Supplementary information

Elevated expression of *S100A8* and *S100A9* correlates with resistance to the BCL-2 inhibitor venetoclax in AML

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Supplementary Materials and Methods

Study material

Bone marrow aspirates or peripheral blood samples were obtained from AML patients and healthy donors after informed consent using protocols approved by a local Institutional Review Board and in accordance with the Declaration of Helsinki. All the AML patients included in the study were venetoclax treatment naïve. Mononuclear cells (MNCs) were isolated by density gradient separation (Ficoll-Paque PREMIUM, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and immediately used for analysis or vitally frozen for later use. Patient characteristics are described in Supplementary Table S3.

Cell lines

AML cell lines MOLM-13, Kasumi-1, SKM-1, NOMO-1, SHI-1 and ML-2 were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen Braunschweig, Braunschweig, Germany). The SHI-1 cell line was maintained in IMDM medium with 20% fetal bovine serum (FBS) and the other cell lines in RPMI 1640 medium supplemented with 10-20% FBS, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL).

Library preparation and RNA sequencing

RNA sequencing-based gene expression analysis was done for 112 AML patient samples and four CD34+ enriched healthy donor BM samples. Briefly, total RNA (2.5-5 µg) was extracted from AML cells using the miRNeasy kit (Qiagen, Hilden, Germany) and depleted of ribosomal-RNA (Ribo-Zero™ rRNA Removal Kit, Epicentre, Madison, WI, USA) after purification, then reverse transcribed to double stranded cDNA (SuperScript™ Double-Stranded cDNA Synthesis Kit, Thermo Fisher, Carlsbad, CA, USA). RNA sequencing

libraries were prepared with Illumina compatible Epicentre Nextera™ Technology and ScriptSeq v2™ Complete kit (Illumina, Inc., San Diego, CA, USA). RNA sequencing libraries were purified with SPRI beads (Agencourt AMPure XP, Beckman Coulter, Brea, CA, USA) and library QC was evaluated on high sensitivity chips using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Paired-end sequencing with 100 bp read length was performed using Illumina HiSeq technology (HiSeq 2000, Illumina, Inc., San Diego, CA, USA).

Gene expression analysis

RNA sequencing data were preprocessed as described previously (1). Briefly, Trimmomatic (2) was used to correct reads for low quality, Illumina adapters, and short read-length. Filtered paired-end reads were aligned to the human genome (GRCh38) using STAR (3) with the guidance of EnsEMBL v82 gene models. Default 2-pass per-sample parameters were used with the overhang on each side of the splice junctions set to 99. The alignments were sorted and PCR duplicates were marked using Picard, feature counts were computed using SubRead (4) and converted to expression estimates using Trimmed Mean of M-values (TMM) normalization (5). Low expressed genomic features with counts per million (CPM) value ≤1.00 were removed. Default parameters were used with exception that reads were allowed to be assigned to overlapping genome features in the feature counting.

Quantitative reverse transcription-PCR (RT-qPCR)

Expression of the *S100* genes was validated for 10 patient samples and *BCL-2* family genes for 7 patient samples by RT-qPCR. The RT-qPCR experiments were performed on 10 ng cDNA prepared from total RNA (SuperScript III Reverse Transcriptase, Thermo Fisher) using iQ SYBR Green Super Mix (Bio-Rad, Hercules, CA, USA) and a CFX96 Real-Time

System (Bio-Rad). Reference genes (*GAPDH*, *LDHA*, *PGK1*) were included in the RT-qPCR experiment and used for normalization purposes. Primer sequences are listed in Supplementary Table S4.

Exome sequencing

Genomic DNA was isolated from patient MNCs and skin biopsies with the DNeasy Blood & Tissue Kit (Qiagen) and 3 µg was used for the sequencing. Exome capture was performed with the Nimblegen SeqCap EZ v2 capture Kit (Roche NimbleGen, Madison, WI, USA) and sequencing was done on HiSeq1500, 2000 or 2500 instruments (6). Data analysis was done as previously described (7).

***Ex vivo* drug sensitivity and resistance testing**

Sensitivity of MNCs from AML patient samples (n = 35) was assessed to 349 different small molecule inhibitors as previously described (6). In short, the drugs were tested in 5 concentrations across a 10,000-fold concentration range by measuring cell viability after 72 h incubation using the CellTiter-Glo (CTG) assay (Promega, Madison, WI, USA) with a PHERAstar FS plate reader (BMG LABTECH, Ortenberg, Germany). A drug sensitivity score (DSS) was calculated based on a modified area under the dose-response curve calculation as described earlier (8). Blast specific responses were presented in comparison to healthy control cells (sDSS).

Prediction of genetic determinants of venetoclax response

To find genes associated with venetoclax response, we applied a linear regression model (9). We assumed that gene expression is affected by confounding factors including age, gender, sequencing batch, RNA extraction method and RNA-sequencing library

preparation. To find the true relationship between gene expression change and drug sensitivity, we corrected the confounding factors in the linear regression model. Genes at false discovery rate (FDR) < 0.05 were considered significant for drug sensitivity association.

Drug combination studies

Drugs (venetoclax, OTX-015) were obtained from ChemieTek (Indianapolis, IN, USA) and added simultaneously at eight different concentrations to AML patient cells and cell lines and incubated for 72 h. Cell viability was measured using the CellTiter-Glo assay (Promega). Data were analyzed with the Zero Interaction Potency (ZIP) model by considering a dose-response matrix where two drugs are tested at 8 concentrations in a serially diluted manner (10). The delta synergy score for each dose pair was determined as the difference between the observed and expected response given by the ZIP model.

Calcium flux assay

Free cytosolic calcium was measured in venetoclax-resistant (OCI-AML3, SHI-1, NOMO-1, SKM-1) and sensitive cell lines (MOLM-13, Kasumi-1, ML-2) using a calcium assay kit (BD Biosciences, Franklin Lakes, NJ, USA). The measurements were done after 4 h exposure to venetoclax (100 nM) or DMSO. Changes in intracellular Ca^{2+} were determined by the ratio of fluorescence intensity to the corresponding DMSO value.

Western blot analysis

Cell lysates were prepared from MNCs, separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Detection antibodies included anti-calgranulin A (C-10, sc-48352), anti-calgranulin B (MRP 1H9, sc-53187) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-MCL-1 (#5453), anti-BCL-XL (#2764), anti-

BCL-2 (#4223), anti-BIM (#42933) (Cell Signaling Technology, Danvers, MA, USA), and anti-β-actin (Merck, Darmstadt, Germany). Data were quantified with the Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Pathway and network analysis

Gene function and network analysis was done for 349 genes negatively associated with venetoclax response using the Enrichr analysis tool (KEGG 2016 and Reactome 2016 cell signaling pathway databases) and Ingenuity pathway analysis (Qiagen). GeneMANIA (version 3.6.0) was used for visualizing a sub-cluster of 29 genes associated with venetoclax resistance.

Statistical analysis

Statistical analyses were performed with R version 3.3.3 (2017-03-06) and Prism 7 (GraphPad, La Jolla, CA, USA). Statistical dependence between two variables was assessed by Pearson's correlation coefficient modeling. The Mann Whitney *U* test was used for analyzing differences between drug responses. *P*-values below 0.05 and false discovery rate (FDR) below 0.05 were considered statistically significant. The Wilcoxon signed-rank test was applied to find significant difference in drug response between wild type and mutated AML patient samples for a given mutation.

Supplementary References

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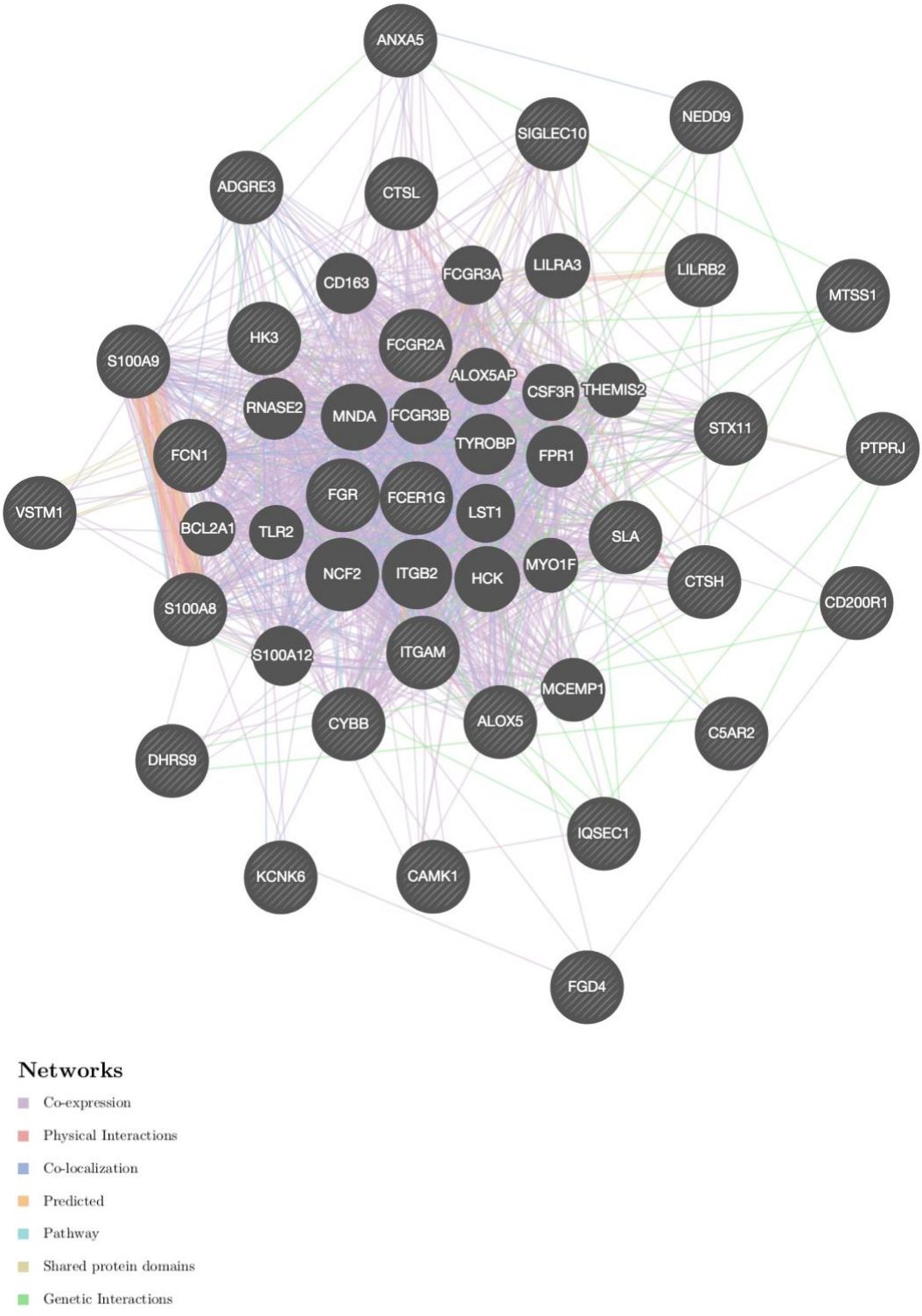
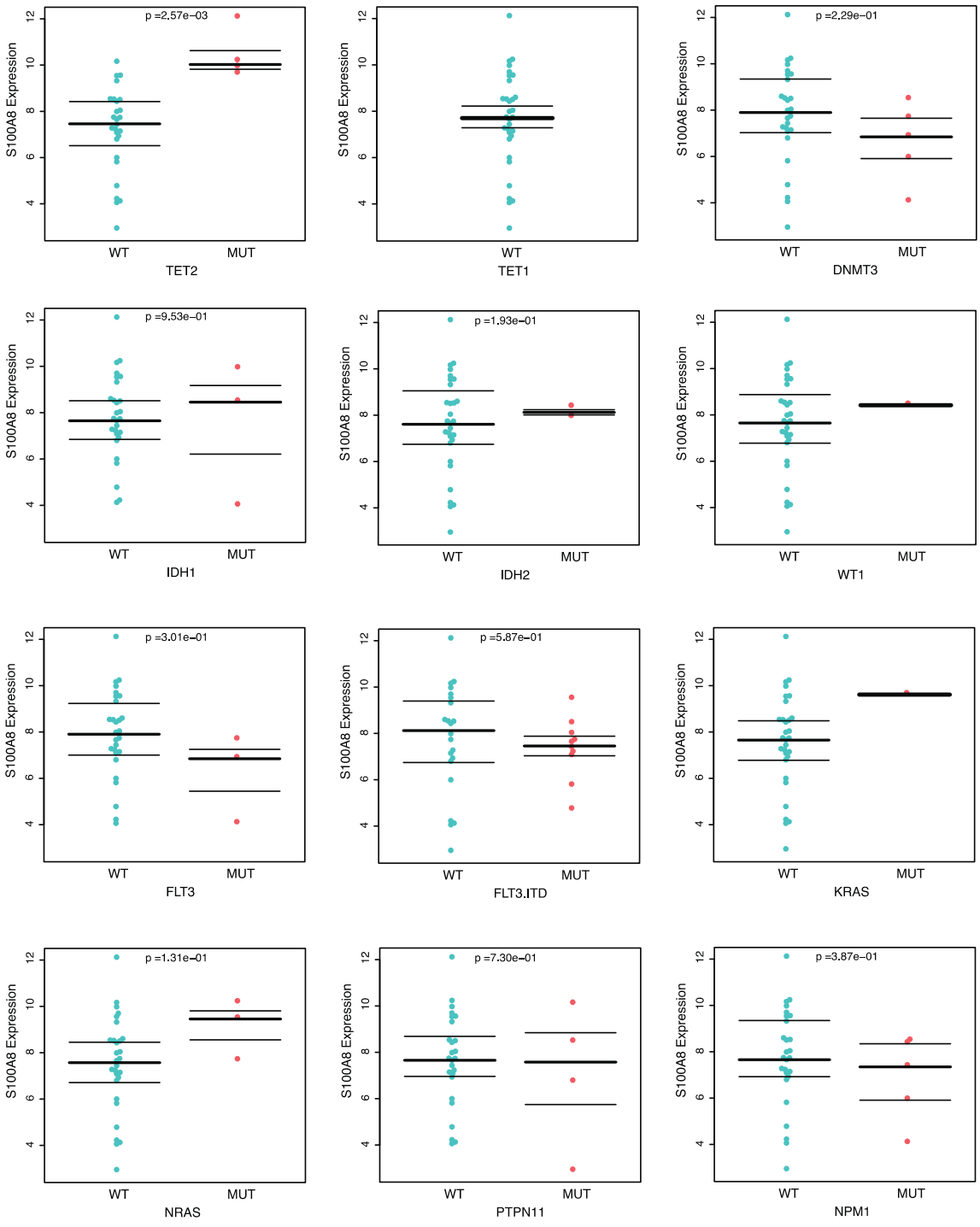
Supplementary Figures



Supplementary Figure S1. Visualization of a sub-cluster of 29 genes associated with venetoclax resistance in AML patient samples by GeneMANIA. Interactions of the overexpressed genes in venetoclax resistant AML patient samples showing upregulation of immune system process related genes and inflammatory response genes.

A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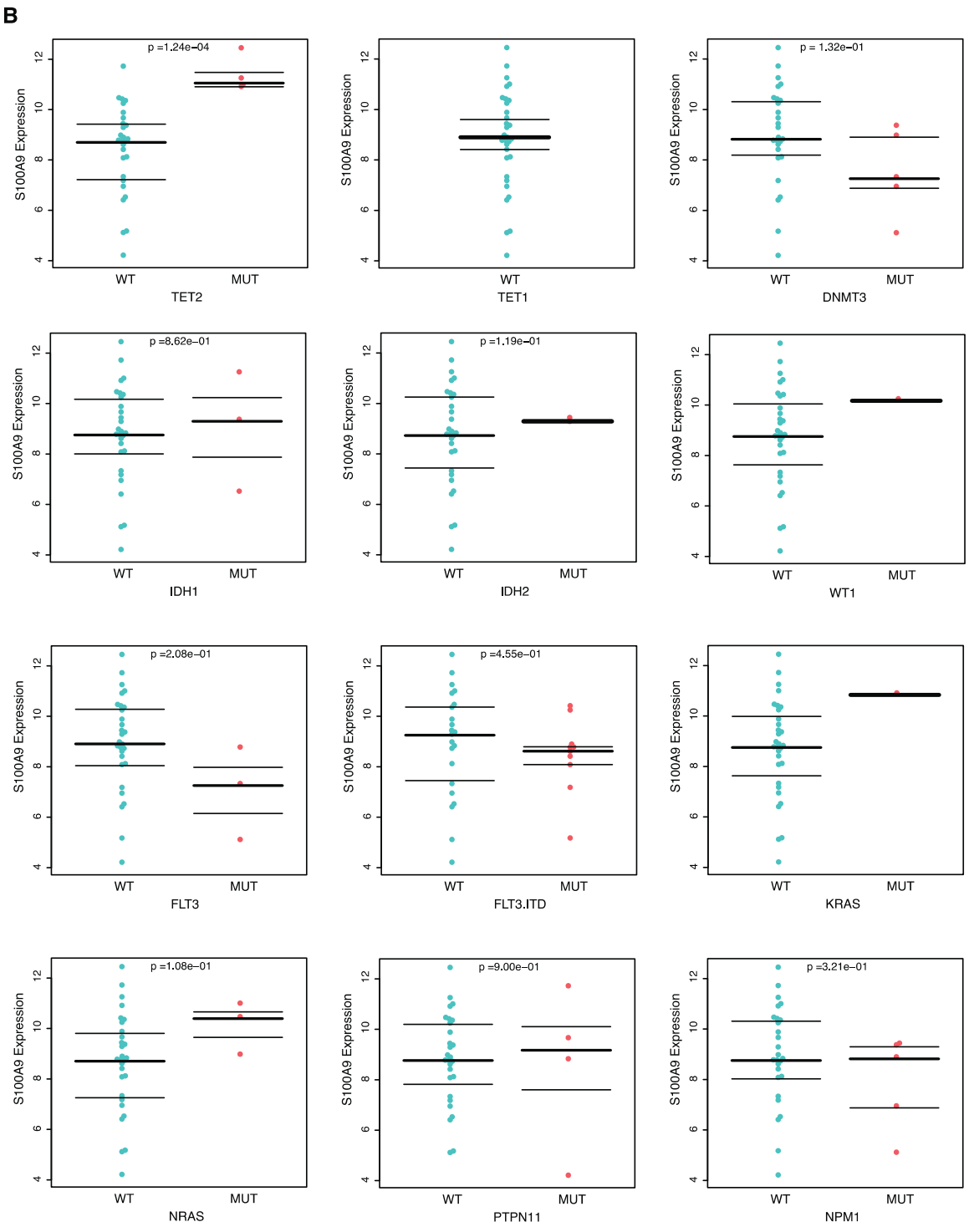
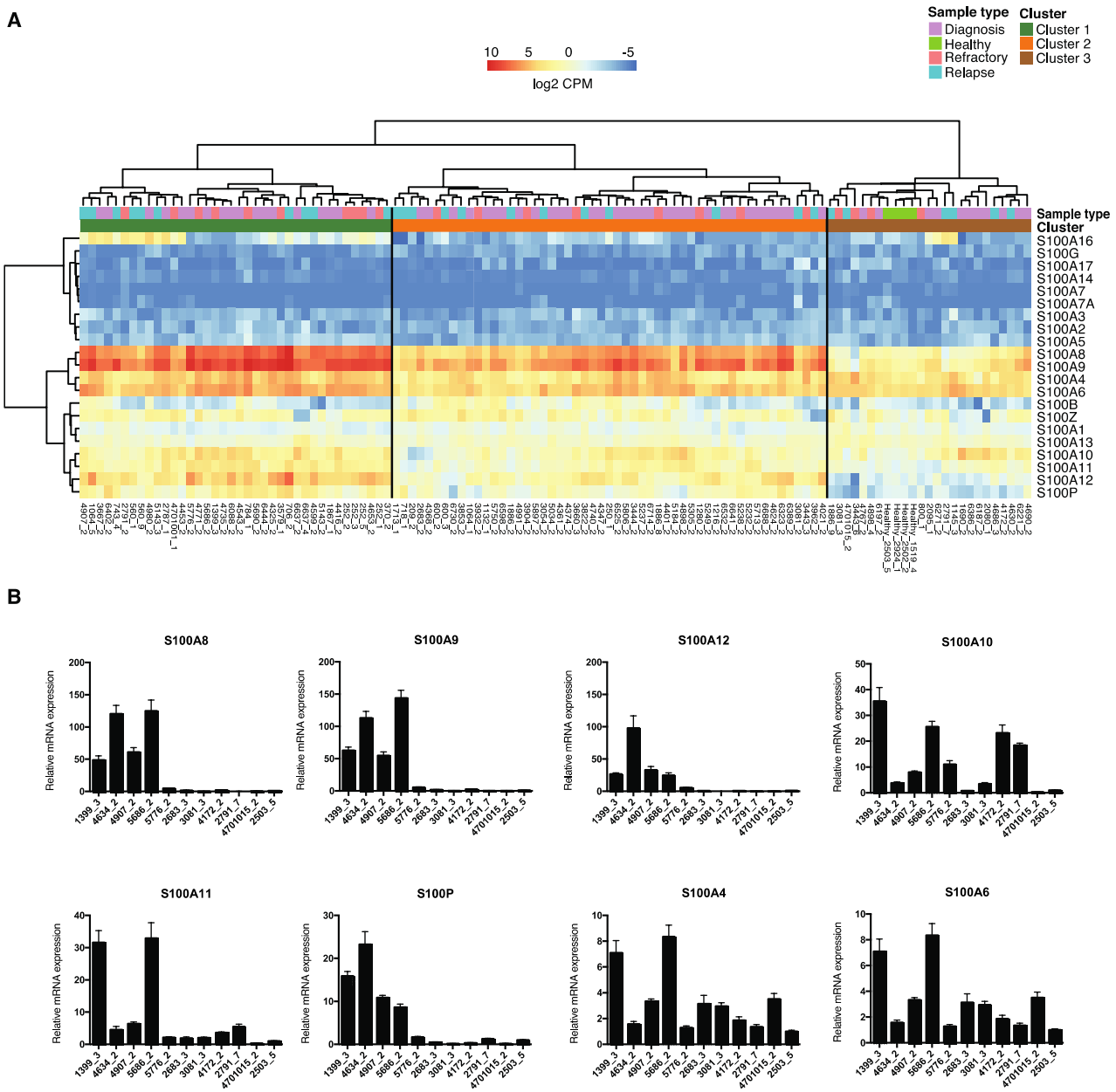
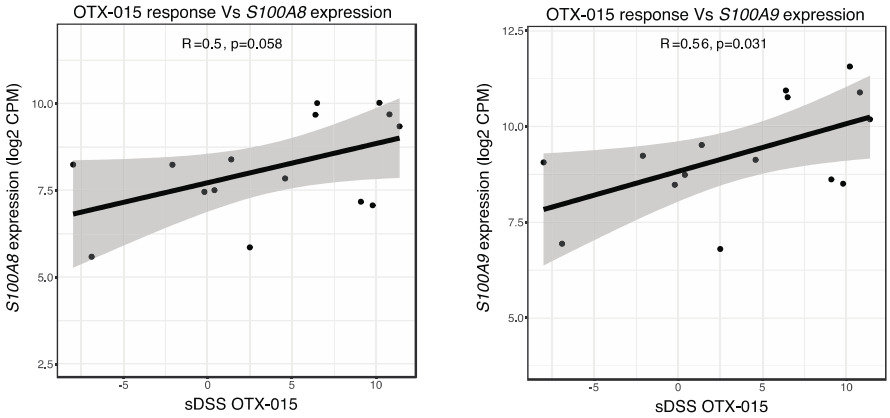


Supplementary Figure S2. Statistical dependence of (A) *S100A8* gene expression and (B) *S100A9* gene expression with somatic mutations. Statistical dependence was assessed by two-tailed *t*-test.



Supplementary Figure S3. Expression profile of *S100* family genes in AML patient samples. (A) Hierarchical clustering of patient samples and *S100* members genes using Euclidean distance matrix and complete clustering method. Mean centered log_2 CPM values of *S100* members in samples from 112 AML patients and 4 CD34+ healthy donors. Red color indicates high and blue low expression, respectively. (B) Quantitative PCR analysis of the expression of *S100* family genes in AML patient samples.

A



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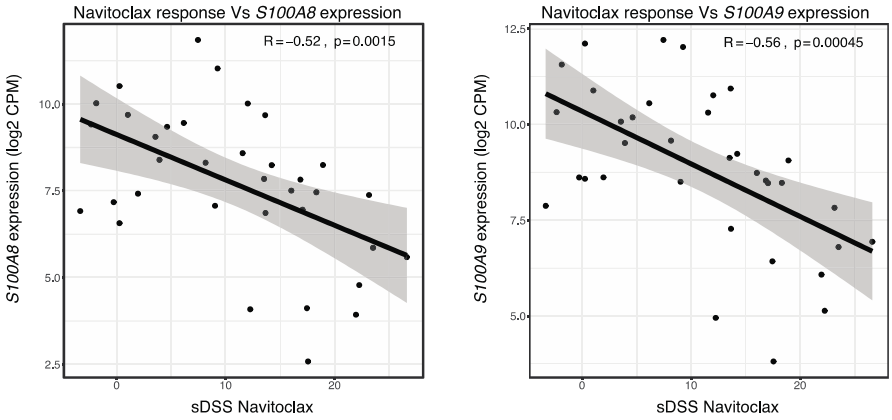
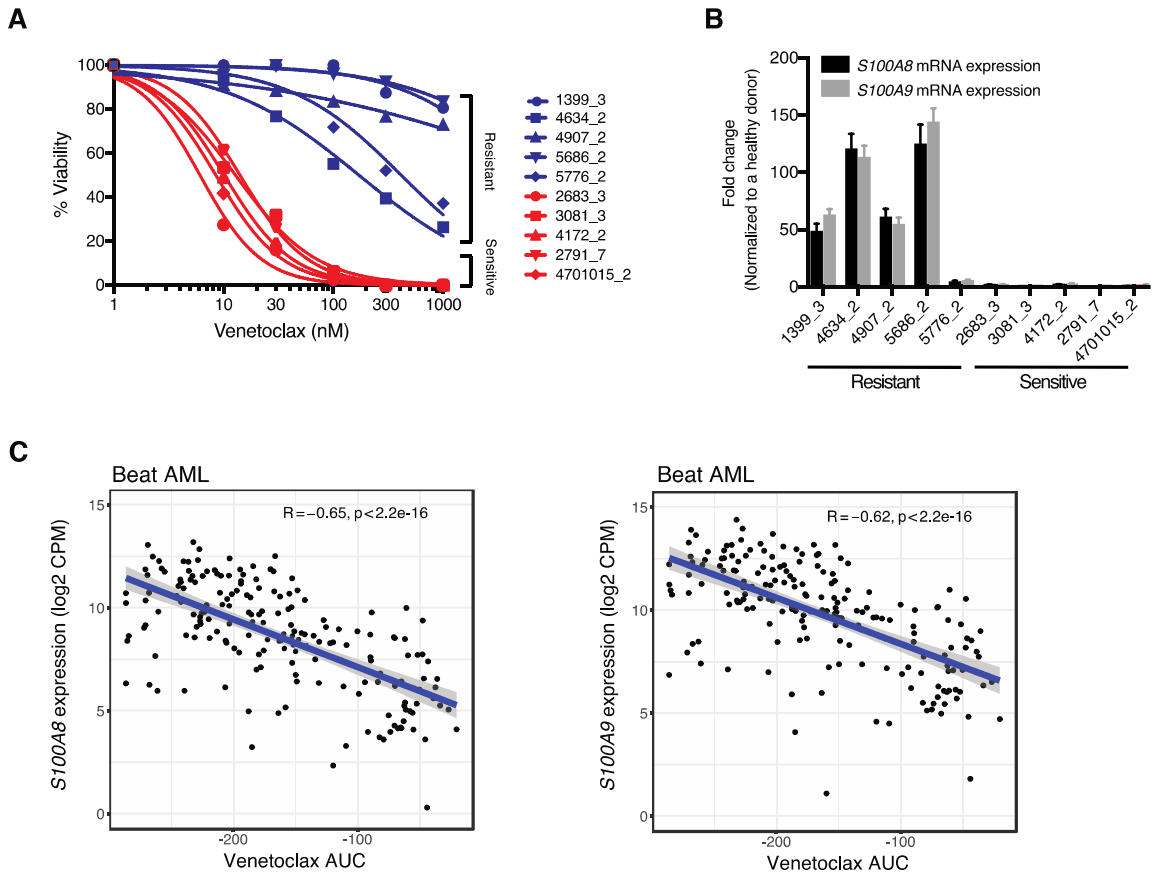
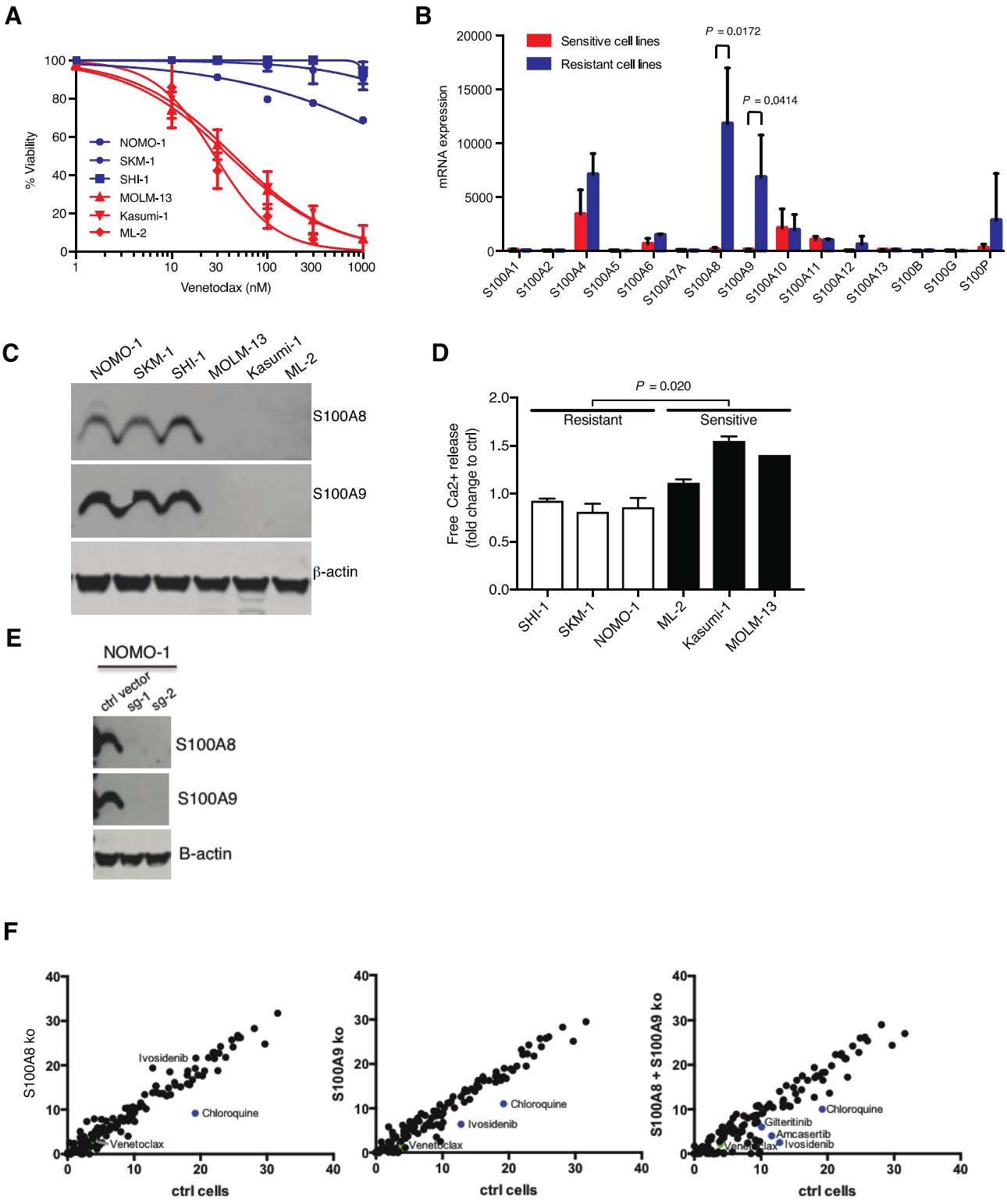


Supplementary Figure S4. Correlation of *S100A8* and *S100A9* expression with *ex vivo* OTX-015 and navitoclax response of AML patient samples. (A) *S100A8* and *S100A9* expression have a positive correlation with sensitivity to BET bromodomain inhibitor OTX-015. (B) Correlation of *S100A8* and *S100A9* expression with *ex vivo* response to navitoclax. *S100A8* and *S100A9* expression and sensitivity to pan-BCL-2 inhibitor navitoclax have an inverse correlation. Statistical dependence was assessed by Pearson's correlation coefficient.

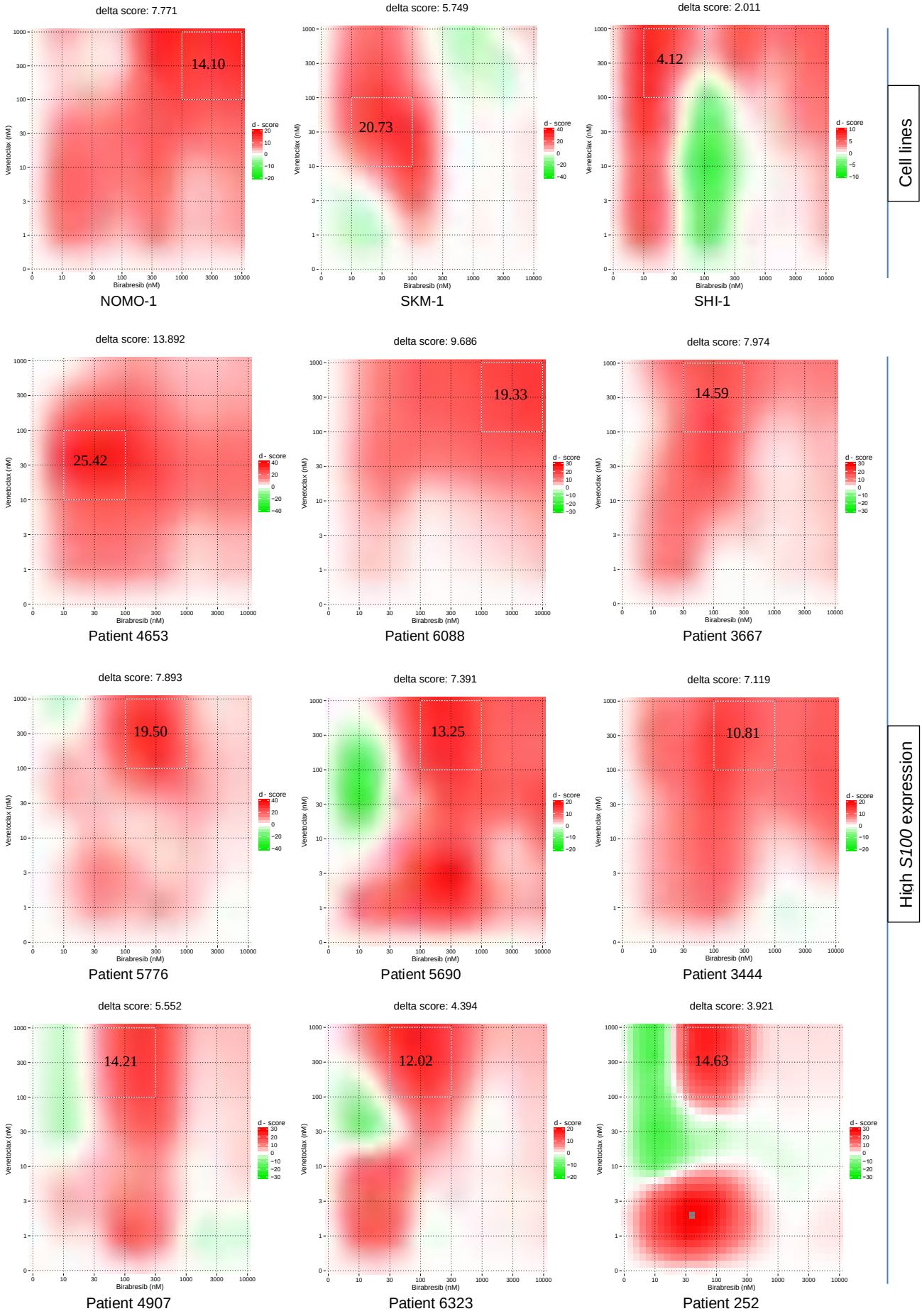


Supplementary Figure S5. **Correlation of *S100A8* and *S100A9* expression with *ex vivo* venetoclax response of AML patient samples.** (A) *Ex vivo* venetoclax response of primary AML patient samples expressing high or low levels of *S100A8* and *S100A9*. AML cells were cultured for 72 h in the presence of 6 concentrations of venetoclax and cell viability measured using the CTG assay. (B) Validation of *S100A8* and *S100A9* expression with RT-qPCR in AML patient samples. Data are normalized against GAPDH expression. (C) Correlation of *S100A8* and *S100A9* expression and venetoclax sensitivity in the Beat AML data set. Statistical dependence was assessed by Pearson's correlation coefficient.



Supplementary Figure S6. ***S100A8*** **and** ***S100A9*** **have a high expression in venetoclax-resistant cell lines compared with sensitive cell lines.** (A) Dose response curves of AML cell lines after treatment with indicated concentrations of venetoclax for 72 h. Cell viability was measured using the CTG assay. (B) mRNA expression of *S100* genes in venetoclax – sensitive (Kasumi-1, MOLM-13, ML-2) and venetoclax-resistant (NOMO-1, SKM-1, SHI-1)

cell lines. *P*-values were calculated with two-tailed *t*-tests. (C) Western blot analysis of S100A8 and S100A9 proteins in AML cell lines. Protein lysates were analyzed by immunoblotting using antibodies to the antigens indicated. (D) Over-expression *of S100A8* and *S100A9* in venetoclax-resistant cell lines is associated with failure to release free-cytosolic Ca^{2+} after exposure to venetoclax (100 nM) for 4 h. Difference in the calcium release was statistically analyzed using the Mann-Whitney *U*-test. (E) Effect of S100A8 and/or S100A9 knockdown on drug sensitivity of NOMO-1 cells. NOMO-1 cells were transduced with lentivirus carrying sgRNAs targeting S100A8 and/or S100A9. (F) Drug sensitivity of the knock out cells was tested for 132 drugs and measured after 72 hours with the CTG assay. Sensitivity is indicated as drug sensitivity scores (DSS).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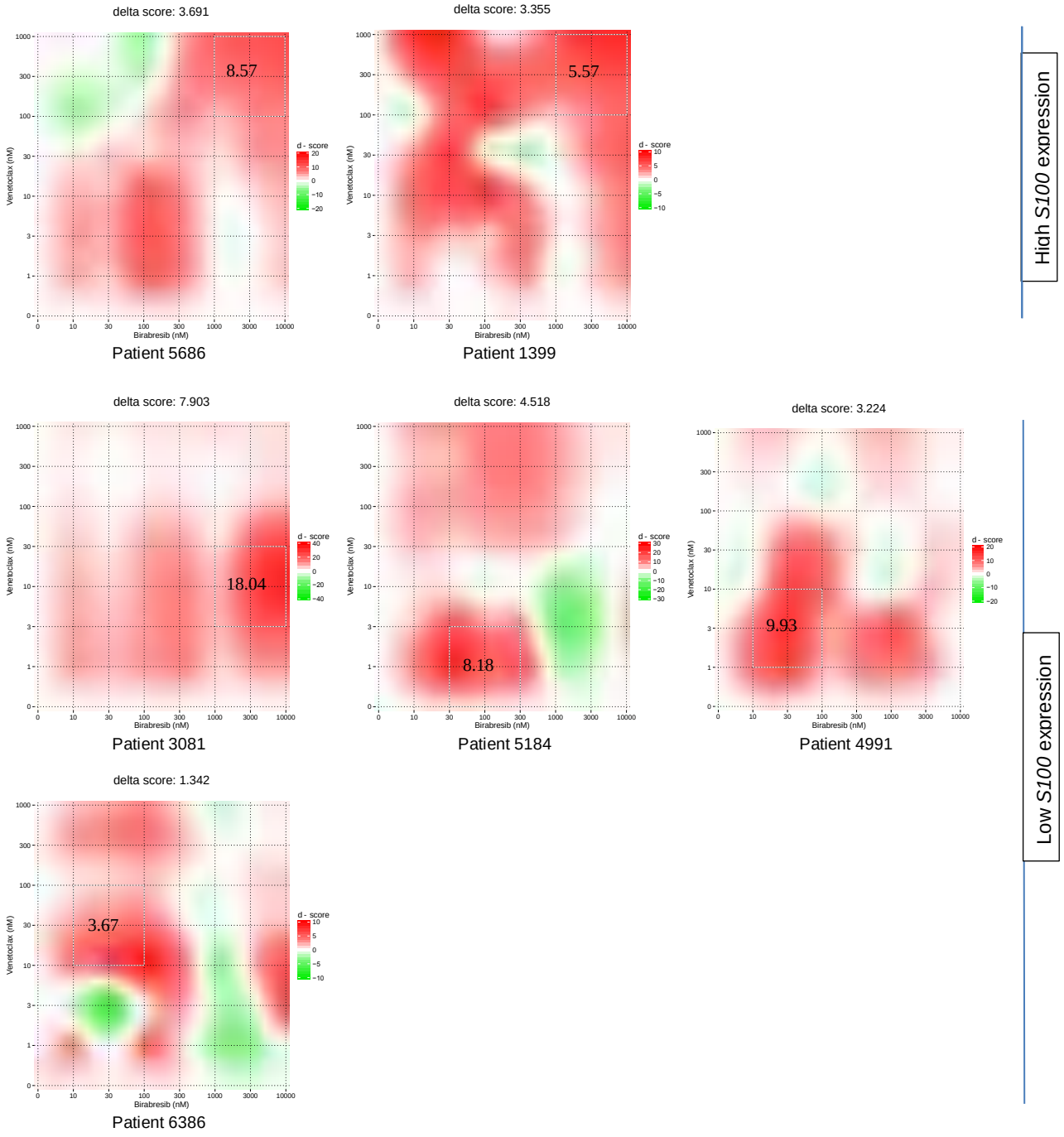
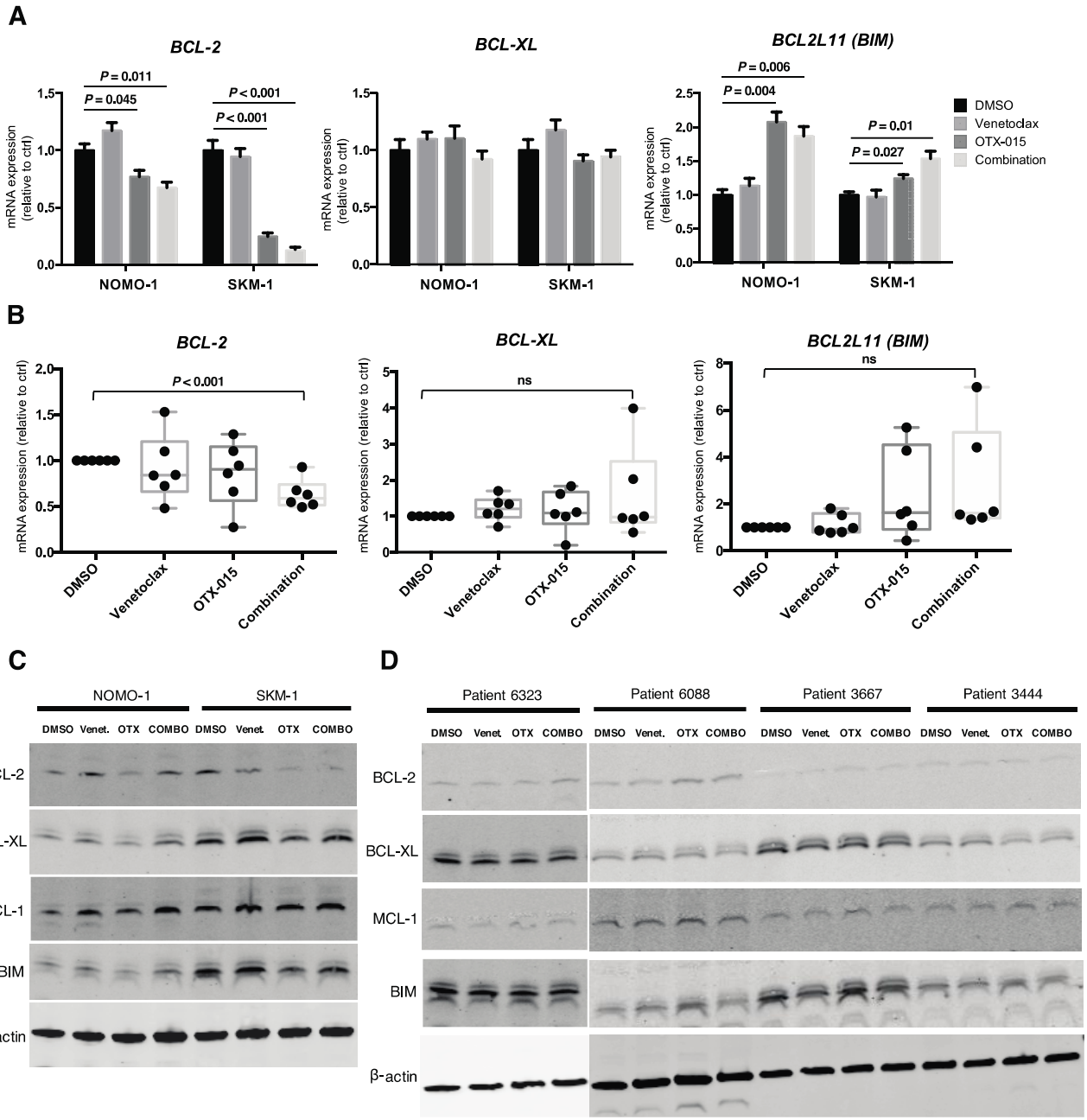
14.10
NOMO-1
delta score: 5.749
20.73
SKM-1
delta score: 2.011
4.12
SHI-1
Cell lines
delta score: 13.892
25.42
Patient 4653
delta score: 9.686
19.33
Patient 6088
delta score: 7.974
14.59
Patient 3667
delta score: 7.893
19.50
Patient 5776
delta score: 7.391
13.25
Patient 5690
delta score: 7.119
10.81
Patient 3444
High S100 expression
delta score: 5.552
14.21
Patient 4907
delta score: 4.394
12.02
Patient 6323
delta score: 3.921
14.63
Patient 252
Venetoclax (nM)
Birabresib (nM)
d - score



Supplementary Figure S7. Combinatorial treatment of AML cells with venetoclax and birabresib (OTX-015) shows synergistic activity in samples overexpressing *S100A8/S100A9*. AML cells were cultured for 72 hours in the presence of various concentrations of the drugs, and cell viability was measured using the CTG assay. For the combination matrices, the interaction landscapes are shown in 2 dimensional. δ, the difference in percentage inhibition compared with the expected additive compound effect calculated by the ZIP model.



Supplementary Figure S8. Mechanistic insights into the synergistic effect of venetoclax and OTX-015 on AML cell lines and patient samples overexpressing *S100A8* and *S100A9*. (A) Analysis of BCL-2 family mRNA transcript levels by RT-qPCR in synergistic AML cell lines and (B) patient samples (n = 6) upon treatment with 100 nM venetoclax, 100 nM OTX-015 or their combination for 48 h. Representative blots showing BCL-2 family proteins in (C) the AML cell lines and (D) four patient samples upon the same treatment. Data are expressed as means ± s.e.m. of three technical replicates. All *P*-values were calculated with two-tailed *t*-tests.