Genomic classification of the RAS network identifies a personalized treatment strategy for lung cancer

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\textbf{ABSTRACT}

Better approaches are needed to evaluate a single patient’s drug response at the genomic level. Targeted therapy for signaling pathways in cancer has met limited success in part due to the exceedingly interwoven nature of the pathways. In particular, the highly complex RAS network has been challenging to target. Effectively targeting the pathway requires development of techniques that measure global network activity to account for pathway complexity. For this purpose, we used a gene-expression-based biomarker for RAS network activity in non-small cell lung cancer (NSCLC) cells, and screened for drugs whose efficacy was significantly highly correlated to RAS network activity. Results identified EGFR and MEK co-inhibition as the most effective treatment for RAS-active NSCLC amongst a panel of over 360 compounds and fractions. RAS activity was identified in both RAS-mutant and wild-type lines, indicating broad characterization of RAS signaling inclusive of multiple mechanisms of RAS activity, and not solely based on mutation status. Mechanistic studies demonstrated that co-inhibition of EGFR and MEK induced apoptosis and blocked both EGFR-RAS-RAF-MEK-ERK and EGFR-PI3K-AKT-RPS6 nodes simultaneously in RAS-active, but not RAS-inactive NSCLC. These results provide a comprehensive strategy to personalize treatment of NSCLC based on RAS network dysregulation and provide proof-of-concept of a genomic approach to classify and target complex signaling networks.

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1. Introduction

Cancer is among the principal causes of disease in the world, with an approximated 12.7 million new cancer cases occurring in 2008 (International Agency for Research on Cancer and Cancer Research UK, 2012). The RAS proto-oncogenes are frequently mutated in human cancers, with constitutively active mutations observed in approximately one third of human tumors (Baines et al., 2011; Downward, 2003; Riely et al., 2009). In humans, three genes encode four different RAS proteins: HRAS, NRAS, KRAS-4A and KRAS-4B, the latter two being alternative splice variants of the KRAS gene (Pylayeva-Gupta et al., 2011). These highly homologous GTPase proteins, sharing 90% amino acid identity (Baines et al., 2011), localize to the cytosolic part of the plasma membrane, where they cycle through “on” (GTP-bound) and “off” (GDP-bound) states (Downward, 2003; Karnoub and Weinberg, 2008). RAS can be switched on through multiple mechanisms, such as via receptor tyrosine kinases like EGFR and other growth factor receptors like PDGFR and IGFR (Bazenet and Kazlauskas, 1993; Chan et al., 2001; Innocenti et al., 2002; Ono and Kuwano, 2006; Repasky et al., 2004), or by obtaining activating mutations in the gene (Downward, 2003). The most common RAS mutations occur in codons 12, 13 and 61 (COSMIC Database; Schubbert et al., 2007), all of which impair hydrolysis of GTP (Downward, 2003). These variants cause RAS to remain in an active GTP-bound state, promoting its oncogenic effects for extended periods of time (Pylayeva-Gupta et al., 2011). Activated RAS can interact with more than 20 effectors to regulate various cellular responses, including cellular proliferation, survival and differentiation (Cox and Der, 2010; Der, 2012; Pylayeva-Gupta et al., 2011; Schubbert et al., 2007). Despite extensive efforts, the RAS proteins have remained undruggable targets (Baines et al., 2011)—no therapies exist in the clinic to directly treat RAS-active tumors (Baines et al., 2011; Gysin et al., 2011).

Targeting the RAS pathway would be beneficial for lung cancer, the leading neoplasm in incidence and mortality in the world (International Agency for Research on Cancer and Cancer Research UK, 2012). KRAS mutations occur in 20–30% of non-small cell lung cancers (NSCLC), which make up 87% of all lung cancer cases; these mutations occur predominantly in the adenocarcinoma subtype of NSCLC (Aviel-Ronen et al., 2006; Graziano et al., 1999; Minna and Larsen, 2011; Roberts et al., 2010), and they represent the most common molecular change in NSCLC (Roberts and Stinchcombe, 2013). In the US, an estimated 228,190 new cases of lung cancer are predicted to have occurred in 2013, with an approximate 70% fatality rate (American Cancer Society, 2013). The 5-year survival rate for lung cancer has only modestly improved since 1975, increasing from 12% in 1975 to 16% in 2007. Lung cancer today still accounts for more deaths than any other cancer in both men and women, killing almost three times as many men as prostate cancer, and almost twice as many women as breast cancer (American Cancer Society, 2013). The survival rate is even worse for KRAS positive lung cancer patients (Guan et al., 2013; Johnson et al., 2012; Meng et al., 2013; Roberts and Stinchcombe, 2013), highlighting the desperate need for novel therapeutics that can treat RAS-active tumors.

The RAS pathway is a large and complicated signaling cascade, comprising a network as opposed to a linear pathway. It is composed of numerous interacting proteins, upstream and downstream of RAS, providing feedback and crosstalk to the different components of the pathway (Stites et al., 2007). Together, these components establish and promote tumorigenic effects in the cell (Cox and Der, 2010; Pylayeva-Gupta et al., 2011). Numerous growth factor receptors, such as EGFR, PDGFR and IGF are among the upstream proteins that can activate the RAS pathway (Bazenet and Kazlauskas, 1993; Chan et al., 2001; Innocenti et al., 2002; Ono and Kuwano, 2006; Repasky et al., 2004); downstream effector proteins include ERK, PI3K, and RPS6, which each have been shown to have primary roles in cell proliferation and survival (Fan et al., 2009; Schubbert et al., 2007). Thus, tumors cells can have an activated RAS pathway by dysregulation of up or downstream pathway components, even without harboring a RAS mutation. Relying on biochemical analysis of RAS through mutation testing and measuring the active form of RAS (GTP-bound form) as means to record RAS-pathway activation status is limited; the existence of mutated RAS does not necessarily predicate an oncogenic addiction to the RAS pathway, as it has been previously shown that KRAS dependency is widely variable in KRAS-mutant cancer cell lines. Therefore, cancer cells may harbor a mutation in RAS, but not necessarily be addicted and dependent on the continual signaling of the pathway (Singh et al., 2009). Furthermore, if RAS is not mutated, it cannot be assumed that the network is “off”, as additional components of the network may still be activated. In addition to redundancy in the network, the relative importance of different RAS network components may be context dependent; thus, focusing on a single protein might limit the ability to accurately reflect activity (Downward, 2009). Therefore, it is critical to study RAS-pathway activation in a comprehensive manner; we use a genomics framework to accomplish this goal.

In order to effectively measure activity in the RAS network, we utilize a RAS gene-expression signature capable of providing a network-scale measurement of activation by measuring the acute transcriptional changes that occur after RAS activation (Bild et al., 2006). As RAS can be activated by many mechanisms, it is critical to measure the RAS network more comprehensively than just by analysis of mutation status in order to obtain a reliable predictor of pathway activation. The need for comprehensive pathway measurements is especially true for complex branching pathways such as RAS (Downward, 2006). To that extent, our RAS gene-expression signature has been previously validated to accurately predict RAS-pathway activity in a variety of diverse settings; including 1) prediction of activity in primary human NSCLC adenocarcinoma tumors (Bild et al., 2006), 2) predictions of RAS activity in a multitude of cancer subtypes represented in Oncomine (Rhodes et al., 2007), 3) measurement of RAS-pathway activity in gastric cancer and ER + breast cancer bone metastasis (Ooi et al., 2009; Zhang et al., 2009) 4) and analysis of K-RAS dependency signature genes and “RAS addiction” in primary lung tumors (Singh et al., 2009). Together, these studies provide support to the robustness
and accuracy of the RAS signature to analyze RAS network activation in multiple settings.

In this study, we develop a network-based genomics framework for drug discovery. Specifically, we use a RAS gene-expression signature to discover therapeutic regimens that target the RAS network in lung cancer. We used this signature to determine RAS activity in a panel of NSCLC cell lines — these RAS-pathway activity measurements for each cancer sample are on a continuous scale, and can be correlated with drug response across the panel of cancer cell lines activity (West et al., 2001). Therefore, we identify compounds whose efficacy correlates to the genomics-based measurement of RAS activity in a drug screen that included 366 known and novel drug compounds. The results from the genomics-based drug screen identified that combined inhibition of EGFR and MEK pathway components most effectively inhibited RAS-active tumor cells. Indeed, there was a highly significant and reproducible correlation between treatment response and RAS-pathway activity in a large panel of lung cancer cell lines, highlighting the ability of this drug combination to selectively block tumor cells with RAS activation independent of the manner in which the RAS pathway is turned on. Additional novel compounds or drug regimens that target other components of the RAS network were not as effective at inhibiting RAS-active tumor cells. We show that in combination, these treatments block both the EGFR-RAS-RAF-ERK and EGFR-Pi3K-AKT-RPS6 nodes of the RAS-pathway network and induce apoptosis, while either drug alone did not effectively inhibit both nodes. These results indicate that RAS-RAS-Raf-ERK and EGFR-Pi3K-AKT-RPS6 are key nodes important for RAS-activated lung cancer tumor cell survival. Our study combines genomic profiling with a high-throughput drug screen to guide the discovery of treatments that can be used to target certain cancer phenotypes. This approach can individualize drug therapies to target signaling pathways more efficiently through gene-expression profiling of network activation (Ascierto et al., 2013; Bentley et al., 2013; Favata et al., 1998; Janne et al., 2013; To et al., 2012).

2. Materials and methods

2.1. Small molecules

Gefitinib, AEW541, erlotinib, trametinib, U0126, sorafenib, and temsirolimus were purchased from Selleckchem and dissolved in 100% DMSO to generate 100 mM stock solutions of each, stored at −80 °C. For erlotinib, the 100 mM stock solution was further diluted to 30 mM in 100% DMSO for complete solubility. Novel compounds were provided by Dr. Chris Ireland and Dr. Sunil Sharma at the University of Utah.

2.2. Genomic data acquisition and normalization

We used gene-expression microarray data that had previously been used to profile the transcriptomic effects of RAS-pathway activation (Barbie et al., 2009; Bild et al., 2006; Boutros et al., 2009; Chang et al., 2009; Kim et al., 2009; Watanabe et al., 2011). We downloaded gene-expression microarray data for lung cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012). Collaborators at Duke University also provided gene-expression data for 56 lung cancer cell lines. This dataset was uploaded to the Gene-Expression Omnibus (GEO) under accession identifier GSE47206. We MAS5 normalized (Hubbell et al., 2002) these datasets using the affy Bioconductor package (Gautier et al., 2004) for our analysis.

2.3. RAS-pathway activation predictions

Using the RAS gene-expression signature (Barbie et al., 2009; Bild et al., 2006; Boutros et al., 2009; Chang et al., 2009; Kim et al., 2009; Watanabe et al., 2011), we predicted RAS-pathway activation for each cell line using the Bayesian binary regression algorithm version 2.0 (BinReg2.0) used as a MATLAB plug-in (West et al., 2001). Prior to making the predictions, the data were log2 transformed and DWD normalized (Benito et al., 2004) to reduce biases that can result from differences in batch processing and microarray platforms. In making the predictions, we used default parameters, except that our signature used 350 genes and 1 metagene (as determined previously to be optimal for the RAS pathway) (Bild et al., 2006). The CCLE dataset was used for the expanded lung and breast cancer cell line predictions, while GSE47206 was used for the 14 lung cancer pilot experiments. For the pilot screen, the SK-MES-1 RAS-pathway activation value was obtained from the CCLE dataset run, as that cell line was not available in the GSE47206 dataset.

2.4. Preliminary genomics-based drug screen assay

Drugs were serially diluted 1:3 in 8 doses of each drug, starting from 30 μM and ending with 13.7 nM. To make the highest doses soluble in aqueous 5% FBS RPMI media solution, the drugs were sonicated twice on ice, and then used for serial dilution. For combinatorial treatments, doses had equal molar concentrations for each compound. All treatment doses were performed in four replicates. Cell viability and growth was measured using CellTiter-Glo (Promega, Madison, Wisconsin) 72 h post-treatment. EC50 values were calculated from dose response data by plotting on GraphPad Prism 4 and using the equation $Y = 1/(1 + 10^{(logEC50 - X)/HillSlope})$ with a variable slope ($Y_{min} = 0$ and $Y_{max} = 1$). Plots were forced to start from the x-axis by plotting for an x-intercept point. Predictions were then correlated against EC50 values of the treatments, and an unbiased approach was used to identify candidate therapies by selecting drugs based on Pearson correlation values of less than or equal to −0.5, significant two-tailed unpaired t-test p-values ($p < 0.05$) and a 95% confidence interval calculated in GraphPad Prism 6.01.

2.5. Novel compounds and fractions drug screen and expanded dose response assays

Cell lines were plated at 1500 cells/well. Detailed information on the cell lines used and their growth conditions is provided in the Supplementary Information sheet. Cell lines were obtained from ATCC. For the dose response assays, known targeted therapeutics were serially diluted 1:3 from 90 μM to the lowest dose of 41.15 nM in media containing 5% FBS.
(Gibco/Life technologies, Carlsbad, CA) and 1× Anti–Anti (Gibco/Life technologies, Carlsbad, CA). To make the highest dose soluble in aqueous media, drugs were sonicated twice on ice. For combination treatments, doses had equal molar concentrations for each compound. Cell viability was measured as described before. Every dose was done at least in duplicate. Dose response curves were generated using the same methodology described above. For novel fractions and compounds used in the screen, two or three doses of each were used respectively. For the novel compounds, the dose most negatively correlated to predicted probability of RAS-pathway activation was represented on the histogram. For the known targeted therapeutics, drug EC50 correlation to RAS-pathway activation was plotted.

2.6. Statistical and multivariate analysis

Linear correlation graphs and box plots were created using Graphpad Prism 6.01, and their corresponding statistical significance tests performed using the software. For correlation plots, a built-in two-tailed significance test calculated by Graphpad Prism 6.01 was used. Graphpad computes a t ratio from the Pearson r and the sample size using the Student’s t-distribution method, and computes p from t values using a standard algorithm. For box plots, a standard two-tailed Mann–Whitney U-test was used when two samples were being compared, with the exception of the box plot diagrams for erlotinib + trametinib, where an unpaired t-test with Welch’s correction was used due to the normality of the data and the unequal standard deviations. When testing for significance across more than 2 samples, we adjusted for multiple comparisons using GraphPad’s built-in Dunn’s multiple comparisons test, which compares the mean rank difference among samples against an α = 0.05. For the Annexin V Apoptosis assay, Dunn’s multiple comparison significance test was used to compare the Annexin V positive cell means of the different drug treatments against the DMSO control, for RAS-active and RAS-inactive cell lines separately. We sought to model the multivariate relationship between gefitinib + U0126 EC50 and the predictor variables: RAS-pathway activity prediction, tumor subtype (adenocarcinoma, large cell carcinoma, or squamous cell carcinoma), KRAS mutation, TP53 mutation, MEK1 mutation, and EGFR mutation. The mutations were coded as (Y/N) based on observations from the CCLL hybrid capture sequencing dataset; we used mutations listed as non-neutral variant SNPs. We then used a two-step approach to construct a parsimonious model. First, a univariate analysis was used to determine the set of predictor variables that were individually associated with gefitinib + U0126 log10 EC50. t-tests were used for binary predictors (mutations), and linear models were used for the continuous predictor (RAS-pathway activation) and for the ternary predictor (tumor subtype). Only those variables that were individually significant (p < 0.05, unadjusted) were included in the multivariate analysis. The multivariate analysis was a linear model. The log10 EC50 values were used in the analysis as they were much less skewed than the μM EC50 values and more closely agreed with the normality assumptions. The multivariate analysis was performed using the “R” statistical computing software, version 2.15.0 (R Development Core Team, 2011).

2.7. Immunostaining and KRAS-GTP pull down

9 NSCLC cell lines with a range of RAS-pathway activation profiles were used for the protein analysis (H1373, LLC-LC-97TM, SK-MES-1, H441, H1944, H1563, H661, H520, H522). Cells were treated with 5 μM of gefitinib, U0126, gefitinib + U0126 and DMSO control in 5% FBS media and 1× Anti–Anti for 6 h. Cells were washed, lysates extracted and western blots run (Supplementary Methods). Primary antibodies for GAPDH (#5174S), EGFR (#4267S), pEGFR-Y1068 (#22345S), pRAF1-S289/296/301 (#9431S), pRAF1-S338 (#9427S), MEK1/2 (#8572S), pMEK1/2-S217/221 (#9154S), pERK1/2-T202/Y204 (#4370S), pAKT-S473 (#4060S), pERK1/2 (#2237S), pRPS6-S240/244 (#5641S) and pPR6-S253/256 (#2588) were obtained from Cell Signaling Technology (Beverly, MA). RAF1 (sc-373722), AKT1/2/3 (sc-8312), ERK1/2 (sc-292838) and KRAS (sc-30) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The KRAS Activation Assay (Cellbioslabs, San Diego, CA) was used to pull down RAS GTP, according to manufacturer’s protocol and run on a western blot. KRAS-GTP was then blotted for using the described KRAS antibody.

2.8. Annexin V apoptosis assay

RAS-active cell lines H358, Calu-3, H2122 and RAS-inactive cell lines H520, H522 and H661 were plated overnight with 400,000 cells per plate in 60 mm plates and grown in their corresponding media (Supplementary Methods). The selected cell lines exhibited high or low probability of RAS-pathway activation, and the highest or lowest response to the combined therapy regimen. All cell lines selected were lines that were part of the drug screens. Cell lines were treated with 1 μM of gefitinib, U0126, gefitinib + U0126 or DMSO control, which was prepared in 5% FBS media and 1× Anti–anti, for 72 h, then washed and collected for Annexin V staining. For the stain, the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & Propidium Iodide for flow cytometry (Life Technologies, Carlsbad, CA) was utilized according to manufacturer’s protocol. Using flow cytometry analysis, the percentage of Annexin V positive cells was recorded, and the means of the positive Annexin V cells (early-stage apoptosis) calculated for the different treatments, separately for the 3 RAS-active cell lines and the 3 RAS-inactive cell lines.

2.9. Erlotinib and trametinib combination dose response assay

The highest drug combination dose was serially diluted 1:3 in 8 doses, starting from 90 μM and ending with 41.15 nM. To make the highest dose soluble in aqueous 5% FBS RPMI media solution, it was sonicated twice on ice, and then used for serial dilution. Doses had equal molar concentrations for each compound. All treatment doses were performed in four replicates. Cell viability was measured and EC50 obtained as described before in the methods. Predictions were then correlated against EC50 values of the combination treatment using Pearson correlation and the built-in GraphPad p-value test for correlation significance and a 95% confidence interval.
3. Results

3.1. Genomics-based drug screen identifies an effective regimen for RAS-pathway inhibition

The goal of this research is to develop a pathway-based genomics framework that can be used to discover drugs to effectively inhibit difficult-to-target oncogenic pathways such as RAS. Complex signaling pathways underlie many diseases, and it remains difficult to align individual patients with therapies that target these pathways effectively. To address this need, we developed a genomic approach to identify inhibitors that target specific pathways (Figure 1). Briefly, we used our validated genomic signature to estimate the RAS-pathway’s activity in NSCLC cells (Bild et al., 2006). This activity is represented by a continuous scale of probability from low to high (0–1 respectively), and produces a quantitative estimate of the pathway’s activity in cells. We correlated these genomics-based pathway activity measurements with cell line response to in vitro treatment of a large catalog of compounds. We then identified the compounds whose efficacy correlated best with genomics-based pathway activity.

The advantages of our drug screening approach are the following: 1) knowledge of the direct drug target is not necessary and instead focuses on the drug’s effect on overall pathway activity rather than on any single pathway component’s activity; 2) measurement of network activity is more comprehensive than just mutation status, thereby capturing all pathway-active cells, and 3) it is relatively high-throughput in the sense that an unlimited number of genomic pathway profiles can be applied to all compounds tested in the drug screen, facilitating the characterization of drug mechanism.

We carried out an initial drug screen on a panel of 14 NSCLC cell lines, and validated our findings in a two larger drug screens that included over 35 NSCLC cell lines each. For our initial screen, we tested several well-characterized small molecule inhibitors that target different components of the growth factor receptor network; these inhibitors include the EGFR inhibitor gefitinib (Mok et al., 2009; Ono and Kuwano, 2006), IGFR inhibitor AEW541 (Garcia-Echeverria et al., 2004), MEK1/2 inhibitor U0126 (Favata et al., 1998) and a mTOR inhibitor temsirolimus (Hudes et al., 2007), which were administered alone and in paired combinations. The drug response data were then correlated to predicted probabilities of RAS-

![Figure 1](image-url) - The overall design of the Screenome approach to find RAS-driven tumor treatments. The RAS gene-expression signature is used to predict the probability of pathway activation in a panel of cancer cell lines. These same cell lines undergo dose response assays. The EC50 values are calculated and correlated against the predicted probability of RAS-pathway activation. The treatments whose effectiveness correlated negatively to RAS activity (the higher the probability of RAS being on, the more sensitive to the drug) are picked out as potential treatments for tumors with an activated RAS pathway.
pathway activation in our panel of NSCLC cell lines, obtained through the use of our RAS gene-expression signature (Bild et al., 2006) (Figure 1). Our preliminary screen showed a significant correlation between predicted RAS-pathway activation and co-inhibition of EGFR and MEK1/2 through the use of gefitinib and U0126, respectively (R = −0.69, p-value = 0.0066) (Supplementary Figure 1A). A significant correlation between RAS activity, as defined by the genomic signature, and drug efficacy did not exist for either of these inhibitors alone (gefitinib: p = 0.0866, U0126: p = 0.2676), suggesting that the drugs must be combined to effectively block RAS activity in NSCLC cells (Supplementary Figure 1B and C). We also investigated the effects of directly targeting the mTOR component of the growth factor receptor and RAS pathway, but mTOR inhibition through temsirolimus did not lead to growth inhibition of RAS-active cells, even with coupled inhibition of EGFR and MEK inhibition (Supplementary Figure 2A−C), and was therefore not pursued further.

3.2 Validation of the genomics-based drug screen results

Next, to validate and further interrogate the relationship between RAS-pathway activity and response to single and combined drug treatments, we performed multiple larger genomics-based screens on an expanded panel of 39 NSCLC cell lines (Figure 2A and Supplementary Figure 3A). In addition to a panel of well-characterized inhibitors, this larger screen also included over 360 novel drug-like compounds. As with the initial genomics-based screen, these experiments validated that the efficacy of EGFR and MEK1/2 co-inhibition through gefitinib and U0126 was most correlated to RAS activity than any other single or combination drug treatments, resulting in a Pearson R score of −0.57 and p = 0.0002 (Figure 2A and C). Specifically, NSCLC cells with high genomics-predicted RAS activity (predicted probability of RAS-pathway activation > 0.5) had significantly lower EC50 scores (drug dose leading to 50% cell survival relative to untreated cells) than cancer cells with low RAS activity (predicted probability of RAS-pathway activation < 0.5), indicating higher drug sensitivity (p < 0.0001, Figure 2B). Cell lines with high RAS averaged an EC50 = 0.436 (log10) = 2.7 μM, while low RAS cell lines averaged an EC50 = 1.07 (log10) = 11.76 μM. As shown in Figure 2C, no other single drug/drop combination showed stronger correlation to RAS-pathway activity. This finding includes other drugs that target the growth factor receptor pathway such as single agent EGFR or MEK inhibitors, as well as single agent or combination therapies such as the RAF inhibitor sorafenib, and novel drug-like compounds (Figure 2C) (Liu et al., 2006). We compared this result against findings from a recently published study that demonstrated efficacy for IGFR and MEK co-inhibition in RAS-mutant cancers (Molina-Arcas et al., 2013). In our data, cell responses for MEK and EGFR co-inhibition correlated more strongly (R = −0.57) to RAS-pathway activity than MEK and IGFR co-inhibition (R = −0.35).

We also investigated the efficacy of the EGFR or MEK inhibitors in isolation. EGFR and MEK inhibition alone had average EC50 values of 11.87 μM and 10.02 μM respectively in the top 50% of responsive cell lines (Supplementary Figure 3B). In comparison, the combined treatment with MEK and EGFR inhibitors had an average EC50 of 1.83 μM (Supplementary Figure 3B). Additionally, as shown in Figure 2C and Supplementary Figure 3C and D, EGFR but not MEK1/2 inhibition showed an individual correlation to RAS-pathway activity, albeit not as strongly as the combined therapy (EGFR inhibition: R = −0.47, p = 0.0027. MEK inhibition: R = −0.26, p = 0.1068). This result is consistent with observations that KRAS dependent NSCLC cells exhibit some sensitivity to EGFR inhibitors (Singh et al., 2009), yet the combination therapy required one-tenth as much drug as gefitinib monotherapy. Furthermore, the BRAF/CRAF inhibitor sorafenib did not show a significant correlation to RAS activity, both alone (p = 0.1247) or in combination with EGFR (p = 0.455) and MEK1/2 (p = 0.5356) inhibitors (Supplementary Figure 4A−C). In support of this finding, sorafenib also failed to show efficacy in patients with KRAS mutated NSCLC in the phase III MISSION trial (Goodman, 2012).

The effectiveness of the EGFR/MEK inhibition as a treatment for NSCLC in general is further highlighted by the overall synergy of these agents in NSCLC cancer cell lines (Supplementary Figures 5A and 6A) and the significantly lower total EC50 scores for combination therapeutics in comparison with monotherapies observed across all the NSCLC cell lines (Supplementary Figure 5B and C). The EGFR + MEK combination exhibited a synergistic response relationship for 29 out of 39 cell lines (74%), signified by a synergy score greater than one (Figure 6A). Synergy was not as prevalent for RAF + EGFR and RAF + MEK inhibitor combinations (Supplementary Figure 6B–D).

Finally, the correlation between EGFR and MEK1/2 inhibitors combined efficacy and RAS-pathway activation was unskewed by the 8 most correlative cell lines (Supplementary Figure 7A) and potentially NSCLC specific, as the relationship between RAS-pathway activity and drug response was not observed in a panel of 35 breast cancer cell lines (Supplementary Figure 7B), possibly due to the dependency of breast cancer on alternate pathways such as PI3K, HER2 and the estrogen receptor pathway (Burstein, 2005; Campbell et al., 2004; Rosen et al., 2010; Sommer and Fuqua, 2001).

3.3 Genomics-based RAS activity predictions, and not RAS mutation status, significantly correlates to EGFR + MEK1/2 inhibitor therapy response

RAS can be aberrantly activated by different mechanisms, including via activating mutations or through dysregulation of other growth factor receptor pathway components (Downward, 2003; Karnoub and Weinberg, 2008; Pylaeva-Gupta et al., 2011). We investigated the relative treatment responsiveness for KRAS mutated cells compared to cells without KRAS mutations. Our studies show that KRAS mutations alone did not account for the responsiveness to combined EGFR/MEK inhibition (p = 0.577, Figure 3A); however, when RAS activity was considered more broadly by measuring pathway activity via our genomic profiling approach, there was a significant correlation between EGFR/MEK drug sensitivity and RAS activity (p = 0.0004, Figure 3B). No significant relationship existed between RAS mutation and drug...
response for any other inhibitor we tested (Supplementary Figures 8 and 9). These results highlight the importance of comprehensively characterizing pathway activity via gene-expression profiles to link pathway activation with drug response.

Next, we performed a single and multivariate analysis to investigate whether the relationship between RAS-pathway activity and EGFR and MEK co-inhibition might be confounded by factors unrelated to RAS-pathway activity or by mutations in EGFR and MEK, the protein targets of gefitinib and U0126 respectively. Potential variables that we evaluated were tumor subtype (adenocarcinoma, large cell carcinoma, or squamous cell carcinoma), KRAS mutation, TP53 mutation, MEK1 mutation, and EGFR mutation, using the adenocarcinoma subtype as the reference sample (Supplementary Table 1). This was especially important since as expected, predicted probability of RAS-pathway activation was the highest in the adenocarcinoma subtype, in comparison to squamous and large cell carcinoma subtypes (Bild et al., 2006) (Supplementary Figure 10).

Figure 2 – Combinatorial inhibition of EGFR + MEK is correlated to RAS-pathway activity in NSCLC. (A) Linear correlation of Gefitinib + U0126 EC50 values with the predicted probability of RAS-pathway activation across 39 NSCLC cell lines. Every dot represents a cell line, with a y value representing the cell line’s Gefitinib + U0126 EC50, and an x value representing the cell line’s predicted probability of RAS-pathway activation. Response to Gefitinib + U0126 is significantly negatively correlated to RAS. Legend = Red: K-RAS-mutant cell lines, Black: K-RAS wild-type cell lines. (B) Cell lines of the 39 NSCLC panel were divided accordingly and box plot diagrams of Gefitinib + U0126 EC50 values were plotted. Box boundaries denote the 25–75th percentiles, while the error bars indicate maximum and minimum values. The line inside the box indicates the median value. Response to Gefitinib + U0126 as measured by EC50 values is shown. Cell lines with elevated probability of RAS activation (predicted probability of RAS-pathway activation > 0.5) were significantly more sensitive to Gefitinib + U0126 treatment than cell lines with low probability of RAS activation (predicted probability of RAS-pathway activation < 0.5). (C) Frequency Distribution of the Correlation of Compounds, Drugs and Fractions with the probability of RAS-pathway activation. A panel of 366 novel small molecules was tested against 39 NSCLC cell lines, and their effects on cell viability correlated to the predicted probability of RAS-pathway activation. These correlation values were plotted on a histogram. Correlation values of temsirolimus, temsirolimus + U0126 and temsirolimus + gefitinib were obtained from the 14 NSCLC cell line preliminary screen and plotted onto the histogram. Gefitinib + U0126 was the treatment most negatively correlated to RAS-pathway activation.
subtype and our RAS-pathway activation predictions as the primary potential predictors of EGFR and MEK1/2 co-inhibition response (Table 1). NSCLC subtype was considered as a single variable with two levels (large cell carcinoma and squamous cell carcinoma), and since one level was significant at \( p = 0.05 \), all levels were included in the multivariate analysis.

Based on the multivariate analysis (Table 2), response to EGFR/MEK inhibition was significantly higher in the NSCLC cell lines with high genomics-based RAS-pathway activity, after adjusting for subtype (\( p = 0.00139 \)). This analysis provides further support to the validity and potential use of our RAS-pathway predictions as a biomarker of response to EGFR + MEK inhibition in NSCLC. Moreover, these results show that the ability of RAS-pathway activity to predict response to combination therapy is not confounded by EGFR nor MEK1 mutations.

3.4. Combined inhibition of EGFR and MEK1/2 blocks key downstream components of the RAS pathway and induces apoptosis in RAS-active, but not RAS-inactive NSCLC cells

Since a variability of response to EGFR and MEK dual inhibition was observed between tumor cells exhibiting an active and inactive RAS pathway, we investigated the potential differences in signaling effects of this drug regimen in NSCLC RAS-active and RAS-inactive tumor cells. We examined the
The phosphorylation of AKT (Figure 3C). This is potentially due to the fact that RPS6 can be phosphorylated by the ERK and AKT arms of the pathway (Roux et al., 2007). Moreover, MEK monotherapy did not inhibit activation of ERK, while MEK inhibition did not inhibit activation of RPS6. Furthermore, a mild decrease in the Ser240/244 phosphorylation mark of RPS6 was observed, as well as a substantial decrease in the activating phosphorylation mark of RAF1 (Ser338) (Supplementary Figure 11A). Lastly, we did not observe the downregulation of ERK activation by MEK inhibition leading to an activation of EGFR upon MEK inhibition (Supplementary Figure 11A), as has been observed in colon cancer, marking a difference between the response of NSCLC and colon cancer to ERK inhibition (Klinger et al., 2013; Prahallad et al., 2012).

Among the two monotherapies and the dual therapy tested, gefitinib was the only treatment to produce RAS inhibition (Supplementary Figure 11B). One explanation for the failure of the combined therapy to inhibit RAS is that the combination of EGFR + MEK inhibition abrogates the effects of RAS inhibition by shutting down EGFR due to the removal of the ERK-mediated negative feedback loop on SOS caused by the complete inhibition of ERK (Shin et al., 2009) (Figure 5). In conclusion, dual inhibition of EGFR + MEK with gefitinib and U0126, respectively, is capable of shutting down both RPS6 and ERK in RAS-active tumors, while single agent treatment only suppresses a single component of the pathway. This is not the case for RAS-inactive tumors, where neither monotherapy nor dual therapy using an EGFR and a MEK inhibitor was able to inhibit activation of ERK, AKT and RPS6.

Next, we studied the ability of combinatorial therapy to induce apoptosis in RAS-active or RAS-inactive NSCLC tumor cells. Interestingly, we observed that upon treating 3 RAS-active and 3 RAS-inactive cell lines with a dose of 1 μM, apoptosis is only induced at significant levels in RAS-active tumor cells via combined inhibition of EGFR and MEK (Figure 4A–C). None of our treatments were capable of inducing apoptosis in RAS-inactive tumor cells (Figure 4B and D). These results were in line with the above observations whereby RAS-inactive cell lines recorded a marked resistance to EGFR + MEK inhibition, and retained activation of ERK, RPS6 and AKT (Figure 3C). These data highlight the importance of identifying the most beneficial cancer phenotypes prior to therapy administration.

In summary, combined inhibition of EGFR and MEK blocks growth of RAS-active NSCLC tumor cells by concurrent inhibition of ERK and RPS6. Combinatorial therapy performed significantly better at targeting RAS-active cells and blocking ERK and RPS6 than single agent therapy, or with therapies that target other nodes of the RAS-pathway network (Figure 5). These observations did not hold true for only RAS- or EGFR-mutant NSCLC tumor cells, providing evidence to the importance of utilizing characterization of pathway activity that allows for multiple mechanisms of pathway activation.

3.5. Determining RAS-pathway activity is crucial to identify tumors that are most responsive to EGFR + MEK dual inhibition

As gefitinib and U0126 are not FDA-approved small molecule inhibitors of EGFR and MEK1/2 respectively, we sought to

<table>
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<tr>
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Effects of EGFR and/or MEK inhibition on components of the EGFR and RAS pathways in a representative panel of 9 NSCLC cell lines, ranging from high to low predicted probability of RAS-pathway activation. In general, combined inhibition of EGFR + MEK led to concurrent inhibition of ERK and RPS6 phosphorylation, with variable effects on AKT activation in cells with higher levels of predicted RAS-pathway activation (Figure 3C). These cell lines also exhibited a general trend towards RAF1 protein loss, while a decrease in RPS6 protein was observed regardless of RAS-pathway activation status (Figure 3C). Conversely, cells with the lowest predicted probability of RAS-pathway activation (H520, H522) maintained RAF1 protein expression and activated ERK, AKT and RPS6 regardless of the treatment, with cell line H522 even upregulating the activation of AKT and RPS6, even with significant RPS6 protein loss (Figure 3C).

While combined inhibition of EGFR and MEK blocked both ERK and RPS6 activation in cells with higher RAS activation probability, EGFR monotherapy on the other hand only inhibited the activation of AKT and RPS6 with little effect on ERK, while MEK monotherapy only inhibited activation of ERK with a small effect on RPS6. Furthermore, EGFR monotherapy did not inhibit ERK, RPS6 or AKT in cells with the lowest probability of RAS-pathway activation, while MEK monotherapy had minimal effects on ERK activation, and even leading to the activation of AKT in cell line H522, highlighting the dual-node effect of the combination therapy.

Interestingly, we observe that the activation pattern of RPS6 did not correlate to probability of RAS-pathway activation. In cell lines H1377, 977TM1, H441 and H661, activation of RPS6 correlated to the phosphorylation of ERK, while in cell lines SK-MES-1, H1944, H1563, H520 and H522 it correlated to phosphorylation of AKT (Figure 3C). This is potentially due to the fact that RPS6 can be phosphorylated by the ERK and AKT arms of the pathway (Roux et al., 2007). Moreover, MEK inhibition decreased the ERK-mediated phosphorylation-dependent feedback inhibition of RAF1 (Ser289/296/301 phosphorylation) as expected (Fritsche-Guenther et al., 2011).
validate the observation of a correlation between RAS-pathway activation and response to EGFR + MEK dual inhibition by using clinically-relevant inhibitors of EGFR and MEK1/2. We tested a combination of erlotinib and trametinib, two FDA-approved inhibitors of EGFR and MEK1/2 respectively (United States Food and Drug Administration, 2013a,b) on a panel of 16 NSCLC cell lines with a varying range of RAS-pathway activation. In support of our previous findings, we recorded a similar strong and significant correlation between RAS-pathway activation and response to the combination therapy (Figure 6A). Moreover, we observe that NSCLC cells with high genomics-predicted RAS activity had significantly lower EC50 scores than NSCLC cells with low RAS activity, indicating higher drug sensitivity (p = 0.025, Figure 6B). Cell lines with predicted high RAS averaged an EC50 = –0.6934 (log10) = 0.20 μM, while low RAS cell lines averaged an EC50 = 0.9545 (log10) = 9.01 μM, a 45-fold difference in sensitivity. As previously observed, there was no difference in response when the cell lines were divided based on KRAS mutation status (p = 0.553, Figure 6C). These results support the clinical relevancy of our previous observations with gefitinib and U0126, and highlight the immediate clinical relevance of this combinatorial regimen to treat RAS-active tumors in the clinic.

In summary, we show that determining RAS-pathway activation status is pivotal to tailor EGFR + MEK dual inhibition therapy to the most responsive NSCLC tumors. In Figures 2A and 6A a strong and highly significant correlation between RAS-pathway activity and response to EGFR + MEK combination therapy is evident. Cell lines with higher probability of RAS-pathway activation responded significantly better to the combination therapy (Figures 2B and 6B). The predictive power of RAS-pathway activity is not confounded by other tested variables, providing further support to the importance of RAS-pathway activation status as a determinant of response to EGFR + MEK inhibition (Tables 1 and 2). This is important to note as KRAS mutation status fails to predict response to EGFR + MEK inhibition (Figures 3A, B and 6C). The strength of this correlation lies in its reproducibility, which is observed in either a small or large sample size, using gefitinib and U0126 (Supplementary Figures 1A, 2A and B) or FDA-approved and clinically used EGFR and MEK inhibitors in the form of erlotinib and trametinib (Figure 6). The identification of RAS-active tumor cells as being most vulnerable to a specific type of combinatorial therapy—in this case EGFR + MEK inhibition—emphasizes a need to reassess the design of clinical studies, with a focus on identifying the potential patient populations that could benefit the most from treatments prior to clinical trial design.

4. Discussion

RAS is a critical target for many solid tumors such as NSCLC; however, targeting the RAS protein directly has proven elusive. Furthermore, RAS can be activated by many mechanisms, making it challenging to identify RAS-active tumors. To discover therapeutics that can target RAS-active tumors, we performed a genomics-based drug screen in which cancer cells are characterized for RAS-pathway activity using a gene-expression signature and then screened against a panel of compounds to identify those drugs whose efficacy correlates to pathway activity. By measuring RAS-pathway activity by a genomics-based biomarker, we are able to more broadly define RAS activity and are not limited to examining mutation status alone. Furthermore, as the pathway predictions generate a continuous scale of RAS activity, we can identify drugs and/or drug combinations whose efficacy correlates to RAS-pathway activity. The genomics-based drug screen has various advantages compared to alternative drug screens such as conventional or siRNA screens as it provides a comprehensive characterization of a pathway’s activity in cells that is not dependent on biochemical testing, and does not require knowledge of the direct drug target.

By using this approach, we have identified two targeted therapies that when combined effectively inhibit growth of RAS-active cancer cells: gefitinib, which inhibits EGFR and U0126, which inhibits MEK1/2. The use of U0126 has been at the forefront of MEK inhibitor research with more than 2500 citations of its parent discovery publication. U0126 is highly selective, with a nanomolar specificity for MEK1/2, and recorded off-target effects when concentrations greater than 10 μM are used (Favata et al., 1998), which is greater than the dose used in our biochemical analyses. Moreover, U0126 shares similar inhibitory characteristics and functions as the recently FDA-approved MEK inhibitor, trametinib (United States Food and Drug Administration, 2013b; Yoshida et al., 2012), potentially extending our observation to the clinic. Indeed, we observe that using the FDA-approved EGFR and MEK1/2 inhibitors erlotinib and trametinib in combination led to similar results, lending support to the clinical relevancy and feasibility of this treatment against RAS-active NSCLC (Figure 6).

EGFR + MEK dual inhibition blocks two arms of the growth factor receptor network in RAS-active NSCLC tumor cells: EGFR-RAS-RAF-ERK and EGFR-P3K-AKT-RPS6, whereas either agent alone failed to achieve this multi-pathway inhibition effect. Of note, KRAS mutation status alone did not predict response to this drug regimen; our analysis provides evidence

*Table 2—Multivariate predictors of Gefitinib + U0126 response (log10 EC50). Given that the dataset consisted of 39 cell lines, the univariate analysis done previously was used to determine the set of predictor variables that were individually significantly related to Gefitinib + U0126 response (log10 EC50). These variables were included in the multivariate analysis. The multivariate analysis was a linear model. Multivariate analysis determined the RAS-pathway activity predictions as the sole predictor of EGFR + MEK inhibition response.*

<table>
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that some lung cancer cell lines with wild-type KRAS exhibit high RAS-pathway activity. This is in line with observations published by Molina-Arcas et al. (2013) that highlighted the IGFR-centric nature of mutant KRAS—but not wild-type KRAS—lung cancer, in activating the PI3K-AKT-RPS6 node of the pathway, with a strong implication of significant input from EGFR in the wild-type KRAS cells. We observed that best responders to EGFR + MEK inhibition were wild-type KRAS lung cancer cell lines with an activated RAS pathway. This result may explain why the response to EGFR + MEK inhibition correlates better to RAS-pathway activity than IGFR and MEK inhibition (Figure 2C).

By studying RAS network activation through the genomics-based biomarker, we have observed the requirement of EGFR + MEK signaling in maintaining activation of the PI3K-AKT-RPS6 and EGFR-RAS-RAF-ERK nodes in RAS-active...
alternative upstream EGFR signaling components such as RAS and AKT. Only by combined treatment of both EGFR + MEK1/2 do we see effective inhibition of both up and downstream growth factor receptor network components. As ERK inhibition is a key regulator of proliferation and survival of cancer cells (Wortzel and Seger, 2011), and RPS6 also plays vital roles important in cell survival as well as translation initiation (Ruvinsky and Meyuhas, 2006), we expect that effectively blocking these two pathways in cancer cells contributes to their synergistic function and decreased cancer cell growth.

Interestingly, although RAS-inactive cell line HS20 displays synergy between gefitinib and U0126 (Supplementary Figure 6A), the cell line still remains significantly recalcitrant to the treatment, requiring a combinatorial EC50 dose of 11.27 μM, more than 4-fold less responsive than the average response in RAS-active cell lines (EC50 = 2.7 μM) (Supplementary Figure 2B and Table 1). This is also the case for the RAS-inactive cell line H661. Although it displays similar overall pharmacodynamic pattern of pathway component inhibition as RAS-active cell lines when treated with gefitinib and U0126, this cell line is also not comparatively sensitive to the treatment (EC50 = 10.28 μM). The observed synergy for these lines is an interesting area for future mechanistic studies. However, our conclusion that RAS-active cell lines are sensitive to combined EGFR/MEK inhibition remains accurate. Indeed, the RAS-inactive cell lines HS20 and H661 are not sensitive to this combined treatment (Supplementary Table 1), independent of their synergy profiles (Figure 3C, Supplementary Figure 6A).

It will be critical in future clinical trials to identify those tumors that are RAS-active independent of the mechanism of RAS activation in order to best treat those patients with a drug regimen that effectively blocks these key nodes in the RAS pathway. By using mutation analysis alone, one overlooks a large population of tumors that do not carry particular mutations yet harbor activated oncogenic pathways. There are currently clinical trials such as recruiting patients for EGFR + MEK inhibitor combinations based on mutation status alone. Our results suggest that mutation status alone cannot always provide optimal selection of responsive patients. By more comprehensively characterizing patients with dysregulated pathway independent of the mechanism of activation, it may be possible to better select patients for clinical trial inclusion.

Gene-expression signatures have recently made their way to the clinical and commercial sectors, laying the foundation for the feasibility of bringing any future gene-expression signatures to the clinic (Arpino et al., 2013). One example is the FDA-approved microarray-based gene-expression profiling signature Mammaprint® which uses tissue core sampled on fresh specimens preserved in RNA later or frozen archived tissue as source material, to output and score low/high risk of tumor metastasis (Arpino et al., 2013; van ’t Veer et al., 2002; van de Vijver et al., 2002). Therefore, the application of a gene-expression signature as a genomic biomarker of RAS-pathway activation is both promising and feasible.

Overall, our data shows the significant correlation between RAS-pathway activity and response to EGFR + MEK inhibition (Figure 2A and B), and the predictive power of our signature

NSCLC. Interestingly, combinatorial inhibition of EGFR with other components of the growth factor receptor network, such as RAF or mTOR, does not correlate to RAS activity in lung cancer cells. One reason the RAF inhibitor sorafenib is not equivalent to MEK inhibition could be due to sorafenib’s inability to block ERK signaling in KRAS-mutant cells (Takezawa et al., 2009; Wilhelm et al., 2004). Together, these studies highlight the complexity of RAS-centric signaling in cancer, and the need to identify an appropriate target population prior to initiation of clinical trials, as data suggests that the different growth factor receptor network combinatorial treatments will be effective only in discrete patient populations.

Although clinical trials, such as the IPASS trial (Mok et al., 2009) have shown efficacy of anti-EGFR tyrosine kinase inhibitors in EGFR-mutant NSCLC, inhibition of growth factor receptors has also been effective in unselected second-line patients. For example, inhibition by anti-EGFR antibody cetuximab was beneficial in EGFR-positive patients in the FLEX and IPASS trials (Mok et al., 2009; Pirker et al., 2009). Indeed, in contrast to colon cancer, KRAS mutations do not predict resistance to EGFR inhibition in lung cancer (Guan et al., 2013; Krejci et al., 2011; Mazzoni et al., 2011; O’Byrne et al., 2011). While not nearly as effective as the gefitinib/U0126 combination, we do see some efficacy of gefitinib alone in our panel of lung cancer cell lines. However, upon investigation of the mechanisms by which these drugs function, we find that EGFR antagonism alone does not significantly block downstream growth factor receptor network components such as RAF, ERK and RPS6. Alternatively, MEK1/2 inhibition does effectively inhibit ERK activity, but often fails to inhibit
which is independent of other factors such as cancer subtype of mutations in key pathway components (Tables 1 and 2). In the clinic, the RAS gene-expression signature could be used to assess RAS-pathway activation status in a patient’s tumor. If the tumor has high pathway activity, that patient may be a candidate to receive EGFR+MEK dual inhibition. Indeed, the clinical potential of our study is supported by the validation of our observed correlation of RAS-pathway activation and EGFR+MEK dual inhibition using FDA-approved inhibitors (Figure 6).

Lastly, it is important to note that while tumor heterogeneity will ultimately be important in identifying and treating refractory subclones within a tumor, our current approach is to analyze the overall RAS activity within a tumor. Thus, higher levels of RAS activity within a tumor will predict greater overall response. Arguably, our approach measures RAS activity within the bulk tumor, where there may be subclones that are a minority population but still have varied RAS activity. Current and future studies are dedicated to addressing this ongoing issue in tumor characterization and treatment.

In summary, we described a genomic-based screen that characterizes RAS-pathway activation and identifies drugs that effectively target the pathway, inducing an apoptotic tumor cell response. By using a genomic approach to characterize oncogenic pathway activity in tumor cells, the ability to find drugs that target and inhibit a specific pathway is increased. Given the complexity and crosstalk of signaling pathways that is unique to individual tumor phenotypes, it is essential to identify and block the relevant pathway components for optimal drug response. By applying a genomic

Figure 6 — Combinatorial inhibition of EGFR + MEK using FDA-approved inhibitors is correlated to RAS-pathway activity in NSCLC. (A) Linear correlation of erlotinib + trametinib EC50 values with the predicted probability of RAS-pathway activation across 16 NSCLC cell lines. Response to erlotinib + trametinib is significantly negatively correlated to RAS. Legend = Red: KRAS-mutant cell lines, Black: KRAS wild-type cell lines. (B) Cell lines of the 16 NSCLC panel were divided accordingly and box plot diagrams of erlotinib + trametinib EC50 values were plotted. Response to erlotinib + trametinib as measured by EC50 values is shown. Cell lines with elevated probability of RAS activation (predicted probability of RAS-pathway activation > 0.5) were significantly more sensitive to erlotinib + trametinib treatment than cell lines with low probability of RAS activation (predicted probability of RAS-pathway activation < 0.5). (C) Response to erlotinib + trametinib with respect to the mutation status of KRAS, as measured by EC50 values. K-RAS mutation does not predict response to erlotinib + trametinib.
approach to discovery of pathway-specific drug regimens, we can identify the patients who may best benefit from those treatment regimens prior to initiation of a clinical trial.

Author contributions

NNE, AHB and PJM conceived the study. NNE and SRP performed the bioinformatic analysis. NNE and KMB performed the experimental work and data analysis. ALC and JTC provided critical input on the concept design. NNE and AHB wrote the manuscript. PJM, SRP, JTC and ALC provided crucial manuscript feedback and suggestions.

Conflict of interests

Authors declare no conflicts of interest.

Acknowledgments

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Appendix A.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.05.005.

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