UNIVERSITY OF TURIN

Phd School in life and Health Sciences
Molecular Medicine

Acquired resistance to targeted therapies in BRAF mutant colorectal cancer

Daniele Oddo

UNIVERSITY OF TURIN

Phd School in life and Health Sciences
Molecular Medicine

XXIX Cycle
Academic Years: 2014-2017

Acquired resistance to targeted therapies in BRAF mutant colorectal cancer

Tutor: Prof. Federica Di Nicolantonio

Daniele Oddo

Coordinator: Prof. Francesco Novelli

Acknowledgements

I would like to express my extreme gratitude to Professor Federica Di Nicolantonio not only for giving me the opportunity to undertake this project but also for her continuing motivation, unwavering support and constant guidance throughout these last years. It has been a great privilege to work with her and I am very grateful for the unique opportunity she gave me.

My Ph.D. thesis was completed with the support of many people, and I am very grateful for the help I have received. I am heartily thankful to Dr. Ludovic Barault for his encouragement, guidance and support along the way. I would like to offer my sincerest thanks to Professor Alberto Bardelli and to the whole molecular oncology laboratory staff for their support and advice. I am also very grateful to Dr. Monica Lamba, Dr. Carlotta Cancelliere, Dr Mariangela Russo, Dr Sabrina Arena and Dr. Giulia Siravegna for their help from the beginning of my Ph.D. experience to the end.

I am also very grateful to Dr. Alice Bartolini, Dr. Michela Buscarino, Benedetta Mussolin and Monica Montone for Next generation sequencing, digital PCR and Sanger sequencing analyses. A special thanks to all the staff of the bio informatics group (BIG) for their analyses and support.

I had the opportunity to work at the Candiolo Cancer Institute, surrounded by fabulous colleagues. Thank you all for the great laughs and scientific discussions.

I would like to offer my sincerest thanks to our collaborators Dr. Filippo Pietrantonio, Professor Salvatore Siena, and Professor Ryan Corcoran for these very nice collaborations.

Finally, I would like to give a posthumous homage to the two colorectal cancer patients who agreed to be involved in this research and without whom this thesis would not have been possible. At the same time I wish to thank their families and relatives for approving their decisions.

Index

1. Abstract

Combinatorial therapies targeting the MAPK pathway and EGFR have shown promising efficacy in clinical trials in *BRAF* mutant colorectal cancer (CRC) patients. However, responses are limited in duration. In this thesis a broad panel of cell lines resistant to seven different clinically-relevant drug combinations was generated in a comprehensive effort to define the molecular landscape of acquired resistance mechanisms in *BRAF* mutant CRC and guide development of therapeutic strategies to overcome resistance. Genotyping of resistant cells identified gene amplification of *EGFR*, *KRAS*, *MET* and mutant *BRAF*, as well as acquired mutations in *KRAS*, *EGFR* and *MAP2K1*. In order to establish the clinical relevance of the molecular mechanisms of drug resistance identified *in vitro*, deep-sequencing was carried out on tumor samples from two *BRAF* mutant metastatic CRC (mCRC) patients who acquired resistance to MAPK inhibitor treatment. In one case emergence of a *KRAS* G12C mutation and increase of mutant *BRAF* V600E allele were detected at relapse from combined treatment with BRAF and MEK inhibitors. In the other patient an increase in *MET* gene copy number was identified at resistance after combined BRAF and EGFR blockade.

The second part of this thesis investigated strategies to overcome acquired resistance. In some cases we identified molecular mechanisms that are targetable such as amplification or mutation of *EGFR* and *MET* gene amplification. The latter also emerged in a tumor biopsy from a patient with clinical acquired resistance to dual EGFR and BRAF blockade. Based on pharmacological data on *BRAF* mutant resistant cells with acquired *MET* amplification, the patient received the BRAF inhibitor vemurafenib combined with the dual ALK-MET inhibitor crizotinib

achieving four months of clinical response. However, despite the initial benefit, disease progressed. Solid and liquid biopsies identified a further increase in *MET* gene hyper-amplification as the most likely mechanism of acquired resistance to the second combination of target therapies. Additionally we investigated strategies to tackle *BRAF* mutant tumors after they had progressed on target therapies independently on the specific molecular mechanism emerged at resistance. Such strategies included ERK inhibitor-based combinations, proteasome degradation impairment by carfilzomib, target therapy drug holidays followed by rechallenging. Pharmacological testing of ERK inhibitor-based combinations or carfilzomib impaired the viability of almost all resistant cells. Preliminary data indicated that discontinuing drug treatment for a month was sufficient to restore drug sensitivity in two out of four resistant cell models. These results suggest possibly strategies to overcome resistance with promising clinical translation.

2. Introduction

2.1. Colorectal cancer – an overview

Colorectal cancer (CRC) is an enormous public health burden. In 2013, 771.000 people died as a result of CRC globally, making the disease the fourth-most common cause of cancer-related death worldwide after lung, liver and stomach cancer [1]. Both genetic and environmental factors play an important part in the etiology of CRC. The majority of CRCs are sporadic and its high incidence in developed countries can be attributed to the increasingly ageing population, unfavorable modern dietary habits and an increase in risk factors, such as smoking, low physical exercise and obesity (reviewed in [2]).

In the colon, the evolution of normal epithelial cells to adenocarcinoma follows a predictable progression of histological and concurrent epigenetic and genetic changes (**Figure 1**). Two pathways have been identified to lead transformation from normal intestinal epithelium to cancer. According to the first, tumors will develop sporadically stepwise from benign adenomatous polyps evolving into an early adenoma and finally into a CRC [3]. These tumors usually arise in the left colon and are characterized by chromosomal instability (CIN) resulting in aneuploidy, with both chromosomal gains and losses [4-7]. CIN are the most frequent and heterogeneous phenotype detected in CRC accounting for about 85% of the total CRCs [8]. At the beginning of the multistep process, aberrant lesion of the intestinal crypt is formed, that is generally due to adenomatous polyposis coli (APC) inactivation. The progression to adenoma and early

carcinoma needs instead first kirsten rat sarcoma (KRAS) activating mutation, then protein 53 (p53) alteration and chromosome 18q loss of heterozygosity (LOH). Other mutations, such as phosphatidylinositol 3-kinase (PI3KCA) activation, can occur at the later stages in a small subset of CRCs. Other features of CIN colorectal tumors are LOH, copy number amplification and chromosomal imbalances in terms of number and size [9]. Genotyping studies have also found that CIN cancers are associated with low or no microsatellite instability, also referred as microsatellite stable (MSS) [10].

The second pathway leading to colorectal malignancies has identified a subset of polyps, called sessile serrated polyps, which arise by molecular and histological events that are distinct from the classic tubular adenomas (**Figure 1**) [6, 7]. Serrated polyps arising in the right colon are commonly not CIN but often exhibit extensive DNA methylation of CpG islands (CIMP). Albeit during cancer formation the methylation levels globally decrease, there is a local hypermethylation of promoters that, in turn, can lead to a downregulation of expression of tumor suppressor genes, such as *APC* and MutL homolog 1 (*MLH1*) [10, 11]. MLH1 is protein involved in the mismatch repair (MMR) system. Impaired MMR during replication gives rise to the accumulation of DNA mutations, which occur, in particular, in short nucleotide repeat of 1-6 bp (called microsatellite), leading to microsatellite instable (MSI) tumors [12]. Additionally, v-RAF murine sarcoma viral oncogene homolog B (BRAF) mutation is considered as a driver in serrated pathway and is overrepresented in sporadic CRC tumors with MSI and CIMP [4, 13, 14].

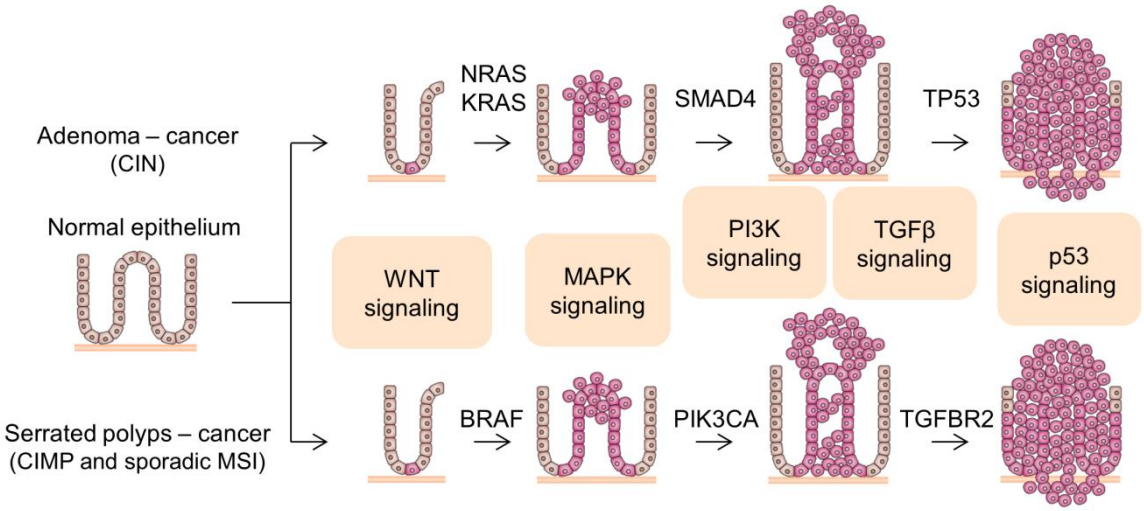

Figure 1. Colorectal cancer progression models. Two different normal colon to colorectal cancer sequences have been identified. Both sequences involve the progression of normal colon epithelial cells to aberrant crypt foci, followed by early and advanced polyps with subsequent progression to early cancer and then advanced cancer. The 'classic' or traditional pathway (top) involves the development of tubular adenomas that can progress to adenocarcinomas. An alternate pathway (bottom) involves serrated polyps and their progression to serrated colorectal cancer. The genes mutated or epigenetically altered are indicated in each sequence; some genes are shared between the two pathways, whereas others are unique (for example, *BRAF* mutations and CpG island methylator phenotype (CIMP) only occur in the serrated pathway). The signaling pathways deregulated during the progression sequence are also shown. Figure adapted from [15].

In 2012, the Cancer Genome Atlas (TCGA) project published the result of full genomic profiling of 276 CRC samples, including exome sequencing, DNA copy number, promoter methylation, mRNA and miRNA expression [4]. This large-scale analysis found that alterations in the WNT–β-catenin, transforming growth factor-β (TGFβ), epidermal growth factor (EGF)–mitogen-activated protein kinase (MAPK) and PI3K signaling pathways are nearly ubiquitous events in CRC [4, 16]. However, tumors with CIN (which are generally non-hyper-mutated) and tumors with MSI (which are typically hyper-mutated) seem to have distinct sequence and pattern of genetic and epigenetic events [17]. A possible example is the activation

of the WNT–β-catenin pathway which is mainly driven by *APC* mutations in MSS samples [4], whereas ring finger protein 43 (*RNF43*) mutations and R-spondin (*RSPO*) family fusions are strongly enriched in MSI CRC [16, 18]. Moreover, genetic alterations of *TP53* and DNA damage response kinase ataxiatelangiectasia (*ATM*) have a mutually exclusive pattern and are predominant in MSS and MSI tumors, respectively [4].

So far, chemotherapy is often the main treatment for advanced CRC and is preceded, in some cases, by surgery at sites of metastasis. Cytotoxic regimens including 5-fluorouracil/leucoverin/oxaliplatin (FOLFOX) and 5-fluorouracil/leucoverin/irinotecan (FOLFIRI) and in selected cases FOLFOXIRI [19] remain the backbone of first-line metastatic CRC therapy [20]. These treatments achieved a median overall survival (OS) of around 20 months when administered together or sequentially [20]. The introduction of biological agents with monoclonal antibodies targeting the vascular endothelial growth factor - VEGF (bevacizumab) and the epidermal growth factor receptor - EGFR (e.g., cetuximab and panitumumab) have improved the survival outcomes, especially when used in combination with cytotoxic chemotherapy [21, 22]. Nonetheless, use of such biologic agents must be preceded by a detailed knowledge of the specific genomic profiling of a given patient. For example, *RAS* mutations, which occur in 45–55% of all patients with metastatic CRC [23], are contraindications to the administration of anti-EGFR therapies, as these activating mutations occur downstream of EGFR and are associated with poor outcomes when used in patients with RAS-mutated metastatic CRC [24, 25].

2.2. The MAPK pathway

The RAS/RAF/MEK/ERK signaling cascade, known as the mitogen-activated protein kinase (MAPK) pathway, regulates cell responses to environmental cues [26] and plays an important role in human cancer [27]. Members of this pathway includes the RAS small guanine-nucleotide binding protein and the protein kinases RAF, MEK (mitogen and extracellular-regulated protein kinase), and ERK. All these family proteins are characterized by multiplicity of their components. In particular there are three *RAS* (*KRAS, HRAS* and *NRAS*), three RAF (*ARAF*, *BRAF*, and *CRAF*), two *MEK* (*MEK1* and *MEK2*), and two *ERK* (*ERK1* and *ERK2*) genes that encode proteins with non-redundant functions. The principal mechanisms involved in the activation of MAPK pathway have been well characterized [28]. Briefly, as depicted in **Figure 2**, the MAPK signaling is initiated when an extracellular ligand binds to a specific receptor tyrosine kinase (RTK) at the plasma membrane (such as EGFR). This binding promotes receptor dimerization and autophosphorylation on intracellular tyrosine residues that in turn act as recognition sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, including the adaptor proteins Shc and Grb2. Son of sevenless (SOS) is then recruited from the cytosol to the plasma membrane through Shc and Grb2 and acts as the major guanine nucleotide exchange factor (GEF) that catalyzes the conversion of inactive RAS-GDP to active RAS-GTP [29]. Activated Ras-GTP can in turn recruit RAF to the inner face of the plasma membrane, where it is activated (for review, [30]). RAF dimerization is a required step in RAS-mediated RAF activation [31]. Like most kinases, the RAF activation is a consequence of a structural conformational change. Under

normal signaling conditions, formation of this active conformation occurs through an allosteric transactivation mechanism that is mediated by RAF dimerization and necessitates RAS binding to promote dimer formation [32]. This allosteric mechanism has been best characterized for RAS-induced BRAF/CRAF heterodimers where BRAF activates CRAF [33, 34]; however, BRAF/BRAF and CRAF/CRAF homodimers have also been observed under physiological conditions, but their activity was lower than that of the heterodimers [35]. Once activated, RAF family members can activate MEK, which in turn activates ERK, which can then be translocated into the nucleus in order to propagate the downstream affect her functions of the MAPK signaling pathway (**Figure 2**) [36]. Activation of ERK triggers an array of negative regulatory events that serve to inhibit the pathway. Indeed, ERK phosphorylates and inhibits receptors [37], the GDP-GTP exchange factor SOS [38], BRAF and CRAF [39]. It also increases the expression of MAPK phosphatases that inhibit the pathway [40]. The duration and intensity of MAPK signaling activity affects how cells respond to extracellular signals [26]. Therefore, the pathway must be carefully controlled to assure appropriate responses to environmental cues. In normal cells, outcomes include survival, proliferation, senescence, and differentiation, but in cancer the constitutive pathway activation favors survival and proliferation [36]. Most cancer-associated lesions that lead to constitutive activation of the MAPK signaling are often mediated by overexpression of receptor tyrosine kinases, activating mutations in receptor tyrosine kinases, sustained autocrine or paracrine production of activating ligands, oncogene mutations (such as *KRAS* and *BRAF*) [41].

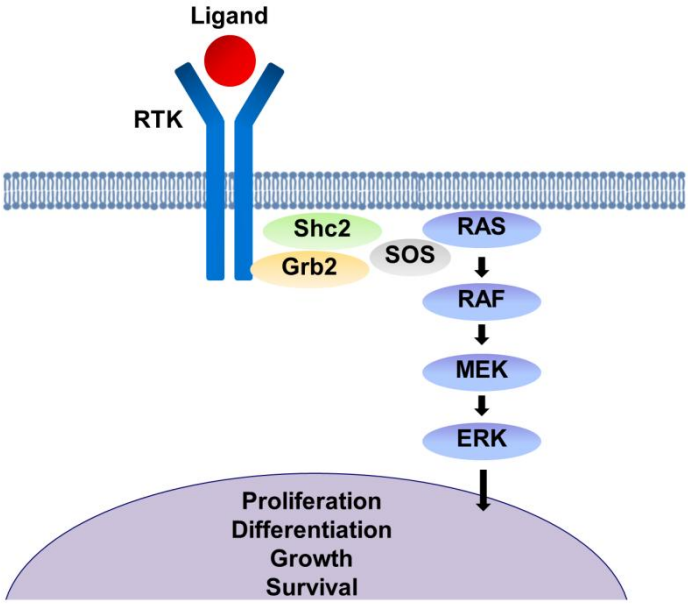

Figure 2. Activation of the MAPK-signaling pathway. Ligand-mediated dimerization of receptor tyrosine kinases (RTKs) triggers the intrinsic tyrosine-kinase activity. This is followed by autophosphorylation of specific tyrosine residues on the intracellular portion of the receptor. These phosphorylated tyrosine residues then bind GRB2. This complex recruits SOS, a cytosolic protein, into proximity to RAS on the plasma membrane. RAS family proteins (HRAS, NRAS and KRAS) cycle between the GDP-bound inactive form and the GTP-bound active form. In an inactivated state, RAS is in the GDP-bound form. The binding of SOS to RAS causes a change in the RAS conformation and leads to the dissociation of GDP and binding of GTP. GTP-bound RAS is the activator of this signaling module. It initiates the signal cascade by phosphorylating RAF, which family include c-RAF1, ARAF and BRAF. RAF, in turn, phosphorylates the MEK (MEK1 and MEK2), which then phosphorylates ERK (ERK1 and ERK2). Activated ERKs then translocate into the nucleus where they phosphorylate specific substrates that are involved in the regulation of various cellular responses. (reviewed in [42]).

2.2.1. RAF protein family

BRAF is a serine-threonine kinase, and together with ARAF and CRAF, forms the RAF protein family in mammals. RAF proteins share three conserved regions: CR1 (which bears a RAS-binding domain (RBD) and a cystein-rich domain (CRD)) and CR2 are both regulatory and present in the N-terminus. Moreover, CR3 is located in the C-terminus and harbor the kinase domain. CR3 contains two regions important for RAF activation: the activation segment and the negatively charged

regulatory region (called N-region) [30]. Though sharing several common structural characteristics, the three RAF isoforms differ considerably in their modes of regulation, tissue distributions and abilities to activate MEK [43]

2.2.2. Regulation of BRAF kinase activity

Trans-phosphorylation and autophosphorylation are important mechanisms by which RAF protein activity are regulated. Activation of BRAF by RAS requires autophosphorylation of threonine 599 and serine 602 [44]. Another serine, S446, which is necessary for the activation of other RAF members, is constitutively phosphorylated in BRAF, while the corresponding serine in ARAF and CRAF is not. Indeed, in BRAF the S446 is located adjacent to two aspartic acids (D448, D449) instead of two tyrosines which are present in ARAF and CRAF [44]. These aspartic acids mimic phosphorylated tyrosines. As a result, BRAF has a stronger basal activity and requires fewer phosphorylation steps to be active that the other RAF proteins.

2.3. *BRAF* mutation in CRC

BRAF is the only RAF protein to be frequently mutated in cancer and its somatic mutations were first reported in 2002 [45]. Among human cancers *BRAF* mutations are found in over 60% melanoma samples [45] and in about 10-15% [46-49]. Approximately 90% of all *BRAF* mutations seen in CRC involve a single amino acid substitution of valine by glutamate at the amino acid level within codon 600 (V600E) [50]. This *BRAF* mutation introduces a negative charge mimicking the phosphorylation events that occur at threonine 599 and serine 602, and

overcoming the requirement for phosphorylation at these sites in activation of BRAF [44]. This amino acid change within the activation segment converts *BRAF* into its active form, functioning as Ras-independent monomers [51], allowing the constitutive activation of the MAPK signaling pathway [44], which in turn promotes tumor cell proliferation and anti-apoptotic activity [52].

BRAF mutation is generally found in a mutually exclusive fashion with *KRAS* and *NRAS* mutations, indicating that a single oncogenic insult to the ERK MAPK pathway is sufficient for promoting tumorigenic activity [45, 46, 53, 54]. A recent exome-wide mutational analysis of 119 *BRAF* mutant CRCs indicated that several other pathways are concomitantly dysregulated in addition to MAPK signaling (**Figure 3**) [49]. Indeed genes involving in the WNT–β-catenin, PI3K, TGFβ and p53 signaling pathways are commonly mutated or epigenetically silenced concomitantly to BRAF. Such a complex landscape might contribute to resistance to BRAF target therapies when given as monotherapy.

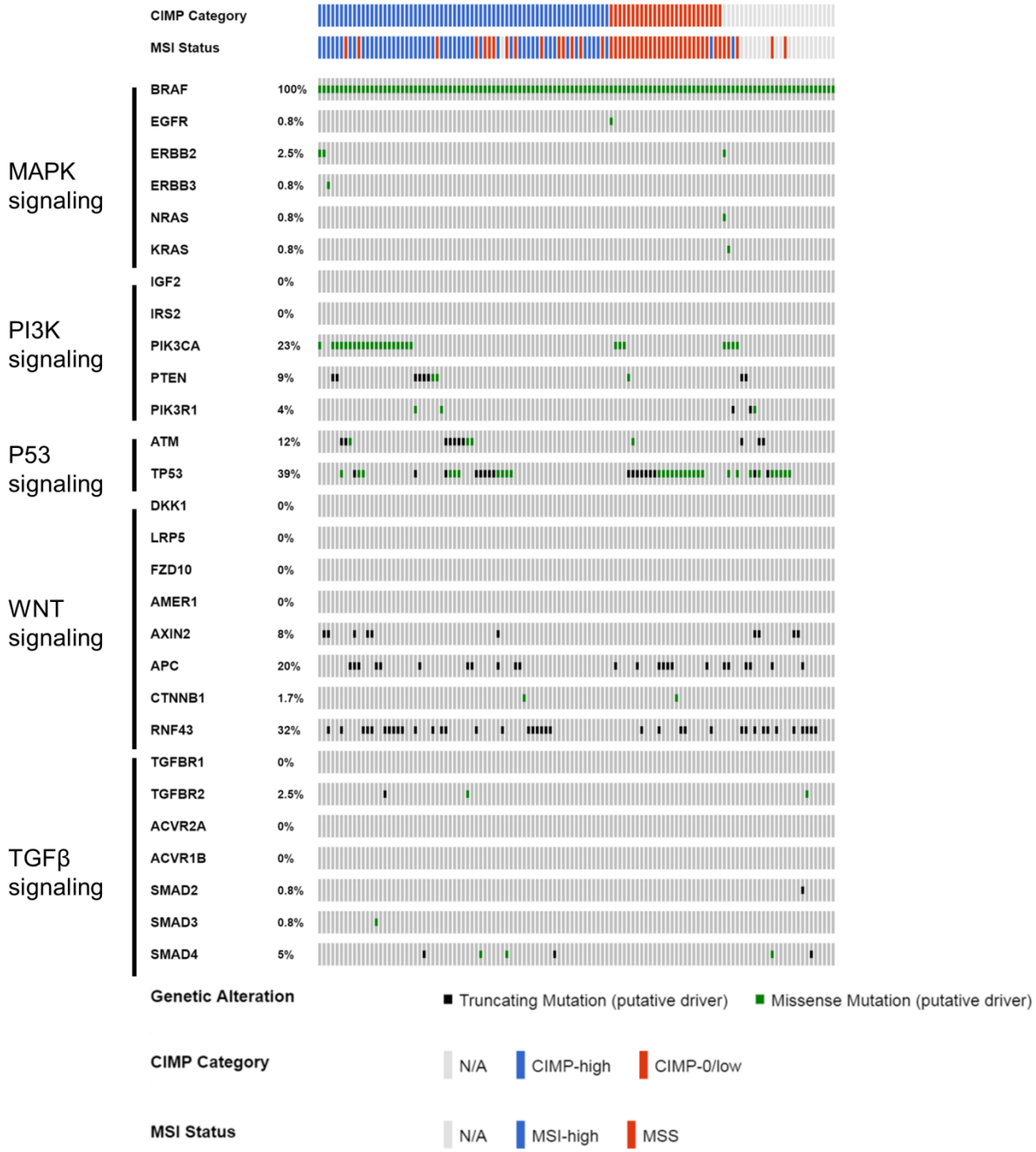

Figure 3. Concomitant genetic alterations in *BRAF* mutant CRC. *BRAF* mutant CRC samples (n=119) were analysed for CIMP, MSI status, and either for truncating and missense mutation (putative driver) on genes involved on MAPK, PI3K, P53, WNT and TGFβ signaling pathways. The most common alterations found in *BRAF* mutant CRC samples affect *PIK3CA*, *TP53* and *RNF43* genes, which in turn dysregulate the PI3K, p53 and WNT pathways. Data were obtained from TCGA [4] and interrogated through CbioPortal (http://www.cbioportal.org) [55, 56].

2.4. Clinicopathologic characteristics of *BRAF* mutant CRC

BRAF mutant colorectal tumors are a specific disease subtype with a unique patient population and associated prognosis. As previously mentioned, *BRAF* mutant cancers are frequently CIMP and are strongly associated with MSI (around 50% of the MSI tumors are BRAF mutated [54]).

Generally, *BRAF* mutant tumors arise prevalently in right-sided proximal colon and are more prevalent in women and in patients of advanced age (typically age >70 years). Additionally these tumors tend to be mucinous histology, serrated and poorly differentiated (reviewed in [50]). Difference among *BRAF* mutant and wild-type CRCs are evident also in the metastatic spread [57]. Indeed, patients with *BRAF* mutations have a metastatic spread that occur more commonly via peritoneal disease (46% *vs.* 24%) or distant lymph node metastasis (53% *vs.* 38%) and less likely to result in lung metastasis (35% *vs.* 49%) when compared to *BRAF* wild-type tumors [57]. This characteristic metastatic spread is highly clinically relevant since these patients are less likely to undergo metastasectomy as their disease is present in sites not favorable to resection. Additionally, *BRAF* mutant CRC patients have a poorer overall survival with a median of 10.4 *vs.* 34.7 months [50]. All these data suggest that *BRAF* mutation may serve as a main driver of right-sided tumor biology, given the strong association between *BRAF* mutations and proximal CRCs and may contribute to the differences in prognosis and metastasis (**Table 1**).

BRAF mutant CRC	
Clinicopathological features	**Molecular features**
Female patients	More prevalent in MSI than in MSS CRC
Age >70 years	CIMP
Right-sided proximal tumors	*MLH-1* methylation
Poorly differentiated	Mutually exclusive to KRAS mutation
Mucinous histology>non-mucinous histology	
More peritoneal and lymph node metastases	
Less lung metastases	

Table 1. Clinicopathologic and molecular features of *BRAF* mutant CRC

2.5. Prognostic role of *BRAF* mutation

Several studies have shown that *BRAF* mutations confer adverse prognosis in CRC. *BRAF* mutations are negative prognostic markers and are associated with short survival after recurrence in stage III disease [58, 59], although this effect is seen mainly in patients with *BRAF* mutant tumors that are MSS [20]. This is consequent of the fact that MSI high tumors are generally associated with a good overall prognosis [54].

Another study that evaluated the prognostic role of *KRAS* and *BRAF* mutations in stage II and III colon cancer patients (N=1,404). They found that patients with *BRAF* mutant tumors had poor overall survival than those with wild-type tumors [48]. The negative prognostic effect was more evident when patients were

stratified by MSS versus MSI because of the relatively good prognosis associated with the latter. Similarly, in another study in which 2,720 patients with stage III disease was enrolled, *BRAF* mutation was associated with a worse overall survival and disease-free survival. Again these differences in survival were particularly clear when microsatellite instability was accounted for [60].

Although some studies [48, 54] suggest that the adverse prognostic association of *BRAF* mutation is limited to MSS tumors, other studies [58, 61, 62] suggest that *BRAF* mutation is also prognostic among MSI cancers. Indeed, in an analysis of 1,253 patients with colon and rectal cancers, in which *BRAF* mutation was present in the 14% of the cases, although patients with MSI tumors had a better prognosis than MSS tumors in general, the MSI/*BRAF* mutant tumors did worse than their MSI/BRAF wild-type counterparts [62].

Further studies have confirmed that *BRAF* mutation is also a negative prognostic marker in the setting of metastatic disease [57, 63]. Importantly, unlike early stage disease, MSI is a negative prognostic factor in metastatic disease. This effect is likely driven by the concomitant presence of *BRAF* V600 mutations, since *BRAF* is mutated in approximately 11% MSI cases in the metastatic setting [57, 64]. In literature only few reports have described metastatic CRC with *BRAF* mutations different from the common V600E (non-V600E BRAF mutations), for which the incidence ranges from 1.6% to 5.1% [65-67]. A recent study in which 9,643 patients with metastatic CRC underwent next generation sequencing (NGS) testing revealed that, the poor prognosis of BRAF is tightly associated with the specific V600E mutation [68]. Indeed the non-V600E BRAF mutations, which occurred in 2.2% of all patients tested, conferred excellent prognosis with

improved overall survival, consistent with an earlier report on codons 594 and 596 [67].

2.6. Predictive role of BRAF mutation

The role of BRAF mutation status as a predictive molecular marker is less clear. The presence of *BRAF* mutations in CRC is thought to play no role in the sensitivity of tumors to standard cytotoxic chemotherapy, such as oxaliplatin and irinotecan [63]. Nevertheless, in the MRC-FOCUS trial, which compared first-line treatments with 5-fluorouracil, 5-fluorouracil/irinotecan and 5-fluorouracil/oxaliplatin *BRAF* mutant CRC patients had a shorter overall survival compared to wild-type CRCs; however, no statistical association was observed between *BRAF* mutations and response to chemotherapy with oxaliplatin versus irinotecan [63]. Additionally, a more recent study reported no differences in PFS of *BRAF* mutant CRC irrespective of whether oxaliplatin- or irinotecan-based chemotherapy was administered in the first-line setting (6.4 versus 5.4 months) [69].

Monoclonal antibodies targeting EGFR (such as cetuximab and panitumumab) have been introduced in the management of metastatic CRC patients with impressive results in the RAS wild type population. Although the predictive role of *RAS* mutations to anti-EGFR therapies is well established, the predictive role of mutated BRAF is less clear. Some retrospective studies hypothesize that *BRAF* mutation may confer resistance to anti-EGFR therapies [47, 70].

Based on these results a number of clinical trials were retrospectively analysed to systematically review the benefit of anti-EGFR therapies according to *BRAF* mutational status [64, 71-73]. Among these studies, only one has shown a

statistically significant detrimental effect of anti-EGFR therapy in *BRAF* mutant metastatic CRC in terms of PFS but not for OS. The major problem of these studies was that they were non-confirmatory since they contained a relatively small number of *BRAF* mutant patients. Therefore, a number of meta-analyses have investigated the predictor role of BRAF in anti-EGFR therapy improving the statistical power of the studies (reviewed in [74]). Overall these studies suggest the role of *BRAF* mutant as predictor of resistance to anti-EGFR monoclonal antibodies and thus the need for *BRAF* mutational status assessment before initiation of treatment with anti-EGFR therapies. This recommendation is consistent with the consensus guidance from ESMO published in 2016 which highlight that the BRAF mutational status should be analysed and reported as standard in all metastatic cases [75].

2.7. BRAF inhibitor insensitivity in *BRAF* mutant CRC

BRAF V600E mutation leads to a constitutive activation of BRAF kinase activity, resulting in phosphorylation and activation of the MEK kinases. Once activated, MEK kinases phosphorylate and activate ERK kinases, which phosphorylate a multitude of cellular substrates involved in cell proliferation and survival. BRAF V600E inhibitors, such as vemurafenib and dabrafenib, induce dramatic response rates of 50–80% *BRAF* mutant melanomas and are clinically approved [76, 77]. However, when CRC patients carrying the same BRAF V600E mutation were treated with vemurafenib in monotherapy, only a 5% response rate was observed [78]. Similarly, treatment with encorafenib, a potent and selective inhibitor specific against mutant BRAF kinase, did not show response when used in monotherapy

[79]. These results were unexpectedly disappointing compared to the clinical activity observed in melanoma.

Pre-treatment and on-treatment biopsies analysis of *BRAF* mutant melanoma patients has revealed that suppression of the MAPK signaling pathway is necessary for tumor response [80]. However, *BRAF* mutant CRC cells showed only a transient MAPK suppression with a rapid re-accumulation of phosphorylated ERK (pERK) within six hours after BRAF inhibitor (BRAFi) treatment [81]. Therefore transient and incomplete MAPK pathway inhibition could be a key factor of BRAFi resistance in CRC. In this tumor setting, the feedback reactivation of MAPK signaling under BRAF or MEK inhibition appears to be driven by EGFR-mediated activation of RAS and CRAF phosphorylation [81, 82]. This molecular feedback could be explained by a model in which BRAF inhibition leads to arrest of MEK and ERK kinase activity, which in turn leads to a reduced activation of CDC25 phosphatases, which ultimately triggers an increase in EGFR phosphorylation (pEGFR) due to decreased dephosphorylation [82].

Melanomas are sensitive to BRAF inhibitors as they originate from the neural crest and do not express EGFR, making this feedback loop ineffective. On the other hand, CRC derive from epithelial cells in which EGFR is generally constitutively expressed [81, 82]. Interestingly, EGFR inhibitors displayed a synergistic effect with BRAFi both *in vitro* and *in vivo* [81, 82]. Particularly, cell lines treated with BRAF and EGFR inhibitors showed abrogation of AKT, MEK and ERK phosphorylation. Moreover, activation of the PI3K signaling pathway has also been hypothesized to explain resistance to BRAF inhibitors in *BRAF* mutant CRC cells [55]. Therefore both EGFR activation and aberrant PI3K pathway signaling may

underlie the limited therapeutic effect of BRAF inhibitor monotherapy in CRC patients. Thus, combining an EGFR inhibitor and/or a PI3K inhibitor may be a rational approach to treating these tumors [83].

2.8. BRAF inhibitor based combinations clinically tested in *BRAF* mutant CRC

Table 2 summarizes the results of the ongoing trials testing EGFR, MAPK and PI3K inhibitors in *BRAF* mutant CRC which are discussed in more details below.

2.8.1. Targeting BRAF and MEK

The first BRAF inhibitor combination trial for *BRAF* mutant CRC involved the combination of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib [84]. This trial started prior to the discovery of EGFR as a major driver of resistance in *BRAF* mutant CRC. The rationale for this trial was based on the finding that the combination of a BRAF inhibitor and a MEK inhibitor could produce more potent and sustained suppression of MAPK signaling in *BRAF* mutant CRC cells, leading to increased efficacy [85]. Forty-three *BRAF* mutant mCRC patients were enrolled in this trial. Although some activity of this combination was confirmed, this is far away from the impressive results in melanoma. The RR was 12% (one complete and four partial responses), 24 patients achieved stable disease (56%), and 10 patients (23%) remained on treatment for more than 6 months. The median PFS was 3.5 months [84].

Drugs	N° of patients	RR (%)	DCR (%)	PFS (months)	Trial	Reference
Vemurafenib	10	5	50	2.1	NCT01524978	Hyman et al. (2015) [86]
Encorafenib	18	0	67	4	NCT01436656	Gomez-Roca et al. (2014) [79]
Vemurafenib + cetuximab	27	4	73	3.7	NCT01524978	Hyman et al. (2015) [86]
Vemurafenib + panitumumab	15	13	83	3.2	NCT01791309	Yaeger et al. (2015) [87]
Dabrafenib + panitumumab	20	10	90	3.4	NCT01750918	Corcoran et al. (2016) [88]
Encorafenib + cetuximab	26	19	77	3.7	NCT01719380	van Geel et al. (2017) [78]
Dabrafenib + trametinib	43	12	68	3.5	NCT01072175	Corcoran et al. (2015) [84]
Dabrafenib + trametinib + panitumumab	83	21	85	NA	NCT01750918	Corcoran et al. (2016) [88]
Encorafenib+ binimetinib + cetuximab	29	48	93	8	NCT02928224	Van Cutsem et al (2018) [90]
Encorafenib + cetuximab + alpelisib	28	18	93	4.2	NCT01719380	van Geel et al. (2017) [89]

Table 2. BRAF inhibitor based combinatorial therapies in *BRAF* mutant CRC. PFS, progression-free survival; RR, response rate; NA, not available.

2.8.2. Targeting BRAF and EGFR

As previously mentioned, clinical trials testing BRAF inhibition as single agent in patients with metastatic CRC carrying *BRAF* mutations revealed disappointing results [78, 79, 86]. However, the central role of EGFR driving the BRAF inhibitor resistance in *BRAF* mutant CRC has led to the development of several clinical trials evaluating combinations of BRAF and EGFR inhibitors. Twenty-seven

patients were enrolled in the BASKET trial evaluating the combination of the BRAFi vemurafenib and the EGFRi cetuximab in *BRAF* mutant CRC [86]. Although, half of these had tumor regression, they did not meet the standard criteria for a partial response. Median PFS and OS for patients receiving combination therapy were 3.7 and 7.1 month, respectively. Another study on 15 patients testing panitumumab and vemurafenib reported a total of 10 tumor regressions, two of which had stable disease lasting over 6 months [87].

Dabrafenib was tested in combination with panitumumab. Analysis of the 20 patients receiving these drugs showed a 10% response rate (RR - one complete and one partial) with an 80% rate of stable disease. Median duration of response was 6.9 months and PFS was 3.5 months [88].

As previously mentioned, encorafenib treatment was ineffective when used in monotherapy [79]. However when encorafenib was tested in combination with cetuximab in 26 *BRAF* mutant CRC patients, a 19% RR (one complete, four partial and one unconfirmed partial response) was observed. Fourteen patients achieved stable disease and the median PFS was 3.7 months [89].

2.8.3. Targeting BRAF, MEK and EGFR

As BRAF inhibition can induce EGFR over activation, adding a MEK inhibitor to the combination of a BRAF and an EGFR inhibitor may allow better MAPK inhibition. This combination was tested on 35 *BRAF* mutant CRC patients [88]. The overall RR was 21%; median PFS is not yet mature. The BEACON phase III trial is currently evaluating the triple combination of encorafenib, binimetinib (MEKi) and cetuximab in patients with *BRAF* mutant mCRC. Interim results have

revealed that this triplet can induce a median PFS of 8 months, with three patients achieving CR and confirmed ORR of 48% [90]. Therefore these data provided evidence of the meaningful clinical activity of the triple compare to double regimen. Additionally, analysis of paired pre-treatment and on-treatment biopsies obtained from *BRAF* mutant CRC patients revealed a 12% and 47% mean decrease in pERK during treatment with BRAF+EGFR inhibitor and BRAF+MEK inhibitor respectively. When BRAF+MEK+EGFR inhibitors were combined together a mean reduction in pERK levels of 69% was observed. This reduction is comparable to the mean 76% decrease observed in *BRAF* mutant melanoma patients treated with dabrafenib alone [91]. Therefore, more robust suppression of MAPK signaling may account for some of the increased efficacy of the triple combination relative to each individual double combination.

2.8.4. Targeting BRAF, EGFR and PI3K

Another triple targeted inhibitor combination has also been evaluated in *BRAF* mutant CRC patients, involving the addition of a PI3K alpha specific inhibitor alpelisib to the BRAF inhibitor encorafenib and the EGFR antibody cetuximab. The addition of a PI3K inhibitor is based on the hypothesis that activation of the PI3K/AKT pathway is an underlying mechanism of both innate and acquired resistance to BRAF inhibitors in *BRAF* mutant CRC [83]. In 28 *BRAF* mutant CRC patients enrolled in a phase I study this triple combination has produced a RR of 18% and a PFS of 4.2 months. The disease control rate was 93% for the triplet versus 77% for double combination. Importantly, the triplet arm, as consequence of alpelisib treatment, had higher toxicities compared to the doublet combination

without improving significantly the benefit. Alpelisib has demonstrated to be ineffective in inhibiting PI3K pathway signaling in the presence of molecular alterations leading to PTEN loss of function [92]. In the *BRAF* mutant CRC these alterations are present in near 40% of the patients thus suggesting a possible mechanism for the poor benefit introduced by alpelisib treatment [4].

3. Aims of the study

Although the BRAF inhibitor based combinations revealed clinical benefit, including prolonged stabilization and partial responses [89, 91, 93, 94], the long-term efficacy of these treatments is limited by the emergence of drug resistance [89, 91, 94, 95]. 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 [96, 97]. 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. In this study, in order 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. Genotyping two *BRAF* mutant CRC patients upon acquired resistance to BRAF based target therapies provided evidence that *in vitro* resistant cell models faithfully recapitulate what occur in clinic and represent valuable tools for key functional studies aimed at identifying effective strategies to overcome drug resistance (**Figure 4**).

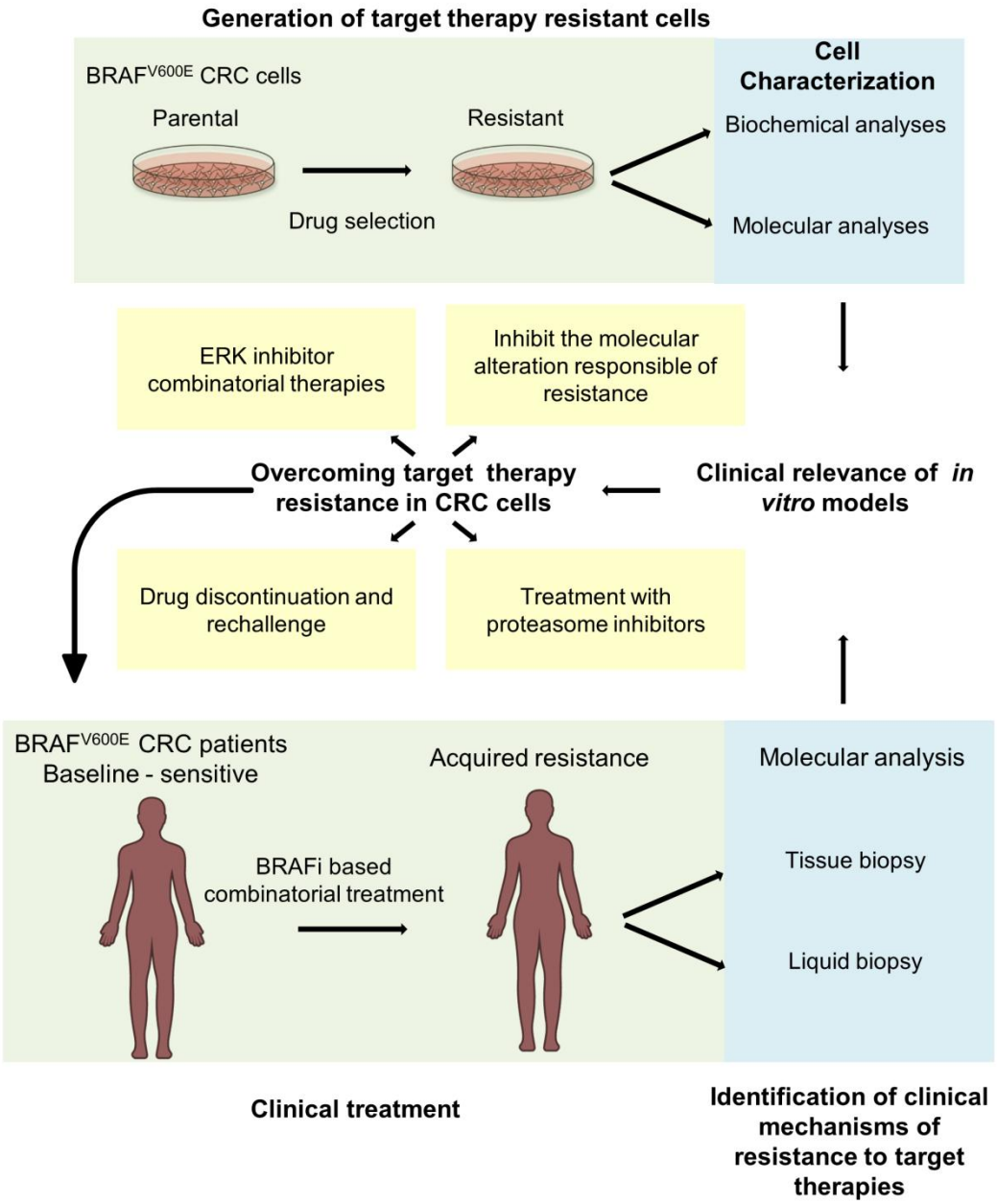

Figure 4. Schematic representation of the aims of the work.

In summary the aims of this study are:

- Develop preclinical models of *BRAF* mutant CRC cells with acquired resistance to target therapy combinations;

- Find molecular mechanisms of acquired resistance to BRAF target therapy combinations in CRC cell models;
- Determine mechanisms of clinical acquired resistance to BRAF target therapy in mCRC patients;
- Identified possible strategies to overcome or delay resistance to BRAF target therapy combinations.

4. Methods

4.1. Cell lines

BRAF mutant WiDr, HROC87 and VACO432 CRC 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). The genetic identity of parental cell lines and their resistant derivatives was confirmed by short tandem repeat profiling (Cell ID System; Promega) at 10 different loci (D5S818, D13S317, D7S820, D16S539, D21S11, vWA, TH01, TPOX, CSF1PO and amelogenin) not fewer than 2 months before drug profiling or biochemical experiments. Cell lines were tested and resulted negative for Mycoplasma contamination with the VenorGeM Classic Kit (Minerva Biolabs).

4.2. Drugs and generation of drug resistant cells

Vemurafenib, encorafenib, selumetinib, trametinib and carfilzomib were purchased from Sequoia Chemicals; alpelisib, crizotinib 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 3**). During drug selection cells were neither passaged nor detached by plates thereby preserving polyclonality.

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

Table 3: List of drug concentrations at which cell lines were made resistant. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); T= Trametinib (MEKi); V= Vemurafenib (BRAFi).

4.3. 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- 96 hour 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). For long-term proliferation assays, cells were seeded in 6-well plates (20,000 cells/well) and cultured in the absence or presence of drugs as indicated. Wells were fixed with 4% paraformaldehyde and stained with 1% crystal violet-methanol solution (Sigma-Aldrich) after 12-14 days. All assays were performed independently at least two times.

4.4. 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, carfilzomib 200 nM, crizotinib 300 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-MET (clone D1C2, 1:1000); anti-phospho MET (Tyr1234/1235, Cat.#3126; 1:1,000); 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); anti-PARP (1:1000); anti-ubiquitin (Santa Cruz Biotechnology; 1:1,000); HSP90 (Santa Cruz Biotechnology; 1:500); Actin (Santa Cruz Biotechnology; 1:1000); anti-vinculin (Millipore; 1:5,000).

4.5. Single cell cloning

Cells were washed with phosphate-buffered saline (PBS), tryspinized, re-suspended in media and count to prepare a suspension of cells containing 1000 viable cells in 10 ml and later diluted to 50 viable cells in 10 ml. A volume of 200 µL was plated into each well of a 96-well culture plate (approximately 1 cell per well). After 7 days wells containing only a single cell clone were marked; wells with no clones or with more than one clone were excluded.

4.6. Gene copy number analysis qPCR

Parental and resistant cell lines were trypsinized, washed with PBS and centrifuged; pellets were lysed and DNA was extracted using Wizard SV Genomic kit (Promega) according to the manufacturer's directions. Real time PCR was performed with 10 ng of DNA per single reaction using GoTaq QPCR Master Mix (Promega) with an ABI PRISM® 7900HT apparatus (Applied Biosytems). Sample analysis was normalized to a control diploid cell line, HCEC [98]. *HER2*, *MET*,

EGFR, *KRAS* and *BRAF* gene copy number was assessed. Primer sequences are reported in **Table 4**.

	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

Table 4: List of primers for gene amplification and sequencing

4.7. 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 **Table 4**.

4.8. Next Generation Sequencing (NGS) on plasma samples

A minimum of 10 ml of whole blood was drawn in EDTA tubes before commencing treatment and at radiological progression. 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. Fragment size distribution of 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 or Next-Seq-500 sequencers (Illumina Inc., San Diego, CA, USA). FastQ files generated by the sequencers were mapped to the human reference genome (assembly version hg19) using BWA-mem algorithm [99] SAMtools package 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 [100, 101]. 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 [102].

4.9. NGS on formalin-fixed and paraffin-embedded (FFPE) samples

Formalin-fixed and paraffin-embedded (FFPE) tumor samples were sliced in 5 µm-thick sections and manually micro-dissected under light microscope control to isolate the highest amount of tumor cellularity compared to contaminating non-neoplastic cells (hopefully 70% or more). Samples were treated with xylene and 100% ethanol to remove all wax traces and DNA was then isolated using the GeneRead DNA FFPE kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Subsequently, DNA amount and quality were controlled by means of NanoDrop platform (Invitrogen, Life Technologies, Foster City, CA, USA) following manufacturer's details. NGS analysis was performed by using small genomic DNA samples (20 ng/µl) and the Ion-Torrent^{TM} Personal Genome Machine platform (Life Technologies-Thermo Fisher Scientific, USA). A detailed description of the applied

T-NGS procedures has been previously published [103]. For this study, we used two custom panels, the first of which designed to amplify 3358 amplicons (246,15 kb) from 110 oncogenes and tumor suppressor genes recurrently mutated in human cancers and a second custom panel to analyse 26 amplicons (2,77 kb) corresponding to the EGFR extra-cellular region (exons from 1 to 14).

4.10. Droplet Digital PCR (ddPCR)

Genomic DNA from CRC cells and isolated circulating free DNA from plasma was amplified using ddPCR™ Supermix for Probes (Bio-Rad) using *BRAF* (PrimePCR™ ddPCR™ Mutation Assay, Bio-Rad) ddPCR assay for V600E mutation and *MET* and *EIF2C1* (reference) for gene copy number variations (PrimePCR ddPCR Copy Number 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 [101].

4.11. Fluorescence *In Situ* Hybridization (FISH) and Silver *In Situ* Hybridization (SISH)

Dual color FISH analysis, both on metaphase chromosomes and interphase nuclei, obtained from cultured cells and on 3 µm FFPE tissue sections, was performed by using Chr7q (7q11.21) / BRAF (7q34) probes (Abnova); Chr7q / EGFR probes (7p12)(Abnova); Chr12q (12q12) / KRAS (12p12.1) probes (Abnova); D7Z1 (7p11.1-q11.1) / c-MET (7q31.2) probes (Cytocell), respectively labelled with FITC and Texas Red.

For both cell lines and clinical samples, dehydration was carried out in ethanol series (70%, 90%, 100%), followed by 3 washes (5' each) and air drying. 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.

Bright field dual-color SISH analysis was performed on 3 μm FFPE tissue sections by using the MET DNP Probe along with the Chromosome 7 DIG Probe (Ventana Medical Systems) on a BenchMark Ultra Platform (Ventana Medical Systems) according to the manufacture's protocol.

For both SISH and FISH analyses the probes signals were counted in at least 100 non overlapping tumor cells nuclei from each case. Two independent molecular pathologists (Annunziata Gloghini and Emanuele Valtorta) scored the slides in a blinded fashion. *MET* gene amplification was defined as positive when: a) *MET*/CEP7 ratio was $\geq$ 2 or b) average number of *MET* signals per tumor cell nucleus was > 6.

4.12. Immunohistochemistry (IHC)

IHC was carried out on a BenchMark Ultra Platform (Ventana Medical Systems, Tucson, AZ) by using the OptiviewDAB Detection Kit (Ventana Medical Systems). MET IHC was evaluated according to a semi-quantitative assessment (H-score)

which combines staining intensity (scored from 0 to 4) with the percentage of positive cells (scored 0–100%). Each individual intensity level is multiplied by the percentage of cells and all values are added to obtain the final IHC score, ranging from 0 to 400. Scores from 0 to 200 are considered negative/low expression and scores from 201 to 400 are considered positive/high expression.

4.13. Preparation of cytoclots

WiDr cytoclots were prepared by pelleting up to $50x10^6$ cells and re-suspending them in 1% agar, followed by polymerization in dry ice for a few seconds, fixation in paraformaldehyde 4% and ethanol 70% at +4°C for 4 hours, and final embedding in paraffin wax.

4.14. 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 **Table 5.** 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.

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

Table 5. Drug concentrations applied in the cross-resistance combinatorial screening depicted in **Figures 26 and 27**. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); T= Trametinib (MEKi); V= Vemurafenib (BRAFi).

4.15. Viral Infection

For Lentiviral particle production, HEK293T cells were co-transfected in 15-cm dishes with the packaging plasmid MDLg/pRRE (2.5 µg) and pRSV.REV (6.25 µg), the vesicular stomatitis virus (VSV) envelope plasmid pMD2.VSV-G (9 µg) and one of the following plasmid: pCCL-MET wild-type (a gift of Elisa Vigna, IRCCS, Candiolo, Turin), hBRAF V600E cDNA (a gift of Maria S. Soengas, CNIO, Madrid, Spain), EGFR WT cDNA (a gift from Dr. C. Sun and Prof R. Bernards, NKI, Amsterdam), EGFR G465R mutant cDNA [104] or lenti-control plasmid (25 µg). Cells were tranduced in six well plates (3 x 10^5 per well in 2 ml of medium) in the presence of polybrene (8 µg/ml) (Sigma).

4.16. Clinical samples

- *Patient 1* is a chemorefractory metastatic CRC patient which was enrolled in the CMEK162X2110 clinical trial (Trial registration ID: NCT01543698) at Niguarda Cancer Center, Milan, Italy. 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.

- *Patient 2*. Tumor samples were collected in accordance with an Institutional Review Board–approved protocol, to which the patient provided written informed consent, and all studies were conducted in accordance with the Declaration of Helsinki. Targeted NGS on FFPE tissues was performed in the Department of

Diagnostic Pathology and Laboratory Medicine at Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. Whole exome sequencing and digital PCR on plasma samples were performed at IRCCS Candiolo, Turin, Italy. The patient's insurance company covered the cost of off-label combinatorial therapies (panitumumab+ vemurafenib; crizotinib+vemurafenib), to which the patient gave informed consent. CT scans were obtained as part of routine clinical care, while FDG-PET/CT scans were performed as part of an ancillary study protocol for patients receiving targeted treatments.

5. Results

5.1. Molecular mechanism of acquired resistance to BRAFi therapy combinations in *BRAF* mutant CRC cells

5.1.1. 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 (**Figure 5**).

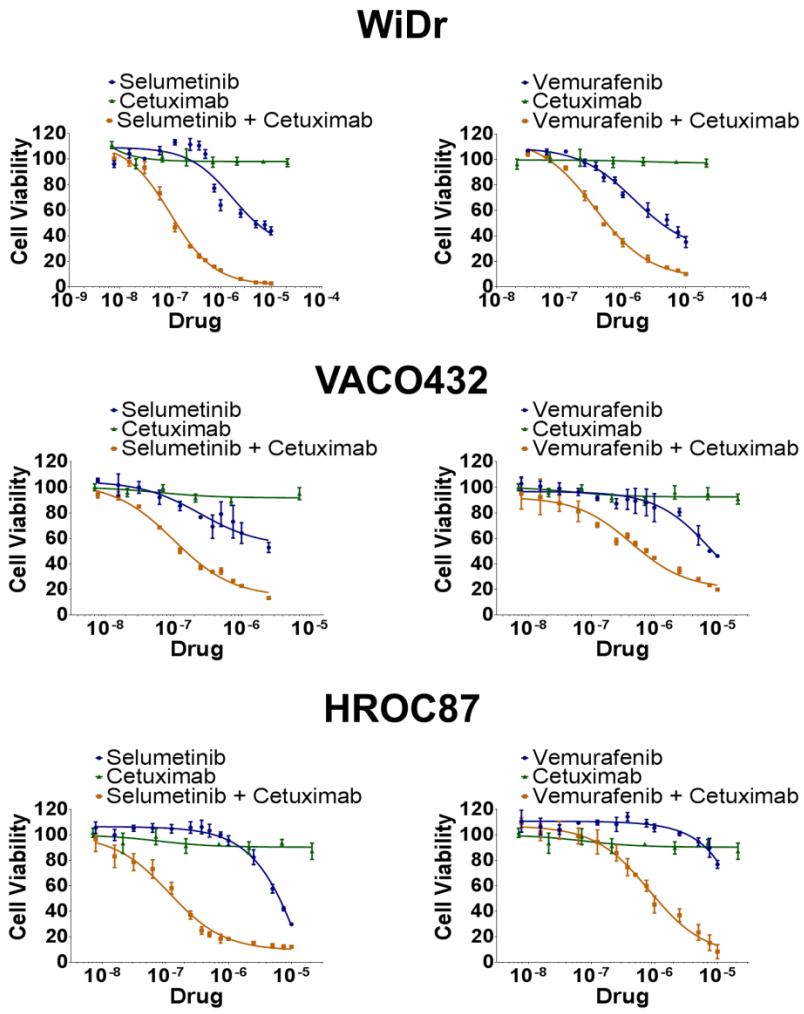

Figure 5. Combinations of MEK inhibitor (selumetinib) or BRAF inhibitor (vemurafenib) with EGFR inhibitor (cetuximab) display synergistic anti-proliferative

activity in *BRAF* mutant WiDr, VACO432 and HROC87 CRC 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.

To gain a comprehensive understanding of potential therapeutic resistance mechanisms in *BRAF* mutant CRC, the above mentioned cell lines were cultured in the presence of seven different drug combinations in clinical trials until resistant derivatives emerged. 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 (**Figure 6**).

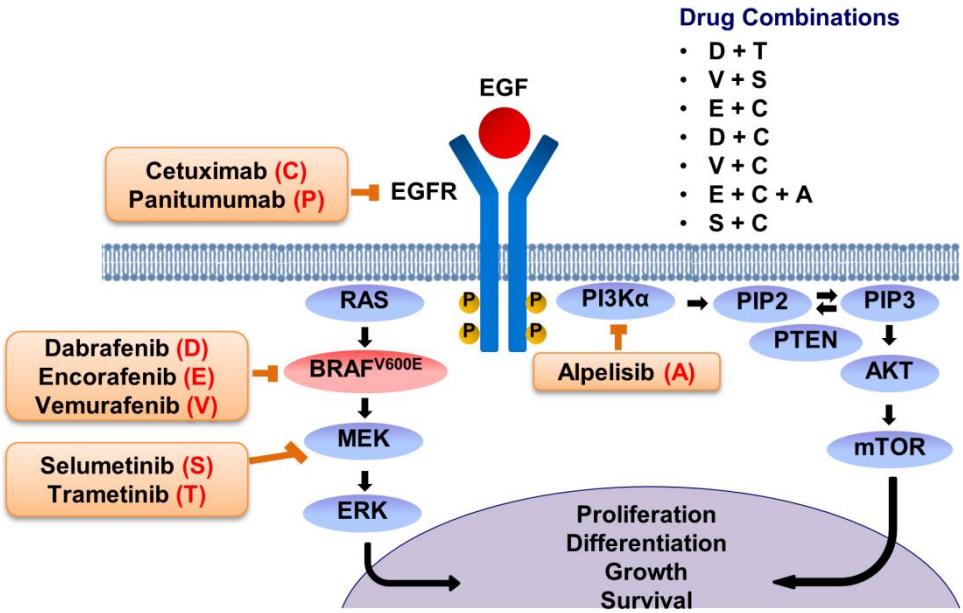

Figure 6. Schematic representation of RAS/RAF/MEK and PI3K/AKT pathways. The orange boxes show the drugs used for generating *BRAF* mutant CRC cell lines resistant to target therapies. The actual list of drug combinations used for generating resistant cell line models is shown on the right; all of these are being evaluated in clinical trials. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); T= Trametinib (MEKi); V= Vemurafenib (BRAFi).

A total of twelve resistant cell line models were generated. Of note, the amount of cells attached to the plate during drug selection did not require any passages until resistant clones emerged. Therefore all resistant cell models maintained their polyclonality. 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 the drug concentrations tested (**Figure 7**).

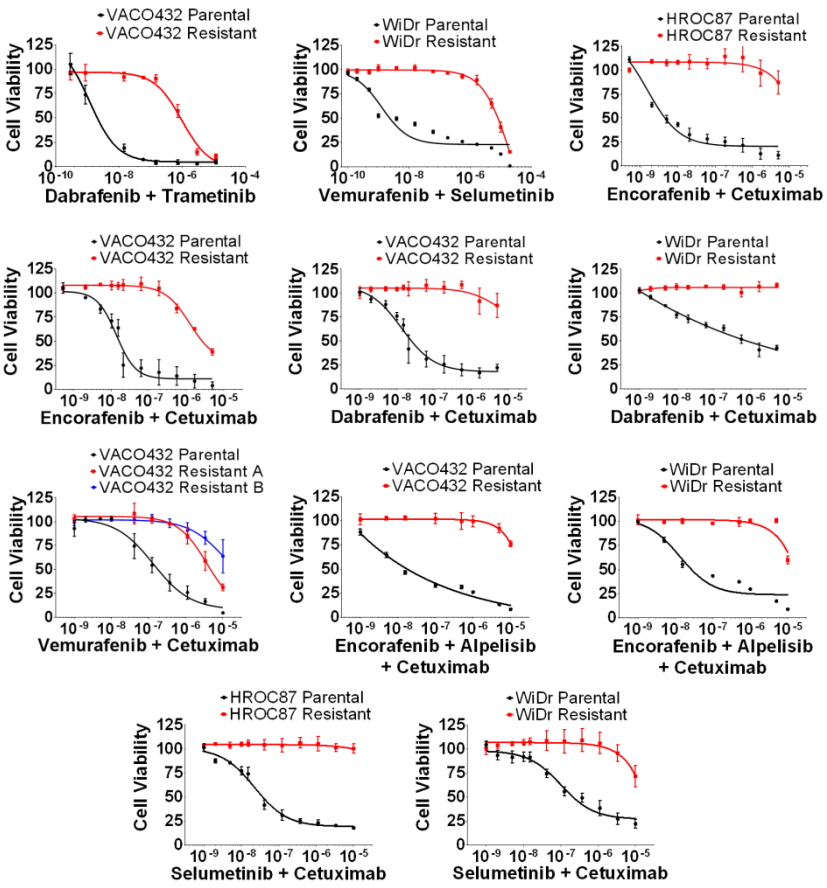

Figure 7. Generation of *BRAF* mutant CRC cells resistant to EGFR targeted monoclonal antibodies and BRAF/MEK or PI3K inhibitors. Parental and resistant cells were treated for 72h with different drug combinations. Cetuximab and BYL719 were given

at a constant concentration of 5 µg/ml and 100 nM, respectively. For generating VACO432 D+C cells, dabrafenib was used at the concentrations reported in figure. For the vemurafenib and selumetinib combination, a fixed 300 nM concentration of selumetinib was applied.

5.1.2. 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 [105-109]. 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 (**Figure 8**). 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 (**Figure 8**).

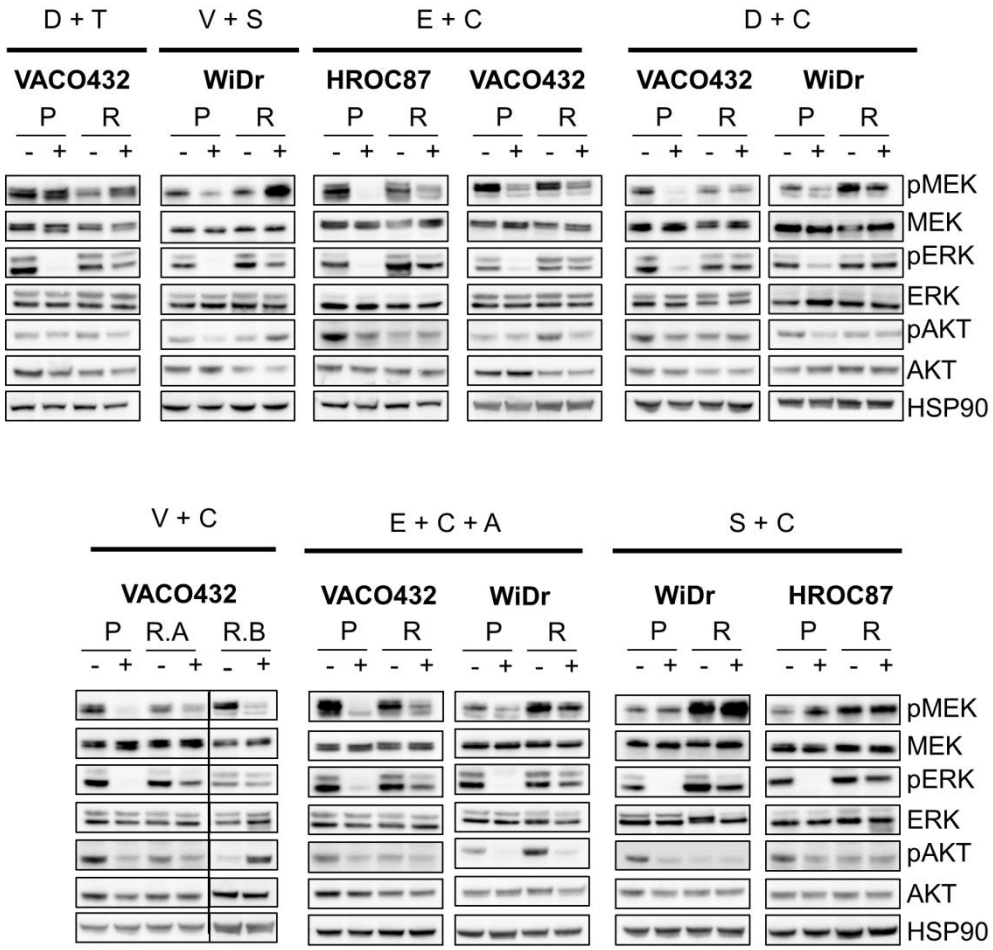

Figure 8. *BRAF* mutant CRC cells with acquired resistance to target therapy maintain ERK1/2 phosphorylation under drug treatment. WiDr, VACO432 and HROC87 parental and resistant cells were treated with different drug combinations as indicated: cetuximab (C, 5 µg/ml); dabrafenib (D, 300 nM); encorafenib (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.

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

In order to identify 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 (qPCR) on genomic DNA extracted from resistant cells showed no changes in *HER2* gene copy number while *MET*, *EGFR*, *KRAS, or BRAF* gene copy number increased in four WiDr derivatives resistant to V+S, E+C+A, D+C or S+C, respectively (**Figure 9A**). All gene amplifications were only found in the resistant cell populations and were confirmed by fluorescence in situ hybridization (FISH) analyses (**Figure 9B**). 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 6**. All resistant cell populations retained the original *BRAF* V600E mutation. All other mutations found in resistant cells were not detected in their parental counterparts.

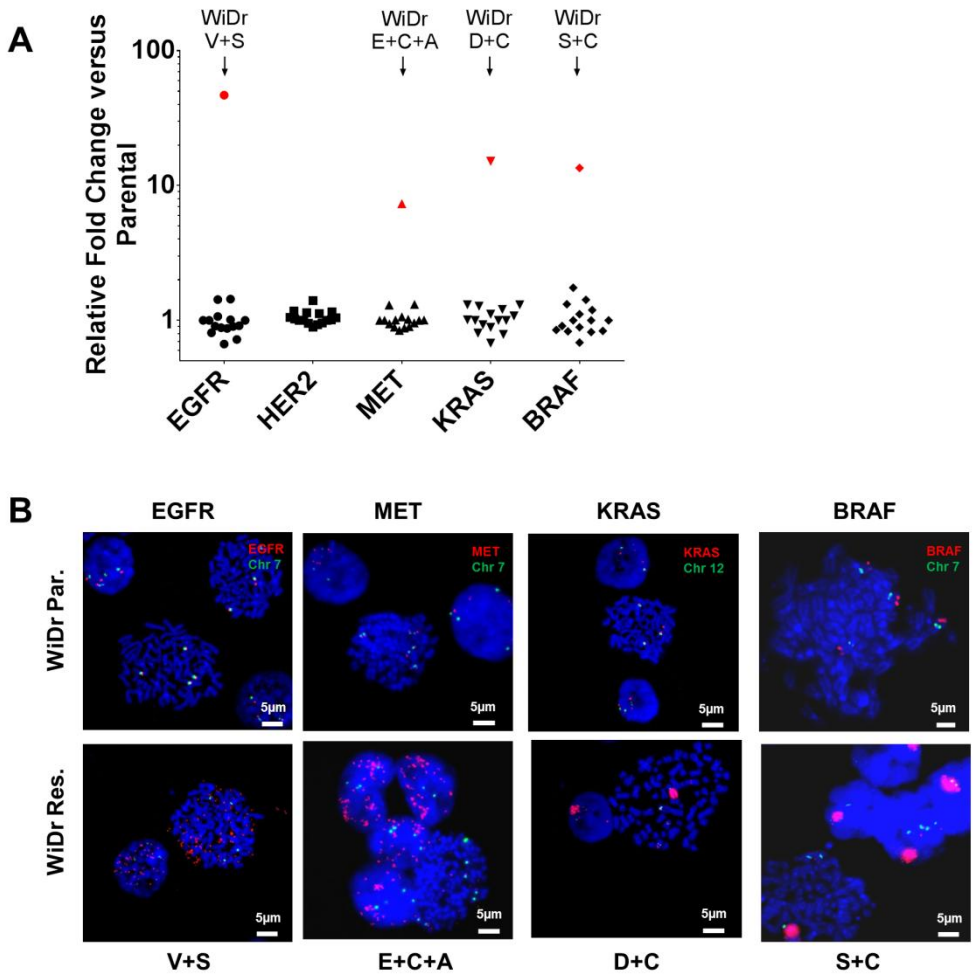

Figure 9. *EGFR*, *MET*, *KRAS* or *BRAF* gene amplification confer acquired resistance to BRAF combination therapies. (A) Quantitative PCR for copy number evaluation of resistant cell lines in respect to their parental counterparts. WiDr V+S, E+C+A, D+C and S+C resistant lines displayed gene amplification of *EGFR*, *MET*, *KRAS* and *BRAF*, respectively. **(B)** FISH analysis on chromosome metaphase spreads confirmed gene amplification. Cell nuclei were colored by DAPI, FISH probes EGFR, MET, KRAS, BRAF were labeled with texas red (red signal) and chromosome 7 (Chr7) and 12 (Chr12) with FITC (green signal). *EGFR* and *MET* gene amplification was found extrachromosomally as double minutes, while a focal intrachromosomal amplification of *KRAS* and *BRAF* loci could be identified. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); V= Vemurafenib (BRAFi).

Drugs	Cell line	EGFR	KRAS	NRAS	MAP2K1	MAP2K2	BRAF/MET 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
	WiDr	WT	WT	WT	WT	WT	MET ampl.
S + C	HROC87	WT	WT	WT	V211D	WT	none
	WiDr	WT	WT	WT	WT	WT	600E ampl.

Table 6. Molecular alterations emerged upon resistance to targeted therapy combinations in *BRAF* mutant CRC cell lines. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); T= Trametinib (MEKi); V= Vemurafenib (BRAFi).

5.1.3.1. *KRAS* alterations

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 [97]. Our data suggest that exon 4 *KRAS* mutations may 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 BRAF/EGFR inhibition [97, 110]. These findings suggest that the cell models generated in this work have the potential to recapitulate clinically-relevant resistance mechanisms.

5.1.3.2. *BRAF V600E* amplification

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 [97], in CRC cell lines with secondary resistance to the MEKi selumetinib [85, 111], as well as in melanoma patients upon progression on the BRAFi vemurafenib [112], 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 (**Figure 10A**). Western blot with a diagnostic antibody specific for the V600E variant showed that the mutant protein was selectively overexpressed (**Figure 10B**). Finally, we validated that ectopic

overexpression of mutant BRAF in WiDr parental cells can confer resistance to combined MEKi+EGFRi (**Figure 10C, 10D**).

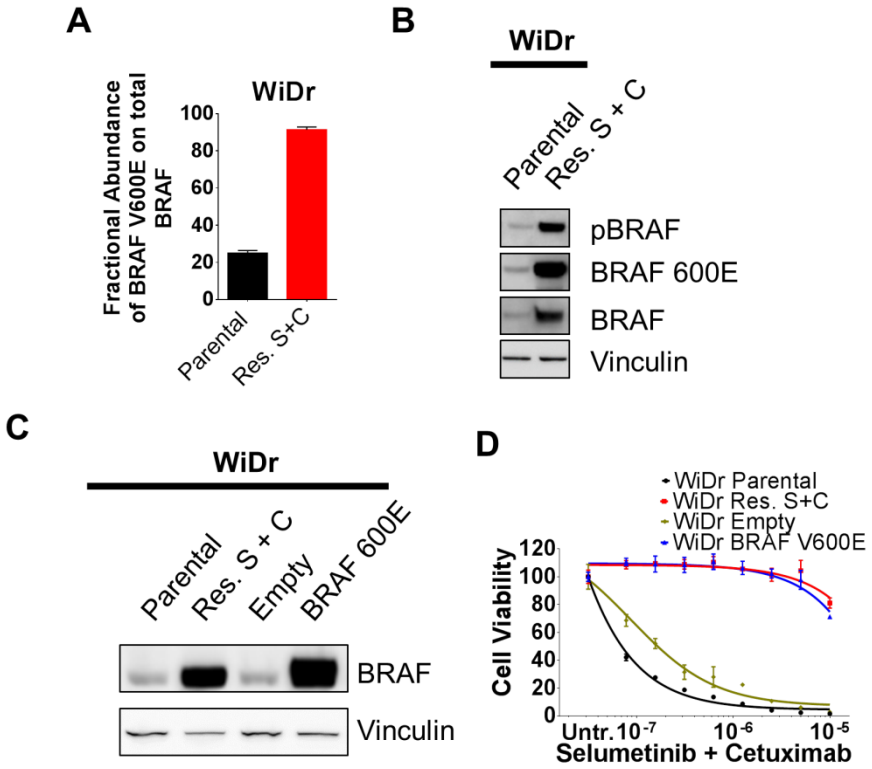

Figure 10. *BRAF* V600E amplification acquired in WiDr resistant to selumetinib and cetuximab (S+C), confers resistance to combination of MEK and EGFR inhibitors in CRC cells. (A) Digital PCR shows selective amplification of the *BRAF* V600E mutant allele in WiDr cells resistant to combined cetuximab 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. Drugs are abbreviated as follow C= Cetuximab (EGFRi); S= Selumetinib (MEKi).

5.1.3.3. *MAP2K1* mutations

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 [106, 113], so they were not subjected to further functional validation.

5.1.3.4. *MET* amplification

MET increased gene copy number and overexpression (**Figure 9**, **Figure 11A**) was identified in WiDr resistant to BRAFi+EGFRi+PI3Ki (E+C+A), a triple combination for which determinants of resistance have not been characterized so far. However, *MET* amplification or MET protein activation have previously been implicated as mechanisms of resistance to targeted agents, including vemurafenib in melanomas and EGFR directed monoclonal antibodies in CRC patients [114-116]. Functional experiments indicated that ectopic expression of MET in WiDr parental cells is able to confer resistance to the triplet regimen that includes PI3K inhibition (**Figure 11B**).

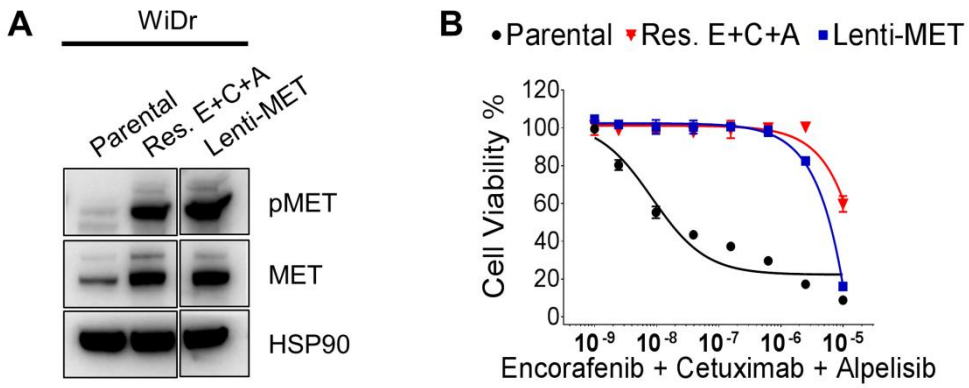

Figure 11. *MET* amplification confers resistance to combination of BRAF, EGFR and PI3KCA inhibitors in *BRAF* mutant CRC cells. (A) Biochemical analysis of WiDr parental and resistant E+C+A , and of WiDr cells transducted with MET cDNA (here called Lenti-MET). HSP90 was used as a loading control. **(B)** WiDr Lenti-MET cells displayed similar viability than the WiDr resistant E+C+A cells upon treatment with encorafenib, cetuximab and alpelisib. 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. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); E= Encorafenib (BRAFi).

5.1.3.5. *EGFR* amplification and mutation

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 [81, 82], *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 [117]. Ectopic overexpression of EGFR in WiDr parental cells was able to confer resistance to combined BRAFi+MEKi or BRAFi+EGFRi (**Figure 12A and 12B**).

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 [104, 118], 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 (**Figure 12C and 12D**).

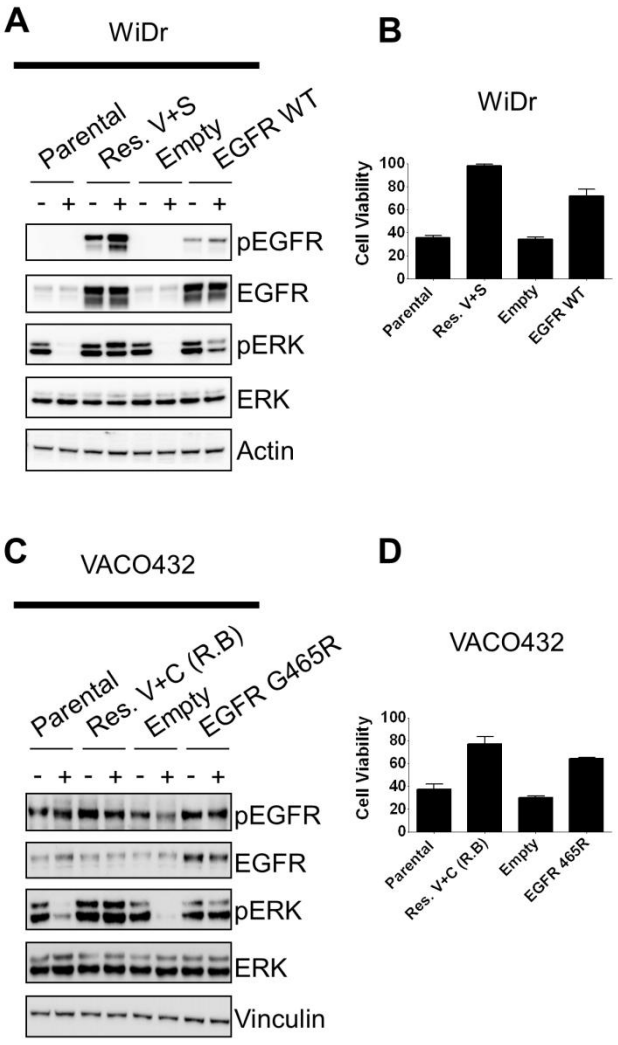

Figure 12. *EGFR* amplification or ectodomain mutations play a causative role in acquired resistance to BRAF combination therapies. (A) Biochemical analyses of

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)** 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. **(D)** 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. In all cases cell viability was assessed by ATP content measurement after 72 hours of treatment. Data are expressed as average ± s.d. of two independent experiments. Drugs are abbreviated as follow C= Cetuximab (EGFRi); S= Selumetinib (MEKi); V= Vemurafenib (BRAFi).

5.1.4. Single clone analyses revealed intercellular heterogeneity upon acquired resistance to MAPK inhibitorial therapies

Genomic instability, a feature observed in a high proportion of solid tumors [119], affects DNA sequence, chromosome structure and chromosome number, generating a high level of intercellular genetic heterogeneity [120]. Although drug pressure triggers a selective evolution of a tumor [121, 122], it is known that for any given therapy there may be multiple mechanisms of resistance [123, 124]. In order to identify whether specific genetic alterations acquired upon resistance were homogeneously shared in the cell population, we performed limiting cell dilution to obtain single cell clones.

BRAF gene copy number was analyzed in clones derived from WiDr resistant S+C cells. Compare to WiDr parental cell line, quantitative PCR for *BRAF* gene copy number revealed a fold change of 7.7 in WiDr resistant S+C cells, and an average of 10.1 (ranging from 2 to 38) in its derivative clones (**Figure 13A**). However, even

if the levels of *BRAF* copy number appeared to be heterogeneous among different clones, all of them displayed selective amplification of the mutant V600E allele (**Figure 13B**).

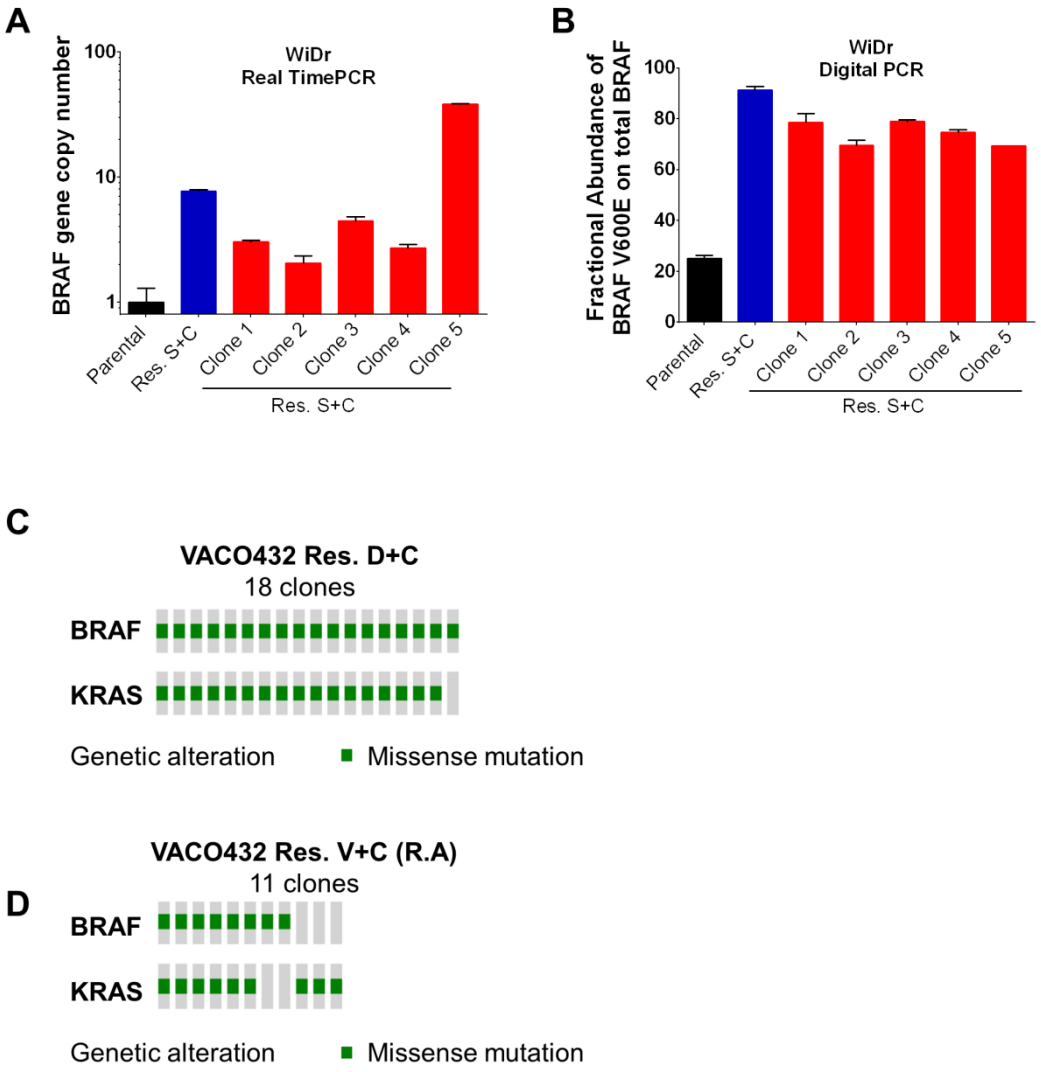

Figure 13. Single clone analyses of *BRAF* gene copy number and BRAF/KRAS mutations revealed intercellular heterogeneity. (A) Quantitative PCR for BRAF copy number evaluation of WiDr cells resistant S+C and from its derivative clones. **(B)** Digital PCR shows that resistant cell clones carry selective amplification of the BRAF 600E mutant allele. **(C-D)** Genetic alteration distribution of BRAF and KRAS in cell clones obtained from VACO432 resistant D+C and V+C (R.A). Genetic data are present as OncoPrint through cBioPortal [55, 56]. Drugs are abbreviated as follow C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); S= Selumetinib (MEKi); V= Vemurafenib (BRAFi).

KRAS mutations were analyzed in two VACO432 resistant cell models. *KRAS* A146T was detected in VACO432 resistant to D+C. Of the 18 clones derived from this population and analyzed by Sanger sequence, 17 (95%) displayed concomitant *BRAF* and *KRAS* mutations and one (5%) showed only *BRAF* mutation (**Figure 13C**). Additionally, analysis of the second VACO432 resistant model, V+C (R.A) clones revealed a similar heterogeneous distribution of *BRAF* and *KRAS* mutations. In detail, among 11 clones, individual *BRAF* or *KRAS* mutation was present in two (18%) and three (27%) cases respectively; concomitant *BRAF* and *KRAS* mutations were identified in 6/11 (55%) VACO432 resistant V+C (R.A) clones (**Figure 13D**). The absence of detectable *KRAS* mutations in some clones suggests that additional undetermined genetic or not genetic alterations triggering resistance might occur in a fraction of cells.

5.2. Clinical acquired resistance to BRAF inhibitor based combinations

In vitro modeling of acquired resistance in cancer cell lines has proven effective in identifying resistance mechanisms that occur clinically [100, 125]. In the previous part of my thesis, I described the molecular alterations acquired upon resistance to BRAFi based therapy in *BRAF* mutant CRC cells (summarized in **Figure 14**). Ultimately, any preclinical work requires validation in the clinic. To test whether the resistance mechanisms identified *in vitro* might recapitulate what is observed in CRC patients, we analyzed, by next generation sequencing, DNA samples (derived from tissue or plasma – liquid biopsy) from two individuals before treatment and upon resistance to target agents. Here we describe two cases that highlight the clinical relevance of the work.

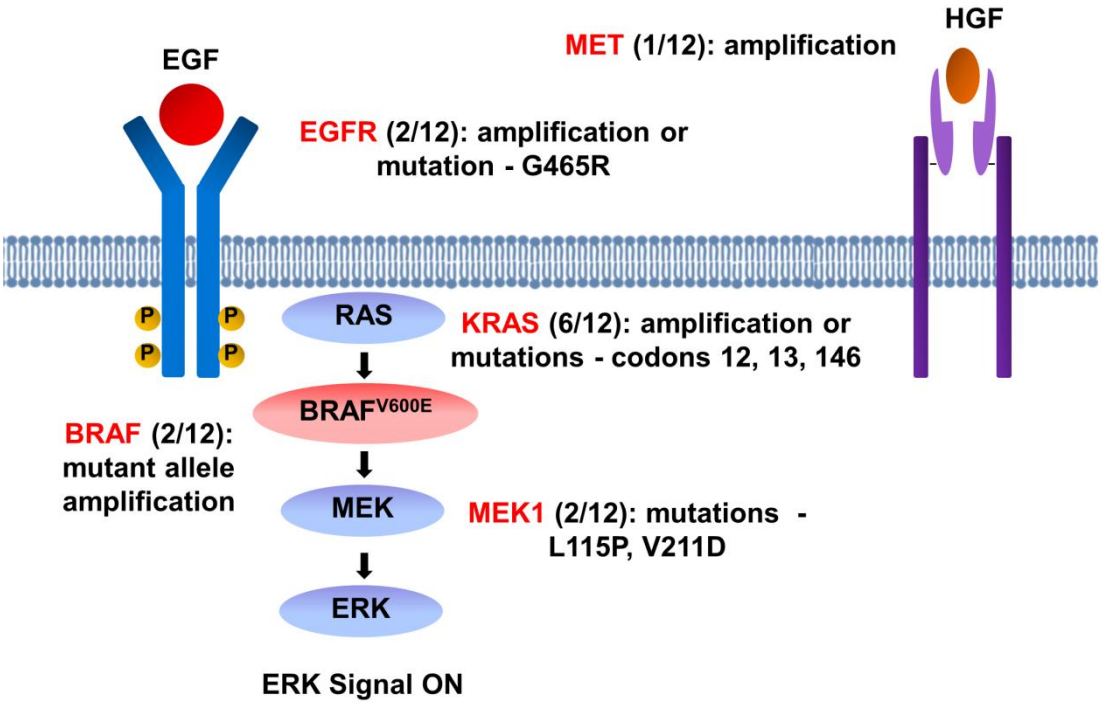

Figure 14. Molecular alterations emerged upon resistance to targeted therapy combinations in *BRAF* mutant CRC cell lines.

5.2.1. Clinical acquired resistance to combined BRAF and MEK targeted treatments - Patient #1

Patient #1 was a *BRAF* mutant metastatic CRC patient who received treatment based on the BRAFi encorafenib (LGX818) in combination with the MEKi binimetinib (MEK162). The patient was treated from September 2013 to March 2014, obtaining a partial response in January 2014, followed by radiological progression in March 2014. No post-treatment tumor tissue was available for this patient, so the identification of clinical acquired resistance mechanisms to targeted therapy combination was performed by genotyping of liquid biopsy samples. Liquid biopsy is non-invasive test that detects in plasma fragments of circulating tumor DNA (ctDNA) that are shed into the blood from primary tumor and metastatic sites.

Plasma samples from Patient #1 were available before treatment and after disease progression. ctDNA was extracted and subjected to molecular profiling by next-generation sequencing (NGS) analysis of a custom panel of 226 cancer related genes [101]. The analysis revealed that the percentage of reads carrying *TP53* p.R282W mutated allele were consistent between the baseline and the progression plasma (**Figure 15**), indicating similar circulating tumor DNA (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. *BRAF* mutant allele amplification and *KRAS* oncogenic alterations were found in several of our resistant cell models to BRAF combinatorial therapies, confirming the clinical relevance of the alteration that was identified in the panel of cells that we generated.

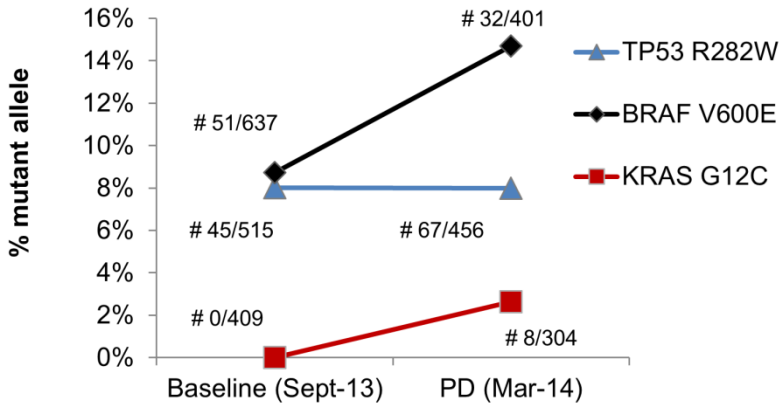

Figure 15. Next generation sequencing of ctDNA of *BRAF* mutant CRC patient #1 at resistance to combined BRAF/MEK inhibition revealed an increase of *BRAF* V600E number of reads and the emergence of a *KRAS* G12C mutation. The lines indicate percentage of reads carrying the variant (mutated) allele over 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.

5.2.2. Clinical acquired resistance to combined BRAF and EGFR targeted treatments - Patient #2

Patient #2 was a *BRAF* V600E CRC metastatic patient with acquired resistance to panitumumab and vemurafenib after 4 months treatment (the patient clinical history is summarized in **Figure 16**)**.** At disease progression, a liver tumor biopsy was obtained in order to investigate molecular mechanisms of acquired drug resistance. Molecular analyses were carried out in parallel both in pre-treatment primary tumor tissue and in post-treatment liver biopsy. Amplicon-based NGS of selected exons in 110 genes did not identify nucleotide variants in genes (such as *EGFR*, *KRAS*, *NRAS* or *MAP2K1*) previously implicated in resistance to EGFR monoclonal antibodies or BRAF target therapies. Immunohistochemical and *in situ* hybridization (ISH) analyses of HER2 and MET on archival rectal sample and

post-progression liver biopsy were performed. No changes in HER2 expression or gene copy number status were observed (data not showed). However, a marked difference was seen in MET expression (**Figure 17**)

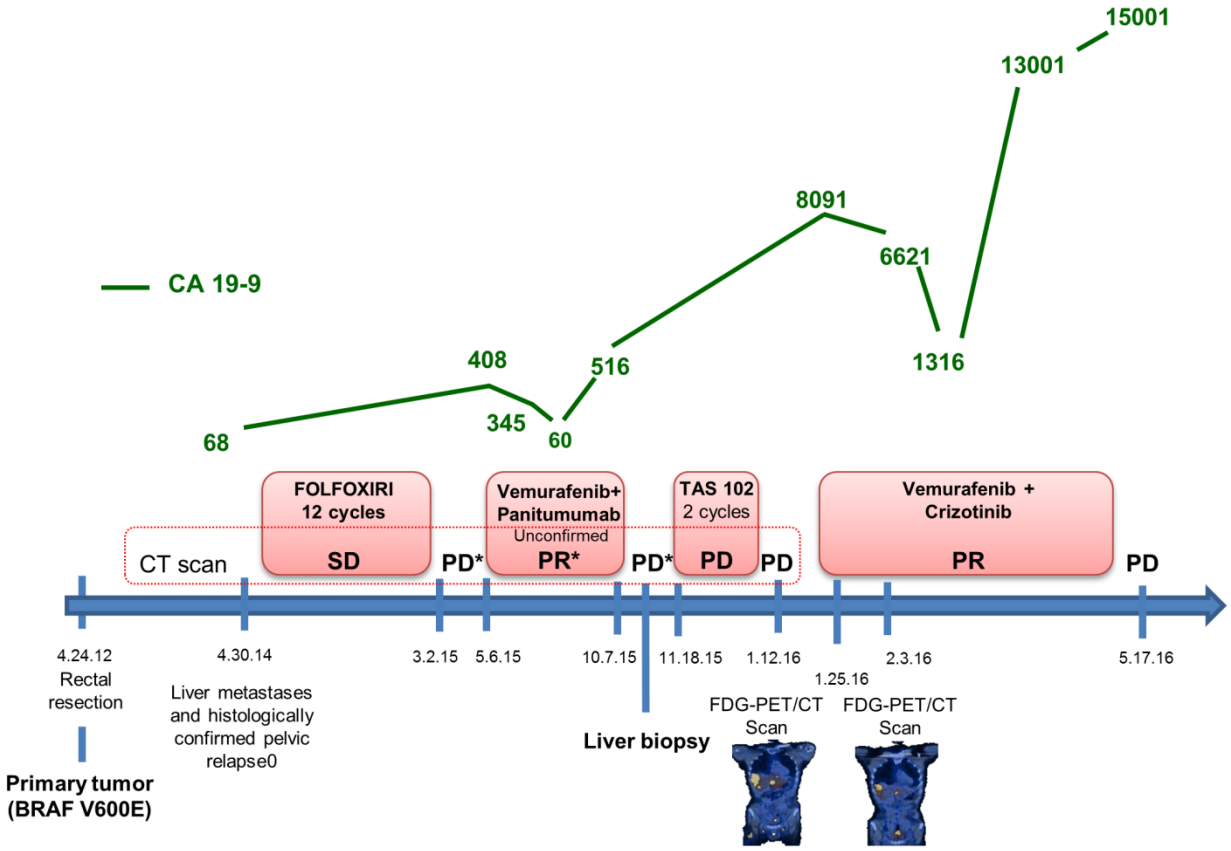

Figure 16. Summary of patient #2 clinical history. The clinical course of patient #2 with colorectal cancer is summarized, with serum cancer antigen 19-9 tumor marker levels shown throughout treatment (green line). Shaded boxes indicate periods of administration of the indicated chemotherapeutic agents. Blue vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsy, as well as dates of tumor assessment by either CT scan or FDG-PET/CT scan.

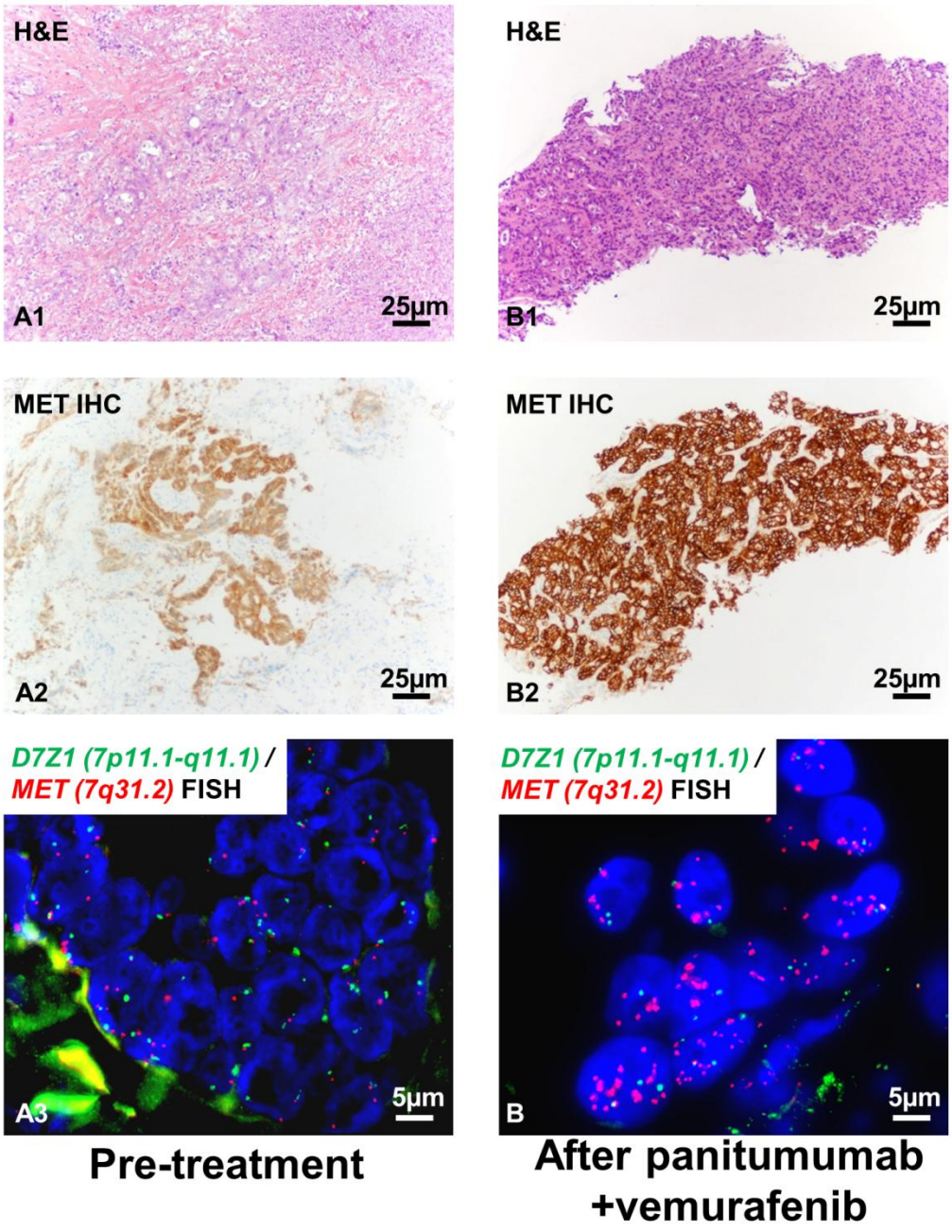

Figure 17. MET amplification is selected for in a *BRAF* mutant CRC biopsy from patient #2 at resistance to combined BRAF and EGFR targeted treatments. In panel A1-4 and B1-4: analyses performed on archival surgical specimen and on post-progression liver rebiopsy, respectively. A1-B1. Conventionally stained section using Hematoxylin & Eosin, showing poorly differentiated carcinoma of the rectum (A1) and liver metastasis (B1) (A1-B1, original magnification x20); A2-C2. Immunohistochemical detection of MET protein. The primary tumor displays heterogeneous immunostaining ranging from weak to moderate (at the center) intensity (A2). Metastatic tumor cells display homogeneous, strong immunostaining (B2) (A2-B2, original magnification X20);

A3-B3. Dual color FISH analysis using D7Z1 (7p11.1-q11.1) / c-MET (7q31.2) probes (Cytocell), respectively labeled with FITC and Texas Red. The primary tumor (A3, original magnification x100) shows heterogeneous, MET gene copy number, ranging from 1 to 8, while metastatic tumors cells (B3, original magnification x100) contain high *MET* gene copy number (ranging from 2 to 30) featuring *MET* gene amplification.

Analysis of the archival rectal sample showed heterogeneous MET immunostaining, with a strong membrane signal in about 50% neoplastic cells, while the remaining cell population showed a weak to moderate membranous staining. Heterogeneity was also observed in *MET* gene copy number. By FISH analyses, we estimated that the signals corresponding to the MET and CEP7 (chromosome seven α-centromeric) probes ranged from 1 to 8 (mean values of 3.52 and 2.89 for MET and CEP7, respectively), with a gene-to-chromosome ratio of 1.2, indicating chromosome 7 polysomy. However, in a small fraction of cells within the pre-treatment sample, the gene-to-chromosome ratio was ≥6, indicating subclonal *MET* gene amplification (**Figure 17** and **Table 7**). In the post-progression liver biopsy, MET was strongly expressed at the membrane level in about 90% neoplastic cells. In this sample, *MET* copy number varied from 1 to 30 (mean 8.5) while CEP7 ranged from 1 to 16 (mean 3.24) with an overall gene-to-chromosome ratio of 2.62. At least 75% cells in the liver biopsy were found to carry *MET* gene amplification (defined by a gene-to-chromosome ratio ≥6), indicating that dual EGFR-BRAF blockade had positively selected for *MET* amplified clones.

	Pre-treatment tumor sample	Post-treatment tumor sample
MET IHC	H-score 150	H-score 270
MET FISH	16% amplified cells	75% amplified cells
HER-2 IHC	Negative	Negative
Next-generation sequencing*	BRAF V600E (allelic frequency 27%) SDHD I40V (allelic frequency 44%)	BRAF V600E (allelic frequency 14%) SDHD I40V (allelic frequency 57%) No additional mutations

*Based on the percentage of mutant reads, both pre-treatment and post-treatment tissue samples displayed substantially comparable tumor purity.

Table 7. Summary of molecular data from the fluorescence in-situ hybridization (FISH) and sequencing analyses conducted on both the pre-treatment primary tumor and the liver biopsy obtained after progression on panitumumab plus vemurafenib treatment from Patient #2.

In order to assess whether MET overexpression alone is causally responsible for resistance to combinatorial vemurafenib and panitumumab treatment, we conducted *in vitro* forward genetic experiments. Ectopic overexpression of MET in WiDr parental cells was able to confer resistance to combined vemurafenib and panitumumab by activating ERK signaling (**Figure 18A**) and sustaining proliferation (**Figure 18B**). Therefore, these results suggest a causal relationship between *MET* amplification and resistance to targeted therapy combinations in *BRAF* mutant CRC.

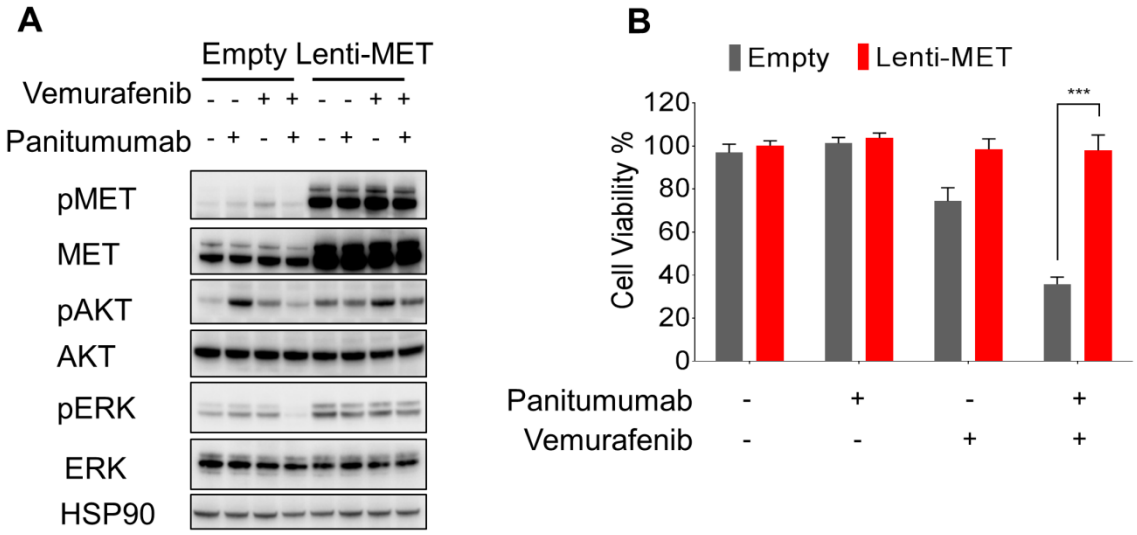

Figure 18. MET overexpression confers resistance to targeted therapy combinations in *BRAF* mutant CRC cells. (A) *BRAF* mutant WiDr cells were transduced with either control (empty) or MET–expressing lentiviral vectors. Cells were then treated with vemurafenib (2 μM), panitumumab (5 μg/ml) and their combination for 5 hours prior to protein extraction and Western blot. **(B)** After 96-hour treatment with panitumumab (5 μg/ml) or vemurafenib (1 μM) or their combinations, the viability of empty or lenti-MET transduced cells was assessed by relative ATP content measurement. Results represent mean ± SD of at least 2 independent observations, each performed in triplicate. ** P<0.01; *** P<0.001 Bonferroni's adjusted ANOVA *p* values.

5.3. Overcoming the emergence of acquired resistance to BRAF inhibitor combination therapies

In the first part of this thesis we identified multiple and convergent resistance mechanisms to BRAF inhibitor combination therapies. Tumors develop resistance to virtually all targeted therapies, including monoclonal antibodies and kinase inhibitors. The mechanisms by which tumors develop acquired resistance to target therapy can broadly be categorized into two classes, which include: secondary alterations in the drug target and activation of bypass signaling pathways. Identifying drug-resistance mechanisms is highly important to establish future treatment strategies to overcome resistance. Therefore, in the second part of this thesis, I have explored preclinical approaches to guide therapeutic development for *BRAF* mutant resistant CRC.

5.3.1. Combinatorial therapies blocking the altered target

5.3.1.1. Overcoming resistance triggered by EGFR alterations

EGFR is druggable through monoclonal antibodies, such as cetuximab or panitumumab, or by small molecule, as gefitinib and erlotinib. Since cetuximab was associated with response to *EGFR* amplification [126], we hypothesized that anti-EGFR therapy could restore sensitivity to BRAFi+MEKi in the EGFR-amplified WiDr V+S resistant model. Single agent or two-drug combination therapies based on BRAF, EGFR or MEK inhibitors did not impair the viability of WiDr V+S. However, when EGFRi was added to BRAFi+MEKi in a triple combination cell viability was drastically reduced (**Figure 19A**). Therefore adding anti-EGFR to

BRAFi+MEKi combination might overcome the resistance mediated by EGFR amplification in *BRAF* mutant CRC.

In VACO432 V+C (R.B) resistance was mediated by EGFR G465R ectodomain mutation. Consistent with its known role in impairing the binding of cetuximab and panitumumab to the extracellular domain of EGFR, combining gefitinib (a small molecule binding the intracellular kinase domain of EGFR) with BRAFi was able to overcome resistance reducing the cell viability of VACO432 V+C (R.B) (**Figure 19B**).

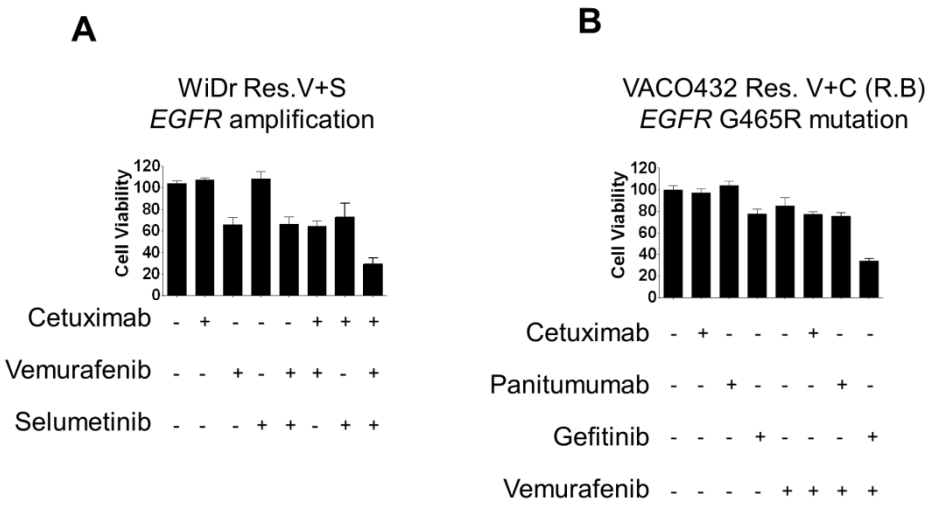

Figure 19. Overcoming resistance mediated by *EGFR* amplification or ectodomain mutations. (A) Effect on cell viability of the addition of cetuximab to V+S treatment in WiDr resistant cells carrying *EGFR* amplification. Cells were treated with vemurafenib (1 µM), selumetinib (0.5 µM) or cetuximab alone or in their combinations. **(B)** 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. Drugs are abbreviated as follow C= Cetuximab (EGFRi); S= Selumetinib (MEKi); V= Vemurafenib (BRAFi).

Globally, these data could be of interest in the future to overcome the emergence of resistance mediated by activated EGFR signaling in patients carrying *BRAF* mutant CRC tumors.

5.3.1.2. Overcoming resistance triggered by MET amplification – from bench to the clinic and back

As described in paragraph 5.1.3.4, we detected the emergence of *MET* increased gene copy number and overexpression in a drug resistant WiDr subline (WiDr Res.E+C+A) obtained by prolonged exposure of parental cells with cetuximab in association with the BRAF inhibitor encorafenib and the selective PI3K-α inhibitor alpelisib (**Figure 9 and 11**), a triplet regimen that is being tested in clinical trials [95]. WiDr resistant E+C+A cells were clearly refractory to EGFR, BRAF and MEK targeted therapies, either as single agents or in combination (**Figure 20A**). Importantly, the dual ALK-MET inhibitor crizotinib alone showed no effects on the proliferation of WiDr resistant cells, whereas its combination with BRAF kinase inhibition by vemurafenib led to a marked decrease of cell viability over prolonged times (**Figure 20A**). Additionally, crizotinib when combined to vemurafenib was able to impair viability in WiDr MET-transduced cells (**Figure 20B**). Altogether, these results suggest a potential therapeutic *in vitro* strategy to overcome MET-mediated resistance.

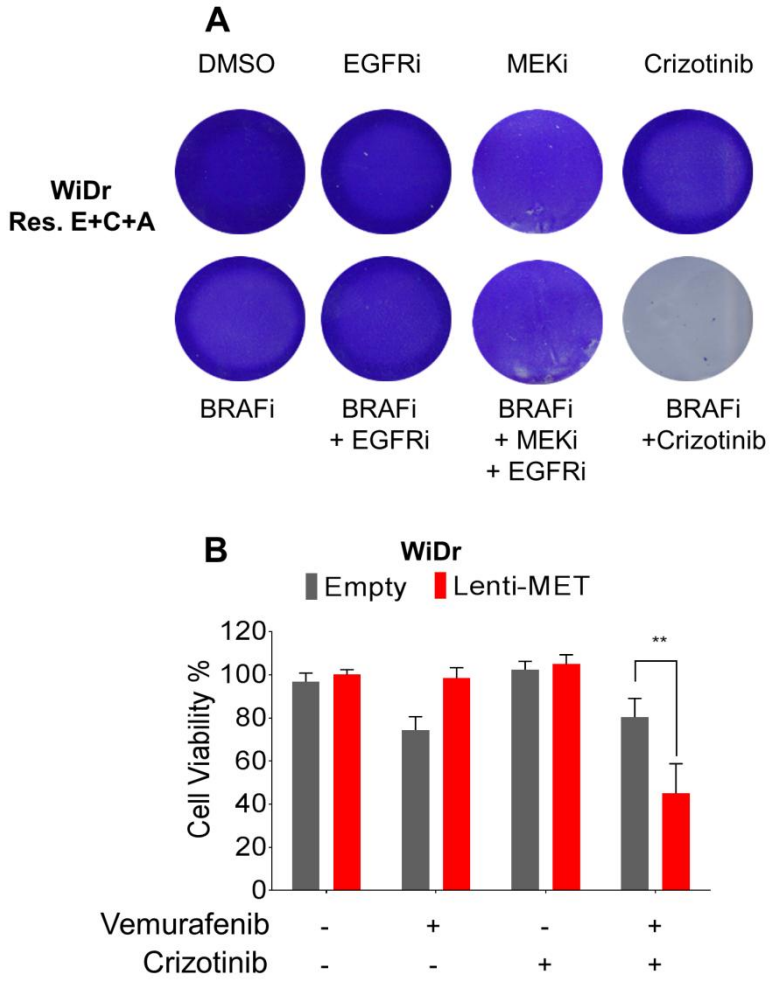

Figure 20. MET inhibition combined with BRAF blockade impairs proliferation of *BRAF* mutant / *MET* amplified CRC cells. (A) Long-term colony forming assay of *MET* amplified encorafenib+cetuximab+alpelisib resistant WiDr cells treated for 12-14 days in the absence or presence of panitumumab (EGFRi, 5 μg/ml), trametinib (MEKi, 10 nM), crizotinib (0.3 μM), vemurafenib (BRAFi, 1.5 μM) or their combinations. Results are representative of at least 2 independent observations. **(B)** *BRAF* mutant WiDr cells were transduced with either control (empty) or MET–expressing lentiviral vectors. After 96-hour treatment with vemurafenib (1 μM), crizotinib (0.3 μM) or their combinations, the viability of empty or lenti-MET transduced cells was assessed by relative ATP content measurement. Results represent mean ± SD of at least 2 independent observations, each performed in triplicate. ** P<0.01; *** P<0.001 Bonferroni's adjusted ANOVA *p* values. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); E= Encorafenib (BRAFi).

5.3.1.3. MET and BRAF inhibitor combination could transiently overcome target therapy resistance in Patient #2

Its known that cancer cell lines could harbor most of the same genetic changes found in patient tumors, and could be used to learn how tumors are likely to respond to new drugs, increasing the success rate for developing new personalized cancer treatments [127]. Therefore, based on the recent phase 1 data about the combination of crizotinib and vemurafenib [128] and the *in vitro* data showing that these drugs can affect viability of *BRAF* mutant / *MET* amplified CRC cells (**Figure 20A**), patient #2 was treated accordingly starting from January 2016. Longitudinal collections of plasma ctDNA samples were analyzed at baseline and during treatment by droplet digital PCR (ddPCR) for *BRAF* V600E mutation and *MET* copy number variation (CNV). After 2 months of treatment, a computed tomography (CT) revealed a reduction in metastatic lesions and a partial response was confirmed (**Figure 21**). In parallel, serum CA19-9 marker declined by >50% after only 2 weeks (**Figure 16**), and the amounts of mutated BRAF V600E alleles in ctDNA declined by ≥ 99% within 3 weeks from treatment initiation.

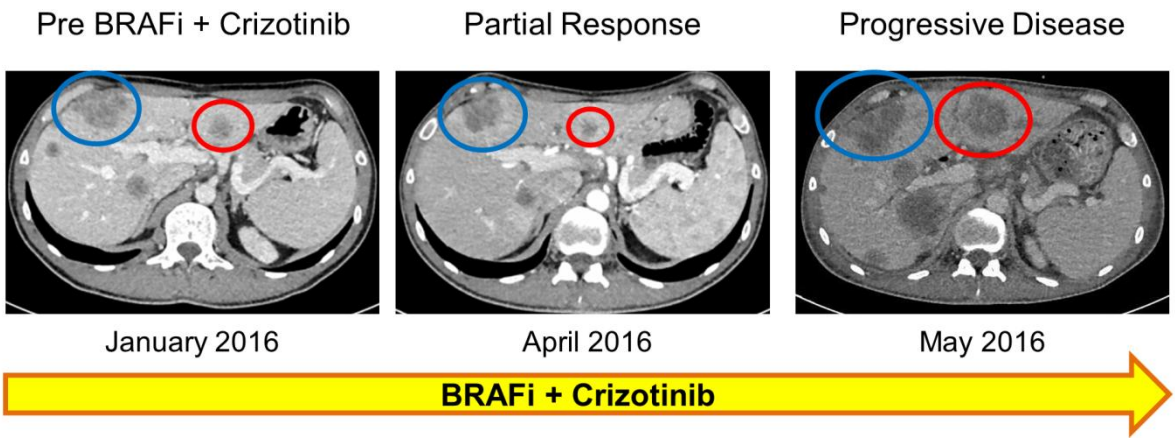

Figure 21. Clinical course of the disease of Patient #2 during treatment with vemurafenib+crizotinib. Computed tomography scans document the disease status before treatment (left panel), after the initial response (middle panel) and at disease progression (right panel).

5.3.1.4. Emergence of hyper-MET amplification conferred further resistance to MET and BRAF inhibitor combination in Patient #2

A subsequent CT scan of Patient #2 obtained after 4 months showed progressive disease (PD) (**Figure 21**) and serum CA 19-9 marker increased by ≥ 90% (**Figure 16**). In order to uncover the potential mechanisms of acquired resistance to the combination of MET and BRAF inhibitors, we performed whole exome sequencing (WES) analysis on plasma ctDNA obtained prior to treatment and at progressive disease. Germline DNA isolated from PBMC was used as reference genome. *BRAF* V600E mutant ctDNA alleles were used as normalizer for the amount of circulating tumor DNA in the pool of all circulating fragments. The number of reads supporting *BRAF* V600E mutation were comparable in the pre-treatment and post-resistance samples (46.4 and 49.45 respectively) suggesting an equal amount of ctDNA (**Figure 22**).

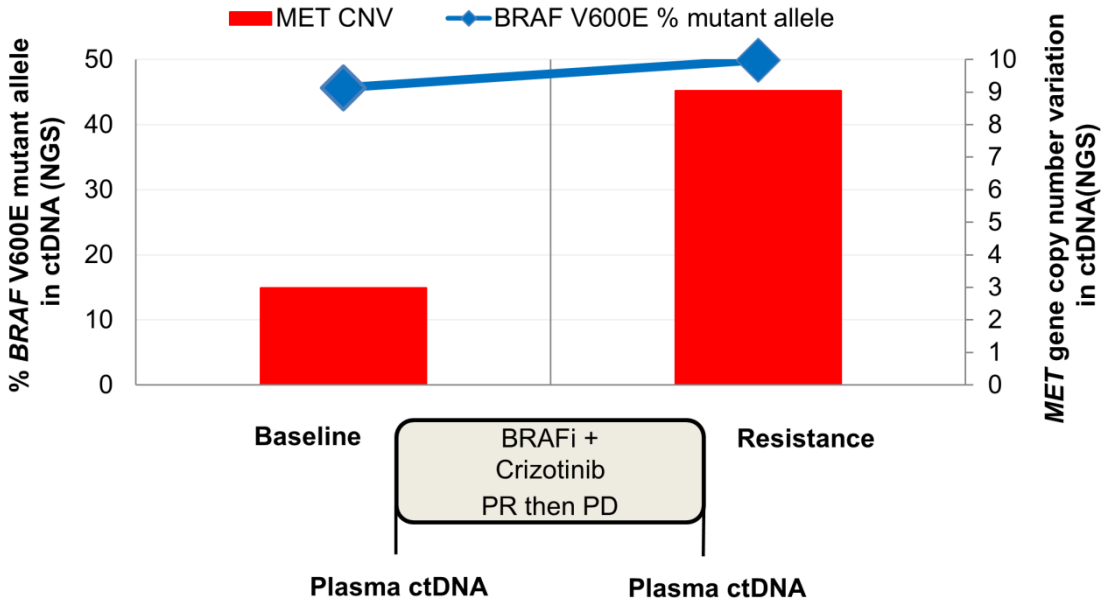

Figure 22. Whole exome sequencing of ctDNA of *BRAF* mutant CRC patient #2 at resistance to combined BRAF/MET inhibition revealed an increase in *MET* gene copy number despite a constant percentage of *BRAF* V600E allele. The blue line indicate percentage of *BRAF* mutant allele and the red bars indicate the *MET* copy number variation, detected by whole exome sequencing in ctDNA at baseline (pre-treatment) and resistance (post-crizotinib+vemurafenib).

Mutational analysis did not identify any genetic alteration (point mutation) potentially triggering drug resistance. Interestingly however, an increased MET CNV was detected at progression. To validate this finding, and to understand clonal dynamics in this patient during treatment, BRAF V600E mutation and MET CNV were longitudinally monitored during treatment in plasma ctDNA by droplet digital PCR (ddPCR) (**Figure 23**). This technology is based on micro compartmentalization of the PCR reaction and can be used to interrogate the mutational status of selected genes with high sensitivity (0.01 to 0.001%) [101]. Therefore this technique could facilitate the detection and quantification of drug resistant subclones in plasma ctDNA. The dynamics of mutant *BRAF* V600E

alleles correlated with clinical and radiographic response. A rapid decrease in *BRAF* V600E mutation and *MET* CNV in plasma ctDNA was observed within two weeks from vemurafenib+crizotinib treatment initiation. The dynamics of mutant *BRAF* V600E allele anticipated radiological progression, as mutant *BRAF* in plasma increased again as early as 8 weeks after starting therapy. At progression, the percentage of *BRAF* V600E alleles were comparable to the pre-treatment sample, while – proportionally – a notable increase in the number of *MET* copies was detected (**Figure 23**), thus validating exome data.

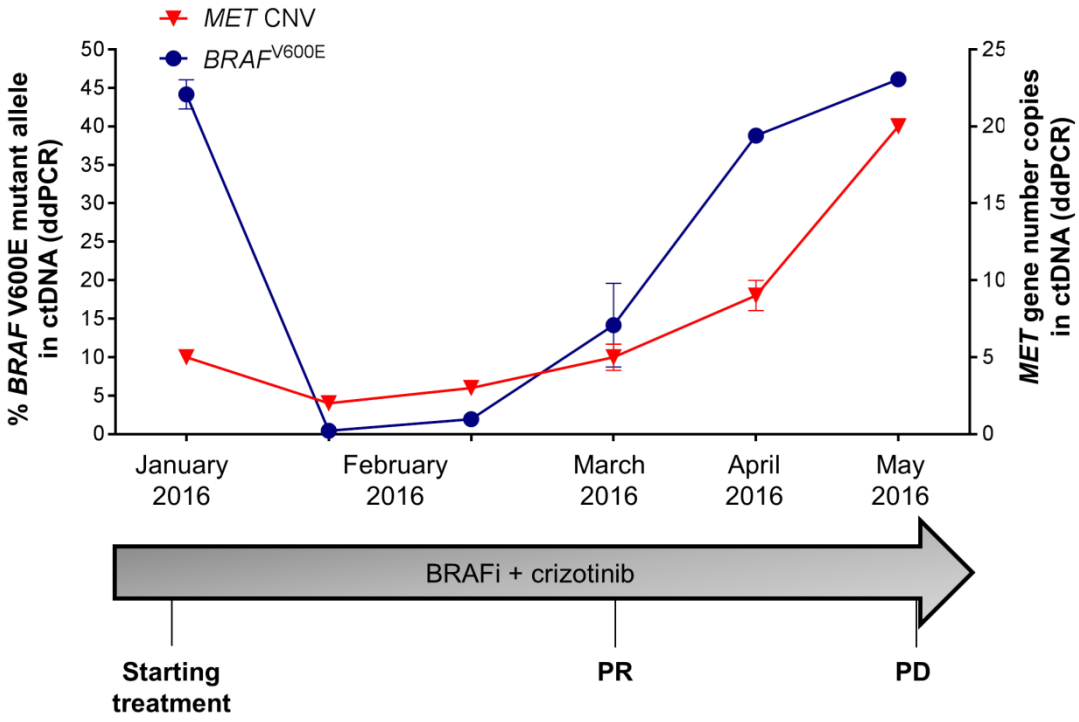

Figure 23. Longitudinal analysis of *BRAF* V600E mutated alleles and *MET* copy number variation in plasma ctDNA samples from patient #2. Blue and the red lines indicate the frequency of *BRAF* mutation (percentage of alleles) and *MET* copy number variation, respectively, detected in ctDNA at the selected time points by ddPCR. Gray arrows represent treatments administered to the patient. CT scan results as RECIST evaluation are indicated below the arrows. PR – partial response; PD – progressive disease.

To further validate *MET* hyper-amplification, and to exclude tumor burden-related *MET* CNV, we performed gene copy number analysis by *MET*/*CEP7* bright field *in situ* hybridization (ISH) in tissue specimens obtained before and after treatment. Upon resistance to BRAFi+crizotinib, the patient consented to inguinal lymph node tumor biopsy, which displayed a further increase in *MET* gene amplification by ISH, compare to the pre-treatment lesion (**Figure 24 – upper panels**). Therefore, the results in liquid and tissue biopsies suggest that clones with higher levels of *MET* gene amplification had been selected by the treatment.

Since evaluation of MET activation has recently been proposed to better correlate with transcription rather than protein expression due to the rapid turnover of the activated protein [129], we investigated *MET* transcript levels in inguinal lymph node tumor tissue by RNA *in situ* hybridization (RNA ISH). Expression of MET transcript was seen in the liver biopsy taken after vemurafenib+panitumumab. Upon resistance to vemurafenib+crizotinib, RNA ISH demonstrated *MET* RNA overexpression (**Figure 24 – bottom panels**). Intriguingly, *MET* hyper-amplification coupled with *BRAF* V600E may have driven a particularly aggressive disease behavior, since the patient died about two months following disease progression.

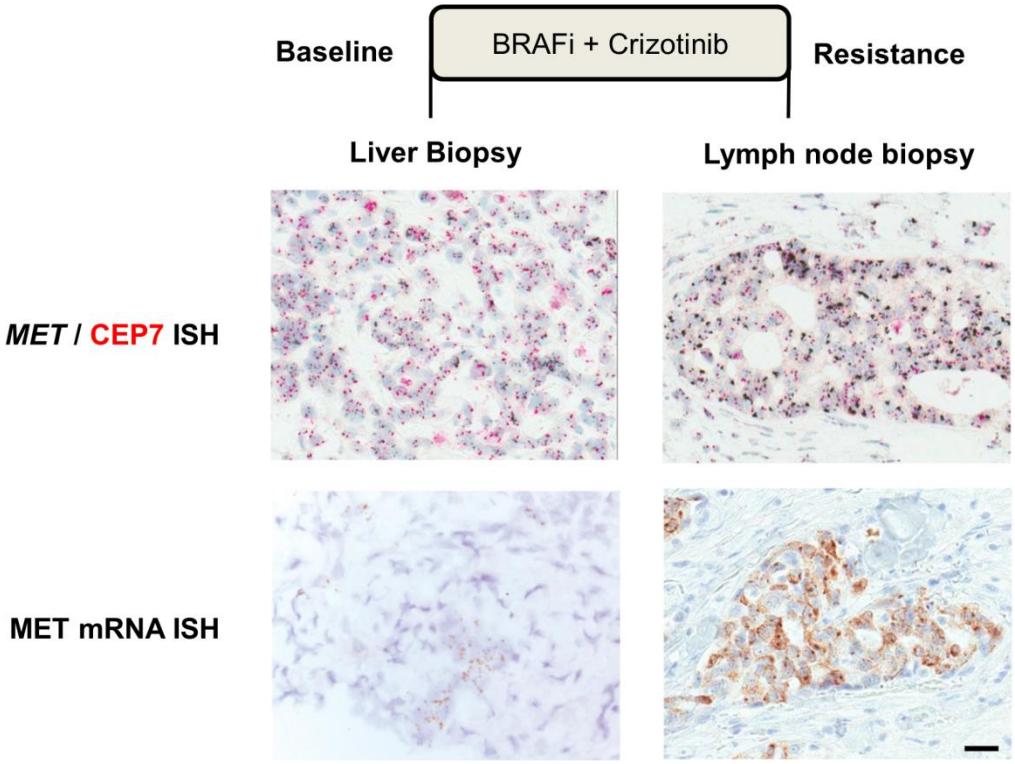

Figure 24. ***MET* gene hyper-amplification and *MET* mRNA expression emerge in patient #2 after target therapy.** *In situ* hybridization analyses for *MET* gene and mRNA levels were performed on a liver biopsy obtained upon resistance to vemurafenib+panitumumab but before vemurafenib+crizotinib (baseline, on the left), as well as inguinal lymph node (on the right) biopsy obtained upon acquired resistance to vemurafenib+crizotinib. **A)** Dual colour bright field in situ hybridization (ISH) for *MET* gene (black dots) and *CEP7* (red dots). The baseline sample obtained at acquired resistance to vemurafenib+panitumumab but prior to vemurafenib+crizotinib (baseline) shows *MET* amplification, with a mean gene copy number of 8.8 (range 2-20). Upon acquired resistance to vemurafenib+crizotinib, tumour cells display *MET* 'hyper-amplification', with a further increase of *MET* gene copy number in inguinal lymph node biopsy. In the bottom part is depicted the *In situ* hybridization for MET mRNA. Although *MET* gene transcription (score = 1) is present in the liver biopsy upon acquired resistance to vemurafenib+panitumumab (on the left – bottom part), this is further increased in the inguinal lymph node obtained upon resistance to vemurafenib+crizotinib (on the right – bottom part), with evidence of strong *MET* mRNA overexpression (score = 4). Scale bar represent 12.5 µm.

Finally, to test whether MET overexpression is causally responsible for resistance to BRAFi+crizotinib combination treatment, we conducted *in vitro* forward genetic experiments. As previously mentioned, WiDr resistant E+C+A was sensitive to BRAFi+crizotinib treatment. We found that exogenous hyper-expression of MET in the same WiDr E+C+A cells (called WiDr E+C+A hyper-MET^{ampl}, **Figure 25A**) could confer resistance to dual BRAF and MET inhibition and prevented drug combination induced cytotoxicity (**Figure 25B, D and E**).

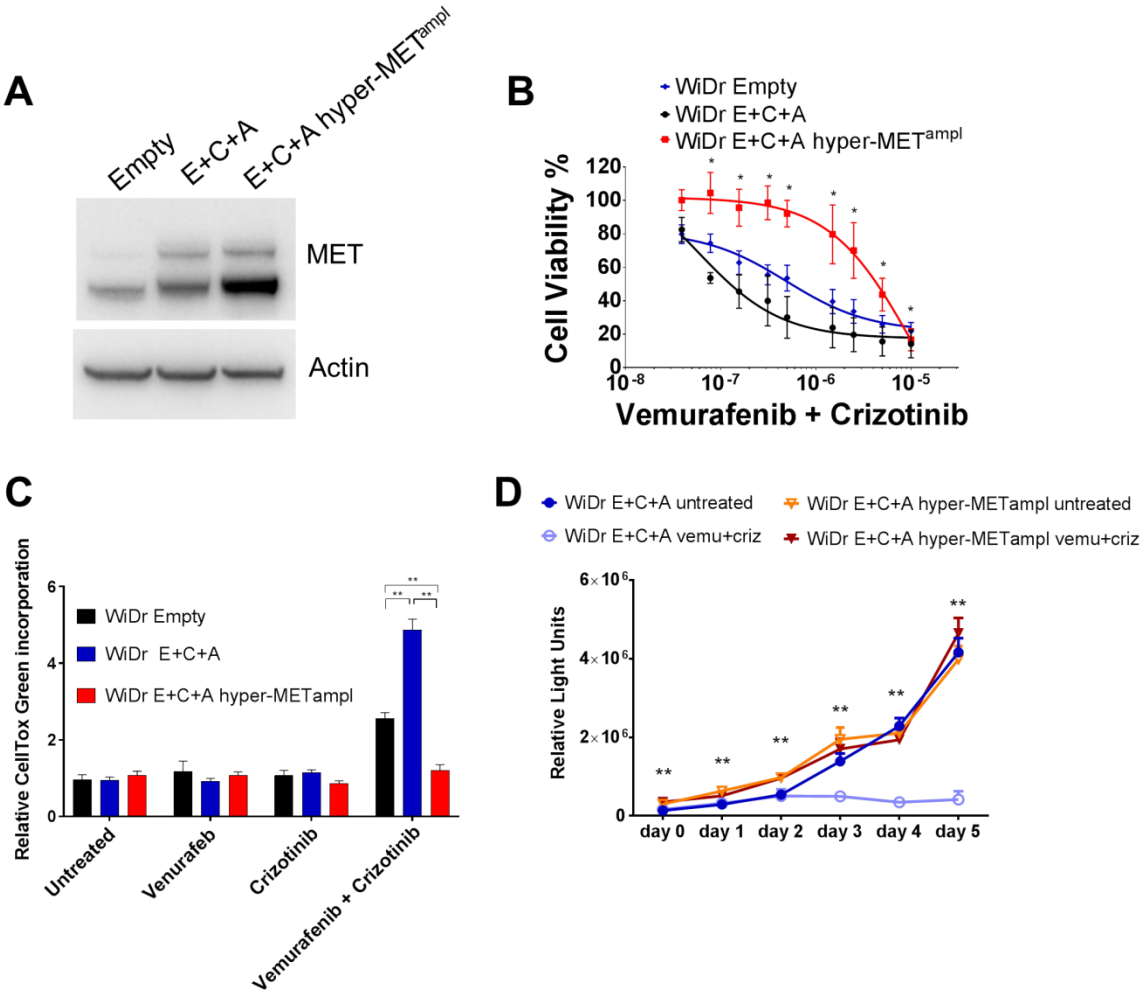

Figure 25. ***MET* gene hyper-amplification confers resistance to combined BRAF and MET inhibition in WiDr cells. (A)** *BRAF*^{V600E} mutant WiDr parental or its *MET* amplified derivative cell line (WiDr resistant E+C+A) were transduced with either control (empty) or MET–expressing lentiviral vector (called WiDr E+C+A hyper-MET^{ampl}), respectively. Protein extraction and Western blotting with total MET antibodies revealed a gradient of

MET protein expression among the three different cell lines. Actin is reported for normalization purposes. **(B)** Cell viability by ATP assay of WiDr empty, E+C+A and E+C+A hyper-MET^{ampl} cells after treatment for 72 hours with the indicated molar concentrations of vemurafenib in association with constant 0.2 μM crizotinib. **(C)** WiDr empty, E+C+A and E+C+A hyper-MET^{ampl} were treated for 72 hours with 1 μM vemurafenib and/or 0.2 μM crizotinib in mono or combinatorial therapies. 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. **(D)** Proliferation rate of WiDr E+C+A and E+C+A hyper-MET^{ampl} cells was measured by ATP assay. Cells were cultured from day 0 to day 5 in absence or presence of the following drug combination: 1 μM vemurafenib and 0.2 μM crizotinib. Data are expressed as relative light units. Results represent mean ± SD of 2 independent observations, each performed in duplicate or triplicate. Statistical differences in *BRAF* mutant cell viability or cytotoxicity between E+C+A and E+C+A hyper-MET^{ampl} cells was determined with the Mann-Whitney *U* test (**P* < .05, ***P* < .01). Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); E= Encorafenib (BRAFi).

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

Not all the acquired alterations that might occur upon resistance are druggable. Indeed, in our cell models resistance is frequently mediated by KRAS, which is currently considered not directly targetable (with the exception of the KRAS G12C mutant [51]). Therefore alternative therapeutic approaches are needed in these instances.

Based on our observations that all resistant cell models show persistent MAPK signaling activation (**Figure 8**), 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 [130]. Additionally, vertical triple blockade of EGFR+BRAF+MEK displayed the highest ability to suppress ERK phosphorylation in *BRAF* V600E CRC cells [131] and this

combination has been shown to induce response rates of up to 40% in *BRAF* mutant CRC patients [91]. Similarly, previously published reports have documented promising preclinical activity of ERK inhibition in BRAFi or MEKi resistant melanoma models [106, 132-134] as well as in MEKi+BRAFi or BRAFi+EGFRi resistant *BRAF* mutant CRC cells [97]. However, it has not yet been widely 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 (**Figure 26**).

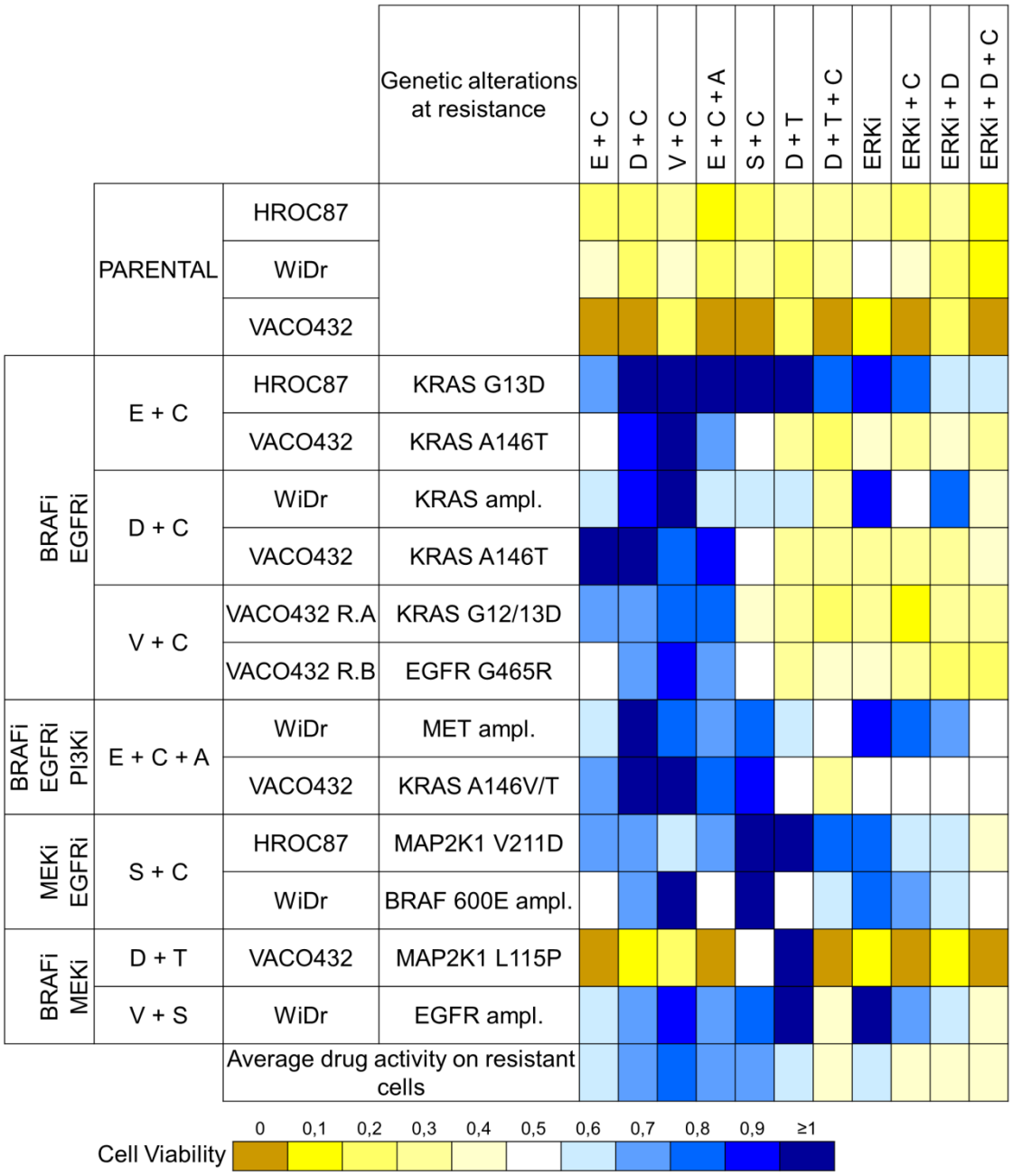

Figure 26. Viability of parental and resistant cell lines treated with different drug combinations targeting EGFR, BRAF, MEK, ERK and PI3K. Survival fraction of cell population was determined by ATP assay after 72h of treatment. Relative survival was normalized to untreated controls. Cell viability is represented as a yellow/blue. Drugs were used at concentrations reported in **Table 5**. Drugs are abbreviated as follows B=BYL719 (PI3K inhibitor); C= cetuximab (EGFRi); D = dabrafenib (BRAFi); E= encorafenib (LGX818, BRAFi); S= selumetinib (MEKi); T= trametinib (MEKi); V= vemurafenib (BRAFi); ERKi: ERK inhibitor. Results represent mean of at least two independent experiments.

As expected, parental cell lines were highly sensitive to all drug treatments (**Figure 26**). 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 [93, 95]. 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. 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 (**Figure 27A and 27B**).

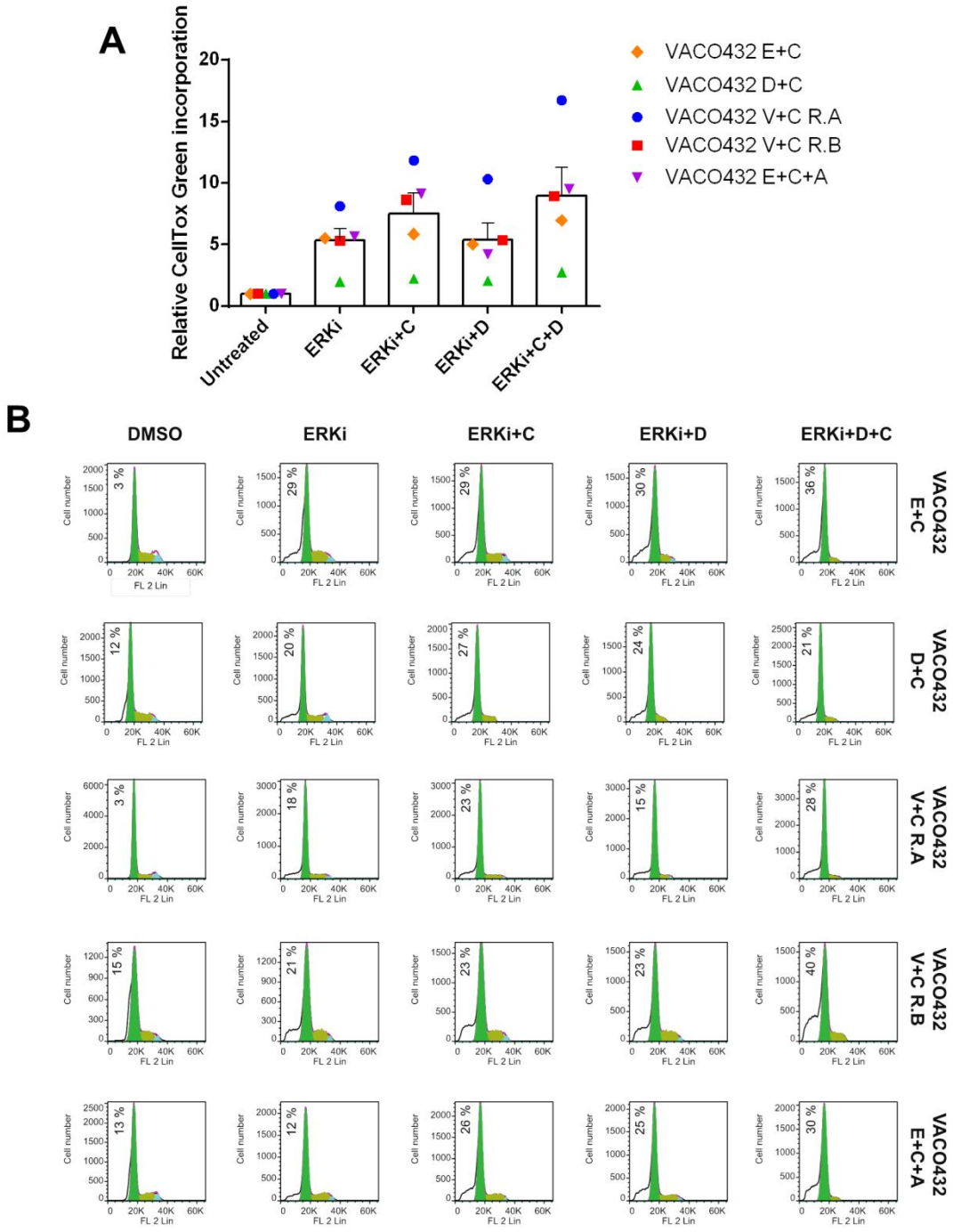

Figure 27. Cytotoxic effects of ERK inhibitor combinatorial therapy on VACO432 resistant derivatives. VACO432 resistant cell lines were cultured without drugs for four days and then treated for 72 hours with 185 nM ERKi, SCH772984, alone or in double or triple combination with 550 nM dabrafenib and/or 5 µg/ml cetuximab, after which

cytotoxicity and cell cycle analysis were assessed. **(A)** CellTox green cytotoxicity 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. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); V= Vemurafenib (BRAFi).

5.3.3. Carfilzomib treatment overcomes resistance to MAPK/PI3K combinatorial therapies.

It was previously reported that *BRAF* mutant CRC cells are particularly sensitive to proteasome inhibition. Vulnerability to proteasome inhibitors such as carfilzomib is dependent on persistent BRAF signaling, because BRAF V600E blockade by vemurafenib can reverse sensitivity to the proteasome inhibitor carfilzomib in *BRAF* mutant CRC cells [135]. Since the resistant models retained the BRAF V600E mutation, we hypothesized that these cells could maintain sensitivity to carfilzomib. We tested this hypothesis treating all resistant cell models with the proteasome inhibitor carfilzomib. We observed that all cell models resistant to MAPK pathway inhibition, after carfilzomib treatment, had higher amount of ubiquitinated proteins when compared to untreated cells; this is consistent with a similar effect observed in parental cells upon carfilzomib exposure (**Figure 28A**). Moreover, resistant cells maintained high sensitivity to carfilzomib in short-term viability assays, with the exception of VACO432 resistant to BRAF+EGFR+PI3K inhibitors (E+C+A) (**Figure 28B**). These results suggest that carfilzomib treatment could be a valid strategy to overcome resistance to MAPK/PI3K targeted

therapies. However, the presence of a non-responder cell model suggests that possible unknown genetic or non-genetic alterations could be acquired during the MAPK/PI3K pathway inhibition resistance process. Given the preliminary nature of these findings, further studies would be necessary to deeply define a biological link between BRAF and proteasome activity.

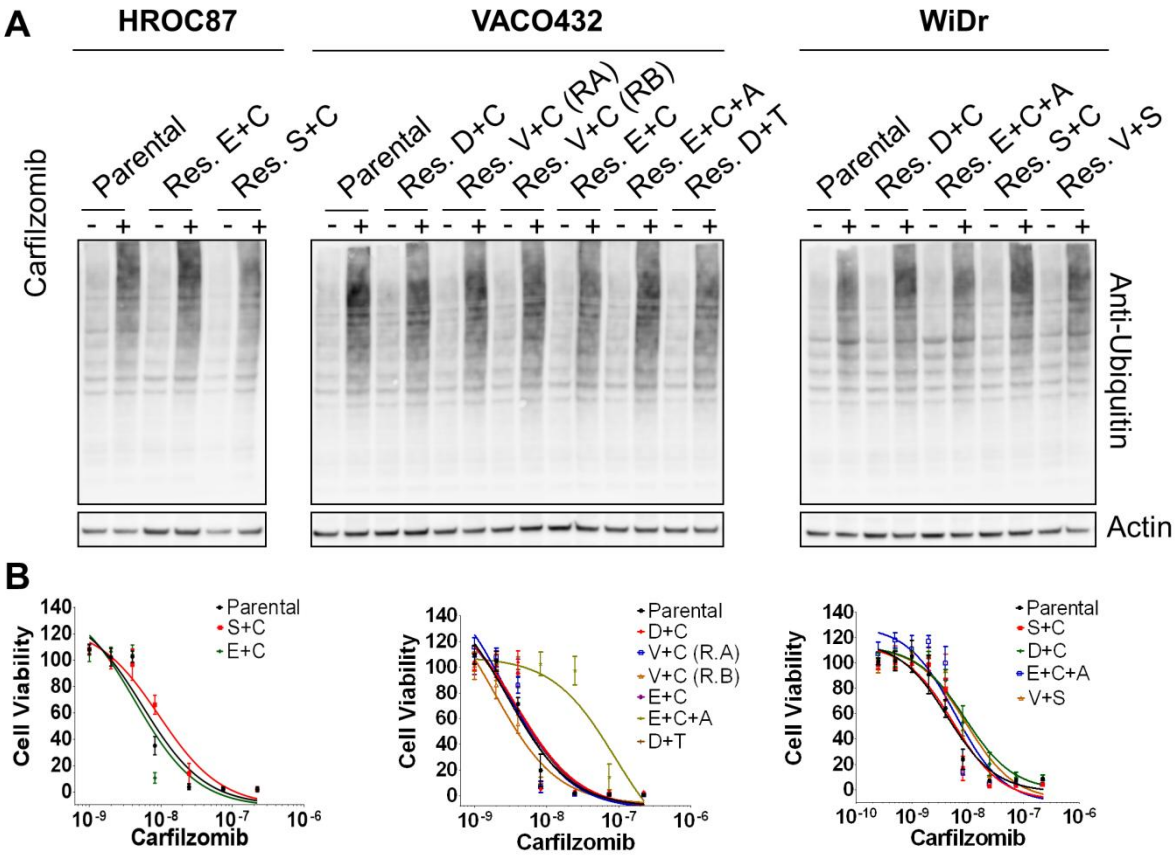

Figure 28. *BRAF* mutant CRC cell lines with acquired resistance to MAPK combinatorial therapies show sensitivity to the proteasome inhibitor carfilzomib. (a) Biochemical analysis revealed ubiquitinylated proteins accumulation after 6 hours treatment with 200 nM of carfilzomib. An antibody against actin was used as a loading control. **(b)** Effect on cell viability after 72h of carfilzomib treatment. Cell viability was estimated by determining ATP content and data are expressed as average ± s.d of two independent experiments. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); T= Trametinib (MEKi); V= Vemurafenib (BRAFi).

5.3.4. Drug discontinuation to reverse sensitivity and prolong treatment benefit

5.3.4.1. Drug discontinuation revealed addiction to MEKi and EGFRi inhibitors in BRAF V600E amplified-WiDr cells

Dependency of tumors on the therapeutic drugs to which they have acquired resistance is supported by observations from cultured cells [117, 136-139], animal models [136, 140] and patients [141-143]. Therefore an intermittent drug-schedule or drug discontinuation upon the emergence of resistance might represent possible approaches to overcome and/or delay the emergence of resistance, with potential applications in cancer treatment.

Based on this hypothesis, we investigated whether the molecular phenotype conferring selective advantage during drug exposure could be counter-selected in the absence of drug. At the time of writing this thesis, pilot experiments have been performed only in 4 WiDr resistant cell models (carrying either EGFR, KRAS, MET or BRAF V600E gene amplification) but we intend to pursue this approach in the future in a wider panel of resistant cell models.

To assess MAPK inhibition (MAPKi) dependency, we seeded 4 WiDr resistant cell lines in absence or presence of drugs combination to which they had become resistant. Our preliminary observations indicate that only the WiDr S+C cells, bearing the BRAF V600E amplification, revealed a rapid loss-of-fitness upon drug discontinuation (**Figure 29A**). Indeed, after 12 days of drug deprivation, cell survival assessed by clonogenic assay was impaired (**Figure 29A**) and apoptosis was evident, as indicated by an increase in PARP cleavage (**Figure 29B**).

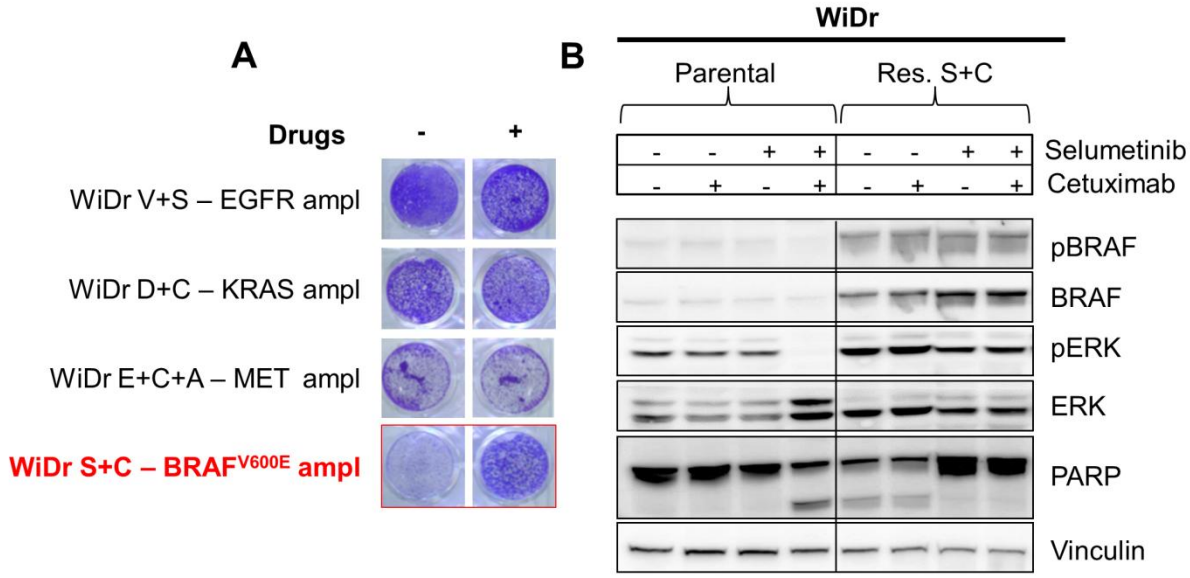

Figure 29. WiDr S+C shows addiction to drug treatment (A) WiDr resistant cells were seeded in 24-well plates (3000 cells for well). After 24 hours drugs, in the same combination for which every models acquired the resistance, were added. The drugs concentration used were: vemurafenib 2μM, dabrafenib 0.5μM, encorafenib, alpelisib and selumetinib 1μM, cetuximab 5μg/ml. For WiDr S+C, selumetinib was used at the concentration of 2μM. After 12 days cells were fixed with PAF 4% and coloured by crystal violet. **(B)** Western blot analysis of full-length PARP or cleaved PARP showed that combination of EGFR and MEK inhibitors and the absence of drug leads to apoptosis respectively in WiDr parental and resistant S+C cells. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); V= Vemurafenib (BRAFi).

5.3.4.2. Resistance mediated by MET or BRAF V600E in WiDr cells may be reversible upon drug withdrawal

To assess possible drug resistance reversion upon drug withdrawal, we generated cells called “release” by growing the 4 WiDr models in absence of drug pressure for 1 month (**Figure 30A**). Such “release” experiments can partly recapitulate what could happen at a molecular and functional level in the tumors of patients who became refractory to the treatment and stopped taking anticancer drugs due to

progression. Although the “release” cells carrying either EGFR or KRAS amplification remained resistant, the “release” cells with MET or BRAFV600E amplification regained drug sensitivity (**Figure 30B**).

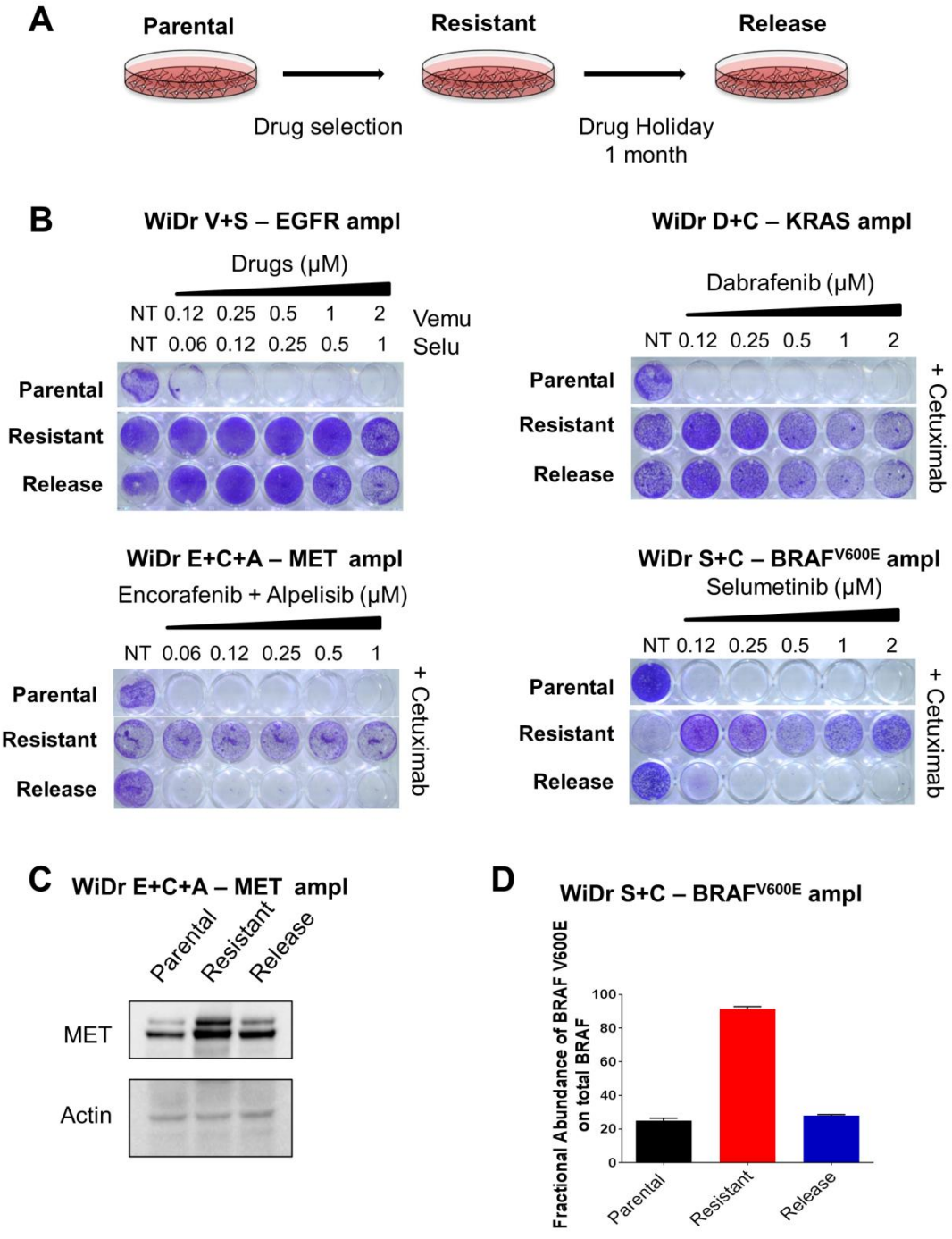

Figure 30. WiDr Res. E+C+A and S+C, after one month of drug withdrawal, regain sensitivity to the same drug combination to which they have become resistant. (A) Schematic representation of the release cells generation. Release indicates WiDr

resistant cells that were cultured absence of drugs for one month. **(B)** WiDr parental, resistant and release cells were seeded in 24-well plates (3000 cells for well) and treated after 24 hours. After 12 days cells were fixed with PAF 4% and colored by crystal violet. Results are representative of at least 2 independent observations. **(C)** Biochemical analysis of WiDr parental, resistant and release E+C+A. Actin was used as a loading control. **(D)** Digital PCR shows a dramatic reduction of the BRAF V600E mutant allele in WiDr Release cells compare to WiDr Res. S+C cells. Drugs are abbreviated as follow A=Alpelisib (PI3K inhibitor); C= Cetuximab (EGFRi); D = Dabrafenib (BRAFi); E= Encorafenib (BRAFi); S= Selumetinib (MEKi); V= Vemurafenib (BRAFi).

Changes in drug sensitivity paralleled variations in the molecular mechanism responsible for acquired drug resistance. WiDr E+C+A “release” cells had a MET protein reduction compared to the resistant cells maintained under drug pressure (**Figure 30C**). In WiDr S+C digital PCR for *BRAF* V600E revealed a reduction of the *BRAF* mutant allele fraction in “release” cells compared to those maintained under treatment (**Figure 30D**). As a consequence, WiDr “release” lost BRAF overexpression and displayed BRAF protein levels comparable to those of parental WiDr cells (**Figure 31**). Notably, combinatorial treatment with cetuximab and selumetinib was able to efficiently restore the abrogation of ERK phosphorylation in WiDr “release” cells (**Figure 31**). These results suggest that drug sensitivity can be restored in resistant cells by releasing pressure on the MAPK pathway.

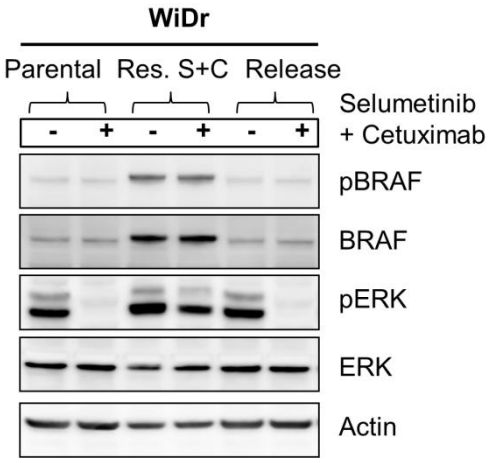

Figure 31. BRAF and ERK phosphorylated and total levels in WiDr Res.S+C cells and their release derivatives. Cells were treated with the combination of cetuximab (5 µg/ml) and selumetinib (5µM) for 5 hours prior to protein extraction. Actin was used as a loading control. Drugs are abbreviated as follow C= Cetuximab (EGFRi); S= Selumetinib (MEKi).

6. Discussion

Clinical trials assessing efficiency of therapies targeting EGFR/BRAF/MEK/PI3K or combining chemo- and target- therapies (such as irinotecan, vemurafenib and cetuximab) in *BRAF* mutant CRC have led to improvement in response rate and progression free survival [84, 86-89, 94]. However following an initial response, acquired resistance inevitably emerges after a few months of treatment [89, 94, 97]. In this thesis, I undertook a comprehensive effort to develop models of secondary resistance to a spectrum of seven clinically-relevant combinatorial therapies and to define the landscape of molecular mechanisms leading to acquired resistance in *BRAF* mutant CRC. While this work was being carried out, other studies have reported acquired mechanisms of drug resistance in *BRAF* mutant CRC patients [78, 94, 97, 110] corroborating our findings.

In the panel of resistant cell lines generated in this thesis, we identified several resistance mechanisms upon acquired resistance in *BRAF* mutant CRC which all converge to increased phosphorylation of ERK as consequence of MAPK signaling pathway reactivation. We identified *EGFR* gene amplification and an G465R ectodomain mutation in cells resistant to MEKi+EGFRi and BRAFi+EGFRi respectively. While the EGFR G465R mutation is known to disrupt antibody binding to the receptor conferring resistance to anti-EGFR monotherapy in *RAS*/*BRAF* wild-type CRC [104], it has not previously been implicated in *BRAF* mutant CRC. 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 not

only to combinatorial treatment involving EGFRi (BRAFi+EGFRi, MEKi+EGFRi) but also to the BRAFi+MEKi combination, which we hypothesized to be a consequence of an increased EGFR signaling flux. The fact that MAPK feedback reactivation during BRAFi monotherapy depends on EGFR expression [82], and its contribution in secondary acquired resistance to MAPK combinatorial inhibition, highlights the crucial role of EGFR in the biology of *BRAF* mutant CRC. However, we also observed that resistance could be driven by signals coming from RTKs other than EGFR, such as MET, that has previously been reported to be activated in *BRAF* mutant CRC cells refractory to BRAFi as well as BRAF wild-type tumors resistant to cetuximab or panitumumab monotherapy [114, 115]. In our study, *MET* amplification was identified as a mechanism of resistance to BRAFi+EGFRi+PI3Ki (E+C+A) and to BRAFi+EGFRi in a cell line and in a patient, respectively. These findings, in addition to our functional experiments, indicate that MET activation could be considered a resistant mechanism to several multi-drug based combinations.

KRAS mutations have a prominent role in the pathogenesis of CRC and mediate clinical resistance to anti-EGFR therapies. Analysis by Sanger sequencing identified *KRAS* and *BRAF* mutations in a mutually exclusive manner [13, 144, 145]. An explanation to these observations could be that concomitant oncogenic activation of KRAS and BRAF would result in activation of cell-cycle inhibitory proteins (e.g.: $P16^{CDKN2A}$/$P14^{ARF}$), leading to oncogenic stress and senescence and subsequent counter-selection during the tumorigenesis [146]. 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, suggesting an intratumoral heterogeneity in *BRAF* and *KRAS* mutational status [78]. We identified *KRAS* alterations in ctDNA of a *BRAF* mutant CRC patient and in a high proportion of our resistant cells. To remark the crucial role of KRAS in CRC, beside mutation, we and others have found wild-type *KRAS* amplification upon resistance to BRAFi+EGFRi in one of our cell model and in *BRAF* mutant CRC patients [97, 110]. Our data suggest that in the future monitoring *KRAS* status would be advisable in *BRAF* mutant CRC patients. This is highly clinically relevant since *KRAS* alterations under BRAFi treatment can not only induce resistance, but cause a paradoxical upregulation of MAPK signaling mediated by RAF dimerization and CRAF activation, promoting tumor growth with deleterious effects for patients [147, 148].

Downstream to KRAS, high level amplification of the *BRAF* 600E mutant allele was found in WiDr resistant to MEKi+EGFRi (S+C). This genetic alteration was recently reported in a *BRAF* mutant CRC patient with acquired resistance to BRAFi+EGFRi [97], in colorectal cancer cell lines with secondary resistance to the MEKi selumetinib [85, 111], as well as in melanoma patients and cells upon progression to the BRAFi vemurafenib [112]. Our data also shows that amplification of oncogenic *BRAF* 600E mutant impairs the therapeutic efficacy of MEKi+EGFRi combination in CRC cells. Of note, during the generation of resistant cell models we avoided any biased selection mediated by cell passaging. Indeed drug pressure might transiently induce a slow cell cycle phenotype [149] which could be lost during passaging and in turn might select cells with higher

proliferation rate. Additionally, during the selection period a dramatic bottleneck effect (i.e. massive cell death) was observed in every case. After some months, a few clones emerged simultaneously within the same plate preserving a relative molecular heterogeneity. Indeed polyclonality of drug selected cells is evidenced by the emergence of multiple resistant mechanisms within the VACO432 refractory to BRAFi+EGFRi+PI3Ki. Although chronic drug treatment is a valuable approach to uncover resistant mechanisms, complementarity strategies can be envisioned. For example, functional genetic screens by shRNA or CRISP libraries could be a valid tool to identify genes whose suppression could restore drug sensitivity [82, 150]. Additionally genome-wide chemical mutagenesis screens could allow the detection of both known and novel drug resistance mutations [151].

Cancer is an evolutionary process during which selective forces act on tumor cells and results in the propagation of tumor clones with a relative fitness advantage that drives the clonal progression of the tumor. According to the recent “Big Bang model” of cancer evolution, private alterations that give rise to intratumor heterogeneity might be generated early after the transition to advanced tumor and will be ‘pervasive’ in the final neoplasm [152]. Therefore, tumor heterogeneity might be already present in an early tumor stage but, under selective drug pressure, specific clones might have a better fitness compared to others with consequent tumor relapse. Similarly, in our resistant models, double *BRAF*/*KRAS* mutant cells might be already present in the parental cell line, but could have an advantage in proliferation and survival only under treatment with drugs quenching activation of the MAPK pathway. Additionally, single clone analyses of these cell

populations revealed a heterogeneous pattern of both *BRAF* and *KRAS* mutations. In most cases, *BRAF* V600E co-occurred with *KRAS* mutations. However, in some cases only BRAF was observed, thus suggesting additional mechanisms of resistance yet unidentified. Intriguingly, in one of the two analyzed models, a small subset of clones solely displayed KRAS mutations without BRAF V600E founder mutation, which we hypothesized to have been lost under adaptive or selective process.

Based on the hypothesis that *KRAS* mutations can co-occur with BRAF V600E prior to treatment, it is highly probable that also other acquired drug resistance mechanisms, such as *MET* or *BRAF* V600E amplification and *MAP2K1* mutation, may also pre-exist in rare tumor subclones. In support to this hypothesis, we identified the presence of a subpopulation of *MET* amplified cells in the primary colon tumor sample, taken from patient #2 before treatment with any targeted therapy. Consequently, we hypothesized that combining upfront EGFR, MET and BRAF inhibition might have led to a more durable response by preventing the clonal expansion of the pre-existing resistant sub-clones.

All these observations suggest that alterations conferring resistance could develop at an early stage of *BRAF* mutant colorectal tumorigenesis, thus laying the seeds for the eventual emergence of acquired resistance. Identification of pre-existing molecular alteration prior to treatment might have deep clinical implications for the selection of the best therapy, avoiding the overuse of inefficient drugs.

However, this approach shows limitation in case of *de novo* mutations which occurred as consequence of genomic instability. Such alterations would normally not confer a fitness advantage in absence of treatment. However, during

pharmacological inhibition such mutations (e.g EGFR G465R, MEK V211D and L115P) prevent the drug-protein interaction limiting therapeutic efficacy.

Some of the molecular alterations occurred in cell lines were also found in patient tumor samples taken at disease progression to BRAFi combinatorial treatment. Previous studies indicate that therapeutic resistance in solid tumors could be driven by multiple different subclones, each harboring distinct resistance-causative mutations usually converging towards similar phenotypic outcomes [119, 123, 153]. In line with this, we identified in Patient #1 two distinct mechanisms conferring resistance, *KRAS* mutation and *BRAF* V600E amplification; both of which lead to reactivation of the MAPK signaling upon BRAFi combinations. In Patient #2 upon progression with BRAFi+EGFRi treatment, resistance was driven by *MET* amplification. Detection in clinical samples of the resistance mechanisms identified *in vitro* (including *KRAS* amplification, *BRAF* V600E and *MET* amplification), demonstrates the reliability of such approach and suggest that further resistance mechanisms, identified in our resistant cell panel, may be found in other patients with *BRAF*-mutant CRC.

The first part of the work of this thesis was aimed at unveiling the molecular alterations upon drug resistance in a panel of resistant cell models and at identifying their molecular clinical relevance. However, elucidating the molecular mechanisms underlying secondary resistance may help in designing further lines of therapy strategies to reverse sensitivity and prolong therapeutic efficacy [118,

153, 154]. For this purpose, in the second part the work, we have explored four different approaches (**Figure 32**).

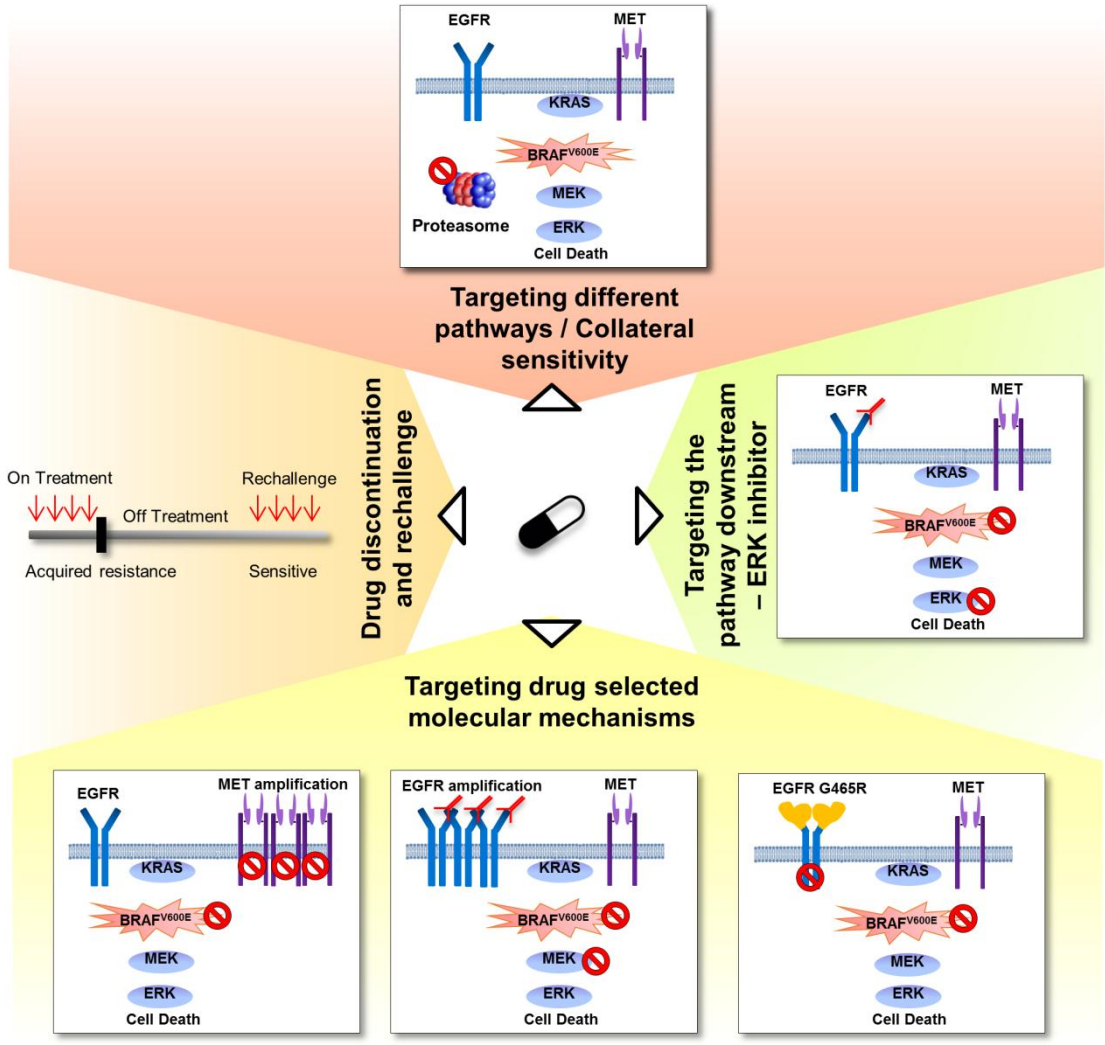

Figure 32: Strategies to overcome acquired resistance to BRAF inhibitor based combinations. In this thesis I provided data on four possibly therapeutic approaches to impair viability of *BRAF* mutant CRC after they had become resistant to target therapy combinations. In some instances, the identification of the molecular mechanism of resistance suggested subsequent and appropriate treatments to regain tumor sensitivity. Additionally, drug discontinuation might impair survival of clones having a good fitness in presence of drugs (the resistant one) but not in their absence; therefore a subsequent drug rechallenge could be a valid approach to overcome acquired resistance. However both approaches are not always affordable, due to undruggable targets or highly aggressive disease. Since all our resistant cell models acquired resistance through MAPK signaling reactivation, its robust inhibition by targeting ERK, BRAF and EGFR revealed a

high efficacy in most of the resistant cell models. Finally, proteasome inhibitors such as bortezomib or carfilzomib that were previously reported to selectively kill the *BRAF* mutant CRC cells (acting on different pathways), demonstrated a potent *in vitro* activity since all but one resistant cell models were sensitive to carfilzomib. Therefore carfilzomib therapy might overcome to BRAF inhibitor combinations resistance independently of the causative molecular mechanism.

Under physiological conditions, signaling through the MAPK pathway is regulated by ERK-mediated feedback inhibition [155]. Through direct phosphorylation events and by increasing the expression of pathway inhibitors, ERK acts to limit RAS-GTP levels, which in turn modulates the amplitude and duration of the MAPK signaling pathway. In *RAS* mutant cells this fine signaling regulation is impaired. In our study, *RAS* alterations were found in half of the resistant models. Although *KRAS* mutant tumors remain largely intractable with target inhibitors, therapeutic strategies relying on the use of pan-RAF inhibitors (drugs that are capable of inhibiting monomeric BRAF V600E as well as wild-type and RAF dimers) might be promising. In fact, melanoma tumors that acquired *RAS* mutations upon BRAFi treatment have been shown to rely on CRAF activation to trigger MAPK signaling pathways [156]. It has been reported that ectopic expression of KRAS G12V in *BRAF* mutant melanoma cells confer resistance to BRAF or MEK inhibition but not to pan-RAF and MEKi combination [157]. Although we have not yet tested pan-RAFi in our resistant models, future studies could determine the benefit of this new class of drugs in *BRAF*/*KRAS* double mutant CRC tumors. If our hypothesis is supported by future experimental data, this therapeutic approach might covering a large proportion of *BRAF* mutant CRC patients that acquired *KRAS* alterations during previous targeted therapies.

In three *BRAF* mutant resistant cell models, drug treatments have selected secondary molecular alterations that could be directly targeted by specific drugs (**Figure 32**). In all these cases alterations involved RTKs, such as *EGFR* and *MET*.

The EGFR mutation G465R affects the epitope recognized by the monoclonal antibodies cetuximab and panitumumab. However, it does not impact binding to the intracellular domain of EGFR by tyrosine kinase inhibitors, such as gefitinib or erlotinib. Consequently, combination of gefitinib with the BRAFi, vemurafenib, restores sensitivity in the *BRAF*/*EGFR* double mutant cells. In another case, resistance to BRAFi+MEKi was mediated by *EGFR* wild-type amplification, which in turn could be overcome introducing the anti-EGFR cetuximab to the treatment.

Notably, we identified *MET* amplification in a resistant cell line and in a patient (previously called patient #2) upon resistance to BRAFi+EGFRi+PI3K and BRAFi+EGFRi respectively. Since the ALK-MET inhibitor crizotinib has shown clinical activity in *MET* amplified cancers and safety when combined with vemurafenib [128, 158, 159], we tested this drug combination on WiDr resistant *MET* amplified cells. Albeit crizotinib alone was ineffective to overcome resistance, its combination with vemurafenib could impair the proliferation of MET-overexpressing cells. Based on this *in vitro* analysis, the patient was treated with BRAFi+METi and achieved a partial response. A recent work correlated *MET* amplification with a higher prevalence of $BRAF^{V600E}$ tumors [160], which may increase the impact of this combination. Unfortunately, after four months of treatment, the tumor relapsed by a further amplification of *MET*, rendering the

inhibitory effect of crizotinib insufficient. Since the patient died about two months following disease progression, we hypothesized that *MET* hyper-amplification coupled with $BRAF^{V600E}$ mutation not only conferred resistance to BRAFi+METi, but might be also responsible for the particularly aggressive disease behavior. Possibly, the extremely high level of MET expression shown by the resistant tumor upon resistance to BRAFi+METi could have been exploited as a Achilles' heel to rationally test as a further salvage line novel anti-MET antibody-drug conjugates, such as ABBV-399, which showed promising activity in *MET* amplified cancer cells and non-small cell lung cancer patients [161, 162].

In tumors, recurrent alterations conferring resistance to targeted therapies appear to converge upon a relatively constrained range of mutations and aberrations, often in multiple parallel clones within the same malignancy [163]. Although our findings support MAPK pathway reactivation as a key event in the development of acquired resistance to BRAF inhibitor combinations, it is striking that several different molecular mechanisms of resistance were identified among different cell models. The diversity of potential molecular alterations leading to acquired resistance suggests that therapeutic approaches targeting individual resistance alterations may be difficult to implement as a generalizable strategy to overcome resistance. However, the common thread among these diverse molecular alterations is that they converge on MAPK pathway reactivation as a mechanism for promoting resistance. Overall by systematically comparing multiple drug combinations 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.

The triple combination of BRAFi+EGFRi+MEKi is currently being evaluated in clinical trials, and preliminary results suggest improved response rate compared to the individual doublet combinations [164], 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. Additionally, as part of a Phase I study of the ERKi GDC-0994 monotherapy (NCT01875705), 13 BRAF V600E CRC patients were treated, of which 2 had a confirmed partial response [165]. Consistent with our findings, previously published reports have documented promising preclinical activity of ERK inhibition in BRAFi or MEKi resistant cell line models [106, 132, 133] and in MEKi+BRAFi and BRAFi+EGFRi resistant *BRAF* mutant CRC cells [97], supporting ERKi as key potential components of future clinical trial strategies for this disease. Although it cannot be ruled out that additional pathways might have a role in resistance of *BRAF* mutant CRC, our data suggest that strong inhibition of MAPK signaling is crucial. Vertical combination therapy designed to more effectively block reactivation of MAPK signaling, possibly through the incorporation of ERK inhibitors, has the potential for improved clinical benefit in patients with this aggressive CRC subtype. This hypothesis is supported by a recent work suggesting that combined RAFi, MEKi and ERKi treatment durably inhibited signaling and proliferation in melanoma cells that were induced to express intermediate or high levels of BRAF V600E [166]. Moreover, this triple-drug

combination revealed a lower toxicity profile than the MEKi+ERKi combination in mice. Indeed, the addition of the BRAFi reduced the inhibitory effect of the other drugs in normal tissue, suggesting that the ability of the BRAFi to paradoxically activate ERK in *BRAF* wild-type cells ameliorates the toxicity of therapy [166].

Another common feature is that the original *BRAF* V600E mutation is maintained in all resistant cell populations. This might have important clinical implication in the future because *BRAF* mutant CRC cells are particularly sensitive to proteasome inhibition. Vulnerability to proteasome inhibitors is dependent on persistent BRAF signaling, because BRAF V600E blockade by vemurafenib was shown to reverse sensitivity to carfilzomib in *BRAF*-mutant CRC cells [135]. Our *in vitro* analyses suggested that the proteasome inhibitor carfilzomib could be a valid approach to overcome acquired resistance to target therapies. Indeed all but one of our resistant cell models were highly sensitive to carfilzomib treatment. It has previously been reported that BRAF V600E mutation can induce a chronic endoplasmic reticulum (ER) stress status in melanoma cells [167]. Therefore, we hypothesize that chronic ER stress could also be characterize *BRAF* mutant CRC cells; in this scenario ER stress could be further induced by proteasome inhibitors to levels incompatible with cell survival by activating autophagic and apoptotic pathways in cancer cells. The mechanisms by which proteasome inhibitors could trigger cell death in *BRAF* mutant CRC cells remain to be elucidated, but the activity of carfilzomib in both parental and resistant cells could be of great interest for the design of future strategies.

Upon the acquisition of resistance to targeted therapies, an intriguing phenomenon called drug addiction sometimes develops [140]. This occurs when cells that are resistant to drug treatment become dependent on the drug for ongoing proliferation. In xenograft models of malignant melanomas treated with vemurafenib, regression of resistant tumors is observed upon withdrawal of the drug [140]. Recent data revealed that drug addicted melanoma cells upon drug withdrawal had a decrease expression of genes associated with cell proliferation, an increased expression of genes associated with metastasis and supra-basal ERK hyper-activation [136, 137]. In three out of four cell models in which drug withdrawal was carried out, we observed no changes on cell proliferation rate. Nevertheless, in a WiDr drug resistant derivative that had developed *BRAF* V600E mutant allele amplification, cell viability and survival were severely impaired after cessation of drug administration. The reason by which only the BRAF V600E amplified cells showed drug addiction is unclear. In melanoma, similarly to our findings in CRC, BRAF V600E amplification drives acquired resistance to MAPK inhibitors via ERK reactivation and was associated with BRAFi [140] or BRAFi+MEKi [138] addiction. Functionally, BRAF V600E overexpression in melanoma cells, upon BRAFi+MEKi withdrawal, enhanced the cell death which depended on a robust phosphorylated ERK rebound. Consequently, a low dose of ERKi was sufficient to block this rebound and reversed drug addiction [137]. Based on these data we can hypothesize that BRAF V600E amplification could peculiarly induce cell death upon drug discontinuation by increasing the MAPK signal flux over the physiological levels. In support to this hypothesis, we indeed showed an increase in ERK phosphorylated protein in BRAF V600E amplified cells

upon drug discontinuation. Nevertheless, further studies in CRC are warranted to elucidate the role of ERK in drug addiction and understanding how and whether drug addiction can be exploited from a therapeutic perspective.

Drug discontinuation and rechallenge has been proposed as a possible approach to regain tumor sensitivity [168]. To explore this possibility in the setting of *BRAF* mutant CRC, we imposed a drug holiday for one month to four WiDr resistant models characterized by *EGFR*, *MET*, *KRAS*, *BRAF* V600E amplification. Of these models only the BRAF V600E amplified cells were drug addicted. Over time, however, clones that were resistant to drug withdrawal spontaneously arose from cell pools. Finally, drugs rechallenge on the four models revealed that the "release" cells coming from the original *MET* or *BRAF* V600E amplified models had regained responsiveness to MAPK pathway inhibition and lost overexpression of MET or BRAF proteins, respectively. Probably this occurred by a counterselection of clones bearing *MET* or *BRAF* amplification, since these amplified cells could have a good fitness in presence of drugs but not in their absence. All this is consistent with the fact that clinical rechallenges with drugs inhibiting the MAPK pathway in melanoma patients whose cancer has progressed on treatment are sometimes successful [168, 169]. Albeit further studies are necessary to fully evaluate the effects of withdrawing treatment, this preliminary data might suggest that rechallenge might be successfully in tumor control of *BRAF* mutant CRC patients that progressed to drugs targeting the MAPK pathway.

Giving target therapies by intermittent dose schedules might be a potential method for reduce toxicity and delay the emergence of acquired resistance [166]. In fact epigenetic changes might drive tolerance under treatment selective pressure [170]. It is therefore possible that a 'drug holiday' could allow reversion to a previous epigenetic profile or could facilitate re-emersion of sensitive clones. In line with this observation, a phase II trial (NCT01894672) is currently testing the safety and efficacy of an intermittent regimen with BRAF inhibitors, in which encorafenib will be administered on a 2-weeks-on/2-weeks-off schedule. During target treatments sensitive melanoma cells might enter in a state of non-mutational tolerance due to a MAPK/PI3K pathway rewiring [171]. Adaptation to target therapy is a temporal process where the initial stage is dependent on the loss of activity of ERK following by epigenetic changes, which in turn increase survival signaling and metabolic adaptation [170]. Since drug tolerance may ensure persistent cell survival and a permissive state for further or enhanced acquisition of resistance, affecting viability of these pre-resistant cells might be a valid strategy to prolong drug efficacy [149]. A possible therapeutic approach may involve modulating the epigenome by the use of BET bromodomain inhibitors. Indeed, the bromodomain inhibitor JQ1 has previously been reported to synergize with BRAFi in melanoma and BRAF mutant CRC cells [149, 172].

This thesis is only limited to the exploration of cancer cell autonomous mechanisms of resistance, which warrant further investigations. Indeed, in the future, studying *in vivo* models would be essential to investigate non-cell autonomous (such as tumor microenvironment-mediated) mechanisms of drug

resistance in *BRAF* mutant CRC. Moreover, non-genetic mechanisms were not explored here. A melanoma study recently demonstrated how the BRAFi, vemurafenib, paradoxically activates the MAPK pathway in BRAF wild-type cells (such as cancer associated fibroblast) mediating the remodeling of the extracellular tumor matrix, contributing to drug adaptation and cell survival. However, my study did not consider the involvement of such mechanism [171]. Therefore, studies translating this hypothesis in the CRC setting under BRAFi based combination are warranted to improve the knowledge on the landscape of *BRAF* mutant resistance to targeted therapy combinations.

Additionally, in the future we plan to assess whether therapeutic strategies based on alternating cycles of chemotherapy (irinotecan or carfilzomib), BRAF inhibitor combinations, epigenetic drugs and drug holiday periods might be a valid strategy to delay the emergence of drug resistance in *BRAF* mutant CRC cells.

Concluding, we characterized the molecular mechanisms of resistance to BRAF inhibitor based combinations. We also provided promising strategies to overcome acquired resistance and, possibly, prolong the survival of the difficult-to-treat metastatic patients with BRAF mutant CRC. We markedly improved our biological understanding of *BRAF* mutant CRC and proposed new addition to the armamentarium against this type of tumor using novel antitumor targeted agents based on rational combination therapies. Although drug combinations improved antitumor efficacy, they increased the monthly cost of these treatments which might limit, in the future, their clinical application depending on the coverage by

National Health Care systems. However, the ultimate key question remains the benefit duration of such therapeutic approaches until acquisition of subsequent refractoriness.

7. References

1. Mortality, G.B.D. and C. Causes of Death, *Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013.* Lancet, 2015. **385**(9963): p. 117-71.
2. Kuipers, E.J., et al., *Colorectal cancer.* Nat Rev Dis Primers, 2015. **1**: p. 15065.
3. Vogelstein, B., et al., *Genetic alterations during colorectal-tumor development.* N Engl J Med, 1988. **319**(9): p. 525-32.
4. Cancer Genome Atlas, N., *Comprehensive molecular characterization of human colon and rectal cancer.* Nature, 2012. **487**(7407): p. 330-7.
5. Noffsinger, A.E., *Serrated polyps and colorectal cancer: new pathway to malignancy.* Annu Rev Pathol, 2009. **4**: p. 343-64.
6. Bettington, M., et al., *The serrated pathway to colorectal carcinoma: current concepts and challenges.* Histopathology, 2013. **62**(3): p. 367-86.
7. Jass, J.R., *Hyperplastic polyps and colorectal cancer: is there a link?* Clin Gastroenterol Hepatol, 2004. **2**(1): p. 1-8.
8. van Geel, R.M., et al., *Treatment Individualization in Colorectal Cancer.* Curr Colorectal Cancer Rep, 2015. **11**(6): p. 335-344.
9. Bogaert, J. and H. Prenen, *Molecular genetics of colorectal cancer.* Ann Gastroenterol, 2014. **27**(1): p. 9-14.
10. Vilar, E. and J. Tabernero, *Molecular dissection of microsatellite instable colorectal cancer.* Cancer Discov, 2013. **3**(5): p. 502-11.
11. Kohonen-Corish, M.R., et al., *Promoter methylation of the mutated in colorectal cancer gene is a frequent early event in colorectal cancer.* Oncogene, 2007. **26**(30): p. 4435-41.
12. Weisenberger, D.J., et al., *CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer.* Nat Genet, 2006. **38**(7): p. 787-93.
13. Tie, J., et al., *Optimizing targeted therapeutic development: analysis of a colorectal cancer patient population with the BRAF(V600E) mutation.* Int J Cancer, 2011. **128**(9): p. 2075-84.
14. Rad, R., et al., *A genetic progression model of Braf(V600E)-induced intestinal tumorigenesis reveals targets for therapeutic intervention.* Cancer Cell, 2013. **24**(1): p. 15-29.
15. Davies, R.J., R. Miller, and N. Coleman, *Colorectal cancer screening: prospects for molecular stool analysis.* Nat Rev Cancer, 2005. **5**(3): p. 199-209.
16. Seshagiri, S., et al., *Recurrent R-spondin fusions in colon cancer.* Nature, 2012. **488**(7413): p. 660-4.
17. Dienstmann, R., et al., *Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer.* Nat Rev Cancer, 2017. **17**(2): p. 79-92.
18. Giannakis, M., et al., *RNF43 is frequently mutated in colorectal and endometrial cancers.* Nat Genet, 2014. **46**(12): p. 1264-6.
19. Loupakis, F., et al., *Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer.* N Engl J Med, 2014. **371**(17): p. 1609-18.

20. Matos, I., et al., *Emerging tyrosine kinase inhibitors for the treatment of metastatic colorectal cancer.* Expert Opin Emerg Drugs, 2016. **21**(3): p. 267-82.
21. Sanz-Garcia, E., et al., *Current and advancing treatments for metastatic colorectal cancer.* Expert Opin Biol Ther, 2016. **16**(1): p. 93-110.
22. Tabernero, J., et al., *Ramucirumab versus placebo in combination with second-line FOLFIRI in patients with metastatic colorectal carcinoma that progressed during or after first-line therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine (RAISE): a randomised, double-blind, multicentre, phase 3 study.* Lancet Oncol, 2015. **16**(5): p. 499-508.
23. Amado, R.G., et al., *Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer.* J Clin Oncol, 2008. **26**(10): p. 1626-34.
24. Van Cutsem, E., et al., *Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer.* N Engl J Med, 2009. **360**(14): p. 1408-17.
25. Lievre, A., et al., *KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab.* J Clin Oncol, 2008. **26**(3): p. 374-9.
26. Marshall, C.J., *Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation.* Cell, 1995. **80**(2): p. 179-85.
27. Gray-Schopfer, V., C. Wellbrock, and R. Marais, *Melanoma biology and new targeted therapy.* Nature, 2007. **445**(7130): p. 851-7.
28. McKay, M.M. and D.K. Morrison, *Integrating signals from RTKs to ERK/MAPK.* Oncogene, 2007. **26**(22): p. 3113-21.
29. Gureasko, J., et al., *Membrane-dependent signal integration by the Ras activator Son of sevenless.* Nat Struct Mol Biol, 2008. **15**(5): p. 452-61.
30. Lavoie, H. and M. Therrien, *Regulation of RAF protein kinases in ERK signalling.* Nat Rev Mol Cell Biol, 2015. **16**(5): p. 281-98.
31. Freeman, A.K., D.A. Ritt, and D.K. Morrison, *Effects of Raf dimerization and its inhibition on normal and disease-associated Raf signaling.* Mol Cell, 2013. **49**(4): p. 751-8.
32. Hu, J., et al., *Allosteric activation of functionally asymmetric RAF kinase dimers.* Cell, 2013. **154**(5): p. 1036-46.
33. Weber, C.K., et al., *Active Ras induces heterodimerization of cRaf and BRaf.* Cancer Res, 2001. **61**(9): p. 3595-8.
34. Garnett, M.J., et al., *Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization.* Mol Cell, 2005. **20**(6): p. 963-9.
35. Rushworth, L.K., et al., *Regulation and role of Raf-1/B-Raf heterodimerization.* Mol Cell Biol, 2006. **26**(6): p. 2262-72.
36. Dhillon, A.S., et al., *MAP kinase signalling pathways in cancer.* Oncogene, 2007. **26**(22): p. 3279-90.
37. Avraham, R. and Y. Yarden, *Feedback regulation of EGFR signalling: decision making by early and delayed loops.* Nat Rev Mol Cell Biol, 2011. **12**(2): p. 104-17.
38. Dong, C., et al., *SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways.* J Biol Chem, 1996. **271**(11): p. 6328-32.
39. Dougherty, M.K., et al., *Regulation of Raf-1 by direct feedback phosphorylation.* Mol Cell, 2005. **17**(2): p. 215-24.

40. Pratilas, C.A., et al., *(V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway.* Proc Natl Acad Sci U S A, 2009. **106**(11): p. 4519-24.
41. Kolch, W., *Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions.* Biochem J, 2000. **351 Pt 2**: p. 289-305.
42. Lake, D., S.A. Correa, and J. Muller, *Negative feedback regulation of the ERK1/2 MAPK pathway.* Cell Mol Life Sci, 2016. **73**(23): p. 4397-4413.
43. Wellbrock, C., M. Karasarides, and R. Marais, *The RAF proteins take centre stage.* Nat Rev Mol Cell Biol, 2004. **5**(11): p. 875-85.
44. Michaloglou, C., et al., *BRAF(E600) in benign and malignant human tumours.* Oncogene, 2008. **27**(7): p. 877-95.
45. Davies, H., et al., *Mutations of the BRAF gene in human cancer.* Nature, 2002. **417**(6892): p. 949-54.
46. Tol, J., I.D. Nagtegaal, and C.J. Punt, *BRAF mutation in metastatic colorectal cancer.* N Engl J Med, 2009. **361**(1): p. 98-9.
47. Di Nicolantonio, F., et al., *Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer.* J Clin Oncol, 2008. **26**(35): p. 5705-12.
48. Roth, A.D., et al., *Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial.* J Clin Oncol, 2010. **28**(3): p. 466-74.
49. Giannakis, M., et al., *Genomic Correlates of Immune-Cell Infiltrates in Colorectal Carcinoma.* Cell Rep, 2016. **17**(4): p. 1206.
50. Clarke, C.N. and E.S. Kopetz, *BRAF mutant colorectal cancer as a distinct subset of colorectal cancer: clinical characteristics, clinical behavior, and response to targeted therapies.* J Gastrointest Oncol, 2015. **6**(6): p. 660-7.
51. Lito, P., et al., *Allele-specific inhibitors inactivate mutant KRAS G12C by a trapping mechanism.* Science, 2016. **351**(6273): p. 604-8.
52. Sebolt-Leopold, J.S. and R. Herrera, *Targeting the mitogen-activated protein kinase cascade to treat cancer.* Nat Rev Cancer, 2004. **4**(12): p. 937-47.
53. Maughan, T.S., et al., *Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial.* Lancet, 2011. **377**(9783): p. 2103-14.
54. Samowitz, W.S., et al., *Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers.* Cancer Res, 2005. **65**(14): p. 6063-9.
55. Gao, J., et al., *Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal.* Sci Signal, 2013. **6**(269): p. pl1.
56. Cerami, E., et al., *The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data.* Cancer Discov, 2012. **2**(5): p. 401-4.
57. Tran, B., et al., *Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer.* Cancer, 2011. **117**(20): p. 4623-32.
58. Ogino, S., et al., *Predictive and prognostic roles of BRAF mutation in stage III colon cancer: results from intergroup trial CALGB 89803.* Clin Cancer Res, 2012. **18**(3): p. 890-900.
59. Taieb, J., et al., *Prognostic Value of BRAF and KRAS Mutations in MSI and MSS Stage III Colon Cancer.* J Natl Cancer Inst, 2017. **109**(5).

60. Sinicrope, F.A., et al., *Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes.* Gastroenterology, 2015. **148**(1): p. 88-99.
61. French, A.J., et al., *Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer.* Clin Cancer Res, 2008. **14**(11): p. 3408-15.
62. Lochhead, P., et al., *Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication.* J Natl Cancer Inst, 2013. **105**(15): p. 1151-6.
63. Richman, S.D., et al., *KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: results from the MRC FOCUS trial.* J Clin Oncol, 2009. **27**(35): p. 5931-7.
64. Smith, C.G., et al., *Somatic profiling of the epidermal growth factor receptor pathway in tumors from patients with advanced colorectal cancer treated with chemotherapy +/- cetuximab.* Clin Cancer Res, 2013. **19**(15): p. 4104-13.
65. Shen, Y., et al., *Effectors of epidermal growth factor receptor pathway: the genetic profiling ofKRAS, BRAF, PIK3CA, NRAS mutations in colorectal cancer characteristics and personalized medicine.* PLoS One, 2013. **8**(12): p. e81628.
66. Ciardiello, F., et al., *Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by next-generation sequencing: findings from the CAPRI-GOIM trial.* Ann Oncol, 2014. **25**(9): p. 1756-61.
67. Cremolini, C., et al., *BRAF codons 594 and 596 mutations identify a new molecular subtype of metastatic colorectal cancer at favorable prognosis.* Ann Oncol, 2015. **26**(10): p. 2092-7.
68. Jones, J.C., et al., *Non-V600 BRAF Mutations Define a Clinically Distinct Molecular Subtype of Metastatic Colorectal Cancer.* J Clin Oncol, 2017. **35**(23): p. 2624-2630.
69. Morris, V., et al., *Progression-free survival remains poor over sequential lines of systemic therapy in patients with BRAF-mutated colorectal cancer.* Clin Colorectal Cancer, 2014. **13**(3): p. 164-71.
70. Loupakis, F., et al., *KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer.* Br J Cancer, 2009. **101**(4): p. 715-21.
71. Peeters, M., et al., *Massively parallel tumor multigene sequencing to evaluate response to panitumumab in a randomized phase III study of metastatic colorectal cancer.* Clin Cancer Res, 2013. **19**(7): p. 1902-12.
72. Karapetis, C.S., et al., *PIK3CA, BRAF, and PTEN status and benefit from cetuximab in the treatment of advanced colorectal cancer--results from NCIC CTG/AGITG CO.17.* Clin Cancer Res, 2014. **20**(3): p. 744-53.
73. Seymour, M.T., et al., *Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial.* Lancet Oncol, 2013. **14**(8): p. 749-59.
74. Orlandi, A., et al., *BRAF in metastatic colorectal cancer: the future starts now.* Pharmacogenomics, 2015. **16**(18): p. 2069-81.
75. Van Cutsem, E., et al., *ESMO consensus guidelines for the management of patients with metastatic colorectal cancer.* Ann Oncol, 2016. **27**(8): p. 1386-422.
76. Flaherty, K.T., et al., *Inhibition of mutated, activated BRAF in metastatic melanoma.* N Engl J Med, 2010. **363**(9): p. 809-19.

77. Long, G.V., et al., *Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma.* N Engl J Med, 2014. **371**(20): p. 1877-88.
78. Kopetz, S., et al., *Phase II Pilot Study of Vemurafenib in Patients With Metastatic BRAF-Mutated Colorectal Cancer.* J Clin Oncol, 2015. **33**(34): p. 4032-8.
79. Gomez-Roca, C.A., et al., *535PENCORAFENIB (LGX818), AN ORAL BRAF INHIBITOR, IN PATIENTS (PTS) WITH BRAF V600E METASTATIC COLORECTAL CANCER (MCRC): RESULTS OF DOSE EXPANSION IN AN OPEN-LABEL, PHASE 1 STUDY.* Annals of Oncology, 2014. **25**(suppl_4): p. iv182-iv183.
80. Bollag, G., et al., *Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma.* Nature, 2010. **467**(7315): p. 596-9.
81. Corcoran, R.B., et al., *EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib.* Cancer Discov, 2012. **2**(3): p. 227-235.
82. Prahallad, A., et al., *Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR.* Nature, 2012. **483**(7387): p. 100-3.
83. Mao, M., et al., *Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents.* Clin Cancer Res, 2013. **19**(3): p. 657-67.
84. Corcoran, R.B., et al., *Combined BRAF and MEK Inhibition With Dabrafenib and Trametinib in BRAF V600-Mutant Colorectal Cancer.* J Clin Oncol, 2015. **33**(34): p. 4023-31.
85. Corcoran, R.B., et al., *BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation.* Sci Signal, 2010. **3**(149): p. ra84.
86. Hyman, D.M., et al., *Vemurafenib in Multiple Nonmelanoma Cancers with BRAF V600 Mutations.* N Engl J Med, 2015. **373**(8): p. 726-36.
87. Yaeger, R., et al., *Pilot trial of combined BRAF and EGFR inhibition in BRAF-mutant metastatic colorectal cancer patients.* Clin Cancer Res, 2015. **21**(6): p. 1313-20.
88. Corcoran, R.B., et al., *Efficacy and circulating tumor DNA (ctDNA) analysis of the BRAF inhibitor dabrafenib (D), MEK inhibitor trametinib (T), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAF V600E–mutated (BRAFm) metastatic colorectal cancer (mCRC).* Annals of Oncology, 2016. **27**(suppl_6): p. 455O-455O.
89. van Geel, R., et al., *A Phase Ib Dose-Escalation Study of Encorafenib and Cetuximab with or without Alpelisib in Metastatic BRAF-Mutant Colorectal Cancer.* Cancer Discov, 2017.
90. Van Cutsem, E., et al., *BEACON CRC Study Safety Lead-in (SLI) in Patients With BRAFV600E Metastatic Colorectal Cancer (mCRC): Efficacy and Tumor Markers.* J Clin Oncol 36, 2018 (suppl 4S; abstr 627).
91. Bendell, J.C., et al., *Efficacy and tolerability in an open-label phase I/II study of MEK inhibitor trametinib (T), BRAF inhibitor dabrafenib (D), and anti-EGFR antibody panitumumab (P) in combination in patients (pts) with BRAF V600E mutated colorectal cancer (CRC).* J Clin Oncol 32:5s, 2014 (suppl; abstr 3515), 2014 ASCO Annual Meeting.

92. Juric, D., et al., *Convergent loss of PTEN leads to clinical resistance to a PI(3)Kalpha inhibitor.* Nature, 2015. **518**(7538): p. 240-4.
93. Van Geel, R., et al., *Phase I study of the selective BRAFV600 inhibitor encorafenib (LGX818) combined with cetuximab and with or without the α-specific PI3K inhibitor BYL719 in patients with advanced BRAF-mutant colorectal cancer.* J Clin Oncol 32:5s, 2014 (suppl; abstr 3514), 2014 ASCO Annual Meeting.
94. Hong, D.S., et al., *Phase IB Study of Vemurafenib in Combination with Irinotecan and Cetuximab in Patients with Metastatic Colorectal Cancer with BRAFV600E Mutation.* Cancer Discov, 2016. **6**(12): p. 1352-1365.
95. Elez, E., et al., *LBA-08 Results of a phase 1b study of the selective BRAF V600 inhibitor encorafenib in combination with cetuximab alone or cetuximab + alpelisib for treatment of patients with advanced BRAF-mutant metastatic colorectal cancer.* Ann Onc, 2015. **26**(suppl 4): p. iv120.
96. Misale, S., et al., *Resistance to anti-EGFR therapy in colorectal cancer: from heterogeneity to convergent evolution.* Cancer Discov, 2014. **4**(11): p. 1269-80.
97. Ahronian, L.G., et al., *Clinical Acquired Resistance to RAF Inhibitor Combinations in BRAF-Mutant Colorectal Cancer through MAPK Pathway Alterations.* Cancer Discov, 2015. **5**(4): p. 358-67.
98. Roig, A.I., et al., *Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate in vitro.* Gastroenterology, 2010. **138**(3): p. 1012-21 e1-5.
99. Li, H. and R. Durbin, *Fast and accurate long-read alignment with Burrows-Wheeler transform.* Bioinformatics, 2010. **26**(5): p. 589-95.
100. Russo, M., et al., *Acquired Resistance to the TRK Inhibitor Entrectinib in Colorectal Cancer.* Cancer Discov, 2016. **6**(1): p. 36-44.
101. Siravegna, G., et al., *Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients.* Nat Med, 2015. **21**(7): p. 827.
102. Forbes, S.A., et al., *COSMIC: exploring the world's knowledge of somatic mutations in human cancer.* Nucleic Acids Res, 2015. **43**(Database issue): p. D805-11.
103. Pelosi, G., et al., *Dissecting Pulmonary Large-Cell Carcinoma by Targeted Next Generation Sequencing of Several Cancer Genes Pushes Genotypic-Phenotypic Correlations to Emerge.* J Thorac Oncol, 2015. **10**(11): p. 1560-9.
104. Arena, S., et al., *Emergence of Multiple EGFR Extracellular Mutations during Cetuximab Treatment in Colorectal Cancer.* Clin Cancer Res, 2015. **21**(9): p. 2157-2166.
105. Poulikakos, P.I., et al., *RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E).* Nature, 2011. **480**(7377): p. 387-90.
106. Morris, E.J., et al., *Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors.* Cancer Discov, 2013. **3**(7): p. 742-50.
107. Ercan, D., et al., *Reactivation of ERK signaling causes resistance to EGFR kinase inhibitors.* Cancer Discov, 2012. **2**(10): p. 934-47.
108. Villanueva, J., et al., *Acquired Resistance to BRAF Inhibitors Mediated by a RAF Kinase Switch in Melanoma Can Be Overcome by Cotargeting MEK and IGF-1R/PI3K.* Cancer Cell, 2010. **18**(6): p. 683-695.

109. Misale, S., et al., *Blockade of EGFR and MEK intercepts heterogeneous mechanisms of acquired resistance to anti-EGFR therapies in colorectal cancer.* Sci Transl Med, 2014. **6**(224): p. 224ra26.
110. Yaeger, R., et al., *Mechanisms of acquired resistance to BRAF V600E inhibition in colon cancers converge on RAF dimerization and are sensitive to its inhibition.* Cancer Res, 2017.
111. Little, A.S., et al., *Amplification of the Driving Oncogene, KRAS or BRAF, Underpins Acquired Resistance to MEK1/2 Inhibitors in Colorectal Cancer Cells.* Sci Signal, 2011. **4**(166): p. ra17.
112. Shi, H., et al., *Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance.* Nat Commun, 2012. **3**: p. 724.
113. Emery, C.M., et al., *MEK1 mutations confer resistance to MEK and B-RAF inhibition.* Proc Natl Acad Sci U S A, 2009. **106**(48): p. 20411-6.
114. Straussman, R., et al., *Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion.* Nature, 2012. **487**(7408): p. 500-4.
115. Bardelli, A., et al., *Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer.* Cancer Discov, 2013. **3**(6): p. 658-73.
116. Wilson, T.R., et al., *Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors.* Nature, 2012. **487**(7408): p. 505-9.
117. Sun, C., et al., *Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma.* Nature, 2014. **508**(7494): p. 118-22.
118. Arena, S., et al., *MM-151 overcomes acquired resistance to cetuximab and panitumumab in colorectal cancers harboring EGFR extracellular domain mutations.* Science Translational Medicine, 2016. **8**(324): p. 324ra14-324ra14.
119. Burrell, R.A. and C. Swanton, *Tumour heterogeneity and the evolution of polyclonal drug resistance.* Mol Oncol, 2014. **8**(6): p. 1095-111.
120. Lengauer, C., K.W. Kinzler, and B. Vogelstein, *Genetic instabilities in human cancers.* Nature, 1998. **396**(6712): p. 643-9.
121. Almendro, V., et al., *Inference of tumor evolution during chemotherapy by computational modeling and in situ analysis of genetic and phenotypic cellular diversity.* Cell Rep, 2014. **6**(3): p. 514-27.
122. Merlo, L.M., et al., *Cancer as an evolutionary and ecological process.* Nat Rev Cancer, 2006. **6**(12): p. 924-35.
123. Blair, B.G., A. Bardelli, and B.H. Park, *Somatic alterations as the basis for resistance to targeted therapies.* J Pathol, 2014. **232**(2): p. 244-54.
124. Holohan, C., et al., *Cancer drug resistance: an evolving paradigm.* Nat Rev Cancer, 2013. **13**(10): p. 714-26.
125. Engelman, J.A., et al., *MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling.* Science, 2007. **316**(5827): p. 1039-43.
126. Laurent-Puig, P., et al., *Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer.* J Clin Oncol, 2009. **27**(35): p. 5924-30.
127. Iorio, F., et al., *A Landscape of Pharmacogenomic Interactions in Cancer.* Cell, 2016. **166**(3): p. 740-754.

128. Kato S, et al., *Overcoming BRAF/MEK resistance using vemurafenib with crizotinib or sorafenib in patients with BRAF-mutant advanced cancers: phase I study.* 2015 AACR Annual Meeting, Abstract 2689.
129. Bradley, C.A., et al., *Transcriptional upregulation of c-MET is associated with invasion and tumor budding in colorectal cancer.* Oncotarget, 2016. **7**(48): p. 78932-78945.
130. Johnson, D.B., et al., *Combined BRAF (Dabrafenib) and MEK inhibition (Trametinib) in patients with BRAFV600-mutant melanoma experiencing progression with single-agent BRAF inhibitor.* J Clin Oncol, 2014. **32**(33): p. 3697-704.
131. Liu, L., et al., *Antitumor effects of dabrafenib, trametinib, and panitumumab as single agents and in combination in BRAF-mutant colorectal carcinoma (CRC) models.* J Clin Oncol 32:5s, 2014 (suppl; abstr 3513), 2014 ASCO Annual Meeting.
132. Carlino, M.S., et al., *Differential activity of MEK and ERK inhibitors in BRAF inhibitor resistant melanoma.* Mol Oncol, 2014. **8**(3): p. 544-54.
133. Hatzivassiliou, G., et al., *ERK inhibition overcomes acquired resistance to MEK inhibitors.* Mol Cancer Ther, 2012. **11**(5): p. 1143-54.
134. Wagle, N., et al., *MAP kinase pathway alterations in BRAF-mutant melanoma patients with acquired resistance to combined RAF/MEK inhibition.* Cancer Discov, 2014. **4**(1): p. 61-8.
135. Zecchin, D., et al., *BRAF V600E is a determinant of sensitivity to proteasome inhibitors.* Mol Cancer Ther, 2013. **12**(12): p. 2950-61.
136. Kong, X., et al., *Cancer drug addiction is relayed by an ERK2-dependent phenotype switch.* Nature, 2017. **550**(7675): p. 270-274.
137. Hong, A., et al., *Exploiting drug addiction mechanisms to select against MAPKi-resistant melanoma.* Cancer Discov, 2017.
138. Moriceau, G., et al., *Tunable-combinatorial mechanisms of acquired resistance limit the efficacy of BRAF/MEK cotargeting but result in melanoma drug addiction.* Cancer Cell, 2015. **27**(2): p. 240-56.
139. Suda, K., et al., *Conversion from the "oncogene addiction" to "drug addiction" by intensive inhibition of the EGFR and MET in lung cancer with activating EGFR mutation.* Lung Cancer, 2012. **76**(3): p. 292-9.
140. Das Thakur, M., et al., *Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance.* Nature, 2013. **494**(7436): p. 251-5.
141. Dooley, A.J., A. Gupta, and M.R. Middleton, *Ongoing Response in BRAF V600E-Mutant Melanoma After Cessation of Intermittent Vemurafenib Therapy: A Case Report.* Target Oncol, 2016. **11**(4): p. 557-63.
142. Seifert, H., et al., *Prognostic markers and tumour growth kinetics in melanoma patients progressing on vemurafenib.* Melanoma Res, 2016. **26**(2): p. 138-44.
143. Abdel-Wahab, O., et al., *Efficacy of intermittent combined RAF and MEK inhibition in a patient with concurrent BRAF- and NRAS-mutant malignancies.* Cancer Discov, 2014. **4**(5): p. 538-45.
144. Rajagopalan, H., et al., *Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status.* Nature, 2002. **418**(6901): p. 934.
145. Fransen, K., et al., *Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas.* Carcinogenesis, 2004. **25**(4): p. 527-33.

146. Cisowski, J., et al., *Oncogene-induced senescence underlies the mutual exclusive nature of oncogenic KRAS and BRAF.* Oncogene, 2015.
147. Heidorn, S.J., et al., *Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF.* Cell, 2010. **140**(2): p. 209-21.
148. Lavoie, H., et al., *Inhibitors that stabilize a closed RAF kinase domain conformation induce dimerization.* Nat Chem Biol, 2013. **9**(7): p. 428-36.
149. Fallahi-Sichani, M., et al., *Adaptive resistance of melanoma cells to RAF inhibition via reversible induction of a slowly dividing de-differentiated state.* Mol Syst Biol, 2017. **13**(1): p. 905.
150. Wang, L., et al., *A Functional Genetic Screen Identifies the Phosphoinositide 3-kinase Pathway as a Determinant of Resistance to Fibroblast Growth Factor Receptor Inhibitors in FGFR Mutant Urothelial Cell Carcinoma.* Eur Urol, 2017. **71**(6): p. 858-862.
151. Brammeld, J.S., et al., *Genome-wide chemical mutagenesis screens allow unbiased saturation of the cancer genome and identification of drug resistance mutations.* Genome Res, 2017. **27**(4): p. 613-625.
152. Sottoriva, A., et al., *A Big Bang model of human colorectal tumor growth.* Nat Genet, 2015. **47**(3): p. 209-16.
153. Russo, M., et al., *Tumor Heterogeneity and Lesion-Specific Response to Targeted Therapy in Colorectal Cancer.* Cancer Discov, 2016. **6**(2): p. 147-53.
154. Bahcall, M., et al., *Acquired METD1228V Mutation and Resistance to MET Inhibition in Lung Cancer.* Cancer Discov, 2016. **6**(12): p. 1334-1341.
155. Lito, P., et al., *Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas.* Cancer Cell, 2012. **22**(5): p. 668-82.
156. Dumaz, N., et al., *In melanoma, RAS mutations are accompanied by switching signaling from BRAF to CRAF and disrupted cyclic AMP signaling.* Cancer Res, 2006. **66**(19): p. 9483-91.
157. Whittaker, S.R., et al., *Combined pan-RAF and MEK inhibition overcomes multiple resistance mechanisms to selective RAF inhibitors.* Mol Cancer Ther, 2015.
158. Kwak, E.L., et al., *Molecular Heterogeneity and Receptor Coamplification Drive Resistance to Targeted Therapy in MET-Amplified Esophagogastric Cancer.* Cancer Discov, 2015. **5**(12): p. 1271-81.
159. Ou, S.H., et al., *Activity of crizotinib (PF02341066), a dual mesenchymal-epithelial transition (MET) and anaplastic lymphoma kinase (ALK) inhibitor, in a non-small cell lung cancer patient with de novo MET amplification.* J Thorac Oncol, 2011. **6**(5): p. 942-6.
160. Jardim, D.L., et al., *Analysis of 1,115 patients tested for MET amplification and therapy response in the MD Anderson Phase I Clinic.* Clin Cancer Res, 2014. **20**(24): p. 6336-45.
161. Strickler, J.H., J.J. Nemunaitis, and C.D. Weekes, *Phase 1, open-label, dose-escalation and expansion study of ABBV-399, an antibody drug conjugate (ADC) targeting c-Met, in patients (pts) with advanced solid tumors.* (ASCO Annual Meeting) J Clin Oncol 34, 2016 (suppl; abstr 2510), 2016.
162. Wang, J., et al., *ABBV-399, a c-Met Antibody-Drug Conjugate that Targets Both MET-Amplified and c-Met-Overexpressing Tumors, Irrespective of MET Pathway Dependence.* Clin Cancer Res, 2017. **23**(4): p. 992-1000.

163. Burrell, R.A. and C. Swanton, *Re-Evaluating Clonal Dominance in Cancer Evolution.* Trends in Cancer, 2016. **2**(5): p. 263-276.
164. Van Cutsem, E., et al., *LBA-07 Updated Results of the MEK inhibitor trametinib (T), BRAF inhibitor dabrafenib (D), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAF V600E mutated (BRAFm) metastatic colorectal cancer (mCRC).* Ann Onc, 2015. **26**(suppl 4): p. iv119.
165. Varga, A., et al., *A first-in-human phase I study to evaluate the ERK1/2 inhibitor GDC-0994 in patients with advanced solid tumors.* European Journal of Cancer, 2016. **69**: p. S11.
166. Xue, Y., et al., *An approach to suppress the evolution of resistance in BRAFV600E-mutant cancer.* Nat Med, 2017. **23**(8): p. 929-937.
167. Corazzari, M., et al., *Oncogenic BRAF induces chronic ER stress condition resulting in increased basal autophagy and apoptotic resistance of cutaneous melanoma.* Cell Death Differ, 2015. **22**(6): p. 946-58.
168. Schreuer, M., et al., *Combination of dabrafenib plus trametinib for BRAF and MEK inhibitor pretreated patients with advanced BRAFV600-mutant melanoma: an open-label, single arm, dual-centre, phase 2 clinical trial.* Lancet Oncol, 2017. **18**(4): p. 464-472.
169. Seghers, A.C., et al., *Successful rechallenge in two patients with BRAF-V600-mutant melanoma who experienced previous progression during treatment with a selective BRAF inhibitor.* Melanoma Res, 2012. **22**(6): p. 466-72.
170. Smith, M.P. and C. Wellbrock, *Molecular Pathways: Maintaining MAPK inhibitor sensitivity by targeting non-mutational tolerance.* Clin Cancer Res, 2016.
171. Hirata, E., et al., *Intravital imaging reveals how BRAF inhibition generates drug-tolerant microenvironments with high integrin beta1/FAK signaling.* Cancer Cell, 2015. **27**(4): p. 574-88.
172. Nakamura, Y., et al., *Targeting of super-enhancers and mutant BRAF can suppress growth of BRAF-mutant colon cancer cells via repression of MAPK signaling pathway.* Cancer Lett, 2017. **402**: p. 100-109.

8. Addendum

Publications

Oddo D, Siravegna G, Gloghini A, Vernieri C, Mussolin B, Morano F, Crisafulli G, Berenato R, Corti G, Volpi C, Buscarino B, Niger M, Dunne P, Rospo G, Valtorta E, Bartolini A, Fucà G, Lamba S, Martinetti A, Di Bartolomeo M, de Braud F, Bardelli A, Pietrantonio F, Di Nicolantonio F. *Emergence of MET hyper-amplification at progression to MET and BRAF inhibition in colorectal cancer*, British Journal of Cancer. 2017 Jul 25;117(3):347-352. doi: 10.1038/bjc.2017.196.

Pietrantonio F*, **Oddo D***, Gloghini A, Valtorta E, Berenato R, Barault L, Caporale M, Busico A, Morano F, Alessi A, Siravegna G, Perrone F, Sartore-Bianchi A, Siena S, Di Bartolomeo M, Bardelli A, de Braud F & Di Nicolantonio F. *MET-driven resistance to dual EGFR and BRAF blockade may be overcome by switching from EGFR to MET inhibition in BRAF mutated colorectal cancer*, Cancer Discovery. 2016 Sep;6(9):963-71. doi: 10.1158/2159-8290.CD-16-0297. *co-first author

Oddo D, Sennott EM, Barault L, Valtorta E, Arena S, Cassingena A, Filiciotto G, Marzolla G, Elez E, van Geel M.J.M., Bartolinin A, Crisafulli G, Boscaro V, Godfrey JT, Buscarino M, Cancelliere C, Linnebacher M, Corti G, Truini M, Siravegna G, Grasselli J, Gallicchio M, Bernards R, Schellens J.H.M, Tabernero J, Engelman J.A, Sartore-Bianchi A, Bardelli A, Siena S, Corcoran R.B, Di Nicolantonio F. *The molecular landscape of acquired resistance to targeted therapy combinations in BRAF mutant colorectal cancer*, Cancer Research. 2016 Aug 1;76(15):4504-15. doi: 10.1158/0008-5472.CAN-16-0396.