

Otto Warburg's contributions to current concepts of cancer metabolism

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Abstract | Otto Warburg pioneered quantitative investigations of cancer cell metabolism, as well as photosynthesis and respiration. Warburg and co-workers showed in the 1920s that, under aerobic conditions, tumour tissues metabolize approximately tenfold more glucose to lactate in a given time than normal tissues, a phenomenon known as the Warburg effect. However, this increase in aerobic glycolysis in cancer cells is often erroneously thought to occur instead of mitochondrial respiration and has been misinterpreted as evidence for damage to respiration instead of damage to the regulation of glycolysis. In fact, many cancers exhibit the Warburg effect while retaining mitochondrial respiration. We re-examine Warburg's observations in relation to the current concepts of cancer metabolism as being intimately linked to alterations of mitochondrial DNA, oncogenes and tumour suppressors, and thus readily exploitable for cancer therapy.

Respiration

The metabolic process by which energy is produced in the presence of O₂ through the oxidation of organic compounds (typically sugars) to CO₂ and H₂O by glycolysis, the citric acid cycle and oxidative phosphorylation.

Es ware möglich, die gesamte Geschichte der Biochemie ... an Otto Warburgs werk aufzuzeigen. (It would be possible to illustrate the entire history of biochemistry ... with the work of Otto Warburg.) (Adolf F. J. Butenandt, 1970)¹

Otto Warburg (FIG. 1) was one of the first true interdisciplinary scientists. Warburg, who spent his entire career in Germany, pioneered work on respiration and photosynthesis during the early twentieth century. During the 1910s, it was thought that the energy-yielding reactions necessary for the growth of cancer cells were lipolysis and/or proteolysis². However, Warburg focused on glycolysis and showed that all of the cancer cells he investigated exhibit a reversed Pasteur effect (the inhibition of fermentation by O₂). In other words, cancer cells produce lactic acid from glucose even under non-hypoxic conditions³, an observation that has come to be known as the Warburg effect⁴ (which is not to be confused with the other Warburg effect: the inhibition of photosynthetic CO₂ fixation by O₂ (REF. 5)). With few exceptions, Warburg's findings were published in German-language journals, and during the latter part of the twentieth century, with the post-Second World War relocation of scientific primacy to English-language institutions and the blossoming of the field of molecular biology, Warburg's contributions became largely disregarded. The discovery in recent decades of a connection between oncogenes and metabolic processes has led to a renaissance of interest

in Warburg's work today⁶, although his findings and conclusions are often misinterpreted. The semantics of Warburg's report that "the respiration of all cancer cells is damaged"⁷ continues to be debated, because the experiments by Warburg and his co-workers, and those of contemporary investigators, indicate that such a conclusion is erroneous.

In this Review, we describe the historical context of Warburg's investigations of lactic acid production by cancer cells and explore the impact of his work on our current conceptual framework of cancer cell metabolism.

Warburg's life

The details of Warburg's life and personality have been gleaned from biographies written by Krebs^{8,9}, Werner^{1,10}, Höxtermann and Sucker¹¹ and Koepcke¹². Otto Heinrich Warburg was born 8 October 1883 in Freiburg im Breisgau. His father, Emil Warburg, was one of the most eminent physicists of his time¹³ and was revered by young Otto. As was common among professors' families, the Warburgs resided at Emil's institute, first at the University of Freiburg and later in Berlin, to allow him to concentrate on research. Thus Otto was raised in an academic environment — Otto's sister Lotte claimed that "Papa weiss nicht einmal, wo Mamas Schlafzimmer ist!" ("Papa doesn't even know where Mama's bedroom is!")¹². Warburg's life and his academic achievements are summarized in the TIMELINE.

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At a glance

- Otto Warburg was a pioneering biochemistry researcher who made substantial contributions to our early understanding of cancer metabolism. Warburg was awarded the Nobel Prize in Physiology or Medicine in 1931 for his discovery of cytochrome *c* oxidase, not for his work on cancer and the formulation of the Warburg hypothesis.
- The Warburg effect is the reverse of the Pasteur effect (the inhibition of fermentation by O₂) exhibited by cancer cells; alteration of the Pasteur effect in cancer is linked to prolyl hydroxylases and hypoxia-inducible factor (HIF).
- Tumour suppressors and oncogenes converge on HIF to reverse the Pasteur effect and thereby induce the Warburg effect.
- Cancer cells carry out aerobic glycolysis and respiration concurrently.
- Tumour suppressors and oncogenes exert direct effects on metabolism: p53 promotes the pentose phosphate pathway and oxidative phosphorylation; MYC induces glycolysis and glutamine metabolism.
- Mutations in metabolic enzymes, specifically isocitrate dehydrogenase 1 (IDH1) and IDH2 and other citric acid cycle enzymes, are causally linked to familial and spontaneous cancers.

Glycolysis

A metabolic pathway that occurs in the cell cytoplasm and involves a sequence of ten enzymatic reactions. These reactions convert glucose to pyruvate and produce the high-energy compounds ATP and NADH.

Pasteur effect

Pasteur's observation that yeast cells consume less sugar when grown in the presence of O₂ than when grown in the absence of it.

Fermentation

The metabolic process by which energy is produced in the absence of O₂ through the oxidation of organic compounds, typically sugars, to simpler organic compounds, such as pyruvate. Pyruvate is further processed to ethanol by alcoholic fermentation or lactic acid by lactate fermentation; see 'glycolysis'.

Warburg effect

A term used to describe two unrelated observations in plant physiology and oncology, both from the work of Otto Warburg. In oncology, the Warburg effect refers to the high rate of glycolysis and lactate fermentation in the cytosol exhibited by most cancer cells, relative to the comparatively low rate of glycolysis and oxidation of pyruvate in mitochondria exhibited by most normal cells. In plant physiology, the Warburg effect is the inhibition of photosynthetic CO₂ fixation by high concentrations of O₂.

An equally important influence in Otto's life was his *Doktorvater* (doctoral advisor) at the University of Berlin, Prof. H. Emil Fischer, who was awarded the Nobel Prize in Chemistry in 1902 for his work on sugar and purine syntheses. Warburg began his studies in chemistry at the University of Freiburg, and had transferred in 1903 to the University of Berlin when his father was invited to join the faculty there. Fischer ruled his institute dictatorially, demanding from his subordinates honour, respect, reliability, frankness, self-responsibility and self-discipline. In 1906, Warburg completed his chemistry Ph.D. dissertation, which was entitled "Über Derivative des Glycocolls, Alanins und Leucins. Über die 1-Brompropionsäure und das 1-Alanlyglycin" ("On derivatives of glycine, alanine and leucine. On 1-bromopropionic acid and alanylglycine"). As a student Otto had already set for himself the lofty goal of curing cancer⁸, so he began to study medicine at the University of Berlin in 1905, and he concluded his studies in medicine at the University of Heidelberg under Prof. von Krehl in 1911. He completed a *Habilitation* in physiology at the University of Heidelberg in 1913 and joined the Department of Physiology of the Kaiser Wilhelm Institute (KWI, which later became the Max Planck Institute) for Biology in Berlin-Dahlem as an independent researcher working on the embryology of sea urchins. He was appointed head of the department in 1914.

Otto Warburg credited his professional success partly to his military experience^{8,10}. At the outbreak of the First World War, Warburg volunteered for military service, and joined the 2nd Regiment Ulanen (3rd Squadron), an elite cavalry unit, first serving as physician and later as aide-de-camp at the headquarters of the 202nd Infantry Division. He served in France and at the Eastern Front in present-day Estonia and Lithuania; he was wounded in 1917, possibly during the battle for Riga, and was awarded the Iron Cross First Class. Near the end of the war, Warburg's mother contacted Albert Einstein, a family friend, and requested that he use his influence to convince her son to fulfil

his patriotic duty doing research rather than serving at the front. Einstein complied⁸ and Warburg agreed and sought release from active service, which was approved in the summer of 1918.

Warburg resumed his scientific activities at the KWI and was concurrently appointed Professor at the Friedrich Wilhelm University in Berlin. Although funding was limited under the Weimar Republic, the full funding of Warburg's one-sentence research proposal speaks to his reputation as an accomplished scientist during the 1920s (FIG. 2). With major support from the Rockefeller Foundation, he established the KWI for Cell Physiology in Berlin-Dahlem in 1931, the same year in which he was awarded the Nobel Prize in Physiology or Medicine for his discovery of the respiratory enzyme cytochrome *c* oxidase.

Although the Warburgs were descended from the Jewish Warburg financiers of Altona, near Hamburg, Otto Warburg's mother was not Jewish, and Emil Warburg had long before converted to Protestantism. After Hitler came to power in 1933, the Nazi's repressive policies negatively affected Otto and his staff. In 1941, he was briefly removed as director of his department, only to be reinstated shortly thereafter. In 1942, he was appointed to a national committee entrusted with fighting cancer, a disease that Hitler morbidly feared. It is plausible that Otto was protected at the highest level because he worked on cancer. It is also clear that Otto chose not to flee Nazi Germany, having sniped to his sister, Lotte, "Ich war vor Hitler da" ("I was here before Hitler")¹². Warburg remained under Nazi scrutiny throughout the period, and it is a wonder that Warburg — given his Jewish ancestry, open disdain for Hitler's regime, and probable homosexuality¹⁰ — was allowed to continue working at all during the Nazi period. After the laboratories sustained damage from Allied air raids¹⁰, the institute was evacuated to Liebenburg in the countryside north of Berlin, where in 1945 the occupying Russian Army appropriated the laboratory equipment⁸. Although the headquarters of the German armed forces classified Warburg's institute as crucial for the war effort, Warburg later refuted that he had ever performed war-related research¹.

After the war, the buildings that housed the KWI for Cell Physiology in Berlin-Dahlem were commandeered as headquarters of the American Army, and Warburg had no research facilities until 1950 when the refurbished institute reopened. Warburg worked there until his death in 1970 at the age of 87. During his later years, Warburg, a non-smoker, adopted a personal cancer-preventive lifestyle that resonates today, consisting of moderate exercise combined with a diet of fresh, home-grown vegetables. He never married, but was accompanied faithfully by his long-standing companion Jakob Heiss.

Like his father and *Doktorvater* before him, Warburg resided in his institute, working 6-day weeks on problems of cell physiology, particularly pertaining to metabolism, cancer and photosynthesis. He often opened his laboratory to academic guests, among them such scientific giants as Otto F. Meyerhof, Hans A. Krebs and Axel H. T. Theorell, but declined



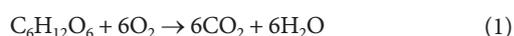
Figure 1 | **Otto Warburg.** Otto Heinrich Warburg in his laboratory of the Kaiser Wilhelm Institute for Biology in Berlin-Dahlem, 1931. Image is reproduced, with permission, from the German Federal Archives: image 102-12525, photographer unknown.

to attach his name to every publication emanating from his institute. He was vigorous and arrogant in his opposition to scientists who questioned his findings, even indulging in unscientific emotional attacks within scientific reports¹, and he was criticized by Krebs for his tendency towards polemics⁹. Warburg preferred to employ instrument makers to whom he taught biochemistry and from whom he tolerated no argument. He enjoyed working with his hands and was a firm believer in quantitative methods. He continually sought means to improve quantification in biological research: he invented the use of thin tissue slices for physiology research¹⁴, improved manometric techniques¹¹ to measure changes in pressure accompanying cell and tissue processes^{14,15}, and is credited as the inventor of the single-beam spectrophotometer¹¹. These contributions were pivotal to his research on metabolism and cancer physiology, which are described in a collection of his early works¹⁵. These publications were groundbreaking because Warburg used quantitative physical-chemical approaches to investigate the rapid growth of cancer cells.

Formulation of the ‘Warburg hypothesis’

Warburg studied and conducted research during a golden age of biochemical discovery (TIMELINE). He could trace his scientific lineage to Adolf von Baeyer (who won the Nobel Prize in Chemistry in 1905), and was thus part of a scientific ‘family’ that includes a dozen Nobel laureates^{1,16}.

In his earlier embryological investigations of sea urchin eggs, Warburg had observed a rapid increase in O₂ uptake and subsequent rapid cell division upon fertilization¹⁷, and he postulated that cancer tissues might also take up more O₂ than normal tissue. To address this hypothesis, Warburg used his improved manometric technique^{14,18} (FIG. 3) to measure O₂ consumption in thin tissue slices metabolizing glucose:



The Warburg manometer was also used to measure CO₂ emission, which is equivalent to lactic acid production, from bicarbonate-containing buffers:



Warburg and co-workers discovered that Flexner–Jobling rat liver carcinoma does not take up more O₂ than normal liver tissue, but that, even in the presence of O₂, such tissue produces lactic acid. This indicates the processing of glucose by lactic acid fermentation, bypassing the entry of pyruvate into the citric acid cycle (respiration)¹⁸. As already mentioned, normal tissue was known to exhibit the Pasteur effect — that is, to stop producing lactic acid in the presence of O₂. Human carcinomas (from throat, intestine, skin, penis and nose) also demonstrated lactic acid production^{19,20}.

Seigo Minami, an academic guest at the KWI for Biology, reported that although the respiration of Flexner–Jobling rat liver carcinoma tissue slices is 20% less than that of normal tissue, which could be attributed to the presence of necrotic cells, approximately tenfold more glucose was metabolized than could be accounted for by respiration. Minami confirmed Warburg’s manometric lactic acid analysis by chemical means¹⁹, and Warburg subsequently determined that the amount of lactic acid produced by cancer cells is two orders of magnitude higher than that produced by normal tissue²⁰.

With these methods, Warburg and co-workers determined how O₂ affects glycolysis and defined the Meyerhof quotient as the molar ratio of the O₂ consumed to the difference in lactic acid production under anaerobic conditions compared with aerobic conditions — that is, a measure of the amount of O₂ required to convert one lactic acid molecule to glucose²⁰. From experiments with thin tumour tissue slices (FIG. 3), they determined a Meyerhof quotient of 1.3, which was equivalent to that determined previously for normal tissues. As such, they concluded that respiration in cancer tissue is normal but inadequate to prevent the formation of lactic acid. It should be noted that, in the experiments performed in the presence of O₂, glucose was present in excess at all times, and the thickness of the tissue slices was limited to <400 μm (FIG. 3) to exclude the possibility that lactic acid was produced because cells became anaerobic. It has since been demonstrated that O₂ consumption in model multicellular spheroids of Chinese hamster fibroblasts is dependent on spheroid diameter, with fourfold reduction of O₂ uptake across the diameter range 200–400 μm²¹. More recent studies of multilayer human choroidal melanoma cells as models of tumour tissues that support Warburg’s calculations pertaining to tissue slices¹⁴ show that O₂ consumption decreases as a function of layer thickness but that thicknesses ≤400 μm are not anoxic²².

Decades later, in 1952, Warburg and Hiepler²³ reported that, per mg of cells, Ehrlich ascites tumour cells from mice produce more lactic acid in normoxic and hypoxic conditions than the thin Flexner–Jobling rat tumour slices (FIG. 3). Chance and co-workers showed

Habilitation

A quasi-independent postdoctoral appointment that is required for further academic advancement in German-speaking countries.

Citric acid cycle

A cyclic series of eight enzymatic reactions that occur in the mitochondrial matrix and that convert acetyl CoA derived from carbohydrates, fatty acids and amino acids to CO₂ and H₂O; also known as the tricarboxylic acid (TCA) cycle or Krebs cycle.

Aerobic glycolysis

The enzymatic transformation of glucose to pyruvate in the presence of O₂; see 'glycolysis'.

that rates of respiration for ascites cells were comparable to those of muscle and yeast cells^{24,25}, thus the enhanced production of lactic acid was not at the cost of respiration. Weinhouse²⁶ also reported that cancer cells exhibit normal rates of respiration and described Warburg's contentions as hypothesis based on "essentially fallacious reasoning", but his account was dismissed by Warburg⁷ and Burk and Schade²⁷.

The lactic acid levels of mouse carcinoma and rat sarcoma tumours *in vivo* were reported by Cori and Cori²⁸ in 1925 to be very much lower than the levels observed in the *in vitro* experiments of Warburg and co-workers^{29,30}. Cori and Cori³¹ further showed that the blood drawn from a vein exiting a Rous sarcoma tumour implanted on one wing of a chicken contained significantly less glucose and more lactic acid than blood passing through the tissues of the corresponding normal wing, and they concluded that, *in vivo*, the excess lactic acid production in tumours is washed out by the blood flow through the tissue. In similar experiments on rats, Warburg and co-workers^{29,30} reported arterial and venous plasma levels of glucose and lactic acid in healthy organs compared with those in Jensen's sarcomas transplanted into the stomach; the glucose content of the veins from control organs was 2–18% less than that of the arteries, compared with a 47–70% drop across the tumours. Arterial versus venous levels of lactic acid from tumours indicate that, on average, 66% of the glucose consumed is converted to lactic acid, whereas healthy organs produced no net lactic acid. Because cancer cells 'recycle' lactic acid under aerobic conditions³², the lactic acid levels recorded in the *in vivo* experiments may be lower than the actual levels produced by tumours^{29,30}. The glucose and O₂ concentration gradient across tissue decrees that the metabolism of tumour cells closer to the arterial blood is more like that of *in vitro* tissue slices, whereas the metabolism of cells deeper in the tumour is limited by diffusion. Thus, the *in vitro* experiments better reflect the *in vivo* conditions of cells close to the metabolic supply side of glucose and O₂. Warburg attempted to address the influence of glucose and O₂ supply to tumour cells *in vivo*^{29,30}, and concluded that it is difficult to inhibit the growth of tumours in living animals through the manipulation of metabolic substrates.

Warburg and co-workers had expected that the O₂ consumption of rapidly dividing cancer cells would be greater than that of normal differentiated tissue, as occurs in embryonic cells. The Meyerhof quotients of approximately 1–2 for thin slices of both normal and cancerous tissues²⁰ indicate that O₂ consumption (that is, respiration) by cancer tissues is the same as that of normal cells. Warburg believed respiration to be fundamentally more complex than glycolysis and, therefore, more vulnerable to injury:

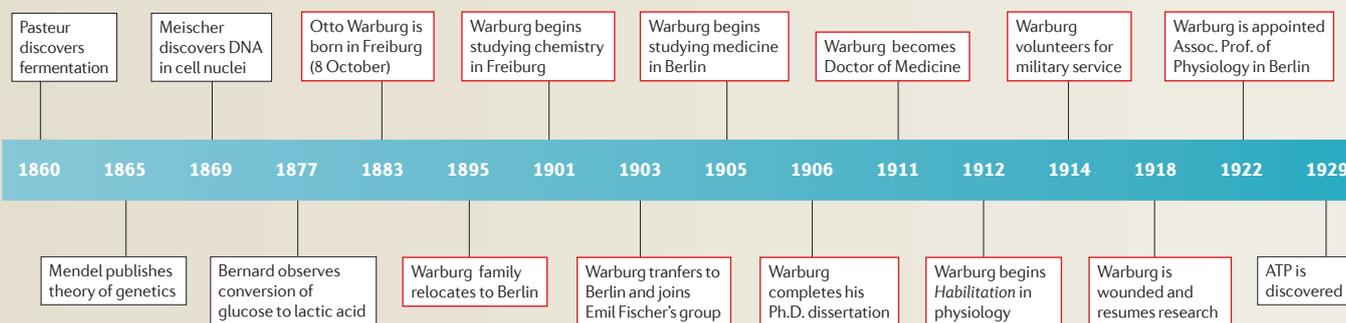
*The origin of cancer lies in the anaerobic metabolic component of normal growing cells, which is more resistant to damage than is the respiratory component. Damage to the organism favours this anaerobic component and, therefore, engenders cancer.*³³

Crabtree³⁴ concurred in 1929: "Warburg postulates a disturbance of respiration as being the fundamental cause of the development of aerobic glycolysis." Warburg reasoned that, since the increased production of lactic acid by cancer cells is not nullified by higher O₂ consumption, respiration must be damaged³³. Today, we understand that the Meyerhof quotient, as defined by Warburg, erroneously links respiration too intrinsically to lactate production; further, Warburg's reasoning about respiration — that higher rates of respiration could reduce the production of lactic acid³⁵ — is incorrect. Sonveaux *et al.*³² recently showed that normoxic cancer cells metabolize lactic acid but anaerobic cells do not. This finding may explain Warburg's observation that oxygenated tumour cells appear to produce less lactic acid (FIG. 3).

Is respiration "damaged"?

The observations that cancer cells simultaneously oxidize and ferment glucose has engendered confusion over the role of respiration in the Warburg effect, particularly as Warburg misinterpreted his own early observations and promoted the erroneous idea that damaged respiration is the *sine qua non* that causes increased glucose fermentation in cancers. The *in vitro* findings of Warburg

Timeline | Significant events in Warburg's life and relevant discoveries in cancer cell metabolism biochemistry



Compiled from information in REFS 1, 9–11. Red boxes refer to events in Warburg's life; black boxes refer to milestones in cancer metabolism research.

Oxidative phosphorylation (OXPHOS). A metabolic process that occurs in mitochondria. It produces energy in the form of ATP from ADP and inorganic phosphate, and is driven by a proton gradient generated by the reactions of the citric acid cycle.

and co-workers²⁰ show that, in the time required for cancer tissue under normoxic conditions to completely metabolize one molecule of glucose to yield 36 molecules of ATP, ten more glucose molecules (FIG. 3) are converted to 20 molecules of lactic acid to yield, at one ATP per lactic acid, an additional 20 molecules of ATP. Under anoxic conditions, cancer cells convert 13 glucose molecules to 26 lactic acid and 26 ATP; thus, in the time it takes a normal cell to produce 36 ATP from one glucose, the aerobic cancer cell produces 56 ATP from 11 glucose, whereas the anoxic cancer cell generates 26 ATP from 13 glucose³⁵. When Warburg and co-workers determined lactic acid levels, they found that the tumour removes 70 mg glucose and releases 46 mg of lactic acid per 100 ml of blood^{29,30}, which, by our reckoning, corresponds to 10% more ATP produced by cancer cells than by normal cells. Recent *in vitro* data on glucose uptake and lactic acid release by human glioblastoma LN18 cells show a similar 13% increase in ATP production³⁷.

In 1956, Warburg reiterated “the respiration of all cancer cells is damaged”⁷⁷, even though findings from his own laboratory¹⁸ and those of others^{24,26} indicated otherwise. In the second collection of his work published in 1962 (REF. 35), Warburg attempted to clarify and modulate his classifications of cancer cells as well as to justify the conclusions he had drawn from his own work, admitting that the description based on insufficient respiration had led to “unfruchtbaren Kontroversen” (“fruitless controversy”). Today, we understand that the relative increase in glycolysis exhibited by cancer cells under aerobic conditions was mistakenly interpreted as evidence for damage to respiration instead of damage to the regulation of glycolysis.

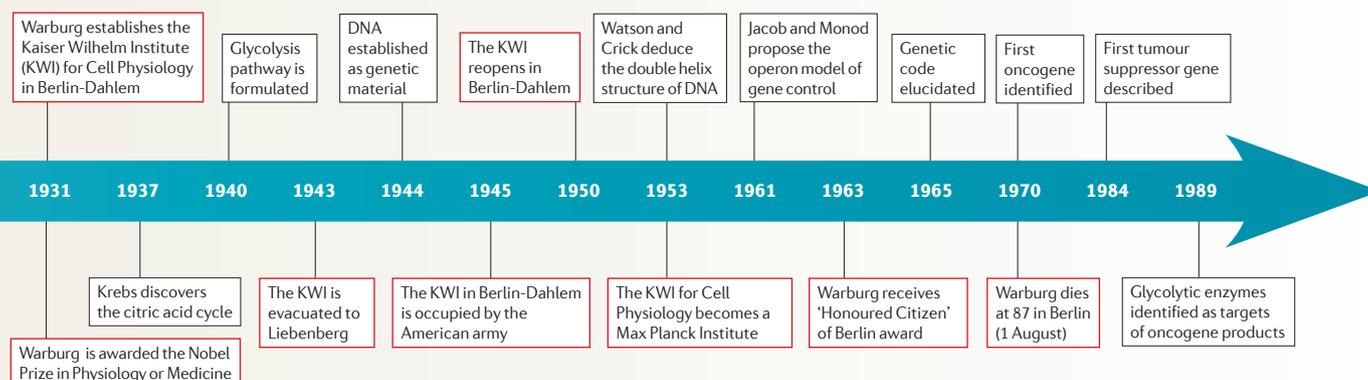
Mitochondrial defects and the Warburg effect

Over the past two decades, the discoveries of oncogenes and tumour suppressor genes have created a paradigm in which cell-autonomous genetic alterations were perceived as the sole driving force for neoplastic transformation^{38,39} and oncogenic alterations of cell metabolism were considered as epiphenomena. However, with the discoveries of oncogenic mutations in mitochondrial metabolic enzymes, such as fumarate hydratase (FH),

succinate dehydrogenase (SDH) and isocitrate dehydrogenase 2 (IDH2), it is now untenable to deny the role of metabolism in tumorigenesis^{40,41}.

Warburg reasoned that respiration must be damaged in cancers because high levels of O₂ are unable to suppress the production of lactic acid by cancer cells⁴². So, are mitochondrial defects sufficient and necessary for tumorigenesis? Although the observations of Chance and Weinhouse^{24–26} negated Warburg’s contention of mitochondrial defects in cancers, many studies over the past several decades have documented oncogenic nuclear and mitochondrial DNA mutations in proteins involved in respiration.

The metabolic profiles of chromaffin tissues, from which paragangliomas and pheochromocytomas arise, must somehow be amenable to tumorigenesis by mutations in these tumour suppressor oxidative phosphorylation (OXPHOS) proteins. Mutations linked to hereditary paragangliomas and pheochromocytomas in nuclear genes that affect mitochondrial respiration have been found in all four subunits (SDHA, SDHB, SDHC and SDHD) of the SDH complex⁴¹. Mutations in SDH5, which is involved in the assembly of SDHD into the complex, were also recently documented in hereditary paragangliomas⁴³ — rare tumours that are not associated clinically with more commonly occurring cancers. This suggests that these germline mutations are insufficient to promote commonly occurring epithelial cancers. Intriguingly, mutations of FH, which is involved in the citric acid cycle downstream of SDH, result in familial leiomyoma, renal cell carcinoma (RCC) and uterine fibroids. Mutations of SDH and FH promote increased levels of succinate and fumarate, which inhibit prolyl hydroxylases that are responsible for the O₂-dependent modification of hypoxia inducible factor 1α (HIF1α) and its degradation. Therefore, even in the presence of normal levels of O₂, these mutations are thought to constitutively increase production of HIF1α to levels that trigger tumorigenesis⁴⁴. In this regard, prolyl hydroxylases (particularly PHD2) confer the Pasteur effect by mediating the degradation of HIF1α in the presence of O₂ (REFS 45,46). Specifically, HIF1, a heterodimer comprising HIF1α and HIF1β (also known as ARNT),



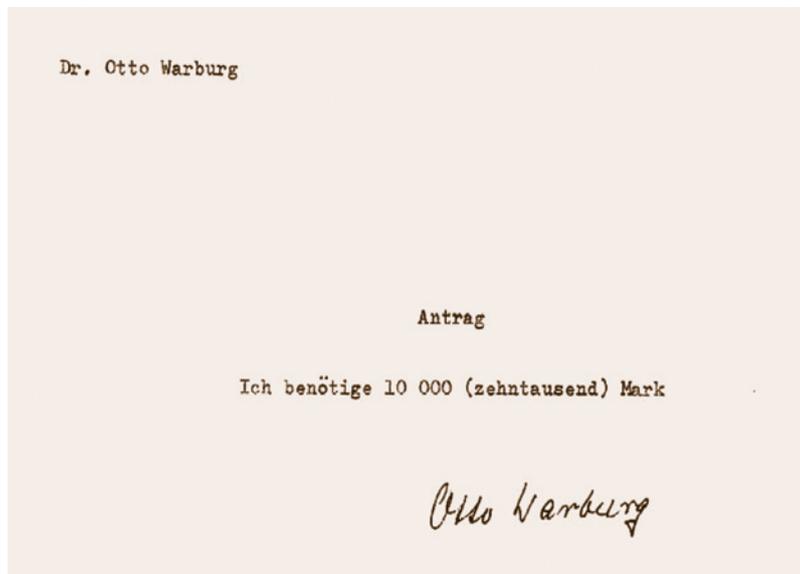


Figure 2 | **Grant proposal.** Facsimile of a research proposal submitted by Otto Warburg to the Notgemeinschaft der Deutschen Wissenschaft (Emergency Association of German Science), probably in 1921. The application, which consisted of a single sentence, “I require 10,000 marks”, was funded in full. This is a reconstruction based on a detailed description from H. Krebs¹.

activates genes that are involved in glycolysis, such as lactate dehydrogenase A (*LDHA*), the product of which contributes to a crucial component of the Warburg effect: the conversion of pyruvate to lactate^{47,48}. Circumstances that increase the levels of HIF1 in non-hypoxic conditions would thus inhibit the Pasteur effect and induce the Warburg effect in cancer cells.

In addition to the familial cancer syndromes associated with OXPHOS mutations, somatic mutations of IDH1 (which is cytosolic) and IDH2 (which is mitochondrial) have been found in 80% of low-grade gliomas and 30% of karyotypically normal acute myelogenous leukaemias^{40,49,50}. Mutations affecting the catalytic sites of IDH1 and IDH2 are thought to be functionally equivalent and were initially thought to cause loss of function that led to diminished conversion of isocitrate to α -ketoglutarate, a metabolic intermediate that is required for the degradation of HIF1 α or HIF2 α (also known as EPAS1)⁵¹. However, the stabilization of HIF1 α by mutant IDH1 or IDH2 has not been independently confirmed. Mutant IDH1 and IDH2 exhibit a neo-enzymatic activity: they convert α -ketoglutarate to 2-hydroxyglutarate (2-HG)⁵², which in turn alters the homeostasis of α -ketoglutarate and reduces its availability as a substrate for the enzymes that methylate DNA and histones. Thus, tumorigenesis is enhanced through the modification of the epigenome^{53,54}.

Although it appears that OXPHOS mutations contribute to tumorigenesis through a simple disruption of glucose metabolism through the alteration of metabolic homeostasis — which in turn affects processes such as HIF1 stabilization and epigenetic regulation — the mechanism is far more complex than this. It is notable, however, that mutations in OXPHOS genes affect a limited range of cancer types. As such, to fully appreciate

this range of cancer gene mutations, we need to better understand the normal genomic and metabolic profiles of the cancer cells of origin.

Somatic mutations in mitochondrial DNA (mtDNA) are found in many human cancers. However, a recent study of mtDNA heteroplasmy demonstrated differences between cancer tissues and normal tissues in heteroplasmic mutations in mtDNA: of the heteroplasmic mutations that frequently arise in normal tissues during embryogenesis, only 33% are in the protein-coding or RNA-coding regions, whereas 85% of heteroplasmic mutations are in these regions in cancer cells⁵⁵. These observations suggest that endogenous mutagenic events occur normally and that somatic mutations of mtDNA in cancers are enriched, perhaps because they confer selective advantage for survival and growth.

Although the prevalence of mtDNA mutations suggests a functional advantage to cancer cells, these mtDNA alterations might be simple bystander mutations. Do mtDNA mutations provide a survival and growth advantage to cancer cells? A compelling study by Wallace and co-workers⁵⁶ documented that 11% of prostate cancers harbour a mitochondrially encoded cytochrome *c* oxidase 1 (*COX1*; also known as *MT-COI*) mtDNA mutation, whereas <2% of non-cancer controls and 7.8% of the general population have a *COX1* mutation. Through the use of cybrid transfer, which generates cell fusions with heterologous nuclei and mitochondria, they documented that the mtDNA *ATP6-T8993G* mutation in PC3 prostate cancer cells confers a sevenfold increase in the size of the xenograft tumours, which produce levels of oxyradicals that are elevated relative to wild-type (*ATP6-T8993T*) cybrids. Similarly, a mitochondrially encoded NADH dehydrogenase 2 (*MT-ND2*) mutation found in head and neck cancers has been reported to enhance the tumorigenicity of HeLa cells⁵⁷, which also produce elevated levels of oxyradicals and lactic acid.

Intriguingly, the extent of mtDNA heteroplasmy versus homoplasmy appears to affect oxyradical formation and tumorigenicity. At heteroplasmic levels, a mutation in *MT-ND5* is associated with increased generation of oxyradicals and tumorigenicity, whereas homoplasmic *MT-ND5* mutations appear to exhibit decreased oxyradical formation and tumorigenicity⁵⁸. Thus, a dosage effect of mtDNA mutations may determine the extent of redox stress and tumorigenicity. Furthermore, an *MT-ND6* mutation introduced by cybrid technology into a mouse tumour cell line is associated with overproduction of oxyradicals and tumour cell metastasis; pretreatment of the tumours with oxyradical scavengers suppressed metastasis, suggesting that alterations of redox balance by mtDNA mutations correlate with tumorigenicity and metastasis potential⁵⁹. These observations might lead us to conclude that normal ambient levels of oxyradicals can cause mtDNA mutations, which in turn could interfere with efficient respiration, lead to increased levels of oxyradicals that would contribute to genomic instability and provide a selective advantage to the cancer cells to progress, apparently independently of a direct effect on glucose metabolism.

Heteroplasmy

The situation in which the many hundreds of mitochondria within a single eukaryotic cell are a mixture of those that contain mutant mitochondrial DNA (mtDNA) and normal mtDNA. Heteroplasmy has a role in the severity of mitochondrial diseases.

Homoplasmy

The situation in which a mutation in mitochondrial DNA is present in all of the mitochondria within a single eukaryotic cell.

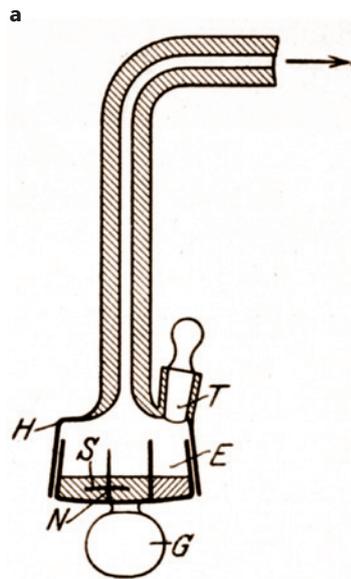


Tabelle I.
FLEXNER-JOBLINGSCHES RATTENCARCINOM.
 37,5°. Ringerlösung. $C_{NaHCO_3} = 2,5 \cdot 10^{-2}$, 0,2 proz. Glucose. 5 proz. CO_2 .
 $p_H = 7,66$.

Nr.	I	II	III	IV	V	VI
	Q_{O_2} (Atmung)	$Q_{CO_2}^{O_2}$ (Glykolyse in Sauerstoff)	$Q_{CO_2}^{N_2}$ (Glykolyse in Stickstoff)	Hemmung d. Glykolyse durch Sauerstoff $(\frac{III-IV}{III})$ %	MEYERHOF- Quotient $(\frac{III-IV}{I})$	Aerobe Glykolyse Atmung $(\frac{II}{I})$
1	— 4,5	+ 21	—	—	—	4,7
2	— 7,8	+ 28	—	—	—	3,6
3	— 11,5	+ 30	—	—	—	2,6
4	— 5,1	+ 18	—	—	—	3,6
5	— 7,5	+ 30,5	—	—	—	4,1
6	— 2,4	+ 17,7	—	—	—	7,4
7	— 4,1	+ 25,6	+ 30,8	18	1,3	5,1
8	— 3,5	+ 19	+ 26,8	29	2,2	5,4
9	— 7,5	+ 22,5	+ 34,6	35	1,6	3,0
10	— 12,8	+ 27	+ 34,5	22	0,6	2,1
11	— 11,8	+ 26	+ 34	24	0,7	2,2
12	— 10,4	+ 22,3	+ 25,3	12	0,3	2,1
13	— 2,5	+ 18,6	+ 28,3	34	3,9	7,6
14	— 9,0	+ 24	+ 30,8	21	0,73	2,7
15	— 11,5	+ 25,5	+ 33,8	25	0,72	2,2
16	— 6,7	+ 27,7	+ 37,0	25	1,4	4,2
17	— 5,5	+ 18	+ 25,6	30	1,4	3,3
18	— 8,9	+ 23,7	+ 27,3	13	0,4	2,7
19	— 4,1	+ 25,7	+ 33,8	24	2,0	6,4
Mittel:	— 7,2	+ 25	+ 31	23	1,3	3,9

Figure 3 | The reaction vessel for tissue slices developed by Otto Warburg and representative data. **a** | The reaction vessel used by Warburg and co-workers¹⁴ to measure O_2 uptake or lactic acid production consisted of a chambered trough in which a tissue slice (S), cut with a razor blade, was mounted on a glass needle (N, fixed to the bottom of the main chamber) and submerged in 0.5 ml Ringer solution. The vessel was closed with a paraffin-coated ground glass joint (H) attached to tubing that connects to a Barcroft manometer. The solid glass bulb (G) served as a handle to facilitate fitting the glass joint, and additions to the reaction trough were made through port T (sealed with a glass stopper during measurements). For measurements of O_2 uptake (which registered as pressure decreases over time), 0.1 ml of 5% potassium hydroxide solution was added to chamber E to absorb CO_2 . Lactic acid production was measured as pressure increases due to CO_2 emission from the Ringer solution, which, for these experiments, contained 24 mM $NaHCO_3$ (REF. 14). O_2 uptake and/or CO_2 release were measured at 37.5 °C for 0.5–1 hour. Warburg¹⁸ calculated that, to avoid anaerobiosis in the centre, the tissue thickness must be smaller than $\sqrt{8c_0DA^{-1}}$, where c_0 is p_{O_2} , D is the diffusion coefficient of O_2 ($1.4 \times 10^{-5} \text{ cm}^3 O_2$ per cm^2 tissue at 38 °C¹²⁹) and A is the O_2 consumption of the tissue; this corresponds to a tissue sample 0.2–0.4 mm in thickness and 2–5 mg in weight. **b** | Results obtained using the apparatus in **a** from experiments with Flexner–Jobling rat carcinoma tissue at 37.5 °C, 0.2% glucose²⁰, at pH 7.41 (not 7.66 as indicated¹³⁰), in which the respiration (per mg of dried tissue) was 7.2 $\text{mm}^3 O_2$ per hour (0.28 μmol per hour). The volume of CO_2 driven out of the Ringer solution by lactic acid during respiration in the presence of O_2 was 25 mm^3 per hour (0.93 μmol per hour), and in the presence of N_2 the volume was 31 mm^3 per hour (1.22 μmol per hour) (values in parentheses calculated for this Review). The uptake of 0.28 $\mu\text{mol } O_2$ per hour implies that 0.047 μmol glucose is oxidized to H_2O and CO_2 (see equation 1 (respiration)). The CO_2 produced during the aerobic and anaerobic experiments corresponds to 0.93 μmol and 1.22 μmol lactic acid (see equation 2 (glycolysis)), respectively, or 0.46 and 0.61 μmol glucose, respectively. Thus, in tumour cells in the presence of O_2 , ten times more glucose is used for glycolysis than for respiration. Image is reproduced, with permission, from REF. 15 © (1926) Springer Science+Business Media.

Deregulated glycolysis and the Warburg effect

The Warburg effect (aerobic glycolysis) could arise from mtDNA mutations and defective respiration; however, as discussed, aerobic glycolysis can occur concurrently with mitochondrial respiration. Hence, if the Warburg effect is evident in cancers with ongoing respiration, what are the mechanisms underlying enhanced conversion of glucose to lactic acid even in the presence of adequate O_2 ?

All major tumour suppressors and oncogenes have intimate connections with metabolic pathways^{60–64} (FIG. 4). Some of the earliest evidence for links between oncogenes and aerobic glycolysis is the stimulation of glucose uptake by activated RAS and the ability of SRC to phosphorylate a number of glycolytic enzymes in fibroblasts^{65,66}. SRC was later implicated in the activation of HIF1 α , which induces glycolysis, but this link appears to be dependent on cell type^{67,68}. The first documented direct mechanistic link between an activated oncogene

and altered glucose metabolism was the transcriptional activation of LDHA by the oncogenic transcription factor MYC (FIG. 5a), which later proved to activate most glycolytic enzyme genes as well as glucose transporters^{69–71}. Pyruvate kinase M2 (PKM2), which converts phosphoenolpyruvate to pyruvate, favours aerobic glycolysis in cellular transformation compared with PKM1, which is encoded by alternative splicing of the PK mRNA^{72,73}. MYC induces the splicing factors that produce PKM2, further underscoring the role of MYC in aerobic glycolysis⁷⁴. MYC and HIF1 share many target glycolytic enzyme genes; however, whereas the normal role of HIF1 is to induce anaerobic glycolysis, MYC can stimulate aerobic glycolysis, as shown when it is overexpressed *in vivo* in transgenic cardiomyocytes^{70,75}.

The AKT oncogenes, which are frequently activated downstream of PI3K, enhance glycolysis through activation of hexokinase 2 and phosphofructokinase 1

Anaerobic glycolysis
 The enzymatic transformation of glucose to pyruvate in the absence of O_2 ; see 'glycolysis'.

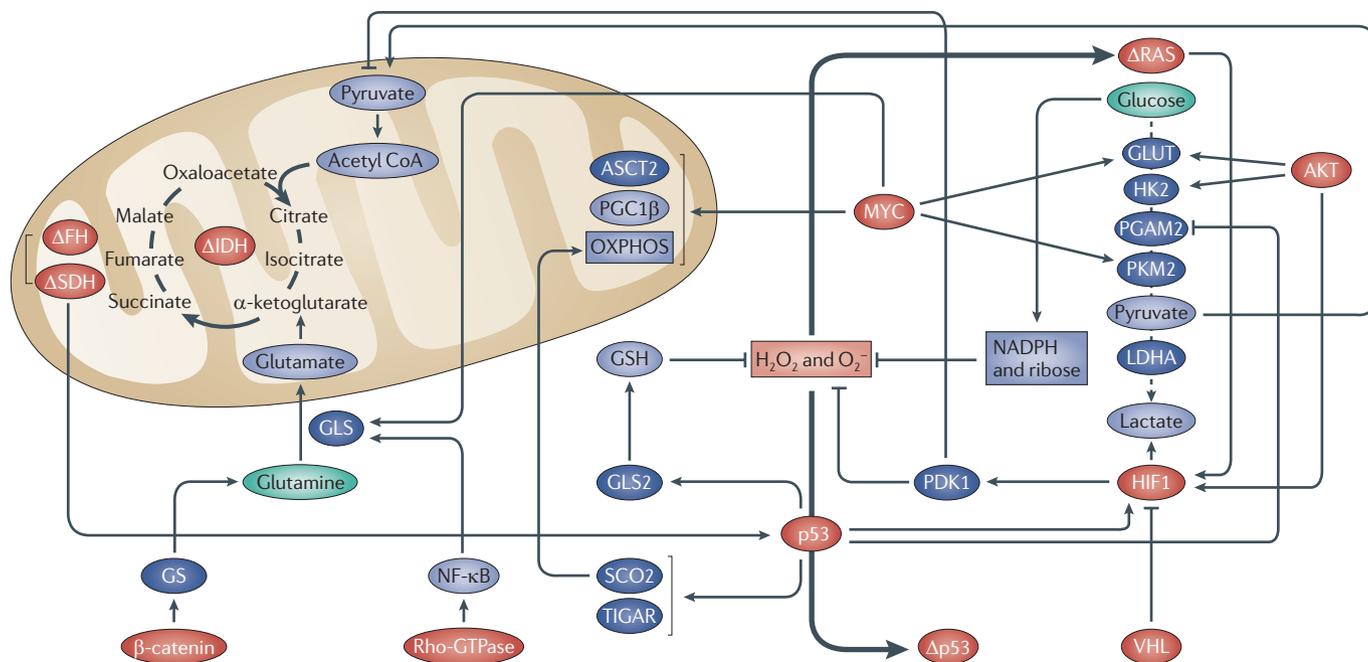


Figure 4 | The regulation of metabolism in cancer. Oncoproteins and tumour suppressors (shown in red) are intimately linked to metabolic pathways through transcriptional or post-transcriptional regulation of metabolic enzymes; arrows in bold depict the conversion of wild-type to mutant (Δ) tumour suppressors or mutant activated oncogenes, presumably by mutational oxidative DNA damage. ASCT2, ASC-like Na^+ -dependent neutral amino acid transporter 2 (also known as ATB(0) and SLC1A5); FH, fumarate hydratase; GLS, glutaminase; GS, glutamine synthetase; GLUT, glucose transporter; GSH, glutathione; HIF1, hypoxia-inducible factor 1; HK2, hexokinase 2; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; NF- κ B, nuclear factor- κ B; OXPPOS, oxidative phosphorylation; PDK1, pyruvate dehydrogenase kinase isoform 1; PGAM2, phosphoglycerate mutase 2; PGC1 β , peroxisome proliferator-activated receptor- γ , co-activator 1 β ; PKM2, pyruvate kinase M2; SDH, succinate dehydrogenase; TIGAR, tumour protein 53-induced glycolysis and apoptosis regulator; VHL, von Hippel-Lindau tumour suppressor.

(PFK1; also known as PFKM) and PFK2 (also known as PFKFB3) and recruitment of glucose transporters to the cell surface^{37,76,77} (FIG. 5a). Although AKT functions independently of HIF1 to induce aerobic glycolysis^{78,79}, it can also increase the activity of HIF1, further enhancing induction of glycolysis⁸⁰. Ectopic expression of AKT or MYC induces aerobic glycolysis in FL5.12 pre-B cells but, unlike MYC, AKT does not increase mitochondrial function⁸¹. Intriguingly, aerobic glycolysis in early passage human breast cancer cells is associated with elevated HIF1 or MYC but not activated AKT⁸². Hence, it is likely that the cellular context and the range of cancer-specific mutations are important for the metabolic manifestations of activated oncogenes such as AKT.

Activated RAS was initially linked to increased cellular glucose transport, but recent studies indicate that the role of RAS in cancer metabolism is more complex. It was recently reported that depriving colon carcinoma cells of glucose increases the mutation rate of RAS, which, thus activated, facilitates glucose import through induction of GLUT1 (also known as SLC2A1), an important glucose transporter⁸³. In a multistep, multigene transformation of human breast epithelial cells, it was documented that the initial transformation of normal epithelial cells by viral oncogenes and telomerase reverse transcriptase is associated with

increased mitochondrial function; with activated KRAS as the final reaction step in this model, the transformed cells exhibit the Warburg effect through high conversion of glucose to lactate⁸⁴. It is notable that activated RAS has been proposed to induce MYC activity and enhance non-hypoxic levels of HIF1, although the precise mechanisms remain to be established^{85,86}. Hence, RAS could mediate its effects on metabolism through HIF1 or MYC (FIG. 5a).

Because HIF1 appears at the crossroads of multiple oncogenes that can stabilize HIF1 under non-hypoxic conditions, it is not surprising that HIF1 also has a pivotal role in the manifestations of tumour suppressors (FIG. 5a). For example, the von Hippel-Lindau (VHL) tumour suppressor protein, which normally mediates proteasomal degradation of HIF1 α , is lost in RCCs, which results in elevated non-hypoxic expression of HIF1 α and HIF2 α ⁸⁷. In RCCs, MYC appears to collaborate with activated HIF2 α to confer tumorigenicity, whereas HIF1 α appears to be expressed in RCCs only when HIF2 α is expressed, suggesting a potential tumour suppressive function of HIF1. Other tumour suppressor genes and proteins have also been implicated as modulators of HIF1 α , and thereby might contribute to the Warburg effect; for example, HIF1-mediated gene expression is facilitated by loss of the *PTEN* tumour suppressor gene⁸⁸. The association of the tumour suppressor

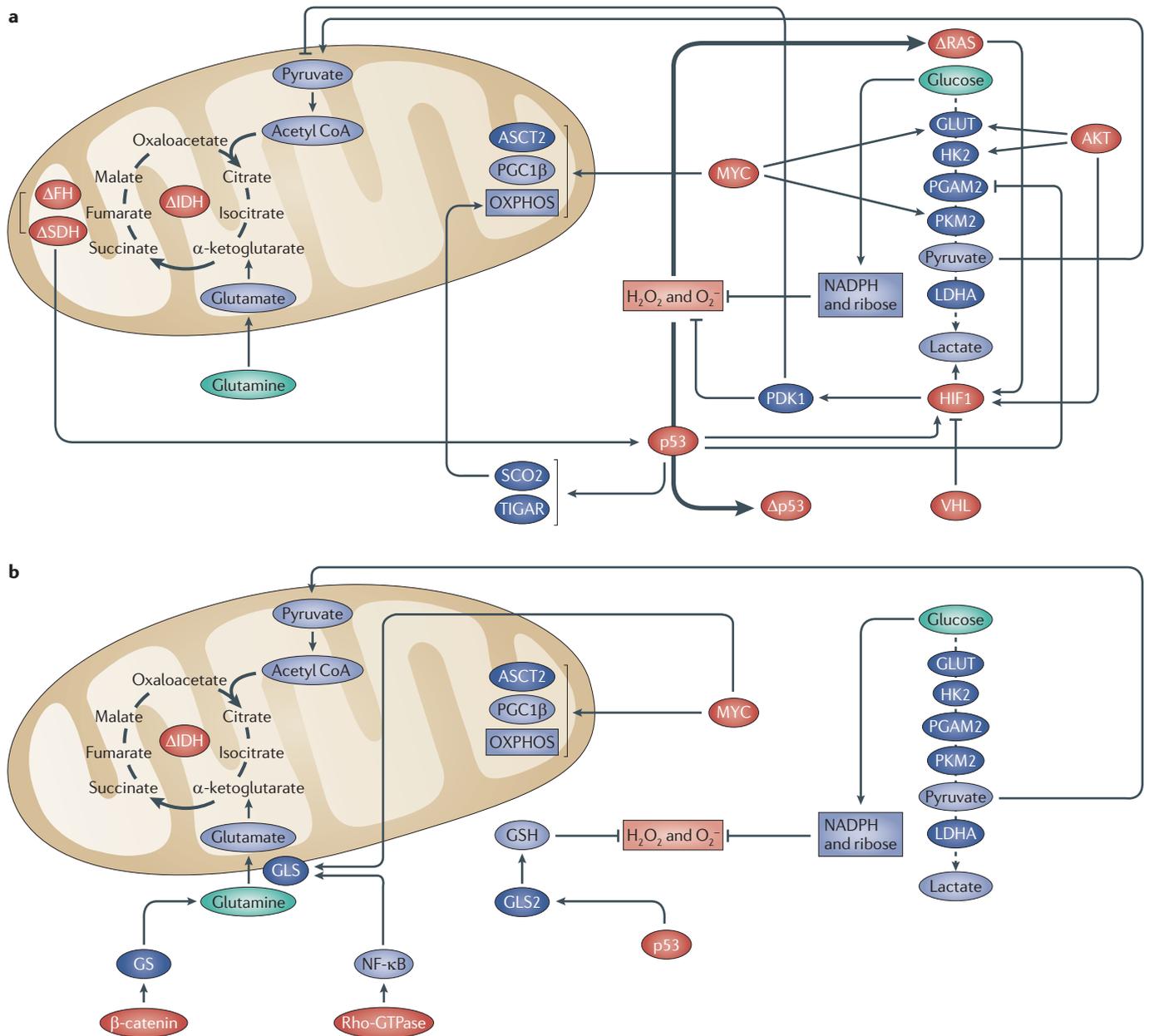


Figure 5 | The effects on glucose and glutamine metabolism. a | The effects of oncoproteins and tumour suppressors (shown in red) on glucose metabolism: MYC, hypoxia-inducible factor 1 (HIF1) and p53 affect gene expression, whereas AKT alters glycolytic proteins post-translationally. **b** | Effects of oncoproteins and tumour suppressors on glutamine metabolism. MYC and Rho-GTPase stimulate glutaminase (GLS), whereas p53 stimulates GLS2 expression; mutant (Δ) β-catenin stimulates glutamine synthetase (GS). ASCT2, ASC-like Na⁺-dependent neutral amino acid transporter 2 (also known as ATB(0) and SLC1A5); FH, fumarate hydratase; GLUT, glucose transporter; GSH, glutathione; HK2, hexokinase 2; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; NF-κB, nuclear factor-κB; OXPHOS, oxidative phosphorylation; PDK1, pyruvate dehydrogenase kinase isoform 1; PGAM2, phosphoglycerate mutase 2; PGC1β, peroxisome proliferator-activated receptor-γ, co-activator 1β; PKM2, pyruvate kinase M2; SDH, succinate dehydrogenase; TIGAR, tumour protein 53-induced glycolysis and apoptosis regulator; VHL, von Hippel-Lindau tumour suppressor.

protein p53 with HIF1α inhibits HIF1-stimulated transcription⁸⁹; however, controversies regarding the link between HIF1α and p53 have not been resolved⁹⁰. Mutations in SDH and FH also stabilize HIF1α in familial syndromes associated with leiomyoma, paraganglioma and pheochromocytoma⁹¹. Hence, constitutively

stabilized HIFs contribute to the Warburg effect and tumorigenesis downstream of bona fide oncogenes and tumour suppressors.

Although the p53 tumour suppressor has been regarded as the ‘guardian of the genome’, its function extends to regulation of cell metabolism^{92,93} through

transcriptional regulation, whereby wild-type p53 stimulates mitochondrial respiration and suppresses glycolysis (FIG. 5a). Activation of SCO2 (which regulates the cytochrome *c* oxidase complex) by p53 increases the efficiency of mitochondrial respiration⁹⁴. Conversely, p53 suppression of phosphoglycerate mutase 2 (PGAM2) and activation of tumour protein 53-induced glycolysis and apoptosis regulator (TIGAR), which has 2,6-fructose biphosphatase activity and depletes PFK1 of a potent positive allosteric ligand, suppresses glycolysis and favours increased NADPH production by the pentose phosphate pathway^{91,95}. Hence, loss of p53 function induces aerobic glycolysis, presumably through increased PGAM and PFK activities.

Cancer metabolism unanticipated by Warburg

Although oncogenic alteration of metabolism generally involves the Warburg effect, the enhanced flux of glucose to lactate is insufficient to promote cell replication⁶¹. Cells are largely comprised of protein and ribonucleic acid, and so are too complex to be supported by a simple glucose carbon skeleton; hence, other metabolic pathways must also be stimulated to provide the building blocks for cell replication. Although previously implicated in the literature^{96–98}, the contribution of glutamine to anabolic carbons and building blocks of a growing cell has been rediscovered and only recently fully appreciated. In fact, citric acid cycle intermediates in proliferating cells are hybrid molecules of glucose and glutamine carbons, with glutamine entering the citric acid cycle through conversion to glutamate by glutaminase (GLS) and then to α -ketoglutarate by either glutamate dehydrogenase or aminotransferases⁹⁹. Furthermore, proliferating cells generate waste and toxic by-products, the removal of which is necessary for cancer cells to maintain redox homeostasis and continue replicating effectively⁸⁴.

MYC has been documented to induce genes involved in mitochondrial biogenesis and glutamine metabolism⁷⁰, specifically those for expression of glutamine transporters and GLS, resulting in increased flux of glutamine carbons through the citric acid cycle^{100,101} (FIG. 5b). Thus, overexpression of MYC in cancer cells renders them sensitive to glutamine withdrawal¹⁰². The ability of MYC to induce both aerobic glycolysis and glutamine oxidation provides cancer cells with ATP, carbon skeletons and nitrogen for nucleic acid synthesis, and hence with the ability to accumulate biomass. Activated Rho-GTPase-mediated transformation is dependent on increased GLS activity, which appears to be modulated by activated nuclear factor- κ B (NF- κ B); chemical inhibition of GLS diminishes transformation by both Rho-GTPase and MYC, showing that key metabolic nodal points can be affected by different oncogenes¹⁰³ (FIG. 5b). Activated RAS was also recently shown to rely on mitochondrial function for cellular transformation, particularly through increased glutamine metabolism, which suggests that the multifaceted roles of oncogenes in metabolism are context dependent¹⁰⁴.

The mutant β -catenin (*CTNNB1*) oncogene increases glutamine synthetase (GS) expression in liver cancers¹⁰⁵ (FIG. 5b); GS produces glutamine from glutamate and

ammonia, hence its expression renders cancer cells independent of extracellular glutamine, although GS appears to be decreased overall in hepatocellular carcinoma (HCC), whereas GLS is elevated^{106,107}. The HCC subtype with GS expression portends a more favourable clinical outcome¹⁰⁸. These collective observations suggest that GS expression in some liver cancers reflects the expression of GS that is required in normal liver cells for ammonia detoxification and glutamine production¹⁰⁹.

GLS2 is transactivated by p53 and is normally expressed in the liver^{110–112} (FIG. 5b). By contrast, GLS is ubiquitously inducible. The increased conversion of glutamine to glutamate by GLS2 is thought to increase the production of glutathione, which in turn attenuates metabolic by-products such as hydrogen peroxide. Hence, beyond the Warburg effect, p53 plays a key part in redox homeostasis through stimulation of NADPH synthesis by the pentose phosphate pathway and stimulation of glutathione synthesis through increased GLS2 expression.

Other alterations favouring oncogenesis include receptor tyrosine kinase activation, such as *ERBB2* (also known as *HER2*) amplification in breast cancer; *ERBB2* can suppress apoptosis resulting from cell detachment from other cells or the substratum (anoikis) in mammary spheroid cultures, in which central mammary epithelial cells that are detached from surrounding cells have diminished glucose uptake and undergo apoptosis¹¹³. It was observed that anoikis is associated with increased oxidative stress that inhibits fatty acid oxidation, resulting in a bioenergetic death that can be rescued by expression of *ERBB2*, which stimulates glucose uptake, NADPH production by the pentose phosphate pathway and fatty acid oxidation, and this consequently diminishes oxidative stress. The role of fatty acids as bioenergetic substrates for cancer is not well understood and deserves more attention.

Perspectives

Although normal cells experience the enhanced aerobic glycolysis of the Warburg effect^{114,115}, there is one distinct metabolic difference between normal and cancer cells that renders cancer cells 'addicted' to the Warburg effect. Normal cells, by virtue of multiple feedback and feedforward regulatory loops, undergo quiescence when deprived of nutrients even in the presence of growth factors. By contrast, oncogenic stimulation of cell growth and proliferation induces both biomass accumulation (such as increased ribosome biogenesis and lipogenesis) and nutrient uptake. When bioenergetic demand is balanced by anabolic supply, cancer cells grow and proliferate. However, oncogenic deregulation of biomass accumulation for cell proliferation creates an increased, sustained bioenergetic demand that addicts cancer cells to an adequate anabolic supply. In this regard, the Warburg effect, in addition to contributing to enhanced lactic acid production, serves to provide anabolic carbons for fatty acid synthesis⁶⁰. For example, MYC-induced ribosome biogenesis and biomass accumulation sensitizes MYC-transformed cells to bioenergetic cell death triggered by glucose or glutamine

deprivation, much like yeast mutants that have constitutively deregulated ribosome biogenesis^{102,116,117}. This pivotal conceptual framework of bioenergetic supply and demand suggests that cancer cells are addicted to the Warburg effect, and nutrient deprivation should trigger an autophagic response, which, if unsustainable, would result in cancer cell death¹¹⁸. Hence, targeting metabolism for cancer therapy holds promise for new classes of anti-neoplastic drugs^{119,120}.

The microenvironmental niches in which cancer cells live are heterogeneous because of ineffective tumour vascularization¹²¹; as such, the genomic and metabolic networks of cancer cells are disrupted not only by cell-autonomous genetic mutations but also by hypoxia¹²². Indeed, it was demonstrated recently that hypoxic tumour cells extrude lactate, which is subsequently recycled to pyruvate for use in mitochondrial OXPHOS by respiring stromal or tumour cells^{32,121,123,124}.

The concepts for cancer cell metabolism framed by Warburg 90 years ago have undergone substantial revision. Taken together, the progress made in the twenty-

first century towards understanding the Warburg effect reveals that genetic alterations of oncogenes and tumour suppressors tend to increase the conversion of glucose to lactate, but glucose is insufficient for cancer cell growth and proliferation. Furthermore, accelerated cancer cell metabolism also produces more waste, such as lactate, superoxide and hydrogen peroxide, for extrusion or neutralization^{125,126}. However, the addiction of cancer cells to the Warburg effect for biomass accumulation can be exploited by therapeutic approaches that uncouple bioenergetic supply from demand or inhibit elimination of metabolic waste products. The Warburg effect itself involves high levels of aerobic glycolysis catalysed by pivotal enzymes that are therapeutically accessible to small drug-like inhibitors that could be aimed at primary and metastatic tumours and monitored in patients by means of metabolic imaging. As such, we are poised to witness the clinical benefits of Warburg's contributions in the next 5 to 10 years, almost 100 years after his initial observations^{103,127,128}.

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Competing interests statement

The authors declare [competing financial interests](#). See Web version for details.

FURTHER INFORMATION

Chi V. Dang's homepage: <http://www.mycrcancergene.org/>

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