Supplementary information

BIF-1 inhibits both mitochondrial and glycolytic ATP production: Its down-regulation promotes melanoma growth

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The supplementary information contains materials and methods, 6 supplementary figures and 1 supplementary table.

Materials and methods

CRISPR/Cas9-mediated down-regulation of *BIF-1* and *ATG7*

A375 and SK-MEL-5 melanoma cells were rendered knockout for *BIF-1* (Gene ID: 51100) and A375 cells were additionally genome edited for loss of *ATG7* (Gene ID: 10533) using a doxycycline-inducible bi-partite CRISPR/Cas9 system as previously described [1]. Briefly, specific guide RNAs (gRNA) were designed using a publicly available tool at http://crispor.tefor.net/ [2]. The *BIF-1* knockout was conditionally prepared using the doxycycline-inducible vector FgH1tUTG and FUCas9Cherry plasmids (gifts from Marco Herold, Melbourne, Australia, and Addgene plasmids # 70183 and #70182, respectively [1]). The gRNA sequences were as follows: *BIF-1* gRNA, 5'-TCC CAG TGA CCT TAA GGA GTG TAG-3' (forward) and 5'-AAA CCT ACA CTC CTT AAG GTC ACT-3' (reverse); *ATG7* gRNA, 5'-TCC CGA AGC TGA ACG AGT ATC GGC-3' (forward) and 5'- AAA CGC CGA TAC TCG TTC AGC TTC-3' (reverse). Primers were synthesized by Microsynth AG (Balgach, DE). Recombinant lentiviral constructs were produced by calcium phosphate transfection together with the envelope vector PMD2.G and the packaging vector psPAX2 (provided by Dr. D. Trono, University of Geneva, Switzerland) in 293T cells [3]. Viruses were harvested 24 hours after transfection, filtered through a 0.22 µm membrane (Merck Millipore, Burlington, Massachusetts, US), and stored at −80 °C until used. A375 and SK-MEL-5 cells were transduced with the desired virus in the presence of 8 µg/mL polybrene. The inducible *BIF-1* knockout (designated thereafter *BIF*$^{-/-}$) and *ATG7* knockout cells were sorted (using fluorescence-activated cell sorting (FACS)) according to their GFP (FgH1UTG) and mCherry (FUCas9Cherry) expression to produce a population of positive cells for both markers. Control A375 and SK-MEL-5 lines were prepared in parallel by infecting them with a non-gRNA sequence containing lentiviruses. Before each experiment, cells were treated with 2.5 µg/mL of doxycycline (Fischer Scientific, BP26531) for 3 to 5 days.

Immunoblot analysis

After cell pellets were collected, cells were lysed with the appropriate amount of lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 2 mM EDTA, 10 mM NaPyrophosphate, 50 mM NaF, 200 µM Na_3VO_4 and 1x PhosSTOP phosphatase inhibitor cocktail (Roche, Rotkreuz, Switzerland)). Lysis buffer was prepared freshly by adding 100 µM phenylmethylsulfonyl and protease inhibitor cocktail (Sigma-Aldrich, P-8340). The protein concentration was measured with the BCA Protein Assay kit (Thermo Fisher Scientific, 23225). 50 µg of cell lysate were separated on 12% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (PVDF; Immobilon-P membrane, Merck Millipore, IPVH304F0). After blocking in 5% milk in TBST (0.1% Tween 20 in 0.20 M Tris, 1.50 M NaCl [pH=7.6]) membranes were incubated with primary antibodies over-night at 4°C. Primary antibodies were purchased as follows: Monoclonal anti-endophilin B1/BIF-1 antibody (Novus Biologicals, NBP2-24733, 1:1’000), monoclonal anti-ATG7 antibody (Cell Signaling, 8558, 1:1’000), monoclonal anti-GAPDH antibody (EMD Millipore, AM4300, 1:5’000), polyclonal anti-phospho-AKT (Cell Signaling, 9271, 1:1’000), polyclonal anti-AKT (Cell Signaling, 9272, 1:1’000), polyclonal anti-p62 (Cell Signaling, 5114, 1:1’000), polyclonal anti-LC3 (Novus Biologicals, NB600-1384, 1:1’000), monoclonal anti-VDAC-1 antibody (Santa Cruz, B-6, 1:300), monoclonal anti-OGDH antibody (Cell Signaling, E1W8H, 1:1’000) and monoclonal anti-alpha-tubulin (Sigma-Aldrich, T5168, 1:5’000). The membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare, NA931-1ML and NA934-1ML) in blocking buffer for 1 h at room temperature. For the detection of HRP, chemiluminescent substrate (ECL-Plus Western Blotting Substrate, Thermo Fisher Scientific, 32132) was used. Immunoblot signals were acquired on an Odyssey Fc (LI-COR Biosciences, Lincoln, Nebraska, US).

Drug treatments and UV irradiation

For *in vitro* anticancer drug treatment, 2 x 10^4 control and *BIF-1* knockout A375 cells were seeded in 24-well plates. Cells were treated for 24 h with 2.5, 10, and 20 µg/ml etoposide (EBEWE Pharma GmbH Nfg. KG, Unterach am Attersee, AT), 0.1, 0.5, and 1 µM doxorubicin hydrochloride (Sigma-Aldrich, D1515), 1, 5, and 10 µM staurosporine (Sigma-Aldrich, 19-123-M). For UV irradiation experiments, 2 x 10^5 control and *BIF-1* knockout A375 cells were seeded in 6-well plates. The media was removed and cells were exposed to 200 x 100 µJ/cm^2 Stratalinker UV Crosslinker (model 1800, Stratagene, La Jolla, California, US) and submitted for cell death analyses.

Cell death analysis

Cells were washed with Annexin V staining buffer (150 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 15 mM HEPES pH 7.2, 2% FCS and 10 mM NaN_3) and incubated with ATTO633-conjugated Annexin V diluted in staining buffer for at least 20 min on ice in the dark. Cells were then washed in staining buffer and resuspended in 200 µL staining buffer containing propidium iodide at a final concentration of 2 µg/mL. Cells were analyzed by flow cytometry using a FACS Lyric (BD Biosciences, Allschwil, CH). Cells double negative for both ATTO633-AnnexinV/propidium iodide were considered as viable cells.

Transmission electron microscopy

Cells were fixed in 2.5% (v/v) glutaraldehyde solution buffered with 0.1 M sodium cacodylate (pH 7.4, 340 mOsm), postfixed in OsO_4 [buffered with 0.1 M sodium cacodylate (pH 7.4, 340 mOsm)], dehydrated in graded ethanol, and embedded in EPON (Sigma-Aldrich). Blocks were cut with the aid of a Diatome ([Diamond Knife], Biel, CH) diamond knife and stained using uranyl acetate and lead citrate. Sections were examined using a Philips (Philips, Eindhoven,

NL) TEM CM12 with 2’650x and 19’500x magnifications. The mitochondrial area, perimeter and length was analyzed using the ImageJ software.

Immunofluorescence

For the immunofluorescence staining procedure, 8 x 10^4 A375 cells were first seeded on coverslips in a 24-well plate. On the following day, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 0.05% saponin followed by acetone treatment at −20°C. Cells were blocked with normal goat serum, bovine serum albumin and PBS at room temperature for 1 h. Cells were then incubated with primary antibody diluted in the blocking solution at 4°C overnight. The primary antibodies were as follows: monoclonal anti-endophilin B1/BIF-1 (dilution 1:200, Novus Biologicals, NBP2-24733) and monoclonal anti-TOMM20 antibody (dilution, 1:200, Novus Biologicals, NBP2-67501). Cells were washed with PBS and incubated with secondary antibodies (goat anti-rabbit 488, goat anti-mouse 555 and goat anti-rabbit 633, Molecular Probes, Invitrogen, A21425, A11070 and A21071) at room temperature for 1 h. Nuclei were stained with Hoechst (Molecular Probes, H3570) and slides were mounted with ProLong™ Gold Antifade Mountant (Invitrogen, P36930). Slides were subsequently analyzed by confocal laser scanning microscopy (LSM 700, Carl Zeiss). The co-localisation analysis was carried out using the Imaris software (Bitplane AG, Zurich, Switzerland) with the Imaris Coloc tool. The % of occupancy of the red channel over the green channel was calculated from three independent experiments. The mitochondrial morphology analysis was carried out using the Imaris software with the Imaris Surpass tool (Surfaces).

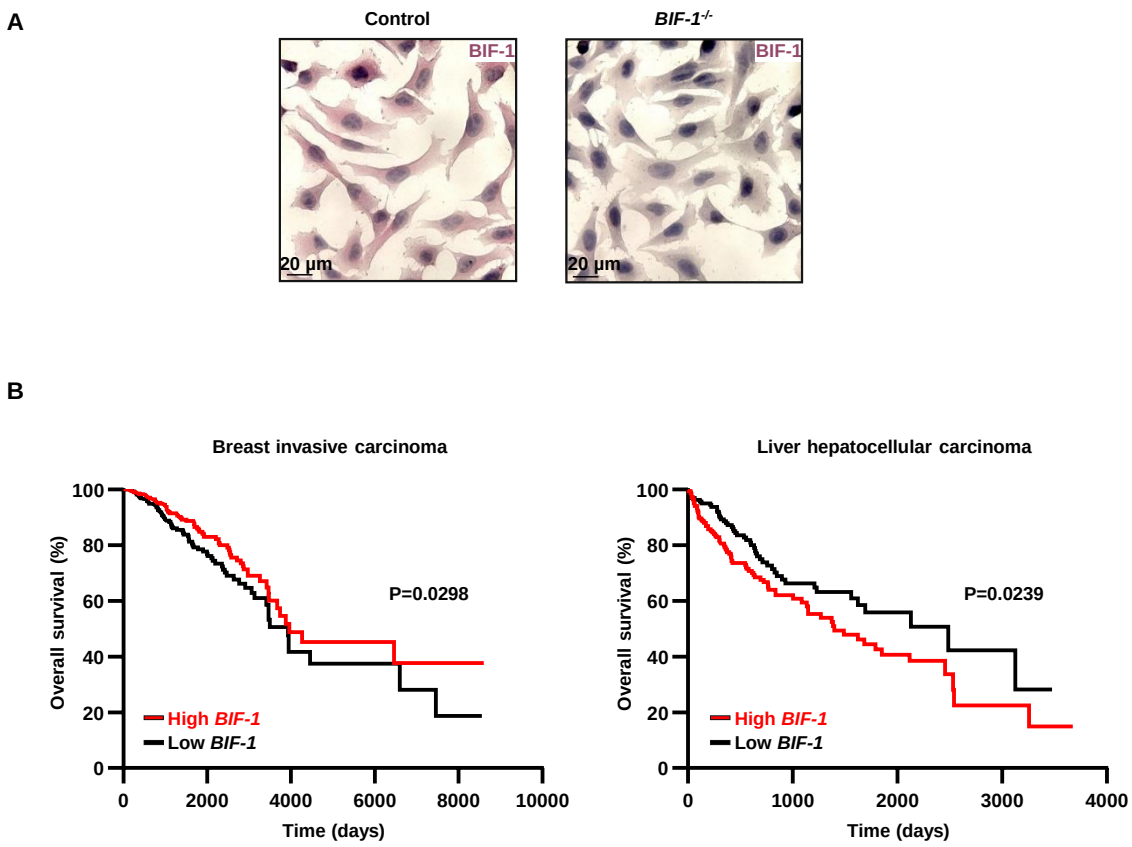
References

1 Aubrey BJ, Kelly GL, Kueh AJ, Brennan MS, O'Connor L, Milla L, et al. An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo. Cell Rep. 2015;10:1422-1432.

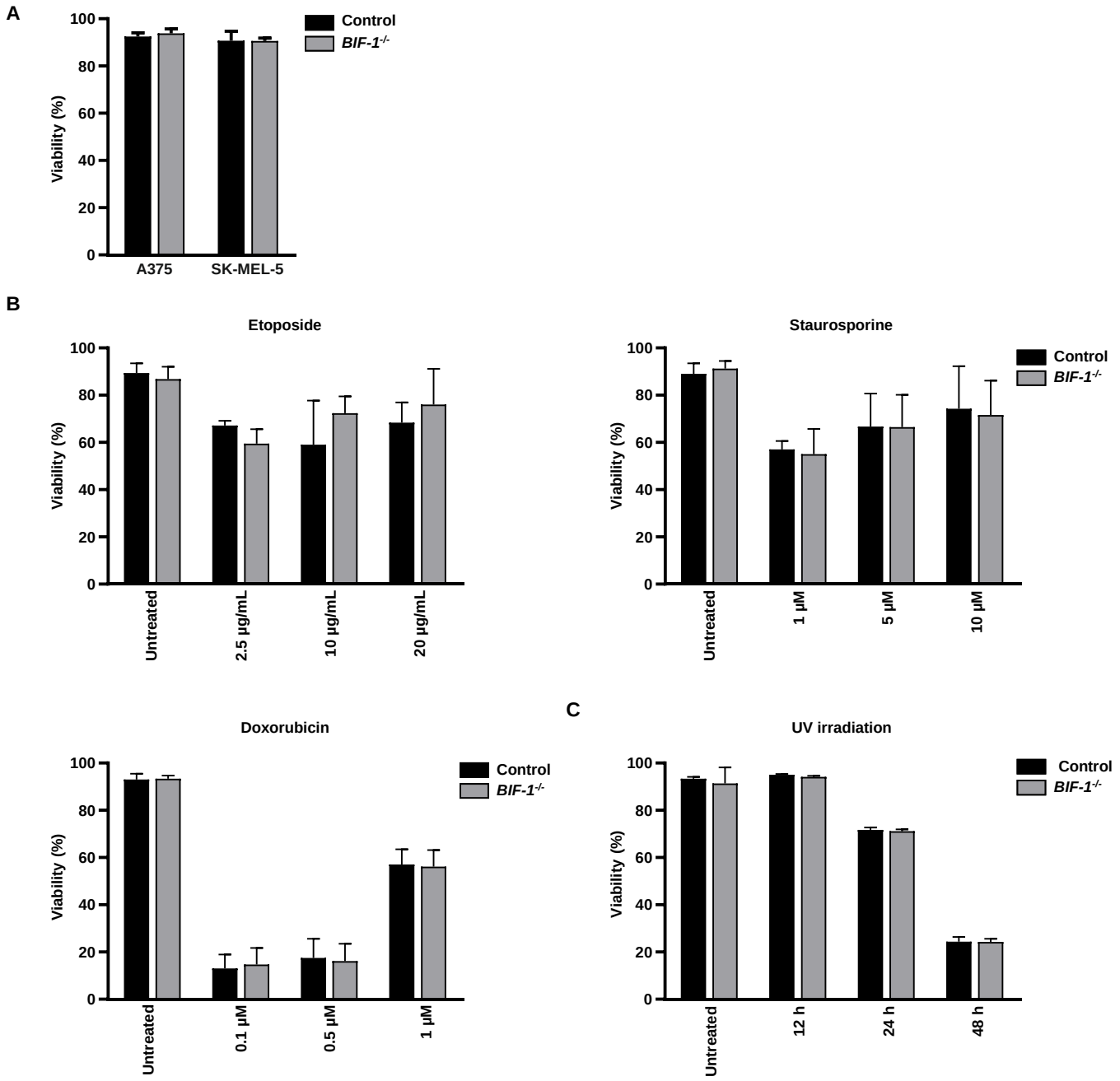
2 Haeussler M, Schonig K, Eckert H, Eschstruth A, Mianne J, Renaud JB, et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 2016;17:148.

3 Yousefi S, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, Scapozza L, et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol. 2006;8:1124-1132.

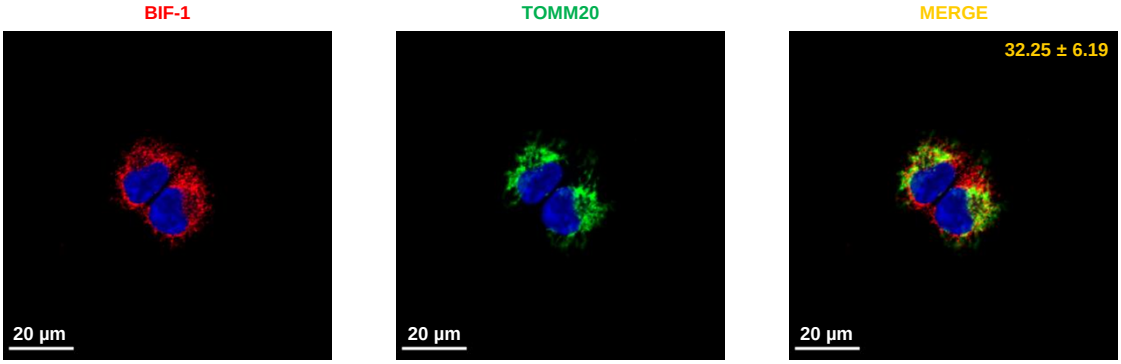
Supplementary Figures



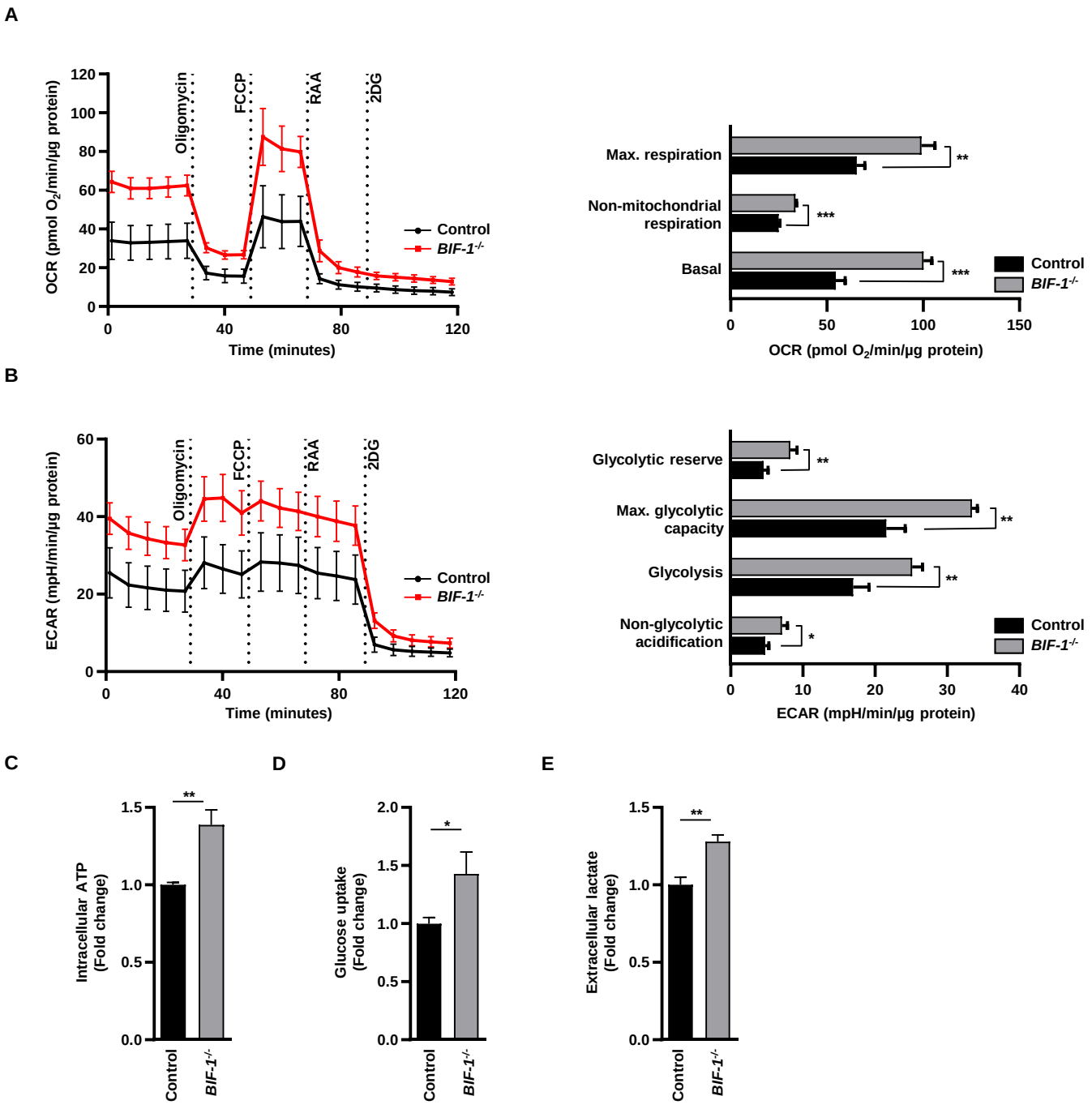
Supplementary Figure 1 *BIF-1* mRNA expression in several cancer types. A Immunohistochemistry. The specificity of the antibody was confirmed by immunohistochemistry using the anti-BIF-1 antibody in control and *BIF-1* knockout A375 melanoma cells. **B** The mRNA expression of *BIF-1* in the breast invasive carcinoma and liver hepatocellular carcinoma was obtained from the TCGA data base and divided into two groups ("high" and "low") based on the mean *BIF-1* mRNA expression in their tumors. Kaplan-Meier curves for overall survival are shown. The hazard ratio for OS in breast invasive carcinoma and liver hepatocellular carcinoma of the group with "high *BIF-1*" expression was 0.7043; the 95% CI was 0.5094 to 0.9650 and 1.503; the 95% CI was 1.055 to 2.110 by log-rank test, respectively.



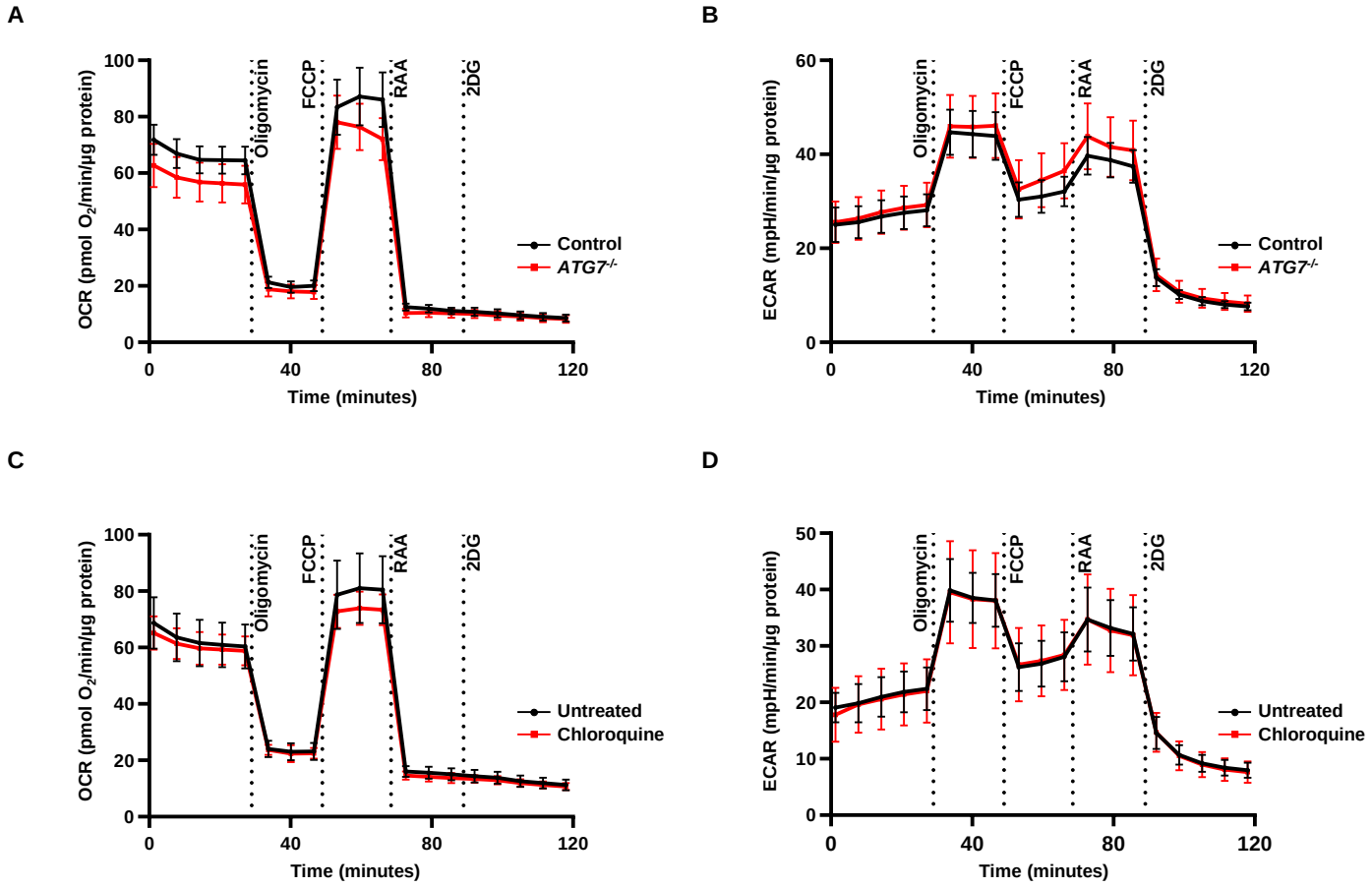
Supplementary Figure 2 *BIF-1* knockout has no effect on A375 melanoma cells apoptosis. A Viability assay. Viability of control and *BIF-1* knockout A375 and SK-MEL-5 melanoma cells was assessed with ATTO633-conjugated Annexin V and PI staining. The Annexin V/PI double negative cells were considered as viable cells. Statistical differences were analyzed by multiple t-test using the Holm-Sidak correction method (n=3). **B** Viability assay. Cells were treated with etoposide (2.5 µg/ml, 10 µg/ml or 20 µg/ml), doxorubicin (0.1 µM, 0.5 µM or 1 µM), or staurosporine (1 µM, 5 µM or 10 µM) for 24 h. Viability was assessed with ATTO633-conjugated Annexin V and PI staining. The Annexin V/PI double negative cells were considered as viable cells. Statistical differences were analyzed by multiple t-test using the Holm-Sidak correction method (n=3). **C** Viability assay. Cells were exposed to 200 x 100 µJ/cm^2 and analyzed after 12, 24 and 48 h. The Annexin V/PI double negative cells were considered as viable cells. Statistical differences were analyzed by multiple t-test using the Holm-Sidak correction method (n=3).



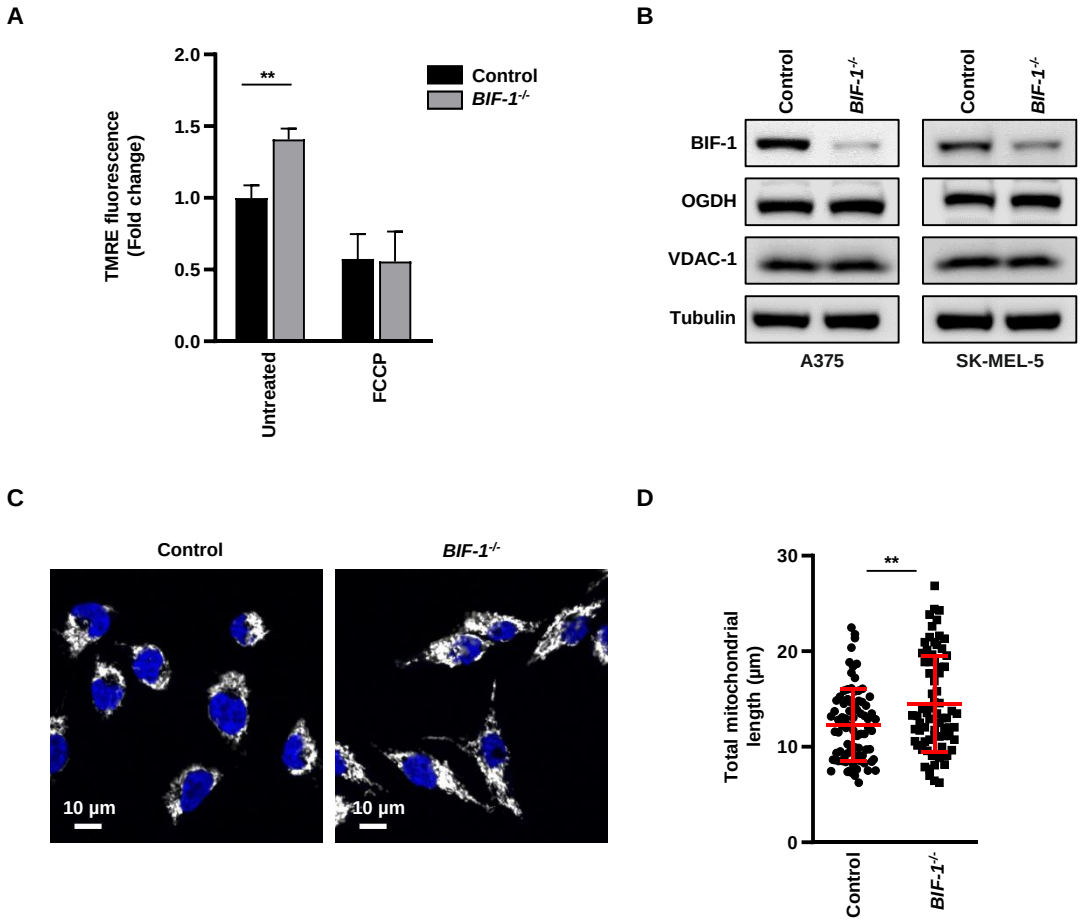
Supplementary Figure 3 BIF-1 localizes on mitochondria. Confocal microscopy. Co-localization of BIF-1 and the mitochondrial marker TOMM20 was determined in A375 control cells using the Imaris software. BIF-1 was stained with anti-BIF-1 antibody (red), mitochondria were stained with anti-TOMM20 antibody (green) and the nuclei with Hoechst (blue). At least 20 cells from each independent experiment were quantified. Values are presented as percentage ± SD (n=3).



Supplementary Figure 4 BIF-1 has an impact on mitochondrial metabolism. A and B OCR and ECAR measurements. During extracellular flux analysis, control and *BIF-1* knockout SK-MEL-5 melanoma cells were sequentially treated with oligomycin = Oligo, FCCP, rotenone/antimycin=RAA and 2-deoxy-glucose=2DG to assess the different OXPHOS (**A**) and glycolytic parameters (**B**). The means ± SD of the mitochondrial stress test on OCR and ECAR are depicted over time. A quantitative analysis of the data is presented on the right. Statistical differences were analyzed by multiple t-test using the Holm-Sidak correction method (n=3). **C** Total intracellular ATP production in control and *BIF-1* knockout SK-MEL-5 melanoma cells was measured by ATP-dependent luciferase activity. Data are presented as the relative luciferase units (RLU) normalized to control SK-MEL-5 melanoma cells. Statistical differences were analyzed by the unpaired t-test (n=3). **D** Glucose-uptake levels in control and *BIF-1* knockout SK-MEL-5 melanoma cells. Cells were incubated in low glucose (2.5 mM) media for 8 h before analyses. Data are presented as the RLU normalized to control SK-MEL-5 melanoma cells. Statistical differences were analyzed by the unpaired t-test (n=3). **E** Lactate levels in control and *BIF-1* knockout SK-MEL-5 melanoma cells. Data are presented as the RLU normalized to control SK-MEL-5 melanoma cells. Statistical differences were analyzed by the unpaired t-test (n=3). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. (Related to Figure 3)



Supplementary Figure 5 Autophagy does not influence glycolysis or oxidative phosphorylation in melanoma cells. During extracellular flux analysis, *ATG7* knockout A375 melanoma cells (**A and B)** and A375 melanoma cells pretreated with chloroquine (10 µM for 2 h) **(C and D)** were sequentially treated with oligomycin = Oligo, FCCP, rotenone/antimycin=RAA and 2-deoxy-glucose=2DG to assess the different glycolytic (**B and D**) and OXPHOS (**A and C**) parameters. (Related to Figure 4)



Supplementary Figure 6 BIF-1 expression impacts on the mitochondrial potential and total mitochondrial length. A Flow cytometry. The mitochondrial potential of control and *BIF-1* knockout SK-MEL-5 melanoma cells was assessed by TMRE (100 nM) fluorescence in the presence and absence of FCCP. Data are presented as relative geometric mean fluorescent intensities normalized to control SK-MEL-5 melanoma cells. Statistical differences were analyzed by the unpaired t-test (n=3). **B** Immunoblot. Expression of mitochondrial proteins OGDH and VDAC-1 in control and *BIF-1* knockout A375 and SK-MEL-5 melanoma cells confirms a similar mitochondrial content between *BIF-1* knockout and control melanoma cells. **C-D** Confocal microscopy. Mitochondria were stained with anti-TOMM20 antibody (white) and the nuclei with Hoechst (blue) in control and *BIF-1* knockout A375 melanoma cells. Representative images are shown (panel **C**). Mitochondria were analyzed using the Imaris software and statistical analyses are provided for the major axis length (panel **D)**. Approximately 80 cells per genotype were quantified. Statistical differences were analyzed by the Mann-Whitney test. **$p \leq 0.01$.

Tables

Supplementary Table 1. The impact of *BIF-1* expression on patients' outcome in melanoma, breast invasive carcinoma and liver hepatocellular carcinoma.

Cancer type	HR (95% CI)[1]	P-value	Mean *BIF-1* levels
SKCM (N=480)	0.7587 (0.5733 to 0.9848)	0.0392	10.94
BRCA (N=1081)	0.7043 (0.5094 to 0.9659)	0.0298	11.26
LIHC (N=362)	1.503 (1.055 to 2.110)	0.0239	10.45

N, Number of patients. [1]The 95% confidence intervals (CI) of the hazard ratio (HR) function of the group with "high *BIF-1*" expression by log-rank test are tabulated.