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⁺, Mohan R. Kaadige¹⁵, Aparna Rao¹, Karen E. Sheppard¹,²,³, Willy Hugo¹⁷, Giulietta M. Pupo¹³, Richard B. Pearson⁴,⁶,⁷,⁸, Sean L. McGee¹¹, Georgina V. Long¹³,¹⁴,¹⁵, Richard A. Scolyer¹³,¹⁴,¹⁵, Helen Rizos¹³, Roger S. Lo¹⁷, Carleen Cullinan²,¹⁸, Donald E. Ayer¹⁸, 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, MYC, and MONDOA (MLXIP), drives glycolysis downstream of BRAF⁶⁰⁰, 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 BRAF⁶⁰⁰ 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, ²Molecular Imaging and Targeted Therapeutics Laboratory, ³Gene Regulation Laboratory, Cancer Therapeutics Program, ⁴Molecular Imaging and Targeted Therapeutics Laboratory, Cancer Therapeutics Program, ⁵Department of Cancer Imaging, Peter MacCallum Cancer Centre, East Melbourne, ⁶Sir Peter MacCallum Department of Oncology, Departments of ⁷Biochemistry and Molecular Biology, and ⁸Pathology, University of Melbourne, Parkville, ⁹Metabolic Remodelling Laboratory, Metabolic Research Unit, School of Medicine, Deakin University, Warrnambool, ¹⁰Department of Medicine, St Vincent’s Hospital, University of Melbourne, Fitzroy, Victoria, ¹¹Westmead Institute for Cancer Research, University of Sydney at Westmead Millennium Institute, Westmead, ¹²Department of Tissue Pathology & Diagnostic Oncology, Royal Prince Alfred Hospital; ¹³Discipline of Pathology, Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia; ¹⁴Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah; and ¹⁵Jonsson 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, Alfred Street East, East Melbourne, VIC 8006, Australia. Phone: 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

Published OnlineFirst January 27, 2014; DOI: 10.1158/2159-8290.CD-13-0440

Downloaded from cancerdiscovery.aacrjournals.org on April 4, 2017. © 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 V600 melanomas rely on RAF–MEK–ERK signaling for growth and survival (7). BRAF V600 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 V600 melanoma, with striking response rates in excess of 50% in patients diagnosed with BRAF V600 metastatic melanoma (10–14). Importantly, BRAF V600 inhibition potently suppresses uptake of the radioactive glucose tracer 2[18F]fluoro-2-deoxy-D-glucose (FDG) in BRAF V600 human melanoma cells and xenografts (15, 16) and in patients with BRAF V600 melanoma (10, 17, 18), indicating that inhibition of glycolysis by BRAF pathway inhibition could be important for clinical responses to BRAFi.

Here, we show that BRAF inhibition potently suppresses 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 V600 melanoma biopsies. We also found that glucose metabolism is restored upon development of BRAFi resistance, a major challenge in the clinical management of BRAF V600 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 V600 melanoma cells and BRAF V600 melanoma biopsies.

RESULTS

To determine the effect of RAF–MEK–ERK signaling on glucose metabolism in melanoma cells, a panel of BRAF WT and BRAF V600 human melanoma cells were treated with the BRAFi vemurafenib. As expected, vemurafenib suppressed [1H]-2DOG uptake (a surrogate marker of glycolytic flux) in BRAF V600 but not BRAF WT 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 (\(r^2 = 0.7355; P = 0.0002\); 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 V600 but not BRAF WT 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 V600 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 V600 cells.

To investigate the molecular mechanisms underlying BRAF V600-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 V600 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 V600-mediated glycolysis regulation occurs at a transcriptional level.

To examine the effect of BRAFi 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 V600 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. SSA–SSF). 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 V600 melanomas after BRAFi therapy (10, 17, 18). On the basis of these data, we hypothesized that melanoma cells require glycolysis for proliferation/survival.

424 | CANCER DISCOVERY APRIL 2014 www.aacrjournals.org
Role of Glycolysis in Responses to BRAF Inhibition

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 by vemurafenib and proliferation IC<sub>50</sub>s for vemurafenib treatment. L-lactate production (C) and ATP production (D) were determined in vemurafenib-treated melanoma cells (expressed as percentage change; control vs. 3 μmol/L vemurafenib; 20 hours). ECAR (E) and OCR (F) in human melanoma cells (percentage of control) determined using a Seahorse XF24 Extracellular Flux Analyzer. G, effect of vemurafenib on protein expression in melanoma cells determined by Western immunoblotting (control vs. 3 μmol/L vemurafenib; 20 hours) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. H, 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. I, 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). J, 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 (MEK inhibitor), 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; †). Affymetrix Human Gene 1.0 ST Arrays (patient 8; ‡), or by RNAseq 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. K, 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. A, C, and D, data 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. E and F, data represent mean ± SEM (n = 5); * P < 0.05. Data were analyzed using a one-way ANOVA coupled with a Tukey multiple comparison post hoc test. B, Pearson correlation, P < 0.001. I, 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 data 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 interrogated 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 metabolism (26, 27), we posited that these are likely to be 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 BRAF 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 BRAF

1 hour increased uncoupled respiration in vemurafenib-resistant cells (Fig. 21 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. 2G 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 BRAF inhibition, as mTOR complex 1 (mTORC1) activity has been shown to be important for responses to BRAF 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 BRAF 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
Role of Glycolysis in Responses to BRAF Inhibition

**Figure 2.** NRAS-mediated resistance to vemurafenib (Vem) is associated with restored glycolysis and can be overcome by combination with a glycolysis inhibitor. A, cell proliferation in melanoma cells transduced with empty vector (pBp) or activated NRAS (NRAS<sup>Q61K</sup>) 0–10 µmol/L vemurafenib; 72 hours). B, [3H]-2DOG uptake in pBp versus NRAS<sup>Q61K</sup> BRAF V600E melanoma cells (control vs. 10 µmol/L vemurafenib; 20 hours). C, ECAI in pBp versus NRAS<sup>Q61K</sup> human melanoma cells (percentage of control) determined using a Seahorse XF24 Extracellular Flux Analyzer. D, membrane versus cytoplasmic GLUT1 and GLUT3 expression in A375- and WM266.4-pBp versus NRAS<sup>Q61K</sup> 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. E, effect of vemurafenib on protein expression in A375- and WM266.4-pBp versus NRAS<sup>Q61K</sup> 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. F, 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 mM/L DCA; 72 hours). M249-AR4 vemurafenib-resistant cells have been previously described and developed an NRAS<sup>Q61K</sup> mutation after long-term selection in 1 µmol/L vemurafenib. G, effect of vemurafenib + DCA on lactate and ATP production in M249-AR4 melanoma cells (0 vs. 15 µmol/L vemurafenib ± 20 mM/L DCA; 24 hours). H, effect of vemurafenib + DCA on protein expression in M249-AR4 melanoma cells (0 vs. 15 µmol/L vemurafenib ± 20 mM/L DCA; 24 hours). L, basal OCR and uncoupled respiration (0 vs. 15 µmol/L vemurafenib ± 20 mM/L DCA; 1 hour) and J, basal ECAR, OCR, and ATP turnover (0 vs. 15 µmol/L vemurafenib ± 20 mM/L DCA; 24 hours) were determined in M249-AR4 melanoma cells. K, 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 mM/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. **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.
changes in gene expression were determined using an Illumina BeadStation (patients 1–7; Δ), Affymetrix Human Gene 1.0 ST Arrays.

MonDoA binding to the TXNIP and ARRDC4 promoters in BRAF V600E A375-pBp or A375-NRAS Q61K cells (0 vs. 3 μmol/L, 24 hours).

Figure 3. BRAF V600E 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 of the MonDoA targets, TXNIP and ARRDC4, 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 ARRDC4 promoters in BRAF V600E A375-pBp or A375-NRAS Q61K cells (0 vs. 3 μmol/L vemurafenib; 24 hours). D, expression of TXNIP, ARRDC4, HIF-1α, and MYC mRNA in melanoma cells (0 vs. 3 μmol/L vemurafenib; 20 hours). E, protein expression/phosphorylation in melanoma cells (n = 3). 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 coupled with a Wilcoxon matched pairs 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; P < 0.05 denotes a statistically significant difference. Igs, immunoglobulin G; FDR, false discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
inhibition (Fig. 3B), demonstrating that MondoA is negatively regulated by BRAFV600E. 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 in vemurafenib-sensitive A375-pB (Fig. 3C). Importantly, NRASQ61K expression suppressed this effect, indicating tight regulation of MondoA promoter-binding activity by mutant BRAF.

We confirmed that vemurafenib treatment increased TXNIP expression and decreased MYC and HIF-1α expression at the mRNA and protein levels in BRAFV600 melanoma cell lines (Fig. 3D and E). Interestingly, BRAFi did not alter total MondoA protein expression (Fig. 3E), indicating that BRAFV600 regulates the association of MondoA with target gene promoters. To examine the mechanism of regulation of MYC and HIF-1α expression, we cotreated melanoma cells with vemurafenib and the proteasome inhibitor bortezomib. Bortezomib limited the effect of vemurafenib on HIF-1α protein expression; thus, BRAFV600 suppresses MYC and HIF1A transcription and HIF-1α degradation (Supplementary Fig. S10). Importantly, we confirmed altered expression of these transcription factors in clinical melanoma specimens after BRAFi treatment. Although overall changes in HIF-1α and MYC expression did not reach statistical significance, their expression was clearly decreased EOT and restored after progression in a subset of patient biopsies. In the cases of MYC and HIF1A, regional microenvironmental and hypoxic variability would significantly affect gene expression, making it difficult to gain an accurate representation of mRNA expression from a small biopsy. We also observed that TXNIP mRNA expression was consistently and significantly increased from baseline during treatment with a BRAFi (P = 0.002) and decreased as compared with on-treatment expression levels (P = 0.016) after disease progression (Fig. 3F and Supplementary Fig. S1A–S1F). Moreover, biopsies from patients who experienced stable disease or a partial response to BRAFi (RECIST criteria) demonstrated significantly greater increases in TXNIP mRNA levels compared with biopsies from patients who experienced disease progression (Fig. 3G; P = 0.02), indicating the potential importance of TXNIP for responses to BRAFi inhibition.

To examine regulation of glucose metabolism by MondoA, MYC, and HIF-1α directly, each transcriptional regulator was targeted with siRNAs. Knockdown of MondoA or HIF-1α phenocopied the inhibitory effect of BRAF knockdown on glucose uptake and cell proliferation, whereas MondoA knockdown significantly increased basal glucose uptake in melanoma cells (Fig. 4A–C). This confirms that MYC and HIF-1α promote glucose uptake, whereas MondoA suppresses basal glucose uptake in BRAFV600 melanoma cells. Inhibition of gene expression using siRNAs showed that MondoA suppresses basal GLUT1 and GLUT3 expression, HIF-1α promotes basal GLUT1 expression, and MYC promotes basal GLUT1 and HK2 expression while suppressing GLUT3 suppression (Fig. 4D), demonstrating that each of these transcription factors controls a different subset of glycolytic targets. To address this function of the BRAF-regulated transcriptional network in responses to vemurafenib, we functionally modulated network components and examined the impact on vemurafenib responses. Suppression of glycolysis and cell proliferation by vemurafenib was partially reversed by siRNA-mediated MondoA knockdown, activation of inducible c-Myc (MycER), or by exposure to hypoxia (that causes HIF-1α stabilization) in BRAFV600 melanoma cells (Fig. 4E–M). 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 BRAFV600 melanoma cells.

**DISCUSSION**

Recent reports have shown a link between BRAFV600 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 BRAFV600 melanoma cells independently of cell-cycle progression or cell death. In some cases, small reductions in the rate of ophos occur in response to vemurafenib; however, these changes are only very modest and do not occur in all vemurafenib cell lines. 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 BRAFV600 melanoma cells in response to BRAFi 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 BRAFV600-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 BRAFV600 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/oophos and suppressing glycolysis (19). DCA restored vemurafenib sensitivity in melanoma cells that display BRAFi resistance via NRAS activation. This agrees with a recent study demonstrating that short hairpin RNAs (shRNA) targeting PDK1 synergize with BRAFi inhibition in transformed human melanocytes and melanoma cells to suppress cell
survival (29). We build on these observations, demonstrating potent induction of reactive oxygen species (ROS) production and mitochondrial hyperpolarization after treatment with vemurafenib + a PDK inhibitor, indicating that mitochondrial dysfunction results from combination treatment. 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 tumorogenesis 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, Kaplon and colleagues (29) demonstrated that PDH is critical for oncogene-induced senescence (OSIS) induced by BRAFV600E in mouse melanocytes and that abrogation of PDH activity overcame BRAFV600E–induced OSIS. This agrees with our observation that BRAF inhibition suppresses PDH activity in BRAF-mutant melanoma cells (evidenced 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 BRAF 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 BRAF 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-032991 (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-Onology 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. Antomi 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 humified, 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.
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 Bioscience). Seahorse XF24 V7 24-well plates were seeded at 5 × 104 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 Kits), 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**

**Conception and design:** T.J. Parmenter, R.B. Pearson, D.E. Ayer, R.W. Johnstone, R.J. Hicks, G.A. McArthur

**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 Möller, Jaclyn Scewney, Gretchen Poortenga, Elaine Sanjir, 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), NC1 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


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.