Vertical Pathway Inhibition Overcomes Adaptive Feedback Resistance to KRAS^{G12C} Inhibition

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ABSTRACT

Purpose: Although *KRAS* represents the most commonly mutated oncogene, it has long been considered an "undruggable" target. Novel covalent inhibitors selective for the KRAS^{G12C} mutation offer the unprecedented opportunity to target KRAS directly. However, prior efforts to target the RAS–MAPK pathway have been hampered by adaptive feedback, which drives pathway reactivation and resistance.

Experimental Design: A panel of *KRAS^{G12C}* cell lines were treated with the KRAS^{G12C} inhibitors ARS-1620 and AMG 510 to assess effects on signaling and viability. Isoform-specific pulldown of activated GTP-bound RAS was performed to evaluate effects on the activity of specific RAS isoforms over time following treatment. RTK inhibitors, SHP2 inhibitors, and MEK/ERK inhibitors were assessed in combination with KRAS^{G12C} inhibitors *in vitro* and *in vivo* as potential strategies to overcome resistance and enhance efficacy.

Results: We observed rapid adaptive RAS pathway feedback reactivation following KRAS^{G12C} inhibition in the majority of KRAS^{G12C} models, driven by RTK-mediated activation of wild-type RAS, which cannot be inhibited by G12C-specific inhibitors. Importantly, multiple RTKs can mediate feedback, with no single RTK appearing critical across all KRAS^{G12C} models. However, coinhibition of SHP2, which mediates signaling from multiple RTKs to RAS, abrogated feedback reactivation more universally, and combined KRAS^{G12C}/SHP2 inhibition drove sustained RAS pathway suppression and improved efficacy *in vitro* and *in vivo*.

Conclusions: These data identify feedback reactivation of wild-type RAS as a key mechanism of adaptive resistance to KRAS^{G12C} inhibitors and highlight the potential importance of vertical inhibition strategies to enhance the clinical efficacy of KRAS^{G12C} inhibitors.

See related commentary by Yaeger and Solit, p. 1538

Introduction

RAS is the most frequently mutated oncogene in cancer, with KRAS mutations being the most predominant of the three RAS isoforms (HRAS, NRAS, and KRAS; ref. 1). In its wild-type form, RAS cycles between the GDP-bound inactive state and GTP-bound active state, and when mutated at the most common G12, G13, and Q61 loci, KRAS is in a constitutively active GTP-bound state. Mutant RAS has long been considered an undruggable target, and thus most therapeutic strategies have focused on targeting downstream effector pathways such as the ERK MAPK cascade (2). However, there has been limited clinical success in targeting downstream effectors, and other approaches of targeting RAS function have been met with limited success (2).

Recently, covalent inhibitors targeting a specific KRAS mutation—Glycine 12 to cysteine (G12C)—have been developed that show encouraging preclinical efficacy in KRAS^{G12C} tumor models (3–5). These inhibitors undergo an irreversible reaction with the mutant cysteine present only in G12C-mutant KRAS, making them highly selective for KRAS^{G12C} versus wild-type KRAS or other RAS isoforms. The inhibitors function by locking KRAS^{G12C} in an inactive GDP-bound state, exploiting the unique property of KRAS^{G12C} to cycle between the GDP- and GTP-bound states (6, 7). The KRAS^{G12C} mutation represents 11% of all KRAS mutations (COSMIC v89; refs. 1, 8), but is the most common RAS mutation in lung cancer and also occurs in many other types of cancer, such as colon and pancreatic cancers. Two KRAS^{G12C} inhibitors have entered clinical trials: AMG510 (NCT03600883) and MRTX1257 (NCT03785249). As the first such agents capable of inhibiting mutant KRAS directly, this class of agents offers an unprecedented therapeutic opportunity to target this critical oncogene.

However, previous efforts to target the RAS–RAF–MEK pathway have been hindered by adaptive feedback reactivation of pathway signaling as a major mode of therapeutic resistance. For example, BRAF inhibition in BRAF^{V600}-mutant cancers leads to loss of negative feedback signals regulated by the MAPK pathway, leading to receptor tyrosine kinase (RTK)-mediated reactivation of MAPK signaling through wild-type RAS and RAF, particularly in specific tumor types, such as colorectal cancer (9–12). Similarly, in KRAS-mutant cancers, MEK inhibitor treatment leads to adaptive feedback activation of RAS signaling, often through EGFR or other human EGFR (HER) family members or FGFR, limiting efficacy (13, 14). Indeed, early preclinical studies with KRAS^{G12C} inhibitors, such as ARS-1620, have suggested a potential role for adaptive feedback as a mechanism of resistance (7, 15). Early clinical data with the KRAS^{G12C} inhibitor AMG510 was recently reported, showing an initial overall response rate of 24% (13/55) in patients with KRAS^{G12C}-mutant cancers, raising the possibility that overcoming adaptive resistance could be a vital next step in improving efficacy (16). Therefore, understanding the potential mechanisms driving adaptive feedback resistance to KRAS inhibition may be critical to the development of future therapeutic strategies.

Here, we evaluate the adaptive feedback response to KRAS^{G12C} inhibition and find evidence of rapid RAS pathway reactivation in the majority of KRAS^{G12C} models. We observe that feedback reactivation

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Translational Relevance

KRAS is the most commonly mutated oncogene in human cancer, and new mutant-specific inhibitors of KRAS, such as covalent inhibitors of $KRAS^{G12C}$, offer the unprecedented opportunity to target mutant KRAS directly. However, prior efforts targeting the RAS–MAPK pathway have been constrained by adaptive feedback reactivation of pathway signaling. We describe how adaptive feedback through multiple RTKs can drive resistance to $KRAS^{G12C}$ inhibition through compensatory activation of wild-type RAS isoforms, which cannot be inhibited by G12C-specific inhibitors. Our data suggest that vertical pathway inhibition strategies, and in particular combinations of $KRAS^{G12C}$ inhibitors with SHP2 inhibitors—which can interrupt feedback from multiple RTKs—may be critical to abrogate feedback reactivation of the RAS pathway following $KRAS^{G12C}$ inhibition and may represent a promising therapeutic approach for $KRAS^{G12C}$ cancers.

is driven in large part by RTK-mediated activation of wild-type RAS (NRAS and HRAS), which is not inhibited by G12C-specific inhibitors. Importantly, we find that this process is mediated by multiple RTKs, and that no single RTK dominates across all $KRAS^{G12C}$ models, suggesting that a strategy cotargeting a single RTK to block adaptive resistance may be ineffective. However, we observe that cotargeting of SHP2 (17, 18), a key phosphatase that mediates signaling from multiple RTKs to RAS, is able to abrogate feedback reactivation of RAS signaling following $KRAS^{G12C}$ inhibition across all models, and that the combination of $KRAS^{G12C}$ and SHP2 inhibitors leads to sustained RAS pathway suppression and improved efficacy *in vitro* and *in vivo*. These data suggest that vertical inhibition strategies to block adaptive RAS pathway reactivation may be key to enhance the efficacy of $KRAS^{G12C}$ inhibitors.

Materials and Methods

Cell lines and inhibitors

Cell lines were obtained from ATCC or the Center for Molecular Therapeutics at the MGH Cancer Center (Boston, MA), which routinely performs cell line authentication testing by SNP and short-tandem repeat analysis, and maintained in DMEM/F12 supplemented with 10% FCS, and were not cultured longer than 6 months after receipt from cell banks. ARS-1620, SHP099, RMC-4550 erlotinib, afatinib, crizotinib, and BGJ398 used for *in vitro* studies were purchased from Selleckchem and AMG 510 was purchased from MedChemExpress. ARS-1620 used in *in vivo* studies was purchased from MedchemExpress and SHP099, afatinib, and BGJ398 used in *in vivo* studies were purchased from Selleckchem.

Inhibitor treatment assays

Short-term sensitivity to ARS-1620 was determined by CellTiter-Glo (Promega). Briefly, cell lines were seeded at $1–2 \times 10^3$ cells/well of a 96-well plate and 24 hours after seeding, a serial dilution of ARS-1620 was added to cells. After 72 hours of inhibitor treatment, plates were developed with CellTiter-Glo and luminescence read on a Plate Reader (Molecular Devices). For long-term viability assays, cells were plated at low density 2×10^2–3×10^3 in 6-well plates and treated with ARS-1620 (1 μmol/L) alone or in combination with SHP099 (10 μmol/L), erlotinib, afatinib, crizotinib, or BGJ398 (all 1 μmol/L) for 10–14 days with drug refreshed every 2–3 days. Assays were fixed and stained with a crystal violet solution (4% formaldehyde), and plates were scanned using a photo scanner and cell growth was quantified using ImageJ software.

Inhibitor treatment and Western blot analyses

Cell lines were treated with ARS-1620, SHP099, or a combination for 4, 24, 48, or 72 hours before samples were collected in NP40 lysis buffer. Whole-cell lysates were resolved on 4%–12% Bis-Tris gels (Thermo Fisher Scientific) and Western blotting was performed using antibodies against phospho-CRAF (S338), phospho-MEK (S217/221), phospho-ERK (T202/Y204), MYC, phospho-AKT (S473; Cell Signaling Technology), phospho-p90 RSK (Abcam), and GAPDH (Millipore). Densitometry analysis was performed using ImageJ software.

RAS-GTP pulldown

After indicated inhibitor treatment, RAS activity was assessed by GST-RAF-RBD pulldown (Cell Signaling Technology), followed by immunoblotting with pan-RAS or RAS isoform–specific antibodies. Pulldown samples and whole-cell lysates were resolved on 4%–12% Bis-Tris Gels, and Western blotting was performed using antibodies against KRAS (Sigma), NRAS (Santa Cruz Biotechnology), HRAS (Proteintech), and pan-RAS (Cell Signaling Technology). Densitometry analysis was performed using ImageJ software.

qPCR

tRNA was isolated using an RNeasy Kit (Qiagen) and reverse transcription was performed using the qScript cDNA SuperMIx (Quantabio). Taqman qRT-PCR was performed on the Light Cycler 480 (Roche) with FAM/MGB–labeled probes against DUSP6 (Hs04329643_s1, Thermo Fisher Scientific) and endogenous control VIC/MGB–labeled β-actin (Thermo Fisher Scientific).

Xenograft studies

SW837 and MIA PaCa-2 cell lines (5×10^6) were injected into 6- to 8-week-old female athymic nude mice (Charles River Laboratories). Treatment of ARS-1620 (200 mg/kg), afatinib (12.5 mg/kg), BGJ398 (20 mg/kg), and SHP099 (75 mg/kg) by daily oral gavage was initiated when tumor size reached 100–200 mm^3 and tumor size was assessed by caliper measurements for 21–25 days. All animal studies were performed through the Institutional Animal Care and Use Committee (IACUC).

Graphical analysis

Data were analyzed using GraphPad Prism 6 software and curve fit and concentration needed to reduce the growth of treated cells to half that of untreated cells (GI_{50}) values were generated as indicated in the Figure Legends.

Results

Feedback reactivation of RAS signaling following $KRAS^{G12C}$ inhibition

To model potential mechanisms constraining the efficacy of $KRAS^{G12C}$ inhibition, we evaluated the effects of the $KRAS^{G12C}$ inhibitor ARS-1620 across a panel of eight $KRAS^{G12C}$-mutant cell lines (**Fig. 1A**). To explore the potential role of adaptive feedback in the setting of $KRAS^{G12C}$ inhibition, we assessed the effects of ARS-1620 in each model over time from 4 hours to 72 hours. In all cell lines, ARS-1620 suppressed MAPK pathway signaling, a key downstream effector pathway of KRAS, as measured by inhibition of phospho-MEK, phospho-ERK, and phospho-RSK at 4 hours (**Fig. 1B** and **C**). In

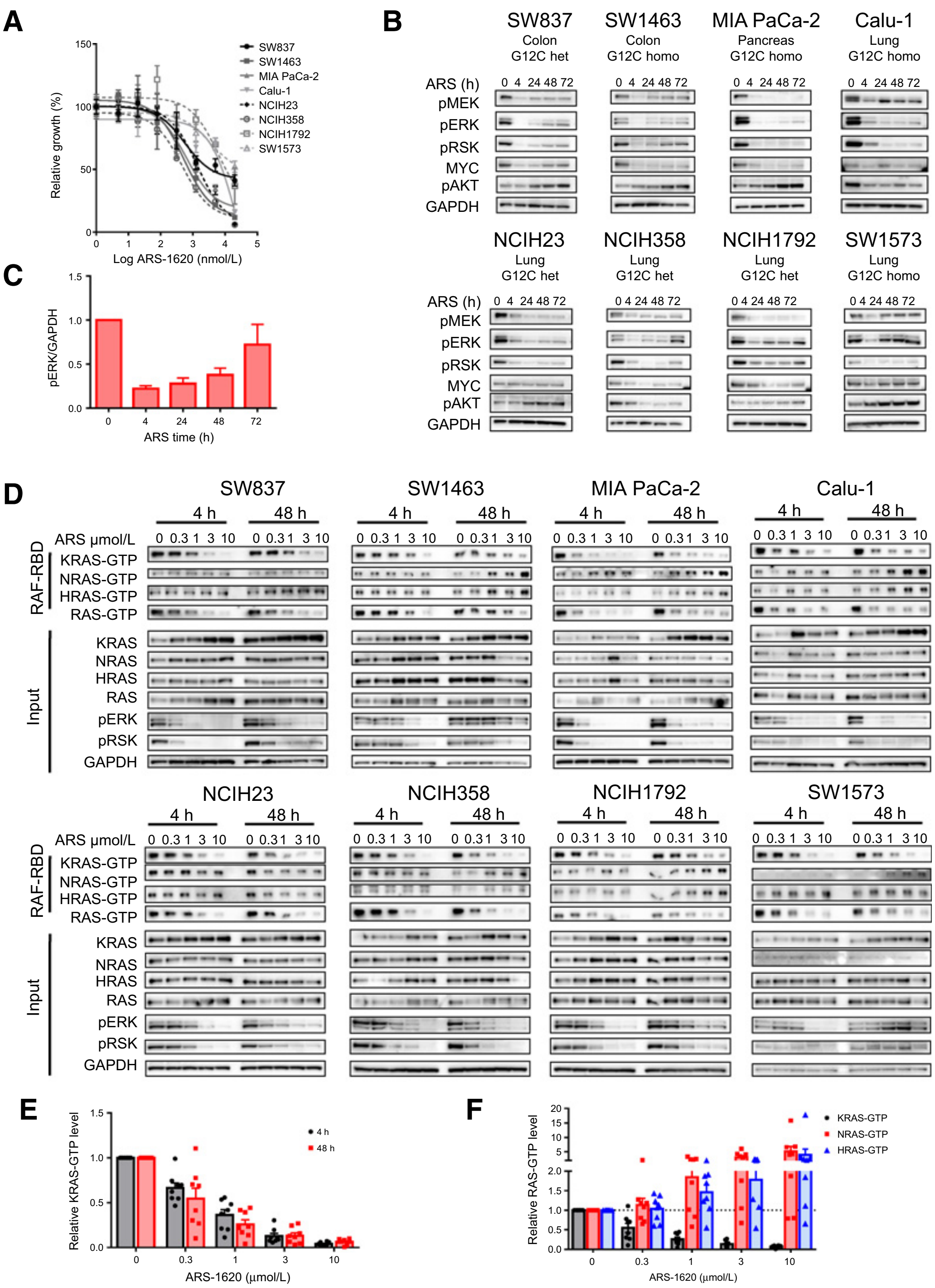

Figure 1.

Feedback reactivation of RAS signaling occurs following KRAS^{G12C} inhibition. **A,** Cell lines were treated for 72 hours with a dose titration of ARS-1620 and viability was measured by CellTiter-Glo. **B,** KRAS-G12C–mutant cell lines were treated with ARS-1620 (1 μmol/L) for 0, 4, 24, 48, and 72 hours. Western blot analysis was performed for phospho- (p)MEK, pERK, pRSK, pAKT, and total MYC with GAPDH as a loading control. **C,** Densitometry of phospho-ERK normalized to GAPDH for blots in, results represent an average of phospho-ERK across all 8 cell lines (**A**). **D,** Cell lines were treated with a dose titration of 0.3–10 μmol/L ARS-1620 for 4 or 48 hours and lysates were subject to a RAF-RBD pulldown and blot analysis of KRAS, NRAS, HRAS, and total RAS as well as pERK, pRSK, and GAPDH for input samples. **E,** Densitometry analysis of 4 and 48 hours KRAS-GTP levels normalized to input KRAS and GAPDH loading control in **D**. **F,** Densitometry analysis of 48 hours KRAS-GTP, NRAS-GTP, and HRAS-GTP levels normalized to input RAS and GAPDH loading control of blots in **D**. Densitometry results in **E** and **F** represent an average across all 8 cell lines.

addition, suppression of total MYC protein levels, which are highly regulated by RAS and ERK activity, was observed (19, 20). However, by 24–48 hours, RAS–MAPK pathway signaling began to rebound from the nadir of pathway suppression, leading to pathway reactivation and incomplete suppression by 72 hours (**Fig. 1B** and **C**). A similar rebound in RAS-MAPK signaling was also observed with the more recently available clinical $KRAS^{G12C}$ inhibitor AMG 510 (Supplementary Fig. S1) Importantly, while some variability in the degree of pathway reactivation was observed across models, with some such as the Calu-1 cell exhibiting little to no rebound, RAS–MAPK pathway activity, as assessed by phospho-ERK levels, rebounded on average to approximately 75% of baseline levels by just 72 hours. The rapid and consistent reactivation of signaling observed following $KRAS^{G12C}$ inhibition suggests that adaptive feedback may have the potential to limit efficacy for this class of inhibitors.

To better understand the mechanism of feedback reactivation in the setting of $KRAS^{G12C}$ inhibition, we assessed the effects of $KRAS^{G12C}$ inhibition with ARS-1620 across a range of concentrations from 0.3 to 10 μmol/L at 4 and 48 hours on levels of active GTP-bound KRAS and wild-type RAS (NRAS, HRAS), as well as downstream signaling (**Fig. 1D**). ARS-1620 effectively suppressed KRAS-GTP levels as measured by a RAF–RAS binding domain (RBD) pulldown assay in all cell lines in a dose-dependent manner, consistent with suppression of $KRAS^{G12C}$ activity (**Fig. 1D** and **E**). However, consistent with our data from **Fig. 1B** and **C**, evidence of a rebound in downstream pathway activation (phospho-ERK, phospho-RSK) was observed by 48 hours, relative to 4 hours. Importantly, this rebound reactivation was observed even at the highest concentrations of ARS-1620, suggesting that pathway rebound is not simply due to inadequate levels of inhibitor. Importantly, in most cell lines, increases in the levels of the active GTP-bound forms of the wild-type RAS isoforms (NRAS-GTP, HRAS-GTP) were noted by 48 hours in a dose-dependent manner (although levels of HRAS and NRAS expression were quite low in some cell models) indicating that induction of wild-type RAS activity may play a key role in feedback reactivation, particularly because no clear rebound in KRAS-GTP levels was noted between 4 and 48 hours of treatment at any inhibitor concentration (**Fig. 1E** and **F**). In fact, on average, we observed a 4- to 5-fold increase in NRAS-GTP and HRAS-GTP levels by 48 hours following treatment with the highest concentrations of ARS-1620 across all models. Similarly, we also observed a strong induction of both NRAS-GTP and HRAS-GTP levels and rebound in downstream MAPK signaling (phospho-ERK, phospho-RSK) upon treatment with AMG 510 (Supplementary Fig. S1). Taken together, these data suggest that adaptive feedback reactivation of pathway signaling following $KRAS^{G12C}$ inhibition corresponds with a rebound in RAS activity, in particular an increase in wild-type RAS activity, although the exact mechanism of RAS reactivation may differ across $KRAS^{G12C}$ cancers. Importantly, these data suggest that blocking adaptive feedback signals driving pathway reactivation may be necessary to enhance the efficacy of $KRAS^{G12C}$ inhibitors.

Adaptive resistance to $KRAS^{G12C}$ inhibition is driven by multiple RTKs

To identify potential strategies to inhibit feedback reactivation following $KRAS^{G12C}$ inhibition, we attempted to define the specific mechanisms driving adaptive pathway rebound. Prior work has demonstrated that adaptive feedback reactivation of RAS-MAPK signaling can be driven by RTKs in response to RAF inhibition and MEK inhibition in BRAF- and KRAS-mutant cancers (11, 13, 21–23). To assess the role of RTKs in feedback reactivation in response to $KRAS^{G12C}$ inhibition, we analyzed the levels of RTK activation at baseline and after 48 hours of ARS-1620 treatment by a phospho-RTK array (Supplementary Fig. S2). We observed high basal levels of several phosphorylated RTKs, including phospho-EGFR in most cell lines. In addition, following 48 hours of treatment with ARS-1620, we observed an adaptive increase in the phosphorylation levels of multiple RTKs, including EGFR, HER2, FGFR, and c-MET, but with a highly heterogeneous pattern across cell line models, suggesting that multiple RTKs may play a role in adaptive feedback to $KRAS^{G12C}$ inhibition. Previous studies have suggested that key RTKs may play dominant roles in driving adaptive feedback to inhibitors of the MAPK pathway, including EGFR in response to BRAF inhibition in $BRAF^{V600E}$ colorectal cancer and EGFR/HER family kinases or FGFR in response to MEK inhibitors in KRAS-mutant lung cancers (13, 14, 21, 24). However, the potential for multiple RTKs to contribute to adaptive resistance in each of the paradigms above may limit efforts to block adaptive feedback by targeting a single RTK. Similarly, the variable induction of multiple RTKs across different $KRAS^{G12C}$ models following ARS-1620 treatment and different levels of phospho-RTKs at baseline suggests that distinct and/or multiple RTKs may drive adaptive feedback across different $KRAS^{G12C}$ cancers, and that strategies targeting a single RTK may not be universally effective. In this scenario, the RTK-associated phosphatase SHP2 may represent a common node through which to inhibit feedback reactivation of RAS signaling by multiple RTKs that may preserve efficacy across heterogeneous $KRAS^{G12C}$ cancers.

Thus, to assess whether vertical inhibition of the RAS–MAPK pathway could block feedback reactivation and enhance the efficacy of $KRAS^{G12C}$ inhibition, we evaluated whether coinhibition of individual RTKs or SHP2 could augment the efficacy of ARS-1620 in suppressing cell viability (**Fig. 2A** and **B**; Supplementary Fig. S3). Interestingly, in each cell line, inhibition of a specific RTK in combination with ARS-1620 led to a marked decrease in cell viability relative to ARS-1620 alone. However, $KRAS^{G12C}$ cell lines demonstrated a heterogeneous response to RTK inhibition, with select inhibitors enhancing antitumor effect in combination, relative to ARS-1620 alone. Overall, the pan-HER inhibitor afatinib and the FGFR inhibitor BGJ398 displayed the greatest and most consistent cooperativity with ARS-1620, although neither combination was universally effective in all models. For example, SW1463 showed primary dependence on EGFR/HER family signaling (erlotinib, afatinib), as compared with FGFR signaling (BGJ398), whereas MIA Paca-2 appeared to be more dependent on FGFR signaling and showed less dependence on EGFR/HER family signaling. Notably, while some cell lines showed heightened dependence on a single RTK, SHP2 inhibition was effective in suppressing adaptive resistance to $KRAS^{G12C}$ inhibition across all cell lines. These results support that multiple RTKs can contribute to adaptive feedback resistance to $KRAS^{G12C}$ inhibition, and that combined inhibition of RTKs or SHP2 and $KRAS^{G12C}$ could represent a more effective therapeutic strategy to overcome resistance and improve efficacy in KRAS G12C–mutant tumors. Furthermore, as strong feedback reactivation of MAPK signaling downstream of RAS is observed following $KRAS^{G12C}$ inhibition, we also assessed potential combinations of $KRAS^{G12C}$ inhibition with MEK and ERK inhibitors, which also displayed potential cooperativity across the panel of $KRAS^{G12C}$ models, although strong induction of RAS activity was observed with these combinations (Supplementary Fig. S5). Together, these data support the potential of vertical pathway inhibition strategies to overcome adaptive resistance to $KRAS^{G12C}$ inhibition.

To test whether individual RTK inhibition in combination with $KRAS^{G12C}$ inhibition might represent a promising therapeutic strategy, we investigated the *in vivo* efficacy of $KRAS^{G12C}$ inhibition in

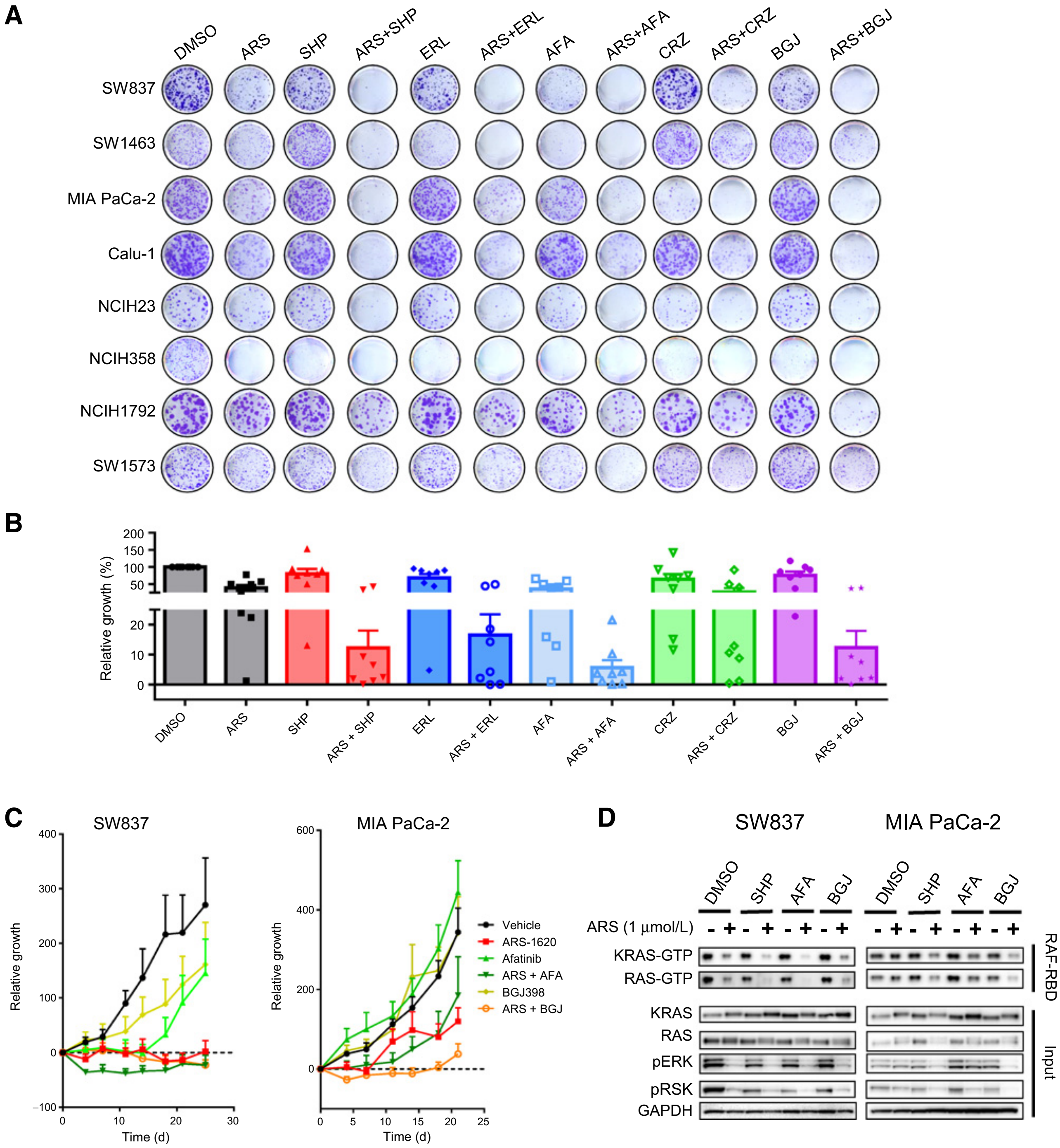

Figure 2.

KRAS^{G12C} inhibitors are subject to adaptive feedback through activation of RTKs. **A,** Cell lines were treated with ARS-1620 (1 μmol/L), SHP099 (10 μmol/L), erlotinib (1 μmol/L), afatinib (1 μmol/L), crizotinib (1 μmol/L), or BGJ398 (1 μmol/L), or a combination for 10–14 days and then stained with crystal violet. **B,** Quantification of assays in **A**. **C,** SW837 and MIA PaCa-2 xenografts treated daily with ARS-1620 (200 mg/kg), afatinib (12.5 mg/kg), BGJ398 (20 mg/kg), or combination for 25 and 21 days, respectively. **D,** SW837 and MIA PaCa-2 cell lines were treated with ARS-1620 (1 μmol/L), SHP099 (10 μmol/L), afatinib (1 μmol/L), or BGJ398 (1 μmol/L), or a combination and lysates were subject to a RAF-RBD pulldown and blot analysis of KRAS-GTP and total RAS-GTP as well as pERK, pRSK, and GAPDH for input samples.

combination with the RTK inhibitors that demonstrated the greatest cooperativity with ARS-1620 *in vitro*: the pan-HER inhibitor afatinib and the FGFR inhibitor BGJ398. ARS-1620 demonstrated modest efficacy as a single agent in both the SW837 and MIA PaCa-2 xenograft models (**Fig. 2C**; Supplementary Fig. S4). Interestingly, while neither reached statistical significance, a clear trend toward enhanced efficacy relative to ARS-1620 alone was observed with a specific RTK combination in each model. However, the predominant RTK dependency of each model differed, with the afatinib combination appearing to produce the greatest effect in SW837 xenografts (although not significant, $P = 0.37$ vs. ARS-1620 alone), in contrast with MIA PaCa-2 xenografts, in which the BGJ398 combination appeared to produce the greatest effect (although not significant, $P = 0.13$ vs. ARS-1620 alone), similar to their *in vitro* profiles (**Fig. 2A**). Consistent with these observations, we found that adaptive feedback reactivation in the setting of $KRAS^{G12C}$ inhibition exhibited a similarly variable RTK dependence in each model for maintaining both KRAS-GTP and total RAS-GTP levels (**Fig. 2D**), with the SW837 model more dependent on HER signaling, and MIA PaCa-2 more dependent on FGFR signaling. However, we found that SHP2 inhibition could enhance suppression of active RAS in combination with ARS-1620 across both models, supporting the potential for SHP2 as a common downstream node through which to inhibit signaling from multiple RTKs. Taken together, these data not only support the potential for vertical inhibition strategies to suppress adaptive feedback resistance to $KRAS^{G12C}$ inhibition but also suggest that coinhibition of individual RTKs may not be broadly effective across $KRAS^{G12C}$ cancers.

SHP2 inhibition reduces feedback reactivation in response to $KRAS^{G12C}$ inhibitors

While our data suggest that SHP2 inhibitors could be a promising combination partner for $KRAS^{G12C}$ inhibitors, SHP2 inhibitors as single agents were recently proposed as a potential therapeutic strategy for $KRAS^{G12C}$ cancers (25). SHP2 acts to dephosphorylate and activate multiple nodes of RAS signaling downstream of RTKs, enhancing signaling through the RAS-RAF-MEK-ERK cascade (25–28). Thus, inhibition of SHP2 has the potential to block the ability of multiple RTKs to activate RAS. Because $KRAS^{G12C}$ actively cycles between its GDP-bound inactive state and its GTP-bound active state, $KRAS^{G12C}$ may be uniquely dependent on some level of basal upstream activation from RTKs to maintain activity. Indeed, SHP2 inhibition alone was shown to have antitumor effects *in vitro* and *in vivo* (25). However, we found that the SHP2 inhibitor SHP099 led to incomplete suppression of KRAS-GTP levels as a single agent (**Fig. 3A**).

However, in all cell lines, concurrent ARS-1620 and SHP099 treatment led to more complete suppression of both KRAS-GTP and total RAS-GTP levels when compared with either agent alone at 48 hours (**Fig. 3A**). SHP099 also reduced NRAS-GTP levels in several cell lines and abrogated the adaptive increase in activated NRAS-GTP levels by 48 hours induced by ARS-1620 in some models. The reduction in KRAS-GTP and RAS-GTP levels translated to further suppression of the MAPK pathway downstream of RAS by 48 hours. Thus, our results suggest that SHP2 inhibition can mitigate the induction of wild-type RAS activity that drives adaptive feedback reactivation of RAS and MAPK pathway signaling in response to $KRAS^{G12C}$ inhibition.

Combined inhibition of $KRAS^{G12C}$ and SHP2 with ARS-1620 and SHP099 also led to a more complete suppression of the MAPK pathway over time in all models, exhibiting a strong reduction in feedback reactivation of the pathway (**Fig. 3B** and **C**; Supplementary Fig. S6). A similar pattern of more complete suppression of the MAPK pathway was also observed with ARS-1620 combined with the SHP2 inhibitor RMC-4550 (Supplementary Fig. S5) and AMG 510 combined with either SHP099 or RMC-4550 (Supplementary Fig. S1). Interestingly, the effects of $KRAS^{G12C}$ or SHP2 inhibition on PI3K/AKT signaling, as measured by phospho-AKT, were highly variable across cell lines, with a reduction noted in some cell lines (SW1463, MIA PaCa-2, NCIH23, NCIH358), and unaffected or increased levels noted in other cell lines, which is consistent with prior studies, suggest that PI3K is not universally tied to RAS activity (15, 29). Combined $KRAS^{G12C}$ and SHP2 inhibition more effectively suppressed tumor cell viability at 72 hours, leading to GI_{50} shifts of approximately 5- to 30-fold in six of eight $KRAS^{G12C}$ models (Supplementary Fig. S7). Interestingly, the effects of combined $KRAS^{G12C}$ and SHP2 inhibition on cell viability were more pronounced in long-term viability assays. While some models (NCIH358, Calu-1) demonstrated marked suppression of viability with ARS-1620 alone, combined $KRAS^{G12C}$ and SHP2 inhibition led to a statistically significant reduction in cell number relative to either agent alone in all $KRAS^{G12C}$ models, while having no effect in the $KRAS^{G12D}$ Ls174T model (**Fig. 3D**; Supplementary Figs. S7 and S8).

Combined $KRAS^{G12C}$ and SHP2 inhibition drives tumor regressions *in vivo*

To better assess combined $KRAS^{G12C}$ and SHP2 inhibition as a potential therapeutic strategy for $KRAS^{G12C}$ cancers, we investigated the efficacy of combined $KRAS^{G12C}$ and SHP2 inhibition *in vivo*. While a reduction in tumor growth relative to vehicle control was observed in both SW837 and MIA PaCa-2 xenograft models with single-agent ARS-1620 or SHP099, the combination of ARS-1620 and SHP099 showed a statistically significant increase in antitumor efficacy compared with each agent alone and led to tumor regressions in the majority of tumors in each model (**Fig. 4A** and **B**; Supplementary Fig. S9). The efficacy of SHP099 monotherapy *in vivo* was more striking than seen in our *in vitro* experiments, supporting previous findings that SHP2 perhaps plays a more important role in $KRAS^{G12C}$-driven tumor growth *in vivo* (27, 28). Because our *in vitro* data suggest that SHP2 inhibition can abrogate adaptive feedback reactivation of RAS pathway signaling following $KRAS^{G12C}$ inhibition, we evaluated the degree of RAS–MAPK pathway inhibition in tumors after 4 days of each treatment by assessing transcript levels of ERK transcriptional targets (30, 31). Consistent with the feedback reactivation observed *in vitro*, we observed modest and incomplete suppression of ERK-dependent transcripts, such as *DUSP6* after 4 days of treatment with either ARS-1620 or SHP099 alone (**Fig. 4E** and **F**; Supplementary Fig. S9). However, combined treatment with ARS-1620 and SHP099 maintained a greater decrease after 4 days of treatment compared with each inhibitor alone. These results support that combined $KRAS^{G12C}$ and SHP2 inhibition may overcome adaptive feedback resistance and may represent a promising therapeutic strategy for future clinical trials in $KRAS^{G12C}$-mutant cancers.

Discussion

$KRAS^{G12C}$ inhibitors represent the first potential opportunity to target mutant KRAS directly in patients. However, adaptive feedback resistance has been a key limitation in prior clinical efforts to target the RAS–MAPK pathway. For example, RTK-driven feedback reactivation of RAS-MAPK signaling has been identified as a key driver of resistance in $BRAF^{V600E}$ cancers treated with BRAF inhibitors (colorectal cancer in particular) and in KRAS-mutant cancers treated with MEK inhibitors (11, 12, 32). Thus, this prior experience predicts that

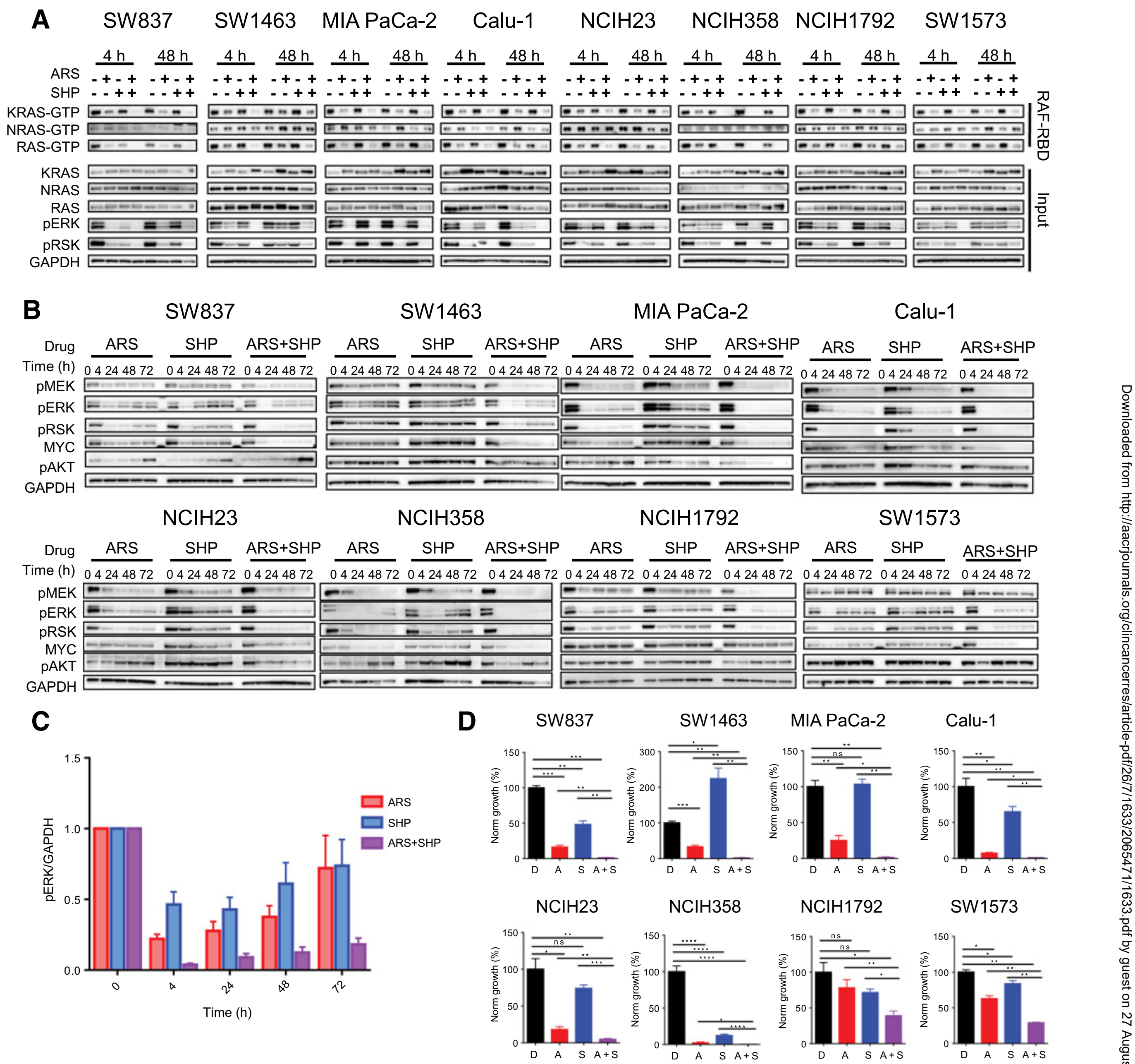

Figure 3.

SHP2 inhibition enhances the efficacy of KRAS^{G12C} inhibition. **A,** Cell lines were treated with ARS-1620 (1 μmol/L), SHP099 (10 μmol/L), or a combination for 4 or 48 hours and lysates were subject to a RAF-RBD pulldown and blot analysis of KRAS, NRAS, HRAS, and total RAS as well as pERK, pRSK, and GAPDH for input samples. **B,** KRAS^{G12C}-mutant cell lines were treated with ARS-1620 (1 μmol/L), SHP099 (10 μmol/L), or a combination for 0, 4, 24, 48, and 72 hours. Western blot analysis was performed for pMEK, pERK, pRSK, pAKT, total MYC with GAPDH as a loading control. **C,** Densitometry analysis of pERK normalized to GAPDH was performed for all cell lines. **D,** Quantification of crystal violet stain of cell lines treated with ARS-1620 (1 μmol/L), SHP099 (10 μmol/L), or a combination for 10–14 days, statistical significance was evaluated by Student *t* test, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

adaptive resistance may pose a key obstacle for KRAS^{G12C} inhibitors, as well, and that strategies to overcome this mechanism may be key to improving clinical efficacy. In line with this hypothesis, we find consistent evidence of rapid feedback reactivation of RAS pathway signaling in KRAS^{G12C} cancer models following treatment with KRAS^{G12C} inhibitors. We observe that feedback reactivation is driven by multiple RTKs and that the pattern of RTK-dependence is highly heterogeneous across KRAS^{G12C} models. RTK-driven feedback may contribute to RAS reactivation through two mechanisms. First, as described by Lito and colleagues, increased RTK activity may lead to increased cycling of KRAS^{G12C} to its active GTP-bound form that hinders the binding of most KRAS^{G12C} inhibitors, which bind specifically to the inactive GDP-bound form (7). Second, in this study we see clear evidence of RTK-driven induction of wild-type RAS (NRAS

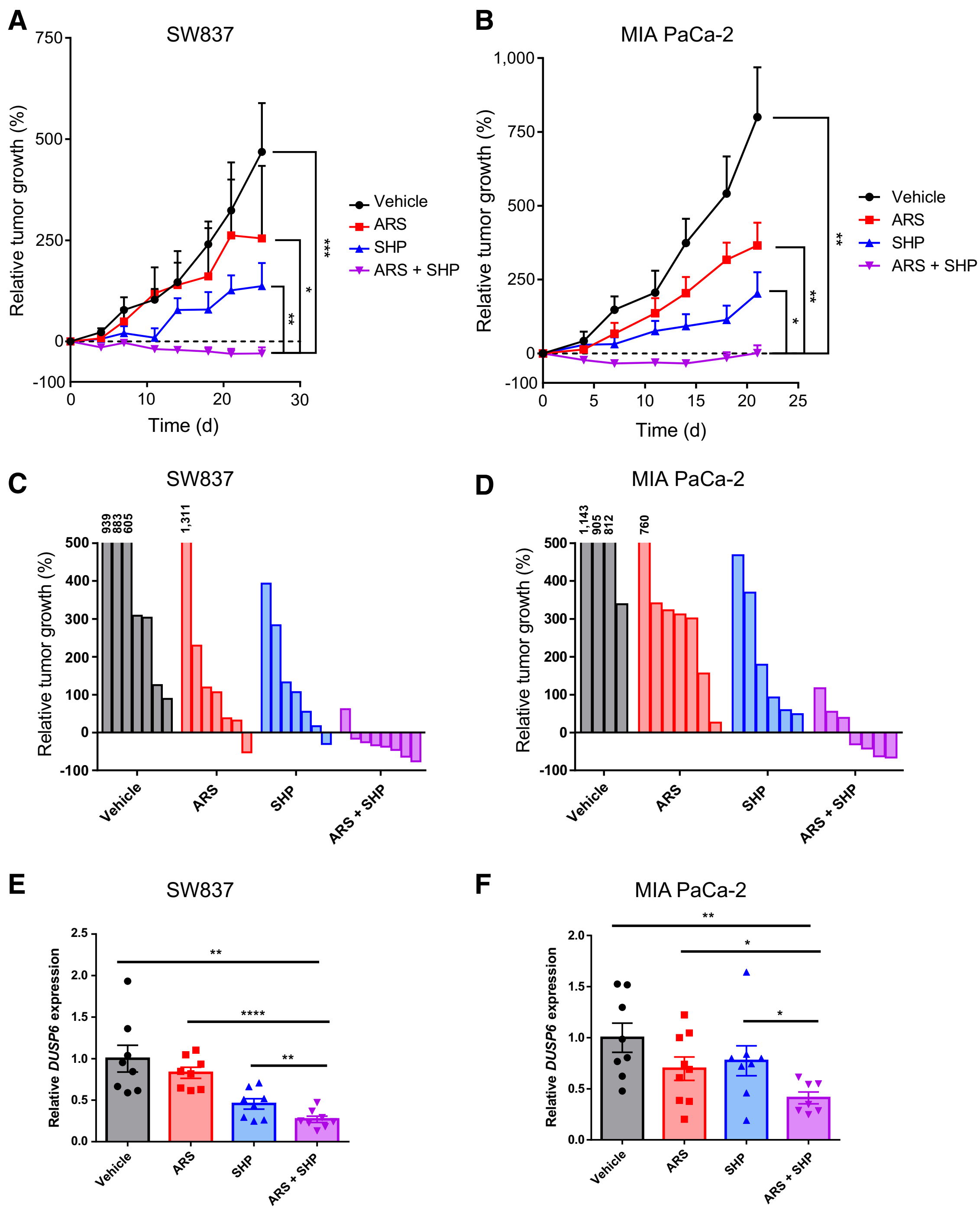

Figure 4.

SHP2 inhibition enhances the efficacy of KRAS^{G12C} inhibition *in vivo*. SW837 (**A**) and MIA PaCa-2 (**B**) xenografts treated daily with ARS-1620 (200 mg/kg), SHP099 (75 mg/kg), or both for 25 and 21 days respectively; statistical significance was evaluated by Mann–Whitney test where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Waterfall plots of SW837 (**C**) and MIA PaCa-2 (**D**) tumors endpoint tumors from **A** and **B**, respectively. SW837 (**E**) and MIA PaCa-2 (**F**) tumors treated with ARS-1620, SHP099, or both for 4 days, and Taqman qPCR was performed to monitor changes in *DUSP6* gene transcription and β-actin was used as an endogenous control; statistical significance was evaluated by Student *t* test, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

or HRAS) activity following KRAS^{G12C} inhibitor treatment, which can reactivate signaling in a KRAS^{G12C}-independent manner. Importantly, while GTP-bound state-selective KRAS^{G12C} inhibitors have been proposed as one possible solution to the first mechanism (increased RTK-driven cycling of KRAS^{G12C} to its active GTP-bound state), this mechanism of action is unlikely to be effective against the second mechanism of adaptive feedback (RTK-driven activation of wild-type RAS; ref. 33). Thus, given this novel potential role for wild-type RAS, our data suggest that interrupting the adaptive feedback loop following KRAS^{G12C} inhibition may be critical to overcoming adaptive resistance.

Targeting a dominant RTK-driven adaptive feedback reactivation is an attractive strategy to suppress adaptive resistance, as it intercepts the critical feedback loop at its most upstream point. Indeed, in BRAF^{V600} colorectal cancer, targeting EGFR—the RTK thought to be the primary driver of feedback reactivation from preclinical studies—in combination with BRAF inhibition, has led to an improvement in clinical efficacy (34–39). However, preclinical and clinical data have demonstrated that other RTKs can drive feedback in an EGFR-independent manner, and that the combination of BRAF and EGFR only suppresses signaling in a subset of patients (34). Similarly, we note that multiple RTKs appear to be involved in feedback reactivation following KRAS^{G12C} inhibition and that the pattern of RTK-dependence is highly variable between KRAS^{G12C}-mutant cancers. Interestingly, our data do indicate that some RTK inhibitors—in particular the pan-HER inhibitor afatinib and the FGFR inhibitor BGJ398—are effective in combination with KRAS^{G12C} inhibitors in many models, suggesting that pan-HER or FGFR cotargeting could be promising strategies. This finding is consistent with prior studies identifying HER family and FGFR signaling as key mediators of adaptive feedback to RAS–MAPK pathway inhibitors (10–14, 21, 22) and recent functional genomic screens (40). However, for each of these inhibitors, there are models in which pathway feedback reactivation appears independent of each specific RTK—that is, MIA PaCa2 for afatinib, and SW1463 for BGJ398 (**Fig. 2A**)—and RTK combinations showed differential effects across *in vivo* models (**Fig. 2C**), suggesting that cotargeting a single RTK is unlikely to be universally effective.

Conversely, targeting SHP2 provides the potential opportunity to intercept a common signaling node linking multiple RTKs to RAS signaling. Indeed, inhibition of SHP2 was recently shown to overcome RTK-driven adaptive feedback mechanisms to MEK inhibition, and enhance the efficacy of MEK inhibitor–induced growth suppression *in vitro* and *in vivo* in RAS-driven cancers (27, 41–44). Furthermore, SHP2 inhibition was also demonstrated to have single-agent efficacy in preclinical KRAS^{G12C} models by reducing cycling to the active GTP-bound state, offering an additional advantage as a potential combination partner (25). Our data support SHP2 as a promising combination partner for KRAS^{G12C} inhibitors, capable of abrogating adaptive feedback reactivation from multiple RTKs to maintain RAS pathway suppression and enhance efficacy *in vitro* and *in vivo*. Although some models showed the greatest sensitivity to KRAS^{G12C} inhibition in combination with a specific RTK inhibitor, combined KRAS^{G12C} and SHP2 inhibition suppressed pathway signaling and enhanced efficacy in all models, supporting its potential as a more universal approach. Moreover, we observed that SHP2 inhibition could consistently block the adaptive increase in wild-type RAS (HRAS, NRAS) activation observed after KRAS^{G12C} inhibition in many models (**Fig. 3A**). While the results of initial clinical trials will demonstrate whether SHP2 inhibitors will have a viable therapeutic index in patients, these data suggest that SHP2 inhibitors, either alone or perhaps in combination with specific RTK inhibitors or downstream inhibitors of the RAS–MAPK pathway, could be promising therapeutic strategies for future clinical trials.

The initial reports of single-agent activity of KRAS^{G12C} inhibitors in patients with KRAS^{G12C} cancers are promising and support that mutant RAS represents a valid clinical target. However, not all patients respond to therapy, and the durability of benefit may be limited, suggesting that strategies to target key resistance mechanisms may improve clinical efficacy. In 55 patients with KRAS^{G12C} cancers treated with the KRAS^{G12C} inhibitor AMG-510, a 24% overall response rate was reported, with 11 responses observed in 23 patients with non–small cell lung cancer (NSCLC), but only 1 response seen in 29 patients with colorectal cancer (16). Interestingly, this pattern of tumor type–specific difference in response is highly consistent with prior examples of adaptive resistance. For instance, BRAF inhibitors led to a monotherapy response rate of >50% in BRAF^{V600E} melanoma and approximately 30% in BRAF^{V600E} NSCLC, but only approximately 5% in BRAF^{V600E} colorectal cancer, likely due to the variable robustness of adaptive feedback networks in each tumor type (39, 45–48). Still, in all cases, combination therapies to suppress adaptive feedback proved key to optimizing the frequency and durability of response in each tumor type (34, 49, 50). Similarly, our data suggest that therapeutic combinations will be key to overcoming adaptive resistance and enhancing clinical benefit of KRAS^{G12C} inhibitors. Initially, combinations may need to focus on improving suppression of RAS signaling by combining KRAS^{G12C} inhibitors with agents targeting adaptive feedback—such as RTK inhibitors, SHP2 inhibitors, or perhaps downstream pathway inhibitors (i.e., MEK or ERK)—and determining the most effective clinical strategy may be a critical first step. However, additional combinations exploring potential synthetic lethal interactions with other pathways may also be key to maximizing efficacy. For example, a study by Misale and colleagues, suggested that targeting the PI3K–AKT pathway, which is often activated independently of RAS, may improve the efficacy of KRAS^{G12C} inhibitors and a study by Molina-Arcas and colleagues suggested targeting IGFR and mTOR (15, 51). Taken together, our data suggest that adaptive feedback is likely to play a key role in resistance to KRAS^{G12C} inhibitors, and that vertical inhibition of the RAS pathway, such as combined SHP2 and KRAS^{G12C} inhibition, may represent a promising strategy for evaluation in future clinical trials.

Disclosure of Potential Conflicts of Interest

R.B. Corcoran is a paid consultant for Amgen, Array Biopharma, Astex Pharmaceuticals, Avidity Biosciences, Bristol-Myers Squibb, C4 Therapeutics, Chugai, Elicio, FOG Pharma, Fount Therapeutics/Kinnate Biopharma, Genentech, Guardant Health, LOXO Oncology, Merrimack, N-of-One, Novartis, nRichDx, Revolution Medicines, Roche, Roivant, Shionogi, Taiho, and Warp Drive Bio, reports receiving commercial research grants from Asana, AstraZeneca, and Sanofi, and holds ownership interest (including patents) in Avidity Biosciences, C4 Therapeutics, Fount Therapeutics/Kinnate Biopharma, nRichDx, and Revolution Medicines. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.B. Ryan, F. Fece de la Cruz, H.A. Shahzade, C.B. Hong, R.B. Corcoran
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Wong, R.B. Corcoran
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