Identifying rational combination therapies for colorectal cancer using functional genetics

Anirudh Prahallad

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Identifying rational combination therapies for colorectal cancer using functional genetics

Het identificeren van rationele combinatietherapieën voor colorectale kanker met behulp van functionele genetica

(met een samenvatting in het Nederlands)

Proefschrift

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Door

Anirudh Prahallad

geboren op 22 juli 1987
te Mysore, Karnataka State, India

Promotor: Prof. dr. R. Bernards

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Opportunities and challenges provided by crosstalk between Signaling pathways in cancer

Adapted from Oncogene 2015

Opportunities and challenges provided by crosstalk between signalling pathways in cancer

A Prahallad and R Bernards

During evolution, connections between the major signalling pathways were established to provide cells with an ability to deal with perturbations of homeostasis. However, these feedback and crosstalk mechanisms can become a liability in the treatment of cancer, as the inhibition of one cancer-relevant signalling pathway can lead to the activation of a secondary survival pathway that interferes with cancer drug efficacy. In this review, we discuss connections between signalling pathways in relation to cancer therapy and we evaluate the use of genetic approaches to identify pathway crosstalk. We also discuss how insight into connections between signalling pathways can be exploited to design powerful synthetic lethal drug combination therapies for the treatment of cancer.

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INTRODUCTION

Cancer treatment over the past decade has increasingly been directed towards targeting specific molecular pathways involved in tumour cell proliferation and survival. Compared with the relatively non-selective cytotoxic chemotherapies, targeting specific molecular pathways provides an opportunity to achieve a high degree of cancer selectivity through biochemical specificity. Next-generation DNA sequencing has been key to identify driver mutations in tumours and match these with the appropriate targeted therapies to achieve favourable therapeutic responses.[1,2] As a consequence of these developments, we are entering into a new era of 'precision medicine' in which cancer is being treated based on the specific oncogenic addictions of the individual cancers instead of the organ or the histology of the tissue in which the tumour arose.[3] For certain cancers that are driven by mutations in genes that regulate core signalling pathways, targeted therapies have delivered remarkable therapeutic benefits. Although single-agent targeted therapies have shown potent initial responses in patients, the emergence of acquired resistance to targeted therapy is inevitable.[4,5] This has led to the realisation that drug combinations will be required to control the disease in a more effective way. This is not surprising considering the instability of the cancer genome, the intra-tumour heterogeneity and the existence of redundancy and crosstalk between signalling pathways that counterbalance treatment effects. The abundance of highly specific targeted cancer drugs poses a major hurdle in the use of combination therapies, as even the number of possible two-way drug combinations is nearly endless.

In this review, we shall discuss how we can use functional genetics to take advantage of 'synthetic lethal' interactions between signalling pathways to identify powerful combination therapies for cancer. We will also review examples of targeted agents that are currently being used as single agents or in combination with chemotherapy in the clinic with limited patient benefit and discuss how one might develop rational combinations that make responses to these targeted agents more durable.

CONVENTIONAL DEVELOPMENT OF COMBINATION THERAPIES FOR CANCER

In the past, most chemotherapeutic agents were developed as monotherapies. When proven active as single agent, their effectiveness was subsequently evaluated in combination. Such studies mostly relied on 'trial and error' approaches, as such studies often lacked a sound molecular rationale to combine the agents. Nevertheless, this empirical work has resulted in a number of effective combination treatment regimens for most of the major cancers. More recently, we have witnessed a similar approach applied to the development of combinations of targeted cancer drugs. One particularly insightful illustration of how such studies can go wrong is provided by the following example. Both the antibody drugs cetuximab (targeting epidermal growth factor receptor (EGFR)) and bevacizumab (targeting the pro-angiogenic factor vascular endothelial growth factor) have been shown to be active against metastatic colon cancer.[6,7] Following the conventional rationale of combining two active agents in the hope that the combination could be more effective, a combination trial was designed. However, the unexpected outcome was that the combination of chemotherapy, cetuximab and bevacizumab resulted in a worse survival compared with cetuximab and chemotherapy only.[8] This example illustrates the urgent need to get smarter about the development of drug combinations, especially of targeted agents. However, the number of targeted agents increases rapidly and the number of possible combinations of targeted agents increases exponentially with the increase in available drugs. This makes it virtually impossible to test all possible drug combinations in all the major cancer genotypes. Below, we describe development of more rational combination therapies, based on unbiased genetic screens that

Division of Molecular Carcinogenesis and Cancer Genomics Netherlands, The Netherlands Cancer Institute, Amsterdam, Netherlands. Correspondence: Professor R Bernards, Division of Molecular Carcinogenesis and Cancer Genomics Netherlands, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1066 CX, The Netherlands.
E-mail: r.bernards@nki.nl
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allow the identification of the most effective drug combinations for cancers having specific oncogenic driver mutations.

SYNTHETIC LETHALITY AS A STRATEGY FOR THE TREATMENT OF CANCER

Synthetic lethality refers to a situation in which the inactivation of two genes (or pathways) individually is not lethal, but the combination of the two is.[9] This provides a potential strategy for the generation of cancer-selective drugs. When a cancer has a mutation in a specific signalling pathway, inactivation of a second gene or pathway that is synthetic lethal with the cancer-specific defect will, by definition, only be lethal to the cancer cells. The discovery that *BRCA1* mutant tumours are very sensitive to PARP inhibitors represented the first demonstration of a potential clinical application of the synthetic lethality concept.[10] In the case of *BRCA1* mutant tumours, the lack of this gene creates a defect in homologous recombination, which is tolerated as long as alternative DNA repair pathways remain available to the cancer cell. When these alternatives are blocked by a drug that acts on the enzymes poly(ADP-ribose) polymerase (PARP) 1 and 2, *BRCA1* mutant cells are unable to repair double-stranded DNA breaks, leading to their death. The recent approval of the PARP inhibitor olaparib in Europe for *BRCA*-mutated ovarian cancer is hopefully only a first of many future clinical applications of this synthetic lethality concept.

The synthetic lethality approach can also be applied to find effective combinations of existing cancer drugs that are of limited clinical benefit when used as single agents. An appealing example is the case of *BRAF*-mutant colon cancer. We have witnessed the recent clinical success in treating *BRAF(V600E)*-mutant melanomas with vemurafenib, a mutant-selective inhibitor of BRAF.[11] About 40–60% of melanoma patients harbouring this mutation have mostly spectacular initial response to treatment, resulting in a 6–9-month increase in progression-free survival and improved overall survival.[12] In contrast, about 5–10% of colon cancers that harbour the same *BRAF(V600E)* mutation do not respond to vemurafenib.[13,14] This highlights that the genotype alone can be a poor predictor of therapy response and emphasises that context in which a mutation occurs does matter. We have recently applied a kinome-centred synthetic lethality genetic screen to identify kinases whose suppression might synergise with BRAF inhibition in *BRAF(V600E)* colon cancer cell lines (Figure 1a). These cell lines replicate the clinical experience in that they also do not respond to vemurafenib monotherapy *ab initio*. Using a collection of short hairpin RNA (shRNA) vectors that collectively target all kinases for suppression, we and others found that inhibition of the EGFR is synthetic lethal with BRAF(V600E) inhibition in colon cancer. Mechanistically, BRAF(V600E) inhibition led to the feedback activation of the EGFR through post-transcriptional processes and reactivated the ERK and the PI3K signalling pathways.[14,15] Inhibition of EGFR or BRAF alone had no effect of cell proliferation, but combining BRAF(V600E) inhibition together with EGFR led to a strong synergistic effect and induced cell death, both *in vitro* and in animal xenografts. This observation has resulted in several clinical trials in which the efficacy of the combination of BRAF or MEK inhibitors and EGFR antibody drugs is tested in *BRAF* mutant colon cancer (Trial identifiers NCT01719380, NCT01750918 and NCT01791309) and indeed, the first positive results from one of these trials has already been reported.[16] Melanoma cells are derived from the neural crest and consequently express only low levels of EGFR, explaining why these tumours do not suffer from intrinsic resistance to vemurafenib, as they lack the target of the feedback loop. It is interesting to note that *BRAF(V600E)* mutant melanomas can acquire resistance to vemurafenib by gaining *EGFR* expression.[17,18]

KRAS-mutant cancers represent a significant population of cancer patients who do not have effective targeted treatment

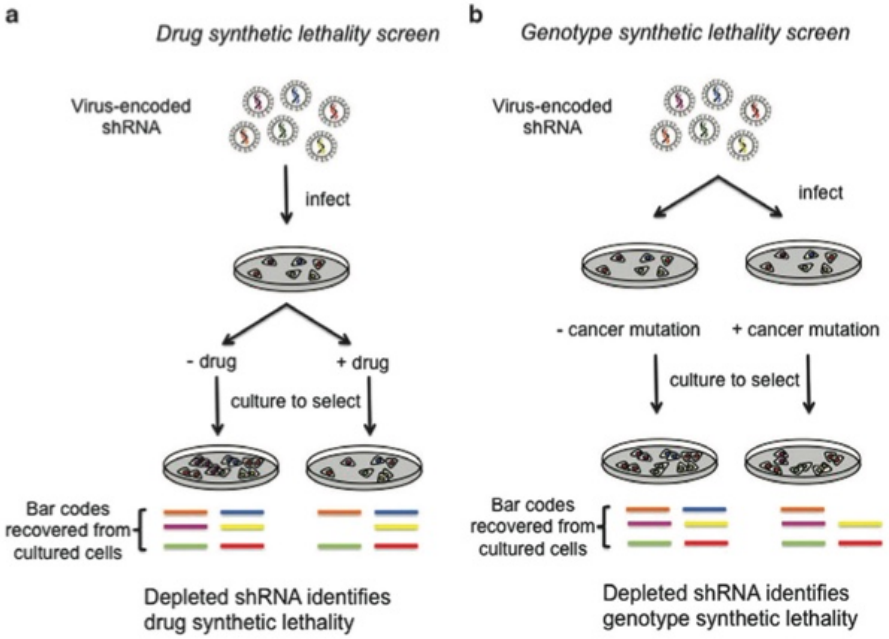

Figure 1. Finding synthetic lethal interactions through functional genetics. (**a**) Collections of shRNA vectors are introduced in a polyclonal format into cells. shRNA vectors that enhance the sensitivity to the cancer drug will become depleted under drug selection compared with the reference population that is not drug-treated. By providing each shRNA vector with a selective DNA 'bar code', the depleted shRNAs can be readily identified through deep sequencing. (**b**) Collections of shRNA vectors are introduced in a polyclonal format into cells that have or lack a cancer-specific mutation. shRNA vectors that are selectively toxic in cells having the cancer-specific mutation will become depleted compared with the reference population that does not carry the mutation and can be identified through deep sequencing.

options, as there are no effective drugs targeting KRAS. As an alternative, targeting the MEK kinases that act downstream of the RAS proteins has been explored as a treatment option for patients with *RAS*-mutant tumours. By analogy to the situation in *BRAF*-mutant melanoma, in which MEK inhibition downstream of BRAF is effective,[19] one would expect that *KRAS*-mutant tumours would similarly respond to MEK inhibition. However, this has been disappointing so far as *KRAS*-mutant tumours are mostly refractory to MEK inhibitors in the laboratory as well as in the clinic.[20–22] To address this, systematic synthetic lethality genetic screens using focused RNAi libraries have been performed to find enhancers of the effectiveness of MEK inhibitors in *KRAS*-mutant cancers. Using a kinome-centred shRNA library, Sun and colleagues[23] recently identified that inhibition of *ERBB3* enhances the effect of MEK inhibition in *KRAS*-mutant lung and colon cancer cell lines. Mechanistically, they found that MEK inhibition resulted in a transcriptional feedback activation of both ERBB3 and ERBB2 receptors that led to the reactivation of both the MAPK and PI3K pathways. These pathways supported tumour growth despite MEK being inhibited. They found that combining afatinib, a potent inhibitor of the two active signalling complexes that ERBB3 can form, with a MEK inhibitor synergistically induced cell death *in vitro* and *in vivo*.[23] They and others have also identified CRAF inhibition as being synthetic lethal with MEK inhibition in the *KRAS*-mutant setting.[24,25] These studies demonstrate that MEK inhibition in *KRAS*-mutant cells can be overcome by relieving a negative feedback that engages the activity of upstream receptor tyrosine kinases to restore MAPK signalling. Another study using a similar genetic screening approach showed that inhibition of *BCL-XL*, an anti-apoptotic gene, was synthetic lethal with MEK inhibition in *KRAS*-mutant setting.[26,27] Even though functional genetic screens have the ability to deliver several clinically relevant targets for combination therapy, not all combinations identified *in vitro* may be feasible in patients. For example, even though the combination of CRAF plus a MEK inhibitor delivers promising anticancer activity *in vitro*, the preclinical compounds targeting CRAF show significant toxicity in mice. To overcome this, second generation MEK inhibitors that inhibit MEK catalytic activity and also impair its reactivation by CRAF by disrupting RAF-MEK complexes are currently being tested in clinical trials.[24,28]

FINDING COMBINATIONS TO INCREASE EFFECTIVENESS OF TARGETED DRUGS APPROVED FOR CLINICAL USE

The current strategies for combining targeted agents fall into three categories: (i) Dual targeting of the same target by multiple drugs (for example, inhibition of HER2 with both trastuzumab and pertuzumab in breast cancer, or EGFR with cetuximab-erlotinib, vascular endothelial growth factor-vascular endothelial growth factor receptor with Bevacizumab-sorafenib). (ii) Vertical targeting of multiple components of the same signalling pathway (for example, treatment of melanoma with BRAF and MEK inhibitors) or (iii) combining targeted agents that hit multiple cellular mechanisms in parallel (for example, proliferation and angiogenesis).[29,30] These strategies have been proposed on the basis of our limited understanding of the complexity of signal transduction pathways. A recent initiative launched by the investigators from the Division of Cancer treatment and Diagnosis of the NCI, launched a set of pilot clinical trials in four different tumour types: solid tumours, melanoma, glioma and renal cell cancer to explore potential combinations using approved targeted agents based on the above-mentioned strategy.[30] Interim analyses of these trials have shown significant clinical benefit for some combinations but not for others, highlighting the empirical nature of this mix and match combination process. For example, the combination of the multi-kinase inhibitor sorafenib and bevacizumab has been explored in ovarian cancer and renal cell carcinoma. Although it is still early to draw firm conclusions, interim analyses of these trials show some trend towards clinical responses, but this combination is associated with severe toxicities.[31,32] Similarly, sorafenib in combination with temsirolimus (an mTOR inhibitor) was explored in glioblastoma multiforme. At the maximum tolerated dose, grade 3 thrombocytopenia was the dose-limiting toxicity, which resulted in the termination of the trial.[33] As pointed out above, these trials lack a strong molecular rationale, which limits the chances of success. Empirical testing of drug combinations can also be performed in patient-derived xenograft models. Such models have the advantage of yielding 'personalised' drug combinations and indeed successes of this type of approach have been described.[34–36]

How should we then approach the identification of combinations with sorafenib? We have discussed above that genetic screens provide an unbiased approach to finding the most potent combinations of pathway inhibitor drugs (Figure 1a). An elegant example of how genetic screens can be used to potentially increase the clinical benefit of currently used cancer drugs was provided recently by Zender and colleagues. His laboratory was interested to find drugs that enhance the response of hepatocellular carcinoma (HCC) to sorafenib. This drug is currently approved for the treatment of advanced HCC, but the overall median survival benefit is only 3 months.[37] This begs the question whether a combination treatment would extend the therapeutic window. Zender and colleagues[38] used a transposon-mediated *in vivo* delivery system of shRNA vectors that enabled them to perform shRNA synthetic lethality screen directly *in vivo* in murine liver carcinomas. They searched for additional genes whose suppression could increase the therapeutic efficacy of sorafenib. They found that the suppression of *MAPK14* (p38α) in combination with sorafenib prolonged the survival of mice with HCC. Their findings indicate that MAPK14-dependent MEK-ERK-ATF2 signalling acts as a resistance mechanism for sorafenib and suggest a combination strategy to overcome sorafenib insensitivity. This elegantly shows the power of functional genetic screens to guide the finding of the best possible drug combination based on a mechanistic rationale to make existing monotherapies more efficacious.[38]

Inhibition of EGFR signalling with monoclonal antibodies cetuximab or panitumumab has proven to be effective for the treatment of *KRAS* wild-type metastatic colon cancer. This is in itself remarkable, given that EGFR is not an oncogenic 'driver' in the classical sense as mutations/translocations/amplifications of the gene encoding *EGFR* are rare in colon cancer.[39,40] As might be expected, activating mutations in the codon 12 or 13 of *KRAS* which acts downstream of EGFR in signalling confer resistance to anti-EGFR therapy. Currently, *KRAS* mutation is therefore used as a predictive biomarker of EGFR therapy response and is used as a diagnostic test to screen patients who could benefit from this therapy. In an attempt to understand the mechanism of secondary resistance, Bardelli and Siena[41] made use of cetuximab-responsive colorectal cancer (CRC) cell line models (Difi and LIM1215) that were wild type for *KRAS*, *BRAF* and *PIK3CA*. They derived cetuximab-resistant variants by continued exposure to cetuximab *in vitro*. Genetic and biochemical characterisation of cetuximab-resistant derivatives of LIM1215 revealed that almost all these resistant variant lines had acquired G13D or G12R mutations in the *KRAS* gene that conferred resistance to cetuximab treatment. This appears to resemble closely the clinical situation in which acquisition of *KRAS* mutation is also the most prominent mechanism of resistance to EGFR therapy. An important question in this respect is whether drug-resistant clones are already present in the tumour before treatment initiation. To test this hypothesis, Misale and Diaz[42] used deep sequencing and high-precision BEAMing (beads, emulsion, amplification and magnetics) on the parental cell population that were used to generate resistant variants to see whether it was possible to detect mutations in *KRAS* gene prior to the start of drug treatment. With this

technology, they were able to detect the *KRAS(G13D)* mutation (rate of 0.2%) in LIM1215-resistant variants and *KRAS* amplification (1:40 000 cells) in the Difi-resistant variants, supporting the notion of pre-existing resistant clones that confer a growth advantage under cetuximab treatment. These mutations were also detected in the cell-free tumour DNA of cetuximab-treated CRC patients who eventually progressed on treatment, suggesting that cell-free tumour DNA analysis may assist in finding drug combination therapies early on to help combat minor subpopulations of cancer cells that already carry a drug resistance mutation. Interestingly, in cell culture, CRC cells that acquired *KRAS* mutation upon cetuximab therapy responded to the combination of a MEK inhibitor and cetuximab, providing a potential treatment option for such drug-resistant patients. This is in contrast to the findings of Sun *et al.*,[23] who found that *KRAS*-mutant CRCs require the combination of a pan-HER inhibitor and a MEK inhibitor, whereas the combination of a MEK inhibitor and an EGFR inhibitor only was ineffective in this setting. Apparently, colon cancer cells that acquired a *KRAS* mutation as part of their oncogenic development respond differently to drugs than colon cancer cells that acquired a *KRAS* mutation late under selective pressure of EGFR therapy. This finding further highlights how important the context is in which any given mutation is present to understand the response to therapy.

TARGETING BOTH THE TUMOUR AND ITS MICROENVIRONMENT AS A COMBINATION STRATEGY

The tumour microenvironment, consisting of a complex system of many cell types, can play a crucial role in malignant progression and response to therapy.[43] Especially tumour-infiltrating immune cells can serve as a source of local and systemic cues to support various hallmarks of a growing tumour including angiogenesis, suppression of antitumour immunity and promoting distant metastasis.[44] For instance, the group of Bentires Alj[45] recently uncovered a complex network of interaction between breast tumour cells and the macrophages associated with them. Mammary tumours secrete the CCL2 chemokine that recruits the CCR2-expressing monocytes from the bone marrow. High level of CCL2 expression is associated with poor prognosis and poor clinical outcome in patients with breast cancer. They demonstrated that treatment with anti-CCL2-neutralising antibody reduces metastasis and blocks the release of monocytes from the bone marrow.

Given the complexity of the interaction between epithelial tumours and its disorganised microenvironment, response to targeted therapy can also be dictated by the tumour microenvironment. A recent example by the groups of Jeffery Settleman and Todd Golub[46,47] illustrates that the tumour microenvironment can indeed confer resistance to therapy. By co-culturing 23 different stromal cells with 45 different cancer cell lines, they screened 35 anticancer drugs to see whether any stromal factor dampened the effect of targeted therapy. Both the groups identified that growth factors such as hepatocyte growth factor, fibroblast growth factor, EGF and NRG1 potently rescued oncogene-addicted cancer cells from targeted therapy treatment. They also observed a clear correlation with lack of clinical response to vemurafenib and strong hepatocyte growth factor staining in the surrounding tumour stroma in *BRAF*-mutant melanoma biopsies, indicating the activation of MET receptor in the tumours that bypass MAPK pathway blockade.

EPIGENETIC DRUG COMBINATIONS

A change in the epigenetic state of the genome can contribute to oncogenesis.[48] These epigenetic changes alter the state of chromatin thereby leading to altered gene expression. Recent cancer genome analyses has uncovered an impressive and still increasing number of epigenetic enzymes that are mutated in cancer (reviewed in Geutjes *et al.*[49]). These mutational changes in the nucleosome remodelling complexes, histone modifiers and DNA-modifying enzymes affect key regulatory signalling pathways required for normal cellular function. Considering the enzymatic activities of these proteins encoded by these genes, targeting them pharmacologically has been of considerable interest to the pharmaceutical industry. However, we still lack a detailed understanding as to where theses drugs could be used effectively as single agents or in combination.[50,51]

There are currently FDA-approved drugs against two classes of chromatin-remodeller enzymes: the DNA methyltransferases and the lysine deacetylases or KDACs (formerly known as histone deacetylases or HDACs). DNA methyltransferases inhibitors antagonize DNA methylation at CpG di-nucleotides. Methylation of the C residues in these sequences leads to gene silencing and DNA methyltransferases inhibitor treatment has been shown to cause re-activation of silenced tumour suppressor genes in colon cancer.[52] This may explain the anti-cancer effects of these drugs. Drugs like decitabine and azacitidine are currently used for the treatment of myelodysplastic syndrome and acute myeloid leukaemia. Two KDAC inhibitors, vorinostat and romidepsin, have gained approval for the treatment of cutaneous T-cell lymphoma.[53,54]

We and others have observed synergy between inhibition of these KDACs and DNA methyltransferase inhibitors in non-small cell lung cancer and CRC,[52,55] which underscores the notion that combinations of epigenetic modifier drugs can be a strategy in the treatment of cancer. The choice of these two enzymes was merely the result of the fact that only for these two gene families, drugs were available and a strong molecular rationale for combining these two drugs is missing. The interplay between DNA methylation, histone modification and nucleosome remodelling is complex, and it is impossible to predict which of the over 600 enzymes that regulate these processes may display synthetic lethal relationships. Unbiased genetic screens using focused shRNA libraries of chromatin-remodeller enzymes represent a logical way forward in finding the most effective combinations of chromatin-remodeller drugs. Indeed, we have already seen small-scale genetic screens showing that in cells that carry a mutation in the *ARID1A* component of the SWI/SNF chromatin-remodeller complex display synthetic lethality with loss of *ARID1B* (approach schematically shown in Figure 1b).[56] By analogy, loss of *BRG1*, which is mutated in some 10% of lung adenocarcinomas, displays synthetic lethality with loss of *BRM*.[57] This latter result is not unexpected, as BRG1 and BRM are the two catalytic subunits of SWI/SNF complexes; they are ATP-dependent helicases, required for nucleosome repositioning. Without helicase activity, nucleosome repositioning cannot take place, explaining the synthetic lethal relationship between these two genes.

It is likely that in the next few years, additional epigenetic drugs will become available for clinical use. The most likely candidates are drugs that target the Polycomb Repressive Complex 2 component EZH2, an enzyme that trimethylates H3K27 and drugs that act as inhibitors of bromodomain proteins.[49] How we should combine these drugs to deliver optimal clinical benefit will require the identification of the interactions and interdependencies between these enzyme families through the use of functional genetic approaches. This task seems daunting, as the cancer epigenome is as different between individual cancers as the cancer genome itself. Thus, interdependencies will be highly context-dependent in ways that are difficult to predict at present.

COMBINATIONS OF EPIGENETIC DRUGS AND TARGETED THERAPIES

Oncogenic pathway inhibition can also lead to certain epigenetic changes that can in turn lead to therapy resistance. These

epigenetic changes are reversible in nature, unlike the changes that are acquired as a consequence of a gene mutation. A study performed in 2010 describes this process by modelling the acute responses to various targeted agents in drug-sensitive cell lines. Settleman and colleagues[58] demonstrated that a small subpopulation of cells displayed a reversible drug-tolerant phenotype. These drug-tolerant persistors displayed more than 100-fold resistance to the targeted therapy and maintained viability through an altered chromatin state requiring the histone demethylase KDM5A. They further demonstrated that targeting the activated IGF1R receptor in the drug-tolerant cells or targeting histone deacetylases eliminated the drug-tolerant cells. Their findings suggest that one might be able to delay resistance development by combining epigenetic drugs with targeted agents.

A second example of epigenetic remodelling during drug resistance development came from the study of BRAF-inhibitor resistance development in melanoma. In some cases, *BRAF*-mutant melanoma cells that express low levels of EGFR owing to their neural crest origin, acquire resistance to BRAF inhibition through transcriptional upregulation of *EGFR*. Remarkably, enforced EGFR expression in melanoma resulted in a slow-growth phenotype *in vitro* and *in vivo* with cells displaying hallmarks of oncogene-induced senescence. This suggested that EGFR expression was only beneficial in the presence of drug, but not in its absence. In trying to understand the molecular mechanism of acquired *EGFR* expression, Sun *et al.*[17] performed a loss-of-function screen using a chromatin modulator shRNA library to identify epigenetic modulators that can induce the expression of *EGFR* under the presence of vemurafenib in *BRAF*-mutant melanoma. They identified that loss of *SOX10,* a neural crest-specific transcription factor, led to increased transforming growth factor-β signalling, leading to a transcriptional increase of *EGFR*. Their data indicate that in a heterogeneous *SOX10* knockdown cell population, vemurafenib administration led to the enrichment of $EGFR^{hi}$ and $SOX10^{low}$ cells and conversely, vemurafenib withdrawal gave rise to $EGFR^{low}$ and $SOX10^{hi}$ cells. These data are consistent with a reversible drug resistance phenotype and is turned back when drug is withdrawn. As such, these data may provide a molecular underpinning for the so-called 'drug holiday' effect, a clinical observation that some patients regain sensitivity to a cancer drug following a break in treatment. Indeed, in animal models of melanoma, intermittent drug dosing proved to be more effective than continuous dosing.[17,58,59]

It remains to be determined how often unstable, non-heritable mechanisms of acquired drug resistance underlie drug resistance in the clinic. It may well be that because of prolific DNA sequencing of tumour DNA, there is a significant reporting bias in the scientific literature in favour of secondary mutations as causal factors in drug resistance. The absence of a secondary mutation in a drug-resistant tumour in such studies is considered a negative result that is not published. Consistent with this notion, a recent review of the literature on reversible drug resistance shows that this is quite prominent in the clinic indeed,[60] suggesting that combinations of targeted and epigenetic drugs may have a prominent place in the cancer clinic of the future.

LIMITATIONS OF DRUG COMBINATIONS

Although stacking of most conventional chemotherapeutic agents leads to cumulative toxicity, we have seen a number of result of recent clinical studies with targeted drugs showing that drug combinations can reduce certain toxicities caused by the monotherapies. For instance, about 15–30% of patients treated with vemurafenib or dabrafenib develop cutaneous squamous cell carcinomas and keratoacanthomas.[61] The molecular mechanism underlying the occurrence of these non-melanoma skin lesions upon vemurafenib treatment is due to the paradoxical activation of CRAF in cells treated with BRAF inhibitors that receive signalling from upstream. As a result, vemurafenib induces the formation of CRAF–BRAF dimers that initiate the hyperactivation of the ERK signalling pathway in *BRAF* wild-type cells.[62–64] It is not the case that vemurafenib initiates malignant transformation, rather it accelerates tumour formation if squamous cells already bear a pre-existing mutation in *RAS* as seen in *HRAS*-driven squamous cell carcinoma cell models. This was confirmed *in vivo* using a two-stage skin carcinogenesis mouse model where squamous cell carcinoma formation initiated by the tropical administration of 7,12-dimethylbenz(a)anthracene and 12-O-tetradecanoylphorbol-13-acetate carcinogens was accelerated by the co-administration of vemurafenib. However, combination of a potent MEK inhibitor together with vemurafenib completely blocked the paradoxical CRAF activation induced by vemurafenib and led to the reduction of squamous cell carcinomas in mice. This combination has reduced the toxicity in patients and has also led to a better overall survival when compared with vemurafenib treatment alone. Clinical and preclinical studies have also shown that combining 5-FU treatment together with vemurafenib significantly led to the decrease in non-melanoma skin lesions.[65–67]

Another example of a drug that also induces the RAF inhibitor paradox is nilotinib, which is used to treat chronic myelogenous leukaemia. Most of the chronic myelogenous leukaemia is driven by the *BCR–ABL* oncogene. In fact, imatinib, nilotinib and dasatinib that target the active site of the BCR-ABL oncoprotein also possess weak off-target activity against RAF. Patients with chronic myelogenous leukaemia respond very well to the anti-BCR-ABL treatment by completely shutting down the ERK signalling pathway. However, patients who progress on the treatment usually acquire a secondary gatekeeper mutation that is not targeted by any of these drugs. As these drugs have a weak off-target effect on the RAF kinase, continuing treatment in patients who have progressed on treatment will lead to the activation of RAF dimers and hence accelerate tumour progression by increased MAPK signalling. However, combining nilotinib with a MEK inhibitor can restore sensitivity to nilotinib.[68]

These examples show that sometimes combinations can reduce toxicity. That targeted therapies can also have synergy in toxicity is also evident. In a recent Phase 1 trial where patients were treated with the combination of ipilimumab (anti-CTLA-4) and vemurafenib, severe toxicity of the liver was observed in most of the patients. The trial was discontinued because of dose-limiting toxic effects of grade 3 elevations in aminotransferase levels developed in patients who received ipilimumab in combination with vemurafenib.[69] It is therefore too early to be optimistic about combinations of targeted therapies in terms of safety and tolerability in the clinic.

CONCLUDING REMARKS

The emergence of resistance to targeted therapies is nearly inevitable and represents one of the most significant hurdles in making real progress in improvements in cancer survival rates. The realisation that combinations of drugs are required to counter drug resistance is not new. In fact, acquired immune deficiency syndrome became a chronic disease only after the implementation of combination therapies. The major obstacle to find successful drug combinations for cancer has been the low 'hit' rate with the conventional 'trial and error' testing of drug combinations. Synthetic lethality genetic screens have the potential to uncover the most powerful combinations of drugs based on an unbiased survey of combinatorial vulnerabilities of the cancer cell. However, this approach is unlikely to be the holy grail of cancer therapy development. Many synthetic lethal interactions identified later turned out to be highly context-dependent,[70–73] which limits their clinical utility. Another limitation of the synthetic lethality concept is that we currently consider

the cancer genotype to be the major factor guiding the choice of targeted therapy. It now becomes increasingly evident that the context in which mutations are present is a major determinant of response also. Synthetic lethal drug interactions may therefore be difficult to predict based on genotype only. Having these reservations in mind, it is nevertheless the case that the first drug based on the concept of synthetic lethality was approved recently and many more are likely to follow. Finally, we need to consider abandoning the old paradigm that drugs are initially developed as single agent therapeutics and only tested in combination after having demonstrated single agent activity. The recent work of the Zender laboratory[38] illustrates this point elegantly: MAKP14 inhibitors may not be successful as single agents in the cancer clinic, but through the synthetic lethal interaction with sorafenib, they may become powerful drugs when used in combination.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter 2

Unresponsiveness of colon cancer to BRAF (V600E) inhibition through feedback activation of EGFR

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Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR

Anirudh Prahallad[1]*, Chong Sun[1]*, Sidong Huang[1]*, Federica Di Nicolantonio[2,3]*, Ramon Salazar[4], Davide Zecchin[2], Roderick L. Beijersbergen[1], Alberto Bardelli[2,3] & René Bernards[1]

Inhibition of the BRAF(V600E) oncoprotein by the small-molecule drug PLX4032 (vemurafenib) is highly effective in the treatment of melanoma[1]. However, colon cancer patients harbouring the same *BRAF(V600E)* oncogenic lesion have poor prognosis and show only a very limited response to this drug[2–4]. To investigate the cause of the limited therapeutic effect of PLX4032 in *BRAF(V600E)* mutant colon tumours, here we performed an RNA-interference-based genetic screen in human cells to search for kinases whose knockdown synergizes with BRAF(V600E) inhibition. We report that blockade of the epidermal growth factor receptor (EGFR) shows strong synergy with BRAF(V600E) inhibition. We find in multiple *BRAF(V600E)* mutant colon cancers that inhibition of EGFR by the antibody drug cetuximab or the small-molecule drugs gefitinib or erlotinib is strongly synergistic with BRAF(V600E) inhibition, both *in vitro* and *in vivo*. Mechanistically, we find that BRAF(V600E) inhibition causes a rapid feedback activation of EGFR, which supports continued proliferation in the presence of BRAF(V600E) inhibition. Melanoma cells express low levels of EGFR and are therefore not subject to this feedback activation. Consistent with this, we find that ectopic expression of *EGFR* in melanoma cells is sufficient to cause resistance to PLX4032. Our data suggest that *BRAF(V600E)* mutant colon cancers (approximately 8–10% of all colon cancers[2,3,5]), for which there are currently no targeted treatment options available, might benefit from combination therapy consisting of BRAF and EGFR inhibitors.

Activating mutations in the *BRAF* oncogene (*BRAF(V600E)*) are seen in some 70% of primary melanomas[6], some 10% of colorectal cancers[7] and some 30–70% of papillary thyroid carcinoma[8–10]. However, clinical responses to the highly selective small-molecule inhibitor of the BRAF(V600E) oncoprotein, PLX4032, differ widely, ranging from a response rate of approximately 80% in melanoma to only 5% in *BRAF* mutant colorectal cancer[2–4]. To investigate the molecular mechanism responsible for the intrinsic resistance of *BRAF(V600E)* colorectal cancers (CRCs) to PLX4032, we first tested a panel of *BRAF(V600E)* mutant melanoma and CRC cell lines for their response to PLX4032. We found that the sensitivity of melanoma and CRC cells in both short-term (Fig. 1a) and long-term (Fig. 1b) proliferation assays *in vitro* mirrors the clinical experience, with melanoma cells being more sensitive to PLX4032 than CRC cells.

RNA interference (RNAi) genetic screens have been used successfully to identify genes that enhance a phenotype[11]. We therefore set out to screen a short hairpin RNA (shRNA) library representing the full complement of 518 human kinases[12] (the 'kinome') and 17 additional kinase-related genes (Supplementary Table 1) for genes whose inhibition confers sensitivity to PLX4032 in *BRAF(V600E)* mutant CRC cells. WiDr cells were infected with the lentiviral kinome shRNA collection and cultured in the absence or presence of PLX4032 for 10 and 18 days, respectively. After this, the relative abundance of shRNA vectors was determined by next generation sequencing of the barcode identifiers present in each shRNA vector (Fig. 1c; see Methods). We arbitrarily considered only shRNA vectors that had been sequenced at least 300 times and which were depleted at least fivefold by the drug treatment. Figure 1d shows that only very few of the 3,388 shRNA vectors in the library met this stringent selection criterion, among which were three independent shRNA vectors targeting the EGFR (see Supplementary Table 2 for all selected shRNAs). This suggested that suppression of EGFR synergizes with BRAF inhibition in these CRC cells. To validate this finding, we infected WiDr cells with each of these three EGFR shRNA vectors (all of which reduced EGFR levels; Fig. 1f) and cultured these cells with or without PLX4032 for 2 weeks. Figure 1e shows that inhibition of EGFR does not significantly affect proliferation of EGFR in WiDr cells, consistent with the clinical observations that *KRAS* or *BRAF* mutant CRC cells do not respond to EGFR-targeted monoclonal antibodies[7,13,14]. In contrast, suppression of EGFR in combination with PLX4032 caused a marked inhibition of proliferation in WiDr cells (Fig. 1e). This suggested that *BRAF(V600E)* mutant CRC cells are responsive to treatment with a combination of BRAF inhibitor plus an EGFR inhibitor.

At present, two classes of anti-EGFR drugs are clinically available; these include the monoclonal antibodies cetuximab and panitumumab, and the small-molecule kinase inhibitors gefitinib and erlotinib. We found that three BRAF mutant CRC cell lines (WiDr, VACO432 and KM20) all lack a significant response to monotherapy with PLX4032, cetuximab or gefitinib. However, strong synergy was seen when PLX4032 was combined with either cetuximab or gefitinib (Fig. 2a and Supplementary Fig. 1A, C) or erlotinib (data not shown), consistent with the notion derived from the shRNA screen that EGFR inhibition is required to elicit a response to BRAF inhibition in CRC cells.

To address the molecular mechanism underlying the synergy between BRAF and EGFR inhibition in colon cancer, we tested lysates of drug-treated cells with phosphoprotein-specific antibodies that identify the activated state of components of the EGFR signalling pathway. To our surprise, we observed that treatment of all three *BRAF* mutant CRC cell lines with PLX4032 resulted in a strong increase in Tyr 1068 phosphorylation of EGFR, which reflects activation of the receptor (Fig. 2b and Supplementary Fig. 1B, D). This observation suggests that a powerful feedback activation of EGFR is elicited by BRAF inhibition. This feedback activation is ligand-dependent, as it does not take place in the absence of serum growth factors (Supplementary Fig. 2B). Co-treatment of these cells with a combination of PLX4032 and either cetuximab or gefitinib prevented this feedback activation of EGFR. PLX4032 treatment inhibited MEK and ERK activation downstream of BRAF but activated AKT, which acts downstream of EGFR in a pathway parallel to BRAF. We note that in all three cell lines treatment with BRAF and EGFR inhibitors caused a more complete inhibition of AKT, MEK and ERK signalling

[1]Division of Molecular Carcinogenesis, Center for Biomedical Genetics and Cancer Genomics Centre, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. [2]Laboratory of Molecular Genetics, Institute for Cancer Research and Treatment, University of Torino, Medical School, Str prov 142 Km 3.95, 10060 Candiolo, Torino, Italy. [3]FIRC-IFOM Institute for Molecular Oncology, Via Adamello 16, 20100 Milan, Italy. [4]IDIBELL, Institut Català d'Oncologia L'Hospitalet de Llobregat, Gran Via s/n, km 2, 7 08907 L'Hospitalet-Barcelona, Spain.
*These authors contributed equally to this work.

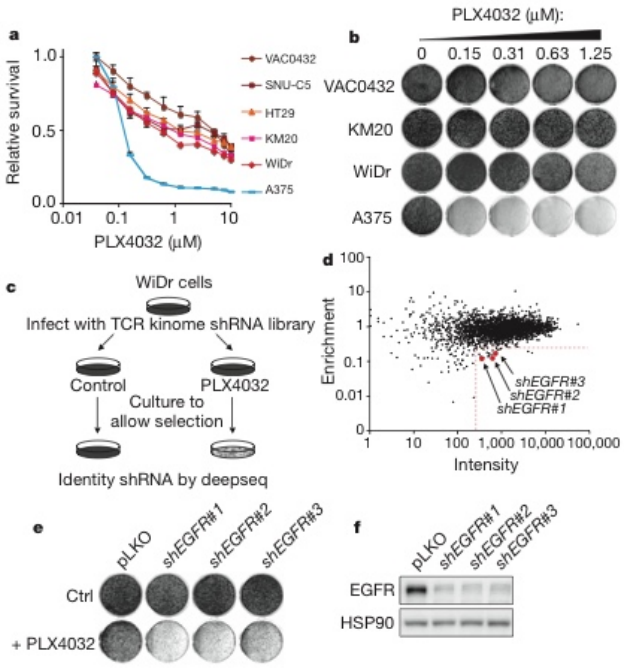

Figure 1 | EGFR inhibition confers sensitivity to BRAF(V600E) inhibition in colon cancer. **a**, **b**, CRC but not melanoma cells harbouring the BRAF(V600E) mutation are resistant to PLX4032 treatment. **a**, Short-term growth-inhibition assay of a cell line panel consisting of CRC (VACO432, SNU-C5, HT29, KM20 and WiDr) and melanoma (A375) cells. Cells were treated with increasing concentrations of PLX4032 for 72 h, and cell viability was determined using CellTiter-Blue by measuring the absorbance at 540 nm in a microplate reader. Error bars show data ± standard error. Means were derived from four replicates ($n = 4$). **b**, Long-term colony formation assay of CRC (VACO432, KM20 and WiDr) and melanoma (A375) cells. Cells were grown in the absence or presence of PLX4032 at the indicated concentrations for 10–14 days. For each cell line, all dishes were fixed at the same time, stained and photographed. **c**, Schematic outline of the 'dropout' RNAi screen for enhancers of PLX4032 sensitivity. Human TRC kinome shRNA library polyclonal virus was produced to infect WiDr cells, which were then left untreated (control) for 10 days or treated with 1 μM PLX4032 for 18 days. After selection, shRNA inserts from both populations were recovered by polymerase chain reaction (PCR) and identified by deep sequencing (deepseq). **d**, Representation of the relative abundance of the shRNA barcode sequences from the shRNA screen experiment depicted in **c**. The *y* axis shows enrichment (relative abundance of PLX4032 treated/untreated) and the *x* axis shows intensity (average sequence reads in untreated sample) of each shRNA. Among the 22 top shRNA candidates (more than fivefold depleted by PLX4032 treatment and more than 300 reads in the untreated condition (as indicated by the red dashed lines), three independent *shEGFR* vectors (in red) were identified. **e**, **f**, Three independent shRNAs targeting *EGFR* enhance response to PLX4032. **e**, The functional phenotypes of non-overlapping sh*EGFR* vectors are indicated by colony formation assay in 1 μM PLX4032. The pLKO vector was used in the control experiment (Ctrl). The cells were fixed, stained and photographed after 14 days. **f**, The level of knockdown of EGFR by each of the shRNAs was measured by examining the EGFR protein levels by western blotting.

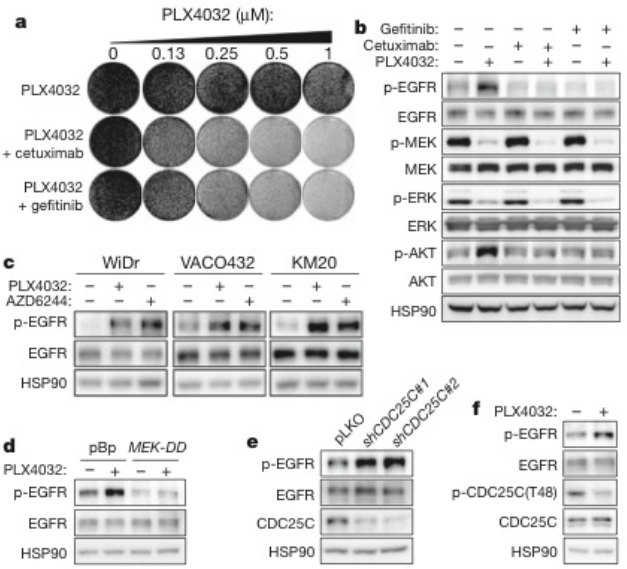

Figure 2 | Functional and biochemical interaction between BRAF and EGFR inhibition in colon cancer. **a**, Synergistic response of WiDr cells to the combination of EGFR and BRAF(V600E) inhibitors. WiDr cells were cultured in increasing concentrations of BRAF inhibitor PLX4032 alone, EGFR inhibitors cetuximab (1.25 mg ml^{-1}) or gefitinib (0.125 μM) alone, or their combinations. The cells were fixed, stained and photographed after 18 days. **b**, Resistance to BRAF(V600E) inhibition in WiDr cells is mediated through feedback activation of EGFR. Biochemical responses of WiDr cells treated with PLX4032, cetuximab or gefitinib, or their combinations, were documented by western blot analysis. Cells were harvested at 6 h after drug treatment. BRAF(V600E) inhibition results in strong upregulation of Tyr 1068 p-EGFR and Ser 473 phosphorylated-AKT (p-AKT), which is abrogated by EGFR inhibitors. Furthermore, combination treatments result in complete inhibition of phosphorylated MEK (p-MEK) and phosphorylated ERK (p-ERK). Heat shock protein 90 (HSP90) served as a control. **c**, **d**, MEK acts downstream of BRAF to mediate the feedback regulation of EGFR in BRAF mutant CRC cells. **c**, MEK inhibitor activates p-EGFR to the same extent as PLX4032. Activation of EGFR in WiDr, VACO432 and KM20 cells treated with PLX4032 or AZD6244 for 6 h was analysed by western blot. **d**, *MEK-DD* prevents the activation of EGFR by PLX4032. Western blot analysis of EGFR in WiDr cells expressing pBabe-*PURO* (pBp) vector control or *MEK-DD* treated with PLX4032 for 1 h. **e**, Western blot analysis showing that suppression of CDC25C by two independent shRNA vectors results in elevated levels of p-EGFR in WiDr cells. **f**, PLX4032 treatment leads to a reduced activation of CDC25C. Feedback regulation of CDC25C and EGFR in VACO432 cells treated with PLX4032 for 1 h were documented by western blot analysis.

as compared to PLX4032 monotherapy, providing a rationale for the observed synergy in growth assays (Fig. 2b and Supplementary Fig. 1B, D).

To begin to address how inhibition of BRAF(V600E) causes activation of EGFR, we treated all three BRAF mutant CRC cells with the selective MEK inhibitor AZD6244. Figure 2c shows that this drug activated EGFR (as judged by phosphorylated EGFR, p-EGFR) to the same extent as PLX4032, indicating that MEK acts downstream of BRAF to mediate the feedback regulation of EGFR. Consistent with this, we observed that expression of an active mutant of *MEK* (*MEK-DD*) in WiDr or VACO432 cells prevented the activation of EGFR by PLX4032 (Fig. 2d and data not shown). In agreement with a central role for MEK in mediating the feedback activation of EGFR, the combination of MEK and EGFR inhibitors also synergized to inhibit growth of VACO432 or WiDr cells (Supplementary Fig. 3 and data not shown). It is unclear how MEK inhibition leads to activation of EGFR through increased phosphorylation of Tyr 1068. It has been shown in *Xenopus* that ERK kinase can phosphorylate Cdc25c on several residues, including Thr 48, leading to activation of its phosphatase activity[15]. Moreover, Cdc25A can bind to and dephosphorylate EGFR[16]. We therefore began by investigating a potential role of CDC25C in the activation of EGFR. We suppressed CDC25C in WiDr cells by shRNA and monitored levels of p-EGFR. We found that two independent *shCDC25C* vectors caused an increase in p-EGFR (Fig. 2e). Moreover, treatment of WiDr cells with PLX4032 inhibited phosphorylation of CDC25C at Thr 48 (Fig. 2f), which has been shown to be required for its phosphatase activity[15]. Together, these data are consistent with a model in which BRAF inhibition leads to inhibition of MEK and ERK kinases, which in turn leads to a reduced activation of CDC25C. Inhibition of CDC25C in turn causes an increase in p-EGFR

due to decreased dephosphorylation (Fig. 2e). Our data do not exclude the related CDC25A, CDC25B, or other phosphatases being involved in this feedback regulation of EGFR.

The EGFR is expressed primarily in epithelial cancers[17]. Because melanomas are derived from the neural crest, we reasoned that the favourable response of melanomas to PLX4032 might result from the paucity of EGFRs on these tumours and hence the absence of the feedback activation of EGFR by BRAF inhibition. We compared EGFR expression in a panel of *BRAF(V600E)* mutant melanoma, colon cancer and thyroid cancer cells. Melanoma cell lines indeed express low levels of EGFR (Fig. 3a and data not shown). Of the ten colorectal cancer cell lines examined, eight express much higher levels of EGFR, but in two (COLO-741 and OXCO-1) EGFR levels were as low as those seen in melanoma. All three thyroid cancer cell lines expressed EGFR at significant levels (Fig. 3a). When we tested the two EGFR^{low} CRC cell lines for their response to PLX4032, we found them to be almost as sensitive as the melanoma cell line A375 in both short-term assays (Fig. 3b) and long-term assays (Fig. 3c), consistent with the notion that EGFR levels determine the response to small-molecule BRAF inhibition. Thyroid cancer cell lines, which express high levels of EGFR, also responded well to the combination of gefitinib and PLX4032, but not to PXL4032 monotherapy (Fig. 3b, c, f and Supplementary Fig. 5). Finally, we tested whether ectopic expression of EGFR was sufficient to confer resistance to PLX4032 in melanoma cells. We transduced EGFR-negative A375 and SK-MEL-28 melanoma cells with a retroviral EGFR expression vector and subjected these cells to treatment with PLX4032 monotherapy or combination with the EGFR inhibitor gefitinib. Figure 3d, e and Supplementary Fig. 4 show that expression of EGFR conferred resistance to PLX4032, but these cells still responded well to the combination of PLX4032 with gefitinib. Together, these data indicate that EGFR levels in *BRAF* mutant CRC, melanoma and thyroid cancer cells are a key determinant of response to BRAF inhibitor monotherapy.

Synergistic anticancer effects of drugs can result from an effect on cell proliferation, cell death, or both. Induction of cell death is preferred to prevent the re-growth of the tumour after the therapy has been terminated. We measured induction of programmed cell death (apoptosis) by measuring the induction of cleaved poly(ADP-ribose) polymerase (PARP), a hallmark of apoptosis[18]. Neither PLX4032 nor EGFR inhibitors alone induced PARP cleavage in WiDr or VACO cells. However, the two drugs combined did induce marked PARP cleavage, indicative of apoptosis induction (Fig. 4a, b). This suggested that BRAF and EGFR inhibition should also be synergistic when combined *in vivo*. To test this, we used immunodeficient mice xenografted with human WiDr and VACO432 CRC tumours. Ten days after injection of tumour cells, palpable tumours were present in all animals, and cohorts of mice were treated with vehicle, the EGFR-targeted drugs cetuximab or erlotinib, the BRAF inhibitor PLX4720

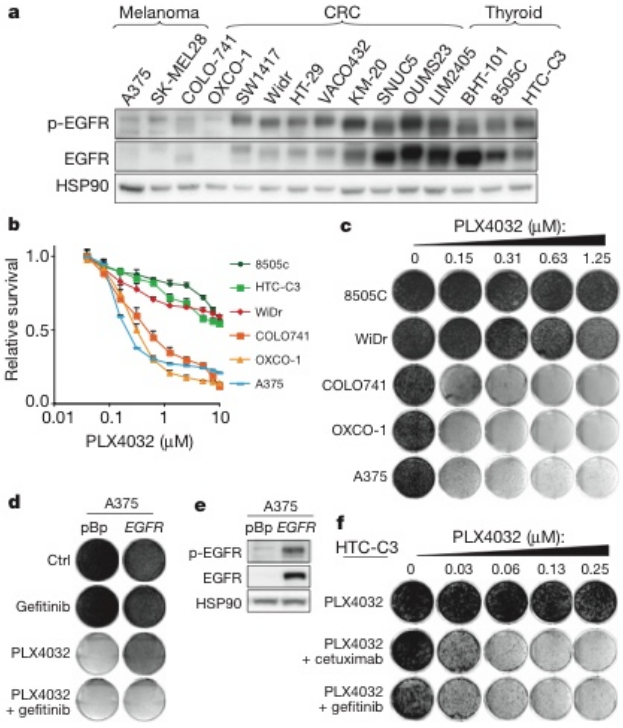

Figure 3 | Correlation between EGFR levels and response to BRAF inhibition in melanoma and CRC. **a**, Western blot analysis of p-EGFR and EGFR levels in a panel of BRAF(V600E) mutant cell lines from melanoma, CRC and thyroid cancer. HSP90 served as a control. **b**, High levels of EGFR expression in tumour types harbouring BRAF(V600E) mutations correlate with PLX4032 resistance. Short-term growth-inhibition assays of a cell line panel consisting of thyroid cancer (HTC-C3 and 8505C), CRC (OXCO-1, COLO741 and WiDr) and melanoma (A375) cells. Cells were treated with increasing concentrations of PLX4032 for 72 h, and cell viability was determined using CellTiter-Blue by measuring the absorbance at 540 nm in a microplate reader. Error bars show data ± standard error. Means were derived from four replicates ($n = 4$). **c**, Long-term colony formation assay of thyroid cancer (8505C), CRC (OXCO-1, COLO741 and WiDr) and melanoma (A375) cells. Cells were grown in the absence or presence of PLX4032 at the indicated concentrations for 10–14 days. For each cell line, all dishes were fixed at the same time, stained and photographed. **d**, Ectopic EGFR expression confers resistance to PLX4032, but not to the combination of PLX4032 and gefitinib in A375 melanoma cells. A375 cells expressing pBabe-*PURO* (pBp) vector control or *EGFR* were cultured in PLX4032 (5 μM), gefitinib (2.5 μM) or their combination. The cells were fixed, stained and photographed after 7 (untreated or gefitinib) or 9 (PLX4032 alone or in combination with gefitinib) days. **e**, Western blot analysis of p-EGFR and total EGFR levels in cells described above. HSP90 served as a control. **f**, Synergistic response of thyroid cancer HTC-C3 cells to the combination of EGFR and BRAF(V600E) inhibitors. HTC-C3 cells were cultured in increasing concentrations of PLX4032 alone, cetuximab (1.25 mg ml^{-1}) or gefitinib (2.5 μM) alone, or their combinations. The cells were fixed, stained and photographed after 18 days.

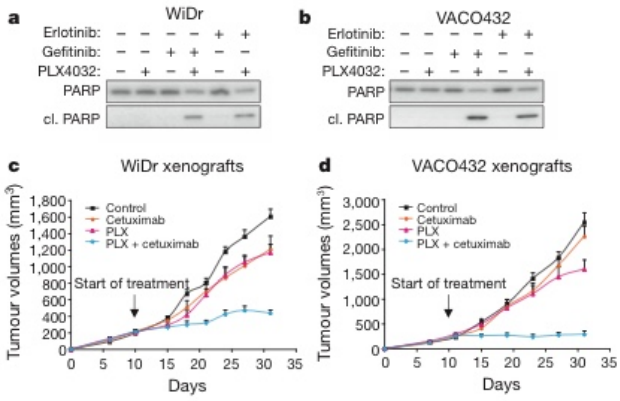

Figure 4 | EGFR and BRAF(V600E) inhibitors synergize to induce apoptosis of CRC cells and to suppress CRC tumour growth in a xenograft model. **a**, **b**, Combination of EGFR and BRAF(V600E) inhibitors leads to apoptosis in CRC cells. Western blot analysis of full-length PARP or cleaved PARP (cl. PARP) in WiDr (**a**) and VACO432 (**b**) cells treated with PLX4032, gefitinib or erlotinib, or their combinations. Cells were harvested at 48 h after drug treatment. **c**, **d**, EGFR inhibitor cetuximab in combination with BRAF(V600E) inhibitor PLX4720 significantly suppresses tumour growth in two different xenograft models. WiDr (**c**) and VACO423 (**d**) cells were grown as tumour xenografts in NOD-SCID mice. After tumour establishment (200–250 mm^3), mice were treated with vehicle, cetuximab (40 mg kg^{-1}), PLX4720 (50 mg kg^{-1}) or cetuximab (40 mg kg^{-1}) plus PLX4720 (50 mg kg^{-1}), for 14 days. Mean tumour volumes ± standard error of the mean are shown ($n = 6$ mice per group).

(highly related to PLX4032, but easier to formulate for *in vivo* use), or the combination of EGFR inhibitor drug plus PLX4720 (see Methods). Figure 4c, d shows that treatment of mice with either PLX4720 or cetuximab monotherapy resulted in marginal growth inhibition. In contrast, the combination of cetuximab and PLX4720 elicited a potent growth inhibition of WiDr and VACO432 CRC tumours. Similarly, WiDr xenografted mice treated with the combination of erlotinib and PLX4720 derived significantly more benefit than those treated with either drug alone, mirroring the observations made *in vitro* (Supplementary Fig. 6).

Our data provide a strong rationale for a clinical trial combining BRAF and EGFR inhibitors in *BRAF*(*V600E*) mutant CRCs, which have a very poor clinical outcome and for which no targeted therapeutic strategies are effective after failure of standard chemotherapeutic regimens[2,3]. At first glance, it would seem counterintuitive to consider treating a *BRAF* mutant colon cancer with an EGFR inhibitor, as multiple clinical studies in colon cancer have shown that EGFR inhibition is without clinical benefit when either *KRAS* or *BRAF* is mutated downstream of EGFR[7,13,14]. The strong synergistic interaction between inhibition of BRAF and EGFR described here is explained by an unexpected and powerful feedback activation of EGFR caused by BRAF inhibition, providing a rationale for the poor clinical response of *BRAF*(*V600E*) mutant colon cancers to PLX4032 monotherapy[4]. The feedback activation of EGFR also implies that *EGFR* expression levels may be a clinically useful biomarker to predict the response to PLX4032 monotherapy in *BRAF* mutant tumours. As *BRAF*(*V600E*) mutations are also common in thyroid papillary carcinomas and hairy-cell leukaemias, *EGFR* expression levels may also help guide the selection of EGFR combination therapy in these cancers[8–10,19]. As some studies have shown EGFR expression in a subset of melanomas[20], (acquired) EGFR expression may also explain some of the clinical resistance against PLX4032 in melanoma.

There are a plethora of targeted drug agents in late-stage clinical development for the treatment of cancer. Given that resistance to monotherapy with these targeted therapies often develops rapidly, there is a trend towards combining targeted agents in clinical trials. However, the number of possible combinations of these agents seems endless. Our results highlight the power of 'synthetic lethality' genetic screens to identify which combinations of pathway inhibition are particularly effective. As such, these screens may help prioritize which combination therapies have the highest likelihood of being successful in the clinic.

METHODS SUMMARY

Pooled 'dropout' shRNA screen. A kinome shRNA library targeting the full complement of 518 human kinases and 17 kinase-related genes was constructed from the The RNAi Consortium (TRC) human genome-wide shRNA collection (TRC-Hs1.0). The kinome library was used to generate pools of lentiviral shRNA to infect WiDr cells. Cells stably expressing shRNA were cultured in the presence or absence of PLX4032. Massive parallel sequencing was applied to determine the abundance of shRNA in cells. shRNAs prioritized for further analysis were selected by the fold depletion caused by PLX4032 treatment.

Mouse xenografts and *in vivo* drug studies. All animal procedures were approved by the Ethical Commission of the Institute for Cancer Research and Treatment and by the Italian Ministry of Health. WiDr cells were injected subcutaneously into the right posterior flanks of 7-week-old immunodeficient NOD-SCID female mice (6 mice per group; Charles River). Tumour formation was monitored twice a week, and tumour volume based on caliper measurements was calculated by the modified ellipsoidal formula: tumour volume = ½ length × width (ref. 2). When tumours reached a volume of approximately 200–250 mm^3, mice were randomly assigned to treatment with vehicle or drug(s).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions R.B. and A.B. conceived the project and supervised all research. R.B., A.P., C.S. and S.H. wrote the manuscript. S.H., R.S. and RL.B. designed the experiments. A.P., C.S., S.H., F.D.N. and D.Z. performed the experiments.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.B. (r.bernards@nki.nl).

METHODS

Cell lines and reagents. A375, SW1417 and HEK293T cells were from the laboratory collection of R.B. SK-MEL-28 cells were a gift from D. Peeper. BHT-101 and HTC-C3 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DMSZ), Germany. These cells were cultured in DMEM supplemented with 8% FCS and 1% penicillin/streptomycin. WiDr and HT-29 cells were purchased from American Type Culture Collection (ATCC). VACO432, KM-20, SNUC5, OUMS23 and LIM2405 cells were from the laboratory collection of A. Bardelli. HTC-C3, 8508C and BHT-101 cells were purchased from DMSZ. These cells were cultured in RPMI supplemented with 8% FCS and 1% penicillin/streptomycin.

PLX4032 (catalogue no. S1267), PLX4720 (catalogue no. S1152) and gefitinib (catalogue no. S1025) were purchased from Selleck Chemicals. Erlotinib (catalogue no. RP01332e) was purchased from Sequoia Chemicals. Cetuximab was obtained from the Hospital Pharmacies at The Netherlands Cancer Institutes and from the Institute for Cancer Research and Treatment.

The TRC human genome-wide shRNA collection (TRC-Hs1.0) was purchased from Open Biosystems. Further information is available at http://www.broadinstitute.org/rnai/public/.

shRNA 'dropout' screen with a custom TRC kinome library. Lentiviral plasmids (pLKO.1) encoding shRNAs that target kinome candidates are listed in Supplementary Table 1. The kinome library consists of seven plasmids pools (TK1–TK7). Lentiviral supernatants were generated as described at http://www.broadinstitute.org/rnai/public/resources/protocols. WiDr cells were infected separately by the seven virus pools (multiplicity of infection <1) and selected with puromycin (2 $\mu g\,ml^{-1}$) for cells expressing integrated shRNA. Cells were then pooled and plated at 300,000 cells per 15 cm dish in the absence or presence of 1 μM PLX4032 (five dishes for each condition) and the medium was refreshed twice per week for 18 days. Genomic DNA was isolated as described[21]. shRNA inserts were retrieved from 8 μg genomic DNA by PCR amplification (PCR1 and PCR2, see below for primer information) using the following conditions: (1) 98 °C, 30 s; (2) 98 °C, 10 s; (3) 60 °C, 20 s; (4) 72 °C, 1 min; (5) to step (2), 15 cycles; (6) 72 °C, 5 min; (7) 4 °C. Indexes and adaptors for deep sequencing (Illumina) were incorporated into PCR primers. 2.5 μl of PCR1 products were used as templates for PCR2 reaction. PCR products were purified using Qiagen PCR purification Kit according to the manufacturer's manual. Sample quantification was performed by BioAnalyzer to ensure samples generated at different conditions were pooled at the same molar ratio before analysis by the Illumina genome analyser.

The shRNA stem sequence was segregated from each sequencing read and aligned to the TRC library. The matched reads were counted and the counts were transformed to abundance that was assigned to the corresponding shRNA.

The primers used are as follows: PCR1_Untreated replicate#1, forward, ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGATCCTTGTGGAAAGGACGAAACACCGG; PCR1_Untreated replicate#2, forward, ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGCTACTTGTGGAAAGGACGAAACACCGG; PCR1_PLX treated replicate#1, forward, ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAGCCCTTGTGGAAAGGACGAAACACCGG; PCR1_PLX treated replicate#1, forward, ACACTCTTTCCCTACACGACGCTCTTCCGATCTTACAAGCTTGTGGAAAGGACGAAACACCGG; PCR1, reverse (P7_pLKO1_r), CAAGCAGAAGACGGCATACGAGATTTCTTTCCCCTGCACTGTACCC; PCR2, forward, AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT; PCR2, reverse (P5_IlluSeq), CAAGCAGAAGACGGCATACGAGAT.

Individual shRNA vectors used were collected from the TRC library. EGFR: TRCN0000039633_GCTGAGAATGTGGAATACCTA; TRCN0000121068_GCCACAAAGCAGTGAATTTAT; TRCN0000121206_GCCAAGCCAAATGGCATCTTT. CDC25C: TRCN0000002433_GAAGAGAATAATCATCGTGTT; TRCN0000002434_GCCTTGAGTTGCATAGAGATT.

Short-term growth inhibition assays. Cultured cells were seeded into 96-well plates (2,000–3000 cells per well). Twenty-four hours after seeding, serial dilutions of PLX4032 were added to cells to final drug concentrations ranging from 0.04–10 μM. Cells were then incubated for 72 h and cell viability was measured using the CellTiter-Blue viability assay (Roche). Relative survival in the presence of PLX4032 was normalized to the untreated controls after background subtraction.

Long-term cell proliferation assays. Cells were seeded into 6-well plates (2×10^4 cells per well) and cultured both in the absence and presence of drugs as indicated. For the EGFR overexpression experiments, A375 and SK-MEL-28 cells were seeded $5–10 \times 10^4$ cells per well. More details are as described[22]. All relevant assays were performed independently at least three times.

Protein lysate preparation and immunoblots. The biochemical responses of cells treated with drugs were analysed by western blot. Cells were plated in medium containing 10% FCS. After 24 h, cells were washed with serum-free medium and cultured for 24 h in medium containing 0.1% serum. After the low serum incubation, cells were treated with drugs for 30 min and stimulated by 10% FCS. The lysates were then collected after 6 h using sample buffer containing 5% β-mercaptoethanol, 150 mM NaCl, 50 mM Tris pH 7.5, 2 mM EDTA pH 8, 25 mM NaF and 1% NP-40, protease inhibitors (Complete, Roche), Phosphatase Inhibitor Cocktails II and III (Sigma). All lysates were freshly prepared and resolved by SDS gel electrophoresis and followed by western blotting. Primary antibodies used are as follows: p-EGFR (Y1068) (AbCam), p-ERK1/2 (T202/Y204), ERK1, ERK2, HSP-90 (Santacruz), p-MEK1/2 (S217/221), MEK1/2, pAKT(S473), ATK1/2, PARP, CDC25C (5H9) and p-CDC25C Thr 48 (Cell Signalling Technology).

Mouse xenografts and *in vivo* drug studies. All drugs for *in vivo* studies were dissolved in DMSO, stored in aliquots at −80 °C and diluted daily in aqueous vehicle before administration. All animals were manipulated according to protocols approved by the Ethical Commission of the Institute for Cancer Research and Treatment and by the Italian Ministry of Health. All experiments were performed in accordance with relevant local and national guidelines and regulations. WiDr cells (6×10^6 cells per mouse) were injected subcutaneously into the right posterior flanks of 7-week-old immunodeficient NOD-SCID female mice (6 mice per group; Charles River). Tumour formation was monitored twice a week, and tumour volume based on caliper measurements was calculated by the modified ellipsoidal formula: tumour volume = ½ length × width (ref. 2). When tumours reached a volume of approximately 200–250 mm^3, mice were randomly assigned to treatment with vehicle (0.2% Tween 80 and 1% methylcellulose in sterile PBS by daily gavage), cetuximab (40 $mg\,kg^{-1}$ of body weight intraperitoneally twice a week), erlotinib (80 $mg\,kg^{-1}\,day^{-1}$ in 0.2% Tween 80 and 1% methylcellulose in sterile PBS by orogastric gavage), PLX4720 (50 $mg\,kg^{-1}\,day^{-1}$ in 0.2% Tween 80 and 1% methylcellulose in sterile PBS by orogastric gavage); or to a drug combination (cetuximab plus PLX720, or erlotinib plus PLX4720), in which each compound was administered at the same dose and scheduled as single agents.

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Supplemental data-Chapter 2

Unresponsiveness of colon cancer to BRAF (V600E) inhibition through feedback activation of EGFR

Nature 483, 100-103, 2012

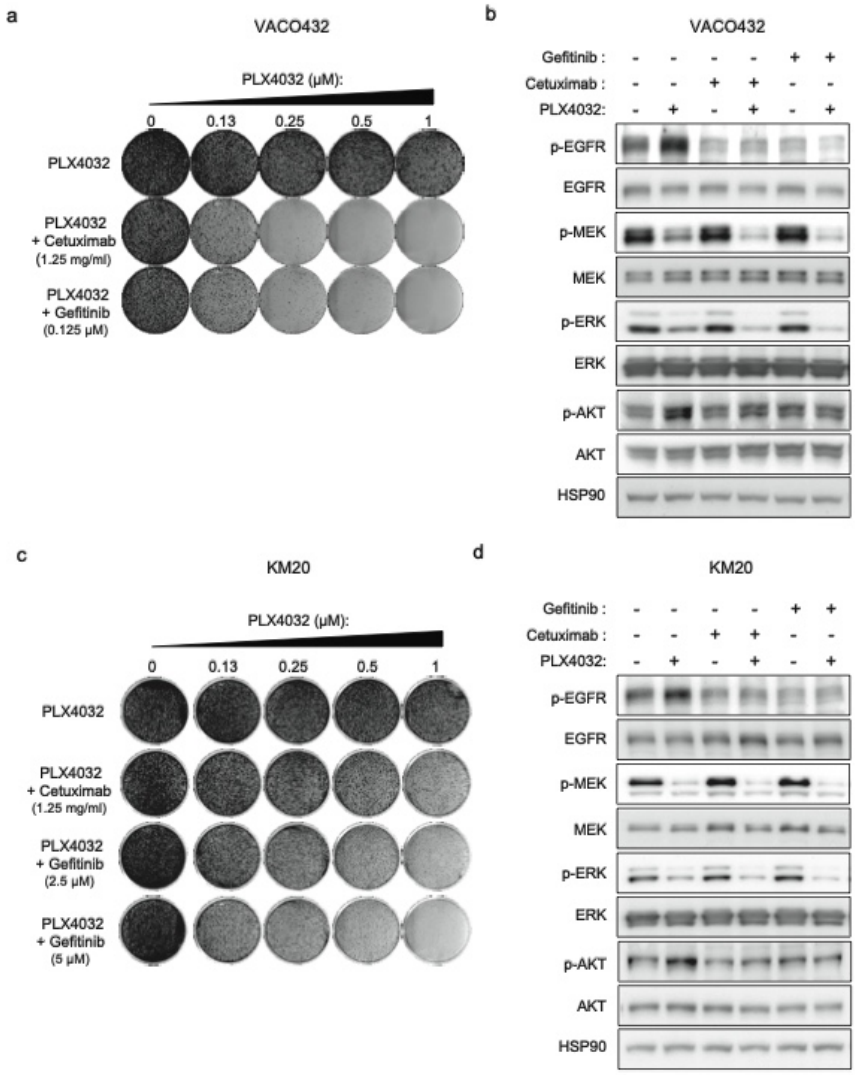

Figure S1. Functional and biochemical interaction between BRAF and EGFR inhibition in additional colon cancer cell lines.

a, Synergistic response of VACO432 cells to the combination of EGFR and $BRAF^{V600E}$ inhibitors. VACO423 cells were cultured in increasing concentrations of BRAF inhibitor

PLX4032 alone, EGFR inhibitors cetuximab (1.25 mg/ml) or gefitinib (0.125 μM) alone, or their combinations. The cells were fixed, stained and photographed after 18 days.

b, Resistance to $BRAF^{V600E}$ inhibition in VACO432 cells is mediated through feedback activation of EGFR. Biochemical response of VACO432 cells treated with PLX4032, cetuximab or gefitinib, or their combinations were documented by western blot analysis. Cells were harvested at 6 h after drug treatment. $BRAF^{V600E}$ inhibition results in strong feedback up regulation of TYR1068-phosphorylated EGFR (p-EGFR) and Ser473-phosphorylated AKT (p-AKT), which is abrogated by EGFR inhibitors. Furthermore, combination treatments result in complete inhibition of phosphorylated MEK (p-MEK) and phosphorylated ERK (p-ERK). Heat shock protein 90 (HSP90) served as a control.

c, Synergistic response of KM20 cells to the combination of EGFR and $BRAF^{V600E}$ inhibitors. KM20 cells were cultured in increasing concentrations of PLX4032 alone, cetuximab (1.25 mg/ml) or gefitinib (2.5 or 5 μM) alone, or their combinations. The cells were fixed, stained and photographed after for 18 days.

d, Resistance to $BRAF^{V600E}$ inhibition in KM20 cells is mediated through feedback activation of EGFR. Biochemical response of KM20 cells treated with PLX4032, cetuximab or gefitinib, or their combinations were documented by western blot analysis. Cells were harvested at 6 h after drug treatment. $BRAF^{V600E}$ inhibition results in strong feedback upregulation of phosphorylated EGFR (p-EGFR) and phosphorylated AKT (p-AKT), which is abrogated by EGFR inhibitors. Furthermore, combination treatments result in complete inhibition of phosphorylated MEK (p-MEK) and phosphorylated ERK (p-ERK).

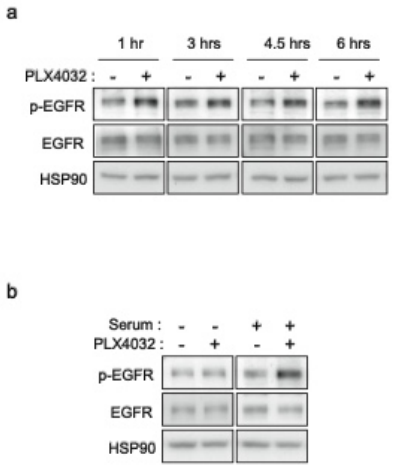

Figure S2. Feedback activation of EGFR is ligand dependent.

a, Time course of the feedback activation of EGFR in respond to $BRAF^{V600E}$ inhibition. Western blot analysis of p-EGFR and total EGFR levels in VACO432 cells treated with PLX4032 for 1, 3, 4.5 and 6 hours. HSP90 served as a control. $BRAF^{V600E}$ inhibition results in strong feedback up regulation of p-EGFR in all the time points.

b, Feedback activation of EGFR is ligand dependent. Western blot analysis of p-EGFR and total EGFR levels in VACO432 cells treated with PLX4032 for 3hours in the absence or presence of serum stimulation. HSP90 served as a control. $BRAF^{V600E}$ inhibition results in strong feedback up regulation of p-EGFR only with serum stimulation.

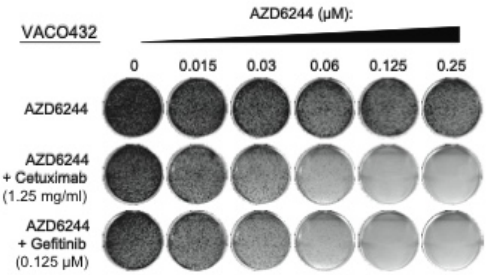

Figure S3. EGFR and MEK inhibitors also synergize to inhibit cell growth of $BRAF^{V600E}$ mutant **CRC cells**

Synergistic response of VACO432 cells to the combination of EGFR and MEK inhibitors. VACO432 cells were cultured in increasing concentrations of AZD6244 alone, cetuximab (1.25 mg/ml) or gefitinib (0.125 μM) alone, or their combinations. The cells were fixed, stained and photographed after for 18 days.

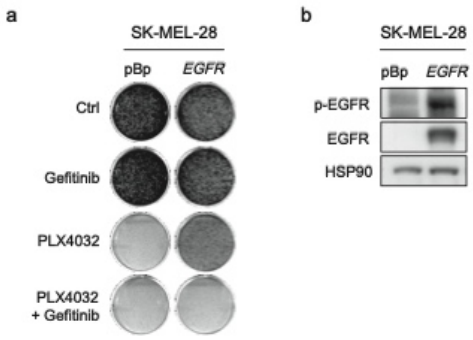

Figure S4. Ectopic expression of *EGFR* in SK-MEL-28 melanoma cells is sufficient to cause resistance to PLX4032.

a, EGFR overexpression confers resistance to PLX4032, but not to the combination of PLX4032 and gefitinib in SK-MEL-28 melanoma cells. SK-MEL-28 cells expressing pBabe vector control or *EGFR* were cultured in PLX4032 (5 μM), gefitinib (2.5 μM) or their combination. The cells were fixed, stained and photographed after for 7 (untreated or gefitinib) or 9 (PLX4032 alone or in combination with gefitinib) days.

b, Western blot analysis of p-EGFR and total EGFR levels in cells described above. HSP90 served as a control.

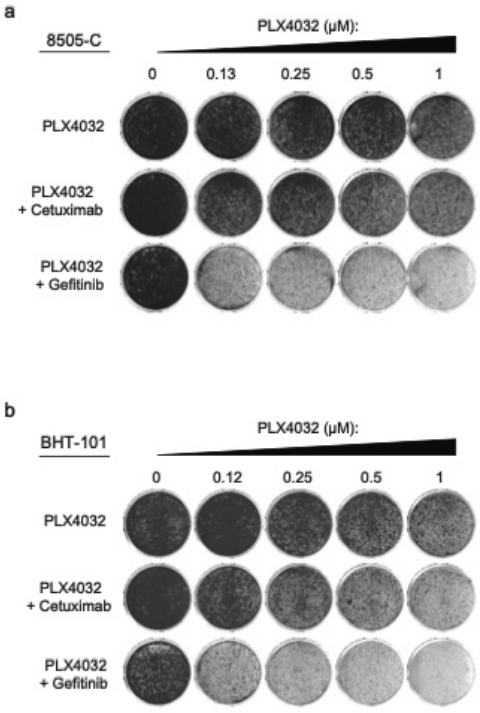

Figure S5. EGFR inhibitor also synergize with PLX4032 to overcome the resistance to BRAF^{V600E} inhibition in thyroid cancer cells that express high levels of EGFR.

a, Synergistic response of 8505 cells to the combination of EGFR and BRAF^{V600E} inhibitors. 8505 cells were cultured in increasing concentrations of PLX4032 alone, cetuximab (1.25 mg/ml) or gefitinib (2.5 μM) alone, or their combinations. The cells were fixed, stained and photographed after for 18 days.

b, Synergistic response of BHT-101 cells to the combination of EGFR and BRAF^{V600E} inhibitors. BHT-101 cells were cultured in increasing concentrations of PLX4032 alone, cetuximab (1.25 mg/ml) or gefitinib (5 μM) alone, or their combinations. The cells were fixed, stained and photographed after for 18 days.

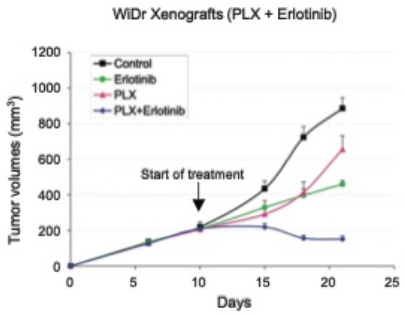

Figure S6. EGFR and BRAF^{V600E} small molecule inhibitor inhibitors synergize to suppress colon cancer tumour growth in a xenograft model.

EGFR small molecule inhibitor erlotinib in combination with BRAF^{V600E} inhibitor PLX4720 significantly suppresses tumour growth in a WiDr xenograft model. WiDr cells were grown as tumour xenografts in NOD-SCID mice. After tumour establishment (200-250 mm^3), mice were treated with vehicle, erlotinib (80 mg/kg), PLX4720 (50 mg/kg) or erlotinib (80 mg/kg) plus PLX4720 (50 mg/kg) for 11 days. The experiment had to be terminated at 21 days due to infection-related death of all animals in one of the monotherapy control arms. Mean tumour volumes ± s.e.m. are shown (n = 6 mice per group).

Chapter 3

PTPN11 is a central node in intrinsic and acquired resistance to targeted cancer drugs

Submitted for publication

PTPN11 is a central node in intrinsic and acquired resistance to targeted cancer drugs

Anirudh Prahallad[1*], Guus.J.J.E.Heynen[1*], Giovanni Germano [2,3], Stefan M. Willems[1, 4], Bastiaan Evers[1], Loredana Vecchione[1], Valentina Gambino[1], Cor Lieftink[1], Roderick L. Beijersbergen[1], Federica Di Nicolantonio[2, 5], Alberto Bardelli[2, 5] and Rene Bernards[1§]

Running title: PTPN11 in drug resistance

Key words: PTPN11, drug resistance, synthetic lethality, BRAF, colon cancer, melanoma

[1]Division of Molecular Carcinogenesis, Cancer Systems Biology Centre and Cancer Genomics Centre Netherlands, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

[2]Candiolo Cancer Institute – FPO, IRCCS, Str prov 142 Km 3.95, 10060 Candiolo, Torino, Italy.

[3]FIRC Institute of Molecular Oncology (IFOM), Milan, Italy

[4]Department of Pathology, University Medical Centre Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands.

[5]University of Torino, Department of Oncology, Str prov 142 Km 3.95, 10060 Candiolo, Torino, Italy.

*These authors contributed equally to this work

[§]To whom correspondence should be addressed E-mail: r.bernards@nki.nl

ABSTRACT

Most *BRAF(V600E)* mutant melanomas are sensitive to selective BRAF inhibitors, but *BRAF* mutant colon cancers are intrinsically resistant to these drugs due to feedback activation of EGFR. We performed an RNA interference-based genetic screen in *BRAF* mutant colon cancer cells to search for phosphatases whose knockdown induces sensitivity to BRAF inhibition. We find that suppression of protein tyrosine phosphatase non-receptor type 11 (*PTPN11*) confers sensitivity to BRAF inhibitors in colon cancer. Mechanistically, we find that inhibition of *PTPN11* blocks signaling from receptor tyrosine kinases (RTKs) to the RAS-MEK-ERK pathway. *PTPN11* suppression is lethal to cells that are driven by activated RTKs and prevents acquired resistance to targeted cancer drugs that results from RTK activation. Our findings identify PTPN11 as a drug target to combat both intrinsic and acquired resistance to several targeted cancer drugs. Moreover, activated PTPN11 can serve as a biomarker of drug resistance resulting from RTK activation.

INTRODUCTION

Intrinsic and acquired resistance to targeted cancer drugs remains a huge problem in the treatment of cancer. As one example, the effects of small molecule inhibitors of the oncogenic BRAF (V600E) protein in *BRAF* mutant colon cancer is negated through the activation of feedback loops that engage the Epidermal Growth Factor Receptor (EGFR) (Prahallad et al., 2012, Corcoran et al., 2012) leading to reactivation of MAPK and PI3K pathways. Similarly, inhibition of the MEK kinases in *KRAS* mutant tumors results in activation of ERBB2 and ERBB3 kinases, which again limits the response to MEK inhibitors (Sun et al., 2014a). In both examples, the synthetic lethal interactions between the drugs and the inhibition of specific signaling pathways were identified through loss-of-function genetic screens, pointing at the utility of this approach to identify effective drug combinations.

Protein tyrosine phosphatases (PTPs) have been implicated in many human diseases including cancer (Hendriks et al., 2013, Hunter, 2009). Somatic mutations in the PTP gene super family are found in different tumor types with *PTPRP* being the most frequently mutated PTP in human cancer. *PTPRT, PTPRF, PTPRG, PTPN3, PTPN13* and *PTPN14*, are found mutated in ~26% of colorectal cancers (CRCs) (Zhao et al., 2014). Protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*, also known as *SHP2*) was the first *bona fide* tyrosine phosphatase to be identified as an oncogene(Bennett et al., 1996, Saxton et al., 1997, Yamauchi et al., 1995). *PTPN11* is ubiquitously expressed and is implicated in transduction of mitogenic, pro-survival and pro-migratory signals from growth factor-, cytokine- and other extracellular matrix receptors (Ostman et al., 2006). PTPN11 is required for the full activation of RAS-MAPK-ERK signaling downstream of most receptor tyrosine kinases (RTKs) (Qu, 2000, Shi et al., 2000). Gain-of-function mutations in *PTPN11* occur in about 50% of Noonan syndrome patients (Tartaglia et al., 2001). Activating mutations in *PTPN11* have also been documented in adult acute myelogenous leukemia, gastric cancer, glioblastoma and anaplastic large cell lymphoma (Bentires-Alj et al., 2004, Chan et al., 2008, Zhan et al., 2009, Higashi et al., 2002).

Using a "phosphatome" centered loss-of-function genetic screen we identify PTPN11 as a drug target to treat both intrinsic and acquired resistance to a number of targeted cancer drugs.

RESULTS

PTPN11 is synthetic lethal with BRAF inhibition in *BRAF* mutant colon cancer.

We set out to identify phosphatases whose suppression can sensitize *BRAF(V600E)* mutant CRC cells to the BRAF inhibitor vemurafenib. We assembled a collection of 1665 shRNA vectors that together target 298 phosphatases (or phosphatase related genes) (Sacco et al., 2012). To find phosphatases whose suppression display synergistic inhibition of proliferation and survival with vemurafenib in *BRAF* mutant CRC cells, we infected *BRAF* mutant Widr CRC cells that are intrinsically vemurafenib resistant (Prahallad et al., 2012) with the phosphatase shRNA library and cultured them in the presence or absence of vemurafenib (**Figure 1a**). After 10 (untreated) or 18 (drug treated) days of selection, cells were harvested and genomic DNA from both the cell populations was harvested. The bar codes contained in the shRNA cassettes were amplified by PCR and the abundance was determined by deep sequencing as described (Prahallad et al., 2012). We only considered genes for which two independent shRNAs could be identified with an average read count of more than 1000 and which were depleted at least 2-fold (log2 scale Y axis -1) by the drug treatment. Figure **1b** shows that only very few of the 298 genes in the library met these selection criteria. *PTPN11*, *CLEC1B* and *PPFIA1* were the only three genes that met these selection criteria, of which *PTPN11* showed the strongest fold depletion (Supplementary Table 1). We therefore focused on *PTPN11* for further investigation. First, we tested additional hairpins from the TRC.2.0 library collection for *PTPN11* knockdown efficiency (data not shown) and decided to use hairpins #5003 (from screen) and #818 (from TRC2.0) for our studies.

To validate the result from the screen, we introduced these two *PTPN11* shRNAs (sh#5003 and sh#818) into the *BRAF* mutant CRC cell lines Widr and Vaco432 and cultured them in the absence or presence of vemurafenib. **Figure 1c** and **1d** show that the control vector-

Fig 1

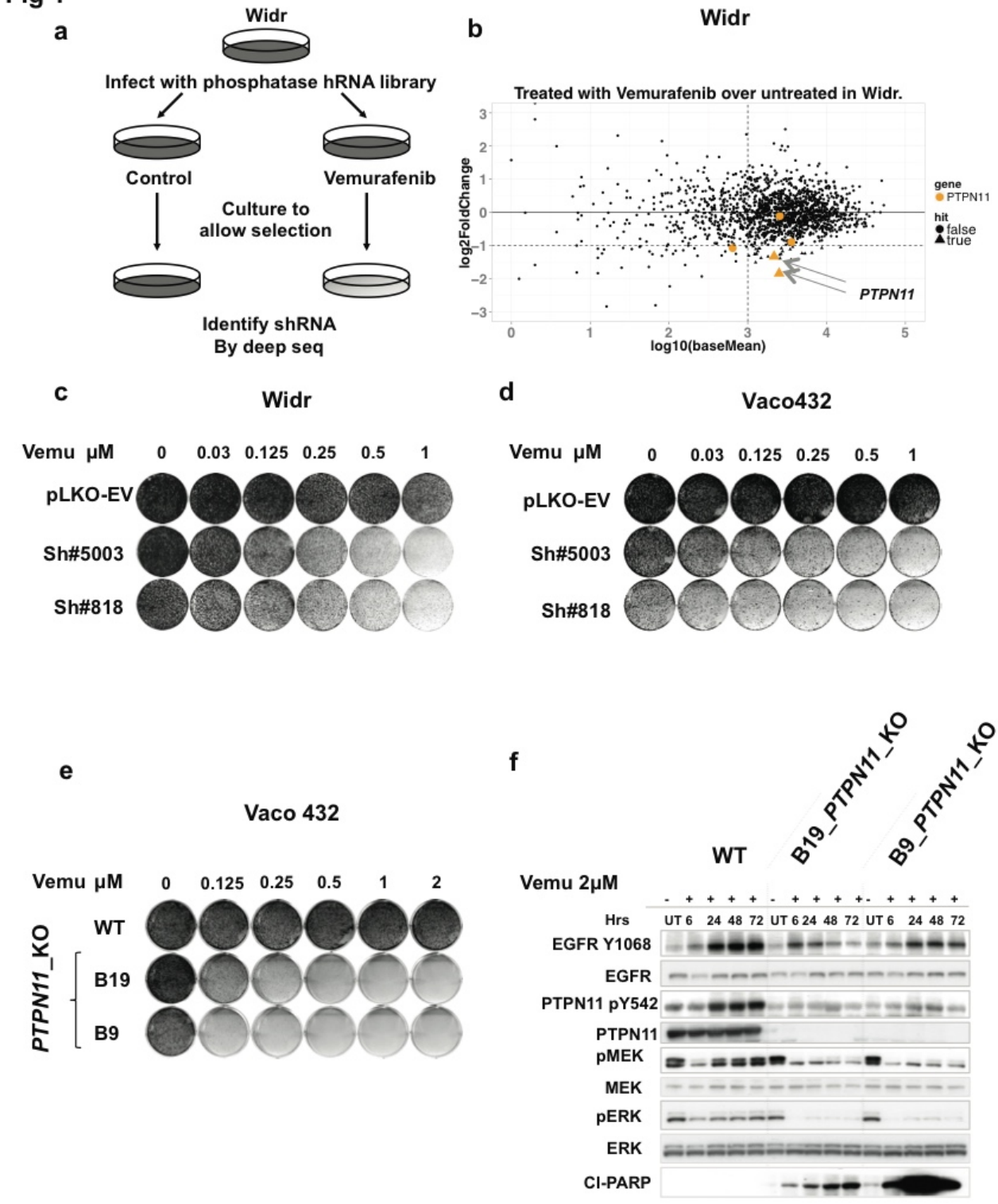
a
Widr
Infect with phosphatase hRNA library
Control
Vemurafenib
Culture to allow selection
Identify shRNA By deep seq
b
Widr
Treated with Vemurafenib over untreated in Widr.
log2FoldChange
log10(baseMean)
gene
PTPN11
hit
false
true
PTPN11
c
Widr
Vemu μM 0 0.03 0.125 0.25 0.5 1
pLKO-EV
Sh#5003
Sh#818
d
Vaco432
Vemu μM 0 0.03 0.125 0.25 0.5 1
pLKO-EV
Sh#5003
Sh#818
e
Vaco 432
Vemu μM 0 0.125 0.25 0.5 1 2
PTPN11_KO
WT
B19
B9
f
WT
B19_PTPN11_KO
B9_PTPN11_KO
Vemu 2μM
Hrs UT 6 24 48 72 UT 6 24 48 72 UT 6 24 48 72
EGFR Y1068
EGFR
PTPN11 pY542
PTPN11
pMEK
MEK
pERK
ERK
Cl-PARP

Figure 1. Inhibition of *PTPN11* confers sensitivity to BRAF inhibition in BRAF mutant colon cancer.

a, Schematic outline of the phosphatome-centered dropout shRNA screen for enhancers of vemurafenib sensitivity.

b, Representation of relative abundance of the shRNA barcode sequences from the shRNA screen depicted in a M/A plot where each dot represents individual shRNA. The y axis shows log2 fold change (relative abundance of vemurafenib treated/untreated) and the x-axis shows intensity (average sequence reads in untreated sample) of each shRNA.

c, Two independent non overlapping shRNA targeting *PTPN11* (#5003 and #818) enhance sensitivity to vemurafenib in both Widr **(c)** and Vaco432 BRAF mutant CRC cells **(d).** Depicted are colony formation assays compared to the pLKO treated control cells that are resistant to vemurafenib. The cells were treated for 14 days and fixed with 4% formaldehyde and stained with 0.1% crystal violet and photographed.

e, PTPN11 knockout was generated using a lentiviral inducible CRISPR-CAS9 vector in the *BRAF* mutant Vaco432 cells. Shown are colony formation assays in the presence of vemurafenib of *PTPN11* knockout Vaco432 cells (clones #B19 and #B9) compared to the parental Vaco 432 cells.

f, Biochemical changes observed under vemurafenib treatment at different time points in Vaco432 cells in comparison with the *PTPN11* knockout clones #B19 and #B9. Vemurafenib treatment of parental Vaco432 cells results in feedback activation of EGFR Y1068 and *PTPN11* Y542 as a consequence driving reactivation of pMEK and pERK signaling. The *PTPN11* knockout cells treated with vemurafenib activated EGFR but were unable to reactivate MEK-ERK signaling and conferred sensitivity to BRAF inhibition. The combined effect of *PTPN11* and BRAF V600E blockade leads to apoptotic cell death as measured by PARP cleavage (Cl-PARP).

infected Widr and Vaco432 cells are intrinsically resistant to PLX4032 (Prahallad et al., 2012). Suppression of *PTPN11* in Widr and Vaco432 cells was efficient (Figures S1a and S1b), but showed no major effect on cell number. However, the combination of *PTPN11* suppression and vemurafenib caused a marked inhibition of cell numbers (Figures **1c, d**). Suppression of *PTPN11* in Widr and Vaco432 cells prevented reactivation of MEK-ERK signaling compared to the control cells (Figures **S1a** and **S1b**). To study this further, we generated knockout of *PTPN11* in the *BRAF* mutant Vaco432 cells using an inducible CRISPR-Cas9 vector. We selected multiple independent clones and validated the loss of PTPN11 expression by western blot analysis. Vaco432 clones #B19 and #B9 had complete loss of PTPN11 protein (Figure **1f**). *PTPN11* knockout had no effect on cell proliferation in the absence of vemurafenib, consistent with the notion that PTPN11 is upstream of mutant BRAF (Figures **1e**, Figure S**2a, b**). However, treatment of knockout cells with vemurafenib had a dramatic effect on proliferation, both in long term and in short term assays (Figure **1e**, **S2a, b**). Similar results were also obtained in *BRAF* mutant Widr CRC cells (Figures **S1 c**).

As reported earlier, in Vaco432 cells vemurafenib treatment induces feedback activation of EGFR as evidenced by increase in tyrosine 1068 phosphorylation (Prahallad et al., 2012) (Fig **1f**). EGFR activation also correlated with phosphorylation of tyrosine 542 of PTPN11, indicative of PTPN11 activation by RTK signaling (Araki et al., 2003). This resulted in restoration of MEK and ERK signaling as evidenced by pMEK and pERK rebound (Figure **1f**). In contrast, *PTPN11* knockout clones of Vaco432 activated EGFR, but had an approximately 80% drop in P-ERK levels after 48-72 hours upon vemurafenib treatment, resulting in massive apoptosis, as evidenced by the appearance of cleaved PARP (Figure **1f**). Identical results were seen in *PTPN11* knockout clones for BRAF mutant Widr cells (Figure **S1c, d**).

Fig 2

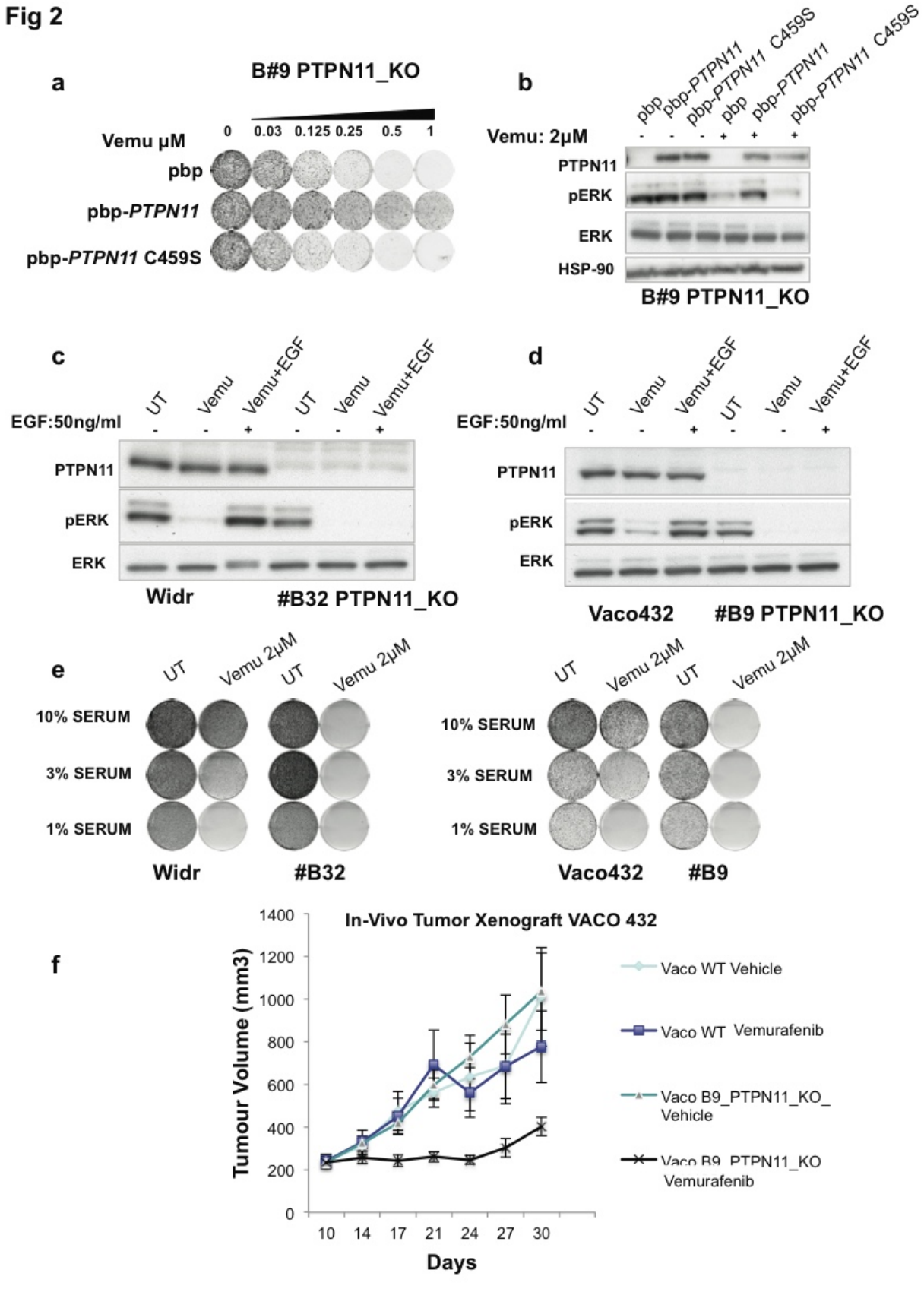
a
B#9 PTPN11_KO
Vemu µM
0
0.03
0.125
0.25
0.5
1
pbp
pbp-PTPN11
pbp-PTPN11 C459S
b
pbp
pbp-PTPN11
pbp-PTPN11 C459S
pbp
pbp-PTPN11
pbp-PTPN11 C459S
Vemu: 2µM
PTPN11
pERK
ERK
HSP-90
B#9 PTPN11_KO
c
EGF:50ng/ml
UT
Vemu
Vemu+EGF
UT
Vemu
Vemu+EGF
PTPN11
pERK
ERK
Widr
#B32 PTPN11_KO
d
EGF:50ng/ml
UT
Vemu
Vemu+EGF
UT
Vemu
Vemu+EGF
PTPN11
pERK
ERK
Vaco432
#B9 PTPN11_KO
e
UT
Vemu 2µM
UT
Vemu 2µM
10% SERUM
3% SERUM
1% SERUM
Widr
#B32
UT
Vemu 2µM
UT
Vemu 2µM
10% SERUM
3% SERUM
1% SERUM
Vaco432
#B9
f
In-Vivo Tumor Xenograft VACO 432
Tumour Volume (mm3)
1400
1200
1000
800
600
400
200
0
10
14
17
21
24
27
30
Days
Vaco WT Vehicle
Vaco WT Vemurafenib
Vaco B9_PTPN11_KO_ Vehicle
Vaco B9_PTPN11_KO Vemurafenib

Figure 2. PTPN11 inhibition is synthetic lethal with BRAF inhibition in vivo.

a, Reconstitution of wild type or phosphatase dead (C459S) mutant of *PTPN11* into *PTPN11* Knockout Vaco432 cells, where pbp denotes the vector pBabe-puro. *PTPN11* Knockout Vaco432 cells are sensitive to vemurafenib; reconstitution with wild type *PTPN11* confers resistance whereas reconstitution with phosphatase dead mutant (C459S) confers sensitivity.

b, Western blot showing the expression of both wild type and C459S mutant to near physiological levels in *PTPN11* knockout VAco432 cells . Wild type *PTPN11* expression reactivates ERK in the presence of vemurafenib whereas; C459S mutants do not reactivate ERK phosphorylation.

c, 2μM vemurafenib treatment for 2hr in overnight serum starved Widr **(c)** and Vaco432 (**d**) *PTPN11* wild-type cells confers complete ERK inhibition upon 2μM vemurafenib whereas addition of EGF (50ng/ml) for 30min to the cells completely reactivates ERK phosphorylation. Treatment of 2μM vemurafenib in overnight serum starved *PTPN11* knockout Widr (#B32 clone) and Vaco432 (#B9 clone) for 2hrs also resulted in complete ERK inhibition and, addition of EGF was not able to restore ERK phosphorylation.

e, colony formation of Widr (parental and *PTPN11* Knockout #B32) and Vaco 432 (Parental and *PTPN11* knockout #B9) cultured under reducing serum concentrations (0.1%, 1%, 3% and 10%) with and without vemurafenib (2μM) treatment for 14days.

f, Vaco432 parental and *PTPN11* knockout clone #B9 were grown as tumor xenografts in NOD-SCID mice. After tumor establishment (200-250mm^3), mice were treated with either vehicle or vemurafenib (60mg/kg), for 30 days. Mean tumor volumes +/- standard error of the mean is shown (n=7 mice per group).

PTPN11 inhibition is synthetic lethal with BRAF inhibition in vivo.

We reconstituted the *PTPN11* knockout Vaco432 clone (#B9) that is sensitive to vemurafenib, with either a wild type *PTPN11* vector or a phosphatase dead mutant of *PTPN11* (C459S). Expression of wild type *PTPN11*, but not the phosphatase dead mutant (C459S), conferred resistance to vemurafenib and restored ERK phosphorylation (Figures **2a, b**). We conclude that the phosphatase activity of PTPN11 is critical for the observed synthetic lethal phenotype with vemurafenib in *BRAF* mutant CRC. Recently, GS493 was identified as a specific inhibitor of PTPN11 that inhibits the catalytic domain of PTPN11 (Grosskopf et al., 2015). We tested the ability of GS493 to resensitize *BRAF* mutant CRC cell lines to vemurafenib. Treatment of Widr and Vaco432 cells with single agent GS493 had no effect on cell proliferation, consistent with the notion that PTPN11 inhibition is upstream of BRAF. However, combining GS493 with vemurafenib synergistically inhibited cell proliferation (Figure **S1e**). Biochemically, inhibition of PTPN11 with GS493 alone did not reduce phosphorylation of ERK, whereas the combination of vemurafenib and GS493 led to a further reduction in downstream ERK phosphorylation (Figure **S1f**).

EGFR activation upon vemurafenib treatment requires its ligand EGF (Prahallad et al., 2012). Consistent with this previous observation, vemurafenib treatment of serum-starved Vaco432 and Widr cells fully inhibited ERK phosphorylation. However, addition of exogenous EGF fully restored ERK phosphorylation even in the presence of vemurafenib (Figures **2c, d**). In contrast, *PTPN11* knockout clones of Vaco432 and Widr failed to reactivate ERK signaling in response to EGF (Figures **2c, d**). Consistent with this, both the *BRAF* mutant CRC cell lines Vaco432 and Widr only showed sensitivity to vemurafenib in the presence of low serum concentrations (3% serum and 1% serum), whereas *PTPN11* knockout derivatives were sensitive to vemurafenib under all conditions (Figure **2e**). Addition of an EGFR inhibitor to vemurafenib in *PTPN11* knockout Widr cells did not further inhibit cell proliferation, indicating that EGFR signaling is fully abrogated in the absence of PTPN11 (Supplementary Figures **S3a, b**). Together, these data indicate that inactivation of *PTPN11* effectively blocks

the effects of EGFR reactivation on the RAS-MEK-ERK pathway and consequently confers robust sensitivity to vemurafenib in the *BRAF* mutant CRC cells.

To test whether the observed synthetic lethal effect of *PTPN11* loss with BRAF inhibition is also observed *in vivo*, we xenografted nude mice with parental Vaco432 cells and a *PTPN11* knockout clone of Vaco432 (#B9). Both the parental Vaco432 cells and the *PTPN11* knockout clone (#B9) formed tumors in mice. However, in *PTPN11* knockout cells, but not in parental Vaco432 cells, vemurafenib treatment potently inhibited tumor growth in vivo (Figure **2f**).

PTPN11 loss is lethal for RTK driven tumors

Activating mutations in RTKs can drive tumorigenesis (Bhargava et al., 2005, Burstein, 2005, Paez et al., 2004, Koivunen et al., 2008). Although there are several selective inhibitors for these activated RTKs, targeting multiple nodes in these RTK signaling pathways may delay the onset of drug resistance. We therefore investigated whether PTPN11 represents an additional drug target in cell lines that harbor activated RTKs. We used three cell lines that harbored specific activations of RTKs, which included *EGFR* amplification (Difi cells), *EGFR* mutation (PC9) and an *EML4-ALK* translocation (H3122). Difi CRC cells manifest elevated activation of PTPN11 as measured by Y542 phosphorylation. Cetuximab, a monoclonal antibody directed against EGFR, inhibits Difi cell proliferation by reducing the phosphorylation of EGFR, PTPN11 and ERK (Figure **3a**, **b**) showing a direct connection between EGFR, PTPN11 and ERK. Suppression of *PTPN11* using shRNA (#5003) also led to marked suppression of Difi cell proliferation (Figure **3a**). PC9 cells, harboring an activating mutation in *EGFR,* fully suppressed PTPN11 Y542 and ERK phosphorylation upon gefitinib treatment (Figure **3d**) and their viability upon suppression of *PTPN11* was severely reduced (Figure **3c**). Similarly, in the *EML4-ALK* translocated lung cancer cell line H3122 crizotinib treatment inhibited cell proliferation by reducing PTPN11 and ERK phosphorylation (Figure

Fig. 3

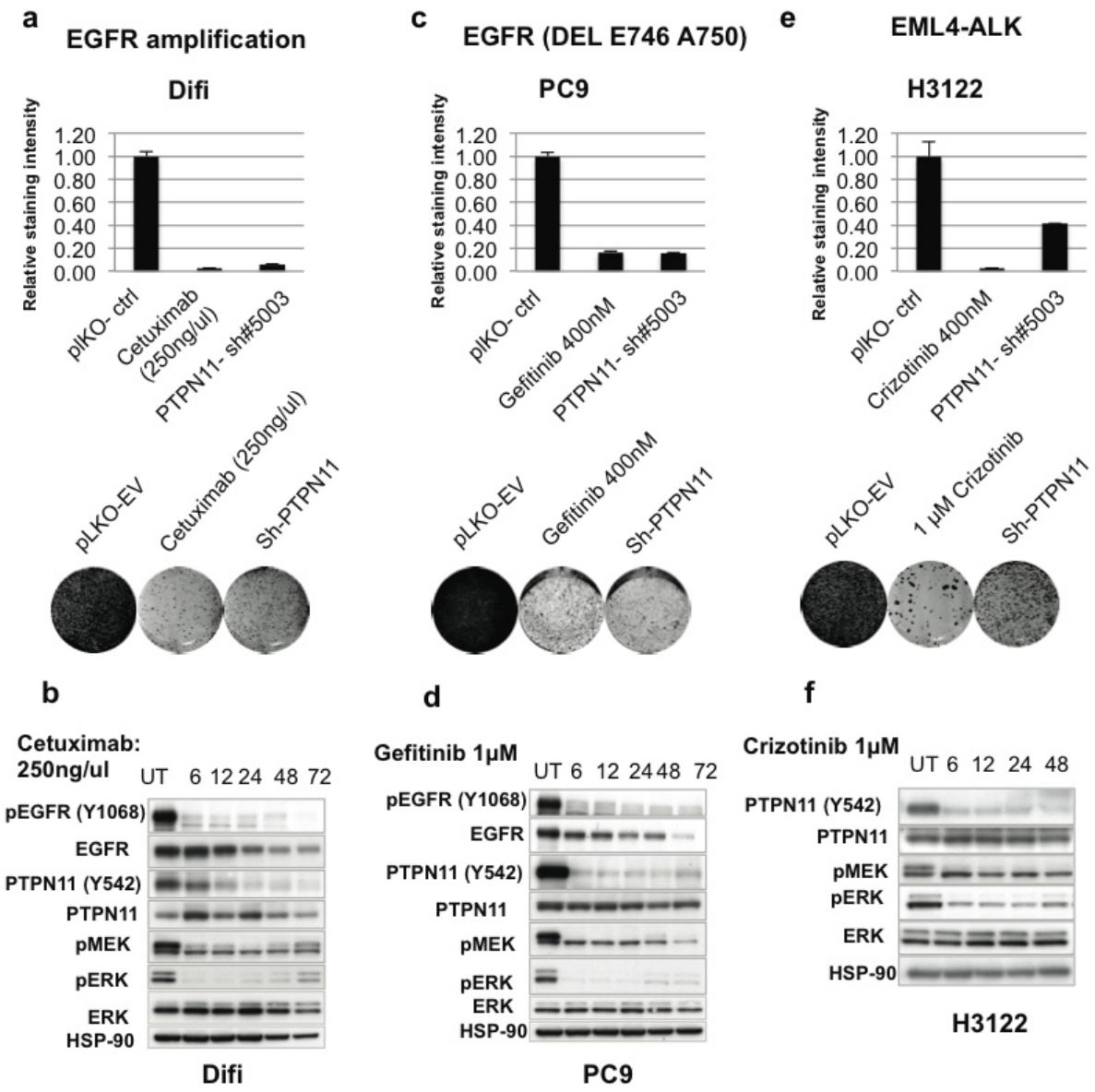
a
EGFR amplification
Difi
Relative staining intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
plKO- ctrl
Cetuximab (250ng/ul)
PTPN11- sh#5003
pLKO-EV
Cetuximab (250ng/ul)
Sh-PTPN11
c
EGFR (DEL E746 A750)
PC9
Relative staining intensity
plKO- ctrl
Gefitinib 400nM
PTPN11- sh#5003
pLKO-EV
Gefitinib 400nM
Sh-PTPN11
e
EML4-ALK
H3122
Relative staining intensity
plKO- ctrl
Crizotinib 400nM
PTPN11- sh#5003
pLKO-EV
1 μM Crizotinib
Sh-PTPN11
b
Cetuximab: 250ng/ul
UT 6 12 24 48 72
pEGFR (Y1068)
EGFR
PTPN11 (Y542)
PTPN11
pMEK
pERK
ERK
HSP-90
Difi
d
Gefitinib 1μM
UT 6 12 24 48 72
pEGFR (Y1068)
EGFR
PTPN11 (Y542)
PTPN11
pMEK
pERK
ERK
HSP-90
PC9
f
Crizotinib 1μM
UT 6 12 24 48
PTPN11 (Y542)
PTPN11
pMEK
pERK
ERK
HSP-90
H3122

Figure 3. PTPN11 inhibition is lethal to RTK driven tumors.

a, *EGFR* amplified Difi cells were grown with and without cetuximab for 14 days. In parallel, shPTPN11 derivatives of Difi were cultured for 14 days, fixed and stained. Top: quantification of staining of dishes shown below.

b, d, f, Biochemical changes seen on western blot upon cetuximab (250ng/ml) (**b**) gefitinib (**d**) or crizotinib (**f**) treatment following time points using pEGFR Y1068, pPTPN11 Y542, pERK and pMEK antibodies.

c, EGFR mutant PC9 cells were grown with and without gefitinib for 14 days. In parallel, shPTPN11 derivatives of PC9 were cultured for 14 days, fixed and stained. Top: quantification of staining of dishes shown below.

e, EML-ALK translocated H3122 NSCLC cells were grown with and without crizotinib for 14 days. In parallel, shPTPN11 derivatives of H3122 were cultured for 14 days, fixed and stained. Top: quantification of staining of dishes shown below.

EML4-ALK translocated lung cancer cells are sensitive to 400nM Crizotinib treatment or sensitive to *PTPN11* inhibition.

3e). These cells display marked sensitivity to crizotinib treatment and to loss of *PTPN11,* suggesting that oncogenic EML4-ALK signaling also requires PTPN11 (Figure **3f**).

***PTPN11* loss abrogates growth factor driven resistance in melanoma.**

BRAF mutant melanomas in general have a favorable response to BRAF inhibition since they mostly lack *EGFR* expression (Prahallad et al., 2012, Sun et al., 2014b). Consistent with this, *BRAF* mutant melanoma cells that are sensitive to PLX4032 do not activate PTPN11, MEK or ERK upon PLX4032 treatment. In contrast, *BRAF* mutant CRC cell lines show strong feedback activation of PTPN11, MEK and ERK upon vemurafenib treatment (Figure **4a, b**). It has been established that certain growth factors can confer resistance to BRAF inhibitors in melanoma (Wilson et al., 2012, Straussman et al., 2012). To investigate whether *PTPN11* loss prevents growth factor-driven resistance in melanoma, we generated *PTPN11* knockout clones of SK-Mel888 *BRAF(V600E)* mutant melanoma cells. We examined the effect of three different growth factors (HGF, FGF9, SCF) that can be secreted by the tumor stroma to confer resistance to vemurafenib in melanoma. Exposure of parental SK-Mel888 cells to any of the three growth factors alone showed no significant effect on cell proliferation in the absence of vemurafenib. However, in the presence of vemurafenib HGF, SCF or FGF9 potently conferred drug resistance. In contrast, two independent *PTPN11* knockout clones of SK-Mel888 (#B11 and #B16) were unable to confer drug resistance to any of these growth factors (Figure **4c, S4a**, S**4c**). Consistent with the effects on proliferation, exposure of parental SK-Mel888 cells to HGF, SCF or FGF resulted in strong activation of PTPN11 as determined by phosphorylation at tyrosine 542, and downstream MEK and ERK, indicating that their receptors engage PTPN11 for signal transduction and MAPK pathway activation. In the presence of vemurafenib, all the three ligands reactivated MEK-ERK signaling in parental SK-Mel888 cells. However, PTPN11 knockout clones of SK-MEL888 (#B11 and #B16) were unable to reactivate MEK-ERK signaling when exposed to any of the three growth factors in the presence of vemurafenib respectively (Figure **4d**, S**4b**, **d**). Inhibition of the phosphatase function of PTPN11 is essential for the effects on growth factor induced drug resistance, since

Fig 4

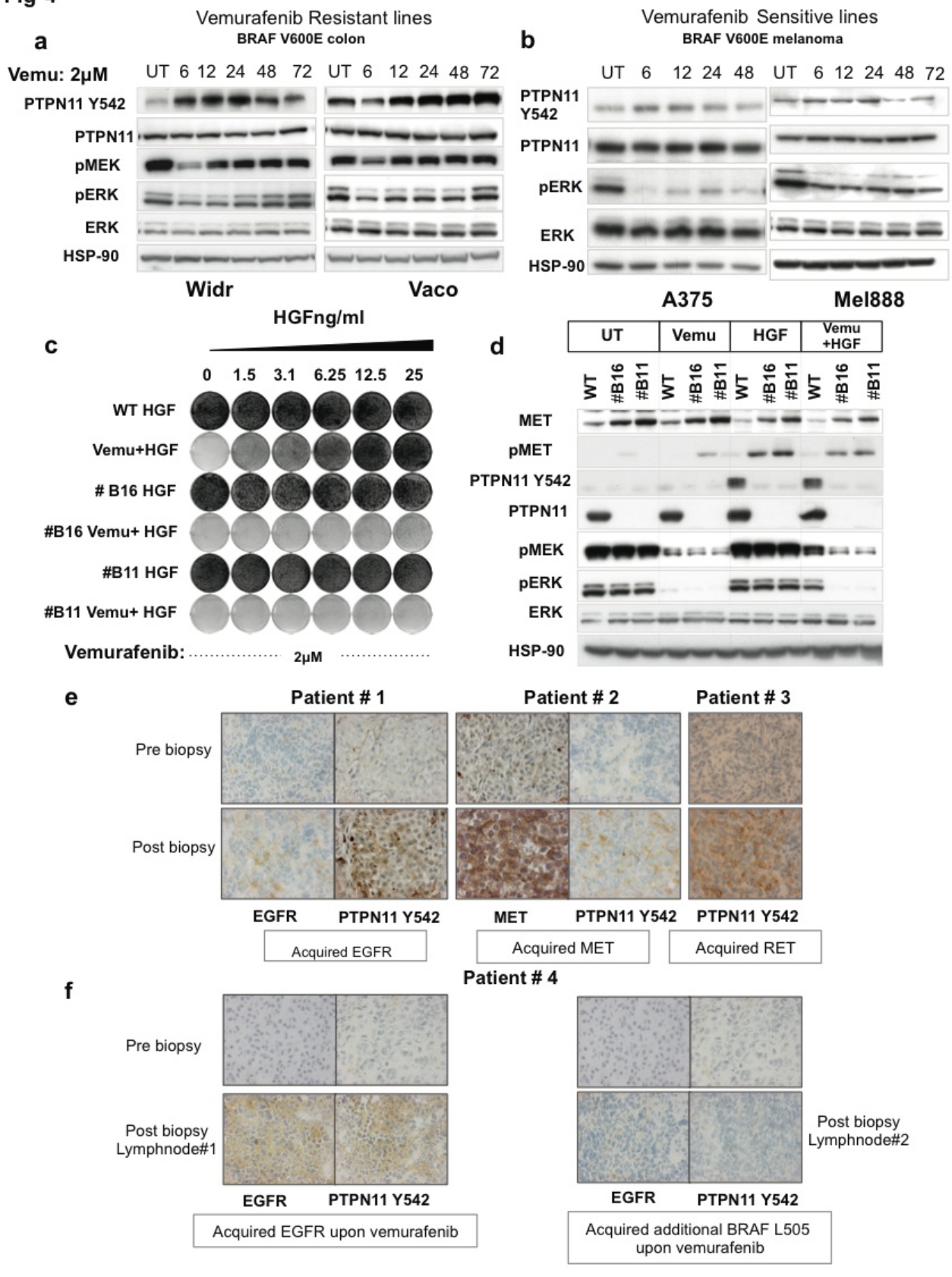
a
Vemurafenib Resistant lines
BRAF V600E colon
Vemu: 2μM
UT 6 12 24 48 72 UT 6 12 24 48 72
PTPN11 Y542
PTPN11
pMEK
pERK
ERK
HSP-90
Widr
Vaco
b
Vemurafenib Sensitive lines
BRAF V600E melanoma
UT 6 12 24 48 UT 6 12 24 48 72
PTPN11 Y542
PTPN11
pERK
ERK
HSP-90
A375
Mel888
c
HGFng/ml
0 1.5 3.1 6.25 12.5 25
WT HGF
Vemu+HGF
B16 HGF
#B16 Vemu+ HGF
#B11 HGF
#B11 Vemu+ HGF
Vemurafenib: 2μM
d
UT
Vemu
HGF
Vemu +HGF
WT #B16 #B11 WT #B16 #B11 WT #B16 #B11 WT #B16 #B11
MET
pMET
PTPN11 Y542
PTPN11
pMEK
pERK
ERK
HSP-90
e
Patient # 1
Patient # 2
Patient # 3
Pre biopsy
Post biopsy
EGFR
PTPN11 Y542
MET
PTPN11 Y542
PTPN11 Y542
Acquired EGFR
Acquired MET
Acquired RET
f
Patient # 4
Pre biopsy
Post biopsy Lymphnode#1
Post biopsy Lymphnode#2
EGFR
PTPN11 Y542
EGFR
PTPN11 Y542
Acquired EGFR upon vemurafenib
Acquired additional BRAF L505 upon vemurafenib

Figure 4. PTPN11 loss abrogates growth factor driven resistance in melanoma.

a, *BRAF* mutant CRC lines Widr and Vaco432 cells display feedback activation of PTPN11 (Y542) upon vemurafenib treatment as a consequence of EGFR feedback activation in a time course experiment.

b, BRAF mutant melanoma cells lack sufficient EGFR expression and show no feedback activation of PTPN11 (Y542) and manifest sustained inhibition of ERK phosphorylation.

c, Hepatocyte growth factor (HGF) activation of the MET receptor can potently rescue Mel888 cells from vemurafenib(2μM) inhibition as seen by colony formation. *PTPN11* knockout clones (#B16 and #B11) prevented the rescue of proliferation upon HGF, and conferred sensitivity to vemurafenib.

d, Biochemical analysis of Mel888 (parental and *PTPN11* knockout #B16 and #B11) treated with vemurafenib(2μM), HGF (25ng/ml) or the combination and analyzed by western blot using pMET, PTPN11 pTYR542, pERK and pMEK.

e, pre and post vemurafenib treated *BRAF* mutant melanoma patient biopsy were stained for PTPN11 pTYR542. Previous genomic copy number analysis on the pre and post treated patient samples indicted a gain in EGFR expression in **Patient #1** which also stained positive for PTPN11 (pTYR542) and EGFR by IHC post vemurafenib treatment, a gain in c-MET receptor expression in **Patient#2** and stained positive for PTPN11 (pTYR542) and MET by IHC and a gain in RET receptor in **Patient#3** stained positive for PTPN11 (pTYR542) by IHC in the post resistant patient samples. One of the two lymph node metastasis biopsies from **patient #4** showed a gain in EGFR expression and positive PTPN11 pTYR542 staining and the second lymph node that had acquired a secondary BRAF mutation (L505H) stained negative for PTPN11 (pTYR542).

expression of a phosphatase dead mutant (C459S) in *PTPN11* knockout SK-Mel888 cells failed to confer resistance to HGF exposure upon vemurafenib treatment (Figure S**4e, f**). Consistent with this, the PTPN11 inhibitor GS493 prevented HGF mediated resistance to vemurafenib in SK-Mel888 cells as seen in long term colony formation (Figure **S4g**). Biochemically, GS493 prevented the reactivation of ERK induced by HGF and conferred sensitivity to vemurafenib (Figure **S4g**).

Melanoma cells often acquire vemurafenib resistance through ectopic RTK expression (Sun et al., 2014b, Girotti et al., 2013, Nazarian et al., 2010). Consistent with this, we find that *PTPN11* loss in A375 melanoma cells delays the emergence of vemurafenib resistant colonies when these cells were cultured in vitro for one month in the presence of high concentration of vemurafenib (Figure **S4h**). Collectively these data suggest that PTPN11 is a central downstream effector of various RTKs whose inhibition could potentially prevent extracellular growth factors from interfering with the tumor cell response to targeted therapies.

PTPN11 activation is a biomarker for acquired drug resistance in melanoma

The data shown above indicate that RTK-driven acquired drug resistance in *BRAF* mutant melanoma activates PTPN11. Consequently, PTPN11 activation could serve as a biomarker to identify whether melanomas acquire vemurafenib resistance via increased RTK signaling or through other means e.g. *MEK* mutation or *BRAF(V600E)* amplification. To investigate this, we obtained biopsies from *BRAF(V600E)* mutant melanoma patients (n = 4) that had progressed upon vemurafenib treatment. Tumor biopsies collected before and after the development of drug resistance were stained for PTPN11 pTYR542. DNA from these tumors has also been analyzed for mutations and copy number alterations to identify the resistance mechanisms. Amplification of *EGFR* was seen in patient 1 and patient 4 (lymph node#1), *MET* amplification in patient 2 and *RET* amplification in patient 3. Immunohistochemical staining of PTPN11 pTYR542 showed that patients who had acquired resistance to vemurafenib by acquiring either *EGFR*, *MET* or *RET* amplification gained strong staining for PTPN11 pTYR542, consistent with the notion that RTK activation correlates with active

PTPN11 (Figure 4**e**). Interestingly, only one of the two lymph node metastasis biopsies from patient 4 had acquired EGFR expression and stained positive for PTPN11 pTYR542. The other lymph node metastasis had acquired a secondary mutation in *BRAF* (L505), which is known to confer vemurafenib resistance (Wagenaar et al., 2014, Choi et al., 2014) and consequently stained negative for PTPN11 pTYR542 (Figure 4e). These data indicate that PTPN11 phosphorylation at Y542 can serve as a biomarker to identify tumors with RTK-driven acquired resistance to BRAF inhibitors.

DISCUSSION

The initial enthusiasm for targeted cancer drugs has been dampened by the rapid onset of resistance in the majority of patients. The mechanisms of resistance to targeted cancer drugs can be broadly subdivided into four categories: secondary mutations in the target itself; downstream pathway activation; upstream pathway activation and activation of parallel pathways (Bernards, 2014). Especially the latter two mechanisms often involve activation of RTKs that fuel reactivation of the inhibited pathway (Duncan et al., 2012, Sun et al., 2014b, Nazarian et al., 2010, Chandarlapaty et al., 2011). This has led to the realization that combining targeted agents that inhibit multiple nodes in the activated signaling pathway could provide longer-lasting therapeutic benefits. This notion has been supported by the strong synergistic effects seen with the combination of EGFR and BRAF inhibitors in *BRAF* mutant CRC (Prahallad et al., 2012) and the observation that dual targeting of BRAF and MEK kinases increases progression free survival for patients with *BRAF* mutant melanoma (Long et al., 2014). This strategy of "vertical" targeting of a pathway is also used by microRNAs to obtain efficient silencing of signaling pathways by partially inhibiting multiple nodes of that pathway (Shirdel et al., 2011). Applying this concept to cancer therapy, the advantage would be that the selective pressure exerted on each of the individual nodes is low, making it difficult for the cancer to escape therapy through secondary mutations. Our current data identify PTPN11 as a protein tyrosine phosphatase whose pharmacological inhibition could be used in such a multi-pronged strategy to inhibit a cancer-activated signaling pathway. We

find that PTPN11 inhibition is synthetic lethal with BRAF inhibition in *BRAF* mutant CRC cell lines and directly lethal in cancer cells driven by activated RTKs. We further find here that loss of PTPN11 in *BRAF* mutant and vemurafenib sensitive melanoma cells could prevent drug resistance driven by multiple growth factors and that loss of PTPN11 delays the development of spontaneous resistance to vemurafenib in *BRAF* mutant melanoma.

While drug development has historically focused primarily on kinases, small molecule inhibitors of phosphatases, including PTPN11, have recently been developed and we show here the utility of one such compound in two combination treatment strategies (Grosskopf et al., 2015, Yu et al., 2013, Chen et al., 2006, Hellmuth et al., 2008). Although it has been documented that loss of PTPN11 in the hematopoietic lineage leads severe loss of hematopoietic stem cells leading to bone marrow aplasia and lethality, PTPN11 inhibitors that have been developed recently are well tolerated in experimental mice (Chan et al., 2011) (Lan et al., 2015) .Our data clearly identify the phosphatase activity of PTPN11 as crucial in conferring drug resistance as reconstitution experiments using the phosphatase dead mutant of PTPN11 (C459S) failed to restore drug resistance in *PTPN11* knockout cells (Figure **2a**). However, most of the phosphatase inhibitors available to date show modest effects on cell proliferation, arguing that such compounds should be used in combination with other pathway-targeted compounds, a notion for which we provide experimental support here. PTPN11 inhibitors could also be useful if multiple RTKs are simultaneously acquired to confer drug resistance. Since PTPN11 is essential for integrating signals from many RTKs, it can be used to suppress the signal of multiple RTKs during acquired resistance.
Finally, we present evidence here that phosphorylated PTPN11 (Y542) can serve as a biomarker to identify melanoma patients that have acquired vemurafenib resistance through RTK activation (Figures **4e, f**). This may prove to be relevant, as our recent data indicate that melanoma patients that have acquired RTK as a drug resistance mechanism may benefit from a “drug holiday” (Sun et al., 2014b). It will be of interest to study whether indeed the PTPN11

(pTYR542)–positive melanoma patients benefit most from a treatment schedule that includes a drug holiday.

EXPERIMENTAL PROCEDURES

A phosphatome centered shRNA library targeting some 451 human phosphatases was assembled from The RNAi Consortium (TRC) human genome-wide shRNA collection (TRCHs1.0). The phosphatase library was introduced into Widr cells by lentiviral transduction. Cells stably expressing shRNA were cultured in the presence or absence of PLX4032. The abundance of each shRNA in the pooled samples was determined by deep sequencing. Each condition in the experiment was done in two replicates. Per hairpin, counts in the Treated and Untreated condition were compared, using DESeq (Anders and Huber, 2010). Hairpins were considered a hit when the log2 fold change was smaller or equal to -1 corrected p value calculated by DEseq was <= 0.1 and the base mean >= 1000. The gene with the strongest fold change was selected for further research. Further details are described in (Prahallad et al., 2012).

Author Contributions

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

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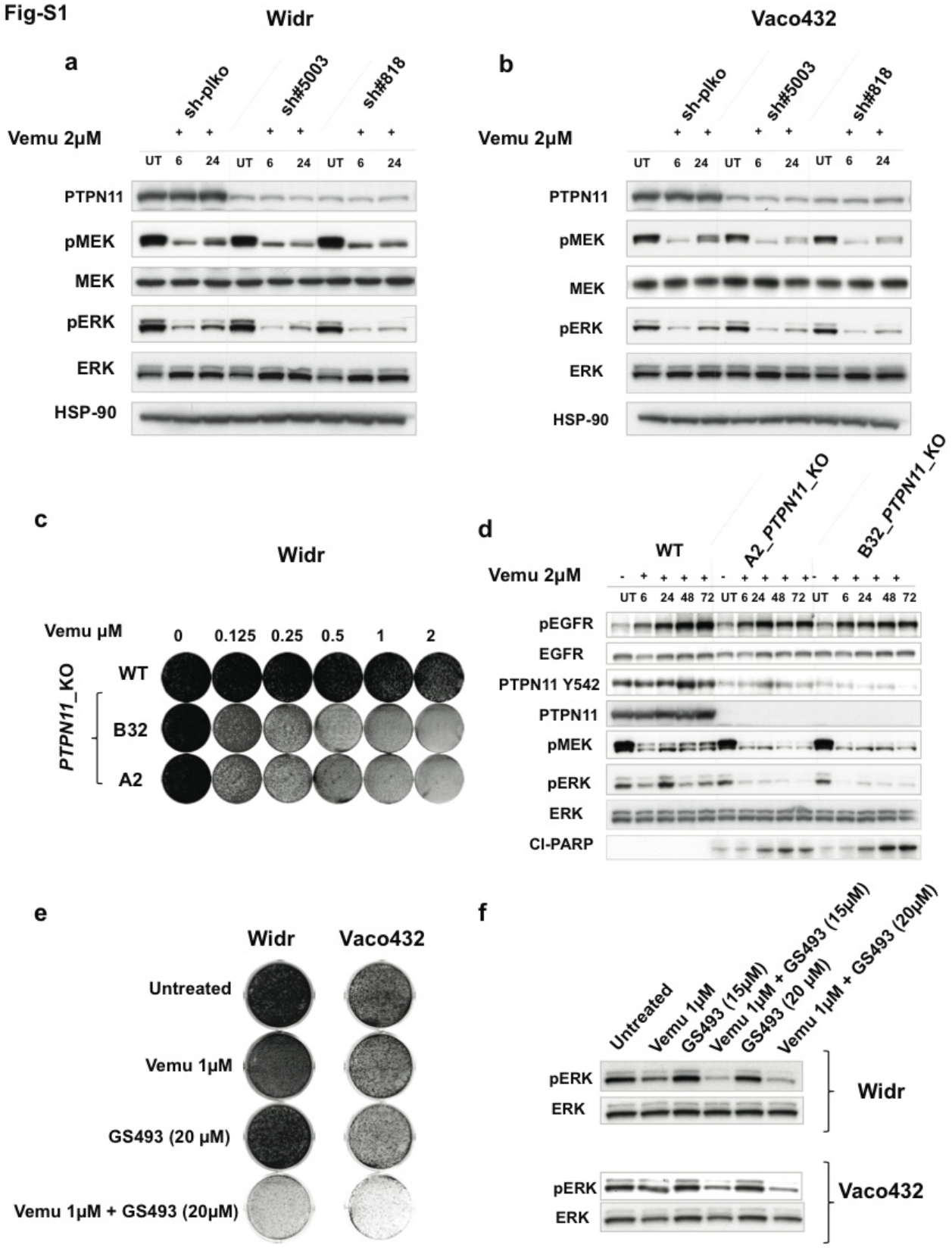
Supplemental data - Chapter 3

PTPN11 is a central node in intrinsic and acquired resistance to targeted cancer drugs

Submitted for publication

Fig-S1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a
b
sh-plko
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UT 6 24
PTPN11
pMEK
MEK
pERK
ERK
HSP-90
c
Widr
Vemu μM
0 0.125 0.25 0.5 1 2
PTPN11_KO
WT
B32
A2
d
WT
A2_PTPN11_KO
B32_PTPN11_KO
Vemu 2μM
UT 6 24 48 72
pEGFR
EGFR
PTPN11 Y542
PTPN11
pMEK
pERK
ERK
Cl-PARP
e
Widr
Vaco432
Untreated
Vemu 1μM
GS493 (20 μM)
Vemu 1μM + GS493 (20μM)
f
Untreated
Vemu 1μM
GS493 (15μM)
Vemu 1μM + GS493 (15μM)
GS493 (20 μM)
Vemu 1μM + GS493 (20μM)
pERK
ERK
Widr
Vaco432

Supplementary Figure 1. a,b, Biochemical analysis of *PTPN11* inhibition with two independent shRNAs targeting the *PTPN11* gene.

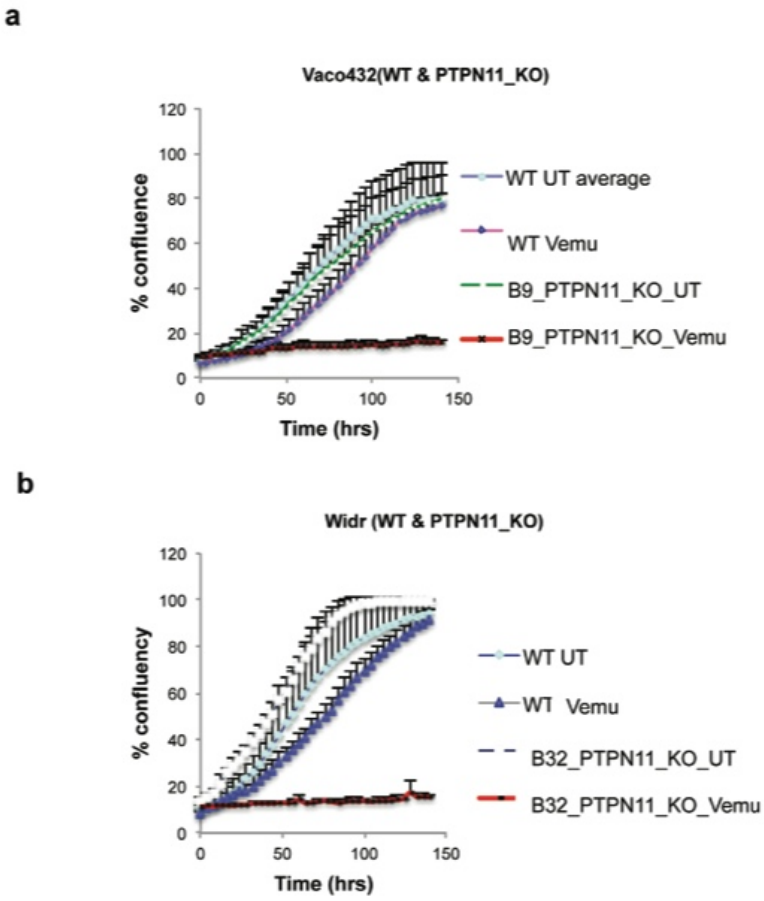
Widr (**a)** and Vaco432 (**b**) cells were lentivirally transduced with **plko**, **sh*PTPN11* #5003** and **sh*PTPN11* #818** and treated with Vemurafenib (2μM) for 6 and 24 hours and analyzed by western blotting using PTPN11, pERK and pMEK antibodies.

c, Knockout of *PTPN11* in Widr (clones #B32 and #A2) using CRISPER –CAS9 technology confers sensitivity to vemurafenib compared to the *PTPN11* wild-type counterparts.

d, Biochemical changes observed on western blot under Vemurafenib (2μM) treatment at different time points in Widr parental cells compared with the PTPN11 knockout counterparts Widr (#B32 and #A2) **(d)** using pEGFR, pPTPN11, pERK, pMEK and cl-PARP antibodies. Refer to table S1 for western blot quantification.

e, long term colony formation of Widr and Vaco432 cells treated with either 1μM of Vemurafenib or 20μM GS493, or in combination. (**f),** Biochemical changes observed on western blot of Widr and Vaco432 cells treated with either Vemurafenib or GS493 or in combination using pERK antibody.

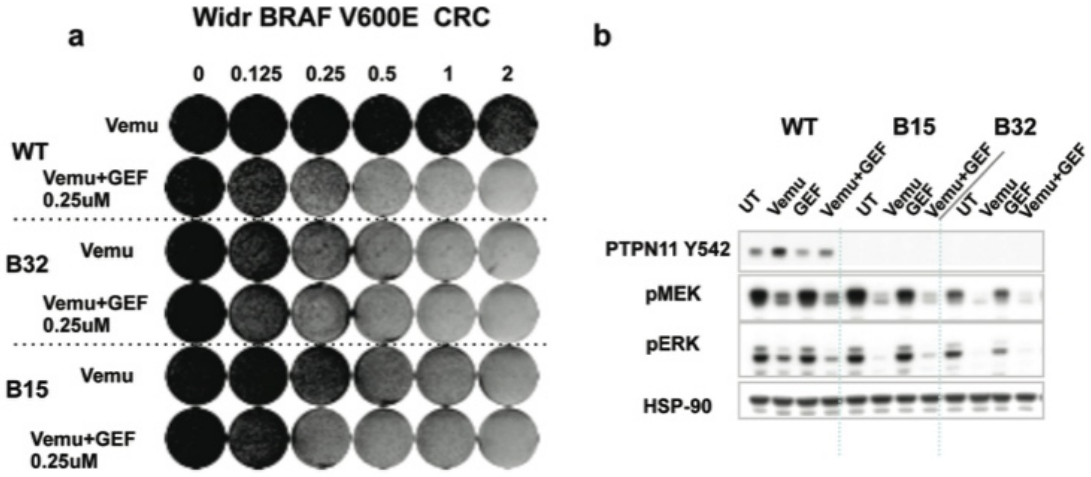
Fig-S2



Supplementary Figure 2.

a,b, incucyte growth curves of *PTPN11* wild type and *PTPN11* knockout Widr (#B32) and Vaco432 (B#9) in the presence and absence of Vemurafenib (2μM).

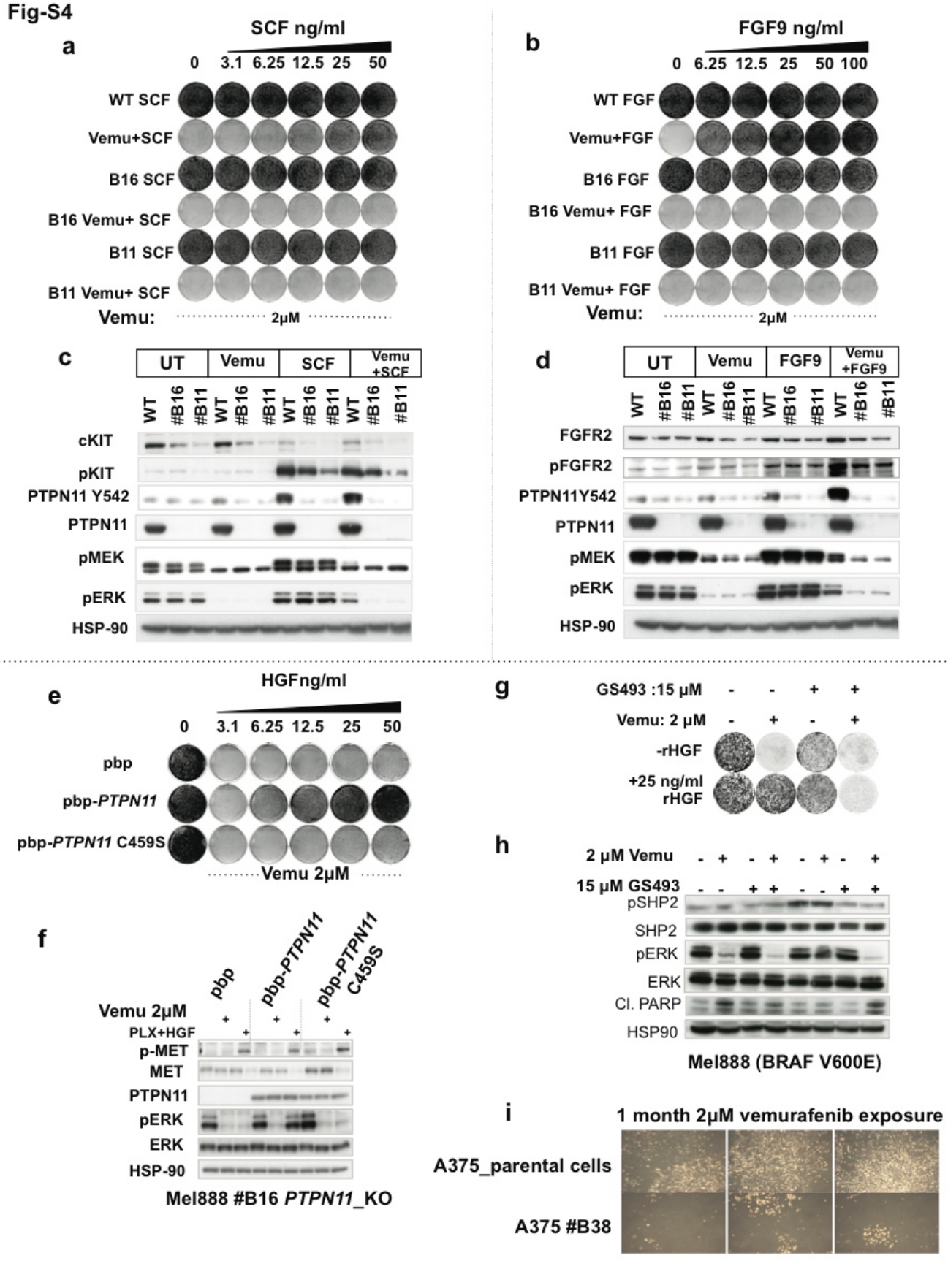
Fig-S3



Supplementary Figure 3. Addition of EGFR inhibitor does not further sensitize *PTPN11* knockout Widr cells to Vemurafenib inhibition.

a, long term colony formation experiment in **(a)** Widr in the presence of increasing concentration of vemurafenib or in combination with 0.25 μM of Gefitinib in both the parental and PTPN11 knockout clones of Widr (#B32 and #B15).

Fig-S4


a
SCF ng/ml
0 3.1 6.25 12.5 25 50
WT SCF
Vemu+SCF
B16 SCF
B16 Vemu+ SCF
B11 SCF
B11 Vemu+ SCF
Vemu: 2μM
b
FGF9 ng/ml
0 6.25 12.5 25 50 100
WT FGF
Vemu+FGF
B16 FGF
B16 Vemu+ FGF
B11 FGF
B11 Vemu+ FGF
Vemu: 2μM
c
UT
Vemu
SCF
Vemu +SCF
WT #B16 #B11 WT #B16 #B11 WT #B16 #B11 WT #B16 #B11
cKIT
pKIT
PTPN11 Y542
PTPN11
pMEK
pERK
HSP-90
d
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Vemu
FGF9
Vemu +FGF9
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pFGFR2
PTPN11Y542
PTPN11
pMEK
pERK
HSP-90
e
HGFng/ml
0 3.1 6.25 12.5 25 50
pbp
pbp-PTPN11
pbp-PTPN11 C459S
Vemu 2μM
f
pbp
pbp-PTPN11
pbp-PTPN11 C459S
Vemu 2μM
PLX+HGF
p-MET
MET
PTPN11
pERK
ERK
HSP-90
Mel888 #B16 PTPN11_KO
g
GS493 :15 μM - - + +
Vemu: 2 μM - + - +
-rHGF
+25 ng/ml rHGF
h
2 μM Vemu - + - + - + - +
15 μM GS493 - - + + - - + +
pSHP2
SHP2
pERK
ERK
Cl. PARP
HSP90
Mel888 (BRAF V600E)
i
1 month 2μM vemurafenib exposure
A375_parental cells
A375 #B38

Supplementary Figure 4. *PTPN11* inhibition in BRAF V600E mutant melanoma prevents SCF and FGF9 mediated resistance to vemurafenib and also delays the onset of resistance to vemurafenib in A375 cells.

a,b, Growth factors SCF and FGF9 activating the KIT and FGF receptor, potently rescue Mel888 cells from Vemurafenib (2μM) inhibition as seen by colony formation. *PTPN11* knockout clones (#B16 and #B11) prevented the rescue of proliferation upon SCF and FGF9, and conferred sensitivity to Vemurafenib.

c,d, Biochemical analysis of Mel888 (parental and *PTPN11* knockout #B16 and #B11) treated with Vemurafenib (2μM), SCF (25ng/ml)**(b)** or FGF9 (25ng/ml) **(d)** or the combination and analyzed by western blot using pKIT, pPTPN11 Y542, pERK and pMEK antibodies. Refer table S1 for western blot quantification.

e, Reconstitution of Wild type *PTPN11* in Mel888 (*PTPN11* knockout #B16) cells confer resistance to HGF. The *PTPN11 (C459S)* does not confer resistance to HGF.

f, Biochemical analysis of Mel888 (*PTPN11* knockout #B16) cells reconstituted with either *PTPN11* wild type or the C459S using pMET, pERK and HSP-90 antibodies.

g, Long term colony formation of Mel888 cells indicating that inhibition of PTPN11 with GS493 prevents HGF induced resistance to vemurafenib. (**h**) Biochemical analysis of Mel888 cells treated with GS493 or Vemurafenib or the combination in the presence or absence of HGF using pERK, pPTPN11 and cleaved PARP antibodies.

i, A375 BRAF V600E mutant melanoma cells (parental and *PTPN11* knockout #B38) cultured in the presence of 2μM Vemurafenib. The resistant colonies were photographed using a bright field microscope.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines, inhibitors and antibodies

Widr, A375, PC9, H3122, Difi, were purchased from American Type Culture Collection (ATCC), Vaco432 and KM-20 were a kind gift from laboratory collection of Alberto Bardelli. SK-Mel888 was from the laboratory collection of Daniel Peeper (NKI-AVL).

Vemurafenib (S1267), gefitinib (S1025), cetuximab (obtained from the NKI-AVL pharmacy), crizotinib (S1068) were purchased from Selleck Chemicals. The PTPN11 inhibitor GS493 was a kind gift from Walter Birchmeier (Max Delbruck Center for Molecular Medicine (MDC)). Human genome wide shRNA collection (TRC-Hs 1.0) was purchased from Open Biosystems (Huntsville AL, USA). Further information is available at

http://www.broad.mit.edu/genome bio/trc/rnai.html.

Antibodies against HSP-90 (H-114), p-ERK (E-4), ERK1 (C-16), ERK2 (C-14), SH-PTP2 (C-18) and Anti-EGFR (1005 sc-03) were purchased from Santa Cruz Biotechnology; for detecting ERK1/2 a mixture of ERk1 and ERK2 antibodies was used. pEGFR (Y1068) (ab5644), pSHP2(Y542) (ab62322) was purchased from Abcam. MET (8198 S), pMET (3077 S), KIT (3074 S), pKIT (3073 S), FGFR2 (11835S), pFGFR2 (3476 S), pMEK (9154S), total MEK (4694S), cl-PARP (5625) were purchased from Cell signaling technologies.

Human recombinant growth factors Hepatocyte Growth Factor (HGF) was purchased from Sigma –Aldrich (H9661), Stem Cell Factor (SCF) (255-SC-050), TGF-beta (240-B-010) and Human recombinant FGF9 (273-F9-025) were purchased from R&D systems.

Cell culture and Lentiviral / Retroviral transduction

All the cell lines were cultured in RPMI supplemented with 10% fetal calf serum (FCS) 1% Glutamine and 1% Penicillin/Streptomycin (Gibco). HEK293T cells were cultured in DMEM with 10% FCS, 1% Glutamine and 1% Penicillin/Streptomycin (Gibco) at 37° C/ 5% CO2. HEK393T cells were used to produce Lentiviral supernatants as described at http://www.broadinstitute.org/rnai/public/resources/protocols. The HEK293T cells were transfected using PEI (1µg/µl) – PEI is Polyethylenimine 25kD linear from Polysciences (cat# 23966-2). The lentiviral-transduced cells were selected by 2µg/ml of puromycin.

Long-term cell proliferation assay

Cells were seeded in 6 well plates at densities ranging from 0.5- 1* 10^4 cells per well and cultured in the absence or presence of drugs as indicated. The cells were fixed using 4% formaldehyde and stained with 0.1% crystal violet (in H2O) after the control cells gained confluency.

Protein lysate preparation

Cell lines were seeded in the medium contain 10% FCS. After 24hr of seeding, cells were grown in 0.1% serum supplemented medium (starvation) overnight. After the starvation, cells were stimulated with medium containing 10%serum and drugs of interest and cell lysates were collected at the desired time points using RIPA buffer supplemented with protease inhibitor (cOmplete, Roche) and Phosphatase Inhibitor Cocktails II and III (Sigma). All the lysates were freshly prepared and quantified using BCA protein quantification kit (Pierce) and resolved by SDS-PAGE using NuPAGE Gel Electrophoresis Systems (Invitrogen).

Image J for quantification of western blot

The band intensities of the western blot images were quantified using Image J software. The data were exported to excel and the percentage signal intensity of a particular phospo epitope signal was compared to the signal of the loading control and their relative ratio was plotted.

shRNA and guide RNA sequences

The following shRNA sequences were used for the suppression of *PTPN11*

sh-*PTPN11* #1- TRCN0000005003- CGCTAAGAGAACTTAAACTTT

sh-*PTPN11* #2- TRCN0000005006- GCAAATATCATCATGCCTGAA

sh-*PTPN11* #3- TRCN0000355818- AGATGTCATTGAGCTTAAATA

gRNA for generating *PTPN11* knockout

gRNA A (PTPN11)

1. Fwd _ CGAGGGAGGAACATGACATCGCGG
2. REV_AAACCCGCGATGTCATGTTCCTCC

gRNA B (PTPN11)

1. FWD_CGAGAACATGACATCGCGGAGGTG
2. REV_ AAACCACCTCCGCGATGTTCATGTT

Xenografts

Vaco432 wild type and SHP2 knockout clone #B9 were injected ($5X10^6$ cells per mouse) subcutaneously in the right flank of 8-week-old immunodeficient CD1 nude female mice (from Charles River Laboratory). Tumor volume was monitored once a week by digital caliper and quantified by the modified ellipsoidal formula (tumour volume = $1/2(\text{length} \times \text{width}^2)$). Mice were randomized (7 mice per group) when they reached a volume of approximately $200mm^3$ and treated for a 34-day period. Vemurafenib (60mg/Kg) was dissolved in 0.2% Tween 80 and 1% methylcellulose (Sigma) and administered daily by oral gavage. Control groups were treated at the same schedule with the vehicle of Vemurafenib. All animal procedures were approved by the Ethical Commission of the University of Turin and by the Italian Ministry of Health and they were performed in accordance with institutional guidelines.

CRISPR-CAS9 mediated knockout of *PTPN11*

A dual vector doxycycline inducible CRISPR/Cas9 system was made on the basis of FH1tUTG (Herold et al., 2008). Briefly, for pLenti-Cas9-T2A-Neo, the GFP cassette was replaced by a neomycin resistance gene and the tetR ORF by a 3XFLAG-NLS-hSpCas9-NLS product amplified from pX260a (Addgene 42229). The existing shRNA cassette was cut out using PacI and re-ligation. To make pLenti-gRNA-tetR-T2A-BSD, a blasticidine resistance gene replaced the GFP cassette of FH1tUTG. Next, existing BfuAI sites were destroyed by replacing fragments containing these with synthetic genes containing point mutations. A synthetic gene encoding a doxycycline inducible H1 promoter, two BfuAI sites and an optimized tracer sequence followed by a 5xT transcriptional stop was then used to replace the existing PacI fragment. BfuAI digestion of the resulting plasmid allowed for directional cloning of annealed oligo pairs containing 20nt specific nucleotides targeting any gene of interest. To prevent premature activation of

CRISPR/Cas9 due to the time the cell needs to build up enough of the tet repressor to efficiently dampen gRNA expression, cells were always first infected with pLenti-gRNA-tetR-T2A-BSD, encoding the repressor, and at least three days later with pLenti-Cas9-T2A-Neo

Supplemental reference:

1 Herold MJ, van den Brandt J, Seibler J, Reichardt HM. Inducible and reversible gene silencing by stable integration of an shRNA-encoding lentivirus in transgenic rats. Proceedings of the National Academy of Sciences of the United States of America 2008; 105: 18507-18512.

Gene Symbol	TRC ID	log2 Fold Change (ratio PLX4032/Untreated)
CLEC1B	TRCN0000062649	-1.12
CLEC1B	TRCN0000062650	-1.04
PPFIA1	TRCN0000002968	-1.09
PPFIA1	TRCN0000002971	-1.08
PTPN11	**TRCN0000005003**	**-1.84**
PTPN11	**TRCN0000005006**	**-1.33**

Supplemental table 1. List of genes identified in the phosphatase screen that had $log_2FC<-1$ and with at least two independent hairpins

Chapter 4

A chromatin modifier genetic screen identifies SIRT2 as a modulator of respo to targeted therapies through regulation of MEK kinase activity.

Oncogene. 2015 Jan 22; 34(4): 531-6

A chromatin modifier genetic screen identifies SIRT2 as a modulator of response to targeted therapies through the regulation of MEK kinase activity

PK Bajpe[1,4], A Prahallad[1,4], H Horlings[1,2], I Nagtegaal[3], R Beijersbergen[1] and R Bernards[1]

Resistance to targeted therapies is a major problem in cancer treatment. The epidermal growth factor receptor (EGFR) antibody drugs are effective in a subset of colorectal cancers, but the molecular mechanisms of resistance are understood poorly. Genes involved in epigenetic regulation are frequently deregulated in cancer, raising the possibility that such genes also contribute to drug resistance. Using a focused RNA interference library for genes involved in epigenetic regulation, we identify sirtuin2 (SIRT2), an NAD^+-dependent deacetylase, as a modulator of the response to EGFR inhibitors in colon and lung cancer. *SIRT2* loss also conferred resistance to BRAF and MEK inhibitors in *BRAF* mutant melanoma and *KRAS* mutant colon cancers, respectively. These results warrant further investigation into the potential role of SIRT2 in resistance to drugs that act in the receptor tyrosine kinase-RAS-RAF-MEK-ERK signaling pathway.

Oncogene advance online publication, 27 January 2014; doi:10.1038/onc.2013.588

Keywords: SIRT2; drug resistance; chromatin modifiers; genetic screen

INTRODUCTION

Recent advances in treating cancer with targeted agents have shown tremendous potential. However, the effectiveness of these drugs is hampered by the frequent emergence of resistance. Understanding the molecular bases of drug resistance is the key to realizing the full benefit of targeted therapies. Cetuximab, a monoclonal antibody, which targets the extracellular domain of epidermal growth factor receptor (EGFR), is used as an effective therapy for metastatic colorectal cancers (CRC).[1,2] However, not all metastatic CRC patients respond to EGFR-targeted agents. For instance, activating mutations occurring in *KRAS* and *BRAF* have been shown to correlate with anti-EGFR monoclonal antibody resistance.[3] In spite of this knowledge, a large number of tumors wild type for *KRAS* and *BRAF* do not respond to cetuximab, necessitating the need to search for additional biomarkers of intrinsic resistance.[3–5]

Changes in epigenetic pathways are frequently associated with tumorigenesis. Indeed, cancer genome analyses have uncovered a flurry of genomic lesions in epigenetic enzymes, establishing a direct link between cancer genetics and cancer epigenetics.[6] For example, many of the components of the SWI/SNF chromatin remodeling complex are either mutated or deleted in a wide variety of cancers.[7,8] Translocation of histone methyltransferase MLL and histone acetyltransferase CBP are dominantly acting oncogenes in leukemia.[9,10] Moreover, overexpression of EZH2 histone methyltransferase, a component of the PRC2 complex, occurs in a variety of cancers.[11,12] Recent studies have identified epigenetic regulators that may modulate drug resistance in lung cancers.[13]

Functional genetic screens serve as powerful tools in identifying novel components of the signaling pathways involved in drug resistance.[14] Here, we report the use of a loss-of-function genetic screen to identify epigenetic regulators that can cause cetuximab resistance in a CRC cell line model. Our work identifies sirtuin2 (SIRT2), a NAD^+-dependent histone deacetylase as a determinant of response to EGFR inhibitors in colon and lung cancer. Moreover, loss of *SIRT2* also confers resistance to other cancer drugs targeting the mitogen-activated protein (MAP) kinase components, like BRAF and MEK.

RESULTS AND DISCUSSION

Loss of *SIRT2* confers resistance to EGFR inhibitors

To identify components of the epigenetic machinery involved in drug resistance pathways, we compiled a library of short hairpin RNAs (shRNAs) targeting some 700 genes, including the KATs (lysine acetyltransferases), KMTs (lysine methyltransferases), KDACs (lysine deacetylases), KDMs (lysine demethylases) and chromatin remodeling complexes like SWI/SNF, ISWI, NuRD and INO80. In this library, we also included a wide array of proteins that harbor one or more chromatin-binding/associated domains. We refer to this library as the 'chromatin regulator library' (Supplementary Table 1). The genes included in the library are each represented on average by five different shRNAs.

The Difi CRC cell line is highly sensitive to the EGFR monoclonal antibody cetuximab. It is wild type for *KRAS*, *BRAF* and *PIK3CA*, but it overexpresses *EGFR* as a result of gene amplification. To identify genetic determinants of cetuximab resistance, we performed a

[1]Division of Molecular Carcinogenesis, Cancer Genomics Center, The Netherlands Cancer Institute, Amsterdam, The Netherlands; [2]Department of Pathology, Academisch Medisch Centrum, Amsterdam, The Netherlands and [3]Department of Pathology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands. Correspondence: Dr R Bernards, Division of Molecular Carcinogenesis, Cancer Genomics Center, The Netherlands Cancer Institute, 121 Plesmanlaan, Amsterdam 1066 CX, The Netherlands.
E-mail: r.bernards@nki.nl
[4]These authors contributed equally to this work.
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loss-of-function genetic screen using the chromatin regulator shRNA library described above. Briefly, we introduced the chromatin regulator shRNA library into Difi cells by lentiviral infection (see Supplementary Methods). The infected cells were plated at low density and cultured with or without cetuximab for 2 weeks. To determine the relative abundance of individual shRNA, genomic DNA was isolated from both cultures and stably integrated shRNAs were polymerase chain reaction (PCR)-amplified using a common set of primers specific for the shRNA cassettes (Figure 1a). To quantify the relative abundance of the shRNAs, the PCR product was deep sequenced as described.[15] An M/A plot was generated using the normalized reads generated from deep sequencing (Figure 1b). The M/A plot represents the relative enrichment of the shRNA. Each dot in the plot represents an individual shRNA; significant outliers (see legend for selection criteria) are located in the upper right quadrant of the M/A plot. We only considered genes for which at least two independent hairpins were enriched more than 5-fold in the cells treated with cetuximab and whose sequence reads were >200 in the untreated control sample. Using these criteria, we identified five different chromatin modifiers, namely *SIRT2*, *INO80B*, *MBTD1*, *RBBP7* and *ZNF546* (Supplementary Table 2). Only *SIRT2* was validated in the subsequent validation round as a candidate gene whose suppression confers resistance to cetuximab (see below). SIRT2 is an NAD^+-dependent deacetylase that has been implicated in maintaining genomic stability and tumor suppression.[16]

To validate that inhibition of *SIRT2* confers resistance to cetuximab, we infected the two identified hairpins (sh36 and sh37) from the screen individually into Difi cells and performed a long-term colony formation assay in the presence or absence of cetuximab. Indeed, as shown in Figure 1c, both hairpins conferred resistance to cetuximab and efficiently suppressed the expression of *SIRT2* at the mRNA level (Figure 1e). As an additional validation, we knocked down *SIRT2* in PC9 lung cancer cells. PC9 is highly sensitive to EGFR inhibition because of an activating *EGFR* mutation,[13] but *SIRT2* knockdown conferred gefitinib resistance in PC9 cells (Figure 1d). As shown in Figures 1e–h, efficient knockdown of *SIRT2* by both hairpins at the mRNA level and at the protein level conferred resistance to gefitinib. A total of five shRNAs exist for *SIRT2* in the chromatin regulator library. The knockdown efficiencies and resistance to cetuximab was assessed for all the shRNAs (Supplementary Figures S2A and B). Sh40 gives efficient knockdown and also confers resistance to cetuximab, but was not picked up in the screen (Supplementary Figures S2A and B) Interestingly, a smaller variant of *SIRT2*, which lacks the N-terminal 37 amino acids, is present in all cell lines we tested (Figures 1g and h). Henceforth, we refer to the full-length *SIRT2* as *SIRT2* long (*SIRT2L*) and the smaller variant as *SIRT2* small (*SIRT2S*). All the shRNAs used in this study target both *SIRT2L* and *SIRT2S*. Taken together, these findings suggest an essential role for SIRT2 in modulating resistance to EGFR inhibitors in both colorectal and lung cancers.

SIRT2 loss leads to increase in ERK phosphorylation levels

To address the underlying mechanism by which *SIRT2* suppression conferred resistance to EGFR inhibitors, we first studied effect of expression of activated components of EGFR signaling, including the MAP kinase/ERK and phosphoinositide 3-kinase/AKT pathways. We stably overexpressed the following activated oncogenes in Difi cells: $BRAF^{V600E}$, *MEK-DD*, $PIK3CA^{H1047R}$ and *Myr-AKT*.[17] Overexpression of $BRAF^{V600E}$ and *MEK-DD* conferred resistance to cetuximab, as assessed by colony-forming assays, whereas expression of $PIK3CA^{H1047R}$ or *Myr-AKT* failed to confer resistance to cetuximab (Figure 2a). We also performed western blot analysis on cell lysates from these stable Difi cell lines with phospho-ERK (pERK) antibodies. Overexpression of $KRAS^{G12V}$, $BRAF^{V600E}$ and *MEK-DD* led to increased pERK levels following drug treatment, whereas overexpression of *Myr-AKT* had no effect on pERK levels (Figure 2b). These data are in agreement with the established dominant role of the RAS-RAF-MEK-ERK pathway in resistance to cetuximab in colon cancer.[3]

To address how *SIRT2* confers resistance to cetuximab, we used a Human Phospho-Kinase Array (R&D Systems, Minneapolis, MN, USA), which allows the identification of activated effector kinases and their substrates. These arrays were probed with lysates prepared from control cells and *SIRT2* knockdown Difi cells with or without cetuximab treatment. Interestingly, a clear activation of ERK1and2 could be seen in *SIRT2* knockdown cells and not in the control cells, suggesting that increased levels of pERK mediate the cetuximab resistance found in *SIRT2* knockdown cells (Figure 2c (black arrow) and Figure 3b). Only a very modest increase in MEK1/2 phosphorylation is also seen in arrays probed with *SIRT2* knockdown lysates, suggesting that SIRT2 acts at the level of MEK or ERK (Figure 2c, red arrow). Taken together, these data indicate that *SIRT2* loss leads to increased pERK levels, which provides a plausible explanation for the observed drug resistance.

SIRT2 loss leads to drug resistance in different cancer types

As *SIRT2* loss leads to increased pERK levels, we asked whether *SIRT2* knockdown could confer resistance to cancer drugs that target other components of the MAP kinase signaling upstream of ERK. We tested two drugs, PLX4032 (vemurafenib), a potent inhibitor of $BRAF^{V600E}$, and AZD6244 (selumetinib), a potent inhibitor of MEK. PLX4032 is very effective in the treatment of melanoma with a $BRAF^{V600E}$mutation and AZD6244 is being tested in clinical trials against several cancers. Loss of *SIRT2* gave potent resistance to PLX4032 in A375 melanoma cells (Figure 3c) and similarly loss of *SIRT2* also gave resistance to AZD6244 in HCA7 CRC cells (Figure 3e). We then probed the lysates of *SIRT2* knockdown cells with pMEK and pERK antibodies. As shown in Figures 3b, d and f, expression of each of the short hairpins sh36 and sh37 resulted in marked increases in pERK levels in Difi, A375 and HCA-7 cells, and these increased levels were maintained in the presence of drugs. When we tested a concentration range of AZD6244, we observed that higher concentrations of AZD6244 are required to inhibit pERK in *SIRT2* knockdown cells, consistent with the notion that activation of a pathway requires higher drug concentrations for effective inhibition (Supplementary Figure 1). Taken together, these data indicate that *SIRT2* loss leads to an increase in pERK levels and resistance to drugs that act upstream of ERK kinase.

Overexpression of *SIRT2* leads to reduced pERK levels and inhibition of growth

As loss of *SIRT2* leads to an increase in pERK levels, we wondered whether an increase in *SIRT2* expression could reduce pERK levels. To address this, we separately cloned the long and the short form of SIRT2 into the pBabe expression vector. As shown in Figure 4a, overexpression of SIRT2L, but not SIRT2S, in Widr colon cancer cells had marked effects on cell proliferation and reduced pERK levels. In contrast, SIRT2S only had mild effect on cell proliferation and almost no effect on pERK levels. These data identify a specific role for SIRT2L in regulating the MEK-ERK signaling cascade.

SIRT2 deacetylates MEK1

MEK1 kinase activity is activated by phosphorylation. The accompanying manuscript by Mayo and co-workers[18] describes the identification of a novel post-translational regulation of MEK1 by showing that acetylation of MEK1 activates its kinase activity. Important for this study, their data also reveal a role for SIRT2 in deacetylation of MEK. On the basis of this, we hypothesized that loss of *SIRT2* expression results in an increase in MEK1 acetylation

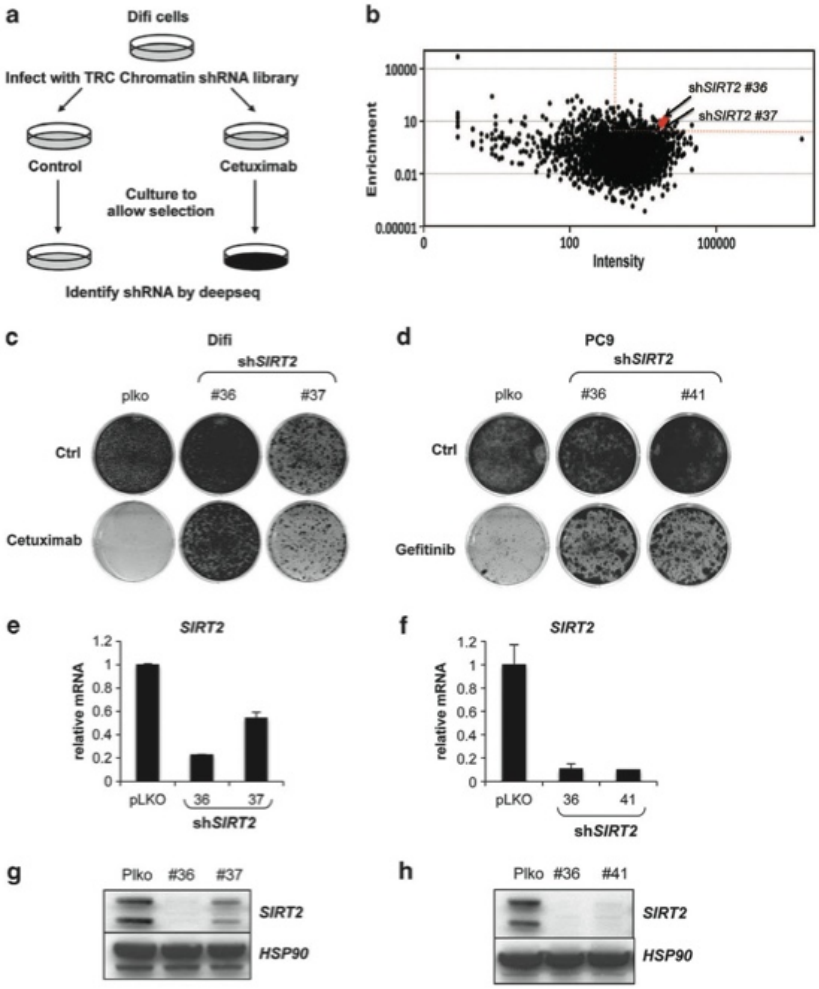

Figure 1. Screen for epigenetic modulators of cetuximab resistance identifies *SIRT2* as a critical determinant of drug response to EGFR inhibitors. (**a**) Schematic representation of the chromatin regulator library screen performed in Difi colon cancer cells. Lentiviral shRNA library targeting the human genome was purchased from Sigma Aldrich, St Louis, MO, USA (TRC Human genome library). Lentiviral plasmids (pLKO.1) encoding shRNAs that target the epigenome (as listed in the Supplementary Table 1) were compiled in a pooled format. The chromatin regulator library consists of six plasmid pools, where each gene is represented by five independent shRNAs. The lentiviral supernatants were produced following the guidelines listed at http://www.broadinsititute.org/rnai/public/resources/protocols. Difi cells were infected with each of the six viral pools independently with a multiplicity of infection 1 (MOI = 1). The library-infected cells were then pooled and plated at 150 000 cells per 15 cm culture dish in the absence or presence of cetuximab 0.25 μg/ml (five dishes per condition). The cells were cultured with and without drugs, changing media two times per week for a period of 3 weeks until drug-resistant colonies appeared in the drug-treated condition. The cells were harvested by the end of the third week and genomic DNA was isolated from both the untreated and treated cells as described.[26] The shRNA cassettes were retrieved from 8 μg of genomic DNA by performing a two-step PCR amplification (PCR1 and PCR2) using the following conditions: (1) 98 °C, 30 s; (2) 98 °C, 10 s; (3) 60 °C, 20 s; (4) 72 °C, 1 min; (5) to step 2, 15 cycles; (6) 72 °C, 5 min; (7) 4 °C. Indices and adaptors for deep sequencing (Illumina, San Diego, CA, USA) were incorporated into PCR primers. From the first PCR 2.5 μl was used as templates for PCR2 reaction. The final PCR products were purified using QIAGEN PCR purification kit (Qiagen, Valencia, CA, USA). To ensure that the samples were pooled at the same molar ratio before performing deep sequencing, we quantified the samples using a bioanalyzer. The shRNA stem sequence was segregated from each sequencing reads and aligned to the TRC library. The matched reads were counted and the counts were transformed to abundance that was assigned to the corresponding shRNA. The primers used are listed in the supplementary document. (**b**) M/A plot representing the relative abundance of the shRNA vectors from the screen. The *y* axis represents the enrichment (relative abundance of cetuximab treated/untreated) and the *x* axis represents the average sequence reads in untreated samples of each shRNA. Among the top shRNA candidates enriched >5-fold in the cetuximab-treated condition (represented by more than 200 reads in the untreated) and represented by two independent hairpins, SIRT2 was one of the strongest. (**c** and **d**) Two independent shRNAs identified by the screen (nos. 36-TRCN0000040218 and 37-TRCN0000040219) were retested for resistance to cetuximab in a long-term colony formation assay in Difi cells (**c**) and for gefitinib in PC9 lung cancer cells (**d**).[27] In PC9 cells, an additional shRNA vector (no. 41, TRCN0000010435) gave potent rescue. (**e** and **f**) Knockdown efficiencies of each of the two *SIRT2* shRNAs were tested by measuring the mRNA levels of SIRT2 by the quantitative real-time PCR (qRT–PCR). The relative mRNA levels of *SIRT2* was normalized to the levels of the housekeeping gene *GAPDH*. The primers were used together with the SYBR green PCR mix reaction (FastStart Universal SYBR green mastermix; Roche, Indianapolis, IN, USA). The sequences of *SIRT2* primers used for the assay were as follows: forward primer, 5′-TCCACCAAGTCCTCCTGTTC-3′, reverse primer, 5′-TGAAGGACAAGGGGCTACTC-3′. (**g** and **h**) Knockdown efficiencies of each of the two *SIRT2* shRNAs were tested using western blot analysis. Protein lysates from Difi cells (**g**) and PC9 cells (**h**) were prepared by directly lysing the cells in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 12.5 mM EDTA, 1% β-mercaptoethanol and 0.02% bromophenol blue). The samples were processed by heating at 98 °C for 6–7 min and used for western blot analysis.

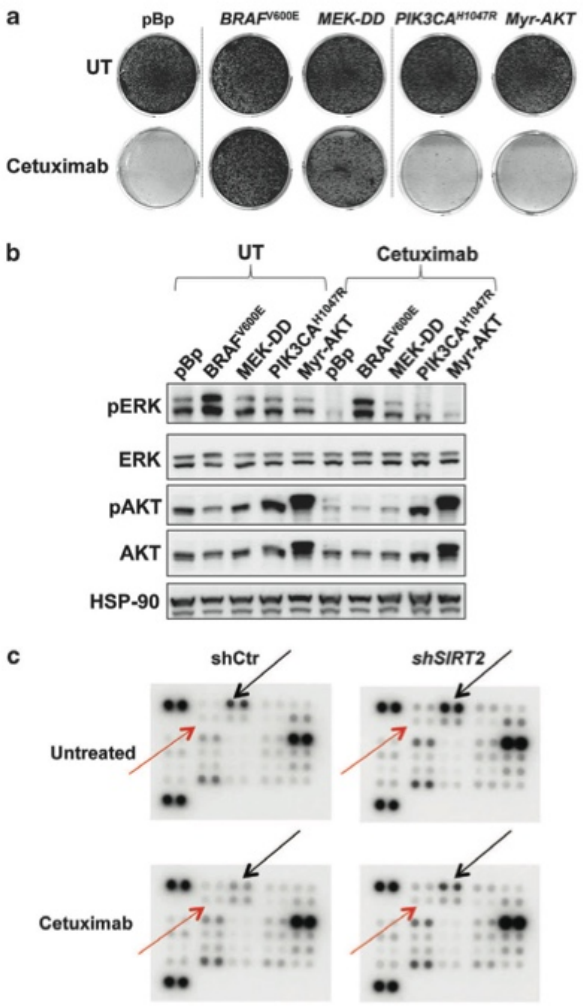

Figure 2. SIRT2 knockdown enhanced pERK levels. (**a**) Difi cells were infected with expression vectors for *BRAF*^{V600E}, *MEK-DD* *PIK3CA*^{H1047R} and *Myr-AKT* and tested for their resistance to cetuximab. The cells were fixed and stained with crystal violet. Empty vector pBABE-puro (pBp) was used as a control. (**b**) Western blot analysis of the overexpression phenotypes shown in (**a**). Difi cells overexpressing *BRAF*^{V600E}, *MEK-DD*, *PIK3CA*^{H1047R} and *Myr-AKT* were grown in the absence or presence of cetuximab for 6 h. Following drug exposure, protein lysates were prepared by directly lysing the cells in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer. The samples were processed by heating at 98 °C for 6–7 min and used for western blot analysis. Upregulation of pERK is seen in *BRAF*^{V600E} and *MEK-DD* cells but not in Myr-AKT and *PIK3CA*^{H1047R} cells. The primary antibodies used for blotting are as follows: HSP-90 (H-114), pERK (E-4), ERK1 (C-16), ERK2 (C-14) were from SantaCruz Biotechnology (Santa Cruz, CA, USA). A mixture of ERK1 and ERK2 antibodies was used for detection of total ERK. (**c**) *SIRT2* knockdown leads to enhanced pERK levels. Difi cells expressing *SIRT2* shRNA and control cells were grown in the absence or presence of cetuximab for 6 h, after which cells were harvested and lysates were prepared. The lysates were prepared using the buffer supplied by the manufacturer of the Human Phospho-Kinase Array (R&D Systems; Catalog no. ARY003B). The lysates were incubated on Human Phospho-Kinase Array and rest of the procedure was followed according to the manufacturer's instructions. *SIRT2* knockdown leads to an increase in pERK levels (black arrow) and a very modest increase in pMEK levels is also seen (red arrow).

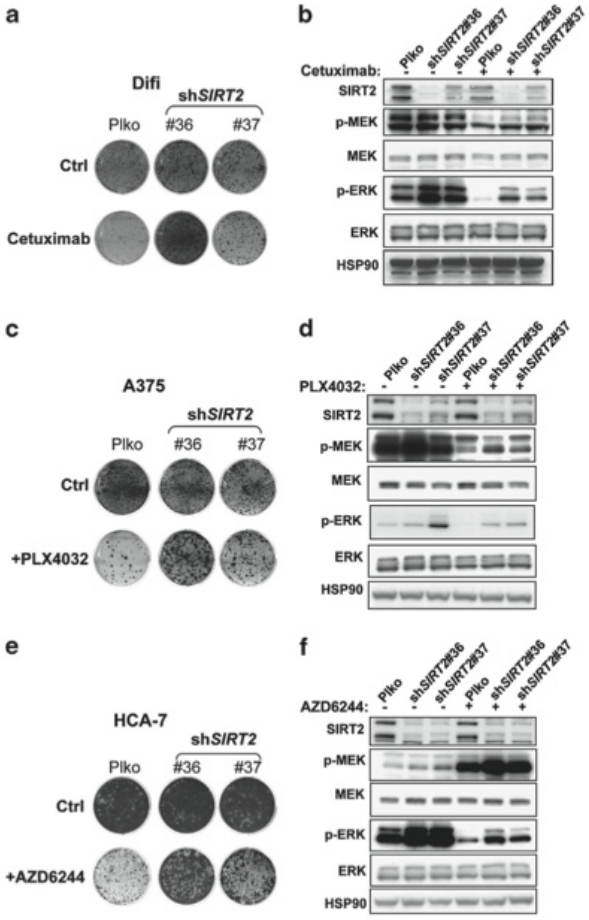

Figure 3. *SIRT2* suppression leads to resistance to multiple MAP kinase pathway drugs and increased pERK levels. (**a**, **c** and **e**) Difi colon cancer cells, A375 melanoma cells or HCA7 colon cancer cells expressing pLKO control vector or *shSIRT2* were seeded in six-well plates at a density of 5–10 × 10^4 cells per well in the presence or absence of drug for long-term colony formation assay as described.[27] The cells were fixed and stained with crystal violet.[27] Following are the drug and cell line combinations. Difi cells—cetuximab, A375 cells—PLX4032 and HCA7 cells—AZD6244. (**b**, **d** and **f**) Difi, A375 and HCA-7 shSIRT2 knockdown cells (shSIRT2 no. 36 and shSIRT2 no. 37) were selected in the presence of cetuximab (**b**), PLX4032 (**d**) and AZD6244 (**f**) for 2 weeks. After 2 weeks of drug selection, the cells were recovered for 48 h in regular RPMI medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. After recovery, the cells were either left untreated or treated as follows for 6 h: 0.25 μg/ml cetuximab for Difi, 1 μM PLX4032 for A375, 1 μM AZD6244 for HCA-7 cells, respectively. Following drug exposure, protein lysates were prepared by directly lysing the cells in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 12.5 mM EDTA, 1% β-mercaptoethanol and 0.02% bromophenol blue). The samples were processed by heating at 98 °C for 6–7 min and used for western blot analysis. The primary antibodies used for blotting are as follows: pMEK1/2 (S217/221, no. 9121) and MEK1/2 (L38C12, no. 4694) are from Cell Signaling Technologies (Danvers, MA, USA). HSP-90 (H-114), pERK (E-4), ERK1 (C-16) and ERK2 (C-14) were from SantaCruz Biotechnology. SIRT2 primary antibody (HPA011165) was purchased from Sigma-Aldrich.

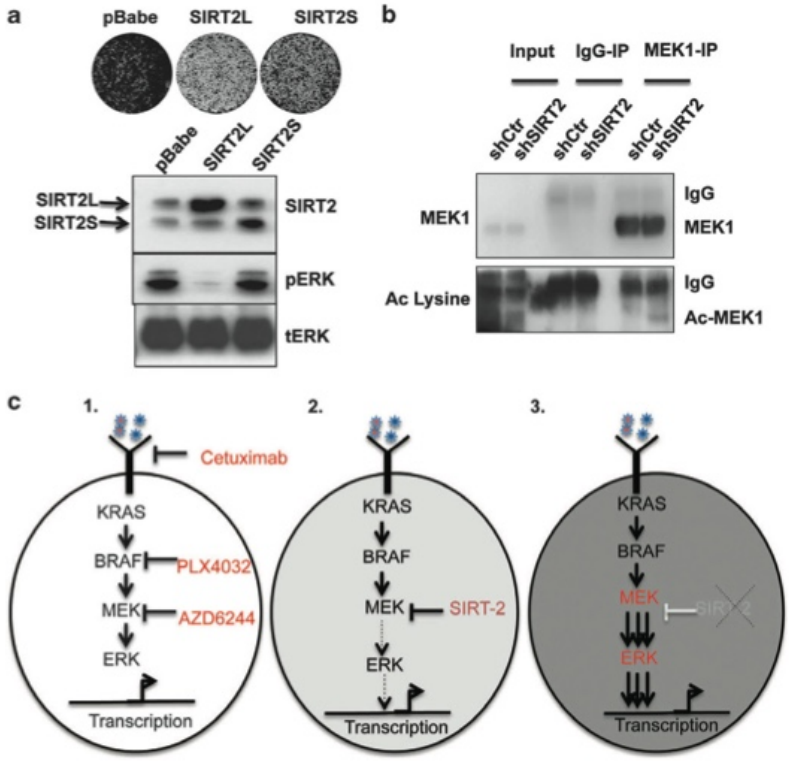

Figure 4. Overexpression of *SIRT2* leads to reduced pERK levels and growth inhibition. (**a**) Widr colon cancer cells, expressing SIRT2L variant, SIRT2S variant and pBabe control vectors were seeded in six-well plates at a density of $5–10 \times 10^4$ cells per well for long-term colony formation assay as described[27] (upper panel). For western blot analysis, protein lysates were prepared by directly lysing the cells in 2 × sodium dodecyl sulfate (SDS) sample buffer (lower panel). (**b**) MEK1 was immunoprecipitated from lysates prepared from control and *SIRT2* knockdown cells using MEK1-specific antibodies. The immunoprecipitated material was analyzed using western blot analysis for MEK1 (upper panel) and pan-acetyl lysine antibody (lower panel). Briefly, control Difi cells and SIRT2 knockdown Difi cells were grown to confluency in RPMI media containing 10% fetal bovine serum in a 10 cm dish. Two hours before harvest, normal RPMI was replaced with glucose-free RPMI. Total cell lysates were prepared using a lysis buffer containing 20 mM, Tris–HCl, pH 8.0, 0.5% NP40, 150 mM KCl, 10% glycerol and 10 mM nicotinamide and 1 μM trichostatin A. Protein concentrations were measured and equal amount of proteins were taken for immunoprecipitation. Control lysate and SIRT2 knockdown lysate each were split into two fractions, one for MEK1 immunoprecipitation (IP) and the other for IgG IP. These antibodies were added to the lysates and incubated for 1 h at 4°C. Protein G-coated Dynabeads (Invitrogen, Carlsbad, CA, USA) were added to the lysates and further incubated at 4°C for 2 h. Protein G immune complexes were washed four times with the lysis buffer and homogenized in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer, after which the samples were run on a 12% SDS–PAGE and analyzed using western blot analysis against MEK1 and pan-acetyl antibodies. (**c**) Model representing the role of SIRT2 in modulating MAP kinase signaling. (1) Represents canonical MAP kinase signaling and the drugs that inhibit the pathway, (2) SIRT2 inhibits MAP kinase signaling by modulating MEK conferring cells sensitive to the drugs and (3) loss of SIRT2 increases MEK kinase activity and enhances MAP kinase signaling output leading to drug resistance.

and activation of its kinase activity. To test this, we asked if MEK1 acetylation is increased in Difi cells following *SIRT2* knockdown. We immunoprecipitated endogenous MEK1 from lysates prepared from both control and *SIRT2* knockdown cells and probed western blot analysis from these immunoprecipitates with an acetyl lysine-specific antibody. Figure 4b indeed shows a clear increase in MEK1 acetylation in *SIRT2* knockdown cells relative to parental Difi cells. Taken together with the data from the accompanying manuscript, these data provide a rationale for the increased ERK kinase activity in cells that have suppressed *SIRT2* expression.

Using a loss-of-function genetic screen with a set of epigenetic regulators, we identify *SIRT2* as a potential player in conferring resistance to several targeted cancer therapies. The important role of epigenetic regulators in cancer becomes increasingly evident as more and more mutations in different epigenetic regulators are discovered. For instance, nearly 50% of clear-cell ovarian carcinomas harbor mutations in the *ARID1A* gene, which is part of the mammalian SWI/SNF complex,[7,19] more than >60% of pancreatic neuroendocrine tumors carry mutations in either *MEN1*, *DAXX* or *ATRX* genes[20] and over half of bladder cancers have genetic aberrations in chromatin remodeling genes.[21] It has also been suggested that epigenetic effects have a role in drug tolerance, most notably a reversible kind, modulating the epigenetic landscape of the cancer cell.[13,22] Here, our work indicates that the loss of *SIRT2* provides drug resistance by upregulating the activity of non-chromatin targets MEK and ERK.

We find here that loss of *SIRT2* confers resistance to EGFR inhibition in colon cancer and lung cancer cell lines. SIRT2 belongs to class III histone deacetylases called sirtuins. Mammalian sirtuins (SIRT1-7) are the homologs of yeast sir2. The special nature of these deacetylases is that these enzymes are NAD^+ dependent and have diverse roles in cellular physiology. The role of sirtuins in aging is well understood, especially in model organisms like *Caenorhabditis elegans* and *Drosophila*. Their role in cancer is beginning to gain interest in light of recent publications describing them both as oncogenes and tumor suppressors. For instance, *SIRT7*, an H3K18 deacetylase, is required for cancer cell maintenance and anchorage-independent growth.[23] On the other

hand, *SIRT6*, an H3K9 deacetylase, is a tumor suppressor that regulates aerobic glycolysis in cancer cells.[24] *SIRT2* itself has been described previously as a tumor suppressor protein, which regulates the activity of anaphase-promoting complex.[16] It has also been shown that *SIRT2* loss leads to increased Aurora kinases A and B, centrosome amplification and aneuploidy.[16]

Our data illustrate another tumor suppressor role for *SIRT2:* its ability to modulate pERK levels (Figures 3b, d, f and 4a). Thus, SIRT2 can keep a check on cell proliferation by inhibiting the MAP kinase pathway and in turn loss of *SIRT2* can contribute to resistance to drugs targeting the receptor tyrosine kinase-RAS-RAF-MEK-ERK pathway. Our data are fully consistent with those of Mayo and co-workers,[18] which demonstrate that MEK1 is activated by acetylation and that this acetylation can be removed by SIRT2. Taken together, our data indicate that loss of *SIRT2* leads to increased MEK1 acetylation and kinase activity. This in turn attenuates the response to upstream inhibition of either EGFR or BRAF.

As *SIRT2* loss conferred resistance to the cancer drugs in the MAP kinase signaling pathway *in vitro*, we asked whether *SIRT2* loss is associated with response to EGFR inhibitors in colon cancers. We stained several tissue microarrays having colon tumors from patients treated with cetuximab for expression of SIRT2 by immunohistochemistry, but failed to establish a strong link between *SIRT2* loss and cetuximab resistance. One of the potential reasons for this could be that most patients receiving cetuximab also received chemotherapy, which confounds the response to cetuximab.[25] In addition, our finding that only SIRT2L isoform, but not the SIRT2S form, has a strong effect on ERK signaling raises the possibility that subtle changes in *SIRT2* isoform expression could modulate drug responses in cancer. We are currently in the process of generating an antibody against the 37 amino acids that are unique to SIRT2L. This reagent will enable a further investigation into the potential role of *SIRT2* in resistance to drugs that act in the receptor tyrosine kinase-RAS-RAF-MEK-ERK pathway.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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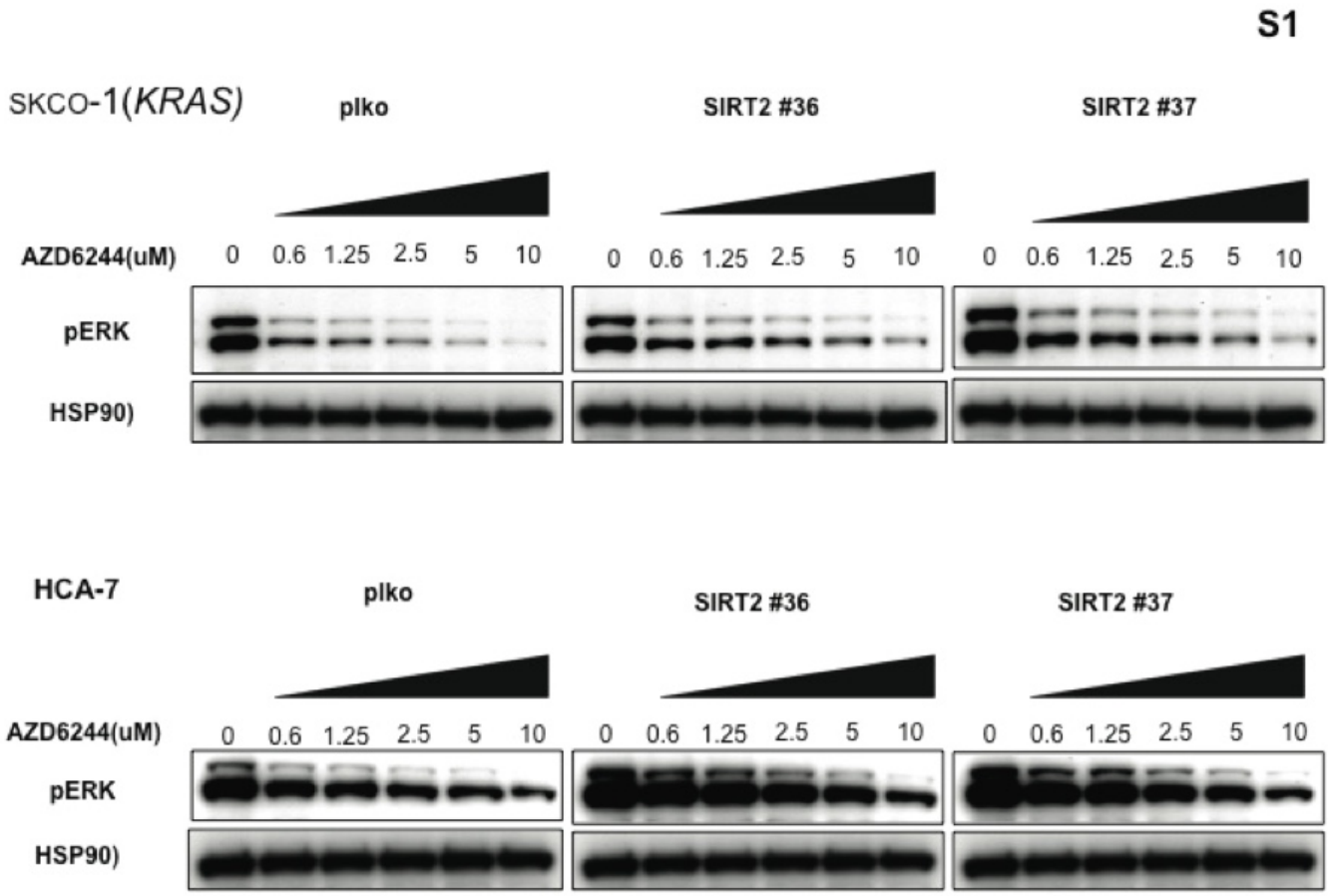
Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Supplemental data-Chapter 4

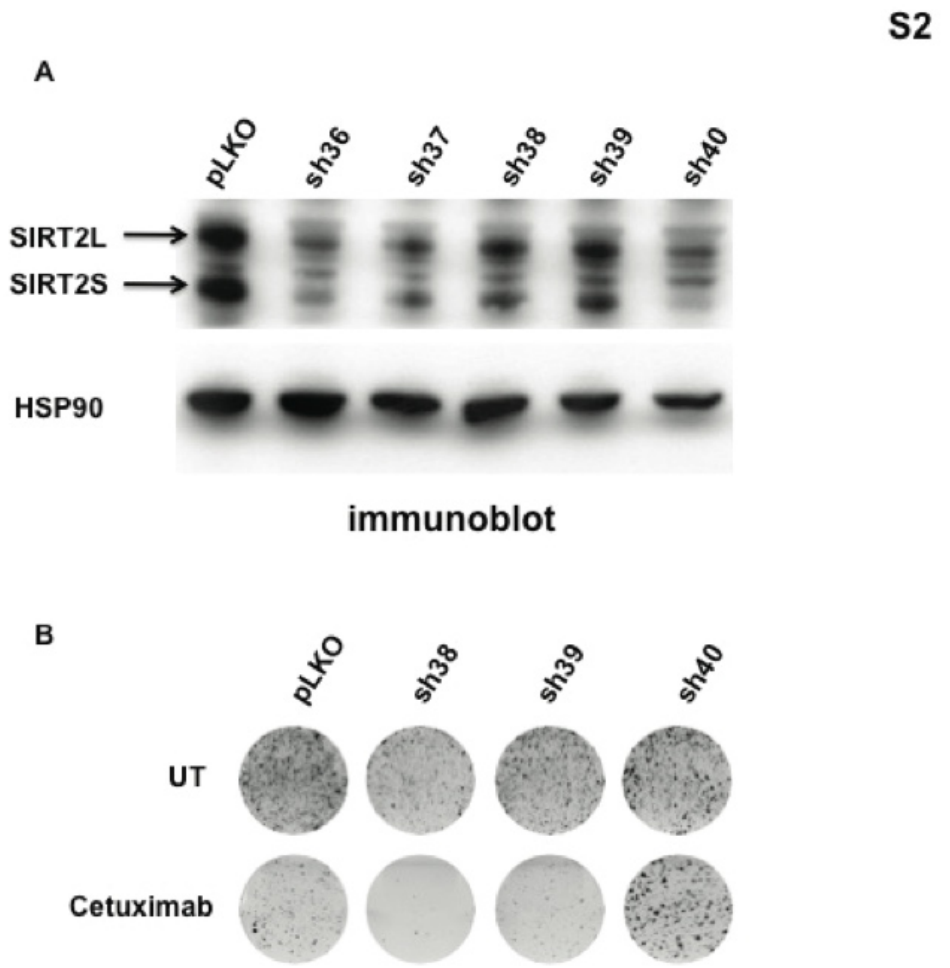
A chromatin modifier genetic screen identifies SIRT2 as a modulator of respo to targeted therapies through regulation of MEK kinase activity.

Oncogene. 2015 Jan 22; 34(4): 531-6



S1: SIRT2 knock down cells need higher concentrations of MEK inhibitor to inhibit p-ERK

Western blots showing inhibition of phospho-ERK over a range of MEK inhibitor (AZD6244) concentrations on two different colon cancer cell lines SKCO-1 (upper panel) and HCA7 (lower panel) in control and *SIRT2* knock down cells. Note that higher concentrations of inhibitor are necessary for inhibition of ERK in *SIRT2* knock down cells.



S2: Knock down efficiencies of SIRT2 using SIRT2 shRNA vectors Knock down efficiencies of all 5 shRNA vectors (sh36, sh37, sh38, sh39 and sh40) for SIRT2 were tested using western blotting with antibodies directed against SIRT2. HSP90 immunoblot was used as loading control.

Genetic Screen with a chromatin regulator shRNA library.

Lentiviral plasmids (pLKO.1) encoding shRNAs that target the epigenome (consisting of chromatin remodelers, chromatin modifiers, histone chaperones, histone variants and many other proteins which share one more domains commonly found in the chromatin associated proteins, as listed in the Supplementary Table 1, were compiled in a pooled format. The chromatin regulator library consists of 6 plasmid pools, where each gene is represented by 5 independent shRNAs. The lentiviral supernatants were produced following the guidelines listed at http://www.broadinsititute.org/rnai/public/resources/protocols.

Difi cells were infected with each of the 6 viral pools independently with a multiplicity of infection 1 (MOI=1). The library-infected cells were then pooled and plated at 150,000 cells per 15cm culture dish in the absence or presence of cetuximab 0.25μg/ml (5 dishes per condition). The cells were cultured with and without drugs, changing media twice per week for a period of 3 weeks until drug resistant colonies appeared in the drug-treated condition. The cells were harvested by the end of the third week and genomic DNA was isolated from both the untreated and treated cells as described (1). The shRNA cassettes were retrieved from 8ug of genomic DNA by performing a 2 step PCR amplification (PCR1 and PCR2) using the following conditions: (1) 98°C, 30 s; (2) 98°C, 10 s; (3) 60°C, 20 s; (4) 72°C, 1 min; (5) to step 2, 15 cycles; (6) 72°C, 5 min; (7) 4°C. Indices and adaptors for deep sequencing (Illumina) were incorporated into PCR primers. From the 1st PCR 2.5μl was used as templates for PCR2 reaction. The final PCR products were purified using QIAGEN PCR purification kit. To ensure that the samples were pooled at the same molar ratio before performing deep sequencing, we quantified the samples using a bioanalyzer.

The shRNA stem sequence was segregated from each sequencing reads and aligned to the TRC library. The matched reads were counted and the counts were transformed to abundance that was assigned to the corresponding shRNA. The primers used are as follows,

PCR1-Forward primers

Illuseq_01_PLKO1_f, ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTGATCTTGTGGAAAGG ACGAAACACCGG, Illuseq_02_PLKO1_f, ACACTCTTTCCCTACACGACGCTCTTCCGATCTACATCGCTTGTGGAAAGG ACGAAACACCGG, Illuseq_03_PLKO1_f, ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCTAACTTGTGGAAAGG ACGAAACACCGG, Illuseq_04_PLKO1_f, ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGTCACTTGTGGAAAGG ACGAAACACCGG, Illuseq_05_PLKO1_f, ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACTGTCTTGTGGAAAGG ACGAAACACCGG, Illuseq_06_PLKO1_f, ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTGGCCTTGTGGAAAGG ACGAAACACCGG

PCR1 reverse primer

CAAGCAGAAGACGGCATACGAGATTTCTTTCCCCTGCACTGTACCC

PCR2 forward primer

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCT

PCR2 reverse primer

CAAGCAGAAGACGGCATACGAGAT

Plasmids

Retroviral expression constructs for MEK-DD and *PIK3CA*^{H1047R} cloned in the pBABE puro construct were purchased from Addgene and sequence verified. The cDNA encoding myr-AKT was cloned into the pBABE puro plasmid. The pBABE-

puro-*BRAF*^{V600E} plasmid was a kind gift from Daniel Peeper. More details of these active alleles of RAS effector pathways were described previously (2).

All lentiviral shRNA vectors were retrieved from the arrayed TRC human genome wide shRNA collection (TRC-Hs 1.0). For additional information please see http://www.broadinstitute.org/rnai/public/clone/search.

For SIRT2, the following shRNAs were used: sh*SIRT2#36* (CCTGTGGCTAAGTAAACCATA), sh*SIRT2#37* (GCCATCTTTGAGATCAGCTAT), sh*SIRT2#41* (TATGACAACCTAGAGAAGTAC).

SIRT2L and *SIRT2S* expression plasmids were generated by amplifying the *SIRT2L* and *SIRT2S* open reading frames using Difi cell cDNA. The primers used are as follows, SIRT2L Forward primer (TTTTTTTGGATCCATGGCAGAGCCAGACCCCTC), *SIRT2L* Reverse primer (TTTTTTTGAATTCTCACTGGGGTTTCTCCCTCT), *SIRT2S* Forward primer (TTTTTTTGGATCCATGGACTTCCTGCGGAACTT) and *SIRT2S* reverse primer (TTTTTTTGAATTCTCACTGGGGTTTCTCCCTCT). The PCR products were cloned into pBABE(Blasticidin) using BamH1/EcoR1 restriction sites and sequence verified.

Cell culture, viral transduction and long-term colony formation assay.

Difi, PC9, A375 and HCA7 cells were cultured in RPMI which was supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1% penicillin and streptomycin (Pen/Strep) at 5% CO_2. HEK293T cells were cultured in DMEM with

10% heat-inactivated FBS and 0.1% Pen/Strep at 5% CO2. Phoenix cells were cultured in DMEM with 10% heat inactivated serum and 0.1% penicillin streptomycin at 5% CO_2.

Phoenix cells were used to produce retroviral supernatants as described at http://www.stanford.edu/group/nolan/retroviral_systems/phx.html. Lentiviruses using the pLKO.1 shRNA plasmids were produced according to the guidelines: http://www.broadinstitute.org/rnai/public/resources/protocols. Calcium phosphate based transfection method was used to transfect the Phoenix and HEK293T cells. Infected target cells were selected with 2μg/ml puromycin. Cells were seeded in 6-well plates at a density of 5-10 * 10^4 cells/ well in the presence or absence of drug for long-term colony formation assay more details are described (3).

Antibodies and Reagents

The primary antibodies used for blotting are as follows: pMEK1/2 (S217/221, #9121) and MEK1/2 (L38C12, #4694), MEK1 (#61B12), acetylated lysine antibody (#9441) were from Cell Signaling technologies. HSP-90 (H-114), p-ERK (E-4), ERK1 (C-16), ERK2 (C-14) were from SantaCruz Biotechnology. A mixture of ERK1 and ERk2 antibodies was used for detection of total ERK. SIRT2 primary antibody (HPA011165) was purchased from Sigma-Aldrich.

Inhibitors such as PLX4032 (S1267), AZD6244 (S1008) were purchased from Selleck Chemicals. Cetuximab (Erbitux) was obtained by the AVL hospital pharmacy.

Protein Biochemistry

Difi, A375 and HCA-7 shSIRT2 knockdown cells (shSIRT2#36 & shSIRT2#37) were selected in the presence of cetuximab, PLX4032 and AZD6244 for 2 weeks. After 2

weeks of drug selection the cells were recovered for 48hrs in regular RPMI medium supplemented with 10% FBS and Pen/Strep. After recovery, the cells were either left untreated or treated as follows for 6 hours: 1:20000 cetuximab for Difi, 1μM PLX4032 for A375, 1μM AZD6244 for HCA-7 for 6hr conditions respectively. Following drug exposure, protein lysates were prepared by directly lysing the cells in SDS_PAGE sample loading buffer (50mM Tris-HCl, pH-6.8, 10% Glycerol, 12.5mM EDTA, 1% β-mercaptoethanol and 0.02% Bromophenol blue). The samples were processed by heating at 98°C for 6-7 min and used for western blot analysis.

Quantitative real time PCR

Quantitative real time PCR (qRT PCR) was carried out to measure the relative levels of RNA of *SIRT2* using 7500 Fast Real-Time PCR System (Applied Biosystems) as described (4). The relative mRNA levels of *SIRT2* was normalized to the levels of the housekeeping gene *GAPDH*. The primers were used together with the SYBR green PCR mix reaction (FastStart Universal SYBR green mastermix, Roche). The sequence of *SIRT2* primes used for the assay are as described. Forward primer-TCCACCAAGTCCTCCTGTTC, Reverse primer-TGAAGGACAAGGGGCTACTC.

Phospho Kinase Array

Phospho proteomic analysis of the downstream effector protein kinases were performed using the Human Phospho-Kinase Antibody Array purchased from R&D Systems (Catalog# ARY003B). The experiment was carried out according to the manufacturers instructions.

Immunoprecipitation

Briefly, control Difi cells and SIRT2 knock down difi cells were grown to confluency in RPMI media containing 10% FBS in a 10 cm dish. 2 hours before harvest, normal RPMI was replaced with Glucose-free RPMI. Total cell lysates were prepared using a lysis buffer containing 20mM, Tris HCl, pH8.0, 0.5% NP40, 150mM KCl, 10% Glycerol and 10mM Nicotinamide and 1uM TSA. Protein concentrations were measured and equal amount of proteins were taken for immunoprecipitation. Control lysate and SIRT2 knock down lysate each were split into two fractions, one for MEK IP and one for pan-acetyl-lysine IP. These antibodies were added to the lysates and incubated for 1 hour at 4 degrees. Protein G coated Dynabeads (Invitrogen) were added to the lysates and further incubated at 4 degrees for 2 hours. Protein G immune complexes were washed 4 times with the lysis buffer and homogenized in SDS-PAGE sample buffer after which the samples were run on a 12% SDS-PAGE and analysed using western blotting against MEK1 and pan acetyl antibodies.

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Chapter 5

General Discussion

General Discussion

Inhibition of deregulated pathways in cancer using targeted agents has proven to be effective in treating cancer in the clinic. However, cell intrinsic and acquired resistance to targeted agents pose a formidable challenge in gaining long-lasting responses. Cell-intrinsic feedback mechanisms or activating mutations in other pathway components can override the response to pathway inhibition. One such example that is clinically relevant is the example of lack of response to BRAF inhibition in *BRAF(V600E)* mutant colorectal cancer. Inhibition of mutant BRAF using vemurafenib in *BRAF* mutant melanomas has led to impressive clinical responses and is associated with extended progression free survival[1]. However, some 5-10% of *BRAF* mutant colon cancers show dismal responses to BRAF inhibition[2]. This is a unique situation where the context rather than the genotype has become the major therapy response predictor. In chapter 2 we have addressed this issue by identifying the mechanism underlying this lack of response. Using an unbiased shRNA screening approach we scarched for additional kinases that, when inhibited, would restore the sensitivity to vemurafenib. We found that inhibition of the epidermal growth factor receptor (EGFR) conferred sensitivity to BRAF inhibition[3]. Mechanistically, BRAF inhibition in BRAF mutant CRC led to a strong feedback activation of the EGF receptor (EGFR) that further resulted in the reactivation of MAP kinase and the PI3K signalling pathways. The feedback engagement of EGFR upon BRAF inhibition was abrogated when the EGFR inhibitors cetuximab or gefitinib were combined with BRAF inhibition. The synthetic lethal relationship between EGFR and BRAF in *BRAF* mutant CRC, suggested a straightforward route of translating this finding to the clinic, since cetuximab is already an FDA approved drug for the treatment of colon cancer[4]. Based on this finding three clinical trials in which the efficacy of the combination of BRAF and EGFR antibody drugs is tested in *BRAF* mutant colon cancer (Trial identifiers NCT01719380, NCT01750918 and NCT01791309) have been initiated. Gratifyingly, the first positive results from one of the trials have already been reported[5]. Relevant to this discussion, most of the *BRAF* mutant colorectal cancer cell lines and some *BRAF* mutant thyroid

cancer cell lines display higher expression of both total and phospho EGFR compared to the *BRAF* mutant melanoma cell lines that show little or no EGFR expression. This is consistent with the notion that both the *BRAF* mutant colon and thyroid cancer cell lines originate from the epithelial lineage compared to *BRAF* mutant melanomas that have a neural crest origin. Hence, EGFR expression is a major predictor of response to BRAF inhibition in *BRAF* mutant CRC. More generally, these findings highlight the notion that the genotype alone is not always a faithful predictor of response to targeted cancer drugs and that the context in which these mutations are found is also relevant.

A similar situation is seen in the case of *KRAS* mutant cancers that are refractory to downstream MEK inhibition[6, 7]. Sun *et al* performed a negative drug selection screen in *KRAS* mutant cells that were resistant to MEK inhibition[8]. They found ERBB3 as synthetic lethal with MEK inhibition. They identified a mechanism where MEK inhibition resulted in a MYC dependent transcriptional upregulation of *ERBB3* and conferred resistance. Drugs that target both EGFR and ERBB2, like afatinib or dacomitinib, were able to synergise with MEK inhibition in *KRAS* mutant cancers. Based on these preclinical findings, this combination is being tested in a clinical trials (NCT02039336, NCT02230553) Based on the initial results patients receiving the combination of dacomitinib and a MEK inhibitor suffered from severe toxicities. Finding alternative combination treatments that are less toxic even at the maximum tolerated dose is a challenging task. Functional genetic screens can identify multiple targets within the same pathway that can be combined in alternative ways together with a targeted agent and could be potentially tested and evaluated in clinical trials. For example in the same loss of function genetic screen that Sun *et al*. performed, they also identified RAF1 as synthetic lethal with MEK inhibition in *KRAS* mutant setting[9]. They demonstrated that inhibition of RAF1 with a preclinical compound AZD628 was highly synergistic with MEK inhibition. However, this drug also showed severe toxicities in mice. Several second generation inhibitors targeting the interaction between MEK and RAF are under investigation[10, 11]. Several others have also found interesting genes that are synthetic lethal with MEK inhibition in the *KRAS* mutant setting however, none of them have proven to be successful in the clinic as of yet[12-14]. Recently, a BRAF inhibitor resensitization screen performed in *BRAF* mutant lung cancer cell line HCC364 revealed YAP1

suppression as synthetic lethal with BRAF inhibition[15]. YAP1 and TAZ are transcriptional co activators and downstream effectors of Hippo signalling pathway[16]. YAP transcriptionally up regulates specific anti-apoptotic components including the BCL2 family member protein BCL-XL[17, 18]. They found that inhibition of YAP1 and MEK /BRAF together led to a strong reduction in BCL-XL expression that explained the synthetic lethal effect. This study highlights an unanticipated functional cross talk between YAP1 and RAF-MEK signalling and promises a potential way of combining drugs targeting these pathways. More generally, this finding highlights that it is essential to know all the druggable components of pathways and the cross talks between signalling pathways to enable clinicians to choose which clinically tolerable combinations to give to patients.

Oncogenic pathway inhibition either upstream (RTKs) in the pathway or downstream (BRAF or MEK inhibition, PI3K or AKT inhibition) can be bypassed either through up regulation of additional RTKs or through acquiring additional mutations in the target itself[19, 20]. This notion is supported by the strong synergistic effects seen with the combination of EGFR and BRAF in *BRAF* mutant CRC.[3]. In a search to find potential phosphatases whose suppression would disrupt the feedback engagement of EGFR in *BRAF* mutant CRC, we identified *PTPN11* (Chapter 3). *PTPN11* is a SRC homology 2 (SH2) containing protein tyrosine phosphatase that is downstream of many receptor tyrosine kinases and cytokine receptors. *PTPN11* is regulated through a auto inhibitory switch. When RTKs are activated through growth factors, the phosphorylated tyrosine on RTKs recruits and activates *PTPN11* through a C terminal phosphorylation (Y542) that activates the phosphatase domain of PTPN11. Its phosphatase function is fully essential to promote RAS activation[21, 22]. We found that suppression of *PTPN11* using shRNAs or CRISPRs in combination with BRAF inhibition led to synergistic cell killing in *BRAF* mutant colon cancer. Since activated receptor tyrosine kinases recruit *PTPN11* for effective signal transduction, we observed a strong feedback activation of both EGFR (Y1068) and PTPN11 (Y542) upon BRAF inhibition and led to downstream MEK-ERK reactivation. However, EGFR engagement of RAS-MEK-ERK signaling was not possible in the absence of PTPN11 and conferred potent sensitivity to BRAF inhibition. We have demonstrated that PTPN11 is downstream of many RTKs (EGFR, MET, HER2, ALK, TGFβR, KIT, and FGFR) and prevents resistance

mediated by the activation of these RTKs when its function is inhibited. Targets like PTPN11 are very attractive since they represent a crucial signalling node that serve to converge extracellular growth factor signals to the downstream effectors pathways. Perhaps vertical targetting of multiple nodes in the same pathway could prevent the emergence of resistance and extend survival[23, 24]. This concept is analogous to microRNAs that inhibit signaling pathways effectively by partial inhibition of multiple nodes of that pathway. For example, Shirdel *et al* examined two separate signalling components of PI3 kinase signalling with respect to microRNA involvement. First the PI3K subunit regulation and second the downstream signalling components of this pathway were examined[25]. PI3K family proteins contain two distinct subunits ; the catalytic subunit PIK3CA/B/C/D and the regulatory subunit PIK3R1/2/3/4/5/6. Mapping the microRNAs targeting genes involved in the assembly of Class1 PI3K subunit regulation revealed a microRNA interactome containing five primary nodes PIK3D, PIK3A, PIK3R1, PIK3R2 and PIK3R3 and 181 secondary nodes and 206 interactions. Strikingly, each primary node is connected to at least two other primary nodes through multiple distinct non overlapping miRNAs. This indicated that the assembly of class1 PI3K subunits is tightly regulated post transcriptionally by the coordination of multiple microRNAs[25]. Mapping the microRNAs targeting genes downstream of the PI3K signalling pathway, revealed a highly connected microRNA network. From this network multiple miRNAs that can co- target potent oncogenes and tumor suppressor genes were enriched. For example, hsa-mir-19b was found to concurrently target PTEN-TSC1-PI3KCA-TP53, and similarly, other microRNAs were able to co-target RPS6-KB1-PDK1-TSC1-PTEN and PTEN-RPS6KB1-FOXO3-TSC1 respectively. There were also miRNAs that targeted pairs of elements of the pathway for example, 15 miRNAs targeted RPS6KB1 and PTEN, 8 miRNAs targeted both RPS6B1 and TSC1 and 4 miRNAs that targeted both EIF4E and RPS6KB1[25].

Another well established example is the *let-7* microRNA that is known to have multiple targets including RAS, MYC, P53, HMGA2 and genes involved in the regulation of cell cycle, proliferation and apoptosis[26-28]. *Let7* is underexpressed in many cancers, and restoration of its expression back to normal physiological levels can have a major impact on reducing the activity of many oncogenes and tumor suppressor genes. Lung cancer, for instance, has several key oncogenic mutations including *p53*, *RAS* and *MYC*, some of which may directly correlate with the reduced expression of *let-7*, and

may be repressed by introduction of *let-7*[29]. A study performed in 2012 investigated the role of *PTPN11* in HER-2 positive and triple negative breast cancer, showed that *PTPN11* suppression was able to prevent stemness and metastasis by down regulating the expression of ZEB1 and MYC[30]. MYC controls the Expression of *let-7* members by binding to their promoters. C-MYC induced LIN28B expression repressed let-7 microRNA and led to overexpression of let-7 targets including RAS and c-MYC. However, Inhibition of PTPN11 led to decreased expression of c-MYC and increased the expression of let-7 and inhibited tumor formation. This suggests that *PTPN11* is a central signaling node that is regulating core-signaling components in the MAPK signaling pathway through the control of let7 expression in breast cancer. Our work places *PTPN11* in the context of feedback activation of RTK during drug resistance. We and others have shown that *PTPN11* is involved in transmitting signals from various RTKs and cytokine receptors and that targeting PTPN11 would prevent the engagement of RTKs while adapting to therapy. Perhaps we could learn and understand better how signalling nodes are interconnected and regulated through miRNAs and leverage this information to drug mutilple nodes to prevent resistance. Since PTPN11 pleiotropically regulates various cellular processes, therapeutic inhibition could lead to toxicities[31]. Perhaps, by analogy to the strategy used by miRNAs, combining drugs that target PTPN11 with various additional drugs that target signalling nodes in the same signaling pathway, where each is used at lower concentration, could reduce toxicity of a combination treatment, while maintaining full pathway inhibition. What is also unique about *PTPN11* is that the pTYR542 activation mark can be used as a biomarker to monitor the involvement of RTKs during drug resistance so that clinicians can decied which appropiate combination treatments to deliver. These findings are paving way for thinking about new ways to target these pathways when using combination therapy with selective PTPN11 inhibitors[32].

Loss of function genetic screens can be employed to idenitfy resistance mechanisms that occur due to loss of expression of certain genes. One such gene that we identified in chapter 4 is SIRT2. The human genome has seven sirtuin proteins (SIRT1–7) that fulfill specialized deacetylase functions based on their cellular compartmentalization to the nucleus, cytoplasm and mitochondria [33, 34]. In

trying to identify molecular mechanisms of resistance to cetuximab treatment in colon cancer, we performed a loss of function genetic screen using a chromatin regulatory shRNA library. We identified that loss of SIRT2 confered potent resistance to cetuximab treatment in colorectal cancer cell line Difi[35]. We and others identified that SIRT2 is implicated in regulation of the MAPK signalling pathway by directly modulating MEK kinase activity[36]. MEK kinase is an important downstream effector protein in the MAPK signalling pathway and is directly downstream of BRAF kinase. Acetylation of MEK kinase by p300 promotes its activation either by stimulating autophosphorylation or enhance EGF induced MEK kinase activation. We found that loss of SIRT2 led to hyper activation of MEK kinase activity due to increased acetylation and this resulted in reactivation of ERK signalling and conferred resistance to EGFR inhibition (cetuximab). In addition, SIRT2 loss conferred resistance to BRAF inhibition in *BRAF(V600E)* mutant melanoma and to MEK inhibitors in some *KRAS* mutant cell lines. Although numerous proteins are known to be deacetylated by sirtuins, this study has revealed a new level of regulation in the MAPK signalling pathway and highlights SIRT2 as a tumor suppressor that regulates inappropriate MEK1 acetylation and subsequent activation.

Concluding remarks

At present cancer cell lines serve as the major model system for functional studies in signalling pathways, as they allow experimental manipulation, detailed mechanistic studies and are amenable for high throughput applications. To realize the objective of personalised cancer medicine, a large number of lineage-specific cell lines are needed to capture the genetic diversity observed in cancer patients in the clinic[37]. Large-scale genomic efforts to fully characterize the cancer cell lines have revealed that most of the genetic features of tumors are represented in cancer cell lines. This also serves as a huge resource and a precise platform for experimental interrogation, predicting drug sensitivity and also identify biomarkers that guide treatment[38, 39] On the other end of the spectrum, tumor heterogeneity can contribute to therapeutic resistance[40]. In this context, patient-derived xenograft (PDX) models can

be generated from a wide range of cancer types and best reflect the heterogenity within and in between different cancer histotypes[41, 42]. They can be implanted as fragments of tumors directly from patients to immunodeficient mice. PDX models also preserve mutation profiles as well as the response patterns to targeted therapies. PDX also recapitulate the post –therapeutic tumor charecteristics such as residual disease and tumor relapse. Furthermore, xenograft models allow the interaction of tumor cells with stromal components. Although the stroma in PDX mouse models is of non-human origin, the composition of the tumor microenvironment in most cases compares well to the structure in human tumors[43]. This is an important aspect to consider since tumor-stroma interactions affect cell signalling, survival and proliferaction and therefore impact drug sensitivity and resistance[44].

Alternatively, tumor-derived organoids can be propogated indefinetly in vitro and are also amenable to several high throughput applications[45]. These tumor-derived organoids also recapitulate the exact genetic makeup of the patients tumor and the living organoid biobank is also amenable to several high throughput applications[46]. It is difficult to say at this point what is a better model to predict genotype-drug response relationships for personalised cancer treatment. Cell lines grown in 2D are in general quite good in predicting responses to targeted cancer drugs seen in the clinic,which still remains to be established for organoids. One recent finding is that *BRAF* mutant colon cancer organoids respond to single agent BRAF inhibitors in 3D culture[46], whereas all *BRAF* mutant 2D CRC cell lines are resistant to BRAF inhibitor monotherapy[3]. In this case, the clinical experience resembles more the responses of 2D cell lines than the 3D organoid cultures, as most *BRAF* mutant CRC patients fail to respond to BRAF inhibitor montherapy[2]. It will be interesting to study how *BRAF* mutant CRC orgnaoids respond to BRAF inhibitors following transplantation into immunodeficient mice. Which in vitro models will turn out to be the most faithful models to predict patient responses in the clinic is an important question that needs significant further study. While 2D cell lines certainly have limitations, the work described in this thesis clearly shows that they do have utility in predicting drug responses in the clinic.

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Addendum

Doctoral research summary

The deployment of cancer therapeutic agents that target specific molecular pathways has been the hallmark of drug development in oncology over the past decade. This has helped us realize the dream of personalized cancer medicine since we can tailor the treatment to individual patients based on the genetic makeup of the tumor. In a limited number of specific instances, a single molecule or antibody directed against an oncogenic target that sustains tumor growth and survival has proved remarkably effective in providing long-term control of a specific disease process. Some of these Molecular Targeted Agents (MTA) include imatinib (BCR-ABL), Vemurafenib (BRAFV600E), Herceptin (HER2), Gefitinib (EGFR TKI) and Cetuximab (EGFRmab) respectively. However, considering the complexity of our genome and the existence of redundancy and crosstalk in signaling pathways, resistance to these MTA is inevitable. We have come to the realization that more combinations of MTAs would be required to maintain a steady response in cancer patients. This is not surprising as multiple combinations of antiviral /antibiotics drugs are administered to patients with HIV or tuberculosis to manage the disease. Since we have an arsenal of MTAs in the pipeline, its difficult to assess which combination treatment will deliver the best therapeutic effect. It is often seen that most of the combination treatments that are experimented in clinical trials often seem to deliver modest clinical responses. This is due to the lack of proper mechanistic insight underlying the complexity of targeting signal transduction pathways. In this thesis we use RNA interference technology to understand signaling crosstalk, resistance mechanism and rational combination therapies for colon cancer.

Chapter 1 is a review that summarizes the challenges and opportunities provided by crosstalk between signaling pathways during therapeutic intervention. This chapter discusses the problem of drug resistance seen with targeted monotherapies and provides a comprehensive over view about identifying rational combinations to fight resistance using functional genetic screening technology.

In **Chapter 2** we have solved a trivial clinical problem i.e. why do BRAF mutant colon cancers respond poorly to BRAF inhibition as against BRAF mutant melanoma? Using a kinome-centered loss of function genetic screening we identified EGFR as synthetic lethal with BRAF inhibition in BRAF mutant CRC. It is counterintuitive to inhibit EGFR, which is upstream of BRAF in the MAPK pathway, but the relief of negative feedback upon BRAF inhibition that is mediated via CDC25C phosphatase, aids in the activation of EGFR upstream

of BRAF. Using a combination of clinically approved EGFR inhibitors and the BRAF inhibitor, we were able to show strong synergistic effect on cell proliferation in-vitro and iv-vivo. Here we have a mechanistic rational to combine EGFR inhibitors with BRAF inhibitor in the context of BRAF mutant CRC. Moreover, this is an elegant example to illustrate that the genotype alone is a bad predictor of therapy response and emphasizes the point of context dependency and tissue specificity[1]. The above study has now led to the design of three clinical trials, in which BRAF mutant CRC patients are being treated with the combination of an EGFR and a BRAF inhibitor. We are beginning to see impressive responses in patients that have been treated so far. ClinicalTrials.gov Identifier: NCT01719380, NCT01750918 and NCT01791309.

In **Chapter 3** we carried out a phosphatase centered loss of function genetic screen in BRAF mutant CRC in order to identify phosphatases that disable the feedback engagement of EGFR upon BRAF inhibition. We identified PTPN11 suppression as highly synergistic with BRAF inhibitors in BRAF mutant CRC. PTPN11 is a positive regulator of receptor tyrosine kinase signaling. We find that inhibition of PTPN11 disrupts the feedback engagement of EGFR signaling in BRAF mutant CRC and also abrogated growth factor driven resistance in melanoma. We show that PTPN11 pTYR 542 can be used as a biomarker to identify the involvement of RTKs during drug resistance.

In **Chapter 4** we focus on identifying resistance mechanism to cetuximab in colorectal cancer. Using a chromatin modifier RNAi library we identified SIRT2 loss as a modulator of cetuximab sensitivity. SIRT2 is a deacetylase of MEK1. Increased Acetylation of MEK1 upon loss of SIRT2 leads to MEK1 hyper phosphorylation and increased MAP kinase pathway signaling and confers resistance to cetuximab. We also show that loss of SIRT2 confers resistance to BRAF and MEK inhibitors in BRAF mutant and KRAS mutant cancers.

In **Chapter 5** we discuss about the general advantages and limitations of cell lines to identify combination treatments and highlight other possible invitro (organoids) or invivo (PDX) models that can be used for preclinical studies.

Samenvatting

Het gebruik van geneesmiddelen die specifieke moleculaire signaleringsroutes remmen is een van de hoogtepunten geweest binnen de oncologie in het afgelopen decennia. Deze ontwikkeling heeft behandeling op maat mogelijk gemaakt: patiënten krijgen een therapie die is afgestemd op de genetische achtergrond van de tumor. Een relatief klein aantal van deze doelgerichte medicijnen heeft geresulteerd in langdurige blokkade van tumorgroei. Het komt helaas veel vaker voor dat patiënten direct of op langere termijn niet meer reageren op een behandeling. Dit wordt veroorzaakt door re-activatie van signaleringsroutes via zogenaamde terugkoppelingsmechanismen. Combinaties van medicijnen zijn daarom nodig om resistentie te voorkomen. Dit is niet verassend, aangezien combinaties van antivirale middelen of antibiotica ook de enige manier zijn om HIV of tuberculose te behandelen. Helaas zijn de meeste combinatietherapieën nauwelijks effectief in klinische studies. Dit is het gevolg van een gebrekkig inzicht in de complexiteit van signaleringsnetwerken die leiden tot resistentie. In deze thesis gebruiken we de RNA interferentie technologie om signaleringsroutes die tot resistentie leiden te ontrafelen met als doel het vinden van rationele combinatietherapieën voor dikke darmkanker.

Hoofdstuk 1 is een literatuuronderzoek waarin ik beschrijf hoe tumoren ongevoelig kunnen worden tegen doelgerichte medicatie. Het gebruik van genetische screens geeft inzicht in de onderliggende resistentiemechanismen. Deze informatie kan gebruikt worden voor het ontwikkelen van slimme combinatietherapieën om resistentie te voorkomen.

In **hoofdstuk 2** hebben we een klinisch probleem opgelost: waarom reageren BRAF gemuteerde dikke darmtumoren slecht op medicijnen die BRAF remmen terwijl melanomen met een identieke mutatie wel goed reageren? Door middel van een genetische screen hebben we ontdekt dat BRAF remming resulteert in activatie van EGFR. Wanneer zowel BRAF als EGFR geremd worden stopt de tumorgroei. Melanomen hebben in tegenstelling tot dikke darmtumoren nauwelijks EGFR en reageren daarom wel op behandeling met enkel een BRAF remmer. Dit onderzoek staat aan de basis van drie klinische studies, waarin patiënten met BRAF gemuteerde dikke darmkanker behandeld worden met een combinatie van een EGFR en een BRAF remmer. De eerste resultaten van deze behandelmethoden zien er veelbelovend uit.

In **hoofdstuk 3** beschrijven we het belang van het gen *PTPN11* in de resistentie tegen BRAF remmers in dikke darmkanker. PTPN11 speelt een cruciale rol in het doorgeven van het groeisignaal van EGFR en andere groeifactorreceptoren. Zodra PTPN11 geremd wordt kan EGFR geen resistentie meer geven tegen BRAF remmers. PTPN11 kan als een 'biomarker' worden gebruikt om de rol van receptoren in resistentie te bepalen.

In **hoofdstuk 4** beschrijven we een resistentiemechanisme tegen de EGFR remmer cetuximab in dikke darmkanker. Met behulp van een genetische screen hebben we ontdekt dat verlies van het gen *SIRT2* leidt tot activatie van MEK1, met als gevolg cetuximabresistentie. We laten daarnaast zien dat verlies van SIRT2 ook ongevoeligheid veroorzaakt tegen BRAF en MEK remmers in respectievelijk BRAF en KRAS gemuteerde dikke darmkanker.

In **hoofdstuk 5** bespreek ik de voor- en nadelen van het gebruik van cellijnen als model om nieuwe combinaties van medicijnen te ontdekken. Daarnaast ga ik in op alternatieve strategieën die gebruikt kunnen worden om resistentiemechanismes te ontdekken: het groeien van tumorcellen in 3D (organoids) en het transplanteren van tumormateriaal afkomstig van patiënten in muizen (patient-derived xenografts).

List of Publications

1 Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D *et al*. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 2012; 483: 100-103.

2 Sun C, Hobor S, Bertotti A, Zecchin D, Huang S, Galimi F *et al*. Intrinsic resistance to MEK inhibition in KRAS mutant lung and colon cancer through transcriptional induction of ERBB3. Cell reports 2014; 7: 86-93.

3 Sun C, Wang L, Huang S, Heynen GJ, Prahallad A, Robert C *et al*. Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. Nature 2014; 508: 118-122.

4 Huang S, Holzel M, Knijnenburg T, Schlicker A, Roepman P, McDermott U *et al*. MED12 controls the response to multiple cancer drugs through regulation of TGF-beta receptor signaling. Cell 2012; 151: 937-950.

5 Prahallad A, Bernards R. Opportunities and challenges provided by crosstalk between signalling pathways in cancer. Oncogene 2015.

6 Bajpe PK, Prahallad A, Horlings H, Nagtegaal I, Beijersbergen R, Bernards R. A chromatin modifier genetic screen identifies SIRT2 as a modulator of response to targeted therapies through the regulation of MEK kinase activity. Oncogene 2015; 34: 531-536.

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Curriculum Vitae

Anirudh Prahallad was born on July 22, 1987 in Mysore, Karnataka State, India. In May 2008 he graduated from Bangalore University with a Bachelor of Science in Life Sciences. In September 2008, he enrolled the international masters degree program Cancer Genomics and Developmental Biology as a recipient of the Utrecht Excellence Scholarship at Utrecht University. He performed his first internship studying brain development in Zebra fish with Dr. Danica Zivkovic at the Hubrecht Institute. In 2009, he joined the group of prof. Rene' Bernards at the Netherlands Cancer Institute in Amsterdam to work on his second internship project co-supervised by Dr. Michiel S van der Heijden. In September 2010, he started to work as a PhD student under the supervision of Prof. Rene Bernards at the Netherlands Cancer Institute. The studies presented in the thesis were performed in the Netherlands Cancer Institute, Amsterdam