Response of BRAF-Mutant Melanoma to BRAF Inhibition Is Mediated by a Network of Transcriptional Regulators of Glycolysis

Tiffany J. Parmenter1, Margarete Kleinschmidt1,2,8, Kathryn M. Kinross1,2,8, Simon T. Bond11, Jason Li2,3, Mohan R. Kaadige1, Aparna Rao1, Karen E. Sheppard1,2,3, Willy Hugo17, Giulietta M. Pupo13, Richard B. Pearson4,8,10,12, Sean L. McGee11, Georgina V. Long13,14,15, Richard A. Scolyer13,14,15, Helen Rizos13, Roger S. Lo17, Carleen Cullinane2,8, Donald E. Ayer16, Antoni Ribas17, Ricky W. Johnstone5,8, Rodney J. Hicks2,6,7,10,12, and Grant A. McArthur1,2,6,7,8,10,12

ABSTRACT
Deregulated glucose metabolism fulfills the energetic and biosynthetic requirements for tumor growth driven by oncogenes. Because inhibition of oncogenic BRAF causes profound reductions in glucose uptake and a strong clinical benefit in BRAF-mutant melanoma, we examined the role of energy metabolism in responses to BRAF inhibition. We observed pronounced and consistent decreases in glycolytic activity in BRAF-mutant melanoma cells. Moreover, we identified a network of BRAF-regulated transcription factors that control glycolysis in melanoma cells. Remarkably, this network of transcription factors, including hypoxia-inducible factor-1α (HIF-1α), MYC, and MONDOA (MLXIP), drives glycolysis downstream of BRAFV600E, is critical for responses to BRAF inhibition, and is modulated by BRAF inhibition in clinical melanoma specimens. Furthermore, we show that concurrent inhibition of BRAF and glycolysis induces cell death in BRAF inhibitor (BRAFi)-resistant melanoma cells. Thus, we provide a proof-of-principle for treatment of melanoma with combinations of BRAFis and glycolysis inhibitors.

SIGNIFICANCE: BRAFis suppress glycolysis and provide strong clinical benefit in BRAFV600E melanoma. We show that BRAF inhibition suppresses glycolysis via a network of transcription factors that are critical for complete BRAFi responses. Furthermore, we provide evidence for the clinical potential of therapies that combine BRAFis with glycolysis inhibitors. Cancer Discov; 4(4); 423–33. © 2014 AACR.

See related commentary by Haq, p. 390.

Authors' Affiliations: 1Molecular Oncology Laboratory, Oncogenic Signaling and Growth Control Program, 2Translational Research Laboratory, Cancer Therapeutics Program, 3Bioinformatics Core Facility, 4The Cancer Signalling Laboratory, Oncogenic Signaling and Growth Control Program, 5Gene Regulation Laboratory, Cancer Therapeutics Program, 6Molecular Imaging and Targeted Therapeutics Laboratory, Cancer Therapeutics Program, 7Department of Cancer Imaging, Peter MacCallum Cancer Centre, East Melbourne, 8Sir Peter MacCallum Department of Oncology, Departments of 9Biochemistry and Molecular Biology, and 10Pathology, University of Melbourne, Parkville, 11Metabolic Remodelling Laboratory, Metabolic Research Unit, School of Medicine, Deakin University, Waurn Ponds, 12Department of Medicine, St Vincent’s Hospital, University of Melbourne, Fitzroy, Victoria, 13Westmead Institute for Cancer Research, University of Sydney, 14Millennium Institute, Westmead, 15Department of Tissue Pathology & Diagnostic Oncology, Royal Prince Alfred Hospital, 16Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah, and 17Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, California

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

Corresponding Authors: Grant A. McArthur, Division of Cancer Research, Peter MacCallum Cancer Centre, Locked Bag 1, Abbekook Street, East Melbourne, VIC 8006, Australia. Phone: 61-3-9656-1408; Fax: 61-3-9656-1408; E-mail: grant.mcarthur@petermac.org; and Rodney J. Hicks, rod.hicks@petermac.org

doi: 10.1158/2159-8290.CD-13-0440

©2014 American Association for Cancer Research.
**INTRODUCTION**

Increased glycolysis in tumor cells compared with normal tissues is observed in most cancers and supports the increased energetic and biosynthetic demands of tumor cells (1). Control of glycolysis by oncogenes and tumor suppressors, such as AKT, TP53, and MYC, is believed to contribute to their tumorigenic activities (2). Treatment of AKT-driven tumor cells or tumors with PI3K/AKT/MTOR inhibitors, such as PF04691502, BEZ235, and ridaforolimus, suppresses glucose uptake and tumor growth/cell survival (3–5). Although the role of glucose metabolism in oncogene-driven tumorigenesis has been well characterized, it remains unclear whether regulation of glucose metabolism by oncogenes is important for tumor responses to oncogene-targeted therapy.

The development of therapies targeting BRAF in melanoma is a clear example of successful targeting of an oncogene for the treatment of cancer. Activating BRAF mutations, particularly the V600 amino acid substitution, have been identified in approximately 50% of metastatic melanomas (6), and BRAF<sup>V600E</sup> melanomas rely on RAF–MEK–ERK signaling for growth and survival (7). BRAF<sup>V600E</sup> expression has been associated with increased glycolytic activity and cell surface glucose transporter 1 (GLUT1 or SLC2A1) expression in colorectal and thyroid cancer cells (8, 9), indicating that glucose metabolism could be important for BRAF-driven tumorigenesis. Recently, RAF–MEK–ERK pathway inhibitors, including the BRAF inhibitors (BRAFi) vemurafenib (RG7204; PLX4032) and dabrafenib (GSK2118436), have been validated for treatment of BRAF<sup>V600E</sup> melanoma, with striking response rates in excess of 50% in patients diagnosed with BRAF<sup>V600E</sup> metastatic melanoma (10–14). Importantly, BRAF<sup>V600E</sup> inhibition potently suppresses uptake of the radioactive glucose tracer 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose (FDG) in BRAF<sup>V600E</sup> human melanoma cells and xenografts (15, 16) and in patients with BRAF<sup>V600E</sup> melanoma (10, 17, 18), suggesting that inhibition of glycolysis by BRAFi pathway inhibition could be important for clinical responses to BRAFi.

Here, we show that BRAF inhibition potently suppressed glycolysis independently of cell-cycle progression and cell death via suppression of hexokinase II (HK2) and GLUT1/3 expression in melanoma cells and clinical BRAF<sup>V600E</sup> melanomas biopsies. We also found that glucose metabolism is restored upon development of BRAFi resistance, a major challenge in the clinical management of BRAF<sup>V600E</sup> melanoma, and that this is overcome by combination with a glycolysis inhibitor. We used microarray experiments to elucidate the mechanisms by which RAF–MEK–ERK signaling promotes glycolysis. This led us to identify and validate a novel network of transcriptional regulators of glycolysis, composed of hypoxia-inducible factor-1α (HIF1A), MYC, and MONDOA, that are altered by BRAFi treatment and development of BRAFi resistance in BRAF<sup>V600E</sup> melanoma cells and BRAF<sup>V600E</sup> melanoma biopsies.

**RESULTS**

To determine the effect of RAF–MEK–ERK signaling on glucose metabolism in melanoma cells, a panel of BRAF<sup>WT</sup> and BRAF<sup>V600E</sup> human melanoma cells were treated with the BRAFi vemurafenib. As expected, vemurafenib suppressed [<sup>14</sup>Cl]-2DOG uptake (a surrogate marker of glycolytic flux) in BRAF<sup>V600E</sup> but not BRAF<sup>WT</sup> melanoma cells (Fig. 1A). Furthermore, the degree to which vemurafenib inhibited glucose uptake correlated significantly with the degree of sensitivity to vemurafenib-mediated suppression of proliferation (r2 = 0.7355; P = 0.0002; Fig 1B). Furthermore, we show that the degree of glycolysis suppression significantly correlates with the degree of inhibition of the transcription of ERK (MAPK3/MAPK1) target genes described herein (Supplementary Fig. S1). This indicates that the degree of ERK pathway inhibition may correlate with the degree of glycolysis inhibition. Treatment with vemurafenib also suppressed lactate and ATP production in BRAF<sup>V600E</sup> but not BRAF<sup>WT</sup> cells (Fig. 1C and D), confirming that BRAFi suppresses glycolytic flux. Importantly, inhibition of glycolysis by BRAFi was not a consequence of altered cell-cycle progression or apoptosis induction (Supplementary Fig. S2A–S2D), indicating that BRAF<sup>V600E</sup> directly promotes glycolysis in human melanoma cells. We also examined glycolytic flux and oxidative phosphorylation (oxphos) determined by measurement of the extracellular acidification rates (ECAR) and oxygen consumption rates (OCR), respectively, in melanoma cells (Supplementary Fig. S3A–S3F). This demonstrated significant decreases in ECAR (Fig. 1E) and small decreases in OCR (Fig. 1F) in BRAF<sup>V600E</sup> cells.

To investigate the molecular mechanisms underlying BRAF<sup>V600E</sup>-driven glycolysis, we examined the effect of BRAFi on glycolytic enzymes (Supplementary Fig. S4). BRAFi increased pyruvate dehydrogenase (PDH) catalytic subunit E1α (PDHA1) phosphorylation at Ser293, which would correspond to decreased enzymatic activity and suppressed oxphos (Fig. 1G; ref. 19). Interestingly, suppression of HK2 protein expression and decreased membrane expression of GLUT1 and GLUT3 (the key GLUT isoforms expressed in human melanomas; ref. 20) was observed in BRAF<sup>V600E</sup> melanoma cells treated with vemurafenib (Fig. 1G and H). These changes were associated with significant reductions in mRNA expression of the genes encoding HK2, GLUT1, and GLUT3 (HK2, SLC2A1, and SLC2A3, respectively; Fig. 1I), indicating that BRAF<sup>V600E</sup>-mediated glycolysis regulation occurs at a transcriptional level.

To examine the effect of BRAF inhibition on markers of glycolysis in a clinical context, we analyzed HK2, SLC2A1, and SLC2A3 mRNA expression in melanoma biopsies from patients diagnosed with BRAF<sup>V600E</sup> melanoma obtained before treatment (Pre), early on treatment (EOT) with a BRAFi (days 3–22), and after disease progression (Prog; Supplementary Table S1). In most cases, expression of the HK2, SLC2A1, and SLC2A3 genes significantly decreased upon BRAFi treatment (P < 0.05) and was significantly restored upon development of drug resistance (Fig. 1J and Supplementary Fig. S5A–S5F). Biopsies from patients who experienced stable disease or a partial response to BRAFi [Response Evaluation Criteria in Solid Tumors (RECIST) criteria] demonstrated significantly greater reductions in tumor SLC2A1 mRNA levels compared with patients who experienced disease progression (Fig. 1K; P = 0.04). This agrees with the potent suppression of FDG uptake in BRAF<sup>V600E</sup> melanomas after BRAFi therapy (10, 17, 18). On the basis of these data, we hypothesized that melanoma cells require glycolysis for proliferation/survival.
**Figure 1.** BRAF<sup>V600E</sup> promotes glycolysis in melanoma cells via regulation of GLUT1, GLUT3, and HK2. A, [3H]-2DOG uptake in melanoma cells [expressed as percentage change; control vs. 3 μmol/L vemurafenib (Vem); 20 hours]. B, Pearson correlation between inhibition of [3H]-2DOG uptake [% of control] and IC<sub>50</sub> (μmol/L) vemurafenib (Vem) treatment. C, l-lactate production [% of control] and ATP production [% of control] for vemurafenib treatment. D, ECAR (% of control) and OCR (% of control) determined using a Seahorse XF24 Extracellular Flux Analyzer. E, effect of vemurafenib on protein expression in melanoma cells (percentage of control) determined using Western immunoblotting (control vs. 3 μmol/L vemurafenib; 20 hours) using malate dehydrogenase (MDH) as a loading control. F, membrane versus cytoplasmic GLUT1 and GLUT3 expression in melanoma cells (control vs. 3 μmol/L vemurafenib; 20 hours). Na<sup+</sup>K<sup+</sup>-ATPase was used as a membrane-specific loading control. G, gene expression of SLC2A1 (GLUT1), SLC2A3 (GLUT3), and HK2 (control vs. 3 μmol/L vemurafenib; 20 hours) was determined by quantitative real-time PCR (qRT-PCR). H, mRNA expression in melanoma biopsies. For all patients, RNA was extracted from fresh-frozen BRAF<sup>V600E</sup> melanoma biopsies obtained from patients pretreatment (Pre), early on dabrafenib (BRAFi) + trametinib (MEKi) treatment, or vemurafenib treatment (BRAFi; EOT) and, in some cases, after disease progression (Prog). Data are included only for patients who showed stable disease or a partial response (RECIST criteria) EOT. Changes in gene expression were determined using an Illumina BeadStation (patients 1–7). I, Affymetrix Human Gene 1.0 ST Arrays (patients 8, 9), or by RINseq for patient 9. For all patients, data are expressed as the mean average signal intensity across all biopsies for an individual patient at each time point. J, change in SLC2A1 gene expression between baseline and EOT in responders [partial response (PR) or stable disease (SD)] versus nonresponders [progressive disease (PD)] to BRAFi + MEKi treatment. K, fold-change in gene expression for single patient pretreatment, and lines represent individual patients. Data were analyzed using a Tukey multiple comparison post hoc test. Data points represent mean ± SEM (n = 3). *P < 0.05. Data were analyzed using a one-way ANOVA coupled with a Tukey multiple comparison post hoc test. Pearson correlation, P < 0.001. Data represent mean ± SEM (n = 3). *P < 0.05. Two-way ANOVA coupled with a Tukey post hoc test. G and H, images are representative of two independent experiments. J, data points represent mean fold-change values across all biopsies from a single patient pretreatment, and lines represent individual patients. Data were analyzed using a t test coupled with a Wilcoxon matched pairs signed-rank test and P < 0.05 denotes a statistically significant difference. K, lines represent mean fold-change in gene expression (EOT vs. Pre) and symbols represent individual patients. Data were analyzed using a t test coupled with a Mann–Whitney test, where P < 0.05 denotes a statistically significant difference.
Consistent with this hypothesis, inhibition of glycolysis via siRNA-mediated knockdown of HK2, GLUT1, or GLUT3 or glucose withdrawal suppressed the proliferation of human melanoma cell lines (Supplementary Fig. S6A–S6D).

Acquired resistance to BRAFi inhibition occurs clinically after a median of 5 to 8 months (14, 21), and several mechanisms of resistance have been identified, including activation of NRAS (22). On the basis of the clinical importance of BRAFi resistance and the observation that HK2, SLC2A1, and SLC2A3 mRNA expression is restored in some patient tumors upon disease progression (Fig. 1I), we investigated the role of glycolysis in BRAFi resistance. We rendered BRAFV600 melanoma cells resistant to vemurafenib by the expression of activated NRAS (NRASQ61K), a clinically validated mechanism of acquired BRAFi resistance that restores MEK–ERK signaling (Supplementary Fig. S7A; ref. 22). NRASQ61K restored not only cell proliferation (Fig. 2A) but also glucose uptake, glycolytic flux, HK2, and GLUT1/3 expression (Fig. 2B–E) in vemurafenib-treated BRAFV600 melanoma cells. To determine whether the dependence of melanoma cells on glucose metabolism could be exploited to overcome BRAFi resistance, we treated BRAFV600 melanoma cells expressing NRASQ61K with vemurafenib alone or in combination with the pyruvate mimetic dichloroacetate (DCA). DCA inhibits PDH kinase (PDK) isoforms that cause downstream reactivation of the catalytic subunit of PDH (PDHE1α), thereby suppressing glycolytic metabolism (19).

This combination was assessed using engineered NRASQ61K-expressing cell lines, M249-AR4 cells that developed an NRAS mutation during long-term selection in vemurafenib and an early-passage cell line (M376) derived from a clinical melanoma specimen with acquired vemurafenib resistance that developed an NRAS mutation (22). Inhibition of PDK using a concentration of DCA that almost completely suppresses PDHE1α phosphorylation produced 21.8% cell death in A375 BRAFV600 melanoma cells (Supplementary Fig. S7B and S7C). However, combination treatment of vemurafenib + DCA induced apoptosis to a greater degree than with either agent alone (Fig. 2F and Supplementary Fig. S7D), concomitant with greater inhibition of lactate/ATP production (Fig. 2G and Supplementary Fig. S7E and S7F). We also observed a significant, albeit less pronounced, enhancement of the effect of the MEK inhibitor PD0325901 (PD901) by DCA on vemurafenib-resistant melanoma cells, indicating that the extent of ERK inhibition is likely to be important for the enhancement produced by glycolysis inhibition (Supplementary Fig. S8).

Combination treatment of vemurafenib-resistant melanoma cells did not enhance the suppression of ERK or PDHE1α phosphorylation by vemurafenib or DCA alone, indicating that the interaction between these drugs does not result from enhancement of drug activity (Fig. 2H and Supplementary Fig. S7G). As expected, 1 or 20 hours of treatment with vemurafenib + DCA increased the basal OCR and decreased the basal ECAR of vemurafenib-resistant melanoma cells, respectively (Fig. 2I and J and Supplementary Fig. S9A–S9D), indicating that vemurafenib + DCA treatment causes reentry of pyruvate into the tricarboxylic acid (TCA) cycle and increases oxphos, resulting in suppressed glycolytic metabolism. Intriguingly, vemurafenib + DCA treatment for 1 hour increased uncoupled respiration in vemurafenib-resistant cells (Fig. 2I and Supplementary Fig. S9A–S9D), suggesting that oxphos has become dysfunctional in these cells. In support of this hypothesis, 20 hours of treatment with vemurafenib + DCA potently suppressed the basal OCR and ATP turnover of vemurafenib-resistant cells (Fig. 2J and Supplementary Fig. S9A–S9D). Furthermore, vemurafenib + DCA potently increased superoxide production and tetramethylrhodamineethylester (TMRE) staining (indicative of mitochondrial hyperpolarization) in vemurafenib-resistant cells (Fig. 2K and Supplementary Fig. S7H).

Initially, we examined the possible involvement of mTOR in glycolytic responses to BRAFi inhibition, as MTOR complex 1 (mTORC1) activity has been shown to be important for responses to BRAFi inhibition in melanoma (23) and may also be important for AKT-driven glycolysis. We found that after 2 hours of treatment with vemurafenib, ribosomal protein S6 phosphorylation was modestly suppressed, but 4EBP1 phosphorylation was unchanged. These observations could be explained by mTORC1-dependent regulation of S6 by ERK, although mTORC1-independent regulation of S6 by ERK has also been described (24). After 24 hours of treatment, stronger inhibition of S6 and 4EBP1 phosphorylation occurred (Supplementary Fig. S10). Because glucose uptake is maximally suppressed within 20 hours of vemurafenib treatment, and because significant inhibition of GLUT1 and GLUT3 mRNA expression occurs within 4 hours of vemurafenib treatment (Supplementary Fig. S10), it is unlikely that these late changes to mTORC1 activity contribute significantly to the regulation of glycolysis by vemurafenib. Previous work has also demonstrated that BRAFV600 regulates LKB1 (STK11)–AMPK (PRKA) pathway activity in melanoma cells (25). Because this pathway is known to regulate energy metabolism, we examined its involvement in BRAFV600-driven glycolysis. We did not observe consistent regulation of LKB1–AMPK signaling by vemurafenib in melanoma cells (Supplementary Fig. S10). Thus, BRAFV600-mediated regulation of glycolysis in melanoma cells occurs by an as-yet-unidentified mechanism. To investigate the mechanism by which BRAFV600 regulates glycolysis in melanoma, we conducted microarrays and used gene set enrichment analysis (GSEA) and candidate gene analysis to identify putative glycolysis-regulating BRAFi targets.

Of the gene sets that were significantly enriched in control versus vemurafenib-treated cells (Supplementary datasets S1 and S2), we identified 15 MYC-regulated and four hypoxia-regulated gene sets, as well as three glycolysis-related gene sets (Fig. 3A and Supplementary Table S2). Because c-Myc and HIF-1α (the key mediator of hypoxia-stimulated gene transcription) are established positive regulators of glucose metabolism (26, 27), we posited that these are likely to be important for regulation of glycolysis by BRAFV600. MONDOA, however, is a critical negative regulator of glucose uptake (28). Although regulation of energy metabolism and the regulation of MONDOA activity by oncogenic signaling pathways are well defined (28), the role of MONDOA in tumorigenesis has yet to be fully elucidated. Here, we describe significant increases in expression of thioredoxin-interacting protein (TXNIP) and arrestin domain-containing 4 (ARRDC4), two direct transcriptional targets of MONDOA (28), in response to BRAFi...
Role of Glycolysis in Responses to BRAF Inhibition

**RESEARCH BRIEF**

Resistant cells have been previously described and developed an NRAS Q61K mutation after long-term selection in human melanoma cells (control vs. 10 μmol/L NRAS Q61K human melanoma cells (percentage of control) determined using a Seahorse XF24 Extracellular Flux Analyzer. Immunoblotting. Na⁺K⁺-ATPase was used as a membrane-specific loading control. Effect of vemurafenib on protein expression in A375- and WM266-4-pBp versus NRAS Q61K melanoma cells (control vs. 10 μmol/L (A375) or 3 μmol/L (WM266-4) vemurafenib; 20 hours). Cytoplasmic and membrane extracts were sequentially prepared from drug-treated cells and equal protein was loaded for Western immunoblotting. Na⁺K⁺-ATPase was used as a membrane-specific loading control. Effect of vemurafenib on protein expression in A375- and WM266-4-pBp versus NRAS Q61K melanoma cells (control vs. 10 μmol/L (A375) or 3 μmol/L (WM266-4) vemurafenib; 20 hours) was determined by Western immunoblotting using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Cell survival (determined by Annexin-V/PI staining) in the presence of vemurafenib or vemurafenib + DCA in M249-AR4 melanoma cells (0–20 μmol/L vemurafenib ± 20 mmol/L DCA; 72 hours). M249-AR4 vemurafenib-resistant cells have been previously described and developed an NRAS Q61K mutation after long-term selection in 1 μmol/L vemurafenib. Effect of vemurafenib + DCA on lactate and ATP production in M249-AR4 melanoma cells (0 vs. 15 μmol/L vemurafenib ± 20 mmol/L DCA; 24 hours). Effect of vemurafenib + DCA on protein expression in M249-AR4 melanoma cells (0 vs. 15 μmol/L vemurafenib ± 20 mmol/L DCA; 24 hours). Basal OCR and uncoupled respiration (0 vs. 15 μmol/L vemurafenib ± 20 mmol/L DCA; 1 hour) and basal OCR, OCR, and ATP turnover (0 vs. 15 μmol/L vemurafenib ± 20 mmol/L DCA; 20 hours) were determined in M249-AR4 melanoma cells. Mitochondrial membrane potential (72 hours) and ROS production (48 hours) were determined by fluorescence-activated cell sorting (FACS) using TMRE and MitoSOX staining, respectively (0 vs. 15 μmol/L vemurafenib ± 20 mmol/L DCA). A–C, G, and I–K, data represent mean ± SEM (n = 3). *P < 0.05. B, t test correct for multiple comparisons using the Holm–Sidak method. C, two-way ANOVA coupled with a Tukey post hoc test. G and I–K, one-way ANOVA coupled with a Tukey multiple comparison post hoc test. D, E, and H, images are representative of two independent experiments.
Figure 3. BRAFV600E promotes HIF-1α and MYC expression and suppresses MONDOA expression in human melanoma cells. A, effect of vemurafenib (Vem) on expression of a glucose transport gene set and expression of the top-ranked MYC and hypoxia gene sets. A375 cells were treated with 0 vs. 3 μmol/L vemurafenib for 24 hours (n = 3) after which RNA was extracted and subjected to microarray analysis of gene expression. GSEA was performed on these data to determine significantly enriched gene sets in either control- or drug-treated cells. B, effect of BRAF inhibition on mRNA expression or the MONDOA targets, TXNIP and ARRD4, in A375 melanoma cells (gene expression data from microarray experiments; vehicle vs. 10 μmol/L vemurafenib; 24 hours). C, MONDOA binding to the TXNIP and ARRD4 promoters in BRAFV600E A375-pBp or A375-NRAS Q61K cells (0 vs. 3 μmol/L vemurafenib; 24 hours). D, expression of TXNIP, ARRD4, HIF1A, and MYC mRNA in melanoma cells (0 vs. 3 μmol/L vemurafenib; 20 hours). E, protein expression/phosphorylation in melanoma cells (vehicle vs. 3 μmol/L vemurafenib; 24 hours). F, mRNA expression in melanoma biopsies. For patients 1–7 (Δ), RNA was extracted from fresh-frozen melanoma biopsies from a single patient pretreatment and lines represent individual patients. Data were analyzed using a signed-rank test and P < 0.05 denotes a statistically significant difference. G, lines represent mean fold-change in gene expression (EOT vs. Pre) and symbols represent individual patients. Data were analyzed using a t test coupled with a Mann-Whitney test, where P < 0.05 denotes a statistically significant difference. IgG, immunoglobulin G; FDR, false discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Role of Glycolysis in Responses to BRAF Inhibition

inhibition (Fig. 3B), demonstrating that MondoA is negatively regulated by BRAF<sup>V600E</sup>. To confirm this, we performed chromatin immunoprecipitation assays to examine binding of MondoA to the TXNIP and ARRD4 promoters and observed that vemurafenib treatment stimulated binding of MondoA to both the TXNIP and ARRD4 promoters. This involved restored GLUT1/3 expression after siRNA-mediated MondoA knockdown, GLUT1 expression by hypoxia, and HK2/GLUT1 expression by MYC overexpression in the presence of vemurafenib in WM266.4 BRAF<sup>V600E</sup> melanoma cells.

**DISCUSSION**

Recent reports have shown a link between BRAF<sup>V600E</sup> and glycolysis in both *in vitro* and *in vivo* models of cancer and in a clinical setting (8, 9, 12, 17, 21). Importantly, BRAFi has been shown to suppress glucose uptake in melanoma cells and xenografts (13, 16) and in patient tumors (10, 15, 17, 18). Here, we show that vemurafenib suppresses glycolysis in BRAF<sup>V600E</sup> melanoma cells independently of cell-cycle progression or cell death. In some cases, small reductions in the rate of oxphos occur in response to vemurafenib; however, these changes are only very modest and do not occur in all vemurafenib-resistant cells. Conversely, inhibition of glucose uptake significantly correlated with vemurafenib sensitivity, indicating that the degree of ERK pathway output profoundly influences the magnitude of glucose uptake in melanoma cells. Expression of HK2 and GLUT1/3 was significantly and consistently decreased in BRAF<sup>V600E</sup> melanoma cells in response to BRAF inhibition, and this is likely to underlie vemurafenib-mediated suppression of glycolysis. Consistent with a role for glycolysis in cell survival, we describe dependence on glucose availability and expression of the glycolytic machinery for melanoma cell proliferation. Importantly, expression of *SLC2A1, SLC2A3*, or *HK2* mRNA was suppressed in melanoma biopsies from patients treated with the BRAFi dabrafenib or vemurafenib and, in some cases, was restored after disease progression. Thus, our data significantly expand on the current understanding of BRAF<sup>V600E</sup>-driven glucose metabolism and suggest a possible role for glycolysis in responses and resistance of melanoma to BRAF-targeted therapies.

On the basis of the restoration of *SLC2A1, SLC2A3*, or *HK2* mRNA expression in some patient biopsies, we examined glycolysis in vemurafenib-resistant melanoma cells. Resistance to vemurafenib develops clinically after a median of 5 to 8 months (14, 21) and poses a significant challenge for the clinical management of BRAF<sup>V600E</sup> melanoma. Vemurafenib-resistant melanoma cells exhibited restored MEK–ERK activation, cell proliferation, HK2 and GLUT1/3 expression, and glucose uptake. Therefore, we determined whether the dependency of melanoma cells on glycolysis could be exploited to overcome vemurafenib resistance. We used the PDK inhibitor DCA that causes downstream reactivation of PDHE1α, thereby increasing pyruvate entry into the mitochondrial citric acid cycle/oxphos and suppressing glycolysis (19). DCA restored vemurafenib sensitivity in melanoma cells that display BRAF resistance via NRAS activation. This agrees with a recent study demonstrating that short hairpin RNAs (shRNA) targeting PDK1 synergize with BRAF inhibition in transformed human melanocytes and melanoma cells to suppress cell...
The effect of vemurafenib (Vem) on [3H]-2DOG uptake in WM266.4 melanoma cells after siRNA-mediated gene knockdown (mock control vs. siRNA; 72 hours after transfection).

C, D, protein expression in WM266.4 melanoma cells after siRNA-mediated gene knockdown (mock control vs. siRNA; 72 hours after transfection).

Figure 4. MondoA, HIF-1α, and c-Myc regulate basal glucose uptake and cell proliferation in melanoma cells and participate in responses to BRAF inhibition. A, [3H]-2DOG uptake and B, cell proliferation in WM266.4 melanoma cells after siRNA-mediated gene knockdown (mock control vs. siRNA; 72 hours after transfection). C and D, protein expression in WM266.4 melanoma cells after siRNA-mediated gene knockdown (mock control vs. siRNA; 72 hours after transfection). 

E, effect of vemurafenib (Vem) on [3H]-2DOG uptake in WM266.4 melanoma cells after siRNA-mediated gene knockdown of mondoA (72 hours after transfection; 0 vs. 1 μmol/L vemurafenib; 8 hours). F, expression of inducible MYC (MycER; 0 vs. 3 μmol/L vemurafenib; 16 hours), or exposure to hypoxia (2% oxygen; 0 vs. 3 μmol/L vemurafenib; 16 hours). G, effect of vemurafenib on cell proliferation in WM266.4 melanoma cells ± siRNA-mediated gene knockdown of mondoA (72 hours after transfection; 0 vs. 1 μmol/L vemurafenib; 48 hours). H, vemurafenib sensitivity (proliferation IC50) after expression of inducible MYC (MycER) or exposure to hypoxia (2% oxygen; 0–10 μmol/L vemurafenib; 72 hours).

M, effect of vemurafenib on protein expression in WM266.4 melanoma cells after siRNA-mediated gene knockdown of mondoA (72 hours after transfection; 0 vs. 1 μmol/L vemurafenib; 8 hours), expression of inducible MYC (MycER; 0, 1, or 3 μmol/L vemurafenib; 16 hours), or exposure to hypoxia (2% oxygen; 0, 1, or 3 μmol/L vemurafenib; 16 hours).

A, B, E–G, data represent mean ± SEM [n = 3]. *P < 0.05. A, B, and G, data were analyzed using t tests. E and F, two-way ANOVA coupled with a Tukey post hoc test. C, D, H–M, images are representative of two independent experiments. NOR, normoxia; HYP, hypoxia.

Because generation of ROS and mitochondrial hyperpolarization can precede apoptotic cell death (30), we hypothesize that these factors underlie the synergistic induction of cell death by combined BRAF and PDK inhibition. A recent study investigated the possible use of DCA for treatment of glioblastoma and, despite some positive results, dose-dependent toxicities limited the application of this inhibitor. Further
Role of Glycolysis in Responses to BRAF Inhibition

To investigate the mechanism by which BRAFV600E regulates glycolysis, we conducted gene expression arrays to identify putative glycolysis-regulating BRAF targets. We identified a network of transcription factors, including MONDOA, HIF-1α, and MYC, which is tightly regulated by BRAFV600E. Expression of these transcription factors is altered by vemurafenib treatment in BRAFV600E melanoma cells and, importantly, in clinical melanoma specimens. Notably, the consistent modulation of TXNIP expression after BRAFi treatment and disease progression in melanoma biopsies indicates that MONDOA is a therapeutically important target of mutant BRAF that is likely to play an important role in the suppression of FDG uptake in the context of BRAF-mutant melanoma observed in patients.

We have also established the functional importance of this transcriptional network for BRAFV600E-driven glycolysis and melanoma cell proliferation. Stabilization of HIF-1α and upregulation of MYC expression has been demonstrated in a huge range of cancers, including melanomas, and regulation of HIF-1α and MYC expression by the RAF–MEK–ERK pathway has been previously described (31, 32). We found that expression of MYC and HIF-1α is required for maintenance of basal glucose uptake in melanoma cells. Conversely, although regulation of MONDOA by oncogenic signaling pathways has been established (28), the role of MONDOA in tumorigenesis is unclear. We show, for the first time, that MONDOA is regulated by BRAFV600E and suppresses basal glucose uptake in melanoma cells. Furthermore, inhibition of MYC and HIF-1α and activation of MONDOA suppression is critical for metabolic and proliferative responses to vemurafenib.

Recently, Kaplan and colleagues (29) demonstrated that PDH is critical for oncogene-induced senescence (OIS) induced by BRAFV600E in mouse melanocytes and that abrogation of PDH activity overcame BRAFV600E-induced OIS. This agrees with our observation that BRAF inhibition suppresses PDH activity in BRAF-mutant melanoma cells (evidence by increased PDH phosphorylation). These observations suggest that regulation of energy metabolism plays a pivotal role important for tumor development, cell survival, and BRAFi responses in the context of BRAF-mutant melanoma.

Our findings show striking reductions in glycolysis and small reductions in the rate of oxygen consumption as an early response to BRAFi (24-hour treatment). A recent study by Haq and colleagues (33), examining later time points (72-hour treatment) and adaptation to BRAFi, demonstrated increased mitochondrial biogenesis and expression of oxphos genes in BRAFi-treated melanoma cells that was associated with increased PGC1α expression. These data are consistent with a model of early treatment response in which potent inhibition of ERK–MAPK pathway activity suppresses glycolysis followed by longer-term adaptive changes, including increased oxphos in cells surviving BRAFi inhibition. Long-term BRAFi inhibition and stimulation of oxphos associated with increased mitochondrial activity might occur as a mechanism to overcome the suppression of glycolysis by BRAFi inhibition described herein. Consistent with this suggestion, Gopal and colleagues found that melanoma cell lines displaying de novo resistance to ERK–MAPK pathway inhibition have a high basal rate of oxphos and increased expression of oxphos genes compared with MEK inhibitor–sensitive cell lines, and that this is associated with high expression of PGC1α (Y. Gopal and M. Davies; personal communication). Taken together, these findings indicate that sensitivity to ERK–MAPK pathway inhibitors in the context of melanoma may be defined by a reliance on glycolysis for survival, and that stimulation of oxphos by ERK–MAPK pathway inhibition or high basal oxphos is associated with de novo and early adaptation and acquired resistance to ERK–MAPK pathway inhibition. Collectively, these data suggest that the metabolic background of a BRAF-mutant melanoma could be pivotal for responses to BRAFi inhibition.

In summary, we have demonstrated that mutant BRAF tightly regulates glycolysis independently of cell-cycle progression or cell death and shown that melanoma cells have a requirement for access to glucose and intact glycolytic machinery for their proliferation. The combination of vemurafenib with the glycolytic inhibitor DCA was shown to restore sensitivity to BRAFi inhibition in NRAS-activated vemurafenib-resistant melanoma cells, not only demonstrating the importance of glycolysis for melanoma cell survival but also providing a proof-of-principle for the combination of targeted therapeutics such as vemurafenib with glycolysis inhibitors to prevent the emergence of drug resistance. Finally, we have identified a network of glycolysis regulators that operate under the control of oncogenic BRAFV600E to modulate glucose uptake in melanoma cells and are altered in clinical melanoma biopsies early during BRAFi treatment and upon development of resistance to BRAFi. For the first time, our data show that inhibition of glycolysis via this network is critical for the suppression of proliferation and glucose uptake induced by inhibition of oncogenic BRAF.

METHODS

See Supplementary Data for a full description of Methods.

Materials and Cells

Vemurafenib and its analog PLX4720 were provided by Plexxikon Inc. Sodium DCA was purchased from Sigma-Aldrich. PD-0332991 (PD991) was provided by Pfizer Inc. HEK-293T, MALME-3M, COLO829, A375, SK-MEL-28, HT144, LOX-IMVI, SK-MEL-2, A2058, CHL1, and MeWo cells were purchased from the American Type Culture Collection and National Cancer Institute (Bethesda, MD). CO89 and D04-M1 cells were obtained from the Australasian Biospecimen Network-OncoCell Cell Line Bank at the Queensland Institute of Medical Research (QIMR). Individuality of the melanoma cell lines was confirmed on early-passage cells by PCR based short-tandem repeat (STR) analysis using six STR loci, and this analysis was routinely performed to confirm the identity of cell lines. M249, M249-AR4, and M376 cell lines were a gift from Dr. Antoni Ribas (Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA). All melanoma cell lines were maintained in RPMI-1640 containing 10% FBS, 2 mmol/L l-alanyl-l-glutamine, 1% penicillin/streptomycin, and 250 ng/mL amphotericin B in a 37°C humidified, 5% CO2 incubator. The BRAF and NRAS mutation status of all cell lines has been reported previously (34, 35) and is described in Supplementary Table S3. M249-AR4 and M376 cells were maintained as above with the addition of 1 μmol/L vemurafenib. HEK-293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10%...
FBS, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 250 ng/mL amphotericin B in a 37°C humidified, 5% CO2 incubator. Melanoma cell lines with a vemurafenib IC50 [determined by sulforhodamine B (SRB) assays; Supplementary Table S3] of greater than 1 μmol/L were considered to be vemurafenib-resistant. The M249 cell line is a BRAFV600E cell line, whereas the M249-AR4 cell line was derived from M249 cells by long-term culture in vemurafenib and was shown to have developed an NRAS mutation (22). The M376 cell line was derived from a patient tumor after relapse on vemurafenib therapy and was also shown to harbor an NRAS mutation (22).

**Analysis of Bioenergetics Using the Seahorse XF24 Extracellular Flux Analyzer**

All extracellular flux analyses were performed using the Seahorse XF24 Extracellular Flux Analyzer (Seahorse Biosoience). Seahorse XF24 V7 24-well plates were seeded at 5 × 10^4 cells per well and, 24 hours later, the cells were treated with either (i) vehicle, vemurafenib (3 μmol/L) for 20 hours, or (ii) vehicle, vemurafenib (15 μmol/L), DCA (20 mmol/L), vemurafenib + DCA in combination for 1 or 20 hours. ECAR and OCR were determined simultaneously (see Supplementary Methods for a full description).

**Microarray Experiments**

A375 BRAFV600E human melanoma cells were treated with 3 μmol/L vemurafenib or vehicle (0.1% dimethyl sulfoxide [DMSO]) for 24 hours, after which RNA was extracted (n = 3). Whole-transcript sense target preparation and labeling (using the GeneChip WT Terminal Labeling and Controls and Ambion WT Expression Kit), hybridization to Affymetrix GeneChip 1.0 ST human gene arrays, and array scanning were completed by The Ramaciotti Centre Microarray Service of the University of New South Wales (Sydney, NSW, Australia). Data analysis is described in the Supplementary Methods. Melanoma cell line microarray data have been deposited in The Gene Expression Omnibus of the National Center for Biotechnology Information (accession number GSE42872).

**Disclosure of Potential Conflicts of Interest**

K.E. Sheppard has received a commercial research grant from Pfizer. G.V. Long has received honoraria from the Speakers Bureau of Roche and is a consultant/advisory board member of Roche, GlaxoSmithKline, and Novartis. R.A. Scolyer is a consultant/advisory board member of Roche, GlaxoSmithKline, and Novartis. A. Ribas is a consultant/advisory board member of GlaxoSmithKline and Roche–Genentech. R.W. Johnstone has received a commercial research grant from Novartis and honoraria from the Speakers Bureau of the same. No potential conflicts of interest were disclosed by the other authors.

**Authors' Contributions**


Development of methodology: T.J. Parmenter, S.L. McGee, G.V. Long, R.J. Hicks, G.A. McArthur

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.J. Parmenter, K.M. Kinross, S.T. Bond, M.R. Kaadige, A. Rao, K.E. Sheppard, G.M. Pupo, S.L. McGee, G.V. Long, R.A. Scolyer, H. Rizos, R.S. Lo, C. Cullinane, A. Ribas


Writing, review, and/or revision of the manuscript: T.J. Parmenter, S.T. Bond, K.E. Sheppard, R.B. Pearson, S.L. McGee, G.V. Long, R.A. Scolyer, H. Rizos, R.S. Lo, C. Cullinane, D.E. Ayer, A. Ribas, R.W. Johnstone, R.J. Hicks, G.A. McArthur

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.J. Parmenter, M. Klein-schmidt, A. Rao, G.V. Long, D.E. Ayer, G.A. McArthur

Study supervision: R.W. Johnstone, R.J. Hicks, G.A. McArthur

**Acknowledgments**

The authors thank Stand Up To Cancer, Burroughs Wellcome Fund, Melanoma Research Alliance, American Skin Association, Harry J. Lloyd Charitable Trust, and The Seaver Institute. The authors also thank Kaylene Simpson and staff at the Victorian Centre for Functional Genomics (VCFG) for guidance in the design of siRNA-based experiments. The authors acknowledge Gideon Bollag and Plexxikon/Roche for providing vemurafenib (PLX4032) and PLX4720, and thank Megan Bywater, Andreas Möeller, Jaclyn Sceney, Gretchen Poortinga, Elaine Sanji, Jeannine Diesch, Kate Hannan, Kerry Ardley, Rachel Walker, Ross Hannan, and Ralph Rossi for technical assistance and conceptual input. The Ramaciotti Centre for Gene Function Analysis, UNSW, Sydney, Australia, assisted with microarrays. Support from the staff at the Melanoma Institute, Australia, is also gratefully acknowledged.

**Grant Support**

This work was supported by grants from the National Health and Medical Research Council of Australia (to R.J. Hicks, A. Rao, R.A. Scolyer, and G.A. McArthur), Cancer Council Victoria (to R.J. Hicks and G.A. McArthur), the Cancer Institute New South Wales (to R.A. Scolyer, H. Rizos, and G.V. Long), NCRI K22CA151638 and 1P01CA168585 (to R.S. Lo) and NIH R01055668 (to D.E. Ayer). G.A. McArthur was a recipient of the Sir Edward Dunlop Fellowship of the Cancer Council of Victoria. S.L. McGee is supported by a National Health and Medical Research Council (NHMRC) Career Development Fellowship (APP1030474).

Received July 29, 2013; revised January 20, 2014; accepted January 22, 2014; published OnlineFirst January 27, 2014.

**REFERENCES**


Role of Glycolysis in Responses to BRAF Inhibition


31. Mills CN, Joshi SS, Niles RM. Expression and function of hypoxia inducible factor-1 alpha in human melanoma under non-hypoxic conditions. Mol Cancer 2009;8:104.


Response of *BRAF*-Mutant Melanoma to BRAF Inhibition Is Mediated by a Network of Transcriptional Regulators of Glycolysis

Tiffany J. Parmenter, Margarete Kleinschmidt, Kathryn M. Kinross, et al.


**Updated version**  Access the most recent version of this article at: doi:10.1158/2159-8290.CD-13-0440

**Supplementary Material**  Access the most recent supplemental material at: http://cancerdiscovery.aacrjournals.org/content/suppl/2014/01/27/2159-8290.CD-13-0440.DC1

**Cited articles**  This article cites 35 articles, 12 of which you can access for free at: http://cancerdiscovery.aacrjournals.org/content/4/4/423.full.html#ref-list-1

**Citing articles**  This article has been cited by 13 HighWire-hosted articles. Access the articles at: /content/4/4/423.full.html#related-urls

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.