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14. ABSTRACT In this study, we perform a targeted kinase inhibitor screen and identify a tool compound, named MTX-216, to be highly effective in blocking NF1-LoF melanoma cells. Single-cell analysis links drug-induced cytotoxicity to effective co-suppression of proliferation marker Ki-67 and the ribosomal S6 phosphorylation. We find the anti-tumor efficacy of MTX-216 to be dependent on its ability to inhibit not only PI3K but also SYK and suppression of a group of genes that regulate mitochondrial electron transport chain. Furthermore, combinations of inhibitors targeting either MEK or PI3K/mTOR with an independent SYK kinase inhibitor or SYK knockdown show favorable effects. These studies provide a path to exploit SYK dependency to selectively block NF1-LoF melanoma cells. In parallel, through systematic experimental measurements, single-cell analysis, and long-duration live-cell microscopy experiments, we have identified potentially novel effective drugs or drug combinations that overcome cell-to-cell variability responsible for incomplete efficacy of currently available MAPK inhibitor treatments across genetically diverse BRAF-mutant melanoma contexts.					
15. SUBJECT TERMS melanoma, MAPK signaling pathway, NF1 loss of function, incomplete drug efficacy, phenotype switching, cell-to-cell variability, small-molecule kinase inhibitors, SYK kinase, mitochondrial electron transport chain, drug discovery, systems biology					
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1. INTRODUCTION:

The overall goal of this project is to identify and target pathway rewiring mechanisms and epigenetic vulnerabilities in genetically different melanomas that exhibit resistance to targeted inhibitors of MAPK signaling. Melanoma is characterized by predominant mutations that activate the MAPK signaling pathway, including mutations in the BRAF and NRAS oncogenes and loss of the tumor suppressor gene NF1, a negative regulator of RAS. Despite genetic heterogeneity, melanomas exhibit significant dependency on MAPK signaling, motivating the use of targeted inhibitors of this pathway as potential therapies. Pathway inhibition in melanoma cells, however, results in a mixture of unpredictable phenotypes, generating heterogeneous populations of drug-tolerant cells. This phenomenon, known as phenotype switching, causes fractional response even in isogenic tumor cells. Although plasticity of melanoma cell states and its consequences for therapy resistance are recognized, it remains unclear how many identifiable functional states there are, what molecular mechanisms – either lineage-specific, oncogene-specific, or mutation-specific – drive switching between those states, and how they determine drug response in individual tumor cells. In this project, we aim to: (1) define the molecular mechanisms of phenotype switching following MAPK pathway inhibition in genetically diverse melanoma cells, (2) Identify new targets for modulation in BRAF-mutant and NF1-deficient melanomas to selectively maximize tumor cell death in combination with MAPK pathway inhibition, and (3) test whether the characterized epigenetic states are predictive of response to therapy in xenograft models of melanoma. If successful, this project will uncover the molecular origins of phenotype switching and adaptation to MAPK pathway inhibition in genetically diverse melanomas, assist in identifying predictive biomarkers and ultimately improve precision melanoma therapy by suggesting strategies to overcome drug resistance.

2. KEYWORDS:

melanoma, MAPK signaling pathway, NF1 loss of function, incomplete drug efficacy, phenotype switching, cell-to-cell variability, small-molecule kinase inhibitors, SYK kinase, mitochondrial electron transport chain, drug discovery, systems biology

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aims (specified in proposal)	Timeline
Major Task 1: Define the molecular mechanisms of phenotype switching following ERK pathway inhibition in genetically diverse BRAF^{V600E}, NRAS^{Q61R} and NF1-deficient melanoma cells.	Months
Subtask 1: Fixed-cell microscopy to characterize drug-induced changes in the state of signaling and phenotypic state in response to MAPK inhibitors. A collection of 10 melanoma cell lines: two BRAF-mutant (COLO858, MMACSF), four NRAS-mutant	1-12

(SKMEL30, WM2032, WM3000, WM3506), and four NF1-deficient cell lines (COLO792, MeWo, SKMEL113, WM3918) provided by commercial sources, including ATCC and Rockland Immunochemicals Inc. will be used. Cells will be treated with a BRAF inhibitor (vemurafenib), one or more of the MEK inhibitors (trametinib and selumetinib), and one or more of the ERK kinase inhibitors (ulixertinib and VTX11e) at 5 doses and 3 time-points (24, 72 and 120 h). This analysis will serve as a platform to identify the appropriate conditions (cell lines, drugs and doses) for the follow-up live-cell microscopy assays (based on the measured markers of resistance phenotypes).	
Subtask 2: Transfect melanoma cells to express genetically encoded reporters, including H2B, ERK activity and cell cycle progression (i.e. geminin). Four cell lines, representing diverse signaling and phenotypic behavior (as measured by fixed-cell assays in subtask 1) will be selected for transfection.	6-18
Subtask 3: Live-cell microscopy of transfected cell lines to characterize the dynamics of drug response and single-cell heterogeneity. Cells will be monitored via real-time, time-lapse imaging for 120 h in selected conditions of BRAF, MEK and ERK inhibitor treatment. Minimum 4 drug conditions and three doses will be tested across the cell lines.	6-18
Subtask 4: Time-lapse single-cell tracking and analysis	6-18
Subtask 5: Measure histone modification states at a single-cell level. Measurements will be performed using immunofluorescence staining of global levels of a set of six histone modifications (including H3K4me3, H3K36me3, H3K4me1, H3K27ac, H3K9me3 and H3K27me3), followed by image analysis and quantification at the single-cell level.	12-24
Subtask 6: Relate drug-induced changes in ERK signaling and phenotypic state transitions to patterns of histone modifications using statistical models	12-24
Subtask 7: Measure genome-wide changes in occupancy patterns of selected histone marks via ChIP-seq. Minimum of two cell lines (with two biological replicates) will be selected for the ChIP-seq experiments performed at least in two time-points of treatment with at least one drug at one dose. The selection of histone marks (between 1 to 6), drug type, and the time-points for the experiments will be based on the data from subtask 5, which will identify the ones with the largest variance and single-cell heterogeneity following drug exposure. Experiments will be initially performed on two cell lines (with the same type of mutation: either NF1 or NRAS), which show diverse phenotypic responses as evaluated in subtask 6. If required (based on downstream analysis), additional cell lines will be tested.	18-30
Subtask 8: Construct data-driven models that link transcription factor networks to consequential changes in epigenetic and phenotypic states in the cell lines, in which ChIP-seq measurements were completed.	24-36
Subtask 9: Validate major model findings using small molecules or RNAi assays. Validation experiments will be performed for at least two biological targets, which remain to be identified.	24-36
Milestones Achieved: At the completion of this major task, we expect to have identified and link the signaling and epigenetic states that could explain differences in phenotypic responses of melanoma cell lines with distinct MAPK mutations to MAPK inhibitors.	
Major Task 2: Identify epigenetic targets for modulation in BRAF^{V600E}, NRAS^{Q61R} and NF1-deficient melanomas to selectively maximize tumor cell death in combination with ERK pathway inhibition.	
Subtask 1: Screen a library of 150 epigenetic modifying compounds in combination with	12-24

selected MEK and ERK kinase inhibitors (trametinib and ulixertinib) using fixed single-cell phenotypic imaging assays in an NF1 or NRAS-mutant cell line. The tested cell line will be one of the cell lines selected for analysis in Subtasks 2-5 of the Major Task 1. The other three cell lines will be tested following cherry picking of a selected number of compounds (see below).	
Subtask 2: Screen a selection of <6 epigenetic modifying compounds for long-duration live-cell microscopy in the four cell lines used in Subtasks 2-5 of the Major Task 1. These compounds will be cherry-picked based on fixed cell assays results (i.e. induction of different phenotypic responses in melanoma cells).	12-24
Subtask 3: Time-lapse single-cell tracking and analysis	12-36
Subtask 4: Infer key cell state transitions using probabilistic computational modeling	12-36
Milestones Achieved: At the completion of this major task, we expect to have identified epigenetic modifying compounds with application for reversing phenotype switching and drug resistance in melanoma.	
Major Task 3: Test whether the characterized epigenetic states are predictive of response to therapy in xenograft models of melanoma.	
Subtask 1: Acquire IACUC and ACURO approval for the animal studies proposed in the project.	6-18
Subtask 2: Establish and grow xenograft tumors in mice. We will begin with the same cell line used in Major Task 2, Subtask 1. However, since not all cell lines would grow as xenograft, we may need to test one or more additional cell lines (all from the collection of cell lines used in Major Task 2, Subtask 2), until we find the one growing as xenograft in nu/nu (nude) mice (Jackson Lab #002019). The established xenografts will be divided in four groups, which will be treated with vehicle control, one MEK inhibitor, one epigenetic drug (to be identified in Major task 2 studies), and the combination of the two drugs, and changes in tumor size will be tracked until tumor recurrence or maximum 60 days (~20 mice per treatment condition, as justified in the proposal).	24-36
Subtask 3: Mice will be euthanized following treatment, and xenografts will be removed and fixed for downstream histological analysis by immunofluorescence. Phenotypic markers (including proliferation and differentiation states) as well as at least two biological targets identified in cell lines will be measured using multiplex immunofluorescence assays in xenograft tissue sections. Xenograft sections from five mice (per condition) will be analyzed.	24-36
Subtask 4: Immunofluorescence image analysis of xenograft sections will be performed to identify single-cell heterogeneity and potential correlation between multiple measured markers and resistance phenotypes, and to assess the inhibitory effect of the tested drug combinations on resistance phenotypes.	24-36
Milestones Achieved: At the completion of this major task, we expect to have validated the effectiveness of identified drug combinations (based on studies from Major Tasks 1 and 2) <i>in vivo</i> .	

What was accomplished under these goals?

Our studies supported by this project (during the period of Sep 1, 2018, through Dec 14, 2022) have led to the following significant results, key outcomes, and major findings with respect to the tasks outlined in the approved SOW. We also cite appended publications, which include data associated with each task.

(Major Task 1, subtasks 1, 5, 6, 7, 8, 9):

See Relevant Publication:

Abecunas C, Whitehead CE, Ziemke EK, Baumann DG, Frankowski-McGregor CL, Sebolt-Leopold JS, Fallahi-Sichani M. Loss of NF1 in Melanoma Confers Sensitivity to SYK Kinase Inhibition. *Cancer Res.* 2023 Jan 18;83(2):316-331. doi: 10.1158/0008-5472.CAN-22-0883. PMID: 36409827; PMCID: PMC9845987.

MTX-216 blocks NF1^{LoF} melanoma cell growth – To identify kinases whose inhibition might elicit efficacious anti-tumor responses in NF1^{LoF} melanoma cells, we assembled a panel of small-molecule inhibitors targeting different kinases that act downstream or upstream of RAS signaling, including: MEK1/2 inhibitor (trametinib), ERK1/2 inhibitor (ulixertinib), pan-PI3K inhibitor (pictilisib), mTORC1/2 inhibitor (AZD8055), CDK4/6 inhibitor (palbociclib), and EGFR inhibitor (gefitinib). We also tested the impact of inhibiting multiple kinases, either by using the combination of two distinct compounds (e.g., ulixertinib plus palbociclib) or by using multi-kinase inhibitors such as the dual PI3K/EGFR inhibitor, MTX-216, and its structural analogue, MTX-211. We profiled the effects of these compounds on cell growth and survival in a panel of nine NF1^{LoF} melanoma cell lines. Among the tested cell lines, six expressed a wild-type (WT) BRAF (BRAF^{WT}/NF1^{LoF}), two possessed BRAF^{V600E}/NF1^{LoF} double mutations, and one carried NRAS^{Q61R}/NF1^{LoF} double mutations. Each inhibitor was tested over a range of four concentrations, including a maximum dose (C_{max}) of approximately 10 times the reported biochemical IC₅₀ for its nominal target. To compare inhibitor sensitivities, we calculated the normalized growth rates by dividing the 7 day-averaged net growth rate for cells treated with each inhibitor (at each dose) to net growth rate for vehicle (DMSO)-treated cells. All the examined inhibitor conditions led to variable responses across NF1^{LoF} cell lines. MTX-216 was, however, consistently cytotoxic, leading to steep dose-response curves. The maximal effect (E_{max}) of MTX-216 was significantly higher than that of other tested compounds, including MAPK inhibitors trametinib and ulixertinib, which exhibited potent but less efficacious responses in NF1^{LoF} cell lines. Among the tested inhibitor combinations, MTX-216 plus trametinib or ulixertinib was most effective, leading to efficacious and potent responses, suggesting that MTX-216 and MAPK inhibitors may target orthogonal vulnerabilities in NF1^{LoF} melanoma cells. To determine whether the efficacy of MTX-216 was specific to NF1^{LoF} cells, we evaluated its effect on non-transformed primary melanocytes as well as on two NF1^{WT}/BRAF^{V600E} melanoma cell lines (A375 and COLO858) that are sensitive to trametinib and ulixertinib. When tested at its highest concentration (1 μM), MTX-216 caused no cytotoxicity in primary melanocytes. It also exhibited a substantially less efficacious response in A375 and COLO858 cells in comparison with any of the nine NF1^{LoF} cell lines tested.

MTX-216 co-suppresses Ki-67 and p-S6 and induces apoptosis in NF1^{LoF} cells – To identify factors influencing the efficacy of MTX-216, we assayed apoptosis in NF1^{LoF} cells using two

distinct methods. First, we exposed MeWo, WM3918 and COLO792 cells to increasing concentrations of MTX-216 for 48 or 72 h. We used the NucView® dye, a fluorogenic substrate of pro-apoptotic effector caspases 3 and 7, and measured substrate cleavage by fluorescence microscopy. Single-cell analysis revealed a dose-dependent increase in the frequency of apoptotic cells (with high caspase activity) induced by MTX-216 in all three NF1^{LoF} cell lines. We further confirmed the induction of apoptosis using a double-staining assay with Annexin-V and propidium iodide (PI) with flow cytometry. 48 h treatment of WM3918 cells with MTX-216 led to a ~3-fold increase in the proportion of early-stage apoptotic (Annexin-V^{High}/PI^{Low}) cells and ~4-fold increase in the proportion of late-stage apoptotic and necrotic (Annexin-V^{High}/PI^{High}) cells in comparison with DMSO-treated samples. To evaluate the effect of MTX-216 on signaling and proliferation, we quantified dose-dependent changes in the phosphorylation state of S6 ribosomal protein (p-S6^{S235/S236}) and expression of Ki-67 protein by immunofluorescence microscopy. p-S6 serves as a surrogate for S6 kinase activity, integrating multiple pro-survival signals downstream of RAS signaling, including MEK/ERK and PI3K/mTOR pathways. Ki-67 is a marker of proliferation, which is expressed at lower levels in quiescent cells than in actively proliferating cells. We analyzed the single-cell expression of p-S6 and Ki-67 and their covariance in three NF1^{LoF} cell lines (MeWo, WM3918 and COLO792) following 72 h treatment with either MTX-216, pictilisib, trametinib or ulixertinib. All inhibitors reduced the expression of both markers. MTX-216, however, was the only compound that consistently drove the largest proportion of NF1^{LoF} cells toward a doubly inhibited p-S6^{Low}/Ki-67^{Low} state in all three NF1^{LoF} cell lines. In contrast, NF1^{WT}/BRAF^{V600E} COLO858 cells (which show higher sensitivity to MEK/ERK inhibitors than to PI3K/mTOR inhibitors) exhibited more effective co-suppression of p-S6 and Ki-67 in response to trametinib and ulixertinib in comparison with MTX-216. Among all treatment conditions, the percentage of p-S6^{Low}/Ki-67^{Low} cells significantly correlated with treatment-induced changes in growth rate. The high efficacy of MTX-216 in blocking NF1^{LoF} melanoma cells was, therefore, associated with its strong ability to co-suppress Ki-67 and p-S6 in these cells. To test whether such high efficacy could be explained by the compound's direct effect on MAPK or AKT/mTOR pathways, we measured changes in p-ERK^{T202/Y204}, p-AKT^{S473}, and p-4EBP1^{T37/46} in MeWo cells following 6 h treatment with different concentrations of MTX-216 using quantitative immunofluorescence microscopy. For comparison, we also treated the cells with other inhibitors of the PI3K/mTOR and MAPK pathways, including pictilisib, AZD8055, MTX-211, and trametinib. Trametinib was the only compound that reduced ERK phosphorylation in a dose-dependent manner, while MTX-211, MTX-216, pictilisib and AZD8055 had no significant effect on p-ERK levels. On the other hand, pictilisib, AZD8055, MTX-216, and MTX-211, but not trametinib, decreased p-AKT levels in a dose-dependent manner. The mTOR inhibitor AZD8055 was the only compound that potentially reduced the levels of p-4EBP1, a marker of mTOR activity, while MTX-216, MTX-211 and pictilisib did not decrease p-4EBP1 levels at concentrations below 1 μ M, and trametinib had no effect on p-4EBP1 levels. Overall, these data confirm the on-target effect of MTX-216 on the PI3K/AKT pathway and suggest that its high efficacy may be driven by mechanisms other than its direct nominal targets.

MTX-216 suppresses the activity of SYK kinase – To identify what is driving the high levels of efficacy following MTX-216 treatment, kinome selectivity testing was carried out against 403 human kinases using two commercial FRET-based kinase assays, including Z'-LYTE which monitors phosphorylation of peptide substrates, and Adapta that quantifies ADP formation and can be used with lipid substrates. We also tested MTX-211 (the structural analogue of MTX-216) to

quantify the differences in target space between the two compounds that might be responsible for their dramatically distinct effects on NF1^{LoF} cells. In the first step, our goal was to filter out kinases that were not inhibited by either compound at a high concentration (i.e., 10 μ M). At this concentration, both compounds inhibited their nominal targets, including different isoforms of the catalytic subunits of PI3K (p110 α , p110 β , p110 δ and p110 γ) and EGFR, by approximately 90-100%. The assay also identified additional kinase targets inhibited by both compounds at 10 μ M, including mTOR, PI4KB and DNA-PK. Repeating the assay for these targets across a $\sim 10^4$ -fold range of concentration showed that they were all inhibited by both compounds to similar degrees (i.e., similar IC₅₀'s ranging from ~ 0.3 nM to 1 μ M). We hypothesized that the superior efficacy of MTX-216 might be due to inhibition of a kinase that is targeted effectively by MTX-216 but not by MTX-211. The most significant candidate was SYK, a non-receptor tyrosine kinase, which was inhibited 94% and 52% by MTX-216 and MTX-211 at 10 μ M, respectively. Comparing the kinase activity of SYK in the presence of various concentrations (from 0.5 nM to 10 μ M) of each compound, we found that MTX-216 inhibited SYK with an IC₅₀ of 281 nM, which was ~ 21 -fold more potent than MTX-211 (with an IC₅₀ of ~ 6 μ M). We then asked whether the difference in the ability of MTX-216 and MTX-211 to inhibit SYK was seen in NF1^{LoF} cells. We exposed MeWo cells to MTX-216 and MTX-211 for 24 h and used Western blotting to measure dose-dependent changes in p-SYK^{Y525/526}, autophosphorylation sites on SYK activation loop that are required for its kinase activity. As a control for SYK inhibition, we used two ATP-competitive SYK inhibitors, R406 and entospletinib. Consistent with its higher potency in inhibition of SYK in biochemical assays, MTX-216 reduced p-SYK^{Y525/526} more effectively in comparison with MTX-211 across both tested concentrations (1 and 3.2 μ M). MTX-216 at 1 μ M inhibited p-SYK expression in MeWo cells by $\sim 50\%$, which was comparable to the effect of R406 and higher than the effect of entospletinib at the same concentration. SYK function has been known primarily in the context of immune signaling in B cells, where it phosphorylates different proteins such as B-cell linker protein (BLNK). We thus set out to test the effect of MTX-216 and MTX-211 (in comparison with R406 and entospletinib) on p-BLNK^{Y96} levels in IgM-stimulated Ramos cells, a B lymphocyte cell line. Western blot analysis showed that SYK inhibitors reduced p-BLNK levels in IgM-stimulated Ramos cells. In addition, MTX-216 was more efficient in inhibition of p-BLNK in comparison with MTX-211. To test whether the difference in inhibitory effects of MTX-216 and MTX-211 on SYK kinase was recapitulated at the level of transcription, we performed RNA sequencing on two NF1^{LoF} melanoma cell lines (MeWo and WM3918) exposed to either MTX-216 (at 1 μ M), MTX-211 (at 1 μ M), or vehicle (DMSO) for 24 h. Genes differentially expressed by at least two-fold by either MTX-216 or MTX-211 in each cell line were selected based on a statistical cutoff of FDR ≤ 0.05 . Among these genes, we focused on the subset differing in enrichment between the two compounds in both cell lines. Genes enriched specifically in MTX-216 treatment (but not in MTX-211 treatment) in both cell lines comprised of 231 down-regulated genes and 467 up-regulated genes. We used these genes in Enrichr and compared them statistically with gene expression signatures extracted from the Gene Expression Omnibus (GEO) database for kinase perturbation. The top kinase perturbation term was SYK drug inhibition, suggesting that MTX-216 at a concentration of 1 μ M (more strongly than MTX-211) affects the expression of genes whose transcription is influenced by SYK inhibition.

Inhibition of SYK underlies increased sensitivity of NF1^{LoF} cells to MTX-216 – To directly evaluate the importance of SYK for growth of NF1^{LoF} cells, we depleted the protein in two NF1^{LoF} melanoma cell lines (MeWo and YUHEF) using four specific siRNAs (targeting SYK exons 10, 11,

12 and 13) combined into a single pool. SYK knockdown led to reduced SYK protein levels in both cell lines. Following 3 and 6 days of inhibition by SYK siRNAs, we measured live cell counts and computed the normalized growth rate in SYK-depleted cells relative to cells treated with non-targeting (control) siRNA. We also compared the normalized growth rates in the presence of different doses of MTX-211, trametinib and pictilisib or vehicle (DMSO). SYK depletion in both MeWo and YUHEF led to a statistically significant decrease in their growth rates and enhanced their sensitivity to MTX-211, trametinib and pictilisib. To rule out the possibility of off-targeting by SYK siRNAs, we also examined the effects of each of the four SYK siRNAs independently. All individual siRNAs reduced SYK expression and growth rate in YUHEF cells. To test the impact of pharmacological inhibition of SYK on NF1^{LoF} melanoma cell growth, we treated three cell lines (MeWo, YUHEF, and WM3918) with the SYK inhibitor R406, individually or in combination with either MTX-211, trametinib or pictilisib. Inhibition of SYK by R406 reduced the growth rate in these cell lines by approximately 2-fold and enhanced their sensitivity to MTX-211, trametinib and pictilisib. Together, these data show that both genetic and pharmacological inhibition of SYK significantly reduced the growth of NF1^{LoF} melanoma cells and increased their sensitivity to PI3K and MEK inhibitors. It is noteworthy, however, that the combination of SYK inhibition with MTX-211 did not exactly phenocopy the impact of MTX-216 alone. We speculate that this might be due to differences in mechanism of SYK inhibition by MTX-216 or possibly inhibition of other potentially significant targets by MTX-216. Because treatment efficacy in NF1^{LoF} cells was correlated with an increase in the proportion of p-S6^{Low}/Ki-67^{Low} subpopulation, we asked how p-S6 and Ki-67 levels would change in NF1^{LoF} cells following SYK inhibition. 72 h treatment with R406 reduced p-S6 and Ki-67 and increased the proportion of p-S6^{Low}/Ki-67^{Low} subpopulation in both MeWo and YUHEF cells. Because SYK inhibition enhanced the sensitivity of NF1^{LoF} cells to MEK inhibition, we also evaluated the effect of combined MEK and SYK inhibition. The combination of R406 with trametinib enhanced co-suppression of p-S6 and Ki-67 and increased the fraction of p-S6^{Low}/Ki-67^{Low} subpopulation.

MTX-216 suppresses genes that regulate mitochondrial electron transport chain in NF1^{LoF} cells – To better understand the mechanisms underlying the superior efficacy of MTX-216 relative to other inhibitors, we extended our RNA sequencing analysis (described above) to treatments with additional inhibitors, including pictilisib (at 1 μ M) and trametinib (at 0.1 μ M). We also included a third NF1^{LoF} cell line (SKMEL113), which was treated for 24 h with either MTX-216 (at 1 μ M), trametinib (at 0.1 μ M), the combination of MTX-216 and trametinib (at 1 μ M and 0.1 μ M, respectively), or vehicle (DMSO). Gene set enrichment analysis (GSEA) of the effect of each individual compound on each cell line (relative to DMSO) confirmed the role of trametinib as an inhibitor of the ERK pathway, and pictilisib, MTX-216, and MTX-211 as inhibitors of the PI3K/AKT/mTOR pathway. To systematically compare gene expression changes across all 42 samples (including 3 cell lines, 6 distinct treatment conditions tested in 3 independent replicates), we used principal component analysis (PCA). PCA captured >80% of variance in the entire dataset by the first five principal components. While the first two principal components (PC1 and PC2) associated mostly with cell line-specific differences, PC3 and PC4 were able to capture treatment-specific variations across the samples. Changes induced by trametinib were revealed by a large shift in scores along PC4 for all three cell lines. MTX-216 treatment, on the other hand, led to a moderate shift along PC4, while inducing a strong shift along PC3. The combination of trametinib and MTX-216 was additive, leading to substantial shifts in both PC4 and PC3, whereas pictilisib and MTX-211 induced only small changes along both principal components. Because PC3 and PC4 separated

the efficacious inhibitors independent of cell lines, we set out to use PC3 and PC4 loadings to identify genes associated with distinct inhibitory effects on NF1^{LoF} cells. The identified genes comprised 315 genes with strong positive PC3 loadings, 414 genes with strong negative PC3 loadings, 491 genes with strong positive PC4 loadings, and 678 genes with strong negative PC4 loadings. The top Gene Ontology (GO) terms associated with significantly negative PC4 loadings included processes involved in cell cycle progression, DNA replication and mitosis. As expected from the PCA scores, these genes were suppressed profoundly in cells exposed to trametinib but were also down-regulated in cells treated with MTX-216. In contrast, genes associated with negative PC3 loadings were substantially down-regulated only in MTX-216 treated cells, but not in cells treated with other inhibitors. The top GO terms associated with these genes included processes involved in cellular respiration and oxidative phosphorylation such as mitochondrial ATP synthesis and respiratory electron transport chain complex biogenesis and assembly. Consistent with these results, an independent GSEA using consolidated Hallmark gene sets identified oxidative phosphorylation to be significantly associated with genes down-regulated by MTX-216. Among the key genes affected were those that encode mitochondrial enzyme subunits involved in the respiratory electron transport chain, such as NADH:Ubiquinone Oxidoreductase Subunits A1 and B1 (NDUFA1, NDUFB1), Ubiquinol-Cytochrome C Reductase Complex III Subunit VII (UQCRCQ) and Cytochrome C Oxidase Subunit 5A (COX5A), which were all consistently down-regulated by MTX-216, but not by trametinib, in all three cell lines. Proliferation hallmarks (such as E2F targets, MYC targets and G2/M checkpoint), however, were down-regulated by both trametinib and MTX-216. Together, these data show that while both MTX-216 and trametinib are effective in inhibition of cell proliferation in NF1^{LoF} melanoma cells, the two compounds are distinguishable with respect to their effect on the suppression of genes that regulate mitochondrial electron transport chain.

Mitochondrial respiratory chain is associated with poor survival in NF1^{LoF} melanoma patients and inhibitable by MTX-216 or SYK inhibition – To test the clinical relevance of SYK kinase and genes encoding mitochondrial electron transport chain (ETC), we assessed their association with NF1 mutation and patient phenotype across melanoma tumors in The Cancer Genome Atlas (TCGA) database. For this purpose, we first grouped patient-derived melanoma tumors based on their NF1 mutation status. Patients bearing “truncating” driver mutations were classified as NF1^{LoF} (39 patients); patients bearing “missense” or “splice” driver mutations were classified as NF1^{Mutant} (9 patients); and patients with no NF1 mutations or only “passenger” NF1 mutations were classified as NF1^{Other} (421 patients). We then analyzed the frequency of SYK mutations across each group of patients. Interestingly, the NF1^{LoF} and NF1^{Mutant} groups showed higher numbers of SYK mutations (including mostly missense mutations) compared to the NF1^{Other} group. From these mutations, however, we were not able to identify any obvious patterns of altered SYK activity. Therefore, we extended our analysis to the gene expression data and asked whether there was any difference in SYK pathway activity between different groups of patients. As a surrogate for SYK activity, we used the SYK drug inhibition gene sets, including genes whose expression changes following treatment with SYK inhibitor. Gene set enrichment analysis showed a slight increase in SYK activity in the NF1^{LoF} and NF1^{Mutant} groups relative to the NF1^{Other} group based on the normalized enrichment score (NES), but the difference was not statistically significant. We also did not observe any significant differences in the overall expression of ETC genes (Respiratory Electron Transport Chain GO term; GO:0022904) in tumors across three groups of melanoma patients. However, when we focused our analysis on each group separately and asked whether the expression of ETC genes correlated with patient survival, we found that within the NF1^{LoF} group (or NF1^{LoF} and NF1^{Mutant}

groups combined), patients with ETC^{High} tumors had a significantly shorter overall survival compared to the patients with ETC^{Low} tumors. Within the NF1^{Other} cohort of patients, however, there was no significant difference between the overall survival and whether tumors belonged to ETC^{High} or ETC^{Low} subgroups. To test whether such association within the NF1^{LoF}/NF1^{Mutant} groups might be due to confounding health risks, we assessed the patients' diagnosis stage, but we did not observe any significant differences among NF1^{LoF}/NF1^{Mutant} patients with ETC^{High} tumors versus those with ETC^{Low} tumors. These data suggest that melanoma tumors with NF1 mutations may rely on mitochondrial respiratory chain for progression. To directly evaluate the importance of mitochondrial respiratory chain for growth of NF1^{LoF} cells, we used IACS-010759, a clinical-grade small molecule inhibitor of complex I of the mitochondrial electron transport chain. IACS-010759 inhibited growth in a dose-dependent fashion in all tested cell lines. Using the area over of the dose-response curve to compare overall drug sensitivity, we found the NF1^{LoF} cell lines to be similarly sensitive to both IACS-010759 and MTX-216, and their sensitivities to these compounds were greater than their sensitivity to R406. When combined with trametinib, however, MTX-216 led to the largest reduction in growth rate. Together, these data support the hypothesis that NF1^{LoF} melanoma cells depend on mitochondrial respiratory chain for growth, and that MTX-216 and trametinib target orthogonal vulnerabilities, leading to enhanced benefit when used in combination. Because genes down-regulated by MTX-216 were involved in the mitochondrial respiratory chain, we asked whether this effect was due to a global reduction in mitochondrial mass or function because of SYK inhibition by this compound. We thus used the fluorescent probes, MitoTracker Deep Red and MitoTracker Red CMXRos, to quantitate the mitochondrial mass and membrane potential in NF1^{LoF} cells, respectively. Measurements were performed by fluorescence microscopy in two cell lines (MeWo and WM3918) following treatment with either SYK siRNA or a non-targeting control siRNA for 72 h, or with MTX-216 (at 1 μ M) or vehicle (DMSO) for 48 h. Both SYK knockdown and MTX-216 treatment shifted cell populations towards lower levels of both mitochondrial markers. To compare the effect of MTX-216 and MTX-211 on mitochondria, we performed the assays in MeWo cells following 48 h treatment with increasing concentrations of either compound. MTX-216 was significantly more potent than MTX-211 in reducing both mitochondrial markers. Together, these results are consistent with RNA sequencing data, suggesting that the efficacy of MTX-216 through SYK inhibition is mediated by the suppression of mitochondrial mass and electron transport chain function. It is noteworthy, however, that R406 and IACS-010759 do not completely recapitulate the phenotypic effects of MTX-216. Therefore, it is possible that other pathways inhibited by MTX-216 may also play a role in determining the MTX-216 activity in NF1^{LoF} melanoma cells.

(Major Task 1, subtasks 2, 3, 4):

See Relevant Publication:

Comandante-Lou N, Khaliq M, Venkat D, Manikkam M, Fallahi-Sichani M. Phenotype-based probabilistic analysis of heterogeneous responses to cancer drugs and their combination efficacy. *PLoS Comput Biol.* 2020 Feb 21;16(2):e1007688. doi: 10.1371/journal.pcbi.1007688. PMID: 32084135; PMCID: PMC7055924.

Estimating probabilistic rate constants using time-lapse live cell microscopy – To experimentally capture stochastic processes of induction of cell death and inhibition of division in drug-treated tumor cell populations, we developed a method based on time-lapse live cell microscopy and by using cells engineered to express two fluorescent reporters. The reporters

included: (i) an H2B-Venus reporter which labels chromatin, allowing identification of nuclei and scoring cell death based on changes in nucleus morphology, and (ii) an mCherry-Geminin reporter for cell cycle progression which allows tracking of cell division events. Using a high-throughput, automated image analysis workflow, the occurrence of individual phenotypic events (death and division) in single cells was tracked in time across a variety of drug treatment conditions. To estimate time-dependent changes in probabilistic phenotype rate constants, the number of cell death and division events (N_{event}) were quantified over a series of uniform time intervals of length Δt . Phenotype rate constants were then estimated via normalizing N_{event} in each time interval to the length (Δt) and the average number of live cells over that time interval $[N_{live}(t \rightarrow t + \Delta t)]_{avg}$ as detailed below:

$$k_{death}(t) = \frac{N_{death}(t \rightarrow t + \Delta t)}{[N_{live}(t \rightarrow t + \Delta t)]_{avg} \Delta t} \quad (\text{Equation 5})$$

$$k_{division}(t) = \frac{N_{division}(t \rightarrow t + \Delta t)}{[N_{live}(t \rightarrow t + \Delta t)]_{avg} \Delta t} \quad (\text{Equation 6})$$

$$k_{stasis}(t) = k_{division}(no\ drug) - k_{division}(with\ drug)(t) \quad (\text{Equation 7})$$

As a proof of concept, we monitored responses of two BRAF-mutated melanoma cell lines (COLO858 and MMACSF) following exposure to a BRAF inhibitor Vemurafenib at 6 doses for a period of ~ 120 h. Heterogeneity in drug response was then visualized through the estimation and analysis of phenotype rate constants, k_{death} and k_{stasis} , as a function of drug dose and time in each cell line. In COLO858 cells, which have been shown to be initially sensitive but rapidly develop adaptive resistance to Vemurafenib, increasing drug concentration enhanced both the amplitude and the rate of increase in k_{death} and k_{stasis} within the first 36 h. After that, these responses were attenuated concurrent with the activation of drug-induced adaptive responses. Responses of MMACSF cells involved a relatively monotonic and dose-dependent decrease in the number of live cells. At the highest drug concentration (3.2 μM), however, we observed two peaks of apoptotic response, one similar to COLO858 cells at $t \approx 36$ h and a higher peak later at $t \approx 108$ h. These data are consistent with previous data reporting high sensitivity of MMACSF cells to 5 days of exposure to Vemurafenib, but also highlight the impact of cell-to-cell heterogeneity and the presence of subpopulations of cells with different levels of drug tolerance. In addition to interrogating dynamic aspects of heterogeneous drug response, we also tested the performance of our automated image analysis workflow by comparing the estimated phenotype rate constants with those measured from data generated by manual single-cell tracking using a MATLAB-based software. The software allowed accurate tracking and cell fate annotation of individual cells across time-lapse images taken over a period of multiple days. Single-cell profiles from manual tracking confirmed heterogeneity in the number and timing of death and division events in cells exposed to drug. In COLO858, for example, cell-to-cell variability ranged from cells that died rapidly (as early as ~ 24 h) in response to high concentrations of Vemurafenib, to cells that survived but did not divide, to cells that slowly divided following a temporary delay in their cell cycle, the proportion and dynamics of which changed with drug dose. By comparing rate constants between two image analysis methods across a variety of conditions in two cell lines, we identified quantitatively similar patterns. This consistency confirms that the automated workflow would be suitable for high-throughput analysis of drug response. We also used single-cell phenotype data to empirically evaluate the assumption of non-stationary *Poisson* process to model drug-induced death and division events. We compared the distribution of phenotypic events measured from time-lapse microscopy experiments with those simulated based on *Poisson* processes using estimates of phenotype rate constants. We observed similarity across patterns of response at the single-cell level and between distributions of events at

the population level, suggesting that a simplified model of non-stationary *Poisson* process for drug-induced death and division events is a reasonable one. Taken together, high-throughput estimation and analysis of phenotype rate constants and their changes with time and dose provide an efficient tool to capture critical dynamic aspects of probabilistic and heterogeneous drug response that would be overlooked in bulk population assays.

(Major Task 2, subtask 1):

See Relevant Publication:

Khaliq M, Manikkam M, Martinez ED, Fallahi-Sichani M. Epigenetic modulation reveals differentiation state specificity of oncogene addiction. *Nat Commun.* 2021 Mar 9;12(1):1536. doi: 10.1038/s41467-021-21784-2. PMID: 33750776; PMCID: PMC7943789.

A chemical screen identifies epigenetic modulators of phenotypic heterogeneity – To systematically search for epigenetic factors that may link heterogeneity in melanoma differentiation state to MAPK dependency, we performed a multi-stage phenotypic screen using a library of 276 epigenetic-modifying compounds. Each compound was used to modulate either a key epigenetic writer, eraser or reader, or a related protein, after which phenotypic responses in the presence or absence of Braf/Mek inhibitors were investigated. For the first stage of the screen, we selected COLO858 and MMACSF cell lines which show distinct patterns of differentiation states. Cells from both cell lines were initially exposed to two different doses (0.2 and 1 μ M) of each of the 276 epigenetic compounds or vehicle (DMSO) for 24 h. Vemurafenib alone (at 100 nM), vemurafenib in combination with trametinib (at 10 nM), or vehicle (DMSO), was then added and cells were grown for a further 72 or 120 h prior to fixation. To differentiate the impact of epigenetic compounds, the growth rates for cells treated with each compound were compared to cells treated without any epigenetic treatment. Statistical analysis identified 58 compounds that led to a significant decrease in normalized growth rate in at least one of the tested conditions. To infer potential variations in the mechanisms of action of the epigenetic compounds, we co-stained cells for p-Erk^{T202/Y204}, p-Rb^{S807/811}, and Mitf, quantifying changes induced by each of the 58 compounds in MAPK signaling, cell cycle progression, and differentiation state, respectively. Unsupervised clustering of the normalized responses revealed remarkable similarities among classes of compounds with common nominal epigenetic targets, suggesting that these responses are most likely the consequence of their on-target effects. Each class of compounds induced responses that were either cell line- or MAPK inhibitor-specific, or common between both cell lines, or independent of MAPK inhibitor condition. For example, CUDC-907 (Fimepinostat), Quisinostat, Panobinostat, Dacinostat, and Trichostatin A were identified as a cluster of histone deacetylase (HDAC) inhibitors that significantly reduced growth rate in both COLO858 and MMACSF cells to similar degrees and independent of the MAPK inhibitor conditions. In contrast, Kdm1a inhibitors inhibited net growth rate selectively in COLO858 cells, and they showed a higher efficacy in the absence of Braf/Mek inhibitors.

(Major Task 2, subtasks 2, 3, 4):

See Relevant Publication:

Comandante-Lou N, Khaliq M, Venkat D, Manikkam M, Fallahi-Sichani M. Phenotype-based probabilistic analysis of heterogeneous responses to cancer drugs and their combination efficacy. *PLoS Comput Biol.* 2020 Feb 21;16(2):e1007688. doi: 10.1371/journal.pcbi.1007688. PMID: 32084135; PMCID: PMC7055924.

Time-lapse single-cell analysis and probabilistic modeling uncover target-specific differences in drug combination efficacies – We applied the probabilistic definition of Bliss independence to evaluate time-dependent changes in the efficacies of a group of 12 compounds in sequential combination with BRAF kinase inhibitor, Vemurafenib, plus MEK kinase inhibitor, Trametinib, in two BRAF-mutated melanoma cell lines COLO858 and MMACSF over the course of five days. Single-cell drug responses were monitored using time-lapse fluorescence microscopy, and changes in probabilistic rate constants k_{stasis} and k_{death} were tracked for the entire period of experiment for each drug condition individually or in combinations. The list of compounds based on their nominal targets included two HDAC inhibitors (Fimepinostat and Givinostat), two BET bromodomain inhibitors (Birabresib and I-BET762), two KDM1A inhibitors (SP2509 and ORY-1001), a pan Jmj-KDM inhibitor (JIB-04), a KDM5 inhibitor (CPI-455), two Tankyrase inhibitors (AZ6102 and NVP-TNKS656), and two CDK4/6 inhibitors (Palbociclib and Abemaciclib). These compounds were selected from two broad classes of anti-cancer drugs, referred to as epigenetic-modifying compounds and cell cycle inhibitors, which have been proposed to be used in combination with standard of care BRAF and MEK inhibitors to overcome drug-adapted subpopulations of cells in BRAF-mutant melanomas. The analysis of variations in Bliss combination index, defined based on probabilistic cytotoxic and cytostatic actions, with drug and time (following unsupervised clustering) led to two major conclusions. First, effective drugs with comparable mechanisms of action (e.g. BET inhibitors, HDAC inhibitors or CDK4/6 inhibitors) exhibited similar dynamic patterns of interaction with BRAF and MEK kinase inhibitors, suggesting that differences in probabilistic drug action and interactions are target-specific. Second, cytostatic and cytotoxic drug interactions among efficacious drug combinations often varied in time and did not necessarily correlate with one another. BET inhibitors, for example, exhibited a strong synergistic cytotoxic interaction ($CI_{death} < 1$) with the combination of BRAF and MEK inhibitors within 48-72 h of treatment in both COLO858 and MMACSF cell lines, whereas their interaction was scored as independent ($CI_{stasis} \approx 1$) with respect to inhibition of cell division. Furthermore, the benefit of BET inhibitors combined with Vemurafenib and Trametinib diminished following 96 h, concomitant with the emergence of a small proliferating subpopulation ($k_{division} > 0$). CDK4/6 inhibitors acted independently with BRAF and MEK inhibitor combination to inhibit cell division within 72-96 h in both cell lines. Surprisingly, however, their effects on cell death appeared to be antagonistic especially in MMACSF cells. This might be due the possibility that upon G0/G1 arrest, BRAF-mutant cells become less responsive to the effect of BRAF and MEK inhibitors, an interesting observation which requires further investigation across more cell lines. Altogether, experimental results and simulation outcomes suggest that dynamic measurements of the phenotype metrics and probabilistic evaluation of combination index reveal distinctive responses of cells to drug combinations that might be indistinguishable when assessed based on conventional assays. Phenotype metrics deconvolve differential (and sometimes opposing) degrees of drug effect on tumor cell killing versus inhibition of cell division, a potentially important consideration in choosing appropriate drug combinations. Furthermore, the probabilistic nature of these metrics makes them sensitive to cell-to-cell heterogeneities which are typically overlooked in conventional bulk drug response assays. They are therefore appropriate choices to assess synergistic efficacy in drug combinations aimed at blocking heterogeneous subpopulations of drug-tolerant cells.

**(Major Task 3, subtasks 1, 2, 3, 4):
See Relevant Publication:**

Khaliq M, Manikkam M, Martinez ED, Fallahi-Sichani M. Epigenetic modulation reveals differentiation state specificity of oncogene addiction. Nat Commun. 2021 Mar 9;12(1):1536. doi: 10.1038/s41467-021-21784-2. PMID: 33750776; PMCID: PMC7943789.

Correlated patterns of melanoma responses to mechanistically distinct epigenetic inhibitors –

To identify potential relationships between the efficacy of each class of epigenetic compounds, we analyzed their effects across a diverse group of cell lines *in vitro* and validated key conclusions *in vivo*. We thus first focused on seven epigenetic inhibitors representing the most effective classes of epigenetic inhibitors (including HDAC inhibitors CUDC-907 and Givinostat, pan-Jmj-KDM inhibitor JIB-04, Tankyrase inhibitor AZ6102, BET inhibitors I-BET762 and OTX015, and Kdm1a inhibitor SP2509) and a group of eight cell lines representing a wider spectrum of differentiation states. By testing each compound in non-transformed human primary melanocytes, we chose effective concentrations of each inhibitor that had little to no effect on healthy cells. To quantify the benefit resulting from combining each of the epigenetic inhibitors with vemurafenib and trametinib, we computed the deviation from Bliss independence (DBI), a metric that compares the observed cellular response to the combination treatment with that expected given independent action for the two individual treatments. We found that the HDAC inhibitor CUDC-907 induced substantial tumor cell killing in all of 8 melanoma cell lines and exhibited additive (independent) to synergistic responses when combined with vemurafenib and trametinib in 6 cell lines. Treatment with the other 6 epigenetic compounds uncovered more heterogeneous patterns of response across cell lines and MAPK inhibitor conditions. For example, BET inhibitors OTX015 and I-BET762 were not effective in any of the cell lines when used as a single agent, but they induced strong cytotoxic and synergistic responses in combination with vemurafenib and trametinib in the majority of cell lines. SP2509, on the other hand, was effective in 5 cell lines, while exhibiting antagonism in combination with vemurafenib and trametinib in all cell lines. To systematically explore potential relationships between patterns of responses to epigenetic inhibitors, we computed all pairwise correlations between the efficacy of seven epigenetic compounds across the eight cell lines. As expected, the efficacy of two BET inhibitors, OTX015 and I-BET762, was strongly correlated (Pearson's $r = 0.99$, $P < 10^{-4}$). In addition, the efficacy of BET inhibitors and the pan-Jmj-KDM inhibitor JIB-04 was positively correlated when cells were co-treated with vemurafenib plus trametinib ($r = 0.85$, $P < 10^{-3}$). In contrast, a negative correlation was observed between the efficacies of JIB-04 and SP2509. We thus asked whether such correlations would be maintained if these compounds were assayed across a larger panel of cell lines. By expanding the analysis to a total of sixteen cell lines, we confirmed the statistical significance of the negative correlation between responses to SP2509 and JIB-04. In more than half of the cell lines tested, five days of treatment with SP2509 induced substantial cell killing. SP2509 sensitivity correlated with sensitivity to SP2577 (seclidemstat; a clinical formulation of SP2509), which also suppressed melanoma cell growth (when used at a daily dose of 80 mg kg⁻¹) in corresponding melanoma xenografts. Interestingly, however, cell lines with the highest level of resistance to SP2509 and SP2577 were sensitive to JIB-04, showing a range of responses from cytostatic to cytotoxic. The triple combination of JIB-04, vemurafenib and trametinib led to additive (independent) to synergistic cell killing in most JIB-04-sensitive cell lines, whereas the combination of SP2509 with Braf/Mek inhibitors was antagonistic in all SP2509-sensitive cell lines. When combined with vemurafenib and trametinib, both BET inhibitors OTX015 and I-BET762 also induced synergistic cell killing in most of the JIB-04-sensitive cell lines, while having minimal effects when used as a single agent. To determine if the epigenetic inhibitor effects persisted with long-term exposure, we extended the duration of growth assays. We found that the

optimal epigenetic treatments identified for three of the MAPK inhibitor-resistant cell lines were highly efficacious, resulting in reduction of up to 1000-fold in live cell count over a period of 20 days. Together, our multi-stage epigenetic screen and systematic correlation analysis identified a set of seemingly distinct epigenetic states whose inhibition, either by the Kdm1a inhibitor SP2509, or by the pan-Jmj-KDM inhibitor JIB-04 or BET inhibitors (when used in combination with Braf/Mek inhibitors), led to tumor cell killing in a melanoma cell line-specific manner.

As describe above, our multi-stage epigenetic screen and systematic correlation analysis on a panel of BRAF-mutated melanoma cell lines led to the identification a set of seemingly distinct epigenetic states whose inhibition, either by the KDM1A inhibitor SP2509, or by the pan-Jmj-KDM inhibitor JIB-04 or BET inhibitors (when used in combination with BRAF/MEK inhibitors), led to tumor cell killing in a melanoma cell line-specific manner. Next, we asked whether such cell line-specific patterns of response may be linked to heterogeneity in MAPK signaling, proliferation and differentiation states. Given the promising *in vitro* data, we set out to evaluate the efficacy of epigenetic treatments *in vivo*. SP2509 has been previously tested in multiple studies in nude mice bearing non-melanoma xenograft tumors. SP2577 (a.k.a. seclidemstat), a clinical formulation of SP2509, recently became available and is currently being evaluated in phase 1 clinical trials for patients with refractory Ewing Sarcoma (NCT03600649) or advanced solid tumors (NCT03895684). Given the improved safety profile of SP2577 and the high correlation of melanoma responses to SP2509 and SP2577, we tested the efficacy of SP2577 to treat AXL^{High} melanoma cells *in vivo*. For xenograft tumor injections, mice were first anesthetized using 3% vaporized isoflurane. 2.5×10^6 cells from either of the melanoma cell lines A375 or WM2664 suspended in 200 μ l of growth factor-reduced Matrigel in PBS (1:1) were injected subcutaneously in the right flank of each mouse. Tumor xenografts were monitored three times a week with digital calipers. Once the tumors were palpable and the mean volume across all tumors reached a volume of approximately 170 mm³, the mice were randomly allocated to two treatment groups of 5 mice per group. SP2577 (at a daily dose of 80 mg/kg) or vehicle (20% DMSO, 20% Cremophor EL, plus 60% sterile water) was then administered to each mouse via I.P. injection of a solution of 200 μ l. Mouse body weight, tumor volume, and general health were monitored three times a week for 12 days. At the endpoint of the study, mice were euthanized by CO₂ asphyxiation and bilateral pneumothorax. In our experiments, A375 and WM2664 tumor xenografts treated with SP2577 (at a daily IP dose of 80 mg/kg) showed significant suppression of tumor growth within 12 days as compared with vehicle-treated xenografts.

Summary and conclusions:

In this project, we performed a targeted compound screen to search for actionable kinases upstream, parallel to, or downstream of MAPK and PI3K/mTOR pathways, whose inhibition individually or in combination with one another lead to lethality of NF1^{LoF} melanoma cells. We identified MTX-216, a dual ATP-competitive inhibitor of PI3K isoforms and EGFR, to kill NF1^{LoF} melanoma cells in a dose-dependent manner. MTX-216 has only minimal effect on non-transformed primary melanocytes or BRAF^{V600E}-mutated cells but induces apoptosis in NF1^{LoF} melanoma cells with a greater efficacy in comparison with equivalent doses of other tested small molecules, including inhibitors of MEK, ERK, pan-PI3K, mTORC1/2, EGFR, and CDK4/6. Single-cell analysis revealed a substantial correlation between drug-induced cytotoxicity and effective co-suppression of Ki-67 (a proliferation marker) and the ribosomal S6 phosphorylation (an integrator of multiple RAS-mediated signaling pathways) in NF1^{LoF} melanoma cells. These effects, however, were not explained merely by the nominal targets of MTX-216. Interestingly, MTX-211, a structural

analogue of MTX-216 with comparable potency against PI3K isoforms and EGFR, did not elicit an efficacious response in NF1^{LoF} melanoma cells. Kinome selectivity analysis integrated with differential gene expression and pathway enrichment analysis led us to identify SYK as an additional target of MTX-216 that is required for NF1^{LoF} melanoma cell growth. SYK is a non-receptor tyrosine kinase conventionally known as a mediator of adaptive immune receptor signaling. Recent studies, however, have implicated SYK localization to mitochondrial intermembrane space and its involvement in the regulation of mitochondrial respiratory chain and oxidative metabolism. Consistent with these studies, we found that MTX-216 treatment in NF1^{LoF} melanoma cells suppressed genes encoding mitochondrial electron transport chain. In agreement with these findings, treatment with IACS-010759, a clinical-grade small-molecule inhibitor of the mitochondrial electron transport chain, also induced cell death in these cells. Therefore, the poly-selectivity of MTX-216, which includes SYK inhibition and the consequential suppression of mitochondrial electron transport chain, causes the unusually high activity of this compound in NF1^{LoF} melanoma cells, and provides a path to exploit SYK dependency to selectively block NF1^{LoF} melanoma cells. In addition, to identify epigenetic features linked to melanoma differentiation states and MAPK dependency, we performed an epigenetic compound screen and identified three classes of compounds that target distinct melanoma cell states associated with either one of the lysine-specific histone demethylases Kdm1a or Kdm4b, or BET proteins. While non-transformed melanocytes remained unaffected by these compounds, their efficacy in melanoma cells depended on a cell's state of MAPK activity and its baseline (drug-naïve) differentiation state. The Kdm1a inhibitor SP2509 was most effective in inhibiting undifferentiated populations of cells that were intrinsically insensitive to Braf/Mek inhibitors, whereas the combination of SP2509 with Braf/Mek inhibitors led to antagonistic interactions.

What opportunities for training and professional development has the project provided?

University of Virginia recognizes the significance of preparing graduate students and postdoctoral scholars for professional development and future success in a diverse range of scientific careers. Through multiple layers of mentorship (with research mentor, graduate advisor, and graduate program chair), career and professional development have been integrated into the PhD curriculum in the Department of Biomedical Engineering. All graduate students are required to create an annual Individual Development Plan (IDP) with help from their mentors. The goal of the IDP is to provide a platform upon which trainees: (1) can explore and define career goals, 2) work with the mentor and/or advisor(s) to develop an individual training plan that meets the graduate program

requirements while supporting progress towards their career goals; and 3) track their progress. Annual updates and re-evaluations are required and monitored through regular one-on-one meetings. All graduate student and postdoctoral trainees have the resources of the University of Virginia Career Center, which provides career development programs, including one-on-one mentoring and problem solving with professional career counselors, career exploration and experiential opportunities, and a series of opportunities to learn about career choices or participate in workshops on a wide range of skills relevant to biomedical scientists.

In addition, this project has provided the following training and professional development opportunities for the PI and the graduate student, whose research was supported by this project during the current reporting period:

- As a faculty member of the University of Virginia (UVA) Comprehensive Cancer Center, Dr. Fallahi-Sichani has been provided ample opportunities to interact with other senior faculty members with expertise in Cancer Biology, Cancer Genetics and Melanoma Biology. Dr. Fallahi-Sichani is currently the Basic Science Lead of the Melanoma Research Team at UVA Cancer Center. He participates in twice-monthly meetings with other leaders with interest in basic, translational, and clinical research on melanoma, to discuss new collaborative ways with a focus on melanoma treatment and prevention through teamwork between basic scientists and clinicians. Dr. Fallahi-Sichani has been invited to present his melanoma research in a variety of internal and external seminars, including talks at the Moffitt Cancer Center (April 2022), Johns Hopkins University (November 2021) as well as for the UVA Department of Biochemistry and Molecular Genetics (April 2022), and the UVA Cancer Center Cancer Biology Program Meeting (November 2021), Systems Approaches to Cancer Biology Meeting (October 2022). Through these presentations, he has received constructive feedback from other faculty members working in complementary areas.

- The PI (Mohammad Fallahi-Sichani) attended the Keystone Symposium on Modern Phenotypic Drug Discovery: From Chemical Biology to Therapeutics 2022, Denver, CO. The conference provided the opportunity for discussion of science related to this project with other cancer biologists and scientists with interest in drug discovery projects. He also presented a poster, describing research supported partly by this project: Khaliq M, Manikkam M, Martinez ED, Fallahi-Sichani M. An integrative screen to identify epigenetic modulators of phenotypic heterogeneity in cancer cells. Dr. Fallahi-Sichani attended the Biomedical Engineering Society Annual Meetings in 2018 through 2022. In these conferences, the PI served as one of the abstract review committee members and as an oral platform session chair in the Computational Systems Biology Tracks. The conference also provided the opportunity for discussion of science related to this project with other cancer systems biologists and bioengineers working on related projects.

- Graduate student, Cara Abecunas (supported by this project) participated in the Biomedical Engineering Society Annual Meetings as well as the Systems Approaches to Cancer Biology and AACR Meetings in 2019 through 2022 and presented talks/posters, describing her research supported by this project.

- Graduate student, Luisa Quesada (supported partly by this project) attended the NCI Junior Investigator Annual Meeting in August 2022. This meeting provided her an opportunity to learn about the cancer (and melanoma) research going on by other labs and network with students and postdocs from other institutions.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Below is the list of all journal publications that include research supported by this project. A copy of each publication has been attached as an appendix. The federal support by this project has been acknowledged in all these publications:

Abecunas C, Whitehead CE, Ziemke EK, Baumann DG, Frankowski-McGregor CL, Sebolt-Leopold JS, Fallahi-Sichani M. Loss of NF1 in Melanoma Confers Sensitivity to

SYK Kinase Inhibition. *Cancer Res.* 2023 Jan 18;83(2):316-331. doi: 10.1158/0008-5472.CAN-22-0883. PMID: 36409827; PMCID: PMC9845987.

Khaliq M, Manikkam M, Martinez ED, Fallahi-Sichani M. Epigenetic modulation reveals differentiation state specificity of oncogene addiction. *Nat Commun.* 2021 Mar 9;12(1):1536. doi: 10.1038/s41467-021-21784-2. PMID: 33750776; PMCID: PMC7943789.

Comandante-Lou N, Khaliq M, Venkat D, Manikkam M, Fallahi-Sichani M. Phenotype-based probabilistic analysis of heterogeneous responses to cancer drugs and their combination efficacy. *PLoS Comput Biol.* 2020 Feb 21;16(2):e1007688. doi: 10.1371/journal.pcbi.1007688. PMID: 32084135; PMCID: PMC7055924.

Khaliq M, Fallahi-Sichani M. Epigenetic Mechanisms of Escape from BRAF Oncogene Dependency. *Cancers (Basel).* 2019 Oct 1;11(10):1480. doi: 10.3390/cancers11101480. PMID: 31581557; PMCID: PMC6826668.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

The list of all conference presentations supported throughout this project are provided below. See Abstracts in the Appendix.

Abecunas C, Whitehead C, Ziemke E, Baumann D, Frankowski-McGregor C, Sebolt-Leopold J, Fallahi-Sichani M. Systematic analysis uncovers SYK dependency in NF1^{LoF} melanoma cells. Biomedical Engineering Society (BMES) Annual Meeting 2022, San Antonio, TX.

Abecunas C, Whitehead C, Ziemke E, Baumann D, Frankowski-McGregor C, Sebolt-Leopold J, Fallahi-Sichani M. Systematic analysis uncovers SYK dependency in NF1^{LoF} melanoma cells. Systems Approaches to Cancer Biology (SACB) Meeting 2022, Marine Biological Laboratory, Woods Hole, MA.

Khaliq M, Manikkam M, Martinez ED, Fallahi-Sichani M. An integrative screen to identify epigenetic modulators of phenotypic heterogeneity in cancer cells. Keystone Symposium on Modern Phenotypic Drug Discovery: From Chemical Biology to Therapeutics 2022, Denver, CO.

Abecunas C, Whitehead C, Ziemke E, Sebolt-Leopold J, Fallahi-Sichani M. Multi-targeted kinase inhibitor MTX-216 exploits dependency on SYK to selectively block NF1^{LoF} melanomas. Biomedical Engineering Society (BMES) Annual Meeting 2021, Orlando, FL.

Khaliq M, Manikkam M, Martinez ED, Fallahi-Sichani M. Epigenetic Modulation Extends the Oncogene Addiction Paradigm on the Basis of Tumor Cell Differentiation State. Biomedical Engineering Society (BMES) Annual Meeting 2020 (Virtual Conference).

Abecunas C, Whitehead C, Ziemke E, Sebolt-Leopold J, Fallahi-Sichani M. Unveiling the mechanism of action of MTX-216 in overcoming drug resistance in NF1-mutant melanomas. Biomedical Engineering Society (BMES) Annual Meeting 2020 (Virtual Conference).

Abecunas C, Whitehead C, Leopold J, Fallahi-Sichani M. Selective Inhibition of NF1 Loss-of-Function Driven Oncogenic Signaling in Melanomas. American Association for Cancer Research (AACR) Annual Meeting 2020, San Diego, CA.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

The list of all individuals and their average research effort throughout the entire period (including no cost extension) of this project are provided below.

Name: Mohammad Fallahi-Sichani

Project Role: PI

ORCID ID: 0000-0003-0917-3525

Nearest person month worked: 2.7

Contribution to Project: Dr. Fallahi-Sichani is the PI overseeing and providing hands-on

assistance for all key aspects of the project (both experimental and computational work), meet daily with lab members and review data and prepare reports for publication and presentations.

Funding Support: this award

Name: Cara Abecunas

Project Role: Graduate student

ORCID ID: 0000-0003-4742-8795

Nearest person month worked: 7.8

Contribution to Project: Cara Abecunas is the PhD student who has been leading the experimental work in the project toward her PhD thesis.

Funding Support: University of Michigan and this award

Name: Mehwish Khaliq

Project Role: Postdoctoral Fellow

ORCID ID: 0000-0001-8757-8013

Nearest person month worked: 2.9

Contribution to Project: Mehwish Khaliq is the postdoctoral fellow, who has been assisting with the genetic experiments.

Funding Support: this award

Name: Mohan Manikkam

Project Role: Research Lab Specialist

ORCID ID: 0000-0002-7033-8977

Nearest person month worked: 1.2

Contribution to Project: Mohan Manikkam assisted with cell culture, drug studies and maintaining the experimental systems.

Funding Support: this award

Name: Luisa Quesada

Project Role: Graduate student

ORCID ID: 0000-0001-7149-048X

Nearest person month worked: 1.4

Contribution to Project: Luisa Quesada has been assisting with the computational/bioinformatics analysis for the project.

Funding Support: this award

Name: Yonatan Degefu

Project Role: Graduate student

ORCID ID: 0000-0002-6540-3148

Nearest person month worked: 1.5

Contribution to Project: Yonatan Degefu has been assisting with the computational/bioinformatics analysis for the project.

Funding Support: this award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Organization Name: University of Michigan

Location of Organization: Ann Arbor, MI, USA

Partner's contribution to the project: Dr. Fallahi-Sichani has an adjunct assistant professor position in the Department of Biomedical Engineering of the University of Michigan, Ann Arbor. His position involves 0.00% effort with no salary support.

- *Financial support:* N/A
- *In-kind support:* N/A

- *Facilities:* Dr. Fallahi-Sichani serves as mentor to the University of Michigan PhD student, Cara Abecunas.
- *Collaboration:* N/A
- *Personnel exchanges:* N/A

Organization Name: University of Michigan

Location of Organization: Ann Arbor, MI, USA

Partner's contribution to the project: Graduate student Cara Abecunas, who conducts research activities in the Fallahi-Sichani lab under the supervision of Dr. Fallahi-Sichani, is affiliated with the University of Michigan. She receives salary, tuition support, and health insurance from the University of Michigan.

- *Financial support:* N/A
- *In-kind support:* N/A
- *Facilities:* N/A
- *Collaboration:* N/A
- *Personnel exchanges:* graduate student working on the project

Organization Name: University of Michigan

Location of Organization: Ann Arbor, MI, USA

Partner's contribution to the project: The small-molecule dual PI3K/EGFR inhibitors MTX-216 and MTX-211, used in this research, are provided by Dr. Judith Leopold from the University of Michigan.

- *Financial support:* N/A
- *In-kind support:* small molecule PI3K/EGFR inhibitors MTX-216 and MTX-211
- *Facilities:* N/A
- *Collaboration:* N/A
- *Personnel exchanges:* N/A

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: QUAD CHARTS:

9. APPENDICES:

See below the abstracts for all conference presentations and copies of journal articles (cited in this report) that resulted from this project throughout its entire period.

Systematic analysis uncovers SYK dependency in NF1^{LoF} melanoma cells

Cara Abecunas*^{1,2}, Christopher Whitehead³, Elizabeth Ziemke⁴, Douglas G. Baumann¹, Christy Frankowski-McGregor⁴, Judith Sebolt-Leopold^{3,4,5} and Mohammad Fallahi-Sichani¹

¹Department of Biomedical Engineering, University of Virginia, Charlottesville, VA

²Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI

³Mekanistic Therapeutics, LLC, Ann Arbor, MI, ⁴Department of Radiology and ⁵Department of Pharmacology, University of Michigan, Ann Arbor, MI

The loss of function (LoF) of NF1 is the third most frequent mutation that drives hyperactivated RAS and tumor growth in >10% of melanomas. While melanomas driven by BRAF^{V600E/K/D} mutations show consistent clinical response to MAPK-targeted therapies, tumors bearing NF1^{LoF} mutations exhibit inconsistent sensitivity to inhibitors of RAS-regulated pathways, such as MAPK and PI3K/mTOR. Thus, there is a need for the identification of more effective strategies to block tumor growth in NF1^{LoF} melanomas, for which efficient targeted therapies are currently unavailable. To search for actionable kinases in NF1^{LoF} melanomas, we performed a compound screen targeting MAPK and PI3K/mTOR pathways. We identified MTX-216, an inhibitor of pan-PI3K and EGFR, to selectively kill NF1^{LoF} melanoma cells and block tumor growth *in vivo*. Interestingly, MTX-211, an analogue of MTX-216 with comparable potency against PI3K and EGFR, does not elicit an efficacious response in NF1^{LoF} melanoma cells. We thus set out to identify mechanisms that might determine the anti-tumor efficacy of MTX-216 in NF1^{LoF} melanomas using systems biology approaches. To identify genes associated with the unique mechanisms of MTX-216, we RNA-sequenced NF1^{LoF} melanoma cell lines (WM3918, MeWo, SKMEL113) treated for 24 h with either MTX-216 (1 μ M), MTX-211 (1 μ M), Trametinib (a MEK inhibitor; 0.1 μ M), Pictilisib (a PI3K inhibitor; 1 μ M), MTX-216/Trametinib combination, or DMSO in triplicate. Differential gene expression analysis was performed to compare and contrast genes regulated by the MTX-216 and -211 analogues. We systematically analyzed variation in gene expression across treatments, cell lines, and replicates by using principal component analysis (PCA). Genes identified from our differential gene analysis and PCA were analyzed in Enrichr for pathway enrichment analysis. We tested our hypothesis regarding MTX-216's mechanisms of action through immunofluorescent microscopy, genetic knockdown, and drug response studies. Transcriptional profiling determined significant upregulated and downregulated genes that were specific to either MTX-216 or MTX-211 across both cell lines. Genes downregulated uniquely by MTX-216 in both cell lines revealed SYK inhibition as the most significant kinase perturbation. We confirmed SYK's role in NF1^{LoF} melanoma by knocking down SYK. SYK siRNA significantly reduced net growth rate in MeWo cells and improved Trametinib drug response. In PCA, MTX-216 scores project significantly along PC3. Gene Ontology (GO) Biological Process revealed that downregulated genes in PC3 were associated with mitochondrial electron transport chain (ETC). MitoTracker mitochondrial dye confirmed that MTX-216 and SYK siRNA reduced mitochondrial mass in NF1^{LoF} melanomas. The polyselectivity of MTX-216, which includes SYK inhibition and the consequential suppression of mitochondrial ETC, causes the efficacious activity of this compound in NF1^{LoF} melanoma cells. Our findings warrant further mechanistic investigation to better understand the role of SYK in NF1^{LoF} melanomas in pursuit to selectively target NF1^{LoF} melanomas.

Systematic analysis uncovers SYK dependency in NF1^{LoF} melanoma cells

Cara Abecunas*^{1,2}, Christopher Whitehead³, Elizabeth Ziemke⁴, Douglas G. Baumann¹, Christy Frankowski-McGregor⁴, Judith Sebolt-Leopold^{3,4,5} and Mohammad Fallahi-Sichani¹

¹Department of Biomedical Engineering, University of Virginia, Charlottesville, VA

²Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI

³Mekanistic Therapeutics, LLC, Ann Arbor, MI, ⁴Department of Radiology and ⁵Department of Pharmacology, University of Michigan, Ann Arbor, MI

The loss of function (LoF) of NF1 is the third most frequent mutation that drives hyperactivated RAS and tumor growth in >10% of melanomas. While melanomas driven by BRAF-V600E/K/D mutations show consistent clinical response to MAPK-targeted therapies, tumors bearing NF1-LoF mutations exhibit inconsistent sensitivity to inhibitors of RAS-regulated pathways, such as MAPK and PI3K/mTOR. Thus, there is a need for the identification of more effective strategies to block tumor growth in NF1-LoF melanomas, for which efficient targeted therapies are currently unavailable. To search for actionable kinases in NF1-LoF melanomas, we performed a multiplex, compound screen targeting MAPK and PI3K/mTOR pathways. We identified MTX-216, an inhibitor of pan-PI3K and EGFR, to selectively kill NF1-LoF melanoma cells and block tumor growth *in vivo*. Interestingly, MTX-211, an analogue of MTX-216 with comparable potency against PI3K and EGFR, does not elicit an efficacious response in NF1-LoF melanoma cells. We thus set out to identify mechanisms that might determine the anti-tumor efficacy of MTX-216 in NF1-LoF melanomas using systems biology approaches. To identify genes associated with the unique mechanisms of MTX-216, we RNA-sequenced NF1-LoF melanoma cell lines (WM3918, MeWo, SKMEL113) treated for 24 h with either MTX-216, MTX-211, Trametinib (a MEK inhibitor), Pictilisib (a PI3K inhibitor), MTX-216/Trametinib combination, or DMSO in triplicate. Differential gene expression followed by Enrichr enrichment analysis was performed to compare and contrast genes regulated by the MTX-216 and -211 analogues. Genes downregulated uniquely by MTX-216 revealed SYK inhibition as the most significant kinase perturbation. We confirmed SYK's role in NF1-LoF melanoma by knocking down SYK. SYK siRNA reduced net growth rate in NF1-LoF cells and improved Trametinib drug response. We systematically analyzed variation in gene expression across treatments, cell lines, and replicates by using principal component analysis (PCA). In PCA, MTX-216 scores revealed significantly downregulated genes associated with mitochondrial electron transport chain (ETC). MitoTracker mitochondrial dye confirmed that MTX-216 and SYK siRNA reduced mitochondrial mass and function in NF1-LoF melanomas. In summary, the polyselectivity of MTX-216, which includes SYK inhibition and the consequential suppression of mitochondrial ETC, causes the efficacious activity of this compound in NF1-LoF melanoma. The discovery of SYK as a vulnerability in NF1-LoF melanomas represents an unconventional approach leveraging mechanistic, poly-pharmacology drug studies to identify novel cancer targets. Our results warrant further mechanistic investigation to better understand the role of SYK in NF1-LoF melanomas in future studies. Such studies may provide a path to exploit SYK dependency to selectively block NF1-LoF melanoma tumors.

An integrative screen to identify epigenetic modulators of phenotypic heterogeneity in cancer cells

Mehwish Khaliq¹, Mohan Manikkam¹, Elizabeth D. Martinez², Mohammad Fallahi-Sichani^{1*}

¹University of Virginia, Charlottesville, VA, USA; ²UT Southwestern Medical Center, Dallas, TX, USA

Lineage-specific epigenetic mechanisms and their reprogramming following inhibition of oncogenic pathways can generate drug-tolerant states that diminish the efficacy of cancer targeted therapies. In BRAF-mutant melanomas, comparable states tolerant of BRAF/MAPK pathway inhibition have been associated with cell-to-cell heterogeneity in differentiation state. To identify epigenetic features linked to melanoma differentiation states and MAPK dependency, we created a new multiplexed, high-content imaging based phenotypic screening platform. We screened a library of 276 epigenetic-modifying compounds, individually and in combination with BRAF/MAPK inhibitors, across both genetically distinct and isogenic populations of melanoma cells from diverse BRAF-mutant melanoma cell lines that represent a wide spectrum of differentiation states. Our goal was to search for actionable epigenetic features that may link single-cell heterogeneity in melanoma differentiation state to BRAF/MAPK dependency. Integrated with multiplexed single-cell measurements, multi-variate computational modeling and genetic experiments, we identified small-molecules that target three seemingly distinct and phenotypically consequential epigenetic states: (1) a lysine demethylase 1A (KDM1A)-dependent state associated with undifferentiated, MAPK inhibitor-resistant cells, (2) a Jumonji histone demethylase (Jmj-KDM)-dependent state associated with neural crest-like cells, and (3) a state induced by BET bromodomain inhibition in MAPK inhibitor-adapted cells, which substantially enhances their requirement for MAPK signaling and, thereby, their sensitivity to BRAF/MEK inhibitors. While melanocytes remained insensitive, the anti-tumor efficacy of each class of inhibitors was predicted based on a melanoma cell's differentiation state and MAPK activity. Together, these results highlight a promising avenue toward identifying actionable epigenetic factors that may extend the oncogene addiction paradigm based on tumor cell differentiation state.

Systematic analysis uncovers SYK dependency in NF1^{LoF} melanoma cells

Cara Abecunas*^{1,2}, Christopher Whitehead³, Elizabeth Ziemke³, Douglas G. Baumann¹, Christy Frankowski-McGregor³, Judith Sebolt-Leopold^{3,4} and Mohammad Fallahi-Sichani¹

¹Department of Biomedical Engineering, University of Virginia, Charlottesville, VA

²Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI

³Department of Radiology and ⁴Department of Pharmacology, University of Michigan, Ann Arbor, MI

The loss of function (LoF) of NF1 is the third most frequent mutation that drives hyperactivated RAS and tumor growth in >10% of melanomas. While melanomas driven by BRAF^{V600E/K/D} mutations show consistent clinical response to MAPK-targeted therapies, tumors bearing NF1^{LoF} mutations exhibit inconsistent sensitivity to inhibitors of RAS-regulated pathways, such as MAPK and PI3K/mTOR. Thus, there is a need for the identification of more effective strategies to block tumor growth in NF1^{LoF} melanomas, for which efficient targeted therapies are currently unavailable. To search for actionable kinases in NF1^{LoF} melanomas, we performed a compound screen targeting MAPK and PI3K/mTOR pathways. We identified MTX-216, an inhibitor of pan-PI3K and EGFR, to selectively kill NF1^{LoF} melanoma cells and block tumor growth *in vivo*. Interestingly, MTX-211, an analogue of MTX-216 with comparable potency against PI3K and EGFR, does not elicit an efficacious response in NF1^{LoF} melanoma cells. We thus set out to identify mechanisms that might determine the anti-tumor efficacy of MTX-216 in NF1^{LoF} melanomas using systems biology approaches. To identify genes associated with the unique mechanisms of MTX-216, we RNA-sequenced NF1^{LoF} melanoma cell lines (WM3918, MeWo, SKMEL113) treated for 24 h with either MTX-216 (1 μ M), MTX-211 (1 μ M), Trametinib (a MEK inhibitor; 0.1 μ M), Pictilisib (a PI3K inhibitor; 1 μ M), MTX-216/Trametinib combination, or DMSO in triplicate. Differential gene expression analysis was performed to compare and contrast genes regulated by the MTX-216 and -211 analogues. We systematically analyzed variation in gene expression across treatments, cell lines, and replicates by using principal component analysis (PCA). Genes identified from our differential gene analysis and PCA were analyzed in Enrichr for pathway enrichment analysis. We tested our hypothesis regarding MTX-216's mechanisms of action through immunofluorescent microscopy, genetic knockdown, and drug response studies. Transcriptional profiling determined significant upregulated and downregulated genes that were specific to either MTX-216 or MTX-211 across both cell lines. Genes downregulated uniquely by MTX-216 in both cell lines revealed SYK inhibition as the most significant kinase perturbation. We confirmed SYK's role in NF1^{LoF} melanoma by knocking down SYK. SYK siRNA significantly reduced net growth rate in MeWo cells and improved Trametinib drug response. In PCA, MTX-216 scores project significantly along PC3. Gene Ontology (GO) Biological Process revealed that downregulated genes in PC3 were associated with mitochondrial electron transport chain (ETC). MitoTracker mitochondrial dye confirmed that MTX-216 and SYK siRNA reduced mitochondrial mass in NF1^{LoF} melanomas. The polyselectivity of MTX-216, which includes SYK inhibition and the consequential suppression of mitochondrial ETC, causes the efficacious activity of this compound in NF1^{LoF} melanoma cells. Our findings warrant further mechanistic investigation to better understand the role of SYK in NF1^{LoF} melanomas in pursuit to selectively target NF1^{LoF} melanomas.

Epigenetic Modulation Extends the Oncogene Addiction Paradigm on the Basis of Tumor Cell Differentiation State

Mehwish Khaliq^{1,2}, Mohan Manikkam¹, Elisabeth D. Martinez^{3,4}, and Mohammad Fallahi-Sichani^{1,5}
¹ Department of Biomedical Engineering, ² Program in Cancer Biology, University of Michigan, Ann Arbor, MI³, Department of Pharmacology, ⁴ Hamon Center for Therapeutic Oncology Research, UT Southwestern Medical Center, Dallas, TX, ⁵ Department of Biomedical Engineering, University of Virginia, Charlottesville, VA

The discovery of driver oncogenes has revolutionized drug development strategies for a large number of cancers. Therapeutic inhibition of oncogenic signaling, however, often leads to variable response rates because of cell-to-cell heterogeneity in the state of oncogene dependency. Such heterogeneity may result from secondary genetic mutations. In many cases, however, it cannot be linked to a clear genetic cause, but rather appears to be associated with epigenetic factors established by a cell's developmental lineage or differentiation state. A paradigmatic example of epigenetic heterogeneity in the state of oncogene dependency is observed among melanomas that carry V600E/D/K mutations in the BRAF oncogene. Despite dramatic responses, BRAF/MEK targeted therapies often fail to eradicate BRAF-mutant melanoma cells. Drug resistance is usually associated with either the enrichment of a subpopulation of drug-tolerant cells that are intrinsically insensitive to the effect of BRAF/MEK inhibition, or the acquisition of adaptive phenotypes with reversibly reduced dependency on MAPK signaling. Numerous studies have associated such fluctuations in the state of MAPK pathway dependency to variations in differentiation state, represented by levels of SOX10, MITF, NGFR and receptor tyrosine kinases such as AXL. Although the consequences of such epigenetic heterogeneities for drug resistance are widely recognized, there is still little known about actionable epigenetic factors that may link heterogeneity in the state of MAPK dependency to plasticity in differentiation state. A system-wide exploration of these factors may reveal novel opportunities for epigenetic treatments that will minimize the emergence of drug resistance. We took a systems pharmacology approach to test the hypothesis that heterogeneity in the state of MAPK pathway dependency may result from a subset of key epigenetic variations across tumor cells of heterogeneous differentiation states. To identify regulator of such variations, we used a library of 276 small molecule epigenetic modulators in BRAF-mutant cell lines that cover a wide spectrum of melanoma differentiation states. We performed a systematic analysis of the effect of each epigenetic modulator (assayed individually and in combination with BRAF and MEK kinase inhibitors) on melanoma cell survival, proliferation, differentiation state, activity of the MAPK signaling pathway, and their variations with treatment dose and time. Integrating multiplexed single-cell analysis of a panel of 16 cell lines with multivariate statistical modeling and genetic experiments, we identified epigenetic features that may link single-cell heterogeneity in melanoma differentiation state to BRAF/MAPK dependency. We identified three classes of small molecules that target seemingly distinct epigenetic states in melanoma cells. These epigenetic states included: (1) a lysine demethylase 1A (KDM1A)-dependent state, predominantly observed in undifferentiated (AXL^{High}), BRAF/MEK inhibitor-resistant (p-ERK^{High}) cells, that is efficiently inhibitable by the reversible KDM1A inhibitor SP2509 (and its clinical formulation SP2577; Seclidemstat), (2) a lysine demethylase 4B (KDM4B)-dependent state, associated with neural crest-like (NGFR^{High}/AXL^{Low}) cells, that is sensitive to JIB-04 (a pan-inhibitor of Jumonji histone demethylases) and induces synergistic cell death upon exposure to the combination of JIB-04 and BRAF/MEK inhibitors, and (3) a state induced by BET bromodomain inhibitors such as OTX015 (Birabresib) in NGFR^{High} cells, which substantially enhances their requirement for MAPK signaling and, thereby, their sensitivity to BRAF/MEK inhibitors. Single-cell analysis of tumor lines shows that these states might co-exist in different combinations and frequencies, highlighting mutual epigenetic vulnerabilities among genetically diverse melanoma cell populations. Importantly, non-transformed primary melanocytes were not sensitive to any of the identified epigenetic inhibitors. Our systems pharmacology approach highlights an avenue toward identifying actionable epigenetic factors that extend the BRAF oncogene addiction paradigm on the basis of tumor cell differentiation state.

Unveiling the mechanism of action of MTX-216 in overcoming drug resistance in NF1-mutant melanomas

Cara Abecunas¹, Christopher Whitehead², Elizabeth Ziemke³, Judith Sebolt Leopold^{2,3,4}, Mohammad Fallahi-Sichani^{1,5}

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²Mekanistic Therapeutics, LLC, Ann Arbor, MI

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Introduction: In contrast to BRAF-mutant melanomas that are responsive to MAPK-targeted therapies, tumors associated with loss-of-function (LoF) mutations in neurofibromin 1 (NF1; a negative regulator of RAS signaling) exhibit inconsistent sensitivity to inhibitors of the MAPK pathway or the parallel PI3K/Akt pathway. To identify actionable vulnerabilities in NF1^{LoF} melanoma cells, we screened the efficacy of a variety of RAS-related pathway inhibitors, used either individually or in combination, through multiplexed and single-cell measurements. We found a dual EGFR/PI3K inhibitor, MTX-216, which selectively kills NF1^{LoF} cells and inhibits tumor growth *in vivo*. Interestingly, MTX-216's analogue, MTX-211 (which is also a dual EGFR/PI3K inhibitor), does not elicit cell death in NF1^{LoF} cells. We sought to apply a systems pharmacology approach to identify the mechanisms through which MTX-216 induces tumor cell killing selectively in NF1^{LoF} cells. Understanding these mechanisms will uncover therapeutic vulnerabilities in NF1^{LoF} melanomas and will ultimately aid in the development of new therapies.

Materials and Methods: To identify genes and pathways associated with the specific action of MTX-216, we used RNA sequencing to transcriptionally profile two NF1^{LoF} melanoma cell lines (WM3918, MeWo), treated for 24 h with either MTX-216 (at 1 μ M), MTX-211 (at 1 μ M), or DMSO in triplicate. We used DESeq2 to perform differential gene expression analysis, and Enrichr for pathway enrichment analysis. To systematically compare kinases that are targeted by each inhibitor, we identified the percentage of inhibition of approximately 400 kinases by each of the compounds when used at 10 μ M using Invitrogen/LifeTech kinase assays. We confirmed our hypothesis regarding the MTX-216's targets using gene knockdown by siRNA and analyzing cellular growth rate.

Results and Discussion: Our analysis led to the identification of SYK, a kinase regulator of RAS signaling, as a target of MTX-216 that is critical for blocking NF1^{LoF} cell growth. Transcriptional profiling determined a number of significant upregulated and downregulated genes that were specific to either MTX-216 or MTX-211 across both cell lines. We used Enrichr to compare the genes downregulated uniquely by MTX-216 to the gene expression signatures associated with kinase perturbations (across the GEO database). Our analysis revealed SYK inhibition as the most significant perturbation. Independent kinase inhibition assays also determined SYK to be inhibited by MTX-216 at a significantly greater level than by MTX-211. Knocking down SYK by siRNA significantly reduced net growth rate in MeWo cells compared to the siRNA control, confirming its key role for NF1^{LoF} cell growth.

Conclusions: Our systems pharmacology approach reveals SYK as a potentially novel vulnerability in NF1^{LoF} melanoma cells.

Selective Inhibition of NF1 Loss-of-Function Driven Oncogenic Signaling in Melanomas

Cara Abecunas ¹, Christopher Whitehead ², Judith Leopold ^{2,3,4} and Mohammad Fallahi-Sichani ¹

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³Department of Pharmacology and ⁴Department of Radiology, University of Michigan, Ann Arbor, MI

Oncogenic activation of the MAPK signaling pathway occurs across >90% of melanomas, motivating the clinical evaluation of MAPK-targeted therapies in these tumors. However, only BRAF^{V600E/K}-mutated melanomas show consistent clinical responses to MAPK-targeted therapies. Inactivation of the tumor suppressor gene neurofibromin 1 (NF1) is among the mechanisms that drive hyperactivated RAS signaling in >10% of melanomas. These tumors, however, have variable dependency on RAS-mediated pathways, such as MAPK and PI3K/mTOR, and thus exhibit inconsistent sensitivity to targeted inhibitors of these pathways. It is of paramount importance, therefore, to identify new therapeutic strategies to block tumor cell growth or improve MAPK- or PI3K-targeted therapy response in NF1 loss-of-function (NF1^{LoF}) melanomas.

To identify actionable vulnerabilities in NF1^{LoF} melanoma cells, we apply a systematic approach, combining multiplexed high-throughput immunofluorescence microscopy, single-cell measurements and computational analysis, to study the responses of differentially mutated human melanoma cell lines to a variety of RAS-related pathway inhibitors.

Our analysis led to the identification of a multi-kinase inhibitor, MTX-216, which is efficacious in induction of tumor cell death and inhibition of NF1^{LoF} melanoma growth both *in vitro* and *in vivo*, but does not exhibit cytotoxicity in BRAF^{V600E} melanomas and wild-type melanocytes. Single-cell analysis of downstream RAS-mediated signaling markers (e.g. phospho-S6) and proliferation markers (e.g. Ki-67) demonstrated cell-to-cell heterogeneity in NF1^{LoF} melanoma cells treated with commercially available kinase inhibitors, resulting in incomplete drug responses. MTX-216 reduces this single-cell variability, providing evidence that co-suppression of signaling and proliferation pathways is correlated with drug-induced cytotoxicity.

Our systematic analysis, together with ongoing studies to uncover the mechanism of action of MTX-216 in NF1^{LoF} melanomas, has the potential to identify new and effective treatment strategies for NF1-mutant melanomas.

Loss of NF1 in Melanoma Confers Sensitivity to SYK Kinase Inhibition

Cara Abecunas^{1,2}, Christopher E. Whitehead³, Elizabeth K. Ziemke³, Douglas G. Baumann¹, Christy L. Frankowski-McGregor³, Judith S. Sebolt-Leopold^{3,4,5}, and Mohammad Fallahi-Sichani^{1,6}



ABSTRACT

Neurofibromin 1 (NF1) loss of function (LoF) mutations are frequent in melanoma and drive hyperactivated RAS and tumor growth. NF1^{LoF} melanoma cells, however, do not show consistent sensitivity to individual MEK, ERK, or PI3K/mTOR inhibitors. To identify more effective therapeutic strategies for treating NF1^{LoF} melanoma, we performed a targeted kinase inhibitor screen. A tool compound named MTX-216 was highly effective in blocking NF1^{LoF} melanoma growth *in vitro* and *in vivo*. Single-cell analysis indicated that drug-induced cytotoxicity was linked to effective cosuppression of proliferation marker Ki-67 and ribosomal protein S6 phosphorylation. The antitumor efficacy of MTX-216 was dependent on its

ability to inhibit not only PI3K, its nominal target, but also SYK. MTX-216 suppressed expression of a group of genes that regulate mitochondrial electron transport chain and are associated with poor survival in patients with NF1^{LoF} melanoma. Furthermore, combinations of inhibitors targeting either MEK or PI3K/mTOR with an independent SYK kinase inhibitor or SYK knockdown reduced the growth of NF1^{LoF} melanoma cells. These studies provide a path to exploit SYK dependency to selectively target NF1^{LoF} melanoma cells.

Significance: A kinase inhibitor screen identifies SYK as a targetable vulnerability in melanoma cells with NF1 loss of function.

Introduction

Neurofibromin 1 (NF1) is a tumor suppressor gene encoding a RAS GTPase-activating protein, whose loss of function (LoF) is the third most frequent mutation present in approximately 13% of melanomas (1–4). The LoF of NF1 abrogates negative feedback on RAS, thereby causing hyperactivation of the downstream MAPK and PI3K/mTOR signaling pathways (5, 6). NF1^{LoF} mutations, however, do not always predict sensitivity to MEK/ERK kinase inhibitors or PI3K/mTOR inhibitors and most NF1^{LoF} melanomas show only partial responses to these therapies (7–9).

Multiple strategies have been tested to enhance treatment efficacy in NF1^{LoF} melanomas. The most commonly explored approaches have been focused on testing combinations of small molecules (e.g., MEK/ERK inhibitors and PI3K/mTOR inhibitors) that target parallel pathways downstream of RAS signaling, to prevent the bypass mechanisms of drug resistance (10). Targeting RAS regulators that function downstream of receptor tyrosine kinases (RTK) has been proposed as another strategy based on evidence that NF1^{LoF} cells

require upstream RTK signals to drive aberrant cell growth (11). SHP2 inhibitors, for example, decouple the RAS/MAPK pathway from external growth signals and thereby reduce tumor growth in NF1^{LoF} melanoma cells (12). An alternative strategy would be to evaluate the effectiveness of multi-targeted inhibitors that block key signaling molecules upstream and downstream of RAS (13, 14). A possible advantage of this approach may be a further increase in treatment efficacy that results from cotargeting the pathways on which NF1^{LoF} cell growth and survival are dependent as well as adaptive pathways that diminish the efficacy of individual MEK/ERK or PI3K/mTOR inhibitors.

In this article, we perform a targeted compound screen to search for actionable kinases upstream, parallel to, or downstream of MAPK and PI3K/mTOR pathways, whose inhibition individually or in combination with one another efficiently block NF1^{LoF} melanoma cells. We identify MTX-216, a dual ATP-competitive inhibitor of PI3K isoforms and EGFR, to kill NF1^{LoF} melanoma cells in a dose-dependent manner and suppress NF1^{LoF} melanoma tumor growth *in vivo*. Single-cell analysis reveals a substantial correlation between drug efficacy in NF1^{LoF} melanoma cells and effective cosuppression of Ki-67 (a proliferation marker) and the ribosomal S6 phosphorylation (an integrator of multiple RAS-mediated signaling pathways). These effects, however, are not explained merely by the nominal targets of MTX-216. Kinome selectivity analysis integrated with differential gene expression and pathway enrichment analysis identify SYK as an additional target of MTX-216 that is required for NF1^{LoF} melanoma cell growth. SYK is a non-RTK conventionally known as a mediator of adaptive immune receptor signaling (15). Recent studies, however, have implicated SYK localization to mitochondrial intermembrane space and its involvement in the regulation of mitochondrial respiratory chain and oxidative metabolism (16–20). Consistent with these studies, we find that MTX-216 treatment in NF1^{LoF} melanoma cells suppresses genes encoding mitochondrial electron transport chain (ETC). In agreement with these findings, treatment with IACS-010759, a clinical-grade small-molecule inhibitor of the mitochondrial ETC (21), also induces cell death in these

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cells. Therefore, the selectivity profile of MTX-216, which includes SYK inhibition and the consequent suppression of mitochondrial ETC, causes the unusually high activity of this compound in NF1^{LoF} melanoma cells.

Materials and Methods

Cell lines and reagents

Melanoma cell lines used in this study were obtained from the following sources: LOXIMVI (CVCL_1381) and COLO858 (CVCL_2005) from MGH Cancer Center, COLO792 (CVCL_1134) from ECACC, MeWo (CVCL_0445), Ramos Burkitt lymphoma (CVCL_0597), and A375 (CVCL_0132) from ATCC, SKMEL113 (CVCL_6074) from Memorial Sloan Kettering Cancer Center, WM3918 (CVCL_C279) from Rockland Immunochemicals, human epidermal primary melanocytes (NHEM-Ad) from Lonza, YUHEF (CVCL_G326), YURED (CVCL_K020), YUHOIN (CVCL_J521), and YUTICA (CVCL_J071) from Yale University Dermatology Cell Culture Facility. All cell lines have been periodically subjected to reconfirmation by short tandem repeat profiling by ATCC and *Mycoplasma* testing by MycoAlert PLUS *Mycoplasma* detection Kit (Lonza). YUHEF, YURED, YUHOIN, and YUTICA cells were grown in Opti-MEM (Gibco, catalog no. 31985088) supplemented with 5% FBS. COLO858, LOXIMVI, COLO792, and SKMEL113 cells were grown in RPMI1640 (Corning, catalog no. 10-040-CV) supplemented with 1% sodium pyruvate (Invitrogen) and 5% FBS. A375 cells were grown in DMEM with 4.5 g/L glucose (Corning, catalog no. 10-013-CV) supplemented with 5% FBS. MeWo cells were grown in EMEM (Corning, catalog no. 10-009-CV) supplemented with 5% FBS. WM3918 cells were grown in 80% MCDB 153 (Sigma-Aldrich, catalog no. M7403), 20% Leibovitz's L-15 (Sigma-Aldrich, catalog no. L1518), 5% FBS, and 1.68 mmol/L CaCl₂. Ramos cells were grown in RPMI1640 supplemented with 1% sodium pyruvate and 10% FBS. NHEM-Ad cells were grown in MGM-4 Melanocyte Growth Medium-4 BulletKit (Lonza, catalog no. CC-3249). We added penicillin (50 U/mL) and streptomycin (50 µg/mL) to all growth media.

Small-molecule inhibitors trametinib, ulixertinib, AZD8055, palbociclib, gefitinib, pictilisib, entospletinib, R406, and IACS-010759 were all purchased from Selleck Chemicals. MTX-216 and MTX-211 were synthesized by Albany Molecular. MTX-211 (HY-107364) is also available from MedChemExpress. Compounds used for cell-based studies were dissolved in the appropriate vehicle (DMSO) at a stock concentration of 5 or 10 mmol/L.

The following primary mAbs and polyclonal antibodies (pAb) with specified animal sources, catalog numbers and dilution ratios, were used in immunofluorescence (IF) staining or Western blot (WB) assays: Ki-67 (AB_2797703; mouse mAb, Cell Signaling Technology, catalog no. 9449, IF, 1:800), p-S6^{S235/S236} (AB_10695457; rabbit mAb, Cell Signaling Technology, catalog no. 4851, IF, 1:400), p-p44/42 MAPK (ERK1/2; T202/Y204; AB_2315112; rabbit mAb, Cell Signaling Technology, catalog no. 4370, IF, 1:800), p-AKT^{S473} (AB_2315049; rabbit mAb, Cell Signaling Technology, catalog no. 4060, IF, 1:400), p-4EBP1^{T37/46} (AB_560835; rabbit mAb, Cell Signaling Technology, catalog no. 2855, IF, 1:200), SYK (AB_628308; mouse mAb, Santa Cruz Biotechnology, catalog no. 1240, WB, 1:1,000), p-SYK^{Y525/526} (AB_2197222; rabbit mAb, Cell Signaling Technology, catalog no. 2710, WB, 1:1,000), p-BLNK^{Y96} (AB_2064915; rabbit pAb, Cell Signaling Technology, catalog no. 3601, WB, 1:1,000), BLNK (AB_626752; mouse mAb, Santa Cruz Biotechnology, catalog no. 8003, WB, 1:1,000), and β-tubulin (AB_2210545) (rabbit pAb, Cell Signaling Technology, catalog no. 2146, WB, 1:1,000). The follow-

ing primary mAb and pAb with specified animal sources, catalog numbers, and dilution ratios, were used in WB assays in xenograft pharmacodynamic studies: SYK (AB_2687924; rabbit mAb, Cell Signaling Technology, catalog no. 13198, WB, 1:1,000), EGFR (AB_2230881; rabbit mAb, Cell Signaling Technology, catalog no. 2646, WB, 1:1,000), AKT (AB_329827; rabbit pAb, Cell Signaling Technology, catalog no. 9272, WB, 1:1,000), β-actin (AB_10950489; rabbit mAb, Cell Signaling Technology, catalog no. 8457, WB, 1:1,000), p-SYK^{Y525/526} (rabbit mAb, Cell Signaling Technology, catalog no. 2710, WB, 1:1,000), p-EGFR^{Y1068} (AB_2096270; rabbit mAb, Cell Signaling Technology, catalog no. 3777, WB, 1:1,000), and p-AKT^{S473} (rabbit mAb, Cell Signaling Technology, catalog no. 4060, WB, 1:1,000). The following secondary antibodies with specified sources and catalog numbers were used at 1:2,000 dilution for IF assays: anti-mouse Alexa Fluor 488 (AB_141607; Thermo Fisher Scientific, catalog no. A21202) and anti-rabbit Alexa Fluor 647 (AB_2536183; Thermo Fisher Scientific, catalog no. A31573). The following secondary antibodies with specified sources and catalogue numbers were used at 1:15,000 dilution for WB assays: anti-rabbit IRDye 800CW (AB_621848; LI-COR Biosciences, catalog no. 926-32213) and anti-mouse IRDye 680RD (AB_10953628; LI-COR Biosciences, catalog no. 926-68072). The following secondary antibody with specified source and catalog number was used at 1:10,000 dilution for WB pharmacodynamic assays: horseradish peroxidase-conjugated anti-rabbit antibody (AB_2313567; rabbit IgG, Jackson ImmunoResearch Laboratories Inc., catalog no. 111-035-003).

High-throughput drug treatment and measurements of drug sensitivity

Growth rate inhibition assays with kinase inhibitors were performed in 96-well clear bottom black plates (Corning, catalog no. 3904). Viable cells were counted using a TC20 automated cell counter (SCR_008426; Bio-Rad Laboratories) and seeded in 200 µL of full growth media at a density of 1,700 to 2,500 cells per well. Cells were treated the next day with small-molecule compounds at reported concentrations or with vehicle control (DMSO) using a D300e Digital Dispenser. At designated timepoints ($t = 24, 72, 120$, and 168 hours), surviving cells were fixed, stained with Hoechst 33342 (Thermo Fisher Scientific, catalog no. H3570), imaged, and counted following image analysis as described in the IF staining and image analysis section.

The net growth rate for cells treated with individual compounds (at different doses), or their combination, or vehicle, was calculated from time-dependent changes in live cell count according to the following equation:

$$k = \frac{\ln\left(\frac{N_{t2}}{N_{t1}}\right)}{t_2 - t_1} \quad (\text{A})$$

where N_{t1} and N_{t2} represent the number of cells measured at timepoints $t = t_1$ and $t = t_2$, respectively, and k describes the net growth rate of cells during the time period between t_1 and t_2 . The net growth rates were then averaged across multiple, consecutive time intervals to determine the mean net growth rate for each condition. To compare drug sensitivity among different cell lines, we normalized the mean net growth rate measured for drug-treated cells (k_{drug}) to that measured for vehicle (DMSO)-treated cells (k_{DMSO}) to calculate the drug-induced normalized growth rate [aka DIP rate (22)]:

$$\text{Normalized growth rate (DIP)} = \frac{k_{\text{drug}}}{k_{\text{DMSO}}} \quad (\text{B})$$

Area over the dose–response curve (AOC; ref. 23) was calculated across all tested inhibitor concentrations (c_i) as follows:

$$\text{AOC} = \int 1 - \text{DIP}(c)dc \cong 1 - \sum_{c_i} \text{DIP}(c_i) \quad (\text{C})$$

IF staining and image analysis

Cells in Corning 96-well plates were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature, washed twice with PBS, and then permeabilized in 100% ice-cold methanol for 10 minutes at -20°C . Cells were rewashed with PBS, blocked using Odyssey Blocking Buffer (LI-COR) for 1 hour at room temperature, and stained with primary antibodies at their appropriate dilutions in Odyssey Blocking Buffer overnight (~ 16 hours) at 4°C . The next day, cells were washed three times with PBS supplemented with 0.1% Tween-20 (Sigma-Aldrich; PBS-T), and then stained with secondary antibodies for 1 hour at room temperature followed by washing once with PBS-T, once in PBS, and incubation with Hoechst 33342 for 20 minutes at room temperature. Cells were washed twice with PBS and imaged with a $10\times$ objective using the ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices) or the Operetta CLS High-Content Imaging System (Perkin Elmer). A total of nine sites were imaged per well. Background subtraction was performed with ImageJ v.2.1.0 software (SCR_003070). Image segmentation and signal intensity quantification was performed with CellProfiler v.3.1.9 (SCR_007358; ref. 24). Single-cell data were analyzed using MATLAB 2019b (SCR_001622). By generating histograms of single-cell data across a variety of conditions for each protein (X), including positive and negative controls, we identified an appropriate binary gate, based on which the percentage of X^{High} versus X^{Low} cells in each condition was quantified.

NucView apoptosis assay

COLO792 and MeWo cells were seeded in 96-well clear bottom black plates in full phenol red–free growth media (100 μL /well). WM3918 were seeded in their usual full growth media. Cells were treated the next day with indicated concentrations of MTX-216 (in duplicate) using D300e Digital Dispenser. After 48 or 72 hours of treatment, a reagent cocktail comprised of Hoechst 33342 and NucView 488 Caspase-3 reporter (Biotium, catalog no. 30029) was added to each well for final concentrations of 1 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{mol}/\text{L}$, respectively. The plates were then incubated in a tissue culture incubator (37°C , 5% CO_2) for 1.5 hours. Prewarmed PFA was then added to each well for a final concentration of 1%. Plates were then sealed using Microseal aluminum foil (Bio-Rad), incubated for 30 minutes at room temperature and imaged with a $10\times$ objective. Image analysis and quantification was performed as described in the IF staining and image analysis section.

Annexin V and propidium iodide double-staining apoptotic assay

WM3918 cells were harvested 48 hours after treatment with inhibitor or DMSO. FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, catalog no. V13242) was used to stain cells for Annexin V and propidium iodide (PI). As per the manufacturer's protocol, samples were washed with ice-cold PBS and suspended in 1x annexin binding buffer. Cell suspensions were mixed with 5 μL FITC Annexin V and 1 μL PI (100 $\mu\text{g}/\text{mL}$) for 15 minutes at room temperature. The mixture was further diluted with 1x annexin binding buffer. Samples were run in BD LSRFortessa Cell Analyzer (SCR_018655; BD Biosciences). Flow data were cleaned up by removing debris and analyzed in FCS Express 7 Research (SCR_016431; DeNovo Software).

Biochemical kinase inhibition assays

To assess their ability to inhibit each of 403 human kinases, MTX-216 and MTX-211 were outsourced to SelectScreen Biochemical Kinase Profiling service at Thermo Fisher Scientific. Each compound was initially screened at 10 $\mu\text{mol}/\text{L}$ using either Z'-LYTE or Adapta kinase activity assay at an ATP concentration of $K_{m,\text{app}}$ for each target. The Z'-LYTE assay monitors phosphorylation of peptide substrates, and Adapta quantifies ADP formation and thus can be used with lipid substrates. For selected targets that were inhibited significantly ($>90\%$) by MTX-216 or MTX-211 at 10 $\mu\text{mol}/\text{L}$ (e.g., SYK), the kinase activity assay was repeated across 10 concentrations. Dose–response plots and the corresponding IC_{50} s were generated using the function doseResponse (developed by Ritchie Smith) available on the MATLAB Central File Exchange (<https://www.mathworks.com/matlabcentral/fileexchange/33604-doseresponse>).

Gene knockdown by siRNA

siRNA knockdown experiments with MeWo and YUHEF cells were performed in antibiotic-free growth media either in cells seeded in 10-cm plates (for the purpose of Western blotting) or in Corning 96-well clear bottom black plates (at a density of 2,500 and 1,000 cells/well, respectively) for the purpose of cell counting and inhibitor sensitivity analysis. In both cases, 24 hours after cell seeding, cells were transfected with Dharmacon's ON-TARGETplus Human SMARTpool siRNA for SYK (L-003176-00-0005), nontargeting control (D-001810-10-05), or the four SYK-specific siRNAs that make up the SYK SMARTpool (J-003176-10, J-003176-11, J-003176-12, J-003176-13) at 25 nmol/L each. DharmaFECT 2 (GE Dharmacon, catalog no. T-2002-01) and DharmaFECT 3 (GE Dharmacon, catalog no. T-2003-01) were used as transfection reagents for MeWo and YUHEF cells, respectively. After 72 hours of culture in the presence of siRNAs, cells in 10-cm plates were transferred to ice, washed with ice-cold PBS, and lysed for the purpose of Western blotting. After 72 hours of knockdown, cells in 96-well plates were either fixed with 4% PFA or treated with kinase inhibitors (trametinib, MTX-211, pictilisib) or DMSO for an additional 72 hours before fixation. All fixed cells were then stained with Hoechst 33342 and counted using quantitative IF microscopy. Time-dependent changes in cell count were used to compute normalized growth rates as described above.

In vivo xenograft assays

Animal handling, care, and treatment were carried out in accordance with procedures approved by the Institutional Animal Care and Use Committee at the University of Michigan (Ann Arbor, MI). For the COLO792 xenograft studies, female 5 to 6 weeks old NCR nude mice (IMSR_TAC:ncrnu; Taconic, CrTac:NCR-Foxn1^{tmu}) were implanted subcutaneously with 2.5×10^6 cells in 1:1 Matrigel:serum-free media. When the average tumor volume was approximately 125 mm^3 (on day 39 postimplantation), mice were randomized into treatment groups and treatments were initiated. MTX-216 was prepared fresh daily as a clear solution in 1:2 propylene glycol:1% Tween80/ Na_3PO_4 , and administered intraperitoneally based upon individual animal body weight (0.2 mL/20 g) on days 1 (i.e., day 39 post implantation), 2, 4, 7, 10, 13, and 16. Tumor volumes were measured on days 1, 3, 6, 8, 12, 15, 18, and 20 posttreatment. For the WM3918 xenograft studies, female 5–6 weeks old NCR nude mice (Taconic) were implanted subcutaneously with 1.5×10^6 cells in 1:1 Matrigel:serum-free media. When the average tumor volume was approximately 115 mm^3 (on day 6 post implantation), mice were randomized into treatment groups and treatments were initiated. MTX-216 was prepared fresh daily as a fine, light brown homogenous

suspension including 5% DMSO and 95% PBS with a pH of 7. MTX-216 was administered once daily intraperitoneally based upon individual animal body weight (0.2 mL/20 g), beginning on day 1 (i.e., day 6 post implantation). Tumor volumes were measured on days 1, 3, 5, 7, 9, and 11. Tumor volumes were calculated by measuring two perpendicular diameters with calipers and using the formula: tumor volume (mm^3) = (length $\times$ width²)/2. Animal body weights were measured two to three times a week. At the completion of the experiment or when vehicle-treated tumor volume exceeded 1,000 mm^3 , the mice were euthanized.

To measure the pharmacodynamic effect of MTX-216 *in vivo*, female 5 to 6 weeks old NCR nude mice (Taconic) were implanted subcutaneously with 3×10^6 WM3918 cells in 1:1 Matrigel:serum-free media. When tumors reached approximately 150 mm^3 , mice were randomized into treatment groups. Mice received a single treatment of vehicle control (5% DMSO/95% PBS), MTX-216 at 50 mg/kg, or MTX-216 100 mg/kg i.p. At 30 and 60 minutes posttreatment, mice were euthanized, and the tumors were harvested and snap frozen in liquid nitrogen.

WBs

Cultured melanoma cells treated with indicated siRNAs or inhibitors were transferred to ice, washed with ice-cold PBS, and lysed with ice-cold RIPA buffer (Thermo Fisher Scientific, catalog no. 89901) consisting of 25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS supplemented with 1 mmol/L Na_3VO_4 , 15 mmol/L NaF, cOMplete ULTRA ethylene diamine tetraacetic acid (EDTA)-free protease inhibitors (Roche, catalog no. 5892953001) and PhosSTOP (Roche, catalog no. 4906837001). Protein samples of cleared lysates were measured using BCA assay (Thermo Fisher Scientific, catalog no. PI23235). Lysates were adjusted to equal protein concentrations for each treatment condition, 4 $\times$ NuPage LDS sample buffer (Invitrogen, catalog no. NP0007) supplemented with 1 μL of 1 M DTT was added, and samples were heated for 10 minutes at 70°C. Samples were then loaded on NuPAGE 3%–8% Tris-Acetate gels (Invitrogen). WBs were performed using iBlot 2 Gel Transfer Stacks PVDF system (Invitrogen). Membranes were blocked with Intercept (TBS) Blocking Buffer (LI-COR, catalog no. 927-60001) for 1 hour and then incubated with primary antibodies (1:1,000 dilution) in 0.5 $\times$ Intercept blocking buffer solution (diluted with TBS, supplemented with 0.1% Tween-20) overnight at 4°C. Secondary antibodies were used at 1:15,000 dilution in 0.5 $\times$ Intercept blocking buffer solution supplemented with 0.01% SDS. Membranes were scanned on an Odyssey CLx scanner (LI-COR) with 700 and 800 nm channels set to automatic intensity at 169 μm resolution. Blot images were processed on Image Studio Lite v.5.2.5 software (LI-COR).

Ramos cells were treated with indicated inhibitors for 6 hours prior to stimulating the cells with 25 $\mu\text{g}/\text{mL}$ IgM (Goat F(ab')₂ Anti-Human IgM, Southern Biotech, catalog no. 2022-01) or BBS control for 3 minutes. Samples were lysed, processed, and run in WBs as described above.

To measure the pharmacodynamic effect of MTX-216 on p-SYK^{Y525/526}, p-EGFR^{Y1068}, p-AKT^{S473} *in vivo*, excised xenograft tumors were rinsed in cold 1 $\times$ PBS before adding complete RIPA lysis buffer. Tumors were homogenized using a douncer and rocked for 1 hour at 4°C. Tumors were then centrifuged at 4°C for 20 minutes at 13.2 rpm. Quantitation of protein lysates was performed using DC Protein Assay (Bio-Rad) according to the manufacturer's instructions. Lysates were adjusted to equal protein concentrations using water, 1 $\times$ LDS Sample Buffer (Life Technologies), and 1 mol/L DTT. Samples

were heated for 10 minutes at 70°C and loaded into NuPage 4%–12% Bis-Tris gel. Proteins were transferred to a 0.45 μm polyvinylidene difluoride membrane (Immobilon-P) for 50 minutes at 20 V in a semidry transfer apparatus containing Transfer Buffer. Membranes were blocked for 1 hour at room temperature in 5% BSA TBS-T or 5% NFDM TBS-T. Blocked membranes were incubated overnight at 4°C with primary antibodies (p-SYK^{Y525/526}, p-EGFR^{Y1068}, p-AKT^{S473}, SYK, EGFR, AKT, β -actin) in 5% BSA TBS-T or 5% NFDM TBS-T, as recommended by the manufacturer. Membranes were incubated for 1 hour at room temperature in horseradish peroxidase-conjugated anti-rabbit antibody in 1% BSA TBS-T or 1% NFDM TBS-T. The membranes were incubated in ECL Prime Western Blotting Detection Reagents (Cytiva) for 30 seconds before exposure to autoradiography film. Blot images were processed on ImageJ.

To quantitatively compare the phosphoprotein data from WBs, the intensities of phosphorylated protein bands were normalized to their respective total protein bands if antibodies were costained on the same blot. For samples that were stained for phosphorylated and total protein levels in separate blots, the band intensities were first normalized to their respective β -actin densitometry values before normalizing phosphorylated levels to total protein levels.

MitoTracker staining, imaging, and quantitation

Cells were seeded at 100 $\mu\text{L}/\text{well}$ in 96-well PerkinElmer ViewPlates (PerkinElmer, catalog no. 6005225) or 96-well Cellvis glass coverslip bottom plates (Cellvis, catalog no. P96-1.5P). Cells were treated the next day with either SYK siRNAs (or nontargeting control), 0.1–1 $\mu\text{mol}/\text{L}$ MTX-216, 0.1–1 $\mu\text{mol}/\text{L}$ MTX-211, or DMSO in triplicate. At the designated time points (72 hours in case of siRNA knockdown and 48 hours in case of inhibitor treatments), MitoTracker Deep Red (Invitrogen, catalog no. M22426) and MitoTracker Red CMXRos (Invitrogen, catalog no. M7512) were added to the wells for a final concentration of 100 nmol/L for each dye. The plates were then incubated in a tissue culture incubator (37°C, 5% CO_2) for 45 minutes. The media including dye solution were aspirated, wells were washed with prewarmed media (including inhibitor or DMSO) and placed in incubator for 1 hour to wash out nonspecific binding of dye. Cells were then washed with PBS followed by 4% PFA fixation for 15 minutes at room temperature. Plates were washed three times (5 minutes each) with PBS followed by Hoechst 33342 staining for 20 minutes. Plates were washed twice with PBS and sealed using Microseal aluminum foil (Bio-Rad). PerkinElmer plates were imaged with 20 $\times$ water-immersion objective and Cellvis glass plates were imaged with 20 $\times$ air objective using the Operetta CLS High-Content Imaging System (Perkin Elmer). A total of 37 sites were imaged per well. Image analysis was performed as described in the IF staining and image analysis section.

RNA extraction, library construction, and RNA sequencing analysis

MeWo, WM3918, and SKMEL113 cells were seeded in 10-cm plates in three replicates, and treated the next day with either DMSO, MTX-216 (at 1 $\mu\text{mol}/\text{L}$), MTX-211 (at 1 $\mu\text{mol}/\text{L}$), pictilisib (at 1 $\mu\text{mol}/\text{L}$), trametinib (at 0.1 $\mu\text{mol}/\text{L}$), or the combination of MTX-216 (at 1 $\mu\text{mol}/\text{L}$) and trametinib (at 0.1 $\mu\text{mol}/\text{L}$), for 24 hours. At the time of harvest, cells were washed once with PBS and then lysed with Rneasy Buffer RLT (Qiagen) in the dish. Lysate samples were then vortexed and frozen. To extract RNA, lysate samples were thawed at room temperature and then processed using QIAshredder (Qiagen, catalog no. 79654) and Rneasy Mini Kit (Qiagen, catalog no. 74104). RNA was Dnase treated (Qiagen, catalog no. 79254) and then eluted. RNA quality was assessed by Bioanalyzer (Agilent). All samples had RNA integrity numbers

(RIN) of 9.0 or higher. Prior to poly-A enrichment, 500 ng of each RNA sample was supplemented with 1 μ L (1:100 dilution) of ERCC spike-in control (Life Technologies, catalog no. 4456740). Poly-A RNA enrichment was performed using NEBNext Poly(A) kit (New England BioLabs). Fragments were end-modified with adaptors using NEBNext Multiplex Oligos for Illumina Set 2 (New England BioLabs), following library amplification (PCR, 11 cycles). Resulting libraries were prepared using NEBNext Ultra II Directional RNA Kit (New England BioLabs). Samples were run on Illumina NovaSeq 6000 (SCR_01638; S4) using paired-end read with 150 cycles. In total, 1.4 billion reads were generated, including approximately 33 million reads per sample. Reads were mapped to the human genome (hg38) using HISAT2 (SCR_015530; ref. 25), transcripts were assembled and quantified using StringTie (SCR_016323; refs. 26, 27), and differential gene expression analysis was performed by DESeq2 (SCR_015687) using the web-based Galaxy platform (SCR_006281; refs. 28, 29).

To identify genes regulated uniquely by MTX-216 versus MTX-211 in WM3918 and MeWo cells, we first selected those genes with base mean ≥ 1 , that changed in abundance by more than 2-fold relative to a DMSO-treated control ($|\log_2(\text{fold change})| \geq 1$; FDR < 0.05) in each cell line. Among these genes, we then identified the subset that was similarly upregulated or downregulated by each compound in both cell lines. Genes enriched specifically by MTX-216 treatment (but not in MTX-211 treatment) in both cell lines were used in Enrichr (SCR_001575; <https://maayanlab.cloud/Enrichr/>; ref. 30) and compared statistically with gene expression signatures extracted from the Gene Expression Omnibus (GEO; SCR_005012) database for kinase perturbation (31).

To systematically perform differential gene expression analysis across three cell lines treated with different compounds (MTX-216, MTX-211, pictilisib, trametinib, MTX-216 plus trametinib, or DMSO), we used principal component analysis (PCA). For PCA, we first organized the gene expression dataset into a matrix with 42 rows (representing three cell lines, six distinct treatment conditions, and three independent replicates) and 16,940 columns (representing genes). The list of these genes was generated after filtering out transcripts with unidentified gene names and functions in StringTie and removing those genes for which all 42 conditions had transcripts per million (TPM) values < 1 . Then $\log_2(\text{TPM}+1)$ values were calculated for each gene and z-scored across all 42 conditions. PCA was then performed using the MATLAB built-in function *pca*.

Because PC3 and PC4 separated the efficacious inhibitors independent of cell lines, we used PC3 and PC4 loadings to identify genes associated with distinct inhibitory effects on cells treated with different compounds. Gene loadings that were differentially enriched along either PC3 or PC4 were selected on the basis of a ratio cutoff of *cotangent* ($\pi/8$) ≈ 2.4 . Among these genes, we identified the subsets along each PC that had loading values of greater than $1.5 \times \text{median}$ for positive loadings, or smaller than $1.5 \times \text{median}$ for negative loadings. These genes were then used in Enrichr to identify significant Gene Ontology (GO) Biological Processes (SCR_002811).

Hierarchical clustering

Following PCA, unsupervised hierarchical clustering of expression levels of genes with negative PC3 or PC4 loadings was carried out using MATLAB 2020b with the Euclidean distance metric and the Average linkage algorithm for computing the distance between clusters.

The Cancer Genome Atlas bioinformatics analysis

The clinical history and the corresponding gene expression (STAR count) data from patients enrolled in the skin cutaneous melanoma

(SKCM) project of The Cancer Genome Atlas (TCGA; ref. 1) were accessed using the cBio Cancer Genomics Portal (ref. 32; <http://cbioportal.org/>) and the Genomic Data Commons Data Portal (SCR_014514; <https://portal.gdc.cancer.gov/>). TCGA-SKCM samples were classified on the basis of their NF1 mutation status. NF1^{LoF} samples were those bearing putative driver “truncating” mutations. NF1^{Mutant} samples were those bearing putative driver “splice” or “missense” mutations. NF1^{Other} samples were those bearing no NF1 mutations or putative “passenger” mutations. Sequencing reads were accessed through *GDCquery* function in the *TCGAbiolinks* package (SCR_017683) in R (SCR_001905; ref. 33). A total of 469 sequenced melanoma tumors were analyzed: NF1^{LoF} (39 samples), NF1^{Mutant} (nine samples), and NF1^{Other} (421 samples). RNA sequencing (RNA-seq) reads were then normalized to adjust for guanine-cytosine (GC)-content effects on read counts and distributional differences between samples using *TCGAanalyze_Normalization* function within the *TCGAbiolinks* package. Genes were then z-scored across all tumors. We then calculated mean z-score values across the genes found within respiratory ETC GO term (GO:0022904; <http://geneontology.org/>). Statistical analysis of ETC expression of NF1^{LoF} patients versus NF1^{Mutant} or NF1^{Other} was analyzed in R using the built-in function *t.test*. Tumors for each NF1 cohort were then stratified into two subgroups, ETC^{High} and ETC^{Low}, based on the top and bottom quartiles. We then used tumors with ETC^{High} and ETC^{Low} signatures in NF1^{LoF} and NF1^{Other} cohorts to compare the overall patient survival between each subgroup based on two-sided *P* values computed by log-rank (Mantel-Cox) test. Stage of diagnosis comparing ETC^{High} and ETC^{Low} within the NF1^{LoF} subgroup was analyzed using the Neoplasm Disease Stage American Joint Committee on Cancer Code database, followed by χ^2 test and Benjamini-Hochberg FDR correction. SYK mutation analysis across TCGA-SKCM was also analyzed with χ^2 test followed by Benjamini-Hochberg FDR correction. All statistical results were calculated and provided by cBio Cancer Genomics Portal.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA; SCR_003199; refs. 34, 35) was performed using GSEA v.4.1.0 or v.4.2.3 software. For the prerank gene analysis, the GSEAPreranked module was used. To analyze the drug effects across the entire Hallmark database (36), genes were preranked on the basis of their $\log_2$ fold change of expression (by MTX-216 or trametinib relative to DMSO) and *P* values generated from DESeq2 for three replicates in each of the three cell lines tested (MeWo, WM3918, and SKMEL113) using the following equation:

$$R_i = -\log_{10} P_i \times \text{sign}[f_i] \quad (\text{D})$$

where R_i represents the rank for gene i , P_i represents the *P* value associated with change of expression for gene i , and f_i represents the $\log_2$ fold change associated with gene i . GSEA was performed with 1,000 permutations across Hallmark gene sets to determine down-regulated and upregulated biological pathways.

To assess the effects of individual drugs compared with DMSO with respect to MAPK and PI3K/mTOR signaling pathways, we ran prerank gene analysis using the $\log_2$ fold change generated from DESeq2. GSEAPreranked was performed with 1,000 permutations across GO ERK1 and ERK2 Cascade (GO:0070371) and Hallmark PI3K AKT MTOR Signaling gene signatures.

To compare SYK activity in NF1^{LoF} or NF1^{Mutant} versus NF1^{Other} patient samples, normalized TCGA-SKCM sequenced samples were run against each other using the default GSEA parameters with

1,000 permutations. Genes downregulated due to SYK drug inhibition from the GEO kinase perturbation database (SYK_druginhibition_282_GSE43510 and SYK_druginhibition_286_GSE43510_downregulated) were used in the enrichment analysis (30, 31).

Statistical analysis and reproducibility

No statistical method was used to predetermine sample size. Sample sizes were chosen based on similar studies in the relevant literature. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All data with error bars were presented as mean values $\pm$ SD or mean values $\pm$ SEM as indicated in figure legends using indicated numbers of independent replicates. The significance of pairwise correlations was evaluated on the basis of *P* values associated with the corresponding two-sided Pearson correlation analysis (r = correlation coefficient). Comparisons between groups were performed using the Student *t* test or ANOVA as described in figure legends. Statistical analyses were performed using MATLAB 2020b software.

Data availability

All data generated in this study are included in this article or its Supplementary Data files and the raw sequencing datasets have been deposited into GEO: GSE213588. Data analysis was performed in MATLAB and R using built-in functions and parameters as described in Materials and Methods.

Materials availability

All cell lines and reagents used in this study are available via commercial or noncommercial resources described.

Results

MTX-216 blocks NF1^{LoF} melanoma cell growth *in vitro* and *in vivo*

To identify kinases whose inhibition might elicit efficacious anti-tumor responses in NF1^{LoF} melanoma cells, we assembled a panel of small-molecule inhibitors targeting different kinases that act downstream or upstream of RAS signaling, including: MEK1/2 inhibitor (trametinib), ERK1/2 inhibitor (ulixertinib), pan-PI3K inhibitor (picilisib), mTORC1/2 inhibitor (AZD8055), CDK4/6 inhibitor (palbociclib), and EGFR inhibitor (gefitinib). We also tested the impact of inhibiting multiple kinases, either by using the combination of two distinct compounds (e.g., ulixertinib plus palbociclib) or by using multi-kinase inhibitors such as the dual PI3K/EGFR inhibitor, MTX-216, and its structural analog, MTX-211 (Fig. 1A; Supplementary Fig. S1A). We profiled the effects of these compounds on cell growth and survival in a panel of nine NF1^{LoF} melanoma cell lines. Among the tested cell lines, six expressed a wild-type (WT) BRAF (BRAF^{WT}/NF1^{LoF}), two possessed BRAF^{V600E}/NF1^{LoF} double mutations, and one carried NRAS^{Q61R}/NF1^{LoF} double mutations (Supplementary Fig. S1B). Each inhibitor was tested over a range of four concentrations, including a maximum dose (C_{max}) of approximately 10 times the reported biochemical IC_{50} for its nominal target. To compare inhibitor sensitivities, we calculated the normalized growth rates (22) by dividing the 7 day-averaged net growth rate for cells treated with each inhibitor (at each dose) to net growth rate for vehicle (DMSO)-treated cells (Fig. 1B; Supplementary Fig. S1C). Normalized growth rates < 0 indicated a net cell loss (i.e., cytotoxicity), a value of 0 represented no change in viable cell number (i.e., cytostasis), and a value of 1 represented no drug effect. All the examined inhibitor conditions led to variable responses across NF1^{LoF} cell lines. MTX-216 was, however, consistently cytotoxic, leading to steep dose-response

curves. The maximal effect (E_{max}) of MTX-216 was significantly higher than that of other tested compounds, including MAPK inhibitors trametinib and ulixertinib, which exhibited potent but less efficacious responses in NF1^{LoF} cell lines (Fig. 1B and C). Among the tested inhibitor combinations, MTX-216 plus trametinib or ulixertinib was most effective, leading to efficacious and potent responses (Supplementary Fig. S1D and S1E), suggesting that MTX-216 and MAPK inhibitors may target orthogonal vulnerabilities in NF1^{LoF} melanoma cells.

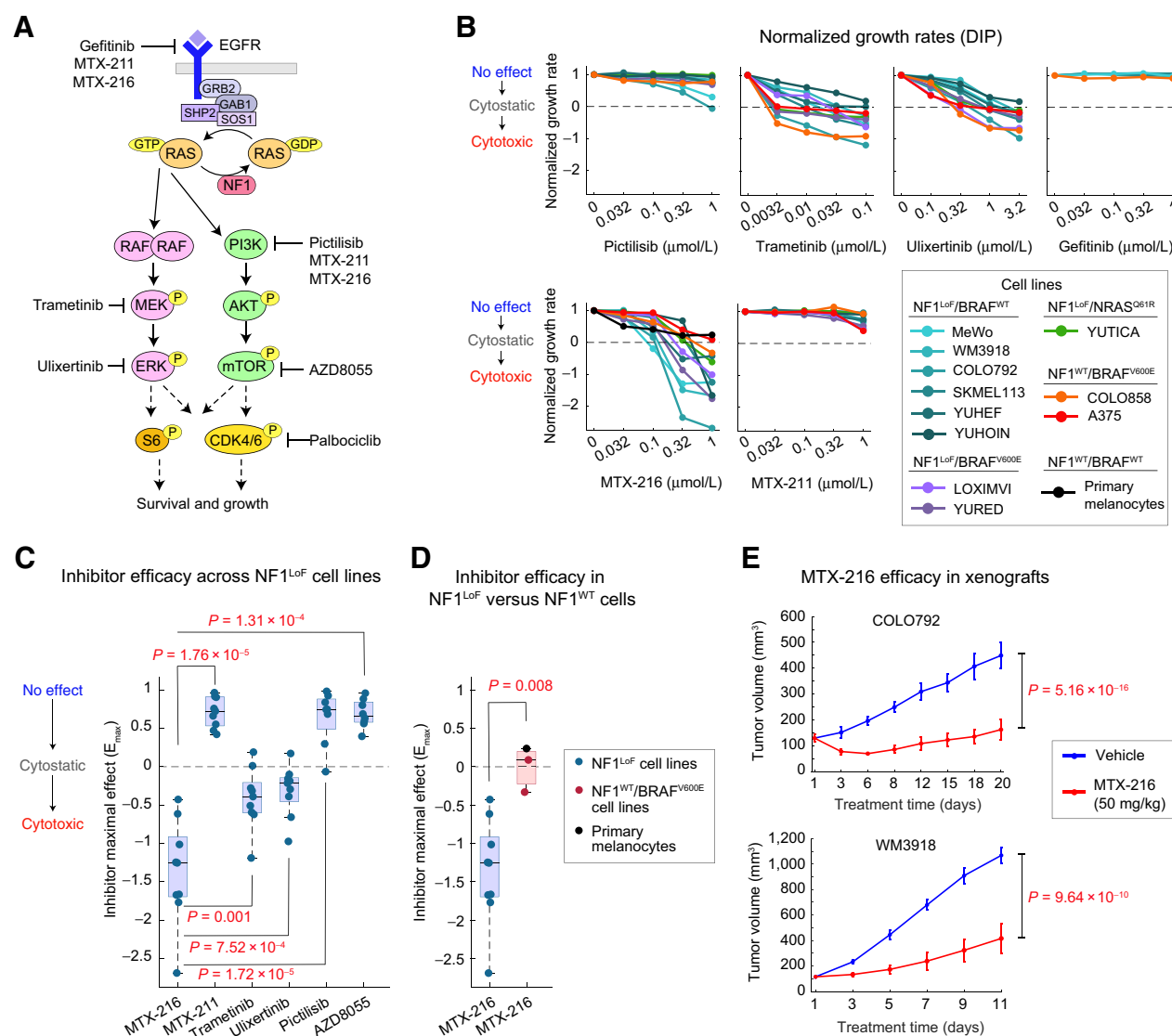
To determine whether the efficacy of MTX-216 was specific to NF1^{LoF} cells, we evaluated its effect on nontransformed primary melanocytes as well as on two NF1^{WT}/BRAF^{V600E} melanoma cell lines (A375 and COLO858) that are sensitive to trametinib and ulixertinib. When tested at its highest concentration (1 μ M/L), MTX-216 caused no cytotoxicity in primary melanocytes (Fig. 1B and D). It also exhibited a substantially less efficacious response in A375 and COLO858 cells in comparison with any of the nine NF1^{LoF} cell lines tested (Fig. 1B and D).

We then asked whether sensitivity to MTX-216 was recapitulated *in vivo*. Athymic nude mice were subcutaneously implanted with either a slowly or rapidly growing NF1^{LoF} cell line, COLO792 or WM3918, respectively. Animals were then treated with MTX-216 (at a dose of 50 mg/kg) or vehicle by intraperitoneal injection. Inhibitor administration in mice was performed every 1 to 3 days for a period of up to 20 days, during which body weight was also monitored. MTX-216 suppressed tumor growth in both cell line-derived xenograft models (Fig. 1E), while animals experienced no major loss in body weight (Supplementary Fig. S1F). Together, these data identify MTX-216 as an efficacious agent that selectively blocks NF1^{LoF} melanoma cells *in vitro* and shows encouraging signs of *in vivo* efficacy. We thus set out to identify the mechanisms that might determine the antitumor efficacy of MTX-216 in NF1^{LoF} melanomas.

MTX-216 cosuppresses Ki-67 and ribosomal S6 phosphorylation and induces apoptosis in NF1^{LoF} cells

To identify factors influencing the efficacy of MTX-216, we assayed apoptosis in NF1^{LoF} cells using two distinct methods. First, we exposed MeWo, WM3918 and COLO792 cells to increasing concentrations of MTX-216 for 48 or 72 hours. We used the NucView dye, a fluorogenic substrate of proapoptotic effector caspases 3 and 7, and measured substrate cleavage by fluorescence microscopy (37). Single-cell analysis revealed a dose-dependent increase in the frequency of apoptotic cells (with high caspase activity) induced by MTX-216 in all three NF1^{LoF} cell lines (Fig. 2A and B). We further confirmed the induction of apoptosis using a double-staining assay with Annexin-V and PI with flow cytometry. A total of 48 hours treatment of WM3918 cells with MTX-216 led to an approximately 3-fold increase in the proportion of early-stage apoptotic (Annexin-V^{High}/PI^{Low}) cells and approximately 4-fold increase in the proportion of late-stage apoptotic and necrotic (Annexin-V^{High}/PI^{High}) cells in comparison with DMSO-treated samples (Supplementary Fig. S2A).

To evaluate the effect of MTX-216 on signaling and proliferation, we quantified dose-dependent changes in the phosphorylation state of S6 ribosomal protein (p-S6^{S235/S236}) and expression of Ki-67 protein by IF microscopy. Ribosomal S6 phosphorylation (p-S6) serves as a surrogate for S6 kinase activity, integrating multiple prosurvival signals downstream of RAS signaling, including MEK/ERK and PI3K/mTOR pathways (38). Ki-67 is a marker of proliferation, which is expressed at lower levels in quiescent cells than in actively proliferating cells (39). We analyzed the single-cell expression of p-S6 and Ki-67 and their covariance in three NF1^{LoF} cell

**Figure 1.**

MTX-216 blocks tumor growth in NF1^{LoF} melanomas *in vitro* and *in vivo*. **A**, A simplified schematic illustration of the function of NF1 in the context of RAS-mediated activation of ERK and PI3K/mTOR signaling. The nominal targets for kinase inhibitors tested in this study are highlighted. **B**, Dose-dependent changes in normalized growth rates (aka DIP rates) across cell lines with different NF1/BRAF/NRAS mutation status or primary epidermal melanocytes. The average net growth rates for each condition were calculated from measurements of live cell count (across two replicates) at four time points (including 1, 3, 5, and 7 days). **C** and **D**, Inhibitor maximal effect (E_{max}), defined as the normalized growth rate evaluated at the highest tested concentration, of different inhibitors across NF1^{LoF} melanoma cell lines (**C**) and between NF1^{LoF} cell lines and NF1^{WT} cells, including two BRAF^{V600E} melanoma cell lines and primary melanocytes (**D**). Statistical significance was determined by two-sided, paired-sample *t* test (**C**) or two-sided, two-sample *t* test (**D**). **E**, Mice bearing xenografts of COLO792 cells (top) and WM3918 cells (bottom) were treated with MTX-216 or vehicle for a period of 20 and 11 days, respectively, to determine the effect on tumor growth. Tumor volume data represent mean values ± SEM across 4 mice per group (in case of COLO792) or 5 mice per group (in case of WM3918). Statistical significance was determined by two-way ANOVA.

lines (MeWo, WM3918, and COLO792) following 72 hours treatment with either MTX-216, pictilisib, trametinib, or ulixertinib. All inhibitors reduced the expression of both markers (Fig. 2C; Supplementary Fig. S2B). MTX-216, however, was the only compound that consistently drove the largest proportion of NF1^{LoF} cells toward a doubly inhibited p-S6^{Low}/Ki-67^{Low} state in all three NF1^{LoF} cell lines. In contrast, NF1^{WT}/BRAF^{V600E} COLO858 cells (which show higher sensitivity to MEK/ERK inhibitors than to PI3K/mTOR inhibitors) exhibited more effective cosuppression of p-S6 and

Ki-67 in response to trametinib and ulixertinib in comparison with MTX-216 (Fig. 2C).

Among all treatment conditions, the percentage of p-S6^{Low}/Ki-67^{Low} cells significantly correlated with treatment-induced changes in growth rate (Fig. 2D). The high efficacy of MTX-216 in blocking NF1^{LoF} melanoma cells was, therefore, associated with its strong ability to cosuppress Ki-67 and p-S6 in these cells. To test whether such high efficacy could be explained by the compound's direct effect on MAPK or AKT/mTOR pathways, we measured changes in p-ERK^{T202/Y204},

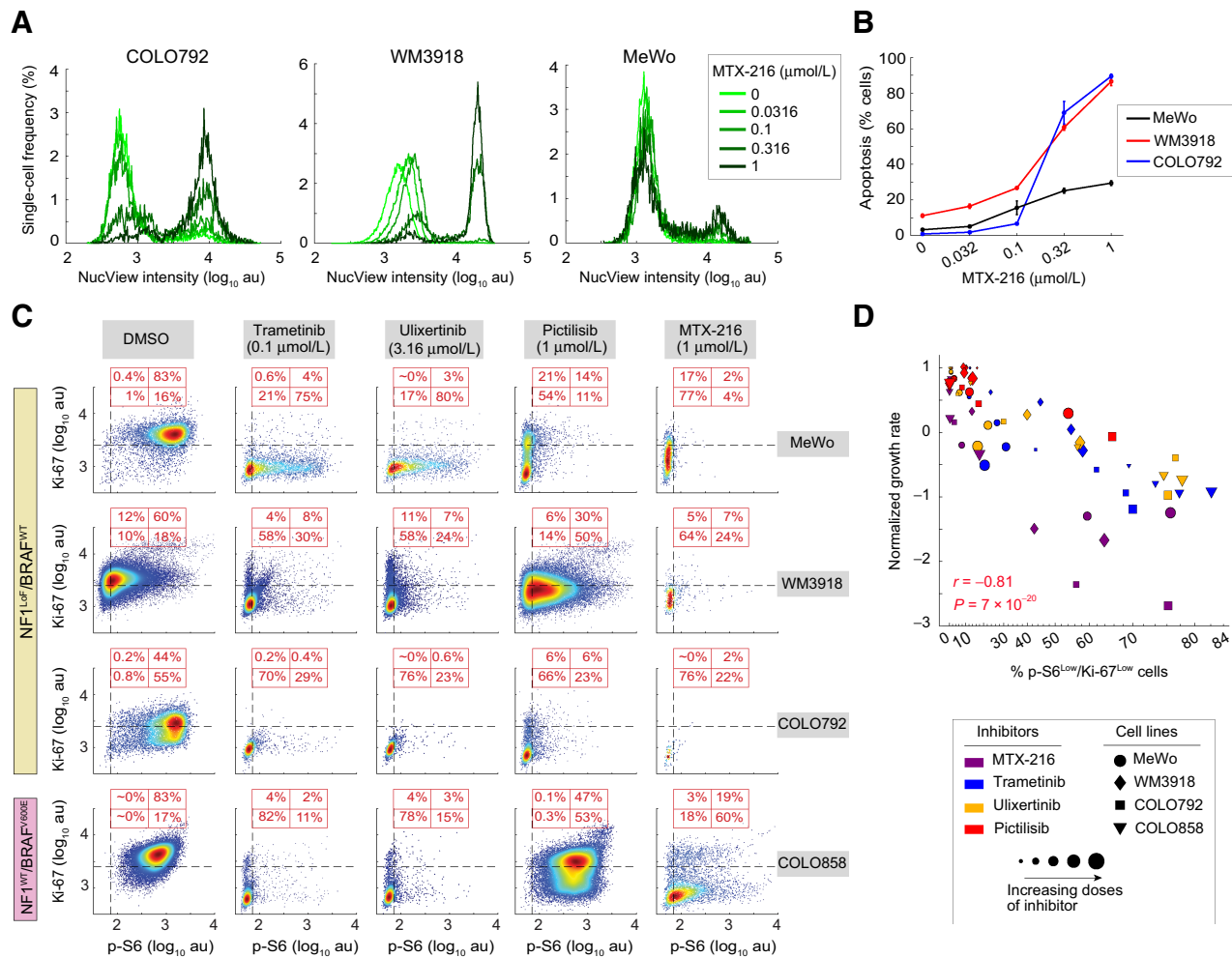


Figure 2.

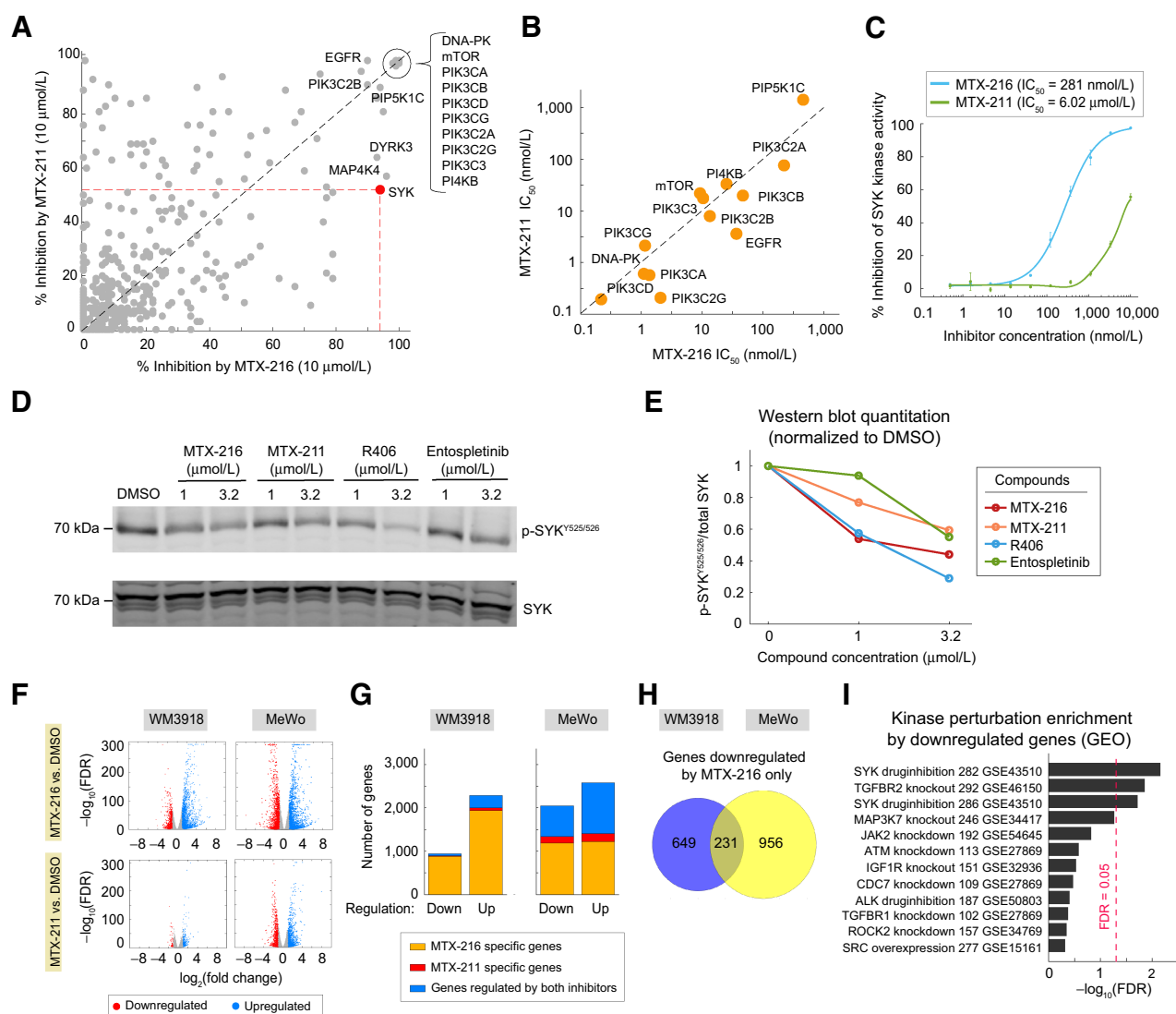
MTX-216 cosuppresses Ki-67 and p-S6 and induces apoptosis in NF1^{LoF} cells. **A** and **B**, MTX-216 dose-dependent changes in apoptotic response of COLO792 cells (after 48 hours) and WM3918 and MeWo cells (after 72 hours), measured using a NucView Caspase-3/7 reporter assay. Single-cell NucView intensities across different inhibitor doses (**A**) and the estimation of the percentage of apoptotic cells based on NucView intensity gating (**B**) are shown for each cell line. Data are presented as mean values $\pm$ SD calculated across $n = 2$ replicates. **C**, Covariate single-cell analysis of p-S6 versus Ki-67 across three NF1^{LoF}/BRAF^{WT} melanoma cell lines (MeWo, WM3918, and COLO792) and one NF1^{WT}/BRAF^{V600E} cell line (COLO858) treated for 72 hours with indicated concentrations of MTX-216, trametinib, ulixertinib, and pictilisib. The horizontal and vertical dash lines were used to gate Ki-67^{High} versus Ki-67^{Low} cells and p-S6^{High} versus p-S6^{Low} cells, respectively. Percentages of cells in each quadrant are shown in red. **D**, Two-sided Pearson correlation analysis between the percentage of p-S6^{Low}/Ki-67^{Low} cells (measured after 72 hours of treatment) and the inhibitor-induced normalized growth rate across three NF1^{LoF}/BRAF^{WT} melanoma cell lines (MeWo, WM3918, and COLO792) and one NF1^{WT}/BRAF^{V600E} cell line (COLO858) treated with MTX-216, trametinib, ulixertinib, and pictilisib (at the same concentrations as shown in **Fig. 1B**).

p-AKT^{S473}, and p-4EBP1^{T37/46} in MeWo cells following 6 hours treatment with different concentrations of MTX-216 using quantitative IF microscopy. For comparison, we also treated the cells with other inhibitors of the PI3K/mTOR and MAPK pathways, including pictilisib, AZD8055, MTX-211, and trametinib (Supplementary Fig. S2C and S2D). Trametinib was the only compound that reduced ERK phosphorylation in a dose-dependent manner, while MTX-211, MTX-216, pictilisib, and AZD8055 had no significant effect on p-ERK levels. On the other hand, pictilisib, AZD8055, MTX-216, and MTX-211, but not trametinib, decreased p-AKT levels in a dose-dependent manner. The mTOR inhibitor AZD8055 was the only compound that potently reduced the levels of p-4EBP1, a marker of mTOR activity, while MTX-216, MTX-211 and pictilisib did not decrease p-4EBP1 levels at concentrations below 1 μ mol/L, and

trametinib had no effect on p-4EBP1 levels. Overall, these data confirm the on-target effect of MTX-216 on the PI3K/AKT pathway and suggest that its high efficacy (in comparison with its structural analog MTX-211 and other compounds tested in **Fig. 1**) may be driven by mechanisms other than its direct nominal targets.

MTX-216 suppresses the activity of SYK kinase

To identify what is driving the high levels of efficacy following MTX-216 treatment, kinome selectivity testing was carried out against 403 human kinases using two commercial fluorescence resonance energy transfer (FRET)-based kinase assays, including Z'-LYTE which monitors phosphorylation of peptide substrates, and Adapta that quantifies ADP formation and can be used with lipid substrates. We also tested MTX-211 (the structural analog of MTX-216) to quantify

**Figure 3.**

SYK kinase activity is more effectively inhibited by MTX-216 than by MTX-211. **A**, High-throughput kinase inhibition assays (using Z'-LYTE and Adapta platforms) reporting percent inhibition of 403 kinases by MTX-216 and MTX-211 (both at 10 $\mu\text{mol/L}$) in the presence of ATP at a concentration of $K_{m,app}$ for each target. Dashed line represents $y = x$. **B**, IC_{50} values for the nominal targets of MTX-216 and MTX-211 (PI3K and EGFR) as well as other kinases that were inhibited significantly (>90%) by both compounds (at 10 $\mu\text{mol/L}$). IC_{50} values were derived from kinase activity assays performed across 10 concentrations of each compound. Dashed line represents $y = x$. **C**, Dose-dependent inhibition of SYK kinase activity by MTX-216 and MTX-211. SYK kinase inhibition was profiled using Z'-LYTE assay in the presence of indicated concentrations of each compound. Data are presented as mean values $\pm$ SD calculated across $n = 2$ replicates. **D** and **E**, WB measurements (**D**) and their quantification (**E**) for p-SYK^{Y525/526} relative to total SYK in MeWo cells treated for 24 hours with indicated concentrations of MTX-216, MTX-211, R406, and entospletinib. Antibodies were contained on the same blot. Data across different treatments conditions are normalized to DMSO-treated cells. **F**, Differentially upregulated or downregulated genes by MTX-216 and MTX-211 (relative to DMSO) following 24 hours treatment in WM3918 and MeWo cells. Significant genes were identified as $|\log_2(\text{fold change})| \geq 1$ and adjusted P value (FDR) ≤ 0.05 . **G**, Number of significant genes upregulated or downregulated by only one of the compounds or by both compounds in each of the WM3918 and MeWo cell lines. **H**, Number of genes downregulated specifically by MTX-216 in either or both of WM3918 and MeWo cell lines. **I**, Top enriched kinase perturbation terms associated with 231 genes downregulated specifically by MTX-216 (but not by MTX-211) in both WM3918 and MeWo cell lines.

the differences in target space between the two compounds that might be responsible for their dramatically distinct effects on NF1^{LoF} cells. In the first step, our goal was to filter out kinases that were not inhibited by either compound at a high concentration (i.e., 10 $\mu\text{mol/L}$). At this concentration, both compounds inhibited their nominal targets, including different isoforms of the catalytic subunits of PI3K (p110 α , p110 β , p110 δ , and p110 γ) and EGFR, by approximately

90% to 100% (Fig. 3A). The assay also identified additional kinase targets inhibited by both compounds at 10 $\mu\text{mol/L}$, including mTOR, PI4KB, and DNA-PK. Repeating the assay for these targets across an approximately 10^4 -fold range of concentration showed that they were all inhibited by both compounds to similar degrees (i.e., similar IC_{50} s ranging from $\sim 0.3 \text{ nmol/L}$ to 1 $\mu\text{mol/L}$; Fig. 3B; Supplementary Fig. S3A). We hypothesized that the superior efficacy of MTX-216

might be due to inhibition of a kinase that is targeted effectively by MTX-216 but not by MTX-211. The most significant candidate was SYK, a non-RTK, which was inhibited 94% and 52% by MTX-216 and MTX-211 at 10 $\mu\text{mol/L}$, respectively (Fig. 3A). Comparing the kinase activity of SYK in the presence of various concentrations (from 0.5 to 10 $\mu\text{mol/L}$) of each compound, we found that MTX-216 inhibited SYK with an IC_{50} of 281 nmol/L, which was approximately 21-fold more potent than MTX-211 (with an IC_{50} of $\sim 6 \mu\text{mol/L}$; Fig. 3C).

We then asked whether the difference in the ability of MTX-216 and MTX-211 to inhibit SYK was seen in NF1^{LoF} cells. We exposed MeWo cells to MTX-216 and MTX-211 for 24 hours and used Western blotting to measure dose-dependent changes in p-SYK^{Y525/526}, autophosphorylation sites on SYK activation loop that are required for its kinase activity (40, 41). As a control for SYK inhibition, we used two ATP-competitive SYK inhibitors, R406 and entospletinib (42, 43). Consistent with its higher potency in inhibition of SYK in biochemical assays, MTX-216 reduced p-SYK^{Y525/526} more effectively in comparison with MTX-211 across both tested concentrations (1 and 3.2 $\mu\text{mol/L}$; Fig. 3D and E). MTX-216 at 1 $\mu\text{mol/L}$ inhibited p-SYK expression in MeWo cells by approximately 50%, which was comparable to the effect of R406 and higher than the effect of entospletinib at the same concentration (Fig. 3E).

SYK function has been known primarily in the context of immune signaling in B cells, where it phosphorylates different proteins such as B-cell linker protein (BLNK; refs. 17, 44, 45). We thus set out to test the effect of MTX-216 and MTX-211 (in comparison with R406 and entospletinib) on p-BLNK^{Y96} levels in IgM-stimulated Ramos cells, a B lymphocyte cell line. WB analysis showed that SYK inhibitors reduced p-BLNK levels in IgM-stimulated Ramos cells (Supplementary Fig. S3B and S3C). In addition, MTX-216 was more efficient in inhibition of p-BLNK in comparison with MTX-211.

To test whether the difference in inhibitory effects of MTX-216 and MTX-211 on SYK kinase was recapitulated at the level of transcription, we performed RNA-seq on two NF1^{LoF} melanoma cell lines (MeWo and WM3918) exposed to either MTX-216 (at 1 $\mu\text{mol/L}$), MTX-211 (at 1 $\mu\text{mol/L}$), or vehicle (DMSO) for 24 hours. Genes differentially expressed by at least 2-fold by either MTX-216 or MTX-211 in each cell line were selected on the basis of a statistical cutoff of $\text{FDR} \leq 0.05$ (Fig. 3F; Supplementary Dataset S1). Among these genes, we focused on the subset differing in enrichment between the two compounds in both cell lines (Fig. 3G). Genes enriched specifically in MTX-216 treatment (but not in MTX-211 treatment) in both cell lines comprised of 231 downregulated genes and 467 upregulated genes (Fig. 3H; Supplementary Fig. S3D). We used these genes in Enrichr (30) and compared them statistically with gene expression signatures extracted from the GEO database for kinase perturbation. The top kinase perturbation term was SYK drug inhibition (Fig. 3I; Supplementary Fig. S3E; Supplementary Dataset S2), suggesting that MTX-216 at a concentration of 1 $\mu\text{mol/L}$ (more strongly than MTX-211) affects the expression of genes whose transcription is influenced by SYK inhibition.

Finally, we evaluated the effect of MTX-216 on its nominal targets and SYK activity *in vivo* by carrying out pharmacodynamic assessment of p-EGFR^{Y1068}, p-AKT^{S473}, and p-SYK^{Y525/526} in xenograft tumors after 0.5 or 1 hour treatment with a single dose of MTX-216 at either 50 or 100 mg/kg (Supplementary Fig. S3F–S3G). WB analysis showed that the pharmacodynamic activity was most pronounced 0.5 hour after dosing of 100 mg/kg. At this dose and timepoint, WM3918 tumors excised from treated mice showed a significant reduction in p-EGFR, p-AKT, and p-SYK levels. Furthermore, MTX-216 was comparatively more effective *in vivo* at reducing the expression of p-SYK compared

with p-EGFR and p-AKT. Together, these data identify SYK as a target of MTX-216.

Inhibition of SYK underlies increased sensitivity of NF1^{LoF} cells to MTX-216

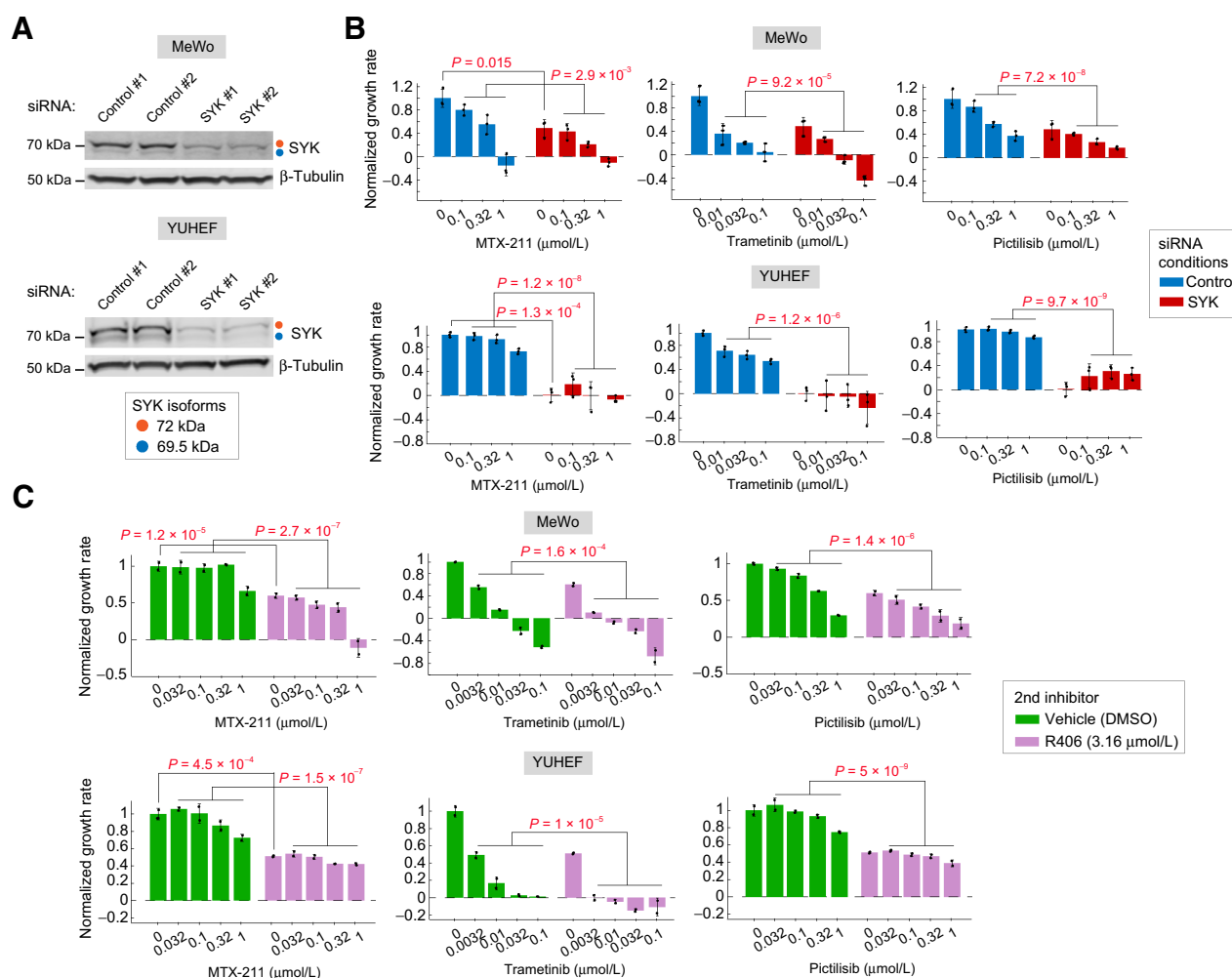
To directly evaluate the importance of SYK for growth of NF1^{LoF} cells, we depleted the protein in two NF1^{LoF} melanoma cell lines (MeWo and YUHEF) using four specific siRNAs (targeting SYK exons 10, 11, 12, and 13) combined into a single pool. SYK knockdown led to reduced SYK protein levels in both cell lines (Fig. 4A). Following 3 and 6 days of inhibition by SYK siRNAs, we measured live cell counts and computed the normalized growth rate in SYK-depleted cells relative to cells treated with non-targeting (control) siRNA. We also compared the normalized growth rates in the presence of different doses of MTX-211, trametinib and pictilisib or vehicle (DMSO). SYK depletion in both MeWo and YUHEF led to a statistically significant decrease in their growth rates and enhanced their sensitivity to MTX-211, trametinib and pictilisib (Fig. 4B). To rule out the possibility of off-targeting by SYK siRNAs, we also examined the effects of each of the four SYK siRNAs independently. All individual siRNAs reduced SYK expression and growth rate in YUHEF cells (Supplementary Fig. S4A and S4B).

To test the impact of pharmacologic inhibition of SYK on NF1^{LoF} melanoma cell growth, we treated three cell lines (MeWo, YUHEF, and WM3918) with the SYK inhibitor R406, individually or in combination with either MTX-211, trametinib, or pictilisib. Inhibition of SYK by R406 reduced the growth rate in these cell lines by approximately 2-fold and enhanced their sensitivity to MTX-211, trametinib, and pictilisib (Fig. 4C; Supplementary Fig. S4C). Together, these data show that both genetic and pharmacologic inhibition of SYK significantly reduced the growth of NF1^{LoF} melanoma cells and increased their sensitivity to PI3K and MEK inhibitors. It is noteworthy, however, that the combination of SYK inhibition with MTX-211 did not exactly phenocopy the impact of MTX-216 alone. We speculate that this might be due to differences in mechanism of SYK inhibition by MTX-216 or possibly inhibition of other potentially significant targets by MTX-216.

Because treatment efficacy in NF1^{LoF} cells was correlated with an increase in the proportion of p-S6^{Low}/Ki-67^{Low} subpopulation, we asked how p-S6 and Ki-67 levels would change in NF1^{LoF} cells following SYK inhibition. 72 h treatment with R406 reduced p-S6 and Ki-67 and increased the proportion of p-S6^{Low}/Ki-67^{Low} subpopulation in both MeWo and YUHEF cells (Supplementary Fig. S4D). Because SYK inhibition enhanced the sensitivity of NF1^{LoF} cells to MEK inhibition, we also evaluated the effect of combined MEK and SYK inhibition. The combination of R406 with trametinib enhanced cosuppression of p-S6 and Ki-67 and increased the fraction of p-S6^{Low}/Ki-67^{Low} subpopulation (Supplementary Fig. S4D).

MTX-216 suppresses genes that regulate mitochondrial ETC in NF1^{LoF} cells

To better understand the mechanisms underlying the superior efficacy of MTX-216 relative to other inhibitors, we extended our RNA-seq analysis (described above) to treatments with additional inhibitors, including pictilisib (at 1 $\mu\text{mol/L}$) and trametinib (at 0.1 $\mu\text{mol/L}$). We also included a third NF1^{LoF} cell line (SKMEL113), which was treated for 24 hours with either MTX-216 (at 1 $\mu\text{mol/L}$), trametinib (at 0.1 $\mu\text{mol/L}$), the combination of MTX-216 and trametinib (at 1 $\mu\text{mol/L}$ and 0.1 $\mu\text{mol/L}$, respectively), or vehicle (DMSO). GSEA of the effect of each individual compound on each cell line (relative to DMSO) confirmed the role of trametinib as an inhibitor of the ERK pathway, and pictilisib, MTX-216, and MTX-211 as inhibitors

**Figure 4.**

SYK inhibition or depletion reduces the growth of NF1^{LoF} melanoma cells and increases their sensitivity to both PI3K and MEK inhibitors. **A**, SYK protein levels assessed by WBs in MeWo (top) and YUHEF cells (bottom) following treatment with siRNAs targeting SYK isoforms or with nontargeting (control) siRNA for 72 hours. Antibodies were costained on the same blot. Each siRNA experiment was performed in two independent replicates. **B**, Normalized growth rates measured in MeWo (top) and YUHEF (bottom) cells following 72 hours treatment with indicated concentrations of MTX-211, trametinib, or pictilisib (all dissolved in DMSO as vehicle) in the presence of siRNAs targeting SYK or nontargeting (control) siRNA (for 72 hours). Data are presented as mean values $\pm$ SD calculated across $n = 3$ replicates. Statistical significance of the effect of SYK siRNA (vs. control siRNA) in the absence of all inhibitors was determined by two-sided, two-sample t test. Statistical significance of the effect of SYK siRNA (vs. control siRNA) in the presence of inhibitors at three indicated concentrations was determined by two-way ANOVA. **C**, Normalized growth rates measured in MeWo (top) and YUHEF (bottom) cells following 7-day treatments with indicated concentrations of MTX-211, trametinib, or pictilisib in combination with either R406 (at 3.16 μ mol/L) or vehicle (DMSO). Data are presented as mean values $\pm$ SD calculated across $n = 2$ replicates. Statistical significance of the effect of R406 (vs. DMSO) in the absence of other inhibitors was determined by two-sided, two-sample t test. Statistical significance of the effect of R406 (vs. DMSO) in the presence of other inhibitors at four indicated concentrations was determined by two-way ANOVA.

of the PI3K/AKT/mTOR pathway (Supplementary Fig. S5A; Supplementary Datasets S3 and S4). To systematically compare gene expression changes across all 42 samples (including three cell lines, six distinct treatment conditions tested in three independent replicates), we used PCA. PCA captured >80% of variance in the entire dataset by the first five principal components (PC; Fig. 5A; Supplementary Datasets S5 and S6). While the first two PCs (PC1 and PC2) associated mostly with cell line-specific differences, PC3 and PC4 were able to capture treatment-specific variations across the samples (Supplementary Fig. S5B). Changes induced by trametinib were revealed by a large shift in scores along PC4 for all three cell lines (Fig. 5B; purple arrows). MTX-216 treatment, on the other hand, led to a moderate shift along

PC4, while inducing a strong shift along PC3 (Fig. 5B; blue arrows). The combination of trametinib and MTX-216 was additive, leading to substantial shifts in both PC4 and PC3 (Fig. 5B; red arrow), whereas pictilisib and MTX-211 induced only small changes along both PCs.

Because PC3 and PC4 separated the efficacious inhibitors independent of cell lines, we set out to use PC3 and PC4 loadings to identify genes associated with distinct inhibitory effects on NF1^{LoF} cells (Fig. 5C). The identified genes comprised 315 genes with strong positive PC3 loadings, 414 genes with strong negative PC3 loadings, 491 genes with strong positive PC4 loadings, and 678 genes with strong negative PC4 loadings. The top GO terms associated with significantly negative PC4 loadings included processes involved in cell-cycle

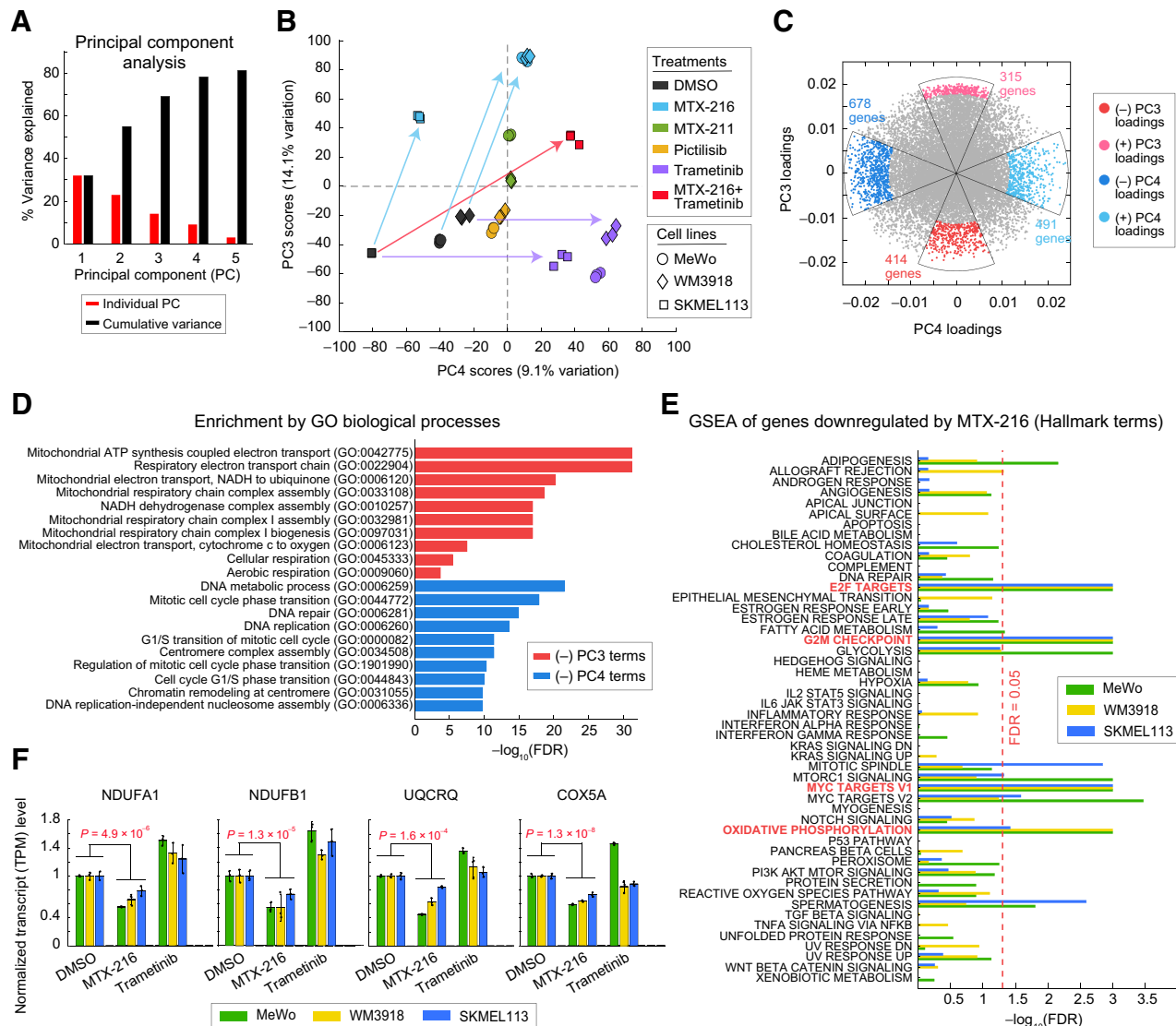


Figure 5. MTX-216 suppresses mitochondrial ETC genes in NF1^{LoF} cells. **A–C**, PCA of gene expression variations across 42 samples, representing three cell lines (MeWo, WM3918, and SKMEL113) and 24 hours treatment conditions with MTX-216 (at 1 μmol/L), MTX-211 (at 1 μmol/L), trametinib (at 0.1 μmol/L), the combination of MTX-216 (at 1 μmol/L), and trametinib (at 0.1 μmol/L), or DMSO, each tested in three independent replicates. **A**, Percentage of variance captured by the first five PCs. **B**, PC3 and PC4 scores capturing treatment-specific variations in gene expression across the samples. Arrows show changes of scores (in PC3-PC4 space) by specific treatments relative to DMSO-treated cells. **C**, PC3 and PC4 loadings highlighting genes associated with distinct inhibitory effects on NF1^{LoF} cells. Gene loadings differentially enriched along either PC3 or PC4 are highlighted in color. **D**, The top GO terms with FDR ≤ 0.05 associated with significantly negative PC3 and significantly negative PC4 loadings. **E**, GSEA of genes downregulated by MTX-216 in three cell lines (MeWo, WM3918, and SKMEL113) using consolidated Hallmark gene sets. Hallmark terms with FDR ≤ 0.05 in all three cell lines are highlighted in red. **F**, DMSO-normalized gene expression (TPM) levels of ETC genes NDUFA1, NDUFB1, UQCRCQ, and COX5A in three cell lines following 24 hours treatment with MTX-216 and trametinib. Data are presented as mean values ± SD calculated across *n* = 3 replicates. Statistical significance was determined by two-way ANOVA.

progression, DNA replication, and mitosis (Fig. 5D, blue bars; Supplementary Dataset S7). As expected from the PCA scores, these genes were suppressed profoundly in cells exposed to trametinib but were also downregulated in cells treated with MTX-216 (Supplementary Fig. S5C). In contrast, genes associated with negative PC3 loadings were substantially downregulated only in MTX-216-treated cells, but not in cells treated with other inhibitors (Supplementary Fig. S5C). The top GO terms associated with these genes included processes involved in cellular respiration and oxidative phosphorylation such as mito-

chondrial ATP synthesis and respiratory ETC complex biogenesis and assembly (Fig. 5D, red bars; Supplementary Dataset S7). Consistent with these results, an independent GSEA using consolidated Hallmark gene sets (36) identified oxidative phosphorylation to be significantly associated with genes downregulated by MTX-216 (Fig. 5E; Supplementary Datasets S1, S8, and S9). Among the key genes affected were those that encode mitochondrial enzyme subunits involved in the respiratory ETC, such as NADH:Ubiquinone Oxidoreductase Subunits A1 and B1 (NDUFA1, NDUFB1), Ubiquinol-Cytochrome C

Reductase Complex III Subunit VII (UQCQRQ) and Cytochrome C Oxidase Subunit 5A (COX5A), which were all consistently downregulated by MTX-216, but not by trametinib, in all three cell lines (Fig. 5F). Proliferation hallmarks (such as E2F targets, MYC targets, and G₂-M checkpoint), however, were downregulated by both trametinib and MTX-216 (Fig. 5E; Supplementary Fig. S5D). Together, these data show that while both MTX-216 and trametinib are effective in inhibition of cell proliferation in NF1^{LoF} melanoma cells, the two compounds are distinguishable with respect to their effect on the suppression of genes that regulate mitochondrial ETC.

Mitochondrial respiratory chain is associated with poor survival in patients with NF1^{LoF} melanoma and inhibitable by MTX-216 or SYK inhibition

To test the clinical relevance of SYK kinase and genes encoding mitochondrial ETC, we assessed their association with NF1 mutation and patient phenotype across melanoma tumors in TCGA database. For this purpose, we first grouped patient-derived melanoma tumors based on their NF1 mutation status. Patients bearing “truncating” driver mutations were classified as NF1^{LoF} (39 patients); patients bearing “missense” or “splice” driver mutations were classified as NF1^{Mutant} (9 patients); and patients with no NF1 mutations or only “passenger” NF1 mutations were classified as NF1^{Other} (421 patients). We then analyzed the frequency of SYK mutations across each group of patients. Interestingly, the NF1^{LoF} and NF1^{Mutant} groups showed higher numbers of SYK mutations (including mostly missense mutations) compared with the NF1^{Other} group (Supplementary Fig. S6A). From these mutations, however, we were not able to identify any obvious patterns of altered SYK activity. Therefore, we extended our analysis to the gene expression data and asked whether there was any difference in SYK pathway activity between different groups of patients. As a surrogate for SYK activity, we used the SYK drug inhibition gene sets, including genes whose expression changes following treatment with SYK inhibitor (Supplementary Dataset S4). GSEA showed a slight increase in SYK activity in the NF1^{LoF} and NF1^{Mutant} groups relative to the NF1^{Other} group based on the normalized enrichment score, but the difference was not statistically significant (Supplementary Fig. S6B). We also did not observe any significant differences in the overall expression of ETC genes (respiratory ETC GO term; GO:0022904; Supplementary Dataset S4) in tumors across three groups of patients with melanoma (Supplementary Fig. S6C). However, when we focused our analysis on each group separately and asked whether the expression of ETC genes correlated with patient survival, we found that within the NF1^{LoF} group (or NF1^{LoF} and NF1^{Mutant} groups combined), patients with ETC^{High} tumors had a significantly shorter overall survival compared with the patients with ETC^{Low} tumors (Fig. 6A; left and middle). Within the NF1^{Other} cohort of patients, however, there was no significant difference between the overall survival and whether tumors belonged to ETC^{High} or ETC^{Low} subgroups (Fig. 6A; right). To test whether such association within the NF1^{LoF}/NF1^{Mutant} groups might be due to confounding health risks, we assessed the patients’ diagnosis stage, but we did not observe any significant differences among NF1^{LoF}/NF1^{Mutant} patients with ETC^{High} tumors versus those with ETC^{Low} tumors (Supplementary Fig. S6D). These data suggest that melanoma tumors with NF1 mutations may rely on mitochondrial respiratory chain for progression.

To directly evaluate the importance of mitochondrial respiratory chain for growth of NF1^{LoF} cells, we used IACS-010759, a clinical-grade small-molecule inhibitor of complex I of the mitochondrial ETC. IACS-010759 inhibited growth in a dose-dependent fashion in all

tested cell lines (Fig. 6B; Supplementary Fig. S6E). Using the area over of the dose-response curve to compare overall drug sensitivity, we found the NF1^{LoF} cell lines to be similarly sensitive to both IACS-010759 and MTX-216, and their sensitivities to these compounds were greater than their sensitivity to R406 (Supplementary Fig. S6E and S6F). When combined with trametinib, however, MTX-216 led to the largest reduction in growth rate (Supplementary Fig. S6E and S6F). Together, these data support the hypothesis that NF1^{LoF} melanoma cells depend on mitochondrial respiratory chain for growth, and that MTX-216 and trametinib target orthogonal vulnerabilities, leading to enhanced benefit when used in combination.

Because genes downregulated by MTX-216 were involved in the mitochondrial respiratory chain, we asked whether this effect was due to a global reduction in mitochondrial mass or function because of SYK inhibition by this compound. We thus used the fluorescent probes, MitoTracker Deep Red and MitoTracker Red CMXRos, to quantitate the mitochondrial mass and membrane potential in NF1^{LoF} cells, respectively (46). Measurements were performed by fluorescence microscopy in two cell lines (MeWo and WM3918) following treatment with either SYK siRNA or a nontargeting control siRNA for 72 hours, or with MTX-216 (at 1 μmol/L) or vehicle (DMSO) for 48 hours. Both SYK knockdown (Fig. 6C and D) and MTX-216 treatment (Fig. 6E and F) shifted cell populations toward lower levels of both mitochondrial markers. To compare the effect of MTX-216 and MTX-211 on mitochondria, we performed the assays in MeWo cells following 48 hours treatment with increasing concentrations of either compound. MTX-216 was significantly more potent than MTX-211 in reducing both mitochondrial markers (Fig. 6G and H; Supplementary Fig. S7). Together, these results are consistent with RNA-seq data, suggesting that the efficacy of MTX-216 through SYK inhibition is mediated by the suppression of mitochondrial mass and ETC function. It is noteworthy, however, that R406 and IACS-010759 do not completely recapitulate the phenotypic effects of MTX-216. Therefore, it is possible that other pathways inhibited by MTX-216 may also play a role in determining the MTX-216 activity in NF1^{LoF} melanoma cells.

Discussion

We performed a targeted kinase inhibitor screen to identify vulnerabilities whose inhibition, individually or in combination with MEK/ERK or PI3K/mTOR inhibitors, would be effective in blocking NF1^{LoF} melanoma cells. We found that the efficacy of kinase inhibitors was determined by their ability to coinhibit the proliferation marker Ki-67 and the p-S6. While MEK/ERK inhibitors and PI3K inhibitors led to heterogeneous responses, we identified a tool compound, MTX-216, that effectively suppressed both Ki-67 and p-S6 in NF1^{LoF} melanoma cells, inducing efficacious antitumor responses in these cells. MTX-216 was originally developed as a dual PI3K/EGFR inhibitor. However, systematic analysis showed that its high efficacy in NF1^{LoF} cells depended on its ability to coinhibit SYK kinase. Therefore, the study of MTX-216 served as a tool to identify SYK as a new vulnerability which when exploited, especially in combination with MEK inhibition, can lead to efficacious responses in NF1^{LoF} melanoma cells. The greater efficacy of MTX-216 in NF1^{LoF} cells, in comparison with BRAF^{V600E} cells, is consistent with this compound being able to inhibit SYK, a non-RTK that can be activated by multiple upstream receptors and initiates downstream signaling through RAS (15, 40, 47–49). Nevertheless, our data prompt additional future investigations to identify the role of SYK and its associated adaptors in relationship to RAS signaling and tumorigenesis in NF1^{LoF} melanomas. Such studies

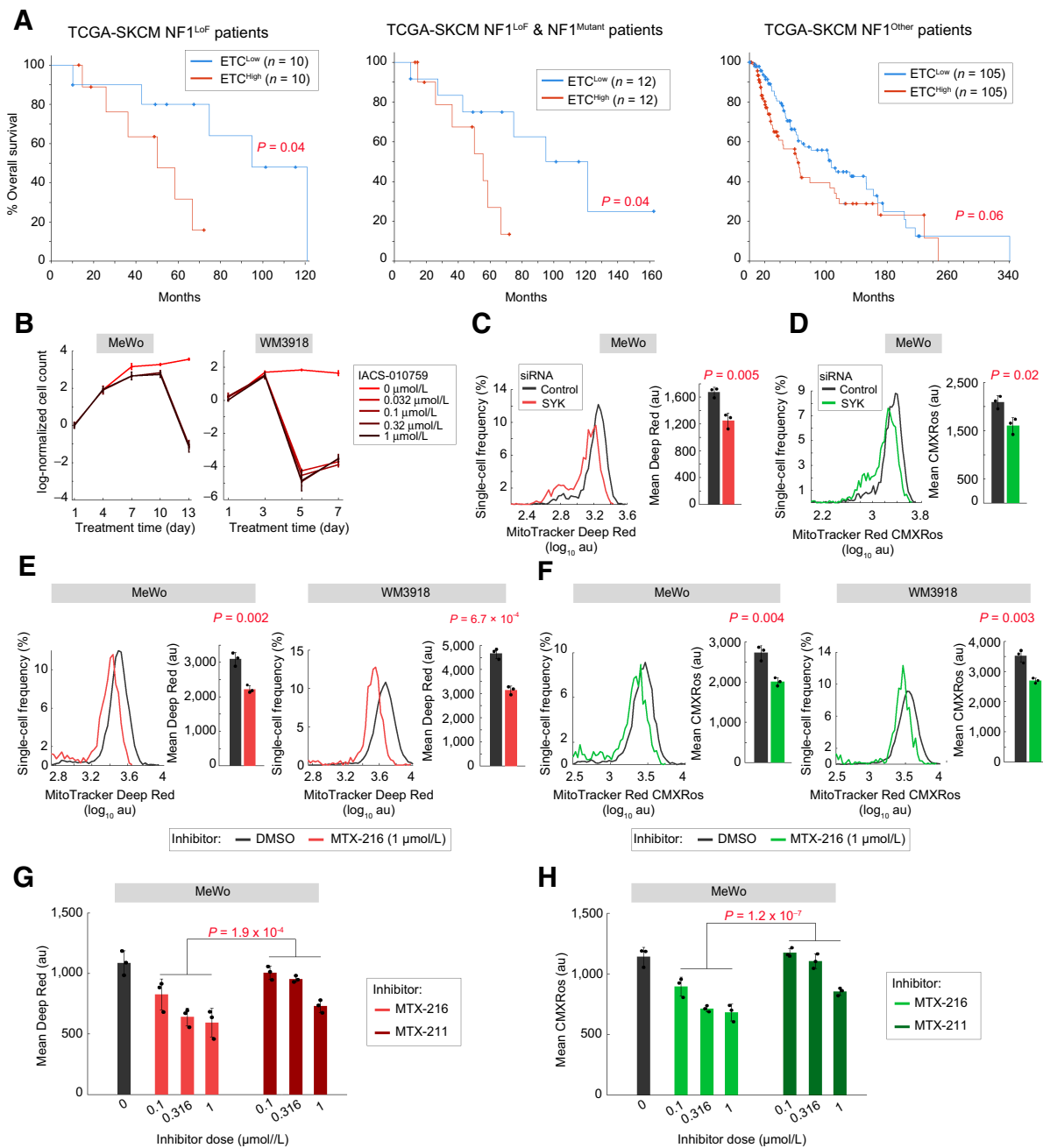


Figure 6.

Mitochondrial respiratory chain is associated with poor survival in patients with NF1^{LoF} melanoma and inhibitable by MTX-216 or SYK inhibition. **A**, Overall survival analysis of patients with ETC^{High} or ETC^{Low} tumors. The analysis was performed in three groups of patients: patients with NF1^{LoF} tumors (left), patients with either NF1^{LoF} or NF1^{Mutant} tumors (middle), and patients with no significant NF1 mutations; NF1^{Other} (right). Statistical significance was determined by two-sided log-rank (Mantel-Cox) test. **B**, Log-normalized changes in live cell count following the exposure of two cell lines MeWo and WM3918 to indicated doses of IACS-010759 for a period of 13 and 7 days, respectively. Data are presented as mean values $\pm$ SD calculated across $n = 2$ replicates. **C** and **D**, Changes in mitochondrial mass measured by MitoTracker Deep Red (**C**) and mitochondrial membrane potential measured by MitoTracker Red CMXRos (**D**) in MeWo cells in the presence of SYK siRNAs or nontargeting (control) siRNA for 72 hours. Single-cell intensities (left) and the population-averaged data (right) are shown for each measurement. Population-averaged data are presented as mean values $\pm$ SD calculated across $n = 3$ replicates. Statistical significance was determined by two-sided, two-sample t test. **E** and **F**, Changes in mitochondrial mass measured by MitoTracker Deep Red (**E**) and mitochondrial membrane potential measured by MitoTracker Red CMXRos (**F**) in MeWo and WM3918 cells following 48 hours treatment with MTX-216 (at 1 μ mol/L) or DMSO. Single-cell intensities (left) and the population-averaged data (right) are shown for each measurement. Population-averaged data are presented as mean values $\pm$ SD calculated across $n = 3$ replicates. Statistical significance was determined by two-sided, two-sample t test. **G** and **H**, Changes in mitochondrial mass measured by MitoTracker Deep Red (**G**) and mitochondrial membrane potential measured by MitoTracker Red CMXRos (**H**) in MeWo cells following 48 hours treatment with different concentrations of MTX-216, MTX-211, or DMSO. Population-averaged data are presented as mean values $\pm$ SD calculated across $n = 3$ replicates. Statistical significance was determined by two-way ANOVA.

may provide a path to exploit SYK dependency to selectively block NF1^{LoF} melanoma tumors.

The discovery of SYK as a vulnerability in NF1^{LoF} melanomas based on the high efficacy of a tool compound (MTX-216) in these cells represents an unconventional approach that leverages mechanistic, polypharmacology drug studies to identify novel cancer targets (14). There are many instances of kinase inhibitors whose antitumor efficacy could be explained by inhibition of kinases other than their originally intended targets (50). This is not surprising because all kinases bind to a common substrate (i.e., ATP), which underlies their great potential for promiscuity. Therefore, kinase inhibitors may lead to unpredicted activities in the combination setting, which could be exploited to discover new mechanisms as well as novel actionable dependencies in cancer cells.

Our analysis provides insight into how MTX-216 promotes antitumor effects in NF1^{LoF} melanoma cells by suppressing a group of genes that regulate mitochondrial ETC and function. The expression of these genes is associated with poor survival in patients with NF1^{LoF} melanomas. Therefore, MTX-216 may block NF1^{LoF} melanoma cells by targeting a mitochondrial vulnerability associated with a specific SYK-dependent signaling context that is required for NF1^{LoF} tumor progression. This is consistent with the sensitivity of NF1^{LoF} melanoma cells to pharmacological inhibition of mitochondrial ETC either by IACS-010759 as demonstrated in this study, or by phenformin as reported previously (10).

The role of SYK kinase in regulation of diverse pathways involved in cellular homeostasis, including mitochondrial function and oxidative metabolism, has been already well documented. In B cells, for example, SYK is activated by the B-cell receptor pathway, regulating multiple signaling pathways involved in differentiation and proliferation, as well as oxidative phosphorylation and mitochondrial oxidation of fatty acids (15, 19, 45). Blocking oxidative metabolism via SYK inhibition has been shown to induce antitumor effects in acute myeloid leukemia, B-cell chronic lymphocytic leukemia, and diffuse large B-cell lymphoma (17–20). Furthermore, SYK activation has been shown to occur, at least in part, in proximity to the respiratory chain, where a pool of SYK localizes to the mitochondrial intermembrane space, controlling cellular response to oxidative stress in diverse cell types (16). Future studies may leverage these findings to elucidate the mechanistic connection between the SYK pathway activity and mitochondrial oxidative metabolism in NF1^{LoF} melanomas.

Authors' Disclosures

C. Abecunas reports personal fees from Ribon Therapeutics outside the submitted work. C.E. Whitehead reports grants from NIH and personal fees and nonfinancial

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Authors' Contributions

C. Abecunas: Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. **C.E. Whitehead:** Conceptualization, resources, formal analysis, investigation, methodology, writing—review and editing. **E.K. Ziemke:** Formal analysis, investigation, visualization, methodology, writing—review and editing. **D.G. Baumann:** Investigation, methodology, writing—review and editing. **C.L. Frankowski-McGregor:** Resources, investigation, methodology, writing—review and editing. **J.S. Sebolt-Leopold:** Conceptualization, resources, supervision, writing—review and editing. **M. Fallahi-Sichani:** Conceptualization, supervision, funding acquisition, writing—original draft, project administration, writing—review and editing.

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Note

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Epigenetic modulation reveals differentiation state specificity of oncogene addiction

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Hyperactivation of the MAPK signaling pathway motivates the clinical use of MAPK inhibitors for *BRAF*-mutant melanomas. Heterogeneity in differentiation state due to epigenetic plasticity, however, results in cell-to-cell variability in the state of MAPK dependency, diminishing the efficacy of MAPK inhibitors. To identify key regulators of such variability, we screen 276 epigenetic-modifying compounds, individually or combined with MAPK inhibitors, across genetically diverse and isogenic populations of melanoma cells. Following single-cell analysis and multivariate modeling, we identify three classes of epigenetic inhibitors that target distinct epigenetic states associated with either one of the lysine-specific histone demethylases Kdm1a or Kdm4b, or BET bromodomain proteins. While melanocytes remain insensitive, the anti-tumor efficacy of each inhibitor is predicted based on melanoma cells' differentiation state and MAPK activity. Our systems pharmacology approach highlights a path toward identifying actionable epigenetic factors that extend the *BRAF* oncogene addiction paradigm on the basis of tumor cell differentiation state.

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Therapeutic inhibition of oncogenic signaling often leads to variable responses due to cell-to-cell heterogeneity in the state of oncogene dependency¹. Heterogeneity may result from secondary genetic mutations, or may be caused by epigenetic differences associated with a cell's developmental lineage or differentiation state^{2–10}. An example of such epigenetic heterogeneity is observed in *BRAF*-mutated melanomas, causing fractional responses to *Braf*/*Mek*-targeted therapies^{11–23}. Numerous studies have associated fluctuations in the state of MAPK dependency to melanoma differentiation state plasticity^{6,18,24–27}. Such plasticity spans a spectrum, ranging from a pigmented melanocytic phenotype associated with transcriptional regulators *Sox10* and *Mitf*²⁸, to a neural crest-like state that expresses *Ngfr*^{12,29}, to an undifferentiated state characterized by high expression of receptor tyrosine kinases such as *Axl*^{19,30}. Melanoma tumors consist of a mixture of these phenotypes at variable single-cell frequencies²⁵. Although the consequences of such heterogeneities for drug resistance are widely recognized, there is still little known about actionable epigenetic factors that may link heterogeneity in the state of MAPK dependency to plasticity in differentiation state. A system-wide exploration of these factors may reveal novel opportunities for epigenetic treatments that will minimize the emergence of drug resistance. In principle, identifying epigenetic treatments that promote cellular requirement for MAPK signaling may enhance the efficacy of *Braf*/*Mek* inhibitors when used in combination^{12,31–33}. Alternatively, treatments that target synthetic lethal partners of the *BRAF* oncogene may serve as a strategy to kill intrinsically drug-resistant cells³⁴.

In this paper, we take a systems pharmacology approach to test the hypothesis that heterogeneity in the state of MAPK dependency may result from a subset of key epigenetic variations across tumor cells of heterogeneous differentiation states. To identify the regulators of such variations, we use a library of 276 small-molecule epigenetic modulators in *BRAF*-mutant melanoma cell lines that cover a wide spectrum of differentiation states. We evaluate the effect of each epigenetic modulator (individually and in combination with *Braf*/*Mek* inhibitors) on cell survival, proliferation, differentiation state, and MAPK activity. Integrating multiplexed single-cell analysis with multivariate modeling and genetic experiments, we identify three classes of small molecules that target seemingly distinct epigenetic states in melanoma cells. These states include: (i) a lysine demethylase 1A (*Kdm1a*)-dependent state, predominantly observed in undifferentiated cells, that is efficiently inhibitable by the reversible *Kdm1a* inhibitor SP2509, (ii) a lysine demethylase 4B (*Kdm4b*)-dependent state, associated with neural crest-like cells, that is sensitive to JIB-04 (a pan-inhibitor of Jumonji histone demethylases), and (iii) a state induced by BET bromodomain inhibitors such as OTX015 (*Birabresib*), which enhances cells' requirement for MAPK signaling. Single-cell analysis shows that these states might co-exist in different combinations and frequencies, highlighting mutual epigenetic vulnerabilities among genetically diverse melanoma cell populations. Importantly, non-transformed primary melanocytes are not sensitive to these inhibitors. These results, therefore, provide a path for identifying actionable epigenetic factors that may extend the *BRAF* oncogene addiction paradigm on the basis of tumor cell differentiation state.

Results

Single-cell analysis uncovers heterogeneities in differentiation, proliferation, and signaling states. To elucidate single-cell heterogeneities in differentiation state and their variations across melanoma cell populations, we utilized high-throughput, multiplex immunofluorescence microscopy. We exposed a panel of 16 *BRAF*^{V600E/D} melanoma cell lines and a batch of non-

transformed human primary epidermal melanocytes to the *Braf* inhibitor vemurafenib (at 100 nM), alone or in combination with the *Mek* inhibitor trametinib (at 10 nM). For the purpose of comparison, we also included an *NRAS*^{Q61K}-mutated variant of the A375 cell line, representing a common mechanism of acquired resistance to *Braf*/*Mek* inhibitors³⁵. All cells were fixed following 3–5 days of treatment and protein levels of three validated differentiation state markers, *Mitf*, *Ngfr*, and *Axl* (Supplementary Fig. 1), were quantified at a single-cell level (Supplementary Figs. 2, 3). To visualize baseline and treatment-induced variations in all three markers, we performed *t*-distributed stochastic neighbor embedding (*t*-SNE) analysis on a total population of 6069 randomly selected cells from all of the 102 tested conditions, covering the entire panel of cell lines, drugs and timepoints (Fig. 1a, Supplementary Fig. 4a, b). Single-cell analysis revealed a continuum of differentiation states ranging from *Mitf*^{High} to *Ngfr*^{High} to *Axl*^{High} cells. We then used the *t*-SNE map as a reference to visualize differentiation state variations in isogenic cell populations from each cell line (Fig. 1b, Supplementary Fig. 4c, d). We also quantified the extent of heterogeneity in each marker by computing the Fano factor, a standardized measure of dispersion of probability distribution (Supplementary Fig. 5). To assess the combined effect of heterogeneity in all three differentiation markers, we determined the average cell-to-cell distance in each cell population (Supplementary Fig. 6). All clonal cell lines exhibited a high degree of heterogeneity in at least one of the differentiation markers and most cell lines expressed substantial plasticity following exposure to *Braf*/*Mek* inhibitors. Interestingly, non-transformed melanocytes also exhibited heterogeneity at a level comparable to melanoma cell lines, suggesting that plasticity in differentiation state is not unique to cancer cells.

Because proliferation of *BRAF*-mutant cells is attributed to their aberrant MAPK signaling, we asked whether heterogeneity in differentiation state was associated with potentially distinct patterns in MAPK signaling. We thus multiplexed single-cell immunofluorescence measurements of p-Erk^{T202/Y204}, Ki67 (a proliferation marker), and p-S6^{S235/S236} (a marker of TORC1 activity that is upregulated in MAPK inhibitor-tolerant cells³⁶) in the same group of cell lines exposed to the same *Braf*/*Mek* inhibitors for 3–5 days (Supplementary Figs. 7, 8). Fano factor, cell-to-cell distance, and *t*-SNE analysis also revealed substantial variability in MAPK signaling (Fig. 1c, d, Supplementary Figs. 9, 10). While vemurafenib led to partial inhibition of MAPK signaling relative to drug-naïve cells, the combination of vemurafenib and trametinib suppressed the pathway more strongly and drove a larger proportion of cells toward a fully inhibited (p-Erk^{Low}/p-S6^{Low}/Ki67^{Low}) state (Fig. 1d). The fraction of fully inhibited cells, however, varied among cell lines. We hypothesized that such variability in MAPK signaling would explain differences in the overall MAPK inhibitor sensitivity and might be related to heterogeneity in melanoma differentiation state.

To compare the overall *Braf*/*Mek* inhibitor sensitivity among cell lines, we computed drug-induced normalized growth rates (a.k.a. DIP rates³⁷) by normalizing the average net growth rate of each cell line in the presence of each drug to that in DMSO-treated cells (Fig. 1e, f and Supplementary Fig. 11a). By correlating normalized growth rates to the state of MAPK signaling across 16 cell lines, we identified a strong correlation between the fraction of fully inhibited (p-Erk^{Low}/p-S6^{Low}/Ki67^{Low}) cells and the overall sensitivity to *Braf*/*Mek* inhibitors (Pearson's $r = -0.65$, $P = 4.3 \times 10^{-9}$) (Supplementary Fig. 11b and Fig. 1g; top left panel). We next extended the systematic correlation analysis to the diversity of differentiation states. We discovered that upon *Braf*/*Mek* inhibition, predominantly undifferentiated (*Axl*^{High}) cell lines generated the smallest

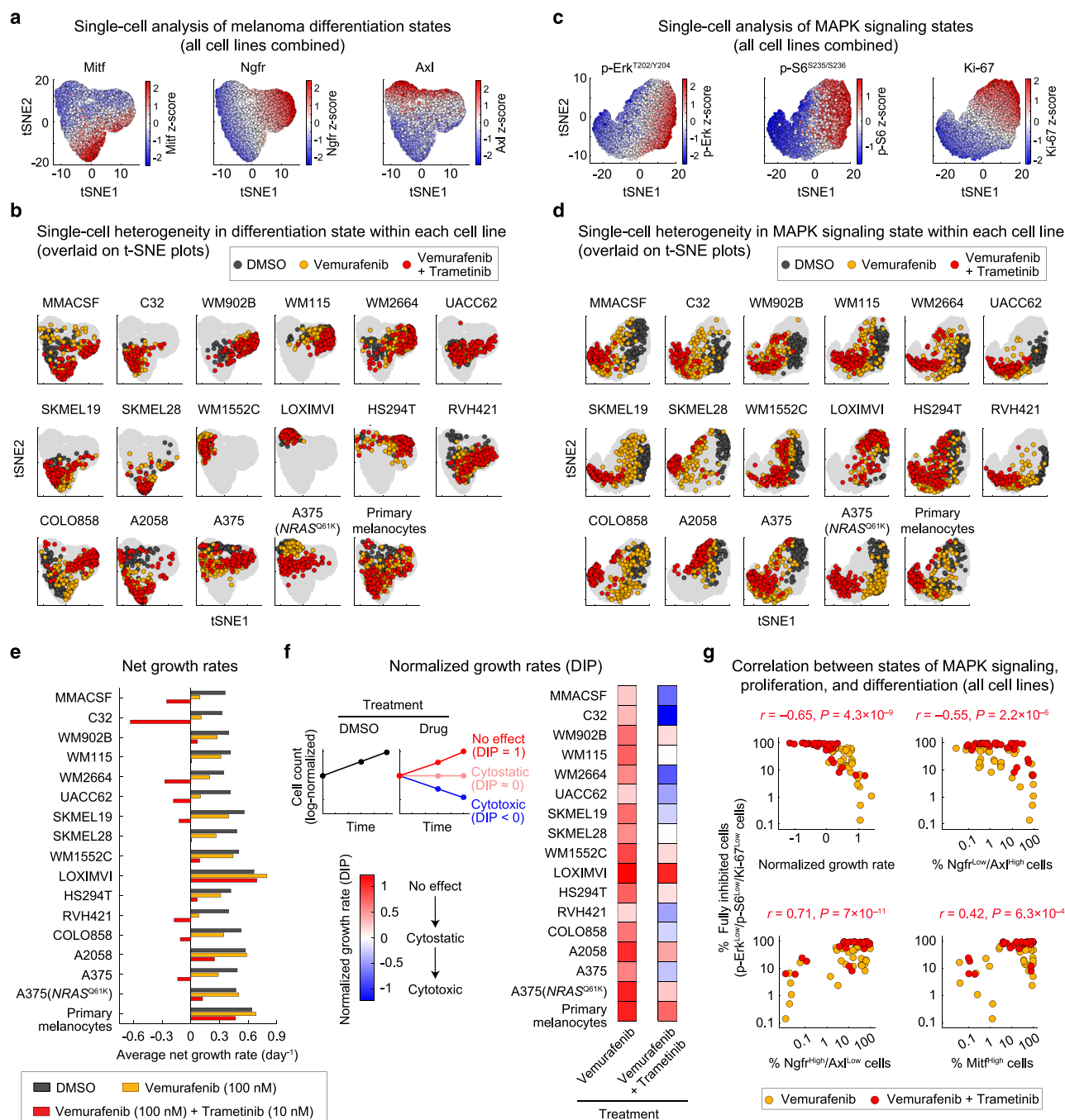


Fig. 1 Single-cell analysis uncovers heterogeneities in melanoma differentiation, proliferation, and MAPK signaling states across a wide range of BRAF/Mek inhibitor sensitivity. **a, b** Single-cell protein levels of three melanoma differentiation state markers, Mitf, Ngfr, and Axl, measured by multiplexed immunofluorescence microscopy and overlaid on t-SNE plots. Cells were exposed to either vehicle (DMSO), BRAF inhibitor (vemurafenib at 100 nM), or the combination of BRAF and MEK inhibitors (vemurafenib at 100 nM and trametinib at 10 nM) for 3 and 5 days. Cells for each experimental condition were randomly selected from the pool of two biologically independent replicates. Single-cell t-SNE maps for all cell lines combined (**a**) and projections of variation within each individual cell line (**b**) are shown. **c, d** Single-cell protein levels of p-Erk^{T202/Y204}, p-S6^{S235/S236}, and Ki-67, measured by multiplexed immunofluorescence microscopy and overlaid on t-SNE plots. Treatment conditions are the same as in (**a, b**). Single-cell t-SNE maps for all cell lines combined (**c**) and projections of variation within each individual cell line (**d**) are shown. **e** Average net growth rates calculated from measurements of live cell count (across at least two biologically independent replicates) at three timepoints (including 0, 3, and 5 days) following exposure to DMSO, vemurafenib (at 100 nM) or vemurafenib (at 100 nM) plus trametinib (at 10 nM). **f** Drug-induced normalized growth rates (a.k.a. DIP rates) calculated by dividing the average net growth rate for drug-treated cells to that for DMSO-treated cells in each cell line. Normalized growth rates <0 indicate a net cell loss (i.e., drug-induced cytotoxicity), a value of 0 represent no change in viable cell number (i.e., cytostasis), a value >0 indicates a net cell gain, and a value of 1 represents no drug effect as cells grow at the same rate as in the DMSO condition. **g** Two-sided Pearson's correlation analysis between the fraction of p-Erk^{Low}/p-S6^{Low}/Ki-67^{Low} cells (referred to as fully inhibited cells) and drug-induced normalized growth rate (top left), the fraction of undifferentiated (Ngfr^{Low}/Axl^{High}) cells (top right), the fraction of neural crest-like (Ngfr^{High}/Axl^{Low}) cells (bottom left), and the fraction of melanocytic (Mitf^{High}) cells (bottom right) across 16 melanoma cell lines treated with BRAF/MEK inhibitors for 3 and 5 days. Source data are provided as a Source data file.

populations of fully inhibited cells (Supplementary Fig. 11b and Fig. 1g; top right panel). Braf/Mek inhibitor resistance in these cells was associated with a high frequency of proliferating (Ki67^{High}) cells exhibiting incomplete inhibition of the MAPK pathway. In contrast, populations of neural crest-like (Ngfr^{High}/Axl^{Low}) cells or differentiated (Mitf^{High}) cells exhibited substantial co-inhibition of p-Erk, p-S6, and Ki67 (Supplementary Fig. 11b and Fig. 1g; bottom panels). Drug adaptation in these populations was, therefore, associated with an overall reduced requirement for MAPK signaling.

Together, these analyses revealed a spectrum of heterogeneities in melanoma differentiation state that corresponded to two previously described but distinct ways through which cells may tolerate the effect of Braf/Mek inhibition: (1) incomplete inhibition of the MAPK pathway (as seen in Axl^{High} cells), and (2) reduced requirement for MAPK signaling (as seen predominantly in Ngfr^{High}/Axl^{Low} cells).

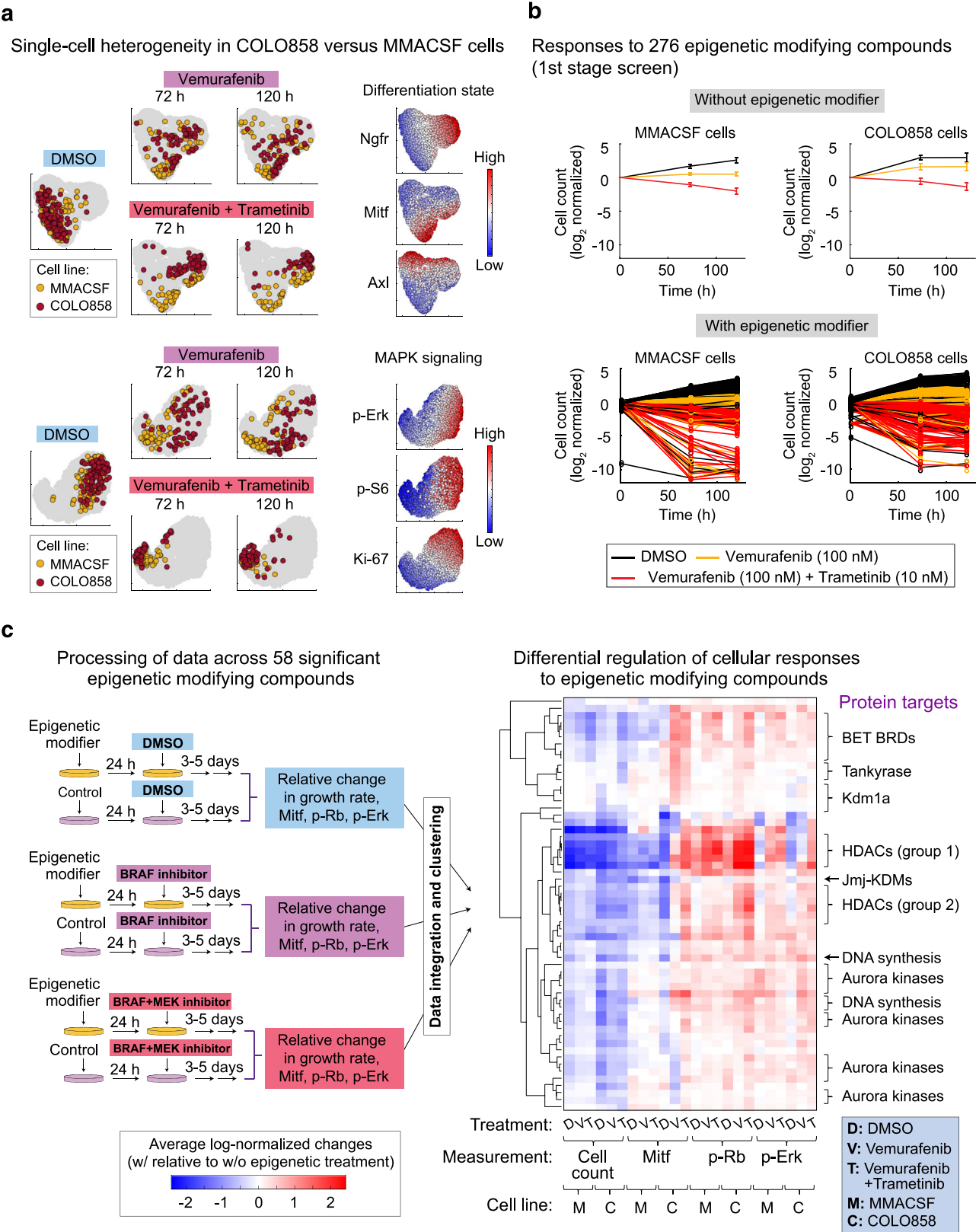
A chemical screen identifies epigenetic modulators of phenotypic heterogeneity. To systematically search for epigenetic factors that may link heterogeneity in melanoma differentiation state to MAPK dependency, we performed a multi-stage phenotypic screen using a library of 276 epigenetic-modifying compounds. Each compound was used to modulate either a key epigenetic writer, eraser or reader, or a related protein, after which phenotypic responses in the presence or absence of Braf/Mek inhibitors were investigated. For the first stage of the screen, we selected COLO858 and MMACSF cell lines which show distinct patterns of differentiation states (Fig. 2a). Cells from both cell lines were initially exposed to two different doses (0.2 and 1 μ M) of each of the 276 epigenetic compounds or vehicle (DMSO) for 24 h. Vemurafenib alone (at 100 nM), vemurafenib in combination with trametinib (at 10 nM), or vehicle (DMSO), was then added and cells were grown for a further 72 or 120 h prior to fixation (Fig. 2b and Supplementary Tables 1, 2). To differentiate the impact of epigenetic compounds, the growth rates for cells treated with each compound were compared to cells treated without any epigenetic treatment. Statistical analysis identified 58 compounds that led to a significant decrease in normalized growth rate in at least one of the tested conditions (Supplementary Figs. 12, 13).

To infer potential variations in the mechanisms of action of the epigenetic compounds, we co-stained cells for p-Erk^{T202/Y204}, p-Rb^{S807/S811}, and Mitf, quantifying changes induced by each of the 58 compounds in MAPK signaling, cell cycle progression, and differentiation state, respectively. Unsupervised clustering of the normalized responses revealed remarkable similarities among classes of compounds with common nominal epigenetic targets, suggesting that these responses are most likely the consequence of their on-target effects (Fig. 2c and Supplementary Fig. 14a). Each class of compounds induced responses that were either cell line- or MAPK inhibitor-specific, or common between both cell lines, or independent of MAPK inhibitor condition. For example, CUDC-907 (Fimepinostat), Quisinostat, Panobinostat, Dacinostat, and Trichostatin A were identified as a cluster of histone deacetylase (HDAC) inhibitors (labeled as group 1 in Fig. 2c) that significantly reduced growth rate in both COLO858 and MMACSF cells to similar degrees and independent of the MAPK inhibitor conditions (Supplementary Fig. 14b). In contrast, Kdm1a inhibitors inhibited net growth rate selectively in COLO858 cells, and they showed a higher efficacy in the absence of Braf/Mek inhibitors.

Correlated patterns of melanoma responses to mechanistically distinct epigenetic inhibitors. To identify potential relationships between the efficacy of each class of epigenetic compounds, we

analyzed their effects across a more diverse group of cell lines. Thus, in the second stage of the screen, we focused on seven epigenetic inhibitors representing the most effective classes (including HDAC inhibitors CUDC-907 and Givinostat, pan-Jmj-KDM inhibitor JIB-04, Tankyrase inhibitor AZ6102, BET inhibitors I-BET762 and OTX015, and Kdm1a inhibitor SP2509) and a group of eight cell lines representing a wider spectrum of differentiation states (Fig. 3a, b). By testing each compound in non-transformed human primary melanocytes, we chose effective concentrations of each inhibitor that had little to no effect on healthy cells (Supplementary Fig. 15). To quantify the benefit resulting from combining each of the epigenetic inhibitors with vemurafenib and trametinib, we computed the deviation from Bliss independence (DBI), a metric that compares the observed cellular response to the combination treatment with that expected given independent action for the two individual treatments³⁸. We found that the HDAC inhibitor CUDC-907 induced substantial tumor cell killing in all of 8 melanoma cell lines and exhibited additive (independent) to synergistic responses when combined with vemurafenib and trametinib in 6 cell lines (Fig. 3b, c). Treatment with the other 6 epigenetic compounds uncovered more heterogeneous patterns of response across cell lines and MAPK inhibitor conditions. For example, BET inhibitors OTX015 and I-BET762 were not effective in any of the cell lines when used as a single agent, but they induced strong cytotoxic and synergistic responses in combination with vemurafenib and trametinib in the majority of cell lines. SP2509, on the other hand, was effective in 5 cell lines, while exhibiting antagonism in combination with vemurafenib and trametinib in all cell lines.

To systematically explore potential relationships between patterns of responses to epigenetic inhibitors, we computed all pairwise correlations between the efficacy of seven epigenetic compounds across the eight cell lines (Fig. 3d). As expected, the efficacy of two BET inhibitors, OTX015 and I-BET762, was strongly correlated (Pearson's $r = 0.99$, $P < 10^{-4}$). In addition, the efficacy of BET inhibitors and the pan-Jmj-KDM inhibitor JIB-04 was positively correlated when cells were co-treated with vemurafenib plus trametinib ($r = 0.85$, $P < 10^{-3}$). In contrast, a negative correlation was observed between the efficacies of JIB-04 and SP2509. We thus asked whether such correlations would be maintained if these compounds were assayed across a larger panel of cell lines. By expanding the analysis to a total of 16 cell lines, we confirmed the statistical significance of the negative correlation between responses to SP2509 and JIB-04 (Fig. 4a). In more than half of the cell lines tested, 5 days of treatment with SP2509 induced substantial cell killing (Fig. 4b and Supplementary Fig. 16a). SP2509 sensitivity correlated with sensitivity to SP2577 (seliclidemstat; a clinical formulation of SP2509), which also suppressed melanoma cell growth (when used at a daily dose of 80 mg kg⁻¹) in corresponding melanoma xenografts (Supplementary Fig. 16b-d). Interestingly, however, cell lines with the highest level of resistance to SP2509 and SP2577 were sensitive to JIB-04, showing a range of responses from cytostatic to cytotoxic (Fig. 4a-c, Supplementary Fig. 16a). The triple combination of JIB-04, vemurafenib, and trametinib led to additive (independent) to synergistic cell killing in most JIB-04-sensitive cell lines, whereas the combination of SP2509 with Braf/Mek inhibitors was antagonistic in all SP2509-sensitive cell lines (Fig. 4c). When combined with vemurafenib and trametinib, both BET inhibitors OTX015 and I-BET762 also induced synergistic cell killing in most of the JIB-04-sensitive cell lines, while having minimal effects when used as a single agent. To determine if the epigenetic inhibitor effects persisted with long-term exposure, we extended the duration of growth inhibition assays. We found that the optimal epigenetic treatments identified for three of the MAPK



inhibitor-resistant cell lines were highly efficacious, resulting in reduction of up to 1000-fold in live cell count over a period of 20 days (Fig. 4d).

Together, our multi-stage epigenetic screen and systematic correlation analysis identified a set of seemingly distinct epigenetic states whose inhibition, either by the Kdm1a inhibitor

SP2509, or by the pan-Jmj-KDM inhibitor JIB-04 or BET inhibitors (when used in combination with Braf/Mek inhibitors), led to tumor cell killing in a melanoma cell line-specific manner. Next, we asked whether such cell line-specific patterns of response may be linked to heterogeneity in MAPK signaling, proliferation, and differentiation states.

Fig. 2 A chemical screen identifies epigenetic modulators of phenotypic heterogeneity. **a** t-SNE maps comparing single-cell heterogeneity in differentiation state (Mitf, Ngfr, and Axl) and MAPK signaling (p-Erk^{T202/Y204}, p-S6^{S235/S236}, and Ki67) within two BRAF^{V600E} melanoma cell lines, MMACSF and COLO858, following exposure to vehicle (DMSO), vemurafenib (at 100 nM), alone or in combination with trametinib (at 10 nM), for 72 and 120 h. **b** Log₂-normalized changes in live cell count following exposure of COLO858 and MMACSF cells to either DMSO, vemurafenib (at 100 nM), or vemurafenib (at 100 nM) plus trametinib (at 10 nM), for a period of 120 h. Cells were pretreated for 24 h with either DMSO (top panels) or two different doses (0.2 and 1 μM) of each of the 276 epigenetic-modifying compounds (bottom panels). Data for treatments without epigenetic modifiers (top panels) are presented as mean values ± s.d. calculated across $n = 276$ biologically independent samples examined over 25 independent experiments. Data for treatments with epigenetic modifiers (bottom panels) are presented as the average of $n = 2$ biologically independent samples. **c** A schematic representation of the overall procedure of data collection, processing (normalization), integration, and hierarchical clustering using measurements of cellular growth rate, Mitf, p-Rb^{S807/S811}, and p-Erk^{T202/Y204} at indicated treatment conditions and timepoints in MMACSF and COLO858 cells. Unsupervised clustering analysis was performed on data collected for 58 epigenetic compounds that led to a statistically significant decrease in normalized growth rate (when used either as a single agent, or in combination with Braf/Mek inhibitors) in either or both cell lines. Prior to clustering, data collected for cells treated with each epigenetic compound and MAPK inhibitor condition (i.e., DMSO, vemurafenib, or vemurafenib plus trametinib) were normalized to cells treated without any epigenetic compound and the same MAPK inhibitor condition. Groups of compounds with similar nominal epigenetic targets are listed on the right side. Source data are provided as a Source data file.

Multivariate modeling identifies predictors of epigenetic inhibitor efficacy. To determine whether molecular markers of differentiation state, MAPK activity, or other phenotypic markers could predict the differential efficacy of epigenetic treatments, we measured both baseline levels and treatment-induced variations in Mitf, Ngfr, Axl, Sox10, p-Erk^{T202/Y204}, p-S6^{S235/S236}, and Ki67 across eight melanoma cell lines. Given the possible contribution of epigenetic histone modifications in DNA damage repair³⁹, we also included phosphorylated Histone p-H2A.X^{S139} (a marker of DNA damage response) in our analysis. Multiplexed immunofluorescence measurements revealed how multiple protein markers were up- or downregulated depending on the cell line, MAPK inhibitor condition, and epigenetic treatment (Fig. 5a). We then used partial least square regression (PLSR) analysis⁴⁰ to generate models that linked epigenetic treatment-induced changes in growth rates (response variables) to input vectors that combined baseline and treatment-induced changes in signaling and phenotypic data. Models were evaluated using leave-one-out cross-validation (Supplementary Fig. 17a). Overall, PLSR models built for JIB-04, SP2509, I-BET762, and OTX015 proved remarkably accurate with an average Pearson's correlation coefficient of 0.85 ± 0.06 between the measured and predicted responses (Fig. 5b and Supplementary Fig. 17b).

To identify those measurements that are predictive of treatment efficacy, we computed the variable importance in the projection (VIP) scores for each PLSR model⁴¹ (Fig. 5c and Supplementary Fig. 17c). Among the most important determinants of treatment efficacy were the baseline differentiation state markers Axl and Ngfr as well as measurements of MAPK activity, including p-Erk, p-S6, and Ki67. Informed by the PLSR results, single-cell analysis across the entire panel of cell lines showed that SP2509 was most effective in inhibiting Ngfr^{Low}/Axl^{High} populations of cells (Fig. 5d; left panels). In contrast, Ngfr^{High}/Axl^{Low} populations were sensitive to the triple combination of JIB-04, vemurafenib, and trametinib (Fig. 5d; right panels). In addition, BET inhibitors I-BET762 and OTX015 (when combined with Braf/Mek inhibitors) enhanced tumor cell killing most significantly in populations of Mitf^{Low}/Axl^{Low} cells (Supplementary Fig. 17d). Interestingly, global changes in differentiation state markers induced by 5 days of treatment with neither of the epigenetic compounds were identified as statistically significant by PLSR models. Single-cell analysis of SP2509-sensitive cells (e.g., A375 and A375-NRAS^{Q61K}), however, revealed partial downregulation of Axl and upregulation of Mitf following treatment with SP2509, which is in agreement with the possibility that Axl^{High}/Mitf^{Low} cells are being selectively eliminated by SP2509 (Supplementary Fig. 18a). In contrast, for cell lines that were sensitive to the combination of JIB-04, vemurafenib, and trametinib (e.g.,

MMACSF and WM115), we observed partial reduction of the frequency of Ngfr^{High} cells when they were treated with the triple-drug combination in comparison with cells treated with vemurafenib and trametinib only (Supplementary Fig. 18b, c).

Together, these data reveal how correlated patterns of responses to SP2509, JIB-04, and BET inhibitors are linked to the state of MAPK signaling and differentiation state. Cells in undifferentiated and neural crest-like states represent two different forms of MAPK inhibitor tolerance observed at variable frequencies across most melanoma tumors. Their selective sensitivity to the identified epigenetic inhibitors, therefore, supports the hypothesis that there are epigenetic features associated with melanoma differentiation state that may be linked to their state of Braf/MAPK dependency.

Kdm4b and Znf217 protein levels predict differentiation state-specific sensitivity to JIB-04 and SP2509. We sought to utilize our knowledge of interactions between JIB-04 and SP2509 and their protein targets to better understand the origins of their selective efficacy in melanoma cells. JIB-04 is known as a pan-inhibitor of Jmj-KDMs, suppressing the activities of Kdm4a, Kdm4b, Kdm5a, and Kdm5b, all with IC₅₀'s < 0.5 μM⁴². We thus asked which of the Jmj-KDM proteins targeted by JIB-04 might explain its inhibitory effect on melanoma cells. We first depleted each of Kdm4a, Kdm4b, Kdm5a, and Kdm5b proteins in two JIB-04-sensitive cell lines (WM115 and WM902B) and one JIB-04-resistant cell line (A2058) using four target-specific siRNAs combined into a single pool (Supplementary Fig. 19). Only depletion of Kdm4b in WM115 and WM902B cells led to a statistically significant decrease in live cell count and enhanced cells' sensitivity to the combination of vemurafenib and trametinib (Fig. 6a). To rule out the possibility of off-targeting by KDM4B siRNAs, we then examined the effects of three constituent KDM4B siRNAs independently. All individual siRNAs reduced Kdm4b expression and cell viability in both WM115 and WM902B, while having no impact on Kdm4a expression in these cell lines (Supplementary Fig. 20a, b). A2058 cells, on the other hand, were sensitive to KDM1A knockout by CRISPR using three independent sgRNAs (Fig. 6b and Supplementary Fig. 20c), which correlated with their sensitivity to SP2509.

We then asked whether the selective efficacy of SP2509 and JIB-04 might be explained by the differentiation state-specific expression of their epigenetic targets. We used mass spectrometry-based proteomics data for BRAF-mutant melanoma lines in the Cancer Cell Line Encyclopedia (CCLE)⁴³ to evaluate possible correlations between the expression of each epigenetic target and the relative expression of Ngfr and Axl. Since SP2509 acts by blocking Kdm1a interaction with its coactivator Znf217⁴⁴,

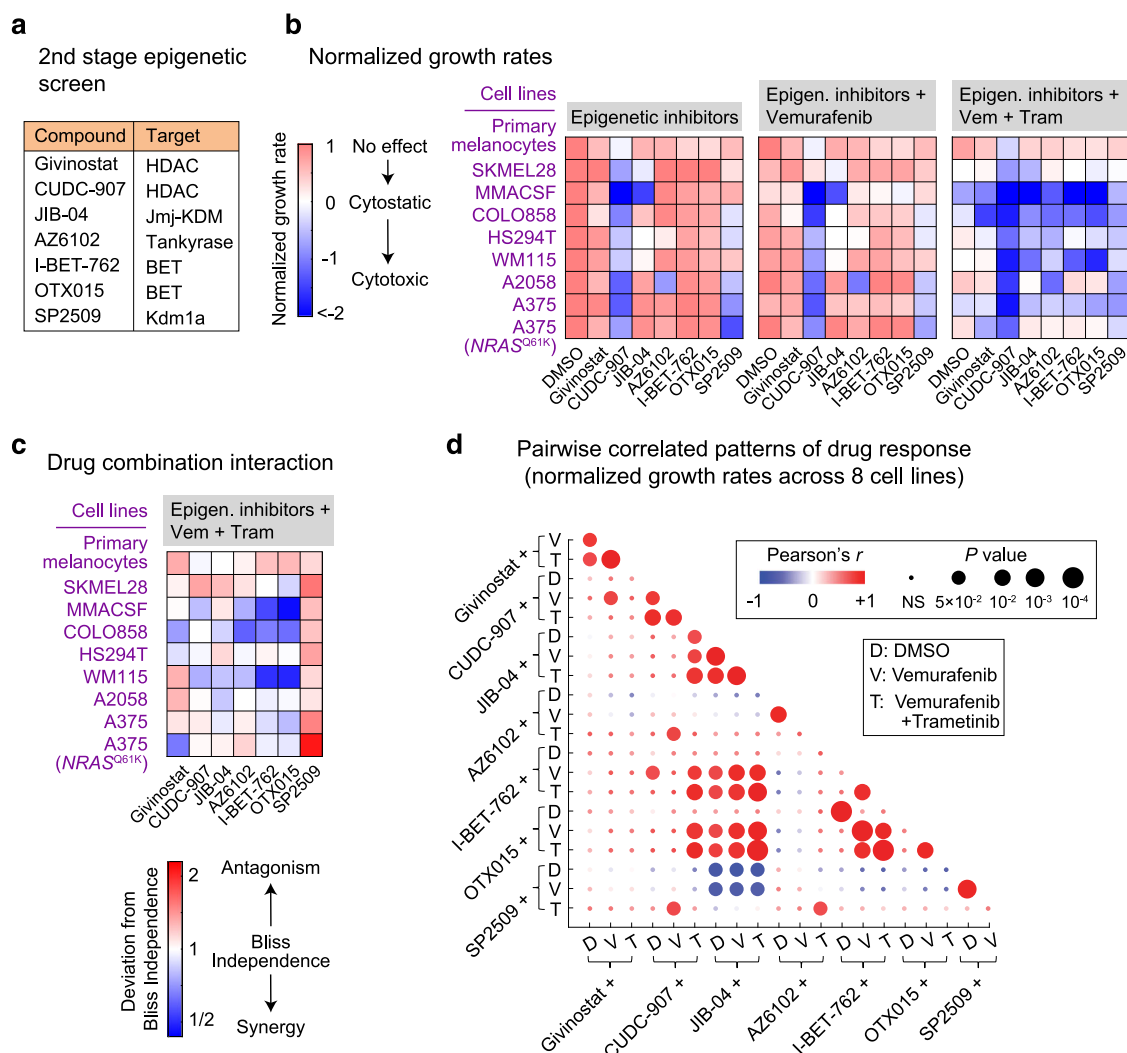


Fig. 3 Patterns of melanoma responses to mechanistically distinct epigenetic inhibitors. **a** Epigenetic compounds (and their nominal protein targets) used in the second stage of the epigenetic compound screen. **b** Measurements of normalized growth rate induced by each epigenetic inhibitor, when used either as a single agent (left), in combination with vemurafenib (middle), or in combination with vemurafenib plus trametinib (right), across eight different *BRAF*-mutant melanoma cell lines and non-transformed primary melanocytes. Treatment doses for different compounds are as follows: Givinostat (200 nM), CUDC-907 (20 nM), JIB-04 (200 nM), AZ6102 (1 μ M), I-BET-762 (1 μ M), OTX015 (0.2 μ M), SP2509 (1 μ M). Cells were pretreated with the indicated epigenetic compounds for 24 h and then treated for a period of 3–5 days with either DMSO, vemurafenib (at 100 nM), or the combination of vemurafenib (at 100 nM) and trametinib (at 10 nM). **c** Deviation from Bliss Independence (DBI) values was computed across diverse epigenetic inhibitor treatments in combination with vemurafenib plus trametinib. DBI = 1 represents an independent (additive) effect equal to what is expected for the combination of drugs that act independently, DBI < 1 represents a combined effect stronger than expected for an independent combination (i.e., synergism), and DBI > 1 represents a combined effect weaker than expected for an independent combination (i.e., antagonism). **d** Two-sided Pearson's correlation between the effects (i.e., normalized growth rates) of mechanistically distinct epigenetic inhibitors (used individually or in combination with vemurafenib or vemurafenib plus trametinib) evaluated across eight *BRAF*-mutant melanoma cell lines. Source data are provided as a Source data file.

we also included this protein in our analysis. While Kdm1a protein levels did not significantly correlate with melanoma differentiation state (Supplementary Fig. 21a), Znf217 was among the top 4% proteins that correlated with the expression of Axl relative to Ngfr (Fig. 6c; left panels). In addition, levels of Kdm4b (but not other JIB-04 targets) were among the top 0.6% proteins whose expression was significantly greater in melanoma cell lines that expressed higher levels of Ngfr relative to Axl (Fig. 6c; right panels, and Supplementary Fig. 21b). These data suggest that the relative levels of Kdm4b and Znf217 proteins and differentiation markers Ngfr and Axl may predict the selective sensitivity of melanoma cells to JIB-04 and SP2509.

To independently test this hypothesis, we first profiled the levels of all four proteins across our original panel of 16

melanoma cell lines plus an additional group of 5 cell lines by immunofluorescence microscopy (Supplementary Fig. 22a–d). Following single-cell analysis, we performed correlation analyses similar to those performed using the mass spectrometry data from CCLE. Our analysis confirmed significant pairwise correlations between the fractions of Ngfr^{Low}/Axl^{High} cells and Znf217^{High} cells on one hand and the fractions of Ngfr^{High}/Axl^{Low} cells and Kdm4b^{High} cells on the other hand (Fig. 6d; top panels). As expected, the sensitivity of melanoma cell lines to SP2509 and the combination of JIB-04, vemurafenib, and trametinib were also correlated with the fractions of Znf217^{High} and Kdm4b^{High} cells, respectively (Fig. 6d; bottom panels). To further validate the predictivity of these protein markers, we divided the panel of 21 cell lines into a test set of 14 cell lines and

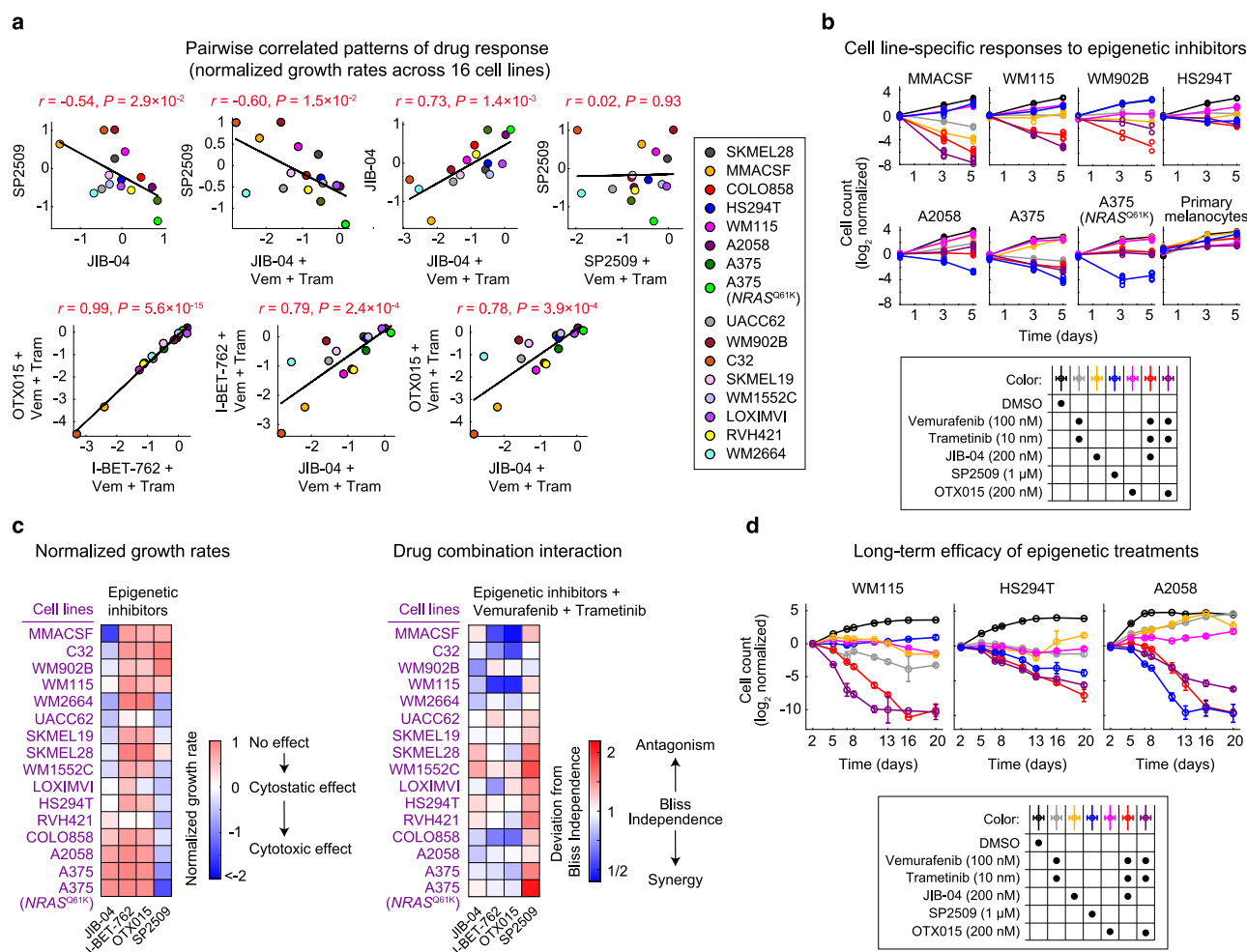


Fig. 4 Correlated patterns of sensitivity to pharmacological inhibition of Kdm1a, Jmj-KDMs, and BET proteins across 16 *BRAF*-mutant melanoma cell lines. **a** Two-sided Pearson's correlation between the effects (i.e., normalized growth rates) of Kdm1a inhibitor SP2509 (at 1 μ M), Jmj-KDM inhibitor JIB-04 (at 200 nM), BET inhibitors OTX015 (at 200 nM), and I-BET762 (at 1 μ M), used individually or in combination with vemurafenib (at 100 nM) plus trametinib (at 10 nM) and evaluated across 16 *BRAF*-mutant melanoma cell lines. **b** Log₂-normalized changes in live cell count following exposure of seven selected melanoma cell lines and non-transformed primary melanocytes to different drugs at indicated doses for a period of 5 days. MMACSF, WM115, and WM902B cell lines represent cell lines that exhibit high sensitivity to the combination of JIB-04 or BET inhibitors with *Braf*/*Mek* inhibitors, while being resistant to SP2509. A2058, A375, and A375(NRAS^{Q61K}) represent cell lines that are highly sensitive to SP2509. HS294T cells show partial sensitivity to either of the compounds. Data are presented as the average of $n = 6$ biologically independent samples (in case of MMACSF, WM115, HS294T, A2058, A375, A375(NRAS^{Q61K}) and primary melanocytes), or the average of $n = 2$ biologically independent samples (in case of WM902B). **c** Measurements of normalized growth rate (left) and deviation from Bliss independence (DBI) values computed across diverse epigenetic inhibitor treatments in combination with vemurafenib plus trametinib. Experimental conditions and the analysis approach are the same as in Fig. 3. **d** Log₂-normalized changes in live cell count following exposure of three selected melanoma cell lines to different drugs at indicated doses for a period of 20 days. Data are presented as mean values $\pm$ s.d. across $n = 4$ biologically independent samples. Source data are provided as a Source data file.

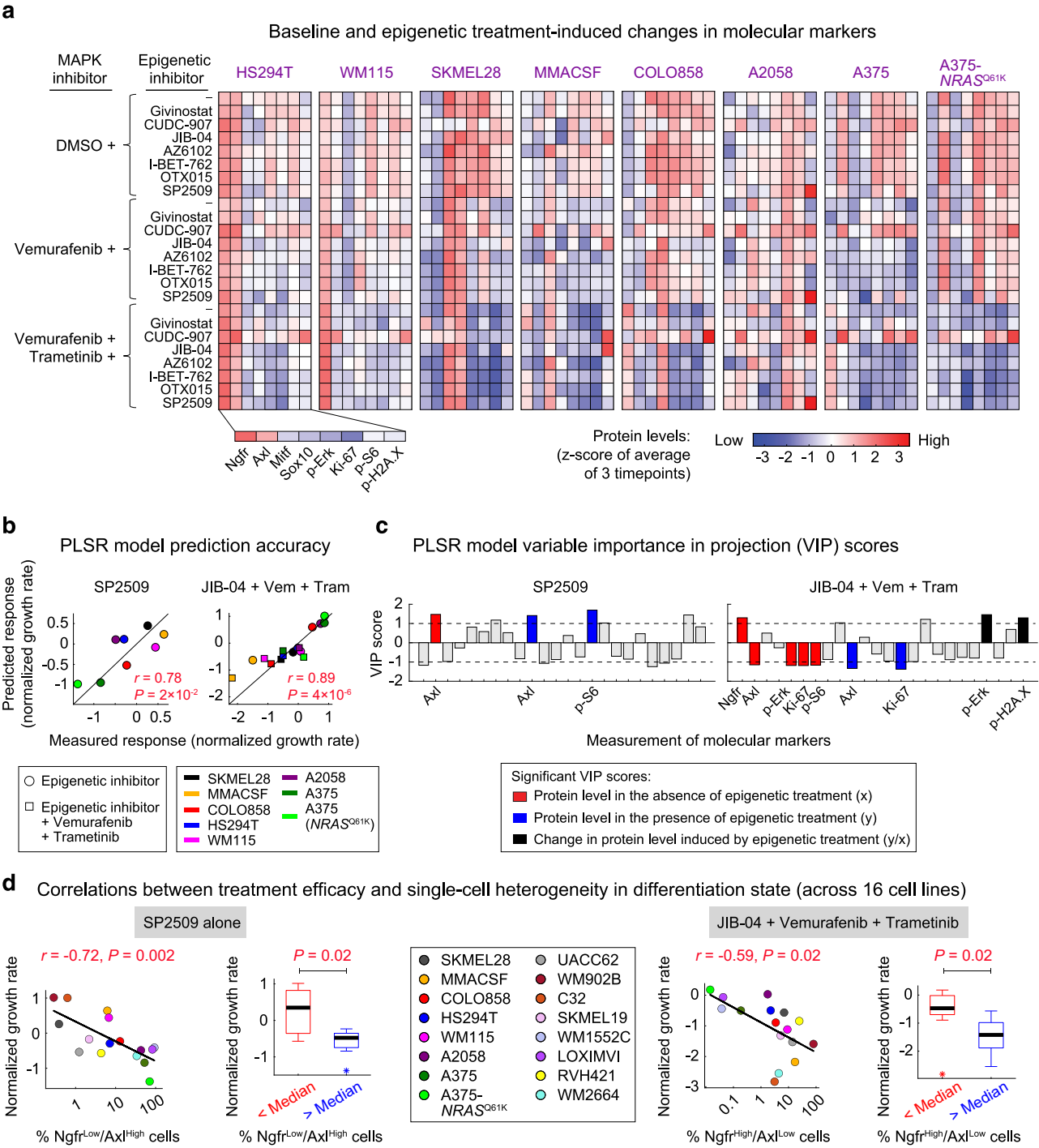
a validation set of 7 cell lines. We then used the training set to develop multi-linear regression (MLR) models of treatment efficacy for SP2509 and JIB-04 using protein levels of *Ngfr*, *Axl*, *Kdm4b*, and *Znf217*. The trained models predicted SP2509 and JIB-04 efficacy in the validation set with high accuracy (Fig. 6e, Supplementary Fig. 22e). Together, these data demonstrate that the sensitivity of *BRAF*-mutant melanoma cells to SP2509 and JIB-04 can be predicted based on their differentiation state (*Ngfr* versus *Axl*) and the relative levels of *Znf217* and *Kdm4b* proteins.

Discussion

Lineage-specific epigenetic mechanisms and their reprogramming following oncogene inhibition can generate drug-tolerant states that diminish the efficacy of cancer-targeted therapies⁴⁵. In

BRAF-mutant melanomas, comparable states tolerant of MAPK inhibition have been associated with variations in differentiation state. Through high-throughput profiling of human melanoma cell lines, we uncovered recurrent patterns of differentiation state heterogeneity that are comparable to those previously seen in patient tumors²⁵. We found that a melanoma cell's ability to tolerate *Braf*/*Mek* inhibitors was associated with its differentiation state. Responses of undifferentiated cells were associated with incomplete inhibition of the MAPK pathway, whereas MAPK-inhibited neural crest-like cells adapted to treatment by reducing their requirement for MAPK signaling.

To identify epigenetic features linked to melanoma differentiation states and MAPK dependency, we performed an epigenetic compound screen and identified three classes of compounds that target distinct melanoma cell states associated



with either one of the lysine-specific histone demethylases Kdm1a or Kdm4b, or BET proteins. While the survival of non-transformed melanocytes remained unaffected by these compounds, their cytotoxic efficacy in melanoma cells depended on their state of MAPK activity and their baseline (drug-naïve) differentiation state. The Kdm1a inhibitor SP2509 was most effective in inhibiting undifferentiated populations of cells that were intrinsically insensitive to Braf/Mek inhibitors, whereas the combination of SP2509 with Braf/Mek inhibitors led to antagonistic interactions. This is consistent with a recent finding regarding the role that Kdm1a may play in disabling *BRAF*^{V600E} oncogene-induced senescence³⁴. Kdm1a inhibition may, therefore, require MAPK signaling to restore senescence in Ngrf^{Low}/Axl^{High} cells. In contrast, Ngrf^{High}/Axl^{Low} populations of cells exhibited additive to

synergistic responses to the combination of Kdm4b and Braf/Mek inhibition. BET inhibitors had minimal effect on melanoma cells when used as a single agent but led to tumor cell killing when combined with Braf/Mek inhibitors. Together, our systematic studies extend previous findings about the potential of each of the epigenetic regulators, Kdm1a, Jmj-KDMs, and BET proteins, as therapeutic vulnerabilities in melanomas^{20,34,46} in two complementary ways. First, by associating these vulnerabilities to the patterns of single-cell heterogeneity in differentiation state, we explain how they may vary from one tumor to another. Second, by linking epigenetic vulnerabilities to the state of Braf/MAPK dependency, we determine whether each epigenetic inhibitor reaches its maximal efficacy when used as a single agent or when combined with Braf/Mek inhibitors.

Fig. 5 Multivariate modeling identifies differentiation state-specific predictors of epigenetic inhibitor efficacy. **a** Baseline and treatment-induced changes in protein measurements of melanoma differentiation state markers (Ngfr, Axl, Mitf, Sox10), MAPK signaling protein modifications (p-Erk^{T202/Y204}, p-S6^{S235/S236}), a proliferation marker (Ki67), and a DNA damage response marker (p-H2A.X^{S139}), across eight melanoma cell lines following treatment with the indicated epigenetic compounds, either individually or in combination with vemurafenib, or vemurafenib plus trametinib. Treatment doses for different compounds are as follows: Givinostat (200 nM), CUDC-907 (20 nM), JIB-04 (200 nM), AZ6102 (1 μ M), I-BET-762 (1 μ M), OTX015 (0.2 μ M), SP2509 (1 μ M), vemurafenib (100 nM), trametinib (10 nM). Protein data shown for each condition represent mean values across three timepoints and two biologically independent samples and are then z-scored across all cell lines and treatment conditions. **b** Two-sided Pearson's correlation between responses (normalized growth rates) to SP2509 (left) or JIB-04 in combination with vemurafenib and trametinib (right), measured for each of the eight melanoma cell lines (x-axis) and corresponding responses predicted by partial least square regression (PLSR) modeling following leave-one-out cross-validation (y-axis). **c** PLSR-derived variable importance in the projection (VIP) scores, highlighting combinations of protein measurements at the baseline (shown in red), following epigenetic inhibitor treatment (shown in blue), or the ratio of change induced by each epigenetic compound (shown in black), that are predictive of efficacy for SP2509 (left) and JIB-04 in combination with vemurafenib and trametinib (right). The sign of VIP score shows whether the change in variable correlated negatively or positively with the treatment-induced response. Only VIP scores of greater than 1 or smaller than -1 with a statistically significant Pearson's correlation ($P < 0.05$) are highlighted. **d** Two-sided Pearson's correlation analysis between the baseline fractions of Ngfr^{Low}/Axl^{High} cells and measurements of normalized growth rate in response to SP2509 when used as a single agent (left), and between the baseline fractions of Ngfr^{High}/Axl^{Low} cells and response to JIB-04 in combination with vemurafenib and trametinib (right), across 16 BRAF-mutant melanoma cell lines. The significance of differences between normalized growth rates was also evaluated based on two-sided Mann-Whitney *U*-test. For this test, 16 cell lines were divided into two groups of eight based on whether the measured variable had a value above or below the median. Data for each group were then presented using box-and-whisker plots, on which the central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to $1.5\times$ the interquartile range as a measure of variance, and datapoints outside the range are plotted individually with asterisks. Source data are provided as a Source data file.

Cell-to-cell heterogeneity in the state of oncogene dependency poses a general challenge to the use of cancer-targeted therapies. Our systems pharmacology approach provides a promising avenue toward the identification of actionable epigenetic factors that may extend the oncogene addiction paradigm on the basis of tumor cell differentiation state. We demonstrate the utility of this approach in BRAF-mutant melanomas by using JIB-04 and SP2509, as investigational tools, to block epigenetically diverse populations of MAPK inhibitor-tolerant cells. We show that the relative baseline levels of Kdm4b and Znf217 (a Kdm1a coactivator), determine differentiation state-specific sensitivity of melanoma cells to their corresponding inhibitors. In cell lines that were highly sensitive to SP2509, 3 to 5 days of treatment with SP2509 reduced the fraction of Axl^{High}/Mitf^{Low} cells in the tumor cell population. In contrast, in cell lines that were sensitive to the combination of JIB-04 and Braf/Mek inhibitors, the frequency of Ngfr^{High} cells was only partially reduced following treatment with the triple-drug combination. A possible explanation for not observing drastic changes in differentiation state markers after epigenetic inhibitor treatments may be that the measured Ngfr and Axl proteins are surrogate markers which, although confirmed to be statistically associated with melanoma differentiation states, are not causally linked to melanoma cell survival^{12,19}. Therefore, additional studies are required to identify key downstream targets of histone demethylases and their interactions with melanoma differentiation state that make them selectively sensitive to each epigenetic inhibitor. This may be accomplished via a combination of transcriptomic, proteomic, and epigenomic profiling of melanoma cells of diverse differentiation states following treatment with each inhibitor in the absence or presence of MAPK inhibitors. The identification of Kdm4b and Znf217/Kdm1a, whose knockdown phenocopies the epigenetic inhibitors' effects, will facilitate such mechanistic studies, e.g., through rationally designed chromatin immunoprecipitation coupled with sequencing (ChIP-seq) experiments.

Our data add to a growing body of research^{18,47–50} showing that even genetically homogenous populations of tumor cells consist of subpopulations at diverse differentiation states that are also different with respect to their state of oncogene dependency. Stochastic fluctuations in the levels of key proteins from pathways that have crosstalk with the oncogenic pathway and differentiation state-specific epigenetic modifications represent possibly

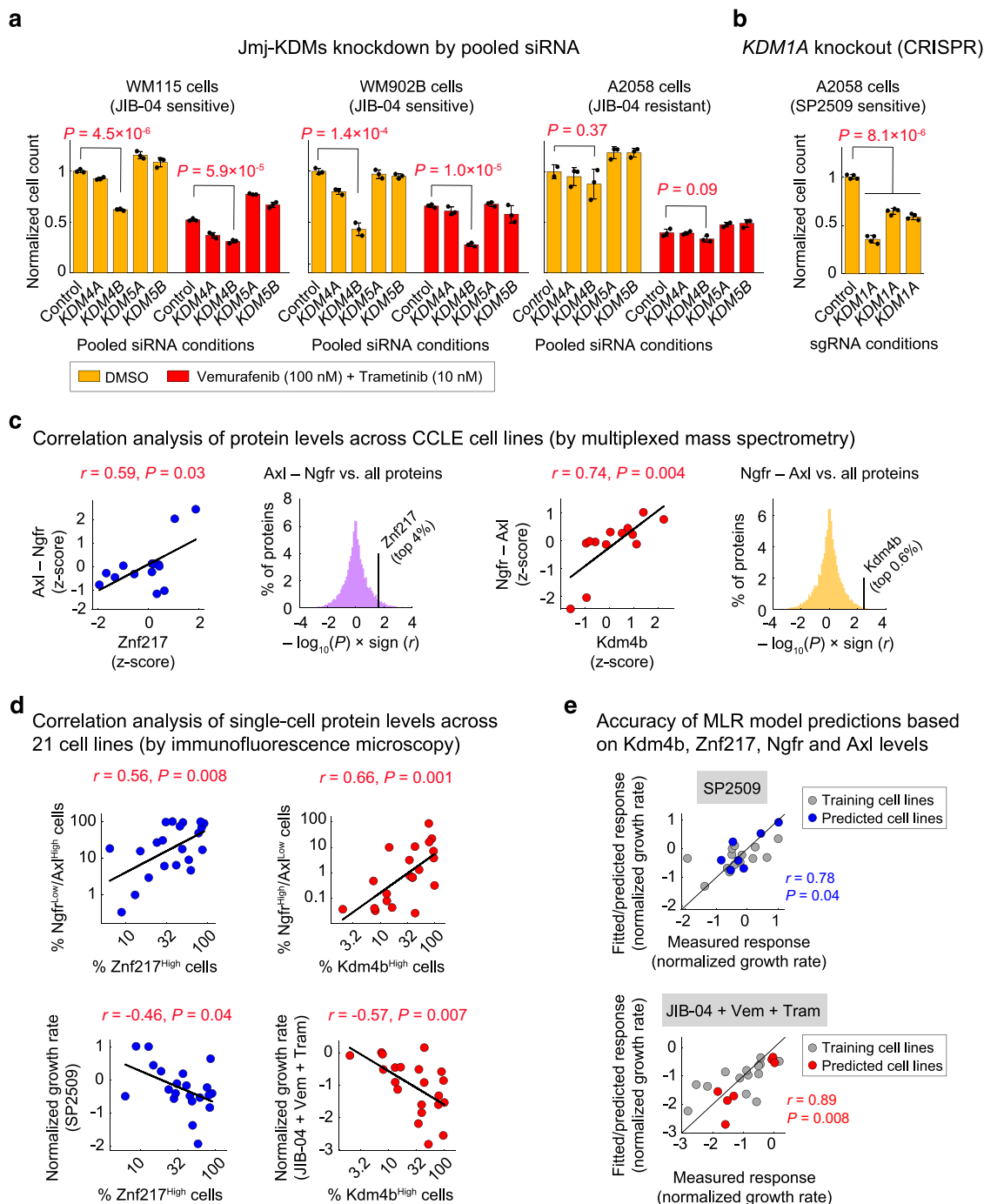
related mechanisms that lead to diverse states of drug tolerance^{2,51,52}. A quantitative understanding of the key drivers of such phenotypically consequential epigenetic states at a single-cell level and uncovering how they are linked to signaling networks that function in feedback regulation of oncogenic signaling, are likely key steps to improving the effectiveness and durability of response to oncogene-targeted therapies.

Methods

Cell lines and reagents. BRAF-mutant melanoma cell lines used in this study were obtained from the following sources: COLO858 (from ECACC), RVH421 (from DSMZ), A375, A375(NRAS^{Q61K}), C32, A2058, WM115, SKMEL28, HS294T, WM1552C, HS695T, RPMI7951, SKMEL5, A101D, IGR39, and human adult primary epidermal melanocytes (all from ATCC), LOXIMV1 (from DCTD Tumor Repository, National Cancer Institute), MMACSF (RIKEN BioResource Center), WM902B and WM2664 (from Wistar Institute), UACC62 and SKMEL19 (from the Cancer Cell Line Encyclopedia). All of the cell lines have been periodically subjected to re-confirmation by short tandem repeat (STR) profiling by ATCC and mycoplasma testing by MycoAlertTM PLUS mycoplasma detection Kit (Lonza). A375, A375(NRAS^{Q61K}), A2058, HS294T, A101D, and IGR39 cells were grown in DMEM with 4.5 g L⁻¹ glucose (Corning, Cat# 10-013-CV) supplemented with 5% fetal bovine serum (FBS). RPMI7951, SKMEL5, and HS695T cells were grown in EMEM (Corning, Cat# 10-009-CV) supplemented with 5% FBS. C32, MMACSF, SKMEL28, and WM115 cells were grown in DMEM/F12 (Gibco, Cat# 11330-032) supplemented with 1% sodium pyruvate (Invitrogen) and 5% FBS. COLO858, LOXIMV1, RVH421, SKMEL19, UACC62, WM1552C, and WM902B cells were grown in RPMI 1640 (Corning, Cat# 10-040-CV) supplemented with 1% sodium pyruvate and 5% FBS. Primary epidermal melanocytes were grown in Dermal Cell Basal Medium (ATCC, Cat# PCS-200-030) supplemented with Adult Melanocyte Growth Kit (ATCC, Cat# PCS-200-042). We added penicillin (50 U mL⁻¹) and streptomycin (50 μ g mL⁻¹) to all growth media.

Small-molecule inhibitors, including a library of 276 epigenetic-modifying compounds, chemicals used in the follow-up cell-based assays (Givinostat, CUDC-907, JIB-04, AZ6102, I-BET-762, OTX015, and SP2509), as well as vemurafenib and trametinib were all purchased from Selleck Chemicals. SP2577 was purchased from MedChem Express. The complete list of compounds used in this study, their catalog numbers and purity, as evaluated by HPLC and MS analysis, are presented in Supplementary Table 1. Compounds used for cell-based studies were dissolved in the appropriate vehicle (either DMSO or water) at a stock concentration of 10 mM. The vehicle for SP2577 used for in vivo studies is described below.

The following primary monoclonal antibodies (mAb, clone name) and polyclonal antibodies (pAb) with specified animal sources, catalog numbers, research resource identifiers (RRID), and dilution ratios, were used in immunofluorescence staining assays: Mitf (mouse mAb, clone D5, Abcam, Cat# ab3201, AB_303601, 1:800), p-Erk^{T202/Y204} (rabbit mAb, clone D13.14.4E, Cell Signaling Technology, Cat# 4370, AB_2315112, 1:800), Ki67 (mouse mAb, clone 8D5, Cell Signaling Technology, Cat# 9449, AB_2715512, 1:1200), Axl (goat pAb, R&D Systems, Cat# AF154, AB_354852, 1:400), p-Rb^{S807/S811} (goat pAb, Santa Cruz Biotechnology, Cat# sc-16670, AB_655250, 1:400), Ngfr (rabbit mAb, clone



D4B3, Cell Signaling Technology, Cat# 8238, AB_10839265, 1:1600), p-S6^{S235/S236} (rabbit mAb, clone D57.2.2E, Cell Signaling Technology, Cat# 4851, AB_10695457, 1:400), Sox10 (mouse mAb, clone SOX10/991, Abcam, Cat# ab212843, AB_2889150, 1:1200), p-H2A.X^{S139} (rabbit mAb, clone EP854(2)Y, Abcam, Cat# ab195188, AB_2889151, 1:700), Kdm1a (rabbit mAb, clone C69G12, Cell Signaling Technology, Cat# 2184, AB_2070132, 1:1600), Kdm4a (rabbit mAb, clone C37E5, Cell Signaling Technology, Cat# 5328, AB_10828595, 1:100), Kdm4b (rabbit mAb, clone D7E6, Cell Signaling Technology, Cat# 8639, AB_11140642, 1:100), Kdm5a (rabbit mAb, clone EPRI8651, Abcam, Cat# ab194286, AB_2889152, 1:1600), Kdm5a (rabbit mAb, clone EPRI2704, Abcam, Cat# ab181089, AB_2889153, 1:100), and Znf217 (rabbit pAb, Thermo Fisher Scientific, Cat# 720352, AB_2716919, 1:200). The following secondary antibodies with specified sources and catalog numbers were used at a 1:2000 dilution: anti-rabbit Alexa Fluor 488 (Thermo Fisher, Cat# A21206, AB_2535792), anti-mouse Alexa Fluor 647 (Thermo Fisher, Cat# A31571, AB_162542), anti-goat Alexa Fluor 568 (Thermo Fisher, Cat# A11057, AB_2534104), anti-mouse Alexa Fluor 568 (Thermo Fisher, Cat# A10037, AB_2534013), and anti-rabbit Alexa Fluor 647 (Thermo Fisher, Cat# A31573, AB_2536183).

Immunofluorescence staining, quantitation, and analysis. Cells in 96-well plates were fixed in either 4% paraformaldehyde (PFA) for 20 min at room temperature or 100% ice-cold methanol for 15 min at -20°C . Cells were then washed with PBS, permeabilized in methanol (in cases where they were fixed with 4% PFA) for 10 min at -20°C , rewashed with PBS, and blocked using Odyssey blocking buffer (LI-COR Biosciences) for 1 h at room temperature. Cells were incubated overnight (~16 h) at 4°C with primary antibodies in Odyssey blocking buffer. The following day, cells were washed three times with PBS supplemented with 0.1% Tween-20 (Sigma-Aldrich) (PBS-T) and incubated for 1 h at room temperature with the secondary rabbit, goat, or mouse antibodies. Cells were then washed twice with PBS-T, once in PBS, and then incubated with Hoechst 33342 (Thermo Fisher, Cat# H3570, 1:20,000) for 20 min at room temperature. Cells were washed twice with PBS and imaged with a $\times 10$ objective using the ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices) or the Operetta CLS High-Content Imaging System (Perkin Elmer). A total of nine sites were imaged per well. Background subtraction was performed with ImageJ. Image segmentation and quantification of signal intensities in the whole cell, nucleus, cytoplasm, or the nucleus/cytoplasm (N/C) ratio were performed with CellProfiler⁵³. Population-

Fig. 6 Kdm4b and Znf217 protein levels predict differentiation state-specific sensitivity to JIB-04 and SP2509. **a** Relative cell viability in WM115 cells (left), WM902B cells (middle), and A2058 cells (right) following treatment with indicated doses of the combination of vemurafenib and trametinib or vehicle (DMSO) in the presence of pools of four siRNAs targeting either *KDM4A*, *KDM4B*, *KDM5A*, or *KDM5B* for 96 h. Viability data for each treatment condition were normalized to cells treated with DMSO and the non-targeting (control) siRNA. Data are presented as mean values $\pm$ s.d. calculated across $n = 3$ biologically independent experiments. Statistical significance was determined by two-sided *t* test. **b** Relative cell viability in Cas9-positive A2058 cells following treatment with three different types of *KDM1A* lentiviral single guide RNA (sgRNA) for 96 h. Viability data were normalized to cells treated with non-targeting (control) sgRNA. Data are presented as mean values $\pm$ s.d. calculated across $n = 4$ biologically independent experiments. Statistical significance was determined by two-sided *t* test. **c** Two-sided Pearson's correlation analysis of variations in the difference between Ngfr and Axl protein levels and Znf217 (left panels) or Kdm4b (right panels) in comparison with the rest of the measured proteome (shown as histograms) across *BRAF*-mutant melanoma cell lines. Protein data are extracted from the Cancer Cell Line Encyclopedia (CCLE) proteomics database (measured by multiplexed mass spectrometry) and z-scored across all of *BRAF*-mutant melanoma cell lines present in the database. **d** Two-sided Pearson's correlation between the baseline fractions of Znf217^{High} cells and Kdm4b^{High} cells and the fractions of Ngfr^{Low}/Axl^{High} cells (top left), the fractions of Ngfr^{High}/Axl^{Low} cells (top right), sensitivity to SP2509 at 1 μ M (bottom left), and sensitivity to the combination of JIB-04 (at 0.2 μ M), vemurafenib (at 100 nM) and trametinib (at 10 nM) (bottom right), as evaluated by normalized growth rates following 5 days of treatment. Protein data were measured by immunofluorescence microscopy, quantified following single-cell analysis, and averaged across $n = 3$ biologically independent samples. **e** Two-sided Pearson's correlation between responses (normalized growth rates) to SP2509 (top) or JIB-04 in combination with vemurafenib and trametinib (bottom), measured for each of the 21 melanoma cell lines (*x*-axis) and corresponding responses fitted by multi-linear regression (MLR) analysis of 14 cell lines (training set; shown in gray) or responses predicted by the trained models for the remaining seven cell lines (validation set; shown in blue or red). Source data are provided as a Source data file.

average and single-cell data were analyzed using MATLAB 2019b. By generating histograms of single-cell data across a variety of conditions for each protein (*X*), including positive and negative controls, we identified an appropriate binary gate, based on which the percentage of X^{High} versus X^{Low} cells in each condition was quantified.

Single-cell analysis and dimensionality reduction. To visualize single-cell heterogeneities in a two-dimensional space, we used a population of 6069 cells assembled following random selection of 60 individual cells (or fewer in the case of the highly drug-sensitive C32 cell line, for which <60 cells survived following treatments with the combination of vemurafenib and trametinib) from each of the 102 tested conditions, covering the entire panel of 17 cell lines, 3 drugs (DMSO, vemurafenib alone, and vemurafenib plus trametinib) and 2 timepoints. Log-transformed single-cell data for multiplexed measurements of differentiation state markers, Mitf, Ngfr and Axl, and for multiplexed measurements of MAPK signaling, p-Erk^{T202/Y204}, p-S6^{S235/S236}, and Ki67, were processed by z-scoring (for each protein measurement) across all 6069 cells, followed by principal component analysis (PCA) using the MATLAB built-in function *pca*. Eighty percent of the variance in the differentiation state single-cell data and 92.5% of the variance in MAPK single-cell data were captured by the first two principal components (PCs). We thus performed *t*-distributed stochastic neighbor embedding (t-SNE) analysis on the scores from the first two PCs of each of the single-cell datasets by applying the built-in *tsne* function in MATLAB 2019b. We used the barneshtut algorithm, learning rate of 1000 for the optimization process, a maximum number of optimization iterations of 2000, perplexity of 480, and exaggeration factor of 4. To analyze the baseline state and treatment-induced changes in the single-cell behavior of each cell line, we used the t-SNE map overlaid with the projection of z-scored single-cell measurements (color-coded between blue and red) for that cell line while showing data for other cell lines in gray.

To compare baseline and treatment-induced heterogeneities in the expression of each protein, we evaluated Fano factor (a.k.a. index of dispersion) by computing the variance-to-mean ratio for single-cell measurements across each population of cells. To compare single-cell heterogeneity in multiple differentiation state markers (Mitf, Ngfr, and Axl) simultaneously, we determined the average cell-to-cell distance by computing the pairwise Euclidean distance among individual cells (after z-scoring the data for each protein marker), and the mean of all possible pairwise distances was reported for each population of cells.

Measurements of growth rate, drug sensitivity, and combination effectiveness.

Growth rate inhibition assays were performed in 96-well clear bottom black plates (Corning, Cat# 3904). Cells were counted using a TC20 automated cell counter (Bio-Rad Laboratories) and seeded in 200 μ l of full growth media at a density of 1200–5000 cells per well, depending on the baseline proliferation rate of each cell line. Using a D300e Digital Dispenser, cells were treated the next day with small-molecule compounds at reported doses or vehicle (DMSO). Measurements of live cell count across multiple timepoints were then used to calculate the net growth rate for each treatment condition. To measure the number of surviving cells at each timepoint, cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were washed twice with PBS and incubated with Hoechst 33342 (Thermo Fisher, Cat# H3570, 1:20,000) for 20 min at room temperature. Cells were washed again with PBS and imaged with a $\times 10$ objective using an ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices) or an Operetta CLS

High-Content Imaging System (Perkin Elmer). A total of nine sites were imaged per well. Nuclear segmentation and cell counting was performed using CellProfiler⁵³.

The net growth rate for cells treated with individual compounds (including MAPK inhibitors or epigenetic inhibitors), or their combination, or vehicle, was calculated from time-dependent changes in live cell count according to the following equation:

$$\mu = \frac{\ln\left(\frac{N_{t_2}}{N_{t_1}}\right)}{t_2 - t_1} \quad (1)$$

where N_{t_1} and N_{t_2} represent the number of cells measured at timepoints $t = t_1$ and $t = t_2$, respectively, and μ describes the net growth rate of cells during the time period between t_1 and t_2 . Average net growth rates for each treatment condition were calculated as the mean of growth rates measured across multiple consecutive timepoints. To compare drug sensitivity among cell lines while correcting for differences in their baseline proliferation rates, drug-induced normalized growth rates (a.k.a. DIP rates³⁷) were computed as follows:

$$\text{Normalized growth rate (DIP)} = \frac{\mu_{\text{drug}}}{\mu_{\text{DMSO}}} \quad (2)$$

As shown above, normalized growth rate for cells treated with a specific drug is calculated by normalizing the average net growth rate measured for drug-treated cells (μ_{drug}) to that measured for vehicle (DMSO)-treated cells (μ_{DMSO}). Normalized growth rates <0 indicate a net cell loss (i.e., cytotoxicity), a value of 0 represents no change in viable cell number (i.e., cytostasis), a value >0 indicates a net cell gain (i.e., population growth), and a value of 1 represents cell growth at the same rate as in vehicle-treated (control) cells.

To quantify the benefit resulting from combining two drugs, e.g., an epigenetic inhibitor (A) with a MAPK inhibitor (B), we evaluated their interactions based on the Bliss independence model³⁸. We first calculated the fraction of cells affected by each treatment as follows:

$$f_a = \frac{1 - \text{DIP}}{1 - \min(\text{DIP})} \quad (3)$$

where $\min(\text{DIP})$ represents the minimum of DIP values reported across all cell lines and drug treatment conditions in this study. Using f_a , we overcome the Bliss metric limitation for the analysis of unbounded drug effects such as the normalized growth rates⁵⁴, while highlighting combined interactions that influence drug efficacy, a parameter that is affected (more obviously than potency) by cell-to-cell heterogeneity and the presence of small subpopulations of drug-tolerant cells⁵⁵. We then used measurements of f_a for individual and combination treatments to compute the deviation from Bliss Independence (DBI) as follows:

$$\text{DBI} = \frac{f_a(A) + f_a(B) - f_a(A)f_a(B)}{f_a(A+B)} \quad (4)$$

where $f_a(A)$ and $f_a(B)$ represent the fraction of cells affected by each drug separately, and $f_a(A+B)$ represents that of the combination treatment (A plus B). Based on this definition, the calculated DBI compares the observed response to that expected given independent action for the two individual treatments. DBI = 1 represents an independent (additive) effect equal to what is expected for the combination of drugs that act independently, DBI < 1 represents a combined effect stronger than expected for an independent combination (i.e., synergism), and DBI > 1 represents a combined effect weaker than expected for an independent combination (i.e., antagonism).

Epigenetic compound screen. COLO858 and MMACSF cells were counted using a TC20 automated cell counter (Bio-Rad Laboratories) and seeded in 96-well clear bottom black plates at a density of 2000 and 3000 cells per well (excluding 36 wells at the edges), respectively. Using the HP D300e Digital Dispenser, cells were treated the next day with either vehicle (DMSO or water) or two different doses (0.2 and 1 μM) of each of the 276 compounds in an epigenetic compound library. After 24 h, cells were either fixed or treated with vemurafenib alone (at 100 nM), or vemurafenib (at 100 nM) plus trametinib (at 10 nM), or vehicle (DMSO). Cells were then grown for a further 72 or 120 h prior to fixation. All experimental conditions with an epigenetic compound treatment (at each dose and timepoint) were tested in two replicates. Experimental conditions that did not include an epigenetic compound treatment (i.e., treatments with only vehicle, vemurafenib, or vemurafenib in combination with trametinib) were repeated in all 96-well plates during the entire period of compound screening, creating a total of 276 replicates that were used to evaluate plate-to-plate and day-to-day experimental robustness. To differentiate the impact of epigenetic compounds on each cell line at different states of MAPK signaling, the growth rates for cells treated with each dose of an epigenetic compound were compared with growth rates for cells treated without any epigenetic compound, following 72 h and 120 h of exposure to each of the MAPK inhibitor treatment conditions (i.e., DMSO, vemurafenib, and vemurafenib plus trametinib). Significant epigenetic compounds were defined as those that led to a statistically significant decrease in normalized growth rate (with an effect size of at least 0.5 day^{-1}) in at least one of the tested conditions (i.e., cell lines, timepoints, or MAPK inhibitor conditions). Statistical significance was evaluated as $P < 0.05$ based on two-sample t -test following correction for multiple comparisons using the Dunn–Sidak method. Additional details about the epigenetic compound screen are available in Supplementary Table 2.

Hierarchical clustering. To infer potential variations in the mechanisms of action of the selected group of 58 epigenetic compounds, treatment-induced changes in growth rate, p-Erk^{T202/Y204}, p-Rb^{S807/S811}, and Mitf across different cell lines and MAPK inhibitor conditions were integrated into a matrix for unsupervised clustering. Growth rates for epigenetic inhibitor conditions were averaged across three timepoints and two doses of the inhibitor and their differences relative to the time-averaged growth rate in the absence of any epigenetic compound were used for each MAPK inhibitor treatment condition (i.e., DMSO, vemurafenib, and vemurafenib plus trametinib). For p-Erk^{T202/Y204}, p-Rb^{S807/S811}, and Mitf, log-transformed signal intensities (from quantitative immunofluorescence microscopy) were averaged across three timepoints and two doses of the inhibitor and their differences relative to the corresponding MAPK inhibitor condition in the absence of any epigenetic compound were used. Unsupervised hierarchical clustering of the integrated data was then carried out using MATLAB 2018b with the Correlation distance metric and the Complete (farthest distance) algorithm for computing the distance between clusters.

Partial least square regression (PLSR) analysis. We used PLSR analysis^{47,40,56} to generate models that linked epigenetic treatment-induced changes in normalized growth rates to baseline and treatment-induced changes in signaling and phenotypic data. By combining data for each epigenetic inhibitor across eight cell lines, we generated one model to predict responses to each epigenetic inhibitor, when used either individually or in combination with Braf/Mek inhibitors. Response variables for each model were defined as normalized growth rates (averaged across 3–5 days of treatment) in the absence or presence of that compound (together with either DMSO, vemurafenib alone, or vemurafenib in combination with trametinib) across the eight cell lines. Input vectors were constructed by combining measurements of a total of eight proteins (including signaling proteins, differentiation state and phenotypic markers) at the baseline (i.e., either DMSO, vemurafenib alone, or vemurafenib in combination with trametinib), following epigenetic inhibitor treatment (together with either DMSO, vemurafenib alone, or vemurafenib in combination with trametinib), and their changes induced by the epigenetic inhibitor which were computed by taking the ratio of the two sets of measurements. All protein measurements were performed by quantitative immunofluorescence microscopy, log-transformed, and averaged across three timepoints (including 24 h after epigenetic inhibitor or DMSO treatment, followed by 72 and 120 h of MAPK inhibitor or DMSO treatment). The data were then z-scored across all conditions and cell lines prior to the application of PLSR analysis using the built-in MATLAB function `plsregress`.

To evaluate the predictability of the linear relationship between the input and output variables in each model, we used leave-one-out cross-validation. The goodness of fit for each model was calculated using R^2 . Prediction accuracy was evaluated by Q^2 and the P values generated from pairwise Pearson's correlations between the measured and predicted responses following cross-validation. For the assessment of relative variable importance in each PLSR model, the information content of each variable was assessed by its variable importance in the projection (VIP)⁴¹.

Multi-linear regression (MLR) analysis. To statistically test the hypothesis that the relative levels of Kdm4b, Znf217, and differentiation state markers Ngfr and Axl can predict the selective sensitivity of melanoma cells to JIB-04 and SP2509, we

performed MLR analysis using the entire panel of 21 cell lines, which were divided into a test set of 14 (to train models) and a validation set of 7 cell lines (to evaluate the model performance). To group the cell lines into training and validation sets, we first sorted the 21 cell lines based on their sensitivity to each compound. Beginning from the most sensitive or resistant cell line, we picked 2 cell lines for the training set and 1 cell line for the validation set, then another 2 cell lines for the training set and 1 cell line for the validation set, etc. We then used the training set to develop MLR models of treatment efficacy for SP2509 and JIB-04 using protein levels of Ngfr, Axl, Kdm4b, and Znf217 measured by quantitative immunofluorescence microscopy. Response variables were defined as normalized growth rates (averaged across 3–5 days of treatment) in the presence of either SP2509 (at 1 μM) or the combination of JIB-04 (at 0.2 μM), vemurafenib (at 100 nM), and trametinib (at 10 nM). Input vectors were constructed by combining baseline (drug-naïve) measurements of the fractions of Ngfr^{High}, Axl^{High}, Kdm4b^{High}, and Znf217^{High} cells in each cell line. We then used the built-in MATLAB function `regress` to return a vector of coefficient estimates for an MLR of the responses to each treatment condition. These coefficients were then used to predict the responses of the validation cell lines to each treatment using an input vector generated for those cell lines. To evaluate the predictability of the models, we evaluated the relationship between the measured and predicted responses for the validation set of cell lines using Pearson's correlation analysis. The goodness of fit for each model was calculated using R^2 of measured versus fitted responses for models generated using all 21 cell lines.

Gene knockdown by siRNA. In the first round of siRNA-mediated knockdown experiments, WM115, WM902B, and A2058 cells were seeded in 200 μl of antibiotic-free growth media in 96-well clear bottom black plates at seeding densities of 3500, 4000, and 1700 cells per well, respectively. After 24 h, cells were transfected using the DharmaFECT 1 transfection reagent (GE Dharmacon T-2001-01) with Dharmacon's ON-TARGETplus Human SMARTpool siRNAs, including four target-specific siRNAs combined into a single pool to increase the likelihood of effective gene silencing. The SMARTpool siRNAs included *KDM4A* (L-004292-00-0005), *KDM4B* (L-004290-00-0005), *KDM5A* (L-003297-02-0005), *KDM5B* (L-009899-00-0005), or non-targeting control (D-001810-10-05) and were used at 25 nM. To rule out the possibility of off-targeting by *KDM4B* siRNAs, we then examined separately the effects of three constituent ON-TARGETplus *KDM4B* siRNAs (J-004290-08-0005, J-004290-09-0005, J-004290-11-0005) at 25 nM. To evaluate the effect of Braf/Mek inhibition in cells in which either of the proteins were knocked down, WM115 and WM902B cells were further treated with vemurafenib (at 100 nM) plus trametinib (at 10 nM) or DMSO 48 h following siRNA addition. After another 48 h, cells were fixed and analyzed using quantitative immunofluorescence microscopy.

Gene knockout by CRISPR-Cas9. A2058 cells were seeded in a 24-well plate at a seeding density of 30,000 cells per well for 24 h. The next day, cells were treated at MOI of 0.3 with the pre-designed Edit-R lentiviral Cas9 nuclease (Dharmacon, Cat# VCAS10124). Polybrene (at 8 $\mu\text{g}/\text{ml}$) was added to enhance the efficiency of the viral infection. Following an incubation period of 72 h with the lentivirus, Cas9-lentiviral-treated cells were selected with blasticidin over a period of 1.5 weeks. Surviving A2058 Cas9-positive cells were subsequently infected with either non-targeting control (Dharmacon, Cat# VSGC10216) or three independent *KDM1A* lentiviral sgRNAs (Dharmacon, Cat# VSGH10142-246522030, VSGH10142-246999191, and VSGH10142-246522028). The efficiency of *KDM1A* knockout and the consequential changes in cell viability were measured via immunostaining for the Kdm1a protein at 96 h, followed by quantitative immunofluorescence microscopy.

siRNA and sgRNA sequences. All siRNA and sgRNA sequences can be found in Supplementary Table 3.

In vivo xenograft assays. All mouse experiments were carried out in accordance with procedures approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan. Athymic, 5–6 weeks old female nude (NU/J) mice were purchased from The Jackson Laboratory. Mice were housed under standard animal housing protocol at the University of Michigan Unit for Laboratory Animal Care (ULAM) in special containment rooms in individual non-ventilated cages at an ambient temperature of 74°F with a humidity of 30–70% and the light-dark cycle of 12 h each. For xenograft tumor injections, mice were first anesthetized using 3% vaporized isoflurane. 2.5×10^6 cells from either of the melanoma cell lines A375 or WM2664 suspended in 200 μl of growth factor-reduced Matrigel (Thermo Fisher, CB-40230C) in PBS (1:1) were injected subcutaneously in the right flank of each mouse. Tumor xenografts were monitored three times a week with digital calipers. Once the tumors were palpable and the mean volume across all tumors reached a volume of $\sim 170 \text{ mm}^3$, the mice were randomly allocated to two treatment groups of 5 mice per group. SP2577 (at a daily dose of 80 mg kg^{-1}) or vehicle (20% DMSO, 20% Cremophor EL, plus 60% sterile water) was then administered to each mouse via I.P. injection of a solution of 200 μl . Mouse body weight, tumor volume, and general health were monitored three times a week for 12 days. At the endpoint of the study, mice were euthanized by CO_2 asphyxiation and bilateral pneumothorax.

Statistics and reproducibility. No statistical method was used to predetermine sample sizes. Sample sizes were chosen based on similar studies in the relevant literature. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All data with error bars were presented as mean values $\pm$ s.d. or mean values $\pm$ s.e.m. as indicated in figure legends using indicated numbers of biologically independent replicates. The significance of pairwise correlations among drug response data were evaluated based on P values associated with the corresponding two-sided Pearson's correlation analysis (r = correlation coefficient). The statistical significance of the effects of epigenetic compounds (in the first stage of the screen) was evaluated based on $P < 0.05$ generated from two-sided t -test following correction for multiple comparisons using the Dunn–Sidak method. To identify the statistical significance of differences between mean or median of measurements within two different groups, P values from two-sided t test or two-sided Mann–Whitney U -test were used, respectively. Statistical significance of the difference in % change in tumor size in mouse experiments was determined by two-way analysis of variance (ANOVA). Statistical analyses were performed using MATLAB 2018b and 2019b.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated in this study are included in this published article and its supplementary information files. Because of the large number and size of raw immunofluorescence microscopy images associated with this study, all relevant image files will be made available by hard drive upon reasonable request to the corresponding author. The Cancer Cell Line Encyclopedia (CCLE) mass spectrometry-based proteomics data analyzed in this study were downloaded from the depmap project portal (<https://depmap.org/portal/download/>). Source data are provided with this paper.

Code availability

All statistical analyses and programming were performed in MATLAB 2018b and 2019b using built-in functions and parameters as described in detail in “Methods”. Custom MATLAB scripts for the analysis of drug response, drug combination interactions based on the Bliss Independence model, t-SNE, PLSR, and MLR analysis are available on GitHub at the following address: <https://github.com/fallahi-sichani-lab/epigeneticModulationAnalysis>.

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Author contributions

M.K. designed and performed the experiments, analyzed the experimental data, performed analyses, and wrote the manuscript. M.M. designed and performed the experiments. E.D.M. synthesized reagents for the experiments. M.F.-S. designed the experiments, performed analyses, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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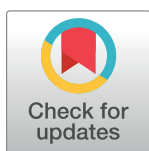
RESEARCH ARTICLE

Phenotype-based probabilistic analysis of heterogeneous responses to cancer drugs and their combination efficacy

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Abstract

Cell-to-cell variability generates subpopulations of drug-tolerant cells that diminish the efficacy of cancer drugs. Efficacious combination therapies are thus needed to block drug-tolerant cells via minimizing the impact of heterogeneity. Probabilistic models such as Bliss independence have been developed to evaluate drug interactions and their combination efficacy based on probabilities of specific actions mediated by drugs individually and in combination. In practice, however, these models are often applied to conventional dose-response curves in which a normalized parameter with a value between zero and one, generally referred to as fraction of cells affected (f_a), is used to evaluate the efficacy of drugs and their combined interactions. We use basic probability theory, computer simulations, time-lapse live cell microscopy, and single-cell analysis to show that f_a metrics may bias our assessment of drug efficacy and combination effectiveness. This bias may be corrected when dynamic probabilities of drug-induced phenotypic events, i.e. induction of cell death and inhibition of division, at a single-cell level are used as metrics to assess drug efficacy. Probabilistic phenotype metrics offer the following three benefits. First, in contrast to the commonly used f_a metrics, they directly represent probabilities of drug action in a cell population. Therefore, they deconvolve differential degrees of drug effect on tumor cell killing versus inhibition of cell division, which may not be correlated for many drugs. Second, they increase the sensitivity of short-term drug response assays to cell-to-cell heterogeneities and the presence of drug-tolerant subpopulations. Third, their probabilistic nature allows them to be used directly in unbiased evaluation of synergistic efficacy in drug combinations using probabilistic models such as Bliss independence. Altogether, we envision that probabilistic analysis of single-cell phenotypes complements currently available assays via improving our understanding of heterogeneity in drug response, thereby facilitating the discovery of more efficacious combination therapies to block drug-tolerant cells.

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Author summary

Resistance to therapy due to tumor cell heterogeneity poses a major challenge to the use of cancer drugs. Cell-to-cell variability generates subpopulations of drug-tolerant cells that diminish therapeutic efficacy, even in populations of cells that are scored as highly sensitive based on drug potency. Overcoming such heterogeneity and blocking subpopulations of drug-tolerant cells motivate efforts toward identifying efficacious combination therapies. The success of these efforts depends on our ability to distinguish how heterogeneous populations of cells respond to individual drugs, and how these responses are influenced by combined drug interactions. In this paper, we propose mathematical and experimental frameworks to evaluate time-dependent drug interactions based on probabilistic metrics that quantify drug-induced tumor cell killing or inhibition of division at a single-cell level. These metrics can reveal heterogeneous drug responses and their changes with time and drug combinations. Thus, they have important implications for designing efficacious combination therapies, especially those designed to block or overcome drug-tolerant subpopulations of cancer cells.

Introduction

In pre-clinical studies, potentially effective drug combinations are usually identified based on evidence of synergy [1–4]. In the case of cancer drugs, synergistic interactions are typically assessed on the basis of bulk cell population measurements, such as relative viability (normalized cell count) and net growth rate inhibition, and their variations with drug dose and combination [5–9]. The benefit of drug combination is then evaluated based on whether using two drugs together improves the potency (via minimizing the dose) or efficacy of treatment (via enhancing the effect) as compared with using either of the drugs alone [10–16]. Such benefit with respect to efficacy and potency, however, may be decoupled [10], as each metric encodes distinct information about cellular response to a drug [17]. Variations in potency are often explained by differences in target engagement (e.g. physicochemistry of drug-target interaction), concentration of drug available to cells (e.g. drug uptake and efflux), or existence of pathway redundancy (e.g. presence of a secondary oncogenic driver), among others [18]. Thus, a more potent drug combination enables engaging the target and achieving the desired effect in a cell population by using lower doses of treatment [19,20]. Efficacy, on the other hand, refers to the maximum response achievable using tolerable doses of a drug. A more efficacious drug or drug combination engages a larger proportion of cells [21,22]. Previous systematic studies have revealed that variation in cancer drug efficacy is associated with the extent of cell-to-cell variability in drug response [17,23], although such heterogeneity is not directly scored in most pre-clinical drug response assays.

Cell-to-cell variability may generate subpopulations of drug-tolerant cells that diminish cancer drug efficacy [24–30]. Heterogeneity is observed following the emergence of adaptive resistance or selection of resistant subclones even in populations of cells that are scored as highly responsive based on drug potency (e.g. EC_{50} measurements) in routine 3 to 5-day assays. In such cases, while more than half (often as many as 90–99%) of cells may respond to treatment (depending on time and dose), the remaining cells give rise to a drug-insensitive subpopulation of survivors that may stay quiescent or divide slowly in the presence of drug [31]. Although not obvious from the most commonly used potency measurements, the emergence of such survivors limits therapeutic efficacy, leading to residual cells from which drug-resistant clones may eventually arise and drive disease progression [32–35].

Overcoming such heterogeneity in drug response and eradicating subpopulations of drug-tolerant cells provide a strong motivation for identifying more efficacious combination therapies [36]. A key step toward this goal is the ability to distinguish how heterogeneous populations of cells respond to individual drugs in short-term assays, and how these responses are influenced by combined drug interactions. However, the standard way in which drugs or their combinations are screened using normalized population assays obscures single-cell and subpopulation effects that likely play a major role in diminishing the therapeutic efficacy [21,37].

Focusing on efficacy, the benefit of drug combination in a heterogeneous population of cells may arise either from its cooperative inhibitory effect on target cells [22], or simply from the increased probability of cells being sensitive to any of the constituent drugs [38]. In both cases, the overall phenotypic consequences of drug interactions may be assessed in cell culture experiments based on null models of non-interaction [3]; synergistic efficacy is typically concluded when the observed combinatorial effect exceeds the expected effect from a given null model. The most commonly used model, Bliss independence, evaluates interactions based on the probability theory for statistically independent drug actions [16]. In cancer treatment, two basic phenotypic events affected by drug action are cell death and division. The effect of a drug on an individual cell changes the probability of its survival or division within a given time interval. However, current application of the Bliss independence typically uses fraction of cells affected (f_a), a number between zero and one defined based on relative viability or net growth rate inhibition normalized to an untreated control at a fixed timepoint, as drug effect [3,7]. We argue that this commonly used approach leads to a bias in the estimation of both drug efficacy and combination effectiveness in heterogeneous cell populations, especially when the ultimate goal is to block or eradicate small subpopulations of drug-tolerant cells. This is because f_a quantities are not equal to the time-dependent probabilities at which cell death or inhibition of cell division are induced by a drug.

In this paper, we discuss evaluating time-dependent drug responses based on probabilistic metrics that quantify drug-induced tumor cell killing and inhibition of division at a single-cell level. Using these phenotype metrics, we re-evaluate criteria for statistical independence of drug interactions based on probability theory. Experimentally, phenotype metrics are measured using time-lapse live cell microscopy via monitoring cells engineered to express fluorescent reporters for nucleus identification (to distinguish live versus dead cells) and cell cycle progression (to score division events). As a proof of concept, we evaluate the performance of the metrics in two BRAF-mutant melanoma cell lines exposed to a range of targeted drugs which have been tested or proposed to be studied in combination with standard of care BRAF and MEK kinase inhibitors. Dynamic measurements of the phenotype metrics reveal distinctive responses of melanoma cells to drug combinations that may not be distinguishable when assessed based on conventional assays. This is because these metrics deconvolve differential degrees of drug effect on tumor cell killing versus inhibition of division, which are not necessarily correlated across various drug treatments and their combinations. Furthermore, these metrics increase the sensitivity of short-term drug response assays to cell-to-cell heterogeneities and thus the presence or emergence of drug-tolerant subpopulations, which are typically overlooked in conventional drug response assays.

Results

Probabilistic description of drug-induced phenotypic events

We model the arrival of phenotypic events, including cell division and death, in a given cell population as independent non-stationary *Poisson* processes with time-varying rate constants (k_{event}). These rate constants are linked to the actual probabilities (P_{event}) with which such

events occur in individual cells within a series of short time intervals (dt):

$$P_{event} = 1 - e^{-k_{event}dt} \approx k_{event}dt \quad (1)$$

At a population level, the occurrence of these phenotypic events can be described by *Poisson* processes of which the time-dependent rates of occurrence are directly related to the probabilities of events at a single-cell level (Fig 1A). Therefore, the distribution of death and division

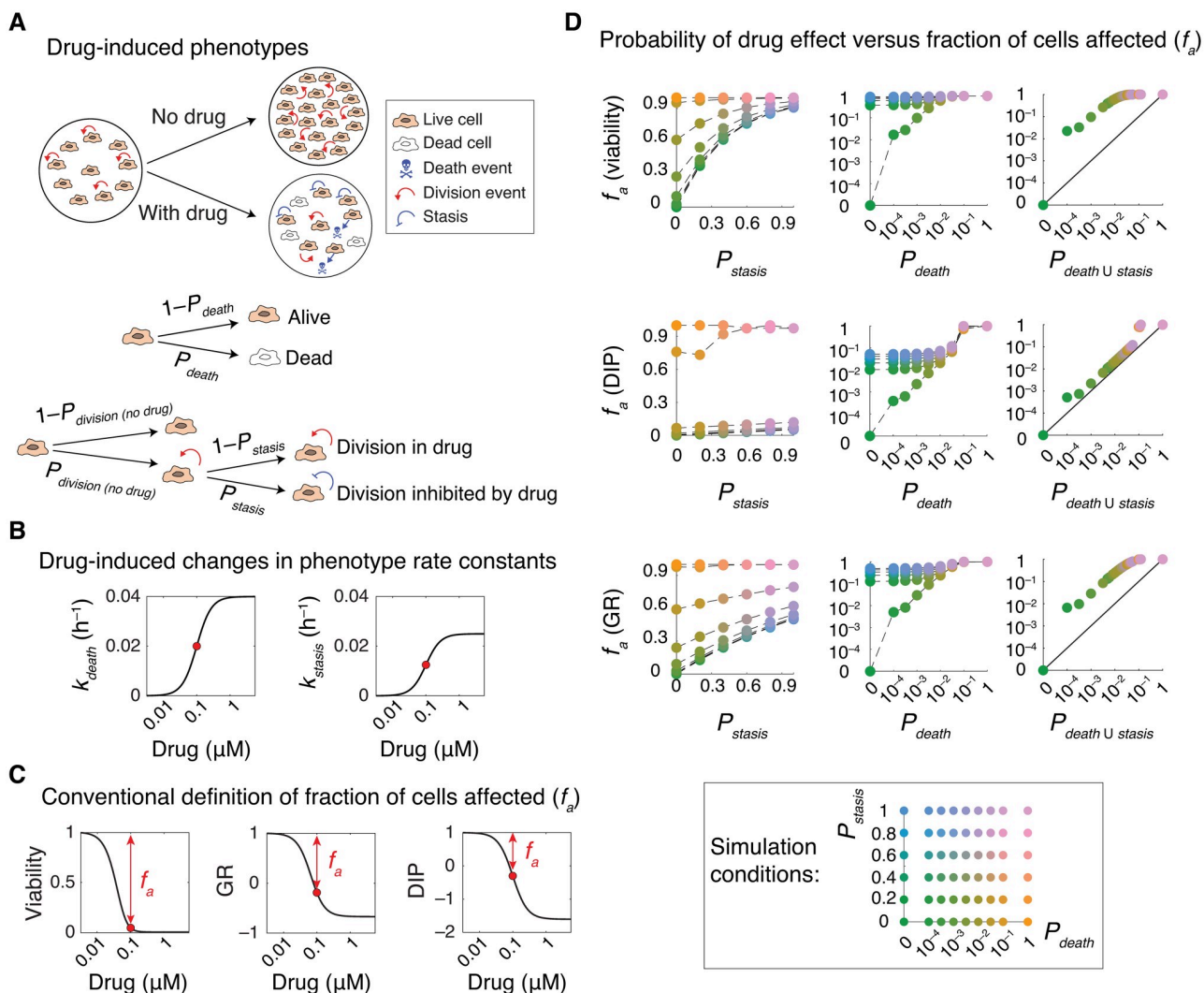


Fig 1. Probabilistic description of drug-induced phenotypic events. (A) Schematic representation of phenotypic effects of drug action in a cell population. Drug effect is described as probabilistic events, involving induction of cell death and inhibition of cell division, at a single-cell level. Cytotoxic effect of a drug on a given cell is described by the probability with which it induces cell death per unit of time (P_{death}). The cytostatic effect of drug on a given cell is described by a conditional probability (P_{stasis}) with which it prevents the cell from dividing given that the same cell would have divided in the absence of drug with a probability of $P_{division (no drug)}$. (B) Dose-dependent changes in phenotype rate constants (k_{death} and k_{stasis}) in simulation of drug effect in a population of cells. (C) Model outputs showing variations in the fraction of cells affected (f_a) at $t = 96$ h corresponding to phenotype rate constant values shown in (B). f_a may be calculated in three different ways based on bulk response metrics such as relative viability and net growth rate inhibition (GR and DIP) following normalization to an untreated control. (D) Simulation results comparing f_a quantities at $t = 96$ h with probabilistic measures of drug action, P_{death} quantified per unit of time (h), conditional probability P_{stasis} , and the overall probability with which a drug induces cell death or inhibits cell division ($P_{death \cup stasis}$) across a variety of conditions, representing drugs with different levels of cytotoxic and cytostatic effect. Each data-point represents the mean of 30 stochastic simulations. Cells grow from an initial number of $N_{live} (t = 0) = 1000$ and at a rate of $k_{division (no drug)} = 0.025 h^{-1}$.

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events observed for a population of N cells during a time period of Δt may be approximated using the following equation:

$$Pr\{N_{event}(t \rightarrow t + \Delta t) = x\} = \frac{(k_{event}(t)N\Delta t)^x}{x!} e^{-(k_{event}(t)N)\Delta t} \quad (2)$$

where $N_{event}(t \rightarrow t + \Delta t)$ is the number of phenotypic events (death or division) occurring during the time interval between t and $t + \Delta t$.

Assuming negligible cell death in the absence of any treatment, the model describes the cytotoxic effect of a drug on a given cell by the probability with which it induces cell death per unit of time ($P_{death} = k_{death}dt$). The cytostatic effect of drug on a given cell is defined by a conditional probability (P_{stasis}) with which it prevents the cell from dividing given that the same cell would have divided in the absence of drug with a probability of $P_{division}(no\ drug) = k_{division}(no\ drug)dt$. The relationships between the conditional probability P_{stasis} and the probability of cell division in the presence of drug ($P_{division}(with\ drug) = k_{division}(with\ drug)dt$) and their associated rate constants are as follows (see [Materials and methods](#) for the derivation details):

$$P_{stasis} = 1 - \frac{P_{division}(with\ drug)}{P_{division}(no\ drug)} = 1 - \frac{k_{division}(with\ drug)}{k_{division}(no\ drug)} \quad (3)$$

$$k_{stasis} = P_{stasis}k_{division}(no\ drug) \quad (4)$$

The model provides a framework to simulate how dose-dependent responses in populations of cells vary with P_{death} per unit of time (h) and P_{stasis} by using input parameters (k_{death} and k_{stasis}) that represent drugs with a wide range of cytotoxic and cytostatic effects. For each condition, the fraction of cells affected (f_a), defined based on changes in relative viability or net growth rate inhibition (using recently developed drug-induced proliferation (DIP) and growth rate (GR) inhibition metrics [5,6]) normalized to an untreated control, are also derived as model outputs ([Fig 1B and 1C](#)). We compared f_a quantities with probabilistic measures of drug action (P_{death} and P_{stasis}) across a number of drug response simulations. Except for extreme cases such as when $P_{death} = P_{stasis} = 0$ (i.e. there is no drug) or when $P_{death} = 1$ (i.e. all cells dying within the first time interval), f_a quantities differed substantially from the probability with which drugs induced cell death or from the probability with which they inhibited cell division ([Fig 1D](#)). f_a gives a closer estimate of the overall probability with which a drug induces either cell death or inhibition of cell division ($P_{death \cup stasis}$), i.e. the probability of a cell being affected ([Fig 1D](#)). However, it still fails to accurately represent the probabilistic nature of drug action in cells. Together, simulation results suggest that using f_a as a metric for probabilistic analysis of drug response or drug combination efficacy (such as in Bliss independence) might lead to unreliable conclusions. Instead, we propose to use direct measures of probabilistic phenotype metrics (P_{death} and P_{stasis} or k_{death} and k_{stasis}) for such analyses.

Probabilistic rate constants capture time-dependent heterogeneities in phenotypic responses

Probabilistic rate constants are estimated based on the frequencies of occurrence of individual phenotypic events. These metrics are expected to exhibit high sensitivity to the presence of cell-to-cell heterogeneities that cause the selection of small subpopulations of drug-tolerant cells. To test this hypothesis, we simulated drug treatment scenarios where the initial cell population consisted of heterogeneous subpopulations, in which a small fraction ($\omega \leq 5\%$) of cells were substantially less sensitive (by up to $r = 16$ -fold) to treatment relative to the majority of the cell population ([Fig 2A](#)). We then defined and calculated “resistance enrichment ratio” for

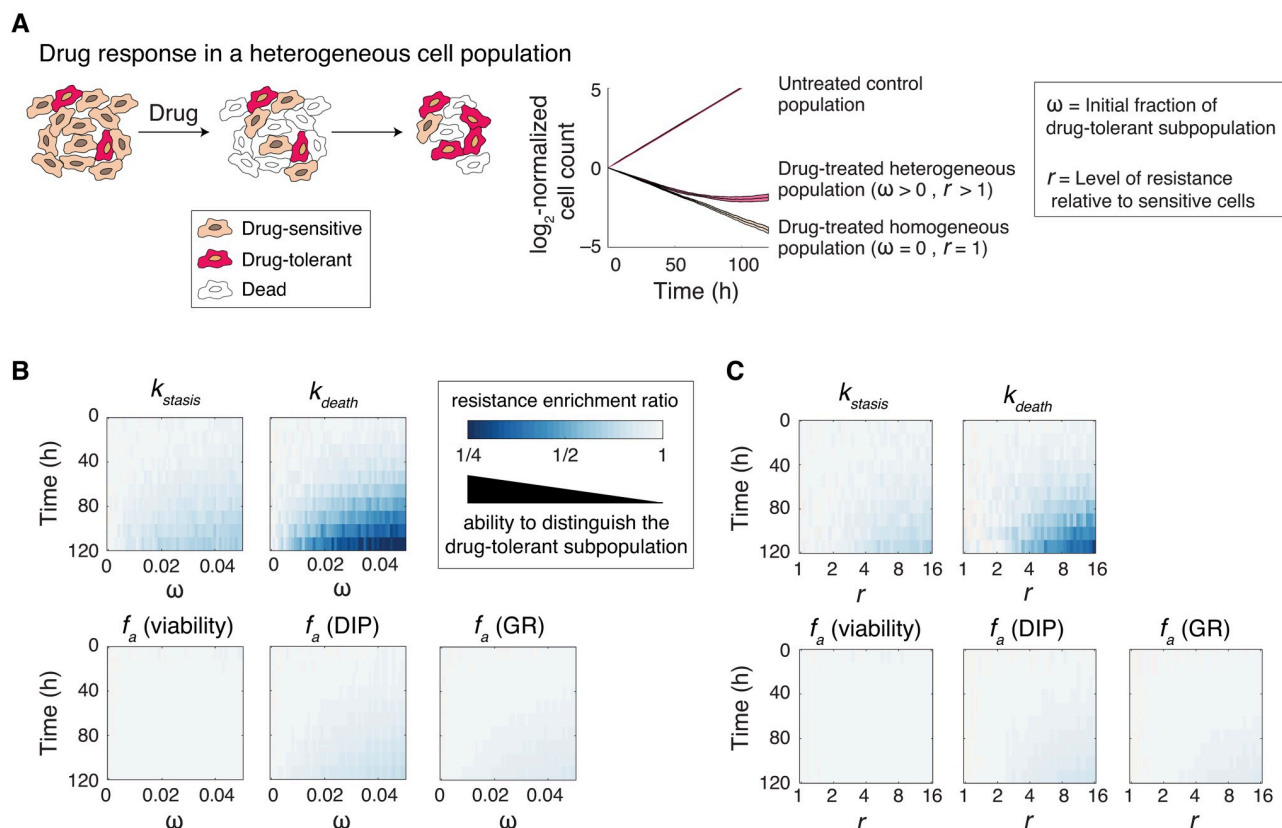


Fig 2. Probabilistic rate constants capture time-dependent heterogeneities in phenotypic responses. (A) Schematic representation of drug response in a heterogeneous cell population. Prior to drug-treatment, cells consist of a dominantly drug-sensitive population plus a small fraction ($\omega \leq 5\%$) of drug-tolerant subpopulation which is r times more drug-resistant than the majority of cells. Upon drug treatment, the drug-tolerant subpopulation is gradually enriched over time. Resistance enrichment ratio for each of the f_a metrics (described based on viability, GR and DIP) or for phenotype rate constants (k_{death} and k_{stasis}) is calculated by normalizing each metric measured for a heterogeneous population to that in a homogeneous population (i.e. $\omega = 0$ or $r = 1$) at different times of treatment. Smaller resistance enrichment ratios represent greater sensitivity to the presence of heterogeneous drug-tolerant cells. (B) Simulation results showing changes in resistant enrichment ratio for each of the f_a metrics or for phenotype rate constants (k_{death} and k_{stasis}) as a function of time and ω (at a fixed value of $r = 16$). (C) Simulation results showing changes in resistant enrichment ratio for each of the f_a metrics or for phenotype rate constants (k_{death} and k_{stasis}) as a function of time and r (at a fixed value of $\omega = 0.03$). Data shown are mean values from 50 simulations. In all simulations, we assumed fixed inherent growth rates for the sensitive and resistant populations: $k_{division}^S$ (no drug) = 0.035 h^{-1} and $k_{division}^R$ (no drug) = 0.02 h^{-1} . All key parameters are described in Materials and Methods.

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each of the f_a metrics (described based on viability, GR and DIP) or for phenotype rate constants (k_{death} and k_{stasis}) by normalizing each metric measured for the heterogeneous population to that in a homogeneous population (i.e. $\omega = 0$ or $r = 1$) at different times of treatment. Smaller resistance enrichment ratios represent greater sensitivity to the presence of heterogeneous drug-tolerant cells.

We first compared the ability of each metric to capture the presence of small subpopulations of drug-tolerant cells by analyzing how resistance enrichment ratio varies with ω and time (Fig 2B and Supporting Information S1A and S1B Fig). Simulation results show that f_a metrics, defined based on either normalized cell viability or growth rate inhibition (GR and DIP), are significantly less sensitive than k_{death} and k_{stasis} to the presence of drug-tolerant cells. Furthermore, for any given initial fraction of drug-tolerant cells (ω), phenotype rate constants captured the emergence of drug resistance at earlier timepoints. Using similar simulations, we also tested how the relative level of drug resistance (r) in a fixed initial fraction of drug-tolerant cells would influence each of the drug response metrics. Simulation results show that for a

given ω , phenotype rate constants detect subpopulations with weaker levels of resistance (i.e. smaller r values) and at earlier timepoints (Fig 2C and Supporting Information S1C and S1D Fig).

Most conventional drug screening assays are performed following exposure of cells to drug for 3 to 5 days. While variations in drug potency are distinguishable in such assays, it is often suggested that longer periods of treatment are essential to detect the phenotypic consequences of drug-tolerant persisters that diminish the efficacy. However, our results show that phenotype rate constants can capture heterogeneities that would otherwise require significantly longer experiments when using population-level f_a metrics that mask such heterogeneities. The benefit of using phenotype rate constants would be especially significant in the case of potent drugs that induce substantial cell death, while sparing a small fraction of drug-tolerant cells (Supporting Information S2A and S2B Fig). In particular, as the efficiency of drug-induced cell killing increases, the sensitivity of f_a metrics to detect drug tolerance in the surviving fraction of cells decreases (Supporting Information S2C Fig).

Estimating probabilistic rate constants using time-lapse live cell microscopy

To experimentally capture stochastic processes of induction of cell death and inhibition of division in drug-treated tumor cell populations, we used time-lapse live cell microscopy and cells engineered to express two fluorescent reporters. The reporters included: (i) an H2B-Venus reporter which labels chromatin, allowing identification of nuclei and scoring cell death based on changes in nucleus morphology, and (ii) an mCherry-Geminin reporter for cell cycle progression [39] which allows tracking of cell division events. Using a high-throughput, automated image analysis workflow (see Materials and methods), the occurrence of individual phenotypic events (death and division) in single cells was tracked in time across a variety of drug treatment conditions (Supporting Information S3 Fig). To estimate time-dependent changes in probabilistic phenotype rate constants, the number of cell death and division events (N_{event}) were quantified over a series of uniform time intervals of length Δt . Phenotype rate constants were then estimated via normalizing N_{event} in each time interval to the length (Δt) and the average number of live cells over that time interval $[N_{live}(t \rightarrow t + \Delta t)]_{avg}$ as detailed below:

$$k_{death}(t) = \frac{N_{death}(t \rightarrow t + \Delta t)}{[N_{live}(t \rightarrow t + \Delta t)]_{avg} \Delta t} \quad (5)$$

$$k_{division}(t) = \frac{N_{division}(t \rightarrow t + \Delta t)}{[N_{live}(t \rightarrow t + \Delta t)]_{avg} \Delta t} \quad (6)$$

$$k_{stasis}(t) = k_{division(no\ drug)} - k_{division(with\ drug)}(t) \quad (7)$$

As a proof of concept, we monitored responses of two BRAF-mutated melanoma cell lines (COLO858 and MMACSF) following exposure to a BRAF inhibitor Vemurafenib at 6 doses for a period of ~120 h. Heterogeneity in drug response was then visualized through the estimation and analysis of phenotype rate constants, k_{death} and k_{stasis} , as a function of drug dose and time in each cell line (Fig 3A and 3B). In COLO858 cells, which have been shown to be initially sensitive but rapidly develop adaptive resistance to Vemurafenib [21,22], increasing drug concentration enhanced both the amplitude and the rate of increase in k_{death} and k_{stasis} within the first 36 h. After that, these responses were attenuated concurrent with the activation of drug-induced adaptive responses (Fig 3A). Responses of MMACSF cells involved a relatively

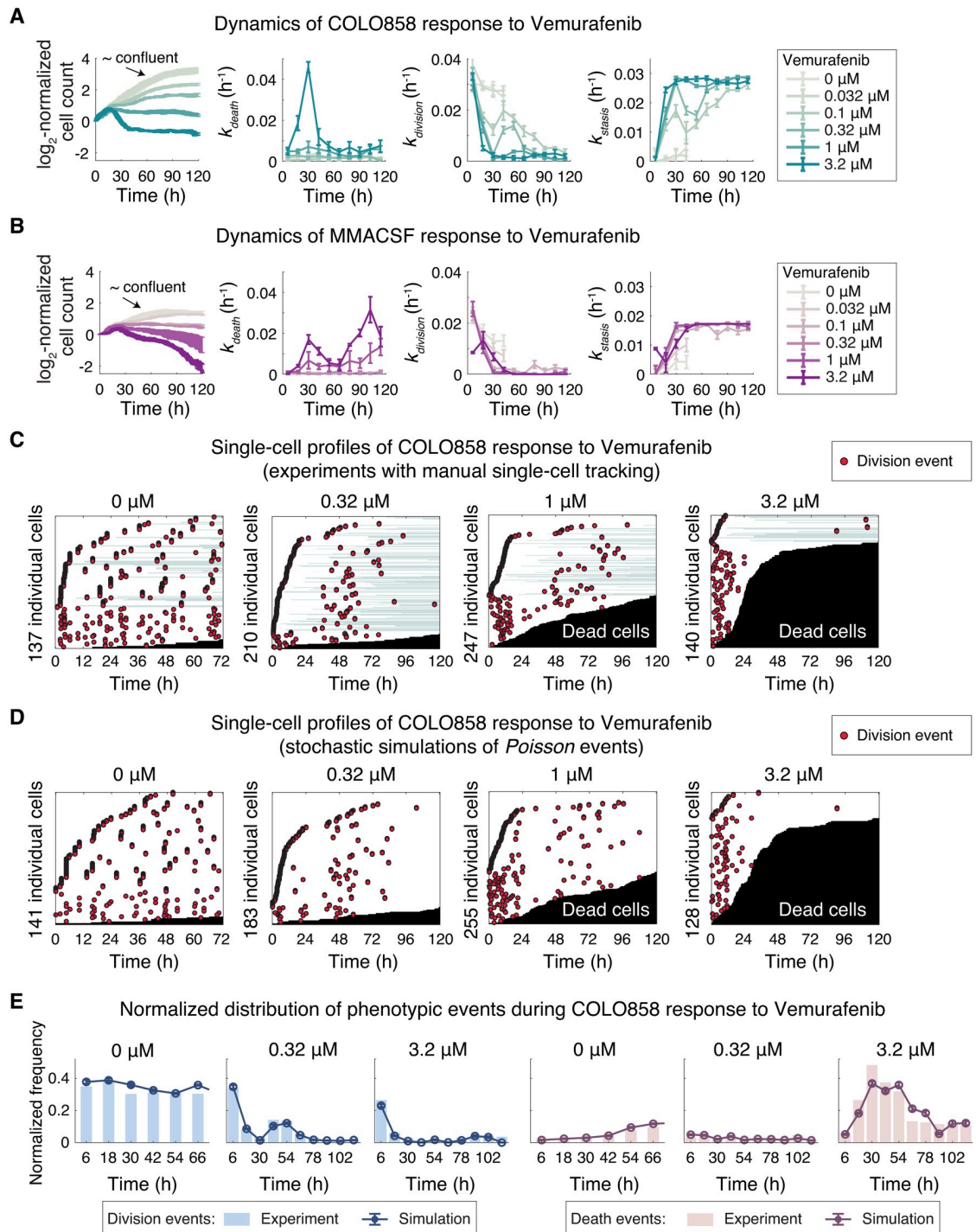


Fig 3. Dynamic analysis of heterogeneous drug response using estimates of probabilistic phenotype rate constants from time-lapse live cell microscopy. (A–B) Dynamics of (A) COLO858 and (B) MMACSF cell responses to BRAF inhibitor Vemurafenib across 6 doses (0–3.2 μM). Time- and dose-dependent changes in live cell count and estimates of k_{death} , k_{division} and k_{stasis} for time intervals of $\Delta t = 12$ h are shown. Experimental data for Vemurafenib concentrations of 0 and 0.032 μM are shown until 48 h, that is when cells reached confluency under these conditions. Data are shown as mean $\pm$ SEM across four replicates. (C) Single-cell profiles of COLO858 response to Vemurafenib depicted based on manual tracking of individual cells exposed to different concentrations of Vemurafenib as described in (A). Each cell track is presented horizontally along time axis. Division events are marked as red circles. Transition from white to black represents a cell death event. Times at which cells spend out of field of view are shown in light green. (D) Single-cell profiles of COLO858 response to Vemurafenib simulated based on *Poisson* processes using rate parameters estimated

from COLO858 experimental data along 12 h time intervals. (E) Comparison of normalized distributions of division and death events along 12 h time intervals between experiments performed in COLO858 cells and the simulated responses for the same conditions. Experimental data-points represent pooled data from all four replicates. Simulated data-points represent mean $\pm$ SEM across 30 simulations.

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monotonic and dose-dependent decrease in the number of live cells. At the highest drug concentration (3.2 μ M), however, we observed two peaks of apoptotic response, one similar to COLO858 cells at $t \approx 36$ h and a higher peak later at $t \approx 108$ h (Fig 3B). These data are consistent with previous data reporting high sensitivity of MMACSF cells to 5 days of exposure to Vemurafenib [21,22], but also highlight the impact of cell-to-cell heterogeneity and the presence of subpopulations of cells with different levels of drug tolerance.

In addition to interrogating dynamic aspects of heterogeneous drug response, we also tested the performance of our automated image analysis workflow by comparing the estimated phenotype rate constants with those measured from data generated by manual single-cell tracking using a MATLAB-based software [40]. The software allowed accurate tracking and cell fate annotation of individual cells across time-lapse images taken over a period of multiple days. Single-cell profiles from manual tracking confirmed heterogeneity in the number and timing of death and division events in cells exposed to drug. In COLO858, for example, cell-to-cell variability ranged from cells that died rapidly (as early as ~ 24 h) in response to high concentrations of Vemurafenib, to cells that survived but did not divide, to cells that slowly divided following a temporary delay in their cell cycle, the proportion and dynamics of which changed with drug dose (Fig 3C). By comparing rate constants between two image analysis methods across a variety of conditions in two cell lines, we identified quantitatively similar patterns (Supporting Information S4 Fig). This consistency confirms that the automated workflow would be suitable for high-throughput analysis of drug response.

We also used single-cell phenotype data to empirically evaluate the assumption of non-stationary *Poisson* process to model drug-induced death and division events. We compared the distribution of phenotypic events measured from time-lapse microscopy experiments with those simulated based on *Poisson* processes using estimates of phenotype rate constants. We observed similarity across patterns of response at the single-cell level and between distributions of events at the population level (Fig 3C–3E), suggesting that a simplified model of non-stationary *Poisson* process for drug-induced death and division events is a reasonable one.

Taken together, high-throughput estimation and analysis of phenotype rate constants and their changes with time and dose provide an efficient tool to capture critical dynamic aspects of probabilistic and heterogeneous drug response that would be overlooked in bulk population assays.

Evaluating statistical independence of drug combination efficacies using probabilistic phenotype metrics

Among the most widely used reference models in evaluation of synergistic efficacy for cancer drug combinations is Bliss independence [16]. The Bliss model assumes that drug effects are consequences of probabilistic processes, and that two drugs act independently if their combined effect confers probabilistic or statistical independence:

$$P_{A+B}^I = P_A + P_B - P_A P_B \quad (8)$$

where P_{A+B}^I describes the expected probability of the combinatorial effect of drugs A and B when they act independently. $0 \leq P_A \leq 1$ and $0 \leq P_B \leq 1$ represent probabilities of effect mediated by drugs A and B when tested individually. The Bliss combination index (*CI*) for drugs A

and B is defined as:

$$CI_{A+B}^{Bliss} = \frac{P_{A+B}^I}{P_{A+B}} \quad (9)$$

where P_{A+B} describes the actual probability of effect induced by drugs A and B when used in combination. Synergistic combination efficacy is concluded if $CI < 1$, i.e. when the observed combinatorial effect exceeds the expected effect from the Bliss independence model. Despite its probabilistic definition, however, the Bliss model is broadly applied to a variety of f_a metrics (such as normalized viability or net growth rate inhibition), thereby leading to unreliable conclusions which are largely due to the following limitations. First, although f_a measurements satisfy the mathematical requirement of $0 \leq f_a \leq 1$, they do not have a probabilistic nature and thus do not necessarily follow the rules of probability theory. Second, f_a quantities are the result of two distinct probabilistic processes, induction of cell death and inhibition of cell division. These processes, even when induced by drugs with the same probabilities, do not necessarily have the same impact on f_a . Third, for drugs A and B with $f_a < 1$, the Bliss model (when applied to f_a) is unable to account for the difference between being affected by drug A, drug B, or both. For example, consider the combined effect of two truly independent and purely cytostatic drugs A and B, whose phenotypic effects (individually) are described by $P_{stasis} = 1$ (and $P_{death} = 0$). By Bliss independence when applied to f_a metrics such as viability or normalized growth rate inhibition, the combination of drugs A and B is expected to exhibit substantial cytotoxic effect and thus their combination would be scored incorrectly as antagonistic ($CI > 1$) (Fig 4A and 4B). To overcome such limitations and to avoid erroneous conclusions about drug combination efficacies, we propose to use probabilities of phenotypic events or their associated rate constants in evaluation of Bliss independence according to its probabilistic definition.

When applied to probabilistic events of drug-induced cell death, Bliss independence for the combined cytotoxic effect of drugs A and B is described as follows:

$$P_{death(A+B)}^I = k_{death(A+B)}^I dt = P_{death(A)} + P_{death(B)} - P_{death(A)} P_{death(B)} \approx (k_{death(A)} + k_{death(B)}) dt \quad (10)$$

where $P_{death(A)}$ and $P_{death(B)}$ represent the probabilities with which drugs A and B induce cell death within a short time interval of dt , respectively. $P_{death(A+B)}^I$ represents the probability of death induced by the combination of drugs A and B when they act independently. $k_{death(A)}$, $k_{death(B)}$ and $k_{death(A+B)}^I$ represent rate constants associated with these probabilistic events, respectively. When applied to the conditional event of inhibition of cell division given that cells divide at a rate of $k_{division(no drug)}$ in the absence of drug, Bliss independence is described as follows:

$$P_{stasis(A+B)}^I = \frac{k_{stasis(A+B)}^I}{k_{division(no drug)}} = P_{stasis(A)} + P_{stasis(B)} - P_{stasis(A)} P_{stasis(B)} \quad (11)$$

where $P_{stasis(A)}$ and $P_{stasis(B)}$ represent the probabilities with which drugs A and B inhibit cell division given that cells would divide with a probability of $P_{division(no drug)} = k_{division(no drug)} dt$ within a short time interval of dt . $P_{stasis(A+B)}^I$ represents the cytostatic effect for the combination of drugs A and B when they act independently. $k_{stasis(A)}$, $k_{stasis(B)}$ and $k_{stasis(A+B)}^I$ represent rate constants associated with these probabilistic events. The Bliss combination index (for each

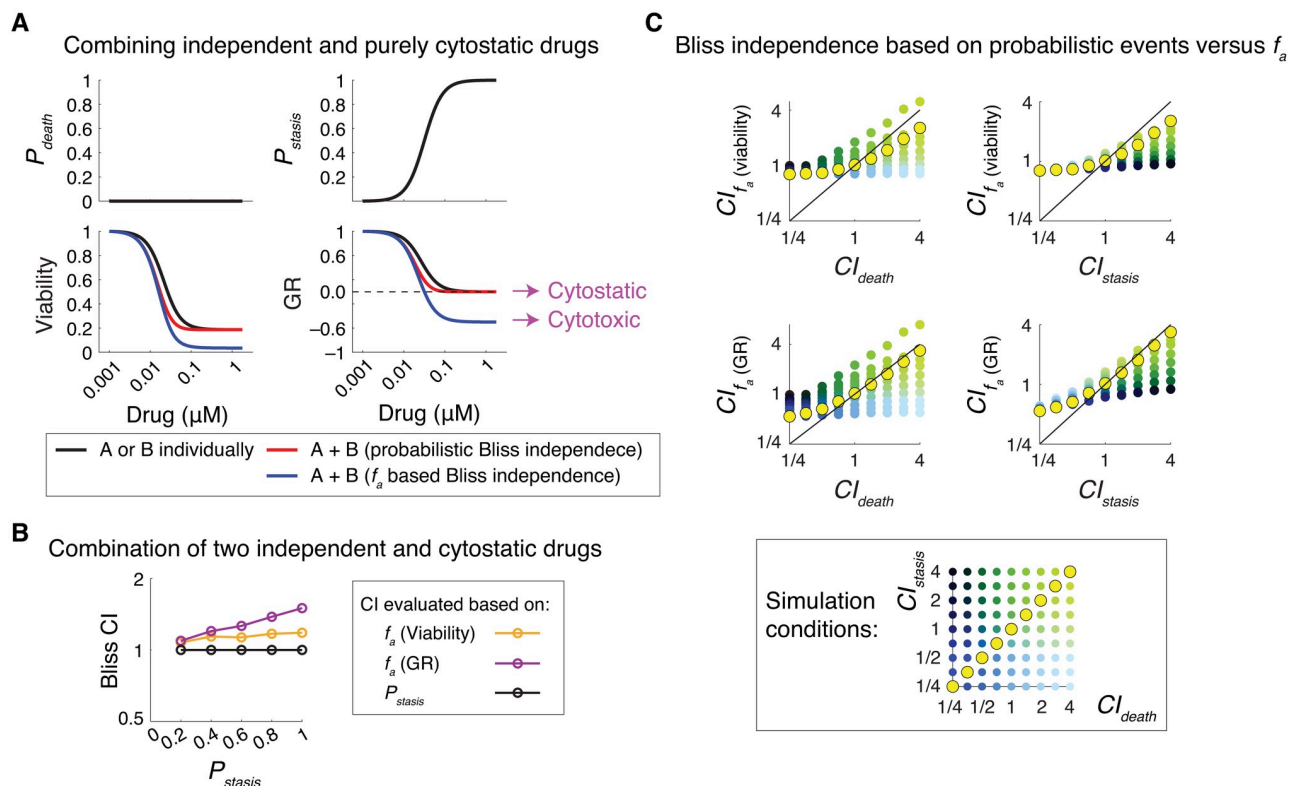


Fig 4. Probabilistic phenotype metrics, but not f_a based metrics, reveal statistical independence in drug combination efficacies. (A) Simulation results shown for the effect of two independent and purely cytostatic drugs, A and B, with identical dose-effect profiles used individually and in combination. Dose-effect profiles of drugs A and B are shown as P_{death} and P_{stasis} , quantified per unit of time (h) and per division event, respectively. Normalized changes in relative viability and growth rate (GR) inhibition are reported for each condition at 48 h and the predicted combination effects are shown for scenarios where either the probabilistic metric P_{death} and P_{stasis} or f_a quantities (based on viability and GR) were used in the evaluation of Bliss independence. (B) Bliss combination index values calculated (at $t = 48$ h) using different drug response metrics, f_a (viability), f_a (GR) and P_{stasis} , in simulations of combined effects of two independent and identical drugs with variable cytostatic effects represented by variations in P_{stasis} . The rate of cell division in the absence of drug was simulated as $k_{division} (no\ drug) = 0.035\ h^{-1}$. (C) Simulation results quantifying Bliss combination index values (at $t = 48$ h) calculated using f_a response metrics, f_a (viability) and f_a (GR), in comparison with probabilistic combination index values (CI_{death} and CI_{stasis}). Each data-point represents the mean of 10 simulations for a drug combination condition with a given set of probabilistic drug interaction condition quantified as CI_{death} and CI_{stasis} . Conditions where $CI_{death} = CI_{stasis}$ are highlighted in yellow. Representative simulations were performed using an initial live cell number of $N_{live} (t = 0) = 2000$, $k_{division} (no\ drug) = 0.035\ h^{-1}$, $k_{death} (drug\ A) = k_{death} (drug\ B) = 0.01\ h^{-1}$, $P_{stasis} (drug\ A) = P_{stasis} (drug\ B) = 0.2$.

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of the drug-induced phenotypic effects) is thus defined as follows:

$$CI_{death(A+B)}^{Bliss} = \frac{P_{death(A+B)}^I}{P_{death(A+B)}} = \frac{k_{death(A+B)}^I}{k_{death(A+B)}} \quad (12)$$

$$CI_{stasis(A+B)}^{Bliss} = \frac{P_{stasis(A+B)}^I}{P_{stasis(A+B)}} = \frac{k_{stasis(A+B)}^I}{k_{stasis(A+B)}} \quad (13)$$

Systematic simulation results show that evaluating probabilistic independence based on drug-induced phenotypic events can distinguish a variety of possible drug interactions that would be otherwise overlooked when assessed on the basis of f_a quantities (Fig 4C). The discrepancy is particularly substantial under conditions where drug combinations have uneven cytotoxic and cytostatic interactions, e.g. when two compounds act synergistically with respect to inhibition of division but act independently or antagonistically with respect to induction of cell death, and vice versa.

Probabilistic phenotype metrics uncover target-specific differences in drug combination efficacies

We applied the probabilistic definition of Bliss independence to evaluate time-dependent changes in the efficacies of a group of 12 compounds in sequential combination with BRAF kinase inhibitor, Vemurafenib, plus MEK kinase inhibitor, Trametinib, in two BRAF-mutated melanoma cell lines COLO858 and MMACSF over the course of five days (see [Methods](#) for details). Single-cell drug responses were monitored using time-lapse fluorescence microscopy, and changes in probabilistic rate constants k_{stasis} and k_{death} were tracked for the entire period of experiment for each drug condition individually or in combinations (Supporting Information [S5](#) and [S6](#) Figs). The list of compounds based on their nominal targets included two HDAC inhibitors (Fimepinostat and Givinostat), two BET bromodomain inhibitors (Birabresib and I-BET762), two KDM1A inhibitors (SP2509 and ORY-1001), a pan JmJ-KDM inhibitor (JIB-04), a KDM5 inhibitor (CPI-455), two Tankyrase inhibitors (AZ6102 and NVP-TNKS656), and two CDK4/6 inhibitors (Palbociclib and Abemaciclib). These compounds were selected from two broad classes of anti-cancer drugs, referred to as epigenetic-modifying compounds and cell cycle inhibitors, which have been proposed to be used in combination with standard of care BRAF and MEK inhibitors to overcome drug-adapted subpopulations of cells in BRAF-mutant melanomas [[10,21,41–49](#)].

The analysis of variations in Bliss combination index, defined based on probabilistic cytotoxic and cytostatic actions, with drug and time (following unsupervised clustering) led to two major conclusions ([Fig 5A](#)). First, effective drugs with comparable mechanisms of action (e.g. BET inhibitors, HDAC inhibitors or CDK4/6 inhibitors) exhibited similar dynamic patterns of interaction with BRAF and MEK kinase inhibitors, suggesting that differences in probabilistic drug action and interactions are target-specific. Second, cytostatic and cytotoxic drug interactions among efficacious drug combinations often varied in time and did not necessarily correlate with one another. BET inhibitors, for example, exhibited a strong synergistic cytotoxic interaction ($CI_{death} < 1$) with the combination of BRAF and MEK inhibitors within 48–72 h of treatment in both COLO858 and MMACSF cell lines, whereas their interaction was scored as independent ($CI_{stasis} \approx 1$) with respect to inhibition of cell division ([Fig 5A and 5B](#)). Furthermore, the benefit of BET inhibitors combined with Vemurafenib and Trametinib diminished following 96 h, concomitant with the emergence of a small proliferating subpopulation ($k_{division} > 0$) ([Fig 5B](#)). CDK4/6 inhibitors acted independently with BRAF and MEK inhibitor combination to inhibit cell division within 72–96 h in both cell lines ([Fig 5A](#), Supporting Information [S5](#) and [S6](#) Figs). Surprisingly, however, their effects on cell death appeared to be antagonistic especially in MMACSF cells ([Fig 5C](#)). This might be due the possibility that upon G0/G1 arrest, BRAF-mutant cells become less responsive to the effect of BRAF and MEK inhibitors, an interesting observation which requires further investigation across more cell lines.

Altogether, experimental results and simulation outcomes suggest that dynamic measurements of the phenotype metrics and probabilistic evaluation of combination index reveal distinctive responses of cells to drug combinations that might be indistinguishable when assessed based on conventional assays. Phenotype metrics deconvolve differential (and sometimes opposing) degrees of drug effect on tumor cell killing versus inhibition of cell division, a potentially important consideration in choosing appropriate drug combinations. Furthermore, the probabilistic nature of these metrics makes them sensitive to cell-to-cell heterogeneities which are typically overlooked in conventional bulk drug response assays. They are therefore appropriate choices to assess synergistic efficacy in drug combinations aimed at blocking heterogeneous subpopulations of drug-tolerant cells.

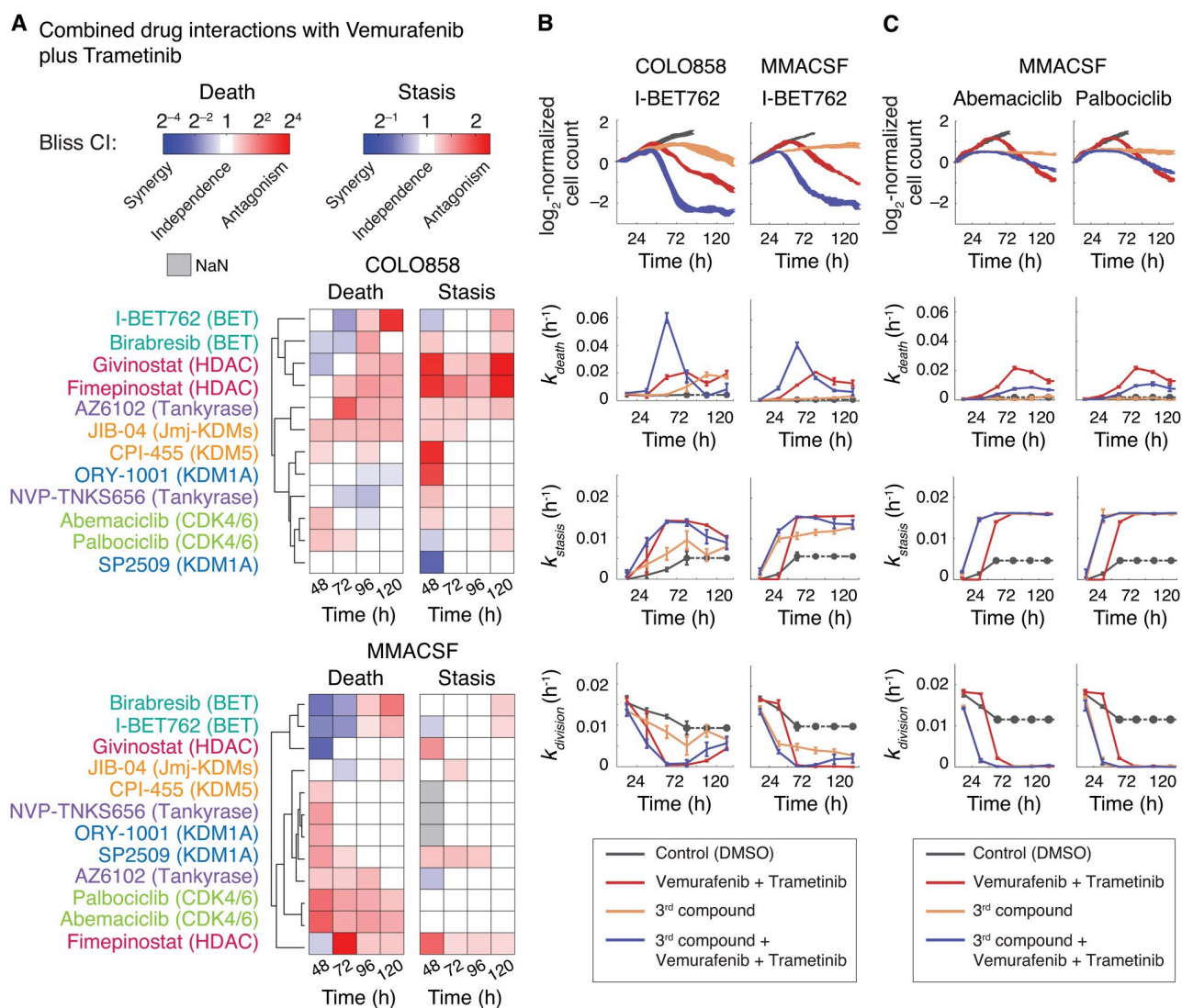


Fig 5. Probabilistic phenotype metrics uncover target-specific differences in drug combination efficacies and their interactions. (A) Unsupervised clustering of Bliss combination index values (CI_{death} and CI_{stasis}) calculated using probabilistic metrics k_{death} and k_{stasis} in COLO858 and MMACSF cells between 48–120 h of exposure to various drugs in sequential combination with Vemurafenib and Trametinib. Cells were treated initially for 24 h with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors (3rd compound) at the following concentrations: Fimepinostat (0.02 μ M), Givinostat (0.2 μ M), Birabresib (0.5 μ M), I-BET762 (1 μ M), SP2509 (1 μ M), ORY-1001 (1 μ M), JIB-04 (0.2 μ M), CPI-455 (5 μ M), AZ6102 (1 μ M), NVP-TNKS656 (1 μ M), Palbociclib (1 μ M), and Abemaciclib (1 μ M). Nominal targets of compounds are highlighted. After 24 h, Vemurafenib at 0.3 μ M plus Trametinib at 0.03 μ M, or DMSO control were added to each treatment condition. Combination index data-points represent mean values across 2–3 replicates. NaN data-points represent conditions where the effect of drug combination or that of the independence model are within measurement error, making the ratio (combination index) unreliable. (B) Estimated dynamics of live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for COLO858 and MMACSF cell responses to the combination of Vemurafenib (0.32 μ M) and Trametinib (0.032 μ M), BET bromodomain inhibitor I-BET762 (1 μ M), their triple combination, or vehicle (DMSO) control. (C) Estimated dynamics of live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for MMACSF cell responses to the combination of Vemurafenib (0.32 μ M) and Trametinib (0.032 μ M), CDK4/6 inhibitors Palbociclib (1 μ M) and Abemaciclib (1 μ M), their triple combination, or vehicle (DMSO) control. $k_{division}$ (no drug) used for the estimation of k_{stasis} for each cell line was estimated using cell division data averaged for the first 24 h in cells treated with DMSO (only). In conditions where confluency was achieved (e.g. DMSO-treated cells after 60 h), data-points were replaced with the last available data-point (dotted line). Data-points represent mean $\pm$ SEM across 2 or 3 replicates.

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Discussion

Synergistic interactions in cancer drug efficacy are typically assessed using Bliss independence or other models (e.g. Highest Single Agent approach [2]). These models are commonly applied

to drug response measurements, whose outcomes are normalized to those measured in untreated controls to identify the fraction of cells affected (f_a) by drugs. Examples of these metrics include drug-induced changes in viability (normalized live cell count) or net growth rate inhibition, which are analyzed across drug doses and combinations. Synergistic efficacy is then concluded when the observed combinatorial effect on f_a metrics exceeds the expected effect from the null model. In this paper, we use basic probability theory and computer simulations to demonstrate that using f_a metrics may bias our estimation of drug combination effectiveness and synergistic efficacy, especially when the ultimate goal is to block heterogeneous drug-tolerant subpopulations. Instead, we propose to use direct measures of time-dependent probabilities of key drug-induced phenotypic events, i.e. induction of cell death and inhibition of cell division, and their associated rate constants (k_{death} and k_{stasis}) to evaluate synergistic efficacy using probabilistic models such as Bliss.

Probabilistic phenotype metrics improve our ability to quantify drug efficacy and characterize drug combination interactions in the following three ways. First, in contrast to the commonly used f_a metrics, phenotype metrics are directly related to the probabilities of drug action in a cell population within any given time interval following drug exposure. Furthermore, they deconvolve differential degrees of drug effect on tumor cell killing versus inhibition of cell division, which may not be correlated in many cases. Second, k_{death} and k_{stasis} dramatically increase the sensitivity of short-term drug response assays to dynamic cell-to-cell heterogeneities and the presence (or emergence) of drug-resistant sub-clones, which are typically overlooked in conventional f_a based drug response analyses. This is a critical issue especially when heterogeneous tumor cell populations consist of cells that are differentially sensitive to drugs and that their sensitivity changes with time. Third, the probabilistic nature of phenotype metrics allows us to use them directly in unbiased evaluation of independence, synergistic or antagonistic efficacy in drug combinations using probabilistic models such as Bliss independence.

While we focused on Bliss independence as a probabilistic framework to study synergistic efficacy, phenotype metrics and their dose- and time-dependent variations could be used in other platforms for broad evaluation of synergy. A recently developed multi-dimensional framework (MuSyC) uses a two-dimensional extension of Hill equation to distinguish synergistic efficacy versus synergistic potency, thereby allowing for a comprehensive understanding of drug interactions. Such understanding not only helps with improving therapeutic efficacy via enhancing effect, but also reducing off-target toxicities via dose reduction [10]. Probabilistic phenotype rate constants follow dose-response patterns suitable to be fit by Hill equation and therefore can be used as input to platforms such as MuSyC.

Estimating probabilistic phenotype metrics requires continuous time-lapse experiments along periods of multiple days, followed by computational single-cell analysis. In this study, we used cell lines engineered to express fluorescent reporters to capture drug-induced changes in cellular death and division events. Such integrative methods may not be necessary for large-scale drug screening projects, in which many drugs are filtered out because of lack of potency. The benefit of these methods is significant, however, when there is a need for identifying more efficacious drugs or drug combinations among a selection of reasonably potent candidates. It has become increasingly evident that cell-to-cell variability is the cause of partial efficacy and incomplete responsiveness of tumor cell populations to a variety of highly potent cytotoxic and targeted therapies. Such heterogeneities are not captured using conventional population-based assays, but may originate residual cells from which drug-resistant clones can arise. Therefore, the probabilistic analysis of single-cell phenotypes has a great potential to improve our understanding of heterogeneity in drug response and facilitate the discovery of more efficacious combination therapies. We envision that the ongoing experimental and computational

advances in single-cell tracking (including dye-based or label-free cell fate tracking and lineage construction by using machine learning and deep learning algorithms) will rapidly improve the efficiency, accuracy and applicability of single-cell approaches to the analysis of cancer drug response.

Materials and methods

Cell culture

BRAF-mutated melanoma cell lines used in this study were obtained from the Massachusetts General Hospital Cancer Center with the following primary sources: COLO858 (ECACC) and MMACSF (Riken Bioresource Center). Each cell line was independently authenticated by Short Tandem Repeats (STR) profiling by ATCC. COLO858 cells were grown in RMPI 1640 (Corning cellgro, Cat. 10–040 CV), and MMACSF cells were grown in DMEM/F-12 (Thermo Fisher Scientific, Cat. 11330–032). For both cell lines, growth media were supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Cat. 26140–079) and 1% sodium pyruvate (Thermo Fisher Scientific, Cat. 11360–070). We added penicillin and streptomycin at 100 U/ml (Thermo Fisher Scientific, Cat. 15140–122) and plasmocin at 0.5 µg/ml (InvivoGen, Cat. ant-mpp) to all growth media. Cells were engineered to stably express H2B-Venus and mCherry-Geminin fluorescent reporters as described previously [21]. Engineered and parental cell lines were confirmed to grow at comparable rates in the absence of any treatment or in the presence of different concentrations of BRAF inhibitor Vemurafenib over 72 hours of treatment.

Reagents

Chemical inhibitors used in this study were obtained from Selleck Chemicals with the following catalog numbers: Vemurafenib (Cat. S1267), Trametinib (Cat. S2673), SP2509 (Cat. S7680), ORY-1001 (Cat. S7795), Palbociclib (Cat. S1116), Abemaciclib (Cat. S7158), AZ6102 (Cat. S7767), NVP-TNKS656 (Cat. S7238), Givinostat (Cat. S2170), Fimepinostat (CUDC-907; Cat. S2759), JIB-04 (Cat. S7281), CPI-455 (Cat. S8287), I-BET762 (Cat. S7189) and Birabresib (OTX-015; Cat. S7360). All chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solution, except Palbociclib of which the stock concentration was 5 mM.

Cell seeding and drug treatment

COLO858 and MMACSF cells expressing fluorescent reporters were seeded into Costar 96-well black clear-bottom tissue culture plates (Corning 2603) in 220 µL full growth medium without phenol red at a density of 2000 and 3000 cells/well, respectively. Cells were counted using a TC20 Automated Cell Counter (Bio Rad). In the case of Vemurafenib dose-response experiments, cells were treated ~24 h after seeding with either DMSO or five different concentrations of Vemurafenib (0.0316, 0.1, 0.316, 1 and 3.16 µM) for a period of ~5 days. In the case of drug combination experiments, cells were treated (also 24 h after seeding) with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors at the following concentrations: Fimepinostat (0.02 µM), Givinostat (0.2 µM), Birabresib (0.5 µM), I-BET762 (1 µM), SP2509 (1 µM), ORY-1001 (1 µM), JIB-04 (0.2 µM), CPI-455 (5 µM), AZ6102 (1 µM), NVP-TNKS656 (1 µM), Palbociclib (1 µM), and Abemaciclib (1 µM); drug concentrations were chosen based on previous reports exhibiting maximal target inhibition in cells. After 24 h, Vemurafenib at 0.3 µM plus Trametinib at 0.03 µM, or DMSO control were added to each treatment condition. All drug treatments were performed in at least 4 replicates using a Hewlett-Packard (HP) D300 Digital Dispenser.

High-throughput time-lapse live cell microscopy

Within 50–60 min after each treatment, cells were imaged every 10 min (for COLO858) and every 15 min (for MMACSF) using a Nikon Ti2-E inverted microscope with motorized stage, Perfect Focus System, 20× objective, and a Photometrics Prime 95B camera followed by 2×2 binning. The process of image acquisition was controlled using NIS element software. Illumination was powered by the Lumencor Spectra X light engine. H2B-Venus fluorescence was captured using 510 nm excitation and 535 nm emission at 25 ms exposure for MMACSF cells and 20 ms exposure for COLO858 cells. mCherry-Geminin fluorescence was captured using 575 nm excitation and a 629.5 nm emission at 80 ms exposure for MMACSF cells and 100 ms exposure for COLO858 cells. Throughout the entire period of image acquisition, environmental conditions were maintained at 37°C, 5% CO₂, and 93% humidity using an OkoLab Enclosure.

Image analysis and automated cell tracking workflow

Images were first processed using Fiji [50] for rolling ball background subtraction with radius of 20 pixels. Background-subtracted images were then analyzed using CellProfiler (3.1.8) for segmentation and classification of cellular phenotypic states, including cells that express high and low levels of Geminin (referred to as Geminin^{high} and Geminin^{low} cells, respectively), or live versus dead cells. Briefly, CellProfiler analysis (Supporting Information S3 Fig) involved: (1) edge enhancement and dark hole feature enhancement of the background-subtracted H2B images to facilitate segmentation; (2) segmenting individual cell nuclei using the Otsu thresholding method; (3) using nuclei segmentations as masks to measure object intensities for all channels as well as object sizes and shapes; (4) classification of phenotypic states of each cell object using the classification model output from CellProfiler Analyst (2.2.1) [51] based on features measured from the previous step. Geminin^{high} versus Geminin^{low} cell classifiers and live versus dead cell classifiers were trained separately using fast gentle boosting algorithm in CellProfiler Analyst with eight and fifteen maximum rules, respectively. The training set used to develop each phenotype classifier was an annotated set, generated via manually sorting the cell object tiles into their corresponding phenotype classes in CellProfiler Analyst. The process of manual sorting followed by model training was iterated until approximately 80% of true positive accuracy was achieved.

Based on phenotype classifications of individual cells for each image output from CellProfiler, corresponding synthetic images were generated in MATLAB (2018b) for each phenotype of interest. Synthetic images contained synthetic pixels at locations of cells. They were used to mark each of the phenotypes of interest (i.e. Geminin^{high} or dead cells) in each image, which were then tracked across series of timepoints using the Fiji plugin TrackMate (3.8.0), without additional need to perform image segmentation. In other words, we combined the power of CellProfiler analysis for accurate and high-throughput image segmentation with the ability of TrackMate to track individual cell phenotypes in a multi-day time-lapse experiment. To achieve this goal, synthetic images for Geminin^{high} cells, dead cells, background-subtracted H2B and Geminin images acquired from the same site were merged into a single multi-channel image composite using Fiji. Image composites were then analyzed using TrackMate with TrackMate extras and Track Analysis extensions [52] for automated tracking. Synthetic pixels of a selected channel were detected by the Laplacian of Gaussian detector and spots were linked with Linear Assignment Problem (LAP) Tracker. Additional spots filtering (based on intensities from multiple channels) and track filtering (based on track duration, track median velocity, and velocity standard deviation) were implemented to optimize tracking results.

Estimating probabilistic phenotype rate constants from individual cell tracking data

Single-cell tracking data generated using TrackMate was analyzed using MATLAB. Transition of a live cell from Geminin^{high} to Geminin^{low} was recorded as a division event, whereas the beginning of a dead cell track was recorded as a death event (Supporting Information S3 Fig). To estimate time-dependent changes in probabilistic phenotype rate constants, k_{death} and $k_{division}$, the number of recorded cell death and division events (N_{death} and $N_{division}$) were quantified over a series of uniformly distributed time intervals ($t \rightarrow t + \Delta t$), where $\Delta t = 12$ h or 24 h. Normalizing N_{death} and $N_{division}$ to the length of each time interval (Δt) and the average number of live cells within the same interval $[N_{live}(t \rightarrow t + \Delta t)]_{avg}$, phenotype rate constant were estimated using Eqs 5–7. As expected, we observed that the magnitude of noise in single-cell tracking data and consequently the relative error in the estimation of $k_{division}$ and k_{death} , increased under conditions where Geminin^{high} and dead cells were highly concentrated, respectively. To mitigate the effect of noise under such conditions, we imposed the following constraints in our estimation of $N_{division}$ (when $N_{death} < N_{division}$) and N_{death} (when $N_{death} > N_{division}$) during each time interval, respectively:

$$N_{division}(t \rightarrow t + \Delta t) = (N_{live}(t + \Delta t) - N_{live}(t)) - N_{death}(t \rightarrow t + \Delta t) \quad (14)$$

$$N_{death}(t \rightarrow t + \Delta t) = N_{division}(t \rightarrow t + \Delta t) - (N_{live}(t + \Delta t) - N_{live}(t)) \quad (15)$$

These constraints are consistent with the assumption that the overall change in the number of live cells during each time interval (Δt) must be equal to the number of division events minus the number of death events during the same time interval.

Verifying the accuracy of automated cell tracking workflow using manual single-cell tracking

To test the performance of our automated image analysis workflow, we compared the phenotype rate constants measured using data from the automated pipeline with those measured using data generated from manual single-cell tracking. This was accomplished using a MATLAB-based software, allowing accurate single-cell tracking and cell fate annotation of individual cells across time-lapse images taken over a period of multiple days [40]. Briefly, the manual tracking method relies on identification of individual cells using intensity and shape information of the nuclear marker (H2B-Venus), track propagation using nearest neighbor criteria, and real-time user correction of tracking, and annotation of cell death and division events based on H2B and Geminin signal intensities. For each condition, about 150–250 cells pooled from four replicates were manually tracked and cell death and division events were recorded. Phenotype rate constants were then calculated using Eqs 5–7.

Estimating fraction of cells affected (f_a) by drug

Currently, evaluation of Bliss independence (and other drug interaction frameworks) is based on fraction of cells affected (f_a), a normalized parameter between zero and one, that represents the fractional effect of drugs individually or in combination [53]. Conventionally, relative viability (or cell count normalized to an untreated control) measured at a fixed time-point (typically 72 or 96 h) following drug treatment has been used to calculate f_a :

$$f_a(\text{viability}) = 1 - \text{viability} \quad (16)$$

Despite its wide-spread usage, however, the relative viability approach in assessing drug response suffers from a fundamental flaw, which is being confounded by variation in cell proliferation rates and assay duration. The reason is that cell count, which is used as a normalization factor in this approach, is non-linearly time-dependent. Therefore, new generation drug response metrics have recently been introduced to correct for this bias [5,6]. The nature of these metrics is based on modeling drug-induced changes in the net growth rate of the cancer cell population (instead of relative viability) as a function of drug dose. These metrics include drug-induced proliferation (DIP) rate [6] and growth rate (GR) inhibition [5], both of which consider and correct for the variability in growth rate that is irrelevant to drug treatment via normalizing the net growth rate of the drug-treated cell population to that of the untreated control. DIP rate and GR inhibition are defined as follows:

$$DIP = \frac{k_{net\ growth(with\ drug)}}{k_{net\ growth(no\ drug)}} \quad (17)$$

$$GR = 2 \frac{k_{net\ growth(with\ drug)}}{k_{net\ growth(no\ drug)}} - 1 \quad (18)$$

where $k_{net\ growth\ (with\ drug)}$ and $k_{net\ growth\ (no\ drug)}$ are the net population growth rates measured in the drug-treated cell population and the untreated control at a particular time-point, respectively.

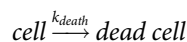
Given the dynamic range of each metric, the definition of fraction of cells affected (f_a) for these new metrics, $f_a(DIP)$ and $f_a(GR)$, is modified as follows so that $0 \leq f_a \leq 1$:

$$f_a(DIP) = \frac{1 - DIP}{1 - \min(DIP)} \quad (19)$$

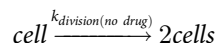
$$f_a(GR) = \frac{1 - GR}{2} \quad (20)$$

Stochastic simulation of cytotoxic and cytostatic drug effects

We modeled phenotypic events in a heterogeneous tumor cell population as a series of independent stochastic reaction processes at a single-cell level. Drug-induced death events were described by the following reaction:



where the rate constant of death k_{death} is defined such that a given cell dies with a probability of $k_{death}dt$ within a reasonably short time interval (dt). Cell division in the absence of drug may be described by the following reaction:



where $k_{division\ (no\ drug)}$ is the inherent rate of division of a given cell. The cytostatic effect of a drug on a cell was described by a conditional probability ($P_{stasis} = P_{inhibition\ of\ division\ (with\ drug) | division\ (no\ drug)}$) with which it prevents a cell from dividing given that it would have divided in the absence of drug with a probability of $P_{division\ (no\ drug)}$. Drugs that do not inhibit cell division and those that accelerate cell division are both characterized by $P_{stasis} = 0$. To satisfy this assumption, we consider an upper-bound limit for the probability of cell division that is equal to $P_{division\ (no\ drug)}$.

For cancer drugs that have inhibitory effect on cell division, the relationship between P_{stasis} and $P_{division (with drug)}$ may be derived as follows:

$$P_{division(no\ drug)} = P_{division(no\ drug) \cap division(with\ drug)} + P_{division(no\ drug) \cap inhibition\ of\ division(with\ drug)} \quad (21)$$

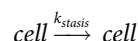
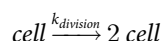
$$P_{division(no\ drug)} = P_{division(with\ drug)} + P_{inhibition\ of\ division(with\ drug) | division(no\ drug)} \cdot P_{division(no\ drug)} \quad (22)$$

$$P_{inhibition\ of\ division(with\ drug) | division(no\ drug)} = \frac{P_{division(no\ drug)} - P_{division(with\ drug)}}{P_{division(no\ drug)}} \quad (23)$$

$$P_{stasis} = 1 - \frac{P_{division(with\ drug)}}{P_{division(no\ drug)}} \quad (24)$$

$$P_{division(with\ drug)} = (1 - P_{stasis}) P_{division(no\ drug)} \quad (25)$$

In the presence of drug, cell division and inhibition of cell division (stasis) may be described by the following reactions, respectively:



where the rate constants are as follow:

$$k_{stasis} = P_{stasis} k_{division(no\ drug)} \quad (26)$$

$$k_{division} = (1 - P_{stasis}) k_{division(no\ drug)} \quad (27)$$

The model assumes that the processes of drug-induced cell death and inhibition of cell division are independent of each other.

At the population level, *Poisson* processes of drug-induced phenotypic events in a tumor cell population were simulated using the Gillespie algorithm. Briefly, the Gillespie algorithm determines the time to the next reaction event in the cell population based on an exponential distribution that statistically characterizes the *Poisson* processes. The algorithm then stochastically determines whether the event is death or division based on probabilities that are proportional to the rates of these two processes (k_{death} and $k_{division}$). If the chosen event is division, then with probability P_{stasis} that division event is rejected.

Validation of non-stationary *Poisson* models for live cell microscopy data

The probabilistic modeling approach used in this study involves a commonly used formulation of stochastic chemical kinetics, describing the time evolution of a reacting system while taking into consideration the fact that individual reaction events (typically between individual molecules) are random point *Poisson* processes [54]. While using a similar formulation, we consider a single-cell event (death or division) as an individual event, rather than modeling any of the drug-induced molecular events that underlie such cellular processes. Previous work (based on live-cell measurement and modeling) has shown that apoptosis, for example, is controlled by a snap action switch at a single-cell level [55]. This switch, however, is associated with a delay that is variable from one cell to another within a population, preventing cells

from dying en masse following exposure to a death stimulus. Such variability makes it possible for an all-or-none response at a single-cell level to be graded at the population level such that the concentration of stimulus (or drug) controls the probability with which a cell responds during a specific period of time. This is in general agreement with the definition of *Poisson* process to model the population behavior, where the rate of a cellular event is directly linked to the *Poisson* rate parameter. Such parameter is expected to be controlled by the concentration of a key set of molecular regulators that are not explicitly modeled. However, changes in cellular state (due to drug adaptation, for example) may influence the concentration of these regulators, causing time-dependent changes in the *Poisson* rate constant, making non-stationary *Poisson* process a potentially suitable framework to model such dynamic, adaptive responses.

To experimentally test whether a simplified model of non-stationary *Poisson* process may explain the distribution of drug-induced death and division events in time-lapse microscopy data, we used maximum likelihood estimation to fit two non-stationary *Poisson* models, one to the single-cell death data and one to the single-cell division data. The rate function $k(t)$ of the non-stationary *Poisson* models used for data fitting was assumed to be a piece-wise function in time, where for each 12 h interval the rate was given by a single parameter. Hence, to capture 120 h of data, we set the rate function for each *Poisson* process with 10 parameters. The log-likelihood function for fitting a non-stationary *Poisson* model is given as follows:

$$l(\theta) = \sum_{j=1}^n \log\{k(t_j; \theta)\} - \int_0^T k(\tau; \theta) d\tau \quad (28)$$

where θ is a vector of the 10 parameters to be estimated from the data, n is the number of datapoints, t_j is the time of the j^{th} event and T is the end time of the experiment. The log-likelihood function was then maximized using the constrained optimization function 'fmincon()' in MATLAB. Using the fit parameters, we then simulated drug responses for 30 times and the normalized mean counts of phenotypic events were compared to that of the same data used for parameter estimation.

Simulations of combined drug responses with variable modes of drug interaction

For combined drug response simulations, we modified the Gillespie algorithm as follows. After determining the time of the next event, the algorithm stochastically determines whether that event is a death event induced by drug A, a death event induced by drug B, or a division event based on probabilities proportional to their rates of occurrence. In cases where the two drugs confer statistically independent cell killing, the probabilities of the next event being drug A-induced death and drug B-induced death are respectively proportional to their single drug-induced rates of death, i.e. $k_{death(A)}$ and $k_{death(B)}$, whereas the probability of the next event being a division event is proportional to the inherent division rate of the cell, $k_{division (no drug)}$. If the next event is division, then with a probability of $P_{stasis(A+B)}$ that division event is rejected. For independent cytostatic interactions, $P_{stasis(A+B)}$ is set to $P_{stasis(A+B)}^I$ as defined in Eq 11. In cases where drug combinations are not independent, $P_{stasis(A+B)}$ and $P_{death(A+B)}$ will be calculated as $P_{stasis(A+B)}^I$ and $P_{death(A+B)}^I$ divided by CI_{death} and CI_{stasis} to simulate different modes of drug interaction, respectively. For the purpose of comparison, we also evaluated Bliss combination index while replacing probabilistic metrics with f_a quantities measured for each drug condition individually and in combination.

Simulations of heterogeneous drug response in the presence of drug-tolerant subpopulations

We simulated drug treatment scenarios where the initial cell population consisted of heterogeneous subpopulations, in which a small fraction of cells was substantially less sensitive to treatment relative to the majority of the cell population. Stochastic arrival of death and inhibition of division events were modeled using Gillespie algorithm as described above, while considering two subpopulations: a larger sensitive (S) subpopulation and a small drug-tolerant or resistant (R) subpopulation. We initialized simulations with 300 cells, a small fraction of which (ω , varied from 0%-5%) had a more resistant phenotype, i.e. a lower death rate constant and a lower probability of stasis than that of the sensitive population, in the presence of drug. We modeled such resistant phenotype by defining the level of resistance ($r \geq 1$, varied from 1–16) as the fold-change in the rates of death and probability of stasis relative to the sensitive population. We used same fold-changes for death and cytostasis rates. We assumed a fixed inherent growth rate for the sensitive population $k_{division}^S (no\ drug) = 0.035\ h^{-1}$, while considering three different possible inherent growth rates for the resistant population $k_{division}^R (no\ drug) = 0.035\ h^{-1}$, $0.02\ h^{-1}$, and $0.009\ h^{-1}$. Drug response parameters for the drug-sensitive population include: $k_{death}^S (drug) = 0.03\ h^{-1}$, $P_{stasis}^S (drug) = 0.8$. Drug response parameters for the drug-tolerant subpopulation are: $k_{death}^R (drug) = k_{death}^S (drug) / r$, $P_{stasis}^R (drug) = P_{stasis}^S (drug) / r$. We assumed that phenotypic responses of both subpopulations are independent of each other and that daughter cells within the same subpopulation inherit the exact same probabilities of phenotypic events as their mother cells. The responses (i.e. number of live cells, death and division events) of the two subpopulations were summed together to show the overall response of the entire cell population. To compare quantitatively the sensitivity of different metrics in capturing the differences in drug effect in the presence of phenotypic heterogeneity, we systematically varied the initial fraction of drug-tolerant subpopulation (ω) and its level of resistance (r) as input parameters in simulations. For each simulation, overall drug effect using different metrics (fraction affected or phenotype rate constants) were calculated. To evaluate the sensitivity of each metric to the presence of drug-tolerant subpopulations, we defined and calculated “resistance enrichment ratio” as the ratio of these metrics between two treatment scenarios, one in the presence of a heterogeneous population (varying $\omega > 0$ and $r > 1$) and one in the absence of heterogeneity ($\omega = 0$ or $r = 1$). The smaller the resistance enrichment ratio becomes, the more significant decrease in drug effect is captured by a given metric in the presence of drug-tolerant cells.

Hierarchical clustering

Unsupervised hierarchical clustering of combination index (CI) values estimated from the application of Bliss independence to probabilistic phenotype rate constants measured for 24 h time intervals of drug treatments was carried out using MATLAB 2018b with the Euclidean distance metric and the Complete (farthest distance) algorithm for computing the distance between clusters.

Sensitivity analysis

To compare the sensitivity of different metrics (with different units), to the variation of parameter P_{death} , we defined fractional sensitivity (S_f) as follows:

$$S_f(f_a, P_{death}) = \frac{\partial f_a / f_a}{\partial P_{death} / P_{death}} \quad (29)$$

$$S_f(k_{death}, P_{death}) = \frac{\partial k_{death}/k_{death}}{\partial P_{death}/P_{death}} \quad (30)$$

S_f values of less than 1 represent reduced sensitivity of the metric to changes in P_{death} .

Statistical analysis

All data with error bars were presented as mean $\pm$ standard error of the mean (SEM) using indicated numbers of replicates.

Code availability

Custom MATLAB scripts for probabilistic simulation of drug response in heterogeneous tumors cell populations (presented in Figs 1, 2 and 4) are available on GitHub at the following address: <https://github.com/fallahi-sichani-lab/probabilisticDrugResponse>.

Supporting information

S1 Fig. Probabilistic rate constants capture time-dependent heterogeneities in phenotypic responses. (A,B) Simulation results showing changes in resistant enrichment ratio calculated for each of the f_a metrics and for phenotype rate constants (k_{death} and k_{stasis}) as a function of ω at a fixed value of $r = 16$ across different times of treatment. Data are shown for fixed inherent growth rates for the sensitive population, $k_{division}^S (no\ drug) = 0.035\ h^{-1}$ and two different rates of inherent growth for the resistant subpopulation: $k_{division}^R (no\ drug) = 0.009\ h^{-1}$ (A) and $k_{division}^R (no\ drug) = 0.035\ h^{-1}$ (B). (C,D) Simulation results showing changes in resistant enrichment ratio calculated for each of the f_a metrics and for phenotype rate constants (k_{death} and k_{stasis}) as a function of r at a fixed value of $\omega = 0.03$ across different times of treatment. Data are shown for fixed inherent growth rates for the sensitive population, $k_{division}^S (no\ drug) = 0.035\ h^{-1}$ and two different rates of inherent growth rate for the resistant subpopulation: $k_{division}^R (no\ drug) = 0.009\ h^{-1}$ (C) and $k_{division}^R (no\ drug) = 0.035\ h^{-1}$ (D). All data represent mean values from 50 simulations. (TIF)

S2 Fig. Sensitivity of f_a metrics decreases as drug cytotoxicity increases. (A) Input dose response profiles used in simulations. The maximum cytotoxic efficacy was varied at three different levels, whereas the cytostatic dose response profiles for all three conditions were held constant. (B) Model output measured from the simulated conditions in (A) at $t = 72\ h$ showing variations in viability, GR and the probabilistic phenotype rate constants. (C) Analysis of metric sensitivity with varying drug cytotoxicity parameter P_{death} , quantified per unit of time (h). Sensitivity analysis was performed on simulations with $P_{stasis} = 0$ and $k_{division}^S (no\ drug) = 0.035\ h^{-1}$. Initial cell number was $N_{live}(t = 0) = 5000$. Data shown are mean $\pm$ SEM across 50 simulations. Probabilistic phenotype rate constants were estimated from a 24 h time-interval centered at 72 h. (TIF)

S3 Fig. Overview of the time-lapse image analysis pipeline to quantify occurrence of single-cell phenotypic events from time-lapse live cell microscopy data. The automated image analysis pipeline involves four steps: (1) Each background (BG) subtracted H2B image was segmented in CellProfiler for nucleus identification. For each nucleus object, a variety of features (e.g. mean signal intensities across multiple channels, area and shape) were measured. (2) To classify the phenotypes of interest (i.e. live or dead cells, Geminin^{high} or Geminin^{low} cells) in each image, classification models were trained in CellProfiler Analyst based on feature measurements of the user-annotated training sets. (3) Based on phenotype classifications of

individual cells for each image output from CellProfiler, corresponding synthetic images were generated in MATLAB for each phenotype of interest. Synthetic images contained synthetic pixels at locations of Geminin^{high} or dead cells. To facilitate tracking of individual cells, relative intensities of the synthetic pixels for each phenotype were scaled with the mean intensity of the signal associated with that phenotype. For example, intensities of death synthetic pixels were scaled with the mean H2B signal intensities of individual cells, whereas intensities of the Geminin^{high} synthetic pixels were scaled with the mean Geminin signal intensities. (4) Synthetic pixels for each phenotype were tracked separately in TrackMate. Since Geminin reporter level drops at the M phase, a division event is marked when the Geminin track ends. The beginning of a death track is also marked as a death event.

(TIF)

S4 Fig. Probabilistic rate constants of phenotypic events measured using automated tracking is consistent with the rate constants acquired from manual single-cell tracking across different cell lines and drug conditions. (A–B) Probabilistic rate constants of death (k_{death}) and division events ($k_{division}$) measured in (A) COLO858 and (B) MMACSF cells treated with Vemurafenib at the indicated doses, using automated tracking analysis pipeline (top row) versus manual tracking (bottom row) on the same set of time-lapse images. For each condition, the automated tracking estimates at each timepoint are the mean values across four replicated wells. Error bars represent SEM. The rate constants calculated from manual tracking data are based on individually tracked cells pooled from four replicated wells, including about 150–220 cells per condition.

(TIF)

S5 Fig. Dynamic responses of COLO858 cells to epigenetic-modifying compounds and cell cycle inhibitors in sequential combination with Vemurafenib plus Trametinib. Estimated dynamics of changes in live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for COLO858 cell responses to the combination of Vemurafenib (0.32 μ M) and Trametinib (0.032 μ M), a 3rd compound (including epigenetic-modifying compounds or cell cycle inhibitors), their triple combination, or vehicle (DMSO) control. Cells were treated initially for 24 h with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors (3rd compound) at the following concentrations: Fimepinostat (0.02 μ M), Givinostat (0.2 μ M), Birabresib (0.5 μ M), I-BET762 (1 μ M), SP2509 (1 μ M), ORY-1001 (1 μ M), JIB-04 (0.2 μ M), CPI-455 (5 μ M), AZ6102 (1 μ M), NVP-TNKS656 (1 μ M), Palbociclib (1 μ M), and Abemaciclib (1 μ M). After 24 h, Vemurafenib at 0.3 μ M plus Trametinib at 0.03 μ M, or DMSO control were added to each treatment condition. $k_{division}$ (no drug) used for the estimation of k_{stasis} is estimated using cell division data averaged for the first 24 h in cells treated with DMSO only. In conditions where confluency was achieved, data-points were replaced with the last available data-point (dotted line). Data-points represent mean $\pm$ SEM across 2 or 3 replicates.

(TIF)

S6 Fig. Dynamic responses of MMACSF cells to epigenetic-modifying compounds and cell cycle inhibitors in sequential combination with Vemurafenib plus Trametinib. Estimated dynamics of changes in live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for MMACSF cell responses to the combination of Vemurafenib (0.32 μ M) and Trametinib (0.032 μ M), a 3rd compound (including epigenetic-modifying compounds or cell cycle inhibitors), their triple combination, or vehicle (DMSO) control. Cells were treated initially for 24 h with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors (3rd compound) at the following concentrations: Fimepinostat

(0.02 μM), Givinostat (0.2 μM), Birabresib (0.5 μM), I-BET762 (1 μM), SP2509 (1 μM), ORY-1001 (1 μM), JIB-04 (0.2 μM), CPI-455 (5 μM), AZ6102 (1 μM), NVP-TNKS656 (1 μM), Palbociclib (1 μM), and Abemaciclib (1 μM). After 24 h, Vemurafenib at 0.3 μM plus Trametinib at 0.03 μM , or DMSO control were added to each treatment condition. $k_{\text{division (no drug)}}$ used for the estimation of k_{stasis} is estimated using cell division data averaged for the first 24 h in cells treated with DMSO only. In conditions where confluency was achieved, data-points were replaced with the last available data-point (dotted line). Data-points represent mean $\pm$ SEM across 2 or 3 replicates.

(TIF)

S1 Table. Numerical data representing dynamic changes in live cell count and estimates of k_{death} and k_{division} measured from time-lapse live cell microscopy experiments for COLO858 cell responses to different doses of Vemurafenib. Presented data are associated with [Fig 3A](#).

(XLSX)

S2 Table. Numerical data representing dynamic changes in live cell count and estimates of k_{death} and k_{division} measured from time-lapse live cell microscopy experiments for MMACSF cell responses to different doses of Vemurafenib. Presented data are associated with [Fig 3B](#).

(XLSX)

S3 Table. Numerical data representing dynamic changes in live cell count and estimates of k_{death} and k_{division} measured from time-lapse live cell microscopy experiments for COLO858 cell responses to different drug combination treatment conditions. Presented data are associated with Supporting Information [S5 Fig](#).

(XLSX)

S4 Table. Numerical data representing dynamic changes in live cell count and estimates of k_{death} and k_{division} measured from time-lapse live cell microscopy experiments for MMACSF cell responses to different drug combination treatment conditions. Presented data are associated with Supporting Information [S6 Fig](#).

(XLSX)

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Review

Epigenetic Mechanisms of Escape from BRAF Oncogene Dependency

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Abstract: About eight percent of all human tumors (including 50% of melanomas) carry gain-of-function mutations in the BRAF oncogene. Mutated BRAF and subsequent hyperactivation of the MAPK signaling pathway has motivated the use of MAPK-targeted therapies for these tumors. Despite great promise, however, MAPK-targeted therapies in BRAF-mutant tumors are limited by the emergence of drug resistance. Mechanisms of resistance include genetic, non-genetic and epigenetic alterations. Epigenetic plasticity, often modulated by histone-modifying enzymes and gene regulation, can influence a tumor cell's BRAF dependency and therefore, response to therapy. In this review, focusing primarily on class 1 BRAF-mutant cells, we will highlight recent work on the contribution of epigenetic mechanisms to inter- and intratumor cell heterogeneity in MAPK-targeted therapy response.

Keywords: oncogene addiction; BRAF-mutant tumors; BRAF and MEK-targeted therapies; phenotype switching; tumor heterogeneity; epigenetic regulation; adaptive drug resistance; histone-modifying enzymes; chromatin regulation; DNA methylation

1. Introduction

The discovery of driver oncogenes has revolutionized therapeutic strategies for an increasing number of cancers. These strategies have been primarily focused on either direct inhibition of a mutated oncoprotein, or targeting a component of its effector pathway [1]. The underlying rationale is that tumor cells sustain a state of pathway dependency, or so-called “oncogene addiction”, where cellular growth and survival become highly dependent on the continued activity of the oncogenic pathway [2]. Despite great promise, however, therapeutic inhibition of oncogenic signaling often leads to variable response rates across tumors that carry the same mutated oncogene [3–6]. A prototypical example of this challenge is observed in cancers that carry alterations in the serine/threonine protein kinase BRAF [7,8]. Oncogenic BRAF alterations lead to hyperactivation of MAP kinase (MAPK) signaling, thereby motivating the development of therapeutic inhibitors of BRAF and downstream MEK kinases [8]. These therapies, however, generate variable cellular and clinical outcomes in BRAF-mutated cancers that originate in distinct tissue types [4,9,10]. For example, they lead to dramatic response rates in melanomas, but their efficacy is limited in thyroid and colorectal carcinomas. Furthermore, despite initially impressive responses [11], BRAF and MEK-targeted therapies ultimately fail to cure most melanomas because of the emergence of therapy resistance [12–15].

Acquired (late) resistance to BRAF and MEK-targeted therapies involves a diversity of genetic mutations in components of the MAPK pathway [12,16–22] or parallel signaling networks such as the PI3K/AKT kinase cascade [14,23]. In many cases, however, the emergence of drug-resistant clones cannot

be explained by genetic mechanisms. It is thought that drug-resistant clones arise following an initially incomplete and heterogeneous response during the early phase of treatment [24–26]. Drug tolerance in this phase could be caused either by enrichment of a subpopulation of undifferentiated or stem-like cells that are intrinsically insensitive to the effect of MAPK inhibition [27–29], or by acquisition of adaptive phenotypes with reduced dependency on BRAF and MAPK signaling [30–36]. Such heterogeneity in the state of BRAF and MAPK dependency appears even among isogenic cells derived from a single clone [35]. Feedback-mediated reactivation of the MAPK pathway or induction of parallel signaling cascades have been recognized as major causes of rapidly induced adaptive resistance to BRAF and MEK-targeted therapies [37–42]. Nevertheless, a number of recent studies have identified heterogeneity in lineage program and differentiation state, both among genetically diverse tumors and within genetically homogeneous populations of cells, to be associated with variability in tumor cells' sensitivity to BRAF and MEK inhibitors [5,35,43–46].

A major contributor to drug resistance is transcriptome plasticity [47], whereby upon drug treatment, distinct subpopulations of drug-tolerant cells emerge with dynamic and differential gene expression profiles. Epigenetic reprogramming following BRAF inhibition can not only induce changes in differentiation state, but also generate new gene expression programs that reduce the cellular requirement for BRAF or MAPK signaling. This phenomenon, referred to as phenotype switching or epigenetic switching, causes fractional response even in genetically homogenous populations of tumor cells. There is still much to learn about how such functionally diverse adaptive phenotypes emerge. However, they appear to be regulated by lineage-dependent, epigenetic mechanisms that continuously reprogram the state of vulnerability in tumor cells [48–52]. These mechanisms complement the genetic code in providing instructions to cells and controlling their responses to internal and external cues in a context-dependent manner. Among these mechanisms are DNA methylation, histone protein modifications, micro(mi)RNAs, and nucleosomal positioning, which together contribute to adopting distinct gene expression states and phenotypes, not only during development and disease but also in response to therapies. The role of only some of these mechanisms has been explored in BRAF-mutant tumors. Nevertheless, a detailed understanding of these mechanisms in BRAF-mutant tumors could be key to increasing the efficacy of MAPK-targeted therapies in these cancers.

In this Review, we discuss the role of epigenetic mechanisms and phenotype plasticity in determining the outcome of MAPK pathway-targeted therapies in BRAF-mutant cancers. We first describe the role of the BRAF kinase, its oncogenic mutations in cancer, and discuss the range of therapies that target BRAF-related malignancies. We then examine the problem of short-lived responses to targeted therapy and the contribution of phenotype plasticity and epigenetic mechanisms to tumor heterogeneity, fractional drug response and drug resistance. We conclude with a discussion of potential therapies with epigenetic inhibitors alone or in combination with MAPK inhibitors to effectively target therapy-resistant BRAF-mutated cancers.

2. BRAF Oncogene: Role in MAPK Pathway, Dysregulation in Cancer, and Potential as a Therapeutic Target

The MAPK pathway transduces crucial extracellular signals into intracellular responses, regulating key physiological processes, such as cell proliferation, differentiation and survival. In mammals, the MAPK family consists of four main subgroups, including extracellular-signal-regulated kinase (ERK), MEK4/7-Jun amino-terminal kinase (JNK), stress-activated protein kinase MEK3/6-(p38/SAPK), and MEK5-ERK5 subfamilies [53,54]. The ERK pathway, in particular, is activated by the binding of mitogens to cognate cytokine receptors, G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs). Upstream activation of membrane-bound RTKs promotes the recruitment of adaptor proteins, such as the guanine nucleotide exchange factor (GEF), son-of-sevenless (SOS). SOS releases GDP from the RAS proteins (HRAS, KRAS, NRAS), a family of small guanine triphosphatases (GTPases), allowing GTP to bind and activate RAS as a result. Active RAS initiates the three-tier RAF-MEK-ERK cascade. First, active RAS recruits the family of rapidly accelerated fibrosarcoma

(RAF) serine/threonine cytosolic kinases (ARAF, BRAF, CRAF) to the plasma membrane, promoting their dimerization [55]. Second, dimerized, catalytically active RAFs directly phosphorylate and activate MEK kinases (MEK1 and MEK2). Third, activated MEK1/2 continue the signaling cascade by phosphorylating and subsequently activating ERK kinases (ERK1 and ERK2) at conserved threonine and tyrosine residues. Activated ERK1/2 kinases phosphorylate several key downstream proteins, including transcription factors and kinases that regulate key cellular processes such as survival, proliferation and differentiation.

Given the importance of the MAPK pathway in maintaining organismal physiology, ensuring a tightly regulated RAS-RAF-MEK-ERK response is vital. In cancer, however, such strictly regulated cellular processes are compromised as a result of MAPK pathway dysregulation. Activating mutations in several components of the MAPK pathway, including RTKs, RAS, RAF, and MEK1/2, have been identified in cancer cells, implicating these modules as oncogenic drivers. In melanomas, for example, malignancy is almost invariably accompanied by mutations that activate this pathway [56]. Among these mutations, BRAF and NRAS are two of the most frequently but mutually exclusively mutated oncogenes with frequencies of >50% and ~30%, respectively. Activating mutations in the MAP2K1 (MEK1) oncogene and loss of the tumor suppressor gene neurofibromin 1 (NF1) are other frequent alterations in melanomas that activate the MAPK pathway, with frequencies of ~6% and ~13%, respectively. Across all human cancers, RAS mutations occur with a frequency of ~15% [57]. MEK and ERK mutations are rare, but almost 8% of all human cancers, including colorectal cancers (10%), papillary thyroid carcinomas (45%), serous ovarian tumors (~30%), non-small cell lung cancers (NSCLCs) (5–10%) and melanomas (>50%) harbor an activating somatic mutation in the BRAF oncogene [57–63].

BRAF kinase mutations are grouped into three classes. Class 1 BRAF mutations refer to a transversion point mutation in the activation segment of the kinase domain at codon 600, resulting in the substitution of glutamic acid, most commonly with valine (V600E), and sometimes with lysine (V600K), arginine (V600R), or aspartic acid (V600D). The mutated BRAF^{V600E} gene produces a constitutively active oncoprotein, which acts as a monomer independently of upstream signals (i.e., RAS), thereby causing the hyperactivation of the MEK-ERK cascade. Of the BRAF mutations observed in melanomas, the V600E mutation is the most common (~90%) [64]. In addition, BRAF^{V600E} mutations are observed in ~10% of colorectal cancers, 5% of lung adenocarcinomas, 40% of papillary thyroid carcinomas, several glioma subtypes, and a subset of hematological malignancies [58,65–68]. Class 2 activating BRAF mutations are also RAS-independent, but they function as dimers, resulting in intermediate kinase activity. Non-V600 class 2 mutations have been found in the activation segment (K601E, L597Q), the DFG motif (F595L) as well as the P-loop (G469A) of the BRAF kinase. In contrast, class 3 BRAF mutations (also non-V600) are RAS-dependent and signal as dimers [69,70], with mutations in either the P-loop (G466), catalytic loop (N581) or the DFG motif (D594) [71]. Class 3 BRAF mutants homodimerize with either wild-type BRAF or heterodimerize with CRAF to signal; these dimers have lower kinase activity than their wild-type counterparts [71–73]. Mutant forms of BRAF with reduced kinase activity have been observed in lung tumors and melanoma [74,75]. Interestingly, class 3 BRAF mutations often co-occur with upstream mutations in RAS or its negative regulator NF1 [72,76]. Mutations can also occur in ARAF and CRAF genes, albeit at a lower rate [77–79]. In addition to point mutations, BRAF gene fusions and in-frame deletions also reveal its characterization as an oncogenic kinase [80,81]. N486-P490-deletion or L485-P490-deletion have been documented in pancreatic, lung, and ovarian cancers [82]. Altogether, these alterations highlight the reliance of multiple cancer subtypes on oncogenic BRAF-MEK-ERK signaling, implicating the BRAF kinase as an exploitable vulnerability and a therapeutic target for such cancers.

3. Current Strategies to Target BRAF-Mutated Cancers

Prior to the identification of BRAF mutations, the first-generation ATP-competitive RAF inhibitor, sorafenib (Nexavar), was developed for targeting RAS-mutant cancers [83]. Sorafenib, however, lacks

robustness against cells that harbor BRAF^{V600E} mutations [84]. The discovery of BRAF^{V600E} mutations in multiple cancers spearheaded efforts to target the mutated BRAF. These efforts culminated in the development and FDA approval of second-generation ATP-competitive inhibitors that target class 1 BRAF mutations: vemurafenib (Zelboraf; PLX4032), dabrafenib (Tafinlar; GSK2118436), and encorafenib (Braftovi; LGX818) [7,11,85–87]. These inhibitors appear to be selective for BRAF^{V600E} mutated cancers because they efficiently inhibit the monomeric form of RAF (i.e., BRAF^{V600E}) that is hyperactivated in these tumors. The stabilization of the structural conformation of α C-helix of the BRAF kinase can determine the effectiveness of RAF inhibitors. For example, binding of an α C-helix-OUT inhibitor molecule, such as vemurafenib, to the first protomer of BRAF stabilizes the α C-helix to an ‘OUT’ conformation; as a result, the second protomer of BRAF is stabilized to an ‘IN’ conformation. This allosteric change in the second protomer prevents binding of a second molecule of BRAF inhibitor [75]. Only at higher concentrations are these α C-helix-OUT BRAF inhibitors able to bind and block both protomers of the BRAF dimer; unfortunately, such extreme concentrations are not feasible for patient treatment. Therefore, α C-helix-OUT inhibitors can inhibit the BRAF^{V600E} kinase, but their impact on cells that lack monomeric BRAF^{V600E} mutations is limited by induction of negative cooperativity and paradoxical activation of ERK signaling [75,88–94]. Thus, these drugs are not effective against tumors that carry class 2 or 3 BRAF mutations, where BRAF signals as a dimer.

Newer groups of BRAF inhibitors that act as “dimer inhibitors” have also been developed [88,94,95]. LXH254 and LY3009120 are examples of such small molecules that inhibit both mutant BRAF monomers and dimers at similar doses, as they do not induce negative cooperativity at the second site of BRAF dimer. In addition, “paradox breakers” such as PLX8394 are next-generation small molecules that disrupt BRAF-containing dimers, including BRAF homodimers and BRAF-CRAF heterodimers, but not CRAF homodimers or ARAF-containing dimers [96,97]. Since PLX8394 does not inhibit wild-type CRAF dimers and has a wide therapeutic window, it is an attractive candidate for the treatment of tumors driven by class 1 and 2 BRAF mutants and BRAF fusions.

To improve patient response, BRAF inhibitors vemurafenib, dabrafenib and encorafenib have been used in combination with small molecule inhibitors of the MEK kinases, such as cobimetinib (Cotellic), trametinib (Mekinist), and binimetinib (Mektovi). All three compounds are allosteric inhibitors that bind to the MEK kinase and induce conformational changes that subsequently block its kinase activity [98,99]. MEK inhibitors inhibit ERK signaling in both BRAF-mutant and BRAF-wildtype cells. The combination of BRAF and MEK inhibitors overcomes the paradoxical activation of ERK signaling with BRAF inhibitors, while also lowering the toxicities associated with the effect of MEK inhibitors in BRAF-wildtype cells [90,100]. As a result, combination therapy with MEK inhibitors (such as trametinib) and class 1 BRAF inhibitors (such as dabrafenib) have become FDA-approved standard of care for patients with metastatic BRAF-mutated melanomas. BRAF^{V600E} melanoma patients have demonstrated improved response rates with combination MAPK therapy [101]. Unfortunately, however, the clinical benefit of such therapies is still short-lived in the great majority of patients due to the emergence of resistance [19,21].

4. Limitations of Current Therapies Targeting BRAF-Mutated Cancers

Despite substantial advances in the development of therapeutics focused on MAPK pathway inhibition in BRAF-mutated cancers, resistance and subsequent tumor relapse remain a major challenge to the durable success of these therapies [16]. For example, despite the initial rapid response rates with BRAF and MEK inhibitor combinations in melanoma patients, resistance typically emerges within the first two years of therapy [3,102]. Intrinsic and acquired resistance to MAPK inhibitors involves a diversity of genetic, non-genetic and epigenetic factors (Figure 1).

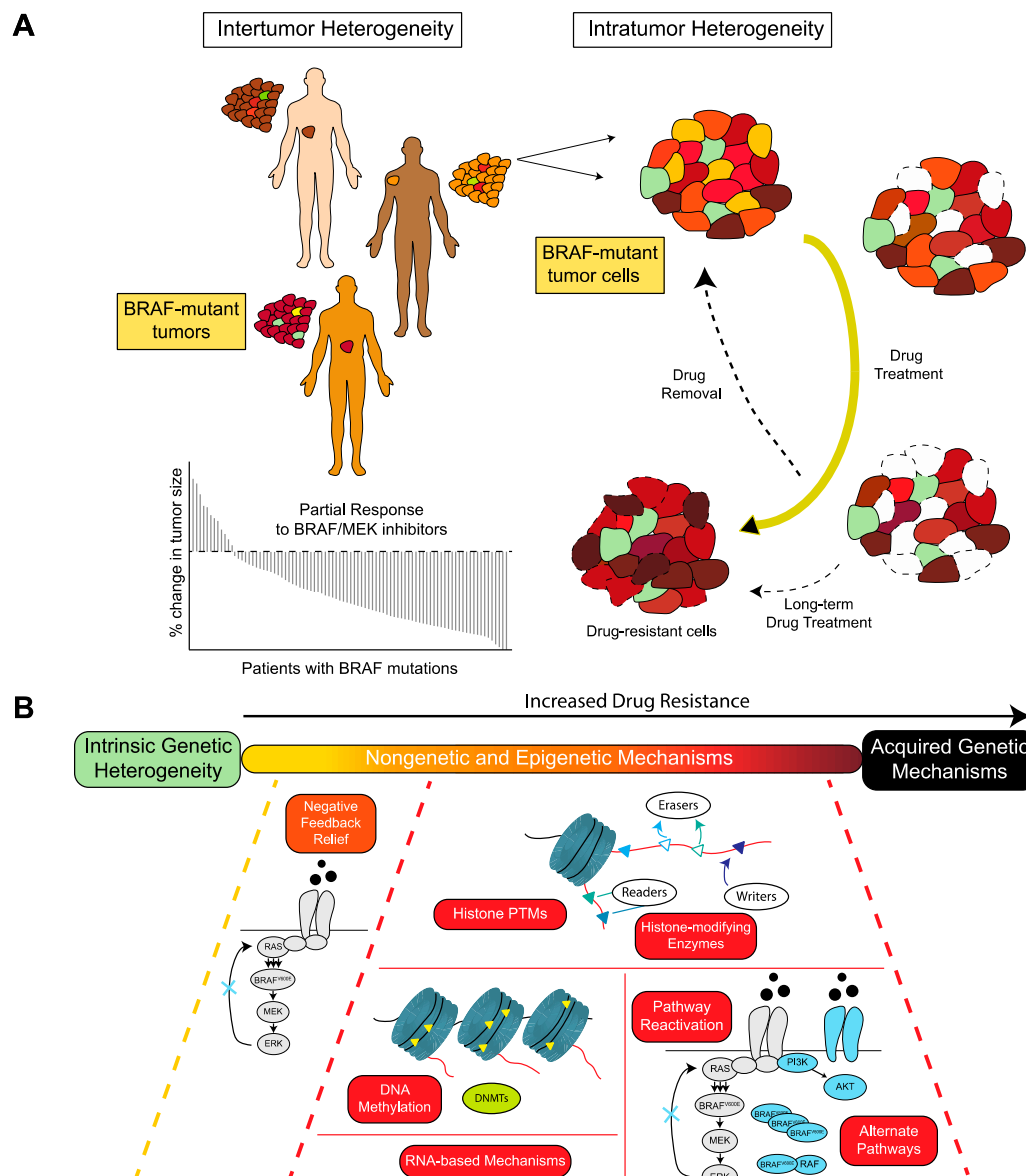


Figure 1. Dynamic mechanisms of inter- and intratumor heterogeneity. (A) Intertumor heterogeneity in patients with BRAF-mutant tumors results in a partial response to BRAF and MEK inhibitor therapy. Intratumor heterogeneity in BRAF-mutant tumors comprises of cells at distinct transcriptional cell states due to genetic (green), nongenetic (e.g., pathway rewiring) and epigenetic mechanisms (gradient from yellow to brown). A cell's BRAF or MAPK dependency can determine its ability to tolerate or adapt to BRAF or MAPK inhibitor therapies. A cell with higher BRAF dependency is more sensitive to short-term BRAF inhibitor therapy (dashed outline), whereas a cell with lower BRAF dependency can better tolerate the drug over time by relying on non-genetic and epigenetic mechanisms. Long-term drug treatment can result in a stable, drug-resistant phenotype, whereas adaptive drug resistance is reversible. (B) Upon drug treatment, drug-tolerant cells can employ non-genetic (relief of MAPK negative feedback, MAPK pathway reactivation, and alternate pathways, such as the PI3K pathway) and epigenetic mechanisms (DNA methylation, histone modifications, histone-modifying enzymes, and RNA-based mechanisms) to escape drug effect. Over time, these drug-tolerant, drug-adaptive mechanisms can ensure cells survive long enough to acquire advantageous mutations that lower the cell's BRAF dependency and lead to long-term resistance.

Genetic mechanisms of resistance comprise of mutations in the MAPK pathway, including NRAS, KRAS and MEK1 mutations, NF1 loss of function, as well as mutations in the AKT/PI3K

pathway, such as AKT mutations and loss of PTEN [16,103–106]. Additional mechanisms of resistance include those that result from overexpression of RTKs (PDGFRB, IGF-1R, EGFR), COT (MAP3K8) kinase, amplification of BRAF or dimerization of alternatively-spliced BRAF isoforms [16,17,107–110]. In addition, treatment with BRAF and MEK inhibitors leads to relief of feedback inhibition of MAPK signaling. In BRAF^{V600E} cancer cells, hyperactivated ERK signaling results in feedback suppression of upstream factors, such as SOS and RTKs, thereby inhibiting RAS activity. When BRAF signaling is pharmacologically inhibited, ERK-mediated feedback is relieved, RAS-GTP levels increase, and subsequently a variety of RAS-mediated signaling pathways get activated [37,111]. By relieving the negative feedback regulation of the MAPK pathway, BRAF and MEK inhibitors attenuate their maximal efficacy through the induction of adaptive resistance mechanisms. Such resistance is often explained by activation of pro-growth pathways, including both cell-autonomous mechanisms, such as up-regulation or rewiring of mitogenic signaling cascades, and non-cell-autonomous changes in the microenvironment. Studies of adaptive responses in BRAF-mutated cancers have shown that these responses are remarkably diverse [38]. They involve different combinations of ERK pathway reactivation and induction of parallel signaling cascades such as the PI3K/AKT, JNK/c-Jun and NF- κ B pathways, with the net effect of reducing drug efficacy. The breadth and complexity of these responses, however, have challenged traditional approaches of developing unified schemes to prevent BRAF and MEK inhibitor therapy resistance in BRAF-mutant tumors.

5. Epigenetic Mechanisms of MAPK Dependency in BRAF-Mutated Tumors

The term “epigenetic” generally refers to non-genetic, heritable mechanisms of gene regulation that contribute to cell fate and phenotype. In such cases, cellular phenotypes are governed by mechanisms that control differential gene expression profiles. Mechanisms of epigenetic regulation include DNA methylation, mRNA processing, micro(mi)RNAs, RNA stability, nucleosomal positioning and occupancy, and chromatin remodeling [112]. Key components involved in shifting chromatin architecture and providing transcription factors access to relevant genes involve DNA methylation and post-translational modifications (PTMs) on core histone proteins. These modifications allow chromatin to exist in an active (i.e., open) or inactive (i.e., closed) state, corresponding with either promoting or inhibiting transcription of relevant genes. In cancer, epigenetic remodeling can promote a dedifferentiated or progenitor-like state, aiding cells with oncogenic mutations and helping to promote tumorigenesis.

Epigenetic mechanisms (together with genetic factors) contribute to both inter- and intratumor heterogeneity. Intertumor heterogeneity occurs between patients diagnosed with tumors sharing the same histology. Intratumor heterogeneity, on the other hand, occurs in tumor cells within the same patient. As a result of such heterogeneity, different subpopulations of tumor cells with a range of epigenetic states may dynamically co-exist within the same tumor. The baseline epigenetic state of a tumor cell is established by its developmental lineage and differentiation state [113,114]. However, microenvironmental perturbations, such as drug treatment or unfavorable metabolic conditions, compel a cell to shift its epigenetic state by inducing modifications in the chromatin structure, modulating gene expression profiles and adjusting its proteomic state. Changes in the proteomic state, as influenced by epigenetic modifications, determine phenotypic consequences by regulating the adaptive or compensatory signals initiated by cells to ensure cell survival. Overall, these mechanisms, present intrinsically or employed by the cancer cells following drug treatment, can lead to their fractional responses to therapies [32,115–118]. They nonetheless provide new therapeutic vulnerabilities that could be exploited to combat drug resistance [119].

In the case of BRAF-mutant melanomas, tolerance to BRAF and MEK kinase inhibitors is associated with distinct phenotype plasticity of melanoma differentiation state and global alterations in gene expression programs [5,26,120]. These programs include: (i) a pigmented melanocytic state associated with transcription factor MITF, a key regulator of melanocyte lineage [121–123], (ii) a neural crest-like state characterized by NGFR and markers of the neural crest, a melanocyte precursor [35,124,125],

and (iii) an undifferentiated state characterized by low levels of the transcription factor SOX10 and high expression of receptor tyrosine kinases such as AXL and EGFR [44,45]. Despite the recurrent composition of differentiation states among melanomas, the frequency of individual states appears to vary from one tumor to the next [46]. Such heterogeneity could influence the nature and relative strength of the pathways through which each tumor responds or adapts to BRAF and MEK inhibition [126]. At the single-cell level, transcriptional heterogeneity and epigenetic reprogramming enable drug resistance in BRAF^{V600E} melanoma cells. Prior to MAPK inhibitor treatment, cells display transcriptional variability with transient fluctuations in resistance genes. This phenomenon allows cells to transition between a non-resistant and pre-resistant phenotype. Upon drug addition, epigenetic reprogramming causes the generation of transiently heritable resistance phenotypes [47]. Long-term drug treatment can ultimately enrich drug-adapted cells with additional mutations that permit cell survival and lead to stable drug resistance [51].

Although both genetic and epigenetic factors contribute to drug resistance, the intricacies of the interplay between the two groups of mechanisms and their control of plasticity and tumor heterogeneity in BRAF-mutant cancers remains to be fully elucidated. What is clear is that they need not be mutually exclusive. Through the emergence of a drug-tolerant, dedifferentiated, slow-cycling cell subpopulation, BRAF-mutant melanomas treated with MAPK inhibitors adapt to drug over time. During the adaptation process, alterations in the transcriptome or epigenome may be transient and reversible upon drug removal [35], but continuous drug treatment can stabilize these transcriptionally and epigenetically-altered phenotypic states [127]. Epigenetic mechanisms that regulate gene expression and phenotypic states may include DNA methylation, histone-modifying enzymes and histone modifications.

6. DNA Methylation in BRAF-Mutated Cancers

In eukaryotes, DNA methylation is a process by which methyl groups (-CH₃) are covalently transferred to the cytosine bases of DNA. The transfer process is catalyzed by three specific enzymes called DNA methyltransferases (DNMTs). DNMTs use the methyl donor, S-adenosyl methionine (SAM) to transfer a methyl group to the 5-carbon of cytosine, producing 5-methylcytosine (5-mC). Additional modifications of cytosines include 5-formylcytosine (5-fC), 5-hydroxymethylcytosine (5-hmC), and 5-carboxylcytosine (5-caC) at CpG sites [128–131]. CpG sites or dinucleotide regions refer to a portion of the genome with a cytosine base preceding a guanine base with the 'p' denoting a phosphodiester bond that is shared between the dinucleotides. CpG sites can be found in coding regions of genes and are usually methylated. CpG islands refer to short DNA sequences that are mainly CG-rich (60%) and remain unmethylated. CpG islands are usually found in gene promoters or transcription start sites of either genes poised for transcription or genes that are highly transcribed [132]. On the other hand, CpG shores, which refer to regions of the DNA that surround CpG islands, contain fewer CG dinucleotides and correlate with gene expression [133,134].

Hypomethylation, defined as the absence or loss of the methyl group from cytosines, can open up the tightly-packed chromatin, leading to genomic instability and chromosomal abnormalities, such as translocations and deletions [135]. Hypermethylation, in contrast, can lead to transcriptional repression. Approximately 80% of the CpG sites in the human genome are methylated [136]. Cancer cells display decreased levels of 5-methylcytosine [137] as well as global DNA hypo- or demethylation and de novo site-specific hypermethylation. About 5–10% of CpG islands at promoters, which are normally unmethylated, become hypermethylated in many cancers [138]. These genes code for tumor suppressors and DNA repair proteins [139,140]. Of the 8–15% of colorectal cancers with an activating BRAF^{V600E} mutation, most have been found to contain genome-wide hypermethylated CpG islands, termed CIMP for CpG Island Methylator Phenotype [141–143]. In a study of serrated polyps and colorectal cancers, the CIMP-high cancers were more frequently identified with the BRAF^{V600E} mutation, implying a significant association between DNA methylation and BRAF mutational status [144]. Although there is a strong association between CIMP and colorectal cancers

harboring BRAF^{V600E} mutations, the relationship between CIMP and BRAF^{V600E} melanomas is less clear. In cutaneous melanoma, TCGA analysis revealed a lower prevalence of BRAF hot-spot mutations in CIMP clusters [56]. Moreover, comparison of BRAF^{V600E} and BRAF^{WT} melanomas revealed no significant difference in the number of hypermethylated peaks [145]. These differences hint at the differential contribution of tissue-specific factors and alternative gene dependencies and programs within each tumor type. Interestingly, the pathway regulating hypermethylation of CIMP genes in BRAF^{V600E} colorectal cancers was also found to be important in epigenetic silencing in BRAF^{V600E} melanomas. This hypermethylation process was mediated by the transcriptional repressor, v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G (MAFG). BRAF^{V600E}-mediated ERK activation resulted in phosphorylation and subsequent preservation of MAFG levels, leading to an increase in MAFG-bound DNA regions. Following DNA binding, MAFG recruited co-repressor molecules, including DNMT3B, BACH1 and CHD8; this co-repressor complex was involved in hypermethylating and silencing the promoter regions of CIMP genes [146].

Besides aberrant DNA methylation marks, mutations in the DNMT enzymes have also been identified in cancer. DNMT3B, considered a de novo DNA methyltransferase for depositing and maintaining methyl marks, has been implicated in tumor progression [147,148]. DNMT3B expression is upregulated in melanoma and is associated with reduced patient survival. In a genetically engineered BRAF^{V600E}, PTEN knockout melanoma mouse model, DNMT3B loss delayed melanoma formation and increased animal survival [149]. In drug-tolerant BRAF^{V600E} melanoma cells that emerged following MAPK inhibitor treatment, DNMT3A, DNMT3B, and DNMT1 were differentially expressed, resulting in low global DNA methylation levels [150]. An integrated epigenetic analysis of human tumors treated with MAPK inhibitors identified drug resistance programs corresponding with transcriptomic and methylomic alterations [15]. Transcriptomic analysis revealed differential mRNA expression in genes such as c-MET, LEF1 and DUSP4 in MAPK inhibitor-resistant tumors. Such gene expression patterns correlated with differential methylation at CpG clusters, suggesting key connections between epigenetic regulation of DNA methylation patterns, gene expression profiles and MAPK inhibitor resistance.

7. Histone-Modifying Enzymes and Post-Translational Modifications in BRAF-Mutated Cancers

In eukaryotic cells, the basic structural unit of chromatin, termed nucleosome, consists of DNA wrapped around an octamer of four core histone proteins. A histone octamer is comprised of two copies each of four histone proteins, including H2A, H2B, H3, and H4. A linker histone, H1, functions to stabilize the nucleosome structure. The N-terminal tails of these histone proteins can be covalently and reversibly altered with a diverse array of post-translational modifications (PTMs), including methylation, acetylation, glycosylation, phosphorylation, ADP-ribosylation, crotonylation, citrullination, sumoylation, and ubiquitylation. These dynamic histone PTMs and the specific histone protein that is modified can promote either transcriptional activation or repression of applicable genes by remodeling the chromatin structure. Chromatin remodelers include histone-modifying enzymes (HMEs), which are categorized into three main groups: (a) readers, enzymes that recognize histone PTMs and facilitate recruitment of protein complexes; (b) erasers, enzymes that remove a histone PTM; and (c) writers, enzymes that deposit the histone PTMs. HMEs that establish histone PTMs include, but are not limited to, histone methyltransferases and histone acetyltransferases (HATs), whereas HMEs that erase PTMs include histone demethylases and histone deacetylases (HDACs). HMEs function in complexes (i.e., Polycomb and Trithorax) and regulate gene expression by facilitating either the opening of condensed chromatin to allow transcriptional machinery access to specific promoters, or the compacting of open chromatin to prevent relevant genes from being transcribed. Besides modifying residues on the histone tails, HMEs can also target non-histone proteins for regulation, such as p53, Rb and Myc. Together, the combination of histone PTMs, constituting the “histone code”, as well as the presence of bivalent histone PTMs, referring to promoters possessing both activating and repressing marks, reveal the complexity of chromatin regulation and remodeling [151,152].

The global pattern of histone PTMs have been used to predict clinical outcome in prostate cancer recurrence, which is indicative of the close relationship between histone PTMs and disease [153]. Changes in the global levels of histone PTMs and mutations in HMEs have also been linked to additional cancers [154–156]. In a zebrafish model of BRAF^{V600E} and p53-mutated melanoma, several genes were found to cooperate with BRAF^{V600E} to accelerate melanomagenesis. SETDB1, a lysine methyltransferase that functions by depositing a methyl mark on histone H3 lysine 9 (H3K9), was found to increase melanoma formation [157]. In addition to lysine methyltransferases, two structurally unrelated H3K9 lysine demethylases, LSD1 and JMJD2C cooperate with BRAF to overcome oncogene-induced senescence (OIS) and contribute to the development of malignant melanoma [48, 158]. Whereas BRAF (or RAS) expression in premalignant melanocytic nevi leads to OIS—which is characterized by cell-cycle arrest and an impediment of tumor formation—the overexpression of JMJD2C or LSD1 in BRAF-mutated cells promotes melanomagenesis by overcoming the dynamic phenotypic state enabled by BRAF-induced senescence. BRAF-mutated cells overcome OIS by utilizing epigenetic reprogramming to their advantage in promoting tumor formation. Such opportunistic methods employed by BRAF-mutant cells also expose their therapeutic vulnerabilities, as evidenced by the sensitivity of BRAF-mutated melanoma cells to LSD1 and JMJD2 inhibitors. These findings are especially powerful since epigenetic dependencies (LSD1 or JMJD2C) in melanoma are not associated with sensitivity to BRAF inhibitors, implicating a potential therapeutic approach for patients relapsing on BRAF-targeted therapies.

Changes in expression levels or activities of key histone-modifying enzymes (HMEs) have been associated with phenotype plasticity and drug tolerance in a variety of cancers. In EGFR-mutant non-small cell lung cancer (NSCLC) cells treated with EGFR inhibitor gefitinib, a reversibly drug-tolerant persister phenotype was reported to be dependent on JARID1A (KDM5A), a Jumonji histone demethylase that removes methyl groups from histone H3 lysine 4 (H3K4) [32]. Similarly, exposure of BRAF^{V600E} melanoma cells to sublethal levels of stress-inducing conditions, such as BRAF and MEK inhibitors, induced the surviving cells to adopt a less differentiated, drug-tolerant state, with elevated expression of histone demethylases such as KDM6A, KDM6B, KDM1B, JARID1A, and JARID1B [28,29,31]. These modifications were concomitant with increased levels of H3K9me3 and lower levels of H3K4me3 and H3K27me3, indicating epigenetic activation and silencing of select genes [31]. Several histone methyltransferases, such as SETDB1 and SETDB2, have also been shown to be upregulated in response to BRAF and MEK inhibition while knockdown of these HMEs restored drug sensitivity [150]. In additional studies of BRAF^{V600E} melanoma cells that were resistant to MAPK inhibitors, expression of the histone deacetylase SIRT6 was shown to be downregulated [116]. Interestingly, only partial, but not complete loss of SIRT6 (i.e., haploinsufficiency) conferred resistance to BRAF and MEK inhibitors. In this case, SIRT6 haploinsufficiency led to upregulation of the IGF-1 receptor (IGF-1R) and subsequent AKT pathway activation.

8. Alternative Combinatorial Treatments to Overcome Therapy-Resistant BRAF-Mutated Cancers

Acquired resistance, due to intratumor heterogeneity, remains an obstacle for the complete eradication of tumor cells. The emergence of reversible drug-tolerant persister (DTP) cells may lead to fully resistant clones. However, these DTP cells also possess novel and exploitable vulnerabilities. For example, cancer cells that lose epithelial markers and obtain a high-mesenchymal cell state not only correlate with the ability to become therapy-resistant [159], but also have increased vulnerability to compounds inducing ferroptosis and statins [115,118]. Specifically, these compounds are more effective in cancer cells with a high-mesenchymal state due to dependency on the lipid glutathione hydroperoxidase, GPX4. If GPX4 is inhibited, the DTP cells, which rely on GPX4, undergo ferroptotic cell death [115,118].

In a similar vein, other vulnerabilities of therapy-resistant cancer cells need to be examined and exploited to harness a more complete and stable response to therapy. In BRAF-mutant melanoma and NSCLC cells, the YAP protein, a component of the Hippo tumor-suppressor pathway, is an

important mediator of resistance to MAPK-inhibitor therapy. Sensitivity of BRAF-mutant cells to MAPK inhibitors increases upon YAP inhibition [160,161]. Drug resistance in YAP-overexpressing BRAF-mutant melanoma cells has been mediated by BET bromodomain proteins. Inhibition of BET proteins in drug-resistant cells reverts these resistant cells to a sensitive state and impairs tumor cell viability [162]. In BRAF^{V600E} melanomas, combination treatment inhibiting BET bromodomains and either BRAF^{V600E} or MEK kinases synergistically promote melanoma cell death and block cell proliferation [95,135,163,164]. BRAF^{V600E} melanoma cells with acquired resistance to BRAF/MEK inhibitors have been shown to exhibit increased production of ROS [127,165]. As a result of high ROS production, drug-resistant cells acquire susceptibility to HDAC inhibitors, which inhibit the cystine/glutamine transporter, SLC7A11, further increasing the ROS levels to lethal quantities (Figure 2A) [127]. While this study highlights the importance of sequential treatment of MAPK and HDAC inhibitors in combating MAPK inhibitor-resistant cells, another study found the simultaneous combination of MAPK inhibitors and Class I HDAC3 inhibitor, entinostat, was able to suppress melanoma tumor growth (Figure 2B) [166]. Besides HDACs, pharmacological inhibition of H3K9 demethylases alone or in combination with MAPK demethylases may also provide benefit for the treatment of BRAF^{V600E} melanomas (Figure 2C) [48].

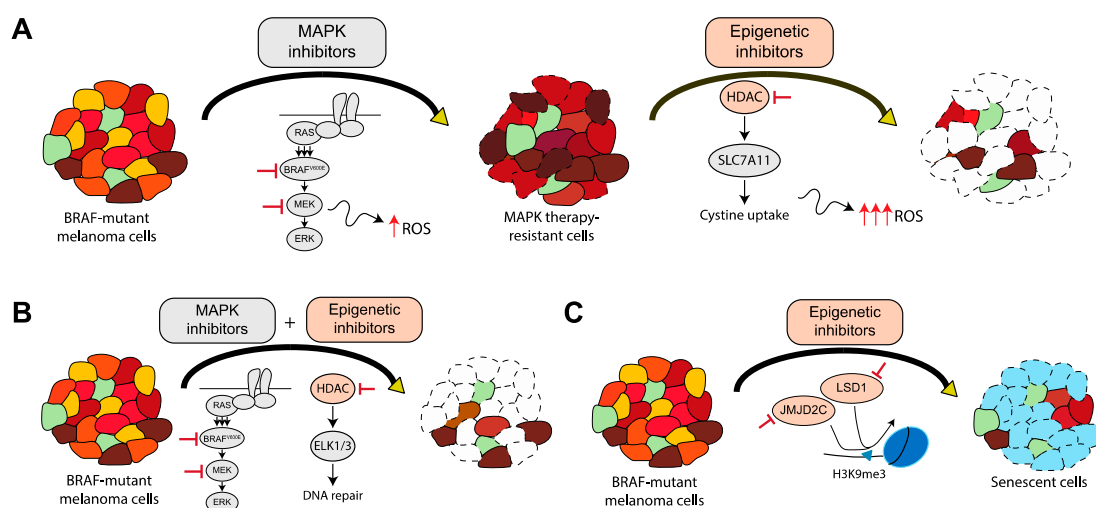


Figure 2. Strategies to target epigenetic mechanisms of drug resistance in BRAF-mutant melanoma cells. (A) BRAF-mutant melanoma cells with acquired resistance to MAPK inhibitor therapy are vulnerable to HDAC inhibitors, which suppress the transporter SLC7A11 and increase reactive oxygen species (ROS) to lethal levels, causing apoptosis. (B) Treatment with a combination of MAPK and HDAC inhibitors is effective in inducing apoptosis in BRAF-mutant melanoma cells. The combination treatment reduces ERK1 and ERK2 levels, resulting in aberrant activity of DNA repair mechanisms and subsequent melanoma cell death. (C) Treatment of BRAF-mutant melanoma cells with LSD1 or JMJD2C inhibitors reduces melanoma cell growth and restores oncogene-induced senescence.

In addition to histone erasers and readers, histone writers are also dysregulated in cancer. A component of the Polycomb Repressive Complex 2 Subunit (PRC2), EZH2 is a histone lysine methyltransferase, which catalyzes trimethylation of H3K27. In melanoma, prostate and breast cancer, EZH2 is overexpressed [167]. When EZH2 is inhibited in melanoma, both cell proliferation and the invasive potential of melanoma cells is reduced [168]. In addition, the transcription factor, NFATc2, is upregulated in melanoma and its inhibition suppresses EZH2 levels. BRAF^{V600E} cell lines, which have intrinsic resistance to vemurafenib, exhibited a marked increase in cell death upon the combination treatment of both NFATc2 and EZH2 inhibitors [169].

The task of identifying novel therapies is multi-faceted and future work requires an in-depth understanding of epigenetic mechanisms, tumor heterogeneity, and drug resistance. This involves elucidating how each component influences or interacts with each other to mediate cellular response and how these data can be applied to unveil novel therapeutic strategies for improving clinical outcomes.

9. Conclusions

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9. Conclusions

Therapy-induced acquired resistance to BRAF and MAPK inhibitors relies on the cooperative communication between genetic, non-genetic and epigenetic mechanisms. Following drug treatment, biochemical and epigenetic reprogramming can dynamically regulate gene expression profiles, leading to complex and fluctuating phenotypic states. These phenotypically plastic cells are selected upon MAPK inhibitor treatment, demonstrating a loss of BRAF dependency in drug-tolerant cells.

Research into the epigenetic mechanisms of BRAF dependency or escape from MAPK pathway dependency in BRAF-mutated cancers is still in its infancy. Progress has been made in understanding transcriptional programs associated with the differentiation states of BRAF-mutated cancers and MAPK inhibitor resistance, but we are only beginning to uncover how differentiation states are regulated and how their dynamic fluctuations can lead to drug tolerance. Systems biology approaches, combining single-cell and molecular analyses, can help to elucidate the contribution of epigenetic states, reprogramming and cellular response in determining BRAF dependency. More importantly, in MAPK inhibitor-resistant cells, the discovery of acquired epigenetic dependencies holds great promise for the development of new, effective therapies.

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