Cell Reports
Article

RAF Suppression Synergizes with MEK Inhibition in *KRAS* Mutant Cancer Cells

Simona Lamba,[1,2,5] Mariangela Russo,[1,2,5] Chong Sun,[3,5] Luca Lazzari,[1,2,4] Carlotta Cancelliere,[2] Wipawadee Grernrum,[3] Cor Lieftink,[3] Rene Bernards,[3] Federica Di Nicolantonio,[1,2,6,*] and Alberto Bardelli[1,2,6,*]
[1]Department of Oncology, University of Torino, Str prov 142 Km 3.95, Candiolo, 10060 Torino, Italy
[2]Candiolo Cancer Institute–FPO, IRCCS, Str prov 142 Km 3.95, Candiolo, 10060 Torino, Italy
[3]Division of Molecular Carcinogenesis, Cancer Systems Biology Centre and Cancer Genomics Centre Netherlands, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 Amsterdam, the Netherlands
[4]FIRC Institute of Molecular Oncology (IFOM), 20139 Milano, Italy
[5]Co-first author
[6]Co-senior author
*Correspondence: federica.dinicolantonio@unito.it (F.D.N.), alberto.bardelli@unito.it (A.B.)
http://dx.doi.org/10.1016/j.celrep.2014.07.033

SUMMARY

KRAS **is the most frequently mutated oncogene in human cancer, yet no therapies are available to treat *KRAS* mutant cancers. We used two independent reverse genetic approaches to identify components of the RAS-signaling pathways required for growth of *KRAS* mutant tumors. Small interfering RNA (siRNA) screening of 37 *KRAS* mutant colorectal cancer cell lines showed that RAF1 suppression was synthetic lethal with MEK inhibition. An unbiased kinome short hairpin RNA (shRNA)-based screen confirmed this synthetic lethal interaction in colorectal as well as in lung cancer cells bearing *KRAS* mutations. Compounds targeting RAF kinases can reverse resistance to the MEK inhibitor selumetinib. MEK inhibition induces RAS activation and BRAF-RAF1 dimerization and sustains MEK-ERK signaling, which is responsible for intrinsic resistance to selumetinib. Prolonged dual blockade of RAF and MEK leads to persistent ERK suppression and efficiently induces apoptosis. Our data underlie the relevance of developing combinatorial regimens of drugs targeting the RAF-MEK pathway in *KRAS* mutant tumors.**

INTRODUCTION

Mutations in the gene encoding the guanosine triphosphatase protein KRAS, the principal of the three isoforms of RAS, are present in approximately 20% of all cancers and are associated with poor prognosis and resistance to therapy (Prior et al., 2012; Pylayeva-Gupta et al., 2011). Oncogenic mutations occur most frequently in codons 12, 13, 61, and 146, and the resulting proteins are resistant to GAP-mediated guanosine triphosphate (GTP) hydrolysis, rendering them constitutively active (Cox and Der, 2010; Pylayeva-Gupta et al., 2011). Efforts to pharmacologically target KRAS directly have been so far unsuccessful. Innovative approaches recently challenged this assumption; nevertheless, development of clinical KRAS inhibitors is not yet within reach (Ostrem et al., 2013; Zimmermann et al., 2013).

KRAS mutations occur in approximately 20% of non-small-cell lung cancer (NSCLC) and 40% of colorectal cancer (CRC) cases, where they are associated with resistance to epidermal growth factor receptor (EGFR)-targeted therapies (Douillard et al., 2013; Karapetis et al., 2008; Linardou et al., 2008). Several strategies have been proposed to target mutant KRAS tumors. Attempts to inhibit single effectors downstream to KRAS (e.g., phosphatidylinositol 3-kinase [PI3K] and MEK) revealed modest or no efficacy (Adjei et al., 2008; Ganesan et al., 2013). Alternative strategies involve targeting MEK together with receptor tyrosine kinases including HER3 or insulin-like growth factor 1 receptor (IGF1R) (Ebi et al., 2011; Turke et al., 2012). Recently, combinatorial treatment with mTOR-targeted agents and BCL-2/BCL-XL inhibitors has also been proposed for *KRAS* mutant CRCs (Faber et al., 2014). All these efforts proved to be less broadly applicable than initially thought, likely due to intrinsic biochemical, biological, and clinical heterogeneity of *KRAS* mutant tumors, thus explaining why a specific drug mix may be effective only in a subset of *KRAS*-mutated tumors.

Previous studies often employed a limited number of *KRAS* mutant tumor cells. The majority of reports frequently relied upon CRC lines displaying microsatellite instability (MSI). It is well established that CRCs exhibiting MSI show a particularly indolent clinical behavior (Roth et al., 2012) and are therefore less prevalent in more-advanced stages of the disease. Patients with metastatic CRC (mCRC) that receive targeted agents, such as anti-EGFR antibodies, show MSI in less than 5% (Smith et al., 2013). Consequently, MSI cell lines do not properly represent the clinical setting where KRAS-targeted therapies are likely to be applied. We sought to overcome these limitations by assembling and characterizing a broad panel of CRC cell lines carrying *KRAS* mutations and lacking MSI.

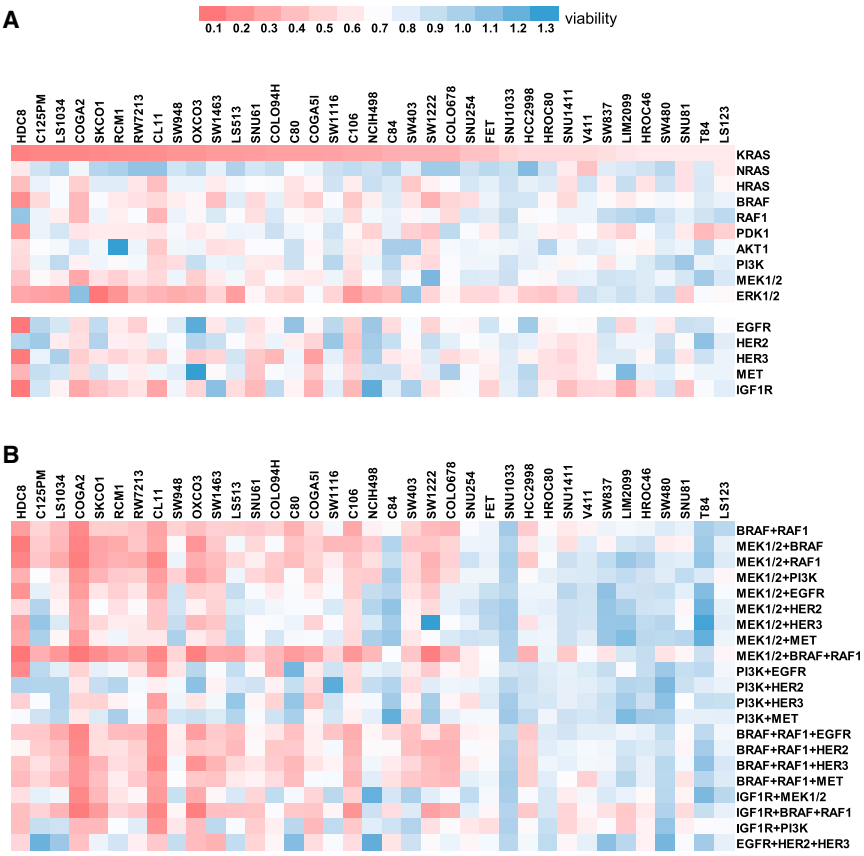

Figure 1. RNAi-Based Suppression of Genes Involved in KRAS Signaling in a Panel of CRC Cells Carrying *KRAS* Mutations

(A) Thirty-seven MSS *KRAS*-mutated CRC cell lines were screened with a library of siRNAs designed to target all three isoforms of RAS (KRAS, HRAS, and NRAS) and their upstream (EGFR, HER2, HER3, MET, and IGF1R) or downstream (BRAF, RAF1, PDK1, AKT1, PI3K, MEK1/MEK2, and ERK1/ERK2) modulators. A pool of four different siRNAs was used for each gene. After 5 days of treatment, the survival fraction was determined by ATP assay. Results represent mean of at least two independent experiments.

(B) Concomitant silencing of two or more effectors involved in KRAS pathways was assessed. After 5 days of treatment, the survival fraction was quantified by measuring ATP content. Results represent mean of at least two independent experiments.

See also Figures S1 and S2.

RESULTS

CRC Cell Lines Harboring KRAS-Activating Mutations Show Differential Sensitivity to Knockdown of Genes Involved in KRAS Pathways

From an initial collection of 72 CRC *KRAS* mutant lines, we selected 39 cell lines with microsatellite stable phenotype (MSS). SNP genotyping was used to determine the genetic status of the entire panel. When multiple cell lines were identified as being derived from the same individual, only one model was included in the final panel, which comprised 37 lines (Tables S1 and S2).

We used a reverse genetics approach to identify genes critical for the growth of *KRAS* mutant cancer cells among those known to be involved in KRAS signaling. To this end, we assembled a library of small interfering RNAs (siRNAs) designed to target all three isoforms of *RAS*, their downstream effectors, and their upstream modulators, such as receptor tyrosine kinases (Figure 1). Each siRNA was individually validated for being capable of suppressing its target as measured by western blotting (Figure S1A). A pool of different siRNAs was used for each gene, and several controls were employed to assess transfection efficiency and to confirm effective target suppression (Figures S1B and S2).

When each gene was individually silenced, suppression of *KRAS* was by far the most effective, being able to restrain the growth of 25/37 (67%) lines by more than 50% (Figure 1A). Suppression of KRAS most-immediate effectors such as BRAF, RAF1, or MEK1+MEK2 was effective in less than 15% cell lines (Figure 1A). These results indicate that the occurrence of mutations generally dictates dependency on KRAS itself, but not on other single signaling molecules.

The reverse genetic screen was then expanded to target two or more genes in combination. Using this strategy, the most effective hit was suppression of MEK1 and MEK2 together with silencing of members of the RAF family (Figure 1B). Simultaneous downregulation of MEK1/MEK2, BRAF, and RAF1 reduced cell viability by more than 50% in a large subgroup of the cells tested (21/37 lines; 57%). This combination was the closest to achieve the same antiproliferative effects observed with suppression of KRAS alone (Figure 2A).

A Reverse Genetic Screen Unveils RAF1 Suppression to Be Synthetic Lethal with MEK Inhibition

A significant fraction of *KRAS* mutant CRC cells (16/37 lines; 43%) are refractory to MEK-RAF combinatorial suppression. We postulated that insensitive cell lines might have distinct signaling features. To gather insights into genes capable of complementing MEK inhibition in *KRAS* mutant cells insensitive to RAF suppression, we performed an unbiased screen using a short hairpin (shRNA) lentiviral library designed to silence 518 kinase and 17 kinase-related genes. Among the refractory lines, we chose SW480, as this cell line proved conductive to large-scale lentiviral-based infection. SW480 was infected with the shRNA library and cultured in the presence or absence of the MEK allosteric inhibitor selumetinib for 14 days. After this, the relative abundance of shRNA vectors was determined by next generation sequencing of the bar code identifiers present in each shRNA vector (Figure S3A).

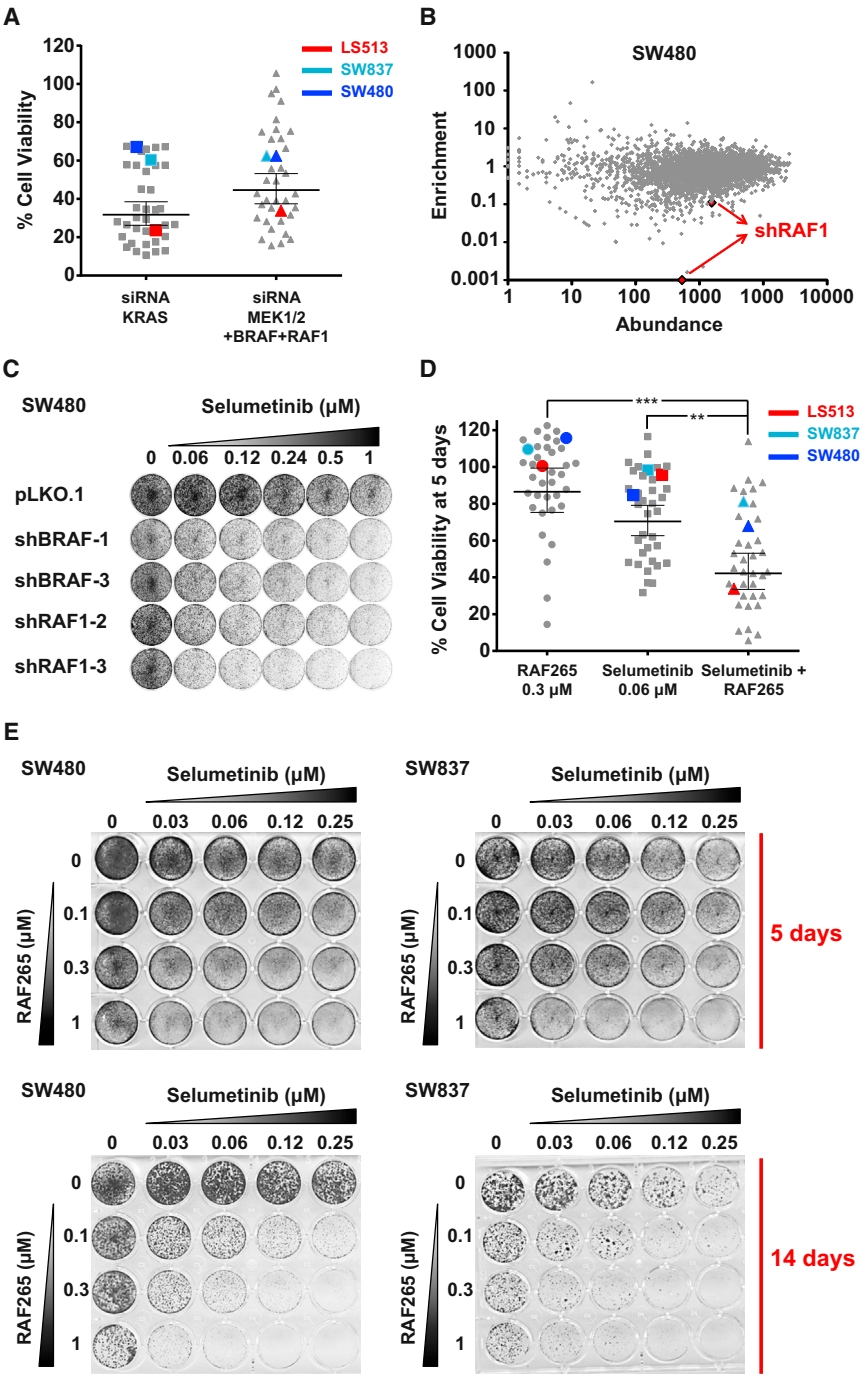

Figure 2. *KRAS* Mutant CRC Cell Lines Are Sensitive to Cosuppression of MEK1/MEK2 and BRAF/RAF1

(A) siRNA-mediated reverse genetic screen identified cell lines sensitive to combinatorial suppression of MEK1/MEK2-BRAF-RAF1. Cell viability was assessed after 5 days of treatment by measuring ATP content. Data points for each cell line are expressed as percentage of viability compared with cells treated with negative control (AllStar). The horizontal line and error bars indicate geometric mean ± 95% confidence interval (CI).

(B) Loss-of-function genetic screen nominates RAF1 suppression as synthetic lethal with MEK inhibition in SW480 cells. Each shRNA from the initial screen experiment is represented as a dot in the plot. The x axis shows the average counts of sequencing reads in the untreated sample. The y axis represents the fold change in abundance of each shRNA in the presence of absence of selumetinib.

(C) Suppression of RAF1 or BRAF by shRNA enhances response to MEK inhibition. SW480 *KRAS* mutant cells were infected with lentiviral shRNAs targeting RAF1 or BRAF. pLKO.1 empty vector served as a control vector. After puromycin selection, cells were seeded into 6-well plate (20,000 cells/well) and cultured in the absence or presence of the MEK inhibitor selumetinib at the indicated concentration for 2 weeks. At the end of the experiment, cells were fixed and stained with crystal violet solution.

(D) Thirty-seven (MSS) *KRAS* mutant cell lines were treated with the MEK inhibitor selumetinib, the pan-RAF inhibitor RAF265, or combination of selumetinib/RAF265 for 5 days. Cell viability was assessed by measuring ATP content. Data points are expressed as percent viability compared with DMSO-only treated cells. The horizontal line and error bars indicate geometric mean ± 95% CI. ***$p < 0.001$; **$p < 0.01$. Cell lines sensitive (LS513 in red) and cell lines insensitive (SW837 in light blue and SW480 in dark blue) to the combinatorial treatment are highlighted.

(E) Cells were treated for 5 or 14 days with increasing concentrations of selumetinib and RAF265. At the end of the experiment, cells were fixed and stained with crystal violet solution.

See also Figures S3 and S4.

We only considered shRNA vectors that had been sequenced at least 300 times in the untreated pool. To limit the off-target effects, hits were selected when two independent shRNAs targeting the same gene were depleted. We rank ordered the shRNAs by their negative selection in the screen. Using this criterion, we identified *RAF1* as the top gene to have two independent shRNAs depleted in the presence of MEK inhibitor (Figure 2B). Of note, *BRAF* was also retrieved in this screening. To validate this—unexpected—finding, we infected SW480 with two independent RAF1 and BRAF shRNAs and cultured them with or without selumetinib for 2 weeks (Figures 2C and S3B). The data confirmed results of the large-scale screening.

RAF-Targeted Agents Synergize with MEK Inhibition in *KRAS* Mutant CRC Cells

To extend the findings obtained by transcriptional suppression of BRAF and RAF1, we performed pharmacological analyses. RAF1-selective inhibitors are not available. However, several pan-RAF inhibitors, with different ranges of action on wild-type

RAF1 and wild-type BRAF have been synthesized, although none has been fully developed for clinical use. For our purposes, we selected RAF265, a small molecule with inhibitory activity against wild-type RAF1/BRAF (Su et al., 2012). We treated the panel of 37 *KRAS* mutant cell lines with RAF265 in the presence or absence of the MEK inhibitor selumetinib. We found that concomitant pharmacological inhibition of MEK and RAF led to a greater than 50% reduction of cell viability in 20/37 (54%) CRC lines (Figure 2D). To rule out that some of the known off-target effects of RAF265 (Su et al., 2012) could contribute to explain its synergy with MEK inhibition, we repeated the cell proliferation assays with AZ628. This compound has been previously reported to be more selective for wild-type RAF kinases and has been widely used for in vitro studies (Montagut et al., 2008; Whittaker et al., 2013). The results obtained with AZ628 in combination with selumetinib are consistent with the observations made with RAF265 (Figure S4A). Notably, the cells that were previously identified as sensitive to combined suppression of RAF1-BRAF and MEK were also affected by the corresponding pharmacological inhibition.

Why are some cells sensitive to inhibition of MEK and RAF in one experimental condition, but not in the other? We noted that, whereas siRNA experiments were based on short-term (5 days) growth assays, the shRNA screening involved a 2-week-long protocol. We therefore repeated the pharmacological screening in a long-term assay with two cell models, SW480 and SW837, which are refractory to the MEK-RAF blockade in the short-term assays (Figures 2A, 2D, and S4A). Both lines were treated with RAF265 and selumetinib as single agents, as well as their combination, for 5 or 14 days (Figure 2E). We found that the time frame (5 versus 14 days) of the experiments plays a critical role. Consistent with the reverse genetics screens, SW480 and SW837 were insensitive in the short-term pharmacological assay, whereas after 14 days, both were effectively inhibited by selumetinib in combination with either RAF265 (Figure 2E) or AZ628 (Figures S4B and S4C).

MEK Allosteric Inhibition Induces RAF Heterodimerization and Sustains ERK Activation

The above results point to a critical role for the kinase activity of BRAF and RAF1 in restraining the effectiveness of MEK inhibitors in *KRAS*-mutated CRC cells. To investigate the biochemical interplay between RAF suppression and MEK blockade, we performed vertical analysis of the pathway on two cell lines, LS513 and SW480, sensitive to the combinatorial inhibition in short-term and long-term assay, respectively. As expected, MEK inhibition alone led to increased pMEK and this was accompanied by incomplete suppression of pERK (Figure 3A). Intriguingly, MEK blockade also triggered phosphorylation of BRAF at its serine 445. A modest increase of RAF1 phosphorylation was also observed. Because KRAS itself is the most well-known activator of RAF proteins, we measured the activity of KRAS upon drug treatment. Selumetinib sustained activation of KRAS-GTP in both LS513 and SW480 cells (Figure 3B) although with a different magnitude of the effect. We reasoned that MEK inhibition could initiate a feedback loop involving RAS hyperactivation, which in turn modulates BRAF and RAF1. To scrutinize further the impact of RAF activation upon MEK inhibition, we performed coimmunoprecipitation experiments. MEK blockade triggered the formation of BRAF-RAF1 complexes, which are known to activate downstream signaling (Figure 3C; Garnett et al., 2005). Induction of heterodimerization is slightly stronger in SW480 than in LS513, paralleling the magnitude of the effect on RAS-GTP induction. We propose that, in a *KRAS* mutant background, MEK inhibition triggers KRAS hyperactivation, leading to engagement of BRAF/RAF1 heterodimers.

Increased (active) KRAS-GTP levels could result from phosphorylation of upstream tyrosine kinase receptors (RTKs), a scenario that has been previously described (Molina-Arcas et al., 2013; Turke et al., 2012). To confirm this in our cell models, we performed western blotting to measure levels of phosphorylated EGFR, HER2, and HER3 proteins upon selumetinib treatment. MEK blockade induced activation of both EGFR and HER3 in LS513 (Figure 3D). None of the tested RTKs were significantly hyperphosphorylated following selumetinib treatment in SW480 cells. However, in this cell line, we noted a persistent increase of activated AKT1 (Figure 3D), which, in turn, may be caused by phosphorylation other RTKs, such as IGF1R or HER3 (Ebi et al., 2011; Sun et al., 2014), or by inhibition of ERK-dependent feedback loops on AKT (Turke et al., 2012).

Synergistic Inhibition of RAF and MEK Abrogates ERK Signaling and Triggers Apoptosis in *KRAS* Mutant CRC Cells

We next investigated signaling changes induced by concomitant pharmacological blockade of MEK and RAF in *KRAS* mutant CRC cells. Whereas selumetinib as a single agent triggered MEK and ERK phosphorylation, the addition of RAF265 resulted in significant reduction of pERK and pAKT levels after 48 hr of treatment in LS513 cells (Figure 4A). The same effect is not observed in SW480 cells, which are refractory to concomitant MEK/RAF blockade in short-term assays (Figure 4B). In line with this, concomitant inhibition of MEK and BRAF/RAF1 biochemically initiates apoptosis, as confirmed by induction of PARP cleavage in LS513, but not in SW480 cells. Of note, MEK—per se—does not lead to PARP activation (Figures 4A and 4B), thus explaining the striking effects on cell viability achieved only by RAF/MEK combinatorial blockade.

Combined RAF-MEK Inhibition Shows Synergy in Lung Cancer Cells Harboring *KRAS* Mutations

We tested whether combined RAF and MEK inhibition could be effective also in other cancer types bearing *KRAS* mutations. To this end, we repeated the unbiased drop-out shRNA screening in two *KRAS* mutant NSCLC cell lines. Notably, as shown in Figures 5A and 5B, we again retrieved suppression of RAF1 as being critical to confer sensitivity to MEK inhibition. The results of the large-scale screening were confirmed by culturing H358 and H1792 lung cells infected with three independent RAF1 or BRAF shRNAs with or without selumetinib (Figures 5C and 5D). We further find that, as in the CRC setting, combinatorial pharmacological inhibition of RAF and MEK is effective in multiple *KRAS* mutant NSCLC cells (Figures 5E, 5F, S5A, and S5B). In line with this, MEK inhibition induces BRAF-RAF1 dimerization and concomitant inhibition of MEK and BRAF/RAF1 initiates apoptosis, as confirmed by induction

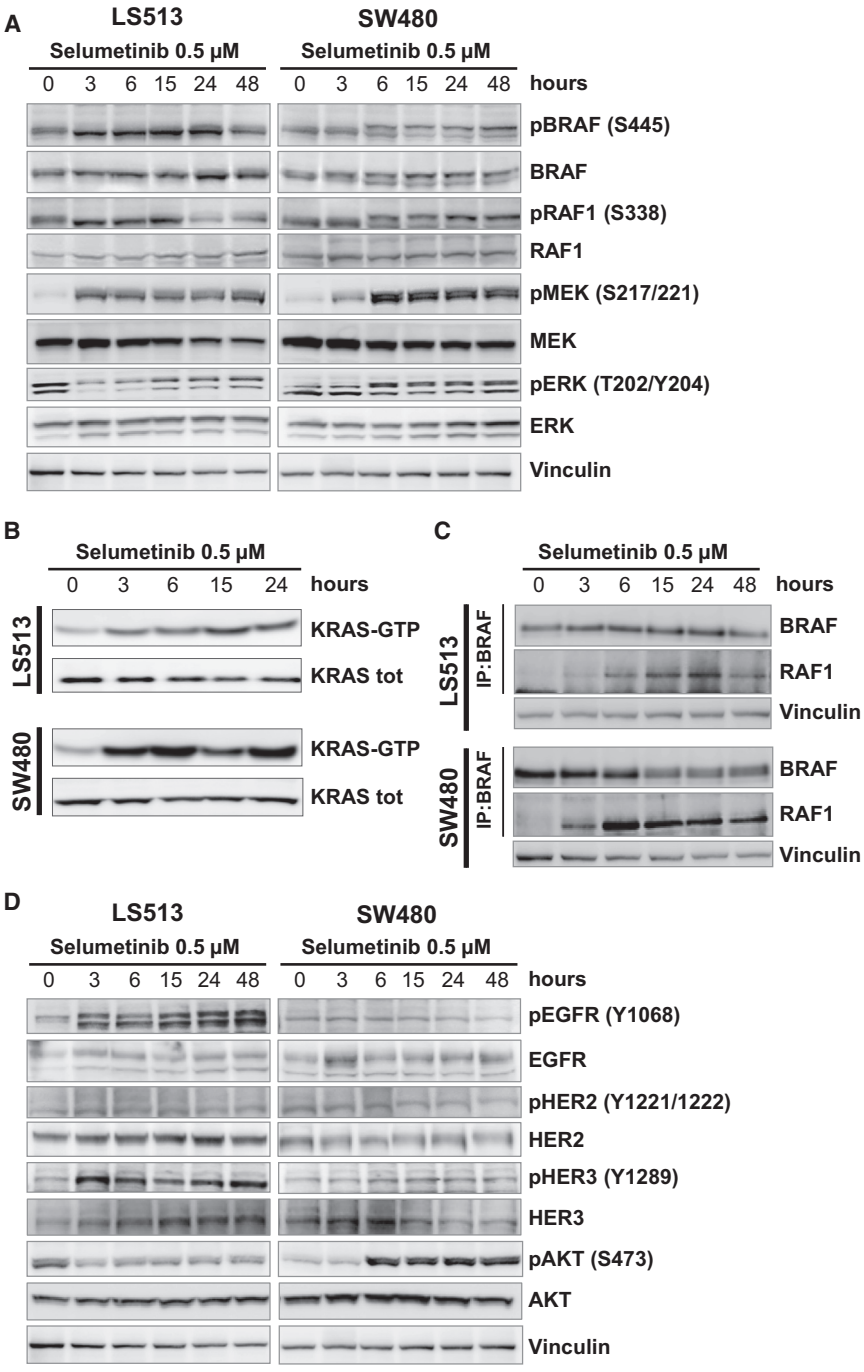

Figure 3. MEK Inhibition Induces KRAS Activation and RAF Heterodimerization in *KRAS* Mutant CRC Cells

(A) Cell lines were treated with selumetinib 0.5 μM at the indicated time points, after which whole-cell extracts were subjected to western blot analysis and probed with indicated antibodies. Vinculin was included as a loading control.

(B) Cell lines were treated with selumetinib 0.5 μM at the indicated time points, after which whole-cell extracts were subjected to pull-down of active KRAS-GTP using the GST-RAF1 Ras-binding domain.

(C) Cell lines were treated with selumetinib 0.5 μM at the indicated time points, after which whole-cell extracts were immunoprecipitated with BRAF antibody and subjected to western blot analysis with the indicated antibodies. Vinculin was included as a loading control. Input controls are shown in (A). IP, immunoprecipitation.

(D) Cell lines were treated with selumetinib 0.5 μM at the indicated time points, after which whole-cell extracts were subjected to western blot analysis and membranes were probed with indicated antibodies. Vinculin was included as a loading control. See also Figure S5.

of PARP cleavage (Figures S5C and S5D). In summary, these results show that the findings obtained in *KRAS*-mutated CRC cells can be extended to lung cancer models bearing *KRAS* mutations.

DISCUSSION

Thirty years after their discovery, *KRAS*-mutated cancers still pose a formidable challenge to researchers and clinicians alike, as efforts for direct pharmacological blockade of RAS have been largely unsuccessful. Approximately 20% of NSCLC and 40% of CRCs carry somatic variants of the *KRAS* gene. Although *KRAS* mutations occur at similar frequencies in microsatellite stable and unstable CRCs, the latter represent a distinct clinical entity. MSI tumors have a more favorable prognosis and are less prone to lymph node spread and metastasis than MSS tumors (Roth et al., 2012). Accordingly, epidemiological analyses indicate that mCRC patients with *KRAS* mutant MSS disease represent the clinical population who would benefit the most from *KRAS* targeting. Based on these premises, we performed a suppression screen for genes involved in KRAS signaling in a collection of 37 MSS *KRAS* mutant cell lines. This approach revealed that a large fraction of CRC cells (67%) were highly dependent on KRAS expression. The screening also highlighted that silencing of individual genes involved in RAS signaling is ineffective on suppressing the growth of *KRAS* mutant CRC cells. This suggests that RAS controls a largely redundant signaling network, which guarantees that interference with an individual effector does not interrupt pathway output (Cox and Der, 2010; Gysin et al., 2011). The architecture of the EGFR-RAS-MEK-signaling pathway is evolutionarily ancient and increased in complexity during evolution (Malumbres and Barbacid, 2003; Yarden and Pines, 2012). In vertebrate cells, this signaling network allegedly encompasses several interconnected routes.

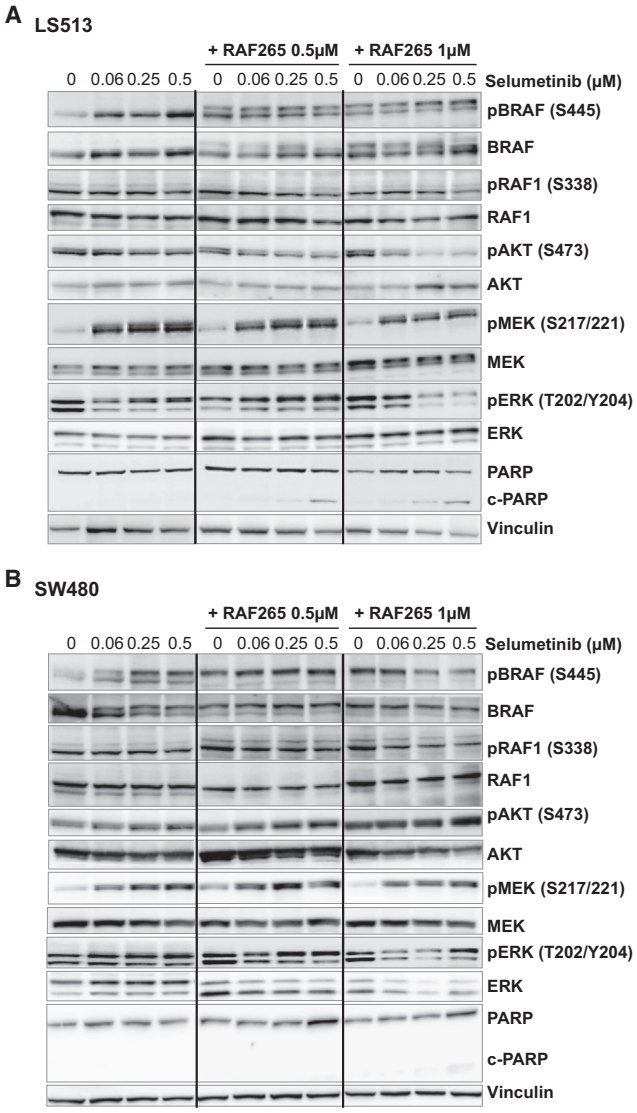

Figure 4. Concomitant Inhibition of MEK and RAF Induce Apoptosis in *KRAS* Mutant CRC Cells

LS513 (A) and SW480 (B) cells were treated with increasing concentrations of selumetinib or selumetinib combined with different concentration of RAF265 for 48 hr. Lysates were subjected to western blot analyses, and membranes were probed with the indicated antibodies. Vinculin was included as a loading control. See also Figure S5.

To identify signaling nodes in the EGFR-KRAS-MEK network whose concomitant blockade might suppress the growth of *KRAS* mutant cells, we performed a combinatorial siRNA screen. The results showed that suppressing MEK is almost invariably necessary but nearly always insufficient to halt growth of *KRAS*-mutated cells. Genes retrieved as being capable of synergizing with MEK silencing could be cataloged in two classes: members of the receptor tyrosine kinase family (RTKs) and elements of the RAF family of serine/threonine kinases. Blockade of RTKs (such as EGFR, HER3, IGF1R, and MET) in conjunction with MEK inhibition for *KRAS* mutant CRCs has been explored in several previous studies (Ebi et al., 2011; Molina-Arcas et al., 2013; Sun et al., 2014; Turke et al., 2012; Van Schaeybroeck et al., 2014). In this work, we focused instead on co-targeting RAFs and MEK. We found that this combination is effective in 21/37 (57%) of *KRAS* mutant MSS CRC cell lines. To understand why the combination was ineffective in a significant proportion of the cell models, one of the refractory lines was subjected to an unbiased drop-out screen to unveil kinase genes synergizing with MEK inhibition. Rather unexpectedly, these experiments retrieved again *RAF1*. Importantly, the same synergistic interaction was confirmed, by shRNA screening, in two NSCLC cell lines bearing *KRAS* mutations. This incongruity was resolved when combined RAF-MEK suppression was tested in short- and long-term assays and showed to be active in a large proportion of mutant *KRAS* CRC and NSCLC cell lines when both conditions were examined. Why should suppression of RAF, which acts immediately upstream of MEK, be necessary to halt growth of *KRAS* mutant CRCs? We found that MEK inhibition increases the GTP-bound fraction of KRAS, promotes the formation of RAF1-BRAF heterodimers (the most active complex among all RAF dimers), and drives constitutive phosphorylation of ERK. These results are in accordance with a recent report showing that RAF1 knockdown enhanced MEK inhibition in a *KRAS* mutant model (Lito et al., 2014). Lito et al. (2014) found that allosteric inhibitors of MEK, such as selumetinib, can induce the formation of RAF-MEK complexes in *KRAS* mutant cells. This effect may be less relevant or not present with new-generation catalytic MEK inhibitors, but additional studies are needed to assess the activity of these MEK-targeted agents in the context of mutant KRAS.

Among the additional hits retrieved by our screen, co-silencing of ERK1 and ERK2 reduced the viability by less than 50% in 15/37 *KRAS* mutant CRC cell lines. ERK inhibitors are still in preclinical development, and their efficacy in *KRAS* mutant cancers has not been extensively investigated. However, previous works suggest that blockade of the EGFR-RAS-MEK axis with agents targeting a single node has often limited impact due to feedback reactivation of the signaling pathway (Ebi et al., 2011; Molina-Arcas et al., 2013; Sun et al., 2014; Turke et al., 2012; Van Schaeybroeck et al., 2014). Of relevance, it has recently been reported that *BRAF* mutant melanoma cells are often resistant to ERK inhibition, because the relief of ERK-dependent negative feedback can activate RAS and PI3K signaling (Carlino et al., 2014). Nevertheless, our data suggest that future studies are needed to investigate the biological effects of ERK inhibition and its biochemical consequences within the context of mutant *KRAS*.

We propose a model whereby *KRAS* mutant cancers can be besieged with two, non-mutually exclusive approaches. The first involves blockade of the upstream RTKs, which presumably are activated through one of the feedback loops that we and others had previously identified (Ebi et al., 2011; Misale et al., 2014; Molina-Arcas et al., 2013; Prahallad et al., 2012; Sun et al., 2014; Turke et al., 2012). For this strategy to be successful, a priori knowledge of the RTK involved in a given tumor/patient is essential or the use of inhibitors capable of intercepting simultaneously multiple RTKs must be applied. This approach is

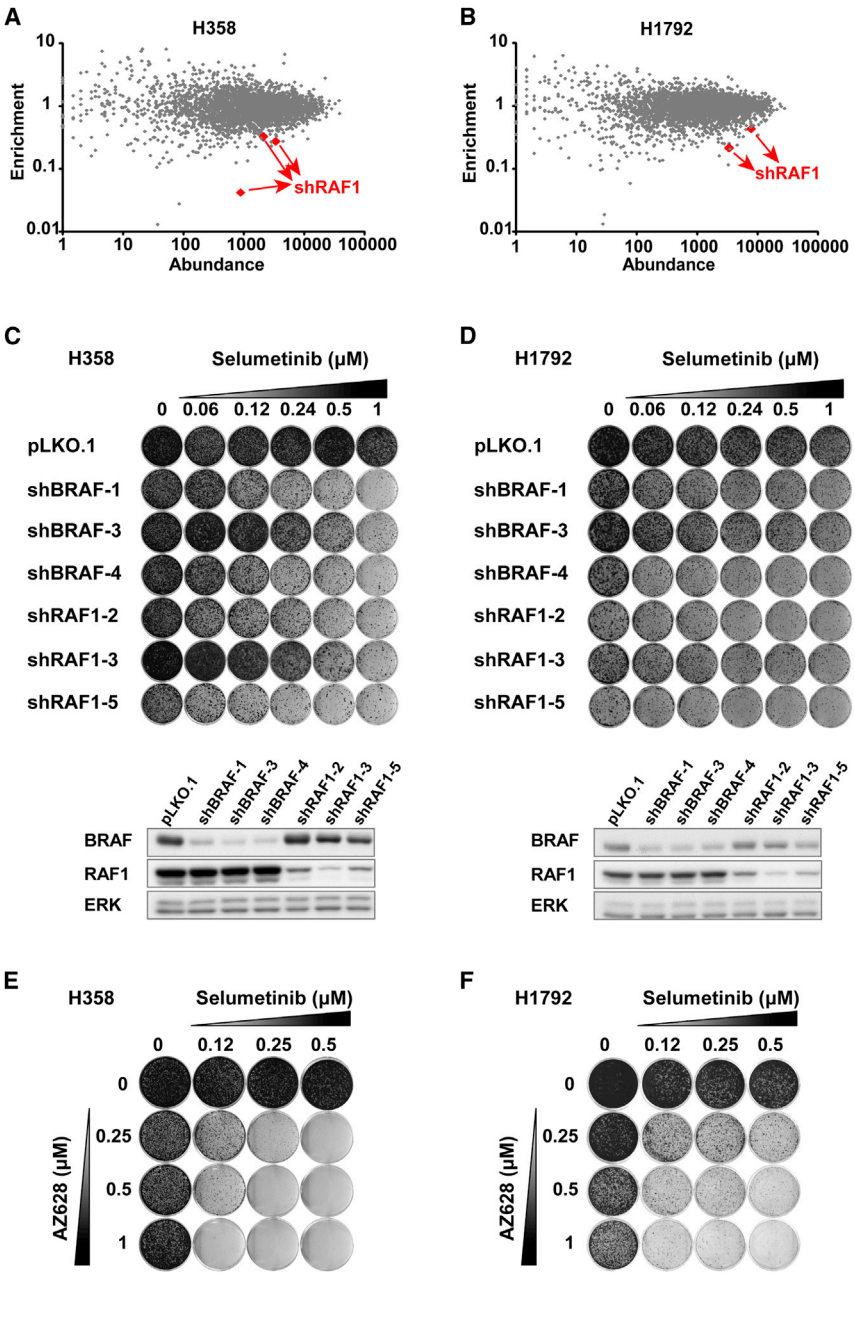

Figure 5. RAF1 Is Synthetic Lethal with MEK Inhibition in *KRAS* Mutant NSCLC Tumors

(A and B) Loss-of-function genetic screen retrieves RAF1 suppression as synthetic lethal with MEK inhibition in H358 (A) and H1792 (B) NSCLC cells. Each shRNA from the initial screen experiment is represented as a dot in the plot. The x axis shows the average counts of sequencing reads in the untreated sample. The y axis represents the fold change in abundance of each shRNA in the presence or absence of selumetinib.

(C and D) Suppression of RAF1 or BRAF by shRNA enhances response to MEK inhibition. H358 (A) and H1792 (B) *KRAS* mutant cells were infected with lentiviral shRNAs targeting RAF1 or BRAF. pLKO.1 empty vector served as a control vector. After puromycin selection, cells were seeded into 6-well plate (20,000 cells/well) and cultured in the absence or presence of the MEK inhibitor selumetinib at the indicated concentration for 2 weeks. At the end of the experiment, cells were fixed and stained with crystal violet solution. The level of RAF1 and BRAF knockdown was determined by western blot. ERK1/ERK2 served as a loading control.

(E and F) NSCLC cells were treated 14 days with increasing concentrations of selumetinib and AZ628. At the end of the experiment, cells were fixed and stained with crystal violet solution.

See also Figure S5.

hindered by the need for biomarkers to reliably assess the feedback loop engaged in each individual tumor; the second strategy is likely associated with toxicity and limited therapeutic index, which often accompany multikinase inhibitors.

An alternative approach, which we have outlined in this work, involves interfering with mutant KRAS by concomitant blockade of MEK and RAFs. The exploitation of this tactic depends on the development of a new class of—clinically suited—inhibitors capable of simultaneously blocking wild-type BRAF and RAF1. Tool compounds having this activity are available mainly for in vitro use, but these compounds are highly toxic, even to experimental animals. Thus, further testing in vivo of the combination therapy proposed here will require the development of a new generation of pan-RAF inhibitors with better pharmacological properties.

EXPERIMENTAL PROCEDURES

Cell Lines

The cell lines used in this study were collected from commercial sources or academic laboratories as indicated in Table S1. Cells were kept in the indicated culture growth media supplemented with 10% FBS, 50 units/ml penicillin, 50 mg/ml streptomycin, and 0.25 mg/ml Fungizone. The genetic identity of each cell line was confirmed by short tandem repeat profiling (Cell ID System; Promega), and the cells were last checked no less than 3 months before performing reverse genetics screen experiments. Results of the analyzed loci for each cell line are provided in Table S2. All CRC cell lines were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

siRNA Screening

The siRNA-targeting reagents were purchased from Dharmacon, as a SMARTpool of four distinct siRNA species targeting different sequences of the target transcript. Cell lines were grown and transfected with SMARTpool siRNAs using Dharmafect 4 (Dharmacon), Lipofectamine2000, or RNAiMAX (Invitrogen) transfection reagents following manufacturer's instructions. Briefly RNAi screening conditions were as follows: on day 1, siRNA were distributed in each well of a 384-wells plate at final concentration of 20 nmol/l. Transfection reagents were incubated in OptiMEM serum-free

media for 20 min and aliquoted at 10 μl/well; after that, 35 μl of cells in media without antibiotics were added to each well. After 5 days, cell viability was estimated with a luminescent assay measuring cellular ATP levels (CellTiter-Glo Luminescent Assay; Promega). Each plate included the following controls: mock control (transfection lipid only), siControl1 (Dharmacon), AllStars (QIAGEN) as negative control, and polo-like kinase 1 (Dharmacon), which served as positive control (Brough et al., 2011). The RNAi screens were carried out in quadruplicate, and survival fraction was calculated as a ratio between the average of experiment well readout and the negative control, nontargeting siRNA (AllStars; QIAGEN).

Loss-of-Function shRNA Screen

The shRNA library used in this study was designed to target 535 human kinases and kinase-related genes. The lentiviral-based vectors were collected from The RNAi Consortium (TRC) human genome-wide shRNA collection (TRC-Hs1.0). SW480 cells were infected by pooled lentivirus generated from the above-mentioned shRNA library. After puromycin selection, cells were cultured in the presence or absence of selumetinib. shRNA sequences were recovered by PCR, and the abundance of each hairpin in pooled samples was determined by deep sequencing.

Drug Proliferation Assay

CRC cell lines were seeded at different densities (1.5–2.5 × 10^3cells/well) in 100 μl complete growth medium in 96-well plastic culture plates at day 0. The following day, serial dilutions of selumetinib and RAF265 were added to the cells in serum-free medium, whereas DMSO-only treated cells were included as controls. Plates were incubated at 37°C in 5% CO_2 for 5 days, after which, cell viability was assessed by measuring ATP content through Cell Titer-Glo Luminescent Cell Viability assay (Promega). For long-term proliferation assays, cells were seeded in 24-well plates (3–5 × 10^3 cells per well) and cultured in the absence and presence of drugs as indicated. Wells were fixed with 3% paraformaldehyde and stained with 1% crystal violet-methanol solution (Sigma-Aldrich) after 2 weeks. All assays were performed independently at least three times. AZ628, selumetinib, and RAF265 were purchased from Selleck Chemicals.

Western Blotting Analysis and Immunoprecipitation

Prior to biochemical analysis, all cells were grown in their specific media supplemented with 10% FBS and treated with selumetinib or RAF265 at indicated time points and concentrations. Total cellular proteins were extracted by solubilizing the cells in boiling SDS buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 1% SDS) or in cold extraction buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and 2 mM EGTA; all reagents were from Sigma-Aldrich, except for Triton X-100 from Fluka) in the presence of 1 mM sodium orthovanadate, 100 mM sodium fluoride, and a mixture of protease inhibitors (pepstatin, leupeptin, aprotinin, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride). Extracts were clarified by centrifugation, and protein concentration was determined using BCA Protein Assay Reagent kit (Thermo Scientific). Western blot detection was performed with enhanced chemiluminescence system (GE Healthcare) and peroxidase conjugated secondary antibodies (Amersham). The following primary antibodies were used for western blotting (all from Cell Signaling Technology, except where indicated): anti-phospho- BRAF (Ser445); anti-BRAF (Santa Cruz); anti-phospho-RAF1 (Ser338); anti RAF1; anti-phospho-p44/42 ERK (Thr202/Tyr204); anti-p44/42 ERK; anti-phospho-MEK1/MEK2 (Ser217/Ser221), anti-MEK1/MEK2; anti-KRAS (Sigma Aldrich); anti-EGFR (clone13G8; Enzo Life Sciences); anti-phospho AKT (Ser473); anti-AKT; anti-phospho EGFR (Tyr1068; Abcam); anti-phospho-HER2 (Tyr1221/Tyr1222); anti-HER2 (Santa Cruz); anti-phosphor-HER3 (Tyr 1289); anti-HER3 (Millipore); antivinculin (Millipore); and antiactin (Santa Cruz). Immunoprecipitation was carried out following cell lysis in extraction buffer in the presence of 1 mM sodium orthovanadate, 100 mM sodium fluoride, and protease and phosphatase inhibitors. Extracts were clarified by centrifugation, normalized with the BCA Protein Assay Reagent kit (Thermo Scientific), and incubated with indicated antibodies for 2 hr at 4°C. Immune complexes were collected with protein A-Sepharose, washed in extraction buffer, and eluted. Extracts were electrophoresed on SDS-polyacrylamide gels and processed as described before.

RAS Activation Assay

GST-RAF1-RAS-binding domain fusion proteins were expressed in *Escherichia coli* by induction with 0.2 mM isopropyl-1-thioβ-D-galactopyranoside for 4 hr at 30°C. The expressed fusion proteins were isolated from bacterial lysates by affinity chromatography with glutathione agarose beads. Six hundred micrograms of whole-cell cleared lysate was incubated with 10 μg of GST-RAF1-Ras-binding domain for 90 min at 4°C. The complexes were collected by centrifugation and washed three times with lysis buffer. Proteins were separated by SDS-PAGE followed by western blot. The KRAS protein was detected with anti-KRAS monoclonal antibody (Sigma-Aldrich). Total lysates (20 μg) from the above cells were immunoblotted with anti-KRAS antibody (Sigma-Aldrich) as a loading control.

Statistical Analysis

Statistical significance for the data of the short-term drug assays was determined by nonparametric Kruskal-Wallis statistics with a Dunn's multiple comparison posttest. This test was chosen because the data did not follow a normal distribution. $p < 0.01$ and $p < 0.001$ were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.07.033.

AUTHOR CONTRIBUTIONS

A.B., F.D.N., R.B., C.S., and S.L. designed the study. S.L., M.R., C.S., L.L., and C.C. performed experiments and analyzed the data. W.G. and C.L. contributed to the analysis of data. A.B., F.D.N., S.L., and M.R. wrote the manuscript. A.B., F.D.N., and R.B. supervised the study.

ACKNOWLEDGMENTS

Supported by the European Community's Seventh Framework Programme under grant agreement no. 259015 COLTHERES (to A.B. and R.B.); Associazione Italiana per la Ricerca sul Cancro (AIRC) IG grant no. 12812 (to A.B.); AIRC MFAG grant no. 11349 (to F.D.N.); grant "Farmacogenomica"—5 per mille 2009 MIUR—Fondazione Piemontese per la Ricerca sul Cancro—ONLUS (to F.D.N.); AIRC 2010 Special Program Molecular Clinical Oncology 5 per mille, project no. 9970 (to A.B.); grant 5 per mille 2011 Ministero della Salute-FPRC onlus (to A.B.); FPRC 5 per mille 2010 Ministero della Salute (to A.B.); Ministero dell'Istruzione, dell'Università e della Ricerca, progetto PRIN (to A.B.); and Progetti di Ateneo-2011, Università di Torino (ORTO11RKTW to A.B.). This work was also supported by grants to R.B. from the European Research Council (ERC), the Dutch Cancer Society (KWF), and the Netherlands Organization for Scientific Research (NWO) to Cancer Genomics Netherlands (CGC.NL).

Received: June 12, 2014
Revised: July 10, 2014
Accepted: July 20, 2014
Published: September 4, 2014

REFERENCES

Adjei, A.A., Cohen, R.B., Franklin, W., Morris, C., Wilson, D., Molina, J.R., Hanson, L.J., Gore, L., Chow, L., Leong, S., et al. (2008). Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. J. Clin. Oncol. *26*, 2139–2146.

Brough, R., Frankum, J.R., Sims, D., Mackay, A., Mendes-Pereira, A.M., Bajrami, I., Costa-Cabral, S., Rafiq, R., Ahmad, A.S., Cerone, M.A., et al. (2011). Functional viability profiles of breast cancer. Cancer Discov. *1*, 260–273.

Carlino, M.S., Todd, J.R., Gowrishankar, K., Mijatov, B., Pupo, G.M., Fung, C., Snoyman, S., Hersey, P., Long, G.V., Kefford, R.F., and Rizos, H. (2014).

Differential activity of MEK and ERK inhibitors in BRAF inhibitor resistant melanoma. Mol. Oncol. *8*, 544–554.

Cox, A.D., and Der, C.J. (2010). Ras history: The saga continues. Small GTPases *1*, 2–27.

Douillard, J.Y., Oliner, K.S., Siena, S., Tabernero, J., Burkes, R., Barugel, M., Humblet, Y., Bodoky, G., Cunningham, D., Jassem, J., et al. (2013). Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N. Engl. J. Med. *369*, 1023–1034.

Ebi, H., Corcoran, R.B., Singh, A., Chen, Z., Song, Y., Lifshits, E., Ryan, D.P., Meyerhardt, J.A., Benes, C., Settleman, J., et al. (2011). Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. J. Clin. Invest. *121*, 4311–4321.

Faber, A.C., Coffee, E.M., Costa, C., Dastur, A., Ebi, H., Hata, A.N., Yeo, A.T., Edelman, E.J., Song, Y., Tam, A.T., et al. (2014). mTOR inhibition specifically sensitizes colorectal cancers with KRAS or BRAF mutations to BCL-2/BCL-XL inhibition by suppressing MCL-1. Cancer Discov. *4*, 42–52.

Ganesan, P., Janku, F., Naing, A., Hong, D.S., Tsimberidou, A.M., Falchook, G.S., Wheler, J.J., Piha-Paul, S.A., Fu, S., Stepanek, V.M., et al. (2013). Target-based therapeutic matching in early-phase clinical trials in patients with advanced colorectal cancer and PIK3CA mutations. Mol. Cancer Ther. *12*, 2857–2863.

Garnett, M.J., Rana, S., Paterson, H., Barford, D., and Marais, R. (2005). Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. Mol. Cell *20*, 963–969.

Gysin, S., Salt, M., Young, A., and McCormick, F. (2011). Therapeutic strategies for targeting ras proteins. Genes Cancer *2*, 359–372.

Karapetis, C.S., Khambata-Ford, S., Jonker, D.J., O'Callaghan, C.J., Tu, D., Tebbutt, N.C., Simes, R.J., Chalchal, H., Shapiro, J.D., Robitaille, S., et al. (2008). K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N. Engl. J. Med. *359*, 1757–1765.

Linardou, H., Dahabreh, I.J., Kanaloupiti, D., Siannis, F., Bafaloukos, D., Kosmidis, P., Papadimitriou, C.A., and Murray, S. (2008). Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol. *9*, 962–972.

Lito, P., Saborowski, A., Yue, J., Solomon, M., Joseph, E., Gadal, S., Saborowski, M., Kastenhuber, E., Fellmann, C., Ohara, K., et al. (2014). Disruption of CRAF-mediated MEK activation is required for effective MEK inhibition in KRAS mutant tumors. Cancer Cell *25*, 697–710.

Malumbres, M., and Barbacid, M. (2003). RAS oncogenes: the first 30 years. Nat. Rev. Cancer *3*, 459–465.

Misale, S., Arena, S., Lamba, S., Siravegna, G., Lallo, A., Hobor, S., Russo, M., Buscarino, M., Lazzari, L., Sartore-Bianchi, A., et al. (2014). Blockade of EGFR and MEK intercepts heterogeneous mechanisms of acquired resistance to anti-EGFR therapies in colorectal cancer. Sci. Transl. Med. *6*, 224ra226.

Molina-Arcas, M., Hancock, D.C., Sheridan, C., Kumar, M.S., and Downward, J. (2013). Coordinate direct input of both KRAS and IGF1 receptor to activation of PI3 kinase in KRAS-mutant lung cancer. Cancer Discov. *3*, 548–563.

Montagut, C., Sharma, S.V., Shioda, T., McDermott, U., Ulman, M., Ulkus, L.E., Dias-Santagata, D., Stubbs, H., Lee, D.Y., Singh, A., et al. (2008). Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. Cancer Res. *68*, 4853–4861.

Ostrem, J.M., Peters, U., Sos, M.L., Wells, J.A., and Shokat, K.M. (2013). K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. Nature *503*, 548–551.

Prahallad, A., Sun, C., Huang, S., Di Nicolantonio, F., Salazar, R., Zecchin, D., Beijersbergen, R.L., Bardelli, A., and Bernards, R. (2012). Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature *483*, 100–103.

Prior, I.A., Lewis, P.D., and Mattos, C. (2012). A comprehensive survey of Ras mutations in cancer. Cancer Res. *72*, 2457–2467.

Pylayeva-Gupta, Y., Grabocka, E., and Bar-Sagi, D. (2011). RAS oncogenes: weaving a tumorigenic web. Nat. Rev. Cancer *11*, 761–774.

Roth, A.D., Delorenzi, M., Tejpar, S., Yan, P., Klingbiel, D., Fiocca, R., d'Ario, G., Cisar, L., Labianca, R., Cunningham, D., et al. (2012). Integrated analysis of molecular and clinical prognostic factors in stage II/III colon cancer. J. Natl. Cancer Inst. *104*, 1635–1646.

Smith, C.G., Fisher, D., Claes, B., Maughan, T.S., Idziaszczyk, S., Peuteman, G., Harris, R., James, M.D., Meade, A., Jasani, B., et al. (2013). Somatic profiling of the epidermal growth factor receptor pathway in tumors from patients with advanced colorectal cancer treated with chemotherapy ± cetuximab. Clin. Cancer Res. *19*, 4104–4113.

Su, Y., Vilgelm, A.E., Kelley, M.C., Hawkins, O.E., Liu, Y., Boyd, K.L., Kantrow, S., Splittgerber, R.C., Short, S.P., Sobolik, T., et al. (2012). RAF265 inhibits the growth of advanced human melanoma tumors. Clin. Cancer Res. *18*, 2184–2198.

Sun, C., Hobor, S., Bertotti, A., Zecchin, D., Huang, S., Galimi, F., Cottino, F., Prahallad, A., Grernrum, W., Tzani, A., et al. (2014). Intrinsic resistance to MEK inhibition in KRAS mutant lung and colon cancer through transcriptional induction of ERBB3. Cell Reports *7*, 86–93.

Turke, A.B., Song, Y., Costa, C., Cook, R., Arteaga, C.L., Asara, J.M., and Engelman, J.A. (2012). MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. Cancer Res. *72*, 3228–3237.

Van Schaeybroeck, S., Kalimutho, M., Dunne, P.D., Carson, R., Allen, W., Jithesh, P.V., Redmond, K.L., Sasazuki, T., Shirasawa, S., Blayney, J., et al. (2014). ADAM17-Dependent c-MET-STAT3 Signaling Mediates Resistance to MEK Inhibitors in KRAS Mutant Colorectal Cancer. Cell Reports *7*, 1940–1955.

Whittaker, S.R., Theurillat, J.P., Van Allen, E., Wagle, N., Hsiao, J., Cowley, G.S., Schadendorf, D., Root, D.E., and Garraway, L.A. (2013). A genome-scale RNA interference screen implicates NF1 loss in resistance to RAF inhibition. Cancer Discov. *3*, 350–362.

Yarden, Y., and Pines, G. (2012). The ERBB network: at last, cancer therapy meets systems biology. Nat. Rev. Cancer *12*, 553–563.

Zimmermann, G., Papke, B., Ismail, S., Vartak, N., Chandra, A., Hoffmann, M., Hahn, S.A., Triola, G., Wittinghofer, A., Bastiaens, P.I., and Waldmann, H. (2013). Small molecule inhibition of the KRAS-PDEδ interaction impairs oncogenic KRAS signalling. Nature *497*, 638–642.