SUPPLEMENTAL DATA FILE

Title: *Survival Advantage of MCL Cells with PIK3CA gain or PTEN Loss Is Mediated By Decreased Dependence on B-Cell Receptor Signaling and Increased Survival after BCL2 Inhibition and Under Hypoxia*

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SUPPLEMENTAL MATERIALS AND METHODS

Cell lines and patient samples

MINO, Z138, UPF1H, UPF19U, JEKO-1 cell lines with transgenic *PIK3CA* (over)expression (*PIK3CA* UP) or with *PTEN* knock-out (*PTEN* KO) were derived as described below. Cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Primary MCL cells were obtained from leukemized blood, infiltrated lymph nodes, malignant ascites, pleural effusion, and infiltrated bone marrow of 28 patients with so far untreated or relapsed / refractory (R/R) MCL. Approval was obtained from all patients according to the (WMA) Declaration of Helsinki. The project was approved by the institutional ethics committee of the General University Hospital in Prague under number 60/20.

Cytotoxic agents

Specific PI3K inhibitors including idelalisib (PI3Kδ inhibitor), duvelisib (PI3K/γ δ inhibitor), AZD8835 (PI3K α/δ inhibitor), AZD8186 (PI3Kβ/δ inhibitor), alpelisib (PI3Kα inhibitor), copanlisib (pan-PI3K inhibitor), and others; capivasertib (pan-AKT inhibitor), ibrutinib (BTK inhibitor), venetoclax (BCL2 inhibitor), S63854 (MCL1 inhibitor), and A1155463 (BCL-XL inhibitor) were purchased from MedChemExpress. 2-Deoxy-D-Glucose (2-DG, inhibitor of glycolysis) was provided by SIGMA.

Cytogenomic analyses

I-FISH analyses were performed with commercially available DNA probes SPEC PIK3CA/CEN 3 DC (**ZytoVision GmbH, Bremerhaven, Germany**), Vysis LSI PTEN/CEP 10 and Vysis LSI TP53 (17p13.1)/CEP 17 (Abbott Molecular, Des Plaines, IL, USA) according to the manufacturers' protocols. At least 200 interphase nuclei were analyzed by two independent observers. The cut off level for positive values were determined on samples obtained from 10 cytogenetically normal persons and were found to be 5% (mean ± 3SD) for losses (deletions, monosomies), and 2.5% (mean ± 3SD) for gains (trisomies, amplifications). aCGH/SNP analyses were performed with a SurePrint G3 Cancer CGH+SNP Microarray, 4x180K (Agilent Technologies, Santa Clara, USA) . The QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany) was used to isolate genomic DNA from bone-marrow cells stored in fixative. The concentration and quality of the isolated DNA were confirmed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The array slides were scanned with a microarray scanner system (G2565CA, Agilent Technologies) and analyzed with Agilent Cytogenomics v5.2.0.20 software (Agilent Technologies).

Generation of cell lines with target genes knock out/knock down

PTEN KO was generated using CRISPR/Cas9 system exactly as we did before(1). Briefly, we combined double strand DNA cut at the *PTEN* translation initiation site and provided repair template plasmid for homologous repair (HR) based insertion of GFP-STOP-pA DNA sequence. In result, GFP is expressed instead of *PTEN* and serves as a marker for viable sorting of *PTEN* KO cells. To introduce double strand DNA break at the *PTEN* translation initiation site, we used a "paired nickases" approach(2). *PTEN* target sequences (PTEN_1 TTGACCTGTATCCATTTCTGCGG and PTEN_2 TTGATGATGGCTGTCATGTCTGG) were cloned into a chimeric plasmids pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A), coding for Cas9 D10A as well as gRNA (Addgene plasmid #42335)(3) and co-electroporated (see below) them with the above-mentioned HR repair template plasmid(1). Western blotting of bulk sorted GFP positive / PTEN KO cells confirmed PTEN KO in UPF1H, Z138, and JEKO-1 cells (Figure 1A and Supplemental Figure 1) and showed PTEN KD in MINO and UPF19U (resulting from heterozygotic KO, Supplemental Figure 1). To generate MINO PTEN KO cells, bulk sorted GFP positive cells were single cell cloned to obtain pure PTEN KO cells (confirmed by western blotting, Figure 1A). If necessary for consequent AKT activity measurement (see below), GFP was knocked out using similar CRISP/Cas9 system with single GFP target sequence (GGGCGAGGAGCTGTTCACCGGGG) cloned into a pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid # 42230, coding for Cas9 as well as gRNA)(3). GFP negative *PTEN* KO cells were then sorted to purity for consequent analyses(4). BCR KO cells were generated as we did previously(1). We used gRNAs targeting the constant region of immunoglobulin heavy (IgH) chain of cell line specific BCR isotype. We used the above mentioned px330 plasmid with the following IgH M target sequence for all tested cell lines: AGATGAGCTTGGACTTGCGGGGG. The BCR KO cell growth and the change of their proportion in cell culture was measured starting three days after electroporation using flow cytometry (staining the BCR with anti-human IgM FITC antibody, ThermoFisher Scientific #H15001). Comparison of growth rates of genetically modified cells was performed using a bead assay as described previously(1).

Generation of cell lines with target genes overexpression

Similarly as we did before, we have used the sleeping beauty transposon system(5) for stable overexpression of *PIK3CA* and to express the AKT activity reporter (see below) in selected cell lines(1, 6). Briefly, the WT PIK3CA cDNA was cloned into donor plasmid pSBbi Pur (Addgene plasmid #60523)(5) and AKT activity reporter was cloned into pSBbi-Pur (Addgene plasmid #60523)(5) or pSBbi-Neo (Addgene plasmid #60525)(5) donor palasmids. These donor plasmids (6 µg) were co-electroporated with 4 µg of transposase coding plasmid pCMV(CAT)T7-SB100 (Addgene plasmid #34879)(7). After three days of culture without antibiotics, selection with appropriate antibiotics was initiated with the

following concentrations: puromycin at final concentration of 2 μg/mL and geneticin at final concentration of 200 μg/mL.

Measurement of AKT activity in living cells

To measure AKT activity in live cells, we used a genetically encoded Förster resonance energy transfer (FRET) biosensor as we developed and used before(1, 4). The AKT activity reporter (Lyn-Akt AR2-EV, Addgene plasmid #125199) has the following structure: cell membrane targeting sequence (from Lyn) - mCerulean3 - FHA1 phospho-amino acid binding domain – EV flexible linker – part of FOXO domain (naturally phosphorylated by AKT) - cpVenus[E172]. AKT mediated phosphorylation of its FOXO part leads to its binding to FHA1 domain, change in reporter confirmation, and increase in FRET. As we published previously, we have used flow cytometry for FRET measurement in living cells using in house R package (fRet) to calculate the absolute FRET efficiency (E) values reflecting the AKT activity(1, 4). FRET measurement and calculations were performed as per the fRet package manual using cells expressing appropriate calibration controls: mCerulean3 only, cpVenus[E172] only, FRET high control, and FRET low control (to calculate necessary set up dependent coefficients for each individually performed experiment)(4).

To compare cells with *PTEN* KO or *PIK3CA* UP to their parental counterparts, five hundred thousand cells per sample were resuspended in fresh media, incubated one hour in regular cell culture incubator (37°C with 5% of CO_2), and FRET measured for each sample immediately after removing them from the incubator using flow cytometry (Cytoflex, Beckma Coulter). To measure the AKT activity after ibrutinib treatment, five hundred thousand cells per each sample were pre-incubated (37°C with 5% of CO_2) for 3 hours with the ibrutinib concentration of 1 μM and 0.1 μM in total amount of 3ml cell culture cell media directly in flow cytometry tubes and FRET measured for each sample immediately after removal from the incubator using flow cytometry (Cytoflex, Beckma Coulter).

Apoptosis and proliferation assays

Apoptosis was measured by standard Annexin-V / propidium iodide (PI) assay. Briefly, on day 1 the cells were resuspended into 96-well plate (100 000 cells per well). Each drug was added in 3 different concentrations. After 24 hours of incubation, Annexin V FITC (Exbio) and PI (Sigma) were added to detect apoptotic and necrotic cells. The measurements were carried out by flow cytometry (BD FACS CANTO II). The percentage of necrotic and apoptotic cells was calculated as previously reported(6).

Proliferation was measured by commercially available WST-8 based cell proliferation assay according to the manufacturer´s recommendations. Briefly, on day 1, the cells were resuspended into 96-well plate (50 000 – 100 000 cells per well). The tested PI3K and AKT inhibitors were added at concentrations 1 and 10 μM (Copanlisib at 0,01 and 0,1 μM), BTK inhibitor at 0,1 and 1 μM and BCL2

family inhibitors at 0,01, 0,1 and 1 µM. After 72 hours of incubation, WST-8 reagent from Quick Cell Proliferation Assay Kit (BioVision) was added, for a further 3 hours incubation. Absorbance of samples was measured on ELISA reader. The proliferation curve was calculated as previously reported(8).

Western blotting

Samples were lysed in Ripa buffer (150 mM NaCl, 0,1 % SDS,1mM EDTA,0,5% Sodium Deoxycholate, 1% Triton X-100, pH 7.4). A protease (Sigma) and a phosphatase inhibitor (Roche) were added. Protein concentration was determined using Pierce BCA Protein Assay (ThermoFisher scientific) .20 µg of sample was mixed with laemelli sample buffer (BioRAD) containing mercaptoethanol and boiled for 5 min. Duplicate samples were separated on 10%,12% and 15% SDS-PAGE gels. After electrophoresis, proteins were blotted onto 0.22 µM PVDF membranes (advansta). Membranes were incubated for 1 h in 1xPBS containing 0.1% Tween-20 and 5% non-fat dried milk. Samples were incubated with primary antibodies overnight then with secondary antibodies for 30 min. To detect bands WesternBright ECL HRP substrate (advansta) was used. The membranes were imaged by ChemiDoc^{TM}MP Imaging system (BioRAD). The following antibodies were used in our study: PIK3CA (# 4249), PTEN (# 5384), phospho-PTEN ser 380 (# 9551), total AKT (# 9272), phospho-AKT ser 473 (# 4060), FOXO3A (# 12829), phospho-FOXO3A ser 253 (# 9466), GSK3-β (# 12566), phospho-GSK3-β ser 9 (# 9336), BIM (# 2933), BAK (# 12105), BAX (# 2774), BCL-XL (# 2764), MCL1 (# 39224) , phospho-BAD ser 136 (# 9295) and HIF1α (# 14179)were from Cell Signaling technology. B-Actin (# 6276), c-MYC (# 32072), and α-Tubulin (# 7291) from ABCAM. BCL2 (# 610539) from BD Transduction Laboratories.BCL2 (#783) from SantaCruiz. GAPDH (# G8795) from SIGMA. BAD polyclonal (# PA5-11403) from Thermo-Fisher Scientific. NOXA polyclonal (# 2437) from ProSci. The densitometric analysis was carried out by Image-Lab Bio-Rad-6.1 software and adjusted total volume values were used for quantification.

Coimmunoprecipitation assay

The immunoprecipitation was performed according to the previously mentioned protocol (9). Anti-BAD, Anti-BIM, anti-BID (# 2002) (cell signaling), anti-BAK and anti BAX were used to target the immunoprecipitated anti-Bcl-2 antibody (# 4223) (cell signaling). Anti-BAD, Anti-BIM, anti-BID (# 2002) (cell signaling), anti-BAK and anti BAX were used to target immunoprecipitated anti-BCL-XL antibody (# 32370) (ABCAM)

In vitro assays under hypoxia

On day 1, 45 x 10^4 cells from each cell line resuspended in 3 ml medium were pipetted into two separate 6-well plates. One plate was incubated under normoxic conditions (O_2 concentration=20%) and one plate under hypoxic conditions (O_2 concentration=1%). After 72 h incubation samples were

taken from both plates and an apoptosis assay was carried out (for more go to paragraph apoptosis assay)

Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) assays

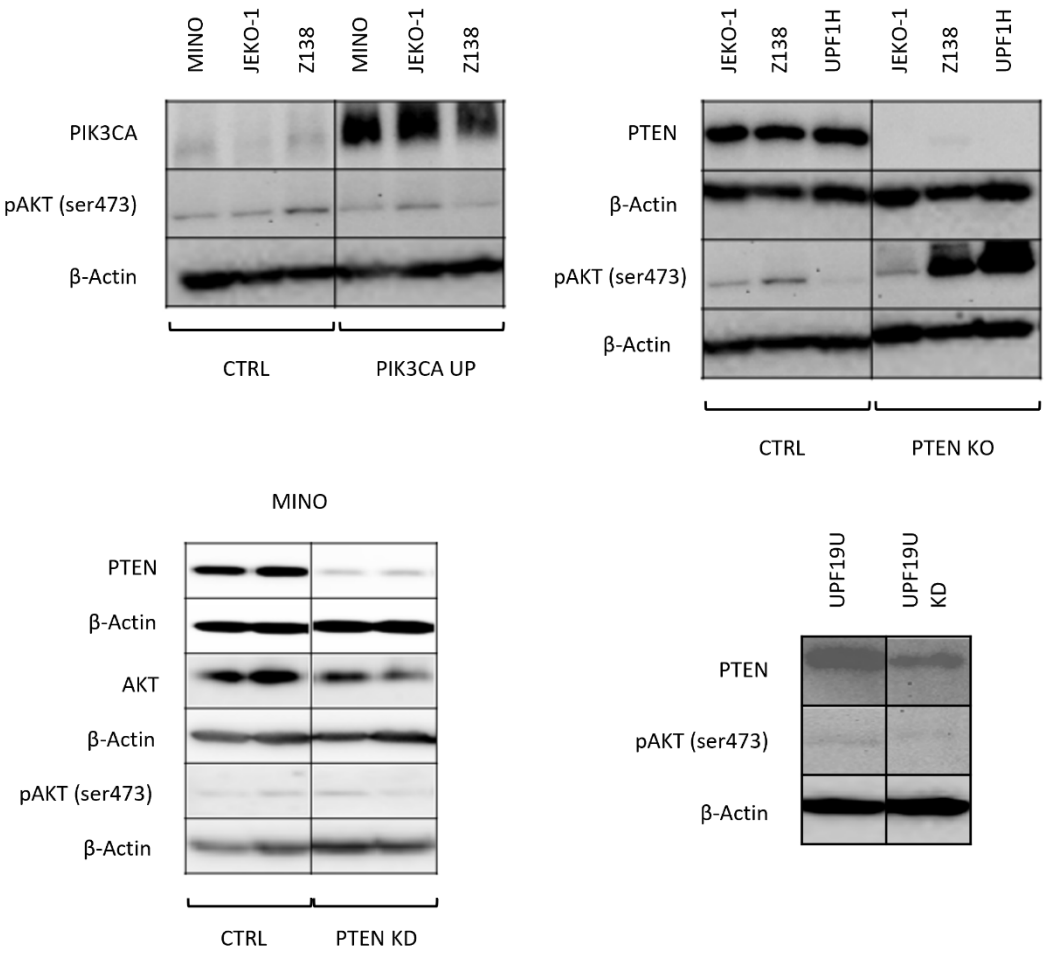
The mito stress test was performed by series of injections, starting with oligomycin (1 µM, ATP synthase inhibitor), followed by CCCP (2 µM, mitochondrial uncoupler), and a combination of antimycin and rotenone (0.5 µM, inhibitors of RCIII and RCI). The glycolytic stress test was run by series of injections, starting with glucose (10mM, substrate of glycolysis), followed by oligomycin (1 µM, inhibits ATP synthase), and 2-deoxy-D-glucose (50 mM, glycolysis inhibitor). OCR/ECAR were measured after each injection. After the assay, the cells were stained with Hoechst and counted using Cytation5 instrument. OCR/ECAR values were normalized to the same cell number.

SUPPLEMENTAL RESULTS

Supplemental table 1. FISH/aCGH of 61 primary MCL patients with > 20% bone marrow infiltration, 43% have *PIK3CA* gain and 7% have *PTEN* monoallelic loss. N/A stands for "not analyzed".

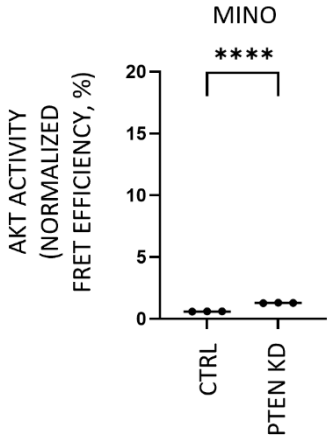
Patient	FISH or aCGH	*PIK3CA*	*PTEN*	*TP53*
1	aCGH	Gain	N	DEL
2	aCGH	N	N	DEL
3	aCGH	N	DEL	DEL
4	aCGH	N	N	DEL
5	aCGH	Gain	N	N
6	aCGH	N	N	DEL
7	aCGH	N	N	N
8	aCGH	N	N	DEL
9	aCGH	N	N	N
10	aCGH	Gain	N	DEL
11	aCGH	Gain	N	DEL
12	aCGH	N	N	DEL
13	aCGH	N	N	DEL
14	aCGH	N	N	N
15	aCGH	Gain	N	DEL
16	aCGH	Gain	DEL	N/A
17	aCGH	N	N	N
18	aCGH	N	N	DEL
19	aCGH	Gain	N	DEL
20	aCGH	N	N	N
21	aCGH	Gain	N	N
22	aCGH	Gain	N	DEL
23	aCGH	Gain	N	N
24	aCGH	Gain	N	N
25	aCGH	N	N	N
26	aCGH	Gain	N	N
27	aCGH	Gain	N	DEL
28	aCGH	N	N	N
29	aCGH	N	N	N
30	aCGH	Gain	N	N
31	aCGH	Gain	N	N

Patient	FISH or aCGH	*PIK3CA*	*PTEN*	*TP53*
32	aCGH	Gain	N	N
33	aCGH	Gain	N	N
34	aCGH	N	N	N
35	aCGH	Gain	N	DEL
36	FISH	Gain	N	N
37	FISH	N	N	DEL
38	FISH	N	N	N
39	FISH	N	N	N
40	FISH	Gain	N	N
41	FISH	N	N	N
42	FISH	N	N	N
43	FISH	Gain	N	N
44	FISH	N	N	N
45	FISH	N	N	N
46	FISH	N	DEL	DEL
47	FISH	Gain	N	N
48	FISH	Gain	N	DEL
49	FISH	N	N	N
50	FISH	Gain	N	N
51	FISH	N	N	N
52	FISH	Gain	N	DEL
53	FISH	Gain	Gain	N
54	FISH	N	N	N
55	FISH	N	N	N
56	FISH	N	DEL	DEL
57	FISH	N	N	DEL
58	FISH	N	N	N
59	FISH	N	N	N
60	FISH	N	N	N
61	FISH	N	N	DEL



184

185 **Supplemental figure 1.** Western blots showing overexpression of *PIK3CA* in MINO, JEKO-1, and Z138
186 *PIK3CA* UP cells, loss of *PTEN* and hyperphosphorylation of AKT in JEKO-1, Z138, and UPF1H *PTEN* KO
187 cells, and. decreased expression of PTEN in MINO and UPF19U *PTEN* KD cells with no changes in the
188 phosphorylation of AKT. For each sample in MINO versus MINO PTEN KD N=2; (N) represents the
189 number of biological replicates.



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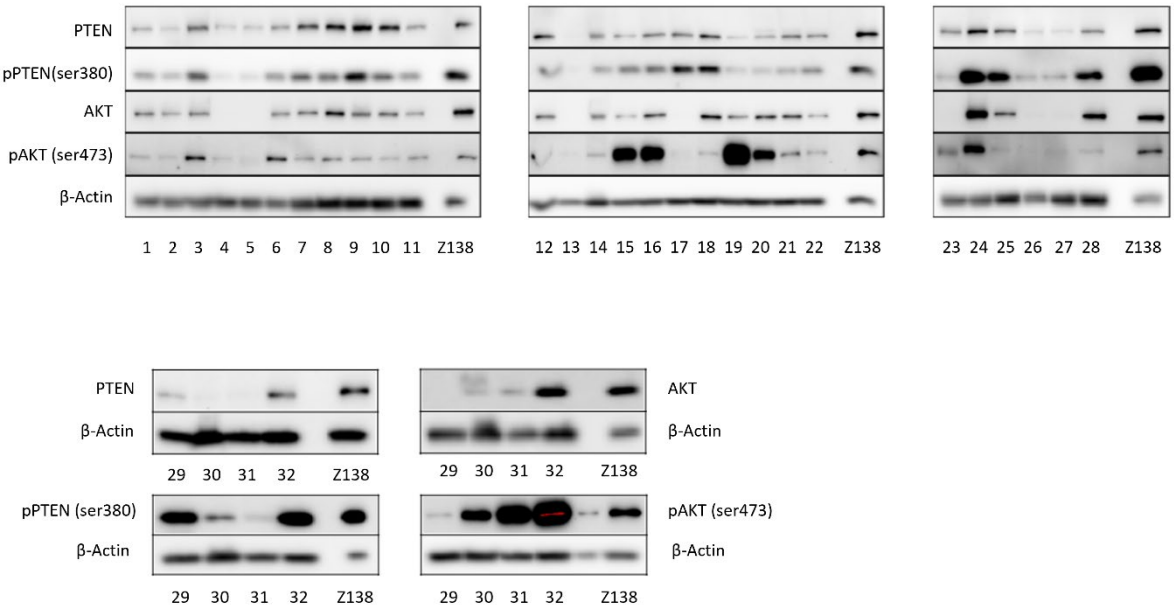
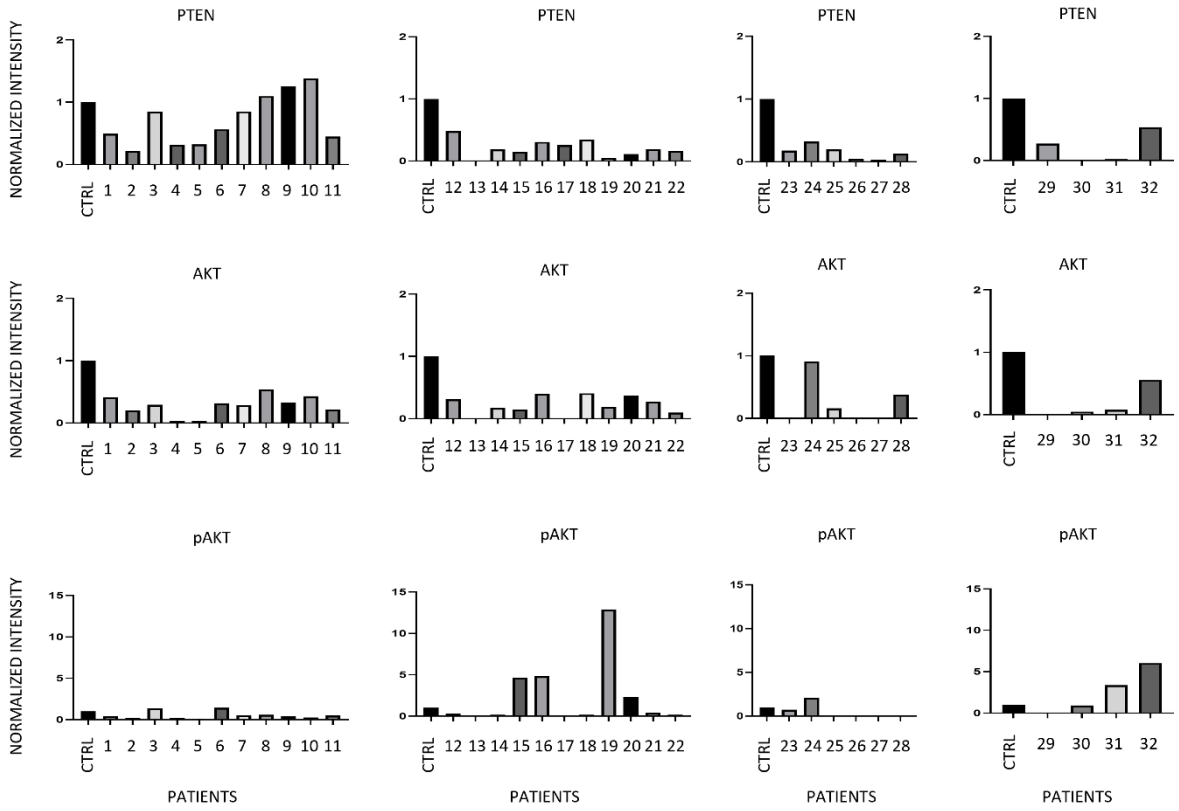
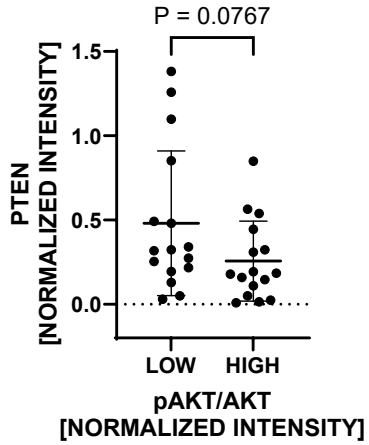
Supplemental figure 2. AKT activity in MINO *PTEN* KD cells. AKT activity as measured using genetically encoded FRET-based biosensor. Increased AKT kinase activity in MINO *PTEN* KD cell line is compared to the respective control cell line, technical triplicates. **** p<0.0001.

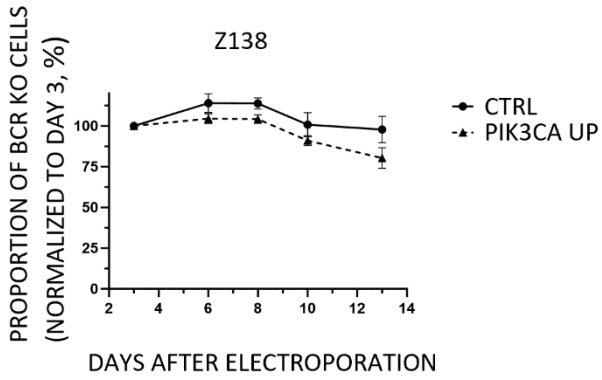


Supplemental figure 3. Western blot analysis of 32 primary MCL samples. Detection of PTEN, pPTEN (ser380), AKT and pAKT (ser473) expressions. Samples P3, 6, 15, 16, 19, 20, 24, and 32 have lower expression of *PTEN* and high phosphorylation of AKT. Samples P13, 30, and 31 show complete loss of PTEN with P31 showing hyperphosphorylation of AKT.

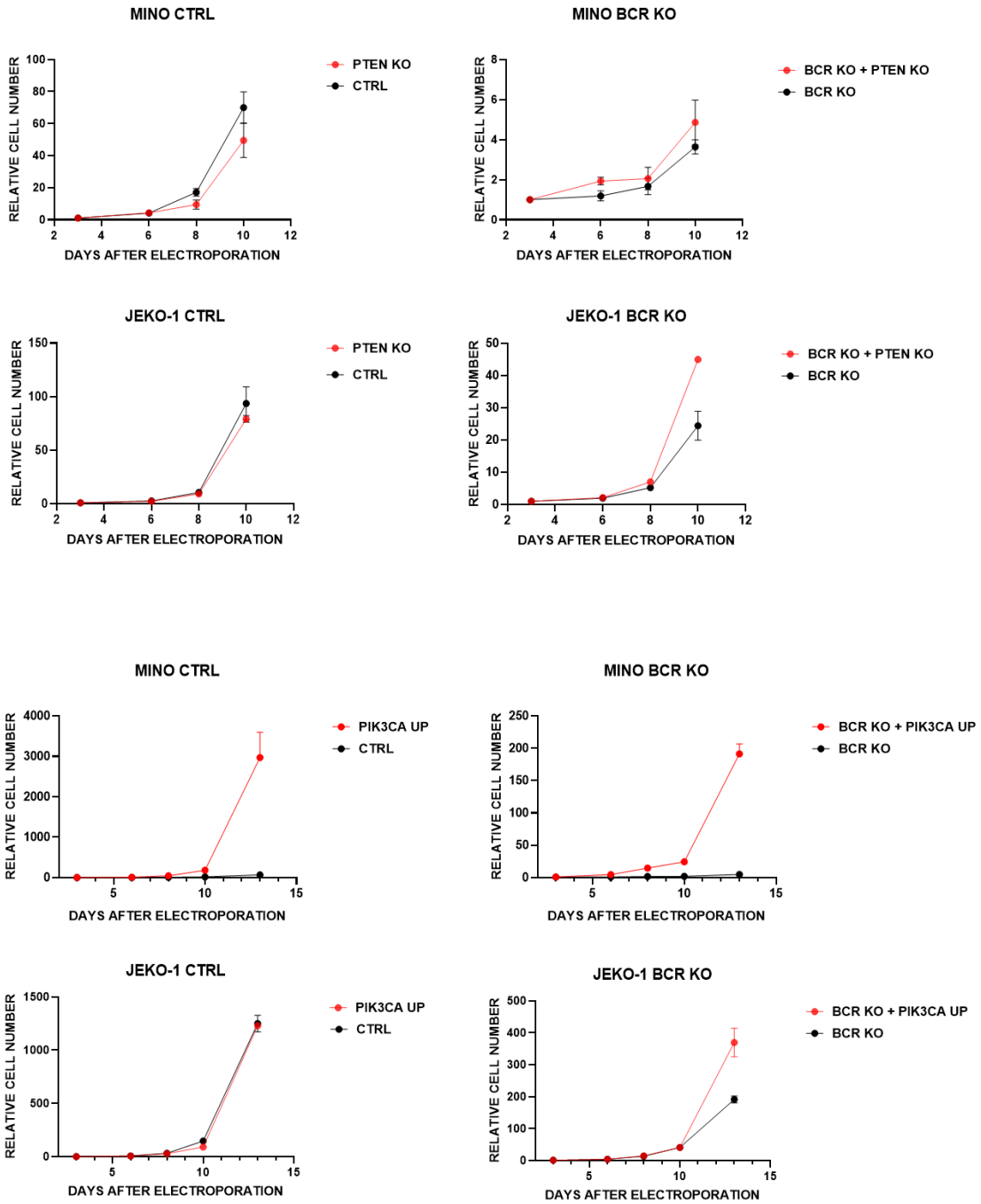




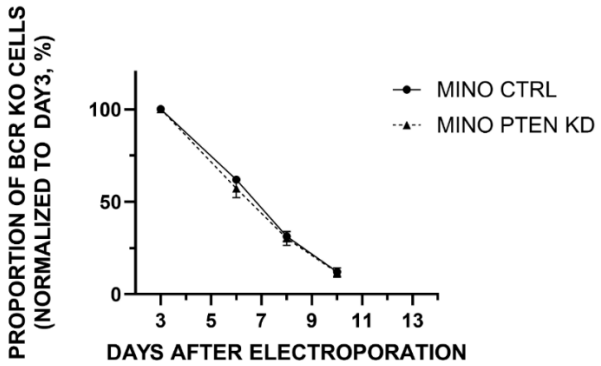
Supplemental figure 4. A. Quantification of the 32 MCL patients' western blot analysis for PTEN, AKT, and pAKT (ser473) levels as normalized to the Z138 cell line (CTRL); B. Comparison of PTEN protein expression levels between pAKT high and low patients' samples based on western blots presented in Supplemental Figures X and X. Samples were divided into two equal groups based on pAKT levels (normalized to total AKT) and normalized western blot PTEN intensities were compared. Means and SD are displayed.



Supplemental figure 5. Knock out of BCR in Z138 cell line demonstrates that Z138 is BCR independent. Averages of three replicates with SD are displayed.



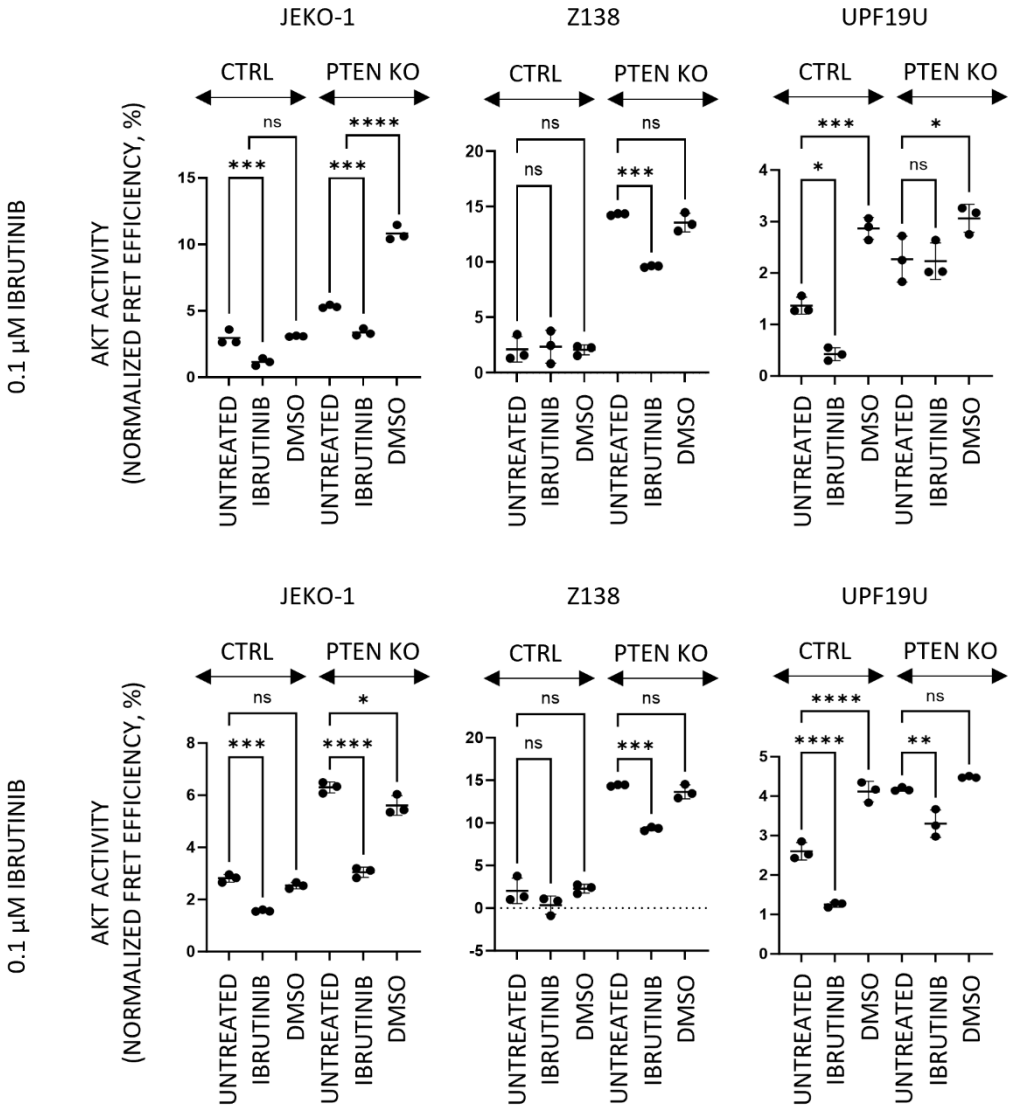
Supplemental figure 6. PTEN KO slightly decreases the growth of MINO and does not affect the growth of JEKO-1 CTRL cell lines but slightly increases growth MINO and JEKO-1 BCR KO variants (top panels). PI3KCA overexpression increases growth of MINO CTRL and as well as MINO BCR KO cells and does not affect growth of CTRL or BCR KO JEKO-1 cells. Cell growth rates were measured in the same experiment as presented in Figure 1D and E. ; N=3; data are represented as means ± SD; (N) represents the number of biological replicates.



221

222 **Supplemental figure 7.** PTEN KD does not compensate for the pro-survival signaling from BCR. PTEN
223 KD did not increase the survival of MCL cells with knockout of BCR gene; N=3; data are represented as
224 means ± SD; (N) represents the number of biological replicates.

225



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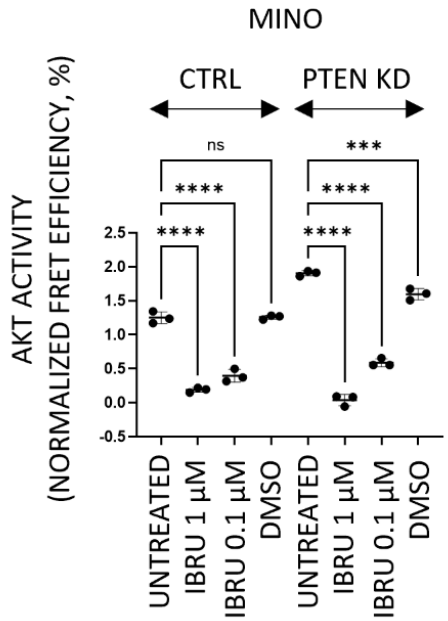
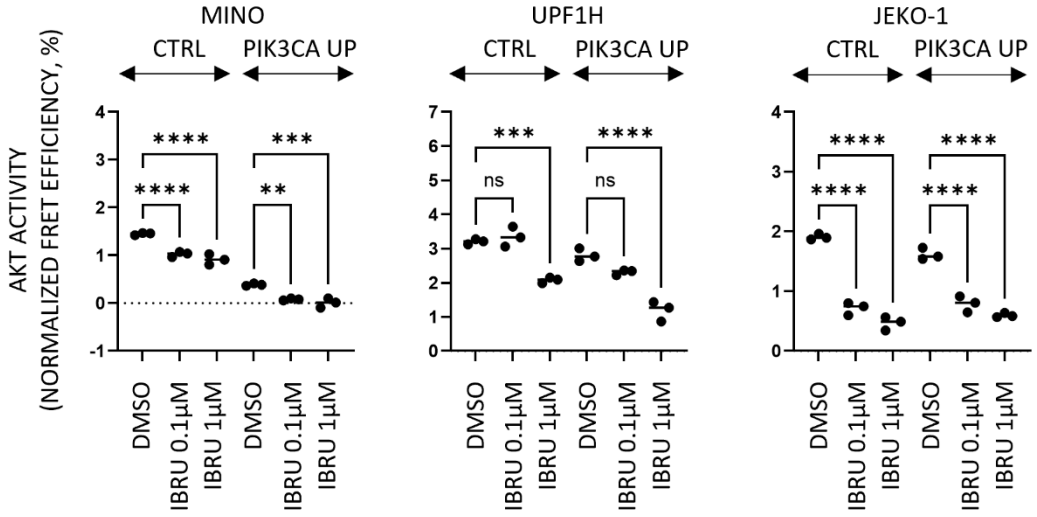
Supplemental figure 8. AKT activity as measured using genetically encoded FRET-based biosensor. AKT activity in *PTEN* KO cells is higher compared to respective unmodified cell lines. After exposure to BTK inhibitor ibrutinib for 3 hours (0.1 µM, 1 µM), AKT activity in *PTEN* KO cells remains higher than AKT activity in the respective CTRL cell lines; "ns" means "not significant", * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; N=3; (N) represents the number of biological replicates.



Supplemental figure 9. AKT activity as measured by genetically encoded FRET-based biosensor in MINO PTEN KD cells after ibrutinib. AKT activity in PTEN KD cells is higher compared to respective unmodified cells. After exposure to BTK inhibitor ibrutinib for 3 hours (0.1 µM, 1 µM), AKT activity in PTEN KD cells remains higher with the 0.1 µM ibrutinib than in the respective CTRL cell line with 0.1 µM ibrutinib; "ns" means "not significant", * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; N=3; (N) represents the number of biological replicates.



240

241 **Supplemental figure 10.** AKT activity is measured using genetically encoded FRET-based biosensor.
242 Measurement of AKT activity after Ibrutinib (3 hours exposure) in 3 *PIK3CA* UP cell lines UPF1H, MINO
243 and JEKO-1 by FRET assay showing that Ibrutinib decreases AKT activity in *PIK3CA* UP cell lines more
244 than in CTRL in two of them; "ns" means "not significant", ** p<0.01, *** p<0.001, **** p<0.0001.
245 N=3; (N) represents the number of biological replicates.

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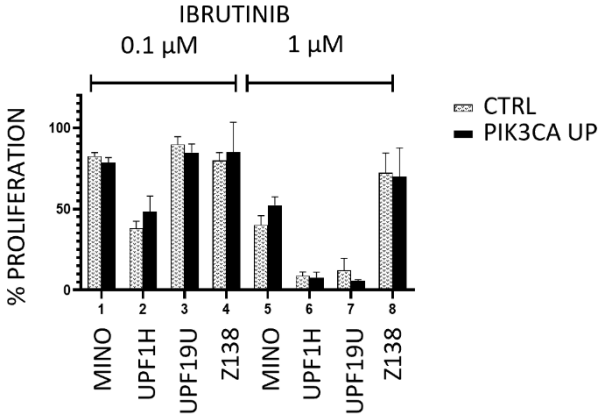

247

248 **Supplemental figure 11.** Proliferation assay implemented 72 hours after exposure to Ibrutinib (0.1, 1
249 µM), the cellular proliferation of the treated cells was normalized to the cellular proliferation of the
250 untreated cells; N=3; data are represented as means ± SD.

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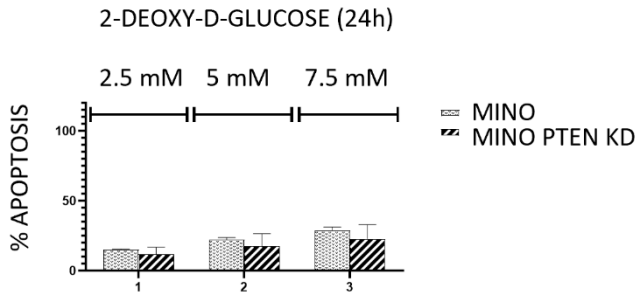

252

253 **Supplemental figure 12.** Number of apoptotic cells 24 hours after exposure of MINO and MINO KD cell
254 lines to the glycolysis inhibitor 2-deoxy-D-glucose (2.5, 5, 7.5 mM). Apoptosis of the treated cells was
255 normalized to the apoptosis of the untreated cells. N=3. Data are represented by means ± SD. (N)
256 represents the number of biological replicates.

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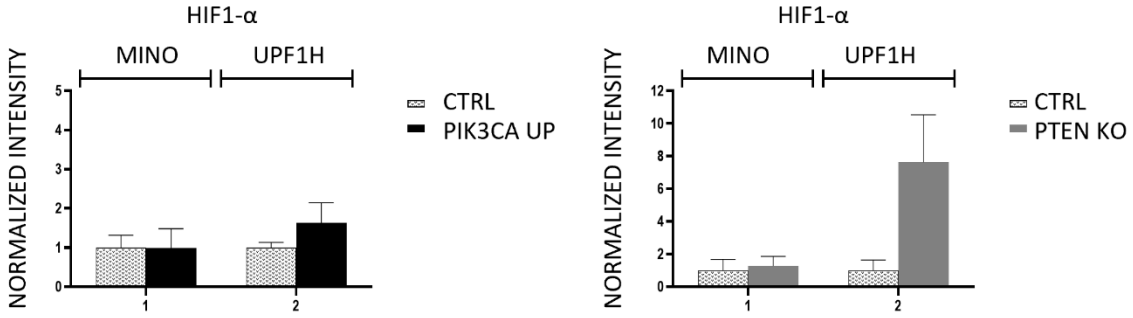

258

259 **Supplemental figure 13.** Quantification of HIF1-α expression in MINO PIK3CA UP, MINO PTEN KO,
260 UPF1H PIK3CA UP, UPF1H PTEN KO and their respective controls. The values are normalized to each
261 cell line's respective control.

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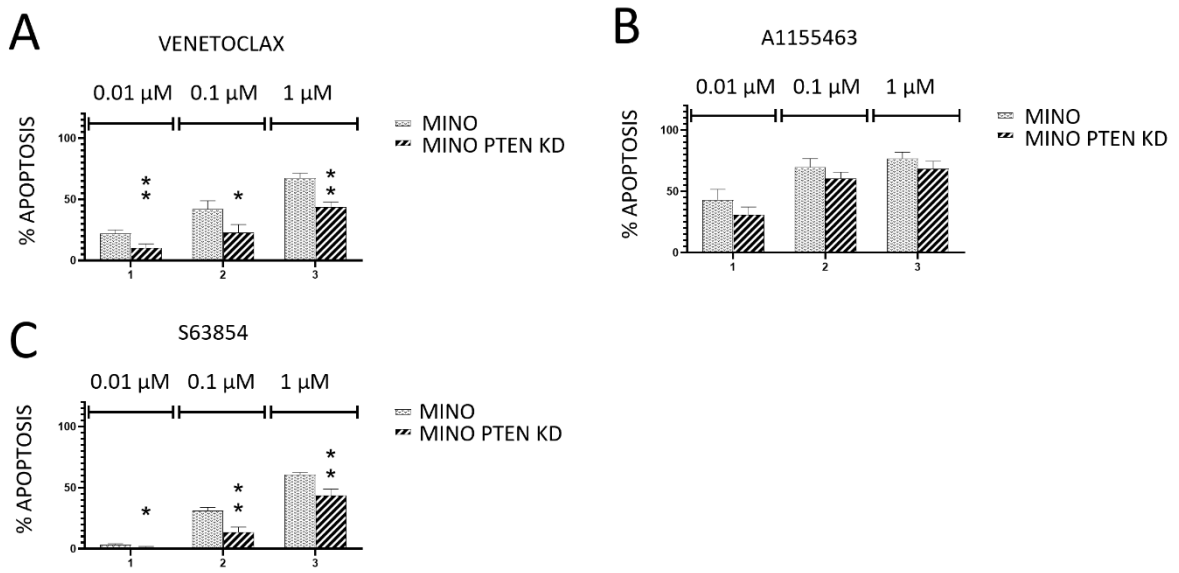
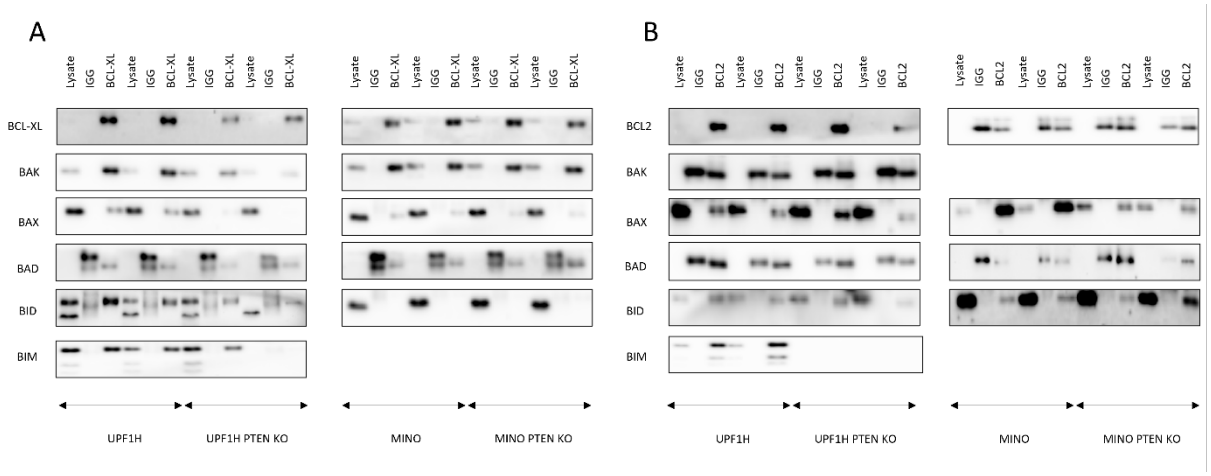
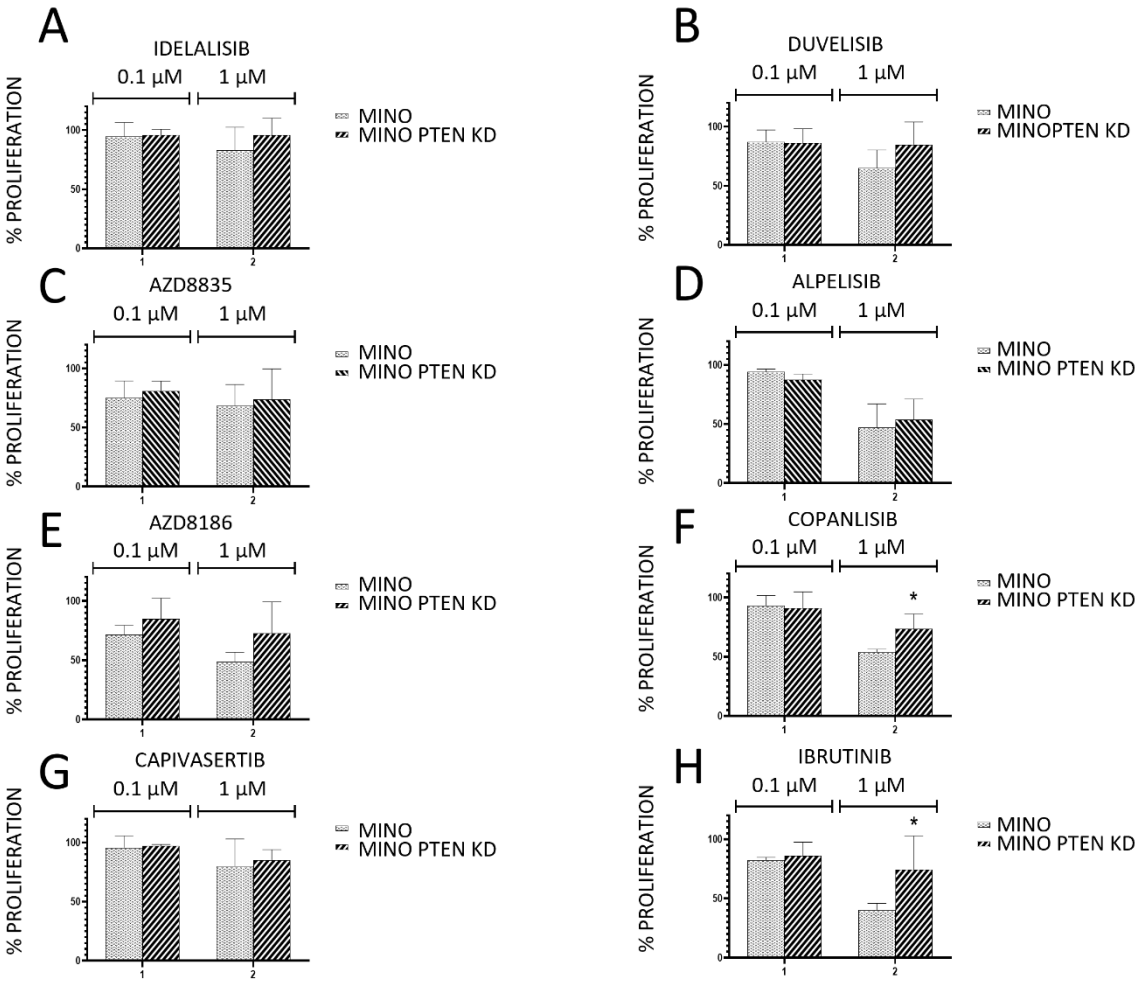


Supplemental figure 14. Number of apoptotic cells 24 hours after exposure of MINO and MINO KD cell lines to the BH3 mimetics venetoclax (0.01, 0.1, 1 µM), S63845 (0.01, 0.1, 1 µM), and A1155463 (0.01, 0.1, 1 µM). Apoptosis of the treated cells was normalized to the apoptosis of the untreated cells. N=3. data are represented by means ± SD; * $p<0.05$, ** $p<0.01$; (N) represents the number of biological replicates.



Supplemental figure 15. Co-immunoprecipitation of BCL-XL and BCL2. (A) Decreased interaction of BIM with BCL-XL in UPF1H and MINO *PTEN* KO. (B) Decreased interaction of BAX with BCL2 in UPF1H and MINO *PTEN* KO. Decreased interaction of BIM with BCL2 in UPF1H *PTEN* KO; for each sample N=2; (N) represents the number of biological replicates. The interaction of BAK with BCL2 was not measured for MINO versus MINO *PTEN* KO since BAK doesn't bind BCL2 in MINO cell line as previously shown (10) MINO has biallelic deletion of BIM.



Supplemental figure 16. Proliferation assays implemented 72 hours after exposure of MINO and MINO KD to the indicated agents: (A) Idelalisib (1, 10 µM), (B) Duvelisib (1, 10 µM), (C) AZD8835, (D)AZD8186 (1, 10 µM), (1, 10 µM), Alpelisib (1, 10 µM), Copanlisib (0.01, 0.1 µM), Capivasertib (1, 10 µM); the cellular proliferation of the treated cells was normalized to the cellular proliferation of the untreated cells; N=3; data are represented as means ± SD; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; (N) represents the number of biological replicates.

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