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ORIGINAL ARTICLE

Abl kinase regulation by BRAF/ERK and cooperation with Akt in melanoma

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The melanoma incidence continues to increase, and the disease remains incurable for many due to its metastatic nature and high rate of therapeutic resistance. In particular, melanomas harboring $BRAF^{V600E}$ and PTEN mutations often are resistant to current therapies, including BRAF inhibitors (BRAFi) and immune checkpoint inhibitors. Abl kinases (Abl/Arg) are activated in melanomas and drive progression; however, their mechanism of activation has not been established. Here we elucidate a novel link between $BRAF^{V600E}$/ERK signaling and Abl kinases. We demonstrate that $BRAF^{V600E}$/ERK play a critical role in binding, phosphorylating and regulating Abl localization and Abl/Arg activation by Src family kinases. Importantly, Abl/Arg activation downstream of $BRAF^{V600E}$ has functional and biological significance, driving proliferation, invasion, as well as switch in epithelial–mesenchymal–transition transcription factor expression, which is known to be critical for melanoma cells to shift between differentiated and invasive states. Finally, we describe findings of high translational significance by demonstrating that Abl/Arg cooperate with PI3K/Akt/PTEN, a parallel pathway that is associated with intrinsic resistance to BRAFi and immunotherapy, as Abl/Arg and Akt inhibitors cooperate to prevent viability, cell cycle progression and *in vivo* growth of melanomas harboring mutant BRAF/PTEN. Thus, these data not only provide mechanistic insight into Abl/Arg regulation during melanoma development, but also pave the way for the development of new strategies for treating patients with melanomas harboring mutant BRAF/PTEN, which often are refractory to current therapies.

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INTRODUCTION

Unlike most cancers, melanoma diagnoses are increasing, particularly in young women (<40), and the disease remains incurable for many with metastatic disease (18%, 5-year survival rate; https://seer.cancer.gov/data/citation.html). Immunotherapies hold promise for increasing the cure rate for a proportion of advanced cases; however, for patients with high metastatic burden, immunotherapy often is not a first-line option, due to the time needed to achieve a response.[1] Moreover, only a subset of patients respond.[2,3] Constitutive activation of the BRAF serine–threonine kinase ($BRAF^{V600E}$) is the most common genetic change in melanoma.[3–5] It is frequently mutated in melanomas derived from intermittent sun exposure (80–90%) and early onset (age $\leqslant 39$; 86%).[4–7] BRAF also is mutated in benign nevi (50–60%) where it promotes senescence due to high-level, sustained ERK activation.[8,9] Activation of the PTEN/PI3K/Akt pathway, which modestly down-modulates BRAF/ERK signaling, promotes escape from oncogene-induced senescence and subsequent progression.[8,9] BRAFi reduce metastatic burden for some patients with $BRAF^{V600E}$-expressing melanomas; however, the majority of responding patients rapidly develop resistance, and combined BRAF/MEK inhibitor therapy (BRAFi/MEKi) only delays resistance.[1,10] Moreover, BRAFi/MEKi often are less effective for melanomas harboring activation of the PI3K/Akt pathway (for example, PTEN loss), which frequently occurs concurrently with BRAF mutations, inducing cytostatic rather than cytotoxic effects, and PTEN mutations also are associated with intrinsic resistance to immunotherapy.[3,11–15] Thus, it is imperative to identify new drug combinations for treating these patients.

Melanomas originate from neural crest-derived melanocytes, and thus, do not undergo classical epithelial–mesenchymal–transition (EMT).[16] Instead, melanoma cells are highly plastic, and switch between differentiated and invasive states, which contributes to the high rate of metastasis and drug resistance.[16] This phenotypic shift has been linked to $BRAF^{V600E}$-induced switch in expression of EMT transcription factors (EMT-TF) from ZEB2 and SNAIL2, which display tumor suppressive properties, to ZEB1 and TWIST1, which cooperate with $BRAF^{V600E}$ to induce invasion and tumor growth.[17] The switch is driven, in part, by ERK-dependent induction and phosphorylation of FRA-1, a member of the AP-1 transcription factor family, which binds *ZEB*1, *ZEB*2 and *TWIST*1 promoters and regulates their transcription.[17] TWIST1 and ZEB1 also are regulated by other pathways as SQSTM1/p62 stabilizes TWIST1 protein; FOXD3, represses *TWIST*1 transcription; and ZEB2 induces microphthalmia-associated transcription factor, which represses *ZEB1*.[18–20]

The Abelson non-receptor tyrosine kinases, Abl (*ABL1*) and Arg (*ABL2*), are most known for their involvement in human leukemia; however, accumulating evidence over the past decade indicates the kinases also have oncogenic roles in solid tumors.[21–23] We and others reported that Abl/Arg are activated in melanoma, and drive invasion, proliferation, survival and late stages of metastasis via unique pathways.[22,24–28] However, to date, little is known regarding the mechanism of Abl/Arg activation. In the current study, we show that $BRAF^{V600E}$ plays a critical role in activating Abl/Arg, which is required for $BRAF^{V600E}$ induction of the EMT-TF

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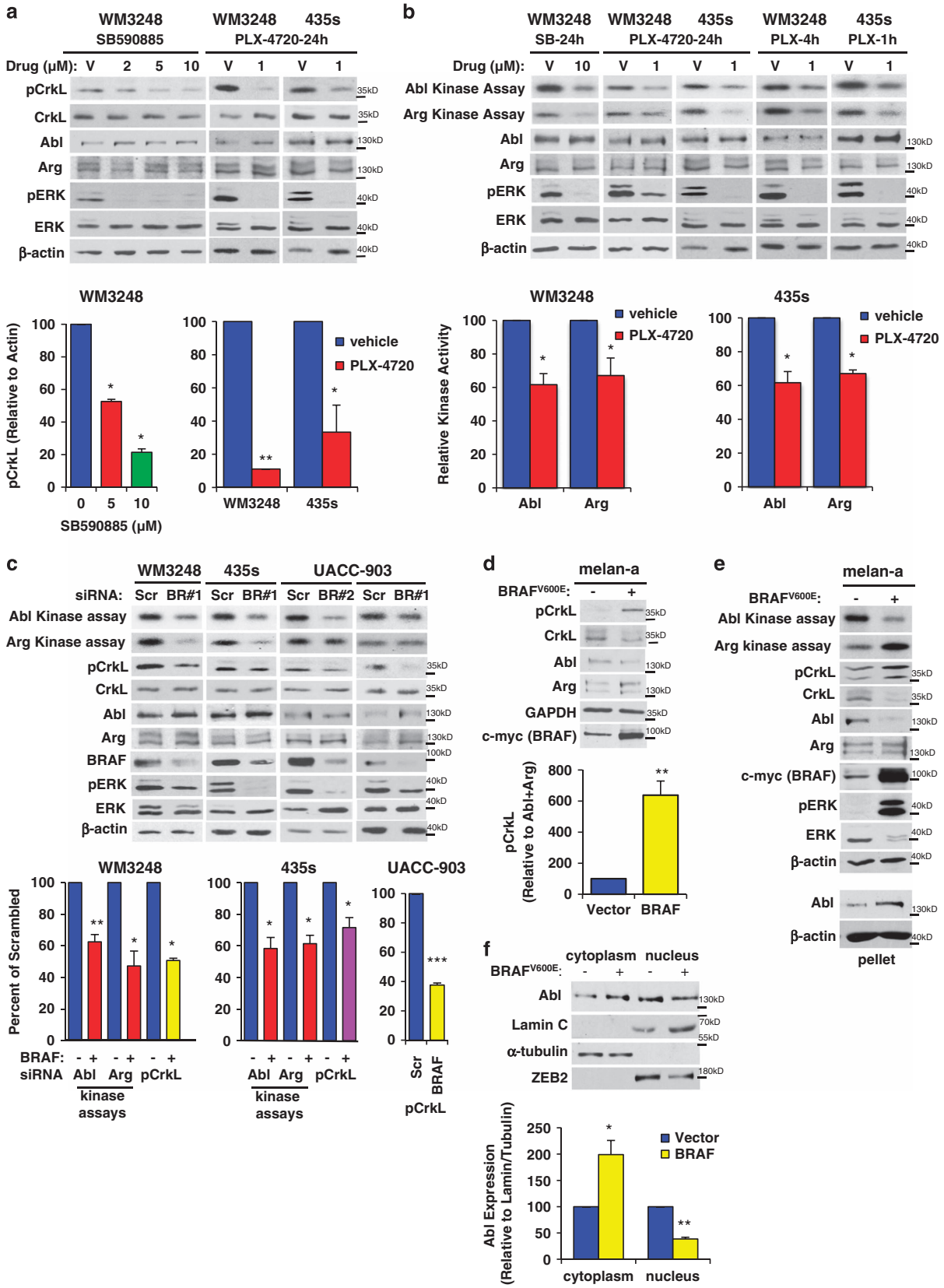
switch, proliferation and invasion, and Abl/Arg also feedback and potentiate BRAF/ERK signaling. Moreover, Abl/Arg synergize with Akt, a parallel, cooperating pathway, to drive survival, cell cycle progression and *in vivo* growth of mutant BRAF/PTEN melanomas. Thus, these data not only uncover a novel link between Abl/Arg and $BRAF^{V600E}$ signaling, but also identify a promising therapeutic strategy for treating patients with melanomas harboring BRAF/PTEN mutations.



RESULTS

Abl/Arg are overexpressed and activated in melanoma cell lines, and promote invasion and proliferation

Previously, we showed that Abl and Arg were highly expressed in six melanoma lines and activated in a subset (60%).[24] Here we expanded these studies to 25 human melanoma cell lines. Abl/Arg were overexpressed in nearly all lines (compared to melanocytes), and a subset (40–60%) also had high basal Abl/Arg activities as determined directly by *in vitro* kinase assay, and indirectly via phosphorylation of endogenous Abl/Arg substrates, Crk and CrkL, on Abl/Arg phosphorylation sites (Supplementary Figure S1A and Supplementary Table S1; denoted pCrkL). pCrkL was highly correlative with Abl+Arg but not Src activity, confirming that it is a reliable readout of Abl+Arg activity (Supplementary Figure S1B).[26,29,30] Previously, we showed that inhibition of Abl and Arg with first (imatinib) or second (nilotinib) generation inhibitors, or silencing Abl/Arg with two independent siRNAs, dramatically reduced proliferation and invasion (matrigel, 3D) of WM3248 and 435s melanoma lines, which have high Abl/Arg activities (Supplementary Figure S1A).[24,25] Here we identified additional lines with high activity (UACC-903 and LOX-IVMI; Supplementary Figure S1A), and demonstrate that Abl/Arg inhibition with nilotinib or GNF-2/GNF-5 (highly specific but less potent allosteric inhibitors),[23,30] or silencing Abl/Arg with siRNAs reduced proliferation and invasion in these lines (Supplementary Figures S1C and D).[24,25] Moreover, stable expression of an shRNA targeting both Abl and Arg also reduced invasion (Supplementary Figure S1E). Thus, Abl and Arg are activated in a subset of melanoma lines and drive proliferation and invasion. Cell lines with high Abl/Arg activities (435s, WM3248, UACC-903, LOX-IVMI) were used for subsequent studies.

$BRAF^{V600E}$ activates Abl/Arg

Previously, we showed that pCrkL, a highly accepted readout of Abl+Arg activities (Supplementary Figure S1A),[26,29,31] was elevated in a subset of primary melanomas (40–60%) using two independent melanoma tissue microarrays.[24,25] Abl/Arg activation was high in patients who were diagnosed with melanomas at an early age ($\leqslant$39; 86%), and in melanomas derived from intermittent sun exposure (61%).[25] Interestingly, BRAF mutations also occur most frequently in early-onset (74%) melanomas, and in melanomas from the intermittent sun exposure subtype (80–90%).[4–7] Thus, we hypothesized that the activities of Abl/Arg and $BRAF^{V600E}$ might be linked. To test whether $BRAF^{V600E}$ contributes to Abl/Arg activation, BRAF activity was blocked with two independent inhibitors (SB590885, PLX-4720-vemurafenib analog), and Abl/Arg activity assessed indirectly (pCrkL; Figure 1a), and directly (*in vitro* kinase assay; Figure 1b), in cell lines harboring $BRAF^{V600E}$ and highly active Abl/Arg (WM3248, 435s). Both drugs have little/no activity toward Abl (*Abl1*).[32,33] Abl/Arg kinase activities and pCrkL were reduced following $BRAF^{V600E}$ inhibition (Figures 1a and b), even as early as 1–4 h after drug treatment (Figure 1b, right). These results were not due to off-target effects or direct inhibition of Abl/Arg by the drugs, as silencing $BRAF^{V600E}$ also reduced pCrkL and Abl/Arg activities (confirmed in a third cell line and with a second siRNA; Figure 1c). Thus, $BRAF^{V600E}$ signaling activates Abl/Arg in melanoma cells.

To examine whether $BRAF^{V600E}$ expression is sufficient to activate Abl/Arg, we performed gain-of-function experiments using murine melan-a melanocytes, since unlike human melanocytes, introduction of $BRAF^{V600E}$ is sufficient to induce their transformation and does not induce senescence.[17] Consistent with our studies in human melanoma cells, introduction of $BRAF^{V600E}$ into melan-a cells induced pCrkL and activated Arg; however, Abl activity was reduced, likely due to decreased Abl protein (Figures 1d and e). Importantly, Abl loss was due to its translocation to a triton-X-insoluble compartment as increased Abl levels were observed in radio-immunoprecipitation assay buffer (RIPA)-solubilized pellets from cells expressing $BRAF^{V600E}$ (Figure 1e, bottom). These data indicate that $BRAF^{V600E}$ might alter Abl localization. Unlike Arg, which is only present in the cytoplasm/plasma membrane, Abl resides in the nucleus, cytoplasm and plasma membrane, and can shuttle between nuclear and cytoplasmic compartments.[34] Activation of nuclear Abl induces apoptosis, which contrasts with its transforming role in the cytoplasm.[22] To examine whether $BRAF^{V600E}$ alters Abl localization, infected melan-a cells were subjected to subcellular fractionation and immunofluorescence. Importantly, introduction of $BRAF^{V600E}$ increased expression of Abl in the cytoplasm and in long membranous extensions, and reduced its levels in the nucleus (Figure 1f and Supplementary Figure S2). These data show for the first time that $BRAF^{V600E}$ signaling plays a crucial role in activating Abl and Arg and impacts Abl localization, which is critical for its function. Consistent with Abl/Arg and $BRAF^{V600E}$ acting within the same pathway, nilotinib and the BRAFi, PLX-4720, did not cooperate to inhibit melanoma proliferation (Supplementary Figure S3).

$BRAF^{V600E}$ binds Abl/Arg SH3 domains, and induces Abl/Arg phosphorylation

To unravel the mechanism by which $BRAF^{V600E}$ promotes Abl/Arg activation, we first tested whether the proteins are in the same complex. Indeed, Abl and Arg bound to $BRAF^{V600E}$ in a heterologous system (Figure 2a), as well as in melanoma cells and ERK was in the same complex (Figure 2b). Importantly, Glutathione-S-transferase (GST) pull-down assays demonstrated that $BRAF^{V600E}$ but not ERK directly bound Abl and Arg SH3 domains (Figure 2c). Abl/Arg are inhibited by SH3 domain-interlinker proline intramolecular interactions, and binding of proteins to the SH3 domain relieves autoinhibition.[21,22] Thus, $BRAF^{V600E}$ binding could induce Abl/Arg autoactivation. However, catalytically inactive BRAF (D594A) retained the ability to bind Abl, indicating that BRAF binding is not sufficient to activate Abl (Figure 2d). Rather, $BRAF^{V600E}$, a serine–threonine kinase, induced Abl threonine phosphorylation (Figure 2e), and tyrosine phosphorylation of kinase-inactive Abl or Arg, which lack the ability to autophosphorylate (Figure 2f). Thus, $BRAF^{V600E}$ likely promotes Abl/Arg phosphorylation by tyrosine kinases that regulate their activities.[21,22] Indeed, Src family tyrosine kinases (SFKs) activate Abl and Arg in melanoma cells (Figure 2g), as we demonstrated in other cell types,[35,36] and SFK inhibition prevented $BRAF^{V600E}$ from

Figure 1. $BRAF^{V600E}$ contributes to Abl/Arg activation in melanoma cells. (**a**, **b**) Human melanoma cell lines were serum-starved and treated with vehicle (V; DMSO) or BRAF inhibitors for 24 h (**a**, **b**, left) or 1–4 h (**b**, right), and phosphorylation of Abl/Arg substrate, CrkL (**a**) (western blot) or Abl/Arg kinase activities (**b**) (*in vitro* kinase assay using GST-Crk as substrate) assessed. Mean ± s.e.m., $n = 3$. $^{*}P < 0.05$, $^{**}P \leqslant 0.01$ using one-sample *t*-tests and Holm's adjustment for multiple comparisons. (**c**) Kinase assays and western blots were performed on lysates from serum-starved, siRNA-transfected cells (72 h). Scrambled = Scr, BRAF = BR. Mean ± s.e.m., $n = 3$. $^{*}P < 0.05$, $^{**}P \leqslant 0.01$, $^{***}P < 0.001$ using one-sample *t*-tests. (**d**, **e**) Western blots (**d**, **e**, bottom) or kinase assays (**e**, top) were performed on RIPA (**d**) or triton-X (**e**) lysates from melanocytes expressing vector (−) or myc-tagged $BRAF^{V600E}$ (48 h) in the presence (**d**) or absence (**e**) of serum. Triton-insoluble pellets were solubilized with RIPA buffer (**e**, bottom). Mean ± s.e.m., $n = 4$. $^{**}P < 0.01$ using a one-sample *t*-test. (**f**) Subcellular fractionation on retrovirally infected melan-a cells. Mean ± s.e.m., $n = 3$. $^{*}P < 0.05$, $^{**}P \leqslant 0.01$; one-sample *t*-tests. Control blots (lamin, α-tubulin) indicate fraction purity. Nuclear ZEB2, which is repressed by BRAF,[17] also is reduced in $BRAF^{V600E}$-expressing cells.

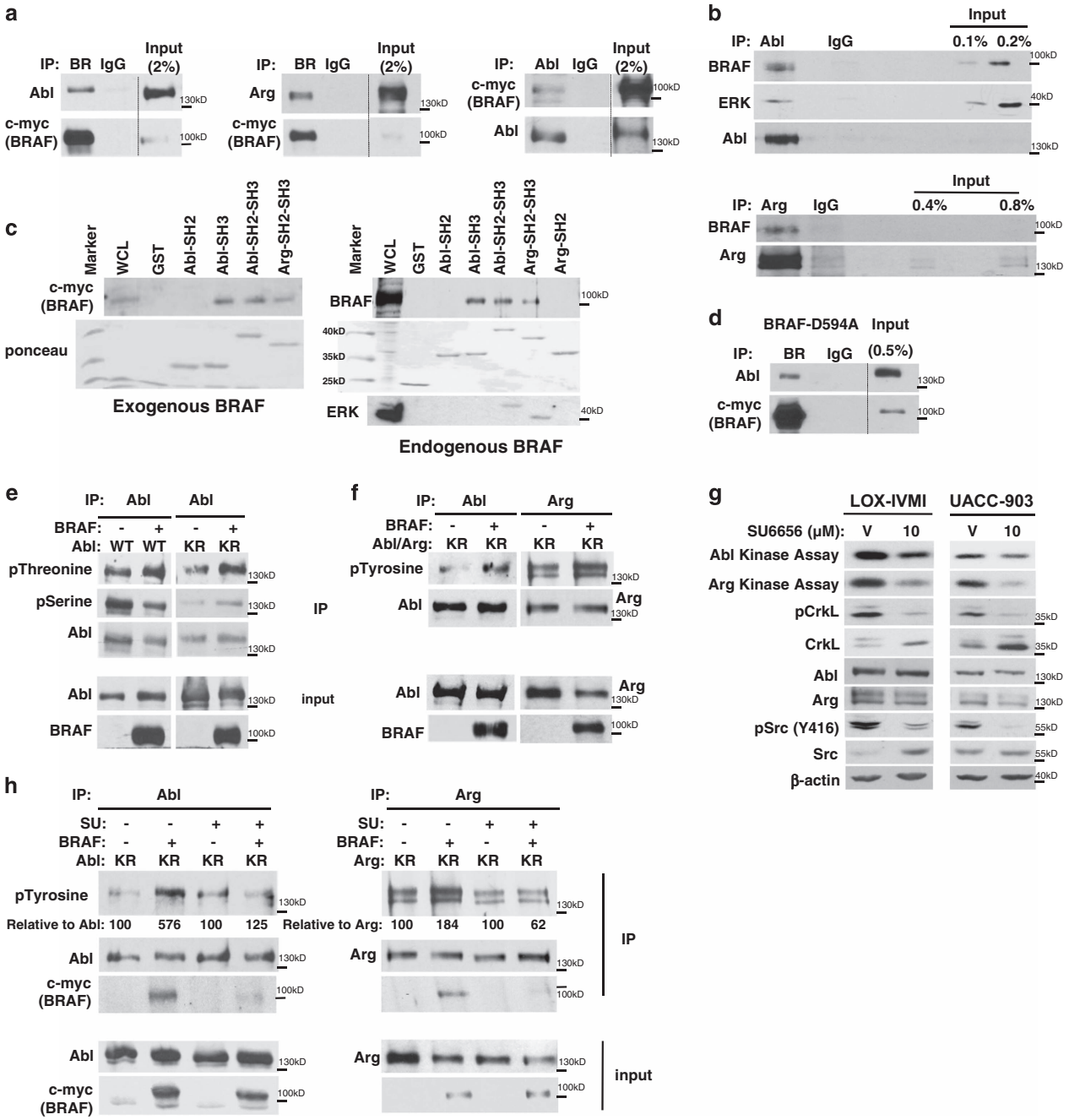


Figure 2. BRAF^{V600E} binds and induces Abl/Arg phosphorylation. (**a**, **b**, **d**) Coimmunoprecipitations. myc-tagged BRAF^{V600E}, Abl or Arg was immunoprecipitated from 293T cells expressing wild-type Abl or Arg (Migr1-Abl-WT, pcDNA-Arg-WT) and myc-tagged BRAF^{V600E} (**a**), or from 293T cells expressing wild-type Abl (pSRα-Abl-WT) and catalytically inactive BRAF (D594A) (**d**) and immunoprecipitates blotted with the indicated antibodies. (**b**) Endogenous Abl or Arg were immunoprecipitated from WM3248 melanoma cell lysate and blotted with the indicated antibodies. IgG isotype-matched control antibody was used in parallel immunoprecipitations. (**c**) GST pull-down assay using lysate from 293T cells transfected with myc-BRAF^{V600E} (exogenous BRAF^{V600E}) or WM3248 cells expressing endogenous BRAF^{V600E} and recombinant fragments of Abl or Arg fused to GST (isolated in bacteria). Bands in long exposure of ERK blot are GST fusion proteins. (**e**, **f**) Immunoprecipitation followed by western blotting using 293T cells transfected with vector (pBabePuro) or myc-BRAF^{V600E} together with wild-type (WT; Migr1-Abl-WT, pcDNA-Arg-WT) or kinase inactive (KR; pSRα-Abl-KR and pcDNA-Arg-KR) Abl or Arg. (**g**) Melanoma cells were treated with vehicle (V; DMSO) or SFK inhibitor, SU6656 (10 μM; 24 h), serum starved and western blot or kinase assays performed on the lysates. (**h**) Abl or Arg immunoprecipitates from vector- (pBabePuro) or myc-BRAF^{V600E}-transfected 293T cells, treated with vehicle (DMSO) or SU6656 (10 μM; 24 h), were blotted with the indicated antibodies.

inducing Abl or Arg tyrosine phosphorylation, and inhibited binding of BRAF ^{V600E} to Abl and Arg (Figure 2h).

BRAF^{V600E} and ERK directly phosphorylate Abl and Arg *in vitro*

To identify the mechanism by which BRAF^{V600E} induces Abl/Arg phosphorylation and activation, first we tested whether kinases downstream of BRAF (MEK, ERK) mediate the effect of BRAF^{V600E} on Abl/Arg activity. Indeed, like the BRAF inhibitor, PLX-4720 (PLX), MEK (U0126) and ERK (SCH772984; SCH) inhibitors also reduced Abl/Arg activities (kinase assays, pCrkL blots; Figures 3a and b), using drug doses that induce similar inhibition of pERK. To test whether BRAF, MEK and/or ERK directly phosphorylate Abl/Arg, we incubated recombinant forms of Abl or Arg with BRAF^{V600E} or ERK

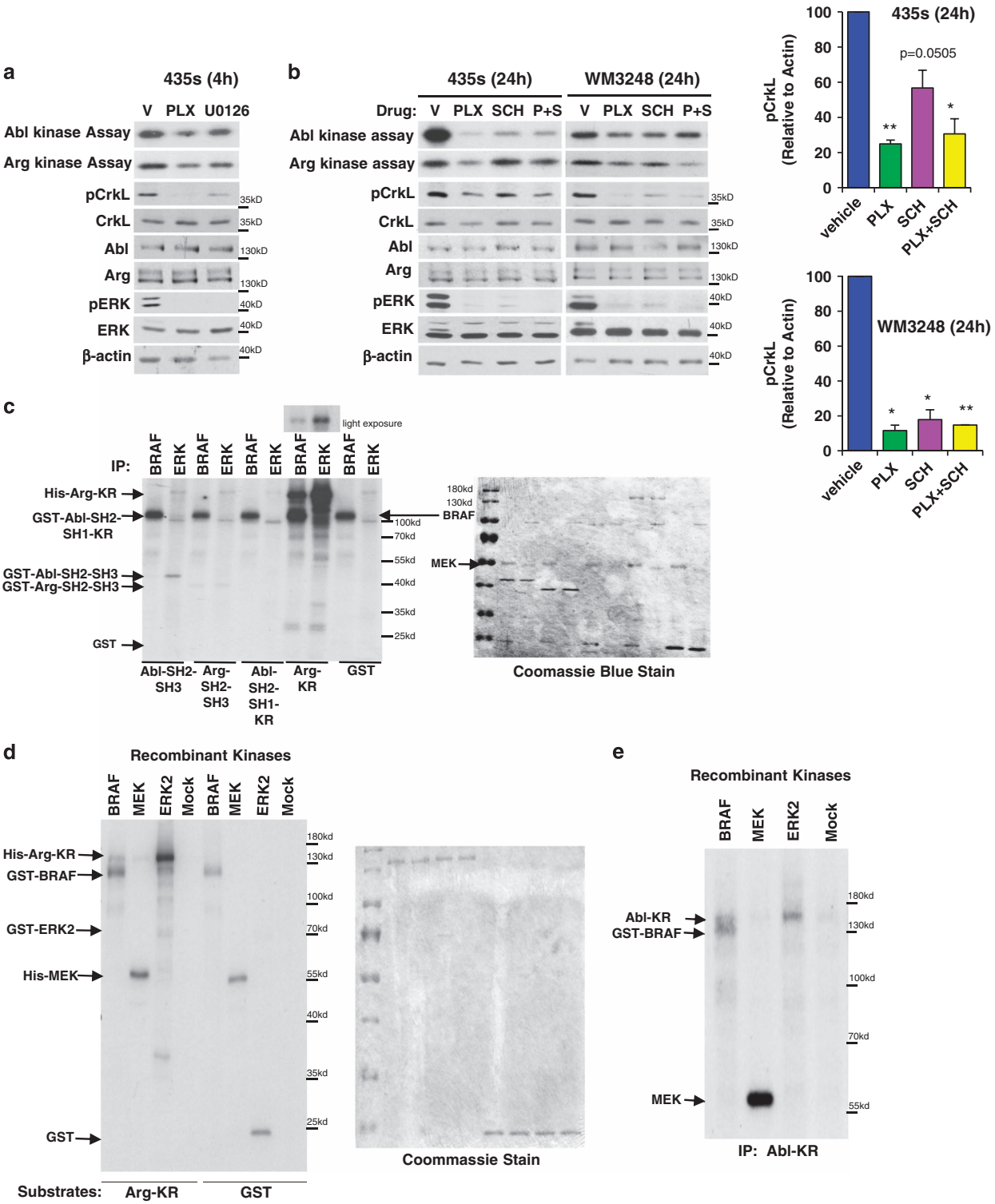


Figure 3. BRAF^{V600E} and ERK directly phosphorylate Abl and Arg *in vitro*. (**a**) Cells were treated with vehicle (V; DMSO), BRAF inhibitor, PLX-4720 (PLX; 1 μM) or MEK inhibitor (U0126; 5 μM) for 4 h, serum-starved and lysates subjected to *in vitro* kinase assay or western blot. (**b**) Cells were treated with BRAF inhibitor (PLX-4720 = PLX; 1 μM), ERK inhibitor (SCH772984 = SCH; 0.1 μM) or the combination for 24 h, serum starved and lysates subjected to *in vitro* kinase assay or western blot. V = vehicle = DMSO. Graphs are mean ± s.e.m. WM3248, $n = 2$; 435s, $n = 3$. $^{*}P < 0.05$, $^{**}P \leqslant 0.01$ using one-sample *t*-tests and Holm's adjustment for multiple comparisons. (**c**) BRAF or ERK immunoprecipitates from 293T cells transfected with myc-BRAF^{V600E}, were incubated with GST-tagged Abl or Arg fragments or with full-length His-tagged, kinase-inactive Arg (His-Arg-KR) in a 'hot' *in vitro* kinase assay. Abl-SH2-SH1-KR contains an inactive kinase domain (KR). The kinase gel was stained with coomassie blue to visualize fragment loading (right), and the dried gel was exposed to film (left). Pan-ERK antibody that recognizes ERK1/2/5 was used for ERK immunoprecipitations. (**d**, **e**) Commercially available, recombinant, full-length GST-BRAF, His-MEK or GST-ERK2 were incubated in 'hot' *in vitro* kinase assays with recombinant His-Arg-KR (**d**) or with kinase-inactive Abl-KR isolated by immunoprecipitation from 293T cells expressing pSRα-Abl-KR (**e**). Recombinant ERK2 also phosphorylated the 'negative' control, GST. However, examination of the sequence revealed the presence of a 'S/TP' ERK phosphorylation site.[54]

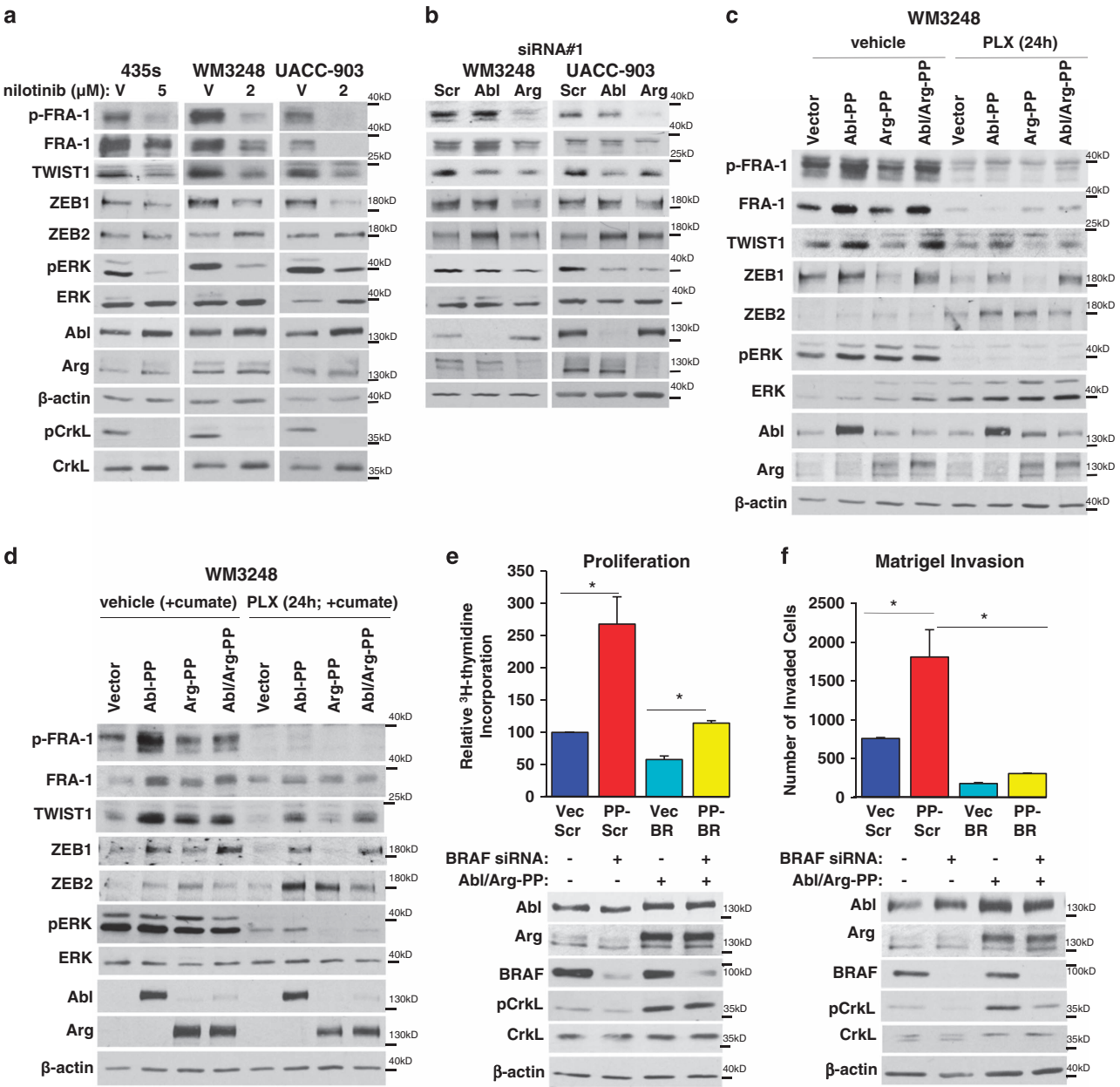


Figure 4. Abl/Arg drive BRAF^{V600E}-mediated processes, and potentiate BRAF^{V600E} signaling. (**a**, **b**) Lysates from vehicle (V; DMSO), nilotinib treated (24 h; **a**) or siRNA-transfected cells (72 h; siRNAs = #1; **b**) were blotted. (**c**, **d**) WM3248 melanoma cells, stably expressing cumate-inducible, Abl-PP/Arg-PP were treated with vehicle (DMSO) or BRAF inhibitor, PLX-4720 (1 μM), in the absence (**c**) or presence (**d**) of cumate (0.5×; 24 h) and lysates blotted. (**e**, **f**) Tritiated thymidine incorporation (**e**) or matrigel boyden chamber invasion (**f**) was assessed using WM3248 cells transfected with vector or Abl-PP+Arg-PP, expressing scrambled or BRAF siRNA (#1). Matrigel invasion was assessed using IGF-1 (10 nM) as chemoattractant at a 48 h timepoint. (**e**) Mean ± s.e.m., $n = 3$. $^*P < 0.05$ using one-sample (vs vector) or two-sample *t*-tests. (**f**) Mean ± s.e.m., $n = 2$, $^*P = 0.05$, using a one-way ANOVA followed by Tukey's HSD test. The homogeneity of variance assumption was assessed by Bartlett's test prior to using ANOVA. Aliquots of cells were lysed and blotted (below). BR, BRAF siRNA; PP, Abl/Arg-PP; Scr, scrambled siRNA; Vec, vector.

immunoprecipitated from BRAF^{V600E}-expressing 293T cells. BRAF^{V600E} and ERK efficiently phosphorylated full-length, kinase-inactive Arg (His-Arg-KR), and ERK modestly phosphorylated an Abl-SH2-SH3 fragment (Figure 3c). Moreover, recombinant forms of BRAF and ERK2 (but not MEK) also phosphorylated His-Arg-KR and kinase-inactive Abl (K290R; KR) (Figures 3d and e). Thus, we propose the following working model. BRAF^{V600E} recruits Abl/Arg to the signaling complex, where ERK (and potentially BRAF) subsequently phosphorylate Abl and Arg. This phosphorylation event likely contributes to Abl cytoplasmic retention, and facilitates tyrosine phosphorylation of Abl and Arg by SFKs.

Abl/Arg are required for BRAF-mediated switch in EMT-TF expression, proliferation and invasion, and potentiate BRAF^{V600E} signaling

To understand the functional and biological significance of Abl/Arg activation by BRAF/ERK, we examined whether Abl/Arg act downstream of BRAF^{V600E} to promote BRAF-driven processes.[17] Expression of BRAF^{V600E} induces ERK-dependent expression and phosphorylation of FRA-1, which causes a switch in EMT-TF expression from ZEB2/SNAIL2 to ZEB1/TWIST1.[17] Consistent with these data, ERK1/2 and FRA-1 were constitutively phosphorylated in melanoma cells that naturally express BRAF^{V600E} (WM3248,

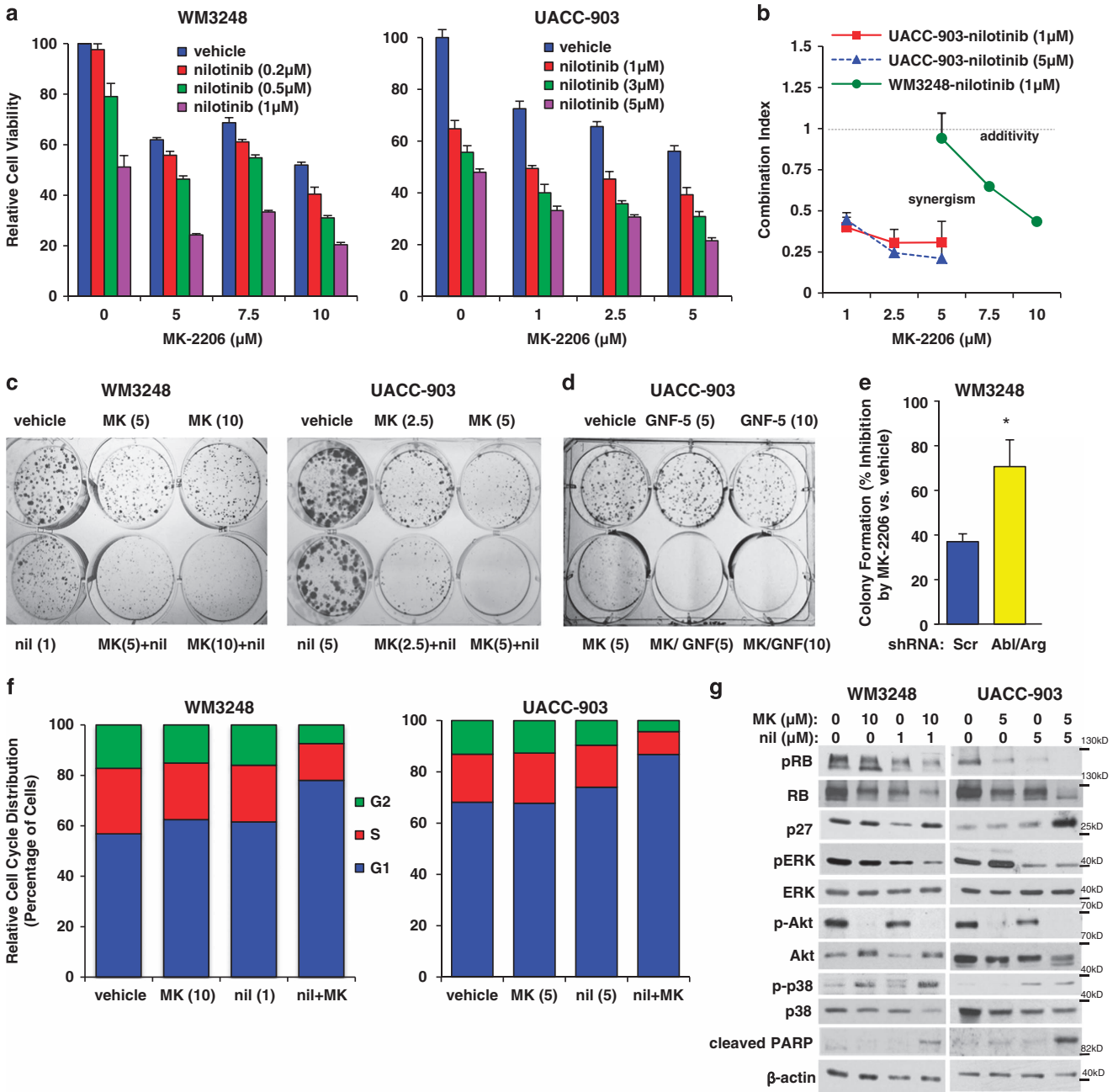


Figure 5. Abl/Arg and Akt pathways synergize to promote melanoma survival, cell cycle progression and colony formation. (**a**) Cell viability (CellTiter Glo) was assessed in treated cells (72 h). Representative experiments of $n=3$. (**b**) Combination indices from $n=3$ from experiments in **a** were obtained using CalcuSyn software. Mean ± s.e.m. Combination indices values >1 = antagonism; $=1$-additivity, <1 = synergy. (**c**, **d**) Drug-treated cells (72 h) were washed and colonies allowed to form for 14 days (**c**) or 7 days (**d**). For **d**, cells were treated for 72 h, 8 days after plating. Drug concentrations are in parentheses (μM). (**e**) Colony-forming assay using WM3248 cells stably expressing a non-targeting shRNA (Scr) or an shRNA that targets Abl and Arg, treated with vehicle or MK-2206 for 5 days. The graph indicates the percent inhibition by MK-2206 as compared to vehicle (DMSO). Mean ± s.e.m., $n=3$. *$P=0.05$, using a two-sample *t*-test. (**f**) BrdU/fluorescence-activated cell sorting cell cycle analysis on treated cells (72 h). (**g**) Western blots on lysates from treated cells (72 h).

UACC-903, 435s; Figure 4a). Transfection of siRNAs targeting Abl or Arg, expression of an shRNA that silences Abl and Arg or treatment with the Abl/Arg inhibitor, nilotinib, reduced pERK1/2, pFRA-1/FRA-1, ZEB1 and TWIST1 expression, and induced ZEB2 (Figures 4a and b and Supplementary Figures S4A and B), indicating that Abl and Arg activation is required for the EMT-TF switch. Consistent with these data, there was a strong trend (Pearson correlation coefficient = 0.3, $P=0.06$; $n=40$) toward a correlation between Arg (*ABL2*) and *TWIST1* mRNA expression in human melanoma metastases (Oncomine Riker data set),[37] which became highly significant when the *n* was increased by including all skin cancer samples (Spearman correlation coefficient = 0.29, $P=0.01$; $n=82$). To test whether Abl and/or Arg mediate the effects of $BRAF^{V600E}$ on the switch, we examined whether expression of constitutively active forms of Abl or Arg (PP)[38] (using a cumate-inducible system), could rescue reversion of the EMT-TF switch induced by inhibiting BRAF in melanoma cells harboring $BRAF^{V600E}$. As expected, treatment

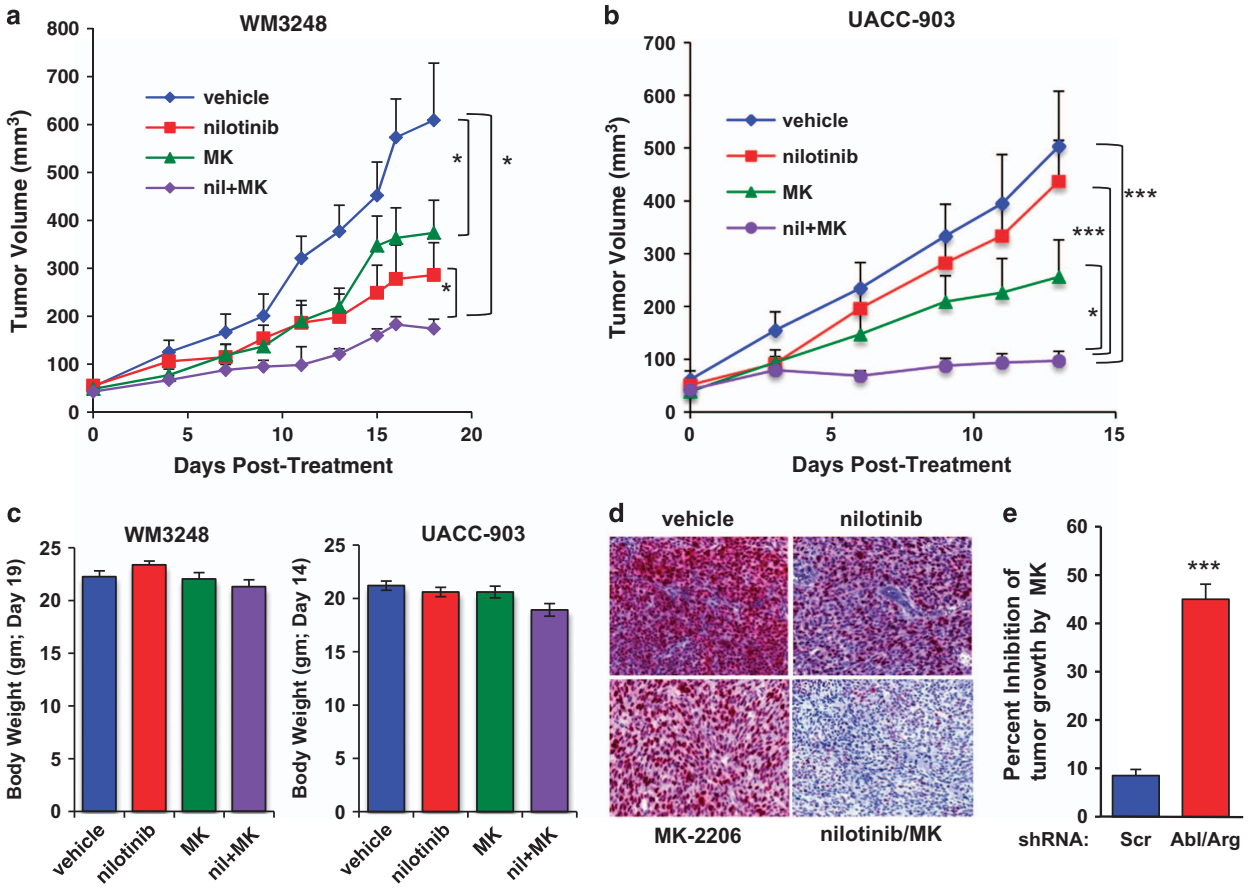


Figure 6. Inhibitors targeting Abl/Arg and Akt pathways cooperate to prevent melanoma growth *in vivo*. (**a**, **b**) Cell lines were orthotopically injected into athymic nude mice, and mice treated with vehicles, nilotinib (33 mg/kg), MK-2206 (90 mg/kg—WM3248; 120 mg/kg—UACC-903) or the combination, 11 (**a**) or 12 (**b**) days following establishment of tumors (graphed as Day 0; tumors were 30–100 mm^3). Tumor volume = $(L \times W^2/2)$, mean ± s.e.m. $^{***}P < 0.001$, $^{*}P < 0.05$, using a linear mixed model with fixed effects of treatment time and their interactions, and random effects of intercept and slope. WM3248: vehicle, $n = 5$; nilotinib, $n = 8$; MK-2206, $n = 8$; nilotinib+MK, $n = 7$. UACC: all groups, $n = 10$. (**c**) Final ending body weight for mice in **a**, **b**. (**d**) Immunohistochemical staining with pRB (S807/S811) antibody on representative UACC-903 tumors (**b**). (**e**) Xenograft assay using WM3248 cells stably expressing non-targeting shRNA (Scr) or Abl/Arg shRNA, treated with vehicle or MK-2206 (120 mg/kg) 9 days after injection (Day 0). Tumor volumes were averaged for treatment days 11, 13, 15, 17 and percent inhibition for control versus Abl/Arg shRNA groups graphed. Mean ± s.e.m. $^{***}P < 0.0001$ using two-sample *t*-test. Vector/vehicle, $n = 6$; vector/MK, $n = 7$; Abl/Arg-shRNA/vehicle, $n = 7$; Abl/Arg-shRNA/MK, $n = 6$.

of vector-transfected cells with the BRAF inhibitor, PLX-4720 (24 h), reversed the EMT-TF switch, inhibiting pERK1/2 and pFRA-1/FRA-1, ZEB1 and TWIST1 expression, and inducing ZEB2 (Figures 4c and d). Notably, expression of Abl-PP rescued the effects of PLX-4720 on TWIST1 and ZEB1, and completely rescued PLX-4720-mediated inhibition of TWIST1 at short-treatment times (2 h; Figures 4c and d, right; Supplementary Figure S4C). Effects were observed in the absence of cumate due to promoter leakiness (Figure 4c), and were enhanced in the presence of cumate (Figure 4d). In contrast, neither Abl-PP nor Arg-PP rescued PLX-4720 effects on pERK or pFRA-1/FRA-1. Thus, Abl is required for BRAF-driven induction of the EMT-TF switch, and induces TWIST1/ZEB1 in an ERK- and FRA-1-independent manner.

Interestingly, Abl-PP and/or Arg-PP expression also increased pFRA-1/FRA-1, pERK1/2, TWIST1 and ZEB1 expression in the absence of PLX-4720 (Figures 4c and d, left), indicating that Abl/Arg also potentiate $BRAF^{V600E}$ induction of the EMT-TF switch, in addition to Abl acting downstream of $BRAF^{V600E}$. In summary, Abl and Arg activation is required to induce the EMT-TF switch; Abl acts downstream of $BRAF^{V600E}$ to regulate TWIST1/ZEB1 expression in an ERK- and FRA-1-independent manner; and Abl/Arg activation potentiates $BRAF^{V600E}$-mediated induction of the EMT-TF switch. These data are the first to link Abl/Arg activation with the EMT-TF switch, which is a critical step in melanoma progression.

Switch in EMT-TF expression is linked to increased melanoma proliferation and invasion.[17,18,39] Thus, we tested whether Abl/Arg are required for BRAF-driven proliferation and invasion. Significantly, Abl-PP+Arg-PP expression completely rescued inhibition of proliferation induced by silencing $BRAF^{V600E}$ (Figure 4e; compare last two bars), indicating that Abl/Arg activation downstream of BRAF is required for BRAF-driven proliferation. In contrast, Abl-PP +Arg-PP expression only partially rescued BRAF siRNA-mediated inhibition of matrigel invasion (Figure 4f, compare last two bars), which likely is due, at least in part, to BRAF siRNA-mediated inhibition of exogenous Abl/Arg activity (pCrkL) in serum-free conditions (Figure 4f, bottom). Interestingly, as we observed with the EMT-TF switch, expression of Abl-PP+Arg-PP also potentiated proliferation and invasion in $BRAF^{V600E}$-expressing cells (Figures 4e and f; compare first two bars), which was dependent on $BRAF^{V600E}$ expression, as silencing BRAF prevented Abl/Arg-mediated potentiation (Figures 4e and f; compare second and fourth bars). Thus, in addition to acting downstream of $BRAF^{V600E}$ and driving BRAF-mediated processes, Abl/Arg also feedback and potentiate $BRAF^{V600E}$ signaling.

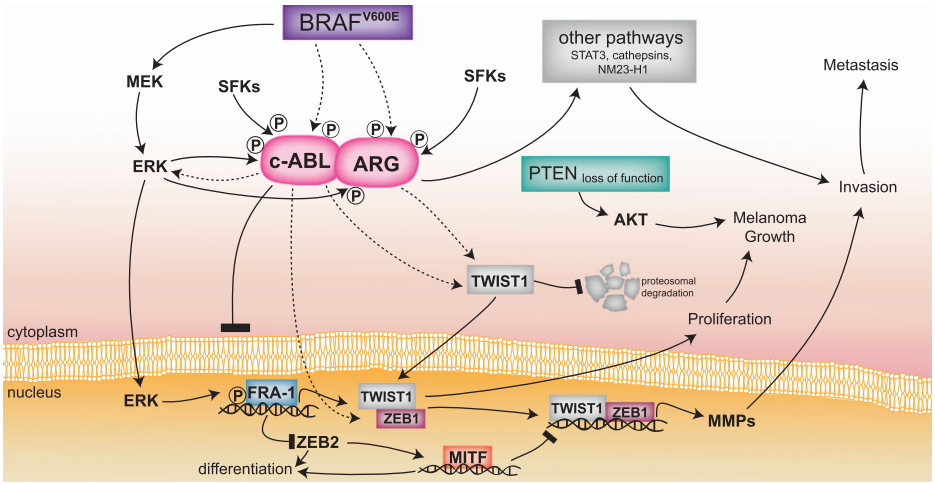


Figure 7. Model for Abl/Arg activation and signaling in melanoma. $BRAF^{V600E}$ contributes to Abl and Arg activation by recruiting Abl/Arg to the signaling complex where ERK (and also potentially BRAF) phosphorylate Abl/Arg. This prevents nuclear targeting of Abl and promotes phosphorylation of Abl and Arg by SFKs. Once activated, Abl/Arg are required for $BRAF^{V600E}$ induction of the EMT-TF switch, invasion and proliferation, and potentiate $BRAF^{V600E}$ signaling, potentially by increasing BRAF expression. Finally, Abl/Arg cooperate with a parallel, compensatory signaling pathway (PTEN loss/Akt activation) to promote melanoma growth and survival.

Abl/Arg cooperate with Akt to promote melanoma growth and survival

Since cancers rapidly develop resistance to targeted agents, the future of targeted therapy lies in targeting cooperating, compensatory pathways. Melanomas with PTEN mutations (activation of Akt), which frequently occurs concurrently with BRAF mutations, often are intrinsically resistant to therapy, indicating a need for new therapies for these patients.[3,11–14] Since Abl/Arg are activated in mutant BRAF/PTEN melanomas (for example, WM3248, UACC-903; Supplementary Figure S1 and Supplementary Table S1), and have little impact on Akt signaling in non-stress conditions,[40] we hypothesized that Abl/Arg and Akt lie in parallel, cooperating pathways. Indeed, inhibitors targeting Abl/Arg (nilotinib; FDA-approved) and Akt (MK-2206; allosteric inhibitor) potently synergized to reduce viability of mutant PTEN melanoma cells expressing highly active Abl and Arg (Figures 5a and b). Nilotinib also cooperated with MK-2206 to block colony formation following drug removal/washout, indicating that the effects are permanent (Figures 5c and d). Moreover, nilotinib's effects were Abl/Arg-dependent and not mediated by off-target or other on-target effects, as GNF-5 treatment (Figure 5d) or expression of an shRNA targeting both Abl and Arg (Figure 5e), also efficiently prevented colony formation of cells treated with MK-2206, even when colonies were allowed to form for 8 days prior to treatment (Figure 5d). Importantly, the mechanism of drug synergy involved G1->S cell cycle arrest (Figure 5f and Supplementary Figure S5), and induction of markers for apoptosis (PARP cleavage) and G1 arrest/senescence/dormancy (↑p27, ↓pRB; Figure 5g).

Combination Abl/Arg and Akt targeting blocks *in vivo* growth of mutant PTEN melanomas

To determine whether Abl/Arg and Akt cooperate to promote melanoma growth, *in vivo*, and to assess whether targeting Abl/Arg and Akt pathways could potentially represent a novel drug combination for treating mutant BRAF/PTEN melanomas, we treated mice harboring mutant BRAF/PTEN melanoma xenografts with vehicle, nilotinib, MK-2206 or the combination. Nilotinib was effective on its own in preventing WM3248 xenograft growth, comparable to effects observed with MK-2206, but was inefficient at reducing the growth rate of UACC-903 xenografts as a monotherapy, consistent with colony-forming assays (Figures 5c right, 6a and b). However, importantly, Abl/Arg and Akt inhibitors dramatically cooperated to prevent WM3248 and UACC-903 growth *in vivo* (Figures 6a and b). Moreover, nilotinib, MK-2206 and the combination did not significantly alter animal body weight (Figure 6c) or induce other signs of toxicity (for example, anemia-pale paws and so on). Similar to *in vitro* results, the drug combination inhibited RB phosphorylation (an indicator of G1 arrest) in the small residual tumors (30–100 mm^3) from combination-treated animals (Figure 6d). Importantly, nilotinib's effects were Abl/Arg-dependent (not due to off-target or other on-target effects) as silencing Abl and Arg with an shRNA targeting both proteins, also significantly sensitized xenografts to MK-2206 treatment (Figure 6e). Taken together, these data indicate that targeting Abl/Arg together with Akt may be an effective treatment strategy for mutant BRAF/PTEN melanomas.

DISCUSSION

This study establishes a novel functional link between $BRAF^{V600E}$ and Abl family kinases. We identify a new mechanism of Abl/Arg activation, and demonstrate that their activation has important functional consequences downstream of $BRAF^{V600E}$ and also feedback and potentiate BRAF/ERK signaling (Figure 7). Moreover, we report data of major translational significance by showing that Abl/Arg and Akt inhibitors potently cooperate to prevent the growth of mutant BRAF/PTEN xenografts.

Abl/Arg proto-oncogenes are tightly regulated, and are kept in an inactive state via intramolecular interactions.[21,22] We show that $BRAF^{V600E}$ directly binds Abl/Arg SH3 domains, which bind conserved PxxP-binding motifs.[41] BRAF contains five PxxP motifs, and one is highly similar to those found in other Abl-SH3-binding proteins.[41] Interestingly, BRAF binding is insufficient to induce Abl/Arg activation, but rather serves to recruit the kinases to the signaling complex, which, interestingly, is dependent on SFK activity.

We provide the first evidence that Abl/Arg drive the EMT-TF switch, which contributes to melanoma metastasis and drug resistance.[16] Abl/Arg activity is required for FRA-1 expression/phosphorylation, induction of TWIST1 and ZEB1 and repression of ZEB2. Moreover, Abl drives TWIST1/ZEB1 expression downstream of $BRAF^{V600E}$ independent of FRA-1/ERK, which indicates that regulation of the switch may not follow a simple linear pathway (FRA-1->ZEB1/TWIST1), and likely involves multiple levels of regulation. Consistent with these data, TWIST1 and ZEB1 also are regulated via other mechanisms, seemingly independent of

ERK/FRA-1 signaling.[18–20] In addition to mediating BRAF induction of the EMT-TF switch, Abl/Arg also are required for BRAF-driven proliferation, which might occur via effects on TWIST1/ZEB1, since TWIST1/ZEB1 not only promote invasion, but also drive melanoma proliferation and cancer stem cell features (Figure 7).[17,18,39,42] Interestingly, in addition to acting downstream of $BRAF^{V600E}$, Abl/Arg also potentiate proliferation, invasion and the EMT-TF switch in the presence of $BRAF^{V600E}$. Importantly, Abl/Arg induce $BRAF^{V600E}$ expression, as Abl-PP and Arg-PP increase $BRAF^{V600E}$ protein, whereas silencing Abl/Arg reduces $BRAF^{V600E}$ expression (Supplementary Figure S6). Thus, Abl/Arg likely potentiate $BRAF^{V600E}$ signaling by increasing $BRAF^{V600E}$ expression. Alternatively, it is also possible that Abl/Arg affect the activity of upstream proteins (for example, Ras or RTKs).[43,44]

BRAFi reduce proliferation and metastatic burden, but often are inefficient at preventing viability, and their lack of permanent effects results in resistance.[10] MEKi extend survival for patients with BRAFi resistance, but have on-target toxicity, and recurrent disease is aggressive and refractory to treatment (including immunotherapy) due to activation of STAT3-dependent invasion.[45–47] Moreover, patients whose melanomas harbor PTEN mutations often are less responsive to BRAFi/MEKi and immune checkpoint inhibitors.[3,11–14] In contrast to BRAFi/MEKi, Abl/Arg inhibitors block STAT3 activation, invasion and metastasis.[24,25] Furthermore, combined inhibition of Abl/Arg and Akt pathways, in melanomas harboring mutant BRAF/PTEN, permanently inhibits colony formation (even when drugs are introduced after colonies develop), induces apoptosis and cell cycle arrest and dramatically prevents melanoma growth *in vivo*. These data are of high translational significance as they indicate that dual inhibition of Abl/Arg and Akt may represent a novel synthetic lethal strategy, and thus, could pave the way for the development of a novel drug combination for patients harboring mutant BRAF/PTEN melanomas (intermittent sun exposure subtype), which often are resistant to therapy. Importantly, Abl and Arg are successful drug targets in other cancer types,[21] and Abl/Arg inhibitors that also block c-Kit activity (imatinib and nilotinib) have been successfully used to treat melanomas harboring c-Kit mutations (acral, mucosal and chronic sun exposure subtypes).[48,49] The availability of a plethora of drugs targeting Abl and Arg, which are relatively non-toxic and several of which are FDA-approved, is likely to facilitate rapid translation of these findings to the clinic.

MATERIALS AND METHODS

Reagents

Cell lines. WM cell lines, 451-LU, 1205-Lu, Mel-1617 and UACC-903 were obtained from Dr Herlyn in 2010 (UACC-903-2014), and authenticated in 2011 (Herlyn lab, Wistar Institute, Philadelphia, PA, USA). MDA-MB-435s-termed 435s was authenticated (genetically identical to M14) in 2012.[24] Melan-a was from Welcome Trust (St George's, University of London, UK; 2015). All other lines were from NCI (NCI-60; 2015, Frederick, MD, USA). Lines were negative for mycoplasma (Lonza MycoAlert; Portsmouth, NH, USA; tested 8/16), and were passaged <1 month. WM3248 cells expressing Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible shRNA targeting Abl and Arg (PLK01-IPTG-3XLacO vector; see Supplementary Materials for plasmid descriptions), non-inducible shRNA targeting Abl and Arg (psiStrike-hygro vector), or Abl-PP and/or Arg-PP (Piggybac cumate vector) were obtained following lentiviral infection (IPTG-shRNA) or transfection (psiStrike-shRNA; Abl/Arg-PP), and selection with puromycin (2.5 μg/ml). For shRNA-expressing cells, clones were picked, expanded, screened for knockdown by western blot and pooled. Inducible shRNA-expressing cells were treated with IPTG (1 mM; 6 days) prior to screening. For cells expressing Abl-PP and/or Arg-PP (Piggyback cumate inducible, transposon vector), polyclonal populations were utilized. Fluorescence-activated cell sorting indicated $>90\%$ of cells were green-fluorescent protein positive.

Drugs. Nilotinib was provided by Novartis (Basel, Switzerland). MK-2206 (Akt inhibitor; *in vitro* studies), GNF-2/GNF-5 (allosteric Abl/Arg inhibitors), SCH772984 (ERK inhibitor) and PLX-4720 (BRAF inhibitor) were from Selleck (Houston, TX, USA). Some studies utilized PLX-4720 from Plexxicon (Berkeley, CA, USA). MK-2206 (*in vivo* studies) was from MedChem Express (Monmouth Junction, NJ, USA). SU6656 was from Millipore (Billerica, MA, USA). Captisol was from Ligand Pharmaceuticals (San Diego, CA, USA). Cumate was obtained from Systems Biosciences and IPTG from Invitrogen (Carlsbad, CA, USA).

Antibodies. Antibodies were obtained from the following companies. Sigma-Aldrich (St Louis, MO, USA): β-actin and Arg (5C6; western). Santa Cruz Biotechnology (Santa Cruz, CA, USA): Abl (K12; kinase assay; K12-AC for endogenous coIP), c-myc (9E10), BRAF (C19 (IP), F-7 (IP, Supplementary Figure S6—western blot), H-145 (all other western blots)), TWIST1 (2C1A), ZEB1 (H-102), ZEB2 (H-160), FRA-1 (R-20; Figure 4b only) and HRP-conjugated secondary antibodies. Cell Signaling (Danvers, MA, USA): FRA-1, pCrkL (recognizes Abl/Arg phosphorylation sites on substrates Crk (Y221) and CrkL (Y207)), CrkL (32H4), pAkt, Akt (#9272), p-p38, p38 (#9212), pRB (S807/S811), RB (4H1), p27 (D37H1), pFRA-1 and phospho-threonine. BD Biosciences (San Jose, CA, USA): Abl (8E9; western blot) and pan-ERK (16/ERK). Promega (Madison, WI, USA): phospho-ERK. Millipore (Billerica, MA, USA): phospho-tyrosine (4G10), phospho-serine (4A4), Src (GD11) and Lamin A/C (clone 14). ThermoFisher (Waltham, MA, USA): α-tubulin (RB9249-P0). The Arg kinase assay antibody and recombinant proteins (Abl/Arg) were previously described.[36,50] Full-length GST-BRAF, His-MEK, GST-ERK2 and siRNAs were from ThermoFisher. siRNAs: Abl: ss866 (10 nM, #1); ss864 (10 nM; #2); 1336 (20 nM, #3); Arg-ss872 (10 nM, #1), ss363 (10 nM, #2), 1478 (20 nM, #3); BRAF: ss2080 (5 nM, #1) and ss2081 (10 nM, #2). shRNA sequence that targets Abl and Arg was 5′-GGGAAATTGCTACCTATGG-3′ (see Supplementary Materials for plasmid descriptions).

Cell transfection

293T cells were transfected with calcium phosphate,[51] Lipofectamine 2000 (Invitrogen) was used for melanoma lines, and melan-a cells were infected with $BRAF^{V600E}$ retrovirus (8 h).[51] WM3248 cells were infected with commercial IPTG-inducible shRNA lentivirus (10 μl per 96-well; non-targeting shRNA $= 2.2 \times 10^7$ Tu/ml, Abl/Arg-shRNA $= 3.7 \times 10^6$ Tu/ml) in the presence of 8 μg/ml polybrene (16 h).

Western blots, coimmunoprecipitations, GST pulldowns and kinase assays

Cells were lysed in kinase lysis buffer (kinase assays, GST pulldowns),[51] RIPA (westerns),[51] or TNEN (coimmunoprecipitations).[52] Proteins were immunoprecipitated or GSTs used for pulldown, and complexes washed (TNEN for coimmunoprecipitations; RIPA/triton-X (+NaCl)/triton-X (-NaCl) for kinase assays).[51] For kinase assays, immunoprecipitates were incubated in Abl/Arg[51] or BRAF[53] kinase buffers containing 1 μM cold ATP, 5 μCi ^{32}P-γ-ATP, 1 μg substrate (Abl/Arg-40′, 25 °C, GST-Crk substrate; BRAF/MEK/ERK-30′, 30 °C, Abl/Arg substrates). For assays using recombinant proteins, 20 ng of BRAF/MEK/ERK and 500 ng of His-Arg-KR were utilized. Kinase assays and westerns were quantitated using a Storm Phosphoimager (GE Healthcare, Pittsburgh, PA, USA) and Image J64, respectively.

Matrigel invasion assays

Assays were performed as described.[24,35] IGF-1 (10 nM; 48 h invasion) was used as chemoattractant for UACC-903 and WM3248 cells, whereas EGF (100 ng/ml; 24 h invasion) was used for LOX-IVMI.

Subcellular fractionation

Cytoplasmic/nuclear lysates were prepared with NE-PER (ThermoFisher).[40]

Proliferation and viability assays

Viability: CellTiter Glo (Promega). Assays were performed using three drug doses (alone/combination).[40]

Proliferation: ^{3}H-thymidine. Tritiated thymidine incorporation was measured in labeled cells (drug treatment = 24 h, label = last 2 h; siRNAs = 72 h transfection, label = 24 h).[40] *BrdU/fluorescence-activated cell sorting*. Cells were treated for 72 h (media/drugs refreshed after 48 h), stained with anti-BrdU antibody (BD Biosciences, Chicago, IL, USA) and analyzed by fluorescence-activated cell sorting (Cell Quest software/Modfit analysis; Verity Software House, Topsham, ME, USA).[40]

Clonogenic assays. Cells were treated (72 h; drugs refreshed after 48 h), washed, media replaced without drugs and colonies fixed (4% paraformaldehyde) and stained (0.5% crystal violet). For some experiments, 72-h treatment was initiated after colonies formed (8 days).

Xenograft assays

WM3248 (3×10^6) and UACC-903 (1×10^6), in HEPES-buffered-saline, were injected subcutaneously in 6-week-old female nude mice (Harlan, Indianapolis, IN, USA). Mice whose tumors grew in 9–12 days (30–100 mm^3) were blindly/randomly assigned to groups. (1) Vehicle: nilotinib-vehicle (0.5% hydroxymethylcellulose/0.05% Tween-80-b.i.d., oral gavage) plus MK-2206-vehicle (30% captisol-3× per week, oral gavage); (2) Nilotinib (33 mg/kg, oral gavage, b.i.d.) plus MK-2206-vehicle; (3) MK-2206 (90 mg/kg per day for WM3248; 120 mg/kg per day for UACC-903 and WM3248-shRNA; 3× per week; oral gavage) plus nilotinib-vehicle; (4) Nilotinib+MK-2206. Tumors were measured 3× per week, and animals killed when largest tumors reached 800 mm^3. Experiments were performed under IACUC protocol #00946M2005, in accordance with University and NIH guidelines.

Power analysis

Ten mice per group provided 85% power to detect a 50 and 75% reduction in tumor volume for single and combination groups, respectively, compared to mean = 600 mm^3 (s.d. = 225 mm^3) in the vehicle group (ANOVA; 1% alpha; adjusted for multiple pairwise testing).

Immunohistochemistry and immunofluorescence

Immunohistochemistry. Antigen retrieval was performed in low pH retrieval solution (Dako, Carpinteria, CA, USA), followed by incubation with pRB antibody (1:50; overnight; 4 °C), amplification with rabbit linker (Dako) and detection with Immpress anti-rabbit-AP and Immpact Vector Red (Vector Laboratories; Burlingame, CA, USA). Slides were scanned on an AperioScope (Vista, CA, USA).

Immunofluorescence. Cells, plated on coverslips, were fixed (4% formaldehyde), permeabilized (0.1% triton-X), blocked (3% BSA) and incubated with Abl (mouse 8E9; 1:50; overnight, 4 °C)[34] and BRAF (rabbit H-145; 1:500; 2 h, 4 °C) antibodies, followed by anti-mouse Alexa-488 and anti-rabbit Alexa-555 secondary antibodies (1:100; Cell Signaling; 1 h), and mounted in ProlongGold antifade (Invitrogen). Images were captured on an Olympus Fluoview FV1000 Confocal microscope, ×60 objective, V1.7 software, using Arg Ion (488 nm excitation for Alexa-488), HeNe (543 nm excitation for Alexa-555) and Diode lasers (405 nm DAPI).

Statistics

Analyses were performed with SAS (V9.3), R (V3.3.1) or the Vassar Website. Tukey HSD test was used for multiple comparison adjustments for ANOVA, whereas Holm's method was used for paired *t*-tests (comparisons against normalized controls) and unpaired Welch's *t*-test (comparisons between groups). All reported values are two-tailed. Variation was presented as s.e.m. for each group. Parametric tests were performed after checking data normality (Shapiro–Wilk tests if samples sizes were sufficient) and homogeneity of variance (Bartlett's tests if necessary). Otherwise, nonparametric tests were performed. Microarray data were downloaded (Oncomine),[37] and Pearson's or Spearman's correlation coefficient used to quantify correlations. Combination indices were calculated with CalcuSyn software (Biosoft; Cambridge, UK).

CONFLICT OF INTEREST

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