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New insight into the role of metabolic reprogramming in melanoma cells harboring BRAF mutations

Anna Ferretta [a,e], Immacolata Maida [a], Stefania Guida [b], Amalia Azzariti [c], Letizia Porcelli [c], Stefania Tommasi [d], Paola Zanna [a], Tiziana Cocco [a], Michele Guida [e,*], Gabriella Guida [a,**]

[a] Department of Basic Medical Sciences Neurosciences and Sense Organs, University of Bari, Bari, Italy
[b] Dermatology Unit, University of Modena and Reggio Emilia, Modena, Italy
[c] Clinical and Preclinical Pharmacology Laboratory, National Cancer Research Centre "Giovanni Paolo II", Bari, Italy
[d] Molecular Genetics Laboratory and Radiology, National Cancer Research Centre "Giovanni Paolo II", Bari, Italy
[e] Medical Oncology Department, National Cancer Research Centre "Giovanni Paolo II", Bari, Italy

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ABSTRACT

This study explores the ^{V600}BRAF-MITF-PGC-1α axis and compares metabolic and functional changes occurring in primary and metastatic ^{V600}BRAF melanoma cell lines.
^{V600}BRAF mutations in homo/heterozygosis were found to be correlated to high levels of pERK, to downregulate PGC-1α/β, MITF and tyrosinase activity, resulting in a reduced melanin synthesis as compared to BRAFwt melanoma cells. In this scenario, ^{V600}BRAF switches on a metabolic reprogramming in melanoma, leading to a decreased OXPHOS activity and increased glycolytic ATP, lactate, HIF-1α and MCT4 levels. Furthermore, the induction of autophagy and the presence of ER stress markers in ^{V600}BRAF metastatic melanoma cells suggest that metabolic adaptations of these cells occur as compensatory survival mechanisms. For the first time, we underline the role of peIF2α as an important marker of metastatic behaviour in melanoma.
Our results suggest the hypothesis that inhibition of the glycolytic pathway, inactivation of peIF2α and a reduction of basal autophagy could be suitable targets for novel combination therapies in a specific subgroup of metastatic melanoma.

1. Introduction

The study of molecular pathways involved in metastatic melanoma (MM) is challenging, and many routes have been reported to support proliferation and survival. Mutations in the BRAF gene have been identified in 50–70% of malignant melanomas. All the mutations occur within the kinase domain, and the specific mutation ^{V600}BRAF (V600E, V600K, V600R and V600D) accounts for approximately 90% of them. This mutation introduces a conformational change in the BRAF activation domain, which results in the constitutive activation of the protein and a major increase in its basal kinase activity. The RAS-RAF-MEK-ERK pathway plays a crucial role in regulating gene expression and cell proliferation, contributing both to melanoma formation and to progression [1].

ERK proteins, through the activation of p90RSK kinase, induce the phosphorylation of the Microphthalmia-associated transcription factor (MITF), increasing its activity, but undermining its intracellular stability. MITF, a master regulator of melanocyte development and melanomagenesis, [2] binds the M box in the tyrosinase promoter region, positively regulating tyrosinase and tyrosinase related protein 1/2 (TRP1/2) gene expression and thereby increasing melanogenesis [3].

MITF expression is also increased following Adenylate Cyclase (AC) stimulation induced by the α-MSH hormone [4]. In turn, the cAMP and

Abbreviations: ATP, adenosine triphosphate; BRAF, serine/threonine-protein kinase B-Raf; cAMP, cyclic adenosine monophosphate; CDK4, cyclin-dependent kinases 4; CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DNP, dinitrophenol; DO, dopa-oxidase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FSK, forskolin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H_2DCFDA, 2′-7′ dichlorodihydrofluorescin diacetate; HIF-1, hypoxia-inducible factor-1; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MBTH, 3-methyl-2-benzothiazolinone; MCT, monocarboxylate transporter; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; MM, metastatic melanoma; NDP-MSH, (Nle4,D-Phe7) alpha-melanocyte stimulating hormone; OCR, oxygen consumption rates; OXPHOS, oxidative phosphorylation; PBS, phosphate buffer saline; PDK1, pyruvate dehydrogenase kinase 1; PDH, pyruvate dehydrogenase; PKA, protein kinase A; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1; PMSF, phenylmethylsulfonyl fluoride; RHC, red hair color; ROS, Reactive oxygen species; TCA, tricarboxylic acid cycle; TH, tyrosine hydroxylase; TRP1/2, tyrosinase related protein 1/2.

* Correspondence to: M. Guida, Medical Oncology Department, National Cancer Research Centre "Giovanni Paolo II", Via Orazio Flacco, 65, 70124 Bari, Italy.
** Correspondence to: G. Guida, Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari, 70124 Bari, Italy.
E-mail addresses: micguida@libero.it (M. Guida), gabriella.guida@uniba.it (G. Guida).

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the RAS-RAF-MEK-ERK pathways cooperate to regulate melanocyte biology and stimulate melanogenesis, and their interplay is essential for MITF activity [5,6].

Another important pathway in melanocytes and melanoma development is the WNT/β-catenin signaling. Its activation, determined by Wnt glycoprotein family members binding to the Frizzled receptors leads to β-catenin activation as a transcription factor and increases C-Myc, PPAR1 and CyclinD1 expression [7].

In addition, ERK activation, constitutive in ^{V600}BRAF mutated melanoma cells, determines an induction of cyclin D1 expression through the activation of the AP1 and ets-2 expression factors [8–12]. In turn cyclin D1, binding CDK4, represses peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α) by phosphorylation, reducing its activity as a transcriptional coactivator [13].

In our study, in particular, attention has been focused on the role of oxidative phosphorylation (OXPHOS), known to be controlled by members of the PGC-1 coactivators family: PGC-1α and PGC-1β. The role of these proteins has been previously studied in metabolic alterations in breast and colon cancers [14–16]. PGC-1α, the most closely studied, is the main positive regulator of mitochondrial biogenesis, increasing the capacity for cellular energy production [17,18].

Although some of its functions overlap with PGC-1α, less is known about PGC-1β, that shows distinct functions in different tissues [19,20]. PGC-1α expression can be influenced by several signaling pathways and has been involved in angiogenesis development [21]. Recently, an important role of PGC-1α has been associated to melanoma resistance to *anti*-BRAF therapies, and its overexpression, driven by MITF in melanoma cells, influences metabolic homeostasis [22–24].

Several decades ago, Otto Warburg observed that cancer cells show a significantly higher glycolytic activity compared to normal cells even in the presence of an adequate oxygen supply [25]. This phenomenon occurs in normal proliferating and tumor cells [26,27], in order to provide building blocks for the synthesis of new macromolecules such as proteins, lipids and nucleic acids. Moreover, in melanoma development and progression, both autophagy and ER stress play an important role in energy regulation, although their true impact is still unclear [28]. Autophagy is the main process for bulk degradation and recycling of damaged intra-cellular components [29–31], providing nutrients during stress conditions, such as nutrient deprivation [32]. Autophagy activation is frequently observed in late stage malignancy. A deregulation of autophagy seems to be a secondary event contributing to melanoma progression, and to play an important role in chemoresistance. Likewise, ER stress may represent a key event in melanoma development. It is activated by the accumulation of unfolded proteins, protein overload or a depletion of ER calcium stores. It is mediated by specific signaling pathways, in order to maintain ER homeostasis promoting the induction of an Unfolded Protein Response (UPR), ensuring a cytoprotective and pro-survival activity [28]. Furthermore, a persistent ER stress condition may induce apoptosis [33].

In this study we analyse the ^{V600}BRAF-MITF-PGC-1α axis and compare metabolic and functional changes, autophagy and ER cellular stress occurring in melanoma cell lines harboring BRAF mutations. In particular, we focus on gaining a better understanding of relevant pathways and their correlation in order to develop new approaches for clinical therapeutic strategies.

2. Materials and methods

2.1. Cell lines

Melanoma cells, hmel1, M3, hmel9, and hmel11, were obtained from human sporadic melanoma biopsy specimens, after obtaining informed consent, and genotyped for NRAS, BRAF and MC1R [34,35] (Table 1). hmel1 and M3 arise from metastases while hmel9 and hmel11 arise from primary lesions. Melanoma cells HBL, used as control for the experiments, and LND1, both BRAFwt, were a gift from Prof. G. Ghanem, Université de Bruxelles, Belgium. Cell culture media used was high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, at 37 °C in a humidified atmosphere at 5% CO_2.

Table 1
Genotyping analysis of BRAF, NRAF and MC1R in melanoma cells lines through direct sequencing or next generation sequencing.

Cell line	Origin	MC1R	NRAS	BRAF exon 15
HBL	Metastasis	WT/WT	WT/WT	WT/WT
LND1	Metastasis	WT/WT	WT/WT	WT/WT
hmel1	Metastasis	WT/WT	WT/WT	V600K/WT
M3	Metastasis	D184H/D184H	WT/WT	V600E/V600E
hmel 9	Primary melanoma	T314T/WT	WT/WT	V600R/V600R
hmel 11	Primary melanoma	T314T/WT	WT/WT	V600R/WT

2.2. Tyrosine hydroxylase (TH) activity

Tyrosinase, as tyrosine hydroxylase (TH) activity, was assessed in cell lysates prepared as described in [34]. The tyrosine hydroxylase activity was determined by a radiometric method. One unit was defined as the amount of enzyme catalyzing the hydroxylation of 1 μmol of L-tyrosine/min in presence of a 50 μM concentration of the substrate and 10 μM L-dopa as cofactor.

2.3. Western blot analysis

Total cell proteins (30 μg) were separated on 13% Tris–Tricine SDS-PAGE according to [36] and transferred onto nitrocellulose membranes. Western blot analysis was performed using the specified primary antibodies against the: CI-39 kDa subunit, CIV-Cox IV subunit, porin (Invitrogen), monocarboxylate transporters MCT1 and MCT4 (Santa Cruz Biotechnology), MITF (Thermo Scientific), eIF2α and peIF2α (Santa Cruz Biotechnology) according to the manufacturer's suggested concentrations. Protein loading was assessed by reprobing the blots with β-actin (Sigma), or Tubulin (Sigma-Aldrich) antibodies. pERK was determined as described in Zanna et al. [35]. The lipid-conjugated form of LC3 (LC3 II) localises to the membranes of autophagosomes and it can be separated from non-conjugated form (LC3 I) by immunoblotting, since LC3 II migrates faster than LC3 I due to the extreme hydrophobicity of LC3 II. For LC3 detection, total cell proteins (45 μg) were separated on 12% Tris-Glycine SDS–PAGE and transferred onto nitrocellulose membranes. Western blot analysis was performed using a specific antibody against LC3B (Cell Signaling Technology).

The secondary antibodies were *anti*-rabbit-HRP, *anti*-mouse-HRP (Millipore) or *anti*-Goat IgG (SIGMA A-8919). Proteins were detected by chemiluminescent LiteAblot reagent (Euroclone) and the signal was quantified by densitometric analysis using Quantity One-4.4.1 imaging software (Bio-Rad Laboratories).

2.4. Cyclic adenosine monophosphate (cAMP) assay

The cAMP levels were determined by the direct cAMP ELISA Kit (Enzo Life Sciences). The cells were treated in the presence of 10^{-7} M α-MSH and 10^{-5} M forskolin for 30 min, used as a positive control, or DMSO used as a vehicle. For cAMP assays, the culture medium was removed and 1 ml of 0.1 M HCl was added to the cell layer, followed by 10 min incubation at 37 °C. The lysed cells were scraped and transferred into Eppendorf tubes. The samples were centrifuged at 1300 × *g* for 10 min. at 4 °C. The supernatants were used for cAMP measurements using a direct immunoassay kit (Assay Designs) according to the manufacturer's instruction. Measurements were performed on a Victor 2030 multilabel reader (PerkinElmer) and normalized on protein content.

2.5. Real-time PCR

Purification of total RNA from fibroblasts was carried out using the RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. One microgram of total RNA was then reverse-transcribed to generate cDNA for PCR using the I Script cDNA Synthesis Kit (Bio-Rad). q-PCR on cDNA was performed as previously described [37], using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin as internal control. Relative quantification was performed using the comparative C_T method ($\Delta\Delta C_T$). Validated primers for qRT-PCR were in Table S1.

2.6. Endogenous respiration rates in intact cells

Mitochondrial oxygen consumption was measured polarographically with a Clark-type oxygen electrode in a water-jacketed chamber (Hansatech Instruments, Norfolk, UK), magnetically stirred at 37 °C as previously described [37].

Cells were grown to 70% confluence and medium was changed the day before the measurement. Cells were collected by trypsinization and centrifugation, washed once in TD (0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris–HCl, pH 7.4), resuspended in the same buffer previously air equilibrated at 37 °C, and transferred into a polarographic chamber, at a final concentration of 1 to 3×10^6 cells per ml. After the native endogenous O_2 consumption rate was recorded, dinitrophenol (DNP) was added at a concentration of 30 μM, followed by 20 nM antimycin A to inhibit the upstream segment of the respiratory chain.

2.7. Total cellular ATP level

The melanoma cells were grown in T25 flasks. Once the cells were at 75% confluence, the ATP level was measured in untreated and 5 μM oligomycin-treated cells incubated for 30 min. After incubation the cells were collected by trypsinization and centrifugation at 500 ×*g* and then resuspended in phosphate-buffered saline, pH 7.4. Cellular ATP content was determined using the PerkinElmer "ATPlite" Kit (PerkinElmer) according to the manufacturer's instructions. Measurements were performed on a Victor 2030 multilabel reader (PerkinElmer) and normalized on protein content.

2.8. Lactate level

Cells were seeded in 60 mm plastic Petri dishes and cultured for 24 h. The amount of lactate in the cell medium was estimated as described in [38].

2.9. Intracellular ROS level

Quantitative analysis of ROS level was performed using the cell permeant probe 2′-7′dichlorodihydrofluorescin diacetate (H_2DCFDA). Cells were incubated with 10 μM H_2DCFDA in a serum-free medium in the dark at 37 °C for 30 min, collected by trypsinization, washed and resuspended in an assay buffer (100 mM potassium phosphate, pH 7.4, 2 mM $MgCl_2$). An aliquot was used for protein determination. The ROS-dependent oxidation of the fluorescent probe (507 nm excitation and 530 nm emission wavelength) was measured by a Jasco FP6200 spectrofluorometer, as described in [37].

2.10. Statistical analysis

Data of quantitative measurements are expressed as means ± SEM, and statistically analyzed with Student's *t*-test; a p value < 0.05 was set for statistically significant differences.

3. Results

3.1. pERK and cAMP levels in ^{V600}BRAF and wt melanoma cells

Table 1 shows the origin of the melanoma cells studied and their MC1R, NRAS and BRAF exon 15 genotype; some of them were already described in [34,35]. HBL and LND1 cells were wild type for (gene analyzed) MC1R, NRAS and BRAF and considered as our control. Instead, hmel1, M3, hmel9, hmel11 melanoma cells, harboring different ^{V600}BRAF mutations, were the experimental cell lines (Table 1). All melanoma cells arise from metastases, except hmel9 and hmel11 which were from primary melanoma lesions. The NRAS genotype was wt in all our analyzed cells.

As previously reported in Zanna et al., [35], we found higher levels of pERK in all melanoma cells harboring the ^{V600}BRAF mutation. In detail, we found the highest pERK levels in M3 and hmel1 cells, the former harboring a BRAF mutation V600E in homozygosis, the latter a V600K in heterozygosis (Fig. 1).

Intracellular cAMP levels activate signaling pathways that finally induce melanogenesis, maintaining the fine balance between proliferation and differentiation and playing an essential role during melanocyte or melanoma development. Therefore we measured basal cAMP levels in the M3 cell line (Fig. 2), in response to NDP-MSH or forskolin treatment, taking into account the previously reported results [35]. In particular, we compared three metastatic cell lines: M3 and hmel1 (^{V600}BRAF mutated) with HBL (BRAFwt) (Fig. 2). In accordance with our previous data [35], cAMP levels in response to NDP-MSH or forskolin were higher in BRAFwt melanoma cells (HBL) compared to ^{V600}BRAF melanoma cells (M3 and hmel1). Thus, both hmel1 and M3 melanoma cells did not show any significant cAMP activation after forskolin treatment. Although M3 carries a MC1R variant (Table 1), cAMP levels after α-MSH or forskolin stimulation were not dissimilar to those of hmel1 (MC1R wt). These results, in accordance with more data obtained on BRAF mutated melanoma cells (data not shown),

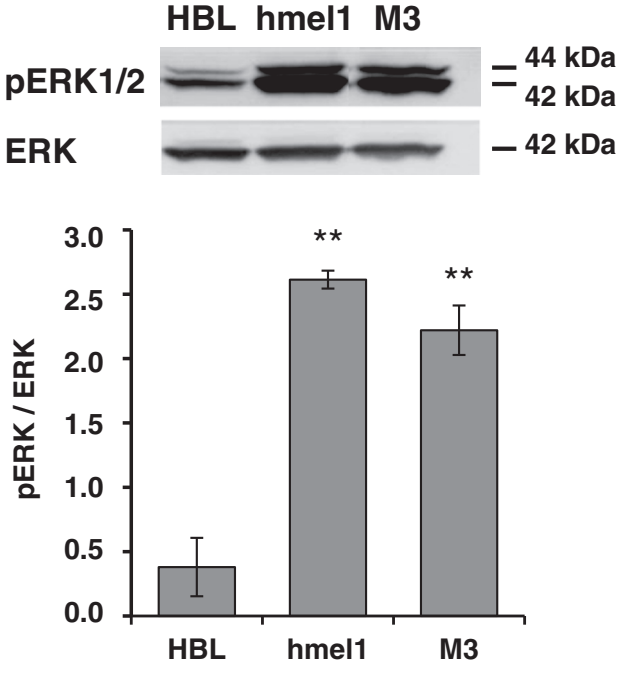

Fig. 1. Phosphorylation protein levels of Erk in wild type and ^{V600}BRAF melanoma cells. Representative Western blot of pERK and ERK levels on whole cell lysates from BRAFwt (HBL) and ^{V600}BRAF (hmel1 and M3) melanoma cells. The other melanoma cells are reported in Zanna et al. [35]. Bar graph shows quantification by densitometric analysis of pERK protein bands normalized to ERK, used as loading control. Values are means ± SEM of three independent experiments; significance was calculated with Student's *t*-test; $^*p < 0.05$, $^{**}p < 0.005$ vs HBL.

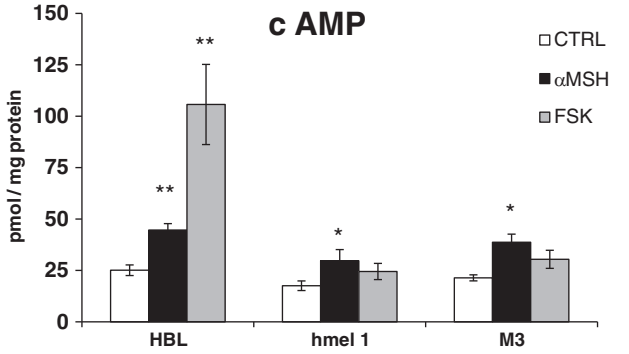

Fig. 2. Effect of ^{V600}BRAF on cAMP levels in total melanoma cell lysates. The graph displays the basal cAMP levels normalized for the total protein content in HBL, hmel1 and M3 melanoma cells (open bar), in the presence of α-MSH (black bar) or forskolin (grey bar). The cAMP levels of the other melanoma cells are reported in Zanna et al. [35]. Values are means ± SEM of three independent experiments; significance was calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.005$ vs HBL.

suggest the hypothesis that the ^{V600}BRAF mutation could affect cAMP levels in response to MC1R stimulation.

3.2. Pigmentation parameters: MITF levels and tyrosinase activity

MITF is considered a master regulator gene for pigment cells, having an important role as a transcription factor involved in melanocytes survival, growth and differentiation. MITF levels are also one of the key players in the phenotypic instability of melanomas [6]. For all melanoma cells we analyzed MITF expression levels by Western blot analysis (Fig. 3A) and their tyrosinase activity (Fig. 3B). ^{V600}BRAF cells displayed a significantly lower MITF expression than BRAFwt HBL and LND1 (Fig. 3A), with MITF values ranging from 30% (hmel1, M3) to 50% (hmel9, hmel11) compared to BRAFwt cells.

A crucial step for melanogenesis in melanoma cells is tyrosinase activity. This activity resulted significantly higher in the melanoma cells HBL and LND1 (NRAS, MC1R, BRAFwt), thus correlated to the pigmented phenotype of their cellular pellets (Fig. 3C).

Moreover, ^{V600}BRAF melanoma cells with lower MITF levels showed a reduced (by >50% in hmel9 and hmel11) or even almost absent (hmel1, M3) tyrosinase activity, compared to the higher levels in BRAFwt melanoma cells.

3.3. Expression levels of PGC-1α and PGC-1β in ^{V600}BRAF and wt melanoma cells

Recently, a"MITF-PGC-1α axis" has been described, involved in melanocyte development as well as in the transformation to melanoma [39]. Haq et al. [23] reported a decreased expression of the mitochondrial master regulator PGC-1α, involved in the regulation of metabolism, differentiation and cell growth in ^{V600E}BRAF cell lines. We investigated the PGC-1α pathways, focusing on the possible metabolic alterations in melanoma cells. First of all, we analyzed mRNA levels of PGC-1α and some of its downstream target genes, directly involved in mitochondrial biogenesis and activity (Fig. 4). Melanoma cells showed a very wide difference in PGC-1α expression related to the BRAF status. A downregulation of this coactivator was found in melanoma cells harboring ^{V600}BRAF (hmel1, M3, hmel9, hmel11), compared to HBL melanoma cells. mRNA levels of TFAM (mitochondrial transcription factor) and cytochrome *c* resulted significantly lower in all ^{V600}BRAF melanoma cells, compared to wt cells (HBL, LND1) (Fig. 4A), confirming an altered PGC-1α activity and transcriptional deregulation of its target genes.

Furthermore, we explored the expression of PGC-1β, a member of the PGC-1 family. PGC-1β shows a similar trend of PGC-1α expression, presenting very low levels in all ^{V600}BRAF melanoma cells (hmel1, M3, hmel9, hmel11), as compared to BRAFwt melanoma cells (HBL, LND1) (Fig. 4B).

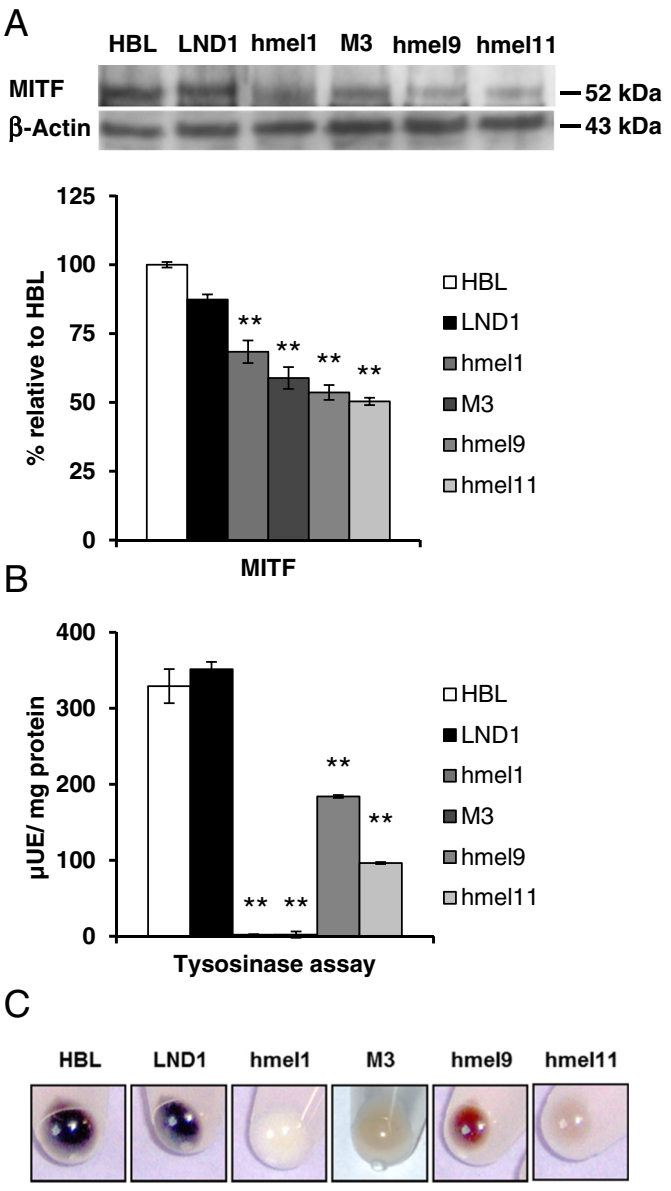

Fig. 3. Effect of ^{V600}BRAF on MITF protein expression and melanogenic enzyme activity. (A) Representative Western blot of MITF levels performed on whole cell lysates. Bar graph shows quantification by densitometric analysis of MITF protein bands normalized to β-actin, used as loading control. Values, means ± SEM of three independent experiments, are expressed as percentage of HBL values. (B) Bar graph shows tyrosinase activity expressed as µUE/mg protein. Values are means ± SEM of three independent experiments. Significance was calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.005$ vs HBL. (C) Pellets of wt and ^{V600}BRAF melanoma cell lines producing different amounts of eumelanin.

3.4. Functional analysis of the bioenergetic metabolism in wt and ^{V600}BRAF melanoma cells

Cancer cells need to regulate their metabolic program to fuel several activities such as unlimited proliferation, resistance to cell death, invasion and metastasis.

Taking into account the existence of a metabolic reprogramming in melanoma cells, with an energy production strategy change from mitochondrial oxidative phosphorylation to cytoplasmic aerobic glycolysis, we analyzed the mitochondrial respiratory function by measuring the oxygen consumption rates (OCR) by endogenous substrates

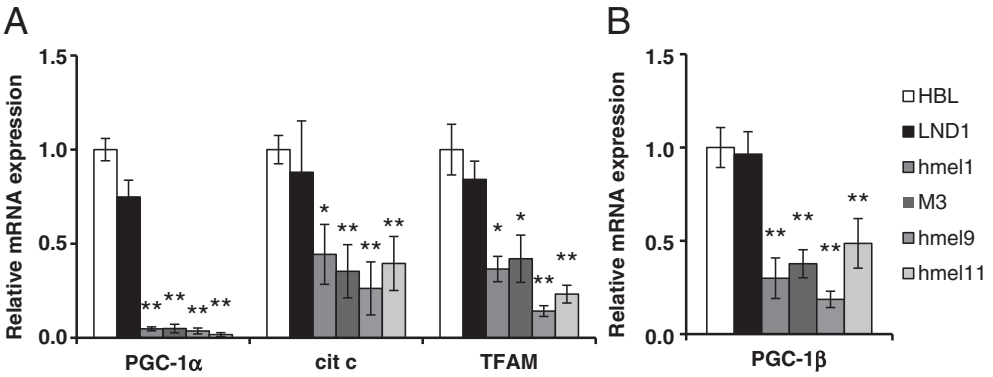

Fig. 4. PGC-1α expression and transcriptional activity in BRAFwt and ^{V600}BRAF melanoma cells. (A) Semi-qRT-PCR analysis of PGC-1α, its target genes (TFAM, cyt *c*) and (B) PGC-1β mRNA levels, normalized to the housekeeping gene GAPDH ($\Delta\Delta C_T$), in BRAFwt (open and filled bars) or ^{V600}BRAF melanoma cells (grey bars). Values are mean ± SEM of five independent experiments performed in duplicate. Significance was calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.005$ vs HBL.

(basal) in intact cells. As shown in Fig. 5A, the basal OCR was significantly lower in the ^{V600}BRAF melanoma cells compared to wt melanoma cells (HBL, LND1), whereas the maximal DNP-uncoupled respiration (DNP-uncoupled), in presence of a proton ionophore (Fig. 5B), was significantly lower exclusively in the primary melanoma cells (hmel9 and hmel11), as compared to the other MM cells (HBL, LND1, hmel1, M3). An important feature emerging from this assay (DNP-uncoupled) is the mitochondrial reserve capacity, calculated by subtracting the basal OCR from the DNP-stimulated rate. A lower maximal respiratory capacity observed in primary melanoma cells (hmel9 and hmel11) could indicate a decreased substrate availability or a compromised mitochondrial integrity (Fig. 5B). To investigate the relative contribution of mitochondrial OXPHOS and of glycolysis to ATP production, we determined the intracellular ATP content in the absence and in the presence of oligomycin, a specific inhibitor of mitochondrial F1F0-ATP-synthase (Fig. 5C). In all ^{V600}BRAF cells (hmel1, M3, hmel9, hmel11), the basal ATP levels did not differ significantly as compared to BRAFwt melanoma cells (HBL, LND1), but a significant decrease was observed in presence of oligomycin (hmel1 25.4 ± 2.8% M3 26.4 ± 1.3% hmel9 23.2 ± 4.3% hmel11 19.7 ± 3.2% of total ATP content) compared to BRAFwt melanoma cells (HBL 36.4 ± 7.4%; LND1 40.0 ± 12.2% of total ATP content).

These data support the hypothesis that the defective OXPHOS ATP production by ^{V600}BRAF cells could be compensated by an increased glycolytic supply. Consistent with the increased glycolytic ATP production, we found higher extracellular lactate levels measured in the culture media of all ^{V600}BRAF cells, as reported in Fig. 5D.

To assess whether an altered expression of mitochondrial proteins could underlie the reduction in the mitochondrial respiratory activity, we evaluated, by semi-quantitative Western blot analysis, the steady-state levels of some OXPHOS subunits (Fig. 5E). The results showed a significant decrease (by about 50%) in the protein expression level of complex I (CI) and complex IV (CIV) subunits, only in primary melanoma cells (hmel9 and hmel11) as compared to the HBL cells, while the mitochondrial outer membrane protein expression (porin) was significantly lower in all the ^{V600}BRAF cells (hmel1: 80 ± 6.7%, M3: 65 ± 12.2%, hmel9: 54 ± 7.4%, hmel11: 56.9 ± 7.9), compared to wt melanoma cells (HBL 100 ± % LND1:110 ± 8.8%).

Since mitochondria commonly metabolize oxygen, thereby producing reactive oxygen species (ROS) as a by-product, we evaluated ROS levels in all melanoma cells. As shown in Fig. 5F, significantly lower ROS levels, by about 50%, were observed in primary melanomas cells (hmel9 and hmel11) compared with MM cells values. These data indicate that during melanoma development in primary and metastatic lesions with the ^{V600}BRAF mutation status, an early activation of the BRAF-MEK-ERK pathway occurs, shifting the energy balance through the inhibition of mitochondrial OXPHOS.

The lactate produced by the enhanced anaerobic glycolysis, described in ^{V600}BRAF melanoma cells, must be eliminated from cells in order to avoid a drop in intracellular pH, which could result in cellular damage. To counteract this effect, HIF-1α, a master-transcriptional activator for a group of genes involved in cell survival, energy metabolism and pH regulation [40] induces the expression of monocarboxylate transporter 4 (MCT4), which mediates lactic acid efflux [40]. In fact, in ^{V600}BRAF melanoma cells, we detected significantly higher HIF-1α mRNA levels compared to HBL cells (HBL: 1; LND1: 1.2; hmel1: 2.9; M3: 1.6; hmel9: 2.8; hmel11: 1.5 fold increase) (Fig. 6A). Furthermore, in these cells, significantly higher levels of MCT4 were observed, in terms of both mRNA (Fig. 6B) and protein expression (Fig. 6C). No significant changes were observed in the protein expression of MCT1, which mediates lactic acid influx, in all ^{V600}BRAF melanoma cells (Fig. 6C).

3.5. ^{V600}BRAF results in increased basal autophagy and chronic ER stress induction

Recent data link oncogenic BRAF-induced chronic ER stress and autophagy [28]. We explored the activation of known pathways linking these two processes. We determined the basal cell autophagy in melanoma cells, performing immunoblot analysis of autophagic markers LC3. As shown in Fig. 7A, a highly significant increase of the LC3II/LC3I ratio was detected in all ^{V600}BRAF cells, hmel1 (96 ± 49%), M3 (113 ± 40%), hmel9 (122 ± 35%) and hmel11 (197 ± 32%) compared with HBL, BRAFwt melanoma cells. These results indicate the accumulation of auto-phagosomes in ^{V600}BRAF melanoma cells. Furthermore, the detection of ER stress was monitored in wt and ^{V600}BRAF cell lines, comparing the phosphorylation status of eIF2α by Western blotting analysis. Significantly high levels of p-eIF2α were observed only in MM ^{V600}BRAF cells, hmel1 (163 ± 7.3%) and M3 (139 ± 20%). Conversely, no significant changes were observed in primary melanoma cells hmel9 and hmel11 as compared with BRAFwt melanoma cells (Fig. 7B).

4. Discussion

Many studies indicate that several melanoma transformations require constitutive activation of the RAS-RAF-MEK-ERK cascade [1,41]. In fact, in ^{V600}BRAF melanoma cells, we found high levels of ERK phosphorylation, compared to BRAFwt cell lines. Activation of the ERK pathway is known to phosphorylate MITF [42], whose expression is regulated by the cAMP/PKA signaling pathway, via phospho-CREB, leading to a reduced melanin synthesis and, consequently hypopigmentation [43].

In fact, we observed that all ^{V600}BRAF melanoma cells have lower levels of cAMP after the stimulation assay, revealing an impaired MC1R coupling to the cAMP pathway, downregulation of MITF expression, a depressed activity of tyrosinase, a key enzyme in melanin biosynthesis, and impaired pigmentation. In detail, in ^{V600}BRAF

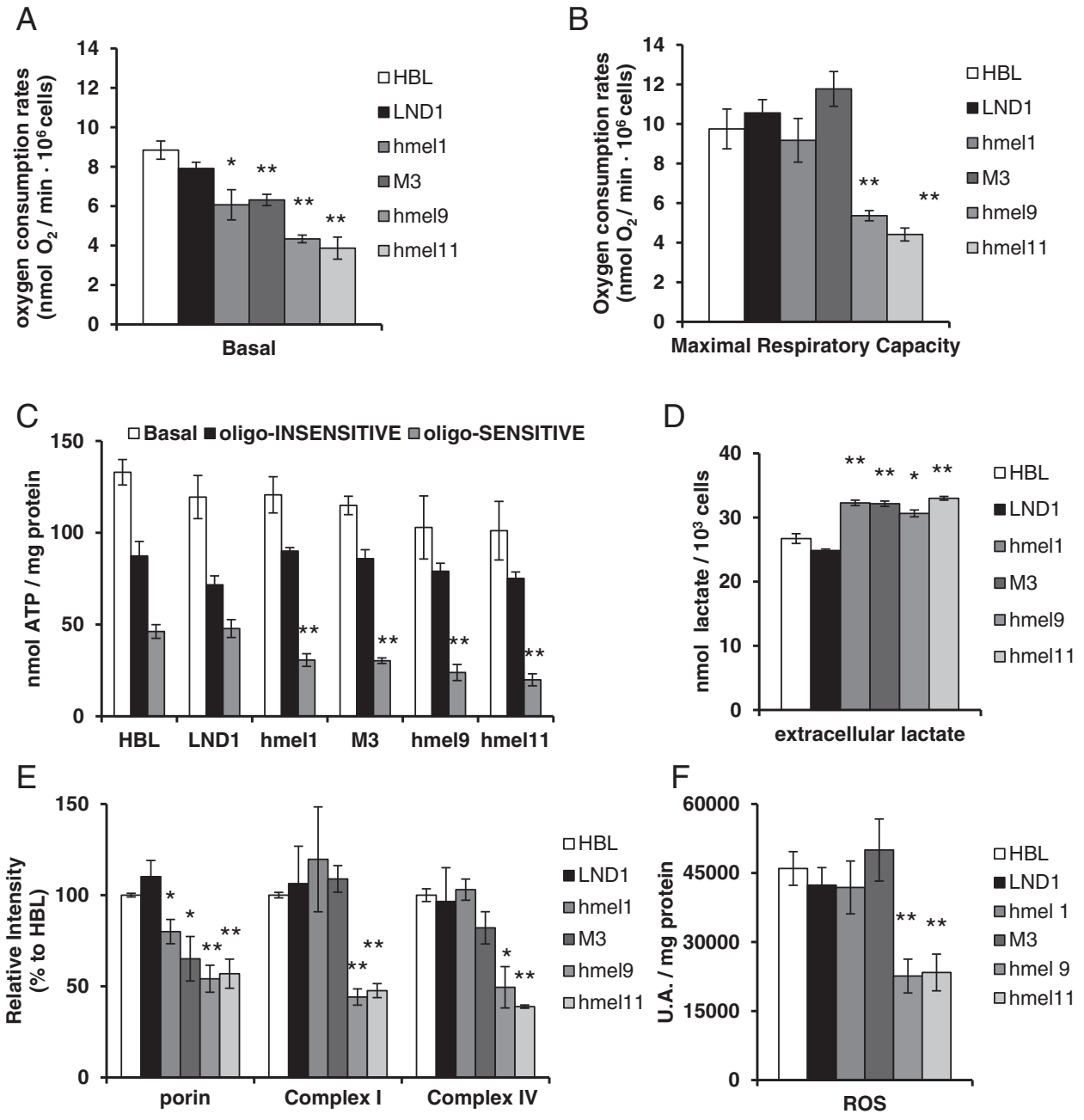

Fig. 5. Mitochondria functional analysis and bioenergetic metabolism in BRAFwt and ^{V600}BRAF melanoma cells. (A) Oxygen consumption rates (OCR) in the presence of endogenous substrates (basal) and (B) DNP (30 μM)-uncoupling agent (Maximal Respiratory Capacity), measured in intact melanoma cells and expressed as nmol O_2/min·10^6 cells. (C) Total cellular ATP content measured under basal conditions, (open bar). The oligomycin-insensitive (oligo-INSENSITIVE) ATP contents were determined in cells incubated for 1 h with oligomycin 5 μM, (filled bar). The oligomycin sensitive (oligo-SENSITIVE) ATP production was calculated by subtracting the oligomycin-insensitive value from the basal ATP content, (grey bar). Values are the means ± SEM of three independent experiment. (D) The extracellular lactate level was measured in the growth medium 24 h after seeding. Values are means ± SEM of three independent experiments. (E) Protein expression of Complex I (39 kDa) and Complex IV (Cox IV) respiratory chain subunits and of porin, performed on whole cell lysates. Bar graph shows quantification by densitometric analysis of mitochondrial protein bands normalized to β-actin, used as loading control. Data, means ± SEM of three independent experiments, are expressed as percentage of HBL values. (F) The intracellular ROS content was detected by DCF fluorescence in cells loaded with DCFH-DA (10 μM) for 30 min. Values are means ± SEM of three independent experiments. Significance was calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.005$ vs HBL.

melanoma cells, ERK activation, involved in the *anti*-melanogenic mechanism, lead to MITF and tyrosinase down-regulation, resulting in a reduction in melanin synthesis.

High levels of ERK phosphorylation, upon nuclear accumulation of ERK, overexpress cyclin D1, which is required for the growth and survival of melanoma cells in vitro and in vivo [44,45]. Bhalla et al. identified PGC-1α as a novel CDK4 substrate and further confirmed that cyclin D1/CDK4 represses PGC-1α activity [13]. Therefore, ^{V600}BRAF sustains the repression of OXPHOS gene expression by cyclin D1, via PGC-1α inactivation. In this context, our study identifies a pathway by which the oncogenic ^{V600}BRAF regulates the bioenergetic metabolism in melanoma, and it underlines how metabolic reprogramming is accompanied by a suppression of MITF and PGC-1α, a major regulator of mitochondrial biogenesis and function.

Furthermore, our findings show that ^{V600}BRAF activation in melanoma cells is associated with OXPHOS dysfunction and an increased glycolytic ATP and lactate production. The decreased OXPHOS will require a shift to other sources of energy generation to ensure the maintenance of cellular ATP levels. Cancer cells rely on the so-called "aerobic

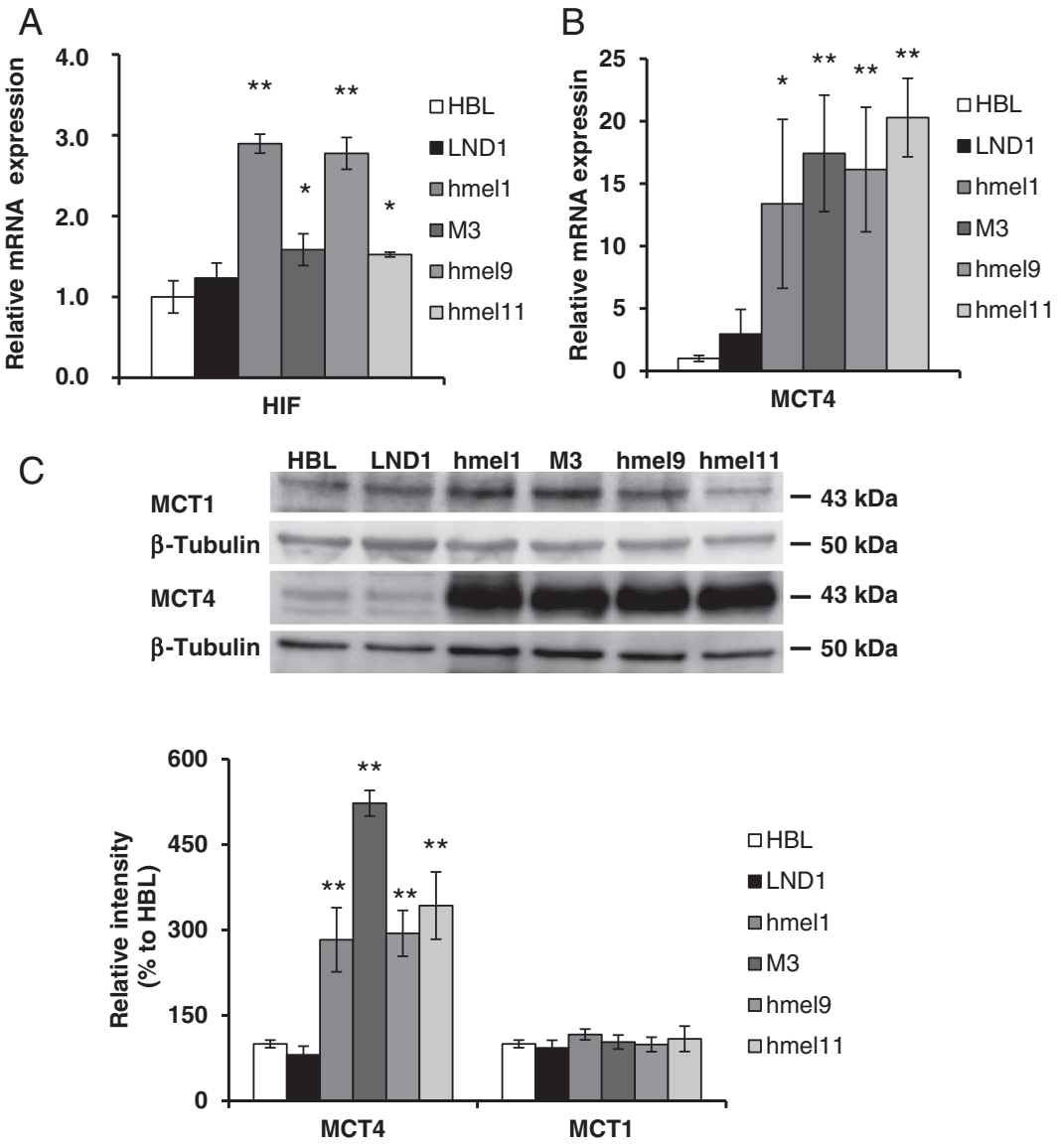

Fig. 6. Effect of HIF-1α on Monocarboxylate transporter (MCT) expression in BRAFwt and ^{V600}BRAF melanoma cells. Semi-qRT-PCR analysis of HIF-1α (A) and MCT4 (B) mRNA levels, normalized to the housekeeping gene GAPDH, in BRAFwt (open and filled bars) or ^{V600}BRAF (grey bars) melanoma cells. Values are means ± SEM of five experiments performed in duplicate. Significance was calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.005$ vs HBL. (B) Representative Western blot of MCT1 and MCT4 levels performed on whole cell lysates, from BRAFwt (open and filled bars) and ^{V600}BRAF (grey bars) melanoma cells. Bar graph shows quantification by densitometric analysis of MCT1 and MCT4 protein bands normalized to β-tubulin, used as loading control. Values are means ± SEM of five independent experiments. Significance was calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.005$ vs HBL.

glycolysis" or 'Warburg effect' [25], characterized by a reduced TCA cycle and OXPHOS with the concomitant production of ATP and lactic acid even in presence of oxygen [46].

It is noteworthy that while significantly lower basal oxygen consumption rates were observed in all ^{V600}BRAF melanoma cells, the DNP-stimulated rate was lower only in primary melanoma cells (hme9 and hmel11) supporting the hypothesis of a lower reserve capacity.

The shift to glycolysis, confirmed by the increased lactate levels, was found to be dependent on HIF-1α expression. In cancer cells, HIF-1α blocked pyruvate dehydrogenase (PDH) activity by activating enzyme pyruvate dehydrogenase kinase 1 (PDK1) [47–49]. This determines the fate of pyruvate, that is converted into lactate, instead of entering into the TCA cycle. This behavior is predominant in ^{V600}BRAF melanoma cells and contributes to the Warburg effect and to the enhancement of the malignant phenotype [50,51].

To counteract the increased lactate levels, HIF-1α induces monocarboxylate transporter 4 (MCT4), which mediates the lactic acid efflux required to preserve a normal intracellular pH [40]. In fact, in ^{V600}BRAF melanoma cells we found a higher HIF-1α expression, higher extracellular lactate levels, and increased MCT4 mRNA and protein levels, compared with BRAFwt cells, confirming the metabolic shift of these cells. It is known that in hypoxic cancer cells, the large amounts of exported lactate create an acidic tumor environment, which fosters cancer cell invasion [52].

It has been reported that the induction of autophagy may represent an adaptation mechanism for cancer cells exposed to an acidic environment [53] and, furthermore, that ^{V600}BRAF induces senescence triggering autophagy, possibly by inhibition of the mTOR pathway [54]. In fact, in our study, we detected the induction of autophagy-related markers expression (LC3), in ^{V600}BRAF melanoma cells as compared to BRAFwt cells.

We can hypothesize that the described ^{V600}BRAF melanoma cells drive a significant upregulation of autophagy as a compensatory survival mechanism to counteract apoptotic signals. Metabolic activity and cell survival are consequently sustained by the recycling of degradation products, allowing a steady supply of nutrients, and removal of

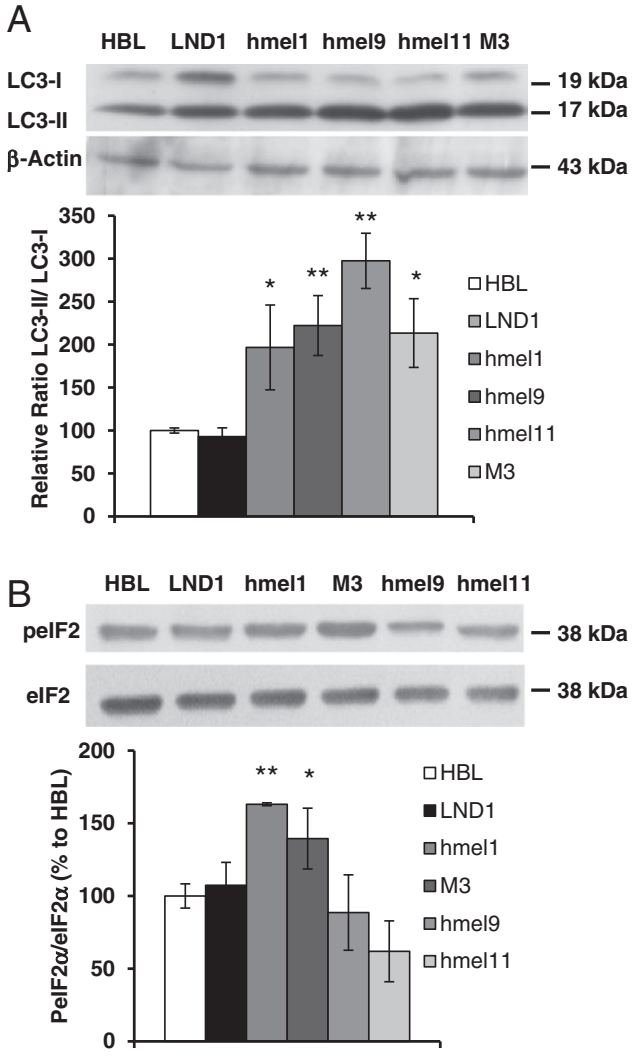

Fig. 7. Autophagy and ER stress in BRAFwt and ^{V600}BRAF melanoma cells. (A) Representative Western blot of LC3-I and LC3-II levels performed on whole cell lysates. Bar graph shows the LC3-II/LC3I ratio calculated by densitometric analysis of the protein bands normalized to β-actin, used as loading control. Value, means ± SEM of five independent experiments are expressed as percentage of the HBL value. (B) Representative Western blot of eIF2α and peIF2α levels performed on whole cell lysates. Bar graph shows the peIF2α/eIF2α ratio calculated by densitometric analysis of the protein bands, using eIF2α as loading control. Value, means ± SEM of three independent experiments are expressed as percentage of the HBL value. Significance was calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.005$ vs HBL.

damaged organelles to oppose the rapid depletion of intracellular nutrients and a buildup of aggregated proteins and damaged organelles [28].

Corazzari et al. described in a recent paper that autophagy and ER stress are activated in melanoma cells, having a cytoprotective and pro-survival activity [28]. Furthermore, it is known that phosphorylation of the α-subunit of the translation initiation factor eIF2 at serine 51 (peIF2α) is a master regulator of cell adaptation to various forms of stress, acting as a molecular switch that dictates either cell survival or death [55].

In our study we highlight an increase of peIF2α in MM but not in primary ^{V600}BRAF cells, thus delineating autophagy activation by ER stress as a pro-survival mechanism in peIF2α-proficient cells (MM ^{V600}BRAF cells). To our knowledge, this is the first report of peIF2α as a marker of a more aggressive phenotype in melanoma.

In conclusion, the innovative findings of our research strongly suggest that metabolic pathways, autophagy and ER stress may be important targets for combination therapies in metastatic melanoma.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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