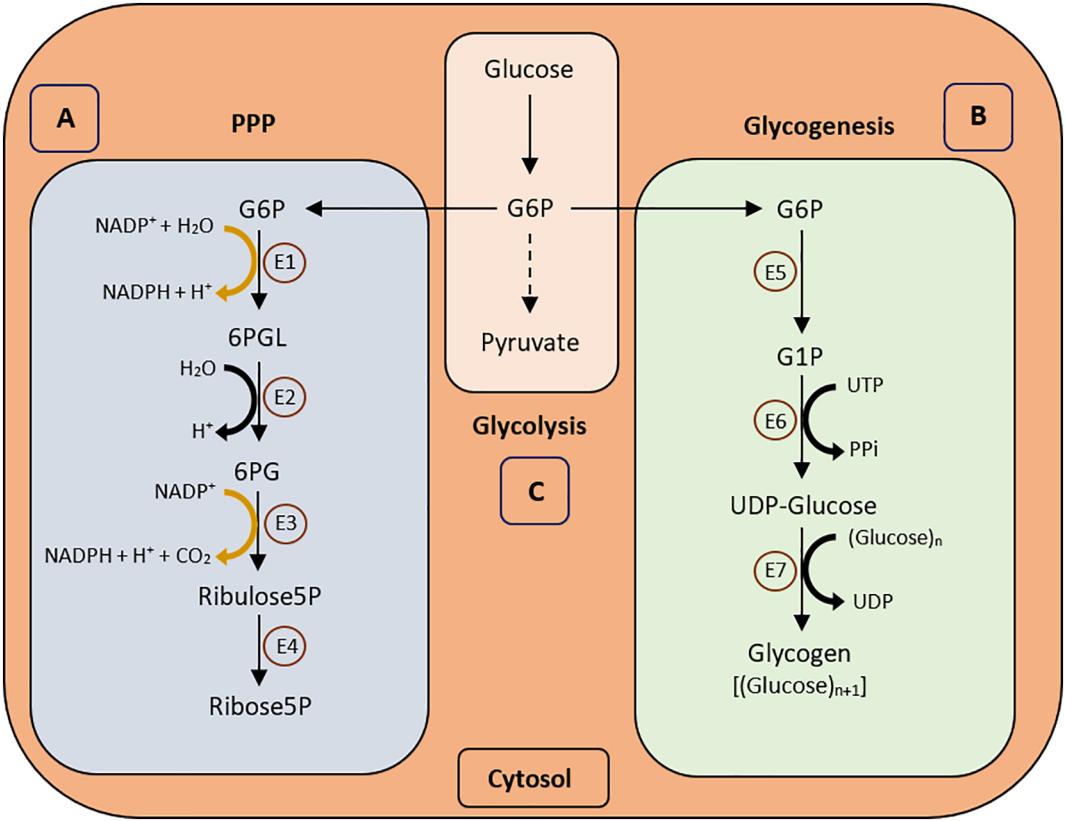
A
PPP
G6P
NADP+ + H2O
E1
NADPH + H+
6PGL
H2O
E2
H+
6PG
NADP+
E3
NADPH + H+ + CO2
Ribulose5P
E4
Ribose5P
Glucose
G6P
Pyruvate
Glycolysis
C
B
Glycogenesis
G6P
E5
G1P
UTP
E6
PPi
UDP-Glucose
(Glucose)n
E7
UDP
Glycogen
[(Glucose)n+1]
Cytosol

Figure 4

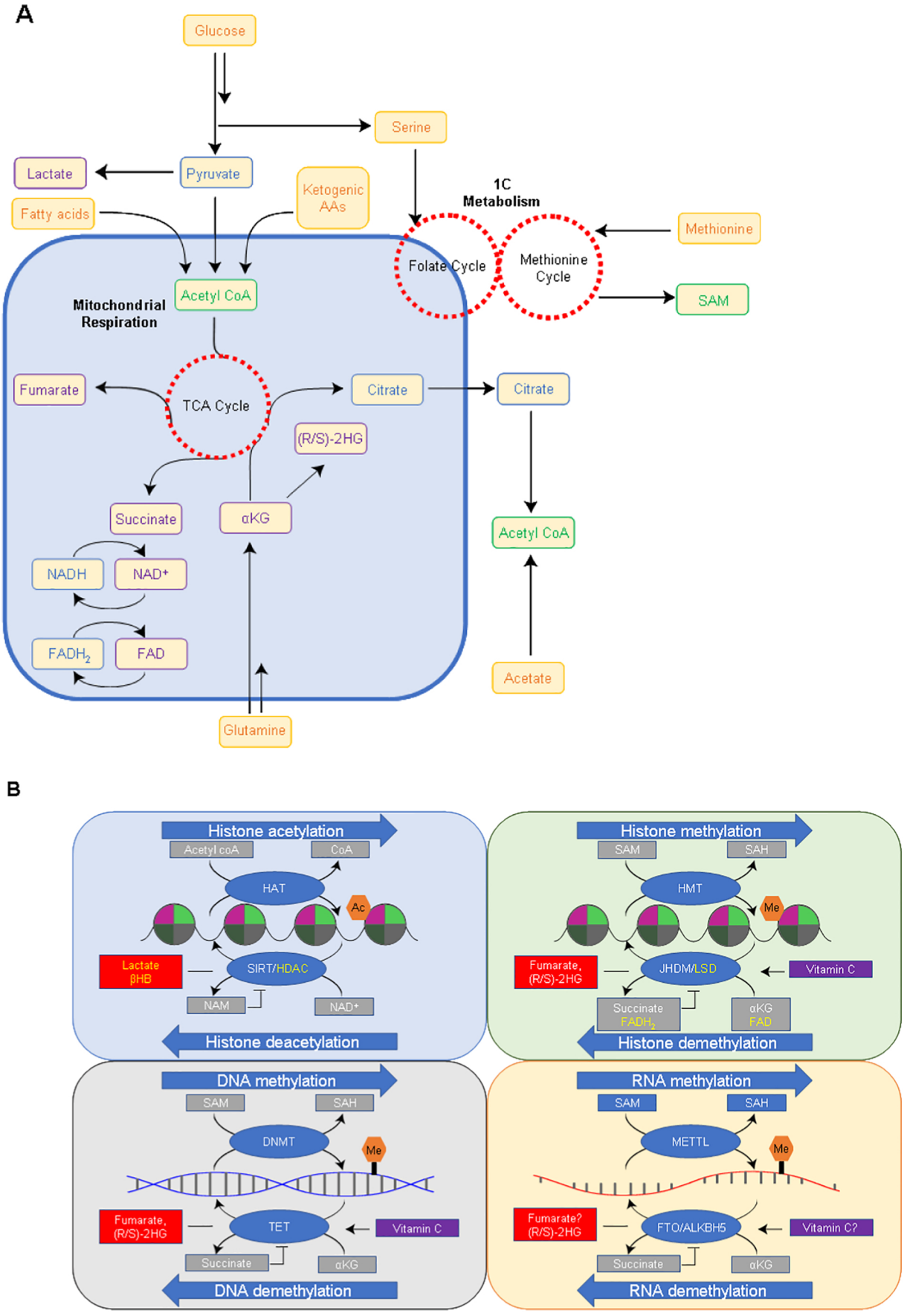
A
Glucose
Serine
Lactate
Pyruvate
Ketogenic AAs
Fatty acids
1C Metabolism
Methionine
Folate Cycle
Methionine Cycle
SAM
Mitochondrial Respiration
Acetyl CoA
Fumarate
TCA Cycle
Citrate
Citrate
(R/S)-2HG
Succinate
αKG
Acetyl CoA
NADH
NAD+
FADH2
FAD
Acetate
Glutamine
B
Histone acetylation
Acetyl coA
CoA
HAT
Ac
Lactate βHB
SIRT/HDAC
NAM
NAD+
Histone deacetylation
Histone methylation
SAM
SAH
HMT
Me
Fumarate, (R/S)-2HG
JHDM/LSD
Vitamin C
Succinate FADH2
αKG FAD
Histone demethylation
DNA methylation
SAM
SAH
DNMT
Me
Fumarate, (R/S)-2HG
TET
Vitamin C
Succinate
αKG
DNA demethylation
RNA methylation
SAM
SAH
METTL
Me
Fumarate? (R/S)-2HG
FTO/ALKBH5
Vitamin C?
Succinate
αKG
RNA demethylation

Figure 5