Short Communication

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BNIP3 contributes to the glutamine-driven aggressive behavior of melanoma cells

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Abstract: Aerobic glycolysis ('Warburg effect') is used by cancer cells to fuel tumor growth. Interestingly, metastatic melanoma cells rely on glutaminolysis rather than aerobic glycolysis for their bioenergetic needs through the tricarboxylic acid (TCA) cycle. Here, we compared the effects of glucose or glutamine on melanoma cell proliferation, migration and oxidative phosphorylation *in vitro*. We found that glutamine-driven melanoma cell's aggressive traits positively correlated with increased expression of HIF1 α and its pro-autophagic target BNIP3. BNIP3 silencing reduced glutamine-mediated effects on melanoma cell growth, migration and bioenergetics. Hence, BNIP3 is a vital component of the mitochondria quality control required for glutamine-driven melanoma aggressiveness.

Keywords: autophagy; bioenergetics; BNIP3; glutamine; melanoma cell; mitochondria.

Cutaneous malignant melanoma (from here onwards, melanoma) is a very aggressive cancer that arises from the malignant transformation of the melanocytes from the skin and that is characterized by a low 5-year survival rate, especially under the presence of metastasis in distant organs (Schadendorf et al., 2015).

Although the 'Warburg effect' – or the ability of cancer cells to ferment glucose into lactate under aerobic conditions (Lu et al., 2015) – is a trait of aggressive cancer cells,

recent evidence indicates that oxidative phosphorylation (OXPHOS) and aerobic glycolysis cooperate together to boost both cancer cell's biomolecule anabolism and the suppressive tumor microenvironment (Pavlova and Thompson, 2016). In particular, fatty acid oxidation and glutaminolysis have been shown to be vital anaplerotic routes that fuel the tricarboxylic acid (TCA) cycle to keep OXPHOS levels high in cancer cells (Icard et al., 2014; Pavlova and Thompson, 2016; Dornier et al., 2017). Recent studies based on glutamine deprivation from the medium (Ratnikov et al., 2015) or glutamine transporters' inhibition (Wang et al., 2014) have underscored that melanoma cells become addicted to glutamine in opposition to melanocytes (Scott et al., 2011). However, the role of these metabolic substrates in melanoma progression remains controversial. On the one hand, it has been suggested that melanoma can be categorized in two different groups according to the ratio between glycolytic and OXPHOS components' expression: one subset would rather rely on aerobic glycolysis (consistent with the 'Warburg effect') whereas another subset would combine a high glycolytic state with increased OXPHOS levels (Feichtinger et al., 2018). Furthermore, melanoma cells that dedifferentiate and switch from OXPHOS to aerobic glycolysis seem to be more resistant to anoikis, eventually promoting metastasis (Bettum et al., 2015). On the other hand, an increased usage of anaplerotic routes such as glutaminolysis and fatty acid oxidation has also been described as a metastatic melanoma cell's feature (Rodrigues et al., 2016). Regardless of its role as a main carbon source, glutamine addiction would require the maintenance of a pool of mitochondria able to assimilate efficiently glutamine-derived intermediates. Therefore, this *in vitro* study was designed to elucidate the contribution of each of these two major carbon sources, glucose and glutamine, to some vital traits of melanoma aggressive behavior, such as growth and migration. Moreover, we aimed to unravel key molecular pathways contributing to the maintenance of mitochondrial homeostasis under different metabolic fuels.

To characterize the effects of the metabolic substrates glucose and glutamine in melanoma aggressiveness, we cultured murine B16-F10 and human A375p melanoma

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cells in medium supplemented with either glucose (indicated in the figures as Glc) and no L-glutamine (from here onwards glutamine) or with glutamine and no glucose (indicated in the Figures as Q), as in previous studies (Ratnikov et al., 2015).

We first assessed the ability of these melanoma cell lines to proliferate and migrate in culture medium supplemented only with glucose (Glc) or glutamine (Q). Whereas BRAF^{WT} murine B16-F10 melanoma cells slowly grew in medium containing only glucose, their proliferation was clearly boosted in glucose-free medium supplemented with glutamine (Figure 1A). Similar results were obtained with the human melanoma cell line A375p, which harbors the BRAF^{V600E} mutation (Figure 1B). These results indicate that – irrespective of their different origin (i.e. murine or human) or BRAF status – melanoma cells prefer to use glutamine as a metabolic substrate to grow, in line with recent reports (Ratnikov et al., 2015). Furthermore, glutamine significantly enhanced the migratory ability of these melanoma cells (Figure 1C). As enhanced proliferation and migration are hallmark features of aggressive melanoma cells, we wondered how glutamine could promote these malignant cellular traits. HIF1 α is a transcription factor upregulated under hypoxia (<1% O₂) which has been described to foster the invasive phenotype of melanoma cells (Widmer et al., 2013). In agreement with this, high HIF1 α levels in metastatic melanoma patients have been positively correlated with poor prognosis (Martinez-Garcia et al., 2017). Interestingly, glutamine significantly increased HIF1 α protein levels in both melanoma cell lines under normoxic conditions, along with its downstream targets BNIP3 and Twist (Figure 1D), key players in the pro-invasive phenotype (Yang et al., 2004; Maes et al., 2014).

The pro-tumorigenic effects induced by glutamine in these melanoma cell lines raised more questions about the metabolic changes behind them. Notably, not only is HIF1 α a well-known driver of glycolysis (Semenza, 2013) but it has also been proposed that, under hypoxia, it would favor glutamine's reductive metabolism (Fendt et al., 2013). To further characterize the bioenergetic profiles of the melanoma cells cultured in the presence of either glucose or glutamine, we analyzed their basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using Seahorse technology. As expected, when compared to medium containing glucose alone, glutamine heightened oxygen consumption (Figure 2A) whereas it significantly decreased medium acidification (mainly driven by lactate production under glycolytic conditions; Figure 2B) in both murine and human melanoma cell lines. This is consistent with the reported increase in

OCRs coupled to ATP synthesis observed in metastatic melanoma cells (Rodrigues et al., 2016).

We next wondered whether intracellular reactive oxygen species (ROS) levels, major OXPHOS byproducts as well as drivers of HIF1 α stabilization (Movafagh et al., 2015), were affected when melanoma cells were grown in the presence of glutamine or glucose. Melanoma cells grown under glutamine conditions showed decreased intracellular hydroxyl/peroxyl ROS (Figure 2C) and superoxide (Figure 2D) levels when compared to glucose-fueled cells. This is in line with the known role of glutamine in maintaining cancer cell's redox state. On the one hand, glutamate production via glutamine catabolism (glutaminases) serves as a crucial precursor of glutathione (Mates et al., 2002). On the other hand, glutamine – by fueling the TCA – allows metabolic intermediates such as citrate to be diverted and exported into the cytosol, where they can be used to generate reducing power in the form of NADPH by either the malic enzyme or isocitrate dehydrogenase (Fendt et al., 2013; Yang et al., 2014; Murai et al., 2017).

Another cellular process supporting cancer progression that has been associated to glutamine metabolism is autophagy (Eng et al., 2010; Strohecker et al., 2013). Autophagy has been extensively described as one of the double-edged processes in melanoma progression: in healthy melanocytes and primary melanoma, autophagy would contribute to restrain malignant transformation by several quality control mechanisms and by inducing senescence (Liu et al., 2013). Instead, in full-blown malignant melanoma, autophagy is upregulated to withstand otherwise lethal conditions found in the increasingly hostile tumor microenvironment or encountered during dissemination, such as hypoxia or anoikis, respectively (Demirsoy et al., 2016). Moreover, not only autophagy supplies through protein degradation essential amino acids like glutamine to support oncogene-driven cancer growth (Strohecker et al., 2013), but also the byproducts of glutamine metabolism increase the autophagic flux in different cancer cell lines (Eng et al., 2010). In particular, cancer cells exposed to glutamine as main carbon source displayed increased oxygen consumption coupled to enhanced mitophagy in order to remove damaged mitochondria via the recruitment of Rheb to the outer mitochondrial membrane (Melser et al., 2013). In both melanoma cell lines used in this study, we observed a slight, yet non-significant increase in the levels of autophagosome-bound LC3B-II and p62 upon glutamine supplementation (Figure 2E and F) when compared to its glucose counterpart. To gain insight into the autophagic flux under these conditions, we used the autophagic flux blocker bafilomycin A (Baf-A), which by altering lysosomal pH prevents the end-stage of autophagy, i.e. degradation

Figure 1: Glutamine fuels melanoma cell growth and migration *in vitro*.

Melanoma cell lines B16-F10 and A375p were routinely maintained in RPMI1640 (B16-F10; Sigma-Aldrich, Bornem, Belgium) or Dulbecco's Modified Eagles Medium (DMEM, A375p; Sigma-Aldrich) supplemented with 10% (v/v) Fetal Bovine Serum (FBS; Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 4 mM (only A375p) L-glutamine (Sigma-Aldrich), respectively, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich). All experiments were conducted in DMEM medium (without glucose, L-glutamine, phenol red, sodium pyruvate and sodium bicarbonate; Sigma-Aldrich), only enriched with 10% (v/v) FBS (Hyclone), 1% (v/v) non-essential amino acids (NEAA, Sigma-Aldrich), 100 units/ml penicillin, 0.1 mg/ml streptomycin supplemented with either glucose (25 mM, Glc; Sigma-Aldrich) or L-glutamine (2 mM, Q). (A) B16-F10 cell number count (trypan blue exclusion method) after 48 h of incubation (37°C, 5% CO₂) with medium containing either glucose or glutamine. Pull of three independent biological experiments performed at least in triplicates is shown. (B) A375p cell number count (trypan blue exclusion method) after 48 h of incubation (37°C, 5% CO₂) with medium containing either glucose or glutamine. Pull of three independent biological experiments performed at least in triplicates is shown. (C) Scratch wound assay performed in B16-F10 cells in glucose-only (25 mM, Glc) or glutamine-only (2 mM, Q) medium. Cells were plated to reach full confluence in their corresponding DMEM medium after 24 h. Prior to the scratching, cells were treated with mitomycin C (5 µg/ml; Sigma-Aldrich) to block cell proliferation. Cultures were further incubated and photographed at 0 h, 12 h and 24 h after scratching using the microscope Olympus IX73 (Olympus, Shinjuku, Japan). Migration distance was quantified with Image J software (NIH, Bethesda, MD, USA). Scale bar represents 400 µm. Pull of three independent biological experiments performed at least in triplicates is shown. The experiment was statistically analyzed using RM one-way analysis of variance (ANOVA) with Tukey's multiple comparison correction test with GraphPad Prism (GraphPad Software, La Jolla, CA, USA). (D) HIF1α, BNIP3, Twist and actin (β-actin) protein levels in B16-F10 (left) and A375p (right) total cell lysates after 48 h incubation with the corresponding supplemented media. Cells were collected in Laemli buffer [125 mM Tris-HCl (Sigma-Aldrich), pH = 6.8; 2% sodium dodecyl sulfate (SDS; Sigma-Aldrich); 20% glycerol (Sigma-Aldrich)] supplemented with protease and phosphatase inhibitors (Roche, now Sigma-Aldrich). Western blots were executed in denaturing conditions, using 4–12% Bis-Tris gels (BioRad, Narareth, Belgium) and nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). Incubation with primary antibodies [HIF1α (Cayman Chemical, Ann Arbor, MI, USA), rodent BNIP3 (Cell Signaling Technologies, Danvers, MA, USA), human BNIP3 (Sigma-Aldrich Protein Atlas), Twist (Active Motif, La Hulpe, Belgium) and actin (Sigma-Aldrich)] was performed overnight at 4°C. After incubation with the corresponding secondary antibodies-either HRP-conjugated (Cell Signaling Technologies) or fluorescently labeled with DyLight680 or DyLight800 (Thermo Fisher Scientific) – specific bands were visualized using the ChemiDoc MP Imaging System (BioRad) or the LICOR Odyssey Clx Western Blot Detection System (Westburg, Leusden, The Netherlands), respectively. Quantifications were performed using either Image J software (NIH) or Image Studio Lite (Westburg) and protein levels were normalized to actin levels. Pull of three independent biological experiments performed at least in triplicates is shown. All data represented are mean ± SEM unless otherwise stated. Experiments were statistically analyzed using unpaired Student's *t*-test with GraphPad Prism (GraphPad Software) unless otherwise stated. *Represents a *p*-value < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, where a *p*-value < 0.05 is considered significant.

Figure 2: Glutamine addition enhances the bioenergetic status of melanoma cells.

(A) Basal oxygen consumption rate and (B) extracellular acidification rate of B16-F10 (left) and A375p melanoma cells (right). Cells were plated at a density of 10 000 (Q) and 20 000 (Glc) cells per well of a XFp Extracellular Flux Analyzer culture plate in glutamine (2 mM, Q) or glucose (25 mM, Glc) supplemented DMEM medium for 24 h. Prior to the assay, cells were switched to the Mitostress assay medium (Seahorse Biosciences, North Billerica, MA, USA) containing either glucose or glutamine and supplemented with 5 mM sodium pyruvate (Sigma-Aldrich), following the manufacturer instructions. Real-time extracellular acidification (ECAR) and oxygen consumption (OCR) during the Mitostress assay were measured with the XFp Extracellular Flux Analyzer (Seahorse Biosciences) following the manufacturer instructions. Data are represented relative to the mean of the corresponding glucose condition. Pull of three independent biological experiments performed at least in duplicates is shown. (C and D) Reactive oxygen species (ROS) levels in B16-F10 (left) and A375p (right) cells after culture with the glucose-only (Glc) or glutamine-only (Q) supplemented medium. After 72 h, cells were incubated with either (C) DCFDA (10 μ M; Life Technologies, Carlsbad, CA, USA) or (D) DHE (10 μ M; Life Technologies) for 30 min to fluorescently label hydroxyl-ROS and peroxyl-ROS (DCFDA) or superoxide (DHE) species. Cells were then trypsinized and collected for analysis using flow cytometry (Attune[®] Acoustic Focusing Cytometer, Applied Biosystems, Foster City, CA, USA). Values represent the average mean fluorescent intensity (MFI) per sample $\pm$ SEM. Pull of three independent biological experiments performed at least in triplicates is shown except for B16-F10 DCFDA (C left), which shows the pull of two independent experiments. (E and F) p62, LC3II and actin levels of B16-F10 (E) and A375p (F) total cell lysate from cells treated with (+) or without (-) bafilomycin A (Baf-A) together with their corresponding metabolic supplementation. Cells were grown in glutamine (2 mM) or glucose (25 mM) supplemented DMEM medium for 24 h, followed by Baf-A (10 nM; Sanbio, Uden, The Netherlands) treatment for another 24 h before collection with Laemli buffer for Western blot analysis (see legend Figure 1). Primary antibodies against p62 (Sigma-Aldrich), LC3B (Cell Signaling Technologies) and actin (Sigma-Aldrich) were probed overnight at 4°C. Protein levels were normalized to actin levels and statistics shown compare all the conditions against glucose condition untreated (Glc Baf-A-, represented with *), glutamine condition untreated (Q Baf-A-, represented by #) or glucose condition treated with bafilomycin A (Glc Baf-A+, represented by \$) using unpaired one-way ANOVA with Tukey's multiple comparison correction test. Pull of two (A375p) or three (B16-F10) independent biological experiments performed in at least triplicates is shown. All data represented are mean $\pm$ SEM. All experiments were statistically analyzed using unpaired Student's *t*-test with GraphPad Prism (GraphPad Software) unless otherwise stated. */\$/# Represents a *p*-value < 0.05, **/## *p* < 0.01, ****p* < 0.001, ****/#### *p* < 0.0001, where a *p*-value < 0.05 is considered significant.

Figure 3: BNIP3 governs glutamine-driven malignant traits of melanoma cells.

(A) BNIP3 protein levels in total cell lysate of B16-F10 transduced with virus containing either empty vector (shCntrl; PLKO.1 vector from Sigma-Aldrich) or shRNA against murine BNIP3 (shBNIP3; Mission shRNA library from Sigma-Aldrich, sequence available upon request) and cultured in glucose (Glc, left) or glutamine (Q, right) supplemented medium. Cells were selected in medium containing 1 mg/ml Puromycin (Sigma-Aldrich). BNIP3 was probed using the rodent specific antibody from Cell Signaling Technologies, following the Western blot protocol detailed in Figure 1. Pull of four independent biological experiments performed in triplicates is shown. Protein levels were normalized to actin levels and statistics shown compare all the conditions against glucose condition from the control cell line (shCntrl Glc) using unpaired one-way ANOVA with Tukey's multiple comparison correction test. (B and C) Cell number count (B) and fold change relative to the corresponding glucose condition per cell line (C) of B16-F10 shCntrl and shBNIP3 cell lines (trypan blue exclusion method) after 48 h of incubation (37°C, 5% CO₂) with medium containing either glucose or glutamine. Pull of three independent biological experiments performed at least in triplicates is shown. (D) Basal oxygen consumption rate of B16-F10 shCntrl and shBNIP3 after culture for 24 h with glucose- or glutamine-supplemented medium (see the legend of Figure 2). Data are represented relative to the mean of the corresponding glucose condition. Pull of three independent biological experiments performed in triplicates is shown. (E) Scratch wound assay performed in B16-F10 shBNIP3 cells in glucose-only or glutamine-only medium (for methodology see the legend of Figure 1). Scale bar represents 400 µm. Pull of three independent biological experiments performed at least in triplicates is shown. All data represented are mean ± SEM. All experiments were statistically analyzed using either unpaired (B–D) or RM (E) one-way ANOVA with Tukey's multiple comparison correction test with GraphPad Prism (GraphPad Software). *Represents a *p*-value < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, n.s. *p* > 0.05, where a *p*-value < 0.05 is considered significant.

of the autophagosomes. Autophagic blockade revealed a higher and significant accumulation of the (undegraded) autophagic substrates LC3B-II and p62 in melanoma cells

exposed to glutamine when compared to those using glucose as substrate (Figure 2E and F), thus showing that autophagic flux is further stimulated by glutamine

supplementation and corroborating previous reports (Eng et al., 2010).

To unravel key molecular components of this glutamine-driven pathway, we subsequently focused on the mitophagy receptor BNIP3 motivated by different reasons: (i) our previous studies showed that BNIP3 in these melanoma cells has a pro-autophagic activity that is required to maintain a healthy pool of mitochondria as well as melanoma cell's proliferative and migrating ability (Maes et al., 2014); (ii) BNIP3 is a known downstream target of HIF1 α , which is stimulated by glutamine and (iii) BNIP3 has been shown to interact with Rheb at the outer mitochondrial membrane thereby promoting autophagy (Li et al., 2007). Altogether, these observations point out that BNIP3 could be implicated in mitochondria homeostasis under glutamine conditions.

To this end, we silenced BNIP3 in the B16-F10 cell line (Figure 3A) and assessed the performance of these cell lines under glucose and glutamine fueling in terms of proliferation (Figure 3B and C), oxygen consumption (Figure 3D) and migration (Figure 3E). Absence of BNIP3 significantly hampered the heightened glutamine-driven proliferation of these melanoma cells when considering their absolute numbers (Figure 3B) whereas it slightly decreased their proliferation relative to their corresponding glucose counterpart (Figure 3C). This hints that the intrinsic proliferation defect caused by BNIP3 knockdown impacts melanoma cells to a higher extent than the selective supplementation with any of these metabolites. Remarkably, BNIP3 silencing almost completely abolished the increase in OCR induced by glutamine (Figure 3D) as well as significantly reduced their migratory ability (Figure 3E). These observations thus indicate that BNIP3 is one of the drivers of the more aggressive melanoma cell phenotype stimulated by glutamine addition, at least under these *in vitro* settings and in both murine and human melanoma cell lines. Moreover, this study highlights HIF1 α -BNIP3 as a potential pro-melanoma axis supporting glutaminolysis.

In fact, the glutamine-mediated upregulation of HIF1 α and its key targets BNIP3 and Twist (Yang et al., 2004; Maes et al., 2014) would foster melanoma cell migration and a mesenchymal-like switch, favoring intravasation and survival in the blood stream, where melanoma cell's glutamine demand could be fully satisfied. In our system, we cannot exclude that the exclusive supplementation of glutamine might cause a cellular status mimicking 'glucose starvation', which would consequently drive HIF1 α upregulation. Indeed, it has been suggested that, upon glucose starvation, colorectal cancer cells upregulate HIF1 α in normoxic conditions in an attempt to evade apoptosis (Nishimoto et al., 2014). Nevertheless, in our

case, melanoma cell proliferation and migration are clearly boosted under glutamine supplementation in contrast with the former study (Nishimoto et al., 2014), clearly pointing at glutamine as the main driver of melanoma cell aggressiveness. Still, more studies are necessary to decipher the molecular contribution of HIF1 α to this metabolic phenomenon as well as how different metabolites modulate its expression.

Interestingly, not only HIF1 α but also cMyc can drive glutaminase-1 (the first enzyme involved in glutaminolysis) expression, again supporting the idea that aerobic glycolysis and glutamine catabolism are more interconnected than initially anticipated (Dang et al., 2008). Whether melanoma cell-associated BNIP3 and mitophagy interface with cMyc should be further explored, also considering that oncogenic levels of cMyc have been shown to foster glutamine catabolism over glycolysis in other cancer types (Munksgaard Thoren et al., 2017).

Notably, previous studies suggest that detached cancer cells would move from glucose to glutamine as carbon source to avoid ROS-induced anoikis (Lu et al., 2015). Thus, it would be interesting to explore further in future studies the dependence on BNIP3-mediated glutamine metabolism in a metastatic model of melanoma. This could better reveal whether BNIP3, or BNIP3-mediated processes could be considered as therapeutic targets in melanoma.

Considering all of the above, it is becoming clear that highly invasive melanoma cells can dynamically switch their metabolic profile to grow and metastasize (Paoli et al., 2013; Rodrigues et al., 2016) and this study unravels BNIP3 as a major player of key malignant traits associated to glutamine metabolism in melanoma cells.

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