LETTER

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

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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

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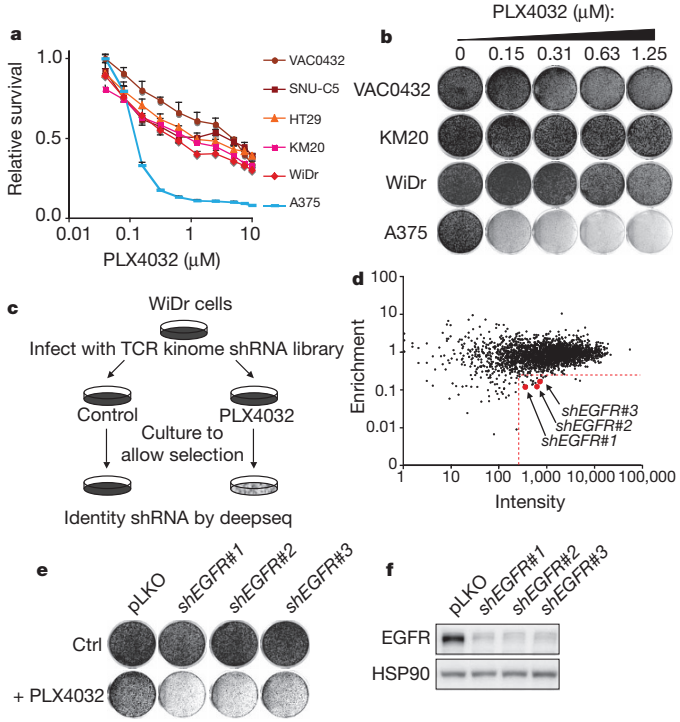

Figure 1 | EGFR inhibition confers sensitivity to BRAF(V600E) inhibition in colon cancer. **a**, **b**, CRC but not melanoma cells harbouring the BRAF(V600E) mutation are resistant to PLX4032 treatment. **a**, Short-term growth-inhibition assay of a cell line panel consisting of CRC (VACO432, SNU-C5, 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.

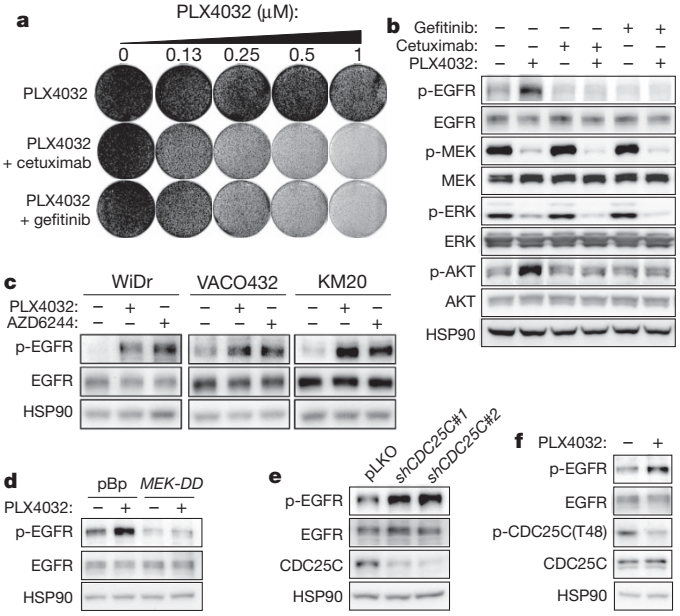

Figure 2 | Functional and biochemical interaction between BRAF and EGFR inhibition in colon cancer. **a**, Synergistic response of WiDr cells to the combination of EGFR and BRAF(V600E) inhibitors. WiDr cells were cultured in increasing concentrations of BRAF inhibitor PLX4032 alone, EGFR inhibitors cetuximab (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.

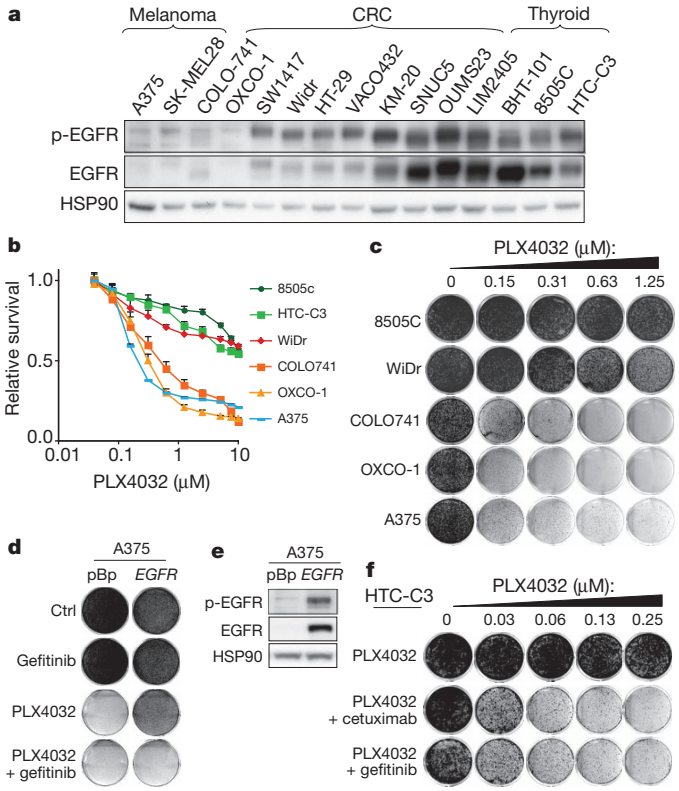

Figure 3 | Correlation between EGFR levels and response to BRAF inhibition in melanoma and CRC. **a**, Western blot analysis of p-EGFR and EGFR levels in a panel of BRAF(V600E) mutant cell lines from melanoma, CRC and thyroid cancer. HSP90 served as a control. **b**, High levels of EGFR expression in 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.

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

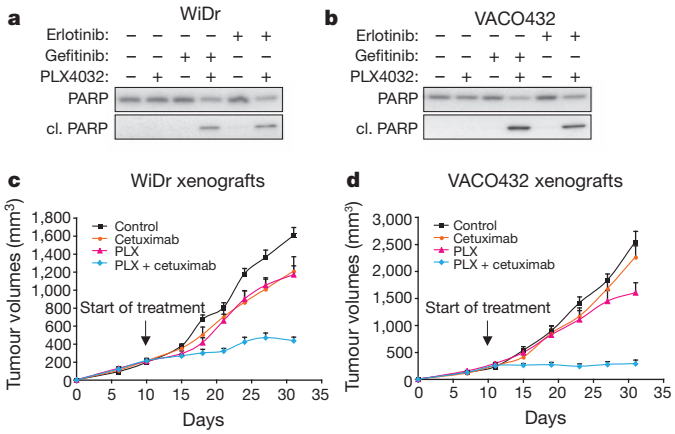

Figure 4 | EGFR and BRAF(V600E) inhibitors synergize to induce apoptosis of CRC cells and to suppress CRC tumour growth in a xenograft model. **a**, **b**, Combination of EGFR and BRAF(V600E) inhibitors leads to apoptosis in CRC cells. Western blot analysis of full-length PARP or cleaved PARP (cl. PARP) 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.

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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.

21. Brummelkamp, T. R. *et al.* An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. *Nature Chem. Biol.* **2,** 202–206 (2006).
22. Huang, S. *et al.* ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome. *Cancer Cell* **15,** 328–340 (2009).