Supplemental Material for

EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of *BRAF* mutant colorectal cancers to RAF inhibition

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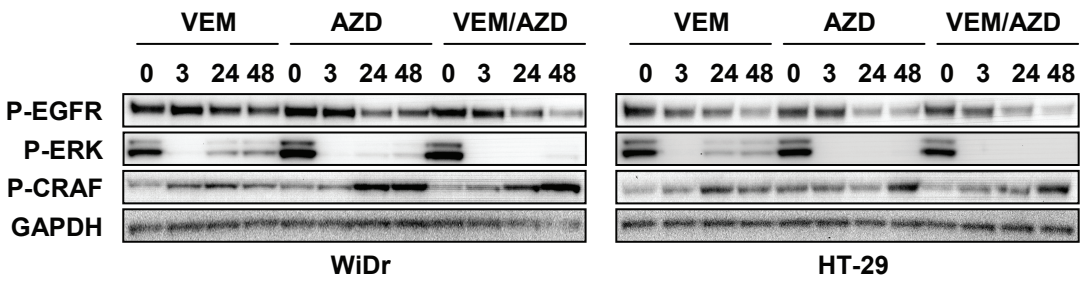
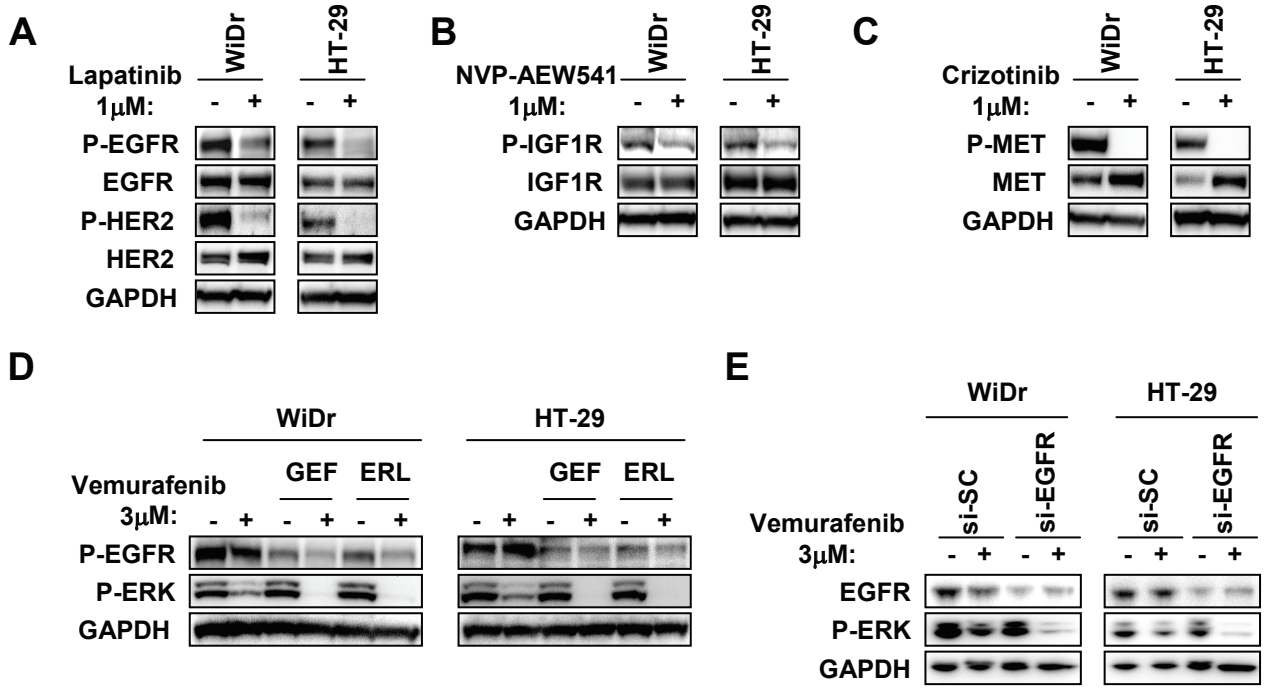
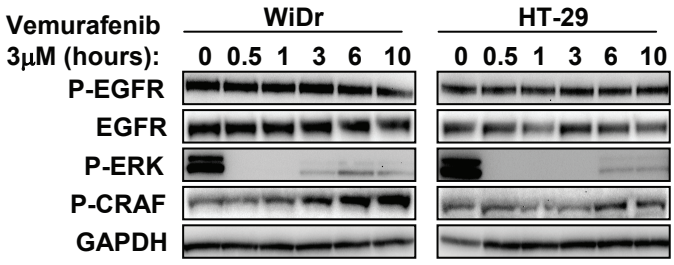
Corcoran et al, Fig. S1



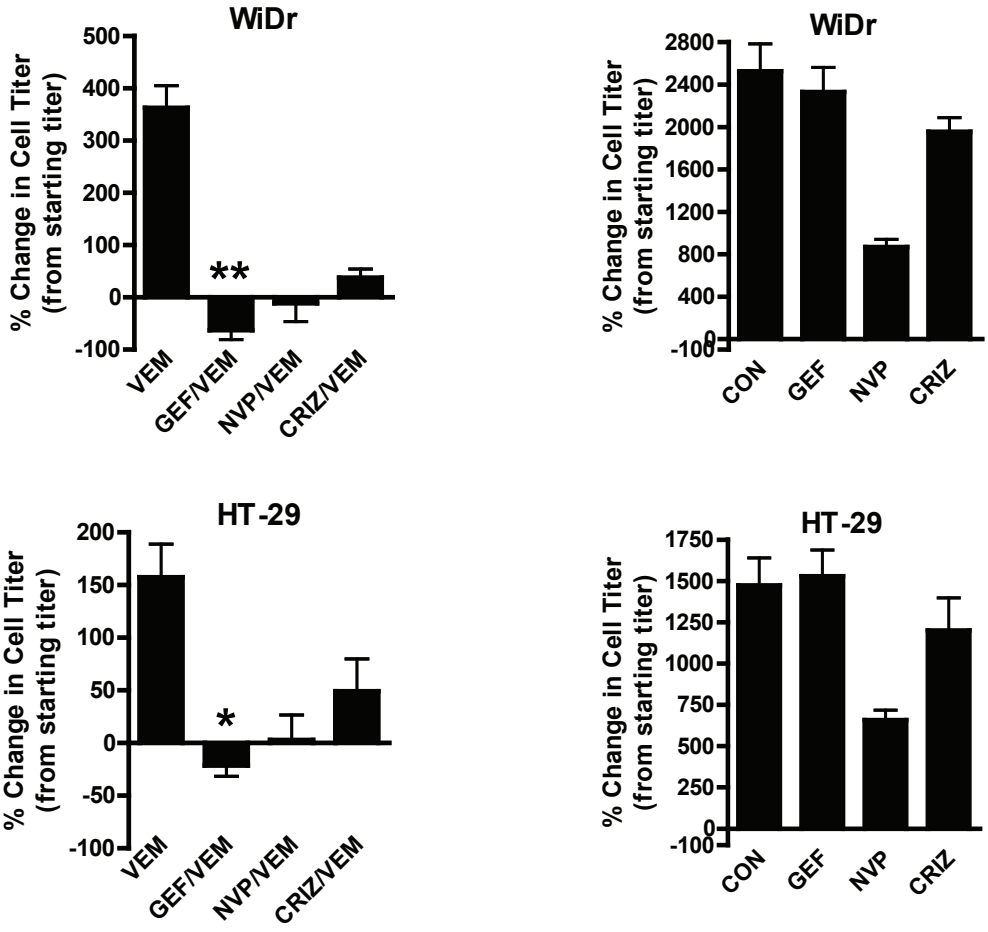
Corcoran et al, Fig. S2



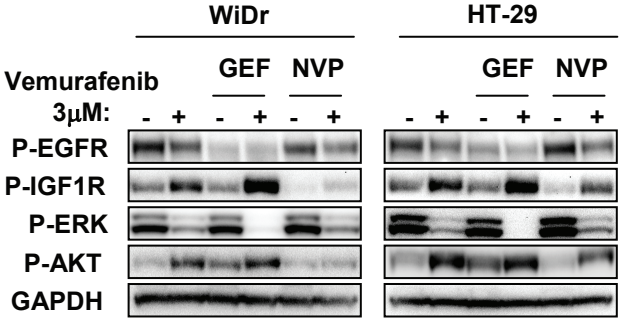
Corcoran et al, Fig. S3



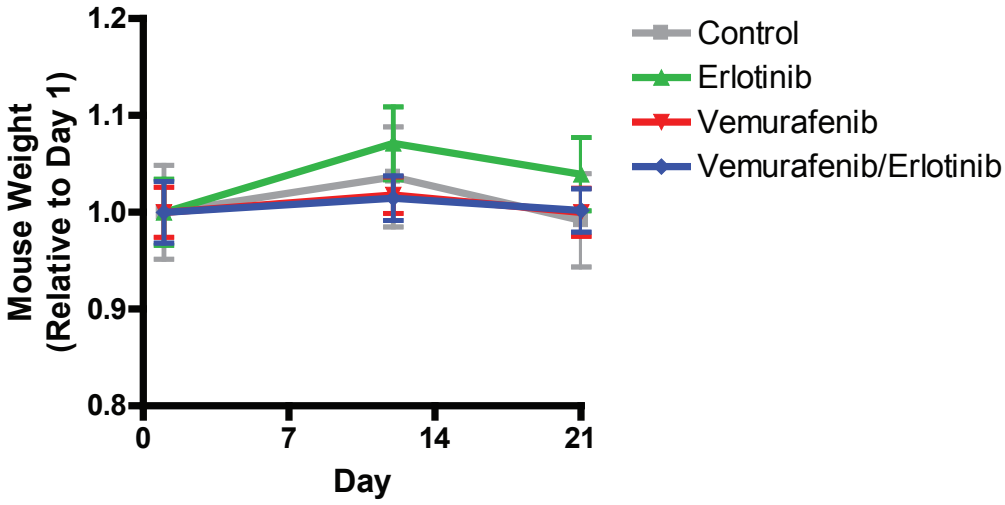
Corcoran et al, Fig. S4



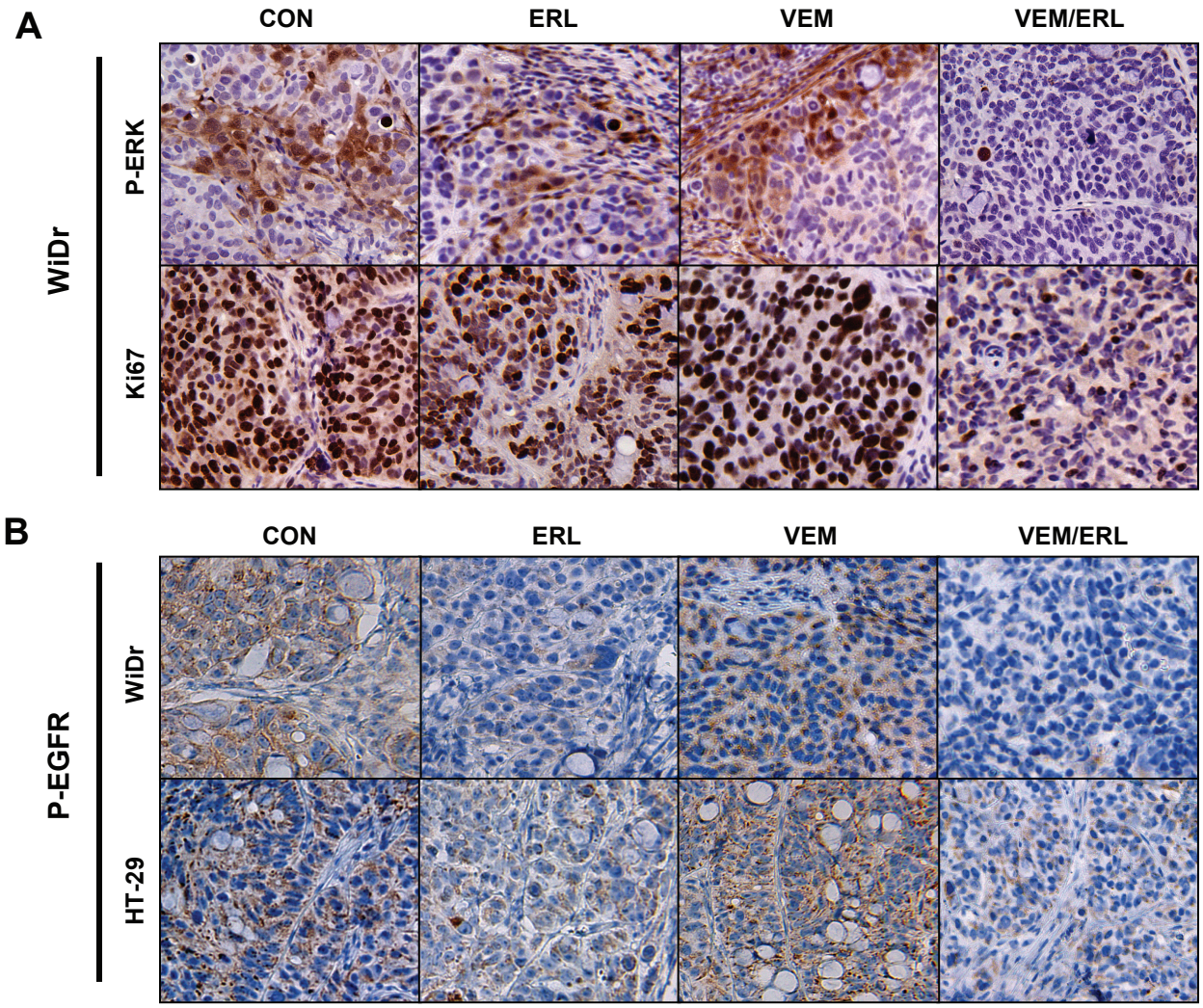
Corcoran et al, Fig. S5



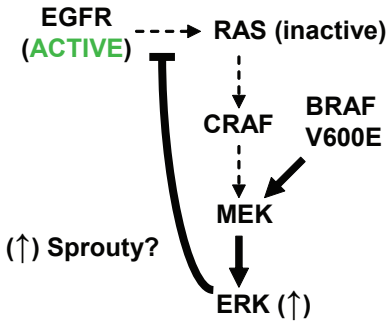
Corcoran et al, Fig. S6



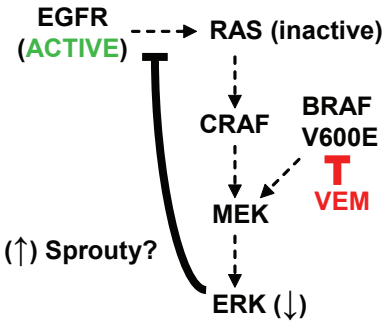
Corcoran et al, Fig. S7



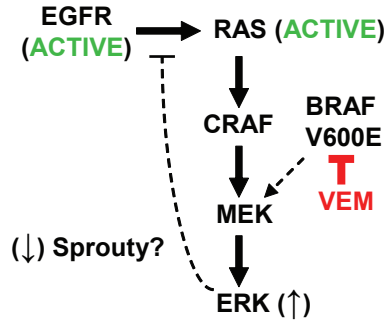
Corcoran et al, Fig. S8



1.) NO TREATMENT
(ERK activated)



2.) VEMURAFENIB ONLY
Early effects (~3 hours)
(ERK transiently inactivated)



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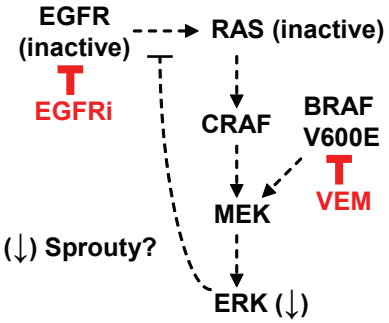
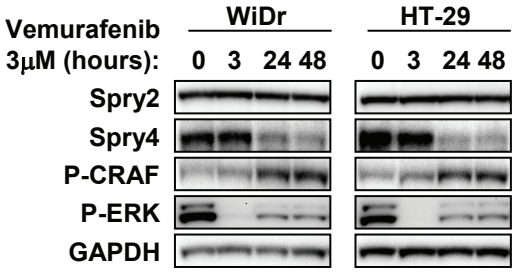
Late effects (~24 hours)
(ERK re-activated)



4.) VEMURAFENIB PLUS EGFR INHIBITOR
(ERK inactivated)

Corcoran et al, Fig. S9



Supplemental Figure Legends

Fig. S1: P-ERK re-accumulation in *BRAF* mutant CRC cells following vemurafenib treatment is MEK-dependent. WiDr and HT-29 cells were treated with vemurafenib (3μM) or AZD6244 (selumetinib, 1μM), alone or in combination, for the indicated times. Lysates were probed with the indicated antibodies.

Fig. S2: RTK inhibition by targeted kinase inhibitors. (A-C) Effective target RTK inhibition in WiDr and HT-29 cells treated for 24h with 1μM lapatinib (A), 1μM NVP-AEW541 (B) or 1μM crizotinib (C) and evaluated by western blot. (D) Cells were treated for 24h with gefitinib (2μM) or erlotinib (1μM) in the presence or absence of vemurafenib (3μM). Lysates were evaluated by western blot. (E) Cells were transfected with control (SC) or EGFR siRNA. After 72h, cells were treated with vemurafenib (3μM) for 24h. Lysates were probed with the indicated antibodies.

Fig. S3: P-EGFR levels in *BRAF* mutant CRC cells do not increase upon vemurafenib treatment. Cells were treated with vemurafenib (3μM) for the indicated times. Lysates were probed with the indicated antibodies.

Fig. S4: Concomitant inhibition of EGFR improves the in vitro efficacy of vemurafenib in *BRAF* mutant CRC cells. WiDr and HT-29 cells were treated for 72h with the above inhibitors at the concentrations specified in the presence or absence of vemurafenib (3μM), and viable cell number was determined. For each cell line, the left panel shows the effect of each inhibitor in combination with vemurafenib, and the right

panel shows the effect of each inhibitor alone. All values shown are from the same experiment, but are shown in separate graphs to allow the effects of each inhibitor in combination with vemurafenib to be viewed in greater detail.

Fig. S5: IGF1R inhibition can abrogate the induction of P-AKT by vemurafenib in *BRAF* mutant CRC cells. Cells were treated with vemurafenib alone or in combination with gefitinib (2μM) or NVP-AEW541 (1μM) for 24h. Lysates were probed with the indicated antibodies.

Fig. S6: Concomitant treatment with vemurafenib and erlotinib is well-tolerated in mice. Average body weights of xenograft-bearing mice treated with vehicle control, erlotinib (100mpk daily), vemurafenib (75mpk twice daily), or both drugs in combination are shown relative to starting body weight. Error bars represent SEM.

Fig. S7: Pharmacodynamic assessment of combined treatment with vemurafenib and erlotinib in *BRAF* mutant CRC xenografts. (A) Tumor tissue from WiDr xenografts treated for 3d as indicated was evaluated by IHC for P-ERK and Ki67. Tumors were harvested 4h after dosing on day 3. (B) Tumor tissue from each treatment group for WiDr and HT-29 xenografts was harvested as in (A), and P-EGFR levels were assessed by IHC.

Fig. S8: Model of EGFR-mediated MAPK pathway re-activation in *BRAF* mutant CRC following vemurafenib treatment. (1) In the absence of treatment, mutant BRAF

(V600E) is the primary activator of MEK and ERK, leading to increased levels of P-ERK. Although EGFR is phosphorylated and active, RAS activation by EGFR is blocked by an ERK-dependent feedback loop, possibly involving ERK-dependent transcriptional targets such as Sprouty proteins. Since RAS is inactive, it cannot directly activate CRAF, and it cannot induce the formation and transactivation of CRAF dimers by vemurafenib. **(2)** Early after treatment with vemurafenib (VEM) P-ERK is suppressed due to inhibition of BRAF V600E. RAS and CRAF remain inactive after initial vemurafenib treatment, likely due to the persistence of an ERK-dependent negative feedback loop that prevents RAS activation by EGFR. **(3)** Continued suppression of ERK leads to de-repression of this negative feedback loop (consistent with loss of expression of an ERK transcriptional target), and EGFR is able to activate RAS. Activated Ras subsequently activates CRAF kinase activity, either by direct CRAF activation, induction and transactivation of CRAF dimers in the presence of vemurafenib, or a combination of both mechanisms. Activation of CRAF kinase activity leads to re-activation of P-ERK. **(4)** However, if vemurafenib is administered in the presence of an EGFR inhibitor (EGFRi), EGFR-mediated activation of RAS and subsequent activation of CRAF is blocked, leading to sustained suppression of P-ERK.

Fig. S9: Effect of vemurafenib treatment on Sprouty protein levels in *BRAF* mutant CRC cells. Cells were treated for the indicated times with vemurafenib (3μM), and lysates were evaluated by western blot.

Corcoran et al, Supplemental Table S1

Cell Line	Type	BRAF	PIK3CA	PTEN (expression by western blot)
HT-29	CRC	V600E	P449T	present
LS411N	CRC	V600E	WT	absent
SW1417	CRC	V600E	WT	present
WiDr	CRC	V600E	ND	present
451Lu	Melanoma	V600E	WT	present
G-MEL	Melanoma	V600E	ND	present
M14	Melanoma	V600E	WT	present
WM164	Melanoma	V600E	ND	present

Table S1: Molecular features of cell lines used in this study. The *BRAF* mutant melanoma and CRC cell lines used are shown above. Mutational status of *BRAF* and *PIK3CA* are shown for each cell line. The presence or absence of PTEN protein expression as determined by western blot is also shown. WT = wildtype. ND = not determined.

Supplemental Methods:

Western Blot Analysis and Antibodies

Western blotting using standard methods and quantification of chemiluminescent signal intensity was performed as previously described (19). Human phospho-RTK arrays were purchased from R&D Systems and used according the manufacturer's guidelines. All antibodies were purchased from Cell Signaling, except for P-EGFR (Y1068) (Abcam), Spry2 and GAPDH (Millipore), and Spry4 (Santa Cruz Biotechnology). For time course experiments, all cells were lysed at the same time. Beginning 48h prior to lysis, all cells received fresh media with or without inhibitor at each timepoint (48h, 24h, and 3h prior to lysis), ensuring that all cells received fresh media and inhibitor 3h prior to lysis. For RAS-GTP pulldown assays, cells were treated for 24h with or without vemurafenib (3μM) and lysed. RAS-GTP was pulled down using the RAS-binding domain (RBD) of Raf-1 from the RAS activation kit (Millipore) according to the manufacturer's protocol. A total-RAS antibody provided in the kit was used for detection of RAS by western blot.

Determination of Cell Titer

Cells were seeded at 2,000 cells per well in parallel 96-well plates. After overnight incubation, one plate was frozen immediately to represent the starting cell titer, and the other plate was treated in for 72h (six wells per condition) and then frozen. Plates were thawed simultaneously, and cell titer was determined using Cell Titer Glo assay (Promega) according to the manufacturer's protocol. Change in cell titer for each treatment condition was calculated relative to starting cell titer.

RNA Interference

Cells were seeded into 6-well plates at a density of $1x10^5$ cells/well. 24hr later, cells were transfected with ON-TARGETplus SMARTpool siRNA against EGFR (Dharmacon) or Silencer negative control #1 siRNA (Ambion) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions, as previously described (16). Transfected cells were cultured at 37 ^oC for 72 hours and then treated with or without vemurafenib (3μM) for an additional 24h prior to lysis.

Statistical Analyses

One-way ANOVA with Tukey post-hoc test was used for Figs. 3D, 4A, and S2D. Chi-square test was used for Fig. 4D. Statistical significance was established for $p<0.05$.