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Oncogenic BRAF regulates oxidative metabolism via PGC1α and MITF

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Summary

Activating mutations in *BRAF* are the most common genetic alterations in melanoma. Inhibition of BRAF by small molecule inhibitors leads to cell cycle arrest and apoptosis. We show here that BRAF inhibition also induces an oxidative phosphorylation gene program, mitochondrial biogenesis, and the increased expression of the mitochondrial master regulator, PGC1α. We further show that a target of BRAF, the melanocyte lineage factor MITF, directly regulates the expression of PGC1α. Melanomas with activation of the BRAF/MAPK pathway have suppressed levels of *MITF* and *PGC1α,* and decreased oxidative metabolism. Conversely, treatment of BRAF mutated melanomas with BRAF inhibitors renders them addicted to oxidative phosphorylation. Our data thus identify an adaptive metabolic program that limits the efficacy of BRAF inhibitors.

Keywords

BRAF; melanoma; vemurafenib; metabolism

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Introduction

Activating mutations in the BRAF protein kinase are the most common genetic alterations in melanoma, found in ~50% of tumors (Davies et al., 2002; Curtin et al., 2005). The most frequent BRAF mutation is the substitution of valine at position 600 by glutamic acid (BRAF V600E) that results in the constitutive activation of its serine/threonine kinase activity and sustained activation of MAP kinase signal transduction pathway (Davies et al., 2002; Wan et al., 2004). BRAF directly phosphorylates the dual-specificity kinases MEK1 and MEK2, which in turn phosphorylate and activate the mitogen-activated protein kinases, ERK1 and ERK2. BRAF has been shown by overexpression and knockdown experiments to be a critical mediator of melanomagenesis. In mice, activation of BRAF in combination with deletion of the tumor suppressor genes *PTEN* or *INK4A* leads to melanoma with complete penetrance (Dankort et al., 2009; Dhomen et al., 2009). Conversely, treatment of BRAF mutant melanomas *in vitro* with chemical inhibitors of BRAF or MEK1/2 promotes cell cycle arrest and apoptosis (Hingorani et al., 2003; Karasarides et al., 2004; Hoeflich, 2006; Wellbrock et al., 2008). Moreover, the BRAF inhibitor vemurafenib (PLX4032) leads to tumor regression and improved overall survival in patients whose melanomas have the BRAF(V600E) mutation, leading to its approval as a treatment for patients with metastatic melanoma (Flaherty et al., 2010; Chapman et al., 2011; Sosman et al., 2012). Despite the promise and dramatic initial effects of BRAF inhibitors in the clinic, patients eventually relapse within several months, suggesting that combination therapies may be needed to overcome intrinsic or acquired resistance (Gray-Schopfer et al., 2007; Poulikakos and Rosen, 2011).

Although melanomas with BRAF mutations have constitutively active growth signals, how they sustain their growth in the setting of nutrient scarcity is not well understood. In 1930, Otto Warburg proposed that cancer cells have a high rate of glycolysis as compared to oxidative metabolism even under conditions of high oxygen, a phenomenon known as the Warburg effect (Warburg, 1956; Vander Heiden et al., 2009). Oxidative phosphorylation depends on the ability of functionally intact mitochondria to metabolize oxygen, whereas glycolysis can occur independently of mitochondria. Warburg theorized that this metabolic switch facilitated the uptake and incorporation of nutrients that were required for cellular proliferation. Although poorly understood in melanoma, the molecular mechanisms of metabolic reprogramming in cancer have been described in other tumor types. *TP53*-deficient tumor cells have diminished levels of the genes *TIGAR* and *SCO2* which regulate glycolysis and assembly of the mitochondrial cytochrome c oxidase complex respectively (Bensaad et al., 2006; Matoba, 2006). Similarly, the dysregulation of the proto-oncogene *MYC* leads to profound effects on tumor metabolism through multiple mechanisms (reviewed in Dang, 2012).

These observations have raised the possibility of targeting key metabolic pathways to inhibit cancer growth. Yun *et al.* demonstrated that several colorectal cancers with KRAS or BRAF mutations have increased glucose uptake and glycolysis and survived better in low glucose conditions compared to non-mutated cell lines (Yun et al., 2009). Suppression of glycolysis with 3-bromopyruvate (a non-active intermediary in the glycolysis pathway) reactivates mitochondrial metabolism in tumor cells, induces their selective killing and suppresses cancer growth. Similarly, suppression of glycolysis by inhibiting conversion of pyruvate to lactate enhances oxidative phosphorylation and suppressed the growth of breast cancer cell lines (Fantin et al., 2006; Bonnet et al., 2007). Thus, while metabolic reprogramming is commonly found in cancer, the mechanism and details of the metabolic alterations in melanoma are unknown.

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Mitochondrial biogenesis and oxidative phosphorylation are well known to be controlled by the members of the peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) family of transcriptional coactivators (PGC1α, PGC1β, PPRC1) (Lin et al., 2005a; Finck, 2006; Handschin, 2009; Leone and Kelly, 2011). The best-studied PGC-1 family member, PGC1α, potently activates coordinated gene expression programs by interacting with transcription factors, the basal transcriptional machinery, histone-modifying enzymes, and the RNA splicing machinery. PGC1α drives mitochondrial biogenesis in multiple contexts, including brown and white adiocytes (Wu et al., 1999; Uldry et al., 2006), skeletal muscle (Lin et al., 2002) and heart (Lehman et al., 2000). PGC1α mRNA expression is sensitive to numerous signaling inputs that are often implicated in cancer biology (Herzig et al., 2001; Yoon et al., 2001; Chinsomboon et al., 2009; Arany et al., 2008). PGC1β shares significant sequence homology and functional overlap with PGC1α, including the activation of mitochondrial biogenesis and oxidative phosphorylation, but also has several distinct functions in different tissues (Lin et al., 2005b; Uldry et al., 2006; Wolfrum and Stoffel, 2006). The regulation of PGC1β has been less extensively studied. Although little is known about the role of the PGCs in melanoma, they have recently been implicated in metabolic shifts in breast cancer cells and colon cancers (Eichner et al., 2010; Bhalla et al., 2011; Sahin et al., 2011; Wang and Moraes, 2011; Girnun, 2012; Klimcakova et al., 2012). Here we comprehensively evaluate the effect of BRAF pathway activation on metabolic gene expression and function in melanoma.

Results

BRAF regulates metabolic reprogramming of melanomas

The mechanisms by which oncogenic BRAF promotes oncogenesis are incompletely understood. Gene Set Enrichment Analysis (GSEA) provides a bioinformatics approach to identify gene signatures among microarray datasets that are induced or suppressed as small coordinated changes in individual genes (Mootha et al., 2003). In order to identify gene expression programs altered by oncogenic BRAF, we evaluated previously published gene expression profiles of BRAF mutant melanomas treated with the BRAF inhibitor vemurafenib (Joseph et al., 2010). As shown in Figure 1a, treatment of BRAF mutant melanomas with vemurafenib resulted in significant increases in the expression the citric acid cycle gene set (Figure 1a) as well as multiple oxidative phosphorylation and ATP synthesis gene sets (Table S1). Similarly, melanoma cells treated with PD0325901, a pre-clinical inhibitor of the MEK1/2 protein kinases (Pratilas et al., 2009; Joseph et al., 2010), also exhibited a trend towards induction of the citric acid cycle and oxidative phosphorylation gene sets (Table S2). In contrast, we did not find enrichment of oxidative phosphorylation, or citric acid cycle gene sets in BRAF-mutant non-melanomas treated with PD0325901 (Table S3). We validated the effects of vemurafenib on OXPHOS gene expression in three melanoma cell lines by quantitative PCR (Figure S1a,b,c).

To directly evaluate the effect of BRAF inhibition on oxidative phosphorylation and mitochondrial number and function, we treated melanoma cell lines with PLX4720, a preclinical analog of vemurafenib (Tsai et al., 2008). PLX4720 increased the mitochondrial density of two BRAF mutant melanomas as detected by MitoTracker Green, which localizes to mitochondria independent of membrane potential. PLX4720 did not affect the mitochondrial density of MeWo cells that are BRAF wild-type (Figure 1b). PLX4720 also induced MitoTracker Red fluorescence, a measure of mitochondrial activity and mass (Figure 1c), and increased the production of mitochondrial oxidative stress measured by the MitoSOX fluorescence assay (Figure 1d). To confirm these findings, we also evaluated mitochondrial number by electron microscopy after treatment of the BRAF mutant melanoma cell line UACC62 with PLX4720. As seen in Figure 1e and Figure S1d, inhibition of BRAF led to a significant increase in the number of mitochondria per cell.

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The conversion of glucose to lactate, as noted by Warburg, can be due to the shunting of pyruvate away from oxidative phosphorylation. In line with our findings above, PLX4720 reduced lactate levels in all BRAF mutant melanomas evaluated (Figure 1f). Lactate levels did not change upon treatment of a melanoma cell line that does not contain the BRAF mutation, consistent with the inability of PLX4720 to suppress ERK signaling in these cells (Figure 1g). Collectively our data suggest that BRAF suppresses oxidative phosphorylation gene expression and mitochondrial density in melanoma.

BRAF and MAPK activation suppresses PGC1α

Since oxidative phosphorylation depends on mitochondrial number and activity, their alteration may contribute to altered tumor metabolism. Candidate pathways, which physiologically regulate mitochondrial content and function include the transcription factors mitochondrial transcription factors A (TFAM) and B (TFB1M, *TFB2M*), nuclear respiratory factor 1 (NRF1), GA binding proteins (*GABPA, GABPB2),* peroxisome proliferator-activated receptors (PPARα, *PPARβ), PPAR-γ* coactivators (PGC1α, PGC1β), and PGC1-related coactivator 1 (PPRC1) (reviewed in Kelly, 2004).

We observed that BRAF(V600E) expression suppressed *PGC1α*, a well-known regulator of mitochondrial metabolism in the microarray datasets above. To validate these observations, we treated a series of BRAF-mutant melanomas and non-melanoma cell lines with PLX4720 and evaluated the effect on *PGC1α* mRNA (Figure 2a). In all melanomas with BRAF mutations, PLX4720 induced 3–14 fold increases in *PGC1α* mRNA. We did not observe any changes in the expression of *PGC1α* in a BRAF wild-type MeWo cell line treated with PLX4720. Surprisingly, we did not observe any effects of PLX4720 on *PGC1α* expression in two BRAF mutant colon cancer cell lines, despite suppression of ERK phosphorylation similar to that seen in melanomas (Figure 2b). We did not observe any change in *PGC1β* mRNA upon treatment with PLX4720 or any effects in a BRAF-wild-type melanoma over 24 hours (Figure S2a,b). These data suggested that there might be lineage-specific differences in the regulation of PGC1α by BRAF. To validate our findings using a structurally unrelated small molecule, we treated several melanoma cell lines with the MEK inhibitor PD0325901. Induction of *PGC1α* mRNA (Figure 2c) and suppression of ERK phosphorylation (Figure 2d) were seen in all cell lines tested including the BRAF wild-type melanoma MeWo, suggesting that the BRAF/MEK/ERK pathway regulates *PGC1α* expression in melanoma cells. These results were also confirmed with additional NRAS-mutant melanoma cell lines treated with a MEK1/2 inhibitor (Figure S2c,d). Finally, we evaluated the expression of PGC1α in an independent dataset of A375 melanoma cells selected for resistance to BRAF inhibitors (Greger et al., 2012). We observed that *PGC1α* expression was 10-fold lower in cells that had acquired resistance to BRAF inhibitors (Figure S2e), likely reflecting their higher demonstrated basal MAPK activity.

We also interrogated a publically available microarray of 12 breast, lung, colon and melanoma cell lines treated with PD0325901 (Joseph et al., 2010). Suppression of MEK only affected PGC1α mRNA in melanoma cell lines (Figure 2e, $p < 0.0001$), suggesting that the regulation of PGC1α mRNA by the BRAF/MEK/ERK pathway is unique to the melanocytic lineage. Consistent with our results, we found that PGC1α expression was significantly correlated with melanocyte-specific antigen expression in a dataset comprising of 105 melanoma cell cultures (Figure 2f).

PGC1α mRNA is directly regulated by the MITF transcription factor

To elucidate how BRAF may regulate *PGC1α*, we compared the expression pattern of all human transcription factors to PGC1α in a large microarray dataset of short-term melanoma cultures (Lin et al., 2008). Among the transcription factors whose expression most closely

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paralleled PGC1α, we observed that both transcription factor EB *(TFEB*) and the micropthalmia-associated transcription factor *(MITF*) were significantly associated with *PGC1α* expression (q < 0.001) (Figure 3a). Both TFEB and MITF are members of a four-member family of distinctly encoded transcription factors (TFEB, TFEC, TFE3 and MITF) that share a common structure, binding recognition sequence, and function (Haq and Fisher, 2011). Whereas TFEB, TFEC, and TFE3 are ubiquitously expressed, MITF is largely restricted to the melanocytic lineage. The correlation of *PGC1α* to *MITF* was therefore interesting in light of our data suggesting BRAF regulation of *PGC1α* in melanoma but not in other lineages (see Figures 2a, 2e). *MITF* expression correlated to *PGC1α* as shown above (see Figure 2f).

We therefore evaluated the requirement of TFE3, TFEB and MITF for the expression of *PGC1α* by siRNA. In both M14 melanoma cells and primary human melanocytes, suppression of MITF but not TFEB or TFE3 (Figure 3b,S3a) led to a significant suppression of *PGC1α* (Figure 3c, S3b) despite similar knockdown efficiency. We were unable to reliably detect *TFEC* expression in the cell lines tested (data not shown). MITF suppression in two other melanomas lines also significantly reduced *PGC1α* expression (Figure S3c), which was validated using two independent shRNAs (Figure S3d).

In sillico analysis of the *PGC1α* promoter identified three putative MITF recognition sequences ('E-boxes'), which were conserved among mammalian species. One E-box was located approximately 420 bp upstream of the transcriptional start site, whereas a proximal Ebox was located within 20 bp of the start site (Figure 3d). Another E-box was located approximately 10 kilobases upstream of these sequences, in the promoter sequences of an alternative exon 1 (PGC1α-alt) (Miura et al., 2008; Chinsomboon et al., 2009). To evaluate if MITF could directly bind to the *PGC1α* promoter, we performed chromatin immunoprecipitation using primers located either near alternative exon 1 or exon 1 (see Figure 3d). As shown in Figure 3e, MITF was found to bind to the proximal *PGC1α* promoter but not to the *PGC1α*-alt promoter in primary melanocytes. Due to limits in the resolution of this assay, we were unable to distinguish if MITF binds to E-box #1, E-box #2 or both. We therefore utilized a *PGC1α* promoter cloned upstream of the luciferase reporter gene (Handschin et al., 2003) and mutated each E-box by site-directed mutagenesis (Figure 3f). MITF overexpression (Figure 3f) or treatment with PLX4720 (Figure 3g) led to the induction of the wild-type promoter, whereas mutation of either of the two E-boxes significantly inhibited this response. Collectively, these data indicate that MITF binds and directly regulates the *PGC1α* gene in the melanocyte lineage. To evaluate if BRAF regulates PGC1α via MITF, we suppressed MITF by siRNA, then treated with PLX4720. As shown in Figure 3h, treatment with PLX4720 strongly induced *PGC1α* mRNA in M14 cells and ~3 fold in UACC62 cells and this induction was absent in cells in which MITF was knocked down by siRNA. These data indicate that BRAF regulates *PGC1α* via MITF.

BRAF negatively regulates MITF activity

The relationship between BRAF and MITF is poorly understood since oncogenic BRAF and the ERK pathway promotes MITF activation but also leads to its degradation (Hemesath et al., 1998; Wu et al., 2000; Wellbrock, 2005; Wellbrock et al., 2008; Boni et al., 2010). We therefore examined the consequences of ectopic BRAF(V600E) expression in immortalized melanocytes. Introduction of oncogenic BRAF was associated with decreased levels of *M-MITF* protein (the melanocyte-specific isoform) but not other isoforms (Figure 4a). Conversely, the MEK inhibitor PD0325901 induced the expression of *TRPM1* and other direct targets of MITF in published microarrays (Figure 4b) and by qtPCR (Figure 4c). These effects on MITF targets were dependent on MITF, since knockdown of MITF blocked induction of *TRPM1* (Figure 4d). In line with these findings, we observed that treatment of

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UACC-257 cells with PLX4720 for 72h lead to increased pigmentation, reflecting MITF's essential role in melanin synthesis and pigmentation (Figure 4e).

We were unable to detect a correlation between BRAF mutation status and *PGC1α* or *MITF* expression in short-term cultures. Given the prevalence of MAPK pathway mutations other than BRAF(V600E) in melanoma, we evaluated the expression of *PGC1α* and *MITF* in melanomas with either high or low expression of a MAPK activation gene signature. Both *MITF* (t-test, $p = 1.4 \times 10^{-14}$) and *PGC1α* (t-test, $p = 1.34 \times 10^{-5}$) expression inversely correlated with MAPK activity (Figure 4f). Collectively, these data suggest that BRAF/MAPK inhibition leads to activation of MITF mRNA and protein and that this induction leads to induction of MITF targets including *PGC1α*.

To evaluate if BRAF suppression alters the BRAF/MITF/PGC1α pathway described here *in vivo*, we obtained serial biopsies from 11 patients prior to treatment with BRAF/MEK inhibitors and 10–14 days after beginning treatment (patient characteristics described in Straussman et al., 2012). Of eight samples that had detectable phosphorylated ERK at baseline (Figure 5a), all had induction of PGC1α upon treatment (Figure 5b). Comparison to separately analyzed levels of *M-MITF* induction following BRAF targeted therapy revealed a significant correlation to PGC1α induction within these patient-derived specimens (Wargo J et al., manuscript in preparation). Together these data indicate that the OXPHOS adaptive response described here exists *in vivo*.

MITF promotes expression of oxidative phosphorylation genes

Up to 30% of melanomas harbor genomic amplifications of *MITF* (Garraway et al., 2005), and MITF is required for the survival of at least a subset of melanomas. Activating point mutations have also been identified in melanoma (Bertolotto et al., 2011; Yokoyama et al., 2011). These data have led to the designation of MITF as a lineage-specific melanoma oncogene (Garraway et al., 2005). Comprehensive expression profiling approaches have identified roles for MITF in promoting cell growth and survival, organelle biogenesis, oxidative stress response and miRNA regulation (Vachtenheim and Borovanský, 2010; Haq and Fisher, 2011). However a role for MITF in regulating metabolism has not been previously described. We therefore tested if *MITF* expression correlated with oxidative phosphorylation by classifying melanomas into two groups based on a previously defined oxidative phosphorylation signature. As seen in Figure 6a, melanomas with high *MITF* expression had significantly higher oxidative phosphorylation gene expression ($p = 5.51 \times 10^{-5}$). Similarly, *MITF* expression correlated with PGC1α -regulated gene expression ($p = 1.42 \times 10^{-15}$).

To evaluate if MITF was sufficient to drive oxidative phosphorylation in melanoma, we evaluated two matched human cell lines derived from primary melanocytes. These two cell lines (termed pmel*+BRAF(V600E) and pmel*+BRAF(V600E)+MITF) are derived from primary normal human melanocytes by immortalization using telomerase, with a constitutively active allele of cyclin-dependent kinase 4 and dominant negative p53 (Garraway et al., 2005). These cells are therefore isogenic with the exception of the expression of *MITF*. Consistent with published reports (Garraway et al., 2005) and our results above, expression of M-MITF protein was undetectable in pmel*+BRAF(V600E) cells, but strongly expressed in the derived MITFexpressing cells, which correlated with PGC1α expression (Figure 6b). BRAF(V600E) cells expressing MITF were able to form tumors in immunocompromised mice whereas control cells were not (Figure 6c), paralleling previous data from soft-agar growth (Garraway et al., 2005). Similar to our findings with BRAF inhibition, Gene Set Enrichment Analysis of microarray data identified a highly significant induction of oxidative phosphorylation gene set in MITF expressing cells compared to control cells ($q \simeq 0.0$, $p < 5 \times 10^{-4}$; see Figure 6c). We found a large majority

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of the oxidative phosphorylation gene set was induced by MITF overexpression (Figure S4a), consistent with the ability of PGC1α to strongly upregulate many oxidative phosphorylation genes (Mootha et al., 2003). We validated the expression of differentially expressed genes (chosen to represent different mitochondrial processes) by qtPCR (Figure S4b) and found significant increases in several oxidative phosphorylation genes. Similarly, expression of the oxidative phosphorylation genes were suppressed by oncogenic BRAF (Figure S4c).

To evaluate the metabolomic consequence of MITF overexpression directly, we evaluated glycolysis and oxidative phosphorylation in the isogenic cells. Overexpression of MITF did not increase glucose uptake (Figure 6d), but strikingly had decreased lactate production (Figure 6e) and oxygen consumption (Figure 6f), consistent with increased mitochondrial metabolism. Conversely, BRAF(V600E) cells had elevated sensitivity to knockdown of pyruvate kinase (muscle isoform), the final step in the glycolysis pathway, compared to isogenic cells expressing MITF (Figure S4d), despite similar degrees of knockdown efficiency in the two cell lines (Figure S4e).

To further validate these findings in patient-derived melanomas, we suppressed MITF by shRNA and performed gene expression profiling. We identified several genes involved in oxidative phosphorylation that were dependent on MITF (Figure S4f), consistent with the above gene expression data. With the exception of PGC1α, which was found to be directly bound by MITF, oxidative phosphorylation targets were not bound by MITF but have been identified as PGC1α targets in other cell lineages (Mootha et al., 2003). We conclude that MITF overexpression is sufficient and necessary to drive oxidative metabolism gene signature and metabolic reprogramming in the melanocyte lineage.

BRAF inhibition leads to bioenergic adaptation by induction of MITF and PGC1α

Collectively, the data above support the notion that BRAF inhibition endangers ATP production, which is rescued by concomitant induction of MITF, PGC1α and oxidative metabolism. To experimentally test this hypothesis, we evaluated the response of the isogenic cell lines expressing BRAF(V600E) with or without M-MITF overexpression to PLX4032. Inhibition of BRAF resulted in 42% drop in ATP in BRAF(V600E) parental cells whereas the magnitude of this drop was reduced in MITF overexpressing cells (Figure 6g), despite similar basal ATP concentrations. Consistent with MITF effects on oxidative metabolism, BRAF(V600E)+MITF cells were significantly more sensitive to the mitochondrial uncoupler 2,4-dinitrophenol (DNP; Figure 7a).

We also evaluated the magnitude of bioenergetic compensation in 8 patient-derived melanoma cell lines. As shown in Figure 7b, in all cases there was induction of PGC1α following vemurafenib treatment, which varied in magnitude by cell line. We also measured ATP levels before and after treatment with vemurafenib, and we observed a significant correlation between induction of PGC1α and ATP levels in BRAF mutant cells ($R = 0.72$, $p = 0.03$; Figure 7c). Strikingly, the two lines with highest ATP levels following BRAF inhibition are the ones with the strongest induction of PGC1α. These data suggest that the metabolic switch to “normal” varies in magnitude among different melanoma cell lines and suggest that the inhibition of BRAF leads to a bioenergetic crisis that can be variably rescued by induction of the MITF/PGC1 α pathway.

We next asked whether oxidative metabolism could be exploited to inhibit the growth of melanoma cells. We compared the sensitivity of melanoma cells to primary melanocytes (Figure S5). All melanoma cells were more sensitive to DNP than primary melanocytes, except A375P. Treatment of UACC257 cells, but not A375P cells with DNP led to a decrease in ATP and increases in lactate, consistent with inhibition of OXPHOS (Figure

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7d,e). To validate the effects of mitochondrial inhibitors on tumor growth *in vivo*, we treated animals bearing tumor xenografts of A375P and UACC257 melanoma cells with DNP. Consistent with the *in vitro* data, we found that longitudinal treatment of UACC257 xenografts profoundly inhibited tumor growth, similar to the effects of PLX4032 (Figure 7f), whereas A375P cells were insensitive to 2,4-DNP (Figure 7g).

Melanomas treated with BRAF inhibitors are addicted to oxidative metabolism

Our observations that BRAF and MITF regulate *PGC1α* expression prompted us to evaluate if oxidative metabolism affected response to BRAF inhibitors. We observed that high levels of PGC1α were associated with poorer prognosis of patients with Stage III melanoma (Figure S6a) (Bogunovic et al., 2009). To this end, forced expression of PGC1α protected cells from PLX4720, as demonstrated using three cell lines that expressed low levels of PGC1α (Figure 8a,b,c). As our data suggesting that BRAF regulates PGC1α and oxidative phosphorylation, we evaluated the effects of inhibitors of oxidative phosphorylation in combination with BRAF inhibitors. In addition, the cells were found relatively insensitive to the mitochondrial uncoupler CCCP, but addition of PLX4720 enhanced the cytotoxicity of this drug (Figure 8d). Melanoma cells treated with 2,4-Dinitrophenol (DNP), or oligomycin A, which inhibit oxidative phosphorylation through different mechanisms additively enhanced the efficacy of PLX4720 *in vitro* (Figure 8e). We also found similar data using rotenone and the complex II inhibitor, TTFA (Figure S6b,c). Together, the data indicate that adaptive induction of oxidative phosphorylation in response to BRAF inhibition limits their efficacy.

Discussion

Previous studies have suggested a role of BRAF signaling in the regulation of tumor metabolism. Clinically, patients with BRAF mutant melanomas have higher levels of serum lactate consistent with diminished oxidative phosphorylation (Board et al., 2009). In addition, BRAF mutant melanomas have an order of magnitude increased uptake of glucose compared to normal tissues *in vivo* as assessed by functional imaging (Bollag et al., 2010). However, the mechanism by which BRAF regulates metabolism in melanoma is poorly understood. This study identifies a pathway by which the oncogenic BRAF pathway regulates energy metabolism in melanoma. Our findings show that BRAF activation is associated with diminished oxidative enzymes, diminished mitochondrial number and function and increased production of lactate. This metabolic reprogramming triggered by BRAF(V600E) is accompanied by a suppression of MITF and PGC1α, a major regulator of mitochondrial biogenesis and function.

We identify the melanocyte master regulator *MITF* as a direct and essential mediator of BRAF-regulated PGC1α transcription. Consistent with the restricted expression of *MITF* to the melanocyte lineage, the ERK pathway does not appear to regulate PGC1α transcription in BRAF mutant cancers that lack *MITF* expression. The MITF-PGC1 connection thus explains the lineage-specific effects of BRAF activation and inhibition.

Although BRAF inhibitors can suppress glycolysis and induce oxidative phosphorylation, we found that MITF only regulates mitochondrial respiration. Consistent with this, MITF and PGC1α expression correlate with oxidative phosphorylation genes in a large series of melanoma short term cultures, but we have not observed an inverse correlation with glycolytic gene expression. We also found that MITF expression did not affect glucose uptake, but decreased lactate and increased oxygen consumption, consistent with oxidative metabolism. Thus, consistent with Warburg's initial hypothesis, the activation of glycolysis and the suppression of oxidative metabolism as shown here to be initiated by oncogenic BRAF, are likely separate processes.

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Overall, our data suggest that MITF is a major regulator of mitochondrial respiration in the melanocyte lineage by acting via PGC1α. Tumors likely generate ATP via both glycolysis and oxidative phosphorylation (Colombi et al., 2010; Weinberg et al., 2010) and tumor cells may require mitochondria for functions other than ATP generation, such as fatty acid synthesis and glutaminolysis in some cases (Wise and Thompson, 2010; Dang, 2012). However, we show here that MITF-expressing melanomas have higher level of oxidative gene expression. These data therefore suggest that MITF expression may serve as a biomarker for greater dependence on mitochondrial function. Since MITF has been difficult to drug directly (Haq and Fisher, 2011), the dependence of MITF dependent melanoma on oxidative phosphorylation thus presents a theoretical therapeutic approach. However, MITF paradoxically can promote tumorigenesis (e.g. Garraway *et al.*, 2005; Yokoyama *et al.* 2011), whereas activation of MITF expression in normal melanocytes typically induces differentiation, which likely antagonizes tumorigenesis (D'Orazio *et al.*, 2006; Carreira S *et al.*, 2006; Haq and Fisher, 2011). A rheostat model has been proposed to explain the apparent paradox of MITF (Goding C, 2011) but definitive evidence of this model remains an area of active investigation. We show BRAF inhibitors induce some, but not all MITF target genes, implying that the context in which MITF is regulated may also contribute to its physiologic effects. Finally, it is also highly likely that MITF has pro-tumorigenic effects outside of its induction of OXPHOS, so that any anti-tumorigenic effect of inducing OXPHOS may well be countered by other pro-tumorigenic effects. In the future it will be of great interest to examine how MITF can coordinate its numerous downstream effects.

Eight patients treated with vemurafenib had induction of PGC1α but a larger sample size will therefore be needed to evaluate the diagnostic and predictive role of PGC1α induction in responses to BRAF inhibitors. Interestingly, mutations in PGC1α have been detected in recent whole genome sequencing efforts (Prickett et al., 2009; Stark et al., 2012). Our data nonetheless suggest that dysregulation of PGC1α may have profound effects on metabolism of melanoma cells, and may contribute to oncogenesis in certain cases.

We found that BRAF-mutant melanomas treated with PLX4720 are dependent on ATP generation by mitochondria. Our data suggest that inhibition of mitochondrial metabolism may be most effective therapeutically as initial therapy, as most patients that have relapsed following BRAF inhibitors have reactivation of the MAPK pathway, which we have shown correlates with decreased level of MITF and PGC1α. While mitochondrial functions would likely be difficult to target therapeutically in many cancer types, agents that exploit bioenergetic and metabolic alterations in mitochondria have been proposed (Fantin and Leder, 2006). We find that mitochondrial uncouplers enhanced the efficacy of PLX4720 in BRAF mutant melanomas, but demonstration of *in vivo* efficacy of this combination remains to be firmly established, which possibly will involve derivation of improved mitochondrial pharmacologic agents. Though available drugs have generally unfavorable pharmacologic properties, 2,4-dintirophenol (DNP) had been used extensively in diet pills (Cutting and Tainter, 1933) and over 100,000 people had been treated world-wide with the drug at the time of its discontinuation (Tainter et al., 1934). Cases of dangerous side effects such as fatal hyperthermia led to its official discontinuation by 1938. Given the toxicities of the drugs, further development of alternative oxidative phosphorylation inhibitors should be considered. Despite the recent successes of BRAF inhibition in the clinical arena, recurrence rates remain high, and survival is only extended several months. While further *in vivo* studies will be crucial, uncouplers such as DNP, or other inhibitors of oxidative phosphorylation may an alternative approach to enhance the effect of BRAF inhibitors in melanoma patients.

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Experimental Procedures

Gene expression and bioinformatics

Immortal melanocytes (pmel*) and their transformed counterparts, pmel*BRAF(V600E)-vector and pmel*BRAF(V600E)+MITF were maintained as described (Garraway et al., 2005). Global gene expression analysis was carried out using HG-U133A microarrays (Affymetrix).

RNA isolation, chromatin immunoprecipitation and quantitative real-time PCR

Chromatin immunoprecipitation (ChIP) was performed in primary human melanocytes using previously described methods (Cui et al., 2007). Chromatin was immunoprecipitated using rabbit polyclonal anti-MITF, or normal rabbit IgG as a control. Results are normalized to input DNA.

Clinical samples

All patients gave informed consent for tissue acquisition as per an IRB-approved protocol (Office for Human Research Studies, Dana-Farber/Harvard Cancer Center). Tumors were biopsied before treatment (day 0), at 10–14 days during treatment.

siRNA delivery and analysis

siRNAs SMARTpools (Dharmacon) were delivered using the lipidoid delivery agent C12-133-B as described (Li et al., 2012).

Promoter assays and luciferase experiments

The murine *PGC1α* promoter was obtained from Addgene. Mutagenesis was performed using the QuickChange Mutagenesis Kit (Stratagene). Mutant promoters were verified by sequencing. UACC-62 cells were transfected with each promoter construct, pRL-CMV Renilla control and a *M*-MITF overexpression vector. PLX4720 treatment was for 48h. Results reported are averages of at least three independent experiments, normalized for transfection efficiency using *Renilla* luciferase.

Xenograft tumor studies

All mouse experiments were done in accordance with Institutional Animal Care and Use Committee (IACUC) approved animal protocols at Dana-Farber Cancer Institute as described in the Supplemental Experimental Procedures.

Electron microscopy

Electron microscopy of UACC-62 melanoma cells treated with vehicle or PLX4720 (3μM, 72hrs) was performed at the PMB Microscopy Core as described in Supplemental Experimental Procedures.

Microarray data

Expression array data are deposited under GEO accession GSE38007.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BRAF suppresses mitochondrial density and oxidative phosphorylation metabolism

MITF directly regulates the mitochondrial biogenesis factor, PGC1α

Melanomas with activation of the MAPK pathway have lower levels of MITF and PGC1α

Mitochondrial uncouplers may have therapeutic use in combination with BRAF inhibitors

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Significance

BRAF mutations are the most common genetic aberrations in melanoma but the mechanisms by which they promote oncogenesis are poorly understood. Here we show that activated BRAF promotes metabolic reprogramming by suppression of oxidative phosphorylation through the actions of the melanocyte lineage factor *MITF* and the mitochondrial master regulator PGC1α. BRAF inhibitors, which transiently suppress melanoma growth *in vitro* and in patients, induce PGC1α and oxidative phosphorylation. This addiction to oxidative phosphorylation in melanomas treated with BRAF targeted therapy therefore suggests that mitochondrial inhibitors should be evaluated in combination with BRAF pathway inhibitors *in vivo*.

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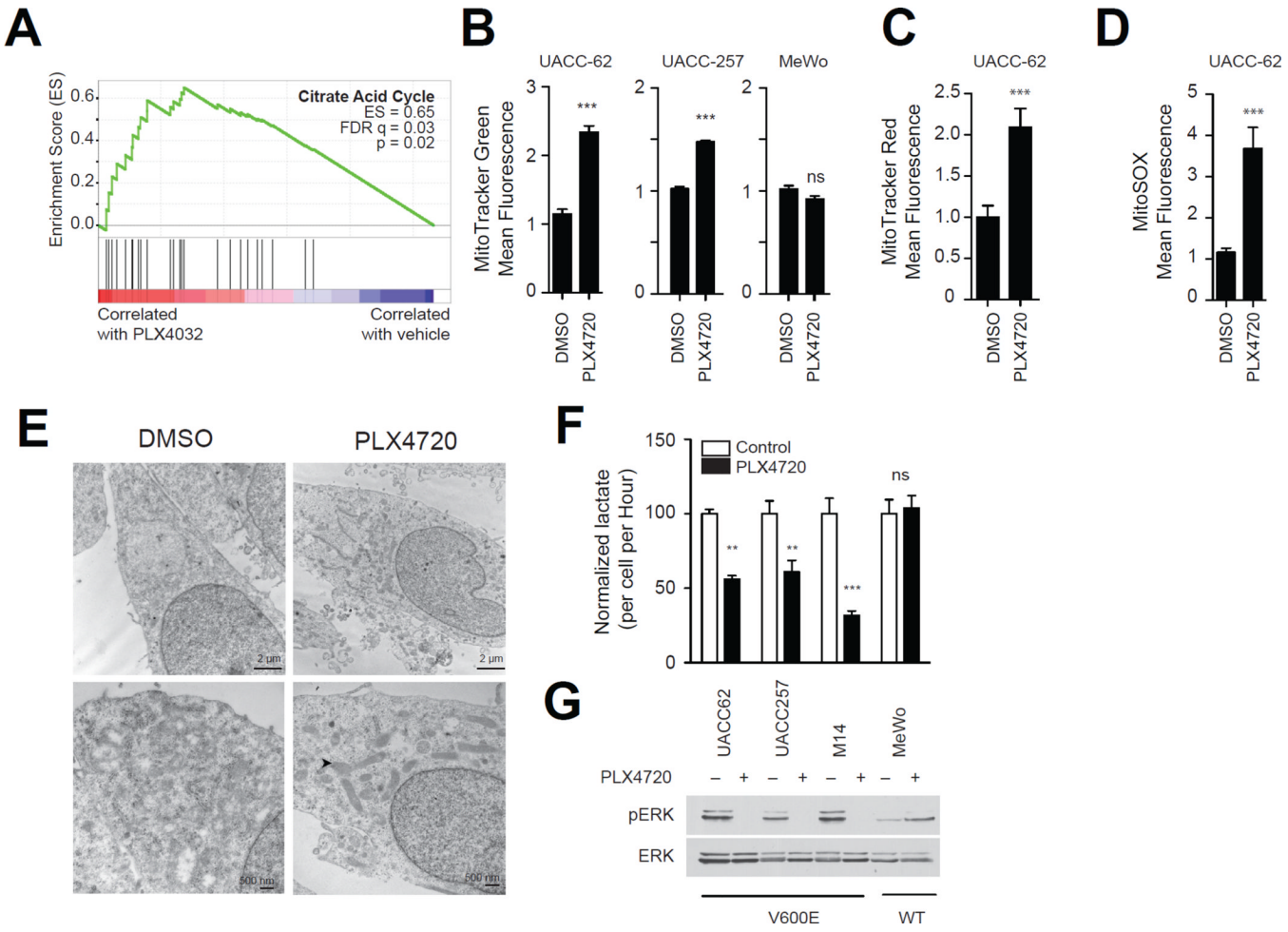

Figure 1.

BRAF inhibitors induce mitochondrial biogenesis and oxidative metabolism. (A) Gene Set Enrichment Analysis plot of melanoma cells treated with vemurafenib showing the most significantly changed gene set. FDR, false-discovery rate; ES, enrichment score. (B) MitoTracker Green fluorescence of BRAF mutant (UACC-62 and UACC-257) or BRAF wild-type (MeWo) melanoma cell lines treated with PLX4720 (1μM, 72h) and subjected to analysis by flow cytometry. (C,D) MitoTracker Red fluorescence (C) or MitoSOX fluorescence (D) of UACC-62 cells treated with PLX4720. (E) Electron micrographs of UACC-62 cells treated with PLX4720 or vehicle control. Representative photographs of cells at 22000× (upper panel) or 44000× (lower panel) are shown. Arrows indicate mitochondria. (F) Lactate levels in media conditioned from the indicated cell lines treated with PLX4720 or vehicle control for 16 hours. (G) Cells in part (F) were concomitantly evaluated for ERK activity by Western blotting using phospho-ERK antibodies. Error bars represent SEM of at least three independent replicates. ***, $p < 0.001$; **, $p < 0.01$. See also Table S1 and Figure S1.

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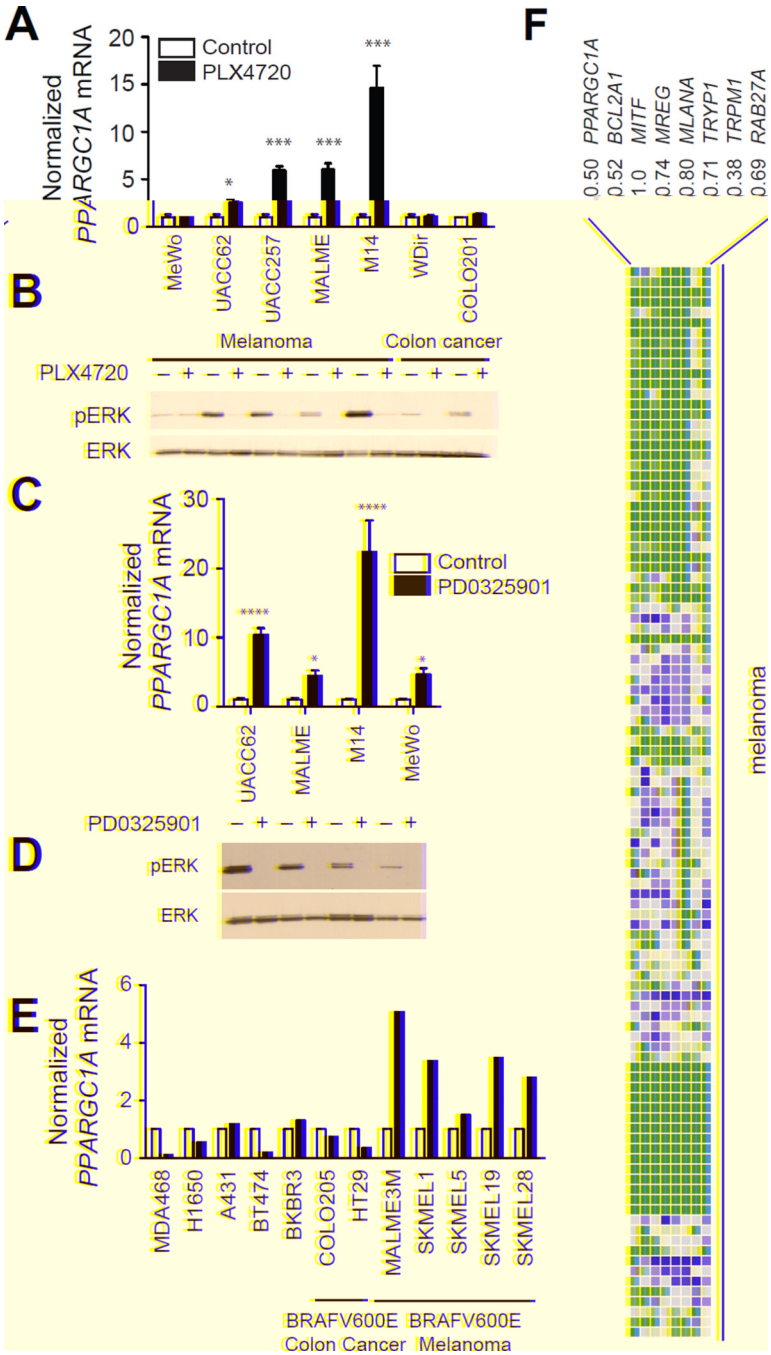

Figure 2.

BRAF inhibitors induce PGC1α expression. PGC1α mRNA (A) and phospho-ERK levels (B) in melanoma or colon cancer cells treated with PLX4720 (1μM) for 24h. PGC1α mRNA (C) and ERK activity (D) in melanoma cells treated with the MEK inhibitor PD0325901 (10nM) for 24h. (E) Microarray analysis (GSE10086) of PGC1α mRNA in cell lines treated with 10nM PD0325901 for 24h. (F) Comparison of *PGC1α* mRNA with MITF, melanocytic markers, and MITF targets in 105 melanoma cell cultures (Hoek et al., 2006). Pearson correlation coefficient is shown below each gene. Error bars represent SEM of at least three independent replicates. ****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.01$. See also Figure S2.

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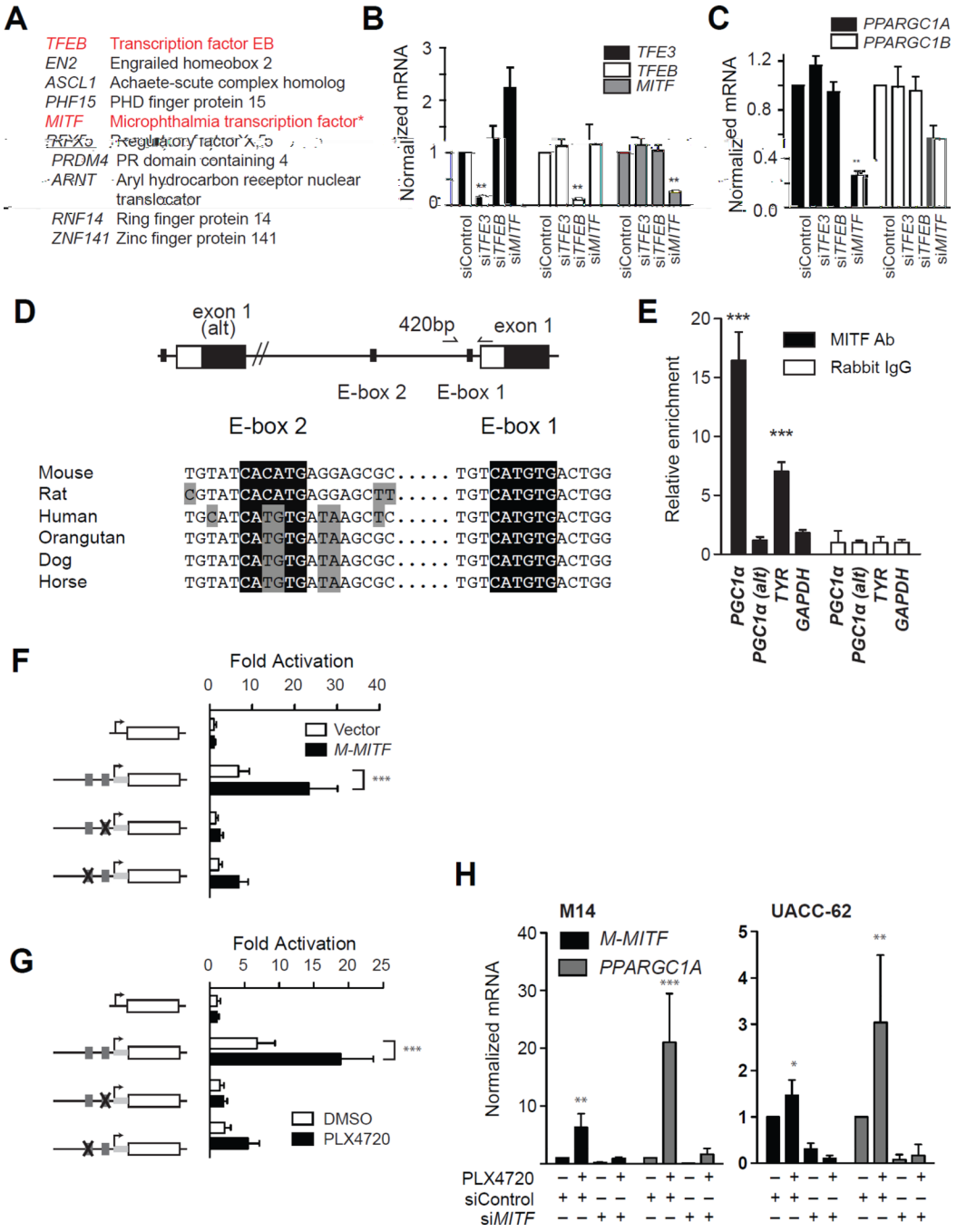

Figure 3.

PGC1α is regulated by MITF in the melanocytic lineage. (A) Top 10 transcription factors correlated to PGC1α mRNA in (Lin et al., 2008). *, q < 0.05. (C) Requirement of MiT family members for PGC1α expression in M14 melanoma cells. Knockdown of each family member is shown in (B). ****, p < 0.0001; **, p < 0.01. (D) Structure of PGC1α promoter in mammalian species showing the location of alterative exon 1 and exon 1. Also depicted are the locations of E-box #1 and E-box #2 and primers used for chromatin-immunoprecipitation. (E) Chromatin immunopreciptation of indicated genomic region with anti-MITF, or rabbit IgG in primary melanocytes. Precipitated DNA was amplified using primers depicted in (D). ***, p < 0.001 compared to Rabbit IgG control. (F,G) Activity of

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PGC1α promoters upstream of the luciferase gene mutated as depicted in response to transfection of MITF (f) or treatment with PLX4720 (G). Grey boxes indicate the location of E-boxes. (H) Expression of PGC1α following knockdown of MITF (48h) and treatment with PLX4720 (1μM, 24h) in M14 cells (left) or UACC-62 cells (right). Error bars represent SEM of at least three independent replicates. ***, $p < 0.001$. See also Figure S3.

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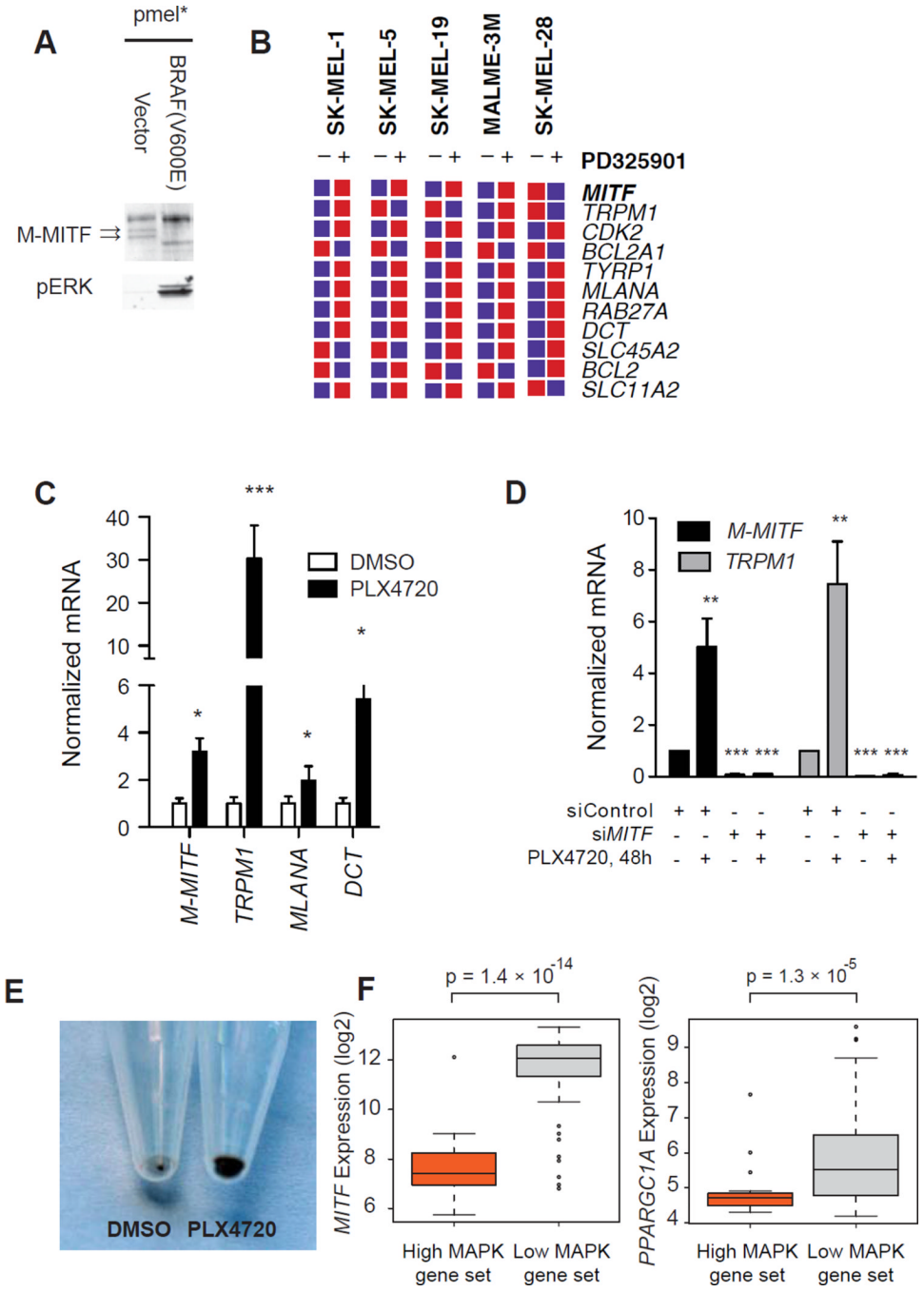

Figure 4.

BRAF suppresses MITF expression and activity. (A) Levels of M-MITF (arrows) and phosphorylated ERK in pmel* and pmel* BRAF (V600E). (B) Effects of MEK inhibitor PD0325901 on *MITF* mRNA and MITF targets by published microarray (Pratilas *et al.*, 2009). (C) Response of MITF and MITF targets to PLX4720 in UACC-257 cells by quantitative PCR. *, $p < 0.01$; ***, $p < 0.001$ relative to DMSO control. (D) Effect of MITF suppression on induction of MITF-target *TRPM1* by PLX4720. **, $p < 0.01$; ***, $p < 0.001$ relative to siControl. Error bars represent SEM of at least three independent replicates. (E) Consequence of PLX4720 (72h) on UACC-257 pigmentation. Equal number of cells were pelleted by centrifugation. (F) Box-plots showing expression of *MITF* (left) and *PGC1α* in

melanoma cells with high or low MAPK activation from 88 short-term melanoma cultures (Lin et al., 2008).

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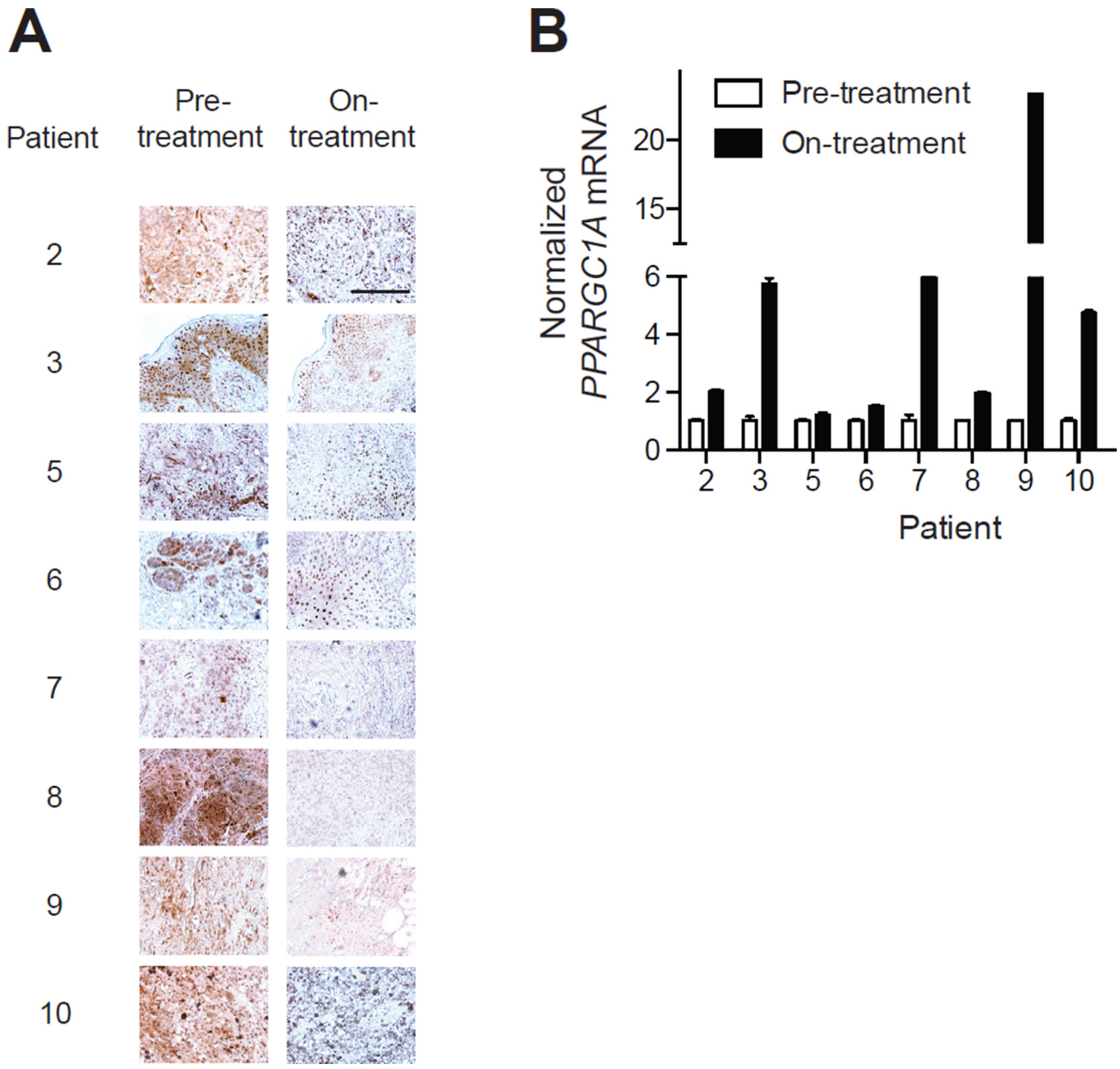

Figure 5.
Validation of induction of PGC1α pathway *in vivo* following BRAF inhibition. (A) Expression of phospho-ERK in eight matched patient biopsies prior or during (10–14 days) of treatment with BRAF/MEK inhibitors. (B) Expression of PGC1α mRNA prior and during treatment with BRAF/MEK inhibitors. Error bars represent SEM of at least three technical replicates. Scale bar represents 100 μm.

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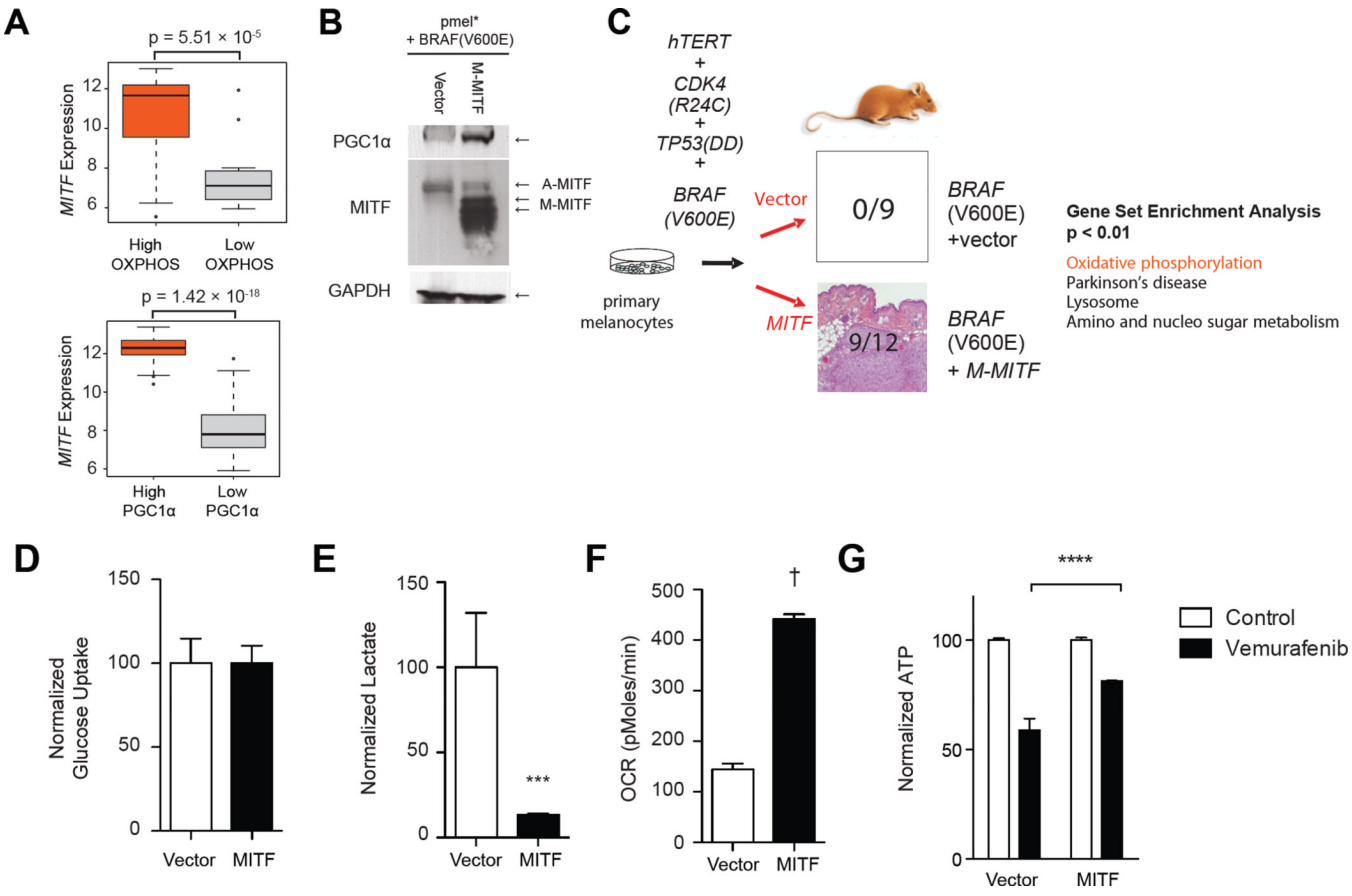

Figure 6.

MITF regulates oxidative phosphorylation. (A) Box plots depicting *MITF* expression in melanomas with high expression of a PGC1α-target gene set (bottom) or oxidative phosphorylation gene set (top). (B) Western blotting showing expression of MITF and PGC1α in pmel* BRAF(V600E) with and without MITF overexpression. (C) Schema showing isogenic system evaluating the effect of MITF overexpression in BRAF(V600E) melanoma cells. The tumorigenicity of the paired cell lines was assessed in FoxN^{nu} mice and the number of formed tumors per injection of each cell line is shown. Gene set enrichment analysis of the paired cell lines with the most highly induced gene sets is shown (right). Glucose uptake (D), lactate levels (E) and oxygen consumption (F) were measured as relative amounts in each cell line, normalized to cell number. (G) ATP levels, normalized to cell number in BRAF(V600E)+vector and BRAF(V600E)+MITF treated with PLX4032 (1μM) for 24h. ***, $p < 0.001$ compared to control cells. Error bars represent SEM of at least three independent replicates. See also Figure S4.

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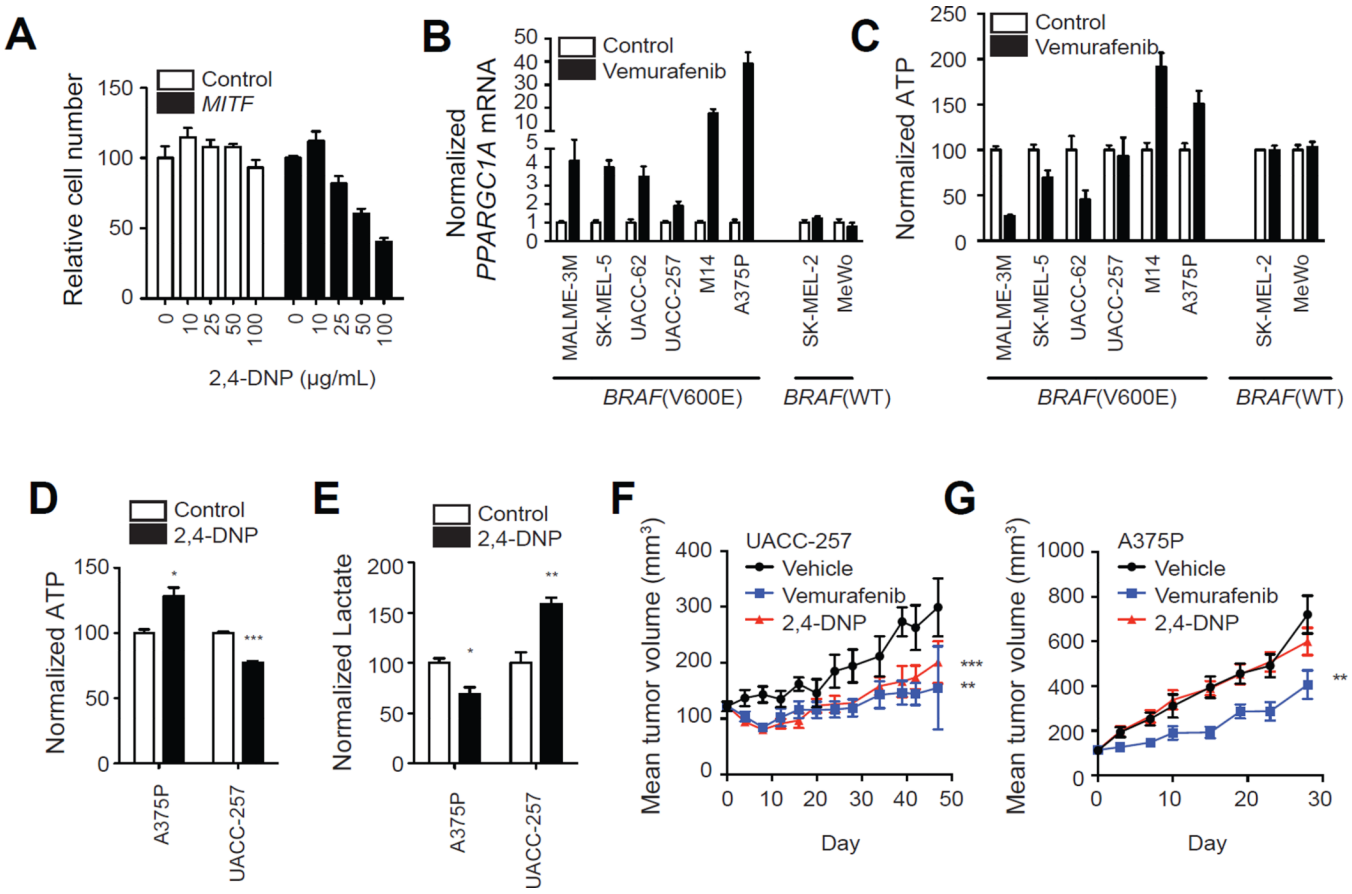

Figure 7.

Effects of 2,4-DNP on growth melanoma cells *in vitro* and *in vivo*. (A) Number of BRAF(V600E)+vector and BRAF(V600E)+MITF melanoma cells following treatment with 2,4- DNP with indicated dose for 72h. Levels of PGC1α mRNA (B) and ATP (C) in melanoma cell lines treated with vemurafenib (1μM) for 24h. Effects of 2,4-DNP (50 μg/mL, 24h) on ATP (D) and lactate levels (E) in indicated cell lines *in vitro*. (F,G) Effect of 2,4-DNP (20 mg/kg/day) or vemurafenib (75 mg/kg/day) on murine xenografts of indicated cell line (N = 7–8 per group). **, $p < 0.01$ compared to vehicle group; ***, $p < 0.001$ compared to vehicle group. Error bars represent SEM of at least three independent replicates. See also Figure S5.

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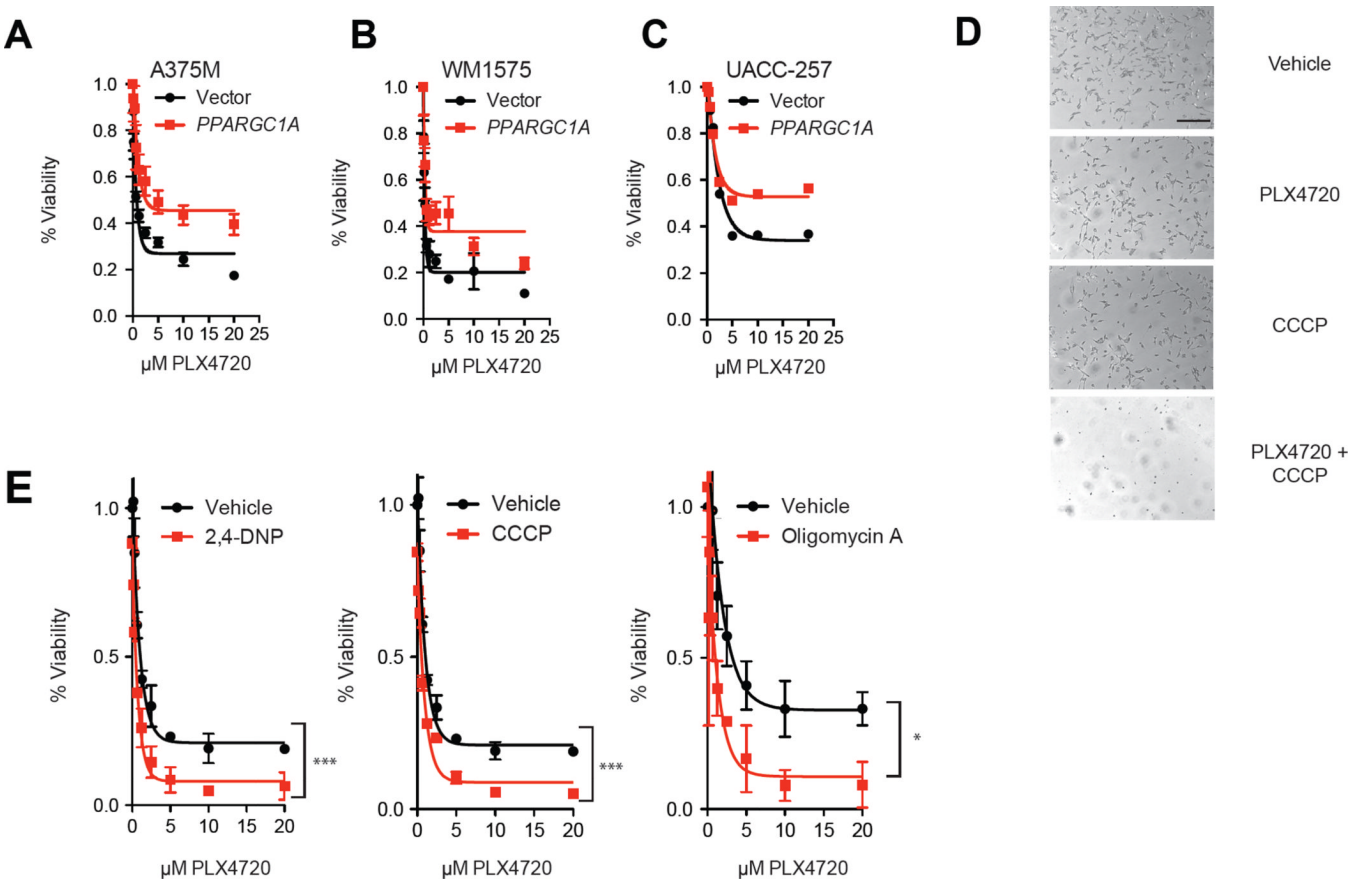

Figure 8.

BRAF inhibitors enhance dependence on mitochondrial metabolism. Sensitivity of A375M (A), WM1575 (B) and UACC-257 (C) melanoma cells overexpressing PGC1α to treatment with PLX4720 for 72 hours. (D) Photograph of M14 cells treated with PLX4720 (5μM), CCCP (20μM) or the combination for 72 hrs. Scale bar represents 100 μm. (E) Cell number following treatment with mitochondrial uncouplers oligomycin A (1μM), CCCP (5μM) or 2,4-DNP (200 μg/mL). Cell number was estimated after 72 hrs of treatment. *, $p < 0.05$ compared to control; **, $p < 0.01$ compared to control; ***, $p < 0.001$ compared to control. Error bars represent SEM of at least three independent replicates. See also Figure S6.