Expanded View Figures

Figure EV1. BNIP3 removal from melanoma cells relents their growth *in vitro* (related to Fig 2).

A Immunoblot detection of ATG5, BNIP3 and ACTIN levels from normoxic lysates ($n = 4$) of B16-F10 cells collected 48 h after plating.

B Proliferation curves and corresponding doubling time of B16-F10 cells ($n = 4$) calculated using the Incucyte technology.

C Immunoblot detection of LC3B (I–II) and ACTIN levels from lysates of B16-F10 cells cultured alone or in the presence of the autophagic blocker BafA (5 nM) for 24 h.

D, E Confocal microscopy imaging (D) and corresponding quantification (E) of the B16-F10 cells transiently transfected with the autophagy flux reporter RFP-GFP-LC3 and cultured alone or in the presence of the autophagic blocker BafA (5 nM) for 24 h. Scale bars represent 10 μm. $N = 10$ for shCntl Baf-, shBNIP3 Baf^{-}, shBNIP3 Baf^{+} and shATG5 Baf^{+}. $N = 6$ for shATG5 Baf^{-}. $N = 8$ for shCntl Baf^{+} condition. Both BafA- and $BafA^{+}$ conditions were analyzed using a Kruskal–Wallis with Dunn's multiple comparisons test.

F Quantification of dead (PI^{+}) B16-F10 cells cultured alone or in the presence of BafA (10 nM) for 24 h ($n = 5$). BafA- conditions were analyzed using Friedman's test with Dunn's multiple comparisons test whereas $BafA^{+}$ conditions were analyzed using a RM one-way ANOVA (Geisser–Greenhouse correction) with Holm–Sidak's multiple comparisons test.

G *Map1lc3b* and *Sqstm1* transcript levels from lysates of normoxic B16-F10 cells ($n = 5$). Transcript expression is represented as the fold change relative to their corresponding shCntl value, and they were analyzed using a one-sample *t*-test against shCntl.

H Immunoblot detection of TFEB and ACTIN levels from normoxic lysates of B16-F10 cells collected 48 h after plating ($n = 5$).

Data information: All quantitative data are mean ± SEM. Densitometric quantifications of all protein levels relative to ACTIN are shown below each corresponding band and analyzed using a one-sample *t*-test against shCntl (A, C, G, H) or shCntl BafA+ (C). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ when compared against shCntl. $\#\#P < 0.01$ when comparing shBNIP3 against shATG5. Unless otherwise specified, each graph or blot represents $n = 4$ biologically independent experiments and they were analyzed using an RM one-way ANOVA (Geisser–Greenhouse correction) with Holm–Sidak's multiple comparisons test.

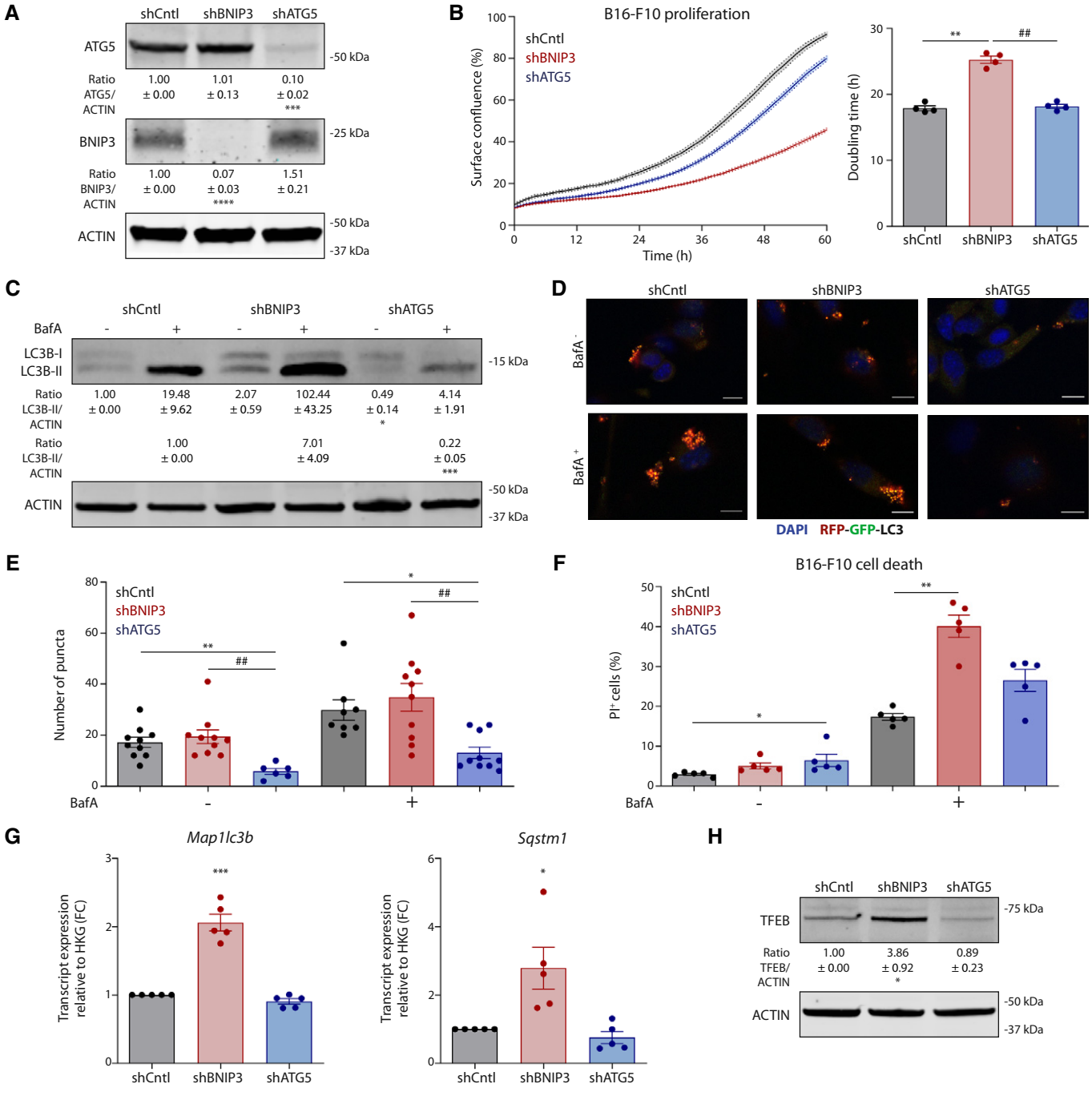

Figure EV1.

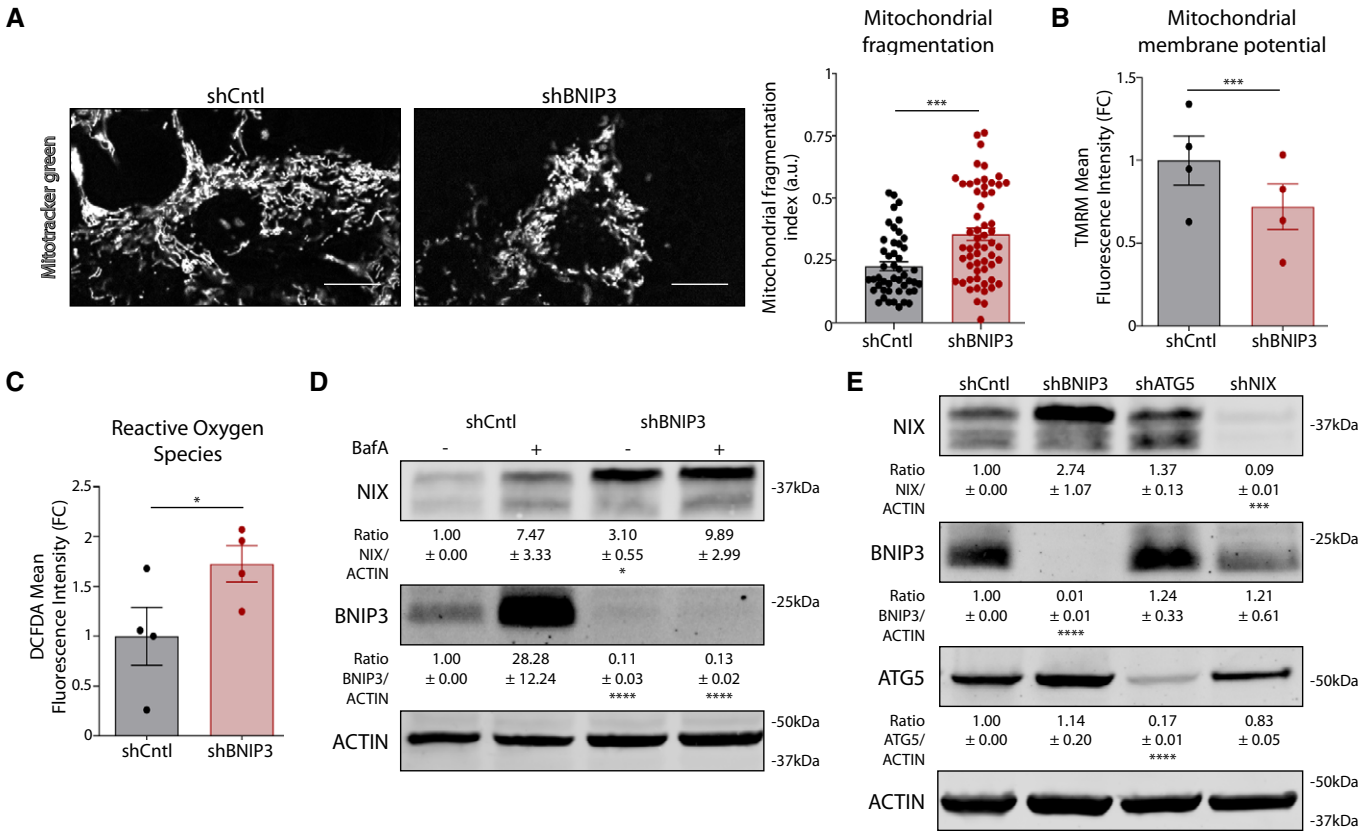

Figure EV2. BNIP3 loss affects mitochondrial homeostasis (related to Fig 3).

A Representative confocal microscopy analysis of mitochondrial morphology in shCntl (n = 47 cells) or shBNIP3 (n = 58 cells) B16-F10 cells incubated 30 min with MitoTracker green coming from 3 biologically independent experiments and analyzed with Mann–Whitney's non-parametric test. Scale bars represent 10 μm.

B Intracellular TMRM mean fluorescence intensity in B16-F10 cells 24 h after plating.

C Intracellular DCFDA mean fluorescence intensity in B16-F10 cells 24 h after plating.

D Immunoblotting for NIX, BNIP3, and ACTIN protein levels in lysates from B16-F10 cells cultured alone or in presence of the autophagic blocker BafA (5 nM) for 24 h (n = 4).

E Immunoblotting for NIX, BNIP3, ATG5, and ACTIN protein levels in normoxic lysates from B16-F10 cells collected 48 h after plating (n = 3).

Data information: All quantitative data are mean ± SEM. Densitometric quantifications of all protein levels relative to ACTIN levels are shown below the corresponding band are were analyzed using a one-sample t-test. *P < 0.05, ***P < 0.001, ****P < 0.0001 when compared against shCntl. Unless otherwise specified, each graph represents n = 4 biologically independent experiments and were analyzed using a paired t-test.

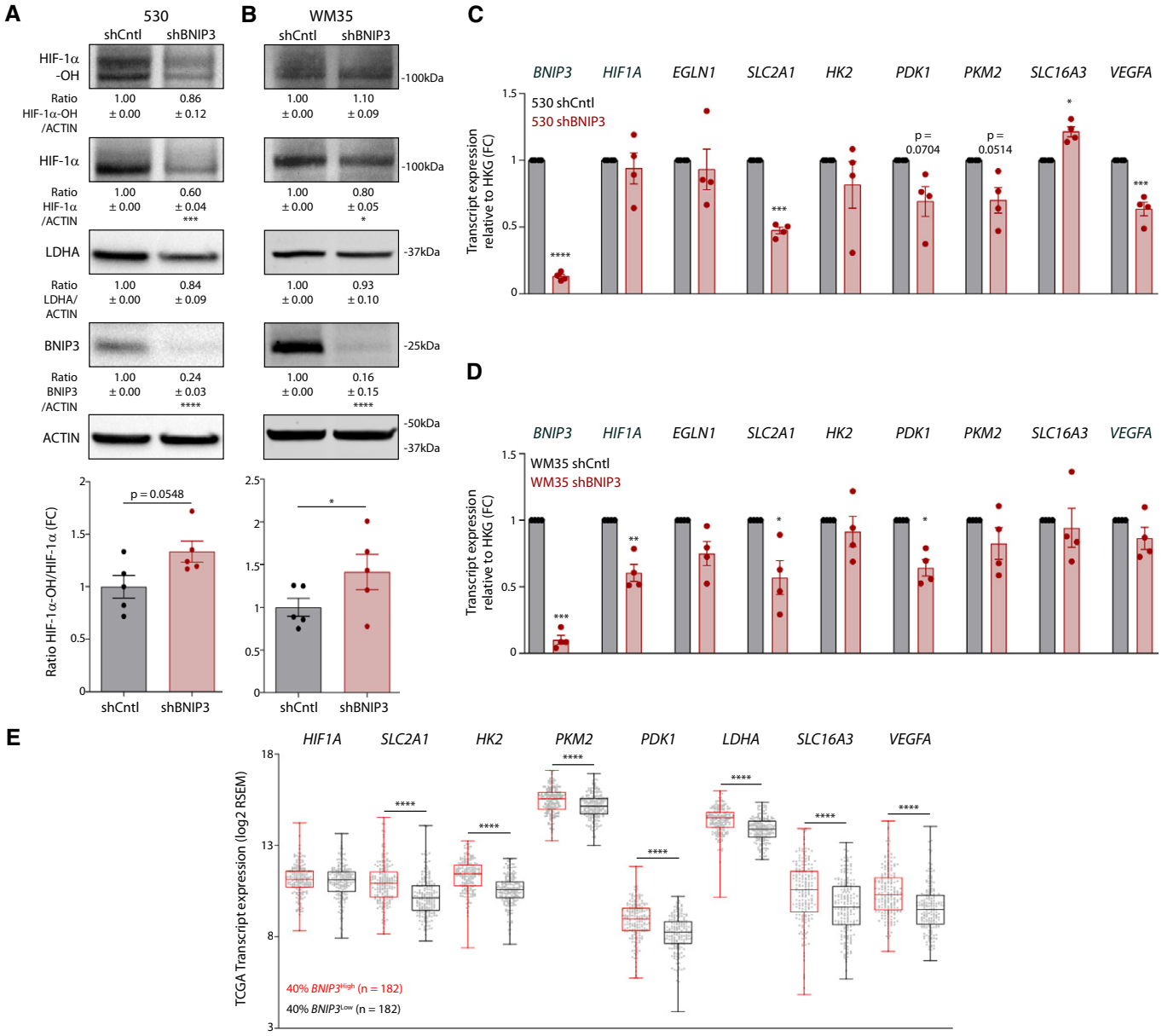

Figure EV3. BNIP3 stabilizes normoxic HIF-1α on a post-translational level in human melanoma cells (related to Fig 4).

A, B Immunoblot detection of Hydroxylated HIF-1α (HIF-1α-OH), total HIF-1α, LDHA, BNIP3, and ACTIN levels from normoxic lysates of the early-stage melanoma cell lines 530 (A) or WM35 (B) transduced with either empty vector (shCntl) or shRNA against BNIP3 (shBNIP3) ($n = 5$). Hydroxylation levels were analyzed using a paired *t*-test.

C, D *BNIP3, HIF1A, EGLN1(PHD2), SLC2A1 (GLUT1), HK2, PDK1, PKM2, SLC16A3 (MCT4),* and *VEGFA* transcript levels from normoxic lysates ($n = 4$) of the early-stage melanoma cell lines 530 (C) or WM35 (D) transduced with either empty vector (shCntl) or shRNA against BNIP3 (shBNIP3). Transcript expression is represented as the fold change relative to their corresponding shCntl value and they were analyzed using a one-sample *t*-test against shCntl.

E *HIF1A, SLC2A1 (GLUT1), HK2, PKM2, PDK1, LDHA, SLC16A3 (MCT4),* and *VEGFA* transcript level comparison between $BNIP3^{high}$ (40%, $n = 182$) and $BNIP3^{low}$ (40%, $n = 182$) in all melanoma patients within the SKCM TCGA cohort, using the unpaired Mann–Whitney's non-parametric test. The box represents the median and the 25^{th}–75^{th} percentile with the whiskers representing the minimum and maximum values.

Data information: All quantitative data are mean ± SEM. Densitometric quantifications of all protein levels relative to ACTIN levels are shown below the corresponding band, and they were analyzed using a one-sample *t*-test against shCntl. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ when compared against shCntl.

Figure EV4. BNIP3 reintroduction, but not metabolites, rescue normoxic HIF-1α levels in BNIP3-deficient melanoma cells (related to Figs 5 and 6).

A *Egln2*, *Egln1*, and *Egln3* transcript levels from lysates of normoxic B16-F10 cells, and they were analyzed using a one-sample *t*-test against shCntl ($n = 4$) except *Phd1* for shBNIP3, which was analyzed using Wilcoxon rank test.

B *Glut1*, *Pdk*, *Mct4*, and *Vegfa* transcript levels from lysates of B16-F10 cells cultured in presence of non-targeting (Scr) or siRNA against PHD2 ($n = 3$).

C, D Relative intracellular succinate (C) and fumarate (D) levels from B16-F10 cells detected using GC-MS ($n = 4$). Data were analyzed using an RM one-way ANOVA (Geisser–Greenhouse correction) with Holm–Sidak's multiple comparisons test.

E 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 in presence or absence of di-sodium fumarate (4 mM) for 24 h ($n = 3$).

F Immunoblot detection of Hydroxylated HIF-1α (HIF-1α-OH), total HIF-1α, LDHA, and ACTIN levels from normoxic lysates of B16-F10 cells transduced with either empty vector (shCntl) or shRNA against NIX (shNIX) ($n = 4$). Hydroxylation levels were analyzed using a paired *t*-test.

G *Hif1a*, *Glut1*, *Pdk1*, *Ldha*, *Mct4*, and *Vegfa* transcript levels from normoxic lysates of B16-F10 cells transduced with either empty vector (shCntl) or shRNA against NIX (shNIX) ($n = 5$).

H Immunoblot detection of NCOA4, LC3B, and ACTIN levels from normoxic lysates of B16-F10 cells transduced with either empty vector (shCntl) or shRNA against BNIP3 (shBNIP3) or NIX (shNIX) and cultured in the presence or absence of BafA 5 μM ($n = 4$).

I *Ncoa4* transcript levels from normoxic lysates of B16-F10 cells transduced with either empty vector (shCntl) or shRNA against BNIP3 (shBNIP3), ATG5 (shATG5), or NIX (shNIX) ($n = 5$).

J Relative intracellular Fe^{2+} levels measured with FerroOrange in normoxic B16-F10 cells transfected in presence of non-targeting siRNA sequences (Scr) or siRNA against NCOA4 ($n = 5$). Data were analyzed using a RM one-way ANOVA (Geisser–Greenhouse correction) with Holm–Sidak's multiple comparisons test.

Data information: All quantitative data are mean ± SEM. Densitometric quantifications of protein levels relative to ACTIN are shown below each corresponding band. Transcript expression is represented as the fold change relative to their corresponding shCntl value. Densitometric quantifications and qPCR data were analyzed using a one-sample *t*-test against shCntl. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared against shCntl. #$P < 0.05$ when comparing shBNIP3 against shATG5.

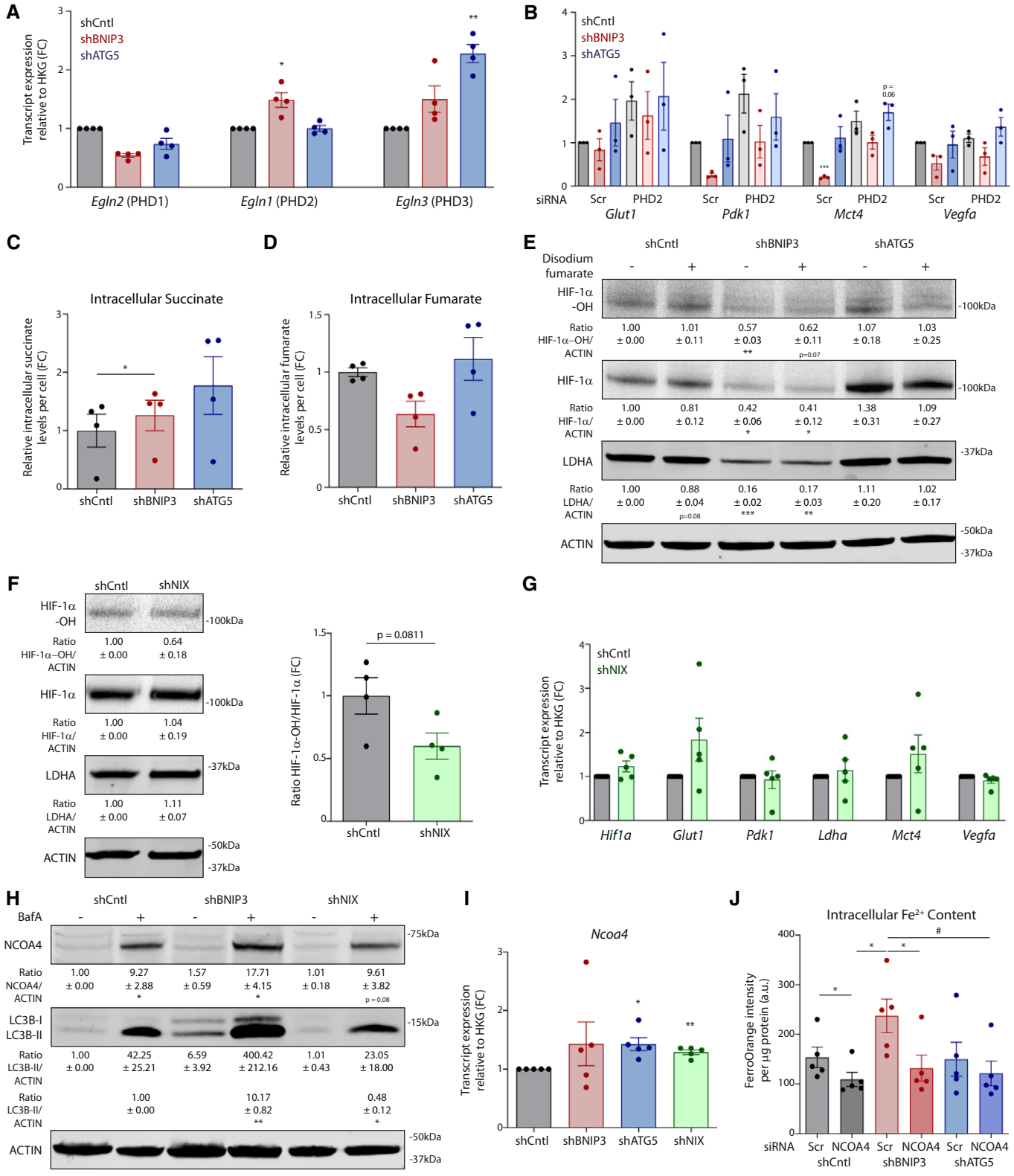

Figure EV4.

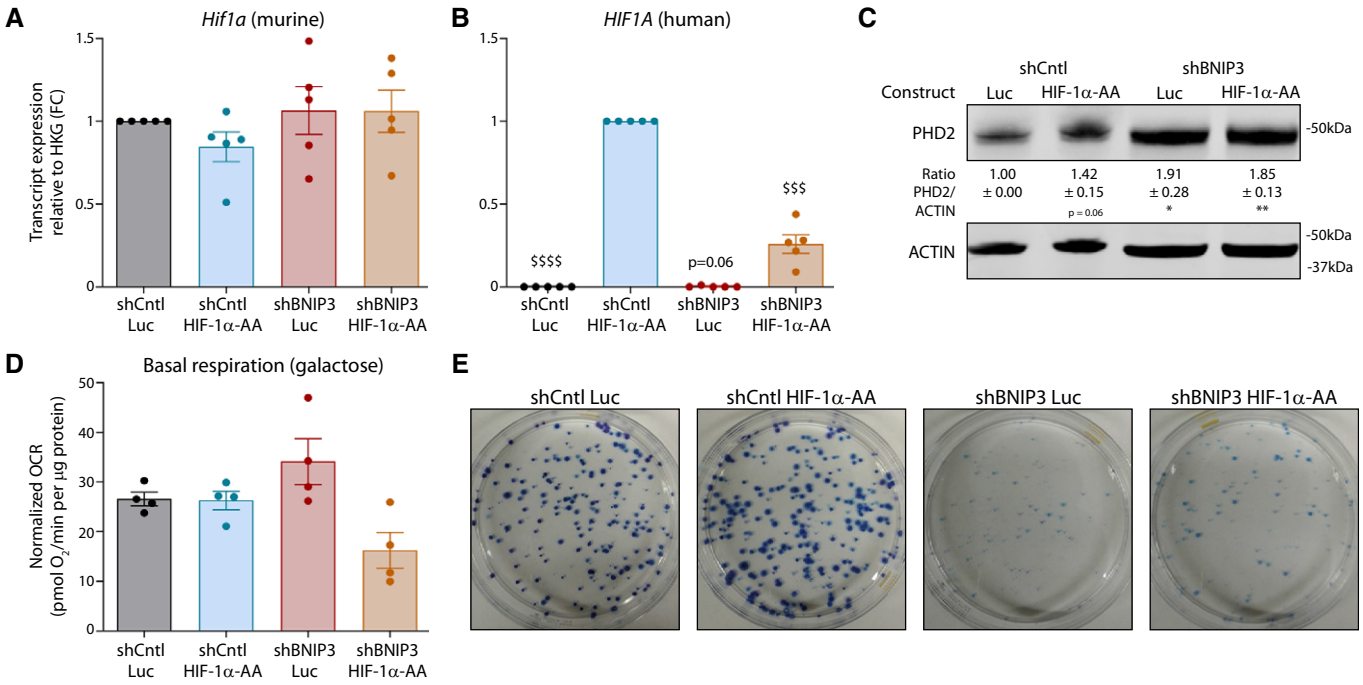

Figure EV5. Recovery of HIF-1α levels in BNIP3-silenced cells restores melanoma glycolytic phenotype and growth *in vitro* (related to Fig 7).

A, B Murine (A) and human (B) *HIF1A* transcript levels measured by qPCR in B16-F10 cell lysates collected 48 h after plating ($n = 5$). They were analyzed against shCntl Luc (A) or shCntl HIF-1α-AA (B) using a one-sample *t*-test except human *HIF1A* in shBNIP3 Luc that was analyzed with Wilcoxon rank test.

C Immunoblot detection of PHD2 and ACTIN levels from B16-F10 cell lysates collected 48 h after plating ($n = 4$). Densitometric quantifications are shown below each corresponding band they were analyzed using a one-sample *t*-test against shCntl Luc.

D Basal respiration rates of B16-F10 cells assayed in galactose (25 mM galactose, 2 mM glutamine) medium using the Seahorse technology ($n = 4$).

E Clonogenic growth of B16-F10 cells 10 days after seeding.

Data information: All quantitative data are mean ± SEM. Transcript expression is represented as the fold change relative to their corresponding shCntl value. *$P < 0.05$ **$P < 0.01$ when compared against shCntl Luc and $$$ $P < 0.001$, $$$$$P < 0.0001$ when compared against shCntl HIF-1α-AA.