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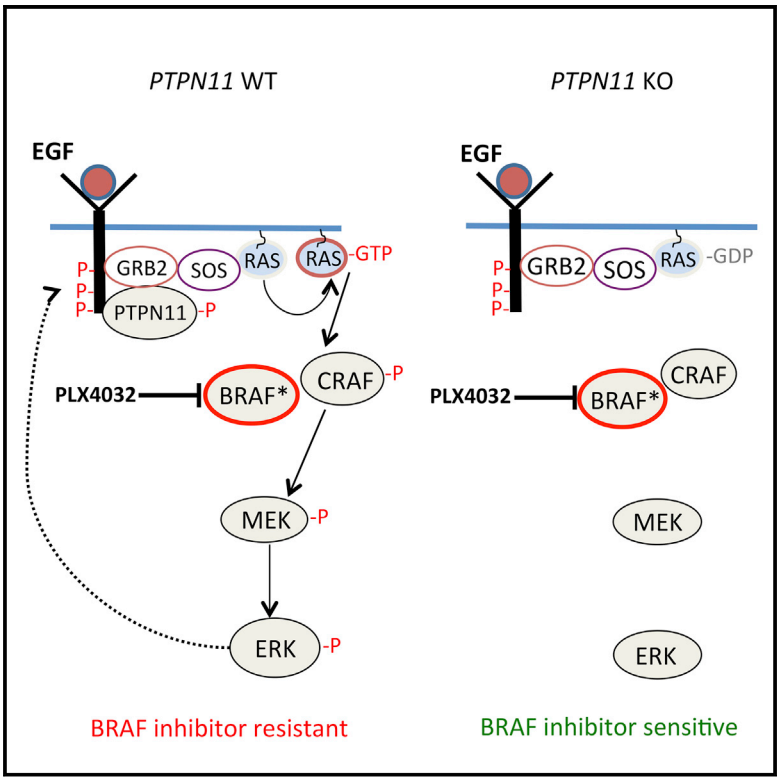
Report

PTPN11 Is a Central Node in Intrinsic and Acquired Resistance to Targeted Cancer Drugs

Graphical Abstract



Highlights

- PTPN11 inhibition is synthetic lethal with BRAF inhibitors in *BRAF* mutant colon cancer
- PTPN11 inhibition blocks the effects of EGFR activation by BRAF inhibitors
- *PTPN11* inhibition is lethal to cancers with activated RTKs
- Phosphorylation of PTPN11 is a biomarker of RTK-driven drug resistance in melanoma

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In Brief

Using a synthetic lethality screen, Prahallad et al. report that *PTPN11* suppression enhances the response to BRAF inhibition in *BRAF* mutant colon cancers by preventing the effects of receptor tyrosine kinase reactivation. These findings identify PTPN11 as a drug target to combat both intrinsic and acquired resistance to targeted cancer drugs.

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PTPN11 Is a Central Node in Intrinsic and Acquired Resistance to Targeted Cancer Drugs

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SUMMARY

Most *BRAF* (*V600E*) mutant melanomas are sensitive to selective BRAF inhibitors, but *BRAF* mutant colon cancers are intrinsically resistant to these drugs because of 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 found that suppression of protein tyrosine phosphatase non-receptor type 11 (*PTPN11*) confers sensitivity to BRAF inhibitors in colon cancer. Mechanistically, we found 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 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. Similarly, inhibition of the mitogen extracellular signal-regulated kinase (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). Somatic mutations in the PTP gene superfamily are found in different tumor types, with *PTPRP* being the most frequently mutated PTP in human cancer (Zhao et al., 2015). PTP, non-receptor type 11 (*PTPN11*, also known as *SHP2*), was the first bona fide tyrosine phosphatase to be identified as an oncogene (Tartaglia et al., 2003; Loh et al., 2004, Mohi et al., 2005). 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). *PTPN11* is ubiquitously expressed and is implicated in the transduction of mitogenic, pro-survival, and pro-migratory signals from growth-factor-, cytokine-, and other extracellular matrix receptors (reviewed in Ostman et al., 2006). PTPN11 is required for the full activation of RAS-MAPK-ERK (extracellular signal-related kinase) signaling downstream of most receptor tyrosine kinases (RTKs) (Qu, 2000; Shi et al., 2000; Bennett et al., 1996).

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 colorectal cancer (CRC) cells to

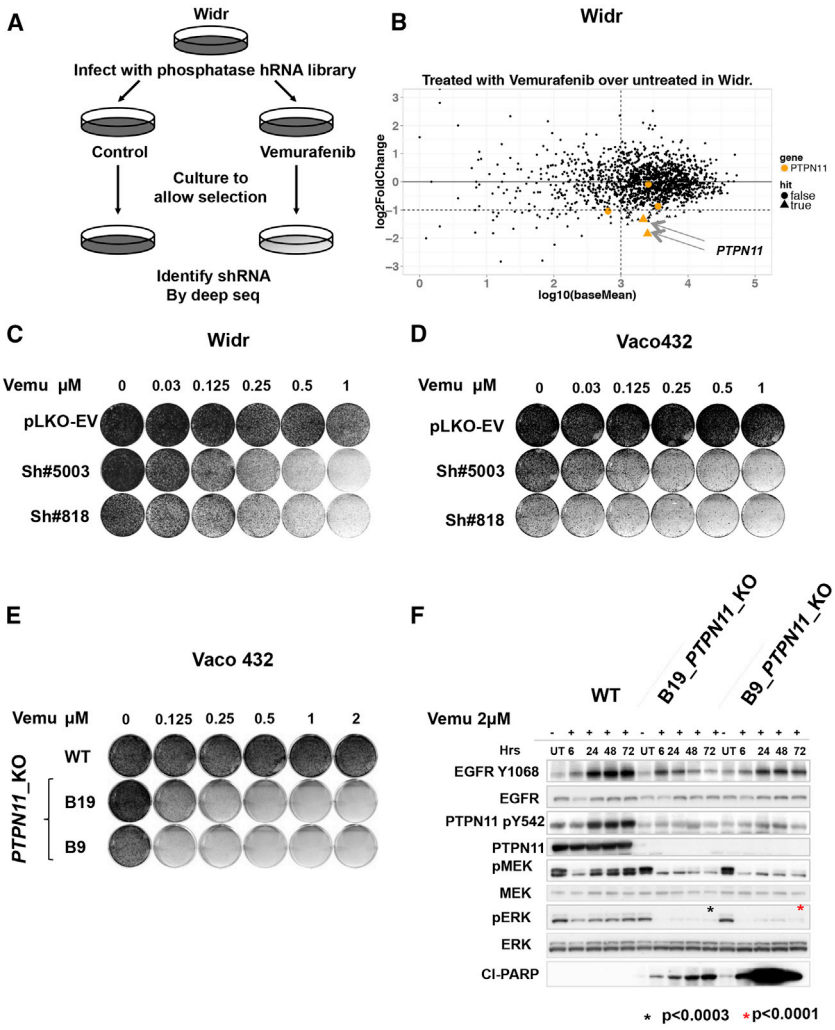

Figure 1. Inhibition of *PTPN11* Confers Sensitivity to BRAF Inhibition in BRAF Mutant Colon Cancer

(A) Schematic outline of the phosphatome-centered dropout shRNA screen for enhancers of vemurafenib sensitivity. seq, sequencing.

(B) Representation of relative abundance of the shRNA bar code sequences from the shRNA screen depicted in an M/A plot where each dot represents individual shRNA. The y axis shows $\log_2$ fold change (relative abundance of vemurafenib-treated/untreated cells), and the x axis shows intensity (average sequence reads in untreated sample) of each shRNA.

(C and D) Two independent non-overlapping shRNA targeting *PTPN11* (#5003 and #818) enhance sensitivity to vemurafenib (Vemu) in both Widr cells (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 (KO) 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 Vaco432 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. Student's t test was performed on three independent experiments to calculate a p value for the change in pERK upon vemurafenib treatment (mean relative AUC [area under the curve] values of the three replicates: 1.64 for 72-hr vemurafenib treatment of Vaco WT cells, 0.29 for 72-hr treatment of *PTPN11* KO clone B19, and 0.35 for 72-hr treatment of *PTPN11* KO clone B9). The combined effects of *PTPN11* and BRAF V600E blockade lead to apoptotic cell death as measured by PARP cleavage (Cl-PARP).

the BRAF inhibitor vemurafenib. We assembled a collection of 1,665 short hairpin RNA (shRNA) vectors that together target 298 phosphatases or phosphatase-related genes (Sacco et al., 2012). To find phosphatases whose suppression displays 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 previously (Prahallad et al., 2012). We only considered genes for which two independent shRNAs could be identified with an average read count of more than 1,000 and which were depleted at least 2-fold ($\log_2$ 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 (Table S1). Therefore, we focused on *PTPN11* for further investigation. First, we tested additional hairpins from the TRC (The RNAi Consortium) 2.0 library collection for *PTPN11* knockdown efficiency (data not shown) and decided to use hairpins #5003 (from screen) and #818 (from TRC 2.0) for our studies.

To validate the result from the screen, we introduced these two *PTPN11* shRNAs (shRNA #5003 and shRNA #818) into the *BRAF* mutant CRC cell lines Widr and Vaco432 and cultured them in the absence or presence of vemurafenib. Figures 1C and 1D show that the control vector-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 reduction in cell numbers (Figures 1C and 1D). 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, S2A, and S2B). However, treatment of knockout cells with vemurafenib had a dramatic effect on proliferation, both in long-term and in short-term assays (Figures 1E, S2A, and S2B). Similar results were also obtained in *BRAF* mutant Widr CRC cells (Figure S1C).

As reported earlier, in Vaco432 cells, vemurafenib treatment induces feedback activation of EGFR as evidenced by an increase in tyrosine 1068 phosphorylation (Prahallad et al., 2012) (Figure 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 phosphorylated MEK (pMEK) and phosphorylated ERK (pERK) rebound (Figure 1F). In contrast, *PTPN11* knockout clones of Vaco432 activated EGFR but had an significant drop of about 80% in pERK levels after 48–72 hr upon vemurafenib treatment, resulting in massive apoptosis, as evidenced by the appearance of cleaved poly(ADP-ribose) polymerase (PARP) (Figure 1F). Identical results were seen in *PTPN11* knockout clones for BRAF mutant Widr cells (Figures S1C and S1D).

PTPN11 Inhibition Is Synthetic Lethal with BRAF Inhibition In Vivo

We reconstituted the *PTPN11* knockout Vaco432 clone (#B9), which is sensitive to vemurafenib, with either a wild-type (WT) *PTPN11* vector or a phosphatase-dead mutant of *PTPN11* (C459S). Expression of WT *PTPN11*, but not the phosphatase-dead mutant (C459S), conferred resistance to vemurafenib and restored ERK phosphorylation (Figures 2A and 2B). We concluded 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 epidermal growth factor (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 and 2D). In contrast, *PTPN11* knockout clones of Vaco432 and Widr failed to reactivate ERK signaling in response to EGF (Figures 2C and 2D). 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 (Figures S3A and S3B). 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 (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. Therefore, we 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 (Figures 3A and 3B), confirming 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 phosphorylation of PTPN11 Y542 and of ERK 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, which was associated with a reduction in PTPN11 and ERK phosphorylation (Figure 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

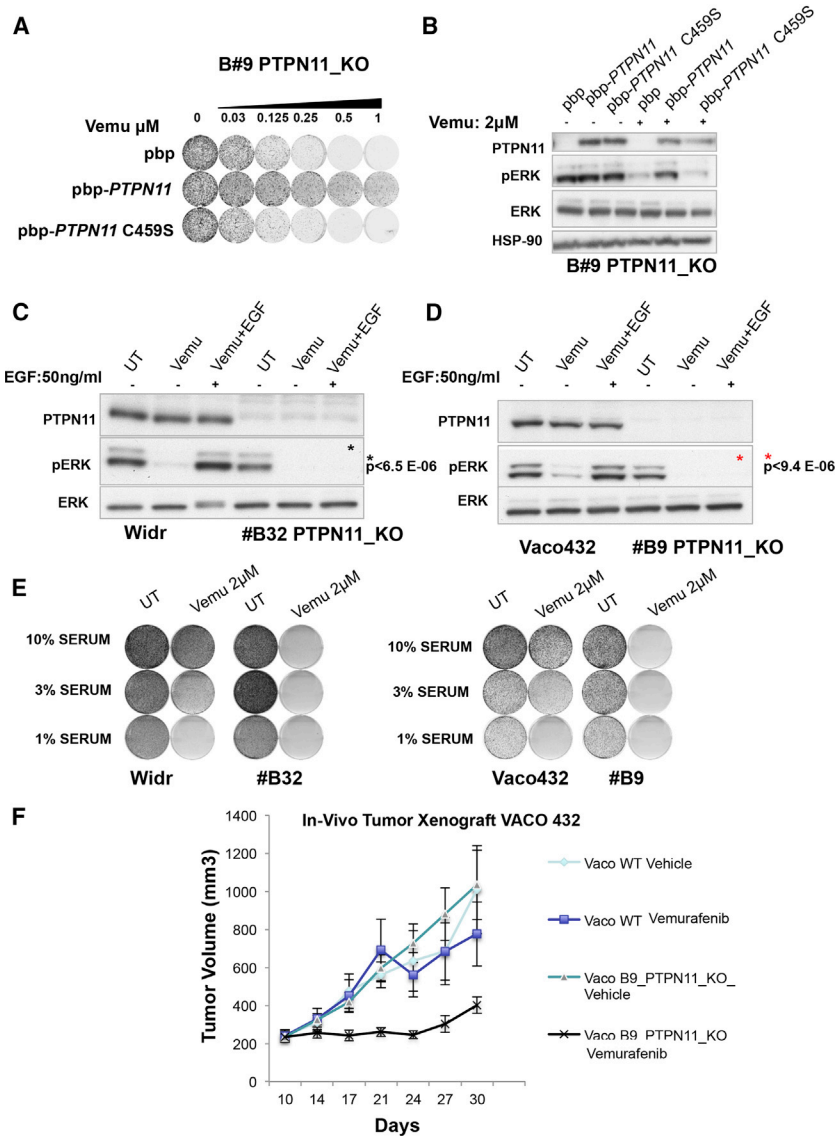

Figure 2. PTPN11 Inhibition Is Synthetic Lethal with BRAF Inhibition In Vivo

(A) Reconstitution of WT or phosphatase-dead (C459S) mutant of *PTPN11* into *PTPN11* knockout (KO) Vaco432 cells, where pbp denotes the vector pBabe-puro. *PTPN11* knockout Vaco432 cells are sensitive to vemurafenib (Vemu); reconstitution with WT *PTPN11* confers resistance, whereas reconstitution with phosphatase-dead mutant (C459S) confers sensitivity.

(B) Western blot showing the expression of both WT and C459S mutant to near physiological levels in *PTPN11* knockout Vaco432 cells. WT *PTPN11* expression reactivates ERK in the presence of vemurafenib, whereas C459S mutants do not reactivate ERK phosphorylation.

(C and D) 2 μM vemurafenib treatment for 2 hr in overnight serum-starved Widr (C) and Vaco432 (D) *PTPN11* WT cells confers complete ERK inhibition upon 2 μM vemurafenib, whereas addition of EGF (50 ng/ml) for 30 min to the cells completely reactivates ERK phosphorylation. Treatment of 2 μM vemurafenib in overnight serum-starved *PTPN11* knockout Widr (#B32 clone) and Vaco432 (#B9 clone) for 2 hr also resulted in complete ERK inhibition, and the addition of EGF was not able to restore ERK phosphorylation. Student's t test was performed on three independent experiments to calculate a p value for the change in pERK upon vemurafenib + EGF treatment (mean relative AUC values of the three replicates: 2.31 for vemurafenib + EGF treatment of Widr WT cells, 0.05 for vemurafenib + EGF treatment of *PTPN11* knockout clone B32, 2.48 for vemurafenib + EGF treatment of Vaco WT cells, and 0.04 for vemurafenib + EGF treatment of *PTPN11* KO clone B9). UT, untreated control.

(E) Colony formation of Widr (parental and *PTPN11* knockout #B32) and Vaco432 (parental and *PTPN11* knockout #B9) cells cultured under decreasing serum concentrations (0.1%, 1%, 3%, and 10%) with and without vemurafenib (2 μM) treatment for 14 days.

(F) Vaco432 parental and *PTPN11* knockout clone #B9 cells were grown as tumor xenografts in non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice. After tumor establishment (200–250 mm^3), mice were treated with either vehicle or vemurafenib (60 mg/kg) for 30 days. Mean tumor volumes ± SEM are shown (n = 7 mice per group).

(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 (Figures 4A and 4B). 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 (hepatocyte growth factor [HGF], fibroblast growth factor 9 [FGF9], and stem cell factor [SCF]) that 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 (Figures 4C, S4A, and S4C). Consistent with the effects on proliferation, exposure of parental SK-Mel888 cells to HGF, SCF, or FGF9 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 but not in the *PTPN11* knockout cells (Figures 4D, S4B, and S4D). Inhibition of the phosphatase function of PTPN11 is essential for the effects on growth-factor-induced drug resistance, since expression of a phosphatase-dead mutant (C459S) in

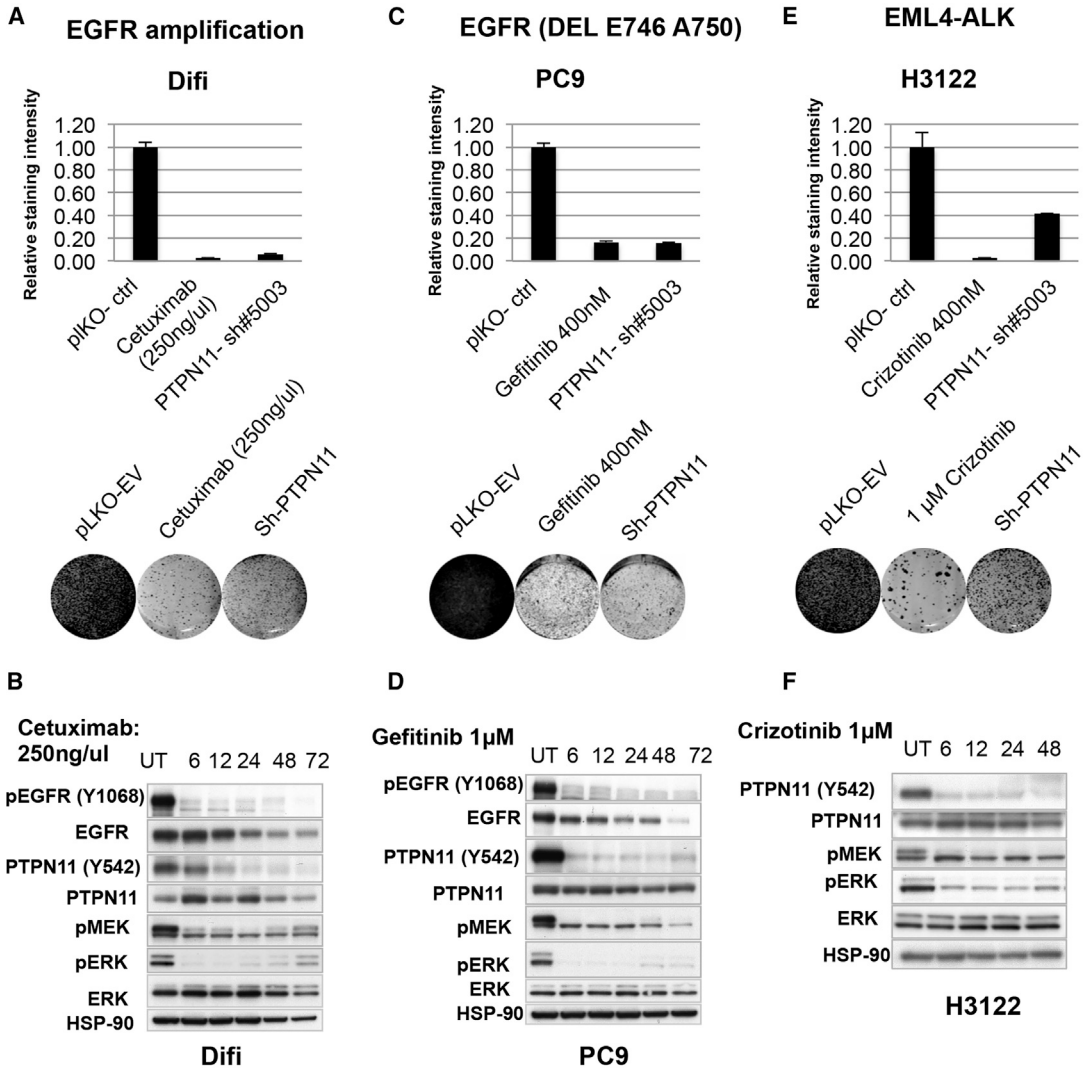

Figure 3. PTPN11 Inhibition Is Lethal to RTK-Driven Tumors

(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 the dishes shown below. ctrl, control.

(B–F) Biochemical changes seen on western blot upon treatment with 250 ng/ml cetuximab (B), gefitinib (D), or crizotinib (F) 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 400 nM crizotinib treatment or sensitive to *PTPN11* inhibition. UT, untreated control.

PTPN11 knockout SK-Mel888 cells failed to confer resistance to HGF exposure upon vemurafenib treatment (Figures S4E and S4F). 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; Nazarian et al., 2010). Consistent with this, we found that *PTPN11* loss in A375 melanoma cells delays the emergence of vemurafenib-resistant colonies when these cells were cultured in vitro for 1 month in the presence of a 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 earlier indicate that RTK-driven acquired drug resistance in *BRAF* mutant melanoma activates PTPN11.

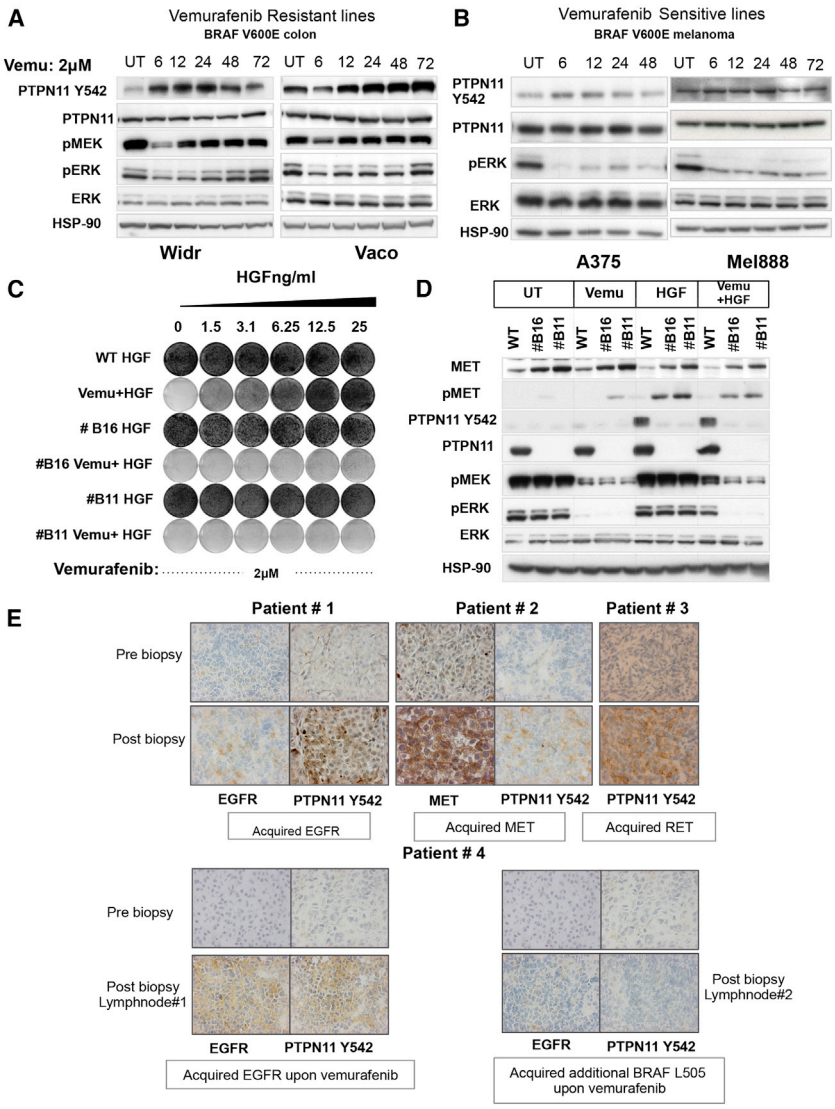

Figure 4. PTPN11 Loss Abrogates Growth-Factor-Driven Resistance in Melanoma

(A) *BRAF* mutant CRC line Widr and Vaco432 cells display feedback activation of PTPN11 (Y542) upon vemurafenib (Vemu) treatment as a consequence of EGFR feedback activation in a time-course experiment. UT, untreated control.

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

(C) HGF activation of the MET receptor can potently rescue Mel888 cells from vemurafenib (2 μM) 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 (2 μM), HGF (25 ng/ml), or the combination and analyzed by western blot using pMET, PTPN11 pTYR542, pERK, and pMEK. Student's t test was performed on three independent experiments to calculate a p value for the change in pERK upon vemurafenib + HGF treatment (mean relative AUC values of the three replicates: 1.37 for vemurafenib + HGF treatment of Mel888 WT cells, 0.08 for vemurafenib + HGF treatment of *PTPN11* KO clone B16, and 0.02 for vemurafenib + HGF treatment of *PTPN11* KO clone B11).

(E) Pre- and post-vemurafenib-treated *BRAF* mutant melanoma patient biopsies 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, whose cells also stained positive for PTPN11 (pTYR542) and EGFR by IHC post-vemurafenib treatment; a gain in c-MET receptor expression in patient #2, whose cells also stained positive for PTPN11 (pTYR542) and MET by IHC; and a gain in RET receptor in patient #3, whose cells also 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).

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) who 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 had 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 was seen in patient 2, and *RET* amplification was seen 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 4E). 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). 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 would have the advantage that the selective pressure exerted on each of the nodes is low, making it difficult for the cancer to escape therapy through secondary mutations. Our present data identify PTPN11 as a PTP 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.

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; Chen et al., 2006; Hellmuth et al., 2008). 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.

Finally, we present evidence here that phosphorylated PTPN11 (Y542) can serve as a biomarker to identify melanoma patients who have acquired vemurafenib resistance through RTK activation (Figures 4E and 4F). This may prove to be relevant, as our recent data indicate that melanoma patients who 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 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 $\log_2$ fold change was smaller or equal to -1, corrected p value calculated using DESeq was ≤ 0.1, and the base mean was $\geq 1{,}000$. The gene with the strongest fold change was selected for further research. Further details are described by Prahallad et al. (2012).

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. The Code for Proper Secondary Use of Human Tissue and The Code of Conduct for the Use of Data in Health Research, as stated by the Federation of Dutch Medical Scientific Societies, were followed for handling patient tissue and clinical data (Federa FMVV, updated 2011; http://www.federa.org/codes-conduct).

Statistical Analysis of Western Blots

The band intensities of the western blot images were quantified using ImageJ software. The data were exported to Excel, and the percent signal intensity of a particular phospho-epitope signal was compared to that of the signal of the loading control, and their relative ratio was calculated. Student's t test was performed on the relative western blot band intensities from three independent western blots to calculate a p value.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.037.

AUTHOR CONTRIBUTIONS

R.B., F.D., A.B., A.P., and G.J.J.E.H. designed the study. A.P., G.J.J.E.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., and R.B. supervised the study. S.M.W. performed immunohistochemistry (IHC) on patient biopsy. B.E. developed the CRISPR system. L.V. and V.G. performed experiments.

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