

Targeting Mitochondrial Oxidative Metabolism in Melanoma Causes Metabolic Compensation through Glucose and Glutamine Utilization

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Abstract

Metabolic targets offer attractive opportunities for cancer therapy. However, their targeting may activate alternative metabolic pathways that can still support tumor growth. A subset of human melanomas relies on PGC1 α -dependent mitochondrial oxidative metabolism to maintain growth and survival. Herein, we show that loss of viability caused by suppression of PGC1 α in these melanomas is rescued by induction of glycolysis. Suppression of PGC1 α elevates reactive oxygen species levels decreasing hypoxia-inducible factor-1 α (HIF1 α) hydroxylation that, in turn, increases its protein stability. HIF1 α reprograms melanomas to become highly glycolytic and dependent on this pathway for survival. Dual suppression of PGC1 α and HIF1 α causes energetic deficits and loss of viability that are partially compensated by glutamine utilization. Notably, triple suppression of PGC1 α , HIF1 α , 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. *Cancer Res*; 74(13); 3535–45. ©2014 AACR.

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 toward 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 anticancer therapies (9, 10).

We recently found that a subset of human melanomas overexpress the transcriptional coactivator PGC1 α that reprograms them to largely depend on mitochondrial oxidation for growth and survival (11). As a consequence, PGC1 α -positive melanoma tumors and derived cell lines are more sensitive to

mitochondrial inhibition (11, 12). On the other hand, PGC1 α -negative melanoma cells rely more on glycolysis and are largely insensitive to suppression of mitochondrial metabolism. These studies suggest that although the subset of PGC1 α -positive melanomas could be targeted by 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 HIF1 α , controls metabolic and cellular programs that support survival and tumorigenesis (13, 14). HIF1 α regulation is largely due to hydroxylation of two proline residues (402 and 564) catalyzed by prolyl hydroxylases enzymes (PHD; ref. 15). Prolyl hydroxylation of HIF1 α 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 HIF1 α stability under hypoxic conditions (16, 17). There is, however, HIF1 α accumulation in normoxic conditions through increases in reactive oxygen species (ROS), 2-oxoglutarate analogs, or iron chelation, which suppress PHD enzymatic activity. HIF1 α stability caused by changes in PHD activities is one of the major mechanisms by which HIF1 α controls expression of gene programs, including glycolysis and angiogenesis, that promote tumor progression (18, 19). In addition, because HIF1 α 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

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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 PGC1 α 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 HIF1 α leading to increased glycolytic rates. Subsequent suppression of HIF1 α results in an alternative metabolic compensation through increases in glutamine utilization that support melanoma growth and survival. Targeting PGC1 α , HIF1 α , 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, H₂O₂, 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-PGC1 α (H-300) were purchased from BioVision Research Products and Santa Cruz Biotechnology. Antibodies against cleaved caspase-3, 9, PARP, proline 564 hydroxylated HIF1 α , and total HIF1 α 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 5 mmol/L L-glutamine containing 10% FBS unless otherwise indicated. Lentiviruses were produced from HEK293T cells as previously described (26). Lentivirus particles were collected 48 hours after posttransfection and used to infect melanoma cells in the presence of 8 μ g/mL polybrene, and then infected cells were selected with 2 μ g/mL of puromycin or 7 μ g/mL blasticidin for 4 days before experiments. Doxycycline inducible shRNA pLKO-expressing melanoma cells were incubated with doxycycline (100 ng/mL) before experiments. GFP control and Flag-HA-PGC1 α adenovirus have been previously described (27).

Western blot analysis

Cells were lysed in a buffer containing 1% IGEPAL, 150 mmol/L NaCl, 20 mmol/L HEPES (pH, 7.9), 10 mmol/L NaF, 0.1 mmol/L EDTA, 1 mmol/L sodium orthovanadate and 1 \times 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 μ g of total RNA was used for cDNA synthesis using a 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 S3.

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 the manufacturer's instructions (Cayman Chemical Company).

Clonogenic assay

For clonogenic assay, 1 \times 10⁴ 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 10 minutes.

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 OD 450 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 OD 405 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 the manufacturer's instructions. Briefly, equal number of cells were seeded in 6-well plates and cultured in phenol-red-free DMEM for 24 hours. For glutamine assay, cells were cultured with 1 mmol/L glutamine and 25 mmol/L 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

A375P cells (1×10^6) stably expressing the indicated shRNAs were injected subcutaneously into the flank of nude mice (Taconic) in 100 μ L of media. Ten days after cell injection, mice were housed with 2 mg/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 (1 mg/kg), compound 968 (150 μ g), 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 nonnecrotic area were used for isolation of nucleus extracts, and then nucleus extracts were used to measure HIF1 α levels.

In vitro hydroxylation

GST-tagged HIF1 α 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 cell-derived PHD2 (50 ng) at 30°C for 1 hour in a reaction buffer containing 40 mmol/L Tris-HCl, pH 7.4, 4 mmol/L 2-oxoglutarate, 1.5 mmol/L FeSO₄, 10 mmol/L KCl, and 3 mmol/L MgCl₂. The hydroxylation at the Pro 564 residue was analyzed by immunoblotting using an anti-Pro564 (OH) antibody.

¹⁴C-L-glutamine oxidation assay

Scrambled, PGC1 α , or HIF1 α shRNA stably expressing A375P cells were cultured for 48 hours with 2 mmol/L glutamine and 25 mmol/L glucose-containing DMEM. At this time, medium was changed with 1 mmol/L L-glutamine and 25 mmol/L glucose-containing DMEM, and cells were further incubated for 12 hours. Then, cells were incubated with 2 μ Ci/mL of ¹⁴C-L-glutamine (PerkinElmer) for 3 hours. 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 PGC1 α (GSE7553) was analyzed with the GSEA algorithm. Thirteen gene sets generated from cells exposed to hypoxia from MsigDB (shown in Supplementary Table S1) were tested for enrichment in the PGC1 α -suppressed cells using the default parameters.

Results

PGC1 α suppress HIF1 α protein stability and glycolysis in melanoma cell lines and tumors

We have recently shown that PGC1 α is strongly over-expressed in a subset of melanoma tumors and derived cell lines (11). Depletion of PGC1 α in these melanoma cells using

a lentivirus encoding a targeting shRNA caused a decrease in cell viability as well as clonogenic cell survival (Fig. 1A). Although approximately 20% of the cells with suppressed PGC1 α died from apoptosis, the cells that survived could be grown for a long term. These PGC1 α -depleted surviving cells had only a slightly reduced growth rate compared with control cells, despite maintaining PGC1 α levels suppressed (Fig. 1A, top right). The surviving PGC1 α -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 HIF1 α promotes glycolysis, we probed the protein stability of this transcription factor. Figure 1B shows that HIF1 α protein levels were strongly increased after shRNA-mediated PGC1 α suppression in three PGC1 α -positive melanoma cell lines. HIF1 α reporter luciferase activity was accordingly elevated in these melanoma cell lines (Fig. 1C). The effect of PGC1 α shRNAs on HIF1 α protein stability was efficiently rescued by ectopic expression of PGC1 α (Fig. 1D). In addition, when melanoma cells were grown *in vivo* as xenografts, the increase in HIF1 α protein levels after PGC1 α suppression was maintained (Fig. 1E). Consistent with HIF1 α elevation, expression arrays performed after suppression of PGC1 α (GSE7553) showed an enrichment of hypoxia-induced signatures in PGC1 α -depleted compared with control melanoma cells (Fig. 1F). Specifically, 11 out of the 13 interrogated hypoxia-induced gene sets from MsigDB were enriched in PGC1 α -depleted cells (Supplementary Table S1). In agreement with this observation, qPCR analysis showed that HIF1 α target genes, including genes encoding for enzymes or proteins linked to glycolysis and lactate production, were increased upon PGC1 α suppression in melanoma cell lines (Figs. 1G and Supplementary Fig. S1B) and xenografted tumors (Fig. 1H). In contrast and as predicted, PGC1 α target genes, including mitochondrial oxidative phosphorylation and ROS detoxification genes, were substantially decreased (Supplementary Fig. S1A). Functionally, increases in HIF1 α target genes caused by knockdown of PGC1 α in cell lines and tumors resulted in increased glucose uptake and lactate production (Fig. 1I and J). Taken together, these results indicate that suppression of PGC1 α results in metabolic and energetic compensation through increases in HIF1 α protein stability and glycolysis.

PGC1 α expression causes reduction of HIF1 α protein stability through suppression of ROS production

As part of the oxidative metabolism program, suppression of PGC1 α results in an increase in intracellular levels of ROS (11). Elevated ROS levels are one of the mechanisms whereby HIF1 α protein is stabilized in normoxic conditions (28, 29). We used the antioxidant N-acetyl cysteine (NAC) to determine whether PGC1 α suppression-dependent HIF1 α protein stability was a result of increased ROS levels (30). Figure 2A shows that increased concentrations of NAC were sufficient to efficiently block HIF1 α protein stability in PGC1 α -suppressed A375P cells. Blockage of HIF1 α induction in these cells translated in a reduction in their ability to induce

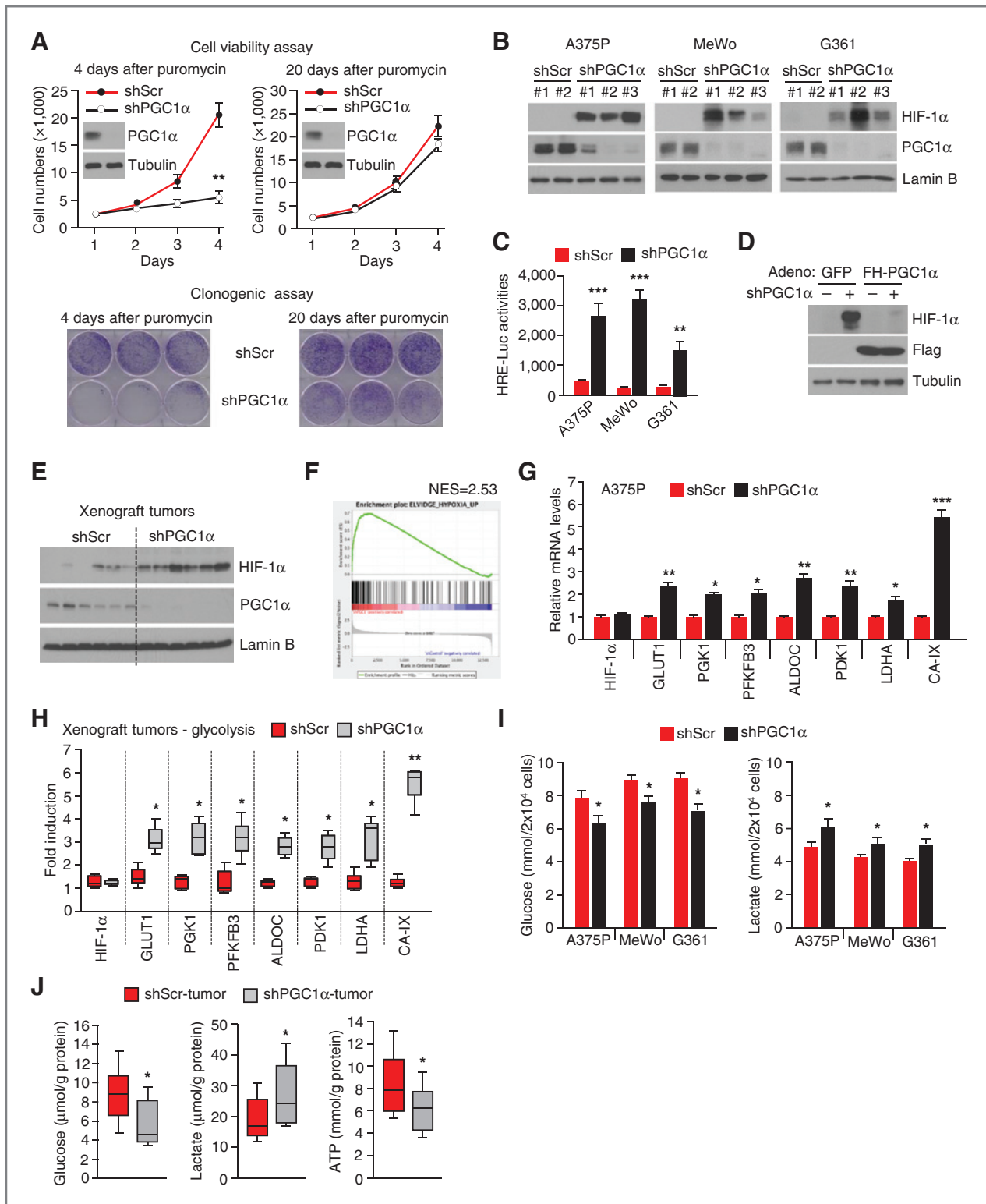


Figure 1. HIF1 α levels and stability in PGC1 α -suppressed cells. **A**, growth curve and clonogenic cell survival of A375P cells with transient (4 days) or chronic (20 days) PGC1 α suppression. **B** and **C**, chronic suppression of PGC1 α elevates HIF1 α protein expression and transcriptional activities in melanoma cells. **D**, HIF1 α levels after rescue of PGC1 α expression in PGC1 α -suppressed A375P cells. Mouse Flag-tagged PGC1 α was expressed exogenously using adenoviruses for 24 hours before harvesting. **E**, HIF1 α levels in A375P xenografts. **F**, GSEA enrichment plot for the top-scoring gene set. (Continued on the following page.)

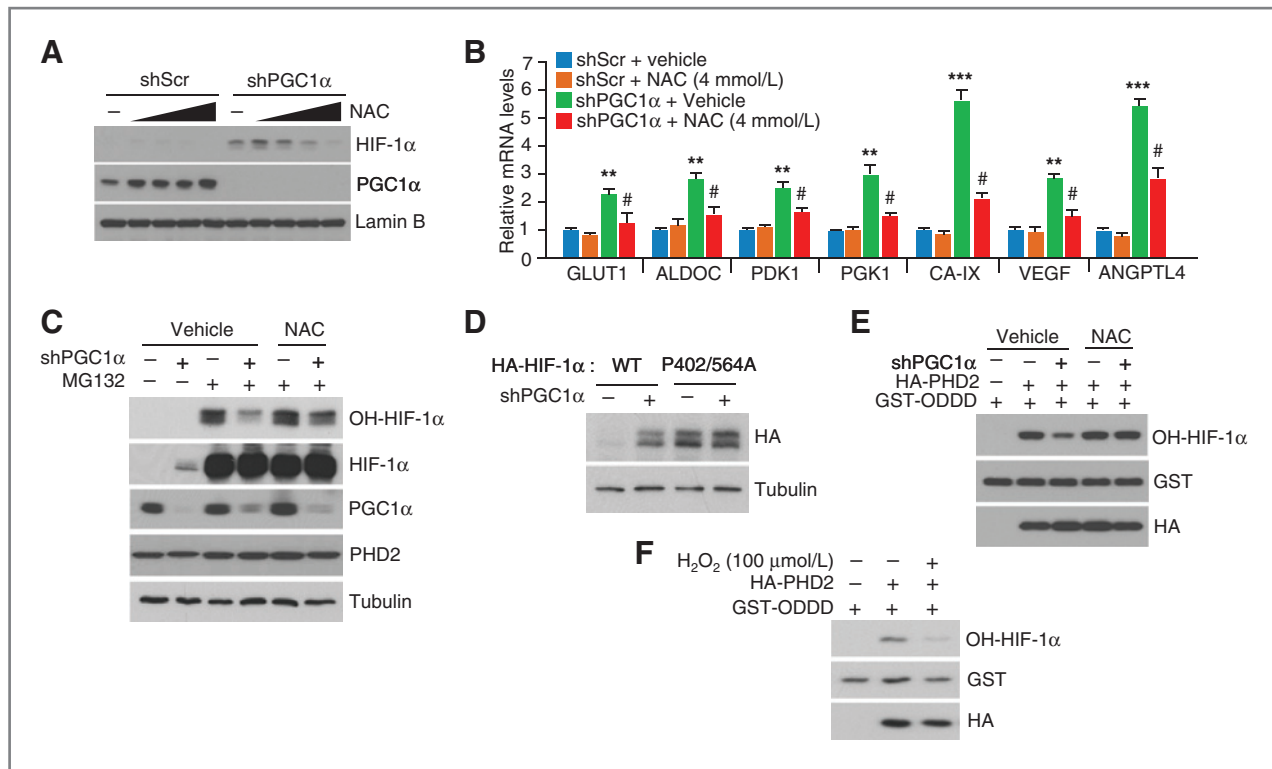


Figure 2. PGC1 α suppression enhances the stability of HIF1 α through increasing ROS levels. **A**, NAC blocks HIF1 α accumulation induced by PGC1 α suppression. PGC1 α -depleted A375P cells were incubated with NAC (0.5, 1, 2, or 4 mmol/L) for 48 hours, and then whole-cell lysates were used to measure HIF1 α protein levels. **B**, NAC attenuates the induction of HIF1 α glycolytic target genes after PGC1 α suppression. Total RNA was isolated from PGC1 α -depleted A375P cells incubated with 4 mmol/L of NAC for 48 hours. **C**, proline hydroxylation of HIF1 α is decreased in PGC1 α -suppressed melanoma cells. PGC1 α -suppressed A375P cells were incubated with 4 mmol/L NAC or vehicle for 48 hours, followed by incubation with 20 μ mol/L of MG132 or DMSO for 3 hours before harvesting. **D**, HIF1 α P402/564A mutant is not further stabilized after PGC1 α suppression. **E** and **F**, oxidative stress induced by PGC1 α depletion (**E**) or H₂O₂ (**F**) inhibits PHD2 enzymatic activity. Values represent mean \pm SD of three independent experiments performed in triplicate; **, $P < 0.01$; and ***, $P < 0.001$ versus control shRNA; and #, $P < 0.05$ versus PGC1 α shRNA.

glycolytic and angiogenic HIF1 α target genes (Fig. 2B). We have recently shown that PGC1 α levels define the metabolic state of melanomas (11). Interestingly, endogenous PGC1 α levels also modulated the magnitude of induction of HIF1 α protein stability in response to either hypoxia or ROS increases through hydrogen peroxide treatment, with a more robust induction of HIF1 α protein in PGC1 α -negative cell lines (Supplementary Fig. S2A–S2C). As expected, the induction of HIF1 α target genes in response to hypoxia or hydrogen peroxide was also more significant in PGC1 α -negative cell lines (Supplementary Fig. S2D and S2E). These results suggest that melanomas also select or switch to the HIF1 α -dependent alternative metabolic route in their natural history.

HIF1 α protein stability is regulated through prolyl hydroxylation of residues 402 and 564 (15). To determine whether

ROS-induced HIF1 α 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 HIF1 α prolyl hydroxylation in the presence of the proteasome inhibitor MG132 upon suppression of PGC1 α . However, this decrease was substantially prevented by treatment with the antioxidant NAC. Moreover, in contrast with the stability of wild-type HIF1 α protein, a defective HIF1 α proline hydroxylation mutant (P402/564A) showed no changes in protein stability after PGC1 α suppression (Fig. 2D). The fact that knockdown of PGC1 α increased HIF1 α protein stability and decreased prolyl hydroxylation suggests that the enzymatic activity of PHD2 was decreased. To test this possibility, we immunoprecipitated PHD2 from control and PGC1 α shRNA melanoma cell lines and measured the PHD activity using recombinant HIF1 α as a substrate (31). Figure 2E shows

(Continued.) Gene expression profile of A375P cells stably expressing control shRNAs or two different shRNAs against PGC1 α (GSE7553) was analyzed with the GSEA algorithm for enrichment of gene sets related to hypoxia. **G**, expression of HIF1 α glycolytic targets after PGC1 α knockdown. **H**, HIF1 α glycolytic target genes are increased in PGC1 α -depleted tumors. **I**, glucose utilization and lactate production in PGC1 α -depleted melanoma cells. **J**, glucose utilization, lactate production, and ATP levels in PGC1 α -depleted tumors. Values represent mean \pm 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.

that PHD2 enzymatic activity was decreased in PGC1 α -suppressed melanoma cells, but this activity was rescued by NAC treatment. In addition, hydrogen peroxide mimicked the effects of PGC1 α suppression, decreasing PHD2 activity (Fig. 2F).

Taken together, these results suggest that suppression of PGC1 α induces HIF1 α protein stability through induction of ROS levels and activation of PHD2 enzymatic activity in melanoma cells.

HIF1 α maintains survival and compensates metabolic and energetic PGC1 α function in melanoma cells and tumors

As illustrated in Fig. 1I, elevated levels of HIF1 α upon suppression of PGC1 α result in a metabolic reprogramming toward a more glycolytic metabolism. These results suggested that these cells might now depend on HIF1 α to maintain their energetic status and survival. To assess this possibility, we suppressed HIF1 α in PGC1 α knockdown cells using a doxycycline-inducible shRNA. Figure 3C shows that both PGC1 α and HIF1 α levels were efficiently suppressed after doxycycline treatment and HIF1 α targets were decreased (Fig. 3A). Of note, doxycycline did not have any effect on the expression of HIF1 α targets in control cells (Supplementary Fig. S3A). Double knockdown of PGC1 α and HIF1 α substantially reduced cell viability (Fig. 3B, left) 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 HIF1 α suppression in PGC1 α -depleted cells; therefore, HIF1 α depletion in these cells caused apoptosis, at least in part, through inhibition of the glycolytic flux. In addition, consistent with HIF1 α increase, hypoxia partially rescued cell death induced by acute PGC1 α knockdown (Supplementary Fig. S3B), further supporting the protective role of HIF1 α for cell survival upon metabolic stress. Next, to test how double HIF1 α /PGC1 α depletion decreased cell survival, we measured changes in metabolites and enzymes associated with ROS activities. Double HIF1 α and PGC1 α knockdown in A375P cells did not change NADPH and glutathione levels (Supplementary Fig. S3C), ROS detoxification gene expression (Supplementary Fig. S3D and S3F), ROS levels (Supplementary Fig. S3E), or antioxidant enzymatic activities (Supplementary Fig. S3G). Moreover, addition of polyethylene glycol (PEG)-conjugated superoxide dismutase or catalase, which repress intracellular oxidative stress (Supplementary Fig. S3H), failed to rescue cell viability in PGC1 α - and HIF1 α -suppressed melanoma cells (Supplementary Fig. S3I). Altogether, these results suggest that the loss of viability in double HIF1 α /PGC1 α -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 PGC1 α /HIF1 α knockdown in tumor maintenance, we injected cells expressing PGC1 α shRNA and inducible HIF1 α shRNA into nude mice and allowed the tumors to form before the induction of the HIF1 α shRNA with doxycycline. Although, suppression of

HIF1 α alone did not compromise the growth of the cells *in vitro*, there was an effect on tumor growth after the tumor reached approximately 500 mm³ in size (Fig. 3F). Consistent with our previous results, PGC1 α suppression resulted in a decrease in tumor growth. However, double knockdown of PGC1 α /HIF1 α 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 PGC1 α causes a HIF1 α -dependent bioenergetic switch toward glycolysis. Moreover, these results show that simultaneously targeting the PGC1 α and HIF1 α 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 PGC1 α sensitizes melanoma tumors to ROS-inducing drugs such as piperlongumine (11). Because levels of antioxidant enzymes were decreased to the same extent in single PGC1 α and double PGC1 α - and HIF1 α -suppressed cells, we determined whether increasing ROS levels would further reduce the growth of these tumors. Notably, piperlongumine completely prevented tumor growth of the double PGC1 α and HIF1 α -depleted cells (Fig. 3I and Supplementary Fig. S3J), which correlated with increased apoptosis markers (Supplementary Fig. S3K). In aggregate, these results indicate that ROS-inducing drugs will be very efficient in melanoma tumors in which PGC1 α and HIF1 α are inhibited.

Glutamine utilization maintains survival and compensates metabolic and energetic HIF1 α and PGC1 α function in melanoma cells and tumors

Despite the reduction of oxidative and glycolytic metabolism, PGC1 α - and HIF1 α -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 PGC1 α /HIF1 α -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 PGC1 α - and HIF1 α -suppressed cells, the 968 glutaminase inhibitor caused a substantial reduction in cell number and ATP levels (Fig. 4E). Next, we used ¹⁴C-glutamine to investigate the rates of glutamine utilization after depletion of PGC1 α /HIF1 α . In contrast with the increase in glucose utilization (Fig. 1I and J), PGC1 α -suppressed cells exhibited a decrease in glutamine utilization measured as release (Fig. 4F) and uptake

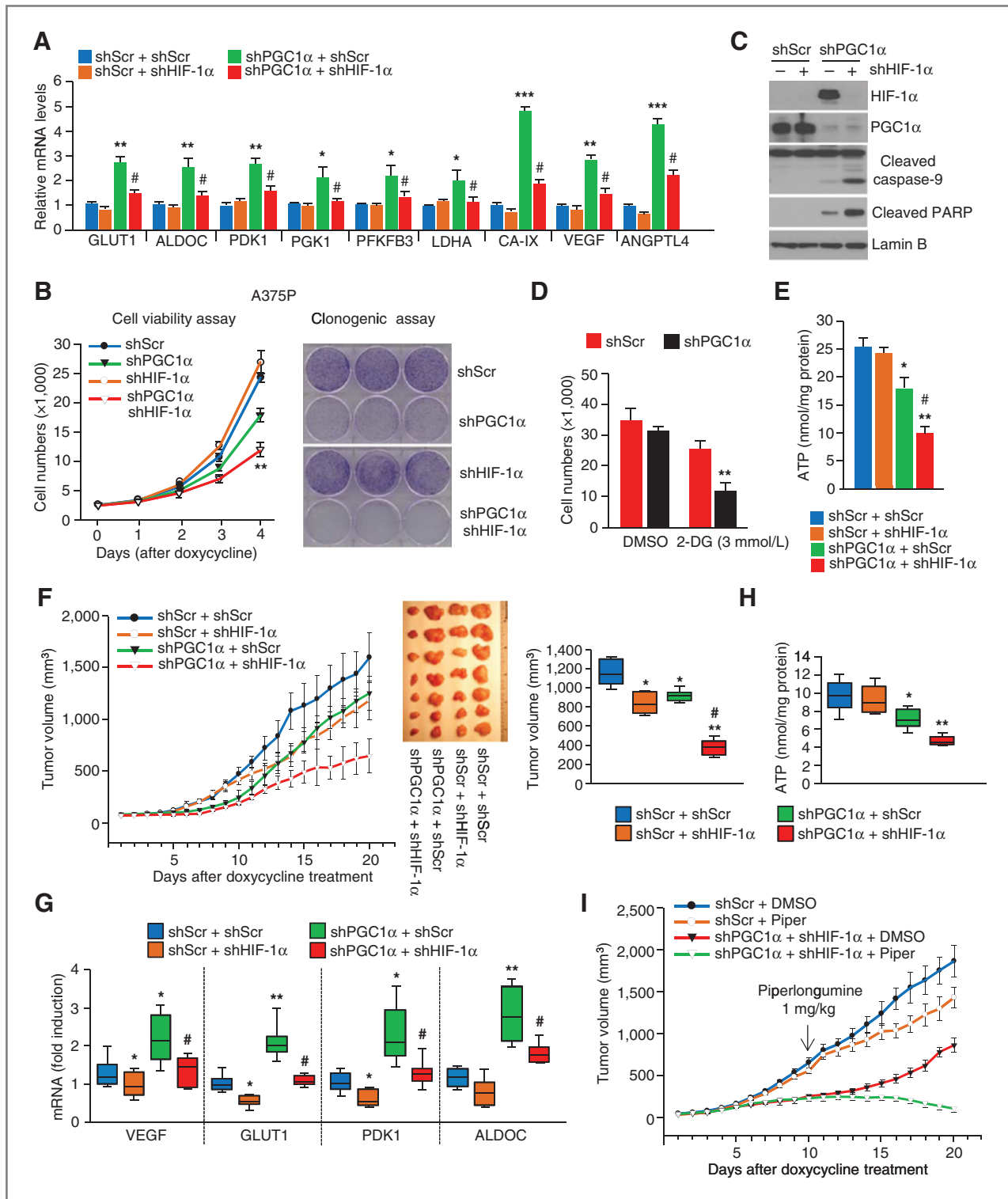


Figure 3. PGC1 α -suppressed melanoma cells are dependent on HIF1 α expression. **A**, expression of glycolytic genes after HIF1 α and PGC1 α suppression in A375P cells. **B**, growth curve and clonogenic cell survival of HIF1 α - and PGC1 α -suppressed A375P cells. **C**, detection of apoptosis in HIF1 α - and PGC1 α -suppressed A375P cells. **D**, cell growth after 2-deoxy-glucose (2DG) treatment in PGC1 α -suppressed A375P cells. **E**, ATP levels in HIF1 α - and PGC1 α -suppressed A375P cells. **F**, tumor growth after suppression of PGC1 α and HIF1 α . The tumor growth curves are plotted as mean \pm SEM ($n = 10$). **G**, inhibition of HIF1 α decreases glycolytic gene expression induced by PGC1 α depletion in tumors. **H**, inhibition of HIF1 α decreases ATP levels in PGC1 α -depleted tumors. **I**, effect of piperlongumine in HIF1 α - and PGC1 α -suppressed tumors. Mice were daily injected with piperlongumine (1 mg/kg) or DMSO for 10 days as described in Materials and Methods ($n = 8$). Values represent mean \pm 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 PGC1 α shRNA. The whiskers in the box plots represent the maximum and the minimum value.

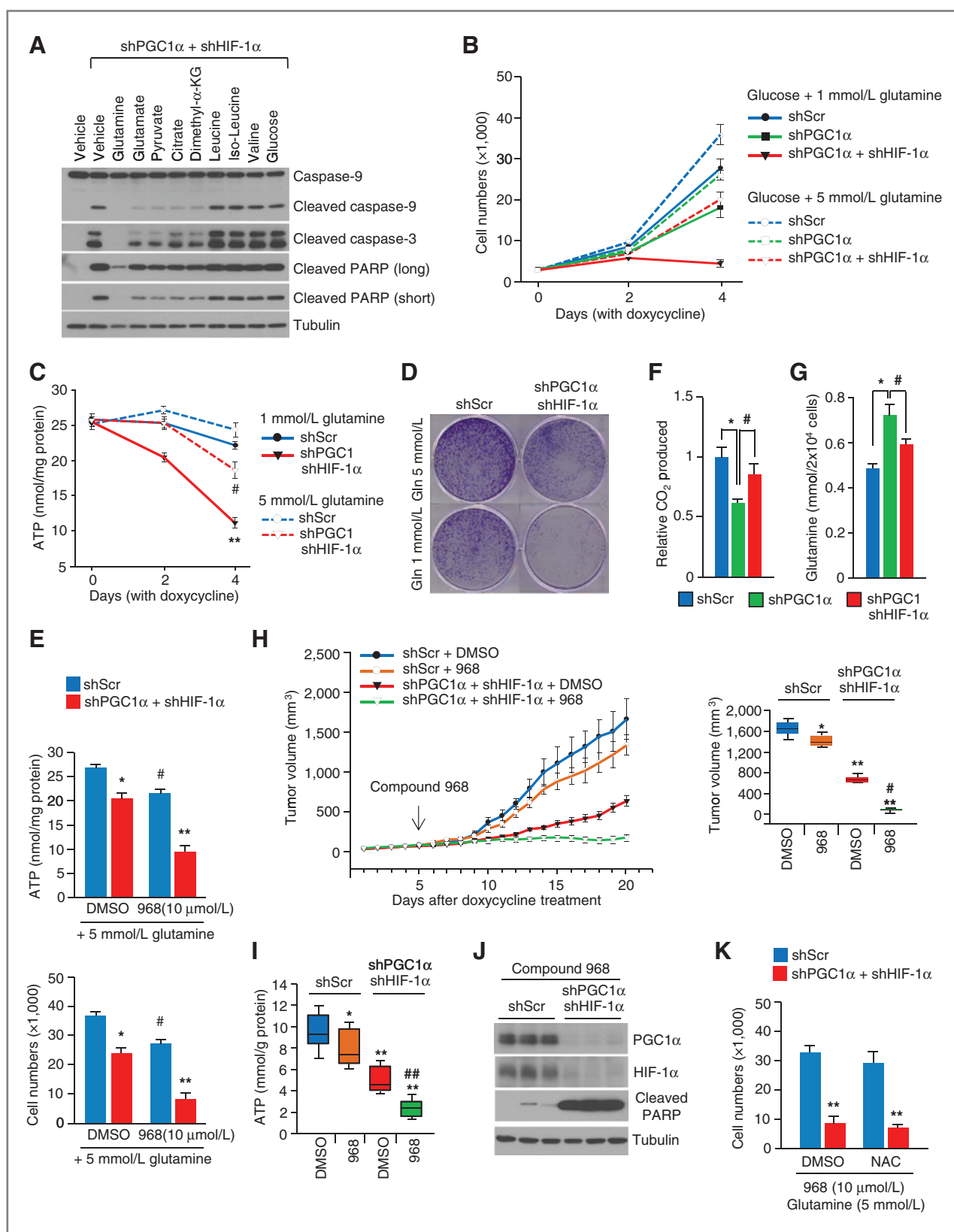


Figure 4. Glutamine utilization after PGC1 α and HIF1 α suppression. A, apoptosis in double PGC1 α /HIF1 α -suppressed cells after exposure to the indicated nutrients. A375P cells expressing PGC1 α shRNA and a doxycycline-inducible shRNA against HIF1 α were incubated for 4 days with 100 ng/mL doxycycline and 5 mmol/L glutamine, 25 mmol/L glucose, or 2 mmol/L of the indicated amino acids or metabolites. (Continued on the following page.)

(Fig. 4G) of $^{14}\text{CO}_2$. However, PGC1 α /HIF1 α -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 PGC1 α or PGC1 α /HIF1 α -suppressed cells with the 968 glutaminase inhibitor. Figure 4H shows that 968 had a small effect on the growth of control tumors but largely prevented tumor growth of PGC1 α /HIF1 α -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. Figure 4K shows that NAC does not rescue cell viability upon glutaminase inhibitor, suggesting that glutamine utilization conferring resistance to apoptosis induced by PGC1 α /HIF1 α 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 in which 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 PGC1 α expression in highly oxidative cells results in elevation of ROS levels, triggering an increase in HIF1 α stability and rewiring toward glycolysis. Subsequent inactivation of HIF1 α 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, PGC1 α and mitochondrial oxidative phosphorylation, HIF1 α 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 nongenetic adaptations,

similar to the adaptations occurring in liver or skeletal muscle. The abundance of alternative pathways and the existence of these nongenetic 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 nongenetic 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 it is possible to elicit a robust response.

A major cellular function of PGC1 α 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 PGC1 α that drives augmented mitochondrial oxidative phosphorylation and ROS detoxification capacities. This subset of tumors largely depends on PGC1 α for growth and survival and is more resistant to oxidative stress (11). Because mitochondrial oxidation generates ROS, the PGC1 α -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 PGC1 α -positive melanoma tumors to ROS-inducing drugs (11). Increased sensitivity to ROS after PGC1 α suppression is partially counterbalanced by the induction of HIF1 α protein stability that maintains the bioenergetic state and survival of the cells. This is consistent with the reported protective effects of HIF1 α in many tumor types (13) and with our data showing that ROS-inducing drugs are more effective after double suppression of PGC1 α and HIF1 α .

Low concentrations of oxygen are the main signal to increase HIF1 α protein stability. However, under normoxic conditions increases in ROS levels can also result in elevated HIF1 α protein stability (18, 35, 36). Our results show that PGC1 α suppression results in ROS-mediated increase in HIF1 α protein stability largely mediated through inhibition of PHD2. Although the mechanisms whereby ROS decreases PHD2 activity are unknown, our data suggest that PHD2

(Continued.) B–D, growth curves (B), ATP levels (C), and clonogenic cell survival (D) in PGC1 α /HIF1 α double-knockdown A375P cells after exposure to different concentrations of glutamine. E, cell growth and ATP levels after pharmacologic inhibition of glutamine utilization in PGC1 α /HIF1 α double-knockdown A375P cells. Cells were incubated with doxycycline, 5 mmol/L glutamine, and 25 mmol/L glucose in the absence or presence of 10 $\mu\text{mol/L}$ of compound 968 for 4 days as indicated. F, glutamine oxidation in PGC1 α /HIF1 α double-knockdown A375P cells. Cells were incubated with 1 mmol/L glutamine and ^{14}C -L-glutamine for 3 hours, and then ^{14}C -labeled CO_2 was measured. G, glutamine utilization in PGC1 α /HIF1 α double-knockdown A375P cells. H, the effects of glutamine utilization inhibition on tumor growth. Fifteen days after 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 PGC1 α /HIF1 α double-depleted tumors described in H. K, NAC does not rescue the growth of PGC1 α /HIF1 α double-knockdown A375P cells upon inhibition of glutamine utilization. Values represent mean \pm 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.

posttranslational modifications and/or differential protein interactions might account for the changes in enzymatic activity. In addition to the HIF1 α increase after PGC1 α suppression, our work also shows that natural PGC1 α -negative melanoma tumors have an increased sensitivity to induction of HIF1 α protein stability, suggesting that targeting PHD2 to inhibit HIF1 α protein stability and compromise energy metabolism could be particularly effective in PGC1 α -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 toward the use of glutamine (7, 8). Our results suggest that suppression of glucose metabolism by drug treatments could also result in a rewiring toward 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 PGC1 α and HIF1 α 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 PGC1 α and HIF1 α 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.-H. Lim, F. Vazquez, P. Puigserver

Development of methodology: J.-H. Lim

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Luo

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-H. Lim, C. Luo, F. Vazquez, P. Puigserver

Writing, review, and/or revision of the manuscript: J.-H. Lim, C. Luo, F. Vazquez, P. Puigserver

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