Background

Inhibitors of the epidermal growth factor receptor (EGFR) were introduced as a targeted therapy because some non–small cell lung cancers (NSCLC) were demonstrated to be dependent on the EGFR oncogene for growth and proliferation.1 Furthermore, it was observed that cells and tumors with KRAS activating mutations were inherently resistant to treatment with EGFR inhibitors (EGFRI).2,3 KRAS-activation mutations (eg, codons 12, 13, and 61) are the most common mutations in lung adenocarcinomas and are observed in 25% to 40% of cases, but currently, there are no therapeutic interventions available to target KRAS directly. We hypothesized that targeting proteins connected to KRAS function rather than targeting KRAS directly could induce cell death in KRAS-active NSCLC cells. To identify potential targets, we leveraged 2 gene expression data sets derived from NSCLC cell lines either resistant and sensitive to EGFRI treatment. Using a Feasible Solutions Algorithm, we identified genes with deregulated expression in KRAS-active cell lines and used STRING as a source for known protein-protein interactions. This process generated a network of 385 deregulated proteins including KRAS and other known mechanisms of EGFRI resistance. To identify candidate drug targets from the network for further study, we selected proteins with the greatest number of connections within the network and possessed an enzymatic activity that could be inhibited with an existing pharmacological agent. Of the potential candidates, the pharmacological impact of targeting casein kinase 2 (CK2) as a single target was tested, and we found a modest reduction in viability in KRAS-active NSCLC cells. MEK was chosen as a second target from outside the network because it lies downstream of KRAS and MEK inhibition can overcome resistance to CK2 inhibitors. We found that CK2 and MEK inhibition demonstrates moderate synergy in inducing apoptosis in KRAS-active NSCLC cells. These results suggest promise for a combination inhibitor strategy for treating KRAS-active NSCLC.

Our group previously identified 2 gene expression signatures of response to the EGFRI, erlotinib, collected under the hypothesis that patients without EGFR-activating mutations may also derive benefit from treatment with EGFRI. Patients with those tumors might be identified by particular gene expression phenotypes and treated with EGFRI.5,10 While this hypothesis was validated using retrospective analysis of gene expression data from colorectal cancers, even more striking were the observations made from bioinformatics analysis of the gene expression signatures.10 Genes from the miRNA signature not only predicted response to EGFRI but also intersected the transforming growth factor beta (TGFβ) signaling cascade as found using pathway enrichment of miRNA targets.9 These data suggested that response to EGFRI may be influenced by activation of TGFβ signaling, and inhibition of this pathway could sensitize EGFRI-resistant tumor cells to erlotinib.9 However, other groups have tested these hypotheses in clinical studies and have been largely unsuccessful in improving response in EGFRI-resistant cancers with concurrent inhibition of TGFβ signaling. Lack of improved response may be due to the competing pro- and antitumorigenic activities along
the TGFβ axis, but efforts to target aberrant TGFβ signaling are ongoing. It was this observation that led us to try new molecular interaction network search approaches over pathway enrichment analyses, which might reveal specific proteins that could be therapeutically targeted in EGFRI-resistant/KRAS-active NSCLC.

We employed a statistical interaction detection method combined with a molecular interaction network analysis to uncover potential novel interactions between the 2 messenger RNA (mRNA) and miRNA signatures. Other groups have demonstrated that pairing expression levels of RNA species in specific disease states may lead to an improved understanding of the disease. This was followed by structured interrogation to uncover enzymatic activities that might intersect and regulate multiple signaling cascades in NSCLC cells. In this report, we describe identification and characterization of casein kinase 2 (CK2) using the bioinformatics process as a potential target. Casein kinase 2 is a multisubunit kinase that can contribute to tumorigenesis when subunit expression is altered. Casein kinase 2 exists mainly as a tetrameric holoenzyme consisting of any α subunits have monomeric kinase activity as well. Moreover, CK2 was shown to be an upstream regulator of AKT/PI3K/mTOR, NFκB, and JAK/STAT signaling cascades irrespective of the receptor tyrosine kinases shown to activate them. As stated above, EGFRI resistance can result from alterations in parallel signaling pathways, including the PI3K/AKT/mTOR and JAK/STAT. Therefore, we postulated that inhibition of CK2 might represent a treatment alternative for NSCLC that are resistant to EGFRI. This strategy may provide some NSCLC patients an additional opportunity for therapeutic intervention.

**Methods**

**Cell culture and western blotting**

A549, H460, and H1650 (NSCLC) cell lines were purchased from ATCC. PC9 cells (NSCLC) were a gift from Eric Haura, MD (Moffitt Cancer Center, Tampa, FL). Cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS; USA Scientific), 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), glucose, and pyruvate and maintained in a humidified incubator at 37°C in 5% CO2 unless otherwise specified. For analysis of protein content by western blot, 3 x 10⁴ cells were plated into a 6-well dish and allowed to adhere in RPMI 1640 containing 10% FBS for 48 hours. Following the adherence period, cells were treated with the concentrations of the CK2 inhibitor, CX-4945 (Silmitasertib; Cayman Chemical), or the MEK1/2 inhibitor, AZD6244 (Selumetinib; AstraZeneca), in RPMI 1640 containing 1% FBS for the treatment durations indicated. Both adherent and nonadherent cells from each sample were collected for total protein. Ten percent of total cell extracts were loaded. Cleaved poly(ADP-ribose) polymerase (PARP) and α-tubulin antibodies were purchased from Cell Signaling.

**Cell viability assay**

Cells were plated at 3 x 10⁴ cells/well in a 96-well plate and allowed to adhere in RPMI 1640 containing 10% FBS for 36 hours. After 36 hours, CX-4945 and AZD6244 were added in the final concentrations indicated in RPMI 1640 containing 1% FBS. Drug treatment persisted for 72 hours. After 72 hours, resazurin was added (100 µM final concentration) to each well, and the plates were gently rocked for 1 minute and then incubated for 3 hours prior to reading. Each plate was read for fluorescence at excitation, 560 nm, and emission, 590 nm, wavelengths using a SpectraMax M5 and corresponding SpectraMax X5 software (SpectraMax). Cellular response to treatment was determined by subtracting signal from corresponding empty wells and then normalized to corresponding untreated cells. Determination of additive or synergistic responses between CX-4945 and AZD6244 was performed using the CompuSyn software. Combination index (CI) values from the CompuSyn analysis were then used to build heat maps. Three biological replicates of all viability assays were performed and were assessed for outliers using a Dixon Q-test. Data were analyzed using Prism Version 7.00 (GraphPad).

**Generation of miRNA and mRNA expression datasets**

Messenger RNA and miRNA expression levels were measured in growing NSCLC cell lines using Affymetrix U133 2.0 microarrays (GSE31625) and TaqMan cards from Applied Biosystems (ABI), respectively, using previously published data. We evaluated interactions among the 1495 mRNA genes and 23 miRNA that are significantly perturbed in erlotinib-sensitive compared with erlotinib-resistant NSCLC cells using a Feasible Solution Algorithm (FSA), as described in the following.

**The FSA statistical methodology and interaction network analysis**

The FSA was used to identify 2-way statistically significant interactions using the expression data. There is no attempt to derive or infer the direction of these potential interactions. We included 1495 mRNA that demonstrated higher expression, representing ~800 probe set identifiers (probeID), in the erlotinib-resistant cell lines. We enumerated the possible solutions with interacting miRNA (n=23), regardless of direction of expression relative to the mRNA (http://CRAN.R-project.org/package=rFSA).

We examined the 100 probeIDs with the highest potential of representing a solution (Supplementary Table 1) for further biological evaluation by overlaying onto a molecular interaction network provided by STRING. Using the miRNA-mRNA interactions found by the FSA, the Affymetrix probeIDs were converted to Ensembl IDs. Files specific to human proteins were downloaded from the STRING database v 10.0 for further processing. Specifically, the Bioconductor v3.0 package
for Affymetrix(R) HGU133-plus2 chips (hgu133plus2.db v3.0.0) was used to translate Affymetrix (R) probeIDs to gene identifiers (symbols, gene names, Entrez IDs, and ENSEMBL Proteins) in R v3.3.2 (2016). From ENSEMBL protein IDs, the species ID 9606 was added to provide STRING protein IDs. The full set of STRING protein-protein interactions (PPI) and indirect associations were filtered to those with an “experimental” evidence score greater than 400. From this subset, the interactions with the original set of genes and their interactors (those genes within one edge or interaction) were extracted from the database (full list of initial genes extracted in Supplementary Table 2). We were interested in interactions G1-X-G2, where G1 and G2 are from our list of proteins and X can be any protein that connects G1 to G2 (full list of genes comprising the expanded network in Supplementary Table 3). For each interaction, only a record that there was an interaction between the 2 proteins was kept with no information on the number of evidences or the score of the interaction. Within this “induced” network (ie, derived subnetwork), communities of genes (Supplementary Table 7) were identified using the cluster_walktrap function in igraph 1.0.1.24,25

Availability of data
All codes for network generation and enrichment analysis and supplemental files are available for download from figshare at: https://figshare.com/s/7e50e9ab2a66b5041451.

Results
A combinatorial analysis of the miRNA and mRNA signatures of EGFR resistance identifies a network of perturbed gene expression related to EGFR resistance

We hypothesized that mRNA and miRNA gene expression data from NSCLC cell lines could be used to identify novel targets for therapy in lung cancer patients resistant to EGFR. We previously identified 2 independent gene expression signatures of response to EGFR using a panel of NSCLC cell lines demonstrating differential resistance to EGFR inhibition as measured by a cell death assay.9 The signatures were culled from a larger set of differentially regulated mRNA and miRNA. Using the larger lists of perturbed gene expressions (1495 mRNA and 23 miRNA), we sought to identify new potential targets for therapy using statistically significant pairs of mRNA and miRNA analyzed with FSA (Supplementary Table 1). FSA first evaluates expression levels of random combinations of mRNA and miRNA pairs and then exchanges the miRNA component to maximize interaction significance with respect to a model of interaction. The algorithm eventually arrives at optimal pairings. Importantly, the pairings are based on expression level similarities, not transient hydrogen bonding of complementary bases that would indicate miRNA targeting of the associated mRNA. Each significant mRNA-miRNA pair was filtered based on higher mRNA expression in the EGFR-resistant cell lines to find targetable gene products within the context of EGFR-resistant tumors. Given this outcome, we hypothesized that ideal, new druggable targets for EGFR-resistant NSCLC may depend on protein interactions with the EGFR signaling network. To investigate this hypothesis, we used the mRNA-derived gene list identified by FSA as optimal protein-coding genes to find additional proteins that physically interact with both the candidate(s) and EGFR.

The 100 mRNA probe IDs with the highest significance (low Prob > F, Supplementary Table 1) were identified by FSA. These 100 probes were translated into 85 Ensembl IDs that demonstrated matches in the STRING v10 network of PPIs.22 To broaden the network, we included EGFR as a node to triangulate the interactions around EGFR resistance. We carried out the protein network expansion analysis, using the scenario G1-X-G2 wherein G1 and G2 were proteins from the original list of 85 Ensembl IDs, while X could be any new protein entity. The “induced” network contained 81 of the 85 original proteins (Supplementary Table 3). However, 304 additional nodes were found (for a total of 385 proteins) that fit in the G1-X-G2 network (Supplementary Table 3).

To identify candidate drug targets from the network for further study, we retained proteins that with the greatest number of edges within the induced network and possessed an enzymatic activity that could be inhibited with an existing pharmacological agent (Figure 1; genes listed in Table 1). We then selected several proteins with potential as therapeutic targets to be evaluated in NSCLC cells (Figure 2D).

CK2 inhibition reduces viability in KRAS-active NSCLC

From the bioinformatics search of perturbed mRNA expression in KRAS-active cells, we found that CK2 may be a novel target in EGFR-resistant NSCLC harboring KRAS-activation mutations. To investigate whether modulation of CK2 activity impacts viability of KRAS-active cells, we performed a viability assay with the CK2 small molecule inhibitor, CX-4945. These experiments demonstrated decreased viability of KRAS-active NSCLC cells in the presence of CX-4945. Specifically, in A549 and H460 cells, we observed approximately 50% cell viability compared with untreated cells after 72 hours of treatment with 30 µM CX-4945 (Figure 2A). We also sought to test the activity of CX-4945 in another genetic context. To do this, we treated 2 different EGFR-sensitive cell lines, H1650 and PC9. In H1650 cells, viability also decreased by approximately 50% after 72 hours of treatment with CX-4945 (Figure 2B). In PC9 cells, the maximal response to 72 hours of treatment with 30 µM of CX-4945 was approximately 75% to 80%. Because treatment with CX-4945 as a single agent was not sufficient to completely impair cell viability in either cell line population, we next
aimed to identify a second target within, or related to, the network containing CK2 that might have additive activity.

**Inhibition of CK2 and the EGFR–RAS–MAPK cascade displays synergy in apoptotic induction in KRAS-active cell lines**

Using the induced network members (n = 366) that are within one edge of CK2 (CK2α/CK2α’ [CSNK2A1/CSNK2A2]), we asked whether any member of the KRAS-MAPK cascade was included in the induced network (Supplementary Table 4). Unexpectedly, CK2α and CK2α’ do not directly interact with any of the members of the EGFR-RAS-MAPK signaling cascade (Supplementary Table 4). Specifically, all members of the EGFR-RAS-MAPK signaling cascade identified by FSA (HRAS, KRAS, NRAS, MAPK1, RAF1) were 2 edges from CK2α/CK2α’ (Supplementary Table 5). To focus selection of a secondary target that might inhibit the activity of the
EGFR-MAPK-ERK pathway without targeting the EGFR receptor, we consulted the literature. Resistance to the CK2 inhibitor, CX-4945, has been demonstrated in head and neck cancers and was shown to be overcome by MEK inhibition. Importantly, CK2α/CK2α′ are in separate network communities from other members of the RAS-MAPK pathway (Supplementary Table 7). These data suggest that CK2 functions independent of the MAPK-ERK cascade and combined inhibition may lead to increased cell death in KRAS-active NSCLC. We then evaluated whether KRAS-active, EGFRI-resistant cells demonstrated decreased viability when treated with a combination of CK2 and MEK inhibition when compared with CK2 inhibition alone.

We first escalated the concentration of the CK2 inhibitor, CX-4945, in combination with a constant concentration of the MEK inhibitor, AZD6244. We observed induction of apoptosis in A549 and H460 cells by western blot analysis using cleaved PARP as an apoptotic marker (Figure 3). Both KRAS-active cell lines examined demonstrated decreased viability when treated with a combination of CK2 and MEK inhibition when compared with CK2 inhibition alone.

We next screened A549 and H460 cells to determine whether treatment with a combination of CX-4945 and AZD6244 resulted in a synergistic response in a cell viability assay (Figure 4). Moderate synergistic activity was observed between the CX-4945 and the AZD6244 inhibitors at a concentration of 3 μM and above in both KRAS-active cell lines (Figure 4; upper left quadrant).

Collectively, these data demonstrate that an intentional approach of coupling the biological phenotypes of drug sensitivity and gene expression levels using bioinformatics analysis focused on integrating potential and known molecular interactions can uncover relevant interactions among genes that can be used to select novel protein targets for therapeutic modulation to overcome resistance to EGFRI in NSCLC.

### Discussion

Patients who have lung tumors harboring KRAS activation or other EGFRI-resistance mutations have few therapeutic options targeting these genes or signaling cascades once failing front line cytotoxic treatment. Newer options are emerging for patients with mutations within EGFR (eg, Osimertinib), but new drug targets and treatment strategies are paramount for lung cancer patients with other, non-EGFR mutations. The goal of this study was to leverage existing gene expression signatures linked by disease and drug response phenotypes and to use a combination of statistical and computational methods to identify interacting pairs of mRNA:miRNA that then yield relevant PPIs. The prior work of Ma and colleagues in addition to other groups have indicated that pairing expression levels of RNA species in specific disease states may lead to an improved understanding of the disease. We used the Feasible Solutions Algorithm to identify statistically interacting pairs of mRNA and miRNA linking KRAS activation and EGFRI response to seed the generation of an induced molecular interaction network that would generate potential pharmacologically
Cancer Informatics

actionable targets linking these 2 molecular phenotypes. We focused on the protein-coding genes as drug targets, rather than the miRNA partner of the pair. The limitation of FSA in this experiment is that not all pharmacologically actionable proteins that contribute to tumorigenesis are captured because we required that the genes of interest be upregulated in KRAS-active cells or tumors. To allow for additional protein targets, we included proteins in a G1-X-G2 relationship with a protein in the original network (G1 and G2).28 Our logic in doing so was that we would expect that proteins/enzymatic functions that joined multiple signaling cascades providing a linchpin, of sorts, would be captured.

We then identified many proteins previously shown to have a role in EGFR resistance in NSCLC (Communities 2 and 4, Supplementary Table 7) and a number of novel putative targets (Supplementary Table 7). By expanding the initial network, we found CSNK2A1 and CSNK2A2, which encode the kinase subunits of protein kinase CK2 (also known as CK2). Casein kinase 2 has been described as a protein that can modulate the activities of many proteins in both EGFR-resistant and -sensitive NSCLC, notably including nuclear factor κB (NFκ-β) and PI3K/AKT.29 Moreover, no oncogenic mutations have been found in CK2 kinase subunits, but deregulation of the activity of this kinase by other mechanisms might contribute to the oncogenic process.30

EGFRI-resistant, KRAS-active NSCLC cells were found to be moderately sensitive to CK2 inhibition (Figure 2A). In comparison, we found that PC9 cells, which harbor an EGFR-activating deletion mutation, were resistant to CX-4945. Interestingly, H1650 cells, which contain an EGFR-activating mutation but display intermediate sensitivity to EGFRI, responded to CX-4945-like KRAS-active A549 and H460 cells (Figure 2B). H1650 cells also contain a PTEN mutation, and CK2 was identified as a possible target for overcoming PTEN-null mutations, potentially explaining this observation.31

| CELL LINE | EGFR Resistance Status | EGFR Mutation Status | KRAS Mutation Status | PI3K/AKT Mutations Status |
|-----------|------------------------|----------------------|----------------------|--------------------------|
| A549      | Resistant              | WT                   | G12S (Active)        | WT                       |
| H460      | Resistant              | WT                   | Q61H (Active)        | PIK3CA E545K (Null)      |
| H1650     | Intermediate           | Exon 19 Del (Activating) | WT                   | PTEN Null (Activating)   |
| PC9       | Sensitive              | Exon 19 Deletion (Activating) | WT                   | WT                       |

Figure 2. NSCLC cells resistant to EGFRI are most responsive to CK2 inhibition. Viability assays were performed on NSCLC treated with CX-4945 (A) KRAS-active, EGFRI-resistant NSCLC (A549 and H460 cells) treated with CX-4945. (B) EGFR-sensitive H1650 cells and PC9 cells. Values are log-transformed (n = 3). (C) Tabular depiction the EGFRI resistance and mutational statuses of each NSCLC analyzed.
Because none of the cell lines tested were exquisitely sensitive to single-agent CK2 inhibition, regardless of EGFR sensitivity, we suspected that our original hypothesis that a single protein that was upregulated in EGFRI-resistant cells and intersected the EGFR signaling cascade was short-sighted. To address this concern, we looked for a second target that, when inhibited, would reduce viability when combined with inhibition of CK2. The KRAS-activation mutation drives RAS-MAPK signaling, but we observed in the induced network that not all of the RAS-MAPK signaling cascade members were represented or directly connected to CK2 as we might expect. It is also possible that the RAS-MAPK cascade operates parallel to CK2 activity and that combining CK2 inhibition with a MAPK pathway inhibitor may overcome compensatory viability signaling. This hypothesis was also founded with the knowledge that MEK inhibition has been used to overcome CX-4945 resistance in head and neck cancers. Furthermore, our lab and others previously demonstrated that inhibition of MEK, concurrently with EGFR inhibition, reduces viability in NSCLC with EGFR T790M. In experiments presented here, we showed that treatment of KRAS-active NSCLC with AZD6244 and CX-4945 increased markers of apoptosis in a dose- and time-dependent manner. Combination of CK2 and MEK inhibition was moderately synergistic and with further validation may represent a novel approach for the treatment of EGFRI-resistant, KRAS-active NSCLC. We plan to validate this observation by assessing viability in a panel of genomically diverse NSCLC cells treated with the combination to determine whether these observations are consistent in other KRAS-active, or parallel-pathway, mutant NSCLC cells. A limitation of this study is limited screening of cells lines with PTEN, PIK3CA, or BRAF mutations, which may have further illuminated mechanism of action.

Our approach used existing genomic and drug response data from NSCLC cell line models to identify and screen new drug targets with novel approaches that combine statistical and graph-computational methods. These methods can be applied to other model systems and allows for disease- or genetic-specific drug discovery. Using gene expression phenotypes of a specific oncogenic driver mutation in NSCLC, we developed a strategy that bypasses the presence of single, activating mutations in EGFR and KRAS as primary therapeutic targets and focuses on downstream proteins that integrate multiple signaling cascades and may represent a novel therapeutic approach for treating a variety of NSCLC tumors. We continue to evaluate additional candidates that lie downstream of KRAS and EGFR in NSCLC.

Conclusions

In many clinical settings, cancer can become a chronic disease with existing therapeutic interventions. In others, new therapies and therapeutic strategies that elicit durable benefit are needed for optimal patient care and management of emerging resistant diseases. We have demonstrated that gene expression signatures descriptive of specific tumor phenotypes can be used to identify candidate targets for new therapeutics or co-therapeutic methodologies. By combining an FSA statistical interaction detection method with an induced subgraph creation method using known molecular interactions from STRING, we derived a network of proteins based on perturbed gene expression in EGFRI-resistant NSCLC. From this network, we identified and tested CK2α/CK2α′ as a therapeutic target for the treatment of EGFRI-resistant NSCLC. Casein kinase 2 inhibition alone did not substantially decrease cell viability. The induced target network suggests that RAS-MAPK signaling and CK2 activity could function exclusively of one another, which directed us to examine the impact of combinatorial CK2 and MEK inhibition. We believe that the combination of MEK and CK2 inhibition could have important implications for the treatment of KRAS-active NSCLC and has potential as an alternative therapy for EGFRI-resistant...
tumors following further validation in a genomically diverse panel of NSCLC cell lines. We also seek to improve this novel pipeline for drug discovery by automating a process that uses gene expression signature as inputs and objectively leverages bioinformatics filtering of prospective targets to minimize wet lab validation.

**Author Contributions**

MKG designed and performed research, analyzed data, and wrote the paper. RMF, JL, HM, and AS analyzed data and edited the paper. EPB designed the research and wrote the paper.

**Supplemental Material**

Supplemental material for this article is available online.

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**Figure 4.** Synergy between CK2 and MEK inhibitors was observed in KRAS-active NSCLC. (A) A549 cells and (B) H460 cells. Cell viability was measured by resazurin viability assay to measure growth in the presence of CX-4945 in combination with AZD6244. Synergism was assessed using combination index (CI) values. CI values were then used to generate the heat maps and quadrant averages for each cell line.
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