Inhibition of mitochondrial translocase SLC25A5 and histone deacetylation is an effective combination therapy in neuroblastoma

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SUPPLEMENTARY MATERIALS & METHODS

1. Mitochondria-associated gene (MAG) list construction

Gene signatures from the molecular signatures database (MSigDB v6.2) were screened using either “mitochondria” or “mitochondrial” search terms (28 gene sets) and then collated and filtered for genes that had valid ensembl gene ID’s to generate a list of 1148 genes.[1-3] An intersection plot of all 28 gene sets was plotted to illustrate the number of unique or overlapping genes in/between each gene set using the *UpSetR* R package.[4]

2. Patient tumour cohort analyses

RNA-seq data, in reads per million (RPM), were obtained for 498 clinically annotated primary NB samples (SEQC NB cohort) from the gene expression omnibus (GEO) with the accession GSE62564.[5] Using the *survival* R package, we ran either univariate or multivariate Cox Proportional Hazards (CoxPH) regression models for genes of interest, by first using median gene expression to dichotomise patient cohorts, followed by regression of covariates associated with adverse NB prognosis such as advanced disease stage (INSS Stage 4), age at diagnosis (> 18 months) and *MYCN* amplification. RPM gene expression values were scaled for each gene using z-score normalisation, multiplied by the univariate CoxPH hazard ratio for each gene, and then summed to produce MAG scores. MAG gene expression profiles were hierarchically clustered across all patients, which yielded 6 main MAG clusters. A simple classifier was then used to group patients into each cluster, wherein the highest average z-score for clusters in a patient was used for grouping. Kaplan-Meier survival curves of

subgroups dichotomised by either; median gene expression, MAG scores or patient classifications were done using the *survival* and *survminer* R packages. All statistical tests concerning Kaplan-Meier analyses were done using log-rank tests, adjusted using the Bonferroni method for multiple hypotheses testing where appropriate. Gene ontologies for each MAG cluster were determined by using the *clusterProfiler* R package with ReactomePA and GO databases.[6-8] Orthogonal analyses were conducted in an independent gene expression microarray dataset, consisting of 649 clinically annotated NB samples (KOCAK cohort) obtained from GEO with the accession GSE45547.[9]

3. ChIP-Seq and ATAC-seq data analysis

ChIP-seq (GSE80151) and ATAC-seq (GSE80152/GSE101294) raw fastq files were obtained directly from the European Nucleotide Archive (ENA) under the study accessions PRJNA318044, PRJNA318043 and PRJNA401531.[10, 11] Reads from fastq files were first quality trimmed using *trimgalore*, followed by alignment to the human genome (GRCh38) using *bowtie2.*[12] *SAMtools* was then used to convert, sort and index alignments.[13] Peaks were then called using *MACS2* either in single-end mode for N-Myc ChIP-seq data or paired-end end mode for ATAC-seq datasets, with an FDR q-value threshold of <0.05.[14] Fold enrichment tracks which represent relative enrichment of the ChIPed protein compared to the genomic input, were generated using *MACS2* and converted to the bigwig format using *BEDtools* for visualisation.[15] *HOMER* was used to annotate peaks (promoters were defined as -1000bp/+100bp from the transcription start site).[16]

4. Project Achilles CRISPRi data analysis

Gene dependency data, in the form of gene dependency probabilities, were obtained from the Project Achilles portal under version 17Q1.[17] Only cell lines with the disease type “Neuroblastoma” were retained and used for downstream analyses. Probability averages were made for each MAG across all 14 NB cell lines, where a value closer to 1 represents a higher probability of a given MAG cluster positively influencing cell viability.

5. Drug sensitivity data mining

Drug sensitivity ($\log IC_{50}$) values were retrieved from the Genomics of Drug Sensitivity in Cancer (GDSC) database for 32 NB cell lines and 251 compounds.[18] Values were first inverted ($1/\log IC_{50}$) and then scaled across all cell lines using z-score normalisation. A higher z-score represents higher sensitivity for a given drug in each cell line. *TP53* mutations in these cell lines were then determined using variant annotations in matching whole exome sequencing (WES) data from the GDSC database (14/32 cell lines had *TP53* mutations). We then calculated average z-scores for each compound in mutant *TP53* cell lines and ranked these compounds by increasing z-score. This data was finally visualised in a heatmap using the *gplots* package in R/Rstudio.

6. Cell viability assays

Cell viability was measured using the Alamar Blue fluorescence assay (Invitrogen) according to the manufacturer's instructions. Assay absorbance was determined using

the Wallac 1420 VICTOR2^{TM} microplate reader at an excitation/emission of 560/590 nm (PerkinElmer). A baseline reading was taken at 0 hours, followed by readings after 6 hours of incubation at 37°C. All data were normalised and compared to a treatment control. IC_{50}'s were determined by fitting non-linear regression curves (Sigmoidal dose-response - variable slope) to normalised data using the GraphPad Prism 7 (Applied BioSystems, Scoresby, VIC, Australia) software.

7. Synergism calculations

Dose range cell viability data were analysed using the software ClacuSyn (Biosoft, Cambridge, UK) to generate Combination Index (CI) values, wherein 0.5<CI<1 was considered moderately synergistic, 0<CI<0.5 strongly synergistic and CI>1 antagonistic.[19] SAHA was combined with PENAO at a ratio of 1:1.5, whilst LBH589 was combined with PENAO at a ratio of 1:300. Correlation coefficients (r) were used to confirm the strength of the median-effect relationships, wherein r>0.95 represented a strong relationship.

8. Cell proliferation assays

Cell proliferation was measured using the Cell Proliferation ELISA, BrdU colorimetric assay (11647229001, Roche) according to the manufacturer's instructions. Changes in cell proliferation were calculated from the absorbance readings at 370 nm (490 nm reference wavelength) on the Benchmark Plus microplate reader (Bio-Rad).

9. Western blot analysis

Cell pellets were lysed in radio-immunoprecipitation assay (RIPA) lysis buffer freshly supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations of the whole cell lysates were determined using the Peirce BCA Protein Analysis Kit (Thermo Scientific, IL, USA) according to the manufacturer's instructions. 30 µg whole protein lysates alongside Precision Plus Protein™ Dual Color Standards (Bio-Rad) were resolved on 10-14% Tris-HCl Criterion gels (Bio-Rad, Gladesville, NSW, Australia). Nitrocellulose membranes (GE Healthcare, Rydalmere, NSW, Australia) were blocked with 10% (wt/vol) nonfat dry milk in Tris-buffered saline with Tween-20 (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20), then incubated over night at 4°C with the following primary antibodies; SLC25A5 (1:1000; Cell Signalling Technologies), MYCN (1:1000; Santa Cruz), β-actin (1:2000; SigmaAldrich), Vinculin (1:2000; abcam), p53 (1:500; Santa Cruz), p21 (1:1000; Cell Signalling Technologies), BAX (1:1000; Cell Signalling Technologies), CHIP (1:1000; Cell Signalling Technologies), MDM2 (1:1000; Cell Signalling Technologies), HSP90 (1:1000; Cell Signalling Technologies) and HDAC6 (1:1000; Cell Signalling Technologies). β-actin and Vinculin were utilised as loading/housekeeper controls. Appropriate horseradish peroxidase conjugated secondary antibodies (anti-mouse or anti-rabbit 1:2000; Thermo Scientific, Surrey Hills, VIC, Australia) were diluted in Tris-buffered saline with 0.1% Tween-20 and membranes were probed on room temperature for 2 hours. Immunoblots were incubated with Super Signal West Pico Chemiluminescence reagents (Pierce) and were visualised using either photosensitive film (GE Healthcare) or the ChemiDoc™ Touch Imaging System (Bio-Rad). Densitometry was conducted using the Quantity One software (Bio-Rad). All samples were normalised to their respective loading control and then to experimental controls.

10. Cycloheximide chase assays

Kelly NB cells were subject to drug treatments for 24 hours and then treated with 100ug/ml cycloheximide (Sigma-Aldrich) for up to 0, 15, 30, 60, 90 and 180 minutes. Cells were immediately lysed at treatment endpoints and protein lysates were analysed by western blot, as described above, to measure changes in TP53 protein stability. Densitometry was conducted, as described above, and each sample was normalised to their respective loading control and then to the 0-minute timepoint for each treatment condition. The half-life (t1/2) was determined by first fitting a one-phase exponential decay curve (least squares regression) to the average values from three biological replicates using the GraphPad Prism 7 (Applied BioSystems, Scoresby, VIC, Australia) software and then extrapolating the time at which 50% of TP53 protein remained.

11. Glutathione quantitation assays

Following 24 hours of treatments NB cell lines were lysed in 150 µl cold 10 mM hydrochloric acid (HCL) (Sigma), followed by two freeze thaw cycles (-80^{0}C for 2 hours and 37^{0}C water bath for 5 minutes). 120 µl of the sample was taken for deproteination whilst the remaining 30 µL was used for a BCA assay as described above to quantify the amount of protein in the cell lysate. Cell lysates were deproteinated by adding 30 µl of cold 6.5% 5-sulfocylic acid (5-SSA) (Sigma) to the 120 µl sample. Samples were pipette mixed, incubated on ice for 10 min, and spun in a centrifuge for 15 min at 5000 rpm whilst at 4^{0}C. The supernatant was taken and used for downstream glutathione quantitation. Both oxidised (GSSG) and reduced (GSH) were quantified using the

Glutathione Assay Kit (#703002, Cayman) according to the manufacturer's instructions, additionally utilising 2-vinylpyridine (2-VP) (Sigma) to derivatise GSH and prevent it from undergoing oxidation, for accurate quantitation. Absorbance was measured using a Benchmark Plus Microplate Reader (Bio-Rad) at 405nm, using the endpoint method, in which a reading was taken at the 0-minute timepoint and then again at the 25-minute assay time point. Following background subtraction glutathione concentrations (measured as GSSG µM) were determined from a standard curve and then normalised to protein levels within each sample, previously determined by the BCA assay.

12. Histology and immunohistochemistry of tissues

Tissues were fixed in 10% neutral buffered formalin for 24 hours followed by 80% ethanol for 5 days. Fixed tissues were then paraffin embedded and sectioned onto glass slides. Slides were stained using haematoxylin-eosin staining solution (Sigma). Immunohistochemical stains included Ki67 (1:150, AB9260, Merck-Millipore), Cleaved Caspase-3 (Asp175) (1:100, Clone 5A1E, 9664, Cell Signalling Technologies) and were counterstained using Mayer's haematoxylin solution (Sigma). Images were captured using an Olympus BX53 light microscope and DP-73 camera with cellSens software.

13. Flow cytometric assays

To detect apoptotic cell death in treated cells, cells were stained with Annexin-V and 7-AAD (559763, BD Pharmingen) according to manufacturer instructions. To measure mitochondrial depolarisation in treated cells, cells were stained using JC-1 (M34152,

Life Technologies) according to manufacturer instructions. Cell cycle phases and apoptosis among treated cells were estimated using Propidium Iodide (PI) (556463, BD Pharmingen) staining as previously described.[20] DNA fragmentation was assessed using the Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay (12156792910, Roche) according to the manufacturer's instructions. To determine cellular and mitochondrial reactive oxygen species (ROS) levels cells were stained with dihydroethidium (D7008, Sigma-Aldrich) or MitoSox™ Red (M36008, Invitrogen) according to manufacturer instructions. Samples were analysed by flow cytometry using the FACSCalibur (BD Biosciences, Macquarie Park, NSW, AU) and the data was analysed using the FlowJo software (Ashland, OR, USA).

14. Microarray analysis

SH-SY5Y cells were treated for a period of 8 hours followed by total RNA extraction (12183018A, Invitrogen) for subsequent microarray profiling on the Affymetrix GeneChip Human Gene 2.0 ST Array (#902113, Applied Biosystems) according to manufacturer instructions. Packages built in R/RStudio were then used to process raw data. Raw probe intensity values were first subject to robust multi-array averaging (RMA) using the oligo package.[21] Probes were annotated with ensemble gene symbols, names and ids using "hugene20sttranscriptcluster.db" annotations alongside the biomaRt package.[22, 23] Genes with multiple probes were collapsed using interquartile range. Differential gene expression between each set of conditions were then calculated using linear regression models in the limma package, p-values were corrected for multiple comparisons using the Benjamini-Hochberg method.[24] Differentially expressed genes between combination and DMSO conditions were

ranked in decreasing order by fold change (log2) and were subsequently used as input into Gene Set Enrichment Analyses (GSEA)[25] using the *fgsea* R package,[26] with HALLMARK gene sets from the molecular signatures database (MSigDb).[2, 3]

Supplemental Tables

Comparison	Animal Number (TH-MYCN$^{+/+}$)	Median Survival (Days)	Log-rank (Mantel-Cox) Test (P-Value)
Saline - PENAO 20mg/kg I.V.	12 – 4	2.5 – 5.5 = +3	0.0056
Saline - PENAO 50mg/kg I.V.	12 – 2	2.5 – 6 = +3.5	0.0216
Saline – PENAO 10 mg/kg 0.G.	12 – 3	2.5 – 3 = +0.5	0.8821
Saline – CDDP 2mg/kg I.P.	12 - 16	2.5 – 28 = +25.5	<0.0001
Saline – CPA 18mg/kg I.P.	12 - 11	2.5 – 35 = +32.5	<0.0001
Saline – VCR 0.2mg/kg I.P.	12 - 16	2.5 – 13.5 = +11	<0.0001
Saline – VP16 6mg/kg I.P.	12 - 10	2.5 – 11 = +8.5	<0.0001
Saline - CDDP 2mg/kg I.P. + PENAO 20mg/kg I.V.	12 - 6	2.5 – 31 = +28.5	0.0001
Saline - CDDP 2mg/kg I.P. + PENAO 10mg/kg 0.G.	12 - 10	2.5 – 36.5 = +34	<0.0001
Saline – CPA 18mg/kg I.P. + PENAO 10mg/kg 0.G.	12 - 10	2.5 – 40 = +37.5	<0.0001
Saline – CPA 18mg/kg I.P. + PENAO 0.5mg/kg S.C.	12 – 6	2.5 – 40.5 = +37.5	0.0001
Saline – VCR 0.2mg/kg I.P. + PENAO 20mg/kg I.V.	12 - 10	2.5 – 10 = +7.5	0.0001
Saline – VCR 0.2mg/kg I.P. + PENAO 10mg/kg 0.G.	12 - 14	2.5 – 11.5 = +9	0.0001
Saline – VP16 6mg/kg I.P. + PENAO 10mg/kg 0.G.	12 - 10	2.5 – 10 = +7.5	<0.0001
CDDP 2mg/kg I.P. - CDDP 2mg/kg I.P. + PENAO 20mg/kg I.V.	16 - 6	28 – 31 = +3	0.3742
CDDP 2mg/kg I.P. - CDDP 2mg/kg I.P. + PENAO 10mg/kg 0.G.	16 - 10	28 – 36.5 = +8.5	0.1934
CPA 18mg/kg I.P. - CPA 18mg/kg I.P. + PENAO 10mg/kg 0.G.	11 - 10	35 – 40 = +5	0.3074
VCR 0.2mg/kg I.P. - VCR 0.2mg/kg I.P. + PENAO 20mg/kg I.V.	16 - 10	13.5 – 10 = -3.5	0.7916
VCR 0.2mg/kg I.P. - VCR 0.2mg/kg I.P. + PENAO 10mg/kg 0.G.	16 - 14	13.5 – 11.5 = -2	0.4478
VP16 6mg/kg I.P. - VP16 6mg/kg I.P. + PENAO 10mg/kg 0.G.	10 – 10	11 – 10 = -1	0.3630
PENAO 20mg/kg I.V. - CDDP 2mg/kg I.P. + PENAO 20mg/kg I.V.	4 - 6	5.5 – 31 = +25.5	0.0011
PENAO 10mg/kg 0.G.- CDDP 2mg/kg I.P. + PENAO 10mg/kg 0.G.	3 - 10	3 – 36.5 = +33.5	<0.0001
PENAO 10mg/kg 0.G. - CPA 18mg/kg I.P. + PENAO 10mg/kg 0.G.	3 - 10	3 – 40 = +37	<0.0001
PENAO 20mg/kg I.V.- VCR 0.2mg/kg I.P. + PENAO 20mg/kg I.V.	4 - 10	5.5 – 10 = +4.5	0.0057
PENAO 10mg/kg 0.G. - VCR 0.2mg/kg I.P. + PENAO 10mg/kg 0.G.	3 - 14	3 – 11.5 = +8.5	<0.0001
PENAO 10mg/kg 0.G. - VP16 6mg/kg I.P. + PENAO 10mg/kg 0.G.	3 - 10	3 – 10 = +7	<0.0001

Table S1.

Log-rank comparisons within each animal study for PENAO treatments with chemotherapeutic agents. For each study in Th-MYCN$^{+/+}$ mice, log-rank statistical comparisons were made between each of the treatment arms involving PENAO, cyclophosphamide (CPA), cisplatin (CDDP), vincristine (VCR) and etoposide (VP16) or their combination. For each comparison, the number of mice in each treatment arm is provided, as well as the difference in median survival time between arms, positive values indicate improved median survival whereas negative values represent poorer median survival. P-values from the log-rank test between individual treatment arms are provided, significant comparisons ($p<0.05$) are shaded.

Cell Line	**Cell Type**	***MYCN* status**	***TP53* status**	***CDKN2A* status**	***MDM2* status**	**PENAO IC50 (µM)**
SH-SY5Y	NB	MNA	WT	WT	WT	0.85
CHP-134	NB	MA	WT	WT	WT	0.94
IMR-32	NB	MA	WT	WT	WT	1.26
SK-N-FI	NB	MNA	737T>G (p.M246R)	WT	WT	2.11
SK-N-DZ	NB	MA	328C>A (p.R110L)	WT	WT	3.56
SK-N-BE(2)-C	NB	MA	404G>T (p.C135F)	WT	WT	3.61
SH-EP	NB	MNA	WT	Homozygous deletion	WT	4.80
SK-N-AS	NB	MNA	Homozygous deletion of exons 10–11	Heterozygous deletion	WT	4.83
Kelly	NB	MA	529C>T (p.P177T)	WT	WT	6.18
LA-N-1	NB	MA	546C>A (p.C182X)	WT	WT	6.74
MRC-5	FB	MNA	WT	WT	WT	12.10
WI-38	FB	MNA	WT	WT	WT	12.88

Table S2. Molecular features of cell lines used in this study. For each cell line; cell type, *MYCN* amplification status, *TP53* mutational status, *CDKN2A* (p14ARF) mutational status, *MDM2* amplification status, associated references and PENAO IC_{50} are provided. MNA= *MYCN* non-amplified, MA = *MYCN* amplified, WT = wild type.

	Cell Line	Combination Index (CI)			Dm	m	r
		IC_{50}	IC_{75}	IC_{90}			
SAHA + PENAO (1:1.5)	SK-N-BE(2)-C	0.78	0.40	0.20	0.60	2.32	0.95
	Kelly	0.85	0.79	0.76	0.66	2.20	0.99
	SH-SY5Y	0.96	0.83	0.73	0.43	2.80	0.98
	CHP-134	0.69	0.50	0.38	0.48	2.81	0.99
	LA-N-1	0.84	0.89	0.95	0.86	2.56	0.98
	SK-N-AS	0.98	1.20	1.48	1.12	1.25	0.98
LBH589 + PENAO (1:300)	SK-N-BE(2)-C	0.96	0.72	0.56	4.44	2.22	0.97
	Kelly	1.02	0.88	0.77	3.58	2.07	0.98
	SH-SY5Y	0.99	0.82	0.69	2.22	2.56	0.97
	CHP-134	0.69	0.48	0.34	2.51	3.09	0.98
SAHA + GSAO (1:5)	SK-N-BE(2)-C	0.56	0.35	0.23	1.86	1.48	0.98
	SH-SY5Y	0.79	0.82	0.86	0.90	1.70	0.99

Table S3. Synergism metrics derived using the Chou-Talalay method. A combination index derived from the Chou-Talalay method (CI) < 1 is considered synergistic, CI = 1 additive and CI > 1 antagonistic. A CI value is provided for different inhibitory concentration of the combination ($IC_{50/75/90}$). Median-effect dose (Dm) represents potency and m indicates how sigmodal a dose-response curve is, r represents the Pearson correlation coefficient of the dose/median-effect relationship.

Gene Symbol	Gene Name	log2 Fold Change	P-Value	Adjusted P-Value
MIR7-3HG	MIR7-3 host gene	4.68	6.09E-06	0.014
EGR1	early growth response 1	3.77	0.00020	0.035
CFAP43	cilia and flagella associated protein 43	3.69	2.71E-05	0.021
DDIT3	DNA damage inducible transcript 3	3.58	1.83E-05	0.019
ANOS1	anosmin 1	3.36	5.28E-06	0.014
CCDC110	coiled-coil domain containing 110	3.26	1.23E-06	0.013
ULBP1	UL16 binding protein 1	3.24	7.02E-05	0.027
SERPINI1	serpin family I member 1	3.15	7.86E-07	0.013
CASC1	cancer susceptibility candidate 1	2.92	1.98E-07	0.010
PCP4L1	Purkinje cell protein 4 like 1	2.85	2.7E-06	0.014
IQUB	IQ motif and ubiquitin domain containing	2.68	1.96E-06	0.014
GPR155	G protein-coupled receptor 155	2.62	2.22E-06	0.014
SNORD14E	small nucleolar RNA, C/D box 14E	2.61	0.00023	0.036
NEAT1	nuclear paraspeckle assembly transcript 1 (non-protein coding)	2.58	0.00016	0.032
FAM126A	family with sequence similarity 126 member A	2.56	9.92E-06	0.017
CFAP54	cilia and flagella associated protein 54	2.52	1.25E-05	0.018
GAS6-AS2	GAS6 antisense RNA 2 (head to head)	2.44	2.68E-05	0.021
CFAP58-AS1	CFAP58 antisense RNA 1 (head to head)	2.42	5.59E-06	0.014
LACTB	lactamase beta	2.41	2.95E-06	0.014
HEPACAM2	HEPACAM family member 2	2.29	2E-06	0.014
CLMP	CXADR like membrane protein	-1.83	2.49E-05	0.020
MIR431	microRNA 431	-1.84	0.00038	0.044
PBDC1	polysaccharide biosynthesis domain containing 1	-1.86	2.14E-05	0.020
MYC	v-myc avian myelocytomatosis viral oncogene homolog	-1.87	0.00040	0.045
PCDHB14	protocadherin beta 14	-1.89	6.47E-06	0.014
NFIC	nuclear factor I C	-1.92	1.16E-05	0.017
SLC6A2	solute carrier family 6 member 2	-1.98	1.41E-05	0.018
FAIM	Fas apoptotic inhibitory molecule	-1.98	1.03E-05	0.017
ASCL1	achaete-scute family bHLH transcription factor 1	-2.05	8.55E-05	0.029
LINC00599	long intergenic non-protein coding RNA 599	-2.08	0.00011	0.030
DKK1	dickkopf WNT signaling pathway inhibitor 1	-2.14	8.02E-05	0.028
ST8SIA3	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 3	-2.15	2.38E-05	0.020
RTL1	retrotransposon-like 1	-2.22	3.94E-05	0.023
MIR433	microRNA 433	-2.24	0.00049	0.049
PHOX2B	paired like homeobox 2b	-2.35	4.18E-06	0.014

LINC00682	long intergenic non-protein coding RNA 682	-2.49	6.34E-05	0.027
TFAP2B	transcription factor AP-2 beta	-2.70	3.89E-07	0.010
MIR136	microRNA 136	-2.79	1.78E-05	0.019
MIR127	microRNA 127	-2.79	6.2E-05	0.027
MIR432	microRNA 432	-3.19	0.00015	0.032

Table S4. Differentially expressed genes after SAHA and PENAO combination therapy. List of top 20 differentially expressed genes either up or down-regulated by the combination treatment in SH-SY5Y cells after 8 hours, excluding uncharacterised genes/loci. The log2 fold change (between treatment and control conditions), p-value and Benjamini-Hochberg (BH) adjusted p-value are provided for each gene.

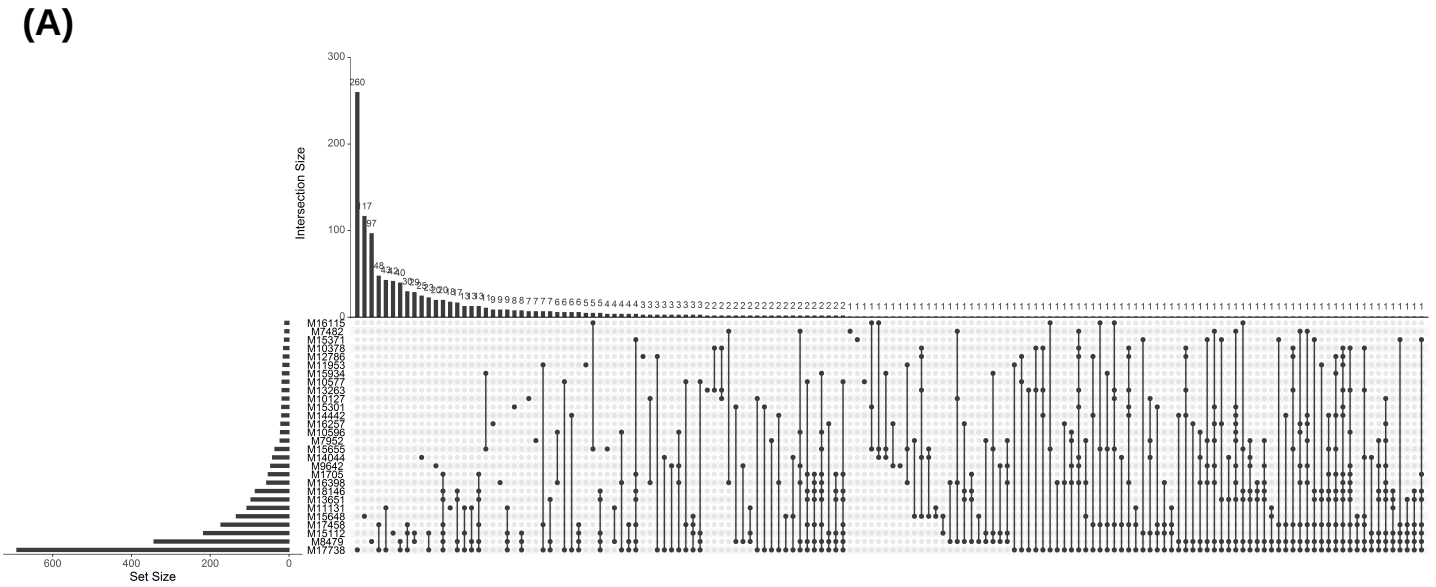
Pathway	NES	P-Value	Adjusted P-Value
HALLMARK_P53_PATHWAY	2.29	0.0015	0.010
HALLMARK_HEME_METABOLISM	2.23	0.0015	0.010
HALLMARK_TNFA_SIGNALING_VIA_NFKB	2.11	0.0015	0.010
HALLMARK_APOPTOSIS	1.89	0.0016	0.010
HALLMARK_CHOLESTEROL_HOMEOSTASIS	1.84	0.0017	0.010
HALLMARK_HYPOXIA	1.83	0.0015	0.010
HALLMARK_UV_RESPONSE_UP	1.82	0.0016	0.010
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	1.78	0.0016	0.010
HALLMARK_ANDROGEN_RESPONSE	1.78	0.0033	0.010
HALLMARK_MTORC1_SIGNALING	1.63	0.0030	0.010
HALLMARK_PROTEIN_SECRETION	1.57	0.0033	0.010
HALLMARK_ESTROGEN_RESPONSE_EARLY	1.55	0.0030	0.010
HALLMARK_ADIPOGENESIS	1.46	0.0091	0.025
HALLMARK_XENOBIOTIC_METABOLISM	1.46	0.0074	0.022
HALLMARK_MYC_TARGETS_V2	-2.28	0.0024	0.010
HALLMARK_G2M_CHECKPOINT	-2.31	0.0030	0.010
HALLMARK_MYC_TARGETS_V1	-2.34	0.0030	0.010
HALLMARK_E2F_TARGETS	-2.66	0.0029	0.010

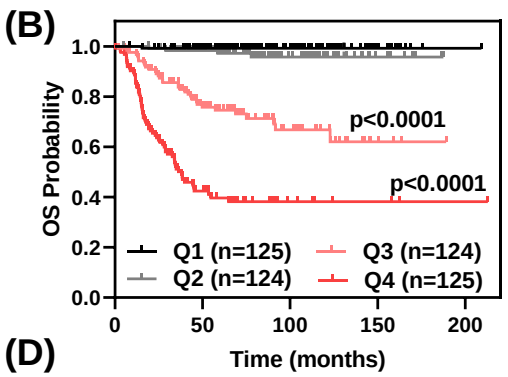
Table S5. GSEA analysis of differentially expressed genes in SH-SY5Y cells after combination treatment using the HALLMARK database. Pathways that were significantly enriched (adj. p-value < 0.05) are displayed. The normalised enrichment scores (NES) are provided for each pathway in each cell line, higher scores indicate higher enrichment of the pathway.

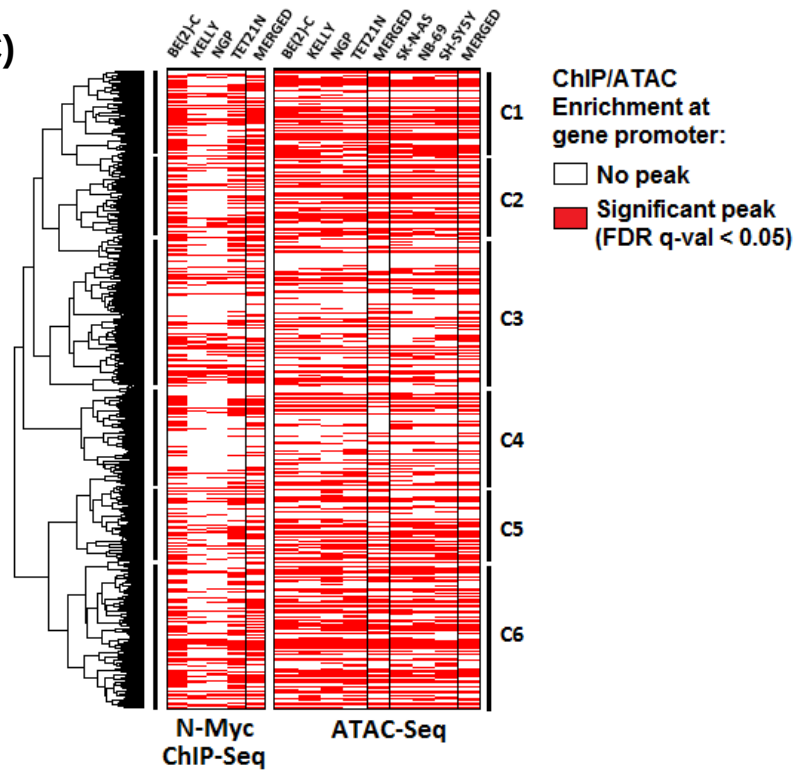
Model	Comparison	Animal Number	Median Survival (Days)	Log-rank Test (P-Value)
Kelly Balb/c Nude Xenografts	Saline – SAHA	9 – 7	15 – 11 = -4	0.0032
	Saline – PENAO	9 - 6	15 – 12 = -3	0.75
	Saline – Combination	9 - 9	15 – 30 = +15	<0.0001
	SAHA- Combination	7 – 9	11 – 30 = +19	<0.0001
	PENAO - Combination	6 – 9	12 – 30 = +18	<0.0001
SK-N-BE(2)-C Balb/c Nude Xenografts	Saline – SAHA	7 – 10	8 – 8.5 = +0.5	0.85
	Saline – PENAO	7 - 9	8 – 9 = +1	0.073
	Saline – Combination	7 - 10	8 – 17 = +9	<0.0001
	SAHA - Combination	10 - 10	8.5 – 17 = +8.5	<0.0001
	PENAO - Combination	9 - 10	9 – 17 = +8	0.0007
Th-MYCN$^{+/+}$ Mice	Saline – SAHA	8 – 10	16.5 – 23 = +6.5	0.43
	Saline – PENAO	8 - 9	16.5 – 21 = +4.5	0.31
	Saline – Combination	8 - 10	16.5 – 27 = +10.5	0.0028
	SAHA - Combination	10 - 10	23 – 27 = +4	0.0058
	PENAO - Combination	9 - 10	21 – 27 = +6	0.020

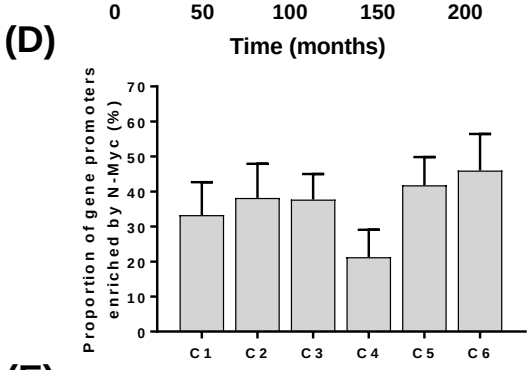
Table S6. Log-rank comparisons within each animal study for SAHA and PENAO treatments. For each study (Kelly, SK-N-BE(2)-C, and Th-MYCN$^{+/+}$), log-rank statistical comparisons were made between each of the treatment arms; Saline control, 17.5 mg/kg/day SAHA, 20 mg/kg/day PENAO and their combination. For each comparison, the number of mice in each treatment arm is provided, as well as the difference in median survival time between arms, positive values indicate improved median survival whereas negative values represent poorer median survival. P-values from the log-rank test between individual treatment arms are provided, significant comparisons ($p<0.05$) are shaded.

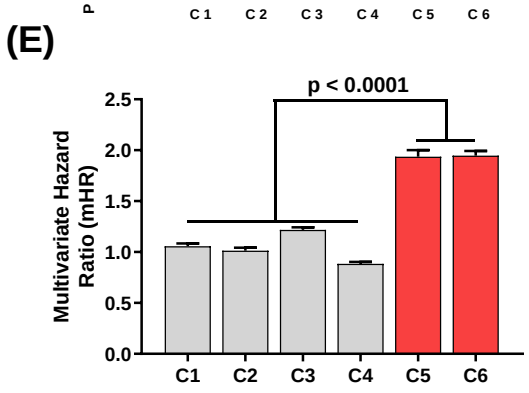
Supplementary Figure 1



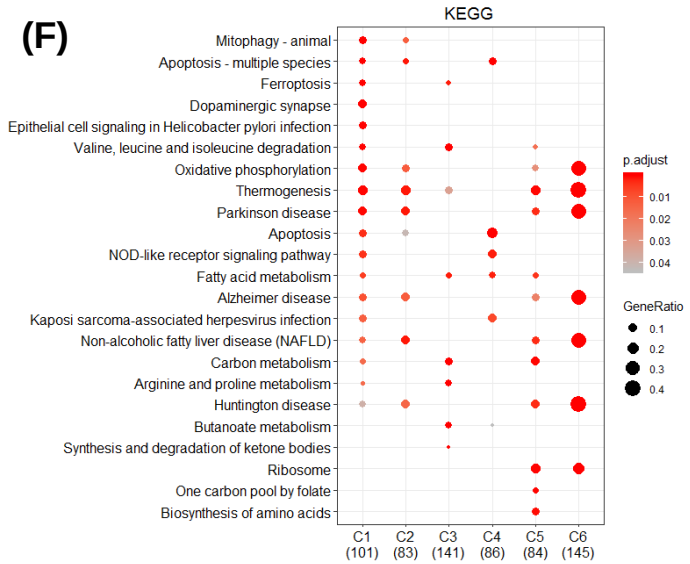


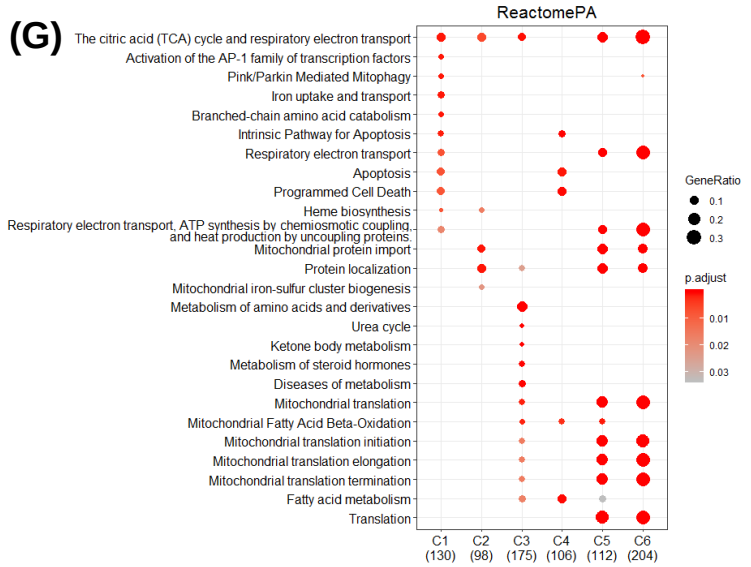


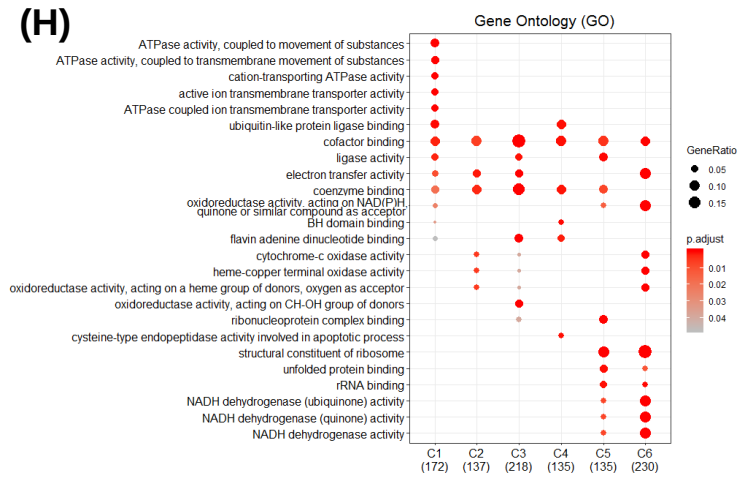




Supplementary Figure 1 cont







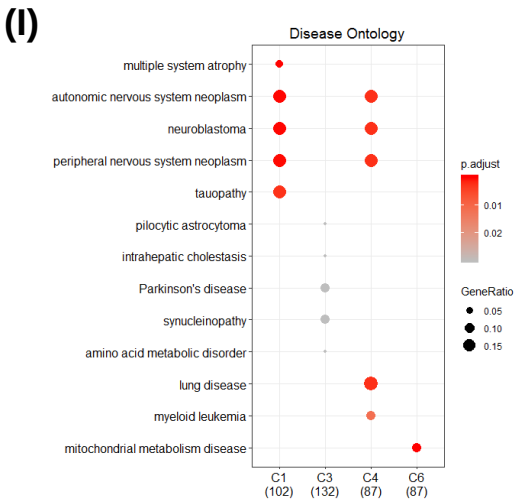
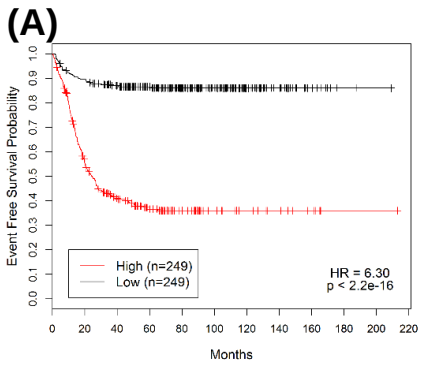
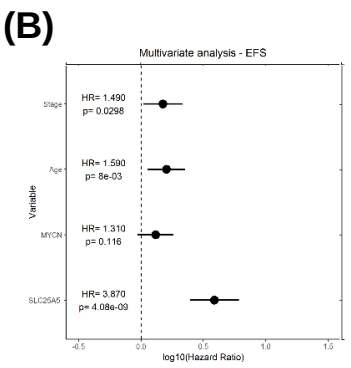
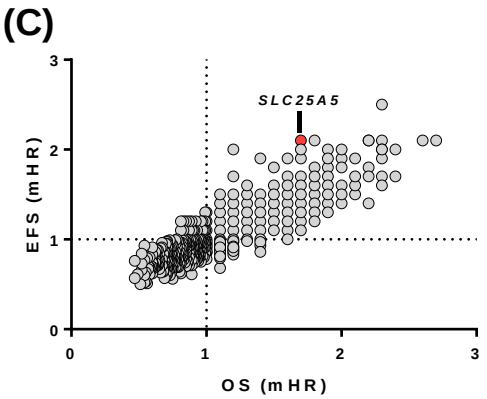


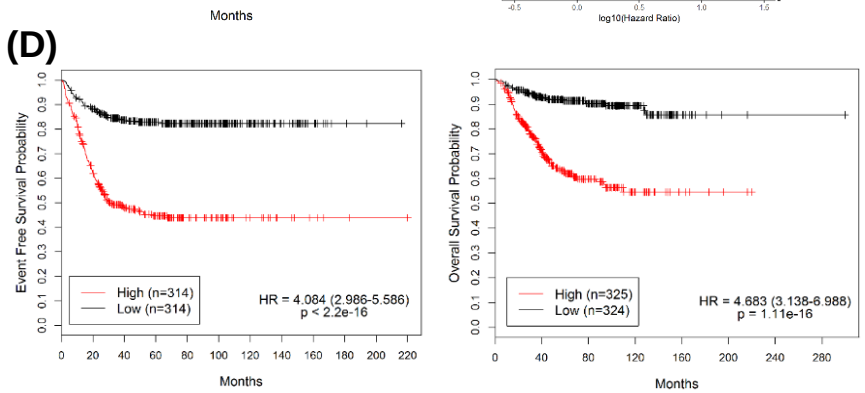
Figure S1.
(A) Intersection plot illustrating the number of intersections between each gene set (n=28) and their contributions to the final pool of mitochondrial associated genes (MAG) (n=1148). (B) Patients from the SEQC NB cohort (n=498) were subdivided (quartiles; Q1-4) based on MAG scores and their survival probability was plotted on a Kaplan-Meier curve. (C) Heatmap displaying significant enrichments (FDR q-value < 0.05) of N-Myc at gene promoters (ChIP-Seq; GSE80151) in 4 MYCN amplified NB cell lines. Significant accessibility (FDR q-value < 0.05) of gene promoters (ATAC-Seq; GSE80152/GSE101294) are also indicated in 7 NB cell lines. 'Merged' columns represents an analysis where all cell lines were considered as pseudo-replicates before peak calling. (D) The average proportion of gene promoters significantly enriched by N-Myc in each cluster with error bars representing the standard error of the mean across all 4 NB cell lines (where a promoter had significant enrichment of N-Myc, FDR q-value < 0.05, -1000/+100bp from TSS). (E) Mean multivariate hazard ratios (mHR) of each cluster are shown with the error bars representing the standard error of the mean of mHRs within a cluster. Cluster-specific gene set enrichment of C1-C6 genes using (F) KEGG, (G) ReactomePA (H) Gene Ontology (GO) and (I) Disease Ontology databases, only significantly enriched gene sets are displayed (adjusted p-value < 0.05), with adjusted p-values shown via colour scale and gene ratio (number of genes present in cluster/total genes in gene set) indicated by dot size. Gene set names are provided as row labels while column labels reference the cluster and the number of genes present in enriched gene sets.

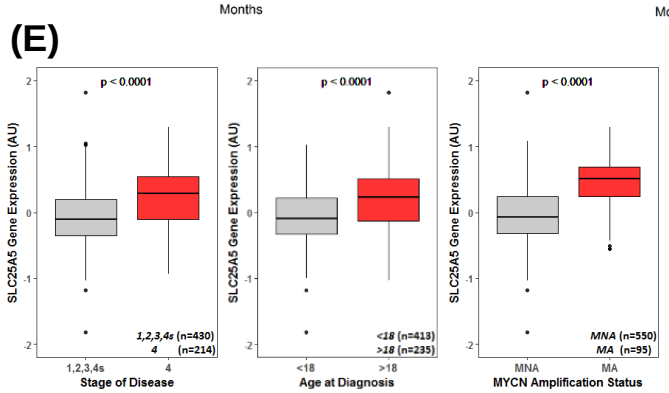
Supplementary Figure 2

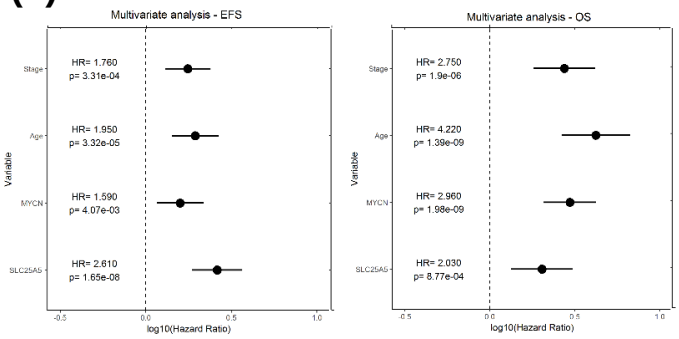


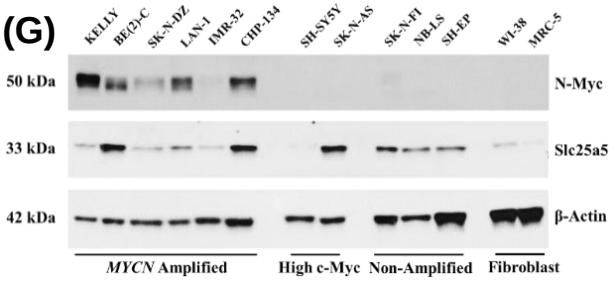


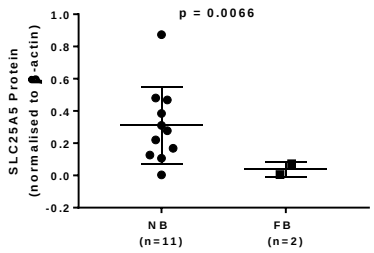












Supplementary Figure 2

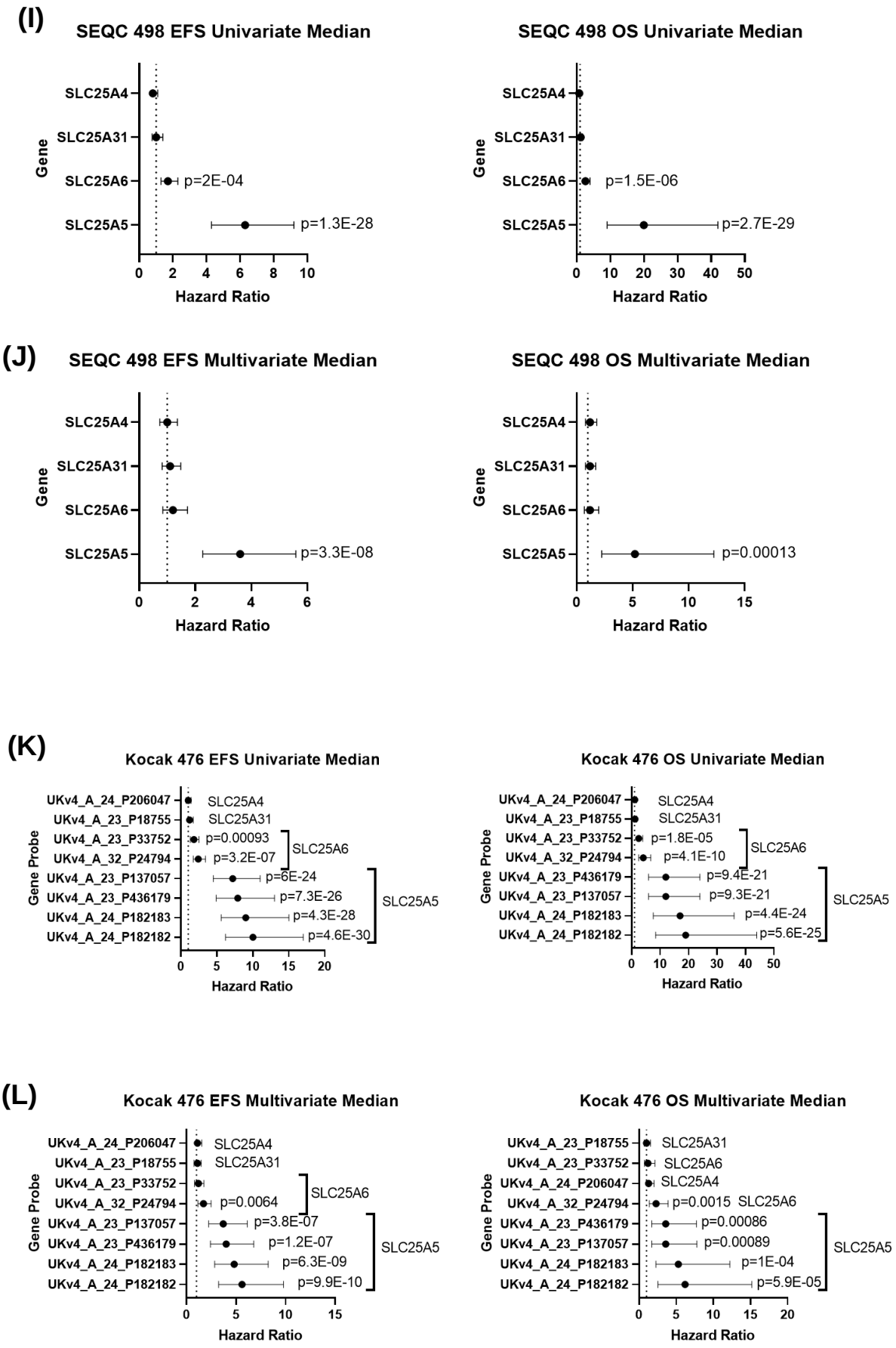
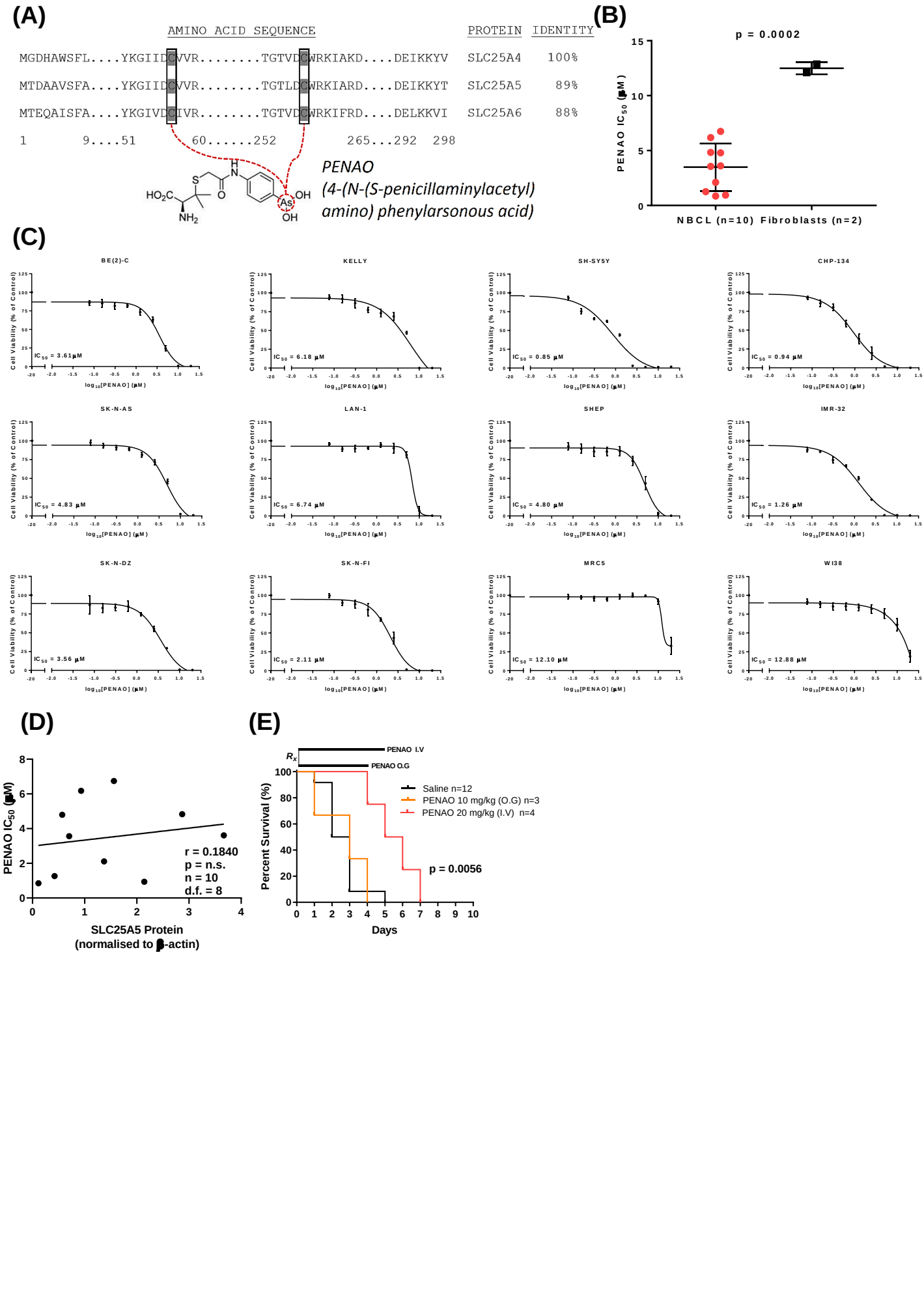


Figure S2.
(A) Kaplan-Meier survival curves showing the EFS probability of patients in the SEQC NB cohort (n=498) when dichotomised by median SLC25A5 gene expression (RNA-Seq). Hazard Ratio's (HR) and log-rank p-values are presented from a univariate CoxPH model. (B) Summaries of the EFS multivariate CoxPH models with SLC25A5 gene expression against classical prognostic predictors. (C) The median gene expression (microarray) of each MAG (n=1148) was considered as a prognostic variable alongside classical predictors of NB patient outcome (MYCN Amplification Status, Stage of Disease & Age) in iterative multivariate CoxPH models using the KOCAK NB patient cohort (n=649). The dot plot represents the multivariate hazards ratio's from the CoxPH models for each gene with regard to event free survival (EFS)/overall survival (OS) probability. (D) Kaplan-Meier survival curves showing the EFS & OS probability of patients in the KOCAK NB cohort (n=649) when dichotomised by median SLC25A5 gene expression (microarray). Hazard Ratio's (HR) and log-rank p-values are presented from a univariate CoxPH model. (E) Box plots of SLC25A5 gene expression against classical prognostic predictors of NB patient outcome. (F) Summaries of the EFS & OS multivariate CoxPH models with SLC25A5 gene expression against classical prognostic predictors in the KOCAK cohort. (G) Western blot of NB and fibroblast (FB) cell lysates followed by staining with anti-N-Myc, anti-SLC25A5 or anti-β-Actin, samples are grouped by MYCN amplification status. (H) Densitometry analysis of western blot in G, with protein levels of SLC25A5 being normalised to a β-Actin loading control, cell lines are grouped into either neuroblastoma cell lines (NB) or fibroblast cell lines (FB). (I-J) Summaries of the EFS and OS univariate and multivariate CoxPH models with SLC25A4, SLC25A5, SLC25A6, and SLC25A31 in the SEQC Cohort GSE62564. (K-L) Microarray analysis (of all probes) showing the EFS and OS univariate and multivariate CoxPH models of the four ANT family members using the KOCAK cohort GSE45547. Only significant analyses ($p<0.05$) are annotated with a p-value.

Supplementary Figure 3



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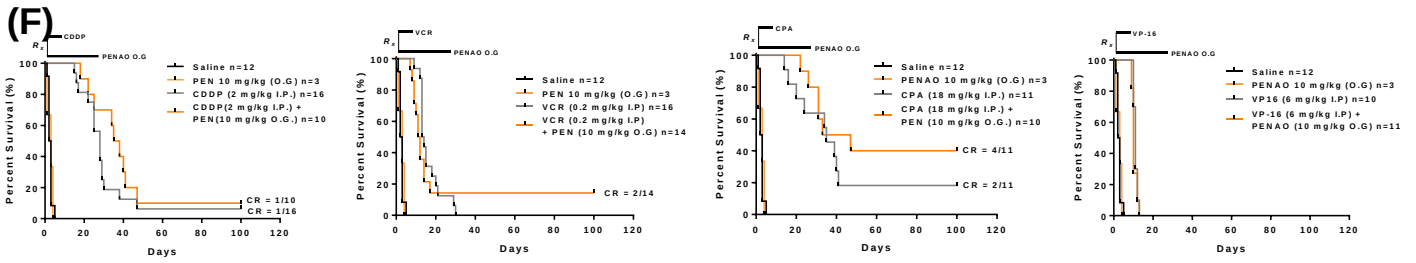
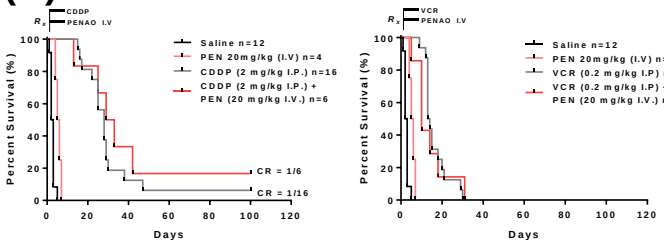
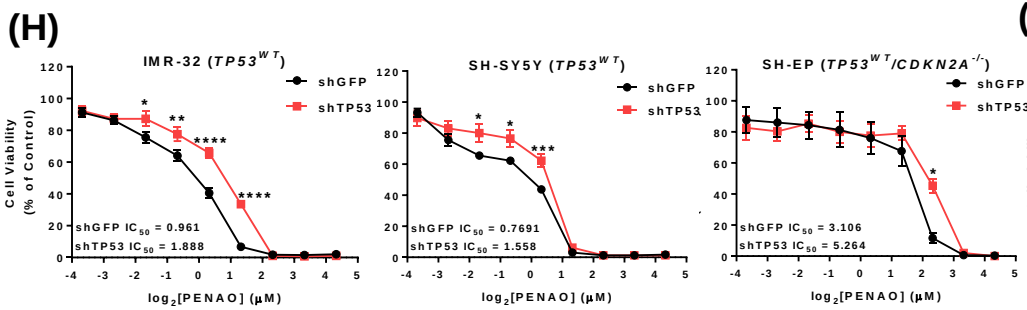
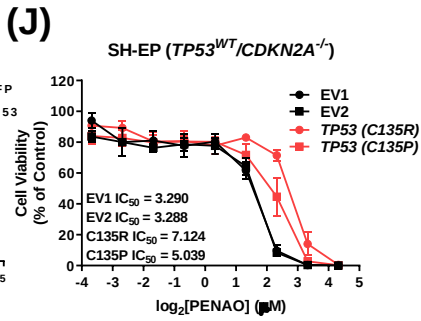
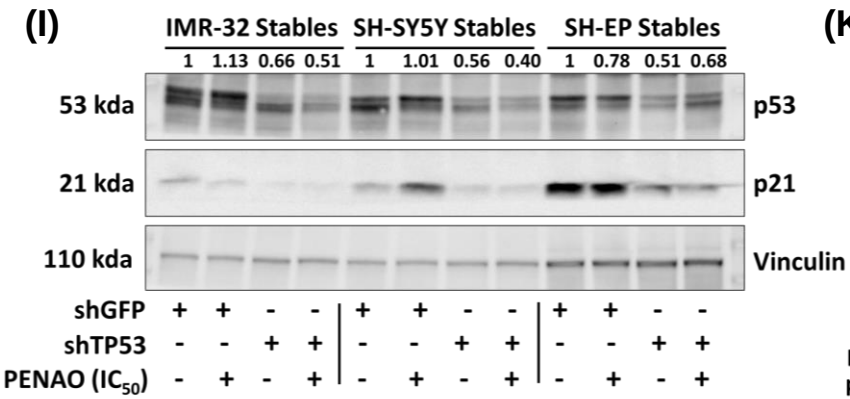










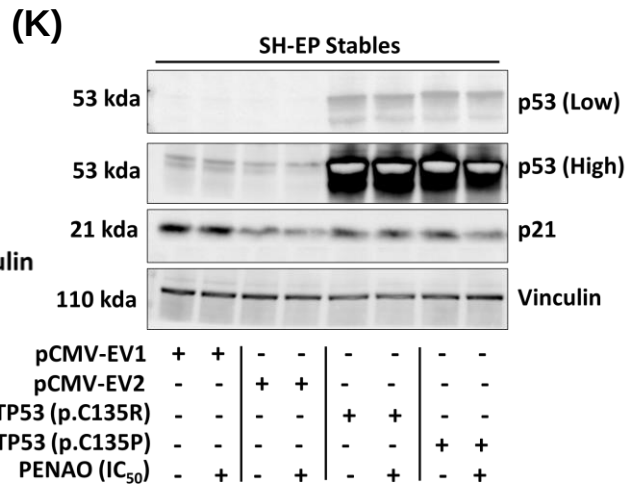
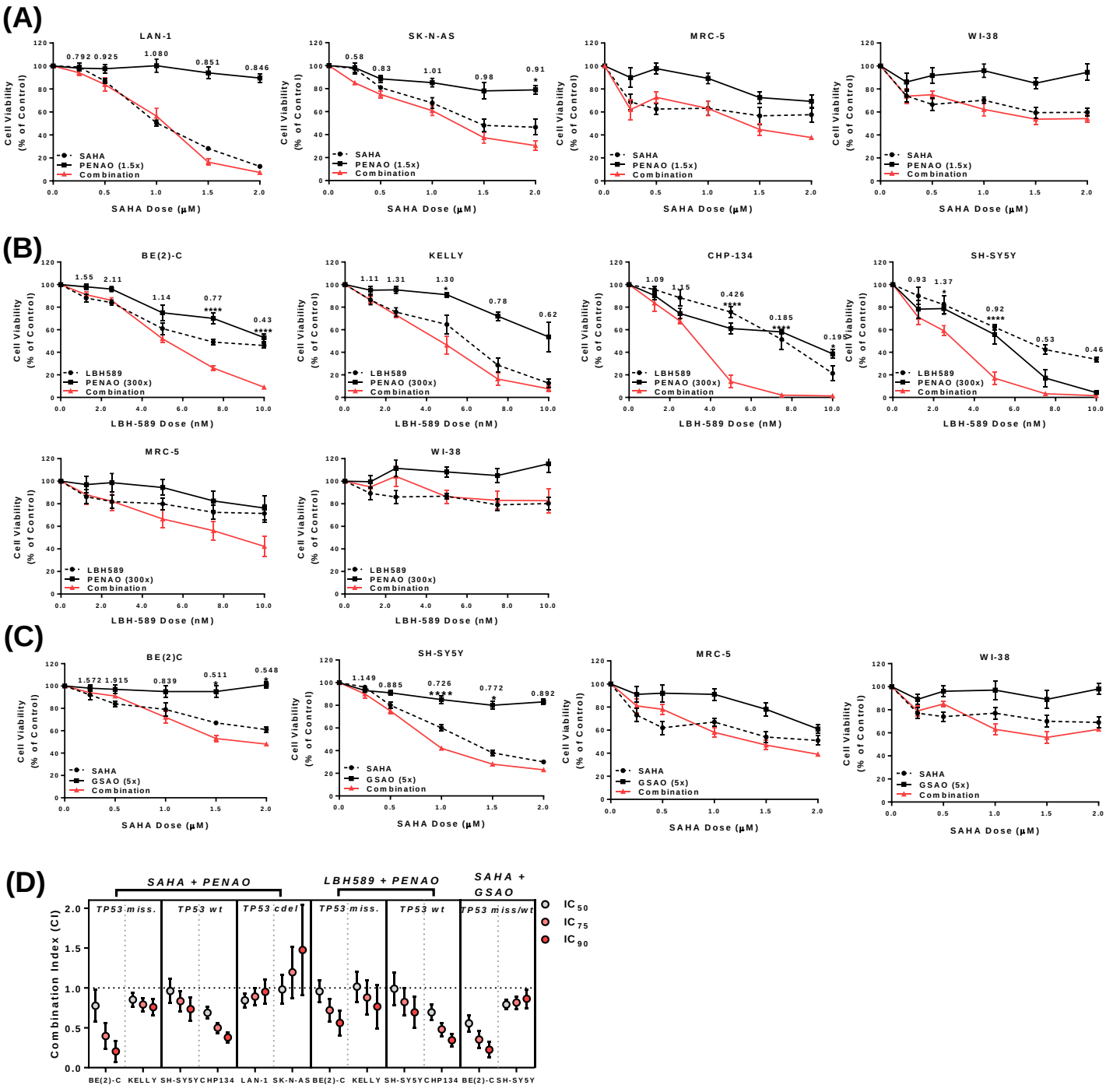


Figure S3.
(A) Representation of amino acid sequences of SLC25A4, SLC25A5 & SLC25A6, amino acid homology/identity is displayed as a percentage. The conserved cysteine residues bound by PENAO (Cys57 & Cys257) for each protein is marked. (B) Comparison of PENAO IC50's in 10 NB cell lines (NBCLs) and 2 Fibroblasts cell lines.
(C) Individual PENAO cytotoxicity curves for each cell line, x-axis represents PENAO dose (0-20 μM) and y-axis; Cell Viability expressed as a percentage of the vehicle control. IC50's are displayed on each graph. (D) Pearson correlation between SLC25A5 protein levels (Fig. S2I) and PENAO IC50. Statistics are reported in the plot. (E) Kaplan-Meier survival curve of Th-MYCN+/+ mice following the administration of PENAO through with either a Saline control, 10 mg/kg PENAO administered by oral gavage (O.G.) or 20 mg/kg PENAO administered by intravenous injections (I.V.). Treatment periods are indicated at the top of the curves. P-value represents log-rank test between 20 mg/kg PENAO I.V. group vs Saline control group. (F) Kaplan-Meier survival curves of Th-MYCN+/+ mice treated with backbone chemotherapy (Cisplatin 2 mg/kg; CDDP, Vincristine 0.2 mg/kg; VCR, Cyclophosphamide 18 mg/kg; CPA and Etoposide 6 mg/kg (VP-16)) alongside PENAO (O.G. 10 mg/kg) administered orally. All chemotherapy backbones were administered by intraperitoneal injections (I.P.). Treatment periods are indicated at the top of the curves. Complete remissions (CR) are annotated. (G) Kaplan-Meier survival curves of Th-MYCN+/+ mice treated with backbone chemotherapy (CDDP, VCR) alongside PENAO (I.V. 20 mg/kg) administered intravenously. Chemotherapy backbones were administered by I.P.. Treatment periods are indicated at the top of the curves. CRs are annotated. (H) PENAO cytotoxicity in 3 neuroblastoma cells lines stably transduced with shRNA constructs targeting GFP (control) or TP53 mRNA after 72 hours of treatment. (I) Immunoblot of cell lysates from IMR-32, SH-SY5Y or SH-EP cells stably expressing shGFP control or shTP53 constructs, after 24 hours of PENAO treatment at the IC50 dose determined in O. Blots were probed using anti-p53 or anti-p21 antibodies with anti-Vinculin serving as a loading control. Densitometry for p53 protein levels are given directly above the p53 blot image, normalised to the vinculin loading control and then again to the shGFP control. (J) PENAO cytotoxicity (0-20μM) curves in SH-EP clones stably expressing TP53 cDNA constructs with point mutations in the DNA-binding domain, after 72 hours of treatment. (K) Immunoblot of cell lysates from SH-EP cells stably expressing empty vector (EV1/EV2) or mutant TP53 constructs (p.C135R/p.C135P), after 24 hours of PENAO treatment at the IC50 dose determined in P. Blots were probed using anti-p53 or anti-p21 antibodies with anti-Vinculin serving as a loading control. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, derived from a one-way ANOVA, error bars represent the standard error of the mean of at least three independent biological replicates.

Supplementary Figure 4



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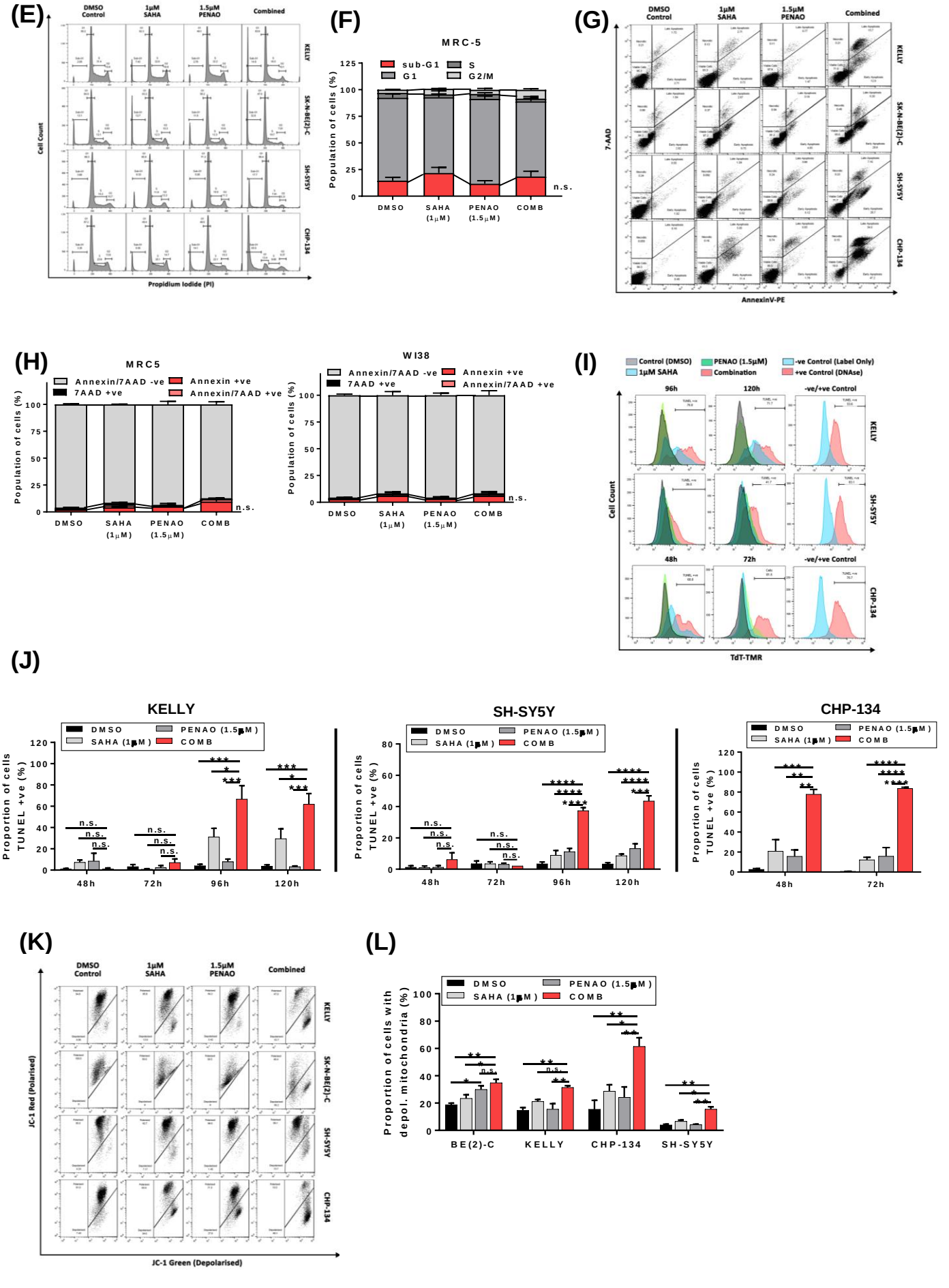

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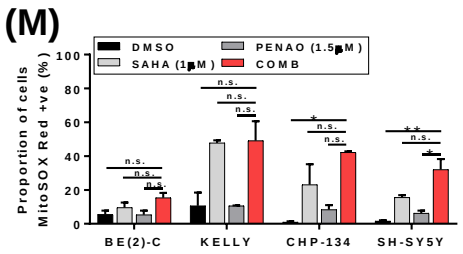

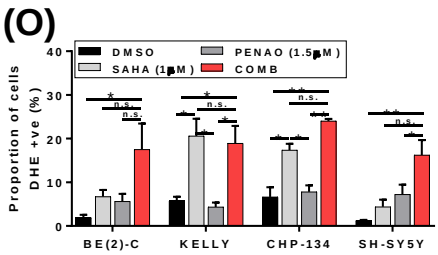

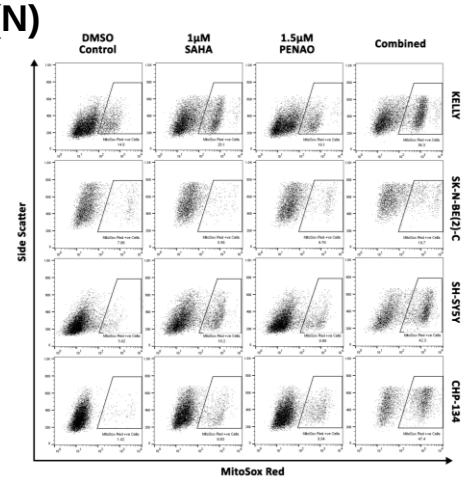

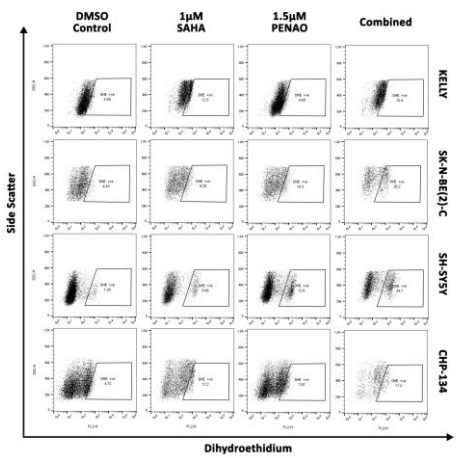

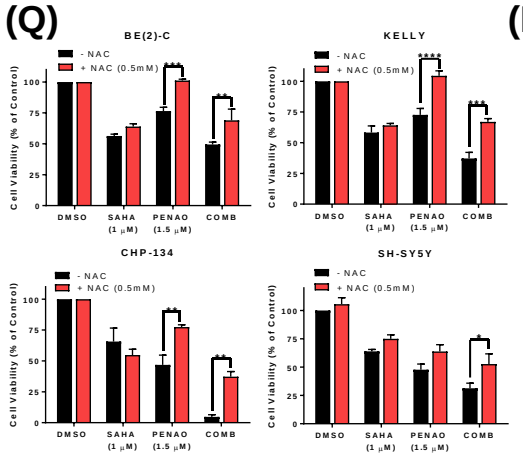

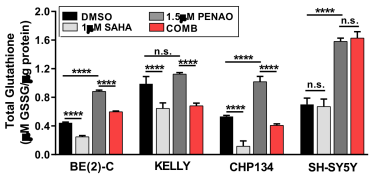

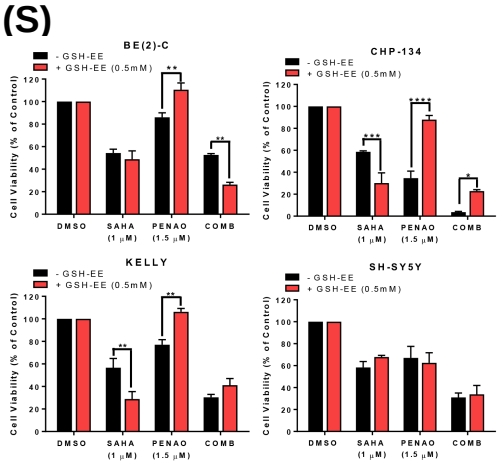

Figure S4.
(A) Cell viability curves of 2 NB (LAN-1, SK-N-AS) and 2 fibroblast cell lines (MRC-5 & WI-38) treated with SAHA (0-2 µM), PENAO (0-3 µM) or in combination at a ratio of 1:1.5. Experimental combination index values (CI) are provided for each dose point, and significant reductions in cell viability for combination compared with single agents are denoted. (B) Cell viability curves of 4 NB and 2 fibroblast cell lines treated with LBH589 (0-10 nM), PENAO (0-3 µM) or in combination at a ratio of 1:300. Experimental combination index values (CI) are provided for each dose point, and significant reductions in cell viability for combination compared with single agents are denoted. (C) Cell viability curves of 2 NB and 2 fibroblast cell lines treated with SAHA (0-2 µM), GSAO (0-10µM) or in combination at a ratio of 1:5. Experimental combination index values (CI) are provided for each dose point, and significant reductions in cell viability for combination compared with single agents are denoted. (D) Combination Index (CI) values at the IC50/75/90 of SAHA+PENAO, LBH589+PENAO, or SAHA+GSAO in NB cell lines grouped by TP53 mutational status (error bars represent S.D.). TP53 miss, refers to TP53 missense mutations (single nucleotide/codon changes), Cdel refers to C-terminal deletion/alteration of the TP53 gene, wt refers to wild type TP53. For all cell viability curves *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, derived from a one-way ANOVA, error bars represent the standard error of the mean of at least three independent biological replicates. (E) Representative gating for cell cycle/apoptosis flow cytometric analysis of NB cell lines stained with Propidium Iodide (PI). Columns represent treatment conditions and rows represent cell lines. (F) Propidium iodide cell cycle assays represented in stacked column graphs representing proportion of cell in each cell cycle phase (including sub-G1) after 72 hours of treatment. (G) Representative quadrant gates for apoptosis analysis of Annexin-V/7AAD stained NB cell lines. Columns represent treatment conditions and rows represent cell lines. (H) Annexin-V/7-AAD apoptosis assays in WI-38 & MRC-5 fibroblasts 72 hours post treatment, represented in stacked column graphs representing the proportion of apoptotic/dead cells. I, Representative gating/analysis of TUNEL stained NB cell lines. Columns represent assay time points following treatment and rows represent cell lines. Positive (DNAse treatment) and negative controls (TdT-TMR label only) are shown in the last column. Treatment conditions are coloured, as indicated at the top of the plot. (J) TUNEL apoptotic assays displaying the proportion of cells with the TdT-TMR fluorophore after 48-120 hours of treatment in KELLY, SH-SY5Y & CHP-134 cell lines. (K) Representative gating/analysis of JC-1 stained NB cell lines. Columns represent treatment conditions and rows represent cell lines. (L) JC-1 assay 24 hours post treatment in NB cell lines, displaying the proportions of cells with depolarised mitochondria. (M) MitoSox Red assays representing the proportion of cells positive for the oxidised MitoSox Red fluorophore after 48 hours of treatment. (N) Representative gating/analysis of MitoSox Red stained NB cell lines. Columns represent treatment conditions and rows represent cell lines. (O) Dihydroethidium (DHE) assays representing the proportion of cells positive for the oxidised DHE fluorophore after 48 hours of treatment. (P) Representative gating/analysis of DHE stained NB cell lines. Columns represent treatment conditions and rows represent cell lines. (Q) Alamar blue cell viability assays of cells co-treated with or without 0.5mM of the reactive oxygen species scavenger N-Acetyl Cysteine (NAC) after 72 hours. (R) Quantitative glutathione assays representing the quantity of total glutathione in cells 24 hours after treatment. Units are expressed as the amount of GSSG (oxidised glutathione) (µM) normalised to the protein input (µg). 1 molecule of GSSG is equivalent to 2 molecules of reduced glutathione (GSH). (S) Alamar blue cell viability assays of treated NB cells with or without 0.5 mM of glutathione mono-ethyl ester (GSH-EE), 72 hours after treatment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, derived from a one-way ANOVA, error bars represent the standard error of the mean of at least three independent biological replicates.

Supplementary Figure 5

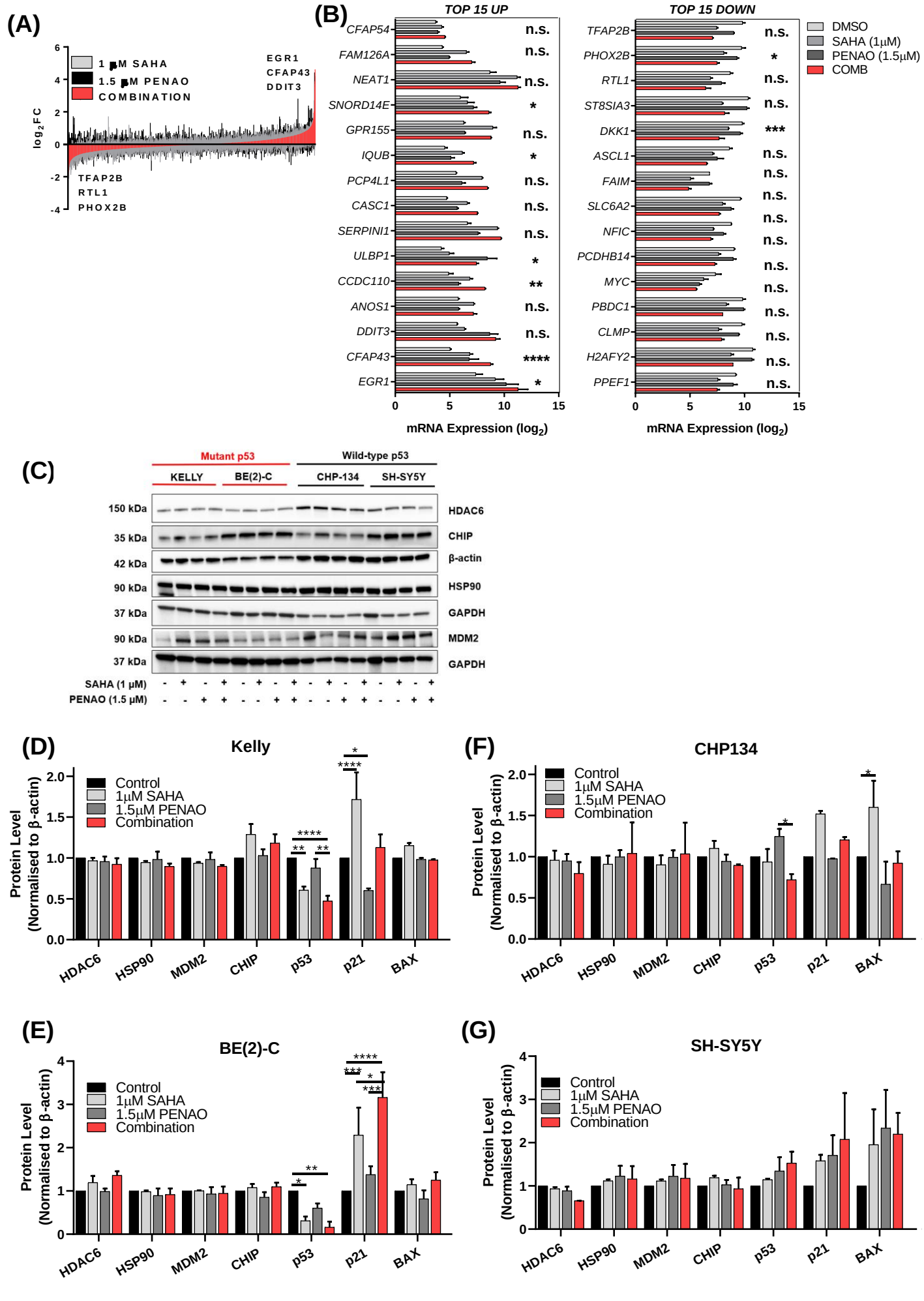

Figure S5.
(A) Rank plot depicting the fold change (log2FC) in mRNA expression of 24716 genes, between the indicated treatment conditions and the DMSO control in wild type TP53 SH-SY5Y cells after 8 hours of treatment (microarray, n=2). Genes are ranked in ascending order by mRNA fold change between the combination and DMSO treatments. (B) Top15 upregulated and downregulated genes in the microarray represented normalised mRNA expression, comparisons are made between the combination treatment and the control/single agents. (C) Immunoblot of NB cell lysates after 24h of the indicated treatments, probed with either; anti-HDAC6, anti-HSP90, anti-MDM2, anti-CHIP or anti-β-actin antibodies. Approximate band sizes are shown to the left of the blots, cell lines used are indicated the top, treatments are indicated below. Both a short and long exposure of anti-p53 probed membrane are provided. (D-G) Densitometry analysis of western blots from treated KELLY, BE2C, CHP-134 & SH-SY5Y cell lysates probed with anti-HDAC6, HSP90, MDM2, CHIP, p53, p21, BAX and β-actin antibodies, with normalisation of values to the β-actin loading control and then to internal vehicle controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, p-values are derived from a two-way ANOVA, error bars represent the standard error of at least two biological replicates.

Supplementary Figure 6

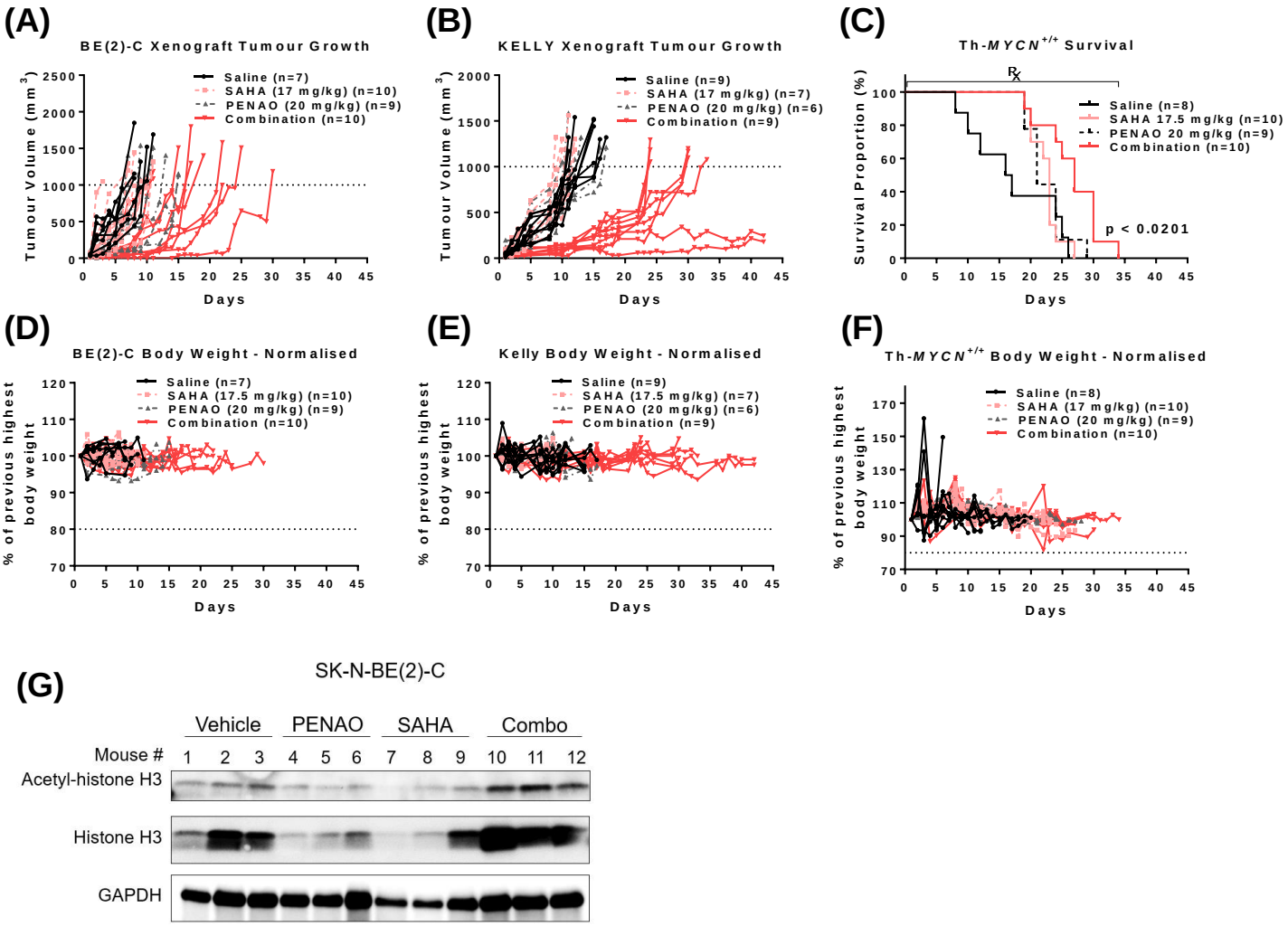

Figure S6.
Tumour growth curves depicting the volume of tumours in each mouse, in each treatment cohort, over the 42 day period in either (A) BE(2)-C or (B) KELLY xenografted mice. (C) Kaplan-Meier survival curves of Th-MYCN+/+ mice at 4 weeks of age after tumour detection (1-2 mm in diameter by palpation) and during the administration of Saline, SAHA (17.5 mg/kg), PENAO (20 mg/kg I.P.) or a combination of both for up to 42 days on a 5 day on/2 day off treatment schedule. P-values are from log-rank statistic tests between the combination and all other treatment groups. Normalised body weights (as a percentage of the previous highest body weight for a given mouse) of (D) BE2C xenografted Balb/c nude mice, (E) KELLY xenografted Balb/c nude or (F) Homozygous Th-MYCN mice during the course of treatment with either Saline, SAHA (17.5 mg/kg), PENAO (20 mg/kg I.P.) or a combination of both for up to 42 days on a 5 day on/2 day off treatment schedule. (G): Western blot analysis of SK-N-BE(2)-C xenograft tumours treated with vehicle, PENAO, SAHA or combination therapy. Hyperacetylation of histone H3 in each tumour sample was detected using anti-histone H3 antibody.

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