Beyond Binary: Mapping the Evolution of Melanoma Across a Discrete Gradient of Acquired Chemoresistance

Emily T. Wang[1], Annabelle Hauss[2], Hiruni Lokuyaddehige[2], Amy E. Nielsen[3], Chun Liang[2]*, Rock J. Mancini[1,3]*

* Corresponding authors: mancinr@miamioh.edu, liangc@miamioh.edu
[1]Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio, 45056, USA
[2]Department of Biology, Miami University, Oxford, Ohio, 45056, USA
[3]Astante Therapeutics Inc., 201 E. Fifth Street, Cincinnati, OH, 45202, USA

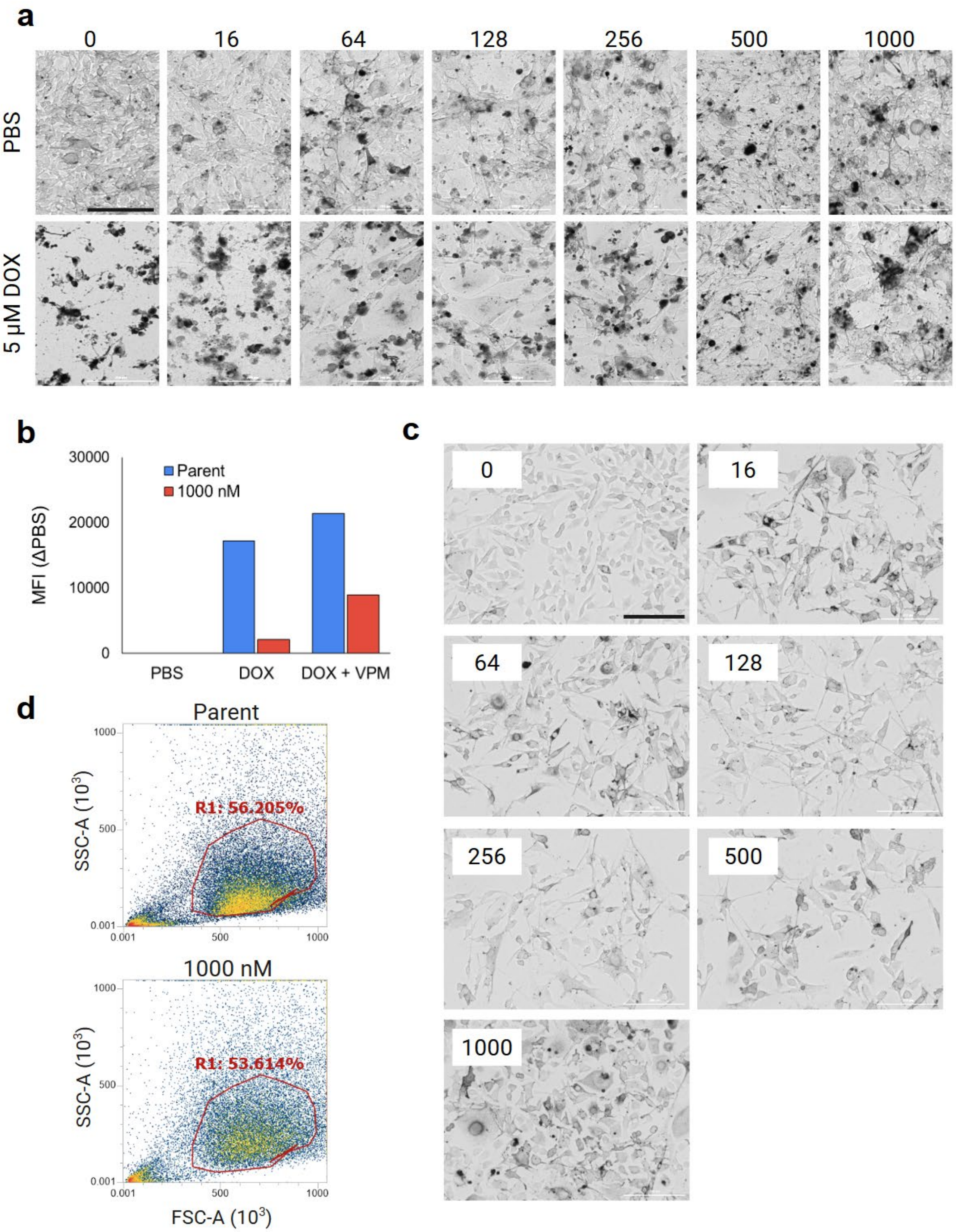
Table of Contents

Antibody	**Catalog Number**	**Dilution (WB)**	**Dilution (IF)**
P-gp	ab170904	1:2,500	1:100
GLUT1	ab115730	1:10,000	1:100
GLUT2	ab54460	1:500	—
GLUT3	ab191071	1:5,000	1:100
GLUT4	ab188317	1:1,000	1:100
β-actin	ab8227	1:5,000	—
HRP secondary	ab97051	1:5,000	—
488 secondary	ab150077	—	1:600

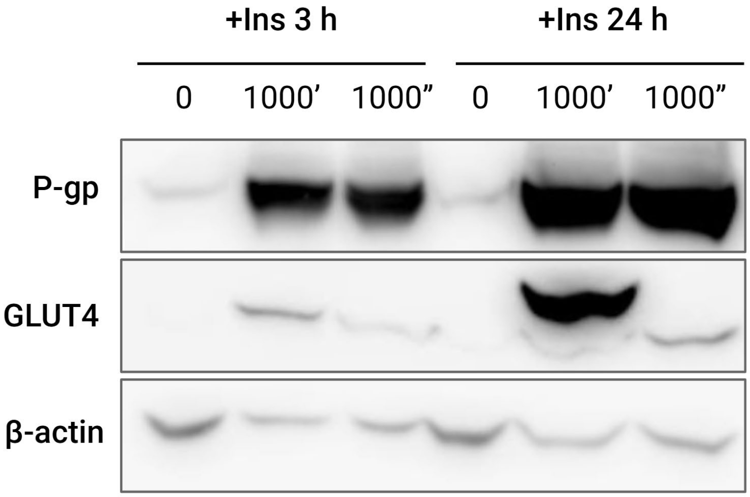
Supplementary Table 1. Antibodies for western blot and immunofluorescence.
All antibodies were purchased through abcam. All primary antibodies come from a rabbit host and react with mouse antigens. All secondary antibodies are goat anti-rabbit.

Primer	**Assay ID**
Slc2a1	Mm00441480_m1
Slc2a2	Mm00446229_m1
Slc2a3	Mm00441483_m1
Slc2a4	Mm00436615_m1
Actb	Mm02619580_g1

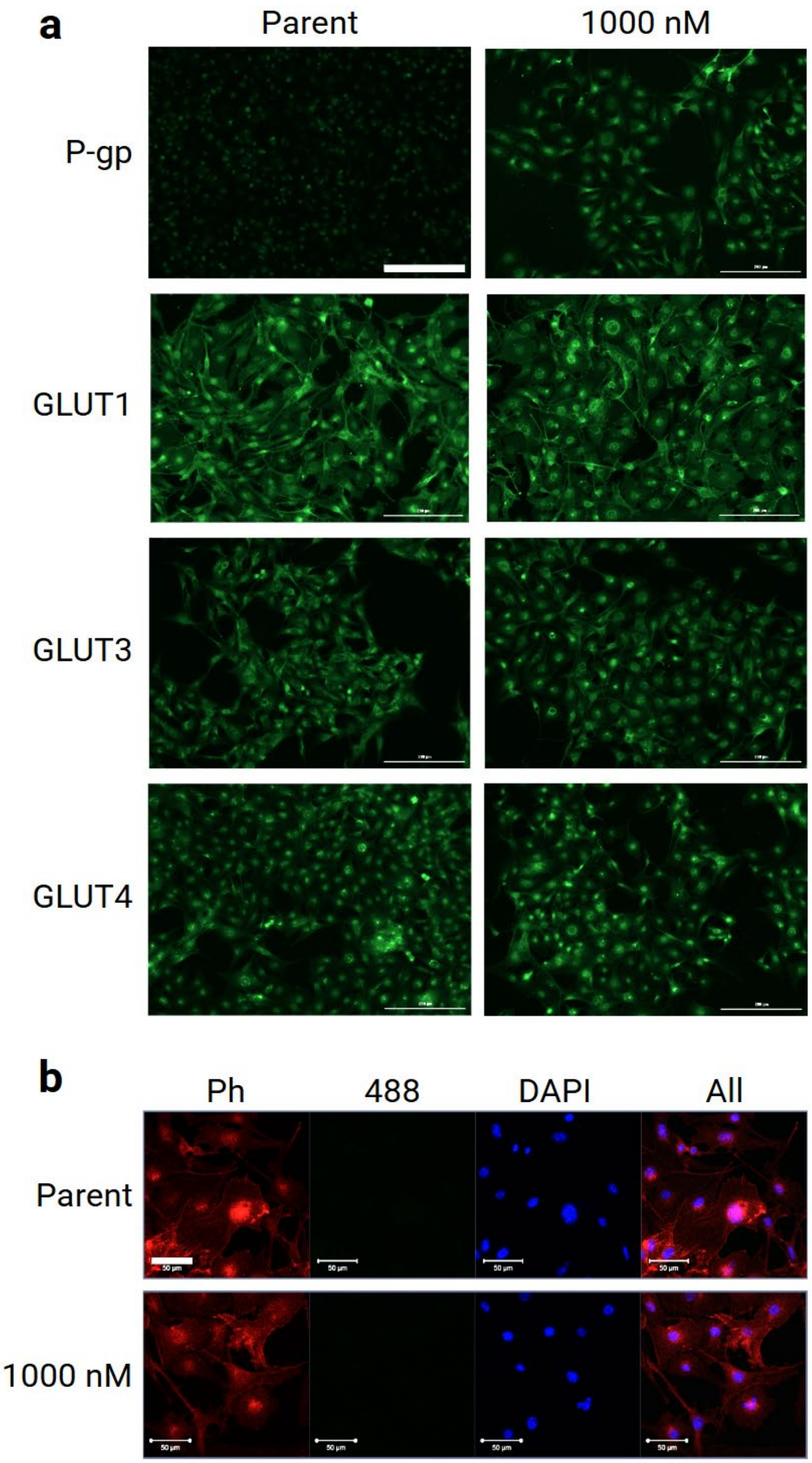
Supplementary Table 2. Primers for qPCR.
All primers were purchased through Life Technologies by ThermoFisher.


a
0
16
64
128
256
500
1000
PBS
5 µM DOX
b
30000
20000
10000
0
MFI (ΔPBS)
Parent
1000 nM
PBS
DOX
DOX + VPM
c
0
16
64
128
256
500
1000
d
Parent
1000
500
0.001
SSC-A (10^3)
R1: 56.205%
0.001
500
1000
1000 nM
1000
500
0.001
SSC-A (10^3)
R1: 53.614%
0.001
500
1000
FSC-A (10^3)

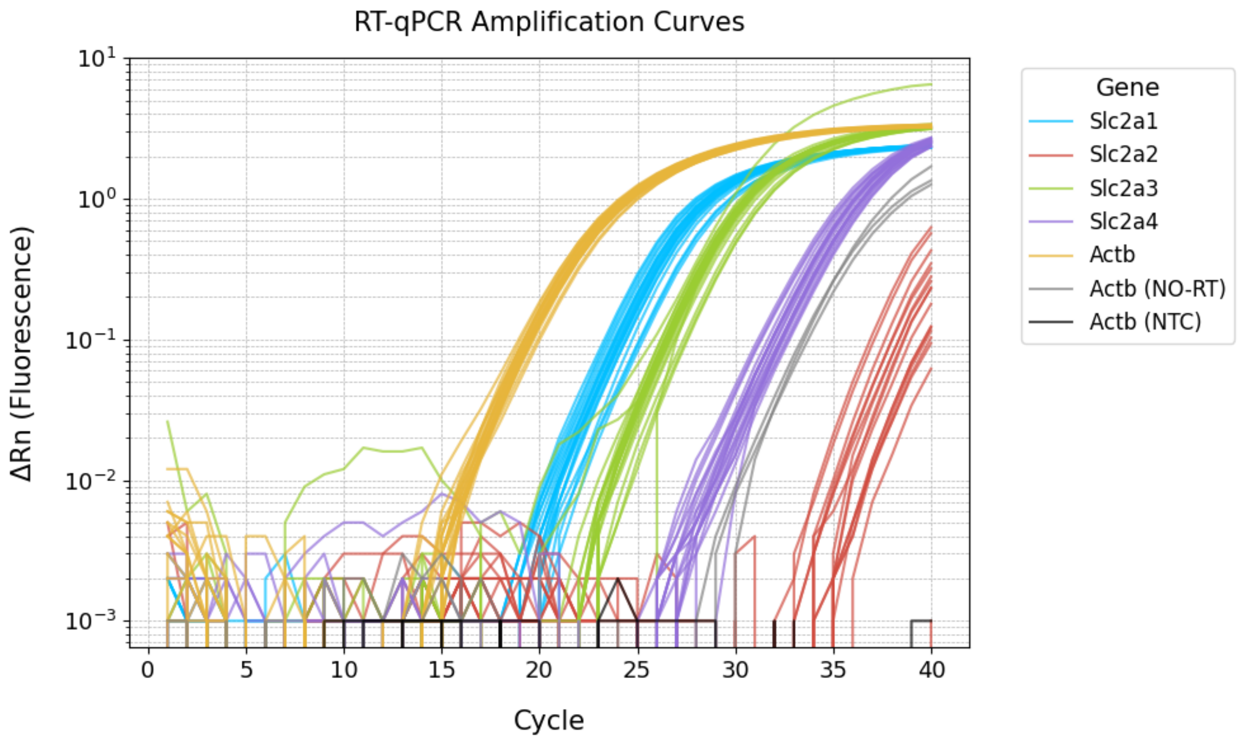
Supplementary Fig. 1. Cell morphology changes with DOX resistance.
(a) The chemoresistance gradient (0, 16, 64, 128, 256, 500, 1000 nM DOX-resistant B16) was subjected to PBS (top row) or 5 μM DOX (bottom row) for 48 h. Brightfield images shown were taken at the end of the incubation period. **(b)** MFI representing DOX fluorescence after parent and 1000 nM-resistant B16 were treated with DOX ± VPM pre-treatment. All data was normalized to PBS. **(c)** Brightfield images of the chemoresistance gradient grown under normal conditions (complete media with their respective DOX concentrations added). For both (a) and (c), images were acquired using a 10X air objective and the scale bar represents 200 μm. **(d)** Flow cytometry density plots for parent and 1000 nM-resistant B16. Each plot includes 30,000 cells in the R1 gate.



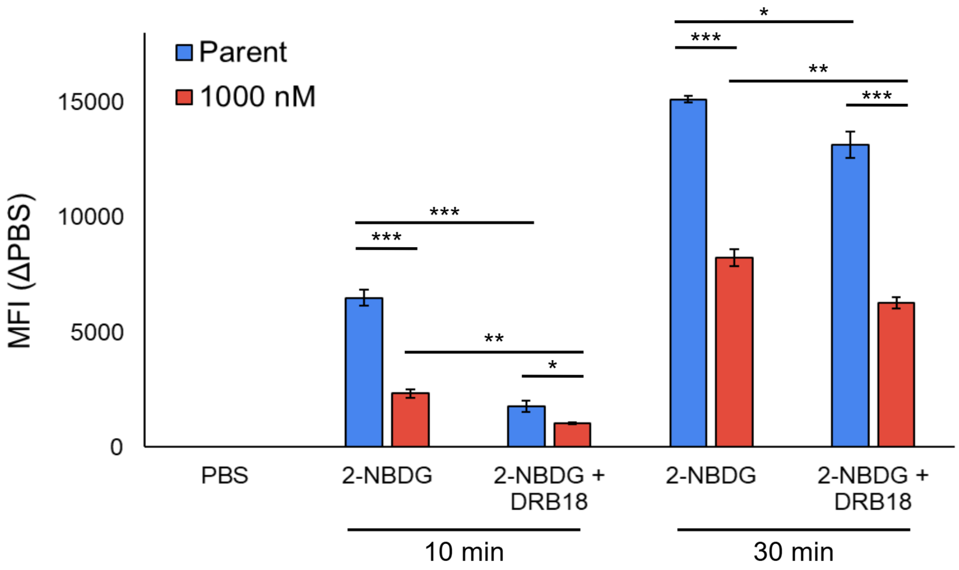
Supplementary Fig. 2. GLUT4 expression following insulin incubation.
Parent B16 (0) and B16 resistant to 1000 nM DOX for 2 passages (1000’) or 6 passages (1000’’) were grown in 6-well plates, two wells each. Upon reaching 80% confluency, one set received 1 µM insulin for 24 h, while the other set received 1 µM insulin for 3 h. Then, cells were lysed, and a western blot was used to visualize P-gp, GLUT4, and β-actin.


a
Parent
1000 nM
P-gp
GLUT1
GLUT3
GLUT4
b
Ph
488
DAPI
All
Parent
1000 nM
50 µm

Supplementary Fig. 3. Additional immunofluorescence images.
(a) P-gp, GLUT1, GLUT3, and GLUT4 visualization on parent and 1000 nM-resistant B16 by antibody staining with an Alexa Fluor 488 fluorophore. Images were acquired by widefield fluorescence microscopy on a 10X air objective. Scale bar represents 200 µm. **(b)** Secondary antibody only controls. Parent and 1000 nM-resistant B16 were stained with Alexa Fluor 488 secondary antibodies, phalloidin-iFluor 594, and DAPI. Images were acquired by confocal microscopy on a 40 X air objective. Scale bar represents 50 µm.

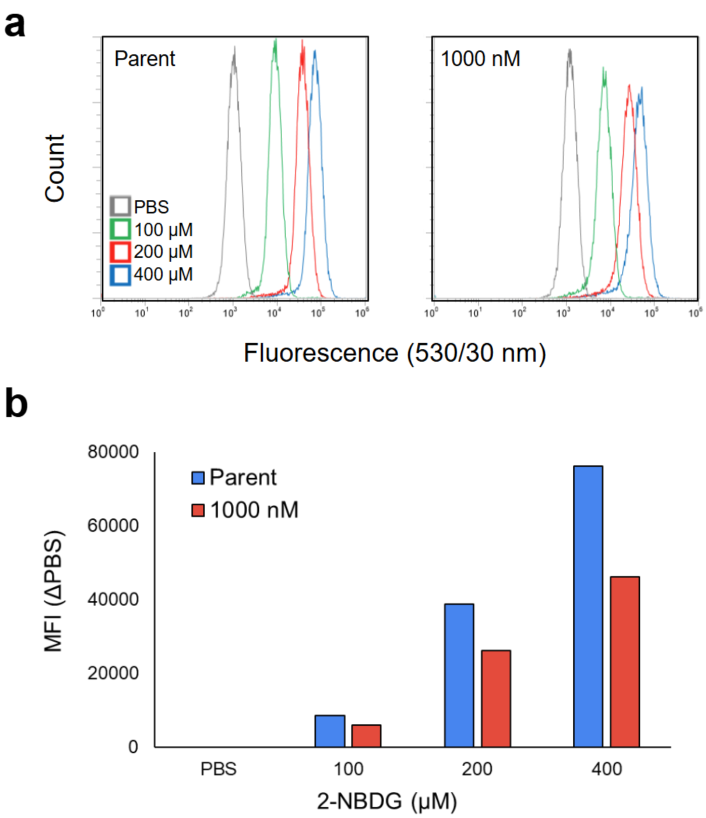


Supplementary Fig. 4. RT-qPCR amplification curves.
Three technical replicates were run for each gene primer across the chemoresistance gradient (0, 16, 64, 128, 256, 500, and 1000 nM-resistant B16). The gray and black curves represent the NO-RT and NTC controls respectively.

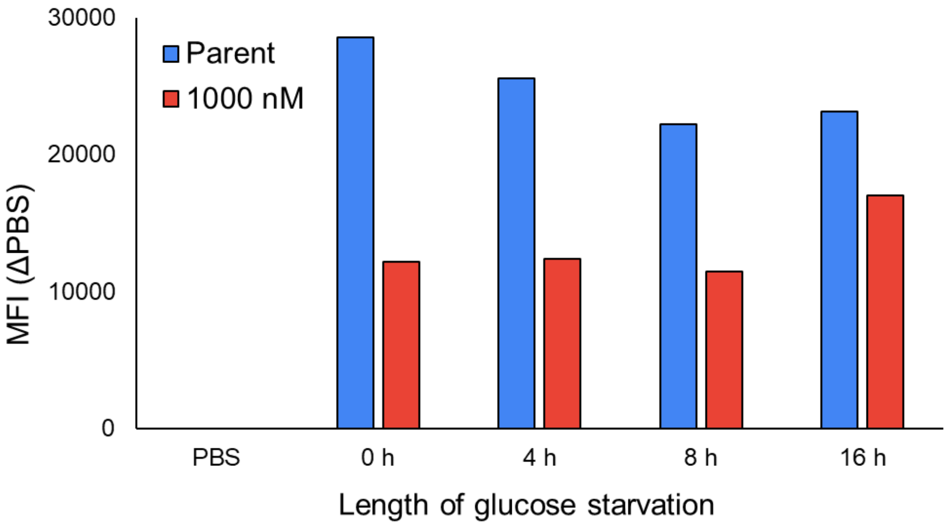


Supplementary Fig. 5. MFI for 2-NBDG ± DRB18 flow cytometry.

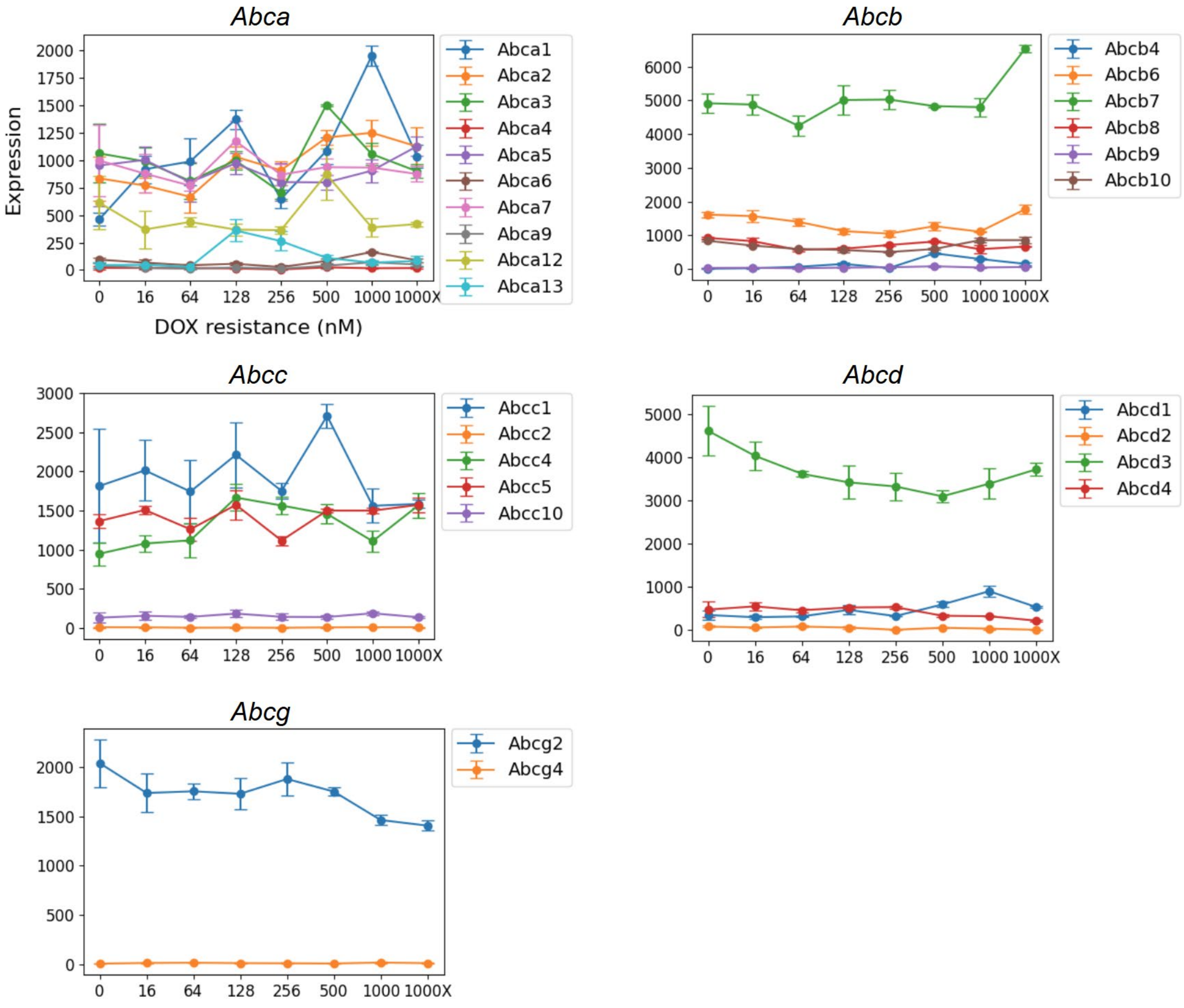
Parent and 1000 nM were pre-incubated with 25 µM of DRB18 for 10 or 30 min at 37C. 100 µM 2-NBDG was then introduced into the media for 30 min, and fluorescence was measured by flow cytometry. Mean fluorescence intensity (MFI) is shown as mean ± SD for three technical replicates. All data was normalized to each sample's mean MFI for PBS. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



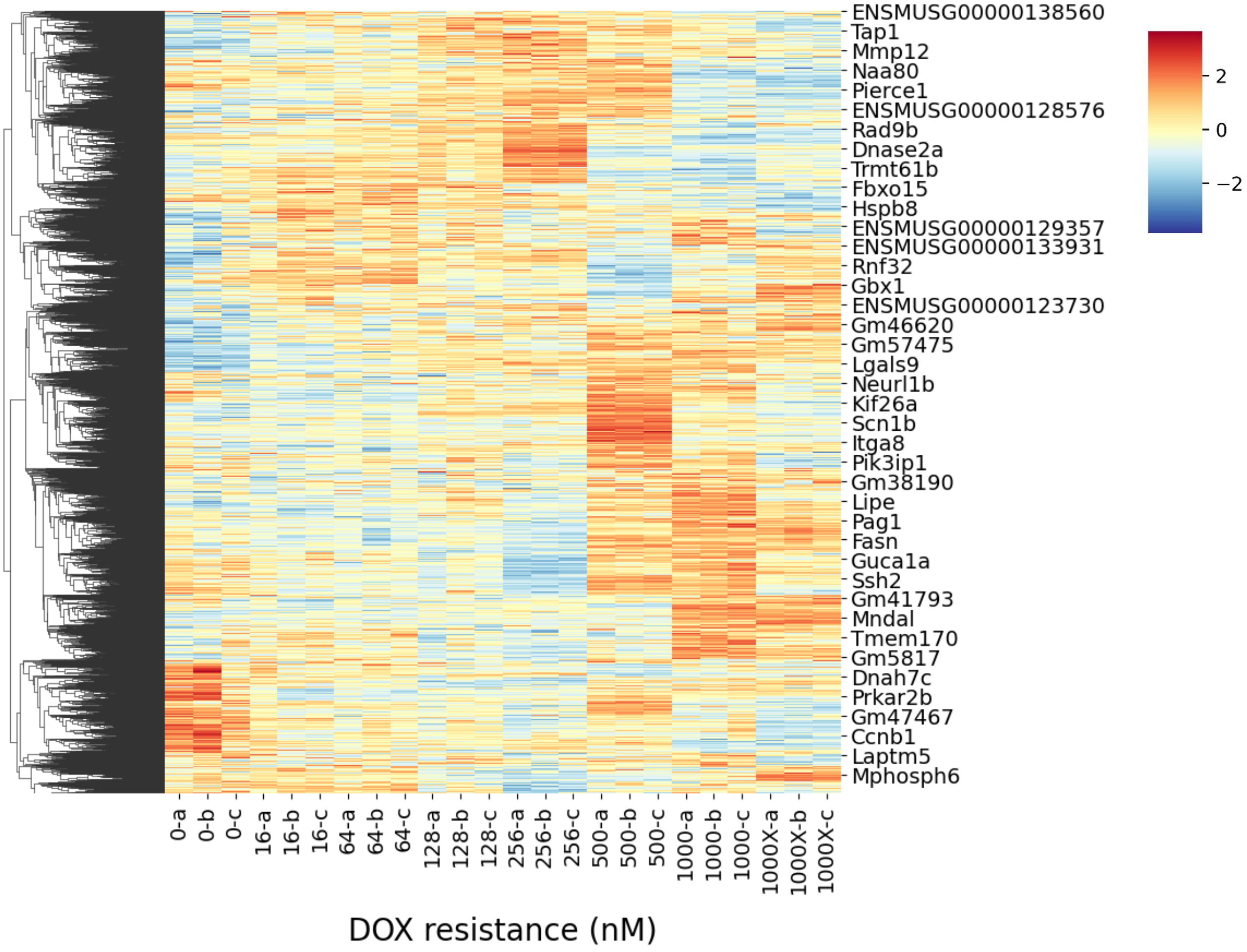
Supplementary Fig. 6. 2-NBDG titrations.
(a) Flow cytometry histograms representing 2-NBDG fluorescence at variable concentrations. Parent and 1000 nM-resistant B16 cells were seeded at 1.5×10^6 cells/well in a 6-well plate and allowed to grow for 72 h. Cells were glucose-starved for 1 h before adding 100, 200, and 400 µM 2-NBDG for 30 min. **(b)** MFI for 2-NBDG fluorescence after normalizing to PBS.



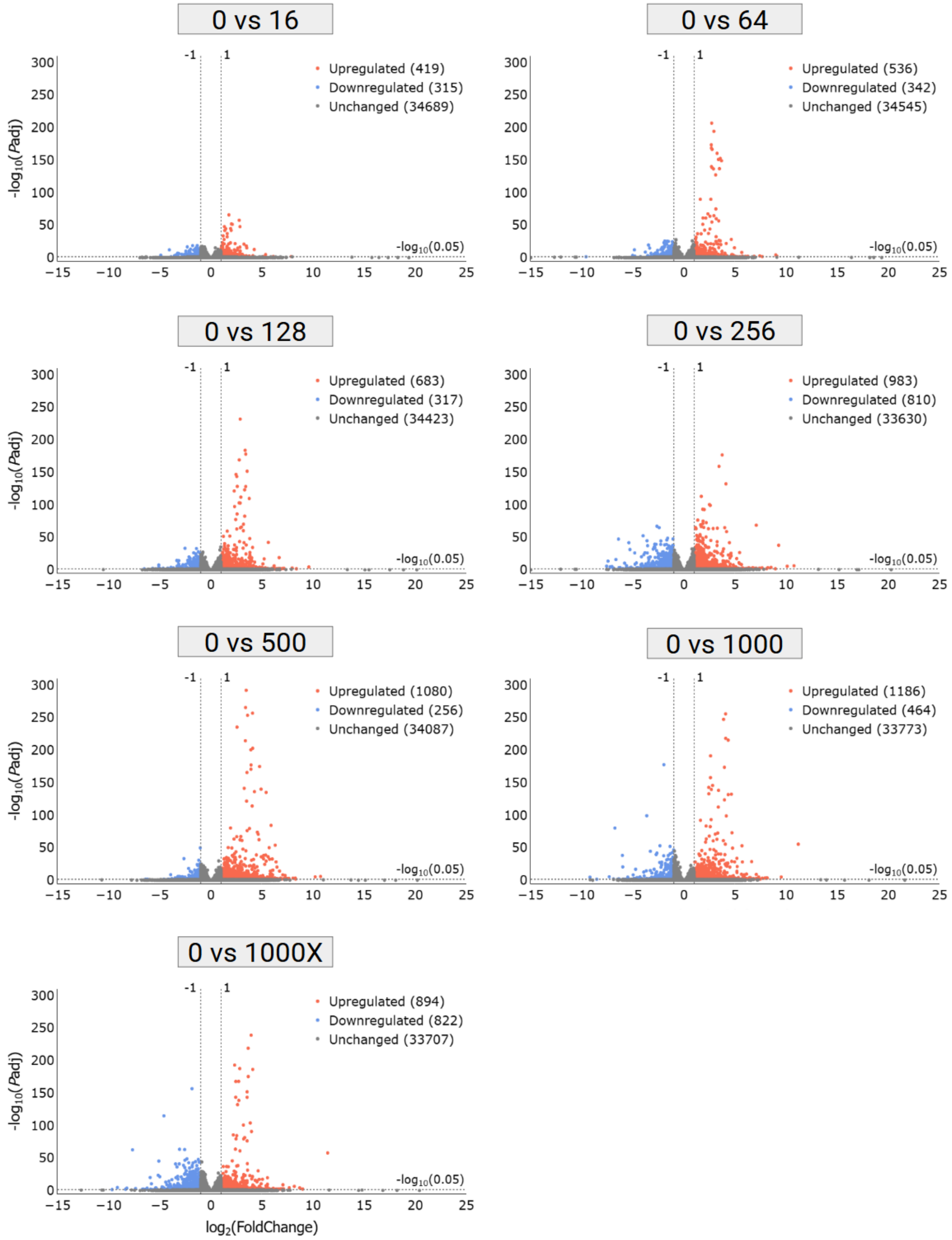
Supplementary Fig. 7. MFI for 2-NBDG with glucose starvation.
Parent and 1000 nM-resistant B16 were glucose-starved for 0, 4, 8, and 16 h before adding 100 μM 2-NBDG for 30 min. Mean fluorescence intensity (MFI) represents fluorescence of 2-NBDG. All data was normalized to PBS.



Supplementary Fig. 8. Expression of ABC transporters by RNA-seq.
All ABC transporter members with expression > 10 in at least one sample across the chemoresistance gradient. Lineplots are divided by the *Abc* subfamilies *Abca*, *Abcb*, *Abcc*, *Abcd*, and *Abcg*. Data represents mean ± SD expression (n=3 biological replicates). Note: *Abcb1a* and *Abcb1b* are not included in the *Abcb* plot for clarity. *Abce* and *Abcf* subfamilies are not included in this plot because while they are in the ABC family, they do not function as transporters.

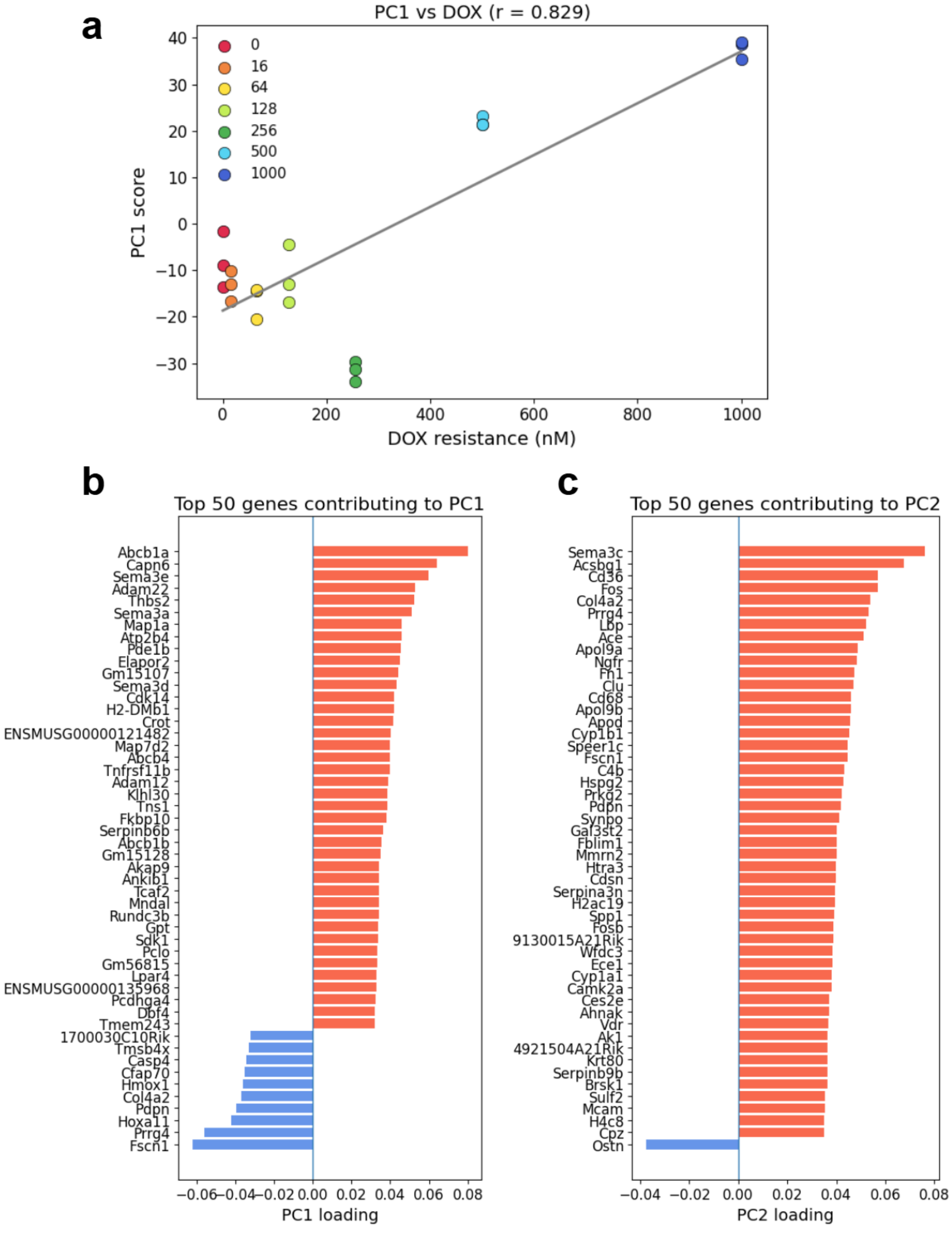


Supplementary Fig. 9. Significant pairwise DEGs.
Heatmap of 6,598 genes identified as differentially expressed between at least one pair of resistance conditions (adjusted $p < 0.05$, $|\log_2 FC| > 1$; Wald test). Expression values are Z-scored across samples and displayed for each biological replicate within each treatment condition.



Supplementary Fig. 10. Volcano plots.

Seven volcano plots comparing parent (0) to the expression profiles of the B16 cells at 16, 64, 128, 256, 500, 1000, and then 1000X nM. Dashed lines denote thresholds for significance: adjusted $p < 0.05$ and $|\log_2 FC| > 1$.

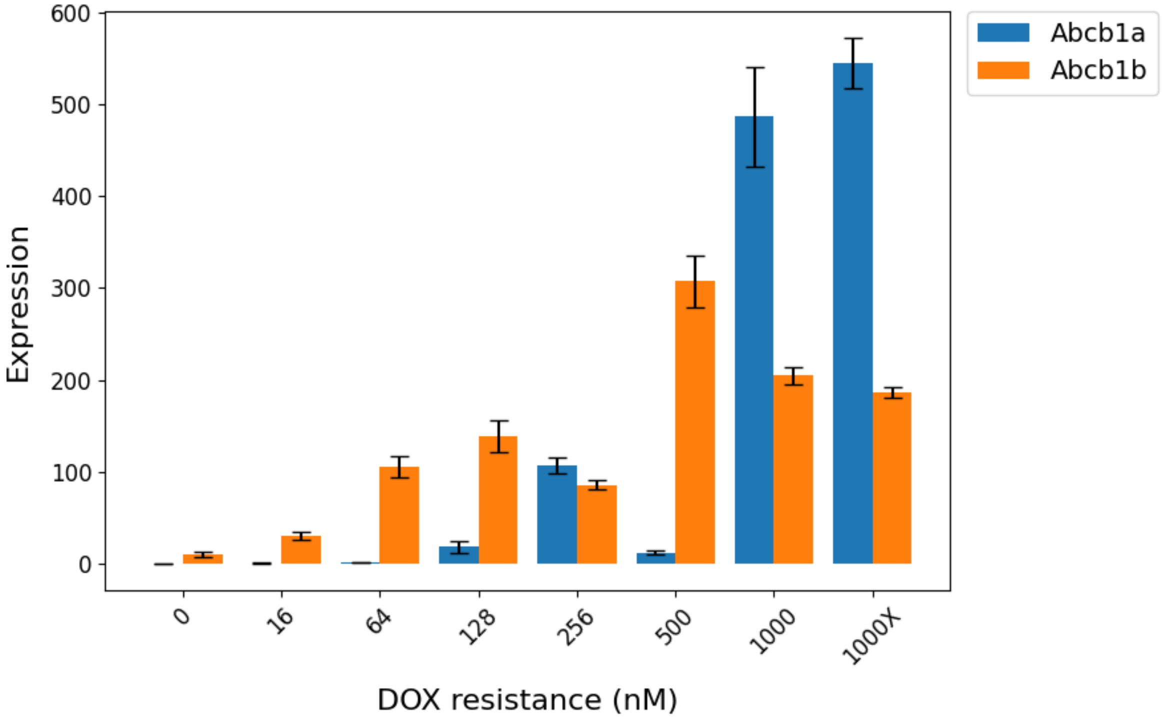


Supplementary Fig. 11. PC1–DOX correlation and top gene loadings.
(a) Scatterplot of PC1 scores versus DOX concentration, with linear regression line and Pearson correlation coefficient (r) shown. Each color represents a distinct resistance level, and each point represents an individual biological replicate (n=3). **(b–c)** Diverging bar plots showing the top 50 genes contributing to PC1 (b) and PC2 (c). Bars represent gene loading values.

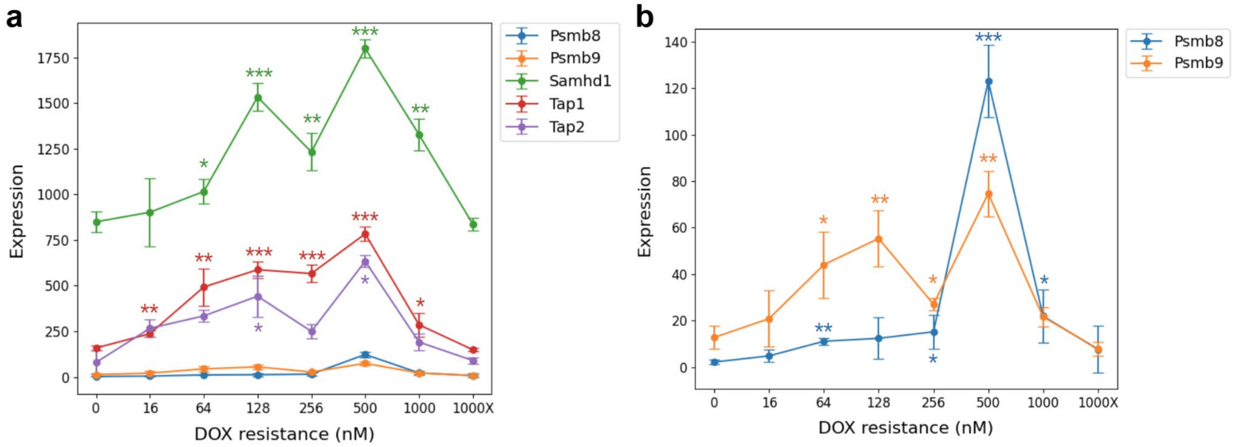
Gene	**Protein**	**Biological Function**	**|log2FC| > 1, p < 0.05**
Hk2	Hexokinase 2	Phosphorylates glucose to glucose-6-phosphate	['0 vs 1000']
Hk3	Hexokinase 3	Phosphorylates glucose to glucose-6-phosphate; less characterized isoform	['0 vs 1000', '0 vs 1000X']
Gpi1	Glucose-6-phosphate isomerase	Converts glucose-6-phosphate to fructose-6-phosphate	['0 vs 64', '0 vs 256', '0 vs 1000X']
Pfkl	Phosphofructokinase, liver	Converts fructose-6-phosphate to fructose-1,6-bisphosphate; liver isoform	['0 vs 64', '0 vs 128', '0 vs 256', '0 vs 1000X']
Pfkp	Phosphofructokinase, platelet	Platelet isoform of PFK1	['0 vs 1000X']
Aldoa	Aldolase A	Cleaves fructose 1,6-bisphosphate into DHAP and GAP; major in muscle	['0 vs 256', '0 vs 1000X']
Aldoc	Aldolase C	Cleaves fructose 1,6-bisphosphate into DHAP and GAP; brain-specific isoform	['0 vs 16', '0 vs 64', '0 vs 128', '0 vs 256', '0 vs 500', '0 vs 1000X']
Tpi1	Triosephosphate Isomerase 1	Converts DHAP to GAP	['0 vs 128', '0 vs 256', '0 vs 1000X']
Gapdh	GAPDH	Catalyzes glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate	['0 vs 64', '0 vs 128', '0 vs 256', '0 vs 1000X']
Gapdhrt	GAPDH Retrogene 1	Putative function similar to GAPDH	['0 vs 128', '0 vs 256', '0 vs 500']
Pgk1	Phosphoglycerate Kinase 1	Catalyzes 1,3-BPG to 3-PG; generates ATP	['0 vs 64', '0 vs 128', '0 vs 256', '0 vs 1000X']
Pgam1	Phosphoglycerate Mutase 1	Catalyzes conversion of 3-PG to 2-PG; widely expressed	['0 vs 1000X']

Eno1	Enolase 1 (Alpha)	Catalyzes 2-phosphoglycerate to phosphoenolpyruvate	['0 vs 64', '0 vs 256', '0 vs 1000X']
Eno1b	Enolase 1B	Retrogene of Eno1; putative similar function in glycolysis	['0 vs 1000X']
Pkm	Pyruvate Kinase M	Catalyzes PEP to pyruvate	['0 vs 64', '0 vs 256', '0 vs 1000X']
Ldha	Lactate Dehydrogenase A	Converts pyruvate to lactate in anaerobic glycolysis	['0 vs 16', '0 vs 64', '0 vs 128', '0 vs 256', '0 vs 1000', '0 vs 1000X']
Ldhd	Lactate Dehydrogenase D	Catalyzes D-lactate to pyruvate	['0 vs 1000', '0 vs 1000X']

Supplementary Table 3. Significant genes involved in glycolysis.
All genes listed are annotated under the glycolytic process gene ontology (GO:0006096) and were identified as significant in at least one treatment condition relative to the 0 nM control ($p < 0.05$, $|\log_2 FC| > 1$). Genes were further filtered to require a minimum expression of >10 normalized counts in at least one sample, as determined by RNA-seq.



Supplementary Fig. 12. TPM expression for *Abcb1a* and *Abcb1b*.
Data are represented as mean ± SD (n=3 biological replicates) and acquired by RNA-seq.



Supplementary Fig. 13. Interferon-stimulated genes are transiently upregulated.

RNA-seq lineplots illustrating DESeq2-normalized expression patterns for (a) *Psmb8*, *Psmb9*, *Samhd1*, *Tap1*, and *Tap2* and (b) *Psmb8* and *Psmb9* only across the MDR gradient. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for independent comparisons to parent (0 nM) (Welch's t-test, n=3, using VST-normalized counts).