Supplementary Materials and Methods

Single cell DNA and protein library preparation and sequencing

Libraries for single cell DNA and protein sequencing for CAL-021 and CAL-013 patient samples were performed using the Tapestri platform (Mission Bio) according to the manufacturer's protocol. Briefly, cryopreserved BM cells were thawed, counted and up to 1 million cells incubated with Cell Staining Buffer (BioLegend), TruStain FcX and Blocking Buffer (Mission Bio) for 15 min on ice. Reconstituted TotalSeq™-D Heme Oncology Cocktail (BioLegend) containing oligo-conjugated antibodies for 45 markers was then added and incubated for 30 min on ice. Cells were then washed with Cell Staining Buffer, filtered and resuspended in Tapestri Cell Buffer for counting. 2,000-4,000 cells/mL was used for cellular lysis, barcoding and library preparation. A custom panel containing 144 amplicons covering *BAX* and 22 genes mutated in AML was used (Supplementary Table 2). Sequencing of equimolar library pools was performed on an Illumina NovaSeq 6000 S1 kit (300 cycles).

Fastq files were processed using the Tapestri Pipeline for adaptor trimming, sequence alignment, barcode correction, cell finding and variant calling. Results were visualized using the Tapestri Insights platform to perform initial filtering steps to remove poor quality genotypes or cells and to identify specific DNA clones present in each sample**.** Variants with clinical implications from known databases (COMSIC and dbSNP) or verified on bulk sequencing were selected. The analytical tool Mosaic (Mission Bio) was used for analysis and visualisation of the DNA and Protein libraries Single cell DNA sequencing for AML388 was performed using the Tapestri platform (Mission Bio) as previously described.[1] Briefly, cryopreserved bone marrow or peripheral blood mononuclear cells were thawed, counted and 2,000-4,000 cells/mL used for cellular lysis, barcoding and library preparation. A custom panel to cover the *BAX* gene and 16 genes mutated in AML was used. Sequencing was performed on an Illumina NextSeq kit (500 cycles). Fastq files were processed using the Tapestri Pipeline for adaptor trimming, sequence alignment, barcode correction, cell finding and variant calling. Results were visualized using the Tapestri Insights platform**.**

BAX targeted next generation sequencing (NGS)

Genomic DNA was prepared from primary AML samples from patients receiving venetoclax-based treatment or standard chemotherapy using the Qiagen DNeasy Blood and Tissue kit (Qiagen #69504). Bulk sequencing for *BCL2*, *BAX* and myeloid genes was performed using a

custom QIAseq Targeted DNA Panel for error-corrected digital DNA sequencing.[2] All *BAX* variants were validated using a second NGS assay using either the KAPA HyperCap Kit and a panel for genes frequently mutated in AML or targeted amplicon sequencing as previously described.[3] All variant calls were manually inspected in integrated genome viewer (IGV) ver2.6.3. Copy number frequency analysis was performed using a hybridization-based NGS panel.[2] Fishplots were constructed using fishplot package for R.[4]

Genome wide copy number analysis

This was performed using the Illumina Infinium Global Screening Array-24 v3.0, build GRCh37: Feb 2009, hg19 platform. Data analysis was undertaken with Illumina KaryoStudio v 1.4.30 software. Copy number and copy-neutral loss of heterozygosity changes were classified and reported according to the recommendations of the American College of Medical Genetics and Genomics and the Cancer Genomics Consortium.

AML cell lines and cell culture

OCI-AML3 cells were cultured in MEM alpha (GIBCO) supplemented with FBS 10% (v/v). MOLM13 and MV4;11 cells were cultured in RPMI (GIBCO) supplemented with FBS 10% (v/v). All media contained penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). All cell cultures were incubated at 37°C at 5% CO_2. All cell lines were determined to be free of mycoplasma contamination.

Drugs and cell viability assays

S55746 and S63845 were provided by Servier Laboratories. A1331852 was provided by Guillaume Lessene (WEHI). Venetoclax was purchased from Selleckchem/Jomar Life Research (cat# S8048). Idarubicin was purchased from Selleckchem (Cat. No. S1228), Cytarabine was purchased from Hospira. Daunorubicin was purchased from Selleckchem (Cat. No. HY-13062A). Doxorubicin was purchased from Selleckchem (Cat. No. HY-15142). AML cell lines were plated at 2.5×10^5 cells/mL (25,000 cells per well) and treated with 6-point 10-fold serial dilution of drugs starting from 10uM. Cell viability was determined after 48 h of drug treatment by FACS analysis of cellular exclusion of SYTOX Blue Dead Cell Stain (Life Technologies Cat No S34857) or Annexin V and 4′,6-diamidino-2-phenylindole (DAPI) staining (Sigma-Aldrich #D9542) using an LSR-Fortessa (BD). FACS data was analyzed using the FlowJo software. GraphPad Prism software was used to calculate drug concentrations causing 50% lethality (LC_{50}).

Generation of OCI-AML3 cells with acquired resistance to BH3 mimetics

Induced resistance to BCL-2 and MCL1 inhibitors was achieved by progressive exposure of OCI-AML3 cells to increasing doses (1nM to 3000nM) of BCL-2i, MCL1i or combined BCL-2 and MCL1i over a period of 3 months. In parallel, OCI-AML3 cells were cultured in increasing doses of DMSO. Once resistance was established, cells were cultured in the absence of drug for 6 weeks (referred to as OCI-AML3 DMSO, BCL-2i R, MCL1i R or combo R) and assayed for sensitivity to BH3 mimetics and chemotherapy agents.

Generation of BAX or BAK knock-out cell lines using CRISPR-Cas9 gene editing

sgRNAs targeting human BAX or BAK were synthesized and cloned into FgH1tUTG (Addgene Plasmid #70183) which permits doxycycline-inducible expression of the sgRNA and constitutive expression of a GFP reporter. All lentiviruses were produced in 293T cells and cells were transduced using established protocols. Stable Cas9-expressing human target cells were generated firstly through transduction of target cells with FuCas9Cherry (Addgene Plasmid #70182), followed by cell sorting for mCherry expression using a FACSAria (Becton Dickinson: BD). Human stable Cas9 cell lines were subsequently transduced with lentiviral supernatants. The loss of Bax or Bak protein expression was confirmed by western blotting following culture of cells for 72h in the presence of 5ug/ml doxycycline (Sigma Cat. No. 17086-28-1).

Western blotting

Protein lysates were generated using NP-40 lysis buffer (10mM Tris-HCl, pH 7.4, 137mM NaCl, 10% glycerol, 1% NP-40) supplemented with PMSF, the complete protease inhibitor cocktail (EDTA-free, Roche, Cat No. 11 836 170 001, 1x) and PhosSTOP phosphatase inhibitor cocktail (Roche Cat. No. 04 906 845 001, 1x). Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose (Amersham Hybond) or PVDF membranes (Immobilon, Millipore). Membranes were blocked in skim milk or BSA and probed overnight with a 1:1000 dilution anti-BCL-2 monoclonal antibody (mAb)(clone Bcl-2-100, WEHI), anti-MCL-1 mAb (clone 19C4-15, WEHI), anti-BCL-xL mAb (clone 44/Bcl-x, BD Transduction Lab), anti-BAX mAb (clone 21C10-23, WEHI), anti-BAK mAb (clone 8F8, WEHI), anti-BIM mAb (clone 3C5, WEHI), anti-GAPDH-HRP mAb (Cell Signaling Technology), or anti-tubulin mAb (Sigma-Aldrich). Filters were then washed, probed with either anti-mouse HRP, anti-rabbit HRP (Dako) or anti-rat HRP

(Souther Biotech) secondary antibodies. Signals were developed using Supersignal chemiluminescent substrates (Thermo Scientific). For MOLM13 WT and DKO cells, protein lysates were prepared in Onyx buffer (20 mM Tris-HCl pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol) containing protease inhibitor cocktail (Roche). Proteins were gel electrophoresed and transferred onto nitrocellulose membranes (Life Technologies). Primary antibodies used were rabbit polyclonal anti-BAX NT (#06-499, Millipore), mouse monoclonal anti-HSP70 (WEHI). Secondary antibodies were (HRP)-conjugated anti-rabbit (#4010-05, Southern Biotech) and anti-mouse (#1010-05, Southern Biotech). Proteins were visualized by Luminata Forte Western HRP substrate (#WBLUF0500, Millipore).

Assessing BAX integration by carbonate extraction

Cells were pre-treated (1 hour) with 25 μM Q-VD (MP Biomedicals Australia) and then incubated with 500 nM venetoclax. After 5 hours, cells were harvested and permeabilized with 0.025% w/v digitonin in MELB buffer (100 mM KCl, 2.5 mM $MgCl_2$, 100 mM sucrose, 20 mM HEPES/KOH pH 7.5) for 10 min on ice and cytosol and mitochondria-enriched heavy membrane were separated by centrifugation. Membrane fractions were then re-suspended in sodium carbonate (0.1M, pH 11.5) and incubated on ice for 20 min before addition of an equal volume of 0.1 M HCL. After treating the samples with DNase I (5 Units/50 μl), the supernatant fraction containing peripheral proteins and pellet fraction containing membrane-integrated proteins were separated by centrifugation. Cytosol, peripheral and integrated fractions were then run on SDS-PAGE and immunoblotted for BAX.

Generating MOLM13 cells expressing BAX WT or BAXP168A by retroviral infection

MOLM-13 WT or BAX/BAK double knockout (DKO) cells were passaged in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) at 37ºC and 10% CO_2. BAX WT or P168A mutant constructs were cloned into the retroviral expression vector pMSCV-IRES-GFP and transduced into MOLM-13 WT or BAX/BAK DKO cells using the packaging cell line HEK293T cells.

Mouse AML xenograft model

All mouse studies were conducted with approval from the Alfred Medical Research and Education Precinct Animal Ethics Committee. For *in vivo* studies, venetoclax was dissolved in 5%DMSO (Sigma-Aldrich, cat#D5879) + 50% PEG 300 (Sigma-Aldrich, cat#202371), 5%

Tween 80 (Sigma-Aldrich, cat#P8192) and 40% distilled water. MCL1-i S63845, was dissolved in 50% (2-hydroxypropyl)-β-cyclodextrin (Sigma #332607) and 50% 50 mM HCI. NOD-SCID IL2Rγ-null mice (NSG, 8-10 weeks old) were obtained from the Animal Resources Centre. Irradiated NSG mice were intravenously injected with $1x10^5$ OCI-AML3, OCI-AML3 combo R, OCI-AML3 $BAX^{-/-}$ or OCI-AML3 $BAK^{-/-}$ cells and dosing commenced on day 4 post-transplant. Mice were gavaged with venetoclax (200 µL 75 mg/kg) or S63845 (200 µL 25 mg/kg) weekly IV or the drug combination, or vehicle for 4 weeks. Non-irradiated NSG mice were intravenously injected with $1x10^5$ MOLM13 WT or MOLM13 BAX/BAK DKO+BAXP168A cells and dosing commenced on day 4 post-transplant. Mice were gavaged with venetoclax (200 µL 75 mg/kg) or S63845 (200 µL 25 mg/kg) weekly IV or the drug combination, or vehicle for 10 days. Drug efficacy was determined by flow cytometric analysis of hCD45 + cells in bone marrow isolated from flushed femurs at end point cull or flow cytometric analysis of hCD45 + cells in bone marrow isolated by intrafemoral sampling.

Immunohistological studies

Mouse sternums and spleens were harvested and fixed in 3.7% formalin for a minimum of 24h before decalcification in phosphate-buffered formalin (3.7% formalin, pH 7.4) containing 20% EDTA for 48h, then embedded in paraffin and sectioned. For immunohistochemical staining of human CD45, slides were prepared using standard procedures and stained with anti-human CD45 mAb (Dako, Cat. No. M0701) in a Dako Autostainer Plus. Slides were imaged and annotated using an Aperio ScanScope and software system (Leica Biosystems).

Data sharing

Processed sequencing data will be made available subject to a data transfer agreement and will be restricted to ethically approved research into blood cell malignancies and cannot be used to assess germline variants

Supplementary Table 1A. *BAX* mutations detected in VEN-treated patients

Patient ID	Age at AML diagnosis	AML (de novo, secondary or therapy related)	Treatment received	Cytogenetics at AML diagnosis	Molecular mutations at AML diagnosis	*BAX* mutations identified (VAF%)
AML108	68	De novo	Venetoclax + LDAC	N/A	*DNMT3A* R882C *DNMT3A* V657M *RUNX1* S314Cfs*285	c.474+2_474+17del; p.? (2.86%)
AML388	78	Secondary (prior MDS)	Venetoclax + LDAC	Trisomy 13	*ASXL1* G646Wfs*12; *ETV6* R129*; *SRSF2* P95_R102del; *RUNX1* V162_V164delins; *BCORL1* 1409_1412delins; *PHF6* R129*	c.280C>T; p.(R94*) (15.5%)
CAL-035	74	Secondary (prior MPN/MDS-u with azacitidine, oral melphalan)	Venetoclax + Intensive chemotherapy	Tetrasomy 8	*SRSF2* P95H; *SETBP1* D868N; *ETV6* T187Nfs*9; *ETV6* F417L; *NRAS* G12S; *NRAS* G12A; *CUX1* Q1023*; *IDH1* R132C; *RUNX1* splice site; *STAG2* D116_N119del; *STAG2* L114Pfs*2	c.502C>G; p.(P168A) (31.5%) c.497G>A; p.(G166E) (1.14%)
CAL-021	70	De novo	Venetoclax + Intensive chemotherapy	Trisomy 4	*IDH1* R132C; *DNMT3A* Y735C; *DNMT3A* R729Q; *NPM1* W288Cfs*12; *NRAS* G12D	c.278dup; p.(R94Pfs*5) (48.6%) c.563G>A; p.(W188*) (0.977%) c.233+2del; p.? (0.968%) c.100C>T; p.R34* (0.675%)
CAL-013	78	De novo	Venetoclax + Intensive chemotherapy	Del(9q)	*NPM1* W288Cfs*12; *FLT3* Y599C; *SF3B1* K700E; *TET2* I873*; *TET2*	c.121dup; p.(E41Gfs*33) (47%)

					Q13569R; *TET2* R1537* *BAX* E41Gfs*33 (VAF 27%)	
CVC-003	67	De novo	Venetoclax + Intensive chemotherapy	Tetraploidy	*IDH2* R140Q; *IDH1* R132H; *SRSF2* P95R; *RUNX1* L98Sfs*24; *KDM6A* S1336*	c.271del; p.(V91Sfs*42) (2.66%) c.280C>T; p.(R94*) (3.46%)
CAL-036	70	De novo	Venetoclax + Intensive chemotherapy	Normal	*DDX41* L426P; *DDX41* D216E	c.121dup; p.(E41Gfs*33) (9.7%) c.327_349dup; p.(A117Gfs*24) (2.9%)

Supplementary Table 1B. *BAX* mutations detected in chemotherapy-treated patients

Patient ID	Age	AML (de novo, secondary or therapy related)	Treatment received	Cytogenetics at AML diagnosis	Molecular mutations at AML diagnosis	BAX mutations identified (VAF%)
AML224	72	De novo	7+3	Normal	*TET2* V636Lfs*3; *TET2* N861Tfs*12; *TET2* L1780Cfs*40; *TP53* Y234H	c.416G>A; p.(W139*) (3.5%)
AML311	63	De novo	7+3	Normal	*NPM1* W288Cfs*12; *DNMT3A* R320*	c.67G>C; p.(G23R) (0.83%)

Supplementary Table 1C. Overview of patient cohorts studied

	Venetoclax failure	Chemotherapy failure
N	41	34
Median age (range)	72 (60-80)	55 (24-78)
Males (%)	23 (56)	19 (56)
AML presentation, n (%)		
De novo	21 (51)	31 (91)
Secondary/therapy-related	20 (49)	3 (9)
Cytogenetic risk, n (%)		
Favorable	-	3 (9)
Intermediate	26 (63)	28 (82)
Adverse	15 (37)	3 (9)
Primary refractory AML, n (%)	7 (17)	-
Relapsed AML, n (%)	34 (83)	34 (100)

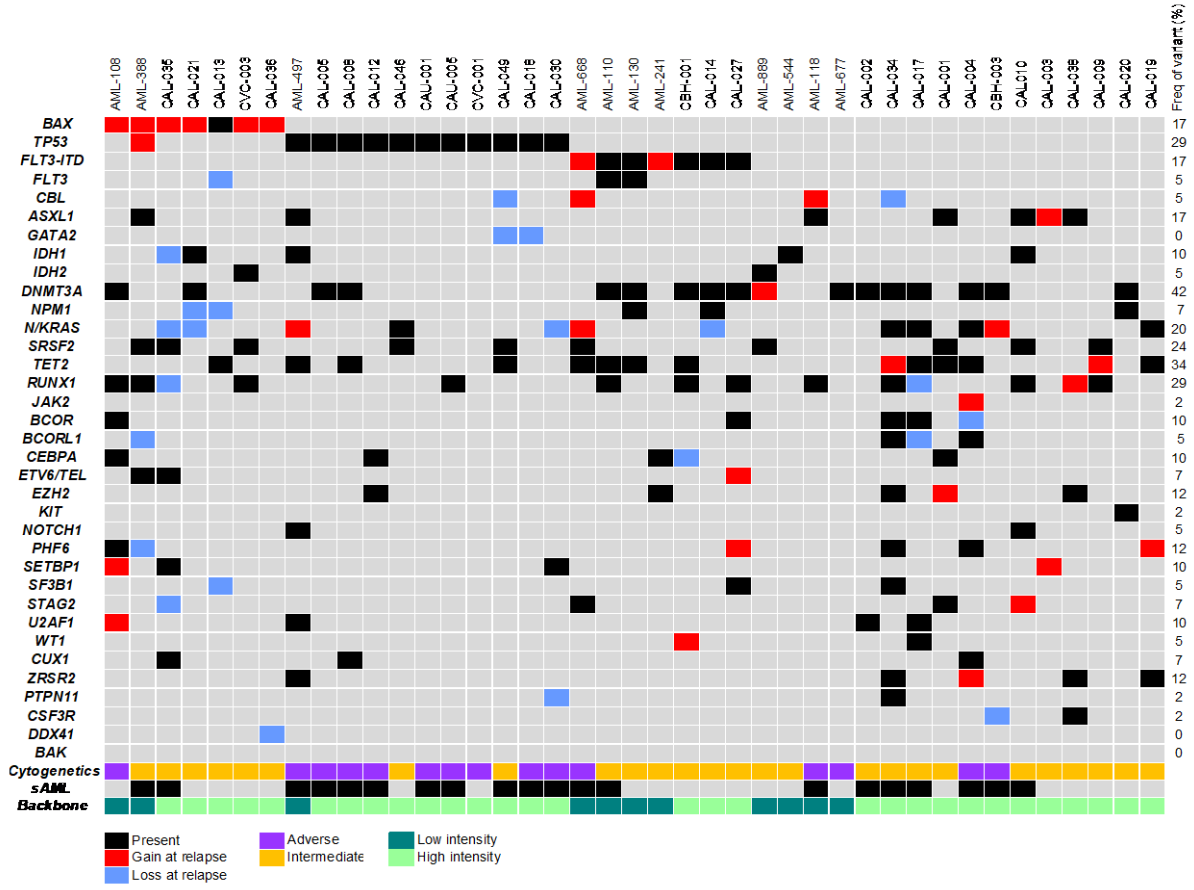
Supplementary Table 2. List of genes in the custom single cell DNA sequencing panel for Tapestri

ASXL1 NM_015338.6	GATA2 NM_032638.5	NPM1 NM_002520.7	SRSF2 NM_003016.4
BAX NM_138761.4	IDH1 NM_005896.4	NRAS NM_002524.5	TET2 NM_001127208.2
CBL NM_005188.4	IDH2 NM_002168.4	PTPN11 NM_002834.5	TP53 NM_000546.6
DNMT3A NM_022552.5	JAK2 NM_004972.4	RUNX1 NM_001754.5	U2AF1 NM_006758.3
EZH2 NM_004456.5	KIT NM_000222.3	SETBP1 NM_015559.3	WT1 NM_024426.6
FLT3 NM_004119.3	KRAS NM_004985.5	SF3B1 NM_012433.4	

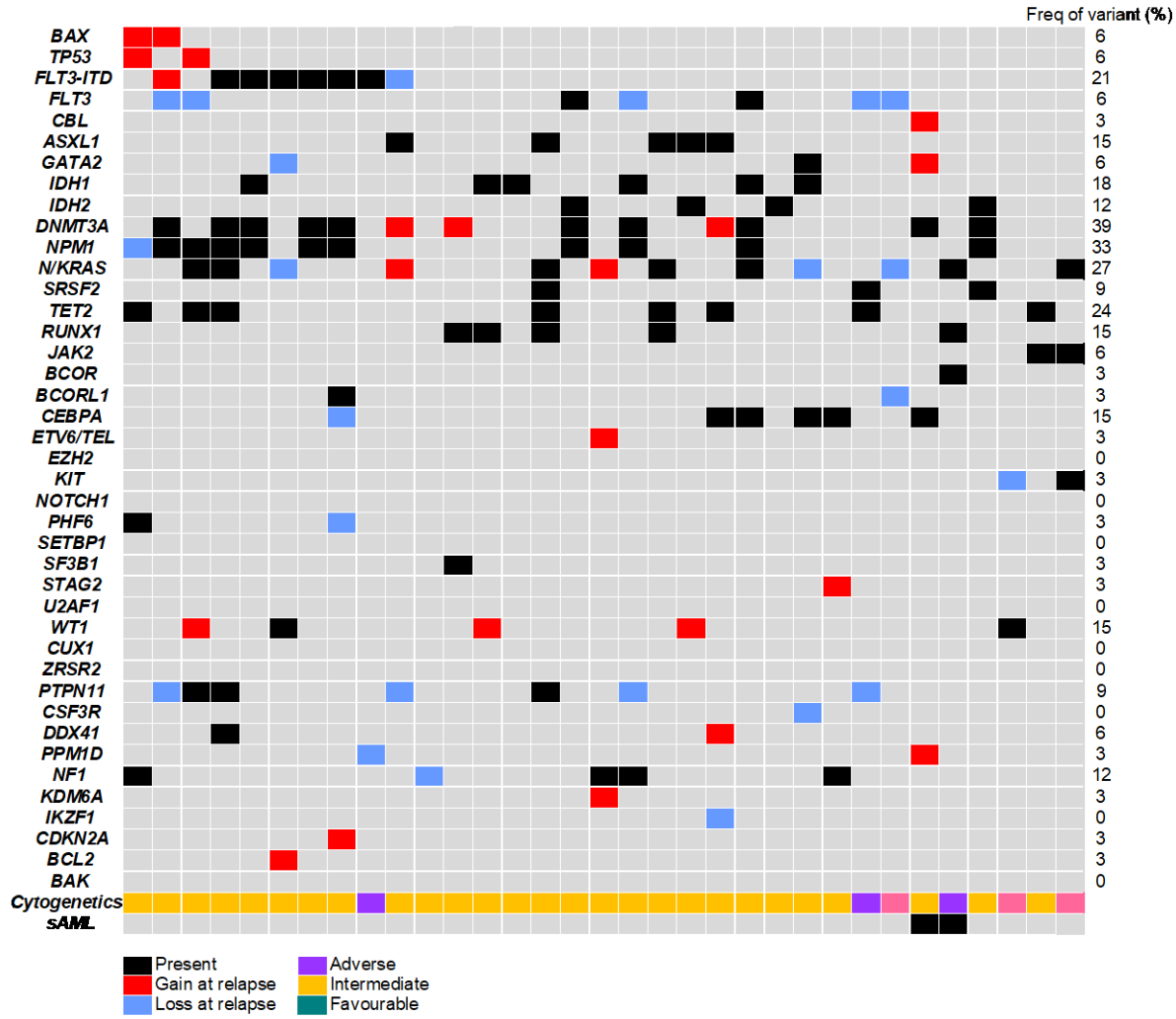
Supplementary figure 1:

Molecular profile of variants detected in patients relapsing after (A) venetoclax (VEN)-based therapy or (B) intensive chemotherapy. Each column represents the results from targeted NGS for myeloid genes on paired samples from diagnosis and relapse. Black squares represent the same genotype at diagnosis and relapse, red squares represent variants gained at relapse and blue squares variants lost at relapse. The frequency at relapse for each variant gene is shown (omitting variants lost and including variants gained). Cytogenetic risk, history of secondary AML (sAML) and the intensity of backbone chemotherapy (high verses low intensity) used in the VEN cohort is indicated.

A.

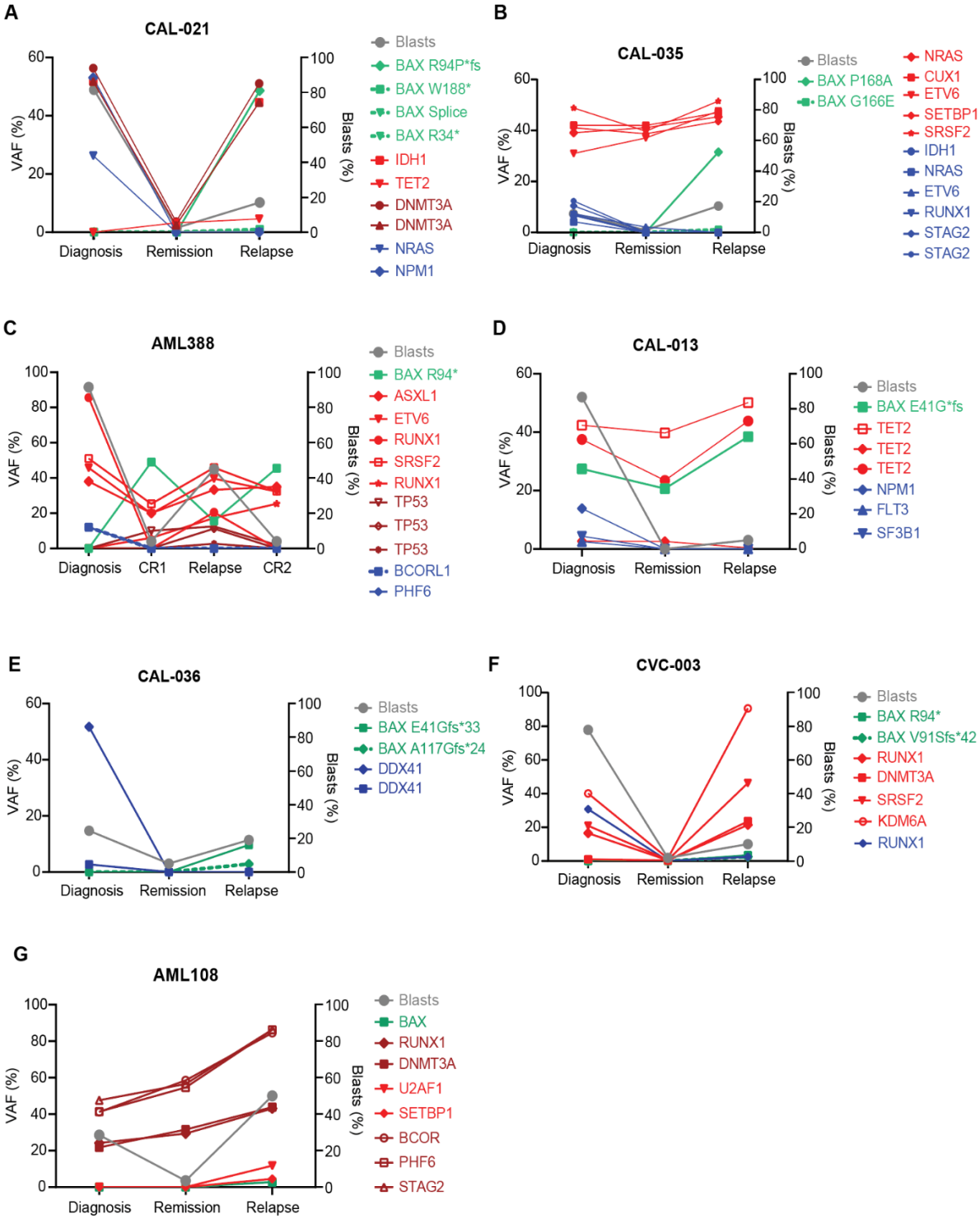


B.


Freq of variant (%)
BAX 6
TP53 6
FLT3-ITD 21
FLT3 6
CBL 3
ASXL1 15
GATA2 6
IDH1 18
IDH2 12
DNMT3A 39
NPM1 33
N/KRAS 27
SRSF2 9
TET2 24
RUNX1 15
JAK2 6
BCOR 3
BCORL1 3
CEBPA 15
ETV6/TEL 3
EZH2 0
KIT 3
NOTCH1 0
PHF6 3
SETBP1 0
SF3B1 3
STAG2 3
U2AF1 0
WT1 15
CUX1 0
ZRSR2 0
PTPN11 9
CSF3R 0
DDX41 6
PPM1D 3
NF1 12
KDM6A 3
IKZF1 0
CDKN2A 3
BCL2 3
BAK 0
Cytogenetics
sAML
Present
Gain at relapse
Loss at relapse
Adverse
Intermediate
Favourable

Supplementary Figure 2:

(A-G) Dynamic changes in clonal architecture from diagnosis to relapse in 7 cases with *BAX* variants above 10% variant allele frequency (VAF). The VAF for each mutation is shown, along with the bone marrow blast count at the corresponding time point.

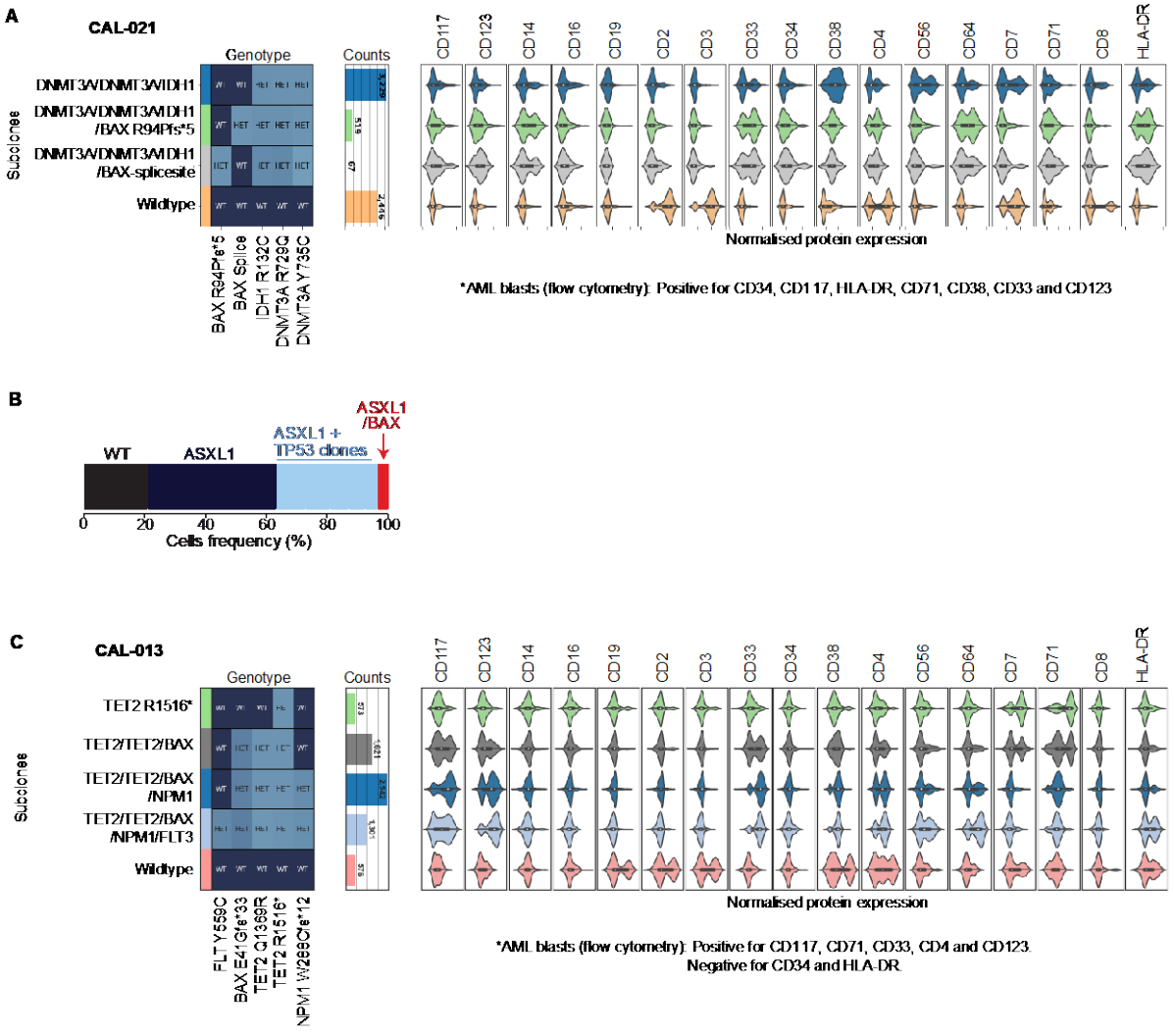


Supplementary Figure 3:

(A) Left: heat map indicates mutation zygosity for each subclone detected in CAL-021 at relapse. Middle: the number of cells for wild type (WT) and each subclone detected. Right: Protein expression of 17 antibody tags for WT cells and subclones with leukemia-containing mutations. *indicates the flow cytometry profile of CAL-021 blasts as determined in the clinical pathology department.

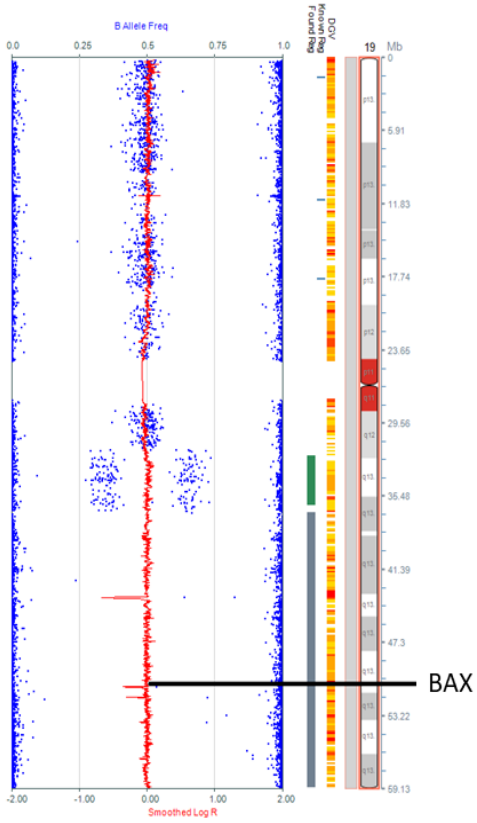
(B) Proportion of wild type and mutation-containing clones in AML388 at relapse. Sequencing at *BAX* p.(R94*) was poorly genotyped. Only cells passing quality filters for BAX have been included.

(C) Left: heat map indicates mutation zygosity for each subclone detected in CAL-013 after one week of venetoclax treatment. Middle: the number of cells for WT and each subclone detected. Right: Protein expression of 17 antibody tags for WT cells and subclones with leukemia-containing mutations. *indicates the flow cytometry profile of CAL-013 blasts as determined in the clinical pathology department.



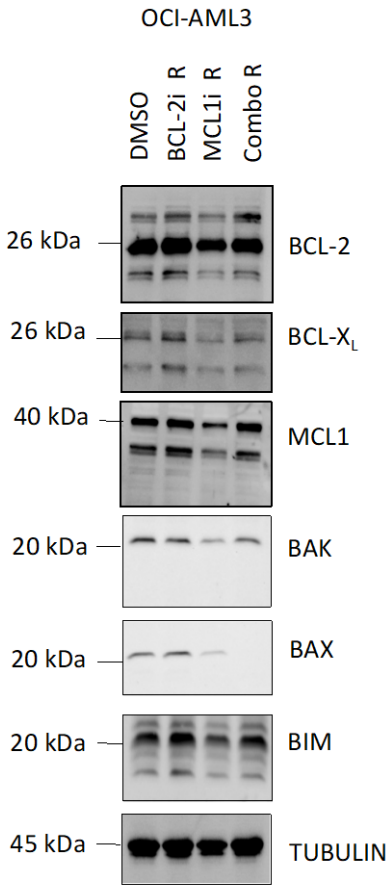
Supplementary Figure 4:

DNA microarray data of OCI-AML3 cells rendered tolerant to 3µM BCL-2i demonstrating a segment of copy number neutral loss of heterozygosity involving the BAX locus on chromosome 19q

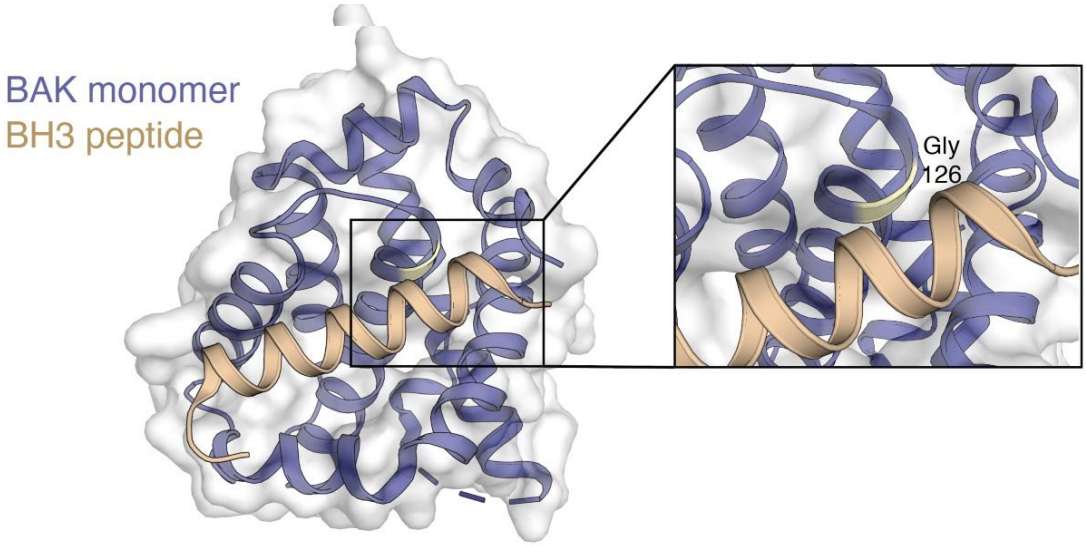


Supplementary Figure 5:

Immunoblot profiling of BCL-2 family proteins in OCI-AML3 cells made tolerant to 1 μM of drug: DMSO, S55746 (BCL-2i R), S63845 (MCL1i R) or S55746 + S63845 (Combo R).

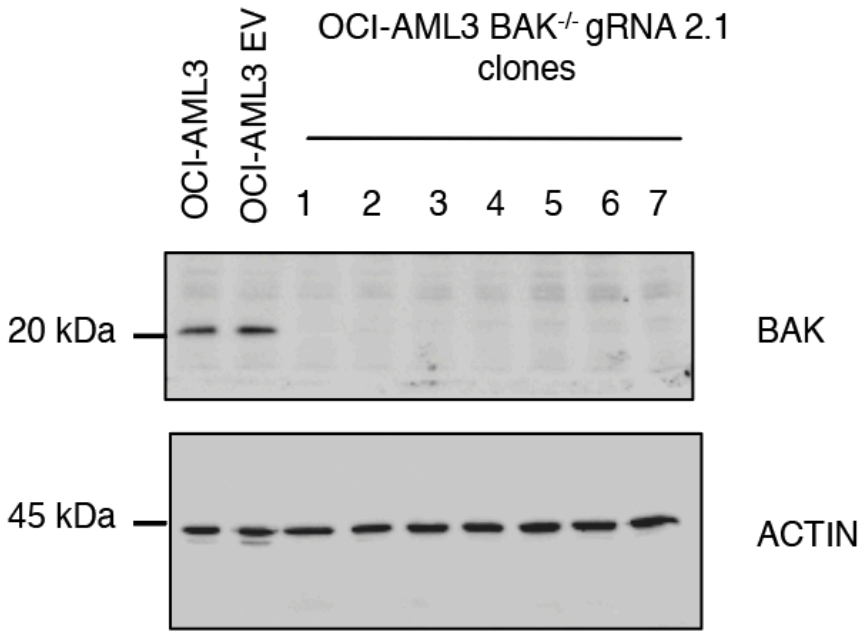


Supplementary Figure 6: The crystal structure of human BAK in complex with a BAK BH3 peptide (PDB ID 7M5C) was visualised using Pymol version 2.5.0. Location of glycine 126 (yellow) in the hydrophobic groove of non-activated BAK (blue) with a BAK BH3 peptide bound (wheat) (PDB ID: 7M5C).



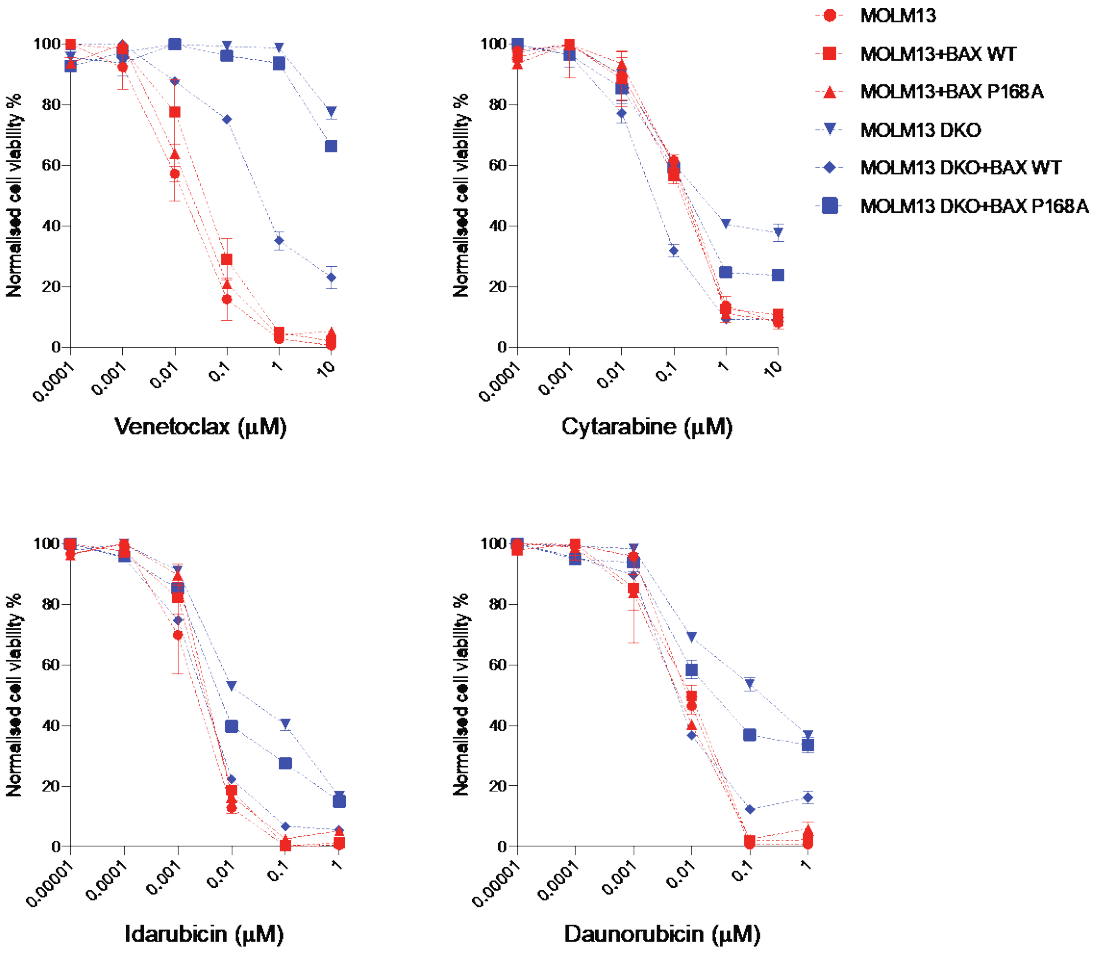
Supplementary Figure 7:

(A-B) BAK expression in CRIPSR-Cas9 edited OCI-AML3 cells. Immunoblot demonstrating CRISPR-Cas9-induced BAK deletion in OCI-AML3 cells using guide RNAs targeting BAK (gRNA 2.1) or a non-targeted (empty vector) control. Cells were treated with 5 μg/ml doxycycline for 72 h to induce BAK loss. OCI-AML3 clone 1 cells were used for subsequent experiments.



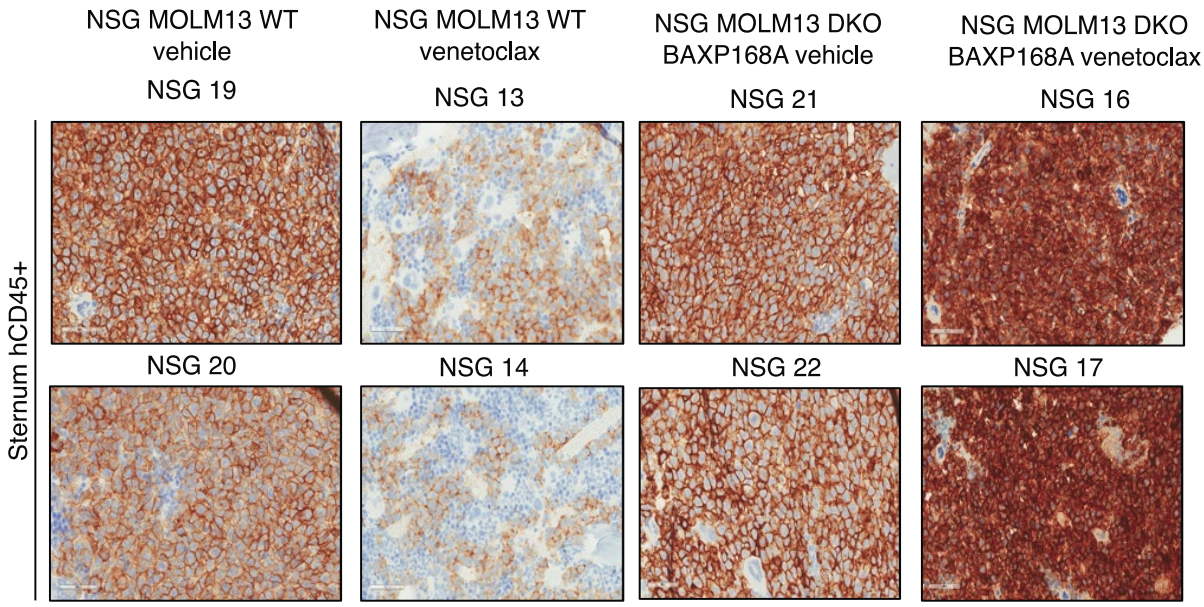
Supplementary Figure 8:

The *BAX* P168A mutation confers resistance to venetoclax but not cytotoxic agents. MOLM13 WT or MOLM13 BAX/BAK DKO cells expressing *BAX* WT or *BAX* P168A were treated with venetoclax, cytarabine, idarubicin or daunorubicin at the indicated doses and cell viability determined after 48 h by flow cytometric enumeration of sytox blue exclusion. Error bars indicate SD of 3 independent experiments



Supplementary Figure 9:

Immunohistochemical analysis for hCD45+ cells performed on sternal bone marrow from cohorts transplanted with $5x10^5$ MOLM13 WT or MOLM13 BAX/BAK DKO cells expressing *BAX* P168A. hCD45+ AML cells were captured at x100 magnification using the Aperio scanscope. Two representative cases from each cohort are shown.



References

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3. Thijssen R, Diepstraten ST, Moujalled D, et al. Intact TP-53 function is essential for sustaining durable responses to BH3-mimetic drugs in leukemias. *Blood, The Journal of the American Society of Hematology*. 2021;137(20):2721-2735.

4. Miller CA, McMichael J, Dang HX, et al. Visualizing tumor evolution with the fishplot package for R. *BMC genomics*. 2016;17(1):1-3.