RESEARCH ARTICLE

Figures and figure supplements

High-content microscopy reveals a morphological signature of bortezomib resistance

Megan E Kelley *et al*.

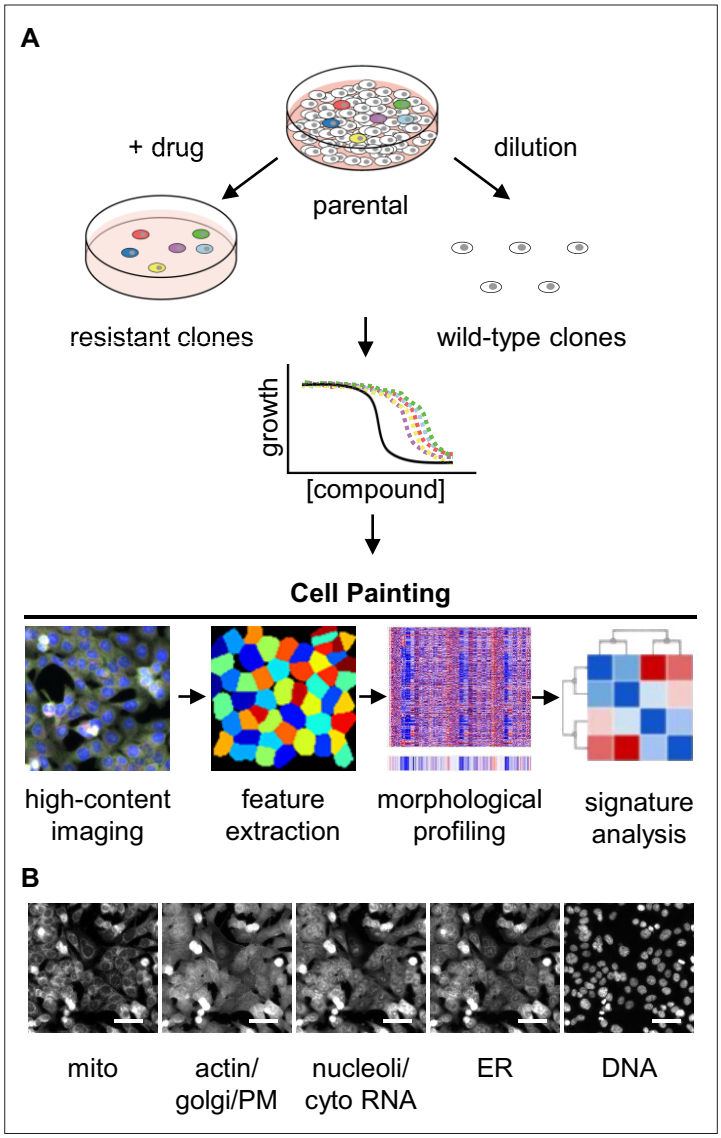

Figure 1. Experimental design for using Cell Painting to examine morphological profiles of drug-resistant cells. (**A**) Graphic of the experimental workflow: we isolated drug-resistant clones by treating parental HCT116 cells with the desired drug and then expanded them for experiments. We isolated drug-sensitive clones by diluting HCT116 cells and then expanded them for experiments. We then performed proliferation assays on select clones to evaluate them for multidrug resistance. Next, we performed Cell Painting on both drug-resistant and -sensitive clones, using multiplexed high-throughput fluorescence microscopy of fixed cells followed by feature extraction and morphological profiling to search for features that contribute to a signature of drug resistance. (**B**) One representative field of view of cells labeled with six fluorescent dyes and captured in five channels used for morphological profiling with Cell Painting. Scale bars, 50 μm.

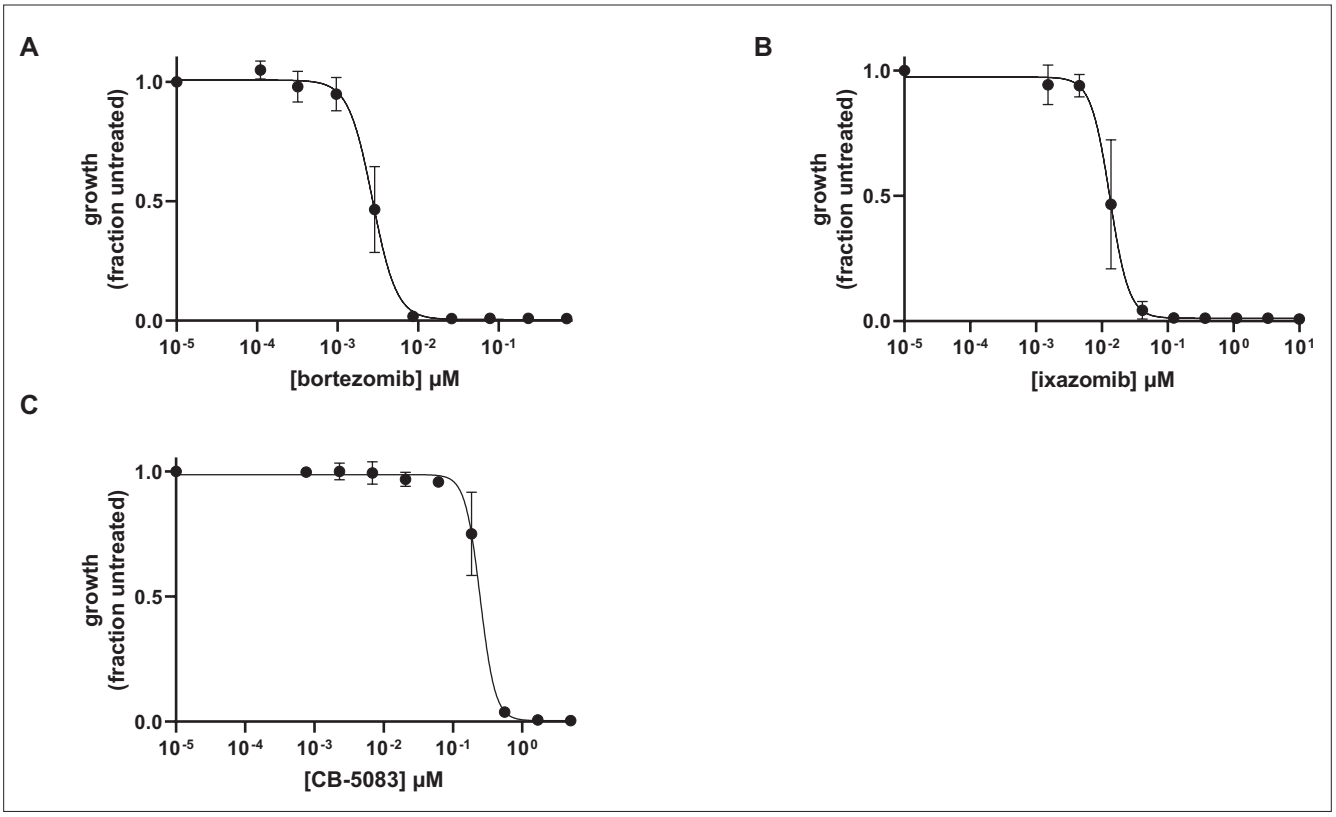

Figure 1—figure supplement 1. Proliferation assays of HCT116 parental cells in drugs used to isolate resistant clones. (**A–C**) Proliferation assays of HCT116 parental cells treated with (**A**) bortezomib, (**B**) ixazomib, and (**C**) CB-5083. Growth is measured relative to untreated cells. Mean ± SD. n = 3 independent experiments (biological replicates) with 3 technical replicates per condition.

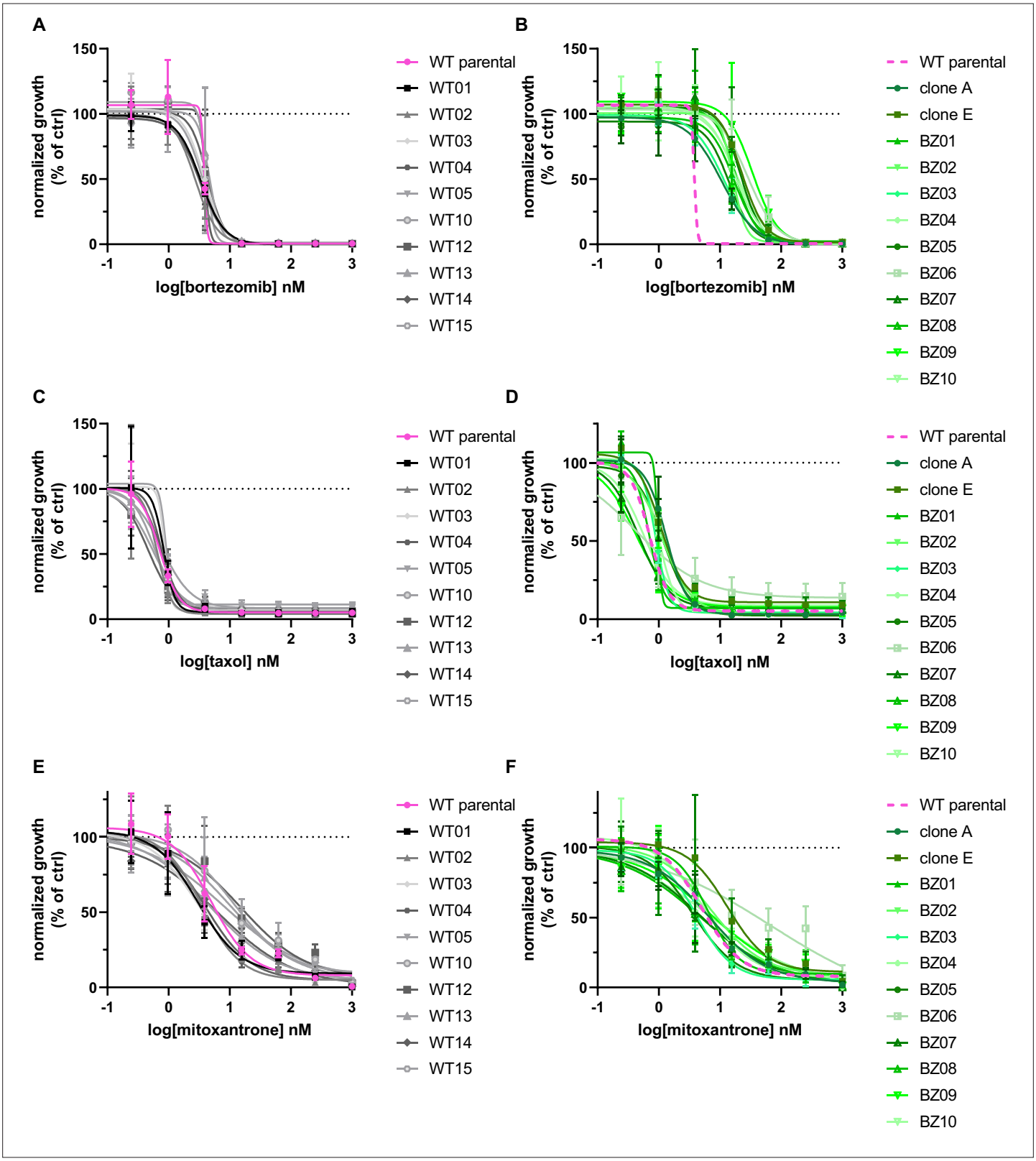

Figure 1—figure supplement 2. Bortezomib-resistant clones do not display strong features of multidrug resistance. Proliferation assays of bortezomib-sensitive (left, WT) and bortezomib-resistant (right, BZ) clones. Cells were treated with (**A and B**) bortezomib, (**C and D**) taxol, or (**E and F**) mitoxantrone. Dashed magenta line in each bortezomib-resistant graph represents data from HCT116 parental cells in the corresponding bortezomib-sensitive graph. Growth is measured relative to untreated control cells (black dotted line). Mean ± SD. n = 3 independent experiments (biological replicates) with 2 technical replicates per condition.

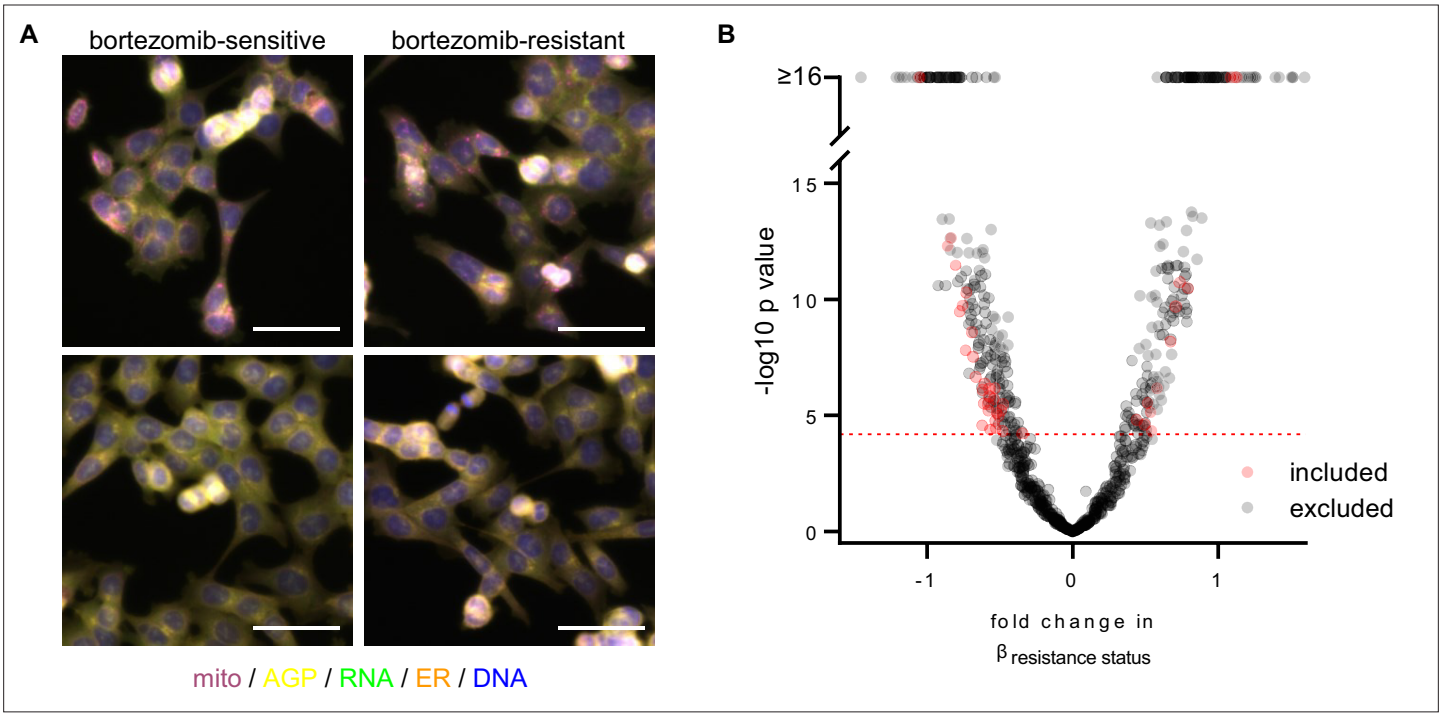

Figure 2. A subset of morphological features contributes to the signature of bortezomib resistance. (**A**) Representative fixed fluorescence microscopy images of two bortezomib-sensitive (WT02 and WT03) and two bortezomib-resistant (BZ02 and BZ03) clones stained and imaged as per the Cell Painting protocol. Channels are labeled as mito (mitochondria; magenta), AGP (actin, golgi, plasma membrane; yellow), RNA (ribonucleic acid; green), ER (endoplasmic reticulum; orange), and DNA (deoxyribonucleic acid; blue). See ***Figure 2—figure supplement 1*** for single-channel images. Scale bars, 50 µm. (**B**) Volcano plot of the variability of morphological features (β) by resistance status. Y-axis -$\log_{10}$p values are from Tukey's Honestly Significant Difference test score (see Materials and methods). Red circles are features included in the final signature of resistance and gray circles are features excluded from the final signature. Features above the red dashed line (-$\log_{10}$[0.05/number of unique features]) were considered significantly varying and those that had not been excluded as technical variables (***Figure 2—figure supplement 3***) were included in the signature of bortezomib resistance. n = 6 independent experiments (biological replicates).

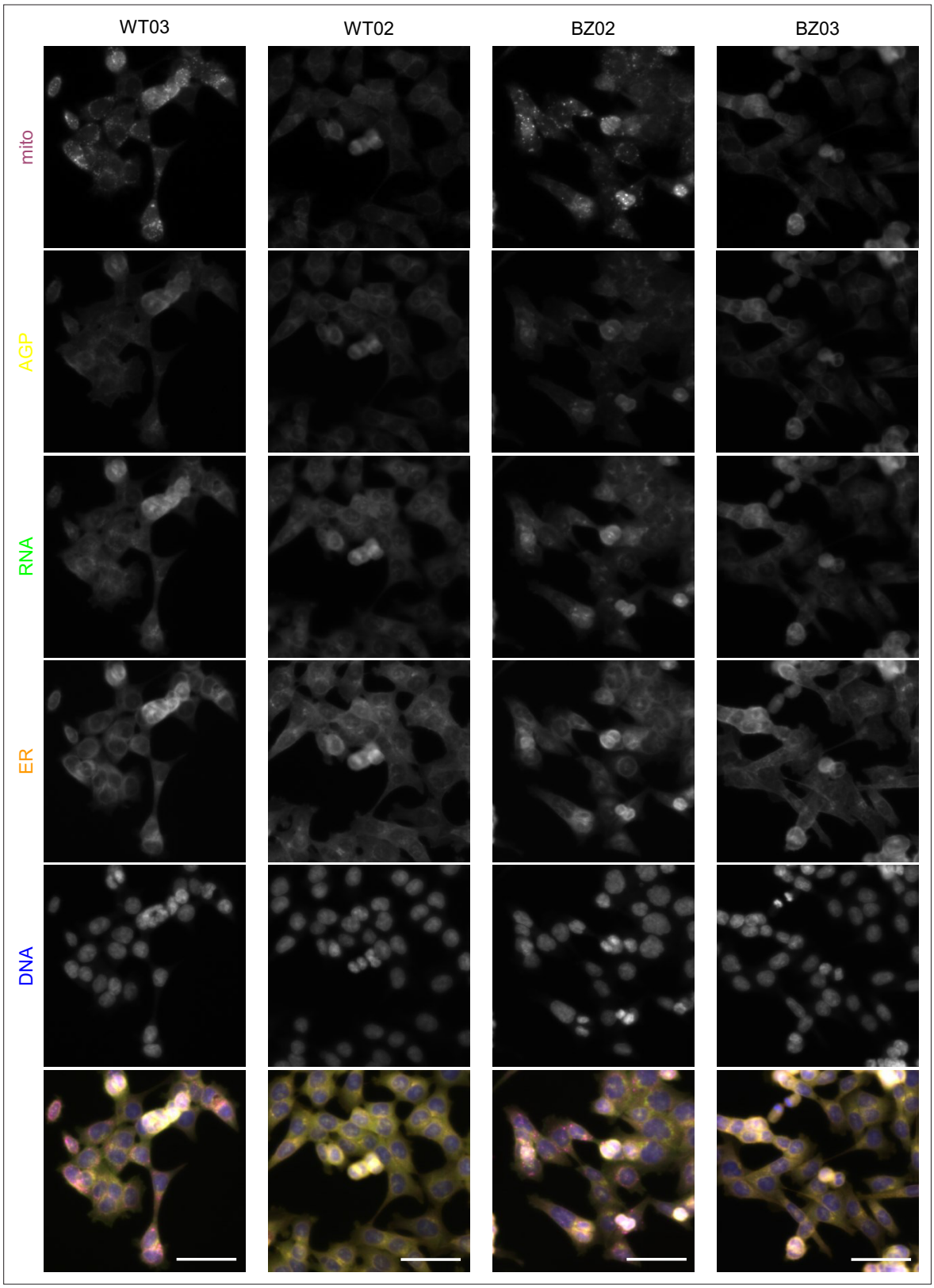

Figure 2—figure supplement 1. Individual channels of bortezomib-sensitive and -resistant clones imaged by Cell Painting. Representative single-channel fixed fluorescence microscopy images of two bortezomib-sensitive (left) and two bortezomib-resistant (right) clones from ***Figure 2A*** stained and imaged as per the Cell Painting protocol. Channels are labeled as mito (mitochondria; magenta), AGP (actin, golgi, plasma membrane; yellow), RNA (ribonucleic acid; green), ER (endoplasmic reticulum; orange), and DNA (deoxyribonucleic acid; blue). Scale bars, 50 μm.

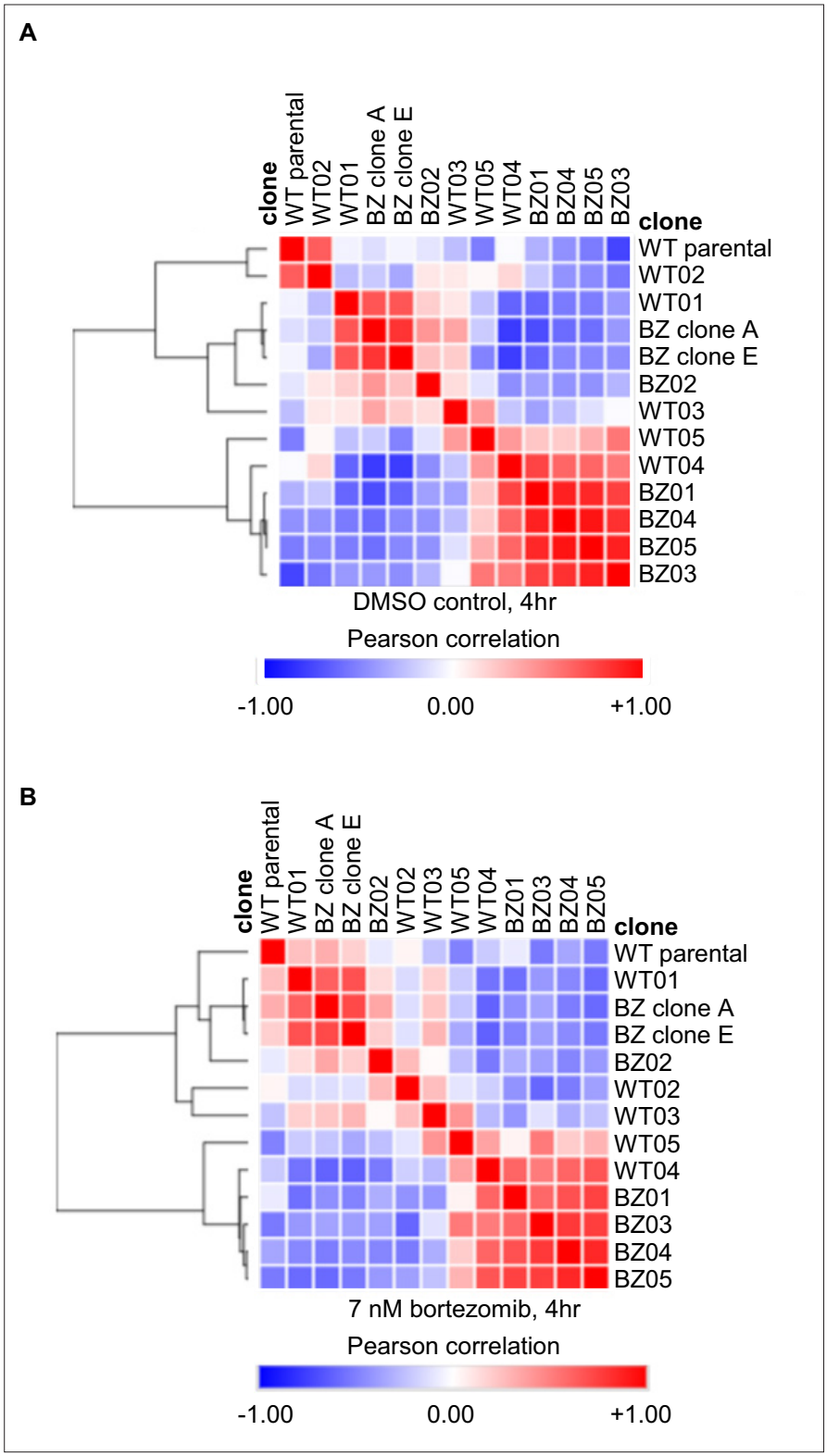

Figure 2—figure supplement 2. Similarity clustering is insufficient to distinguish bortezomib-resistant from -sensitive clones. Similarity matrices of pairwise Pearson correlation coefficients for morphology profiles of bortezomib-resistant and -sensitive clones treated for 4 hr with either (**A**) 0.1% DMSO or (**B**) 7 nM bortezomib. Dendrograms display hierarchical clustering of pairwise similarity.

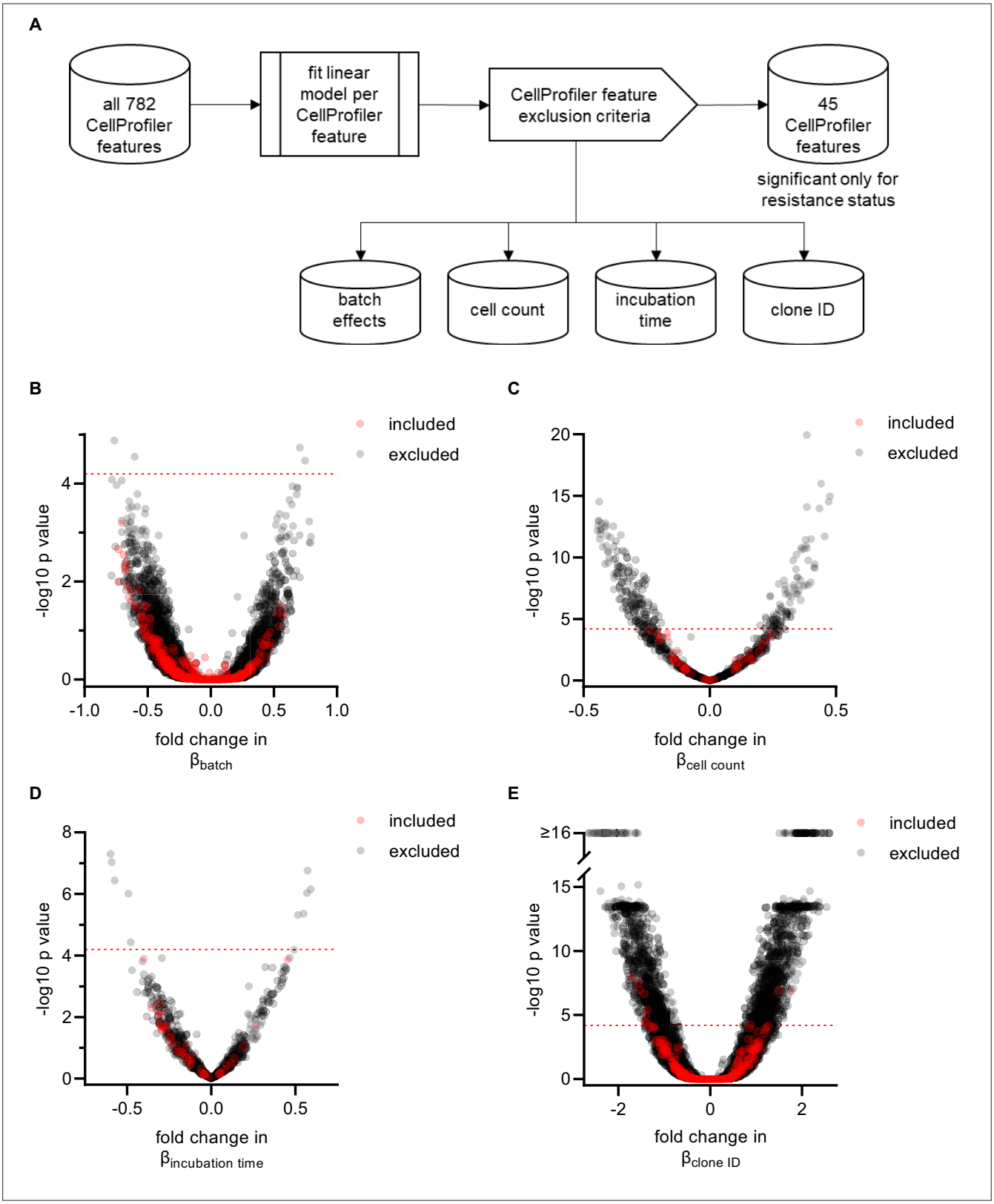

Figure 2—figure supplement 3. Technical variables are controlled for and excluded from analyses. (**A**) Model of the workflow to exclude CellProfiler features not related to resistance. (**B–E**) Volcano plots of the variability of morphological features (β) by (**B**) batch, (**C**) cell count, (**D**) incubation time, and (**E**) clone ID. Y-axis -$\log_{10}$p values are from Tukey's Honestly Significant Difference test score (**B and D–E**) and linear regression analysis (**C**). Red circles are features included in the final signature of resistance and gray circles are features excluded from the final signature. For technical variables (**B–E**), features

Figure 2—figure supplement 3 continued on next page

Figure 2—figure supplement 3 continued

above the red dashed line (-$\log_{10}$[0.05/number of unique features]) were considered significantly varying and were excluded from the signature of bortezomib resistance. Note that some features above the red line in (**E**) vary between only one pair of bortezomib-sensitive clones and were therefore not necessarily excluded from the final signature. n = 6 independent experiments.

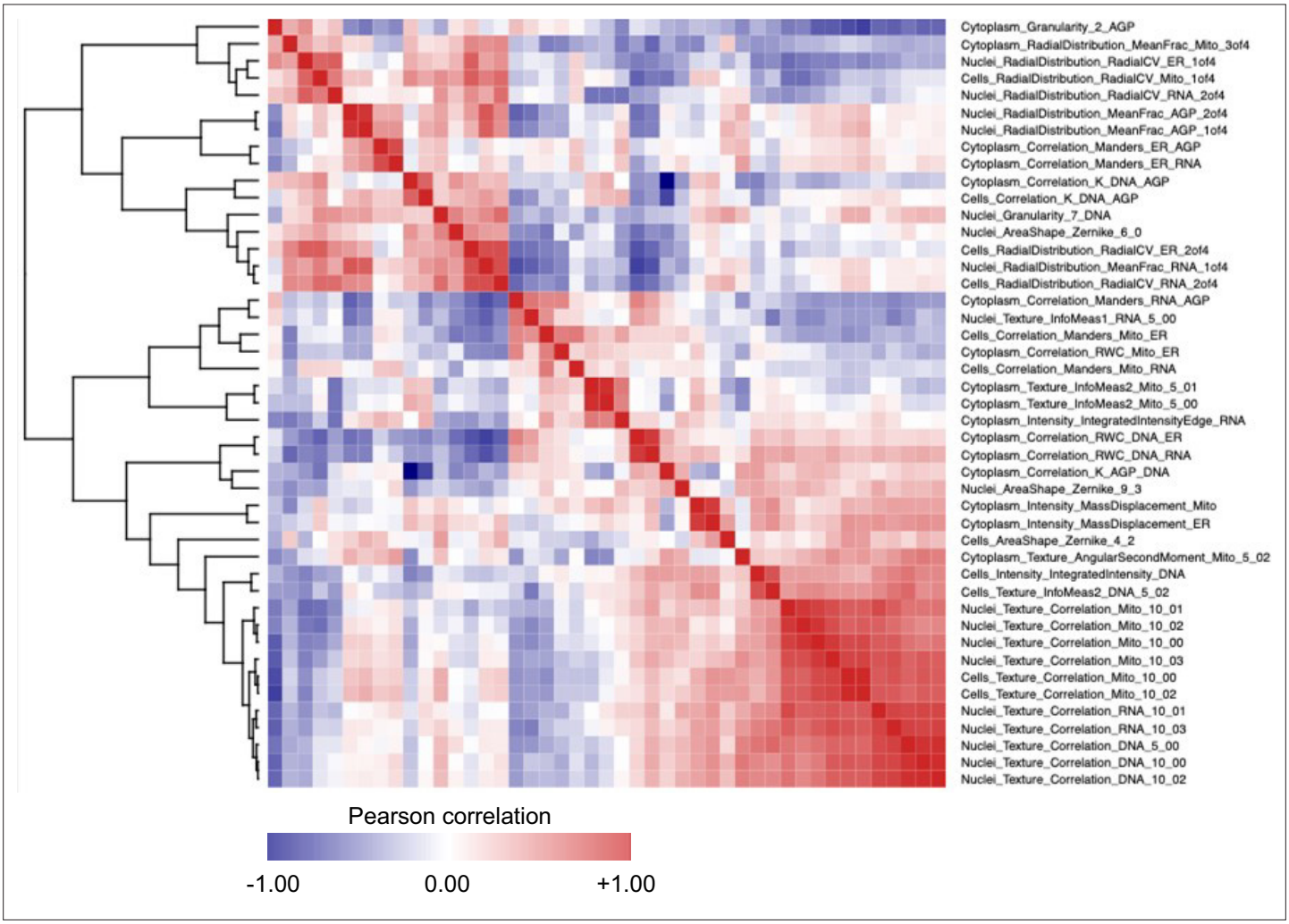

Figure 2—figure supplement 4. Features contributing to the Bortezomib Signature do not universally correlate. Similarity matrix of pairwise Pearson correlation coefficients of all 45 Bortezomib Signature features. Dendrogram displays hierarchical clustering of pairwise similarity.

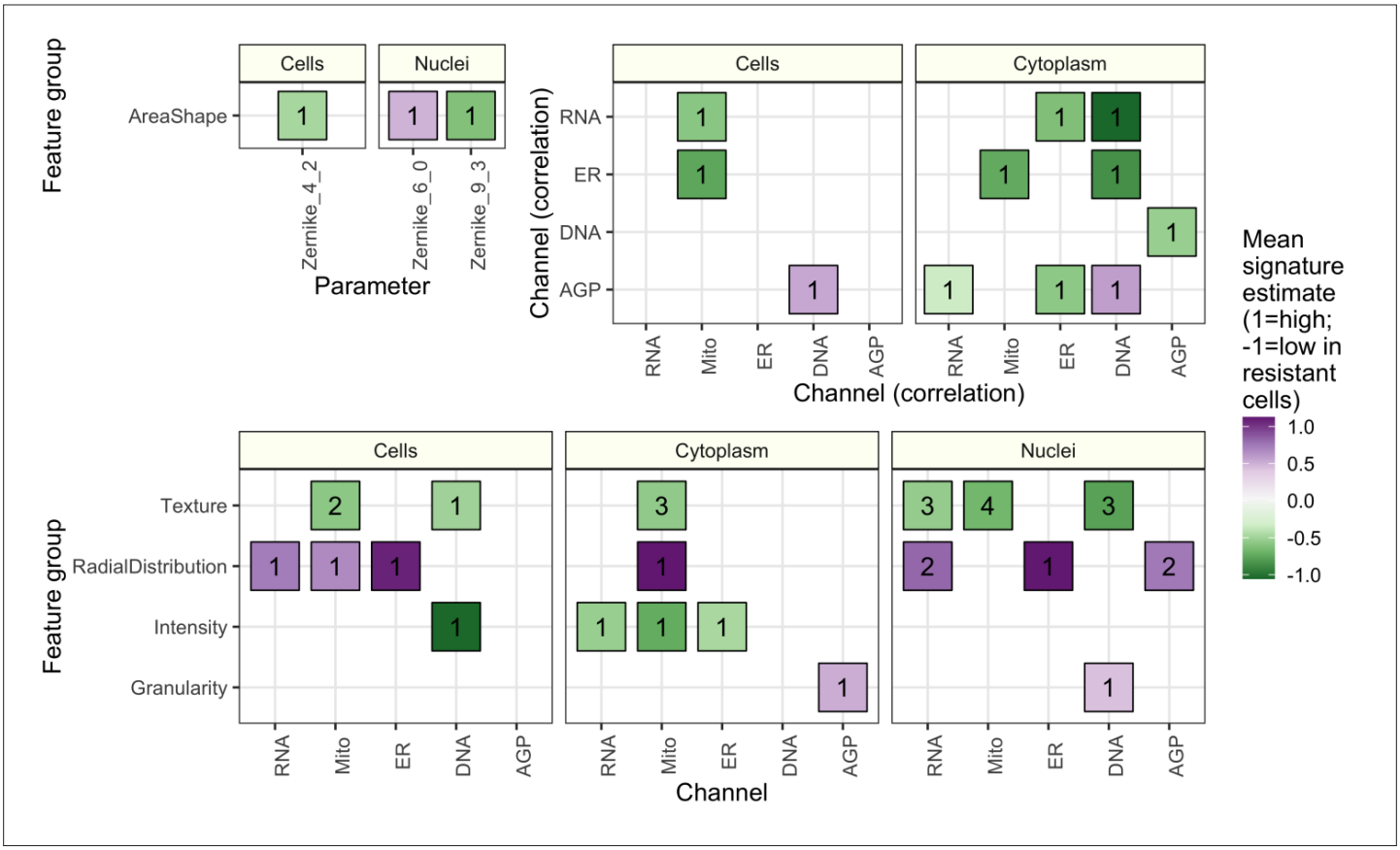

Figure 2—figure supplement 5. Bortezomib Signature visualized by CellProfiler features. Visualization of CellProfiler features contributing to the Bortezomib Signature. Features with high values (mean signature estimates) in resistant cells are purple while features with low values in resistant cells are green. The mean signature estimates were based on Tukey's Honestly Significant Difference test score and the number in each box represents the number of features used to calculate the mean signature estimate.

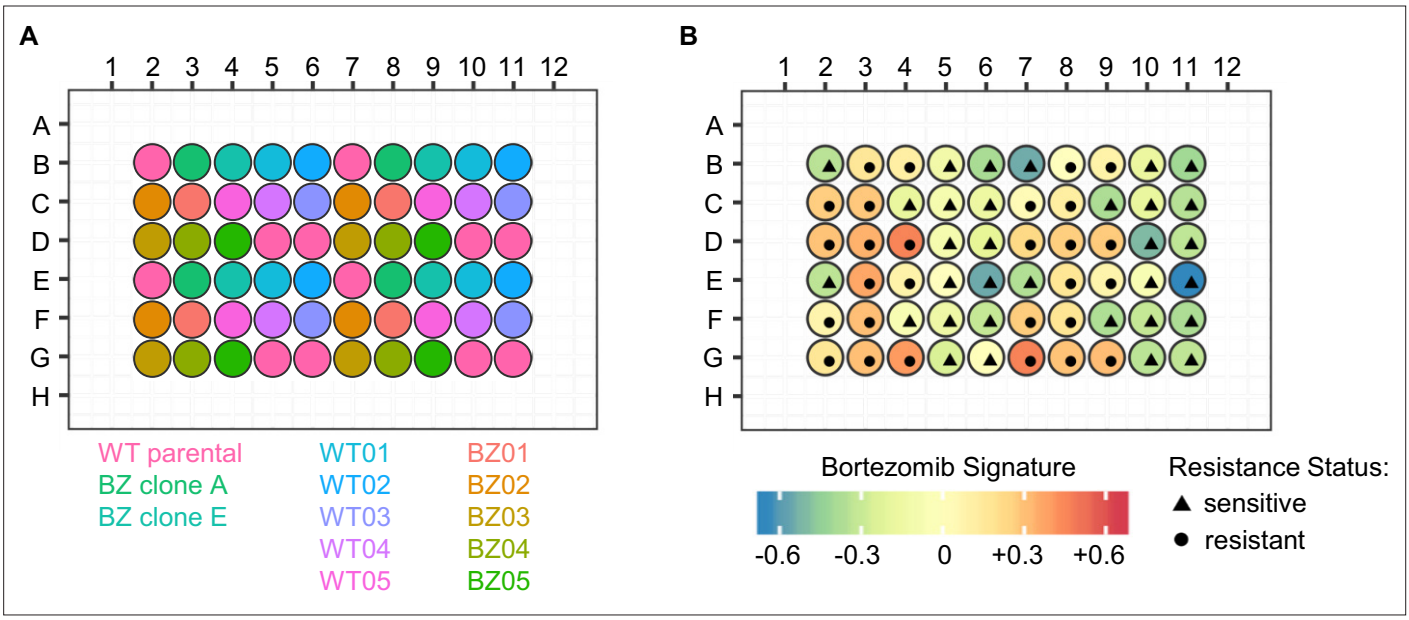

Figure 2—figure supplement 6. Well location does not strongly correlate with Bortezomib Signature. (**A**) Schematic of the plating pattern for cells in a representative Cell Painting assay. Different clones are distinguished by color. (**B**) Bortezomib Signature for each clone in (**A**).

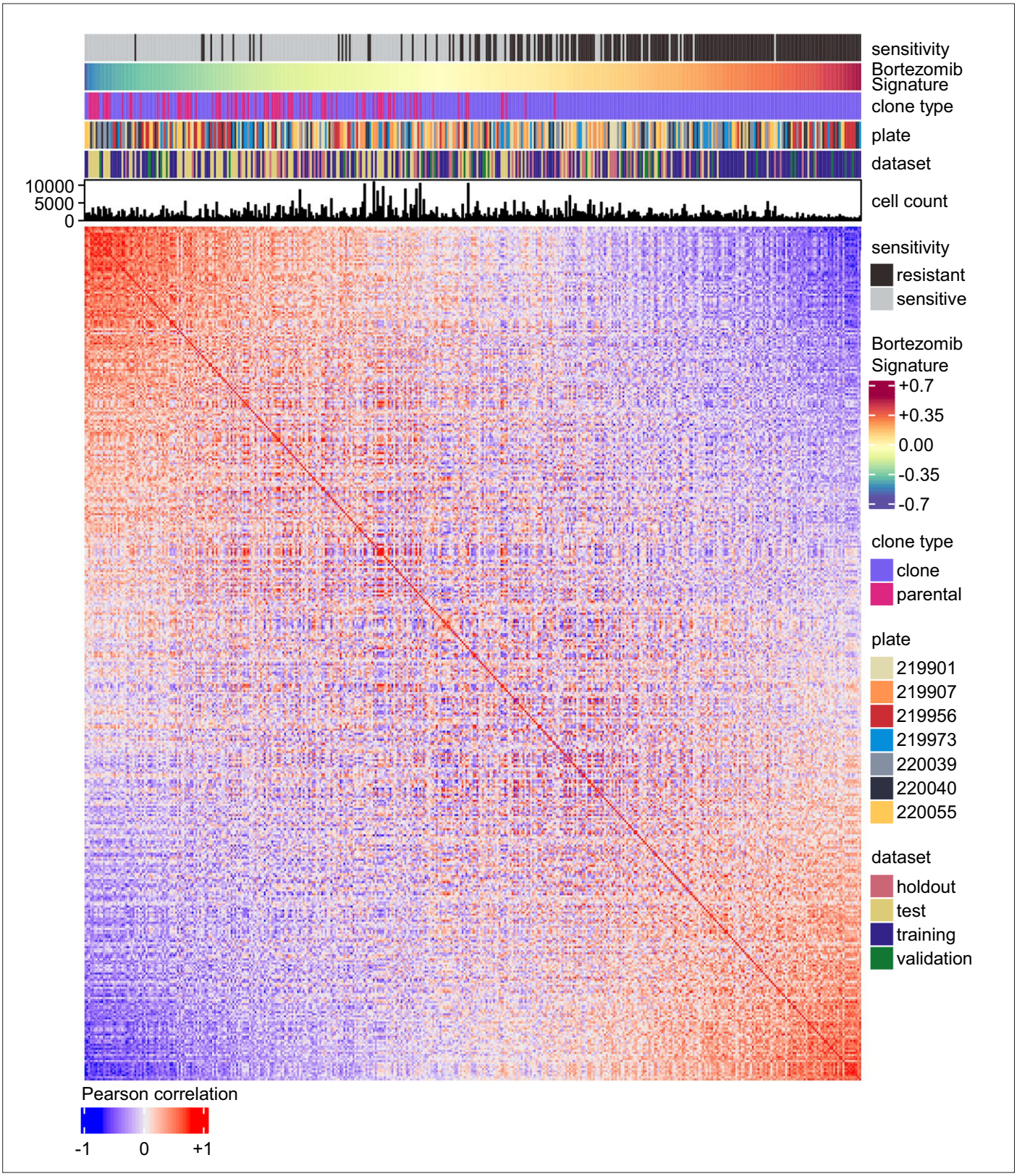

Figure 2—figure supplement 7. Bortezomib Signature identifies resistant clones and not technical variables. Similarity matrix of Pearson correlation coefficients for well-level profiles of clones. Drug sensitivity, Bortezomib Signature, clone type (polyclonal HCT116 parental or clonal), plate number (batch), dataset, and cell count are indicated.

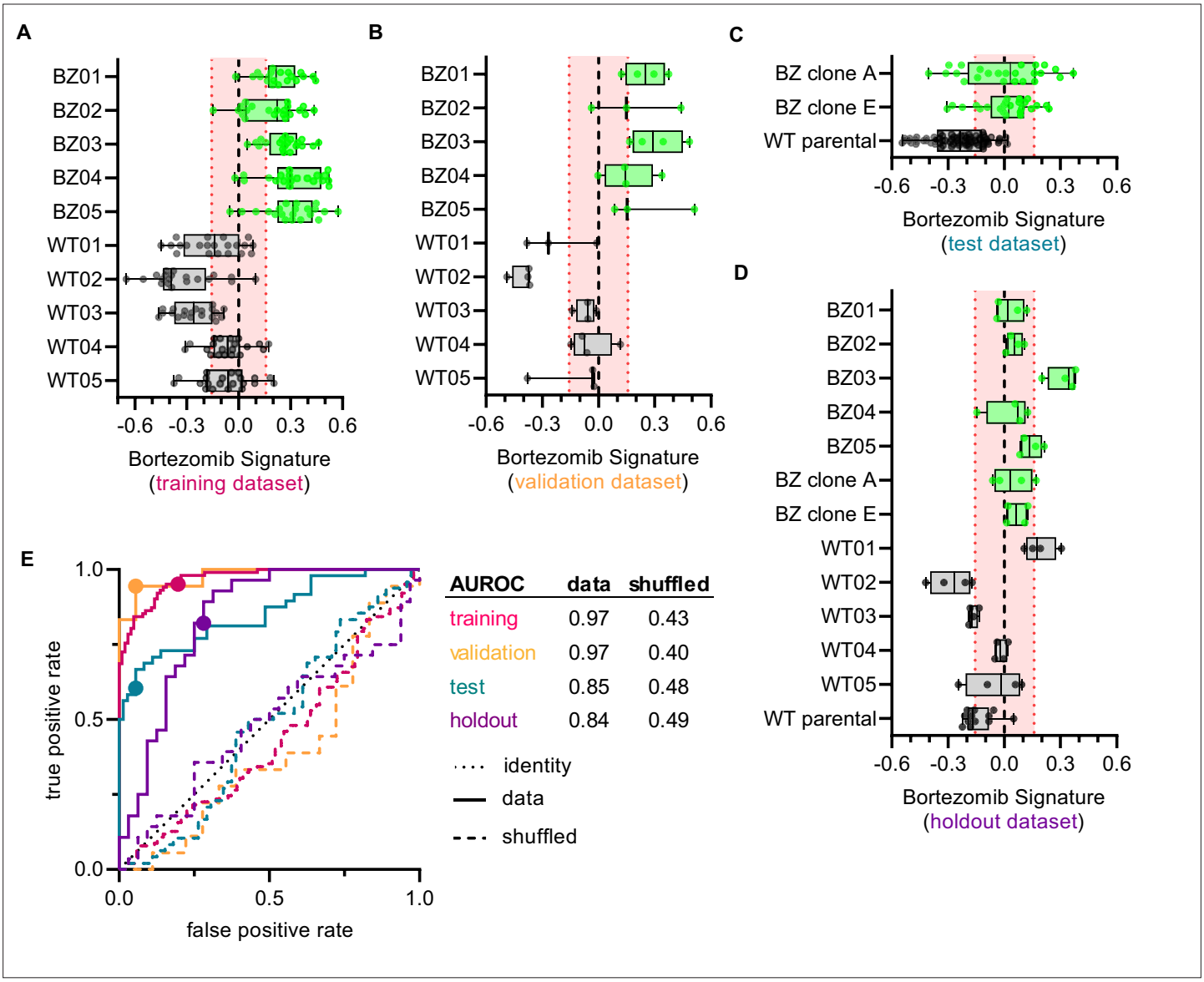

Figure 3. Cell morphology predicts the bortezomib sensitivity of clones across datasets. Box plots of Bortezomib Signatures for clones in the (**A**) training, (**B**) validation, (**C**) test, and (**D**) holdout datasets. Plots show values for individual well profiles (points), range (error bars), 25th and 75th percentiles (box boundaries), and median. Dashed vertical black line is Bortezomib Signature = 0, dashed vertical red lines are the 95% confidence interval for Bortezomib Signatures of 1000 random permutations of the data. (**E**) ROC curves for the performance of the Bortezomib Signature on the indicated dataset (solid line) or its shuffled counterpart (dashed line). Datasets are designated by color: training (magenta), validation (orange), test (teal), and holdout (purple). Colored points are the corresponding false positive and true positive rates at the absolute minimum thresholds for each respective dataset. Black dotted line is the identity line where false positive rate = true positive rate. AUROC values reported for data and shuffled data. See ***Figure 3—source data 1*** for breakdown of profiles and experiments per dataset.

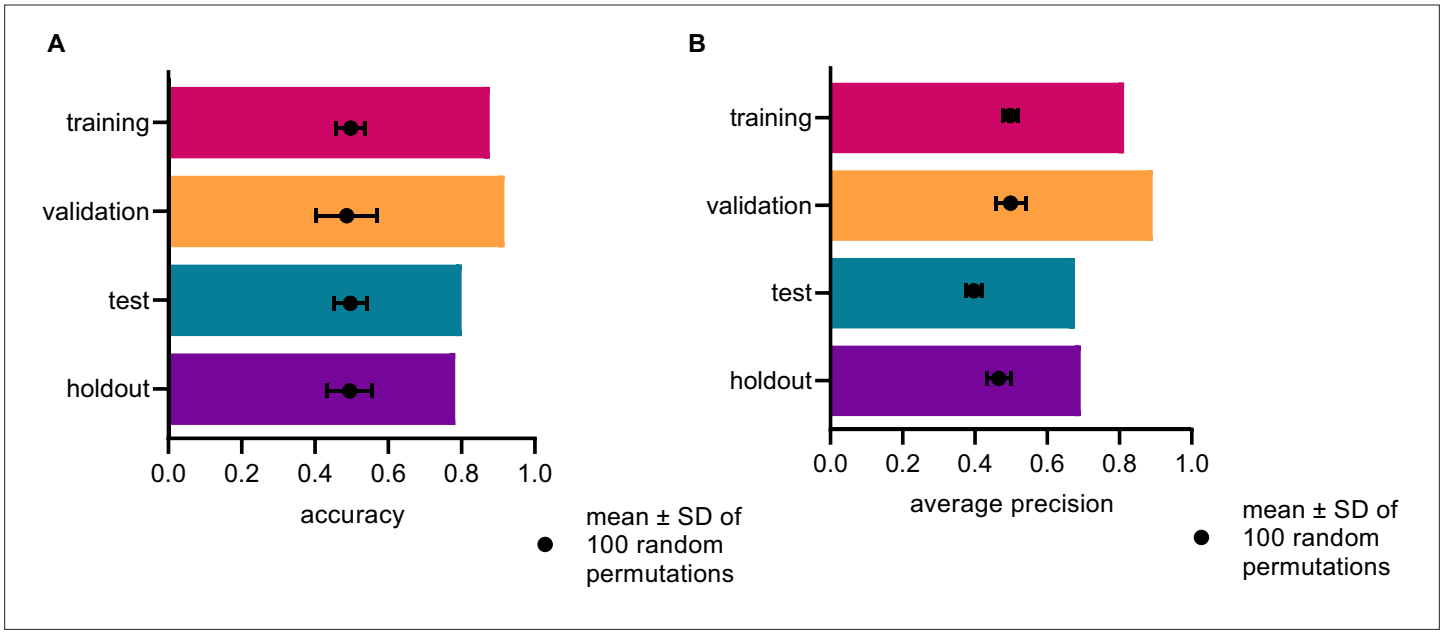

Figure 3—figure supplement 1. Accuracy and average precision of the Bortezomib Signature. Plots showing the (**A**) accuracy (the number of correctly classified clones divided by the total number of clones) and (**B**) average precision (see Materials and methods) of the Bortezomib Signature when classifying the resistance status of clones separated by dataset (colored bars). Black symbols and error bars are the means of 100 random permutations of the data ± SD. See ***Figure 3—source data 1*** for breakdown of profiles and experiments per dataset.

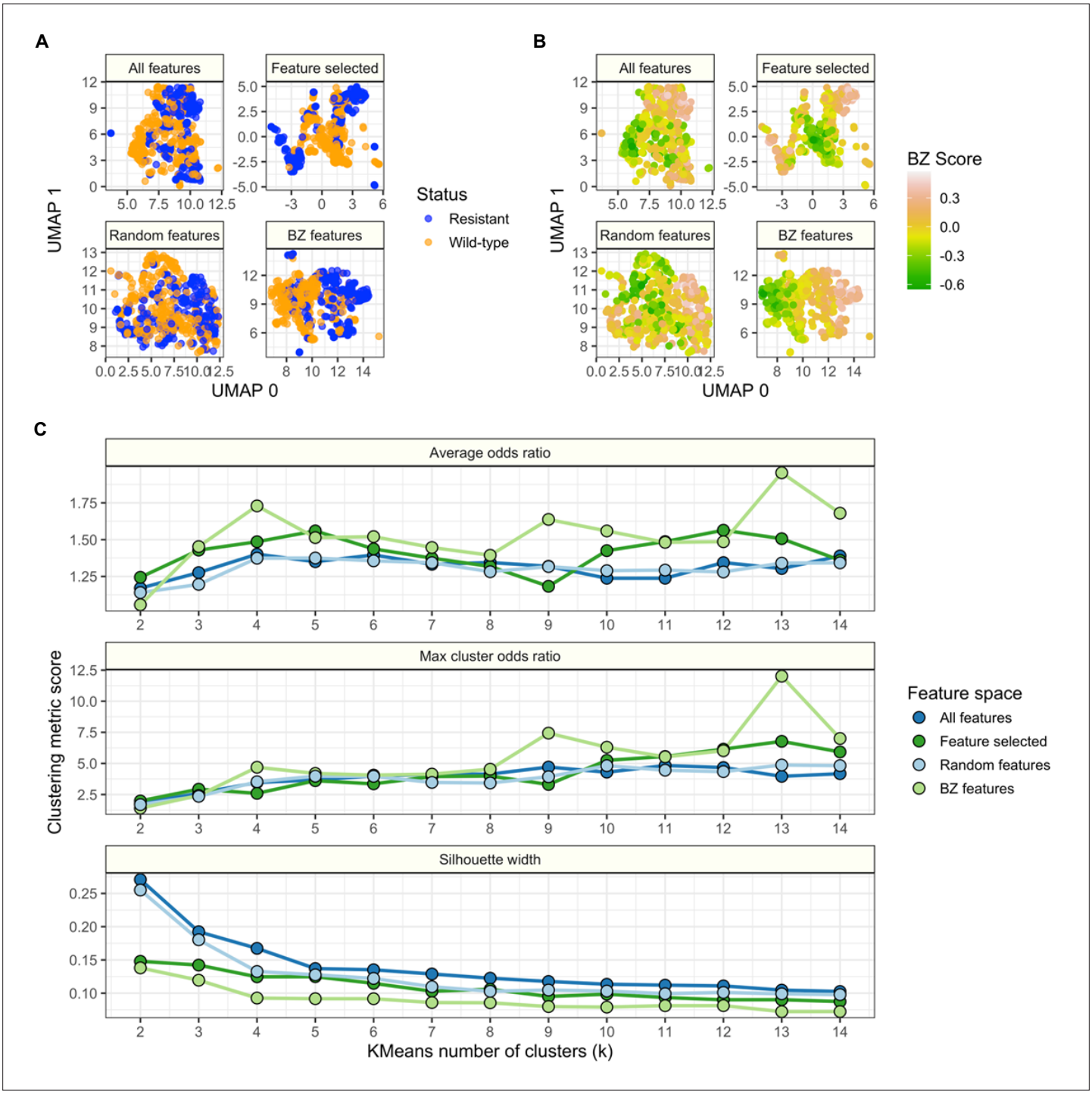

Figure 3—figure supplement 2. Benchmarking linear-modeling feature selection to separate clones by bortezomib resistance. Uniform Manifold Approximation and Projection (UMAP) analysis of the qualitative separability of (**A**) resistance status and (**B**) Bortezomib Signature scores across four different feature spaces. (**C**) k-means clustering from k = 2 to k = 14 of average odds ratio, maximum odds ratio (Fisher's exact test), and Silhouette width using Bortezomib Signature features.

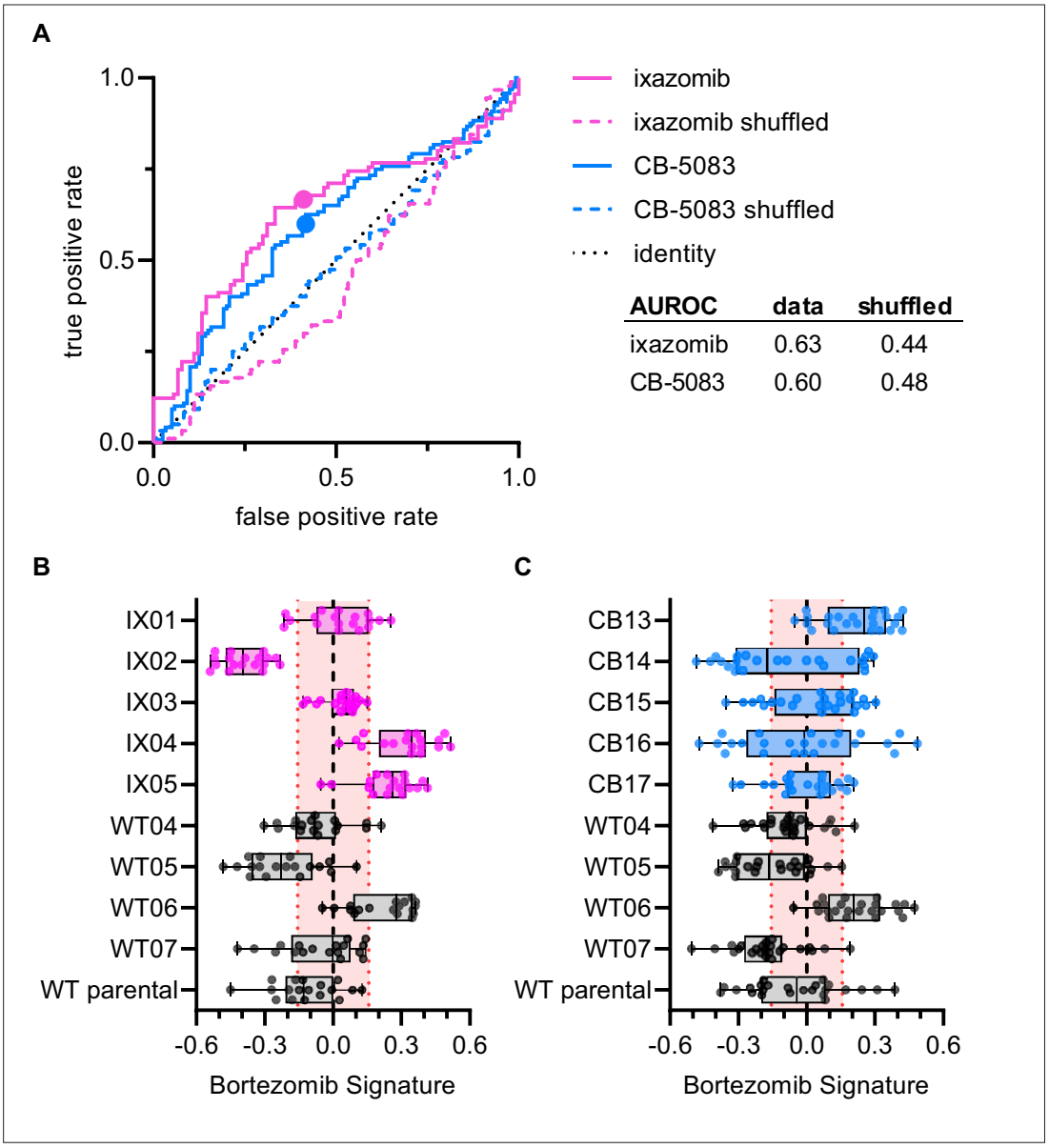

Figure 4. Bortezomib Signature has limited ability to characterize clones resistant to other UPS-targeting drugs. (**A**) ROC curves for ixazomib-resistant (magenta) and CB-5083-resistant (blue) experimental data. Colored solid lines are the actual data while colored dashed lines are the shuffled data for each set of clones. Colored points are the corresponding false positive and true positive rates at the absolute minimum thresholds for each respective cell type. Black dotted line is the identity line where false positive rate = true positive rate. AUROC reported for the data and shuffled data. Box plots of Bortezomib Signatures for (**B**) ixazomib-resistant and bortezomib-sensitive clones (n = 18 profiles, 3 independent experiments) and (**C**) CB-5083-resistant and bortezomib-sensitive clones (n = 24 profiles, 4 independent experiments). Plots show values for individual well profiles (points), range (error bars), 25th and 75th percentiles (box boundaries), and median. Dashed vertical black line is Bortezomib Signature = 0, dashed vertical red lines are the 95% confidence interval for Bortezomib Signatures of 1000 random permutations of the data.

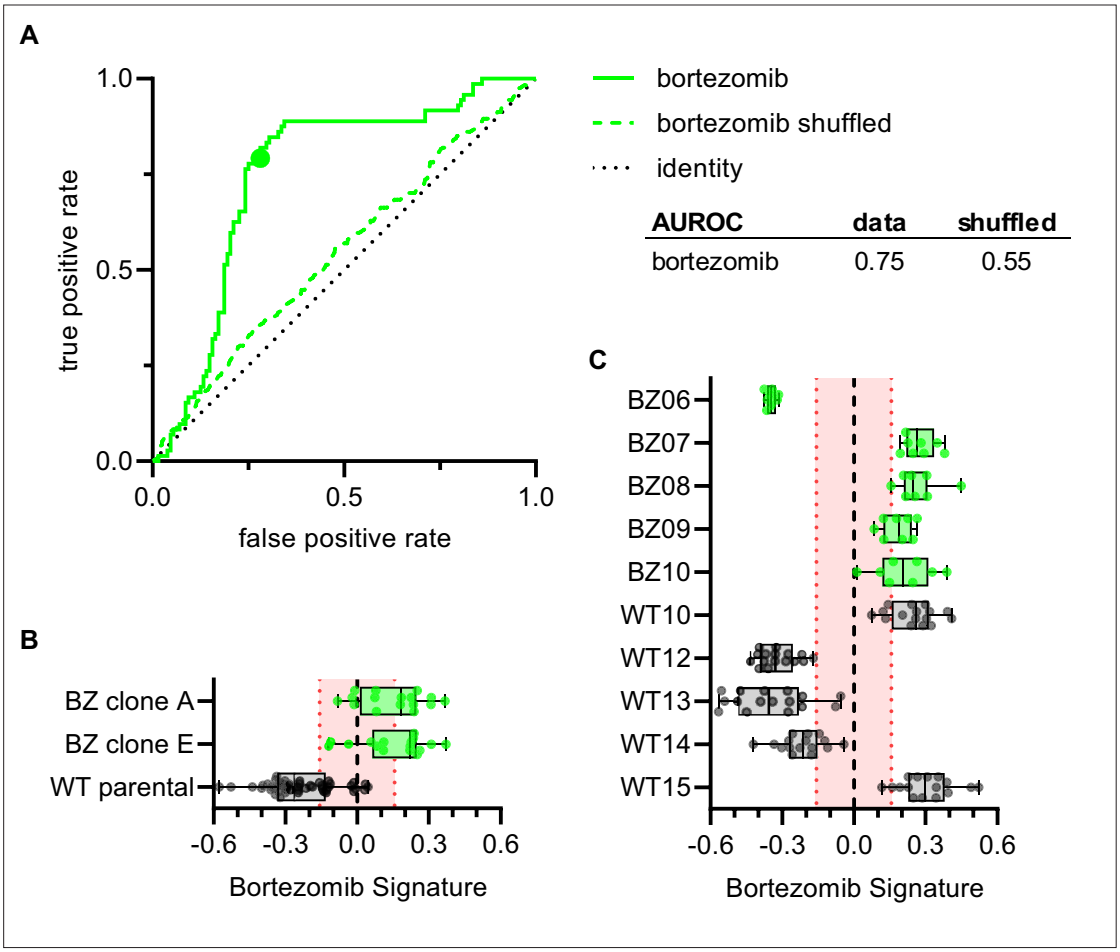

Figure 5. Bortezomib Signature correctly characterizes bortezomib sensitivity of seven out of ten clones not included in the training, validation, test, or holdout datasets. (**A**) ROC curve for the Bortezomib Signature of clones in (**B**) and (**C**) (solid line) and shuffled data (dashed line). Colored point is the corresponding false positive and true positive rate at the absolute minimum threshold. Black dashed line is the identity line where false positive rate = true positive rate. AUROC reported for the data and shuffled data. (**B**) Box plots of Bortezomib Signatures for bortezomib-resistant clones A and E (n = 16 profiles each) and HCT116 parental cells (n = 48 profiles). (**C**) Box plots of Bortezomib Signatures for bortezomib-sensitive clones WT10, WT12-15 (n = 16 profiles each) and bortezomib-resistant clones BZ06-10 (n = 8 profiles each). Plots show values for individual well profiles (points), range (error bars), 25th and 75th percentiles (box boundaries), and median. Dashed vertical black line is Bortezomib Signature = 0, dashed vertical red lines are the 95% confidence interval for Bortezomib Signatures of 1000 random permutations of the data. 4 independent experiments (biological replicates).

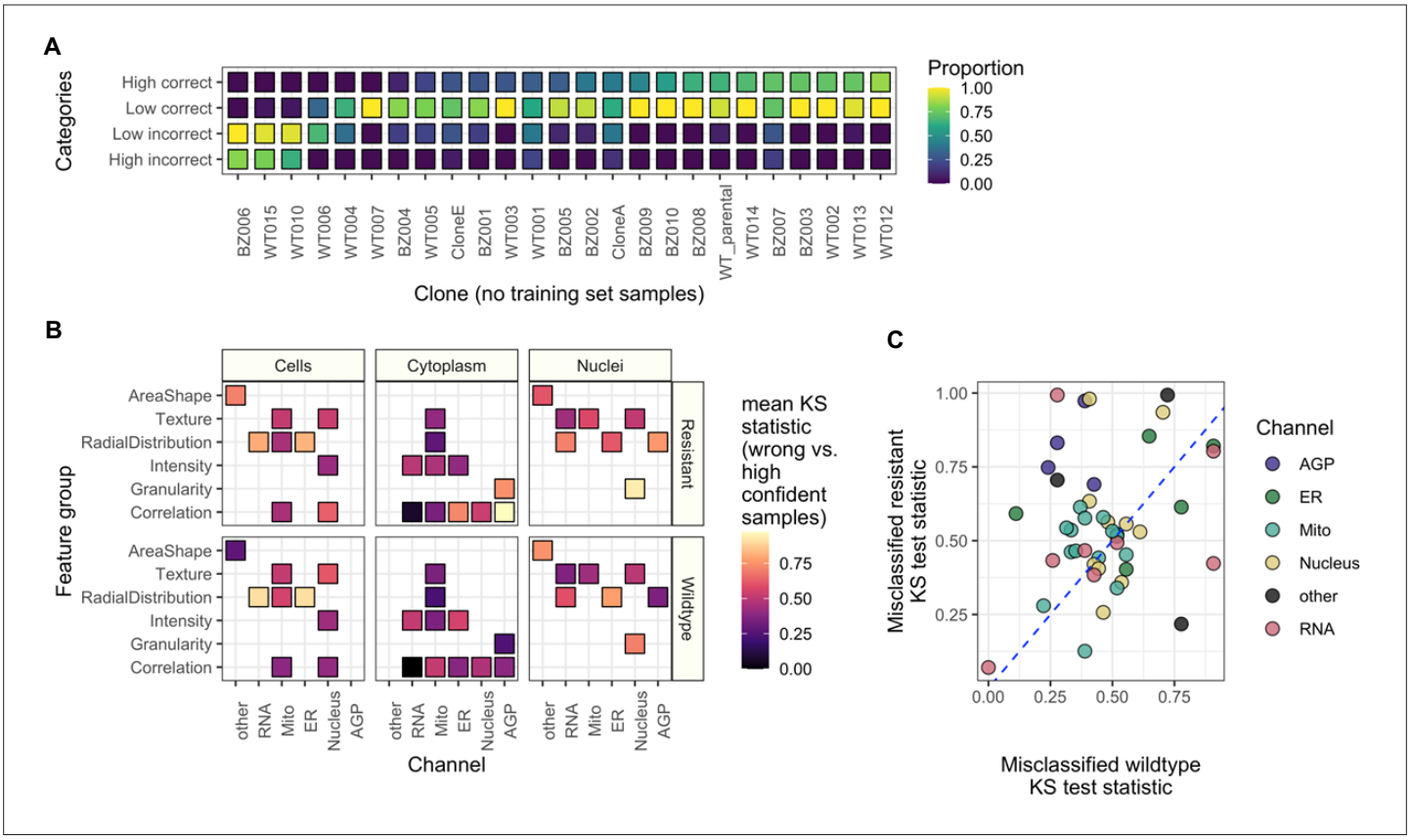

Figure 5—figure supplement 1. Examining the accuracy of clone classification and misclassification of clones. (**A**) Proportion of high-confidence correct, low-confidence correct, low-confidence incorrect, and high-confidence incorrect predictions of well profiles across clones in the test, holdout, and validation sets. High-confidence predictions (high) had a Bortezomib Signatures greater (resistant clones) or less than (sensitive) the 95% confidence interval of randomly permuted data while low-confidence predictions (low) had Bortezomib Signatures within the 95% confidence interval of randomly permuted data. (**B**) Visualization of Kolmogorov-Smirnov (KS) test statistic means of feature groups across channels and cellular compartments. (**C**) Plot of the KS test statistic means for feature groups in bortezomib-resistant vs. -sensitive cells. Each feature group is color coded by the imaging channel.