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Metabolic rewiring in melanoma

Boris I. Ratnikov, David A. Scott, Andrei L. Osterman, Jeffrey W. Smith, and Ze'ev A. Ronai
Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla CA, 92037

Abstract

Oncogene-driven metabolic rewiring is an adaptation to low nutrient and oxygen conditions in the tumor microenvironment that enables cancer cells of diverse origin to hyperproliferate. Aerobic glycolysis and enhanced reliance on glutamine utilization are prime examples of such rewiring. However, tissue of origin as well as specific genetic and epigenetic changes determines gene expression profiles underlying these metabolic alterations in specific cancers. In melanoma, activation of the MAPK pathway driven by mutant BRAF or NRAS is a primary cause of malignant transformation. Activity of the MAPK pathway, as well as other factors, such as HIF1 α , Myc and MITF, are among those that control the balance between non-oxidative and oxidative branches of central carbon metabolism. Here, we discuss the nature of metabolic alterations that underlie melanoma development and affect its response to therapy.

Introduction

Melanoma is the most aggressive and, until recently, the most treatment-resistant form of skin cancer⁶⁶. Melanomas arise from neural crest progenitor-derived, melanin-producing cells called melanocytes, which reside in the skin, eye, mucosal epithelia and meninges of the brain and spinal cord. In cutaneous melanoma, MAPK signaling is often turned on due to activating mutations in genes regulating the pathway. The largest genomic subtype exhibits mutations in BRAF (52%)¹¹, most of which are V600E, followed by the significantly less frequent V600K and V600R mutations. The second most prevalent oncogenic mutations, which occur in 28% of melanomas, target the NRAS gene primarily at residue Q61 (Q61R, Q61K, Q61L and Q61H). The third most commonly mutated gene, also in the MAPK pathway, is NF1, which is altered in 14% of patient samples. Remaining melanomas comprise the so-called triple wild-type subtype: among them are tumors bearing mutations and amplification of the tyrosine kinase c-Kit, a member of the MAPK pathway, and microphthalmia-associated transcription factor MITF⁶⁴, both of which play a major role in normal melanocytic development. In uveal melanoma, which is biologically distinct from its cutaneous counterpart, somatic mutations occur mostly in in genes encoding guanine nucleotide-binding protein G(q) subunit alpha (GNAQ) and guanine nucleotide-binding protein subunit alpha-11 (GNA11), two closely related large GTPases of the G α q family^{110, 111}.

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Correspondence - zeev@ronailab.net; bratnikov@sbpDiscovery.org.

Metabolic rewiring is a hallmark of malignant transformation across all cancer types^{10, 56, 115, 132}. The tumor microenvironment, which is often poorly vascularized, subjects cancer cells to oxygen and nutrient deprivation, both of which drive metabolic changes promoting their survival and growth under such stressful conditions. Rapid proliferation increases demand for energy required for macromolecular biosynthesis as well as for precursors of amino acids, nucleotides and lipids. As a result, central carbon metabolism is frequently altered in transformed cells^{17, 21, 30, 114}. Given the prevalence of BRAF mutations in melanoma and their importance in metabolic reprogramming², we focus primarily on metabolic alterations mediated by oncogenic BRAF. Metabolic rewiring in melanoma is also governed by MITF, which, interestingly, can be either upregulated (or amplified)³² or mutated (or downregulated)¹²⁸ in melanoma, the latter more associated with metastatic and therapy-resistant tumors⁸³. This observation suggests that too much or too little of MITF elicits oncogenic transformation, likely by perturbing different transcriptional programs. Another E-box binding transcription factor amplified in a subset of melanomas is c-Myc^{55, 80}. Here we review recent literature relevant to metabolic rewiring in melanoma in the context of oncogenic BRAF^{25, 85}, HIF1 α , MITF⁴⁰ and c-Myc, focusing on the interplay of non-oxidative and oxidative branches of central carbon metabolism in cytosolic versus mitochondrial compartments.

Metabolic changes in glycolysis and related pathways

Highly proliferative phenotypes exhibited by cancer cells are supported in part by aerobic glycolysis (the Warburg effect¹¹⁴), which supplies energy and building blocks for macromolecule biosynthesis^{18, 69}. In normoxia, melanoma cells of varying oncogenic backgrounds, like other tumor cells or untransformed cells, often display highly glycolytic phenotypes in which 60–80% of glucose is converted to lactate, an activity enhanced to 90% or more in hypoxia⁹⁹. Oxygen availability is one of the major factors determining the metabolic state of mammalian cells. Under low oxygen, Hypoxia Inducible Factors (HIF) induce a transcriptional program adapting the cell to hypoxic stress, which has a profound impact on central carbon metabolism⁷¹. Hypoxia induced accumulation of HIF1 leads to increased glycolytic rates, accompanied by loss of glucose carbon utilization in the TCA cycle due HIF-mediated expression of Pyruvate Dehydrogenase Kinase 1 (PDK1)⁵³, thereby decreasing mitochondrial respiration⁸⁷. Stability of HIF1 α protein in hypoxia and normoxia can be affected by oncogenes, such as Ras⁷³, Src⁴⁹ as well as activation of the RAS/MAPK pathway¹⁰⁰. In cancer cells HIF1 α stimulates glycolysis by upregulating expression of genes involved in glucose uptake and subsequent utilization along the pathway²¹. Malignant melanoma cells often exhibit constitutive HIF1 activity even under normoxia⁵⁷. Glucose transport, which is upregulated in many cancer types (melanoma included), is facilitated by increased expression of the transporter protein GLUT1 (SLC2A1)⁵⁴ (Fig. 1). In addition, high rates of aerobic glycolysis enhance lactate production due to increased expression of lactate dehydrogenase A (LDHA), promoting formation of tetrameric LDH isoforms 3, 4 and 5⁴⁴. The latter favor formation of lactate from pyruvate, while isoforms 1 and 2 preferentially catalyze lactate-pyruvate interconversion due to presence of LDHB in the tetramer⁹⁰. The gene that encodes LDHA is a known HIF1 α ^{21, 29} target. Thus, in hypoxic conditions, the contribution of glucose to TCA cycle metabolite pools decreases

dramatically, and they are instead mostly derived from glutamine, an important carbon source used by diverse tumor cells¹²¹. Notably, under hypoxia, fatty acid biosynthesis is partially supported by glutamine carbon through reductive carboxylation of alpha-ketoglutarate due to reversal of the α -ketoglutarate-to-citrate segment of the TCA cycle^{28, 99} (Fig. 1). This phenomenon has been observed in other cancer cells⁷⁶ and mechanistically requires activity of the isocitrate dehydrogenase 1 and 2 (IDH1 and 2)-dependent pathway, which is predominant at low oxygen levels^{28, 76}. Interestingly, although both IDH isoenzymes require NADP⁺, IDH1 is expressed in the cytosol and IDH2 in mitochondria, and both support reductive carboxylation. NAD⁺-dependent IDH3 functions only in the oxidative direction⁷⁶. Mutations at positions R132 and R172 of IDH1 and 2 respectively or at R140 of IDH2 render these enzymes incapable of generating α -ketoglutarate (α -KG) and instead they begin synthesizing an oncometabolite 2-hydroxoglutarate (2-HG) from isocitrate¹²³. Loss of α -KG leads to increase in HIF-1 α levels due to insufficient activity of α -KG-dependent prolylhydroxylases (PHD)¹³⁴. In addition, 2-HG acts a competitive inhibitor of α -KG-dependent dioxygenases including the TET family of 5-methylcytosine (5-mC) hydroxylases, thereby affecting histone demethylation and 5mC hydroxylation^{13, 123}. Loss of 5-hydroxymethylcytosine (5-hmC) associated with inhibition of the TET family of 5-mC hydroxylases has been reported as an epigenetic hallmark of melanoma, likely due to IDH2 downregulation⁶³. Another report indicates that 10% melanomas harbor mutant IDH1 or 2, thereby providing *in vivo* growth advantage¹⁰¹.

The serine and glycine biosynthetic pathway plays a significant role in metabolic rewiring in many cancers to enable proliferative phenotypes^{3, 58, 68, 126}. Melanoma cells are no exception: the gene that encodes the first enzyme in the serine biosynthetic pathway, phosphoglycerate dehydrogenase (PHGDH), is amplified in a significant percentage of melanoma tumor samples, more frequently than in most tumor types^{6, 82}. Importantly, PHGDH has been identified as one of the few putative metabolic oncogenes⁸², and the region of human chromosome 1p12 harboring it, which is devoid of other known oncogenes, is frequently amplified in melanoma⁶. Serine is synthesized via the pathway branching from glycolysis and consisting of three steps, starting with oxidation of 3-phosphoglycerate to 3-phosphohydroxypyruvate and generating one reducing equivalent in the form of NADH from NAD⁺ by PHGDH activity (Fig. 1). The next steps involve transamination of 3-phosphohydroxypyruvate to phosphoserine, followed by dephosphorylation to serine (Fig. 1). PHGDH gene silencing in cells with aberrantly high PHGDH expression decreases serine biosynthesis and proliferation⁶⁷. Although PHGDH's oncogenic properties are still a matter of debate, it is well known that serine is a vital intermediate in several biosynthetic pathways³. One report demonstrated the importance of 3-phosphohydroxypyruvate transamination in generating α -ketoglutarate for TCA cycle anaplerosis⁹¹. However, this mechanism does not appear to play a major role in melanoma cells, in which alanine and aspartate transaminases together with glutamate dehydrogenase serve as major contributors to glutamate carbon flux into the TCA cycle^{93, 99}. Surprisingly, supplementation of cell culture medium with exogenous serine does not rescue proliferation in melanoma cells following PHGDH knockdown⁸². Therefore, in addition to controlling intracellular serine levels, activity of the serine biosynthetic pathway and related pathways may confer growth advantages to tumor cells harboring amplified PHGDH. Those advantages could include

generation of reducing equivalents in the form of NADH, which would alter the cytosolic redox balance, and production of cytosolic α -ketoglutarate, which is important for many biochemical processes, including amino acid transamination. Serine is used in transfer of one carbon unit to tetrahydrofolate, leading to formation of 5,10-methylene-tetrahydrofolate, which is later converted to 10-formyltetrahydrofolate and contributes to purine and pyrimidine biosynthesis³ (Fig. 1). Glycine may also be a significant source of methyl groups for one carbon units and is essential for glutathione biosynthesis (Fig. 1)³.

Notably, serine controls activity of pyruvate kinase isoform M2 (PKM2), the most abundant metabolic enzyme in proliferating cells⁷⁴. When its levels are high, serine allosterically activates PKM2¹²⁵, accelerating the rate of aerobic glycolysis. When serine is deficient, lower PKM2 activity allows glycolytic intermediates to back up and fuel serine biosynthesis and the pentose phosphate pathway. Whether the pyruvate kinase M2 isoform is required for tumor cell proliferation remains unclear, although PKM2 depletion accelerates mammary tumor growth in a mouse model⁴⁷. PKM1 expression in untransformed primary cells deficient in PKM2 promotes a metabolic state marked by unsupported DNA biosynthesis, thus impacting proliferation⁷⁰. Detailed reviews of pyruvate kinase M2 regulation and function in cell proliferation are available^{36, 74}. Melanoma cells reportedly can also redirect glucose carbon toward serine and glycerol-3-phosphate pathways by upregulating expression of the cytosolic gluconeogenic enzyme phosphoenolpyruvate carboxykinase (Fig. 1), promoting proliferation of tumor-repopulating cells with stem-like properties⁶² without activating gluconeogenesis, and surprisingly, strengthening glycolytic flux by increasing glucose uptake and lactate production. Silencing or inhibition of phosphoenolpyruvate carboxykinase suppresses tumorigenesis in vivo and cell proliferation in vitro⁶².

Alterations in nucleotide biosynthetic pathways can modulate melanoma aggressiveness. Downregulated expression of guanosine monophosphate reductase (GMPR) promotes invasive phenotypes in melanoma cells¹¹⁶. GMPR participates in de novo purine biosynthesis by converting guanosine monophosphate (GMP) to inositol monophosphate, thereby fueling adenosine monophosphate synthesis and diverting GMP away from GTP generation. This activity decreases both intracellular GTP pools and levels of GTP-bound activated Rho GTP-ases, limiting invasiveness of metastatic melanoma cells. Expression of guanosine monophosphate reductase is downregulated in human invasive melanoma samples¹¹⁶.

Pyruvate – the gatekeeper of mitochondrial metabolism

Pyruvate is at the crossroads of glycolysis and the TCA cycle. In melanoma, pyruvate is reportedly a pivotal metabolite in BRAF^{V600E}-driven tumors, and the way it is utilized either promotes oncogene-induced senescence (OIS), via a shift toward oxidative metabolism, or tumorigenesis, by fueling aerobic glycolysis^{51, 85} (Fig. 1). In the absence of additional oncogenic insult, the BRAF^{V600E} mutation induces growth arrest and leads to senescence in human naevi⁷⁷. Studies conducted in BRAF^{V600E}-mutant human fibroblasts demonstrate that OIS is accompanied by enhanced utilization of pyruvate in the TCA cycle facilitated by activity of the pyruvate dehydrogenase complex (PDH)⁵¹. Among other mechanisms, PDH activity is regulated by modification by either pyruvate dehydrogenase kinases (PDKs) or

phosphatases (PDPs)⁸⁸. In senescent cells, the balance shifts toward lower PDK1 activity and higher PDP2 activity, enhancing pyruvate-driven oxidative metabolism due to increased PDH activity. Importantly, PDH activity is required to maintain OIS, as PDK1 overexpression in BRAF^{V600E} melanocytes is sufficient to promote tumorigenesis and abrogate senescence⁵¹. Silencing PDK1 in a xenograft model decreases tumorigenesis or promotes regression of established tumors. These findings are in line with observations that dichloroacetate, a PDK inhibitor, suppresses tumor growth in vitro and in vivo⁷⁸. Significantly, PDK1 knockdown enhances sensitivity of BRAF^{V600E} melanoma cells to BRAF inhibitors, suggesting that combining PDK1 and BRAF inhibition could have clinical application.

Pyruvate transport into mitochondria occurs via the carrier proteins MPC1 and MPC2, and in cancer cells their expression modulates pyruvate oxidation⁹⁶. MPC1 loss is correlated with poor patient outcomes, and increasing its expression in tumor cells significantly inhibits anchorage-independent cell growth without altering growth in adherent culture. Interestingly, suppression of pyruvate transport in myoblasts dramatically decreases glucose consumption and pyruvate oxidation but does not alter cell growth, TCA cycle activity, or mitochondrial respiration¹⁰⁸, likely due to enhanced TCA cycle glutaminolysis via malic enzyme and pyruvate dehydrogenase activity, and elevated branched chain amino acid and fatty acid beta oxidation. As a metabolic adaptation, mitochondria switch to synthesizing pyruvate internally in the absence of pyruvate transport from the cytosol¹⁰⁸. In contrast, inhibition of PDH or mitochondrial complex I does not induce a compensatory response. These observations indicate significant flexibility in substrate utilization to derive pyruvate when its availability from glycolysis is diminished as well as in activating alternative pathways to generate Acetyl-CoA to sustain TCA activity. Another study utilizing glioma cells established that blockade of pyruvate mitochondrial transport activated glutamate dehydrogenase, which was accompanied by rerouting of glutamine carbon in the TCA cycle via reductive carboxylation to produce oxaloacetate and Acetyl-CoA¹²⁴. Inhibitors of glutamate dehydrogenase have antiproliferative effects in cultured cells and cause synthetic lethality when combined with inhibitors of mitochondrial pyruvate transport.

Glutamine metabolism is uniquely altered in melanoma

Glutamine addiction is a hallmark of oncogenic transformation, melanoma included²⁷, and glutamine withdrawal reportedly induces tumor cell apoptosis^{131, 133}. In addition to being one of the 20 proteinogenic amino acids, glutamine is an anaplerotic substrate, one whose carbon is used to maintain pools of TCA cycle intermediates and for biosynthesis of glutamate, aspartate, proline and asparagine. Glutamine alpha nitrogen is used for transamination of alpha keto acids following glutamine conversion to glutamate, and glutamine gamma nitrogen is used for purine, hexosamine, and asparagine and as a vital redox carrier NAD and NADP biosynthesis¹⁹. It is not surprising then that cancer cells are critically dependent on glutamine, which is the most abundant amino acid in blood. To serve as an energy substrate in the TCA cycle via glutaminolysis, a primary pathway utilized by cancer cells, glutamine is deamidated to glutamate in mitochondria by the mitochondrial splice variant (GAC) of kidney type glutaminase (GLS) (Fig. 1)¹², whose expression is essential for tumor cell growth¹⁰⁹. Glutamate is then converted to α -ketoglutarate by

deamination by glutamate dehydrogenase (GDH) or transamination by alanine or aspartate aminotransferases (GPT or GOT, respectively). A systematic study of glutamine utilization in melanoma lines harboring diverse mutations revealed a heretofore unappreciated major mode of energy-producing glutaminolysis, whereby glutamine carbon enters and exits the TCA cycle via the same intermediate - glutamate. This occurs via both GDH activity, which feeds glutamine carbon into the TCA cycle, and GPT and GOT, both of which supply and export glutamine carbon in and out of the TCA cycle⁹³. This mechanism is significant, as previously glutaminolysis was considered primarily to constitute a way to convert glutamine to lactate via malic enzyme¹⁷. The requirement for glutamine for optimal melanoma cell growth is mostly driven by energy-producing TCA cycle anaplerosis and asparagine biosynthesis⁹³ (Fig. 1). Complementing these findings, another study using tumor cells from different tissues of origin found that asparagine played a critical role in regulating cellular adaptation to glutamine deficiency, primarily by blocking the apoptotic arm of the ATF4-mediated amino acid starvation response¹³³. Aspartate, which functions in purine and pyrimidine biosynthesis, cannot be effectively salvaged in melanoma cells and must be synthesized internally⁹³, making its generation via anaplerotic use of glutamine critical for highly proliferative melanoma cells. In agreement, recent elegant studies addressing the role of mitochondrial respiration in proliferating cells demonstrated an essential function of the mitochondrial electron transport chain (ETC) in supporting aspartate biosynthesis^{8, 106}. Interestingly, following ETC inhibition, cytosolic rather than mitochondrial aspartate biosynthesis is enabled by reverse action of the cytosolic isoform of glutamate to oxaloacetate transaminase (GOT1), a process stimulated by addition of extracellular pyruvate⁸. This important finding provides a mechanistic basis for the long-standing observation that very high pyruvate levels rescue cellular proliferation when the TCA cycle is disrupted⁴². The authors provide further evidence that cells use salvaged pyruvate to generate NAD⁺ necessary to activate the cytosolic isoform of malate dehydrogenase (MDH1), as MDH1 knockdown blocks the ability of pyruvate to rescue TCA inhibition, an outcome rescued by MDH1 overexpression. In the presence of NAD⁺, MDH1 acts on malate to generate NADH and oxaloacetate, which is then converted to aspartate by cytosolic GOT1.¹¹²

A notable feature of glutamine metabolism in melanoma cells is upregulation of genes participating in proline biosynthesis from glutamate, resulting in substantially increased proline generation by tumor cells relative to melanocytes¹⁶. MYC is an oncogene contributing to many human malignancies and greatly affecting central carbon metabolism¹⁴. C-Myc undergoes amplification in a subset of melanomas⁵⁵. Glutamine utilization and proline biosynthetic pathways are reportedly upregulated by c-Myc in many cancer cells⁶⁵. Analysis of proline synthetic pathways, which converge at pyrroline-5 carboxylate, identified distinct functions for isoforms of pyrroline-5 carboxylate reductase (PYCR1-3). Isoforms 1 and 2 catalyze proline biosynthesis in mitochondria from glutamine-derived pyrroline-5 carboxylate via glutamate using NADH as cofactor, while PYCR3 uses an arginine-derived intermediate via ornithine as a substrate to generate proline in the cytosol and shows a significant preference for NADPH¹⁶ (Fig. 1). Differential regulation of enzymatic activity of each isoform by extracellular proline is a notable feature of this enzyme family. Cytosolic PYCR3 activity is unaffected by proline levels of up to 1 mM,

while mitochondrial PYCR2 is intermediately sensitive to product inhibition, retaining more than half of its activity at 0.5 mM proline, a concentration sufficient to completely inhibit the PYCR1 mitochondrial isoform. Melanoma cells synthesize proline even when it is abundant in the medium, and given that its concentration in blood ranges from 50 to 350 μM ¹²², pathway activity rather than product levels may be critical in tumorigenesis, possibly by concurrent generation of oxidizing equivalents in the form of NAD^+ in mitochondria and NADP^+ in the cytosol.

Ketogenesis promotes BRAF^{V600E}-mediated activation of MEK-ERK signaling

A screen performed using melanoma lines expressing the BRAF^{V600E} mutant revealed the mitochondrial ketogenic enzymes HMG-CoA synthase 1 (HMGCS1) and 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) to be synthetic lethal partners of oncogenic BRAF in a metabolic gene knockdown screen, implicating the ketogenesis pathway as a potential target in that tumor context⁵⁰. Ketogenesis normally occurs in liver in response to starvation, prolonged strenuous exercise, or pathologically, in uncontrolled diabetes⁷⁵. Expression of oncogenic (V600E) but not wild-type BRAF causes upregulation of HMGCL at mRNA and protein levels. In primary tumor human tissue samples, HMGCL overexpression on a BRAF^{V600E} background is accompanied by elevated phosphorylation and activation of MEK1 and ERK relative to tumor cells with wild-type BRAF⁵⁰. Elevated HMGCL expression accompanied by increased MEK1 and ERK phosphorylation is also seen in primary human leukemia cells derived from patients with hairy cell leukemia whose tumors harbor the somatic BRAF^{V600E} mutation. This observation provides independent confirmation of the connection between oncogenic BRAF and HMGCL overexpression seen in melanoma patients. Importantly, HMGCL is required for BRAF^{V600E}-induced cell transformation, as knockdown of either mutant BRAF or HMGCL decreases cell proliferation and tumor growth in xenograft models. The mechanistic link between HMGCL activity and BRAF^{V600E}-driven cell transformation suggests selective enhancement of MEK-ERK activation, as evidenced by attenuated cell proliferation of BRAF^{V600E}- but not wild-type BRAF-expressing cells following HMGCL knockdown, the former accompanied by loss of MEK1 and ERK1/2 phosphorylation. Of interest, acetoacetate was identified as a factor promoting BRAF^{V600E}-activated signaling. Addition of acetoacetate but not 3-hydroxybutyrate to the medium of cultured melanoma cells selectively promoted proliferation of BRAF^{V600E} but not BRAF wild-type cells and rescued growth phenotypes induced by HMGCL knockdown in the former. Rescue of growth phenotypes by acetoacetate was accompanied by increased MEK1 and ERK1/2 phosphorylation, while addition of 3-hydroxybutyrate had no effect on either. In vitro studies showed that acetoacetate selectively promotes MEK1 phosphorylation in the presence of V600E but not wild-type BRAF. Treatment of cells with acetoacetate but not 3-hydroxybutyrate reportedly increased both binding and phosphorylation of MEK1 by oncogenic but not wild-type BRAF, providing the molecular basis for the observed phenotype. BRAF^{V600E} knockdown or inhibition decreased both HMGCL mRNA and protein levels, an effect not observed in the presence of wild-type BRAF or following MEK1 inhibition. This suggests that induction of HMGCL expression is independent of MAPK pathway activity. Furthermore, Oct1 was

identified as a driver of HMGCL transcription through an Oct1-specific response element on its promoter (Fig 2). The ability of Oct1 to stimulate HMGCL expression is apparently controlled by BRAF^{V600E} activity, as both BRAF^{V600E} knockdown or its inhibition by PLX4032 attenuated Oct1 binding to the identified promoter region. Overall, BRAF^{V600E} activates Oct1, leading to HMGCL expression. In turn, generation of its product acetoacetate selectively enhances BRAF^{V600E}-mediated signaling via the MEK-ERK pathway.

Oncogenic BRAF regulates oxidative metabolism in melanoma

It is well established that oncogenic BRAF regulates metabolic reprogramming in melanoma⁴⁰. Treatment of BRAF^{V600E} cells with the BRAF inhibitor vemurafenib results in increased expression of several genes functioning in the TCA cycle as well as that of genes associated with oxidative phosphorylation and ATP synthesis, effects not seen in wild-type BRAF melanoma cells. Similarly, treatment of BRAF-mutant but not wild-type cells with the BRAF inhibitor PLX4720 increases mitochondrial density and ROS production and decreases glucose conversion to lactate, suggesting that glucose carbon is rerouted away from glycolysis and towards oxidative phosphorylation in those cells. Activation of BRAF or MAPK in melanoma cells downregulates expression of PGC1 α (PPARGC1A), a member of a small family of transcriptional co-activators (including PGC1b and PRC) that promotes mitochondrial biogenesis and enhances oxidative phosphorylation⁹². Conversely, treatment of any melanoma cells carrying oncogenic BRAF with PLX4720 significantly increases PGC1 α mRNA levels⁴⁰. Acquired resistance of melanoma cells to BRAF inhibitors downregulates PGC1 α expression, in agreement with elevated basal MAPK activity. Interestingly, treatment of BRAF^{V600E}-mutant colon cancer cells with PLX4720 does not alter PGC1 α expression, despite decreased ERK phosphorylation, suggesting that the BRAF/PGC1 α link is lineage-specific.

MITF³⁹ is a lineage-specific oncogene amplified in 30% of melanomas³² and harboring a point mutation (E318K) leading to impairment in SUMOylation and predisposing to melanoma and renal carcinoma^{7, 128}. MITF was found to promote expression of metabolic genes functioning in oxidative phosphorylation by upregulating PGC1 α in a subset of melanomas, inducing changes in oxidative metabolism^{40, 113} (Fig. 2). The same metabolic genes were found to be PGC1 α -responsive in other lineages⁸¹. Both MITF and PGC1 α levels are inversely proportional to MAPK pathway activity based on the MAPK activation signature seen in several melanomas carrying oncogenic BRAF⁴⁰. These data indicate that inhibition of BRAF/MAPK signaling triggers MITF expression followed by PGC1 α induction in melanoma. Elevated MITF and PGC1 α levels are also observed in biopsies from melanoma patients who have been treated with BRAF/MEK inhibitors. BRAF inhibition reportedly causes energetic adaptation of melanoma cells via MITF and PGC1 α induction, such that low ATP levels due to downregulated glycolysis are partially restored by MITF expression via PGC1 α -driven mitochondrial biogenesis and increased oxidative metabolism, thus making cells more sensitive to the mitochondrial uncoupler 2,4-dinitrophenol⁴⁰. Induction of oxidative phosphorylation limits BRAF inhibitor efficacy, as forced expression of PGC1 α in BRAF^{V600E} melanoma cells desensitizes them to treatment, and high PGC1 α expression positively correlates with poorer survival of patients with stage III melanoma^{9, 40}. Overall, these observations suggest that induction of oxidative

metabolism limits BRAF inhibitor efficacy but that combining BRAF and mitochondrial inhibitors has an additive effect. Phenformin, a known inhibitor of AMP-activated protein kinase, reportedly enhances the therapeutic efficacy of BRAF^{V600E} in melanoma^{84, 129}, effects thought to result from synergy with PLX4720 in inhibiting mTOR and inducing apoptosis. Significantly, phenformin selectively inhibits growth of slow cycling melanoma cells expressing JARID1B (an H3K4 demethylase), while PLX4720 targets JARID1B-negative cells¹²⁹. Expression of JARID1B is important for maintenance of long-term melanoma tumor growth⁹⁴. Combined PLX4720 plus phenformin treatment significantly delays acquisition of resistance to BRAF inhibitors in cultured melanoma cells and induces tumor regression in xenograft and BRAF^{V600E}/PTEN^{-/-}-driven melanoma mouse models¹²⁹. Metabolic analysis revealed that treatment with biguanides (such as phenformin and metformin) prevents the boost in glycolytic and TCA cycle intermediates during oncogenic transformation, effects consistent with inhibition of mitochondrial complex I⁴⁸. Accordingly, another study reports that BPTES, an inhibitor of the mitochondrial glutaminase, synergizes with BRAF inhibitors due to the increased reliance of BRAF^{V600E}-inhibited melanoma cells on glutamine anaplerosis^{4, 43}. Glutaminase inhibition significantly delayed if not prevented acquisition of resistance, similar to synergistic effects observed following PLX4720/phenformin treatment. Overall, these studies independently confirm that combined targeting of mitochondrial oxidative metabolism and oncogenic BRAF signaling synergistically suppresses melanoma progression.

Although almost ~20% of cutaneous melanomas have activating NRAS mutations¹¹, surprisingly little is known about the metabolic consequences of aberrant NRAS signaling. However, it should be noted that somatic mutations in KRAS account for most human cancer-associated mutations^{5, 98} and multiple studies address the metabolic rewiring arising from them in pancreatic and other cancers highlighting changes in glutamine utilization^{31, 104}, anabolic glucose metabolism^{31, 127, 130}, mitochondrial metabolism and ROS¹¹⁸.

Mitochondrial ROS contributes to melanoma metastasis

Increased ROS generation due to high or dysregulated metabolic activity is a stress factor that plays a significant role in tumorigenesis³⁷. Depending on context, ROS generation can enhance¹¹⁷ or block tumor progression^{23, 107} by either promoting genomic instability and stimulating cell proliferation or inducing cellular toxicity, respectively. Moderately elevated ROS levels function in signaling and are necessary for normal cell physiology, while high levels induce oxidative stress⁹⁷. Thus, modulation of ROS is considered an anticancer strategy³⁵. Activation of antioxidant scavenging enables tumor cells to adapt to and protect themselves against elevated ROS^{86, 103}. PGC1 α reportedly functions to control mitochondrial ROS generation¹⁰⁵, and its expression increases in response to upregulated metabolic activity together with induction of mitochondrial biogenesis and oxidative capacity³⁸ (Fig. 2). Melanoma cells exhibiting high PGC1 α levels likely depend on its expression for survival and tumorigenesis¹¹³: PGC1 α knockdown in those cells activates the intrinsic apoptotic pathway. PGC1 α loss also promotes decreased mitochondrial membrane potential accompanied by elevated ROS and lowered levels of glutathione (GSH), cystathionine, and 5-adenosylhomocysteine¹¹³. Activation of apoptosis in melanoma cells is

largely suppressed by antioxidant treatment, indicating that ROS levels in the absence of PGC1 α expression promote cell death. In agreement, expression of several genes functioning in ROS detoxification decreases upon PGC1 α knockdown. Loss of PGC1 α target genes, such as components of mitochondrial respiratory complexes, also increases ROS and decreases GSH levels, suggesting that in addition to downregulation of detoxification genes, uncontrolled ROS generation in melanoma cells occurs due to defects in mitochondrial bioenergetics following PGC1 α depletion. Conversely, increased PGC1 α levels in response to ectopic expression of either MITF or PGC1 α itself elevate expression of the mitochondrial ROS detoxification genes SOD2 and GPX1, a finding supporting PGC1 α function in activating the ROS detoxification program and driving melanoma cell survival¹¹³.

Although antioxidants are promoted as beneficial anticancer agents for patients, their use has been controversial^{33, 119, 120}, as some clinical trials suggest they increase cancer risk^{1, 34, 60}. There are reports that antioxidants stimulate tumor initiation and progression^{20, 22, 41, 95}, while two recent studies demonstrate that oxidative stress inhibits metastasis but not primary tumor growth in mouse models of melanoma^{59, 89}. Investigators showed that following intravenous or splenic injection of patient-derived melanoma cells into mice, tumor formation was significantly suppressed relative to subcutaneous implantation, particularly in the case of inefficiently metastasizing cells⁸⁹. Melanoma cells in the bloodstream and internal organs undergo oxidative stress not observed in established subcutaneous tumors. Statistical analysis of metabolic profiles from tumor samples shows that data obtained from metastatic nodules derived from liver, pancreas or kidney tends to cluster together but separately from subcutaneous tumors. Notably, GSH/GSSG (reduced to oxidized glutathione) ratios are always higher in subcutaneous than in circulating tumor cells or metastatic nodules, in concordance with elevated GSH consumption that occurs during oxidative stress in metastasizing cells relative to established tumors⁸⁹. In agreement with that, cytosolic ROS levels were higher and mitochondrial membrane potential lower in melanoma cells found in the bloodstream and distant metastatic sites compared to subcutaneous tumors. Treatment of mice with the antioxidant N-acetyl cysteine (NAC) had no effect on growth of subcutaneous tumors but significantly increased the number of tumor cells in blood as well as metastatic spread. In a BRAF^{V600E}/PTEN^{-/-} mouse melanoma progression model¹⁵, NAC administration in drinking water had no impact on tumor burden but significantly increased the number of lymph node and (to a lesser degree) lung metastases⁵⁹. Interestingly, NAC treatment also does not alter proliferation but increases migration and invasion of human malignant melanoma cells in vivo in this model⁵⁹. Migratory and invasive phenotypes were positively correlated with an increased GSH/GSSG ratio and were abolished by simultaneous administration of an inhibitor of de novo glutathione biosynthesis (L-BSO). The inability of antioxidants to induce migratory and invasive phenotypes in the presence of BSO indicates that glutathione biosynthesis, not just GSSG reduction, is necessary for the effect. Changes in redox status also reportedly alter nucleotide binding by the RHO family of GTP-ases^{45, 46, 79}. GTP loading of RHOA but not RAC1 increases consistently following treatment with antioxidants like NAC and the vitamin E analog Trolox. Inhibition of RHOA-associated kinase (ROCK), the downstream signaling partner of RHOA, prevents antioxidant-induced tumor cell migration without

affecting basal migration. NADPH is required to reduce GSSG to GSH^{26, 35, 61, 72} (Fig. 3), and increased NADP and NADPH levels are consistently observed in metastatic cells relative to subcutaneous tumors⁸⁹. Lower NADPH/NADP ratios in metastatic cells are observed less consistently; thus the degree of oxidative stress could explain why elevated NADP and NADPH levels do not necessarily alter the ratio of the two. Folate metabolism is a major source of NADPH for oxidative stress management^{26, 61, 126}. Metastatic cells contain higher levels of purine biosynthetic pathway intermediates compared to subcutaneous tumor cells from the same mice, suggesting that the folate pathway promotes distant metastasis (Fig. 3). Serine biosynthesis can contribute to activity of the folate pathway (Fig 1 and 3). Stable labeling of mouse tumors derived from patient melanoma cells with ¹³C-glucose indicates significantly increased ¹³C enrichment in serine and glycine in cells from metastatic tumors relative to their subcutaneous counterparts⁸⁹, with no ¹³C enrichment in 3-phosphoglycerate or lactate. These observations suggest that upregulated folate pathway activity underlies the increased contribution of glucose carbon to tissue serine and glycine levels. Analysis of expression of folate pathway genes revealed upregulation of the NADPH-producing oxidoreductase ALDH1L2 (Fig. 3) at the protein level in liver metastatic nodules relative to subcutaneous tumors, regardless of whether the latter were established using cells from metastatic sites or not, indicating that gene expression changes are reversible. Treatment of mice bearing patient-derived subcutaneous tumors with low doses of methotrexate, an inhibitor of dihydrofolate reductase (Fig. 1), in combination with thymidine and hypoxanthine to avoid side effects related to nucleotide biosynthesis, significantly reduced the number of tumor cells in circulation as well as the metastatic burden, further suggesting that NADPH generation is the folate pathway step most relevant to enhanced metastasis. Silencing of both ALDH1L2 and MTHFD1 (another NADPH-producing enzyme in the folate pathway (Fig. 3)) reduced the number of tumor cells in the bloodstream and in metastases. Thus, survival of metastasizing cells depends on their ability to combat oxidative stress, an activity afforded in part by the folate pathway. Interestingly, ALDH1L2 is a mitochondrial enzyme, while MTHFD1 resides in the cytosol (Fig. 3), indicating that NADPH production via the folate pathway in both compartments plays a role in ROS management in melanoma. A recent elegant study on compartmentalization of NADPH metabolism demonstrated that conversion of serine to glycine occurs primarily in the mitochondria of non-small lung carcinoma and alveolar basal adenocarcinoma, suggesting that mitochondrial SHMT2- but not cytosolic SHMT1-driven serine conversion to glycine (Fig. 1) is primarily responsible for NADPH generation in that system.⁶¹ In melanoma, an interplay between mitochondrial and cytosolic compartments apparently functions in ROS management, as both are reportedly involved in NADPH regeneration via the folate pathway. It is noteworthy, that the location of metastatic sites may influence the metabolic program executed by the invading cells as has been demonstrated for breast cancer cells²⁴.

Concluding remarks

In recent years significant advances have been made in treating metastatic melanoma^{52, 66}. The advent of immunotherapies and BRAF inhibitors has significantly improved survival and for a subset of patients produced durable therapeutic benefits. The main issues still

plaguing the field are lack of or poor response to immunotherapies and development of resistance to BRAF inhibition. These issues leave significant room for developing alternative approaches based on targeting auxiliary functions, such as metabolic enzymes and pathways, altered by malignant transformation, either individually or in combination with immune or chemotherapies.

Metabolic reprogramming in melanoma occurs in response to oncogenic stimuli and as an adaptation to tumor microenvironment-dependent stress. Cytosolic and mitochondrial compartments often show patterns of reciprocal adaptation to accommodate changes in one versus the other. The cytosolic serine pathway is often upregulated in melanoma to drive one carbon metabolism important to mitigate ROS generated in mitochondria. Inhibition of one carbon metabolism is already a therapeutic strategy in cancer⁶⁸, and combined with other agents could prove useful in combating melanoma. Specifically, inhibiting the serine biosynthetic pathway reduced glutathione and NADPH levels to induce oxidative stress in metastatic cells., Higher ROS levels could decrease invasiveness of metastatic melanoma cells due to poor RHOA/GTP loading and activity.

Hypoxia reduces glucose flux into the TCA cycle, enhancing glutamine carbon utilization for fatty acid biosynthesis via reductive carboxylation. Downregulation of ATP-generating glycolysis following inhibition of oncogenic BRAF leads to upregulation of oxidative phosphorylation via a PGC1 α -driven mitochondrial generation program to restore ATP levels required for proliferative phenotypes. Combining BRAF inhibitors with inhibitors of mitochondrial function is one promising approach to block tumor cell proliferation, as supplementation with the biguanides metformin and phenformin or with the glutaminase inhibitor BPTES significantly delays onset of resistance in vitro and or in vivo. There is growing evidence that combining orthogonal approaches such as BRAF inhibition with targeting oxidative metabolism can result in significant synergy against melanoma, which could greatly improve disease outcomes.

Alternative last paragraph:

Finally, reports summarized here suggest that combining BRAF inhibitors with inhibitors of mitochondrial function is a promising approach to block tumor cell proliferation, as supplementation with the biguanides metformin and phenformin or with the glutaminase inhibitor BPTES significantly delays onset of chemoresistance in vitro and or in vivo. There is growing evidence that BRAF inhibitors plus reagents that target oxidative metabolism act synergistically to antagonize melanoma. If so, these approaches could greatly improve patient outcomes.

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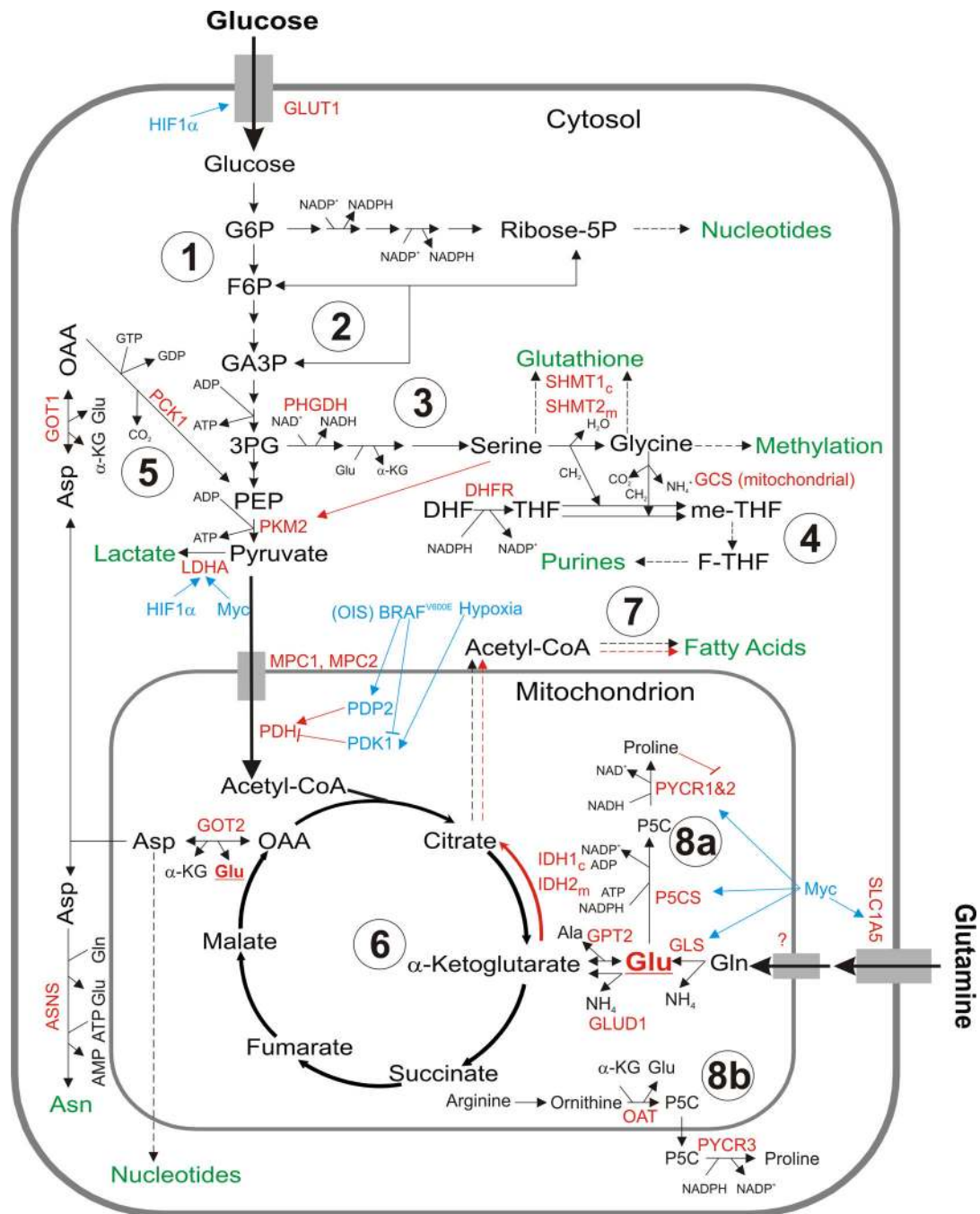


Figure 1. Metabolic rewiring in melanoma

Pathway end products are shown in green, factors affecting expression of metabolic genes or their regulators in blue (letters and arrows). OIS – oncogene induced senescence. Allosteric activation of PKM2 by serine is shown as red arrow. Metabolic enzyme and metabolite carrier names are in red. Glutamate as the major metabolite participating in both influx and outflow of glutamine carbon from the TCA cycle is shown in red bold letters and is also underlined. Dotted arrows indicate multistep reactions.

Metabolic pathways: 1. Glycolysis, 2. Pentose phosphate pathway, 3. Serine and glycine biosynthesis, 4. Folate pathway, 5. Gluconeogenesis, 6. Tricarboxylic (TCA) acid cycle, 7. Fatty acid biosynthesis, 8. Proline biosynthesis: a – from glutamate, b – from arginine.

Metabolites: G6P – glucose 6 phosphate, F6P – fructose 6 phosphate, GA3P – glyceraldehyde 3 phosphate, PEP – phosphoenolpyruvate, OAA – oxaloacetate, DHF – dihydrofolate, THF – tetrahydrofolate, me-THF - 5,10-methylene-THF, F-THF - 10-formyltetrahydrofolate, P5C – pyrroline-5 carboxylate,

Genes: GLUT1 (SLC2A1) - facilitated glucose transporter member 1, PHGDH - phosphoglycerate dehydrogenase, SHMT1&2 – serine hydroxymethyl transferases 1 (cytosolic) and 2 (mitochondrial), GCS – glycine cleavage system, DHFR – dihydrofolate reductase, PKM2 – pyruvate kinase isoform M2, PCK1 - phosphoenolpyruvate carboxykinase, LDHA – lactate dehydrogenase A, GOT1 and 2 – glutamate to oxaloacetate aminotransferase isoforms 1 and 2, MCP 1 and 2 – mitochondrial pyruvate carriers 1 and 2, PDH – pyruvate dehydrogenase complex, PDK1 – pyruvate dehydrogenase kinase, PDP2 - pyruvate dehydrogenase phosphatase 2, ASNS – asparagine synthetase, IDH1&2 – isocitrate dehydrogenases 1 (cytosolic and peroxisomal) and 2 (mitochondrial), GLS – kidney type glutaminase, GLUD1 – glutamate dehydrogenase 1, GPT2 – glutamate to pyruvate aminotransferase 2, PYCR – pyrroline-5 carboxylate reductase: 1 – isoform 1, 2 – isoform 2, 3 – isoform 3, OAT – ornithine aminotransferase, SLC1A5 – glutamine plasma membrane carrier.

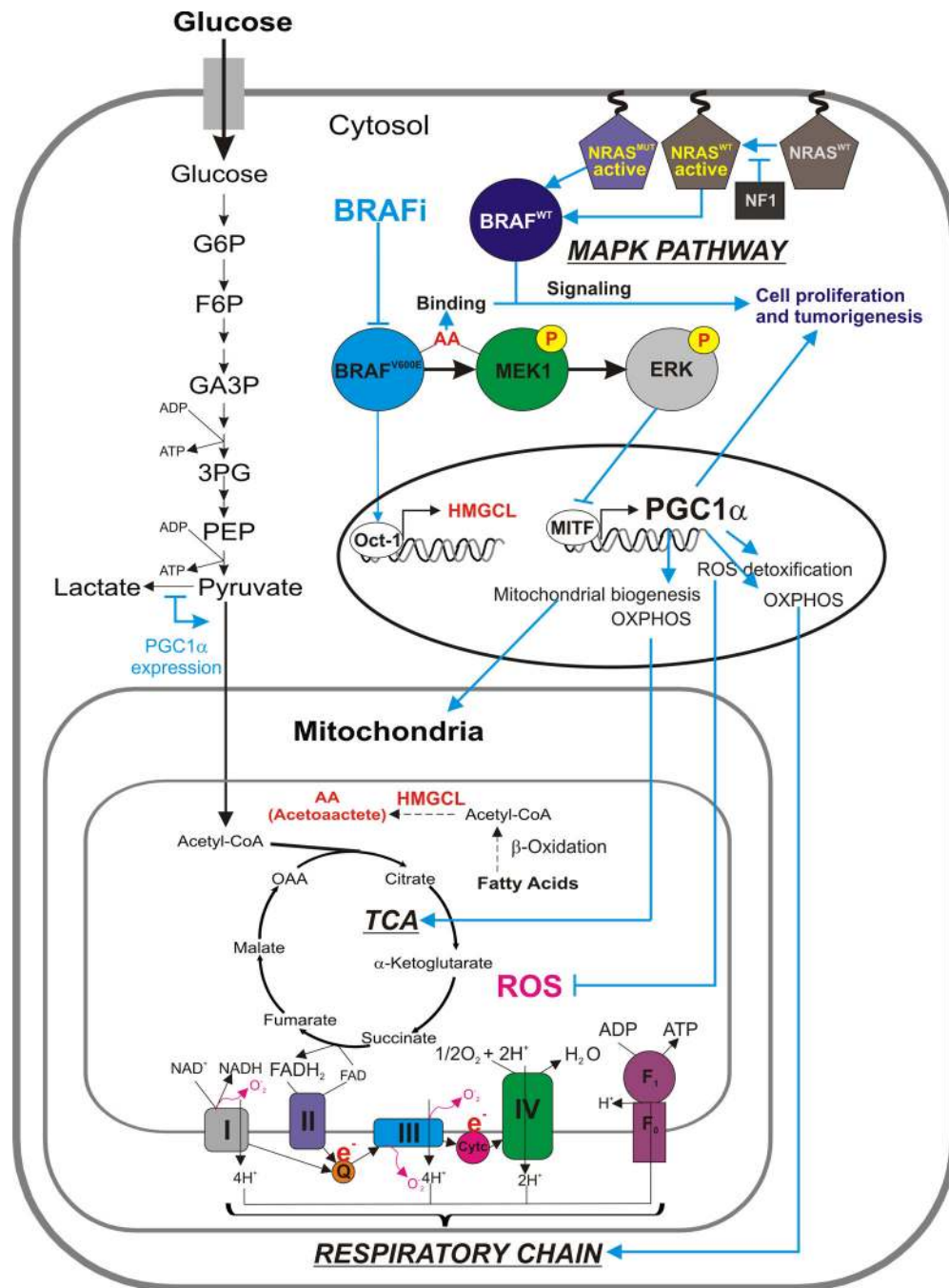


Figure 2. MAPK pathway activity, oncogenic BRAF and oxidative metabolism in melanoma
 Oncogenic BRAF activity induces formation of ketone body acetoacetate, which enhances MAPK pathway activity by promoting binding between BRAF^{V600E} and MEK1. Inhibition of oncogenic BRAF de-represses MITF expression leading to increased mitochondrial biogenesis and oxidative phosphorylation due to enhanced expression of the master mitochondrial regulator PGC1 α , which also suppresses glycolysis. High PGC1 α levels are critical for survival and tumorigenesis of a subset of melanomas relying heavily on oxidative metabolism by upregulating expression of ROS detoxification genes, thus mitigating

oxidative stress. NRAS activation by mutations or loss of activity of the NF1 tumor suppressor¹⁰² can contribute to the activation of the MAPK pathway. Abbreviations: HMGCL - 3-hydroxy-3-methylglutaryl-CoA lyase, OXPHOS – genes encoding proteins participating in TCA cycle activity, oxidative phosphorylation and ATP production in mitochondria, ROS – reactive oxygen species, BRAFi – inhibitors of BRAF^{V600E} activity, I – V – mitochondrial complexes 1 through 5.

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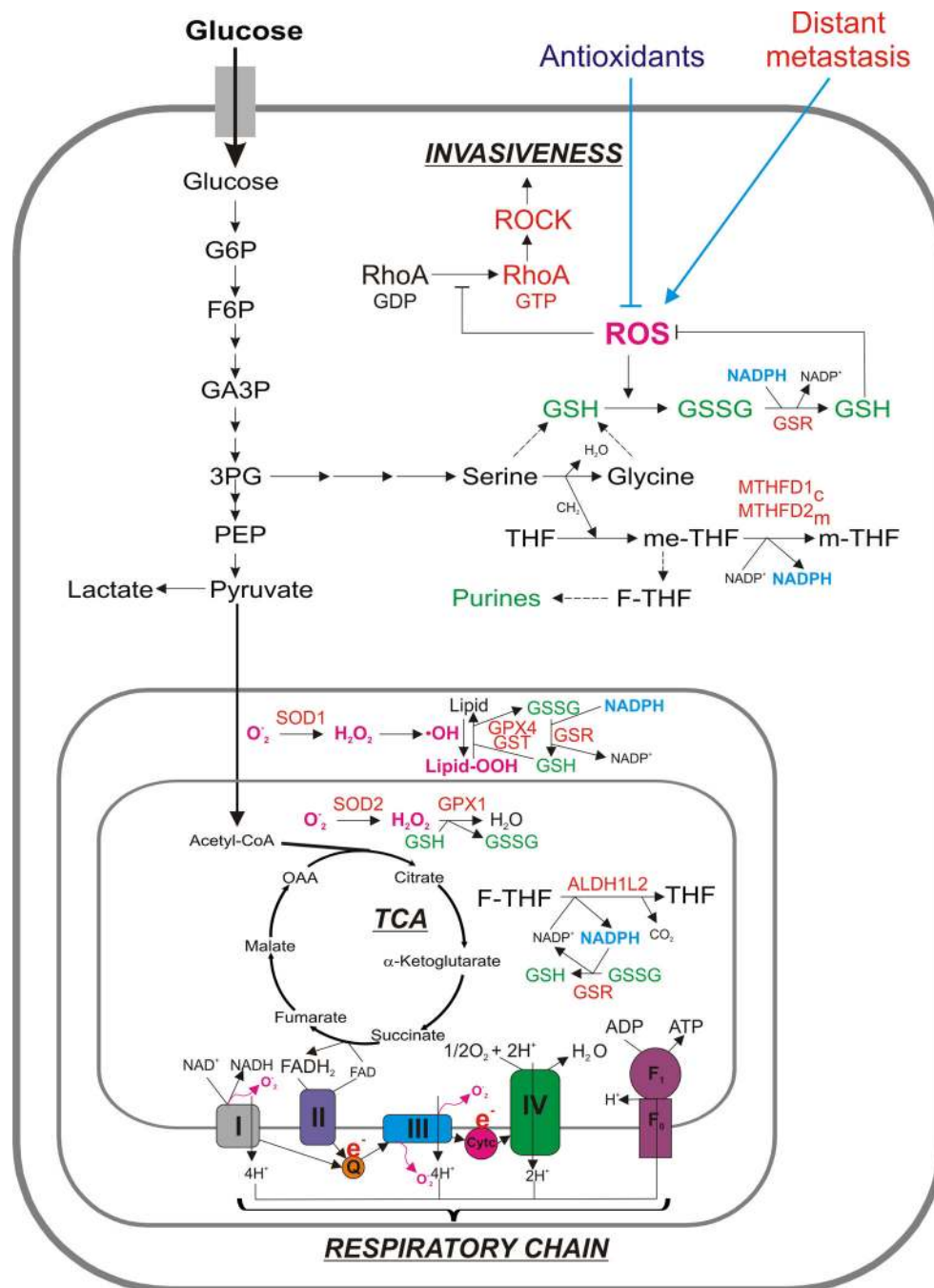


Figure 3. ROS levels affect metastasis in melanoma

Distant metastasis causes oxidative stress in melanoma cells. Treatment with antioxidants promotes metastasis by enhancing GTP loading of RhoA GTPase and activating Rho-associated protein kinase (ROCK) thus inducing invasiveness. Activity of the folate pathway is important for mitigating oxidative stress by providing NADPH necessary for generation of reduced glutathione – a primary ROS detoxification agent.

Abbreviations: GSH – reduced glutathione, GSSG – oxidized glutathione, MTHFD - methylenetetrahydrofolate reductase 1 (cytosolic) and 2 (mitochondrial), SOD – superoxide

dismutase, GPX – glutathione peroxidase, GSR – glutathione reductase, 10-formyltetrahydrofolate dehydrogenase

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