BNIP3 promotes HIF-1α-driven melanoma growth by curbing intracellular iron homeostasis

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Abstract

BNIP3 is a mitophagy receptor with context-dependent roles in cancer, but whether and how it modulates melanoma growth in vivo remains unknown. Here, we found that elevated BNIP3 levels correlated with poorer melanoma patient’s survival and depletion of BNIP3 in B16-F10 melanoma cells compromised tumor growth in vivo. BNIP3 depletion halted mitophagy and enforced a PHD2-mediated downregulation of HIF-1α and its glycolytic program both in vitro and in vivo. Mechanistically, we found that BNIP3-deprived melanoma cells displayed increased intracellular iron levels caused by heightened NCOA4-mediated ferritinophagy, which fostered PHD2-mediated HIF-1α destabilization. These effects were not phenocopied by ATG5 or NIX silencing. Restoring HIF-1α levels in BNIP3-depleted melanoma cells rescued their metabolic phenotype and tumor growth in vivo, but did not affect NCOA4 turnover, underscoring that these BNIP3 effects are not secondary to HIF-1α. These results unravel an unexpected role of BNIP3 as upstream regulator of the pro-tumorigenic HIF-1α glycolytic program in melanoma cells.

Keywords: BNIP3; ferritinophagy; HIF-1α; melanoma; metabolism

Subject Categories: Autophagy & Cell Death; Cancer; Metabolism

Introduction

Metabolic plasticity is a common trait of established tumors including melanoma, an aggressive skin cancer relying on heightened autophagy for stress adaptation and progression (Kimmelman & White, 2017; Rinaldi et al., 2018; Di Leo et al., 2019). Emerging evidence indicates that oncogenic drivers co-opt autophagy to endow cancer cells with the metabolic versatility required for their bioenergetic and anabolic needs and to adjust to the fluctuating microenvironment (Galluzzi et al., 2015; Kimmelman & White, 2017). During tumorigenesis, lysosomal cargo degradation and recycling of nutrients through autophagy operate as fundamental cancer cell-autonomous mechanisms to mitigate metabolic stress, by supplying fuel sources feeding into multiple metabolic and biosynthetic pathways (Kimmelman & White, 2017). Furthermore, the selective targeting and degradation of mitochondria, through the process of mitophagy, help cancer cells to preserve the cellular pool of healthy and respiring mitochondria (Vara-Perez et al., 2019a). However, how mitophagy and its regulators functionally interface with oncogene-driven pathways orchestrating metabolic networks in cancer cells remains ill-defined.

The hypoxia-inducible factor 1 alpha (HIF-1α) target BCL-2 19 kDa interacting protein 3 (BNIP3) is an outer mitochondria-associated atypical BH3-only member of the B-cell lymphoma 2 (BCL2) family that operates as a mitophagy receptor. BNIP3 harbors an
LC3B-interacting region (LIR) motif, which facilitates autophago- 
mal targeting of the mitochondria for lysosomal degradation (Vara-
Perez et al, 2019a). Under hypoxia, HIF-1α-driven BNIP3 expression 
contributes to reducing the overall cellular oxygen consumption 
by eliciting mitophagy, thus favoring metabolic adaptation to low 
oxygen concentrations (Lee et al, 2020).

High BNIP3 and HIF-1α levels often correlate with poor patient 
prognosis in a number of cancer types, suggesting that BNIP3 contrib- 
tutes to the tumor-promoting role of HIF-1α in these cancers. 
However, the precise mechanism of action of BNIP3 is largely 
unknown and appears to be mainly tumor type-specific (Vara-Perez 
et al, 2019a). In an MMTV-PyMT breast cancer model, loss of BNIP3 
resulted in reactive oxygen species (ROS)-mediated stabilization of 
HIF-1α, due to the inability of cancer cells to perform mitophagy, 
which then fostered a HIF-1α-driven glycolytic shift and a more 
invasive breast cancer phenotype (Chourasia et al, 2015). This study 
therefore revealed a tumor suppressor role for BNIP3 by restricting 
HIF-1α signaling through mitophagy. Instead, BNIP3 levels posi- 
tively correlated with poor prognosis in uveal melanoma (Jiang 
et al, 2018) and BNIP3 supported melanoma cell’s migratory abili-
ties (Maes et al, 2014a) and their glutamine dependency (Vara-Perez 
et al, 2019b) in vitro. Together with the fact that constitutive HIF-1α 
levels have also been associated with poor prognosis in melanoma 
(Martínez-García et al, 2017), these studies suggest that BNIP3 and 
HIF-1α jointly coordinate key metabolic and signaling nodes driving 
the aggressive phenotype of melanoma cells, yet whether and how 
the loss of BNIP3 affects HIF-1α signaling and melanoma growth in 
vivo have not been addressed. To shed light on these questions, 
we studied the effects of BNIP3 deletion on melanoma cell’s mito-
chondria quality control, metabolic profile, and growth in vivo.

**Results**

**BNIP3 expression in melanoma is associated with poor prognosis**

To assess the impact of BNIP3 levels on the prognosis of melanoma 
patients, we interrogated the skin cutaneous melanoma (SKCM) 
cohort from The Cancer Genome Atlas (TCGA) database comprising 
469 melanoma samples. We compared SKCM patients harboring 
“high” BNIP3 levels versus “low” BNIP3 levels using different gene 
expression cutoffs based on the distribution of BNIP3 expression 
levels throughout the whole cohort (Fig 1A, Appendix Fig S1A and 
B). This analysis showed that SKCM patients with higher BNIP3 
expression exhibited significantly reduced overall survival (OS). 
Only a subset of patients (with BNIP3 expression in median ± 10% 
log2 range, equivalent to 91 out of 455 patients) did not conform to 
this trend, possibly due to a lack of sufficiently different BNIP3 
expression relative to median cutoff (Appendix Fig S1A and B). 
BNIP3 expression did not differ between primary (103 patients) and 
malignant specimens (366 patients) (Fig 1B). In both patient 
groups, there was a consistent trend (albeit non-significant) associ- 
ating higher BNIP3 levels with lower OS (Appendix Fig S1C–H).

To substantiate the TCGA analyses at the protein level, we 
immunohistochemically stained a subset of benign nevi (n = 5) and 
paired (n = 7) primary and metastatic skin cutaneous melanoma 
patient samples (Fig 1C; Appendix Table S1). The histopathological 
analysis revealed a gradient pattern of BNIP3 expression in benign 
nevi coherent with the maturation pattern of the nevus (Fig 1C). As 
the melanocytes in the nevus mature toward a more neural phenot-
type in the deeper part of the lesion, they presented a lower 
intensity of BNIP3 compared with the melanocytes toward the 
dermo-epidermal junction (Fig 1C). In contrast, BNIP3 expression 
was uniformly distributed in all primary melanoma sections, in 
agreement with the absence of maturation that is typical of mela-
oma lesions. Melanomas were divided by BNIP3 expression in two 
groups: those with an intensity comparable to the junctional part 
of the benign nevi (+, Fig 1C) and those with the strongest positivity 
(+++, Fig 1C). We observed a strong concordance between the 
primary and their corresponding metastases: a stronger BNIP3 
expression in the primary melanoma was found also in the associ-
ated metastatic lesion (Fig 1C). Together, these data suggest that 
the prognostic impact of BNIP3 in these SKCM patient cohorts may be 
driven by the primary tumor samples.

Furthermore, analysis of tissue microarrays (TMAs) consisting of 
158 cases of metastatic melanomas (Appendix Table S1) stratified 
according to increasing BNIP3 staining intensity (Fig 1D) high-
lighted an inverse relationship between patients’ OS and BNIP3 
staining intensity, whereby higher BNIP3 expression was associated 
with lower OS (Fig 1D). It should be noted that nearly 92% (145/158) 
of the metastatic melanoma samples showed a BNIP3 medium 
(score 2)-to-high (score 3) staining intensity, further underscoring a 
strong association between high BNIP3 levels and disease progres-
sion. This analysis supports an inverse relationship between BNIP3 
expression and SKCM patients’ OS, as previously found in uveal 
melanoma (Jiang et al, 2018).

Together, these data show that increased BNIP3 expression is an 
early event during melanoma progression to metastasis.

**Loss of BNIP3 delays melanoma growth in vivo**

Melanoma cells lacking essential autophagy genes, such as ATG5, 
have been shown to display reduced growth (Maes et al, 2014b; 
Mgdritchian et al, 2017), but how BNIP3 deficiency affects mela-
oma growth in vivo remains ill-defined. BNIP3 functions as a mito-
chondrial receptor for the recruitment of the autophagosomal 
machinery to the mitochondria (Vara-Perez et al, 2019a). Since 
autophagosomal formation requires ATG5 (Galluzzi et al, 2015), we 
asked to what extent defects caused by BNIP3 silencing in mela-
noma cells were epistatic to defects in general autophagy.

Hence, we subcutaneously transplanted BNIP3- or ATG5-compe-
tent (shCntl) or deficient (shBNIP3 or shATG5, respectively) B16-
F10 cells (Fig EV1A) in immunocompetent (syngeneic) mice and 
collected tumors (named CntlRD, BNIP3RD, and ATG5RD from here 
outwards to distinguish them from the cell lines) when they reached 
1,000 mm3 (Fig 2A and B). All further analysis was performed in 
size-matched tumors. Not only B16-F10-BNIP3KD tumors showed a 
significant and pronounced growth delay compared with B16-F10-
CntlRD but also to the B16-F10-ATG5KD counterparts (Fig 2B). 
Consistent with previous studies (Maes et al, 2014b), B16-F10-
ATG5KD tumors displayed a trend toward increased cell death (Fig 2 
C and D) rather than a decrease in proliferation (Fig 2E and F). In 
contrast, B16-F10-BNIP3KD tumors showed a smaller proportion of 
actively proliferating cells (Fig 2E and F), in line with the defective 
proliferation observed in vitro (Fig EV1B). As expected, B16-F10-
ATG5KD tumors displayed a more diffuse LC3B immunostaining,
indicating impaired autophagosome formation (Fig 2G and Fig EV1C). In contrast, loss of BNIP3 caused an increase in the tumoral LC3B punctuated pattern (Fig 2G) suggestive of an altered LC3B turnover or enhanced autophagic flux in vivo. In vitro analysis showed that in contrast to the shATG5 cells, in the shBNIP3 cells basal autophagic flux was indeed functional, as indicated by (i) the
Figure 2.
accumulation of LC3B-II by Western blot when lysosomal acidification was altered by Bafilomycin A1 (BafA; Fig EV1C) and (ii) upon transfection with the autophagy flux reporter RFP-GFP-LC3, with the presence of red fluorescent puncta (identifying autophagolysosomes which have lost GFP-fluorescence upon acidification) turning into yellow puncta (identifying underlying autophagolysosomes) upon BafA treatment (Fig EV1D and E). Additionally, we found that BafA treatment increased cell death predominantly in BNIP3-silenced cells (Fig EV1F), suggesting that silencing BNIP3 levels renders melanoma cells particularly reliant on autophagy/lysosomal degradation for their survival, as also shown in previous studies using a breast cancer model (Chourasia et al, 2015). Interestingly, Map1lc3b and Sqstm1 (coding for the autophagic mediators LC3B and p62, respectively) were significantly increased both in vitro (Fig EV1G) and in vivo (Fig 2H and I). This suggests that BNIP3 downregulation in melanoma cells might stimulate a compensatory transcriptional mechanism sustaining elevated expression of pro-autophagic genes. Consistent with this, along with Map1lc3b and Sqstm1, which in murine cells have been shown to belong to TFEB’s transcriptional program (Wang et al, 2019), shBNIP3 cells displayed elevated protein levels of TFEB (Fig EV1H).

Together, these data highlight the reliance of melanoma cells on BNIP3 for tumor growth in vivo.

BNIP3 loss impairs mitochondria clearance and rewire the glycolytic metabolism of melanoma cells

Both the in vitro data and the histological analysis of the tumors described above suggest that loss of BNIP3 may affect melanoma growth by altering specific cancer cell-autonomous processes.

Previous studies in melanoma (Maes et al, 2014a) and breast (Chourasia et al, 2015) cancer cells indicated the relevance of BNIP3 for mitochondria clearance and homeostasis. Consistent with these studies, under baseline conditions, BNIP3 silencing significantly impaired mitophagy, as shown by the reduced co-localization of GFP-LC3 with the mitochondrial marker TOMM20 (Fig 3A). BNIP3-deprived cells displayed a fragmented mitochondrial network, which was associated with loss of mitochondrial membrane potential and an overall accumulation of intracellular ROS (Fig EV2A–C), as reported previously (Maes et al, 2014a). BNIP3 silencing resulted in the accumulation of the outer mitochondrial membrane mitophagy receptor NIX, a functional BNIP3 homolog (Vara-Perez et al, 2019a; Lee et al, 2020), in the absence of BafA, whereas BNIP3-proficient cells displayed increased amounts of both mitophagy receptors upon blockade of lysosomal degradation (Fig EV2D). In contrast, while ATG5 silencing increased the presence of both mitochondrial receptors, NIX knockdown did not alter BNIP3 levels above control (Fig EV2E), suggesting that these mitochondrial proteins undergo degradation in association with BNIP3-regulated mitophagy. Together, while these results do not exclude the contribution of alternative clearance pathways (Saita et al, 2013; Vincow et al, 2019), they indicate that the absence of BNIP3 disturbs mitochondrial homeostasis leading to the accumulation of dysfunctional mitochondria, under baseline/replete conditions.

Defects in mitochondrial clearance or general autophagy not only impact mitochondrial functionalities but could alter the metabolic performance of melanoma cells. Considering that in BNIP3-silenced cells mitochondrial clearance and homeostasis were impaired but autophagic flux was still operational, we then evaluated the effects of BNIP3 or ATG5 depletion on the metabolic profiles of melanoma cells by combining different metabolomic approaches (Fig 3B). ShBNIP3 cells respiring equally well as their shCntl counterparts in the presence of full medium (25 mM glucose, 2 mM glutamine) whereas shATG5 cells showed an overall decrease in basal oxygen consumption rate (OCR; Appendix Fig S2A), as reported in previous studies (Kimmelman & White, 2017; Jiao et al, 2018). However, under galactose, which forces the cells to rely further on mitochondrial oxidative phosphorylation (OXPHOS) (Aguer et al, 2011), shBNIP3 melanoma cells exhibited an increased basal OCR compared with their control and shATG5 counterparts (Fig 3C, Appendix Fig S2B). Interestingly, we observed a significant reduction in extracellular acidification rate (ECAR, which is often used as a proxy for lactate release) in BNIP3-deprived cells (Fig 3D), suggestive of impaired glycolytic activity.

To further validate these findings and discriminate the impact of defective BNIP3 or ATG5 expression on the metabolic profile of melanoma cells, we next analyzed melanoma cells’ steady-state metabolism using complementary approaches: by proton nuclear magnetic resonance (1H-NMR; steady-state) and by gas chromatography coupled to mass spectrometry (GC-MS; steady-state 13C glucose labeling). ShBNIP3 cells secreted (Fig 3E, Appendix Fig S2C) significantly less lactate, whereas they accumulated glucose...
Figure 3.
both extracellularly and intracellularly (Fig 3F and G) as assessed by ¹H-NMR. Furthermore, ¹³C-labeled lactate (mααα, isotopologue) was significantly reduced in shBNIP3 cells when compared to both shCntl and shATG5 conditions (Fig 3H), indicating that less glucose is contributing to lactate production, as expected from cells with defective glycosylation. Altogether, these results indicate that—opposite to the metabolic effects observed upon blockade of canonical autophagy—silencing BNIP3 in melanoma cells results in a decreased glycolytic and fermentation ability.

We then evaluated whether this specific BNIP3 effect could be the result of a transcriptional downregulation of key components of the glycolytic pathway. Consistent with this, we found that the expression level of several glycolytic enzymes (Hk2, Pdk1, Pkm1, Pkm2, Ldhα, and Ldhβ), as well as of the glucose and lactate transporters Glut1 and Mct4, respectively, was significantly diminished in BNIP3-deficient cells (Fig 3I). Consistent with previous reports (Jiao et al, 2018), ATG5 removal induced a trend toward an upregulation—rather than downregulation—of some components of this pathway (Fig 3I), in line with the higher glycolytic metabolism of these autophagy-compromised melanoma cells.

Collectively, these data support a specific role for BNIP3 in promoting the glycolytic metabolism of these melanoma cells.

**BNIP3 maintains HIF-1α levels in melanoma in vivo**

Our findings that BNIP3 depletion in melanoma cells reduced glycolytic fermentation and forced cells to rely on OXPHOS seemed at first inconsistent with the accumulation of their abnormal mitochondria and with the glycolytic shift usually observed in cells with compromised mitophagy (Vara-Perez et al, 2019a). Since the metabolic effects of BNIP3 were orchestrated at the transcriptional level, we then postulated that depletion of BNIP3 could cause the downregulation of a master transcription factor regulating glycolysis in melanoma cells. We focused on HIF-1α, rather than on other transcription factors, for several reasons including: (i) Glut1, Hk2, Pdk1, Pkm2, and Ldhα are well-established HIF-1α targets and HIF-1α is a master regulator of the Warburg effect in melanoma (Soni & Padwad, 2017; Lee et al, 2020); (ii) melanoma cells harbor constitutively high levels of HIF-1α (Kuphal et al, 2010; Martínez-García et al, 2017) suggesting regulatory mechanisms of HIF-1α stability operating under normoxia, and last but not least (iii) BNIP3 is a well-established HIF-1α target (Lee et al, 2020).

BNIP3 silencing, but not the knockdown of ATG5, caused a significant downregulation of HIF-1α protein levels, which was accompanied by a reduction in the LDHA protein expression (Fig 4A). Lentiviral transduction of a myc-tagged BNIP3 full-length construct in the shBNIP3 cells led to a partial recovery of BNIP3 protein levels (Appendix Fig S3A), likely due to fast protein turnover of the BNIP3 full-length tagged mutant under baseline conditions (Park et al, 2013). In spite of this, we observed a proportional rescue of HIF-1α protein levels and its target LDHA (Appendix Fig S3B), thus supporting the specific effects of BNIP3 on HIF-1α levels.

We then assessed the effects of BNIP3 silencing on HIF-1α levels in vivo as detected by Western blotting of tumor lysates (comprising normoxic and hypoxic areas) (Fig 4A). HIF-1α staining showed a fainter pattern in BNIP3KD tumors than in CntlKD or ATG5KD (Fig 4B). The effects of BNIP3 silencing on HIF-1α were mitigated but not completely abrogated under hypoxia (Appendix Fig S3C), suggesting that BNIP3-regulated mechanisms become overshadowed by the powerful HIF-1α stabilization under low oxygen levels (1% O₂). Yet, qPCR analysis from tumor tissues showed a concomitant reduction in the levels of various key HIF-1α target genes including Glut1, Pdk1, and Vegfa (Fig 4C and D). Along with the reduced HIF-1α and LDHA protein levels detected by immunohistochemistry and Western blot in BNIP3-silenced tumors, these data show that the BNIP3-HIF-1α axis is still operational in vivo. In contrast, shATG5 cells or B16-F10-ATG5KD tumors remained HIF-1α proficient both in vitro and in vivo (Fig 4A). These BNIP3 effects were not caused by alterations of Hif1a mRNA levels (Appendix Fig S3D), suggesting that the activity of the oxygen-sensing prolyl hydroxylases (PHDs) might be differentially regulated.

Prolifer hydroxylases hydroxylate the alpha subunit of HIF-1 in two proline residues, tagging HIF-1α for proteasomal degradation (Lee et al, 2020). We then looked at the ratio hydroxylated/total
HIF-1α (by Western blotting) both in vitro and in vivo. Consistent with this, HIF-1α was more hydroxylated (on P564) upon BNIP3 knockdown in vitro and in vivo (Fig 4A, Appendix Fig S3B). Moreover, proteasomal blockade via MG132 treatment rescued HIF-1α, but not LDHA, protein levels (Appendix Fig S3E), as hydroxylated HIF-1α cannot enter the nucleus to activate transcription (Semenza, 2007). Increased HIF-1α hydroxylation and downregulation of its main target genes were also observed in two human early-stage melanoma cell lines, the BRAFWT 530 and BRAFV600E mutant WM35, upon BNIP3 silencing (Fig EV3A–D), although these BNIP3-mediated effects were of a lower magnitude as compared to the murine B16-F10 cell line. Interestingly, analysis of TCGA data from the BNIP3high and BNIP3low melanoma patient clusters (Fig 1B) showed that, irrespective of HIF1A transcript levels that were unchanged in both cohorts (Fig EV3E), there was a significant association between low expression levels of BNIP3, reduced HIF-1α transcriptional signature, and higher OS (Fig EV3E). This trend is in stark contrast to what was reported in breast cancer patients (Chourasia et al, 2015), where low BNIP3 levels and high HIF1A predicted poor patient survival better than high HIF1A alone. These
observations suggest the existence of a cancer type-specific functional outcome of the BNIP3-HIF-1α axis.

Given that the downregulation of HIF-1α signaling included the expression of Vegfa, the main effector of HIF-1α driven angiogenesis (Lee et al, 2020), both in vitro and in vivo (Fig 4D, Appendix Fig S3F), we questioned whether loss of BNIP3 was associated with defects in tumor angiogenesis. We then analyzed tumoral vessel morphology and maturation by (co)staining tumor sections with the endothelial cell marker CD31 and the mural cell marker αSMA, identifying vessel coverage by pericytes (Fig 4E). In B16-F10-BNIP3KD tumors, while the number of vessels per field slightly increased, the vessel size was significantly diminished compared with control tumors and even more compared with B16-F10-ATG5KD (Fig 4F and G). Double staining for CD31 and αSMA did not reveal gross, albeit significant, differences in vessel maturation across conditions (Fig 4 H) and tumors displayed similar levels of hypoxia (Fig 4I).

In aggregate, these results corroborate the view that the specific depletion of BNIP3 restrains HIF-1α signaling both in vitro and in vivo.

**BNIP3 silencing exacerbates PHD2-mediated HIF-1α downregulation through modulation of intracellular iron levels**

Key effects of the depletion of BNIP3 in melanoma cells were associated both in vitro and in vivo with changes in HIF-1α hydroxylation. We then analyzed the role of the PHDs and the factors that could elevate their HIF-1α hydroxylation activity in response to BNIP3 deprivation. We focused on PHD2 because, among the three PHD isoforms, PHD2 showed a specific trend toward an upregulation upon loss of BNIP3 on RNA (Fig EV4A) and protein level both in vitro and in vivo (Fig 5A). Moreover, previous studies on the differential selectivity among PHDs indicated PHD2 as the main HIF-1α hydroxylating enzyme (Di Conza et al, 2017; Lee et al, 2020).

The reaction catalyzed by PHD2 requires alpha-ketoglutarate (αKG) as donor substrate, iron (Fe2+), and oxygen as co-factors and yields succinate and CO2 together with the hydroxylated proteins (Lee et al, 2020). We first validated the functional role of PHD2 in our settings by chemical and genetic interference.

The PHD2-chemical inhibitor IOX2 (Chowdhury et al, 2013) and the iron chelator deferoxamine (DFO) significantly decreased the ratio HIF-1α-OH/HIF-1α levels in all the cell lines (Fig 5B–D, Appendix Table S2) and recovered HIF-1α signaling in BNIP3-silenced cells to a similar extent as observed in shCntl and ATG5-depleted melanoma cells (Fig 5B–D, Appendix Table S2). The siRNA-based PHD2 knockdown (Fig 5E and Fig EV4B, Appendix Table S2) exerted similar rescuing effects though their magnitude was milder as compared to the PHD2 pharmacological inhibition and depended on the PHD2 knockdown efficiency. These results together indicate PHD2 as the predominant HIF-1α hydroxylating enzyme in these melanoma cells.

Beyond increased expression, we also examined possible mechanisms underlying the exacerbated PHD2 hydroxylation activity upon loss of BNIP3. The TCA metabolites succinate and fumarate have been described as PHD inhibitors (Koivunen et al, 2007; Koivunen et al, 2013; Lee et al, 2020). Instead, intracellular fumarate levels were reduced, although not significantly, by BNIP3 silencing, which would however suggest PHD2 inhibition and depended on the PHD2 pharmacological inhibition and depended on the PHD2 knockdown efficiency. These results together indicate PHD2 as the predominant HIF-1α hydroxylating enzyme in these melanoma cells.

We then reasoned that the specific changes in mitochondrial homeostasis, oxygen consumption, and clearance induced by the loss of BNIP3 could underlie the effector mechanism promoting PHD2-mediated HIF-1α downregulation. The emerging crosstalk between mitochondria clearance, autophagy, iron homeostasis, and HIF-1α signaling suggested that BNIP3 could control PHD2 activity through changes in the intracellular iron content. In line with this, only BNIP3-depleted cells—but not autophagy-compromised cells—displayed a significantly higher content of intracellular Fe2+ as imaged and quantitated by FerroOrange, a probe reacting specifically with intracellular Fe2+ ions (Fig 6A). Furthermore, to validate this finding we performed highly sensitive capillary electrophoresis combined

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Figure 5. BNIP3 stabilizes normoxic HIF-1α on a post-translational level in melanoma.

A Immunoblot detection of PHD2 and ACTIN protein levels from lysates of normoxic cultures (n = 4) or B16-F10 tumors [CntlKD (n = 5), BNIP3KD (n = 7), and ATGSKD (n = 6)]. Denisitometric quantifications relative to ACTIN levels are shown below each corresponding band. In vitro data were analyzed using a one-sample t-test against shCntl whereas the in vivo data were analyzed using a one-way ANOVA with Tukey’s multiple comparisons test.

B Immunoblot detection of Hydroxylated HIF-1α (HIF-1α-OH), total HIF-1α, LDHA, and ACTIN protein levels from normoxic lysates of B16-F10 cells cultured alone or in the presence of the PHD2 inhibitor IOX2 (100 µM) for 24 h.

C Glut1, Pdk1, Mct4, and Vegfa transcript levels from B16-F10 cells cultured alone or in the presence of the PHD2 inhibitor IOX2 (100 µM) for 24 h (n = 4). Data were analyzed using a one-sample t-test against shCntl, except for Pdk1 shBNIP3 IOX2 which was analyzed using the non-parametric Wilcoxon test.

D Immunoblot detection of Hydroxylated HIF-1α (HIF-1α-OH), total HIF-1α, LDHA, and ACTIN protein levels from normoxic lysates of B16-F10 cells cultured alone or in the presence of the iron chelator DFO (200 µM) for 24 h.

E Immunoblot detection of Hydroxylated HIF-1α (HIF-1α-OH), total HIF-1α, LDHA, PHD2, and ACTIN protein levels from normoxic lysates of B16-F10 cells transfected in presence of non-targeting siRNA sequences (Scr) or siRNA against PHD2.

Data information: All quantitative data are mean ± SEM. Denisitometric quantifications of protein levels relative to ACTIN for (B, D, E) are shown in Appendix Table S2. *P < 0.05, **P < 0.01, ***P < 0.001 when compared against shCntl. (B, D, E) provide a representative blot from n = 3 biologically independent experiments.

with inductively coupled plasma mass spectrometry (CE-ICP-MS), from shCntl, shBNIP3, and shATGS melanoma cells, which allowed for quantitative Fe²⁺/Fe³⁺ speciation analysis (Michalke et al., 2019). This analysis showed that while the total iron content across these cell lines remained constant (Fig 6B; Appendix Table S3), shBNIP3 cells harbored a significantly higher proportion of Fe²⁺ when compared to Fe³⁺ (Fig 6B, Appendix Table S3).

The cytoplasmic pool of labile iron is regulated by ferritinophagy, which utilizes the nuclear receptor coactivator-4 (NCOA4) to target ferritin to the lysosomes through the autophagosome, thereby leading to the export of Fe²⁺ in the cytoplasm (Mancias et al., 2014). NCOA4, as well as other receptors for specific autophagic cargo, has been shown to be among the most rapidly degraded proteins (Meij-vang et al., 2018), although a comprehensive understanding of the degradation pathways contributing to the overall control of NCOA4 turnover is still missing.

We then compared NCOA4 and ferritin (typified by its light chain, FTL) protein expression in the absence or presence of BafA to...
Figure 6. BNIP3 modulates intracellular iron content to support HIF-1α levels in melanoma cells.
A Relative intracellular Fe²⁺ levels in normoxic B16-F10 cells (n = 4). Representative pictures of FerroOrange intensity are shown on the left and analyzed using a RM one-way ANOVA (Geisser–Greenhouse correction) with Holm–Sidak’s multiple comparisons test. Scale bars represent 50 µm.
B Intracellular total iron content (left) and Fe²⁺ to Fe³⁺ ratio (right) measured by CE-ICP-MS in normoxic B16-F10 cells (n = 3). Data were analyzed using a one-way ANOVA with Tukey’s multiple comparisons test.
C Immunoblot detection of NCOA4, FTL, and ACTIN levels from normoxic lysates of B16-F10 cells cultured in the presence or absence of BafA 5 nM for 24 h (n = 3). Data were analyzed using a one-way ANOVA with Tukey’s multiple comparisons test.
D Immunoblot detection of NCOA4, PHD2, and BNIP3 levels after immunoprecipitation of endogenous BNIP3 protein from normoxic lysates of B16-F10 shCntl cells (representative of n = 3 immunoprecipitations). A lysate from B16-F10 shCntl cells cultured in the presence of BafA was used for band reference. Lad. stands for the reference ladder.
E Intracellular Fe²⁺ to Fe³⁺ ratio measured by CE-ICP-MS in normoxic B16-F10 cells transfected in presence of non-targeting siRNA sequences (Scr) or siRNA against NCOA4 (n = 4). Data were analyzed using a one-way ANOVA with Tukey’s multiple comparisons test.
F Immunoblot detection of Hydroxylated HIF-1α (HIF-1α-OH), total HIF-1α, LDHA, NCOA4, and ACTIN levels from normoxic lysates of B16-F10 cells transfected in presence of non-targeting siRNA sequences (Scr) or siRNA against NCOA4 (n = 4).

Data information: All quantitative data are mean ± SEM. Densitometric quantifications of protein levels relative to ACTIN for (C, F) are shown in Appendix Table S2. **P < 0.01, ***P < 0.001, ****P < 0.0001 when compared against shCntl. #P < 0.05, ##P < 0.01, ###P < 0.001 when comparing shBNIP3 against shATG5.
unravel its lysosomal turnover. In line with this, baseline levels of NCOA4 in the melanoma cells were increased by altering lysosomal acidification and degradation upon treatment with BafA (Fig 6C, Appendix Table S2), while this was not the case when ATG5 was silenced. NCOA4 can be degraded also by alternative ATG8 (Goodwin et al, 2017) and ATG5/ATG7 (MeiJvang et al, 2018)-independent pathways, suggesting that a similar mechanism operates under basal conditions in these melanoma cells. In the presence of BafA, we observed reduced levels of FTI as compared to non-BafA-treated cells, which may highlight the contribution of alternative degradative (proteosomal) pathways as observed previously (De Domenico et al, 2006; Du et al, 2019; Gammella et al, 2020). However, and irrespective of this, both NCOA4 and FTI accumulation were higher in shBNIP3 cells compared with shCntl or ATG5-depleted cells upon alkalization of the lysosomes (Fig 6C, Appendix Table S2), thus suggesting that loss of BNIP3 enhances ferritinophagy.

We then questioned whether the knockdown of the close BNIP3 homolog and mitophagy receptor NIX (Vara-Perez et al, 2019a; Lee et al, 2020) would phenocopy the effects of the BNIP3 silencing on HIF-1α signaling and intracellular iron content. However, the absence of NIX did not affect either HIF-1α stabilization (Fig EV4F), HIF-1α-driven transcription (Fig EV4G) or NCOA4 turnover (Fig EV4H), thus demonstrating that the observed effects are BNIP3 specific.

We then investigated whether BNIP3 regulated Ncoa4 levels. However, BNIP3 knockdown did not affect Ncoa4 mRNA levels (Fig EV4I) while it increased PHD2 (Egln1) expression (Fig EV4A). Further, we explored the possibility that BNIP3 could directly interact with NCOA4 or PHD2, thereby regulating their signaling functions. To this end, we immunoprecipitated endogenous BNIP3 in B16-F10 shCntl cells and probed for the presence of NCOA4 or PHD2 by Western blotting. Because baseline levels of NCOA4 in the input were nearly undetectable, shCntl cells treated with BafA to block NCOA4 turnover, thereby increasing its detection, were loaded on the same gel as reference. Notably, the immunoprecipitation of endogenous BNIP3 pulled down NCOA4 but not PHD2 (Fig 6D). This suggests that BNIP3 regulates the availability of the cytosolic pool of NCOA4 that can be engaged in ferritinophagy under normoxic conditions, by its interaction with NCOA4.

We then questioned whether the heightened ferritinophagy in the BNIP3-depleted condition could contribute to enhancing the PHD2-mediated degradation of HIF-1α. To this end, we down-regulated NCOA4 by siRNA-mediated knockdown and analyzed iron content and HIF-1α levels in these melanoma cells by using both FerroOrange (Fig EV4J) and CE-ICP-MS-based Fe2+/Fe3+ speciation analysis (Fig 6E). In line with this, NCOA4 silencing diminished intracellular Fe2+ levels (Fig 6E and Fig EV4J and Appendix Table S4) and completely abolished the striking increase in the Fe2+/Fe3+ ratio observed in the BNIP3-depleted cells. NCOA4 silencing also partially rescued the levels of HIF-1α and its down-stream target LDHA in the BNIP3-depleted melanoma cells, whereas did not exert major effects in the shCntl and shATG5 (Fig 6F, Appendix Table S2).

Although we cannot exclude that other mechanisms controlling overall iron metabolism are affected, these data indicate that BNIP3 depletion in melanoma cells is associated with an exacerbated ferritinophagy flux, which increases the bioavailability of free iron operating as the main co-factor for the PHD2-mediated HIF-1α hydroxylation.

Rescuing HIF-1α levels compromises the anti-melanoma effects of BNIP3 deficit

We next wished to elucidate the extent to which increased HIF-1α degradation in BNIP3-silenced melanoma cells was responsible for metabolic alterations and impaired tumor growth in vivo.

To this end, we transduced the melanoma cell lines with constructs overexpressing either a decoy protein (luciferase, Luc) or a human HIF-1α mutant which is no longer susceptible to PHD-mediated degradation, since both proline residues targeted for hydroxylation have been mutated into alanine residues (HIF-1α-AA), thereby leading to a constitutive HIF-1α transcriptional activation (Di Conza et al, 2017).

Successful expression of the human HIF-1α-AA mutant in the murine B16-F10 cell lines (Fig EV5A and B) elevated overall HIF-1α levels, but not its hydroxylation, in shCntl and corrected HIF-1α protein levels in BNIP3-silenced cells, without overtly causing an overexpression (Fig 7A). Most importantly, in the BNIP3-silenced melanoma cells (Fig 7A), HIF-1α-AA expression significantly rescued (i) HIF-1α glycolytic and pro-angiogenic (Vegfa) target genes/proteins (Fig 7A and B) and without affecting their basal PHD2 levels (Fig EV5C; which also suggests that the increase in PHD2 in BNIP3 silenced cells is possibly an adaptive downstream response to changes in iron homeostasis, rather than downstream of HIF-1α) and (ii) induced a drop in OCR concomitant with a rise in ECAR, thus restoring glycolysis-driven metabolism (Fig 7C and D, and Fig EV5D).

In contrast, BNIP3-mediated effects on the lysosomal NCOA4 turnover (ferritinophagy flux) were not affected by the expression of HIF-1α-AA (Fig 7E). Taken together, these data show that in the BNIP3-depleted cells the glycolytic defect is secondary to HIF-1α downregulation and suggest that the effects on ferritinophagy are BNIP3-coordinated upstream events, ultimately stimulating PHD2 expression and function.

The expression of HIF-1α-AA in control cells fostered their clonogenic growth in comparison with the shCntl Luc cells but did not exert major effects on the colony formation defects of the BNIP3-deficient cells (Fig EV5E). This is likely explained by the reported inability of BNIP3-depleted melanoma cells to form clones in vitro, due to the major effects of BNIP3 on the cytoskeleton (Maes et al, 2014a). We next evaluated the tumor growth ability and impact on the tumor microenvironment of the HIF-1α-AA expressing shCntl (CnlKD HIF-1α-AA) and of the shBNIP3 melanoma cells (BNIP3KD HIF-1α-AA) and compared them with their respective empty-vector expressing shCntl (CnlKD Luc) and shBNIP3 melanoma cells (BNIP3KD Luc) (schematically shown in Fig 7F). Remarkably, compared with the BNIP3KD Luc which showed the observed severe tumor growth impairment, the BNIP3KD HIF-1α-AA cells recovered their tumor-forming ability to a similar degree as the CnlKD Luc (Fig 7G). The growth of the CnlKD HIF-1α-AA melanoma cells with fully operational HIF-1α signaling including the enforced expression of BNIP3 (Fig 7A), possibly mimicking a pseudo-hypoxic status, was significantly exacerbated in these in vivo settings (Fig 7G), further suggesting that a feedforward BNIP3-HIF-1α axis reinforces its pro-tumorigenic action (Fig 7F).

In line with the finding that expression of HIF-1α-AA in shCntl and BNIP3-silenced melanoma cells promoted or rescued prevalently their glycolytic phenotype (Fig 7A–D), we did not observe major morphological or functional changes in the vasculature of
the HIF-1α-AA expressing tumors that could explain their heightened growth rate (Fig 7H). Instead, the accelerated growth rate of the CntlKD HIF-1α tumors further suggests that beyond the tumor-promoting role of glucose metabolism in melanoma, a feed-forward BNIP3-HIF-1α axis supports tumor growth through additional mechanisms.

Together these results show that, secondary to the increase in NCOA4-mediated ferritinophagy induced by the depletion of BNIP3, PHD2-mediated downregulation of HIF-1α ablates tumor glycolysis and growth in BNIP3-silenced melanoma cells.

Discussion

In this study, we show that BNIP3 supports HIF-1α stabilization and its pro-tumorigenic program in melanoma. We propose a model in which BNIP3, due to its ability to regulate the intracellular availability of iron by directly controlling NCOA4-mediated ferritinophagy, maintains HIF-1α-driven glycolytic program and establishes a feed-forward BNIP3-HIF-1α axis that fosters melanoma growth. This bidirectional loop between BNIP3 and HIF-1α is an unexpected finding of this study, given that BNIP3 is a hypoxia-responsive gene thought
to operate as a downstream target of HIF-1α rather than being a positive regulator of this transcription factor.

**BNIP3 supports HIF-1α glycolytic program and melanoma growth**

Mechanistic studies comprehensively examining the role of BNIP3 in cancer are scarce. In this study, we show that depletion of BNIP3 decreases mitochondria clearance causing the accumulation of dysfunctional mitochondria. Despite this, BNIP3-silenced melanoma cells still retain their OXPHOS ability in combination with dysregulated glycolysis and compromised fermentation. This is in contrast to the effects mediated by the reduction in general autophagy in the ATG5-silenced melanoma cells, which result in the stimulation of aerobic glycolysis, as observed in other studies (Kimmelman & White, 2017; Jiao et al., 2018). We show that the reduced aerobic glycolysis of the BNIP3-silenced cells is secondary to their inability to support normoxic HIF-1α levels and its overall transcriptional glycolytic program. Furthermore, rescuing the basal expression of HIF-1α (by expressing an undegradable HIF-1α mutant) in BNIP3-silenced cells restores their glycolytic capability and correct their delayed tumor growth in vivo.

Thus, compromising mitochondrial clearance while concomitantly preventing aerobic glycolysis in melanoma cells through the removal of BNIP3 has oncoseuppressive ability. Consistent with this, studies in multiple tumor mouse models have shown that defects in mitophagy can cause tumor regression (Porporato et al., 2018; Vara-Perez et al., 2019a). In our melanoma model, BNIP3-silenced cells are still able to sustain their heightened autophagic flux, which ultimately supports their survival, at least in vitro. Future studies using autophagy blockers in vivo will be important to understand whether inhibiting autophagic flux contributes to accelerate tumor cell death and completely block the growth (or relapse) of the BNIP3-depleted tumors.

Finally, the metabolic rewiring observed in the BNIP3-silenced cells could impact the tumor microenvironment. We found that BNIP3-depleted tumors have smaller tumor vessels, which together with lower LDHA levels (reduced acidosis), could render the tumor microenvironment less immunosuppressive (Cascone et al., 2018).

Considering that melanoma glycolysis is associated with immune resistance to adoptive T-cell therapy (Cascone et al., 2018), the potential contribution of BNIP3 on immune resistance mechanisms (and T cell effector functions, in particular) should be explored in future studies.

Notably, in an MMTV-PyMT breast cancer model, loss of BNIP3 was shown to mitigate mitophagy, elevate ROS, and maintain autophagic flux under normoxic conditions (Chourasia et al., 2015), as observed in this study. Yet, in the context of breast cancer, BNIP3 had a pro-tumorigenic role. This in vivo dichotomous BNIP3 role is related to HIF-1α. In the MMTV-PyMT breast cancer model, mitochondrial dysfunction promotes—rather than suppresses—HIF-1α stabilization and aerobic glycolysis (Chourasia et al., 2015). The stabilization of HIF-1α eventually feeds breast cancer growth, angiogenesis, and dissemination (Chourasia et al., 2015), supporting the view that BNIP3 restricts breast cancer progression. A clear understanding of these discrepancies is still lacking and may reflect additional roles of BNIP3 that are cancer cell type-specific or related to alterations of other catabolic pathways, such as ferritinophagy (see below) or to cancer cell-specific mitochondrial circuitries.

Irrespective of this unknown, and supporting our patient and mechanistic data, high BNIP3 levels have been shown to promote cancer cell survival and metastatic dissemination in uveal melanomas (Jiang et al., 2018). Our TCGA and TMA data show that, in early-stage melanomas and further in metastasis, BNIP3 levels are elevated, correlate with the HIF-1α transcriptional signature and associate with shorter patient survival. A limitation of this analysis is the inability to discriminate normoxic and hypoxic HIF-1α stabilization in vivo since it is very difficult to assess the origin of HIF-1α levels in whole tissue proteomic or transcriptomic analysis (such as the TCGA or our own tumor lysates). Despite this, BNIP3 could be considered as a novel prognostic biomarker for at least a subset of melanoma patients. Moreover, since HIF-1α levels contribute to therapeutic failure in melanoma (Zbytew et al., 2013), a better insight into the BNIP3-mediated pathways regulating the HIF-1α glycolytic program could be important to design new therapeutic avenues.
**Lack of BNIP3 enhances PHD2-mediated HIF-1α degradation by stimulating ferritinophagy**

Using pharmacological and genetic tools, we show that BNIP3 depletion increases the hydroxylation of HIF-1α both in vitro and in vivo by elevating the expression and activity of PHD2. This BNIP3-regulated mechanism operates prevalently under conditions of oxygen availability and is mitigated when HIF-1α is firmly stabilized by hypoxia. HIF-1α is classically associated with the transcriptional response to oxygen deprivation (Lee et al., 2020); however, in the past years, several studies have highlighted mechanisms of normoxic or pseudo-hypoxic HIF-1α stabilization in cancer (Semenza, 2010). High normoxic HIF-1α levels in glioblastoma cells were supported by the accumulation of glycolytic metabolites, such as pyruvate and lactate (De Saedeleer et al., 2012). Together with pyruvate, other metabolites such as succinate or fumarate (Hewitson et al., 2007; Lee et al., 2020) have been proposed as indirect HIF-1α stabilizers, although the precise mechanism remains controversial (Hewitson et al., 2007). Similar mechanisms were described in response to the loss of PDK1 in head and neck squamous cell carcinoma (McFate et al., 2008) or PDK2 in lung and breast cancer cells (Sutendra et al., 2013). Although we cannot rule out that, upon loss of BNIP3, subtle changes in the concentrations of cellular metabolites affecting PHD2 activity may further accentuate HIF-1α downregulation, our data suggest that deregulated iron metabolism is a crucial BNIP3-associated effector mechanism.

Ferritinophagy is activated largely in response to changes in the cytoplasmic concentration of Fe²⁺ and is controlled by the cargo receptor NCOA4 (Santana-Codina & Mancias, 2018). We found that BNIP3 silencing in melanoma cells exacerbated the lysosomal turnover of NCOA4, resulting in an increase in the cytoplasmic Fe²⁺/Fe³⁺ ratio. Moreover, we also found that BNIP3 and NCOA4 physically interact and that the removal of NCOA4 rescued iron levels in BNIP3-silenced cells. Hence, the elevated iron level of the BNIP3-depleted cells is likely resulting from increased availability of cytosolic NCOA4 engaged in homeostatic ferritinophagy, which is caused by disrupting BNIP3-NCOA4 interaction. Intracellularly, iron is mobilized by ferritinophagy and by both mitophagy and non-canonical (e.g. endosomal microautophagy) lysosomal pathways, but the molecular mediators of these pathways are still elusive (Goodwin et al., 2017; Mejlvang et al., 2018; Santana-Codina & Mancias, 2018). It is tempting to propose that, as a consequence of BNIP3 depletion and the disrupted interaction between BNIP3 and NCOA4, the elevated turnover of NCOA4-mediated ferritinophagy compensates for the reduced mitophagy in order to supply levels of Fe²⁺ compatible with the maintenance of vital mitochondria OXPHOS functions (Vara-Perez et al., 2019a). This is an intriguing possibility that requires further investigation.

Boosting the pool of labile iron then favors PHD2 activity and HIF-1α degradation, as shown by the complete rescue of HIF-1α levels and downstream targets in the BNIP3-silenced cells treated with the iron chelator DFO. Although our data support a role for PHD2 as an important effector of the BNIP3-HIF-1α axis, we cannot exclude that elevation of intracellular iron in the BNIP3-depleted cells favors the concerted activity of other prolyl hydroxylases and may affect other iron-dependent enzymes and pathways.

Lastly, the elevation of the labile iron pool observed upon loss of BNIP3 suggests that melanoma cells harboring high levels of BNIP3 may be less vulnerable to ferroptosis, an emerging iron-mediated and lipid peroxidation-driven necrotic cell death with possible therapeutic implications in melanoma (Tsai et al., 2018). These are interesting conjectures that need further molecular investigations in order to identify the iron-sensing mechanisms and effectors regulated by BNIP3.

In summary, this study unravels an unexpected loop whereby BNIP3 and HIF-1α mutually reinforce each-other pro-tumorigenic function in melanoma, which may provide the basis for new therapeutic options.

**Materials and Methods**

**Experimental design**

The objective of this study was to determine the role of BNIP3 in melanoma progression. We investigated mRNA and protein expression of BNIP3 in melanoma patient-derived material by analysis of public RNAseq datasets together with IHC-TMA and paired primary-metastases samples. Subcutaneous implantation of a murine melanoma cell line with different BNIP3 or ATG5 levels in syngeneic mice aimed to establish the pro-tumorigenic function of BNIP3 in vivo and the potential difference between BNIP3 and a canonical autophagy gene. *In vitro/in vivo* studies using immuno(cyt/o)histochemistry, flow cytometry, metabolic flux readouts (Seahorse, ¹H-NMR, GC-MS), protein and transcript levels, and subsequent mechanistic validation studies evaluated the role of BNIP3 in melanoma progression, with particular focus on the regulatory feedforward loop of HIF-1α.

**Chemicals and reagents**

Cell culture reagents (unless otherwise stated), antimycin A, β-mercaptoethanol, bovine serum albumin (BSA), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), deeroxamine mesylate (DFO), D-glucose, dimethyl sulfoxide (DMSO), disodium-fumarate, galactose, gelatin from bovine skin, glycerol, glycine, gelatin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hydrogen peroxide solution, IOX2, methylene blue, oligomycin, polybrene, sodium doceyl sulfate (SDS), Tris, Triton X, and Tween 20 were obtained from Sigma-Aldrich (Bornem, Belgium). Sodium deoxycholate, NP-40, phenylmethylene sulfonyl fluoride or phenylmethylsulfonyl fluoride (PMSF), glutamine and orthovanadate were purchased from Sigma-Aldrich (Taufkirchen, Germany). FeCl₃·6H₂O standard was from Sigma-Aldrich Chemie (Steinheim, Germany). Sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl₂), and calcium chloride (CaCl₂) were purchased from ACROS organics (Fisher Scientific, USA). Chloroform, ethanol, methanol, and xylene were obtained from VWR International (Oud-Heverlee, Belgium). Bafilomycin A (BafA) was purchased from Sanbio (Uden, The Netherlands). Puromycin dihydrochloride was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Hygromycin B was obtained from Invivogen (San Diego, CA, USA). Tetramethylthiourea (TMAH) and HCl suprapure were purchased from Merck (Darmstadt, Germany). Argon gas was purchased from Air Liquide (Düsseldorf, Germany). FeCl₂·4H₂O was purchased from AppliChem GmbH (Darmstadt, Germany).
Germany). The cOmplete™ Protease Inhibitor Cocktail was from Roche, Mannheim, Germany. Whenever concentrations are included throughout the methods, they refer to the final concentration.

### Cell culture

Melanoma cell lines B16-F10 (murine) and WM35 (human) were obtained from ATCC. WM35 was validated by STR profiling. Murine cell lines cannot be reliably validated by STR profiling at this moment. Patient-derived early-stage human melanoma cell line 530 was a kind gift from Dr. G. N. P. van Muijen (Radboud University, The Netherlands). Murine melanoma cells (B16-F10) were maintained in RPMI-1640 medium containing 10% (v/v) Fetal Bovine Serum (FBS; Hyclone, Thermo Fisher Scientific) and penicillin (100 units/ml)/streptomycin (0.1 mg/ml). Human melanoma cells 530 and WM35 were maintained in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM glutamine and penicillin (100 units/ml)/streptomycin (0.1 mg/ml). All cells were maintained routinely in 5% CO₂ and 95% air at 37°C. Cells were routinely checked for mycoplasma contaminations using the Plasmotest kit (Invivogen) according to the manufacturer’s instructions.

For protein and RNA extraction, cells were seeded at a density of 150,000 (B16-F10 shCntl, B16-F10 shATG5), 200,000 (all human melanoma cell lines), or 300,000 (B16-F10 shBNIP3) cells/well in a 6-well plate (Greiner Bio-One, Vilvoorde, Belgium) and cultured for 48 h (normoxic condition). For hypoxic conditions (1.5% O₂, 5% CO₂, 93.5% N₂), the cells were kept in an Invivo2400 hypoxia workstation (Ruskin, Heusden-Zolder, Belgium) for 24 h after 24 h of plating. In case of a 24-h treatment, cell medium was refreshed after 24 h of plating including the chemical of interest [IOX2 (in DMSO, 0.1% v/v)] was added 4 h before fixation.

### Generation of shRNA stable clones of melanoma cells

Lentiviral shRNA vectors against human BNIP3 or murine BNIP3, ATG5, and NIX were purchased from the MISSION™ shRNA library (Sigma-Aldrich). An empty pLKO.1-puro control vector was used as a control (shCntl). To generate lentiviral particles, HEK 293T cells were seeded in 10 cm² dishes at 1.5 × 10⁶ cells per 6 ml and transfected the following day by the calcium phosphate method with 4 µg of pLKO.1-puro carrying the respective shRNAs or with empty pLKO.1-puro. Each transfection also included 1.2 µg of a plasmid encoding VSV-G (pMD2-VSV-G, Tronolab) and 2.6 µg of a plasmid encoding packaging proteins (pCMVdR8.9, Tronolab). VSV-G pseudotyped virus was collected 48 h after transfection, processed through 0.45 μm filters (Millipore, Burlington, MA, USA), and then added to the exponentially growing melanoma cell cultures in the presence of 8 mg/ml of polybrene. The cells were expanded and selected by puromycin treatment (10 µg/ml). Target downregulation was confirmed by immunoblotting and qPCR.

### Generation of undegradable HIF-1α and BNIP3 stable clones of B16-F10 melanoma cells

Human HIF-1α mutant cDNA was PCR-amplified from the plasmid pLenti/V5-HIF-1α-mPPN and cloned into in-house pCHMWS-eGFP-ires-hygro after removal of eGFP. Murine Myc-BNIP3 sequence was obtained from Addgene plasmid #100796. The cDNA was ordered as a gBlocks Gene Fragment at IDT (Leuven, Belgium). These fragments were cloned into our in-house pCHMWS-GFP-ires-hygro transfer plasmid via BamHI and Nhel restriction sites after removal of eGFP. In both cases, Firefly luciferase was used as decoy protein in the control cell lines. Lentiviral vectors were produced by the Leuven Viral Vector Core as described in (Ibrahimi et al., 2009). 100,000 cells/well were plated in a 24-well plate, and viral transduction was performed during 48 h. Cells were selected with hygromycin (200 µg/ml) until all non-transduced cells died. Target overexpression was confirmed by immunoblotting and qPCR.

### Cell transfection

Cells were transiently transfected with different immunofluorescently labeled LC3B constructs: RFP-EGFP-LC3 tandem (Addgene plasmid #21074) and LC3B-GFP (Addgene plasmid #22405) using TransFect X2 (Mirus Bio LLC, Madison, WI, USA) transfection reagent in serum-free DMEM medium according to the manufacturer’s indications. 24 h after transfection, the transfection medium was removed and was replaced by full medium to let the cells recover. After 24 h, surviving cells were seeded in glass coverslips (Thermo Fisher Scientific) coated with 1% (w/v) gelatin in PBS and allowed to grow for an additional 24 h before fixation. When necessary, BafA was added 4 h before fixation.

Cells were transiently transfected with 40 µM siRNA from the SMARTpool: ONtarget plus library from Dharmacore (Lafayette, CO, USA) either non-targeting (siScr, D-001810-10-05), targeting murine PHD2 (siPHD2, L-040757-01-0005) or murine NCOA4 (siNCOA4, L-049515-01-0005) using Dharmafect1 (Dharmacon) transfection reagent in serum-free medium according to the manufacturer’s indications. 24 h after transfection, a second transfection was performed to ensure enough protein degradation. 24 h after the second transfection, medium was removed and was replaced by full medium to let the cells recover. 36 h after the change of medium, surviving cells were collected for protein and RNA extraction. 12 h after the change of medium, cells were plated for the FerroOrange assay in a dark 96-well plate (see below).

### Mice experiments

Animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven (ECD P237/2015) and were performed following the institutional and national guidelines and regulations. Female C57BL/6 mice of 6 weeks of age were provided by the KU Leuven mouse facility or were purchased from Janvier (France). For all experiments, mice were group-housed in standard cages under a 12 h light/dark cycle with ad libitum access to water and food. Only females were used in the experiments. To assess subcutaneous tumor growth, 150,000 B16-F10 murine melanoma cells proficient or deficient for ATG5 and BNIP3 were injected subcutaneously into the right flanks of immunocompetent syngeneic (C57BL/6) mice. Tumor volumes were measured at least three times per week with a caliper using the formula \[ V = \pi \times d^2 \times t/6 \], where D is the major tumor axis, d is the minor tumor axis, and t is the thickness of the tumor. The mice were sacrificed when tumors reached a maximum size of 1,000 mm³ depending on the experimental setup. Tumors were collected for histological
analysis, gene expression, or protein analysis. To detect tumor hypoxia, tumor-bearing mice were injected 60 mg/kg pimonidazole hydrochloride (Hypoxyprobe kit, #HP3-200Kit, Chemicon-Millipore, Billerica, MA, USA) 1 h before tumor collection.

**Immunoblotting**

A modified Laemli sample buffer (125 mM Tris–HCl, pH 6.8 buffer containing 2% SDS and 20% glycerol) containing 10% (v/v) protease and phosphatase inhibitors (Pierce Protease Inhibitor/Phosphatase Inhibitor Tablets, Thermo Fisher Scientific) was used to lyse the cells. Frozen tumors were smashed in Laemli buffer with a metal mortar (Usbeck Metal Products, Radevormwald, Germany), centrifuged 15 min, at 20,000 g at 10°C, and the supernatant was collected for protein assays. Protein concentration in cell and tumor lysates was determined using the BCA protein assay reagent (Pierce, Thermo Fisher Scientific). Precision Plus Protein™ ladder (#1610374, Bio-Rad Laboratories, Hercules, CA, USA) was used for protein size reference. Samples were separated by SDS–PAGE on the Criterion system (Bio-Rad Laboratories) on a 4%–12% Bis-TRIS gel and electrophoretically transferred to Amersham Protran 2 μm-pored nitrocellulose paper (GE Healthcare, Chicago, IL, USA). The blots were blocked for 60 min at RT in TBS-T buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk (w/v) and then incubated with selected primary and secondary antibody solutions prepared in blocking buffer unless otherwise stated.

Primary antibodies for ATG5 (1:1000, #12994), murine BNIP3 (1:1000, #3769), HIF-1α-ÖH P396 (1:500, #3434), LC3B (1:1000, #3868), LDHA (1:1000, #2012), NIX (1:1000, #12396), PHD2 (1:1000, #4835), and TFEB (1:500, #4240) were purchased from Cell Signaling Technologies (Danvers, MA, USA) and prepared in 5% (w/v) BSA in TBS-T buffer. HIF-1α antibody (1:1000, #10006421) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). FTL antibody (1:1000, #ab109373) was purchased from Abcam (Cambridge, UK). Human BNIP3 (1:1000, #HPA003105) is from the Sigma-Aldrich’s Protein Atlas antibody repertoire. Luciferase antibody (1:1000, NB600-307) is from Novus Biologicals (Centenals, CO, USA). (Beta-)Actin (1:2000, #A5441) is from Sigma-Aldrich. NCOA4 (1:1000, A302-272A) was purchased from Bethyl Laboratories (Montgomery, TX, USA).

Appropriate secondary antibodies for chemical (HRP-based) detection were from Cell Signalling Technologies and for infrared detection were from Thermo Fisher Scientific. Signal detection was performed using the Typhoon infrared-imaging system (GE Healthcare) or Chemidoc™ MP system (Bio-Rad Laboratories) using the ECL solution from Pierce (Thermo Fisher Scientific). Whenever necessary, membranes were stripped for 20 min in ReBlot Plus Mild Antibody Stripping Solution (Millipore) 1X in dH2O and reblocked in 5% milk in TBS-T before the addition of the new primary antibody. Quantifications by densitometry of the bands were performed using the software Image Studio (Li-Cor Biosciences, Lincoln, NE, USA).

**Immunoprecipitation of endogenous BNIP3**

48 h after plating, B16-F10 shCntl cells were collected through scraping and lysed in lysis buffer (1% (w/v) CHAPS, 50 mM Tris–HCl, 100 mM KCl, 150 mM NaCl, 10% (v/v) protease inhibitor) for 30 min at 4°C. Cells were centrifuged to remove debris for 5 min (max. speed, 4°C), and the supernatant was collected for protein quantification with the BCA protein assay reagent. 1,250 μg of proteins were precleared for 1 h at room temperature (RT) using the Protein A/G-agarose beads (#sc2003, Santa Cruz Technologies). From the precleared solution, 500 μg of proteins were combined with 4 μl from either an isotype immunoglobulin G (IgG) control (#sc2025, Santa Cruz Technologies) or anti-BNIP3 (#ab10433, Abcam) primary antibody overnight (ON) at 4°C.

Protein–antibody complexes were captured by the addition of Protein AG Magnetic Beads (#88802, Pierce, Thermo Scientific) for 1.5 h at RT. Protein AG Magnetic Beads with captured protein–antibody complexes were washed three times with lysis buffer. Proteins were eluted in loading buffer, boiled at 100°C for 5 min, and loaded on a gel for Western blot analysis. 50 μg of precleared protein solution were used as input, and 50 μg of a B16-F10 shCntl treated with BafA lysate were used as band reference. For the immunoprecipitation blots, HRP-based detection using as secondary antibody the Veriblot antibody (#ab131366, Abcam; 1:1000) and the Clarity and Clarity Max solutions (Bio-Rad Laboratories) to develop the membranes in an Amersham Imager 600 (GE Healthcare).

**RNA extraction, cDNA generation, and qPCR**

Cells destined for RNA extraction were collected in RLTplus buffer (Qiagen, Hilden, Germany) supplemented 1/100 (v/v) with β-mercaptoethanol. RNA extraction was performed using the RNeasy Mini Kit (Qiagen) following the manufacturer’s indications. Tumor RNA was extracted first by homogenizing the tissue with a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) in the presence of TRIzol™ (Life Technologies, Thermo Fisher Scientific) and precipitating it with chloroform. After centrifugation, RNA was further purified using the PureLink™ RNA Mini Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer’s instructions.

RNA concentration was measured using a Nanodrop 3000 (Thermo Fisher Scientific) and cDNA was synthesized using 0.5 μg RNA per sample as template and the QuantiTect Kit (Qiagen) following the manufacturer’s instructions. After diluting the resulting cDNA 1:5 in sterile water, expression analysis was performed using home-designed or literature-based primers (Integrated DNA Technologies, Haasrode, Belgium) and ORA Sybr Green Mastermix (Highgu, Kraichtal, Germany) in an ABI7500 (Applied Biosystems, Thermo Fisher Scientific) machine. Primer sequences are available in Appendix Table S5. CT values were calculated using the software of the machine and the geometric mean of two housekeeping genes 

$$\text{Expression Value} = \frac{1}{(2^{-\Delta CT})_{\text{Gene Of Interest}} / (2^{-\Delta CT})_{\text{HKG}}}$$

where GOI stands for “Gene Of Interest” and HKG for “Housekeeping Genes”. Afterward, the fold change relative to their respective control was calculated.

**Determination of proliferation, cell death, and colony formation**

Proliferation curves were generated using an IncuCyte ZOOM system (Eisen BioScience), starting from cells seeded at a density of 3,000 cells per well in a 96-well plate (Greiner, Vilvoorde, Belgium),
based on phase-contrast images taken at 2-h intervals for the duration of the experiments. The interval comprising 10–80% of confluence was used to calculate the doubling time of the cells per well using the exponential fitting provided by Prism software (GraphPad Software, La Jolla, CA, USA).

Cell death was determined by the propidium iodide (PI) exclusion method. Briefly, cells were trypsinized at the indicated time points and incubated with the vital dye PI (Sigma-Aldrich) for 15 min at RT. PI-positive (dead) cells were quantified via flow cytometry (Attune Cytometer, Invitrogen, Life Technologies, Paris, France) and analyzed using FlowJo (BD Biosciences).

For the colony formation assay, cells were plated as single-cell suspension at very low density (500 cells in 15 ml medium on a 10 cm dish). After growth for 10 days, cells were fixed in methanol containing 1% (w/v) methylene blue.

**Determination of mitochondrial parameters**

Mitochondrial depolarization and ROS levels were detected separately after incubation of the cells with either 20 nM TMRM (Molecular probes, Invitrogen) or 7.5 mM CM-H2DCFDA (Molecular probes, Invitrogen), respectively, 30 min at 37°C. Fluorescence was measured using an Attune flow cytometer and analyzed using FlowJo software by gating first alive cells in the FSC/SSC display and singlets in the FSC-A/FSC-H display. Mean fluorescence intensity (MFI) was calculated from the final population and plotted as fold change of the corresponding shCntl MFI value.

Mitochondrial morphology was detected after incubation of the cells with 100 nM MitoTracker Green (Molecular Probes, Invitrogen) 30 min at 37°C. After one wash in PBS, live cells were imaged in Krebs solution (containing 150 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM HEPES (pH 7.3), 11.5 mM glucose, and 1.5 mM CaCl₂), using an LSM880 confocal microscope in a temperature, CO₂-controlled incubator (Carl Zeiss, Munich, Germany).

**Immunostaining and Immunohistochemistry**

Cells were grown and treated on gelatin-coated glass coverslips (as described before). Cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 (v/v) in PBS, blocked with 5% (v/v) FBS, 1% (w/v) BSA, 2.25% (w/v) glycine in PBS and incubated with selected primary (anti-TOMM20, 1:100, #ab186735, Abcam) and secondary antibodies (1:200, #A27040, Life Technologies) in 5% (v/v) FBS, 1% (w/v) BSA, 2.25% (w/v) glycine in PBS and incubated with the vital dye PI (Sigma-Aldrich) for 1 h at RT and then incubated overnight at 4°C with the primary antibody in TNB buffer: anti-Ki67 (1:100, #MA5-14520, Thermo Fisher Scientific), anti-HIF-1α (1:300, #10006421, Cayman Chemicals), anti-LC3B (1:100, #3868, Cell Signaling Technologies), anti-CD31 (1:100, #550274, BD Biosciences), anti-smooth muscle actin-Cy3 (1:250, #C6198, Sigma-Aldrich), or anti-hydroxyprobe (1:100, HP3-200Kit, Chemicon-Millipore).

For the immunoperoxidase technique, the Vectastain Elite ABC kit (Vector Labs, Peterborough, UK) combined with the chromogen ImmPACT DAB Peroxidase substrate kit (Vector Labs) were used according to the manufacturer’s protocol. Slides were counterstained in Harris Haematoxylin (Diapath), dehydrated in ethanol series, cleared in xylene, and permanently mounted with a resinous mounting medium (DPX, Diapath). For immunofluorescence, sections were then incubated with the appropriate peroxidase-labeled IgGs (Dako), followed by amplification with Cy3 or Cy5-tyramide signal amplification (TSA) kit (Perkin Elmer). DAPI was used as nuclear counterstain and the slides were mounted with Prolong Gold antifade reagent. TNT buffer was used as washing buffer in between steps.

Cell death was detected and quantitated via the transferase-mediated dUTP nick end-labeling (TUNEL) technique using the “In situ Cell Death Detection” Kit (Roche, USA) according to the manufacturer’s instructions.

**Imaging and analysis**

For cell death and hypoxia measurements, 10 × mosaic pictures were taken with an Olympus IX73 (Olympus, Berchem, Belgium) and the positive area was quantified using Olympus’ CellSense imaging software and expressed as a percentage of the total area of the analyzed tumor. For proliferation measurements, 20 × mosaic pictures were taken with an Axioscan.Z1 (Carl Zeiss) and the positive cells were quantified using QuPath software (Bankhead et al, 2017) and expressed as a percentage of total cell number within the area of the analyzed tumor. For the LC3B immunohistolocial staining, the pictures were acquired with the LSM780 confocal microscope, 25 × objective (Carl Zeiss, Munich, Germany).

For the co-localization and the mitochondrial morphology analysis, the pictures were acquired with the LSM880 confocal microscope, 60 × objective (Carl Zeiss).

For the GFP-LC3-TOMM20 co-localization analysis, images were quantified by manual counting using ImageJ/Fiji software. GFP-LC3 puncta were defined as round structures found only in the green channel with no corresponding structure in the other channels. Co-localization analysis was performed by counting all GFP-LC3 puncta per cell overlapping with a mitochondrial structure, defined by TOMM20 staining and calculating the percentage of colocalizing puncta over the total number/cell. The mitochondrial morphology was analyzed with the macro “Mito-morphology” from ImageJ/Fiji Software. Since the confocal pictures contained a variable number of cells, these values were considered non-parametric for statistical
purposes and outlier values were removed using the Rout method before statistical analysis.

**Metabolic measurements**

**Seahorse**

Cells were plated at a density of 30,000 (shCntl, shATG5) and 50,000 (shBNIP3) cells per well of an XFp Extracellular Flux Analyzer culture plate in culture medium overnight. Before the assay, cells were switched to the Mitostress assay medium (Seahorse Biosciences, North Billerica, MA, USA) containing either glucose or galactose (25 mM) and supplemented with 2 mM glutamine and 1 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. Following the Mitostress assay, 8 μM oligomycin, 4.5 μM FCCP and 5 μM antimycin A diluted in assay medium were added whenever appropriate. Once the run was finished, all the medium was removed from the well and all the cells were collected in 10 μl of Laemli buffer for BCA-based protein quantification (as explained before).

**1H-NMR-based metabolic measurements**

Cells were plated and grown to confluency in T175 flasks. 24 h before collecting the samples, medium was replaced by fresh new medium and all the cells were collected in 10 μl of Laemli buffer for BCA-based protein quantification (as explained before).

SIMCA-P + 12.0 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) followed by a partial least square discriminant analysis (PLS-DA) were carried out on the dataset. Only the descriptors with a Variable Importance in the Projection (VIP) above 1 were considered in this study. Their corresponding metabolites were identified using the Chenomx NMR suite software (version 8.1.1).

**GC-MS-based metabolic measurements**

**Metabolite extraction and derivatization method**

Metabolite quenching and extraction were performed as described before (Elia et al, 2017). Briefly, samples were collected in 800 μl of 60% ice-cold methanol containing 90 ng/ml of glutaric acid as an internal standard. Subsequently, a volume of 500 μl of precooled chloroform was added to each sample. During the collection procedure, the samples were always refrigerated in a mixture of dry and wet ice. The samples were then vortexed for 10 min at 4°C and centrifuged for 10 min (max. speed, 4°C) to achieve phase separation. After centrifugation, the metabolites were separated into two phases divided by a protein layer: polar metabolites in the methanol/water (upper) phase and the lipid fraction in the chloroform (lower) phase. The upper and lower phases were collected in separate tubes and dried at 4°C overnight using a vacuum concentrator.

The samples were derivatized and measured as described before (Elia et al, 2017; Lorendeau et al, 2017). Briefly, polar metabolites were resuspended and derivatized in 20 μl of 20 mg/ml methoxyamine in pyridine per sample for 90 min at 37°C. Subsequently, 15 μl of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide, with 1% tert-butyldimethylchlorosilane were added to 7.5 μl of each derivative and incubated for 60 min at 60°C.

The metabolites were analyzed by gas chromatography (7890A GC system) coupled to mass spectrometry (5975C Inert MS system) from Agilent Technologies. Metabolites were separated with a DB35MS column (30 m, 0.25 mm, 0.25 μm) using a carrier gas flow of helium fixed at 1 ml/min for the analysis of polar metabolites. A volume of 1 μl of sample was injected with a split ratio 1–3 with an inlet temperature set at 270°C. For the detection of polar metabolites, the gradient started at 100°C for 1 min ramped to 105°C at 2.5°C/min, then to 240°C at 3.5°C/min and finally to 320°C at 22°C/min. For the measurement of metabolites by mass spectrometry, the temperatures of the quadrupole and the source were set at 150°C and 230°C, respectively. An electron impact ionization fixed at 70 eV was applied and either a scan or a selected-ion monitoring (SIM) mode was used for the measurement of polar metabolites.

After the acquisition by GC-MS, an in-house MATLAB M-file was used to extract mass distribution vectors and integrated raw ion chromatograms. The natural isotope distribution was also corrected using the method developed by Fernandez et al, as described before (Elia et al, 2017; Lorendeau et al, 2017). The peak area was subsequently normalized to the internal standard glutaric acid and to cell number to account for sample loss during the metabolite extraction procedure and differences in cell density in the samples, respectively.

**Intracellular Fe²⁺ measurements (FerroOrange)**

Intracellular iron content was measured using the dye FerroOrange (F374-10, Dojindo EU GmbH, Munich, Germany) according to the manufacturer’s instructions. Briefly, B16-F10 cells were seeded in 100 μl of medium (20,000 cells per well for shCntl/shATG5 and 30,000 shBNIP3) and incubated overnight. After washing with HBSS...
with 10 mM HEPES (HBSS-HEPES buffer) three times, cells were incubated with FerroOrange 1 μM in HBSS-HEPES buffer for 30 min. After the incubation, the plate was immediately read using a Synergy H1 M Multi-Mode reader (BioTek, Winooski, VT, USA), with the excitation set at 543 nm and emission at 580 nm. Afterward, if microscopy images were needed, cells were imaged in the wells using an Olympus IX73 microscope with a 20 × objective. Both wells containing cells without dye and dye in empty wells were used for background correction of the plate reader intensity. For protein normalization, background wells seeded in parallel that were not incubated with the dye were used. All the cells were collected in 10 μL of Laemli buffer for BCA-based protein quantification (as explained before).

Intracellular total iron and iron redox species (Fe²⁺, Fe³⁺) measurements (CE-ICP-MS)

Cells were lysed in modified RIPA lysis buffer containing PBS pH 7.4, 0.5% sodium deoxycholate, 1% NP-40 containing 1 mM PMSF, 1 mM orthovanadate, and 1x cOmplete™ Protease Inhibitor on ice for 45 min with gentle agitation. Cell lysates were centrifuged at 10,000 g for 10 min, and supernatants were sent on dry ice to Helmholtz Center Munich. Speciation and quantification of Fe²⁺, Fe³⁺, and total iron were performed by capillary electrophoresis inductively coupled plasma mass spectrometry (CE-ICP-MS) as described previously (Michalke et al., 2019). Briefly, samples were analyzed on a “PrinCe 706” CE system equipped with an uncoated capillary (85 cm × 50 μm ID) and a laboratory-constructed CE-ICP-MS interface for element selective iron quantification of separated iron redox species at ICP-DRC-MS. Fe²⁺/Fe³⁺ separation and quantification were performed in 20 mM HCl-electrolyte at +25 kV separation voltage and 56Fe isotope detection at ICP-DRC-MS. DRC technology with NH₃ as reaction gas was employed for interference-free detection of the 56Fe isotope. Data were presented as Fe²⁺/Fe³⁺ ratio, and absolute values are presented in Appendix Tables S3 and S4.

Bioinformatic analysis (TCGA)

RNAseq level3 data and associated patient information from the SKCM cohort were downloaded from the TCGA website on 04/Nov/2016 and it contained 469 SKCM cases. 15 samples were discarded because of lack of available follow-up data, and one was discarded due to data disparity. The remaining 453 cases were analyzed using the R environment. Briefly, samples were separated in primary or metastatic according to the TCGA information (if necessary) and ranked according to their log₂ transformed BNIP3 levels using the function rttile. Once the expression groups were formed, Kaplan–Meier analysis or associated gene analysis (log₂ transformed) was performed using GraphPad Prism.

Patient sample acquisition and processing

Biopsies from benign nevi, primary, and metastatic melanoma were collected at the University Hospital of Leuven with patients’ informed consent, in accordance with the Declaration of Helsinki and with the approval of the Medical Ethics Committee of the University Hospital. The use of samples for the present study was approved by the Medical Ethics Committee of the University Hospital (project number S58320). The TMA consisting of 181 cases of melanoma metastases was constructed previously at the UZ Leuven (van Kempen et al. 2016). From the original 181 cases, 16 did not have tissue available and the other 7 did not have complete survival data. These 23 cases were excluded from the analysis, resulting in 158 cases. Appendix Table S1 compiles the clinical information associated with both the paired melanoma slides and the TMA samples. Immunohistochemistry was performed on the Leica BOND-MAX automatic immunostainer (Leica Microsystems). Antigen retrieval was performed on board using the Bond Epitope Retrieval Solution 2 (pH 9.0; Leica) according to the manufacturer’s instructions followed by 5 min of peroxidase blocking, 30 min of anti-BNIP3 (1:100 dilution; HPA003015, Protein Atlas, Sigma-Aldrich) primary antibody incubation, 30 min of secondary antibody (Dako, ready to use), and 30 min of AEC (Dako, ready to use) or Bond Polymer Refine Red Detection (Leica) as substrate, both resulting in pink/red immunoreactivity. Melanoma-specific overall survival was defined as the interval from diagnosis of the primary tumor to death from melanoma or date of the last follow-up. Sections were analyzed by conventional light microscopy, and the results were analyzed in a semiquantitative manner both for paired slides (+,−, weak and gradient staining; +, weak and homogeneous staining; +++, intense and homogeneous staining) and TMA (0, absence of staining; 1, weak staining; 2, moderate staining; 3, intense staining).

Statistics

Data represent mean ± SEM of pooled biologically independent experiments, and each experiment was repeated at least three times. n values represent the number of independent experiments performed (in vitro data), the number of animals per condition (in vivo), or the number of patients per condition (patient-derived data). Normality was checked using both Shapiro–Wilk and Kolmogorov–Smirnov tests. To account for the inter-experimental technical variation among biological replicates from the in vitro experiments, the values coming from the same experiment were considered as matched values and, consequently, analyzed with the corresponding paired test, as specified in the figure legend. Statistical significance was calculated using the statistical software Prism software (GraphPad Software). A P < 0.05 was considered significant.

Data availability

This study includes no data deposited in external repositories.

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**Author contributions**

Experiment design, analysis, and manuscript writing: MV-P and PA; Experiments: MV-P, HM, ER, and KR; Data analysis: MV-P, HM, and ER; Performing and analyzing GC-MS steady-state stable isotope metabolic experiments: MR and S-MF; Performing and analyzing confocal imaging: MV-P and MLS; Generation of the Luc, BNIP3-FL, and Hif1α-AA overexpressing plasmasids: CVDH; Performing and analyzing the CE-ICP-MS experiments: VV and BM; Performing and analyzing the 1H-NMR metabolic experiments: CS and JMC; Staining, scoring, and analyzing the human patient material: MV-P, JW, FMB, and JVDvD; Assistance in TCGA data analysis: AGO; Assistance in tumor vasculature staining and analysis: AIO and MM; Helpful comments and discussion: WA, PV, JVS, SMF, and MM; Project and funding: PA; Manuscript revision and comments: All the authors.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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