Targeting mitochondrial oxidative metabolism in melanomas causes metabolic compensation through glucose and glutamine utilization

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Abstract
Metabolic targets offer attractive opportunities for cancer therapy. However, their engagement may activate alternative metabolic pathways that can still support tumor growth. A subset of human melanomas relies on PGC1α-dependent mitochondrial oxidative metabolism to maintain growth and survival. Herein, we show that loss of viability caused by suppression of PGC1α in these melanomas is rescued by induction of glycolysis. Suppression of PGC1α elevates ROS levels decreasing HIF-1α hydroxylation that, in turn, increases its protein stability. HIF-1α reprograms melanomas to become highly glycolytic and dependent on this pathway for survival. Dual suppression of PGC1α and HIF-1α causes energetic deficits and loss of viability that are partially compensated by glutamine utilization. Notably, triple suppression of PGC1α, HIF-1α and glutamine utilization results in complete blockage of tumor growth. These results show that due to high metabolic and bioenergetic flexibility, complete treatment of melanomas will require combinatorial therapy that targets multiple metabolic components.

Introduction
Cancer cells rely on activated metabolic routes to support cell proliferation and survival (1-3). The wiring and directional fluxes of these metabolic pathways is regulated through signaling/transcription mechanisms that are targets of oncogenic or tumor suppressor activities (4-6). For example, the oncogene c-myc reprograms carbon metabolism towards the use of glutamine as a main substrate to maintain ATP levels and promote cell growth (7, 8). The fact that tumor cells
are highly dependent on specific metabolic and energetic routes could be exploited to develop anti-cancer therapies (9, 10).

We recently found that a subset of human melanomas overexpress the transcriptional coactivator PGC1α that reprograms them to largely depend on mitochondrial oxidation for growth and survival (11). As a consequence, PGC1α positive melanoma tumors and derived cell lines are more sensitive to mitochondrial inhibition (11, 12). On the other hand, PGC1α negative melanoma cells rely more on glycolysis and are largely insensitive to suppression of mitochondrial metabolism. These studies suggest that although the subset of PGC1α positive melanomas could be targeted blocking mitochondrial oxidation, alternative metabolic pathways exist for melanoma growth that could compensate the inhibition of mitochondria metabolism.

The family of hypoxia-inducible transcription factors (HIF), which includes HIF-1α, controls metabolic and cellular programs that support survival and tumorigenesis (13, 14). HIF-1α regulation is largely due to hydroxylation of two proline residues (402 and 564) catalyzed by Prolyl Hydroxylases enzymes (PHDs) (15). Prolyl hydroxylation of HIF-1α causes polyubiquitination through binding of the von Hippel-Lindau (VHL) protein, which is part of an E3 ligase complex, and degradation by the proteasome. The fact that PHDs catalytic activity requires oxygen, iron and 2-oxoglutarate makes these enzymes oxygen sensors that cause increased HIF-1α stability under hypoxic conditions (16, 17). There is, however, HIF-1α accumulation in normoxic conditions through increases in Reactive Oxygen Species (ROS), 2-oxoglutarate analogues or iron chelation which suppress PHD enzymatic activity. HIF-1α stability caused by changes in PHD activities is one of the major mechanisms by which HIF-1α controls expression of gene programs including glycolysis and angiogenesis that promote tumor progression (18, 19). In addition, since HIF-1α increases glycolytic fluxes it
can function as a metabolic alternative pathway to maintain energetic and cellular tumor growth (20).

Many oncogenic signals drive bioenergetic requirements in tumor cells through increases in glucose metabolism. In some tumor types or conditions, glutamine, the most abundant amino acid in blood, is utilized to promote tumor growth (7) and certain glucose PET negative tumors exhibited high rates of glutamine consumption (21, 22). In cancer cells, intracellular glutamine exceeds the requirements for protein synthesis and is used for different metabolic activities including ATP synthesis and lactate production, nucleotide, lipid and glutathione synthesis (8). As a consequence of this metabolic and bioenergetic pleiotropy, glutamine is an important nutrient that supports tumor growth and survival (3, 6). In fact, glutaminase inhibitors have been shown to be potent inhibitors of several types of malignancies (23, 24). Glutamine is efficiently used for mitochondrial metabolism and is decoupled from glycolysis providing an alternative bioenergetic and anabolic substrate (25).

Here we report that inhibition of mitochondrial metabolism through suppression of PGC1α in melanomas results in the emergence of sequential metabolic and bioenergetic compensatory pathways that allow cell survival and tumor progression. The first compensation involves a ROS-dependent activation of HIF-1α leading to increased glycolytic rates. Subsequent suppression of HIF-1α results in an alternative metabolic compensation through increases in glutamine utilization that support melanoma growth and survival. Targeting PGC1α, HIF-1α and glutamine utilization is required to completely block tumor growth. In addition, we show that, these alternative metabolic pathways are induced not only by exogenous perturbations but also selected naturally as alternative mechanisms for tumor growth. These results underscore the metabolic plasticity of cancer cells and show that a combinatorial therapy will be required to treat tumors using metabolic targets.
Materials and Methods

Reagents and Antibodies. *N*-acetyl-L-cysteine, H$_2$O$_2$, amino acids, MG132, dimethyl-α-ketoglutarate, anti-HA, PEG-SOD, PEG-catalase, anti-FLAG and FLAG-M2 affinity beads were purchased from Sigma-Aldrich. Piperlongumine and Anti-PGC1α (H-300) were purchased from BioVision Research products and Santa Cruz Biotechnology. Antibodies against cleaved-caspase-3, 9, PARP, Proline 564 hydroxylated HIF-1α and total HIF-1α were purchased from Cell Signaling Technology. Anti-tubulin and lamin B antibodies were purchased from Millipore.

Cell Culture and Lentivirus Production and Infection. Melanoma cells were cultured in high glucose DMEM with 5mM L-glutamine containing 10% FBS unless otherwise indicated. Lentiviruses were produced from HEK293T cells as previously described (26). Lentivirus particles were collected 48h after post-transfection and used to infect melanoma cells in the presence of 8 μg/ml polybrene, and then infected cells were selected with 2 μg/ml of puromycin or 7 μg/ml blasticidin for 4 days prior to experiments. Doxycycline inducible shRNA pLKO expressing melanoma cells were incubated with doxycycline (100ng/ml) prior to experiments. GFP control and Flag-HA-PGC1α adenovirus have been previously described (27).

Western Blot. Cells were lysed in a buffer containing 1% IGEPAL, 150 mM NaCl, 20 mM HEPES (pH7.9), 10 mM NaF, 0.1 mM EDTA, 1 mM Sodium orthovanadate and 1X protease inhibitor cocktail. Cell lysates were electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon-P membrane (Millipore).

Quantitative real time-PCR. Total RNA was isolated with Trizol (Invitrogen) and 2 μg of total RNA was used for cDNA synthesis using high capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCRs were
carried out using SYBR Green PCR Master Mix (Applied Biosystems). Primers used for PCR are list in Supplementary Table 3.

**Measurement of antioxidant activity.** Infected cells were grown for 24 hours and cell extracts were used to measure human catalase, superoxide dismutase, and glutathione peroxidase activities. Enzymatic activities were measured according to manufacturer’s instructions (Cayman Chemical Company, USA).

**Clonogenic assay.** For clonogenic assay, 1X10^4 melanoma cells were seeded in 6-well plate and maintained in the medium as indicated in individual experiment. After 7 days, cells in the plate were fixed by 10% formalin, followed by staining with crystal violet for 10min.

**Glutathione and NADPH level.** The levels of NADPH, NADH+, reduced glutathione (GSH), and oxidized glutathione (GSSG) in cultured cells were determined using a NADP+/NADPH quantification Kit, glutathione colorimetric detection kit (BioVision), and oxiselect glutathione assay kit (Cell BioLabs), following the manufacturer’s instructions. Briefly, cell lysates were prepared in an NADP+/NADPH extraction buffer, and then NADPH and NADP+ levels were measured by spectrometry at OD450 nm. For measurement of glutathione levels, cell lysates were prepared in extraction buffer without thiol compounds such as dithiothreitol (DTT) or β-mercaptoethanol, and then glutathione levels were measured by spectrometry at OD405 nm.

**Glucose Consumption, Lactate Production, Glutamine Consumption and ATP Levels.** Lactate, glucose and glutamine assay kits (BioVision Research Products) were used to measure extracellular lactate, glucose and glutamine following manufacturer’s instructions. Briefly, equal number of cells were seeded in 6-well plates and cultured in phenol-red free DMEM for 24h. For glutamine assay, cells were cultured with 1mM glutamine and 25mM glucose containing Phenol-red free DMEM. Intracellular ATP levels were determined in cell lysates.
using a luciferin-luciferase based ATP determination kit (Invitrogen) according to the manufacturer's instructions and all values were normalized to cellular protein concentration.

**Tumor xenograft assay.** 1 X 10^6 A375P cells stably expressing the indicated shRNAs were injected subcutaneously into the flank of Nude mice (Taconic) in 100 μl of media. 10 days after cell injection, mice were housed with 2mg/ml of Doxycycline and 5% of sucrose containing water until the end of the experiment. For the small molecule treatments, 20 days after cell injection, mice were injected daily with piperlongumine (1mg/kg), compound 968 (150 μg) or DMSO for 10 days. Tumor volumes were monitored with a caliper and calculated using the equation volume = \(ab^2/2\), where “a” is the maximal width and “b” is maximal orthogonal width. All procedures were conducted in accordance with the guidelines of the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Outside of tumors contained non-necrotic area were used for isolation of nucleus extracts, and then nucleus extracts were used to measure HIF-1α levels.

**In-vitro hydroxylation.** GST-tagged HIF-1α ODDD (Oxygen dependent degradation domain) and full-length PHD2 proteins were expressed in Escherichia coli BL21 cells and A375P cells, and proteins were purified using GSH-affinity chromatography and HA-affinity beads. For the in-vitro proline hydroxylation assay, GST-ODDD (100 ng) was incubated with A375P cells derived PHD2 (50 ng) at 30°C for 1 h in a reaction buffer containing 40 mM Tris–HCl, pH 7.4, 4 mM 2-oxoglutarate, 1.5 mM FeSO4, 10 mM KCl and 3 mM MgCl2. The hydroxylation at the Pro 564 residue was analyzed by immunoblotting using an anti-Pro564 (OH) antibody.

**14C-L-glutamine oxidation assay.** Scrambled, PGC1α or HIF-1α shRNA stably expressing A375P cells were cultured for 48 h with 2 mM glutamine and 25 mM glucose contained DMEM. At this time, medium was changed with 1 mM L-
glutamine and 25 mM glucose containing DMEM, cells were further incubated for 12 h. Then, cells were incubated with 2 μCi/ml of 14C-L-glutamine (Perkin Elmer) for 3 h. 14C labeled CO2 was captured in phenylethylamine-soaked Whatman paper and measured on a scintillation counter.

GSEA analysis
A previously published gene expression profile of A375P cells stably expressing control shRNAs or two different shRNAs against PGC1α (GSE7553) was analyzed with the GSEA algorithm. 13 Gene-sets generated from cells exposed to hypoxia from MsigDB (shown in Supplementary Table 1) were tested for enrichment in the PGC1α suppressed cells using the default parameters.

RESULTS
PGC1α suppress HIF-1α protein stability and glycolysis in melanoma cell lines and tumors
We have recently shown that PGC1α is strongly overexpressed in a subset of melanoma tumors and derived cell lines (11). Depletion of PGC1α in these melanoma cells using a lentivirus-encoding a targeting shRNA caused a decrease in cell viability as well clonogenic cell survival (Fig. 1A). Although ~20% of the cells with suppressed PGC1α died from apoptosis, the cells that survived could be grown long term. These PGC1α-depleted surviving cells had only a slightly reduced growth rate compared to control cells, despite maintaining PGC1α levels suppressed (Fig. 1A, upper right panel). The surviving PGC1α suppressed cells displayed a reduction in mitochondrial oxidative phosphorylation but increased glycolysis and lactate production (11) – suggesting that a metabolic switch to glycolysis might be an underlying mechanism that rescued viability. Because HIF-1α promotes glycolysis, we probed the protein stability of this transcription factor. Fig. 1B shows that HIF-1α
protein levels were strongly increased after shRNA mediated PGC1α suppression in three PGC1α−positive melanoma cell lines. HIF-1α reporter luciferase activity was accordingly elevated in these melanoma cell lines (Fig. 1C). The effect of PGC1α shRNAs on HIF-1α protein stability was efficiently rescued by ectopic expression of PGC1α (Fig. 1D). In addition, when melanoma cells were grown in vivo as xenografts, the increase in HIF-1α protein levels after PGC1α suppression was maintained (Fig. 1E). Consistent with HIF-1α elevation, expression arrays performed after suppression of PGC1α (GSE7553) showed an enrichment of hypoxia-induced signatures in PGC1α depleted compared to control melanoma cells (Fig 1F). Specifically, 11 out of the 13 interrogated hypoxia-induced gene-sets from MsigDB were enriched in PGC1α depleted cells (Supplementary Table 1). In agreement with this observation, qPCR analysis showed that HIF-1α target genes -including genes encoding for enzymes or proteins linked to glycolysis and lactate production- were increased upon PGC1α suppression in melanoma cell lines (Figs. 1G and S1B) and xenografted tumors (Fig. 1H). In contrast and as predicted, PGC1α target genes including mitochondrial oxidative phosphorylation and ROS detoxification genes were substantially decreased (Fig. S1A). Functionally, increases in HIF-1α target genes caused by knockdown of PGC1α in cell lines and tumors resulted in increased glucose uptake and lactate production (Figs. 1I and 1J). Taken together, these results indicate that suppression of PGC1α results in metabolic and energetic compensation through increases in HIF-1α protein stability and glycolysis.

PGC1α expression causes reduction of HIF-1α protein stability through suppression of ROS production

As part of the oxidative metabolism program, suppression of PGC1α results in an increase in intracellular levels of ROS (11). Elevated ROS levels are one of the mechanisms whereby HIF-1α protein is stabilized in normoxic conditions (28, 29). We used the antioxidant N-Acetyl Cysteine (NAC) to determine if PGC1α
suppression-dependent HIF-1α protein stability was a result of increased ROS levels (30). Figure 2A shows that increased concentrations of NAC were sufficient to efficiently block HIF-1α protein stability in PGC1α suppressed A375P cells. Blockage of HIF-1α induction in these cells translated in a reduction in their ability to induce glycolytic and angiogenic HIF-1α target genes (Fig. 2B). We have recently shown that PGC1α levels define the metabolic state of melanomas (11). Interestingly, endogenous PGC1α levels also modulated the magnitude of induction of HIF-1α protein stability in response to either hypoxia or ROS increases through hydrogen peroxide treatment –with a more robust induction of HIF-1α protein in PGC1α negative cell lines (Figs. S2A, S2B and S2C). As expected, the induction of HIF-1α target genes in response to hypoxia or hydrogen peroxide was also more significant in PGC1α negative cell lines (Figs. S2D and S2E). These results suggest that melanomas also select or switch to the HIF-1α-dependent alternative metabolic route in their natural history.

HIF-1α protein stability is regulated through prolyl hydroxylation of residues 402 and 564 (15). To determine if ROS-induced HIF-1α stability in melanoma cells was due to changes in prolyl hydroxylation, we measured this chemical modification using specific antibodies. Figure 2C shows a decrease in HIF-1α prolyl hydroxylation in the presence of the proteasome inhibitor MG132 upon suppression of PGC1α. However, this decrease was substantially prevented by treatment with the antioxidant NAC. Moreover, in contrast to the stability of wild type HIF-1α protein, a defective HIF-1α proline hydroxylation mutant (P402/564A) showed no changes in protein stability after PGC1α suppression (Fig. 2D). The fact that knockdown of PGC1α increased HIF-1α protein stability and decreased prolyl hydroxylation suggests that the enzymatic activity of Prolyl Hydroxylase 2 (PHD2) was decreased. To test this possibility, we immunoprecipitated PHD2 from control and PGC1α shRNA melanoma cell lines and measured the Prolyl Hydroxylase activity using recombinant HIF-1α as a substrate (31). Fig. 2E shows that PHD2 enzymatic activity was decreased in PGC1α suppressed melanoma cells, but this activity was rescued by NAC.
treatment. In addition, hydrogen peroxide mimicked the effects of PGC1α suppression decreasing PHD2 activity (Fig. 2F).

Taken together, these results suggest that suppression of PGC1α induces HIF-1α protein stability through induction of ROS levels and activation of PHD2 enzymatic activity in melanoma cells.

**HIF-1α maintains survival and compensates metabolic and energetic PGC1α function in melanoma cells and tumors**

As illustrated in Figure 1I, elevated levels of HIF-1α upon suppression of PGC1α result in a metabolic reprogramming towards a more glycolytic metabolism. These results suggested that these cells might now depend on HIF-1α to maintain their energetic status and survival. To assess this possibility, we suppressed HIF-1α in PGC1α knockdown cells using a doxycycline-inducible shRNA. Fig. 3C shows that both PGC1α and HIF-1α levels were efficiently suppressed after doxycycline treatment and HIF-1α targets were decreased (Fig. 3A). Of note, doxycycline did not have any effect on the expression of HIF-1α targets in control cells (Fig. S3A). Double knockdown of PGC1α and HIF-1α substantially reduced cell viability (Fig. 3B left panel) and clonogenic cell survival (Fig. 3B right panel), which correlated with increased apoptotic markers including cleavages of caspase 9 and PARP (Fig. 3C) and decreased intracellular ATP levels (Fig. 3E). Interestingly, inhibition of glucose utilization by 2-deoxy-glucose led to reduced viability (Fig. 3D), mimicking the effect caused by HIF-1α suppression in PGC1α-depleted cells; therefore, HIF1α depletion in these cells caused apoptosis, at least in part, through inhibition of the glycolytic flux. In addition, consistent with HIF1α increase, hypoxia partially rescued cell death induced by acute PGC1α knockdown (Fig. S3B), further supporting the protective role of HIF-1α for cell survival upon metabolic stress. Next, to test how double HIF1α/PGC1α depletion decreased cell survival, we measured changes in metabolites and enzymes associated with ROS activities. Double HIF-1α and PGC1α knock-down in A375P cells did not change NADPH and glutathione
levels (Fig. S3C), ROS detoxification gene expression (Figs. S3D and S3F), ROS levels (Fig. S3E) or antioxidant enzymatic activities (Fig. S3G). Moreover, addition of polyethylene glycol (PEG)-conjugated superoxide dismutase or catalase, which repress intracellular oxidative stress (Fig. S3H), failed to rescue cell viability in PGC1α and HIF-1α suppressed melanoma cells (Fig. S3I). Altogether, these results suggest that the loss of viability in double HIF-1α/PGC1α suppressed cells is not triggered by an increase in ROS levels and is likely due to a failure to maintain cellular energy levels.

To determine the effects of the double PGC1α/HIF-1α knockdown in tumor maintenance, we injected cells expressing PGC1α shRNA and inducible HIF-1α shRNA into nude mice and allowed the tumors to form prior to the induction of the HIF-1α shRNA with doxycycline. Although, suppression of HIF-1α alone did not compromise the growth of the cells in vitro, there was an effect on tumor growth after the tumor reached ~500 mm³ in size (Fig. 3F). Consistent with our previous results, PGC1α suppression resulted in a decrease on tumor growth. However, double knockdown of PGC1α/HIF-1α had a more pronounced effect on the growth of the tumor. The effects observed on tumor growth were consistent with substantial decreases in the expression of glycolytic and angiogenic genes (Fig. 3G) and on ATP levels (Fig. 3H). These results indicate that suppression of PGC1α causes a HIF-1α-dependent bioenergetic switch towards glycolysis. Moreover, these results show that simultaneous targeting the PGC1α and HIF-1α arms results in a more pronounced effect on cell survival and tumor growth than targeting either arm alone.

We have recently shown that suppression of PGC1α sensitizes melanoma tumors to ROS inducing drugs such as piperlongumine (11). Since levels of antioxidant enzymes were decreased to the same extend in single PGC1α and double PGC1α and HIF-1α suppressed cells, we determined if increasing ROS levels would further reduce the growth of these tumors. Notably, piperlongumine completely prevented tumor growth of the double PGC1α and HIF-1α depleted cells (Figs. 3I and S3J) which correlated with increased apoptosis markers (Fig.
S3K). In aggregate, these results indicate that ROS inducing drugs will be very efficient in melanoma tumors in which PGC1α and HIF-1α are inhibited.

**Glutamine utilization maintains survival and compensates metabolic and energetic HIF-1α and PGC1α function in melanoma cells and tumors**

Despite the reduction of oxidative and glycolytic metabolism, PGC1α and HIF-1α suppressed cells were able to grow –albeit to a slower rate- and form small tumors. To investigate the energetic source that contributed to the growth of these cells, we exposed them to single carbon sources and measured their ability to prevent apoptosis. Figure 4A shows that glucose and branched chain amino acids were unable to block apoptosis –shown by the levels of caspases or PARP cleavage. Other carbon sources including glutamate, pyruvate and TCA intermediates attenuated the levels of apoptotic markers. Strikingly, however, glutamine dramatically prevented the induction of these apoptotic markers to undetectable levels. Reduction in apoptosis correlated with increased cell number (Fig. 4B), elevated levels of intracellular ATP (Fig. 4C) and clonogenic cell survival (Fig. 4D), which was more pronounced in PGC1α/HIF-1α suppressed cells than in control cells. To further explore the role of glutamine in melanoma cell viability we used a specific glutaminase inhibitor, compound 968 (24). Consistent with the effect of glutamine promoting cell survival in PGC1α and HIF-1α suppressed cells, the 968 glutaminase inhibitor caused a substantial reduction on cell number and ATP levels (Fig. 4E). Next, we used $^{14}$C-glutamine to investigate the rates of glutamine utilization after depletion of PGC1α/HIF-1α.

In contrast to the increase in glucose utilization (Figs. 1I and 1J), PGC1α suppressed cells exhibited a decrease in glutamine utilization measured as released of $^{14}$CO₂ (Fig. 4F) and uptake (Fig. 4G). However, PGC1α/HIF-1α suppressed cells increased the levels of glutamine utilization (Fig. 4F) and uptake (Fig. 4G).

To further support the relevance of glutamine utilization in vivo, we treated mice xenografted with PGC1α or PGC1α/HIF-1α suppressed cells with the 968
glutaminase inhibitor. Fig. 4H shows that 968 had a small effect on the growth of control tumors but largely prevented tumor growth of PGC1α/HIF-1α suppressed xenografts. The effects seen on tumor growth correlated with strong reduction of ATP levels (Fig. 4I) and the induction of cell death markers (Fig. 4J). Because inhibition of glutamine utilization also increases ROS levels, we tested whether reduced cell viability by glutaminase inhibitor depends on ROS accumulation. Fig. 4K shows that NAC does not rescue cell viability upon glutaminase inhibitor, suggesting that glutamine utilization conferring resistance to apoptosis induced by PGC1α/HIF-1α suppression is independent of ROS. Together, these results indicate that glutamine is an alternative energy source to maintain cell survival in melanoma tumors, particularly in conditions where mitochondrial metabolism and glycolysis are reduced.

**DISCUSSION**

In this study, we report that melanomas develop alternative metabolic compensatory strategies for survival and growth. Inhibition of mitochondrial metabolism through suppression of PGC1α expression in highly oxidative cells results in elevation of ROS levels triggering an increase in HIF-1α stability and rewiring towards glycolysis. Subsequent inactivation of HIF-1α reactivates glutamine utilization to maintain the cellular bioenergetic state that is necessary for survival after inhibition of oxidative phosphorylation and glycolysis. These results reveal three alternative compensatory metabolic/bioenergetic routes, PGC1α and mitochondrial oxidative phosphorylation, HIF-1α and glycolysis and glutamine utilization, underscoring the flexibility of cellular metabolism in melanomas and possibly other tumors.

Cellular metabolism has evolved to be highly flexible and rapidly rewired to adapt to nutrient and energy fluctuations. For example, in fasting conditions skeletal muscle or liver cells quickly switch to utilize fatty acids instead of glucose as an energy source (32). Similar metabolic flexibilities occur in cancer cells that reprogram their metabolism to meet high bioenergetic and biosynthetic demands...
to proliferate and survive in disparate nutrient and hypoxic conditions (3, 33). In cancer cells, such metabolic and energetic rewiring is the result of both genetic changes and non-genetic adaptations, similar to the adaptations occurring in liver or skeletal muscle. The abundance of alternative pathways and the existence of these non-genetic adaptations make it difficult to exploit metabolic targets for cancer therapy and it remains unclear that targeting a single metabolic component could be an effective anticancer therapy. Our results provide experimental evidence of these non-genetic adaptations when targeting critical metabolic pathways in melanomas and show that, in this context, targeting a single metabolic node is not sufficient to cause a robust response. Fortunately, it is likely that the number of alternative metabolic pathways that the cancer cell can use is limited and that rational combination therapy targeting all possible nodes could maximize the effect of the treatment. Our results provide an example of such rational combination therapy and show that by blocking all key metabolic nodes is possible to elicit a robust response.

A major cellular function of PGC1α is to promote mitochondrial biogenesis and to increase the cellular energetic state while in parallel strongly protecting against oxidative stress. A subset of melanoma tumors (and likely other tumor types) overexpresses PGC1α that drives augmented mitochondrial oxidative phosphorylation and ROS detoxification capacities. This subset of tumors largely depends on PGC1α for growth and survival and is more resistant to oxidative stress (11). Since mitochondrial oxidation generates ROS, the PGC1α-mediated increase in ROS detoxification enzymes is essential to maintain survival. In addition, this augmented ROS detoxification capacity facilitates the cell’s oxidative phosphorylation function as elevated ROS levels suppress ATP synthesis (34). This is clearly exemplified by the higher resistance of PGC1α positive melanoma tumors to ROS-inducing drugs (11). Increased sensitivity to ROS after PGC1α suppression is partially counterbalanced by the induction of HIF-1α protein stability that maintains the bioenergetic state and survival of the cells. This is consistent with the reported protective effects of HIF-1α in many
tumors types (13) and with our data showing that ROS-inducing drugs are more effective after double suppression of PGC1α and HIF-1α.

Low concentrations of oxygen are the main signal to increase HIF-1α protein stability. However, under normoxic conditions increases in ROS levels can also result in elevated HIF-1α protein stability (18, 35, 36). Our results show that PGC1α suppression results in ROS-mediated increase in HIF-1α protein stability largely mediated through inhibition of PHD2. Although the mechanisms whereby ROS decreases PHD2 activity are unknown, our data suggest that PHD2 posttranslational modifications and/or differential protein interactions might account for the changes in enzymatic activity. In addition to the HIF-1α increase after PGC1α suppression, our work also shows that natural PGC1α negative melanoma tumors have an increased sensitivity to induction of HIF-1α protein stability, suggesting that targeting PHD2 to inhibit HIF-1α protein stability and compromise energy metabolism could be particularly effective in PGC1α negative melanoma tumors.

In clinical settings, PET negative tumors tend to be positive for glutamine utilization, suggesting that glutamine instead of glucose is their main nutrient source (21). It is known that some mutational events drive tumors towards the use of glutamine (7, 8). Our results suggest that suppression of glucose metabolism by drug treatments could also results in a rewiring towards the use of glutamine. Under these conditions glutaminase inhibitors could be used in combination therapy. The mechanism by which glutamine utilization rescues cell death caused by dual inhibition of PGC1α and HIF-1α is unknown. Enhanced glutamine oxidation as observed in our studies is likely to be one mechanism. In addition, reductive glutamine metabolism that can either increase fatty acid synthesis, as reported to occur under hypoxic conditions (37), or change the α-ketoglutarate/citrate ratio (38), might also contribute to melanoma survival after PGC1α and HIF-1α suppression.
In summary, our studies reveal a series of metabolic compensatory mechanisms that occur in melanoma and denote the energetic versatility and plasticity that these cells exhibit. These metabolic nodes could be targets for combinatorial cancer therapy.

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References


FIGURE LEGENDS

Figure 1. HIF-1α levels and stability in PGC1α suppressed cells. A, Growth curve and clonogenic cell survival of A375P cells with transient (4 days) or chronic (20 days) PGC1α suppression. B and C, Chronic suppression of PGC1α elevates HIF-1α protein expression and transcriptional activities in melanoma cells. D, HIF-1α levels after rescue of PGC1α expression in PGC1α-suppressed A375P cells. Mouse flag-tagged PGC1α was expressed exogenously using adenoviruses for 24 h before harvesting. E, HIF-1α levels in A375P xenografts. F, GSEA enrichment plot for the top scoring Gene-set. Gene expression profile of A375P cells stably expressing control shRNAs or two different shRNAs against
PGC1α (GSE7553) was analyzed with the GSEA algorithm for enrichment of gene sets related to hypoxia. **G**, Expression of HIF-1α glycolytic targets after PGC1α knockdown. **H**, HIF-1α glycolytic target genes are increased in PGC1α-depleted tumors. **I**, glucose utilization and lactate production in PGC1α-depleted melanoma cells. **J**, glucose utilization, lactate production and ATP levels in PGC1α-depleted tumors. Values represent mean ± SD of three independent experiments performed in triplicate; *p < 0.05, **p < 0.01 and ***p < 0.001 versus control shRNA. The whiskers in the box plots represent the maximum and the minimum value.

**Figure 2.** PGC1α suppression enhances the stability of HIF-1α through increasing ROS levels. **A**, NAC blocks HIF-1α accumulation induced by PGC1α suppression. PGC1α-depleted A375P cells were incubated with NAC (0.5, 1, 2 or 4 mM) for 48 h, and then whole cell lysates were used to measure HIF-1α protein levels. **B**, NAC attenuates the induction of HIF-1α glycolytic target genes after PGC1α suppression. Total RNA was isolated from PGC1α-depleted A375P cells incubated with 4 mM of NAC for 48 h. **C**, Proline hydroxylation of HIF-1α is decreased in PGC1α-suppressed melanoma cells. PGC1α-suppressed A375P cells were incubated with 4 mM NAC or vehicle for 48 h, followed by incubation with 20μM of MG132 or DMSO for 3 h prior to harvesting. **D**, HIF-1α P402/564A mutant is not further stabilized after PGC1α suppression. **E** and **F**, Oxidative stress induced by PGC1α depletion (E) or H₂O₂ (F) inhibits PHD2 enzymatic activity. Values represent mean ± SD of three independent experiments performed in triplicate; **p < 0.01 and ***p < 0.001 versus control shRNA and #p < 0.05 versus PGC1α shRNA

**Figure 3.** PGC1α-suppressed melanoma cells are dependent on HIF-1α expression. **A**, Expression of glycolytic genes after HIF-1α and PGC1α suppression in A375P cells. **B**, Growth curve and clonogenic cell survival of HIF-1α- and PGC1α-suppressed A375P cells. **C**, Detection of apoptosis in HIF-1α-
and PGC1α-suppressed A375P cells. D, Cell growth after 2-deoxy-glucose (2DG) treatment in PGC1α-suppressed A375P cells. E, ATP levels in HIF-1α- and PGC1α-suppressed A375P cells. F, Tumor growth after suppression of PGC1α and HIF-1α. The tumor growth curves are plotted as mean ± SEM (n=10). G, Inhibition of HIF-1α decreases glycolytic gene expression induced by PGC1α-depletion in tumors. H, Inhibition of HIF-1α decreases ATP levels in PGC1α-depleted tumors. I, Effect of piperlongumine in HIF-1α- and PGC1α-suppressed tumors. Mice were daily injected with piperlongumine (1mg/kg) or DMSO for 10 days as described in Materials and Methods (n=8). Values represent mean ± SD of three independent experiments performed in triplicate; *p < 0.05, **p < 0.01 and ***p < 0.001 versus control shRNA and #p < 0.05 versus PGC1α shRNA. The whiskers in the box plots represent the maximum and the minimum value.

Figure 4. Glutamine utilization after PGC1α and HIF-1α suppression. A, Apoptosis in double PGC1α/HIF-1α−suppressed cells after exposure to the indicated nutrients. A375P cells expressing PGC1α shRNA and a doxycycline-inducible shRNA against HIF-1α were incubated for 4 days with 100ng/ml doxycycline and 5 mM glutamine, 25 mM glucose or 2 mM of the indicated amino acids or metabolites. B, C and D, Growth curves (B), ATP levels (C) and clonogenic cell survival (D) in PGC1α/HIF-1α double knockdown A375P cells after exposure to different concentrations of glutamine. E, Cell growth and ATP levels after pharmacological inhibition of glutamine utilization in PGC1α/HIF-1α double knockdown A375P cells. Cells were incubated with doxycycline, 5 mM glutamine and 25 mM glucose in the absence or presence of 10μM of compound 968 for 4 days as indicated. F, Glutamine oxidation in PGC1α/HIF-1α double knockdown A375P cells. Cells were incubated with 1mM glutamine and 14C-L-glutamine for 3 h, and then 14C labeled CO₂ was measured. G, Glutamine utilization in PGC1α/HIF-1α double knockdown A375P cells. H, The effects of glutamine utilization inhibition on tumor growth. Fifteen days post injection, mice were injected daily with compound 968 or DMSO and the tumor volume was
measured (n=8). I and J, ATP levels (I) and apoptosis (J) in PGC1α/ HIF-1α double depleted tumors described in (H). K, NAC does not rescue the growth of PGC1α/HIF-1α double knockdown A375P cells upon inhibition of glutamine utilization. Values represent mean ± SD of three independent experiments performed in triplicate; **p < 0.01, #p < 0.05; the whiskers in the box plots represent the maximum and the minimum value.
Figure 1

A. Cell Viability assay

- 4 days after puromycin
  - shScr vs. shPGC1α
  - Tubulin

- 20 days after puromycin
  - shScr vs. shPGC1α
  - PGC1α vs. Tubulin

B. Clonogenic assay

- 4 days after puromycin
  - shScr vs. shPGC1α

- 20 days after puromycin
  - shScr vs. shPGC1α

C. HIF-1α, PGC1α, Lamin B

D. Adeno: GFP FH-PGC1α

E. Xenograft tumors

- shScr vs. shPGC1α
  - HIF-1α
  - PGC1α
  - Lamin B

F. NES=2.53

G. Relative mRNA levels

H. Xenograft tumors - Glycolysis

- Fold induction
  - shScr vs. shPGC1α
  - HIF-1α, GLUT1, PGK1, PFKFB3, ALDOC, PKI, LDHA, CA-IX

I. Glucose (mmol/2x10⁴ cells) and Lactate (mmol/2x10⁴ cells)

J. Glucose (μmol/g protein), Lactate (μmol/g protein), ATP (μmol/g protein)

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Figure 2

A

B

C

D

E

F

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NAC
HIF-1α
PGC1α
Lamin B

Relative mRNA levels

GLUT1
ALDOC
PDK1
PGK1
CA-IX
VEGF
ANGPTL4

shScr + Vehicle
shScr + NAC (4mM)
shPGC1α + Vehicle
shPGC1α + NAC (4mM)

**
#

C

D

E

F

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HA

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OH-HIF-1α
HIF-1α
PGC1α
PHD2
Tubulin

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OH-HIF-1α
GST
HA

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Figure 3

A. Relative mRNA levels of various genes in different conditions.

B. Cell viability assay showing A375P cell numbers (×1000) over days after doxycycline treatment.

C. Western blot showing protein expression levels of HIF-1α, PGC1α, Cleaved Caspase-9, Cleaved-PARP, and Lamin B.

D. Clonogenic assay showing clonogenic cell numbers (×1000) for different conditions.

E. ATP levels (nmol/mg protein) for different conditions.

F. Tumor volume (mm³) over days after doxycycline treatment for different conditions.

G. mRNA fold induction for VEGF, GLUT1, PDK1, and ALDOC in different conditions.

H. Tumor volume (mm³) over days after doxycycline treatment for different conditions.

I. Tumor volume (mm³) over days after doxycycline treatment with different treatments.

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Figure 4

(A) Western blot analysis showing the expression of Caspase-9, Cleaved-Caspase-9, Cleaved-Caspase-3, Cleaved-PARP (Long), and Cleaved-PARP (Short) after treatment with glucose + 1mM glutamine or glucose + 5mM glutamine.

(B) Graph showing the cell numbers (X 1000) over days (with Doxycycline) for different treatments: shScr, shPGC1α, shPGC1α + shHIF-1α, Glucose + 1mM Glutamine, Glucose + 5mM Glutamine.

(C) Graph showing the ATP levels (nmol/mg protein) over days (with Doxycycline) for 1mM Glutamine and 5mM Glutamine treatments.

(D) Photograph of cells treated with shScr, shPGC1α, or shPGC1α + shHIF-1α.

(E) Graph showing the ATP levels (nmol/mg protein) for DMSO, 968 (10μM), and 968 (10μM) + 5mM Glutamine treatments.

(F) Graph showing the relative CO2 produced for different treatments: shScr, shPGC1α, shPGC1α + shHIF-1α.

(G) Graph showing the glutamine levels (mmol/2×10^4 cells) for different treatments: shScr, shPGC1α, shPGC1α + shHIF-1α.

(H) Graph showing the tumor volume (mm^3) over days after doxycycline treatment for different treatments: shScr + DMSO, shScr + 968, shPGC1α + shHIF-1α + DMSO, shPGC1α + shHIF-1α + 968.

(I) Graph showing the cell numbers (X 1000) for DMSO, 968 (10μM), and 968 (10μM) + 5mM Glutamine treatments.

(J) Western blot analysis showing the expression of PGC1α, HIF-1α, Cleaved PARP, and Tubulin after treatment with Compound 968.

(K) Graph showing the cell numbers (X 1000) for DMSO, NAC, 968 (10μM), and Glutamine (5mM) treatments.
Targeting mitochondrial oxidative metabolism in melanomas causes metabolic compensation through glucose and glutamine utilization

Ji-Hong Lim, Chi Luo, Francisca Vazquez, et al.

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