

Molecular Pathways: BRAF Induces Bioenergetic Adaptation by Attenuating Oxidative Phosphorylation

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Abstract

Cancers acquire mutations in cooperating pathways that sustain their growth and survival. To support continued proliferation, tumor cells adapt their metabolism to balance energy production with their augmented biosynthetic needs. Although most normal differentiated cells use mitochondrial oxidative phosphorylation (OXPHOS) as the bioenergetic source, cancer cells have been proposed to rely principally on cytoplasmic glycolysis. The molecular basis for this shift, termed the Warburg effect, is the subject of intense investigation, because mechanistic understanding may lead to novel approaches to target the altered metabolism of cancer cells. Recently, mutations BRAF(V600E) have emerged as a major regulator of metabolic homeostasis. Melanoma cells may use a metabolic shift to circumvent BRAF(V600E)-induced senescence though limiting their reliance on OXPHOS and promote proliferation. Furthermore, BRAF(V600E) acts to suppress expression of the melanocyte master regulator microphthalmia-associated transcription factor (MITF) and the mitochondrial biogenesis coactivator PGC1 α . Accordingly, therapeutic inhibition of BRAF(V600E) reverses metabolic reprogramming in melanoma cells and elevates OXPHOS through increased MITF–PGC1 α levels. BRAF-targeted drugs modulate the metabolic state of malignant melanoma cells, and counteracting these adaptive responses using pharmacologic agents may prove useful in combinatorial therapeutic strategies. *Clin Cancer Res*; 20(9); 2257–63. ©2014 AACR.

Background

In 2002, the COSMIC team at the Sanger Center in the United Kingdom identified frequent gain-of-function BRAF (V600E) mutations in melanoma (1). Oncogenic mutations in the *BRAF* gene induce constitutive activation of its serine/threonine kinase activity and activation of the downstream mitogen-activated protein kinase (MAPK) signal transduction pathway. BRAF mutations are also documented in other malignancies, for example, papillary thyroid carcinomas (2), hairy cell leukemias (3), non-small cell lung (4) and colorectal carcinomas (5), thus highlighting this pathway as a key oncogenic driver in multiple anatomic tissues. Overall, BRAF(V600E) is the most prevalent missense mutation in human cancer that in addition can be targeted therapeutically.

Identification of oncogenic driver mutations in human cancers such as the *KRAS* and *BRAF* genes has accelerated

the development of small-molecule inhibitors along the RAS–RAF–MEK–MAPK signaling pathway. Importantly, the first-in-class BRAF-specific inhibitor vemurafenib (PLX4032; ref. 6) was approved in 2011 and extends melanoma survival by 4 to 6 months compared with standard chemotherapy (7, 8). Despite dramatic initial tumor shrinkage in patients with melanoma, long-term efficacy is thwarted due to emergence of drug resistance (9). Alternative BRAF inhibitors, such as dabrafenib (GSK2118436, ref. 10), have shown similar response in patients with melanoma followed by drug resistance and progression (11). However, the antitumor activity of vemurafenib in patients with BRAF(V600E)-mutant papillary thyroid or colorectal cancers remains dismal, possibly due to existence of compensatory pathways, such as epidermal growth factor receptor (EGFR) signaling (12). Targeting the downstream target MEK1/2 has shown survival benefit in patients with melanoma (13) and has been approved for treatment of patients with BRAF-mutant melanoma. Collectively, targeted inhibition of the BRAF–MEK axis in malignant melanoma has extended patient survival, but responses are limited by emergence of drug resistance. Understanding how to prevent onset of resistance to oncogene-targeted therapies and increase their initial therapeutic efficacy may provide key insights toward developing sustainable treatment against BRAF-mutant cancers in general and melanoma in particular. Because cancer cells have distinct metabolic demands from non-malignant cells, combinatorial inhibition of tumor-critical

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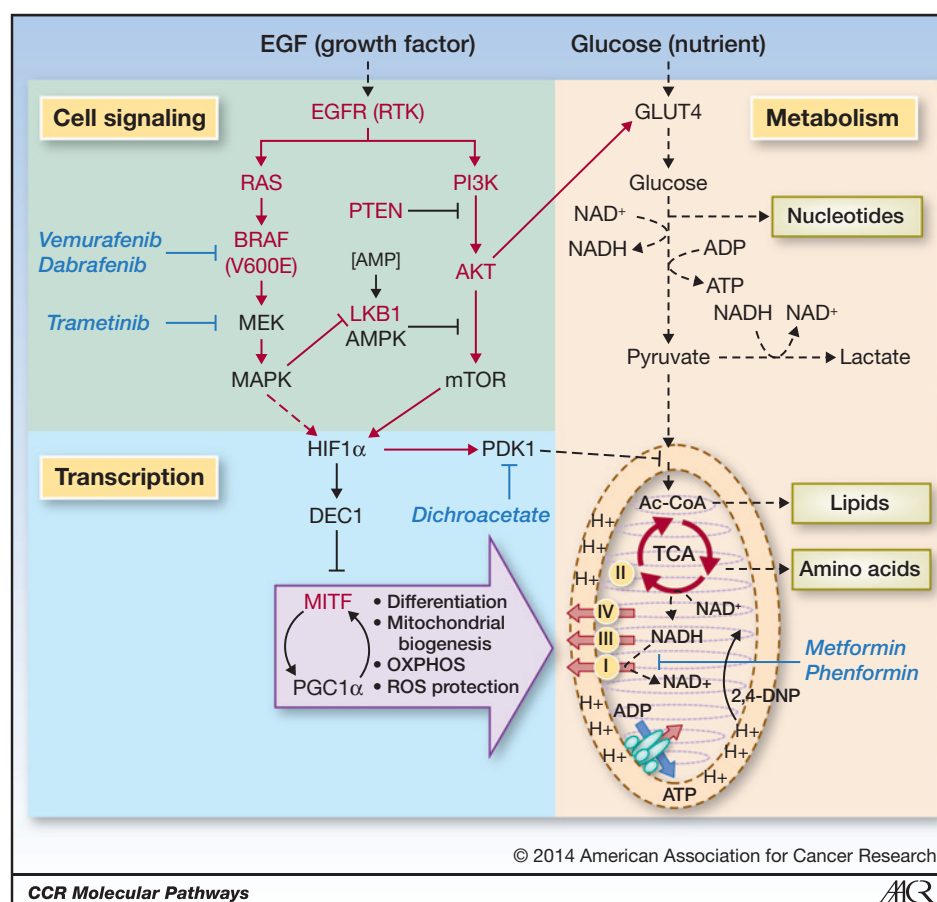


Figure 1. Intersection of signaling, transcription, and metabolism regulates generation of ATP and building block availability to sustain survival and growth, respectively. Growth factor-mediated activation (EGF) of a receptor tyrosine kinase (EGFR) leads to downstream pathway signaling along the RAS–RAF–MEK–MAPK and PI3K–AKT–mTOR pathways. Key convergence points are MAPK-mediated suppression of LKB1/AMPK energy sensing, and AKT-activated translocation of the glucose transporter GLUT4 to the plasma membrane leading to augmented glucose import for anabolic (nucleotides, lipids, and amino acids) in addition to catabolism toward ATP regeneration in the mitochondria using the proton gradient. Furthermore, MAPK stabilizes and mTOR increases translation of HIF-1 α , triggering the transcriptional function with effects on reducing pyruvate decarboxylation/oxidation to acetyl-CoA (Ac-CoA) destined for use in the TCA cycle. In melanoma cells, HIF-1 α activity reduces expression of MIF–PGC1 α levels and thereby causes effects on reducing mitochondrial bioactivity (OXPHOS, ROS protection) in addition to differentiation-associated cascades. Frequently mutant components (and pathways) in human cancers are indicated in red. Inhibitors of indicated pathway components are indicated in blue.

metabolic pathways is an attractive therapeutic approach to extend the usefulness of BRAF- and MAP-ERK kinase (MEK)-targeted therapies.

Metabolic shift in the transition from normal to cancer cell

In normal aerobic cells, glucose is the quintessential nutrient for energy production (Fig. 1). Metabolic breakdown of glucose (glycolysis) drives catabolic ATP production through a pathway that couples the Krebs/tricarboxylic acid (TCA) cycle with oxidative phosphorylation (OXPHOS). In essence, glycolysis produces pyruvate, which is transported to the mitochondria for oxidation into acetyl-CoA to fuel the TCA cycle. Successive oxidation of acetate in the TCA cycle leads to transfer of electrons to NAD⁺ for the generation of NADH, which in turn is consumed by oxygen-dependent reactions (respiration) producing carbon dioxide (CO₂), water (H₂O), and chemical energy. The released chemical

energy, in the form of a proton gradient over the inner mitochondrial membrane, subsequently drives ATP regeneration from ADP. Overall, the TCA cycle and OXPHOS is a highly efficient mitochondrial chemical energy conversion pathway.

Proliferating cells must produce ATP-like normal nondividing cells, but also generate biomass and duplicate its genome to enable cell division. Hence, rapidly dividing cells, such as tumors and stem cells, must balance their catabolic ATP demand with anabolic building block production to maintain survival and sustain their proliferation, respectively. Therefore, it is not surprising that cancer cells display a different metabolic phenotype and nutrient usage when compared with normal cells. Already observed by Dr. Otto Warburg (Max Planck Institute, Berlin, Germany) in the early twentieth century, tumors metabolize glucose anaerobically despite plentiful oxygen present to generate lactate (14), a phenomenon now commonly referred to as

the "Warburg effect." Specifically, lactate dehydrogenase consumes pyruvate to regenerate NAD^+ from NADH produced by glucose breakdown, and hence enables a high rate of glycolysis to take place. Consequently, lactate is a byproduct in a reaction that sustains rapid production of building block intermediates and ATP sufficiency in proliferating cells. As lactate can easily be transported out and in between cells, and lactate dehydrogenase can generate NADH and pyruvate in reverse, this reaction is able to fuel mitochondrial oxidative phosphorylation among adjacent cells (15).

Role of mitochondrial oxidative phosphorylation in cancer

Despite the prevailing emphasis on glycolysis in cancer metabolism, leukemias, prostate and breast cancers, as well as melanomas, require oxidative phosphorylation for their growth (16–19). In fact, some melanomas have significantly higher oxygen consumption than melanocytes (20), indicating that OXPHOS itself may be an important metabolic target in some tumor contexts. Moreover, inhibition of OXPHOS using elesclomol provokes apoptosis initially, whereas prolonged *in vitro* selective growth drives enhanced glycolysis. Hence, this study indicates that melanomas are acutely dependent on OXPHOS, while they are able to adapt to alternate metabolism. Using a mouse malignant melanoma model, it has also been demonstrated that selectively depleting mitochondrial DNA (mtDNA), which encodes key components of the electron transport chain and thus compromises OXPHOS, delays subcutaneous tumor growth and prevents metastasis (21). Similar results have been reported in a human breast cancer cell line (22). Together, these observations suggest that in addition to a general glycolytic phenotype due to anabolic demands, there is significant heterogeneity in the metabolic requirements of cancer cells. Therefore, a molecular understanding of how OXPHOS is regulated by oncogenic pathways is critical for its rational therapeutic exploit in cancer treatment.

Activation of oncogenes and the shift to anabolic metabolism

Normal cells have a number of programs in place to reduce the likelihood for renegade growth (23). In particular, oncogene-induced senescence (OIS) provides such a barrier that leads to premature terminal growth arrest in response to mutant RAS and RAF, which depends on intact pRB (*RB1*) and p53 (*TP53*) pathways (24–26). Interestingly, OISs by mutant RAS have been causally connected to induction of reactive oxygen species (ROS; ref. 27). Because the major source for ROS is OXPHOS, aberrant metabolism may be important in triggering OIS. To escape OIS, cells may choose to reroute their metabolism toward glycolytic metabolism to reduce ROS. A key regulator that drives this metabolic shift is hypoxia-induced factor-1 α (HIF-1 α), which in response to low oxygen gets stabilized and activates a program that reduces mitochondrial utilization through its function as a transcription factor. Specifically, HIF-1 α upregulates pyruvate dehydrogenase kinase 1

(PDK1; refs. 28, 29) that inactivates the pyruvate kinase complex, which is responsible for conversion of pyruvate to the main fuel for the TCA cycle: acetyl-CoA. Consistent with reducing OIS during hypoxic conditions (27), HIF-1 α acts as an oxygen sensor and drives cellular metabolism toward anabolic pathways, which is in line with Warburg's original observation (14). Hence, adaptation to oncogenic mutations along the RAS–RAF–MEK pathway is causally linked to the metabolic shift observed in cancer cells.

Oncogenic BRAF(V600E) and senescence-like benign nevi

Seemingly a paradox at first, benign nevi were found to harbor BRAF(V600E) mutations in a frequency similar to that of malignant melanomas (30), offering some doubts about its function as a cancer driver. However, subsequent functional studies using various models substantiated this oncogene as a melanoma formation driver and verified it as a key therapeutic target (31–35). Additionally, benign nevi have been demonstrated to exhibit hallmarks of OIS due to BRAF(V600E) activation (36), highlighting that fail-safe barriers to tumorigenesis could be visualized *in vivo*. Because oncogenic BRAF is not sufficient to induce tumorigenesis in the absence of additional genetic alterations, the vast majority of nevi represent an end-stage of local melanocyte proliferation. Hence, cooperating mutations such as PTEN loss-of-function mutations, which lead to constitutive activation of the phosphoinositide 3-kinase (PI3K) v-akt murine thymoma viral oncogene (AKT)–mTOR pathway, are found in a substantial fraction of melanomas coexisting with mutant BRAF (37). Activation of the PI3K–mTOR pathway promotes glycolysis by inducing the activity of HIF-1 α (38), and therefore also indicates a consistent "Warburg effect" to allow for continued proliferation through bypassing OIS (39).

Furthermore, recent data have revealed that BRAF (V600E)-induced senescence is accompanied by increased pyruvate entry into the TCA cycle via increased pyruvate dehydrogenase (PDH) complex activity (40). This effect was correlated to suppression of the PDH-inhibitory enzyme PDK1 and induction of the PDH-activating enzyme pyruvate dehydrogenase phosphatase 2 (PDP2). Reversal of PDH activation enabled bypass of OIS and tumorigenic growth. Whether HIF-1 α activation would enable bypass of OIS in this context was not analyzed, but because PDK1 is a *bona fide* downstream target gene (28, 29), it seems highly plausible and would favor Warburg's original observation. Nonetheless, these data highlight that BRAF(V600E) as an oncogene provokes OIS that requires coordination of metabolic reprogramming to drive tumor initiation.

The oncogene MITF controls melanocyte development and melanoma metabolism

It is of critical importance to determine whether the effects of oncogene-targeted therapies intersect with the inherent cellular wiring of cancer cells to provide feedback control that limits or affords therapeutic effects. Given that melanoma cells are inherently sensitive to therapeutic

inhibition of BRAF(V600E)-targeted agents, whereas papillary thyroid, non-small cell lung and colorectal carcinomas are not, a plausible explanation is that the metabolic state is dramatically different between these tumor cell types.

Among mutations that cosegregate and cooperate with BRAF(V600E) in melanoma is amplification of the melanocyte master regulator microphthalmia-associated transcription factor (MITF; ref. 35). MITF is required for melanocyte development (41, 42) and regulates differentiation-associated programs of pigment synthesis and transport, as well as survival cues via the BCL-2 family of antiapoptotic proteins (43, 44). As an oncogene in melanoma, MITF is target of frequent gene amplification (35) and point mutation within sporadic and germ-line predisposition (45, 46), which consistently lead to its elevated transcriptional activity. Surprisingly, MITF expression is often reduced in melanomas as compared with normal melanocytes, suggesting that it may have both pro- and anticancer effects depending on the context. MITF is regulated by multiple signaling pathways, including the metabolic regulator HIF-1 α . Specifically, HIF-1 α suppresses expression of *MITF* in melanoma cells through its transcriptional effects on the repressor *DEC1* (47, 48).

Contrasting the effects of HIF-1 α to promote anabolic metabolism, MITF has recently been linked to promote catabolic metabolism by directly regulating the mitochondrial biogenesis coactivators PGC1 α and PGC1 β (49–51). Specifically, MITF's transcriptional regulation of PGC1 α promotes mitochondrial respiration and resistance to oxidative stress in a defined subset of melanoma tumors with high MITF and PGC1 α expression (49, 50). Furthermore, BRAF and MEK inhibitors enhance *MITF* expression, leading to elevation of PGC1 α levels and an increase in OXPHOS activity (50). Hence, through simultaneous control of metabolism and cell survival cues, MITF poses to balance the effects of BRAF- and MEK-targeted therapies as a genuine melanoma oncogene and lineage master regulator (35, 45, 46).

Control of metabolism by cellular sensing mechanisms

The general metabolic sensing pathway in cells involves the tumor suppressor liver kinase B1 (LKB1; STK11) acting on 5' AMP-activated protein kinase (AMPK) to respond to alterations in AMP/ATP levels in cells (52). Because activation of AMPK by LKB1 suppresses growth through inactivation of signaling onto mTOR when nutrients are scarce and ATP levels remain constant in cells, the activity is essentially affected by AMP levels alone. Interestingly, growth factors through their receptor tyrosine kinases and BRAF(V600E) have been shown to alter LKB1–AMPK signaling, leading to reduced AMP sensing in melanoma cells (53, 54). Specifically, downstream activation of MAPK and RSK has been shown to directly phosphorylate LKB1, leading to compromised ability to activate AMPK and thus decouples metabolic sensing (53), which have been shown to exert important effects on melanoma growth. In addition, during conditions when the RAS–RAF–MEK pathway

is not constitutively activated or mutated, metabolic sensing through AMPK may lead to specific suppression of BRAF activity (55). Hence, constitutive RAS–RAF–MEK signaling decouples energy sensing and may confer a sensitivity that offers mechanistic clues about how cancer cells become sensitive to metabolic inhibition. Furthermore, mouse embryonic fibroblasts from LKB1 null mice are resistant to transformation by oncogenic RAS (56), suggesting that LKB1 tumor suppressor function may in fact be required to drive the metabolic shift, which further places emphasis on the intersection between energy sensing and cancer development.

Clinical–Translational Advances

The growing recognition of altered metabolism as a hallmark of cancer has kindled interest in approaches that target metabolic pathways in combination with current available anticancer treatment modalities (23). Chemical screens from over 20 years ago pointed to approaches to modulate OXPHOS, and given the recognition of this pathway in cancer, it will be worthwhile to reevaluate these data in light of the current era of genomics and targeted therapies. In particular, recent work has revealed a number of metabolic intervention opportunities with regard to forestall resistance to targeted therapy against mutant BRAF.

Targeting the metabolic shift that prevents OIS

BRAF mutations are detected in more than half of all melanomas, and NRAS is mutant in another 15% to 25%, underscoring the NRAS–BRAF axis as causally linked to melanocytes neoplasia. As outlined above, the source of the metabolic shift seen in human cancer cells is believed to be caused by OIS. With particular interest on pRB's known effects on cellular metabolism (57) and governing OIS (24, 25, 26), reactivation of its function using a CDK4/6 inhibitor in melanoma cells has been shown to cause senescence (58). A current active area of exploration is to determine whether CDK4/6 inhibitors can synergize with BRAF- and MEK-targeted agents to increase therapeutic efficacy (59).

Deregulation of HIF-1 α contributes to the Warburg effect, and, consequently, therapeutic intervention of its activity and deregulated pathways that converge on its function is under intense scrutiny for cancer therapy (60). This pursuit has recently been accelerated as mutations in key metabolic enzymes in the TCA cycle are believed to deregulate important metabolites, such as succinate, which is expected to increase the stability of HIF-1 α due to interfering with its oxygen-dependent degradation.

In addition, as OIS induced by BRAF(V600E) was recently shown to be dependent on increased pyruvate oxidation suggests that targeting PDK may be one possible approach (40). The preclinical data suggest that the generic inhibitor of PDK, dichroacetate, may in fact synergize with BRAF and MEK inhibitors to enhance the efficacy of inhibiting melanoma growth, even to resolve targeted drug resistance (40).

Metabolic intervention strategies with regard to modulation of OXPHOS

The recognition that melanoma cells may adapt to BRAF and MEK inhibitors by driving oxidative metabolism through MITF-*PGC1 α* suggests that targeting this pathway may be an alternative approach (50). Specifically, mitochondrial uncoupling using poisons of the electron transport chain complexes, such as cyanide, do not have sufficient therapeutic index. The mitochondrial uncoupler, 2,4-dinitrophenol (2,4-DNP), which leads to dissemination of the proton gradient and generation of heat, has been used extensively in diet pills but was discontinued due to dangerous side effects such as fatal hyperthermia. Although alternative mitochondrial uncouplers may be useful, they will likely lead to significant toxicities that possibly preclude their use for anticancer treatment regimens.

Well-tolerated inhibitors of OXPHOS may have greater therapeutic potential for cancer treatment, in particular the biguanides metformin and phenformin used in treatment of type II diabetes. These drugs are believed to act by inhibiting complex I of the electron transport chain (61). Although biguanides have demonstrated anticancer activity both *in vitro* and *in vivo* (62), the efficacy of metformin may be limited by the expression of organic cation transporters required for uptake and OXPHOS inhibition (63). Notwithstanding, LKB1 mutant non-small cell lung cancer cells as well as an *in vivo* cancer model demonstrate robust tumor growth inhibition by phenformin (64). Given that constitutive RTK-RAS-BRAF imparts decreased LKB1 function in melanoma cells, phenformin was recently shown to enhance the effects of BRAF inhibitors both *in vitro* and in a mouse melanoma model (65). Hence, inhibition of OXPHOS using well-tolerated biguanides, such as metformin and phenformin, may offer therapeutic effects in combination with BRAF and MEK inhibitors.

In addition, the repurposing of existing drugs (approved by the U.S. Food and Drug Administration) that target OXPHOS (66) could offer additional approaches. Of particular interest is the use of antibiotics such as antiparasitic

drugs such as atovaquone that inhibits cytochrome oxidase complex III of the electron transport chain (67), or the antimicrobials tigecycline that inhibits mitochondrial protein translation with shown antileukemic activity *in vitro* and *in vivo* cancer models (16).

Summary

Recent work has underscored that BRAF(V600E) attenuates OXPHOS consistent with Warburg's initial hypothesis of the metabolic shift of cancer cells. However, new mechanistic insights have revealed that the metabolic shift is caused by evasion of oncogene-induced senescence with important parallels to response to BRAF- and MEK-targeted therapy in melanoma cells. In addition, modulating metabolic dependencies may have combinatorial efficacy. It is the current hope that targeting these metabolic pathways may prevent the onset of drug resistance, lead to durable melanoma treatment responses, and extend the use of current BRAF-targeted agents to other human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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