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Molecular landscape of acquired resistance to targeted therapy combinations in *BRAF* mutant colorectal cancer

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Summary

Although recent clinical trials of BRAF inhibitor combinations have demonstrated improved efficacy in *BRAF* mutant colorectal cancer, emergence of acquired resistance limits clinical benefit. Here, we undertook a comprehensive effort to define mechanisms underlying drug resistance with the goal of guiding development of therapeutic strategies to overcome this limitation. We generated a broad panel of *BRAF* mutant resistant cell line models across seven different clinically-relevant drug combinations. Combinatorial drug treatments were able to abrogate ERK1/2 phosphorylation in parental sensitive cells, but not in their resistant counterparts, indicating that resistant cells escaped drug treatments through one or more mechanisms leading to biochemical reactivation of the MAPK signaling pathway. Genotyping of resistant cells identified gene amplification of *EGFR, KRAS* and mutant *BRAF*, as well as acquired mutations in *KRAS*, *EGFR,* and *MAP2K1*. These mechanisms were clinically relevant, as we identified emergence of a *KRAS* G12C mutation and increase of mutant *BRAF* V600E allele frequency in the circulating tumor DNA of a patient at relapse from combined treatment with BRAF and MEK inhibitors. In order to identify therapeutic combinations capable of overcoming drug resistance, we performed a systematic assessment of candidate therapies across the panel of resistant cell lines. Independent of the molecular alteration acquired upon drug pressure, most resistant cells retained sensitivity to vertical MAPK pathway suppression when combinations of ERK, BRAF, and EGFR inhibitors were applied. These therapeutic combinations represent promising strategies for future clinical trials in *BRAF* mutant colorectal cancer.

INTRODUCTION

Activating mutations in the *BRAF* oncogene occur in approximately 7% of human malignancies, including 50-60% of melanomas and 5-8% of colorectal cancers (CRCs) (1). The most frequent *BRAF* mutation (V600E) affects the kinase domain, mimics BRAF phosphorylated state, and leads to constitutive activation of the protein (1). In CRC, *BRAF* mutations are associated with hypermethylated tumor subtypes and are linked with aggressive, less-differentiated and therapy-resistant disease (2). Metastatic CRC (mCRC) patients with *BRAF* V600E mutant tumors show poor sensitivity to the EGFR-targeted monoclonal antibodies panitumumab and cetuximab and display poor prognosis with a median overall survival of only about 6-9 months (3).

BRAF V600E mutant tumor types do not respond uniformly to BRAF-targeted therapy (4). Targeted inhibitors of mutant *BRAF* alone, or in combination with inhibitors of its downstream effector MEK, induce high response rates in *BRAF* mutant melanoma (5,6); by contrast, a phase I study of mCRC patients has shown that the BRAF inhibitor (BRAFi) vemurafenib has no clinical benefit when given as monotherapy (7). The molecular basis of this discrepancy has been partly explained by dissimilar EGFR expression levels between these two malignancies. Intrinsic resistance of CRC cells to BRAF or MEK targeted agents is mediated by the release of a feedback loop which activates EGFR signaling, leading to reactivation of MAPK signaling and often to upregulation of parallel PI3K-AKT pathways, triggering proliferation and survival (8-10). Melanomas are sensitive to BRAFi as they originate from the neural crest and do not express EGFR, making this feedback loop

ineffective. On the other hand, CRCs arise from epithelial cells in which EGFR is generally constitutively expressed.

These preclinical studies have provided the rationale for testing dual/triple vertical blockade of the MAPK pathway by targeting EGFR, BRAF, and MEK in *BRAF* mutant mCRC patients. Combinations targeting EGFR, BRAF, and the pro-survival PI3K pathways are also being explored. Clinical objective responses have been seen in 20-40% of patients treated with doublet or triplet combinatorial regimens (11-13).

Nevertheless, preliminary clinical evidence from phase Ib trials shows that responses are limited in duration (4,11-16). The molecular basis underlying intrinsic or acquired resistance to these drug combinations in *BRAF* mutant mCRC has not been comprehensively defined. The mechanisms by which cancer cells evade targeted therapies are usually molecularly heterogeneous, but they often converge downstream in the pathway which was originally blocked by the targeted agent. For instance, cell lines and mCRC patients that become resistant to single-agent cetuximab or panitumumab show a variety of molecular mechanisms that converge in reactivating the MAPK pathway, including mutations in the drug binding sites of *EGFR*, *RAS*/*RAF* amplification or mutations, or genetic alterations leading to activation of alternative receptor tyrosine kinases (RTKs) such as MET or HER2 (reviewed in (17)). Similarly, *BRAF* mutant melanomas that become refractory to BRAF and/or MEK inhibitors (MEKi) also show a variety of molecular mechanisms leading to reactivation of MAPK and/or AKT signaling. These include increased expression of RTKs such as PDGFRβ, IGF-1R and EGFR; overexpression of the

COT kinase; mutation of MEK1 (*MAP2K1*) and MEK2 (*MAP2K2*) kinase; *MITF* or *NRAS* mutations; amplification, or alternative splicing of the *BRAF* gene; *CDKN2A* loss; or genetic alterations in the PI3K-PTEN-AKT pathway (reviewed in (18)).

On these premises, we hypothesized that heterogeneous genetic alterations leading to reactivation of the MAPK pathway could be responsible for acquired resistance to regimens co-targeting EGFR, BRAF, MEK, and PI3K in CRC patients, despite vertical pathway suppression at multiple key nodes. To perform a comprehensive assessment of the landscape of potential acquired resistance mechanisms, we cultured *BRAF* mutant CRC cell lines in the presence of seven distinct clinically-relevant combinatorial regimens until the emergence of resistant derivatives. These cell lines were subjected to genetic, biochemical, and functional analyses to identify molecular alterations underlying drug resistance. Since *in vitro* modeling of acquired resistance in cancer cell models has proven effective in identifying resistance mechanisms that occur clinically (19-21), these findings may predict those mechanisms of resistance likely to arise in patients. These preclinical models also represent valuable tools for key functional studies aimed at identifying effective strategies to overcome drug resistance.

Materials and Methods

Generation of drug resistant cell lines

WiDr parental cells were a gift from Dr René Bernards (Amsterdam, The Netherlands) in July 2011. HROC87 parental cells were shared by Michael

Linnebacher (Rostock, Germany) in September 2011. VACO432 parental cells were obtained from Horizon Discovery (Cambridge, United Kingdom) in March 2011. The genetic identity of parental cell lines and their resistant derivatives was confirmed by short tandem repeat profiling (Cell ID System; Promega) not fewer than 2 months before drug profiling experiments. *BRAF* mutant HROC87, VACO432 and WiDr cells were seeded in 100-mm dishes at a density of $5x10^6$/plate and treated with drug combinations as indicated in **Supplementary Table S1**. Additional information is provided in Supplementary Materials and Methods.

Drug sensitivity assay

Cell proliferation and cytoxicity were determined by cellular ATP levels (CellTiter-Glo Luminescent Assay; Promega) and DNA incorporation of a fluorescent cyanine dye (CellTox Green; Promega) after 72 hours' drug treatment, respectively. Additional information is provided in Supplementary Materials and Methods and **Supplementary Table S2**.

Western Blot analysis

Protein quantification, SDS-PAGE, western blotting and chemiluminescent detection were performed as previously described (19). Detailed information is provided in Supplementary Materials and Methods.

Gene copy number analysis qPCR

Cell line DNA (10 ng) was amplified by quantitative PCR using the GoTaq QPCR Master Mix (Promega) with an ABI PRISM 7900HT apparatus (Applied Biosytems). *HER2*, *MET*, *EGFR*, *KRAS* and *BRAF* gene copy number was assessed as

previously described (19). Data were normalized to a control diploid cell line, HCEC (22) and expressed as the ratio between resistant and the corresponding parental cells. Primer sequences are reported in **Supplementary Table S3.**

Fluorescence *In Situ* Hybridization (FISH)

Dual color FISH analysis was performed using: Chr7q (7q11.21) / *BRAF* (7q34) probes; Chr7q / *EGFR* (7p12) probes; Chr12q (12q12) / *KRAS* (12p12.1) probes (Abnova); all probe pairs labelled with FITC and Texas Red, respectively. Details are provided in Supplementary Materials and Methods.

Candidate-gene mutational analysis

Cell line DNA was extracted by Wizard® SV Genomic DNA Purification System (Promega) according to manufacturer's directions. The following genes and exons were analyzed by automated Sanger sequencing by ABI PRISM 3730 (Applied Biosystems): *KRAS* (exons 2, 3 and 4), *NRAS* (exons 2 and 3), *BRAF* (exon 15), *EGFR* (exon12), *MAP2K1* (exons 2 and 3), *MAP2K2* (exon 2). Primer sequences are listed in **Supplementary Table S3**.

Droplet Digital PCR (ddPCR)

Genomic DNA from CRC cells was amplified using ddPCR™ Supermix for Probes (Bio-Rad) using BRAF V600E assay (PrimePCR™ ddPCR™ Mutation Assay, Bio-Rad). ddPCR was then performed according to manufacturer's protocol and the results reported as percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild type) DNA alleles, as previously described (23).

Viral Infection

The lentivirus production, cell infection, and transduction procedures were performed as previously described (24). WiDr cells were transduced with a lenti-control vector or a lentiviral vector carrying a mutated hBRAF V600E cDNA (a gift of Maria S. Soengas, CNIO, Madrid, Spain) or EGFR WT cDNA (a gift from Dr. C. Sun and Prof R. Bernards, NKI, Amsterdam). VACO432 cells were transduced with a lentiviral vector carrying EGFR G465R mutant cDNA (25).

Clinical samples

A chemorefractory metastatic CRC patient was enrolled in the CMEK162X2110 clinical trial (Trial registration ID: NCT01543698) at Niguarda Cancer Center, Milan, Italy. The patient was treated with the BRAFi encorafenib (LGX818) in combination with the MEKi binimetinib (MEK162) from September 2013 to March 2014, obtaining a partial response in January 2014, followed by radiological progression in March 2014. Blood samples from this patient were obtained at baseline (September 2013) and at progression (March 2014) through a separate liquid biopsy research protocol approved by the Ethics Committee at Ospedale Niguarda, Milan, Italy. The study was conducted according to the provisions of the Declaration of Helsinki, and the patient signed and provided informed consent before sample collection.

Next Generation Sequencing (NGS) analysis

Germline DNA was obtained from PBMC (Promega, ReliaPrep Tissue Kit), while cell free circulating DNA of tumor origin (ctDNA) was extracted from 2 ml plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's

instructions. Libraries were prepared with Nextera Rapid Capture Custom Enrichment Kit (Illumina Inc., San Diego, CA, USA), according to the manufacturer's protocol, as previously described (23). The custom-panel included the coding region of 226 genes, as previously detailed (23). Further details are provided in Supplementary Materials and Methods.

Bioinformatics analysis

NGS bioinformatics analysis was performed as previously described (23). Mutational analyses were the result of comparison between pre- and post-treatment samples. Details are provided in Supplementary Materials and Methods.

Results

Generation of models of acquired resistance to combinatorial therapies targeting EGFR-BRAF-MEK-PI3K

We selected three BRAF V600E mutant CRC cell lines, HROC87, WiDr and VACO432, that are resistant to single-agent BRAFi or MEKi, but sensitive to combined BRAFi/MEKi or their combinations with cetuximab (**Supplementary Fig. S1**). To gain a comprehensive understanding of potential therapeutic resistance mechanisms in *BRAF* mutant CRC, cell lines were cultured until resistant derivatives emerged in the presence of seven different drug combinations currently being explored in clinical trials. The drugs included the BRAFi dabrafenib, encorafenib, and vemurafenib; the MEKi selumetinib and trametinib; the EGFR-targeted antibody cetuximab; and the selective PI3K-α inhibitor (PI3Ki) alpelisib (**Fig. 1A**). A total of eleven resistant cell line models were generated. Two independent resistant cell

populations were obtained by growing VACO432 cells with vemurafenib and cetuximab (V+C) and these were therefore indicated as resistant A (R.A) and resistant B (R.B). Resistance to drug treatment was confirmed by cell viability assay comparing parental and resistant cell derivatives. All resistant cell models were clearly refractory at all drug concentrations tested (**Fig. 1B**).

Cells with acquired resistance to BRAF inhibitor combinations display biochemical reactivation of MAPK signaling

Prior studies indicate that tumors with acquired resistance to BRAF or EGFR targeted agents in monotherapy maintain sustained levels of MEK/ERK or (occasionally) AKT phosphorylation even in the presence of drug (19,26-29). We tested whether the same biochemical rewiring could occur in cells made resistant to combinations of therapies targeting EGFR-BRAF-MEK-PI3K. Amounts of total MEK, ERK, or AKT proteins were not substantially different between parental cells and their resistant counterparts. However, variation of their phosphorylation levels (pMEK, pERK, or pAKT) was evident after drug treatment. Some, but not all, resistant models displayed increased phosphorylation of AKT at Ser473 upon drug treatment. However, every resistant model showed sustained levels of ERK phosphorylation despite drug treatment, in stark contrast to parental cells in which robust inhibition of ERK phosphorylation was observed with all treatments (**Fig. 2**).

Overall, these analyses indicate that combinatorial EGFRi/BRAFi/MEKi/PI3Ki treatments abrogate ERK phosphorylation in parental sensitive cells, but that their resistant counterparts can sustain MAPK signaling in the presence of these therapeutic combinations (**Fig. 2**).

Acquired molecular alterations in *BRAF* mutant CRC cell lines confer resistance to BRAF inhibitor combinations

In order to identify likely candidate drug resistance mechanisms leading to biochemical reactivation of MAPK signaling, we focused our analysis on components of the MAPK pathway by performing copy-number analyses of *HER2*, *EGFR*, *MET*, *KRAS* and *BRAF* and Sanger sequencing of the most pertinent exons of *EGFR, KRAS, NRAS*, *BRAF*, *MAP2K2* and *MAP2K1*.

Quantitative PCR on genomic DNA extracted from resistant cells showed no changes in *HER2* or *MET* gene copy number while *EGFR*, *KRAS, or BRAF* gene copy number increased in three WiDr derivatives resistant to V+S, D+C or S+C, respectively (**Fig. 3A**). All gene amplifications were only found in the resistant cell populations and were confirmed by fluorescence in situ hybridization (FISH) analyses (**Fig. 3B**). Sanger sequencing of hotspot regions of *EGFR* (exon 12), *KRAS* (exons 2, 3, and 4), *NRAS* (exons 2 and 3), *BRAF* (exon 15), *MAP2K1* (exons 2 and 3) and *MAP2K2* (exon 2) revealed acquired gene mutations in eight cell lines, as summarized in **Table 1**. All resistant cell populations retained the original *BRAF* V600E mutation. All other mutations found in resistant cells were not detected in their parental counterparts by conventional Sanger sequencing.

Alterations in *KRAS* were the most common resistance mechanisms observed. Acquired *KRAS* mutations affecting exons 2 and 4 (G12D, G13D and A146T/V) were found in five different cell line models resistant to doublet BRAFi+EGFRi or triplet E+C+A. In one case, multiple *KRAS* mutations were concomitantly present in the

resistant cell population, suggesting polyclonality. Prior functional studies in cell models have already demonstrated a causative role of exon 2 *KRAS* mutations in driving resistance to BRAFi+EGFRi (30). Our data suggest that exon 4 *KRAS* mutations can also promote resistance. Additionally, *KRAS* amplification was identified in WiDr resistant to BRAFi+EGFRi (D+C). *KRAS* amplification was found in the post-treatment biopsy of a CRC patient with acquired resistance to the combination of encorafenib and cetuximab (30). These findings suggest that the cell models generated in this work have the potential to recapitulate clinically-relevant resistance mechanisms.

Increased *BRAF* gene copy number was seen in WiDr resistant to MEKi+EGFRi (S+C). Selective amplification of mutant BRAF V600E allele was previously identified in a *BRAF* mutant CRC patient with acquired resistance to BRAFi+EGFRi (30), in CRC cell lines with secondary resistance to the MEKi selumetinib (31,32), as well as in melanoma patients upon progression on the BRAFi vemurafenib (33), but not yet implicated in refractoriness to combined MEKi+EGFRi. To assess whether *BRAF* gene amplification had occurred in an allele selective manner, we performed digital PCR analyses. WiDr parental cells carried 1 mutant and 3 wild-type alleles of BRAF, while their S+C resistant derivatives displayed a 9:1 mutant/wildtype ratio (**Supplementary Fig. S2A**). Western blot with a diagnostic antibody specific for the V600E variant showed that the mutant protein was selectively overexpressed (**Supplementary Fig. S2B**). Finally, we validated that ectopic overexpression of mutant BRAF in WiDr parental cells can confer resistance to combined MEKi+EGFRi (**Supplementary Fig. S2C and S2D**).

Two different *MAP2K1* mutations leading to the V211D and L115P amino acid changes were identified in HROC87 and VACO432 resistant to MEKi+EGFRi (S+C) and BRAFi+MEKi (D+T), respectively. These mutations have previously been reported to confer resistance to MEK allosteric inhibitors in melanoma and CRC by preventing drug binding (27,34), so they were not subjected to further functional validation.

Interestingly, amplification of *EGFR* was found in WiDr resistant to BRAFi+MEKi (V+S). Although EGFR signaling has been implicated in intrinsic resistance to BRAFi monotherapy in *BRAF* mutant CRC (8,9), *EGFR* gene amplification has not previously been established as a potential resistance mechanism in *BRAF* mutant CRC. This result is consistent with previous observations that induction of EGFR protein expression can drive resistance to BRAFi or MEKi in melanoma (35). Ectopic overexpression of EGFR in WiDr parental cells was able to confer resistance to combined BRAFi+MEKi or BRAFi+EGFRi (**Fig. 4A and 4B**). Importantly, however, the triple combination of BRAFi+EGFRi+MEKi was able to restore sensitivity in resistant cells carrying *EGFR* amplification (**Fig. 4C**).

A single point mutation affecting the ectodomain of EGFR (G465R) was found in VACO432 V+C (R.B). Although this variant has previously been shown to disrupt receptor-antibody interaction, leading to cetuximab or panitumumab resistance in RAS/BRAF wild-type CRCs (25), mutations affecting the EGFR ectodomain have not been reported previously as potential resistance mechanisms in the context of *BRAF* mutant tumors. To investigate the role of this mutation, we induced ectopic expression of *EGFR* G465R in VACO432 parental cells. Analysis of transduced cells

indicated that the *EGFR* G465R mutation is able to sustain ERK phosphorylation and cell proliferation in the presence of combined V+C treatment (**Fig. 4D and 4E**). Cross-resistance to the combination of vemurafenib and panitumumab was seen. However, consistent with its known role in disrupting anti-EGFR antibody binding, the ability of the *EGFR* G465R mutation to promote resistance was specific to BRAFi+EGFRi combinations with anti-EGFR antibodies only, and kinase inhibition of EGFR by gefitinib was able to restore sensitivity in combination with BRAFi (**Fig. 4F**).

Clinical acquired resistance to combined therapy with BRAF and MEK inhibitors

Identification of clinical acquired resistance mechanisms to targeted therapy combinations was performed by genotyping of liquid biopsy samples. Plasma samples taken before treatment and after disease progression were collected from a patient with *BRAF* V600E mutant colorectal cancer who had achieved a partial response on a RAF/MEK inhibitor combination (Trial registration ID: NCT01543698). Circulating tumor DNA (ctDNA) was extracted and subjected to molecular profiling by NGS analysis of a custom panel of 226 cancer related genes (23). The analysis revealed that the percentage of reads carrying *TP53* p.R282W mutated allele were consistent between the baseline and the progression plasma (**Fig. 5**), indicating similar ctDNA content in both samples. By contrast, the proportion of *BRAF* V600E mutant reads at resistance was twice as much as those in the baseline, suggesting selective amplification of the *BRAF* mutant allele. NGS analysis revealed concomitantly the emergence of a *KRAS* G12C allele, which was undetectable in the pretreatment sample. These results indicate that the mechanisms of resistance to

target inhibitors identified in cell lines could faithfully recapitulate those found in clinical samples.

Overall, we observed that a diverse array of molecular mechanisms can drive acquired resistance to clinically-relevant therapeutic combinations targeting the EGFR-BRAF-MEK-PI3K pathways in *BRAF* mutant CRC. However, we also found that each of these heterogeneous resistance mechanisms converges on a common signaling output to promote resistance-reactivation of MAPK signaling — suggesting that it may be possible to devise a universal targeted combination strategy capable of overcoming multiple resistance mechanisms.

Vertical combined suppression of the MAPK pathway has residual activity on drug resistant cells

Based on our observations that all resistant cell models show persistent MAPK signaling activation (**Fig. 2**), we postulated that they could retain sensitivity to suppression of the pathway downstream. In this regard, previous data indicate that some melanomas with acquired resistance to BRAFi monotherapy can benefit from additional treatment based on combined BRAFi and MEKi blockade (36). Additionally, vertical triple blockade of EGFR+BRAF+MEK displayed the highest ability to suppress ERK phosphorylation in *BRAF* V600E CRC cells (37) and this combination has been shown to induce response rates of up to 40% in *BRAF* mutant CRC patients (11). Similarly, previously published reports have documented promising preclinical activity of ERK inhibition in BRAFi or MEKi resistant melanoma models (27,38,39) as well as in MEKi+BRAFi or BRAFi+EGFRi resistant *BRAF* mutant CRC cells (30). However, it has not yet been established whether ERK

inhibitors might exhibit improved ability to overcome resistance when given as monotherapy, or in combination with BRAFi and/or EGFRi. Accordingly, we hypothesized that acquired resistance to BRAFi combinations could be overcome by more profound MAPK pathway suppression achieved by triplet combinations or by the incorporation of ERK inhibitor-based combinations. In order to test these hypotheses, the effect on viability was systematically tested across all resistant cell line models for all drug combinations used to generate resistant derivatives, as well as combinations incorporating the ERK inhibitor (ERKi) SCH772984 and the vertical cetuximab+dabrafenib+trametinib (BRAFi+MEKi+EGFRi) triplet combination (**Fig. 6**).

As expected, parental cell lines were highly sensitive to all drug treatments (**Fig. 6**). In general, resistant cell lines derived from one BRAFi+MEKi combination (D+T or V+S) showed cross-resistance to the other BRAFi+MEKi combination; and cell lines resistant to cetuximab in combination with encorafenib, dabrafenib or vemurafenib were cross-resistant to other BRAFi+EGFRi combinations, irrespective of the specific drug used in the selection protocol. This suggests that resistance mechanisms emerging under the selective pressure of these specific drug combinations are capable of conferring resistance to that class of inhibitors, and are unlikely to be related to any unique properties of the specific drugs used.

Interestingly, the addition of PI3Ki to BRAFi+EGFRi treatment did not robustly affect viability in any of the resistant cells relative to BRAFi+EGFRi alone. This finding is consistent with initial results of a clinical trial comparing encorafenib and cetuximab to encorafenib, cetuximab, and alpelisib, which have not demonstrated a clear benefit in response rate or progression-free survival with the addition of the PI3K

inhibitor alpelisib (12,40). In marked contrast, the triple combination of BRAFi+EGFRi+MEKi showed improved efficacy in many models relative to either BRAFi+EGFRi, BRAFi+MEKi, or MEKi+EGFRi alone. Finally, the addition of BRAFi and/or EGFRi to ERKi appeared to improve efficacy in some resistant models relative to ERKi alone, suggesting that ERKi may best be administered as part of therapeutic combinations in future clinical trials for *BRAF* mutant CRC. Indeed, analysis of resistant cell lines indicated that ERK inhibition could induce cytotoxicity, which was further enhanced when combined with BRAFi and/or EGFRi (**Supplementary Figures 3A and 3B**).

Discussion

Over the past few years, BRAF inhibitors have demonstrated striking clinical efficacy in patients with *BRAF* mutant melanoma. However, BRAF inhibitors are not equally effective in other *BRAF* mutant cancer histologies (4). Preclinical studies defining EGFR and MAPK pathway reactivation as key drivers of BRAF inhibitor resistance in *BRAF* mutant CRC have provided the rationale for testing double or triple combinations of therapies targeting EGFR/BRAF/MEK/PI3K in this disease (11-13,15,16).

Unfortunately, while these approaches have led to improvements in response rate in *BRAF* mutant CRC patients, preliminary clinical observations have indicated that, following an initial response, acquired resistance in *BRAF* mutant CRC patients typically emerges after a few months of treatment (11-13,15). The mechanisms underlying acquired resistance in *BRAF* mutant CRC cells remain poorly

characterized. In this study, we undertook a comprehensive effort to develop models of secondary resistance to a spectrum of seven clinically-relevant combinatorial therapies in order to more robustly define the landscape of molecular mechanisms leading to acquired resistance in *BRAF* mutant CRC. Our results indicate that the mechanisms leading to acquired resistance to these combinations can be genetically heterogeneous, but appear to converge on the reactivation of the MAPK signaling pathway at the biochemical level, suggesting that it might be possible to develop universal combination strategies capable of overcoming multiple resistance mechanisms. We acknowledge that no *in vivo* models were generated or assessed in this study, thus limiting our observations to cancer cell autonomous drug resistance mechanisms. However, analyses of plasma samples at baseline and at acquired resistance to BRAF combinatorial therapy, in a *BRAF* mutant CRC patient, revealed genetic alterations consistent with those identified in cell models, thus underscoring the clinical relevance of the broad panel of resistant lines generated in this work. Since microenvironment and non-genomic mechanisms of drug resistance may also occur, future studies based on the analysis of BRAF mutant murine models and patient samples will be needed to expand our knowledge on this aspect.

In our resistant cell line panel, we identified several novel mechanisms of acquired resistance not previously reported in *BRAF* mutant CRC. In particular, we identified an *EGFR* G465R ectodomain mutation in a cell line with acquired resistance to the combination of a BRAFi and anti-EGFR antibody. While this mutation has been established as a mechanism of acquired resistance to anti-EGFR antibody monotherapy in RAS/BRAF wild-type CRC due to disruption of antibody binding (25), this class of mutations has not previously been implicated in *BRAF* mutant CRC. Our

observation warrants assessing for *EGFR* ectodomain mutations in *BRAF* mutant CRC patients upon acquired resistance to BRAFi and anti-EGFR antibody combinations. Importantly, we found that a resistant model harboring this mutation retained sensitivity to BRAFi and an EGFR kinase inhibitor, as well as to downstream inhibitor combinations, such as BRAFi+MEKi. We also identified *EGFR* amplification as a novel potential mechanism of acquired resistance in *BRAF* mutant CRC. Interestingly, unlike the *EGFR* ectodomain mutation, *EGFR* amplification conferred cross-resistance to BRAFi+EGFRi, BRAFi+MEKi, and MEKi+EGFRi combinations, likely as a consequence of increased EGFR signalling flux, and retained sensitivity only to the triple combinations of BRAFi+EGFRi+MEKi and ERKi+BRAFi+EGFRi. The finding that EGFR signaling leads to MAPK feedback reactivation and resistance during BRAFi monotherapy, but also can contribute to acquired resistance to MAPK combinatorial inhibition, highlights the central role of EGFR in the biology of *BRAF* mutant CRC.

Molecular analyses of our resistance cell line panel also identified the presence of several resistance mechanisms previously identified in the setting of acquired resistance in *BRAF* mutant CRC—including *KRAS* mutation or amplification, *BRAF* V600E amplification, and *MAP2K1* mutation (30), thereby underscoring the likely importance of these specific mechanisms within the spectrum of acquired resistance in *BRAF* mutant CRC and supporting the likelihood that these specific alterations may be frequently observed in patients. This is also supported by the identification of two different genetic alterations identified at resistance to BRAF/MEK inhibition in plasma sample of a *BRAF* mutant CRC patient, i.e., the emergence of a *KRAS* mutation and a likely amplification of mutant *BRAF* V600E. In our resistant cell

models, *KRAS* alterations were the most common resistance mechanism. The high prevalence of *KRAS* mutations in CRC and its role in resistance to anti-EGFR therapies underpin a central role for KRAS in this disease. Analysis by standard sensitivity sequencing has typically identified *KRAS* and *BRAF* mutations in a mutually exclusive fashion in CRC (41-43). In order to explain these observations, it has been suggested that concomitant oncogenic activation of KRAS and BRAF would be counter-selected during tumorigenesis, as it would result in activation of cell-cycle inhibitory proteins of the Ink4/Arf locus, leading to oncogenic stress and senescence (44). Nevertheless, the use of more sensitive techniques, such as droplet digital PCR, have recently revealed that low-allele frequency *KRAS* mutations could coexist with *BRAF* V600E in CRC samples (7). These rare subclones may be present but might possess an unfavorable fitness compared to clones with only mutant *BRAF*. However, the selective pressure of BRAF-directed therapy may improve the proliferation rate of the double mutant clones while reducing the viability of cells bearing only mutant *BRAF*, thus driving outgrowth of resistant *BRAF*/*KRAS* double mutant clones. Indeed, a recent study analyzing tumor biopsies from *BRAF* mutant CRC patients obtained prior to BRAF-directed therapy revealed that more than 50% bear low frequency *KRAS* mutations (7). This finding might be explained by the 'Big Bang' model (45), whereby tumors grow predominantly as a single expansion producing numerous intermixed subclones, where the timing of an alteration rather than clonal selection for that alteration is the primary determinant of its pervasiveness. Similarly, it is possible that some of the other common acquired resistance mechanisms we have observed in *BRAF* mutant CRC, such as *BRAF* V600E amplification and *MAP2K1* mutation, may also pre-exist in rare tumor subclones. Indeed, we previously found that rare tumor cells with *BRAF*

amplification could be identified in pre-treatment tumor biopsies from *BRAF* mutant CRC patients (32). Altogether, these observations suggest that *KRAS* as well as other resistance alterations could develop at an early stage of *BRAF* mutant colorectal tumorigenesis, thus laying the seeds for the eventual emergence of acquired resistance. In a resistant cell model and in our patient, BRAF combinatorial therapies have resulted in the appearance of at least two concomitant resistance mechanisms. Indeed, the lower percentage of *KRAS* mutant allele in comparison with to the *TP53* founder mutation suggested that this variant may have been present in only a fraction of tumor cells distinct from the *BRAF* V600E amplified subset. These data are consistent with previous reports in melanomas resistant to BRAFi, either as monotherapy or in combination with MEKi, in which multiple resistance mechanisms have been described to co-occur in individual patients (46,47).

The observation that all resistance mechanisms identified in our cell panel converge to reactivate MAPK signaling has important clinical implications. Since it may not be practical to design specific therapeutic strategies against each of the individual acquired resistance mechanisms observed in *BRAF* mutant CRC, there would be clear clinical advantages to developing a more “universal” therapeutic strategy targeting a common signalling output that would be capable of overcoming a spectrum of potential resistance mechanisms. By systematically comparing multiple drug combinations designed to achieve more optimal MAPK pathway suppression across the molecular landscape of acquired resistance mechanisms in *BRAF* mutant CRC, we were able to identify the most promising therapeutic candidates to overcome resistance. Although a few resistant cell lines showed only modest

sensitivity to these combinations, suggesting the possibility that these models might harbor additional MAPK-independent resistance mechanisms, overall we observed that the combination of BRAFi+EGFRi+MEKi or ERKi in combination with BRAFi and/or EGFRi displayed superior activity across the vast majority of resistant models. Therefore, these combinations may represent the most promising strategies for evaluation in clinical trials for patients with *BRAF* mutant CRC. Notably, the triple combination of BRAFi+EGFRi+MEKi is currently being evaluated in clinical trials, and preliminary results suggest improved response rate and progression-free survival compared to the individual doublet combinations (48), which is consistent with our findings, and suggests that improved activity against the common resistance mechanisms in *BRAF* mutant CRC may account in part for the improved clinical efficacy observed.

Consistent with our findings, previously published reports have documented promising preclinical activity of ERK inhibition in BRAFi or MEKi resistant cell line models (27,38,39) and in MEKi+BRAFi and BRAFi+EGFRi resistant *BRAF* mutant CRC cells (30), supporting ERKi as key potential components of future clinical trial strategies for this disease. While it is likely that secondary mutations in ERK1/2 may limit the long-term efficacy of ERKi (49), it remains an important and unanswered question as to whether it is best to administer ERKi as monotherapy or whether ERKi might be more effective as part of drug combinations in *BRAF* mutant CRC. Indeed, it is possible that ERK inhibition alone might trigger survival-promoting feedback loops through alternative pathways that might be optimally suppressed with therapeutic combinations. In order to help guide future clinical trial strategies, our study begins to address this critical question, and suggests that ERKi appear to be

more effective against the spectrum of acquired resistance mutations in *BRAF* mutant CRC when administered in combination with BRAFi and/or EGFRi inhibitors. In fact, the triplet combination of ERKi+BRAFi+EGFRi appeared to be the most effective combination strategy overall across our panel of resistant cell line models. Thus, our study suggests that initial clinical trials of ERKi in *BRAF* mutant CRC patients should prioritize therapeutic combinations with BRAFi and EGFR inhibitors.

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Table 1. Molecular alterations acquired upon resistance to targeted therapy combinations in *BRAF* mutant CRC cell lines.

Drugs	**Cell line**	EGFR	KRAS	NRAS	MAP2K1	MAP2K2	BRAF gene CNV
D + T	VACO432	WT	WT	WT	L115P	WT	none
V + S	WiDr	EGFR ampl.	WT	WT	WT	WT	none
E + C	HROC87	WT	G13D	WT	WT	WT	none
	VACO432	WT	A146T	WT	WT	WT	none
D + C	VACO432	WT	A146T	WT	WT	WT	none
	WiDr	WT	KRAS ampl.	WT	WT	WT	none
V + C	VACO432 R.A	WT	G12D	WT	WT	WT	none
	VACO432 R.B	G465R	WT	WT	WT	WT	none
E + C + A	VACO432	WT	A146V A146T	WT	WT	WT	none
S + C	HROC87	WT	WT	WT	V211D	WT	none
	WiDr	WT	WT	WT	WT	WT	600E ampl.

Legend to Figures

Figure 1. Generation of *BRAF* mutant CRC cells resistant to EGFR targeted agent and BRAF/MEK or PI3K inhibitors (A) Schematic representation of RAS/RAF/MEK and PI3K/AKT pathways. The orange boxes show the drugs used to generate resistant cell lines. List of the drug combinations used for generating resistant cell lines is shown on the right; all of these have been or are being evaluated in clinical trials. Drugs are abbreviated as follows: A=Alpelisib (PI3K inhibitor, PI3Ki); C= Cetuximab (EGFRi); D= Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); T= Trametinib (MEKi); V= Vemurafenib (BRAFi). **(B)** Parental and resistant cells were treated for 72 hours with the indicated molar drug concentrations. Cetuximab and alpelisib were given at a constant concentration of 5 μg/ml and 100 nM, respectively. In the vemurafenib and selumetinib combination, selumetinib was used at a constant concentration of 300 nM.

Figure 2. Resistant cells maintain ERK1/2 phosphorylation after treatment. WiDr, VACO432 and HROC87 parental and resistant cells were treated with different drug combinations as indicated: cetuximab (C, 5 μg/ml); dabrafenib (D, 300 nM); encorafenib (E, 400 nM); alpelisib (A, 1 μM); vemurafenib (V, 2 μM); selumetinib (S, 1 μM) and trametinib (T, 30 nM). Drug treatment was given for 5 hours prior to protein extraction.

Figure 3. *EGFR*, *KRAS* and *BRAF* gene amplification confer acquired resistance to BRAF combination therapies. (A) Quantitative PCR for copy number evaluation of resistant cell lines in respect to their parental counterparts.

WiDr V+S, D+C and S+C resistant lines displayed gene amplification of *EGFR*, *KRAS* and *BRAF*, respectively. **(B)** FISH analysis on chromosome metaphase spreads confirmed gene amplification. Cell nuclei were colored by DAPI, FISH probes EGFR, KRAS, BRAF were labeled with texas red (red signal) and chromosome 7 (Chr7) and 12 (Chr12) with FITC (green signal). *EGFR* gene amplification was found extrachromosomally as double minutes, while a focal intrachromosomal amplification of *KRAS* and *BRAF* loci could be identified.

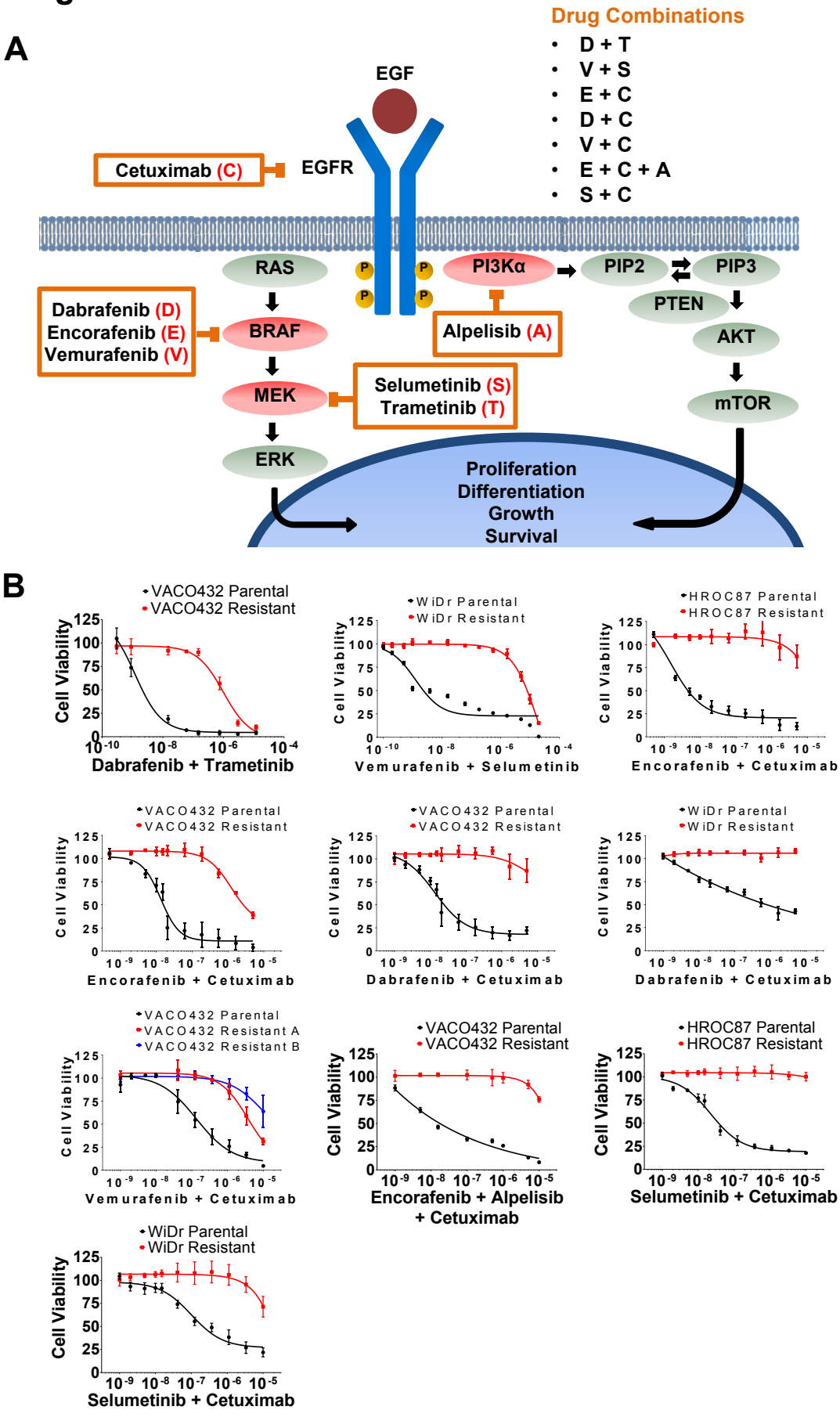
Figure 4. *EGFR* amplification or ectodomain mutations play a causative role in acquired resistance to BRAF combination therapies. (A) Biochemical analyses of WiDr parental and V+S resistant cell lines, and of WiDr cells transduced with either GFP cDNA or EGFR WT cDNA. Cells were treated with vemurafenib and selumetinib before protein extraction. Actin was used as a loading control. **(B)** Effect of vemurafenib (at the indicated molar concentrations) in combination with selumetinib (0.5 μM) on the viability of WiDr cells transduced with EGFR WT cDNA. **(C)** Effect on cell viability of the addition of cetuximab to V+S treatment in WiDr resistant cells carrying *EGFR* amplification. Cells were treated with vemurafenib (1 μM), selumetinib (0.5 μM) or cetuximab alone or in their combinations. **(D)** EGFR and ERK expression and phosphorylation in VACO432 parental and resistant B cells, and in cells transduced with either GFP cDNA or EGFR G465R cDNA variants. VACO432 cells were treated with vemurafenib and cetuximab for 5 hours before protein extraction. Vinculin was used as a loading control. **(E)** Effect of vemurafenib (at the indicated molar concentrations) in combination with cetuximab (5 μg/ml) on the viability of VACO432 cells transduced with EGFR G465R cDNA. **(F)** VACO432 with acquired *EGFR* G465R mutation upon treatment with vemurafenib and cetuximab

retain sensitivity to vemurafenib and gefitinib treatment. All survival data were assessed by ATP content measurement after 72 hours of treatment. Data are expressed as average ± s.d. of two independent experiments.

Figure 5. Next generation sequencing of ctDNA of a *BRAF* mutant CRC patient at resistance to combined BRAF/MEK inhibition revealed an increase of *BRAF* V600E number of reads and the emergence of a *KRAS* G12C mutation. Data labels indicate number (#) of mutant reads over the total number of reads covering that position, detected by next generation sequencing in circulating tumor DNA (ctDNA) at baseline and resistance. PD, progressive disease.

Figure 6. Acquired resistance to target therapy combinations can be overcome by vertical MAPK pathway suppression. The viability of parental and resistant cell lines treated with different drug combinations targeting EGFR, BRAF, MEK, ERK and PI3K was determined by ATP assay after 72 hours incubation. Relative survival was normalized to the untreated controls. Relative cell viability is depicted as indicated in the bottom color bar. Drugs were used at the concentrations listed in Supplementary Table S2. Results represent mean of at least two independent experiments, each performed in triplicate.

Figure 1


A
Drug Combinations
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• V + S
• E + C
• D + C
• V + C
• E + C + A
• S + C
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EGFR
Cetuximab (C)
RAS
BRAF
MEK
ERK
PI3Kα
PIP2
PIP3
PTEN
AKT
mTOR
Dabrafenib (D)
Encorafenib (E)
Vemurafenib (V)
Selumetinib (S)
Trametinib (T)
Alpelisib (A)
Proliferation
Differentiation
Growth
Survival
B
VACO432 Parental
VACO432 Resistant
Cell Viability
Dabrafenib + Trametinib
WiDr Parental
WiDr Resistant
Vemurafenib + Selumetinib
HROC87 Parental
HROC87 Resistant
Encorafenib + Cetuximab
Encorafenib + Cetuximab
Dabrafenib + Cetuximab
Dabrafenib + Cetuximab
VACO432 Resistant A
VACO432 Resistant B
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Encorafenib + Alpelisib + Cetuximab
Selumetinib + Cetuximab
Selumetinib + Cetuximab

Figure 2

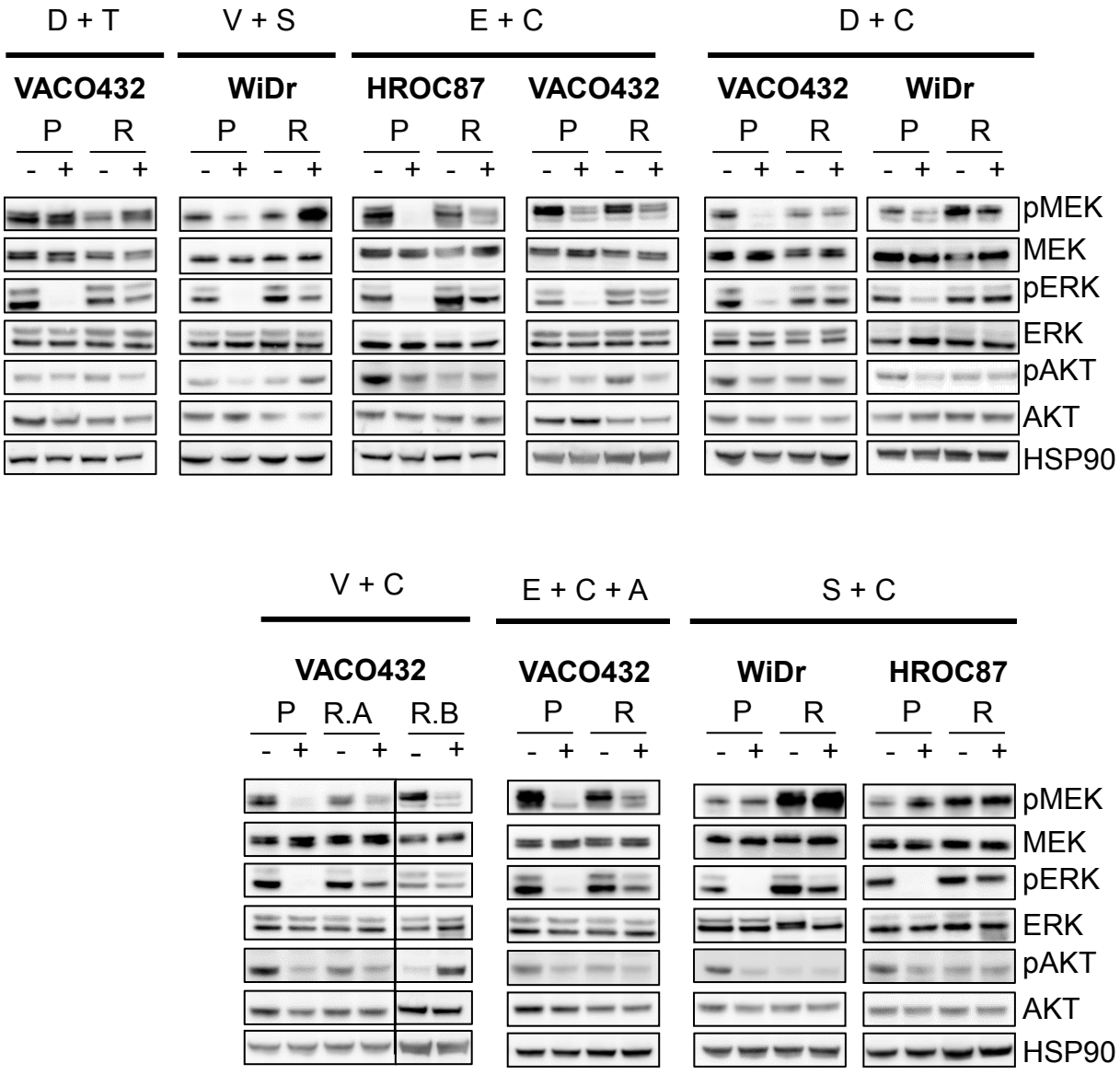
D + T
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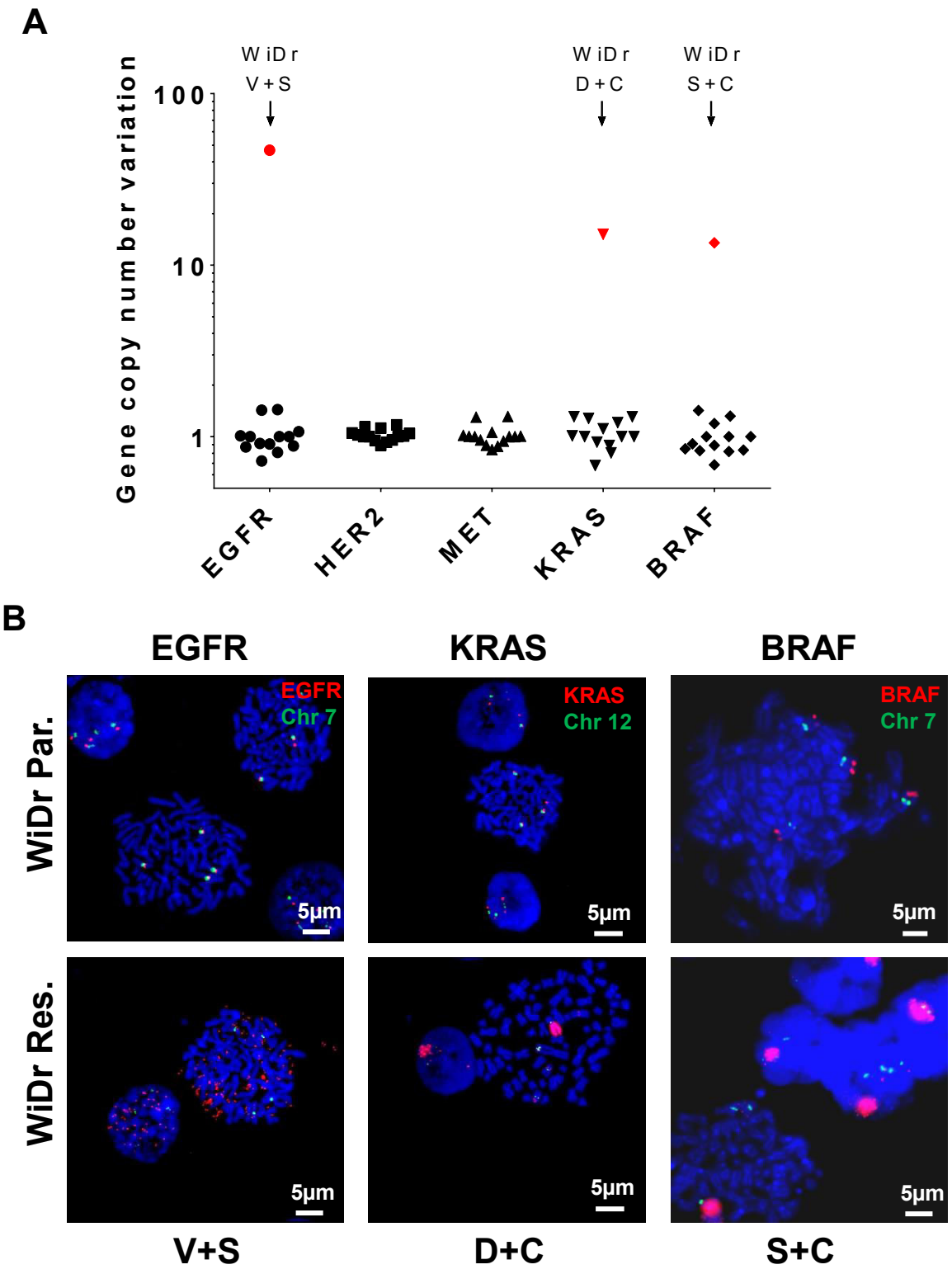
Figure 3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Gene copy number variation
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HER2
MET
KRAS
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B
EGFR
KRAS
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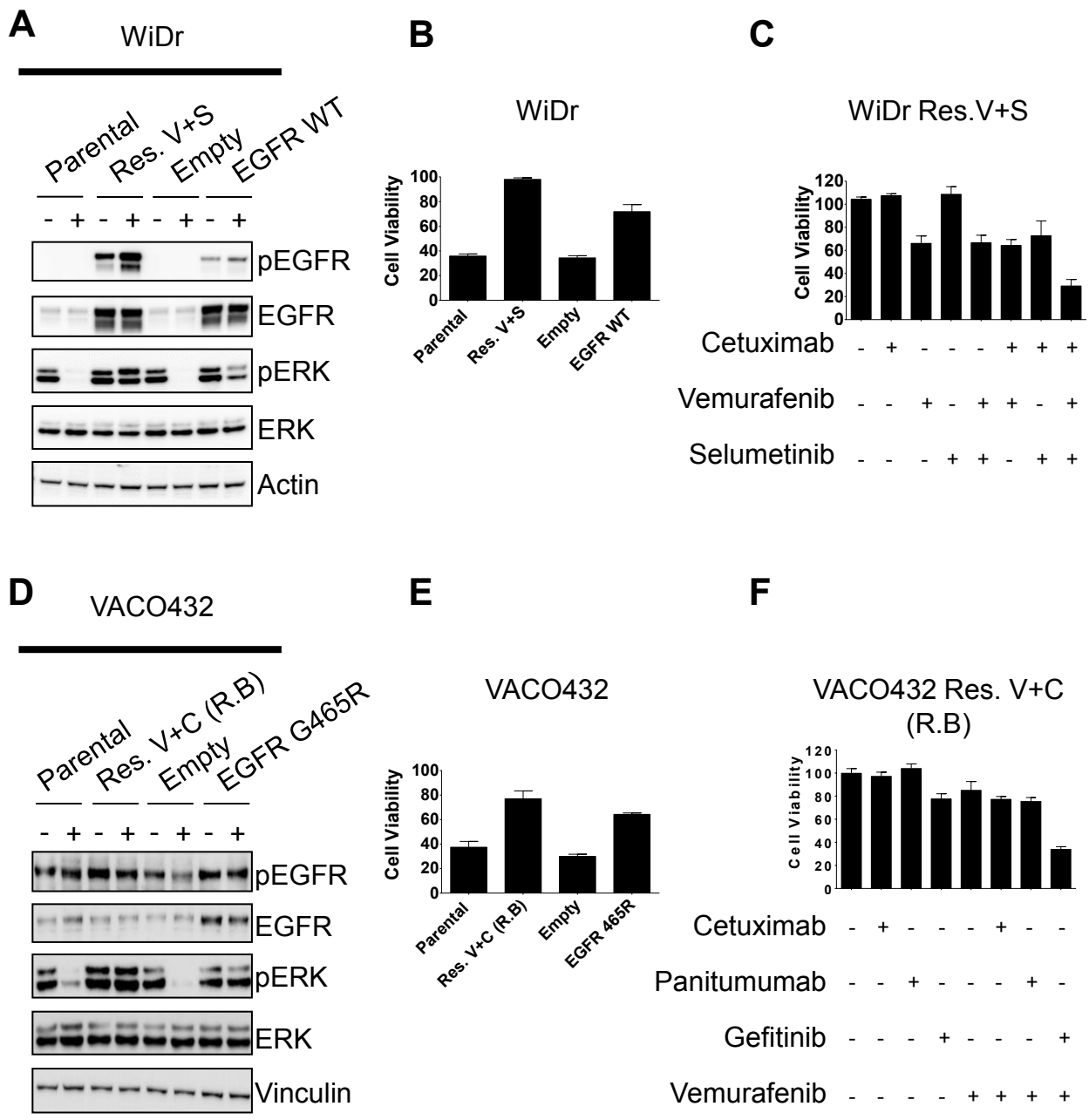
S+C

Figure 4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Cell Viability
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0
Parental
Res. V+C (R.B)
Empty
EGFR 465R
F
VACO432 Res. V+C (R.B)
Cell Viability
120
100
80
60
40
20
0
Cetuximab - + - - - + - -
Panitumumab - - + - - - + -
Gefitinib - - - + - - - +
Vemurafenib - - - - + + + +

Figure 5

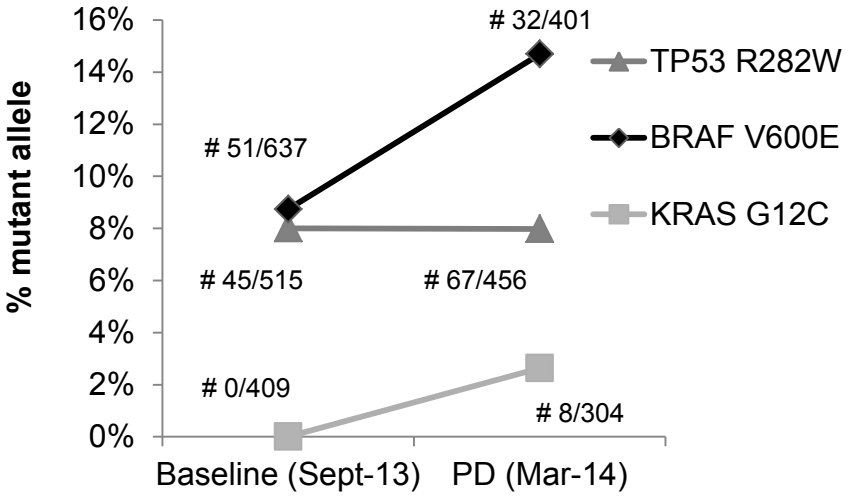
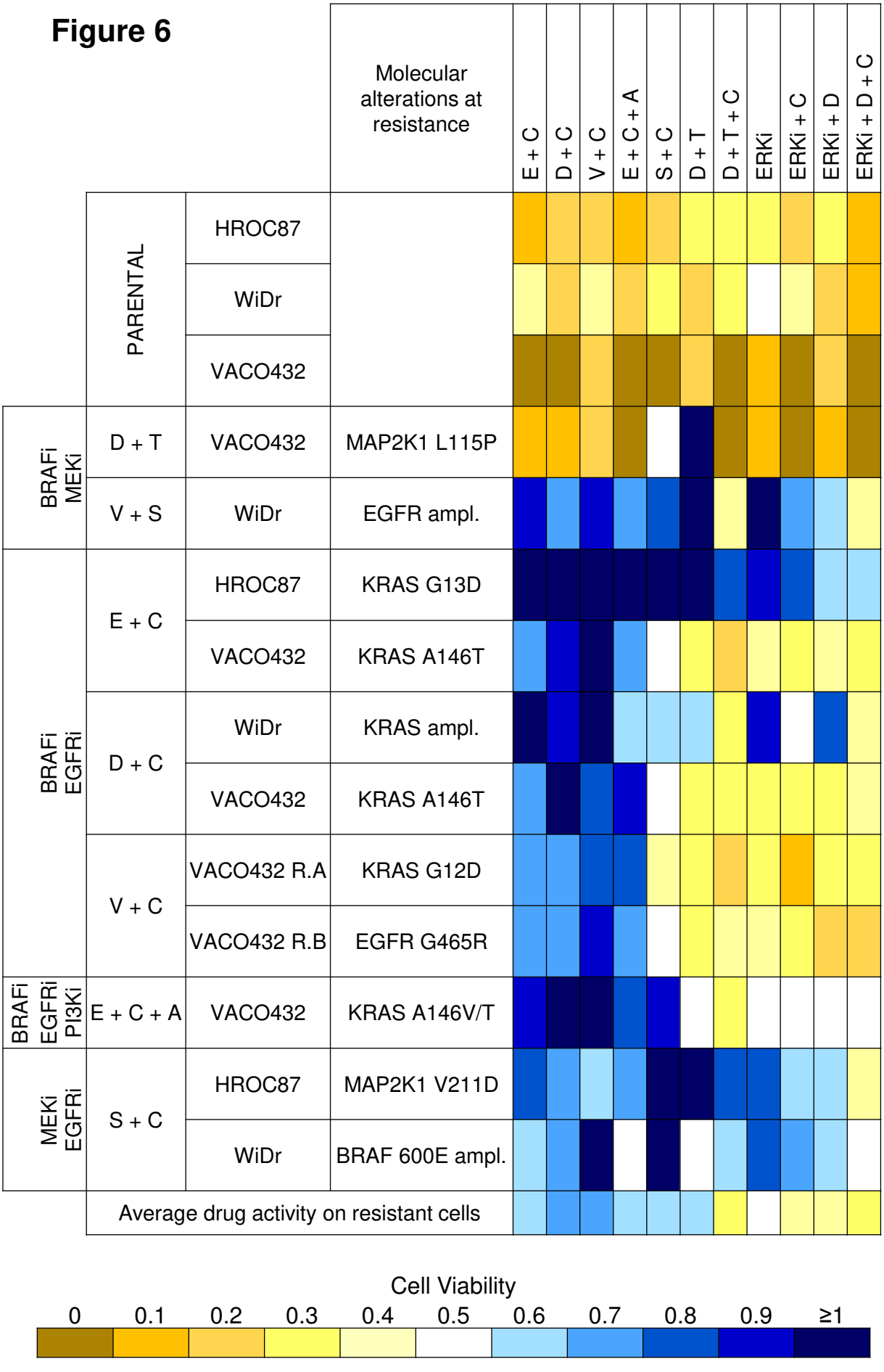

% mutant allele
16%
14%
12%
10%
8%
6%
4%
2%
0%
32/401
51/637
45/515
67/456
0/409
8/304
TP53 R282W
BRAF V600E
KRAS G12C
Baseline (Sept-13)
PD (Mar-14)

Figure 6


Molecular alterations at resistance
E + C
D + C
V + C
E + C + A
S + C
D + T
D + T + C
ERKi
ERKi + C
ERKi + D
ERKi + D + C
PARENTAL
HROC87
WiDr
VACO432
BRAFi MEKi
D + T
VACO432
MAP2K1 L115P
V + S
WiDr
EGFR ampl.
BRAFi EGFRi
E + C
HROC87
KRAS G13D
VACO432
KRAS A146T
D + C
WiDr
KRAS ampl.
VACO432
KRAS A146T
V + C
VACO432 R.A
KRAS G12D
VACO432 R.B
EGFR G465R
BRAFi EGFRi PI3Ki
E + C + A
VACO432
KRAS A146V/T
MEKi EGFRi
S + C
HROC87
MAP2K1 V211D
WiDr
BRAF 600E ampl.
Average drug activity on resistant cells
Cell Viability
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
≥1

Molecular landscape of acquired resistance to targeted therapy combinations in *BRAF* mutant colorectal cancer

Supplementary Materials and Methods

Cell lines

WiDr, HROC87 and VACO432 cells were cultured at 37°C and 5% CO_2 in RPMI 1640, DMEM/F-12 and McCoy's (Invitrogen), respectively, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Cell lines were tested and resulted negative for Mycoplasma contamination with the Venor GeM Classic Kit (Minerva Biolabs).

Drugs and generation of drug resistant cells

Vemurafenib, encorafenib, selumetinib and trametinib were purchased from Sequoia Chemicals; alpelisib and dabrafenib mesylate were from ChemieTek; gefitinib and SCH772984 were from Selleck Chemicals. The EGFR targeted monoclonal antibodies cetuximab and panitumumab were obtained from the Pharmacy at Ospedale Niguarda, Milan. Cetuximab was administered at a constant concentration of 5μg/ml, while vemurafenib, encorafenib, dabrafenib, selumetinib, and trametinib have been initially given at a concentration of 2 μM, 500 nM, 90 nM, 2 μM, 30 nM, respectively. The concentrations of chemical inhibitors were increased by discrete

intervals until reaching a final concentration at which the cells showed resistance (**Table S1**).

Drug proliferation assays

Cell proliferation experiments were carried out in 96-well plates in triplicate. Cells were plated (3,000 cells/well for VACO432 and WiDr, 4,000 cells/well for HROC87) in 100 µl complete growth medium. At 24 hours post-seeding, 100 µl of serum-free medium with or without cetuximab (5 µg/ml) was manually added to the cells. All other drugs were added directly on the plate by TECAN D300e digital dispenser (HP). After 72 hours' treatment cell viability was assessed by ATP content using CellTiter-Glo Luminescent Assay (Promega). Viability was normalized as a percentage of control untreated cells. Data from growth-inhibition assays were plotted using the nonlinear regression curve fit modelling from GraphPad Prism-5 (GraphPad Software).

Western Blot analysis: drug treatments and antibodies

Before biochemical analysis, cells were grown in their respective media devoid of drugs for four days and then treated for the times indicated in figure legends with cetuximab 5 µg/ml, alpelisib 1 µM, dabrafenib 300 nM, encorafenib 400 nM, vemurafenib 2 µM, selumetinib 1 µM, trametinib 30 nM, unless otherwise stated. Total cellular proteins was extracted by lysing cells in boiling Laemmli buffer (1% SDS, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl) or in cold extraction buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1% Triton-X-100, 10% glycerol, 5 mM EDTA, 2 mM EGTA; all reagents were from Sigma-Aldrich) 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). The following primary antibodies were used (all from Cell Signaling Technology, except where otherwise indicated): anti-EGFR (clone13G8, Enzo Life Sciences; 1:100); anti-phospho EGFR (Tyr 1068; 1:1,000); anti-BRAF (Santa Cruz Biotechnology; 1:1,000); anti-pBRAF (Ser445; 1:1000); anti-BRAF V600E (Ventana 1:500); anti-phospho-MEK1/2 (Ser217/221; 1:1,000); anti-MEK1/2 (1:1,000); anti-phospho p44/42 ERK (Thr202/Tyr204; 1:1,000); anti-p44/42 ERK (1:1,000); anti-phospho AKT (Ser473; 1:1,000); anti-AKT (1:1,000); HSP90 (Santa Cruz Biotechnology; 1:500); Actin (Santa Cruz Biotechnology; 1:1000); anti-vinculin (Millipore; 1:5,000).

Fluorescence *In Situ* Hybridization

FISH analyses on metaphase chromosomes and interphase nuclei were provided by the Division of Pathology, Ospedale Niguarda. Samples were fixed in a mixture of methanol and acetic acid and dehydrated at room temperature prior to probe hybridization. Probes and target DNA were co-denatured for 5 min at 75 °C and then hybridized overnight at 37 °C. Slides were washed with washing solution I (0.4x SSC, 0.3% NP-40) for 5 min at 73 °C, for 1 min with washing solution II (2x SSC/0.1% NP-40) at room temperature (Abnova) and finally counterstained with 4′,6-diamidino-2-phenylindole (DAPI). FISH signals were evaluated with a Zeiss Axioscope Imager.Z1 (Zeiss) equipped with single and triple band pass filters.

Cytotoxicity and cell cycle analysis assays

For cell cycle flow cytometric analysis, VACO432 resistant cells were allowed to grow for 24 hours followed by 72 hours of treatment with SCH772984 alone or in

combination with dabrafenib and/or cetuximab. Drugs were used at the concentrations listed in **Supplementary Table S2.** Cells were fixed and stained with propidium iodide (DNA Con3 – CONSUL TS, Orbassano, Italy) overnight. All samples were acquired with the CyAn ADP (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

For cytotoxicity assays VACO432 resistant cells were seeded at 4,000 cells/well in 96-well black optical-bottom plates (Nunc, Life Technologies). After 24 hours cells were treated with the same drug combinations and concentrations applied for cell cycle analysis. The CellTox Green cytotoxicity assay was performed according to manufacturer instructions and fluorescence was read by TECAN Spark 10M plate reader at 535 nm. As toxicity control, Lysis Solution was added (4 µl per 100 µl of cells) 30 minutes prior to reading. Subsequently, the amount of viable cells for each well was quantified by CellTiter-Glo Luminescent Assay (Promega). Background fluorescence (medium and CellTox Green dye) was subtracted and data were first normalized to the amount of cells and after to untreated control.

Plasma processing and Next Generation Sequencing (NGS) analysis

A minimum of 10 ml of whole blood was drawn in EDTA tubes before commencing treatment in September 2013 (baseline) and at radiological progression in March 2014. Plasma was separated within 2 h through two different centrifugation steps (1,600g followed by 3,000g both centrifugations for 10 minutes at RT). PBMC were also obtained which served as a reference control for germ-line genomic DNA.

Preparation of libraries was performed using up to 150 ng of plasma ctDNA and 100 ng germ-line DNA obtained from PBMC. Germ-line gDNA was fragmented using transposons, adding simultaneously adapter sequences. For ctDNA libraries

preparation was used NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England BioLabs Inc., Ipswich MA), with optimized protocol. Germ-line gDNA from PBMC after the tagmentation step, and ctDNA were used as template for subsequent PCR to introduce unique sample barcodes. Fragments' size distribution of the DNA was assessed using the 2100 Bioanalyzer with a High Sensitivity DNA assay kit (Agilent Technologies, Santa Clara, CA). Equal amount of DNA libraries were pooled and subjected to targeted panel hybridization capture. Libraries were then sequenced using Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA).

Bioinformatics

FastQ files generated by Illumina MiSeq sequencer were mapped to the human reference genome (assembly version hg19) using BWA-mem algorithm (1) SAMtools package (3) was used to sort aligned reads and to remove PCR duplicates. We used a custom script for NGS in order to call somatic variations when supported by at least 1.5% allelic frequency and 5% significance level obtained with a Fisher's Test, as previously described (2,3). Mutations were annotated by a custom pipeline printing out gene information, number of normal or mutated reads, the allelic frequencies and the variation effect (synonymous, nonsynonymous, stop-loss and gain). Each of these entries was associated with the corresponding number of occurrences in the COSMIC database (4).

Supplementary References

1. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 2010;26(5):589-95.
2. Russo M, Misale S, Wei G, Siravegna G, Crisafulli G, Lazzari L, et al. Acquired Resistance to the TRK Inhibitor Entrectinib in Colorectal Cancer. Cancer Discov 2016;6(1):36-44.
3. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med 2015;21(7):827.
4. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res 2015;43(Database issue):D805-11.

Molecular landscape of acquired resistance to targeted therapy combinations in *BRAF* mutant colorectal cancer

Supplemental figure legends

Supplementary Figure 1. Combinations of MEK inhibitor (selumetinib) or of BRAF inhibitor (vemurafenib) with EGFR inhibitor (cetuximab) display synergistic anti-proliferative activity in *BRAF* mutant WiDr, VACO432 and HROC87 colorectal cancer cells. WiDr, VACO432 and HROC87 parental cells were treated with increasing concentration of selumetinib or vemurafenib with or without cetuximab (5 µg/ml). Cell viability was assayed after by an ATP assay. Data points represent the mean ± s.d. of two independent experiments, each performed in triplicate.

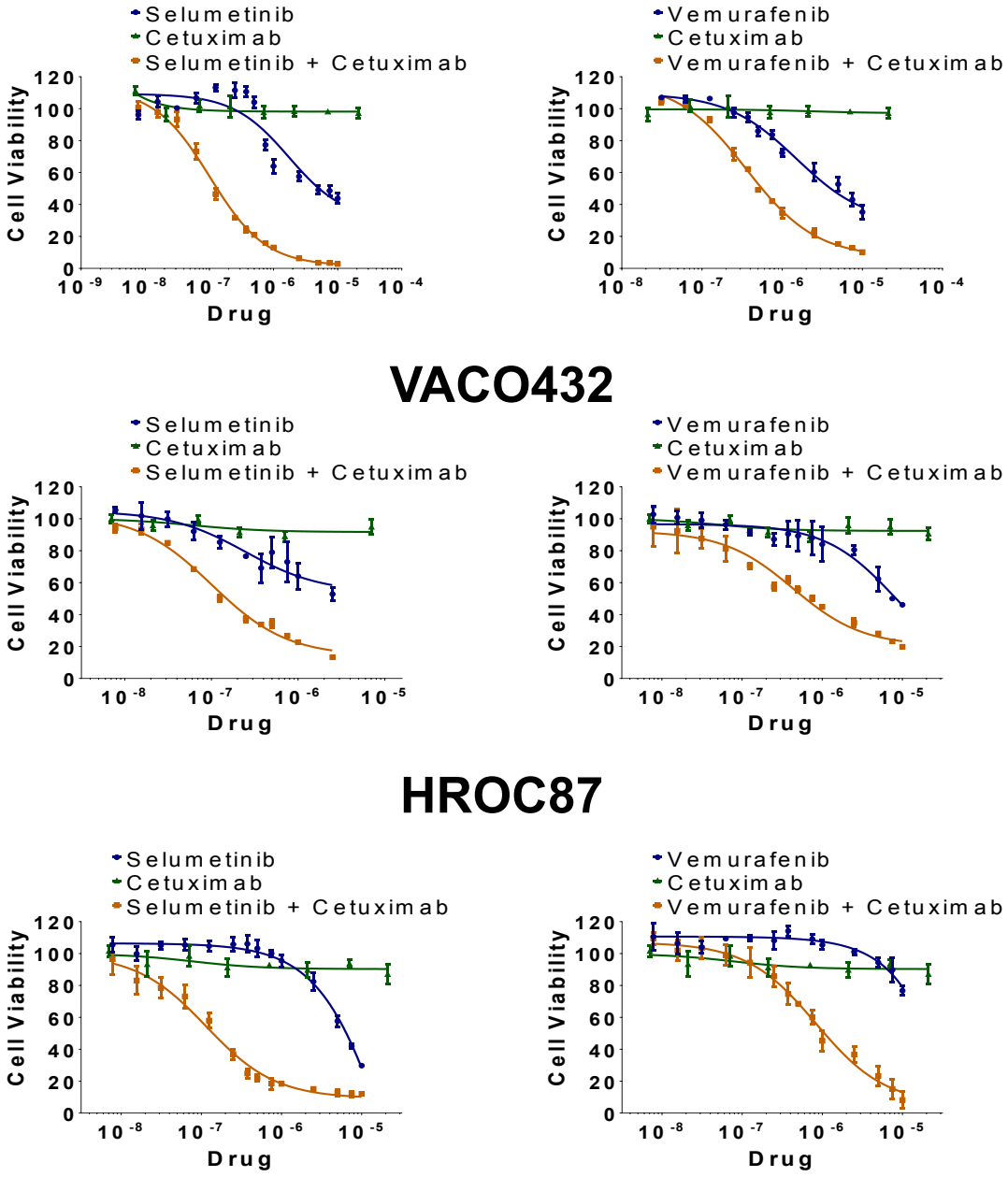
Supplementary Figure 2. *BRAF* V600E amplification acquired in WiDr resistant to selumetinib and cetuximab, confers resistance to combination of MEK and EGFR inhibitors in CRC cells. (a) Digital PCR shows selective amplification of the *BRAF* V600E mutant allele in WiDr cells resistant to combined cetuximab and selumetinib. **(b)** Biochemical analysis on WiDr parental and resistant S+C was performed with the indicated antibodies. **(c)** Western blot analysis of proteins extracted from WiDr cells 5 days after transduction with a lentiviral vector expressing the hBRAF V600E cDNA. **(d)** WiDr-BRAF V600E transduced cells displayed similar viability than the WiDr resistant cells upon treatment with MEK and EGFR inhibitors. Five days after transduction, cells were treated for 72 hours before viability was assessed by measuring ATP content. Data are expressed as average ± s.d. of two independent experiments, each performed in triplicate.

Supplementary Figure 3. ERK inhibition induces cytotoxicity in VACO432 resistant to BRAF combination therapies. VACO432 resistant cell lines were cultured without drugs for four days and then treated for 72 hours with 185 nM ERKi, SCH772984, alone or in double or triple combination with 550 nM dabrafenib and/or 5 μg/ml cetuximab, after which cytotoxicity and cell cycle analysis were assesed. **(A)** CellTox green cytoxicity assay was performed to identify cells with compromised membrane integrity characteristic of cell death. Data are expressed as fold change relative to DMSO treated control cells. Histograms and error bars indicate mean ± s.e.m of three independent experiments. **(B)** VACO432 resistant cell lines were analyzed for cell cycle and sub-G1 group by flow cytometry. Plots of cell number versus propidium iodide fluorescence intensity are shown. The percentage indicate the sub-G1 fraction.

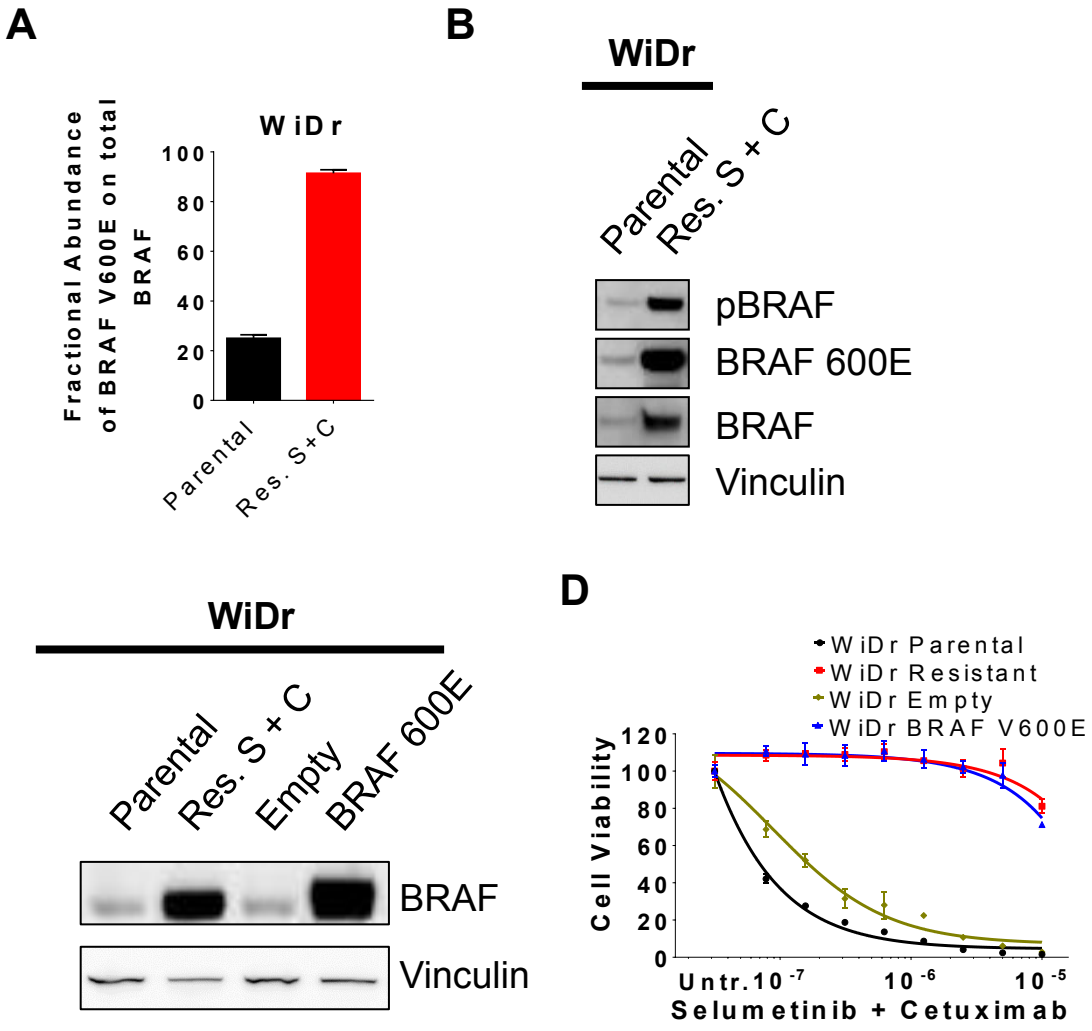
Molecular landscape of acquired resistance to targeted therapy combinations in *BRAF* mutant colorectal cancer

Supplementary Figures and Tables

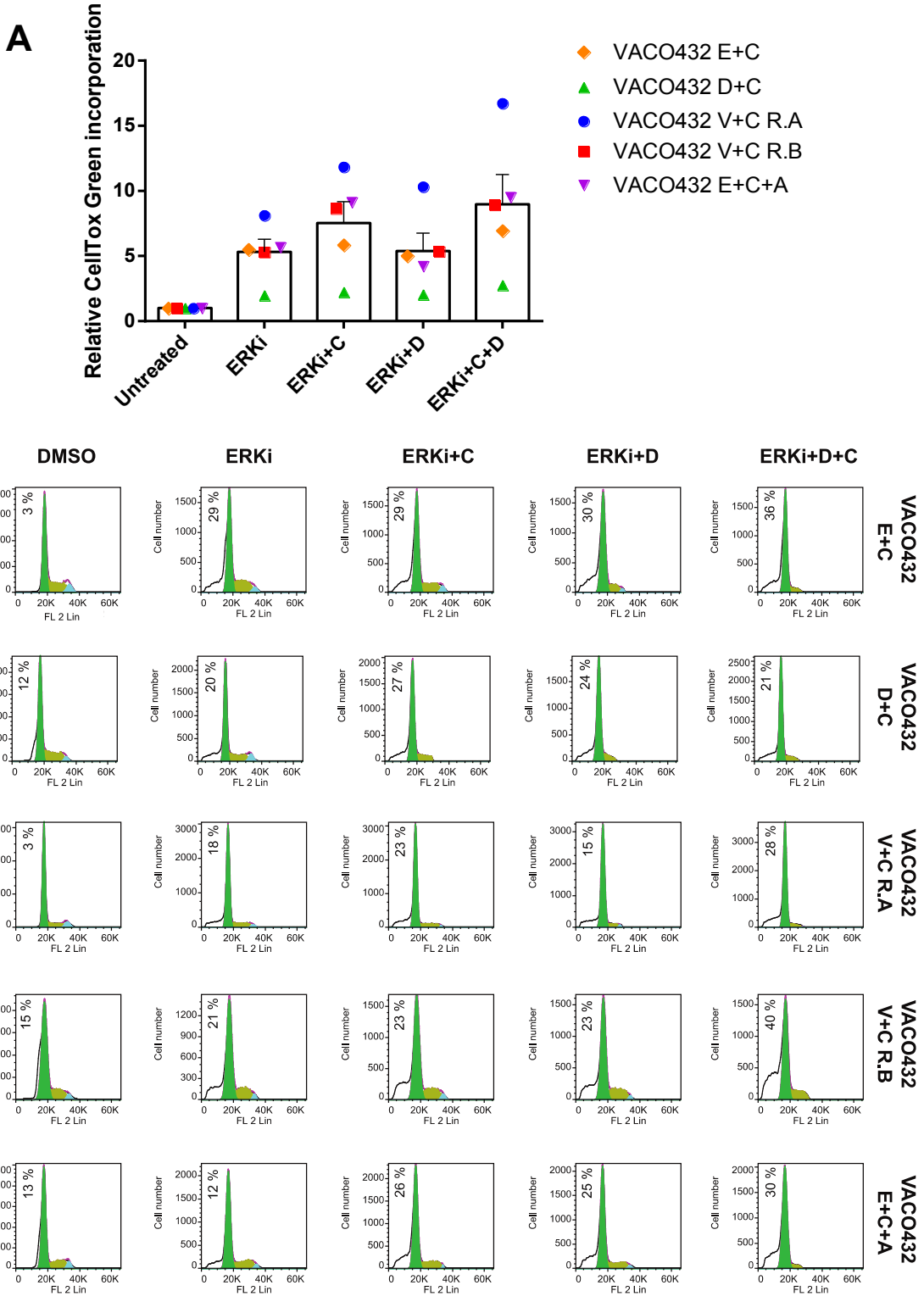
Supplementary Fig. S1



Supplementary Fig. S2



Supplementary Fig. S3



Supplementary Table S1

Supplementary table S1. List of drug concentrations at which cell lines were made resistant.

Drug treatment	Cell Line	Final Drug concentration
S + C	HROC87	S 5 µM + C 5 µg/ml
	WiDr	S 5 µM + C 5 µg/ml
D + C	WiDr	D 5 µM + C 5 µg/ml
	VACO432	D 5 µM + C 5 µg/ml
V + C	VACO432 (R.A)	V 5 µM + C 5 µg/ml
	VACO432 (R.B)	V 3 µM + C 5 µg/ml
E + C	HROC87	E 2 µM + C 5 µg/ml
	VACO432	E 2 µM + C 5 µg/ml
V + S	WiDr	V 3 µM + S 1 µM
D + T	VACO432	D 0.25 µM + T 0.12 µM
E + C + A	VACO432	E 1 µM + C 5 µg/ml + B 0.5 µM

Supplementary Table S2

Supplementary table 2. Drug concentrations applied in the cross-resistance combinatorial screening depicted in Figure 6.

Drugs	Drug concentrations
Encorafenib + Cetuximab	E 550 nM + C 5 µg/ml
Dabrafenib + Cetuximab	D 550 nM + C 5 µg/ml
Vemurafenib + Cetuximab	V 1.1 µM + C 5 µg/ml
Encorafenib + Cetuximab + Alpelisib	E 550 nM + C 5 µg/ml + A 300 nM
Selumetinib + Cetuximab	S 1.1 µM + C 5 µg/ml
Dabrafenib + Trametinib	D 185 nM + T 30 nM
Dabrafenib + Trametinib + Cetuximab	D 185 nM + T 30 nM + C 5 µg/ml
SCH772984 (ERKi)	ERKi 185 nM
SCH772984 (ERKi) + Cetuximab	ERKi 185 nM + C 5 µg/ml
SCH772984 (ERKi) + Dabrafenib	ERKi 185 nM + D 550 nM
SCH772984 (ERKi) + Dabrafenib + Cetuximab	ERKi 185 nM + D 550 nM + C 5 µg/ml

Supplementary Table S3

Supplementary Table S3: List of primers for gene amplification and sequencing

	Primer name	Sequence
Gene Copy Number	HER2 gDNA FW	TATGCAGGGCTGACGTAGTGC
	HER2 gDNA REV	AATGTGTGCCACGAAACTGCT
	KRAS gDNA FW	CTGAGCTCCCCAAATAGCTG
	KRAS gDNA REV	AGGTTAGGGCTAGGCACCAT
	MET gDNA FW	TGTTTTAAGATCTGGGCAGTG
	MET gDNA REV	AATGTCACAACCCACTGAGG
	EGFR gDNA FW	TCCAGGAGGTGGCTGGTTA
	EGFR gDNA REV	CTAAGGCATAGGAATTTTCGTAGTACATATT
	BRAF gDNA FW	GGGAAGTAAAGACAGGGAGGT
	BRAF gDNA REV	AGAGAGGTAGGAAAGGGCAAG
	CHR12 gDNA FW	GGGATCTTATGATGTGTCAGG
	CHR12 gDNA REV	ACTCTTGGTCTCAGTCTGCC
	STS gDNA FW	CCTTCAAGAGAAAGACGACAG
	STS gDNA REV	AGGACTTATAAAAGGCAAGGG
	ULK2 gDNA FW	TTTGTGTGTGTGACGGAGTCT
	ULK2 gDNA REV	AGGCTAAGGCAGGAGAATGAG
Sequencing	BRAF ex 15 FW	TGCTTGCTCTGATAGGAAAATG
	BRAF ex 15 RV	AGCATCTCAGGGCCAAAAAT
	KRAS ex 2 FW	GGTGGAGTATTTGATAGTGTATTAACC
	KRAS ex 2 RV	AGAATGGTCCTGCACCAGTAA
	KRAS ex 3 FW	AAAGGTGCACTGTAATAATCCAGAC
	KRAS ex 3 RV	ATGCATGGCATTAGCAAAGA
	KRAS ex 4 FW	TGGACAGGTTTTGAAAGATATTTG
	KRAS ex 4 RV	ATTAAGAAGCAATGCCCTCTCAAG
	NRAS ex 2 FW	GTACTGTAGATGTGGCTCGC
	NRAS ex 2 RV	AGAGACAGGATCAGGTCAGC
	NRAS ex 3 FW	CTTATTTAACCTTGGCAATAGCA
	NRAS ex 3 RV	GATTCAGAACACAAAGATCATCC
	EGFR ex 12 FW	CCTCAAGGAGATAAGTGATGGAG
	EGFR ex 12 RV	AAAGGACCCATTAGAACCAACTC
	MAP2K1 ex 2 FW	TCCCGCGAAATTAATACGACTTGACTTGTGCTCCCCACTT
	MAP2K1 ex 2 RV	GCTGGAGCTCTGCAGCTAAGGCAAACTCACCTTTCTGGC
	MAP2K1 ex 3 FW	TCCCGCGAAATTAATACGACGTGCCAATGCCTGCCTTAGT
	MAP2K1 ex 3 RV	GCTGGAGCTCTGCAGCTACCACCCAACTCTTAAGGCCA
	MAP2K1 ex 6 FW	TCCCGCGAAATTAATACGACGCCTTGGTGTACAGTGTTTGC
	MAP2K1 ex 6 RV	GCTGGAGCTCTGCAGCTAACATGTAGGACCTTGTGCCC
	MAP2K2 ex 2 FW	TCCCGCGAAATTAATACGACAGGTAGCTAACCCCTACCCT
	MAP2K2 ex 2 RV	GCTGGAGCTCTGCAGCTAAATCAGAATGCAGAGACCCG